Evolutionary footprints of cold adaptation in arctic-alpine Cochlearia (Brassicaceae) – evidence from freezing experiments and electrolyte leakage

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Abstract

As global warming progresses, plants may be forced to adapt to drastically changing environmental conditions. Arctic-alpine plants have been among the first to experience the effects of climate change, as regions at high latitudes and elevations are over-proportionally affected by rising temperatures. As a result, cold acclimation and freezing tolerance may become increasingly crucial for the survival of many plants as winter warming events and earlier snowmelt will cause increased exposure to occasional frost. Studying the evolution of cold adaptation allows us to make assumptions about the future responses of different species to climate change. The tribe Cochlearieae from the mustard family (Brassicaceae) offers an instructive system for studying cold adaptation in evolutionary terms, as the two sister genera Ionopsidium and Cochlearia are distributed among different ecological habitats throughout the European continent and the far north into circumarctic regions. By applying an electrolyte leakage assay to leaves, the freezing tolerance of different Ionopsidium and Cochlearia species was assessed by experimentally estimating lethal freezing temperature values (LT50 and LT100), thereby allowing for a comparison of different accessions in their responses to cold. We hypothesized that, owing to varying selection pressures, geographically distant species would differ in freezing tolerance. Despite Ionopsidium being adapted to hot and dry Mediterranean conditions and Cochlearia species preferring cold habitats, all accessions exhibited similar cold responses. Whether this phenomenon has resulted from an evolutionary adaptation of a common ancestor of the two taxa or has evolved from parallel evolution is yet to be investigated. The results presented in this study may, however, indicate that adaptations to different stressors, such as salinity and drought, may confer an additional tolerance to cold; this is because all these stressors induce osmotic challenges, as demonstrated via metabolomic analysis.

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Evolutionary footprints of cold adaptation in arctic-alpine *Cochlearia* (Brassicaceae) – evidence from freezing experiments and electrolyte leakage

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ABSTRACT

As global warming progresses, plants may be forced to adapt to drastically changing environmental conditions. Arctic-alpine plants have been among the first to experience the effects of climate change, as regions

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KEYWORDS

Brassicaceae, climate change, *Cochlearia* , cold adaptation, electrolyte leakage assay, *Ionopsidium*

1 INTRODUCTION

With the recently published 6th report of the Intergovernmental Panel on Climate Change, IPCC (IPCC 2022; https://www.ipcc.ch/report/ar6/wg2/), we have again been warned that ongoing global warming will expose plants and animals to conditions exceeding the limits of their historical and eventual evolutionary experience. Species inhabiting polar and alpine regions that are already being influenced by a warming climate will face challenges induced by these changes, including population decline (Descamps et al., 2017), habitat loss, and even extinction. These challenges surpass the limits of adaptation, even for less vulnerable species and systems (e.g. Román-Palacios & Wiens, 2020). Studying the potential of plant species to adapt to differing and rapidly changing conditions is now more relevant than ever. Habitats at the coldest margins of the Earth, such as Arctic and alpine ecosystems, are considered most affected by rapidly proceeding global warming (Ernakovich et al., 2014); this is especially true for arctic-alpine species at the limit of their distribution range (Lescia et al., 2004). Even though temperatures are rising on a global scale, winter at high latitudes and elevations is over-proportionally affected by climate change, as average winter temperatures are increasing more rapidly in Arctic and alpine habitats (ACIA, 2005; Kreyling, 2010). Winter warming has led to a decline in the amount and duration of snow cover, which is crucial to the survival of subnivean species in several regions. During the last 30 years, Arctic snow cover has already declined by 10%, and model projections expect an additional decrease of 10-20% until the end of this century (ACIA, 2005; Kreyling, 2010). In Europe, a 40-80% decline in days with snow coverage is predicted (Kreyling, 2010). The rate of snow cover reduction is among the most dramatic signs of climate change, and its impacts are severe. Snow cover is one of the most important factors affecting the survival of arctic and alpine plants. Its availability, depth, and duration determine environmental variables such as soil moisture, temperature, and freezing depth (Wipf & Rixen, 2010). Snow has an insulating effect because it contains a large amount of air, which protects species living underneath from wind and extreme winter temperatures (Pomeroy & Brun, 2001). This dynamic creates a relatively mild microclimate with temperatures only slightly below 0°C, whereas ambient temperatures may drop as low as -45°C, thereby ensuring the survival of many plant species (Bokhorst et al., 2009; Armstrong et al., 2015). Earlier snowmelt and decreased snow depth caused by a reduction in snowfall and mid-winter thawing events followed by cold periods are consequences of global warming that can threaten the survival of arctic and alpine plants. Plants may be exposed to considerably lower temperatures, cold winds, and temperature fluctuations, which can cause substantial frost damage and may lead to plant die-offs (Sonesson & Callaghan, 1991; Bokhorst et al., 2009). Winter warming has led to

a lengthening of the vegetative growing season in the Northern Hemisphere, especially at higher latitudes (Parmesan, 2006). As plants often react to warming with increased productivity and less investment in protection, frost events after a period of warming can be fatal in many cases because they occur more frequently. Considering this dynamic, plant species inhabiting areas experiencing changes in snow cover may paradoxically require an increased tolerance to freezing temperatures, as these changes counteract the overall effect of global warming (Kreyling, 2010). As climatic conditions change drastically, plant populations may no longer be best adapted to ambient conditions (Franks et al., 2014). Reactions of plant species to changes in ecological conditions include strategies such as migration and adaptation through evolutionary changes or developing ample phenotypic plasticity (Aitken et al., 2008; Williams et al., 2008; Franks et al., 2014). Migration allows for the exploration of more suitable conditions. This is, however, a limited option, as migration attempts may not keep pace with rapidly progressing changes, and such explorations may require the movement of entire ecosystems (Loarie et al., 2009). The expected response of northern plant species to rising temperatures is relocation (ACIA, 2005). With improvements in livability for warm-loving plant species in arctic regions, poleward migrations are expected, leaving arctic species at risk of displacement; this risk is especially pressing for species whose northward migration is hindered by the Arctic Ocean. Similarly, a documented upward shift in the distribution of montane species has been caused by the inability of species to adapt to local conditions at their current elevation. In addition, successful colonization increases with longer growing seasons and higher temperatures (Walther et al., 2005; Parolo et al., 2008). These shifts in distribution patterns have led to increased species richness in both Arctic and high alpine regions, which may lead to increased competition for species originally inhabiting these areas (Loarie et al., 2009). If species currently adapted to the coldest regions and highest mountain summits, where no space for further migration is left, fail to adapt to unfavorable conditions in time, the eventual eradication of these species is a real possibility and a serious threat. Therefore, local adaptation may be a quicker and more efficient response to changing conditions. Several plant populations may need to respond to climate change through phenotypic plasticity, which refers to the ability of a genotype to express different phenotypes or adaptive evolution (Franks et al., 2014). The extent to which plant populations can adapt to locally changing conditions is highly relevant in predicting future responses and the chances of their survival in periods of rapidly progressing global warming. Nevertheless, these responses may be insufficient to keep pace with the current rate of climate change (Franks et al., 2014).

An important adaptation of plants inhabiting cold-characterized regions is their ability to protect against frost damage induced by minimum winter temperatures through a process called cold acclimation. Minimum winter temperatures remain a major factor in determining species distribution ranges (Armstrong et al., 2020). Tolerance to freezing temperatures is a trait most common in temperate as well as arctic and alpine plants, which are frequently exposed to sub-zero temperatures, whereas most tropical and subtropical plants suffer injuries from temperatures below 10 °C (Xin & Browse, 2000). Freeze-induced cellular dehydration caused by membrane destabilization is commonly accepted as the primary cause of freezing damage (Uemura et al., 1995). The formation of ice crystals in extracellular spaces and cell walls leads to decreased water potential outside the cells, thereby drawing water and electrolytes out by osmosis and eventually leading to cell death. Because freezing damage primarily occurs in cell membranes, changes in membrane behavior are critical for developing freezing tolerance (Xin & Browse, 2000). In nature, temperate plants can increase their ability to withstand freezing temperatures after being exposed to low but non-freezing temperatures for a certain period, which makes them tolerant against seasonal changes and protects them against freezing damage in winter and early spring (Ritonga & Chen, 2020). The process of cold acclimation is mostly initiated by decreasing temperatures in late fall and can take a few days to several weeks, depending on the plant species (Xin & Browse, 2000). For *Arabidopsis thaliana* , an acclimation period of a few days is sufficient to improve cold tolerance (Gilmour et al., 1988), whereas 2-4 weeks of cold acclimation is necessary for wheat or rye to develop an increased frost tolerance (Brule-Babel & Fowler, 1989). The molecular basis of cold acclimation has long been investigated but has not yet been fully resolved. The process involves regulating hundreds of genes and is accompanied by complex biochemical and physiological changes, as well as structural changes (Xin & Browse, 2000; Thomashow, 2010). Such changes include the accumulation of soluble sugars, which act as cryoprotectants for enzymes, prevent excessive dehydration, and promote membrane stability,

as well as compositional and structural changes in the cell membrane (Reyez-Díaz et al., 2006; Uemura et al., 2006); cold acclimation is therefore clearly a multifactorial process in which many genes act in parallel to ensure maximum freezing tolerance (Renaut et al., 2005). There is natural variation in the freezing tolerances of different plants and their potential to acquire and increase tolerance following cold acclimation; this variation exists among species and even populations within species (Xin & Browse, 2000; Hannah et al., 2006); Some of this variation may be linked to the species' or populations' distribution (Armstrong et al., 2020). Varying environmental conditions in different geographic regions may expose plants to different selection pressures, which may lead to differences in phenotypic adaptation to cold in geographically distant populations (Zuther et al., 2012). Therefore, an increased selection for cold tolerance is to be expected in northern populations, whereas in southern regions, there may be less selection for cold tolerance (Armstrong et al., 2020). This cline has been reported for accessions of different *Arabidopsis* species (Hannah et al, 2006; Zuther et al., 2012), whereas other studies have not confirmed this phenomenon (Armstrong et al., 2015; Armstrong et al., 2020). Freezing tolerance may also be pronounced along longitudinal and altitudinal gradients (Zuther et al., 2012; Davey et al., 2018). Cold acclimation is not a constitutive trait but instead must be induced by specific environmental cues. Several researchers have hypothesized that the process may, therefore, be biologically costly, thereby leading to trade-offs between the degree of cold tolerance and other traits such as productivity. Constitutive cold tolerance may be costly in environments that rarely encounter freezing events, as extensive physiological and biochemical changes must be maintained (Zhen et al., 2011). For species with a distribution spanning steep temperature gradients, the same degree of cold tolerance would not be beneficial across the full distribution range (Meireles et al., 2017). This cost of cold tolerance has been reported for *Olea europaea* (Arias et al., 2017). For herbaceous plants such as *Arabidopsis thaliana* , there is no indication of a trade-off between the level of protection and productivity (Zhen et al., 2011; Wos & Willi, 2015; Wos & Willi, 2018). The ability to prepare for freezing events through cold acclimation is especially important for arctic and alpine plant species because they are exposed to a wider temperature range (Davey et al., 2018).

Although several studies have intensively investigated the freezing tolerance and acclimation potential of different *Arabidopsis*accessions and ecotypes (e.g. Hannah et al., 2006), minimal information is known about this trait in diverse but evolutionarily related species assemblages, such as the tribe Cochlearieae from the Brassicaceae family (mustards). This tribe belongs to one of the few isolated lineages of the Brassicaceae family, comprising approximately 30 species (Koch, 2012). It represents a promising system to study cold adaptation, as it shows distinctive traits that have evolved over a short time span. These traits include adaptations to extreme bedrock types, heavy metal soils, salt habitats, and high alpine and Arctic regions (Wolf et al., 2021). The tribe includes two genera, *Cochlearia* and *Ionopsidium* , which were separated by a deep evolutionary split during the mid-Miocene, approximately 13.8 million years ago (Koch, 2012). Whereas*Ionopsidium* species adapted to western Mediterranean bioclimatic conditions, progenitors of present-day *Cochlearia* expanded their distribution range to the northern hemisphere and rapidly diversified during the Pleistocene to ecological conditions in central and northern Europe and the circumarctic (Koch, 2012; Wolf et al., 2021). Even though these taxa are closely related, they have colonized substantially different ecological niches, such as salt marshes, sand dunes, coastal areas, cold and calcareous springs, and high alpine and arctic environments. Separating most *Cochlearia* species from*Ionopsidium* is the coldness of their habitats (Koch, 2012). Most*Cochlearia* species are already endangered, as suitable habitats have become increasingly rare, and two species have become extinct in the wild (*Cochlearia polonica* , Cieślak et al., 2007; Cieślak et al., 2010; *C. macrorrhiza* , Koch et al., 2003; Koch & Bernhardt, 2004). Most *Cochlearia* species, having been forced into cold-adapted and geographically isolated niches, may face extinction if they are unable to quickly adapt to warming conditions. Evaluating the different *Cochlearia* and *Ionopsidium* taxa in their physiological responses to freezing temperatures offers the opportunity to gain insights into the evolution of cold adaptation and tolerance in the tribe Cochlearieae.

We previously demonstrated that both *Cochlearia* and*Ionopsidium* show a strong but very similar cold metabolome response to cold treatment (Wolf et al., 2021). We hypothesized that adaptations to drought (Mediterranean habitats), salinity (coastal line habitats), and cold (arctic-alpine environments, cold spring

water) may be achieved via the same primary response in herbaceous (non-woody) plants, thus posing the question of an evolutionary shared molecular-physiological trait among *Ionopsidium* and *Cochlearia* .

The focus of this study was therefore to elaborate on this idea and to test explicitly for cold tolerance of the living system across evolutionary scales (genera and species), genomic complexity (diploids versus polyploids), and biogeographic and bioclimatic gradients (Mediterranean to the Arctic). We elaborate on the hypothesis that *Cochlearia* species are caught in its cold-adapted niche, and successful escape from this bioclimatic cage may require a complete shift in life history strategies, a shift from perennial/polycarpic to annual/monocarpic growth, which is the most severe difference between *Cochlearia* and *Ionopsidium* .

2 MATERIALS AND METHODS

2.1 Taxon sampling, plant material, and growth conditions

Because *Cochlearia* and *Ionopsidium* species are rare and often endangered, our study is based upon our germplasm collections. These collections were compiled over the last three decades (*BrassiBase* [https://brassibase.cos.uni-heidelberg.de/]: Koch et al., 2012; Kiefer et al., 2014; Koch et al., 2018) because our taxon sampling required sufficient and high-quality seed material from wild populations to propagate the respective material used in the freezing experiments. *Cochlearia* species under study were selected from a range of different habitats, including arctic regions (*C. groenlandica*), alpine habitats (endemic *C. excelsa* from Austrian South-Eastern European Alps/endemic *C. tatrae* , High Tatra Mountains), cold calcareous springs and creeks (Central European *C. pyrenaica* /polish *C. polonica*), coastal dune areas, and salt marshes (*C. danica* /*C. aestuaria* /*C. anglica*).*Cochlearia danica* , which originally adapted to coastal sand dune areas, migrated along road routes into central Europe, where it remains today (Koch, 1996; Koch, 1997). Accessions of *Ionopsidium* came from Mediterranean habitats. A total of 34 different accessions, comprising 13 species, were used in the experiments (Fig. 1, Suppl. Material Table 1). Plants were grown and cultivated in a growing chamber for 34-38 weeks with a day/night cycle of 14/10 h and a 30 min transition period, during which the light intensity was gradually lowered. The growth conditions included a permanent temperature of 20°C (± 2°C) and a relative humidity of 50%. Plants were watered regularly and no light or drought stress was omitted. For each accession, 20 individuals were propagated to finally select three randomly selected individuals each (non-flowering and healthy rosette-forming plants) for the two acclimation treatments: (i) three individuals were acclimated at 4°C in a climate chamber for five days at a 65% relative humidity (a slight increase in humidity, compared to 20°C growth conditions, was implemented because of the lower temperature). (ii) Three randomly selected individuals were not acclimated and remained in the original growth chamber at 20°C as a control. The same day/night regime was applied for both treatments, as was chosen for the initial cultivation. Six week old individuals of the *Arabidopsis thaliana* Col-0 ecotype were used as an internal control.

The aim of this study was to performed electrolytic conductivity measurements on leaves subjected to a freezing regime from 0°C to -10°C at different time points and to calculate lethal experimental freezing temperatures (LT) for representative species from both genera.LT_{50} and LT_{100} (50% and 100% cell membrane damage) values were calculated then using logistic functions fitted to the measured electrolytic leakage values (PEL).

2.2 Electrolytic assays

Electrolytic leakage of detached leaves is commonly used to quantitatively assess the freezing tolerance and cold acclimation potential of plants (Armstrong et al., 2020; Hincha & Zuther, 2014; Wos & Willi, 2015, 2018). The cell membranes are the primary sites of freezing damage. When cell membranes are damaged, the cell's contents leak out; this leakage can be detected by measuring electrical conductivity because the ionic composition of the water in which the leaves were immersed changes. Therefore, electrolytic leakage is expressed in terms of relative conductivity (Lee & Zhu, 2010; Hatsugai & Katagiri, 2018; Armstrong et al., 2020). Electrolytic leakage was measured following cold exposure and again after boiling at 100°C to ensure 100% leaf damage. The relative leakage, or percentage of leakage, was calculated from the ratio of the two measurements. The lethal temperature (LT_{50}), which refers to the temperature that causes 50% electrolytic

leakage from cells (or otherwise causes 50% leaf damage), was derived based on the relative leakage. LT_{100} is the temperature at which 100% of the electrolytes have leaked from the cells and is calculated from the percentage of electrolytic leakage. The electrolytic leakage was measured according to the method described by Thalhammer et al.. al (2014), with some modifications outlined below in detail.

For the electrolyte leakage assay, six different freezing temperatures (0°C, -2°C, -4°C, -6°C, -8°C, -10°C) were selected to follow a respective temperature cline for further regression analyses. One leaf per temperature treatment (0, -2, -4, -6, -8, and-10°C) and three leaves for negative controls were harvested from acclimated (4°C) and non-acclimated individuals (20°C). In total, nine leaves of similar size (appr. 1 cm^2) were harvested from each of the three individuals from a single accession, which resulted in 54 leaves and measurements for each accession. Leaves of approximately the same size and thickness were chosen and placed into ddH_2O-filled (3 ml) 10 ml DURAN glass tubes that were closed tightly afterwards with metal lids. The negative controls were incubated at 4°C on a shaker at 100 rpm. To control the down-cooling freezing of other samples, tubes were placed in a cooling bath (LAUDA RP2045; LAUDA Scientific, Lauda-Königshofen, Germany). An automatic temperature ramping program was used to ensure a steady, standardized temperature change from 0°C to -10°C for all samples. The temperature was lowered by 2°C during a 3 min period between 45 min stable cooling intervals. Sample collection commenced at the end of each cooling interval, just before the 3 min cooling period started.

Crystallization of the remaining samples was induced using liquid nitrogen 20 min after cooling at-2 °C. Inoculation loops (steel, 0.5 mm) were used to initiate the crystallization. Loops were immersed in ddH_2O and subsequently in liquid nitrogen until ice crystals formed; the loops were then placed carefully into the tubes, carefully damaging the samples to induce immediate crystallization of the entire sample. The samples were then removed at various freezing temperatures (0°C to -10°C) and incubated for 48 h at 4°C and 100 rpm on a shaker. Wires were carefully removed, and an additional 2 ml cold ddH_2O was added to the tubes. The measurements were performed 24 h later. The electrical conductivity of the solution was measured using a conductivity meter (METTLER TOLEDO LE703; Mettler-Toledo, Albstadt, Germany) after the samples were brought to room temperature. Measurements were performed before (EL_0) and after (EL_1) boiling the solution for two h at 100°C. Boiling ensured complete destruction of the leaves, leading to 100% electrolytic leakage out of the cells. Finally, the analysis of 34 accessions resulted in 1836 measurements.

2.3 Analysis of electrolytic leakage data

The following equations were used to calculate the percentage of electrolytic leakage (PEL):

$$EL = EL_0/EL_1 \quad (2.1)$$

$$EL_{control} = EL_{0,control}/EL_{1,control} \quad (2.2)$$

$$PEL = (EL – mean(EL_{control})) \times 100 \quad (2.3)$$

Equations 2.1 and 2.2 calculate the ratio of electrical conductivity of un-boiled (EL_0) and boiled (EL_1) samples for frozen and control samples. Equation 2.3 calculates the percentage of electrolytic leakage, which is corrected by subtracting the mean $EL_{control}$ value for each pretreatment, as leaf damage could occur from harvesting, incubating in water, and cooling at 4°C. Negative PEL values were corrected to a 0.00% leakage. This correction occurred occasionally for samples cooled at 0°C or -2°C, as leaf damage remained relatively low at these temperatures compared to the control samples. This made it possible for control values to increase.

All calculations were performed in the R statistical environment using R Studio (Version R.4.1.2; R Core Team, 2021). A self-starting model was used to apply a logistic function to the measured PEL values as follows:

$$PEL = A/[1 + e^{(xmid- T/scal)}] \quad (2.4)$$

The inflection point (xmid) gives the LT50, and scal is a scale factor. The asymptote (A) was set to 100%, and the input(T) was the temperature. For this purpose, the stats package in R (Version 4.1.2; R

Core Team, 2021) implementing functions nls() and SSlogis() was used to write an r-script (Supplementary Material File 1). Values were derived using predict(); this process was performed for measured PEL values of each accession for acclimated and non-acclimated samples, separately. Using this method, the lower asymptote of the curve approached a 0% electrolytic leakage (or zero leaf damage) and the upper asymptote approached a 100% electrolytic leakage (or maximum leaf damage). LT_{50} and LT_{100} values for the acclimated and non-acclimated samples were calculated using the model data. Because some leaf damage was caused by the harvesting, incubation in water, and cooling at 4degC, the mean $EL_{control}$ of each pretreatment was subtracted from 100%, and the resulting percentage was used to calculate LT_{100} values. To assess the variation in the measurements, the mean PEL value and standard error were calculated from replicates of each temperature. The difference between the acclimated LT and non-acclimated LT values was computed to calculate the ΔLT_{50} and ΔLT_{100} values. Measured values and calculated values were exported, and the script allowed the computation of a graph showing all PEL values and mean values of the measured data, LT_{50} ,LT_{100} , and sigmoidal curves for acclimated and non-acclimated sample accessions.

Further statistical analyses to test differences in LT_{50} and LT_{100} values, both acclimated and non-acclimated, were performed using t-tests. To confirm whether variation in lethal values existed among species, an analysis of variance (ANOVA) was applied. A correlation analysis of the LT_{50} and LT_{100} values with geographic coordinates (latitude and longitude) as well as with ploidy level of the different species (diploid versus polyploid) was performed using R Studio (Version R.4.1.2; R Core Team, 2021); the data were checked for normality using the Shapiro-Wilk normality test. This was performed using the shapiro. test() function of the stats package. Information on chromosome number and ploidy level is provided in Suppl. Material Table 1 (Koch et al., 1996; Koch et al., 1998; Koch et al., 1999; Koch, 2002; Koch et al., 2003; Koch & Bernhardt, 2004; Cieslak et al., 2007; Koch, 2012; Wolf et al., 2021).

Pearson's correlation measures a linear dependence between two variables that have a normal distribution: lethal values and latitude/longitude. For visualization, scatterplots were produced using ggscatter() from the ggpubr package (Version 0.4.0), showing lethal values of acclimated and non-acclimated samples at different latitudes and longitudes. A simple regression line was calculated (through add = "reg.line").

2.4 BioClim data analyses of investigated accessions

Principal coordinate analysis was performed to identify different species groups according to the bioclimatic characteristics of the accession habitats. For this purpose, nineteen bioclimatic variables were downloaded from the WorldClim climate data grid (https://www.worldclim.org; Hijmans et al., 2005) for all *Cochlearia* and *Ionopsidium* accessions. These included temperature-related (BIO1-BIO11) as well as precipitation-related variables (BIO12-BIO19), namely annual mean temperature (BIO1), mean diurnal range (BIO2), isothermality (BIO3), temperature seasonality (BIO4), maximum temperature of warmest month (BIO5), minimum temperature of coldest month (BIO6), temperature annual range (BIO7), mean temperature of wettest quarter (BIO8), mean temperature of driest quarter (BIO9), mean temperature of warmest quarter (BIO10), mean temperature of coldest quarter (BIO11), annual precipitation (BIO12), precipitation of wettest month (BIO13), precipitation of driest month (BIO14), precipitation seasonality (BIO15), precipitation of wettest quarter (BIO16), precipitation of driest quarter (BIO17), precipitation of warmest quarter (BIO18), and precipitation of coldest quarter (BIO19). Principal coordinate analysis (PCoA) was performed using the multivariate statistical package (MVSP 3.22; Kovach, 2007). Bioclimatic variables and accession numbers were imported in the csv format, data were centered and standardized, and Kaiser´s rule was used to extract the axes that retained factors with eigenvalues greater than one.

3 RESULTS

3.1 Bioclimatic niche characteristics of analyzed *Cochlearia* and *Ionopsidium* accessions show severe differences

The principal coordinate analysis revealed a cumulative proportion of variance of 91% for the first three principal components (PC1,65.9%; PC2,19.2%; PC3,5.9%). As shown in Fig. 2a, four *Cochlearia* ecotype groups were defined, with *Ionopsidium* forming a fifth and separate group of accessions. Mostly along PC1,

alpine accessions (*C. excelsa* and *C. tatrae*) were grouped into cluster 1. Cluster 2 was comprised primarily by inland taxa such as *C. polonica* and *C. pyrenaica* , but also included arctic *C. groenlandica* . A third cluster combined polycarpic coastal taxa (*C. anglica* and *C. aestuaria*). Monocarpic accessions of*C. danica* were considered as a separate cluster 4, which shows closest affinities with Mediterranean *Ionopsidium* representing cluster 5 also defined exclusively by monocarpic taxa. These results are consistent with the findings of Wolf et al. (2021), who identified the same groups based on nine of the 19 bioclimatic variables. These results show that *Cochlearia* and *Ionopsidium* accessions are exposed to varying environmental conditions, which is expected considering their geographically distant distribution.*Ionopsidium* accessions can be aggregated into a single group, suggesting that they inhabit regions with similar precipitation rates and temperatures. *Cochlearia* accessions, however, were separated into different groups, suggesting a wider distribution and habitat sites that vary in their bioclimatic character along PC1. The orientation and length of loading vectors of variables indicate the method through which variables contribute to principal components, and therefore to separate different clusters (Fig. 2b). Notably, precipitation- and temperature-dominated variables separate clusters on the left (mostly polycarpic *Cochlearia* accessions) from clusters on the right (monocarpic coastal, Mediterranean *Ionopsidium*) in a very similar way. The differentiation of clusters along axis 1 suggests that the habitats of alpine and inland *Cochlearia* species may be strongly influenced by the amount and regularity of precipitation under cold conditions, whereas for coastal and Mediterranean species, temperature, especially warm temperatures, may play a more prominent role in ecological separation. This raises the question of whether continentality, which is defined by a strong seasonality of temperature and lower precipitation (Bruch et al., 2011), strongly influences the inland distribution of *Cochlearia* species. Based on these results, it would be expected for different ecological groups to vary in their physiological adaptation to cold; this is especially relevant for groups that were most clearly separated, such as alpine*Cochlearia* and *Ionopsidium* accessions, as differences in these groups´ bioclimatic niches are supposedly the strongest.*Cochlearia danica* and coastal *Cochlearia* species may exhibit a cold response that is more similar to *Ionopsidium*species, as these groups were positioned closely together in the PCoA analysis.

3.2 Cold acclimation similarly enhanced the freezing tolerances of*Ionopsidium* and *Cochlearia* accessions

Cold acclimation for five days at 4°C enhanced the freezing tolerance of all *Cochlearia* and *Ionopsidium* accessions.LT_{50} and LT_{100} values were significantly lower for acclimated samples than for non-acclimated samples (LT_{50} : t = -6.5886, df = 61.131,p -value = 5.824e-09; LT_{100} t = -5.8435, df = 79.099, p -value = 5.437e-08). AcclimatedLT_{50} ranged from -2.82°C to -12.82°C with a mean value of -7.06°C, whereas non-acclimated LT_{50}values were generally higher, ranging from -2.34°C to -9.24°C with a mean of -4.17°C (Table 1). This was also true forLT_{100} values, with acclimated values ranging from -4.24°C to -18.09°C (mean: -11.56°C) and non-acclimated values ranging from -2.12°C to -11.78°C (mean: -7.37°C) (Table 2). There was substantial variation in the lethal values for all the measured accessions (Table 2). Cold acclimation potential is indicated by the difference between LT values of acclimated and non-acclimated samples, as it shows how the freezing tolerance of individuals increases through exposing plants to low but non-freezing temperatures for a certain period (in this study, 4°C for five days). This difference was expressed in terms of the LT_{50} and ΔLT_{100} values (Tables 1 and 2). The larger this difference, the greater the freezing tolerance of plants through cold acclimation. The LT_{50} andLT_{100} values were exclusively positive, which supports the observation that acclimated lethal values were considerably lower than non-acclimated values. There was substantial variation in the cold acclimation potential of different accessions, withLT_{50} ranging from 0.48°C to 6.59°C (mean:2.91°C) and LT_{100} ranging from 0.06°C to 10.22°C (mean:4.18°C). Generally, ΔLT_{100} values were significantly higher than Δ LT_{50} values (p -value 0.004), which could be expected because freezing damage is not a linear function. Examples of freezing tolerance measurement experiments are provided in Fig. 3, and individual measurements are shown in Suppl. Mat. Table 2. The mean values for our internal control*Arabidopsis thaliana* Col0 were -8.1°C (SD:0.7) and -4.1 (SD:0.3) (LT_{50} , acclimated and non-acclimated, respectively), which is also within the range of previously reported values for this ecotype (-9.7°C and -5.5°C; Hannah et al., 2006).

3.3 Freezing tolerance variation within and between species: not taxonomic group specific

Substantial variation in LT_{50} andLT_{100} values were observed within species (Tables 1 and 2). The highest range of LT_{50} values was exhibited by acclimated *C. tatrae* samples, spanning a difference (min-max) of 6.92°C, and non-acclimated *I. abulense* samples, at 4.6°C. For LT_{100} values, *C. tatrae* showed the highest range of values among species for both acclimated and non-acclimated samples, at 8.21°C and 9.3°C, respectively. If standard deviations are used to compare variability in the data, considering varying sample sizes, there was also substantial variation within (and among) species (Fig. 4). The various species showed, on average, a much lower standard deviation in non-acclimated LT_{50}values compared to all other values. For acclimated samples, *C. tatrae* had the highest standard deviation for bothLT_{50} and LT_{100} values. This species also showed the highest standard deviation forLT_{50} values of the non-acclimated samples. Although LT_{50} values of non-acclimated samples presented the lowest standard deviation values, there was also minimal difference among species, with only *I. abulense* showing the highest value. Notably, a small sample size did not necessarily result in a higher standard deviation, as was expected. *Cochlearia pyrenaica* (n = 9) and *C. tatrae* (n = 6) showed higher standard deviation values than some species with lower sample sizes, such as*C. excelsa* (n = 2). This suggests that, besides varying sample sizes, other differences, such as genetic variation within species, likely influence the variation in the measured data. Because only one accession was measured for *I. glastifolium* and *I. megalospermum* , no standard deviation could be calculated for these species. Given that these two species are genetically very similar and are sometimes referred to as subspecies (Vogt, 1987; Koch, 2012) and as they are also distributed in the same regions, they may be compared here as if they were a single species. A similar cold response with very similar lethal values was observed for these two taxa (Fig. 4).

Comparisons among species revealed that *C. danica* (coasts of the Atlantic Ocean and Northern Sea) and *I. abulense* (Spain mainland) showed the lowest LT_{50} values for acclimated samples (-10.72°C and -9.96°C, respectively) (Table 1). Both species are adapted to hot, dry, and in the case of *C. Danica* , coastal, high-salt conditions (Fig. 1). This result supports the assertion that*C. danica* exhibits a similar cold response compared to*Ionopsidium* owing to the similar environmental conditions of their habitats (Fig. 3). This was contrary to the expectation that these species would display the lowest cold tolerance. We expected that species such as arctic *C. groenlandica* and high alpine *C. tatrae* and *C. excelsa* exposed to the lowest ambient temperatures would display the highest frost tolerance and the lowest lethal values. Interestingly, a different species pair showed the lowest acclimatedLT_{100} values: *I. abulense* with -15.48°C and *C. excelsa* with -15.26°C, which shows that even though*C. excelsa* did not show the lowest LT_{50} value, it may still be able to withstand more extreme temperatures than other species.

Coastal *C. danica* , which also occurs in Portugal and Spain, may exhibit a cold response similar to that of *Ionopsidium* species, as these species may be exposed to similar environmental conditions. However, this assumption was not supported by the measured data (Fig. 4). Because lethal values vary considerably within the*Ionopsidium* group, comprehensive comparisons of this group other species proved difficult. Notably, *I. abulense* showed much lower lethal values (both LT_{50} andLT_{100}) than the remaining species of this group. As stated above, *C. danica* did exhibit lethal values similar to those of *I. abulense* . Coastal *C. anglica* and *C. aestuaria* exhibited high lethal values that were most similar to those of other *Ionopsidium* species. However, no obvious distinction between alpine/arctic *Cochlearia* species and coastal species, such as *Ionopsidium* , could be identified. ANOVA showed that, generally, there was a significant difference in lethal values between species (LT_{50} acclim: ***,LT_{50} non-acclim: **, LT_{100}acclim: **, LT_{100} non-acclim: **, df = 12). However, multiple t-tests revealed mostly insignificant differences. As Table 3 shows, only nine of the 55 comparisons were significant.

The two evolutionary lineages leading to the sister genera*Cochlearia* and *Ionopsidium* diverged from each other approximately ten million years ago. However, comparisons of lethal values between the two genera revealed insignificant differences, as shown in Fig. 5 (LT_{50} acclimated, $p = 0.822$; LT_{100} acclimated, $p = 0.883$;LT_{50} non-acclimated, $p = 0.0599$;LT_{100} non-acclimated, $p = 0.249$). This further substantiates the finding that *Cochlearia* and*Ionopsidium* species respond similarly to freezing temperatures. This is contrary to the expectation that, as western Mediterranean*Ionopsidium* forms a separate bioclimatically defined group (Fig. 2), the genus would respond differently to freezing temperatures. Similarly, there were no differences

when comparing polycarpic versus monocarpic and diploid versus polyploid accessions (Table 4).

3.3 Freezing tolerance shows weak geographically defined trends

Low temperatures, especially winter minimum temperatures, are important in determining the geographic boundaries of plant species distributions. Therefore, it is expected that a species' tolerance to freezing temperatures is often correlated with its geographic distribution (Armstrong et al., 2020). There is indeed an environmental gradient strongly associated with temperature, which may create a gradient in natural selection with strong selection pressures for an increased cold tolerance towards the north (Wos & Willi, 2015; Armstrong et al., 2020). A significant correlation between lethal values and longitude has been demonstrated for *A. thaliana* accessions, suggesting that there may also be a continentality factor influencing the response to cold, as conditions become colder and drier with increasing distance from the coast (Bruch et al., 2011; Zuther et al., 2012). Therefore, geographically distant taxa are expected to differ in their responses to cold (Davey et al., 2018). As shown in the previous chapter,*Cochlearia* and its sister clade *Ionopsidium* exhibit similar responses to freezing temperatures, despite being distributed in different geographic regions with varying bioclimatic conditions. To elaborate further on this spatial distribution,LT_{50} and LT_{100} values for the different *Cochlearia* and *Ionopsidium* accessions have been plotted on a map (Fig. 6). The range of lethal values (acclimated and non-acclimated) is indicated by a color gradient. We determined that accessions at high latitudes would display higher cold tolerances and therefore show lower lethal values than accessions at low latitudes, thereby forming a gradient of cold tolerance. As inland species may be exposed to a colder winter climate than coastal species owing to continentality, a longitudinal gradient may also occur. No such gradient was initially identified for non-acclimated samples, as these plants did not undergo the process of cold acclimation that would naturally occur. Acclimated LT_{50} values showed a weak trend towards a gradient that spanned from southwest to northeast with lower lethal values, indicating an increase in freezing tolerance; this was partly mirrored by LT_{100} values for acclimated samples. However, the northwestern arctic *C. groenlandica* was an outlier in both cases, showing the highest freezing tolerance. In addition, *I.abulense* accessions located in the southwest showed a comparatively high freezing tolerance, thereby suggesting that the supposed gradient is extremely weak. This also suggests that a combination of latitude and longitude might influence freezing tolerance instead of a single factor. Correlation analysis supports these observations. Both the correlation between lethal values and latitude (LT_{50} acclim: R = -0.3; p = 0.057,LT_{100} acclim: R = -0.2; p = 0.22,LT_{50} non-acclim: R = 0.023; p = 0.88,LT_{100} non-acclim: R = -0.12; p = 0.44) and the correlation between lethal values and longitude (LT_{100} acclim: R = 0.056; p = 0.73,LT_{50} non-acclimated: R = 0.3; p = 0.059,LT_{100} acclim: R = -0.0094; p = 0.95,LT_{100} non-acclim: R = 0.21; p = 0.19) were not significant. However, in reviewing the correlation analysis between lethal values and these two factors, the lethal values of the acclimated samples seemed to be slightly more correlated with latitude.

4 DISCUSSION

Cold acclimation is an inducible process, and researchers have demonstrated that induction of the cold acclimation pathway occurs within the first 15 min of exposure to low, non-freezing temperatures such as 4°C for the monocarpic and annual Brassicaceae species*Arabidopsis thaliana* . The highest frost tolerance was reached in a few days (2-5) in previous studies as multiple mechanisms worked in parallel, sometimes interacting to confer maximum tolerance to frost (Gilmour et al., 1988; Xin & Browse, 2000; Hincha et al., 2014). For all *Cochlearia* and *Ionopsidium* individuals assessed in this study, an acclimation period of five days at a temperature of 4°C was sufficient for the studied species to develop a profound cold acclimation and a largely enhanced freezing tolerance. This tolerance is built upon similar physiological principles and primary metabolites (Wolf et al., 2021), especially carbohydrates and amino acids. These significant increases in carbohydrate levels, a widely known reaction to cold stress in plants, have been demonstrated in *Cochlearia* and*Ionopsidium* through metabolome analyses (Wolf et al., 2021). Carbohydrates play a crucial role as cryoprotectants and signalling molecules in plant cold responses (Janská et al., 2010; Davey et al., 2008). Similarly, among analyzed amino acids, proline is recognized for its role in plant responses to various abiotic stresses, including low temperatures (Ashraf & Foolad, 2007). Increased levels of glutamic acid and aspartic acid are associated with a typical stress response, which is consistent with the cold metabolomes of *A.*

thaliana (Kaplan et al., 2004).

In contrast to *A. thaliana* sampled across Europe and analyses of various "ecotypes" with a demonstrably low within-accession variation (e.g. Hannah et al., 2006; Zuther et al., 2012), there was a substantially larger variation in the freezing tolerance within the accessions of *Cochlearia* and *Ionopsidium* analysed herein. In contrast to *A. thaliana* ecotypes with comparably minimal within-accession genetic variation (often inbred lines obtained from stock centers), the material used in this study most often reflects its natural genetic diversity because (i) the seeds originated from the wild, (ii) most species are outbreeding, and (iii) polyploidy may contribute to increased genetic variation. Furthermore, we may also assume a larger phenotypic plasticity, at least for polycarpic *Cochlearia* species, compared to annual taxa. However, we did not find any significant difference ($p > 0.01$ neither for ploidal-level variation (LT_{50} accl.,LT_{50} non-acclim., LT_{100}accl., LT_{100} nonaccl.; $p = 0.152/0.489/0.019/0.138$) or in comparing monocarpic and polycarpic life forms (LT_{50} accl., LT_{50}non-acclim., LT_{100} accl.,LT_{100} nonaccl.; $p = 0.315/0.361/0.623/0.437$) in the *Cochlearia* /*Ionopsidium*alliance; this suggests that multiple factors contributed to its higher plasticity compared to *A. thaliana* . Additional electrolytic leakage data are available for *Arabidopsis lyrata* , a polycarpic species spanning a distribution range from lowland sites in Central Europe to the Arctic region across the Northern Hemisphere (Schmickl et al., 2010; Hohmann et al., 2014; Koch, 2018; Hohmann & Koch, 2017). For this polycarpic, diploid, and largely outbreeding taxon, significant differences in the survival of sub-zero temperatures from different geographic regions have been demonstrated, with the majority of plants not surviving temperatures below -10°C (Davey et al., 2018). In North America, *A. lyrata* (Wos & Willi, 2015) demonstrated that resistance to frost and heat varies significantly with latitude. However, in this study, aside from resistance as quantified by leaf damage (electrolytic leakage), tolerance to frost and cold measured as the phenotypic plasticity of an entire plant grown under varying temperature regimes did not increase in the northern region; therefore, the cost of frost tolerance may be an important component of the limits of species distributions (Wos & Willi, 2015).

Cochlearia and *Ionopsidium* can be clustered into separate groups according to the bioclimatic character of their habitats, proving that different species experience varying bioclimatic environmental conditions. In the distribution range of *Ionopsidium* , hot and dry conditions prevail, and arctic-alpine *Cochlearia* species may be exposed to extremely low winter temperatures paired with a strong temperature seasonality and winter dryness. Inland as well as coastal *Cochlearia* species experience conditions between these extremes. We assumed that varying selection pressures in these geographically distant habitats would lead to differences in the species´ responses to cold. Even though considerable variation in lethal values was detected within species assessed in this thesis, there was minimal significant variation in freezing tolerance among species. Even though the species supposedly experience different environmental conditions, they exhibited a similar responses to freezing temperatures in this study. We expected that northern species such as *C. groenlandica* would display a much higher freezing tolerance compared to southern *Ionopsidium*species, proving the existence of a latitudinal gradient of selection for or against cold tolerance. However, the data measured in this experimental setting did not support this hypothesis. Northern *Cochlearia* species did not show considerably higher lethal values than southern *Ionopsidium* species (see Fig. 6). Temperature seasonality and winter dryness intensify with increasing distance from the coast, which may increase the need for cold tolerance in inland species. Continentality can therefore create a longitudinal gradient of frost tolerance. However, the analysis indicated that a combination of latitude and longitude may influence the freezing tolerance of different accessions. There was only a demonstrably weak trend in decreasing lethal values following a southwest to northeast gradient. Considering this result, key adjustments of the *Cochlearia* species may instead be adaptations to winter dryness, as the response to low temperatures seemingly did not vary significantly between most *Cochlearia* species. Accordingly, principal coordinate analysis (Fig. 2) showed that the distributions of northern, alpine, and inland Cochlearia species were strongly influenced by precipitation variables. Arctic and alpine *Cochlearia* species may be covered by an insulating layer of snow during winter, which suggests another reason for the lack of differences in freezing tolerance between these and other species. Snow protects plants growing underneath from freezing damage as it creates a relatively mild microclimate. Plants growing underneath a layer of snow are sheltered from ambient temperatures that can drop as low as -45°C,

whereas temperatures below the snow cover may only be as low as -5°C (Bokhorst et al., 2009; Armstrong et al., 2015). This suggests that northern and alpine species may not be exposed to lower temperatures than other *Cochlearia* species, thereby decreasing the need for increased frost tolerance compared to other species. Even though *Cochearia* species are spatially widespread, they mostly inhabit cold-characterized habitat sites (Wolf et al., 2021). The lack of a correlation between latitude or longitude and the species´ response to cold supports the distribution of *Cochlearia*species among often azonally distributed habitats, such as cold calcareous springs, wet meadows, or wet bedrock, where local conditions are formative rather than climate zones. Even though the ambient climate of the greater region may be warmer, plants growing in or along cold-characterized habitats share a need for cold adaptation, similar to arctic and alpine species. Central European *Cochlearia* species, except for *C. danica* , are highly endangered and are mostly threatened by habitat loss. In the alpine system, however, *C. excelsa* occurs only as a southeastern alpine-endemic species at two high mountain peaks (Seckauer Zinken, Eisenhut; Austria); during the last 25 years, scattered populations have declined rapidly and elevational occurrence has lost roughly 250 m at its lower distribution limits from 1900 m to 2150 m a.s.l. (Koch, unpublished data); this is in accordance with general observations of alpine flora shifts affected by global warming (e.g. Auld et al., 2022; and reference provided therein).

The similar cold responses exhibited by different *Cochlearia*species may therefore be a result of several factors acting in concert. It still unknown why *Ionopsidium* species respond similarly to*Cochlearia* species in mitigating freezing temperatures despite being exposed to hot and dry conditions. Although cold acclimation is likely a useful tool in protecting against freezing damage in most*Cochlearia* species, *Ionopsidium* species should not be expected to require cold acclimation, considering their Mediterranean distribution. Researchers have hypothesized that cold acclimation comes with a biological cost to the plant in environments that rarely encounter freezing events, such as the Mediterranean (Zhen et al., 2011; Meireles et al., 2017). As the process of cold acclimation includes extensive physiological and biochemical changes, a trade-off should be expected between the degree of cold tolerance and other metabolically challenging processes, such as growth or reproductive rates. The cost of cold tolerance may explain latitudinal selection gradients. If cold tolerance does not come at a cost to the plant, it should be generally high, even in species that are seldom exposed to freezing temperatures (Armstrong et al., 2020). This cold tolerance cost has not been observed in several studies evaluating the freezing tolerances of different plant species (Zhen et al., 2011; Wos & Willi, 2018; Armstrong et al., 2020). This could explain why *Ionopsidium* species exhibit similar cold responses to those of *Cochlearia* species. Constitutive cold tolerance in *Ionopsidium* could be maintained, even though these species rarely encounter freezing events. Wolf et al. (2021) revealed that although different *Cochlearia* and *Ionopsidium* species can be clustered into ecological groups, they do not show significantly different metabolomic responses to cold stress. The same ecological groups were identified herein and support the observations of Wolf et al., in that all species seem to exhibit a similar tolerance to cold. We speculate that the observed magnitude of the cold response may predate the origin of *Cochlearia* and *Ionopsidium* and may have resulted from an ancient preadaptation in a remote tribe (Cochlearieae) in the Brassicaceae family (Walden et al., 2020). Wolf et al. (2021) argued the continuous connection of *Cochlearia* to cold-characterised habitats since its diversification during the Pleistocene glaciation and deglaciation cycles, in which it migrated to the northern regions. This early-evolved cold tolerance may have not been lost secondarily in *Cochlearia* explaining the low lethal values in southern and coastal species such as *C. danica* . However, cold tolerance appears to be accompanied by sensitivity to increased temperature, which was not analyzed in this study. However, some species, such as *C. pyrenaica* and *C. polonica* , are critically endangered according to the IUCN Red List of Endangered Species, as their habitats have become increasingly rare. With global warming, endemic species such as *C. polonica* may face extinction, they may not have enough time to adapt to rapidly warming conditions.

Cochlearia danica and the genus *Ionopsidium* may exemplify the escape route needed to migrate from increased temperature and drought, as all species are monocarpic and may survive uncomfortable seasons as seeds in the soil seed bank. *Cochearia danica* , for example, is highly adapted to coastal sand dune habitats and can manage high levels of salinity, drought, and disturbance (Koch, 2012). This species exhibited the highest freezing tolerance, which supports the assertion that adaptations to salt or drought stress may also

confer tolerance to cold. Other studies have evaluated the ability of*Cochlearia* seedlings to cope with salt stress (Levi Yant, Nottingham, unpublished), showing that salt stress can be managed by cold-adapted species that do not naturally occur in regions with high salinity. Unfortunately, all present-day *Ionopsidium* are monocarpic; therefore, we cannot test the hypothesis of convergent evolution (as referred to in *C. danica*) changing the life cycle, such as with the onset of the Mediterranean salinity crisis (MSC) appr. 6 -5.3 mya during the Late Miocene (Mascle & Mascle, 2019).

5 CONCLUSION

The genus *Ionopsidium* is adapted to hot and dry Mediterranean conditions, whereas *Cochlearia* species largely prefer cold habitats. However, all species evaluated in this study exhibited similar and severe cold responses. Whether this similarity has resulted from an evolutionary adaptation of a common ancestor of the two taxa or from parallel evolution is unclear. However, the results of the present study indicate that adapting to different stressors, such as salinity and drought, also confers tolerance to cold, as all these stressors induce osmotic challenges. From a present-day perspective, all taxa were subjected to higher temperatures (*Ionopsidium* and *C. danica*), and eventually accelerated drought stress was adapted to at a macroevolutionary scale with monocarpic growth. This pattern was more widely distributed in the entire Brassicaceae family (e.g., tribe Arabideae; Karl xxx), which, therefore, might indicate a more general evolutionary path to temporarily escape from a changing environment towards increasing temperature and drought. However, this also leads to a pessimistic scenario for the long-term survival of various polycarpic Central European *Cochlearia* species.

AUTHOR CONTRIBUTIONS

Marcus A. Koch: conceptualization (lead), formal analysis (lead), investigation (lead), methodology (lead), resources (lead), writing – original draft (lead), writing – review and editing (lead), funding acquisition (lead), and project administration (lead).**Sarina Jabbusch:** data curation (supporting), investigation (supporting), and methodology (supporting). **Karolin Eisenschmid:** data curation (supporting), formal analysis (supporting), investigation (equal), methodology (supporting), and writing – first draft (equal).

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CONFLICT OF INTEREST

The authors declare no conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data presented in this study has been uploaded to Dryad (htpps://doi.org/10.5521/dryad.12311)

- Sampling locations

- Climate data (BioClim) and input files

- Measurements/results of electrolytic leakage analyses

- R-scripts used to analyse data

These documents are found with the online version of the manuscript:

Suppl. Material Table 1: Accession information (Taxon, coordinates, HEID-No., ploidal level and chromosome number)

Suppl. Material Table 2: Measurements of individual freezing damage

Supplementary Material File 1: R-scripts for data processing

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Fig. 1

Geographic distribution of accessions and species of *Cochlearia*and *Ionopsidium* analysed for electrolytic leakage. Further accession details are provided with Suppl. Material Table 1.

Fig. 2

Results of the analysis of bioclimatic variables grouping accessions into distinct clusters. (a) Results of Principal Coordinate Analysis for the first two principal components for all analysed accessions and 19 WorldClim variables related to temperature and precipitation. Ecological groups are indicated by coloured circles. Dark-blue: alpine species (*C. excelsa* /*tatrae*), Yellow: inland species (*C. pyrenaica* /*polonica* /*groenlandica*), Light-blue: coastal species (*C. anglica* /*aestuaria*), Red: *C. danica* , Green: mediterranean species (*Ionopsidium*). (b) Respective biplot showing the respective loading vectors. Out of the 19 WorldClim bioclimatic variables 11 are related to temperature (orange), and 8 are related to precipitation (blue) as named as follows:

temperature-related: BIO1 annual mean temperature, BIO2 mean diurnal range, BIO3 isothermality, BIO4 temperature seasonality, BIO5 max. Temperature of warmest month, BIO6 min Temperature of Coldest Month, BIO7 Temperature Annual Range, BIO8 Mean Temperature of Wettest Quarter, BIO9 Mean Temperature of Driest Quarter, BIO10 Mean Temperature of Warmest Quarter, BIO11 Mean Temperature of Coldest Quarter.

Precipitation: BIO12 Annual Precipitation, BIO13 Precipitation of Wettest Month, BIO14 Precipitation of Driest Month, BIO15 Precipitation Seasonality, BIO16 Precipitation of Wettest Quarter, BIO17 Precipitation of Driest Quarter, BIO18 Precipitation of Warmest Quarter, and BIO19 Precipitation of Coldest Quarter

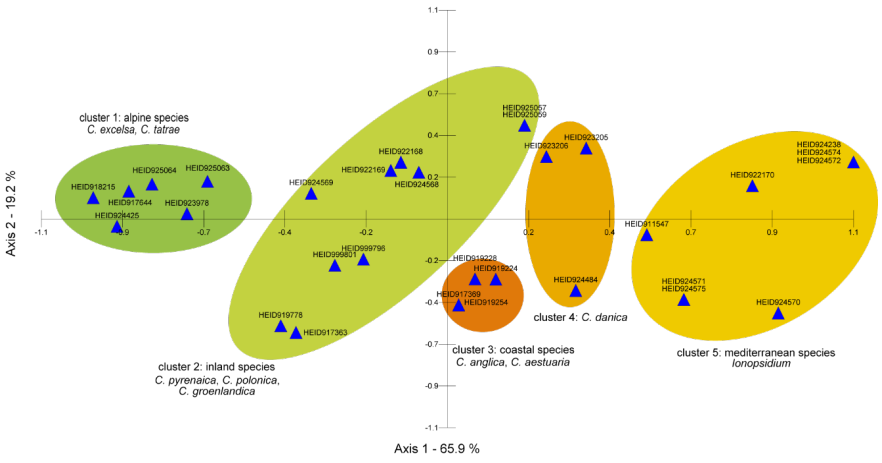

Fig. 3

Effect of sub-zero temperatures on the freezing tolerance of four selected accessions. Freezing tolerance of acclimated and non-acclimated samples was evaluated by measuring electrolytic leakage after exposing detached leaves to sub-zero temperatures. Acclimation occurred at 4°C. The graphs show estimates of LT_{50}

andLT_{100} values which are derived from a logistic function fitted to the measured data (shown by data points). Empty points present measured data, filled points show mean values of measured data. Dotted lines indicate the LT_{50} values

Fig. 4

Mean LT_{50} and LT_{100} values of each *Cochlearia* and *Ionopsidium* species before (orange) and after (blue) cold acclimation. LT values were estimated by fitting a logistic function to measured PEL values (see examples in Fig. 3). Error-bars indicate the standard deviation. For *I. glastifolium* and *I. megalospermum* no standard deviation could be derived as sample size is n = 1 each.

Fig. 5

Comparison of LT_{50} andLT_{100} values before and after cold acclimation between the genus *Cochlearia* and *Ionopsidium* . Numbers above bars indicate p -values of t -tests between genera.

Fig. 6

Spatial distribution ofLT_{50} /LT_{100} values. The dots represent the spatial distribution of different *Cochlearia*and *Ionopsidium* accessions (see Fig. 1).LT_{50} and LT_{100} values are indicated using a color gradient where light colors (yellow, green) indicate higher values and dark colors (blue, dark-blue) indicate lower values.

Table 1

Lethal temperatures (LT_{50}) of acclimated and non-acclimated samples for different *Cochlearia* and*Ionopsidium* accessions. LT_{50} values are derived from a logistic function fitted to measured PEL values. Here,LT_{50} refers to the temperature at which 50% of electrolytes have leaked from cells. ΔLT_{50} are calculated from the difference between acclimated and non-acclimatedLT_{50} values.

The accession codes refer to the collection/voucher code at the Heidelberg Botanical Garden and Herbarium (HEID) (see also Suppl. Material Table 1).

Species	**Accession**	LT_{50}**-acclim**	LT_{50}**-nonacclim**	$\boldsymbol{\Delta LT_{50}}$
C. tatrae	HEID925064	-7,25	-3,89	3,36
C. tatrae	HEID925064	-9,68	-3,91	5,77
C. tatrae	HEID925063	-8,7	-3,51	5,19
C. tatrae	HEID925063	-10,43	-5,45	4,98
C. tatrae	HEID918215	-5,02	-3,47	1,55
C. tatrae	HEID917644	-3,51	-3,42	0,09
C. pyrenaica	HEID999801	-7,45	-6,11	1,34
C. pyrenaica	HEID999796	-5,02	-2,98	2,04
C. pyrenaica	HEID924569	-5,86	-3,36	2,5
C. pyrenaica	HEID924568	-7,03	-3,41	3,62
C. pyrenaica	HEID922169	-4,21	-3,43	0,78
C. pyrenaica	HEID922168	-4,21	-3,43	0,78
C. pyrenaica	HEID919781	-6,08	-3,29	2,79
C. pyrenaica	HEID917363	-7,46	-4,34	3,12
C. pyrenaica	HEID924569	-4,04	-2,64	1,4
C. danica	HEID924484	-10,05	-3,46	6,59
C. danica	HEID923206	-11,23	-4,79	6,44
C. danica	HEID923205	-10,88	-2,86	8,02
C. anglica	HEID919254	-5,44	-3,53	1,91
C. anglica	HEID917369	-7,48	-4,87	2,61
C. aestuaria	HEID919228	-4,7	-3,3	1,4
C. aestuaria	HEID919224	-3,59	-3,5	0,09

Species	**Accession**	LT_{50}**-acclim**	LT_{50}**-nonacclim**	$\boldsymbol{\Delta\Lambda T_{50}}$
C. polonica	HEID925059	-6,26	-3,45	2,81
C. polonica	HEID925057	-8,77	-4,43	4,34
C. groenlandica	HEID919778	-10,11	-4,81	5,3
C. groenlandica	HEID919778	-9,76	-5,73	4,03
C. excelsa	HEID924425	-7,98	-4,45	3,53
C. excelsa	HEID923978	-8,03	-5,18	2,85
I. prolongoi	HEID924574	-5,11	-3,24	1,87
I. prolongoi	HEID924574	-4,03	-3,5	0,53
I. prolongoi	HEID924572	-6,82	-3,6	3,22
I. prolongoi	HEID924238	-5,32	-3,32	2
I. prolongoi	HEID924238	-4,42	-4,94	0,52
I. megalospermum	HEID922170	-7,54	-4,66	2,88
I. glastifolium	HEID911547	-6,93	-3,32	3,61
I. abulense	HEID924575	-12,82	-9,24	3,58
I. abulense	HEID924575	-7,76	-4,64	3,12
I. abulense	HEID924575	-10,98	-6,45	4,53
I. abulense	HEID924571	-8,26	-7,06	1,2
I. acaule	HEID924570	-6,48	-3,75	2,73
I. acaule	HEID924570	-2,82	-2,34	0,48

Table 2

Lethal temperatures (LT_{100}) of acclimated and non-acclimated samples for different *Cochlearia* and*Ionopsidium* accessions. LT_{50} values are derived from a logistic function fitted to measured PEL values. Here,LT_{100} refers to the temperature at which 50% of electrolytes have leaked from cells. ΔLT_{100} are calculated from the difference between acclimated and non-acclimatedLT_{100} values.

The accession codes refer to the collection/voucher code at the Heidelberg Botanical Garden and Herbarium (HEID) (see also Suppl. Material Table 1).

Species	**Accession**	LT_{100}**-acclim**	LT_{100}**-nonacclim**	$\boldsymbol{\Delta\Lambda T_{100}}$
C. tatrae	HEID925064	-13,25	-7,76	5,49
C. tatrae	HEID925064	-16,69	-7,82	8,87
C. tatrae	HEID925063	-11,9	-4,76	7,14
C. tatrae	HEID925063	-16,4	-11,42	4,98
C. tatrae	HEID918215	-12,34	-2,12	10,22
C. tatrae	HEID917644	-4,24	-4,18	0,06
C. pyrenaica	HEID999801	-10,45	-9,1	1,35
C. pyrenaica	HEID999796	-11,59	-3,88	7,71
C. pyrenaica	HEID924569	-10,51	-4,36	6,15
C. pyrenaica	HEID924568	-6,52	-4,89	1,63
C. pyrenaica	HEID922169	-10,81	-4,31	6,5
C. pyrenaica	HEID922168	-10,69	-4,87	5,82
C. pyrenaica	HEID919781	-6,36	-5,09	1,27
C. pyrenaica	HEID917363	-10,28	-4,2	6,08
C. pyrenaica	HEID924569	-10,4	-10,14	0,26
C. danica	HEID924484	-10,56	-4,6	5,96
C. danica	HEID923206	-14,28	-8,13	6,15
C. danica	HEID923205	-13,36	-10,1	3,26
C. anglica	HEID919254	-16,99	-9,86	7,13

Species	**Accession**	LT_{100}**-acclim**	LT_{100}**-nonacclim**	$\boldsymbol{\Delta\Lambda T_{100}}$
C. anglica	HEID917369	-13,55	-9,69	3,86
C. aestuaria	HEID919228	-16,97	-12,84	4,13
C. aestuaria	HEID919224	-13,35	-11,14	2,21
C. polonica	HEID925059	-13,88	-11,12	2,76
C. polonica	HEID925057	-15,02	-11,78	3,24
C. groenlandica	HEID919778	-9,44	-4,26	5,18
C. groenlandica	HEID919778	-9,97	-8,82	1,15
C. excelsa	HEID924425	-8,24	-4,08	4,16
C. excelsa	HEID923978	-4,42	-4,06	0,36
I. prolongoi	HEID924574	-10,74	-4,3	6,44
I. prolongoi	HEID924574	-7,48	-4,48	3
I. prolongoi	HEID924572	-13,45	-5,87	7,58
I. prolongoi	HEID924238	-9,07	-4,14	4,93
I. prolongoi	HEID924238	-8,71	-7,98	0,73
I. megalospermum	HEID922170	-11,69	-8,49	3,2
I. glastifolium	HEID911547	-11,42	-9,49	1,93
I. abulense	HEID924575	-9,6	-8,04	1,56
I. abulense	HEID924575	-8,04	-4,78	3,26
I. abulense	HEID924575	-18,09	-11,12	6,97
I. abulense	HEID924571	-13,14	-9,52	3,62
I. acaule	HEID924570	-17,02	-12,6	4,42
I. acaule	HEID924570	-13,66	-11,34	2,34

Table 3

Result of multiple t-test of lethal values between species. Significance is labelled as follows: ***: 0.0001 < p < 0.001; **: 0.001 < p < 0.01; *: 0.01 < p < 0.05, n.s: p > 0.05. Only significant comparisons are shown in this Table. *Ionopsidium glastifolium* and *I. megalospermum* were excluded from the analysis due to insufficient sample size for a t-test (sample size = 1).

Treatment Species 1 Species 2 Significance

LT_{50} acclim *C. danica C. pyrenaica***

LT_{50} acclim *C. danica I. prolongoi***

LT_{50} acclim *C. excelsa C. pyrenica**

LT_{50} acclim *C. groenlandica C. pyrenaica* **

LT_{50} acclim *C. groenlandica I. prolongoi* *

LT_{100} non-acclim *C. anglica C. pyrenaica* *

LT_{100} non-acclim *C. polonica C. pyrenaica* **

LT_{100} non-acclim *C. polonica I. prolongoi* *

LT_{100} non-acclim *C. pyrenaica I. aucaule* *

Table 4

Mean LT_{50} and LT_{100} values (in °C) for grouping (i) diploids versus polyploids and (ii) monocarpic versus polycarpic *Cochlearia* /*Ionopsidium* species and accessions.

acclimated non acclimated

	LT_{50}	LT_{100}	$LT_{50}LT_{100}$
ploidal level			
polyploids	-7.4	-12.5	-4.3 -7.9
diploids	-6.4	-10.0	-4.0 -6.4
p-value	0.15	0.19	0.49 0.19
life form			
monocarpic	-6.7	-11.4	-3.9 -7.1
polycarpic	-7.6	-11,9	-4.4 -7.8
p-value	0.31	0.62	0.36 0.43