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Adaptations of plant species to environmental changes

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Forse il più bel fiore è la rosa. Ma il giglio è così pieno di mistero!

Géza Gárdonyi

Abstract

The future climate change represents the biggest challenge for plant and animal species. The scenarios forecasted by the IPCC predict a global increase in temperatures and regional changes in rainfall during this century. Therefore, studying the relationship between climate and several traits of species is crucial in our understanding of the effects that future changes may have on plants. Species growing along a wide environmental gradient are a suitable study model to verify the relationship between intraspecific variability and both current and future climate. The aim of my PhD project is to analyse the relationship between environmental variability and variation in morphological traits, germination capacity and genetic structure of *Lilium pomponium*, using a multidisciplinary approach. The results of this study may enhance our understanding about the possible responses of this threatened species to environmental changes. *Lilium pomponium* is an endemism of the Ligurian and Maritime Alps spanning along a wide altitudinal gradient (100 and 2000 m a.s.l.), ranging from a Mediterranean to a subalpine climate. Our results show that the populations exposed to different environmental pressures have variations in floral characteristics, these variations likely play an important role in reducing variability among populations in reproductive output. Germination tests show that the increase in temperature may negatively affect seeds germination capacity. Nevertheless, projections of thermal requirement for seed germination under future climatic conditions suggest that populations will probably respond to future temperature increase shifting their germination phenology. Moreover, in the future the species may find suitable ecological conditions for seed germination in new areas at higher altitudes. Finally, genetic analysis suggests that there is no clear pattern of diversity and differentiation between populations, which may be related to the topographic complexity of the areas and to the biogeographical history of the species. Overall, the results obtained suggest that high among-populations variability found in *L. pomponium* may be a bet-hedging strategy to cope with unpredictable environmental conditions occurring in Mediterranean climate and that it might also represent a successful strategy to face the future environmental change.

Riassunto

Il futuro cambiamento climatico rappresenta la sfida più grande per le specie vegetali e animali. Gli scenari previsionali dell'IPCC annunciano un aumento globale delle temperature e dei cambiamenti regionali delle precipitazioni nel corso di questo secolo. Pertanto, lo studio della relazione tra il clima e i diversi tratti delle specie è fondamentale per comprendere gli effetti che i futuri cambiamenti potrebbero avere sulle piante. Le specie che crescono lungo un ampio gradiente ambientale possono essere un modello di studio adatto per verificare la relazione tra la variabilità intraspecifica e il clima attuale e futuro. Lo scopo del mio progetto di dottorato di ricerca è quello di analizzare la relazione tra variabilità ambientale e variazione dei tratti morfologici, della capacità germinativa e della struttura genetica di *Lilium pomponium*, attraverso un approccio multidisciplinare. I risultati di questo studio possono migliorare la nostra comprensione delle possibili risposte di questa specie minacciata al cambiamento ambientale. La specie è un endemismo delle Alpi Liguri e Marittime distribuita lungo un ampio gradiente altitudinale (100 e 2000 m s.l.m.), da aree con clima mediterraneo fino ad aree con clima subalpino. I nostri risultati mostrano che le popolazioni esposte a diverse pressioni ambientali presentano variazioni delle caratteristiche floreali, che probabilmente svolgono un ruolo importante nel ridurre la variabilità della produzione riproduttiva tra le popolazioni. I test di germinazione mostrano che l'aumento della temperatura potrebbe influire negativamente sulla capacità germinativa dei semi. Tuttavia, le proiezioni dei requisiti termici per la germinazione dei semi in condizioni climatiche future suggeriscono che le popolazioni risponderanno probabilmente al futuro aumento della temperatura spostando la loro fenologia di germinazione. Inoltre, in futuro la specie potrebbe trovare condizioni ecologiche adeguate per la germinazione dei semi in nuove aree ad altitudini più elevate. Infine, l'analisi genetica suggerisce che non esiste un chiaro modello di diversità e differenziazione tra le popolazioni, che può essere correlato alla complessità topografica delle aree e alla storia biogeografica della specie. Nel complesso, i risultati ottenuti suggeriscono che l'elevata variabilità riscontrata tra le popolazioni di *L. pomponium* potrebbe rappresentare una

strategia per far fronte a condizioni ambientali imprevedibili, frequenti nel clima Mediterraneo, inoltre potrebbe rappresentare una strategia di successo per affrontare il futuro cambiamento ambientale.

Chapter 1.

Introduction

Understanding how plant species respond to the rapid and complex environmental changes represents one of the major challenges to biologists in conserving biodiversity. According to the Millennium Ecosystem Assessment (MEA, 2005) climate change is the largest forthcoming threat to biodiversity, and many authors (McCarty 2001; Parmesan & Yohe 2003; Root et al. 2003; Walther et al. 2002) recognize it as one of the major threats for the plants survival. During the 20th century global average temperature has risen by approximately 0.7 °C, because of gas emissions by human activities (IPCC 2007). It is expected that increase in temperatures will continue in the coming decades, in fact, using different climatic scenarios and emission models the IPCC report asserts that global average temperatures increase will range from 2 to 5 ° C by 2100 (IPCC 2013). "The predicted increase in temperature and altered rainfall will have dramatic consequences are expected for biodiversity" (Williams et al. 2003; Colwell et al. 2008), species will face new conditions that will be likely lethal unless species feature enable to respond adequately.

Natural plant populations can respond to climatic change in four ways: they may migrate, adapt to new environmental conditions, decline into relictual populations like some tertiary endemics of the Mediterranean flora (Thompson 2005) or become extinct. The importance of these different pathways as ways to cope with environmental changes can vary on the base of different factors including the dispersal capability of species, the availability of alternative habitats, the rate and extent of environmental change. Clearly, a combination of multiple answers cannot be ruled out (Davis & Shaw 2001). The most common response of species to climate change is the shift to higher altitude or latitude to keep pace with the shift in climatic conditions to which they are adapted. However, it was suggested that plant adaptation may play an important role in species survival as the rapid rate of current climate change makes many plant species incapables of tracing the climate to which they are currently adapted (Gienapp et al. 2008). Nevertheless, our knowledge of the potential adaptation of species is still rather limited (Parmesan 2006).

Reproductive fitness (i.e., number of seeds per flower, seed size and seed set) and performance (i.e., genetics, physiology, morphology and demography) may vary across the geographical range, decreasing from places with optimal ecological condition to places where survival is difficult. The theory of a gradual decline in fitness and performance arose in the early 1980s (Hengeveld & Haeck 1982; Brown 1984) as "centre-periphery hypothesis" (CPH), later called also ‘abundant centre’ (Sagarin. 2002) or ‘central-marginal’ hypothesis (Eckert et al. 2008). The CPH predicts that populations at geographical periphery are smaller, less abundant and fragmented, more prone to extinction and genetically less diverse than populations at the centre of distributional range (Pironon et al. 2015) because they occur in harsh ecological conditions. Therefore, to fully understand the response of species to future environmental change, it is fundamental to figure out the relationship between environments and plant traits both in central and in marginals populations.

For this reason, the purpose of my Ph.D. thesis is to investigate the relationship between intraspecific variability and environment in *Lilium pomponium* L., a species threatened by future climate change (Casazza et al., 2014). To raise our awareness of how plant traits affect reproductive performance of populations in different bioclimatic conditions along an altitude range may help us to best understand the possible response of species to future environmental changes. *Lilium pomponium* is a suitable species model because it has a restricted geographical range and a wide altitudinal distribution spanning from Mediterranean to subalpine climate, these features allow us to easily sample the entire intraspecific variability.

In particular, this thesis has four main research goals: first, to examine the phenotypic and reproductive responses of *L. pomponium* to environmental variation along this distribution range **(chapter II)**; second, to characterise the spatial and temporal model of the response of seed germination to temperature and its possible displacement caused by future climate change **(chapter III)**; third, to determine whether the seed mass and germination capacity change along an altitudinal gradient and whether the temperature affects the germination of the seeds of *Lilium pomponium* **(chapter IV)**; fourth, assessing the genetic structure of *Lilium pomponium* population **(chapter V).**

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Chapter 2

Effects of environmental heterogeneity on the phenotypic variation of a lily endemic to the Maritime and Ligurian Alps

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Abstract

Geographical limits are assumed to be coincident with ecological margins, but this assumption might not always be true. Highly heterogeneous environments selection, like Alpine and Mediterranean ecosystems, may favour high phenotypic variability and increase the adaptative potential in peripheral populations. Floral traits are often found to be less variable and less affected by environmental heterogeneity than vegetative traits because variation in floral morphology may have negative effects on the reproductive functionality of individual plants. All these intraspecific variations might cause plants to adjust their resource allocation strategies in response to environmental change. For this reason, it is important to quantify variation in floral traits and plant fecundity. The objective of the present study is to examine the phenotypic and reproductive responses of a lily endemic to the Maritime and Ligurian Alps to environmental variation across this distribution range. In *Lilium pomponium*, marginal climatic populations resulted both at marginal geographical extremes and close to the centre of the distributional range of the species, showing that geographical and environmental gradients are not necessarily concordant. The selection on floral traits is dependent on local contexts in different environmental condition where populations occur because the array of interaction among resource availability, pollinator-mediated selection and population size may differentially affect floral traits. Contrary to the general expectation, in this study all groups had a similar and moderate seed production because each group was limited by different factors and it resulted to be condition by the life-history strategy of the species.

Key words

Lilium pomponium, climatic niche, phenotypic response, environmental variation, marginal populations

Introduction

There is much interest in ecology and evolution in the occurrence of trait (genetic, physiological, morphological and demographical) variation across from the centre towards the geographical periphery of species' distributions (Sagarin 2002; Eckert et al. 2008; Pironon et al. 2015). In particular, as the centre-periphery hypothesis (CPH) predicts, geographically isolated peripheral populations are expected to be divergent from central populations and to be smaller, less abundant and more isolated from each other than central populations, features that are likely to significantly affect levels of both neutral and adaptive genetic diversity when compared to central populations (Pironon et al. 2016). Such genetic and morphological divergence from central populations could trigger important speciation events (Lesica et al. 1995).

In many studies geographical limits are assumed to be coincident with ecological margins, but this assumption might not always be true (Soulé 1973; Pironon et al. 2016). First, peripheral populations may occur in conditions similar to those in the centre of the range (Piñeiro et al. 2007; Kropf et al. 2008). Second, environmental factors may impose ecologically marginal conditions in any part of the species' range (Soulé 1973 – hereafter "marginal populations"). Third, geographically peripheral populations may not occur in marginal conditions but simply in different ecological conditions (Papuga et al. 2018). In particular, in Alpine and Mediterranean ecosystems, environmental factors change over very short distances because of the high topographic complexity (Körner 2003; Thompson 2001), leading to differences in abiotic and biotic resources. Therefore, these highly heterogeneous environments selection may favour high phenotypic variability and increase the adaptative potential (Graae et al. 2018). For all these reasons, geographically peripheral populations have been considered very important for species studies (Lesica 1995; Hampe and Petit 2005).

Floral traits are often found to be less variable and less affected by environmental heterogeneity than vegetative traits because variation in floral morphology may have negative effects on the reproductive functionality of individual plants (Berg 1960; Frazee and Marquis 1994). Nevertheless, both abiotic and biotic factors may induce a selective pressure acting on the floral phenotype (Galen et al. 1999). Intraspecific variation has been detected in anther-stigma distance (Griffin and Willi, 2014; Papuga et al. 2018), in floral display (Dai et al. 2017; Lambrecht et al. 2017), and in pollen – ovules ratio (Guo et al. 2010; Dai et al. 2017). All these intraspecific variations might cause plants to adjust their resource allocation strategies in response to environmental change (Matesanz et al. 2010; Merilä and Hendry 2013). For this reason, it is important to quantify variation in floral traits and plant fecundity (pollen grains and seed set) across a species' range in order to understand factors shaping the distributional patterns of species in response to climate change (Gaston, 2003). Nevertheless, relatively few studies have been conducted to understand relationship between floral polymorphism and environment in range restricted species that are particularly prone to extinction risk as a result of climate change.

Lilium pomponium L. is a perennial, endemic geophyte to the Maritime and Ligurian Alps (Figure 2.1) that grows only on calcareous outcrops from 100 to 2,000 m of altitude, from a typical Mediterranean climate to a cool-summer continental type climate in subalpine habitats. The objective of the present study is to examine the phenotypic and reproductive responses of *Lilium pomponium* L. to environmental variation across this distribution range. Specifically, the goals of this study were: (i) to assess whether geographical periphery and ecological marginality are coincident; (ii) to assess whether peripheral (ecologically marginal) populations differ from central populations in floral trait variation, reproductive output and mating system.

Material and Methods

Study species

Lilium pomponium has hermaphrodite flowers, although male flowers can occur (ca. 10%, personal observation). Anthesis usually lasts from May to July and capsules develop from late July to

September, according to local climatic conditions. Reproductive output is low, flowers are self-incompatible (Casazza et al. 2018) and show ‘*approach herkogamy*’, that is, the stigmas are above the anther levels (Fryxell 1957; Webb and Lloyd 1986). These traits limit self-pollination and promote outcrossing (Webb and Lloyd 1986) and reduce sexual interference (Barrett 2002).

Study occurrences and climatic data

The distributional range of *L. pomponium* extends from Neva Valley in northwest Italy to the Verdon Valley in southwest France (Figure 2.1).

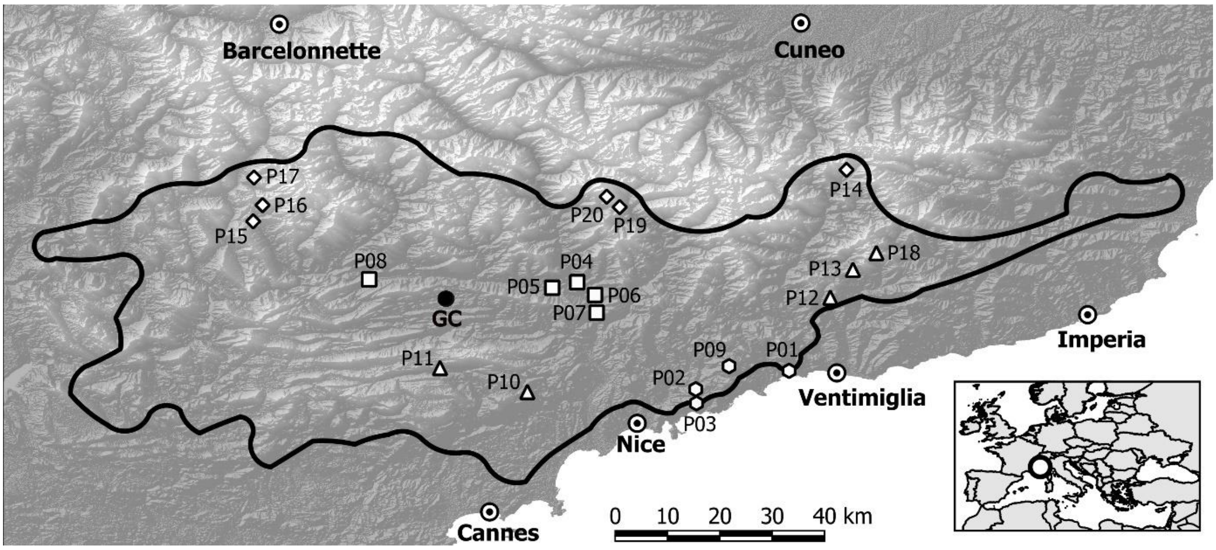

Figure 2.1. Distribution area and selected populations of *Lilium pomponium* L. Populations’ symbols: hexagon = Mediterranean marginal; triangle = central populations; square = continental marginal; rhombus = subalpine marginal. Filled circle = Geographic centre (GC). Populations’ codes are those reported in Table 2.1

The distribution of *L. pomponium* is almost continuous and there are no clear physical barriers. Species occurrences were obtained from field surveys (performed by the authors) and from local databases: SILENE (Conservatoire Botanique National Méditerranéen de Porquerolles; flore.silene.eu/) and LiBiOss (Regione Liguria; http://www.cartografiarl.regione.liguria.it/Biodiv/Biodiv.aspx). Occurrences were spatially filtered and those closer than 1 km to each other were removed, resulting in a final data set of 809 occurrences. To study climatic conditions of populations we downloaded nineteen bioclimatic variables representative of the period 1979–2013 from the CHELSA climate database website (http://chelsa-climate.org/) at 30-s (c. 1 km) spatial resolution (Karger et al. 2017).

Definition of climatically central and marginal populations

To distinguish ecologically marginal and central populations we first characterized the climatic niche of *L. pomponium* carrying out a principal component analysis (PCA) of the bioclimatic variables using the ‘ade4’ package implemented in R (R Core Team 2018). We considered “central” and “marginal” the populations falling into and outside the 70% of confidence ellipse, respectively. Then, we grouped the marginal populations in different climate groups according to the PCA quadrants where they fall. Moreover, we tested differences among groups of populations because of mean annual temperature and annual precipitation using the non-parametric Kruskal-Wallis test. Kernel density plots were used to visualize the distribution of each variable.

Correlation between geographical and climatic distance

To test whether populations that are geographically peripheral are also ecologically different or marginal we calculated the Euclidean distance from each population to the climatic optimum in the PCA and the Euclidean distance from each population to the centre of the distributional range. We calculated the correlation between the two distances using Kendell-tau coefficient.

Floral polymorphism

To assess whether different groups of populations differ in floral traits we measured 566 flowers from six to 38 randomly chosen individuals per populations (according to the population size) during the years 2017–2018 (252 and 314 flowers in 2017 and 2018 respectively). The number of flowers analysed per population ranged from 10 to 81 (Table 2.1).

Table 2.1. Study site of *Lilium pomponium* populations. MM: Mediterranean marginal; CM: continental marginal; CC: central and SM: subalpine marginal.

Pop	Group	Country	Lat	Long	Alt	Pop size	N° measured flowers
P01	MM	Baisse Saint-Paul, Castellar (IT)	43.793	7.526	434	~ 100	25
P02	MM	Plateau Tercier, Sainte-Thècle (FR)	43.755	7.369	554	~ 250	33
P03	MM	Fort de la Revère (FR)	43.737	7.367	661	~ 40	16
P04	CM	Les Pras, La Tour (FR)	43.944	7.163	243	~ 50	10
P05	CM	Ciamp du Var, Maisson (FR)	43.935	7.120	267	~ 30	10
P06	CM	Route de la Tinée, Tournefort (FR)	43.919	7.187	230	~ 200	27
P07	CM	Route de Grenoble, Utelle (FR)	43.905	7.196	203	~ 100	20
P08	CM	Entrevaux (FR)	43.949	6.805	586	~ 20	17
P09	MM	Col de la Madone de Gorbio, Peille (FR)	43.801	7.423	915	~ 200	30
P10	CC	Col De Vence, Vence (FR)	43.757	7.0775	955	~ 200	31
P11	CC	Greolieres (FR)	43.797	6.9276	1014	~ 150	25
P12	CC	Mt. Comune, Pigna (IT)	43.918	7.5975	1123	~150	30
P13	CC	Mt. Lega, Pigna (IT)	43.966	7.6363	1326	~ 200	23
P14	SM	Castel Tournou, Tenda (FR)	44.125	7.6254	1334	~ 30	14
P15	SM	Méailles (FR)	44.037	6.6068	1313	~ 250	35
P16	SM	Peyresq (FR)	44.964	6.6231	1466	~ 70	20
P17	SM	Ondres (FR)	44.112	6.6082	1565	~ 200	31
P18	CC	Mt. Grai, Pigna (IT)	43.995	7.6772	1759	~ 500	40
P19	SM	L'adrechas, La Colmiene (FR)	44.075	7.2255	1646	~ 400	80
P20	SM	La Colmiene (FR)	44.078	7.2174	1789	~ 200	43

We analysed two traits involved in pollinator attraction: the number of flowers per inflorescence and the corolla surface. In particular, corolla surface was calculated as the surface of an oblate spheroid (Figure 2.2), measuring height and width of the corolla (the latter measured three times, one for each pair of tepals – Figure 2.2 a and b). We also analysed a trait involved in flower–pollinator interaction: the spatial separation of pollen presentation and pollen receipt; in particular, we measured the height of the stylous + stigma (Figure 2.2 c), the height of the six stamens (Figure 2.2 d) and anthers (Figure 2.2 e). We classified flowers in three categories: i) flowers showing approach herkogamy, stigma above the anther; ii) flowers showing reverse herkogamy, stigma below the anther; and iii) flowers without herkogamy, stigma among the anthers. Floral measurements were obtained in field with a digital caliper (error = ±0.01 mm).

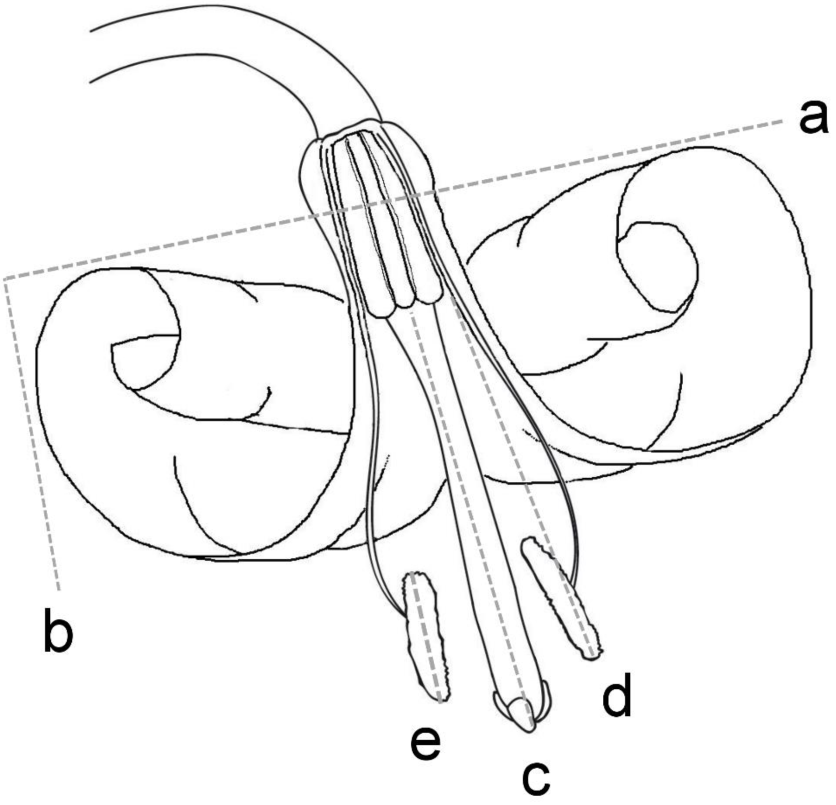

Figure 2.2 Floral measurements. **a**: corolla width; **b**: corolla length; **c**: stigma length; **d**: stamen length; **e**: anther length. Measurements of c and d were taken from the top of the ovary

Pollen limitation and reproductive performances

To test whether the self-fertilization rate was different between groups of populations we bagged 183 flower buds using non-woven fabric bags. Furthermore, to test whether the degree of pollen limitation leading to reduced seed production was different between central and marginals populations we assigned a total of 181 flowers to supplemental hand pollination.

For each population we quantified seed number on naturally pollinated flowers (Po) and supplementary-pollinated flowers (Ps) and thus obtained a value for pollen limitation (PL) using the formula of Baskin and Baskin (2017): PL = (Ps−Po)/Pmax [Ps or Po]. Values of PL range from 1 to -1. Positive values indicate a lower seed set in natural than in pollen-supplemented flowers, negative values indicate a higher seed set in natural than in pollen-supplemented flowers. Because the number of pollen donors may affect the reproductive success (Schemske and Pautler 1984), we collected pollen from at least three donors.

To estimate Ps (seed set in pollen-supplemented flowers; n=104) and Po (seed set in open-pollinated flower; n=374) mature capsules were collected before dehiscence, preventing seed dispersion. Seeds were counted under a Leica M205 C stereomicroscope. We calculated seed set as filled seeds/total number of ovules (filled seeds + aborted seeds + unfertilized ovules).

Statistical analyses

To test whether the groups of populations differed significantly in number of flowers per inflorescence, flower size, and pollens and ovules production, we applied the non-parametric Tukey-Kramer-Nemenyi post hoc test using the R ‘PCMMRplus’ package (Pohlert 2014), implemented in R (R Core Team 2018). To test whether the groups of populations differed significantly in the percentage of flowers belonging to different groups of populations, we used chi-squared or exact Fisher test when the expected frequencies was less than five in some cells.

Because seed production follows a binomial distribution, lacking the property of linearity and additivity, the effects of marginality of populations on plant fitness were analysed by fitting factorial generalized linear mixed models (GLMMS, logit link function, binomial distribution) to the seed set data with group of populations as fixed predictors, and populations as random factor. Statistical analyses were performed using the ‘lme4’ package (Bates et al. 2015) implemented in R (R Core Team 2018). Post hoc tests were conducted to evaluate pair-wise differences in measured traits between treatments using the ‘glht’ function in the ‘multicomp’ package (Hothorn et al. 2008) implemented in R (R Core Team 2018).

Results

Central and marginal populations

The first two axes of the PCA explain 76.84% of the variation of the whole data set. The ellipses drawn include 70% of the data of the group. This allowed us to recognize five central populations (hereafter CC) and 15 marginal populations (Figure 2.3).

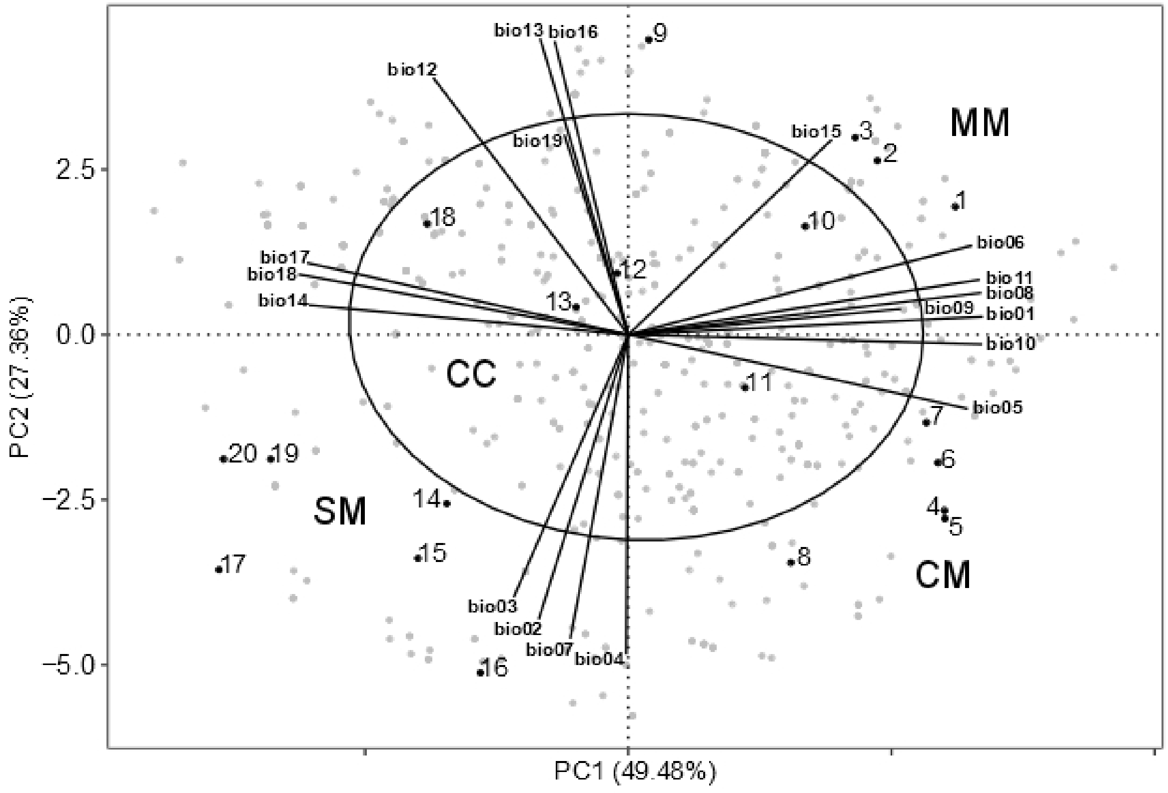

Figure 2.3. Principal component analysis of climate data for all known population locations (grey dots). Ellipses include 70 % of each class variance. Central population are inside the ellipse while the marginal populations are outside. Numbers indicate the sample populations. P01, P02, P03, P09: Mediterranean marginal (MM); P04, P05, P06, P07, P08: continental marginal (CM); P10, P11, P12, P13, P18: central (CC); P14, P15, P16, P17, P19, P20: subalpine marginal (SM). Populations’ codes

are those reported in Table 1. The nineteen bioclimatic variables (BIO01-BIO19) are those from the CHELSA climate database website

The marginal populations were further subdivided into three different groups growing under different climatic conditions (Figure 2.4). The first group, hereafter called Mediterranean marginal (MM) included four populations growing under Mediterranean conditions (warm and seasonal aridity; Figure 2.3 and 2.4). The second group, hereafter called continental marginal (CM) included five populations growing under continental conditions (warmest and driest conditions; Figure 2.3 and 2.4). Finally, the third group, hereafter called subalpine marginal (SM) included six populations growing under subalpine conditions (cold and wet conditions; Figure 2.3 and 2.4). Distance from the geographical centre and distance from the climatic centre were negatively correlated (tau: -0.22).

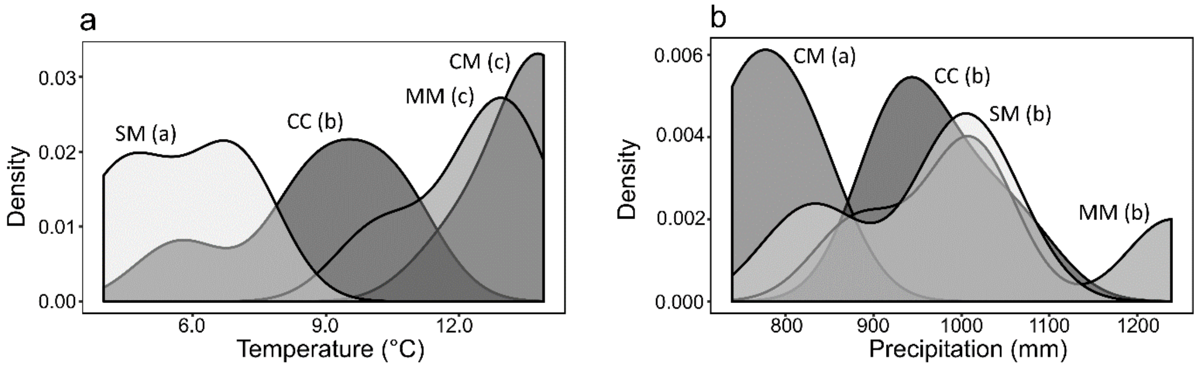

Figure 2.4. Kernel density plots of Temperature and Precipitation of the annual averages (**a** and **b**). MM = Mediterranean marginal populations; CM = central marginal populations; CC = central populations; SM = subalpine marginal populations. Letters in brackets are referred to statistical difference, *P*-values= 0.05

Floral polymorphism

Flowers were significantly larger in MM and CM (Figure 2.5 a); the mean flower size decreased from 3.14 mm^2 (MM), 3.03 (CM), 2.70 (CC) to 2.49 (SM) mm^2. The number of flowers per inflorescence was significantly lower in MM and CC (Figure 2.5 b). In particular, MM and CC plants bore 1.61 (sd=1.07) and 2.12 (sd=1.86) flowers, while SM and CM plants bore 2.89 (sd=4.64) and 2.90

(sd=2.06) flowers. In the majority of flowers (86 %) the stigma was placed at the level of anthers (i.e., no herkogamy); 12 % of flowers showed approach herkogamy and 2% of flowers showed reverse herkogamy. A significant difference in percentage of flowers with a separation between stigma and anthers was detected only between MM and the other groups (Figure 2.5 c). In particular, MM showed at the same time (Figure 2.5 c) the highest percentage of flowers with approach herkogamy (25.27%) and thus the lowest percentage of flowers without herkogamy (73.63%).

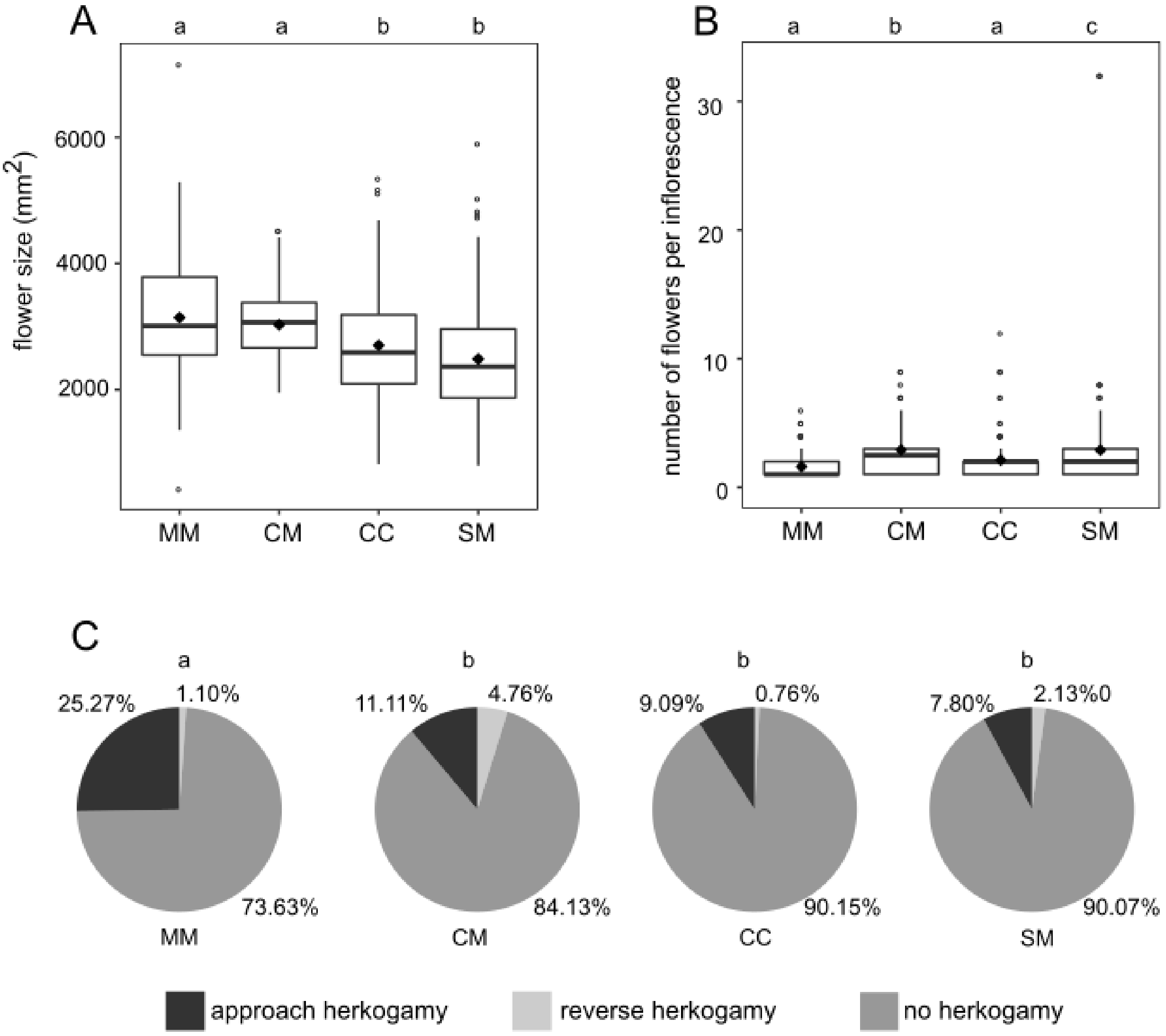

Figure 2.5. **a**: Boxplot of corolla surface. **b**: Boxplot of number of flowers per inflorescence. **c**: percentage of flowers with a separation between stigma and anthers. Different letters indicate statistical differences, *P*-values= 0.05

Pollen limitation and reproductive performances

No significant differences were detected in seed set among groups of populations. Seed set was pollen-limited mainly in MM (PL=0.20) and in CM (PL=0.12). In CC the mean value of PL was close to zero (PL=0.005), suggesting no pollen limitation. Differently, the mean PL value of SM (Figure 2.6) was weakly negative (PL=-0.09). In the self-pollination treatment, only one out of the 93 flowers produced a fruit.

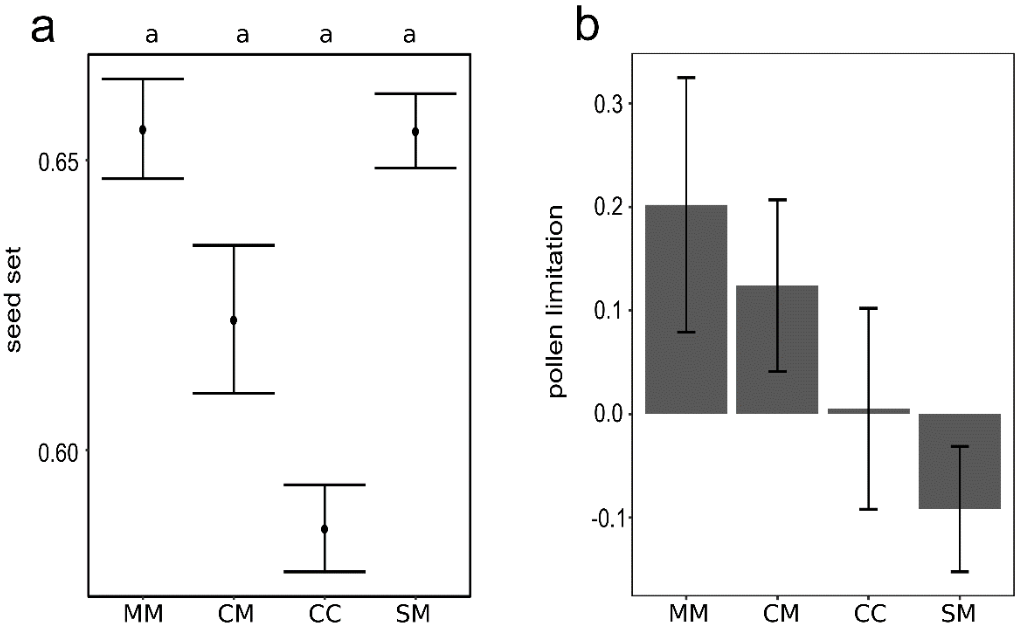

Figure 2.6. a: Boxplot of seed set (vital seed/ aborted seed + vital seed). Different letters indicate statistical differences, *P*-values= 0.05. **b**: effect of pollen supplementation

Discussion

In general, species are predicted to show higher performance in the centre of their distributional range where habitat conditions are expected to be more favourable and stable than in peripheral or marginal populations (Hengeveld and Haeck 1982; Brown 1984). Therefore, a concordance between geographical extremes and ecological margins is expected (Antonovics 1976; Lesica, 1995; Van Rossum and Prentice 2004). In *L. pomponium,* marginal climatic groups MM and SM occur respectively at the southern and northern geographical extremes of the distributional range, while the CM group occurs close to the centre of the distributional range of the species (Figure 2.1). In particular, ecologically marginal populations growing in warm and dry conditions are located both at southern (i.e., MM group) and lower altitudinal (i.e., CM group) limits, while marginal populations growing in cold and wet conditions (i.e., SM group) are located at highest altitude in the northern limit of the distributional range (Table 2.1 and Figure 2.4). This result is in line with the lack of correlation between geographical and climatic distances (i.e. p-value: 0.19 and tau: -0.22), suggesting that populations near the geographical centre are not necessarily near the ecological centre and vice versa. In particular, at low altitude ecologically marginal conditions occur in CM group growing near the centre of distribution of the species in the driest and warmest habitats (Figure 2.4). These results support the idea that geographical and environmental gradients are not necessarily concordant (Ribeiro and Fernandes 2000; Herilhy and Eckert 2005; Herrera and Bagaza 2008; Villelas et al. 2012; Pironon 2015; Dallas et al. 2017) and that factors such as topography may impose marginal ecological conditions near the geographical centre (Soulé 1973).

In general, unfavourable environmental conditions occurring at the ecological margins are expected to reduce reproductive performances of populations (Sagarin et al. 2006; Sexton et al. 2009). Thus, ecologically marginal populations are expected to diverge from central populations because they have smaller and fewer flowers, reduced stigma-anther separation, and high self-fertilization rate because of a reduction in pollinators visit and outcross pollination (Herlihy and Eckert 2005; Mimura and Aitken 2007). In *L. pomponium* not all ecologically marginal groups of populations are different from central populations. In particular, flower size is significantly wider in MM and CM than in SM and CC (Figure 2.5 a). Similarly, MM and CM are pollen limited contrary to SM and CC (Figure 2.6 b). The largest flower size detected in pollen limited groups (i.e., MM and CM) is congruent with the theory that pollen limitation may favour attractive traits (i.e. corolla dimension). Indeed, pollen limitation may select for large flowers that enhances visibility and pollinator attraction (Thompson 2001; Arista and Ortiz 2007; Barrio and Teixido 2014), favouring the reliability of pollinator visits (Haig and Westoby 1988; Totland 2001; Teixido and Aizen 2019). The selective pressure due to pollen limitation may be particularly relevant in *L. pomponium* because large flower are particularly successful in attracting Lepidoptera (Thompson 2001), the main pollinators (Casazza et al. 2018). Differently, the number of flowers per inflorescence is highest in CM and SM (Figure 2.5 b). The small but rather numerous flowers detected in SM (Figure 25 a and b) are in line with the general expectation of a trade-off between flowers size and number because of energetic constraints (Sargent et al. 2007). Moreover, in this group the high number of flowers per inflorescence might be a bet-hedging strategy to assure reproduction in unpredictable environments (Koops et al. 2003). In fact, despite their cost, the late-blooming flowers may act as a reserve when the earlier-blooming ones are lost early in the season (Brown 1984), because of late spring frosts. Contrary to the general expectation, CM has large and numerous flowers (Figure 2.5 a and b). In this group the high number of flowers per inflorescence may be related to the small size of populations (Table 2.1). The high number of flowers may however increase the frequency of within-plant pollinator movements favouring geitonogamy (Mustajärvi et al. 2001; Iwaizumi and Sakai, 2004) and thus reduce outcrossing (Lloyd 1992; Harder and Barrett 1995), it might be a strategy which permits small sized populations to produce an adequate number of seeds to assure seedbank (Roberts et al. 2014). Finally, only MM presents a higher percentage of flower with approach herkogamy (Figure 2.5 c) with a protruding stigma that reduces self-pollen deposition (Webb and Lloyd 1986). This is favourable in pollen limited populations of a non-autogamous species, like *L. pomponium* (Casazza et al. 2018), where the self-pollen deposition causes self-interference reducing female fitness (Webb and Lloyd 1986; Li et al. 2013). Taken together, our result suggests that selection on floral traits is dependent on local contexts in different environmental condition where populations occur because the array of interaction among resource availability, pollinator-mediated selection and population size may differentially affect floral traits.

Reproductive fitness is expected to gradually decrease and become more variable towards the margin of the range, because populations occur in unfavourable ecological conditions (Sagarin et al. 2006; Sexton et al. 2009; Abeli et al. 2014). Contrary to the general expectation, in our study all groups have a similar and moderate seed production (seed-set = 0.586-0.655, Figure 2.6 a). In many-flowered species like *L. pomponium*, the chance for a flower to mature all ovules in seeds depends simultaneously on the number of pollinated flowers (Charlesworth 1991), on the number of ovules per flower, on pollination ecology and on resource availability (Strelin and Aizen 2018). Therefore, under different environmental conditions seed set may be limited by different factors. For example, the moderate seed set of MM and CM may be explained by pollen limitation; in addition, by occurring in warm and dry conditions, these populations may suffer a drought stress during seed development, potentially reducing the number seeds. In contrast, the moderate seed production in CC and SM growing in wetter conditions may be explained by other non-mutually exclusive factors such as seed predation and herbivory (Knight et al. 2006; Straka and Starzomski 2015). In particular, these groups may be more prone to damages by the lily beetle (*Lilioceris lilii* Scopoli, 1763) that prefers environmental conditions (shaded, cool and moist areas; Majka and LeSage 2008) occurring in mountain and subalpine habitats where CC and SM occur. Hence, even though different reproductive output is expected in marginal and central populations due to variation in environmental conditions, the seed set recorded in *L. pomponium* may be similar because each group is limited by different factors, as observed elsewhere (Garwood and Horvitz 1985). Such similar seed set may also be condition by the life-history strategy of *L. pomponium*; like other long-lived herbaceous perennials, the species may have a strategy of annually limited but inter annually constant seed production, in which sub-maximal seed production is a part of a size-dependent strategy that maximises life-time seed production without compromising adult survival (Garcia and Zamora 2003; Andrieu et al. 2007).

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Chapter 3.

The response of germination to climate change: the study case of *Lilium pomponium*.

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Introduction

Global temperature is projected to likely exceed 1.5°C above pre-industrial levels in the more optimistic climatic scenarios for the end of the 21st century (Pachauri *et al.* 2014). In particular, Mediterranean Basin is recognized as a hotspot of climate change (Giorgi 2006), because in this region the increase in temperature will be roughly 0.5°C higher than the average increase forecasted at global scale (Guiot & Cramer 2016). This human-induced change in climate is one of the major threats to plants, because changes in temperature may affect a range of key biological features (Franklin 2009; Bita & Gerats 2013) among which the reproduction of plants (Hedhly et al. 2009; Zinn et al. 2010), fundamentally influencing species distributions (Bykova et al 2012). Temperature matches seed germination and emergence with environmental conditions optimal for subsequent seedling establishment. Then understand the response of seed germination to temperature changes plays a pivotal role in predict species response to climate change.

Several reproductive traits like seed aging, the dormancy timing and loss and germination rate (Roberts 1988) have serious effects on population dynamics (Donohue et al. 2010). Even if phenotypic plasticity may enable species to tolerate short-term variability in climate, the rate of adaptation may be inadequate to ensure long-term persistence (Gomulkiewicz et al., 2010). So, in response to global warming, species may locally persist shifting their germination phenology (Bradshaw & Holzapfel 2008) or shift altitudinally (Gworek et al. 2006; Parolo & Rossi 2008) to keep peace with their optimal temperature. In species living below their thermal optimum an increase in temperature may move them closer to their optimum increasing their fitness. Alternatively, in species currently living at their thermal optima, a temperature increase may move them beyond it, resulting in a performance decline (Miller & Stillman 2012). Nevertheless, even if the thermal requirements for germination will occur in the current distributional range, temperature change may induce a shift in the timing of germination (Cochrane 2016) having drastically effect on plant fitness (McNamara et al. 2011). Nevertheless, the shift in the period of seeds emergence may expose them to conditions unfavourable for their survival (Mondoni et al. 2012). Information on regeneration under climate change is urgently needed for plants conservation. However, the overall impact of climate change on plant regeneration has largely been neglected (Hedhly et al., 2009).

The present study is aimed at characterizing the spatial and temporal pattern of germination response to temperature and its possible shift due to future climate change. More specifically, we are asking the following questions. (i) What is it the spatio-temporal germination pattern of *L. pomponium*? (ii) How would germination change in time and space under predicted climate change scenarios? (iii) Does an increase in temperature inhibit or promote germination in different season across the year? The results of the study should allow us to better understand the possible response of plants to future climate change

Materials and Methods

Species distribution and populations selection

Occurrence data were collected from the database "SILENE" of the Conservatoire Botanique National Méditerranéen de Porquerolles (CBNMED; flore.silene.eu/) and from our own field surveys. A final data set of 809 presence records were used. The mean annual temperature at each population was estimated by using the geographic coordinates to extract data from the WorldClim interpolated climate surface.

Seed collection and germination tests

In the summer 2017 and 2018 (from August to September), a total of 8,046 mature seeds were collected from the 14 populations of *L. pomponium* (Figure 3.1).

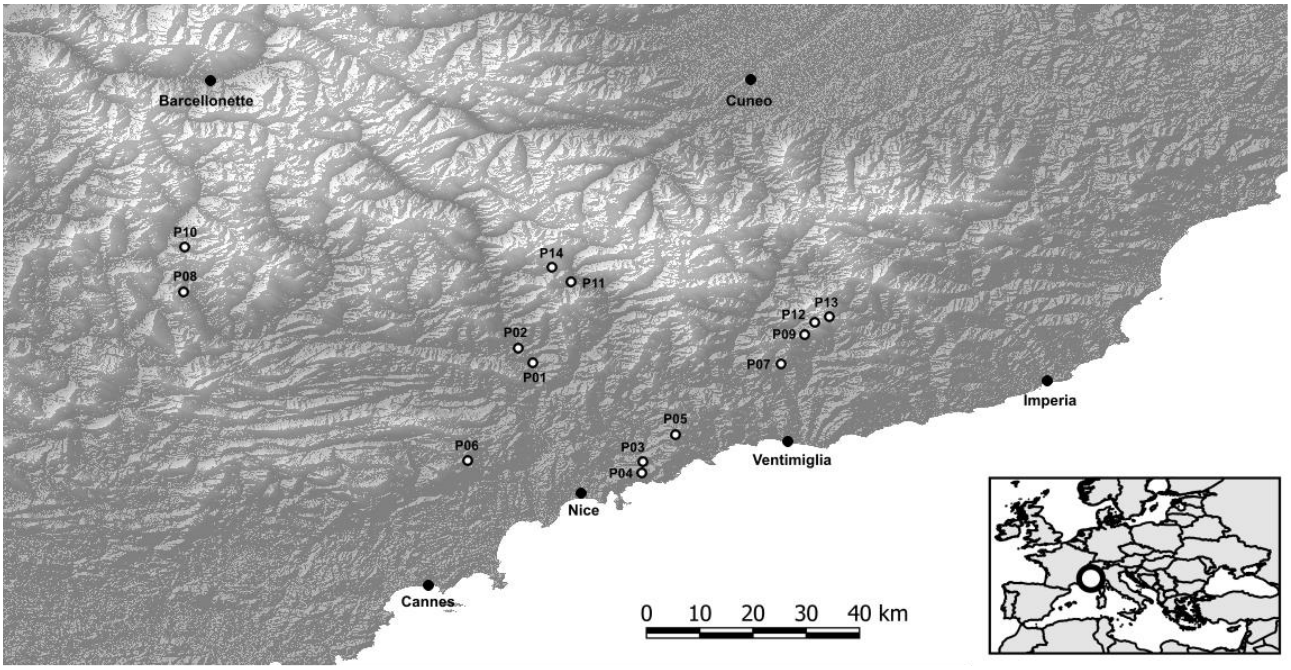

Figure 3.1. Selected population of *Lilium pomponium*

The number of seeds per population used in the experiment ranged from 400 to 700, depending on the availability of seeds. According to Mascarello *et al.* (2011), seeds were sown, in aseptic conditions, in Petri dishes (9 cm of diameter) containing sterilized distilled water solidified by 6 g/L of agar. To examine variation in germination among populations from different climatic conditions seeds were sown at seven different constant temperatures (9, 12, 15, 18, 21, 24 and 27°C ±1°C) in dark conditions. Experiments were carried out with four replicates per population. Germination pattern was checked three times a week for 60 days and seeds were considered germinated when radicle had emerged through the integument.

Climatic data

We downloaded the monthly values of precipitation and monthly average temperature for both current (i.e., 1979-2013) and future (i.e., 2061-2080) climate at about 1x1 km spatial resolution from the CHELSA v.1.2 dataset (Karger et al. 2017a; 2017b; www.chelsa-climate.org). For the future climate, we chosen two Representative Concentration Pathways (RCPs) representing moderate and extreme possible future emission trajectories and coded according to a possible range of radiative forcing values in the year 2100 relative to preindustrial values (+2.6 and +8.5 W/m2, hereafter optimistic and pessimistic scenarios, respectively; IPCC, 2014). We used RCPs projections from five general circulation models, which represent physical processes in the atmosphere, ocean, cryosphere and land surface: CESm1-CAM5, FIO-ESM, IPSL-CM5A-MR, MIROC5 and MPI-ESM-MR. Models were chosen following the recommendations of Sanderson et al. 2015, in order to minimize the model interdependence.

Data analysis

In order to fit seed germination to the spatial and temporal pattern of temperatures, the germination response to temperature in each population was analysed by using the Timson's index (Timson 1965) for each replicate computed as follows:

$$\sum n = \sum_{i=1}^{t} Gi,$$

where *Gi* is the cumulative germination percentage in time interval *i* and *t* is the total number of time intervals. This metric was chosen from a variety of different analysis tools because it considers both the germination rate and percentage. The Timson's index values were normalized and according to Müller et al (2017) its response to temperature was described by fitting either a quadratic, Gaussian, Kumaraswamy and Log-normal function to the data, using function nlsLM() for solving algorithm, with support for lower and upper bounds of parameters, as implemented in the R package MINPACK.LM (Elzhov et al. 2013). The function with the best fit was selected by using the Akaike Information Criterion (Akaike, 1973). Based on this curve, we calculated the predicted Timson's index values for each one-tenth of a degree and normalized them to range from 0 to 1. To reconstruct the spatial distribution of germination response of the populations in each month, we projected the predicted Timson's index values of each population on the maps of monthly mean temperature. Then we assessed the germination response of species to temperature calculating among populations average and standard deviation of predicted Timson's index values. We estimated the reliability of the model calculating the root-mean-squared error (RMSE) on the mean annual values of predicted germinability, as implemented in R package ROCR (Sing et al 2015). We converted continuous output into a prediction of suitable (1) or unsuitable (0) considering suitable the grid cells that have the temperature falling in the range of temperatures across which the species achieved ≥50% of its predicted maximum performance. Then we calculated for each pixel where species occur the first month when species achieved the 50% of its predicted maximum performance and the number of months for which the predicted performance was higher than 50% of the maximum. Because in some grid cells the threshold may be achieved in two different periods, we calculated these values for each period. Moreover, we tested whether altitude is different between populations having one or two germination seasons under current and future climate by using non-parametric Kruskal–Wallis test, followed by the Nemenyi's non-parametric all-pairs comparison test. We used Kendall rank correlation to test for relationships between onset and length of germination season, and altitude. Eventually, to explore the relationship between altitude and both onset and duration of germination season we fitted a smoothed curve through the scatterplot with local polynomial regression fitting. Precipitation and temperature data were used to calculate the aridity index for each season by using the formula $4P/(T + 10)$ (De Martonne, 1926), where P is cumulative seasonal precipitation in mm and T the average seasonal temperature in °C. The De Martonne's classification is widely used to asses aridity and is effective in characterize moisture levels in southern Europe (Paltineanu et al 2007). Moreover, the aridity is calculated as a function of time allowing assessment of temporal change.

Results

Gaussian function provided the best fit to the thermal performance data in all analysed populations of *L. pomponium* (Table 3.1).

Table 3.1. Akaike Information Criterion values for four functions relating normalized Timson's index to temperature for the 14 analysed populations of *L. pomponium*.

Function	**df**	**P01**	**P02**	**P03**	**P04**	**P05**	**P06**	**P07**
Gaussian	4	377.67	439.15	239.37	408.88	371.27	414.19	430.32
Quadratic	4	345.95	434.59	228.14	406.36	362.24	377.04	387.3
Log-normal	3	405.27	476.09	251.53	441.38	396.37	436.13	464.21
Kumaraswamy	5	409.26	438.81	254.55	411.18	400.05	440.13	465.35

Function	**df**	**P08**	**P09**	**P10**	**P11**	**P12**	**P13**	**P14**
Gaussian	4	401.16	457.31	322.52	390.89	380.25	410.18	367.99
Quadratic	4	380.57	456.81	298.74	362.96	375.69	394.18	338.91
Log-normal	3	432.51	482.37	344.44	422.62	409.58	444.11	393.74
Kumaraswamy	5	436.5	461.44	348.42	426.45	380.56	447.54	397.72

According to this thermal curve (Figure 3.2) the thermal optimum was at 13.9°C (sd=1.0) and the temperatures across which the species achieved the 50% of predicted species performance ranged from 10.0 to 17.8°C (sd=1.2 and sd=2.5, respectively).

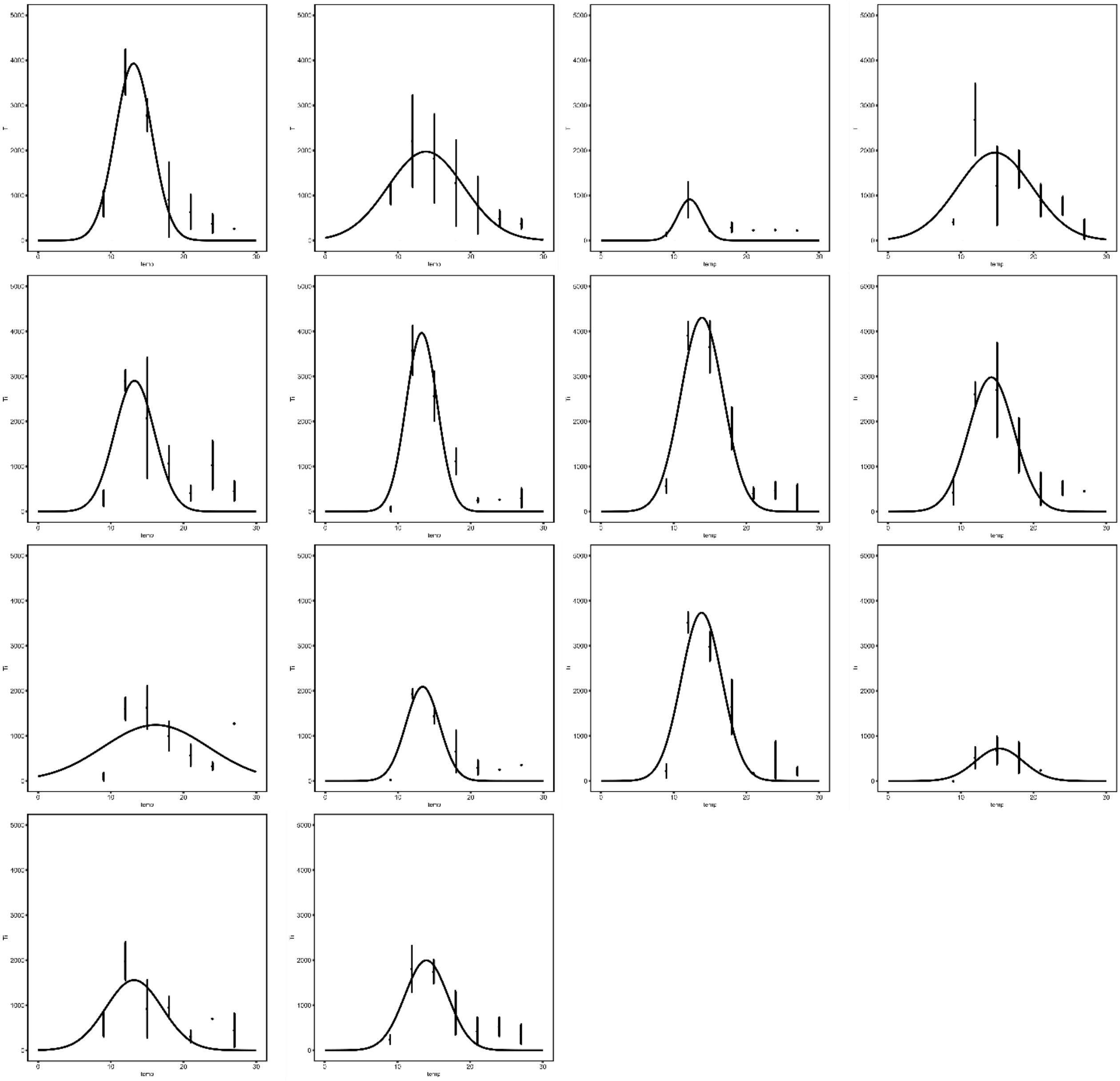

Figure 3.2. Observed Timson’s index values (±SD) for each temperature tested in the climate chambers (points) and Timson’s index values curves (lines) fitted using the best fit function selected by using the Akaike Information Criterion were reported for each analysed population of *L. pomponium*.

The among populations standard deviation is low at high elevations and is highest mainly during the summer period at middle elevation reaching the maximum value of roughly 0.3 under all climates (Figure 3.3).

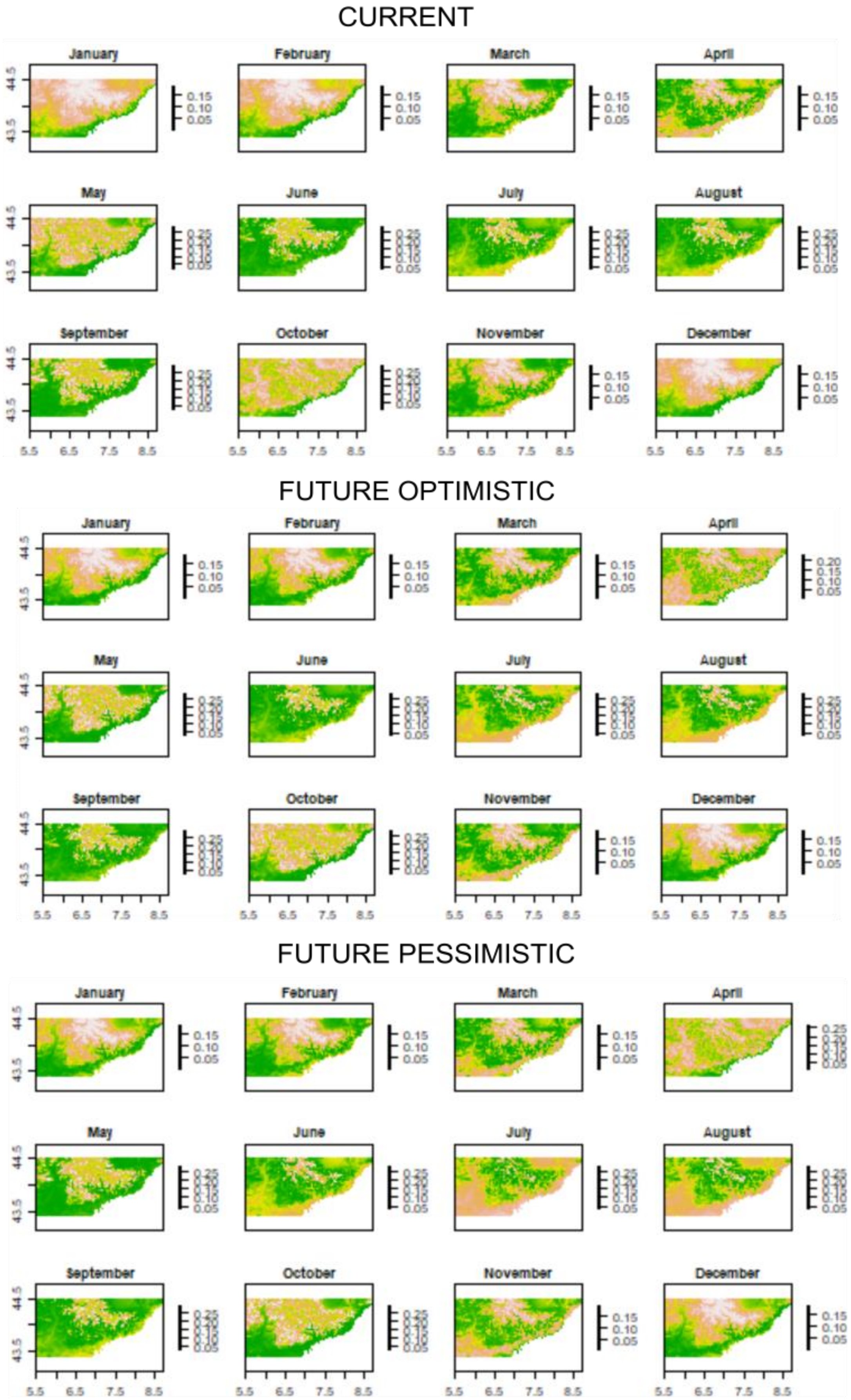

Figure 3.3. Among populations standard deviation of Timson's index fitted for average monthly temperature under current and both optimistic and pessimistic scenarios

The RMSE value is 0.33, indicating a high agreement between predicted germination response and the observed proportions of sites occupied by the species. The populations growing below 1,000 m a.s.l. were within the range of temperatures across which the species achieved ≥50% of its predicted maximum performance both during Spring and Autumn (points between horizontal black lines in Figure 3.4 A) when climate ranged from humid or extremely humid (Figure 3.4 B), the populations growing above 1,000 m a.s.l. were within the range during Summer when climate ranged from sub-humid to very humid (Figure 3.4 A and B).

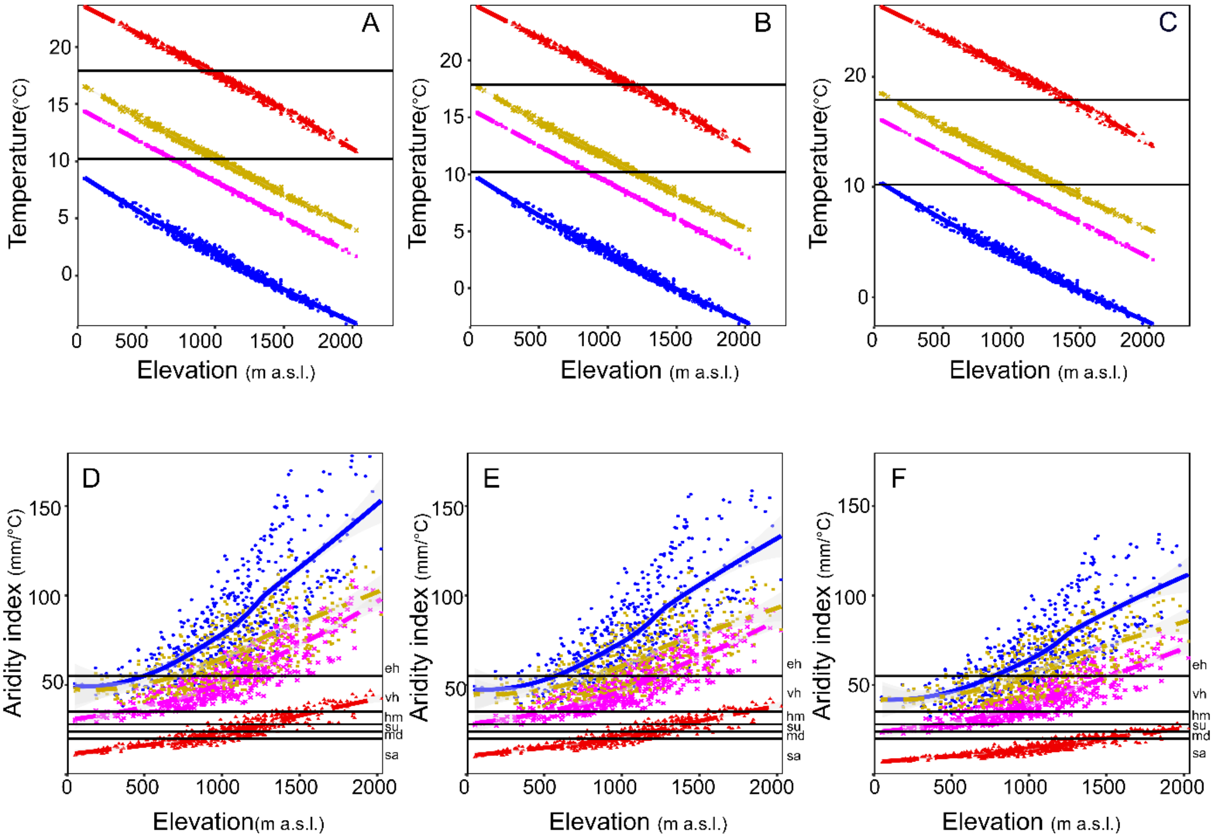

Figure 3.4. Relationship between altitude and both seasonal mean temperature (A, B and C) and seasonal aridity index (D, E and F) in currently know populations of *L. pomponium* under current (A and D), future optimistic (B and E) and future pessimistic (C and F) climate. The predicted Loess regression (lines) is reported for each season. Horizontal lines show the range of temperatures across which the species achieved ≥50% of its predicted maximum performance (A) and the type of climate according to the de Martonne aridity index (B) according to Croitoru et al. (2013): sa=semi-arid; md=Mediterranean; su=sub-humid; hm=humid; vh=very humid; eh=extremely humid. Blue circles and solid line=winter; violet squares and dashed line=spring; red triangle and long dashed line=summer; dark yellow crosses and dash-dotted line =autumn.

Under current climate, roughly the 70% of populations meet the temperatures suitable for seed germination both in spring and autumn (Table 3.2, Figure 3.5). The percentage was forecasted to drastically rise under future climate both in optimistic and pessimistic scenario. The climate change will affect more drastically the duration of spring germination season and the onset of autumn germination season (Figure 3.5). In fact, our results suggest that future climate will hasten the onset of spring season of seed germination of four days in optimistic scenario and of twelve days in pessimistic one. Moreover, it will shorten the duration of spring germination season of ten and fifteen days in optimistic and pessimistic scenario, respectively. (Table 3.2). The autumn germination season will be delayed of four and eleven days and will be shorten of fifteen and eighteen days in optimistic and pessimistic scenario, respectively (Table 3.2).

Table 3.2. For present and future climate were reported the percentage of grid cell having germination season both in spring and autumn (P), the mean date of onset (GB) and the duration in days (GD) of both germination seasons and the mean total duration in days (TD) of the germination seasons.

Scenario	P	GB_spring	GD_spring	GB_autumn	GD_autumn	TD
current	70.94%	03-May	76 days	03-Sept.	51 days	112 days
optimistic	87.38%	26-Apr.	66 days	07-Sept.	50 days	107 days
pessimistic	95.03%	21-Apr.	51 days	14-Sept.	46 days	95 days

The altitude is significantly different between populations having one or two possible germination seasons under all climates (Figure 3.6). In particular, under current climate populations growing roughly below 1,000 m a.s.l. meet their optimal germination temperatures two times a year during spring and summer under humid aridity conditions (Figure 3.4 and 3.6) while populations growing above 1,000 m a.s.l. meet their optimal germination temperatures once time a year during summer under Mediterranean aridity conditions (Figure 3.4 and 3.7). Under future climates, the altitude of populations reaching their optimal germination temperatures two times a year (spring and autumn) will shift upwards in both scenarios up to the 1,500 m a.s.l. (Figure 3.4 and 3.7). This altitudinal shift of germination phenology will allow populations growing at intermediate elevation (between 1,000 and 1,500 m a.s.l.) to avoid the summer aridity (figure 3.7). Altitude was positively significantly correlated with the onset of spring germination season under current and future climate (current, τ=0.726 $p \leq 0.01$; optimistic scenario, τ=0.726 $p \leq 0.01$; pessimistic scenario, τ= 0.738 $p \leq 0.01$). On the contrary, it was negatively significantly correlated with the onset of autumn germination season under current and future climate (current, τ=-0.618 $p \leq 0.01$; optimistic scenario, τ=-0.743 $p \leq 0.01$; pessimistic scenario, τ=-0.733 $p \leq 0.01$). The length of spring germination season was positively significantly correlated with altitude only under current and future optimistic climate (current, τ=0.590 $p \leq 0.01$; optimistic scenario, τ=0.502 $p \leq 0.01$; pessimistic scenario, τ=0.03 p=0.32). Differently, the length of autumn germination season was weakly significantly correlated with altitude under and both future climates (current, τ=-0.05 p=0.26; optimistic scenario, τ=0.264 $p \leq 0.01$; pessimistic scenario, τ=0.196 $p \leq 0.01$).

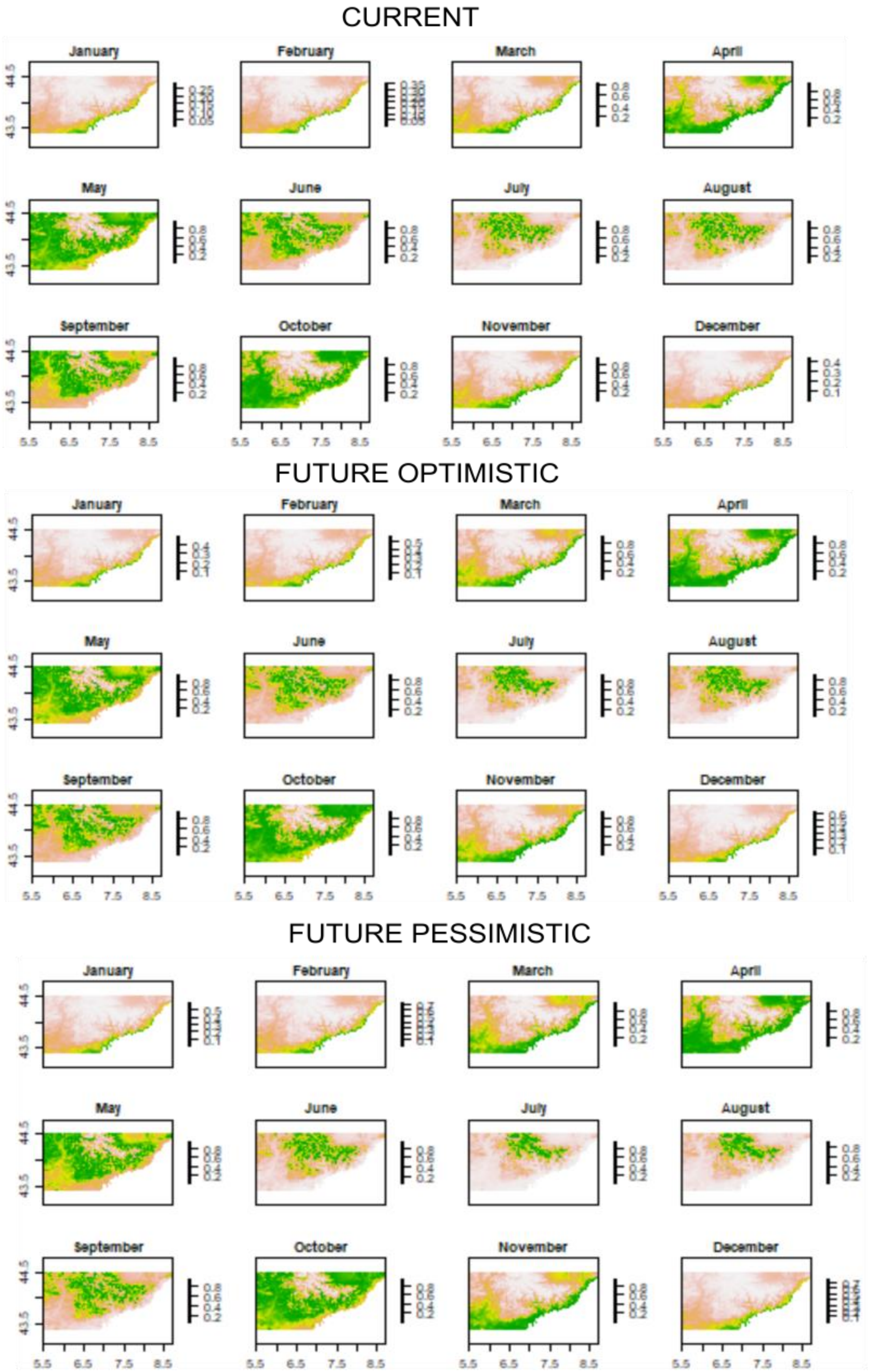

Figure 3.5. Average populations Timson's index fitted for average monthly temperature under current and both optimistic and pessimistic scenarios.

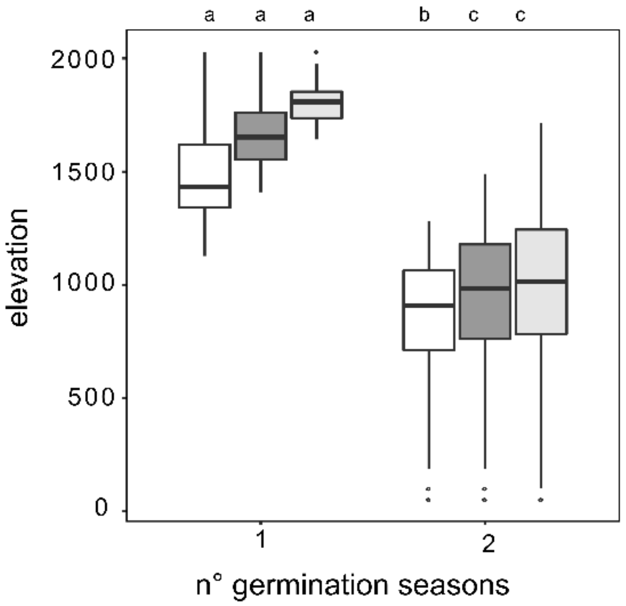

Figure 3.6. Boxplots of measurements of elevation per currently know populations of *L. pomponium* having or not a biseasonal germination pattern under current climate (white) and under both optimistic (light grey) and pessimistic (dark grey) future climates. Boxplots with the same letter are not significantly different ($p > 0.05$), according to the Nemenyi's non-parametric all-pairs comparison post hoc test.

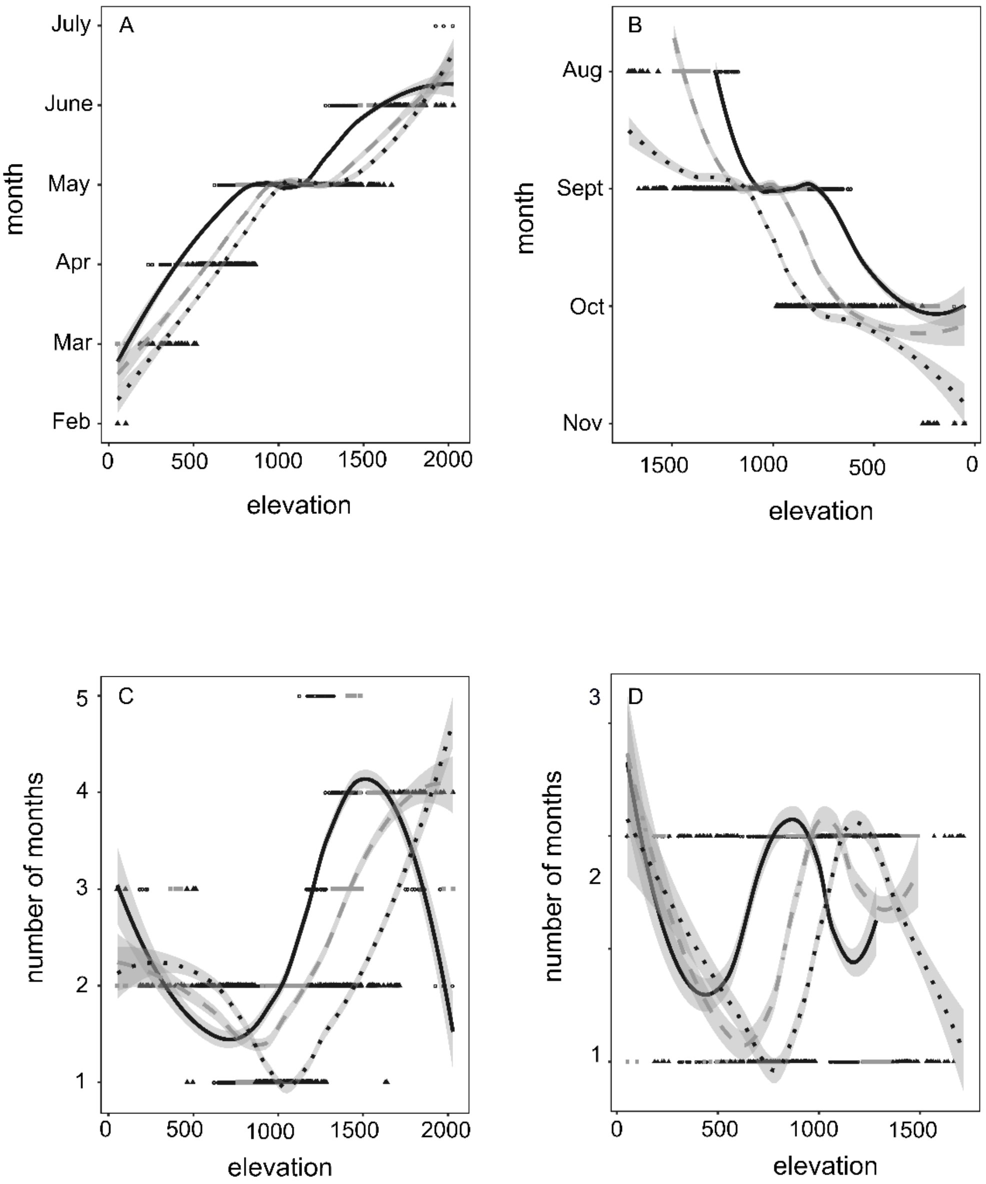

Figure 3.7. Relationship between altitude and both month of onset (A and B) and duration (C and D) of germination in currently know populations of *L. pomponium* under current and future climates. Data are reported both for spring or summer (A and C) and autumn (B and D) germination season. Lines represent the Loess regression and grey bands the 95% confidence interval. Withe circles and black solid line=current climate; grey squares and dashed line=future optimistic scenario; black triangle and dotted line=future pessimistic scenario.

In agreement with these results, the onset of spring germination season is in March at low altitude and in July at high altitude under current and optimistic climate, while under pessimistic scenario the onset of the season will be in June also at high altitude (Figure 3.7 A). The onset of autumn germination season is in August at high altitude and in October at low altitude under current and optimistic climate, while under pessimistic scenario at low altitude the onset of germination season will be in November (Figure 3.7 B). Under current climate, the duration of spring germination season is longest in populations growing at roughly 1,500 m of altitude while under future climate will be longest in populations growing at roughly 2,000 m of altitude (Figure 3.7 C). Under current climate, the duration of autumn germination season is longest in populations growing both at lowest altitude and between 500 and 1,000 metres of altitudes, while populations growing above roughly 1,200 m do not show the autumn germination season (Figure 3.7 D). Under future optimistic climate, the duration of autumn germination season will be longest in populations growing at 1,000 and 1,500 metres, while under pessimistic climate will be longest in populations growing at roughly 1200 metres (Figure 3.7 D).

Discussion

Germination under current climate

Seeds of *L. pomponium* require low temperature for germination (Figure 3.2). Moreover, our results suggest that under current climate the germination phenology of *L. pomponium* changes according to the elevation. Roughly the 70% of populations (Table 3.2) growing below 1,000 m a.s.l. have a biseasonal germination pattern that enable them to avoid the warm and dry Summer season: a relatively short germination season (1 or 2 months) both during the Autumn and the Spring (Figure 3.4 and 3.7). Differently, populations growing above 1,000 m a.s.l. have a one-season pattern, the germination onset is in late Spring or early Summer and lasts several months (more than 4), this enable seeds to avoid the autumn and spring frost that may occur at high elevation (Figure 3.4 and 3.7).

The low temperature required for seed germination (Figure 3.2) fits with the requirement expected for a Mediterranean and subalpine species. In fact, in Mediterranean environments, germination is favoured at relatively low temperatures (roughly 15°C) in order to avoid summer drought (Thompson 1970; Bell et al. 1993; Doussi & Thanos, 2002). Similarly, low temperature favours germination in subalpine species enabling seeds to germinate during snowmelt (Baskin & Baskin, 2014; Fernández-Pascual et al. 2017a) and to use of the growing season very early (Donohue et al. 2010). Differently, Alpine and some Mediterranean orophyllous plants require higher temperatures to germinate to avoid germination in early spring or autumn when a high probability of frost events may result in a low probability of seedling establishment (Cavieres & Arroyo 2000; Giménez-Benavides et al. 2005).

In *L. pomponium* environmental condition are critical for determining the seed germination phenology, as observed in other species (Walk & Hidayati 2004). In particular, populations from low and middle elevation subject to hot and dry summer showed response patterns that combined potential for germination in spring and autumn, avoiding the arid summer (Figure 3.7 A and D). Because *L. pomponium* blooms in spring, the seeds dispersed in late spring/early summer and mainly germinated in the following autumn, as previously detected in several spring-flowering geophytes (Thompson 1977; Vandelook and Van Assche, 2008) Nevertheless, asynchronous germination as observed in these populations was previously detected in species occurring in habitat prone to disturbing environmental constraints such as drought and flood and it may reduce both the risk of seed regeneration failure (Hölzel & Otte 2004) and the competition intensity in offspring during unfavourable season (Yang et al. 2019). Similarly, in *L. pomponium* this pattern may be a bet-hedging strategy to avoid unpredictable heat waves causing aridity in spring and autumn and to reduce competition between seedling during the dry summer. Moreover, in geophytes, autumn emergence may be an adaptation to ensure seedling to grow quickly when temperatures begin to rise in early spring (Mondoni et al 2008). In *L. pomponium*, autumn seeds emergence may be advantageous for low elevation seeds where freezing winter is very rare and for intermediate elevation seeds (until

1000-1200) that may be shielded from damaging frosts by a thick layer of leaf litter and by snow cover. Differently, *L. pomponium* populations at high elevation showed a response pattern for germinating only during summer season. This pattern allows seeds of high elevation populations to germinate early after snow melt using the humid and warm summer and to avoid the frost that may still occur in spring and autumn. Our results suggest that despite the germination niche of *L. pomponium* seems to be shaped to avoid dry summer in Mediterranean climate, it enables the species to grow at high altitude in north Mediterranean mountain, shifting its germination phenology and taking advantage of the relatively warm and wet summer. This result is in line with the idea that the reproduction niche may plays an important role in determining plant distribution (Bykova et al 2012; Rosbakh & Poschlod 2015).

Germination under future climate

Under both simulated future climate will decrease the duration of germination seasons and will shift the timing of seed germination in L. *pomponium* (Table 3.2) according to elevation (Figure 3.7). In particular, the spring germination season will onset earlier, the autumn germination season will onset later and the duration of the germination seasons will be reduced. Moreover, the elevation limit between populations having bi-seasonal germination will shift upward from roughly 1,000 to 1,500 m a.s.l. (Figure 3. 4 and 3.7). Similarly, the maximum length of germination season will shift upward from 1,500 to 2,000 m a.s.l. (Figure 3.7 C). This upwards shift suggests that newly suitable areas for *L. pomponium* due to temperature rise may be available at high altitude favouring an upwards shift of its distributional range. This shift is in line with the so-called “thermophilization” of high elevation plant communities occurring in most Mediterranean mountain systems (Gottfried et al. 2012; Rumpf et al. 2018).

Differently, middle elevation populations will probably prone to less favourable conditions because of a reduction of germination season because of the increase in the length of summer warm and aridity period. Germination phenology may be the most important driver of community shifts in response to climate change being the first filter on which the changes act (Kimball et al 2010). In Alpine species global warming is expected to dramatically shift seeds emergence from mostly spring to autumn (Mondoni et al 2012). In Artic species climate warming may result in an earlier germination and in a longer growing season, improving the chance of seedling survival (Milbau et al 2009). Nevertheless, this shift will enable also in the future seeds to avoid germinating during too arid or too cold periods (Figure 3.4 B, C, E and F). Shift in germination phenology may favour species with lower temperature optima for germination like *L. pomponium* enabling them to germinate avoiding arid conditions (Kimball et al 2010). Overall, our results suggest that the shift in germination phenology because of the climate change will enable seedlings to remain under conditions they are able to tolerate.

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Chapter 4.

Effects of altitude on seed size and germination of an endemic species of Maritime Alps

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Paper under preparation

Introduction

Change in global surface temperature forecasted for the end of the 21st century is projected to likely exceed 1.5°C above pre-industrial levels in the more optimistic climatic scenarios (Pachauri *et al.* 2014). Human activities are simultaneously modifying abiotic and biotic agents of selection resulting in growing discrepancies between current and optimal phenotypes (Etterson 2004). This current, rapid human-induced change in climate is a major threat to biodiversity, particularly in mountain ecosystems, which are often important centres of plant endemism (Médail *et al.* 1997). Montane endemic plants have shown to be very sensitive to change in temperature because it affects a range of key biological features (Franklin 2009; Bita & Gerats 2013, Cotto *et al.* 2017). In particular, temperature increase is expected to have a strong effect on plants fitness (Hedhly *et al.* 2009; Zinn *et al.* 2010) as it may induce an early onset of sexual reproductive development (Sherry *et al.* 2007). Temperature increase may increase (mainly in Arctic or Antarctic species), decrease (mainly in temperate and mountain species; Arft *et al.* 1999; Liu *et al.* 2012; Meineri *et al.* 2013) or do not affect reproductive output (Iler *et al.* 2019). The effects of temperature change can affect both seeds mass and germination process (Probert 2000), having serious effects on population dynamics.

Among-populations traits variation along a climatic gradient may affect plant performance under different environmental conditions (Rathcke & Lacey 1985; Messier et al. 2010) and consequently the response of populations to climate change (Dawson et al. 2011). Despite their importance for plants conservation, variations in seed traits along a climatic gradient and how they may affect the response of populations to climate change remain poorly understood, especially in narrow-ranged or threatened species (but see Giménez-Benavides *et al.* 2005; Cochrane *et al.* 2014; De Castro *et al.* 2015) and in Mediterranean-climate regions (Cochrane *et al.* 2015). Therefore, a comparison of the germination capacity of seeds from different altitudes and the effect of seed size on germination both with an increase of temperature is necessary in studies on reproductive efficiency of the species (Vera 1997; Vandelook 2009).

The present study is aimed at investigating whether seed mass and germination change along an altitudinal gradient and whether the temperature affects the germination between populations of *Lilium pomponium* L. occurring in different altitudinal belts. The species is endemic to south-western Alps (Provence, Maritime and Ligurian Alps), and grows in xeric stony or rocky calcareous habitats between 100 and 2,000 metres of altitude, spanning from a hot-summer temperate climate typical of Mediterranean hills to a cool-summer boreal climate typical of subalpine habitats (Tison *et al.* 2014). More specifically, we are asking the following questions: (1) Does seed germination response changes among different incubation temperatures? (2) Are seed mass and germination different among seed sources distributed along an altitudinal gradient? (3) Is seed germination response to incubation temperature affected to altitude of seed source? The results of this study should allow us to better understand how the populations of this endangered endemic species will response to climate change assuring the survival of the plant on the future.

Materials and Methods

Study species

Lilium pomponium L. (Liliaceae) is listed in the Annex V of the EU Habitat Directive (Council Directive 92/43/EEC 1992), and it is one out of the six *Lilium* species included in the IUCN Global Red List (last accession 26.07.2018). The species was assessed as Endangered (EN) in Italy (Abeli *et al.* 2013) and Least Concern (LC) at worldwide level (Gargano 2015), and it is predicted to be threatened with extinction because of high amounts of range loss induced by future climate change (Casazza *et al.* 2014). Other major threats are overgrazing, abandonment of pastoral systems, forest planting and the illegal collection of plants for gardening (Gargano 2015).

Selection of population

Species occurrences data were collected from the database “SILENE” of the Conservatoire Botanique National Méditerranéen de Porquerolles (CBNMED; flore.silene.eu/), LiBiOss (Regione Liguria; http://www.cartografiarl.regione.liguria.it/Biodiv/Biodiv.aspx) and from our own field surveys. Occurrences were spatially filtered and those closer than 1 km to each other were removed. We selected a total of 15 population (Table 4.1) along the altitudinal range representing the different environmental conditions in which the species occurs.

Table 4.1. Details for sampled populations of *L. pomponium.* Code and locality name, altitude (alt), number of capsules collected, and number of seeds used in the experiment

Code	Locality	Lat	Long	Alt	N° capsules	Mean seeds per fruit	N° seeds	Seed mass
P01	Route de Grenoble, Utelle (FR)	43.905	7.196	203	9	155.37 ±19.82	700	12.045 ± 2.54
P02	Route de la Tinée, Tournefort (FR)	43.919	7.187	230	31	162.84 ±21.23	700	11.063 ± 2.10
P03	Les Pras, La Tour (FR)	43.944	7.163	243	12	129.41 ±35.80	700	10.026 ± 1.44
P04	Plateau Tercier, Sainte-Thècle (FR)	43.755	7.369	554	39	137.48 ±18.55	700	10.393 ± 1.70
P05	Fort de la Revère (FR)	43.737	7.367	661	9	133.66 ±13.75	504	12.388 ± 2.05
P06	Col de la Madone de Gorbio, Peille (FR)	43.801	7.423	915	45	142.56 ±19.82	700	8.364 ± 3.20
P07	Col De Vence, Vence (FR)	43.757	7.0775	955	42	157.88 ±31.30	700	8.408 ± 2
P08	Mt. Comune, Pigna (IT)	43.918	7.5975	1123	31	144.72 ±18.65	700	5.647 ± 1.52
P09	Méailles (FR)	44.037	6.6068	1313	39	147.95 ±19.30	700	7.576 ± 2.64
P10	Mt. Lega, Pigna (IT)	43.966	7.6363	1326	46	156.25 ±27.52	700	6.364 ± 3.20
P11	Ondres (FR)	44.112	6.6082	1565	34	125.25 ±19.47	700	7.658 ± 2.46
P12	L'adrechas, La Colmiene (FR)	44.075	7.2255	1646	55	140.88 ±23.40	700	6.397 ± 1.87
P13	Passo dell'Incisa, Pigna (IT)	43.987	7.65428	1674	42	156.74 ±28.54	700	6.648 ± 1.32
P14	Mt. Grai, Pigna (IT)	43.995	7.6772	1759	60	170.45 ±18.63	700	6.703 ± 2.2
P15	La Colmiene (FR)	44.078	7.2174	1789	31	159.85 ±26.86	700	5.839 ± 1.36

Seed collection and germination tests

In the summer 2017 and 2018 (from August to September), 78,823 mature seeds were collected in the field from the 15 selected populations of *L. pomponium*. The number of seeds per population used in the experiment ranged from 400 to 6000, depending on the availability of fruits (Table 4.1). In laboratory, the mean seed weight per populations was obtained by weighting ten seeds per fruits randomly chosen (Table 4.1). To examine variation in germination among populations from different climatic conditions seeds were sown at seven different constant temperatures (9, 12, 15, 18, 21, 24 and 27°C ±1°C) in darkness conditions according to Mascarello et al. (2011). Experiments were carried out with four replicates per population. Germination was checked every two days for 120 days.

Statistical analysis

The germination rate was characterized by the onset of germination time (T0) and the time to obtain 50% germination (T50). The T_{50} was calculated according to the formulae of Coolbear et al. (1984) modified by Farooq *et al.* (2005):

$$T_{50} = \frac{t_i + (\frac{N}{2} - n_i)(t_j - t_i)}{(n_j - n_i)}$$

where N is the final number of germinating seeds and n_j and n_i are the cumulative number of seeds germinated by adjacent counts at times t_j and t_i, respectively, when $n_i < N/2 < n_j$.

The relationship between final proportion of germinated seeds, T0, T50 and the altitudinal belt was analysed by linear regression to the germination data at different temperature as fixed predictors and altitudinal belts as factor (Bolker *et al.* 2009). Statistical analyses were performed using the lme4 package (Bates *et al.* 2015) implemented in R (R Core Team 2018).

We used linear mixed models (LMM) to determine how seed size was influenced by altitude, by taking altitude as fixed factor and populations as random factors, using the lmerTest package (Kuznetsova *et al.* 2019) implemented in R (R Core Team 2018).

Prior to the statistical analysis, all percentages were arcsine transformed. Germination tests were carried out on the apparently viable seeds, i.e. seeds whose embryos were white and firm.

Roughly the 60% of the seeds were considered viable.

Results

The highest percentage of germinated seeds was observed at 12°C (68%), furthermore germination is reduced both with the increase (10% for 24 and 27°C) and with the decrease (23%) in temperatures (Fig 4.1 A). At the lowest tested temperature and the highest the T0 increases respectively by 74 day at 9°C and 26 days at 27°C (compared to an average of 11 day) (Fig 4.1 A). Low temperatures affect the achievement of T50, in fact the mean at 9 and 12°C was respectively 34 and 22 days (compared to an average of 8 day).

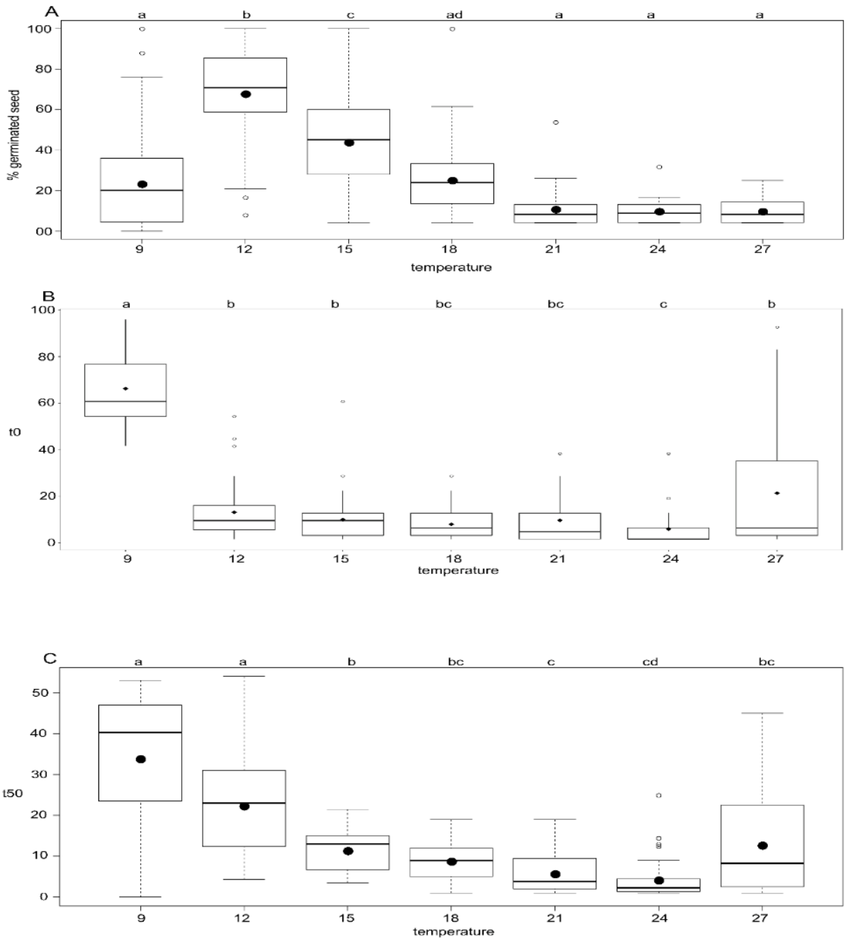

Figure 4.1. Differences in germination percentage (A), T0 (B) and T50 (C) among seeds from different altitudes. Different letters indicate statistical differences, p-values ≤ 0.05

Effect of altitude on seed mass and germination

In *L. pomponium*, seeds size decreases with altitude even if the trend is no significant. Nevertheless, the seeds size is significantly greater in low than in high altitude populations (Fig. 4.2).

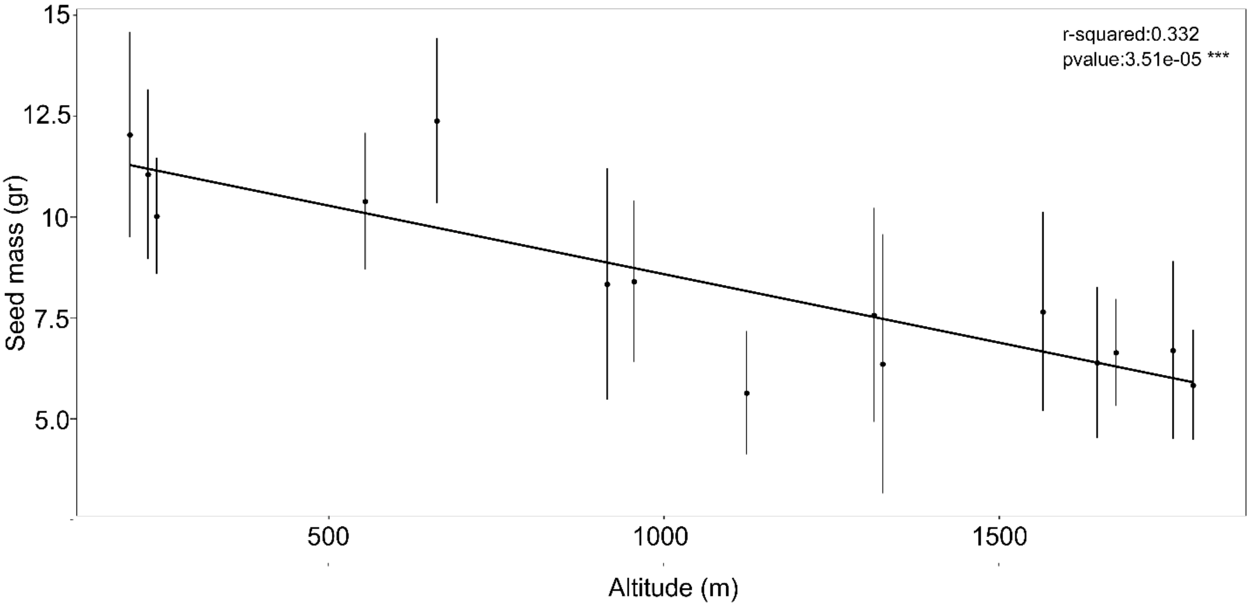

Figure 4.2. Correlation between seeds size and altitudinal belts. p-values= 0.05

The germination rate was never significantly correlated with altitude; nevertheless, it decreases with altitude at lowest and highest temperature (Fig 4.3 A, E and F) and increase at the other germination temperatures (Fig. 4.3 from B to G).

The mean germination time ranged from four to 70 days for T0 and from four to 34 days for T50 (Fig. 4.4 and 4.5). The T0 increased with altitude in all tested temperature and the trend was significant in intermediate temperature (i.e. 12, 15, 18, 21°C. Fig. 4 B-E). Similarly, T50 was positively correlated with altitude in all tested temperature except for the lowest (i.e., 9°C), even if the trend was no significant in highest temperature (i.e., 24 and 27°C. Fig. 5 F and G).

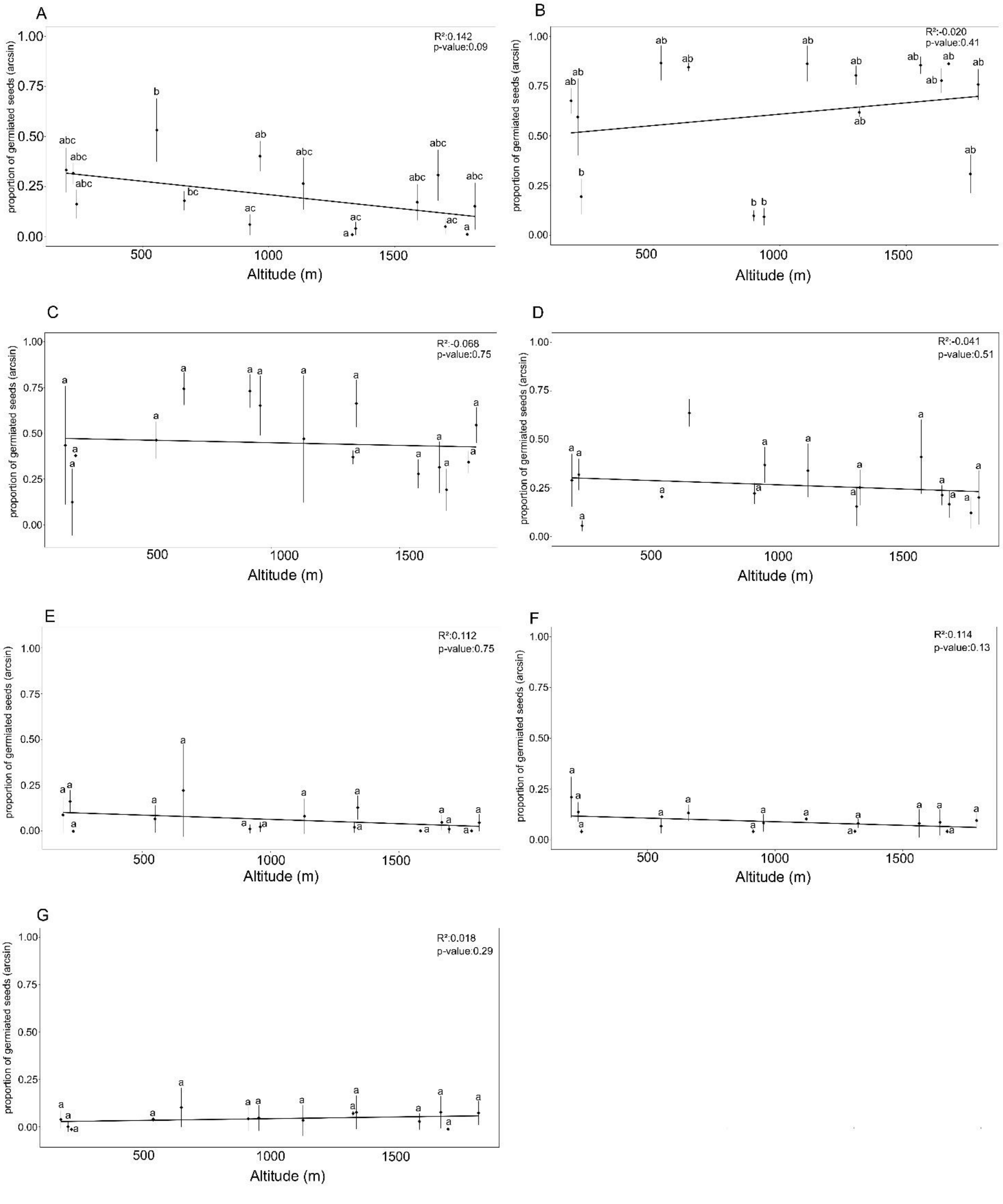

Figure 4.3. Correlation between final seed germination and altitude of seed source at different incubation temperatures: 9°C (A), 12°C (B), 15°C (C), 18°C (D), 21°C (E), 24°C (F) and 27°C (G). Different letters indicate statistical differences, p-values= 0.05.

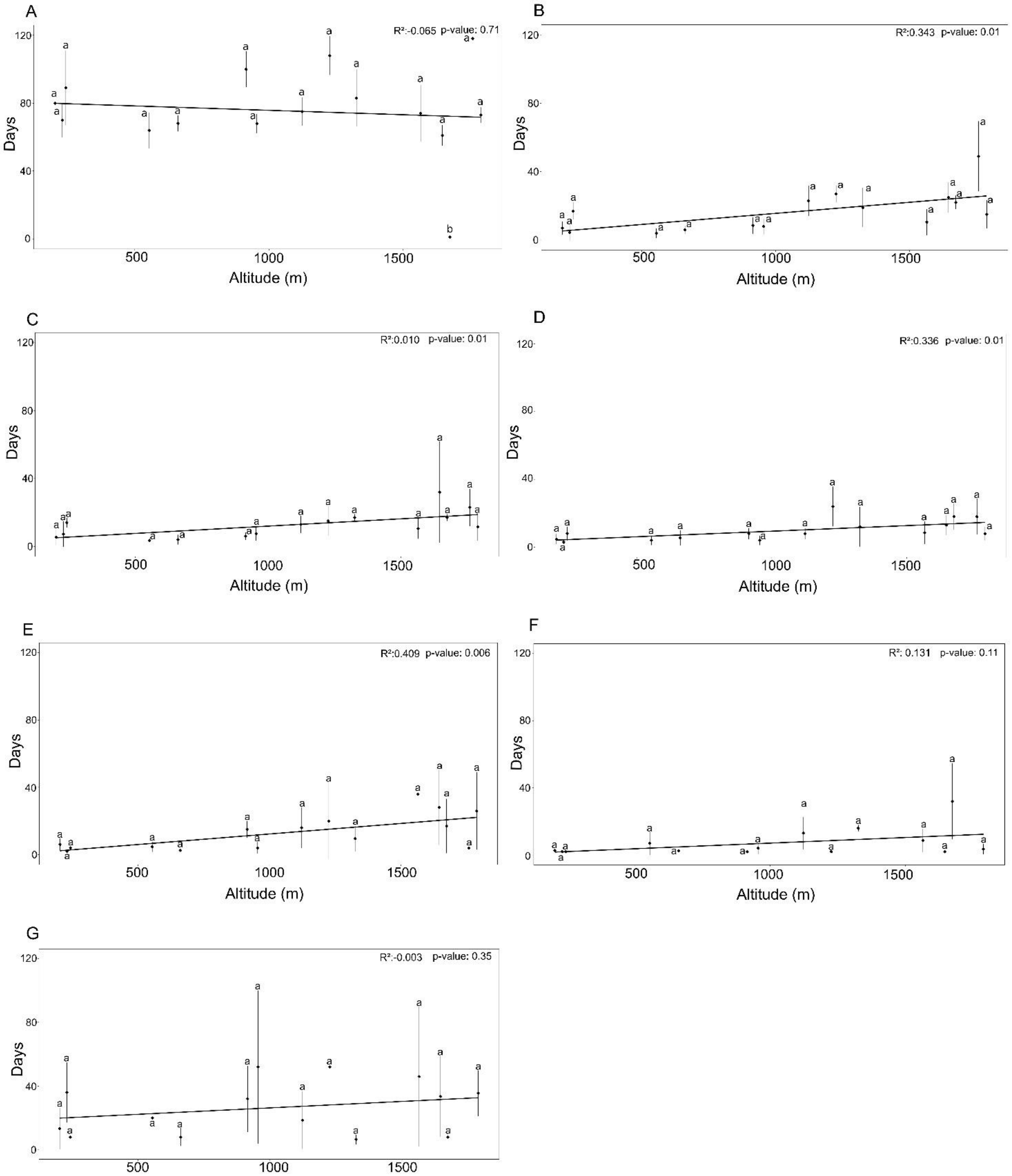

Figure 4.4. Correlation between time to onset the germination and altitude at different incubation temperatures: 9°C (A), 12°C (B), 15°C (C), 18°C (D), 21°C (E), 24°C (F) and 27°C (G). Different letters indicate statistical differences, p-values= 0.05.

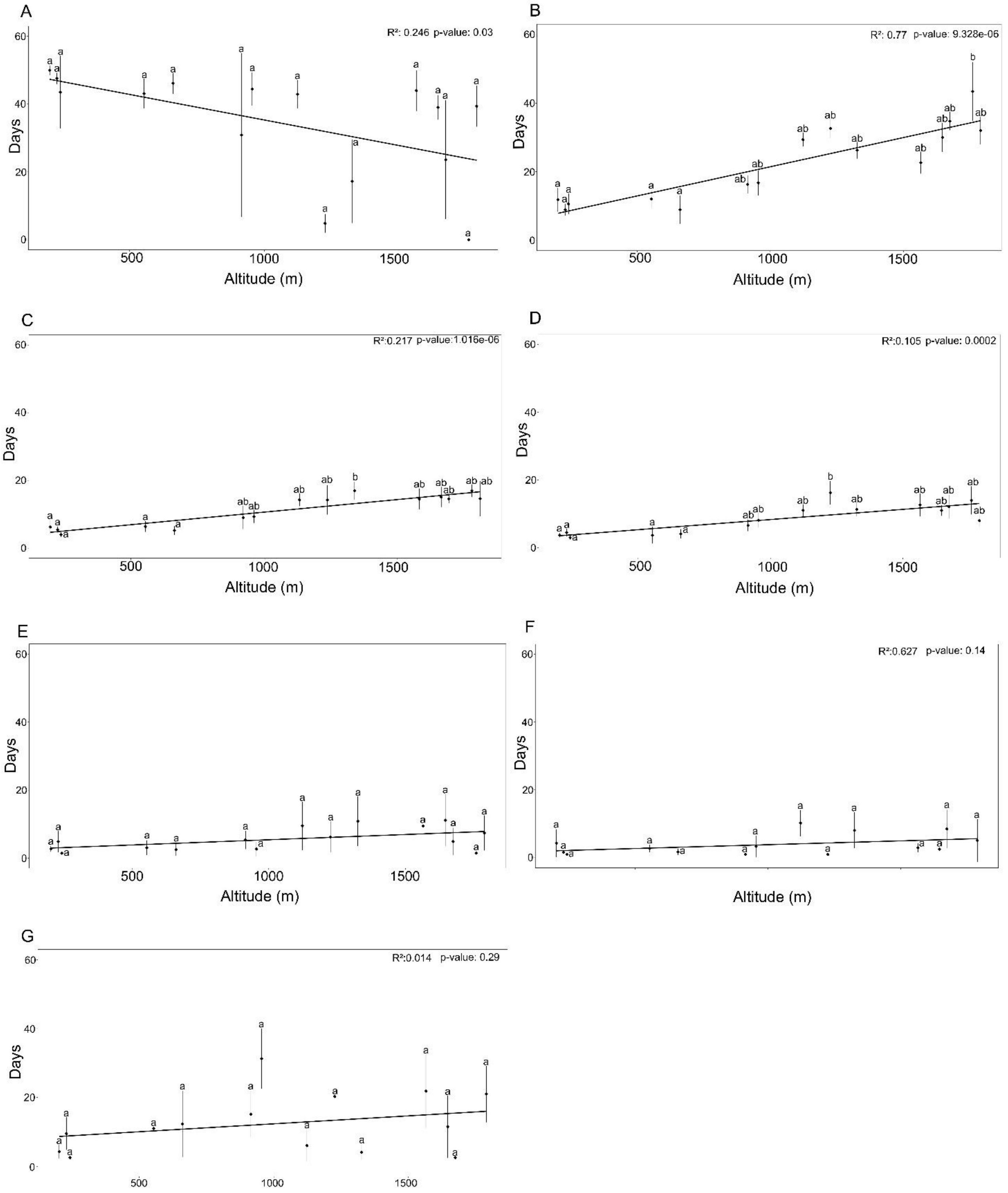

Figure 4.5. Correlation between time to obtain the 50% of germination and altitude at different incubation temperatures: 9°C (A), 12°C (B), 15°C (C), 18°C (D), 21°C (E), 24°C (F) and 27°C (G). Different letters indicate statistical differences, p-values= 0.05

Discussion

Effect of temperature on seed germination

Temperature is one of the most important environmental factors affecting seed germination (Bewley et al. 2013). In particular, temperature play an important role in affecting germination of several Mediterranean geophytes (Doussi & Thanos 2002; Marques & Draper, 2012). In line with the general expectation of a regulatory effect of temperature on germination, our results show that, in *L. pomponium*, temperature affects the final proportion of germinating seeds (Figure 4.1 A). On the contrary, it weakly affects the germination rate (Figure 4.2) probably because germination rate indices are based on the fraction of germinated seeds. For this reason, if the total proportion of germinating seeds differs, different percentiles of the total seed population are compared, and possibly underestimating the differences in germination rates (Bewley et al. 2013). The highest proportion of germinating seeds was observed at 12°C (Figure 4.1 A) and it sharply dropped with the increase in temperature. This optimum temperature restricts the germination window of *L. pomponium* to two short period during the wet Mediterranean seasons (autumn and spring), suggesting a biseasonal germination strategy. In species having a biseasonal germination strategy the surviving of spring seedlings is usually higher than that of autumn seedlings (Masuda & Washitani, 1992; Picó, 2012). Nevertheless, autumn seed germination permits to take advantage of the period of autumn and spring rains avoiding the summer drought, while spring seed germination may assure the germination when few autumn-germinated seedlings survived winter as a second chance. In fact, the low germination temperature suggests a late autumn germination and a short germination window before winter, assuring the building of a seed bank in the soil, as detected in other mountain species (Stöcklin & Bäumler 1996; Schütz 2002). This bet-hedging strategy may increase fitness in unfavourable year (i.e., low spring precipitation) to the detriment of fitness in favourable year (Venable & Brown 1988). This capability is very important under a climate highly variable and unpredictable like that of

Mediterranean mountain, where delayed frost or early drought may occur. In summary, the germination strategy of *L. pomponium* is in line with that expected for a Mediterranean species and for a species with the annual cycle like geophytes (i.e. *Stembegia lutea*, Dafni 1981) that usually survive winter period by underground storage organs and have a quite short aboveground growth period in spring or autumn.

Effect of altitude on seed mass and germination

In *L. pomponium* seed mass does not significantly increase with altitude (Table 4.1, Figure 4.2). Nevertheless, despite a lack of a clear trend, seed mass is higher in population at low than at high altitude (Figure 4.2). In general, wide seeds needs less moisture to reproduce successfully favouring seedling survival in arid environments (Lloret et al., 1999) or in environments with unpredictable drought (Venable and Brown, 1988; Metz et al., 2010). In line with this general expectation, we detected the highest seed size in low altitude population growing under Mediterranean climatic conditions having highly variable rainfall trends. On the contrary, the high-altitude populations, growing under more humid oro-submediterranean climate, had the lowest seed size. Moreover, smaller seeds germinate more slowly than larger seeds (Venable & Brown 1988) and they are expected to be more persistent in soil seed bank (Metzner et al. 2017). A large seed bank may be advantageous in higher altitudes where delayed or early frost may threat seedlings. Therefore, the seed mass in *L. pomponium* seems to be related to environmental conditions and the lack of a linear relation between seed mass and altitude may be because Mediterranean mountains are characterized by large topographic complexity and by high heterogeneity in water availability strongly affecting local site conditions. Small seeds have higher base temperature than large seeds (Aréne et al. 2017), for this reason, large seeds of low altitude may onset germination at lower temperature than small seeds of high altitude. This may enable seeds, in Mediterranean environment, to germinate early in order to avoid summer drought, while in mountain environment, to germinate late to avoid frost.

In *L. pomponium*, the germination rate in all tested temperatures did not change among altitudes of seed source (Figure 4.3); on the contrary, germination velocity (both T0 and T50) increased with altitude of seed source, even if the trend was significant mainly in incubation temperature near to the optimal germination temperature for the species (Figure 4.4 and 4.5). Overall, our results suggest that along the altitudinal gradient the germination percentage did not change but a variation in germination velocity was recorded and both germination proportion and rate were not affected by incubation temperature. The lack of relationship between altitude of source seeds and germination proportion suggests that in *L. pomponium*, the proportion rate is not under a strong environmental pressures contrary to the germination rate. In general, the variability of germination characteristics may be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions (Gutterman 1994; Kigel 1995) and it will reduce the risk of seedlings being subjected to poor growing conditions, due to the establishment of intense competition hierarchies. In *L. pomponium* seed source may explain differences in germination rate but not in germination proportion, as observed in other mountain Mediterranean plants (Giménez-Benavides et al 2007). The timing of germination may affect the subsequent seedling survival under environment with high seasonal variations (Baskin & Baskin, 1998; Shimono & Kudo, 2003), like Mediterranean mountains where aridity may occur during the growing season. The high germination rate in low altitude populations may be a strategy to enable seedlings to growth rapidly before the onset of drought stress during the summer (Fernández-Pascual et al. 2017). In fact, because mechanisms regulating the timing of transition from seed to seedling would be under strong selective pressure (Meyer et al 1997), seed germination patterns, reducing the probability to germinate under environmental conditions not appropriate for seedling survival, may be favoured. For example, in *Phacelia secunda* J.F.Gmel. the among populations variation in germination response is correlated with the duration of snow cover (Cavieres & Arroyo 2000). In *Campanula americana* L. cold temperatures reduce germination across populations, but this decrease was greater for northern than for southern populations; this strategy was supposed to be a bet-hedging strategy to decrease risk of frost damage in northern areas with later snow melt (Zettlemoyer et al. 2017). Similarly, in *L. pomponium*, growing mainly in xeric stony or rocky calcareous habitat of a Mediterranean mountain system, the main risk of seedling death is probably the drought, which rapidly increases over the summer, more than the late frost in spring.

Conclusion

Taken together our results suggest that germination requirement of *L. pomponium* is that expected for a Mediterranean species. However, seed mass and germination rate differ among different seed sources linked to altitude. Even if, from one side this variability may be a survival strategy for a species growing under a variety of environmental conditions like those occurring along a sharp altitudinal gradient in Mediterranean mountains, on the other side same variability does not follow the differences among populations in seed mass and germination rate that are genetically based. Nevertheless, this variability may reduce the probability of extinction in populations that will occur under new climatic conditions leaving enough time for populations to move to another adaptive peak (Price et al. 2003; Ghalambor et al. 2007). A severe increase in temperature might result in a decrease of seed germination and then in changes in population dynamics (Walck et al. 2011; Ooi 2012).

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Chapter 5.

Assessing population genetic structure and variability with AFLP in *Lilium pomponium* L

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Introduction

Quantifying the variation of genetic traits of natural plant populations in the geographical range of a species is crucial for understanding both the factors that have modelled the current distribution patterns (Gaston, 2003) and the processes acting in maintaining and/or expanding the range limits (Thomas et al., 2001). Furthermore, this could enable us to understand whether species will be able to adapt to environmental changes (Parmesan, 2006). The main theory that explains the variation (genetics, physiology, morphology and demography) in the range of a species is the centre-periphery hypothesis (CPH; Hengeveld and Haeck 1982; Brown 1984), also called abundant centre (Sagarin and Gaines, 2002) or central-marginal hypothesis (Eckert et al., 2008). The CPH postulates that species generally have the highest performance at the geographical centre of the range, while toward the edge of a species’ range performance tend to decrease because of harsh marginal ecological conditions (Brown, 1984; Curnutt et al., 1996; Sagarin and Gaines, 2002; Gaston, 2003; Vucetich and Waite, 2003; Hampe and Petit, 2005), which may influence or have influenced levels of neutral and adaptive genetic diversity compared to central populations (Pironon et al., 2015; 2017). In particular, according to the CPH, gene flow is expected to be lower in peripheral populations because of diminished habitat connectivity (Eckert et al., 2008), resulting in an isolation-by-distance pattern (IBD – Wright, 1943). Nevertheless, contrary to this expectation, because peripheral populations are exposed to different selection pressures compared to central populations, gene flow may be strong among similar environments resulting in an isolation-by-environment effect (IBE - Cooke et al., 2012; Zellmer et al., 2012; Bradburd et al., 2013; Wang et al., 2013). So, when ecological margins and geographical periphery are coincident, as assumed by the CPH, both IBE and IBD may be expected.

Furthermore, according to the CPH, geographically peripheral populations are expected to exhibit lower genetic diversity and higher genetic differentiation than central populations (Eckert et al., 2008). However, in peripheral populations, patterns of genetic diversity are usually strongly shaped by past climate-driven range dynamics (Hewitt 2000; 2004). As a consequence, peripheral populations in rear (i.e., stable refuge areas – Pironon et al., 2017) and leading (i.e., recently colonized areas – Pironon et al., 2017) edge of distributional range are expected to have different genetic patterns. Hampe and Petit (2005) suggested that in populations at the leading edge of species distribution the genetic structure is largely shaped by rare long-distance dispersal events followed by exponential population growth, resulting in a decrease of genetic diversity both within and among populations. Differently, in populations located at rear edge of species distribution the small size and the prolonged isolation have resulted in reduced within-population genetic diversity and in disproportionately high levels of genetic differentiation among populations (Hampe and Petit, 2005).

However, the main CPH assumption of a concordance between geographical peripherality and ecological marginality (Brown, 1984) might not always be true (Soulé, 1973; Pironon et al., 2017). In fact, geographically peripheral populations may occur in conditions similar to those in the centre of the range (Piñeiro et al., 2007; Kropf et al., 2008) or in different but not marginal ecological conditions (Papuga et al., 2018). Moreover, environmental factors may impose ecologically marginal conditions in any part of the species' range (Soulé, 1973 – hereafter "marginal populations").

When geographical peripherality and ecological marginality are not concordant, marginal populations are anyway expected to be less genetically diverse and more genetically differentiated, while the among-populations genetic distance is expected to be correlated to the environmental distance and not to the geographic distance.

Nevertheless, little is known about patterns of gene flow across species ranges, and whether there are prevailing patterns with respect to spatial and environmental gradients (Sexton et al., 2013). Species spread along an environmental gradient are particularly appropriate for addressing questions regarding patterns of gene flow and population genetic variations. In this study we used Amplified Fragment Length Polymorphism (AFLP) to explore genetic polymorphism of *Lilium pomponium* L. an endemic plant of Maritime and Ligurian Alps across its wide environmental range, spanning from Mediterranean to Alpine habitats. More specifically, we are asking the following questions: (i) Are environmental and/or geographic distances correlated to genetic distance?? (ii) Is the genetic diversity higher in central than in ecologically marginal populations of *L. pomponium*? (iii) Is there a difference in genetic diversity and genetic differentiation among the marginal populations? To answer these questions may allow to inform the management of populations under environmental stress particularly during periods of rapid environmental change.

MATERIAL AND METHODS

Study species

Lilium pomponium is a perennial geophyte endemic to the Maritime and Ligurian Alps that grows only on calcareous outcrops from 100 to 2,000 m of altitude. The species has a self-incompatible outcrossing breeding system, with a poor capacity for selfing (Casazza et al., 2018).

In a previous study (Macrì et al., submitted; see Chapter 2) 20 populations were differentiated in ecologically marginal and central populations on the basis of the bioclimatic variables using a principal component analysis (PCA). This allowed to recognize five central populations (CC) and 15 marginal populations. The marginal populations were further subdivided into three different groups growing under different climatic conditions: Mediterranean marginal (MM), included four populations; continental marginal (CM) included five populations; and subalpine marginal (SM), included six populations. The marginal climatic groups MM and SM occur respectively at the southern and northern geographical extremes of the distributional range, while the CM group occurs close to the centre of the distributional range of the species (Figure 5.1).

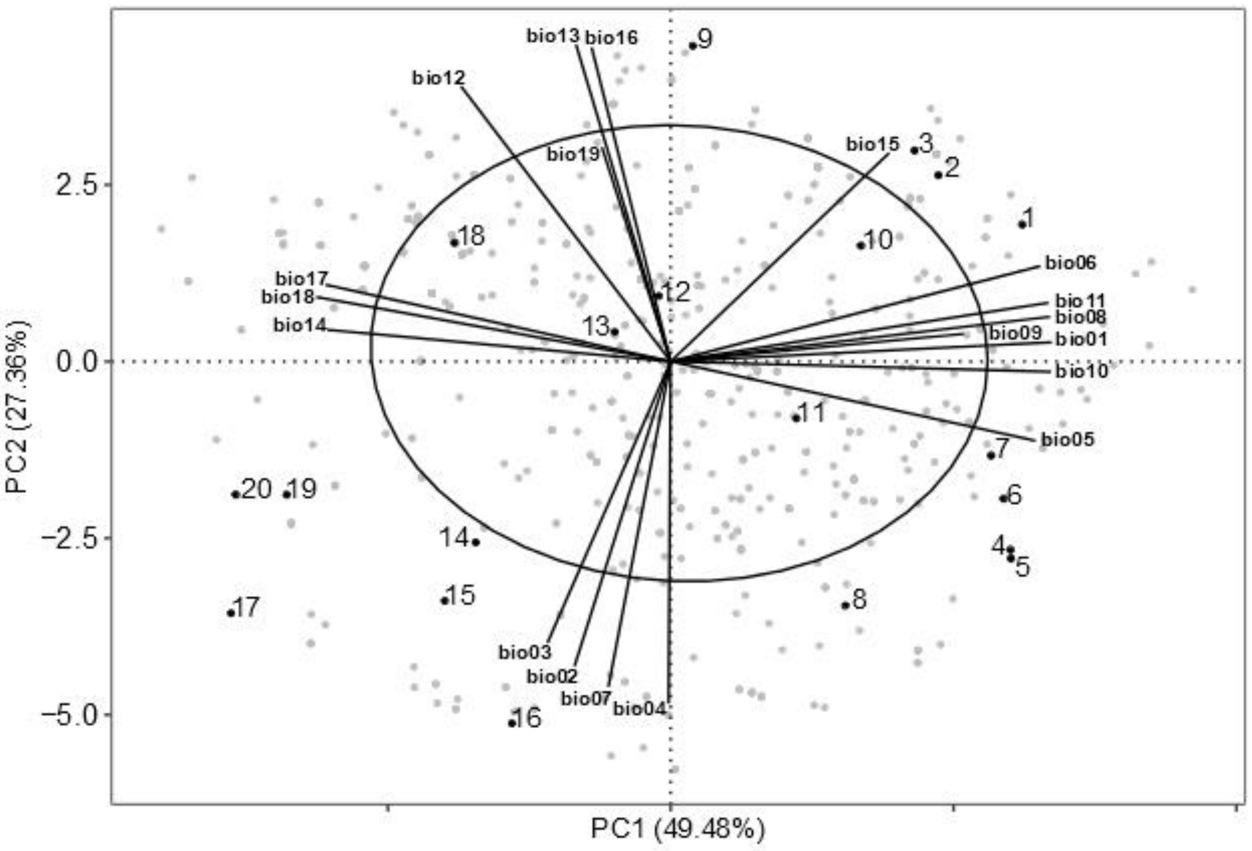

Figure. 5.1. Principal component analysis of climate data. Ellipses include 70 % of each class variance. Central population are inside the ellipse while the marginal populations are outside. Numbers indicate the sample populations. P01, P02, P03, P09: Mediterranean marginal; P04, P05, P06, P07, P08: continental marginal; P10, P11, P12, P13, P18: central; P14, P15, P16, P17, P19, P20: subalpine marginal

Study occurrences and climatic data

Species occurrences were obtained from field surveys (performed by the authors) and from local databases: SILENE (Conservatoire Botanique National Méditerranéen de Porquerolles) and LiBiOss (Regione Liguria). Occurrences were spatially filtered and those closer than 1 km to each other were removed, resulting in a final data set of 809 occurrences. For climatic conditions we downloaded nineteen bioclimatic variables representative of the period 1979–2013 from the Chelsa climate database website (http://chelsa-climate.org/) at 30-s (c. 1 km) spatial resolution).

Sampling and DNA extraction

We collected ten leaf samples from the 20 populations differentiated in ecologically marginal and central populations, in order to cover the entire geographical (Figure 5.2). Genomic DNA has been isolated from fresh leaves according to Kobayashi et al. (1998). The AFLP method has been performed as describe by Vos et al. (1995) whit the modification proposed by Sakazono et al. (2012), as the genome of *Liliaceae* is much larger (Muratović et al., 2010; *L. pomponium* C-value: 34,357 Mbp) than that of a regular plant (C-value: 3,423-13,692 Mbp) and the common protocol for AFLP analysis is not suitable because numerous peaks are detected.

Frozen leaves samples were ground in a mixer 150 mill "TissueLyser" (Quiagen-Retsch). Total DNA was extracted using NucleoSpin Plant II 151 Kit (Macherey & Nagel, Germany). DNA concentrations 172 were measured using a photometer (Biophotometer, Eppendorf, Germany).

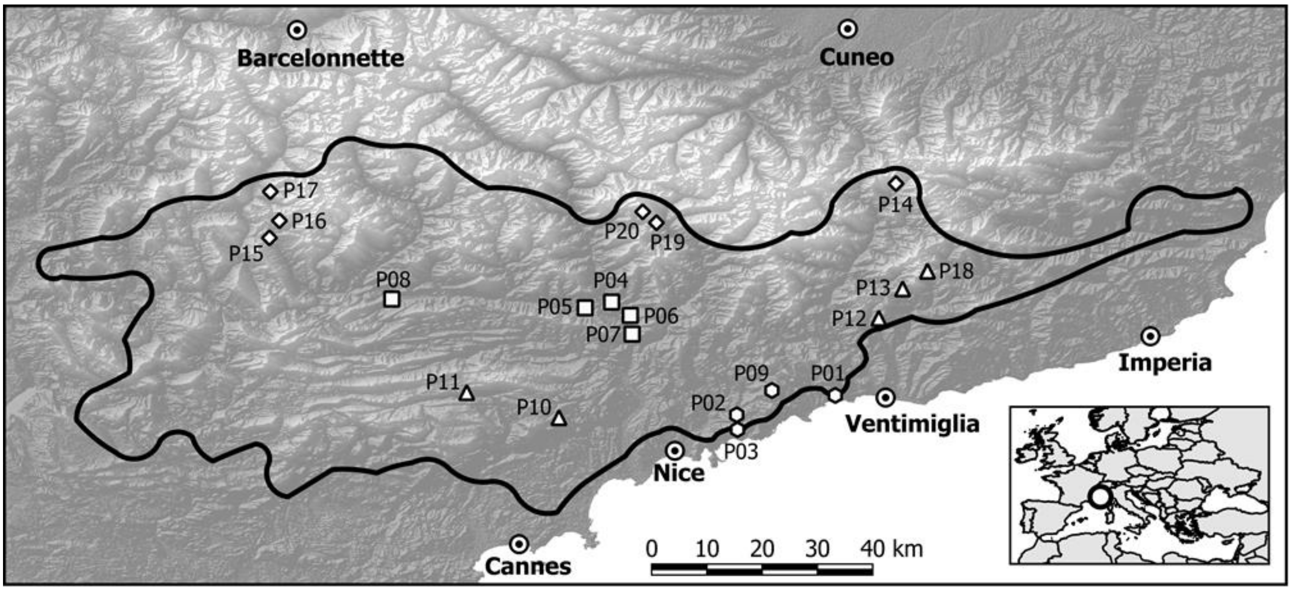

Figure 5.2. Selected population of *L. pomponium*. Populations' symbols: hexagon = Mediterranean marginal; triangle = central populations; square = continental marginal; rhombus = subalpine marginal. Filled circle = Geographic centre (GC).

AFLP genotyping

For the AFLP genotyping we followed the protocol of Baumel et al. (in preparation). According to Vos *et al.* (1995), 100 ng of DNA was digested using the restriction enzymes *Eco RI* and *Tru 9I* (Fisher Scientific, France) for 3 h at 37°C and then for 3h at 65°C in a total volume of 25 µl (15 µL + 10µl of DNA). Digestion products were ligated to 0.5 µL Eco and 25 µL Mse adaptors for 3 h at 37°C and treated with T4 DNA Ligase and 0.1 µL of 100 mM ATP to a final volume of 25 µL (5 µL + 20 µl of restriction products). Ligation products were diluted eight times and pre-selective PCR amplification was performed using *EcoR1*+A, Mse+C primers and *Taq* DNA polymerase in a 44.5 µL volume. The profile of pre-amplification thermocycle was 94°C for 2 min, followed by 20 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 1 min and 72°C for 10 min. For the selective amplification, three primer combinations were chosen for PCR: ASII: EcoR1-AGG/ MseI -CGG, ASIII: EcoR1-AGC/ MseI -CAG, ASVII: EcoR1-AGC/ MseI -CTG dyed with 6-FAM fluorescence at 5′ Eco end (Eurofins Genomics, Ebersberg, Germany). Hundred times diluted pre-amplification products were used to perform selective amplification in a final volume of 20 µL (15 µL + 5 µL of diluted pre-amplification products). For selective amplification thermocycle, we used 94°C for 2 min, 10 cycles of 94°C for 30 s, 65°C for 30 s (step -1°C per cycle), 72°C for 1 min, followed by 22 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, 72°C for 5 min and 4°C for 2h. The fragment length produced by the amplification was separated and quantified by electrophoresis using an ABI 3730xl DNA analyser (Applied Biosystems, Foster City, California, U.S.A.) with GS600 LIZ size marker.

Data analysis

Peaks were scored in Peak Scanner V 1.0 Applied Biosystems) as present (1), absent (0), or “no data” (NA). We utilised Raw Geno 2.0 (Arrigo et al., 2009) to select the fragments longer than 100 bp and smaller than 200 bp. Maximum binning between peaks was set at 1.75 and minimum at 1.5.

For each population we calculated the following parameters of genetic diversity: (i) Nei's diversity index of expected heterozygosity (H_e) estimated using package "Poppr" (Kamvar et al., 2019), (ii) index of observed heterozygosity (H_o) and (iii) the inbreeding coefficient (F_{IS}) estimated using package "Hierfstat" (Goudet et al., 2015) implemented in R (R Core Team 2018). Post-hoc tests were conducted to evaluate pair-wise differences in genetic diversity parameters among central and peripheral populations using the posthoc.kruskal.dunn.test function in the R '*PMCMR*' package (Pohlert, 2018).

Genetic differentiation among populations was compared by calculating fixation index (F_{ST}) pairwise distance matrix using ARLEQUIN (ver 3.5.2.2, Excoffier et al., 2010). The difference among groups was tested using Multiple Response Permutation Procedure (MRPP) in the R package "vegan" (Oksanen et al., 2019). We performed the Analysis of Molecular Variance (AMOVA) to detect genetic variation between the groups, among populations within the groups and individuals using ARLEQUIN.

The correlations between genetic differentiation and both geographical and ecological distance matrix were determined by a combination of Mantel tests (using the R package "vegan" - Oksanen et al., 2019) and matrix regression analysis with a distance matrix (using the R package '*ecodist'* - Goslee et al., 2017). Results were displayed by using package "*scatterplot3D*" (Ligges et al., 2018) implemented in R.

Results

The five AFLP primer pairs resulted in 134 polymorphic fragments (Table 5.1). Within population, an average of 2.29 ± 0.05 of polymorphic fragments were detected.

Table 5.1. Summary of the number of AFLP fragments scored for different primer pairs used in selective amplifications

*Eco*RI primer	*Mse*1primer	Number of loci
E-AGC	M-CGG	78
E-AGC	M-CAG	48
E-AGC	M-CTG	88
total		214
mean		71.3

Average Shannon diversity (H) of tested populations ranged from 2.079 to 2.303, and expected heterozygosity, He, from 0.189 to 0.367 (Table 5.2).

Table 5.2. Genetic diversity estimates in total dataset populations based on AFLP data. Pop: Population name, Group: njhgurahgiuj, N: Sample size, H: Shannon-Wiener Index of MLG diversity (Shannon, 2001), He: Nei's diversity index of expected heterozygosity, Ho: index of observed heterozygosity, FIS (inbreeding coefficient).

Pop	**Group**	**N**	**H**	**He**	**Ho**	**Fis**
P01	MM	10	2.303	0.189	0.138	-0.106
P02	MM	10	2.303	0.301	0.241	-0.142
P03	MM	10	2.303	0.302	0.257	-0.174
P04	MM	10	2.303	0.313	0.259	-0.164
P05	CM	10	2.303	0.304	0.254	-0.164
P06	CM	10	2.303	0.234	0.195	-0.157
P07	CM	10	2.303	0.318	0.318	-0.230
P08	CM	10	2.303	0.335	0.336	-0.242
P09	CM	10	2.303	0.339	0.335	-0.230
P10	CC	10	2.303	0.317	0.405	-0.312
P11	CC	8	2.079	0.360	0.410	-0.301
P12	CC	9	2.197	0.341	0.485	-0.389
P13	CC	10	2.303	0.367	0.415	-0.293

P14	CC	10	2.303	0.357	0.409	-0.299
P15	SM	10	2.303	0.353	0.326	-0.219
P16	SM	10	2.303	0.313	0.284	-0.203
P17	SM	10	2.303	0.285	0.445	-0.398
P18	SM	10	2.303	0.288	0.307	-0.242
P19	SM	10	2.303	0.298	0.394	-0.330
P20	SM	10	2.303	0.278	0.221	-0.145

Nei's gene diversity per population ranged from 0.19 to 0.37 with a mean of 0.31 (±0.04). In addition, Nei's gene diversity was significantly lower in MM and MS populations than in the CC ones (Figure 5.3 A). Observed heterozygosity within populations ranged from 0.14 to 0.48 with a mean of 0.32 (±0.09). In MM populations H_o was significantly lower than in the central ones (Figure 5.3 B). The inbreeding coefficient is negative for all populations ranging from -0.40 to -0.10 with a mean of -0.24 (±0.08). In addition, F_{IS} is significantly lower in CC than in MM populations (Figure 5.3. C).

The AMOVA showed that the majority of genetic variation (90 %) resided within populations, among groups only 4 %, while 5 % of the total genetic variation was detected among populations within group (Table 5.3).

Table 5.3 Analysis of molecular variance (AMOVA) displaying the genetic variation between the groups, among populations within the groups and individuals of *Lilium pomponium*.

Source of variation	df	Sum of squares	Percentage of variation
Among groups	3	201.4	4.02
Among populations within groups	16	457.8	5.68
Within populations	177	3128.0	90.30 *
Total	196	3787.2	

** P< 0.01

Results of MRPP indicates that pairwise F_{ST} is significantly different among groups (A= 0.1934, p=0.0003) and CM and MM populations have lower values of pairwise F_{ST} (respectively 0.2882 and 0.2857) compare to CC and SM ones (respectively 0.4027 and 0.4149).

Weak and significant correlation was found between genetic distance and geographical distance (Mantel test r: 0.265, p-value: 0.002). On the contrary negative and non-significant correlation was found between genetic distance and environmental distance (Mantel test r: -0.042, p-value: 0.672). The same result was confirmed by the combined model of geographical and environmental distances implemented with MMRR, showing that IBD ($r = 0.452$, $P < 0.001$) was more important in explaining the genetic pattern than IBE ($r = -0.313$, $P < 0.001$). Our results suggest a lack of ecological isolation among populations of *L. pomponium*, as geographically distant populations can be ecologically similar (Figure 5.4)

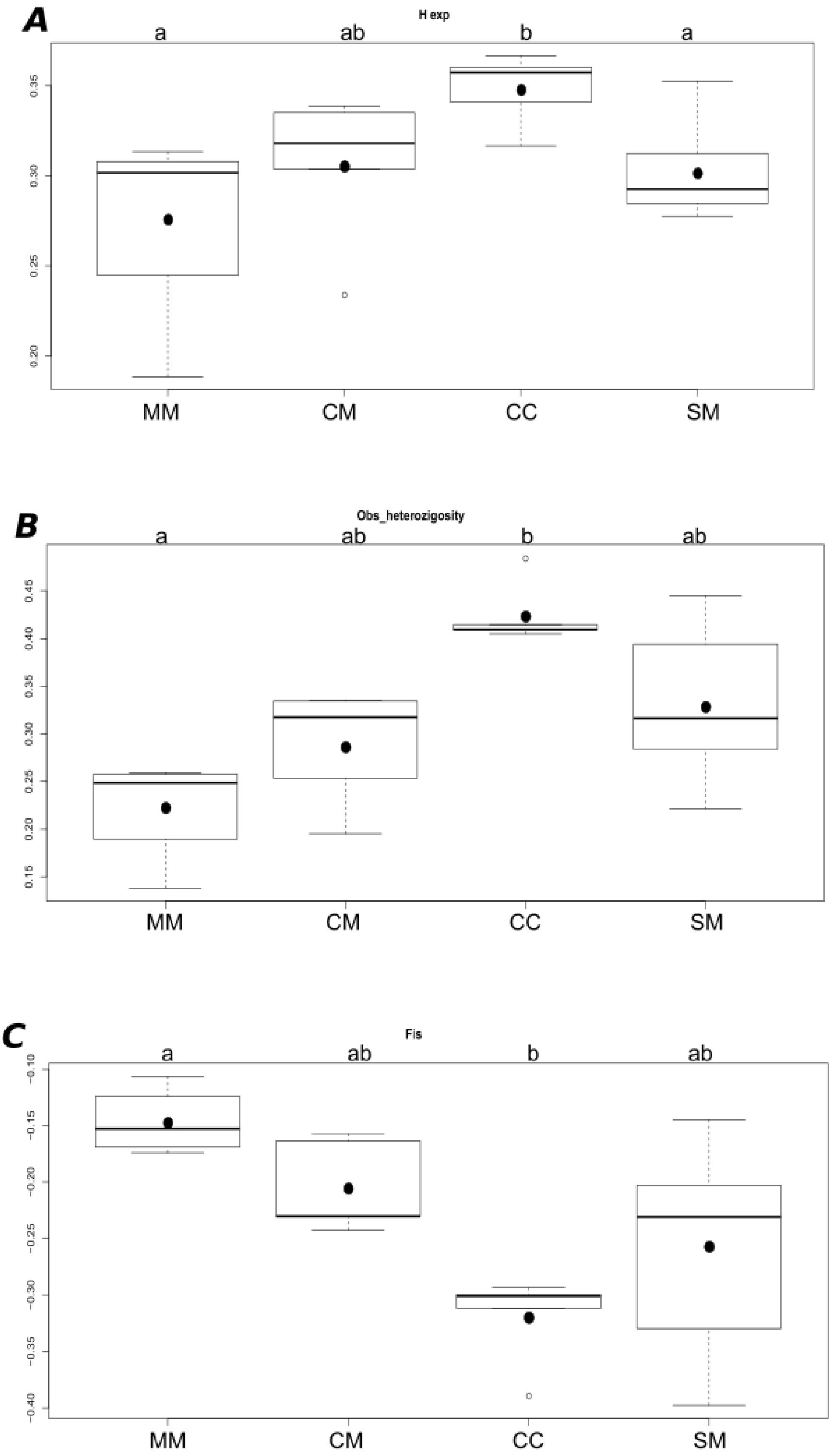

Figure 5.3. A: Boxplot of expected heterozygosity. B: Boxplot of observed heterozygosity. C: Boxplot of inbreeding coefficient. Different letters indicate statistical differences, p-values= 0.05.

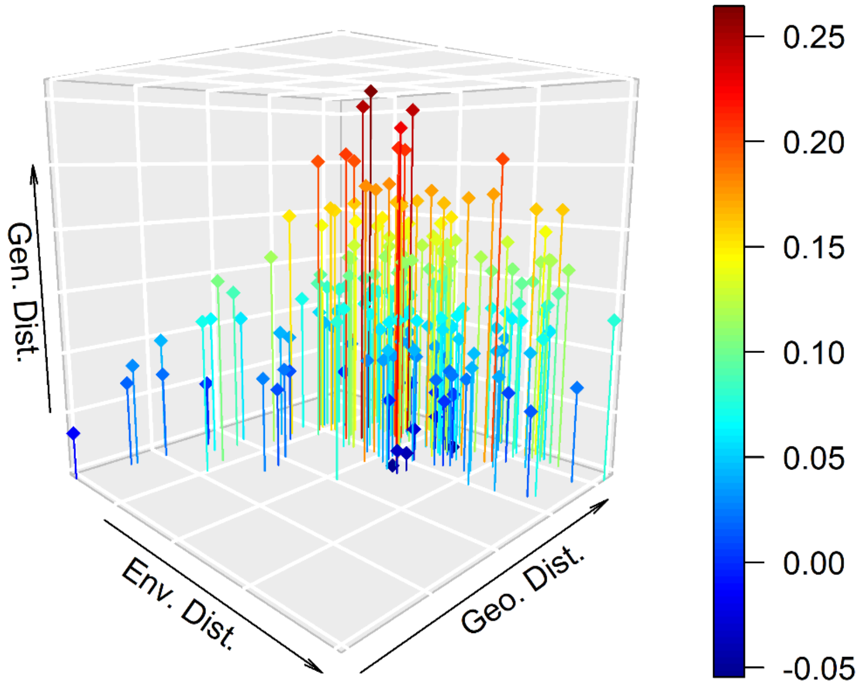

Figure 5.4. 3D scatterplots showing the relationship of genetic, environmental and geographical distance. High correlation values are indicated by red colour, on the contrary low correlation values are indicated by blue colour.

Discussion

Are environmental and geographic distance correlated to genetic distance?

Our results suggest a pattern of isolation by distance, while we found a negative, but not significant correlation between environmental and genetic distances, suggesting that high gene flow may occurs also among dissimilar environments. This is congruent with the partial correspondence between the geographical periphery and ecological marginality. In fact, in Alpine and Mediterranean ecosystems, such as the area where *L. pomponium* occurs, environmental factors change over very short distances because of the high topographic complexity (Körner, 2003; Thompson, 2001), leading to differences in abiotic and biotic resources. Therefore, these highly heterogeneous environments may impose ecologically marginal conditions in any part of the species' range (Soulé 1973). In particular for *L. pomponium* ecologically marginal condition were found close to the geographical centre of the species (i.e., MC), and this likely results in the negative correlation observed between environmental and genetic distances. Moreover, a negative correlation might occur also if differences in the production of propagules (e.g., Kirkpatrick and Barton 1997; Barton 2001) or dispersal in one direction (e.g. prevailing winds pushing propagules up or down a slope (May et al. 1975) cause strong directional gene flow from central to marginal populations along gradients (Sexton et al., 2013).

Is gene diversity higher in central than in ecologically marginal populations of L. pomponium?

According to general CHP expectation, we found that CC populations have higher genetic variability and lower inbreeding than marginal ones (Table 5.2, Figure 5.2), in particular the gene diversity was significantly lower in MM and SM populations compare to CC ones (Figure 5.2) In general, our results support the predictions that genetic diversity is higher in central populations than in marginal populations (Brussard, 1984; Eckert et al., 2008). However, we did not find difference between CM and CC populations, this might be due to the presence of gene flow between these two geographically close groups, as supported by the pattern of isolation by distance found in this study.

Is genetic differentiation higher in rear edge populations growing at the warm margin?

According to the general expectation refugial populations have a reduced within-populations genetic diversity, but disproportionately high levels of genetic differentiation among populations (Hampe and Petit, 2005) compare to recently colonized populations, which are expected to have decrease genetic diversity also among populations, as they represent the front of recent colonization. Differently to the expectation for warm refugia during glaciation, genetic differentiation in MM and CM populations was lower, though not significantly, than SM populations (Table 5.2) and MM and CM populations resulted less differentiated than SM ones (results of MRPP). These results seem to suggest that SM populations might be refugial populations, while the MM and CM populations might represent recently colonized populations. However, the observed genetic structure in MM and CM populations might result from recent bottlenecks that have increased genetic drift and led to the loss of genetic diversity because of the the severe urbanization and landscape fragmentation along the strongly human-populated areas where they occur (Olivier et al., 1995; Médail and Diadema, 2006). In general, the observed pattern may be due to a complex phylogeographical history of *L. pomponium*, in fact, in the Maritime and Ligurian Alps, the less severe ice effects (Médail and Diadema, 2009; Seguinot et al., 2018) combined with heterogeneous topography and high diversity of microhabitats (Casazza et al., 2008; Szövényi et al., 2009) likely allowed *in situ* survival via short-distance dispersal during climatic shifts altitudinal shifts of the species' distribution (Patsiou et al. 2014; Casazza et al. 2016), resulting in accumulation of the responses of species to successive palaeoenvironmental changes (Migliore et al., 2012). Further studies are necessary in order to understand the observed pattern.

Taken together, our results support only partially the CPH, as suggested by the pattern of isolation by distance found and the higher genetic diversity in the CC populations compare to the SM and MM ones. However, we found gene flow among dissimilar environments and a not clear pattern of genetic differentiations among marginal populations, suggesting that environmental complexity, biogeographical history and recent anthropogenic pressures play a role in shaping the spatial distribution of genetic variation in a species with a broad environmental range.

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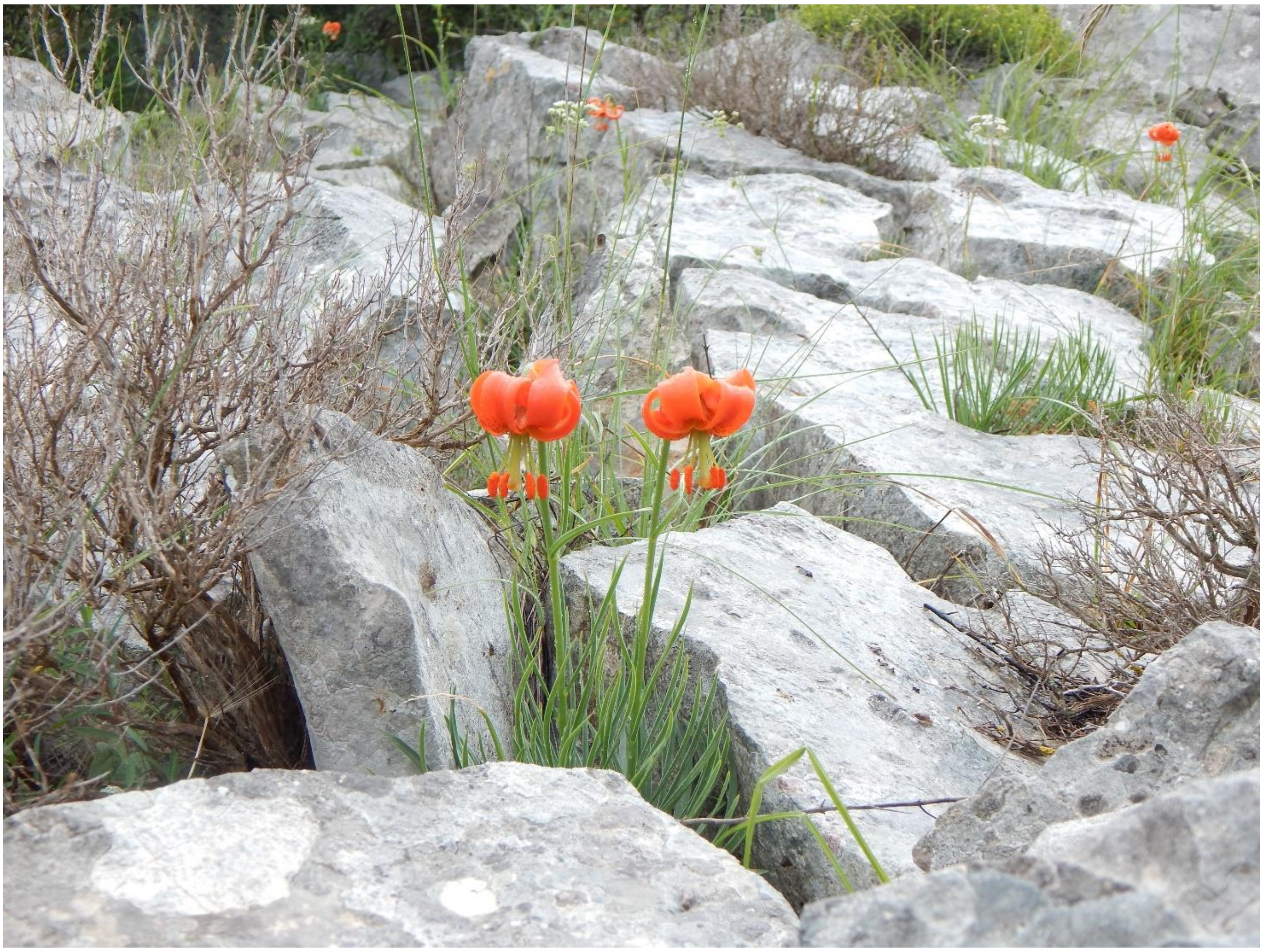
Chapter 6.

Conclusion



"Now, here, you see, it takes all the running you can do,
to keep in the same place.
If you want to get somewhere else,
you must run at least twice as fast as that!"
Lewis Carroll, *Through the Looking Glass*

The aim of my research was to determine if already today it was possible to observe differences in floral phenology, reproductive fitness, and genetics structure in populations of *Lilium pomponium* along its wide altitudinal and environmental range to understand the species' ability to respond to future environmental changes.

The results of this doctoral thesis support the importance to take into account the different life stages of plants to fully understand the relationship between species and both current and future environments. The use of a multidisciplinary approach has been fundamental, since starting from the results obtained from the single analysis (where even the response of a single effect could cause levels of uncertainty about the compensation of the species' response), we were able to evaluate, comprehensively, the possible response of the species to future environmental changes.

First, in *L. pomponium*, climatic marginal populations are both at geographical periphery and close to the centre of the distributional range of the species, suggesting that geographical and environmental gradients are not necessarily concordant and that other factors, like topographic complexity, may impose ecologically marginal conditions near the geographical centre. Moreover, the array of interaction among resource availability, pollinator-mediated selection and population size may induce local conditions which differentially affect floral traits and result in a similar reproductive output because it is likely limited by different factors.

Second, the response of seed germination to temperature supports the idea that the reproductive niche of *L. pomponium* may play an important role in determining plant distribution boundary, although other biotic and abiotic factors contribute to determine the actual distribution. Moreover, as a consequence of future temperature increase, *L. pomponium* will probably shift its germination phenology enabling seeds to germinate avoiding arid conditions and seedlings to remain under conditions they are able to tolerate. The among-populations variability detected in seed germination seems not strictly related to altitudinal gradient. This variability may be a bet-hedging survival strategy for a species growing under high spatially heterogeneous environmental conditions like those occurring along a sharp altitudinal gradient in Mediterranean mountains.

Third, we observed that in populations occurring in warm and dry conditions genetic diversity and differentiation is lower compared to other populations. We assessed that gene flow is correlated whit the distance between populations while environmental isolation does not affect the flow gene. So, in different environments gene flow may be higher, compared to populations in similar environments and geographically close to each other. Thus, our results suggest that the genetic structure of *L. pomponium* populations may be conditioned by the topographic complexity of the areas and by both historical and contemporary biogeographic changes of the life history of the species. Future environmental change could impoverish the genetic structure of the species, especially in populations where we have already observed reduced diversity and differentiation.

Taken together our results suggest that intraspecific variability we observed may be partially explained by CHP but several other factors such as topography complexity, environmental heterogeneity, biogeographical history and anthropogenic pressures may affect the morphological, reproductive and genetic variability of the species. This complexity results in a high intraspecific variability that may favour the species to cope with the unpredictable environmental conditions, frequent in the Mediterranean climate. On the one hand, this variability might also represent a successful strategy to face future environmental change. On the other hand, phenotypes variability due to stressful environments as observed in ecologically marginal populations might result in a phenotype being further away from the favoured optimum under new conditions, favouring populations extinction. In conclusion, our findings suggest that the species shows all the potential characteristics to respond to future environmental changes but probably the answer may not be the same in all populations as the effect of environmental change may act differently on populations along the altitudinal gradient.