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Migration to colder climates in grasses involves pre-existing and adaptive traits

By

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A Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The University of Sheffield

Faculty of Sciences

Department of Animal and Plant Sciences

March 2019

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Acknowledgements

I would like to express my gratitude to my supervisors, Professor Colin Osborne and Dr Pascal-Antoine Christin for a chance to do the PhD and expand my research field into biogeography and genomics. I am enormously thankful for their invaluable support, advice and guidance. Their commitments have helped me to grow in knowledge and skills.

Thank goes to Dr Erik Murchie for providing me with the fluorometer with useful advice.

I have been lucky to have many supportive colleagues in various disciplines. I would like to thank Irene Johnson for her support on seed germination, Dr Luke Dunning and Dr Matheus Bianconi for their help and advice on bioinformatics, Dr Emma Jardine and Dr Kimberley Simpson for their advice on geospatial analysis. I would like to thank also the members of Osborne's and Christin's labs who provided me with insight on broad aspects of evolution from ecology to genomics with great discussions.

I am also grateful to the stuff of the Department of Animal and Plant Sciences, especially stuff of Molecular Ecology Lab, the AWEC and Annexe for their continuous support.

Many thanks to all of my wonderful and amazing friends in Sheffield who supported me and fulfilled my life abroad. Special thanks to my housemates who shared precious moments with wonderful dinners.

Lastly, I would like to deeply express my gratitude to my beloved family for their unconditional love and support along my PhD journey.

This research was funded by the Royal Thai Government Scholarship.

Summary

A large number of transitions between climates have been found in phylogenetic trees, but some clades with particular features are more likely to shift to new climates than others. Several traits have previously been associated with these multiple transitions. In order to understand how traits are involved in transitions between climates, the enabling effects of traits on transitions and the evolution of traits in new climates need to be identified. The diversity of species that have made multiple transitions between climates in grasses allows the phylogenetic comparative method to be used to address this issue. In this thesis, I investigated the morphological, physiological and genomic traits which have been hypothesised to be enablers of, or adaptations to, the transitions between climates. I provide evidence that pre-existing traits are important factors in facilitating the migration between climates. Using a biogeographical analysis, I first showed that the evolution of C_4 photosynthesis in tropical climates facilitates transitions into cooler climates and expansion into warmer climates. This is consistent with modelling analyses which show that the benefits of C_4 photosynthesis for canopy carbon gain are maximised at high temperatures, but remain significant at low temperatures if leaves can resist chilling and freezing. Using an experimental approach, I next showed that transitions into cold climates were facilitated by additional pre-existing traits that provide constitutive chilling and freezing resistances. These also arise initially in tropical species. Freezing resistance was determined by osmotic pressure, moisture content and cold acclimation, which influence the transition to cold climates, while chilling resistance was associated with culm height and leaf width. Therefore the combined facilitating effects of pre-existing traits determined the initiation of transitions into new climates. However, the adaptive evolution of traits to improve the efficiency of a plant after migration to new environments may also be required for specialism in cold climates. I found that cold acclimation to increase freezing tolerance evolved after migration to cold climates. This suggests that cold acclimation may enhance the efficiency of freezing resistance. In particular, I found signatures of adaptive evolution to cold climates in chloroplast genes encoding proteins which function in the structural stability of the photosystems. These findings suggest that pre-existing traits facilitate migration to new climates, but their efficiency depends on the total effect of related traits. Once in new climates, the additional adaptive evolution of multiple traits is required for a plant to become successful.

Chapter 1

General introduction

1.1 Climate and plant geography

Scientists have long used the association between plant species and environments to categorise and predict large-scale patterns and changes in vegetation (Humboldt & Bonpland 1805; Woodward 1990; Mucina 2019). For example, across altitudinal gradients, the variation in climate determined by changes in temperature and precipitation causes differences among plants from lowland to alpine areas (Humboldt & Bonpland 1805). To explain these relationships, climatic factors are thought to influence distributions because plants have to cope with environmental stresses. The capacity to cope with theses stresses reflects the morphology, anatomy and physiology of plants which indicates adaptation to a particular environment (Schimper 1903; Woodward 1990; Mucina 2019). This environment in which plants can persist and maintain populations called the niche (Hutchinson 1957). The niche may be conserved within a group of plants because of the evolutionary specialisation of species or clades (Wiens & Graham 2005; Wiens *et al.* 2010). During the last decade, studies of ecological adaptation have documented strong phylogenetic signals in niche-related traits, indicating niche conservatism (Losos 2008; Wiens *et al.* 2010). At the same time, more and more rapid niche shifts have been discovered (Ackerly *et al.* 2006; Edwards & Donoghue 2013). Studies of how climatic transitions evolve in plants suggest that particular traits or conditions act as pre-adaptions which facilitate the migration of plants into novel environments (Fisher-Reid *et al.* 2012; Donoghue & Edwards 2014; Weeks *et al.* 2014). Sclerophyllous leaves, for example, evolved in high-rainfall habitats but have several advantageous traits for drought, such as protection from leaf xylem cavitation, which facilitated migration into arid habitats (Salleo *et al.* 1997; Donoghue & Edwards 2014).

Apart from being the result of other pre-adaptive factors, climatic transitions also cause evolutionary changes in plants so that they acquire adaptations to become successful in novel environments (Edwards & Donoghue 2013; Donoghue & Edwards 2014). For instance, the selection from seasonal rainfall moved the vascular bundles

toward the adaxial or inner surface of leaves. This allowed leaves to unroll in the savannah wet season to increase gas exchange and photosynthetic efficiency in the summer, while species in arid regions have fixed rolled leaves which tolerate year-round drought (Toon *et al.* 2015). However, general understanding of the evolutionary trajectories underlying climatic transitions of plants to novel environments and the consequences of these climatic transitions remain incomplete, despite the importance of this knowledge for predicting the effects of climate changes or invasive species on ecosystem and biodiversity (Wiens *et al.* 2010; Araújo *et al.* 2013; Lavergne *et al.* 2013; Atwater *et al.* 2018).

1.2 Coping with cold conditions

The challenges of adapting to cold climates arise from coping with the negative impacts of chilling, which encompasses low temperatures from approximately 10 or 12 to 0 °C, and ice formation from freezing temperatures (Lyons 1973; Pearce 2001). When temperature decreases, the metabolism of plants is limited by the lower efficiency of chemical reactions and enzyme activities (Goldanskii 1976; More *et al.* 1995; Fitter & Hay 2002). This limits cell expansion and cell division, slowing plant growth and development (Harrison *et al.* 1998). The rate of photosynthesis also decreases in a similar way when the temperature falls, such that low temperature significantly inhibits photosynthetic functions. In particular, a lack of electron acceptors arises from an imbalance between the electrons generated by light-capture and the regeneration of electron acceptors at low temperature. This leads to the flow of electrons to Oxygen molecule and then the formation of reactive oxygen species which damage the photosynthetic apparatus (Prasad *et al.* 1994; Moon *et al.* 2006). At low temperatures, the lipid components of membranes also change from a liquid-crystalline state to a solid gel. This causes a decrease in fluidity and permeability of membranes that decrease the stability of cell form and function (Lyons 1973; Quinn 1985; Schulze *et al.* 2005; Lambers *et al.* 2008).

When the temperature drops below zero, ice formation adds to the negative effects on plants. Ice can form inside or outside plant cells, depending on various factors, such as the rate of cooling. If the temperature decreases rapidly, ice forms in intercellular spaces which contain more water than the cytosol within cells. This process begins from

the xylem and spreads throughout the other tissues (Pearce 2001; Larcher 2003; Schulze *et al.* 2005; Arora 2018). On the other hand, if the cytosol has high water content, ice crystals can form within cells (Burke *et al.* 1976). In both cases, ice formation can destroy tissues and potentially the whole plant (Mazur 2003). Ice formation within the cells directly destroys cell structure. However, extracellular ice formation may also cause cellular damage, since it withdraws water from the protoplast. This occurs because vapour pressure is lower over ice than over liquid water at the same temperature (Hansen & Beck 1988; Arora 2018). Membranes can also be damaged severely because of additional dehydration from ice formation (Guy 1990; Thomashow 1999). Lipid membranes disintegrate into micelles through the removal of the surrounding water film. This is caused by the loss of hydrophobic interactions that maintain the bilayer (Guy 1990; Schulze *et al.* 2005). Moreover, when temperature increases after deep freezing, small bubbles will occur in xylem conduits and expand until the conduit becomes fully embolised, thereby blocking the transportation of water in the xylem (Langan *et al.* 1997; Utsumi *et al.* 1999). This can cause hydraulic failure and tissue desiccation, leading to death (Hammel 1967).

1.3 Adaptation to cold climates

Plants adapted to cold climates have evolved various mechanisms to sustain their lives. To avoid the negative effects of cold climates, plants may escape from the worst conditions during winter. For example, annual plants may grow and finish their life cycle within the summer, producing seeds to pass through winter that are more tolerant of cold conditions (King & Heide 2009). Perennial plants such as temperate trees and herbaceous plants also become dormant by scenesing all leaves, developing dormant organs and reducing metabolic rate in order to pass winter (Perry 1971; Larcher 2003). However, the essential structures such as seeds and buds in these plants need to develop mechanisms to survive during winter. This survival may come from structural adaptations or adjustments to metabolism and water content. For instance, seeds and dormant buds are more resistant to freezing than other organs because they have low tissue water content in combination with high levels of carbohydrates and proteins (Sakai & Larcher 1987). Moreover, plants have evolved mechanisms to escape cold environmental conditions by maintaining tissue temperatures while the temperature in the surrounding environment

becomes lower. Reduction of heat loss is thus an important protective mechanism, with plants using structures such as hairs or thorns, or compact life forms, such as the cushion form (Sakai & Larcher 1987; Arroyo *et al.* 2003). Other mechanisms involve improved heat gain, either through the capture of solar energy or internally generated heat from increased respiration (Vojnikov *et al.* 1984; Sakai & Larcher 1987; Lambers *et al.* 2008). Plants may also avoid damage from ice formation by preventing ice crystallization. Supercooling protect tissues by increasing the concentration of solutes in the cells or having the nucleation barriers, such as thick and rigid cell walls, or anti-freeze proteins which delay the ice formation at subzero temperatures. If plants are not able to totally avoid ice formation, water may be moved to form ice in less important or more resistant parts such as intercellular spaces or vacuoles containing substances that reduce the available water (Larcher 2003; Wisniewski *et al.* 2014). The side effect of this mechanism is dehydration, which plants may need to mitigate by developing drought tolerance to maintain cells. Moreover, freeze-thaw embolism in the xylem tissues is avoided by using small conduits which cause small bubbles and fast recovery from embolism (Yang & Tyree 1992; Davis *et al.* 1999; Hacke & Sperry 2001). On the other hand, small conduits also limit plant productivity because they lead to low hydraulic and photosynthetic capacities (Choat *et al.* 2011).

In addition to the acquirement of avoidance mechanisms, the ways in which phenology is synchronised with the timing of cold conditions are also important mechanisms for cold adaptation. Cold acclimation and vernalization, for example, synchronise the life cycle with cold weather using low temperatures and photoperiod as triggers (Sandve *et al.* 2011; Preston & Sandve 2013). Cold acclimation involves biological modifications which increase freezing tolerance during a period of chilling temperatures. This process increases non-photochemical dissipation of excess energy which reduces photoinhibition and improves photosynthetic efficiency under low temperatures (Thomashow 1999). Further mechanisms to protect plants from the damage of freezing are also induced by cold acclimation. For example, ice-recrystallization forces many new small crystals to form rather than allowing a large crystal to grow, thereby reducing damage from large ice crystals (Capicciotti *et al.* 2012). The accumulation of fructans in the cell also increases chilling and freezing tolerance by increasing membrane stability (Valluru & Van den Ende 2008). Venalization is the process that detects a change

in the length of day and night to prevent flowering in the cold conditions of winter and permit flowering in the favorable conditions of spring (Kim *et al.* 2009).

Some studies have provided evidence that these mechanisms relate to the migration of plants to colder environments. The acquisition of mechanisms such as a herbaceous life form, small xylem conduits and annual growth facilitate plant migration to colder climates whereas some others, for example, deciduous leaves and a cushion form, are the consequences of adaptation to colder climates after migration (Zanne *et al.* 2014; Ogburn & Edwards 2015; Boucher *et al.* 2016). Moreover, the origin of a gene network to control vernalization has been hypothesised to facilitate the transition to cold climates (McKeown *et al.* 2016). Conversely, some mechanisms which are advantageous in warm climates may limit migration to colder ones, restricting the niche space that species can access. C_4 photosynthesis, for instance, enhances the efficiency of plant productivity at high temperatures, facilitating plant migration and range expansion into warmer climates. However, it is widely thought that C_4 photosynthesis limits plant distributions in colder climates because of its lower efficiency than C_3 photosynthesis under low temperatures (Ehleringer 1978; Edwards *et al.* 2010; Christin & Osborne 2014).

1.4 Phylogenetic comparative approaches for studying adaptation

Phylogenetic comparative methods have become widely used to answer ecological and evolutionary questions because conventional statistical methods are violated by the non-independence of species data values. The non-independence of species values are influenced by the relatedness between species because closely related species are likely to share similar trait values and to inhabit similar environments. Phylogenetically independent contrasts were one of the first methods developed to address this problem, by considering that the evolutionary divergences or the differences between species are independent (Felsenstein 1985). To apply these methods, a phylogeny which provides the relationship between organisms is constructed with molecular data, and incorporated into comparative methods to deal with the non-independence of species. The phylogenetic method is able to address the association between traits among species regardless of statistical and historical issues (Harvey & Pagel 1991). Using phylogenetically independent contrasts, for example, the correlation or regression coefficients between

contrasts of traits may be tested statistically because the contrast in each interspecific trait is statistically independent. The phylogenetic approach has been widely applied to study adaptation, including the assessment of environmental effects on traits. Similar to trait-trait correlations, the changes of traits and environments across the phylogeny are treated as independent values and the correlation assessed statistically. Moreover, since the environment is not likely to be influenced by traits, it can be inferred that, under natural selection, a particular environment affects the evolution of traits (Harvey & Pagel 1991; Nunn 2011).

1.5 Grasses as a model group

The grass family is one of the large plant families, consisting of more than 11,000 species (Gibson 2009). The group is distributed across nearly all the areas in the world from the savannah to tundra biomes and covers up to 40% of the terrestrial area, contributing approximately 33% of primary productivity (Gibson 2009; Beer *et al.* 2010). Moreover, a large number of crops such as rice, wheat and maize are in this group. The grass family has been split into two major clades, PACMAD and BEP, with three additional outgroups, the subfamilies Anomochlooideae, Pharoideae and Puelioideae (Grass Phylogeny Working Group II 2012). The clade PACMAD comprises of subfamilies Panicoideae, Aristidoideae, Chloridoideae, Micrairoideae, Arundinoideae and Danthonioideae (Grass Phylogeny Working Group II 2012). The centres of diversity for most groups of PACMAD grasses are in the tropics, with the exception of Danthonioideae which are found in temperate climates of the southern hemisphere (Bouchenak-Khelladi *et al.* 2010; Visser *et al.* 2014). The BEP clade contains the subfamilies Bambusoideae, Ehrhartoideae and Pooideae. BEP grasses are found in both tropical and temperate climates depending on the groups. Ehrhartoideae commonly occupy tropical biomes while Pooideae are dominant in both Northern and Southern temperate climates. Bambusoideae contains both tropical and temperate bamboo species (Bouchenak-Khelladi *et al.* 2010; Edwards & Smith 2010; Visser *et al.* 2014).

The ecological success of grasses is influenced by multiple factors. C_4 photosynthesis is considered an important mechanism that allows grasses to fix carbon dioxide efficiently in hot and low CO_2 conditions, and this adaptation has increased diversification (Spriggs *et al.* 2014; Bouchenak-Khelladi *et al.* 2015) and links to the

successful distribution of grasses in hot, open and seasonally dry or arid tropical environments (Osborne & Freckleton 2009; Edwards & Smith 2010; Linder *et al.* 2018). On the other hand, the dominant grasses in cold climates are in Pooideae and Danthonioideae clades and have acquired adaptations to chilling and freezing. These two subfamilies are likely to have migrated to colder climates since the origin of the clades (Edwards & Smith 2010; Sandve *et al.* 2011; Humphreys & Linder 2013). Danthonioideae originated in the Oligocene and made the transition into the temperate zone during cooling of the global climate. This group has evolved cold tolerance beyond that expected from the low temperature limits of its distribution (Bouchenak-Khelladi *et al.* 2010; Humphreys & Linder 2013). The ancestor of Pooideae also migrated to temperate climates during a global cooling period of the Eocene-Oligocene transition, earlier than in Danthoniodeae (Zachos *et al.* 2001; Bouchenak-Khelladi *et al.* 2010; Sandve & Fjellheim 2010). Cold tolerant mechanisms such as vernalization and ice recrystalization have been studied in Pooideae, including in important temperate crops, providing evidence to explain how grasses adapted to cold climates (Sandve *et al.* 2011; Preston & Sandve 2013; McKeown *et al.* 2016; Schubert *et al.* 2017). Although other subfamilies in PACMAD are largely adapted to warm climates, recent research has found that several species occur in cold climates, indicating that more migration into cold climates has occurred within grasses but remains unexplored (Sage *et al.* 2010; Aagesen *et al.* 2016). Therefore, the grass family is an outstanding group in which to address the generality of factors that facilitate climatic transitions and the adaptive evolution of climatic transitions, especially transitions from the tropics to cold climates.

1.6 Thesis aims and structure

The aim of this thesis is to investigate the evolutionary process of transitions between climates in plants, including morphological, physiological and genomic changes. The transition to cold climates was the main focus of this study because of the physiological barriers to obtaining cold adaptations and their importance for explaining plant geography. The grass family was used as a study group because grasses are distributed across nearly all areas on Earth, covering wide ranges of climatic types. This variation in distribution in relation to climate and the diversity of species in this group makes them suitable for the study of how climatic shifts evolve. Moreover, multiple transitions to cold

climates in grasses have allowed me to use the comparative method to test the relationships between traits including genomic characters and climatic transitions, accounting for phylogenetic history. Chloroplast genomes which are small and simple, but important for plants, were selected to be representative of genomic characters.

In chapter 2, I investigate the causal relationship between the evolution of C_4 photosynthesis, which is one of the most important physiological changes in plants, and transitions between climates. C_4 photosynthesis has long been believed to promote migration to warmer climates but there remains controversy about the limitations to C_4 plants in colder climates. I use phylogenetic approaches incorporated with occurrence data to identify these relationships across multiple climatic transitions. I first test for an effect of temperature on evolutionary transitions between the C_3 and C_4 photosynthetic types as well as the influence of these photosynthetic types on the migration of plant lineages among climatic zones. I then evaluate quantitatively the effect of the photosynthetic type on temperature niche breadth. The results indicate that C_4 photosynthesis facilitates the transition to cooler climates. The work was published as Watcharamongkol *et al.* (2018).

The results from chapter 2 also suggested that there are other factors apart from C_4 photosynthesis that influence the transitions to cold climates. Therefore, in chapter 3, I investigate the morphological and physiological traits related to cold resistance. I conducted a physiological experiment in controlled environmental chambers to determine the chilling and freezing resistances of species from tropical and cold climates. Morphological traits were integrated into the study in order to investigate the relationships between structure and function. These data combined with the phylogeny of 28 species and at least 9 climatic transitions were used to test whether constitutive chilling and freezing resistances differ between species from tropical and cold climates, whether cold acclimation increases freezing resistance, and which traits contribute to chilling and freezing resistances. The results suggest that constitutive cold resistance which evolved in the tropics pre-adapts plants for migration into cold climates, and then adaptive evolution of cold acclimation increases freezing resistance to allow particular groups to become specialists.

In chapter 4, the interest is shifted from finding the factors influencing migration to cold climates to the adaptive evolution of chloroplast genomes after the transition to cold climates. I sequenced and assembled the chloroplast genomes of 22 species to

increase the number of natural replicates of transitions between tropical and cold climates across the phylogenetic tree. Using all available genomic data, I then investigated the relationships between genome composition and the transition to cold climates under the expectation that temperature affects the structure of genomes directly by selecting on the nucleotide composition and influencing its directional change. Chloroplast gene sequences are also expected to be selected under the transition to cold climates and I test whether this expectation is met. With adaptive changes in proteins that stabilise the photosystems during the transition to cold climates, this chapter highlights the important consequences of the transition.

In conclusion, these three chapters have broadened our perspective on the processes involved in transitions to novel climates. The findings presented here suggest that multiple factors initially facilitate the migration, with adaptive evolution subsequently causing specialisation within a particular environment.

Chapter 2

C_4 photosynthesis evolved in warm climates but promoted migration to cooler ones

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This work is a modified version of the published manuscript:

Watcharamongkol, T., Christin, P.-A. & Osborne, C.P. (2018), C_4 photosynthesis evolved in warm climates but promoted migration to cooler ones. *Ecol. Lett.*, 21, 376–383.

Personal contribution:

I co-designed the study, generated and analysed the data. I wrote the paper with the help of all the authors.

2.1 Abstract

C_4 photosynthesis is considered an adaptation to warm climates, where its functional benefits are greatest and C_4 plants achieve their highest diversity and dominance. However, whether inherent physiological barriers impede the persistence of C_4 species in cool environments remains debated. Here, we use large grass phylogenetic and geographic distribution data sets to test whether (1) temperature influences the rate of C_4 origins, (2) photosynthetic types affect the rate of migration among climatic zones, and (3) C_4 evolution changes the breadth of the temperature niche. Our analyses show that C_4

photosynthesis in grasses originated in tropical climates and that C_3 grasses were more likely to colonise cold climates. However, migration rates among tropical and temperate climates were higher in C_4 grasses. Therefore, while the origins of C_4 photosynthesis were concentrated in tropical climates, its physiological benefits across a broad temperature range expanded the niche into warmer and enabled diversification into cooler environments.

2.2 Introduction

Temperature varies significantly over the surface of the Earth and through time and is considered the primary factor determining the global distributions of plant species (Woodward 1990; Larcher 2003). During evolution, plants have colonised almost all possible temperature niches (Kier *et al*. 2005; Araújo *et al*. 2013) via a variety of biochemical, physiological and growth adaptations to either cool or warm temperatures (Sakai & Larcher 1987; Larcher 2003). One particular dimension of the temperature niche is the capacity to increase net benefits from photosynthesis under the ambient temperature conditions. Net photosynthetic gains are damped at higher temperatures because CO_2 fixation by Rubisco is offset by competition with O_2 fixation, increasing the rate of photorespiration (Ehleringer & Björkman 1977; Sage & Kubien 2007). Some plants have evolved CO_2-concentrating mechanisms that minimise photorespiration, including C_4 photosynthesis, which has been very successful in land plants (Ehleringer & Björkman 1977; Sage & Monson 1999; Still *et al.* 2003; Sage *et al.* 2012).

C_4 photosynthesis is a physiological process resulting from a series of biochemical and anatomical modifications over the ancestral C_3 photosynthetic type (Hatch 1987). Together, these concentrate CO_2 around Rubisco, thereby increasing its efficiency and reducing photorespiration (von Caemmerer & Furbank 2003; Sage *et al.* 2012). However, the extra biochemical reactions required for C_4 photosynthesis increase the energetic cost of carbon fixation, so that C_4 photosynthesis is predicted to outperform C_3 only when photorespiration is significant, especially under high temperatures (Ehleringer & Björkman 1977; Osborne & Beerling 2006). The improved performance of C_4 plants at high temperatures predicted from physiological measurements and theory is supported by global distribution patterns (Ehleringer & Björkman 1977; Ehleringer 1978; Griffith *et al*. 2015). Indeed, while trees are almost all C_3, open biomes are predominantly occupied

by C_4 species in hot to warm climates, and cooler open biomes are dominated by C_3 species (Sage *et al*. 1999b; Edwards *et al*. 2010).

More than half of C_4 species belong to the grass family (Poaceae) (Sage *et al*. 1999a). At large scales, C_3 and C_4 grasses sort largely according to temperature (Ehleringer & Björkman 1977; Ehleringer 1978; Sage *et al*. 1999a; Osborne *et al*. 2014), and C_4 grasses dominate most open biomes in tropical and subtropical areas, where they achieve greater biomass and higher growth rate (Still *et al*. 2003; Edwards *et al*. 2010; Atkinson *et al*. 2016). Despite these general patterns, low temperatures do not completely exclude C_4 grasses. Several C_4 grass species are found in alpine, steppe or high-latitude habitats where they survive cold conditions during the growing season, with some species developing physiological cold acclimation to tolerate freezing (Long *et al*. 1975; Beale & Long 1995; Sage & Sage 2002; Márquez *et al*. 2006; Liu & Osborne 2008, 2013; Sage *et al*. 2010). These examples demonstrate that C_4 species can survive and compete with C_3 species in cold environments, at least under some circumstances.

The rarity of C_4 plants in cold climates could be explained by inherent physiological constraints on the amounts or activities of key photosynthetic proteins (Sage & Kubien 2007). However, it is also possible that the current geographical distributions reflect historical processes, with C_4 plants evolving in tropical and subtropical climates and inheriting traits that are poorly suited for cooler climates (Long 1999). Differentiating these scenarios requires large-scale comparisons of C_3 and C_4 lineages, while considering their evolutionary history. Past comparative work has shown that C_4 grasses emerged from tropical C_3 lineages (Edwards & Still 2008; Edwards & Smith 2010), and suggested that C_4 evolution in specific clades enabled a niche expansion into both cooler and warmer climates (Christin & Osborne 2014; Lundgren *et al*. 2015; Aagesen *et al*. 2016). However, systematic tests for an effect of photosynthetic types on transition rates among different climatic zones are lacking.

In this study, we use phylogenetic and geographical distribution data for 2,133 grass species (a fifth of all grass species), including 948 C_4 species (a fifth of all C_4 grass species) representing 18 independent C_4 lineages (most of the 24 C_4 groups), to assess the relationships between photosynthetic types and temperature niches. Using comparative analyses, we first test for an effect of temperature on evolutionary transi tions between the C_3 and C_4 photosynthetic types as well as the influence of these photosynthetic types on the migration of plant lineages among climatic zones. We then evaluate quantitatively

the effect of the photosynthetic type on temperature niche breadth. Our investigations shed new light on the interplay between physiology and evolutionary history in determining the sorting of plants across ecological space.

2.3 Methods

Climate dataset

All available geo-referenced occurrence records (~14.3M) for the grass family (Poaceae) were extracted from the Global Biodiversity Information Facility (GBIF) web portal (http://www.gbif.org, accessed 10th December 2015). Records were cleaned to filter out unreliable location data using the following steps. First, duplicate records from the same location were removed. Potentially incorrect geographical data were then excluded, including those with coordinates outside the map, where the country did not match the coordinates, within 20 km of the GBIF headquarters, where longitude and latitude were exactly the same because these may represent a typo or default value, or where the georeference was recorded to a precision fewer than three decimal places. The species names were then checked against the Kew grass synonymy database (Clayton *et al*. 2006) via the software package Taxonome (Kluyver & Osborne 2013), and records without a valid name were discarded. The species with less than ten occurrences were also excluded to increase the accuracy of the temperature range estimated for each species. For each set of coordinates, we extracted the average mean, minimum and maximum temperature of the coldest and warmest months from WorldClim version 2, 30-arc seconds resolution data (Fick & Hijmans 2017). The median and 5th and 95th percentiles from each species were considered in further analyses, to investigate range limits while avoiding extreme outliers. The temperature range occupied by each species was estimated as the difference between the 5th percentile of the coldest month and 95th percentile of the warmest month (Quintero & Wiens 2013).

Phylogenetic tree

We used a published time-calibrated phylogenetic tree that includes 3,595 species of grasses, covering the whole family and using a time-calibration hypothesis based upon macrofossils and microfossils (Spriggs *et al*. 2014). Taxa without occurrence data after filtering were pruned from the tree, resulting in a data set of 2,133 species with both

phylogenetic and distribution data. Comparison of the proportions of C_3 and C_4 species in each climatic region between those available in GBIF and the subset included in the phylogenetic tree shows that the filtering by the phylogeny did not bias the dataset (Table S2.1 and Fig. S2.1). Each analysis was first conducted on the whole grass family. However, C_4 origins only exist within one of the two major clades of grasses, the PACMAD clade (Grass Phylogeny Working Group II 2012). We therefore repeated the analyses considering only this clade of 1,197 species to check the robustness of results.

Modelling transition rates between photosynthetic and climatic types

The aims of this analysis were to test whether climatic regions influence the rates of transitions among photosynthetic types, and whether photosynthetic types in turn influence the rates of transitions among climatic types. The photosynthetic type of each species was assigned based on the literature (Osborne *et al*. 2014). Climate was categorised using the Köppen-Geiger classification, as the analysis required binary characters, and this is a well-established and widely known climate scheme (Peel *et al*. 2007). Specifically, we used three climatic types, based on temperature: tropical (mean temperature of the coldest month above 18 °C); temperate (mean temperature of the coldest month between 0 and 18 °C); and cold (mean temperature of the coldest month below 0 °C, which includes continental, polar and alpine climates). The temperate range was further divided into freezing and non-freezing conditions, since freezing represents a particular physiological challenge to plants (Sakai & Larcher 1987). Minimum temperature of the coldest month was used to identify regions that are exposed to freezing. Climate types were assigned based on species median values.

Transition rates among photosynthetic and climatic types were estimated for each pair of climate classes that are adjacent on the temperature gradient: (1) tropical vs. temperate without freezing; (2) temperate without freezing vs. temperate with freezing; and (3) temperate with freezing vs. cold. For each independent combination of adjacent climates, Pagel's method (Pagel 1994, 1999; Pagel & Meade 2006) was used to model the eight possible evolutionary transitions between the four states (two adjacent climates multiplied by two photosynthetic types). The model was fitted using a maximum likelihood method to derive point estimates of log-likelihoods in the package BayesTraits (Pagel & Meade 2006).

By fixing some parameters, we tested four hypotheses for each pair of adjacent climates using likelihood ratio tests on nested models. First, we tested whether the rate of transitions from warmer to cooler climates (i.e. tropical to temperate without freezing, temperate without freezing to temperate with freezing, or temperate with freezing to cold climates) differs between C_3 and C_4 lineages (by contrasting rates of 1→2 and 5→6, 2→3 and 6→7, or 3→4 and 7→8, Fig. 2.1). Second, we tested whether the rate of reverse transitions from cooler to warmer climates differs between C_3 and C_4 lineages (by contrasting rates of 2→1 and 6→5, 3→2 and 7→6, or 4→3 and 8→7, Fig. 2.1). Third, we tested whether the rate of transitions from C_3 to C_4 states differs between warmer and cooler climates (by contrasting rates of 1→5 and 2→6, 2→6 and 3→7, or 3→7 and 4→8, Fig. 2.1). Finally, based upon previous work suggesting that reversions from C_4 to C_3 photosynthesis are unlikely (Christin *et al*. 2010; Grass Phylogeny Working Group II 2012), we tested whether transitions from C_4 to C_3 are possible in either of the two climates (by contrasting rates of 5→1, 6→2, 7→3, and 8→4 with a rate fixed to zero, Fig. 2.1).

Bias in the underlying species sampling could theoretically influence the results of these tests if either C_3 or C_4 species within a particular climate regime were under- or over-represented. The GBIF database has a known bias, with particular regions being well sampled (e.g. Europe, North America, Australia) and other regions being poorly covered, especially in the tropics (e.g. India, parts of Africa). However, this bias only creates problems for our analysis if C_3 or C_4 species are differentially sampled within tropical regions, and we can think of no reason why this should be the case. On the other hand, the phylogenetic tree is likely biased as taxa judged interesting for a variety of reasons would be preferentially sequenced. However, because the sampling of species from the phylogeny is filtered by the availability of GBIF, the final dataset is representative of GBIF without any bias from phylogeny (Table S2.1 and Fig. S2.1).

To visualise the historical transitions between climatic types of C_3 and C_4 species, ancestral values were computed for climatic types using the ace function in the ape package in R (Pagel 1994; Paradis *et al*. 2004) and the most likely climate of ancestors was mapped for each node on the phylogenetic tree using the ggtree package in R (Yu *et al*. 2017).

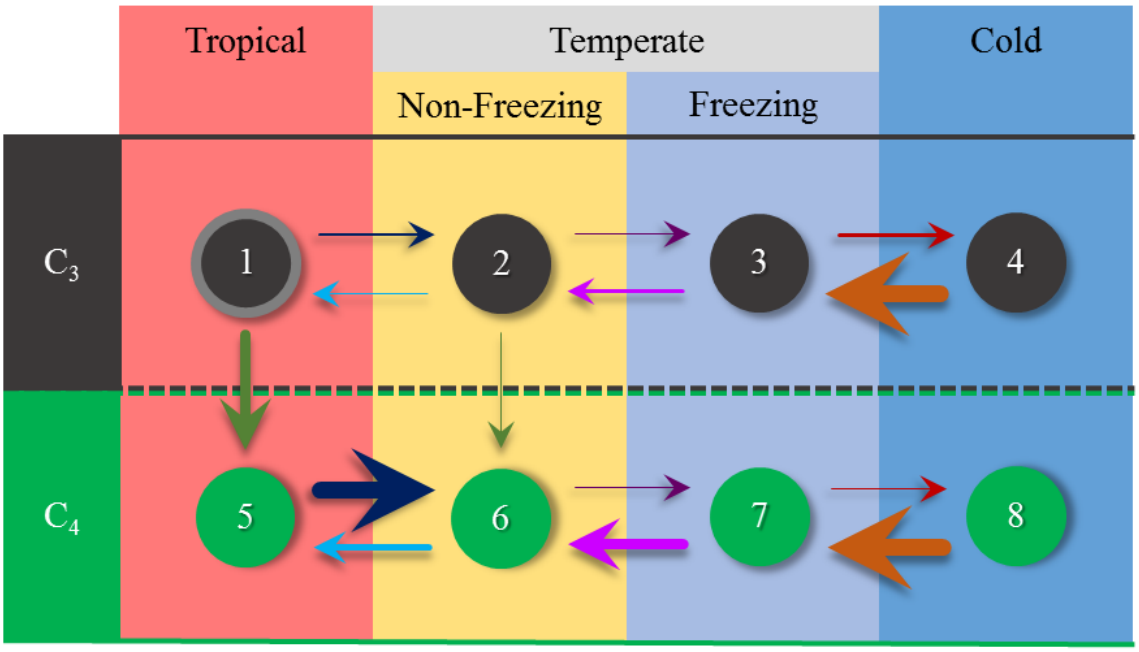

Figure 2.1 Model of coevolution of photosynthetic types and temperature niches. Sizes of arrows indicate transition rates among climate and photosynthetic types. The most likely ancestral condition is indicated by the grey outer circle.

Phylogenetic comparisons of temperature niches

Phylogenetic generalised least squares (PGLS) were used to confirm that the photosynthetic type influences thermal maxima and minima as well as the breadth of the temperature niche, as suggested previously with smaller data sets (Lundgren *et al*. 2015; Aagesen *et al*. 2016). Temperature was the independent variable, with photosynthetic type as the categorical predictor. Phylogeny was incorporated to control for phylogenetic dependence and to estimate the phylogenetic signal using Pagel's λ (Pagel 1999; Freckleton *et al*. 2002).

2.4 Results

Climatic distributions of C_3 and C_4 species

The percentage of C_4 species decreases from tropical to cold climates (Table S2.2), with more than 10% of C_4 species available in GBIF occur in freezing temperate or cold climates (Table S2.1). Our data set of 2,133 species includes perennial C_4 species from

three different subfamilies that colonised cold climates (Table S2.2), including a number of perennial species. The predominance of C_3 species in cold climates mainly reflects the success of members of the Pooideae subfamily, although other groups are also represented (Table S2.2). Yet only members of Pooideae can inhabit areas where the mean temperature of the coldest month is lower than –10 °C (Fig. 2.2).

Rates of transition among photosynthetic types and climates

Models were used to test whether climate influences transitions among photosynthetic types. They supported the hypothesis that C_4 origins are more frequent in tropical than temperate climates (rates of 1→5 > 2→6; $P < 0.001$; Fig. 2.1; Table 2.1). The ancestral state reconstructions confirmed that C_4 photosynthesis evolved from C_3 PACMAD ancestors in tropical climates, while the ancestor of Danthonioideae moved to temperate climates, with descendants that remained C_3 (Fig. 2.3). The rate of C_4 origins is not significantly different from zero in freezing temperate and cold climates (rates of 3→7, 4→8 = 0; $P > 0.05$, Fig. 2.1; Table 2.1), but it is greater than zero in non-freezing temperate climates (rate of $2 \rightarrow 6 \neq 0$; $P < 0.05$; Fig. 2.1; Table 2.1). Based on our models, the rate of transition from C_4 to C_3 types is not significantly different from zero under any climatic conditions (rates of 5→1, 6→2, 7→3, 8→4 = 0; $P > 0.05$; Fig. 2.1; Table 2.1), which is consistent with previous conclusions that the rate of reversal from C_4 to C_3 is extremely low or null in grasses (Christin *et al.* 2010; Grass Phylogeny Working Group II 2012).

The same models were used to test whether the photosynthetic type influences transitions between climatic zones. The rates of transitions between tropical and non-freezing temperate climates across the whole family and in the PACMAD clade are significantly higher in both directions in C_4 than C_3 taxa (rates of 5→6 > 1→2 and 6→5 > 2→1; $P < 0.001$; Fig. 2.1, S2.2; Table 2.1, S2.3). The ancestral state reconstructions indicate that these transitions occurred many times since the split of Chloridoideae, Panicoideae and Aristidoideae (Fig. 2.3). Moreover, C_4 photosynthesis increases the rate of transition from temperate climates with freezing to those without freezing (rates of 7→6 > 3→2; $P < 0.001$; Fig. 2.1; Table 2.1). The shift to occupy temperate climates without freezing during the winter therefore occurred more frequently in C_4 than C_3 taxa (Fig. 2.3). The rate of transition from temperate climates without freezing to climates with freezing was also higher in C_4 than C_3 types, but only in the analysis of the PACMAD

clade (rates of 6→7 > 2→3; $P < 0.05$; Fig. S2.2, Table S2.3). The transition from non-freezing to freezing conditions was found commonly in C_4 PACMAD lineages, but the shift also occurred frequently in C_3 Pooideae (Fig. 2.3), leading to equal rates when considering the grass family as a whole (rates of 6→7 = 2→3; $P > 0.05$; Fig. 2.1; Table 2.1). However, transitions from temperate to cold climates are more frequent within C_3 than C_4 lineages (rates of 7→8 < 3→4; $P < 0.001$; Fig. 2.1, Table 2.1), while the rate of transitions in the opposite direction, from cold to temperate climates, is independent of the photosynthetic type, with both C_3 and C_4 grasses moving at the same rate (rates of 4→3 = 8→7; $P > 0.05$; Fig. 2.1; Table 2.1). Phylogenetic reconstructions suggest a few transitions to cold climates within C_4 groups, while the large Pooideae C_3 clade migrated early to cold climates and diversified there (Fig. 2.3).

Evolution of temperature niche breadth

In our study, temperature extremes and ranges were used to confirm that differences exist between C_3 and C_4 plants in the breadth of the temperature niche. The PGLS analyses indicate that C_4 evolution led to an expansion of the species-level temperature niche (Table 2.2). C_4 photosynthesis is specifically associated with increases in the upper bound of the species range toward higher temperatures during the warmest month (Table 2.2), mirroring previous conclusions with a smaller species sampling (Aagesen *et al*. 2016). By contrast, the lower bounds of the temperature ranges within species did not differ significantly between C_4 and C_3 groups, which indicates that C_4 evolution does not affect the lower range of the temperatures that are occupied (Table 2.2). The conclusions remained the same whether the comparison was made across all grasses or just the PACMAD clade, which includes all C_4 lineages (Table 2.2).

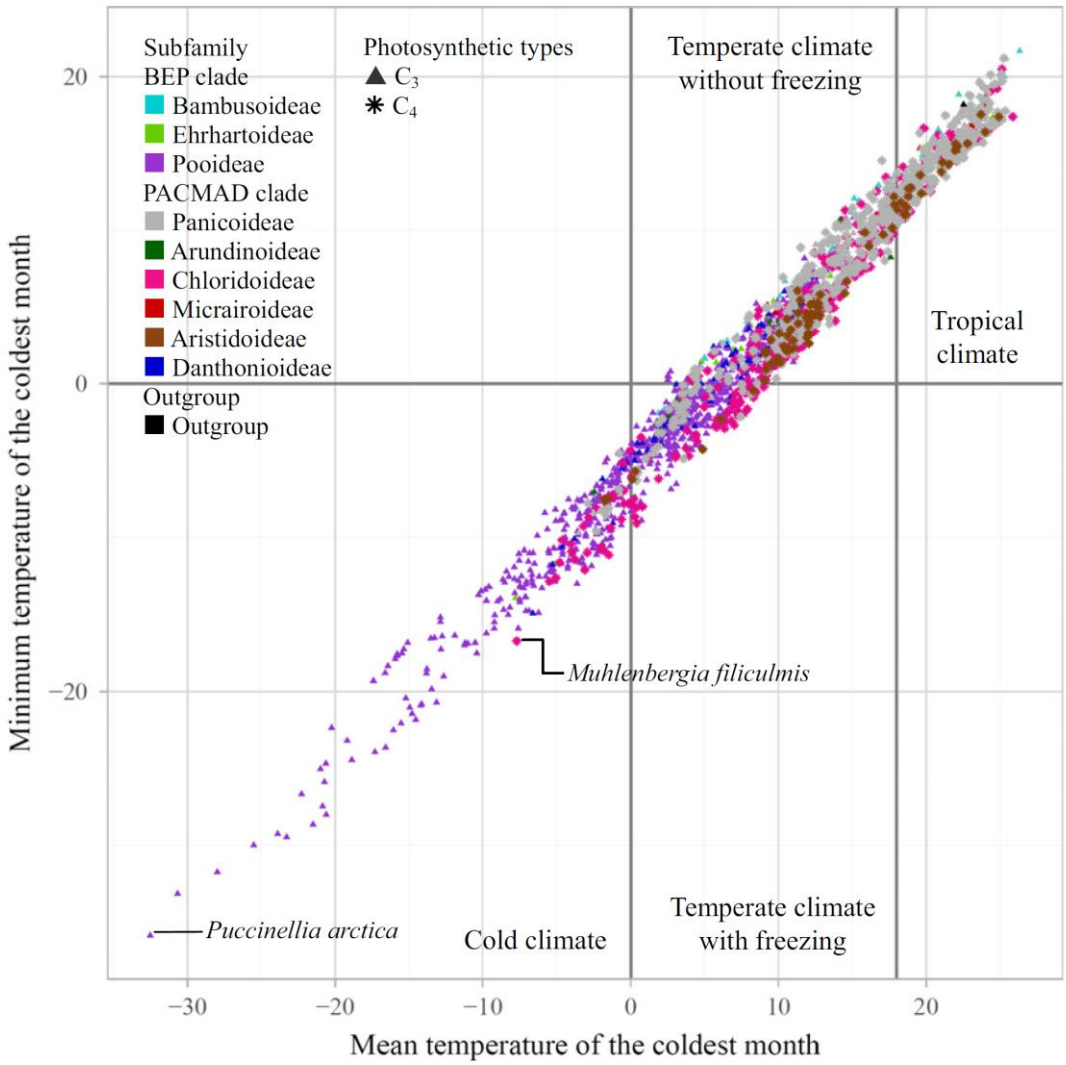

Figure 2.2 Climatic distributions of C_4 and C_3 species from various subfamilies indicated by temperature regimes. The thick grey lines indicate the boundaries between climatic types. The C_3 and C_4 species in the lowest temperature regimes are indicated.

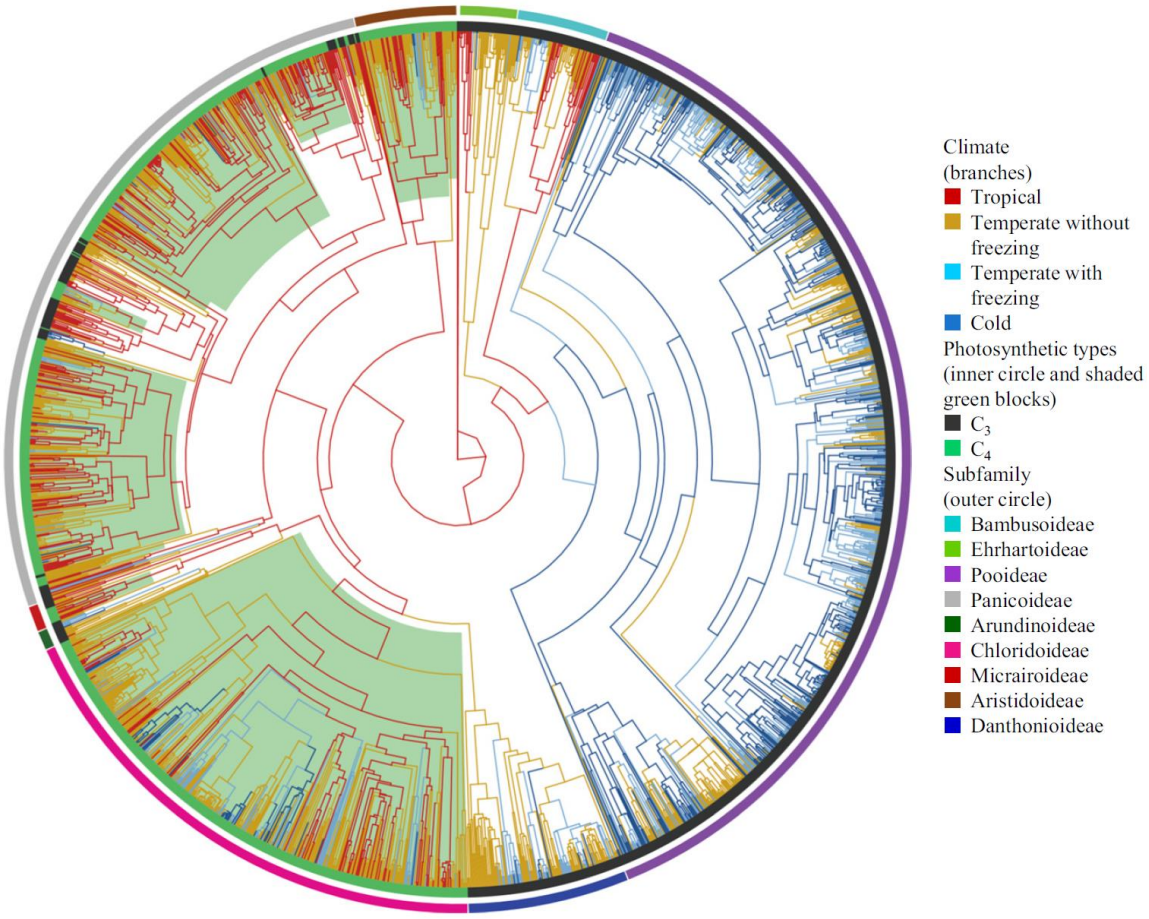

Figure 2.3 Maximum likelihood reconstruction of the transitions between climatic regions: tropical, temperate without freezing, temperate with freezing and cold climates. Photosynthetic types and subfamilies are indicated.

Table 2.1 Rates of transitions determined from point estimates of models.

State	Rate	Estimated rates
Tropical vs. Temperate climates and C_3 vs. C_4 photosynthesis		
Transition from tropical to temperate climate		
C_3	$Rate_{1\rightarrow2}$	0.02200
C_4	$Rate_{5\rightarrow6}$	0.11663
$C_4 - C_3$	$Rate_{5\rightarrow6} - Rate_{1\rightarrow2}$	0.09464***
Transition from tropical to temperate climate		
C_3	$Rate_{2\rightarrow1}$	0.00004
C_4	$Rate_{6\rightarrow5}$	0.04316
$C_4 - C_3$	$Rate_{6\rightarrow5} - Rate_{2\rightarrow1}$	0.04313***
Transition from C_3 to C_4 photosynthesis		
Tropical	$Rate_{1\rightarrow5}$	0.00749
Temperate	$Rate_{2\rightarrow6}$	0.00016
Tropical – Temperate	$Rate_{1\rightarrow5} - Rate_{2\rightarrow6}$	0.00734***
Temperate = 0	$Rate_{2\rightarrow6} - 0$	0.00016*
Transition from C_4 to C_3 photosynthesis		
Tropical	$Rate_{5\rightarrow1}$	0
Temperate	$Rate_{6\rightarrow2}$	0
Tropical = Temperate = 0	$Rate_{5\rightarrow1} + Rate_{6\rightarrow2} - 0$	0^{ns}
Temperate climates without freezing vs. with freezing and C_3 vs. C_4 photosynthesis		
Transition from temperate climate without freezing to with freezing		
C_3	$Rate_{2\rightarrow3}$	0.01095
C_4	$Rate_{6\rightarrow7}$	0.01356
$C_4 - C_3$	$Rate_{6\rightarrow7} - Rate_{2\rightarrow3}$	0.00262^{ns}
Transition from temperate climates with freezing to without freezing		
C_3	$Rate_{3\rightarrow2}$	0.03054
C_4	$Rate_{7\rightarrow6}$	0.07954
$C_4 - C_3$	$Rate_{7\rightarrow6} - Rate_{3\rightarrow2}$	0.04900***
Transition from C_3 to C_4 photosynthesis		
Non-freezing	$Rate_{2\rightarrow6}$	0.00355
Freezing	$Rate_{3\rightarrow7}$	0.00000
Non-freezing – Freezing	$Rate_{2\rightarrow6} - Rate_{3\rightarrow7}$	0.00355***
Freezing = 0	$Rate_{3\rightarrow7} - 0$	0^{ns}
Transition from C_4 to C_3 photosynthesis		
Non-freezing	$Rate_{6\rightarrow2}$	0
Freezing	$Rate_{7\rightarrow3}$	0
Non-freezing = Freezing = 0	$Rate_{6\rightarrow2} + Rate_{7\rightarrow3} - 0$	0^{ns}
Temperate vs. Cold climates and C_3 vs. C_4 photosynthesis		
Transition from temperate to cold climates		
C_3	$Rate_{3\rightarrow4}$	0.03118
C_4	$Rate_{7\rightarrow8}$	0.00421
$C_4 - C_3$	$Rate_{7\rightarrow8} - Rate_{3\rightarrow4}$	-0.02697***
Transition from cold to temperate climates		
C_3	$Rate_{4\rightarrow3}$	0.13183
C_4	$Rate_{8\rightarrow7}$	0.13628
$C_4 - C_3$	$Rate_{8\rightarrow7} - Rate_{4\rightarrow3}$	0.00445^{ns}
Transition from C_3 to C_4 photosynthesis		
Temperate	$Rate_{3\rightarrow7}$	0.00233
Cold	$Rate_{4\rightarrow8}$	0.00000
Temperate – Cold	$Rate_{3\rightarrow7} - Rate_{4\rightarrow8}$	0.00233*
Cold = 0	$Rate_{4\rightarrow8} - 0$	0^{ns}
Transition from C_4 to C_3 photosynthesis		
Temperate	$Rate_{7\rightarrow3}$	0
Cold	$Rate_{8\rightarrow4}$	0
Temperate = Cold = 0	$Rate_{7\rightarrow3} + Rate_{8\rightarrow4} - 0$	0^{ns}

Asterisk indicates the differences between rates of transitions, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ^{ns} indicates no differences between rates of transitions.

Table 2.2 Results from phylogenetic generalised least square regression testing for an association between photosynthetic pathway and climate, and making statistical comparisons between photosynthetic types.

Clade	Variable[1]	C_3	C_4	*P*-value	λ
All grasses	MTCM max	17.0	17.7	0.5154	0.78
	MTCM min	9.0	8.0	0.5191	0.80
	MTWM max	26.0	27.8	0.0142*	0.69
	MTWM min	20.1	20.8	0.3995	0.81
	Range	15.3	18.3	0.0126*	0.79
PACMAD	MTCM max	17.7	18.3	0.5577	0.68
	MTCM min	9.6	8.4	0.3873	0.72
	MTWM max	26.7	28.4	0.0031**	0.64
	MTWM min	20.3	21.0	0.3748	0.75
	Range	16.2	19.4	0.0057**	0.68

[1] MTCM max = 95th mean temperature of the coldest month; MTCM min = 5th mean temperature of the coldest month; MTWM max = 95th mean temperature of the warmest month; MTWM min = 5th mean temperature of the warmest month; Range = difference between MTWM max and MTCM min.

* $P < 0.05$, ** $P < 0.01$.

2.5 Discussion

C_4 plants evolved in tropical climates, expanded to warmer and shifted to cooler environments

Our analyses of evolutionary transitions across the whole grass phylogeny provide general statistical support for the hypothesis that C_4 photosynthesis in grasses evolved in tropical climates (Figs. 2.1 and 2.3), confirming previous work (Sage 2004; Edwards & Still 2008; Edwards & Smith 2010). Hot climates, under the low CO_2 atmosphere that prevailed for the last 30 million years (Pagani *et al*. 2005) exacerbated photorespiration, providing a selective pressure for novel photosynthetic physiologies that decrease the net cost of this process (Ehleringer & Björkman 1977; Osborne & Beerling 2006; Christin *et al.* 2008a). Current models indicate that C_4 photosynthesis evolved via a series of intermediate stages, including photorespiratory bypasses and weak C_4 cycles, which progressively decreased the adverse effects of photorespiration (Sage 2004;

Heckman*n et a*l. 2013; Mallman*n et a*l. 2014). While it has been questioned whether extant taxa with an intermediate physiology are similar to those that enabled C_4 evolution, with some arguing that they might instead result from hybridization events (Kadereit *et al*. 2017), these intermediates are concentrated in hotter climates (Lundgren & Christin 2017). If states that preceded C_4 evolution were similarly restricted to hotter climates, C_4 origins would consequently be concentrated in warm climates, as observed here (Fig. 2.1). However, because the physiological effects of C_4 photosynthesis are broader than those of these intermediates (Vogan & Sage 2011; Christin & Osborne 2014), the ecological consequences of C_4 evolution might not be limited to warm climates (Christin & Osborne 2014). Our analyses support this hypothesis.

Our modelling analysis shows for the first time that C_4 photosynthesis accelerated the migration of grass taxa between tropical and temperate climates in comparison with C_3 lineages (Fig. 2.1). Therefore, C_4 photosynthesis presents no inherent physiological barrier to the colonisation of temperate environments. It has been hypothesised that cold acclimation in C_4 plants may be impeded by their leaf anatomy, which provides insufficient cellular volume to accumulate Rubisco protein (Sage & Kubien 2007), although this is debated (Long & Spence 2013). It has also been proposed that C_4 species should be excluded from low temperature regions by competition with C_3 plants, which have a higher photosynthetic efficiency than the C_4 type in cool environments, especially in low light conditions within dense leaf canopies or under cloudy skies (Ehleringer & Björkman 1977; Ehleringer 1978). However, modelling suggests that, under cloudless, high light conditions, the cost resulting from the extra C_4 reactions may be more than compensated at the canopy scale by light-saturated photosynthetic rates in sunlit leaves (Long 1999; Long & Spence 2013). In addition, the C_4 syndrome provides advantages besides carbon-fixation efficiency. These include greater nitrogen- and water-use efficiencies than the C_3 type (Long 1999), and increased net assimilation rates enable investment into different growth strategies (Atkinson *et al*. 2016). Together, these properties might contribute to the success of C_4 species across a range of temperature conditions.

However, our analysis also shows that C_4 species are overall less likely than C_3 ones to migrate into continental, polar or alpine climates (grouped as “cold climates” in our analysis). A colonisation of cold climates has previously been inferred early during the history of C_3 Pooideae (Edwards & Smith 2010), the group that dominate cold and temperate climates (Fig. 2.3; Table S2.2). This suggests that adaptation in this group to

survive under prolonged cold conditions has been enabled by traits that evolved early during their history and may not characterise other grass lineages (Sandve & Fjellheim 2010; Vigeland *et al*. 2013; Spriggs *et al*. 2014). The early migration and adaptation to cold climates allowed the subsequent diversification of Pooideae in cold and temperate climates (Table S2.2, Fig. 2.3).

Biogeography affects the current distribution of C_4 plants

Evolutionary history, coupled with biogeographical pattern, explains the higher frequency of C_4 species in tropical or temperate climates (Table S2.2). C_4 origins happened predominantly in tropical climate regions (Fig. 2.1), allowing grasses to tolerate higher temperatures and expanding the temperature niche (Table 2), as shown previously (Christin & Osborne 2014; Lundgren *et al*. 2015; Aagesen *et al*. 2016; Bena *et al*. 2017). However, our analysis is the first to show that C_4 photosynthesis also increased the rate of transitions among climate types, within frequent migration into temperate climates without freezing (Fig. 2.1). The rate of C_4 plant migration into freezing temperate climates was also considerable, and was higher than that of close C_3 relatives within the PACMAD clade. When considered across the grass family as a whole it was comparable to the rates in Pooideae, indicating that C_4 lineages are physiologically prone to colonise cold environments.

Geographical barriers could have played important roles in limiting the expansion of some C_4 groups into cold climates. Most tropical climate regions are geographically distant from cold climates (Donoghue 2008), presenting the little opportunity for tropical plants to migrate into cooler environments (Edwards & Donoghue 2013). C_4 species of cold climates are therefore found mostly in high altitude habitats located at low latitudes, and only rarely at high latitudes (Long 1999; Sage & Monson 1999; Sage *et al.* 2010), but we argue that this pattern does not stem from physiological limitations, instead being the direct consequence of the increased rate of C_4 origins in tropical regions.

2.6 Conclusions

Using a large phylogeny for the grasses, we show for the first time that C_4 photosynthesis evolved primarily in tropical climates, and subsequently enhanced the rates of evolutionary transitions between tropical and temperate climates. When compared to close relatives, C_4 plants were furthermore more likely to colonise freezing environments. Our conclusions therefore contradict previous work based solely on geographical distributions and physiological theory. The macroevolutionary processes revealed in our large comparative study underpin the high ecological diversity and global expansion of C_4 species. Although there appear to be no physiological barriers to prevent C_4 plants from colonising cooler environments, C_4 grass clades have still migrated less frequently from temperate to cold climate regions than members of some C_3 lineages. This pattern arises from the recent origins of C_4 photosynthesis in warm climates, in lineages with warm adapted traits, which contrasts with the ancient origin of cold adaptation in Pooideae. C_4 plants must therefore have both the time and the opportunities to acquire further traits needed to successfully colonise cold climates.

2.7 Acknowledgements

This work was funded by a studentship from the Royal Thai Government. PAC is supported by a Royal Society University Research Fellowship (URF120119).

2.8 Supplementary data

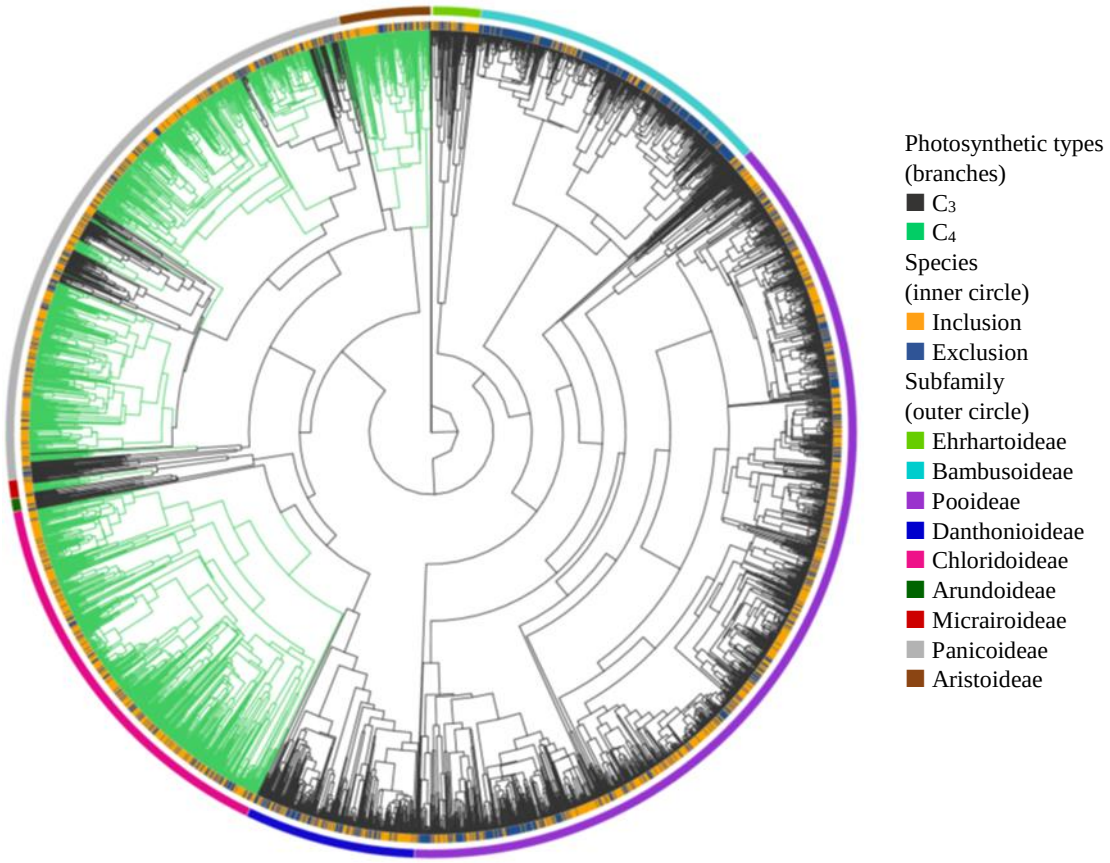

Figure S2.1 Phylogenetic tree shows the distribution of the excluded species due to lacking occurrence data.

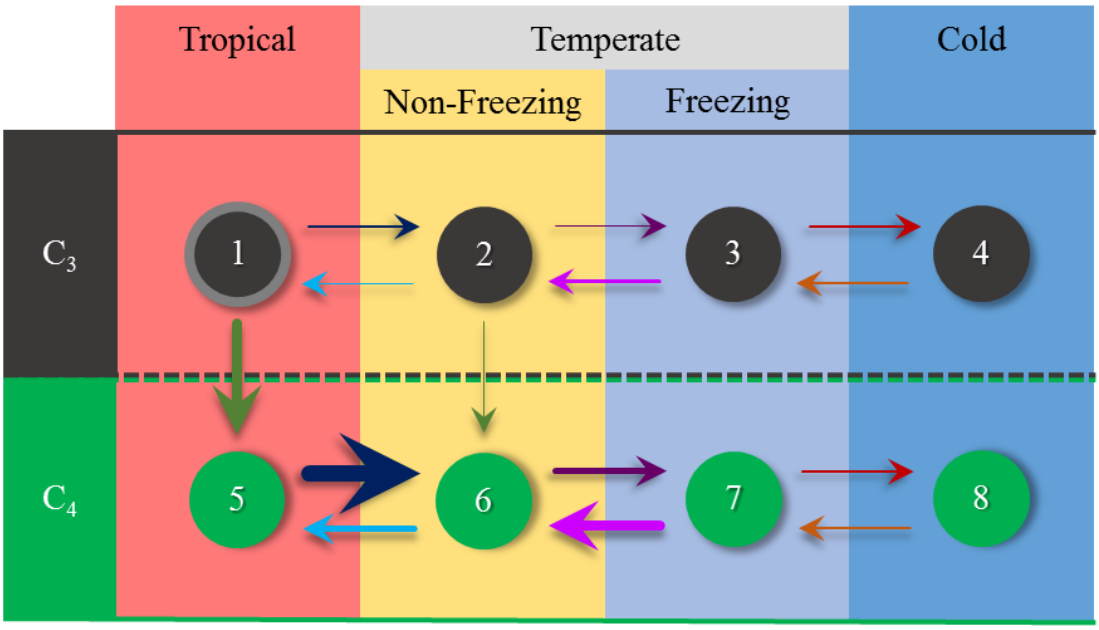

Figure S2.2 Model of coevolution of photosynthetic types and temperature niches in PACMAD clade. Size of arrows indicates transition rates among climate and photosynthetic types. The most likely ancestral condition is indicated by the grey outer circle.

Table S2.1 Distribution of C_3 and C_4 photosynthesis among climatic types before and after fitting into the phylogenetic tree.

Group	Number of species				
	Tropical	Temperate		Cold	Total
		Non-freezing	Freezing		
Before fitting into phylogenetic tree					
C_3	210 (5%)	859 (21%)	623 (15%)	475 (11%)	2,167 (52%)
C_4	710 (17%)	1,097 (26%)	154 (4%)	60 (1%)	2,021 (48%)
Total	920	1,956	777	535	4,188
After fitting into phylogenetic tree					
C_3	83 (4%)	439 (21%)	370 (17%)	293 (14%)	1,185 (56%)
C_4	263 (12%)	542 (25%)	105 (5%)	38 (2%)	948 (44%)
Total	346	981	475	331	2,133

Table S2.2 Distribution of grass subfamilies and C_4 photosynthesis among climatic types

Group	Number of species (Number of C_4 species)			
	Tropical	Temperate		Cold
		Non-freezing	Freezing	
Bambusoideae	18 (0)	37 (0)	12 (0)	3 (0)
Ehrhartoideae	10 (0)	28 (0)	3 (0)	3 (0)
Pooideae	0 (0)	234 (0)	314 (0)	272 (0)
Panicoideae	227 (179)	288 (243)	41 (36)	11 (7)
Arundinoideae	1 (0)	9 (0)	4 (0)	1 (0)
Chloridoideae	57 (57)	244 (244)	64 (64)	29 (29)
Micrairoideae	10 (7)	10 (5)	0 (0)	0 (0)
Aristidoideae	21 (20)	50 (50)	5 (5)	2 (2)
Danthonioideae	0 (0)	81 (0)	32 (0)	10 (0)
Outgroup	2 (0)	0 (0)	0 (0)	0 (0)
Total	346 (263)	981 (542)	475 (105)	331 (38)

Table S2.3 Rates of transitions in the PACMAD clade determined from point estimates of models.

State	Rate	Estimated rates
Tropical vs. Temperate climates and C_3 vs. C_4 photosynthesis		
Transition from tropical to temperate climate		
C_3	$Rate_{1\rightarrow2}$	0.02355
C_4	$Rate_{5\rightarrow6}$	0.11088
$C_4 - C_3$	$Rate_{5\rightarrow6} - Rate_{1\rightarrow2}$	0.08733***
Transition from tropical to temperate climate		
C_3	$Rate_{2\rightarrow1}$	0.00104
C_4	$Rate_{6\rightarrow5}$	0.03975
$C_4 - C_3$	$Rate_{6\rightarrow5} - Rate_{2\rightarrow1}$	0.03872***
Transition from C_3 to C_4 photosynthesis		
Tropical	$Rate_{1\rightarrow5}$	0.01161
Temperate	$Rate_{2\rightarrow6}$	0.00100
Tropical – Temperate	$Rate_{1\rightarrow5} - Rate_{2\rightarrow6}$	0.01061***
Temperate = 0	$Rate_{2\rightarrow6} - 0$	0.00100**
Transition from C_4 to C_3 photosynthesis		
Tropical	$Rate_{5\rightarrow1}$	0
Temperate	$Rate_{6\rightarrow2}$	0
Tropical = Temperate = 0	$Rate_{5\rightarrow1} + Rate_{6\rightarrow2} - 0$	0^{ns}
Temperate climates without freezing vs. with freezing and C_3 vs. C_4 photosynthesis		
Transition from temperate climate without freezing to with freezing		
C_3	$Rate_{2\rightarrow3}$	0.00510
C_4	$Rate_{6\rightarrow7}$	0.01390
$C_4 - C_3$	$Rate_{6\rightarrow7} - Rate_{2\rightarrow3}$	0.00881**
Transition from temperate climates with freezing to without freezing		
C_3	$Rate_{3\rightarrow2}$	0.04660
C_4	$Rate_{7\rightarrow6}$	0.08201
$C_4 - C_3$	$Rate_{7\rightarrow6} - Rate_{3\rightarrow2}$	0.03541*
Transition from C_3 to C_4 photosynthesis		
Non-freezing	$Rate_{2\rightarrow6}$	0.00695
Freezing	$Rate_{3\rightarrow7}$	0.00000
Non-freezing – Freezing	$Rate_{2\rightarrow6} - Rate_{3\rightarrow7}$	0.00695*
Freezing = 0	$Rate_{3\rightarrow7} - 0$	0^{ns}
Transition from C_4 to C_3 photosynthesis		
Non-freezing	$Rate_{6\rightarrow2}$	0
Freezing	$Rate_{7\rightarrow3}$	0
Non-freezing = Freezing = 0	$Rate_{6\rightarrow2} + Rate_{7\rightarrow3} - 0$	0^{ns}
Temperate vs. Cold climates and C_3 vs. C_4 photosynthesis		
Transition from temperate to cold climates		
C_3	$Rate_{3\rightarrow4}$	0.01196
C_4	$Rate_{7\rightarrow8}$	0.00403
$C_4 - C_3$	$Rate_{7\rightarrow8} - Rate_{3\rightarrow4}$	-0.00793^{ns}
Transition from cold to temperate climates		
C_3	$Rate_{4\rightarrow3}$	0.24525
C_4	$Rate_{8\rightarrow7}$	0.12936
$C_4 - C_3$	$Rate_{8\rightarrow7} - Rate_{4\rightarrow3}$	-0.11590^{ns}
Transition from C_3 to C_4 photosynthesis		
Temperate	$Rate_{3\rightarrow7}$	0.00596
Cold	$Rate_{4\rightarrow8}$	0.00000
Temperate – Cold	$Rate_{3\rightarrow7} - Rate_{4\rightarrow8}$	0.00596^{ns}
Cold = 0	$Rate_{4\rightarrow8} - 0$	0^{ns}
Transition from C_4 to C_3 photosynthesis		
Temperate	$Rate_{7\rightarrow3}$	0
Cold	$Rate_{8\rightarrow4}$	0
Temperate = Cold = 0	$Rate_{7\rightarrow3} + Rate_{8\rightarrow4} - 0$	0^{ns}

Asterisk indicates the differences between rates of transitions, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ^{ns} indicates no differences between rates of transitions

Chapter 3

Ecological shifts from tropical to cold climates require constitutive cold resistance before acclimation

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I co-designed the study with Colin Osborne and Pascal-Antoine Christin. I performed the experiments and analysed the data. Erik Murchie provided the chlorophyll fluorometer and supervision on its usage. I wrote the manuscript with the the help of Colin Osborne and Pascal-Antoine Christin.

3.1 Abstract

Grasses first evolved in warm habitats, but today have a near-ubiquitous global distribution, from tropical savannahs to polar and alpine tundras. It has been hypothesised that colonisation and diversification within cold climates require adaptations to low and freezing temperatures which are difficult to evolve. In the grass family, two lineages acquired such cold adaptations millions of years ago, and subsequently diversified to achieve widespread dominance of cold habitats. However, phylogenetic analyses suggest numerous more recent origins of cold adaptations in the group that remain largely

unexplored. In this study, we experimentally evaluated the chilling and freezing resistance of grasses originating from various climates and covering the phylogenetic diversity of the group to test the hypothesis that transitions to cold habitats involved acclimation mechanisms and increases of cold tolerance. As expected, grasses originating from cold climates were more resistant to chilling and freezing than tropical ones. There was however little evidence of cold acclimation responses in any of the assessed species, and consequently no increase in osmotic pressure. The adverse effects of chilling were particularly pronounced in some tropical species, progressively inhibiting photosynthesis over a two-week period. Moreover, high tissue moisture content of tropical species limited their freezing resistance. However, other species from tropical climates that had low tissue moisture content showed constitutive freezing resistance that mimicked that observed in species from cold climates. These results suggest that migration to cold environmental conditions was based on the co-option of constitutive cold resistance, which can arise in some tropical taxa because of changes in their leaf moisture content. The initial transitions were followed by further specialisations, including specific acclimation mechanisms, which improved the success of these species in cold climates.

3.2 Introduction

Adaptation to cold climates has occurred multiple times independently in angiosperms (Donoghue & Edwards 2014). The cold adaptive traits that evolved in a number of clades allowed these plants to pass through the ecological filter imposed by cold conditions, such that the ability to survive a particular low temperature threshold sorts plant distributions from tropical to cold climates across both latitudinal and altitudinal gradients (Woodward 1990; Preston & Sandve 2013; Körner 2016). Low temperatures, including freezing, pose a number of challenges for growth and survival. Exposure to temperatures in the range from 0 to 10 – 12 °C is known as chilling, and has a number of adverse effects. First, the reduction of energy from decreased respiration affects cytoplasmic streaming, thereby limiting water and nutrient uptakes (Lyons 1973). Photosynthetic performance is also reduced by photoinhibition because low temperatures limit photochemical energy use and, if it is not dissipated, the surplus energy produces damaging free radicals, limiting biosynthesis and growth (Sonoike 1996; Harrison *et al.* 1998). Moreover, chilling temperatures change the membrane structure from a semifluid state into a semi-crystalline

state causing loss of fluidity and function. These effects develop progressively during chilling episodes and the full extent of chilling damage can cause death. Freezing causes additional damage via ice formation, which kills cells if the protoplast contains a high water content and ice forms internally, or dehydrates the cell if ice forms in extracellular spaces (Sakai & Larcher 1987; Woodward 1990; Larcher 2003). Freezing also produces air bubbles in the hydraulic system which lead to embolisms that limit water transport (Krause *et al.* 1988). Although mechanisms that have evolved to avoid or tolerate these challenges have been identified, it is currently unknown whether there are general mechanisms that explain how species have adapted to low temperatures. Some morphological and anatomical adaptations such as deciduous leaves, small conduits, sclerophyllous leaves and a herbaceous life form have been identified as key strategies enabling plants to migrate and become successful in cold habitats, while some others such as cushion life form, and toothed and lobed leaves, arise as adaptations after the colonisation of cold habitats (Salleo *et al.* 1997; Zanne *et al.* 2014; Boucher *et al.* 2016; Edwards *et al.* 2016).

Grasses are an interesting group in which to study adaptation to cold climates because, despite their tropical ancestors, they have occupied almost all terrestrial habitats from savannahs to tundras (Hartley 1950; Gibson 2009; Edwards & Smith 2010) with an atypical latitudinal diversity gradient that arises (Visser *et al.* 2014) because particular groups (Pooideae and Danthonioideae) have specialised in temperate and cold climates (Chapter 2; Cross 2007; Edwards & Smith 2010; Linder & Bouchenak-Khelladi 2017). Various traits and mechanisms have been associated with the evolution of cold adaptation in Pooideae. In this subfamily, cold acclimation is one of the important adaptive mechanisms to improve frost tolerance and survival in cold climates. At low temperatures, cold acclimation alters numerous biochemical, physiological and metabolic functions (Thomashow 1999; Sandve *et al.* 2011). In particular, the photosynthetic apparatus is modified in order to minimise the inhibitory effect of photoinhibition at low temperatures (Huner *et al.* 1993; Ouellet 2007). Additionally, anti-freeze proteins and fructans are accumulated to protect plant cells and functions from ice crystallization during freezing (Hincha *et al.* 2000; Sidebottom *et al.* 2000; John *et al.* 2009). These mechanisms are controlled by cold responsive genes which play a crucial role in adaptation to cold climates (Sandve *et al.* 2011). Apart from acclimation, survival to sudden freezing can be promoted by supercooling mechanisms that reduce the freezing

point to become sub-zero, thereby preventing damage caused by ice formation within cells (Sakai & Larcher 1987). The Pooideae and Danthonioideae groups of grasses evolved cold adaptations during the Eocene and Oligocene that allowed them to migrate and become successful in cold climates (Bouchenak-Khelladi *et al.* 2010; Sandve & Fjellheim 2010; Humphreys & Linder 2013). The mechanisms allowing transitions to cold conditions in other groups of grasses remain however largely unexplored.

Cold adaptations evolved relatively recently in a number of groups of grasses, many of which use C_4 photosynthesis, which has generally been considered a physiological barrier to low temperature adaptation (Chapter2; Long *et al.* 1975; Kubien & Sage 2004; Sage *et al.* 2010). Although C_4 photosynthesis is typically less efficient than C_3 photosynthesis at low temperatures, a number of C_4 grasses are found in cold climates such as in alpine and high-latitude habitats where they co-occur with Pooideae and Danthoniodeae (Ehleringer & Björkman 1977; Sage *et al.* 2010; Linder *et al.* 2013; Preston & Sandve 2013). These species have evolved survival mechanisms similar to those observed in Pooideae, including freezing resistance and cold acclimation (Liu & Osborne 2008, 2013; Sage *et al.* 2010; Sandve *et al.* 2011). These previous studies suggest that the clades with recent cold adaptation may share mechanisms with the older lineages to migrate and become successful in cold climates, which may indicate a shared common cold adaptive mechanisms in grasses. However, systematic tests for an association between specific cold resistant mechanisms and migration to cold climates in grasses are lacking.

In this study, we assess the chilling and freezing resistances of grasses from recently cold adapted clades to test the hypothesis that transitions to cold climates involved increases in these properties. We compare within a phylogenetic framework species originating from different climates to separate the effects of the phylogeny from those of the climate. Data obtained in controlled conditions were used to test: (1) whether chilling and freezing resistances differ between species from tropical and cold climates; (2) whether cold acclimation increases freezing resistance; and (3) which traits contribute to chilling and freezing resistances in grasses.

3.3 Materials and methods

Experimental design

The experiment was carried out in controlled environment growth chambers and was designed to assess: (1) chilling resistance in the short and long terms; (2) freezing resistances with and without acclimation, and (3) mechanisms induced by cold acclimation. It was therefore separated into two periods during which plants were exposed to chilling and freezing conditions, respectively (Fig. 3.1). All plants were first grown under control conditions with a day/night temperature of 25/20 °C. During the chilling period, half of the plants from each species were transferred to a low temperature treatment of 15/5 °C day/night for 14 days, while the other half remained in control conditions (Fig. 3.1). To measure resistance of the photosynthetic system to chilling, the difference in maximum quantum efficiency of photosystem II (PS2) between the chilling and control treatments was measured on the 1st ($\Delta F_v/F_m$ AC1), 7th ($\Delta F_v/F_m$ AC7) and 14th ($\Delta F_v/F_m$ AC14) day (Fig. 3.1). The efficiency of PS2 is a good metric for photoinhibition (Maxwell & Johnson 2000). On the 14th day, leaf osmotic pressure (Π) was measured in the chilling treatment and control to evaluate its role in any cold acclimation (Fig. 3.1).

Freezing was applied to all plants on the 15th day. First, plants from both the chilling treatment and control were transferred to chilling conditions under a low light intensity to minimise photoinhibitory effects. In the chamber, the temperature was gradually reduced from 15 to –5 °C overnight, after which temperature was increased to the control conditions (Fig. 3.1). The difference between the maximum quantum efficiency of PS2 in the control plants before and after freezing ($\Delta F_v/F_m$ AF) was to test for constitutive freezing resistance, while comparison between the maximum quantum efficiency of PS2 in the chilling and control treatments after freezing was tested for effects of acclimation on freezing resistance (i.e. cold acclimation) (Fig. 3.1). The effect of cold acclimation on freezing resistance was also investigated by comparing electrolyte leakage (Leakage) between the chilling and control treatments after freezing (Fig. 3.1).

This study also investigated the identity of traits explaining chilling and freezing resistance, so general morphology and water related traits were retrieved from a database (culm height, leaf width and length) or measured in the experiment (leaf water content

and osmotic pressure). Leaf water content was measured on plants growing in control conditions.

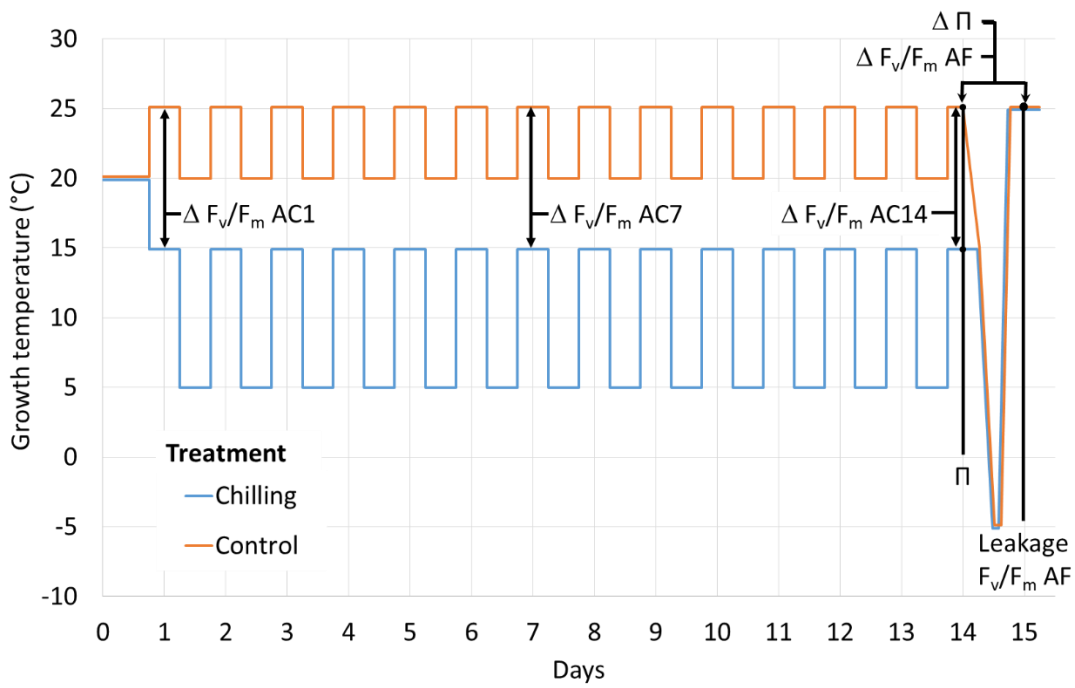

Figure 3.1 Overview of experimental conditions and measurements.

Plant material and environmental conditions

The experiment compared closely related species from tropical and cold climates. The climatic type was described using a method similar to that used chapter 2, which followed the Köppen-Geiger classification. Briefly, plants originating from tropical (mean temperature of the coldest month above 18 °C) and cold (mean temperature of the coldest month below 0 °C, which includes continental, polar and alpine) climates were chosen to capture a number of climatic contrasts. The final set of species is based on (1) availability of seeds, (2) climate type, and (3) phylogenetic patterns. Specifically, for each sister groups differing in the climatic origin based on the results from chapter 2, we selected at least one species from each of the two groups. Tropical species in Bambusoideae and Ehrhartoideae were included to encompass both C_3 and C_4 species. In total, 28 were included in this experiment, which captured at least 9 transitions between tropical and cold climates (Table S3.1).

Most of the species were germinated from seeds except *Miscanthus sinensis*, *Miscanthus sacchariflorus*, *Miscanthus* × *giganteus* and *Saccahrum officinarum* that were obtained as mature plants from a nursery (Table S3.1). The seeds were germinated in sterile agar for one week, after which they were transferred to pots with a high nutrient compost (Levington Advance Pot & Bedding M3, ICL, Suffolk, UK) for an extra two weeks. All plants were then grown in pots (10 × 10 × 17 cm^3) containing six parts of M3 compost, one part of sand (Chelford 52, Sibelco, UK) and one part of Perlite (Sinclair Pro, Cheshire, UK) in a controlled environment chamber (storage chamber) set to the control treatment with a 12 h photoperiod with a photon flux density (PFD) of 600 µmol m^{-2} s^{-1} measured at plant height, day/night temperature of 25/20 °C, and relative humidity of 60/80% for 12 weeks. The plants were watered daily with deionised water and twice a week with 40% Long Ashton solution to provide sufficient nutrients and moisture.

For the chilling period, plants were transferred from the storage chamber to a growth chamber applying the chilling treatment of 15/5 °C for 14 days, while the other half were transferred from the storage chamber to another growth chamber applying the control treatment of 25/20 °C. One replicate of each species was transferred on consecutive days, so that each transition between treatments and set of measurements were undertaken on different days for each replicate. Plants were exchanged between cabinets midway through the experiment to minimise the confounding effects of treatment and cabinet.

For the freezing period, each replicate from the chilling period was transferred to a walk-in controlled environment cabinet set to 15 °C and a PFD of 300 µmol m^{-2} s^{-1} at 14:00h. Plants were exposed to these conditions for 5 hours before the lights were turned off. The temperature was ramped downwards gradually by 4 °C per hour to reach 0 °C, then decreased more slowly to reach a minimum temperature of –5 °C at 07:00h the following day. After 1 hour at –5 °C, the lights were turned on to deliver a PFD of 300 µmol m^{-2} s^{-1}. The temperature was then ramped gradually upward to reach 15 °C at 13:30h.

Leaf water content

Nine mature leaves of each species in the control environment (25/20 °C) were collected and weighed to consecutively obtain fresh mass (M_f), turgid mass (M_t) after rehydration

overnight and dry mass (M_d) after oven drying at 70 °C for 3 days. Leaf moisture content (MC) and leaf relative water content (RWC) were determined as $MC = [(M_f - M_d) / M_d] \times 100$ and $RWC = [(M_f - M_d) / (M_t - M_d)] \times 100$, respectively.

Leaf osmotic pressure

Osmotic pressure (Π) of expressed sap for each species under the chilling and control treatments were measured (Fig. 3.1) using a vapor-pressure osmometer (Vapro, Wescor Inc, Logan, Utah, USA). The plants were watered at dawn three hours before sampling. Small pieces of the middle part of the leaf without the mid-vein were collected in 1.5 ml microtubes, immersed in liquid nitrogen and subsequently thawed. Expressed sap from thawed samples was passed through a fine hole in the base of the microtube into another microtube by evenly distributing the force from two ball bearings of combined weight 1.4 g above samples and centrifuging at 2000 RFC (g) for 3 min. The osmolality of expressed sap was determined using the vapor-pressure osmometer that was calibrated daily with standard NaCl solutions. Osmotic potential of expressed sap was calculated from the osmolality of expressed sap $(n/V)_{ES}$ using the Van't Hoff equation: $\Pi = (n/V)_{ES}RT$ where R is the universal gas constant (8.314472) and T is temperature (293 K).

Maximum quantum efficiency

The measurement of photosynthetic efficiency in chilling treatment and control environment was carried out after 24 hours, 7 and 14 days, and after the freezing treatment (Fig. 3.1), using a PAM chlorophyll fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) to calculate the maximum quantum efficiency of PS2 for each species. A mature leaf of each sample was adapted in the dark for 30 min and then exposed to a low-intensity activating beam to obtain the zero fluorescence level (F_0) and followed by a saturation pulse to obtain the maximum fluorescence level (F_m). Each sample was measured at least two times with consistent value. Maximum quantum efficiency (F_v/F_m) was calculated using $F_v/F_m = (F_m - F_0)/ F_m$. The percentage change of F_v/F_m was obtained from the calculation [(F_v/F_m after treatment – F_v/F_m control)/ F_v/F_m control]*100.

Electrolyte leakage

Electrolyte leakage was used to determine leaf freezing injury. After the freezing treatment, a couple of 2 cm long middle sections were cut from each of the leaves. The total area was standardised to be 4 cm^2 by a number of leaf segments. Leaf segments of each sample were rinsed three times with deionised water and put into a vial containing 10 ml deionised water. The vial was shaken overnight at room temperature and the electrical conductivity measured using a conductivity meter (Jenway 3540, Cole-Parmer, Staffordshire, UK) to obtain conductivity after freezing (C_f). The conductivity of killed leaves was measured as a reference after the sample had been autoclaved and cooled down to obtain total conductivity (C_t). The degree of leakage was estimated by relative conductivity which was determined as Leakage = $[C_f / C_t] \times 100$.

Plant traits

Quantitative descriptions were compiled from GrassBase (Clayton *et al.* 2006). Culm height, leaf length and leaf width were reported as the mean of maximum and minimum values of each species. The occasional extreme values were excluded from analyses.

Statistical analysis

A phylogenetic generalised linear regression model (PGLS) was fitted using climatic types as a predictor and leaf water contents, leaf osmotic potential, maximum quantum efficiency and electrolyte leakage as variables to test whether these traits and responses differ between tropical and cold climates. The model was also used to test for relationships among traits, and among traits and chilling and freezing resistances. The analysis used a published phylogenetic tree with a time-calibration hypothesis based on macrofossils and microfossils (Spriggs *et al.* 2014) following the previous climatic transition study (Chapter 2) to control for phylogenetic non-independence and to estimate phylogenetic signal λ with the Caper package in R (Pagel 1999; Freckleton et al. 2002). An ordinary linear model (OLS) was used to test whether physiological responses differed between chilling and control (chilling pre-treatment) in each species.

3.4 Results

Correlation between climatic origin and cold resistances

Photosynthetic efficiency in our experiment was used as an indicator of chilling and freezing resistances. As expected, phylogenetic comparative analyses showed that chilling inhibited photosynthesis progressively in tropical species through the two-week duration of this treatment (Fig. 3.2, Table 3.1) and freezing dramatically lowered the photosynthetic performance further (Table 3.1). Although chilling and freezing also decreased photosynthetic efficiency in species from cold climates, the reduction of photosynthesis in species from cold climates was less pronounced than that of species from tropical climates (Table 3.1, Fig. 3.3). Moreover, the gap between the inhibitory effects of chilling on photosynthesis from tropical and cold climates widened over time (Table 3.1).

Table 3.1 Results of phylogenetic generalised linear models testing for the effects of climatic types on plant traits, and physiological responses after transferring from control (warm) conditions to chilling and freezing treatments (denoted by Δ). Significance level: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, n refers to sample size and λ refers to Pagel's estimator of phylogenetic signal.

Variable	n	λ	Cold climates	Tropical climates	*P*-value
Traits					
Culm height (cm)	28	0.933	196	224	0.4204
Leaf length (cm)	28	0.000	32	36	0.6852
Leaf width (mm)	28	0.045	8	12	0.2823
Moisture content (%)	27	0.555	241	320	0.0085***
Π (MPa)	28	0.783	1.54	1.42	0.1148
Responses to chilling					
$\Delta F_v/F_m$ AC1 (%)	28	0.767	–0.1	–4.1	0.0558
$\Delta F_v/F_m$ AC7 (%)	28	0.428	–12.4	–29.9	0.0001***
$\Delta F_v/F_m$ AC14 (%)	28	0.000	–15.6	–34.8	0.0004***
ΔΠ (%)	28	0.750	10.0	12.9	0.652
Responses to freezing					
Leakage (%)	27	0.756	37.2	66.5	0.0016**
$\Delta F_v/F_m$ AF (%)	27	0.343	–26.4	–58.3	0.0019**

In addition to photosynthetic performance, electrolyte leakage, which measured the damage of membranes after freezing, was also used to assess freezing resistance. Electrolyte leakage was consistent with the reduction of photosynthetic efficiency after freezing (Table 3.1). These results indicate that, as expected, cold climate species are on average more resistant to chilling and freezing than tropical ones (Fig. 3.3, Table 3.1). Surprisingly however, two species from tropical climates were as resistant to chilling and freezing as species from cold climates (Fig. 3.3), even though the plant material in this study originated from tropical climate locations (Table S3.1). However, the geographical distribution of these species indicated that they can colonise cold environments in high tropical mountains where a few populations are found even though most of these species occur in warmer regions (Figs. S3.3 and S3.4). A strong phylogenetic signal was detected only in electrolyte leakage (Table 3.1), meaning that different clades of species from tropical and cold climates differ in their freezing resistance. The phylogenetic signal of both chilling and freezing resistance was not significantly different from zero, indicating that cold resistance tends not to be independent of the phylogeny (Table 3.2).

Table 3.2 Phylogenetic signal of plant traits and physiological responses after transferring from control (warm) conditions to chilling and freezing treatments. Significance level: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, n refers to sample size and λ refers to phylogenetic signal.

Variable	n	λ	$P_{(\lambda=0)}$	$P_{(\lambda=1)}$
Traits				
Culm height (cm)	28	**0.952**	0.011*	0.326
Leaf length (cm)	28	0.000	1	0.000***
Leaf width (mm)	28	0.000	1	0.000***
Moisture content (%)	27	0.000	1	0.000***
Π (MPa)	28	**0.719**	0.008**	0.108
Responses to chilling				
$\Delta F_v/F_m$ AC1 (%)	28	0.627	0.845	0.000***
$\Delta F_v/F_m$ AC7 (%)	28	0.000	1	0.000***
$\Delta F_v/F_m$ AC14 (%)	28	0.000	1	0.000***
ΔΠ (%)	28	0.733	0.152	0.004**
Responses to freezing				
Leakage (%)	27	0.673	0.148	0.041*
$\Delta F_v/F_m$ AF (%)	27	0.154	0.600	0.028*

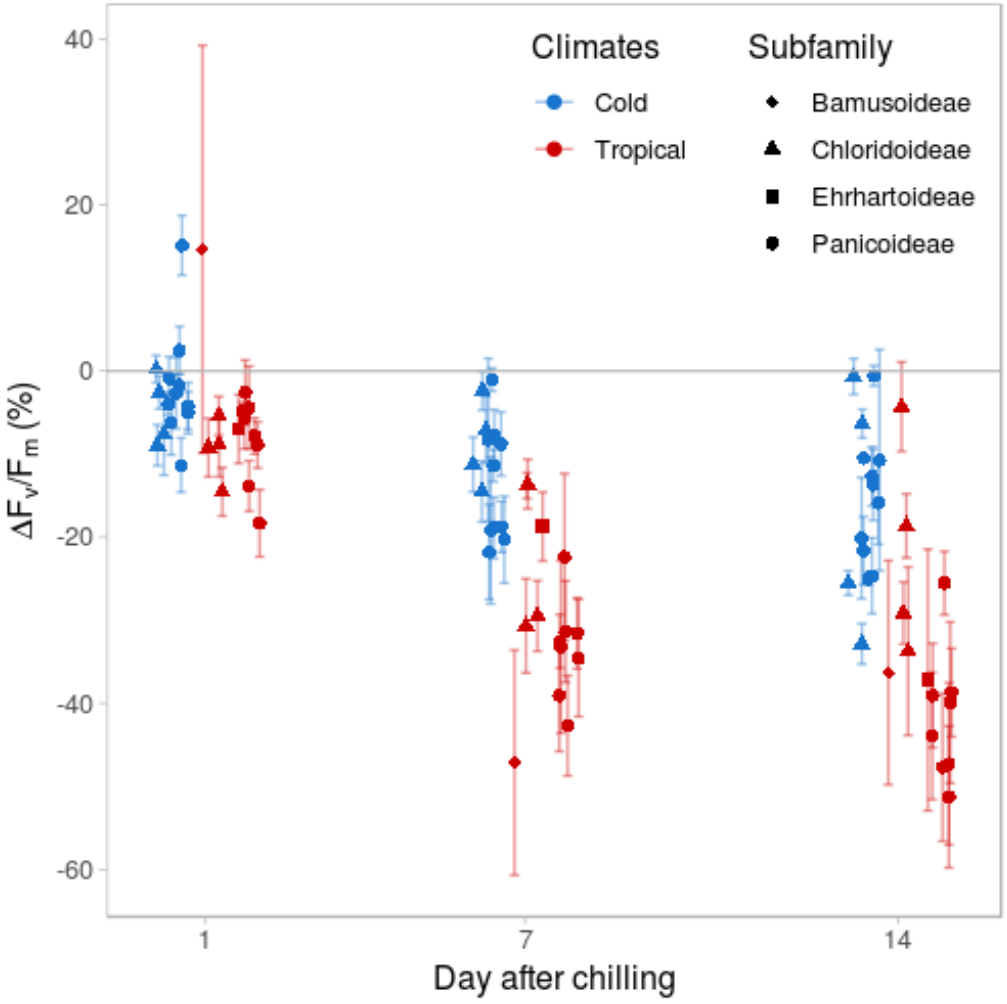

Figure 3.2 Change of maximum quantum yield of photosystem II ($\Delta F_v/F_m$) after 1, 7 and 14 days of the chilling treatment, for species originating from tropical and cold climates and from various subfamilies.

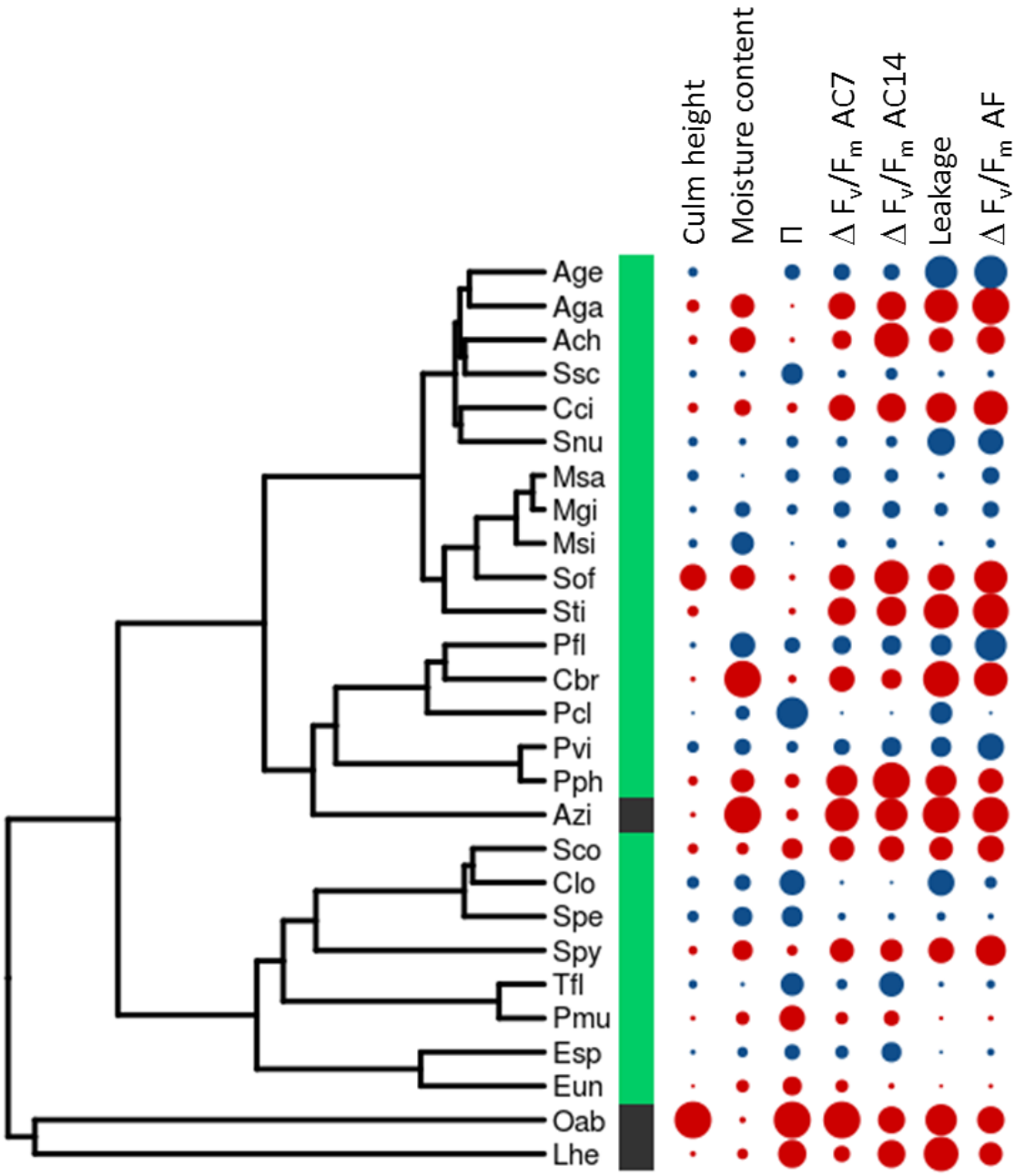

Figure 3.3 Phylogenetic distribution of climatic origin, values of plant traits, and freezing and chilling resistances. Green denotes C_4 species, while black shows C_3 ones. The red circles show value for species from tropical climates, while blue circles indicate those for species from cold climates. The relative values of traits are indicated by the size of the circles. Abbreviations are: osmotic pressure (Π); effect of chilling on the maximum quantum yield of PS2 on the 7^{th} ($\Delta F_v/F_m$ AC7) and 14^{th} ($\Delta F_v/F_m$ AC14) days of the treatment; and effect of the freezing event on the maximum quantum yield of PS2 for plants from the control treatment ($\Delta F_v/F_m$ AF).

Cold acclimation and adaptation to cold climates

Cold acclimation was expected to be an important strategy for increasing freezing survival. As expected, cold acclimation in leaves was found in several species from cold climates. The chilling pre-treatment significantly reduced the freezing damage assessed by measurement of electrolyte leakage in *Andropogon gerardii* (Age), *Calamovilfa longifolia* (Clo), *Leersia hexandra* (Lhe), *Pennisetum clandestinum* (Pcl), *Spartina pectinata* (Spe), and *Schizachyrium scoparium* (Ssc). The largest effect of cold acclimation was in *Calamovilfa longifolia* (Clo) with a 90% reduction of electrolyte leakage in plants subjected to the chilling treatment compared with plants directly transferred from warm conditions. However, most of the cold acclimation responses in this experiment reduced the electrolyte leakage by less than 50% in both freezing resistant (*Spartina pectinata*; Spe, *Schizachyrium scoparium*; Ssc) and sensitive (*Andropogon gerardii*; Age) species from cold climates. No effect of cold acclimation, as indicated by no effect of the chilling treatment on freezing damage, was also found in a number of species originating from cold climates, including *Eragrostis spectabilis* (Esp), *Tridens flavus* (Tfl), *Miscanthus sinensis* (Msi), *Miscanthus* × *giganteus* (Mgi) and *Miscanthus sacchariflorus* (Msa) which were highly freezing resistant, irrespective of the pre-treatment. However, a number of the species originating from cold climates that lacked cold acclimation were freezing sensitive, notably *Pennisetum flaccidum* (Pfl), *Panicum virgatum* (Pvi) and *Panicum virgatum* (Snu). Furthermore, cold acclimation did not protect photosynthetic efficiency after freezing (Fig. 3.4). Instead, there was evidence that exposure to chilling worsened the impact of freezing on photosynthesis for some species originating from tropical climates (Fig. 3.4).

Our results show that cold acclimation is not a general requirement for species to inhabit cold climate regions, despite its potential importance for freezing survival, in some species. Conversely, most of the tropical species were sensitive to freezing without cold acclimation. Interestingly, cold acclimation was found in the tropical *Leersia hexandra* (Lhe), although it only reduced electrolyte leakage slightly (Fig. 3.4). This suggested that cold acclimation may be present but inefficient in tropical species.

It was hypothesised that cold acclimation induces the accumulation of compatible osmolytes that increase osmotic pressure and subsequently freezing resistance. Our results showed that the chilling pre-treatment did increase the osmotic pressure (Π) in some species from cold climates (Fig. 3.4). However, the increase of osmotic pressure

after cold acclimation in these species was not associated with greater freezing resistance (Fig. 3.4). Unexpectedly, cold acclimation increased osmotic pressure in the tropical species, but there was no correspondence to the increase of freezing resistance except in *Leersia hexandra* (Lhe) (Fig. 3.4). Therefore, the improvements in freezing resistance arising from cold acclimation must typically result from other mechanisms.

Trait contribution to chilling and freezing resistances

Our PGLS analyses showed that only one plant trait was related to climatic origins; the moisture content. The higher moisture content in species from tropical than cold climates was associated with the lower freezing resistance in tropical species, indicated by the correlation of moisture content with both electrolyte leakage and the reduction of photosynthetic efficiency after freezing (Figs. 3.3 and 3.5). Species from the tropical climates that had a moisture content as low as closely related species from cold climates were as chilling and freezing resistant as their cold climate counterparts (Fig. 3.3).

Apart from traits related to climatic origins, chilling and freezing resistances significantly correlated with culm height, leaf width and osmotic pressure. These three traits and climatic origin can be used as a predictor for chilling and freezing resistances; approximately 44 % of the variation in changes of photosynthetic efficiency after one day of chilling are explained by a combination of culm height and leaf width ($P < 0.001$). This result suggests that large plants with narrow leaves are more resistant after 24 hours to the cold shock imposed by a sudden change to chilling than small plants with broad leaves. On the other hand, the variation in other measures of freezing and chilling resistances were associated with the climatic origin. The variation in the change of photosynthetic efficiency after 14 days of chilling and electrolyte leakage after freezing was explained only by the climatic origin ($R^2 = 0.39$, $P < 0.001$ and 0.33, $P < 0.01$, respectively). The change of photosynthetic efficiency after 7 days of chilling was explained by a combination of culm height and climate origin ($R^2 = 0.6$, $P < 0.001$; Table 3.3). This means that the larger plants from cold climates were more likely to be resistant to chilling than small plants from tropical climates in this condition. The change of photosynthetic efficiency after freezing was by a combination of osmotic pressure and climatic type ($R^2 = 0.44$, $P < 0.001$; Table 3.3). This shows that the high osmotic pressure

in species from cold climates is associated with high freezing resistance of photosynthesis (Table 3.3).

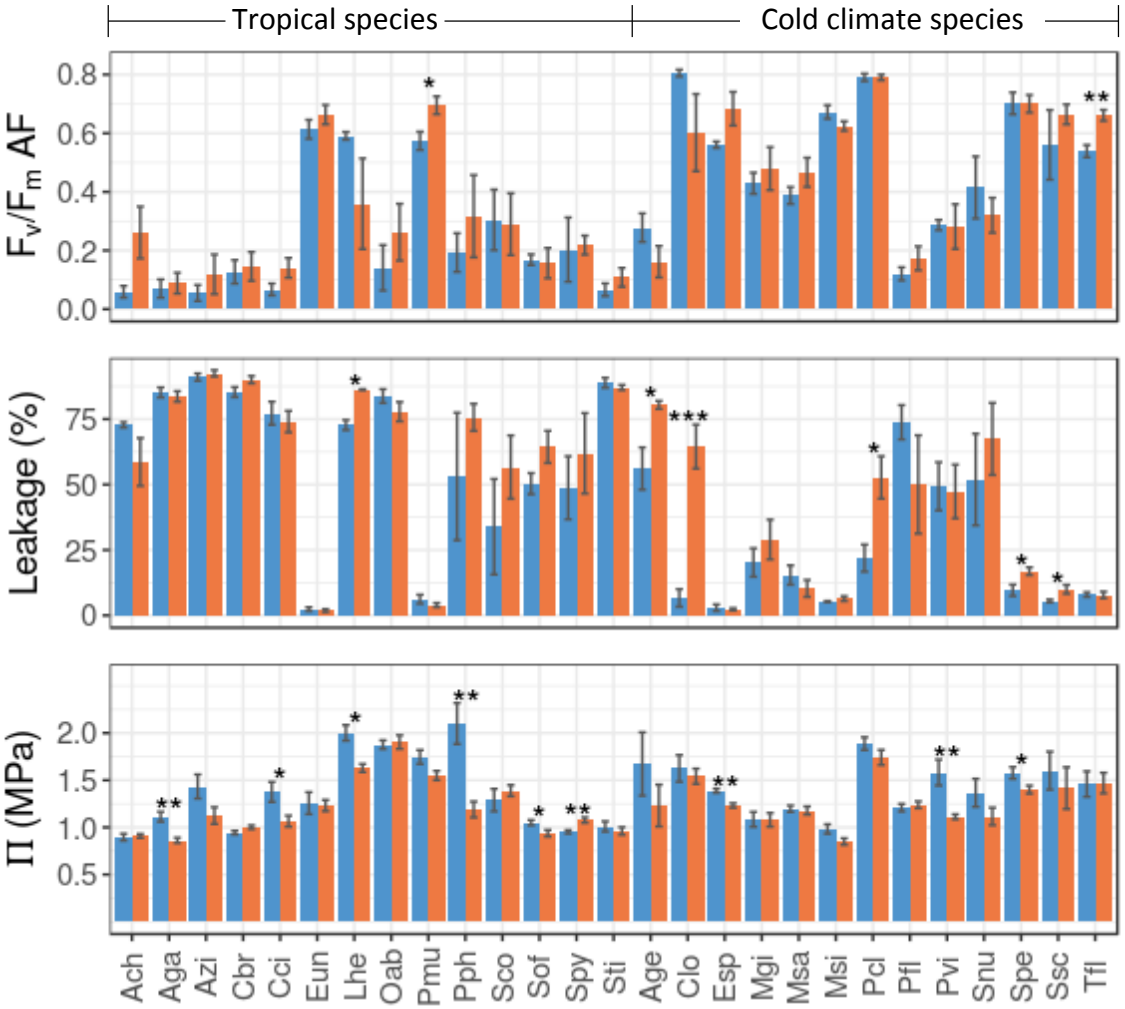

Figure 3.4 Comparison between the effects of chilling pre-treatment (blue bars) and control (orange bars) on osmotic pressure (Π), electrolyte leakage (Leakage) and maximum quantum yield of PS2 after the freezing event (F_v/F_m AF). Abbreviations for species names are as in Table S3.1. Values are mean ± SE. Significance level for the effect of chilling pre-treatment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

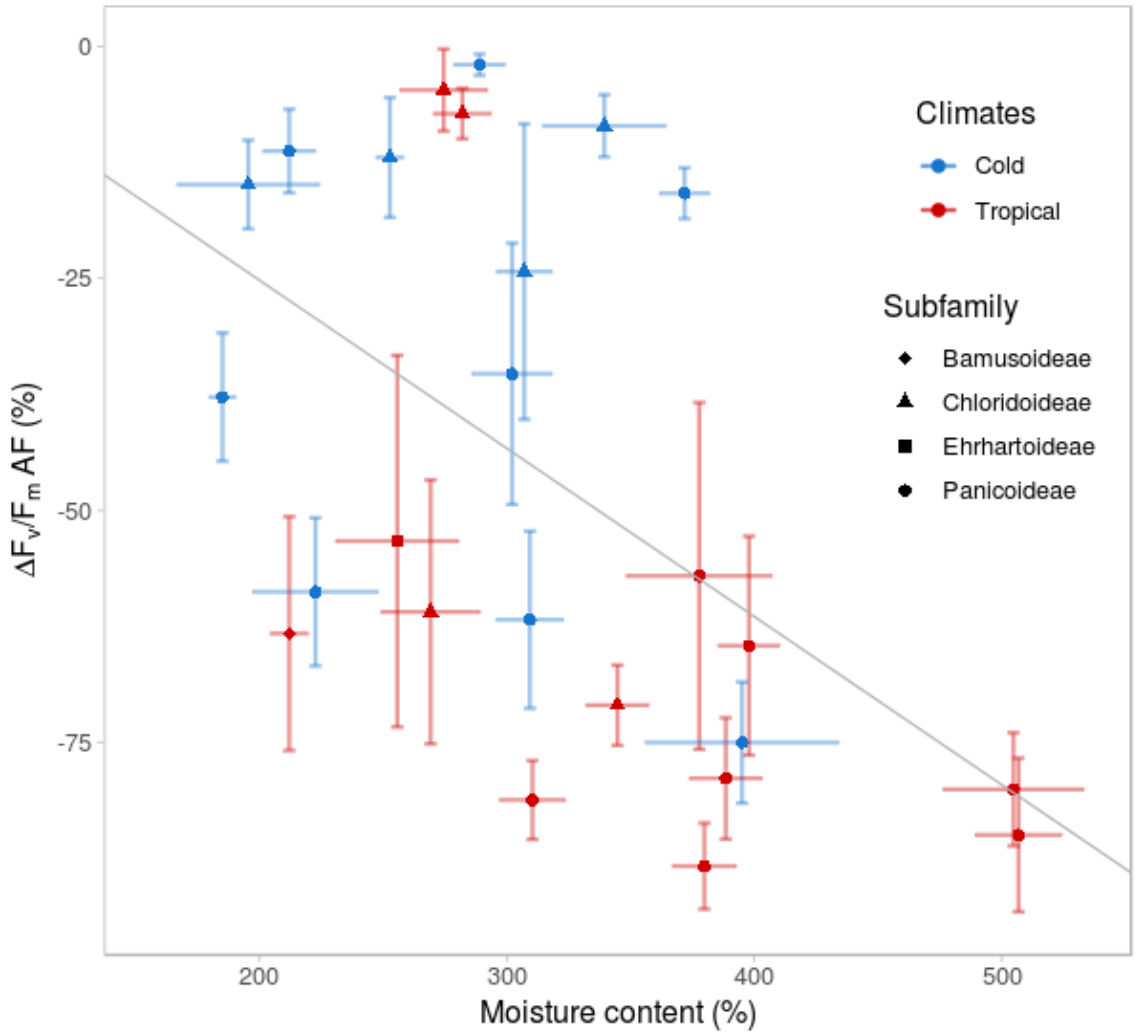

Figure 3.5 Relationship between moisture content and the change of maximum quantum yield in photosystem II after freezing ($\Delta F_v/F_m$ AF) in grasses from tropical and cold climates and various subfamilies.

Table 3.3 The contributions of climate and plant traits to chilling and freezing responses, as determined by phylogenetic generalised linear models. In combination, climatic types and species mean trait values of culm height, leaf length, leaf width, moisture content and osmotic pressure (Π) predicted the change of maximum quantum yield in photosystem II on the 1st ($\Delta F_v/F_m$ AC1), 7th ($\Delta F_v/F_m$ AC7) and 14th ($\Delta F_v/F_m$ AC14) day of chilling, electrolyte leakage (Leakage) and the change of maximum quantum yield in photosystem II after freezing ($\Delta F_v/F_m$ AF). Significance level: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Variable	Climate	Culm height	Leaf length	Leaf width	Π	R^2
$\Delta F_v/F_m$ AC1	ns	0.04***	ns	-0.50**	ns	0.44***
$\Delta F_v/F_m$ AC 7	-16.23***	-0.03*	ns	ns	ns	0.61***
$\Delta F_v/F_m$ AC14	-19.00***	ns	ns	ns	ns	0.39***
Leakage	29.29**	ns	ns	ns	ns	0.33**
$\Delta F_v/F_m$ AF	-26.72**	ns	ns	ns	45.84*	0.44***

3.5 Discussion

Traits affecting cold tolerance are infrequent in tropical grasses

Our phylogenetic analyses comparing plants with different climatic origins confirmed that tropical plants are sensitive to chilling and freezing because of physiological malfunction, so that distribution in cold climates is limited by a lack of chilling and freezing resistances in tropical species (Lyons 1973; Sakai & Larcher 1987; Woodward 1990; Larcher 2003). Moreover, our analyses indicated that climatic origin exerted a greater influence over chilling and freezing resistances than phylogenetic history (Table 3.1 and 3.2).

Tropical origin also affected traits such as moisture content, which is higher in species from tropical than cold climates (Table 3.1). This may be a factor influenced by tropical climates that acts to indirectly limit distribution into cold climates because a high tissue moisture content reduces freezing resistance (Fig. 3.5). Moisture content is important for freezing resistance because a higher water content in tissues increases ice formation, which damages cells (Beck *et al.* 1984; Goldstein *et al.* 1985; Lipp *et al.* 1994; Pescador *et al.* 2016). Because dry matter content is inversely correlated to moisture

content, previous work has also shown that ecotypes which adapt to high temperature showed higher water content/lower dry matter content than ecotypes from low temperature regions. For example, dry matter content was lower in a C_4 population of *Alloteropsis semialata* originating from a sub-tropical climate than in a C_3 population which has migrated into cooler climates (Osborne *et al.* 2008). Similarly, *Arabidopsis* spring ecotypes have a lower leaf dry matter content than winter ones (Tomeo & Rosenthal 2018). Moreover, the two species in this experiment that originated from tropical climates, but were constitutively freezing resistant, consistently had low moisture contents. This trait may allow these two species to migrate from the tropical lowlands to high altitudes despite their main populations remaining in the tropics. However, the size of the high altitude populations is limited possibly because of the lower efficiency in suboptimal temperature affected by tropical origin (Stewart 1945; Roseveare 1948).

Apart from traits influenced by climatic origin, plant size and leaf width were also associated with chilling resistance, while osmotic pressure was linked to freezing resistance. Large plants with narrow leaves may tolerate a chilling shock because small leaves lose energy more slowly than larger ones (Jordan & Smith 1994; Wright *et al.* 2017) but the plant size result contradicts the prediction that small plants are associated with low temperatures (Jordan & Smith 1994; Moles *et al.* 2009). The high osmotic pressure seems to relate to high freezing resistance in the case of photosynthetic efficiency because high concentrations of osmolytes will stabilise the structure and function of the cell (Sakai & Larcher 1987; John *et al.* 2016).

Cold acclimation is not compulsory for migration to cold climates

The cold acclimation developed in some species in this experiment suggested that exposure to low temperature was able to increase the freezing resistance in grasses from cold climates (Fig. 3.4), but it is not needed to inhabit cold climates (Table 3.2). This data, combined with previous studies (Rowley 1976; Liu & Osborne 2008, 2013), shows that cold acclimation evolved independently at least nine times during recent cold adaptation in grasses. However, most of the cold climate species in this study were constitutively resistant to freezing, suggesting that constitutive freezing resistance is more important than acclimation in recent cold adaptation. Moreover, some of the species from cold climates were sensitive to freezing, and had no acclimation strategy. In these cases,

the leaves of these perennial plants may not persist during winter. Instead, rhizomes and shoots underground must survive and then produce new leaves next summer, in a cold avoidance strategy (Sakai & Larcher 1987; Preston & Sandve 2013; Körner 2016). One of the mechanisms through which cold acclimation reduces ice formation is via the accumulation of osmolytes which increase osmotic pressure to lower the freezing point (Sakai & Larcher 1987). Although osmotic pressure increased in this study after chilling, indicating an osmotic adjustment in relation to cold acclimation, our results still found leaf damage after freezing which showed that the level of adjustment was insufficient to avoid ice formation. To reach equilibrium at –5 °C in this experiment, theoretically plants had to increase osmotic pressure to at least 5 MPa (Beck *et al.* 1984; Goldstein *et al.* 1985), a level that exceeding all of the values measured here (Fig. 3.4).

On the other hand, constitutive freezing resistance seems to be an important pre-adaptation for enabling plants to migrate into cold climates, allowing them to tolerate a particularly low temperature limit. Moreover, this study indicated that the low moisture content observed in particular lineages increases constitutive freezing resistance (Fig. 3.3), consequently enabling these plants to colonise environments where freezing is encountered. The low moisture content in tropical species may be an adaptation to dry habitats because the trait allows these species to survive in dry conditions for a longer time than species with a high moisture content (Poorter & Markesteijn 2008). This is consistent with the observation that small xylem conduits seem to pre-adapt plants to dry habitats prior to migration into freezing habitats (Zanne *et al.* 2014). Species containing small conduits are more resistant to drought than species containing large conduits because the dimension of xylem conduits affects the vulnerability to embolism under dry conditions, which causes hydraulic failure (Tyree & Sperry 1988; Yang & Tyree 1992; Blackman *et al.* 2010). Similarly, an embolism can also happen after freezing, so this trait also increases freezing resistance (Tyree & Sperry 1989; Choat *et al.* 2011). Moreover, genes from drought tolerance pathways seem to have been recruited for cold acclimation and cold resistance (Preston & Sandve 2013).

Based on these findings, we propose that pre-adaptation to dry habitats provided partial benefits for constitutive freezing resistance. Cold acclimation subsequently evolved via the recruitment of genes from drought tolerance pathways, to improve cold resistance on top of that provided constitutively. This mechanism would allow plants to become more specialised and shift their thermal niche to lower temperatures. The

evidence of cold acclimation in this study indicated that this mechanism facilitated recent migration to cold climates in only a small number of lineages, paralleling the situation in the older, cold-adapted Pooideae lineage (Preston & Sandve 2013; Zhong *et al.* 2018).

3.6 Conclusions

Our experimental comparative study demonstrates that many grass species from tropical climates lack chilling and freezing resistance, and typically have a high moisture content which contributes to freezing intolerance. However, phylogenetic variation in moisture content means that some tropical lineages have constitutive freezing resistance. Low moisture content is, therefore, a pre-adaptation that has enabled some grass lineages to migrate recently from tropical to cold climates, thereby expanding their temperature niches to lower temperatures. Other lineages migrated to cold habitats despite a high moisture content, and therefore had to modify their leaf properties during the transition. Because cold acclimation does not characterise all cold adapted groups, we suggest it evolved in some lineages as a subsequent adaptation that made them more specialised to cold climate environments.

3.7 Acknowledgements

We thank Irene Johnson for advice on seed germination. This work was funded by a studentship the Royal Thai Government.

3.8 Supplementary data

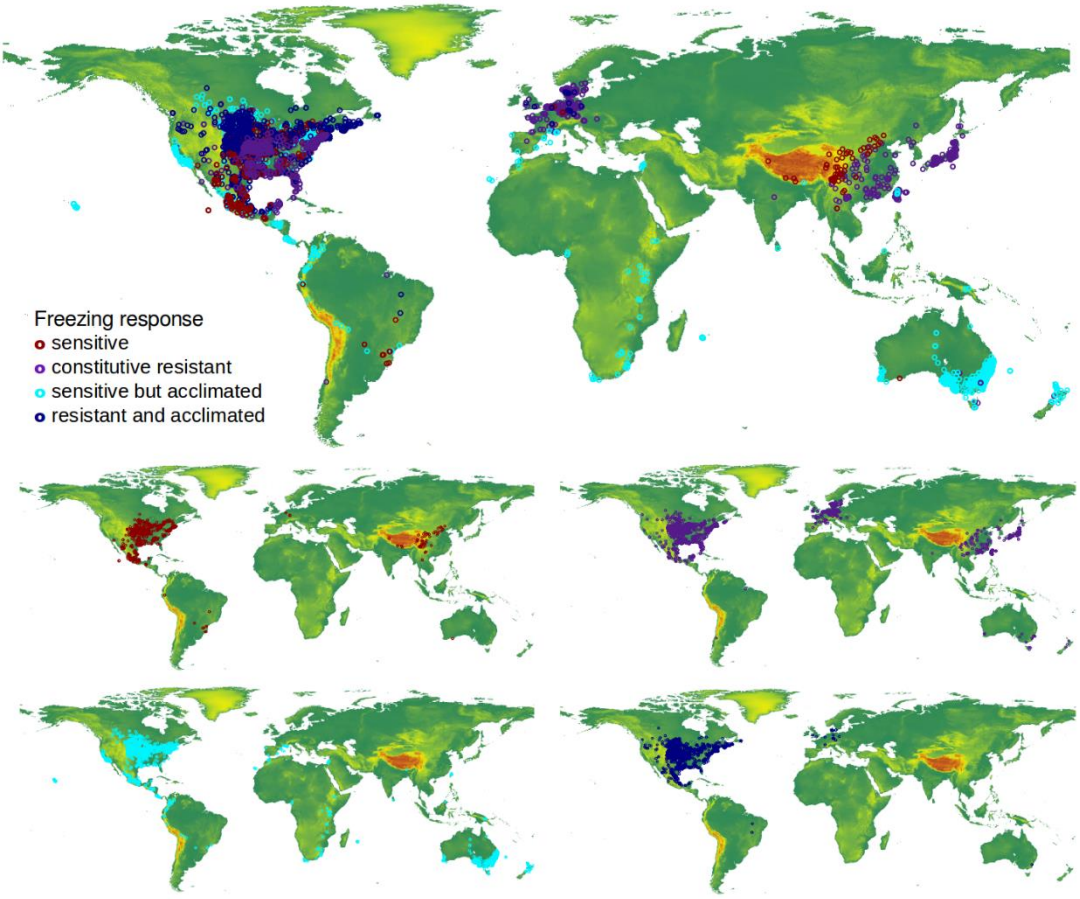

Figure S3.1 The distributions of cold climate grasses which showed various physiological responses.

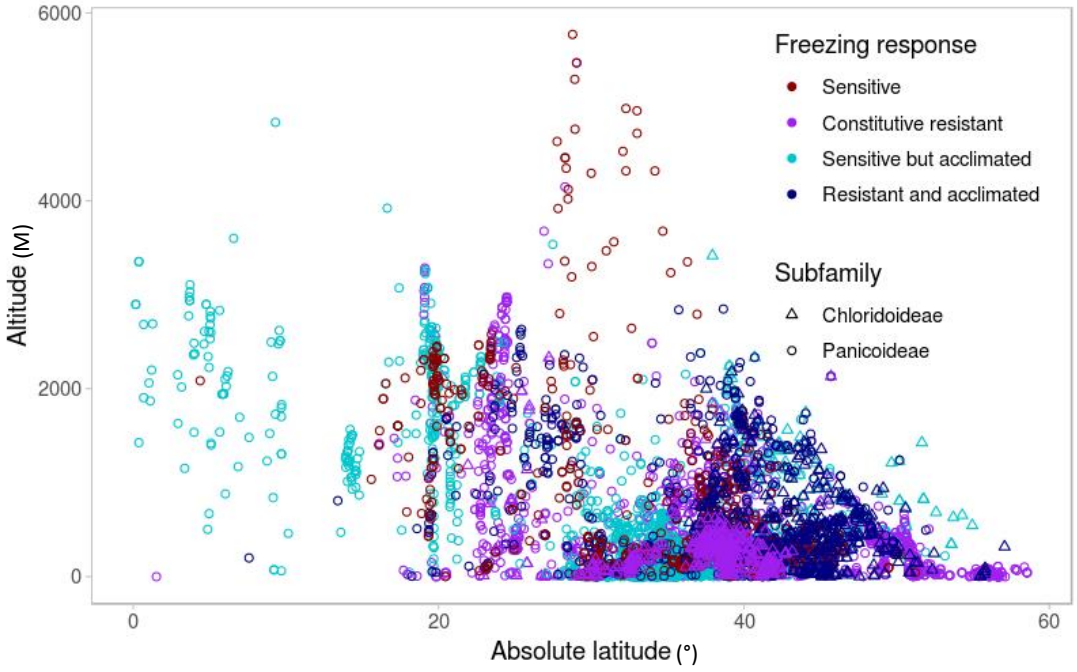

Figure S3.2 The distribution along latitude and altitude (metre) of cold climate grasses and physiological responses.

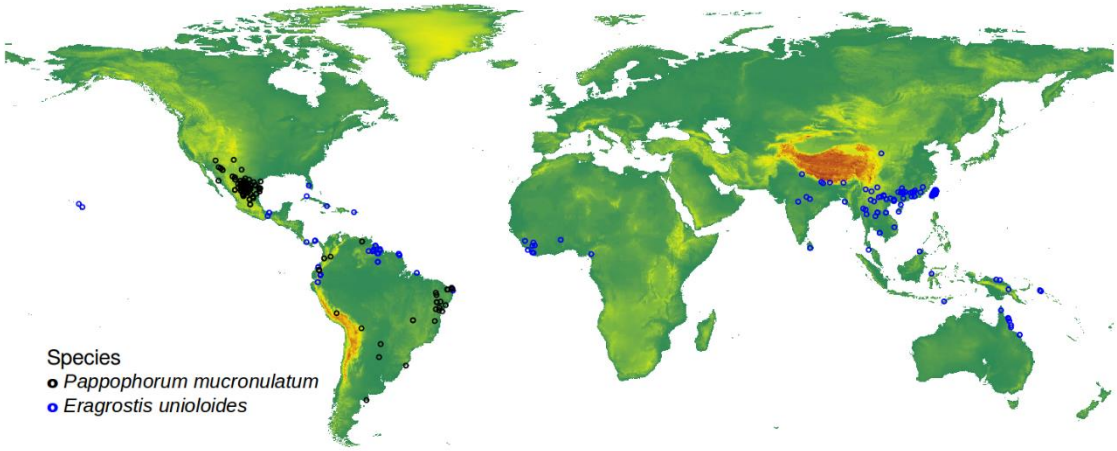

Figure S3.3 The geographical distributions of tropical grasses with constitutive freezing resistance.

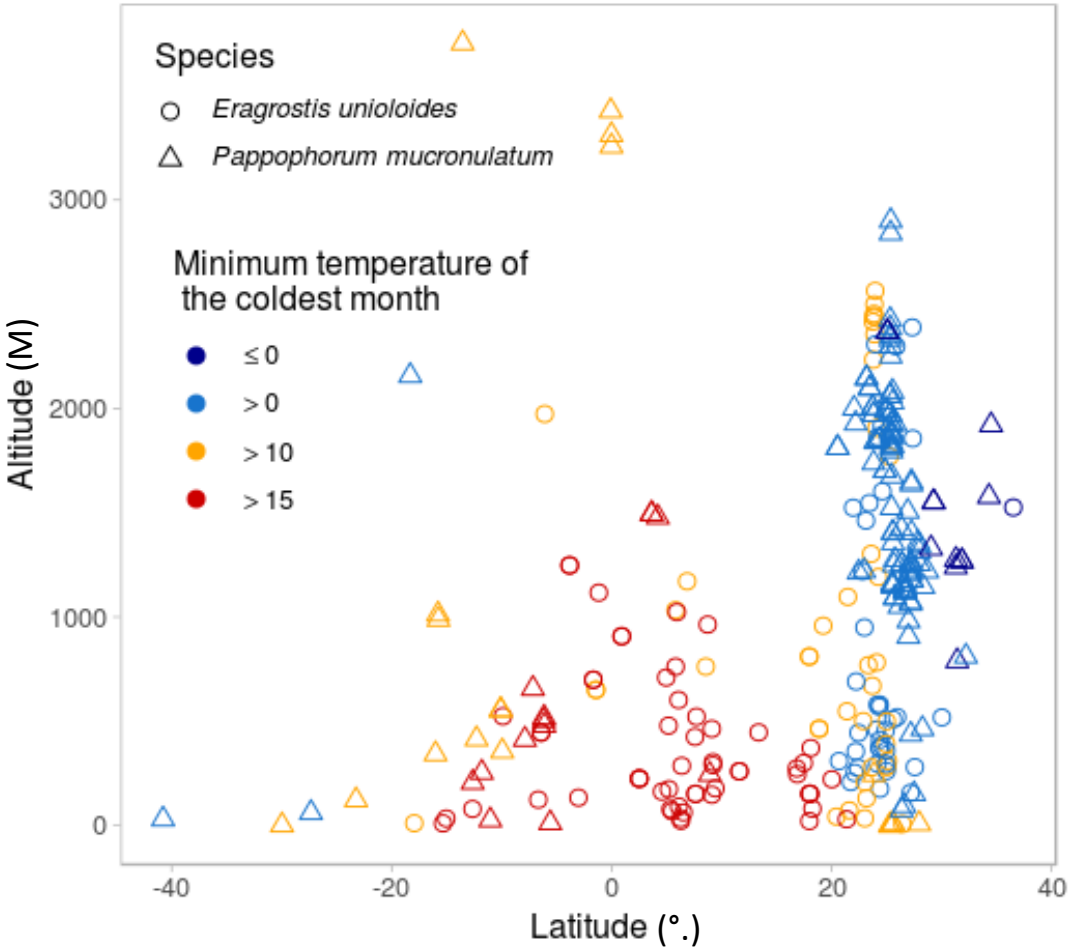

Figure S3.4 The distributions along latitude and altitude gradients of tropical grass species with constitutive freezing resistance and the minimum temperature of the coldest month in each case.

Table S3.1 Species tested for physiological tests with abbreviations (Abb.), along with climatic types, subfamily, photosynthetic type (C_3/C_4) and accession number

Species	Abb.	Climate	Subfamily	C_3/C_4	Material	Plant source	Accession number
Andropogon chinensis	Ach	tropical	Panicoideae	C_4	seed	AusTRCF	309303
Andropogon gerardii	Age	cold	Panicoideae	C_4	seed	Prairie Moon Nursery	-
Andropogon gayanus	Aga	tropical	Panicoideae	C_4	seed	AusTRCF	24575
Acroceras zizanioides	Azi	tropical	Panicoideae	C_3	seed	AWEC collection	0567505
Cenchrus brownii	Cbr	tropical	Panicoideae	C_4	seed	AusTRCF	37127
Cymbopogon citratus	Cci	tropical	Panicoideae	C_4	mature plant	Victoriana Nursery Gardens	-
Calamovilfa longifolia	Clo	cold	Chloridoideae	C_4	seed	ARS GRIN NPGS	PI477995
Eragrostis spectabilis	Esp	cold	Chloridoideae	C_4	seed	B & T World Seeds	40177
Eragrostis unioloides	Eun	tropical	Chloridoideae	C_4	seed	ARS GRIN NPGS	PI213254
Leersia hexandra	Lhe	tropical	Ehrhartoideae	C_3	seed	Kew	0081094
Miscanthus giganteus	Mgi	cold	Panicoideae	C_4	mature plant	Knoll Gardens	-
Miscanthus oligostachyus	Mol	cold	Panicoideae	C_4	seed	B & T World Seeds	530513
Muhlenbergia racemosa	Mra	cold	Chloridoideae	C_4	seed	B & T World Seeds	438584
Miscanthus sacchariflorus	Msa	cold	Panicoideae	C_4	mature plant	Knoll Gardens	-
Miscanthus sinensis	Msi	cold	Panicoideae	C_4	mature plant	Knoll Gardens	-
Oxytenanthera abyssinica	Oab	tropical	Bamusoideae	C_3	seed	Kew	0322902
Pennisetum clandestinum	Pcl	cold	Panicoideae	C_4	seed	Herbiseed	-
Pennisetum flaccidum	Pfl	cold	Panicoideae	C_4	seed	ARS GRIN NPGS	PI434640
Pappophorum mucronulatum	Pmu	tropical	Chloridoideae	C_4	seed	ARS GRIN NPGS	PI477097
Panicum phragmitoides	Pph	tropical	Panicoideae	C_4	seed	Kew	0493208
Panicum virgatum	Pvi	cold	Panicoideae	C_4	seed	Prairie Moon Nursery	-
Sporobolus consimilis	Sco	tropical	Chloridoideae	C_4	seed	Kew	0109187
Panicum virgatum	Snu	cold	Panicoideae	C_4	seed	B & T World Seeds	32879
Saccharum officinarum	Sof	tropical	Panicoideae	C_4	stalk	ARS GRIN NPGS	PI67507
Spartina pectinata	Spe	cold	Chloridoideae	C_4	seed	ARS GRIN NPGS	W6 30925
Sporobolus pyramidalis	Spy	tropical	Chloridoideae	C_4	seed	Kew	0090043
Schizachyrium scoparium	Ssc	cold	Panicoideae	C_4	seed	B & T World Seeds	38434
Sorghum timorense	Sti	tropical	Panicoideae	C_4	seed	AusTRCF	302529
Tridens flavus	Tfl	cold	Chloridoideae	C_4	seed	ARS GRIN NPGS	PI648975

Table S3.2 Results of phylogenetic generalised linear models testing for the effects of climatic types on cold acclimation (Acc) which improves the freezing resistance detected by the change of electrolyte leakage (Leakage Acc) and photosynthetic efficiency (F_v/F_m Acc).

Variable	n	λ	Cold climates	Tropical climates	*P*-value
Leakage Acc (%)	27	0.000	45.2	8.5	0.4357
F_v/F_m Acc (%)	27	0.000	3.9	2.5	0.9364

Chapter 4

Adaptive evolution of chloroplast genomes to cold climates may improve the structural stability of proteins but not the genome

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Personal contribution:

I co-designed the study, generated and analysed the data. I wrote the paper with the help of all authors.

4.1 Abstract

The evolution of genomes results from the interplay between mutations, selection on genome structure, and selection on the encoded proteins. Organellar genomes, trapped in their hosts, must moreover co-evolve with the native nuclear genomes. However, which of these factors dominate during ecological shifts remains poorly understood. In this study, we test the non-exclusive hypotheses that migration to colder climates altered the evolutionary trajectories of chloroplast genomes in grasses by (1) changes in GC content as the trade-off between building cost and stability was altered or (2) adaptive changes of proteins encoded by key genes. Our analyses showed no relationship between GC content and temperature, but provided evidence of positive selection related to cold climates on photosystem and ribosomal genes. The genes under positive selection mainly encode proteins which have functions in the structural stability of the photosystems, with a few proteins also directly involved with photosynthetic and translational performances. We

conclude that adaptation to cold climates requires increased stability of proteins and the chloroplast apparatus, but no significant changes in the chloroplast genome structure.

4.2 Introduction

The evolution of genomes is dictated by the interplay between mutations and selection, as mutations generate the variation on which selection acts (Vries 1909; Kimura & Maruyama 1966; Nei & Nozawa 2011). Transitions to novel environments alter selection pressures, with potential effects on both genome structure and the encoded proteins (Curtis-McLane *et al.* 2008; Gorter *et al.* 2017; Willoughby *et al.* 2018). As one of the main ecological factors affecting organismal survival, the temperature has been shown to affect genomic characteristics to provide beneficial effects in both eukaryotes and prokaryotes. In prokaryotes, species which grow at high temperature are more likely to have high GC contents, and it has been proposed that the triple hydrogen bonds between GC pairs provide greater genomic stability at high temperature than the double hydrogen bonds between AT pairs (Jonvel & Andermann 1983; Galtier & Lobry 1997). However, the evidence of positive correlation between temperature and GC content has only been found in free-living prokaryotes (Hurst & Merchant 2001; Mann & Chen 2010). This constraint may arise because of trade-offs between cost and efficiency of GC pairings, which counteracts the relationship between temperature and GC content. For example, endosymbiotic prokaryotes that require nutrients from their hosts show no correlation between temperature and GC content, suggesting that the higher cost of synthesis for GC pairs might be detrimental in low nutrient environments (Rocha & Danchin 2002; Mann & Chen 2010). In contrast, nuclear genomes of eukaryotes show negative correlations between temperature and GC contents (Šmarda *et al.* 2014). The adaptive evolution of prokaryotic genomes therefore differs from that of eukaryotic genomes under the same selective pressures, possibly because of the differences in genome structure, organisation and evolutionary history between prokaryotes and eukaryotes (Vellai & Vida 1999; Bendich & Drlica 2000; Martin *et al.* 2015). The organellar genomes are especially interesting in this regard because of their shared features between eukaryotic and prokaryotic genomes. The structure of organellar genomes is similar to prokaryotic genomes, but their evolution is also dictated by co-evolution with nuclear genomes. In particular, the transfer of genes among organelles and from the organelles to the nuclear

genomes affect their GC contents, as coding genes have higher GC content than non-coding sequences (Howe *et al.* 2003; Barbrook *et al.* 2010). Therefore, the adaptive evolution of organellar genomes is a good study system in which to broaden our understanding of how climatic shifts influence genome adaptation.

The chloroplast genome originated from cyanobacterial endosymbionts to provide the eukaroyotic cell with photosynthetic capacity (Mereschkowsky 1905; Howe *et al.* 2008). Chloroplasts still retain functional genes, including those encoding key photosynthetic enzymes and the apparatus to express them, but the endosymbiosis has affected their evolution. The genes in the chloroplast can be either retained, lost or transferred to the nucleus, depending on their host (Howe *et al.* 2003; Raven & Allen 2003). Ecological transitions of host plants further cause positive selection on some chloroplast genes, including *matK*, *ndhF*, and *rbcL* (Miller 2003; Christin *et al.* 2008b; Young *et al.* 2012; Galmés *et al.* 2015; Daniell *et al.* 2016; Orr *et al.* 2016; Zhao *et al.* 2017). Recent advances in sequencing technology now allow the analyses of full plastomes. In this paper, we take advantage of this advance to make a comparative evaluation of how transitions into cold climates have influenced the evolution of chloroplast genome structure.

Adaptation to cold climates poses a number of challenges for plants, yet it happened numerous times across the phylogeny of flowering plants. The grass family (Poaceae) has been the focus of previous chloroplast analyses, because a large number of lineages in this group have diversified in hot climates, sometimes associated with C_4 evolution (Edwards & Smith 2010; Piot *et al.* 2018). However, other lineages of grasses in parallel colonised very cold climates, and today the family dominates most open biomes from the Arctic tundras to tropical savannahs, which results from numerous transitions among temperature habitats (Chapter 2; Hartley 1950; Gibson 2009). Because the family contains numerous crops and species of economic interest, numerous genomic resources are available (Saarela *et al.* 2018). The family, therefore, constitutes an excellent system to evaluate how transitions to cold climates affect the evolution of chloroplast genomes, as independent transitions to cold climates provide natural replicates of evolutionary processes. We have recently shown that C_4 photosynthesis boosts the rate of transitions between tropical and temperate climates in grass species (Chapter 2). Here, we evaluate how these transitions to colder climates influenced chloroplast evolution.

We used 433 chloroplast genomes retrieved from public databases, adding 22 newly sequenced chloroplast genomes to increase the number of contrasts between species from tropical and cold climates across the whole grass phylogeny. Climatic niches were extracted for the same species, and a phylogenetic tree based was inferred from the 76 protein-coding genes. These data were combined to provide a subset of 356 species for which both genomic and climatic data were available. The dataset was used to: (1) evaluate the relationships between genome composition, temperature niche and photosynthetic type; and (2) quantify the selective pressures acting on all 76 protein-coding genes from the chloroplast genome, testing the hypothesis that these selective pressures were altered following transitions from tropical to cold climates. Our work sheds new light on the selective pressures that shape interspecific variation in organellar genomes.

4.3 Materials and methods

Plastome assembly and phylogenetic tree

Twenty two species were selected to maximise the numbers of contrasts between tropical and cold-adapted climates, as previously inferred based on phylogenetic trees (Chapter 2; Spriggs *et al.* 2014). Samples for these species were obtained from the leaves of plants growing in controlled environmental chambers in the previous experiment (Chapter 3), and DNA was extracted using Qiagen DNesy Plant Kit. Whole genomes were then sequenced using a shotgun approach (low coverage sequencing), which generated 125bp paired-end reads with an Illumina HiSeq 2000 (Illumina, San Diego, USA). The chloroplast fraction of reads was used to assemble chloroplast genomes (plastomes) from these species, using the NOVOPlasty pipeline (Dierckxsens *et al.* 2016). Each protein-coding gene in the plastid genome of *Oryza sativa* was used as a seed to initiate assemblies. All potential contigs or whole plastomes were mapped onto the sequences of the whole plastome of *Oryza sativa* and available species in the same genus in order to assemble and validate the *de novo* assemblies. When the protein-coding genes of *Oryza sativa* remained unassembled, sequences of each species were assembled using the same pipeline with available protein coding genes of the same species or close relatives available in public databases being the seeds to initiate assemblies. All potential contigs or whole plastomes were treated in the same way as protein-coding sequences to obtain

de novo assemblies. The original reads were then mapped onto the *de novo* assemblies, and errors were corrected by hand, by removing sequences and assembling errors. The newly assembled plastomes were annotated using *Zea mays* (NC_001666.2) as a reference, and protein-coding genes were then extracted using Geneious 11.2 (Biomatters Ltd., Auckland).

Complete plastomes and sets of protein-coding genes for a total of 433 species were retrieved from NCBI databases and combined with data from the 22 newly assembled plastomes, leading to a dataset of 454 grasses including *Joinvillea ascendens* (as outgroup), which included two accessions of each of *Molinia caerulea* and *Schizachyrium scoparum*, and multiple varieties of both *Oryza sativa* and *Saccharum officinarum*. Each of the 76 coding genes was aligned using the MAFFT L-INS-I algorithm with 1000 cycles of iterative refinement (Katoh & Standley 2016). The 76 alignments were concatenated, and a maximum likelihood phylogenetic tree was inferred using IQ-TREE (Nguyen *et al.* 2015), while estimating the best fitting model of sequence evolution from the data. The best fitting model was inferred to be the general time-reversible model with free eight-rate categories and unequal base frequency being counted directly from the alignment (GTR+F+R8) by Bayesian information criterion. Support was evaluated with 100 bootstrap pseudoreplicates. The phylogenetic tree was rooted with *Joinvillea ascendens*, the only non-grass in the dataset (Grass Phylogeny Working Group II 2012).

Climate data

Grass geographical records were extracted from the Global Biodiversity Information Facility (GBIF) web portal (http://www.gbif.org, accessed from 18 September 2017) and cleaned to exclude identical records, records where the country did not match the coordinates and records containing less than three decimal coordinates. The species names from the records was checked and corrected using software package Taxonome (Kluyver & Osborne 2013) based on the Kew grass synonymy database (Clayton *et al.* 2006) and then using package Taxostand in R (Cayuela *et al.* 2012) based on The Plant List (http://www.theplantlist.org) database when the species name did not match in the first package. The records without a valid name in any databases were discarded. Species with less than five occurrences were also excluded to increase the accuracy of temperature

niche estimates. Mean annual temperature and mean temperature of the coldest month for each location were extracted from WorldClim version 2, 5-arc minute resolution (Fick & Hijmans 2017) to obtain median values across the geographical range of each species. The mean annual temperature and temperature of the coldest month indicates the general thermal regime, while mean temperature of the coldest month specifically relates to the lowest temperatures that a species must survive in a particular area. Mean temperature of the coldest month was also used to categorise climatic types with the Köppen-Geiger classification criteria (Peel *et al.* 2007).

Phylogenetic analyses of GC content and genome size

Chloroplast GC content and genome sizes were estimated from whole plastomes as well as for the protein-coding sequences using the package Seqinr in R (Charif & Lobry 2007). The GC contents and genome size of the nuclear genome of 31 species were obtained from reports from genome assemblies in the NCBI database. The GC contents were observed for the whole genome (GC WGS), the total GC content of all 76 protein-coding genes (GC CDS) and the GC content in the third position of all 76 protein-coding genes (GC3 CDS), which are less affected by selection. The photosynthetic type of each species in the dataset was determined based on published databases (Spriggs *et al.* 2014) and GrassBase (Clayton *et al.* 2006). These data were incorporated with the 445 species tree and the climatic data to obtain the subset of 356 species in total which contained both genomic and climatic data for further analysis. Phylogenetic least squares analyses (PGLS), as implemented in the Caper package in R (Orme 2013), were used to test for an effect of the temperature niche and photosynthetic type on either the GC content or the genome size. The analyses were first conducted on the total 356 plastomes and then on the 31 nuclear genome dataset. In every case, Pagel's λ phylogenetic signal was estimated to control for phylogenetic dependence (Pagel 1999).

Positive selection test

Past selective pressures were evaluated using the ratio between the rate of fixation of non-synonymous mutations and that of synonymous mutations (Yang & Bielawski 2000). Because the aim was to test for positive selection specifically linked to adaptation to cold climates, we considered only species from tropical and cold climates (following the

Köppen-Geiger climate classification and excluding species from arid and temperate climates), leading to a reduced dataset of 159 species. Previous analyses have suggested numerous, bidirectional transitions between temperate climates and either the tropics or cold climates (e.g. Chapter 2), rendering *a priori* identification of branches associated to shifts difficult. By contrast, transitions from cold climates to tropical ones are at best very rare. In a tree containing only tropical and cold-habitat grasses, transitions can, therefore, be assumed to occur only from tropical to cold habitats, and match all branches leading to clades composed only of cold-adapted species.

For each of the 76 protein-coding genes, different codon models were optimised using the software codeml from the package PAML (Yang 2007). The null model is a site model (M1a), in which selective pressures are constant throughout the tree and sites are either under purifying or relaxed selection. The alternative site model (M2a) allows a third category of sites, which are under positive selection throughout the whole phylogenetic tree. By contrast, two branch-site models include sites that are under purifying or neutral selection throughout the whole tree, while other sites shift from purifying or neutral to relaxed or positive selection, in models MA1 and MA, respectively. In these branch-site models, branches where the shift in selective pressure occurs have to be identified *a priori*, in this case as those leading to clades composed solely of cold-habitat species. The four models were compared statistically using likelihood ratio tests (LRT), using a false discovery rate of 0.05, which takes into account multiple testing (Benjamini & Hochberg 1995). For each gene, LRT was used to compare the MA model to both MA1 and M1a and to compare the M2a model to the M1a. If both MA and M2a model were significantly better than the M1a model, AIC scores were used to identify the best-fit model. For those genes under positive selection according to the LRT, the Bayes empirical Bayes approach was used to identify those sites under positive selection (Zhang *et al.* 2005).

4.4 Results

No effect of temperature or photosynthetic type on plastome composition

PGLS analyses from 356 species showed no evidence of relationships between GC content in the chloroplast genome and temperature niches, when considering GC WGS,

GC CDS or GC3 CDS, and either the mean temperature of the coldest month or the mean annual temperature (Table 4.1, Fig. S4.1). The highest GC content in the whole chloroplast genome (GC WGS) was 39.1 in a tropical Ehrhartoideae grass (*Streptogyna americana*), whereas the lowest was 37.2 in a temperate Pooideae grass (*Aegilops geniculata*). Across the 76 protein-coding sequences in this study, the total GC content (GC CDS) and GC in the third position (GC3 CDS) were highest at 39.5 in *Buergersiochloa bambusoideae* (Bambusiodeae) and 31.1 in *Pseudosasa japonica* (Bambusoideae), respectively. These are a tropical and a temperate bamboo, respectively. On the other hand, the lowest values were 38.7 for GC CDS in *Aegilops geniculata* (Pooideae) and 29.3 of GC3 CDS in *Chondrasum gracile* (Chloridoideae). These two species are from temperate climates. The values of GC CDS and GC3 CDS were more likely to be lower in core Pooideae and Chloridoideae than in other groups (Fig. 4.1).

In contrast with the chloroplast GC WGS, the nuclear GC WGS from 31 species was inversely correlated to temperature (Table 4.1, Fig. S4.1), as previously reported (Šmarda *et al.* 2014). This suggested that the temperature niche affected GC WGS differently in the chloroplast and the nucleus (Table 4.1). The analysis of phylogenetic signal also indicated different evolutionary histories of GC WGS in the chloroplast and the nucleus. There was strong phylogenetic signal in chloroplast GC WGS ($\lambda = 1.000$ and 0.864) but weak phylogenetic signal in nuclear GC WGS ($\lambda = 0.000$). The analysis also found a relationship between photosynthetic type (C_3 vs. C_4) and GC content, but only in the 76 protein-coding sequences. GC CDS and GC3 CDS were lower in C_4 than C_3 species (Table S4.1).

Apart from GC content, the largest genome size in this study was 158 kilobases in *Aegilops cylidrica* (Pooideae) which is found in cold climates, while the smallest was 113 kilobases in *Paspalum paniculatum* (Panicoideae) which is found in the tropics. The smallest chloroplast genome sizes seem to be in Chloridoideae, Danthonioideae, Ehrhartoideae and particular groups of Pooideae, but the largest ones are in Panicoideae (Fig. 4.1). However, temperature niches were not related to either chloroplast or nuclear genome sizes in grasses (Table 4.1). Furthermore, the genome size of the chloroplast was unrelated to the photosynthetic type (Table S4.1).

Table 4.1 Results from phylogenetic generalised least square regression testing for the effects of climatic variables on GC content and genome size in grasses

Variable	part	slope	n	λ	*P*-value
GC content in the whole genome (GC WGS)					
Annual Mean Temperature	nucleus	–0.099	31	0.000	0.043*
	chloroplast	0.001	31	1.000	0.856
	chloroplast	0.002	356	0.864	0.276
Mean temperature of the coldest month	nucleus	–0.075	31	0.000	0.036*
	chloroplast	0.003	31	1.000	0.537
	chloroplast	0.001	356	0.864	0.210
GC content in coding sequence (GC CDS)					
Annual Mean Temperature	chloroplast	0.000	356	0.976	0.803
Mean temperature of the coldest month	chloroplast	0.000	356	0.976	0.923
GC3 content in coding sequence (GC3 CDS)					
Annual Mean Temperature	chloroplast	0.000	365	0.997	0.873
Mean temperature of the coldest month	chloroplast	0.000	365	0.997	0.790
Genome size (Size WGS)					
Annual Mean Temperature	nucleus	–0.044	31	1.000	0.127
	chloroplast	0.000	31	1.000	0.786
	chloroplast	0.000	356	0.629	0.330
Mean temperature of the coldest month	nucleus	-0.042	31	1.000	0.515
	chloroplast	0.000	31	1.000	0.804
	chloroplast	0.000	356	0.629	0.207

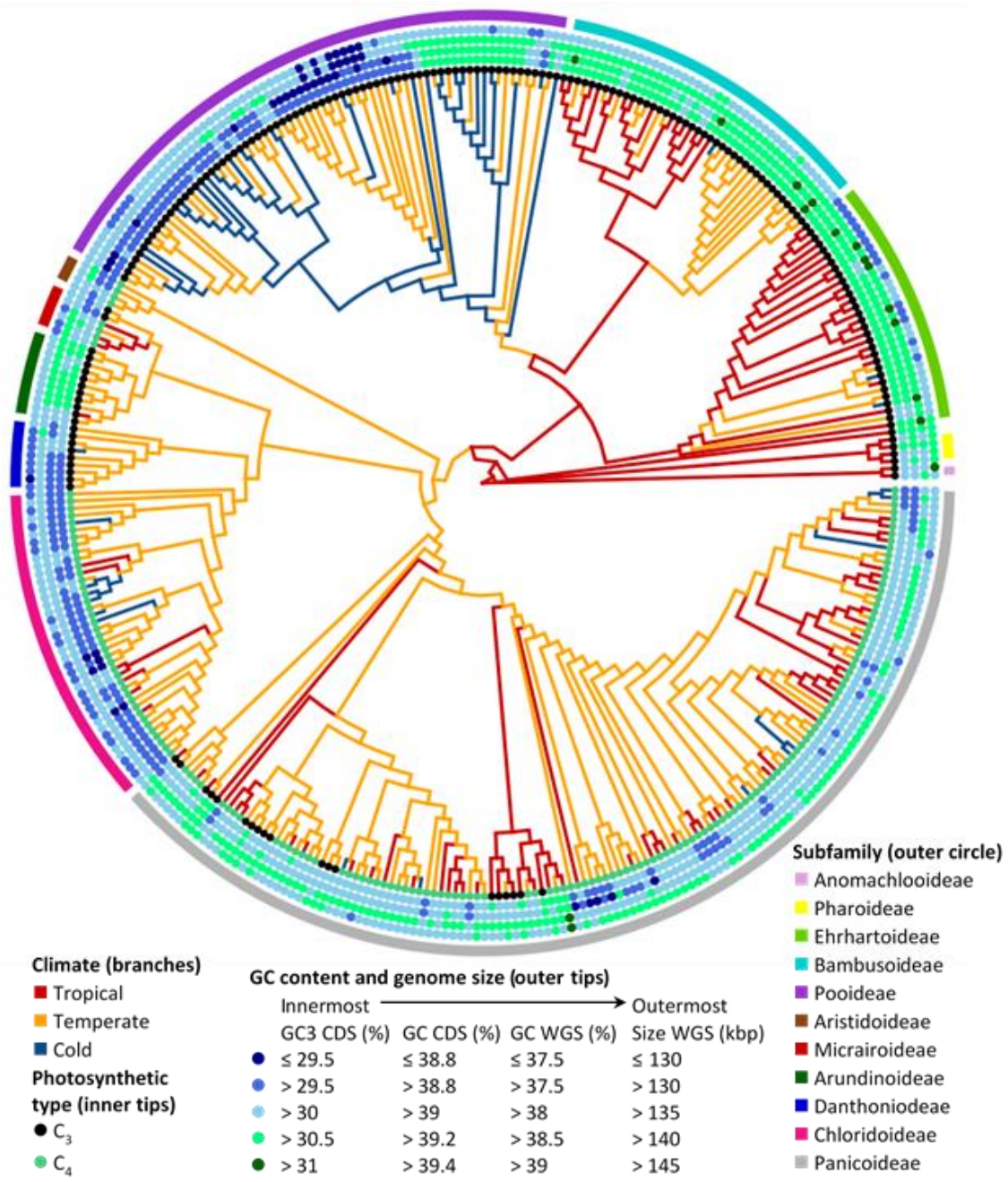

Figure 4.1 The evolutionary transitions between climatic regions across the phylogenetic tree with chloroplast GC content, genome size and photosynthetic type.

Evolutionary adaptation of chloroplast genes

The codon model tested on the 76 chloroplast genes revealed that 25 genes were under positive selection across the whole tree and 6 genes were under positive selection only on cold-climate branches (Table 4.2). The 25 genes under positive selection across the whole tree were proved statistically in LRT and were consistent with the best fit model in AIC (Table 4.2). These genes encode proteins which cover roughly all the categories of chloroplast-encoded proteins, including basic functions such as transcription and translation, through to the complex functions of photosynthesis. Nearly one-third of genes under positive selection across the whole tree encode proteins which function in transcription and translation and nearly half of them function in photosynthesis, including the genes encoding ATP synthase, NAD(P) dehydrogenase, cytochrome complex, photosystem I, photosystem II and ribulose bisphosphate carboxylase proteins (Table 4.2 and 4.3).

The six genes under positive selection only on cold-climate branches were indicated by MA being the best fitted model according to AIC, and significant differences of MA from MA1 and M1a (Table 2). The proteins encoded by these six genes are involved in photosystem I, photosystem II and ribosomes (Table 4.3). The Bayes empirical Bayes approach identified the amino acid sites under positive selection, indicating recurrent mutation at the same sites in independent cold climate lineages (Figs. 4.2, 4.3 and 4.4). Transitions amongst hydrophobic amino acids (Valine, Isoleucine, Leucine) were more likely to be found than other groups of amino acids in positively selected genes of photosystems I and II (7 positions in 5 genes), but there was no bias toward particular amino acids (Figs. 4.2 and 4.3). A pattern was found similarly in the transition between a small amino acid (Alanine) and nucleophilic amino acids (Serine or Threonine) for the genes *psaI* in position 25 and gene *psbA* in position 346 (Figs. 4.2 and 4.3). Additionally, transitions from Aspartic acid to Asparagine or Histidine, which added extra nitrogen to amino acids, were identified in position 79 of *rps14* (Fig. 4.4). The transitions of amino acids in all positively selected genes were similar between the cold-adapted Pooideae clade and lineages in other subfamilies. This means that convergent changes of amino acids (Figs. 4.2, 4.3 and 4.4) occurred during the cold-adaptation of grass lineages separated by more than 70 Myr of evolutionary divergence (Christin *et al.* 2014).

Table 4.2 Akaike information criterion difference (AIC) for the four codon substitution models and likelihood ratio test (LRT) with false discovery rate correction for multiple testing between models applied separately to 76 protein-coding genes of the chloroplast genome

No	Gene	ΔAIC				*P*-value in LRT		
		M1a	M2a	MA	MA1	M2a vs M1a	MA vs MA1	MA vs M1a
1	atpA	**0**	4	3	**0**	1.000	1.000	1.000
2	atpB	7	**0**	7	4	**0.014***	1.000	0.590
3	atpE	**0**	4	4	**0**	1.000	1.000	1.000
4	atpF	5	**0**	8	5	**0.033***	1.000	1.000
5	atpH	**0**	4	4	**0**	1.000	1.000	1.000
6	atpI	6	10	**0**	2	1.000	0.307	**0.036***
7	ccsA	229	**0**	227	222	**0.000*****	1.000	0.332
8	cemA	16	**0**	20	18	**0.000*****	1.000	1.000
9	clpP	7	11	6	**0**	1.000	1.000	0.357
10	infA	14	**0**	18	16	**0.001****	1.000	1.000
11	matK	193	**0**	161	191	**0.000*****	**0.000*****	**0.000*****
12	ndhA	14	**0**	18	16	**0.000*****	1.000	1.000
13	ndhB	57	**0**	61	59	**0.000*****	1.000	1.000
14	ndhC	**0**	3	5	3	0.967	1.000	1.000
15	ndhD	**0**	4	2	2	1.000	1.000	1.000
16	ndhE	**0**	4	3	**0**	1.000	1.000	1.000
14	ndhF	168	**0**	169	166	**0.000*****	1.000	1.000
18	ndhG	27	**0**	31	29	**0.000*****	1.000	1.000
19	ndhH	6	2	10	**0**	0.052	1.000	1.000
20	ndhI	13	**0**	15	12	**0.001****	1.000	1.000
21	ndhJ	1	**0**	5	3	0.167	1.000	1.000
22	ndhK	**0**	4	3	1	1.000	1.000	1.000
23	petA	**0**	4	4	1	1.000	1.000	1.000
24	petB	195	**0**	148	197	**0.000*****	**0.000*****	**0.000*****
25	petD	36	**0**	40	38	**0.000*****	1.000	1.000
26	petG	**0**	4	3	1	1.000	1.000	1.000
27	petL	2	**0**	6	4	0.118	1.000	1.000
28	petN	**0**	4	4	2	1.000	1.000	1.000
29	psaA	13	**0**	18	15	**0.001****	1.000	1.000
30	psaB	10	14	**0**	5	1.000	0.082	**0.006****
31	psaC	**0**	4	8	1	1.000	1.000	1.000
32	psaI	12	10	**0**	9	0.114	**0.007****	**0.004****
33	psaJ	2	**0**	6	4	0.110	1.000	1.000
34	psbA	17	17	**0**	14	0.249	**0.001****	**0.001****
35	psbB	4	**0**	8	5	0.060	1.000	1.000
36	psbC	4	8	**0**	1	1.000	0.410	0.126
37	psbD	**0**	4	4	**0**	1.000	1.000	1.000

No	Gene	ΔAIC				*P*-value in LRT		
		M1a	M2a	MA	MA1	M2a vs M1a	MA vs MA1	MA vs M1a
38	psbE	**0**	4	4	**0**	1.000	1.000	1.000
39	psbF	1	5	2	**0**	1.000	1.000	1.000
40	psbH	**0**	1	4	2	0.478	1.000	1.000
41	psbI	7	**0**	11	9	**0.011***	1.000	1.000
42	psbJ	6	10	**0**	8	1.000	**0.015***	**0.036***
43	psbK	11	8	**0**	13	0.061	**0.001****	**0.005****
44	psbL	**0**	4	5	1	1.000	1.000	1.000
45	psbM	12	16	**0**	14	1.000	**0.001****	**0.005****
46	psbN	2	**0**	6	4	0.133	1.000	1.000
47	psbT	**0**	5	4	2	1.000	1.000	1.000
48	psbZ	**0**	4	4	2	1.000	1.000	1.000
49	rbcL	193	**0**	168	195	**0.000*****	**0.000*****	**0.000*****
50	rpl14	**0**	4	4	2	1.000	1.000	1.000
51	rpl16	50	**0**	53	49	**0.000*****	1.000	1.000
52	rpl2	**0**	4	4	2	1.000	1.000	1.000
53	rpl20	**0**	1	4	2	0.404	1.000	1.000
54	rpl22	**0**	3	4	2	1.000	1.000	1.000
55	rpl23	17	19	3	**0**	0.678	1.000	**0.002****
56	rpl32	**0**	4	9	2	1.000	1.000	1.000
57	rpl33	3	**0**	6	5	0.082	1.000	1.000
58	rpl36	**0**	4	4	5	1.000	0.357	1.000
59	rpoA	43	**0**	49	45	**0.000*****	1.000	1.000
60	rpoB	29	**0**	33	31	**0.000*****	1.000	1.000
61	rpoC1	24	**0**	26	26	**0.000*****	0.644	1.000
62	rpoC2	216	**0**	215	208	**0.000*****	1.000	0.340
63	rps11	**0**	4	4	2	1.000	1.000	1.000
64	rps12	197	**0**	200	199	**0.000*****	0.980	1.000
65	rps14	9	13	**0**	11	1.000	**0.003****	**0.011****
66	rps15	6	4	2	**0**	0.114	1.000	0.069
67	rps16	3	4	**0**	4	0.404	0.086	0.134
68	rps18	38	**0**	41	40	**0.000*****	1.000	1.000
69	rps19	**0**	2	4	2	0.725	1.000	1.000
70	rps2	1	**0**	5	3	0.179	1.000	1.000
71	rps3	2	4	1	**0**	0.651	1.000	0.432
72	rps4	**0**	4	4	2	1.000	1.000	1.000
73	rps7	12	**0**	16	14	**0.001*****	1.000	1.000
74	rps8	1	**0**	5	3	0.197	1.000	1.000
75	ycf3	**0**	3	3	1	1.000	1.000	1.000
76	ycf4	18	**0**	22	20	**0.000*****	1.000	1.000

Asterisks indicate significance level; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 4.3 Groups of genes showing evidence of positive selection which shared the same functions

Positive selection	Function	Gene
Whole tree	ATP synthase	*atpB, atpF*
	NAD(P) dehydrogenase	*ndhA, nadB, ndhF, ndhG, ndhI*
	cytochrome complex	*petB, petD*
	photosystem I	*psaA*
	photosystem II	*psbI*
	ribulose bisphosphate carboxylase	*rbcL*
	transcription and translation	*matK, rpoA, rpoB, rpoC1, rpoC2, rpl33, rps7, rps12, rps18*
Cold climate clades	photosystem I	*psaI*
	photosystem II	*psbA, psbJ, psbK, psbM*
	transcription and translation	*rps14*

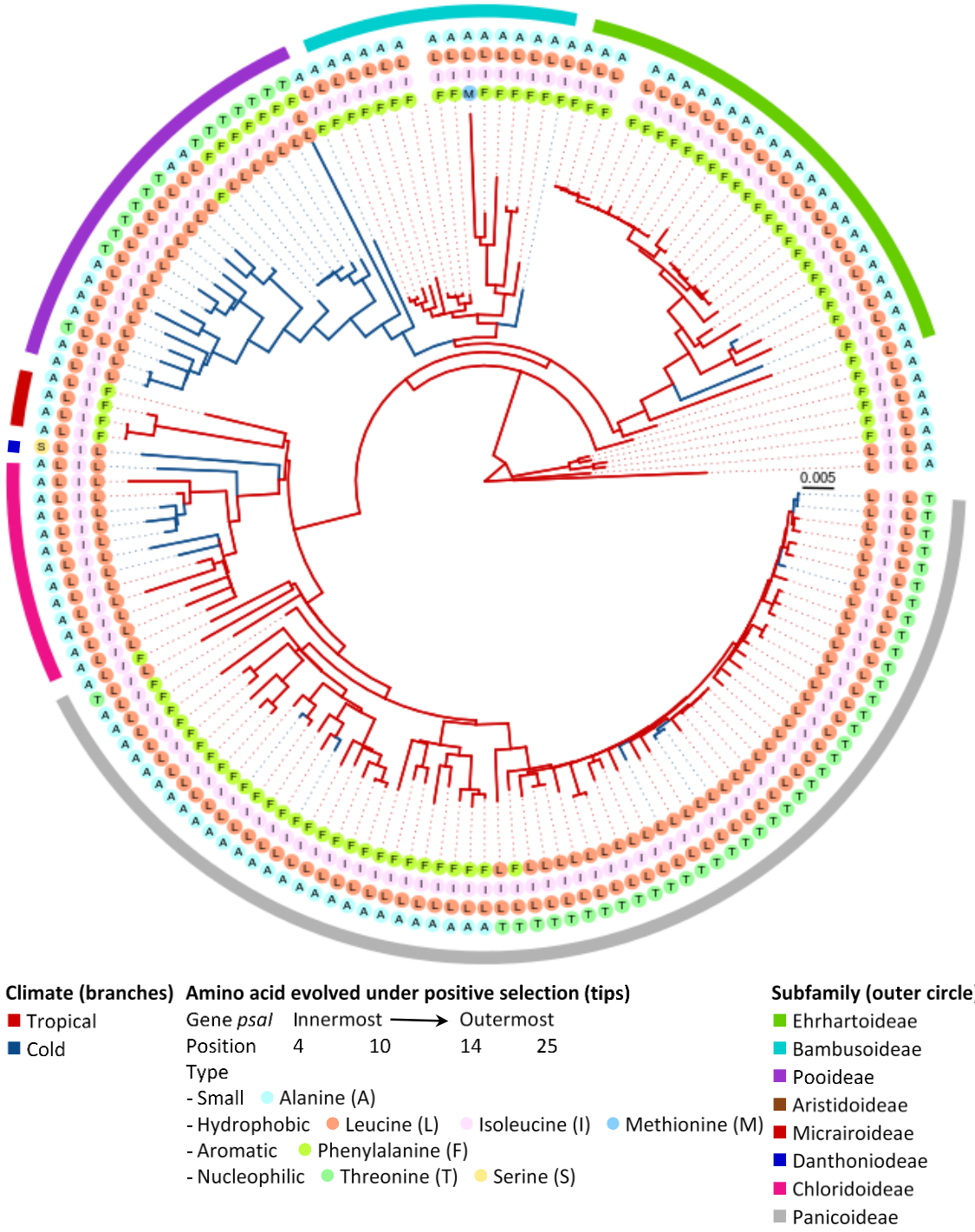

Figure 4.2 Amino acid positions of the *psaI* gene identified to have evolved under positive selection in cold-climate species shown in the phylogenetic tree inferred from 76 protein-coding genes.

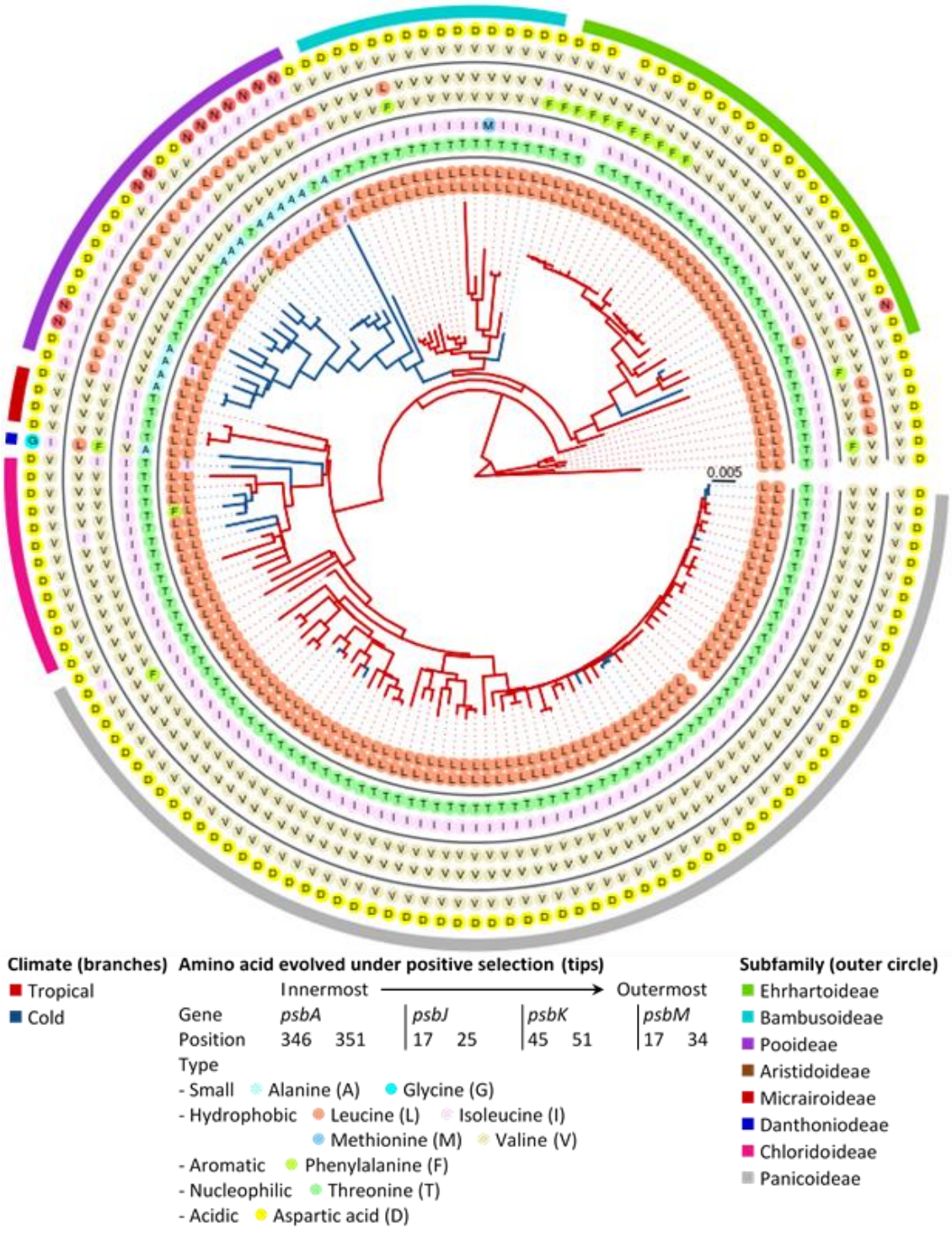

Figure 4.3 Amino acid positions of genes related to photosystem II identified to have evolved under positive selection in cold-climate species shown in the phylogenetic tree inferred from 76 protein-coding genes.

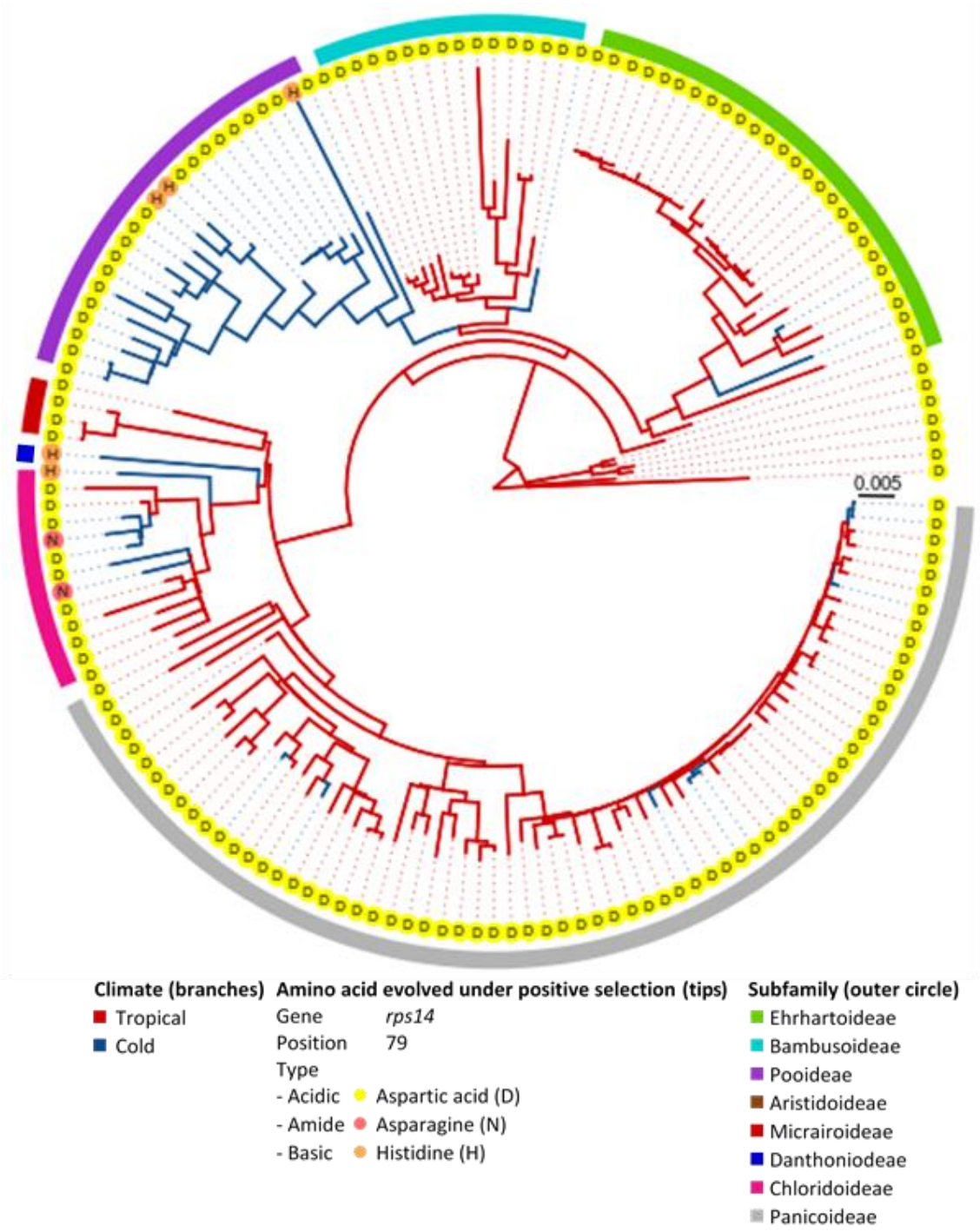

Figure 4.4 Amino acid positions of the *rps14* gene identified to have evolved under positive selection in cold-climate species shown in the phylogenetic tree inferred from 76 protein-coding genes.

4.5 Discussion

No direct effects of temperature niche on chloroplast GC content or genome size

In this study, GC content and the genome size of chloroplasts were unrelated to temperature niches, but nuclear GC content negatively correlated to temperature niches, which is consistent with previous work (Table 1; Šmarda *et al.* 2014). This shows that chloroplasts have evolved differently from nuclear genomes under the same climatic conditions, supporting the hypothesis that selective pressures act differently on prokaryotic and eukaryotic genomes within the same organism (Vellai & Vida 1999; Bendich & Drlica 2000; Martin *et al.* 2015).

Chloroplasts are descended from cyanobacterial endosymbionts, and so the structures and functions of this organelle still share common features with its prokaryotic ancestor (Mereschkowsky 1905; Zoschke & Bock 2018). From the perspective of free-living prokaryotes, it has been proposed that the optimum proportion of GC content in genomes increases at higher temperatures because of the stronger bound between G and C than between A and T pairs (Jonvel & Andermann 1983). However, GC content is limited by the higher energy cost required to synthesise these nucleotides than is needed for AT (Rocha & Danchin 2002). Therefore, the positive correlation between temperature and GC contents seems to be found only in free-living groups (Mann & Chen 2010) implying that resource-limitation is less important for the genome structure of these species. On the other hand, plastids are descended from endosymbiotic prokaryotes and have to obtain resources from their hosts, which imposes an additional constraint on the evolution of genome structure (Table1; Mann & Chen 2010). Furthermore, chloroplastic and endosymbiotic prokaryotes are protected by their plant or other hosts, which may reduce the negative effect of temperature on genome structure. Overall, this suggests that a trade-off between thermostability and energy cost under a low selective pressure drives the adaptation of GC content to a particular environment. On top of that, the reduction of plastid genome size and high conservation in this genome also limit the potential for GC content to change (Palmer 1987) because of the small genome size and low occurrence of duplication in chloroplast genes (Xiong *et al.* 2009). In addition, the non-recombination and uniparental inheritance that is typical for plastid genomes (Greiner *et al.* 2015) decrease mutation rates, reducing the variation on which selection can act, and leading to slow changes in genome composition (Smith 2012; Xiao-Ming *et al.* 2017).

Although temperature did not influence the change of GC contents in the chloroplast genome directly, C_4 photosynthesis which originated in tropical climates (Chapter 2: Kubien & Sage 2004; Edwards & Still 2008; Edwards & Smith 2010), showed a small, but statistically significant, decrease of GC content in coding sequences (Table S1). This finding from chloroplast analysis conflicts with the hypothesis from nuclear gene analysis that C_4 photosynthesis increases the GC content because of the higher requirement for nitrogen atoms in GC pairs than in AT pairs. This pattern is expected to arise because the C_4 pathway provides a higher nitrogen use efficiency than the C_3 type (Kelly 2018). However, as with adaptation to temperature, this suggests that there are differences in the processes that influence genome structure in the coding sequences of nuclear and chloroplast genomes. The chloroplast genome undergoes more rounds of replication than the nuclear genome during cell division (Birky 2002), which predisposes it to higher replication errors. Moreover, reactive oxygen species generated during the normal functioning of the chloroplast are able to cause damage and lead to errors in the plastid genome (Boesch *et al.* 2011; Kumar *et al.* 2014). The mechanisms to repair such errors cause a bias toward high AT content (Cerutti *et al.* 1995; Khakhlova & Bock 2006; Smith 2012). These factors may cause C_4 species occupying habitats where environmental conditions generate high concentrations of reactive oxygen species to have a higher AT content than C_3 ones.

Selection of cold climates on stabilizing the protein structures

Our results showed that five genes encoding proteins which function in photosystem I (*psaI*) and II (*psbA, psbJ, psbK, psbM*) were under positive selection (Figure 2 and 3). These genes are important for sustaining photosynthetic functions in various ways, suggesting that migration to cold climates affected the adaptive evolution of photosynthetic proteins.

The most fundamental and best described protein under selection is D1, a core protein of photosystem II encoded by *psbA* which plays a role in photosynthetic electron transport. Low temperatures directly limit photosynthetic metabolism, which in turn limits the energy sinks for photochemistry. When photochemistry is limited in this way, alternative energy sinks generate ROS (Huner *et al.* 1998; Allen & Ort 2001). The high production of ROS which damages the protein forces the plant to increase the rate of

synthesis to substitute for the loss, causing selection on the D1 protein under low temperatures (Singh 2000).

For the other proteins in photosystem II, their functions are to stabilise various structural components of the photosystem II supercomplex. For example, the protein encoded by *psbM* is required directly for the formation and indirectly for the structural stability of photosystem II dimers (Kawakami *et al.* 2011). Similarly, it has been experimentally proved that the roles of proteins encoded by *psbK* and *psbJ* are for the stable assembly of the core complex of photosystem II, not for structures involved with photosynthetic electron transport (Takahashi *et al.* 1994; Hager *et al.* 2002; Wei *et al.* 2016). Furthermore, the identity of the only protein in photosystem I under positive selection in cold climates supports the idea that the structural stability of photosystems is required for cold adaptation. Studies of the role of the protein encoded by *psaI* in photosystem I indicate that the protein is important for photosynthetic efficiency and structural stability in conditions where large amounts of reactive oxygen species are produced. Therefore, when missing this gene, photosynthetic efficiency could not recover after chilling (Schöttler *et al.* 2017). We might hypothesise that the changes in amino acid sequence seen convergently in these proteins might serve to maintain photosynthesis under attack from ROS when photochemical energy sinks are limited by low temperatures (Huner *et al.* 1998; Allen & Ort 2001).

Only one ribosomal protein encoded by *rps14* showed positive selection in cold-habitat species (Figure 4). This protein is required for translations in the chloroplast, and plant growth and development are limited when this gene is knocked out (Ahlert *et al.* 2003; Kindgren *et al.* 2018).

4.6 Conclusions

Numerous lineages of plants independently colonised cold climates. While the effects on genome evolution of transitions to warmer habitats are known, the genomic consequences of the challenges presented by cold have largely remained unexplored. Using comparative genome analyses, we recovered the previously reported inverse correlation between temperature and GC content for the nuclear genome. However, this relationship was not present in the chloroplast genomes of the same species, which shows that different

genomes of the same organism respond differently to the same shift of the external temperature niche. From a functional point of view, we showed that multiple chloroplast genes encoding proteins with functions related to the stability of the photosystems have been under positive selection specifically in cold climates. This shift of selective pressures, accompanied by parallel amino acid replacements across independent lineages, suggests that cold climates drove modifications of the encoded proteins, potentially to maintain the stability of the photosynthetic machinery at low temperatures. We conclude that the effects of low temperature are mainly observed on specific genes, without global genomic effects as previously reported.

4.7 Acknowledgements

We thank Luke Dunning for helping us on selection test and bioinformatics analyses. This work was funded by a studentship of the Royal Thai Government.

4.8 Supplementary data

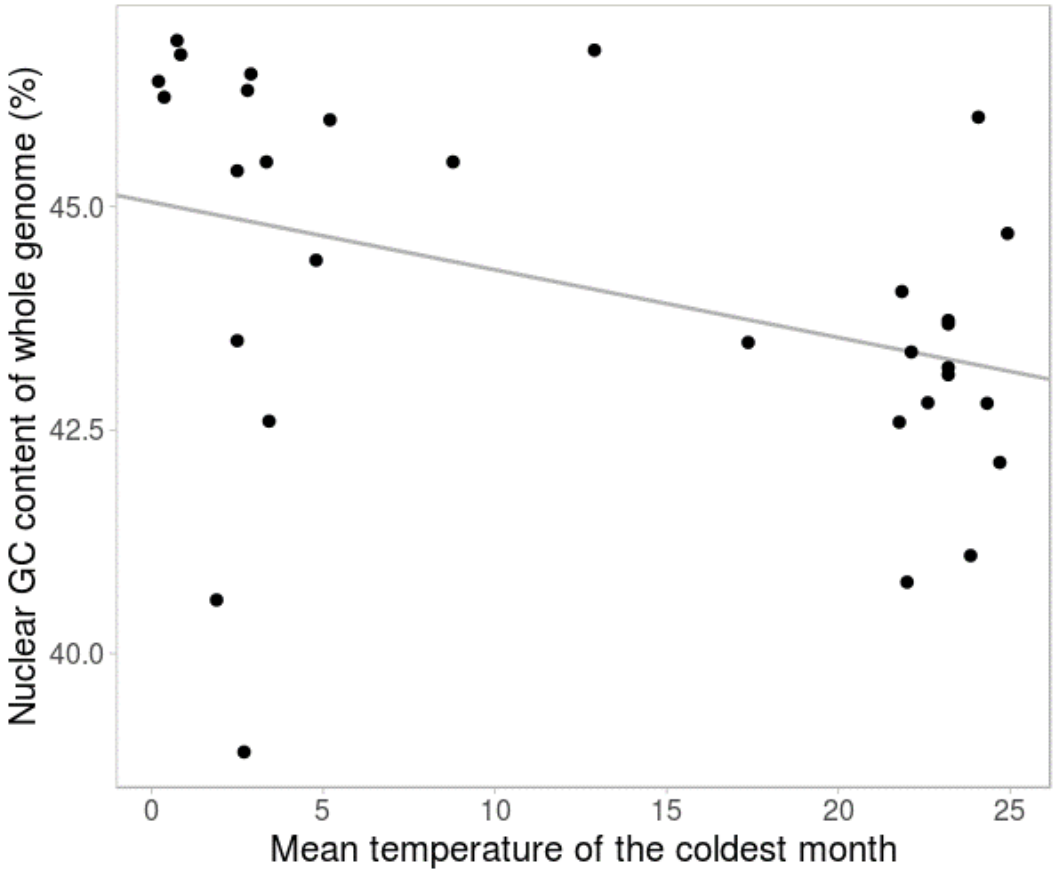

Figure S4.1 Regression line obtained from PGLS showing the inverse relationship between nuclear GC content of the whole genome and mean temperature of the coldest month.

Table S4.1 Results from phylogenetic generalised least square regression testing for an association between climatic variables, GC content and genome size in grasses

Variable	n	C_3	C_4	λ	*P*-value
GC content in the whole genome (GC WGS)					
nucleus	31	43.98	44.20	0.028	0.802
chloroplast	31	38.63	38.50	1.000	0.809
chloroplast	356	38.51	38.47	0.860	0.283
GC content in coding sequence (GC CDS)					
chloroplast	356	39.20	39.11	0.974	0.002**
GC3 content in coding sequence (GC3 CDS)					
chloroplast	365	30.54	30.38	0.997	0.003**
Genome size (Size WGS)					
nucleus (mbp)	31	314	690	1.000	0.716
Chloroplast (kbp)	31	133	133	1.000	0.672
Chloroplast (kbp)	356	142	142	0.643	0.671

Asterisks indicate significant level; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Chapter 5

General discussion

5.1 Factors influencing the climatic transition in grasses

It has been hypothesised that the probability of transition between climates arises from the combination of three factors (Donoghue & Edwards 2014). The first is the intrinsic potential of the lineage to be ecologically successful in novel climatic conditions (Marazzi *et al.* 2012; Donoghue & Edwards 2014; Pyron *et al.* 2015). Pre-existing traits can allow plants to overcome abiotic or biotic limiting factors in new environments, if they become used for a function that differs from the original one, in an example of exaptation *sensu* Gould & Vrba (1982). The traits that evolved for a different purpose may provide tolerance of a novel abiotic environment or enhance competition for space with the species that already inhibit the new habitats (Armbruster *et al.* 2009; Heibl & Renner 2012; Donoghue & Edwards 2014). The presence of such traits might explain why clades with a particular feature are more or less likely to shift to particular climates than others (Edwards & Donoghue 2013). The second factor includes extrinsic physical limitations that determine the opportunity for movement. For example, geographical barriers such as seaways or mountains commonly set limits to distributions, and geographical proximity and access to a novel environment are therefore important determinants of colonisation potential (Wiens & Donoghue 2004; Donoghue 2008). Finally, biotic factors may limit the opportunities to colonise novel habitats. For example, competition for space in the new environment can limit the success of incoming species (Donoghue & Edwards 2014). The complex interactions amongst these factors influence the invasion success of novel environments by particular groups and therefore control the likelihood of evolutionary transitions among climatic zones.

In this work, I focused on the first of these factors; the intrinsic properties that act to minimise or maximise the effects of extrinsic abiotic and biotic factors. Grasses are one of the remarkable groups that have become successful across a broad range of climatic conditions, with a diversity of species that independently made the transition between climates (Chapter 2; Hartley 1950; Gibson 2009; Edwards & Smith 2010). When

considering the physical limits to these transitions, pre-existing environmental tolerance and geographical proximity are important for determining transitions between climates. There are four possible situations (Fig. 5.1). One possible case is a large environmental difference coupled with a short geographical distance and limited barriers to dispersal (Fig. 5.1a). This situation is consistent with altitudinal gradients which dramatically change climatic niche variables such as temperature within short geographical distances. In this case, it is likely that climatic transitions have been mainly driven by changes in the climatic niche. Therefore, I investigated a number of features in grasses, including morphology and physiology that are hypothesised to be associated with climatic transitions. The comparative approach adopted by this study provided multiple examples of traits that promoted or limited the transitions between climatic niches in grasses. Transitions between tropical and temperate climates were promoted by the evolution of C_4 photosynthesis (Chapter 2). Although C_4 species were less likely than C_3 species to further migrate into cold climates, several C_4 grass species are found in cold climates such as alpine and high latitude habitats (Chapter 2; Sage *et al.* 2010). Moreover, freezing tolerance which allows C_4 plants to survive in cold climate was demonstrated in some of these species (Sage & Sage 2002; Márquez *et al.* 2006; Liu & Osborne 2008, 2013). Using an experimental approach, I also identified further traits which promoted and traits which limited migration to cold climates (Chapter 3). Constitutive freezing resistance potentially occurs in tropical species, allowing plants to migrate into cold climates, whereas high tissue moisture contents counteract this constitutive freezing resistance and subsequently limit migration (Chapter 3). This finding suggests that there are pre-existing traits which minimise the negative effects of low temperature with no extra cost, facilitating multiple migrations to colder climates.

Several traits in various pieces of previous research are also consistent with this study. Constitutive freezing resistance may arise from the combination of multiple traits to minimise the negative effects of sub-zero temperatures. Low moisture content is one factor, which increases freezing resistance because it decreases the water available to become ice and damage the cell (Chapter 3; Burke *et al.* 1976; Pearce 2001; Sierra-Almeida & Cavieres 2012). The small diameter of xylem conduits is another trait which may reduce the cavitation after freezing, which blocks the entire xylem leading to complete failure of water transport (Sperry & Sullivan 1992; Utsumi *et al.* 1999; Zanne *et al.* 2014). These two traits also increase drought tolerance which leads to the hypothesis

that pre-adaptation to arid habitats influences constitutive freezing resistance, thereby allowing plants to survive freezing (Tyree & Sperry 1988; Poorter & Markesteijn 2008). Furthermore, the herbaceous habit also acts as a key strategy of plants migrating into freezing habitats, which is consistent with the observation of alpine tree- and shrub-lines which represent the inability of woody plants to occupy the tops of the high mountains (Myers-Smith *et al.* 2011; Zanne *et al.* 2014).

If a large geographical distance is involved in the differences between climatic niches, the combination of these two factors will decrease the opportunity to shift to new climates (Fig. 5.1b). This situation is represented particularly by latitudinal gradients in which large geographical distances are combined with drastic changes of climatic niches, especially temperature, and the photoperiod. However, the negative effects of latitude on climatic shifts may be reduced by additional traits which increase the ability to disperse long distances. The ubiquitous and global distribution of grasses (Osborne *et al.* 2014) suggests that grasses generally have a good dispersal ability, but the role of grass dispersal in the biogeography of the group has not been extensively studied (Linder *et al.* 2018). Although grasses have a good dispersal ability, the multiple climatic variables that change with latitude require multiple traits to facilitate migration (Sakai & Larcher 1987; Preston & Sandve 2013). Rapid growth in the warm conditions of summer may allow grasses to migrate to high latitudes (Nippert *et al.* 2007), especially for C_4 species in which photosynthetic benefits are maximised in hot conditions (Chapter 2). This may explain the pattern of productivity for C_4 grasses in North America which dominate productivity in temperate grassland during summer while C_3 grasses dominate productivity during spring (Sage & Monson 1999). This is also consistent with studies indicating a lower diversity of C_4 species growing in the same temperature at high latitude than at high altitude (Sage & Monson 1999), suggesting that geographical distance, in combination with other environmental factors such as photoperiod, combine to reduce the likelihood of transitions to cold climates at high latitudes.

In the case that a small environmental difference is coupled with a large geographical distance (Fig. 5.1d), geographical barriers limit migration to similar climatic niches in another area. The gaps between continents (continental disjunctions) are particularly important for limiting climatic shifts (Pyron *et al.* 2015). The effects of this factor are minimised by human-mediated dispersal. For example, *Miscanthus* species are native grasses in Asia, but were introduced into Europe and North America to produce

biomass and grow well in similar climates to those in their native range (Clifton-Brown *et al.* 2015; Sage *et al.* 2015). This indicates that the limitation preventing this clade from migrating to Europe and America arose from the low possibility of crossing geographical distances and barriers. Moreover, invasive species which were recently introduced to a new area are good examples to indicate the increase in opportunities to shift to new climates brought by humans (Atwater *et al.* 2018).

The last case is low environmental and geographical differences (Fig. 5.1c). In this case, all species in the same area are likely to have an equal possibility to migrate to adjacent areas. This may not require particular traits to facilitate migration. However, biotic interactions are likely to be important. For biotic factors, the effects of organisms in a new habitat can be combined with abiotic factors influencing the transition to new climates. A higher probability of shifting into new climates occurs in new environments with less competition from native plants which share the same resources. For example, the analysis in chapter 2 showed that grasses are more likely to shift from tropical into temperate than from temperate to tropical climates. The global pattern of species richness of all vascular plants follows the classic latitudinal diversity gradient pattern, whereby the highest richness is in the tropics and decreases toward the poles (Hawkins & Felizola Diniz-Filho 2004; Hillebrand 2004; Kier *et al.* 2005). The high diversity in the tropics causes high interspecific competition which limits migration from temperate climates (Wright 2002). This factor may explain the observation that there have been no transitions back to tropical environments in Danthonioideae and Pooideae which migrated to temperate and cold climates soon after the origins of each clade (Fig. 2.3). However, intense competition in tropical climates limits incoming species, so that the priority effect may be the main driver for the successful climate transition (Cavender-Bares *et al.* 2016). The priority in arrival into temperate areas may also be applied to explain the less successful distribution of Danthonioideae than Pooideae in temperate areas of the northern hemisphere (Inda *et al.* 2008; Linder *et al.* 2013; Visser *et al.* 2014). Pooideae originated in Eurasia during the Eocene and migrated through the Bering Bridge to North America and then occupied the whole American continent, while Danthonioideae originated in temperate areas of southern Africa later in the Oligocene and migrated more recently through Australia and southern America to North America and Europe (Inda *et al.* 2008; Linder & Bouchenak-Khelladi 2017). As a result, Danthonioideae needed to

compete with Pooideae which had already occupied Europe and North America continents, leading to less successful migration in this group (Visser *et al.* 2014).

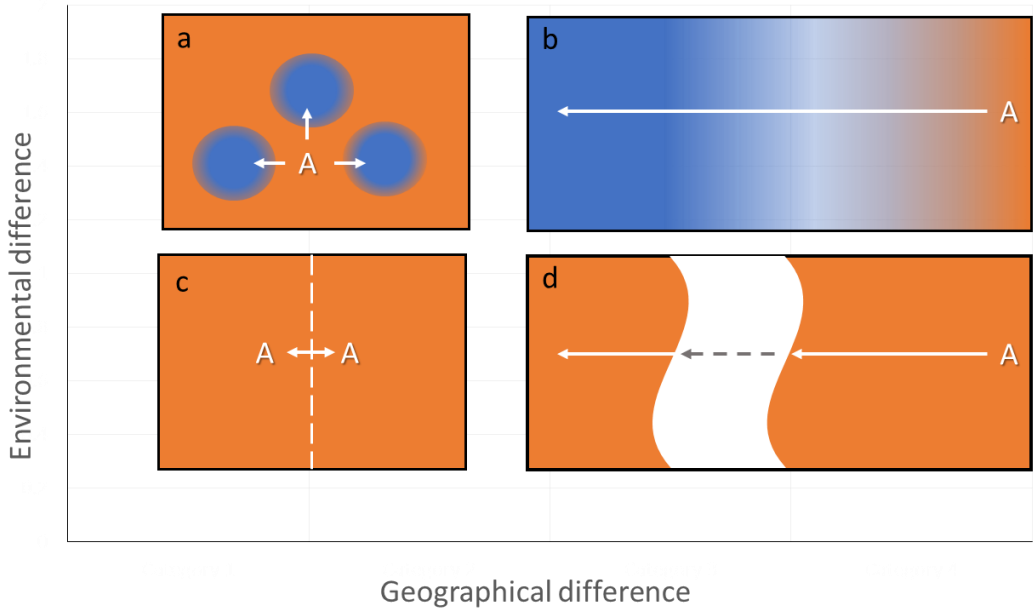

Figure 5.1 Illustration of the extrinsic abiotic factors that limit migration with various combinations of environmental and geographical distances. The climatic shift of clade A is shown in conditions with (a) high environmental difference and small geographical difference, (b) high environmental and large geographical differences, (c) low environmental and small geographical differences or (d) low environmental difference and large geographical distance. The length of the arrow indicates long distance migration. The broken arrow refers to the low likelihood of migration because of physical barriers (modified diagram based on Edwards & Donoghue 2013; Donoghue & Edwards 2014; Pyron *et al.* 2015).

5.2 Adaptive evolution of grasses in cold climates

In this thesis, I assumed that chilling and freezing temperatures filter the plants that can survive in cold climates. Using a comparative approach, I provide evidence that pre-existing traits providing an advantage at low temperature facilitated migrations to

temperate and cold climates. These traits include C_4 photosynthesis (Chapter 2) as well as low moisture content, which is associated with a constitutive freezing resistance (Chapter 3). The effect of these traits likely adds to those of other properties that increase the rate of transition to cold habitats, such as herbaceous life form, small xylem conduits and annual growth (Zanne *et al.* 2014; Ogburn & Edwards 2015) so that the probability that a given plant lineage makes the transition from warm to cold climates depends on the sum of its properties,

Evolution takes place within populations, and ecological transitions as observed along phylogenetic trees are initially triggered by the successive dispersal of a few individuals. In the case of freezing tolerance, I have shown that populations of some species originating from tropical climates with constitutive freezing resistance can colonise higher altitudes, expanding the species range toward colder climates (Chapter 3; Stewart 1945; Roseveare 1948). However, their low population density in high altitude which has been reported (Stewart 1945; Roseveare 1948) indicating the decreases with temperature (Shelford 2006), which may suggest that, while pre-existing traits allow plants to survive in freezing conditions, the success of the individuals decreases in the extremes of the range. Therefore, the persistence of such populations can still offer an opportunity for *in situ* adaptation, with the acquisition of novel attributes that gradually increases their success in cold climates. If the warm and cold climate populations remain connected by frequent gene flow, the potential for local adaptation would remain low. However, if gene flow is reduced because of geographical distance or the acquisition of reproductive barriers, the populations from the cold extremes would become increasingly adapted to cold climates, and might therefore expand their range extremes into even colder climates. Over time, this would lead to further migration into different types of environments from the cold climate or into even colder climates.

While my approach focused on macroevolutionary transitions, I have obtained evidence that some specialisations are acquired after plants transitioned to cold climates. For example, cold acclimation is presented only in some of the species from cold climates, and therefore likely evolves once the plants are already in cold habitats as an *in situ* adaptation (Chapter 3). Because cold acclimation triggered by chilling temperature improves freezing resistance (Chapter 3), it will increase the ability of a given species to colonise colder habitats, likely allowing further expansions of the range extremes into colder environments. Besides the observable phenotypic changes, my genomic work also

suggests that adaptation to cold environments involve sustained positive selection of specific genes. In the case of chloroplast genomes studied in chapter 4, I have shown that adaptation of genes involved in the structural integrity of photosystems is especially prevalent in grasses from cold climates. Because chloroplast genomes represent only a small proportion of the genetic material of plants, I predict that secondary adaptations to cold climates involve positive selection of a large number of genes encoding a variety of traits, which ultimately transform pre-adapted tropical ancestors into descendants specialised to cold climates.

In grasses, adaptation to cold climates is best-studied in Pooideae, where previous work has demonstrated both the adaptive evolution of cold acclimation and vernalization via molecular adaptation of cold- and daylength-responsive genes (Sandve *et al.* 2008; McKeown *et al.* 2016; Schubert *et al.* 2017; Zhong *et al.* 2018). It has been proposed that the multiple successive evolutionary steps from ancestral cold-stress-responsive genes, which are conserved in grasses, to co-options of specific cold-responsive genes in Pooideae may play a crucial role in the successful distribution of this group (Sandve *et al.* 2008; Zhong *et al.* 2018). Gene duplication and functional divergence of the co-opted genes may drive these gene family expansions, leading to novel adaptive traits. For example, the gene family encoding ice-recrystallisation inhibition proteins (IRIP) evolved through multiplication of repeated motifs that shared a common ancestor with a gene encoding a leucine-rich repeat protein in rice, suggesting adaptive novel traits from pre-existing molecular components (Sandve *et al.* 2008, 2011). In the C-repeat binding factor (*CBF*) gene family, the copy number of this gene expanded during the cooling period of the Eocene-Oligocene, which leads to the hypothesis that low temperature during this period put selection pressure on ancestral stress-responsive genes, to increase the likelihood that duplicates of these genes are retained followed by functional diversification (Sandve & Fjellheim 2010; Zhong *et al.* 2018). In Danthonioideae, selection for increased cold tolerance occurred multiple times after the migration to temperate climates since the origin of the clade (Humphreys & Linder 2013). This is consistent with the pattern of selection for increased cold resistance in Pooideae (Sandve & Fjellheim 2010; Vigeland *et al.* 2013). These different studies show that specialisation to cold habitats involves numerous changes besides the ones observed in the plants that transitioned recently to cold climates, and which were the focus of this dissertation.

Together, the different lines of evidence suggest that, although the initial transitions to colder climates may be relatively easy for species with the necessary pre-adaptations, subsequent success across cold climates requires follow-up adaptations. The secondary colonisation of extremely cold areas might be an especially difficult evolutionary endeavor that requires the emergence of novel traits and consequently requires time (Vigeland *et al.* 2013; Zhong *et al.* 2018). The fact that most groups of grasses migrated to cold climates considerably later than Pooideae may therefore explain why the species richness at high latitudes and altitudes of the latter by far exceeds that of the former (Chapter 1). Because the grass lineages I studied in this dissertation include recent transitions to cold climates, the amount of changes is likely to differ from those observed in the large, old cold-climate lineages. However, this hypothesis will need to be rigorously tested, and the data produced in chapter 4 can be used in the future to test the transferability of the conclusions based on Pooideae to other lineages, and therefore their universality.

5.3 C_4 evolution and climatic transitions

C_4 photosynthesis is considered one of the most efficient biochemical mechanisms to assimilate carbon in higher plants, increasing water- and nitrogen-use efficiency by decreasing photorespiration (Hatch 1987; Sage *et al.* 2012). It has been hypothesised that a decline in atmospheric CO_2 caused carbon starvation and an increase in photorespiration, exerting selection pressure on C_3 ancestors that led to C_4 evolution (Ehleringer *et al.* 1997). Other factors that increase photorespiration such as high temperature and high light may also play parts in C_4 evolution (Sage *et al.* 2012), and it is generally accepted that C_4 photosynthesis first evolved in open habitats within tropical climates (Chapter 2; Sage 2001; Osborne & Freckleton 2009; Edwards & Smith 2010; Christin *et al.* 2013; Christin & Osborne 2014). A number of traits may have enabled these transitions. High vein density, for example, has been found multiple times in close C_3 relatives of C_4 clades, and may have originally functioned to reduce the negative effects of high temperature by increasing hydraulic flux and evapotranspiration (McKown & Dengler 2007; Osborne & Sack 2012; Christin *et al.* 2013; Griffiths *et al.* 2013). The reduction of vein space between bundle sheath and mesophyll would have facilitated the exchange of metabolites between them, enabling a fully functioning C_4 cycle to arise

afterwards (Osborne & Sack 2012; Sage *et al.* 2012; Hattersley 2017). This suggests C_4 evolution occurred under high photorespiration conditions in open tropical habitats under atmospheres with low CO_2 concentrations through the long geological period starting in the Oligocene (Christin *et al.* 2008a; Sage *et al.* 2012), consistent with the findings of chapter 2.

The advantages of C_4 photosynthesis for efficiency provided benefits beyond the environmental conditions under which this trait evolved, allowing C_4 plants to compete with C_3 relatives and expand their climatic niche with rapid dispersal across a wider range of environments and distances (Chapter 2; Lundgren *et al.* 2015; Aagesen *et al.* 2016; Bena *et al.* 2017). The most obvious expansion of the niche occurred into warmer habitats without withdrawal from cold ones (Chapter 2) which was consistent with previous analysis carried out for the Panicoideae (Aagesen *et al.* 2016) but it is inconsistent with results obtained in Gomphrenoideae (Amaranthaceae), which expanded into cooler climates after evolving C_4 photosynthesis (Bena *et al.* 2017). Therefore, C_4 photosynthesis may initially facilitate niche expansion to reach new environmental limits depending on the local availability of this novel niche space. No expansion into cold climates or withdrawal from cold climates has been found previously in grasses indicating similar low temperature limits for C_4 and C_3 species (Chapter 2; Aagesen *et al.* 2016). However, this thesis shows that, although C_4 species were less likely to migrate to cold climates than C_3 ones, they were more likely to migrate between tropical and temperate climates than C_3 species (Chapter 2). This suggested that C_4 photosynthesis promotes the transition into cooler climates but there are additional factors influencing the successful migration to cold climates (Chapter 2).

The results from chapter 3 identify complex connections between several traits involved in the migration to cold climates in grasses indicating the potential adaptation of C_4 species to cold climates (Fig. 5.2). From the concept of migration facilitated by pre-existing traits, the results from chapter 3 showed that some populations originated from tropical climates possess a constitutive freezing resistance, which allows them to survive in cold climates without major changes. This may indicate a potential for niche expansion into cold climates. However, constitutive freezing resistance was still partially limited by tropical origin (Fig. 5.2). For example, high moisture content leads to freezing sensitivity, preventing many tropical plants from passing freezing selection and limiting their distribution in non-freezing habitats (Chapter 3). Therefore, the multiple factors that

affect the change of freezing resistance negatively, such as high moisture content, and positively, such as cold acclimation and high osmotic pressure, will subsequently influence migration to cold climates (Fig. 5.2). In contrast, high constitutive chilling resistance over a short period was found in all species, while the ability to tolerate chilling over a long period is the factor that distinguishes species especially originating from cold climates and likely influences migration to cold climates (Chapter 3). Moreover, some traits such as culm height and leaf width are associated with chilling resistance, although this association seems most likely to facilitate migration to cold climates only indirectly (Fig. 5.2).

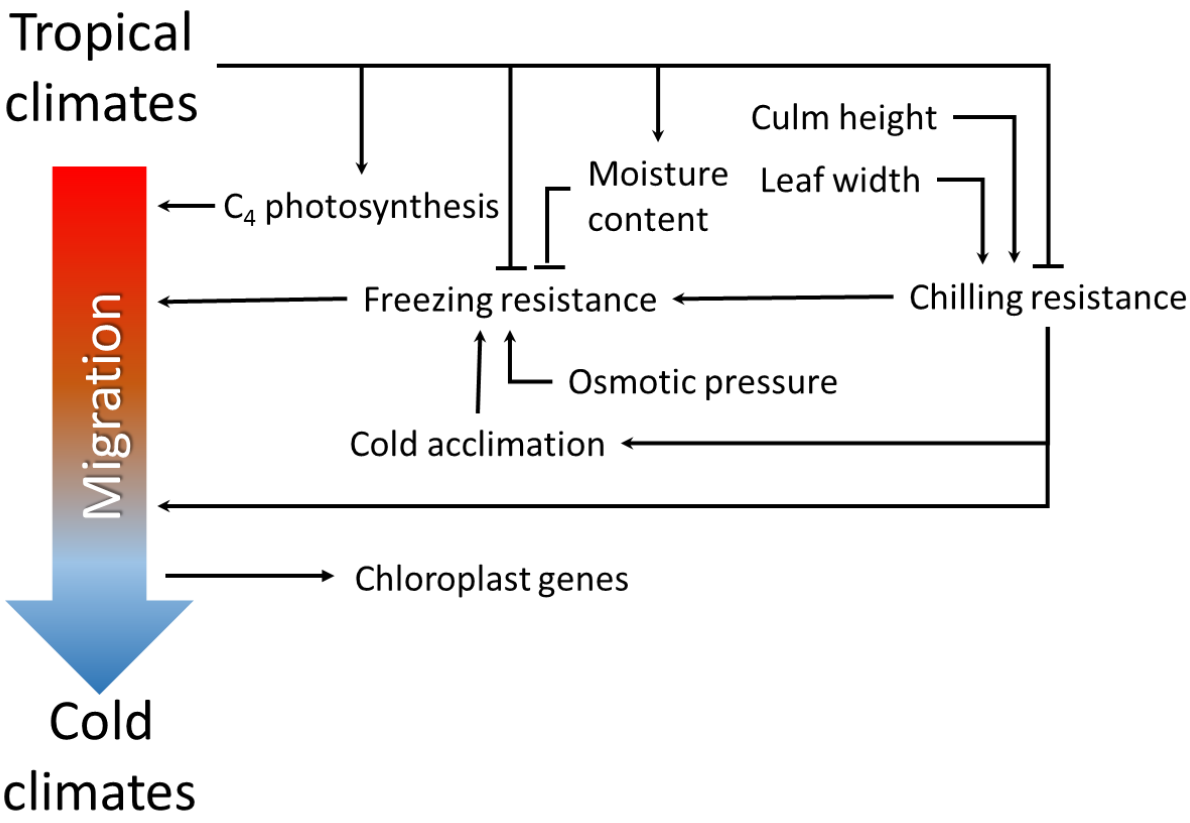

Figure 5.2 The main factors influencing migration to cold climates in grasses and the effect of migration on chloroplast genes. An arrow indicates a positive effect of the factor while a blunt arrow indicates a negative effect of the factor.

Besides genetically determined traits, plastic phenotypes that increase low temperature resistances may also increase the probability of migration to cold climates. For example, the increase of water content in the growing season decreases freezing resistance, reducing the likelihood of survival if freezing occurs during that period (Sakai & Larcher 1987; Sklenář 2017). My study also provided examples of how cold acclimation increases freezing resistance in accordance with previous works (Chapter 3; Liu & Osborne 2008, 2013). The fact that these traits such as cold acclimation can arise independently multiple times in C_4 species indicates that there is no inherent limitation preventing C_4 species from adapting and migrating to cold climates. It has been previously argued that lower plasticity of C_4 photosynthesis compared to the C_3 type may limit its adjustment to environmental conditions, leading to low competitiveness in cold climate habitats (Sage & McKown 2006). These findings may explain why C_4 species are present but not common in cold environments. Chapter 2 showed the occurrence of several C_4 species in cold climates, with multiple transitions to cold climates in C_4 grasses, but C_3 species still dominate these cold climates more successfully than C_4 species (Ehleringer 1978; Sage *et al.* 2010; Osborne *et al.* 2014). Therefore, the successful distribution of C_4 species in cold climates may require the adaptive evolution of multiple traits and the successive evolution of novel traits to increase efficiency. For instance, the adaptive evolution of chloroplast genes may be needed in cold climates to increase the structural stability of the photosystems (Chapter 4).

5.4 Comparisons of potential biofuel crops

The data from chapter 3 also provides evidence to evaluate the potential of bioenergy species to grow in cold climates. The responses of bioenergy species in this experiment suggested that photosynthesis under chilling and after freezing as well as cell membrane damage should be considered together in order to select suitable cold resistant species to grow in cold climates. Of the species that have been utilised for bioenergy, *Spartina pectinata* (Spe) had the highest resistance to chilling and freezing, followed by *Miscanthus* × *giganteus* (Mgi), which is a proven biofuel feedstock and its relatives (Msi and Msa), *Panicum virgatum* (Pvi) and *Andropogon gerardii* (Age) respectively (Fig. 3.3). Photosynthetic performance was high in chilling conditions and after freezing, with a low electrolyte leakage after freezing, in *Spartina pectinata* (Spe) leaves (Fig. 3.3; Sage

et al. 2015). Conversely, the photosynthetic efficiencies of *Miscanthus* species (Mgi, Msi and Msa) were reduced under chilling and after freezing, although low electrolyte leakage showed a high resistance of cellular membranes to freezing (Fig. 3.3). This may involve the ability to synthesise and assemble D1 protein after degradation under low temperatures, which recovers and sustains the photosynthesis function, suggesting a cold adaptation of photosynthetic performance in low temperatures (Wang *et al.* 2008; Głowacka *et al.* 2014; Spence *et al.* 2014). The two most cold sensitive bioenergy species are *Andropogon gerardii* (Age) and *Panicum virgatum* (Pvi), which show a high reduction of photosynthetic efficiency and leakage (Fig. 3.3; Gu *et al.* 2008). These data suggest that *Spartina pectinata* should be explored as an alternative bioenergy feedstock that is more cold tolerant than *Miscanthus* × *giganteus*, consistent with a suggestion in previous work (Friesen et al. 2015).

Chapter 6

General conclusions

In this thesis, I have applied a phylogenetic comparative approach to various genomic, physiological, morphological and geographical datasets to elucidate the evolutionary processes underlying transitions between climates in grasses. The multiple transitions between climates were underpinned by correlations between traits and climates. However, comparative evidence showed that these traits can be either factors that influence migration or consequences of adaptive evolution under novel climates.

Firstly, several pre-existing traits were identified that likely facilitated migration between climates. I showed that C_4 photosynthesis evolved in tropical climates but subsequently promoted migration to temperate climates. However, further transitions from temperate to cold climates were less likely in C_4 plants than C_3 plants. This finding indicates that there are further requirements to migrate into cold climates. I also found that constitutive chilling and freezing resistances may arise initially in tropical species and later serve as exaptations that allow plants to later migrate to cold climates. Moreover, other several traits indirectly affect these resistances in both negative and positive ways, subsequently influencing the migration to cold climates. Therefore migration to cold climates is determined by the sum of positive and negative effects of multiple pre-existing traits.

Adaptive evolution later causes changes in traits that improve the efficiency of plants in the new environments. In particular, the result suggested that cold acclimation mechanisms evolved after migration to cold climates. These mechanisms increase the freezing resistance, which is the key to migrate to cold climates, to increase fitness in specific conditions. I also showed that the adaptive evolution of chloroplast genes was likely involved in improving the structural stability of photosystem proteins. I conclude that the evolution of cold tolerance in lineages that recently made the transition to cold habitats involves the co-option of existing traits followed by the sustained adaptation of the physiological responses and chloroplast genomes. While this has not been studied

here, I assume that similar adaptation will be observed in the nuclear genome, potentially as an expansion of gene families, as reported in the Pooideae.

Numerous questions related to the transition between climates remain unexplored. For example, why do several pre-existing traits that facilitate the migration to cold climates, such as constitutive chilling and freezing resistances, evolve in tropical climates, and which are the main factors causing this constitutive resistance? Further studies will need to test for an association between these traits and other ecological factors, especially drought. I suggest large-scale comparative investigations capturing a variety of tropical taxa to determine whether some of the traits identified here as important for cold adaptation are more prevalent in some tropical environments. The moisture content, which was identified here as an important determinant of freezing tolerance, is especially amenable to such studies as it can be easily measured on a large number of samples. Because I have shown that it affects freezing resistance independently of the climatic history of the taxa, future research on this property will not necessarily need to include freezing experiments. Moreover, the anatomical characters and physical properties of tissue underlying constitutive resistance need to be studied. I suggest large-scale comparative analyses of leaf anatomy in grasses. Because large datasets have been produced in the past for other purposes (e.g. Christin *et al.* 2013; Griffiths *et al.* 2013), such studies would be feasible with additions of a modest number of species to capture more transitions among the physiological states identified here.

Summarizing all cold environments by the temperature gives only a limited picture of their diversity. Fully understanding the dynamics that determine the long-term success of different lineages in cold habitats depends on the intricate interactions between the numerous factors that define habitats and the multitude of features that characterise a plant

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