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Can drought tolerance be a preadaptation to frost tolerance?

Evolution of responses to drought and sudden frost within the grass subfamily Pooideae.

Sylvia Pal Stolsmo
Master of Science in Management of Natural Resources

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Sylvia Pal Stolsmo

«Du ska itte trø i graset.
Spede spira lyt få stå.
Mållaust liv har og e mening
du lyt sjå og tenkje på.»

Einar Skjæraasen 1945

Abstract

The grass family (Poaceae) is one of the most economical and ecological important plant family on earth. The largest of the Poaceae grass subfamilies is Pooideae. This subfamily contains many of our crop and forage grasses and it dominates the grass flora in the temperate and arctic regions. Many studies indicate that Pooideae spread from the Tropics to the temperate zone due to acquisition of frost tolerance. However, little is known about what could be the preadaptation that led to evolution of frost tolerance. Since drought responses resembles frost responses in plants, drought tolerance is a good candidate as a precursor to frost tolerance. This study asks whether drought tolerance could be a preadaptation to frost. Plants from a phylogenetically diverse set of Pooideae species were subjected to drought and sudden frost separately, and then the evolutionary history of drought and sudden frost tolerance were reconstructed by ancestral state reconstruction. The results suggest that drought tolerance is not a preadaptation to sudden frost tolerance within the subfamily Pooideae because freezing resistance is determined by phylogenetic history, but drought resistance is not. The phylogenetic signal was stronger for frost tolerance compared to the weak phylogenetic signal from drought tolerance. Further, the core Pooideae has a higher sudden frost tolerance but a lower drought tolerance compared to the early diverging linages of Pooideae. These findings suggest that drought tolerance was not a significant precursor for development of frost tolerance within Pooideae.

Key words: Pooideae, evolution, ancestral state reconstruction, drought tolerance, sudden / episodic frost tolerance, preadaptation, water content, conductivity, fluorescence, regrowth.

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1. Introduction

1.1 Temperate grasses – from the Tropics to the north

Pooideae, one subfamily of the grass family Poaceae, dominates the grass flora in the temperate and northern latitudes (Hartley, 1973; Visser et al., 2014). It contains cereal crops such as barley (*Hordeum vulgare*), oat (*Avena sativa*), wheat (*Triticum aestivum*), rye (*Secale cereal*) and the forage grasses such as fescues (*Festuca*), ryegrass (*Lolium perenne*) and timothy (*Phleum pratense*). Thus, Pooideae is of importance for both agriculture and economy. Since Poodieae constitute 90 % of the grass flora in the temperate region, it also has an important ecological role (Hartley, 1973). Because of the Pooideae grasses' economic and ecological importance and a changing global climate, examining how they evolved to survive and thrive in temperate and arctic climates is timely. These climates are characterized by short growing seasons, highly variable temperatures according to seasons, episodic and periodic frost and drought and variable precipitation patterns. These challenging environments require complex adaptations of all plants living there.

Despite the fact that Pooideae are widespread in temperate and arctic regions today, studies indicate that the ancestor of the Pooideae was adapted to a tropical climate (Bouchenak-Khelladi et al., 2010; Strömberg, 2011). By using fossils, Schubert et al. (2018) dated the origin of the Pooideae to be about 69 million years ago (Mya). This indicates that Pooideae evolved during a warm period (Zachos et al., 2001) in the late Cretaceous (Bouchenak-Khelladi et al., 2010; Schubert et al., 2018). However, Pooideae experienced cold climate when the temperature on earth had a precipitous drop approximately 34 Mya (Pound & Salzmann, 2017; Zachos et al., 2001). This decrease in temperature may have initiated a period of global cooling during late Eocene and early Oligocene and an expansion of the temperate region when seasonality simultaneously started to increase due to slight changes in the earth's orbit (Zachos et al., 2001). One possible explanation for this biome shift for the Pooideae, from a tropical to a temperate climate, could be that Pooideae managed to evolve phenological and physiological adaptations to frost and short growing seasons (Fjellheim et al., 2014; McKeown et al., 2016; Sandve et al., 2011). These adaptations allowed the Pooideae to diversify and successfully expand in the northern temperate and arctic regions, as nearly the only subfamily of Poaceae to do so (Bouchenak-Khelladi et al., 2010; Kellogg, 2001).

Most of the tribes distributed in the most northerly regions belong to the "core Pooideae" (Hultén & Fries, 1986). The core Pooideae consists of the most species rich tribes; Bromeae, Triticeae and Poeae (with Avenea; Figure 1) (Soreng & Davis, 1998). The rest of the Pooideae is commonly referred to as the early diverging linages (Soreng & Davis, 1998), e.g. the grasses in the tribes Stipeae, Meliceae and Lygeeae. Since most of the economic important grasses belongs to the core Pooideae, much information is known about physiological and molecular mechanisms for frost and drought tolerance for the core Pooideae. On the other side, little is known about the early diverging linages in regard to frost and drought responses.

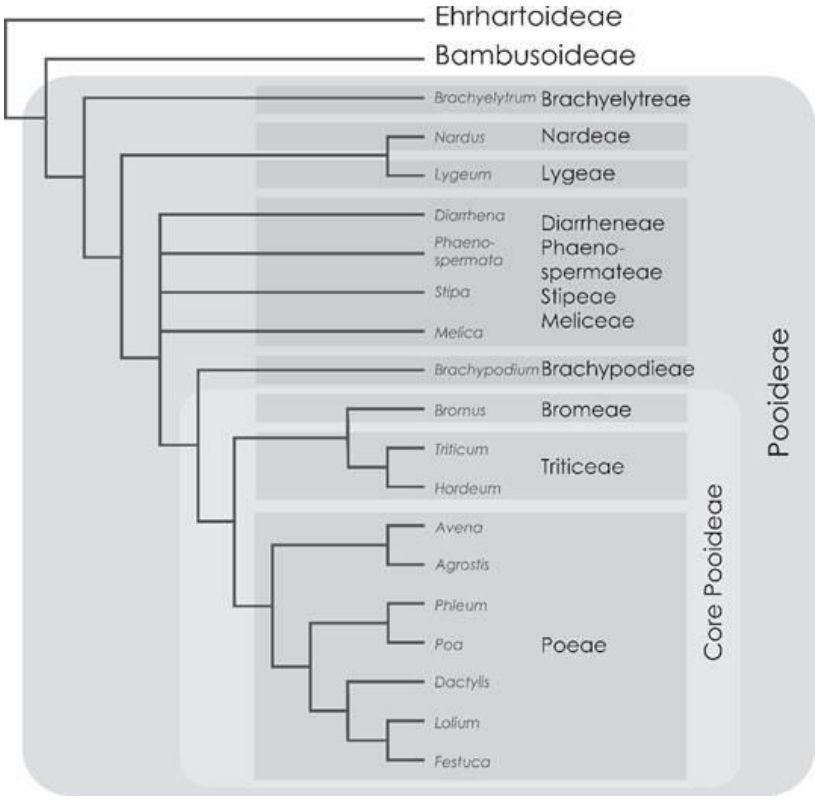

Figure 1: Simplified phylogeny of Pooideae with selected genera. The tribes that the genera belong to are named in the dark grey boxes. The light grey box indicates the tribes and genera that belong to the core Pooideae.

1.2 Evolution of frost tolerance

Of the global land area, about one third is totally free from freezing temperatures and most of these places are restricted to the tropical area (Larcher, 2005). It is also here the most species rich areas are found (Kier et al., 2005). Since only a few plant linages have transitioned to the northern latitudes (Bouchenak-Khelladi et al., 2010; Kellogg, 2001), it is assumed that the physiological adaptations required for frost tolerance are difficult to evolve (Donoghue, 2008).

It has been suggested that frost tolerance has evolved multiple times independently because temperate species are found within many different plant families (Preston & Sandve, 2013; Ricklefs & Renner, 1994). Preston and Sandve (2013) also found that some of the cold stress responses are conserved in distantly related species and that similar ancestral pathways have been repeated to evolve these cold stress responses. This implies that plants might have been preadapted to tolerate frost. Since frost responses have many molecular mechanisms in common with drought when dealing with water deficit (Preston & Sandve, 2013; Sakai & Larcher, 1987; Shinozaki & Yamaguchi-Shinozaki, 2000; Shinozaki et al., 2003), drought tolerance is suggested as the most possible preadaptation to frost.

Frost tolerance relies on the ability to avoid intracellular freezing, which means to avoid ice crystal formation that can puncture the cell membranes, by either supercooling or extracellular freezing. Supercooling is a phenomenon were the cool water inside the cell remains liquid, even though it is exposed to freezing temperatures. This allows the cell to maintain metabolic activity at temperatures below freezing and avoid dehydration (Cavender-Bares, 2005; Larcher, 2000; Wolfe et al., 2002). Freezing avoidance by supercooling can occur in small vacuoles of pure water were no ice formation starters (nucleators) are present (Wisniewski & Fuller, 1999), or induced by increased viscosity of the water, making ice crystal formation less likely to occur due to slow rotation of the water molecules (Cavender-Bares, 2005; Wolfe et al., 2002). Accumulation of osmotic active solutes in the vacuole can make the water more viscose and depress the freezing point and thus lower the threshold supercooling temperature (-40 °C, (Cavender-Bares, 2005; Sakai & Larcher, 1987). However, ice nucleation of the water will occur when reaching the threshold supercooling temperature, resulting in cell membrane puncture due to intercellular ice formation. Extracellular freezing occurs when ice crystal formation takes place outside the cell and makes the cell experience water deficit. This will lead to withdrawal of water from the protoplast and make the protoplast shrink and experience dehydration. This also happens during drought, where the water deficit is a result of little or no precipitation. When the protoplast shrinks, the concentration of cellular solutes will be higher than normal and can lead to toxification if the concentrations get high enough (Larcher, 2005). When the desiccation has reached a certain point, the cell will collapse. Morin et al. (2007) suggested that species with less water content are more frost tolerant and resistant to dehydration. Since less water available for freezing during frost lower the chance of cell membrane damage and might increase the frost tolerance.

The physiological processes are different between sudden and periodic frost (Körner, 2016). Periodic frost is predictable periods of frost, for instance low temperature in winter in the temperate region. The ability to cope with periodic frost usually relies on cold acclimation induced by the length of the photoperiod (McKenzie et al., 1974; Williams et al., 1972). During cold acclimation the freezing tolerance is increased by synthesis of anti-freezing proteins, stabilizing lipids for the cell membrane and alterations of the sugar content of the cell (Janská et al., 2010; Preston & Sandve, 2013). However, frost tolerance is not present throughout the year and short day induces cold acclimation.

On the other side with sudden frost (episodic frost or acute frost), the plant is not pre-induced by photoperiod and is not cold acclimated. The length of the frost periods can vary during sudden frost and probably the best way to cope with this type of frost might be to induce a rapid freezing point depression by alteration of the concentration of active solutes in the cell (Sakai & Larcher, 1987). Since sudden frost is unpredictable it can cause damage and be both lethal and sublethal to many plants, especially for plants in an active vegetative state during spring (Inouye, 2000; Sakai et al., 1981). Sudden frost can occur during summer in the arctic, in both spring and autumn in the temperate region, during night in the subtropical desserts and high mountains (Sakai & Larcher, 1987).

Some studies have seen a relationship between drought and frost tolerance. For instance, plants that have been exposed to drought and then subjected to frost showed increased frost tolerance (Pisek & Larcher, 1954) and plants from humid mountains had a lower frost tolerance compared to plants form arid mountains (Sierra-Almeida et al., 2016). Sakai and Larcher (1987) suggested one route for the plant transition from the moist tropic to the cold temperate region with seasonality, that goes through the tropical mountains. This plant migration pattern implies that drought tolerance might be crucial for adaptations to cold temperature and seasonality. First, the plant must tolerate cold temperatures above freezing, then develop further supercooling ability to withstand temperatures below freezing and finally achieve increased dehydration tolerance to survive winter freezing (Sakai & Larcher, 1987).

1.3 Motivation and research question

In this experiment sudden frost tolerance is tested, since Sakai and Larcher (1987) suggest that the plants which evolved frost tolerance took the route by the high mountains and possibly experienced sudden frost before evolving periodic frost tolerance necessary to survive in the

temperate region. Other studies have also found that Pooideae originated in Eurasia (Bouchenak-Khelladi et al., 2010; Schubert et al., 2018) and Schubert et al. (2018) suggest a mountain habitat in the nascent Alpine orogeny in Eurasia as the place of origin.

In this study, I aim to find out if drought is a preadaptation to sudden frost by examining the evolution of drought and sudden frost tolerance within the grass subfamily Pooideae. This is done by exposing the plants to sudden frost at -1 °C and -3 °C, and to drought, and then interpreting the results in a phylogenetic and paleoclimatic context, by doing an Ancestral State Reconstruction (ASR). I hypothesize that the Pooideae species evolved frost tolerance from drought tolerance. To test this hypothesis, I first predict that (I) species with high sudden frost tolerance will have high drought tolerance and (II) that drought tolerance evolved before sudden frost tolerance.

2. Materials and methods

2.1. Species selection

Species were selected based on their distribution in different climate zones and to cover the Pooideae phylogeny of Schubert et al. (2018), aiming to have species within each tribe. This resulted in 94 species, with two accessions each for *Brachypodium pinnatum*, *Phleum pratense, Nassella pubiflora and Melica ciliata,* and four accessions of *Piptatherum miliaceum* that were sown (Appendix I. A). However, due to lack of germination, I managed to cover seven of the ten Pooideae tribes in the phylogeny and only *Phleum pratense* had two accessions used in the experiment (Appendix I. A and C). According to The Online World Grass Flora (Clayton et al., 2002 onwards), the majority of the species in the experiment are perennials. The annuals are given in Appendix I. B. For the species names, the accepted names from the The Plant List (2013) were used (Appendix I. A).

2.2. Germination and growth

The germination, growth and regrowth of the plants took place in a greenhouse at Vollebekk, Ås, Norway (59°39'42.4"N 10°45'01.5"E) in the period from 14th of September until 14th of December 2018. The greenhouse held an average temperature of 17 °C and long day conditions with 16 hours of light. The light (200 µmol) was a mix of natural light through the windows and light from metal halide lamps with both Philips MASTER HPI-T Plus light bulbs (400W/645 E40 1SL) and Osram POWERSTAR HQI-BT light bulbs (400W/ D PRO).

To promote synchronized germination the seeds were stratified in humid soil at 4 °C for 4 days and then transferred to 25 °C for 24 hours. This was done in the dark and the pots were covered with black plastic to keep the humidity and ensure complete darkness. The seeds were then moved back to the greenhouse for germination and the plastic was removed. When plants were big enough (~5 cm, approximately two-three weeks after seeding), single tillers were pricked out in 8x8 cm square pots filled with soil ("Gartner jord", Tjerbo Torvfabrikk, Rakkestad, Norway). This was done for all the species within a two-week period to ensure that they were almost equal in size and developmental stage. Overall, 56 tillers per species were pricked out and the 48 most viable (best looking) individuals were used in the experiment. The plants were assigned an identification number, making it possible to identify each individual and which species it belongs to. For some species fewer then 48 tillers were used. Species with fewer than 30 tillers were not included in the experiment. In order to avoid table effects the plants were randomly rotated among the tables every week.

The plants were watered once with fertilized water after they were pricked out. The fertilized water contained a mix of 800 g/100L Kristalon Indigo (9 % N + 5 % P + 25 % K) and 600 g/10L YaraLiva Calcinit (15.5 % N + 19 % Ca), both produced by Yara. The conductivity was 1.7 mS/cm. Thereafter, they were watered daily with regular water. During the week before the start of the sudden frost and drought experiments they were again watered with the fertilizer solution daily.

Two days before the start of the experiment the plants were randomly divided into four treatment groups by using random numbers from Excel: sudden frost -1 °C and -3 °C, drought and control. For most species there were 10 individuals per species in each treatment group and in addition 4 individuals per species for initial electrolyte leakage and 4 individuals per species weight measurements (Appendix I. C). The plants were randomly placed in the trays by again using random numbers from Excel.

2.3. Measurements

2.3.1. Water content

To measure the water and dry mass content of each species, the plants were cut at the base and the aboveground biomass was weighed. Then the cut off plant parts were placed in paper bags and dried in a Unitherm drying oven (Russell-Lindsey Engineering Ltd., Birmingham, UK) at

90 °C for 14 hours and weighed again. Formula (1) was used to calculate the water content of the total aboveground biomass:

Formula (1) $$\text{Percentage } water\ content\ = \frac{WW-DW}{WW} * 100\ \%$$

where WW is wet weight and DW is dry weight.

2.3.2. Electrolyte leakage and conductivity measurements

When a cell gets damaged, it will release electrolytes (Hincha et al., 1987). Conductivity (mS) is a measurement that indicates how many electrolytes that are released by the damaged leaf. High conductivity indicates high cell damage. Approximately 1 cm^2 of a representative leaf of the plant was cut and placed in a tube with 10 mL distilled water. Then it was shaken at room temperature for 10 hours before the conductivity was measured with CWO Volmatic Mesur EC (Senmatic A/S DGT Volmatic, Søndersø, Denmark). The conductivity of the shaken samples was then divided by the maximum conductivity (formula (2)). To obtain the maximum electrolyte leakage per species the leaf sample in the tube was boiled at approximately 97 °C for 11 minutes and the conductivity was measured again when the tubes reached room temperature (25 °C). To get the percentage conductivity after each treatment, formula (2) was used per individual per species:

Formula (2) $$Percentage\ conductivity\ = \frac{CS}{CB} * 100\ \%$$

where CS is conductivity after shaking and CB is conductivity after boiling. To see if the treatments had any effect compared to the control group formula (3) was used (Fujikawa & Miura, 1986):

Formula (3) $$Percentage\ damage\ = \frac{100(\%\ CT - \%\ CC)}{100 - \%\ CC}$$

where $\%\ CT$ and $\%\ CC$ is the percentage conductivity by using formula (2) for the treatments and control group respectively. Formula (3) was also used to check if something had happened to the control group by comparing the control group to the start conductivity.

2.3.3. Soil moisture

Since species have different rates of water uptake (Taiz et al., 2015) and the soil content might differ slightly in the pots, soil moisture was measured in every pot in the drought experiment and a drought zone was defined as ≤ 5 % soil moisture. A HH2 Moisture Meter (Delta-T Devices Ltd, Cambridge, UK) was used to measure the moisture by placing it in the soil. Measurements in the same pot were taken on opposite sides to avoid getting a too low moisture value due to holes in the soil. When necessary, three measures at different corners were done for a pot and the average was used. To determine when the plants had been 4-5 days in the drought zone, the soil moisture decline /water uptake rate had to be estimated first by using the start soil moisture and the last soil moisture measurement of ≤ 20 % in formula (4):

Formula (4) $$Soil\ moisture\ decline\ rate\ (r) = \frac{MS-ML}{n}$$

where *MS* is the start moisture, *ML* is the last moisture recorded and *n* is number of days. The soil moisture decline rate was then used to estimate an approximate date when soil moisture was ≤ 5 % (formula (5)).

Formula (5) $$Remaining\ days\ until\ drought\ zone\ is\ reached\ = ML\ \% - r\frac{\%}{day} * x\ day = 5\ \%$$

where *ML* is the last moisture recorded, *r* is the soil moisture decline rate found by using formula (4) and *x* is the number of days until the species hits the drought zone.

2.3.4. Fluorescence

To compare the fluorescence data with the control, fluorescence was measured on both the control and drought plants. The drought and control groups were measured on the same day for every species. The fluorescence measurements were carried out using FluorPen FP100 (Photon Systems Instruments, Drasov, Czech Republic) with the OJIP fluorescence transient analysis program. This program measures Fv/Fm, which is the maximum quantum yield of photosynthesis. If the value of Fv/Fm is low, it can indicate that the plant is damaged due to low photosynthesis (Gilbert & Medina, 2016). The measurements were taken in the middle of a representative leaf per plant. To ensure an accurate measure of photosynthesis and to avoid light contamination, the plants were placed in a dark room for 25-35 minutes before the

fluorescence measurements were taken in the dark. Formula (6) was used to get the fluorescence of drought plants in relation to the control plants:

Formula (6) $$Percentage\ fluorescence = \frac{FD}{FC} * 100\%$$

where *FD* is the last fluorescence measurement of the plant in the drought zone before it was cut and *FC* is the average fluorescence measurements of the control throughout the whole experiment.

2.3.5. Regrowth

After the drought and sudden frost experiments the plants including the control plants were cut down to approximately 2-4 cm and the regrowth was scored on a scale from 0 – 9, where 0 is dead and 9 is normal growth. This was done two and three weeks after the plants were cut. Formula (7) was used to get the regrowth of the treatment plants in relation to the control plants:

Formula (7) $$Percentage\ regrowth = \frac{RT}{RC} * 100\ \%$$

where *RT* is the average regrowth after two and three weeks for the plants that were subjected to treatment and *RC* is the average regrowth after two and three weeks for the control plants.

2.4. Control group

Both the sudden frost and drought experiments were carried out simultaneously which allows for the use of the same control for all the treatments. The control plants were placed in the greenhouse at Vollebekk. The greenhouse had an average temperature of 17 °C and the same light mix as described above. The trays were randomized within the table every week. During the drought experiment, fluorescence was measured on the control plants. After the experiment, electrolyte leakage was measured, and regrowth was scored.

2.5. Drought experiment

The drought experiment took also place in the greenhouse at Vollebekk with the same light and temperature conditions described above. Soil moisture and fluorescence were measured before the drought experiment started and then every fourth day until the plants had stayed in the drought zone for 4-5 days. This procedure ensured that all the plants have been subjected to the same amount of drought stress, even though they reached the drought zone at different time

points. After the end of the drought period, leaves of 10 individuals per species were harvested for conductivity measurements and the plants were watered and cut down to approximately 2-4 cm height. Regrowth was scored after two and three weeks.

During a heavy rainfall some of the plants got wet and the moisture returned to or exceeded the starting point. These individuals were removed from the experiment. For *Ammophila arenaria,* more than five individuals got wet, resulting in exclusion of the species from the analysis.

2.6. Sudden frost experiment

The sudden frost experiment took place in the frost chambers at "Senter for klimaregulert planteforskning", Ås, Norway (59°40'08.7"N 10°46'07.6"E) without additional light than the window in the chambers. To test how the plants responded to sudden frost, they were not cold acclimated. The lowest temperatures for mild and severe sudden frost were -1 °C and -3 °C, respectively. Following the protocol of Alm et al. (2011), the starting temperature was set to 0 °C for 12 hours and then lowered with 1 °C per hour to the lowest temperature, where it was kept for 24 hours. Then the temperature increased by 1 °C per hour back to 0 °C. Further on, the plants were watered and placed in a room at +3 °C to thaw. Sampling of leaves from 4 individuals per species was done and electrolyte leakage was measured as described above. After 24 hours at +3 °C, the plants were moved back to the greenhouse and cut down to approximately 2-4 cm height. Regrowth was scored after two and three weeks.

2.7. Statistical and phylogenetic analyses

In total 62 accessions from 61 species were used in the statistical analyses (Appendix I. C). All data analyses were done with RStudio version 1.1.383 (RStudio Team, 2016), based on R version 3.5.2 (R Core Team, 2018).

2.7.1. Phylogeny

The Pooideae phylogeny from Schubert et al. (2018) was pruned to retain only the species in the present experiment. The species in the experiment that did not exist in the phylogeny were assigned to tips of their closest relative in the tree. To find the closest relative to the species in the experiment among the species in the Schubert et al. (2018) phylogeny, other phylogenies containing species from both the experiment and the Schubert et al. (2018) phylogeny were consulted. For instance, the phylogenies of Hamasha et al. (2012) and Cialdella et al. (2007) were used for the Stipeae tribe, Grebenstein et al. (1998) for *Helictotrichon* and Gillespie et al. (2007) for *Poa*. When I could not find a phylogeny with species from both the experiment and

Schubert et al. (2018) phylogeny, the species in the experiment were placed within their respective genus. See Appendix I. D for an overview of the replacements.

Since the control group of *Avena fatua* did not survive the cutting (showed no regrowth), the species was excluded from analyses. The outgroup *Ehrharta calycina* was also omitted because only one outgroup is not representative of all other grasses.

2.7.2. Phylogenetic signal (λ)

I first checked if the traits showed phylogenetic signal. According to Pagel (1999) the phylogenetic signal (λ), which varies on a scale from 0 to 1, indicates if the observed trait variance is correlated with the phylogenetic distance among species (λ=1), or if it is independent of phylogeny (λ=0). To estimate the phylogenetic signal of each trait, fit of different models with distinct assumptions of λ was tested. The Brownian Motion (BM) model assumes λ = 1 and the white-noise (non-phylogenetic) model assumes λ = 0. The lambda model estimates λ based on the observed values. The best model per trait was determined based on the sample-size corrected Akaike Information Criterion (AICc) (Akaike, 1974), calculated using the R package *geiger* (Harmon et al., 2008). A low AICc indicates a better fit for the model. To distinguish between the models, I used a difference in AICc of at least two (Anderson & Burnham, 2004).

2.7.3. Covariation and correlation among experimental variables

Next, a principal component analysis (PCA) was done to get an overview of covariation among experimental variables (traits) and to see how trait variation was partitioned among and within tribes. The PCA was plotted using R package *ggbiplot* (Vu, 2011).

Further on, a series of pairwise regressions was performed to test which experimental variables that were correlated with each other. To test whether I needed to run a phylogenetically corrected regression, I tested whether the residuals among the traits were autocorrelated. All trait combinations were tested. For the trait combinations with a significant autocorrelation of residuals, a phylogenetic regression was done by using the R package caper (Orme et al., 2018). By doing this, I checked if there still was a correlation when the phylogenetic relationship was taken into account. For the traits where the residuals were not autocorrelated, pairwise correlation tests (Pearson's correlation test) were done. Pairwise correlations were done using the function "cor.test" in R (R Core Team, 2018).

2.7.4. Choosing traits as proxies for drought and sudden frost tolerance

The experimental variables to be used as proxies for drought and frost tolerance were chosen based on the results of the pairwise correlation and phylogenetic signal tests above. Traits that were significantly correlated with other variables, and therefore carried information about several experimental measurements, and that showed a phylogenetic signal, and are therefore more interesting in an evolutionary perspective, were selected as proxy (conductivity for drought and regrowth after sudden frost at -3 °C for sudden frost tolerance). Previous studies, e.g. Knaupp et al. (2011), have showed that fluorescence and conductivity can indicate the same level of damage. This gives the opportunity to use only one trait. Statistical analyses were also done on the traits that were not chosen as proxies (regrowth and conductivity after -1 °C, conductivity after -3 °C and regrowth and fluorescence after drought), and the results were put in Appendix III.

2.7.5. Ancestral State Reconstruction (ASR)

The ASR was done under the best fitting model tested above (BM, lambda or white). This was achieved by reconstructing ancestral states using BM (assuming $\lambda = 1$), having first rescaled the branch lengths of the phylogeny according to the phylogenetic signal of the trait in question (estimated λ). Ancestral states were reconstructed using the "ace" function in *ape* (Paradis & Schliep, 2018) and phylogenetic branches were rescaled using "rescale" in *geiger* (Harmon et al., 2008). Finally, the reconstructed ancestral states were visualized on the original pruned phylogenetic tree by using the R packages *ggtree* (Yu et al., 2017), *cowplot* (Wilke, 2019) and *ggplot2* (Wickham, 2016).

2.7.6. Rate shifts

The term rate shift can be used if drought or sudden frost tolerance evolves faster or slower in some clades than the rest of the phylogeny. I tested whether drought and frost tolerance evolved at a single rate (BM, $\lambda = 1$) or under multiple rates using the R package *motmot.2.0* (Puttick et al., 2018). I tested for a maximum of five rate shifts and the minimum clade size was set to five species.

2.7.7. Water content

To test if there was a correlation between water content of the plants and drought and frost tolerance a pairwise regression was performed. An autocorrelation test of the residuals was also done. Since there was significant autocorrelation among the residuals, a phylogenetic regression

was used to test if there was a significant correlation between water content and drought tolerance. The residuals were not autocorrelated between water content and frost tolerance, thus a Pearson's correlation test was performed. The water content was also checked for phylogenetic signal and an ASR was performed, as described in 2.7.2. and 2.7.4. respectively.

2.7.8. Bioclimatic variables

To see how the experimental variables relate to climate characteristics of the species' native ranges, I used mean values for each species for each species distribution range for 19 bioclimatic variables available through WorldClim (Table 1) (Hijmans et al., 2005). Species distributions were based on GBIF occurrence records for all the species. The occupied climate for each species was summarized as the mean value per bioclimatic variable. The dataset was downloaded from Schubert et al. (2018) and reduced to the original species in the pruned phylogeny used in this experiment, omitting also *Hystrix patula* and *Koeleria glauca* due to lack of geographic data. Then a PCA was done on all the experimental and bioclimatic variables to see if some of the bioclimatic variables covaried with any experimental variables. Next, PCAs were done separately for regrowth after sudden frost at -3 ^{o}C and the temperature variables, and conductivity-drought and the precipitation variables.

The PCAs were repeated for the experimental variables and bioclimatic variables of the site where each accession was sampled. The sample sites were either given as coordinates from the seed distributors or, when only country or region were given, the midpoint coordinates were taken from Google maps. For accessions where no information about sample site was given (Appendix I. A), GBIF was used to locate their distribution and coordinates for one population were taken. Then the bioclimatic variables for each locations were obtained from WorldClim - Global Climate Data by using the R packages *raster* (Hijmans, 2019) and *maps* (Becker et al., 2018).

Table 1: A list over the code of the bioclimatic variables used in the PCA analysis. The bioclimatic variables are taken from WorldClim v1.4 dataset (Hijmans et al., 2005).

Code	Bioclimatic variable
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

3. Results

3.1. Distribution of the experimental variables

Almost all individuals regrew after sudden frost at -1 °C and drought treatment (Appendix II.), thus the regrowth expressed little variation. Regrowth after sudden frost at -3 °C showed a skewed distribution towards no regrowth (Appendix II.). A skewed distribution is also seen towards low conductivity for both sudden frost treatments, but sudden frost at -3 °C also show more variation (Appendix II.). Conductivity after drought shows a bimodal distribution. Fluorescence after drought shows a normal distribution (Appendix II.).

3.2. Phylogenetic signal (λ)

The model selection resulted in either the white-noise model or the lambda model as the best fitting model for the traits of the drought treatment (Table 2). The model with lowest AICc score is the model that gives the best fit for the trait. The white-noise model was the best fit for both regrowth and fluorescence of the drought treatment (Table 2). For conductivity, the AICc value was indistinguishable between the white-noise model and the lambda model (Table 2). This allowed the use of the lambda model that showed a phylogenetic signal for the drought treatment (Table 3).

For mild sudden frost, the lambda model was only best fitting for regrowth, which thus had a λ-value higher than 0 (Table 3). For conductivity, the white-noise model was the best fit. For

the conductivity measures after the severe sudden frost, the AICc values for the white-noise model and lambda model were not distinguishable and both models could be used. For regrowth after sudden frost at -3 °C, the lambda model was the best fitting model (Table 2) and gave a λ-value higher than 0 (Table 3).

The λ expressed by the drought variables are lower compared to the λ expressed by the sudden frost variables, which suggest a stronger phylogenetic signal for sudden frost tolerance than for drought tolerance (Table 3)

Table 2: AICc values for different models for the experimental variables, water content, the second (PC2) and the third principal (PC3) component. The best fitting model for each trait, with lowest AICc, is marked in bold type and red. When the AICc for two models were indistinguishable, both were marked in red. BM is equivalent to λ=1, white is λ=0 and lambda is where λ is estimated from the data.

VARIABLE	MODEL BM	MODEL WHITE	MODEL LAMBDA
PRINCIPAL COMPONENT			
PC2	240	**211**	213
PC3	241	**193**	195
SUDDEN FROST -1 °C			
Regrowth	-28	-51	**-61**
Conductivity	513	**447**	449
SUDDEN FROST -3 °C			
Regrowth	31	19	**5**
Conductivity	561	**551**	**550**
DROUGHT			
Regrowth	-50	**-64**	-62
Conductivity	640	**602**	**603**
Fluorescence	83	**38**	40
WATER CONTENT	343	327	**318**

Table 3: The best fitting model for each trait and the phylogenetic signal (λ). When the AICc for two models were indistinguishable, both models and their phylogenetic signal were given.

VARIABLE	MODEL	λ
PRINCIPAL COMPONENT		
PC2	White	0
PC3	White	0
SUDDEN FROST -1 °C		
Regrowth	Lambda	0.47
Conductivity	White	0
SUDDEN FROST -3 °C		
Regrowth	Lambda	0.47
Conductivity	White / Lambda	0 / 0.63
DROUGHT		
Regrowth	White	0
Conductivity	White / Lambda	0 / 0.11
Fluorescence	White	0
WATER CONTENT	Lambda	0.45

3.3. Principal component analysis (PCA), correlations and proxies

The PCA shows that several of the traits covary (Figure 2A). For instance, fluorescence varies in the opposite direction of conductivity after drought. This means that species with high fluorescence would have low leaf damage (conductivity). Conductivity and fluorescence have also minor loadings from regrowth after drought. Moreover, both the regrowth variables for sudden frost covary in the same direction and in the opposite direction of conductivity for sudden frost. This indicates a strong correlation between both sudden frost treatments, and that species with high regrowth had low leaf damage after sudden frost. There was a low loading between the drought and sudden frost variables, indicating low covariation. Five principal components were needed to explain more that 90 % of the variance.

The PCA also revealed that the tribes are not separated into distinct clusters but overlap to a large extent (circles in Figure 2). Lack of clustering can indicate that there are other factors than drought and frost tolerance that drive the evolutionary genetic separation into tribes. All the traits covary in the same direction (arrows pointing down) in the third principal component axis (PC3; although with very low loadings for fluorescence and conductivity drought; Figure 2B). The second principal component axis (PC2) splits the regrowth for both frost and drought, and fluorescence into the same direction. In addition, PC2 makes all the conductivity variables varies in the opposite direction of regrowth and fluorescence. This indicates that species at the lower extreme of PC2 axes had a high tolerance of both frost and drought (left upper and lower corner in Figure 2B). Therefore, I tested whether there was phylogenetic signal to PC2 or PC3, which would allow performing further evolutionary analyses on this variable. However, the best fitting model was white-noise for both (Table 2) and therefore no further analyses were performed for PC2 and PC3.

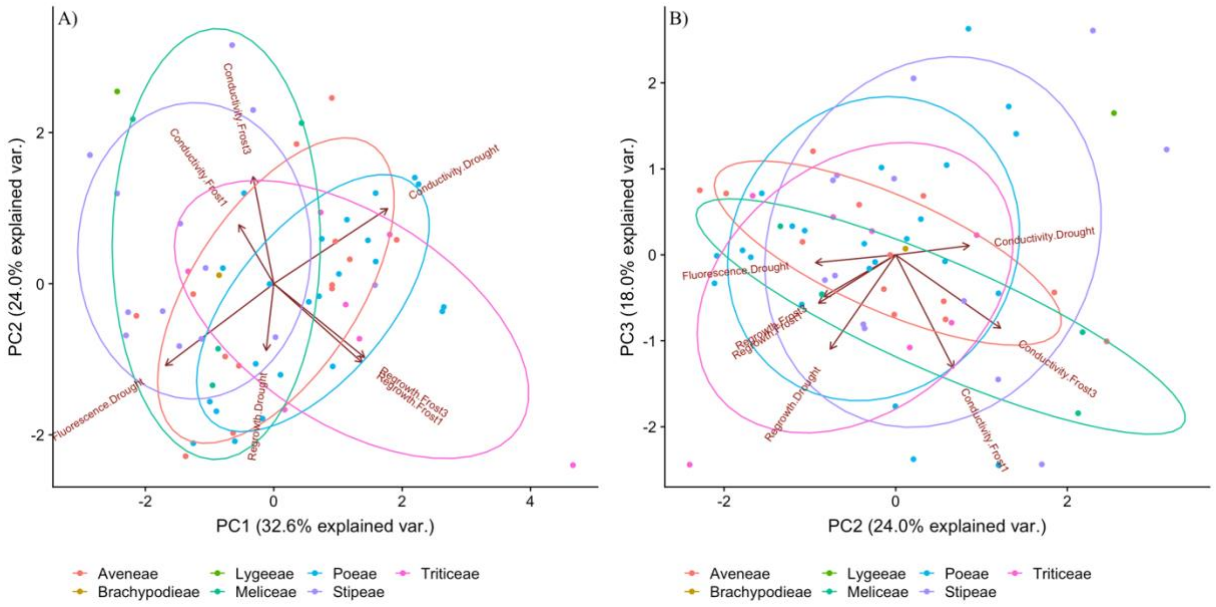

Figure 2: Principal component analysis (PCA) of the experimental variables. A) shows the first two principal components (PC1 and PC2), while B) shows PC2 and PC3. The dots are species and the circles are the distribution of the tribes. The arrows with the traits show in which direction and how much (length of the arrow) each trait contributes to the distribution of the species, in relation to the other traits.

There was autocorrelation among the residuals for the linear regression between regrowth at -1 °C and -3 °C ($P = 0.02$). The phylogenetic regression gave a significant positive correlation between regrowth at -1 °C and -3 °C ($P << 0.001$, $R^2 = 0.32$). In other words, species with high regrowth after mild sudden frost will probably have a higher regrowth after severe sudden frost irrespective of their evolutionary history. For the other trait combinations where there was autocorrelation among the residuals for the pairwise regressions, the phylogenetic regression was not significant ($P > 0.05$, see Appendix III. A for table over these trait combinations).

Pearson's correlation test gave conductivity and fluorescence of the drought treatment a significant, negative correlation ($P << 0.001$, correlation = -0.90). This suggests that when the plant gets damaged (high conductivity) it will have a lower photosynthesis (low fluorescence). In addition, regrowth after sudden frost at -3 °C was significantly, positively correlated with conductivity after drought treatment ($P < 0.05$, correlation = 0.27). This means that species with better regrowth after severe sudden frost treatment have higher conductivity, i.e. are more damaged, after drought treatment. The last significant correlation was for conductivity after sudden frost at -1 °C and -3 °C ($P < 0.05$, correlation = 0.39). No other trait combinations were significantly correlated ($P > 0.05$).

Conductivity for the drought treatment was used in further analyses as a proxy for drought tolerance, since it was significantly, negatively correlated with fluorescence and conductivity showed a phylogenetic signal, whereas fluorescence did not (Table 3). Regrowth after sudden frost at -3 °C was used as a proxy for sudden frost tolerance, due to a significant, positive correlation between regrowth at -1 °C and -3 °C. Both these variables had phylogenetic signal (Table 3) but the more severe frost treatment distinguished responses of the species better, resulting in a response variable (regrowth) with greater variance and a more normal distribution (Appendix II.). Regrowth was chosen instead of conductivity because regrowth showed a phylogenetic signal at both levels, which conductivity did not (Table 3).

3.4. Ancestral stat reconstruction (ASR)

The ASR for drought tolerance shows that the Stipeae and Lygeeae are ancestrally more drought tolerant (lower conductivity; yellow nodes) compared to the rest (Poeae, Aveneae, Triticeae, Brachypodieae and Meliceae; green nodes; Figure 3). This suggests divergence between Stipeae and Lygeeae and the other tribes in the evolution of drought tolerance.

This divergence is also seen in the ASR for sudden frost at -3 °C. Stipeae and Lygeeae have ancestral nodes indicating lower regrowth ability (blue nodes) than for the rest (Poeae, Aveneae, Triticeae, Brachypodieae and Meliceae, green nodes). This pattern is the same in the ASR for sudden frost at -1 °C, only with difference in magnitude of regrowth ability (Appendix III. B1). There is also a slightly higher ancestral frost tolerance in Triticeae and the *Poa* clade of Poeae after sudden frost at -3 °C (more yellow-green, Figure 3).

The pattern in the ASR for drought tolerance is concurrent with the pattern in the ASR for sudden frost tolerance (Figure 3). This pattern indicates that the Stipeae and Lygeeae have higher drought tolerance and lower sudden frost tolerance – both today and ancestrally – to the other tribes (Poeae, Aveneae, Triticeae, Brachypodieae and Meliceae). The other tribes have higher ancestral frost tolerance. There is more variation between the species in the other tribes, for instance in Poeae, compared to Stipeae when looking at the damage (conductivity) caused by drought. However, the overall response for the other tribes is lower tolerance to drought (green nodes) and a higher tolerance for sudden frost (green nodes) than for the Stipeae and Lygeeae (yellow nodes for drought and blue nodes for sudden frost). The most recent common ancestor of Pooideae shows an intermediate frost tolerance relatively to the frost tolerance of the other nodes (regrowth ability after frost; blue-green) and intermediate drought tolerance relatively to the drought tolerance of the other nodes (leaf damage after drought; yellow-green).

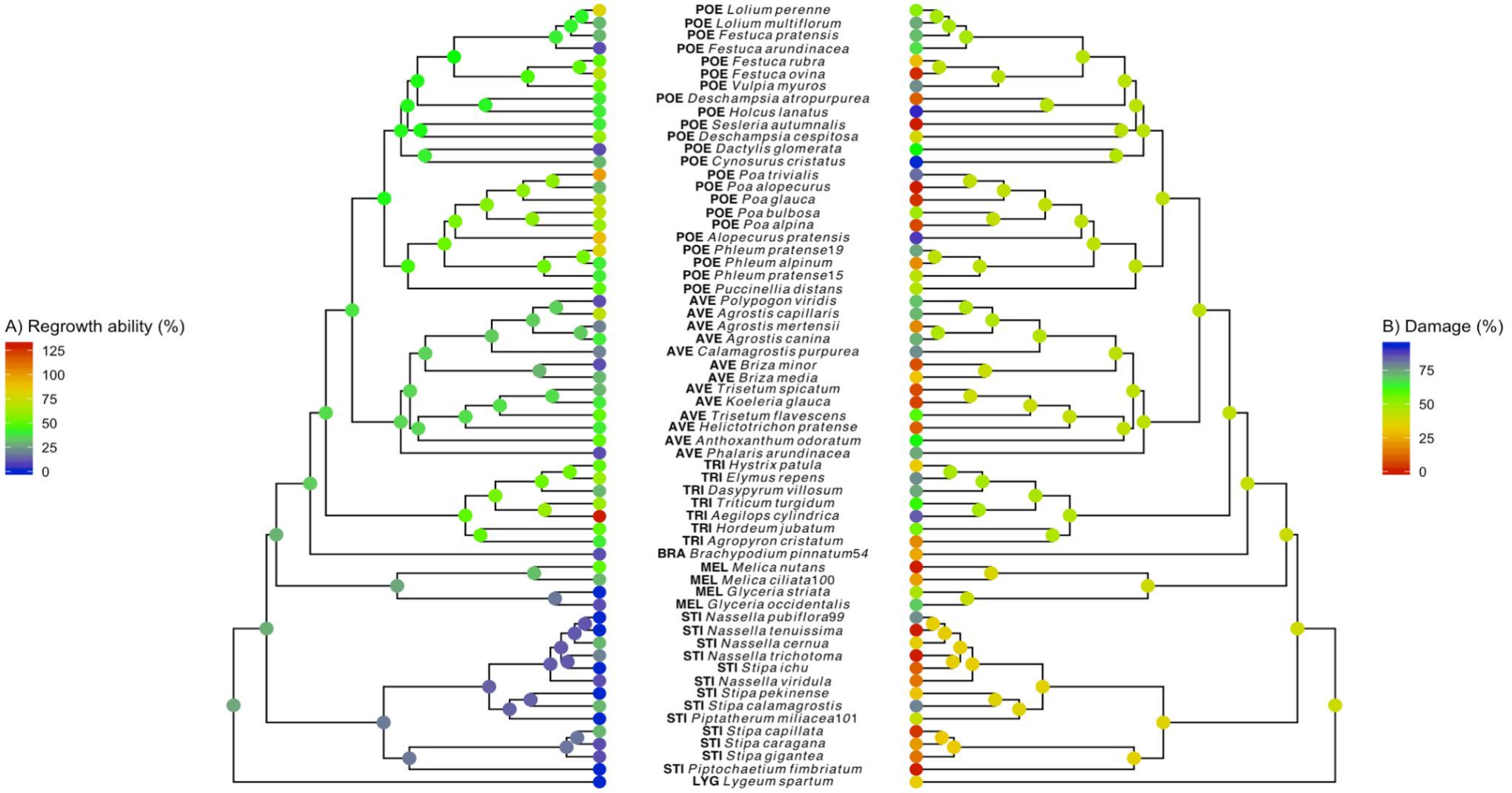

Figure 3: Ancestral State Reconstruction of A) regrowth ability for sudden frost at -3 °C (λ = 0.47) and B) leaf damage (conductivity) for drought (λ = 0/0.11). Red indicates low damage/conductivity and high regrowth, while blue indicates high damage/conductivity and low regrowht. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae. For species where more than one accession was sown, the accession number is indicated.

3.5. Rate shift

For the proxies used, no rate shifts (single rate) were detected in the evolution of drought or sudden frost tolerance (Appendix III. C).

3.6. Water content

There was significant autocorrelation among the residuals of the linear regression for water content and conductivity after drought treatment ($P < 0.05$), and the phylogenetic regression gave a significant result ($P < 0.05$, $R^2 = 0.16$). The regrowth ability after the severe sudden frost treatment had no significant correlation with water content after the Pearson's correlation test ($P > 0.05$).

Due to the lowest value of AICc the lambda model gave the best fit for water content. The phylogenetic signal gave a $\lambda = 0.45$. Figure 4 shows the ASR for water content. As seen above, there is still a divide between the Stipeae and Lygeeae, and the core Pooideae (Poeae, Aveneae and Triticeae), where the core Pooideae has a higher ancestral water content compared to Stipeae and Lygeeae. The ancestral node of Pooideae has an intermediate water content relative to the other nodes.

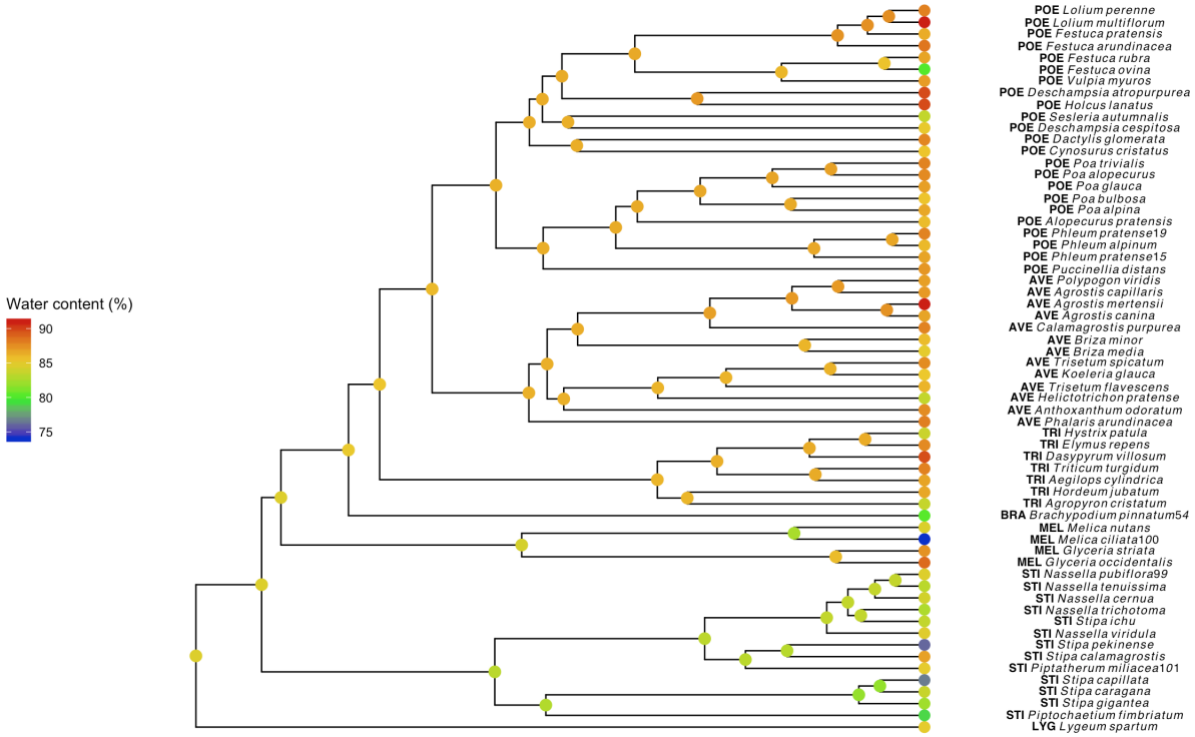

Figure 4: Ancestral State Reconstruction of water content of aboveground biomass ($\lambda = 0.45$)). Red indicates high water content while blue indicates low water content. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae. For species where more than one accession was sown, the accession number is indicated.

3.7. Bioclimatic variables in comparison to the experimental variables

Figure 5 shows the PCA of the experimental and bioclimatic variables for both the average calculated across each species' distribution and the specific locality at which each accession was collected. All the tribes (circles) are overlapping, and there are no distinct clusters among tribes. Both PCAs show that the traits of frost covary with the majority of temperature variables (BIO1-11), while the traits of drought covary with the majority of precipitation variables (BIO12-19). This confirms that the experimental variables carry the expected signatures related to temperature and precipitation, respectively. For both PCAs, eight principal components were needed to explain more that 90 % of the variance.

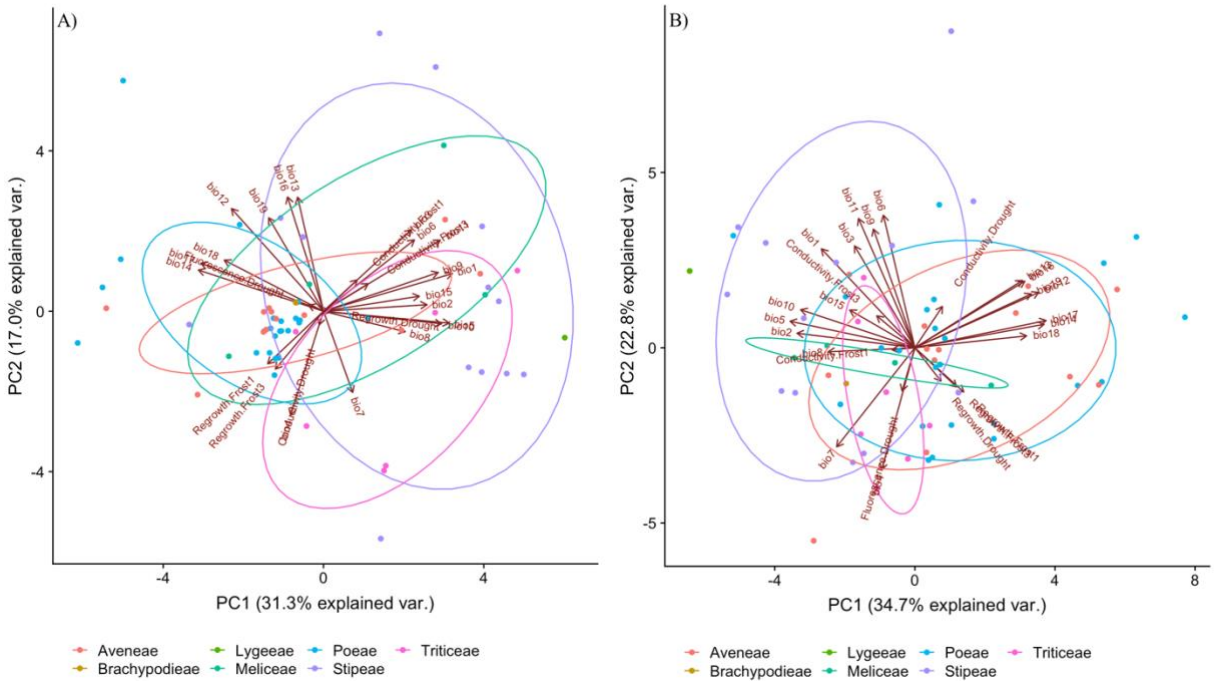

Figure 5: Principal component analysis (PCA) of the experimental traits and A) average bioclimatic variables and B) local bioclimatic variables. The dots are species and the circles are the distribution of the tribes. In relation to each other, the arrows with the traits and bioclimatic variables show in which direction and how much (length of the arrow) each trait and each bioclimatic variable contribute to the distribution of the species. The value increases to the tip of the arrows. **BIO1** = Annual Mean Temperature, **BIO2** = Mean Diurnal Range (Mean of monthly (max temp - min temp)), **BIO3** = Isothermality (BIO2/BIO7) (* 100), **BIO4** = Temperature Seasonality (standard deviation *100), **BIO5** = Max Temperature of Warmest Month, **BIO6** = Min Temperature of Coldest Month, **BIO7** = Temperature Annual Range (BIO5-BIO6), **BIO8** =Mean Temperature of Wettest Quarter, **BIO9** = Mean Temperature of Driest Quarter, **BIO10** = Mean Temperature of Warmest Quarter, **BIO11** = Mean Temperature of Coldest Quarter, **BIO12** = Annual Precipitation, **BIO13** = Precipitation of Wettest Month, **BIO14** = Precipitation of Driest Month, **BIO15** = Precipitation Seasonality (Coefficient of Variation), **BIO16** = Precipitation of Wettest Quarter, **BIO17** = Precipitation of Driest Quarter, **BIO18** = Precipitation of Warmest Quarter, **BIO19** = Precipitation of Coldest Quarter (Hijmans et al., 2005).

Figure 6 shows the PCA for all the temperature variables (BIO1-11) and the selected proxy for sudden frost tolerance (regrowth after sudden frost at -3 °C), for both the average calculated across each species' distribution and the specific locality at which each accession was collected. Both PCAs shows that the experimental variable varies in the opposite direction to the direction of all the temperature variables, with only minor loadings from BIO4 (temperature seasonality) and BIO7 (temperature annual range). This means that species that generally experience warm summers and mild winters had poor regrowth ability following severe frost treatment (primarily those in Stipeae and Triticeae), while species that generally experience cool summers and cold winters had high regrowth ability following severe frost treatment (primarily those in Poeae and Aveneae). Overall, however, the different tribes largely overlapped. For both PCAs, four principal components were needed to explain more that 90 % of the variance.

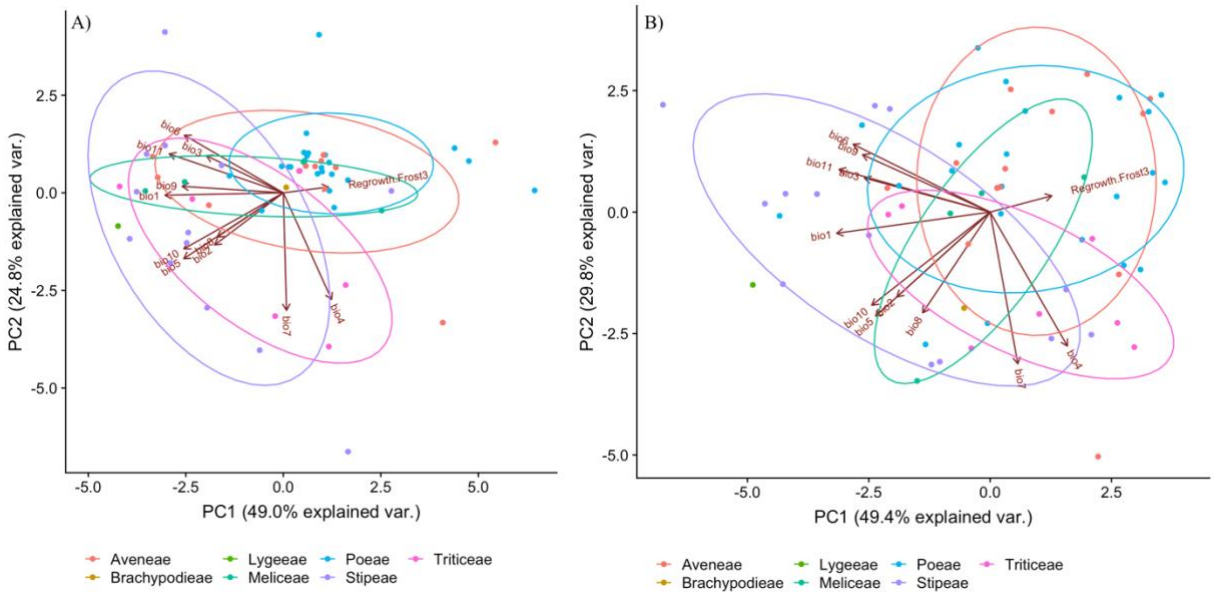

Figure 6: Principal component analysis (PCA) of the regrowth after frost -3ºC and A) average temperature variables and B) locally temperature variables. The arrows with the regrowth after sudden frost and temperature variables show in which direction and how much (length of the arrow) the regrowth and each temperature variable contribute to the distribution of the species, in relation to the other temperature variables. **BIO1** = Annual Mean Temperature, **BIO2** = Mean Diurnal Range (Mean of monthly (max temp - min temp)), **BIO3** = Isothermality (BIO2/BIO7) (*100), **BIO4** = Temperature Seasonality (standard deviation*100), **BIO5** = Max Temperature of Warmest Month, **BIO6** = Min Temperature of Coldest Month, **BIO7** = Temperature Annual Range (BIO5-BIO6), **BIO8** =Mean Temperature of Wettest Quarter, **BIO9** = Mean Temperature of Driest Quarter, **BIO10** = Mean Temperature of Warmest Quarter, **BIO11** = Mean Temperature of Coldest Quarter (Hijmans et al., 2005).

Figure 7 shows the PCA for all the precipitation variables (BIO12-19) and the proxy for drought tolerance (conductivity), for both the average distribution and locally per accession. For the average bioclimatic variables, the experimental variable covaries with BIO14 and 17 and in the opposite direction of BIO13, 15 and 16 (Figure 7A). This suggests that species that responded well to the drought treatment (low conductivity) tend to have most of their rain in a wet season (i.e. a marked dry season with low rainfall), while species that responded poorly to the drought treatment (high conductivity) have a lot of rain in the driest season (i.e. less precipitation seasonality). There are very low loadings of rest of the bioclimatic variables (BIO12,18 and 19), indicating low covariation.

For the local bioclimatic variables, the experimental variable varies in the opposite direction as before and covaries with BIO12, 13, 15, 16, and 19, with the strongest loadings for PC2 for BIO 12, 15 and 16 (Figure 7B). The rest of the bioclimatic variables (BIO14, 17 and 18) have almost a straight angle with the experimental values indicating no covariations. There is no obvious separation of the tribes in either plot. For both PCAs, three principal components were needed to explain more that 90 % of the variance.

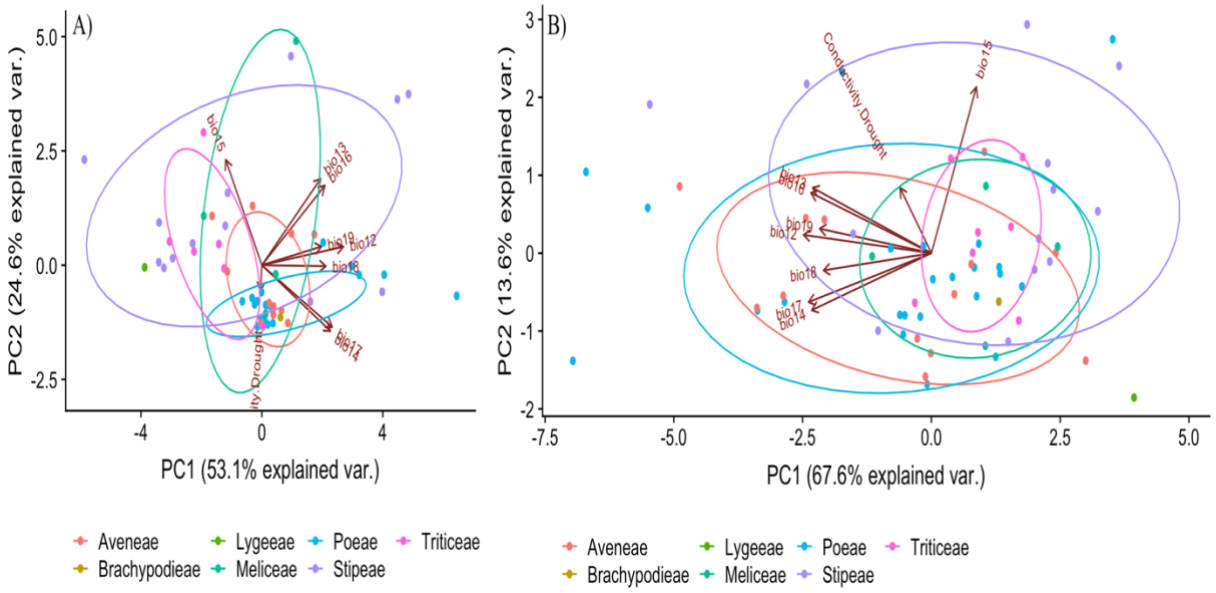

Figure 7: Principal component analysis (PCA) of conductivity after drought and A) average precipitation variables and B) locally precipitation variables. The arrows with the conductivity of drought and precipitation variables show in which direction and how much (length of the arrow) the conductivity and each precipitation variable contribute to the distribution of the species, in relation to the other precipitation variables. **BIO12** = Annual Precipitation, **BIO13** = Precipitation of Wettest Month, **BIO14** = Precipitation of Driest Month, **BIO15** = Precipitation Seasonality (Coefficient of Variation), **BIO16** = Precipitation of Wettest Quarter, **BIO17** = Precipitation of Driest Quarter, **BIO18** = Precipitation of Warmest Quarter, **BIO19** = Precipitation of Coldest Quarter (Hijmans et al., 2005).

4. Discussion

4.1. Evolution of responses to drought and sudden frost

In the drought experiment, most of the core Pooideae have more leaf damage compared to the drought tolerant tribe Stipeae (Figure 3). Bajji et al. (2002) tested whether leaf conductivity could separate between drought tolerant and sensitive durum wheat (*Triticum durum*). They found that the drought sensitive durum wheat had a higher leaf damage (conductivity) compared to the drought tolerant durum wheat. They also argued that their result was in line with other field studies of drought resistance in durum wheat. This indicate that conductivity is a good proxy for drought tolerance and that core Pooideae species are less drought tolerant than species of the Stipeae. However, one weakness of Bajji et al. (2002)'s study, is that they only subjected a segment of the leaf to drought and not the whole plant, which could give different results. Even though my results are in line with what they found, the whole plant was subjected to drought in my experiment. All the plants regrew after the drought treatment (Appendix II) and this result could indicate that only a leaf damage assessment (conductivity) is not enough to tell if the plant is drought tolerant or not. For instance, there could be other mechanisms, e.g. an extensive root system, that enables species to access more water and survive. On the other hand, the drought treatment in this experiment might not have lasted long enough to separate the species based on drought tolerance, and the conductivity may be an indication of what to expect after a more severe drought treatment.

Core Pooideae had a higher water content compare to Stipeae (Figure 4). The higher drought tolerance in the species of the Stipeae compared to the core Pooideae may be explained by the water content of the leaves, which was significantly positively correlated with conductivity (Figure 3 and 4 and correlations test). On the other hand, studies done on wheat types with known different drought resistance, found that wheat with higher initial or relative water content had a higher drought tolerance (Araghi & Assad, 1998; Lonbani & Arzani, 2011; Schonfeld et al., 1988). This might indicate an ability to keep the water and reduce water loss in drought tolerant wheat. However, as most of the plants in my experiment were unaffected by the drought treatment in survival and regrowth (Appendix II. and Appendix III. B2), both results might be right and could reflect different strategies to tolerate drought. Wheat belongs to the core Pooideae, which may have a different evolutionary history when evolving water content level and drought tolerance than species from the Stipeae (Figure 3 and 4). High water content during drought might be a good sign of drought tolerance for the core Pooideae indicating that they have adapted to reduce water loss, whereas the drought tolerance in Stipeae might arise

from other adaptations to tolerate drought. There are different kinds of drought adaptations such as leaf shape, thickness and size, cuticula, hair, water use efficiency, life cycles etc. (Lambers et al., 2008; Taiz et al., 2015) illustrating that it is possible that within Pooideae different types of drought adaptation could evolve. For example, the Stipeae species might have a bigger root system compared to the core Pooideae, because they allocate more of the photosynthetic product to the root than to the shoot, which is the opposite of what the core Pooideae do (Lindberg unpubl.pers.com). This allows for more efficient water search. It could be that less water content would make it possible to obtain CO_2 without losing too much water through the stomata, but this must be verified for the Pooideae in a future study. Stipeae also have more narrow leaves, which is shown to be dominating in open and more dry habitats (Gallaher et al., 2019). Narrow and thick leaves have a lower surface area to volume ratio, which is an adaptation to drought conditions (Aroca, 2012). Drought tolerant plants are also shown to have smaller xylem (Craine et al., 2013). Smaller xylem might indicate less water content, but this is not certain, and future studies of xylem size and how it effects drought tolerance in Pooideae would be of interest.

It has been hypothesized that plant species in dry conditions have evolved drought tolerance via evolution of small xylem conduits, as these lowers the risk of embolism (xylem bubbles, (Blackman et al., 2010; Tyree & Sperry, 1988; Yang & Tyree, 1992)). Embolism can also occur after frost, thus small conduits can also increase freezing tolerance (Choat et al., 2011; Tyree & Sperry, 1989). Watcharamongkol (unpubl.pers.com) found that low water content in cold adapted linages from the so called tropical PACMAD clade (including the grass subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristioideae and Danthonioideae; (Soreng et al., 2015)) increases constitutive freezing tolerance. If this was a physiological mechanism also in Pooideae I would expect that the low water content for the Stipeae would give a high sudden frost tolerance, but low frost tolerance is shown instead. On the contrary, sudden frost tolerance was not dependent on water content, as water content did not show a significant correlation with sudden frost tolerance.

The results of my study showed a strong correlation between photosynthetic activity and leaf damage are in line with Knaupp et al. (2011). This means that when the leaf gets damaged it will reduce photosynthesis. The criteria for not using fluorescence is that it would not be possible to perform an ASR, due to lack of a phylogenetic signal. This would make it difficult to answer the research question. However, it should be noted that another study concludes that

fluorescence is a better measure for drought tolerance compared to conductivity, because it potentially gave more reliable estimates and it was easier to distinguish between high and low tolerance (Sayar et al., 2008).

4.2. Species tolerance and habitat climate

Schubert et al. (2019) found that *Stipa lagasceae* and *Nardus stricta* from the early diverging lineages had high intrinsic frost tolerance compared to core Pooideae species. Based on this I hypothesized that earlier diverging lineages would have higher resistance to sudden frost. I further hypothesized that this may be related to low seasonality as species in such areas would experience sudden frost more often without being acclimated, especially if the temperature difference between day and night is high. However, the PCA (Figure 6) shows the opposite: species found in climates with low seasonality are associated with warmer climate. These species have low frost tolerance (e.g. Stipeae), whereas species with high sudden frost tolerance (e.g. Poeae and Aveneae) are found in places where there are more changes between seasons and more severe and long winters. This might indicate that more complex frost tolerance mechanisms enable the plants also to better cope with sudden frost. Moreover, my results reflect that species in areas with low precipitation have higher resistance to drought due to low leaf damage (Figure 7). These results show that adaptations to climatic niches follow species distributions. However, based on these results it is difficult to say if species evolve adaptations in place as climate changes, or if they evolved adaptations first and then moved into the climatic niches.

The PCAs which are based on the average bioclimatic variables were more reliable than the bioclimatic variables taken from the more local sites, because most of the species lacked coordinates for their sample site or a country midpoint coordinate from the sample site was taken (Appendix I. A). Thus, the average bioclimatic variables have been used in the interpretation of the proxies.

4.3. Species with high sudden frost tolerance did not have high drought tolerance

In contrast to prediction I (that species with high sudden frost tolerance will have high drought tolerance), the results show that species with high sudden frost tolerance had low drought tolerance (Figure 3 and correlation test). Thus, it does not seem as frost tolerance has evolved from drought tolerance. One interesting finding in my results is the divide in both drought and frost tolerance between core Pooideae and the early diverging linages (Figure 3). The core

Pooideae had a higher sudden frost tolerance compared to the early diverging linages. This is the opposite of what Schubert et al. (2019) found, where the early diverging linages showed a higher frost tolerance when not acclimated compared to the core Pooideae. This difference could be explained by the number of species tested. Schubert et al. (2019) looked at few species (9 species representing 6 tribes), while in this study I have used many species (61 species representing 7 tribes). Furthermore, different species were used. For instance, *Nardus stricta* was not a part of the statistical analyses in my study due to lack of germination, while it was representing one of the three early diverging species in the study of Schubert et al. (2019). A higher sudden frost tolerance for the core Pooideae is in line with the species distribution. Most of the species in the northernmost latitudes belongs to the core Pooideae tribes, which are adapted to frost (Fjellheim et al., 2014) and they will probably experience more of both episodic and periodic frost, while most of the early diverging linages do not enter the subarctic zone (Bouchenak-Khelladi et al., 2010; Hultén & Fries, 1986; Schubert et al., 2018). This is also reflected in the analysis of climatic data showing that the species with the highest frost tolerance indeed was found in areas with cold climate and strong seasonality (Figure 6).

Another possible explanation to the observed pattern between core Pooideae and the Stipeae could be that core Pooideae and the early diverging linages started to separate phylogenetically over 45 Mya (Schubert et al., 2018) and this led to independent evolution of frost and drought tolerance. The geographically separation of the major lineages at the turn of the Eocene approximately 34 Mya (Schubert et al., 2018) in combination with a new and more severe temperature drop at approximately 15 Mya (Zachos et al., 2001), could have enhanced the differentiation in frost and drought tolerant mechanisms between core Pooideae and Stipeae (Figure 8). To adapt to cold, sub-zero conditions requires evolutionary changes in a suite of complex mechanisms (Larcher, 2003). This require energy and the drought tolerance might have been lost as a tradeoff for the core Pooideae.

It should be mentioned that the majority of *Stipa* species are found in places where sudden frost often occurs (Larcher, 2005). However, for most of the *Stipa* in this experiment the sample location is unknown (Appendix I. A). If the majority of these species that represent the Stipeae tribe are found in warm locations with little sudden frost, and none comes from locations with frost, the evolutionary signal would probably show low frost tolerance and high drought tolerance. This is a limitation of the study and could have an impact. Yet, it is not likely that none of the *Stipa* included in this experiment have experienced frost. Ideally, the species

representing one tribe should cover the climatic zones from where the tribe is distributed. More tribes from the early diverging linages should also have been included in the study.

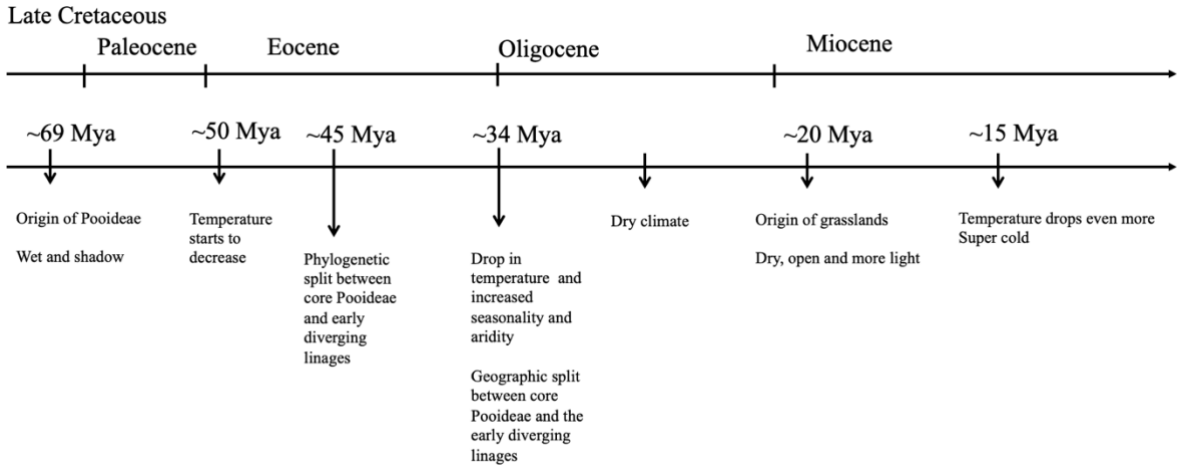

Figure 8: Thought course of events that might have caused frost and drought tolerance within Pooideae. Dates for evolutionary and climatic events are taken from (Bouchenak-Khelladi et al., 2010; Schubert et al., 2018; Strömberg, 2011). The timeline is not scaled.

4.4. Did drought tolerance evolve before sudden frost tolerance (prediction II)?

I predicted that if tolerance to frost has evolved from a drought tolerance response, tolerance to drought should have evolved before tolerance to frost (Prediction II). This prediction is not supported in this study because the most ancestral node shows a sudden frost and drought tolerance somewhere in between frost and drought tolerance of the core Pooideae and the early diverging linages (bluegreen color for sudden frost and yellowgreen for drought tolerance, Figure 3). Furthermore, no rate shift was detected that could indicate different rate of evolution of drought or sudden frost tolerance within Pooideae. From these results, it is impossible to distinguish if drought tolerance evolved before or after sudden frost tolerance.

However, there are some indications that frost tolerance evolved before drought tolerance in Pooideae. The medium drought tolerance expressed by the ancestral node of Pooideae in my experiment corroborate with a shift towards a drier habitat, through forest margins, for both the core Pooideae and the Stipeae later in the evolution of Pooideae during Oligocene(Bouchenak-Khelladi et al., 2010). In line with my findings, other studies have shown that the common ancestor of Pooideae evolved in closed forest habitats (Bouchenak-Khelladi et al., 2010; Gallaher et al., 2019; Strömberg, 2011), indicating more humid environments. The increased

seasonality and aridity during Eocene (~34 Mya) in companion with a cooler climate led to a drier Oligocene (Bouchenak-Khelladi et al., 2010). Several studies has shown that Pooideae diversified into open and more dry habitats, and grasslands came to be dominating, during Oligocene or in Miocene approximately 20 Mya (Bouchenak-Khelladi et al., 2010; Strömberg, 2011). This shift from wet to dry habitats may indicate that drought tolerance evolved after frost tolerance, since the temperature had already started to decrease during Eocene and early Oligocene (Zachos et al., 2001). The major tribes within Pooideae were already established when Pooideae diversified into open habitats (Bouchenak-Khelladi et al., 2010; Schubert et al., 2018), implying that drought adaptations evolved independently in different lineages.

Again, it seems more likely that sudden frost tolerance evolved before drought tolerance. In this experiment, frost resistance is inherited while the drought resistance is likely due to local adaptations. Figure 3 shows that freezing resistance is determined by phylogenetic history, but drought resistance is not, and the phylogenetic signal was stronger for frost tolerance compared to the weak phylogenetic signal from drought tolerance. While Zanne et al. (2014) suggest that evolving small xylem conduits preadapted plants to dry habitats before they migrated into cold climates, my results seem to indicate the opposite (see 4.1.). Schubert et al. (2018) found that the ancestors of all the Pooideae linages experienced freezing temperatures, possibly in a cold, microclimatic niche in emerging mountains in the middle East (Schubert et al., 2018). The set of 16 genes with a shared cold responses also relate to drought responses (Schubert et al., 2019) and could indicate a common drought tolerance within Pooideae. This is not contradicted by my results (Figure 3), but the drought tolerance we see in the most tolerant groups here, i.e. the Stipeae is of more recent origin (Figure 3). Pooideae has since a possible primitive frost-response in a drought-tolerant Pooideae ancestor evolved much more advanced freezing tolerance mechanisms (Bouchenak-Khelladi et al., 2010; Strömberg, 2011), thus abandoning or refining the initial response. Furthermore, the transcriptomic responses to cold acclimation in five diverging Pooideae species show that very few responses to cold were conserved (Schubert et al., 2019). This is not surprising since all major tribes diverged before the steep drop in temperature at the E-O split 34 Mya. A similar result may be expected for a comparison of transcriptomic responses to drought across Pooideae, since all the large tribes had diverged before dry climate emerged.

5. Further perspectives

Since drought tolerance, in different ways, is assumed to be a key factor in evolving land plants from water plants (Oliver et al., 2000; Zhao et al., 2019), drought tolerance might have been evolved, gone dormant or lost and regained several times in evolutionary history and cannot be disregarded as a preadaptation to frost tolerance. To get a clearer answer to the question, weather drought tolerance could be a preadaptation to frost tolerance within Pooideae, it might be a good idea to look beyond the subfamily and into whole grass family instead. Furthermore, a drought experiment which better discriminate between drought tolerance among species at the level of regrowth and/or survival could also give more insight. Root measurements in combinations with leaf damage or photosynthesis and regrowth could help enlighten the whole plant responses to drought and frost. A large-scale, multi-species transcriptomic experiment could compare genetic responses to frost and drought within the same species and reveal if there are the same genes that play a role in both drought and sudden frost responses. Following this, the number of genes that are common for the responses in different species across the phylogeny could be compared to see if evolution of these responses is similar in phylogenetically diverse species.

This study provides the first large-scale study of drought and sudden frost responses in a large set of phylogenetically diverse species in Pooideae and has given new insight into the evolution of stress-responses in a group of grasses inhabiting the most extreme climates. There are few, if any, other large-scale, phylogenetic studies on this topic present today and this study may serve as an example for similar studies in other plant groups. Drought and frost responses have complex mechanisms and in future studies it might be useful to compare with other plant families to see if the responses evolve in the same way.

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Appendix

Appendix I. Species lists

A. Table of species selected, sown and analyzed in the experiment. The table shows the experimental population number, tribe, the accepted scientific name, seed ID, source of the seeds and the country where they origin from. Species included in the statistical analyses are printed in bold type. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, DIA = Diarrheneae, MEL = Meliceae, PHA= Phaenospermateae, LYG = Lygeeae, NAR = Nardeae and STI = Stipeae. Outgroup are species from tribes outside of the subfamily Pooideae.

Number	Tribe	Species name from source	Accepted name from The Plant List	Seed ID	Source	Country
SR1	POE	*Phippsia algida*	*Phippsia algida*	-	Sampled in wild	Norway
SR2	POE	*Deschampsia flexuosa*	*Deschampsia flexuosa*	-	Sampled in wild	Norway
SR3	**POE**	***Poa trivialis***	***Poa trivialis***	**18304,1**	**NGB**	**Finland**
SR4	**POE**	***Deschampsia cespitosa***	***Deschampsia cespitosa***	**11127,2**	**NGB**	**Norway**
SR5	**POE**	***Poa alpina***	***Poa alpina***	**1197,2**	**NGB**	**Sweden**
SR6	**POE**	***Phleum alpinum***	***Phleum alpinum***	**1342,3**	**NGB**	**Sweden**
SR7	**POE**	***Lolium perenne***	***Lolium perenne***	**4262,2**	**NGB**	**Norway**
SR8	**POE**	***Dactylis glomerata***	***Dactylis glomerata***	**7723,1**	**NGB**	**Norway**
SR9	**POE**	***Poa alopecurus***	***Poa alopecurus***	**0662293**	**RBG Kew**	**Falkland islands**
SR10	**POE**	***Poa bulbosa***	***Poa bulbosa***	**0176493**	**RBG Kew**	**Jordan**
SR11	**POE**	***Festuca pratensis***	***Festuca pratensis***	**0055789**	**RBG Kew**	**Switzerland**
SR12	POE	*Milium effusum*	*Milium effusum*	7296	Plant World Seeds	Unknown
SR13	**POE**	***Sesleria autumnalis***	***Sesleria autumnalis***	**GRA3624**	**IPK**	**Germany**
SR14	**POE**	***Vulpia myuros***	***Vulpia myuros***	**GRA2908**	**IPK**	**Spain**
SR15	**POE**	***Phleum nodosum***	***Phleum pratense***	**PI319076**	**Grin**	**Spain**
SR16	**POE**	***Puccinellia distans***	***Puccinellia distans***	**PI502580**	**Grin**	**Russian Federation**
SR17	**POE**	***Festuca rubra***	***Festuca rubra***	**PI595056**	**Grin**	**Norway**
SR18	**POE**	***Festuca arundinacea***	***Festuca arundinacea***	**PI601418**	**Grin**	**USA**
SR19	**POE**	***Phleum pratense***	***Phleum pratense***	**PI321682**	**Grin**	**France**
SR20	**POE**	***Holcus lanatus***	***Holcus lanatus***	**PI442500**	**Grin**	**Belgium**
SR21	**POE**	***Festuca ovina***	***Festuca ovina***	**PI676237**	**Grin**	**Germany**
SR22	**POE**	***Cynosurus cristatus***	***Cynosurus cristatus***	**16615,2**	**NGB**	**Sweeden**
SR23	**POE**	***Alopecurus pratensis***	***Alopecurus pratensis***	**13377,1**	**NGB**	**Norway**
SR24	**POE**	***Lolium multiflorum***	***Lolium multiflorum***	**13320,1**	**NGB**	**Denmark**
SR25	**POE**	***Vahlodea atropurpurea***	***Deschampsia atropurpurea***	**-**	**Sampled in wild**	**Norway**
SR26	**POE**	***Poa glauca***	***Poa glauca***	**-**	**Sampled in wild**	**Norway**
SR27	AVE	*Avena fatua*	*Avena fatua*	9271,3	NGB	Norway
SR28	**AVE**	***Anthoxanthum odoratum***	***Anthoxanthum odoratum***	**18256,2**	**NGB**	**Finland**
SR29	**AVE**	***Phalaris arundinacea***	***Phalaris arundinacea***	**4199.3**	**NGB**	**Norway**
SR30	**AVE**	***Calamagrostis purpurea***	***Calamagrostis purpurea***	**2172.1**	**NGB**	**Norway**
SR31	**AVE**	***Agrostis canina***	***Agrostis canina***	**4356,2**	**NGB**	**Sweden**
SR32	**AVE**	***Polypogon viridis***	***Polypogon viridis***	**0081773**	**RBG Kew**	**Lesotho**
SR33	**AVE**	***Helictotrichon pratense***	***Helictotrichon pratense***	**GRA513**	**IPK**	**Germany**
SR34	AVE	*Ammophila arenaria*	*Ammophila arenaria*	GRA2692	IPK	Polen
SR35	**AVE**	***Koeleria glauca***	***Koeleria glauca***	**W613215**	**Grin**	**Kazakhstan**
SR36	**AVE**	***Trisetum flavescens***	***Trisetum flavescens***	**PI422495**	**Grin**	**Germany**
SR37	**AVE**	***Briza minor***	***Briza minor***	**PI204410**	**Grin**	**Turkey**
SR38	**AVE**	***Briza media***	***Briza media***	**PI350681**	**Grin**	**Netherlands**
SR39	**AVE**	***Agrostis capillaris***	***Agrostis capillaris***	**4209,2**	**NGB**	**Norway**
SR40	**AVE**	***Trisetum spicatum***	***Trisetum spicatum***	**-**	**Sampled in wild**	**Norway**
SR41	**AVE**	***Agrostis mertensii***	***Agrostis mertensii***	**-**	**Sampled in wild**	**Norway**
SR42	TRI	*Leymus arenarius*	*Leymus arenarius*	9977,1	NGB	Iceland
SR43	**TRI**	***Elymus repens***	***Elymus repens***	**90282.2**	**NGB**	**Former Soviet Union**

SR44	**TRI**	***Triticum turgidum***	***Triticum turgidum***	**22751,1**	**NGB**	**Sweden**
SR45	**TRI**	***Aegilops triuncialis***	***Aegilops triuncialis***	**AE1557**	**IPK**	**Unknown**
SR46	TRI	*Bromus erectus*	*Bromus erectus*	PI598591	Grin	Kazakhstan
SR47	TRI	*Elymus hystrix*	*Hystrix patula*	W649580	Grin	USA
SR48	**TRI**	***Hordeum jubatum***	***Hordeum jubatum***	**-**	**Impecta**	**Unknown**
SR49	TRI	*Hordeum fuegianum*	*Hordeum fuegianum*	6471,3	NGB	Chile
SR50	**TRI**	***Dasypyrum villosum***	***Dasypyrum villosum***	**6594,1**	**NGB**	**Greece**
SR51	TRI	*Leymus alaicus*	*Leymus alaicus*	90432,4	NGB	Tadzhikistan
SR52	**TRI**	***Agropyron cristatum***	***Agropyron cristatum***	**90257,1**	**NGB**	**Former Soviet Union**
SR53	BRA	*Brachypodium pinnatum*	*Brachypodium pinnatum*	0036898	RBG Kew	Italy
SR54	**BRA**	***Brachypodium rupestre***	***Brachypodium pinnatum***	**PI440172**	**Grin**	Russian Federation
SR55	DIA	*Diarrhena americana*	*Diarrhena americana*	405986	B and T World Seeds	Unknown
SR56	MEL	*Melica rigida*	*Melica rigida*	PI 477090	Grin	Uruguay
SR57	**MEL**	***Melica nutans***	***Melica nutans***	**GRA512**	**IPK**	**Germany**
SR58	**MEL**	***Glyceria striata***	***Glyceria striata***	**W650682**	**Grin**	**USA**
SR59	MEL	*Glyceria canadensis*	*Glyceria canadensis*	W648862	Grin	USA
SR60	MEL	*Melica ciliata*	*Melica ciliata*	PI253453	Grin	Former Serbia and Montenegro
SR61	**MEL**	***Glyceria occidentalis***	***Glyceria occidentalis***	**Ames31334**	**USDA ISU**	USA
SR62	**STI**	***Nassella hyalina***	***Nassella hyalina***	**PI 289543**	**Grin**	Argentina
SR63	STI	*Nassella pubiflora*	*Nassella pubiflora*	PI478575	Grin	Peru
SR64	**STI**	***Stipa capillata***	***Stipa capillata***	**ZA394**	**Jelitto Perennial Seeds**	**Unknown**
SR65	**STI**	***Stipa extremiorientalis***	***Stipa pekinense***	**ZA398**	**Jelitto Perennial Seeds**	**Unknown**
SR66	**STI**	***Stipa gigantea***	***Stipa gigantea***	**ZA400**	**Jelitto Perennial Seeds**	**Unknown**
SR67	**STI**	***Stipa ichu***	***Stipa ichu***	**ZA399**	**Jelitto Perennial Seeds**	**Unknown**
SR68	STI	*Stipa pennata*	*Stipa pennata*	ZA402	Jelitto Perennial Seeds	Unknown
SR69	STI	*Stipa pulcherrima*	*Stipa pulcherrima*	ZA404	Jelitto Perennial Seeds	Unknown
SR70	**STI**	***Stipa tenuissima***	***Nassella tenuissima***	**ZA407**	**Jelitto Perennial Seeds**	**Unknown**
SR71	**STI**	***Stipa trichotoma***	***Nassella trichotoma***	**ZA406**	**Jelitto Perennial Seeds**	**Unknown**
SR72	STI	*Stipa ucrainica*	*Stipa zalesskii*	ZA408	Jelitto Perennial Seeds	Unknown
SR73	**STI**	***Piptochaetium fimbriatum***	***Piptochaetium fimbriatum***	**0093527**	**RBG Kew**	**USA**
SR74	STI	*Stipa comata*	*Stipa comata*	0170893	RBG Kew	USA
SR75	STI	*Stipa diegoensis*	*Stipa diegoensis*	0440763	RBG Kew	USA
SR76	STI	*Oryzopsis hymenoides*	*Oryzopsis hymenoides*	0201276	RBG Kew	Unknown
SR77	STI	*Oryzopsis contracta*	*Oryzopsis contracta*	0393478	RBG Kew	Unknown
SR78	STI	*Piptatherum miliaceum*	*Piptatherum miliaceum*	0049845	RBG Kew	Greece
SR79	**STI**	***Nassella cernua***	***Nassella cernua***	**0527992**	**RBG Kew**	**USA**
SR80	STI	*Piptatherum munroi*	*Piptatherum munroi*	0013574	RBG Kew	India
SR81	STI	*Oryzopsis miliacea*	*Piptatherum miliaceum*	7296	Plant World Seeds	Unknown
SR82	**STI**	***Achnatherum calamagrostis***	***Stipa calamagrostis***	**GRA2848**	**IPK**	**Spain**
SR83	STI	*Ampelodesmos mauritanica*	*Ampelodesmos mauritanica*	62975	B and T World Seeds	Unknown
SR84	STI	*Hesperostipa neomexicana*	*Stipa neomexicana*	W627071	Grin	USA
SR85	STI	*Macrochloa tenacissima*	*Stipa tenacissima*	PI315875	Grin	Slovakia
SR86	STI	*Piptochaetium napostaense*	*Piptochaetium napostaense*	PI202062	Grin	Argentina
SR87	STI	*Austrostipa scabra*	*Stipa scabra*	2AUSSCAB	AustraHort	Unknown
SR88	STI	*Austrostipa bigeniculata*	*Stipa bigeniculata*	2AUSBIGE	AustraHort	Unknown
SR89	**STI**	***Stipa conferta***	***Stipa caragana***	**0775014**	**RBG Kew**	Kyrgyzstan
SR90	STI	*Achnatherum bromoides*	*Stipa bromoides*	0053109	RBG Kew	Greece
SR91	PHA	*Duthiea brachypodium*	*Duthiea brachypodium*	W623539	Grin	China
SR92	**LYG**	***Lygeum spartum***	***Lygeum spartum***	**0185109**	**RBG Kew**	**Egypt**
SR93	NAR	*Nardus stricta*	*Nardus stricta*	GRA936	IPK	Germany
SR94	Out - group	*Molinia caerulea*	*Molinia caerulea*	-	Sampled in wild	Norway

SR95	Out-group	*Danthonia sericea*	*Danthonia sericea*	0480734	RBG Kew	USA
SR96	Out-group	*Cenchrus ciliaris*	*Cenchrus ciliaris*	0098496	RBG Kew	Botswana
SR97	Out-group	*Acroceras calcicola*	*Acroceras calcicola*	0099378	RBG Kew	Madagascar
SR98	STI	*Piptatherum miliaceum*	*Piptatherum miliaceum*	-	Research group	Unknown
SR99	**STI**	***Nassella pubiflora***	***Nassella pubiflora***	**PI478575**	**Grin**	**Peru**
SR100	**MEL**	***Melica ciliata***	***Melica ciliata***	**PI494705**	**Grin**	**Romania**
SR101	**STI**	***Oryzopsis miliacea***	***Piptatherum miliaceum***	**PI207772**	**Grin**	**Israel**
SR102	Out group	*Ehrharta calycina*	*Ehrharta calycina*	PI284803	Grin	Australia

B. Table of the annual species sown for the experiment. Species included in the statistical analyses are printed in bold type. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, STI = Stipeae. Outgroup are species from tribes outside of the subfamily Pooideae. The number is the experimental population number.

Number	Tribe	Species
SR14	**POE**	***Vulpia myuros***
SR24	**POE**	***Lolium multiflorum***
SR27	AVE	*Avena fatua*
SR37	**AVE**	***Briza minor***
SR44	**TRI**	***Triticum turgidum***
SR45	**TRI**	***Aegilops triuncialis***
SR50	**TRI**	***Dasypyrum villosum***
SR85	STI	*Stipa tenacissima*
SR97	Outgroup	*Acroceras calcicola*

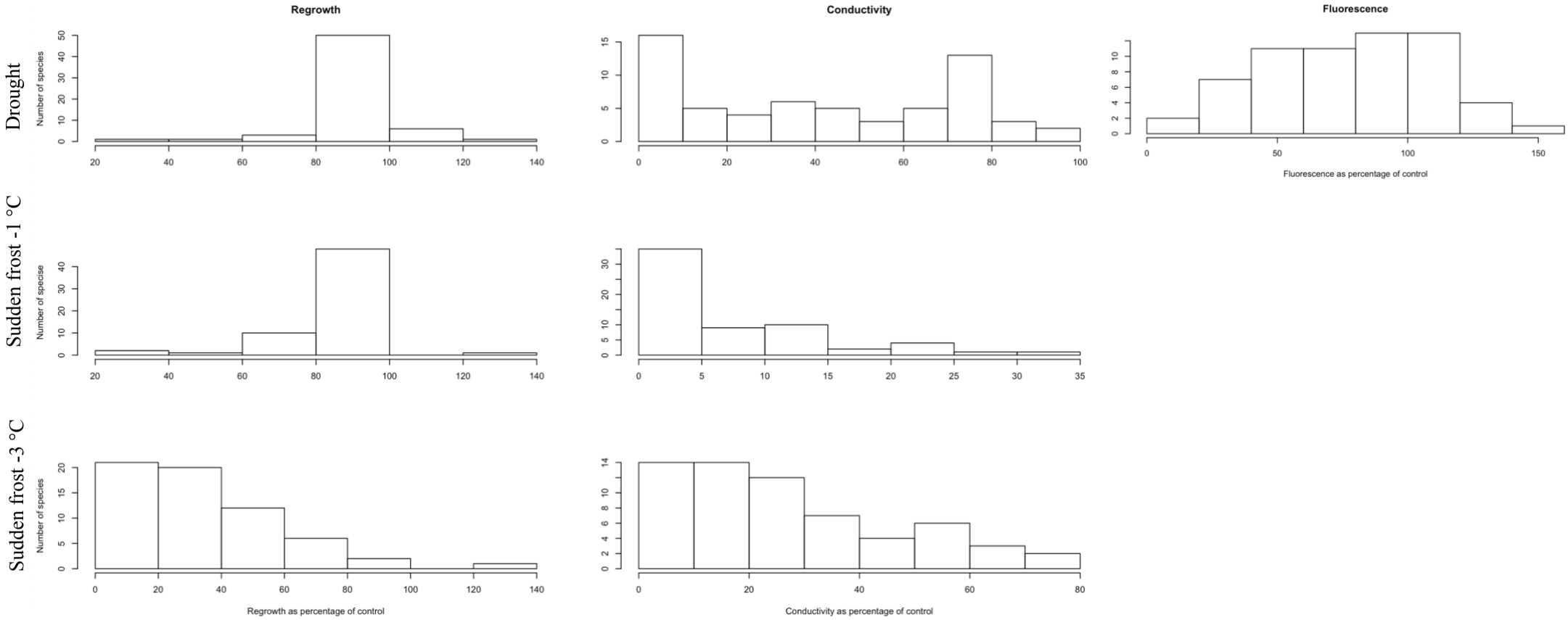
C. Table of the number of individuals per species in each treatment group used in the statistical analyses. The number is the experimental population number.

Number	Species	Control	Drought	Sudden frost -1	Sudden frost -3	Weight	Start conductivity	Total
SR3	Poa trivialis	10	10	10	10	4	4	**48**
SR4	Deschampsia cespitosa	10	10	10	10	4	4	**48**
SR5	Poa alpina	11	10	10	10	4	4	**49**
SR6	Phleum alpinum	10	10	10	10	4	4	**48**
SR7	Lolium perenne	10	10	10	10	4	4	**48**
SR8	Dactylis glomerata	10	10	10	10	4	4	**48**
SR9	Poa alopecurus	6	8	9	9	4	4	**40**
SR10	Poa bulbosa	10	10	10	10	4	4	**48**
SR11	Festuca pratensis	5	9	9	9	4	4	**40**
SR13	Sesleria autumnalis	10	10	10	10	4	4	**48**
SR14	Vulpia myuros	10	10	10	10	4	4	**48**
SR15	Phleum pratense	10	10	10	10	4	4	**48**
SR16	Puccinellia distans	10	10	10	10	4	4	**48**
SR17	Festuca rubra	9	10	10	10	4	4	**47**
SR18	Festuca arundinacea	10	10	10	10	4	4	**48**
SR19	Phleum pratense	10	10	10	10	4	4	**48**
SR20	Holcus lanatus	10	10	10	10	4	4	**48**
SR21	Festuca ovina	10	10	10	10	4	4	**48**
SR22	Cynosurus cristatus	10	10	10	10	4	4	**48**
SR23	Alopecurus pratensis	10	10	10	10	4	4	**48**
SR24	Lolium multiflorum	10	10	10	10	4	4	**48**
SR25	Deschampsia atropurpurea	10	10	10	10	4	4	**48**
SR26	Poa glauca	10	10	10	10	4	4	**48**
SR28	Anthoxanthum odoratum	10	10	10	10	4	4	**48**
SR29	Phalaris arundinacea	10	10	10	10	4	4	**48**
SR30	Calamagrostis purpurea	10	10	10	10	4	4	**48**
SR31	Agrostis canina	10	10	10	10	4	4	**48**
SR32	Polypogon viridis	7	8	8	8	4	4	**39**
SR33	Helictotrichon pratense	10	9	10	10	4	4	**47**
SR35	Koeleria glauca	10	9	10	10	4	4	**47**
SR36	Trisetum flavescens	10	10	10	10	4	4	**48**
SR37	Briza minor	10	10	10	10	4	4	**48**
SR38	Briza media	10	10	10	10	4	4	**48**
SR39	Agrostis capillaris	10	10	10	10	4	4	**48**
SR40	Trisetum spicatum	8	10	10	10	4	4	**46**
SR41	Agrostis mertensii	5	5	6	6	4	3	**29**
SR43	Elymus repens	10	10	10	10	4	4	**48**
SR44	Triticum turgidum	10	10	10	10	4	4	**48**
SR45	Aegilops triuncialis	10	10	10	10	4	4	**48**
SR47	Hystrix patula	10	10	10	10	4	4	**48**
SR48	Hordeum jubatum	10	10	10	10	4	4	**48**
SR50	Dasypyrum villosum	10	10	10	10	4	4	**48**
SR52	Agropyron cristatum	6	9	9	9	4	3	**40**
SR54	Brachypodium pinnatum	10	10	10	10	4	4	**48**
SR57	Melica nutans	10	10	10	10	4	4	**48**
SR58	Glyceria striata	10	10	10	10	4	4	**48**
SR61	Glyceria occidentalis	10	10	10	10	4	4	**48**
SR62	Nassella hyalina	10	10	10	10	4	4	**48**
SR64	Stipa capillata	10	10	10	10	4	4	**48**
SR65	Stipa pekinense	10	10	10	10	4	4	**48**
SR66	Stipa gigantea	10	10	10	10	4	4	**48**
SR67	Stipa ichu	10	10	10	10	4	4	**48**
SR70	Nassella tenuissima	9	9	9	9	4	4	**44**
SR71	Nassella trichotoma	10	10	10	10	4	4	**48**
SR73	Piptochaetium fimbriatum	6	6	6	6	4	4	**32**
SR79	Nassella cernua	10	10	10	10	4	4	**48**
SR82	Stipa calamagrostis	10	10	10	10	4	4	**48**
SR89	Stipa caragana	6	9	9	9	4	4	**41**
SR92	Lygeum spartum	5	8	8	8	4	4	**37**
SR99	Nassella pubiflora	10	10	10	10	4	4	**48**
SR100	Melica ciliata	10	9	10	10	4	4	**47**
SR101	Piptatherum miliaceum	10	10	10	10	4	4	**48**
Total		**583**	**598**	**603**	**603**	**248**	**246**	**2881**

D. The experimental names and the names in the phylogeny of Schubert et al (in press). The names printed in bold type are species placed at different species in the phylogeny. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, PHLYG = Lygeeae, and STI = Stipeae. The number is the experimental population number.

Tribe	Number	Species update	Name in phylogeny
POE	**SR3**	***Poa trivialis***	***Poa pratensis***
POE	SR4	*Deschampsia cespitosa*	*Deschampsia cespitosa*
POE	SR5	*Poa alpina*	*Poa alpina*
POE	SR6	*Phleum alpinum*	*Phleum alpinum*
POE	SR7	*Lolium perenne*	*Lolium perenne*
POE	SR8	*Dactylis glomerata*	*Dactylis glomerata*
POE	**SR9**	***Poa alopecurus***	***Poa billardierei***
POE	**SR10**	***Poa bulbosa***	***Poa annua***
POE	SR11	*Festuca pratensis*	*Festuca pratensis*
POE	SR13	*Sesleria autumnalis*	*Sesleria autumnalis*
POE	SR14	*Vulpia myuros*	*Vulpia myuros*
POE	**SR15**	***Phleum pratense***	***Phleum arenarium***
POE	SR16	*Puccinellia distans*	*Puccinellia distans*
POE	SR17	*Festuca rubra*	*Festuca rubra*
POE	SR18	*Festuca arundinacea*	*Festuca arundinacea*
POE	SR19	*Phleum pratense*	*Phleum pratense*
POE	SR20	*Holcus lanatus*	*Holcus lanatus*
POE	SR21	*Festuca ovina*	*Festuca ovina*
POE	SR22	*Cynosurus cristatus*	*Cynosurus cristatus*
POE	SR23	*Alopecurus pratensis*	*Alopecurus pratensis*
POE	SR24	*Lolium multiflorum*	*Lolium multiflorum*
POE	SR25	*Deschampsia atropurpurea*	*Vahlodea atropurpurea*
POE	**SR26**	***Poa glauca***	***Poa palustris***
AVE	SR28	*Anthoxanthum odoratum*	*Anthoxanthum odoratum*
AVE	SR29	*Phalaris arundinacea*	*Phalaris arundinacea*
AVE	**SR30**	***Calamagrostis purpurea***	***Calamagrostis canadensis***
AVE	SR31	*Agrostis canina*	*Agrostis canina*
AVE	SR32	*Polypogon viridis*	*Polypogon viridis*
AVE	SR33	*Helictotrichon pratense*	*Helictotrichon bromoides*
AVE	**SR35**	***Koeleria glauca***	***Koeleria albida***
AVE	SR36	*Trisetum flavescens*	*Trisetum flavescens*
AVE	SR37	*Briza minor*	*Briza minor*
AVE	SR38	*Briza media*	*Briza media*
AVE	SR39	*Agrostis capillaris*	*Agrostis capillaris*
AVE	SR40	*Trisetum spicatum*	*Trisetum spicatum*
AVE	**SR41**	***Agrostis mertensii***	***Agrostis vinealis***
TRI	SR43	*Elymus repens*	*Elymus repens*
TRI	SR44	*Triticum turgidum*	*Triticum turgidum*
TRI	**SR45**	***Aegilops triuncialis***	***Aegilops cylindrica***
TRI	**SR47**	***Hystrix patula***	***Elymus trachycaulus***
TRI	SR48	*Hordeum jubatum*	*Hordeum jubatum*
TRI	SR50	*Dasypyrum villosum*	*Dasypyrum villosum*
TRI	SR52	*Agropyron cristatum*	*Agropyron cristatum*
BRA	SR54	*Brachypodium pinnatum*	*Brachypodium pinnatum*
MEL	SR57	*Melica nutans*	*Melica nutans*
MEL	**SR58**	***Glyceria striata***	***Glyceria fluitans***
MEL	SR61	*Glyceria occidentalis*	*Glyceria occidentalis*
STI	**SR62**	***Nassella hyalina***	***Nassella viridual***
STI	**SR64**	***Stipa capillata***	***Stipa juncea***
STI	SR65	*Stipa pekinense*	*Achnatherum pekinense*
STI	**SR66**	***Stipa gigantea***	***Stipa lagascae***
STI	SR67	*Stipa ichu*	*Jarava ichu*
STI	SR70	*Nassella tenuissima*	*Nassella tenuissima*
STI	**SR71**	***Nassella trichotoma***	***Jarava media***
STI	**SR73**	***Piptochaetium fimbriatum***	***Piptochaetium avenaceum***
STI	**SR79**	***Nassella cernua***	***Nassella clarazii***
STI	SR82	*Stipa calamagrostis*	*Achnatherum calamagrostis*
STI	**SR89**	***Stipa caragana***	***Stipa barbata***
LYG	SR92	*Lygeum spartum*	*Lygeum spartum*
STI	**SR99**	***Nassella pubiflora***	***Nassella filiculmis***
MEL	**SR100**	***Melica ciliata***	***Melica minuta***
STI	SR101	*Piptatherum miliaceum*	*Oloptum miliaceum*

Appendix II. Distribution of the experimental values



A. Distribution of the experimental variables. The columns in each treatment group (drought, sudden frost at -1 °C and -3 °C) show the number of species with regrowth / conductivity / fluorescence in percentage of the regrowth / conductivity / fluorescence in the control group.

Appendix III. Additional results

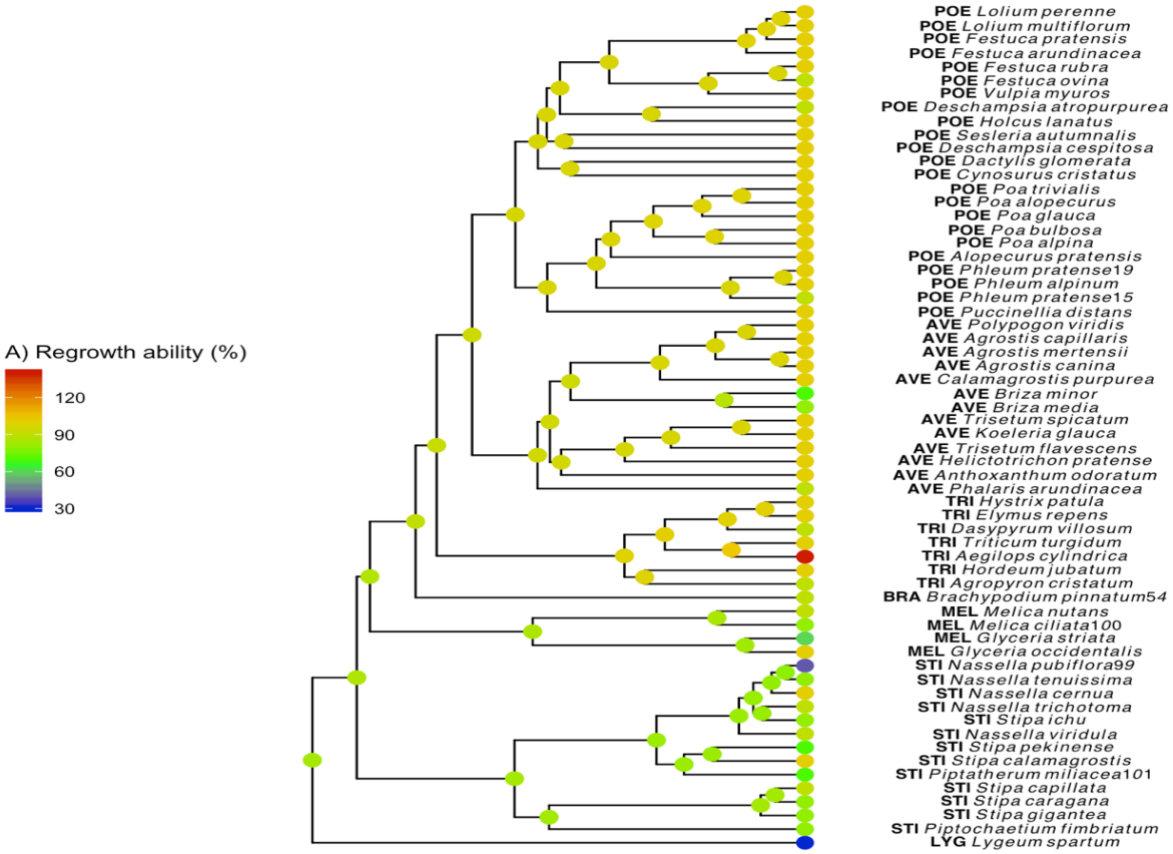
A. Autocorrelation and phylogenetic regression

A1. Table of the trait combinations with a significant autocorrelation among the residuals ($P < 0.05$), but with no significant correlation after the phylogenetic regression ($P > 0.05$).

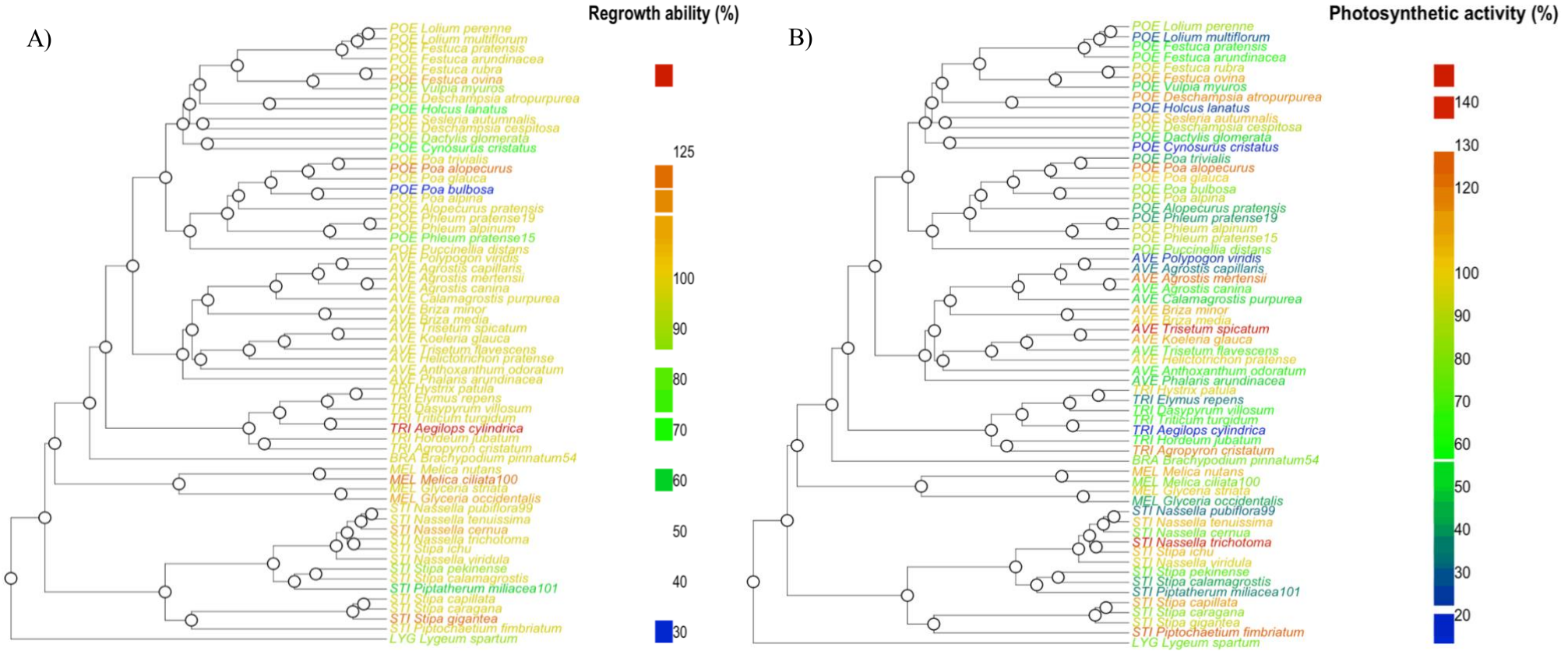
Trait 1	Trait 2
Sudden frost -1 °C regrowth	Drought conductivity
Sudden frost -1 °C regrowth	Drought fluorescence
Sudden frost -1 °C regrowth	Drought regrowth
Sudden frost -1 °C regrowth	Sudden frost -3 °C conductivity
Sudden frost -1 °C regrowth	Sudden frost -1 °C conductivity
Sudden frost -3 °C regrowth	Drought regrowth
Drought regrowth	Drought fluorescence
Drought regrowth	Drought conductivity

B. Phylogenetic distribution of drought and sudden frost tolerance expressed by variables that were not chosen as proxies

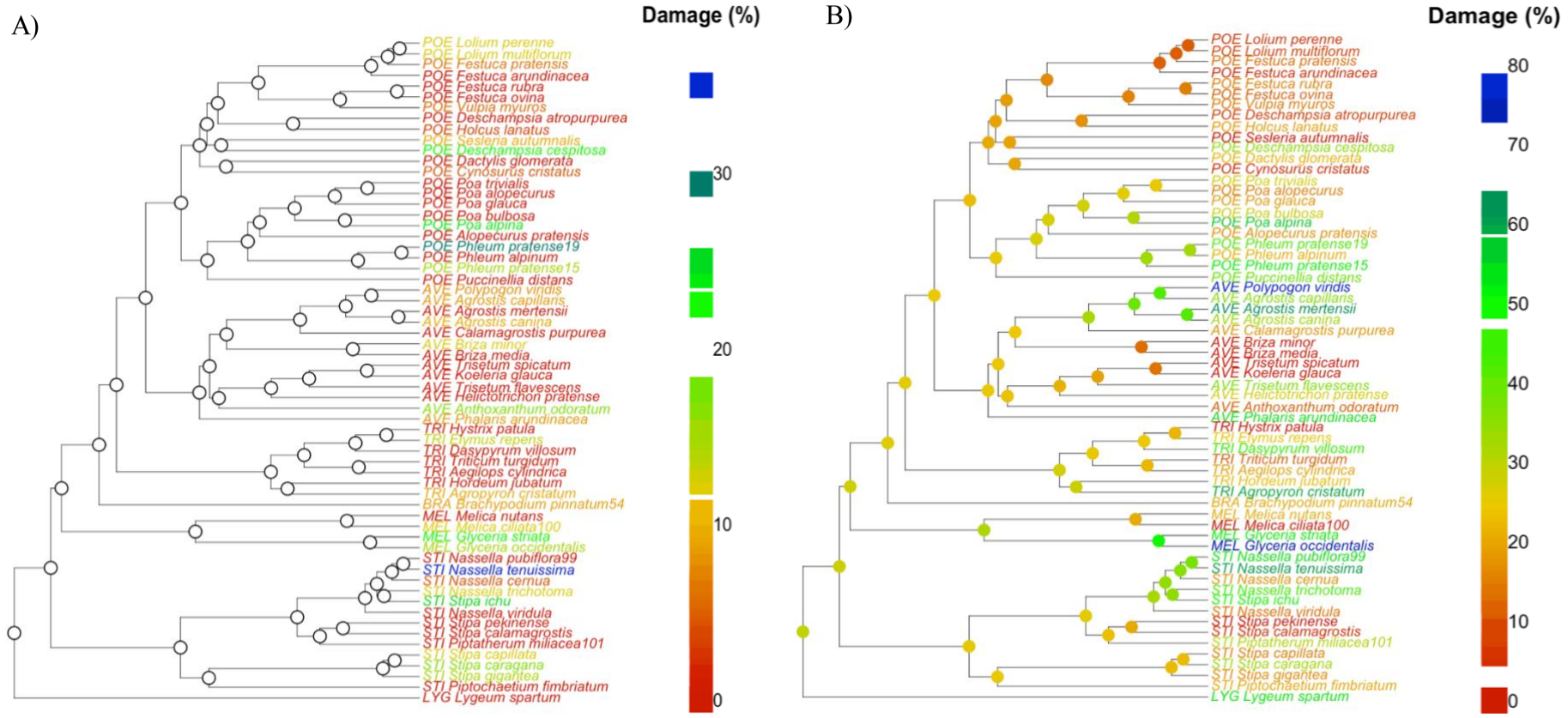
Since it is not possible to do an ASR on a tree with λ = 0, the traits that had the white-noise model as the best fitting model, the original pruned phylogenetic tree was used to plot the traits and the nodes were colored white. See Table 3 for the best fitting model and λ per trait.



B1. Ancestral State Reconstruction of regrowth ability for sudden frost at -1 °C (λ = 0.47). Red indicates high regrowth ability (i.e. high frost tolerance), blue indicates low regrowth ability. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae. For species where more than one accession was sown, the accession number is indicated.



B2. Drought A) regrowth and B) fluorescence. White nodes indicate that $\lambda = 0$. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae. For species where more than one accession was sown, the accession number is set behind the species name.



B3. Conductivity for A) sudden frost at -1 °C and B) sudden frost at -3 °C (λ = 0.63). White nodes indicate that λ = 0. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae. For species where more than one accession was sown, the accession number is set behind the species name.

C. Rate shifts

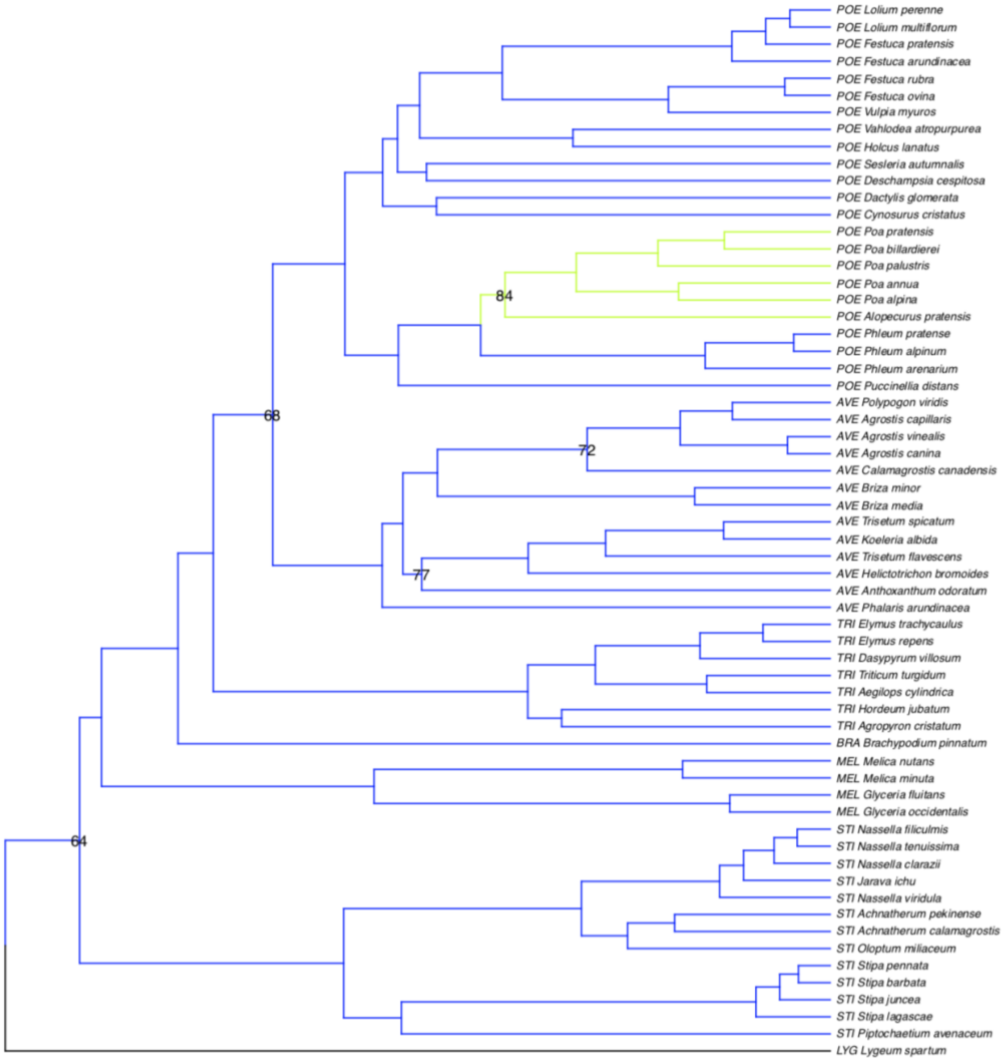
Evolutionary rate shifts were detected in regrowth ability and conductivity measures for sudden frost at -1 °C (Table C1 and Figure C3-4). For conductivity measures of sudden frost at -3 °C, an evolutionary rate shift was only detected in one nod in Poeae (Table C1 and Figure C5). For the majority of the rate shifts there were no lower confidence interval given (Table C2), making it difficult to indicate the rate of the shifts and if it is significant or not. This could be a result of too few species. No rate shift (single rate) were detected in the evolution of drought tolerance (Table C1).

C1. Rate shifts in the evolution of each trait per treatment. Both the number of rate shift search for and the minimum clade size was put to 5, except for regrowth after frost -1 °C where the number of rate shift was set to 6.

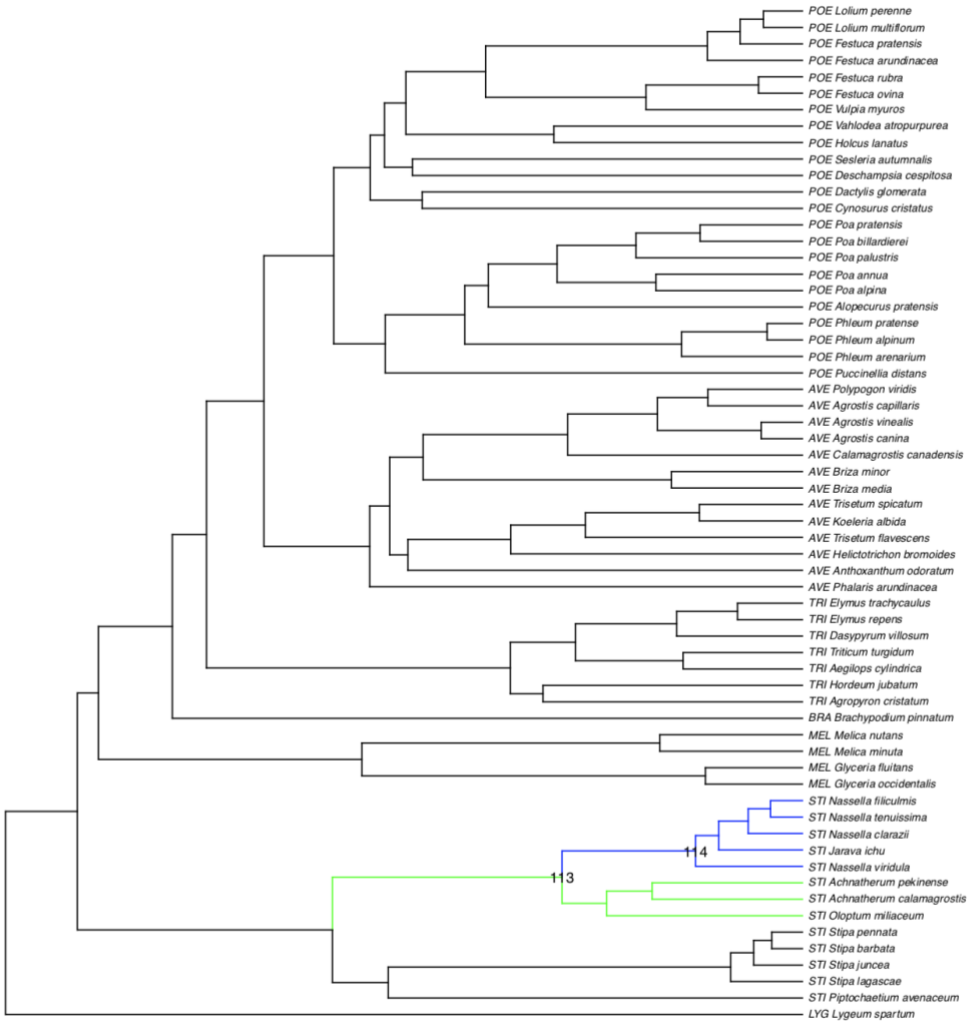
TREATMENT	RATE SHIFT
SUDDEN FROST -1 °C	
Regrowth	One clade in Poeae, two clades in Aveneae, one clade in the core Pooideae and in the node that separates the Stipeae from the rest.
Conductivity	Two clades in the Stipeae
SUDDEN FROST -3 °C	
Regrowth	Single rate
Conductivity	One clade in Poeae
DROUGHT	
Regrowth	NA
Conductivity	Single rate
Fluorescence	Single rate

C2. Table of which node a rate shift occurred, the maximum likelihood rate (ML) and the lower and upper confidence interval (CI) for each treatment and trait are given.

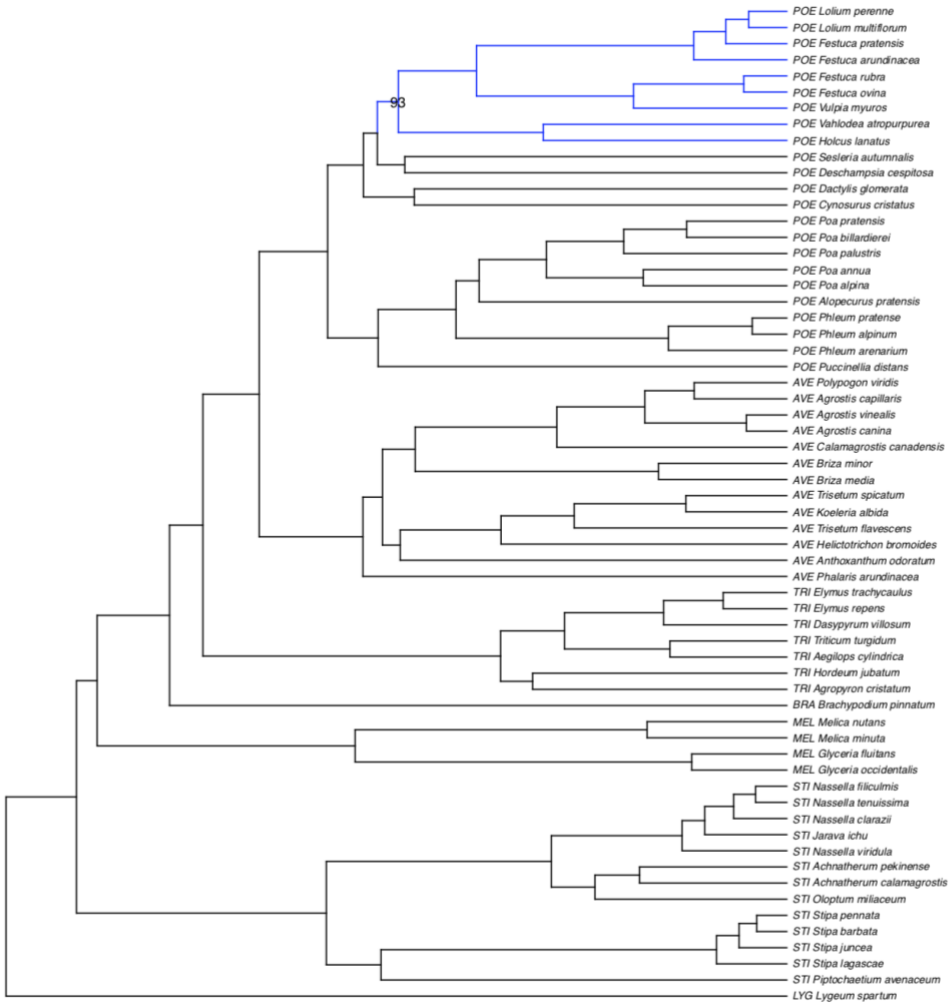
Treatment/Trait	Node	ML rate	Lower CI	Upper CI
SUDDEN FROST -1 °C				
Regrowth	84	1000	NA	NA
Regrowth	72	65.268	NA	170.459
Regrowth	77	1e-08	NA	1e-08
Regrowth	68	1e-08	NA	1e-08
Regrowth	64	1e-08	NA	NA
Conductivity	113	17.076	4.218	47.192
Conductivity	114	1e-08	NA	1e-08
SUDDEN FROST -3 °C				
Conductivity	93	0.080	0.031	0.268



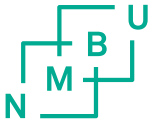
C3. Rate shift in the evolution of regrowth ability after sudden frost at -1 °C. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae.



C4. Rate shift in the evolution of the trait conductivity after sudden frost at -1 °C. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae.



C5. Rate shift in the evolution of the trait conductivity after sudden frost at -3 °C. Tribes are abbreviated as: POE = Poeae, AVE = Aveneae, TRI = Triticeae, BRA = Brachypodieae, MEL = Meliceae, STI = Stipeae and LYG = Lygeeae.



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway