

Comparative seed germination traits in alpine and subalpine grasslands: higher elevations are associated with warmer germination temperatures

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Abstract

Seed germination traits in alpine grasslands are poorly understood, despite the sensitivity of these communities to climate change. This article tests the hypothesis that germination traits predict plant species occurrence along the alpine-subalpine elevation gradient. A phylogenetic comparative analysis was performed using fresh seeds from twenty-two plant species typically found in alpine and subalpine grasslands (1600 – 2400 m) of the Cantabrian Mountains, northern Spain (43° N, 5° W). Laboratory experiments were conducted with seeds from each species to characterize germinability, optimum germination temperature and the effect of cold and warm stratification on dormancy breaking. Variability in the estimated traits was reduced by Phylogenetic Principal Component Analysis (phyl.PCA). Phylogenetic Generalised Least Squares regression (PGLS) was used to fit a model in which species average elevation was predicted from the species position in the PCA axes. Most subalpine species germinated in snow-like conditions, whereas most alpine species needed an accumulation of warm temperatures. Phylogenetic signal was low. The first PCA axis ordered germination traits according to overall germinability, whilst the second axis ordered them according to the preference for warm or cold germination treatments. The second axis significantly predicted species occurrence in the alpine-subalpine elevation gradient, as higher elevation species tended to have warmer germination preferences. Our results show that germination traits in high-mountain grasslands are closely linked to the alpine-subalpine gradient. Alpine species, especially those from stripped and wind-edge communities, have a preference for warmer germination niches, suggesting that summer emergence prevents frost damage during seedling establishment. In contrast, alpine snowfield and subalpine grassland plants have cold germination niches, indicating that winter emergence may occur under snow to avoid drought stress.

Keywords: Altitude, functional traits, germination temperature, high-mountain flora, phylogenetic comparative methods, Phylogenetic Generalised Least Squares (PGLS), physiological seed dormancy.

Nomenclature: Castroviejo et al. (1986–2015), except for Compositae and Gramineae, which follow the Euro+Med PlantBase (<http://www.emplantbase.org/home.html>, accessed 21 Aug 2015).

Introduction

Alpine plant communities *sensu* Körner et al. (2011) are those that dwell above the treeline, beyond a worldwide ecological boundary defined by low soil temperature (Körner & Paulsen 2004). Alpine environments subject plants to stress through short growing seasons, frost damage and cold drought (Ellenberg 1988). To cope with this, alpine communities are mainly dominated by short grasses and cushion plants, which stick to the ground to benefit from its benign microclimate (Körner 2003). However, such cold-adapted plants are particularly sensitive to the ongoing global change (Nogués-Bravo et al. 2007). Climate warming reduces suitable species' ranges (Dullinger et al. 2012), forces plants to migrate upwards (Chen et al. 2011) and alters community composition (Jiménez-Alfaro et al. 2014b). These community changes operate partly through early life

stage processes, related to the plant regeneration niche. As the timing of seed germination is controlled by temperature and water availability, it will respond to climate change (Briceño et al. 2015). For example, warming may shift the alpine germination season from spring to autumn (Mondoni et al. 2012) and change the species assemblages emerging from the alpine soil seed bank (Hoyle et al. 2013).

Species-specific responses to climate – and their translation to community patterns – will depend on species traits. However, defining alpine germination traits has proved an elusive quest for plant ecologists. Körner (2003) proposed that, because of the sharply-defined alpine seasons, physiological seed dormancy (Baskin & Baskin 2014) may not be essential to prevent mid-winter germination. Instead, a high temperature requirement for germination would suffice to ensure

summer emergence. However, this interpretation has not been entirely backed by experimental investigations. Pioneer studies from North America reported that alpine species either germinated well without any pre-treatment at warm temperatures, or did not germinate at all, which was interpreted as dormancy (Bliss 1958; Amen 1966; Sayers & Ward 1966; Marchand & Roach 1980). More recently, Schwienbacher et al. (2011) pointed out that deep physiological dormancy and low germination is prevalent in alpine plants, but non-dormant species also exist. This disparity of results led Schwienbacher et al. (2011) to conclude that a common alpine set of germination traits cannot be described, a view shared by Körner (2003). After all, on top of the generally adverse conditions, alpine topography determines a mosaic of microhabitats ranging from protected snowfields to highly-demanding windswept crests (Jiménez-Alfaro et al. 2014c). Nonetheless, efforts to associate different germination traits to specific alpine conditions have had mixed results. In Japan, Shimono & Kudo (2005) did not find different germination strategies between snowfields and fellfields. Conversely, Schwienbacher et al. (2012) reported that moraine pioneers from the Alps germinated at colder germination temperatures than dominants. In the Mediterranean high mountains of central Spain, Giménez-Benavides et al. (2005) related germination traits to biogeography, with Mediterranean endemics being less dormant than arctic-alpine species.

Based on these works, the comprehensive review of Baskin & Baskin (2014) suggested that alpine species are less dormant and have warmer germination temperatures than subalpine species (i.e., species that live close to the potential treeline and in the lower alpine belt). Nonetheless, it also highlighted that there could be a wrong estimation of dormancy status in alpine species, caused by several methodological constraints found in the existing literature. In the earlier studies, cold stratification was generally applied to dried seeds (Bliss 1958; Sayers & Ward 1966; Marchand & Roach 1980), but now it is known that seed imbibition is usually essential to break physiological dormancy (Baskin & Baskin 2014). Other studies germinated seeds only at high summer temperatures (Bliss 1958; Schwienbacher et al. 2011), and thus it cannot be concluded whether their species had conditional dormancy, i.e. if their germination temperature range increased towards colder temperatures after stratification (Baskin & Baskin 2014). Also, studies dealing with dormancy loss were interpreted by comparing non-treated (i.e., recently sown) vs. cold-stratified seeds (i.e., after several weeks of chilling), without exposing seeds to a similar period of warm stratification. Thus, it is not possible to know if these species were dormant and dormancy loss required cold overwintering, or if they just needed a long time to germinate. Finally, several studies on alpine species were performed using dry-stored instead of freshly collected seeds, limiting their ecological interpretation (Baskin & Baskin 2014). For these reasons, Baskin & Baskin (2014) concluded their review stating that alpine dormancy loss and germination require further study.

Surprisingly, very few studies have compared the germination traits of high-mountain species under a single protocol inspired in species occurrences in natural habitats. Phylogenetic comparative analyses of seed germination are relatively rare, maybe because of the necessary statistical treatment of multispecies data (Garamszegi 2014). Here we perform one such analysis, benefitting from recent developments in phylogenetic statistical methods (Revell 2009; Revell 2010). We argue that such an approach can be a useful step towards understanding the functional ecology of alpine plant communities. To add to the growing picture of seed traits in alpine plants, we wanted to assess variation related to one of the main environmental drivers in alpine environments: the elevation gradient. We performed a comparative investigation of seed germination traits using 22 species with optimal distribution at either the alpine or the subalpine belt in a mountain region of northern Spain. Our specific aim was to test the hypothesis that species seed germination traits can predict species occurrence in the alpine-subalpine gradient.

Material and methods

Study system

This study took place in alpine and subalpine plant communities of the Cantabrian Mountains, Spain (43° N, 5° W). Regional climate is temperate oceanic, with a relative Mediterranean influence. In the study sites, precipitation falls as snow from November to May, and soils can remain snow-covered for most of that time when topographic conditions are favourable (Jiménez-Alfaro et al. 2014c). The warmest month is August, with estimated day/night air temperatures (Hijmans et al. 2005) ranging from 21/8 °C at the warmest study site to 17/4 °C at the coldest. The coldest month is February, with air temperatures from 4/-3 °C to 1/-7 °C. Soil temperatures in the alpine belt were recorded from 2009 to 2014 with eight M-Log5W dataloggers (GeoPrecision GmbH, Ettlingen, Germany). August soil temperatures ranged from 20/12 °C to 14/9 °C. In snow-beds, soil temperatures remained close to 0 °C from November to May. In windswept places, where snow rarely accumulates, temperatures dropped to -3/-4 °C in February.

In the study area, the alpine belt is restricted to the higher massifs (above 2000 m) and represents an important outpost of European alpine vegetation, hosting a flora composed of arctic-alpine species, high-mountain Mediterranean plants and regional endemics (Remón Aldabe et al. 2009; Jiménez-Alfaro et al. 2014a). Subalpine communities are widely represented between 1600 m and 1900 m asl. The subalpine belt lacks dominant tree species due to cloudy oceanic summers and centuries of cattle grazing and is covered by a mosaic of scrub and grasslands (Marinas Murillo et al. 2009).

Twenty-two high-mountain species were selected for this study (Table S1), all of them typically found in alpine or subalpine grasslands of the area. Each species was

assigned an average elevation for the Cantabrian Mountains, using a floristic database of species occurrences with at least 20 values per species (Atlas of Priority Cantabrian Flora, Jardín Botánico Atlántico, Xixón, Spain). This value was meant to represent the average point of the elevation gradient to which each species is adapted, which is useful to assess variation among species assuming low intra-specific variation within the alpine-subalpine gradient (in our study, from 1600 to 2300 m). In order to perform phylogenetically-informed statistical tests (see below), a phylogeny of the species (Supporting information 1) was created using Phylomatic v3 (Webb & Donoghue 2005). The genus *Arenaria* was resolved using traditional taxonomy (Castroviejo 1986-2015). Branch lengths were adjusted using the 'bladj' function in Phylocom (Webb et al. 2008) with the Wikström node ages (Wikström et al. 2001).

Seed germination traits

Seeds of each study species were collected in 2012-13 between 1600 and 2300 m asl. For each species, one population was sampled at its time of dispersal (Table S1). Seed sampling regularly covered the entire area of occupancy of the studied populations, and included all spotted individuals bearing ripe seeds (i.e., fully matured dispersal units that could be easily detached from the mother plant). After collection, dispersal units spent three weeks in the laboratory (21 °C, 50 % RH) to ensure homogeneous maturation; subsequently they were cleaned and germination experiments began.

Laboratory germination experiments (Figure S1) were conducted inside incubators (Grow-S 360, Ing. Climas, Barcelona, Spain). Seeds were sown on Petri dishes holding 1 % distilled water agar, sealed with Parafilm to prevent desiccation. Four Petri dishes with 25 seeds each were used per experimental treatment. For each species, 24 dishes were prepared. To assess seed dormancy at dispersal and dormancy loss without overwintering, half of the dishes were subjected to a move-along experiment with three steps: (1) Four weeks at one of three germination temperature regimes (see below); (2) eight weeks of warm stratification at a common temperature of 25 °C; and (3) four additional weeks at the same temperatures as used in step 1. Simultaneously, to assess dormancy loss by overwintering, the other half of the dishes were subjected to cold stratification at 3 °C for twelve weeks; and then to four weeks at one of three germination temperature regimes (see below). Warm and cold stratification were performed in darkness. Germination temperature regimes were three daily alternating treatments: 14/4 °C (representing spring/cold summer); 22/12 °C (warm summer); and 30/20 °C (unusual heat in nature, which may promote germination in some species). These germination temperatures were coupled to a 12 h-light/12 h-darkness photoperiod (c. 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the light phase, provided by six Philips TLD30W/54-765 cool fluorescent tubes). It must be stressed that both the germination temperature at 30/20 °C and the warm stratification at 25 °C are

markedly outside the field temperature range. These overly hot temperatures were selected to elicit a potential physiological response, and to assess dormancy loss and germination in the absence of temperatures that occur in the field.

During incubation, germination was scored weekly as radicle emergence, and seedlings were removed. After the end of the experiments, non-germinated seeds were cut open and classified as apparently viable, empty and fungus infected. Germination percentages and further statistical analyses were calculated only with potentially germinable seeds (germinated + apparently viable, Supporting information 2). Some treatments that lacked variance (i.e., germination = 0 or 100 %) had to be transformed (to germination = 1 or 99 %) so they could be included in the Generalised Linear Models (see below), but the correct values are shown in the figures. In several species, seeds germinated in darkness during the warm or cold stratifications; these germinations were added to the final counting. In *Carex asturica*, germination was zero in all pre-treatments and temperatures. The germination experiment was repeated with *Carex asturica* seeds from another population, but germination was still zero. Assuming that no germination in the experimental conditions was a species trait (i.e., deep dormancy), rather than an artefact (i.e., low quality seed collection), *Carex asturica* was kept for further analyses.

All statistical analyses were performed in R (R Core Team 2014). First, germination data was analysed separately for each species using Generalised Linear Models (GLM) with binomial error distribution and logit link function (Supporting information 3), in order to identify significant effects of germination temperature (14/4 °C, 22/12 °C and 30/20 °C) and pre-treatment. Three pre-treatments were considered: fresh (germination in the move-along experiment after the first step); warm-stratified (total germination in the move-along experiment after the third step); and cold-stratified (germination in the cold stratification experiment). It must be remembered however that seeds in the fresh and warm-stratified pre-treatments were the same. For this reason, two models were fitted separately for each species: (1) fresh vs. cold-stratified seeds, to check dormancy at dispersal; and (2) warm-stratified vs. cold-stratified seeds, to assess the necessity for cold overwintering in order to break dormancy. In each case, a full factorial model was fitted first, including germination temperature, pre-treatment and their interaction. Subsequently, the model was simplified by stepwise deletion of non-informative model parameters, until a minimal adequate model for each case was selected. These models were used to estimate the mean germination proportion and 95 % binomial confidence interval of each significantly different treatment. The estimated germination proportions were used to construct a matrix of germination traits per species, in which every species was assigned ten germination traits (estimated mean germination at each of the nine pre-

treatment x germination temperature combinations + dark germination during cold stratification, Supporting information 4).

Phylogenetic comparative analysis

A phylogenetic comparative analysis was performed to test the hypothesis that germination traits could predict species occurrence in the alpine-subalpine gradient (Supporting information 5). The variability in the germination traits matrix was reduced using Phylogenetic Principal Component Analysis (phyl.PCA) (Revell 2009), computed with the covariance matrix and the lambda method using the package 'phytools' (Revell 2012). Then, the position of each species in the main PCA axes was used to predict the average elevation of each species, by fitting a Phylogenetic Generalised Least Squares regression (PGLS) (Grafen 1989) with the package 'nlme' (Pinheiro et al. 2015). It must be noted, however, that the average elevation and the collection elevation differed in some species (Table S1). Therefore, PGLS analysis was repeated twice, first with the average elevation and then with the collection elevation, to confirm the validity of any detected relationship. Both phyl.PCA and PGLS were calculated using the approach proposed by Revell (2010), in which phylogenetic signal in the residual error and analysis parameters are estimated simultaneously. For a review on the correctness of this approach (instead of calculating first the phylogenetic signal, and only use a phylogenetic-explicit analysis in the event that the signal is significant) see Garamszegi (2014). A multivariate regression with all relevant PCA axes was fitted first. Subsequently, non-informative axes were eliminated to arrive to a minimal adequate model.

Results

Germination during cold stratification

In most species (17 out of 22), some seeds germinated during cold stratification, i.e. in darkness at 3 °C (Fig. 1). These germinated seeds were added to subsequent germination during light incubation to produce the final results of the cold-stratified treatment. Otherwise, in some species it would artificially seem that the germination of cold-stratified seeds was less than the germination of fresh or warm-stratified seeds. However, this means that responses to germination temperature after cold stratification have to be interpreted with caution. This is especially relevant in those species with high germination during stratification. For example, all *Teesdaliopsis conferta* seeds germinated during cold stratification. Thus, the 100 % germination shown for cold-stratified seeds at all germination temperatures (Fig. 2) does not actually mean that cold-stratified seeds could germinate at 30/20 °C; these seeds germinated during, not after, stratification.

Fresh vs. cold-stratified models

The fresh vs. cold-stratified models (Figure 2a, Table S2) compared the germination of recently collected seeds with seeds after 12 weeks of simulated overwintering. In

the majority of species, germination was significantly different between fresh and cold-stratified seeds. Cold stratification significantly improved germination in 13 out of 22 species: *Arenaria grandiflora*, *A. purpurascens*, *Carex sempervirens*, *Festuca eskia*, *Fritillaria legionensis*, *Helictochloa marginata*, *Kobresia myosuroides*, *Lychnis alpina*, *Patzkea paniculata*, *Rumex suffruticosus*, *Silene acaulis*, *Silene ciliata* and *Teesdaliopsis conferta*. Cold stratification also interacted positively with temperature in some species, indicating an increase of the germination temperature range: towards 14/4 °C in *Carex sempervirens* and *Lychnis alpina*, and towards 30/20 °C in *Arenaria erinacea*, *A. grandiflora*, *Helictochloa marginata*, *Jurinea humilis* (only marginally), *Polygonum viviparum* and *Teesdaliopsis conferta*. Cold stratification had the opposite effect, decreasing germination, in five out of 22 species: *Androsace villosa* (only marginally), *Arenaria erinacea*, *Jasione cavanillesii*, *Jurinea humilis* and *Polygonum viviparum*. In some cases, it also had a negative interaction with temperature, decreasing the germination temperature range towards 14/4 °C in *Arenaria erinacea*, *A. grandiflora* and *Jurinea humilis*. Finally, cold stratification did not have a significant effect in the germination of four out of 22 species: *Armeria cantabrica*, *Carex asturica*, *Saxifraga oppositifolia* and *Scorzoneroides cantabrica*.

In the fresh vs. cold-stratified models, germination temperature did not have an effect in nine out of 22 species, indicating that seeds germinated the same at the three tested temperatures: *Androsace villosa*, *Carex asturica*, *Festuca eskia*, *Fritillaria legionensis*, *Jasione cavanillesii*, *Patzkea paniculata*, *Rumex suffruticosus*, *Saxifraga oppositifolia* and *Silene ciliata*. In the rest of the species, germination was significantly higher in some part of the germination temperature range: at 14/4 °C in *Arenaria grandiflora* and *Armeria cantabrica*; at 14/4 °C and 22/12 °C in *Helictochloa marginata* and *Teesdaliopsis conferta*; at 22/12 °C in *Arenaria erinacea*, *A. purpurascens*, *Jurinea humilis* and *Polygonum viviparum*; at 22/12 °C and 30/20 °C in *Kobresia myosuroides*, *Lychnis alpina*, *Scorzoneroides cantabrica* and *Silene acaulis*; and at 30/20 °C in *Carex sempervirens*.

Warm vs. cold-stratified models

The warm vs. cold-stratified models (Figure 2b, Table S3) compared the germination of seeds after 12 weeks of simulated overwintering with seeds that had underwent the same period of incubation at warm temperatures. The two types of stratification produced significantly different results in the majority of species. Warm stratification had a significantly better effect in 11 out of 22 species: *Androsace villosa*, *Arenaria erinacea*, *Armeria cantabrica*, *Carex sempervirens*, *Helictochloa marginata*, *Jasione cavanillesii*, *Jurinea humilis*, *Polygonum viviparum*, *Scorzoneroides cantabrica*, *Silene acaulis* and *Saxifraga oppositifolia*. Conversely, cold stratification produced significant increases in germination in eight out of 22 species: *Arenaria*

grandiflora, *A. purpurascens*, *Festuca eskia*, *Fritillaria legionensis*, *Kobresia myosuroides*, *Patzkea paniculata*, *Rumex suffruticosus* and *Teesdaliopsis conferta*. Cold stratification also interacted with germination temperature in several cases. On the one hand, it increased the germination temperature range towards 14/4 °C in *Armeria cantabrica*, and towards 30/20 °C in *Arenaria erinacea*, *A. grandiflora*, *Helictochloa marginata* and *Polygonum viviparum*. On the other hand, it decreased the germination temperature range towards 14/4 °C in *Arenaria erinacea*, *A. grandiflora*, *Kobresia myosuroides*, *Scorzoneroides cantabrica*, *Jurinea humilis* and *Silene acaulis*; and towards 30/20 °C in *Jasione cavanillesii* and *Jurinea humilis*. The effects of warm and cold stratification were not significantly different in only three out of 22 species: *Carex asturica*, *Lychnis alpina* and *Silene ciliata*.

In the warm vs. cold-stratified models, germination temperature had in general the same effects as in the fresh vs. cold-stratified models, with the following exceptions. No effect of temperature was found for *Scorzoneroides cantabrica* and *Silene ciliata*, which had the same germination at the three temperatures. The best germination temperature shifted to 14/4 °C after warm stratification in *Kobresia myosuroides*. Highest germination occurred now at 14/4 °C in *Patzkea paniculata*; at 14/4 °C and 22/12 °C in *Arenaria purpurascens*, *Jurinea humilis* and *Polygonum viviparum*; at 22/12 °C and 30/20 °C in *Carex sempervirens*; and at 30/20 °C in *Jasione cavanillesii* and *Silene ciliata*.

Phyl.PCA ordination of germination traits

Phylogenetic signal in the phyl.PCA was low (Pagel's $\lambda < 0.001$), indicating a low phylogenetic constraint in the relationships between traits. The first two axes (Fig. 3) accounted for 78 % of the total variance in germination traits. Axis one (56 % of variance explained) correlated negatively (Pearson's $r > -0.48$) with all germination traits. This axis corresponded with overall germinability (or germination 'size'), separating those species with very poor results (e.g. *Carex asturica*) from those that germinated readily across treatments (e.g. *Teesdaliopsis conferta*) (Fig. 1). Axis two (22 % of variance explained) correlated negatively with warm treatments; especially germination at 30/20 °C of fresh (Pearson's $r > -0.61$) and warm-stratified seeds (Pearson's $r > -0.56$). At the same time, axis two correlated positively with cold treatments; especially germination at 3 °C during cold stratification (Pearson's $r > 0.85$), and at 14/4 °C after cold stratification (Pearson's $r > 0.70$). Thus, axis two separated species that responded better to colder treatments (e.g. *Fritillaria legionensis*) from those that responded to warmer germination treatments (e.g. *Carex sempervirens*) (i.e., germination 'shape').

PGLS regression of germination traits vs. elevation

PGLS was fitted first using the first two phyl.PCA axes, but axis one was not significant and was dropped. The final model significantly predicted average elevation

from the values of axis two ($b = -155.813 \pm 49.103$, $t(20) = -3.173$, $p = 0.005$, Pagel's $\lambda = -0.012$). Species with lower axis two scores, representing a warmer seed germination niche, had higher average elevations (Fig. 4). When the analysis was repeated using the elevation values of the collection sites, instead of the species average elevations, the results were similar (PC1 not significant; for PC2 $b = -180.648 \pm 84.701$, $t(20) = -2.133$, $p = 0.046$, $\lambda = 0.063$). The phylogenetic signal (Pagel's λ) associated with the regression residual errors was close to zero in both cases, again indicating a weak phylogenetic constraint in the relationship between the germination traits and altitude. In the case of low phylogenetic signal, PGLS performs equally to an ordinary least squares regression (OLS), and it is not necessary or desirable to carry out both test and compare their results (Garamszegi 2014) (However, OLS can be easily calculated using the raw data and the R script, Supplementary information 4 and 5).

Discussion

This study demonstrates how the seed germination traits of high-mountain species differ between elevation belts. Overall, we found that alpine species generally show a preference for warm germination niches, whilst subalpine species germinate better at cold temperatures. These results confirm the trend suggested by Baskin & Baskin (2014), who recorded a higher average germination temperature in alpine (18 °C) vs. subalpine (13.4 °C) species. This is in line with the preference for warm germination temperatures suggested in the early works on alpine and arctic species (Bliss 1958; Amen 1966; Sayers & Ward 1966; Marchand & Roach 1980) and in the recent work of Walder & Erschbamer (2015). Moreover, our results indicate that dormancy loss by cold overwintering may be less relevant than generally expected in high mountains, a statement previously suggested in the review of Körner (2003) but neglected in the recent literature. It must be stressed that, in most of the species in which cold-stratified seeds germinated better than both fresh and warm-stratified seeds, most of the germination occurred during and not after stratification. Therefore, the significant improvement of their germination in the cold-stratified treatment might be an artefact; i.e. these species could have very low optimum germination temperatures (c. 3 °C), rather than a need for cold stratification. To our knowledge, this is the first time that cold stratification has been compared to a similar period of warm stratification in alpine species, providing empirical evidence for the role of temperature in dormancy loss in alpine communities. However, we must stress that our cold stratification period (3 months) was shorter than the snow cover expected in the field. Therefore, it could be possible that longer stratification was needed to fully break dormancy, especially in those species that showed little germination (e.g., *Saxifraga oppositifolia*).

It must also be taken into account that a long period of warm stratification at 25 °C (or germination at 30/20 °C) are clearly unrealistic conditions in the study area.

Therefore, the response to these treatments does not imply that seeds naturally germinate when soil temperatures stay at 30/20 °C for several weeks, which is unlikely to occur in the study habitat. Rather, it suggests that these species have a relatively high base temperature for germination, and that germination requires the accumulation of degrees-day above this base temperature (Pritchard et al. 1999). Under the average low temperatures and the short summers of the study area, this accumulation would probably take several years. Nevertheless, it must be taken into account that at the micro-scale, alpine root and surface temperatures can become much higher than expected, depending on local topography and cloud cover (Scherrer & Körner 2010). A better understanding of how this warm strategy controls the germination timing will require detailed studies of germination thermal time coupled with field emergence (Porceddu et al. 2013).

This warm and slow germination strategy, requiring a long accumulated exposure to warm days, seems to characterise indicator species of alpine exposed sites. This is especially relevant in low-germinating species of stripped communities (*Androsace villosa*, *Jasione cavanillesii*, *Saxifraga oppositifolia* and *Silene ciliata*), which occur in rocky and windswept spots subjected to cryoturbation and relatively short periods of snow cover (Remón Aldabe et al. 2009; Jiménez-Alfaro et al. 2014a). Also, but to a lesser extent, in species of wind-edge swards (*Kobresia myosuroides*). In these habitats, plants are subjected to extreme conditions of temperature fluctuation, as corroborated by soil temperature loggers (see materials and methods), and widely known to occur in exposed alpine communities (Ellenberg 1988). In these conditions, cold stress seems to be the dominant constraint on seed germination traits, forcing seeds to germinate only in warm mid-summer temperatures. Indeed, very low germination of *Saxifraga oppositifolia* from windswept spots has been reported in the Alps (Schwienbacher & Erschbamer 2002; Schwienbacher et al. 2010; Schwienbacher et al. 2011), and *Silene ciliata* needed long periods of stratification to promote germination in central Spain (García-Fernández et al. 2015). In the mountains of Asia Minor, where cryoturbation processes are common, Arslan et al. (2011) found *Androsace villosa* to germinate at warm temperatures but only in darkness, and proposed that this strategy would restrict germination to crevices that accumulate water and offer protection from frost in the absence of snow cover. In contrast, more generalist species of the alpine belt (*Carex sempervirens*, *Lychnis alpina* and *Silene acaulis*), with higher frequencies across different topographies (Jiménez-Alfaro et al. 2014a), also have a preference for warm temperatures, but are relatively less dormant and can germinate better in cold treatments.

Cold seed germination is preferred by regional indicator species of acidophilous subalpine grasslands (*Festuca eskia*, *Fritillaria legionensis*, *Helictochloa marginata*, *Patzkea paniculata* and *Teesdaliopsis conferta*)

(Castroviejo 1986-2015). This agrees with field observations from the Swiss Prealps, where *Patzkea paniculata* starts to germinate as soon as February (Kupferschmid et al. 2000). These species have optimal germination conditions in darkness and at close to zero temperatures, and so seedling establishment likely takes place under the protective cover of snow. Another subalpine species, *Scorzoneroides cantabrica*, can also germinate at cold temperatures without pre-treatment, although its optimal germination is at warmer treatments. These subalpine communities occur in sites with early snowmelt subjected to a significant summer drought (Marinas Murillo et al. 2009). Therefore, water stress seems to be the main constraint for seedling establishment here, favouring seeds to germinate under snow or during snowmelt. Germination under snow, or in stratification conditions that simulate snow cover, has been reported both in field and laboratory in different mountain regions (Giménez-Benavides et al. 2005; Shimono & Kudo 2005; Baskin & Baskin 2014). This supports that this is a regeneration strategy generalizable across mountain regions. Nevertheless, other species of subalpine sites have very limited germination. *Rumex suffruticosus* has low germination percentages, which may be advantageous in the disturbed screes it occupies. *Carex asturica* did not germinate in any treatment, and may need a special signal that we were unable to provide in this study.

Despite the general pattern of warmer germination temperatures in alpine rather than subalpine species, there were exceptions. Low dormancy and a strong response to germination conditions that simulate snow cover is shown by indicator species of snowfields (*Armeria cantabrica* and *Polygonum viviparum*) (Remón Aldabe et al. 2009; Jiménez-Alfaro et al. 2014a). In these communities, snow cover is guaranteed to last and protect plants from frost and drought, but at the same time shortens the growing season (Ellenberg 1988). Therefore, germinating under snow becomes less risky and is favoured by fecundity selection, as it allows plants to make use of the growing season from the very beginning (Donohue et al. 2010). More strikingly, a capacity to germinate in the cold temperatures of autumn, snow cover or snowmelt is also shown by some species of alpine stripped communities (*Arenaria erinacea*, *A. grandiflora*, *A. purpurascens* and *Jurinea humilis*), where a more conservative frost-avoiding regeneration strategy could be expected. However, these species are strongly related to the Mediterranean mountains of the Iberian Peninsula (Castroviejo 1986-2015), and their cold germination is in agreement with the general behaviour of Mediterranean species (Cochrane et al. 2011).

These exceptions suggest that the elevation gradient is just one of several environmental drivers, and more detailed studies are needed to paint a complete picture of alpine plant regeneration by seed. It would be especially informative to analyse the relative importance of cold germination as a response to drought in alpine areas without Mediterranean influence, as well as in purely

Mediterranean mountains. At the same time, a more detailed plot-based study could assess both micro-topographical differences (Shimono & Kudo 2005) and differences between dominant and pioneer species (Schwienbacher et al. 2012). Finally, it will be necessary to take into account intraspecific variability, given that different populations may show different germination behaviours (Cochrane et al. 2015). Although we only tested one population per species, our results were the same when using the average elevation or the collection elevation values, suggesting that germination temperature preferences are stable species traits within the study area, at least when analysing a wide range of germination treatments as we did. Potentially, intraspecific clinal variation in germination traits along the elevation gradient would obscure the relationship between species average elevation and germination traits, but we were nonetheless able to detect such relationship.

Summarising our findings, it seems that two contrasting functional types, warm and cold germinators, can be identified within the high-mountain communities that occur in a same mountain region. We propose that these types are associated to the main environmental filters for seedling establishment, frost or water stress, and that they can be partly related to the alpine-subalpine elevation gradient. Contrary to the study of Hoyle et al. (2013), our conclusions point to an increase in earlier emergence of alpine species in a climate warming context, in line with Mondoni et al. (2012). This increase could be deleterious if it exposes seedlings to otherwise unfavourable emergence environments, although recent investigations suggest that a longer establishment season would be beneficial (Mondoni et al. 2015). However, serious disruptions of emergence patterns can contribute to the on-going decline of high-mountain specialists (Gottfried et al. 2012; Pauli et al. 2012; Jiménez-Alfaro et al. 2014b), supporting the idea that seed germination traits play an essential role to understand the ecology of alpine plant communities and their response to climate change.

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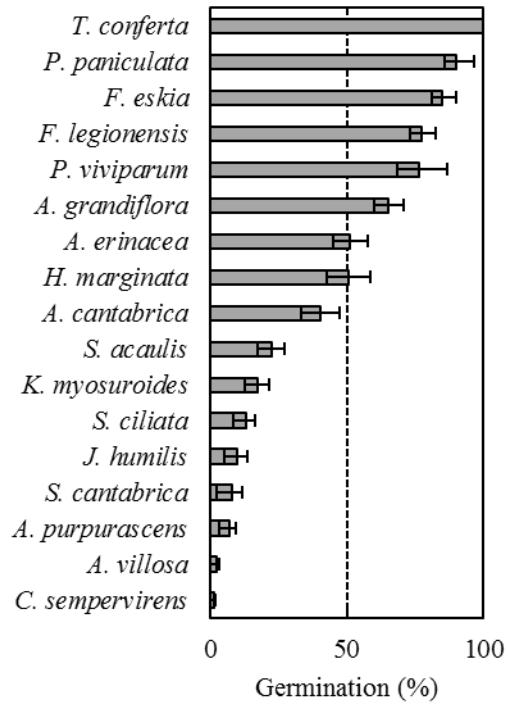


Figure 1 Germination during cold stratification. Final germination percentages after 12 weeks at 3 °C in darkness. Bars represent means proportions, brackets are the 95% binomial confidence interval. Only species with at least one germinated seed are shown.

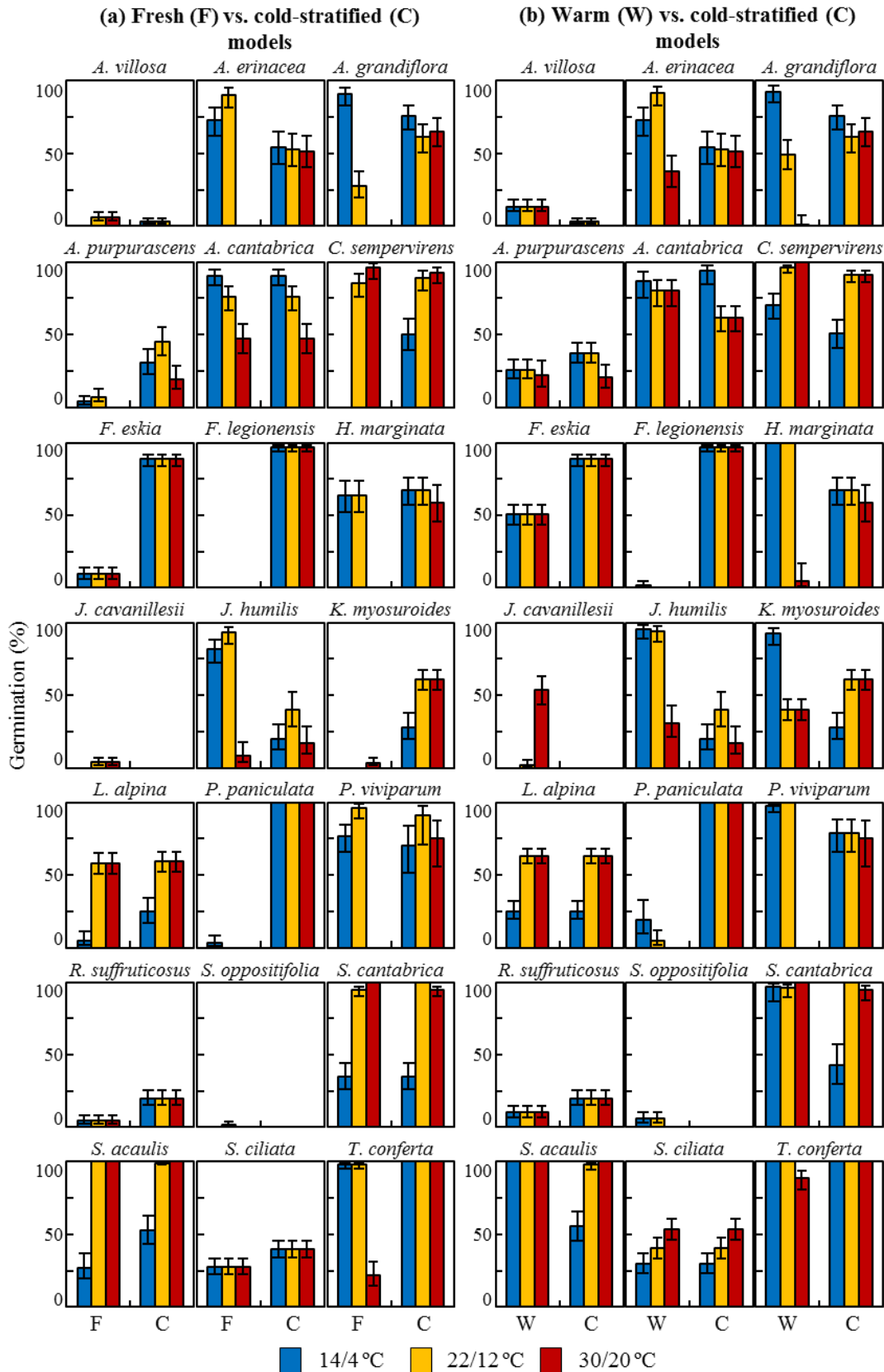


Figure 2 Germination traits per species. Results were estimated by GLM (binomial distribution, logit link). Two separated models were fitted for each species: Fresh vs. cold-stratified seeds (Fig. 2a) and warm vs. cold-stratified seeds (Fig. 2b). Bars represent mean proportions across treatments that were not significantly different, brackets the 95 % binomial confidence interval. Results are grouped per pre-treatment (F = fresh; C = cold-stratified; W = warm-stratified); and within pre-treatment per germination temperature.

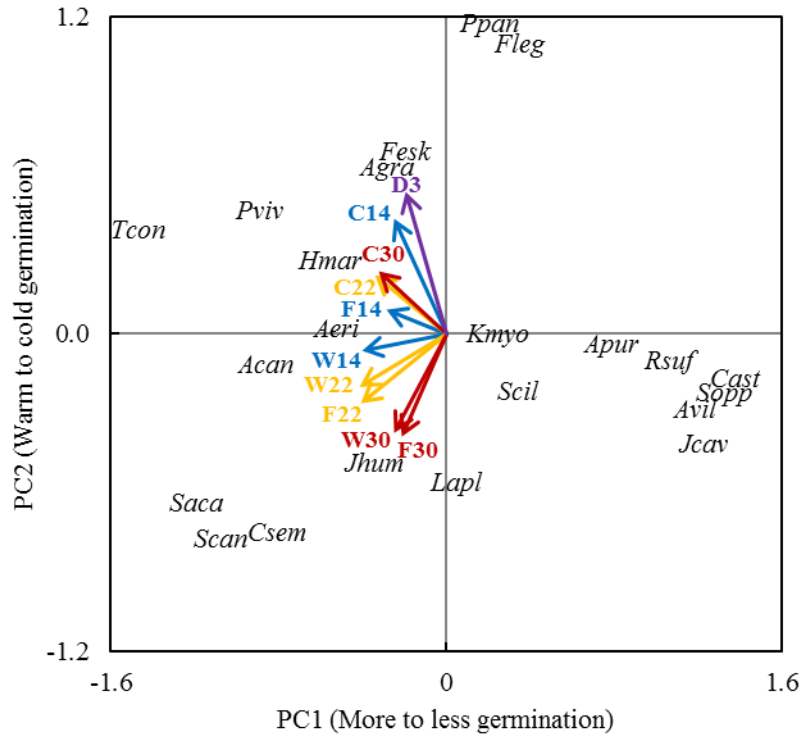


Figure 3 Phylogenetic PCA of the variation in germination traits. For each species, germination behaviour was characterized by ten traits. Vectors represent the contribution of each germination trait to the axes. Traits are coded with letters and numbers: letters represent the 3 pre-treatments (F = fresh seeds; C = cold-stratified; W = warm-stratified); numbers represent the 3 germination temperatures (14 = 14/4 °C, 22 = 22/12 °C, 30 = 30/20 °C); D3 represents germination in darkness during cold stratification at 3 °C.

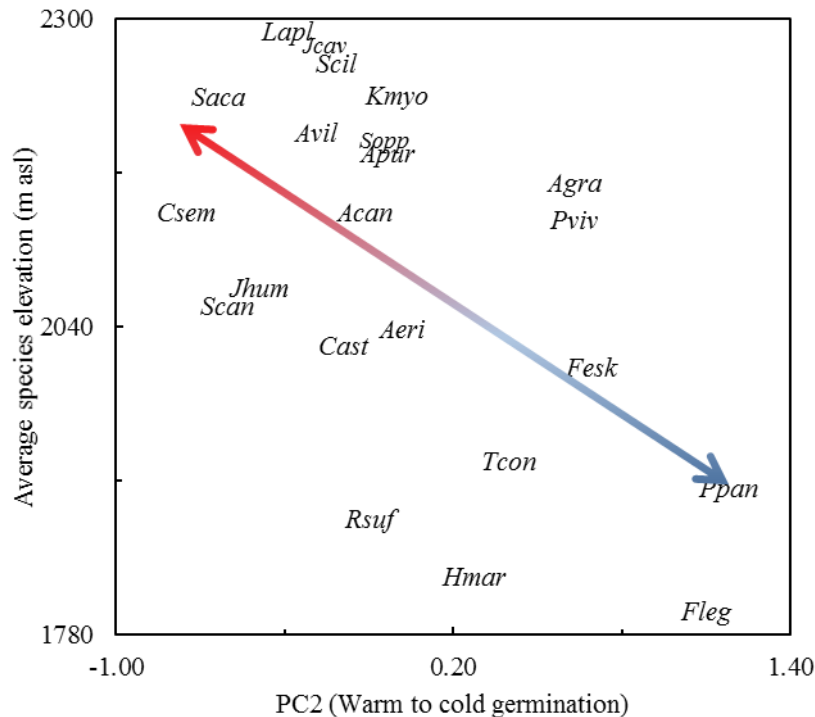


Figure 4 PGLS regression of germination traits vs. the average elevation of each species. Axis x is the position of each species in the second PCA axis (Fig. 3), which ordered species according to their response to a warm or cold germination niche. The arrow-headed line is the fitted PGLS regression line