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INTEGRATED *IN SITU* AND *EX SITU* APPROACH FOR CONSERVATION OF *Gentiana lutea* L. subsp. *lutea*

BIO/03 Botanica Ambientale e Applicata

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A MI FAMILIA POR EL AMOR Y LA PACIENCIA DEDICADA

Il forse è la parola più bella del vocablario italiano,
perché apre delle possibilità, non certezze.
Perché non cerca la fine,
ma va verso l'infinito…
Giacomo Leopardi

CONTENTS

GENERAL INTRODUCTION

Integrating *in-situ* and *ex-situ* aspects, in the design of a multidisciplinary research program, is an example of a holistic approach to be applied for effective efforts in plant conservation (Silva *et al*. 2015). A multidisciplinary approach can be very useful in order to accurately determine a cause-effect relationship. The main task of conservationists is to design, construct, and manage protected areas or species; this belief is based on the correct perception that conservation of nature requires whole ecological systems, with their dynamic, changing, complex interactions over space and through time (Soulé and Orians 2001). This research presents a holistic study dedicated to *Gentiana lutea* L. subsp. *lutea* in Sardinia (Italy), which have encompassed various aspects: species distribution models (SDMs), seed ecophysiology, reproductive biology, study of threats and evaluation of conservation *status* (through regional assessment IUCN). In addition, the inclusion of future predictions, associated to different IPCC scenarios, improved the information for more effective management, recovery programmes and conservation investments. Undoubtedly, the need to address different aspects involved in the assessment and restoration of endangered plants arises directly from the Global Strategy for Plant Conservation (GSPC), namely from its Objective 1 (*Plant diversity is well understood, documented and recognized*, CBD 2012).

A special attention for this species, included in Directive 92/43/EEC, is necessary to prevent future extinctions on the boundary of its distribution and to propose detailed measures/guidelines for conservation. Due to the limited distribution range in Sardinia and to the degree of isolation and growing sites fragmentation, this *taxon* is particularly vulnerable in this area and, consequently, it's important to highlight the reliability of this study and the possibility of reproducing this framework for further *taxa* with a similar conservation status and/or distribution.

The Gentianaceae family is a cosmopolitan group of 87 *genera* and more than 1600 *taxa* widely distributed throughout the world (Renobales 2012). *Gentiana* L. is a *genus*, comprising ca. 360 *taxa*, being the largest in this family. Most *Gentiana* species are found in temperate and alpine regions of the World (Struwe and Albert 2002). *Gentiana lutea* L. (yellow gentian) is a rhizomatous perennial herb, whose native range includes alpine and sub-alpine pastures of central and southern Europe (Yankova and Yurokova 2009). Four subspecies belong to this species (Tutin 1972, Pignatti 1982, Renobales 2012), with two of them being reported in Italy: *G. lutea* L. subsp. *lutea* and *G. lutea* L. subsp. *symphyandra* Murb (Pignatti 1982).

This study was focused on *G. lutea* subsp. *lutea*, a *taxon* that grows in grasslands, meadows, clear beech, heather and broom in the upper montane and subalpine; 900−2500 m. (Renobales 2012). Its distribution range is through Central-South of Europe: Sardinia, Corsica, Iberian, Italian and Balcan Peninsulas and Alps, rarely on Caucasus and Anatolia (Pignatti 1982, Renobales 2012, Jeanmonod and Gamisans 2013). Concretly, the plant distribution range in Sardinia is restricted to Gennargentu Massif (Chiappini and Angiolino 1983, Gentili *et al.* 2013). Gennargentu Massif (Fig. 1), situated in Central-Eastern Sardinia, is an independent biogeographical sector with a surface of ca. 721 km^2 and consists of a system of summits and windy ridges at 1400−1500 m a.s.l., with four peaks at more than 1800 m a.s.l. (Fenu *et al.* 2014). Metamorphic rocks are by far the most represented outcrops (Carmignani *et al*. 2001). According to the Rivas-Martínez's bioclimatic classification, most of the Gennargentu Massif has a temperate-submediterranean climate, with thermotypes ranging from the lower supratemperate to the lower orotemperate, and ombrotypes from the upper subhumid to the upper humid. The Mediterranean climate is only found on the eastern and southern slopes of the massif, with a lower supramediterranean thermotype and ombrotype ranging from the upper subhumid to the lower humid (Bacchetta *et al.* 2009).

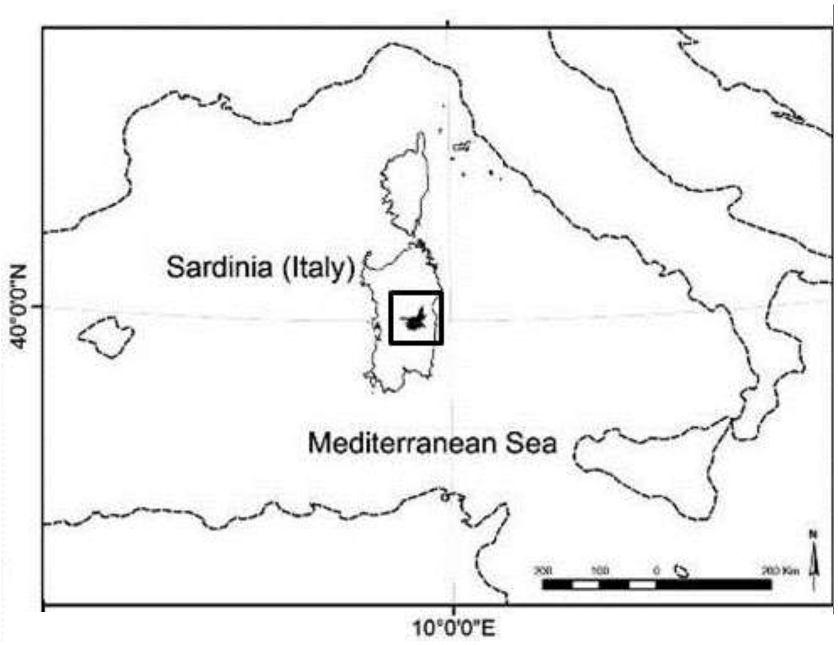

Figure 1. Location of Gennargentu Massif, Sardinia (Italy).

Gentiana lutea subsp *lutea* (Fig. 2) is a long-lived geophyte, which usually develops one unbranched stout stem (rarely two or three) measuring up to 190 cm tall; it shows a basal rosette formed from lanceolate-elliptic leaves measuring 190–350 × 55–150 mm (Renobales 2012). Fertile stems bloom in summer (June-July), and show several tens of bisexual and actinomorphic flowers grouped in pseudo-whorls. Flowers present a bicarpellate ovary fixed over a split calix, a stigma with two lamellae and (4–8) petals fused on the basis. Corollas have an open structure, which facilitates pollinator access (mainly insects belonging to Hymenoptera and Diptera orders) to flower nectaries. *G. lutea* fruits are capsules, which hold a great number of elliptic, flattened and winged seeds, measuring 2.5–4.5 mm, which ripen in summer (Renobales 2012). Wind is the main seed dispersal agent (Struwe and Albert 2002).

Yellow gentian has a high medicinal value for the intensely bitter properties residing mainly in the root, being the main vegetable bitter employed in homeopathy. Its medicinal traits were known to the ancient people, Illyrian King Gentian was the first to indicate them in 200 BC (Millaku *et al*. 2012). The roots are a rich source of bitter glycosides such as gentiopikrin and amarogencine (Pérez-García *et al.* 2012) and for the liqueurs production (Mabberley 2008). Wild populations continued to be exploited for commercial uses, which has led to its decline in some areas. Traditional harvesting of gentian roots implies large recovery periods for wild populations.

However, most of the raw material (90%) continues to be derived from wild resources and wild collections exceed the sustainable quantity available from the natural resources, thus threatening this species (Yonkova *et al.* 2010). This situation has compelled almost all developing countries to enact laws governing the production and distribution of medicinal plants material to safeguard the survival of species in nature for future generation (Pérez-Garcia *et al*. 2012). Hence, *G. lutea* is presented in the List of endangered medicinal plants in the Annex D to the Council Regulation (EC) No. 338/97 of EU, whose purpose is the protection of the plant species by control of their trade. In addition, this species is included in the Annex V of the Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora (European Commission 1992) and has legal protected *status* in several Countries of Europe.

Figure 2. Male step in proterandric flowers of *G. lutea* L. subsp. *lutea* (left), open corolla (centre) with visitor *Diptera* L. genus and (right) female step.

Due to a lack of knowledge on the current *G. lutea* distributional information in Sardinia, Species Distribution Models (SDMs) could be even an important tool to limit search efforts by selecting the areas where field surveys are to be carried out for guiding discoveries of new localities and to evaluate the influence of extant and extinct localities **[Chapter 1]**. These methods are useful when applied to endangered and/or rare species with a poor known distribution area, especially due to difficulties in plant detection and in reaching the study areas (Rebelo and Jones 2010).

Once distribution knowledge was updated, further activities performed on representative localities, including *ex situ* and *in situ* studies were carried out.

Firstly, our contribution to conserve the *G. lutea* in Sardinia, included *ex situ* aspects that provided an alternative and complementary method for preventing immediate extinction (Godefroid *et al.* 2011) and support further interventions (Cogoni *et al.* 2013). The experimental examination of the time when germination occurs in natural sites, the understanding of the seed behaviour in the soil, together with the investigation of the germination response under laboratory conditions **[Chapter 2],** are all crucial for an effective management of plant species (Hesse *et al.* 2007). In particular, the isolated and on the boundary Sardinian population can differ genetically and morphologically from central populations because of their smaller population size and greater physical and ecological distances from the centre of the range, and may contain genotypes adapted to extreme environmental conditions; it is therefore important to check the effect on seed ecophysiology germination on small and spatial isolated populations (Mimura and Aitken 2007).

Previous studies have found that the germination of many mountain plants was promoted by cycles of cold-wet stratification that released seed dormancy in transient and permanent seed banks (Giménez-Benavides *et al.* 2005, Shimono and Kudo 2005, Giménez-Benavides and Milla 2013, Porceddu *et al.* 2013). In this way, dormancy played a key role in optimizing germination success by controlling the timing of germination (Penfield and King 2009). Nevertheless, there is a dearth of data about its ecology, especially in the Mediterranean mountains. Thus, this research would contributed to this species conservation, suggesting the optimal protocol of germination and multiplication and, on the other hand, the information of the different kinds of seed dormancy **[Chapter 3].** This latter property is important to understand the evolutionary relationship and the natural selection to favour germination patterns that reduce the probability of facing adverse environmental conditions for seedling establishment (Baskin and Baskin 2014).

In situ conservation measures are the best methods for preserving plant diversity (UNEP 2002). *G. lutea* is reported as being threatened not only by root harvesting practices, but also by global climatic warming due to its distribution, which is restricted mainly to the upper sectors of the mountains (Gentili *et al.* 2013). Especially in mountain areas, climate warming is projected to shift species' ranges to higher elevations (Grabherr *et al*. 2010). Plant species have responded to global warming through a generally accelerated phenology, enhanced growth and increased reproductive effort (Cleland *et al.* 2007). Even fewer studies have addressed such response of the populations at the boundary of species range distributions (Macias *et al.* 2006), which are thought to be particularly sensitive to climate change, hence it was evaluated the effect of anomalous temperatures (extremely warm) during the year of 2015 on phenology and reproductive successful **[Chapter 4].** Monitoring the underlying drivers of this variation in phenological shifts will contribute to a mechanistic understanding of the biological effects of climate change (Iler *et al.* 2013).

Finally, the criteria established by the IUCN (2001) that are widely employed as the gold standard for information on the conservation *status* of species (*e.g.* De Grammont and Cuarón 2006, Rodrigues *et al.* 2006) were applied at regional scale for *G. lutea*. The assessment through the IUCN Criteria and Categories at global level was updated by Bilz *et al.* (2011) as Least Concern (LC) and at Italian level was recently made by Rossi *et al.* (2013) as Near Threatened (NT) for the Italian Red List. At regional level, Sardinian population were only assessed by Conti *et al.* (1997) as Critically Endangered (CR). An updating of local conservation status of *G. lutea* was thus provided by the analysis of its decline and comparing its potential suitable habitats **[Chapter 5]** based on the emissions scenarios presented by the Intergovernmental Panel on Climate Change (IPCC) for 2050 and 2070.

THESIS STRUCTURE

This research consists in five chapters, divided into two main sections encompassing *ex situ* and *in situ* conservation aspects.

Due to its obsolete knowledge and difficulties in finding its presence in Sardinia, this research programm firstly was implemented with distributional information through several field excursions guided by results of species distribution modelling.

The main aim was to use the Species Distribution Models (SDMs) trained by small sample data for guiding discoveries of new localities.

[CHAPTER 1] describes the development of Maximum-Entropy (MaxEnt) models for this *taxon* in Sardinia with the aims of guiding survey efforts. Besides the Area Under Curve (AUC) values, it was used the Observed Positive Predictive Power (OPPP; observed/modelled positive localities ratio) to compare results arisen from 8, 24 and 58 presence-only data points. Even with the initial small and biased sample data, it was found that surveys could be effectively guided by our results achieving to focus our research on 48% of our initial 721 Km^2 study area. The high OPPPs values additionally proved the reliability of our results in discovering 16 new localities of *G. lutea*.

Nevertheless, the predictive models should be considered as a complementary tool rather than a replacement for expert knowledge.

***EX-SITU* CONSERVATION [SECTION 1]**

Aims:

- To examine the seed germination ecophysiology of *G. lutea* subsp. *lutea* and the influence of growing sites on seed germination.
- To investigate field seed germination time in the soil by seed burial experiments (in Sardinia and North of Spain).
- To determine the class and the type of seed dormancy and relate the dormancy breaking through embryo growth and radicle emergence.
- To characterize the thermal requirements for seed germination (optimal protocol of germination).

This section is composed of two chapters:

[CHAPTER 2] describes the research carried out on seed ecophysiology. Seeds from four natural growing sites, two at the Southern (Sardinia) and two at the Western border (Spain) of the species' distribution range, were tested for germination requirements under a range of different temperatures and by comparing the results with the ones obtained under both cold stratification at 5°C and GA_3 treatment. The identification of germination traits in the laboratory and under natural conditions is necessary in order to better understand its ecology among different growing sites. At the same time, germination tests were carried out in the collecting sites to investigate the germination responses under natural conditions. Our results indicated that germination of *G. lutea* subsp. *lutea* seeds under controlled conditions was limited by high temperatures (25°C), below which GA_3 has a remarkable effect on final germination percentages. Cold stratification did not have an influence on the germination response, suggesting that a temperature of 5°C is not sufficient to break seed dormancy. Germination in the field was not always coherent with the germination response observed in the laboratory, while a considerable degree of physiological difference among localities was recorded under natural conditions. According to these results, implications for the conservation of this species were discussed.

[CHAPTER 3] concerns seed dormancy as one of the adaptive plant mechanisms used to promote survival of the species by dispersing germination in space and time until environmental conditions are favourable for germination. The main goals of this chapter were to evaluate if a pre-chilling temperature at 0°C was effective in breaking seed dormancy and in promoting embryo growth, to identify the class and level of seed dormancy, to evaluate the influence of different locality sizes on the germination response and finally to suggest an optimal germination protocol for this *taxon.* Seeds were subjected to various pre-treatments, including cold stratification (0 and 5°C), warm stratification (25/10°C) and different combinations of them, and after incubated under a range of different temperatures. Embryo growth during pre-treatments and incubation conditions were assessed at different times by measuring the embryo to seed length ratio (E:S ratio). Fleshy, mature seeds had linear underdeveloped embryos. Morphophysiological dormancy (MPD) was identified for *G. lutea* subsp. *lutea* seeds. Cold stratification at 0°C promoted germination and the embryo grew during this pre-treatment. The high optimal germination temperatures found for seeds, as well as its pre-chilling requirement, demonstrated that this species is well adapted to a temperate climate, which is consistent with the centre of origin for this genus. As highlighted by our results, low germination values were correlated with a reduction of locality size.

IN-SITU CONSERVATION [SECTION 2]

Aims:

- To investigate in which way vegetative and reproductive stages (phenology) were connected with climatic conditions and to examine how they conditioned the *G. lutea* reproductive successful.
- To deepen the vulnerability of *G. lutea* analysing the local trend of its localities among past, present and predicted future.
- To assess the conservation *status* following the IUCN criteria A and B owing to fine-quality distribution data.

This section is composed of two chapters:

[CHAPTER 4] makes the bridge between *ex situ* and *in situ* methodology. Our analyses were focused on the phenological and reproductive responses related with climatic factors (temperatures and precipitations). Considering the global warming trend, this information could allow us to bring up some conclusions connected on the effect of climate change on the Mediterranean mountain plants living at the edge of their distribution and to consider these results for next steps towards their conservation. The main aims of this chapter were to investigate in which way warming climatic conditions were connected with the variation of *G. lutea* phenology (vegetative and reproductive stages) and to examine how they conditioned the reproductive successful. In a such climatically unstable condition which we are currently experiencing, phenological temporal changes can be attributed year by year. This chapter provided an example of which problems could occur with plant on the boundary of their distribution associated at global warming. In particular, warm temperatures during the cold period or vernalization (typically autumn and winter) are as important as spring or summer temperatures on flowering and reproductive processes.

[CHAPTER 5] concerns the conservation *status* assessment of *G. lutea* subsp. *lutea* in Sardinia according to the IUCN guidelines. To updated regional distribution by using a series of proved extant and extinct localities. Trends in extinction risk were estimated under IUCN criteria A and B; it was performed to provide local stakeholders the possibility of using this assessment to establish a territorial list of protected species, as proposed by IUCN (2011). Additionally, it was used MaxEnt to compare its potential suitable habitats in 2050 and 2070 under two different emissions scenarios of Intergovernmental Panel on Climate Change in the recent fifth Coupled Model Intercomparison Project (IPCC-CMIP5). Owing to a suffered population reduction, *G. lutea* in Sardinia could be considered as Endangered (EN), according to the IUCN criteria A and B. In addition, models on future climate changes scenarios upraised that most threatened localities will be the ones at the edge of the distribution and elevation gradient; following these results, this *taxon* seems to tend to reduce its elevational range towards higher altitudes. This chapter show a comprehensive analysis which could help the development of a necessary efficient conservation management of *G. lutea* in Sardinia.

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______CHAPTER 1_______

A PRACTICAL METHOD TO SPEED UP THE DISCOVERY OF UNKNOWN POPULATIONS USING SPECIES DISTRIBUTION MODELS

1 Chapter I – A practical method to speed up the discovery of unknown populations using species distribution models

1.1 Introduction

Although the constantly increasing number of threatened species (plants and animals) call for prioritization (Pimm *et al*. 1995), even funds and experts availability for discovering biodiversity are decreasing (Grieneisen *et al*. 2014) due to the current financial crisis; this trend has also been detected for Sardinian threatened flora (Fenu *et al*. 2015). Thus, a critical issue for research is nowadays the optimization of the efforts (Stroud *et al.* 2014). In this framework, several software packages implementing Species Distribution models (SDMs) might be of great help since they are often open source and they can be used to carry out statistical analyses without excessive costs.

Consequently, SDMs have become common place in biological studies, as a tool for exploring basic ecological questions (*e.g.* Guisan and Zimmermann 2000, Ashcroft *et al.* 2011, Bucklin *et al*. 2015), paleo-ecological scenarios (e.g. Varela *et al*. 2011, Patsiou *et al*. 2014, Russo *et al*. 2014), future ecological changes (*e.g.* Randin *et al*. 2009, Araújo *et al.* 2011) and providing support to species conservation or reserve planning (*e.g.* Araújo and Williams 2000, Bosso *et al*. 2013, Guisan *et al.* 2013). SDMs also have practical applications to environmental management, such as detecting unknown populations of endangered species (*e.g.* Jarvis *et al*. 2005, Jiménez-Valverde *et al*. 2008, De Siqueira *et al.* 2009, Williams *et al*. 2009, Rebelo and Jones 2010).

The utility of such models depends on many factors; *e.g*. in case of rare and\or difficult-to-detect species, most of these models are mainly connected to the quantity and quality of initial distributional data. Indeed, they are often limited to small samples of observed localities due, for example, to scarce recent field survey efforts and to the lack of precise localities and bio-ecological data associated with some museum specimens (Graham *et al*. 2004, Soberón and Peterson 2004, Tessarolo *et al.* 2014). These problems are particularly frequent when data come from poorly known ecosystems (*e.g*. tropical) where distributional data are scarce (Pearson *et al*. 2007, Lomba *et al.* 2010, Bosso *et al.* 2013). Some of the methods employed in ecological modelling require absence data to generate SDMs, *e.g.* General Linear Model (GLM) and Random Forest (RF), whereas others are exclusively based on presence data, *e.g*. Maximum Entropy (MaxEnt) and Genetic Algorithm for Rule-set Prediction (GARP). Modelling species with presence-only data has been particularly used for such species with a scarce knowledge and small distributional range (Pearson *et al*. 2007, Shcheglovitova and Andersonn 2013). However, the lack of surveyed locality still affect the model performance and validation can be problematic (Pearson et al. 2007, Wisz *et al.* 2008, Chen and Lei 2012).

Such analyses have been used for population discoveries of either cryptic, rare or endangered animals (*e.g.* Raxworthy *et al.* 2003, Rebelo and Jones 2010, Verovnik *et al*. 2014) or wild plants (*e.g.* Bourg *et al*. 2005, Jarvis *et al.* 2005, De Siqueira *et al.* 2009). This is the case with the species addressed in our study: the yellow gentian (*Gentiana lutea* L. subsp. *lutea*) in Sardinia.

G. lutea deserves special attention because it has been included in the international CITES convention and in the European Habitats Directive; furthermore, the species was assessed as Least Concern (LC) according to the IUCN methodology both at European (Bilz *et al.* 2011) and at national level (Gentili *et al.* 2013). The roots are traditionally used to prepare bitters and liqueurs (Pérez-García *et al*. 2012), as well as pharmaceuticals such as anti-inflammatory agents and diuretics (Nastasijević *et al.* 2012). Consequently, an excessive harvesting and a subsequent decrease in abundance of this species has been observed in several European territories (*e.g.* Kery *et al.* 2000, Gentili *et al*. 2013). In Sardinia, the plant distribution range is characterized by small groups or scattered individuals located at the edge of its distribution range, as a typical peripheral and isolated plant population (PIPP). In addition, due to a lack of knowledge, the current distributional information on *G. lutea* in Sardinia is incomplete and biased.

Owing to these limits, presence-only modelling appeared best suited to deal with its potential distribution in order to reduce survey efforts. We also tried to identify the historical extinct localities through a deep investigation guided by model results and ecological field investigations.

In this study, we developed a Maximum Entropy (MaxEnt, Phillips *et al.* 2006) presence-only distribution model for *G. lutea* to reach the following goals: (1) to use the SDMs trained by small sample data for guiding discoveries of new localities; (2) to evaluate the influence of extant and extinct localities addition on model; and (3) to evaluate the models using the positive predictive power values calculated from a post-test observed data.

1.2 Materials and Methods

1.2.1 Study area and data collecting

According to bibliographic data (Chiappini and Angiolino 1983, Gentili *et al*. 2013), herbarium specimens (CAG, CAT, FI, RO, SASSA, SS, TO *Herbaria*), and unpublished data by the authors, we selected the Gennargentu Massif as the whole distributional area of *G. lutea* subsp. *lutea* in Sardinia (Western Mediterranean Basin). The Gennargentu Massif (Fig. 1), located in the Central-Eastern part of the Island, has a surface of c. 721 km^2 and consists of a system of summits and windy ridges at 1400–1500 m a.s.l., with four peaks at more than 1800 m a.s.l. This area has been recently defined as an independent biogeographical sector based on the peculiar presence of its endemic flora and geomorphological units principally constituted by metamorphic outcrops (Bacchetta *et al*. 2013, Fenu *et al*. 2014).

First localities used for the SDMs indicated by the scarce bibliographic sources (n = 8, Chiappini and Angiolino 1983, Gentili *et al*. 2013) were confirmed by field surveys and georeferred with a hand-held GPS receptor (Garmin e-Trex 20, Schaffhausen, SW). The same methodology was thus used for the subsequent discoveries.

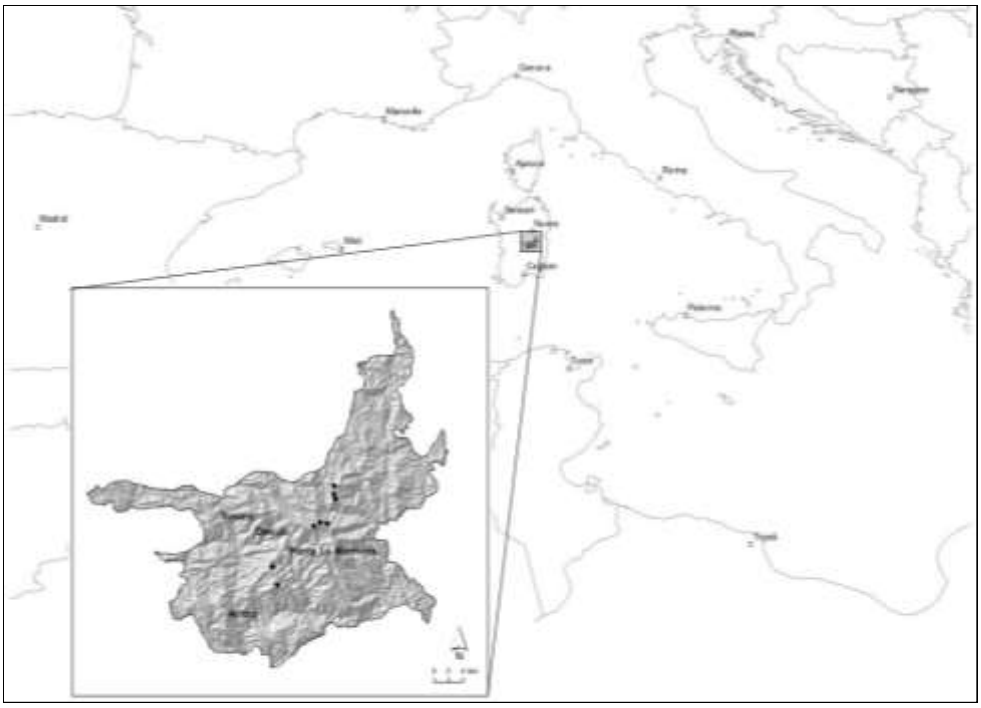

Figure 1. Study area and geographical context. Marked localities of Aritzo, Desulo and Tonara are municipalities included in the Gennargentu massif. Previous known occurrence data are shown as black points.

1.2.2 Distribution modelling

The model comparison was very complicated due to the fact that our study case was based on a plant species with a poorly known distribution, and investigations started based only on occurrence data. We therefore chose *a priori* the most applied method for modelling species distributions with scarce presence-only data, *i.e.* the Maximum Entropy modelling (MaxEnt; Phillips *et al.* 2006) ver.3.3.3k (http://www.cs.princeton.edu/~schapire/maxent). This method usually results in good predictive models compared to other presence-only models (*e.g.* Elith *et al.* 2006, Wisz *et al*. 2008, Ramírez-Villegas *et al.* 2014). Besides the predictive qualities of the technique, it is a generative approach, rather than a discriminative, which can be an inherent advantage when the amount of training data is limited (Phillips *et al*. 2006). Furthermore, it has a good ability to predict new localities for poorly known species (Pearson *et al*. 2007, Rebelo and Jones 2010, Verovnik *et al.* 2014).

Recommended default values were used for the convergence threshold (10^{-5}) and maximum number of iterations (500), and the analysis of variable importance was measured by jackknife, response curves and random seed. Suitable regularization multiplier (fixed at 1), included to reduce overfitting, were also selected automatically by the MaxEnt program (Phillips *et al*. 2006). The form of replication used was the cross-validation; as suggested by Pearson *et al*. (2007) for testing small samples, this run type makes it possible to replicate n sample sets removing each time one locality.

1.2.3 Eco-Geographical variables

According to our research goals, the extension of the study area, the previous ecological knowledge and sample size, we chose to avoid the promiscuous use of variables (*i.e.* model complexity) in order to reduce model overfitting (Anderson and Gonzalez 2011). In addition, we used the finer resolution as possible which usually provides better predictions, especially for fixed or very locally mobile organisms (Guisan and Tuiller 2005). Environmental data at different spatial resolution (10, 250 and 1000 m) were obtained from the web: Digital Elevation Model (DEM) (http://www.sardegnageoportale.it) and Moderate Resolution Imaging Spectroradiometer (MODIS) data (http://modis.gsfc.nasa.gov). Topographic variables (altitude, slope and aspect) were derived from a 10-m resolution DEM. In addition, we computed four layers that represented estimates of vegetation cover and surface temperatures. Two variables were generated from the MODIS 16-day Enhanced Vegetation Index (EVI) and the 16-day Normalized Difference Vegetation Index (NDVI) at 250-meter spatial resolution. Layers of June (*G. lutea* flowering period, Gentili *et al*. 2013) for the last five years (2010-2014) were downloaded using USG MODIS Reprojection Tool (https://lpdaac.usgs.gov/tools/modis_reprojection_tool) and the ensemble of years were computed by BiodiversityR package (Kindt *et al.* 2008) in R (R Development Core Team 2010). The same packages were also used to process the further variables at a lower resolution (1000 m). These were obtained by the daytime MODIS 8-Day land surface temperature: the surface temperature of June (Tjune) and February (Tfeb) were generated by the ensemble of two extreme dates of each month (since 2000 to 2006). All data and coverages were re-sampled to 250 m using raster R package (Hijmans and Van Etten 2012).

Even if Maxent algorithm is able to discriminate the variables by itself, possible overfitting was reduced through the use of high-resolution variables and by analyzing the Variance Inflation Factor (VIF) and percent of contribution indexes. We calculated VIF values to exclude the correlation between all predictor variables through a stepwise procedure. We used the *vifcor* function of usdm R package (Naimi *et al*. 2014), which first finds a pair of variables which has the maximum linear correlation and excludes one of them which has greater VIF. The procedure is repeated until no variable with a high correlation coefficient with other variables remains. Despite of low VIF values (Table 1), surface temperature of February was excluded due to a consistent correlation with the NDVI ($R^2 = 0.73$, Table 2).

Table 1. Variance Inflation Factor (VIF) and Percent of Contribution values. All VIFs values ranged from 1.032 to 2.67, indicating that multicollinearity is not a likely threat to the parameter estimates in our study. Only variable with a percent of contribution (P_contribution) > 0.49 (in bold) were used for each model (M1, M2, M3). Because of high correlation with NDVI, the Temperature of February was not employed (n.e.).

Variables	VIF	P_Contribution (M1)	P_Contribution (M2)	P_Contribution (M3)
EVI	1,730292	0	0	0
NDVI	2,132307	**0,5**	**5**	0,1
Tjune	1,731615	0,2	**0,7**	0,2
Tfeb	2,676252	n.e.	n.e.	n.e.
Altitude	1,566500	**90,6**	**91,8**	**96**
Aspect	1,032645	0	0,2	**1,1**
Slope	1,052781	**8,7**	**1,8**	**0,9**

For each experimental SDM, we used the contribution information automatically provided by Maxent; a first model with all variables was carried out, then only features with a contribution up to 0.5 were included for the final modelling process (Table 1).

Table 2. Correlation matrix for the predictor variables. Bold numbers represent the highest correlation coefficient values (more than 0.7). See text for abbreviations.

	EVI	NDVI	Tjune	Tfeb	Altitude	Aspect	Slope
EVI	1	0,25	0,65	0,27	0,14	0,0097	0,029
NDVI		1	0,26	**0,73**	0,41	0,024	0,019
Tjune			1	0,27	0,003	1,1e-0,5	0,01
Tfeb				1	0,59	0,041	0,079
Altitude					1	0,091	0,099
Aspect						1	0,014
Slope							1

1.2.4 Model evaluation and discovery of unknown localities

As previously tested for small sample size (*e.g.* Hernandez *et al.* 2006, Pearson *et al.* 2007, Ferraz *et al.* 2012), we used a ‘leave-one-out’ method (Fielding and Bell 1997) to evaluate the SDMs. The number of models generated was equal to the number of localities (n) available for that dataset. The n-fold average test AUC was used to measure the ability of predictions to discriminate between observed presence and absence.

We chose to investigate only the predicted areas; we therefore did not take into account negative rates (especially false negatives) since indexes using these values could be biased. Thus, we decided to calculate the post-test Positive Predictive Power (PPP) at the Lowest Presence Threshold value (LPT) corresponding to the “minimum training presence threshold” in Maxent results. The LPT method identifies the minimum predicted area possible whilst maintaining zero omission error in the training data set; also, it is appropriate for applications that aim to identify unknown distributional areas (Pearson *et al.* 2007). The PPP is the rate between true positive (TrP) and total predicted positive (TotP) values of the confusion matrix (Fielding and Bell 1997). In our case TotP is the number of predicted cells, TrP is the number of cells confirmed by direct observations (discovered population and confirmed historical reports), thus we termed the rate “Observed Positive Predicted Power” (OPPP; Table 3).

Table 3. Confusion matrix with simulated under the lower presence threshold (LPT) vs. observed presences and absences. Below it the formula to calculate the observed positive predictive power - OPPP. OPPP = True Positive/Total Positive, where Total Positive (TotP) = True Positive (a) + False Positive (b). Simulated absences were not recorded (N.R).

	Observed *Presence*	**Data** *Ausence*
Data *Presence*	(a) TRUE POSITIVE	(b) FALSE POSITIVE
Simulated *Absence*	FALSE NEGATIVE (N.R.)	TRUE NEGATIVE (N.R.)

AUC and OPPP computation was repeated for three different datasets constituted either by eight data points (only previous known extant occurrences); 24 data points (previous and discovered extant occurrences) and 58 data points (all sites including historical extinctions, Table 4); furthermore, we computed the OPPPs for three different grid scales (0.25, 1, 4 km^2, Table 5). Instead of considering the inference of different scale as a “low-resolution bias” (Boschetti *et al.* 2004), we related these differences to the level of accuracy of the prediction. In order to reduce the sampling bias (Boria *et al.* 2014), we filtered the data by removing nearby localities (distance < 0.5 km).

First model (M1) reliability and the number of the environmental variables were evaluated by AUC values. A great part of the detected suitable areas of M1 (with the LPT threshold) was visited during the months of June and July 2014 (expected *G. lutea* flowering period) in order to detect previously unknown occurrences. During the same guided field surveys, all the historical reports were verified to detect the extinct historical presences. Such areas were recorded as extinct localities only where the model prediction was confirmed by the actual presence of the ecological conditions. All sites were georeferenced using a hand-held GPS receptor (Garmin e-Trex 20, Schaffhausen, SW).

After georeferencing all field surveys data, new occurrence points were employed to build M2 and M3 models and to compute the OPPP values. We used QGIS 1.7.4 (Quantum GIS Development Team 2012) to evaluate the OPPP values.

1.3 Results

Models were analysed according to the two different outcomes and sample size. We firstly wanted to test the utility of M1 to reduce field surveys efforts and costs. M1 showed high AUC values (±standard deviation); the best one was recorded using only three variables (Alt, Slope, NDVI; Table 4). From an initial study area of 721 Km^2 (corresponding to 121 grid cells of 4 km^2), we focused our research on the 49% of the total area (59 grid cells; Table 5).

Table 4. Results of Maxent models according to different number of occurrences. AUC is an estimate of the model accuracy based on the area under the curve, standard deviation (sd) of replicates AUCs is reported inside parentheses. The OPPP at LPT value is the rate between Total Positive (TotP) and True Positive (TrP). See text for further explanations.

Model	AUC (±sd)	TotP	TrP	OPPP at LPT	Resolution (km^2)
M1	0,982 (0.019)	528	66	0.11	0.25
		193	51	0.228	1
		59	33	0.48	4
M2	0.974 (0.016)	501	66	0.11	0.25
		166	51	0.266	1
		55	33	0.491	4
M3	0.978 (0.015)	246	66	0.236	0.25
		91	51	0.484	1
		42	33	0.738	4

After several field surveys covering 97% of the detected suitable areas (228 km^2), 49% of them were also recorded as discoveries of unknown localities (12 grid cells) and extinct confirmed localities (16 grid cells; Table 5); thus, 16 new current occurrences of *G. lutea* (included in 12 grid cells of 4 km^2) were found. From a high fragmented pattern composed by three poorly known and isolated stands (Fig. 1), we achieved to increase the observed distribution knowledge and to suppose a connection between the groups (Fig. 2d, 2e, 2f). This information was also corroborated by the modelled (*i.e.* potential) distribution (Fig. 2c).

M2 and M3 also showed high AUCs values, thus, the choice of the best-fitted model was difficult because of the previously reported problems of small sample size data training. The OPPPs at LPT showed optimal performance when using the largest sample size (including also confirmed extinct localities); although an increase of the independent variables allowed the model to reduce the errors in false positive detection (*i.e.* commission). The OPPP value of M1 confirmed the utility of modelling even with small sample sizes. Indeed, in 47% of the cases it was possible to confirm the goodness of the predicted results (Table 5). No significant evidence indicated the increase of trained localities between M1 and M2, whereas a consistent difference in OPPPs was detected between M3 and the others; this was also confirmed by standard deviations of AUCs. Comparison between of OPPPs with different grid size highlighted the influence of spatial resolution on the accuracy level of the predictive model (Table 5).

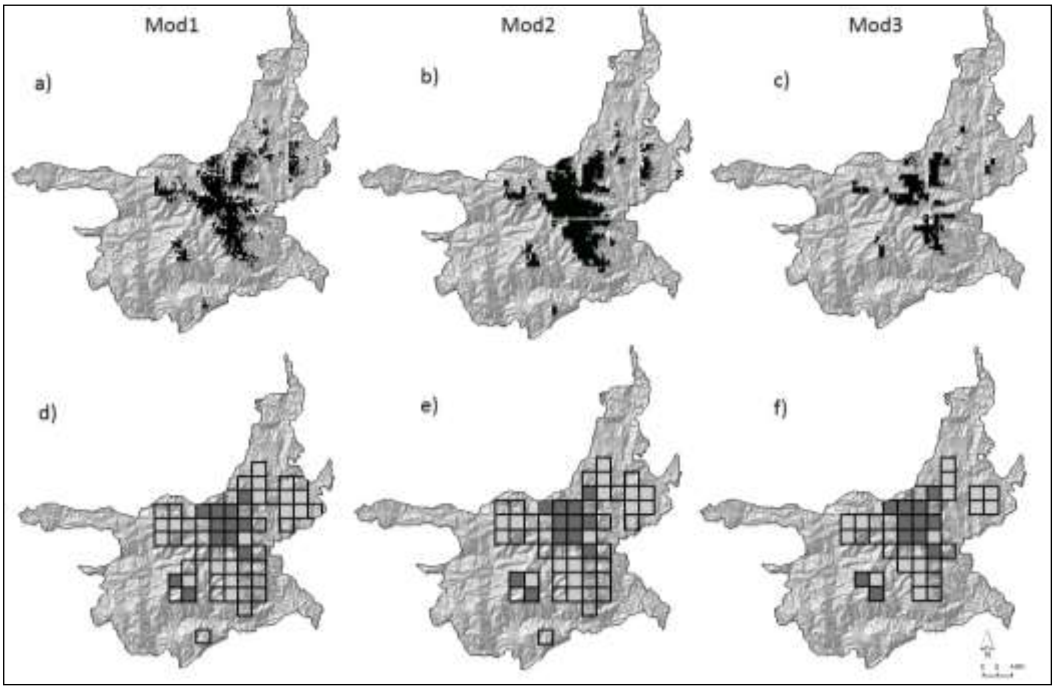

Figure 2. Potential area of occupancy estimated for *Gentiana lutea* along its known distribution area in Sardinia, using three data sets obtained from bibliography (M1; a,d) and field surveys (M2; b, e and M3; c, f). Dark colors (a; b; c) show model-based estimates using maximum entropy algorithm and the minimal predicted area as probability threshold. Grids (2 km × 2 km) with black contour represent the potential distribution areas, dark gray cells represent the current presences and light gray the confirmed extinct localities.

Table 5. Summary of characteristics of the three models. The first model (M1) was trained only by previous known localities, they increase in second (M2) and third (M3) models due to the addition of discovered and extinct localities. The variables[b] are in order of percent contribution.

Model	N. Localities[a]	Discovered localities	Extinct localities	Variables
M1	8	0	0	Alt, Slope, NDVI
M2	24	16	0	Alt, NDVI, Slope, Tjune
M3	58	16	34	Alt, EVI, Asp, Slope

[a] the number of the occurrence points used for each model.
[b] See text for abbreviations

1.4 Discussion

Previous studies have identified considerable differences between predictions obtained from different modelling algorithms, emphasizing the importance of careful selection of appropriate methods and the need to assess results from more than one approach (*e.g.* Thuiller *et al.* 2004).

Despite this, our results reinforced the choice to use Maxent models for incomplete and biased presence samples (*e.g.* Rebelo and Jones 2010). The predictive power of models was firstly supported by high AUC values (> 0.75; Elith *et al.* 2002). Thus, we also gathered practical evidence of our models performance by identifying previously unknown areas of presence for *G. lutea* in Sardinia.

Because of the alluded limits, especially for M1, we initially had some doubts on model reliability. Nevertheless, as previously demonstrated (Bourg *et al.* 2005, Jarvis *et al.* 2005, Pearson *et al*. 2007, Williams *et al*. 2009), our modelling effort (advised by a previous ecological knowledge) was successful at guiding field surveys and the subsequent discovery of unknown localities. Considering our results, we stress the usefulness of SDMs as a tool which significantly reduces both the extent of planned field surveys and the efforts related to time and economic resources. Due to its particular biological (it grows in difficult-to-reach places and it is detectable only during its short flowering period) and historical traits (prolonged harvesting of wild plants), we started from an incomplete distribution dataset of *G. lutea*. Despite this, we achieved to discover 16 unknown extant localities (doubling the previous known occurrences) and we better defined the historical distribution through the investigation of confirmed extinct localities during only one field season (approx. two months), thus improving significantly our knowledge on the conservation status and distribution of *G. lutea*. Therefore, our results encourage us to apply SDMs for several threatened and poorly investigated plants spread through the Sardinian territory. These methods could be particularly useful for conservation planning (*e.g*. management, restoration, conservation status assessment) in areas such as the Gennargentu Massif, which is especially rich of endemic species (Cañadas *et al*. 2014).

The use of AUC values was driven by previous successful works in comparing models with the same species and extent of the area of study (Peterson *et al.* 2007, Lobo *et al*. 2008, Elith and Graham 2009, Lobo *et al.* 2010).

Due to our difficulties in model discrimination through this threshold independent value, we searched for further indexes of performance. In some cases, the lack of occurrence records meant that no independent test of model quality was carried out (*e.g*. Ortega-Huerta and Peterson 2004); in other cases, a *P* value is calculated (Pearson *et al*. 2007). This threshold-dependent index is also useful for model evaluation of rare species (Hernandez *et al*. 2006, Pearson *et al.* 2007) but it still sharing the assumption that randomly selected samples from original data constitute independent observations.

The choice to evaluate models *a posteriori* through the OPPPs values allowed us to confirm the goodness of models, especially when only few points are trained, through real external data. In particular, OPPPs values gave a concrete measure of the utility of SDMs for guiding unknown population discovery and enforced the low differences detected by AUCs and their standard deviations.

1.5 Conclusion

Our results were consistent with previous studies that found model performance increases with sample size (Pearce and Ferrier 2000 Stockwell and Peterson 2002, Hernandez *et al.* 2006; Wisz *et al.* 2008). This kind of information was difficult to detect by AUCs values and only the lower AUC standard deviation (sd) confirmed the best OPPP value of M3. In particular, the greater number of occurrence points allowed the algorithm to better identify the actual distribution range of the species and to avoid a high false positive rate (*i.e.* low OPPP). Thus, we suppose that the information contained by the additional confirmed extinct localities allowed to reduce biased data (due to an indiscriminate and stochastic harvest) and to better define the ecological niche of the species (Peterson *et al*. 2011).

Limits of model predictions were still evident when trying to get results with higher resolution. When using small sample size, a relative low prediction power was detected even at fine resolution (2 km), while it increased significantly when using all known localities (47.4% of modelled suitable habitats coincided with all proved occurrence data). At very high resolution (0.5 km), an acceptable level of accuracy was obtained only when using as many as possible number of known occurrence data (*i.e.* after further researches on species distribution).

Our results represent a practical and easily applicable example of how useful SDMs could be for biodiversity discovery, especially when dealing with threatened and endangered plants, as well as with *taxa* whose distribution is poorly known. Due to the intrinsic characteristics of rare and threatened species, the commonly applied statistical methods for model evaluation could be insufficient and we argue that only the use of indexes computed by post-test external data could increase the reliability of results. We therefore would like stress that predictive models represent a potential useful tool that must be used with caution (especially for some goals such as conservation assessment) and they must be regarded as a complement of rather than a replacement for expert knowledge.

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_______CHAPTER 2_______

SEED GERMINATION OF *Gentiana lutea* L. subsp. *lutea*: COMPARISION OF NATURAL *versus* CONTROLLED CONDITIONS

2 Chapter II – Seed germination of *Gentiana lutea* L. subsp. *lutea*: comparison of natural *versus* controlled conditions

2.1 Introduction

The broad objective of a seed germination ecologist is to explain how the timing of germination is controlled in nature, the ecological and evolutionary origins and the consequences of them (Baskin and Baskin 2014). Information on seed germination also is important because it contribute to a better understanding of certain biological concepts such as plant reproductive strategies, life history traits, adaptation to habitats and physiological process (Baskin and Baskin 2014). In seasonal climates (as our study case), temperature is usually the main environmental factor controlling seed germination in moist soils (Fenner and Thompson 2005). Plants in environments that are exposed to cold field temperatures have been found to be more likely to possess some form of seed dormancy than species living in milder environments (Jurado and Flores 2005). Seed dormancy prevents precocious seedling emergence after seed dispersal and avoids damage during winter conditions (Körner 2003), this type of seed dormancy is naturally broken through exposure to low-temperature conditions characterised by the snow cover period and time of snowmelt and, experimentally, through cold stratification methods (Baskin and Baskin 2014).

Gentiana lutea L. is a species whose distribution range includes the mountains of Central and Southern Europe, which suggests that its seeds may have a cold stratification requirement for germination (Favarger 1953). Generally, germination of Gentianaceae has been shown to be difficult under laboratory conditions and seeds have been reported to be dormant (Baskin and Baskin 2014). Several species of this family can exhibit morphological (MD) and morphophysiological dormancy (MPD) (Baskin and Baskin 2005, 2014). Controversial information on the type of dormancy were found for *G. lutea* seeds, Nikolaeva *et al.* (1985) reported a non-deep complex MPD, while Pérez-García *et al.* (2012) showed that seeds of this species exhibit non-deep PD. Once the gentian seeds have lost their dormancy, they will germinate under a wide range of temperatures, as reported for many temperate climate species around the world (Vandelook and Van Assche 2008, Baskin and Baskin 2014). Pérez-García *et al.* (2012) showed that moist chilling at 5°C did not significantly increase the final germination percentages over the non-chilled seeds of the different accessions of *G. lutea* seeds belonging to North-Western Spain, while GA_3 enhanced seed germination drastically in all the accessions. A similar behaviour was observed by González-

López and Casquero (2014) in seeds of *G. lutea* var. *aurantiaca*. It is widely known that gibberellins play an important role in a number of physiological processes of plant development (Finch-Savage and Leubner-Metzger 2006). In particular, GA_3 can enhance dormancy release, embryo growth, increment the germination rate and promote seed germination under a wide range of temperatures (Mattana *et al.* 2012).

As regards germination in natural sites, seeds of some temperate Gentianaceae may retain their viability for at least three years in soil seed banks and may germinate in spring or early summer (Thompson *et al.* 1997). However, seeds of several herbaceous *Gentiana* species from the Alps were classified as having transient soil seed banks, persisting for less than one year (Cerabolini *et al*. 2003). In fact, most mountain species are "programmed to germinate in spring/summer" (Mondoni *et al*. 2012). The experimental examination of the time when germination occurs in natural sites, the understanding of the seed behaviour in the soil, together with the investigation of the germination response under laboratory conditions are all crucial for an effective management of plant species (Hesse *et al.* 2007) and the germination percentage can be used as an indicator of recruitment success (Walder and Erschbamer 2015).

Pérez-García *et al.* (2012) found intraspecific differences in seed mass and germination among Spanish *G. lutea* populations. Variation in seed mass has been observed in many species among populations, among plants within populations and within single plants, which sometimes appear to be favoured in particular environments (Giles 1990). Fenner and Thompson (2005) reported that seed size may affect the germination percentage. In particular, in alpine and mountain species seed mass appears to be positively related to the germination rate (Schwienbacher and Erschbamer 2002). According to Pluess *et al.* (2005), the seed mass increases with altitude, and heavier seeds germinate more rapidly and perform better than light seeds (Erschbamer *et al.* 2010).

Populations from similar habitats may have different germination responses, and intraspecific differences among populations of the same species can arise from environmental variation during seed maturation and the effect of maternal genotype (Wulff 1995).

In this work, we examined the intraspecific variation of *G. lutea* subsp. *lutea* germination response by studying the effects of pre-chilling and GA_3 on dormancy breaking and seed germination in laboratory conditions and evaluating the germination behaviour in natural sites. We were specifically interested in one main question: are there any differences in the seed germination behaviour of *G. lutea* subsp. *lutea* seeds under controlled and natural conditions? As additional aims, we wanted to verify the presence of intraspecific variation on seed mass and germination ability.

2.2 Materials and Methods

2.2.1 Study species

Gentiana lutea subsp. *lutea* (hereafter *G. lutea*) is a perennial herbaceous plant over one meter tall, with rhizomatous and branched roots. In summer the plant produces inflorescences up to 120 cm, with yellow flowers (3–4 cm) grouped in pseudo-whorls with corolla divided into five open lobes (Gentili *et al.* 2013). This *taxon* has been included in the European Habitats Directive (92/43/EEC) Annex V, in the EU Wildlife Trade Regulation No.338/97 and in the EU Policy species (Allen *et al.* 2014).

In this study, four natural growing sites were chosen (Table 1), two in the Gennargentu Massif (CE-Sardinia), where it can be found in small groups or scattered individuals in open grasslands (Fois *et al.* 2015), and two in the Cantabrian mountains (N-Spain), where it grows in small groups in wet meadows, hay meadows and open woods (Renobales 2012). This species grows on sloping territories and it is found in the plant communities referable to the *Carici-Genistetea lobelii* (Klein 1972) Pignatti *et* Nimis 1980 in Sardinia (Pignatti *et al.* 1980) and *Cytisetea scopario-striati* Rivas-Martínez 1975 in Northern Spain (Rivas-Martínez *et al.* 2002).

Table 1. Locations, collection data and seed lot details for the Sardinian and Spanish growing sites of *G. lutea* under study.

Locality (Region, State)	Locality code	Collection date	Altitudinal range (m a.s.l.)	Substrate type	Coordinates (WGS84 datum)	Aspect	Seed mass (mg ± SD)	Seed water content (aw)
Is Terre Molentes, (Sardinia, Italy)	IS	20/08/2013	1460 - 1505	Metamorphic (phillite)	N 40°02’43” E 09°19’91”	35° NE	0.154 ± 0.008	0.546
Trainu Murcunieddu, (Sardinia, Italy)	TM	21/08/2013	1324 - 1372	Metamorphic (phillite)	N 40°03’29” E 09°19’25”	280° NW	0.153 ± 0.006	0.533
Peña Santa Lucia, (Castilla y León, Spain)	SL	27/08/2013	1409 - 1429	Metamorphic (metaquarzite)	N 42°54’38” O 04°38’15”	270° NW	0.112 ± 0.005	0.525
Peña Carazo, Castilla y León, Spain)	PC	25/08/2013	1491 - 1526	Metamorphic (phyllite)	N 42°57’34” O 04°34’25”	15° NE	0.117 ± 0.009	0.512

2.2.2 Seed lot details

Mature fruits (capsules) of *G. lutea* containing well-developed ripe seeds were sampled in August 2013 from four representative wild growing sites (Table 1), two from the Gennargentu Massif, located in Central-Eastern Sardinia (Is Terre Molentes "IS" and Trainu Murcunieddu "TM") and two from the Natural Park Fuentes Carrionas of Palencia province in Northern Spain (localities of Peña Santa Lucia "SL" and Peña Carazo "PC"). All of them are found at an altitudinal range of 1300–1500 m a.s.l. and are characterized by siliceous metamorphic substrate (Table 1). In addition, the two sites of each region are exposed one to the North-West and the other one to the North-East, respectively (Table 1). Seeds were taken from at least 30 randomly selected plants in each growing site. The collected seeds showed a similar degree of ripeness, as observed from their colour and hardness. Seeds were manually cleaned, discarding any visually malformed seeds (empty, parasitized or not completely formed), and then stored at room temperature (ca. 20°C and 40% relative humidity) until they were used in the germination tests. The seed mass (mg/seed) of the different accessions was quantified in an analytical balance with a precision of 0.1 mg (Crystal series, Gibertini, Italy) before starting the experiments by weighing 10 replicates of 20 seeds each one. The initial seed water content was determined through the indirect method to measure *water activity* (*aw*) by using a Hygropalm AW-DIO (ROTRONIC Measurement Solutions International, Table 1).

2.2.3 Germination tests under controlled conditions

For each accession, four replicates of 25 seeds were sown on the surface of 1% agar water in 90 mm diameter plastic Petri dishes and incubated in the light (12 h light/12 h dark) under a range of constant temperatures (5, 10, 15, 20 and 25°C) and under an alternating temperature regime (25/10°C). In the alternating temperature regime, the 12 h light period coincided with the higher temperature period. The effect of cold stratification (CS) at 5°C (1% agar water in 90 mm diameter plastic Petri dishes) was tested for a period of three months, after which the seeds were incubated as detailed above. The effect of gibberellic acid (GA_3, $250 \cdot \text{mg L}^{-1}$) in the agar substrate was tested under the same range of germination temperatures. Germinated seeds were scored three times a week and germination was defined as visible radicle emergence (≥ 1 mm). All germination tests (Control, CS and GA_3) started in September 2013 (ca. one week after seed collection) and were conducted at the same time using the same environmental test chamber (Sanyo MLR-351) equipped with white fluorescent lamps (FL40SS.W/37 70-10 µmol m^{-2} s^{-1}). At the end of the germination tests (for a minimum of 90 days), when no additional germination had occurred for two weeks, a cut test was carried out to determine the firmness of the remaining seeds and firm seeds were considered viable (Porceddu *et al.* 2013).

2.2.4 Germination tests under natural conditions

Within two weeks from the date of collection, a total of 15 replicates of 35 seeds were placed in fine-mesh polyester envelopes and buried in the soil at a depth of ca. 3 cm in each of the four natural growing sites (Table 1). Three replicates were exhumed at intervals of about three months from September 2013 to June 2014 (with two intermediate spring exhumations in April and May 2014, for a total of five exhumations; Table 2). Retrieved envelopes were analysed in the laboratory, where they were washed under running water and opened. The number of germinated seeds was recorded, and a cut test was carried out to check the viability of any remaining non-germinated seeds. Seeds with fresh white endosperm and healthy embryos were considered viable.

Soil temperatures at the level of the envelopes were recorded at 90 min intervals using data loggers (TidbiTw v2 Temp logger, Onset Computer Corporation, Cape Cod, MA, USA).

Table 2. Date of sowing and category results (mean ± SD) of *G. lutea* seeds obtained at each exhumation time in each of the growing sites (Is Terre Molentes, IS; Trainu Murcunieddu, TM; Peña Santa Lucia, SL; Peña Carazo, PC) and average temperature recorded during each exhumation date.

Locality (Region)	Date of sowing	Date of exhumations (days after sowing)	Average temperature day at exhumation time (°C)	Germination (%± SD)	Viability (%± SD)	Dead seeds (%± SD)	Empty seeds (%± SD)
IS (Sardinia)	24/08/2013	14/12/2013 (112)	0.56	0	92.38 ± 1.65	0.95 ± 1.65	6.67 ± 1.65
		22/03/2014 (210)	1.41	0.95 ± 1.65	97.14 ± 2.86	1.90 ± 1.65	0
		19/04/2014 (238)	7.77	5.71 ± 2.86	79.05 ± 8.25	2.86 ± 0.00	12.38 ± 5.95
		23/05/2014 (272)	11.98	7.62 ± 5.95	67.62 ± 1.65	7.62 ± 5.95	17.14 ± 2.86
		26/06/2014 (306)	16.89	0	9.52 ± 1.65	11.43 ± 4.95	79.05 ± 3.30
TM (Sardinia)	21/08/2013	14/12/2013 (115)	1.93	0	95.23 ± 1.65	2.86 ± 2.86	1.90 ± 3.30
		22/03/2014 (213)	4.50	71.43 ± 9.90	12.38 ± 8.73	4.76 ± 4.36	11.43 ± 2.86
		19/04/2014 (241)	10.02	73.33 ± 14.00	12.38 ± 4.36	2.86 ± 0.00	11.43 ± 10.30
		23/05/2014 (275)	13.85	75.24 ± 6.60	11.43 ± 4.95	4.76 ± 1.65	8.57 ± 2.86
		26/06/2014 (309)	16.45	0	13.33 ± 4.36	0.95 ± 1.65	85.71 ± 2.86
SL (Castilla y León)	19/09/2013	23/12/2013 (95)	0.12	0	96.19 ± 1.65	1.90 ± 1.65	1.90 ± 1.65
		28/03/2014 (190)	1.29	0	86.67 ± 1.65	8.57 ± 2.86	4.76 ± 1.65
		04/05/2014 (227)	10.15	60.00 ± 17.38	22.86 ± 7.56	3.81 ± 4.36	13.33 ± 7.19
		28/05/2014 (251)	11.29	60.95 ± 10.82	15.24 ± 19.02	12.38 ± 4.36	11.43 ± 5.71
		28/06/2014 (282)	14.54	1.90 ± 3.30	3.81 ± 4.46	3.81 ± 3.30	90.48 ± 4.36
PC (Castilla y León)	21/09/2013	24/12/2013 (94)	1.72	0	96.19 ± 1.65	0.95 ± 1.65	2.86 ± 2.86
		29/03/2014 (189)	1.74	0	91.43 ± 2.86	2.86 ± 2.86	5.71 ± 1.65
		08/05/2014 (229)	6.56	15.24 ± 3.30	58.10 ± 4.36	6.67 ± 4.36	20.00 ± 5.71
		29/05/2014 (250)	6.32	24.76 ± 8.73	60.00 ± 4.95	1.90 ± 3.30	13.33 ± 1.65
		29/06/2014 (281)	11.01	8.57 ± 4.95	40.00 ± 2.86	2.86 ± 2.86	48.57 ± 4.97

2.2.5 Data analyses

For each germination trial under controlled conditions, the final germination percentage (FGP) and the germination rate (T_{50}) were calculated. The FGPs were calculated as the mean of the four replicates ± standard deviation (SD) on the basis of the total number of filled seeds. Germination rate (T_{50}) was determined as the time in days required to reach 50% of germination (Bewley *et al*. 2013); when the 50% of germination was not reached, the value was not calculated (empty seeds were excluded).

2.2.6 Statistical analyses

Generalized Linear Models (GLMs) were used to evaluate the effect of treatments and incubation temperature on the FGP and the T_{50}. Significant differences highlighted by GLM were then analysed by a *post hoc* pairwise comparison *t*-test (with Bonferroni adjustment). A log link function and Poisson error structure was used for analysing the T_{50}, while a logit link function and quasibinomial error structure was used for analysing the FGP. GLM with a logit link function and quasibinomial error structure and F tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used in order to overcome residual over dispersion (Crawley 2007). All statistical analyses were carried out using R v. 3.1.3 (R Development Core Team 2015).

2.3 Results

2.3.1 Seed mass

Seed mass varied significantly ($P < 0.001$) among accessions belonging to Sardinian or Spanish localities, with Sardinian seeds having a higher mass (Table 1). The differences among Sardinian localities were not significant ($P > 0.05$). The same was observed in the Spanish localities ($P > 0.05$). Seeds from Sardinia were heavier than seeds from Spain ($P < 0.001$, Table 1)

2.3.2 Seed germination under controlled conditions

GLMs highlighted a statistically significant ($P < 0.001$) effect on final seed germination percentage (FGP; dependent variable) for all three tested factors (Treatment; Temperature; Locality), as well as for their two-way and three-way interactions (Table 3).

Table 3. GLM results of the final germination percentage (FGP) as dependent variable of the following factors 'Treat' (Treatment: Control, cold stratification and GA_3, 250·mg L^{-1}), 'Temp' (Temperatures of incubation: 5, 10, 15, 20, 25 and 25/10 °C) and 'Loc' (Experimental site: IS, TM, SL and PC) and their interactions.

Variables	d.f.	Deviance	Residual d.f.	Residual deviance	*P* (> Chi)
Null			287	21587.7	***
Treat	2	17295.3	285	4292.4	***
Temp	5	2613.1	280	1679.3	***
Loc	3	84.1	277	1595.1	***
Treat × Temp	10	275.8	267	1319.3	***
Treat × Loc	6	78.0	261	1241.3	***
Temp × Loc	15	477.8	246	763.5	***
Treat × Temp × Loc	30	123.8	216	639.7	***

'***' Significate codes: $P < 0.001$

The FGP (see Fig. 1) of the control test was < 3% for all temperatures and accessions. Cold stratification did not significantly increase the FGP relative to control seeds, with no significant differences among them ($P > 0.05$). Conversely, GA_3 treatment had a significant effect ($P < 0.001$) on the FGP (Fig 1), with more than 50% of germinated seeds at a temperature range from 5 to 20°C.

More specifically, the highest germination under this treatment was generally observed at 10°C and 15°C. SL was also the only locality to show the highest germination percentage (ca. 91%) at 15°C. FGPs at 5°C ranged from ca. 66% (PC) to 93% (TM); at 15°C it ranged from ca. 74% (PC) to 97% (TM), and at 20°C from ca. 72% (IS) to 85% (PC). A considerable reduction in the germination ability was found in all accessions at 25°C and at 25/10°C; almost all of these values were statistically different compared to those obtained at 5–20°C ($P < 0.05$ by *post hoc* pairwise *t*-test; Tables 3).

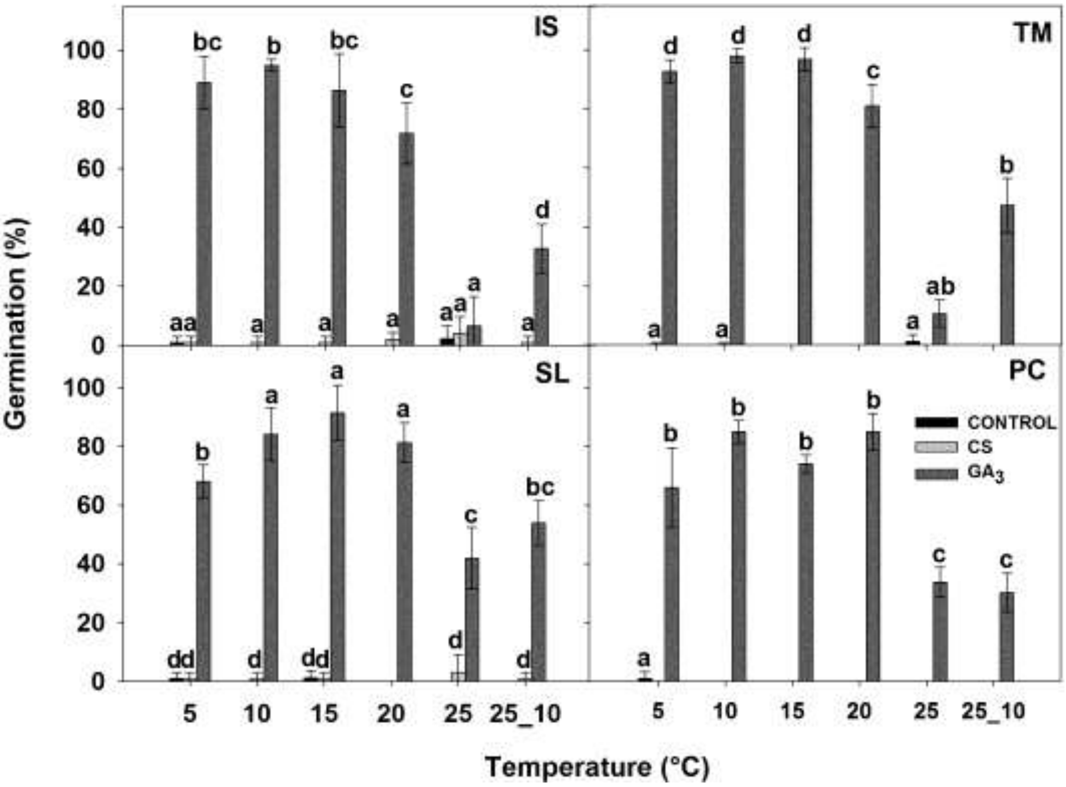

Figure 1. FGP achieved at the end of germination tests after each pre-treatment (Control; CS, 5°C for three months; GA_3, 250 mg l^{-1} of GA_3 in the germination substrate). Data are the mean of four replicates (± SD). Temperatures, treatments and their interaction are statistically significant ($P < 0.001$) by GLM. *Post hoc* pairwise *t*-test comparisons in each locality were carried out for each germination temperature, and bars with different letters indicate significant differences ($P < 0.05$).

T_{50} values within the range of temperatures from 5 to 20°C decreased with increasing incubation temperatures (Fig. 2), showing the same pattern in both the Spanish and the Sardinian accessions.

More specifically, from 5 to 20°C, they ranged from 120 to 52 days for IS, from 68 to 25 days for TM, from 66 to 18 days for SL and from 69 to 30 days for PC (Fig. 2). T_{50} values at 25 and 25/10°C could not be calculated since the 50% of germination was not reached; the only exceptions to this pattern were found in SL (which showed T_{50} values of 20 days at 25°C and 34 days at 25/10°C, respectively) and TM (68 days at 25/10°C; Fig. 2).

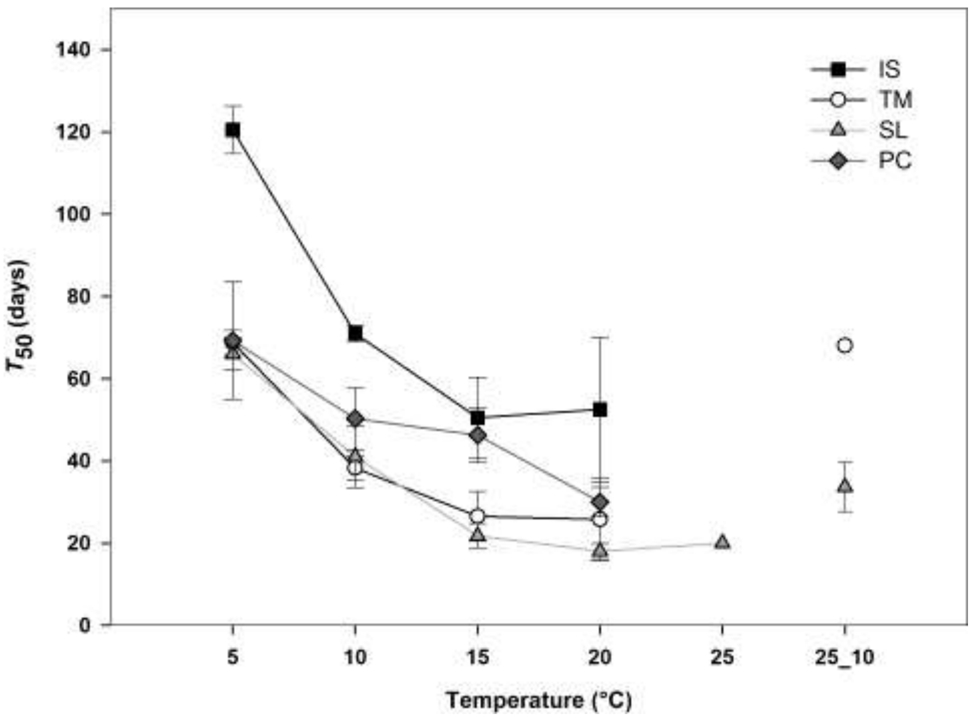

Figure 2. T_{50} (days ± SD) of *G. lutea* seeds treated with GA_3 (250 mg L^{-1} in the germination substrate) collected in each locality.

2.3.3 Seed germination under natural conditions

Seeds of *G. lutea* buried in each experimental site (Table 1) were exposed to a warm autumnal period (lasting ca. 44 days in Sardinian and ca. 40 days in Spanish populations), with a mean recorded soil temperature of ca. 7–9°C (Fig.3). In the Sardinian sites, the maximum soil temperature recorded in this period was ca. 15°C (4th October 2013; Fig. 3), while the minimum was ca. 0°C (15th December 2013; Fig. 3). As regards the Spanish sites, the mean soil temperature was ca. 7–8°C with a maximum of ca. 13°C in SL (23rd September 2013; Fig. 3) and 11°C in PC (16th October 2013; Fig. 3) and a minimum near 0°C in both SL (since 14th December 2013; Fig. 3) and PC (18th December 2013; Fig. 3). After the warm autumnal period, seeds experienced a cold period (*i.e.*, mean daily soil temperatures ≤ 5°C) with a duration varying from 128 (PC) to 152 days (SL) (Fig.3). At the time of the first exhumation (December 2013) seeds had remained dormant in all experimental sites (Table 2). At the time of the second exhumation (March 2014) the great majority of seeds were still dormant but viable in three out of four experimental sites. Germination occurred from April to May 2014 in IS, SL and PC (third exhumation), when the mean daily soil temperatures were ca. 9°C in IS and SL and ca. 7°C in PC (Fig. 3; Table 2). At the time of the fourth exhumation (May 2014), the germination was ca. 88% for IS, 75% for TM, 61% for SL and

25% for PC (Fig. 3; Table 2). Finally, at the time of the last exhumation (June 2014) the percentages of empty seeds were high in all sites (approx. 48–90%), probably due to the death of the seedlings inside the polyester envelopes (Table 2).

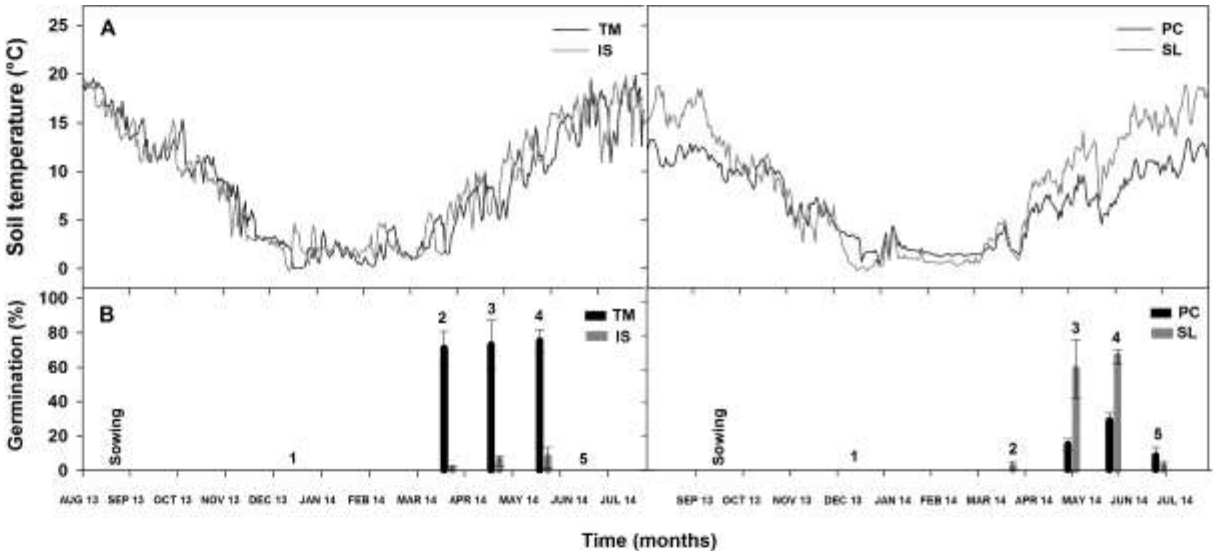

Figure 3. Soil temperatures and germination in the field for the Sardinian (TM; IS) and the Spanish (PC; SL) sites. (A) Annual trends of mean daily temperatures recorded in the soil and (B) field germination (three replicates of 35 seeds each) at the time of each exhumation. 1, 2, 3, 4 and 5 correspond to the time of seeds exhumation.

The maximum germination percentages obtained in the field were found in TM and SL, as also recorded under controlled conditions. GLMs highlighted a statistically significant (*P* < 0.001) effect on field germination (dependent variable) for both the Date and the Locality (Loc) factors, as well as for their two-way interaction (Loc × Date; *P* < 0.001; Table 4).

Table 4. GLM results for the effect on seed germination in the field (dependent variable) of the ‘Date’ (Date of the five exhumations) and ‘Loc’ (Localities: IS, TM, SL and PC) factors.

	Df	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>*F*)**
Null			59	3267.5		
Date	4	1053.51	55	2214.0	91.933	***
Loc	3	1560.50	52	653.5	181.565	***
Date × Loc	12	536.67	40	116.8	15.611	***

‘***’ Significate codes: *P* < 0.001

2.4 Discussion

2.4.1 Relationship between seed mass and germination

Previous studies (*e.g.* Fenner and Thompson 2005, Giménez-Benavides *et al*. 2005) have indicated that seed germination behaviour usually has some relationships with habitat, seed mass and life cycle. In our accessions seed mass was not correlated with the FGP, but is probably related with the origins of seeds. In addition, differences on seed mass related with altitudinal variation were excluded in this study. Our results are in contrast to those obtained by Kery *et al.* (2000), who argued that seed mass in *G. lutea* had a significant effect on germination and reported that smaller seeds incremented germination, and to the study of Pérez-García *et al.* (2012), who reported a slightly positive correlation between seed mass and final germination percentage. In our experiment, the accessions with the heaviest seeds (the Sardinian sites) reached highest FGP under controlled conditions but, as recorded in IS, the same behaviour was not observed in the natural sites. Intraspecific seed variation in germination may depend on genetic differences, local weather during growth of mother plant, soil quality, or other naturally occurring factors (Karlsson and Milberg 2008). Seeds from the nearby (< 10 Km) collecting sites (both in Sardinian and Spanish localities) did not show the same pattern in the natural sites, as high germination were found in TM (Sardinia) with heavy seeds and SL (Spain) with light seeds. These results therefore suggest that the differences in seed mass and FGP were probably influenced by the specific ecological conditions characterizing each site.

2.4.2 Seed germination under controlled conditions

Untreated seeds of *G. lutea* did not germinate due to seed dormancy. Also, cold stratification at 5°C did not affect seed germination in all the accession. This is in accordance with Pérez-García *et al.* (2012), who found moist chilling in seeds of this species did not enhance germination, and with González-López and Casquero (2014), who observed a similar pattern in *G. lutea* var. *aurantiaca*. Seed germination of all accessions were promoted by GA_3; in fact, high germination percentages were generally obtained at each tested temperature for all localities. However, a decrease in the germination ability was detected at high (*i.e.* 25°C) and at alternate (25/10°C) temperatures. Also, seeds did not experience a high temperature period in the growing sites, thus suggesting that high temperatures represent a limit for the seed germination of this species also in the presence of GA_3.

The soil temperatures recorded by data loggers our experiments illustrate that seeds of *G. lutea* from different sites were exposed to intermediate temperatures (~ 7°C) post seed dispersal and to a cold period (~ 0–2°C) before germination; we therefore suppose that 5°C, the temperature which is most often used in this kind of research for pre-chilling treatments (Baskin and Baskin 2014), was probably not enough to break seed dormancy in this species, and that a temperature of about 0°C might be more effective.

2.4.3 Seed germination under natural conditions

We found physiological (seed germination) variability among *G. lutea* accessions. In addition, our study confirmed that *G. lutea* created a short-term persistent seed bank, and seeds mainly germinated in their first year, as also reported by Hesse *et al.* (2007). Germination in the natural sites occurred during spring and after a natural cold stratification period, when the average soil temperatures were ca. 5–12°C. This is in accordance with the results obtained in the laboratory, where the optimal FGPs were generally recorded at a temperature comprised between 5 and 15°C.

We found a one-month delay in the starting of germination in Spanish localities with respect to the Sardinian ones. It is well known that temperature is the main environmental factor regulating seed germination (Fenner and Thompson 2005, Baskin and Baskin 2014), therefore the delay observed in Spanish sites is very likely the result of the lower soil temperature experienced by seeds, which could have influenced the time of dormancy release and the beginning of seed germination.

2.4.4 Intraspecific seed germination variation

The localities under study differ among them on aspect, while the altitude is similar in each of them; however, the results differed in their seed germination percentages. Specifically, higher germination percentages in the field were found in TM and SL, both of them characterized by a North-Western orientation, while the other two localities (IS and PC) are characterized by a North-Eastern orientation. Populations from similar habitats have different germination responses, which can arise from environmental variation during seed maturation and the effect of maternal genotype (Wulff 1995) and could reflect local adaptation to particular environments (Andersson and Milberg 1998, Pérez-García *et al.* 2006). Indeed, intra-specific variation in the germination characteristics could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions (Baskin and Baskin 2014). It is well known that some mountain plants show high variation in germination behaviour, which is not always attributable to habitat characteristics (Giménez-Benavides *et al.* 2005). However, in this work, all sites under study have similar environmental characteristics and the localities appear to be stable (personal observation). Scherrer and Körner (2011) showed that mountain topography can cause temperature differences over very short horizontal distances, and this could be also the case for the sites under study. The only dissimilarity observed in our experimental sites that might explain the differences in the germination behaviour, and therefore the field germination response of the different sites, regards aspect. Interestingly, the potential of small-scale topographic complexity to drive microclimatic variation (Opedal *et al*. 2015) could cause the different intra-specific germination responses. In this framework, there is still relatively little information about the extent of intraspecific trait variation at small scales across sites differing in topographic complexity (Albert *et al*. 2010, Boucher *et al*. 2013). More studies are needed in order to gain further insights on the role that this environmental factor play in the intraspecific variation of *G. lutea*, as well as to verify the effect of any other key factor that might not have been taken into account up to now (see Stöcklin *et al.* 2009).

2.5 Conclusions

These results are consistent with several previous studies investigating the relevance of the differences in the behaviour of seeds belonging to different accessions of the same *taxon*. The importance of such a variability might be particularly high for those plant *taxa* that have a relatively wide distribution range but that at the same time have a high conservation value, such as *G. lutea*.

Our experiments highlighted the existence of a considerable degree of physiological difference among seeds belonging to different growing sites (*e.g.* the difference in the final seed germination percentages recorded both under controlled and natural conditions). These differences must definitely be taken into account when planning *ex situ* conservation actions on this *taxon* and emphasize the importance of collecting and preserving seeds from multiple origins in order to maximize the genetic diversity of seed collections stored in germplasm repositories. Furthermore, information on seed germination potentially has great monetary value. A knowledge of what controls the timing of germination enhances: planning for the effective propagation of threatened and/or economically important plant.

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______CHAPTER 3_______

SEED GERMINATION AND EMBRYO DEVELOPMENT RESPONSE TO DIFFERENT STRATIFICATION PERIODS AND THERMAL REGIME IN *Gentiana lutea* L. subsp. *lutea*

3 Chapter III – Seed germination and embryo development response to different stratification periods and thermal regime in *Gentiana lutea* L. subsp. *lutea*

3.1 Introduction

Seed germination is a crucial process for seedling establishment and survival in nature (Fenner and Thompson 2005). There are a number of mechanisms that regulate germination; among these, one of the most important is seed dormancy, which is used by plants to promote survival, dispersing germination in space and time until environmental conditions are favourable for germination (Baskin and Baskin 2014). Variation in these mechanisms, both within and between species, has been interpreted as an adaptation to specific habitat conditions at local and regional scales (Meyer *et al.* 1995, 1997). In this context of adaptations to particular habitats, Peripheral and Isolated Plant Populations (PIPPs) can differ genetically and morphologically from central populations because of their smaller population size and greater physical and ecological distances from the centre of the distributional range. Furthermore, they may contain genotypes that are adapted to extreme environmental conditions; it is therefore important to check the effect of these factors on the seed ecophysiology of these populations (Mimura and Aitken 2007).

In this study we focused our efforts on the understanding of the seed germination requirements of *Gentiana lutea* L. subsp *lutea*, a *taxon* which is considered a PIPP in Sardinia (Fois *et al.* 2015). *Gentiana lutea s.l.* is a perennial herb with wide latitudinal, altitudinal and distributional ranges throughout the Central-Southern European mountains, where it can be found at an altitude of 800–2500 m. a.s.l. (Anchisi *et al.* 2010). This species is included in Annex D of the European Habitats Directive and is also reported as being threatened as a result of root harvesting practices and of global climatic warming due to its distribution, which is restricted mainly to the upper sectors of the mountains (Gentili *et al.* 2013).

In order to understand the reproductive mechanisms of *G. lutea*, the study of its germination ecophysiology is fundamental. Embryos in some Gentianaceae seeds are small at the time of dispersal and may increase by 57–182% before the radicle emerges from the seed (Baskin and Baskin 2005). Martin (1946) included seeds of Gentianaceae in the “dwarf” seed category. Baskin and Baskin (2007) revised Martin’s embryo type classification system and included this family within the “linear underdeveloped” embryo. The presence of underdeveloped embryos would mean that seeds may have either morphological (MD) or morphophysiological (MPD) dormancy, depending on whether physiological dormancy (PD) occurs in the embryo or not (Nikolaeva 1969, Baskin and Baskin 2004). Seeds of many *Gentiana* species need stratification for germination (Baskin and Baskin 2014). Nikolaeva *et al.* (1985) reported a non-deep complex MPD in seeds of *G. lutea*, while Pérez-García *et al.* (2012) showed that seeds of this species exhibit non-deep PD.

Seeds from populations encountering long periods with snow cover and adverse winter conditions would require longer periods of cold stratification for germination than those from populations exposed to milder winters (Jurado and Flores 2005). Recently, Cuena-Lombraña *et al.* **[Chapter 2]** reported that a chilling temperature of 5°C alone is not enough to break seed dormancy in *G. lutea* subsp. *lutea*. For many species, 5°C is optimal for dormancy breaking, but in some cases temperatures below 5°C are more effective (Baskin and Baskin 2014). For example, the temperature of 0°C was more effective in overcoming physiological dormancy in seeds of *Aegopodium podagraria* L. after the embryo had become fully developed (Phartyal *et al*. 2009); *Gentiana purpurea* L. germinated around 60% after treatment of 0°C for 7 months in darkness (Orsenigo *et al.* 2015) and *Erythronium dens-canis* L. radicles began to emerge 28 days after transfer from autumn to winter conditions (0⁄5°C) and growth was completed 56 days later (Mondoni *et al.* 2012).

Previous studies on *G. lutea* (Kery *et al.* 2000) suggest that small populations may face an increased short-term risk of extinction because of reduced reproduction and an increased long-term risk because they are less able to respond to environmental changes. Another example of these deleterious effects regards the congeneric species *G. pneumonanthe* L., whose small populations have been reported to be particularly susceptible to deteriorating environmental conditions and reduced offspring fitness (Oostermeijer *et al.* 1994).

The main aim of this study was to investigate the seed germination ecophysiology of *G. lutea* subsp *lutea* in order to: (i) evaluate if a chilling temperature of 0°C is effective in breaking seed dormancy and embryo growth; (ii) identify the class and level of seed dormancy; (iii) suggest an optimal germination protocol for this species; and (iv) evaluate the influence of different locality sizes on the germination performance.

3.2 Material and methods

3.2.1 Study area and species

Gentiana lutea subsp. *lutea* (hereafter *G. lutea*) is a perennial rhizomatous herb with a European distribution, although it is mainly present in the mountain ranges of Central-Southern Europe, *i.e.* in Sardinia, Corsica, Iberian, the Italian and Balcan Peninsulas and in the Alps, rarely in the Caucasus and in Anatolia (Pignatti 1982, Renobales 2012, Jeanmonod and Gamisans 2013). The distribution range in Sardinia consists of a vast population located in the Gennargentu Massif, where it is found in different nuclei or scattered individuals (Fois *et al.* 2015). The Gennargentu Massif (Fig. 1), situated in Central-Eastern Sardinia, is an independent biogeographical sector with a surface of ca. 721 km^2 and consists of a system of summits and windy ridges at 1400–1500 m a.s.l., with four peaks at more than 1800 m a.s.l. (Fenu *et al.* 2014).

During August-September 2014 mature fruits (capsules) of *G. lutea*, containing well-developed ripe seeds, were sampled in three localities with different size (Fig. 1): Is Terre Molentes (IS), situated at 1460–1505 m. a.s.l. and with an area of approximately 10.000 m^2 and producing more than 5.000 flowering stems, here defined as “large size”; Trainu Murcunieddu (TM), at 1324–1372 m. a.s.l., an area of approximately 2.000 m^2 (here defined as “medium size”) and producing between 2.500–5.000 flowering stems; and Bruncu Spina (BS), at 1743–1750 m. a.s.l., the highest locality reported for Sardinia with an area of 84 m^2 (here defined “small size”), producing 9–13 flowering stems. Seeds were collected from at least 50 randomly selected plants in each site (when available). The collected seeds showed a similar degree of ripeness, as observed from their colour and hardness. Seeds were manually cleaned, discarding any visually malformed seeds, and stored at room temperature (ca. 20°C and 40% of relative humidity) until the start of the germination tests in September 2014.

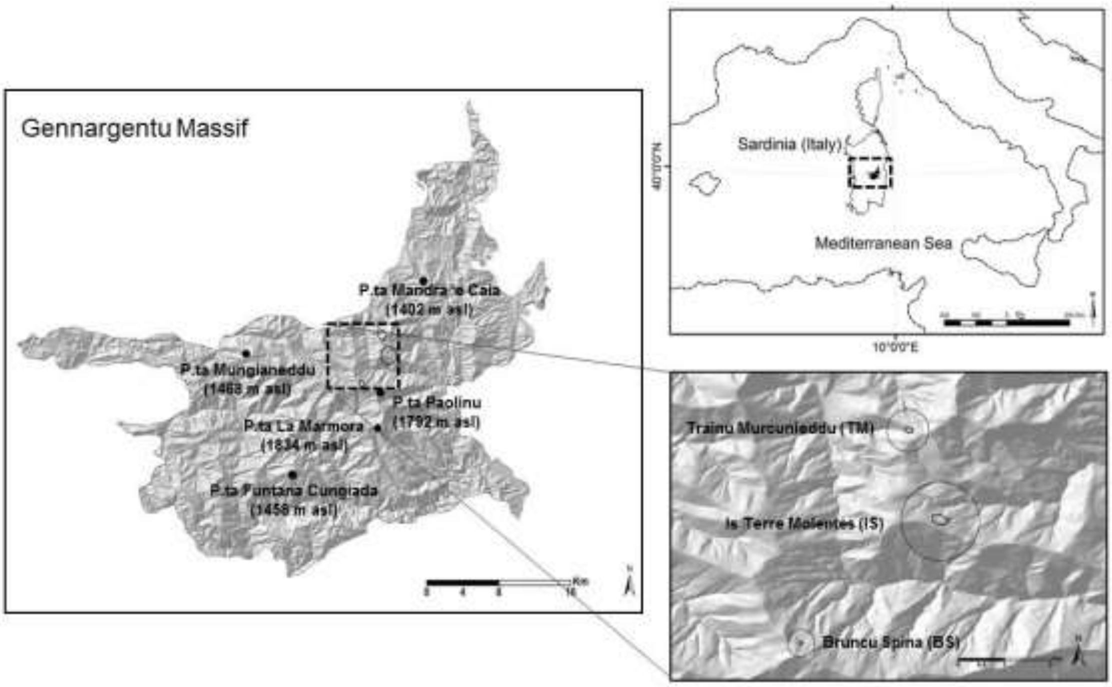

Figure 1. Geographical location of Sardinia in the Mediterranean context, toponyms of the main peaks included in the Gennargentu Massif and sampling sites of *G. lutea*: Is Terre Molentes (IS; large size); Trainu Murcunieddu (TM; medium size) and Bruncu Spina (BS; small size).

3.2.2 Seed germination test

The following pre-treatments were applied to seeds of *G. lutea*: *i)* control at 5, 10, 15, 20, 25°C and 25/10°C *ii)* cold stratification at 0°C for three months (C0); *iii)* cold stratification at 5°C for three months (C5); *iv)* warm stratification at 25/10°C for three months (W); *v)* warm stratification (25/10°C for three months) followed by five months of cold stratification at 5°C (W+C5) and *vi)* warm stratification followed by two different cold stratification periods, the first at 5°C for one month and the second at 0°C for three months (W+C5+C0). The pre-treatment C0 was performed in dark conditions (0 h light/24 h dark) in order to simulate the snow cover period.

For all germination conditions four replicates of 25 seeds were sown on the surface of 1% agar water in 90 mm diameter plastic Petri dishes and incubated in the light (12 h light/12 h dark) under a range of constant temperatures (5, 10, 15, 20 and 25°C) and under an alternating temperature regime (25/10°C). In the alternating temperature regime, the 12 h light period coincided with the higher temperature period. Light was provided by white fluorescent lamps (FL40SS.W/37 70–10 μmol m^{-2} s^{-1}). All germination tests were conducted at the same time and started within two weeks after collection. Germinated seeds were scored three times a week and germination was defined as visible radicle emergence (≥ 1 mm). At the end of the germination tests (for a minimum of 90 days), when no additional germination had occurred for two weeks, a cut test was carried out to determine the firmness of the remaining seeds and the number of empty seeds. Firm seeds were considered viable (ISTA 2006).

For each germination trial, the final germination percentage (FGP) and the germination rate (T_{50}) were calculated. Germination rate (T_{50}) was determined as the time (expressed in days) required to reach 50% of the germination percentage; this value was only calculated when the 50% of germination was reached. The FGPs were calculated as the mean of the four replicates (± SD) on the basis of the total number of filled seeds (empty seeds were excluded).

3.2.3 Embryo measurements

Embryo growth during pre-treatments was assessed at different times by measuring 10 seeds for each sample interval, with the exception of seeds from BS locality due to low number of available seeds. Embryo and seed lengths were determined by cutting the seeds longitudinally using laboratory tweezers and a scalpel, both during and after the pre-treatments (see Table 1 for details).

Ten seeds were sectioned in half under a dissecting microscope and images of embryos acquired using a Zeiss SteREO Discovery.V8, with an objective Achromat S 0.63x, FWD 107mm (Carl Zeiss MicroImaging GmbH) at 1.0 × magnification, coupled to a Canon (Power shot G11) digital camera. Embryo and seed lengths were measured using the image analysis software ImageJ 1.41 (National Institutes of Health, Bethesda, MA, USA). Seed length was measured ignoring the seed coat (Mattana *et al.* 2012). The embryo to seed length ratio (*i.e.* E:S ratio) was calculated. The initial E:S ratio was calculated by measuring 20 randomly selected seeds before the start of the experiments; in order to take these measurements the seeds were sowed for 24 hours at room temperature on the surface of 1% agar water in 90 mm diameter plastic Petri dishes. The critical E:S ratio (*i.e.* the E:S ratio at the moment immediately prior to germination, when the seeds had a split seed coat but no radicle protrusion) was determined as the average E:S ratio of 20 seeds. The critical E:S ratio was also considered for those seeds that had germinated before measurements were taken (Vandelook *et al.* 2007).

Table 1. Description of the pre-treatments applied and experimental design for embryo growth measurements.

Pre-treatment		**Embryo growth measurements**	
Code	**Description**	**Number of measu-rement**	**Measurement timing**
0	Control	4	After 15, 30, 60 and 90 days
C0	0°C for 3 months (0/24 hours of light)	7	After 30, 60 and 90 days during cold stratification and after 15, 30, 60 and 90 days after sowing for germination.
C5	5°C for 3 months (12/12 hours of light)	7	After 30, 60 and 90 days during cold stratification and after 15, 30, 60 and 90 days after sowing for germination.
W	25/10°C for 3 months (12/12 hours of light)	7	After 30, 60 and 90 days during warm stratification and after 15, 30, 60 and 90 days after sowing for germination.
W+C5	25/10°C for 3 months + 5°C for 5 months (12/12 hours of light)	10	After 30, 60 and 90 days during warm stratification, after 30, 60 and 90 days during cold stratification and after 15, 30, 60 and 90 days after sowing for germination.
W+C5+C0	25/10°C for 3 months + 5°C for 1 month (12/12 hours of light) + 0°C for 3 months (0/24 hours of light)	11	After 30, 60 and 90 days during warm stratification, after 30 days during cold stratification at 5°C, after 30, 60 and 90 days during cold stratification at 0°C and after 15, 30, 60 and 90 days after sowing for germination.

3.2.4 Statistical analyses

Generalized Linear Models (GLMs) were used to evaluate the effect of pre-treatments and incubation temperature on the E:S ratio, the FGP and the T_{50}. Significant differences highlighted by GLM were then analysed by a *post hoc* pairwise comparison *t*-test (with Bonferroni adjustment). A log link function and Poisson error structure was used for analysing the T_{50}, while a logit link function and quasibinomial error structure was used for analysing the FGP. A log link function and quasipoisson error structure was used for analysing the E:S ratio. Quasibinomial and quasipoisson error structures and *F* tests with an empirical scale parameter instead of *chi*-squared on the subsequent ANOVA were used in order to overcome residual overdispersion (Crawley 2007). All statistical analyses were carried out using R v. 3.1.3 (R Development Core Team 2015).

3.3 Results

3.3.1 Effect of pre-treatments on embryo growth

GLMs indicated that both the pre-treatment and the locality factors had a significant effect on embryo growth ($P < 0.001$; Table 2), while the interactions between pre-treatments and localities were not statistically significant ($P > 0.05$; Table 2). The mean length of embryos from freshly mature seeds were 0.12 ± 0.01 cm in IS and 0.09 ± 0.01 cm in TM locality, while the seed lengths were 0.27 ± 0.02 and 0.24 ± 0.03 cm, in IS and TM, respectively. Hence, the initial E:S ratio in mature seeds was 0.44 ± 0.04 in IS and 0.39 ± 0.05 in TM (Fig. 2).

Table 2. GLMs results of the effects of the pre-treatments (initial E:S ratio and values at the end of C0, C5, W, W+C5, W+C5+C0) and localities (IS, TM) factors on the E:S ratio. C0 = Cold stratification at 0°C for three months; C5 = Cold stratification at 5°C for three months; W = Warming stratification, 25/10°C for three months; W+C5 = 25/10°C for three months + 5°C for five months; W+C5+C0 = 25/10°C for three months + 5°C for one month + 0°C for three months. TM = Trainu Murcunieddu and IS = Is Terre Molentes.

Embryo length	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
Null			119	2.74746		
Pre-treatment	5	1.98431	114	0.76315	72.4683	< 2.2e-16 ***
Locality	1	0.13264	113	0.63051	24.2213	3.099e-06 ***
Pre-treatment × Locality	5	0.0445	108	0.58490	1.6656	0.1491

The final mean embryo length of cold stratified seeds at 0°C was 0.17 ± 0.02 cm (E:S 0.64 ± 0.07) in IS and 0.16 ± 0.02 cm (E:S 0.60 ± 0.06) in TM, while at 5°C it was 0.14 ± 0.02 cm (E:S 0.51 ± 0.07) and 0.12 ± 0.02 cm (E:S 0.46 ± 0.05) in IS and TM, respectively; no embryos achieved the embryo critical length for germination (Fig. 2) during these pre-treatments. Embryos in W stratified seeds reached mean lengths of ca. 0.14 cm (E:S of ca. 0.52) in both localities. Embryos that suffered two cycles of pre-treatment (*i.e.* W+C5 and W+C5+C0) did not reach a critical E:S ratio for seed germination, but W+C5+C0 reached the highest value for the E:S ratio (0.72 ±0.05 in IS and E:S 0.71 ± 0.07 in TM) probably due to the long duration of the pre-treatment.

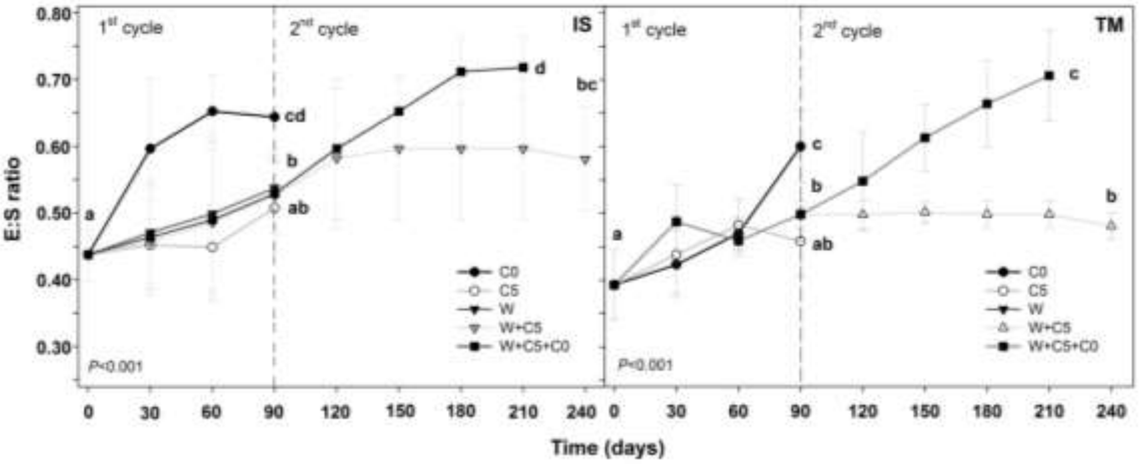

Figure 2. The effect of pre-treatment on embryo growth. Embryo:Seed (E:S) ratio at the beginning of the experiment and during pre-treatments. E:S ratio values are the mean of 10 seeds (±SD). GLMs were carried out, values with the same letter are not statistically different at $P > 0.05$ by *post hoc* pairwise *t*-test comparisons. C0 = Cold stratification at 0°C for three months; C5 = Cold stratification at 5°C for three months; W = Warming stratification, 25/10°C for three months; W+C5 = 25/10°C for three months + 5°C for five months; W+C5+C0 = 25/10°C for three months + 5°C for one month + 0°C for three months. TM = Trainu Murcunieddu and IS = Is Terre Molentes.

In the first cycle no statistical differences ($P > 0.05$) were found between the initial E:S ratio and the final E:S in C5, while statistical differences were found in the other pre-treatments ($P < 0.05$) in both localities (Fig. 2). Considering the second cycle, the pre-treatment W+C5+C0 was statistically different with respect to W+C5 ($P < 0.05$); the latter was statistically similar ($P > 0.05$) to C5 and W in both sites. The statistical results indicated that the effect of C0 and W+C5+C0 on embryo growth was similar ($P > 0.05$) in both localities (Fig. 2).

3.3.2 Effect of incubation temperature on embryo growth during germination tests

The mean critical E:S ratio were 0.79 ± 0.08 for IS and 0.81 ± 0.06 for TM (Fig. 3). The embryo growth during the germination tests in the control treatment did not reach the mean critical E:S ratio in none of the localities and under none of the temperature conditions. The same occurred in the pre-treatments C5, W and W+C5. In the C0 pre-treatment the critical E:S ratio was achieved after 15 days at 5, 10, 15 and 20°C in both localities. At 25°C and alternate temperatures (25/10°C) more days were required to reach it, *i.e.* 90 and 60 days for IS and TM, respectively. In W+C5+C0 the critical E:S ratio was achieved during the first two weeks of incubation in both localities and at all temperatures (Fig. 3). In general, the mean critical E:S ratio was achieved at all incubation temperatures only after the seeds had undergone a period at a temperature of 0°C (C0 pre-treatment).

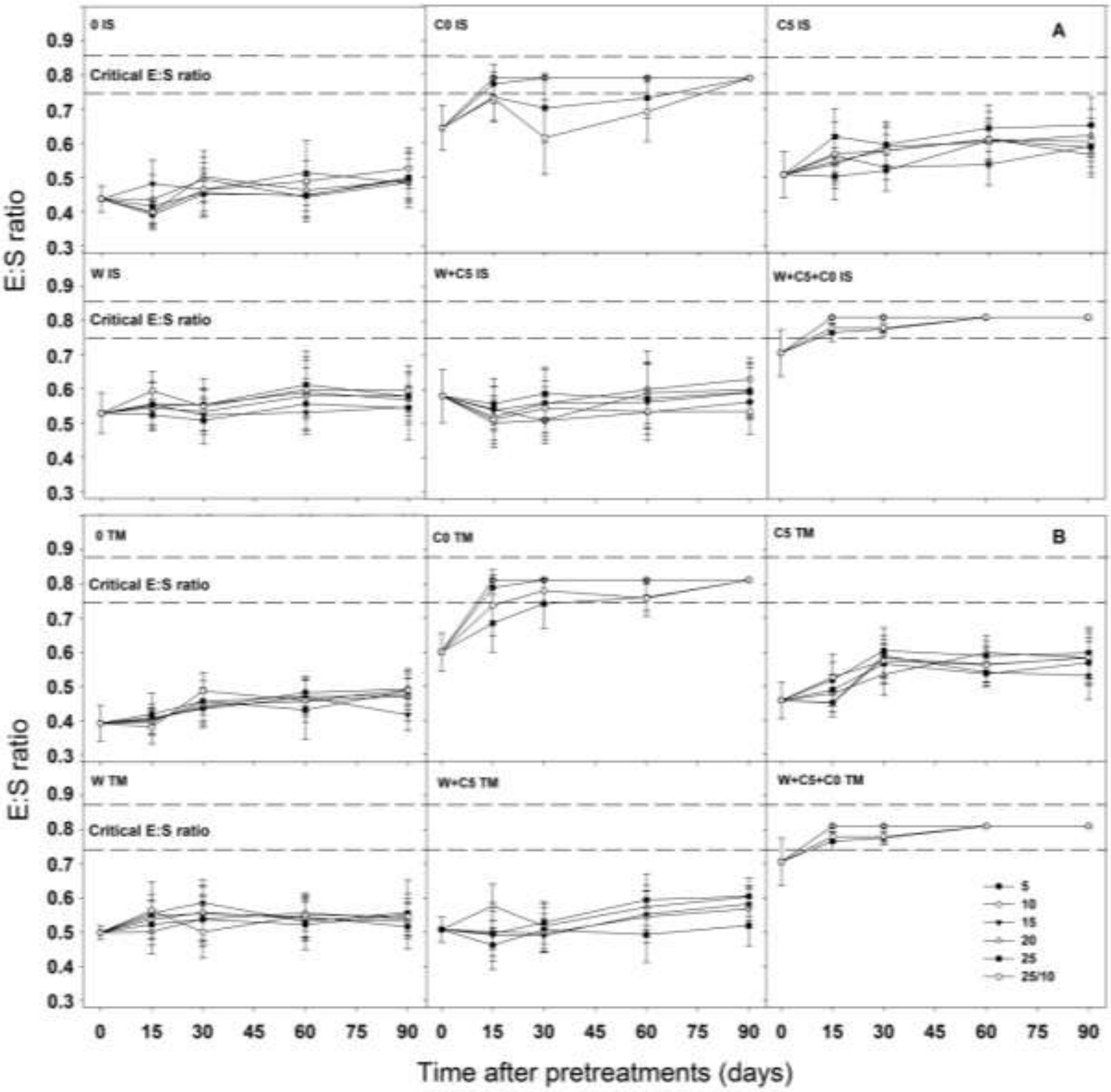

Figure 3. Effect of germination temperature conditions on the E:S ratio in all pre-treatments. 5, 10, 15, 20, 25 and 25/10°C were the incubation temperatures during the germination tests. Upper graphics regard (A) IS, Is Terre Molentes locality and lower graphics (B) TM, Trainu Murcunieddu locality. The range of critical E:S ratio are indicated by dashed lines. C0 = Cold stratification at 0°C for three months; C5 = Cold stratification at 5°C for three months; W = Warming stratification, 25/10°C for three months; W+C5 = 25/10°C for three months + 5°C for five months; W+C5+C0 = 25/10°C for three months + 5°C for one month + 0°C for three months.

3.3.3 Effect of pre-treatments on seed germination

Seeds treated for 90 days at 5°C did not germinate; W and W+C5 were also ineffective on seed germination. In general, all the seeds included in the experiment with the pre-treatments C0 and W+C5+C0 achieved high germination percentages (Fig. 4). The differences in effectiveness of this two last pre-treatments were not significant ($P > 0.05$; Table 3) for FGP and significant for the T_{50} ($P = 0.001$; Table 4). The differences among localities under the same pre-pretreatment were statistically significant for the FGP ($P < 0.001$; Table 3) and for the T_{50} ($P < 0.05$; Table 4), as well as for the incubation temperatures ($P < 0.001$; Table 3 and 4).

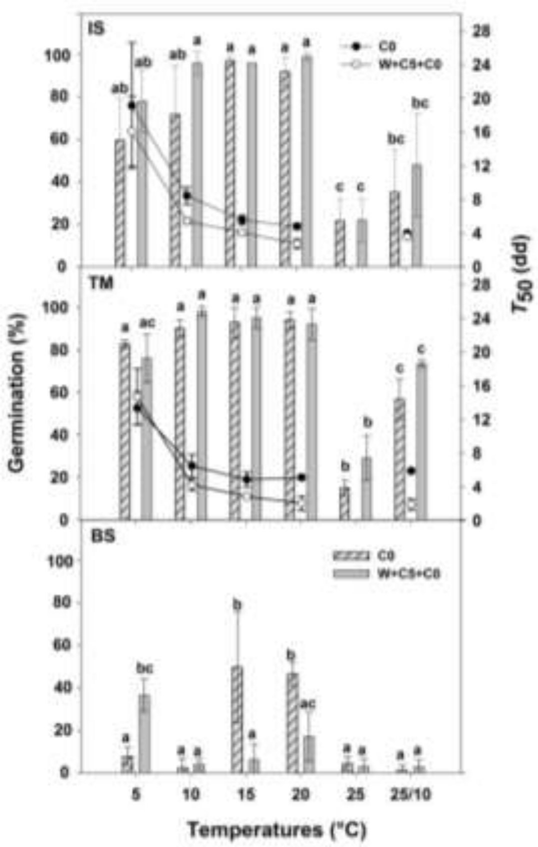

Figure 4. FGP (bars) and T_{50} values (points and lines) achieved at the end of the germination tests, after each pre-treatment (only the pre-treatment where germination occurred are included in this figure); C0 = Cold stratification at 0°C for three months, W+C5+C0 = 25/10°C for three months + 5°C for one month + 0°C for three months. Temperatures and localities are statistically significant ($P < 0.001$ by GLM). *Post hoc* pairwise *t*-test comparisons were carried out for each germination temperature and bars with different letters indicate significant ($P < 0.05$) differences. TM = Trainu Murcunieddu; IS = Is Terre Molentes; BS = Bruncu Spina. Data are the mean of four replicates (±SD).

Table 3. GLMs results of seed germination (FGP) of the following factors: Pre-treatment (C0, W+C5+C0), Temperature (5, 10, 15, 20, 25, 25/10 °C) and Locality (TM, IS, BS). C0 = Cold stratification at 0°C for three months and W+C5+C0 = 25/10°C for three months + 5°C for one month + 0°C for three months. TM = Trainu Murcunieddu; IS = Is Terre Molentes; BS = Bruncu Spina.

	Variables	**d.f.**	**Deviance**	**Residual d.f.**	**Residual deviance**	***F***	***P*(>F)**
FGP	Null			143	9990.5		
	Pre-treatment	1	12.6	142	9977.9	0.9261	0.3376
	Locality	2	4385.8	140	5592.1	160.7075	$< 2.2^{e-16}$ ***
	Temperature	5	3728.7	135	1863.4	54.6525	$< 2.2^{e-16}$ ***

Table 4. GLMs results of T_{50} of the following factors: Pre-treatment (C0, W+C5+C0), Temperature (5, 10, 15, 20, 25/10 °C) and Locality (TM, IS). C0 = Cold stratification at 0°C for three months and W+C5+C0 = 25/10°C for three months + 5°C for one month + 0°C for three months. TM = Trainu Murcunieddu and IS = Is Terre Molentes.

	Variables	**d.f.**	**Deviance**	**Residual d.f.**	**Residual deviance**	***P* (>Chi)**
T_{50}	Null			71	233.094	
	Pre-treatment	1	10.263	69	217.808	0.001 **
	Locality	1	5.022	70	228.071	0.025 *
	Temperature	4	187.350	65	30.458	$< 2.2^{e-16}$ ***

In IS after C0, more than 60% of FGPs were reached at temperatures of 5, 10, 15 and 20°C (Fig. 4). At 25°C the FGP was ca. 20% and at 25/10°C it was > 40%. The T_{50} values decreased (19.15, 8.43, 5.56, 4.80 days) from 5 to 20°C and at 25/10°C it was reached in ca. 4 days. In TM, high FGPs (> 80%, Fig. 4) were achieved at 5, 10, 15 and 20°C after C0. At 25°C the FGPs were less than 20% and increased up to about 60% in the alternate temperatures. As for the time to achieve 50% of the final germination, the values decreased (13.35, 6.45, 4.87 and 5.12 days, Fig. 4) with the increase of the incubation temperature from 5 to 20°C, while in the alternate temperatures regime (25/10°C) the T_{50} was reached after 5.90 days. In BS locality, the FGPs were < 50% at all temperatures (Fig. 4). The highest values of FGP were ca. 49% at 15°C and ca. 46% at 20°C.

The effects of W+C5+C0 highlighted a reduction in the time needed for germination in all localities and, in the majority of temperatures, it increased the FGP in IS and TM, but not in BS. As regards IS (Fig. 4), the FGP was ca. 80% at 5°C. At 10, 15 and 20°C the FGP reached values near 100%, whereas at 25°C it decreased to 22% and at 25/10°C to 48%. At 5°C the T_{50} was 16.07 days, while at 10, 15 and 20°C it was 5.45, 4.06 and 2.74 days, respectively, and at 25/10°C it was 3.58 days (Fig. 4). In TM more than 70% of the seeds germinated at a temperature range comprised between 5 and 20°C and at alternate temperatures (25/10°C, Fig. 4); at 25°C the FGPs were less than 30%. As for the T_{50}, at 5°C it was 14.67 days, while at 10, 15, 20 and 25/10°C it decreased to 4.24, 2.86, 2.04 and 1.90 days, respectively (Fig. 4). As regards BS, the FGP at 5°C was ca. 36%, while in the other incubation temperatures it was always < 20%.

3.3.4 Variability of seed germination response based on different locality sizes

We found significant differences ($P < 0.001$) by GLM on seed germination among localities, and the *post hoc* pairwise *t*-test highlighted non-significant differences ($P > 0.05$) between large size (IS) and medium size (TM) localities, while both these localities were statistically different ($P < 0.05$) with respect to small size locality (BS; Fig. 5).

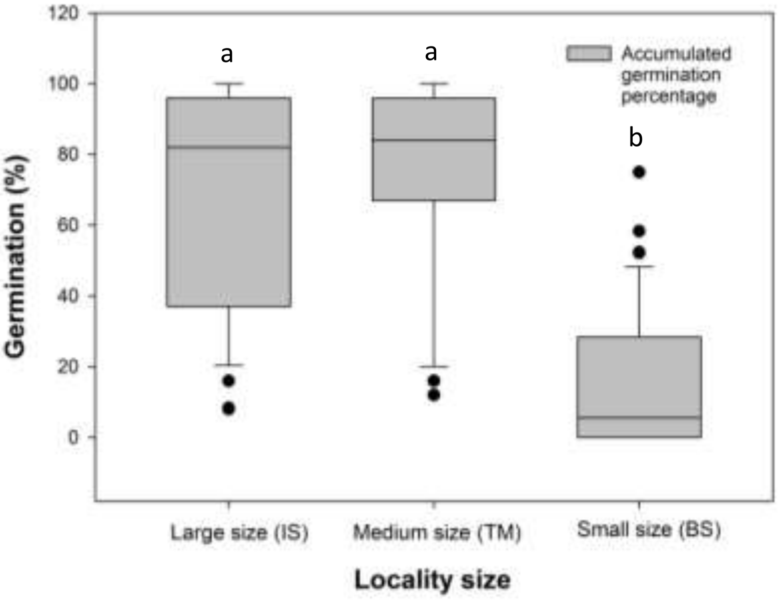

Figure 5. Accumulated final germination percentage under all temperature conditions and under the pre-treatments C0 (cold stratification at 0°C for three months) and W+C5+C0 (25/10°C for three months + 5°C for one month + 0°C for three months), separated by locality size. Different letters above boxplots indicate significant differences at $P < 0.05$ by *post hoc* pairwise *t*-test comparisons. Large size = Is Terre Molentes (IS), medium size = Trainu Murcunieddu (TM), small size = Bruncu Spina locality (BS).

3.4 Discussion

3.4.1 Class of dormancy and dormancy-breaking temperature

Gentiana lutea has linear underdeveloped embryos. In general, if embryo growth and radicle emergence are completed in about 30 days under suitable conditions, seeds have only morphological dormancy (MD). On the other hand, if germination is delayed for more than 30 days and seeds require a dormancy-breaking treatment such as exposure to moist cold (0–10°C) and/or moist warm (≥ 15°C) stratification to germinate, they have morphophysiological dormancy, MPD (Nikolaeva 1977, Baskin and Baskin 2004). In the simple kinds of MPD, embryos grow at relatively high temperature (≥ 10°C), while in complex kinds of MPD, embryos grow during cold stratification (Baskin and Baskin 2004, Baskin *et al.* 2008). In *G. lutea* fleshy mature seeds, both the root and the shoot emerged after cold stratification at 0°C, this temperature was effective in interrupting seed dormancy and promoting embryo growth and germination. To help determine the class of dormancy it is necessary to obtain information also on the effectiveness responses to plant hormones on seed germination, in particular to gibberellic acid (Baskin and Baskin 2014). Previous studies on *G. lutea* seeds **[Chapter 2]** demonstrated the positive effect of gibberellic acid (GA_3) on seed germination (FGP > 60%). Following the classification system *sensu* Baskin and Baskin (2014), we argue that *G. lutea* seeds have an intermediate complex MPD.

Our results are in accordance with the concept of natural selection favouring germination patterns that reduce the probability of facing adverse environmental conditions for seedling establishment (Baskin and Baskin 2014). Seeds must be adapted to germinate soon after winter, thus avoiding unfavourable conditions, and when temperature and soil moisture may be appropriate for germination (Meyer and Monsen 1991, Jurado and Flores 2005). The same pattern has been reported for some temperate species growing in high mountains of Sardinia, such as *Rhamnus persicifolia* Moris, which required cold stratification to break dormancy and relatively low temperatures for seed germination (Porceddu *et al.* 2013). This highlights an increasing threat from global warming, which could reduce the level of natural emergence in the field (Mattana *et al.* 2012).

In the case of seeds with PD and MPD, low temperatures in winter and the moist but still cold environment during snowmelt provide after-ripening and cold stratification conditions to break dormancy (Schwienbacher *et al.* 2011). Cuena-Lombraña *et al.* **[Chapter 2]**, through the study of soil temperatures recorded by data loggers from 2013 to 2014 buried in the natural sites, detected a warm autumn post-dispersal period (September, October and November) with mean daily temperatures of ca. 10°C before the beginning of winter, followed by one month of ca. 5°C and ca. three months of temperatures near 0°C. Coherently with this pattern, seed dispersal of *G. lutea* occurs in late summer, they form a short transient soil seed bank (during autumn-winter), and germinate in the following spring, after experiencing the low winter temperatures that break dormancy **[Chapter 2]**.

3.4.2 Optimal germination protocol

G. lutea is a species of high economic importance; in several European countries, the bitter substances contained in the roots are used to prepare bitters and liqueurs, as well as pharmaceuticals such as anti-inflammatory agents and diuretics (Carnat *et al*. 2005, Nastasijević *et al.* 2012). Nevertheless, there is a dearth of data about its ecology and germination requirements, especially in the Mediterranean mountains, which represent the southern part of its distributional range (Catorci *et al*. 2014). On the basis of this results, we suggest that the optimal germination protocol for this species consists of a period (ranging from one to three months) of cold stratification at ca. 0°C in dark conditions, followed by seed incubation at 10–20°C under photoperiod conditions of 12/12 hours. Considering the few statistical differences on FGP and T_{50} between the two pre-treatments that promoted seed germination (*i.e.* C0, with a three-months duration and W+C5+C0, seven months), our study suggests that they both have the same effect on the seed germination response, therefore highlighting the importance of cold stratification near 0°C to break physiological dormancy.

3.4.3 Variability of seed germination response in relation to different locality size

Our results suggest that locality size may be a factor which influences the germination capacity, as also highlighted by Kery *et al.* (2000), who reported that the reduction of population size resulted in reduced fecundity and decline in offspring performance in *G. lutea*. However, the higher seed abortion reported for this *taxon* in small localities **[Chapter 4]** could be the result of an increased mortality of developing seeds due to inbreeding, whose deleterious effects are often expressed during seed development (Kery *et al.* 2000). In addition, the small locality under study (Bruncu Spina) produces a lower number of fruits and seeds per plant and these seeds are smaller **[Chapter 4]** than those from other localities. These patterns and the lower seed germination in

Bruncu Spina could be due to pollen limitation, or to increased inbreeding and loss of genetic variation in small populations (Ågren 1996, Fischer and Matthies 1998). In addition, lower habitat quality or habitat fragmentation might play a key role in this sense, as the Bruncu Spina locality is found in an area characterized by the development of skiing infrastructures. It is already known that increased habitat fragmentation and the associated isolation may cause a decline in seed yield (Steffan-Dewenter and Tscharntke 1999, Putz *et al.* 2015). In the case of *G. lutea*, which is an obligate outcrossing species, the disruption of pollinator-plant mutualisms in small populations (Rossi *et al*. 2014) might play an important role in the reduction of the germination ability in population with small size such as Bruncu Spina. Bumblebees, which are important pollinators for this species, have been shown to pollinate a higher proportion of flowers in large than in small populations (Sih and Baltus 1987).

A reduction of seed germination in small populations of *G. lutea* may have negative consequences (*i.e*. reduced recruitment of new individuals) even for the short-term population dynamics of such a long-lived species, while in the medium term it may decrease the chance to colonize unoccupied habitat patches. The consequence of a reduced sexual reproduction could compromise the ability of a small population to respond to changing environmental conditions in the long term and therefore increase its extinction risk (Kery *et al.* 2000). Moreover, PIPPs are particularly vulnerable and consequently we should give more attention to their conservation *status* (Putz *et al.* 2015). More studies are needed to delve into the mechanisms of this potential association between seed germination response and locality size.

3.5 Conclusions

These results are relevant to conservation of this species listed in the Habitats Directive and also considering the threat represented by global warming, as its distribution mainly regards the upper sectors of mountains. Our study shows that seeds of *G. lutea* are characterized by intermediate complex morphophysiological dormancy and that temperature is a critical environmental factor for germination to occur.

Seeds belonging to different size localities of *G. lutea* showed a considerable degree of physiological (final germination percentage) variability, which would be relevant when conserving and germinating seeds from multiple origins. The optimal protocol of germination for isolated populations should therefore be taken into account when developing a conservation strategy involving the *ex situ* cultivation of plants, an action that could effectively contribute to the reduction of the root harvest pressure on wild populations.

The decline in seed germination observed in small localities leads us to recommend paying more attention to and monitoring small nuclei because of their increased vulnerability and extinction risk.

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______CHAPTER 4_______

INTER-ANNUAL CONSEQUENCES OF WARMING ANOMALIES IN PHENOLOGICAL AND REPRODUCTIVE PERFORMANCE OF *Gentiana lutea* L. subsp. *lutea*

4 Chapter IV – Inter-annual consequences of warming anomalies in phenological and reproductive performance of *Gentiana lutea* L. subsp. *lutea*

4.1 Introduction

Phenology is the scientific study of periodic plant life cycle events and how these are influenced by seasonal and interannual variations in climate (Zhao *et al.* 2013). In seasonal climates, such as the Mediterranean climate, temperature is considered to be the major cue that initiates the onset of flowering (Blionis and Vokou 2001) and of many plant development processes (Khanduri *et al.* 2008). Phenological changes from year to year may be a sensitive and easily observable indicator of environmental changes, and have a wide range of consequences for ecological processes, agriculture, forestry, human health, and the global economy (Khanduri *et al.* 2008). In this sense, climate warming is expected to change seasonal biological phenomena such as plant growth and flowering (Khanduri *et al.* 2008), and could be thus detected by plant responses (Arft *et al.* 1999, Dormann and Woodin 2002, Cleland *et al.* 2007). In particular, climate warming in mountain areas is projected to shift species' ranges to higher elevations (Grabherr *et al.* 2010).

Increases in temperature have been predicted and reported for the Mediterranean mountain ranges due to climate change (Peñuelas and Boada 2003, Giménez-Benavides *et al.* 2007); for instance, changes in plant phenology related to heat waves (*sensu* WMO 2003) have been detected in several studies by the end of the 21st century (*e.g.* Ciais *et al.* 2005, Abeli *et al*. 2012).

Plant populations at the margins of their geographical and ecological ranges are thought to be particularly sensitive to climatic anomalies and global warming in general (Abeli *et al*. 2012, Rehm *et al.* 2015). As the distance to the edge of the range for a species decreases, individuals often experience increasingly stressful climatological conditions resulting in fewer, smaller patches of suitable habitat, or in decreased reproduction (Parmesan *et al.* 2000). Accordingly, individuals living along range boundaries might be at the edge of their species' physiological tolerances and thus are more likely to experience stressful, harmful, or lethal weather events than those in the core of their distribution (Hoffman and Parsons 1997, Parmesan *et al.* 2000). Otherwise, such general trend was in some cases unconfirmed and species-specific studies are thus needed in order to corroborate this hypothesis (Abeli *et al.* 2014).

Using *Gentiana lutea* L. subsp. *lutea* as case of study, we analysed the phenological and reproductive performance anomalies of two phenological cycles (2013/2014 and 2014/2015) and we correlated them with soil temperatures and precipitation. In particular, we focused our attention on the hypothetical shifting plant phenology and reproductive performance in response to global warming on the Peripheral Isolated Plant Population (PIPP) of Sardinia (Western Mediterranean Basin). Special attention was paid for the drastic phenological changes experienced in the last cycle (2014/2015), which has been also reported as one of the warmest years since 1961 (NOAA 2015).

In particular, 2015 tied with 2011 as the warmest year in the 51-year period of record in the Mediterranean Basin. Uncertainties relative to earlier periods are larger and more difficult to estimate (WMO 2015).

Our main aims were to investigate in which way warming climatic conditions were related to the experienced variation of vegetative and reproductive stages and to examine how they conditioned the *G. lutea* reproductive success. Considering the global warming trend, we put use of the anomalies occurred during 2014/2015 to obtain information useful to bring up conclusions related to the effect of climate change on the Mediterranean mountain plants living at the edge of their distribution and to consider these results for next steps towards their conservation.

4.2 Methods

4.2.1 Species description and study area

Gentiana lutea L. subsp. *lutea* (therefore *G. lutea*) is a rhizomatous long-lived plant. It presents an unbranched stout stem, growing up to ca. 1.5 meters tall. The plant develops into a basal rosette during spring, and may further grow some lateral rosettes in the following years (Hesse *et al.* 2007). Flowering stems carry up to 5 pseudo-whorls containing numerous pediculate flowers (about 20) between June and July. Fruit is a many-seeded capsule (Struwe and Albert 2002) composed of two carpels and ripening in August. Seeds are circular to elliptic, attended and winged, the wing is often absent at the hilum/micropile. *G. lutea* is self-incompatible and thus depends on pollination by insects (mainly by *Hymenoptera* and *Diptera* genus) to produce seeds (Kery *et al.* 2000). Our field surveys suggest that most seeds germinate in early spring **[Chapter 2]**. *G. lutea* could also multiply through vegetative propagation: the spreading of rhizome assures population persistence and growth, hence even large populations are often represented by few individuals (Hesse *et al.* 2007).

This species mainly grows on calcareous (sub)-alpine pastures (800–2500 m a.s.l.), from the Pyrenees to Minor Asia (Anchisi *et al.* 2010). As far as our study case is concerning, *G. lutea* in Sardinia is present as PIPP on the Gennargentu massif (Fois *et al.* 2015), which is considered a

Mediterranean glacial *refugium* (Bacchetta *et al.* 2013). From a climatic point of view, it is characterised by a dry summer and, by contrast, the winter can be very wet (Bacchetta *et al.* 2009, 2013). According to the Rivas-Martínez’s bioclimatic classification (2011), most of the Gennargentu massif has a temperate-submediterranean climate, with thermotypes ranging from the lower supratemperate to the lower orotemperate, and ombrotypes from the upper subhumid to the upper humid. The Mediterranean climate is only found on the eastern and southern slopes of the massif, with a lower supramediterranean thermotype and ombrotype ranging from the upper subhumid to the lower humid (Bacchetta *et al.* 2009). The ecologic and bioclimatic isolation, added to the geographic insularity of Sardinia let to identify the Gennargentu massif as an independent biogeographic sector (Fenu *et al.* 2014) and one of the main “micro biodiversity hotspot” of Sardinia (Cañadas *et al.* 2014).

4.2.2 Temperature variables and precipitations

Predictor climatic variables considered for this experiment were inferred from soil temperatures and precipitations. The study was conducted on four representative localities named Trainu Murcunieddu (TM), Nodu e Littipori (NL), Is Terre Molentes (IS) and Bruncu Spina (BS), situated at different elevations (TM = 1356 m asl; NL = 1428 m asl; IS = 1556 m asl; BS = 1778 m asl). All temperatures were obtained from data of soil temperatures, which were measured by automatic loggers (TidbiTw v2 Temp logger, Onset Computer Corporation, Cape Cod, MA, USA) that were set in the central part 3–5 cm below the soil surface of three locations (TM, IS, BS), NL was excluded for most of analyses due to a data logger failure after the snowmelt time. Temperatures were recorded at 90 min. intervals during the experimental period. Besides the Growing Degree Days (GDD), accounted as the sum of daily mean temperatures at soil surface using a threshold value of 5ºC (Kudo and Suzuki 1999), we used the soil temperature information to extrapolate eight general variables and six variables, subdivided into three referred to cold (from December to February) and three to warm periods (from June to August). The eight general climatic variables by cycles (from 1st August 2013 to 1st August 2014 and from 1st August 2014 to 1st August 2015) were: (1) minTyear and (2) MaxTyear, referred to the minimum and maximum temperatures recorded during the two cycles, (3) the number of days with snow cover (assumed as a consecutive period with stable mean temperatures within the range of 0–2°C and a maximum of 4°C; Mattana *et al*. 2012), (4) sum of mean temperatures during spring (sum_spring) and mean temperatures of (5) spring, (6) winter, (7) autumn and (8) summer. Trends derived for the cold period were computed as

(1) the sum of the daily differences between the maximum and minimum temperatures of the coldest month (January), (2) the sum of total days with temperatures ≤ 1°C (Tcdd) and (3) the consecutive period of days with temperatures ≤ 1°C (Tcd). The same set of three temperatures variables were also calculated for the warm period: (1) the sum of the daily differences between the maximum and minimum temperatures of the warmest month (July), (2) the sum of total days with temperatures ≥ 25°C (Twdd) and (3) the consecutive period of days with temperatures ≥ 25°C (Twd).

Precipitation data were calculated by summing daily rainfall (mm); these data were registered by the station of Separadorgiu (Fonni, 1427 m asl.) and provided by the Sardegna-Clima ONLUS association (http://www.sardegna-clima.it).

4.2.3 Phenological and reproductive variables

The general pattern of flowering and fruiting phenology was monitored during the entire reproductive season at 10 days intervals from the year 2013 to 2015. The reproductive stage in four localities (TM, IS, BS NL) was categorised following the experiments of Kawai and Kudo (2011) by visual observations in each locality as percentage of reproductive stages: (1) flowering initiation (1–25% plants opening flowers), (2) peak flowering (about 25–75% plants opening), (3) late flowering (>75% plants finished flowering), (4) developing fruits (fruiting but no seed dispersal), (5) and fruit maturation (seed dispersal).

Following a tested sample methodology (*e.g.* Watkinson *et al.* 1998, Fenu *et al.* 2011), differences on the number of flowering individuals (Fec 1) were measured within a variable number of random plots of 2 × 1 m in each locality depending on their different sizes. The remaining aspects of reproductive performance were estimated by randomly selecting 100 reproductive stems with floral buds in the two bigger localities (IS and TM) and 55 reproductive stems in NL and all 13 stems in BS before the flowering season. To test "fitness plant hypotheses", we carried out a natural experiment within an open pollination system (Wang *et al.* 2014) examining the variation of the number of fruits (Fec 2) and seeds per plant (Fec 3), number of seeds per fruit (Fec 4), number of viable seeds per fruit (Fec 5) and seeds weight in milligrams (mg; Fec 6). Random samples of ripe fruits (one per internode each stem) were taken in the four localities. We harvested fruits when the colour of them began to change but it still closed in order to count the number of seeds by fruit. All seeds were counted in each fruit and weighed on a microbalance. Viable or not viable seeds were distinguished in the laboratory by their size, shape and colour. The number of seeds per plant was determined by multiplying the average number of seeds per fruit by the number of fruits per stalk.

4.2.4 Data analyses

Statistical analyses were conducted using R statistical software (version 3.2.2, R Development Core Team 2015). Following Walker *et al.* (2012), the Wilcoxon signed-rank test for paired samples was used to test the difference in mean, minimum, maximum temperatures and daily precipitations between the two sampling cycles (2013/2014 and 2014/2015) and between cold and warm periods of each locality (TM, BS and IS). Significance was reported at the $P < 0.05$ level. The same Wilcoxon signed-rank test was used to evaluate differences on reproductive performance (Fec 1–6) between the sampling cycles. Linear regression analyses were used to measure the strength of the association (by R^2 values) between the snowmelt day and the starting date of vegetative and reproductive stages of each locality and also between the accumulated soil temperatures of the period comprising the snowmelt time and the start of vegetative and reproductive stages.

To determine the effect of climatic variables on reproductive performance were used Generalized Linear Models (GLMs). Depending on the error distribution of each response variable, we set the most appropriate link function (Luzuriaga *et al.* 2006). GLMs with Gaussian family, using the ‘identity’ link function and setting, were applied when the distribution of the response variable was considered as normal (*i.e.* Fec 3, Fec 5, Fec 6). A Poisson estimation, using a ‘log’ link function, was used when the distribution of the response variables were Poisson-like (*i.e.* Fec 1, Fec 2, Fec 4). In order to determine the relative importance of each significant factor ($P < 0.05$) which was identified by the previous GLMs analyses, we used Hierarchical Partitioning (HP) in the ‘hier.part’ package in R (Walsh and Nally 2008). HP, as currently implemented in the ‘hier.part’ package, assumes a monotonic relationship between the dependent and the explanatory variables (Luoto *et al.* 2006, Luzuriaga *et al.* 2006). For each reproductive response (Fec 1–6), the independent contribution of each explanatory variable was returned. The ‘hier.part’ package produced a matrix and a barplot of percentage distribution of Independent Effects only (IE). The amount of deviance explained adjusted for the number of observations and parameters (D^2_{adj}; Guisan and Zimmermann 2000) by each GLM was also assessed using ‘Dsquared’ function in the modEvA package for R (Barbosa *et al.* 2013).

4.3 Results

4.3.1 Climatic differences between the two cycles

Soil temperatures of all tested localities (Fig. 1) registered an annual mean of 8.20±0.91°C in the cycle 2013/2014 and an annual mean of 9.74±0.76°C in the cycle 2014/2015; these data confirmed the increment of temperatures in the 2014/2015, with 1.54°C of difference between the sampling cycles. Reductions in cold period duration were caused by the increment of temperatures during autumn and spring. Concretely, the increase of mean daily temperatures in 2014/2015 was 0.47°C in TM, 1.61°C in BS and 0.41°C in IS during the autumn while the increase during spring was 2.84°C in TM, 1.78°C in BS and 2.32°C in IS. Contrarily, the variation of mean daily temperatures during winter were negative in 2015 with differences of -0.72°C in TM, -0.12°C in BS and -1.11°C in IS. On the other side, the greater variation of temperatures was found in summer with a mean daily increment of 4.27°C in TM, 3.62°C in BS and 3.24°C in IS.

Snow cover continuously persisted in the highest BS locality for 134 days (from November to March) in the first cycle and for 106 days in the second cycle (from December to March). Regarding the other lower localities, snow cover generally had a shorter duration and slightly increased during the second cycle (from 35 to 46 days in TM and from 36 to 49 days in IS).

The total annual precipitation in the cycle 2013/2014 was 816.3 mm with a decrease of 90.5 mm during the second cycle.

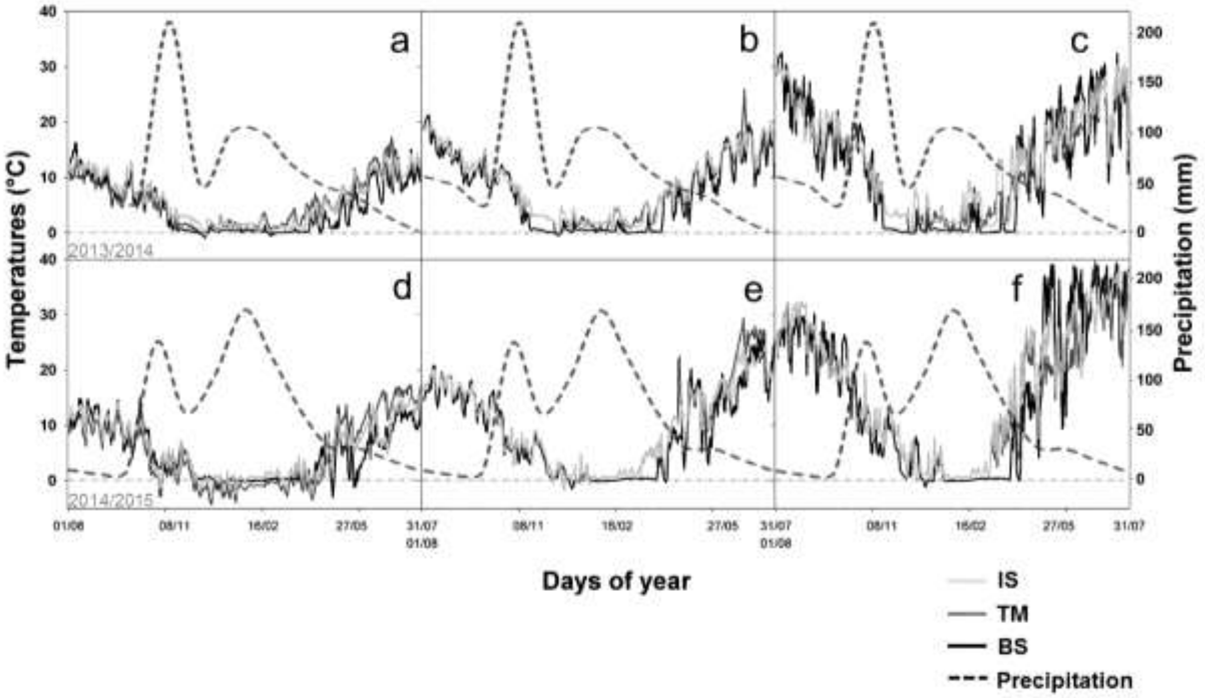

Figure 1. Soil temperatures (°C) registered from 01/August/2013 to 31/July/2015 in the study localities. Upper graphs (a-c) represent cycle from 01/08/2013 to 31/07/2014 and lower graphs (d-f) represent the cycle from 01/08/2014 to 31/07/2015 on minimum (a,d) mean (b,e) and maximum (c,f) temperatures for three localities. Dashed lines represent the monthly cumulated precipitations (mm) for each cycle registered by station of Separadorgiu, Fonni at 1427 m a.s.l. TM = Trainu Murcunieddu (1356 m a.s.l.); IS = Is Terre Molentes (1556 m a.s.l.) and BS = Bruncu Spina (1778 m a.s.l.).

Wilcoxon test results (Table 1) highlighted differences among cycles on mean, minimum, maximum daily soil temperatures. Annual mean temperatures, as well as the ones relative to the warm period, were all significantly different in all tested localities (*P* < 0.001; Table 1). Otherwise, a similarity on mean daily temperatures of the cold period was detected for BS (*P* > 0.05; Table 1). Maximum temperatures were statistically different among cycles (*P* < 0.001; Table 1) on annual temperatures and warm periods for all localitiess; only IS showed significant differences between cycles for the cold period (*P* < 0.001; Table 1). Wilcoxon test on annual minimum temperatures showed no statistical differences for TM and BS (*P* > 0.05; Table 1) and significant for IS (*P* < 0.05; Table 1). Minimum temperatures of cold periods were significantly different (*P* < 0.001) in TM and IS, while warm periods were statistically similar (*P* > 0.05, Table 1) in all localities. These differences in temperatures variables were not associated with a significant variation on precipitations. Wilcoxon test on annual daily precipitations (N=360) and separately by warm and cold periods (N=90) did not showed significant differences (*P* > 0.05) between the two analysed cycles.

Table 1. Results of Wilcoxon signed-rank test indicating, for each tested locality (TM, BS and IS), differences between the two cycles (significant *P* < 0.05 in bold) on mean, maximum and minimum temperatures of annual (N=360 days), cold (from December to February, N=90 days) and warm periods (from June to August, N=90 days).

	TM			**BS**			**IS**		
	Mean	**Max**	**min**	**Mean**	**Max**	**min**	**Mean**	**Max**	**min**
Annual Temp.	< **0.001**	< **0.001**	>0.05	< **0.001**	< **0.001**	>0.05	< **0.001**	< **0.001**	< **0.05**
Temp. Cold period	< **0.05**	>0.05	< **0.001**	>0.05	>0.05	>0.05	< **0.001**	< **0.001**	< **0.001**
Temp. Warm period	< **0.001**	< **0.001**	>0.05	< **0.001**	< **0.001**	>0.05	< **0.001**	< **0.001**	>0.05

4.3.2 Vegetative and reproductive stages differences between the two cycles

A delay in second cycle (2014/2015) on snowmelt was detected in all tested localities (Fig. 2a). The day of snowmelt was positively correlated with the time of the beginning vegetative stage (Fig. 2b, $R^2 = 0.779$). On the other hand, the period comprising the snowmelt and the start of vegetative stage (pre-vegetative time) slightly decreased in the second cycle (27–54 days against 28–67 days of the first cycle; Fig. 2); this reduction on pre-vegetative time in the second cycle was contrasted by an increment of accumulated soil temperatures ranging from 142.86 to 204.56 °C in first cycle and from 152.75 to 293.15°C in second one (Fig.2a). These accumulated soil temperatures during the pre-vegetative time were negatively correlated with the beginning of vegetative stage (Fig. 2c, $R^2 = 0.602$).

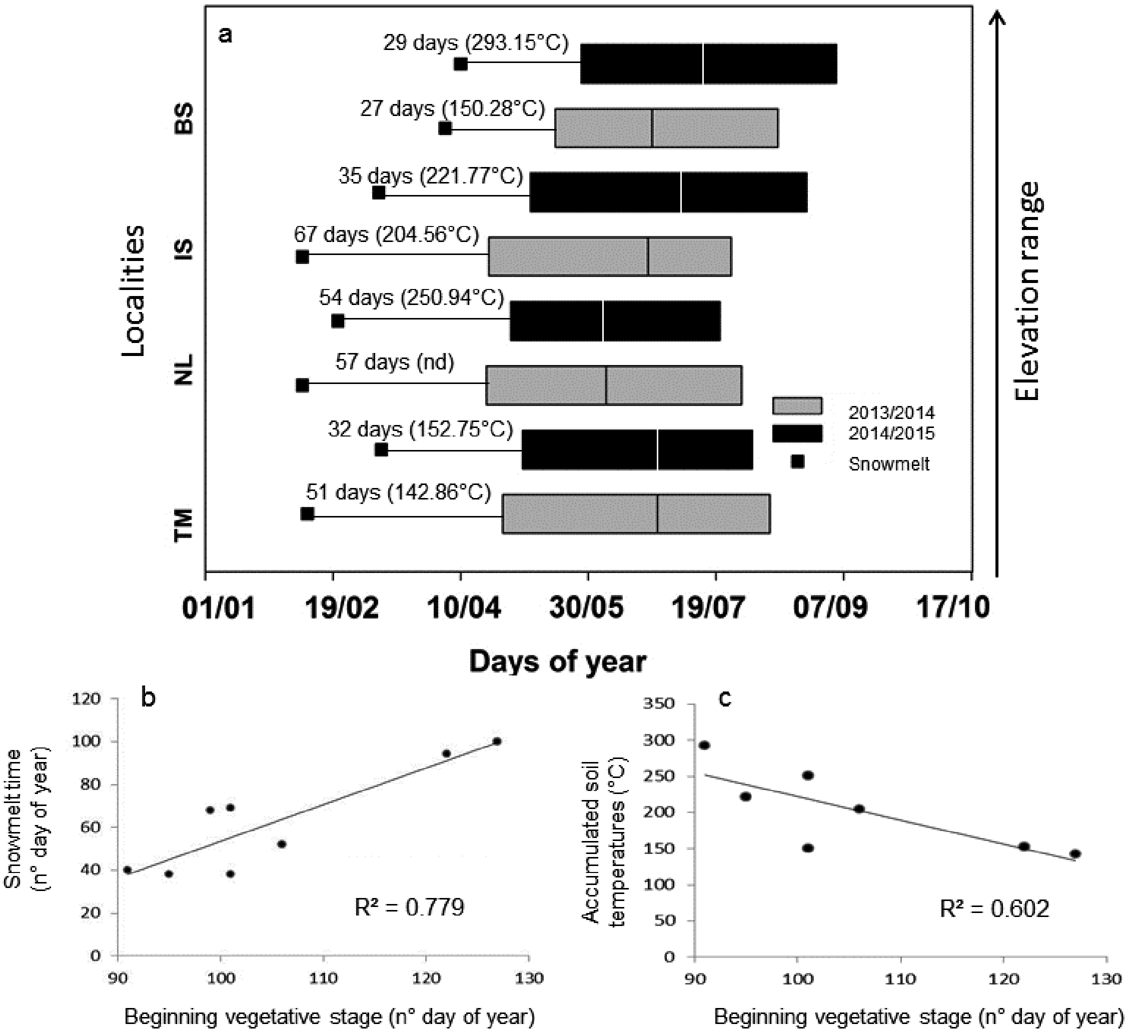

Figure 2. Variability on the vegetative stage duration between cycles (2013/2014 and 2014/2015) and localities (TM, NL, IS, BS) considering the duration of vegetative stage (a). Black squares represent the time of snowmelt (day of year). Details on the pre-vegetative time (number of days) and the sum of mean daily soil temperatures (°C) during this period are reported in the figure (a). “Nd” indicates unrecorded temperatures due to a data logger failure. Correlations of linear regressions between the snowmelt time and the beginning of vegetative stage (b) and between the accumulated soil temperatures of pre-vegetative time and the beginning of vegetative stage (c) are evaluated by r-squared values (R^2). TM = Trainu Murcunieddu (1356 m asl); NL = Nodu ‘e Litipori (1428 m asl); IS = Is Terre Molentes (1556 m asl) and BS = Bruncu Spina (1778 m asl)

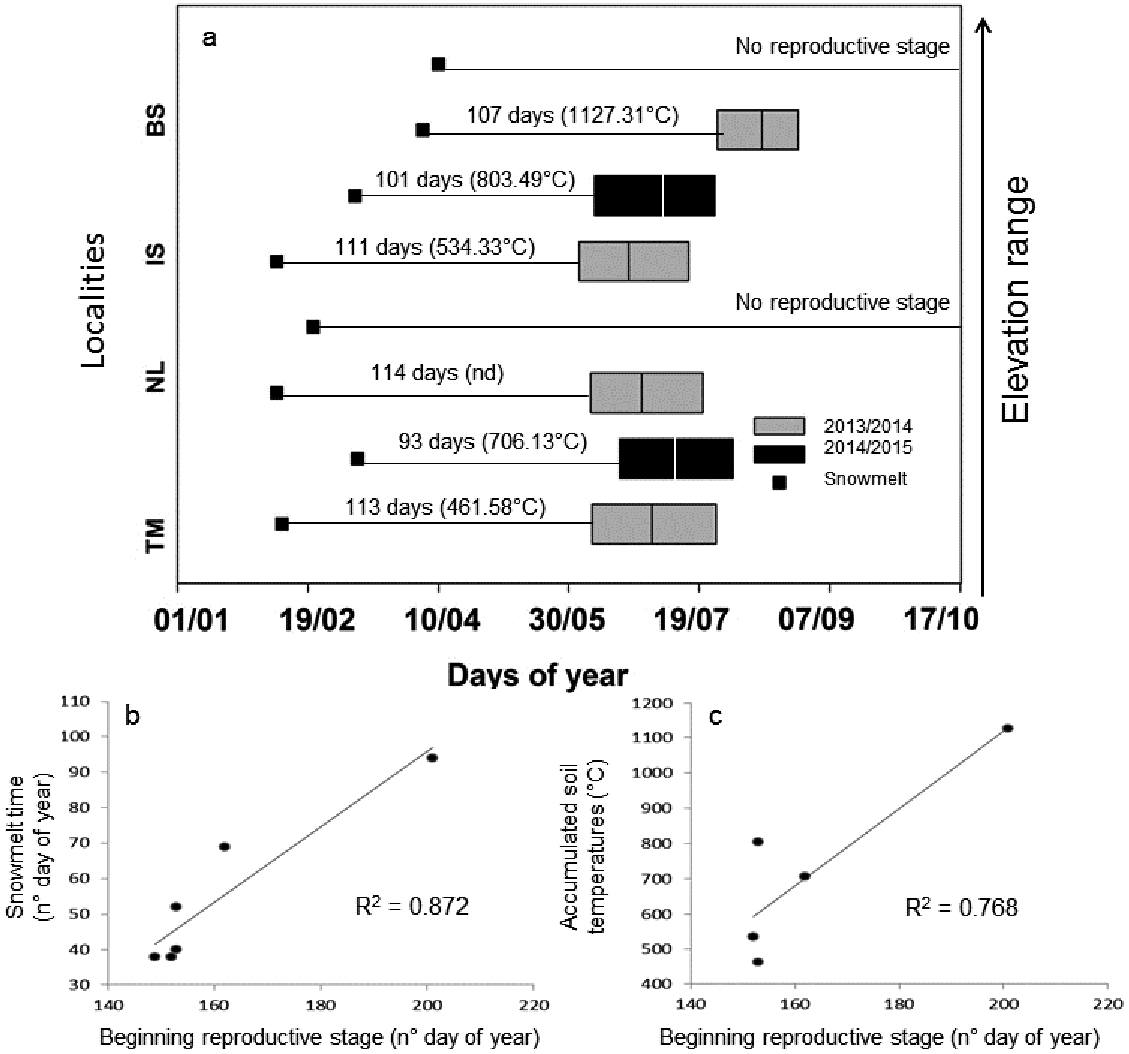

Figure 3. Variability on the reproductive stage duration between cycles (2013/2014 and 2014/2015) and localities (TM, NL, IS, BS) from the flowering initiation (1-25% plants opening flowers) to the end of fruit maturation (seeds dispersal). Black squares represent the time of snowmelt (day of year). Details on the pre-reproductive time (number of days) and the sum of mean daily soil temperatures (°C) during this period are reported in the figure (a). "Nd" indicates unrecorded temperatures due to a data logger failure. BS and NL localities during the second cycle did not reach the reproductive stage. Correlations of linear regressions between the snowmelt time and the beginning of reproductive stage (b) and between the accumulated temperatures of pre-reproductive time and the beginning of reproductive stage (c) are evaluated by r-squared values (R^2). TM = Trainu Murcunieddu (1356 m asl); NL = Nodu 'e Litipori (1428 m asl); IS = Is Terre Molentes (1556 m asl) and BS = Bruncu Spina (1778 m asl)

A decrease in number of days was generally detected for the pre-reproductive time during the second cycle (from 113 to 93 days for TM and from 111 to 101 days for IS), with a variation of accumulated soil temperatures from 461.58 to 706.13°C and 534.33 to 803.49 °C respectively (Fig.

3a). The date of reproductive stage beginning was positively correlated with the snowmelt time (R^2 = 0.872; Fig. 3b) and with accumulated soil temperatures (R^2 = 0.768; Fig. 3c).

4.3.3 Variation on reproductive performance between the two cycles

In the case of flowering occurrences during 2015 (TM and IS), differences in reproductive performance among cycles for both localities were showed in Figure 4 and the results of Wilcoxon test were showed in Table 2. The inflorescence production (Fec 1, Fig. 4a) differed significantly in both localities ($P < 0.001$, Table 2) with a decrease in the second cycle from 0.4 and 1.65 to 0.1 and 0.05 reproductive individuals per m^2 in IS and TM, respectively.

Table 2. Results of Wilcoxon Signed-Rank Test (*P* value) for the variation in reproductive responses by the two localities (TM, BS) where the flowering occurred in both cycles (2013/2014–2014/2015). Significant correlations ($P < 0.05$) are reported in bold. Fec 1, number of flowering individuals inside plots; Fec 2, number of fruits per plant; Fec 3 number of seed per plant; Fec 4, number of seeds per fruit; Fec 5, number of viable seeds per fruit and Fec 6 seed weight.

	Fec 1	Fec 2	Fec 3	Fec 4	Fec 5	Fec 6
TM	**< 0.001**	0.069	**0.013**	**0.048**	**0.020**	0.241
IS	**< 0.001**	0.509	0.761	**0.017**	**0.035**	**0.006**

The variation of the number of fruits per plant between the two cycles (Fec 2, Fig. 4b) were not statistically significant ($P > 0.05$) in both localities. The number of seeds per plant (Fec 3, Fig. 4c) was statistically different in TM ($P < 0.05$) with a decrease in second cycle 2014/2015, while it was similar in IS ($P > 0.05$, Table 2). Nevertheless, the number of seeds per fruit (Fec 4, Figure 4d), decreased significantly ($P < 0.05$; Table 2) in both localities during the 2014/2015. The proportion of viable seeds per fruit (Fec 5, Fig. 4e) was statistically different among cycles in TM and IS ($P < 0.05$; Table 2). Differences between first and second cycles of seed mass (mg, Fec 6; Fig. 4f) were significant only in IS ($P < 0.01$; Table 3).

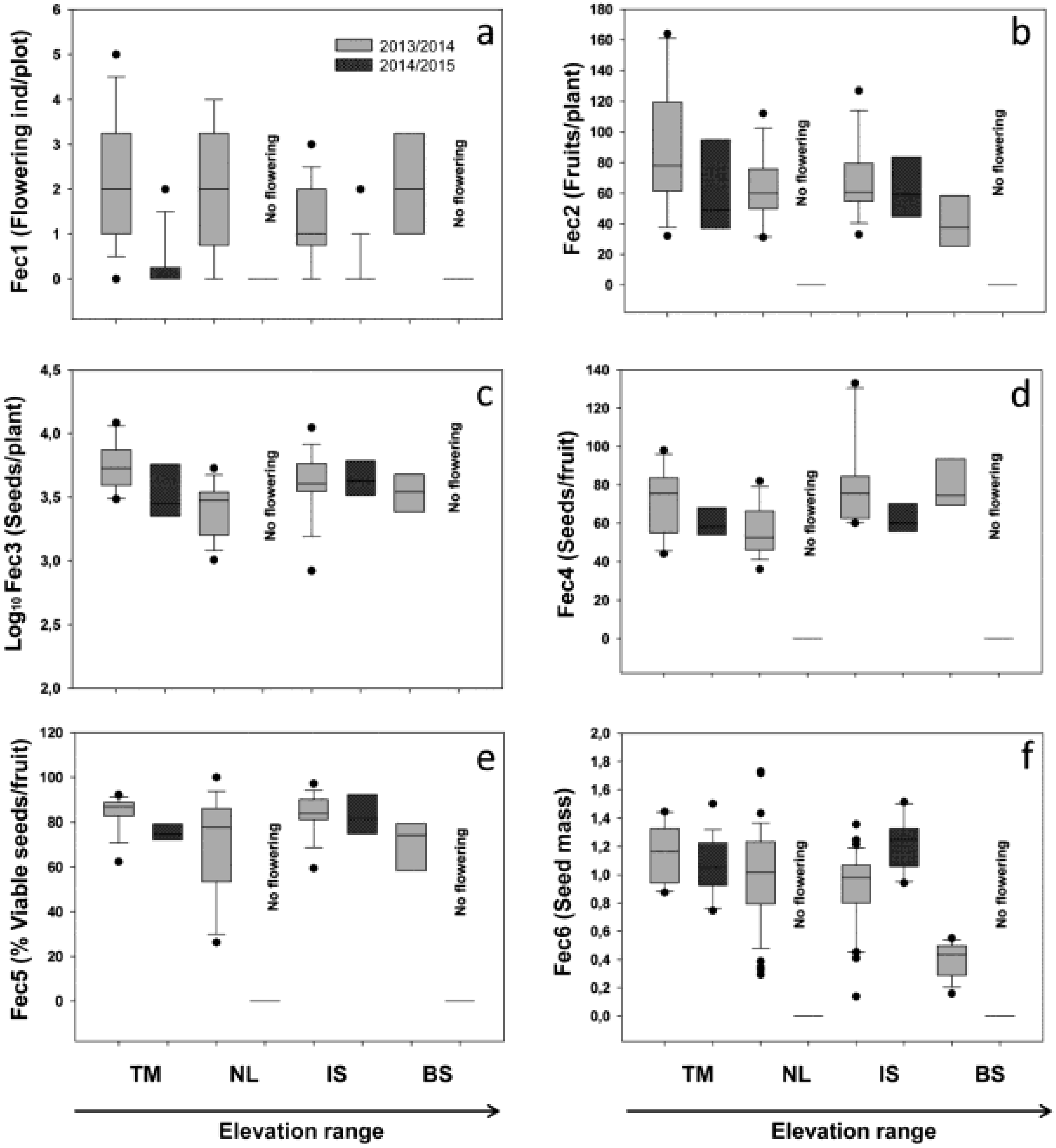

Figure 4. Box plot of reproductive performance variables (Fec 1−6) derivated by open pollination in the two study cycles 2013/2014 and 2014/2015. TM = Trainu Murcunieddu; NL = Nodu ‘e Litipori; IS = Is Terre Molentes and BS = Bruncu Spina.

4.3.4 Reproductive success related with warm and cold climatic variables

HP and D^2_{adj} were evaluated only for the significant correlations ($P < 0.05$; see Table S1 in Appendix 1). D^2_{adj} of each GLM highlighted that climatic factors only explained a high percentage of the variance for Fec 1 (D^2_{adj} = 0.897, Fig. 5f) and for Fec 6 (D^2_{adj} = 0.527, Fig. 5). While deviances were only marginally explained by climatic factors (D^2_{adj} < 0.3, see Fig. 5) for the other reproductive variables (Fec 2–5). The reduction of number of flowering individuals, Fec 1 (Fig. 5, D^2_{adj} = 0.897) was mainly determined by MaxTyear (IE = 17.57%), autumn (IE = 15.93%), sum of mean temperatures during spring and mean temperatures of spring (sum_spring, IE = 13.37%) and July (IE = 13.23%). The Independent Effects (IE) on the variation on seed mass, Fec 6 (Fig. 5, D^2_{adj} = 0.527), were principally determined by the sum of total days with temperatures ≥ 25°C (Twdd; IE = 15.15%), the consecutive period of days with temperatures ≥ 25°C (Twd; IE = 14.86%) and MaxTyear (IE = 12.28%).

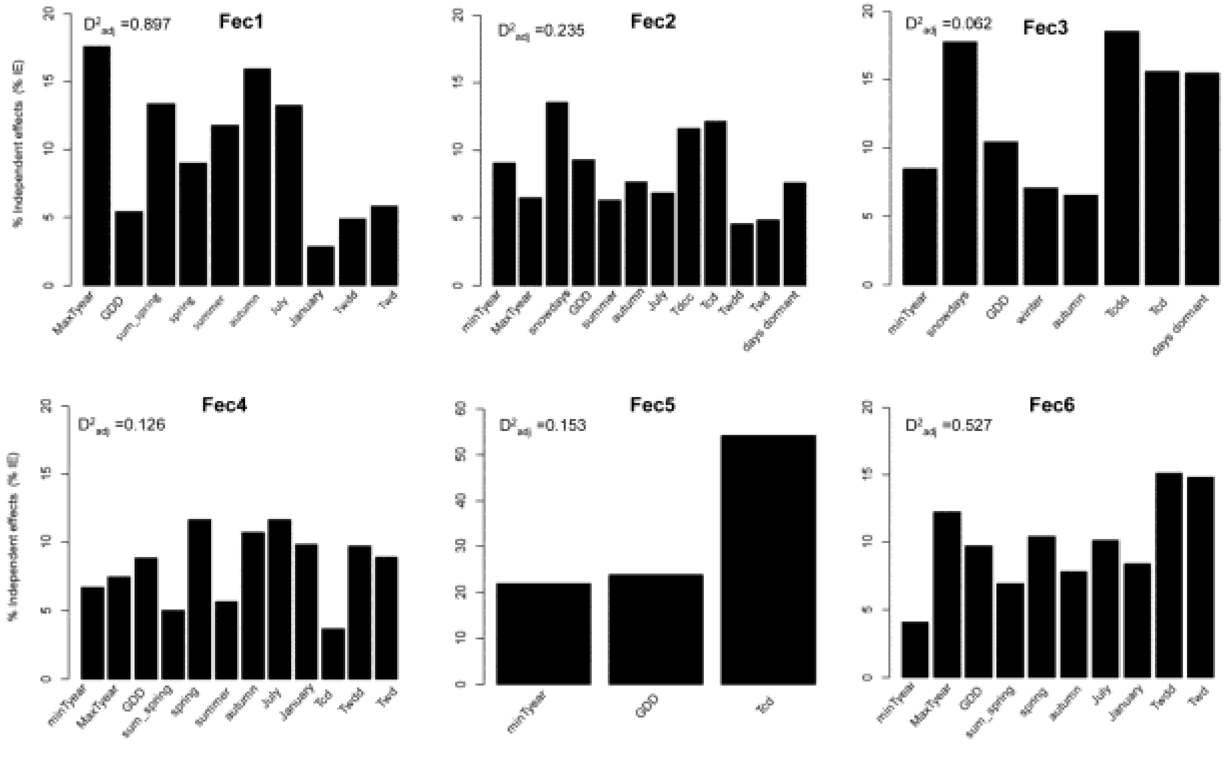

Figure 5. Independent Effect (IE) to the total variance explained on the six reproductive performance variables (Fec 1−6) and amount of deviance explained adjusted for the number of observations and parameters (D^2_{adj}) accounted for each significant key factor (*P* < 0.05; Appendix 1, Table S1). Fec 1, number of flowering individuals inside plots; Fec 2, number of fruits per plant; Fec 3, number of seed per plant; Fec 4, number of seeds per fruit; Fec 5, number of viable seeds per fruit and Fec 6, seed weight.

4.4 Discussion

This study showed how sensitive is *G. lutea* in Sardinia to a variation of temperatures between two consecutive cycles. Differences in terms of phenology and temperatures, but not precipitations, were so evident that it was possible to obtain significant results only comparing two cycles. As demonstrated by other authors for the same genera (Kawai and Kudo 2011) and for other Mediterranean mountain plants (Giménez-Benavides *et al*. 2007, Porceddu *et al*. 2013), our results indicated that both vegetative and reproductive stages were correlated with the snowmelt time and accumulated soil temperatures. In this particular case, a delay of snowmelt produced a delay of the beginning of both vegetative and reproductive stages. On the other hand, increased accumulated soil temperatures during pre-vegetative and pre-reproductive stages were likely to cause an anticipated sprouting and, at the same time, a delayed reproductive stage. According to the R^2 values, the snowmelt time was the most important factor in influencing the start of both stages; additionally, the accumulated soil temperatures were more correlated to the reproductive than the vegetative stage. These results were similar to the ones obtained for alpine ecosystems in which the disappearance of the snowpack was a fundamental factor on wildflowers growth initiation (Inouye 2008), while the floral development were more affected by the ambient temperatures between sprouting and flowering (Kawai and Kudo 2011).

As regard as the reproductive performance, the more remarkable difference between cycles was the drastic reduction of the number of flowering plants (Fec 1) in all study localities and a significant increment on seed mass (Fec 6) in the second cycle 2014/2015. Results from the deviance explained by models on Fec 1 and climatic variables, found that phenological changes were mainly determined by maximum temperatures, autumn temperatures and the sum of the daily differences of the warmest month (July). In accordance with these results, also Abeli *et al.* (2012) demonstrated that flowering production of mountain plants in their range marginality within the Mediterranean area greatly fluctuated and was significantly affected by the variation of the mean temperature of June and July. Our study implemented this assumption with the importance of temperatures during autumn (pre-vegetative time), suggesting that focusing only on spring warming sensitivities would lead to incomplete interpretations and predictions for species that may rely both on autumn/winter chilling and spring forcing (Cook *et al.* 2012). We thus suggest that also in *G. lutea*, cold period or vernalisation, which is commonly related only with the plant dormancy (chilling), played a role in the second 'forcing' phase of what is often abstracted into a two-phase system (chillig-forcing; Caffarra and Donnelly 2011).

According to our results, also the increasing seed mass was related with warming temperatures, although this kind of response was explained by the analysed variables only for the 52.7% of the deviance ($D^2_{adj} = 0.527$) suggesting that further unanalysed factors, such as intraspecific competition (Völler *et al.* 2012) and pollinator efficiency (Rossi *et al.* 2014), might concur with it. In the case of *G. lutea*, which is an obligate outcrossing species, the disruption of pollinator-plant mutualisms might play an important role in the reduction of the reproductive success (Rossi *et al.* 2014). Bumblebees, which are important pollinators for this species, have been shown to pollinate a higher proportion of flowers in large than in small populations and/or in years with more flowering occurrences (Sih and Baltus 1987). Seed mass is strongly connected, among others, to plant dispersal ability and the establishment success and competitive ability of seedlings, and it is considered to be one of the fundamental dimensions of plant ecological strategy (Westoby *et al*. 2002). Otherwise, because of its relationship with several factors, seed mass is likely to be a difficultly determined plant response to climate change (Diaz and Cabido 1997).

As shown by the analysis on independent effects, responses of reproductive success variables to climatic conditions was contrasting. In one hand, the variation of the number of fruits (Fec 2), seeds per plant (Fec 3) and the number of viable seeds (Fec 5) were more influenced by cold variables (Snowdays, Tcdd and Tcd). In accordance with these results, the experimental studies of Cook *et al.* (2102) suggested that warm temperatures during the cold period or vernalisation (typically autumn and winter) can delay dormancy or the fulfilment of chilling requirements, thereby delaying spring events, such as flowering and sexual process (Schawartz and Hanes 2010). On the other hand, the number of flowering individuals (Fec 1), variation of seeds per fruit (Fec 4) and seed mass (Fec 6) were more influenced by warm variables (sum_spring, July, Twdd, Twd and MaxTyear). As far as warming spring is concerned, prolonged exposure to high temperatures can reduce the reproductive performance in many mountain species, as a consequence of morphological modifications and reduced activity of physiological processes (Abeli *et al.* 2012). Also Hedhly (2011) demonstrated that flowering and sexual processes are especially sensitive to temperature stress both during their development before pollination and during the post-pollination stage.

In general, researches on plant phenology responses to global warming that included multispecies studies from any location, reported neutral, negative and positive results (Parmesan and Yohe 2003). These ambiguous results did not help to understand at local level what response of a widespread species could have in the boundary of its distribution, where is often needed prioritization to conservation (Hampe and Petit 2005). In our case, the flowering density of *G. lutea* at the southern boundary of its range was not favoured by increased temperatures. Thus, the possibility that a long-term effect of climate on *G. lutea* reproductive performance might alter the population dynamics cannot be ruled out. In this scenario, *G. lutea* in Sardinia is likely to have a high sensitivity to climate warming, which is typical to peripheral flora of Mediterranean mountains and is often connected with high rates of species loss (Hampe and Petit 2005, Cleland *et al*. 2007, Grabherr *et al*. 2010).

4.5 Conclusions

The main conclusion of the present study is that *G. lutea* vegetative and reproductive stages are controlled by snowmelt time and temperatures and, as a consequence, in a such unstable climatic condition that we are currently experiencing, phenological changes can be attributed year by year. Monitoring the underlying drivers of this variation in phenological shifts will contribute to a mechanistic understanding of the biological effects of climate change (Iler *et al.* 2013). The unreached reproductive stage was probably conditioned by increments of temperatures; this provided an example of which problems could occur with plants on the boundary of their distribution associated with global warming (Gordo and Sanz 2010). In particular, warm temperatures during the cold period or vernalisation (typically autumn and winter) are as important as spring or summer temperatures on flowering and reproductive processes. The reduction of sexual reproduction, connected with the loss of genetic diversity within populations, represents one of main threats of global warming with negative consequences on both biodiversity conservation (for endangered species) and economical resources (for crop species). A large literature could allow to state the causes of climatic global changes, but effects of these changes are still uncertain. Besides the specific conservation aspects, here we have shown how the phenology could be an excellent sentinel of climatic changes. Considering the importance of this issue and the ease and cost effectiveness of phenological monitoring, we thus argued that researches in this sense could be a key tool for the enhancement of crucial targets for the future as the biodiversity loss mitigation associated to climate change.

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Appendix 1

Table S1 Summary results of Generalized Linear Models (GLMs) of the six reproductive performance variables (Fec 1−6) and the 16 climatic independent factors (details below). According to the error distribution type, Poisson or Gaussian link functions were used.

minTyear and MaxTyear referred to the minimum and maximum temperatures recorded during the two cycles (2013/2014-2014/2015), snowdays referred the number of days with snow cover (assumed as a consecutive period), Growing Degree Days (GDD), accounted as the sum of daily mean temperatures at soil surface using a threshold value of 5ºC, sum of mean temperatures during spring indicated as sum_spring and mean temperatures of spring, winter, autumn and summer. Three temperatures variables were calculated for the warm period: the sum of the daily differences between the maximum and minimum temperatures of the warmest month (July), the sum of total days with temperatures ≥ 25°C (Twdd) and the consecutive period of days with temperatures ≥ 25°C (Twd) and three temperatures variables were also calculated for the cold period: the sum of the daily differences between the maximum and minimum temperatures of the coldest month (January), the sum of total days with temperatures ≤ 1°C (Tcdd) and the consecutive period of days with temperatures ≤ 1°C (Tcd), in addition we calculated for each locality the number of days during plant dormancy (days dormant). Fec 1, number of flowering individuals inside plots; Fec 2, number of fruits per plant; Fec 3, number of seed per plant; Fec 4, number of seeds per fruit; Fec 5, number of viable seeds per fruit and Fec 6, seed weight.

	FEC 1 (Poisson Error Distribution)				**FEC 2 (Poisson Error Distribution)**			
	Estimate	**Std. Error**	**z value**	***P***	**Estimate**	**Std. Error**	**z value**	***P***
minTyear	-0.0098	0.1196	-0.083	0.9340	-0.1090	0.0184	-5.913	$3.35e^{-09}$
MaxTyear	-0.1185	0.0225	-5.268	$1.38e^{-07}$	-0.0071	0.0020	-3.427	0.0006
snowdays	-0.0009	0.0040	-0.224	0.8220	-0.0067	0.0007	-9.676	$<2e^{-16}$
GDD	0.01215	0.0036	3.376	0.0007	0.0030	0.0004	7.099	$1.26e^{-12}$
sum_spring	-0.0064	0.0011	-5.552	$2.83e^{-08}$	-0.0007	0.1390	-0.053	0.9580
spring	-0.4806	0.1091	-4.404	$1.06e^{-05}$	0.0150	0.0142	1.057	0.2900
winter	-0.1807	0.2169	-0.833	0.4050	0.0002	0.0260	0.011	0.9910
summer	-0.5120	0.1040	-4.922	$8.59e^{-07}$	-0.0370	0.0077	-4.810	$1.51e^{-06}$
autumn	-0.7904	0.1347	-5.869	$4.38e^{-09}$	0.0747	0.0244	3.052	0.0022
july	-0.0124	0.0025	-4.860	$1.18e^{-06}$	-0.0007	0.0001	-3.983	$6.82e^{-05}$
january	-0.0231	0.0085	-2.709	0.0067	-0.0006	0.0009	-0.650	0.5160
Tcdd	0.0047	0.0031	0.015	0.9880	-0.0044	0.0004	-9.181	$<2e^{-16}$
Tcd	-0.0104	0.0070	-1.495	0.1350	-0.0122	0.0012	-9.572	$<2e^{-16}$
Twdd	-0.0882	0.0278	-3.173	0.0015	-0.0048	0.0018	-2.664	0.0077
Twd	-0.0676	0.0299	-2.261	0.0237	-0.0068	0.0020	-3.301	0.0009
daysdormant	-0.0341	0.0216	-1.580	0.1140	-0.0230	0.0033	-6.866	$6.59e^{-12}$

	FEC 3 (Gaussian Error Distribution)				FEC 4 (Poisson Error Distribution)			
	Estimate	**Std.Error**	**t value**	***P***	**Estimate**	**Std. Error**	**z value**	***P***
minTyear	-0.1093	0.0310	-3.524	0.0008	-0.0568	0.0180	-3.148	0.0016
MaxTyear	-0.0012	0.0038	-0.335	0.7390	-0.0092	0.0021	-4.378	$1.2e^{-05}$
snowdays	-0.0013	0.0009	-1.372	0.1770	0.0011	0.0005	2.249	0.0245
GDD	0.0013	0.0006	1.879	0.0667	0.0020	0.0004	4.897	$9.74e^{-07}$
sum_spring	0.0001	0.0002	0.553	0.5820	-0.0003	0.0001	-2.74	0.0061
spring	-0.0129	0.0253	-0.509	0.6130	-0.0848	0.0139	-6.087	$1.15e^{-09}$
winter	0.0060	0.0465	0.130	0.6229	0.0404	0.0253	1.595	0.1110
summer	-0.0071	0.0125	-0.566	0.5740	-0.0225	0.0072	-3.108	0.0018
autumn	-0.0010	0.0429	-0.025	0.9811	-0.1311	0.0227	-5.752	$8.8e^{-09}$
july	-0.0004	0.0003	-1.308	0.1970	-0.0011	0.0001	-6.111	$9.88e^{-10}$
january	-0.0012	0.0017	-0.714	0.4790	-0.0053	0.0009	-5.515	$3.48e^{-08}$
Tcdd	-0.0012	0.0007	-1.603	0.1160	-0.0005	0.0004	-1.341	0.1800
Tcd	-0.0034	0.0020	-1.680	0.0999	-0.0025	0.0011	-2.143	0.0321
Twdd	-0.0044	0.0030	-1.464	0.1500	-0.0112	0.0018	-6.011	$1.84e^{-09}$
Twd	-0.0056	0.0034	-1.656	0.1050	-0.0119	0.0021	-5.610	$2.02e^{-08}$
daysdormant	-0.0055	0.0056	-0.997	0.3240	-0.0019	0.00311	-0.635	0.5250

	FEC 5 (Gaussian Error Distribution)				FEC 6 (Gaussian Error Distribution)			
	Estimate	**Std.Error**	**t value**	***P***	**Estimate**	**Std.Error**	**t value**	***P***
minTyear	-5.2750	2.0180	-2.614	0.0113	$-5,26e^{-02}$	$2,35e^{-02}$	-2.243	0.0286
MaxTyear	0.0540	0.2385	0.227	0.8210	$1,17e^{-02}$	$2,27e^{-03}$	5.164	$2.99e^{-06}$
snowdays	-0.1522	0.0410	-3.708	0.0005	$-8,80e^{-04}$	$7,87e^{-04}$	-1.118	0.2690
GDD	0.0811	0.0326	2.483	0.0168	$-2,15e^{-03}$	$4,98e^{-04}$	-4.31	$8.79e^{-05}$
sum_spring	0.0255	0.0161	1.588	0.1175	$6,61e^{-04}$	$1,68e^{-04}$	3.939	0.0002
spring	1.3110	1.2070	1.086	0.2830	$7,92e^{-02}$	$1,71e^{-02}$	4.622	$3.2e^{-05}$
winter	4.3950	2.1380	2.056	0.0456	$-4,68e^{-02}$	$3,74e^{-02}$	-1.252	0.2170
summer	0.0528	0.6052	0.087	0.9310	$9,70e^{-03}$	$1,02e^{-02}$	0.952	0.3460
autumn	3.5090	1.9960	1.758	0.0855	$1,16e^{-01}$	$3,05e^{-02}$	3.797	0.0004
july	-0.0071	0.0159	-0.447	0.6570	$1,06e^{-03}$	$2,20e^{-04}$	4.810	1.73e-05
january	0.0824	0.0837	0.984	0.3300	$4,91e^{-03}$	$1,24e^{-03}$	3.962	0.0002
Tcdd	-0.1263	0.0331	-3.807	0.0004	$7,07e^{-04}$	$6,40e^{-04}$	1.104	0.2750
Tcd	-0.3191	0.0910	-3.505	0.0010	$2,49e^{-03}$	$1,71e^{-03}$	1.459	0.1520
Twdd	-0.0772	0.1504	-0.513	0.6100	$1,16e^{-02}$	$1,89e^{-03}$	6.144	$1.91e^{-07}$
Twd	-0.1670	0.1675	-0.997	0.3240	$1,31e^{-02}$	$2,12e^{-03}$	6.178	$1.7e^{-07}$
daysdormant	-0.8526	0.2411	-3.536	0.0009	$1,99e^{-03}$	$4,63e^{-03}$	0.429	0.6700

______CHAPTER 5______

CONSERVATION STATUS ASSESSMENT OF *Gentiana lutea* L. subsp. *lutea* AT REGIONAL LEVEL: PAST, PRESENT AND PREDICTED FUTURE PERSPECTIVES

5 Chapter V – Conservation status assessment of *Gentiana lutea* L. subsp. *lutea* at regional level: past, present and predicted future perspectives

5.1 Introduction

The International Union for Conservation of Nature (IUCN) Red Lists are internationally recognised as the standard for assessments of species extinction risk and are instrumental in analyses of biodiversity change (Grammont de and Cuarón 2006, Mace *et al.* 2008). Conservation assessments at a global scale can help to justify and provide a big-picture perspective on the current and projected status of biodiversity on the planet (Ferrier *et al.* 2004); otherwise, listing species for protection that are globally common but locally endangered may lack pertinence for conservation planning due to their marginal nature in a particular region (Gärdenfors *et al*. 2001, Vazquez *et al.* 2008). In this sense, administrative divisions can have an important influence on conservation recommendations and cost efficiency (Kark *et al*. 2009) and a reliable key task could thus be to assess the geographic and administrative levels necessary for priority setting (Gauthier *et al*. 2010).

According to the regional responsibility criterion (*sensu* Gauthier *et al.* 2010), a local priority list should be created in order to better identify the target species for conservation measures (Gauthier *et al*. 2010, Bacchetta *et al*. 2012). The question of assessing priorities at different spatial scales is particularly clear for species in peripheral parts of their distribution range (Abeli *et al.* 2009, Fenu *et al.* 2015a) which, as suggested by international organizations (*e.g.* IUCN, European Council, Planta Europa), should be considered an important resource for biodiversity and should therefore be included in conservation programmes. According to the most recent IUCN guidelines (IUCN 2014), regional isolated populations could be *per se* assessed applying the same criteria used for an endemic *taxon*. In general, distribution data is crucial for measuring the size of species geographic ranges by using Extent of Occurrence (EOO) and Area of Occupancy (AOO), which are globally accepted as surrogates of extinction risk under IUCN Red List criteria (Gärdenfors *et al.* 2001).

Among direct threats listed by IUCN (2012), climate change is one of the most influent and, at the same time, debated one. For instance, several prominent global analyses (*e.g.* Araújo *et al*. 2011, Warren *et al*. 2013) predicted that a percentage ranging from 30% to 60% of species would be endangered or extinct by 2080; differences of results are reflected by differences among taxonomic groups and their distribution patterns. From a geographical point of view, peripheral populations and narrow plant species are more sensitive to climate change than others (Thuiller *et al*. 2005). Projections for the Mediterranean Basin predicted a long-term downward trend in rainfall and an increase in temperature, especially during the hot season (Hutchings 2010), which should cause greater summer aridity (Giorgi and Lionello 2008). In particular, mountain plant species are supposedly destined to a general upward migration under a warming climate (Crawford 2008, Gentili *et al* 2015). In this sense, Species Distribution Models (SDMs) could be used to assess the vulnerability to climate changes, as also suggested by IUCN guidelines (2014). Several methods of SDMs have been implemented throughout the last decades; otherwise, Maximum Entropy models (MaxEnt; Philips *et al.* 2006) have been successfully tested in contrast to other methods and especially recommended for small sample sizes and for restricted extents of the study area (*e.g.* Elith *et al.* 2006, Pearson *et al*. 2007, Fernández *et al*. 2015). Accordingly, MaxEnt models were used for regional assessments through EOO and AOO computations (*e.g.* Sérgio *et al*. 2007, Syfert *et al*. 2014).

This study was focused on the conservation status of *Gentiana lutea* L. subsp. *lutea* (hereafter *G. lutea*) in Sardinia (Italy, Western Mediterranean Basin). Its distribution in this region is characterized by small groups or scattered individuals located at the edge of its distribution range as a typical Peripheral and Isolated Plant Population (PIPP). Up to date, the only conservation status assessment (*sensu* IUCN) of *G. lutea* in Sardinia is by now outdated (Conti *et al.* 1997).

Nonetheless, its potential niche and the current and historical distributional information were recently implemented for the Sardinian territories (Fois *et al.* 2015). Also considering that border populations are usually more prone to local extinctions because of their isolation and restriction to marginal habitats (Case *et al.* 2005, Crawford 2008, Fenu *et al*. 2015a), we argued that an update on the IUCN regional category, based on its historical and predicted trends, was necessary for an effective conservation planning and management.

Our main aims were thus to analyse the vulnerability of *G. lutea* considering the local trend of its geographic ranges among past, present and predicted future and, following the IUCN criteria A and B, to assess the conservation status of this plant according to variations in EOO and AOO over the time. These results will implement the information about the distribution range and the current and potential threats to *G. lutea* in Sardinia, providing a useful tool for next conservation activities.

5.2 Methods and Material

5.2.1 Study species and area

Gentiana lutea L. is a long-lived rhizomatous plant which grows in mountainous and alpine grasslands (800–2500 m a.s.l.) from the Pyrenees to Minor Asia (Tutin *et al*. 1972, Rossi M *et al.* 2015). Four subspecies have been identified: *G lutea* L. subsp. *lutea* (distributed in the southern European high mountains, from Spain to Greece up to the North-West of Turkey and except for the Balkan Peninsula and the eastern Alps), *G. lutea* subsp. *symphyandra* (Murb.) Hayek (which spontaneously grows in the eastern part of the Alps and in the Balkan Peninsula), *G. lutea* subsp. *vardjanii* T.Wraber (endemic to south-eastern Alps) and *G. lutea* subsp. *montserratii* (Vivant ex Greuter) Romo (endemic to Pyrenees).

Pharmaceuticals and traditional uses led to an excessive harvesting of roots and to the decrease in abundance of this species in several regions of Europe (Catorci *et al*. 2014). At European level, this *taxon* was catalogued as Least Concern (LC, global scale, Bilz *et al*. 2011) and included in the CITES convention and European Habitats Directive (92/43/ECC). As far as the Italian peninsula is concerned, root harvesting and, as its distribution mainly regards the upper sectors of mountains, global climatic warming were identified as main conservation threats (Gentili *et al*. 2013, Catorci *et al*. 2014). Accordingly, it was assessed as Near Threatened (NT) in the Italian Red List (Rossi G *et al.* 2015) and, less recently, as Critically Endangered (CR) for Sardinia (Conti *et al*. 1997).

For all these reasons above mentioned, we focused this study on the PIPP of *G. lutea* in Sardinia, where the whole distributional area was limited to the Gennargentu Massif (Fig. 1), located in the central-eastern part of the island and constituted of a system of summits and windy ridges at 1300–1500 m a.s.l., with four peaks at more than 1800 m a.s.l. and a prevalence of metamorphic substrata (Bacchetta *et al*. 2013). Limits of this area were recently defined as an independent biogeographic sector by analysing its endemic and differential vascular flora in concomitance of its environmental and climatic characteristics (Fenu *et al*. 2014).

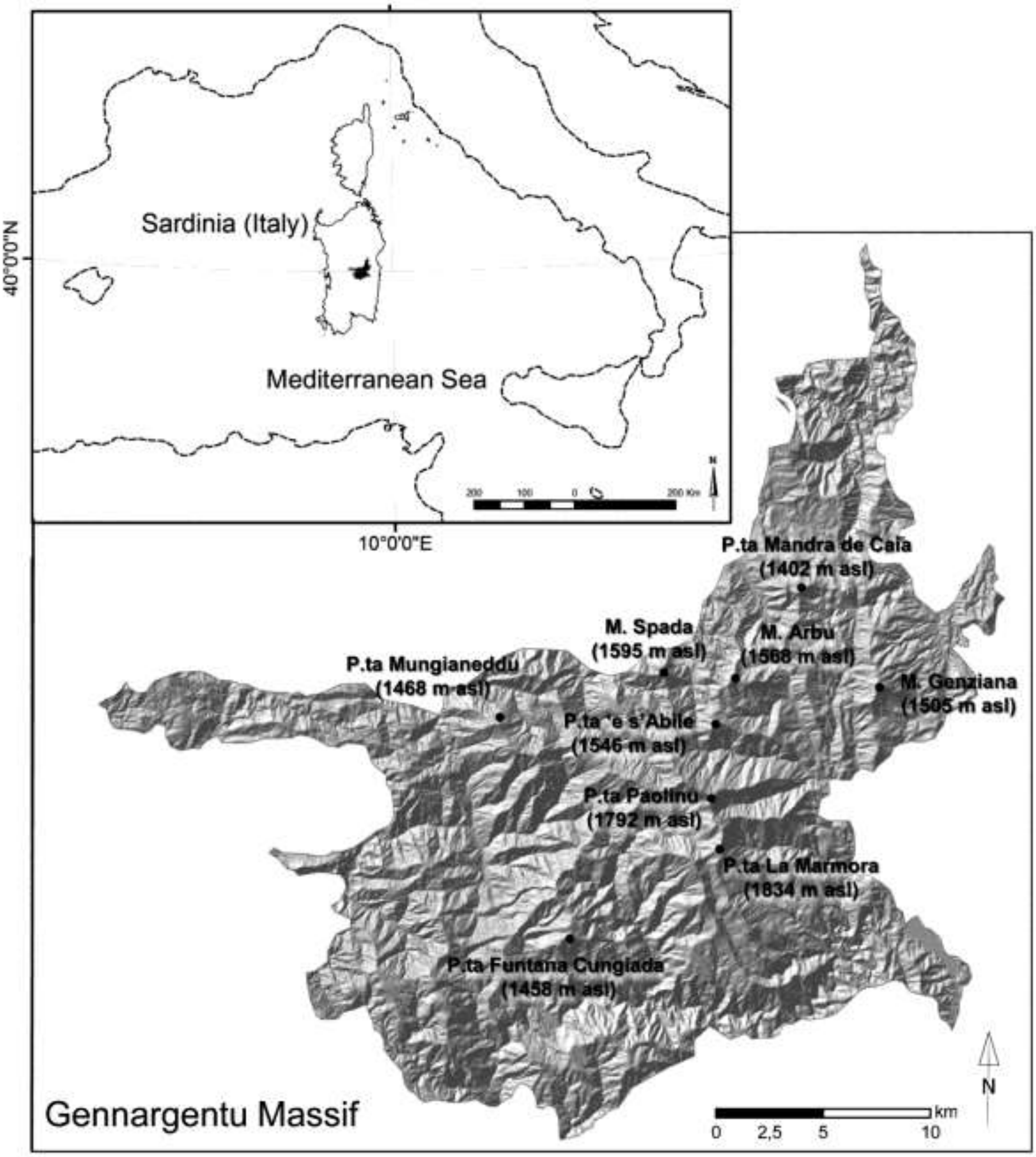

Figure 1. Geographical position of the Sardinian island in the Mediterranean context and toponyms of main peaks included in the Gennargentu Massif.

5.2.2 Historical and present distribution data

Up to recent years, the distribution in Sardinia of *G. lutea* was poorly known and consisting on few bibliographic data (*e.g.* Chiappini and Angiolino 1983), herbarium specimens (CAG, CAT, FI, RO, SASSA, SS, TO Herbaria) and historical reports. Recently, Fois *et al*. (2015) verified them through a deep field investigation guided by SDMs results **[Chapter 1]**; this work permitted to implement the current presence data points from the previous eight known localities with new 16 occurrences and 34 historical extinct localities (Fig. 2a, b). For the IUCN assessment at regional level, we included the 24 localities where *G. lutea* currently grows in the Gennargentu Massif. According to the threats connected to roots harvesting, we will avoid to specify all these localities which still currently mostly unknown to the majority of people.

5.2.3 Conservation status assessment based on historical and present distribution data

The conservation status assessment by historical and present distribution data was based on two criteria (A and B). Firstly, major threats were determined through field observations and categorized following the version 3.2 of IUCN classification scheme (IUCN 2012). Following the IUCN guidelines (IUCN 2014), *G. lutea* was assessed according to negative trends in population size (criterion A) and current geographical distribution size (criterion B). Generation time periods (i.e. the average age of parents of the current regional cohort; IUCN 2014) were used to scale the decline rate threshold for the species' life history and for the application of the criterion A. The geographical distribution size was measured by the EOO and AOO. The EOO, defined as the area contained within the shortest continuous imaginary boundary that can be drawn to encompass all the known sites of a *taxon*'s occurrence, was calculated as the extent of a Minimum Convex hull Polygon (MCP) applying the method implemented by GEOCAT (Bachman *et al.* 2011). The AOO, defined as the area within the extent of occurrence, was calculated using the Italian standard grid of 2 × 2 km (Rossi G *et al.* 2015) superimposed onto the occurrences data points. The comparison of historical EOO and AOO with the current ones were carried out in order to assess the species decline according to the sub-criterion A2. The assessment based on the criterion B was also carried out using both EOO (sub-criterion B1) and AOO (sub-criterion B2).

5.2.4 Conservation status assessment based on future predictions

Future predictions were based on the emissions scenarios presented by the Intergovernmental Panel on Climate Change in the recent fifth Coupled Model Intercomparison Project (IPCC-CMIP5). Climatic variables, at 30 arc-sec resolution (~ 1 km) and constituted by two climate change projections for 2050 and 2070, were implemented by the WorldClim web-site

(Hijmans *et al.* 2005). Among the 19 Global Circulation Models (GCMs) presented by IPCC-CMIP5, we choose the GCMs implemented by three Institutes: Pierre-Simon Laplace (France; IPSL-CM5A-LR), Max Planck Institute for Meteorology (Germany, MPI-ESM-LR) and Met Office Hadley Centre (UK, HadGEM2-ES). These three GCMs resulted to be the less correlated among the ones implemented by European Institutes (Flato *et al.* 2013). Following Guerrina *et al*. (2015), we selected the two most extreme Representative Concentration Pathways emission scenarios (RCP 2.6 and 8.5), representing the least and the greatest estimated greenhouse gas emissions, respectively (Moss *et al*. 2010). As suggested by Thuiller (2004), we computed a consensus of the three GCMs to reduce the decrease uncertainties and we only compared the two scenarios between two projections (2050 and 2070). Bioclimatic variables selection was based on previous analyses in which *G. lutea* in Sardinia was likely to be mostly influenced by elevation and warm climatic conditions (Fois *et al*. 2015). The subset used to calibrate the SDMs consisted of seven bioclimatic variables combined with the constant topographic variable of elevation (Table 1). In order to avoid the inclusion of highly correlated variables and to minimize overfitting, we computed the Variance Inflation Factor values (VIF values; Marquardt 1970). We used the *vifcor* function from R package usdm (Naimi *et al.* 2014), which first found a pair of variables with the maximum linear correlation and then excluded the one with the greater VIF value. This procedure was repeated until no variable with a high correlation coefficient with other variables remained; consequently, the four selected bioclimatic variables (BIO5, BIO10, BIO15, BIO17) and elevation were used to construct SDMs.

Table 1. List and description of bioclimatic and topographic variables analysed in the study, derived from the temperature and rainfall values provided by the worldclim website and from the Digital Elevation Model provided by the official website of the Autonomous Region of Sardinia.

Name	Resolution	Description	Source
Elevation	10 meters	Meters above the sea level	http://www.sardegnageoportale.it
BIO5	1 kilometer	Max Temperature of Warmest Month	http://www.worldclim.org
BIO9	1 kilometer	Mean Temperature of Driest Quarter	http://www.worldclim.org
BIO10	1 kilometer	Mean Temperature of Warmest Quarter	http://www.worldclim.org
BIO14	1 kilometer	Precipitation of Driest Month	http://www.worldclim.org
BIO15	1 kilometer	Precipitation Seasonality (coefficient of variation)	http://www.worldclim.org
BIO17	1 kilometer	Precipitation of Driest Quarter	http://www.worldclim.org
BIO18	1 kilometer	Precipitation of Warmest Quarter	http://www.worldclim.org

We tested models' ability to discriminate among presence localities and other localities in the environment predictions *via* Area Under the Curve (AUC) in a Receiver Operating

Characteristic (ROC; Fielding and Bell 1997). AUCs were averaged from 100 bootstrap iterations by randomly resampling 25% of test points with replacement. An AUC value of 1.0 indicates perfect discrimination ability and a value of 0.5 or less indicates a prediction no better than random (Philips *et al.* 2006). According to Fernández *et al.* (2015), MaxEnt is prone to overfitting especially when presence data are scarce and too many environmental variables are included in the model and recommended to evaluate models using different values of the regularization multiplier; otherwise, some authors have shown that default settings perform as well as adjusted settings (*e.g.* Pearson *et al*. 2007, Phillips and Dudík 2008). In our particular case, we already tested *G. lutea* with different settings and datasets in a recent research (Fois *et al*. 2015) and we noticed that, with a previous variable selection excluding correlated variables, default setting resulted the best fitting solution. Although using both extinct and current localities was proved to improve the capacity of prediction models (Fois *et al.* 2015), we selected for this analyses only the current 24 extant ones. Indeed, we cannot exclude that climate change was almost a contributory cause of past extinctions.

Predicting extinctions by SDMs requires choosing a threshold value at which to discriminate presences from absences; maps of projected SDMs were initially converted into integer grids and reclassified as either 0 (unsuitable area) or 1 (suitable area) for ease of comparison using threshold values that allowed a maximum of 5% omission error based on the calibration data (Liu *et al.* 2005). We thus calculated the MCP superimposing the reclassified MaxEnt outputs and considering as extinctions the current occurrences out of future suitable areas. Although other techniques for the calculation of the EOO, such as MaxEnt output and α-hull polygons, were experimented by several authors (*e.g.* Sergio *et al*. 2007, Syfert *et al*. 2014), their results suggested at the same time that the use of convex polygons was the most conservative method to reduce possible overestimations of extinction risks.

5.3 Results

5.3.1 Conservation status assessment based on historical and present distribution data

According to the IUCN classification scheme, three historical and current threats were hierarchized as following: Threat 5.2.1, gathering terrestrial plant as intentional uses, was considered as the principal threat according to historical and current information, while Threats 2.3.1 and 2.3.2, nomadic and small-holders grazing ranching or farming were only marginally affecting the regional conservation status of *G. lutea*. On the other hand, all potential impacts connected with climate change (Threat 11, climate change and severe water) could be one of main extinction cause in next future. Otherwise, locations *sensu* IUCN (2001) were cautiously determined only by the current main threat (Threat 5.2.1), which was not present in some small and difficult to reach areas. For this reason, a number of two locations were accounted.

Considering the comparison of both EOO and AOO values (Fig. 2a and 2b respectively) obtained from confirmed extinct and extant occurrences localities, the reduction of *G. lutea* could be estimated as greater than the 50% (Table 2); thus, following the IUCN sub-criterion A2, this species could be considered as Endangered (EN) in Sardinia. Owing to the number of two identified locations (*sensu* IUCN 2001, 2014), the evaluation of *G. lutea* as EN in Sardinia was also confirmed using the sub-criteria B1 and B2 due to an EOO of 76 km^2 (EOO < 5000 km^2 and the individuation more than one location for EN) and an AOO of 48 km^2 (AOO < 500 km^2 and the individuation more than one location for EN).

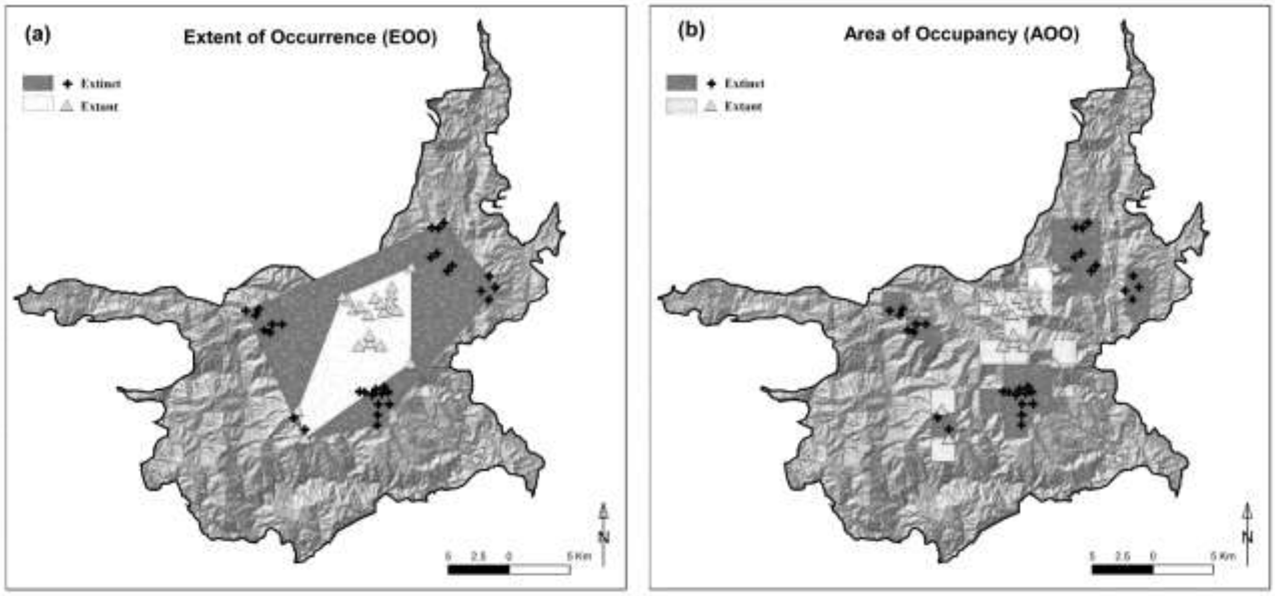

Figure 2. EOO (a) and (b) AOO reductions from past (including extinct localities) and current extant localities. The EOO was the extent of the Minimum Convex hull Polygon (MCP) which encompassed all the known sites of past and current occurrences while the AOO was calculated using a standard grid of 2 × 2 km superimposed onto the occurrences data points of *G. lutea* in the Gennargentu Massif (Sardinia).

Table 2. Extent of Occurrence (EOO) and the Area of Occupancy (AOO) calculated using also localities which are now extinct (Past) and only the current extant localities (Present). Reductions of current EOO and AOO from the past are reported in percentages (%).

	EOO (km^2)	AOO (km^2)
Past	203	144
Present	76	48
Reduction	62%	67%

5.3.2 Conservation status assessment based on future predictions

For all SDMs under the future emission scenarios of IPCC-CMIP5, AUC values were higher than 0.9, indicating good model performances and making them suitable for deriving future projections.

Changes on the potential range of *G. lutea* were detected among the selected scenarios and projections. Indeed, also with the projected scenario to 2050 with the optimistic estimated greenhouse gas emission (RCP = 2.6), three predicted extinctions (localities situated at 1250, 1300 and 1350 m asl; Fig 3a) would cause a reduction of 35% of EOO and 8% of AOO (Table 3); according to the less optimistic emission scenario (RCP = 8.5) for the same time period, a further one extinction (at 1275 m asl; Fig. 3b) would increase the reduction up to 45% and 17% for the EOO and AOO, respectively (Table 3).

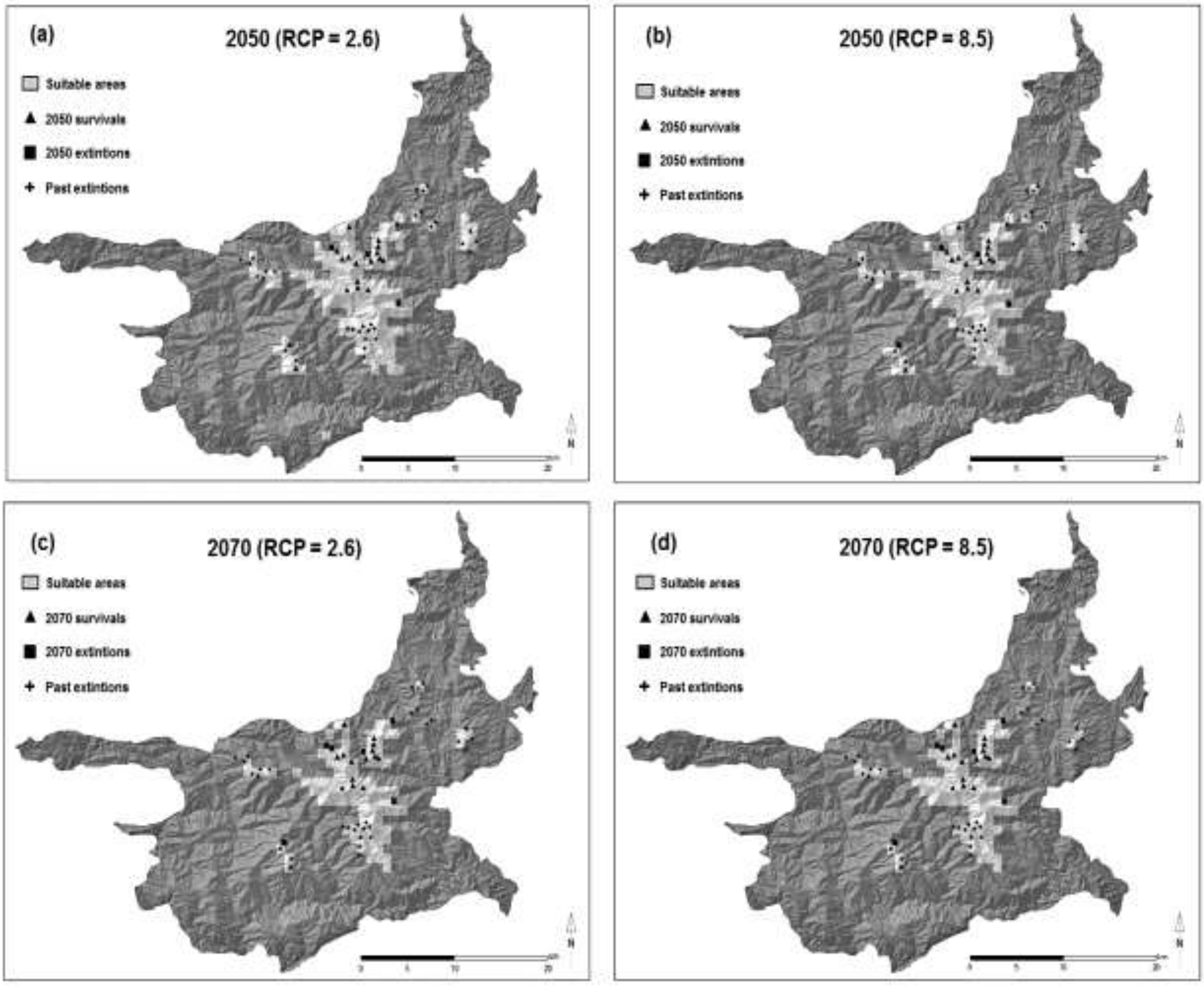

Figure 3. High future suitable areas for *G. lutea* in Sardinia among two projections 2050 (a,b) and 2070 (c,d); and based on two Representative Concentration Pathways emission scenarios, RCP 2.6 (a,c) and 8.5 (b,d).

The number of extinct localities would increase in 2070; even with the most optimistic estimated greenhouse gas emission (RCP = 2.6) additional two extinctions (at 1425 and 1450 m asl; Fig 3c-d) would predict a reduction of 55% and 25% of the EOO and AOO (Table 3). In line with the expectations, all extinctions would occur at the edge of the distribution and elevation gradient; according to it, *G. lutea* seems to tend to reduce its elevational range towards higher altitudes (Table 3). Due to the expected EOO reductions greater than 50% within a time period lower than three generations (RCP = 2.6 and 8.5, projection for 2070), *G. lutea* could be also assessed as EN according to the criterion A3 (IUCN 2001).

Table 3. Current and future trends of *G. lutea* with the number of extinctions (Ext) under four consensus global circulation models based on two Representative Concentration Pathways emission scenarios (RCP 2.6 and 8.5) for two projections (2050 and 2070). The three most influencing variables are reported in order of their percentage of contribution. The Extent of Occurrence (EOO) and the Area of Occupancy (AOO) in square kilometers were calculated taking into account the predicted extinctions; their relative percentage of reduction from the current EOO and AOO are also reported between brackets.

Scenarios	Ext	Altitudinal range m asl	BIO5 (°C)	BIO10 (°C)	EOO (km^2)	AOO(km^2)
2015	-	1250-1775	21.0-24.2	15.3-18.4	75.6	48
2.6 2050	3	1257-1775	24.4-27.2	18.4-21.0	49.0 (35%)	44 (8%)
8.5 2050	4	1400-1775	25.8-28.6	19.5-22.3	41.3 (45%)	40 (17%)
2.6 2070	6	1400-1775	26.4-29.3	20.4-22.9	34.4 (55%)	36 (25%)
8.5 2070	6	1400-1775	27.4-30.2	21.1-23.7	34.4 (55%)	36 (25%)

5.4 Discussion

This research is in line with the EU Member States agreement to monitor and report the conservation status of all species listed in the Habitats Directive; accordingly, this work is in addition to a series of similar conservation activities carried out for other policy species of Sardinia (*e.g.* Fenu *et al.* 2011, Fenu *et al*. 2015a). Considering that the only previous assessment of *G. lutea* in Sardinia date back to 1997 (Conti *et al.* 1997) and the commitment to protecting this species in compliance of the Habitats Directive 92/43/EEC, this work will also contribute to the implementation of knowledge and conservation management of the peripheral and endangered Sardinian localities of *G. lutea*.

Several authors (*e.g.* Akçakaya *et al.* 2006, Guerrina *et al*. 2015) suggested to proceed with caution when such IUCN assessments have to be used for concrete conservation measures.

Accordingly, we assessed the conservation status of *G. lutea* in Sardinia by ‘canonical’ methods implemented by official IUCN guidelines (IUCN 2014) and following as much as possible number of criteria based on the EOO and AOO measures of geographical distribution size. Besides the past and current EOO and AOO declines, our projections predicted a reduction that could permit to assess *G. lutea* as Endangered (EN, *sensu* IUCN 2012) also by climate change. Some of past extinctions could be adducible to an intense human harvesting (Desole 1960, Gentili *et al.* 2013); otherwise, further threats, as the loss of habitat and climate change, should not be excluded *a priori*.

Considering the possible contribution of further factors (*e.g.* competition with established vegetation, stochastic events and human impacts), the 55% of EOO reduction by 2070 could be also greater, especially for such small and isolated populations. As recommended by IUCN (2014), we considered reductions over three generations for assessing a species as EN; in our particular case, we had documented and unpublished certainties that these events were occurred since the 1950s when, due to the expansion of malaria disease, the plant was widely harvested for its therapeutic uses (Desole 1960, Gentili *et al.* 2013). Thus, being *G. lutea* a rhizomatous long-lived plant, we argued that extinctions were surely occurred before the limit of three generations. Furthermore, the plant still currently used and harvested for liquors productions and also other factors, such as the high presence of wild and domestic animals and the habitat loss, are still having a negative influence on the status of this species. Although assessments at too local scale are discouraged by IUCN (2003), the same organization (IUCN 2003, 2014) have recognized the importance of regional assessments for isolated and peripheral populations which are seldom important under ecological and genetic points of view (Case *et al.* 2005, Gentili *et al*. 2015). Because PIPPs are often threatened, but not in decline, their global assessments, based on IUCN criteria, could not accurately reflect their extinction risks (Mace *et al.* 2008, Abeli *et al*. 2009). In confirmation of it, our results highlighted differences among the assessments at global, national and regional level.

Indeed, an increasing risk category for this species was also previously determined from wide to local levels. As far as the Sardinian PIPP is concerned, the decrease of previous category assessment from CR (Conti *et al*. 1997) to EN, due to the new discovered localities during last years (Fois *et al*. 2015), confirmed the necessity of a conservation status revision.

Although the use of SDMs is the most straightforward way to red-list species threatened by climate change, making IUCN classifications under future climates should be considered with caution (Akçakaya *et al.* 2006). Besides the uncertainties of such scenarios, climate is only one of several determinants of future species distribution which also may result from dispersal limitation, competition with established vegetation, effects of past, large-scale events and human pressures (Thuiller *et al*. 2005). Nevertheless, several studies in mid-latitude mountains (*e.g.* Thuiller *et al*. 2005, Guerrina *et al*. 2015, Gentili *et al*. 2015) identified climate change as one of main extinction causes in the next future. In addition, the predictive power of SDMs is usually higher in case of species and/or populations with small geographic ranges and limited environmental tolerances (Elith *et al*. 2006), such as *G. lutea* in Sardinia; accordingly, we also found high values of AUC.

Although high AUC values could be biased by sample sizes, influencing the contrast between presence and pseudo-absences in a determined space (Elith *et al*. 2006, Stokland *et al.* 2011), we argued that our modelling methods and performances for the same study case were enforced and confirmed by a post-hoc validation on field, previously implemented by Fois *et al.* (2015).

In conclusion, we are quite confident to assert that further measures for the conservation of this plant are necessary in order to avoid a continuous decline that started in the past and probably will continue in the future.

5.4.1 Conservation implications

As also confirmed by previous results obtained for other Sardinian *taxa* (see review of Fenu *et al.* 2015b), the propaedeutic practice of an exhaustive regional assessment is seldom necessary for a cost-effective conservation planning which should also include genetic characterisation and *in situ*/*ex situ* activities (Fig. 4).

Besides the mere IUCN assessment, according to past, current and predicted geographical distribution sizes declines, further information could be pointed out by these latter analyses; as expected, all six predicted extinctions were depicted at the edge of its distribution and at lower elevations. Lacking an extensive knowledge about the dispersal ability of this plant, the *ex situ* conservation could represent an effective complementary method to face the potential loss and/or shifting of climatic suitability (Serra-Diaz *et al*. 2014, Fenu *et al.* 2015b). Otherwise, according to our results from SDMs under future climate scenarios, the majority of extinct and currently extant localities would still be suitable even in the future. In these cases, *in situ* activities, such as monitoring, fence protections and translocations should be the priority.

This work confirmed the reliability of IUCN Red List Categories for assessments of species extinction risk; although conservation assessments at a global scale can help to justify and provide a big-picture perspective, since some species may require different protection throughout their distribution, the reliability of regional assessments for that species, such as *G. lutea*, which are differently threatened throughout their distribution area was here confirmed. Also considering that regional analyses should be carried out by local researchers that are supposedly the best experts of their own territory, we argued that such high-resolution practices are in many cases a very effective tool for the conservation management of biodiversity.

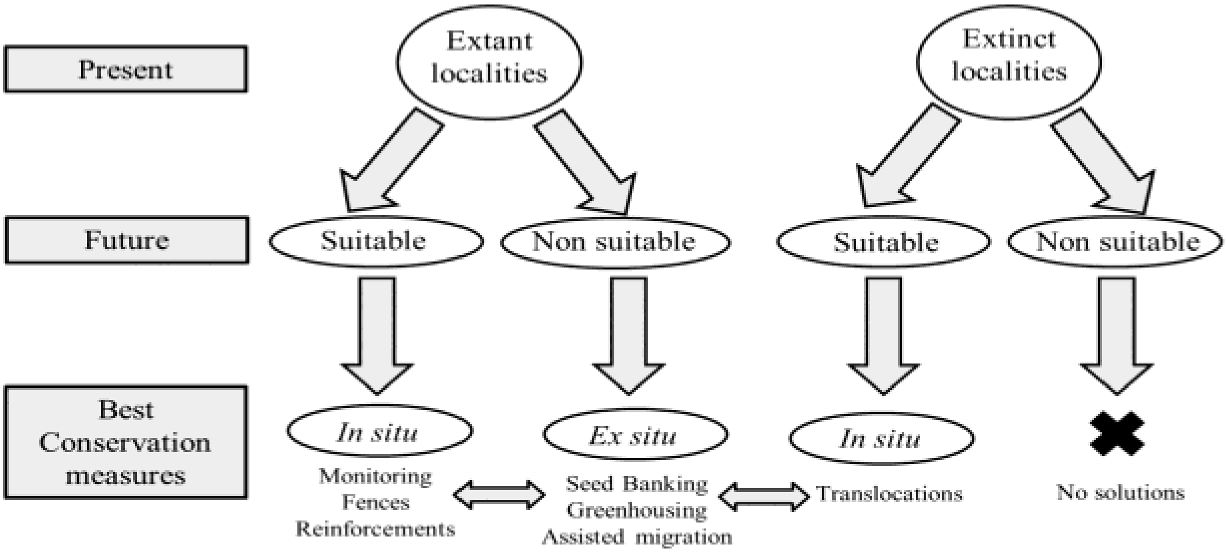

Figure 4. Methodological chart of the conservation measures guided by assessments at regional level. Starting from the current state of the art, future projections highlighted the possible future conservation status of all known extant and extinct localities. Accordingly, best theoretical conservation measures could be suggested for each particular case.

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GENERAL CONCLUSIONS

This thesis, through a multidisciplinary approach, achieved the following general conclusions:

1. The usefulness of Species Distribution Models (SDMs) for biodiversity discovery. This models could be an important tool to limit search efforts by selecting the areas where field surveys are to be carried out **[Chapter 1]**.
2. The choice to evaluate models *a posteriori* through the Observed Positive Predictive Power (OPPP) values. This index allows to give a concrete measure of the utility of SDMs for guiding unknown population discovery, especially when only few points are trained **[Chapter 1]**.
3. The existence of intraspecific seed germination variation among localities. These differences must definitely be taken into account when planning *ex situ* conservation actions to emphasize the importance of collecting and preserving seeds from multiple origins in order to maximize the genetic diversity of seed collections stored in germplasm repositories **[Chapter 2]**.
4. The creation of short-term persistent seed bank permit breaking seed dormancy during winter (cold stratification period). Temperature is a critical environmental factor to control germination, delaying it for the next spring **[Chapter 2]**.
5. The usefulness of the optimal germination protocol. This should be used as an strategy to *ex situ* conservation and multiplication of *G. lutea* **[Chapter 3]**, contributing to the reduction of the root harvest pressure on wild populations through future farmland to commercial uses.
6. The reduction on seed germination related to small locality sizes **[Chapter 3].** This may have negative consequences for short term (new recruitments), medium term (decreasing the chance to colonize unoccupied habitat patches) and for long term population dynamics (compromising the resilience to changing environmental conditions and therefore increasing its extinction risk).
7. The influence of snowmelt time and temperatures on *G. lutea* phenology. The increments of temperatures and climatically unstable conditions apparently cause the unreached reproductive stage. In particular, warm temperatures during the cold period or vernalization (typically autumn and winter) are important as spring or summer temperatures on flowering and reproductive process for this *taxon* **[Chapter 4]**.

8. The importance of phenology as sentinel of climatic change. Considering the importance of this issue and the ease and cost effectiveness of phenological monitoring **[Chapter 4]**, researches in this sense could be a key tool towards the enhancement of biodiversity conservation associated to climate changes consequences.
9. The reliability of regional assessment. This should be a powerful method for conservation management of biodiversity and in many cases a cost-effective practice related to *in situ\ex situ* conservation plannings **[Chapter 5]**.
10. The multidisciplinary approach with *ex situ* conservation and *in situ* activities (such as monitoring, fence protections and translocations) as practical solutions to maximize the persistence of all genetic diversity, according to the specifical threats of each site **[Chapter 5]**.

The usefulness of a given multidisciplinary specific approach arise out of the complexity of plant population processes and effect of numerous environmental variables. The incorporation of field data, ecology, ecophysiology, modelling, future projections and assessment information, permit us provide useful guidelines for management conservation of this threatened *taxon*.

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