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**An integrated approach to the conservation of the threatened
plant *Malcolmia littorea* (Brassicaceae)**

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CHAPTER 1

INTRODUCTION

1.1. Conservation biology in an European context

Conservation biology has emerged as a vital component of the social response to the contemporary wave of global environmental change that is threatening to extinguish a very large fraction of the world's biological diversity (Soulé and Kohm 1989). As stated by Soulé and Kohm (1989), "never in the history of our species has the biological diversity of the planet been so threatened; never in the history of the planet has one species so dominated the biosphere and so changed environmental conditions". Conservation biology is a synthetic, multidisciplinary science that evolved since the 1980s, feeding on a variety of other areas of biology, notably ecology, demography, population biology, population genetics, biogeography, landscape ecology, environmental management and economics, and has also spawned new areas such as conservation genetics, metapopulation ecology dynamics and biology, restoration biology and ecology, fragmentation biology and patch dynamics (Heywood and Iriondo 2003). Conservation biology is often termed a crisis discipline, in that it arose in response to the dramatic loss of biodiversity that was being documented across the world (Soulé and Kohm 1989; Heywood and Iriondo 2003). Conservation biologists view their main task as providing the intellectual and technological tools that will anticipate, prevent, minimize, and/or repair the ecological damages caused by habitat alteration, fragmentation and destruction which threaten populations, species, communities and ecosystems (Soulé and Kohm 1989). The past 50 years have witnessed a major evolution in our understanding of conservation and its relationship with the elusive goal of sustainable development. This period has also been marked by two antagonistic trends. On the one hand, there has been the rise of environmentalism and the establishment of conservation-orientated institutions and organisations and the negotiation and implementation of a series of treaties and other instruments that affect the ways in which

most countries address these issues. On the other hand, the scale and intensity of human interactions with the environment have led to progressive and widescale habitat loss of species and genetic variability (Heywood and Iriondo 2003). Because of the key role they play in earth's ecosystems, plants should have the highest priority in conservation efforts (Ellstrand and Elam 1993).

The context of conservation changed significantly following the United Nations Conference on the Environment and Development at Rio in 1992, the culmination of a remarkable series of major international initiatives in the preceding 30-40 years. It represents the first step, in a global scale, for biodiversity conservation and defines the guidelines for the elaboration of common strategies for safeguard of animals, plants, and habitats. The subsequent ratification by most of the world's governments of the *Convention on Biological Diversity* (CBD) marked a turning point and has placed the subject of biodiversity firmly on the political agenda. Conservation ceased to be an optional extra and became official, global and national policy (Heywood and Iriondo 2003). The CBD promotes biodiversity conservation, sustainable use of its components and the equitable sharing of the benefits arising from the use of biodiversity. Specifically, in relation to plants, at its sixth conference of the parties in 2002, the CBD Secretariat promoted the Global Strategy for Plant Conservation (GSPC), a strategic plan for plant conservation at global level (CBD 2002a). The CBD *2010 Biodiversity Target* (CBD 2002b), committed the parties to achieve by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional and national level as a contribution to poverty alleviation and to the benefit of all life on earth. This target has not been achieved. In 2010, at the tenth conference, CBD updated the GSPC (Updated Global Strategy for Plant Conservation 2011-2020), and this new version addressed 16 main objectives, e.g., the *in situ* conservation of at least 75% of the endangered plants and the *ex situ* collection of 75% of the endangered plants, for restoration purposes (CBD 2010). Of particular relevance is target 12: "By 2020 the extinction of known threatened species has been prevented and their conservation status, particularly of those most in decline, has been improved and sustained" (CBD 2010).

In order to coordinate the implementation of the GSPC at European level, the European Strategy for Plant Conservation (ESPC) was adopted. The first European Strategy was developed in 2001 by the Council of Europe and Planta Europa (2001) and was valid until 2007. Targets both in the Global Strategy and the European Strategy address five major objectives: (1) understanding and documenting plant diversity; (2)

conserving plant diversity; (3) using plant diversity sustainably; (4) promoting education and awareness about plant diversity and (5) building capacity for the conservation of plant diversity. At the fifth Planta Europa Conference, the Strategy was reviewed and renewed and has set targets for the period 2008-2014 (Planta Europa 2008) which were aligned to the GSPC. Target 2 was of major relevance as it requested “A preliminary assessment of the conservation status of all known plant species at national, regional and international levels”. The production of the European Red Lists (Bilz et al. 2011) contributed to the target and in particular the sub-target ESPC 2.1, providing an assessment of 1,826 European plant species. It was also useful for the completion of Target ESPC 7.1, which states “60% of species of European conservation priority plant and fungal species, including crop wild relatives, conserved *in situ* by 2014 through the implementation of national strategies for conserving priority species”.

EU nature conservation policy is based on two main pieces of legislation: the EU Birds Directive (Council Directive 79/409/EEC of 2 April 1979 on the conservation of wild birds) and the EU Habitats Directive (Council Directive 92/43/EEC on the conservation of natural habitats and of wild flora and fauna). The main aim of this nature conservation policy is to ensure the favourable conservation status of the habitats and species found in the EU, and one of the main tools to enhance and maintain this status is the Natura 2000 network of protected areas, which is made of sites designated and protected under both directives, combining long-term conservation with economic and social activities as part of a sustainable development strategy. The Bern Convention, a binding international agreement that aims to conserve wild flora and fauna and their natural habitats, was the basis for designing the Habitats Directive (Bilz et al. 2011).

The Habitats Directive contains a series of Annexes that identify habitats and species of European Community concern, and each member state proposed a national list of sites for evaluation in order to form a European network of sites of community importance (SCIs). Regarding the establishment of protected areas for vascular plant conservation, the identification of Important Plant Areas (IPAs) plays an important role. Whilst the role of IPAs is more important for countries outside of the EU, IPA data can also provide valuable information for priority setting for site conservation inside the EU 27, either by pointing out existing gaps in the current Natura 2000 network of sites, or by identifying sites for threatened plant species not listed in Annex II of the EU Habitats Directive. Plant micro-reserves (PMRs) are another valuable tool for conserving small populations with restricted

ranges and several hundred such micro-reserves have already been set up in the EU to date (Planta Europa 2008), notably through several LIFE and LIFE+ projects.

Another noteworthy international instrument for biodiversity conservation, which was subscribed by the European countries and EU member states, is the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which regulates the international trade in endangered species and is legally binding to its parties. This legislation controls the trade of plants, seeds and other propagules for the most endangered species on Earth.

The current biodiversity crisis is one of the most important challenge of our generation and the current habitat loss and extinction spasm, due to the influence of human activity, have to be minimized through the political pressure, human and financial resources, and the application of the modern principles of conservation biology (Young 2000), such as the identification of extinction threats and their removal or reduction (IUCN 2013). A better understanding of the ways in which ecosystems function and of which of their component species play a key role would help in focusing conservation efforts and resources (Heywood and Iriondo 2003).

1.2. The processes of habitat alteration and the major threats to plant extinction

The accelerating destruction of natural habitats and consumption of natural resources by rapidly expanding human populations has caused huge impacts to ecosystems across the globe (Defeo et al. 2009). Fragmentation, habitat destruction, and environmental stresses limit or reduce the size of plant populations favouring a patchy distribution (Ellstrand and Elam 1993; Schemske et al. 1994; Thompson 1999). Predicting extinction or, conversely, ensuring the persistence of species, requires ecological and evolutionary information at more than one spatial scale (Schemske et al. 1994). Nowadays the dominant influence on biodiversity are humans and the scale of transformation, management and utilisation of ecosystems in the past two to three centuries is so massive that no part of the world can be considered as truly “undisturbed” (Heywood and Iriondo 2003). However, among the factors responsible of habitats change and destruction and of plant species decline and disappearance, not all of them are human induced threats, but could also be geological events such as landslides and avalanches. Europe has arguably the most highly fragmented landscape of all continents, and only a tiny fraction of its land surface can be considered as

wilderness (Bilz et al. 2011). In the European Red Lists (Bilz et al. 2011), the main threats to the extinction of the plant species are reviewed (Fig. 1.1).

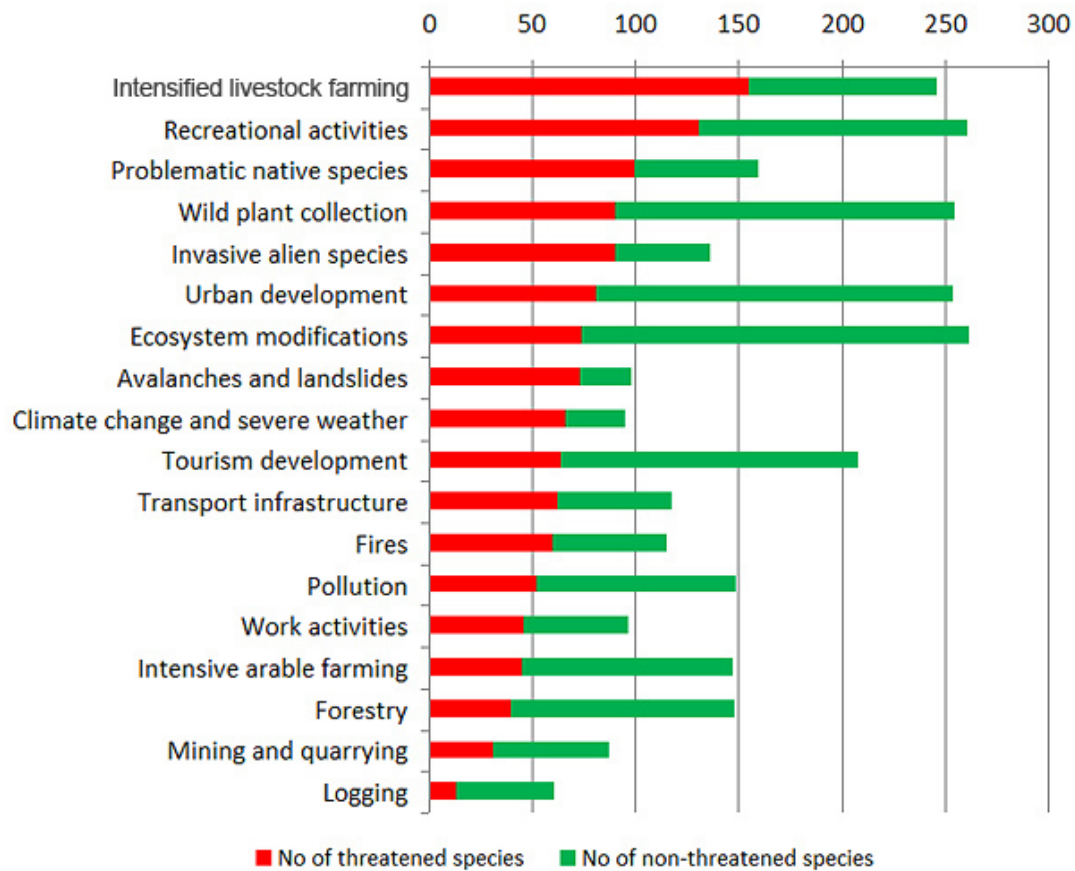


Figure 1.1. Major threats to plants in Europe (source: European Red Lists, Bilz et al. 2011).

Intensified livestock farming, and especially intensive grazing activities have the worst impacts on plant species, because these are directly affected through livestock eating them or due to trampling (Rossi et al. 2009) and increased nitrification. The conversion of grasslands into agricultural land for livestock, arable farming or forestry is a serious threat leading to habitat loss or at least degradation. Whereas an intensification of agricultural activities leads to the disappearance of those species on one hand, the complete abandonment of these activities poses also a serious threat (listed under ecosystem modifications). The abandonment leads to changes in the vegetation dynamic and succession of woody plants and shrubs that lead to an increased competition and therefore disappearance of plant species. Recreational activities are the second biggest factor causing species to be directly or indirectly threatened. Ecosystems frequented by humans often notice a decrease in quality and the plants are in danger of being trampled. Many plant species are very attractive and therefore collected for their beauty. This collection ranges

from the occasional picking to systematic collection for the horticultural trade, medicinal use, or for food. Here the threat is not only the removal of the species from the wild but the additional impact of reduced reproductive success due to over-collection.

Invasive alien species and problematic native species are particularly threatening the flora on the Canary Islands and Madeira but also in the Mediterranean. Introduced plants such as *Carpobrotus edulis* and *Opuntia ficus-indica* aggressively compete for space, light and other resources with native species in a way that often leads to the disappearance of the latter. A major driver of habitat loss is urban and tourism development as well as transport infrastructure. It is not only the fact that the plants cannot cope with a change of its habitat due to an increased use by humans, but the expansion of urban environments or the development of new tourist complexes or roads is creating impermeable, sealed surfaces and a loss of soil habitat for the species. Fires, which can occur naturally or be set by humans can have devastating effects on plant populations. Pollution comes in the form of water pollution and of garbage disposal. Water pollution is mainly caused by run-off from agricultural fields and the application of fertilisers, pesticides and herbicides which can be harmful to other plants or change the native species dynamic and increase competition. The effects of climate change are manifested in the form of increased droughts, particularly in the Mediterranean area. For several species human disturbance in form of work activities such as cleaning of beaches, mowing activities at inappropriate times, removal of forest undergrowth or roadside vegetation, etc. is causing population declines. Most plants are faced by more than one threat and a combination of these can worsen their persistence conditions. Furthermore, unsustainable human mismanagement of the environment is causing loss, degradation and/or increased fragmentation of habitats (Bilz et al. 2011). Many ecosystems around the world are currently undergoing dramatic loss or changes in species composition due to the influence of human activity (Heywood and Iriondo 2003).

The Mediterranean Basin, with its 11.8 endemic plants per 100 Km², has been recognised as one of the priority regions for conservation in Europe and it has been identified as one of the 25 most important “biodiversity hotspots” of the planet (Myers et al. 2000). Human activity in the Mediterranean region has led to large environmental changes and has further affected plant population structure, for example through degradation and fragmentation of suitable habitats (Thompson 1999; Cozzolino et al. 2003). Such changes may alter selection pressures acting on species, for example, through increased salinity in the soil, and may alter levels of gene flow among populations, for example, through increased isolation of remnant populations (Cozzolino et al. 2003). In the

Mediterranean region, sandy coastal habitats are considered among the most endangered environments (Curr et al. 2000; Lomba et al. 2008; Carboni et al. 2009; Defeo et al 2009; Prisco et al. 2013).

1.3. The coastal sand dune habitats

Coastal dune systems, which cover relatively small areas, can however host a high level of biodiversity compared with other natural ecosystems (Carranza et al. 2008) and provide a wide range of ecosystems services, many of which are essential to support human uses of sandy coasts. The most important ecosystem services include: sediment storage and transport; wave dissipation and associated buffering against extreme events; dynamic response to sea-level rise; breakdown of organic materials and pollutants; water filtration and purification; nutrient mineralisation and recycling; water storage in dune aquifers and groundwater discharge through beaches; maintenance of biodiversity and genetic resources; nursery area for juvenile fishes; nesting sites for turtles and shore birds, and rookeries for pinnipeds; prey resources for birds and terrestrial wildlife; recreational opportunities; and functional links between terrestrial and marine environments in the coastal zone (Defeo et al. 2009). Despite their narrow width, a complex sea-to-inland environmental gradient develops along the coastal sand dune habitats (Fenu et al. 2013a), making them physically dynamic and critical habitats inhabited by highly diverse and specialised biotic assemblages that are structured mainly by these physical forces (Defeo et al. 2009; Prisco et al. 2013). According to this environmental gradient, structurally and floristically different vegetation types develop, from pioneer communities on the beach to shrublands and macchias on fixed dunes (Acosta et al. 2006; Fig. 1.2).

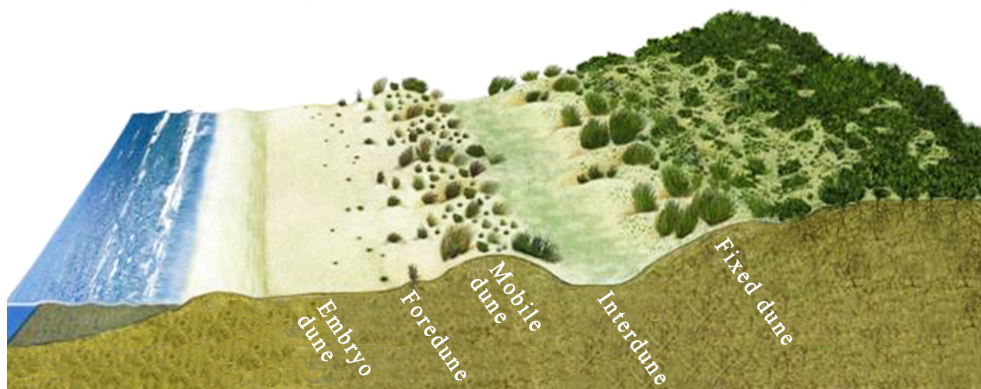


Figure 1.2. Coastal sand dune vegetation zonation (Acosta and Izzi 2007, modified).

Seventeen habitat types in annex I of the Habitats Directive describe the important environmental heterogeneity of coastal sand dune habitats in Europe (European Commission 2013).

Threats to sandy coastal habitats arise from a range of stressors that span a spectrum of impact scales from localised effects (e.g. trampling; Maun 1994; Santoro et al. 2012; Fenu et al. 2013b) to a truly global reach (e.g. sea-level rise due to global climate change; Defeo et al. 2009). In the last two centuries, they have been strongly altered by human activity (Cozzolino et al. 2003; Defeo et al. 2009; Fenu et al. 2013a), being severely fragmented or destroyed primarily as a result of urbanization and industrialization (Acosta et al. 2000). Coast-bound tourism, in particular, became a mass phenomenon after World War II and is now considered the principal cause of degradation of coastal dunes (Acosta et al. 2000). As a result, plant populations have been fragmented and affected in their structure (Thompson 1999; Cozzolino et al. 2003), with a risk of local extinctions and invasions of alien species (Carranza et al. 2010), and in places where disturbances have been particularly intense or prolonged, coenoses have often completely disappeared, being substituted by replacement communities (Acosta et al. 2006).

Extreme physical stress and disturbance factors act at very small spatial scales shaping plant community zonation along the dune profile (Acosta et al. 2006). Rain, inundation by seas, and depth of water table play a prominent role in regulating coastal halophytic communities (Aziz and Khan 1996), as even salinity (Maun 1994; Del Vecchio et al. 2012a) and availability of nutrients (Maun 1994; Lammerts et al. 1999; Escaray et al. 2010). Coastal dune plants, also called psammophytes, have developed adaptations to face with the prohibitive environmental conditions, such as the presence of trichomes to protect against desiccation, high temperatures and herbivores, or the presence of the aerenchyma tissue, an adaptation useful to reduce time or frequency of stomata opening and, consequently, water loss (Ciccarelli et al. 2009). However, in this kind of habitat, the transformation of a seed to a seedling is especially hazardous because of the tremendous microenvironmental variability mediated by wind and wave action which creates harsh conditions for seed germination, emergence of seedlings and their establishment (Maun 1994; Cogoni et al. 2012). Moreover, sand movement is a regular feature in coastal dune systems where seeds, seedlings and adult plants experience regular burial in sand to various depths (Maun 1994, 1998; Ma and Liu 2008). On the open sand dunes, maximum surface temperatures are essentially similar to those of the desert environment and may cause the death of a large number of young seedlings (Maun 1994). Sand has low field

capacity and, as a result, plants regularly encounter severe moisture stress (Maun 1994). Concerning soil moisture, Maun (1994) observed several adaptive responses favouring seedling establishment in dry environments such as a rapid vertical root elongation and an emergence when moisture is plentiful. Finally, salinity may inhibit germination even at low concentrations, and its increase induces both a reduction in the percentage of germinating seeds and a delay in the initiation of the germination process (Meot-Duros and Magné 2008).

In recent years, the concepts of coastal zone management and ecosystem conservation have received special emphasis (Taveira Pinto 2004; Ma and Liu 2008; Defeo et al. 2009; Prisco et al. 2013) and for threatened species important conservation strategies may include augmenting existing populations or reintroducing new viable populations (Bowles and Whelan 1994; Guerrant and Pavlik 1998; IUCN 1998, 2013; Lawrence and Kaye 2011; Rossi et al. 2013a).

1.4. The disadvantages of small and isolated populations

Large-scale destruction of natural habitats has reduced many species distributions to scattering of small and isolated populations, for which losses in genetic variation are likely (Menges 1991). An important notion within conservation biology is that small and isolated populations for a number of reasons, may be characterized by reduced population viability and persistence times (Shaffer 1981; Menges 1991). The disadvantages suffered by small and isolated populations involve both reduced genetic variation and greater sensitivity to nongenetic forces such as environmental and demographic stochasticity (Menges 1991; Ouborg and Van Treuren 1995; Fischer and Matthies 1998a). Individuals from small populations are more susceptible to inbreeding depression, genetic drift, and the accumulation of deleterious mutations, and are predicted to be less fit than those from large populations (Barrett and Kohn 1991; Ellstrand and Elam 1993; Fischer and Matthies 1998a). Reduced and isolated populations may also be disadvantaged because of reduced pollinator activity and effectiveness, and changes in pollen sources (Menges 1991; Jacquemyn et al. 2001). In fact, in small populations fewer pollinators can be observed compared to larger populations and both pollen quantity and quality appear to be contributing to lower seed set in small populations (Ågren 1996; Jacquemyn et al. 2001).

In population of small size (e.g. < 100), gene frequencies can undergo large fluctuations in different generations, leading to loss of alleles and loss of polymorphism at neutral loci through stochastic forces (Barrett and Kohn 1991). Inbreeding in any form (selfing, sib-mating, etc.) increases homozygosity, and its major effect is inbreeding depression, which can be defined as the relative reduction in fitness of selfed offspring compared with outcrossing offspring, in which, conversely, random mating always restores Hardy-Weinberg genotype frequencies (Barrett and Kohn 1991). The most commonly assumed basis for inbreeding depression is the presence in a gene pool of lethal or highly deleterious recessive alleles (Barrett and Kohn 1991; Ellstrand and Elam 1993). In outcrossing population, these deleterious recessive alleles are maintained by the balance between mutation and selection, and when an individual self-fertilizes (or mates with a relative), these alleles are often made homozygous, resulting in inbreeding depression (Barrett and Kohn 1991). Genetic erosion, due to the combined negative effects of isolation, inbreeding and genetic drift can only influence the probability of extinction in as far as it affects the variation in demographic events or the sensitivity of individuals to environmental variation (Ouborg and Van Treuren 1995). The clearest effects of genetic erosion on fitness might be expected in species that have historically large populations that have declined to a few individuals within a few generations (Ouborg and Van Treuren 1995). Under some circumstances, however, inbreeding will tend to purge populations of enough deleterious recessive mutations to reduce inbreeding depression and the extent of purging depends on many factors such as the size of mutational effects, the degree of dominance, interactions among loci, the breeding system, and population size (Byers and Waller 1999).

Gene flow is usually considered beneficial in conservation biology, preventing inbreeding depression and depletion of genetic variation in small populations (Ellstrand and Elam 1993). A drop in gene flow due to habitat fragmentation or loss of pollinators may lead to problems that can be solved by gene flow augmentation. Since migration is hampered by the severe isolation between small populations of endangered species, plant conservation genetic management should maintain gene flow by transplantation, by transport of seeds or spores, or by cross-pollination among populations (Ellstrand and Elam 1993). However caution must be used when augmenting gene flow, because under certain conditions it can also be detrimental for small populations reducing local variation, preventing local adaptive differentiation, and reducing fitness through outbreeding depression (Ellstrand and Elam 1993).

In the long term, populations with reduced levels of genetic variability have a reduced potential for future adaptation to change in environmental conditions, while in the short term, low levels of genetic variation may indirectly affect fitness through increased levels of inbreeding and inbreeding depression (Barrett and Kohn 1991, Ellstrand and Elam 1993). Understanding the ecological factors which regulate small populations is critical for the conservation of rare species (Quilichini and Debussche 2000).

Furthermore, plant populations isolated at the border of their distribution are particularly important from both ecological and genetic points of view and require more attention from conservation biologists (Abeli et al. 2009; Cires et al. 2011). In fact, peripheral populations are likely to have lower and more variable finite rate of increase than a population from a species core, with the result of a higher probability of extinction. On the other hand, plants from peripheral populations may have specific adaptations to complex pattern of site availability, disturbance regime, predation and competition (Volis et al. 2004). According to the European Strategy for Plant Conservation (Planta Europa 2008), these peripheral populations should be considered of high interest, with the need of urgent conservation measures.

1.5. Objectives of the study

Malcolmia littorea (L.) R. Br. is a plant species which grows on sandy coastal dune habitats of the southwestern Europe and northern Africa and is characterized by a disjoint distribution along Mediterranean and Atlantic coasts. The high level of habitat alteration, mainly due to human activities, is enhancing the fragmentation, isolation and decline of its population in several regions of its range. In particular, the peripheral European populations of *M. littorea*, located in northern Spain and central Italy are severely threatened with extinction, as reported by local and national red lists and scientific reports. Immediate and effective conservation measures have to be taken in order to avoid the extinction of the wild populations of this species from a considerable portion of its range and the European/Global conservation status should be assessed. The critical conservation status of the Italian *M. littorea* populations, and their high extinction risk, drew scientific attention and interest towards an understanding of the causes (biological/anthropogenic) of its conservation status. Until now, rather few experimental studies have focused on this threatened species (Giovi 2004; Gratani et al. 2011; Del Vecchio et al. 2012b; Novoa et al.

2012; Mira et al. 2015) and none of them covered population genetics and comparative seed ecology across European populations, while only a few studies dealt with reintroduction and revegetation techniques (Escaray et al. 2010; De Vitis 2011; De Vitis et al. 2013). However, these studies have been useful to understand interesting traits of the target species and to support some findings of the present research.

Several tools may be used to investigate the biological and ecological traits of an endangered species and to produce useful data in support of its *in situ* conservation and management. In particular, experimental comparisons of populations of varying size may help elucidate the relationships between population size and both the amount of genetic variation within populations (Barrett and Kohn 1991; Fisher and Matthies 1998a; Gijbels et al. 2015) and plant fitness (Menges 1991; Jacquemyn et al. 2001). Genetic information may be useful for establishing conservation priorities when only a subset of the current populations can be protected (Schemske et al. 1994). Estimating and monitoring levels of genetic variation in populations of concern could provide information regarding the genetic structure of the populations and should prove helpful for a species management (Ellstrand and Elam 1993). Moreover, consideration of the ecological aspects of reproduction by seed is essential to an understanding of germination requirements of a target species and may help its conservation (Cochrane et al. 2002). Laboratory research is a valid study contributing vital information on the prediction of these germination requirements (Baskin and Baskin 1988).

Furthermore, an understanding of species specific attributes, such as *in situ* seed longevity and the propensity to form a persistent seed bank (Thompson et al. 1993), and requirements, such as cultivation and reintroduction needs, is the key to many aspects of practical management for the effective *in situ* conservation.

In the light of these considerations, the main aims of the present study were: (1) to provide useful information about biological and ecological traits of *M. littorea*, in order to address specific management actions; and (2) to ensure effective *in situ* and *ex situ* conservation of the species, in particular for those populations or genotypes that are threatened in terms of genetic erosion and reduced fitness.

The main objectives were to investigate the genetic variability of a set of European *M. littorea* populations to identify the priority units for the conservation of the species in Europe; to investigate the reproductive biology of the species both under field and laboratory conditions; and to contribute to the development of a reintroduction protocol. The specific objectives of the present study were (1) to identify, through the cross-genus

amplification, a set of nuclear and chloroplast microsatellite markers to evaluate the genetic diversity of the study species; (2) to quantify the genetic variation within and among a set of European populations of *M. littorea*; (3) to infer the pattern of differentiation among these populations by assessing their genetic structure; (4) to identify the basic requirements for the species seed germination (storage, light, temperature, seed burial); (5) to investigate the differences among the seed germination requirements of a set of European populations; (6) to investigate the propensity of the species to form a soil seed bank; (7) to assess if the population size affects the genetic variability and the reproductive fitness of *M. littorea* populations; and (8) to evaluate the species' response to different reintroduction methods.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. THE STUDY SPECIES: *MALCOLMIA LITTOREA*

The genus *Malcolmia* R. Br. is a member of the family Brassicaceae and in Europe is represented by 13 taxa (source: Euro+Med PlantBase, <http://ww2.bgbm.org/EuroPlusMed>), most of which inhabit coastal habitats. *Malcolmia ramosissima* (Desf.) Thell is a diagnostic species for the EU Habitat 2230 “*Malcolmietalia* dune grasslands”, an association with many small annuals and often abundant ephemeral spring bloom of deep sands in dry interdunal depressions of the coasts (European Commission 2013).

Malcolmia littorea is a suffruticose chamaephyte, short to medium perennial herb, densely white-pubescent or -tomentose with stellate hairs. Stems numerous, prostrate or ascending, woody at base (Fig. 2.1a). Leaves 2-4 x 10-20 mm, elliptical and entire to sinuate-dentate, mostly stalkless. Flowers hermaphrodite in racemes: sepals 9 mm, erect and grey; petals 14-22 mm, purple; median nectaries absent; style absent or indistinct; stigma 2-6 mm, deeply 2-lobed or retuse, the lobes erect and connate; siliqua 30-65 x 1-1.5 mm, not torulose, the apex attenuate (Fig. 2.1b); $2n = 20$ (Pignatti 1982; Greuter et al. 1986).

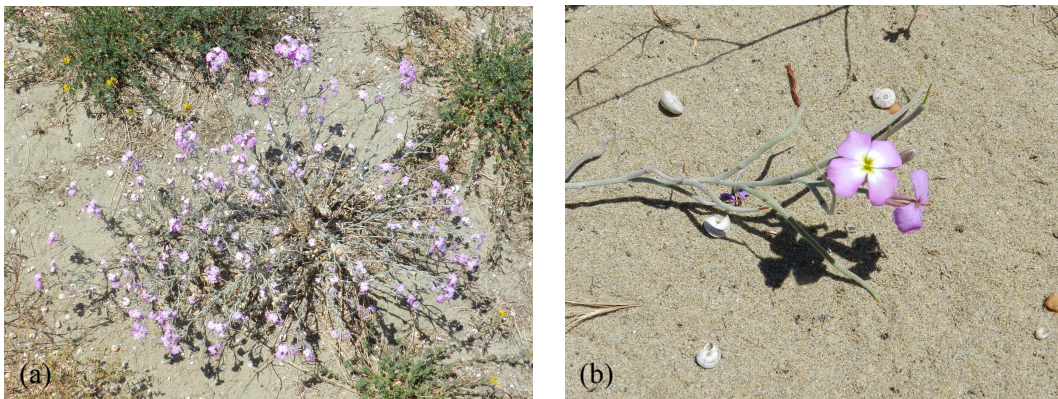


Figure 2.1. (a) A flowering individual of *M. littorea*, and (b) a reproductive stem with flowers and fruits (San Felice Circeo, Italy; photo: M. De Vitis).

Seeds in one row in each loculus, as most of the Brassicaceae, have a spatulate jackknife-bent embryo without endosperm. The embryo is also classified as foliate. That means it is large, generally central rather than peripheral, with accumbent expanded cotyledons (Martin 1946; Fig. 2.2).

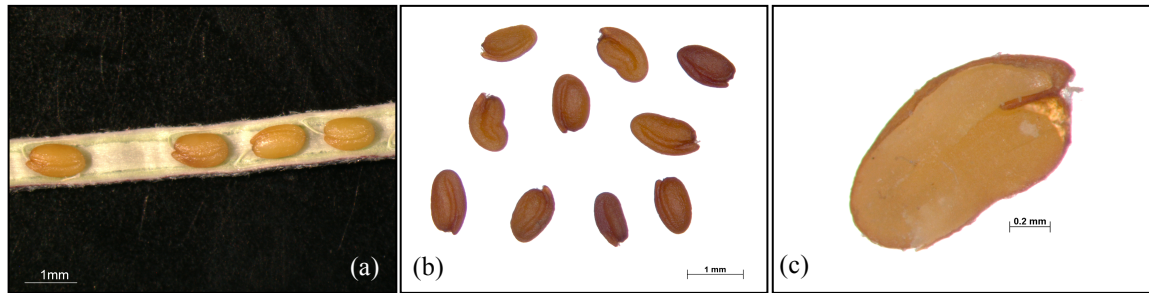


Figure 2.2. (a, b) Seeds of *Malcolmia littorea* and (c) the detail of a longitudinal section (photo: M. De Vitis).

2.1.1. Reproductive biology

In the months following seed germination, the seedling develops into a rosette shape which can reach a diameter of 5 cm (Fig. 2.3a). With the arrival of the reproductive season, each shoot shifts to an erect habit and becomes a reproductive apex (Fig. 2.3b). The reproductive apex appears stretched, thin and during the reproductive season it loses all the leaves except for the distal end.

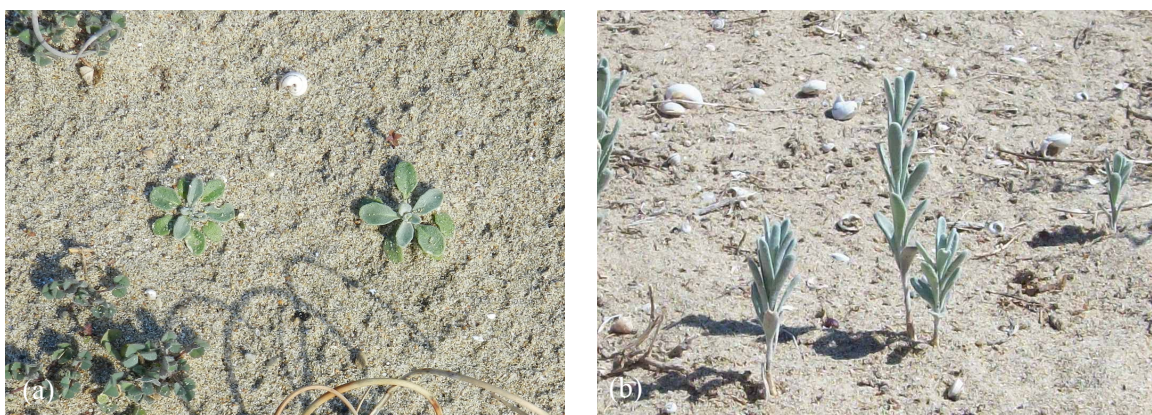


Figure 2.3. (a) Winter and (b) spring habit of seedlings (San Felice Circeo, Italy; photo: M. De Vitis).

After the reproduction, the apex loses the apical dominance, decays and in the same time all the axillary shoots in the first cm of the stem are activated (Fig. 2.4a). Some of

these shoots (3-5) will become the vegetative apexes, developing a rosette habit, and then the described scheme is repeated at each reproductive season for each new vegetative shoot. After several years, the plant takes the shape of a hemispherical shrub (Fig. 2.4b), which occupies less than 1 m diameter and 50-60 cm in height with ca. 50 branches. The adult plant is woody at the base with numerous non-flowering stems and a root system poorly branched, but very deep (even more than 1 m) and perfectly adapted to the drought conditions (Giovi 2004). The propagation by cuttings has been experimented, but without positive results, while the vegetative reproduction has never been observed (Giovi 2004).

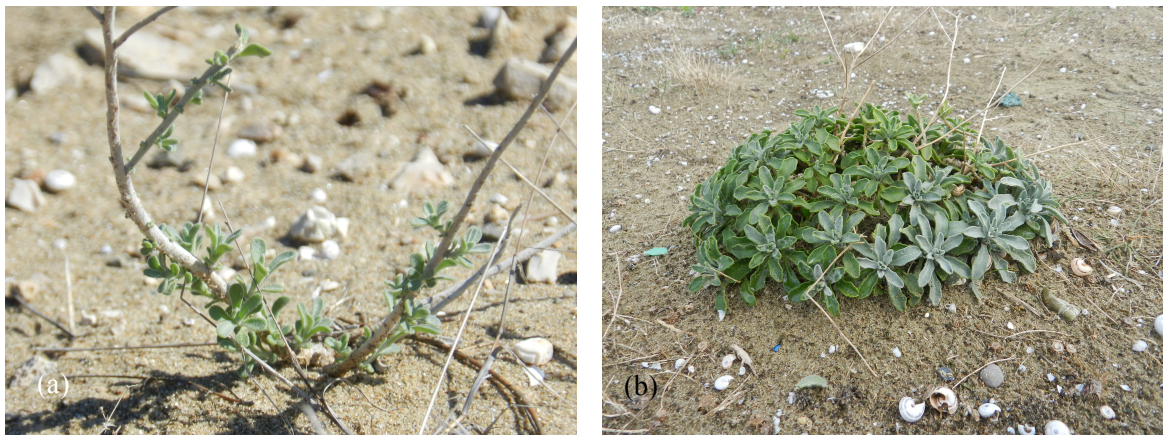


Figure 2.4. (a) Axillary shoots activated after the reproductive season (autumn) and (b) hemispheric shape of an adult plants of *M. littorea* during winter (San Felice Circeo, Italy; photo: M. De Vitis).

2.1.2. Ecology

M. littorea grows in sandy coastal habitats, in particular it takes part to the consolidation of the transition and fixed dune. It prefers low and sparse vegetation, suffering when surrounded by tall bushes and herbs (Giovi 2004). In bioclimatic terms, it lives in the Mediterranean bioclimate, except the populations in the northern Spain, which is considered to be a temperate bioclimate (Rivas-Martínez et al. 2011). The species is related to different phytosociological syntaxa, depending on the different phytogeographical province (*sensu* Rivas-Martínez et al. 2001).

The analysis of climatic parameters, conducted by Del Vecchio et al. (2012b) suggested that *M. littorea* distribution in Europe is driven by the minimum winter temperature and the total rain in summer, making it sensitive to hard winters and summer drought. The species is also characterized by a low tolerance to high and low air temperatures: high net photosynthesis and respiration decreases during drought period and

during winter are indicative of its low competitive ability compared to the other species that lives on sand dunes (Gratani et al. 2011).

The pollination is entomophilous with several insects observed to be responsible for the pollination such as beetles (Fig. 2.5a), butterflies, such as *Colias croceus* (Fig. 2.5b), and *Apis* spp. (Fig. 2.5c). Each adult can produce ca. 13 fruits per shoot and ca. 50 seeds per fruit. The splitting of the fruit, when it ripens, is not explosive and the seeds may be dispersed by the wind (anemochory) or mainly by barochory, but with a low colonization potential. *Malcolmia littorea* also acts as host plant for several insects, such as the beetle *Psylliodes maroccanus* (Bensusan and Pérez 2006) and *Eurydema* spp. (Fig. 2.5d). Furthermore, for the south-western Spain, seed dispersal by endozoochory, specifically, by European rabbit, *Oryctolagus cuniculus*, has been reported. The plant is eaten by the mammalian herbivore and the seeds can germinate from the faecal pellets or can enter the seed bank, as they are resistant to the passage through the digestive system of the rabbit (Dellafiore et al. 2007, 2010). The rabbit can promote the increased coverage of the species dispersed within the same sandbar, and enable the colonisation of other distant sandbars (Dellafiore et al. 2007, 2010), playing an important role in the plant population dynamics.



Figure 2.5. (a) Pollination by an unidentified beetle (probably *Epicauta* spp.), (b) the butterfly *Colias croceus* and (c) a bee (*Apis* spp.) (San Felice Circeo, Italy) and (d) breeding of beetles at Plage des Aresquiers (Frontignan, France) on *Malcolmia littorea* (photo: M. De Vitis).

2.1.3. Distribution

M. littorea is a stenomediterranean species, distributed along the coasts of South-West Europe (Italy, France, Portugal and Spain) and North-West Africa (Algeria and Morocco) (Pignatti 1982; Greuter et al. 1986). In Europe its distribution appears to be disjointed along the Mediterranean coasts of Italy, France and Spain, occurring in a single region of the central Italy (Latium), then in the southern coast of France (regions of Provence-Alpes-Côte d’Azur and Languedoc-Roussillon), with a gap between the most western population in France and the eastern one in Spain (in the province of Castellón). Then, it is more abundant and its distribution is more uniform from the southern Spanish province of Almeria to the northern Spanish province of Corunna, across the southern and western coasts of Portugal, becoming again discontinuous on the northern coast of Spain (Fig. 2.6).

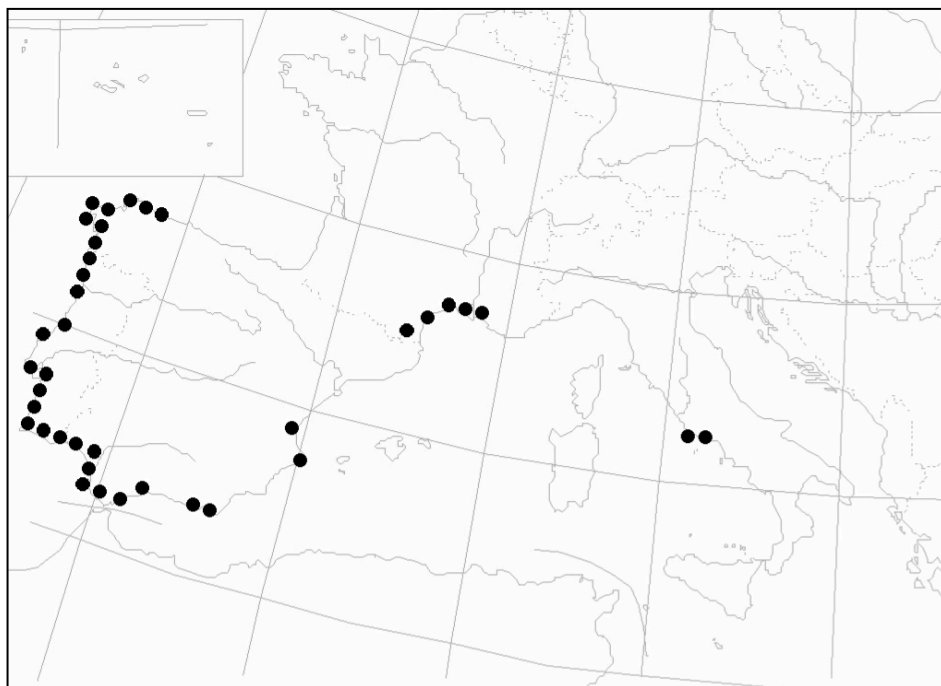


Figure 2.6. European distribution of *Malcolmia littorea* (Atlas Florae Europaeae, Jalas and Suominen 1972–1996).

In Portugal, *M. littorea* occurs in all the coastal provinces: Algarve, Baixo Alentejo, Beira Litoral, Douro Litoral, Estremadura and Minho (Castroviejo et al. 1993).

In Spain, the species occurs in the autonomous communities of Andalusia (provinces of Almeria, Cádiz, Granada, Huelva, Malaga), Asturias, Basque Country, Cantabria, Galicia (provinces of Corunna, Lugo and Pontevedra; source: www.asturnatura.com) and Valencia (provinces of Alicante, Castellón, and Valencia; Castroviejo et al. 1993; Aizpuru

et al. 2010). The plant distribution appears disjointed, since the species is absent in the autonomous communities of Catalonia (on the border with the French department of the Eastern Pyrenees) and Murcia (between Valencia and Andalusia), and being represented by very few populations along the northern coast (Asturias, Cantabria, Basque Country; Cadiñanos Aguirre et al. 2006; Aizpuru et al. 2010). The flowering period is reported from February to June in the western Andalusia by Valdes et al. (1987), from February to December in all the Iberian Peninsula by Castroviejo et al. (1993), and from April to July in the Alicante province by Laliga (2007).

In France, it occurs in the southern regions of Provence-Alpes-Côte d'Azur (department Bouches du Rhône), Languedoc-Roussillon (departments Gard, Hérault, Aude, Eastern Pyrenees), and Aquitaine (department Pyrénées-Atlantiques; source: www.tela-botanica.org), where it flowers from May to September (source: www.tela-botanica.org).

In Italy, the species occurs in only three small populations standing in a limited portion of the Latium coast, between Sabaudia and Terracina (Latina). The overall occupied area is ca. 2 ha (Giovi et al. 2012). The minimum distance between two populations is 4 km while the maximum is 13 km. Pignatti (1982) reported the species occurrence in Latium (as rare, from Tor Vaianica to Terracina), Sicily, Sardinia and Liguria. Its occurrence in the other regions outside Latium has never been confirmed. In the checklist of the Italian vascular Flora (Conti et al. 2005) it was also recorded as “no longer recorded” for Sicily. Mulder (1993), studying the coastal landscapes and the vegetation types in Latium, and using the abundance-cover scale of van der Maarel (1 minimum/ 9 maximum; 1979), recorded *M. littorea* in the herbaceous layer of Tor Vaianica (2), Rio Torto (2), Lido di Capo Portiere (7) and Litorale Sabaudia (2), and in the shrub layer of Tor Vaianica (2) and Caprolace (5). Although the Italian populations were located near the Circeo National Park, for many years they were little known or unknown. From the biogeographic point of view, the Italian distribution of the species seems to be limited by geographic obstacles represented by the Circeo Promontory to the North and the rocky coasts to the South (Giovi 2004). It flowers from April to July (sometimes the flowering lasts up to September).

Del Vecchio et al. (2012b) conducted a DCA on 139 phytosociological relevés carried out in all European countries where *M. littorea* grows, and the results showed that the species is related to different phytosociological syntaxa, depending on the different phytosociological province (*sensu* Rivas-Martínez et al. 2001), but it is more frequent on

the Atlantic side of its distribution range (Portuguese and Spanish relevés), even if in all the cases it grows in a similar habitat along the coastal zonation, preferring more sheltered coastal environments (transition dune and fixed dunes) with respect to those of the foredune and mobile dunes.

In Morocco, *M. littorea* is reported as common on littorals from Tanger to Safi (Lewalle and Montfort 1997), while in Algeria occurs in the provinces of Mostaganem and Oran (Battandier and Trabut 1884). *M. littorea* var. *multicaulis* (Pomel) Maire is reported for Mostaganem and Oran (Algeria); var. *sinuata* Rouy & Foucaud for the Moroccan regions of Tangier-Tetouan, Gharb-Chrarda-Béni Hssen and Grand Casablanca; var. *spathulata* Camus for the Moroccan region Tangier-Tetouan (Maire 1952). The varieties *spathulata* and *multicaulis* were reported as endemic to North Africa (Maire 1952). However, the existence of all these varieties seems to have been rejected (source: African Plant Database, Conservatoire et Jardin Botaniques Ville de Genève, <http://www.ville-ge.ch/musinfo/bd/cjb>). The flowering in North Africa occurs from March to July (Maire 1952; Lewalle and Montfort 1997), even if Battandier and Trabut (1884) reported it only from May to June in Algeria.

2.1.4. Conservation status

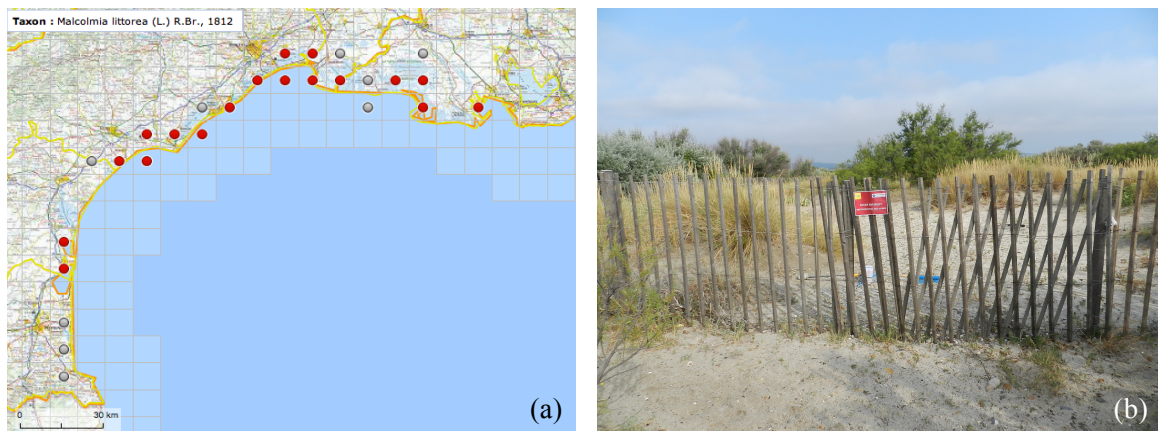
Malcolmia lacera subsp. *gracillima* (Samp.) Franco is the only *Malcolmia* spp. included in the European Red List and categorized as data deficient (DD; Bilz et al. 2011), while the conservation status of *M. littorea* (L.) R. Br., although is a declining species in several regions of its European distribution, has not been evaluated yet. Its conservation status seriously needs to be assessed across Europe, or better across its entire distribution.

For Portugal and North Africa, no lists of threatened plant species were available.

The species is not included in the Red List of Spanish vascular flora (Moreno 2008). However, it is included in the “Catalogo Regional de Flora Amenazadas de Asturias” as endangered due to the occurrence of only three small and fragmented populations on the western border of the autonomous community and to the high alteration of its habitat by human activities (sources: www.asturnatura.com, www.asturias.es, Gobierno del Principado de Asturias; Díaz Gonzáles et al. 2005), and it is reported as *vulnerable* (IUCN category: VU) for the province of Alicante, where it is threatened by the urbanization, which is causing the fragmentation of the sand dune habitat, and by alien plants (Laliga

2007). In the Basque Country, it is considered of high naturalistic interest even if it occurs only in two populations, both standing inside protected areas (Aizpuru et al. 2010; Fernández Prieto et al. s.a.), and is included in the Red List as DD (Data Deficient; Aizpuru et al. 2010). Here and in Cantabria a colonization of the species has started since very few individuals have been recorded for the first time in 2002-2003 (Cadiñanos Aguirre et al. 2006; Aizpuru et al. 2010). In the western Andalusia, it is reported as very frequent (Valdes et al. 1987). In the province of Valencia, an important restoration project has been carried out on the sand dune system of the Devesa de l'Albufera (Olmos 2004; Escaray et al. 2010), where the species occurs.

In France, the abundance of the species declined of 30% in the last 25 years (www.silene.eu; Figure 2.7a). However, the species is not reported in the *Livre Rouge de la flore menacée de France* (Olivier et al. 1995). Along the Mediterranean French coast, several conservation actions have been applied, such as sand dune habitat protection and restoration, and good examples of these actions occur at the Plage des Aresquiers (Frontignan, Hérault; Fig. 2.7b) and at the Plage du Grand Travers (La Grande-Motte, Hérault).



Figures 2.7. (a) Record of observations of the study species in the French department: red dots denote current observations while grey dots stay for observations prior to 1990 (www.silene.eu). (b) A fence protecting the sand dune vegetation in the Plage des Aresquiers (Frontignan, France), with a signboard where it is written “Conservatoire du littoral, Accès interdit, Restauration des dunes” (photo: M. De Vitis).

In Italy, it is considered *critically endangered* (IUCN category: CR) according to the Italian Red List (Rossi et al. 2013b). The IUCN criteria B2ab(ii,iii,v) + C2a(ii) were applied, which summarize a small area of occupancy (AOO), the occurrence of the species in only one location, a decline of the occupied area and of the habitat quality, a small and declining number of reproductive individuals, and all the reproductive individuals living in

the same population (Giovi et al. 2012). The Italian conservation status of the species was probably evaluated before the finding of other two small (ca. 170 and 130 reproductive individuals) populations in 2013, and in 2014 a total of 580 reproductive individuals was counted in Latium. The immediate and high extinction risk of these populations is mainly due to the huge stress that its habitat experiences, especially during summer. The Latium sandy coasts are affected by tourism and trampling, and also by habitat destruction for urbanization (Acosta et al. 2000, 2006; Carranza et al. 2008). The disappearance of the suitable area previously occupied by the species is shown in ortophotos, available from the Italian Environmental Ministry: while in 1994 a well developed coastal dune vegetation can be observed, in 2006 part of the area was levelled out with the removal of the vegetation, and in 2008 was substituted by human settlements (Del Vecchio et al. 2012b). The Italian populations probably do not exchange DNA with the other Mediterranean populations, due to the geographic isolation (Giovi 2004). Until today, no *in situ* conservation measures have been taken for the species in the Italian territory, except for an experimental reintroduction carried out in 2009-2011 (De Vitis 2011). As part of the present study, two other reintroduction trials were performed in collaboration with National Authorities for Nature Conservation (see paragraphs 2.3., 5.2.2. and 5.2.3.).

2.2. POPULATION SELECTION AND SAMPLING

In 2011 in Italy, one native, one *ex situ* cultivated and one reintroduced *M. littorea* populations were sampled for seeds harvesting, in order to carry out experiments on the basic germination requirements. Details about sampling and storage method for these seeds are reported in paragraph 4.2.1.

In 2013, a total of 15 wild populations of *M. littorea* were selected and sampled for fresh leaves and ripe fruits across Europe. These populations were selected on the basis of the information available from local experts about the distribution of *M. littorea* populations in each European country and according to the economic resources available for the field trips. In Portugal, Prof. Carlos Silva Neto (University of Lisbon) kindly sampled two populations, one northern and one southern; in Spain, it was possible to visit only the autonomous community of Valencia, where five populations were selected on the basis of their size, trying to sample populations of different sizes. In France, five populations were sampled using the same criterion, and most of the French distribution was covered. Finally in Italy, all the three existing populations were sampled.

Details of each population sampled, such as the specific locality of sampling and the geographic coordinates of the population centre, are reported in Table 2.1, while their georeferenced positions are illustrated in Figure 2.8.

For each population, the main threats to their habitat, or directly to their persistence, were identified and classified following the *List of Threats and Pressures* of the European Environment Agency (EEA 2011). The same tool is also used for threats classification in Habitats Directive and is similar to the IUCN *Unified Classification of Threats and Actions* (Salafsky et al. 2008; IUCN 2012). The main factors threatening the sampled populations were found in the following EEA categories: agriculture (A), mining and extraction of materials and energy production (C), transportation and service corridors (D), urbanisation, residential and commercial development (E), human intrusions and disturbances (G), invasive or other problematic species and genes (I) and natural system modifications (J). For an appropriate interpretation of categories and subcategories see Appendix 1. All these populations occur under the Mediterranean bioclimate (*sensu* Rivas-Martínez et al. 2011; Fig. 2.9).

Table 2.1. Locality, code, coordinates (expressed in decimal degrees), and altitude of *Malcolmia littorea* populations sampled in the present study.

Country	Locality (Province)	Code	Lat.	Long.	Altitude (m a.s.l)
Portugal	Vila do Conde (Oporto)	PT-1	41.34	-8.75	3
Portugal	Praia dos Salgados (Albufeira)	PT-2	37.09	-8.34	3
Spain	Playa de Moncófar (Castellón)	ES-1	39.78	-0.15	1
Spain	Puerto de Sagunto (Valencia)	ES-2	39.66	-0.21	0
Spain	Playa de Tavernes de Valldigna (Valencia)	ES-3	39.08	-0.21	1
Spain	Albufera Natural Park (Valencia)	ES-4	39.36	-0.32	2
Spain	Oliva (Valencia)	ES-5	38.89	-0.04	4
France	Fos-sur-mer	FR-1	43.43	4.86	2
France	Beauduc (Camargue Regional Nature Park, Arles)	FR-2	43.38	4.59	0
France	Portiragnes	FR-3	43.28	3.36	4
France	Plage des Aresquiers (Frontignan)	FR-4	43.46	3.82	0
France	Plage du Grand Travers (La Grande-Motte)	FR-5	43.56	4.03	2
Italy	Torre Olevola (San Felice Circeo, Latina)	IT-1	41.25	13.12	3
Italy	Terracina (Latina)	IT-2	41.27	13.16	4
Italy	Sabaudia (Latina)	IT-3	41.30	13.01	13

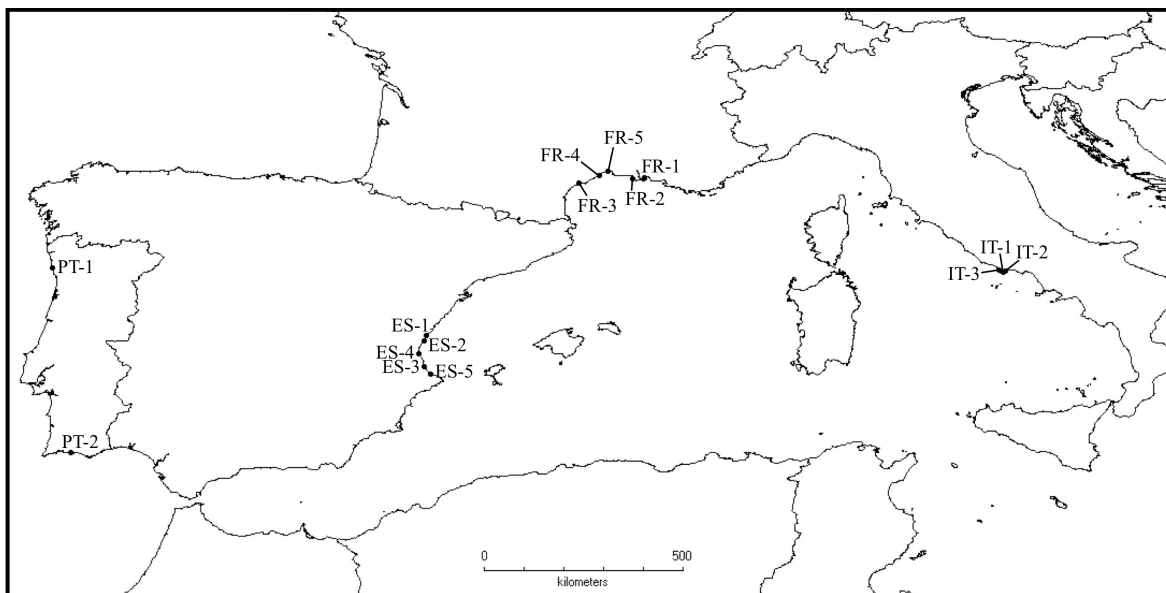


Figure 2.8. The 15 European populations of *Malcolmia littorea*, sampled in this study and georeferenced with a Diva-GIS software.

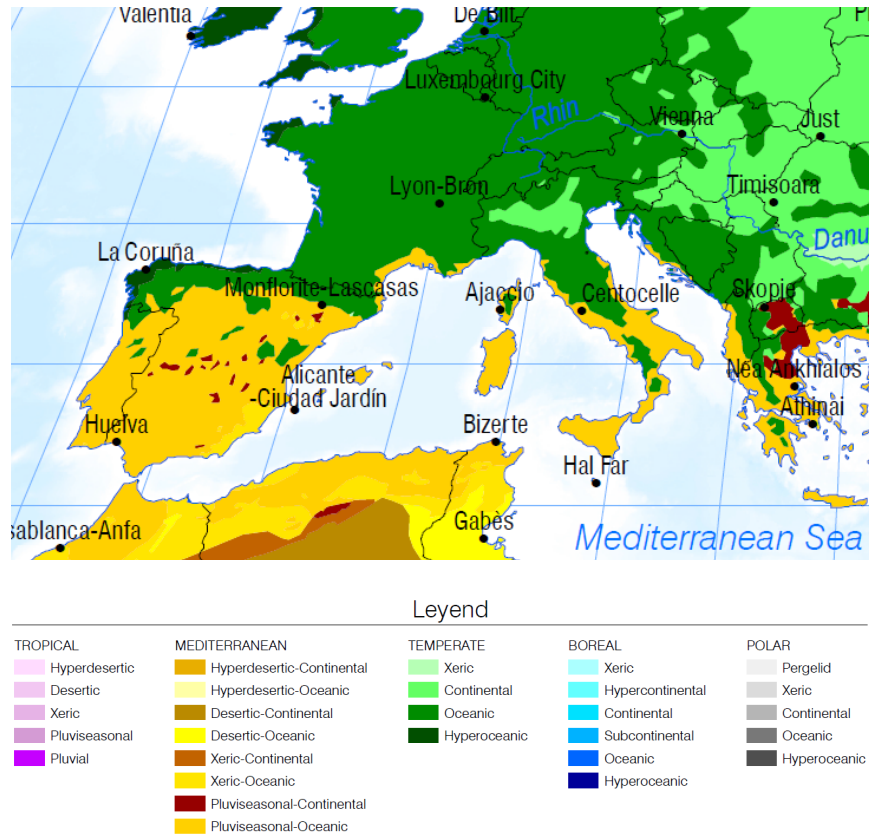


Figure 2.9. World Bioclimates map (Rivas-Martínez et al. 2011).

Since it was not possible to estimate the population sizes by counts for all the populations, the area occupied by each population was taken as a measure of its size. In particular, the extremes of each populations were mapped through a GPS and then inserted in Google Earth Pro, where the areas of the polygons containing the individual population were calculated (Fig. 2.10).



Fig 2.10. The polygon containing the area occupied by the *M. littorea* population at Puerto de Sagunto (Valencia, Spain).

Fresh leaves and ripe fruits were sampled to carry out population genetics and seed ecology investigations, respectively. Details on sampling methods were reported in specific paragraphs (3.2, 4.2.1 and 4.2.2).

2.2.1. Characteristics of European sites

The calculated areas occupied by each population, the threats affecting the populations according to the *List of Threats and Pressures* of the European Environment Agency (EEA 2011; Appendix 1), and the accession codes of the seed samples stored at the Tuscia Germplasm Bank are reported in Table 2.2.

Table 2.2. Occupied area (ha), disturbances and accession code in the Tuscia Germplasm Bank (BGT) for each *M. littorea* population considered in the present study. The disturbances threatening the populations are categorized according to the *List of Threats and Pressures* of the EEA (2011). For the interpretation of the subcategories see Appendix 1. A + was given to those populations for which some conservation measure is in act.

Population	Area (ha)	Disturbance/s	BGT code
PT-1	~7.50	I01, E01, G01	A 270 13
PT-2	~62.06	+	A 271 13
ES-1	~0.08	E01, +	A 265 13
ES-2	~2.54	D03.01.04, E02, G05.01, G05.05	A 266 13
ES-3	~15.49	I01, E01, G01, G05.05,	A 267 13
ES-4	~171.12	G01, +	A 268 13
ES-5	~9.51	E01, G01, G05.05, I01,	A 269 13
FR-1	~1.09	C02, D01	A 260 13
FR-2	~180.00	+	A 261 13
FR-3	~8.57	E01, G01, G02.08, G05.05	A 262 13
FR-4	~2.07	G01, +	A 263 13
FR-5	~13.34	D01, G01 +	A 264 13
IT-1	~0.02	E01, E03.01, G01, J03	A 258 13
IT-2	~0.30	A01, E01, J03	
IT-3	~0.01	D01, J03	A 259 13

2.3. SITE SELECTION FOR *IN SITU* TRIALS

During the last three years, a seed longevity experiment and two reintroduction/translocation trials were carried out in order to understand if the species is able to form a soil seed bank and to test different reintroduction methods to develop an effective reintroduction strategy.

The seed longevity experiment was carried out at the IT-1 (Table 2.1) population site. This site was chosen for the higher accessibility, compared to the other two Italian populations.

Fiedler and Laven (1996) defined four classes of site-selection criteria for reintroduction projects: physical, that include both large-scale geomorphic properties and finer-scale factors, such as a specific soil type; biological, that include both autoecological and synecological factors; logistical criteria such as access by researchers and land-use management plans; and the historical ones that include whether the taxon is known to have occupied the site in the past, but also look to the long-term future prospects of a site providing suitable habitat. On the basis of these criteria, two suitable sites for *M. littorea* experimental reintroduction/translocation were identified in 2012: the Site of Community Importance (SCI IT6030048; according to the EU Directive 92/43/EEC) *Litorale di Torre Astura* (Nettuno, Rome, Italy; Figure 2.11), and the coastal site at *Rio Martino* (Latina, Italy; Figure 2.11), which was under the Circeo National Park Authority, and managed by the National Forest Service.

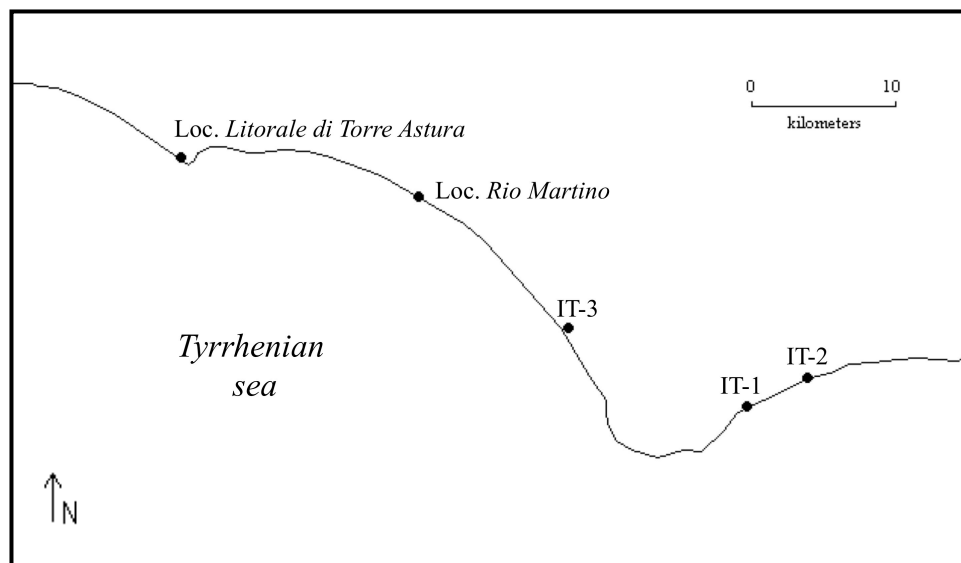


Figure 2.11. The portion of Italian coast (Lazio region) where the native (IT-1, IT-2, IT-3) and reintroduced population sites of *Malcolmia littorea* occur. The sites were georeferenced with Diva-GIS software.

2.3.1. *Environmental Impact Assessment (EIA) procedure*

In order to conduct the reintroduction trials in the above mentioned sites, individual formal proposals were submitted to the *Biodiversity and Geodiversity Section* of the *Regional Park Agency* (ARP, *Regione Lazio*) and to the Circeo National Park Authority, respectively. Then, a feasibility study was prepared for each action and it was required to the *Parks and Natural Reserves Section* of the *Regional Direction of Infrastructures, Environment and Housing policies* (*Regione Lazio*) to conduct the *Environmental Impact Assessment* (EIA) procedure (introduced in Europe in 1985 by the EU Directive 85/337/EEC). A feasibility study includes a balance of the conservation benefits against the costs and risks of both the specific action and alternative conservation actions, covering the full range of relevant biological and non-biological factors (IUCN 2013).

Both *in situ* conservation proposal were approved by the *Parks and Natural Reserves Section* of the *Regional Direction of Infrastructures, Environment and Housing policies* (*Regione Lazio*; documentation about the procedure is enclosed in Appendix 2). In particular, for the action proposal at the SCI *Litorale di Torre Astura*, the response arrived on October 2013, and in the same month that reintroduction trial started; while for the action proposal at Rio Martino the EIA procedure response arrived in May 2014, and that reintroduction trial started in the following autumn, on October 2014.

CHAPTER 3

POPULATION GENETICS STUDIES AS A TOOL FOR IDENTIFICATION OF CONSERVATION PRIORITIES

3.1. INTRODUCTION

One of the most important unresolved issues in conservation biology is the management of fragmented wild populations of threatened species (Frankham 2010). Thus, understanding the extent and distribution of diversity is critical to designing effective and appropriate conservation strategies (Scheldeman and van Zonneveld 2010). Several complementary approaches can be used to investigate the genetic diversity of the species and make recommendations for its conservation. One way consists in investigating the genetic variation within the study species using molecular markers, which can clarify the impact of evolutionary forces such as drift and migration, and provide, with adequate experimental design, basic information on the biology of the species (Muller et al. 2009).

Microsatellite markers, or simple sequence repeats (SSR), are popular and high efficiency tools that have been used for studies in population genetics, phylogeography and systematics (Bell and Ecker 1994; Provan 2000; Plieske and Struss 2001; Clauss et al. 2002; Suwabe et al. 2002; Cozzolino et al. 2003; Provan and Campanella 2003; Dobeš et al. 2004; Ceplitis et al. 2005; Selkoe and Toonen 2006; Barbará et al. 2007; Edh et al. 2007; Muller et al. 2009; Skrede et al. 2009; Liu et al. 2013; Mattioni et al. 2013; Lusini et al. 2014) due to their reproducibility and polymorphism (Russell et al. 1997; Provan et al. 2001; Arroyo-García et al. 2002). They are sequences of repetitive DNA where a single motif consisting of one to six base pairs is repeated tandemly a number of times (Navascués and Emerson 2005; Selkoe and Toonen 2006). The DNA surrounding a microsatellite locus is termed the flanking region, which is generally conserved across

individuals of the same species and sometimes of different species (Selkoe and Toonen 2006). Microsatellite sequences have been identified in both nuclear and chloroplast genomes (Powell et al. 1995) and are mostly derived from noncoding regions, so they are likely to evolve neutrally (Edh et al. 2007).

The combination of chloroplast and nuclear microsatellite markers is becoming a common approach to study genetic variation in plant species (Dobeš et al. 2004; Edh et al. 2007; Muller et al. 2009; Liu et al. 2013), because it provides a more comprehensive method to study population structure and information useful to implementation of a conservation strategy and restoration of populations based on recent and ancient evolutionary processes (Crandall et al. 2000; Petit et al. 2003, 2005). While nuclear microsatellite loci are usually highly polymorphic, codominant and inherited in a Mendelian mode, chloroplast microsatellites are uniparentally inherited and linked because of the nonrecombinant nature of the chloroplast chromosome (Navascués and Emerson 2005). The genotyping of chloroplast microsatellites will result in haplotypes that will be composed of the combination of alleles found at each chloroplast microsatellite locus (Navascués and Emerson 2005). Furthermore, chloroplast microsatellites show levels of polymorphism quite variable across loci and across species, and some loci have been found to be monomorphic in all the species studied (Navascués and Emerson 2005). Their maternal transmission in the majority of angiosperms coupled with the smaller effective population size of the haploid genome means that chloroplast-specific markers should be more geographically structured and thus represent good indicators of historical gene flow (Provan and Campanella 2003). On the other hand, the relatively low mutation rates associated with organelle genomes means that it has often been difficult to detect sufficient levels of variation below the species level in studies employing organelle-specific markers (Provan et al. 2001; Provan and Campanella 2003).

Unfortunately, it is expensive and time consuming to develop microsatellite markers for each study organism (Clauss et al. 2002), thus, it is desirable if they can be transferred across species and genera (Skrede et al. 2009). Weising and Gardner (1999) designed a set of 10 microsatellite primers, based on *Nicotiana tabacum* chloroplast genome, and found a high cross-genus amplification (almost 100%) within the Solanaceae family. Testing the same primers on other families species, they found that 8 of the 10 primer pairs generated products for the majority of tested species from other dicotyledonous angiosperms families (Actidiniaceae, Brassicaceae, Fabaceae, Myrtaceae, Rosaceae), while amplification of monocotyledons was somewhat less efficient (Weising and Gardner 1999). Barbará et al.

(2007) reviewed 64 microsatellite primer notes and found a transfer success in eudicot plants of 60% within genera and 10% between genera within families. Even if the microsatellite transferability into distantly related species seems to be limited (Plieske and Struss 2001), their distribution among different species of the Brassicaceae family showed a high variation potential making them valuable resources for ecological and evolutionary studies (Clauss et al. 2002; Bon et al. 2005; Burrell and Pepper 2006). Clauss et al. (2002) tested 42 microsatellite primers for amplification in different *Arabidopsis* species and found that amplification was successful for all primers in *A. thaliana* and *A. petraea*, for 98% of primers in *A. halleri* and *A. lyrata* and 79% of primers in the more distantly related *Arabis drummondii*. Suwabe et al. (2002) found a high cross-species transferability (almost 100%) of 36 microsatellite primers in *Brassica* spp. while 75% of them amplified the loci within *Raphanus sativus* and *Sinapis alba* and 50% of them amplified in *A. thaliana*. Bon et al. (2005) found a microsatellite transferability of ca. 70% between closely related *Lepidium* spp. Burrell and Pepper (2006) tested 10 microsatellite primers on a collection of 21 specific and subspecific taxa belonging to the genera *Caulanthus*, *Streptanthus*, and *Guillenia* and found that each microsatellite primer pair produced an amplification product in ca. 95% of the taxa tested. Skrede et al. (2009) developed 65 microsatellite markers for *Draba nivalis* and found a 77% cross-species transfer within *Draba*, and a cross-genus transfer of 18% and 6% between *Draba* and *Cardamine* and *Smelowskia*, respectively.

No specific nuclear and chloroplast microsatellite markers have been developed for the genus *Malcolmia* up to now, but they have abundantly been developed for several species in the Brassicaceae family (Bell and Ecker 1994; Weising and Gardner 1999; Provan 2000; Clauss et al. 2002; Suwabe et al. 2002; Bon et al. 2005; Basic and Besnard 2006; Burrell and Pepper, 2006; Skrede et al. 2009).

The evaluation of different genetic indices such as the heterozygosity (also called heterogeneity index or gene diversity; Nei and Roychoudhury 1974), the genetic distance between two populations (Nei 1972), the mean number of alleles per locus, the percentage of polymorphic loci, and the heterozygote deficit can help to highlight species/populations/gene pools to be preserved. Parameters that allow assessment of inbreeding may help *in situ* conservation in focusing on viable populations, where inbreeding and subsequent loss of alleles are minimal (van Zonneveld et al. 2012). Petit et al. (1998) suggested to give a high priority also to the allelic richness (the number of alleles per locus), which should be a better indicator of past demographic changes, and thus of interest in the context of conservation. van Zonneveld et al. (2012), together with allelic

richness, considered the locally common alleles an important tool to assign conservation priority to a species' population.

Recently a new discipline called landscape genetics improved understanding and monitoring of biodiversity. This field of research combines spatial analysis and population genetics and can contribute significantly to the formulation and implementation of more targeted, and hence more effective, conservation strategies (Scheldeman and van Zonneveld 2010). Outputs from spatial studies provide critical information on the diversity present in specific geographic areas and can be used to evaluate the current conservation status of plant species and to identify key sites for carrying out *ex situ* and *in situ* conservation activities (priority areas for collection and protection), particularly since resources allocated for conservation are frequently scarce, limiting the possibility to conduct conservation actions in several areas (Petit et al. 1998; Scheldeman and van Zonneveld 2010; Cires et al. 2011).

The objectives of this study were to:

- investigate the present genetic diversity and structure of *Malcolmia littorea* European native populations;
- identify populations/areas with conservation priority;
- provide useful information for conservation and management programs.

3.2. MATERIALS AND METHODS

For genetic analysis, fresh leaves were sampled from a minimum of 20 adult individuals per population. The choice of sampling only adults was taken to ensure the collection of genotypes effectively established within the populations. When the population size and its site were large enough, individuals separated by at least 5 m were randomly selected to avoid selection of siblings. Five to ten leaves were collected from each plant, were put in individual breathable tea-bags (Fig. 3.1a), which were put in air-tight plastic bags containing silica gel, needful to dry the leaves (Fig. 3.1b).

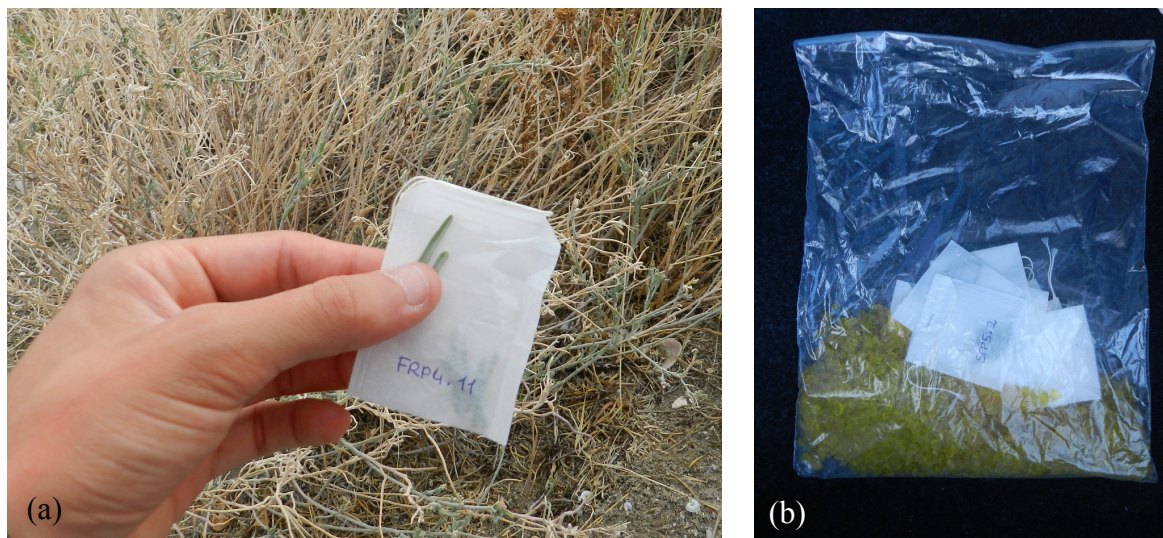


Figure 3.1. (a) The individual breathable tea-bag for leaves collection and (b) the air-tight plastic bag containing silica gel for leaves drying.

Genetic analyses were conducted at the Institute of Agro-environmental and Forest Biology of the National Research Council (Ibaf-CNR, Terni, Italy).

3.2.1. DNA extraction and microsatellite analysis

Genetic analysis were carried out on a subset of populations: PT-1, PT-2, ES-1, ES-4, ES-5, FR-1, FR-3, FR-4, FR-5, IT-1, IT-2, and IT-3 (Table 2.1.).

DNA was extracted from 50-100 mg of leaf tissue, using the DNeasyTM Plant Mini Kit (Qiagen, Valencia, California, USA; Fig. 3.2a,b) following the manufacturer's protocol. Extraction products were visualized on 1% agarose/TBE gel, using a 123 bp ladder

(Thermo Fisher Scientific, Waltham, Massachusetts, USA) as standard, stained with GelRedTM (Biotium, Hayward, California, USA; Fig. 3.2c).

A cross-transfer approach was used in this study to find microsatellite markers for *M. littorea*. Primers previously developed, or just tested, in other species within the Brassicaceae family, were tested in *Malcolmia*. Some of these primers were already tested for cross-genus or cross-family transfer. In particular, nine chloroplast and 37 nuclear microsatellite primers (Appendix 3) were obtained from Bell and Ecker (1994), Weising and Gardner (1999), Provan (2000), Clauss et al. (2002), Suwabe et al. (2002), Bon et al. (2005), Ceplitis et al. (2005), Basic and Besnard (2006), Burrell and Pepper (2006), and Skrede et al. (2009) and were screened over a subset of samples of the Italian populations.

The DNA samples of the different *M. littorea* populations were analysed for length polymorphism using the microsatellite markers that succeeded in amplification. All forward primers were given a tail with the M13 sequence (5'-CACGACGTTGTAAAACGAC-3'; Schuelke 2000) and used in combination with reverse primer and a third M13-primer dyed with FAM, NED or VIC (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

For all the microsatellite primers, PCR amplification was carried out in 10 µL reaction mix, with 3 µL DNA, 1 µL Buffer 10X, 0.3 µL MgCl₂, 2 µL dNTP, 1 µL F primer, 0.5 µL of R primer and M13-primer, and 0.04 µL polymerase DNA Taq, completed with sterile water.

Conditions of the PCR amplification were as follows: 94°C (5 min), then 30 cycles at 94°C (30 s) / 56°C (45 s) / 72°C (45 s), followed by 8 cycles at 94°C (30 s) / 53°C (45 s) / 72°C (45 s), and a final extension at 72°C for 10 min (Schuelke 2000).

Amplification products (1 µL) were added to 9.8 µL formamide and 0.2 Genescan-500 LIZ. The samples were run on ABI PRISM 3130 DNA sequencer (Fig. 3.2d). The resulting row data were collected applying GeneMapper v. 5.0 (Applied Biosystems, Foster City, California, USA), which was also used to size and score the microsatellite profiles.



Figures 3.2. (a, b) DNA extraction phases, (c) preparation of an electrophoresis gel for visualization of extraction products, and (d) ABI PRISM 3130 DNA sequencer for capillary electrophoresis of the amplification products.

3.2.2. Data analysis

3.2.2.1. Chloroplast microsatellites

A set of measures of intra- and interpopulations genetic statistics were calculated using the program GeneA1Ex 6.5 (Peakall and Smouse 2012).

For chloroplast loci the following parameters were estimated: the total ($Na_{cp,tot}$), average within-population (Na_{cp}), and effective ($Ne_{cp} = 1/\sum p_i^2$, where p_i is the frequency of the i th allele; Brown and Weir 1983) number of alleles, the last one representing an estimate of the number of equally frequent alleles in an ideal population; the haploid genetic diversity ($h = 1 - \sum p_i^2$; Nei 1978) which provides an indication of the probability that two random individuals will be genetically different; the unbiased haploid genetic diversity ($uh = [n/(n-1)][1 - \sum p_i^2]$, where n is the sample size; Nei 1987), which is weighted on the number of samples; and the information index ($I_{cp} = \sum p_i \ln p_i$), which is a

measure of allelic diversity. Further, the chloroplast genetic diversity over all loci (H_{cp}) was estimated, with the unbiased one (uH_{cp}).

Allelic information from single chloroplast microsatellite loci were combined to define the chloroplast haplotypes. Then, the frequency of each haplotype was calculated over the populations and within each population.

Within each population, the average ($Na_{cp,pop}$) and effective ($Ne_{cp,pop}$) number of alleles per locus, the gene diversity ($H_{cp,pop}$), with its unbiased version ($uH_{cp,pop}$), and the information index ($I_{cp,pop}$) were computed. A dissimilarity matrix based on Nei's genetic distance (Nei 1972) was computed. One-way ANOVA (for normally distributed data) or Kruskal–Wallis (for data not normally distributed or when the assumption of homoscedasticity was rejected), were used to compare the estimated genetic parameters among the populations.

To estimate the hierarchical partitioning of genetic variation within and among populations and groups (or simply the chloroplast genetic structure), and to test population differentiation, analyses of molecular variance (AMOVA, Excoffier et al. 1992) was performed with 999 permutations. The global and pairwise Φ_{PT} (the differentiation index for haploid data) were calculated and, on the basis of the Φ_{PT} matrix resulting from the AMOVA test, a principal coordinates analysis (PCoA) was performed to visualize the pattern of genetic differentiation in such a matrix. Furthermore, the standardized Φ_{PT} (Φ'_{PT}), which represents a 0-1 scaled estimator of differentiation (Meirmans 2006), and the mean haploid number of migrants ($Nm_{cp} = [(1/\Phi_{PT}) - 1]/2$) over all populations, were calculated. The correlations between the size of the occupied sites (as a measure of population size) and different indexes of genetic variability, such as $H_{cp,pop}$, $uH_{cp,pop}$ and the molecular variance within populations (AMOVA sum of squares), were calculated.

The spatial pattern of distribution of chloroplast genetic diversity was studied by means of a Mantel test (Sokal and Rohlf 1995), which estimates the correlation between the matrices of geographical distances and genetic distances between individuals, using 999 random permutations. The pairwise, individual-by-individual linear geographic distances matrix was generated from X and Y coordinates, while the calculation of pairwise genetic distances followed the method of Huff et al. (1993).

3.2.2.2. Nuclear microsatellites

Several genetic diversity statistics were computed for nuclear microsatellites loci, using GeneAIEx 6.5 (Peakall and Smouse 2012): the total number of alleles, ($Na_{nuc,tot}$), the average number of alleles (Na_{nuc}), the effective number of alleles (Ne_{nuc}), the observed heterozygosity ($Ho_{nuc} = \text{No. of Hets}/n$), and expected ($He_{nuc} = 1 - \sum p_i^2$; Nei, 1978) heterozygosity with its unbiased version ($uHe_{nuc} = [2N/(2N-1)]He_{nuc}$; Nei and Roychoudhury 1974) were calculated at each locus. Wright's F-statistics (Weir and Cockerham 1984), within-population inbreeding coefficient (F_{IS}), total-population inbreeding coefficient (F_{IT}) and among-population genetic differentiation coefficient (F_{ST}), were estimated for each locus over all populations.

For each population, the following parameters were estimated as means over all loci: the number of alleles per locus ($Na_{nuc,pop}$), the effective number of alleles per locus ($Ne_{nuc,pop}$), the observed, expected and unbiased expected heterozygosity ($Ho_{nuc,pop}$, $He_{nuc,pop}$, and $uHe_{nuc,pop}$, respectively), the information index ($I_{nuc,pop}$). One-way ANOVA (for normally distributed data) or Kruskal–Wallis test (for data not normally distributed or when the assumption of homoscedasticity was rejected), were used to compare the estimated genetic parameters among the populations.

The estimation of mean number of alleles per locus as a measure of allelic richness can be affected by differences in sample size. For this reason, allelic richness (R_s) and private allele richness (PR), which are independent of sample size (El Mousadik and Petit 1996), were computed by the rarefaction method with HP-RARE software (Kalinowski 2005). This approach uses the frequency of alleles at a locus to estimate the expected number of alleles and/or private alleles in a subsample of n individuals selected at random from a sample of N individuals in each population.

The genetic structure of nuclear microsatellites was estimated using different approaches. Using ARLEQUIN 3.11 (Excoffier et al. 2005), a hierarchical locus-by-locus analysis of molecular variance (AMOVA, Excoffier et al. 1992), was performed to compute the unbiased estimators of Wright's F-statistics (Weir and Cockerham 1984), within-population inbreeding coefficient (F_{IS}), total-population inbreeding coefficient (F_{IT}) and among-population genetic differentiation coefficient (F_{ST}), for each population over all loci. The statistical significance of F_{IS} , F_{IT} and F_{ST} was tested using a non-parametric approach described in Excoffier et al. (1992) with 999 permutations. A principal coordinates analysis (PCoA) was performed using the F_{ST} pairwise matrix, to visualize the

differentiation among the populations. The mean number of diploid migrants ($Nm_{nuc} = [(1/F_{ST}) - 1]/4$) over all populations, and the correlations between the size of the occupied sites and different indexes of genetic variability, such as $Ho_{nuc, pop}$, $He_{nuc, pop}$, $uHe_{nuc, pop}$ and the molecular variance within populations (AMOVA sum of squares), were calculated.

Because the presence of null alleles can affect the estimation of population differentiation, for instance, by reducing the genetic diversity within populations and overestimating the population differentiation, null allele frequencies were estimated for each locus and population following the Expectation Maximization (EM) algorithm of Dempster, Laird and Rubin (1977), implemented in FreeNA software (Chapuis and Estoup 2007). When a locus was suspected to be affected by the presence of null alleles, the AMOVA and the computation of the parameters F_{IS} , F_{IT} and F_{ST} were repeated without this locus to compare the results.

The STRUCTURE 2.3.3 program (Pritchard et al. 2000) was used to estimate the number of clusters (K) and assigning the total individuals to the clusters, using Bayesian clustering algorithms. This method attempts to assign individuals to several genetic groups in order to minimize within group linkage disequilibrium and deviation from the Hardy-Weinberg equilibrium. Six runs of STRUCTURE were done by setting K from 1 to 14 (the number of sampled populations plus two). Each run consisted in a burn-in period of 10 000 steps followed by 10^5 Markov chain Monte Carlo (MCMC) replicates. No prior information was used to define the clusters. Furthermore, the ad-hoc statistics ΔK , defined by Evanno et al. (2005), was used to detect the most likely number of populations. The ΔK statistics is based on the second order rate of change $L(K)$ (the posterior probability of the data among given K) between successive K values over six replicates. As demonstrated by Evanno et al. (2005), it is possible to identify the number of clusters corresponding to the uppermost hierarchical level of genetic partitioning among populations. The six runs from the most probable number of clusters were averaged applying the Full Search algorithm provided by CLUMPP software 1.1.2 (Jakobsson and Rosenberg 2007). The corresponding matrices were graphically displayed by DISTRUCT software (Rosenberg 2004).

As for chloroplast microsatellites, the spatial pattern of distribution of genetic diversity was studied by means of a Mantel test, which was used to estimate the correlation between the matrix of geographical distances between populations and that of the genetic distances.

Furthermore, the nuclear microsatellite data were used to perform a spatial diversity analysis with the software Diva-GIS 7.5 (Hijmans et al. 2012). First, a species richness analysis was performed through the command *Analysis/Point to Grid/Richness*, using the

Rarefaction option, and a grid cell of 0.05 x 0.05. The rarefaction method, which recalculates the diversity measured at each subunit of the study area to a standardized identical number of samples, has been developed to compare richness among cells that have a dissimilar number of observations or samples (Petit et al. 1998; Leberg 2002). Then, a geographical distribution of individual alleles was estimated. Based on their frequencies and geographic distributions, different types of alleles can be identified, and among them, *locally common alleles* are the most important from a conservation standpoint as they can indicate adaptation to local conditions and due to their restricted distribution more vulnerable to losses than broadly distributed alleles (Frankel et al. 1995). In this case the command *Analysis/Distance/Statistics by Class* was used, and the locally common alleles were identified as alleles repeatedly observed in a small area. The probability that this phenomenon occurs repeatedly and within a limited area is much lower than the probability of observing single alleles only once, and this explains why, for conservation purposes, this type of alleles are considered more important than private alleles (van Zonneveld et al. 2012). To assist in defining priority conservation areas for *Malcolmia littorea*, the command *Analysis/Point to Grid/Reserve Selection* was used with the options *Complementarity* and *Equal weight*, which give the same weight to each allele. Furthermore, the *Reserve Selection* procedure (Rebelo and Siegfried 1992) uses an optimization algorithm and combines the information on molecular diversity to define the minimum number of geographic units needed to conserve all species' genetic diversity, and to identify, in sequence of importance, the geographic units that should be prioritized for conservation. Finally, the cluster analysis was used to assess differences in allelic composition between raster cells, providing further insight in the genetic structure across the species' geographical distribution. In this case, the command *Analysis/Point to Grid/Richness* was used with the option *Presence/Absence* to generate a group of presence/absence rasters. Then, a stack file was created starting from these rasters, and was selected for the *Stack/Cluster* analysis. This analysis differs from the STRUCTURE analysis, because the last one assigns genotypes to clusters based solely on allelic frequencies, independent of the population of the study in which they are located or any other *a priori* definition of populations. Using Diva-GIS, the distance between two different raster cells is calculated on the basis of the composition of alleles found in each cell, using the *Binary* option (presence/absence) and the *Dice* method (also known as the *Nei-Li* coefficient; De Vicente et al. 2004), which calculate the matrix of distances.

3.3. RESULTS

3.3.1. Cross-amplification

Among the 46 tested primers, only five chloroplast (ATCP7905, ATCP28673, ATCP31017, ATCP46615, ATCP70189,) and six nuclear (AthCDPK9, AthDET1, AthGAPAb, Dnb101, ICE5, SLL2) amplified in *M. littorea* (Fig. 3.3). All five chloroplast primers were used in this analysis, while among the nuclear ones, only four of them were used, discarding the primers which produced a weak amplification (AthDET1 and Dnb101).

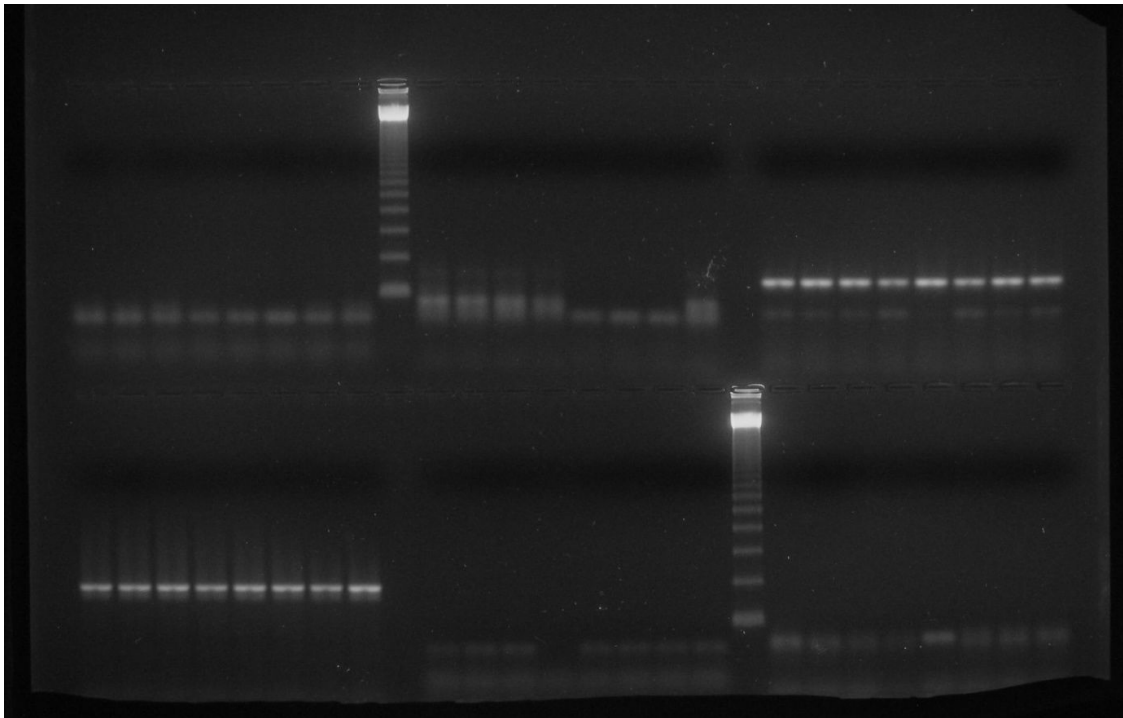


Figure 3.3. Electrophoretic gel for the visualization of PCR amplification products.

3.3.2. Chloroplast microsatellites

Only two of the five cpSSR (chloroplast microsatellite) loci examined were polymorphic (ATCP28673 and ATCP31017), both showing four alleles (Table 3.1). Over all the populations, the average number of alleles ($N_{a_{cp}}$) for ATCP29673 and ATCP31017 was 2.50 and 1.83, respectively, and the effective number of alleles ($N_{e_{cp}}$) was 1.58 and 1.40; the haploid genetic diversity (h) was 0.31 for the first locus and 0.23 for the second,

with the unbiased genetic diversity (uh) being 0.33 and 0.24, respectively; and the information index (I_{cp}) 0.55 and 0.36 (Table 3.1).

Table 3.1. Specific dye of the M13-primer, allele size range (pb), and estimates of total ($Na_{cp,tot}$), average within-population (Na_{cp}), and effective (Ne_{cp}) number of alleles, haploid genetic diversity (h), unbiased haploid genetic diversity (uh), and the information index (I_{cp}) at five chloroplast microsatellite loci in *Malcolmia littorea*.

Locus	Dye	Range (pb)	$Na_{cp,tot}$	Na_{cp}	Ne_{cp}	h	uh	I_{cp}
ATCP7905	FAM	152	1	1.00	1.00	0.00	0.00	0.00
ATCP28673	VIC	161-164	4	2.50	1.58	0.31	0.33	0.55
ATCP31017	NED	226-230	4	1.83	1.40	0.23	0.24	0.36
ATCP46615	FAM	115	1	1.00	1.00	0.00	0.00	0.00
ATCP70189	NED	134	1	1.00	1.00	0.00	0.00	0.00

Combining the different alleles of the two polymorphic loci, 11 haplotypes were identified and numbered on the basis of their frequency (Table 3.2), from the most frequent, H1 (34.30%), to the less frequent, H10 and H11 (0.48%).

Table 3.2. Haplotypes deriving from the combination of the different alleles found at the two polymorphic loci, numbered from the most to the less frequent.

cpSSR locus			
Haplotype	ATCP28673	ATCP31017	Frequency (%)
H1	161	228	34.30
H2	161	229	30.92
H3	161	230	10.63
H4	163	229	6.76
H5	164	229	6.76
H6	164	228	3.86
H7	163	228	2.42
H8	163	230	2.42
H9	162	229	0.97
H10	161	226	0.48
H11	162	228	0.48

H1 was the most abundant haplotype in French and Portuguese populations; it also occurred in one Italian population but did not in any Spanish one; H2 occurred frequently in both Italian and Spanish populations but was also present in three French and one Portuguese (PT-1) populations; H3 occurred abundantly among the Italian populations and was also present in PT-1; H4 and H5 were shared only by some Spanish and French populations; H6 was exclusive of the Spanish populations; H7 was shared by two French

(FR-1, FR-5) and one Portuguese (PT-2) populations; H8 occurred with a low overall frequency, but was exclusive of the Italian populations; H9 was exclusive of two Spanish populations (ES-1 and ES-4), while H10 and H11 were exclusive of PT-1 and PT-2, respectively (Table 3.3, Figure 3.4).

Table 3.3. Haplotype variation and abundance within *Malcolmia littorea* populations.

Population	Haplotype										
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11
PT-1	11	1	1							1	
PT-2	13						1				1
ES-1		17		1					1		
ES-4		5		3	10				1		
ES-5		10		6	2						
FR-1	11	5		2			1				
FR-3	10	1		2	1	3					
FR-4	12					3					
FR-5	13	1			1	2	3				
IT-1	1	6	9					2			
IT-2		5	8					2			
IT-3		13	4					1			

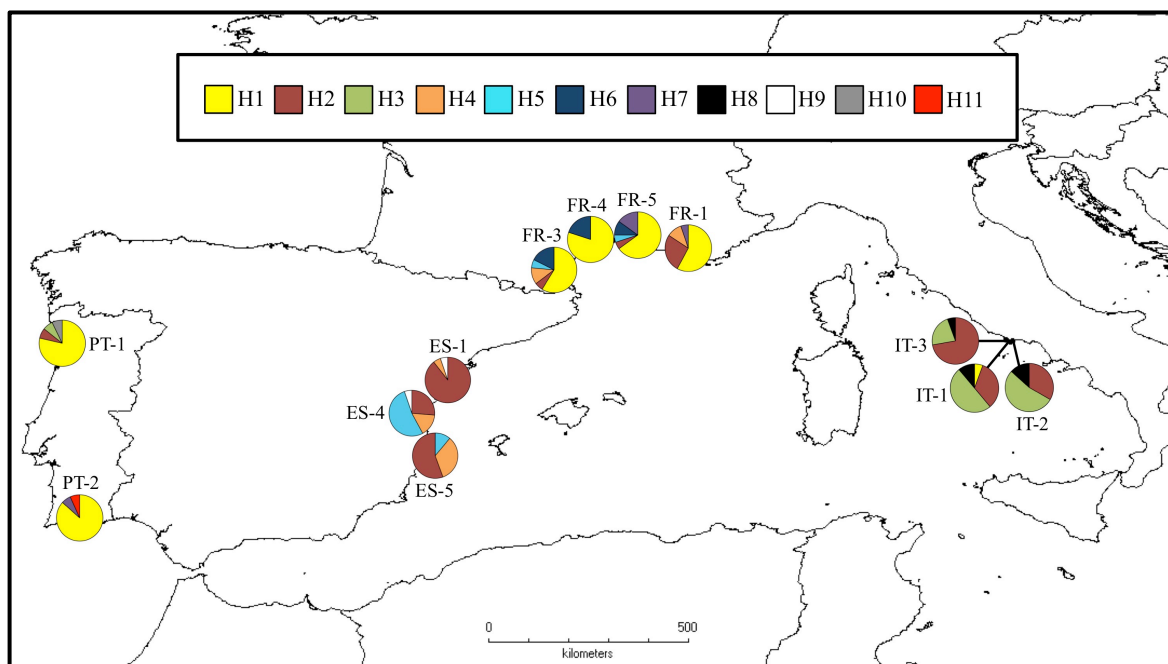


Figure 3.4. Geographic distribution of chloroplast haplotypes in populations of *Malcolmia littorea*. Pie graphs indicate the frequencies of the different haplotypes in each population.

Among the 12 populations of *M. littorea*, the average number of alleles ($Na_{cp,pop}$) ranged from the minimum of 1.20 for FR-4 to a maximum of 1.60 for several populations (PT-1, ES-4, FR-3, FR-5, IT-1). All the other populations presented the intermediate value of 1.40. Looking at the effective number of alleles ($Ne_{cp,pop}$), both the minimum and the maximum values were found in Spanish populations, being 1.05 and 1.34 in ES-1 and ES-4, respectively. The Italian populations showed intermediate values (1.26, 1.22, 1.16 for IT-1, IT-2 and IT-3, respectively; Table 3.4). Even if with a small extent, the unbiased values of gene diversity ($uH_{cp,pop}$) were always higher than the biased ones ($H_{cp,pop}$), except for PT-2, for which it was lower. For parameters $H_{cp,pop}$, $uH_{cp,pop}$, and $I_{cp,pop}$ the highest values were found in FR-3 (0.17, 0.19 and 0.28, respectively), while the lowest values were found in ES-1 (0.04, 0.04 and 0.08, respectively; Table 3.4). By means of Kruskal-Wallis test, no significant differences were found comparing $H_{cp,pop}$ and $uH_{cp,pop}$ values among the populations ($P > 0.05$).

The Nei genetic distance varied from 0.00 (between IT-1 and IT-2) and 0.34 (between PT-2 and ES-4). The chloroplast genetic diversity H_{cp} over all loci and populations was 0.11.

Table 3.4. Number of individuals genotyped (N_{cp}) and estimates of mean ($Na_{cp,pop}$) and effective ($Ne_{cp,pop}$) number of alleles per locus, genetic diversity ($H_{cp,pop}$), unbiased genetic diversity ($uH_{cp,pop}$) and information index ($I_{cp,pop}$) for chloroplast microsatellite loci in 12 *Malcolmia littorea* populations.

Pop.	N_{cp}	$Na_{cp,pop}$	$Ne_{cp,pop}$	$H_{cp,pop}$	$uH_{cp,pop}$	$I_{cp,pop}$
PT-1	14	1.60	1.12	0.07	0.08	0.15
PT-2	15	1.40	1.06	0.08	0.05	0.10
ES-1	19	1.40	1.05	0.04	0.04	0.08
ES-4	19	1.60	1.34	0.13	0.13	0.23
ES-5	18	1.40	1.26	0.11	0.12	0.19
FR-1	19	1.40	1.25	0.15	0.15	0.22
FR-3	17	1.60	1.32	0.17	0.19	0.28
FR-4	15	1.20	1.09	0.06	0.07	0.10
FR-5	20	1.60	1.22	0.13	0.14	0.23
IT-1	18	1.60	1.26	0.14	0.15	0.24
IT-2	15	1.40	1.22	0.14	0.15	0.21
IT-3	18	1.40	1.16	0.10	0.11	0.16

The AMOVA analysis indicated that 56% of the variation was found within populations, whereas 44% of the variation among populations. The overall value of Φ_{PT} was 0.44 ($P = 0.001$), while Φ'_{PT} was 0.50, and the average Nm_{cp} was estimated to be 0.63.

The PCoA, performed using the matrix of Φ_{PT} , highlighted the significant differentiation of the populations and three groups emerged: the French-Portuguese, the Italian and the Spanish groups (Fig. 3.5). The first and second axes of the PCoA explained 57.91 and 21.64% of the variability, respectively. The Φ_{PT} values deriving from the pairwise comparison ranged between 0.000 (between FR-3 and FR-5, FR-4 and FR-5, IT-1 and IT-2) and 0.813 (between PT-2 and ES-1; Table 3.5).

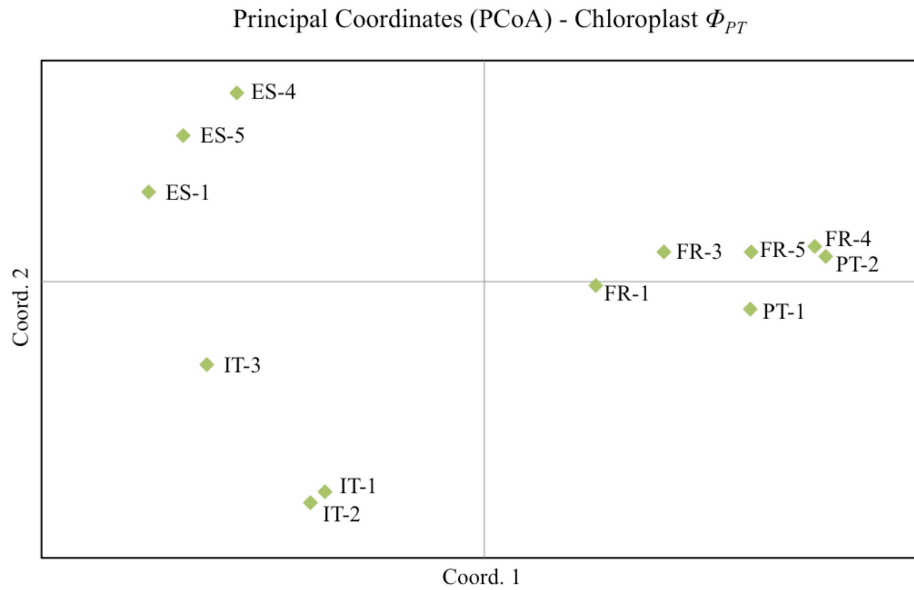


Figure 3.5. Principal coordinates analysis (PCoA) performed with the differentiation index, Φ_{PT} . The main differentiation, between the Italian-Spanish group and the French-Portuguese one, is underlined by the first axis, which explained 57.91% of the variability.

Table 3.5. Φ_{PT} values are shown above diagonal, while p -values based on 999 permutations are shown below diagonal. Populations are considered significantly differentiated at $P < 0.05$ (significant p -values in bold).

	PT-1	PT-2	ES-1	ES-4	ES-5	FR-1	FR-3	FR-4	FR-5	IT-1	IT-2	IT-3
PT-1	0.000	0.063	0.721	0.674	0.630	0.072	0.093	0.109	0.074	0.415	0.470	0.521
PT-2	0.051	0.000	0.813	0.728	0.705	0.167	0.105	0.022	0.023	0.555	0.604	0.655
ES-1	0.001	0.001	0.000	0.426	0.173	0.439	0.531	0.792	0.648	0.452	0.495	0.141
ES-4	0.001	0.001	0.001	0.000	0.150	0.483	0.456	0.693	0.585	0.501	0.513	0.413
ES-5	0.001	0.001	0.025	0.015	0.000	0.381	0.432	0.686	0.556	0.404	0.422	0.226
FR-1	0.106	0.023	0.001	0.001	0.001	0.000	0.021	0.187	0.072	0.292	0.335	0.295
FR-3	0.072	0.040	0.001	0.001	0.001	0.247	0.000	0.049	0.000	0.351	0.384	0.399
FR-4	0.034	0.428	0.001	0.001	0.001	0.010	0.184	0.000	0.000	0.547	0.592	0.642
FR-5	0.060	0.226	0.001	0.001	0.001	0.072	0.383	0.404	0.000	0.441	0.478	0.515
IT-1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000	0.134
IT-2	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.343	0.000	0.161
IT-3	0.001	0.001	0.016	0.001	0.002	0.001	0.001	0.001	0.001	0.038	0.016	0.000

The area of the site occupied by the populations was not significantly correlated with any index of molecular variability.

The correlation between geographic and chloroplast genetic distance was positive, but very weak ($R^2 = 0.02$; $R_{xy} = 0.13$; $P = 0.001$; Figure 3.6).

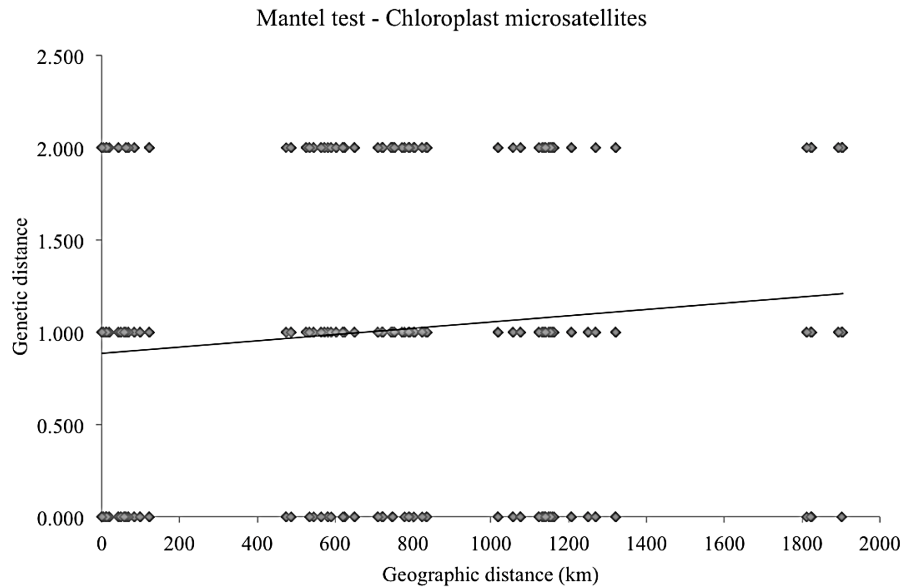


Figure 3.6. Relationship between geographic and chloroplast genetic distances among populations of *M. littorea*, estimated by means of a Mantel test (Sokal and Rohlf 1995). Matrices of both geographic and genetic distances were calculated using GeneA1Ex 6.5 (Peakall and Smouse 2012).

3.3.3. Nuclear microsatellites

In *M. littorea*, the four nuclear microsatellite loci used in this study were all polymorphic, with the number of alleles per locus ranging between two, for both AthCDPK9 and SLL2, and six for both AthGAPAb and ICE5 (Table 3.6).

Table 3.6. Specific dye of the M13-primer, allele size range (pb), and estimates of total ($N_{a_{nuc,tot}}$), average ($N_{a_{nuc}}$), and effective ($N_{e_{nuc}}$) number of alleles, observed ($H_{o_{nuc}}$), expected ($H_{e_{nuc}}$), and unbiased expected ($uH_{e_{nuc}}$) heterozygosity, within-population inbreeding coefficient (F_{IS}), total-population inbreeding coefficient (F_{IT}) and among-population genetic differentiation coefficient (F_{ST}) at four nuclear microsatellite loci in *Malcolmia littorea*.

Locus	Dye	Range (pb)	$N_{a_{nuc,tot}}$	$N_{a_{nuc}}$	$N_{e_{nuc}}$	$H_{o_{nuc}}$	$H_{e_{nuc}}$	$uH_{e_{nuc}}$	F_{IS}	F_{IT}	F_{ST}
AthCDPK9	FAM	125-127	2	1.08	1.01	0.00	0.01	0.01	1.00	1.00	0.05
AthGAPAb	VIC	189-198	6	3.83	2.54	0.46	0.59	0.60	0.22	0.36	0.18
ICE5	VIC	120-126	6	2.42	1.69	0.53	0.41	0.42	-0.32	-0.30	0.01
SLL2	FAM	176-177	2	1.92	1.52	0.32	0.30	0.31	-0.04	0.19	0.22

The within-population mean ($N_{a_{nuc, pop}}$) and effective ($N_{e_{nuc}}$) number of alleles per locus ranged between 1.75 (IT-3) and 3.00 (FR-4) and between 1.43 (IT-3) and 1.87 (IT-1), respectively (Table 3.7). Private alleles were found in ES-1, ES-5, FR-4 and IT-1 ($P_R = 0.02, 0.08, 0.71, \text{ and } 0.40$, respectively). The same populations showed the highest values of allelic richness ($R_s = 2.55, 2.70, 2.96, \text{ and } 2.62$, respectively). The observed heterozygosity ($H_{o_{nuc, pop}}$) ranged between 0.20 (FR-4) and 0.45 (FR-5), the expected heterozygosity ($H_{e_{nuc, pop}}$) ranged between 0.23 (IT-3) and 0.37 (FR-5), and the unbiased expected heterozygosity ($uH_{e_{nuc, pop}}$) ranged between 0.23 (IT-3) and 0.38 (FR-5). No significant differences were found comparing the mean values of $H_{o_{nuc, pop}}$, $H_{e_{nuc, pop}}$ and $uH_{e_{nuc, pop}}$ among the populations (Kruskal-Wallis test: $P > 0.05$).

Considering all the nuclear loci examined, the analysis of molecular variance (AMOVA) for the total population showed that 12.78% of the variation occurred among populations, 0.98% among individuals within populations, and 86.24% within individuals. According to these results, the overall F_{IS} , F_{IT} and F_{ST} were 0.01 ($P > 0.05$), 0.13 ($P < 0.0001$) and 0.13 ($P < 0.0001$) respectively, showing overall low within-population inbreeding and differentiation among populations. The average Nm_{nuc} was 1.71.

The software FreeNA detected a high frequency of null allele in the locus AthCDPK9, so the AMOVA and the calculation of the Wright's F-statistics was repeated excluding this locus. Considering only the other three loci, the percentages of variation changed slightly, being 12.86, 0.38 and 86.76% the variation among populations, among individuals within populations and within individuals, respectively, while the Wright's F-statistics and their significance did not change.

From the within-population analysis, non-significant differences emerged between the F_{IS} values calculated with and without the locus AthCDPK9 (Table 3.7). The F_{IS} value showed by FR-4 (0.46) was the highest and the only significantly positive ($P < 0.0001$). Among the Portuguese populations, PT-1 showed a positive value, while PT-2 showed a negative one; all the Spanish populations showed negative values ranging between -0.09 and -0.16; all the French and Italian populations showed positive values except FR-5 and IT-3. The nuclear genetic diversity H_{nuc} over all loci and populations was 0.33.

Table 3.7. Number of individuals genotyped (N_{nuc}) and estimates of mean ($Na_{\text{nuc,pop}}$) and effective ($Ne_{\text{nuc,pop}}$) number of alleles per locus, allelic richness (Rs), private allelic richness (PR , standardized to 13 individuals from the original number of plants per population), observed ($Ho_{\text{nuc,pop}}$), expected ($He_{\text{nuc,pop}}$) and unbiased expected ($uHe_{\text{nuc,pop}}$) heterozygosity, information index ($I_{\text{nuc,pop}}$) and the inbreeding coefficient calculated over all loci ($F_{\text{IS},4}$) and over three loci not affected by null alleles ($F_{\text{IS},3}$) for nuclear microsatellite loci in 12 *Malcolmia littorea* populations.

Pop.	N_{nuc}	$Na_{\text{nuc,pop}}$	Ne_{nuc}	Rs	PR	$Ho_{\text{nuc,pop}}$	$He_{\text{nuc,pop}}$	$uHe_{\text{nuc,pop}}$	$I_{\text{nuc,pop}}$	$F_{\text{IS},4}$	$F_{\text{IS},3}$
PT-1	13	2.25	1.58	2.25	0.00	0.27	0.32	0.33	0.52	0.19	0.19
PT-2	13	2.00	1.62	2.00	0.00	0.37	0.33	0.35	0.52	-0.06	-0.06
ES-1	20	2.75	1.86	2.55	0.02	0.41	0.35	0.36	0.61	-0.15	-0.15
ES-4	19	2.25	1.85	2.25	0.00	0.40	0.33	0.34	0.57	-0.16	-0.16
ES-5	20	2.75	1.69	2.70	0.08	0.36	0.33	0.33	0.60	-0.09	-0.09
FR-1	20	2.00	1.69	1.91	0.00	0.36	0.36	0.36	0.52	0.01	0.01
FR-3	20	2.00	1.66	2.00	0.00	0.29	0.34	0.35	0.53	0.18	0.18
FR-4	14	3.00	1.64	2.96	0.71	0.20	0.34	0.35	0.62	0.46***	0.46***
FR-5	20	2.00	1.73	2.00	0.00	0.45	0.37	0.38	0.56	-0.19	-0.19
IT-1	19	2.75	1.87	2.62	0.40	0.30	0.33	0.34	0.59	0.10	0.03
IT-2	19	2.25	1.69	2.17	0.00	0.25	0.28	0.29	0.49	0.13	0.13
IT-3	18	1.75	1.43	1.75	0.00	0.26	0.23	0.23	0.36	-0.13	-0.13

The area of the site occupied by the populations was not significantly correlated with any index of molecular variability.

The first two axes of the principal coordinates analysis (PCoA), based on the F_{ST} pairwise matrix, explained respectively 64.84 and 20.61% of the variability, and again three main gene clusters emerged. In particular one cluster was formed by the northern Portuguese and three French populations, another one consisted of the southern Portuguese and the Spanish populations, and the last genetic cluster was formed by the Italian populations. The pairwise F_{ST} values ranged between 0.000 (ES-1 vs ES-4, ES-4 vs ES-5, and IT-1 vs IT-2), and 0.404 (between PT-1 and IT-3; Figure 3.7; Table 3.8).

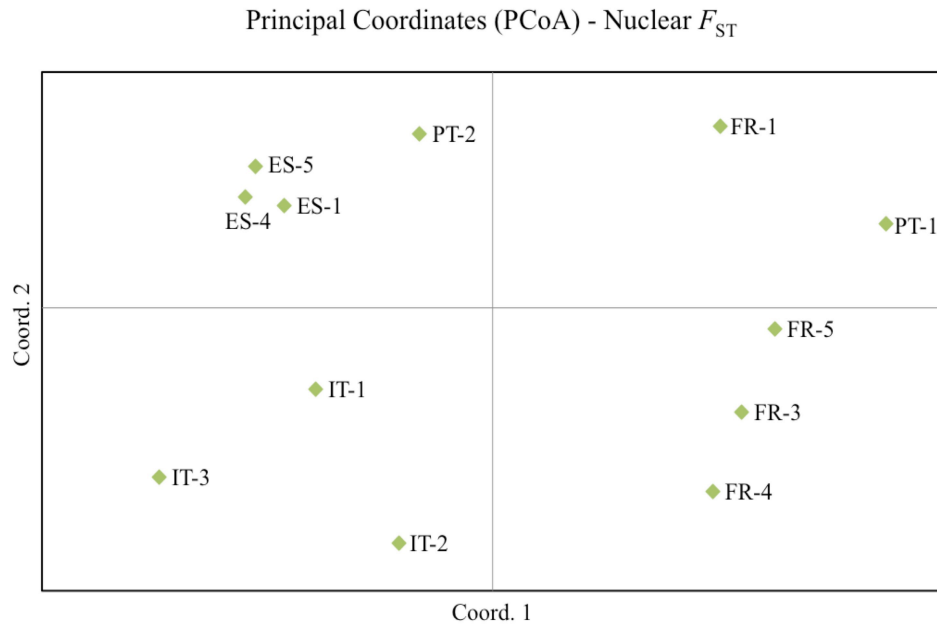


Figure 3.7. Principal coordinates analysis (PCoA) of 12 *M. littorea* populations based on F_{ST} pairwise values.

Table 3.8. F_{ST} values are shown above diagonal, while p -values based on 999 permutations are shown below diagonal. Populations are considered significantly differentiated at $P < 0.05$ (significant p -values in bold).

	PT-1	PT-2	ES-1	ES-4	ES-5	FR-1	FR-3	FR-4	FR-5	IT-1	IT-2	IT-3
PT-1	0.000	0.166	0.236	0.272	0.269	0.021	0.023	0.071	0.020	0.238	0.243	0.404
PT-2	0.003	0.000	0.036	0.046	0.004	0.044	0.114	0.137	0.109	0.031	0.098	0.167
ES-1	0.001	0.074	0.000	0.000	0.011	0.147	0.160	0.177	0.176	0.038	0.098	0.096
ES-4	0.001	0.034	0.396	0.000	0.000	0.170	0.193	0.192	0.206	0.031	0.100	0.114
ES-5	0.001	0.354	0.205	0.404	0.000	0.142	0.191	0.193	0.191	0.018	0.095	0.116
FR-1	0.169	0.065	0.001	0.001	0.001	0.000	0.053	0.092	0.024	0.144	0.175	0.307
FR-3	0.138	0.004	0.001	0.001	0.001	0.011	0.000	0.003	0.001	0.124	0.101	0.245
FR-4	0.019	0.002	0.001	0.001	0.001	0.001	0.325	0.000	0.025	0.102	0.055	0.276
FR-5	0.176	0.007	0.001	0.001	0.001	0.100	0.356	0.118	0.000	0.145	0.134	0.289
IT-1	0.001	0.089	0.023	0.056	0.114	0.001	0.001	0.001	0.001	0.000	0.000	0.078
IT-2	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.011	0.001	0.431	0.000	0.124
IT-3	0.001	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.004	0.001	0.000

From the analysis conducted with STRUCTURE software, the most probable division was $K = 2$, with the highest ΔK value, followed by $K = 12$ and $K = 9$ (Figure 3.8).

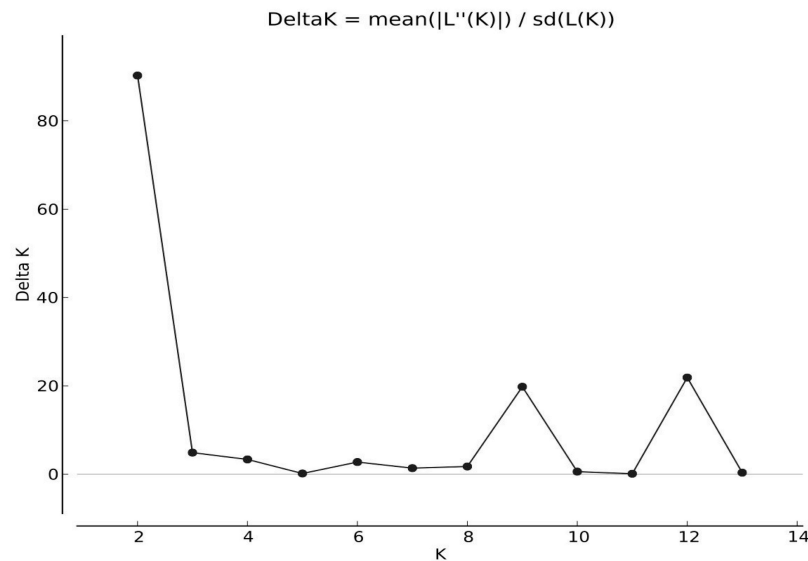


Figure 3.8. Inference of K , the most probable number of clusters, using STRUCTURE software, based on microsatellite analysis of 215 total samples of *Malcolmia littorea*. In the figure it is showed the second order of change of the log-likelihood of the data (ΔK) as a function of K , calculated over six replicates.

Based on $K = 2$, the Portuguese and French populations were predominantly composed of a single cluster (blue cluster; Figure 3.9) whereas the Spanish and one Italian populations (IT-3) were mainly composed of the other one (red cluster). The other two Italian populations (IT-1 and IT-2) showed intermediate features between the two clusters. The estimated population structure inferred from this analysis is shown in Figure 3.9. These results seem to be in accordance with F_{ST} values, underlying a differentiation between Portuguese-French and Spanish-Italian groups.

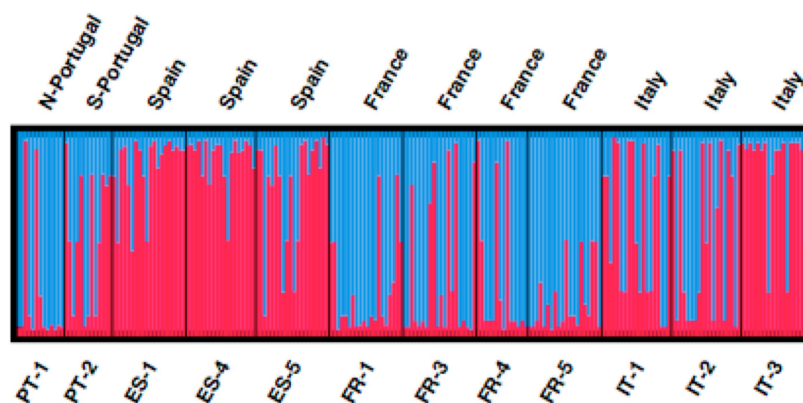


Figure 3.9. Population structure inference for 215 samples of *Malcolmia littorea* by Bayesian assignment using STRUCTURE software. Each individual is represented by a vertical line, and populations are separated by a vertical black line. Different colours in the same line indicate the individual's estimated membership percentage in each cluster.

As for chloroplast data, the correlation between geographic and nuclear genetic distances was positive but very weak ($R^2 = 0.01$, $R_{xy} = 0.09$, $P = 0.001$; Figure 3.10).

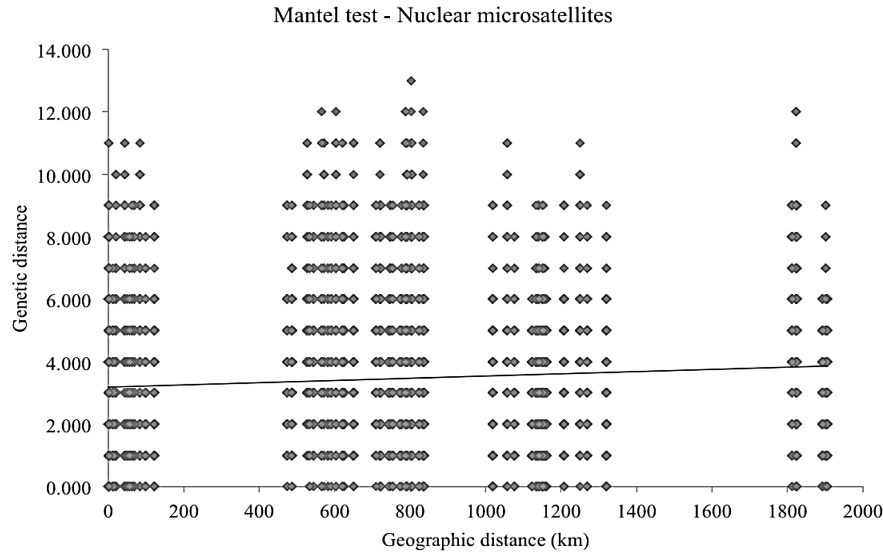


Figure 3.10. Relationship between geographic and nuclear genetic distances among populations of *Malcolmia littorea*, estimated by means of a Mantel test. Matrices of both geographic and genetic distances were calculated using GeneAIEx 6.5.

The richness analysis conducted with Diva-GIS, showed results comparable with the R_s values calculated with HP-RARE software (Table 3.6). The results were visualized on a georeferenced map (Figure 3.10a). Through the analysis of geographic distribution of individual alleles, AthGAPAb-196 and AthGAPAb-198 were identified as locally common alleles (Figure 3.11a). Moreover, the populations which should be prioritized for conservation were identified, with the following priority sequence: FR-4, IT-1 and ES-1 (Figure 3.10a). From the cluster analysis, a slightly different set of clusters than STRUCTURE was observed across the European populations. In particular, four principal clusters were detected: one cluster included the Portuguese populations, FR-1, FR-3, FR-5, IT-2 and IT-3; another one was formed by the three Spanish populations; while the remaining two populations (FR-4 and IT-1) were divergent from all the other populations and represented individual clusters. This grouping was better represented on the map using the Diva-GIS pruning option to visualize the dendrogram which considers different genetic distances. In this case the best representation was obtained at genetic distance of 0.06 (Figure 3.11b).

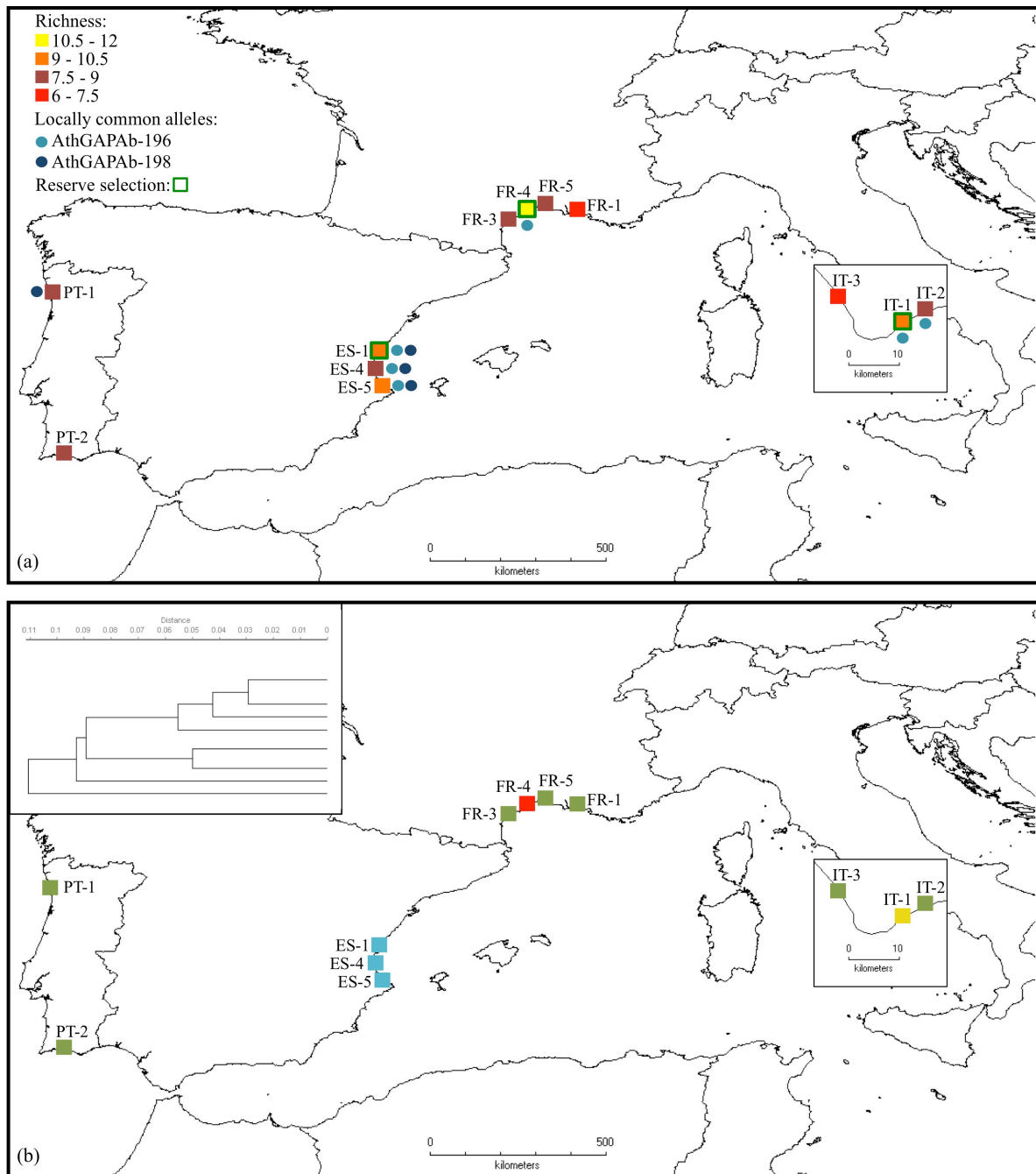


Figure 3.11. Results of the spatial analysis conducted with Diva-GIS software, using nuclear microsatellite data of 12 *Malcolmia littorea* populations: (a) the allelic richness visualized by four levels (yellow, orange, brown and red squares), the occurrence of the locally common alleles (AthGAPAb-196 represented by a light blue round and AthGAPAb-198 represented by a dark blue round), and the populations identified as conservation priorities (marked with a green outline); and (b) the results of the cluster analysis, in which four different clusters emerged at the genetic distance of 0.06, as shown in the dendrogram in the upper left corner of the figure. The Italian coastal portion where the populations occur, was emphasized to allow a better visualization of the results.

3.4. DISCUSSION

This study is the first report on genetic variability of European populations of *Malcolmia littorea*. The combined use of chloroplast and nuclear microsatellite loci provided important information on the genetic diversity and structure of the studied populations.

3.4.1. Cross-amplification

The cross-genus amplification was highly effective for the chloroplast genome since five of nine microsatellite primers gave a strong amplification. This result can be explained by the relatively low mutation rate and the high degree of sequence conservation associated with organelle genomes (Provan et al. 2001; Provan and Campanella 2003), that allow the use of the same primers in unrelated species. On the other hand they may represent a difficulty in detecting sufficient levels of variation below the species level due to a species-specific component of variation (Weising and Gardner 1999; Provan et al. 2001; Provan and Campanella 2003). According to these characteristics of the chloroplast genome, in *M. littorea* three of the five chloroplast microsatellite loci were monomorphic.

Similarly to *M. littorea*, loci ATCP7905 and ATCP46615 were found to be monomorphic in *Capsella bursa-pastoris* (Ceplitis et al. 2005) and in three *Brassica* spp. (Zamani-Nour et al. 2013), while conversely they showed polymorphism in *Arabidopsis thaliana* (Provan and Campanella 2003). Locus ATCP28673 showed polymorphism in *M. littorea* as in *A. thaliana* (Provan and Campanella 2003) and in four *Brassica* spp. (Edh et al. 2007; Zamani-Nour et al. 2013), while it was found to be monomorphic in *C. bursa-pastoris* (Ceplitis et al. 2005). At locus ATCP31017, four alleles were detected in *M. littorea* as in *C. bursa-pastoris* (Ceplitis et al. 2005). Finally, locus ATCP70189 was monomorphic in *M. littorea* as in four *Brassica* spp. (Edh et al. 2007; Zamani-Nour et al. 2013) while it was polymorphic in *A. thaliana* (Provan 2000) and in *C. bursa-pastoris* (Ceplitis et al. 2005).

For the nuclear genome the cross-genus amplification was not so effective, since only 11% of the tested primers gave a strong amplification in *M. littorea*. However, the four amplified loci were all polymorphic. Similarly to *M. littorea*, the same four loci were found to be polymorphic in *A. lyrata* (Gaudeul et al. 2007). Loci AthGAPAb and SLL2

were found to show polymorphism in *A. thaliana* too (Carlsen et al. 2010). Locus SLL2 was also studied in three *Boechea* spp. by Dobeš et al. (2004), who found it to be polymorphic in *Boechea* x *divaricarpa* and *B. holboellii* while monomorphic in *B. stricta*. Studies by Clauss et al. (2002) and Skrede et al. (2009) also reported results about amplification of the same loci in the genera *Arabidopsis*, *Draba*, *Cardamine* and *Smelowskia*, but they are not reliable since the results are based on the amplification of one or two individuals.

3.4.2. Within-population genetic diversity and population size

Combining allelic information from the two variable chloroplast microsatellite loci, 11 haplotypes were found across the 12 *M. littorea* European populations with no population fixed for a single haplotype. The haplotype distribution allowed the identification of different lineages: the more evident lineage, with H1 spread and dominant, had a Portuguese-French distribution; another lineage may be represented by two Spanish and one Italian populations, in which H2 was dominant. However, H2 was found in at least one population per country. These results indicated a high level of diversity if compared with similar studies. Provan and Campanella (2003), combining allele data across eight loci, found that 17 worldwide *A. thaliana* populations were all fixed for a unique haplotype, except for one population with two haplotypes. Ceplitis et al. (2005), studying the evolutionary history of *C. bursa-pastoris*, found a rather limited molecular diversity in the species chloroplast genome with only eight haplotypes emerged from 59 worldwide accessions. From allele data of three polymorphic loci in six *B. cretica* populations from Crete, Edh et al. (2007) found the occurrence of five haplotypes and that all populations were fixed for a particular haplotype. In the study of Zamani-Nour et al. (2013), on the chloroplast diversity of three *Brassica* spp., applying 14 primers pairs to a collection of 49 genotypes, it emerged that *B. oleracea* was the most diverse with 13 different haplotypes, followed by *B. napus* with 12 haplotypes and then by *B. rapa* with a total of six haplotypes.

The within-population chloroplast gene diversity ($H_{cp, pop}$, $uH_{cp, pop}$) did not significantly differ among populations, and this result was explained by the fact that three of five loci were monomorphic and their correspondent diversity parameters were equal to zero for all the populations. However, the lowest diversity (H_{cp}) was found for one of the smallest

populations (ES-1), while the highest for a French population (FR-3). *M. littorea* average H_{cp} , over all loci (0.11) was lower than the worldwide *C. bursa-pastoris* (0.19; Ceplitis et al. 2005) but higher than the island *B. cretica* (0.00) where no chloroplast variation was found within populations since all the alleles were fixed within a population (Edh et al. 2007).

Similarly to chloroplast loci, the within-population nuclear variability ($Ho_{nuc, pop}$, $He_{nuc, pop}$, $uHe_{nuc, pop}$) did not differ among the investigated *M. littorea* populations. The nuclear genetic diversity over all loci was 0.33, similar (Clauss et al. 2002; Gaudeul et al. 2007) or higher (Edh et al. 2007) to those found for other perennial Brassicaceae species. One of the Italian populations (IT-3) showed the lowest diversity (He) and allelic richness (R_s) values, indicating that the genetic drift may have a greater influence in this population, since it is a very small population. Conversely, one of the French peripheral populations (FR-1) that showed a low value of allelic richness, exhibited a F_{IS} value close to 0 and one of the highest values of gene diversity (He), indicating a possible founding event from the nearby populations. Populations ES-1 and IT-1, despite being among the smallest populations, showed a genetic situation similar to a larger French population (FR-4), with quite high genetic diversity and allelic richness values and the occurrence of private alleles. The main difference was that this French population showed a high and significant value of F_{IS} . Similarly, all the other French populations, except FR-5, showed positive F_{IS} , that is a likely indication of a long-term separation of this area from the other areas of the species' range. Similarly, all Italian populations except IT-3 showed positive F_{IS} , while conversely, all Spanish populations were found to show a negative F_{IS} , that may be explained by a higher gene flow between this area and the southern Spanish populations not considered in the present study. Gijbels et al. (2015), reviewing 28 studies about the relationship between orchid population size and genetic diversity, showed that the genetic diversity decreased with decreasing population size, even if F_{IS} did not significantly change with population size. A possible reason for a lack of a relationship between F_{IS} and population size is a selection process against less fit homozygotes in small orchid populations (Gijbels et al. 2015).

For *M. littorea* populations investigated in this study, the within-population genetic variability, both at chloroplast and at nuclear loci, was not correlated with the area occupied by the populations, which was an indirect estimator of the population size. Using computer simulation, Lacy (1987) found that genetic drift was the predominantly force controlling the loss of heterozygosity over 100 generations in populations ranging in size

from 20 to 500. According to this simulation, Fischer and Matthies (1998a) found that *Gentianella germanica* (Gentianaceae) populations consisting of less than ca. 1000 fruiting individuals had reduced levels of genetic variability, with a significant positive correlation between molecular genetic variation and actual population size, probably due to the loss of variation in small populations through genetic drift.

The emerged lack of correlation between diversity levels and population size in the considered populations of *M. littorea* may relate to their histories. Populations that remain small for long periods are likely to be considerably less diverse than those that have only recently become small (Barrett and Kohn 1991), so it is possible that the small populations of *M. littorea* numerically declined only recently. Unfortunately, their histories were not known. It is clear that knowledge of the history of the populations, such as the extent of the historical distribution, is of vital importance both for formulating expectations and for interpretation of results (Ouborg and Van Treuren 1995).

Another reason for such a level of genetic diversity in the small and isolated Italian populations of *M. littorea*, similar to the larger and less isolated populations, may be the presence of a reintroduced population in the vicinity of the wild populations (De Vitis 2011). This reintroduced population, which consisted of ca. 190 reproductive individuals in 2013 and which originated from local seed sources, may have facilitated gene flow among the three native populations, allowing the maintenance of quite high levels of genetic diversity, but without altering the local genetic identity, as it is evident from the results of this study.

3.4.3. Population structure

A comparable population structure was observed using both chloroplast and nuclear data. In particular, visualizing Φ_{PT} and F_{ST} values with a principal coordinates analysis, three main clusters emerged: one cluster includes Portuguese and French populations, one includes the Spanish populations, and the last one with the Italian populations. The results obtained with the software STRUCTURE, partially confirmed the PCoA pattern at the nuclear microsatellite loci. In fact, at $K = 2$, the presence of two distinct genetic clusters was highlighted, with one of them including the northern Portuguese and the French populations and the other one formed by the Spanish populations and one Italian population (IT-3). The southern Portuguese and the other two Italian populations (IT-1 and

IT-2) showed intermediate traits between the two clusters. Even the cluster analysis conducted with Diva-GIS, based on Nei genetic distance, underlined the similarity between the French and the Portuguese populations, and the occurrence of a cluster formed only by the Spanish populations. Isolation-by-distance effect was not significant due to the similarity between distant populations, such as French and Portuguese populations. Despite the significant differentiation among populations, the variation within populations was higher than among populations for both chloroplast and nuclear microsatellites, which may indicate the lack of a clear geographical structure and a high level of gene flow. In fact, the overall differentiation values ($\Phi_{PT} = 0.44$, $F_{ST} = 0.13$), and the average migration rates ($Nm = 0.63$ and 1.71 for chloroplast and nuclear microsatellite loci, respectively) were lower and higher, respectively, than in other perennial Brassicaceae species, indicating higher levels of gene flow in *M. littorea*. High F_{ST} estimates of both chloroplast (1.00) and nuclear (0.63) microsatellite loci found by Edh et al. (2007) in *Brassica cretica*, together with an average migration rate of 0.29, clearly revealed an exceptional degree of genetic differentiation among *B. cretica* populations from Crete, and so that they have been heavily influenced by genetic drift. Gaudeul et al. (2007) investigated the genetic diversity of Scandinavian populations of *A. lyrata* spp. *petraea* and found a strong among-population differentiation ($F_{ST} = 0.28$) based on nuclear diversity. Cires et al. (2011), studying five isolated populations of *Cochlearia pyrenaica* through AFLP markers, found a regional divergence with high genetic differentiation values both at chloroplast (0.47) and at nuclear (0.39), with unlikely gene flow among the populations.

As chloroplast genome is maternally inherited in most plant species, the more limited dispersal ability of seeds compared to pollen may further enhance population differentiation of chloroplast loci, compared to nuclear loci, and, as a result, F_{ST} estimates for chloroplast markers are often higher than for nuclear markers (Petit et al. 2005). This was confirmed for *M. littorea*. The high level of gene flow detected across the European populations, especially for nuclear genome, could be due to a long-distance pollen flow acted by pollinators such as the migratory butterfly *Colias croceus*, that is a pollinator of *M. littorea* and is known to migrate from south-western Europe up to Britain (Sparks et al. 2007). Bensusan et al. (2014) suggested that this migratory butterfly species, migrating between Africa and Europe, uses stopover sites, such as Gibraltar, to refuel and to successfully reach more northerly sites.

3.4.4. Possible phylogeography of *Malcolmia littorea*

The present distribution of plant species is determined not only by current suitable climatic and biotic conditions but also by historic events (Vogel et al. 1999), and the identification of refugia may be important for conservation plans, since these areas represent climatically stable areas and key areas for the long-term persistence of species and genetic diversity (Médail and Diadema 2009). From several studies in which postglacial colonization routes in Europe were compared, it emerged that each species has its individualistic migration behaviour and has responded independently to Quaternary cold periods (Taberlet et al. 1998; Petit et al. 2003). However, some common patterns emerged. Several authors hypothesised that Mediterranean peninsulas (Iberian, Italian, and Balkans-Greece) have served as refugia during the warm and cold stages in the Quaternary (Taberlet et al. 1998; Vogel et al. 1999; Hewitt 2004; Petit et al. 2003; Médail and Diadema 2009). Here taxa may have survived and it can be assumed that large areas of Europe (Scandinavia, the British isles and Central Europe, north of the Alps) were colonized from refugia in the Mediterranean area following the retreats of the ice after the Weichselian glaciation (Vogel et al. 1999).

Petit et al. (2003), investigating patterns of chloroplast DNA diversity in 22 woody species sampled in 25 European forests, suggested that several species, originating from Italy or the Balkans, entered Iberia from the north. Médail and Diadema (2009), after having selected and analysed 80 papers about intraspecific plant phylogeography, identified 52 refugia within the Mediterranean region, including the regions of Beira Litoral, Estremadura, Algarve (Portugal), Cadiz/Algeciras, Serrania de Ronda, Sierra Nevada/Gata and Valencia (Spain). These regions are nowadays inhabited by *M. littorea* and may have act as refugia for this species in Europe.

Coastal plant taxa offer several advantages when trying to infer distributional history and recognize the effect of species-specific biology on geographical patterns of genetic variation: they often are azonally distributed and have both latitudinally and longitudinally large geographical ranges, which are likely to still contain both refugial and recolonized areas; in contrast to inland species, they show a linear distribution range which limits the spatial options for migration to take place along the coasts, or across the seas where suitable dispersers occur (Kadereit et al. 2005). This drastically reduces the number of potentially possible migration routes into and out of refugial areas and easily allows reconstruction of distributional limits in glacial times (Kadereit et al. 2005). Kadereit et al.

(2005) compared the phylogeography of five coastal plants at a European scale, based on AFLP variability, and found that all the species, except one, shared an Atlantic Ocean/North Sea/Baltic Sea cluster, that reached from Gibraltar along the entire coasts of these seas, and that was separated from the eastern Spain cluster. The role of the Strait of Gibraltar, as biogeographical barrier, has acted as a filter to plant migration in accordance with certain life-history and dispersal traits of species (Lavergne et al. 2013). The role of biogeographical barriers such as the Strait of Gibraltar would be expected to influence phylogeographic patterns, though different views have been put forward (Juan et al. 2012).

The phylogeography of *M. littorea* is still unclear, but in the light of the results from this study, it may be possible that this species had its refugia in central Italy and in the Iberian peninsula, and that the present distribution is the result of fragmentation and differentiation processes. The absence of a strong geographical structure and the occurrence of a high gene flow level may point to the existence of a single European metapopulation (Juan et al. 2012). However, the disjunct distribution of *M. littorea* across Europe, as a common attribute of many Mediterranean species, may reflect the geological complexity of the Mediterranean basin and/or the movement and isolation of tectonic microplates in the Tertiary (Thompson 1999). The interpretation of disjunct distributions has been centred on two hypotheses: first, the existence of populations of the same species in disjunct areas may result from a barrier arising within a previously more widespread distribution of a single taxon; and second, the disjunct distributions may result from dispersal of organisms across pre-existing physical and/or ecological barriers from a central zone of origin (Thompson 1999).

3.5. CONCLUSIONS AND IMPLICATIONS FOR CONSERVATION

The identification of conservation units (Cires et al. 2011), or genetic clusters, is necessary so that management and monitoring programmes can be efficiently targeted (Juan et al. 2012). On the basis of the genetic data derived from 12 European native populations of *M. littorea* and the statistical results, the subdivision of the populations into three main genetic clusters emerged. In particular, inside each one of these clusters, one population was identified as characterised by peculiar genetic attributes which make it a priority for conservation purposes. Among these three populations, only the French one at *Plage des Aresquiers* (FR-4) is physically protected, while the other two populations (ES-1, *Playa de Moncófar*, Castellón and IT-1, *Torre Olevola*, Latina) are very small, with genetic drift maybe playing a considerable role in reducing their fitness, and are threatened by human activities. Furthermore, the Italian one (IT-1, *Torre Olevola*, Latina), together with the other two Italian populations, form a highly isolated genetic cluster. Although populations are capable of purging their genetic load under some circumstances, purging appears neither consistent nor effective enough to reliably reduce inbreeding depression in small and inbred populations (Byers and Waller 1999). However, the possible occurrence of inbreeding depression was not evident from neutral markers which are not affected by selective pressures. Thus, further studies involving adaptive genes may be useful to investigate the impact of populations decline on their fitness. Furthermore, good fossil records, such as pollen records, together with a solid knowledge of distributional history of a species, may be helpful to distinguish historical and extant factors determinants of genetic structure and to speculate on the location of refugia (Hewitt 2000, Kadereit et al. 2005).

The unexpected high gene flow detected among *M. littorea* populations was maybe due to long-distance pollinator movements. The goal of management should be to preserve this natural network of genetic connections, rather than just distinct populations within the network, in order to preserve fundamental evolutionary processes (Crandall et al. 2000).

CHAPTER 4

INVESTIGATION ON SEED GERMINATION REQUIREMENTS AND THE FACTORS AFFECTING REPRODUCTIVE FITNESS

4.1. INTRODUCTION

Consideration of the ecological aspects of reproduction by seed of a target species is essential to understand its germination requirements which may be helpful for its maintenance in the wild (Cochrane et al. 2002). From the time that the seed is formed, it becomes liable to a succession of hazards in which seed size is relevant: it may be eaten by predators or attacked by pathogens while it is on the plant or after it is shed; it may be buried too deep or not deep enough, and if it germinates, the seedling may find itself in a hostile environment or involved in a struggle for existence with other plants (Harper et al. 1970). Seed mass is a key trait in plant reproductive biology and population dynamics and is linked to many other species attributes. It can be used for the assessment of dispersal and colonization ability and thereby contribute to conservation biology of species in fragmented landscapes (Eriksson 2000). Seed mass is a likely indicator of quality, which is manifested both as germinability and rate of germination (Greipsson and Davy 1995).

In most plant species, the seeds vary in their degree of germinability among and within populations and among and within individuals (Greipsson and Davy 1995; Ouborg and Van Treuren 1995; McKee and Richards 1996; Gutterman 2000; Jacquemyn et al. 2001), and these variations could be correlated to the population fitness (e.g. Menges 1991) or to the environmental conditions (e.g. McWilliams et al. 1968; Probert et al. 1985a,b; Daws et al. 2004; Pendleton and Meyer 2004; Orrù et al. 2012).

In small and isolated populations, fitness reductions due to higher frequencies of intraplant pollinations and to lowered genetic variation could be exposed early in the life of

the sporophyte plant (e.g. fruit set, seed set, and germination), and thus, germination differences among small and large populations could well reflect inbreeding effects (Menges 1991; Ellstrand and Elam 1993). Inefficient germination of seeds is a likely result of the effects of fragmented-caused reduced genetic variation, and a laboratory seed germination experiment could detect it (Menges 1991).

On the other hand, much of the germination variation among populations it is known to be phenotypic and caused by the micro-environmental conditions under which the seeds matured (Gutterman 2000; Pendleton and Mayer 2004; Donohue et al. 2005). Weather during the months of pollination and seed maturation has also been reported to be an important influence on seed production (McKee and Richards 1996).

The recognized relationships between seed germination requirements and ecophysiological characteristics will assist in the identification of the relevant conditions necessary for the stimulation of the target species (Cochrane et al. 2002). Several key factors influence the germination of a vital seed, including seed ageing (Pemadasa and Lovell 1975), temperature (Garcia-Huidobro et al. 1982; Probert 2000), light (Thanos et al. 1994; Luna et al. 2004), seed burial (Pemadasa and Lovell 1975; Ma and Liu 2008), salinity (Meot-Duros and Magné 2008; Santo et al. 2014) and soil moisture (Maun 1994).

Seed ageing may result in a rise in the maximum temperature at which germination can occur (Pemadasa and Lovell 1975). Different species exhibit differing mechanisms of delaying germination until the optimal requirements are fulfilled (Maun 1994) and mechanism of dormancy breakage appear to be important for determining the timing of germination (Donohue et al. 2005).

Seed germination responses to temperature can be summarized in terms of three cardinal temperatures, namely the base, or minimum (T_b), the optimum (T_o), and the ceiling, or maximum (T_c) temperatures (Bradford 1995; Yan and Hunt 1999), which define the thermal response of a seed sample (Garcia-Huidobro et al. 1982). The progressive germination of seeds within a sample can be presented by plotting the germination fraction (G) from zero to a maximum fraction, and when the sample is large enough, G increases with time both smoothly and reproducibly. The most basic way to define the germination time (t) is the time to 50% germination, and the corresponding rate of germination, $1/t$ often increases linearly with temperature T up to an optimum T_o , beyond which the rate decreases. When the rate is linear, extrapolation defines a base temperature, T_b , at which the rate is zero (Garcia-Huidobro et al. 1982). Both the rate of germination and the fraction of germinated seeds after a fixed period of time are important in understanding and

predicting the effects of environment on germination, and temperature affects them in different ways. In particular, the rate of germination has a sharply defined optimum (T_o), whereas the highest values of the maximum fraction of seeds germinated are achieved over a range of temperatures (Garcia-Huidobro et al. 1982). Garcia-Huidobro et al. (1982) supposed that a batch of seeds contains a large number of sub-sets of identical individuals, each sub-set having a thermal response defined by distinctive values of T_b and being identified by a value of G (for example, $G = 0.50$ identifies the sub-set which germinates immediately after 50% of the population has already germinated). In the sub-optimal temperature range, the general situation for nondormant seeds is that there is little variation in T_b among individual seeds and sub-sets (Ellis et al. 1987; Garcia-Huidobro et al. 1982).

The optimal temperatures for seed germination of coastal Mediterranean species are typically within the range of 5-15 °C, with germination percentages decreasing at higher temperatures (Thanos et al. 1989, 1995; Tlig et al. 2008). Mediterranean species are also characterised by having a slow germination at cool temperatures which becomes more rapid with increasing temperatures (Skordilis and Thanos 1995; Thanos et al. 1995; Doussi and Thanos 2002) as an adaptation to the moisture plentiful during autumn and winter and to the unpredictable rainfalls and low soil moisture during spring and summer (Pemadasa and Lovell 1975; Maun 1994).

The light response can control the timing of germination in the field, a crucial factor for the survival of the resulting seedlings (Luna et al. 2004). Ellis et al. (1989), studying the response of seed germination in seven genera of Brassicaceae to white light, showed that the action of light on the seed germination was positive in the case of low energy reaction (LER), and negative in the case of the high irradiance reaction (HIR). In coastal species of Mediterranean climates, light-inhibited seed germination has been reported (e.g. Thanos et al. 1989, 1991, 1994). This mechanism is of adaptational value in the sea-shore or sandy habitats, since germination on the surface of the sand might present a great hazard for newly germinated seedlings, due to rapid evaporation and large temperature fluctuations (Thanos et al. 1989).

For any seed size there is an optimal depth of burial for any given soil type (Harper et al. 1970). Sand burial results in seed concentration in deep soil layers and plays a key role in shaping the spatiotemporal pattern of the seed bank of active sand dunes and therefore is important for seedling emergence, recruitment, and the subsequent growth of pioneering annual dune plant species (Ma and Liu 2008). Furthermore, the seed burial increases soil

contact and relative humidity around the seeds, thus enhancing the probability of germination (Maun 1998).

Under the typical Mediterranean climate, characterised by irregular rainfall events and a harsh dry summer period, salinity may inhibit, even at low concentration, seed germination especially during summer (Maun 1994; Meot-Duros and Magné 2008; Tlig et al. 2008). On coastal sand dune systems, seed germination and seedling establishment occur when soil salinity is at low levels owing to leaching caused by high rainfall (Maun 1994). For Mediterranean species, the highest soil temperature are usually encountered in the summer months when dune soils may experience exceptionally high temperatures, with a very low moisture status. Presumably, in this period, the moisture and temperature regimes are not favourable for the germination of these species (Pemadasa and Lovell 1975). The seasonal pattern of coastal dune species emergence suggests that the peak emergence is when the soil temperatures are low and the moisture is not a limiting factor (Maun 1994). In seeds that dehydrate at maturity, the germination process is initiated by the imbibition of water by the dry seed (Bradford 1995). Sometimes, specific conditions defined by a combination of the above-mentioned factors, is necessary to promote seed germination (Barazani et al. 2012). When comparing the germination of different species or different populations of the same species, seeds to be used in the experiments should be stored following a standardized protocol, to avoid the influence of different conditions on seed storage (Keller and Kollmann 1999).

Until the beginning of the present study, few studies investigated specific traits of *M. littorea* seed ecology such as the effect of moisture, pH and salinity on seed germination (Novoa et al. 2012) and the effect of water content and temperature on seed longevity (Mira et al. 2015). Light, temperature and burial requirements for seed germination were unknown, and laboratory research were useful to obtain vital information about these requirements. Knowledge of the differences among different plant genotypes in their temperature requirements for the germination is an important aspect when endeavouring to understand differential interactions between plant genotype and environments (Yan and Hunt 1999). Moreover, germination differences among populations could well reflect inbreeding effects, so to consider the possible effects of fragmented-caused reduced genetic variation, the population differences in seed germination must be correlated with population size (Menges 1991).

The objectives of this germination study were to:

- investigate the seed traits and the basic requirements for seed germination of *Malcolmia littorea*;
- evaluate how different seed provenances can affect germination;
- assess how population size and environmental factors affect the germination response;
- interpret the findings in the context of implementing conservation and management programs.

4.2. MATERIALS AND METHODS

The seed ecology analyses were carried out at both Tuscia Germplasm Bank laboratory (Tuscia University, Viterbo, Italy), and seed science laboratories of Royal Botanic Gardens, Kew, Wakehurst Place (Ardingly, UK).

In a first study, the basic requirements for seed germination (light, temperature, maximum burial depth for seedling emergence) were investigated, since they were unknown for *Malcolmia littorea*, using seeds collected from IT-1.

During 2009–2011, using seeds produced by the Italian native populations, an experimental reintroduction was performed by *in situ* seed sowing and plant transplanting (*ex situ* seed sowing and transfer of the grown plants), and resulted in an experimental population (*sensu* Caplow 2004) of the species, established inside its historical range (De Vitis 2011), 100 m far from IT-1. The temperature response of IT-1 was compared with the above mentioned reintroduced population and with one *ex situ* cultivated population.

Subsequently, a comparative study on seed ecology was conducted among the European populations sampled in 2013 (Table 4.1).

4.2.1. Germination tests

In all the germination experiments, five replicates of 20 seeds per population were sown on the surface of 1% water-agar, which provided a solid, non-sterile medium for germination, in 90 mm plastic Petri dishes. Germination was defined as visible radicle emergence. At the end of the germination tests, a cut-test was carried out to determine the viability of the remaining seeds (soft or firm) and the final germination percentage was calculated on the basis of the total number of filled seeds as the mean of the five replicates $\pm$ standard error (SE).

4.2.2. Seed germination basic requirements

The following populations of *M. littorea* were sampled for ripe siliquas in 2011, at the time of natural dispersal: one native population (IT-1; Table 2.1), one population cultivated at the Botanical Garden of Rome (BG) and one reintroduced population (IT-R), formed by

plants deriving by both *in situ* sowing (RE1) and *ex situ* sowing followed by transplanting of the grown plantlets (RE2). All the fruits were cleaned in the laboratory and the seeds were stored in glass vials at room conditions (+20 °C) for three months until the experiments started. During summer 2013 seeds of IT-1 were collected again and sown two days after collection. Hereafter, stored seeds of IT-1 are called IT-S, while the fresh ones are called IT-F (Table 2.3). Average seed mass was calculated for each seed lot by weighing five replicates of 50 seeds each.

Table 4.1. Provenance, habitat, altitude, population size and the average seed mass ($\pm$ standard deviation) for each seed lot of *M. littorea* investigated in this study. One-way ANOVA was performed to compare average seed mass; values with different letters are significant at $P < 0.05$.

Seed Lot	Provenance	Plant Habitat	Altitude (m a.s.l.)	Population size	Seed mass (mg)
IT-S	Native population (stored seeds)	Coastal sandy dune	3	30	0.14 $\pm$ 0.01b
IT-F	Native population (fresh seeds)	Coastal sandy dune	3	30	0.15 $\pm$ 0.01b
BG	Botanical Garden of Rome	Living collection	17	10	0.16 $\pm$ 0.01b
RE1	Reintroduced population (by sowing)	Coastal sandy dune	2	120	0.15 $\pm$ 0.01b
RE2	Reintroduced population (by transplanting)	Coastal sandy dune	2	13	0.17 $\pm$ 0.00a

4.2.2.1. Effect of light irradiance, temperature and storage

Stored (IT-S) and fresh (IT-F) seeds belonging to the native population were incubated at 15 °C under different light conditions. In particular, seeds were exposed to a 12 h photoperiod (12 h) with a ratio of red:far red of ca. 2.0, a light quantity of ca. 7 $\mu\text{mol}/\text{m}^2/\text{s}$ and density of 50–100 W/m^2 , and in the dark, achieved by wrapping the dishes in two layers of aluminium foil. Seeds were scored for germination every two days. In the darkness, seeds were either scored under safe green light (GL; light density of 14.0 W/m^2) or only at the end of the experiment (D; 26 days).

Seeds belonging to IT-S, BG, RE1 and RE2 were incubated in the dark and scored every two days under safe green light at both constant (5, 10, 15, 20 and 25 °C) and alternating (15/5, 20/10 and 25/15 °C) temperatures. As more seeds of IT-S were available for experimental work, they were also germinated at constant temperatures of 30 and 35 °C.

4.2.2.2. Experimental seed burials

Five replicates of 20 seeds of IT-S were placed on top of the 55 mm diameter Whatman no. 1 filter paper and buried at different depths in six black plastic boxes (174 × 115 × 60 mm) with transparent lids. Each box was filled with 1060 g of sand, with grain sizes between 0.8 and 0.005 mm, pH 6.0–7.5 (ISTA 2006), and moistened with 105 ml of distilled water, calculated on the basis of previous experiments with seeds of comparable size (unpublished internal report of the Seed Conservation Department, Royal Botanic Gardens, Kew, UK). Replicates were buried at 1, 5, 10, 15, 20 and 25 mm. The boxes were incubated at the constant temperature of 15 °C and 12 h photoperiod (ratio of red:far red of ca. 2.0, a light quantity of ca. 7 $\mu\text{mol}/\text{m}^2/\text{s}$ and density of 50–100 W/m^2) and checked every two days for seedlings to emerge beyond the sand surface. Emerged seedlings were removed. After 26 days, the replicates were exhumed and the numbers of germinated non-emerged seedlings were scored and the viability of the non-germinated seeds was checked by a cut-test. The percentage of emerged seedlings was then recalculated on the basis of the filled seeds, as the mean of the five replicates $\pm$ standard error (SE).

4.2.3. Comparative study on European populations

The comparative study on seed ecology was conducted using seeds of all the populations reported in Table 2.1, except one Italian population (IT-2), due to a poor fruiting in 2013.

Ripe siliques were harvested in each population from 10-50 adult plants, according to the population size, at the time of natural dispersal (July-August). They were stored in paper bags until they were cleaned in the laboratory. Then, seeds were stored for one month in a dry room at +15 °C and 15% RH (relative humidity) in individual glass vials for each population and then they were stored at -20 °C for one year and four months, until the experiments started.

Seeds remaining from the experiments were stored at -20 °C for long-term seed banking in both Tuscia Germplasm Bank (Tuscia University, Viterbo, Italy) and Millennium Seed Bank (Royal Botanic Gardens, Kew, Wakehurst Place).

4.2.3.1. Seed mass, moisture content, oil content, x-ray analysis and the effect of temperature on seed germination

Seeds of all populations were defrosted in a dry room (15 °C, 15% RH) for 24 h, then for each population average seed mass was calculated by weighing five replicates of 50 seeds each (fresh seed mass: *FSM*), using the Mettler Toledo UMT2 analytical balance (four decimal places). Then, the seed moisture content (*MC*) was calculated by drying these replicates at 103°C for 17 h, and then weighing them again (dry seed mass: *DSM*). The *MC* was calculated by the following formula:

$$MC\% = [(fresh\ mass - dry\ mass) / fresh\ mass] \times 100.$$

The seed oil content (*OC*) was measured by time domain nuclear magnetic resonance (TD-NMR). For each population the oil content was quantified on three replicates of seeds which had been stored at 15 % RH (relative humidity). Each replicate consisted of at least 29.3 mg of seeds (ranging from 29.3 – 390.3 mg). The seeds were analysed using a Bruker mq20 minispec with a 0.47 Tesla magnet (20 MHz proton resonance frequency) operating at 40 °C. Because of the small samples sizes, a 10 mm probe assembly was used. Samples were analysed using a sunflower oil calibration where the method consisted of the acquisition of 16 scans with a recycle delay of 2 seconds.

One-hundred seeds of each population were analysed with x-ray to evaluate the quality of the seed lot, identifying empty and infested seeds.

Furthermore, seeds of each population were incubated in the dark at eight constant temperatures (0, 5, 10, 15, 20, 23, 25, and 27 °C) and scored every day under green safelight (light density of 14.0 W/m²). The dark was achieved by inserting the dishes inside light-proof aluminium foil bags. The germination experiment lasted for a maximum of 30 days, at which time no further germination was observed.

4.2.4. Data analysis

Average seed mass, moisture content, oil content and final germination percentage values were transformed when necessary and analysed with a t-test (paired and unpaired; for normally distributed data) or with the Wilcoxon (paired) and Mann–Whitney (unpaired) tests (for data not normally distributed) to compare two groups of data. When more than two groups were compared, a one-way ANOVA (for normally distributed data)

or Kruskal–Wallis (for data not normally distributed or when the assumption of homoscedasticity was rejected), was followed by a post-hoc test (Tukey's or Mann–Whitney pairwise comparison). Two-way ANOVA was also performed to identify a significant effect of independent variables, and their interaction, on a dependent variable.

Germination rate (GR), defined as the reciprocal of the time to reach a specific percentage of the maximum germination in one replicate, was calculated considering each seed lot to be composed of three subpopulations (Garcia-Huidobro et al. 1982). In particular, days required to achieve 25, 50 and 75% germination (t_{25} , t_{50} and t_{75}) and their respective germination rates (GR_{25} , GR_{50} , and GR_{75}) were calculated for each population. Then, germination rate of each subpopulation was plotted as a function of temperature and regressed using an ordinary least squares (OLS) linear model, to estimate the base temperature (T_b) as the intercept between x -axis and the sub-optimal model. The theoretical T_b , below which germination rate is equal to zero, was calculated for each population as the mean of the three subpopulations.

In the comparative study of European populations, regression lines calculated on the basis of GR_{50} were also statistically tested for homogeneity of slopes (Huitema 2011) and analysed using an analysis of covariance (ANCOVA) to test differences of germination rates in the response to constant temperature among the 14 populations, setting the temperature as independent variable, or covariate (x), and the germination rate as dependent variable (y).

Furthermore, all the dependent variables estimated in the comparative study were tested for correlation with several independent variables, such as climatic and geographic parameters or the area occupied by the populations ($AREA$), to understand which are the driving forces of the differences among populations. In particular, using the linear regression analysis methods and transforming data when necessary, the following correlations were explored: fresh seed mass (FSM), dry seed mass (DSM), moisture content (MC), and oil content (OC) with min (T_{min}), max (T_{max}) and mean (T_{mean}) temperature and mean precipitation (Prec.) of April, May and June (the months in which the flowering and the seed production occur) over the period 1950–2000, min (T_{min}), max (T_{max}) and mean (T_{mean}) temperature and relative humidity (RH) of April, May and June 2013, latitude (LAT) and $AREA$; final germination percentages ($G\%$), germination rates (GR) and base temperature (T_b) with T_{min} , T_{max} and T_{mean} and Prec. of October, November and December (the months in which the germination could occur) over the period 1950–2000, T_{min} , T_{max} and T_{mean} and RH of October, November and December 2013, LAT and $AREA$. When a

significant correlation was found, the appropriateness of the linear regression model was assessed examining the residual plots.

Mean temperature and precipitation values over the period 1950-2000 were extrapolated for the specific populations' localities from WorldClim climate grids (Hijmans et al. 2005) using the software Diva-GIS 7.5 (Hijmans et al. 2012), while temperature and relative humidity mean values of the year 2013 registered by the meteorological stations closest to each population site were obtained from the National Climatic Data Center (NCDC) of the National Oceanic and Atmospheric Administration (NOAA, Washington, DC, USA).

Furthermore, the correlation structure of fruiting adaptation was investigated by principal components analysis (PCA) on a data matrix with standardised (z-transformed) variable values, using the parameters *DSM* (dry seed mass), *MC* (moisture content), *OC* (oil content), and *LAT* (latitude). Similarly, the correlation structure of germination response to temperature was investigated by a PCA using the final germination percentages at eight constant temperatures of each population. Correlation between single variables and PCA-axis scores were examined.

Software Past 2.13 (Hammer et al. 2001) was used to analyse data.

4.3. RESULTS

4.3.1. Seed germination basic requirements and their adaptation

4.3.1.1. Effect of light irradiance, temperature and storage

At 15 °C, exposure to 12 h photoperiod resulted in a very low germination percentage (1%) both for stored (IT-S) and fresh (IT-F) seeds, with no significant differences between them (Mann-Whitney $U = 12.50$, $P > 0.05$). Under dark condition with scoring every 2 days under green light (GL), seeds of IT-F reached their maximum germination ($37 \pm 8.9\%$) in 14 days, while those of IT-S in 6 days with a significantly higher germination percentage ($68 \pm 3.4\%$; $t = 3.30$, $P < 0.05$). In contrast, under continuous darkness (D) germination of IT-F ($13 \pm 3.4\%$) was significantly higher than that of IT-S (0%; Mann-Whitney $U = 0.50$, $P < 0.05$; Figure 4.1).

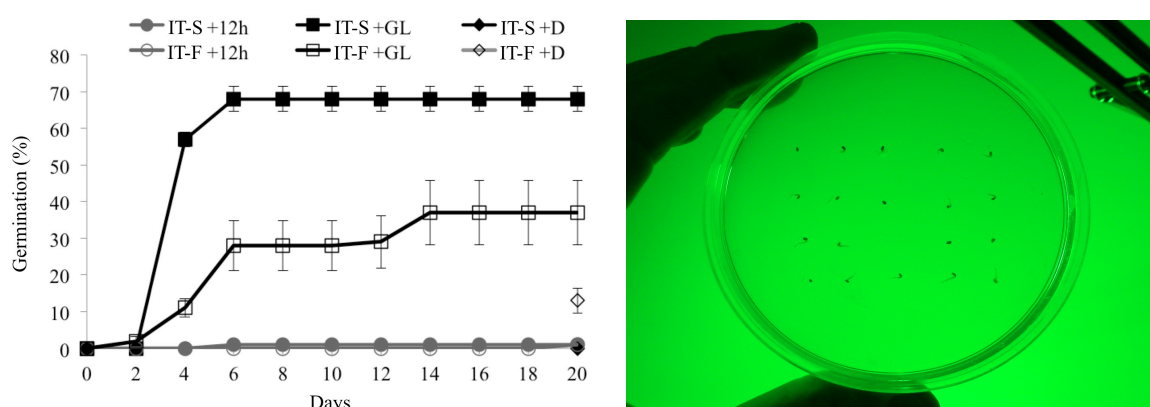


Figure 4.1. On the left: germination percentages (mean $\pm$ SE) of the natural population (IT-1) under 15 °C and different light (exposure to 12 h of white light, 12 h; dark with short exposure to green safelight for scoring, GL; and continuous darkness with scoring at the end of the experiment, D) and storage conditions (stored, IT-S; and fresh, IT-F). On the right: a Petri dish with germinating seeds of *Malcolmia littorea*, scored under the green safelight.

At constant temperatures, seeds from IT-S showed the highest final germination at 5 °C ($75.0 \pm 2.7\%$) and 10 °C ($64.0 \pm 7.6\%$) and a statistically significant decreasing trend (ANOVA/Kruskal-Wallis: $P < 0.001$) with increasing temperature, reaching less than 2.0% germination at 30 and 35 °C (Fig. 4.2). In contrast, the lowest germination percentage was detected at 5 °C in the other seed lots (RE1, RE2 and BG), with maximal germination between 10 and 25 °C (ANOVA/Kruskal-Wallis: $P < 0.05$; Fig. 4.2). A two-way ANOVA, applied on final germination percentages of all seed lots at 5–25 °C, highlighted that the

effect of provenance (Pr), temperature (T) and their interaction (Pr $\times$ T) were highly significant ($P < 0.0001$).

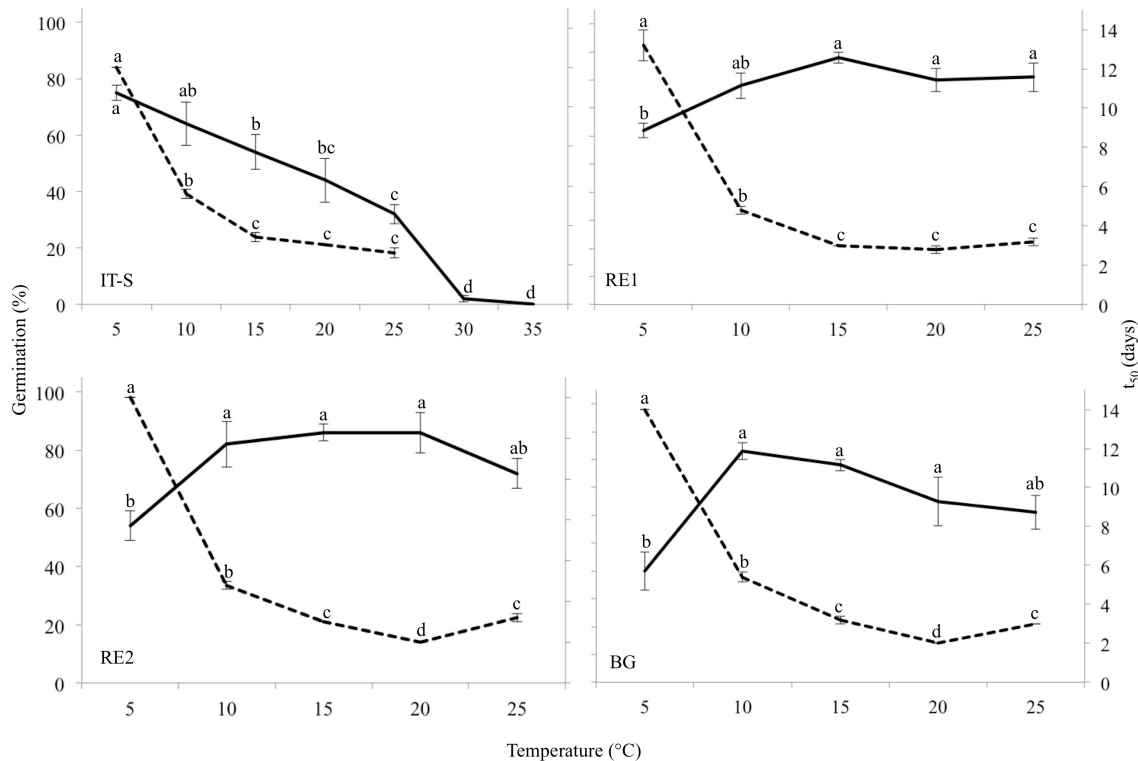


Figure 4.2. Germination percentage (G%; solid line; mean $\pm$ SE) and t_{50} (dotted line; mean $\pm$ SE) under constant temperatures in the dark with scoring under green safelight for IT-S, RE1, RE2 and BG. Within each seed lot, a significant difference amongst G% and t_{50} was detected by one-way ANOVA (for normally distributed data) and Kruskal–Wallis (for data not normally distributed or when the assumption of homoscedasticity was rejected) at $P < 0.01$. Bars with different letters are significant at $P < 0.05$ (post-hoc tests: Tukey's for normally distributed data and Mann–Whitney for data not normally distributed).

Under alternating temperatures, RE1, RE2 and BG showed high germination percentages in all the tested conditions, ranging from ca. 70 to ca. 90%, but significantly lower germination percentages of IT-S at 15/5 and 25/15 °C. A paired comparison of the germination percentages between alternating temperatures and their corresponding average constant temperatures showed that alternating temperatures did not affect the germination of IT-S and BG. For RE1, germination was higher at 15 °C than at 20/10 °C (Wilcoxon: $W = 15$, $P < 0.05$) and for RE2, germination was higher at 20 °C than at 25/15 °C ($t = 2.8$, $P < 0.05$; data not shown).

Under constant temperature regimes, t_{50} of all seed lots were significantly longer at 5 °C after which t_{50} shortened with increasing temperature. A two-way ANOVA applied on t_{50} values of all seed lots at 5–25 °C, showed that the effect of provenance (Pr) was not

significant, while the effects of temperature (T) and of the interaction of the two variables ($\text{Pr} \times \text{T}$) were highly significant ($P < 0.0001$). Alternating temperatures did not decrease the t_{50} in comparison with their corresponding average constant temperatures in any seed lot (data not shown).

4.3.1.2. Experimental seed burials

The highest percentage of seedling emergence was detected between 1 and 10 mm in depth (Fig. 4.3). From below 10 mm very few seedlings emerged (1% of all the buried seeds at depths ≥ 15 mm). At 1 mm, $20.0 \pm 3.8\%$ of the seeds were dead and $9.0 \pm 8.3\%$ of seedlings died after germination and all the emerged seedlings ($51.0 \pm 9.7\%$) were found to have an elongate hypocotyl under the very fine sand layer. At 5 mm $69.0 \pm 2.7\%$ of the seeds germinated and emerged and $25.0 \pm 5.0\%$ seeds germinated but did not emerge. At 10 mm the percentage of emerged seedlings was $41.0 \pm 11.1\%$ and the percentage of germinated non-emerged seedlings was $21.0 \pm 1.7\%$. At the depth 15 mm, $45.0 \pm 7.4\%$ of the seeds died and $48.0 \pm 5.5\%$ germinated without reaching the surface; at 20 mm, $31.0 \pm 7.3\%$ of the seeds germinated without emerging, a small percentage of seeds died ($9.0 \pm 4.2\%$) and a greater percentage remained viable under the sand ($57.0 \pm 11.9\%$); while at 25 mm almost all seeds died ($90.0 \pm 2.5\%$) and a few seeds germinated but did not emerge ($7.0 \pm 3.4\%$).

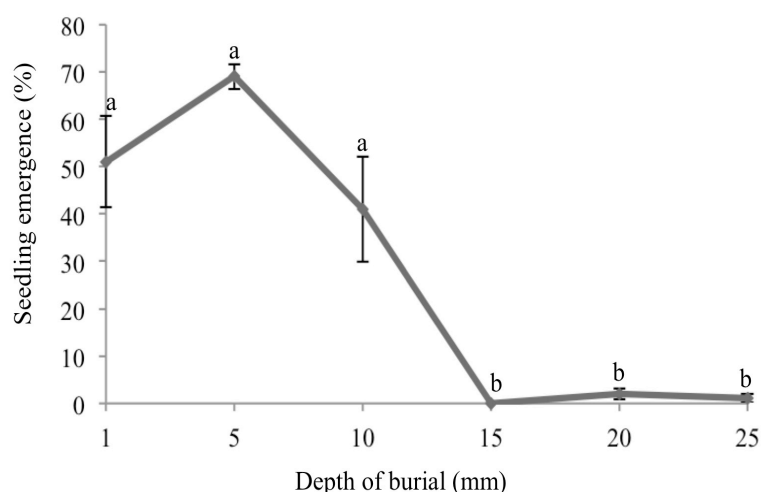


Figure 4.3. Seedling emergence of five replicates (mean $\pm$ SE) at 15 °C for the tested burial depths. Significant differences were tested by Kruskal–Wallis test ($P < 0.001$); bars with different letters are significant at $P < 0.05$ (by Mann–Whitney pairwise comparison).

4.3.2. Comparative study on European populations

4.3.2.1. Seed mass, moisture content, oil content and x-ray analysis

The comparison of the average fresh seed masses of the European populations revealed a significant difference among them (Kruskal-Wallis: $P < 0.0001$), as for dry seed mass (Kruskal-Wallis: $P < 0.0001$), moisture content (Kruskal-Wallis: $P < 0.0001$) and oil content (Kruskal-Wallis: $P < 0.01$) values (Table 4.2).

From x-ray analysis, FR-5 showed the lowest percentage of viable seeds (65%; Table 4.2, Fig. 4.4), while among the other populations the percentages of viable seeds were never lower than 81%.

Table 4.2. Mean values ($\pm$ standard error) of fresh (*FSM*) and dry (*DSM*) seed mass (mg), moisture content (*MC*) and oil content (*OC*) are reported for each of the 14 *Malcolmia littorea* populations. The percentage of intact, viable seeds is also reported on the basis of x-ray analysis on 100 seeds per population.

Seed lot	<i>FSM</i> (mg)	<i>DSM</i> (mg)	<i>MC</i> (%)	<i>OC</i> (%)	x-ray (%)
PT-1	0.15 $\pm$ 0.00	0.15 $\pm$ 0.00	5.02 $\pm$ 0.04	41.48 $\pm$ 1.17	97
PT-2	0.14 $\pm$ 0.00	0.13 $\pm$ 0.00	4.70 $\pm$ 0.06	40.07 $\pm$ 0.43	93
ES-1	0.13 $\pm$ 0.00	0.13 $\pm$ 0.00	4.81 $\pm$ 0.06	40.17 $\pm$ 0.03	100
ES-2	0.13 $\pm$ 0.00	0.12 $\pm$ 0.00	4.80 $\pm$ 0.04	40.17 $\pm$ 0.14	88
ES-3	0.14 $\pm$ 0.00	0.14 $\pm$ 0.00	5.32 $\pm$ 0.05	39.18 $\pm$ 0.13	95
ES-4	0.13 $\pm$ 0.00	0.12 $\pm$ 0.00	5.29 $\pm$ 0.36	40.70 $\pm$ 0.17	98
ES-5	0.14 $\pm$ 0.00	0.13 $\pm$ 0.00	5.14 $\pm$ 0.04	40.61 $\pm$ 0.12	95
FR-1	0.09 $\pm$ 0.00	0.09 $\pm$ 0.00	4.89 $\pm$ 0.07	42.79 $\pm$ 0.73	97
FR-2	0.12 $\pm$ 0.00	0.11 $\pm$ 0.00	4.29 $\pm$ 0.10	41.25 $\pm$ 0.72	81
FR-3	0.14 $\pm$ 0.00	0.14 $\pm$ 0.00	3.32 $\pm$ 0.36	38.62 $\pm$ 0.13	87
FR-4	0.13 $\pm$ 0.00	0.12 $\pm$ 0.00	5.08 $\pm$ 0.10	38.38 $\pm$ 0.16	83
FR-5	0.13 $\pm$ 0.00	0.12 $\pm$ 0.00	5.20 $\pm$ 0.53	34.79 $\pm$ 0.15	65
IT-1	0.14 $\pm$ 0.00	0.14 $\pm$ 0.00	5.62 $\pm$ 0.28	39.51 $\pm$ 0.52	83
IT-3	0.17 $\pm$ 0.00	0.16 $\pm$ 0.00	5.24 $\pm$ 0.06	37.16 $\pm$ 0.39	86

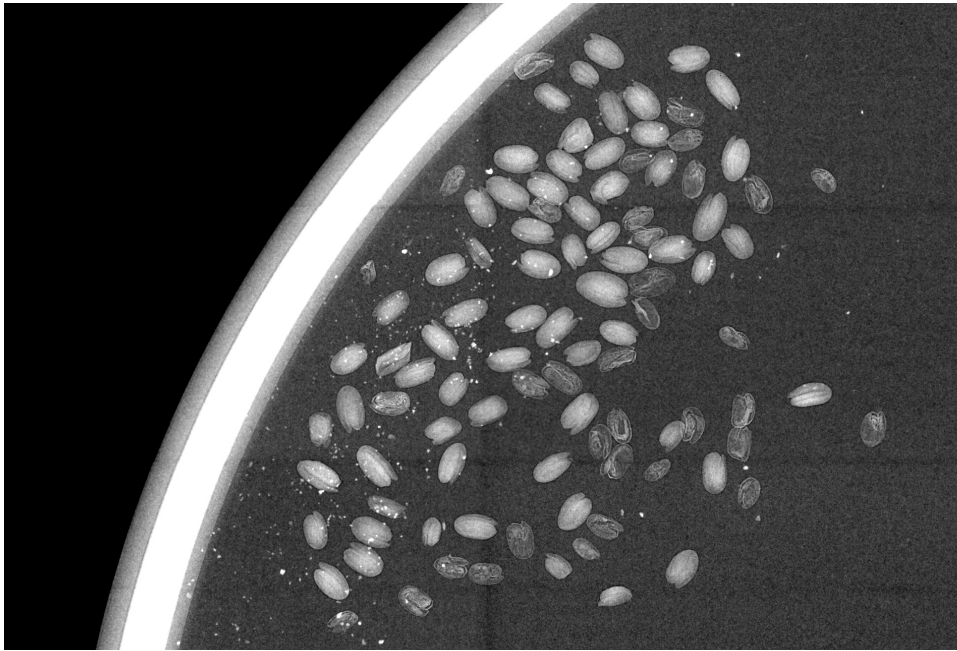


Figure 4.4. X-ray screening of seeds belonging to FR-5. Intact and firm seeds are recognizable by the white and uniform colour, while empty or infested seeds appear partially transparent and dark.

None of these dependent variables (fresh and dry seed mass, seed moisture and oil content) was significantly correlated with the climatic parameters, nor with the latitude or the occupied area.

From the PCA performed with the variables *DSM*, *MC* and *OC*, it did not emerge any significant gradient or pattern and no significant correlations were shown when the first two axes scores of the PCA were regressed against the latitude, occupied area, and the climatic parameters of 1950-2000 and 2013.

4.3.2.2. The effect of temperature on germination across Europe

At all the tested temperatures, the germination percentages ($G\%$) were significantly different both within (ANOVA/Kruskal-Wallis: $P < 0.001$) and among (ANOVA/Kruskal-Wallis: $P < 0.001$) the 14 populations (Table 4.3; Fig. 4.5 and 4.6) with IT-1 showing the lowest percentages when compared to the other populations. However, similar within-population trends emerged: the highest germination percentages were shown at 10 and 15 °C while the lowest were exhibited at 27 °C for most populations, and in some cases at 0 °C (Table 4.3; Fig. 4.5 and 4.6).

Table 4.3. Germination percentages (mean $\pm$ ES) of 14 *Malcolmia littorea* populations at eight constant temperatures. Within each population, a significant difference amongst $G\%$ was detected by one-way ANOVA (for normally distributed data) and Kruskal-Wallis (for data not normally distributed or when the assumption of homoscedasticity was rejected) at $P < 0.001$. Values with different letters are significant at $P < 0.05$ (post-hoc tests: Tukey's and Mann-Whitney after ANOVA and Kruskal-Wallis, respectively).

Seed lot	0°C	5°C	10°C	15°C	20°C	23°C	25°C	27°C
PT-1	57.0 $\pm$ 4.1 b	89.0 $\pm$ 3.7 a	96.0 $\pm$ 1.9 a	87.0 $\pm$ 2.5 a	68.0 $\pm$ 2.0 b	57.0 $\pm$ 4.1 b	25.0 $\pm$ 6.5 c	16.0 $\pm$ 1.9 c
PT-2	48.0 $\pm$ 4.1 b	77.6 $\pm$ 3.0 a	73.7 $\pm$ 2.9 a	86.0 $\pm$ 1.9 a	78.0 $\pm$ 3.4 a	77.4 $\pm$ 6.8 a	67.9 $\pm$ 6.1 ab	53.0 $\pm$ 4.6 b
ES-1	34.0 $\pm$ 1.9 bc	55.0 $\pm$ 5.2 ab	61.0 $\pm$ 4.3 a	55.0 $\pm$ 6.1 ab	55.0 $\pm$ 4.7 ab	35.0 $\pm$ 4.2 bc	26.0 $\pm$ 6.2 cd	12.0 $\pm$ 3.7 d
ES-2	74.8 $\pm$ 1.4 ab	80.3 $\pm$ 5.0 ab	87.5 $\pm$ 4.3 a	86.7 $\pm$ 4.2 a	73.6 $\pm$ 4.2 ab	74.5 $\pm$ 4.4 ab	59.1 $\pm$ 3.5 b	31.9 $\pm$ 2.0 c
ES-3	71.0 $\pm$ 2.4 c	85.7 $\pm$ 3.1 ab	89.8 $\pm$ 1.6 a	93.0 $\pm$ 3.0 a	89.0 $\pm$ 1.9 a	90.9 $\pm$ 1.9 a	73.4 $\pm$ 4.4 bc	56.4 $\pm$ 4.6 c
ES-4	67.7 $\pm$ 5.0 cd	81.0 $\pm$ 3.7 abc	85.0 $\pm$ 1.6 ab	96.0 $\pm$ 1.9 a	73.0 $\pm$ 3.4 bcd	61.0 $\pm$ 2.9 de	52.0 $\pm$ 4.4 e	31.0 $\pm$ 1.9 f
ES-5	44.5 $\pm$ 2.8 c	53.8 $\pm$ 7.1 bc	72.7 $\pm$ 2.5 a	64.6 $\pm$ 3.8 ab	62.0 $\pm$ 4.6 ab	63.0 $\pm$ 3.0 ab	37.4 $\pm$ 2.5 c	26.8 $\pm$ 2.7 d
FR-1	84.6 $\pm$ 4.3 bc	99.0 $\pm$ 1.0 a	98.0 $\pm$ 1.2 a	98.0 $\pm$ 2.0 a	93.0 $\pm$ 2.5 ab	79.0 $\pm$ 1.0 c	59.0 $\pm$ 5.3 d	24.0 $\pm$ 4.3 e
FR-2	87.0 $\pm$ 3.4 b	99.0 $\pm$ 1.0 a	99.0 $\pm$ 1.0 a	99.0 $\pm$ 1.0 a	99.0 $\pm$ 1.0 a	92.0 $\pm$ 4.1 ab	80.0 $\pm$ 4.2 bc	67.0 $\pm$ 3.4 c
FR-3	45.8 $\pm$ 5.9 e	88.0 $\pm$ 3.4 bc	93.0 $\pm$ 2.6 ab	97.0 $\pm$ 2.0 a	97.0 $\pm$ 1.2 a	85.0 $\pm$ 2.2 bcd	84.0 $\pm$ 1.9 cd	66.0 $\pm$ 7.3 de
FR-4	74.7 $\pm$ 1.6 b	96.0 $\pm$ 1.9 a	100.0 $\pm$ 0.0 a	100.0 $\pm$ 0.0 a	97.0 $\pm$ 2.0 a	97.0 $\pm$ 2.0 a	91.0 $\pm$ 4.0 a	68.9 $\pm$ 2.8 b
FR-5	79.8 $\pm$ 1.0 b	85.9 $\pm$ 2.9 b	96.9 $\pm$ 1.2 a	95.0 $\pm$ 2.2 a	96.9 $\pm$ 2.1 a	98.0 $\pm$ 1.2 a	57.2 $\pm$ 4.9 c	35.0 $\pm$ 4.2 d
IT-1	15.1 $\pm$ 3.1 c	44.0 $\pm$ 4.9 a	50.9 $\pm$ 5.4 a	44.7 $\pm$ 4.5 a	36.8 $\pm$ 4.9 ab	22.5 $\pm$ 3.5 bc	22.5 $\pm$ 2.3 bc	12.1 $\pm$ 2.5 c
IT-3	42.8 $\pm$ 1.9 b	46.4 $\pm$ 6.3 b	57.0 $\pm$ 3.7 ab	69.0 $\pm$ 4.8 a	60.0 $\pm$ 2.7 ab	54.0 $\pm$ 5.8 ab	45.6 $\pm$ 4.0 b	21.6 $\pm$ 3.8 c

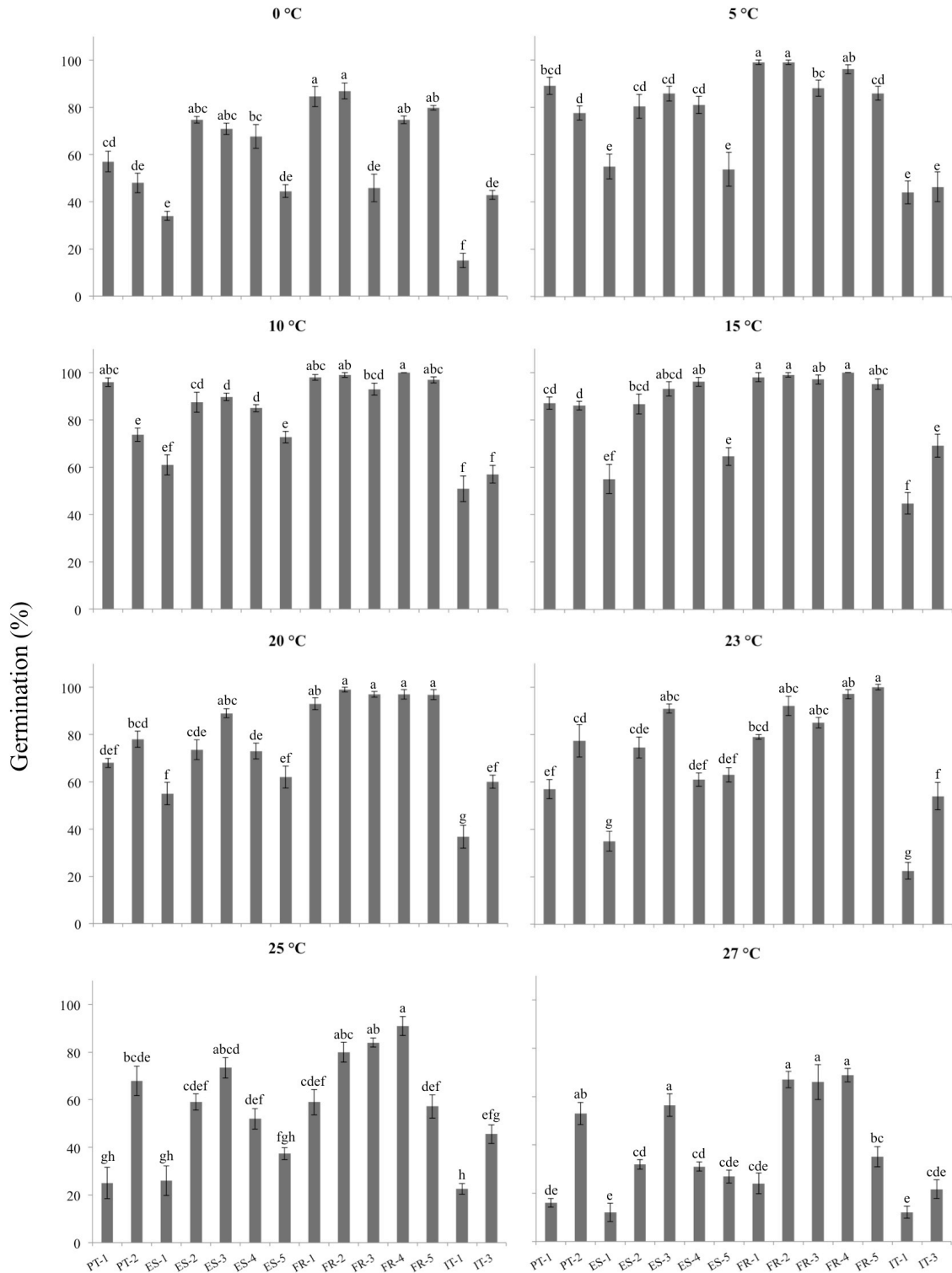


Figure 4.5. Germination percentages ($G\%$; mean $\pm$ SE) under constant temperatures (0-27 °C) in the dark with scoring under green safelight for the 14 *Malcolmia littorea* European populations. Within each temperature treatment, a significant difference amongst $G\%$ was detected by one-way ANOVA (for normally distributed data) and Kruskal-Wallis (for data not normally distributed or when the assumption of homoscedasticity was rejected) at $P < 0.0001$. Bars with different letters are significant at $P < 0.05$ (post-hoc tests: Tukey's for normally distributed data and Mann-Whitney for data not normally distributed).

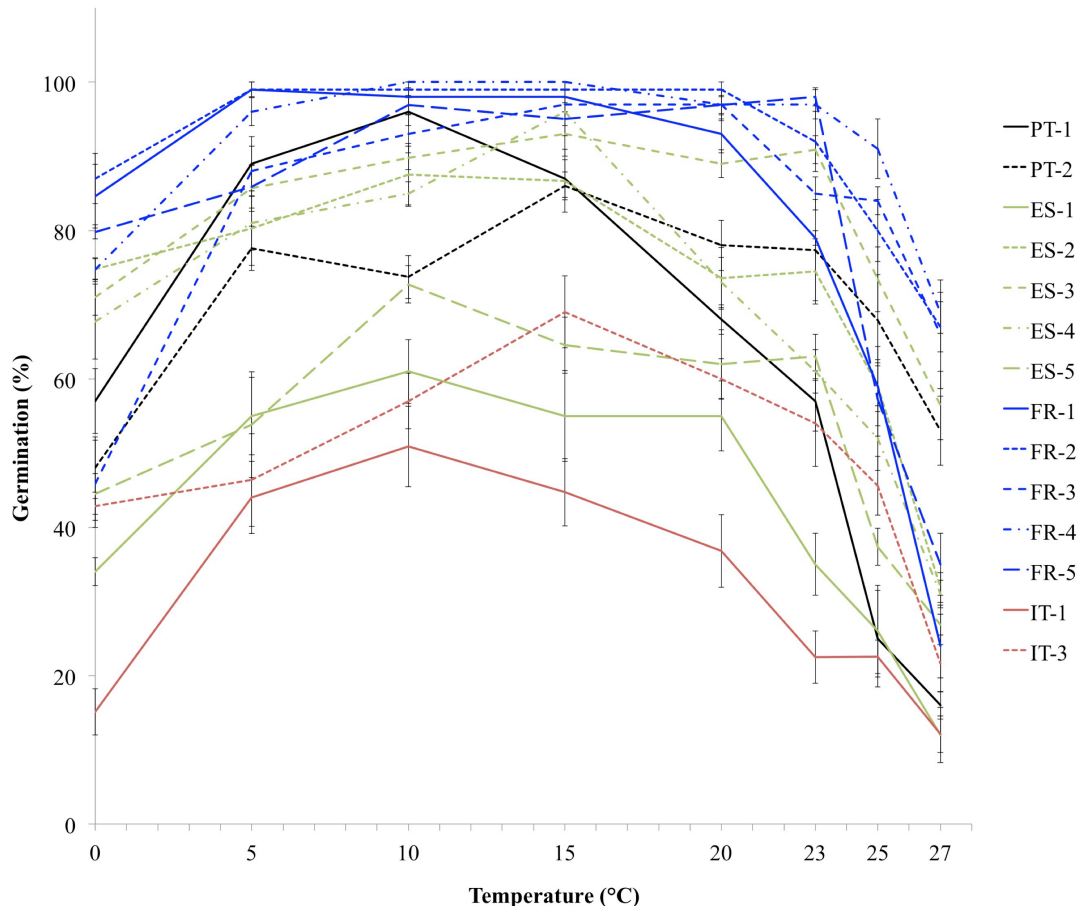


Figure 4.6. Germination percentages (mean $\pm$ SE) of the 14 *Malcolmia littorea* European populations under constant temperatures (0-27 °C) in the dark with scoring under green safelight.

Within the temperature range considered in this study (0-27 °C), *FSM* (fresh seed mass) and *G%* (germination percentages) showed a decreasing negative Pearson's *r* coefficient of correlation with increasing temperature, being significant only at 0 and 5 °C ($r = -0.68$, $P < 0.01$ and $r = -0.58$, $P < 0.05$, respectively). For the parameter *DSM* (dry seed mass) the same situation occurred ($r = -0.67$, $P < 0.01$ and $r = -0.54$, $P < 0.05$ for 0 and 5 °C, respectively). The moisture content (*MC*) was significantly negatively correlated with *G%* at 27 °C ($r = -0.54$, $P < 0.05$), while the oil content was not found to be correlated with any *G%* at the temperature tested.

The log-transformed *AREA* (occupied area) was significantly positively correlated with the germination percentages (*G%*) at all the tested temperatures, except 25 °C. In particular, the *AREA* and *G%* at 5, 10, and 15 °C were correlated with a Pearson's *r* coefficient ranging between 0.68 and 0.71 at $P < 0.01$, while at 0, 20, 23 and 27 °C the *r* coefficient ranged between 0.58 and 0.64 at $P < 0.05$. Figure 4.7 shows the results of the linear regressions between germination percentages at seven constant temperatures (all the

tested temperatures except 25 °C) for each population, and the area of the occupied site (log-transformed) of the respective population.

The test of homogeneity of slopes (parallelism) showed that the slopes of the regression lines were not significantly different ($F = 0.08$, $P = 0.99$), justifying application of ANCOVA, which showed a significant difference among the seven regression lines ($F = 16.59$, $P < 0.001$), underlying the significant effect of temperature on germination.

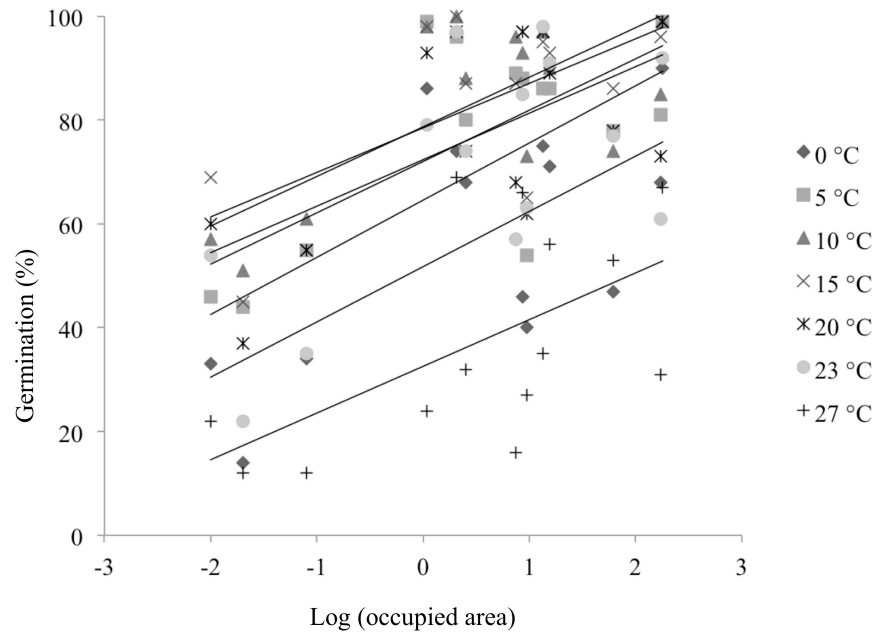


Figure 4.7. Germination percentages of 14 *Malcolmia littorea* populations at seven constant temperatures (0, 5, 10, 15, 20, 23 and 27 °C) as a function of area of the occupied site (log-transformed). The lines are the regression functions significant at $P < 0.01$ (5, 10, and 15 °C) and at $P < 0.05$ (0, 20, 23 and 27 °C). The germination percentages at 25 °C were not considered since they were not significantly correlated with the area of the occupied site. The ANCOVA test showed a significant difference among the seven regression lines ($F = 16.59$, $P < 0.001$).

The first two axes of the principal component analysis (PCA), performed using the germination percentages at all the tested temperatures as variables, explained 83.4 and 11.0 %, respectively, of the variation in the data set. The first axis mainly represented variation in the $G\%$ at 20 and 23 °C, while the second axis was mainly determined by the $G\%$ at the extreme temperatures (0 and 27 °C). The population scores on the first two PCA axes are presented in Figure 4.8. The correlation between the first PCA component and the germination percentages ranged between 0.80 and 0.98. The first PCA axis scores were significantly correlated with the log-transformed site area (Pearson's $r = 0.68$, $P < 0.01$),

with the November $T_{\min}$ of both 1950-2000 (Pearson's $r = -0.57$, $P < 0.05$) and 2013 (Pearson's $r = -0.61$; $P < 0.05$), with T_{mean} of November 2013 (Pearson's $r = -0.58$, $P < 0.05$), and with $T_{\min}$ of December 1950-2000 (Pearson's $r = -0.55$, $P < 0.05$).

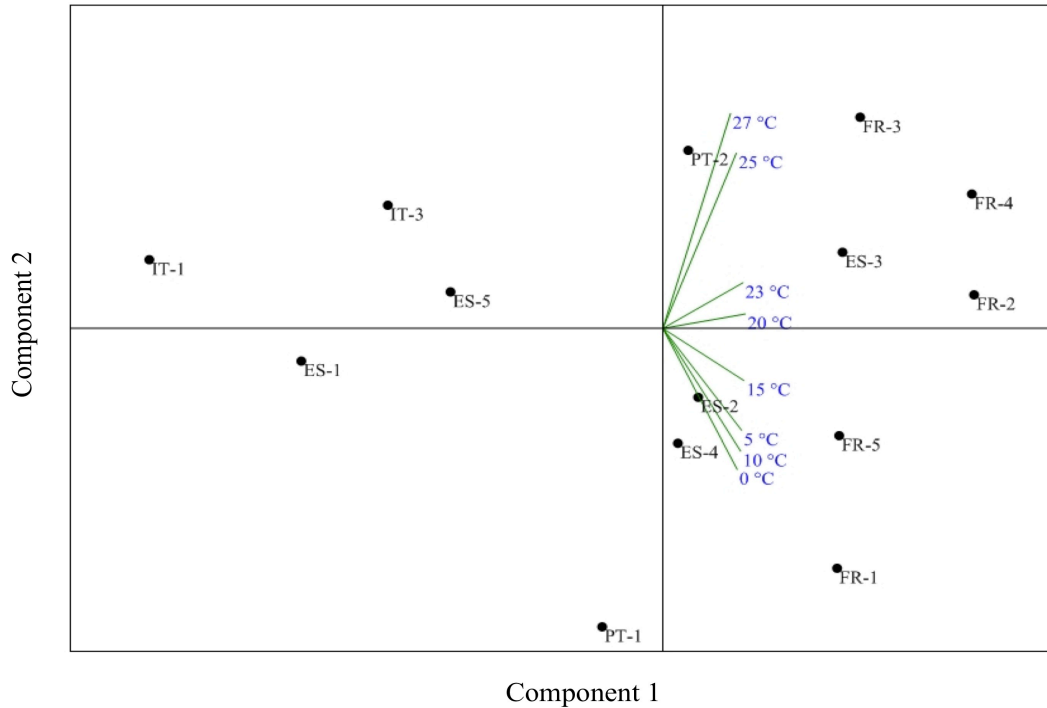


Figure 4.8. Distribution of populations in principal components analysis (PCA) ordination space along axes 1 and 2. In the figure the biplot displaying the correlation structure of germination percentages at the different temperatures tested along the axes 1 and 2 is also shown.

At 0 and 5 °C, the GR_{50} were significantly different among the 14 populations (ANOVA/Kruskal-Wallis: $P < 0.001$), ranging between 0.04 and 0.06 and 0.09 and 0.13, respectively (Table 4.4). At 10 °C, the GR_{50} of ES-1, IT-1 and IT-3 (0.21, 0.20 and 0.19, respectively) were similar and significantly lower (Kruskal-Wallis: $P < 0.001$) than the values of the other populations (0.24 ÷ 0.25). At 15 °C, only the GR_{50} of IT-1 (0.28 ± 0.02) was significantly different (Kruskal-Wallis: $P < 0.05$) from the values showed by ES-3, ES-5 and FR-4 (0.40 ± 0.04 , 0.43 ± 0.04 and 0.40 ± 0.04 , respectively). A similar situation occurred at 20 °C where GR_{50} value of IT-1 (0.37 ± 0.03) differed significantly (Kruskal-Wallis: $P < 0.05$) from all the other populations, which showed the same value (0.50 ± 0.00). At 23 and 25 °C no differences were detected, while at 27 °C, the only significant difference (Kruskal-Wallis: $P < 0.05$) occurred between GR_{50} of IT-3 (0.70 ± 0.12) and PT-1 (0.36 ± 0.07 ; Table 4.4).

Table 4.4. Germination rates (mean $\pm$ SE) relative to t_{50} (time to reach 50% of maximum germination in each replicate) for 14 *Malcolmia littorea* European populations at 0-27 °C. Where the standard error was not reported it was 0.00.

Seed lot	0°C	5°C	10°C	15°C	20°C	23°C	25°C	27°C
PT-1	0.05	0.11	0.24 $\pm$ 0.01	0.33	0.50	0.50	0.40 $\pm$ 0.10	0.36 $\pm$ 0.07
PT-2	0.06	0.12	0.25	0.33	0.50	0.50	0.50	0.50
ES-1	0.04	0.12 $\pm$ 0.01	0.21 $\pm$ 0.01	0.33	0.50	0.60 $\pm$ 0.10	0.50	0.50
ES-2	0.05	0.11	0.25	0.33	0.50	0.50	0.50	0.50
ES-3	0.06	0.11	0.25	0.40 $\pm$ 0.04	0.50	0.50	0.50	0.50
ES-4	0.05	0.12	0.25	0.37 $\pm$ 0.03	0.50	0.50	0.50	0.50
ES-5	0.06	0.12	0.25	0.43 $\pm$ 0.04	0.50	0.50	0.50	0.50
FR-1	0.05	0.13	0.25	0.33	0.50	0.50	0.50	0.50
FR-2	0.04	0.13	0.25	0.33	0.50	0.50	0.50	0.50
FR-3	0.05	0.11	0.25	0.33	0.50	0.50	0.50	0.50
FR-4	0.06	0.12	0.25	0.40 $\pm$ 0.04	0.50	0.50	0.50	0.50
FR-5	0.05	0.12	0.25	0.33	0.50	0.50	0.50	0.50
IT-1	0.05	0.09	0.20	0.28 $\pm$ 0.02	0.37 $\pm$ 0.03	0.50	0.50	0.34 $\pm$ 0.10
IT-3	0.05	0.09	0.19 $\pm$ 0.01	0.33	0.50	0.50	0.50	0.70 $\pm$ 0.12

Fresh seed mass was found to be significantly negatively correlated with GR_{50} (germination rates) at 5 °C (Pearson's $r = -0.74$, $P < 0.01$) and 10 °C (Pearson's $r = -0.54$, $P < 0.05$). Dry seed mass was significantly correlated with GR_{50} only at 5 °C (Pearson's $r = -0.72$, $P < 0.01$). Moisture and oil content values were not significantly correlated with any GR_{50} at the temperature tested.

For the 14 populations, there was a significant correlation between the site area (log-transformed) and the GR_{50} at 5 °C (Pearson's $r = 0.76$, $P < 0.01$) and at 10 °C (Pearson's $r = 0.87$, $P < 0.001$).

Among the mean T_b values of the 14 populations, no differences emerged (Table 4.5) and they ranged from -1.74 ± 0.50 °C to 0.03 ± 0.09 °C.

The assumption of homogeneity of slopes (parallelism) was met for the regression lines relative to GR_{50} ($F = 0.43$, $P = 0.95$), and the analysis of covariance (ANCOVA) applied to them did not show a significant difference in the sub-optimal range among the regression equations of 14 populations ($F = 1.38$, $P = 0.19$; Fig. 4.9).

The mean T_b values evaluated for each population were found to be significantly correlated with the oil content of the respective populations (Pearson's $r = 0.67$, $P < 0.01$).

Table 4.5. Regression equations, p -values (**: $P < 0.01$, ***: $P < 0.001$), and r^2 estimated by linear sub-optimal model for GR_{50} calculated for each population. The T_b (base temperature) values were estimated as mean ($\pm$ ES) of three sub-populations (GR_{25} , GR_{50} and GR_{75}).

Seed lot	Model	p -value	r^2	T_b ($^{\circ}\text{C}$)
PT-1	$y = 0.023x + 0.019$	**	0.98	-0.78 ± 0.31
PT-2	$y = 0.022x + 0.034$	**	0.98	-1.74 ± 0.50
ES-1	$y = 0.024x + 0.003$	***	0.98	0.00 ± 0.30
ES-2	$y = 0.022x + 0.026$	**	0.98	-1.38 ± 0.61
ES-3	$y = 0.023x + 0.030$	***	0.98	-1.56 ± 0.63
ES-4	$y = 0.023x + 0.026$	***	0.99	-1.32 ± 0.57
ES-5	$y = 0.024x + 0.036$	**	0.97	-1.68 ± 0.65
FR-1	$y = 0.022x + 0.029$	***	0.98	-1.14 ± 0.22
FR-2	$y = 0.022x + 0.029$	***	0.99	-1.31 ± 0.19
FR-3	$y = 0.023x + 0.021$	***	0.98	-0.79 ± 0.27
FR-4	$y = 0.023x + 0.032$	***	0.99	-0.90 ± 0.24
FR-5	$y = 0.022x + 0.030$	**	0.98	-0.77 ± 0.44
IT-1	$y = 0.019x + 0.016$	***	0.97	-0.42 ± 0.21
IT-3	$y = 0.023x + 0.003$	***	0.95	0.03 ± 0.09

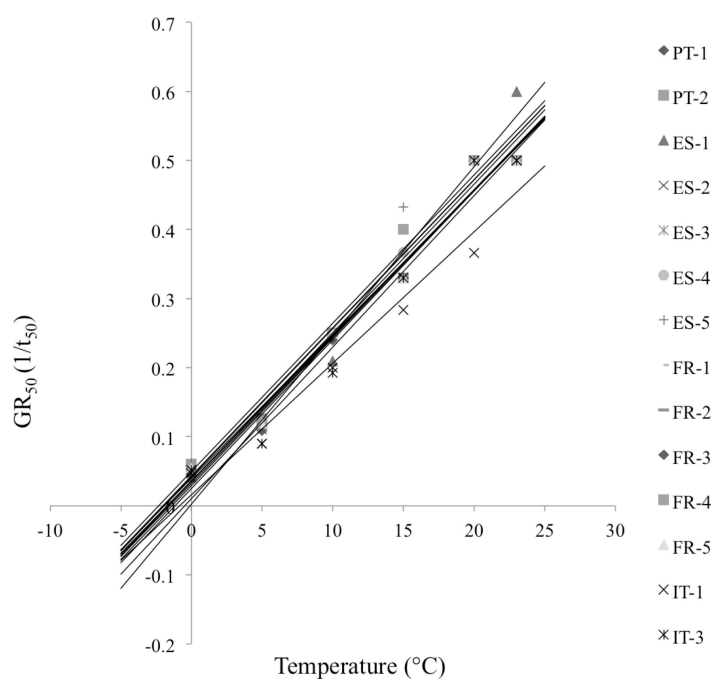


Figure 4.9. Germination rates (GR_{50}) for the 14 *Malcolmia littorea* populations in the sub-optimal range of temperatures (0 – 23 $^{\circ}\text{C}$). Points correspond to the actual data and solid lines indicate the fitted lines from the linear regressions. $P = 0.58$ for the sub-optimal models (0 – 23 $^{\circ}\text{C}$) by ANCOVA. Data are the means of five replicates ($\pm$ ES).

The significance levels of correlation between $G\%$ (germination percentages) and climatic variables (mean minimum temperature: $T_{\min}$, mean mean temperature T_{mean} , mean maximum temperature: $T_{\max}$, mean relative humidity: RH or mean precipitation: Prec.) of October, November and December relative to the period 1950-2000 and to the year 2013 are shown in Table 4.6, as the significance levels of correlation between GR_{50} (germination rates) and the same climatic variables. The Bagnouls-Gaussen ombrothermic diagrams are reported for the areas where the studied populations occur (Fig. 4.10).

Table 4.6. Significance levels of correlation (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$) between $G\%$ (germination percentages) and GR_{50} (germination rates) at constant temperatures 0-27 °C, and the climatic variables (var.) registered in October, November and December of both the historic series 1950-2000 and the year 2013 (mean minimum temperature: $T_{\min}$, mean mean temperature T_{mean} , mean maximum temperature: $T_{\max}$, mean relative humidity: RH , mean precipitation: Prec.).

		0 °C		5 °C		10 °C		15 °C		20 °C		23 °C		25 °C		27 °C	
1950-2000	Var.	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR
Oct.	$T_{\min}$	*		*		***				*							
	T_{mean}	*		*		*											
	$T_{\max}$			*													
	Prec.																
Nov.	$T_{\min}$																
	T_{mean}																
	$T_{\max}$																
	Prec.					*											
Dec.	$T_{\min}$																
	T_{mean}																
	$T_{\max}$			*		*		**		*		*					
	Prec.																
2013	Var.	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR	$G\%$	GR
Oct.	$T_{\min}$	**		**	***	**	*			*	*						
	T_{mean}														*		
	$T_{\max}$														*		
	RH														*		
Nov.	$T_{\min}$	***		***	***	***	*	**		***	*	**					
	T_{mean}																
	$T_{\max}$																
	RH				*												
Dec.	$T_{\min}$			*	*												
	T_{mean}																
	$T_{\max}$																
	RH														*		

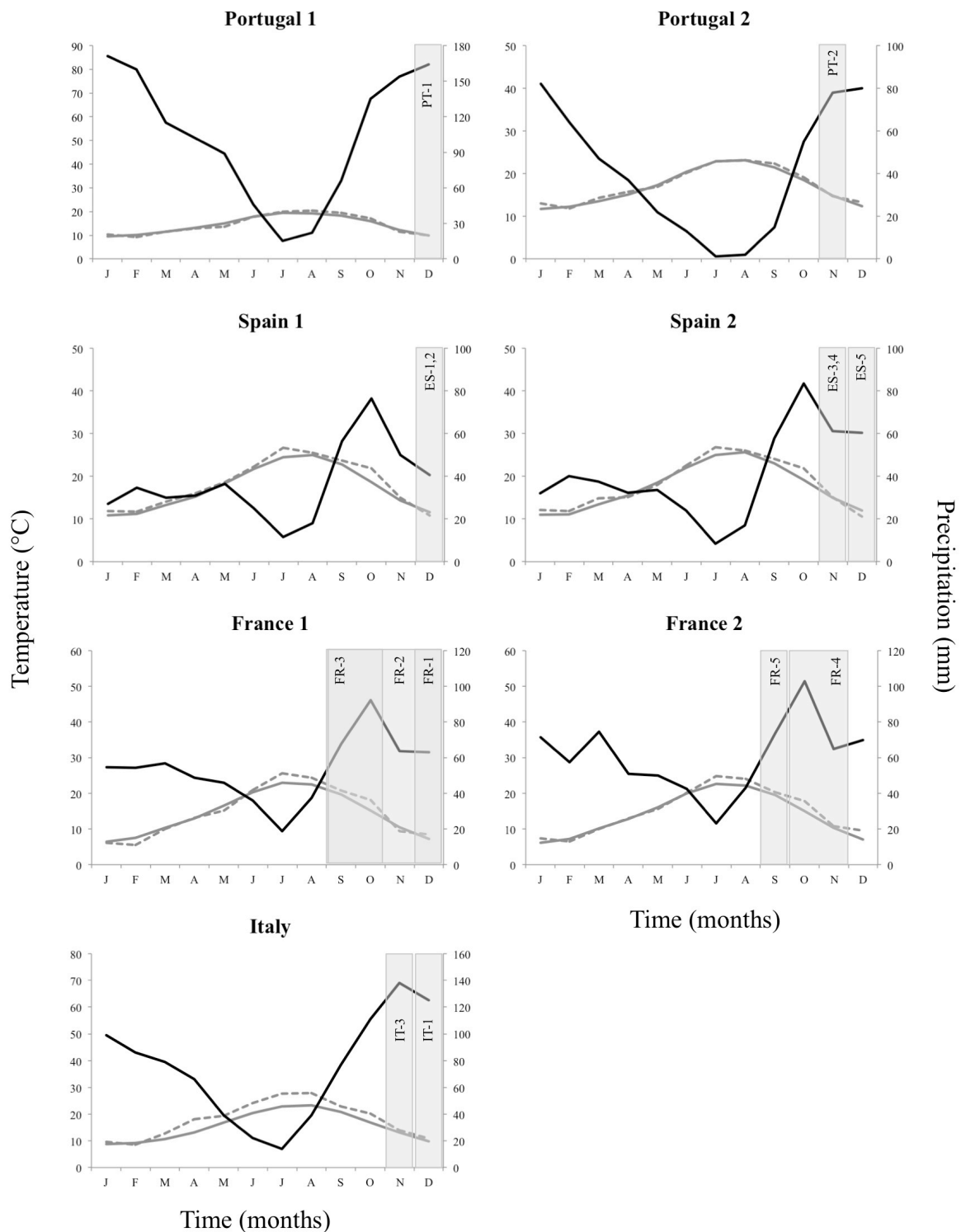


Figure 4.10. Bagnouls-Gaussen ombrothermic diagrams for seven areas of *Malcolmia littorea* distribution. Spanish, French and Italian populations have been grouped according to the climatic characteristics of their sites. The solid lines indicated the average trends of precipitation (black) and mean temperature (grey) over the period 1950-2000, while the broken line represented the mean temperature trend in the year 2013. The grey blocks indicated the highest germination for each population and are placed on the month with the corresponding mean temperature.

4.4. DISCUSSION

4.4.1. Seed germination basic requirements and their adaptation

This is the first study that reports temperature, light and burial requirements for *Malcolmia littorea* seed germination (De Vitis et al. 2014; Appendix 4).

4.4.1.1. Germination behaviour and soil depth-sensing of the Italian native population

Information on seed storage and germination requirements of threatened species must be considered in order to ensure their long term conservation and management. For seeds of the threatened *Malcolmia littorea*, the lower germination of fresh seeds in comparison to stored seeds suggest that physiological dormancy is likely, broken by after-ripening in dry storage (*sensu* Baskin and Baskin 1998, 2004). Short exposure (e.g. a few minutes every two days) of seeds in the dark to the green safelight was found to positively affect seed germination rather than a condition of complete darkness, without receiving any light impulse, or the exposure to 12 h of white light. Purportedly, short bursts of light may stimulate germination in the late stages of dormancy breakage (Baskin et al. 2006). Mediterranean species have previously been reported to be sensitive to green light. Luna et al. (2004) evaluated the effect of green safelight with respect to absolute darkness on the germination response of a set of 12 Mediterranean species; starting the germination tests several months after seed collection, the germinations of six species were sensitive (five positively and one negatively) to short-duration exposures to green safelight.

Coastal Mediterranean species typically germinate within the range of 5–15 °C, therefore limiting germination to the winter so plants can establish before the onset of the summer drought (Thanos et al. 1989, 1995). At 5–10 °C, the germination of the native population (IT-1) of *M. littorea* was the highest amongst the tested temperatures, conferring to this species ecological adaptation to the unpredictable rainfall pattern of species living under “typical” Mediterranean climate conditions, reducing the risk of losing a cohort of plants.

Rapid germination has also been suggested to be an adaptation in advance of soil drying in unpredictable environments (Daws et al. 2002), such as under summer conditions in the Mediterranean. Compared to other Mediterranean species such as *Glaucium flavum*,

Papaveraceae (t_{50} of ca. 30 days for untreated seeds at 10 °C; Thanos et al. 1989), *Muscari* spp., Asparagaceae (t_{50} ranging from 12 to 16 days at 10 °C; Doussi and Thanos 2002), *Centranthus ruber*, Valerianaceae (t_{50} of 4.5 days at 15 °C; Mattana et al. 2010) and *Dianthus morisianus*, Caryophyllaceae (t_{50} ranging from 4 to 5 days at 15 °C; Cogoni et al. 2012), IT-1 showed faster germination, with t_{50} values of 3.4 days at 15 °C and 5.6 days at 10 °C.

In Mediterranean climates, germination on the soil surface is unfavourable due to high irradiance. Many species employ surface-avoiding mechanisms (Thanos et al. 1989), based on diurnally fluctuating temperatures and light quality, to detect depth of seed burial (Koutsovoulou et al. 2014), as germination from too deep within the soil will lead to seedling death by seedling exhaustion before surface emergence (Benvenuti et al. 2001). Furthermore, the seed burial increases soil contact and relative humidity around the seeds, thus enhancing the probability of germination (Maun 1998). The application of alternating temperature regimes did not improve the final germination percentage nor the t_{50} of IT-1 in comparison with constant temperatures, and this is consistent with previous studies where seeds smaller than 2 mg did not respond to alternating temperatures (Cogoni et al. 2012; Mattana et al. 2010; Rojas-Arèchiga et al. 2001). Bond et al. (1999) found that there is an allometric correlation between seed mass and the maximum depth at which seedling emergence occurs [maximum depth (mm): $27.3 \times \text{seed weight}^{0.334} \text{ (mg)}$]. According to this model, *M. littorea* seeds should not be able to emerge from depths > 15 mm. Laboratory experiments confirmed the model validity for *M. littorea*, as very few seedlings emerged from burial depths > 15 mm (1%), while a considerable emergence of seedlings was evident between 1 and 10 mm. Further interpretation of the seedling emergence experiment is precluded by the fact that the irradiance of the incubator lighting was lower than expected in nature. Nonetheless, *M. littorea* appears to germinate as a typical Mediterranean species avoiding high irradiance and by germinating at cooler temperatures.

4.4.1.2. Impact of maternal environment

Seeds produced and matured on the mother plants in the wild (IT-1) had a different germination response to those produced from the experimental populations, suggesting that seeds may show a rapid alteration of fitness within one generation. This has previously been shown in seeds of the cactus *Stenocereus stellatus* (Rojas-Arèchiga et al. 2001). If the

latitude. In *M. littorea* no particular pattern or gradient in the seed mass trait was evident, which varied slightly (not even twofold) among the populations. The absence of correlation between seed mass and the climatic/geographic variables may be explained by the small geographic range considered (latitude ranging between 37.09 and 43.56 decimal degrees), with all the populations occurring under the Mediterranean bioclimate (*sensu* Rivas-Martínez et al. 2011). Harper et al. (1970) stated that, even if within a species mean seed size may be influenced by plants density, stress, disease, selective pressure of predation, specific site and season, it is a stable component due to homeostasis which affects the most important organ for maintaining continuity between the generations. Some of the variation in individual seed weight produced by a plant is due to differences in embryo genotype; in fact differences between the size of seeds derived from reciprocal crosses between inbred lines often show a powerful maternal influence not only on seed size, but also on variability (Harper et al. 1970). Furthermore, the rather high values of heritability so often found indicate that seed size is a readily selected trait (Harper et al. 1970). On the other hand, some authors have shown that seed size is a relatively plastic feature with low heritability and that it may vary depending on several factors, such as seed production, environmental conditions, and site characteristics. For example, McWilliams et al. (1968), studying *Amaranthus retroflexus* (Amaranthaceae), demonstrated that, among 17 different worldwide sources, seed weight was correlated with latitude, clearly dependent upon the genotype of the particular race or ecotype and also upon the environmental preconditioning to which the seeds have been subjected, such as the temperature during seed maturation. McKee and Richards (1996), studying the variation in seed production of *Phragmites australis* (Poaceae) among 39 British and French sites, found that seed weight was significantly different among populations and positively correlated with seed set, which, in its turn, was influenced by the site area and weather. In particular, a small site size was an indicator of paucity of resources availability for adult plants, and mean temperatures and rainfalls of the months of pollination and seed maturation had an important influence on seed production (McKee and Richards 1996). Fischer and Matthies (1998b) found that in *Gentianella germanica* (Gentianaceae), population size had a strong influence on reproduction, explaining a significant variation in the number of seeds per fruit and per plant and that mean seed mass was negatively correlated with the number of fruits per plant. Susko and Lovett-Doust (2000), studying 14 populations of *Alliaria petiolata* (Brassicaceae) along a latitudinal transect in central North America, found that the seed mass varied nearly eightfold with significantly lower values

maternal environment during seed development is determinant for the germination behaviour of *M. littorea*, then the source of seeds could have a major impact on the success of conservation programmes. Therefore, seeds for reintroduction should be harvested from plants produced in the same environment into which they will be sown.

4.4.2. Comparative study on European populations

4.4.2.1. Seed traits and their relationship with germination

In *Malcolmia littorea* the mean seed mass differed significantly among the European populations, as the seed moisture and oil content values. The seed oil content values (35–43%) of the 14 *M. littorea* populations was higher than the value detected for the same species by a previous study (32%; Mira et al. 2015) for the Spanish population of *El Perellonet* (Valencia), very close to the population of *Albufera Natural Park* (Valencia; ES-2). However, this difference may be due to the different methods used, since Mira et al. (2015) determined the seed oil content using a modified version of the Bligh and Dyer (1959) protocol, which implies the seeds grinding and mixing in a chloroform:methanol solution while in the present study it was assessed by the non destructive method of time domain nuclear magnetic resonance.

Among the 14 European populations considered in the present study, no relationships emerged between seed mass and the climatic/geographic variables or the population site area. Similarly, Ouborg and Van Treuren (1995) showed that the mean seed weight and the proportion of both nonviable and optimal-sized seeds varied significantly among four populations of *Salvia pratensis* (Lamiaceae) and that these parameters were not related to population size. Daws et al. (2004) studying seedlots deriving from five European (Greece-Scotland) populations of the recalcitrant species *Aesculus hippocastanum* (Sapindaceae), found that the seed mass varied fivefold among them, decreasing along the south-north gradient while, along the same gradient the moisture content varied in the opposite way, suggesting that environmental conditions can affect a range of seed traits in recalcitrant species. From the study of Pakeman et al. (2008), which comprised data collected during 2003 and 2004 on herbaceous vegetation from 12 sites in nine European countries from Scandinavia to Mediterranean, it emerged that weighted seed mass was unrelated to rainfall and mean temperature, while it was significantly negatively correlated with

in populations closer to the geographic periphery of the species' range than in the center. Jacquemyn et al. (2001) showed that in small populations of *Primula elatior* (Primulaceae) mean seed mass per fruit depended highly on the number of seeds per fruit, whereas the same dependence was less evident in large populations. Gijbels et al. (2015), reviewing the 28 studies on relationship between population size and fruit set in 26 orchid species, and exploring data with a meta-analysis, found that orchid fruit set decreased with decreasing population size. These results indicate that seed mass may be affected by seed set, which is often correlated with weather and population size. In particular, a small population may be less attractive to pollinators (Ouborg and Van Treuren 1995) and the lower proportion of pollinator movements among individuals could result in reduced seed set and fitness of seed progeny (Menges 1991; Ågren 1996).

At 0 and 5 °C, the mean seed mass of 14 *M. littorea* populations was negatively correlated with germination percentages and at 5 and 10 °C it was negatively correlated with the germination rates, meaning that, at low temperatures, small-seeded populations showed a higher and faster germination than the large-seeded populations. Similarly, from the study of Greipsson and Davy (1995) on seed mass and germination behaviour of 36 populations (34 in Iceland and 2 in Britain) of the dune-building grass *Leymus arenarius* (Poaceae), an inverse relationship between mean seed mass (which varied sixfold) and median time to germination emerged, while contrary to *M. littorea*, there was a positive relationship between mean seed mass and total germination percentage. McKee and Richards (1996), studying the variation in germinability of *Phragmites australis* among 39 sites, found that heavier seeds had a greater germination potential. Furthermore, they found a tendency for seeds from southern sites to germinate to a higher percentage than those from northern sites (McKee and Richards 1996). This conclusion was also expressed by Ouborg and Van Treuren (1995), who found that seed size had a profound influence on both germination and seedling size in *Salvia pratensis*. Differences in seed size and germination were presumably a consequence of differences in maternal environment, which was also underlined by the significant effect of sampling year on differences in percentage germination among *Salvia pratensis* populations (Ouborg and Van Treuren 1995). Jacquemyn et al. (2001) found that germination percentage of *Primula elatior* was shown to be highly influenced by seed weight, with large seeds showing larger germination percentages.

Most of the above-mentioned studies show that the seed mass is positively correlated with germination percentages, and this phenomenon may be explained by a higher amount

of resources stored in a larger seed than in a small one, which may be translated in a higher seed vigour and a quicker response to the environmental stimuli. However, Lacey et al. (1997) stated that it cannot be assumed that larger seeds have more storage tissue (i.e. more embryo or endosperm tissue) than smaller seeds, because in *Plantago lanceolata* (Plantaginaceae) they found that low parental temperature increases seed mass by increasing coat mass rather than by increasing embryo/endosperm mass, which could be the cause of a reduced germination. Seed coat thickness and chemical composition have been shown to regulate germination (Guterman 2000). For *M. littorea* it is likely that the heavier seed weight is due to a higher coat thickness which affects the germination, especially at low temperatures (0-10 °C). Another consequence of a difference in seed mass is that small seeds are usually more easily buried in soil than large ones (Cerabolini et al. 2003) and this could mean that large seeds may not be effectively buried under the sand, with a risk of predation.

4.4.2.2. Similar germination patterns across Europe and high performance at low temperatures

Under eight constant temperatures, the germination behaviour of the 14 *M. littorea* European populations was similar, without considering the absolute values but considering the within-population germination trend. The germination percentages and rates increased with temperature, being the highest between 5 and 23 °C and between 20 and 27 °C (often decreasing after 23 or 25 °C), respectively. The rapid germination under high temperatures may be explained by the seed adaptation of taking advantage when a summer rain occurs. At the extreme temperatures (0 and 27 °C) the germination significantly decreased within all populations, and it is likely that the over-riding factor limiting the germination during summer is the relatively low and fluctuating water availability. However, in several populations it was high as well (> 50%) and this result was surprising, especially at 0 °C. Here the germination was continuous (indicated by a slow germination rate), meaning that seeds require a long period of continuous hydration for germination. Among the reviewed studies on seed germination of coastal dune species, none of them tested the germination at 0 °C. Thus, it was not possible to know if a similar response occur in other coastal species. On the other hand, the study of Cogoni et al. (2012) reported a T_b ranging from ca. 0 to 2.29 °C for the Mediterranean coastal *Dianthus morisianus* (Caryophyllaceae). Dürr et al.

(2015) reviewed the ranges of critical temperature for the germination of species worldwide, and found that several plants, among tree, crops and wild species, showed T_b values lower than 0 °C. These species originated from cool growing areas and had the ability to germinate in cold temperatures (Dürr et al. 2015).

However, it is worth noting that after storage the seeds may give a higher percentage of germination at a faster rate than the fresh seeds and the germination performance is equally high at a wider range of experimental temperatures (Pemadasa and Lovell 1975). In the European locations where *M. littorea* lives, the minimum temperatures never reached or dropped below 0 °C during 2013. Even among the average minimum temperatures relative to the period 1950-2000, 0 °C was never reached. Even if these temperature values were relative to the air and not to the soil, it is unlikely that the soil temperature dropped to 0 °C in a coastal habitat.

For *M. littorea* it can be expected the occurrence of a cross-tolerance mechanism. In the study of Mira et al. (2015) *M. littorea* dehydrated seeds showed an exponentially increasing seed longevity with decreasing water content, with a critical water content of 0.02-0.03 g·H₂O·g⁻¹ DW (on a dry weight basis) at 45 °C, corresponding to 5.5 % RH (relative humidity) in equilibrium at 25 °C. Further, after drying below the critical water content (0.01 g·H₂O·g⁻¹ DW), no damage was detected for its seeds (Mira et al. 2015). The mechanism involved in the extreme resistance of seeds to desiccation might provide the germinating seed with cross-tolerance to other abiotic stresses, such as temperature (Stupnikova et al. 2006). The high resistance of *M. littorea* seeds to a critical dehydration, and even their enhanced longevity, detected by Mira et al. (2015) means that they are able to survive and to remain viable under the stressful conditions typical of the Mediterranean summers, when the soil moisture is below the minimum for germination. Under these conditions seeds are forced to remain dormant until the increased precipitation in autumn increases the soil moisture regime to a favourable level allowing germination (Pemadasa and Lovell 1975).

The similar general germination behaviour observed for the 14 *M. littorea* populations investigated was probably due to genotypic influences of the plant species, which ensure that the adaptation of the plant to its habitat conditions is such that germination is likely to occur in the right season and in the right place (Gutterman 2000).

4.4.2.3. A reduced reproductive fitness in small populations

For *M. littorea*, the germination percentages of the 14 European populations were significantly correlated with their site area at each of the tested constant temperatures, except at 25 °C. Furthermore, the correlation between the first PCA axis scores and the site area was significant and gave a further evidence for the population site area to be a strong driving force for the population reproductive fitness. These results are indicative of a reduced fitness in the smaller populations of *M. littorea*, probably due to inbreeding depression. In small, fragmented and isolated habitat patches, population size may become so small that pollinators do not visit them anymore and/or that the remaining pollinators are less effective (Ouborg and Van Treuren 1995; Fischer and Matthies 1998b; Jacquemyn et al. 2001). In very small populations, most pollinator movements may occur within, rather than between, plants, so a greater proportion of seeds will be produced by within-plant crosses and may suffer of inbreeding depression (Menges 1991). A study of Menges (1991) showed that, among 23 populations of *Silene regia* (Caryophyllaceae), the germination, at optimal conditions for the species, was lower than 50% only for populations smaller than 100 plants, while larger populations (> 150 plants) had uniformly higher percentages of seed germination. In the same study, a curvilinear regression fit implied that population size effects on germination operate mainly among smaller populations, suggesting the importance of effects of habitat fragmentation and destruction on fitness components, such as seed germination (Menges 1991). Fischer and Matthies (1997) observed in *Gentianella germanica* (Gentianaceae) that offspring resulting from selfing and near neighbours crosses showed inbreeding depression in germination, early growth, and survival compared with offspring of the 10 meters pollination treatment, and that pollinator limitation may affect plant fitness only in very small populations. The reduced performance of *G. germanica* plants from smaller populations was not an effect of habitat quality, but reflects the effect of loss of genetic variation with an accumulation of mildly detrimental mutations and an increased level of inbreeding (Fischer and Matthies 1998b). Jacquemyn et al. (2001) did not find any direct relationship between seed germination percentage and population size in *Primula elatior*, even if, as mentioned above, they found that in small populations the seed size-seed number trade-off occurred and that germination percentage was influenced by seed mass. Hence there may be an interaction between these variables, closely related to population size, that indirectly link it with the fitness expressed through the germination potential.

4.4.2.4. Relationship and adaptation to the environmental factors

As revealed in this discussion, both environmental and genetic factors play an important role influencing the germination response and its seasonal pattern. The phenotypic influences, including environmental and maternal effects, during maturation on seed germination ensure that, even under optimal conditions, only a portion of the seed population will germinate in one rain event or in one season. This heteroblasty prevents catastrophes of mass germination after a relatively heavy rain, followed by a long dry period, which would cause all the seedlings to die out (Guterman 2000). The environmental factors influence a series of biochemical patterns and the accumulation of different relative amounts of chemicals in the seeds which may be the responsible of the differences in seed germinability within a population. These chemicals, such as enzymes and hormones, could lead to the development of seed coats with different degrees of water impermeability and thickness, according to the day length and age effects; could accumulate in the embryo and may be activated to produce germination inhibitors, such as polyphenols, which accumulate in the fruit or in the seed coat (Guterman 2000). McWilliams et al. (1968) cultivated 17 populations of *Amaranthus retroflexus* deriving from different worldwide sources and found that the variation in percentage of germination between populations appeared largely genetic, since there were detected differences between populations both from the same and from different latitudes. This difference in response was largely explained by a difference in dormancy between populations and the most likely explanation for variation in dormancy between populations is an evolutionary divergence in germination requirements in relation to local environments (McWilliams et al. 1968). Similarly, Probert et al. (1985a,b) regenerated several European populations of *Dactylis glomerata* (Poaceae) at Wakehurst Place (UK), to study their germination response. They found that the germination behaviours of the different European populations were clearly related to origin: the northern populations showed adaptation to a temperate oceanic or continental climate with a tendency towards a winter dormant growth habit, while the southern populations were adapted to a Mediterranean climate with a tendency towards a summer dormant growth habit. Probert et al. (1985a,b) concluded that, in view of the wide geographical background of the populations studied, the microclimate differences between habitats have resulted in selection for differences in germination response which probably reflect adaptation to specific environmental pressures. Also Barazani et al. (2012), studying the seed germination requirements of five Israeli

populations of *Eruca sativa* (Brassicaceae), found that populations from arid sites possess deeper primary dormancy than those from mesic Mediterranean sites. Pendleton and Meyer (2004) demonstrated the existence of habitat-correlated variation in seed germination response for 26 populations of *Coleogyne ramosissima* (Rosaceae); most of the variation in germination response of their experiments could be explained in terms of collection site elevation and geographic origin of the seeds. Furthermore, the difference between years in dormancy status at particular sites suggests that there is considerable plasticity in germination patterns, perhaps in response to environmental conditions during ripening (Pendleton and Meyer 2004).

The germination behavior of a single genotype may depend on the photoperiod during seed maturation, the season of dispersal, and the geographic location to which it was dispersed (Donohue et al. 2005). Fernández-Pascual et al. (2013) found that seed dormancy in northern Spain *Centarium somedanum* (Gentianaceae) exhibits a cline along a local gradient of altitude and climate, and that this cline is related both to genetic composition and to the seed maturation environment. The timing of germination is highly responsive to environmental conditions and consequently, if environments change, germination behavior is likely to change as a direct and immediate response (Donohue et al. 2002). According to the results and conclusions of the above mentioned studies, germination response of the 14 *M. littorea* populations showed a stronger correlation with the climatic parameters of the same year of collection (2013), rather than with the mean climatic parameters calculated for the period 1950-2000. In particular, the final germination percentage of each population in the temperature range 0-23 °C showed a highly significant correlation with the minimum temperature of November 2013 recorded for the respective localities, indicating that this parameter is another important driving force of the germination response. In fact, the month of November is the period in which germination may naturally occur.

Even if at the lowest temperatures the germination rates of the small populations were significantly lower among the 14 *M. littorea* populations, the comparisons among the regression lines in the sub-optimal temperature range and among the base temperatures (T_b), showed a similar behaviour maybe due to the environmental factors which are similar since all the populations live in the sandy coastal habitats. Several studies confirmed that the T_b is a constant within a population (Covell et al. 1986; Ellis et al. 1987; Orrù et al. 2012), while this value may slightly or significantly change among populations of the same species (i.e. Ellis et al. 1987, Daws et al. 2004; Orrù et al. 2012), especially when the population over time has differing levels of dormancy. In studies dealing with populations

of the same species or with related species, the sub-optimal model and the T_b have often been found to change mostly according to the environmental conditions. For example, Daws et al. (2004) found that, among five European populations of *Aesculus hippocastanum*, spread along a south-north gradient of ca. 19° latitude (Greece-Scotland), the T_b increased with latitude, with the major driving factor being the heat sum accumulated by the seeds during their development. Mattana et al. (2010), studying the germination ecology of two *Centranthus* species (*C. amazonum* and *C. ruber*), of which seeds were collected at 1300 and 50 m a.s.l., respectively, found significant differences in the sub-optimal models and the T_b , due to the different habitat of seed production, with *C. ruber* seeds being better adapted for rapid establishment in harsh conditions. Orrù et al. (2012) found that four different Sardinian populations of *Vitis sylvestris*, varying in altitude from ca. 90 to 780 m a.s.l., differed in T_b according to altitude, with the high-altitude populations showing higher T_b .

4.5. CONCLUSIONS AND IMPLICATIONS FOR CONSERVATION

Malcolmia littorea appears to germinate as a typical Mediterranean species avoiding high irradiance and the summer drought and by germinating at cooler temperatures. The adaptive significance of the germination at 0 °C remains to be clarified, since this temperature condition is rarely met in Mediterranean coastal dune habitats. However, it is possible that both the high tolerance to desiccation and the effect of storage might provide the seeds with the ability to germinate at such low temperature.

Even if the species genotype ensure the adaptation of the plant to its specific habitat conditions across its European distribution, the seasonal and yearly environmental fluctuations play an important role in affecting seed germination pattern, maybe through the conditions experienced by the mother plant and by the seed during its maturation.

However, the environmental conditions are not the only factor affecting seed germination. From this study it emerged that *M. littorea* is a susceptible species to decreasing population size in terms of reproduction. Small populations suffer for a reduced reproductive fitness, which is reflected in a reduced seed germinability, probably due to the inbreeding depression. In particular, two populations (ES-1, *Playa de Moncófar*, Castellón and IT-1, *Torre Olevola*, Latina) exhibited low germination percentages along the entire range of tested temperatures and occupied very small areas. In these populations, a low genetic variability in the reproduction-related genes could be the cause of their reduced reproductive fitness. Immediate conservation measures have to be taken for these populations in order to avoid a further decline. For a example, the physical protection from disturbances, such as trampling and habitat destruction, together with augmentation actions may be useful in order to ensure their long-term persistence and restore a minimal viable population. If the protection or the restoration of the current sites is not possible, a reintroduction inside a nearby protected area is suggested, paying attention to the genetic origin and composition of the seed source used in these actions.

CHAPTER 5

IN SITU TRIALS: REGENERATION BIOLOGY OF *MALCOLMIA LITTOREA*

5.1. INTRODUCTION

In situ conservation, the preservation of a plant species in its natural environment, is generally considered to be the most efficient conservation strategy (Degreef et al. 2002). Investigation on soil seed bank densities and dynamics is the key to many aspects of practical land management to the effective conservation of many rare species (Thompson et al. 1993), because the accumulation of a bank of seeds may provide a small but significant reduction in the risk of extinction (Satterthwaite et al. 2007). The widespread occurrence of seed banks in many plant species may ensure that a single successful colonization event is sufficient to ensure long-term population persistence in the face of short-term environmental fluctuations (Turnbull et al. 2000).

Several classifications of seed persistence in the soil exist. The classification proposed by Bakker (1989), described by Thompson (1992) and followed by several authors (Thompson et al. 1993; Bakker et al. 1996; Cerabolini et al. 2003) described three classes of seed persistence: transient, persisting in the soil for less than one year; short-term persistent, persisting for more than one but less than five years; and long-term persistent, persisting for at least five years. Thompson et al. (1993) reviewed a database of ca. 200 published studies which inferred seed persistence both from direct estimation of seed longevity and from the depth distribution of seeds in the soil; measurement were made for 97 British natives or naturalized alien species, and almost all were herbaceous. Their results showed that compact diaspores which weigh less than 3 mg are all persistent in the soil and a strong phylogenetic effect also emerged with families in which the diaspores are

normally small and compact (e.g. Caryophyllaceae, Brassicaceae) are generally persistent, while families in which the diaspores are large and either flattened or elongated (e.g. Umbelliferae) are mostly short lived. The same result was found by Cerabolini et al. (2003) who analyzed data on seed mass, dimensions and persistence in the soil of 259 species of the Italian flora. Seed size and shape determine the likelihood to be buried and to persist in the soil, since the mechanism of burial is linked to the relative freedom from predation and seeds do not persist for long periods on the soil surface (Thompson 1987; Thompson et al. 1993). However, persistence depends not only on seed size and shape, but also on other variables such as germination physiology, longevity and chemical and physical defences against predators and pathogens (e.g., Thompson et al. 1998).

There are two main methods to study the soil seed bank: the soil core and the artificial burying. The soil core method has been useful to reveal vital information about plant reproduction and the existence of soil seed banks in specific areas (e.g. Aziz and Khan 1996; Arroyo et al. 2004). However, with this method it is not possible to distinguish clearly between the transient and persistent components of the soil seed bank because soil cores may contain seed produced over a number of years, and very little can be said about the longevity of seeds beyond one year (Bakker et al. 1996; Arroyo et al. 2004). The alternative approach is to experimentally bury batches of seeds of known age in the soil, retrieving and examining samples for viability and/or germinability at a series of programmed future dates (Baskin and Baskin 1998; Arroyo et al. 2004). The ability of forming a soil seed bank has been reported for several species characteristic of sandy coastal habitats (Aziz and Khan 1996; Quilichini and Debussche 2000; Sternberg et al. 2004; Yan et al. 2009). Here sand burial and wind erosion play key roles in shaping the spatiotemporal pattern of the seed bank with erosion resulting in the concentration of seeds in shallow soil layers, while burial resulting in seed concentration in deep soil layers (Ma and Liu 2008).

Reintroductions and translocations are useful *in situ* conservation tools to counteract species extinction (Maunder 1992; IUCN 1998, 2013). The first refers to the intentional movement and release of an organism inside its indigenous range from which it has disappeared, while the second is the human-mediated movement of living organisms from one site for release in another (IUCN 1998, 2013). In particular, the reintroduction of plants into natural or semi-natural habitats is becoming an increasingly utilized strategy for plant conservation, but it should not be seen as a substitute for primary habitat retention or community oriented *in situ* conservation (Maunder 1992). A proper reintroduction strategy

aims to maximize the probability of long-term success by designing founding populations that, by their composition and care, will minimize extinction risks and maximize population growth rates in the short-term (Guerrant 1996; IUCN 1998). An ideal natural seed source for reintroduction is one that is geographically close and ecologically similar to that of the reintroduction site, and from which seeds can be collected without damage it (Zeiter and Stampfli 2008; Lawrence and Kaye 2011). If multiple suitable seed sources are available, initial high levels of genetic diversity can be obtained by mixing them (Guerrant 1996). Several studies have shown that mixing the seeds from different native units may positively affect the demographic performance of the reintroduced population maximizing its genetic diversity (Kephart 2004; McEachern et al. 1994).

Fiedler and Laven (1996) defined four classes of site-selection criteria for reintroduction projects: physical, biological, logistical and historical. Conservation plans should also consider potentially suitable but presently unoccupied sites (Eriksson 2000). Regarding the optimum life stage, there are, in theory, a wide variety of horticultural options from which to choose in a reintroduction program, but the basic choice is often between transplanting plants and sowing seeds (Guerrant 1996; Guerrant and Kaye 2007). On the one hand, transplanting propagated adult plants can speed the establishment of population cohorts, avoid the losses due to seed predation, and can lower the extinction risk (McEachern et al. 1994; Guerrant 1996); on the other hand *in situ* germination allows natural selection to operate at the dispersal stage and to add all life-history stages to the experimental reintroduction (McEachern et al. 1994). Furthermore, *in situ* sowing experiments could be very important in a reintroduction project because they offer a direct way of identifying whether suitable, unoccupied habitat patches exist at a regional scale, also providing a way to study dispersal limitation (Turnbull et al. 2000). However, the appropriate choice is strongly context dependent, and any species has to be studied individually before spending resources (Guerrant 1996). Any reintroduction project should be conducted in an experimental framework to understand the factors that may influence its success (Heywood and Iriondo 2003; Maschinski et al. 2004), and meaningful results can only be determined through careful monitoring and evaluation (Guerrant and Pavlik 1998; Clewell et al. 2000). In fact, failure to achieve success is likely a result of our poorly developed understanding of species' biology (Schemske et al. 1994; Maschinski and Duquesnel 2006) and of the ecological, genetic, and human-related factors that can affect the reintroduction of threatened plants (Lawrence and Kaye 2011; Maschinski et al. 2004). Thus, prior to reintroductions, acquiring knowledge of the taxonomy, reproductive biology,

demography, horticulture and ecology of the target species is recommended (Schemske et al. 1994; Clewell et al. 2000; Rossi et al. 2013a). Then, the created populations not only reduce the risk of extinction, but also amplify our understanding of the target species and increase our ability to successfully manipulate its natural populations (Heywood and Iriondo 2003). Furthermore, there should be strong evidence that the threats that caused any previous extinction have been correctly identified and removed or sufficiently reduced (IUCN 2013). Hence, it is recommended that reintroduction is undertaken inside a protected area (Maunder 1992; Clewell et al. 2000; Maschinski et al. 2004), in which the new population could persist without human disturbances, in order to avoid the risk of wasting the reintroduction efforts. Monitoring is the most time consuming and expensive portion of any reintroduction, but without appropriate monitoring there will be no way of evaluating the success of the intervention and to understand to what degree the reintroduction objectives have been attained (Clewell et al. 2000; Turnbull et al. 2000; Caplow 2004). Even if the annual count of reproductive individuals is part of a monitoring process, it would not provide all the information needed to evaluate the success of the reintroduction (Caplow 2004). A reintroduction is not successful if plants are not able to reproduce and establish successive generations, so a monitoring plan must focus on the reproductive traits and on plant regeneration ability, considered useful tools to identify limits to recruitment and their causes (Caplow 2004; Maschinski et al. 2004; Fenu et al. 2013b; Ulian et al. 2013).

Finally, an understanding of environmental policy regulating rare plant management both at the national and local level is needed since plant conservation cannot success unless political and economic factors are incorporated in management strategies. Furthermore, communication and interaction are crucial among the diverse national, local and private agencies involved in plant conservation because would gain from sharing biological data of rare plants and details of the successes and failures of recovery measures (Schemske et al. 1994).

The objectives of this study were to:

- investigate the *in situ* seed longevity of *Malcolmia littorea* through a burial experiment and the ability of forming a soil seed bank;
- evaluate and compare the efficiency of different reintroduction methods for *Malcolmia littorea*;
- study the *in situ* germination response to different ecological conditions;
- translate the findings into practical advices for the implementation of a reintroduction strategy for *M. littorea*.

5.2. MATERIALS AND METHODS

5.2.1. Seed longevity experiment

On September 2013, seeds were collected from plants of one Italian native population (IT-1) and were randomly selected to form five replicates of 50 seeds each. These replicates were immediately stored in nylon mesh envelopes (Fig. 5.1a) and buried within the first 10 mm of soil in five different points spread inside the area occupied by the native population. These replicates were left under the soil, without any manipulation, for one year.

On September 2014, only two of the five replicates buried the year before were recovered. These two replicates (called A and B), were exhumed (Fig. 5.1b) and brought to the laboratory where the envelopes were carefully opened. All the 50 seeds/seed coats of each replicate were found in the envelopes. The status of the seeds was investigated under a stereomicroscope (Nikon SMZ 1000) and the seeds were scored as germinated (where an empty seed coat occurred), or non-germinated (where the entire seed occurred).

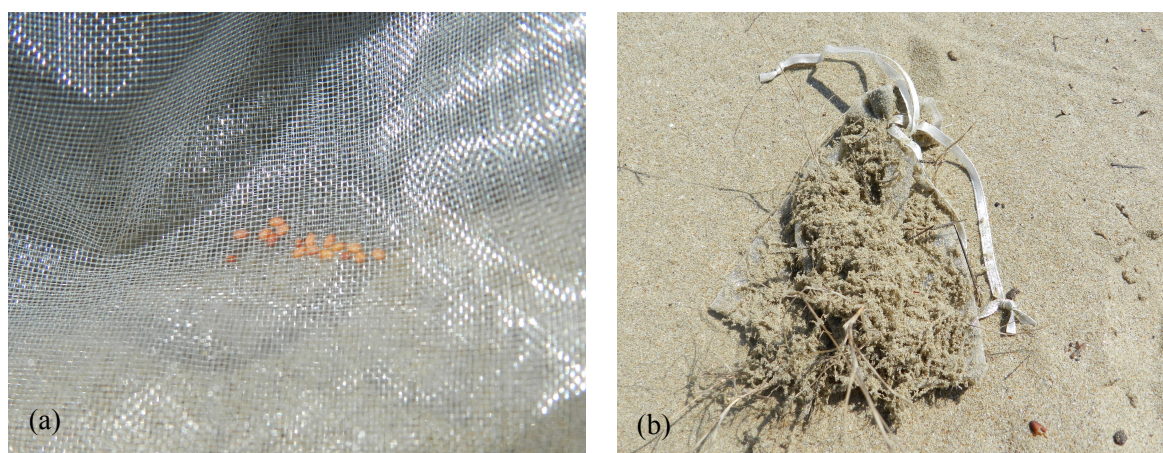


Figure. 5.1. (a) Seeds introduced into the fine grain, nylon mesh envelopes before being buried on September 2013, and (b) an exhumed replicate on September 2014 (photo: M. De Vitis, San Felice Circeo, Italy).

The non-germinated seeds of each replicate were divided into two sub-replicates (A.1, A.2, B.1, B.2), sown on Wathman no. 2 filter papers moistened with distilled water in 60 mm plastic Petri dishes, and incubated in the darkness at 10°C to test for their germinability. The darkness was achieved by wrapping the dishes in two layers of aluminium foil. The germination was scored every two days under the daylight, and the germination experiment lasted for a maximum of 30 days, after which time no further

germination was observed. Germination was defined as visible radicle emergence. At the end of the germination tests, a cut-test was carried out to determine the viability of the remaining seeds (soft or firm) and the final germination percentage was calculated on the basis of the total number of filled seeds as the mean of the five replicates $\pm$ standard error (SE).

5.2.2. Reintroduction trial - *Litorale di Torre Astura*

5.2.2.1. Site description

The site selected for the reintroduction trial was a portion of 2000 m² (Lat: 41.41, Long: 12.76) of the sand dune system occurring in the SCI *Litorale di Torre Astura*, which was included in the Nettuno Artillery Training Area. This military area was established in 1888 on 1550 ha along the Tyrrhenian coast, and represents one of the most important residual patches of semi-natural landscape of the coastal Latium. The area was destroyed for most of its extension by land reclamation in the 1930s and by subsequent intensive farming and urbanization (Filibeck and Lattanzi 2008). It preserves many rare and endangered plant species and several habitat types, including sand dunes. Here, well preserved perennial plant communities of the inland side of mobile dunes, dominated by chamaephytic and herbaceous species, can be found. These are attributable to the EU Habitat 2210 “*Crucianellion maritimae* fixed beach dunes” and EU Habitat 2220 “Dunes with *Euphorbia terracina*” (*sensu* Habitats Directive 92/43/EEC; European Commission 2013).

5.2.2.2. Plant propagation

On September 2012, the propagation of *M. littorea* plants started in one of the glasshouses of the Botanical Garden of Rome using seeds collected from the Italian native populations in the previous reproductive season. The propagation was also repeated seven months later on April 2013. The seeds deriving from the different populations were mixed and sowed randomly on the surface of both plastic and peat pots, containing a mixture of soil and sand. The two different kind of pots were used to understand if the cultivation in

peat pots could avoid some stress during transplant process, facilitating the establishment of plants. In fact, growing the plants in peat pots allows to transplant a plant together with the degradable pot, avoiding the stresses of extracting it from the pot and of exposing its root system (Fig. 5.2a,b).

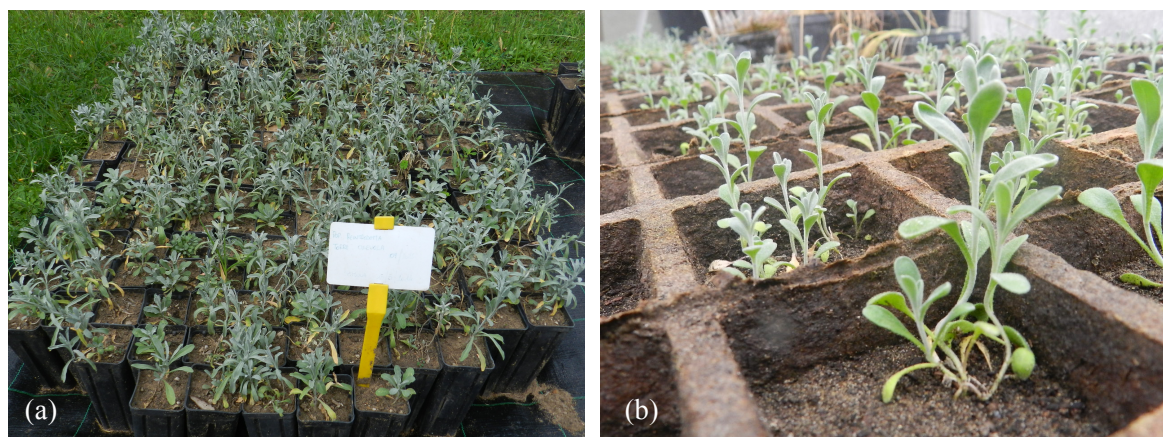


Figure 5.2. Plant propagation of *M. littorea* in (a) plastic and (b) peat pots.

5.2.2.3. Experimental design

On October 2013, 200 adults (13 months old) and 200 seedlings (six months old) were available for the transplantation in the reintroduction site. Both for adults and seedlings, half of them were grown in plastic pots and half in peat pots. They were transplanted in groups of 10 plants (plots) mixing neither adults and juveniles nor plants grown in plastic and peat pots. Twenty plots (ten for the adults and ten for the seedlings) were established on the slope of the fixed dune exposed to the wind and salt spray (Fig. 5.3a), while other twenty plots (ten for the adults and ten for the seedlings) were set on the sheltered slope of the mobile dune (Fig. 5.3b and 5.4).

In the same date, 25 sowing plots were set under different conditions along the sand dune habitat: five on the slope of the fixed dune exposed to the wind and salt spray, five on the sheltered slope of a mobile dune, five on the foredune and ten in two areas (five in each area) characterised by a high density of alien plants (especially *Agave Americana* and *Carpobrotus* spp.; Fig. 5.3c,d). In each plot, 100 seeds, collected in summer 2013 from the Italian populations, were randomly sowed (Fig. 5.4).

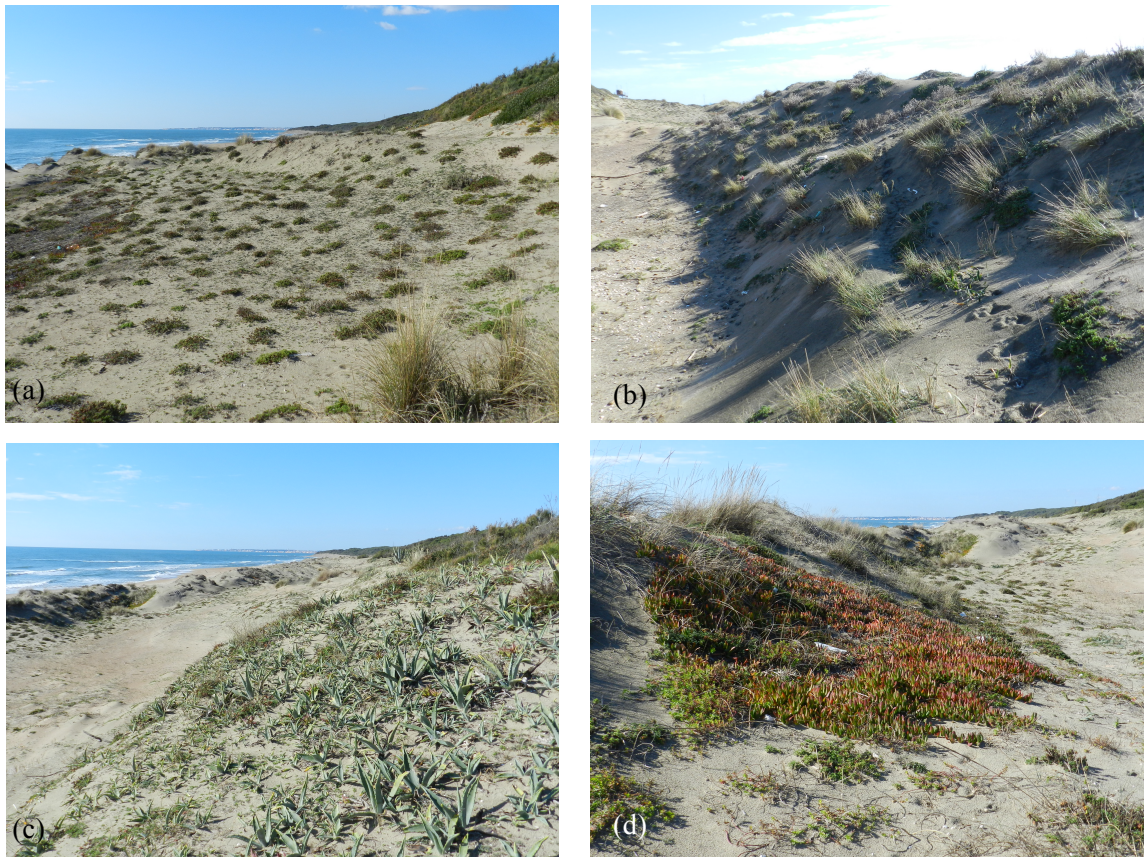


Figure 5.3. *Litorale di Torre Astura*: (a) slope of the fixed dune exposed to the sea, (b) slope of the mobile dune sheltered from the sea, (c) exposed site with high density of *Agave Americana*, and (d) sheltered site with high density of *Caprobrotus* spp. (photo: M. De Vitis).

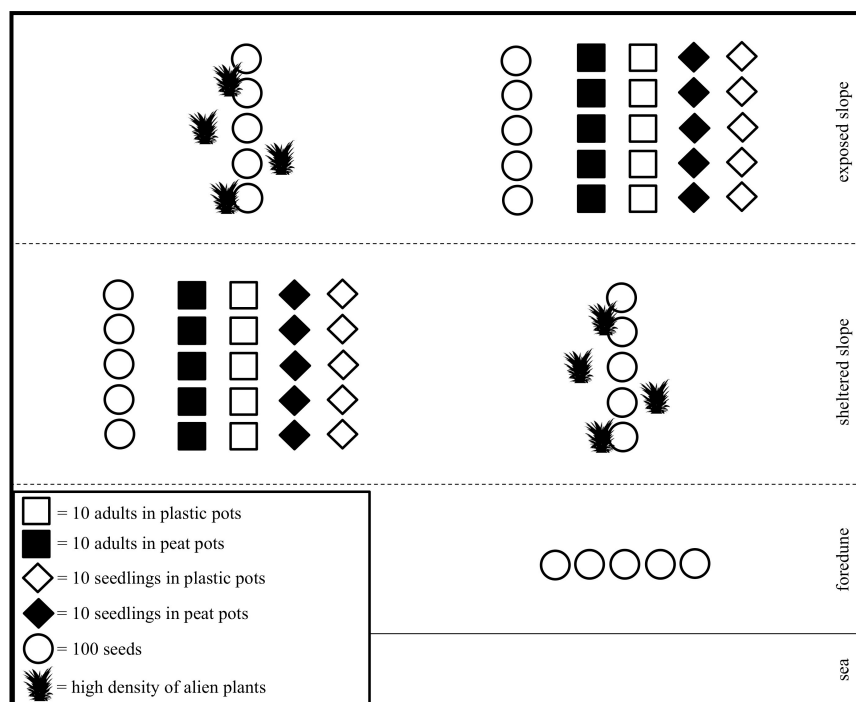


Figure 5.4. Experimental design of the reintroduction trial at *Litorale di Torre Astura*. Two hundreds adult plants and 200 seedlings were transplanted in plots (squares and diamonds, respectively) of ten and 2500 seeds were sown in plots (circles) of 100.

After the trial started, every month plants survival and seed germination were monitored in the study site. Transplants survival was calculated for each treatment (plastic pot + exposition, plastic pot + shelter, peat pot + exposition, peat pot + shelter) as the mean number of living plants over the five replicates ($\pm$ standard deviation), both for adults and seedlings. Then, the mean survival was compared among treatments. During the monitoring of June 2014, 14 adult plants from each of the different microsites (sheltered and exposed dune) were specifically observed and measured: for each plant the phenology was recorded, the number of stems was counted and the longest stem was measured.

For sowing, the germination percentage under each condition was calculated as the mean number of germinated seeds over the five replicates ($\pm$ standard deviation). Then the conditions were compared.

5.2.3. Reintroduction/translocation trial - *Rio Martino*

5.2.3.1. Site description

The site selected for the reintroduction trial is a portion of a transition dune of ca. 100 m² (Lat: 41.39, Long: 12.91) in Rio Martino locality. This site is part of the Circeo National Park, which is situated in southern Latium. The coastal dune system included in the Park has a relatively simple structure that usually consists of a main dune ridge with some embryo dunes 40-50 m from the drift line. The system is bounded by the Tyrrhenian Sea to the west and four coastal lagoons (Fogliano, Monaci, Caprolace and Paola) to the east. The coastal dune belt has an average width of 250 m and an average altitude of ca. 15 m. The section of the beach most frequented by people is cleaned and smoothed over two or three times a year, eliminating pioneer vegetation from the embryo dunes. People usually cross the dunes on foot, which causes shifting of sand. In some places, erosion generates a mosaic of steep dunes, narrow canyons and mobile sand deposits (Acosta et al. 2000).

5.2.3.2. Plant propagation

On March 2014, the propagation of *M. littorea* plants started in the nursery of the National Forest Service (Sabaudia, Latina, Italy). As for the first trial, seeds collected in the previous reproductive season (2013) from the Italian populations were cleaned, mixed and randomly sowed in plastic pots containing a mixture of soil and sand. From ca. 1,000 germinated seeds, very few plants survived during the following months and the summer drought. On October 2014, only 50 *ex situ* grown seedlings (seven months old) were available for the reintroduction trial at *Rio Martino*.

5.2.3.3. Experimental design

The plantlets were transplanted in ten plots (five plants each) established in the dune strip between the mobile and fixed dune of the reintroduction site (Fig. 5.5a and 5.6). In the same date, a sowing experiment was carried out (Fig. 5.5b), sowing three replicates of 100 seeds for three different seed provenances: two Italian native populations (IT-1 and IT-3) and one population reintroduced in Italy in 2010 (IT-R; De Vitis 2011). This design was drawn to detect the differences in *in situ* germination among the different seed provenances and to compare them with *ex situ* germination results (see paragraphs 4.2.2.1 and 4.3.1.1). Where the sowing plots were established, vegetation gaps were created to facilitate seed germination and seedling establishment (De Vitis et al. 2013).

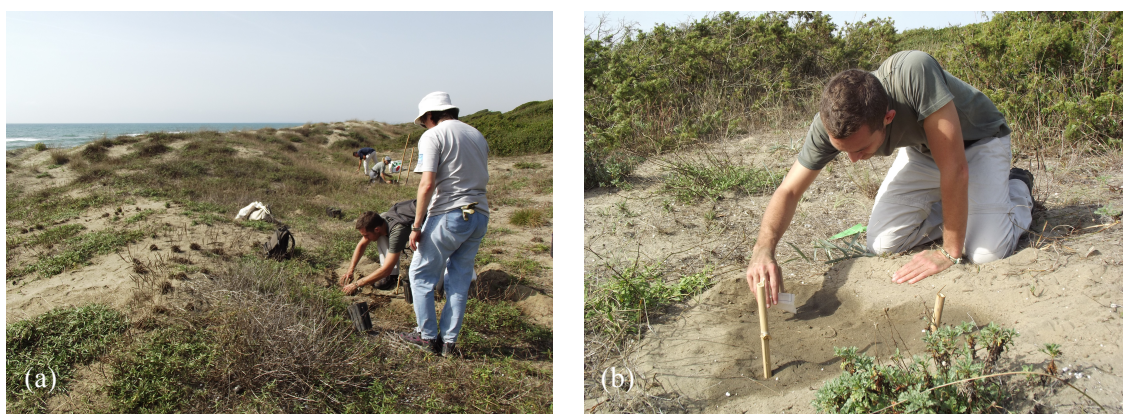


Figure 5.5. *In situ* (a) transplanting and (b) sowing at *Rio Martino* (photo: S. Magrini).

Furthermore, 30 adult plants were translocated from the most imperilled Italian native population (IT-1) to a site adjacent and ecologically similar to the reintroduction site. The plants were transplanted in six plots of five plants each (Fig. 5.6).

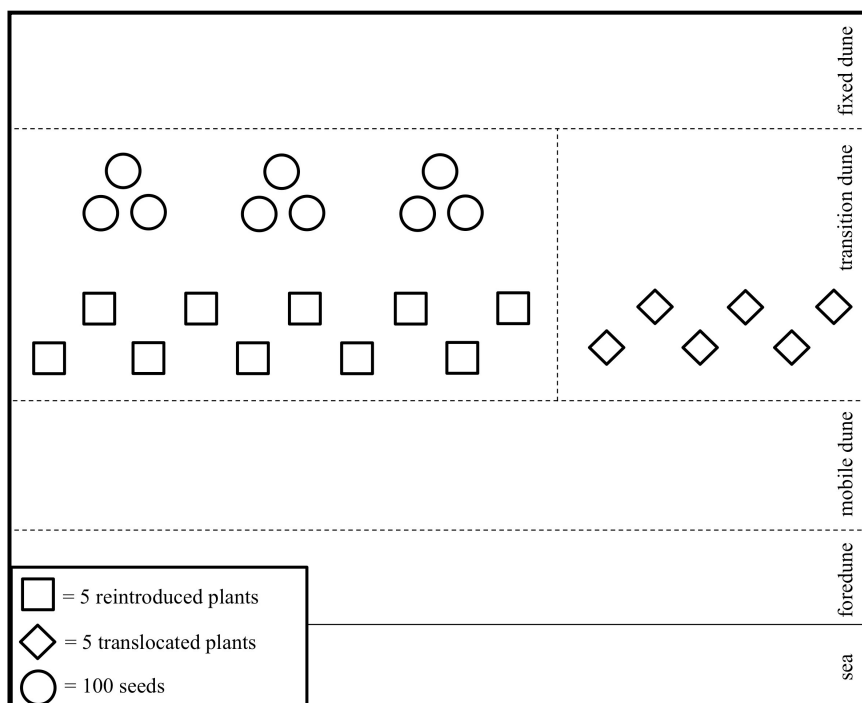


Figure 5.6. Experimental design of the reintroduction/translocation trial at *Rio Martino*. Fifty *ex situ* grown plants were transplanted in plots (squares) of five; 30 plants were translocated from a native population (IT-1) in six plots (diamonds) of five; and three replicates of three sowing plots (circles) were set: in each replicate the seeds of IT-1, IT-3 and IT-R were sown, respectively.

After the trial started, plants survival and seed germination were monitored monthly at the study site. The survival of transplanted/translocated plants was calculated as the mean percentage of living plants among the replicates ($\pm$ standard deviation). For sowing, the germination percentage of each provenance was calculated as the mean number of germinated seeds of the three replicates ($\pm$ standard deviation). Then the germination percentages of the different provenances were compared by Kruskal-Wallis test.

5.3. RESULTS

5.3.1. Seed longevity experiment

For both replicates A and B, the percentage of empty coat (assumed as germinated seeds) was low (14.0 and 10.0%, respectively), with a mean ($\pm$ ES) of $12.0 \pm 2.0\%$. The percentages of non-germinated seeds were 86.0 and 90.0% respectively, with a mean of $88.0 \pm 2.0\%$.

When the non-germinated seeds were incubated in the darkness at 10 °C, the mean germination percentage achieved by replicate A within 30 days was $93.1 \pm 2.2\%$, while $100.0 \pm 0.0\%$ was reached by replicate B. The overall mean ($n = 4$) was $96.5 \pm 2.2\%$.

5.3.2. Reintroduction trial - *Litorale di Torre Astura*

At the date of the last monitoring (March 2015), the mean percentage ($\pm$ standard deviation) of surviving adult plants overall different sites was $4.4 \pm 1.1\%$, while an overall $0.8 \pm 0.6\%$ of alive seedlings was recorded, with a significant difference between them (Mann-Whitney: $U = 6$, $P < 0.001$). All the surviving plants were those grown in plastic pots. In fact, for both life stages, no plants grown in peat pots survived until the last monitoring date. In particular, adults and seedlings grown in peat pots died within four and three months, respectively.

Concerning the adult plants, in the sheltered site a mean ($\pm$ standard deviation) of 2.6 ± 1.1 plants per plot survived, while in the exposed site the mean number of survived plants per plot was 1.8 ± 0.8 , with a significant difference between them ($t = -4$, $P < 0.05$; Fig. 5.7a). Between May and June 2014, a military celebration of the 70th anniversary of the landing at Anzio (1944) occurred at the *Litorale di Torre Astura*. Four-hundreds soldiers landed in this site driving amphibious landing crafts and tanks on the dunes, damaging the vegetation. Even if a certain mortality was registered between the monitoring of May and June 2014, it was low and quite similar for plants on both the exposed and the sheltered sides of the dune and it was not possible to evaluate the entity of the damages caused by the tanks.

On June 2014, 78.6 and 35.7% of the plants observed in the sheltered and in the exposed sites respectively, were in the reproductive phase. The mean ($\pm$ standard deviation) number of stems per plants was 4.3 ± 4.4 in the sheltered site and 2.4 ± 1.1 in

the exposed site, with no significant difference between them. The mean length of the longest stem was significantly higher ($P < 0.001$) in plants established in the sheltered site (25.1 ± 12.4 cm) than those in the exposed site (9.2 ± 6.5 cm). At the last monitoring (March 2015), recruitment was visible near the adult plants which flowered and fruited in the previous reproductive season. In particular, in the plots of the adult plants situated in the leeward site, the mean number of seedlings per plot was 4.8 ± 6.1 , while in the windward site plots it was 4.4 ± 5.0 , without a significant difference between them.

Regarding the sowing experiments, after six months since the trial started, on the overall quantity of seeds (2500) introduced in the study site only 11 germinated, which corresponded to 0.44%. In particular, 10 of them germinated in the area on the exposed slope of the dune with high density of *Agave americana* (Fig. 5.7b) and the remaining one was found on the sheltered slope of the dune. From June 2014, the germinated seedlings started to die until they were not recorded anymore on September 2014. Then, during the successive autumn/winter, new germinated seedlings started to be recorded. At the last monitoring of March 2015, 62 seedlings were found in the sowing plots, which corresponded to 2.49% of the total quantity of seed introduced. In particular, nine of them were found in the leeward site plots, 12 germinated in the windward site plots, and the other 41 were found in the plots on the exposed slope of the dune with high density of *Agave americana*. No seeds germinated in the foredune and in the site with high density of *Carpobrotus* spp.

Comparing the climatic data from October to December for 2013 and 2014, it emerged that there was not a significant difference of $T_{\min}$, T_{mean} and $T_{\max}$ between October 2013 and 2014, but a higher mean RH (relative humidity) percentage was detected for October 2013 (85%) than 2014 (75%; $P < 0.01$). For the month of November, it occurred an opposite situation, showing a significant difference in $T_{\min}$, T_{mean} and $T_{\max}$ but not in $RH\%$ between the years 2013 and 2014. In particular, November 2014 showed significantly higher mean values of $T_{\min}$ (11.6 °C; $P < 0.05$), T_{mean} (16.5 °C; $P < 0.05$) and $T_{\max}$ (20.1 °C; $P < 0.01$) than 2013 ($T_{\min} = 9.4$ °C, $T_{\text{mean}} = 13.9$ °C and $T_{\max} = 17.2$ °C). For December, no differences were detected between values registered in 2013 and 2014.



Figure 5.7. (a) A transplanted plant in the site *Litorale di Torre Astura* (photo: P. Di Bacco) and (b) a germinated seedling in the site with high density of *Agave americana* (photo: M. De Vitis).

5.3.3. Reintroduction/translocation trial - *Rio Martino*

During the last monitoring (March 2015), the mean ($\pm$ standard deviation) percentage of surviving reintroduced plants was $8.0 \pm 10.3\%$ (Fig. 5.8a), while a mean of $10.0 \pm 24.5\%$ of alive translocated plants was recorded. Several translocated plants had ripe fruits on their stems during their transfer from the native population to the experimental site. The seeds retained in those fruits, representing a canopy seed bank (*sensu* Ma and Liu 2008), were dispersed in the experimental site and germinated in the following months. Recruitment occurred near the translocated plants, with a mean number of germinated seedlings per plot of 5.3 ± 9.7 .

Concerning the sowing experiments, no germinated seeds of the population IT-1 were recorded, while in the IT-3 and IT-R plots, 6.0 ± 5.6 (mean $\pm$ standard deviation) and 1.0 ± 0.0 seeds germinated, respectively (Fig. 5.8b). On March 2015, a total of seven adults and 53 juvenile plants were registered.

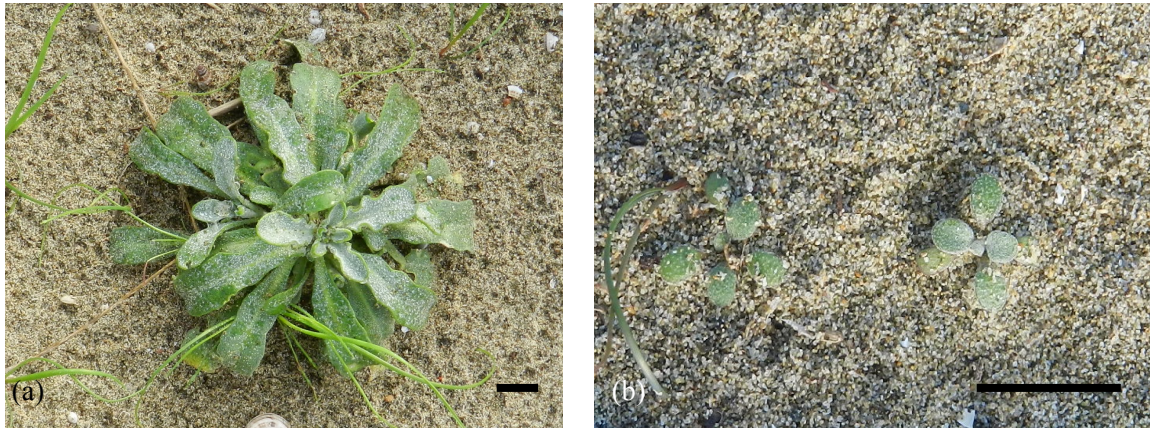


Figure 5.8. (a) A nine months old seedling reintroduced at *Rio Martino* and (b) seedlings germinated at *Rio Martino* (scale bar: 1cm; photo: M. De Vitis).

5.4. DISCUSSION

5.4.1. Seed persistence in the soil and reintroduction chances

Since the *in situ* burying experiment lasted one year, it was not possible to infer anything about longer-term persistence of *Malcolmia littorea* seeds in the soil. Nonetheless, after this period, a high percentage of exhumed seeds was viable and able to germinate under laboratory conditions. This result confers to *M. littorea* the ability to form at least a short-term persistent soil seed bank. It is well known that some seeds experience an annual dormancy cycle when buried (Baskin & Baskin 1998). This phenomenon of intermittent germination, where seeds of the same fruiting season may germinate in different times, is found in species that survive under unpredictable conditions (Baskin and Baskin 1998), which characterise, for example, the sand dune habitats. Several studies have found the ability of forming a seed bank in plant species inhabiting such habitats (Sternberg et al. 2004; Yu et al. 2007; Ma and Liu 2008; Yan et al. 2009). In particular, Sternberg et al. (2004), studying the vegetation of an Israeli Mediterranean sand dune, found a high potential for producing persistent seed banks in the crucifer functional group.

In many plant species, different maternal and environmental factors may increase the phenotypic diversity, which ensures that, during or after a rainfall, only a portion of the total seed bank of a certain plant species will germinate (Guterman 2000). The presence of buried viable seeds in the soil is commonly associated with a phenomenon of dormancy, which prevents the seeds from germinating even under favourable environmental conditions (Degreaf et al. 2002), and the persistence of these seeds in the soil seed bank ensures the recovery of a population after regular crashes due to environmental fluctuations (Turnbull et al. 2000). The study of Volis et al. (2004) on the annual *Hordeum spontaneum* (Poaceae), demonstrated that in occasional and extremely dry years when amount or timing of rainfall is insufficient for seed to germinate, a short-term persistent seed bank becomes the most important demographic stage ensuring population existence. The seed longevity analysis allowed an estimate of seed germination of the native *M. littorea* population in its natural site. It was about 12%, presumably an overestimation, since every empty seed coat was considered as a germinated seed, but, it may be possible that some of those seeds were eaten by predators or attacked by pathogens, rather than germinated.

The ability of the study species to form a persistent seed bank also emerged from the reintroduction trial at *Torre Astura*. A very small portion of the total amount of seeds

sowed in autumn 2013 germinated during the same year and another small portion of the same seeds germinated during the successive year. An improvement in the germination ability with seed ageing is also believed to be owing to physiological changes occurring in the seed, referred as after-ripening and/or softening of the hard seed coat. The variation between species in the period of after-ripening necessary to achieve maximum germination ability is considerable, and this variation appears to be related to the time of seed production in the field (Pemadasa and Lovell 1975). Although the percentage of germinated seeds, with respect to the total amount of seeds sown, was very low in both years, in the second year it was a little bit higher than the first. Other studies reported low *in situ* seed germinations in Mediterranean arid habitats (e.g., García-Fayos and Gasque 2006). A difference in the temperatures in November was registered, with higher minimum, average and maximum temperatures in 2014 than 2013, between the two years (2013 and 2014). Even in this case, the temperature in November appeared to influence the seed germination (see paragraph 4.2.2.3). In particular, very few seeds can germinate below a specific threshold of temperature during November. All the seedlings germinated in 2013 at *Torre Astura* died by the end of the summer. Similarly, Aziz and Khan (1996) while studying the seed bank dynamics and recruitment of a coastal shrub community in a sandy beach of Karachi coast (Pakistan), observed that only few seeds of *Cressa cretica* (Convolvulaceae) germinated after the rains but these seedlings failed to establish due to rapid drying of the soil.

However, recruitment following seed addition in the unoccupied sites indicated that a regeneration niche existed, and the lack of the species in the plant community can be attributed to dispersal limitation (Turnbull et al. 2000).

5.4.2. Effect of alien plants on germination

At *Torre Astura*, alien plants affected germination in different ways. In the site with high density of *Agave americana* (Asparagaceae), the germination of *M. littorea* was higher than the other sites, maybe due to the soil compaction acted by the alien plant rhizomes, which may limit the wind erosion and provide microsites which favor *M. littorea* seed germination. The high clonal reproduction of agaves on sandy soils, found by Badano and Pugnaire (2004) in the southeastern Spain, suggested that this soil type provides a niche that enhances their population growth rate, with the possibility of limiting space and

resources for native communities. Furthermore, their results suggested that the occurrence of *Agave* spp. on sand may modify the proportional cover of some species rather than their richness, favoring the performance of a specific plant over other native species. In the site with high density of *Carpobrotus* spp. (Aizoaceae), no seeds germinated. Novoa et al. (2012), while studying the impact of *C. edulis* on *M. littorea* found an allelopathic function of *C. edulis* litter against *M. littorea* germination and root growth. Hence, it is possible to conclude that in presence of high density of *Carpobrotus* spp., *M. littorea* germination is inhibited.

5.4.3. Effect of salinity and moisture level in the soil on germination

At *Torre Astura*, no seeds germinated on the foredune, maybe due to the highly unfavorable conditions of the site, with higher salinity and sand burial than in the transition dune where *M. littorea* usually lives. In fact, its radicle growth has been found to be stimulated by relatively low levels of salinity (Novoa et al. 2012).

Pemadasa and Lovell (1975) studying seven annual species living in the Aberffraw (UK) sand dune system, found out that high temperatures were effective in imposing seed dormancy and that germination performance was clearly dependent on the availability of moisture. In particular, they found fresh seeds showing considerable decline in both the ultimate percentage and rate of germination with decreasing soil moisture regime, and desiccation of germinating seeds effectively delaying germination. For unimpeded germination the seeds require both an adequate and continuous supply of moisture (Pemadasa and Lovell 1975). It may be possible that *M. littorea* seeds needed higher moisture level and continuity during autumn and winter than those registered in the years 2013 and 2014, more than a specific temperature range in the month of November. Novoa et al. (2012), with their laboratory germination experiments, showed that, when moisture, salinity and pH levels were tested together, moisture emerged as the most important factor determining the seed germination in *M. littorea*. In particular, seed germination and root growth were higher at high moisture levels and they were not influenced by different levels of pH and salinity (Novoa et al. 2012). However, salinity has also its own role in affecting the germination. Del Vecchio et al. (2012a) found a salt induced dormancy and an increased sensibility to salt at high temperatures in *Crucianella maritima* (Rubiaceae). This mechanism might represent an advantage in harsh ecosystems, helping seed to germinate

during the period which guarantees higher seedling survival and a successful seedling establishment (Del Vecchio et al. 2012a).

5.4.4. Differences between *in situ* and *ex situ* germination

Among the few seeds germinated at *Rio Martino*, most of them were of one wild provenance (IT-3), very few were recorded for the reintroduced provenance (IT-R), while no seeds were recorded for the other native source (IT-1). Under laboratory conditions, IT-R (RE1 and RE2, see paragraph 3.2.1.1.) seeds showed higher germination percentages than IT-1 and IT-3 (even if the comparison between these seedlots may be affected by the different year of collection). However, experiments in incubators, under controlled conditions, have certain limitations, because many physical and ecological factors are missing and/or are different from natural conditions. For example, inside a Petri dish, light intensity is several times lower than full sunlight; moisture is constant and more optimal for germination; the temperature is constant too and, at the seed level, can be higher than the value set for the chambers, especially for dark seeds (Keller and Kollmann 1999); there is no wind nor salt spray affecting seedling growth, no predation nor human impact; above all, there is no competition with other seedlings and other species for space, light, and nutrients in the soil. In isolation, germination results can be misleading if they take no account of seedling emergence and competition and plant mortality. For example, Moot et al. (2000) showed that, under laboratory conditions, *Trifolium repens* (Fabaceae) had a more rapid germination than *Lolium perenne* (Poaceae), but the latter species had a higher seedlings growth rate which makes it more competitive in the field.

5.4.5. Comparison between transplanting and sowing for reintroduction purposes

The high mortality of transplants in both the reintroduction and in the translocation trials might have been due to the transplanting stress. In particular, from the *Torre Astura* trials it emerged that the peat pots did not help the plants against transplanting stress, but conversely, they affect negatively the plants survival, maybe obstructing the root system development and the water supply. Moreover, the juveniles (six months old plants) were not sufficiently resistant to be transplanted. Similarly, Guerrant (1996) found that

reintroduced populations derived from the youngest plants were much more extinction prone than populations derived from larger plants. It is possible that the cultivation of the seedlings, inside a glasshouse, affected negatively their growth. In fact, the seedlings transplanted at the other reintroduction site (*Rio Martino*), just one month older than those transplanted at *Torre Astura*, were grown outdoor and subjected to a natural hardening process. This process could be responsible of the higher survival percentage of seedlings at *Rio Martino* than at *Torre Astura*, even if the values are both very low. From the reintroduction of another psammophilous species, *Dianthus morisianus* (Caryophyllaceae), it emerged that transplanting in a protected area with hardened plants and during an optimal period, according to the species adaptations and to the habitat characteristics, results in a low mortality rate (Cogoni et al. 2013). From the *Torre Astura* trial, furthermore, a significant preference in microsite for adult plants emerged. In the slope of the dune sheltered from wind and salt sprays, on June 2014, a higher percentage of adult plants, with longer stems, was flowering compared to the exposed dune slope. Also in the study of Zuccarini et al. (2015), the preference of *M. littorea* for the leeward face of the dune has been reported.

The sowing method resulted in 62 and 21 individuals emerging at *Litorale di Torre Astura* and *Rio Martino*, respectively. These numbers are low, especially if considered as proportions of the starting amounts of seeds introduced, but the effort, in terms of time and economic resources spent, was almost null. In fact, it just required seed collection, germplasm storing, and the *in situ* sowing. From an ecological point of view, *in situ* germination allows natural selection to operate at the dispersal stage (McEachern et al. 1994) and the resulting plants may be more adapted than the *ex situ* grown ones. The strategy of augmenting the starting amounts of seeds may not be the right solution, especially because the collection of larger quantity of seeds could be destructive for the small Italian populations imperiling their regeneration. A different sowing strategy may be to spread the seeds inside the reintroduction site, rather than sow them in small plots, where predators, pathogens or other not favorable factors may also occur. Even if in the present study the low number of germinated seeds at *Rio Martino* did not allow to appreciate the gap creation strategy, through the removal of litter and vegetation, and through the soil ploughing, its positive effect on seed germination has been reported by several studies (Zobel et al. 2000; Jusaitis et al. 2004; Sternberg et al. 2004; Satterthwaite et al. 2007; Jogar and Moora 2008; Mitchell et al. 2008; Hellstrom et al. 2009), and even for the same species (De Vitis al. 2013).

Compared to the sowing method, transplanting required more time and economic resources. In fact, for transplanting more materials and steps such as the *ex situ* cultivation (for which a greenhouse, pots, soil, water, etc.) and the transfer of the plants (which occupy a significant larger space than seeds) were needed. However, using this method it is possible to optimize the biological material since the optimal conditions for seed germination are known. Under controlled conditions, it is possible to obtain a plant from each viable seed. The disadvantage is that *ex situ* grown plants may be so poorly adapted to the actual conditions under which they will be transferred that they may die after few days in the reintroduction site. To make this method an advantageous one, in terms of time and economic resources, the transplant mortality must be reduced by improving the *ex situ* cultivation methods.

5.5. CONCLUSIONS AND IMPLICATIONS FOR REINTRODUCTION

The transition from seed to seedling is likely to be a critical stage in the plant life-history of *M. littorea*, and might be affected by the environmental factors typical of Mediterranean sand dune habitats, such as the unpredictable temperature fluctuations and the low field capacity of water retention, play a key role. In particular, they can regulate the percentage of germinated seeds and established seedlings. The fraction of non-germinated seeds may remain viable in the soil for at least one year and germinate in the year successive to its production and dispersal. The ability of the species to form a persistent soil seed bank is an important outcome that will be helpful in practical *in situ* conservation and management actions. The existence of a soil seed bank represents an escape in time from the temporarily unfavourable conditions, especially for an isolated and small plant population (Quilichini and Debussche 2000) and may provide a small but significant reduction in the risk of extinction (Satttherthwaite et al. 2007). However, factors disturbing the soil, such as the frequent trampling (Rossi et al. 2009), may destroy the fragile soil structure and lead to a spatial instability of the seed distribution and their burial depth (Quilichini and Debussche 2000), and, as a result, the seed bank density may be reduced (Sternberg et al. 2004). More studies are needed to evaluate the actual and potential entity of the soil seed bank in *M. littorea*. For example, longer time burying experiments, soil core experiments to study the spatio-temporal pattern of the soil seed bank, and comparative studies among different populations.

The results of these trials and *in situ* experiments represented the first step in the development of the strategy for a successful reintroduction of *M. littorea*. However, further studies are needed to improve it. Since germination requirements for the species are known, the *ex situ* propagation and plant transplanting can speed the establishment of population cohorts and avoid the losses due to high mortality of juveniles (Guerrant 1996; Maschinski and Duquesnel 2006) and to seed predation (McEachern et al. 1994; Calviño-Cancela 2007), and the possibility of a very low seed germination due to unfavourable climate conditions. From this study it emerged that it is better to transplant more resistant adult rather than young plants, and that it is better to cultivate the plants in plastic pots, because the peat pots may degrade over long times representing an obstacle to root development. However, the study species was not so prone to *ex situ* cultivation and suffered for transplantation. *In situ* sowing trials showed that germination was dependent upon multiple factors, such as climate conditions, the presence of alien plants and the

excessive exposition to salt spray. Even if in the first year very few seeds germinate, their longevity allows the ungerminated ones to remain viable and to possibly germinate the subsequent year. Thus, *in situ* seed sowing is an important action to facilitate the formation of a soil seed bank in an unoccupied site.

CHAPTER 6

CONCLUSIONS: TOWARDS AN INTEGRATED CONSERVATION OF *MALCOLMIA LITTOREA*

With the large number of species at risk and the limited amount of time and resources available, general rules must be developed and employed to ensure the conservation of threatened species (Ellstrand and Elam 1993).

The present study represented the first report on the conservation biology of the Mediterranean coastal plant *Malcolmia littorea*, and the data produced and the results will be useful for the development of an efficient strategy for its long-term conservation in Europe.

This species is characterised by a disjoint distribution, which is a common trait of many Mediterranean species, with the Italian populations representing a cluster isolated from the rest of the European populations. Despite this situation of isolation, the Italian populations showed levels of genetic diversity comparable to other larger and less isolated populations. On the other hand, these populations, together with another small Spanish population, showed low germination percentages, reflecting a likely effect of inbreeding depression (Barrett and Kohn 1991; Ellstrand and Elam 1993). The loss of genetic variation through stochastic forces and the deleterious effects of inbreeding in small populations are the major threats that compromise attempts to maintain the long-term viability of populations of rare plant species (Barrett and Kohn 1991).

The relatively high genetic diversity in small populations which show reduced fitness in terms of seed germination, and the lack of correlation between diversity level and population size, may be explained by the populations history. In species with historically large populations that have declined to a few individuals within a few generations, the effect of genetic erosion on fitness might be expected (Ouborg and Van Treuren 1995),

while populations that remain small for long periods are likely to be considerably less diverse than those that have only recently become small (Barrett and Kohn 1991). Thus, it is likely that the small populations of *M. littorea* declined to small sizes recently and it appears important to minimize their further genetic erosion by genetic drift. Adaptive alleles may be lost from the population due to genetic drift (Ouborg and Van Treuren 1995).

The management of plant populations should aim to increase the number of plants in small populations and the gene flow among populations through artificial dispersal of seeds (Fischer and Matthies 1998a). While in the present study only neutral marker were studied, fitness related genes should be analysed in future, so as to genetically characterise the fitness traits, such as the seed germination, among different populations.

On the basis of both chloroplast and nuclear markers analysis, three main genetic clusters emerged across Europe. The origin of the species and its glacial refugia remain unclear, but it is likely that, as other Mediterranean species, *M. littorea* has its European refugia in the Iberian and Italian peninsulas. The identification of these refugia may be important to species conservation, since they represent climatically stable areas and key areas for the long-term persistence of species and genetic diversity (Médail and Diadema 2009). Palynological fossil records may be used to obtain knowledge of the distributional history of the species and to speculate on the location of refugia and to support possible migration routes (Hewitt 2000; Kadereit et al. 2005; Juan et al. 2012).

The identification of conservation units is necessary for efficient management and monitoring programmes (Cires et al. 2011; Juan et al. 2012). The spatial analysis, based on genetic data, revealed three populations (FR-4, IT-1 and ES-1), each one belonging to a different genetic cluster, as conservation priorities. These populations retain most of the genetic diversity of the species in Europe and hence they require particular conservation attention. Seed banking of these populations is a priority for the long-term conservation of the species and seed collection could be useful for restoration programmes of the respective nearby areas. However, population from most of the Portuguese region, northern Spain and northern Africa were not considered in this study and they may also host some important populations for the conservation of this species.

The genetic differentiation emerged among these genetic clusters indicates that the management of the populations should focus on maintaining their genetic identity. On the other hand, a high gene flow was detected among *M. littorea* populations, and was maybe due to long-distance pollinators' movements. The goal of management should be to

preserve this natural network of genetic connections, rather than just distinct populations within the network, in order to preserve fundamental evolutionary processes (Crandall et al. 2000). However, pollinators are known to be more attracted by large plant populations and by a diverse and dense vegetation patterns (Ouborg and Van Treuren 1995). Hence, this gene flow level is expected to decrease in the small populations, together with the decrease in seed production and plant fitness. Moreover, *M. littorea* is known to be characterised by a limited dispersal ability. Species which are characterized by a poor dispersal ability are of course threatened in a landscape where their habitats are becoming increasingly fragmented (Eriksson 2000).

Surprisingly, two of the populations identified as genetically relevant are the same two populations for which low germinability levels were detected. These populations occur in very small areas along the Spanish and the Italian coasts, respectively. Through the laboratory experiments on seed germination, a susceptibility of *M. littorea* to decreasing population size in terms of reproduction was detected. Therefore, small *M. littorea* populations are likely to become extinct soon because of their reduced regeneration ability.

The primary method for the conservation of a native species should be represented by the correct *in situ* management of its populations. Often, physical protection is needed in order to ensure the survival and the long-term persistence of a species, even if this might not be sufficient to avoid the decline of a population. A micro-reserve was established through a LIFE project (93/NAT/E/000766) to conserve and protect the plant community at *Playa de Moncófar* (Castellón, Valencia, Spain; Fig. 6.1a) where *M. littorea* occurs (population ES-1). It is evident that the formal action of assigning to a specific area a particular conservation status, without physically protecting it, does not ensure its effective conservation. Therefore, other conservation actions are needed, such as species augmentation, reintroduction or translocation, especially because this population represents an important genetic resource for the whole species and showed a reduced germination ability, probably related to its small size. Obeso and Aedo (1992) stated that, there is not obvious dune size to suggest for the establishment of nature reserves, since extinction occurs on dune systems of all sizes; they should be large enough for species that require large areas and should include particular dune systems where area-independent species are present. Another example of ineffective conservation of a sand dune plant community occurs at *Portiragnes* (France; Fig. 6.1b). Here, despite a sign saying that the natural area

is protected, there is no physical protection, therefore the conservation of the habitat relies on the common sense of the people and/or environmental education.



Figure 6.1. Ineffective conservation measures: (a) the micro-reserve at the *Playa de Moncófar* (Castellón, Spain) and (b) a protection sign at the sand dune habitat of Portiragnes (France; photo: M. De Vitis).

Although it is commonly believed that fencing portions of habitat is not attractive and may even induce people to climb over, it might still be very useful in order to limit vegetation trampling, extirpation of rare and endangered species, and the possible waste disposal, allowing the ecosystem to remain healthy and restore itself. A healthy ecosystem will provide ample opportunity for the historical assemblage of species to reach their natural densities without costly and untested management (Schemske et al. 1994). Several cases of fenced sand dune habitats have demonstrated its positive action, and so, a recommendation for the most imperilled *M. littorea* populations is to fence them, at least until there is not a critical extinction risk anymore.

There are also cases of formally and physically protected but mismanaged sites. Such sites could potentially be perfect nature reserves, but poor management plans applied on them or their use for a specific service can cause their alteration. An example is the SCI *Litorale di Torre Astura*: this SCI is included in the Nettuno Artillery Training Area where usually soldiers practice in shooting and mine exploding. Rarely, memorial celebrations are held using tanks which are driven on the vegetation. As a result, some habitats of this SCI, like the sand dune habitat, are rich in metal depositions and the occurring vegetation communities may be affected by them and may be partly damaged by the passage of heavy vehicles.

When the site protection of an endangered species is not possible, a translocation or a reintroduction inside a nearby protected area should be suggested. Even if for *M. littorea*

several reintroduction trials have already been performed (De Vitis 2011; De Vitis et al. 2013), the best strategy for its reintroduction was still unknown. Carrying out experimental reintroductions was useful to our understanding of several factors that can influence the success of such a plan. Conservation plans that use experimental approaches are more likely to succeed and well-planned experimental reintroductions could increase the likelihood of success (Barrett and Kohn 1991). In particular, transplantation of *ex situ* grown *M. littorea* plants did not result in a satisfactory number of established plants. The high mortality of both transplanted and translocated plants was maybe due to the high plant sensitivity to transplanting. Hence, propagation and transplantation methods should be improved, in order to avoid wasting cultivation efforts and plant material. From the reintroduction trials carried out in this study, as from the seed longevity experiment, it emerged that *M. littorea* seeds are able to remain viable in the soil for one year after dispersion, maybe due to a physiological dormancy. This mechanism of dormancy induction and maintenance is likely to be a result of both the maternal environment under which the seeds have ripened and the specific climate conditions encountered after it is dispersed. This attribute plays an important role in the regeneration biology of the species, providing the possibility for seeds to germinate in a successive period when the optimal environmental conditions occur and to reduce the risk of extinction.

Since *M. littorea* grows in similar habitats and under similar conditions from Italy to northern Portugal, the populations genotypes show significantly similar adaptation to temperature requirements for their regeneration through seed germination. This may be helpful in predicting the germination behaviour of a distant population. However, stored seeds or seeds produced by plants cultivated *ex situ* can show an alteration in the germination pattern with enhanced performance, such as, for example, a high germination at 0 °C, which is an unlikely encountered condition in the Mediterranean coastal habitats.

Furthermore, the seasonal climate patterns, especially the temperatures in November, affect significantly the annual recruitment of the species and its ability to germinate.

With respect to climate change and the predicted increase in temperatures, the germination of species that grow in high temperature environments could be more affected than those growing in cooler places, as the range of sub-optimal germination temperatures of the latter is larger (Dürr et al. 2015). Some of the boreal and Mediterranean species with a narrow range of sub-optimal temperatures may not be adapted to survive future temperature increases (Dürr et al. 2015).

In species that typically germinate in the early autumn, such as *M. littorea*, protracted summer drought conditions could cause germination to be delayed until later in the autumn. Such a delay in germination timing not only may have direct fitness consequences, but it may also affect other aspects of the plants life history, such as seed production and the deriving progeny (Donohue et al. 2002).

Considering various key factors (climate change, the high level of fragmentation and degradation experienced by the Mediterranean coastal dune habitats, the poor dispersal ability of *M. littorea*, its low performance under cultivation conditions, its low competitive ability against other coastal species, and its low *in situ* germination and seedling establishment), the *ex situ* conservation of *M. littorea* appears appealing as it will provide an alternative, yet complementary, way to prevent its immediate extinction and to preserve different genotypes, until a more efficient *in situ* conservation strategy can be developed. *Malcolmia littorea* seeds collected from the different European populations are currently stored in two different seed banks (Tuscia Germplasm Bank of Viterbo and Millennium Seed Bank, RGB, Kew). Where habitats are in immediate danger of destruction, the collection from the wild of plant material (e.g. seeds) and its maintenance in seed banks and botanical gardens become necessary (Barrett and Kohn 1991). In the populations which are known to have unique or rare alleles, emergency preservation plans should be applied, involving seed collection, dehydration, and banking *ex situ* (Degreef et al. 2002). Furthermore, seeds are relatively easy to collect, can represent a range of genetic diversity in the species if harvested from many individuals of the population and can be stored in a relatively small space (Li and Pritchard 2009).

The seed collection, in particular from the European populations identified as genetically peculiar, and their banking together with further studies on the biology of *M. littorea*, will help refining the strategy for its effective *in situ* conservation. However, there should be strong evidence that the threats that caused any previous extinction or that endangered the populations have been correctly identified and removed or sufficiently reduced. Studying the biological and ecological aspects of endangered species would not be sufficient to conserve them, unless threats are removed or minimized.

For *M. littorea*, urgent conservation measures have to be undertaken to stop its local decline across its European distribution. These measures should include habitat protection and restoration, population augmentation, and reintroduction, and must be applied especially for the peripheral populations of each geographic region. The peripheral populations may be characterized by a lower finite rate of increase than a population from

the core of the distribution area, and might have a higher probability of extinction (Volis et al. 2004; Rossi et al. 2009). However, since they may have specific adaptations to particular environmental conditions occurring at the periphery of the distribution (Volis et al. 2004), they should be considered of high conservation interest. For populations that live in very small habitat patches both protection and habitat restoration have to be performed because it has been argued that larger dunes support more species and are less exposed to human pressure, wind, and sand abrasion than smaller ones (Obeso and Aedo 1992).

Particular attention should be given to situation in Italy, as the species was found to be in a critical state. Here, all the populations are severely threatened with extinction mainly due to human activities and their disappearance will mean the disappearance of unique genotypes from the wild.

In addition to the understanding of the biology and ecology of endangered plants, the political and economic factors that can limit the feasibility and effectiveness of recovery efforts must be taken into account. Clark (1989) emphasized that a major weakness of recovery programs for endangered species is a failure to recognize that “numerous non-biological factors and forces have direct, immediate and paramount significance to endangered species recovery”.

To conclude, the present study yielded the following findings:

- Three main genetic clusters have been identified across the European distribution of *Malcolmia littorea* with a significant differentiation occurring among them;
- On the basis of the genetic information, three populations have been identified as the units needed to conserve all the diversity of this species and hence to be prioritized for conservation;
- *Malcolmia littorea* germinated as a typical Mediterranean species avoiding high irradiance and the summer drought and by germinating at cooler temperatures;
- Under the Mediterranean bioclimate all the populations showed similar patterns of germination in response to temperature;
- Stored seeds or seeds produced by plants cultivated *ex situ* can show an alteration in the germination pattern with enhanced performance;
- *M. littorea* seeds are able to persist in the soil for at least one year after dispersal;
- *M. littorea* is susceptible to habitat fragmentation and to decreasing population size, in terms of reproductive fitness;

- The reintroduction success is affected by the cultivation and transplanting methods, by the germplasm source, and by the microclimatic and ecological conditions of the reintroduction site, such as the exposition to the sea, the density of alien plants and the protection;

and suggests the following further actions and investigations:

- Sampling of other *M. littorea* populations from northern Africa, northern Spain and Portugal;
- Genetic and ecological analysis on African, northern Spanish and other Portuguese populations of *M. littorea* using the same methods and techniques used in the present study;
- Development of species-specific microsatellite markers;
- Molecular analysis of *M. littorea* involving adaptive genes;
- Genetic analysis of the reintroduced populations;
- Germination tests for moisture and salt stress;
- Seed burial and soil core experiments at different sites of the distribution;
- Specific investigations on recruitment limitation (soil characteristics, predation, competition);
- Demographic analysis on a set of selected European populations;
- Monitoring of the reintroduced and endangered native populations;
- Restoration of the sand dune habitats and physical protection of the populations, especially the most imperilled;
- Germplasm collection every year from the priority populations, without depauperate them;
- Pressure on the authorities for a correct and efficient conservation of the species and its habitat;
- Divulgate the conservation principles and collaborate with national and local entities for the conservation of the species and its habitat.

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APPENDICES

APPENDIX 1. List of Threats and Pressures (EEA 2011)

A	Agriculture
A01	Cultivation
A02	modification of cultivation practices
A02.01	agricultural intensification
A02.02	crop change
A02.03	grassland removal for arable land
A03	mowing / cutting of grassland
A03.01	intensive mowing or intensification
A03.02	non intensive mowing
A03.03	abandonment / lack of mowing
A04	grazing
A04.01	intensive grazing
A04.01.01	intensive cattle grazing
A04.01.02	intensive sheep grazing
A04.01.03	intensive horse grazing
A04.01.04	intensive goat grazing
A04.01.05	intensive mixed animal grazing
A04.02	non intensive grazing
A04.02.01	non intensive cattle grazing
A04.02.02	non intensive sheep grazing
A04.02.03	non intensive horse grazing
A04.02.04	non intensive goat grazing
A04.02.05	non intensive mixed animal grazing
A04.03	abandonment of pastoral systems, lack of grazing
A05	livestock farming and animal breeding (without grazing)
A05.01	Animal breeding,
A05.02	stock feeding
A05.03	Lack of animal breeding
A06	annual and perennial non-timber crops
A06.01	annual crops for food production
A06.01.01	intensive annual crops for food production/ intensification
A06.01.02	non- intensive annual crops for food production
A06.02	perennial non-timber crops
A06.02.01	intensive perennial non-timber crops/intensification
A06.02.02	non-intensive perennial non-timber crops
A06.03	biofuel-production
A06.04	abandonment of crop production
A07	use of biocides, hormones and chemicals
A08	Fertilisation
A09	Irrigation
A10	Restructuring agricultural land holding
A10.01	removal of hedges and copses or scrub
A10.02	removal of stone walls and embankments
A11	Agriculture activities not referred to above
B	Sylviculture, forestry
B01	forest planting on open ground
B01.01	forest planting on open ground (native trees)
B01.02	artificial planting on open ground (non-native trees)
B02	Forest and Plantation management & use
B02.01	forest replanting
B02.01.01	forest replanting (native trees)
B02.01.02	forest replanting (non native trees)
B02.02	forestry clearance
B02.03	removal of forest undergrowth
B02.04	removal of dead and dying trees

B02.05	non- intensive timber production (leaving dead wood/ old trees untouched)
B02.06	thinning of tree layer
B03	forest exploitation without replanting or natural regrowth
B04	use of biocides, hormones and chemicals (forestry)
B05	use of fertilizers (forestry)
B06	grazing in forests/ woodland
B07	Forestry activities not referred to above

C Mining, extraction of materials and energy production

C01	Mining and quarrying
C01.01	Sand and gravel extraction
C01.01.01	sand and gravel quarries
C01.01.02	removal of beach materials
C01.02	Loam and clay pits
C01.03	Peat extraction
C01.03.01	hand cutting of peat
C01.03.02	mechanical removal of peat
C01.04	Mines
C01.04.01	open cast mining
C01.04.02	underground mining
C01.05	Salt works
C01.05.01	abandonment of saltpans (salinas)
C01.05.02	conversion of saltpans
C01.06	Geotechnical survey
C01.07	Mining and extraction activities not referred to above
C02	Exploration and extraction of oil or gas
C02.01	exploration drilling
C02.02	production drilling
C02.03	jack-up drilling rig
C02.04	semi-submersible rig
C02.05	drill ship
C03	Renewable abiotic energy use
C03.01	geothermal power production
C03.02	solar energy production
C03.03	wind energy production
C03.04	tidal energy production

D Transportation and service corridors

D01	Roads, paths and railroads
D01.01	paths, tracks, cycling tracks
D01.02	roads, motorways
D01.03	car parks and parking areas
D01.04	railway lines, TGV
D01.05	bridge, viaduct
D01.06	tunnel
D02	Utility and service lines
D02.01	electricity and phone lines
D02.01.01	suspended electricity and phone lines
D02.01.02	underground/submerged electricity and phone lines
D02.02	pipe lines
D02.03	communication masts and antennas
D02.09	other forms of energy transport
D03	shipping lanes, ports, marine constructions
D03.01	port areas
D03.01.01	slipways
D03.01.02	piers / tourist harbours or recreational piers
D03.01.03	fishing harbours
D03.01.04	industrial ports
D03.02	Shipping lanes
D03.02.01	cargo lanes
D03.02.02	passenger ferry lanes (high speed)
D03.03	marine constructions

D04	airports, flight-paths
D04.01	airport
D04.02	aerodrome, heliport
D04.03	flight paths
D05	Improved access to site
D06	Other forms of transportation and communication
E	Urbanisation, residential and commercial development
E01	Urbanised areas, human habitation
E01.01	continuous urbanisation
E01.02	discontinuous urbanisation
E01.03	dispersed habitation
E01.04	other patterns of habitation
E02	Industrial or commercial areas
E02.01	factory
E02.02	industrial storage
E02.03	other industrial / commercial area
E03	Discharges
E03.01	disposal of household / recreational facility waste
E03.02	disposal of industrial waste
E03.03	disposal of inert materials
E03.04	Other discharges
E03.04.01	costal sand suppletion/ beach nourishment
E04	Structures, buildings in the landscape
E04.01	Agricultural structures, buildings in the landscape
E04.02	Military constructions and buildings in the landscape
E05	Storage of materials
E06	Other urbanisation, industrial and similar activities
E06.01	demolishment of buildings & human structures
E06.02	reconstruction, renovation of buildings
F	Biological resource use other than agriculture & forestry
F01	Marine and Freshwater Aquaculture
F01.01	intensive fish farming, intensification
F01.02	suspension culture
F01.03	bottom culture
F02	Fishing and harvesting aquatic ressources
F02.01	Professional passive fishing
F02.01.01	potting
F02.01.02	netting
F02.01.03	demersal longlining
F02.01.04	pelagic longlining
F02.02	Professional active fishing
F02.02.01	benthic or demersal trawling
F02.02.02	pelagic trawling
F02.02.03	demersal seining
F02.02.04	purse seining
F02.02.05	benthic dredging
F02.03	Leisure fishing
F02.03.01	bait digging / collection
F02.03.02	pole fishing
F02.03.03	spear-fishing
F03	Hunting and collection of wild animals (terrestrial)
F03.01	Hunting
F03.01.01	damage caused by game (excess population density)
F03.02	Taking and removal of animals (terrestrial)
F03.02.01	collection of animals (insects, reptiles, amphibians,)
F03.02.02	taking from nest (falcons)
F03.02.03	trapping, poisoning, poaching
F03.02.04	predator control
F03.02.05	accidental capture
F03.02.09	other forms of taking animals

F04	Taking / Removal of terrestrial plants, general
F04.01	pillaging of floristic stations
F04.02	collection (fungi, lichen, berries etc.)
F04.02.01	hand raking
F04.02.02	hand collection
F05	Illegal taking/ removal of marine fauna
F05.01	dynamite
F05.02	date mussel-fishing
F05.03	poisons
F05.04	poaching
F05.05	shooting
F05.06	removal for collection purposes
F05.07	other (i.e. drift nets)
F06	Hunting, fishing or collecting activities not referred to above
F06.01	game/ bird breeding station
G	Human intrusions and disturbances
G01	Outdoor sports and leisure activities, recreational activities
G01.01	nautical sports
G01.01.01	motorized nautical sports
G01.01.02	non-motorized nautical sports
G01.02	walking, horse-riding and non-motorised vehicles
G01.03	motorised vehicles
G01.03.01	regular motorized driving
G01.03.02	off-road motorized driving
G01.04	mountaineering, rock climbing, speleology
G01.04.01	mountaineering & rock climbing
G01.04.02	speleology
G01.04.03	recreational cave visits
G01.05	gliding, delta plane, paragliding, ballooning
G01.06	skiing, off-piste
G01.07	scuba diving, snorkelling
G01.08	other outdoor sports and leisure activities
G02	Sport and leisure structures
G02.01	golf course
G02.02	skiing complex
G02.03	stadium
G02.04	circuit, track
G02.05	hippodrome
G02.06	attraction park
G02.07	sports pitch
G02.08	camping and caravans
G02.09	wildlife watching
G02.10	other sport / leisure complexes
G03	Interpretative centres
G04	Military use and civil unrest
G04.01	Military manouvres
G04.02	abandonment of military use
G05	Other human intrusions and disturbances
G05.01	Trampling, overuse,
G05.02	shallow surface abrasion/ mechanical damage to seabed surface
G05.03	penetration/ disturbance below surface of the seabed
G05.04	Vandalism
G05.05	intensive maintenance of public parks /cleaning of beaches
G05.06	tree surgery, felling for public safety, removal of roadside trees
G05.07	missing or wrongly directed conservation measures
G05.08	closures of caves or galleries
G05.09	fences, fencing
G05.10	overflying with aircrafts (agricultural)
G05.11	death or injury by collision

H	Pollution
H01	Pollution to surface waters (limnic, terrestrial, marine & brackish)
H01.01	pollution to surface waters by industrial plants
H01.02	pollution to surface waters by storm overflows
H01.03	other point source pollution to surface water
H01.04	diffuse pollution to surface waters via storm overflows or urban run-off
H01.05	diffuse pollution to surface waters due to agricultural and forestry activities
H01.06	diffuse pollution to surface waters due to transport and infrastructure without connection to canalization/sweepers
H01.07	diffuse pollution to surface waters due to abandoned industrial sites
H01.08	diffuse pollution to surface waters due to household sewage and waste waters
H01.09	diffuse pollution to surface waters due to other sources not listed
H02	Pollution to groundwater (point sources and diffuse sources)
H02.01	groundwater pollution by leakages from contaminated sites
H02.02	groundwater pollution by leakages from waste disposal sites
H02.03	groundwater pollution associated with oil industry infrastructure
H02.04	groundwater pollution by mine water discharges
H02.05	groundwater pollution by discharge to ground such as disposal of contaminated water to soakaways
H02.06	diffuse groundwater pollution due to agricultural and forestry activities
H02.07	diffuse groundwater pollution due to non-sewered population
H02.08	diffuse groundwater pollution due to urban land use
H03	Marine water pollution
H03.01	oil spills in the sea
H03.02	toxic chemical discharge from material dumped at sea
H03.02.01	non-synthetic compound contamination
H03.02.02	synthetic compound contamination
H03.02.03	radionuclide contamination
H03.02.04	introduction of other substances (e.g. liquid, gas)
H03.03	marine macro-pollution (i.e. plastic bags, styrofoam)
H04	Air pollution, air-borne pollutants
H04.01	Acid rain
H04.02	Nitrogen-input
H04.03	other air pollution
H05	Soil pollution and solid waste (excluding discharges)
H05.01	garbage and solid waste
H06	excess energy
H06.01	Noise nuisance, noise pollution
H06.01.01	point source or irregular noise pollution
H06.01.02	diffuse or permanent noise pollution
H06.02	Light pollution
H06.03	Thermal heating of water bodies
H06.04	Electromagnetic changes
H06.05	Seismic exploration, explosions
H07	Other forms of pollution
I	Invasive, other problematic species and genes
I01	invasive non-native species
I02	problematic native species
I03	introduced genetic material, GMO
I03.01	genetic pollution (animals)
I03.02	genetic pollution (plants)
J	Natural System modifications
J01	fire and fire suppression
J01.01	burning down
J01.02	suppression of natural fires
J01.03	lack of fires
J02	human induced changes in hydraulic conditions
J02.01	Landfill, land reclamation and drying out, general
J02.01.01	polderisation
J02.01.02	reclamation of land from sea, estuary or marsh

J02.01.03	infilling of ditches, dykes, ponds, pools, marshes or pits
J02.01.04	recultivation of mining areas
J02.02	Removal of sediments (mud...)
J02.02.01	dredging/ removal of limnic sediments
J02.02.02	estuarine and coastal dredging
J02.03	Canalisation & water deviation
J02.03.01	large scale water deviation
J02.03.02	canalisation
J02.04	Flooding modifications
J02.04.01	flooding
J02.04.02	lack of flooding
J02.05	Modification of hydrographic functioning, general
J02.05.01	modification of water flow (tidal & marine currents)
J02.05.02	modifying structures of inland water courses
J02.05.03	modification of standing water bodies
J02.05.04	reservoirs
J02.05.05	small hydropower projects, weirs
J02.05.06	wave exposure changes
J02.06	Water abstractions from surface waters
J02.06.01	surface water abstractions for agriculture
J02.06.02	surface water abstractions for public water supply
J02.06.03	surface water abstractions by manufacturing industry
J02.06.04	surface water abstractions for the production of electricity (cooling)
J02.06.05	surface water abstractions by fish farms
J02.06.06	surface water abstractions by hydro-energy
J02.06.07	surface water abstractions by quarries/ open cast (coal) sites
J02.06.08	surface water abstractions for navigation
J02.06.09	surface water abstractions for water transfer
J02.06.10	other major surface water abstractions
J02.07	Water abstractions from groundwater
J02.07.01	groundwater abstractions for agriculture
J02.07.02	groundwater abstractions for public water supply
J02.07.03	groundwater abstractions by industry
J02.07.04	groundwater abstractions by quarries/open cast (coal) sites
J02.07.05	other major groundwater abstractions from groundwater for agriculture
J02.08	Raising the groundwater table /artificial recharge of groundwater
J02.08.01	discharges to groundwater for artificial recharge purposes
J02.08.02	returns of groundwater to GWB from which it was abstracted
J02.08.03	mine water rebound
J02.08.04	other major groundwater recharge
J02.09.	Saltwater intrusion of groundwater
J02.09.01	saltwater intrusion
J02.09.02	other intrusion
J02.10	management of aquatic and bank vegetation for drainage purposes
J02.11	Siltation rate changes, dumping, depositing of dredged deposits
J02.11.01	Dumping, depositing of dredged deposits
J02.11.02	Other siltation rate changes
J02.12	Dykes, embankments, artificial beaches, general
J02.12.01	sea defence or coast protection works, tidal barrages
J02.12.02	dykes and flooding defence in inland water systems
J02.13	Abandonment of management of water bodies
J02.14	Altered water quality due anthropogenic changes in salinity
J02.15	Other human induced changes in hydraulic conditions
J03	Other ecosystem modifications
J03.01	reduction or loss of specific habitat features
J03.01.01	reduction of prey availability (including carcasses)
J03.02	anthropogenic reduction of habitat connectivity
J03.02.01	reduction in migration/ migration barriers
J03.02.02	reduction in dispersal
J03.02.03	reduction in genetic exchange
J03.03	reduction, lack or prevention of erosion
J03.04	applied (industrial) destructive research

K	Natural biotic and abiotic processes (without catastrophes)
K01	abiotic (slow) natural processes
K01.01	Erosion
K01.02	Silting up
K01.03	Drying out
K01.04	Submersion
K01.05	Soil salinization
K02	Biocenotic evolution, succession
K02.01	species composition change (succession)
K02.02	accumulation of organic material
K02.03	eutrophication (natural)
K02.04	acidification (natural)
K03	Interspecific faunal relations
K03.01	competition
K03.02	parasitism
K03.03	introduction of disease (microbial pathogens)
K03.04	predation
K03.05	antagonism arising from introduction of species
K03.06	antagonism with domestic animals
K03.07	other forms of interspecific faunal competition
K04	Interspecific floral relations
K04.01	competition
K04.02	parasitism
K04.03	introduction of disease (microbial pathogens)
K04.04	lack of pollinating agents
K04.05	damage by herbivores (including game species)
K05	reduced fecundity/ genetic depression
K05.01	reduced fecundity/ genetic depression in animals (inbreeding)
K05.02	reduced fecundity/ genetic depression in plants (incl. endogamy)
K06	other forms or mixed forms of interspecific floral competition
L	Geological events, natural catastrophes
L01	volcanic activity
L02	tidal wave, tsunamis
L03	earthquake
L04	avalanche
L05	collapse of terrain, landslide
L06	underground collapses
L07	storm, cyclone
L08	inundation (natural processes)
L09	fire (natural)
L10	other natural catastrophes
M	Climate change
M01	Changes in abiotic conditions
M01.01	temperature changes (e.g. rise of temperature & extremes)
M01.02	droughts and less precipitations
M01.03	floodings and rising precipitations
M01.04	pH-changes
M01.05	water flow changes (limnic, tidal and oceanic)
M01.06	wave exposure changes
M01.07	sea-level changes
M02	Changes in biotic conditions
M02.01	habitat shifting and alteration
M02.02	desynchronisation of processes
M02.03	decline or extinction of species
M02.04	migration of species (natural newcomers)
X	No threats or pressures
XO	Threats and pressures from outside the Member State
XE	Threats and pressures from outside the EU territory
U	Unknown threat or pressure

APPENDIX 2. Documentation about Reintroduction trials

1) Result of the *Environmental Impact Assessment* (EIA) procedure for the site *Litorale di Torre Astura*.

038984

REGIONE LAZIO

DIREZIONE REGIONALE INFRASTRUTTURE, AMBIENTE E POLITICHE ABITATIVE
Area Parchi e Riserve Naturali

DIPARTIMENTO DI SCIENZE ECOLOGICHE E BIOLOGICHE (DEB)
VITERBO

ARRIVO

Prot. N°

21 OTT. 2013

A: Università della Tuscia
Dipartimento di Scienze Ecologiche e Biologiche (DEB)
Largo dell'Università s,n,c
Blocco C/D
01100 VITERBO
c.a. Dott. Marcello De Vitis

e.p.c. Agenzia Regionale Parchi (ARP)
Via del Pescaccio 98
00166 ROMA

Oggetto: Procedura di valutazione d'incidenza ai sensi dell'art. 5 del DPR 8 settembre 1997, n. 357 e s.m.i. sul progetto "Rafforzamento della popolazione italiana di *Malcolmia littorea* (L.) R. Br., specie gravemente minacciata di estinzione" in loc. "Torre Astura" nel Comune di Nettuno (VT). Richiedente: Dr. Marcello De Vitis-Dipartimento di Scienze Ecologiche e Biologiche-Università della Tuscia. (Q64/13)

Si fa riferimento alla nota prot. n. 980/13 del 16/09/2013, acquisita al protocollo regionale n. 363339/35/18 del 2/10/2013, con cui Codesto Dipartimento richiede se per l'intervento menzionato in oggetto, ricadente all'interno del Sito di Importanza Comunitaria(SIC) IT6030048 Litorale di Torre Astura, sia necessario attivare la procedura di valutazione di incidenza ai sensi dell'art. 5 del DPR n. 357/1997 e s.m.i.

Si puntualizza innanzitutto che quanto segue attiene esclusivamente alla competenza di quest'Area in materia di Rete Natura 2000 e procedura di valutazione di incidenza, di cui alle Direttive Comunitarie 79/409/CEE "Uccelli", sostituita dalla Direttiva 2009/147/CE, e 92/43/CEE "Habitat" nonché al DPR n. 357/1997 e s.m.i.

Si prende atto degli allegati alla sopra citata nota:

- Progetto di Ricerca per il corso di Dottorato in Ecologia e Gestione delle Risorse Biologiche titolo *Conservazione e gestione di Malcolmia littorea* (L.) R. Br., specie mediterranea minacciata di estinzione.
- De Vitis M., Fabrini G. and Magrini S. "Seed introduction and gap creation facilitate restoration of *Malcolmia littorea* (L.) R. Br." Poster EVS 22th Workshop Roma 8-11 Aprile 2013.
- Studio di Fattibilità del Progetto di Ricerca "Rafforzamento della popolazione italiana di *Malcolmia littorea* (L.) R. Br., specie gravemente minacciata di estinzione"
- Cartografia del sito d'intervento (Allegato I; in rosso è evidenziata l'area di intervento di ca. 6000-metri quadrati);

Considerato che gli interventi previsti consistono nella creazione di un nuovo nucleo di individui di *M. littorea*, all'interno del SIC IT6030048 "Litorale di Torre Astura" tramite diversi metodi traslocazione, con successivo monitoraggio del nucleo stesso.

Tra gli scopi principali del progetto vi sono:

ASSESSORATO ALLE INFRASTRUTTURE, ALLE POLITICHE ABITATIVE E ALL'AMBIENTE
DIPARTIMENTO ISTITUZIONALE E TERRITORIO
DIREZIONE REGIONALE INFRASTRUTTURE, AMBIENTE E POLITICHE ABITATIVE
AREA PARCHI E RISERVE NATURALI

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rot. n. 0001115 del 23/10/2013 - [Ufficio: Dipartimento di scienze ecologiche e biologiche - DEB - Classif. VII/4] Pag. 1

- rafforzare in maniera consistente la popolazione italiana così da diminuirne il rischio di estinzione;
- definire un protocollo efficace di reintroduzione della specie, contribuendo alla produzioni di dati utili e di tecniche riproducibili.

Considerato che la specie oggetto, *M. littorea*, è scomparsa in gran parte del suo areale storico italiano e il suo stato di conservazione è estremamente critico (categoria IUCN *Critically endangered* **estremamente minacciata**).

Preso atto che la superficie d'intervento come riportato negli elaborati ricade all'interno dell'habitat d'interesse comunitario codice 2210 "*Crucianellion maritimae* fixed beach dunes" e che è stata individuata un'area di circa 6000 metri quadrati dove

individui di *Malcolmia littorea* saranno messi a dimora attraverso diversi metodi, tra cui trapianto e semina. Tale nucleo verrà periodicamente monitorato per valutare e quantificare l'efficacia dei diversi metodi di reintroduzione e verificare lo stabilimento della specie nella comunità.

Considerato che la specie *M. littorea* è riconosciuta come non invasiva e debolmente competitiva rispetto ad altre specie dello stesso habitat.

Preso atto che presso l'Orto Botanico di Roma sono stati propagati nel 2012 circa 1000 individui, a partire da semi raccolti dalla popolazione spontanea, che saranno traslocati nel sito di rafforzamento durante la stagione autunnale (settembre-ottobre 2013), per permettere un miglior attecchimento delle piante ed evitare la mortalità associata con la stagione calda e arida,

e che sarà inoltre praticata una semina sempre durante la stagione autunnale, periodo della naturale dispersione, utilizzando circa 10000 semi raccolti dalla popolazione spontanea e conservati nella Banca del Germoplasma della Toscana.

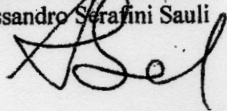
Considerato che l'intervento possa ritenersi una reintroduzione, in quanto la porzione di litorale laziale presso Torre Astura rientra nell'areale storico di *M. littorea*, ossia il litorale laziale che va da Torvaianica a Terracina (Pignatti 1982; Mulder 1993).

Ritenuto che gli interventi in oggetto non comportino incidenze negative significative, dirette o indirette, sui valori ambientali tutelati della Rete Natura 2000.

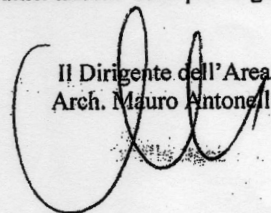
Si comunica pertanto che l'istruttoria in merito agli interventi in argomento è da considerarsi favorevolmente conclusa con la presente comunicazione senza che sia necessario attivare ulteriori fasi della procedura di valutazione di incidenza ai sensi dell'art 5 comma 3 del DPR n. 357/1997 e s.m.i.

Si invita Codesto Dipartimento a fornire una relazione di sintesi alla Scrivente Area dei risultati e monitoraggi al termine del periodo di studio, al fine di acquisire documentazione utile per la gestione ed il monitoraggio degli habitat e specie di interesse comunitario.

Il Funzionario Tecnico estensore
Dott. Alessandro Serafini Sauli



Il Dirigente dell'Area
Arch. Mauro Antonelli



2) Result of the *Environmental Impact Assessment* (EIA) procedure for the site *Rio Martino*.

 REGIONE LAZIO	DIPARTIMENTO DI SCIENZE ECOLOGICHE E BIOLOGICHE (DEB) VITERBO 16 GIU 2014 ARRIV 688
DIREZIONE REGIONALE INFRASTRUTTURE, AMBIENTE E POLITICHE ABITATIVE AREA PARCHI E RISERVE NATURALI	
Prot. n. <u>297393</u>	Roma <u>22 MAG. 2014</u>
A: Università della Tuscia Dipartimento di Scienze Ecologiche e Biologiche (DEB) Largo dell'Università s.n.c Blocco C/D 01100 VITERBO c.a. Dott. Marcello De Vitis	
Parco Nazionale del Circeo Via Carlo Alberto, 104 04016 SABAUDIA (LT)	
Comando Provinciale del Corpo Forestale dello Stato di Latina Via dei Volsci, 36 04100 LATINA	

Oggetto: Procedura di valutazione d'incidenza ai sensi dell'art. 5 del DPR 8 settembre 1997, n. 357 e s.m.i. sul progetto "Intervento di conservazione in situ della popolazione italiana di *Malcolmia littorea* (L.) R. Br., specie gravemente minacciata di estinzione" all'interno del Parco Nazionale del Circeo presso la loc. Rio Martino in Comune di Latina (LT) Richiedente: Dr Marcello De Vitis Dipartimento di Scienze Ecologiche e Biologiche Università della Tuscia. (Q2/I4ter)

Si fa riferimento alla nota prot. n. 1316/13 del 16/12/2013, acquisita al protocollo regionale n. 24698/03/18 del 16/1/2014, con cui il Dott. Marcello De Vitis dottorando di Codesto Dipartimento richiede se per l'intervento menzionato in oggetto, ricadente all'interno del Sito di Importanza Comunitaria (SIC) IT6040018 Dune del Circeo e nella Zona Speciale di Conservazione (ZPS) IT6040015 Parco Nazionale del Circeo, sia necessario attivare la procedura di valutazione di incidenza ai sensi dell'art. 5 del DPR n. 357/1997 e s.m.i.

Preso atto che con nota prot. PNC/DIR/2014/1387 del 16/4/2014 acquisita al protocollo regionale n. 257854/03/18 del 5/5/2014 il Parco Nazionale del Circeo comunicava che con Deliberazione del Presidente n. 3/2014 veniva approvato un protocollo d'intesa tra l'Ente Gestore del PNC e l'università della Tuscia sul progetto in oggetto.

Si puntualizza innanzitutto che quanto segue attiene esclusivamente alla competenza di quest'Area in materia di Rete Natura 2000 e procedura di valutazione di incidenza, di cui alle Direttive Comunitarie 79/409/CEE "Uccelli", sostituita dalla Direttiva 2009/147/CE, e 92/43/CEE "Habitat" nonché al DPR n. 357/1997 e s.m.i.

Si prende atto degli allegati alla sopra citata nota:

- Progetto di Ricerca per il corso di Dottorato in Ecologia e Gestione delle Risorse Biologiche titolo *Conservazione e gestione di Malcolmia littorea* (L.) R. Br., specie mediterranea minacciata di estinzione.
- De Vitis M., Fabrini G. and Magrini S. "Seed introduction and gap creation facilitate restoration of *Malcolmia littorea* (L.) R. Br." Poster EVS 22th Workshop Roma 8-11 Aprile 2013.
- Studio di Fattibilità del Progetto di Ricerca "Rafforzamento della popolazione italiana di *Malcolmia littorea* (L.) R. Br., specie gravemente minacciata di estinzione"

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- Cartografia del sito d'intervento (Allegato I; in rosso è evidenziata l'area di intervento di circa 6000 metri quadrati);

Considerato che gli interventi previsti consistono nella creazione di un nuovo nucleo di individui di *M. littorea*, all'interno del SIC IT6040018 "Dune del Circeo" tramite diversi metodi traslocazione, con successivo monitoraggio del nucleo stesso.

Tra gli scopi principali del progetto vi sono:

- rafforzare in maniera consistente la popolazione italiana così da diminuirne il rischio di estinzione;
- definire un protocollo efficace di reintroduzione della specie, contribuendo alla produzioni di dati utili e di tecniche riproducibili.

Considerato che la specie oggetto, *M. littorea*, è scomparsa in gran parte del suo areale storico italiano e il suo stato di conservazione è estremamente critico (categoria IUCN *Critically endangered*– *gravemente minacciata*). Preso atto che la superficie d'intervento come riportato negli elaborati ricade all'interno dell'habitat d'interesse comunitario codice 2210 "Crucianellion maritimae fixed beach dunes", e che è stata individuata un'area di circa 6000 metri quadrati dove

individui di *Malcolmia littorea* saranno messi a dimora attraverso diversi metodi, tra cui trapianto e semina. Tale nucleo verrà periodicamente monitorato per valutare e quantificare l'efficacia dei diversi metodi di reintroduzione e verificare lo stabilimento della specie nella comunità.

Considerato che la specie *M. littorea* è riconosciuta come non invasiva e debolmente competitiva rispetto ad altre specie dello stesso habitat.

Considerato che l'intervento possa ritenersi una reintroduzione, in quanto la porzione di litorale laziale presso Rio Martino rientra nell'areale storico di *M. littorea*, ossia il litorale laziale che va da Torvaianica a Terracina (Pignatti 1982; Mulder 1993).

Ritenuto che gli interventi in oggetto non comportino incidenze negative significative, dirette o indirette, sui valori ambientali tutelati della Rete Natura 2000.

Si comunica pertanto che l'istruttoria in merito agli interventi in argomento è da considerarsi favorevolmente conclusa con la presente comunicazione senza che sia necessario attivare ulteriori fasi della procedura di valutazione di incidenza ai sensi dell'art 5 comma 3 del DPR n. 357/1997 e s.m.i.

Si invita Codesto Dipartimento a fornire una relazione di sintesi alla Scrivente Area dei risultati e monitoraggi al termine del periodo di studio, al fine di acquisire documentazione utile per la gestione ed il monitoraggio degli habitat e specie di interesse comunitario.

Il Responsabile del Procedimento
Dott. Alessandro Serafini Sauli

Il Dirigente Regionale
Arch. Mauro Antonelli

Il Direttore
Ing. Bruno Placidi

APPENDIX 3. Details of the 46 microsatellite markers screened in *M. littorea*: forward and reverse primer sequences, genome (nc, nuclear; cp, chloroplast), and literature source.

Locus	Primer sequence (5'- 3')	Genome	Source
AthATPASE ^{a, d}	F: CTGGGAACGGTTCGATTCGAGC R: GTTCACAGAGAGACTCATAAACCA	nc	Bell & Ecker 1994
AthCTR1 ^{a, l, m}	F: TATCAACAGAAACGCACCGAG R: CCACTTGTTTCTCTCTCTAG	nc	Bell & Ecker 1994
nga249 ^{a, d}	F: TACCGTCAATTTTCATCGCC R: GGATCCCTAACTGTAAAATCCC	nc	Bell & Ecker 1994
ccmp6 ^{b, k}	F: CGATGCATATGTAGAAAGCC R: CATTACGTGCGACTATCTCC	cp	Weising & Gardner 1999
ccmp10 ^{b, k}	F: TTTTTTTTTAGTGAACGTGTCA R: TTCGTCGDCGTAGTAAATAG	cp	Weising & Gardner 1999
ATCP7905 ^{c, h}	F: CGAACCCTCGGTACGATTAA R: TGGAGAAGGTTCTTTTCAAGC	cp	Provan 2000
ATCP28673 ^{c, h, k}	F: GCGTTCCTTTCATTTAAGACG R: TGCACTCTTCATTCTCGTTCC	cp	Provan 2000
ATCP30287 ^{c, h}	F: CCCTATACCCTGAAATTTGACC R: CAGCTCGGCCCAATAATTAG	cp	Provan 2000
ATCP46615 ^{c, h}	F: AATTTTTTTCATTGCACATTG R: TCAGAAATAGTCGAACGGTCG	cp	Provan 2000
ATCP66701 ^{c, h}	F: TCCACATCCTCCTTCTTTTTT R: CATTTGAAAACGTAAAGGCC	cp	Provan 2000
ATCP70189 ^{c, h, k}	F: CGGGTTGATGGATCATTACC R: GCAATGCACAAAAAAGCCT	cp	Provan 2000
F20D22 ^d	F: CCCAAGTGACGTCTGGTTTC R: AACAAAATGAGTTTCTCTGCATG	nc	Clauss et al. 2002
ICE10 ^d	F: AACATCCACAAGTTTCTAAAACAATC R: GACTCTTATGGGTAAGCTCCTTG	nc	Clauss et al. 2002
ICE13 ^d	F: GATCCTTCACCGGGTCTTG R: GTGGTGGAGACTCTTCGAGC	nc	Clauss et al. 2002
F19G10 ^d	F: AGTTGGTCTCGAGCTCTCC R: AAGAACTTAATTTCTCTCACCCG	nc	Clauss et al. 2002
AthZFPg ^d	F: TTGCGTTTCCACATTTGTTT R: TGGGTCAATTCACATGTAGAGA	nc	Clauss et al. 2002
SLL2 ^{d, f, m}	F: CATGTACTGGGATTCAGTGTCC R: CGTCCTTTGTGTGGTTACACG	nc	Clauss et al. 2002
ADH1 ^d	F: ACCACCGGACAGATTATTCG R: CCCAGAAGTAAACATCGGTGTG	nc	Clauss et al. 2002
MDC16 ^d	F: GAGTGGCCTCGTGTAGAGAAAG R: TGTCACCTCTTTTCTCTGGTTTG	nc	Clauss et al. 2002
AthGAPAb ^{d, m}	F: CACCATGGCTTCGGTTACTT R: TCCTGAGAATTCAGTGAAACCC	nc	Clauss et al. 2002
AthDET1 ^d	F: TTCAAACACCAATATCAGGCC R: GGTGAAAATGGAGGAGACGA	nc	Clauss et al. 2002
ICE5 ^d	F: CTTGCAACCGCCAACTCAATCG R: CCTGTCTCGCTCCCGCACG	nc	Clauss et al. 2002

AthCDPK9 ^d	F: TCAATCATTGTCCAAAACCTTGG R: GAAACTGACTTGGAGAAGGCA	nc	Clauss et al. 2002
ICE2 ^d	F: CTCGGGTCAAAATTAGGGTTTCG R: GCTACCAGATCCGATGGTAAGATG	nc	Clauss et al. 2002
AthPHYC ^d	F: CTCAGAGAATTCCCAGAAAAATCT R: AAACCTCGAGAGTTTTGTCTAGATC	nc	Clauss et al. 2002
MHJ24 ^d	F: CCGTCCTTGATCCTTGAGATTCTGAG R: CAATTCCGAAAATCATATTCATGCACC	nc	Clauss et al. 2002
BRMS008 ^{e, m}	F: AGGACACCAGGCACCATATA R: CATTGTTGTCTTGGGAGAGC	nc	Suwabe et al. 2002
BRMS020 ^e	F: AACAAGAGAAGGAGAGCCACCG R: CGCTTATAAAATGGCAGTCGCA	nc	Suwabe et al. 2002
BRMS025 ^e	F: TGAAAACAAGCGCTACATGTGG R: CAAGCAAGCATGACAAGCAACA	nc	Suwabe et al. 2002
BRMS033 ^{e, m}	F: GCGGAAACGAACACTCCTCCCATGT R: CCTCCTTGCTTTCCCTGGAGACG	nc	Suwabe et al. 2002
BRMS044 ^{e, m}	F: AGGCGAGGAGAAGACAACACAA R: TACGGGTGGTTTGAATCAGCAG	nc	Suwabe et al. 2002
Ledr02 ^g	F: TGATGACAGGCAACAGG R: CAAGGATGGCAAATTC	nc	Bon et al. 2005
Ledr11 ^g	F: TACCTGCGGTTGAACAC R: TCCTCTCATTTAGCTTCTC	nc	Bon et al. 2005
ATCP31017 ^h	F: GCCTACCGCATCGAAATAGA R: CAAGAAAGTCGGCCAGAATC	cp	Ceplitis et al. 2005
Tc-ZNT5 ⁱ	F: AATCACACAAAACGTTAAGCTC R: AAGGTATGGCGGCGATCTTG	nc	Basic & Besnard 2006
Tc-IRT1 ⁱ	F: CTTGCGATATCGAGTCATTGC R: TCCAATGACCACAGAGTGAAC	nc	Basic & Besnard 2006
Tc-WRKY ⁱ	F: TTCTCCGGAAAAGTCTCCGG R: CTCACGGTTCTCTTCATCCG	nc	Basic & Besnard 2006
Tc-AGAMOUS ⁱ	F: CCTCCATTGTTGTTAATGTCTG R: TACTCTCACTTACCATCACATG	nc	Basic & Besnard 2006
Tc-bHLH ⁱ	F: CTTGGAAACATTGGTGTAAAGG R: GATTCCATCTCAAATCCGGTC	nc	Basic & Besnard 2006
Ca46 ^j	F: GACTCTGCTACAGTTCAACCAC R: ACTCTTCACGTTGTGGATCTATC	nc	Burrell & Pepper 2006
Ca62 ^j	F: CTTTCTCACTCTGCAACTCTTC R: CTGCATCTGCGTCCATGATC	nc	Burrell & Pepper 2006
DnA222 ^m	F: GTGGCAATTTGCTTCCAACC R: GCGCAGTGAGATGGATTCTGG	nc	Skrede et al. 2009
DnB101 ^m	F: TGGCTTACCATTGCTGTCC R: CCGCATTGTGTTGTTCTTG	nc	Skrede et al. 2009
DnB207 ^m	F: GGACGGCTGCATTTTCAC R: TCAGCTTCACACCAAACAATTC	nc	Skrede et al. 2009
DnB220 ^m	F: GCAAAGCAGAGCGTAGAATGG R: ACTCGGACGTCTCAATCAGC	nc	Skrede et al. 2009
AthSO392 ^{l, m}	F: GTTGATCGCAGCTTGATAAGC R: TTGGAGTTAGACACGGATCTG	nc	Skrede et al. 2009

- ^a Primers amplifying in *Arabidopsis thaliana* (Bell and Ecker 1994).
- ^b Primers amplifying in *Nicotiana tabacum*, *N. silvestris*, *N. acuminata*, *N. benthamiana*, *Lycopersicon hirsutum*, *L. peruvianum*, *L. esculentum*, *Petunia hybrida*, *Solanum tuberosum*, *Actinidia arguta*, *A. polygama*, *A. chrysantha*, *A. chinensis*, *A. deliciosa*, *Brassica oleracea*, *Sinapis alba*, *Arabidopsis thaliana*, *Pisum sativum*, *Metrosideros excelsa*, *Malus domestica*, *Hordeum vulgare*, *Lolium multiflorum*, *Cordyline australis* (Weising and Gardner 1999).
- ^c Primers amplifying in *Arabidopsis thaliana*, *Brassica oleracea*, *B. rapa*, *B. napus*, *B. nigra*, *B. carinata*, and *B. juncea* (Provan 2000).
- ^d Primers amplifying in *Arabidopsis thaliana*, *A. halleri*, *A. lyrata* ssp. *lyrata*, *A. lyrata* spp. *petraea*, and *Arabis drummondii* (Clauss et al. 2002).
- ^e Primers amplifying in *Arabidopsis thaliana*, *Brassica carinata*, *B. juncea*, *B. napus*, *B. nigra*, *B. oleracea*, 19 cultivars of *Brassica rapa*, *Raphanus sativus*, and *Sinapis alba* (Suwabe et al. 2002).
- ^f Primer amplifying in *Boechera stricta*, *B. X divaricarpa*, and *B. holboellii* (Dobeš et al. 2004).
- ^g Primers amplifying in *Lepidium draba* spp. *draba*, *L. draba* spp. *chelepanse*, and *L. appelianum* (Bon et al. 2005).
- ^h Primers amplifying in *Capsella bursa-pastoris* (Ceplitis et al. 2005).
- ⁱ Primers amplifying in *Thlaspi caerulescens*, *Thlaspi arvense*, *Arabidopsis thaliana*, *Arabis hirsuta*, *Brassica napus*, *Sinapis arvensis*, *Erophila praecox*, *Capsella bursa-pastoris* and *Biscutella laevigata* (Basic and Besnard 2006).
- ^j Primers amplifying in *Caulanthus amplexicaulis* var. *amplexicaulis*, *C. amplexicaulis* var. *barbarae*, *C. heterophyllus* var. *heterophyllus*, *C. heterophyllus* var. *pseudosimulans*, *C. inflatus*, *Guillenia flavescens*, *G. lasiophylla*, *G. lemmonii*, *Streptanthus albidus* spp. *peramoenus*, *S. barbiger*, *S. batrachopus*, *S. bracteatus*, *S. campestris*, *S. carinatus*, *S. farnsworthianus*, *S. glandulosus* spp. *pulchellus*, *S. glandulosus* spp. *secundus*, *S. insignis*, *S. niger* and *S. tortuosus* (Burrell and Pepper 2006).
- ^k Primers amplifying in *Brassica cretica* (Edh et al. 2007).
- ^l Primers amplifying in *Cardamine blaisdellii*, *C. digitata*, *C. microphylla*, and *C. purpurea* (Jørgensen et al. 2008).
- ^m Primers amplifying in *Cardamine blaisdellii*, *C. digitata*, *Draba nivalis*, *D. fladnizensis*, *Smelowskia borealis* and *S. porsildii* except for DnA222 and SLL2 which did not amplify in *D. fladnizensis* and for BRMS044 which did not amplify in *Cardamine* spp. and *Smelowskia* spp. (Skrede et al. 2009).

APPENDIX 4.

De Vitis M., Seal C.E., Ulian T., Pritchard H.W., Magrini S., Fabrini G., and Mattana E. 2014. Rapid adaptation of seed germination requirements of the threatened Mediterranean species *Malcolmia littorea* (Brassicaceae) and implications for its reintroduction. *South African Journal of Botany* 94, 46-50.



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Short communication

Rapid adaptation of seed germination requirements of the threatened Mediterranean species *Malcolmia littorea* (Brassicaceae) and implications for its reintroduction



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ABSTRACT

Malcolmia littorea (Brassicaceae) is a threatened species growing in the coastal sandy dunes of the west-Mediterranean basin. In this study, the seed germination and seedling emergence requirements of this species were investigated in the only remaining native population in Italy. The highest germination percentage was achieved in darkness with scoring under safe green light at 5–10 °C. Seedling emergence was highest when seeds were buried between 1 and 10 mm in depth. The results suggest that germination and seedling emergence are adapted to Mediterranean coastal habitats by employing a common mechanism of light-inhibited germination and by germinating at cooler temperatures before the onset of the summer drought. Seeds were also collected from plants cultivated at a botanical garden and from plants reintroduced by sowing or by transplanting. For those populations, germination was maximal between 10–25 °C, suggesting that the thermal germination behaviour may be affected by the maternal environment of seed production within one generation. It is suggested to use seeds produced in the same environment to which they will be used for the reintroduction of this species.

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1. Introduction

Mediterranean coastal habitats are particularly vulnerable to disturbance, being subjected to a dynamic equilibrium due to several natural factors and human activities (Carboni et al., 2009; Curr et al., 2000; Lomba et al., 2008; Ma and Liu, 2008). In places where disturbance has been particularly intense or prolonged in time, plant populations have been fragmented with a risk of local extinction of species, and coenoses have often completely disappeared (Acosta et al., 2006; Carranza et al., 2010).

It is important to understand which factors restrict the establishment and growth of rare species to support their in situ conservation (Schemske et al., 1994) such as through reintroduction. While species reintroduction from seed is usual for many commonly occurring plants, little is known about the germination characteristics of many rare, native plant species (Cochrane et al., 2002). Several key factors influence

seed germination, including temperature and light (Probert, 2000; Thanos et al., 1994). The optimal temperatures for total germination of coastal Mediterranean species are typically within the range of 5–15 °C, with germination percentages decreasing at higher temperatures (Thanos et al., 1989, 1995; Tlig et al., 2008). These species are also characterised by having a low germination rate and being negatively affected by prolonged chilling (Doussi and Thanos, 2002; Skordilis and Thanos, 1995).

The stimulation of seed germination by alternating temperatures is also common in species from arid zones (Mahmoud et al., 1983; Probert, 2000). The requirement for alternating temperatures and light may represent an adaptation of small-seeded species to ensure that germination occurs close to the soil surface in vegetation gaps (Probert, 2000). These responses can be interpreted in terms of the ecology of the species, in that larger seeds can emerge from greater depths, and thus there is less need for depth-sensing mechanisms (Murdoch, 1983). In coastal species of Mediterranean climates, light-inhibited seed germination has been reported (e.g. Thanos et al., 1989, 1991, 1994). This mechanism is of adaptational value in the sea-shore or sandy habitats, since germination on the surface of the sand might present a great hazard for newly germinated seedlings, due to rapid evaporation and large temperature fluctuations (Thanos et al., 1989).

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In this study, the seed germination behaviour of *Malcolmia littorea*, a suffruticous chamaephyte belonging to the Brassicaceae family, was investigated. This species grows in sandy coastal habitats along transition and fixed dunes (Del Vecchio et al., 2012), and has a west-Mediterranean distribution (Pignatti, 1982), occurring in Italy, France, Spain, Portugal, Morocco and Algeria (Greuter et al., 1986). In Italy the species is represented by just one native population in the Latium region. Severe urbanisation of the area and tourist pressure are the most intensive threats for this isolated population (Del Vecchio et al., 2012) and as a result, it is listed as critically endangered in the Italian Red List (Rossi et al., 2013). During 2009–2011, using seeds produced by the Italian native population, an experimental reintroduction was performed by in situ seed sowing and plant transplanting (ex situ seed sowing and transfer of the grown plants), and resulted in an experimental population (sensu Caplow, 2004) of the species, established inside its historical range (De Vitis, 2011).

The main aim of this study was to characterise the seed germination behaviour of the native population of *M. littorea* by (1) determining light, storage and temperature effects on seed germination, and (2) assessing the optimal burial depth for seed germination and seedling emergence. As the environment during seed production and cultivation is likely to affect the germination performance (Evans, 1993; Rojas-Arèchiga et al., 2001), and therefore reintroduction success, the germination of seeds from the native population was also compared with seeds from three experimental populations.

2. Material and methods

2.1. Seed lot details

Ripe siliques of *M. littorea* were collected directly from plants in the native population (San Felice Circeo, Latina, Italy: NA1), in one population cultivated at the Botanical Garden of Rome (BG) and in two reintroduced populations (formed by in situ sowing (RE1), and ex situ sowing followed by transplanting of the grown plantlets (RE2)), at the time of natural dispersal in summer 2011 (Table 1). All the fruits were cleaned in the laboratory and the seeds were stored in glass vials at room conditions (+20 °C) for three months until the experiments started. During Summer 2013 seeds from plants in the native population were collected again and sown two days after collection (NA2). In each population, siliques were collected from 10 to 50 individuals, according to their population size (Table 1), as specified below. Average seed mass was calculated for each seed lot by weighing five replicates of 50 seeds each.

2.2. Germination tests

In all the experiments, five replicates of 20 seeds were sown on the surface of 1% water-agar, which provided a solid, non-sterile medium for germination, in 90 mm plastic Petri dishes and incubated at different temperatures and irradiances as specified below. Experiments lasted for a maximum of 26 days, at which time no further germination was observed. Germination was defined as visible radicle emergence. At the end of the germination tests, a cut-test was carried out to determine

the viability of the remaining seeds (soft or firm) and the final germination percentage was calculated on the basis of the total number of filled seeds as the mean of the five replicates $\pm$ standard error (SE).

2.3. Effect of light irradiance, temperature and storage

Stored (NA1) and fresh (NA2) seeds belonging to the native population were incubated at 15 °C under different light conditions. In particular, seeds were exposed to a 12 h photoperiod (12 h) with a ratio of red:far red of ca. 2.0, a light quantity of ca. 7 $\mu\text{mol}/\text{m}^2/\text{s}$ and density of 50–100 W/m^2 , and in the dark, achieved by wrapping the dishes in two layers of aluminium foil. Seeds were scored for germination every two days. In the darkness seeds were either scored under safe green light (GL; light density of 14.0 W/m^2) or only at the end of the experiment (D; 26 days).

Seeds belonging to NA1, RE1, RE2 and BG were incubated in the dark and scored every two days under safe green light at both constant (5, 10, 15, 20 and 25 °C) and alternating (15/5, 20/10 and 25/15 °C) temperatures. As more seeds of NA1 were available for experimental work, they were also germinated at constant temperatures of 30 and 35 °C.

2.4. Experimental seed burials

Five replicates of 20 seeds of NA1 were placed on top of the 55 mm diameter Whatman no. 1 filter paper and buried at different depths in six black plastic boxes (174 $\times$ 115 $\times$ 60 mm) with transparent lids. Each box was filled with 1060 g of sand, with grain sizes between 0.8 and 0.005 mm, pH 6.0–7.5 (ISTA, 2006), and moistened with 105 ml of distilled water, calculated on the basis of previous experiments with seeds of comparable size (unpublished internal report of the Seed Conservation Department, Royal Botanic Gardens, Kew, UK). Replicates were buried at 1, 5, 10, 15, 20 and 25 mm. The boxes were incubated at the constant temperature of 15 °C and 12 h photoperiod (ratio of red:far red of ca. 2.0, a light quantity of ca. 7 $\mu\text{mol}/\text{m}^2/\text{s}$ and density of 50–100 W/m^2) and checked every two days for seedlings to emerge beyond the sand surface. Emerged seedlings were removed. After 26 days, the replicates were exhumed and the numbers of germinated non-emerged seedlings were scored and the viability of the non-germinated seeds was checked by a cut-test. The percentage of emerged seedlings was then recalculated on the basis of the filled seeds, as the mean of the five replicates $\pm$ standard error (SE).

2.5. Data analysis

Differences amongst final germination and t_{50} (time to reach 50% of the maximum germination in one replicate) were analysed with a t-test (paired and unpaired; for normally distributed data) or with the Wilcoxon (paired) and Mann–Whitney (unpaired) tests (for data not normally distributed) to compare two groups of data. When more than two groups were compared, a one-way ANOVA (for normally distributed data) or Kruskal–Wallis (for data not normally distributed), was followed by a post-hoc test (Tukey's or Mann–Whitney pairwise comparison). Two-way ANOVA was also performed to identify a

Table 1

Provenance, habitat, population size, number of sampled individuals and the average seed mass ($\pm$ SD) for each seed lot of *M. littorea* investigated in this study. One-way ANOVA was performed to compare average seed masses; values with different letters are significant at $P < 0.05$.

Seed lot	Provenance	Plant habitat	Altitude (m a.s.l.)	Population size	Sampled individuals	Seed mass (mg)
NA1	Native population	Coastal sandy dune	3	30	20	0.14 $\pm$ 0.01b
NA2	Native population	Coastal sandy dune	3	30	20	0.15 $\pm$ 0.01b
RE1	Reintroduced population (by sowing)	Coastal sandy dune	2	120	50	0.15 $\pm$ 0.01b
RE2	Reintroduced population (by transplanting)	Coastal sandy dune	2	13	10	0.17 $\pm$ 0.00a
BG	Botanical Garden of Rome	Living collection	17	10	10	0.16 $\pm$ 0.01b

significant effect of independent variables, and their interaction, on a dependent variable. Software Past 2.13 (Hammer et al., 2001) was used.

3. Results

3.1. Effect of light irradiance and storage

At 15 °C, exposure to 12 h photoperiod resulted in a very low germination percentage (1%) both for stored (NA1) and fresh (NA2) seeds, with no significant differences between them ($U = 12.50$, $P > 0.05$). Under dark condition with scoring every 2 days under green light (GL), seeds of NA2 reached their maximum germination ($37 \pm 8.9\%$) in 14 days, while those of NA1 in 6 days with a significantly higher germination percentage ($68 \pm 3.4\%$; $t = 3.30$, $P < 0.05$). In contrast, under continuous darkness (D) germination of NA2 ($13 \pm 3.4\%$) was significantly higher than that of NA1 (0%; $U = 0.50$, $P < 0.05$; Fig. 1).

3.2. Effect of temperature

At constant temperatures, seeds from NA1 showed the highest final germination at 5 °C ($75.0 \pm 2.7\%$) and 10 °C ($64.0 \pm 7.6\%$) and a statistically significant decreasing trend ($P < 0.001$) with increasing temperature, reaching less than 2.0% germination at 30 and 35 °C (Fig. 2). In contrast, the lowest germination percentage was detected at 5 °C in the other seed lots (RE1, RE2 and BG), with maximal germination between 10 and 25 °C ($P < 0.05$; Fig. 2). A two-way ANOVA, applied on final germination percentages of all seed lots at 5–25 °C, highlighted that the effect of provenance (Pr), temperature (T) and their interaction ($Pr \times T$) were highly significant ($P < 0.0001$).

Under alternating temperatures, RE1, RE2 and BG showed high germination percentages in all the tested conditions, ranging from ca. 70 to ca. 90%, but significantly lower germination percentages of NA1 at 15/5 and 25/15 °C (Fig. 3). A paired comparison of the germination percentages between alternating temperatures and their corresponding average constant temperatures showed that alternating temperatures did not affect the germination of NA1 and BG. For RE1, germination was higher at 15 °C than at 20/10 °C ($W = 15$, $P < 0.05$) and for RE2, germination was higher at 20 °C than at 25/15 °C ($t = 2.8$, $P < 0.05$).

Under constant temperature regimes, t_{50} of all seed lots were significantly longer at 5 °C after which t_{50} shortened with increasing temperature (Fig. 2). A two-way ANOVA applied on t_{50} values of all seed lots at 5–25 °C, showed that the effect of provenance (Pr) was not significant, while the effects of temperature (T) and of the interaction of the two variables ($Pr \times T$) were highly significant ($P < 0.0001$). Alternating

temperatures did not decrease the t_{50} in comparison with their corresponding average constant temperatures in any seed lot (data not shown).

3.3. Effect of burial depth

The highest percentage of seedling emergence was detected between 1 and 10 mm in depth (Fig. 4). From below 10 mm very few seedlings emerged (1% of all the buried seeds at depths ≥ 15 mm). At 1 mm, $20 \pm 4\%$ of the seeds were dead and $9 \pm 8\%$ of seedlings died after germination and all the emerged seedlings ($51 \pm 10\%$) were found to have an elongate hypocotyl under the very fine sand layer. At 5 mm $69 \pm 3\%$ of the seeds germinated and emerged and $25 \pm 5.0\%$ seeds germinated but did not emerge. At 10 mm the percentage of emerged seedlings was $41 \pm 11\%$ and the percentage of germinated non-emerged seedlings was $21 \pm 2\%$. At the depth 15 mm, $45 \pm 7\%$ of the seeds died and $48 \pm 6\%$ germinated without reaching the surface; at 20 mm, $31 \pm 7.3\%$ of the seeds germinated without emerging, a small percentage of seeds died ($9 \pm 4\%$) and a greater percentage remained viable under the sand ($57 \pm 12\%$); while at 25 mm almost all seeds died ($90 \pm 3\%$) and a few seeds germinated but did not emerge ($7 \pm 3\%$).

4. Discussion

4.1. Germination behaviour and soil depth-sensing of the native population

Information on the seed biology and ecology of threatened species must be considered in order to ensure their long term conservation and reintroduction. For seeds of the threatened *M. littorea*, the lower germination of fresh seeds in comparison to stored seeds suggest that physiological dormancy is likely, broken by after-ripening in dry storage (sensu Baskin and Baskin, 1998, 2004). Short exposure (e.g. a few minutes every two days) of seeds in the dark to the green safelight was found to positively affect seed germination rather than a condition of complete darkness, without receiving any light impulse, or the exposure to 12 h of white light. Purportedly, short bursts of light may stimulate germination in the late stages of dormancy breakage (Baskin et al., 2006). Mediterranean species have previously been reported to be sensitive to green light. Luna et al. (2004) evaluated the effect of green safelight with respect to absolute darkness on the germination response of a set of 12 Mediterranean species; starting the germination tests several months after seed collection, the germination of six species were sensitive (five positively and one negatively) to short-duration exposures to green safelight.

Coastal Mediterranean species typically germinate within the range of 5–15 °C, therefore limiting germination to the winter so plants can establish before the onset of the summer drought (Thanos et al., 1989, 1995). At 5–10 °C, the germination of the native population (NA1) of *M. littorea* was the highest amongst the tested temperatures, conferring to this species ecological adaptation to the unpredictable rainfall pattern of species living under “typical” Mediterranean climate conditions, reducing the risk of losing a cohort of plants. Rapid germination has also been suggested to be an adaptation in advance of soil drying in unpredictable environments (Daws et al., 2002) as prevalent in the Mediterranean. Compared to other Mediterranean species such as *Glaucium flavum* (t_{50} of ca. 30 days for untreated seeds at 10 °C; Thanos et al., 1989), *Muscari* spp. (t_{50} ranging from 12 to 16 days at 10 °C; Doussi and Thanos, 2002), *Centranthus ruber* (t_{50} of 4.5 days at 15 °C; Mattana et al., 2010) and *Dianthus morisianus* (t_{50} ranging from 4 to 5 days at 15 °C; Cogoni et al., 2012), NA1 showed faster germination, with t_{50} values of 3.4 days at 15 °C and 5.6 days at 10 °C.

In Mediterranean climates, germination on the soil surface is unfavourable due to high irradiance. Many species employ surface-avoiding mechanisms (Thanos et al., 1989), based on diurnally fluctuating temperatures and light quality, to detect depth of seed burial (Koutsovoulou et al., 2014), as germination from too deep within the

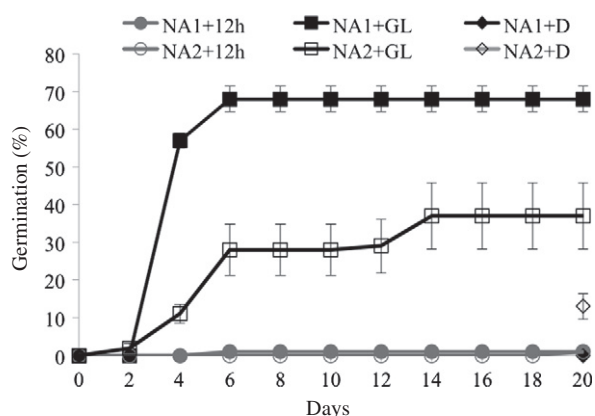


Fig. 1. Germination percentages (mean $\pm$ SE) of the natural population under 15 °C and different light (exposure to 12 h of white light, 12 h; dark with short exposure to green safelight for scoring, GL; and continuous darkness with scoring at the end of the experiment, D) and storage conditions (stored, NA1; and fresh, NA2).

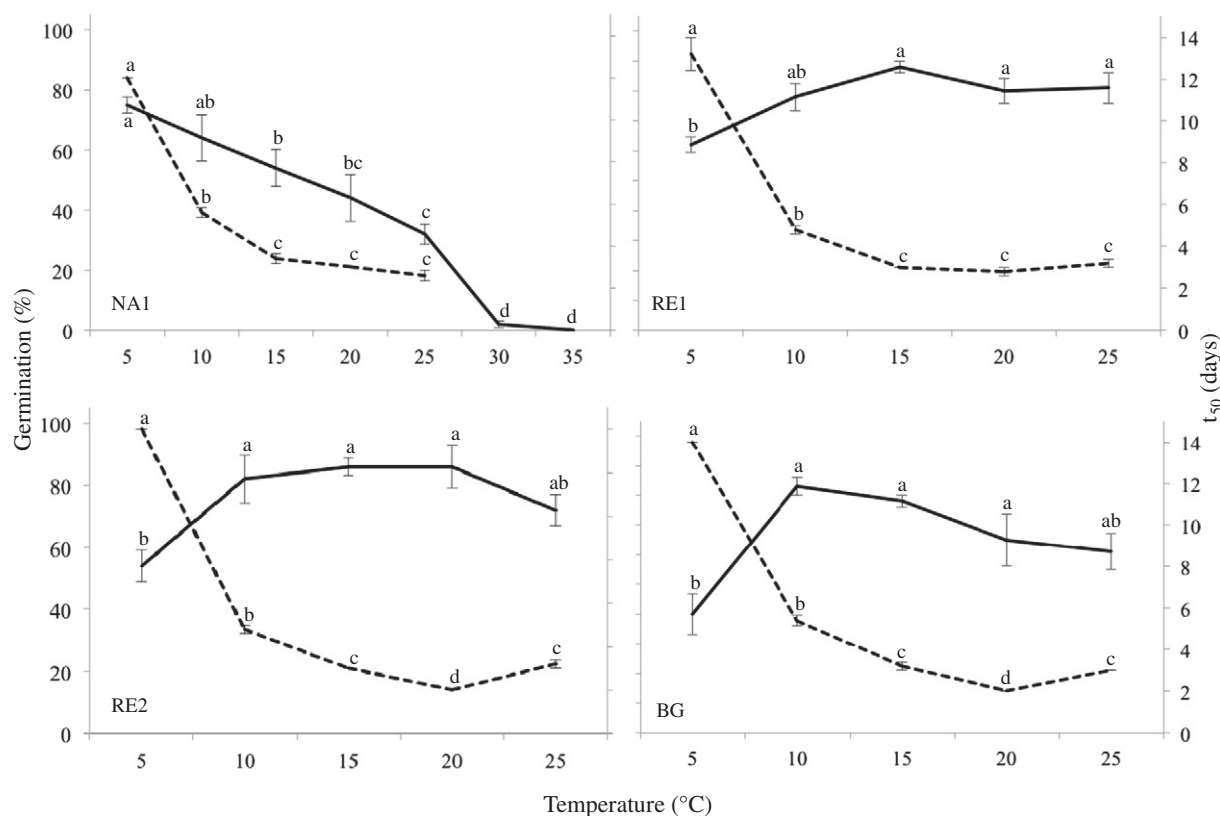


Fig. 2. Germination percentage (G%; solid line; mean $\pm$ SE) and t_{50} (dotted line; mean $\pm$ SE) under constant temperatures in the dark with scoring under green safelight for NA1, RE1, RE2 and BG. Within each seed lot, a significant difference amongst G% and t_{50} was detected by one-way ANOVA (for normally distributed data) and Kruskal–Wallis (for data not normally distributed) at $P < 0.01$. Bars with different letters are significant at $P < 0.05$ (post-hoc tests: Tukey's for normally distributed data and Mann–Whitney for data not normally distributed).

soil will lead to seedling death by seedling exhaustion before surface emergence (Benvenuti et al., 2001). The application of alternating temperature regimes did not improve the final germination percentage nor the t_{50} of NA1 in comparison with constant temperatures, and this is consistent with previous studies where seeds smaller than 2 mg did not respond to alternating temperatures (Cogoni et al., 2012; Mattana et al., 2010; Rojas-Arèchiga et al., 2001). Bond et al. (1999) found that there is an allometric correlation between seed mass and the maximum depth at which seedling emergence occurs [maximum depth (mm): $27.3 \times \text{seed weight}^{0.334}$ (mg)]. According to this model, *M. littorea* seeds should not be able to emerge from depths > 15 mm. Laboratory experiments confirmed the model validity for *M. littorea*, as very few seedlings emerged from burial depths > 15 mm (1%), while a considerable

emergence of seedlings was evident between 1 and 10 mm. Further interpretation of the seedling emergence experiment is precluded by the fact that the irradiance of the incubator lighting was lower than expected in nature. Nonetheless, *M. littorea* appears to germinate as a typical Mediterranean species avoiding high irradiance and by germinating at cooler temperatures.

4.2. Impact of maternal environment and implications for conservation

Seeds produced and matured on the mother plants in the wild (NA1) had a different germination response to those produced from the experimental populations, suggesting that seeds may show a rapid alteration of fitness within one generation. This has previously been shown in seeds of the cactus *Stenocereus stellatus* (Rojas-Arèchiga et al., 2001). If the maternal environment during seed development is a determinant

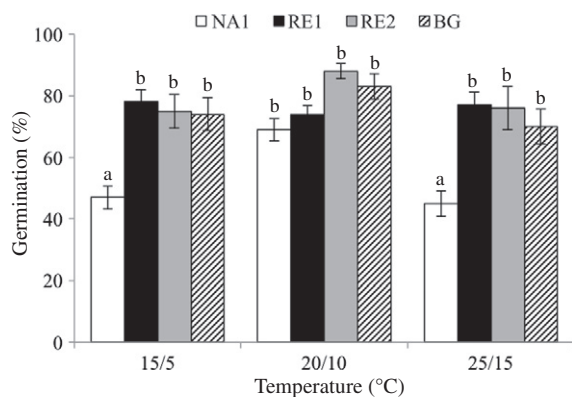


Fig. 3. Final germination percentages (mean $\pm$ SE) of the different seed lots under alternating temperature treatments. One-way ANOVA detected a significant difference amongst them ($P < 0.001$); bars with different letters are significant at $P < 0.05$ (post-hoc Tukey's test).

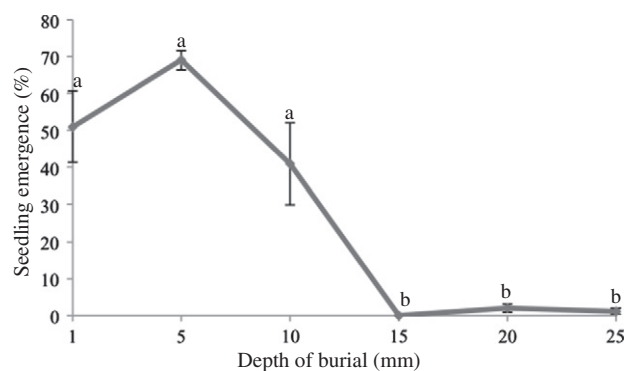


Fig. 4. Seedling emergence of five replicates (mean $\pm$ SE) at 15 °C for the tested burial depths. Significant differences were tested by Kruskal–Wallis test ($P < 0.001$); bars with different letters are significant at $P < 0.05$ (by Mann–Whitney pairwise comparison).

for the germination behaviour of *M. littorea*, then the source of seeds could have a major impact on the success of conservation programmes. Therefore, seeds for reintroduction should be harvested from plants produced in the same environment into which they will be sown. If seeds from the native population must be collected, it is suggested that only small quantities are removed so as to reduce the risk of depauperation, and then sown ex situ to produce plants for reintroduction. Finally, we suggest seeds should be dried upon collection, after-ripened to break any physiological dormancy, and sown in situ within 1 to 10 mm of the soil surface at 10–15 °C.

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