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Comparative studies on the *Silene mollissima* aggregate (Caryophyllaceae)

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Bene, vediamo un po' come fiorisci,
come ti apri, di che colore hai i petali,
quanti pistilli hai, che trucchi usi
per spargere il tuo polline e ripeterti,
se hai fioritura languida o violenta,
che portamento prendi, dove inclini,
nel morire infradici o insecchisci,
avanti su, io guardo, tu fiorisci.

Da Poesie, Patrizia Cavalli 1999

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ABSTRACT

The *Silene mollissima* aggregate (*S. italica* complex; *Siphonomorpha* Otth. section; Caryophyllaceae family), with 11 closely related endemics of the western Mediterranean Basin, is a group of chasmo-comophyte species mainly growing in coastal habitats, in particular on islands and islets, and often presenting scattered or narrow distribution areas, as probable consequence of a post-Messinian fragmentation of a common ancestor range (Boquet *et al.* 1978). The majorities of these taxa are rare or endangered, however information supporting human decision-making on their conservation is still scarce.

In this Ph.D. thesis, a multidisciplinary approach was adopted to contribute to the knowledge of some *taxa* of the *S. mollissima* aggregate and to provide useful information and data to support conservation measures. In particular, the specific aims were: 1) to evaluate, by means of quantitative analysis of seed morphological characters, the taxonomic treatment and inter-population variability in the Tyrrhenian *taxa* of the studied aggregate, and 2) to investigate the ecological requirements of some rare and/or endangered species of the aggregate, both through of *ex situ* investigations, performing seed germination and seedlings growth tests under laboratory conditions, and *in situ* studies on ecological niche.

In Chapter I, a statistical classifier for the Tyrrhenian *taxa* of the *S. mollissima* aggregate (*Silene badaroi* Bestr., *Silene ichnusae* Brullo, De Marco & De Marco, *Silene velutina* Pourr. ex Loisel., *Silene oenotriae* Brullo, *Silene hicesiae* Brullo & Signorello), based on 132 morpho-colorimetric variables describing seed size, shape and color of seed, was tested at inter- and intra-specific levels. The applied methodology was useful in provide key information about species and population differentiation, highlighting criticalities at the systematic level. In particular, the performed analysis revealed that shape, color and seed coat features have a diagnostic value in the studied species. Moreover, the systematic treatment at section and species level was confirmed, although further investigations, both regarding the taxonomic position of *S. hicesiae* into the whole aggregate and the differentiation of *S. ichnusae* from *S. velutina,* would be needed. Indeed, at the population level the Linear Discriminant Analysis (LDA) allowed to identify both the presence of connections and high differentiation rates among populations. In particular, the most differentiated populations, both for *S. velutina* and *S. badaroi*, were those at the edge of the species' distribution area; indeed, for both species, seeds from populations of the core areas of distribution ranges were less different among each other.

In Chapters II and III, relevant information was reported about phenology and ecological requirements of some Tyrrhenian (*S. velutina*, *S. ichnusae* and *S. badaroi*) and Ibero-Levantine species (*Silene mollissima* and *Silene hifacensis* Rouy ex Willk.) of the *S. mollissima* aggregate, providing germination and seedling growth protocols useful for their *ex situ* propagation and conservation. The investigated species occur in coastal areas, islands and islets on rocky habitats and/or sandy dunes. For all species the inter- and intra-specific variability in the responses to light, constant (5-25°C) and alternating temperatures (25/10°C), different NaCl concentrations, and recovery during seed germination were evaluated. Moreover, for the Tyrrhenian species the effect of KNO_3 on seed germination under salinity was also tested; while, for the Ibero-Levantine endemics the biomass production and the growth rate during the early stages of seedlings development were measured in relation to the same factor tested on seed germination. Seeds of the three Tyrrhenian species were non-dormant. Their germination was improved by light and occurred with high percentages at the low temperatures (5-15°C) and under the alternating temperature regime. *Silene velutina* and *S. ichnusae* seeds germinated until 300 mM NaCl,

while *S. badaroi* up to 100 mM. Except for *S. badaroi*, salt did not affect seed viability and recovery. However, inter-population variability both in salt tolerance and recovery was detected for *S. velutina*. The addiction of KNO_3 did not affect germination of the three species and their recovery under saline conditions, resulting unable to alleviate salt stress. Our results suggest, for the three Tyrrhenian species an optimum of field germination during autumn-winter, when under the Mediterranean climate water availability is highest and soil salinity levels are minimal. However, both *S. velutina* and *S. ichnusae* can be also able to germinate until early spring. Seeds of the two Ibero-Levantine species were non-dormant too, but contrarily to the Tyrrhenian species, the photoperiod did not affect their germination. For both *S. mollissima* and *S. hifacensis* seeds, germination rates were higher at the lowest temperatures (10 and 15°C), while at the highest temperatures (20 and 25°C) inter-specific differences were observed. The two species were able to germinate up to 250 mM NaCl, but inter-population differences were detected in the tolerance limit. As well as observed for Tyrrhenian species, the highest germination occurred in the non-saline control and decreased with increasing salinity. Independently from the tested temperature, *S. mollissima* and *S. hifacensis* seeds totally recovered their germination after the NaCl exposure. For both *S. mollissima* populations the increasing temperature promoted the seedling dry weight and the growth rate, while this pattern was observed only for one *S. hifacensis* population. These results are consistent with a field germination in a period from autumn until spring for *S. mollissima* and limited to the autumn-winter months for *S. hifacensis*.

In Chapter IV, by taking into account several environmental and population parameters, the microniche variation and the niche breadth dynamics, at local and regional scales, were investigated in Mediterranean islands of different size (large and small islands; LI and SI, respectively) for the Sardinian-Corsican endemic *S. velutina*. This taxon is a priority species listed in the Annex II of the Habitat Directive 92/43 EEC and included in the Bern Convention. Moreover, it is also included in the IUCN Red Lists as near threatened (NT), and considered vulnerable (VU) and endangered (EN) according the French and Italian Red Lists, respectively. As regards SI populations, a realized niche characterized by harsh and homogeneous environments, with low disturbance levels and potentially low competition was detected, where the life-history strategy of populations was based more on the persistence of adult individuals than on recruitment of juveniles. In contrast, LI populations showed a highly heterogeneous ecological niche, characterized by higher levels of biodiversity, plant cover and the presence of woody vegetation. Such a realized niche on LI appeared to be even more diversified because of the presence of several disturbances to which populations seem to respond by the increase of juveniles and showing a more reliance on the regeneration niche. Concerning the niche breadth, at the regional scale wider niche were observed on LI, probably for the high spatial heterogeneity, which was positively correlated to island size. In contrast, at the local scale, SI showed a wider niche breadth which appears to be due to a release from competition.

In conclusion, this Ph.D. thesis contributed to the knowledge of some rare and/or endangered *taxa* of the *S. mollissima* aggregate, providing new findings for the recovery and conservation planning of species under study. Firstly, the taxonomic treatment of *taxa* was confirmed and additional data on population differentiation were provided; secondly, optimal protocols of germination and seedling growth were detected for same species and their related populations, which result to be essential elements not only for their propagation aimed at conservation, but also to increase knowledge of their *in situ* ecology and phoenology; thirdly, the ecological niche dynamics, for the Sardinian-Corsican endemic *S. velutina*, in two different geographical contexts and two spatial scales, were detected.

GENERAL INTRODUCTION

Biodiversity and endemism in the Mediterranean

As recognized by the Convention on Biological Diversity (CBD), signed by more than 150 nations at the United Nations Conference and Development held in Rio de Janeiro in 1992, "Biological Diversity" means variability among living organisms from all sources, including terrestrial, marine and other aquatic ecosystems, and the ecological complexes of which they are part. This concept includes different levels of a progressively smaller scale (e.g. ecosystems, community, species, populations, organisms, genes etc.) and only preserving biodiversity at each level its conservation can be guaranteed (CBD 1992).

Because of historic, geographic and climatic factors, the biological richness is often concentrated in particular areas, where a significant number of species can be found in a relatively small surface. To quickly reduce the biodiversity loss and to guide investments in conservation measures, these areas have only recently drawn considerable interest and attention. The identification of "biodiversity hotspots" is in fact one of the most successful strategies developed to safeguard the biologically richest in the most endangered areas on Earth (Myers *et al.* 2000). Two strict criteria must be satisfied to qualify a region as a hotspot: 1) it must contain at least 1,500 species of vascular plants as endemics and 2) it has to have lost at least 70% of its original habitat (Mittermeier *et al.* 2004; Williams *et al.* 2011).

The Mediterranean Basin is one of the 35 biodiversity hotspots in the world (Williams *et al.* 2011). Although it occupies only the 1.6% of the Earth's land mass, it includes about the 10% of all the currently known vascular plants (Médail & Quézel 1999). Moreover, with the presence of about 13,000 endemic species, it is the third region in the world for vascular plant endemism rate (Myer *et al.* 2000). As the 44% of these endemics is included in only the 22% of its area, to better assess plant conservation priorities, ten biodiversity hotspots were identified within the Basin (Médail & Quézel 1997; Fig. 1). However, recently, two additional regions (northern Algeria-Tunisia and Adriatic Islands) has been recognized as hotspots, for a total of 12 areas (Vela & Benhouhou 2007; Nikolic *et al.* 2014). Moreover, reducing the spatial scale, smaller hotspots can also be identified within larger hotspots, because the endemic-plant richness is not uniformly distributed, but largely depends on local conditions (Fenu *et al.* 2010; Cañadas *et al.* 2014). For example, within the "macro hotspot" of Tyrrhenian islands some "meso hotspots" (e.g. Sardinia) are identified, which in turn including some "micro hotspots" (e.g. the Supramontes region), in which some "nano hotspots" (such as La Marmora Peack) were recognized (Fenu *et al.* 2010; Cañadas *et al.* 2014).

For its particular shape, the Mediterranean Basin can be divided into two sub-basins separated by the strait of Sicily. The western part is characterized by high endemism rates, in particular of relict type, due to the age of its geological platform; while, in the eastern portion of the Basin, vicariant endemisms prevail, mainly related to the Quaternary glacial events and to the type of rocky substrata (Verlaque *et al.* 1997). However, at a smaller scale, the complex topography and geo-morphology causes high levels of isolation in every part of the Basin. Consequently, many species in the region are site-specific (narrow) endemics and show highly restricted ranges of distribution (Thompson 2005). These exclusive endemics of a single-site mainly grow on islands and islets, high mountain peaks, peninsulas and rocky cliffs (Fineschi *et al.* 2002; Hellwig 2004, Lavergne *et al.* 2004; Bacchetta *et al.* 2012; Fenu *et al.* 2014; Fois *et al.* 2015), such as *Silene ichnusae* Brullo, De Marco & De Marco (Sardinia), *Apium bermejoi* L. Llorens (Minorca), *Arenaria*

bolosii (Cañig.) L. Sáez & Rosselló (Majorca), *Anchusa sardoa* (Illario) Selvi *et* Bigazzi (Sardinia).

In addition to the presence of high species richness and high rates of overall and regional endemism (with the predominance of narrow endemics), the Mediterranean flora also shows a high frequency of disjoint distributions of closely related species with the same chromosome number (Verlaque *et al.* 1997; Deboussche & Thompson 2003). These species groups are frequently constituted of several restricted endemics (e.g. genus *Gymnospermium* in Eurasia, see Tan *et al.* 2011; genus *Aquilegia* in Sardinia, see Garrido *et al*. 2012), or of one widespread species with one or more restricted endemic species outside of its large distribution area (Küpfer 1974; Contandriopoulos & Favarger 1975; Cardona & Contandriopoulos 1979; Verlaque *et al.* 1991; Bacchetta & Brullo 2005). This pattern of distribution has been termed "schizo-endemic" and could be explained by a monophyletic origin followed by a geographical fragmentation. The *Silene mollissima* aggregate is a typical example of this phenomenon in the western part of the Mediterranean (Boquet *et al.* 1978).

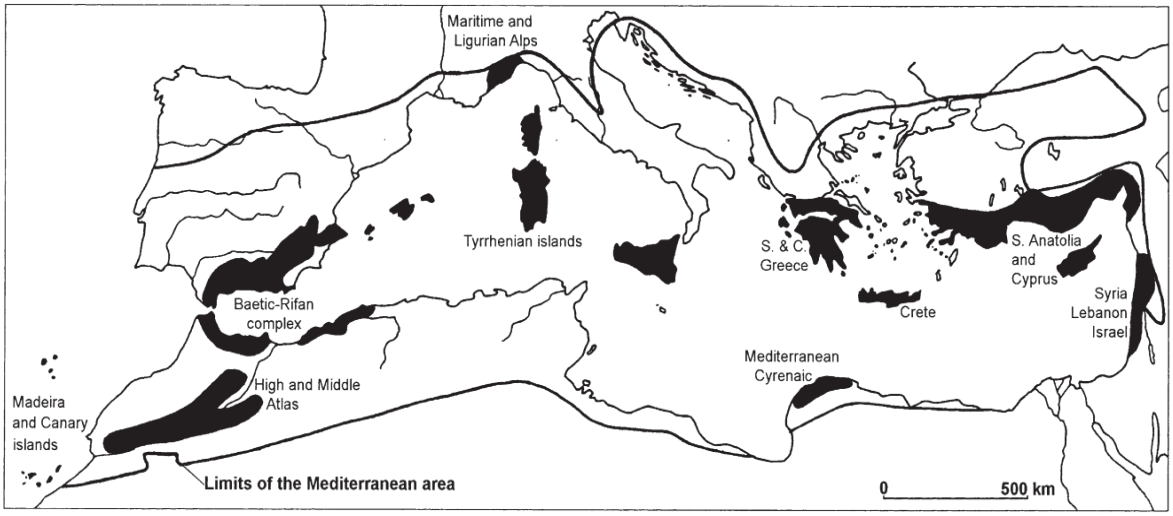

Figure 1- The biogeographic Mediterranean region, with highlighted the area included in the Mediterranean hotspot (black line) and these constituting the 10 mini-hotspots (black areas) identified by Médail & Quézel (1999). These lattest cover about 22% of the total Basin's area, comprising almost 5,500 endemic plants, i.e. about 47% of total Mediterranean endemics. New mini-hotspots (northern Algeria-Tunisia and Adriatic Islands) has been recently identified in the Basin by Vela & Benhouhou (2007) and Nikolic *et al.* (2014).

Plants biodiversity in the Mediterranean is not only due to the intense process of plants speciation in the region, but also to the fact that the Basin has been a refuge area for many species (Quézel 1978, 1985; Verlaque *et al.* 1997; Habel & Assman 2009; Habel *et al.* 2010). Indeed, a recent study delimited 52 refugia within the Basin, significantly associated with the 10 regional hotspots formerly identified (Médail & Diadema 2009). These refugia are thought to have persisted through different paleo-geographical and climatic events, some of which can be supposedly traced back to the Tertiary tectonic history (Magri *et al.* 2007). The 60% of the identified refugia, are situated in Mediterranean submontane and mountain margins (Gentili *et al.* 2015). In these areas, numerous small and isolated populations of herbs and shrubs, even mesothermic species, survived during glacial periods. This is evidenced by the presence of several boreo-alpine or temperate plants in the Basin: e.g. *Poa alpina* L. and *Luzula spicata* (L.) DC. in the Moroccan High Atlas Mountains (Alaoui Haroni *et al*. 2009), *Campanula scheuchzeri* Vill., *Minuartia verna* L.

in the mountains of Sicily (Raimondo *et al.* 2010) or *Gentiana lutea* L. subsp. lutea and *Juniperus communis* L. subsp. *alpina* Gray in the Gennargentu massif (Bacchetta *et al.* 2013) in Sardinia.

The Mediterranean flora is not a random assemblage in terms of plants systematic affinities, biology, habitat requirements, and geographical distribution, but is the expression of the landscape diversity which characterizes this area, and of the complicated paleo-geographic and climatic events that have occurred until now (Thompson 2005). In particular, the current diversity and the origins of the Mediterranean biota are strongly related to both the region's location, which is at the intersection of two large landmasses (Eurasia and Africa), and to the Basin's violent geological history, which produced a high topographical and geographical diversity (e.g. presence of high mountains, several peninsulas and one of the largest archipelagos in the world) (Thompson 2005). Nevertheless, a wide range of local climates, with mean annual rainfall ranging from 100 mm up to 3,000 mm (Blondel & Aronson 1999), as well as the long history of human presence in the area (including the current impact of about 300 millions of people), have certainly played an major role in the evolution of the Mediterranean biodiversity (Hansen *et al.* 2012).

Therefore, although numerous *taxa* became extinct, especially during the Middle Miocene, the Middle/Late Pliocene, the First/Middle Pleistocene and Quaternary glaciations (Hewitt 1996; Peyron *et al.* 1998; Thompson 2005), the current flora is still extraordinarily rich and original, both because of its diverse origin, shown by the presence of elements of ancient tropical, temperate and boreo-alpine floras, and also because of the great impulse to *in situ* differentiation, caused by the high environmental heterogeneity. However, although species richness is extremely high in this area, endemism at higher levels is greatly reduced, with only one endemic family (Aphyllanthaceae) holding only a single species (Médail & Myers 2004). All these aspects are important to define the current Mediterranean flora, which has been essentially described as a complex admixture of Mediterranean woody plants, mainly belonging to pre-Mediterranean lineages (Tertiary) (Verdú *et al.* 2003), and numerous localized neo-endemics, composed predominantly of herbs and sub-shrubs of several families (e.g. Asteraceae, Brassicaceae, Caryophyllaceae, Cistaceae, Fabaceae, Lamiaceae, Poaceae, Ranunculaceae, among others) (Médail & Myers 2004).

The *Silene mollissima* aggregate

With about 700 species, *Silene* L. is one of the largest genera of the world's flora (Melzheimer 1980; Zhou *et al.* 2001; Morton 2005). It comprises mainly herbaceous plants and, more rarely, small shrubs or sub-shrubs (Mamadalieva *et al.* 2014), and also includes a number of cultivated species and widespread weeds (Eggens *et al.* 2007). This genus is widespread in Eurasia, America and Africa, although it shows a greater variety of ecological and morphological characters in the Mediterranean area and in the Middle East (Oxelman & Lidén 1995). The most authoritative taxonomic revisions, subdivided the *Silene* genus in 44 sections (Chowdhuri 1957) and, most recently, in 43 sections (Lazkov 2003). However, different molecular phylogenetic studies did not agree with either of these classifications (Oxelman & Lidén 1995; Desfeux & Lejeune 1996; Oxelman *et al.* 1997; Eggens *et al.* 2007; Popp & Oxelman 2007; Rautenberg *et al.* 2012).Within the genus, more than three quarters of the rare or endangered species are included in the section *Siphonomorpha* Otth. (Walter & Gillett 1998), a group of perennial or rarely biennial plants, characterized by a rosettes habitus, and producing pyramidal inflorescences and

flowers with cylindrical-clavate calyces (Chowdhuri 1957; Jeanmonod & Mascherpa 1982). The section comprises about 35 species distributed across the entire Mediterranean Basin (Leuzinger *et al.* 2014) and was subdivided, according to morphological and ecological criteria, in four groups of unequal sizes (Jeanmonod 1984a): two independent species (*S. viridiflora* L. and *S. nutans* L.), the *S. paradoxa* aggregate and the largest complex of *S. italica*, a circum-Mediterranean group of about 29 species (Leuzinger *et al.* 2014), in turn subdivided into *S. mollissima* and *S. italica* aggregates (Jeanmonod 1985a).

Figure 2 - Flowers of *Silene hifacensis*, *S.ichnusae* and *S. velutina*.

Considering the nomenclatural revisions and the recently described species, the *S. mollissima* aggregate currently comprises 11 schizoendemic species (2n=24) of the western Mediterranean Basin (Tab. 1), which often present scattered or narrow distributions (Murru *et al.* 2015). These perennial plants are characterized by dense rosettes, subcorimbose-subpyramidal inflorescences, flowers with white, reddish, purplish or violet petals (Fig. 2) and seeds with plane faces (Jeanmonod 1984a). These species are considered as vicariant chasmo-comophytes in coastal areas, islands and islets within their distribution area (Jeanmonod 1984a; Murru *et al.* 2015, 2017). For the allopatric and parapatric characteristics of the *taxa* and the highly adaptive value of their characters, these species can be considered a model of a clinal speciation (Jeanmonod 1984a), and were also used as a typical example of flora distribution pattern explained by the Messinian model (Boquet *et al.* 1978). In particular:

- *Silene badaroi* Bestr. is an endemic species of the coasts of the Provence (southern France), Liguria and of some islands of the Tuscan Archipelago (Italy), where it occurs on calcareous and metamorphic substrates. It is considered a vulnerable species (VU) in the French Red Lists (Olivier *et al.* 1995).

- *Silene ichnusae* Brullo, De Marco & De Marco is a recently described endemic species, with a very narrow distribution, located in a single site of the north-western coast of Sardinia (Italy), where it grows on metamorphic rocky cliffs and glareicolous habitats facing the sea (Brullo *et al.* 1997; Murru *et al.* 2015).

- *Silene hicesiae* Brullo & Signorello is a narrow endemic of Panarea and Alicudi islands (Aeolian Archipelago; Sicily) and occurs in rocky slopes of volcanic origin (Brullo & Signorello 1984). It is included as a priority species in Annexes II and IV of the Habitats Directive 92/43 EEC and is categorized vulnerable (VU) in the IUCN Red Lists (Domina & Troia 2011).

- *Silene oenotriae* Brullo is a narrow endemic species of the Pollino Massif (Italy) where it can be found at different elevations (from 260m to about 2000m a.s.l.) in rocky and glareicolous habitats on limestones (Brullo 1997; Peruzzi *et al.* 2007).

- *Silene velutina* Pourr. ex Loisel. is an endemic species of southern Corse and north-eastern Sardinia and occurs in coastal habitats and on some islets surrounding the two largest islands (Corrias 1985). It is able to grow from rocky to sandy substrates, derived both from siliceous and calcareous bedrocks (Murru *et al.* 2015). It is listed as a priority species in the Habitats Directive 92/43, included in the IUCN Red Lists as near threatened (NT; Buord *et al*. 2011) and considered vulnerable (VU) and endangered (EN) respectively in the French and Italian Red Lists (Olivier *et al.* 1995; Conti *et al.* 1997; Pisanu *et al.* 2014).

- *Silene hifacensis* Rouy ex Willk. is endemic from Alicante and Teulada provinces (Iberian Peninsula, Spain) and from western Ibiza (Balearic Islands, Spain), but also occurs in some islets within this area: S'Espartar, Es Vedrà and Illeta Mitjana. (Pilar Blasco *et al.* 2011; Murru *et al.* 2017). It grows both in calcareous crags and cliffs near to the sea and on better developed soils at the foot of the cliffs (Pilar Blasco *et al.* 2011). It is listed in the Habitats Directive EEC 92/43, included in the IUCN International Red Lists as an endangered taxon (EN; Pilar Blasco et al. 2011) and inserted with the same degree of threat in the Red List of Spanish plants (Lozano, 2000; Bañares *et al.* 2010).

- *Silene mollissima* (L.) Pers. is endemic of calcareous rocky habitats of Mallorca and Menorca (Balearic Islands, Spain), and it is able to grow both in front of the sea and in the inland rocky cliffs. It is not current under protection and urgently needs an assessment of the conservation status.

- *Silene tomentosa* Otth. is endemic of Gibraltar (Spain) where it grows on limestone rocky outcrops. It is an exceptionally rare species, and it was considered extinct since 1991, when very few individuals were re-discovered in the wild. After that, several individuals were conserved in the Gibraltar Botanic Gardens and a reinforcement of the wild population was carried out. It is currently protected by the low of Gibraltar under the Nature Protection Act, 1991, while is a not evaluated species (NE) by the IUCN.

- *Silene andryalifolia* Pomel. is the species with the most extended distribution range of the whole section *Siphonomorpha,* and it is endemic of the Betic-Rifan region, from South-eastern Spain to the north-western of Africa (northern Morocco and Algeria) and grows on limestone substrates (Lopez *et al.* 2011).

- *Silene auricolifolia* Pomel. is a narrow endemic of the Oran region (Algeria). There are no ecological data on this species.

- *Silene gazulensis* A. Galán, Cortés, Vicente Orellana & Morales Alonso presents a single population located in the Cadiz region in Andalusia (Spain).This species lives in fissures of limestone rocks with exclusively N and NW orientation (Iriondo 2011). It is critically endangered (CR) both in the Spanish and in the international Red Lists (Iriondo 2011; De Vega *et al*. 2006), however it is not affected by any legal protection measures.

  Despite the great interest in this group from an evolutionary and biogeographically standpoint, only few studies of morphology, biology and ecology were carried out for these species (Jeanmonod, 1984; Paradis, 2006; Faggio, 2006; Pretince *et al.* 2003; Escriba *et al.* 2011), although the majority of them are endangered and/or rare species.

Table 1 - Distribution, presence in Habitat Directive 92/43 (priority species are indicated by asterisk *) and assessment of conservation status for the species of the *Silene mollissima* aggregate.

Species	**Distribution**	**Habitat Directive 92/43**	**IUCN Assessment**
Silene mollissima (L.) Pers.	Endem. Bl	absent	_
Silene hifacensis Rouy ex Willk.	Endem. Hs-Bl	present*	EN (IUCN Red List) EN (Spanish Red List)
Silene tomentosa Otth.	Endem. Hs	absent	_
Silene gazulensis A. Galán, Cortés, Vicente Orellana & Morales Alonso	Endem. Hs	present	CR (IUCN Red List) CR (Spanish Red List)
Silene hicesiae Brullo & Signorello	Endem. Si	present*	VU (IUCN Red List)
Silene oenotriae Brullo	Endem It	absent	_
Silene badaroi Bestr.	Endem Ga It At	absent	VU (French Red List)
Silene ichnusae Brullo, De Marco & De Marco	Endem. Sa	absent	_
Silene velutina Pourr. ex Loisel.	Endem. Sa Co	present*	NT (IUCN Red List) VU (French Red List) EN (Italian Red list)
Silene auricolifolia Pomel.	Endem. Ag	absent	_
Silene andryalifolia Pomel.	Endem. Ma Ag	absent	_

TOPICS UNDER STUDY

The importance of quantitative studies of seed characters

The correct identification of *taxa* as well as the assessment of species and population differentiation are needed to guarantee and improve conservation actions on endangered plants (Quilichini *et al.* 2004; Hamrick *et al.* 1991; Holsinger & Gottlieb 1991; Ellstrand & Elam 1993; Wise 1997; Thompson 1999; Morim & Lughadha 2015). Morphological characters can be considered "the earliest genetic markers" used for species identification and to investigate on variation among organisms (Dettori 2013). Indeed, the study of physical features is of great importance for taxonomic purposes and it has been successfully applied not only for the classification of *taxa* but also as indicator of relatedness (Zareh 2005; Bacchetta *et al.* 2008; Bacchetta *et al.* 2011a, 2011b). There are several sets of physical characters for different crops at different developmental stages (e.g. seed, juvenile, adult, flower and fruit), and although the state of some of these characters is strongly dependent on the environment, the seed coat features are surprisingly little affected by the environmental conditions under which a plant grows (Barthlott 1984).

Several studies found that shape, size, coat features and color of seeds are all diagnostic characters useful to distinguish *taxa* (Chowdhuri 1957; Chuang & Ornduff 1992; Yildiz 2002; Minuto *et al.* 2006; Salmeri *et al.* 2011; Bacchetta *et al.* 2014), which sometimes can be satisfactory, even if considered alone (Dadandı *et al.* 2009). In fact, several authors reported that seed morphology is particularly useful in providing additional information for solving taxonomic and evolutionary problems in different families (Bergreen, 1981; Akbari & Azizian, 2006; Abid & Ali, 2010; Rajbhandary & Shrestha, 2010; Grillo *et al.* 2010; Ackin & Binzet, 2011). This has been abundantly documented for several genera of the Caryophyllaceae family such as: *Agrostemma, Cerastium, Dianthus, Gypsophila, Lychnis, Minuartia, Moenchia, Petrorhagia, Stellaria* and *Velezia* (Fedotova & Ardjanova 1992; Kovtonyuk 1994; Crow 1971; Wofford 1981; Wyatt 1984; Volponi 1993; Poyraz & Ataşlar 2010; Kaplan *et al.* 2009; Minuto *et al.* 2006), but especially for the *Silene* genus (Melzheimer 1987; El-oqlah 1990; Keshavarzi *et al.* 2015; Yildiz 2002, 2006a,2006b; Hong *et al.* 1999; Fawzi *et al.* 2010, Camelia 2011, Bacchetta *et al.* 2014). However, in many cases, difficulties in quantifying same characters (e.g. color), and also in taking several measurements, for example because of the reduced seed size in some families (i.e. Cynomoriaceae, Primulaceae, Rubiaceae, Scrophulariaceae, etc.), such as in the *Silene* genus, could well hinder a limit relatively to results reliability (Bacchetta *et al.* 2008).

The application of morpho-colorimetric analysis techniques, by means of the acquisition of digital images, is a non-destructive method which allows obtaining precise and repeatable measurements of color, size and shape of seeds (Grillo *et al.* 2010). Moreover, the methodology applied in this thesis, is a standardized and quantitative analysis of seed characters, and thank to the use of a software and hardware support for image processing, it allows to: 1) survey a very large number of seeds and their relative parameters, 2) statistically identify the most important variables in the discrimination among *taxa* and/or populations, and 3) ensure a reduction of both cost and time of analysis (Lo Bianco *et al.* 2015). The image analysis technique performed was in fact, also successfully used to study species of agricultural interest (Chen & Sun, 1991; Venora *et al.* 2009) and for archaeological investigations (Sabato *et al.* 2015; Ucchesu *et al.* 2016). However, it can also be extremely attractive for studies of wild plants, in particular to assess patterns of variability, not only at different systematic levels, but also in relation to geographical distribution of allied *taxa* and/or critical taxonomic groups (Mattana *et al.* 2008; Bacchetta *et*

al. 2011a; Bacchetta *et al.* 2011b). Nevertheless, quantitative studies of the degree of morphological differentiation among closely related endemics have rarely been carried out, although they could yield important information about systematic and evolutionary history of *taxa* and could also provide useful data for plan conservation measures (Hamrick *et al.* 1991; Holsinger & Gottlieb 1991; Ellstrand & Elam 1993; Wise 1997; Thompson 1999; Debussche & Thompson 2002; Quilichini *et al.* 2004).

Seed germination and seedling growth, two critical phases of plants life cycle

Seed germination and seedling growth are among the most complex and vulnerable phases of the plant life cycle. A detailed knowledge of how environmental factors can influence these processes is crucial to ensure the conservation of rare and/or endangered species (Baskin & Baskin 1998; Pinna *et al.* 2014). Consequently, a large number of studies have dealt with these issues (Aranda *et al.* 2005; Farris *et al.* 2009; Pisanu *et al.* 2012; Cogoni 2013; Cuena Lombrana 2016).

A seed is a propagating organ formed in the sexual reproductive cycle of Gymnosperms and Angiosperms, consisting of a protective coat enclosing an embryo and food reserves (Baskin & Baskin, 1998). Seed germination is a process by which a seed embryo develops into a seedling (Leck *et al.* 2008) and involves the reactivation of the metabolic pathways that lead to growth and the emergence of the radicle and plumule (Fenner & Thompson 2005). The visible protrusion of the radical tip is considered the completion of germination, and physiologically represents the transition point from seed to seedling, which is characterized by the loss of desiccation tolerance. Although the seed is a discrete phase of the plant life cycle, such as flowering, fruiting, etc., seedling may be more arbitrarily delimited because its end point is along a growth continuum and is more difficult to recognize (Leck *et al.* 2008).

In coastal species, both from rupicolous and psammophilous habitats (as are the species under study), the most crucial life stages of a plant are seed germination and seedling growth (Ungar 1982). Therefore, the habitat specificity in the studied species, might have a marked effect, especially in these two sensitive phases of their life cycle, and consequently influences the species and populations fitness. Coastal habitats, in fact, are characterized by very harsh environmental conditions (Maun 2009; Thanos *et al.* 1991), and the main limitations for the plants life are the high salt concentrations in the substrate and the shortage of soil (Santo 2013), which in rocky cliffs is accumulated only in a few cracks, while in sand dunes its presence depends to the vegetation ability to both produce and retain it. Moreover, in these habitats, the combination of strong sunlight and the constant winds (in addition to the high levels of salinity), determines conditions of exceptional dryness, and thus species living in coastal habitats show typical xerophylous adaptations. Finally, in the specific case of sandy dunes, the spatial and temporal variation in substrate and the micro-environmental changes mediated by wind and wave actions, contribute to create uncertain and variable conditions (Maun 1994).

Variations in seed germination and dormancy, as well as in seedlings growth, reflect adaptations to specific ecological conditions (Grime *et al.* 1981; Nishitani & Masuzawa 1996; Baskin & Baskin 2014). In coastal habitats, considering the numerous abiotic factors affecting seed germination and seedling growth (light, temperature, soil moisture, nutrient availability, soil salinity etc.) (Khan & Ungar 1984; Baskin & Baskin 1998; Nicotra *et al.* 1999; Santo at al. 2014a, 2014b), the micro-site characteristics strongly influence seeds

probability of germination and their subsequent survival. Nevertheless, as expressed by the seed-seedling conflict theory, environmental conditions which are favourable for seeds are not always favourable for seedlings (Shupp 1995).

Several studies have highlighted the presence of inter- and intra-specific variations in seed germination and seedling growth (e.g. Andersson & Milberg, 1998; Keller & Kollman, 1999; Santo *et al.* 2015a, 2015b) and have attributed this phenomenon to genetic variations, environmental differences or both (Degreef *et al.* 2002; Cruz *et al.* 2003). For example, both different populations and different phylogenetically related species can respond in a similar way to some environmental variables, and indeed respond differently compared to other variables (Ellison, 2001; Murru *et al.* 2015; Santo *et al.* 2015a Murru *et al.* 2017). A detailed knowledge of ecological requirements at different biological levels, during these critical phases, can help us to better understand and explain species' distribution and rarity (Ramírez-Padilla & Valverde 2005). Moreover, detecting the presence of local adaptations processes can be very useful in the perspective to preserve the species aptitude to persist and adapt in their environment, this is crucial to activate successful *in situ* and *ex situ* conservation strategies.

The ecological niche and habitat specialisation, key elements to understand population dynamics and species distribution

Understanding how populations respond to changes of environmental conditions is a central issue for species conservation (Thompson 2005). Population ecology deals with the study of populations in relation to their environment, and attempts to give answers to several questions, such as the distribution tendencies, the habitat specialization, the width of the ecological niche, the causes and mechanisms which control the different demographic strategies, etc. A first step to understand population dynamics may be to investigate the environmental conditions to which they are subjected to, or rather their ecological niche. In fact, the ecosystem components (abiotic and biotic factors), affecting the life of organisms, can be considered the set of environmental variables constituting their niche. In particular, according to Hutchinson (1957), the fundamental niche can be geometrically represented by a hypervolume in a multi-dimensional space, where each environmental variable represents a different spatial dimension. This concept basically defines the organisms requirements, or rather the full range of environmental conditions and resources that they can possibly occupy and use, which guarantee the persistence of their populations. However, species, populations, or more in general individuals, as a consequence of competitive exclusion, are not able to use their entire fundamental niche, but only a portion of it, i.e. the realized niche (Hutchinson 1957; Pulliam 2000).

Habitat specialization is closely related to the ecological niche, and has often used as a synonymous of a narrow niche breadth (Futuyma & Moreno 1988; Devictor 2010, Poisot *et al.* 2011; Bulangeat *et al.* 2012). Therefore, species occurring in narrow ranges of environmental conditions are considered habitat specialists (or having a restricted ecological niche). In particular, specialisation reflects the range of environmental tolerance and the ability to exploit resources, and like the ecological niche, it can be defined for different biological levels (e.g. individuals, populations, species, etc.). The strong selection imposed by conditions characterizing the "preferred habitat/s" by species is likely to cause habitat specificity (Bazzaz 1991). However, the dynamics of specialization is often unclear and its overall understanding depends on the clarification of the mechanisms behind observed distributions of species and on the identification of the scale at which they function (Levin 1992).

A variety of mechanisms have been proposed to explain niche width (or habitat specialization), such as physiological tolerance (Robson & Maze 1995) and competitive ability (Van Valen 1965). Furthermore, these factors can act alone or in combination, increasing their effect (Futuyma & Moreno 1988), and become important at different spatial and temporal scales or in particular geographical contests (e.g. niche expansion in islands; Levin 1992; Givnish 1997; Gillespie & Clague 2009). Different authors, in fact, agree that there is no one "best" scale for ecological researches, and overemphasizing that the small- rather than the largest scale can be misleading (Huston 1999; Blackburn & Gaston 2002). Therefore, a hierarchical approach can be often necessary (Whittaker *et al.* 2001; Willis & Whittaker 2002), because several processes are nested according to both spatial and temporal scales. However, few studies of ecological niche and habitat specialisation have considered the spatial scale.

Investigating the causes of species distributions and determining which factors influence species range limits and population patterns is an ongoing challenge for ecologists (Guisan & Thuiller, 2005; Colwell & Rangel, 2009). Studies of the ecological niche are particularly useful to provide new insights on the mechanisms driving population dynamics and species distributions (Guisan & Thuiller 2005; Costa *et al.* 2010; Barve *et al.* 2011). In fact, habitat specialization and narrow niche breadth were widely cited as a potential cause of species rarity (Kruckeberg & Rabinowitz 1985; Hubbell & Foster 1986; Prober & Austin 1991). Therefore, a better knowledge of these ecological aspects, in particular at different spatial scales and for different geographical contexts, is extremely important not only theoretically but also practically, both because of lack of information on these topics and for the useful information which it can provide for the management and conservation of threatened species.

AIMS

In this Ph.D. thesis, a multidisciplinary approach was conducted to obtain useful information and data to support conservation measures for some rare and/or endangered species of the *S. mollissima* aggregate for which, to our knowledge, no study or only few studies were previously carried out.

In particular, this thesis aimed *in primis* to evaluate the taxonomic treatment of some studied *taxa*, and secondly to investigate some ecological and eco-physiological aspects, focusing both on the inter- and intra-specific variability of investigated *taxa*.

In detail, the different targets of the thesis were:

1) to implement and validate the statistical classifier, based on seed morphometric and colorimetric parameters, for the Tyrrhenian species belonging to the *S. mollissima* aggregate, with the aim to compare the results with the current taxonomic treatment and evaluate the differentiation degree among species and populations.

2) to investigate the intra- and inter-specific variability on seed germination ecology and seedling growth of some Tyrrhenian (*S. ichnusae*; *S. velutina*; *S. badaroi*) and Ibero-Levantine species (*S. mollissima*; *S. hifacensis*), with the specific purpose to identify the optimal conditions useful to elaborate protocols of germination and seedling growth. In particular, the considered abiotic parameters were: photoperiod, thermoperiod, salinity and nutrient availability. Moreover, the ability of seeds to recover successfully after the salt stress was evaluated.

3) to characterize the ecological niche of the Corsican-Sardinian endemic *S. velutina*, by investigating on the niche differentiation between populations living on large and small Mediterranean islands and quantifying the niche breadth at two different spatial scales (regional and local).

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CHAPTER I

Seeds morpho-colorimetric analysis on the Tyrrhenian species of the *Silene mollissima* aggregate

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ABSTRACT

With the aim to evaluate the systematic treatment of Tyrrhenian species of the *Silene mollissima* aggregate (*Silene badaroi, S. ichnusae, S. hicesiae, S. oenotriae, S. velutina*), including several endemic *taxa* of the western Mediterranean, and to investigate the relationships among species and populations, seed morpho-colorimetric analyses were carried out applying computer-aided image analysis techniques. In keeping with findings of other authors, our study showed that shape, color and seed coat features have a diagnostic value. The applied methodology provided key information about the differentiation of the analyzed endemic *taxa* and of their populations, highlighting critical issues at systematic level. In particular, our results confirmed the current systematic treatment at section and species levels. Nevertheless, further investigations, regarding the taxonomic position of *S. hicesiae* into the whole aggregate, and also the differentiation of *S. ichnusae* from *S. velutina*, would be needed. Furthermore, the employment of the analytical tool at population level, enabled to detect both the presence of connections and high differentiation rates among populations.

Keywords: Caryophyllaceae, endemic species, Mediterranean vascular flora, seed image analysis.

INTRODUCTION

With about 700 species, *Silene* L. is one of the largest genera in the World's flora (Greuter 1995) and shows a great variety of ecological and morphological characters, especially in the Mediterranean area and in the Middle East (Greuter 1995; Oxelman & Lidén 1995). In the Chowdhuri taxonomic revision (1957), the genus included 44 sections, while more recently Lazkov (2003), considering a higher number of species, subdivided it into 43 sections. However, both classifications show a degree of uncertainty because of the inconsistencies with recent molecular studies (e.g., Oxelman & Lidén 1995; Desfeux & Lejeune 1996; Oxelman *et al.* 1997; Oxelman *et al.* 2001; Eggens *et al.* 2007; Popp & Oxelman 2007; Petri & Oxelman 2011; Rautenberg *et al.* 2012).

The section *Siphonomorpha* Otth. is represented by perennials or rarely biennial plants, characterized by dense rosettes and considerable hairiness, exhibiting panicolate or subpiramidal inflorescences with short and opposite pedicels, tubular chalices and seeds with flat faces (Brullo & Signorello 1984). This section, reviewed several times following both a morphological (Rohrbach 1868; Williams 1896; Boissier 1867; Chowdhuri 1957; Jeanmonod 1984b) and a molecular approach (Cotrim 2001; Cotrim *et al.* 2005), includes the *Silene italica* (L.) complex (Jeanmonod 1985), a large circum-Mediterranean group of species, subdivided into *Silene italica* and *S. mollissima* aggregates.

By embracing 11 closely related species, often presenting scattered or narrow distribution, *S. mollissima* aggregate is a group of chasmocomophytes growing in coastal areas and in particular on islands and islets of the western Mediterranean Basin (Murru *et al.* 2015). As documented by the allopatric and parapatric characteristics of the *taxa* and the highly adaptive value of their characters, it is considered a model of a clinal speciation (Jeanmonod 1984a), and was also used as an example of flora distribution pattern explained by the Messinian model (Boquet *et al.* 1978). The group evolution, according to the same model, mainly occurred during the Messinian salinity crisis, with the directional expansion (north-south), from northern Italy to Tunisia, of an ancestral species compatible with *S. italica* (L.) Pers. (Jeanmonod 1984a). In a similar scenario, the Tyrrhenian area would have been the most probable colonization route and the differentiation center of the group. As a consequence, five species out of 11 of the aggregate are endemic species of this area.

Silene badaroi Bestr. is an endemic to the coast of the south France, Liguria and the Tuscan Archipelago (Italy) and can be found both in calcareous, granitic and metamorphic substrates. It is protected in Italy by the Tuscan low 56/2000.

Silene ichnusae Brullo, De Marco & De Marco is a recently described narrow endemic species growing in metamorphic rocky cliffs near to the sea of the Stintino peninsula (NW Sardinia).

Silene hicesiae Brullo & Signorello is endemic to the Aeolian Islands and grows on the rocky slopes of two small volcanic islets: Panarea and Alicudi (Sicily). It is listed as a priority species in Annexes II and IV of the Habitats Directive 92/43 and is categorized critically endangered (CR) in the IUCN Red Lists (Domina & Troia 2011).

Silene oenotriae Brullo is a narrow endemic species from the Pollino Massif (Italy) where can be found at different elevations in cliffs and rocky habitats on limestones.

Silene velutina Pourr. ex Loisel. is an endemic of coastal habitats and same islets of north-eastern Sardinia and of southern Corse and grows in a variety of substrata (from rocky to sandy) derived both to siliceous and calcareous bedrocks. It is listed as a priority species in the Habitats Directive 92/43, in the Bern Convention, and it is included in the IUCN Red Lists as near threatened (NT; Buord *et al*. 2011), considered vulnerable (VU) and endangered (EN) respectively in the French and Italian Red Lists (Olivier *et al.* 1995; Conti *et al.* 1997; Pisanu *et al.* 2014), and near threatened (NT) in the Corsican Red List (Delage & Hugot 2015).

Whilst meticulous morphological studies (Jeanmonod 1984b) allowed to distinguish these *taxa*, mainly thanks to the identification of same important diagnostic characters (e.g. form of basal leaves, hair type and plant height), the analysis of seed morphology was a critical aspect because of the misleading results obtained both in the *S. italica* and in the *S. mollissima* aggregate (Jeanmonod 1984a, 1984b).

Seed morphological studies can be alternative or additional methods for delimiting *taxa*, albeit sometimes the seed characters alone may be satisfactory (Dadandı *et al.* 2015). In fact, several authors studied seed morphology in different genera of Caryophyllaceae (Fedotova & Ardjanova 1992; Kovtonyuk 1994; Crow 1979; Wyatt 1984; Volponi 1993; Poyraz & Ataşlar 2010; Kaplan *et al.* 2009; Minuto *et al.* 2006) and especially in the *Silene* genus, finding that shape, size, testa features and color of seeds are useful diagnostic characters to distinguish *taxa* (Chowdhuri 1957; Melzheimer 1987; El-oqlah 1990; Keshavarzi *et al.* 2015; Yildiz 2002, 2006a,2006b; Hong *et al.* 1999; Fawzi *et al.* 2010, Camelia 2011, Bacchetta *et al.* 2014).

For studies of critical taxonomic groups, the seed morpho colorimetric analysis was shown to be a useful tool to evaluate the patterns of variability both at different systematic levels and in relation to geographical distribution of allied *taxa* (Mattana *et al.* 2008; Bacchetta *et al.* 2011a; Bacchetta *et al.* 2011b; Grillo *et al.* 2013; Pinna *et al.* 2014). The potential of biometric and colorimetric indices of seeds is currently well known (Granitto *et al.* 2003; Kiliç *et al.* 2007; Grillo *et al.* 2010; Orrù *et al.* 2012; Santo *et al.* 2015) and the application of image analysis techniques allows to perform precise and repeatable measurements in color, size and shapes of seeds. Several authors have successfully applied this technique to distinguish different *taxa* and populations (e.g., Mebatsion *et al.* 2012; Orrù *et al.* 2013; Smykalova *et al.* 2011; Smykalova *et al.* 2013; Lo Bianco *et al.* 2017a; Lo Bianco *et al.* 2017b). Furthermore, a seed image analysis application on autochthonous Mediterranean species of the Caryophyllaceae family (including species of *Siphonomorpha* section), evidenced a high performances of inter-specific discrimination (Bacchetta *et al.* 2008).

Consequently, the aims of this study are to: (1) implement and validate the statistical classifier, for the Tyrrhenian species belonging to the *S. mollissima* aggregate, based on seed morphometric and colorimetric parameters; (2) compare our results to the current taxonomic treatment; and (3) evaluate the differentiation degree at intra-specific/inter-specific level.

MATERIAL AND METHODS

Seed lot details

A total of 1845 seeds of 19 accessions belonging to five *Silene* tyrrhenian species of the *S. mollissima* aggregate: *S. badaroi, S. hicesiae, S. ichnusae, S. oenotriae* and *S. velutina,* and one accessions of *S. colorata* Poir. used as out-group, were collected (Tab. 1). Sampling was conducted in such a way as not to prejudice the genetic resources *in situ*, on a representative sample of intra-population genetic diversity, following internationally recognized protocols and the guidelines expressed in the APAT manual for the collection, study, conservation and *ex situ* management of germplasm (Bacchetta *et al.* 2006). As showed in Fig. 1, all the germplasm retrieved has been cleaned, selected and stored in the Sardinian Germplasm Bank (BG-SAR), according to Bacchetta *et al.* (2008).

Seed collections in Sardinia were carried out after obtaining permits from the "Ministero dell'Ambiente e della Tutela del Territorio e del Mare (MATTM)", as required by the European and Italian laws for the species listed in the appendices of the Habitat Directive 92/43 EEC; seed collections in the Tuscan Archipelago were conducted as ruled out by the authorisation granted by Angelino Carta from the Tuscan Archipelago National Park; while seeds from Corsica were provided after obtaining permits from the "Direction Régionale de l'Environnement, de l'Aménagement et du Logement (DREAL) and from the Comité Consultatif de la Réserve Naturelle des Bouches de Bonifacio.

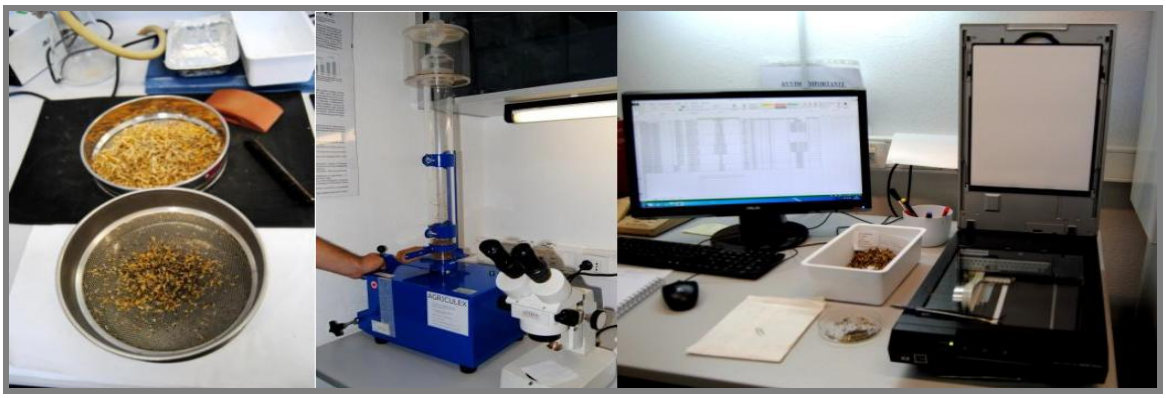

Figure 1- Cleaning and scanning seeds at the Sardinian Germplasm Bank (BG-SAR)

Image analysis

Digital images of seeds (Fig. 2) were acquired using the same equipment and following the same procedure reported in Lo Bianco *et al.* (2015) and processed using the software package KS-400 V. 3.0 (Carl Zeiss, Vision, Oberkochen, Germany). A macro specifically developed for the characterization of seeds (Venora *et al.* 2009), was modified to perform automatically all the analysis procedures, reducing the execution time and contextually mistakes in the analysis process.

Moreover, the macro was enhanced by adding algorithms able to compute the Elliptic Fourier Descriptors (EFDs) for each seed analysed, obtaining further parameters useful to discriminate among the studied *taxa* (Orrù *et al.* 2013) and to improve the classification performance. As described by Orrù *et al.* (2013), this method allows for the description of

the boundary of the seed projection, as an array of numbers that correspond to the pixel positions of the seed boundary. According to many authors (Yoshioka *et al.* 2004; Lootens *et al.* 2007; Iwata *et al.* 2009; Hâruta 2011), regarding the use of a number of harmonics for an optimal description of seed outlines, in order to optimize the efficiency of shape reconstruction and to minimize the measurement errors, 20 harmonics were used to define the seed boundaries, obtaining a further 78 parameters that were useful to discriminate among the seeds studied (Orrù *et al.* 2012).

In addition, following the same procedure described by Lo Bianco *et al.* (2015), the macro was further improved by including algorithms able to compute 11 Haralick's descriptors and the relative standard deviations for each seed analysed (Haralick *et al.* 1973, 1979; Haralick & Shapiro 1991). The 11 Haralick's descriptors measured on each seed to mathematically describe the surface texture are reported as supplementary material (Suppl. material 1). A total of 132 morphometric, colorimetric and textural characters were measured on each seed (Suppl. material 2).

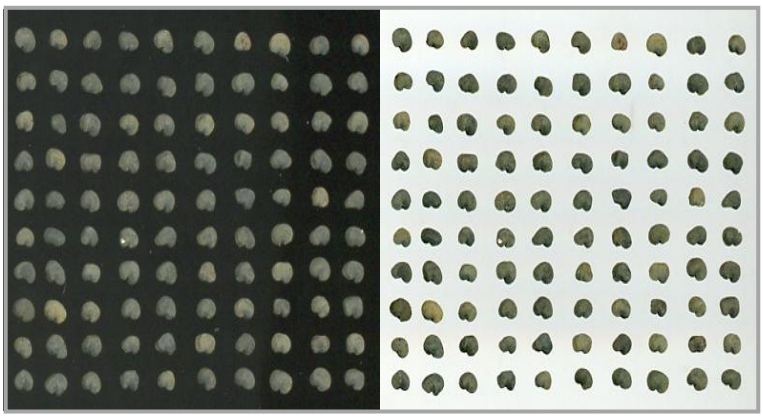

Figure 2 - Example of seed scan on wite and black backdrop.

Table 1 - Population data and seed lot details.

Species	Code	Location	Seed amount	Coordinate N	Coordinate E	Elevation (m)	Substrate
Silene badaroi Beistr.	VM 01/13	Capo Noli - Liguria	98	44°11'	8°25'	240	Limestones
	VM 05/13	Is. Capraia - Tuscan Archipelago - Tuscany	59	43° 2'	9°50'	35	Volcanites
	VM 06/13	Is. Elba - Tuscan Archipelago - Tuscany	98	42°48 '	10°8'	8	Granites
	VM 08/13	Presqu'ile de Giens - Hyères - Provence	104	43° 2'	6°5'	3	Phyllites
Silene colorata Poir.	171/12	Poetto - Cagliari - Sardinia	100	39°12'	9°10'	4	Sand dunes
Silene ichnusae Brullo, De Marco & De Marco	VM 216/13	Capo Falcone - Stintino - Sardinia	99	40°57'	8°11'	60	Metamorphytes
Silene hicesiae Brullo & Signorello	VM 02/13	Panarea - Aeolian Islands - Sicily	100	38°38'	15°3'	310	Volcanites
Silene oenotriae Brullo	VM 04/13	Massiccio del Pollino - Calabria	100	39°49'	16°19'	350	Limestones
Silene velutina Pourr. ex Loisel.	VM 212/13	Abbatoggia - Is. della Maddalena - La Maddalena Archipelago - Sardinia	100	41°15'	9°24'	4	Sand dunes
	194/06	Abbatoggia - Is. della Maddalena - La Maddalena Archipelago - Sardinia	99	41°15'	9°24'	5	Sand dunes
	VM 213/13	Is. Baccà - La Maddalena Archipelago - Sardinia	100	41°11'	9°26'	3	Granites
	181/06	Is. Stramanaro - La Maddalena Archipelago - Sardinia	100	41°17'	9°21'	6	Granites
	VM 13/14	Is. Stramanaro piccolo- La Maddalena Archipelago - Sardinia	90	41°17'	9°21'	6	Granites
	VM 14/14	Is. Colombo- La Maddalena Archipelago - Sardinia	100	41°14'	9°23'	7	Granites
	VM 214/13	Riu di Li Saldi - Aglientu - Sardinia	100	41° 7'	9° 4'	2	Sand dunes
	VM 215/13	Casetta Bianca - Porto Vecchio - Corse	99	41°35'	9°18'	2	Granites
	CO 13/33	Is. Tamaricciu - Porto vecchio - Corse	100	41°32'	9°18'	3	Granites
	CO 13/34	Tamaricciu - Porto vecchio -Corse	99	41°33'	9°19'	6	Sand dunes
	CO 13/20	Capu di Fenu - Ajaccio - Corse	100	41°57'	8°35'	45	Granites

STATISTICAL ANALYSIS

The achieved results were used to build a database including morpho-colorimetric, EFDs and Haralick's descriptors. Statistical elaborations were executed using SPSS software package release 16.0 (SPSS Inc. for Windows, Chicago, Illinois, USA 2007), and the stepwise Linear Discriminant Analysis (LDA) method was applied to identify and discriminate among the investigated *Silene* accessions.

This approach is commonly used to classify/identify unknown groups characterized by quantitative and qualitative variables (Duda *et al.* 2000; Fisher 1936, 1940; Fukunaga 1990), finding the combination of predictor variables with the aim of minimizing the within-class distance and maximizing the between-class distance simultaneously, thus achieving maximum class discrimination (Hastie *et al.* 2001; Holden *et al.* 2011; Kuhn & Johnson 2013; Rencher & Christensen 2012). Then, the stepwise procedure identifies and selects the most statistically significant features among the 132 measured on each seed (Grillo *et al.* 2012). Finally, a cross-validation procedure was applied to verify the performance of the identification system, testing individual unknown cases and classifying them on the basis of all others (SPSS, 2007).

All the raw data were standardized before starting any statistical elaboration. Moreover, in order to evaluate the quality of the discriminant functions achieved for each statistical comparison, the *Wilks' Lambda*, the percentage of explained variance and the canonical correlation between the discriminant functions and the group membership, were computed. The *Box's M* test was executed to assess the homogeneity of covariance matrices of the features chosen by the stepwise LDA while the analysis of the standardized residuals was performed to verify the homoscedasticity of the variance of the dependent variables used to discriminate among members of groups (Haberman 1973; Morrison 2004). *Kolmogorov-Smirnov's* test was performed to compare the empirical distribution of the discriminant functions with the relative cumulative distribution function of the reference probability distribution, while the *Levene's* test was executed to assess the equality of variances for the used discriminant functions calculated for members of groups (Gastwirth *et al.* 2009; Lopes 2011). To graphically highlight the differences among groups, multidimensional plots were drawn using the first three discriminant functions.

Supplementary material 1. Haralick's descriptors measured as reported in Haralick *et al.* (1973).

Code	Feature	Equation
Har 1	Angular second moment	$\sum_i \sum_j p(i,j)^2$
Har 2	Contrast	$\sum_{n=0}^{N_g-1} n^2 \left\{ \sum_{i=1}^{N_g} \sum_{j=1}^{N_g} p(i,j) \right\}, \lvert i,j \rvert = n$
Har 3	Correlation	$\frac{\sum_i \sum_j (ij) p(i,j) - \mu_x \mu_y}{\sigma_x \sigma_y}$ where μ_x, μ_y, σ_x and σ_y are the means and the standard deviations of p_x and p_y.
Har 4	Sum of square: variance	$\sum_i \sum_j (i-\mu)^2 p(i,j)$
Har 5	Inverse difference moment	$\sum_i \sum_j \frac{1}{1+(i-j)^2} p(i,j)$
Har 6	Sum average	$\sum_{n=2}^{2N_g} i p_{x+y}(i)$ where x and y are the coordinates (row and column) of an entry in the co-occurrence matrix, and $p_{x+y}(i)$ is the probability of co-occurrence matrix coordinates summing to $x+y$.
Har 7	Sum variance	$\sum_{i=2}^{2N_g} (i-f_8)^2 p_{x+y}(i)$
Har 8	Sum entropy	$-\sum_{i=2}^{2N_g} p_{x+y}(i) \, log\{p_{x+y}(i)\} = f_8$
Har 9	Entropy	$-\sum_i \sum_j p(i,j) \, log[p(i,j)]$
Har 10	Difference variance	$\sum_{n=0}^{N_g-1} i^2 p_{x-y}(i)$

Har 11 Difference entropy $$-\sum_{n=0}^{N_{g-1}} p_{x-y}(i)\ log\{p_{x-y}(i)\}$$

The basis for these features is the gray-level co-occurrence matrix (*G* in equation 1). This matrix is square with dimension *Ng*, where *Ng* is the number of gray levels in the image. Element [*i,j*] of the matrix is generated by counting the number of times a pixel *(p)* with value *i* is adjacent to a pixel with value *j* and then dividing the entire matrix by the total number of such comparisons made. Each entry is therefore considered to be the probability that a pixel with value *i* will be found adjacent to a pixel of value *j*.

$$G = \begin{bmatrix} p(1,1) & p(1,2) & \cdots & p(1,N_g) \\ p(2,1) & p(2,2) & \cdots & p(2,N_g) \\ \vdots & \vdots & \ddots & \vdots \\ p(N_g,1) & p(N_g,2) & \cdots & p(N_g,N_g) \end{bmatrix} \quad (1)$$

Supplementary material 2 - List of morpho-colorimetric features measured on seeds, excluding the 78 Elliptic Fourier Descriptors (FD) calculated according to Hâruta (2011).

	Feature	**Description**
$\boldsymbol{A}$	Area	Seed area (mm^2)
$\boldsymbol{P}$	Perimeter	Seed perimeter (mm)
$\boldsymbol{P_{conv}}$	Convex Perimeter	Convex perimeter of the seed (mm)
$\boldsymbol{P_{Crof}}$	Crofton's Perimeter	Perimeter of the seed calculated using the Crofton's formula (mm)
$\boldsymbol{P_{conv}/P_{Crof}}$	Perimeter ratio	Ratio between convex and Crofton's perimeters
$\boldsymbol{D_{max}}$	Max diameter	Maximum diameter of the seed (mm)
$\boldsymbol{D_{min}}$	Min diameter	Minimum diameter of the seed (mm)
$\boldsymbol{D_{min}/D_{max}}$	Feret ratio	Ratio between minimum and maximum diameters
$\boldsymbol{Sf}$	Shape Factor	Seed shape descriptor = (4 x π x area)/$perimeter^2$ (normalized value)
$\boldsymbol{Rf}$	Roundness Factor	Seed roundness descriptor = (4 x area)/(π x max $diameter^2$) (normalized value)
$\boldsymbol{Ecd}$	Eq. circular diameter	Diameter of a circle with an area equivalent to that of the seed (mm)
$\boldsymbol{EA_{max}}$	Maximum ellipse axis	Maximum axis of an ellipse with equivalent area (mm)
$\boldsymbol{EA_{min}}$	Minimum ellipse axis	Minimum axis of an ellipse with equivalent area (mm)
$\boldsymbol{R_{mean}}$	Mean red channel	Red channel mean value of seed pixels (grey levels)
$\boldsymbol{R_{sd}}$	Red std. deviation	Red channel standard deviation of seed pixels
$\boldsymbol{G_{mean}}$	Mean green channel	Green channel mean value of seed pixels (grey levels)
$\boldsymbol{G_{sd}}$	Green std. deviation	Green channel standard deviation of seed pixels
$\boldsymbol{B_{mean}}$	Mean blue channel	Blue channel mean value of seed pixels (grey levels)
$\boldsymbol{B_{sd}}$	Blue std. deviation	Blue channel standard deviation of seed pixels
$\boldsymbol{H_{mean}}$	Mean hue channel	Hue channel mean value of seed pixels (grey levels)
$\boldsymbol{H_{sd}}$	Hue std. deviation	Hue channel standard deviation of seed pixels
$\boldsymbol{L_{mean}}$	Mean lightness channel	Lightness channel mean value of seed pixels (grey levels)
$\boldsymbol{L_{sd}}$	Lightness std. deviation	Lightness channel standard deviation of seed pixels
$\boldsymbol{S_{mean}}$	Mean saturation channel	Saturation channel mean value of seed pixels (grey levels)
$\boldsymbol{S_{sd}}$	Saturation std. deviation	Saturation channel standard deviation of seed pixels
$\boldsymbol{D_{mean}}$	Mean density	Density channel mean value of seed pixels (grey levels)
$\boldsymbol{D_{sd}}$	Density std. deviation	Density channel standard deviation of seed pixels
$\boldsymbol{S}$	Skewness	Asymmetry degree of intensity values distribution (grey levels)
$\boldsymbol{K}$	Kurtosis	Peakness degree of intensity values distribution (densitometric units)
$\boldsymbol{H}$	Energy	Measure of the increasing intensity power (densitometric units)
$\boldsymbol{E}$	Entropy	Dispersion power (bit)
$\boldsymbol{D_{sum}}$	Density sum	Sum of density values of the seed pixels (grey levels)
$\boldsymbol{SqD_{sum}}$	Square density sum	Sum of the squares of density values (grey levels)

RESULTS

Data obtained by measuring 132 morpho-colorimetric quantitative variables describing seed size, shape and color, were analysed by stepwise LDA, and statistical classifiers were developed in order to distinguish the studied *taxa*.

The five tyrrhenian species of the *S. mollissima* aggregate were well identified and classified, with percentage of correct identification ranged between 77.8% (*S. ichnusae*) and 99.0% (*S. hicesiae*) (Tab. 2). Figure 3A report a graphical distribution of the taxonomical groups, on the basis of the three available discriminant functions. In order to validate the comparison among the studied *taxa*, *S. colorata* was included in this analysis as out-group, resulting highly distinguished from the other species. The histogram of the standardized residuals (Fig. 3B), the normal probability plot (Fig. 3C) and the dispersion plot of the standardized residuals (Fig. 3D) were also included to better understand the normal distribution of the data.

An additional analysis was implemented among the *S. badaroi* populations, in order to assess the inter-population variability. An overall percentage of correct identification of 80.6% was reached, with performances included between 93.3% (Hyères, Provence) and 60.2% (Elba, Toscana). The highest misattributions were recorded among the populations of Elba (Toscana), Capraia (Toscana) and Capo Noli (Liguria) (Tab. 3).

Analyzing the relationship among *S. velutina* populations, not particularly high percentages were reached for each population, although an overall performance higher than 70% was recorded (Tab. 4). Moreover, in order to compare the *S. velutina* populations with the only one population of *S. ichnusae*, the seed-lot from Capo Falcone (Stintino, Corse) was added to the previous comparative analysis (Tab. 5). The seed-lots of *S. ichnusae* reached a correct identification percentage of 60.6%, and were mainly misattributed to the population from Riu di Li Saldi (Aglientu, Sardinia) and Casetta Bianca (Porto Vecchio, Corsica) in 12.1% and 18.2% of the cases, respectively (Tab. 5).

For each of these statistical comparisons, the best five discriminant variables chosen by the stepwise method are shown in Tab. 6. In the comparison among the six studied species, the most discriminant variables were related to the seed shape, while for discrimination of S. badaroi populationscolor and texture descriptors resulted to be the most relevant. While, in the evaluation of the parameters that most influenced the discrimination process of *S. velutina* distinguished by population as well as in the comparison between *S. ichnusae* and *S. velutina* distinguished by population, the most important variables were strongly related to the seed color and textural information, resulting particularly powerful in the discrimination process (Tab. 6).

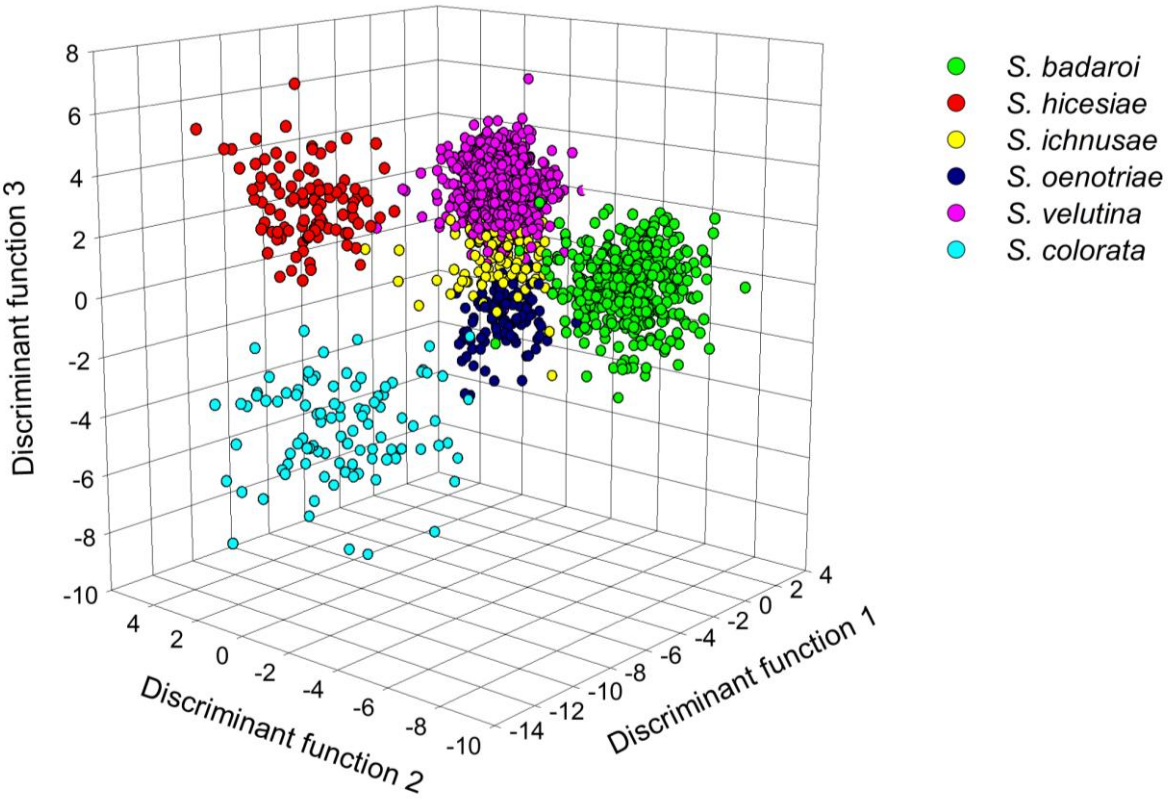

Figure 3a - Distribution of the taxonomical groups on the basis of three available discriminant functions.

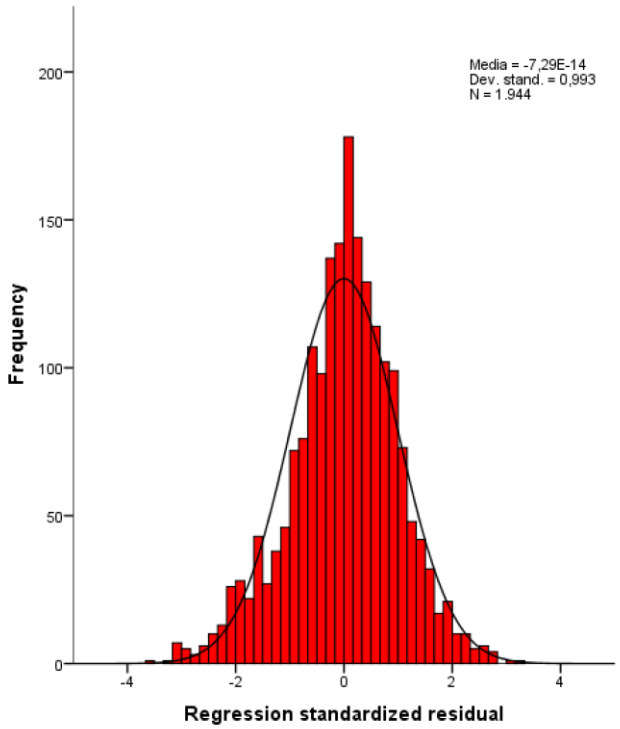

Figure 3b - Histogram of the standardized residuals.

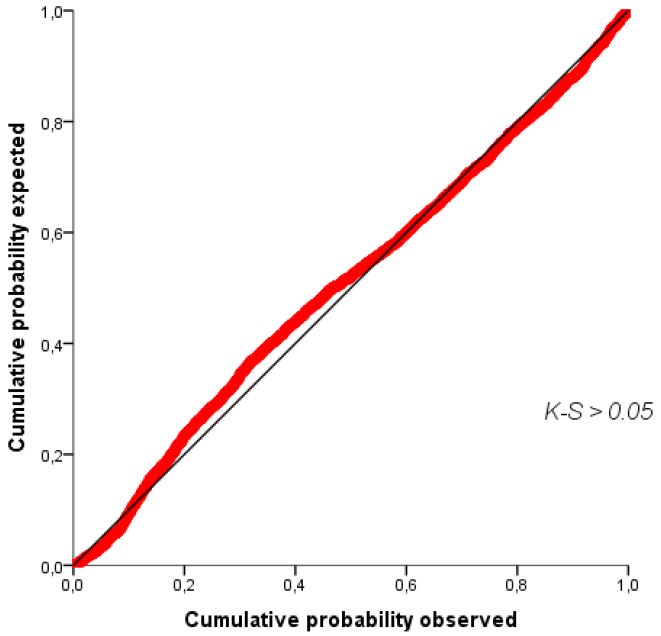

Figure 3c - The normal probability plot.

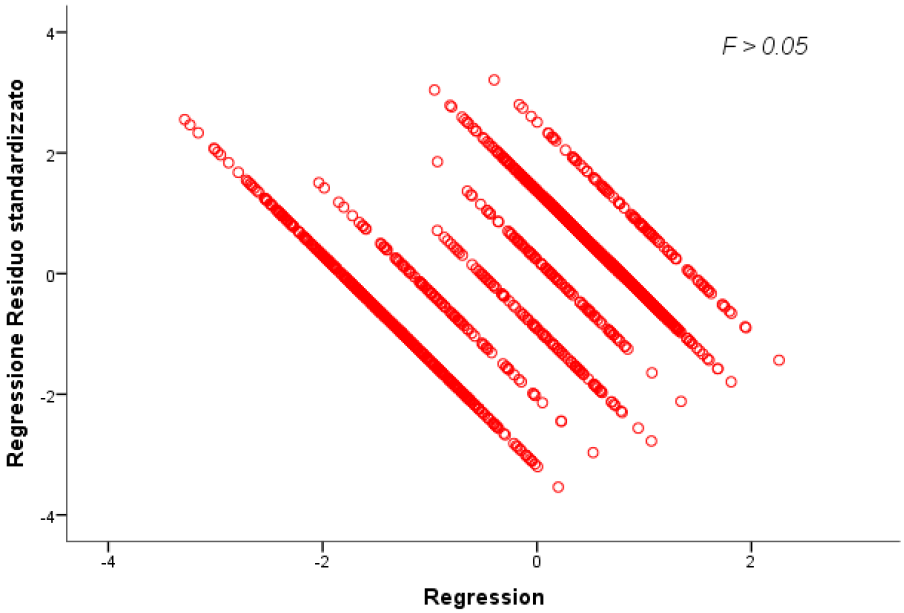

Figure 3d - The dispersion plot of the standardized residuals.

Table 2 - Comparison among study *taxa*. Percentage (in bold) and number of seeds (in brackets) correctly identificate.

	S. badaroi	*S. hicesiae*	*S. ichnusae*	*S. oenotriae*	*S. velutina*	*S. colorata*	Total
S. badaroi	**89.8 (412)**	-	5.7 (26)	3.3 (15)	1.3 (6)	-	100.0 (459)
S. hicesiae	-	**99.0 (99)**	-	-	-	1.0 (1)	100.0 (100)
S. ichnusae	2.0 (2)	-	**77.8 (77)**	-	20.2 (2)	-	100.0 (99)
S. oenotriae	1.0 (1)	-	1.0 (1)	**90.0 (90)**	8.0 (8)	-	100.0 (100)
S. velutina	0.7 (8)	-	8.9 (97)	8.3 (90)	**82.0 (891)**	0.1 (1)	100.0 (1087)
S. colorata	1.0 (1)	1.0 (1)	2.0 (2)	1.0 (1)	-	**94.9 (94)**	100.0 (99)
Overall							**85.5 (1944)**

Table 3 - Comparison among *S. badaroi* populations. Percentage (in bold) and number of seeds (in brackets) correctly identificate.

	Capo Noli (Liguria)	Hyères (Provence)	Is. Capraia (Tuscany)	Is. Elba (Tuscany)	Total
Capo Noli (Liguria)	**72.4% (71)**	2.0% (2)	8.2% (8)	19.4% (19)	100.0 (98)
Hyères (Provence)	1.0% (1)	**96.6% (100)**	1.0% (1)	1.9% (2)	100.0 (104)
Is. Capraia (Tuscany)	6.8% (4)	3.4% (2)	**76.3% (45)**	13.6% (8)	100.0 (59)
Is. Elba (Tuscany)	15.3% (15)	6.1% (6)	9.2% (9)	**69.4% (68)**	100.0 (98)
Overall					**79.1 (359)**

Table 4 - Comparison among *S. velutina* populations. Percentage (in bold) and number of seeds (in brackets) correctly identificate.

	Abbatoggia	Is. Baccà	Is. Stramanaro	Is. Colombo	Riu di Li Saldi	Casetta Bianca	Is. Tamaricciu	Tamaricciu	Capu di Fenu	Total
Abbatoggia (La Maddalena Arch., Sardinia)	**68.8% (137)**	14.6% (29)	2.0% (4)	-	5.0% (10)	3.5% (7)	1.5% (3)	0.5% (1)	4.0% (8)	100.0 (199)
Is. Baccà (La Maddalena Arch., Sardinia)	9.0% (9)	**71.0% (71)**	-	2.0% (2)	8.0% (8)	6.0% (6)	4.0% (4)	-	-	100.0 (100)
Stramanari (La Maddalena Arch., Sardinia)	0.5% (1)	-	**64.2% (122)**	2.6% (5)	0.5% (1)	-	13.2% (25)	11.6% (22)	7.4% (14)	100.0 (190)
Is. Colombo (La Maddalena Arch., Sardinia)	1.0% (1)	-	4.0% (4)	**78.0% (78)**	-	-	9.0% (9)	8.0% (8)	-	100.0 (100)
Riu di Li Saldi (Aglientu, Sardinia)	2.0% (2)	-	-	1.0% (1)	**83.0% (83)**	12.0% (12)	2.0% (2)	-	-	100.0 (100)
Casetta Bianca (Porto Vecchio, Corse)	6.1% (6)	6.1% (6)	1.0% (1)	1.0% (1)	6.1% (6)	**76.8% (76)**	1.0% (1)	1.0% (1)	1.0% (1)	100.0 (99)
Is. Tamaricciu (Porto Vecchio, Corse)	-	-	11.0% (11)	12.0% (12)	1.0% (1)	-	**59.0% (59)**	13.0% (13)	4.0% (4)	100.0 (100)
Tamaricciu (Porto Vecchio, Corse)	-	-	5.1% (5)	9.1% (9)	-	-	15.2% (15)	**61.6% (61)**	9.1% (9)	100.0 (99)
Capu di Fenu (Ajaccio, Corse)	2.0% (2)	-	3.0% (3)	4.0% (4)	1.0% (1)	1.0% (1)	6.0% (6)	6.0% (6)	**77.0 % (77)**	100.0 (100)
Overall										**70.3 (1087)**

Table 5- Comparison among all the *S. velutina* populations and the only *S. ichnusae* population. Percentage (in bold) and number of seeds (in brackets) correctly identificate.

	Capo Falcone	Abbatoggia	Is. Baccà	Is. Stramanaro	Is. Colombo	Riu di Li Saldi	Casetta Bianca	Is. Tamaricciu	Tamaricciu	Capu di Fenu	Total
Capo Falcone (Stintino, Corse)	**60.6% (60)**	4.0 (4)	1.0% (1)	1.0% (1)	-	12.1% (12)	18.2% (18)	3.0% (3)	-	-	100.0% (99)
Abbatoggia (Arch. della Maddalena, Sardinia)	**1.0% (2)**	**67.3% (134)**	14.6% (29)	2.5% (5)	-	4.5% (9)	5.5% (11)	2.0% (1)	0.5% (1)	3.0% (6)	100.0% (199)
Is. Baccà (Arch. della Maddalena, Sardinia)	-	12.0% (12)	**68.0% (68)**	-	2.0% (2)	7.0% (7)	7.0% (7)	4.0% (4)	-	-	100.0% (100)
Is. Stramanaro (Arch. della Maddalena, Sardinia)	1.1% (2)	-	-	**59.5% (113)**	2.6% (5)	-	0.5% (1)	14.2% (27)	14.7% (28)	7.4% (14)	100.0% (190)
Is. Colombo (Arch. della Maddalena, Sardinia)	-	-	-	5.0% (5)	**77.0% (77)**	-	1.0% (1)	7.0% (7)	8.0% (9)	1.0% (1)	100.0% (100)
Riu di Li Saldi (Aglientu, Sardinia)	3.0% (3)	2.0% (2)	1.0% (1)	-	-	**82.0% (82)**	10.0% (10)	2.0% (2)	-	-	100.0% (100)
Casetta Bianca (Porto Vecchio, Corse)	9.1% (9)	4.0% (4)	8.1% (8)	1.0% (1)	-	5.1% (5)	70.7% (70)	-	1.0% (1)	1.0% (1)	100.0% (99)
Is. Tamaricciu (Porto Vecchio, Corse)	-	-	-	11.0% (11)	14.0% (14)	2.0% (2)	1.0% (1)	49.0% (49)	15.0% (15)	8.0% (8)	100.0% (100)
Tamaricciu (Porto Vecchio, Corse)	-	-	-	8.1% (8)	10.1% (10)	-	-	13.2% (13)	57.6% (57)	11.1% (11)	100.0% (99)
Capu di Fenu (Ajaccio, Corse)	1.0% (1)	1.0% (1)	-	3.0% (3)	4.0% (4)	1.0% (1)	2.0% (2)	5.0% (5)	5.0% (5)	78.0 % (78)	100.0% (100)
Overall											**66.4% (1087)**

Table 6. Number of groups, discriminant steps and performance of identification. Ranking of the best five discriminant parameters and the percentage of correct classification are reported for each of the implemented statistical comparisons (C1= comparison among species; C2= comparison among *S. badaroi taxa* distinguished by populations; C3= comparison among the *S. velutina* distinguished by population; C4= comparison between *S. ichnusae* and *S. velutina* distinguished by populations).

For each parameter, the tolerance, F-to-remove and Wilks' lambda values are reported in brackets.

	C1	**C2**	**C3**	**C4**
N of groups	6	4	9	10
N of steps	50	39	33	34
1st discriminant parameter	*FD37* (0.96; 0.018; 0.008)	*FD61* (0.95; 0.049; 0.087)	D_{mean} (0.01; 24.112; 0.019)	D_{mean} (0.01; 23.887; 0.017)
2nd discriminant parameter	*FD67* (0.97; 0.014; 0.008)	*S* (0.10; 0.030; 0.087)	Har_6 (0.01; 21.950; 0.000)	Har_6 (0.01; 23.585; 0.000)
3rd discriminant parameter	*FD71* (0.96; 0.013; 0.007)	G_{sd} (0.05; 0.006; 0.088)	*E* (0.2; 20.932; 0.000)	*E* (0.02; 18.942; 0.000)
4th discriminant parameter	*FD50* (0.97; 0.012; 0.008)	*FD1* (0.93; 0.005; 0.088)	Har_{11} (0.1; 20.295; 0.000)	Har_{11} (0.01; 18.253; 0.000)
5th discriminant parameter	D_{sd} (0.01; 0.005; 0.008)	L_{mean} (0.01; 0.004; 0.088)	G_{mean} (0.01; 17.591; 0.000)	G_{mean} (0.01; 18.037; 0.000)
Percentage of correct identification between groups	85.5%	79.1%	70.3%	66.4%

\\

DISCUSSION

This study represents a complementary approach to investigate the taxonomic relationship and quantify the inter-specific and inter-population differentiation in the Tyrrhenian species of the *S. mollissima* aggregate. The discrimination process allowed to reach good percentages of correct identification, at the species level, according to the results obtained by Bacchetta *et al.* (2008), reporting a percentage of 84.3% of correct discrimination among different Caryophyllaceae species. The most discriminating parameters were descriptive of the seed shape, although the low F-to-remove values (from 0.005 to 0.018) showed that none of the measured parameters were particularly peculiar. Indeed, as also demonstrated by Camelia (2011) and Dadandi & Yldiz (2015), parameters related to seed shape are particularly important in discrimination of same *Silene* species. In agreement with the current taxonomic treatment *S. colorata* (*Dipterospermae* section), selected as out-group, was highly distinguishable (94.9%) from all other species, confirming its clear differentiation compared to the species of *Siphonomorpha* section. For species of *S. mollissima* aggregate, the results of the LDA showed a closer affinity among *taxa* from the central Tyrrhenian area (Fig.1A). A few misattributions among the seeds of *S. velutina, S. ichnusae* and *S. oenotriae*, suggested that *S. velutina*, with a wider distribution area than the two *taxa*, was probably the most ancestral species within this sub-group. The other two species of the group, *S. badaroi* and *S. hicesiae*, were shown to be well differentiated (89.8% and 99%, respectively). As regards *S. badaroi*, our results are in accordance with those obtained from the comparative analysis of the morphological characters of the aggregate (Jeanmonod 1984b), supporting the hypothesis of an ancient differentiation of this species. On the other hand, the results reached for *S. hicesiae* seem to be in contrast with those of Jeanmonod (1984b), showing a close affinity of this *taxa* with *S. velutina*, probably due to its recent differentiation in a volcanic island of quaternary origin. From the present study, in fact, no common characters between *S. hicesiae* and the other species of the group were found, but rather a certain independence of this *taxa* was observed. Further details are needed to better understand the systematic position of *S. hicesiae* in the whole aggregate.

The comparison among the four populations of *S. badaroi* yelded high performance of correct identification (80.6%). Differently from previous comparison among species, color and texture descriptors were shown to be relevant, although none of the most important features (FD61, S, Gsd, FD1, Lmean) had high F-to-remove values. The Elba population showed the greater similarities with the others accessions and in particular with the Capraia population (Tab. 3). In addition, for both populations of the Tuscan Archipelago, a high percentage of seeds (10.2; 22.4%, respectively) were misidentified as Capo Noli, a population located in a central position within the distribution area of the species. In contrast, the most differentiated population was Hyères which showed only the 1.9% of seeds misclassified within Capo Noli population. Therefore, the Tuscan populations, closer to one another, resulted to be the similar, while the Hyeres population showed a tendency to segregate. The tool used was effective in discriminating the different populations providing results that were consistent with the geographical distribution and the relevant distances among the *S. badaroi* populations. In fact, it is known that the maintenance of population differentiation depends on the balance between genetic drift and natural selection, whereas a reduced population differentiation depends on the presence of gene flow which homogenizes variation (Quilichini *et al.* 2004; Grillo *et al.* 2013).

As regards the inter-population variability of *S. velutina*, a high percentage of correct attribution was observed (70.3) and the most important characters were strongly related to the color and texture of seeds (D_{mean}, Har6, E, Har11, G_{mean}), resulting particularly

powerful in the discrimination process (Tab. 6). The most differentiated populations were Capo di Fenu, the only one of western Corsica, and Riu di li Saldi, the westernmost population in Sardinia (Tab. 4). These results were in agreement with different authors (Debussche & Thompson 2002; Thompson *et al.* 2005; Quilichini *et al.* 2004) that found high levels of differentiation both in peripheral populations and in geographically isolated populations of species with disjoint distributions.

To better understand the relationship between *S. velutina* and *S. ichnusae*, considering the morphological similarity and the geographical proximity between the two *taxa*, the comparison among all accessions of both species was carried out, achieving a lower identification performance than that obtained by the analysis carried out exclusively on *S. velutina* (Tab. 5). With a percentage of correct identification of 60.6%, the narrow endemic *S. ichnusae* showed closed affinities both with Corse (e.g. 18.2% of seeds wrongly classified as Casetta Bianca) and Sardinian populations (eg. 12.1% of seeds misattributed to Riu di li Saldi, the westernmost population in Sardinia) of *S. velutina*. Color and texture descriptors were shown to be the most useful parameters to discriminate among the populations (Tab. 6). Additional studies will be needed to investigate the differentiation degree between the two *taxa*.

CONCLUSION

The applied methodology was useful to provide key information on the differentiation and evolution of the studied endemic *taxa* and their populations, and also highlighted critical issues at the taxonomic level. Particularly, we confirmed the current taxonomic treatment both at section and species level, although further investigations, regarding the taxonomic position of *S. hicesiae* into the whole aggregate and the differentiation of *S. ichnusae* from *S. velutina*, would be needed. Furthermore, at population level we were able to highlight the presence of both close connections and high differentiation rates, as demonstrated both for *S.badaroi* and *S. velutina*. In fact, seeds of populations located at the edge of the distribution range of species were more differentiated among them than seeds of populations located at the core areas. In agreement with several authors (Hamrick *et al.* 1991; Holsinger & Gottlieb 1991; Ellstrand & Elam 1993; Wise 1997; Thompson 1999), we suggest that a better knowledge of population and species differentiation could also be useful for rare and/or endangered species, such as those of the studied group, as it could provide valuable information for their management and conservation.

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CHAPTER II

Seed germination, salt stress tolerance and effect of nitrate in three Tyrrhenian coastal species of the *Silene mollissima* aggregate (Caryophyllaceae)

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ABSTRACT

Silene mollissima aggregate is part of the section *Siphonomorpha* Otth. and currently comprises 11 narrow endemic species of the Western Mediterranean Basin. Three of these *taxa* have a distribution range centred in the Northern Tyrrhenian area; these are *Silene velutina* Pourr. ex Loisel., *Silene ichnusae* Brullo, De Marco & De Marco f. and *Silene badaroi* Bestr., all occurring in coastal habitats. Inter- and intra-specific variability in the responses to light, constant (5-25°C) and alternating temperatures (25/10°C), NaCl (0-600 mM), KNO_3 (20 mM) under salinity stress and recovery of seed germination were evaluated for these species to more effectively support their *in situ* and *ex situ* conservation. Our results highlighted that the seeds of these three *taxa* were non-dormant, and light significantly improved their germination, which showed high percentages (> 80%) at low temperatures (5-15°C) and under the alternating temperature regime (25/10°C), decreasing significantly at the highest temperature (25°C). *Silene velutina* and *S. ichnusae* seeds germinated in up to 300 mM NaCl, and *S. badaroi* germinated until 100 mM. For all species except *S. badaroi*, salt did not affect seed viability and recovery did not decrease with increasing salinity and temperature, except for *S. badaroi*. Inter-population variability both in salt tolerance and recovery was detected for *S. velutina*. The addition of KNO_3 did not affect germination or recovery under salt conditions. The lack of effect of KNO_3 suggests that nutrient availability is not a requirement for seed germination in these species. Our results show that all species experience optimum period of germination during autumn-winter, when water availability is highest and soil salinity levels are minimal due to the Mediterranean rainfalls, but *S. velutina* and *S. ichnusae* are also able to germinate until spring.

Keywords: endemism, germination ecology, Mediterranean, NaCl, potassium nitrate

INTRODUCTION

The Mediterranean vascular flora is characterised by the presence of high species richness and high rates of endemism and shows elevated frequencies of disjoined distributions of related species (Thompson 2005). These distributions frequently consist of narrow endemics (often located on islands) or a widespread species with some restricted endemics outside of its distribution area (Küpfer 1974; Verlaque *et al.* 1991; Cañadas *et al.* 2014). Many such *taxa* are identified as "schizo-endemics" with distribution patterns mainly ascribed to allopatric speciation (Gielly *et al.* 2001). In the Western Mediterranean, the *Silene mollissima* aggregate may be considered an effective example of this phenomenon (Boquet *et al.* 1978; Jeanmonod 1984).

This aggregate is included in the section *Siphonomorpha* Otth. and currently comprises 11 endemic species of the Western Mediterranean Basin. Five of these species (*Silene badaroi* Bestr., *Silene ichnusae* Brullo, De Marco & De Marco f., *Silene hicesiae* Brullo & Signorello, *Silene oenotriae* Brullo and *Silene velutina* Pourr. ex Loisel.) have a distribution centred in the Tyrrhenian area. For the purposes of this study, only the species with North Tyrrhenian distribution (*S. velutina*, *S. ichnusae*, *S. badaroi*) were considered. Among these species, *S. velutina* is the only one under protection, although it is present in a greater number of stations with respect to the other species.

Seed germination is one of the most important and complex stages of a plant's life cycle, and a detailed understanding of how environmental factors influence this process is important to ensure the conservation of rare and/or endangered species (Baskin and Baskin 1998). Several studies highlight the presence of inter- and intra-specific variations in seed germination and dormancy (e.g. Andersson and Milberg 1998; Keller and Kollman 1999; Santo *et al.* 2015*a*, 2015*b*), attributing this phenomenon to environmental differences, genetic variations or both (Degreef *et al.* 2002; Cruz *et al.* 2003). In phylogenetically related species, such as congeneric species, it is well known that several environmental factors (e.g. light, temperature, moisture, and soil composition) have a direct impact on germination behaviour (Ellison 2001), which reflects their ecological adaptations and therefore may explain their distribution and rarity (Ramírez-Padilla and Valverde 2005). This aspect is even more significant for plants growing in coastal habitats, where harsh environmental conditions occur (e.g. high insolation, temperature and soil moisture fluctuations, strong winds, high salt concentrations, low nutrient availability) (Maun 2009; Thanos *et al.* 1991). In these complex ecosystems several species adopt the strategy of seed dormancy, waiting for favourable conditions to germinate (Baskin and Baskin 1998).

Although light and temperature are the main factors that influence seed germination, salinity is also considered as one of the selective forces (Baskin and Baskin 1998). Salt stress can cause changes in the mechanisms producing the balance of germination regulators, thereby inducing a physiological secondary dormancy (Ungar 1978; 1984). In particular, salt may inhibit seed germination either by creating a low osmotic potential, which prevents water uptake, or through the toxic effects of Na^+ and Cl^- ions on the metabolic processes (Khajeh-Hosseini *et al.* 2003; Kaya *et al.* 2006). In the literature, nitrogenous compounds, and more specifically potassium nitrate (KNO_3), are reported to alleviate salt stress and promote germination under saline conditions (Khan 2003; Zehra *et al.* 2013). Seeds unable to germinate at high salinity levels might survive during salt exposure and maintain the ability to germinate later (recovery), when salinity decreases due to various environmental events, such as autumnal rainfall (Baskin and Baskin 1998). Seeds of several species treated with high salt concentrations recovered their germination following transfer to distilled water. The ability of seeds to recover is species-specific

(Song *et al.* 2005), and variations in seed recovery percentages were often attributed to differences in the temperature regimes to which they were exposed (Pujol *et al.* 2000).

Several studies on the seed germination ecology of *Silene* L. species were conducted with the aim to investigate their responses to light, temperature, and the effect of seed scarification (Thompson 1970, 1975; Flocca *et al.* 2004; Menges 2005; Camelia 2011). However, there are few studies on the effect of NaCl and nitrates (Woodell 1985). Despite this, these compounds could play an important role on the germination of three species highlighted in the present study, which are exposed to a salt gradient in their habitats, due to their proximity to the sea and to nitrates in the soil, originating both from the decomposition of *Posidonia oceanica* (L.) Delile leaves and the guano produced by seabirds (seagulls, shearwaters, etc.). In particular, the presence of seabird colonies and their excrement was considered as a threat for *S. velutina* (Paradis *et al.* 2001), the only species, among the three considered in this work, that has already been the object of other studies (Bacchetta 2001; Paradis 2006). Nevertheless, in the literature, the relationship between nitrates and salinity is poorly understood and varies among species. Moreover, no published works exist about the ecology and biology of the studied species, or the other species of the aggregate.

Therefore, the present study was conducted with the aim to investigate and compare: 1) seed germination requirements in terms of light and temperature, 2) the effect of NaCl and KNO_3 on seed germination and its recovery at different temperatures and 3) intra- and inter-specific variability in seed germination under the different treatments, for the three different species (*S. velutina*, *S. ichnusae* and *S. badaroi*). The responses to these factors could be useful to better understand the distribution and rarity of these schizo-endemic species and to plan appropriate *in situ* and *ex situ* conservation actions.

MATERIALS AND METHODS

Study species

The three investigated species belonging to the *S. mollissima* aggregate are perennial chamaephytes characterised by dense rosettes, flowering from April to June and fruiting from May to July. Moreover, *Silene* species produce orthodox seeds with a peripheral embryo (Martin 1946; Royal Botanic Gardens Kew 2015). All three species occur in coastal habitats of the Northern Tyrrhenian area. In particular, *S. badaroi* has a narrow and scattered range. It is endemic to sandy and rocky areas of the Provence region (France), Liguria and the Tuscan Archipelago (Italy), therefore having a disjunct distribution. *Silene ichnusae* is a narrow endemic occurring only on rocky and glareicolous habitats in North Western Sardinia (Italy). *Silene velutina* is a Sardinian-Corsican endemic species of rocky and sandy habitats in the Northern Sardinia (Italy), Central-Southern Corse (France) and some surrounding islets. *Silene velutina* is listed in the Habitats Directive 92/43/EEC as a priority species, in the Bern Convention and it is included in the IUCN International Red Lists as near threatened (NT) (Buord *et al*. 2011), in the French and Italian Red Lists, respectively as vulnerable (VU) and endangered (EN) (Olivier *et al.* 1995, Conti *et al.* 1997, Pisanu *et al.* 2014, Rossi *et al.* 2015), and in the Corsican Red List as near threatened (NT) (Delage & Hugot 2015).

Seed lot details

Capsules of each species were collected from 30 individuals randomly selected in their natural populations at the time of natural dispersal (Tab. 1). Seed collections of *S. velutina* in Sardinia were carried out after obtaining permits from the "Ministero dell'Ambiente e della Tutela del Territorio e del Mare (MATTM)", as required by the European and Italian laws for the species listed in the appendices of Habitat Directive 92/43/EEC, while seeds from Corsica were collected and provided after obtaining permits from the Direction Régionale de l'Environnement, de l'Aménagement et du Logement (DREAL). Seeds were separated from the fruits, selected by hand and stored under controlled conditions (20°C and 50% of relative humidity) for two weeks before starting germination tests. The mean seed mass (± 1 standard deviation, hereafter SD) was calculated by weighing 10 replicates of 20 seeds each (Tab. 1).

Table 1 - Population data and seed lot details. In the last column the different experiments carried out for each seed lot are reported (L = Light; T = Temperature; NaCl = Salinity; KNO_3= Nitrate under salinity).

Species	Population code	Coordinates (UTM, WGS84)	Substrate	Distance from sea (m)	Date of collecting	Mean seed mass (mg ± SD)	Experimental trials
S. velutina	Svel1	41°14' N 09°24' E	Aeolian sands	10	09/08/2013	0.98 ± 0.06	L; T
S. velutina	Svel2	41°35' N 09°18' E	Conglomerates	2	28/07/2013	1.09 ± 0.09	L; T ; NaCl ; KNO_3
S. velutina	Svel3	41°11' N 090°26' E	Granites	2	11/08/2013	1.26 ± 0.09	L; T ; NaCl; KNO_3
S. ichnusae	Sich1	40°57' N 08°11' E	Metamorphytes	10	29/07/2013	0.98 ± 0.08	L; T; NaCl; KNO_3
S. badaroi	Sbad1	44°11' N 08°25' E	Limestones	25	01/07/2013	0.74 ± 0.06	L; T ; NaCl; KNO_3

Germination tests

Germination tests were performed under laboratory conditions at the Sardinian Germplasm Bank (BG-SAR). A preliminary test was carried out for each seed lot in order to evaluate the effect of light on seed germination. Seeds were sown on 1% water agar substrate in plastic Petri dishes of 60 mm diameter and then incubated in growth chambers (SANYO MLR–351, Japan) at 15°C, both in the light (12 h of irradiance per day) and in total darkness. Light in each growth chamber was provided by nine fluorescent lamps with white light (Mitsubishi OSRAM 40; 53 Watt for each), and darkness was achieved by wrapping the dishes in two layers of aluminium foil, immediately after the seeds were sown and before water imbibition. For each condition, four replicates of 25 seeds each were used. The criterion for germination was visible protrusion of the radicle (≥ 2 mm). Seeds incubated in the light were scored daily, and germinated seeds discarded. Seeds incubated in the dark were scored only at the end of the test to avoid any exposure to irradiance (Baskin *et al.* 2006). When no additional germination occurred for two consecutive weeks, tests were stopped, and the viability of each remaining seed was checked by a cut test using a scalpel and subsequent observation of the endosperm under a binocular microscope. In order to evaluate the effect of temperature and inter- and intra-

population variability, germination tests were conducted for each seed lot only in the light. Four replicates of 25 seeds per each treatment were incubated in growth chambers in a range of constant (5, 10, 15, 20, 25°C) and alternating (25/10°C) temperatures. In particular, in this latter temperature regime, the higher temperature coincided with the light period.

To evaluate the effect of salt stress on seed germination, seeds from two *S. velutina* seed lots and from the unique seed lots of both *S. ichnusae* and *S. badaroi* (Table 1) were sown and treated with different NaCl concentrations (0, 100, 200, 300, 400, 500, 600 mM) and incubated in a range of constant temperatures (5, 10, 15 and 20°C) in the light. In order to evaluate the effect of potassium nitrate (KNO_3) on the seed germination of each species, seeds from selected seed lots (Tab. 1) were sown in different NaCl concentrations (0, 100, 200, 300 mM) with the addition of 20 mM of KNO_3 and incubated at the set of constant temperatures of 10, 15 and 20°C.

After two consecutive weeks without additional germination under control conditions (0 mM NaCl), non-germinated seeds were washed with distilled water and then sown in new Petri dishes containing 1% water agar substrate for an additional 30 days period (recovery phase) at the same incubation temperatures. Seeds treated with KNO_3 were compared using the correspondent germination in the same NaCl concentration at the same incubation temperature as the control.

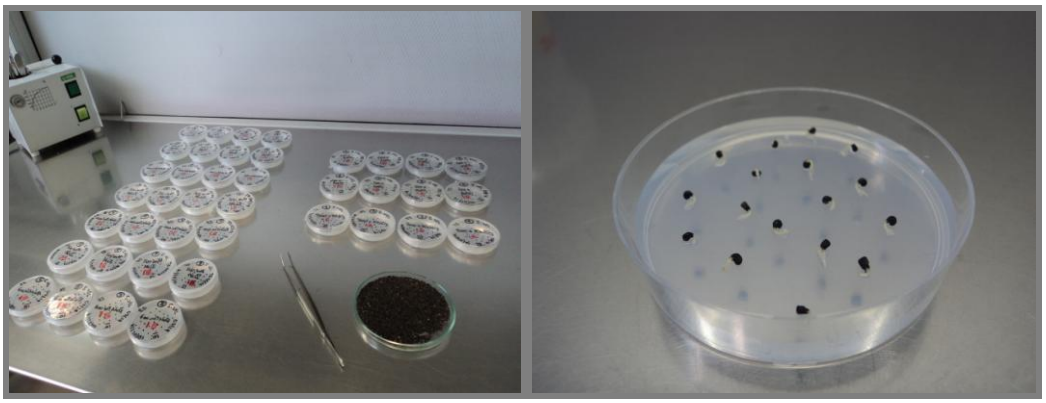

Figure 1 - Germination tests with KNO_3 and germinated seeds.

DATA ANALYSIS

Final germination percentages were calculated as the mean of four replicates (± 1SD), and recovery percentages (hereafter RP) were calculated according to the following equation (1) (Pujol *et al.* 2000):

$$(1)\ RP = \{[(a\text{-}b)/(c\text{-}b)] \times 100\},$$

where *a* is the total number of seeds germinated in salt solutions plus those that recovered and germinated in fresh water, *b* is the total number of seeds germinated in saline solutions and *c* is the total number of seeds. Germination percentages and RP were analysed using the non-parametric Kruskal–Wallis test, followed by a Mann–Whitney *U* test. Graphs were realised using Sigmaplot 11.0 software (Systat Software Inc., London, UK), and all

statistical analyses were carried out using Statistica 7.0 for Windows (Software Statsoft Release 7).

RESULTS

Effect of light on seed germination

The preliminary light experiment was performed for all the seed lots at a temperature of 15°C. *Silene velutina* seed germination in the light ranged from 98.0 ± 2.3% (Svel3) to 99.0 ± 5.2 (Svel2), while in the dark from 0% (Svel2) to 30.0 ± 11.5% (Svel3). *Silene ichnusae* germinated with 99.0 ± 2.3% and 26.0 ± 6.9%, in the light and in the dark, respectively. *Silene badaroi* seeds germinated with percentages of 99.0 ± 9.8 % in the light and 40.0 ± 10.8% in the dark. The Kruskal–Wallis test for all the seed lots (Tab.2) showed that final germination percentages were significantly higher ($p < 0.05$) in the light (ca. 100%) than in the dark (up to ca. 40%). Therefore, all subsequent germination tests were conducted in the light.

Table 2 - Final germination at 15°C in the light (12/12) and total darkness for the five Silene seed lots investigated in this study. Kruskal-Wallis test was conducted for each seed lot to detect the effect of light (L) on seed germination. Mann Whitney U-test was conducted to identify significant differences at $p < 0.05$. Data are the mean of four replicates (± 1 standard deviation). Values with the same letters are not different at $p < 0.05$. See Table 1 for the explanation of the population codes.

Species	Germination (%)	
	Light	Dark
S. velutina (Svel1)	99.0 ± 3.3a	3.0 ± 3.8 b
S. velutina (Svel2)	99.0 ± 5.2a	0 ± 0 b
S. velutina (Svel3)	98.0 ± 2.3a	30.0 ± 11.5 b
S. ichnusae (Sich1)	99.0 ± 2.3a	26.0 ± 6.9 b
S. badaroi (Sbad1)	99.0 ± 9.8a	40.0 ± 10.8 b

Effect of temperature and inter-population variability in seed germination

At 5°C final germination differed among the seed lots and the highest germination percentages (up to ca. 99%) were detected for the three *S. velutina* populations. The lowest germination was detected for Sbad1 and it was significantly different ($p < 0.05$) from all others. At 10 and 15°C, germination was higher than 85% for all the seed lots (Fig. 2). At 20°C the highest germination was detected for Svel2, Svel3 and Sich1 (with percentages of ca. 90%), which was significantly different ($p < 0.05$) from the lowest germination observed for Svel1 and Sbad1 (ca 40%; Fig. 2). At 25°C the highest germination occurred for Svel3 and Sich1 (ca. 20%) while the lowest was detected for Sbad1 (ca. 5%). No germination at this temperature was observed for seed lots Svel1 and Svel2. At the alternating temperature regime of 25/10°C, the highest germination occurred for Svel3 (ca. 95%), and this value was not significantly different ($p > 0.05$) only from Sich1. Seeds from Svel1, Svel2 and Sbad1 germinated with percentages of ca. 80%.

For all the seed lots, final germination at 25°C was significantly lower than at all other temperatures, or in some cases (Svel1 and Svel2) no germination occurred. Significantly lower germination percentages were also detected at 20°C for Svel1 and Sbad1 (ca. 50% and 35%, respectively). High germination (> 80%) occurred at 5, 10, 15 and 25/10°C for all populations.

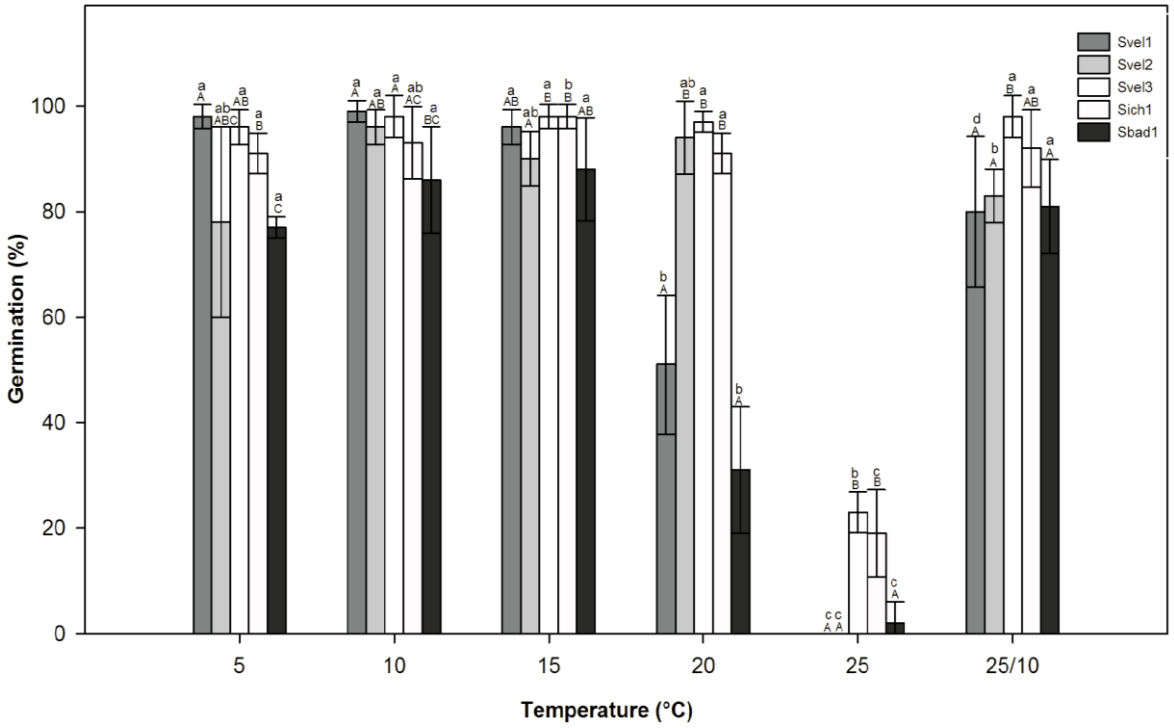

Figure 2 - Germination in the light (12/12 h) at constant (5-25°C) and alternating (25/10°C) temperatures for all seed lots of the species investigated in this study. Kruskal-Wallis tests were conducted to detect differences among populations at the same temperature (capital letters, by Mann Whitney *U*-test) and the effect of different temperatures for each population (lower-case letters, by Mann Whitney *U*-test). Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney *U*-test). Data are the mean of four replicates (± 1SD). See table 1 for the explanation of the population codes.

NaCl and recovery on seed germination

Svel2

The highest germination percentages were observed under control conditions (0 mM NaCl), and significant differences ($p < 0.05$) were detected among the tested temperatures. Seed germination decreased with increasing salinity at all temperatures; however, germination at 100 mM NaCl at 10 and 15°C did not show significant differences ($p > 0.05$) with that detected at 0 mM (ca. 90%). At 100 mM, the lowest germination occurred at 5 and 20°C (ca. 70% for both). At concentrations above 200 mM, germination was completely inhibited at all temperatures. Significant differences ($p < 0.01$) among germination percentages under the different NaCl concentrations were detected at each tested temperature (Tab. 3A).

Independent of the tested temperature, RP did not show significant differences ($p > 0.05$) at 100, 200 and 300 mM NaCl. At 400 mM, RP were higher than 80% at all temperatures and showed the highest values at 10 and 15°C (ca. 93%). At 500 mM, the highest recovery occurred at 15°C (ca. 90%) while the lowest RP at 5 and 20°C (ca. 45% and 70%, respectively) were observed. At 600 mM RP did not differ among the tested temperatures and showed values above 90%. Significant differences ($p < 0.05$) were detected among RP at 5 and 20°C, and RP did not differ at 10 and 15°C ($p > 0.05$).

Svel3

The highest germination percentages (> 70 %) were detected at 0 and 100 mM NaCl (Tab. 3B). Significant differences ($p < 0.01$) were detected under the control among the four tested temperatures. This seed lot germinated at a concentration of 300 mM at 15°C (ca. 6%), and germination at all other temperatures was inhibited at concentrations above 100 mM. No significant differences ($p > 0.05$) were detected at 100, 200 and 300 mM for germination at different temperatures.

RP did not show significant differences ($p > 0.05$) among the tested temperatures, with the exceptions of 200 and 400 mM, at which RP ranged from ca. 90% to ca. 100% ($p < 0.05$). At all temperatures, RP did not show significant differences at different NaCl concentrations.

Table 3 - Germination (G) and recovery percentages (RP) at each temperature regime (5-20°C) and under different saline conditions (0-600 mM NaCl) for *Silene velutina* (A: Svel2 and B: Svel3). Kruskal-Wallis tests were conducted to detect the effect of the same temperature and salinity concentration on germination percentages and RP; [p values were considered not significantly different ($p > 0.05$, ns), significantly ($p < 0.05$, *; $p < 0.01$**), by Kruskal-Wallis test]. Data are the mean of four replicates (± 1SD). Capital letters in columns are related to the same NaCl concentration, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at $p < 0.05$ (by Mann-Whitney *U*-test). See Table 1 for the explanation of the population code.

(A)

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
5	G	96.0 ± 3.3 Aa	72.0 ± 7.3 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	25.1 ± 23.0 a	82.0 ± 10.1 bc	90.0 ± 2.3 bd	81.0 ± 3.8 Acd	44.0 ± 17.0 Aa	91.0 ± 7.6 bcd	*
10	G	91.0 ± 6.0 ABa	91.0 ± 3.8 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	0	82.0 ± 12.0	89.0 ± 5.0 b	92.0 ± 5.7 BC	86.0 ± 2.3 B	95.0 ± 2.0	ns
15	G	83.0 ± 3.8 Ba	89.0 ± 3.8 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 b	**
	RP	-	0	94.0 ± 5.2	91.0 ± 3.8	94.0 ± 4.0 B	91.0 ± 2.0 C	91.0 ± 3.8	ns
20	G	96.0 ± 3.3 Aa	72.0 ± 7.3 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	25.1 ± 23.0 a	97.0 ± 3.8 b	92.0 ± 6.5 bc	83.0 ± 3.8 ACc	67.0 ± 10.5 Ad	91.0 ± 8.2 bc	*
	G	*	**	ns	ns	ns	ns	ns	
	RP	-	ns	ns	ns	*	**	ns	

(B)

Temperature (°C)	Percentage (%)	NaCl concentration (mM)							
		0	100	200	300	400	500	600	
5	G	90.0 ± 2.3 Aa	92.0 ± 10.8 Aa	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	88.9 ± 19.2	90.0 ± 5.2	82.0 ± 9.52	88.0 ± 3.3 A	96.0 ± 4.6	88.0 ± 3.3	ns
10	G	92.0 ± 5.7 Aa	98.0 ± 2.3 Aa	0 Ab	0 Ab	0 Ab	0 Ab	0 b	**
	RP	-	-	100.0 ± 0.0	93.3 ± 7.9	91.0 ± 3.8 A	93.0 ± 2.0	90.0 ±7.7	ns
15	G	70.5 ± 13.8 Ba	96.0 ± 0 Ab	1.0 ± 2.0 Ac	6.0 ± 9.52 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	-	99.0 ± 2.1	77.7 ± 16.5 A	100.0 ± 0 B	89.0 ± 8.2	69.0 ± 21.3	ns
20	G	99.0 ± 2.0 Ca	92.0 ± 5.7 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	87.5 ± 25.5	96.0 ± 5.7	86.0 ± 9.5	87.0 ± 7.6 A	92.0 ± 6.5	69.0 ± 21.3	ns

G	**	ns	ns	ns	ns	ns	ns	
RP	-	ns	*	ns	*	ns	ns	

Sich1

Under control conditions (0 mM NaCl), no significant differences ($p > 0.05$) were observed among the tested temperatures (Tab. 4A). Significant differences ($p < 0.05$) were detected at 100 mM and germination ranged from ca. 80% to ca. 95%. The highest germination occurred at 0 mM and 100 mM but, at 5°C, germination at 100 mM was significantly ($p < 0.05$) lower than at 0 mM. At 15°C, seeds showed their ability to germinate up to 300 mM, while germination at all other temperatures was inhibited at concentrations above 100 mM. RP at all NaCl concentrations showed no significant differences ($p > 0.05$) among the tested temperatures.

Sbad1

Under control conditions (0 mM NaCl) significant differences ($p < 0.01$) were detected among the tested temperatures, and germination ranged from ca. 50% to ca. 95%. At 100 mM, the lowest germination occurred at 5°C (ca. 20%) while the highest occurred at 10 and 15°C (ca. 85%). Germination was inhibited at concentrations above 100 mM at all tested temperatures (Tab. 4B). Significant differences were detected among RP at all NaCl concentrations, except at 200 mM. At each of the tested temperatures, RP showed significant differences ($p < 0.05$) among the NaCl concentrations.

Table 4 - Germination (G) and recovery percentages (RP) at each temperature regime (5-20°C) and under different saline conditions (0-600 mM NaCl) for *Silene ichnusae* (A: Sich1) and *S. badaroi* (B: Sbad1). The statistical tests were the same used to analyze the data of Table 2. Data are the mean of four replicates (± 1SD). Capital letters in columns are related to the same NaCl concentration, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at $p < 0.05$ (by Mann-Whitney *U*-test). See Table 1 for the explanation of the population code.

(A)

Temperature (°C)	Percentage (%)	NaCl concentration (mM) 0	100	200	300	400	500	600	
5	**G**	90.0 ± 4.0 Aa	80.0 ± 3.3 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	75.8 ± 19.5	89.0 ± 3.8	81.0 ± 6.0	88.0 ± 8.6	70.0 ± 9.5	89.0 ± 14.4	ns
10	**G**	90.0 ± 5.16 Aa	91.0 ± 3.8 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	33.0 ± 0	82.0 ± 12.0	89.0 ± 5.0	92.0 ± 5.7	86.0 ± 2.3	95.0 ± 2.0	ns
15	**G**	97.0 ± 3.8 Aa	95.0 ± 3.8 Ba	1.0 ± 2.0 Ab	2.0 ± 2.3 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	-	93.9 ± 4.1	85.7 ± 9.8	94.0 ± 2.3	88.0 ± 9.8	89.0 ± 6.0	ns
20	**G**	93.0 ± 6.8 Aa	94.0 ± 5.2 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	83.3 ± 28.9	98.0 ± 2.3	87.0 ± 6.0	92.0 ± 7.3	88.0 ± 9.8	89.0 ± 6.0	ns
	G	ns	*	ns	ns	ns	ns	ns	
	RP	-	ns	ns	ns	ns	ns	ns	

(B)

Temperature (°C)	Percentage (%)	NaCl concentration (mM) 0	100	200	300	400	500	600	
5	**G**	48.0 ± 14.2 Aa	18.0 ± 5.2 Ab	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	85.4 ± 3.5 a	78.0 ± 6.9 b	74.0 ± 6.9 Ab	25.0 ± 13.2 Ac	25.0 ± 15.8 Ac	30.0 ± 18.0 Ac	*
10	**G**	95.0 ± 5.0 Ba	84.0 ± 6.5 Bb	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	25.0 ± 28.9 a	88.0 ± 3.3 b	83.0 ± 7.6 Abc	71.0 ± 6.8 Bc	77.0 ± 3.8 Bc	75.0 ± 10.5 Bc	*
15	**G**	70.0 ± 9.52 ACa	84.0 ± 6.5 Ba	0 Ab	0 Ab	0 Ab	0 Ab	0 Ab	**
	RP	-	25.0 ± 28.9 a	79.0 ± 11.2 b	83.0 ± 7.6 Abc	70.0 ± 15.0 Bb	77.0 ± 3.8 Bb	78.0 ± 14.8 Bb	*
20	**G**	78.0 ± 7.7 Ca	44.0 ± 11.8 Cb	0 Ac	0 Ac	0 Ac	0 Ac	0 Ac	**
	RP	-	75.5 ± 17.4 a	75.0 ± 10.5 a	42.0 ± 14.0 Bb	52.0 ± 5.7 Bb	39.0 ± 13.2 Ab	43.0 ± 16.1 Ab	*
	G	**	**	ns	ns	ns	ns	ns	
	RP	-	*	ns	*	**	**	**	

Effect of KNO3 on seed germination under salinity

For each seed lot, germination both under control conditions (only NaCl) and with the addition of potassium nitrate (NaCl + KNO_3) was inhibited at all concentrations above 100 mM NaCl, except for Svel3 and Sich1 under control conditions at 15°C. For all seed lots, no significant differences in germination percentages ($p > 0.05$) were detected between the control conditions and nitrate treatment (Fig. 3). The only exceptions were for Svel3 (0 mM NaCl at 15°C), where KNO_3 improved seed germination, and at 100 mM NaCl (Svel2 at 20°C, Svel3 at 15°C, Sich1 and Sbad1 at 10°C), where the addition of nitrate negatively affected germination.

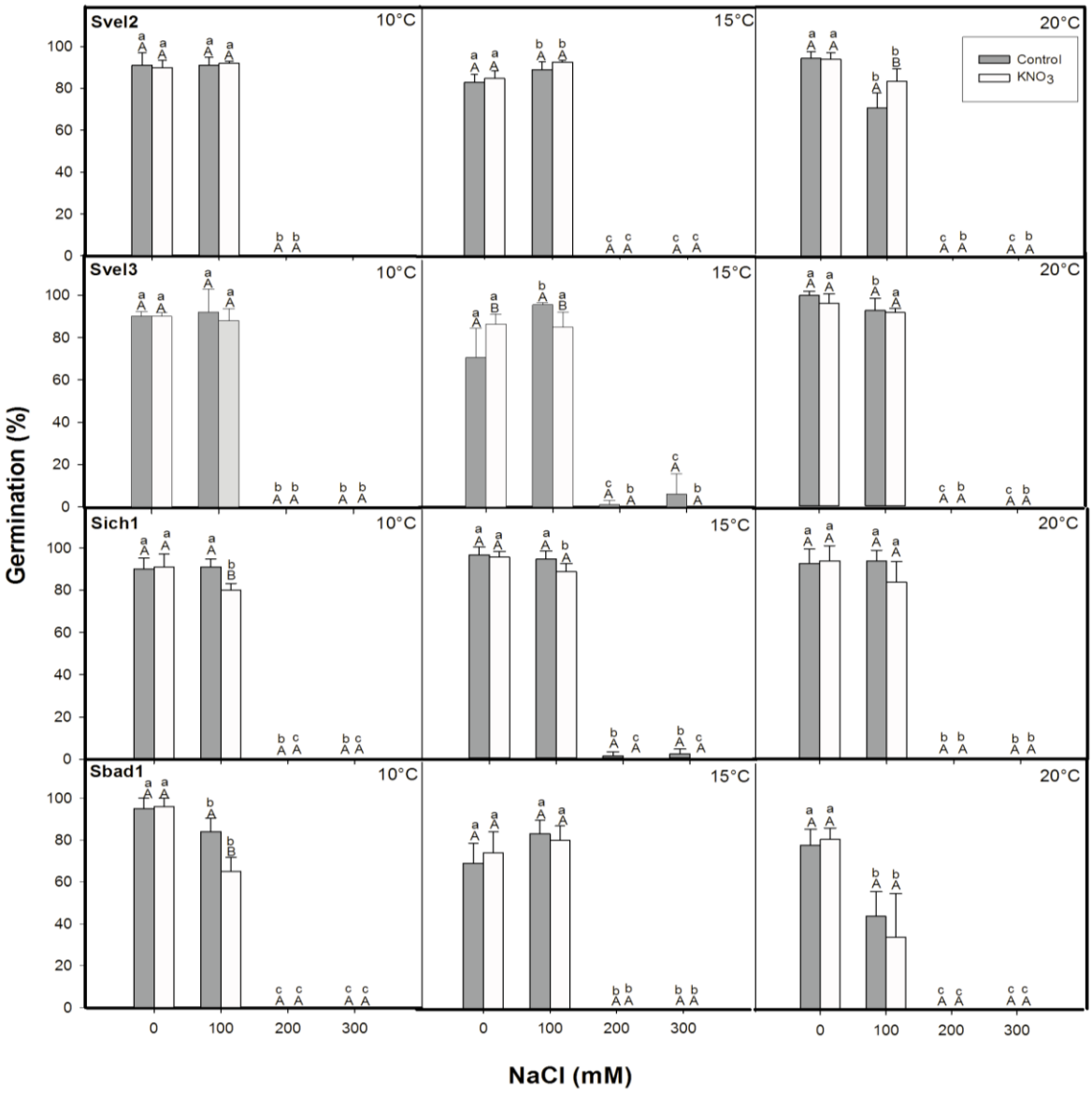

Figure 3 - Effect of KNO_3 (20 mM) on seed germination under NaCl (100-300 mM) at constant temperatures (10-20°C) for *S. velutina* (Svel2, Svel3), *S. ichnusae* (Sich1) and *S. badaroi* (Sbad1). Kruskal-Wallis tests were conducted to detect differences between the nitrate treatment and the control (capital letters, by Mann Whitney *U*-test) and the effect of different temperatures for each population (lower-case letters, by Mann Whitney *U*-test). Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney *U*-test). Data are the mean of four replicates (± 1SD). See Table 1 for the explanation of the population codes.

Effect of KNO_3 under salinity on recovery

For the four tested seed lots and at all temperatures, germination recovery under control conditions (NaCl) increased significantly ($p > 0.05$) following exposure to 100 mM to 200 and 300 mM NaCl, except for Sich1 at 20°C, which did not show significant differences ($p > 0.05$) at different salt concentrations (Fig. 4). Recovery at 15°C for all seed lots (except Sbad1) was not performed because all seeds germinated or died under the previous phase of treatment. Germination recovery at 200 and 300 mM was also significantly higher ($p < 0.05$) than at 100 mM NaCl, with the exception of Sich1 at 10°C (for which there were no differences), and 20°C (for which RP at both 100 and 300 mM NaCl were significantly lower than at 200 mM NaCl). In most cases, no significant differences ($p > 0.05$) were detected in the RP between control conditions and nitrate treatment. The only differences were observed for Svel3 at 200 mM NaCl at 15°C and Sich1 and Sbad1 at 100 mM at 10° and 20°C, respectively.

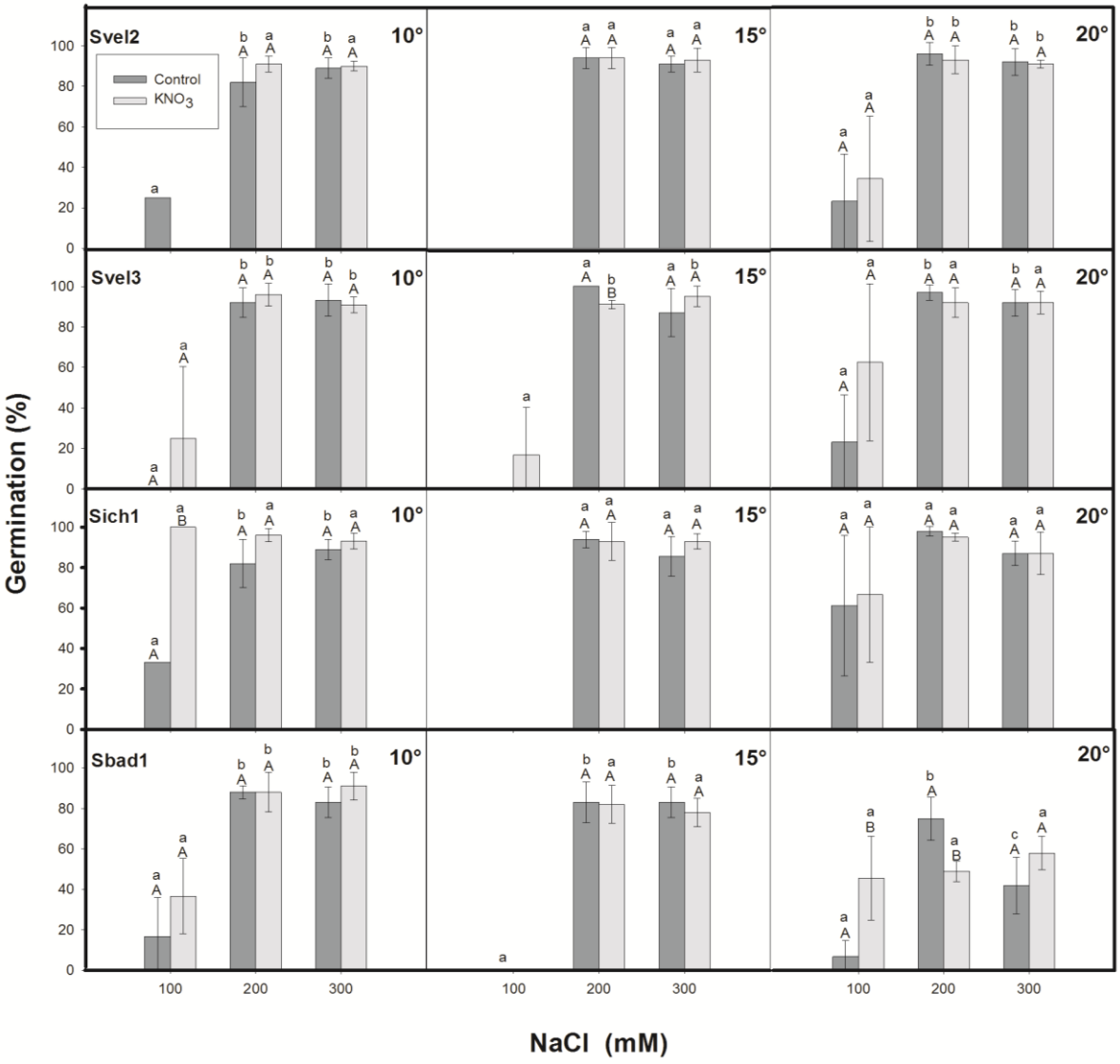

Figure 4 - Recovery percentages (RP) of seeds exposed to KNO3 (20 mM) under different salinities (100-300 mM) at constant temperatures (10-20°C) for *S. velutina* (Svel2, Svel3), *S. ichnusae* (Sich1) and *S. badaroi* (Sbad1). Kruskal-Wallis tests were conducted to detect differences between treatment and control (capital letters, by Mann Whitney U-test) and the effect of different NaCl concentrations for each population (lower-case letters, by Mann Whitney U-test). Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney U-test). Data are the means of four replicates (± 1SD). See table 1 for the explanation of the population codes.

DISCUSSION

Thanos *et al.* (1989; 1995) found that the germination of several Mediterranean coastal species is photo-inhibited, highlighting a surface avoidance mechanism that enables seeds to avoid germinating under the harsh conditions of the soil surface. However, seeds of the three *Silene* species investigated in the present study, which achieved high germination percentages in the light, did not display this kind of response, therefore they were not photo-inhibited for germination. Moreover, for all species, the higher germination percentages detected in the range of 5-15°C and the significant decrease in germination at the highest temperature (25°C) are in accordance with Thanos *et al.* (1989; 1995), who observed that germination at low temperatures is a widely extended trait in many Mediterranean coastal species. However, for *S. badaroi*, the germination response at 20°C was significantly lower than that detected at the colder temperatures. *Silene velutina* and *S. ichnusae* seeds also showed their capability to germinate at high percentages at 20°C. Therefore these results may correspond to field germination in the period from autumn until late spring for *S. velutina* and S. *ichnusae*, and mainly during winter months for *S. badaroi*. Germination during the period from autumn to spring (when water availability, soil moisture and rainfall are high, and temperatures are not prohibitive for germination and the establishment of consequent seedlings) may ensure ecological success in an unpredictable climate such as that found in the Mediterranean Basin (Thanos *et al.* 1995; Kadis and Georghiou 2010, Santo *et al.* 2014*b*).

Probert (1992) suggested that responding to alternating temperatures represents an adaptation of small-seeded species, which ensures that germination occurs only close to the soil surface because light is only able to penetrate 4-5 mm into the soil in physiologically significant quantities (Tester and Morris 1987). Seeds of all three *Silene* species responded positively to the tested alternating temperature regime, showing that field germination occurs preferably in the soil layers near to the surface, where the effect and influence of the alternate temperatures are greater.

The tested *Silene* species showed different levels of tolerance to salinity, which were 300 mM NaCl for *S. velutina* and *S. ichnusae* and 100 mM for *S. badaroi*. For all the species, lower germination percentages were observed in the salt substrate, compared to the higher germination percentages observed under control conditions (0 mM NaCl). Temperature did not influence germination under salt stress in any of the species. Many studies report that percentages of germination decrease with increasing salinity stress, and the highest germination occurs in the absence of NaCl in the substrate (Khan and Ungar 1984; Baskin and Baskin, 1998; Santo *et al.* 2014*b*). The limit of tolerance to salt varies among different species (Ungar 1995), for example, 200 mM NaCl in *Halopyrum mucronatum* (L.) Stapf and *Sporolobus arabicus* Boiss. (Gulzar *et al.* 2001), 300 mM in *Silene maritima* With. (Woodell 1985), 310 mM in *Briza maxima* L. (Lombardi *et al.* 1998), 344 mM in *Puccinellia nuttalliana* (Schult.) Hitchc. (Macke and Ungar 1971) and *Hordeum vulgare* L. (Badger and Ungar 1989), 400 mM in *Diplachne fusca* (L.) Beauv. (Morgan and Myers 1989), 500 mM in *Urochondra setulosa* (Trin.) C.E. Hubb. (Gulzar *et al.* 2001) and up to 1712 mM NaCl in Neo*kochia americana* (S.Wats.) G.L. Chu & S.C. Sand. (Clarke and West 1969). Differences in tolerance to salinity were detected in the present study between the two tested populations of *S. velutina* (100 mM for Svel1 and 300 mM for Svel2). However intra-specific variability in germination patterns has been reported for several species and investigated in various studies (Bischoff *et al.* 2006; Kremer *et al.* 2009; Bischoff and Müller-Schärer 2010). Moreover, differences in salt stress response were also observed among populations of *Panicum turgidum* Forssk. (El-Keblawy *et al.* 2010), *Spartina patens* (Aiton) Muhl (Hester *et al.* 1996) and *Rouya polygama* (Desf.) Coincy

(Santo *et al.* 2014*a*), but not in *Crucianella maritima* L. (Del Vecchio *et al.* 2012). According to Gutterman (1994) and Kigel (1995), the variability of germination requirements could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions.

Similarly to *Silene maritima* (Woodell 1985), *S. velutina* and *S. ichnusae* showed a high capability to recover their germination after exposure to salt, independent of the temperatures and NaCl concentrations to which the seeds were exposed in the previous salt phase. Conversely, *S. badaroi* seed recovery after salt conditions decreased with increasing salinity at the extreme temperatures of 5°C and 20°C. The different NaCl tolerance and recovery response behaviour among the three *Silene* species confirm the assertion of Khan and Ungar (1984) that tolerance and recovery from salinity and temperature stress are species-specific. Seeds of some species do not recover or show little recovery response when subjected to high salinity and temperature stress (Khan and Gul 2006). However, for all three species the effects of NaCl did not influence seed mortality, inasmuch as seeds of each species also survived high salt concentrations, demonstrating their ability to wait for favourable germination conditions, after high salt exposure.

Many studies have demonstrated that nitrates are capable of alleviating salinity stress in several species. For example, Gull and Weber (1998) found that the addition of nitrate (20 mM) alleviated the inhibitory effect of salt on germination in *Allenrolfea occidentalis* (S. Watson) Kuntze at different salt concentrations (200, 400, 600 and 800 mM NaCl); similarly, an equal concentration of nitrate promoted seed germination in *Atriplex prostrata* Boucher ex DC. at 100, 200, 300 mM NaCl (Khan 2003), and in *Crithmum maritimum* L., 10 mM KNO_3 enhanced germination at 100 and 200 mM NaCl (Atia *et al.* 2009). On the contrary, nitrate did not alleviate seed dormancy and germination in *Suaeda salsa* (L.) Pallas, *Descurainia sophia* (L.) Webb ex Prantl (Li *et al.* 2005), *Halogeton glomeratus* (M.Bieb.) C.A.Mey., *Lepidium latifolium* L. and *Peganum harmala* L. (Ahmed *et al.* 2013) under various salinity treatments (from 0 until 400 mM NaCl). We observed a similar response in the three *Silene* species investigated in this study, for which, at any tested NaCl concentration, germination was not significantly promoted or affected by nitrate.

Our results highlight that the investigated species experience optimum germination during autumn-winter, when, water availability is highest and soil salinity levels are minimal under the Mediterranean climate. *Silene ichnusae* and *S. velutina* are also able to germinate in spring, while *S. badaroi*, responding less to the constant temperature of 20°C, showed a narrower germination window, demonstrating the ability to germinate mainly during winter months. All three species demonstrated that their germination in the field may occur when, due to rainfall, salt concentration decreases in the soil. However, their seeds may tolerate relatively high salinity (600 mM) values in the substrate, recovering their germination after the salt exposure. While they cannot be considered halophilous species, this property may be considered a way to avoid ecological competition with other coastal plant species. The addition of nitrate in the substrate did not affect seed germination of the three species, suggesting both that the nutrient availability is not a requirement for seed germination and that the presence of KNO_3 in the habitat due to seabirds does not constitute necessarily a threat for them. Further studies are needed, possibly extended to the whole *S. mollissima* aggregate, to better understand the ecology of all species and explain their distribution and rarity.

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CHAPTER III

Comparative germination ecology and seedling growth of two Ibero-Levantine endemic species belonging to the *Silene mollissima* aggregate (Caryophyllaceae)

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ABSTRACT

The present study was focused on the seed germination and seedling growth of *Silene mollissima* and *Silene hifacensis*, both endemic of the Mediterranean Ibero-Levantine biogeographic province, with the aim to better understand their germination requirements and, consequently, their distribution and rarity. Inter- and intra-specific variability in the responses to light (12/12 hours) and darkness, constant and alternating temperatures, salinity tolerance and germination recovery after washing seeds with distilled water were evaluated. Moreover, the biomass production and the growth rate during the early stages of seedlings development were measured at several temperatures. Seeds of the two species were non-dormant and light did not affect their germination. For both species, germination was high at the lowest tested temperatures, while at the highest temperatures inter-specific differences were observed. Seeds of the two species were able to germinate up to 250 mM NaCl, furthermore the highest germination occurred in the non-saline control and decreased with increasing salinity. Independently from the tested temperature, *S. mollissima* and *S. hifacensis* seeds totally recovered their germination after the NaCl exposure. For both *S. mollissima* populations the increasing temperature promoted the seedling dry weight and the growth rate, while this pattern was observed only for one *S. hifacensis* population. Our results suggest a field germination from autumn until early spring for *S. mollissima* and limited to the autumn-winter months for *S. hifacensis*, when soil moisture is high and salinity levels are low due to the seasonal washout by rainfall. These findings highlighted the ecological adaptation to habitat in which these species occur and partially explain the narrow distribution of the two species, although further investigation about their ecological requirements and tolerance limits to differents environmental factors during other phases of life cycle would be needed.

Keywords: endemic coastal species, islands, Mediterranean Basin, recovery, salinity, seed germination.

INTRODUCTION

The Iberian Peninsula and the Balearic Archipelago are among the floristically richest areas in the Mediterranean Basin (Domínguez-Lozano *et al.* 2000; Rita & Bibiloni 2013; Fos *et al.* 2014). In particular, the Balearic Islands show highly original flora and rich of endemism (Alomar *et al.* 1997; Aedo *et al.* 2013). Specific biodiversity in this area can be related to climatic characteristics, geomorphological complexity, and the isolation produced by the insularity conditions (Domínguez-Lozano *et al.* 1996, 2000), but also by paleogeographic events, such as Pleistocene glaciations, during which many Mediterranean areas served as refugia for numerous *taxa*, allowing the persistence and differentiation of isolated populations (Rosselló *et al.* 2007; Médail & Diadema 2009; Molins *et al.* 2011). For these reasons, these islands harbour numerous narrowly endemic *taxa*, many of which, today, show regressive distribution (Alomar *et al.* 1997; Mateo-Sanz & Crespo-Villalba 1998). Some of these species are often present in conservative habitats, such as coastal cliffs or mountain peaks.

In the western Mediterranean Basin, the *Silene mollissima* aggregate, which is included in the sect. *Siphonomorpha* Otth., comprises 11 endemic species (Murru *et al.* 2015). the most of the *taxa* of this aggregate occurs in coastal habitats, in particular on sea cliffs, and many of them are threatened or have anarrow or disjointed distribution. Little is known about the biology and ecology of these species, and recent studies only aimed to investigate their seed germination ecology (Escribá *et al.* 2006; Murru *et al.* 2015), by testing their responces to some natural factors typical of coastal habitats, such as light, temperature, salt stress and nutrient availability. However, this research highlighted the need to expand these studies to the other species of the same aggregate.

It is well known that seed germination and seedling growth are the most complex and vulnerable stages of plant life, and detailed knowledge of how environmental factors influence these processes is crucial for *in situ* species conservation. In coastal habitats, several environmental factors, typical of these environments may influence seed germination, therefore this process is still more obstructed in these habitats, for example by high soil moisture fluctuations or by highly variable temperatures in the soil (Santo 2013; Baskin & Baskin 2014; Bhatt *et al.* 2016). Light is an extremely important factor influencing the seed germination process, especially mediated by phytochrome (Baskin & Baskin 2014), and its presence could either inhibit germination completely (Benvenuti *et al.* 2004), partially (Zia and Khan, 2004) or have no effect (Wei *et al.* 2008). Temperature can also interact with light, modifying the seed sensitivity to this latter factor (Sugahara & Takaki 2004). For species growing in coastal habitats, soil salinity is another important factor (Minissale *et al.* 2011), and many species usually reduce or delay their seed germination under high values of salinity (Tlig *et al.* 2008). In several studies has been observed that the tolerance to salinity during the germination process is influenced by temperature, and usually high temperatures increased the deleterious effects of salts (Santo *et al.* 2014a). When a seed does not have the ability to germinate at high salinity, the ability to maintain its viability and germinate when salts are leached, for example due the rainfall, is essential. This property of seeds is known as recovery and is species-specific (Baskin & Baskin 2014). Environmental conditions of natural habitats where species grow, may also influence the ability of seeds to recover germination (Pujol *et al.* 2000).

The present study investigated the seed germination and seedling growth of *Silene mollissima* (L.) Pers. and *Silene hifacensis* Rouy ex Willk., two closely related endemics of the Mediterranean Ibero-Levantine Province (Martínez & Arregui 1999). Data about seed germination are reported only for *S. hifacensis* by Escribá *et al.* (2006), which observed a great inter-population variability in its germination requirements. However, such work,

considering a high number of species, did not deepen specifically the seed ecology of *S. hifacensis* and did not investigate on same important factors influencing its seed germination, such as salt concentration in the substrate. In particular, this study was aimed to investigate the intra- and inter-specific variability of the two endemic species *S. mollissima* and *S. hifacensis* in terms of: 1) their seed germination requirement of light and temperature, 2) the salt tolerance of seeds and their germination recovery at different temperatures, and 3) the biomass production and the growth rate at different temperatures during the early seedling developmental stages.

MATERIALS AND METHODS

Study species and seed lot details

S. mollissima and *S. hifacensis* are calcicolous chamaephytes, flowering between April and June, fruiting from May to July (Jeanmonod 1984; Escribá *et al.* 2006) and producing orthodox seeds (Royal Botanic Gardens Kew, 2015). *Silene mollissima* is endemic of limestone cliffs of Mallorca and Menorca (Balearic Islands, Spain), while *S. hifacensis* is endemic of coastal rocky cliffs in the province of Alicante and Teulada (Iberian Peninsula, Spain), and the island of Ibiza (Balearic Islands, Spain) (Jeanmonod and Boquet, 1981;). *Silene mollissima* is currently not protected, whereas *S. hifacensis* is listed in the EEC Habitats Directive 92/43, included in the IUCN International Red Lists as an endangered (EN) taxon (Pilar Blasco *et al*., 2011), and inserted with the same degree of threat in the Red List of Spanish plants (Lozano 2000; Bañares *et al.* 2010).

Table 1 - Populational data and seed lot details. In the last column the different experiments carried out for each seed lot are reported (L = Light; T = Temperature; NaCl = Salinity; SG = Seedling growth).

Species	Population code	Locality	Coordinates (WGS84, UTM)	Mean elevation (m a.s.l.)	Distance from the sea (m)	Date of collecting	Mean seed mass (mg)	Experimental trials
S. mollissima	Smol1	Coma Freda (Mallorca)	39° 48' N 02° 52' E	650	6507	06/08/2014	0.81 ± 0.02	L; T; NaCl; SG
	Smol2	Monte Toro (Mallorca)	39° 59' N 04° 06' E	334	4722	08/08/2014	0.64 ± 0.02	NaCl; SG

S. hifacensis	Shif1	Morro de Toix (Alicante)	38° 37' N 00° 01' E	56	71	01/07/2014	0.78 ± 0.02	L; T; NaCl; SG
	Shif2	Sa Casa Redona (Ibiza)	39° 02' N 01° 18' E	113	115	23/08/2014	0.72 ± 0.02	NaCl; SG

For both species, capsules were collected from two different natural populations at the time of dispersal (Tab. 1). Seed collections were carried out after obtaining permits as required by European laws for the species listed in the appendices of the Habitat Directive 92/43/EEC. The two *S. hifacensis* seed lots were provided by the Centro para la Investigación y Experimentación Forestal (CIEF) of the Generalitat Valenciana and the Institut Balear de la Natura (IBANAT) of Balears, while the two *S. mollissima* seed lots were provided by the Institut Menorquí d'Estudis (IME) and IBANAT. Seeds of each seed

lot were extracted from their fruits, and stored under controlled conditions (20 °C and 50% relative humidity) for two weeks. After this period germination tests started. The mean seed mass (± standard error, hereafter SE) of each seed lot was calculated by weighing 10 replicates of 20 seeds each (Tab. 1).

Germination tests and seedling growth

Germination tests were performed under laboratory conditions at the Sardinian Germplasm Bank (BG-SAR) of the Hortus Botanicus Karalitanus (HBK) of the University of Cagliari (Italy) (Fig.1). To evaluate the effect of light on seed germination a preliminary test was carried out on one seed lot for each species (Smol1 and Shif1; Tab. 1). Seeds were sown in 60 mm diameter plastic Petri dishes on a 1% water agar substrate and then incubated at 15 °C in growth chambers (MLR-351, SANYO), both in the light (12 h of irradiance per day, photosynthetic photon flux density of 40 µmol m-2 s-1) and in total darkness. Darkness was simulated by wrapping dishes in two aluminium foils. For each condition, three replicates of 20 seeds each were used. A seed was considered to be germinated when the radicle length was ≥ 2 mm. Germinated seeds were counted and removed daily from light-exposed treatments, while unexposed treatments were evaluated at the end of the total germination period. At the end of the tests, each un-germinated seed was cut with a scalpel and its endosperm was observed under a microscope.

To evaluate the effect of thermoperiod and differences among populations in seed germination, three replicates of 20 seeds each per treatment were incubated at a range of constant (10, 15, 20, 25 °C) and alternating temperatures (25/10 °C). The alternating thermoperiod had day/night temperatures which lasted for 12 hours and corresponded with day/night photoperiod. To assess the salinity tolerance during germination, seeds were sown under different NaCl concentrations (0, 125, 250, 500 mM) and incubated in the light at three constant temperatures (10, 15, 20 °C). After 15 days without germination in the non-saline control, non-germinated seeds were washed with deionized water and re-sown on agar without salt for a further 30 day period (recovery).

To evaluate the effect of temperature on seedling growth for both species, 20 seeds for each seed lot (germinated on the same day in growth chambers at different temperatures of 10, 15, 20, 25/10 °C) were transplanted on a 1% water agar substrate in cylindrical plastic pots (diameter 90 mm). After 19 days of incubation in the growth chambers, seedlings were dried in an oven (FP 115, BINDER) at 80 °C per 72 hours (Yu *et al.* 2008), and samples were weighed at room temperature (20 ± 1 °C) with an electronic balance (Crystal 100 CAL, GIBERTINI) to measure the mean seedling dry weight and the growth rate (mg/day). When the minimum number of seedlings (20) was not reached at one temperature, seedling growth was not computable.

Figure 1 - Germination test, germinated seed and seedling.

DATA ANALYSIS

Final germination percentages were the mean of three replicates, and recovery percentages (RP) were calculated following the equation (Pujol *et al.* 2000):

$$RP = \{[(a\text{-}b)/(c\text{-}b)] \times 100\},$$

where *a* is the total number of seeds germinated in salt solutions plus those that recovered to germination in the fresh water, *b* is the total number of seeds germinated in salt substrates, and *c* represents the total number of seeds of each Petri dish. For each temperature and population, the growth rate (velocity of seedling growth, expressed in mg/day) was calculated dividing the dry weight of each seedling at the end of the experiment by the total duration of growth monitoring (19 days). The normality of all the data, was analysed by the Shapiro-Wilk test (Shapiro & Wilk, 1965). Germination percentages of the preliminary light experiment and the mean seed weights and growth rate were arcsine transformed and analysed by one-way ANOVA and Fisher's Least Significant Differences (LSD) *post-hoc* test (Cazalla *et al.* 1999). Recovery and germination percentages under different temperatures and in salt conditions were analysed by the non-parametric Kruskal-Wallis test, followed by a Mann-Whitney *U*-test, due the non-satisfaction of the ANOVA assumptions (Breslow 1970). All statistical analyses were carried out using the statistical software Statistica 7.0 for Windows (Software Statsoft Release 7).

RESULTS

Mean seed weight and seed germination

For both species, seeds from different populations showed differences in mean seed weight (Fig. 2). For *S. mollissima*, the mean seed mass of Smol1 (0.81 ± 0.05 mg) was higher than for Smol2 (0.64 ± 0.07 mg). For *S. hifacensis*, the mean seed mass of Shif1 was higher than for Shif2 (0.78 ± 0.05 mg and 0.72 ± 0.03 mg, respectively).

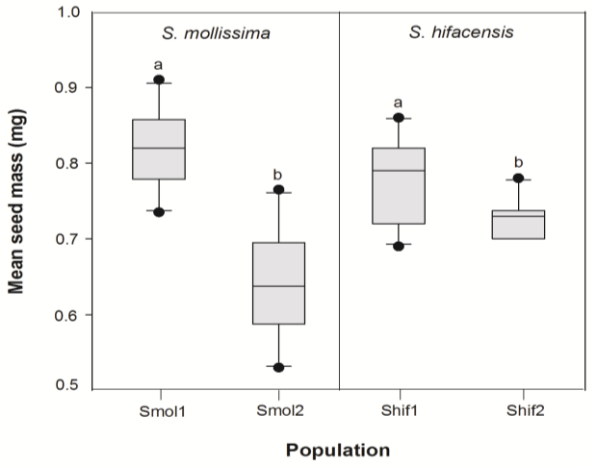

Figure 2 - Mean seed mass (mg), for *Silene mollissima* and *Silene hifacensis* the their tested populations. A one-way ANOVA was conducted for each species in order to identify differences among populations. Values with different letters are significantly different at $p < 0.05$ (Fisher's Least Significant Difference post hoc test). See Table 1 for the explanation of the population codes.

For both species, light did not affect seed germination and no differences were observed between light- and dark-conditions (Fig. 3). Germination percentages detected during a light-preliminary test at 15 °C, showed no differences between the two photoperiods, for both species (for *S. mollissima* 83.3 ± 2.9% and 93.3 ± 13.2%, in light and in darkness, respectively; for *S. hifacensis* 95.0 ± 8.7% in light and 80.0 ± 18.5% in darkness). Therefore, all subsequent germination tests were conducted in the light.

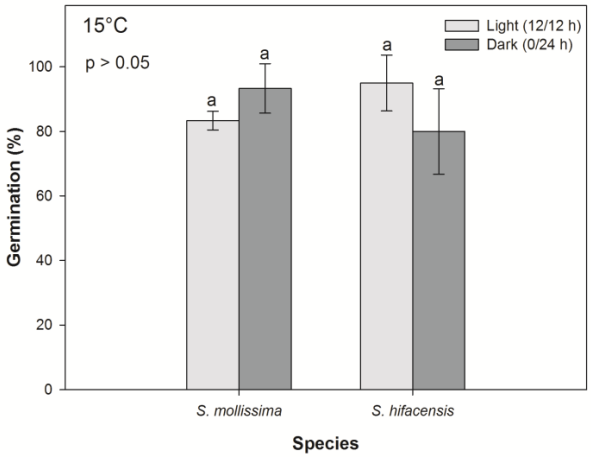

Figure 3 - Final germination at 15°C in the light (12/12 h) and total darkness for *S. mollissima* (Smol1) and *S. hifacensis* (Shif1). A one-way ANOVA was conducted for each species in order to identify differences between the two conditions. Values with the same letters are not significantly different at $p > 0.05$ (Fisher's LSD *post hoc* test). Data are the means of three replicates (± 1 standard deviation). See Table 1 for the explanation of the population codes.

For *S. mollissima*, the highest germination occurred in the range 10–20 °C and at 25/10 °C, while, at 25 °C, germination percentages considerably decreased (ca. 18%; Fig. 4). *Silene hifacensis* seeds showed the highest germination at the low temperatures of 10–15°C, with germination percentages significantly decreasing at 20 and 25 °C (ca. 20 % and 3 %, respectively). For the latter species the alternating temperature regime did not promote seed germination (only ca. 40%) (Fig. 4).

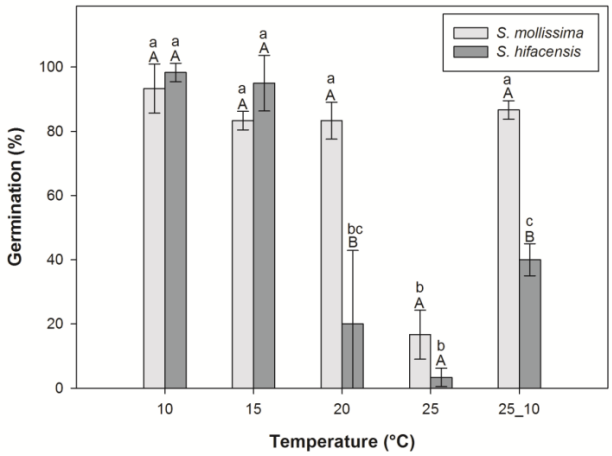

Figure 4 - Germination in the light (12/12 h) at constant (10-25°C) and alternating (25/10°C) temperatures for *S. mollissima* (Smol1) and *S. hifacensis* (Shif1). Kruskal-Wallis tests were conducted to detect differences between the two species at the same temperature (capital letters, by Mann Whitney *U*-test) and the effect of different temperatures for each species (lower-case letters, by Mann Whitney *U*-test). Values with different letters were used to indicate significant differences at $p < 0.05$ (Mann Whitney *U*-test). Data are the means of three replicates (± 1SD).

NaCl effects and recovery

Silene mollissima

For Smol1, at all salt concentrations, germination did not differ among the temperatures (Tab. 2a). The highest germination percentages were observed in the non-saline control and at 125 mM NaCl, independently of the temperature. In particular, at 0 mM, the highest values were observed at the lowest tested temperature (10 °C), while at 125 mM at the intermediate temperature (15 °C). Seed germination decreased with increasing salt concentration at all temperatures. Except at 15 °C, no differences were observed between germination under control conditions and at the lowest salt concentration (125 mM). Seeds were able to germinate up to 250 mM NaCl with percentages not higher than ca. 10%. At 500 mM, no germination occurred at all the tested temperatures.

Recovery percentages were not different among the temperatures and salinities (Table 2a). The highest recovery was detected at the lowest temperature (10 °C).

Smol2 at all NaCl concentrations did no differ in seed germination among the tested temperatures (Tab. 2). The highest percentages were observed under 0 mM NaCl, but at 10 and 15 °C they did not differ from those detected at 125 mM NaCl. Independently of the tested temperature, seed germination decreased with increasing salinity, and seeds were not able to germinate above 125 mM NaCl. Recovery did not differ both among the temperatures and the different salt concentrations and recovery percentages ranged from ca. 10% to ca. 75% (Tab. 2a).

Silene hifacensis

For Shif1, the highest germination was observed without NaCl, and no differences were detected among temperatures at all the salinities (Tab. 2b). However, at 125 mM NaCl, germination was inhibited at 20 °C, while it was higher than 90% at 10 and 15 °C. At NaCl concentrations of 250 mM and above, germination was totally inhibited at all temperatures. Seed germination decreased with increasing salinity at all temperatures, but at 15 °C no differences were observed between the non-saline control and 125 mM NaCl. Recovery did not differ among the tested temperatures or among the salt concentrations. Except for 125 mM at 10 °C (with recovery percentages of ca. 80%), more of the 90% of seeds recovered.

For Shif2, temperature had no effect for all the tested NaCl concentrations (Tab. 2b). Seed germination decreased with increased salinity at all tested temperatures. The highest germination occurred at 0 mM NaCl, but no differences were observed between this condition and 125 mM NaCl at 10 and 15 °C. Except at 15 °C, germination was inhibited at all temperatures and salt concentrations at 250 mM and above. Recovery percentages ranged from ca. 70% to ca. 100%, and no differences were detected among the tested temperatures or NaCl concentrations.

During the salt experiments, seed mortality was not higher than 2% for both species, demonstrating the ability of *S. mollissima* and *S. hifacensis* seeds to tolerate exposure to the highest salinity concentrations.

Table 2 - Germination (G) and recovery percentages (R) at the tested temperature range (10–20°C) and under different salt concentrations (0–500 mM NaCl) for two populations of (a) *Silene mollissima* (Smol1 and Smol2) and (b) *S. hifacensis* (Shif1 and Shif2). Kruskal-Wallis tests were conducted to detect the effect of the same temperature and salinity concentration on G and R; [p values were considered not significant ($p > 0.05$, ns) and significant ($p < 0.05$, *) by Kruskal-Wallis test]. Data are the mean of three replicates (± SE). Capital letters in columns are related to the same NaCl concentration, while lower-case letters in rows to the same temperature. Values with different letters were used to indicate significant differences at $p < 0.05$ (by Mann-Whitney *U*-test).

a) ***Silene mollissima***

Smol1

Temperature (°C)	Percentage (%)	NaCl concentration (mM)				
		0	**125**	**250**	**500**	
10	**G**	93.3 ± 3.8 a	80.0 ± 6.6 a	10.0 ± 2.5 b	0 c	*
	R	-	-	91.0 ± 4.0	86.7 ± 1.4	ns
15	**G**	83.3 ± 1.4 b	95.0 ± 2.5 a	3.3 ± 1.4 c	0 c	*
	R	-	-	86.1 ± 8.0	76.7±6.3	ns
20	**G**	83.3 ± 2.9 a	73.3 ± 2.9 a	1.7 ± 1.4 b	0 b	*
	R	-	27.8 ± 12.7	81.2 ± 4.1	85.0 ± 6.6	ns
	G	ns	ns	ns	ns	
	R	ns	-	ns	ns	

Smol2

Temperature (°C)	Percentage (%)	NaCl concentration (mM)				
		0	**125**	**250**	**500**	
10	**G**	76.7 ± 5.2 a	60.0 ± 9.0 a	0 b	0 b	*
	R	-	-	53.3 ± 9.5	73.3 ± 3.8	ns
15	**G**	63.3 ± 6.3 a	58.3 ± 7.6 a	0 b	0 b	*
	R	-	9.7 ± 5.0	75.0 ± 0	61.7 ± 9.5	ns
20	**G**	66.7 ± 8.7 a	28.3 ± 2.9 b	0 c	0 c	*
	R	-	42.7 ± 8.8	58.3 ± 12.6	55.0 ± 6.6	ns
	G	ns	ns	ns	ns	
	R	ns	ns	ns	ns	

b) Silene hifacensis

Shif1

Temperature (°C)	Percentage (%)	NaCl concentration (mM)				
		0	**125**	**250**	**500**	
10	**G**	96.7 ± 1.4 a	90.0 ± 2.5 Ab	0 c	0 c	*
	R	-	83.3 ± 14.4	93.3 ± 1.4	95.0 ± 2.5	ns
15	**G**	96.7 ± 1.4 a	96.7 ± 1.4 Aa	0 b	0 b	*
	R	-	-	95.0 ± 2.5	95.0 ± 2.5	ns
20	**G**	80.0 ± 2.5 a	0 Bb	0 b	0 b	*
	R	-	95.0 ± 2.5	95.0 ± 2.5	95.0 ± 2.5	ns
	G	ns	*	ns	ns	
	R	ns	ns	ns	ns	

Shif2

Temperature (°C)	Percentage (%)	NaCl concentration (mM)				
		0	**125**	**250**	**500**	
10	**G**	100 ± 0 a	93.0 ± 2.9 a	0 b	0 b	*
	R	-	100 ± 0	98.3 ± 1.4	91.7 ± 2.9	ns
15	**G**	98.3 ± 1.4 a	91.7 ± 1.4 a	1.7 ± 1.4 b	0 b	*
	R	-	100 ± 28.8	96.6 ± 1.5	93.3 ± 2.9	ns
20	**G**	98.3 ± 1.4 a	85.0 ± 4.3 b	0 c	0 c	*
	R	-	70.0 ± 13.2	85.0 ± 6.6	100 ± 0	ns
	G	ns	ns	ns	ns	
	R	ns	ns	ns	ns	

Seedling growth

For both *S. mollissima* populations, differences were detected among the mean dry weights of seedlings grown at different temperatures (Fig. 5). In particular, for Smol1, higher biomass values were observed at the higher temperatures (20 and 25/10 °C; ca. 1.48 mg), while the lowest were observed at 10 °C (ca. 0.75 mg). For Smol2, the dry weight of seedlings grown at 20 °C (ca. 1.23 mg) differed from those detected at the lower temperatures of 10 and 15 °C (ca. 0.60 mg for both).

S. hifacensis seedlings from population Shif1 showed a dry mass of about 0.72 mg at the end of the experiment, without any difference among temperatures (Fig. 5). Instead, the increase in temperature influenced the biomass of Shif2 seedlings, which showed higher values (ca. 0.92 mg) at the higher temperatures of 15 and 20 °C (Fig. 5).

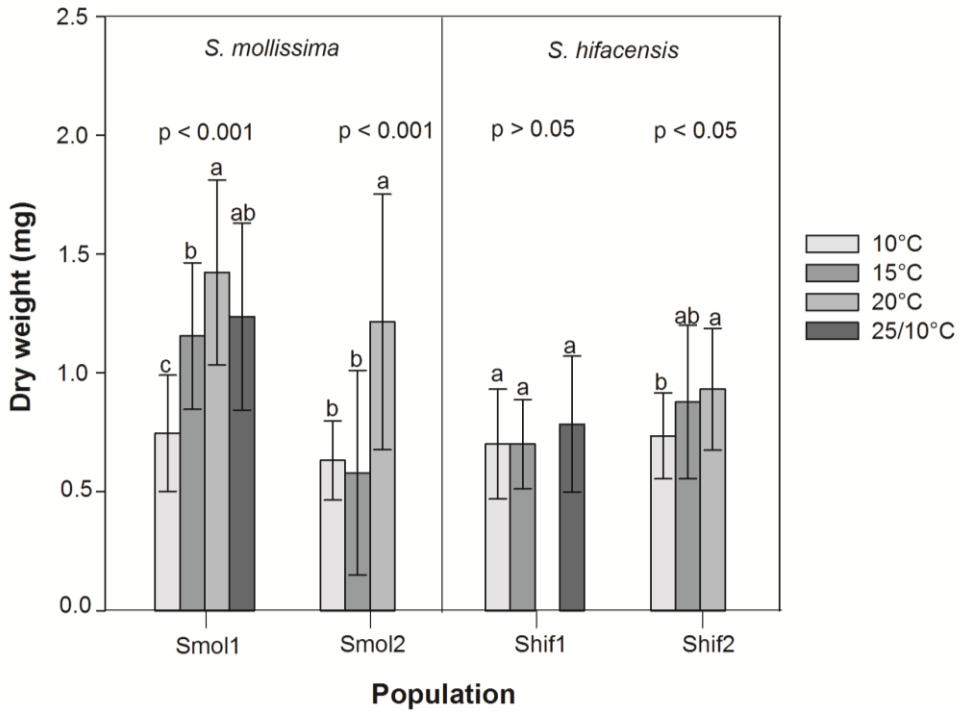

Figure 5 - Dry weight of *S. mollissima* and *S. hifacensis* seedlings, obtained from seeds germinated at different temperatures (10, 15, 20, 25/10°C) and measured after 19 days from germination. A Kruskal-Wallis test was conducted, for each population, to detect the effect of temperature on seedlings growth. Different letters were used to indicate significant values at $p < 0.05$ (Mann Whitney *U*-test). For each population, only data of temperatures for which the minimum number of seedlings (20) was available are reported. See Table 1 for the explanation of the population codes.

S. mollissima seedling growth rate showed highly differences in terms of temperature, indeed for both populations, growth rate was lowest at 10 °C, but increased nearly two-fold at 20 °C. For *S. hifacensis*, different responses in seedling growth rates to temperature were detected between the two tested populations, with Shif1 showing mean growth rates of ca. 0.04 mg/day at all temperatures, while Shif2 showed a growth behaviour similar to that detected in the two *S. mollissima* populations, however with a lower relative increase in response to rising temperatures (Tab. 3).

Table 3 - Growth rate of *S. mollissima* (Smol1 and Smol2) and *S. hifacensis* (Shif1 and Shif2) seedlings at different temperatures (10, 15, 20, 25/10°C). A Kruskal-Wallis test was conducted for each population in order to detect differences (column p value) among the growth rates at the tested temperatures. Different letters were used to indicate significant values at $p < 0.05$ (Mann Whitney U-test). For each population, are reported only data of temperatures for which the minimum number of seedlings (20) was available. See Table 1 for the explanation of the population codes. The abbreviation "n.c" stays for "not calculable".

Population	Temperature (°C)	Growth rate (mg/day)	p value
Smol1	10	0.04 ± 0.01a	p < 0.001
	15	0.06 ± 0.01b	
	20	0.07 ± 0.02c	
	25/10	0.06 ± 0.02bc	
Smol2	10	0.03 ± 0.01a	p < 0.001
	15	0.03 ± 0.02b	
	20	0.06 ± 0.03c	
	25/10	n.c.	
Shif1	10	0.04 ± 0.01a	p > 0.05
	15	0.04 ± 0.02a	
	20	n.c.	
	25/10	0.04 ± 0.01a	
Shif2	10	0.04 ± 0.01a	p < 0.05
	15	0.05 ± 0.02ab	
	20	0.05 ± 0.01b	
	25/10	n.c.	

DISCUSSION

Silene mollissima and *S. hifacensis* seeds were non-dormant and achieved high germination percentages both in the light and in the dark; therefore, they are not photo-inhibited for germination and candemonstrated to be able to germinate also in the harsh conditions of the soil surface, differently to several coastal species in the Mediterranean area (Thanos *et al.* 1989, 1991). A similar behavior was observed for seeds of *S. velutina*, *S. ichnusae* and *S. badaroi*, same Tyrrhenian species of the *S. mollissima* aggregate, which also resulted non-dormant and not photo-inibited (Murru *et al.* 2015). The germination pattern observed in the present study was confirmed by the seed mass of both species (average of ca. 0.74 mg for both). In fact species with seed weight < 0.10 mg often require light for germination, and their dependence on light decreases with increasing size of seeds (Pearson *et al.* 2002). Despite this, in several species, was observed that the probability of burial seems to depend both on the reduction of the seed mass and the progressive

rounding of dispersules (Cerabolini *et al.* 2003). Therefore these species could have high potential to create persistent soil seed banks.

Temperature influenced both *S. mollissima* and *S. hifacensis* seed germination. The former, similar to *S. velutina* and *S. ichnusae* (Murru *et al.* 2015), germinated in the range of temperatures (10-20 °C) corresponding to the field germination period from autumn to early spring. *Silene hifacensis* showed its optimum germination at low temperatures (10–15 °C), but considerably lower at high temperatures (20 °C and 25 °C). In the field, this pattern might correspond to germination in the autumn to winter period and reflects the typical behaviour of many Mediterranean coastal plants for which germination temperatures between 5 °C and 15 °C represent the optimal conditions (Thanos *et al.* 1989).

S. mollissima seeds, unlike *S. hifacensis*, germinated better under alternating temperatures, and therefore field germination of this species might occur near the topsoil whenever influence of the temperature alternation is higher.

S. mollissima and *S. hifacensis* seeds were able to germinate up to 250 mM NaCl and independently of temperature. Both species showed the lowest germination percentages when there was the presence of NaCl in the substrate, while the highest germination rates were observed at 0 mM NaCl. Several studies reported that germination decreases when salinity is higher and optimal germination occurs without salts (Baskin & Baskin 2014; Khan & Ungar, 1996). At concentrations higher than 250 mM NaCl, both species did not germinate. Several studies asserted that each species has its limit in the ability to tolerate salinity (Ungar 1995).

The results of laboratory tests under salinity provide important findings also on the field ecology of the study species, highlighting that their seeds are salt tolerant, until the concentration of sea wather, and have moderate ability to germinate in salty soils, typical of the habitats where effectively both species grow. Moreover, the seed property to recover germination after exposure to high NaCl concentrations, suggest a field germination after the period of autumnal rainfalls. Recovery was very high (up to > 90%) for both *S. mollissima* and *S. hifacensis*, both independently of temperature and salt concentrations. In the field, the interactions between salinity and temperature have important eco-physiological implications and they might influence the exact time of germination of a species (Ungar 1995). When temperatures are high, salinity could cause a loss of viability and, therefore, poor recovery. Tolerance to a high salinity concentration, in particular when temperature is an added stressing factor, and the subsequent ability of seeds to recover from these stresses also depends on the species (Khan & Ungar, 1997). In our case, the seeds of both species showed very low mortality during germination tests, but after exposition to the highest salinity concentrations and tested temperatures, they showed a great ability to recover their germination.

Variability among populations in seed germination patterns has been investigated in several studies (Bischoff *et al.* 2006; Santo *et al.* 2015a, 2015b); for example, in relation to the seed provenance, *Polypogon monspeliensis* (Atia *et al.* 2011), *Silene velutina* (Murru *et al.* 2015) and *Cakile maritima* (Megdiche *et al.* 2007) differed in their salt tolerance when several populations were considered as did *Rouya polygama* (Santo *et al.* 2014b) and *Panicum turgidum* (El-Keblawy *et al.* 2010). In *S. mollissima* and *S. hifacensis*, inter-population differences were detected in the salt tolerance of their seeds but these were not related to their distance from the coastline. However, it is well known that differences in the salt tolerance during the seed germination phase among populations of the same species depends not only on the distance from the sea but also on other environmental

factors, such as soil composition, rainfall pattern in the site of each population, presence of canopy, etc. (Baskin & Baskin 2014).

Silene mollissima and *S. hifacensis* seedling dry mass and growth rates increased with increasing temperatures, highlighting the presence of inter-population differences for *S. hifacensis*. Seedling growth usually ceases above 50 °C and below 0 °C. Within these extremes, however, a considerable variation in growth rate can occur depending upon the temperature (Beevers & Cooper 1964). The optimum seedling growth temperature of 20°C detected for *S. mollissima*, and 15-20°C for *S. hifacensis*, observed in our study are in accordance with the optimal germination conditions detected during our laboratory experiments. Seedling establishment is the most sensitive phase during the life cycle of a plant and certainly the evolution of these species favoured the occurring of seed germination in season with not excessively extreme temperatures, so that seedlings may find the best conditions for their growth and successful establishment in the field.

CONCLUSIONS

Our results are consistent with a field germination from autumn to early spring for *S. mollissima* and autumn to winter months for *S. hifacensis*. Both species showed the ability to germinate under salinity conditions up to a concentration of 250 mM NaCl. Salt did not influence seed viability and both salinity concentration and temperature increases did not affect recovery response, therefore salinity cannot be considered as an environmental factor limiting the distribution of these two species, because the seasonal rainfall typical of the Mediterranean climate is able to wash seeds, which have a high ability to germinate also after salt exposure. These patterns indicate that high water availability in the soil and minimal soil salinity levels represent an advantageous ecological adaptation to the unpredictable Mediterranean rainfall pattern to support seedling establishment in both species. These results have contributed to increasing the ecological knowledge of two closely related endemics, which present a partial overlap of their distributional area and of their ecological niche. In particular, it has been highlighted that although they have different germination needs, they share several common traits in the investigated phases of their life cycle. Similar studies, may allow to highlight the role of the same environmental factors on different ecological and phytogeographycal aspects of plants, such as the ecological niche and the distribution range; moreover, when conducted on systematically related *taxa* they may also be useful to better understand patterns of ecological vicariance on phylogenetically related species. Studies are in progress to enlarge and compare the seed ecology to other species of the *S. mollissima* aggregate.

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CHAPTER IV

Niche differences between large and small Mediterranean islands at different spatial scales in endemic Silene velutina (Caryophyllaceae).

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ABSTRACT

When compared to mainland areas, islands often show differences in population and community dynamics. Moreover, since the formulation of the first biogeographical theories, the presence of atypical patterns between large (LI) and small islands (SI) have been detected. However, to date there are no studies which have compared the ecological niche of a plant species between populations located on LI and SI, and few researches consider the spatial scale effect. By taking into account several environmental and population parameters, this study investigates microniche variation in populations of coastal endemic *Silene velutina* Pourr. ex Loisel. living on Mediterranean islands of different size (LI; SI), and the niche breadth dynamics at local (site) and regional scales (group of islands). Concerning niche characterisation, the effect of island size was detected for several variables related to substrate characteristics, biodiversity, plant coverage, community composition and presence of disturbance, illustrating a clear niche shift between populations on large and small islands, which was associated with different demographic strategies. In particular, it was observed a more reliance on the regeneration niche on LI and on persistence niche on SI. For niche breadth, at the regional scale we detected wider niche on LI probably due to spatial heterogeneity, which was positively correlated to island size. Environmental heterogeneity in fact, is one of the main factors used to indirectly explain same ecological patterns on islands of different size and was often associeted to the presence of species with wide ecological niches. In contrast, at the local scale, SI showed a wider niche breadth which appears to be due to a release from competition at this scale (low biodiversity levels and plant coverage and absence of phanerophytes). This is in accordance with the *niche variation hypotesis*, holding that populations can reduce or extend their niche breadth when exposed respectively to high or low levels of interspecific competition, with a potential niche expansion on islands due to reduced competition.

Key words: Demographic strategies, ecological release, persistence niche, plant populations, niche breadth, regeneration niche, spatial heterogeneity.

INTRODUCTION

The western Mediterranean, with the presence of the three major islands of the whole basin and about 1100 minor islands (Fois *et al.* 2016), represents an ideal natural laboratory for studies of insular ecology and biogeography. When compared to the mainland areas, islands often show alterations of population and community dynamics, such as demography, species composition, niche shift and niche breadth (Crowell 1983; Wardle *et al.* 1997; Adler and Levins 1994; Cody 2006, Yu *et al.* 2012). One of the first and most authoritative paradigms on the topic of insularity, *The Theory of Island Biogeography* (MacArthur & Wilson 1967), explained the patterns of biodiversity and the extinction and colonization dynamics in insular geographical contexts. However, since the formulation of the theory, "anomalous" patterns on species-area relationship (SAR) have been highlighted on small islands. These, were subsequently described as the *small island effect* and consist in the absence of a significant effect of area on species richness below a certain island size (Whitehead & Jones 1969; Lomolino & Weiser 2001). The presence of different patterns between large and small islands suggests that, in these areas, dynamics of same phenomena are poorly understood, both theoretically and empirically. Therefore, because such patterns were not completely explicated by the biogeographical viewpoint (MacArthur & Wilson 1967), other causes should be searched for their better understanding. In spite of this, except for the numerous investigations regarding SAR, only few studies concerned the effect of the island size on species and community parameters (Linhart & Feinsinger 1980; August 1985; McClanahan 1986). Moreover, we found no studies that specifically compared large and small islands.

As suggested by several authors, habitat diversity or environmental heterogeneity is one of the main factors that can indirectly explain ecological phenomena on islands of different size (e.g. Williams 1964; Kohn & Walsh 1994; Ricklefs & Lovette 1999; Triantis *et al.* 2003; Hortal *et al.* 2009). This was already highlighted by MacArthur & Wilson (1967), who expressed the need to incorporate environmental heterogeneity into theories and models concerning SAR. Habitat diversity is often positively correlated to the island size (Williams 1964; MacArthur 1972; Kohn & Walsh 1994; Rosenzweig 1995 Ricklefs & Lovette 1999) and is considered one of the main drivers influencing population and community dynamics (Tilman 1982; Chisholm *et al.* 2014). In fact, an increase in environmental heterogeneity with island size, can promote ecological complexity which often takes the form of a landscape mosaic of different environments (Thompson 2005), in which interactions between organisms can assume a more significant role at the local scale than biogeographical factors (Van Valen 1965; Callaway & Walcker 1997; Bruno *et al.* 2003). Such a framework can be locally complicated by the presence of disturbance, which can alter the environmental condition and relationships among individuals. In several cases in fact, its impact permits to reduce competition by producing new open areas and thus interfering with niche dynamics (Farris *et al.* 2009; Sheil 2016).

Several studies indicate that generalist species can preferably occur in heterogeneous and disturbed environments whereas specialist species prefer more homogeneous and stable habitats (Futuyma and Moreno 1988, Devictor *et al.* 2008). Specialization is a concept that is closely related to the ecological niche, in fact it was often used as a synonymous of a narrow niche breadth (Futuyma and Moreno 1988; Devictor 2010, Poisot et al 2011; Bulangeat *et al.* 2012). Although several definitions has been postulated, specialisation is considered as an intrinsic property of organisms, reflecting their range of environmental tolerance and their ability to exploit resources, while the fundamental niche defines organisms' requirements, representing the set of environmental conditions and resources that they can possibly occupy and use, and that can guarantee persistence of their

populations (Hutchinson 1957). However, organisms are rarely able to use their entire fundamental niche and they usually exploit only a portion of it, the realized niche (Hutchinson 1957; Pulliam 2000).

Specialisation and niche breadth may vary across locations. This shift can be determined by the spatial fluctuations of both environmental conditions and biological interactions (Devictor 2010), to which populations can respond with different demographic dynamics (Garcia & Zamora 2003; Baumel *et al*. 2008). In particular, different population strategies, based on *in situ* maintenance of established individual plants (persistence) or alternatively on replacement of individuals by seedlings (regeneration), can occur in different habitats, along a gradient of environmental stress or inter-specific competition (Bellingham & Sparrow 2000; Bond & Midgley 2001; Garcia & Zamora 2003). Consequently, populations can reduce or extend their niche breadth when exposed, for example, to different disturbance regimes and/or different competition levels (Van Valen 1965; Bolnick *et al.* 2010). In particular, niche expansion due to reduced competition (ecological release) was often illustrated for islands' species. In fact, several studies identified such geographical units as typical locations in which ecological release can be manifested (Givnish, 1997; Schluter, 2000; Gillespie & Clague 2009).

There are several methods for the estimation of niche characteristics, all presenting advantages and disadvantages (Slatyer *et al.* 2013; Pannek *et al.* 2016), however most of them take into account the responses of populations to environmental changes. In the ecological studies, both specialisation and niche breadth are conditioned to the set of considered variables and to the spatial scale at which they are evaluated (Devictor *et al.* 2010; Baumel *et al*. 2008). Therefore, species may be specialist for certain variables, or at a given spatial scale, and generalist for other variables, or at a different spatial scale (Gaston *et al.* 1997; Hughes 2000; Pannek *et al.* 2016). The same concept can be equally applied to the niche width. Nevertheless, few studies have considered the spatial scale effect on niche breadth; moreover studies on niche parameters were generally based on few variables that are relatively easy to measure or to detect from spatial datasets (Vetaas, 2002; Chase & Leibold, 2003). Detecting a high number of niche axes, instead, can help to statistically identify the most important variables and thus explain the observed patterns.

Given the lack of information regarding dynamics of vascular plant niche in relation to island size, and the importance of the spatial scale for its characterisation, in this work we analysed the realized niche of the Sardinian-Corsican endemic *Silene velutina* Pourr. ex Loisel., taking into account several environmental and population parameters. The objective of this study is two-fold. First, we would quantify whether there is niche differentiation between populations on large and small islands, and verify the presence of heterogeneity among sites. Second, based on the whole set of analyzed variables, we quantified the niche breadth at two different spatial scales (local and regional), both on large and small islands.

MATERIALS AND METHODS

Study species

Silene velutina is endemic to the coasts and several islets of the North Eastern Sardinia and Southern Corse. It grows to a maximum of 80 cm and is characterized by a rosette habitus and a dense pubescence of stems and leaves. The production of floral stems occurs in early spring, the flowering in late spring, while the fruiting in summer, with the production of dehiscent capsules. No data are available about the time of germination in the wild, but studies on in vitro germination ecology indicate that it can occur until early spring (Murru *et al.* 2015). The species is chamaephytic and can grow in a considerable variety of substrata (from rocky to sandy), derived both from siliceous and calcareous bedrock degradation, as well as on soil, moreover their seeds are able to tolerate salinity similar to the sea concentrations (Murru *et al.* 2015). *Silene velutina* is protected by the Berne Convention and listed with priority status in Annex II of the EU ‘‘Habitat’’ Directive. It is considered vulnerable (VU) in France and endangered (EN) in Italy (Olivier *et al.* 1995, Conti *et al.* 1997), but not evaluated (NE) in the IUCN International Red Lists (Buord *et al*. 2011).

Sampling design

For our study we distinguished between populations located on the large islands (LI) of Sardinia, Corsica and La Maddalena and several small islets (SI) around the LI. Within each group, on a list of locations where the species occurs, six sites were randomly selected (Tab. 1; Fig. 1and Fig. 2). For each one of them within the surface occupied by the species, 10 plots 1 x 2 m, 25 cm grilled (45 nodes x plot) were randomly placed.

Table 1 - Sampled sites for the large islands (LI) and the small islets (SI). The location, the municipality and the administrative region (Sa = Sardinia; Co = Corse) are reported in the column site. Elevation and distance from the sea are indicated as the mean of the location where the plant occurs.

	Site code	Site	Coordinates (UTM, WGS84)		Elevation (m)	Distance from the sea (m)	Substrate
			N (m)	E (m)			
LI	*Abt*	Abbatoggia, La Maddalena, Sa	4566551	533799	6	34	Granitic sands
	Agl	Riu di Li Saldi, Aglientu, Sa	4552859	506905	3	15	Granitic sands
	Cbi	Casetta Bianca, Porto vecchio, Co	4604557	525515	1	4	Granitic sands and conglomerates
	Src	Saint Roch, Bonifacio, Co	4581716	513411	80	37	Limestone rocks
	Cfn	Capu di Fenu, Ajaccio, Co	4644685	466736	45	30	Limestone rocks
	Tmr	Tamaricciu, Porto Vecchio, Co	4600105	526527	2	5	Granitic sands
SI	*Ibc*	Isolotto Baccà, La Maddalena, Sa	4560226	537021	3	4	Granitic rocks
	Ist1	Isolotto Stramanaro 1, La Maddalena, Sa	4570287	530454	7	27	Granitic rocks
	Ist2	Isolotto Stramanaro 2, La Maddalena, Sa	4570286	530551	6	16	Granitic rocks
	Icl	Isolotto Colombo, La Maddalena, Sa	4566544	533344	4	8	Granitic rocks
	Ipr	Isolotto Porro, La Maddalena, Sa	4566546	533231	4	7	Granitic rocks
	Ids	Ilot du Silene, Bonifacio, Co	4577307	521375	5	36	Granitic rocks

Figure 1 - Sites on LI: Abbatoggia (a), Riu di li Saldi (b) Casetta Bianca (c), Saint Roch (d), Capu di Fenu (e) and Tamaricciu (f).

Figure 2 - Sites on SI: Isolotto Baccà (a), Isolotto Stramanaro 1 (b) Isolotto Stramanaro 2 (c), Isolotto Clombo (d), Isolotto Porro (e) and Ilot du Silene (f).

Response variables

In each plot we quantified a wide range of environmental and population parameters. The response variables and the method of sampling are reported in Tab. 2. The abiotic variables (Tab. 2a) concerned both geo-morphological (e.g. slope), geographical (e.g. distance from the sea), and substrate characteristics (e.g. drainage, rockiness, presence of litter) of each plot.

Table 2 - Detected environmental and population variables. Response variables that were detected as presence/absence per plot (N=1) and frequency per plot (N = 45) are indicated with * and **, respectively. See sampling design for method of sampling.

Variable	**Sampling (1X Plot)**	**Sampling (45 X Plot)**
a) ABIOTIC	Elevation (m)	Stone **
	Distance from the sea (m)	Rock **
	Slope (< 30°; ≥ 30°) *	Soil **
	Drainage (Low; Good) *	Sand **
		Litter **
b) BIOTIC	Alfa diversity	Species (vascular plants)
	Shannon index	Life form of species
	Evenness index	
	Total coverage	
	Silene velutina coverage	
	Other species coverage	
	Number of species for life form	
	Coverage for life form	
c) DISTURBANCE	Phytophagi and parasites *	
	Grazing *	
	Trampling *	
	Fire *	
	Exotic species *	
	Garbage *	
	Other disturbances *	
d) POPULATION	Abundance of individuals (seedling, sapling, adults, total)	
	Number of floral stems x adult	
	Number of mature fruits x floral stem	
	Number of seeds x fruit	
	Seed set x adult	

For the study of the biotic variables (Tab. 2b), at each plot node all vascular plants were recorded with the aim to calculate the biodiversity indexes (total number of species, Shannon index and Evenness) and the coverage of vascular plants (total coverage, *S. velutina* cover and other species cover) per plot. The Shannon index (H') was calculated according to Equation 1:

$$1) \qquad H' = \sum_{i=1}^{s} - (Pi * \ln Pi)$$

where S is the number of species in the plot and Pi is the species relative abundance per plot.

The Pielou's Evenness index (J) was calculated using Equation 2:

2) $J = H'/H_{max}$

where H' is the Shannon index per plot and H_{max} is the maximum possible value of H' (if every species was equally likely) that is equal to lnS, where S is the total number of species per plot.

In order to observe whether the community was constituted mostly of rare or dominant species, the species frequency in one, two and three or more plots was also detected. Therefore, every recorded species was classified according to its life form with the aim to calculate both the number of plant species and the coverage for each life form per plot.

With the aim to detect population structure of *S. velutina*, all individuals of the species at each plot were numbered, measured and attributed to one of three life stages: 1) *seedlings*: individuals with cotyledons often with one or two pair of leaves and with foliage diameter < 3cm, 2) *saplings*: non reproductive individuals in the year of the study with foliage diameter ≥ 3 cm, 3) *adults:* all reproductive individuals in the year of the study. In order to study the reproductive success, we detected the number of floral stems for each adult, estimated the number of mature fruits for each floral stem and the seed set for each adult individual. Because of the dehiscence of capsules, the number of seeds per fruit was calculated including the seed loss that was estimated for each site. With the aim to calculate the percentage of seeds lost since the time of sampling, six fruits for 15 individuals at each site were enveloped with a little bag of teaseled cotton at the beginning of the fruiting. Field work was conducted in July 2014, when all populations were at the same phenological phase or rather at the end of fruiting.

DATA ANALYSIS

In order to verify whether the abiotic variables (Tab. 2a) and the different types of disturbance (Tab. 2c), respectively detected as frequency and presence/absence per plot, were dependent to the size of the island (LI vs SI), χ^2 tests were carried out to compare the observed values of variables with the expected values. Similarly, heterogeneity tests were conducted to compare the same variables among the different sites on each type of island. For the quantitative response variables (both abiotic and biotic, Tab. 2a-2b), ANOVA (analysis of variance) was used to test the effect of site and island size, and to estimate the effect of each factor on the structure and the reproductive success of populations (Tab. 2d). In ANOVA, island size was considered as a fixed factor; site as a random factor and nested within island size. The homogeneity of variances was tested a priori using Cochran's C-test, if necessary data were appropriately transformed. Student-Newman-Keuls (SNK) tests were carried out to compare the mean values of all significant factors (Underwood 1997).

The stepwise Linear Discriminant Analysis (LDA) method was applied to characterize and discriminate the realized niche among sites and groups of islands. This method is useful to identify unknown groups characterized both by quantitative and qualitative variables (Duda *et al.*, 2000; Fisher 1936, 1940; Fukunaga 1990). A combination of predictor variables was found with the aim of minimizing the within-group distance and maximizing the between-groups distance simultaneously, thus achieving maximum group discrimination (Hastie *et al.*, 2001; Holden *et al.*, 2011; Kuhn & Johnson, 2013; Rencher & Christensen, 2012).

With the aim to reduce the overall complexity, differences in ecological niche of *S. velutina* between the LI and the SI were drawn by means of Principal Components

Analysis (PCA) and graphically represented in a three-dimensional graph using the first 3 discriminant functions corresponding to the coordinate system. Finally, ANOVA on the Coefficient of Variation (CV) was conducted to test the effect of the island size on the niche breadth at the local and regional spatial scale.

χ^2 and Heterogeneity tests were conducted using an excel spreadsheet, ANOVAs were performed with GMAV5 software package (University of Sydney), PCA and LDA were elaborated by means of SPSS software package release 16.0 (SPSS Inc. for Windows, Chicago, Illinois, USA).

RESULTS

Abiotic factors

Plot localization

Elevation and distance from the sea were not significantly different for sites on LI and SI (Fig. 3a-3b). On LI there were significant differences ($p < 0.05$) among sites for both variables. The *Src* and *Cfn* sites (see Tab. 1 for population codes) showed both an elevation (ca. 60 m a.s.l.) and a distance from the sea (ca. 40 m) higher than the other sites. On SI differences among sites were found only for the distance from the sea ($p < 0.05$). With regard to the land slope (Fig. 3c), we did not find significant differences ($p > 0.05$) between LI and SI. Both on LI and SI the slope was highly heterogeneous among sites ($p < 0.01$).

Substrate

As regards to the substrate, in both groups of islands highly-drained substrates were more common than the lowly-drained substrates (51 plots on LI; 48 plots on SI), with no significant differences ($p > 0.05$) in their frequency on LI and SI (Fig. 4a). A significant heterogeneity was observed among sites both on the LI ($p < 0.01$) and the SI ($p < 0.02$), where drainage was significantly higher in five and four (out of six) sites, respectively. With regard to substrate composition (Fig. 4b-4d), on the LI we observed a significantly higher ($p < 0.01$) stoniness and sandiness (respectively 9.7% and 31.8%) than SI (2.6% and 0%, respectively) and a significantly lower ($p < 0.01$) rockiness (18.7% vs 59.7%). Concerning soil cover (Fig. 4e) there was no significant difference ($p > 0.05$) between LI and SI (ca. 39% of soil coverage in both groups of islands) while the litter cover on LI (63.4%) was significantly higher ($p < 0.01$) than the SI (47.1%, Fig. 4f). On LI, the frequency of stones, rocks, sand, soil and litter were highly heterogeneous ($p < 0.01$) among sites (Fig. 4b-4f). On SI stone cover ($p < 0.05$) and rockiness, soil and litter cover ($p < 0.01$) were significantly heterogeneous whereas sand was absent from all monitore sites (Fig. 4b-4f).

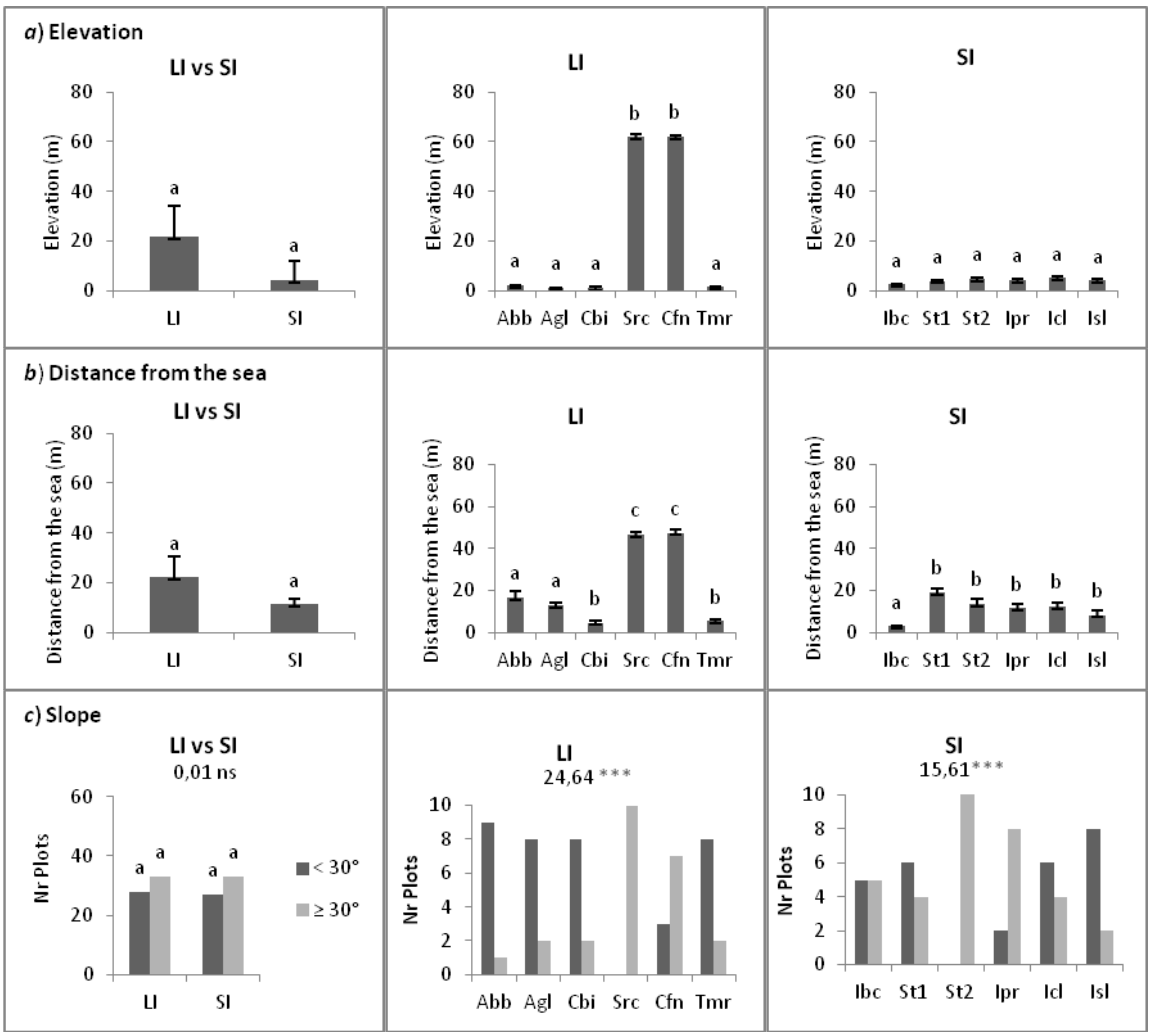
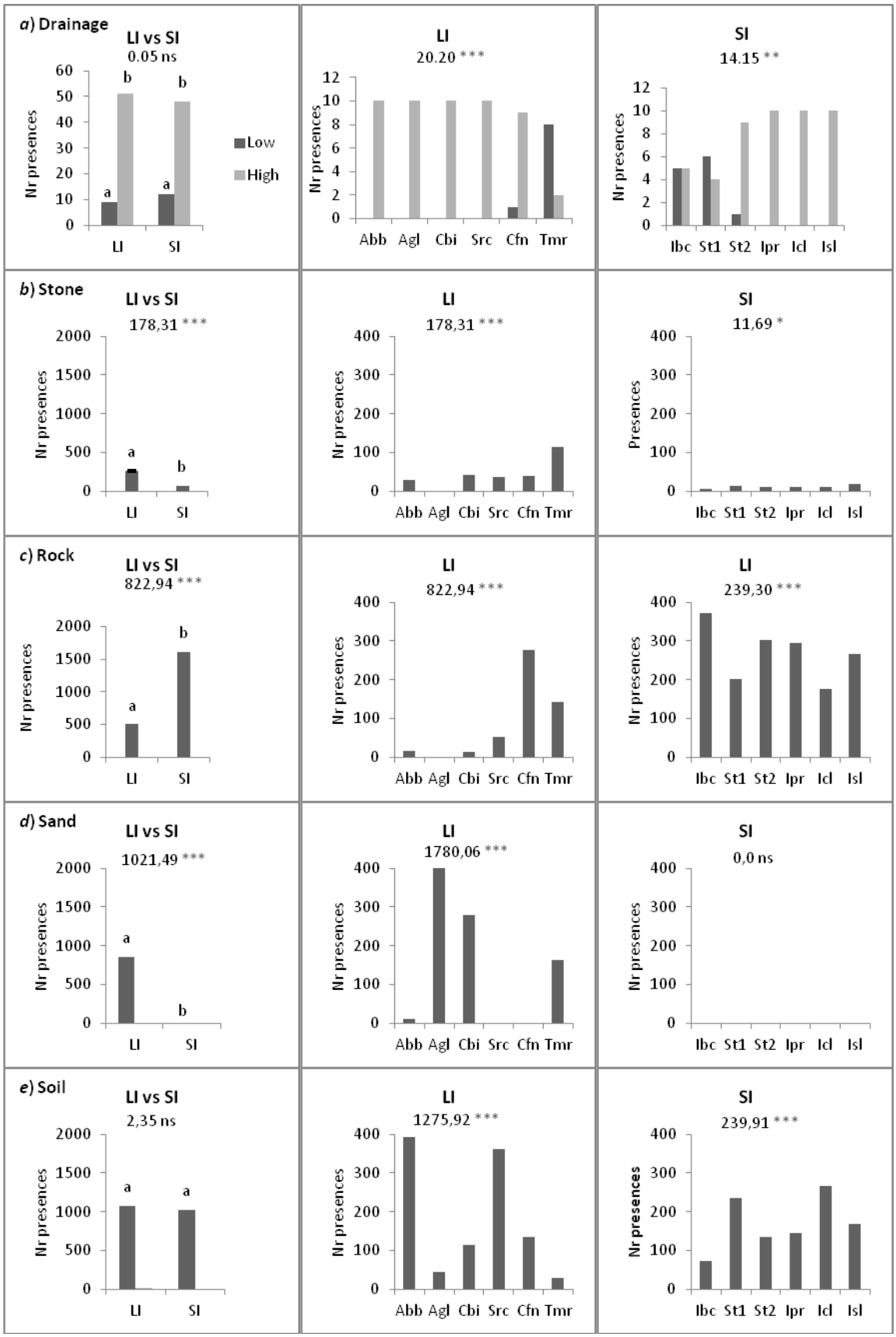


Figure 3 - Elevation (a), distance from the sea (b) and slope (c) on large (LI) and small (SI) islands). A two-way Anova was conducted to detect differences between the two groups of islands and among sites in *a* and *b*; data are the mean of six sites (± 1SE) and of ten plots (± 1SE), respectively; different lower-case letters show significant differences at $p < 0.05$ (SNK test). In *c* a $\chi 2$ test and a Heterogeneity test were conducted to detect differences between the two groups of islands and among sites, respectively; data are detected as presence/absence of the response variable for LI and SI (60 replicates) and for site (ten replicates); different lower-case letters show significant differences at $p > 0.05$ between LI and SI; p values were considered not significantly different ($p > 0.05$, ns) or significantly different ($p < 0.05$, *; $p < 0.02$, **; $p < 0.01$***), by $\chi 2$ and Heterogeneity tests. See Tab. 1 for the explanation of the site code.


a) Drainage
LI vs SI
0.05 ns
Nr presences
Low
High
LI
20.20 ***
SI
14.15 **
Abb Agl Cbi Src Cfn Tmr
Ibc St1 St2 Ipr Icl Isl
b) Stone
LI vs SI
178,31 ***
LI
178,31 ***
SI
11,69 *
Presences
c) Rock
LI vs SI
822,94 ***
LI
822,94 ***
LI
239,30 ***
d) Sand
LI vs SI
1021,49 ***
LI
1780,06 ***
SI
0,0 ns
e) Soil
LI vs SI
2,35 ns
LI
1275,92 ***
SI
239,91 ***
LI
SI

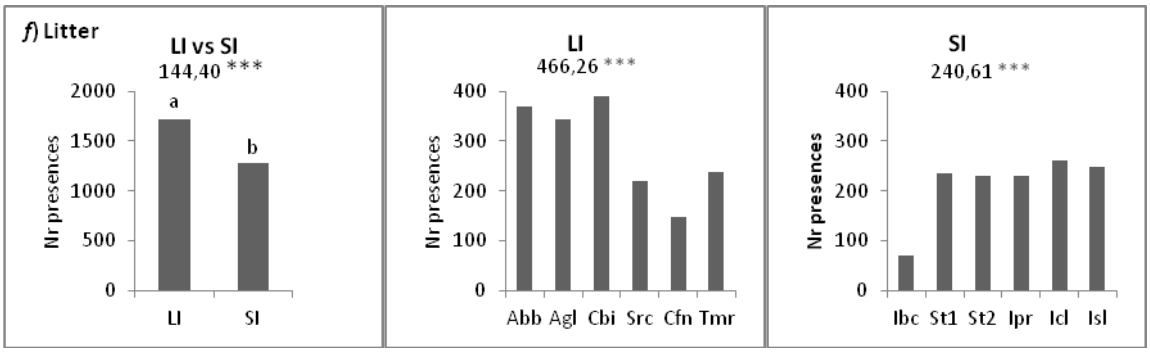


Figure 4 - Substrate characteristics in terms of (low-high) drainage (a) and of coverage of stone (b), rock (c), sand (d), soil (e) and litter (f) on large (LI) and small (SI) islands and the different study sites. A χ^2 test was conducted to detect differences between the two groups of islands (lower-case letters), while a Heterogeneity test was conducted to detect differences among sites within groups; 60 replicates in 2a and 2700 replicates in 2b-2f were considered for each group of islands; 10 replicate in 2a and 450 replicates in 2b-2f were considered for each site; each plot was 2 m^2 (45 nodes x plot). See Tab. 1 for the site code.

Biotic factors

Biodiversity

The biodiversity indexes were calculated per plot (2 m^2 of surface). The Shannon index (Fig. 5a) and the total number of species (Fig. 5b) were significantly higher ($p < 0.02$) on LI (respectively 1.6 ± 0.2 and 5.5 ± 0.6) than SI (respectively 0.9 ± 0.0 and 3,0 ± 0.1), while there was no effect of island size on the Evenness index ($p > 0.05$, Fig. 5c). For the Shannon index (ranged from ca. 1.0 to ca. 2.3 on LI and from ca. 0.9 to ca. 1.0 on SI) and the total number of species (ranged from ca. 3.5 to ca. 7.6 on LI and from ca. 2.8 to ca. 3.2 on SI) significant differences among sites ($p < 0.05$) were found on the LI, for which the *Agl* and *Src* sites showed the highest values (respectively 7.1 ± 0.5; 7.6 ± 0.4). The Evenness index did not show statistically significant differences among sites.

Plant cover

With regard to the plant coverage per plot (2 m^2), both the total (56,4% on LI; 41.1% on SI, respectively) and the *S. velutina* coverage (13.6% on LI; 22.4% on SI, respectively) were not significantly different between the two types of islands, while the other species coverage was significantly higher ($p < 0.01$) on LI (42.9%) than on SI (17.3%). We observed highly significant differences ($p < 0.02$) among sites only for the other species coverage on SI (ranging from ca. 7.6% of *Ibc* to ca. 45.3% of *Ids*).

Species frequency

Concerning the species frequency in 1, 2 and 3 or more plots, the LI showed a significantly higher values than SI; moreover, in both groups of islands the number of species detected in 3 or more plots was significantly higher than those of the species detected in one or 2 plots .

Life forms

The number of species per plot (2 m^2) did not differ between LI and SI for the Therophytes, Geophytes, Hemicryptophytes, and Chamaephytes, but for the Phanerophytes

it was statistically higher on LI (1.3 ± 0.3) than SI (0.1 ± 0.0). The number of species per plot was statistically different ($p < 0.05$) among sites for Therophytes (ranged from ca. 0.6 of *Abt* to ca. 12.9 of *Tmr*), Hemicryptophytes (ranged from ca. 0.8 of *Tmr* to ca. 6.1 of *Cfn*), Chamaephytes (ranged from ca. 0.9 of *Cbi* to ca. 11.5 of *Tmr*) on LI , while we did not find significant differences ($p > 0.05$) among sites for SI for any life form. The cover percentage per plot (2 m^2) of life forms between the two groups of islands, similarly to the number of species, was statistically different at $p < 0.05$ only for Phanerophytes, for which the LI showed higher values (ca. 23.7%) than the SI (ca. 0.9%). The coverage was statistically different ($p < 0.05$) among sites for the Therophytes on LI (ranged from ca. 4.0% to ca. 27.1%) and for Geophytes on SI (ranged from 0% to ca. 8.7%). Percentages of total coverage per group of islands (120 m^2, Fig. 6) showed higher values of Phanerophytes (40%), followed by Hemicryptophytes (26%) and Chamaephytes (18%) on LI, while on SI there was a very high prevalence of Hemicryptophytes (71%).

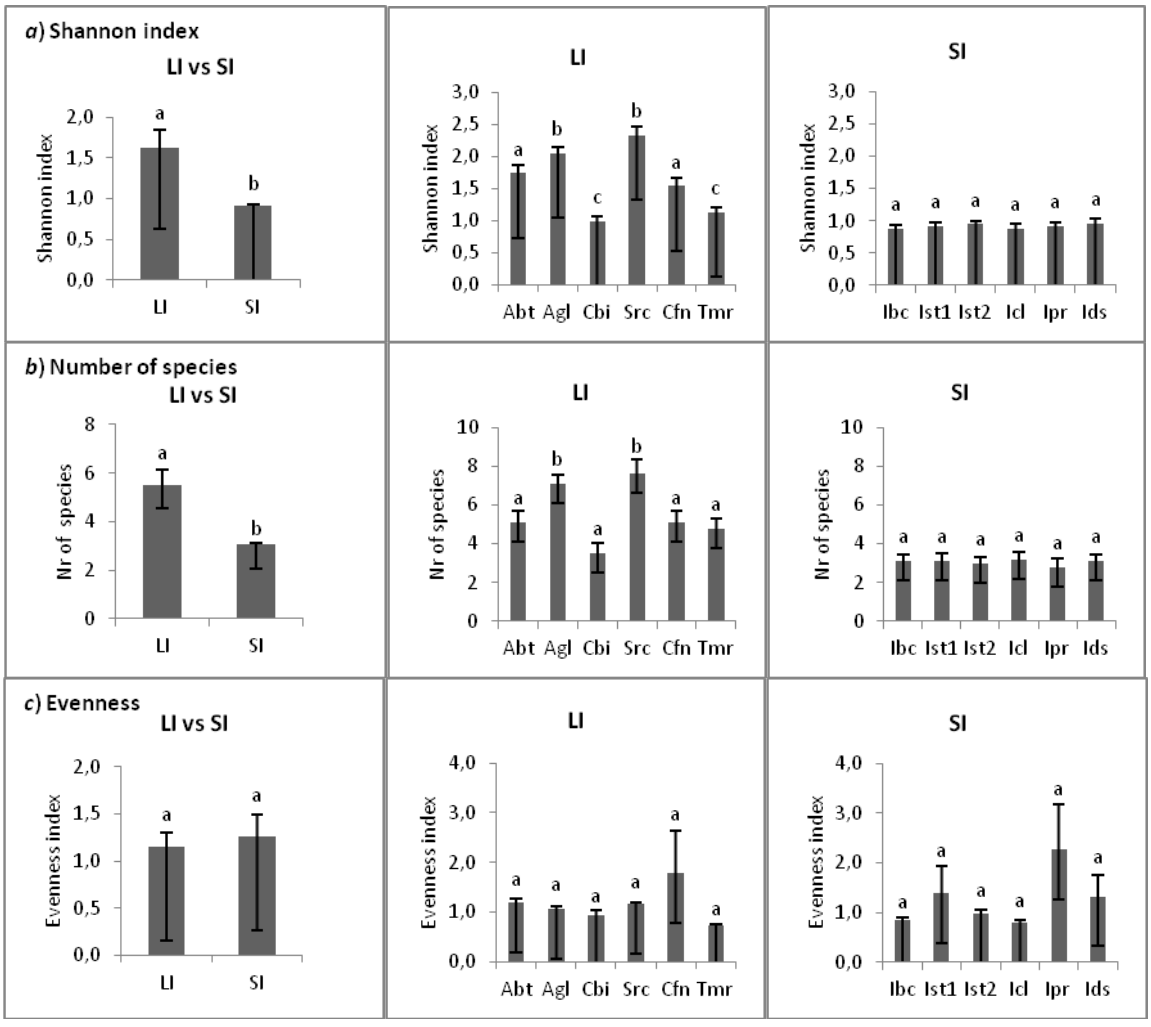


Figure 5 - Biodiversity indexes [Shannon index (a), number of species (b) and Evenness index (c)] for the LI, SI and related sites. A two-ways Anova was performed to detect the effect of the size of island and of different sites; data in the graphs are the mean of six sites (± 1SE) and of ten plot (± 1SE), respectively; plot surface was 2 m^2. Different lower-case letters show significant differences at $p < 0.05$ (by SNK test). See Tab. 1 for the site code.

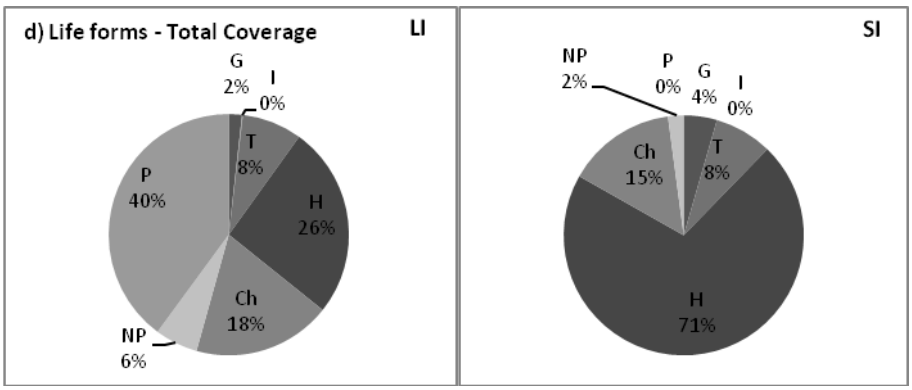


Figure 6 - Total coverage for life form on LI and SI.

Disturbance

As regard the disturbances detected at each plot, the presence of fire and phytophagi did not differ between LI and SI ($p > 0.05$), while significant differences ($p < 0.05$) were detected for grazing (LI ca. 12%; SI ca. 0%), trampling (LI ca. 37%; SI ca. 3%), presence of exotic species (LI ca. 20%; SI ca. 0%) and garbage (LI ca. 25%; SI ca. 7%), and highly significant differences ($p < 0.01$) were observed for the other types of disturbance (LI ca. 30%; SI ca. 0%). In all cases, the disturbance was significant higher on LI than SI. Regarding the heterogeneity per site, on LI were detected significant differences ($p > 0.025$) for all disturbances except for fire; on the contrary on SI there were not differences among sites in any case and we detected the absence of grazing, fire, exotic species and other disturbances.

Population parameters

Population structure

The number of seedlings, saplings and the total number of individuals of *S. velutina* were not significantly different ($p > 0.05$) between LI and SI, while significant lower values ($p > 0.05$) in the number of adults were detected on LI (1.1 ± 0,2 individuals 2 m^{-2}) than on the SI (1.7 ± 0.2 individuals 2 m^{-2}; Fig. 7).

Adult size

As regard the size of adult individuals (major axis, minor axis, height) there were no significant differences ($p > 0.05$) between the two groups of islands. On LI the major axis ranged from 47.3 ± 1.2 cm at *Agl* to 92.6 ± 10.9 cm at *Cbi*, the minor axis ranged from 28.5 ± 6.5 cm at *Cfn* to 64.7 ± 3.7 cm at *Cbi* and the height ranged from 19.7 ± 1.1 cm at *Agl* to 37.8 ± 4.7 cm at *Tmr*. Significant differences among sites were highlighted for LI islands, while no differences were found on SI.

Reproductive success

The reproductive success of adult individuals was estimated by means of the number of floral stems, mature fruits, seeds per fruit and seed set per adult individual. No variable showed significant differences ($p > 0.05$) between LI and SI. Both mature fruits and seeds per fruit did not show significant differences ($p > 0.05$) among sites on LI, while the number of floral stems (ranged from 5.3 ± 0.64 at *Agl* to 33.7 ± 6.0 at *Cbi*) and the seed

set per adult individual (ranged from 831.4 ± 181.8 at *Agl* to 9964.7 ± 2167.7 at *Cbi*) significantly differed ($p < 0.05$) among sites. No significant differences ($p > 0.05$) were found in the reproductive efficiency among SI sites.

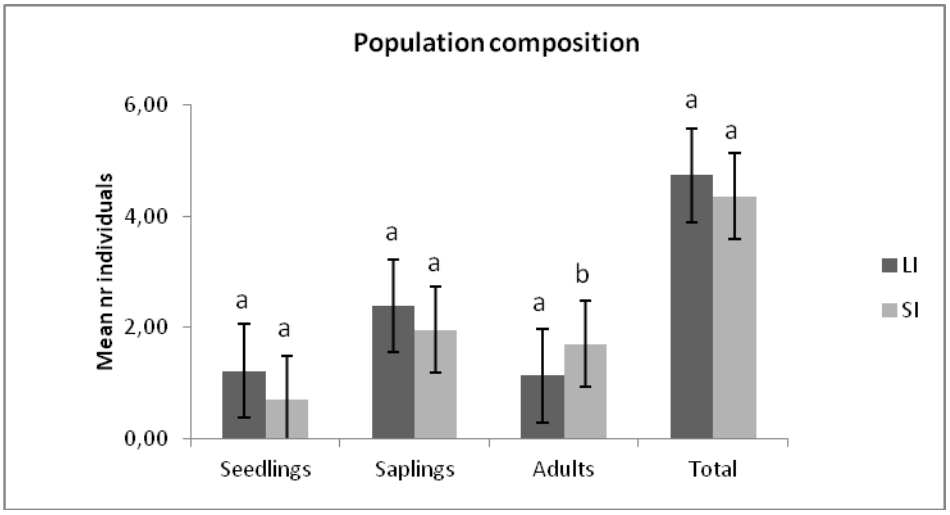


Figure 7 - Population composition (mean number of individuals for the different life stages: seedlings , saplings, adults, and total) per plot (2 m^2) for the LI and SI. A one way Anova was performed to compare the number of individuals of the different age class. Different lower-case letters show significant differences at $p < 0.05$ (by SNK test).

Ecological niche

Linear Discriminant Analysis (LDA) allowed us to better investigate on the niche differentiation between groups and among sites. The two groups of islands were well identified and classified, with percentage of correct identification of 91.6%, and a low percentage of misleading results LI were correctly identified at 86.7% while SI at 96.6% both with original data and after cross validation (Tab. 3). Elevation, sand, trampling, exotic species and rockiness were the five variables having a greater weight in the group distinction. In addition, we found a correct discrimination among sites of 32.7% (considering LI and SI together; data not showed), while analysis for individual group, showed a correct identification of sites of 58.3% (ranged from 40% for *Agl* to 0% for *Tmr*) for LI (Tab. 4) and of 25.0% (ranged from 40% for *Agl* to 0% for *Tmr*) for SI (Tab. 5).

Fig. 8 represents the distribution of the two groups of islands (LI and SI) on the basis of three principal components, which together explained the 53% of the variance. The first axis (PC1) was mainly influenced by the biotic factors (total number of species, Evenness, coverage of other species and total), the second axis was strongly influenced by the substrate characteristics (slope, stoniness and sandy coverage) and disturbance (exotic species and trampling), the third axis was principally influenced by *S. velutina* coverage and the presence of and grazing. The PCA graph allowed identifying the LI and the SI as two well-defined groups in space, although they showed a partial niche overlap. LI produced a wider point cloud than SI, showing a great variability of environmental conditions if compared to the relative homogeneity of SI.

Fig. 9 shows the niche breadth at two different spatial scales tested by means of CV analysis. At the local scale mean CV for several abiotic (elevation, distance from the sea, slope and litter coverage) and biotic (*S. velutina* coverage, other species coverage) variables and the disturbances presence (phytophagi) were higher on SI than LI. At the

regional scale, for the total abiotic factors, inverse patterns were detected, in fact the mean CV was higher on LI than SI.

Table 3 - LDA: Group classification. Percentages after cross-validation.

	LI	SI	Total
LI	**86.7**	13.3	100.0
SI	3.4	**96.6**	100.0

Table 4 - LDA: LI sites classification. Percentages after cross-validation.

	Abt	Agl	Cbi	Src	Cfn	Tmr	
Abt	**60.0**	10.0	20.0	-	.-	10.0	100.0
Agl	-	**40.0**	-	40.0	20.0	-	100.0
Cbi	10.0	-	**60.0**	-	-	30.0	100.0
Src	-	40.0	-	**60.0**	-	-	100.0
Cfn	10.0	20.0	10.0	.0	**60.0**	-	100.0
Tmr	-	-	10.0	.0	20.0	**70.0**	100.0

Table 5 - LDA: SI sites classification. Percentages after cross-validation.

	Ibc	St1	St2	Icl	Ipr	Ids	Total
Ibc	**20.0**	-	-	10.0	60.0	10.0	100.0
St1	60.0	**10.0**	-	30.0	-	-	100.0
St2	50.0	10.0	**20.0**	-	-	20.0	100.0
Cbi	40.0	10.0	-	**30.0**	-	20.0	100.0
IPr	70.0	20.0	-	-	-	10.0	100.0
Icl	-	30.0	-	-	-	**70.0**	100.0

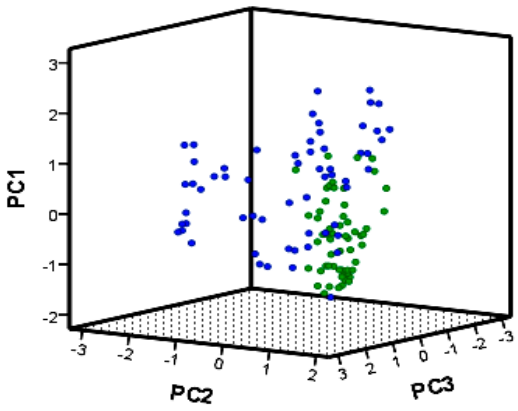


Figure 8 - PCA of Environmental variables (abiotics, biotics and disturbances). Each graph axis is represented by a principal component (PC1, PC2, PC3). Blue dots are LI, green dots SI.

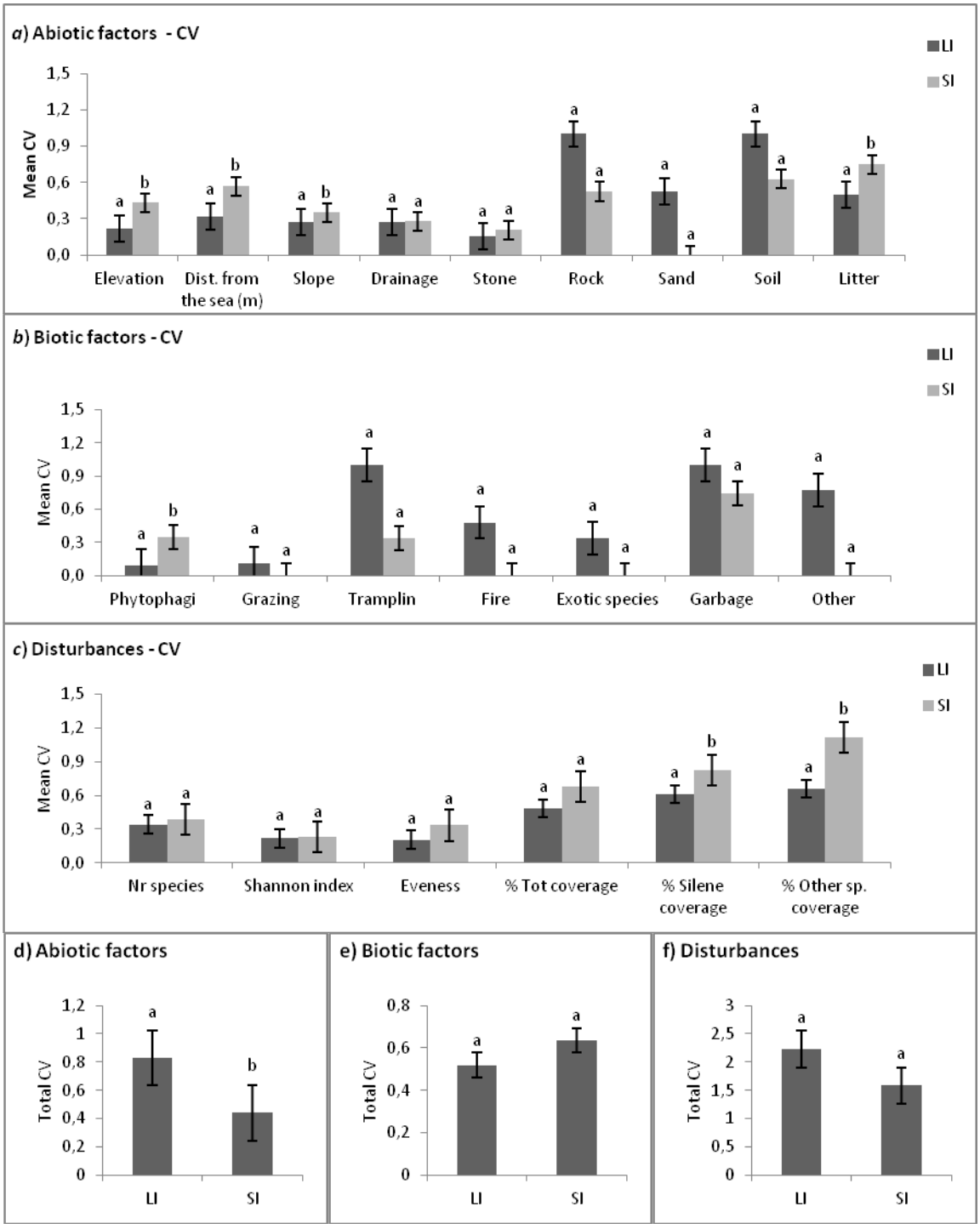


Figure 9- Coefficient of variability (CV) at local (a, b, c) and regional scale (d, e, f) for the abiotic and biotic factors and disturbances considered. A one-ways Anova was conducted to detect differences between the two groups of islands (LI; SI); data are the mean of six sites (± 1SE) at the local scale and the mean of CV of all variables for the regional scale. Different lower-case letters show significant differences at $p < 0.05$ (by SNK test). See Tab. 1 for the site code.

DISCUSSION

Characterisation of niche shift

Our results showed a niche shift between populations of LI and SI. In particular, we found a significant effect of island size on several paremeters related to substrate characteristics, biodiversity, plant coverage, community composition and the presence of some disturbances. LDA results highlighted a marked niche differentiation between the two groups of islands, showing a high accuracy in the identification (91.6%) of LI and SI populations, which was essentially due to the different patterns of abiotic factors (elevation, sand cover, rockiness) and the presence of some disturbances (trampling and exotic species). Moreover, the PCA revealed a high weight of some biotic factors (total number of species, evenness, coverage of other species and total cover) on the identification of similarities among populations of each islands group, and a higher variability of environmental conditions on LI than SI. Niche shift, in fact, is often determined by the spatial fluctuations of both environmental conditions (including disturbance regime) and biological interactions, such as for example competition (Bolnick *et al.* 2010; Devictor 2010). However, there are also other factors, which were not detected in this study, such as local adaptation and phenotypic plasticity, which may have a role, at the local level, in niche differentiation (Devictor 2010).

Large islands

On LI we detected a greater stone, sand and litter coverage than SI, and a high heterogeneity among sites for all abiotic variables considered. Such substrate composition variability and the presence of abundant organic matter were associated both with high diversity (Shannon index, total number of species) and cover of other species, and also the presence of habitats in advanced successional stages (woody vegetation). Therefore, on LI populations, individuals of *S. velutina* proved to be able to fit into highly different ecological conditions among sites, and to occur, both in sandy and rocky coastal locations, along the environmental stress gradient, from the early successional stages nearest to the sea, to the inland edge of the mature stages (communities with *Juniperus* sp.), where environmental stress decreases, the availability of resources increases, but competition is supposed to be more intense (Bertness & Callaway 1994). Such realized niche appears to be even more diversified because of the presence of several disturbances (grazing, trampling, exotic species, garbage and other disturbances) to which populations seems to respond by the increase of juveniles. The dominance of sapling individuals and a lower number of adult individuals on LI, in fact, suggest the presence of a life-history strategy based on rectuitment of seedlings. Examples of regeneration niche strategy (Grubb 1977) were already reported for species and populations from disturbed environments (Grime 2001). It is known in fact that a disturbance event can create local dynamics of extinction-colonization able to provide empty habitat patches that may be efficiently colonized by species/populations/individuals presenting a local fitness advantages (Farris *et al.* 2009, Büchi & Vuilleumier 2016). In particular, disturbance can favour species with high dispersal ability (e.g. high number of juveniles and propagules) and high fecundity (Büchi & Vuilleumier 2016), but can also produce positive effects for several non-competitive species (Farris *et al.* 2009).

Small islands

On SI we observed a high presence of rocky habitats, the absence of sandy substrates and low litter coverage. Moreover, in this environments, characterised by low/dwarf and sparse vegetation, as attested by the hemicriptophyte dominancy (71% of coverage), the absence of phanerophytes and the reduced coverage of other species, we detected lower biodiversity levels than LI, although the nearly complete absence of human induced disturbance. Therefore, the realized niche on SI populations was characterized by harsh and homogeneous environments (absence of heterogeneity among sites in 14 environmental variables out of 22), with low disturbance levels and potentially low competition. Similar ecological niche was reported for other narrow Mediterranean endemics, which were typically found in rocky areas on relatively unfertile substrates, and were associated to stressful habitats with low competition levels and infrequent human perturbations (Debussche & Thompson 2003; Lavergne *et al.* 2004). Concerning population composition, although there were no differences in the abundance of individuals between populations of the two islands groups, the proportion of adult individuals was higher on small islands than larger islands, hence, *S. velutina* on islets probably has a life-history strategy based more on persistence of adult individuals than on recruitment of juveniles, as instead occurs on LI. In particular, the persistence niche strategy (Bond & Migdley 2001) was found in populations of abiotically harsh and/or nutrient-poor environments such as rocky cliffs (Larson *et al.* 1999, 2000), where resprouting is prevented by resource limitation, but also in habitats with low levels of disturbance (Grime 2001).

Niche breadth

By means of CV analysis, at the regional scale we showed, for some abiotic and biotic variables and some disturbances, wider niche in *S. velutina* populations on LI than SI (Fig. 9d, 9e, 9f), while at the local scale inverse patterns were observed (Fig. 9a, 9b, 9c).

At the regional scale this was mainly due to spatial heterogeneity, which resulted positively correlated to the island size, but also, probably, to disturbance occurrence. It was reported in fact that disturbance can strongly modify the species niche breadth, affecting the dispersal abilities of individuals, because it is able to produce areas free from competition (Büchi & Vuilleumier 2016). In agreement, several studies reported that generalist species are most frequent in heterogeneous and disturbed environments, while specialist species in homogeneous and poorly disturbed habitats (Futuyma and Moreno 1988, Devictor *et al.* 2008).

At the local scale, SI showed a higher niche breadth than LI. Such a result may be coherent with a niche expansion associated with low biodiversity, plant coverage and absence of phanerophytes on SI, and niche reduction maybe due to the increase of these factors on LI. In the presence of interspecific competition in fact, species can show a space use restriction and a niche shift (Van Valen 1965; Bolnick *et al.* 2010). Moreover, several studies have shown that, when exposed to low levels of competition, species can expand their occupied niche space because of a competitive release (Van Valen 1965; Bolnick *et al.* 2010). Consequently, species do not always occupy the part of their fundamental niche that corresponds to conditions in which individuals have higher fitness (Smith 2007), but only the part in which they are competitively dominant (Bolnick *et al.* 2010). Similar results have been found for both animals and plants (Grace & Wetzel 1982; Shimizu & Tabata 1991, Mesquita *et al.* 2007, Pannek *et al.* 2016).

CONCLUSIONS

Our study provides novel results concerning the realized niche of the coastal endemic *S. velutina*, showing niche differences between populations located on large and small islands. Moreover, we detect variation in niche breadth at two different spatial scales, a regional (group of islands) scale and a local (site) scale. To date, we did not find any study which investigated on the niche dynamics in relation to the island size, and only few studies which examined niche breadth at different spatial scales, even though the realized niche is a highly scale dependent (Gaston *et al.* 1997; Hughes 2000).

This kind of study can provide useful information, not only from a theoretical viewpoint but also at the management level, which is urgent to acquire for rare species like *S. velutina*, for which population dynamics are poorly understood, even in protected areas. Therefore, for this priority species (under Habitats Directive EEC 92/43), confined to few coastal sites in Sardinia and Corsica, the options to menage different populations could be rapidly optimized, taking into account how vary the demographic strategies of populations (e.g. life-hystory based more on persistence or regeneretion), in relation to changes in community composition (e.g. phanerophyte presence/absence), interspecific competition levels and disturbance regime, which resulted to be associated to different geographycal contexts where the species occurs (large or small islands).

This work clearly shows the need to consider detailed quantitative data, concerning both environmental and population parameter, to correctly characterize species niche, which should be detected in population located in different ecological and/or geographical contexts, within the distribution range of species; moreover, it underline the importance to detect population dynamics associated to changing of environmental parameters at different spatial scales.

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GENERAL CONCLUSIONS

Thanks to its multidisciplinary approach, this Ph.D. thesis contributed to the knowledge of some rare and/or endangered *taxa* of the *S. mollissima* aggregate, for which, to our knowledge, no study or only few studies were previously carried out. In particular, with the overall aim to support both their *in situ* and *ex situ* conservation, the current taxonomic treatment was assessed by means of seed morpho-colorimetric analysis, while the ecological requirements of some species and their populations, were explored both *ex situ* (performing seed germination and seedlings growth tests under laboratory conditions), and *in situ*, through the ecological niche study.

The results reported in Chapter I confirmed the validity of the applied seed image analysis technique in providing key information about the identification of the investigated *taxa* and their inter-population differentiation. We confirmed the current taxonomic treatment for the Tyrrhenian species of the *S. mollissima* aggregate, both at section and species level, although further investigations (regarding the systematic position of *S. hicesiae* into the whole aggregate and the effective differentiation degree of *S. ichnusae* from *S. velutina*) would be necessary. At population level, the employed method was able to detect both the presence of close connections and/or high differentiation rates among populations, as shown, respectively, both for the Tuscan (Elba and Capraia) and Hyères (France) populations of *S. badaroi*, and for those of core and edge areas of the distribution range of *S. velutina*. Moreover, as already found by several authors (Chowdhuri 1957; Melzheimer 1987; El-oqlah 1990; Keshavarzi *et al.* 2015; Yildiz 2002 2006a, 2006b; Hong *et al.* 1999; Fawzi *et al.* 2010, Camelia 2011, Bacchetta *et al.* 2014), our study confirmed that shape, color and seed coat features represent diagnostic characters useful to distinguish *taxa*.

The results of the tests reported in Chapter II and III, provided both useful details for *ex situ* propagation and conservation of the studied species, and some important information about their phenology and ecological requirements, during some critical phases of their life cycle. In particular, the optimal protocols for seed germination and seedling growth of some species of the *S. mollissima* aggregate were identified, as well as inter- and intra-specific variability were identified in relation to the studied parameters. Seeds of all the investigated species were non-dormant, and inter- and intra-specific differences in the responses to light, salinity and recovery were observed. In the studied Tyrrhenian *taxa*, in contrast to other Mediterranean coastal species (Thanos *et al.* 1989; Doussi & Thanos 2002), the germination was significantly improved by light. For all the seed lots of the three investigated species, the higher germination percentages were detected in the range 5-15°C, in accordance with Thanos *et al.* (1989; 1995), for whom germination at low temperatures is an extended trait in several Mediterranean coastal species. In spite of this, *S. velutina* and *S. ichnusae* seeds showed the capability to germinate with high percentages also at 20°C, differently to *S. badaroi*. These results may correspond to a field germination in a period from autumn to early spring for *S. velutina* and S. *ichusae* and mainly concentrated during the autumn and winter months for *S. badaroi*. Moreover, for all the Tyrrhenian species, the positive responses to the alternating temperature regime suggested that their field germination can occur preferably in the soil layers near the surface, where the effect and the influence of the alternation of temperatures is higher. The three *Silene* species had different salt tolerance limits (300 mM NaCl for *S. velutina* and *S. ichnusae* and 100 mM for *S. badaroi*) and despite the detected intra-specific differences, their germination was in all cases higher under control conditions (0 mM NaCl). *Silene velutina* and *S. ichnusae* showed a high ability to recover their germination after the exposure to salt, while seed germination recovery of *S. badaroi* decreased to increasing salinity at the temperatures of 5°C and 20°C. These results showed that seeds of these species may also

tolerate relatively high salinity values in the substrate, but that their germination in the field may occur only when, due to rainfalls, salts concentration decreases in the soil. Moreover, the addition of KNO_3 did not affect germination and recovery under salt conditions, and was unable to alleviate salt stress, contrary to findings ofdifferent authors for other coastal species (Khan 2003; Zehra *et al.* 2013). Conversely, for the Ibero-Levantine species examined in the Chapter III, light did not affect seed germination percentages. Concerning temperature, the two studied species showed different optimal ranges. *Silene hifacensis* germinated more at the lowest temperatures (10 and 15°C), while for *S. mollissima* germination was low only at the highest temperatures (25°C). Both species showed the ability to germinate under salinity conditions up to concentration of 250 mM NaCl, although intra-specific differences were showed. The highest germination occurred, also for these species, in the control treatement (no salt) and decreased with increasing salinity. Irrespective of the tested temperature, *S. mollissima* and *S. hifacensis* seeds were able to recover their germination totally after the NaCl exposure. Therefore, for the two Ibero-Levantine endemics, our results are consistent with a field germination from autumn to early spring for *S. mollissima*, and limited to the autumn-winter months for *S. hifacensis,* when the soil salinity decreases due to the increase of rainfall. A regards the dry weight and the growth rate of seedlings, those of both *S. mollissima* populations were promoted by the increasing temperature, while this pattern was observed only for one *S. hifacensis* population.

In Chapter IV were detected the effects of both the island size and the spatial scale on the microniche change of populations of the coastal endemic *Silene velutina*, a species listed with a priority status in the Habitat Directive 92/43 EEC, included in the IUCN Red Lists as near threatened (NT; Buord *et al*. 2011) and considered vulnerable (VU) and endangered (EN) in the French and Italian Red Lists, respectively (Olivier *et al.* 1995; Conti *et al.* 1997; Pisanu *et al.* 2014). As for the niche characterization, on small islands (SI) we found essentially the presence of less disturbed but harsher environments than large islands (LI), with the presence of low and sparse vegetation, while LI resulted to be more heterogeneous among sites, regarding the abiotic factors, with a highest biodiversity and plant coverage than SI and the presence of woody vegetation. As regards the niche breadth, at the regional scale we found a wider niche on LI than on SI. This pattern was mainly imputed to environmental heterogeneity, which resulted positively correlated to the island size. On the contrary, at the local scale, we detected a larger niche on SI than LI. These results may be coherent with a niche expansion phenomenon in SI, associated to release of competition, and with a niche reduction on LI probably due to the increase of competition.

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