

The relationship between fundamental and realized niches in plant species' frost hardiness

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von Frau Dipl.-Biol. Maria Hofmann (geb. Auerswald)
geboren am 21.06.1985 in Schlema

Gutachter

1. Prof. Dr. rer. nat. habil. Helge Bruelheide
2. PD Dr. rer. nat. habil. Matthias Hoffmann
3. Prof. Dr. rer. nat. habil. Jürgen Kreyling

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Summary

A species' niche comprises two components, the fundamental and the realized niche. The fundamental niche is defined as the range of environmental conditions under which a species is potentially able to occur without biotic interactions, while the realized niche is defined as the range of environmental conditions under which a species actually occurs. Based on this niche concept, the fundamental niche of any species is defined by its physiology and the realized niche is represented by the species' geographical range. Defining the relationship between the two niche types is one of the main research topics in biogeography. An important component of the fundamental and the realized niches of many plant species is frost hardiness which consequently is also relevant to its distribution range. In this thesis the relationship between fundamental and realized niche was investigated in four studies, all of which focused on the impact of freezing temperatures as a limiting factor for species distribution. The overarching hypothesis is that a species' frost hardiness is related to associated macroclimatic variables. The hypothesis is tested both at the interspecific and the intraspecific levels, asking whether species and provenances distributed across colder regions are more frost resistant than species and provenances distributed in warmer regions.

A total of 85 plant species were analysed across the different studies. The species' fundamental niche, with respect to its frost hardiness, were assessed by the electrolyte leakage method, resulting in LT_{50} -values that indicated the temperature at which 50% of maximum tissue damage occurred. The species' realized niche, expressed by its geographical distribution range, was determined by climate envelope modelling, within which species distribution maps are related to macroclimatic variables.

In general, there was no or only a weak correlation between a species' frost hardiness and the macroclimatic variables that limited its geographical distribution range in any of the four studies. The first study included all 85 species and focused on the differences in the relationship between LT_{50} -values and macroclimatic variables between species of different life form and leaf habit type. As expected, the relationship between frost hardiness and macroclimatic variables differed between the investigated life forms and leaf habit types, with only a small subset of evergreen needle-leaved species showing a positive relationship.

Relating frost hardiness to the species' phylogeny showed that frost hardiness was highly phylogenetically conserved while macroclimatic variables were not. The second study focused on the intraspecific variability of frost hardiness between provenances of *Fagus sylvatica*, with a total of 20 provenances being investigated from a common garden. Although there was substantial variability in frost hardiness among provenances, contrary to expectation, frost hardiness was unrelated to the climatic conditions of the provenances' origin. The third study focused on the relationship between frost hardiness and different phenological stages of eight tree species in spring. Frost hardiness before budburst differed between the species, but immediately after budburst, frost hardiness decreased to nearly the same level in all species. Unexpectedly, frost hardiness was not reflected in the species' geographical distribution range, neither before nor after budburst. The fourth study focused on the variability of frost hardiness between different developmental stages of eight tree species. As expected, seedlings were less frost resistant than adult individuals, but seedlings' frost hardiness did not differ between the different developmental stages. Furthermore, frost hardiness for seedlings and adults was not related to species' distribution range.

The main finding of this thesis is that inter- and intraspecific variability in frost hardiness is unrelated to macroclimatic variables describing the species distribution ranges. The according lack of correspondence between species' fundamental and realized niches may have several explanations. First, the studies revealed a complex influence of the phenological and developmental stages of individuals on variation in frost hardiness, which brings into question the use of one value in frost hardiness to describe a species' fundamental niche. Second, the macroclimatic variables that limit a species' distribution range might be inadequate to describe the temperatures that actually act on plants in the field. Third, a further possibility that is the generally assumed match between both types of niche with respect to frost hardiness may not exist, thereby implying that low temperatures do not seem to play the key role in limiting the geographical distribution range of the investigated species.

Zusammenfassung

Die Nische einer Art umfasst zwei Bestandteile: die fundamentale und die realisierte Nische. Die fundamentale Nische wird definiert als der Bereich von Umweltbedingungen, unter denen eine Art potenziell ohne biotische Interaktionen vorkommen kann, während die realisierte Nische definiert wird als der Bereich von Umweltbedingungen, unter denen eine Art tatsächlich vorzufinden ist. Basierend auf diesem Nischenkonzept ist die fundamentale Nische einer Art durch ihre Physiologie bestimmt, während die realisierte Nische deren geographisches Verbreitungsgebiet repräsentiert. Das Verhältnis beider Niscentypen zueinander ist eines der Hauptuntersuchungsgebiete der Biogeographie. Ein wichtiger Bestandteil der fundamentalen und realisierten Nische von vielen Pflanzenarten ist die Frosthärte, welche relevant für deren Verbreitungsgebiet ist. In dieser Arbeit wurde das Verhältnis zwischen fundamentaler und realisierter Nische in vier Studien untersucht, um den Einfluss von Frosttemperaturen als begrenzender Faktor für die Verbreitung von Arten zu charakterisieren. Dabei ist die Haupthypothese, dass die Frosthärte einer Art in Beziehung zu makroklimatischen Variablen steht. Diese Hypothese wurde sowohl auf inter- als auch auf intraspezifischer Ebene getestet. Untersucht wurde dabei, ob Arten und Provenienzen, welche in kälteren Gebieten verbreitet sind, frosthärter sind als solche, die in wärmeren Gebieten vorkommen.

Insgesamt 85 Pflanzenarten wurden in den verschiedenen Studien untersucht. Die fundamentale Nische der Art in Bezug auf die Frosthärte wurde mittels der Elektronenausflussmethode bestimmt. Die daraus resultierenden LT_{50} -Werte zeigen die Temperatur an, bei der 50 % der maximalen Gewebeschäden vorkommen. Die realisierte Nische der Art, also deren geographisches Verbreitungsgebiet, wurde durch „climate-envelop“-Modellierung beschrieben, bei der Verbreitungsvorkommen mit makroklimatischen Variablen in Beziehung gesetzt werden.

In allen vier Studien gab es keine oder nur eine schwache Korrelation zwischen der Frosthärte einer Art und den makroklimatischen Variablen, welche deren geographisches Verbreitungsgebiet begrenzen. Die erste Studie umfasste alle 85 Arten und fokussierte auf die Variabilität in der Beziehung zwischen LT_{50} -Werten und makroklimatischen Variablen von Arten mit unterschiedlicher Lebensform und Blattausdauer. Die Beziehung von Frosthärte

und makroklimatischen Variablen unterschied sich wie erwartet zwischen den untersuchten Lebensformen und Blattausdauerarten, wobei nur für die kleine Untergruppe der immergrünen nadelblättrigen Arten eine Korrelation gefunden werden konnte. Eine phylogenetische Untersuchung zeigte, dass die Frosthärte der Arten stark phylogenetisch konserviert ist, während makroklimatische Variablen dies nicht sind. Die zweite Studie fokussierte auf die intraspezifische Variabilität der Frosthärte zwischen Provenienzen von *Fagus sylvatica*, wobei insgesamt 20 Provenienzen aus einer Versuchsanpflanzung untersucht wurden. Obwohl eine große Variabilität der Frosthärte zwischen den Provenienzen gefunden wurde, stand die Frosthärte nicht in Beziehung zu den klimatischen Bedingungen im Herkunftsgebiet der Provenienz. Die dritte Studie fokussierte auf das Verhältnis zwischen Frosthärte und verschiedenen phänologischen Phasen von acht Baumarten im Frühling. Vor dem Austreiben der Knospen unterschied sich die Frosthärte zwischen den Arten, aber direkt nach dem Austreiben der Knospen ging die Frosthärte aller Arten auf ein etwa gleiches Level zurück. Entgegen der Erwartung spiegelte sich die Frosthärte nicht im geographischen Verbreitungsgebiet der Arten wider, weder vor noch nach dem Austreiben der Knospen. Die vierte Studie fokussierte auf die Variabilität der Frosthärte zwischen verschiedenen Entwicklungsstufen von acht Baumarten. Wie erwartet waren Keimlinge weniger frosthart als adulte Individuen, jedoch unterschied sich die Frosthärte innerhalb der Keimlinge nicht zwischen den verschiedenen Entwicklungsstufen. Außerdem zeigt die Frosthärte der Keimlinge und der adulten Individuen keinen Bezug zum Verbreitungsgebiet der Art.

Das Hauptergebnis dieser Arbeit ist, dass die inter- und intraspezifische Frosthärte nicht in Beziehung zu makroklimatischen Variablen steht. Das daraus resultierende Fehlen von Übereinstimmung von fundamentaler und realisierter Nische einer Art kann verschiedene Erklärungen haben. Erstens zeigten die Studien deutlich einen komplexen Einfluss von phänologischen Phasen und Entwicklungsstufen des Individuums auf die Variation der Frosthärte, was die Nutzung eines Frosthärtewertes, um die fundamentale Nische einer Art zu beschreiben, fraglich macht. Zweitens scheinen die makroklimatischen Variablen, welche die Verbreitung einer Art begrenzen, unzureichend zu sein, um die Temperaturen zu beschreiben, welche tatsächlich auf die Pflanze im Freiland einwirken. Drittens muss infrage gestellt werden, ob der generell angenommene Zusammenhang zwischen beiden Nischen überhaupt für Frosthärte existiert. Dies impliziert, dass niedrige Temperaturen möglicherweise keine wichtige Rolle in der Begrenzung des geographischen Verbreitungsgebietes der untersuchten Arten spielen.

1 Introduction

1.1 Ecological niche

Niche concept

A principal focus in ecology is to investigate the relationship between species' environmental requirements and their geographical distribution. Grinnell (1917), Elton (1927) and Gause (1936) used niche concepts to describe such relationships and, one century later, the niche concept is still of great importance to ecologists (Chase & Leibold 2003, Wiens & Graham 2005). Hutchinson (1957) advanced preceding concepts and defined a niche from the perspective of the organism, whereby the niche is conceived as a multidimensional volume of limiting environmental factors for a given species (Pulliam 2000, Colwell & Rangel 2009, Wiens et al. 2010). In the context of Hutchinson's niche concept, the range of environmental conditions under which a species is physiologically able to occur is defined as the fundamental niche (e.g. Colwell & Fuentes 1975, Pulliam 2000, Pearman et al. 2008). In contrast, the realized niche is defined as the range of environmental conditions under which a species is able to occur under natural conditions, i.e. when including biotic factors (e.g. Wiens & Graham 2005, Alexander & Edwards 2010). In particular, the realized niche represents a subset of the fundamental niche, which is reduced by biotic interactions and dispersal limitations (e.g. Colwell & Rangel 2009, Alexander & Edwards 2010, Araújo et al. 2013). A close relationship has been shown between both niches, for example, in marine pelagic species (Helaouët & Beaugrand 2009), beetles or limnic organisms (see review by Holt 2009). However, such studies were conducted on only a few species since abiotic fundamental niche identification can only be estimated by using costly and often time-consuming manipulative experiments (Pearman et al. 2008). Since a species' distribution range can be considered as the geographical expression of its realized niche (Thompson et al. 1998, Holt et al. 2005, Sexton et al. 2009), the analysis of a species' fundamental niche is essential to understanding of species' geographical range limitation (Pearman et al. 2008, Guisan et al. 2014).

Realized niche and climate

Abiotic factors as well as biotic interactions are known to be limiting factors for a species' performance and thereby for species distribution (Colwell & Fuentes 1975, Seynave et al. 2008, Chuine 2010). The persistence of a species in a given area indicates that it is able to adapt to the given abiotic and biotic environmental conditions (Pulliam 2000). The tolerance to climatic stress factors seems to represent a key process in determining species distribution (e.g. Beuker et al. 1998, Guisan & Zimmermann 2000, Normand et al. 2011). For example, Woodward & Williams (1987) found that global distribution limits of major vegetation types can be predicted by species responses to extreme minimum temperatures as well as to water availability. A species' distribution boundaries are influenced by abiotic environmental factors, such as precipitation, minimum temperatures, mean annual temperature or length of the growing season (e.g. Jump et al. 2007, Seynave et al. 2008, Cailleret & Davi 2011, Lenz et al. 2014). As there are interspecific differences in responses to climatic conditions, differences in the geographical distribution of species emerge (Davis & Shaw 2001). Many studies have shown that the natural distribution limits of numerous plant species is in congruence with isotherms (e.g. Jäger 1975, Dahl 1998, Saxe et al. 2001). For instance, Jeffree & Jeffree (1994) proposed that the complete geographical distribution limits of a species can be described by only four isotherms: two isotherms for the upper and the lower temperature limits of the warmest month and two isotherms for the upper and the lower temperature limits of the coldest month in the year. In addition, George et al. (1974) found a correlation between the northern distribution boundary and the minimum temperature isotherm for 49 deciduous tree species in North America. Low temperatures are considered to be one of the main factors limiting species distribution worldwide and therefore represent a main component in defining a species' climatic niche (e.g. Inouye 2000, Xin & Browse 2000, Wisniewski et al. 2014).

Freezing temperatures as a limiting factor for species distribution

Coldness is a widespread phenomenon, since 64 % of the global landmass exhibits a mean minimum air temperature of below 0°C and only 25 % of the landmass is free from frost (Sakai & Larcher 1987). In particular, in the boreal and temperate zones of the northern hemisphere, and especially in Europe, the northern and eastern range boundaries as well as the upper altitudinal boundaries of plant species are considered to be limiting with respect to low temperatures (e.g. Pither 2003, Cunningham & Read 2006, Kreyling 2010). Hence, temperate and boreal species have evolved numerous morphological and physiological

mechanisms to cope with freezing temperatures, defined as frost hardiness (e.g. Burke et al. 1976, Lenz et al. 2013, Zanne et al. 2014). It is generally assumed that species with higher physiological frost hardiness are distributed further in colder regions than species with lower frost hardiness. For example, herbaceous and woody species in the Alps that exhibit higher frost hardiness have a higher altitudinal distribution boundary than species with lower frost hardiness (Taschler & Neuner 2004). However, such studies covering the relationship between a species' fundamental niche and its macroclimatic niche with respect to frost hardiness across different species are still rare. Many of these studies reveal contradictory results, whereby general conclusions can not be drawn. For example, Bannister & Polwart (2001) found a correlation between frost hardiness of different ericoid species and their distribution on the British Isles, while the restricted distribution of rarer *Erica* species on the British Isles was not caused by frost hardiness. Because of such inconsistent results, there is a demand for further studies on the relationship between frost hardiness and distribution that incorporates a larger pool of co-occurring species. For this purpose, the major hypothesis tested in this thesis is whether a species' fundamental niche, expressed as frost hardiness, is related to its realized niche, expressed as macroclimatic variables from the distribution range. If proven, species distributed in colder regions should be more frost resistant than species distributed in warmer regions.

1.2 Frost hardiness

Freezing temperatures affect plants at different levels ranging from the cell to the whole organism (Weiser 1970, Pearce 2001). Freezing temperatures can damage individuals indirectly, by dehydration, and directly, by ice formation (e.g. Sakai & Larcher 1987, Beck et al. 2004). Extracellular ice formation induces dehydration of the cells but is not necessarily lethal (e.g. Burke et al. 1976, Beck et al. 2004). In contrast, intracellular ice formation causes the disintegration of cellular membranes, resulting in a plasma efflux, which is inevitably lethal (e.g. Pearce 2001, Neuner 2014). The electrolyte leakage method can be used to measure this efflux as an increase in conductivity of the solution and to determine frost hardiness of plant tissues by estimating LT_{50} -values (Murray et al. 1989, Sutinen et al. 1992, Nunes & Smith 2003), a value which is defined as the temperature at which 50 % of the investigated plant material is destroyed (e.g. Visnjic & Dohrenbusch 2004, Azzarello et al. 2009, Sjøgaard et al. 2009). The method thereby allows for an efficient assessment of differences in frost hardiness within and among species (Nunes & Smith 2003, Thomas & Sporns 2009).

Many studies have demonstrated the effect of phylogenetic conservatism in the evolution of different plant traits (e.g. Peterson et al. 1999, Ackerly 2004, Pearman et al. 2008, Wiens et al. 2010). Studies looking at similar environmental requirements of closely related species (e.g. Qian & Ricklefs 2004, Crisp et al. 2009, Humphreys & Linder 2013) indicate that frost hardiness contains a phylogenetic component (Losos 2008, Pérez et al. 2014). However, the level of phylogenetic conservatism relating to frost hardiness has not yet been quantified across a large set of species (compare Prinzing 2001, Pérez et al. 2014). Therefore, one further hypothesis tested in this thesis is whether species' frost hardiness is phylogenetically conserved; i.e. closely related species more commonly share frost hardiness as a trait compared to more distantly related species.

Interspecific variation of frost hardiness

Since plants have developed numerous mechanisms to deal with low temperatures, frost hardiness varies between species within the same region (e.g. Taschler et al. 2004, Morin et al. 2007, Thomas & Sporns 2009). For example, data compiled by Araújo et al. (2013) on 1,816 plant species from across the globe demonstrate large interspecific differences in frost hardiness among species.

Lineages of different life forms developed different strategies to protect their overwintering organs from lower temperatures (e.g. Sakai & Larcher 1987, Zanne et al. 2014). The overwintering meristems of herbaceous species (i.e. hemicryptophytes and cryptophytes) are located belowground or near to the soil surface (Bruehlheide & Heinemeyer 2002, Briceño et al. 2014), where plants can be protected by leaf litter or snow cover from more extreme freezing temperatures (e.g. Ungar 1975, Rixen et al. 2010, Neuner 2014). For such species, even a low level of frost hardiness can be sufficient to prevent frost damage. In contrast, woody species must acclimate to lower freezing temperatures (Larcher 2001, Zanne et al. 2014), since their overwintering organs are usually exposed to ambient temperatures and winter air temperature at the canopy height, which are assumed to be much lower than temperatures near the soil surface (Körner & Paulsen 2004).

In addition, species with different types of leaf habit develop different strategies to cope with lower temperatures (e.g. Sakai & Larcher 1987, Larcher 2005), which is often explained by the trade-off between maximizing the length of the growing season and minimizing the risk of frost damage (Huner et al. 1998, Chuine 2010). Evergreen species are able to extend their

photosynthetically active vegetation period into the cold season, with broad-leaved evergreen species being less frost resistant (Cavender-Bares et al. 2005) than needle-leaved evergreen species, which can survive even extreme low temperatures (Larcher 2001, Strimbeck et al. 2007). In contrast, broad-leaved deciduous species shed their foliage in autumn and become dormant during winter. As a consequence, the dormant buds of these species are usually highly resistant to freezing temperatures (Weiser 1970, Cavender-Bares et al. 2005, Zanne et al. 2014).

The outcome of comparative studies can be highly biased and even contradictory if different life forms or leaf habit types are included. Thus, to draw general conclusions of the relationship between frost hardiness and distribution range it is essential to use a large species set that includes different life forms and leaf habit types. As yet, only a few studies have used a large species pool. One of the few multi-species studies is the study of Araújo et al. (2013), which indicated, that the realized niche will tend to underestimate species tolerance limits to climate as a consequence of fundamental niche conservatism. Additionally, there is currently a lack of studies that determine a species' frost hardiness with standardized laboratory methods and many cases, frost hardiness is modelled from climatic variables of a species' actual distribution range. For the analysis of the relationship between frost hardiness and distribution range, the species' frost hardiness should not be derived from the species' distribution range, but should be independently determined of the species' distribution range. As such, a further hypothesis tested in this thesis is whether frost hardiness varies between species of varying life form and differing leaf habit type. In particular, it was expected that herbaceous species and evergreen species are more frost sensitive than woody species and deciduous species, respectively.

Intraspecific variation of frost hardiness between different provenances

In addition to interspecific differences, intraspecific differences in frost hardiness are known (e.g. Lawes et al. 1995, Beuker et al. 1998, Lennartsson & Ögren 2003, Charrier et al. 2013). A broad geographical distribution range usually implies substantial variation in local environmental conditions, resulting in locally adapted ecotypes within plant species (e.g. Visnjic & Dohrenbusch 2004, Alberto et al. 2013, Kreyling et al. 2014). Such intraspecific differences can be analysed through provenance trials, which compare the intraspecific variation in performance of different provenances of the same species under controlled conditions in common garden experiments (Lawes et al. 1995,

Varelides et al. 2001, König 2005). A number of studies have demonstrated a correlation between frost hardiness and the climatic conditions of the provenance's origin (e.g. Bannister & Polwart 2001, Jensen & Deans 2004, Kreyling et al. 2012b, Leiblein-Wild et al. 2013), with frost hardiness often varying considerably between provenances (e.g. Gömöry & Paule 2011, Andivia et al. 2011, Humphreys & Linder 2013). Accordingly, provenances from northern, colder regions or from higher altitudes have been shown to be better acclimated to lower temperatures than provenances from southern, warmer regions or lower altitudes (e.g. Weng & Parker 2008, Aldrete et al. 2008, Kathke & Bruelheide 2011). In contrast, some other studies found no evidence of local adaptation (e.g. Hannerz & Westin 2000, Schraml & Rennenberg 2002). One major criticism in many of the previous studies is that they investigated only a small set of provenances, representing only a fraction of the macroclimatic gradient covered by the entire distribution range (e.g. Deans & Harvey 1996, Ducousso et al. 1996, Andivia et al. 2011). As such, there is a need for studies that include provenances from the whole distribution range covering the complete macroclimatic gradient of a species' realized niche. Another hypothesis tested in this thesis is whether a species' frost hardiness varies between different provenances.

Intraspecific variation of frost hardiness between different phenological stages

Intraspecific variation is also reflected in variation of frost hardiness depending on the phenological stage of the species (e.g. Fuller & Telli 1999, Stevenson et al. 1999, Bigras et al. 2004). Especially, species originating from temperate and boreal regions have to modulate their frost hardiness to the seasonal changes of environmental conditions, such as annually recurring frost events (e.g. Leinonen & Hänninen 2002, Beck et al. 2007, Vitasse et al. 2014). The term *cold acclimation* or *hardening* describes the seasonal changes from a sensitive to a more resistant state of frost hardiness (Weiser 1970, Burke et al. 1976, Huner et al. 1998). Increasing tolerance against freezing temperatures can be achieved by an accumulation of carbohydrates, resulting in dehydration of cells, as well as by structural changes in proteins and membrane lipids (e.g. Thomas et al. 2004, Larcher 2005, Charrier et al. 2013). Plant hardening is only possible during growth cessation periods and varies among tissues and organs (Hannah et al. 2006, Martin et al. 2010, Charrier et al. 2013). Hardening in autumn is a very complex process of biochemical and physiological changes, which is mainly induced by both decreasing temperatures and the shortening photoperiod in autumn (e.g. Repo et al. 2001, Li et al. 2002, Beck et al. 2004). In contrast, dehardening in spring is much faster and is mainly induced by increasing temperatures (e.g. Leinonen et al. 1997, Hänninen et al. 2007, Kalberer et al. 2006).

Frost damage often occurs at the time of budburst and leaf unfolding, which is the most frost sensitive phenological phase (e.g. Kang et al. 1998, Taschler et al. 2004, Dittmar et al. 2006, Inouye 2008). Therefore, late frost events in spring affect plant performance during the growing season and may limit species distribution (e.g. Sakai & Larcher 1987, Augspurger 2009, Kollas et al. 2014, Vitasse et al. 2014). A recent study demonstrated a positive correlation between late frost sensitivity and the minimum temperatures in May for different populations of *Fagus sylvatica* (Kreyling et al. 2012a). Hence, early dehardening in spring is related to an increasing risk of frost damage, but also to an increasing length of the growing season as well as greater carbon yield (e.g. Leinonen & Hänninen 2002, Linkosalo et al. 2006, Lenz et al. 2013). In consequence, early-budding species should be more frost resistant than late-budding species, due to higher risk of being exposed to frost events. For example, northern populations of *Pinus greggii* showed higher frost hardiness as well as earlier budburst than southern populations (Aldrete et al. 2008). Similarly, the growth of *Fagus sylvatica* was negatively related to early budburst along an elevation gradient in France, suggesting a high risk of spring frost damage in that species (Maxime & Hendrik 2011).

Most studies analysing the impact of phenology on frost hardiness focus on different provenances of one single species (e.g. Beuker et al. 1998, Charrier et al. 2011, Gömöry & Paule 2011), often during a single phenological stage, i.e. during budburst (e.g. Ducouso et al. 1996, Prozherina et al. 2003, Vitasse et al. 2014). Studies investigating frost hardiness of different co-occurring species and comparing different phenological stages at the onset of the growing season are lacking, and a further hypothesis tested in this thesis is whether frost hardiness varies consistently across species between different phenological stages, in particular whether early-budding species are more frost resistant at the time of budburst than late-budding species.

Intraspecific variation of frost hardiness between different developmental stages

Intraspecific variation is also reflected in variation of frost hardiness between different developmental stages (e.g. Menzel et al. 2003, Beck et al. 2004, Bigras et al. 2004). Among the life stages, the seedling stage is the most sensitive phase, as frost hardiness is expected to increase with increasing plant development (e.g. Joosen et al. 2006, Ningre & Colin 2007, Lim et al. 2014). This has already been demonstrated by Morin et al. (2007) for several European *Quercus* species, whereby seedlings were found to be significantly more

frost sensitive than adults. In another study, the herbaceous species *Digitalis purpurea* was found to be approximately four times more likely to suffer freezing injuries during the seedling stage than during the adult stage (Bruehlheide & Heinemeyer 2002). Freezing temperatures can severely affect seedling establishment, growth and survival (Funkenberg et al. 2012, Kreyling et al. 2012b, Marcante et al. 2012), which may in turn have a significant effect on the distribution of species. For example, frost damage limits the growth of *Quercus robur* seedlings and can be regarded as a limiting factor for the northern distribution range of the species (Repo et al. 2008).

Frost hardiness of seedlings has been investigated in numerous studies, but frost hardiness of seedlings and adults of the same species has not been compared in a joint analysis, with most existing experiments having only been conducted on seedlings or saplings (e.g. Hannerz & Westin 2000, Czajkowski & Bolte 2006, Aldrete et al. 2008). Thus, there is a need for studies that focus on different developmental stages within single species, and the hypothesis tested here is whether a species' frost hardiness varies consistently across species between different developmental stages, i.e. whether seedlings are more frost sensitive than adult individuals of the same species.

1.3 Objectives and aims of the thesis

This thesis investigates the relationship between the fundamental niche of a species, with respect to its frost hardiness, and the realized niche, expressed by the geographical distribution range of different plant species (see Tab. 1.1 at the end of this chapter). In particular, it focuses on the role of freezing temperatures as a limiting factor for species distribution. The overarching hypothesis is that frost hardiness is related to macroclimatic variables, both on the interspecific and the intraspecific levels, which were tested in all four of the studies composing the thesis (see Fig. 1.1). Accordingly, species and provenances with distribution ranges extending into colder regions should be more frost resistant than species and provenances limited to warmer regions, respectively.

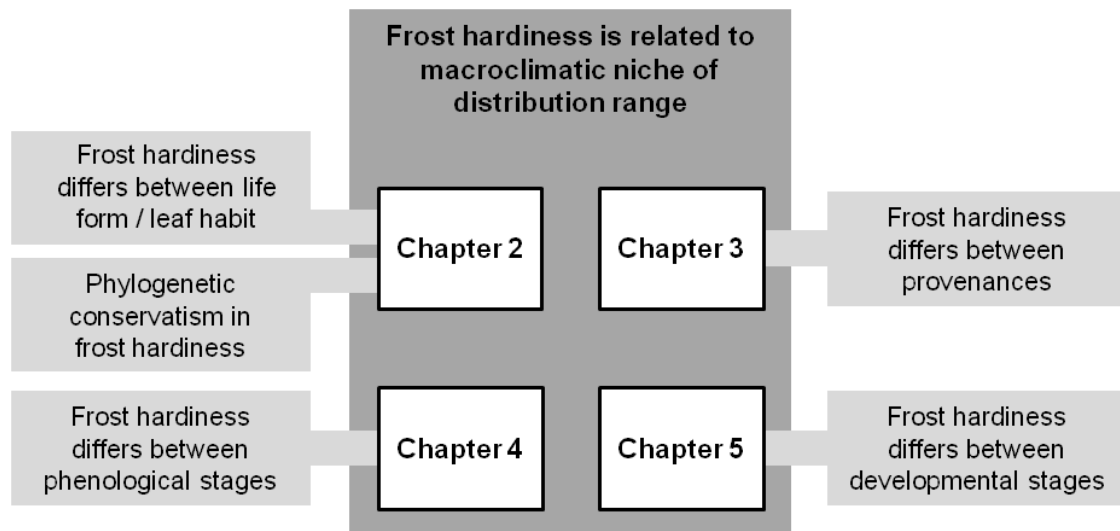


Figure 1.1 The major hypothesis (dark grey) and the specific hypotheses (light grey) tested in the present thesis and their affiliation to the different studies.

Chapter 2 examines the relationship between frost hardiness and climatic conditions in the geographical distribution range and frost hardiness and phylogeny across a set of 85 plant species. The hypothesis is then tested as to whether the relationship between frost hardiness and macroclimatic variables differs between different life forms and different leaf habit types (Fig. 1.1). Additionally, a further tested hypothesis is whether frost hardiness is phylogenetically conserved (Fig. 1.1), i.e. whether closely related species exhibit a more similar degree of frost hardiness than more distantly related species.

Chapter 3 examines intraspecific variation of frost hardiness in *Fagus sylvatica* provenances. To investigate variability within this species, the frost hardiness of 20 provenances was measured and related to the prevailing climatic conditions at the provenances' geographical origin. The hypothesis tested is whether frost hardiness differs between provenances (Fig. 1.1).

Chapter 4 examines the role of phenology on the spring frost hardiness of eight tree species. The frost hardiness of buds from early- and late-budding species was investigated before and directly after budburst. The hypothesis tested is whether a species' frost hardiness differs between phenological stages (Fig. 1.1), i.e. whether early-budding species are more frost resistant at the time of budburst than late-budding species.

Chapter 5 examines the role of plant age on frost hardiness, focussing on the correspondence of frost tolerance of seedlings and adult individuals in eight tree species. The hypothesis tested here is whether a species' frost hardiness varies between developmental stages (Fig. 1.1), i.e. whether seedlings are more frost sensitive than adult individuals.

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Table 1.1 Species used in the present thesis, family and use in the different studies.

Species	Family	Use in chapter			
		2	3	4	5
<i>Acer campestre</i> L.	Aceraceae	x			
<i>Acer monspessulanum</i> L.	Aceraceae	x			
<i>Acer negundo</i> L.	Aceraceae	x			x
<i>Acer platanoides</i> L.	Aceraceae	x			x
<i>Acer pseudoplatanus</i> L.	Aceraceae	x			
<i>Acer saccharinum</i> L.	Aceraceae	x			
<i>Achillea millefolium</i> L.	Asteraceae	x			
<i>Achillea pannonica</i> SCHEELE	Asteraceae	x			
<i>Aesculus hippocastanum</i> L.	Hippocastanaceae	x			
<i>Alnus alnobetula</i> (EHRH.) K. KOCH	Betulaceae				
<i>Alnus glutinosa</i> (L.) P. GAERTN.	Betulaceae	x			x
<i>Apollonias canariensis</i> (WILLD.) NESS	Lauraceae	x			
<i>Arbutus unedo</i> L.	Ericaceae	x			
<i>Betula alleghaniensis</i> BRITT	Betulaceae	x			
<i>Betula pendula</i> ROTH	Betulaceae	x		x	x
<i>Brachypodium sylvaticum</i> (HUDS.) P. BEAUV.	Poaceae	x			
<i>Bromus benekenii</i> (LANGE) TRIEMEN	Poaceae	x			
<i>Bromus ramosus</i> HUDS. S. STR.	Poaceae	x			
<i>Buxus sempervirens</i> L.	Buxaceae	x			
<i>Carlina biebersteinii</i> BERNH. EX HORNEM.	Asteraceae	x			
<i>Carlina vulgaris</i> L. S. STR.	Asteraceae	x			
<i>Carpinus betulus</i> L.	Betulaceae	x		x	
<i>Celtis biondii</i> PAMP.	Ulmaceae	x			
<i>Centaurea scabiosa</i> L. S. L.	Asteraceae	x			
<i>Centaurea stoebe</i> L. S. L.	Asteraceae	x			
<i>Choerospondias axillaris</i> (ROXB.) B.L.BURTT & A.W.HILL	Anacardiaceae	x			
<i>Cinnamomum camphora</i> L.	Lauraceae	x			
<i>Dianthus carthusianorum</i> L.	Caryophyllaceae	x			
<i>Dianthus deltoides</i> L.	Caryophyllaceae	x			
<i>Distylium myricoides</i> HEMSL.	Hamamelidaceae	x			
<i>Fagus sylvatica</i> L.	Fagaceae	x	x	x	x
<i>Ficus henryi</i> WARBURG EX DIELS	Moraceae	x			
<i>Fraxinus excelsior</i> L.	Oleaceae	x		x	
<i>Fraxinus pennsylvanica</i> MARSHALL	Oleaceae	x			
<i>Hedera helix</i> L.	Araliaceae	x			
<i>Helleborus foetidus</i> L.	Ranunculaceae	x			
<i>Helleborus lividus</i> subsp. <i>corsicus</i> (BRIQ.) YEO	Ranunculaceae	x			
<i>Hordelymus europaeus</i> (L.) JESSEN EX HARZ	Poaceae	x			
<i>Hypericum maculatum</i> CRANTZ S. L.	Clusiaceae	x			
<i>Hypericum pulchrum</i> L.	Clusiaceae	x			
<i>Ilex aquifolium</i> L.	Aquifoliaceae	x			
<i>Illicium lanceolatum</i> A.C.SM.	Illiciaceae	x			
<i>Inula conyzae</i> (GRIESS.) MEIKLE	Asteraceae	x			
<i>Inula hirta</i> L.	Asteraceae	x			

Table 1.1 (continued)

Species	Family	Use in chapter			
		2	3	4	5
<i>Juglans nigra</i> L.	Juglandaceae	x			
<i>Juglans regia</i> L.	Juglandaceae	x		x	
<i>Koeleria macrantha</i> (LEDEB.) SCHULT.	Poaceae	x			
<i>Koeleria pyramidata</i> (LAM.) P. BEAUV.	Poaceae	x			
<i>Laurus nobilis</i> L.	Lauraceae	x			
<i>Magnolia acuminata</i> L.	Magnoliaceae	x			
<i>Magnolia stellata</i> (SIEB. & ZUCC.) MAXIM.	Magnoliaceae	x			
<i>Mahonia repens</i> (LINDL.) G. DON	Berberidaceae	x			
<i>Meliosma flexuosa</i> PAMPANINI	Sabiaceae	x			
<i>Picea abies</i> (L.) H. KARST.	Pinaceae	x			x
<i>Pinus cembra</i> L.	Pinaceae	x			
<i>Pinus nigra</i> J. F. ARNOLD	Pinaceae	x			
<i>Pinus strobes</i> L.	Pinaceae	x			
<i>Pinus sylvestris</i> L.	Pinaceae	x			
<i>Pittosporum illiciodes</i> MAKINO	Pittosporaceae	x			
<i>Potentilla incana</i> P. GAERTN., B. MEY. & SCHERB.	Rosaceae	x			
<i>Potentilla tabernaemontani</i> ASCH.	Rosaceae	x			
<i>Prunus avium</i> L.	Rosaceae	x			
<i>Prunus padus</i> L.	Rosaceae	x			
<i>Prunus serotina</i> EHRH.	Rosaceae	x			
<i>Pseudotsuga menziesii</i> (MIRB.) FRANCO	Pinaceae	x			x
<i>Quercus ilex</i> L.	Fagaceae	x			
<i>Quercus robur</i> L.	Fagaceae	x			
<i>Quercus rubra</i> L.	Fagaceae	x			
<i>Rhaphiolepis indica</i> (L.) LINDL.	Rosaceae	x			
<i>Rhododendron catawbiense</i> MICHX.	Ericaceae	x			
<i>Rhododendron ponticum</i> L.	Ericaceae	x			
<i>Salix x rubens</i> SCHRANK	Salicaceae	x		x	
<i>Scabiosa columbaria</i> L.	Dipsacaceae	x			
<i>Scabiosa ochroleuca</i> L.	Dipsacaceae	x			
<i>Silene otites</i> (L.) WIBEL	Caryophyllaceae	x			
<i>Sorbus aria</i> (L.) CRANTZ S. STR.	Rosaceae	x			
<i>Sorbus domestica</i> L.	Rosaceae	x			
<i>Sorbus torminalis</i> (L.) CRANTZ	Rosaceae	x			
<i>Styrax dasyanthus</i> PERKINS	Styracaceae	x			
<i>Syringa vulgaris</i> L.	Oleaceae	x			
<i>Syzygium buxifolium</i> HOOKER & ARNOTT	Myrtaceae	x			
<i>Taxus baccata</i> L.	Taxaceae	x			
<i>Tilia cordata</i> MILL.	Tiliaceae	x		x	x
<i>Tilia platyphyllos</i> SCOP.	Tiliaceae	x			
<i>Ulmus laevis</i> PALL.	Ulmaceae	x		x	

2 Can frost hardiness explain plant species' distribution ranges?

Maria Hofmann, Oliver Purschke, Gunnar Seidler & Helge Bruelheide

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2.1 Abstract

The objective of this study was to investigate the relationship between frost resistance and the macroclimatic niche across a large set of plant species. While physiological frost resistance reflects a major component of a plant species' fundamental niche, the macroclimatic niche represents the realized niche and can be derived from species' geographical distribution range. We hypothesized that species with distribution ranges that extend into colder regions have a higher frost resistance than species confined to warmer regions. Frost resistance was assessed on 85 plant species in a standardized way, exposing the buds, leaves or needles to different freezing temperatures in a climate test chamber and deriving LT_{50} -values from electrolyte leakage measurements. The relationship of frost resistance to the 1%-percentiles both of minimum temperature of the coldest month and mean annual temperature in the species' distribution range was tested with linear models, also taking into account the species' life form (herbaceous vs. woody) and leaf habit (evergreen vs. deciduous). Additionally, we related frost resistance, life form, leaf habit and macroclimatic characteristics to the phylogeny of the species. There was only a weak relationship between the LT_{50} -values and macroclimatic niche variables across all species, which was only driven by evergreen trees, including needle-leaved conifers. In contrast, herbaceous species showed no relationship between frost resistance and range characteristics. The strong relationship encountered in evergreen species was caused by including needle-leaved species in the subset, and vanished, when the analysis was restricted to broad-leaved evergreen species. We found frost resistance to be highly phylogenetically conserved, whereas the macroclimatic niche variables were not. In consequence, fundamental and realized niche of the investigated species were not congruent. This implies that physiological frost resistance can only be considered a good predictor for geographic distribution ranges in certain groups of species.

Keywords: electrolyte leakage, fundamental niche, LT_{50} , macroclimatic niche, phylogeny, realized niche

2.2 Introduction

The relationship between a species' requirements and tolerances of environmental factors and the distribution of these factors is of great interest to ecologists. The niche concept of Hutchinson (1957) provides the theoretical background for understanding species distribution (Pulliam 2000) and can be seen as a useful tool for describing the pattern and limits of species survival (Colwell & Fuentes 1975). The range of environmental conditions under which a species is physiologically able to become established, grow and survive is defined as fundamental niche. Under natural conditions, the fundamental niche is reduced by biotic interactions and dispersal limitation, resulting in the range of environmental conditions under which a species actually occurs, which has been termed the realized niche (Hutchinson 1957, Colwell & Fuentes 1975, Pulliam 2000). As the presence of a species in a given area indicates that this species is able to cope with the environmental conditions in that location (Pulliam 2000), the sum of all locations where a species occurs, i.e. its geographical distribution range, can be seen as the geographical expression of the species' realized niche (Thompson et al. 1998, Holt et al. 2005). For the geographical range of a species the term γ niche has also been coined (Silvertown et al. 2006).

A key question in niche theory is whether there is a match between fundamental and realized niche. If that was not the case, fundamental niche factors would not determine the large-scale distribution patterns of plants, as has been assumed by many authors (e.g. Woodward 1987, Pearson & Dawson 2003, Chuine 2010). In particular, differences in species tolerance limits to minimum temperatures can be invoked to explain distribution patterns. It has long been recognized in plant geography that northern distribution boundaries tend to coincide with winter temperatures (e.g. Dahl 1998, Jäger 1972). For example, George et al. (1974) indentified a relationship between the northern boundaries of the distribution range of 49 deciduous tree species in North-America and the minimum temperature isotherm. Similarly, there are many case studies on single species, which have made winter temperatures responsible for distribution limits. For example, the distribution of *Fagus sylvatica* was found to be restricted from regions with a mean temperature in January below -3°C (Bolte et al. 2007). It is well known that the mean temperatures of the coldest month are highly correlated with the absolute minima (Prentice et al. 1992). Consequently, from such correlative findings is generally assumed that absolute minima are the main limiting factor for the northern distribution boundaries of plant species in the northern hemisphere (e.g. Sakai & Larcher 1987, Repo et al. 2001, Saxe et al. 2001). The same applies to high-altitudinal boundaries. Comparing the realized niches of native and *ex situ* garden

locations of four *Rhododendron* species, Vetaas (2002) demonstrated that extreme frost temperatures provide a good explanation not only for northern but also high-altitudinal range boundaries. However, all the studies mentioned are only correlative and do not allow conclusions on the species' fundamental climatic niche.

Assessing the fundamental niche in frost hardiness requires experimental testing. Low temperatures can affect plants directly by ice formation in cells, tissues and organs as well as indirectly by freeze dehydration (Sakai & Larcher 1987, Pearce 2001, Beck et al. 2004). Intracellular ice formation is inevitably lethal, and therefore, plant species have developed different mechanisms to withstand freezing temperatures (e.g. Huner et al. 1998, Xin & Browse 2000). The mechanisms differ between the species' life forms (Sakai & Larcher 1987). While in herbaceous plants the overwintering meristems are located belowground or near to the soil surface, where protection is provided by a cover of foliage or snow (Larcher 2001, Bruelheide & Heinemeyer 2002), the hibernating growth-points of woody species are usually exposed to ambient temperatures, and thus require higher frost resistance (Larcher 2001). As hibernation precludes carbon assimilation, there is a trade-off between maximizing the length of the growing season and minimizing the risk of frost damage (Huner et al. 1998, Chuine 2010). This trade-off is often invoked to explain the different strategies between broad-leaved evergreen species which have a positive carbon balance also in the cold season, but in consequence are less frost-tolerant (Cavender-Bares et al. 2005), and broad-leaved deciduous species which during hibernation tolerate much lower temperatures (Weiser 1970). In contrast, needle-leaved evergreen conifers exhibit both an extended photosynthetically active vegetation period and a high frost resistance of the green needles in winter (Larcher 2001, Strimbeck et al. 2007).

One major question in evolutionary biology is the evolution of ecological characteristics over time scales (e.g. Peterson et al. 1999, Losos 2008, Crisp & Cook 2012). When testing for a match of physiological frost resistance and geographic distribution, a crucial aspect is to which degree both characteristics are evolutionarily constrained. Many studies have shown conservative trait evolution for numerous functional traits (e.g. Ackerly 2004, Wiens et al. 2010). As frost resistance involves complex adaptations (Sakai & Larcher 1987), strong phylogenetic conservatism would be expected. In contrast, species distribution ranges might be much less conservative because speciation in most cases is allopatric, which would automatically cause differences in the climate space occupied by the daughter species compared to that of the parent taxon. However, it has been pointed out that also climatic distributions are phylogenetically conserved (Silvertown et al. 2006, Schuldt et al. 2014).

For example, Humphreys & Linder (2013) found closely related species of the Danthonioideae to have similar climate niches. Nevertheless, the degree of conservatism might be lower in traits related to the species geographical distribution ranges rather than to their physiological traits. This might be concluded from the fact that some Ellenberg indicator values (EIVs) have been shown to be less phylogenetically conserved than others (Prinzing 2001). Indices related to the species climatic distribution (i.e. the continentality and temperature EIVs) showed a much lower degree of conservatism than those related to habitats (soil moisture and nutrient EIVs). Support for this view does also come from a comparative study on 12 Chilean *Myrceugenia* species by Pérez et al. (2014) who found that both foliar freezing resistance and minimum temperature in the species distribution range showed a significant phylogenetic signal, which however, was stronger in freezing resistance than the macroclimatic characteristic. Still, differences in the degree of phylogenetic conservatism can still result in significant relationships between the fundamental and realized niche (Pérez et al. 2014).

The correspondence between fundamental and realized niche regarding the resistance of minimum temperatures seems to be so basic to our ecological understanding that it has not yet been properly tested with comprehensive data sets. It is generally assumed that species with higher physiological frost resistance are distributed further into cold regions than species with lower frost resistance. We set out to test this assumption, using a set of 85 species, for which we have measured frost resistance with standardized laboratory methods (Kathke & Bruelheide 2011, Hofmann et al. 2013, 2014) and compiled global distribution data. We hypothesized i) that physiological frost resistance is related to minimum temperature of the coldest month in the species' distribution range, and ii) this relationship differs between the different types of life form and leaf habit. Finally, iii) we hypothesized that niche conservatism is stronger in frost resistance than macroclimatic range characteristics.

2.3 Material and methods

Study species

We analyzed frost resistance and macroclimatic niches of 85 plant species from 33 families. A total of 25 and 60 species were herbaceous and woody, and 48 and 37 were deciduous and evergreen, respectively (Tab. 2.1). Samples were taken from species growing in the surrounding of Halle (Saale) or from the Botanical Garden in Halle (Saale).

Table 2.1 Species used in the present study as well as family, LT_{50} -value, life form (LF) and leaf habit (LH).
D – deciduous, E – evergreen, H – herbaceous, W – woody.

No.	Species	Family	LT_{50}	LF	LH
1	<i>Acer campestre</i> L.	Aceraceae	-31.29	W	D
2	<i>Acer monspessulanum</i> L.	Aceraceae	-14.77	W	D
3	<i>Acer negundo</i> L.	Aceraceae	-32.17	W	D
4	<i>Acer platanoides</i> L.	Aceraceae	-32.56	W	D
5	<i>Acer pseudoplatanus</i> L.	Aceraceae	-42.60	W	D
6	<i>Acer saccharinum</i> L.	Aceraceae	-37.42	W	D
7	<i>Achillea millefolium</i> L.	Asteraceae	-22.25	H	E
8	<i>Achillea pannonica</i> SCHEELE	Asteraceae	-16.81	H	D
9	<i>Aesculus hippocastanum</i> L.	Hippocastanaceae	-33.56	W	D
10	<i>Alnus alnobetula</i> (EHRH.) K. KOCH	Betulaceae	-34.59	W	D
11	<i>Alnus glutinosa</i> (L.) P. GAERTN.	Betulaceae	-36.48	W	D
12	<i>Apollonias canariensis</i> (WILLD.) NESS	Lauraceae	-16.05	W	E
13	<i>Arbutus unedo</i> L.	Ericaceae	-9.54	W	E
14	<i>Betula alleghaniensis</i> BRITT	Betulaceae	-24.25	W	D
15	<i>Betula pendula</i> ROTH	Betulaceae	-32.91	W	D
16	<i>Brachypodium sylvaticum</i> (HUDS.) P. BEAUV.	Poaceae	-13.26	H	D
17	<i>Bromus benekenii</i> (LANGE) TRIEMEN	Poaceae	-25.18	H	D
18	<i>Bromus ramosus</i> HUDS. S. STR.	Poaceae	-26.26	H	D
19	<i>Buxus sempervirens</i> L.	Buxaceae	-30.10	W	E
20	<i>Carlina biebersteinii</i> BERNH. EX HORNEM.	Asteraceae	-30.95	H	D
21	<i>Carlina vulgaris</i> L. S. STR.	Asteraceae	-16.60	H	E
22	<i>Carpinus betulus</i> L.	Betulaceae	-28.18	W	D
23	<i>Celtis biondii</i> PAMP.	Ulmaceae	-9.28	W	D
24	<i>Centaurea scabiosa</i> L. S. L.	Asteraceae	-10.94	H	D
25	<i>Centaurea stoebe</i> L. S. L.	Asteraceae	-9.07	H	D
26	<i>Choerospondias axillaris</i> (ROXB.) B.L.BURTT & A.W.HILL	Anacardiaceae	-11.03	W	D
27	<i>Cinnamomum camphora</i> L.	Lauraceae	-12.15	W	E
28	<i>Dianthus carthusianorum</i> L.	Caryophyllaceae	-28.72	H	E
29	<i>Dianthus deltoides</i> L.	Caryophyllaceae	-30.70	H	E
30	<i>Distylium myricoides</i> HEMSL.	Hamamelidaceae	-10.52	W	E
31	<i>Fagus sylvatica</i> L.	Fagaceae	-32.18	W	D
32	<i>Ficus henryi</i> WARBURG EX DIELS	Moraceae	-10.03	W	E
33	<i>Fraxinus excelsior</i> L.	Oleaceae	-31.81	W	D
34	<i>Fraxinus pennsylvanica</i> MARSHALL	Oleaceae	-19.36	W	D
35	<i>Hedera helix</i> L.	Araliaceae	-16.83	W	E
36	<i>Helleborus foetidus</i> L.	Ranunculaceae	-12.09	H	E
37	<i>Helleborus lividus</i> subsp. <i>corsicus</i> (BRIQ.) YEO	Ranunculaceae	-13.19	H	E
38	<i>Hordelymus europaeus</i> (L.) JESSEN EX HARZ	Poaceae	-11.94	H	E
39	<i>Hypericum maculatum</i> CRANTZ S. L.	Clusiaceae	-15.65	H	E
40	<i>Hypericum pulchrum</i> L.	Clusiaceae	-16.38	H	E
41	<i>Ilex aquifolium</i> L.	Aquifoliaceae	-24.78	W	E
42	<i>Illicium lanceolatum</i> A.C.SM.	Illiciaceae	-12.93	W	E
43	<i>Inula conyzae</i> (GRIESS.) MEIKLE	Asteraceae	-13.85	H	E
44	<i>Inula hirta</i> L.	Asteraceae	-16.65	H	D
45	<i>Juglans nigra</i> L.	Juglandaceae	-16.57	W	D
46	<i>Juglans regia</i> L.	Juglandaceae	-17.06	W	D

Table 2.1 continued.

No.	Species	Family	LT ₅₀	LF	LH
47	<i>Koeleria macrantha</i> (LEDEB.) SCHULT.	Poaceae	-29.08	H	D
48	<i>Koeleria pyramidata</i> (LAM.) P. BEAUV.	Poaceae	-35.47	H	D
49	<i>Laurus nobilis</i> L.	Lauraceae	-20.27	W	E
50	<i>Magnolia acuminata</i> L.	Magnoliaceae	-21.07	W	D
51	<i>Magnolia stellata</i> (SIEB. & ZUCC.) MAXIM.	Magnoliaceae	-29.57	W	D
52	<i>Mahonia repens</i> (LINDL.) G. DON	Berberidaceae	-17.91	W	E
53	<i>Meliosma flexuosa</i> PAMPANINI	Sabiaceae	-12.82	W	E
54	<i>Picea abies</i> (L.) H. KARST.	Pinaceae	-28.31	W	E
55	<i>Pinus cembra</i> L.	Pinaceae	-39.68	W	E
56	<i>Pinus nigra</i> J. F. ARNOLD	Pinaceae	-29.41	W	E
57	<i>Pinus strobes</i> L.	Pinaceae	-42.84	W	E
58	<i>Pinus sylvestris</i> L.	Pinaceae	-47.02	W	E
59	<i>Pittosporum illicioides</i> MAKINO	Pittosporaceae	-11.99	W	E
60	<i>Potentilla incana</i> P. GAERTN., B. MEY. & SCHERB.	Rosaceae	-14.90	H	D
61	<i>Potentilla tabernaemontani</i> ASCH.	Rosaceae	-12.95	H	E
62	<i>Prunus avium</i> L.	Rosaceae	-24.24	W	D
63	<i>Prunus padus</i> L.	Rosaceae	-29.58	W	D
64	<i>Prunus serotina</i> EHRH.	Rosaceae	-27.67	W	D
65	<i>Pseudotsuga menziesii</i> (MIRB.) FRANCO	Pinaceae	-36.61	W	E
66	<i>Quercus ilex</i> L.	Fagaceae	-7.60	W	E
67	<i>Quercus robur</i> L.	Fagaceae	-35.85	W	D
68	<i>Quercus rubra</i> L.	Fagaceae	-35.45	W	D
69	<i>Rhaphiolepis indica</i> (L.) LINDL.	Rosaceae	-21.29	W	E
70	<i>Rhododendron catawbiense</i> MICHX.	Ericaceae	-36.86	W	E
71	<i>Rhododendron ponticum</i> L.	Ericaceae	-21.81	W	E
72	<i>Salix x rubens</i> SCHRANK	Salicaceae	-26.01	W	D
73	<i>Scabiosa columbaria</i> L.	Dipsacaceae	-17.31	H	D
74	<i>Scabiosa ochroleuca</i> L.	Dipsacaceae	-20.33	H	D
75	<i>Silene otites</i> (L.) WIBEL	Caryophyllaceae	-11.09	H	D
76	<i>Sorbus aria</i> (L.) CRANTZ S. STR.	Rosaceae	-37.63	W	D
77	<i>Sorbus domestica</i> L.	Rosaceae	-36.14	W	D
78	<i>Sorbus torminalis</i> (L.) CRANTZ	Rosaceae	-31.76	W	D
79	<i>Styrax dasyanthus</i> PERKINS	Styracaceae	-10.47	W	E
80	<i>Syringa vulgaris</i> L.	Oleaceae	-25.45	W	D
81	<i>Syzygium buxifolium</i> HOOKER & ARNOTT	Myrtaceae	-33.91	W	E
82	<i>Taxus baccata</i> L.	Taxaceae	-20.50	W	E
83	<i>Tilia cordata</i> MILL.	Tiliaceae	-40.45	W	D
84	<i>Tilia platyphyllos</i> SCOP.	Tiliaceae	-48.41	W	D
85	<i>Ulmus laevis</i> PALL.	Ulmaceae	-27.70	W	D

Frost experiment

Frost resistance was investigated on leaves, needles or buds in winter (see Appendix 2.1) and investigated in a climate test chamber (SANYO Atmos Chamber MTH-4400) following the procedure described in Hofmann et al. (2013). In short, the freshly harvested plant material was stored at +4 °C (control level) and then successively exposed to 10 temperature levels, decreasing from -3 °C up to -196 °C (see Appendix 2.1). The plants were exposed to the control level and to every temperature level for 45 minutes, with cooling rates between levels of 0.2 to 2.6 °C/min. For every species and level, eight replicates were used, resulting in 88 sample units per species. At the end of each level, the eight replicates per species were removed from the climate test chamber and allowed to thaw at +4 °C in a freezer, with however, unknown warming rates. Frost damage was measured as electrolyte leakage according to Murray et al. (1989). Intracellular ice formation destroys the cell membrane, resulting in the effusion of osmotica, which is measurable as an increase in electric conductivity C of the surrounding solution (Ashworth & Pearce 2002). After frost exposure on the subsequent day, the plant material was transferred into test tubes with 3%-isopropanol solution. The temporal increase of electrical conductivity was measured at six points in time: first immediately after preparing the plant material to define a baseline for conductivity (C_0), followed by four measurements after 4 h, 24 h, 48 h and 72 h (C_t) after the initial measurement, and a final measurement after boiling the samples for 20 minutes, by which all cell membranes were completely destroyed, showing the maximum conductivity (C_b) of that particular sample.

Based on these measurements relative conductivity (RC) was calculated according to formula 1 (Murray et al. 1989).

$$RC = \frac{C_t - C_0}{C_b - C_0} = 1 - e^{-k \cdot t} \quad (1)$$

The rate of electrolyte leakage (k values) of every species was calculated from an exponential regression according to formula (1). In a next step, k values were regressed to the temperature levels the samples had been exposed to in a 4-parametric sigmoid regression according to formula (2):

$$k = f(T) = c + \frac{a}{1 + e^{-\left(\frac{T - LT_{50}}{b}\right)}} \quad (2)$$

where the parameter a describes the plateau of k -values reached at lowest temperatures, b is the steepness of the curve and c is the k -value of the control. The regression parameter LT_{50} describes the inflection point (Fig. 2.1) and is the temperature at which 50% of the

maximum electrolyte leakage was reached. Thus, LT_{50} is a standardized measure of frost resistance across different species and plant organs that differ in the total amount of electrolytes.

Distribution data and phylogeny

The geographical distribution characteristics were based on detailed digitized distribution maps of all investigated species in ArcMap 9.1 (ESRI). The maps were produced based on the data provided by Meusel et al. (1965), Meusel & Jäger (1992), floristic atlases and online databases (for details see Hofmann et al. 2013). Macroclimatic data of species range were extracted from the WORLDCLIM dataset (Hijmans et al. 2005), by deriving percentiles of all the 2.5 arc minutes grid cells, in which a species was present. For the following analyses we used the lower 1%-percentile of the minimum temperature of the coldest month (BIO6, in the following: MTCM) as well as the median of the mean annual temperature (BIO1, in the following: MAT). Although the WOLRDCLIM dataset does not provide absolute minima, we are confident these are highly correlated with the minimum and mean temperatures used in our study (Prentice et al. 1992).

A phylogenetic tree for the 85 species in our study was extracted from a dated, ultrametric supertree for Central European vascular plant species (Daphne 1.0, Durka & Michalski 2012, Fig. 2.5). Missing species that were not included in the supertree (*Acer saccharinum*, *Apollonias canariensis*, *Betula alleghaniensis*, *Celtis biondii*, *Choerospondias axillaris*, *Cinnamomum camphora*, *Distylium myricoides*, *Ficus henryi*, *Illicium lanceolatum*, *Juglans nigra*, *Magnolia acuminata*, *Magnolia stellata*, *Mahonia repens*, *Meliosma flexuosa*, *Pittosporum illiciodes*, *Potentilla verna*, *Rhaphiolepis indica*, *Rhododendron catawbiense*, *Scabisa ochroleuca*, *Styrax dasyanthus*, *Syzygium buxifolium*) were added manually into the phylogeny based on taxonomy.

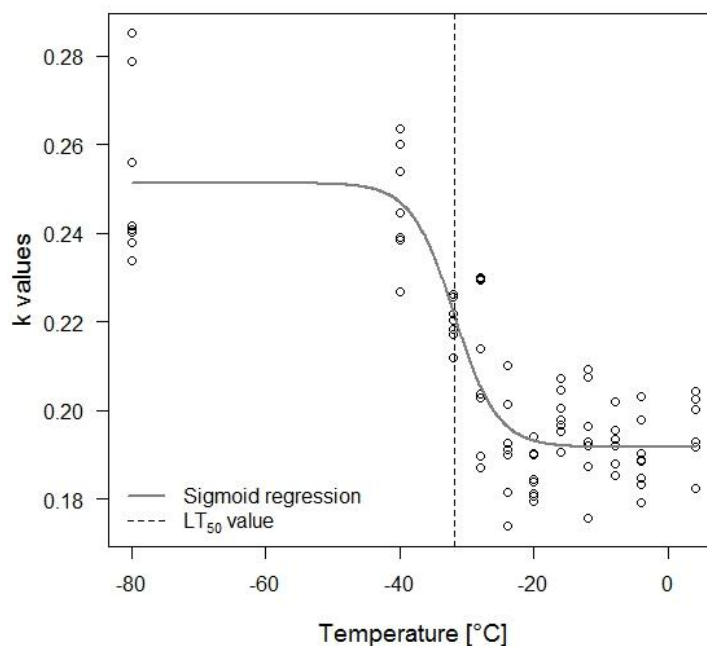


Figure 2.1 Sigmoid regression of k -values and temperature and the resulting regression parameter LT_{50} -value of *Fraxinus excelsior*.

Statistics

The relationship between LT_{50} -values and MTCM as well as between LT_{50} -values and MAT was tested with linear regression models. Differences in LT_{50} -values, MTCM and MAT between life form and leaf habit was tested with ANOVAs. We also tested for relationships between LT_{50} -values and MTCM or MAT within life form and leaf habit with linear regression models. Regression tree analysis was used to identify the variables that explained best the differences in LT_{50} -values among species.

To assess whether closely related species shared similar trait values we assessed the phylogenetic signal in LT_{50} , MTCM, MAT, life form and leaf habit, using three measures: Blomberg's K (Blomberg et al. 2003), Abouheif/Moran's I (Abouheif 1999, Pavoine et al. 2008) and Pagel's λ (Pagel 1999). Values of Blomberg's K and Pagel's λ close to zero indicate that species' traits are randomly distributed on the phylogeny, while values >1 indicate stronger phylogenetic signal than would be expected from a Brownian motion model of trait evolution, implying that closely related species are ecologically similar. Abouheif/Moran's I represents a measure of phylogenetic autocorrelation and, in contrast to K and λ , is not based on an underlying evolutionary model. P-values for Blomberg's K and Abouheif/Moran's I were obtained based on 999 randomizations, and likelihood ratio tests were used to assess significance of Pagel's λ .

The sigmoid regressions were calculated with Sigmaplot 11.0 (Systat Software 2008), while all the other statistical analyses were conducted using R 3.0.3 (R Development Core Team, 2014; packages: party (Hothorn et al. 2006), adephylo (Jombart et al. 2010) and phytools (Revell 2012)).

2.4 Results

Frost resistance and climate

Across all species, frost resistance as described by LT_{50} -values ranged from -48.4 to -7.6 °C and the 1%-percentile of the minimum temperature of the coldest month (MTCM) from -51.7 to 2.3 °C (Fig. 2.2). A total of 15 out of the 85 species was located above the 1:1 line of LT_{50} -values and the 1%-percentile of MTCM, while the majority was located below, indicating that most species were able to tolerate lower temperatures in the experiment than suggested by MTCM. The species tested also covered a wide range in mean annual temperature (MAT) from -1.6 to 18.1 °C. According to our first hypothesis, we found a significant positive correlation between LT_{50} -values and MTCM ($p = 0.005$, Tab. 2.2, Fig. 2.2) as well as between LT_{50} -values and MAT ($p < 0.001$, Tab. 2.2, Appendix 2.2). However, there was a considerable scatter in LT_{50} -values, resulting in low coefficients of determination of only 0.092 and 0.192, respectively (Tab. 2.2).

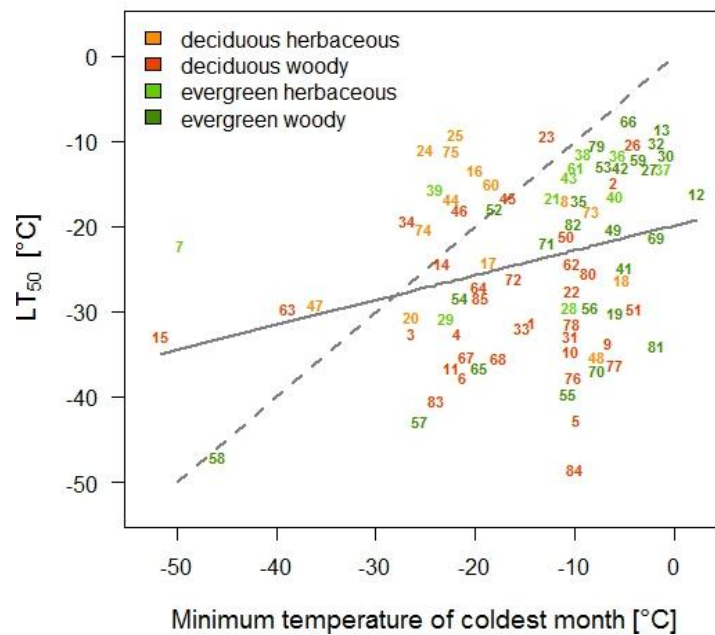


Figure 2.2 Frost resistance expressed as LT_{50} -values as a function of the 1%-percentile of the minimum temperature of the coldest month (MTCM). The solid line shows the regression line ($p = 0.005$, see Tab. 2.2), the broken line is the 1:1 relationship. Species are coded by numbers (see Tab. 2.1).

Table 2.2 Coefficients of determination (R^2) and p-values of the linear regression models relating LT_{50} -values to the 1%-percentile of the minimum temperature of the coldest month (MTCM) or to mean annual temperature (MAT) across all 85 investigated species, as well as for different subsets (n = number of the subset).

	n	MTCM		MAT	
		R^2	p-value	R^2	p-value
Total dataset	85	0.092	0.005	0.192	< 0.001
without conifers	78	0.053	0.042	0.132	0.001
Herbaceous species	25	0.033	0.382	0.099	0.126
evergreen species	14	0.199	0.169	0.299	0.082
deciduous species	11	0.012	0.713	0.005	0.815
Woody species	60	0.201	< 0.001	0.421	< 0.001
broad-leaved species	53	0.137	0.006	0.357	< 0.001
evergreen species	26	0.440	< 0.001	0.526	< 0.001
broad-leaved species	19	0.029	0.489	0.109	0.167
conifers	7	0.494	0.078	0.509	0.072
deciduous species	34	0.010	0.578	0.189	0.010
Evergreen species	37	0.256	0.001	0.327	< 0.001
without conifers	30	0.054	0.216	0.080	0.13
Deciduous species	48	< 0.001	0.922	0.029	0.246

The regression tree analysis only revealed two significant predictors for LT_{50} -values, MAT and life form (LF) (Fig. 2.3). Across all species, the most frost sensitive species were characterized by a $MAT > 12.53$ °C, which showed an arithmetic average in LT_{50} -value of -15 °C. Species from colder climates ($MAT \leq 12.53$ °C) were more frost tolerant when they were trees rather than herbs, with mean LT_{50} -value, -30.7 and -19.1 °C, respectively. Further splits in the regression tree analysis were produced both in the branches of herbs and trees at MAT of 5.5 °C and within the tree branch for evergreen and deciduous leaf habit, but they all were not significant.

According to our second hypothesis, we divided the total data set into four subsets (Tab. 2.2). Evergreen herbaceous species were the most frost sensitive subset (mean $LT_{50} = -17.7$ °C), closely followed by deciduous herbaceous species (mean $LT_{50} = -19.8$ °C). In contrast, deciduous woody species were the most frost resistant subset (mean $LT_{50} = -29.3$ °C). The subset of evergreen species showed a large variability in frost resistance (minimum $LT_{50} = -7.6$ °C, maximum $LT_{50} = -47.0$ °C, mean $LT_{50} = -22.3$ °C), with broad-leaved species being clearly more frost sensitive than conifers (mean $LT_{50} = -17.8$ °C and -34.9 °C, respectively).

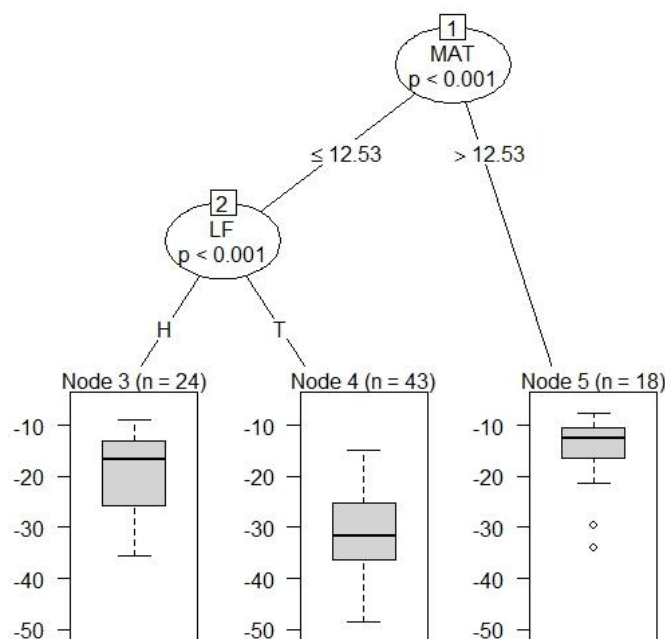


Figure 2.3 Regression tree for predicting LT_{50} -values, MTCM, MAT, life form and leaf habit. Bold values at the tree tips are mean LT_{50} -values across the equal group of species. The left site of the knot shows the group of species, which belong to this trait and the right site shows the group of species, which do not belong to this trait.

The subset of woody species as well as evergreen species showed significant relationships of LT_{50} -values to the 1%-percentile of MTCM and MAT (Tab. 2.2). Removing the conifers from the dataset strongly decreased the response of LT_{50} -values to MTCM and MAT, which was also the case within the subset of woody species (Tab. 2.2). In contrast, the significant relationship of LT_{50} -values to MTCM and MAT disappeared in the group of evergreen woody species, when only broad-leaved species were considered (Tab. 2.2, Fig. 2.4d).

Frost resistance and phylogeny

LT_{50} -value, life form and leaf habit all showed significant phylogenetic according to Abouheif/Moran's I , Pagel's λ and Blomberg's K (Tab. 2.3, Fig. 2.5). Thus, related species were more similar in these traits than expected from a Brownian motion model. The highest trait conservatism was found for life form, while LT_{50} -value and leaf habit showed signals of similar magnitude. At least part of the conservatism in LT_{50} -value and leaf habit was brought about by the conifer species, which in our data set were all evergreen and uniformly showed low LT_{50} -values. With respect to the macroclimatic range characteristics, all three statistics trait did not find significant deviations from randomness for MTCM, while a phylogenetic signal in MAT was only found by the Abouheif/Moran's I index and Pagel's λ (Tab. 2.3, Fig. 2.5).

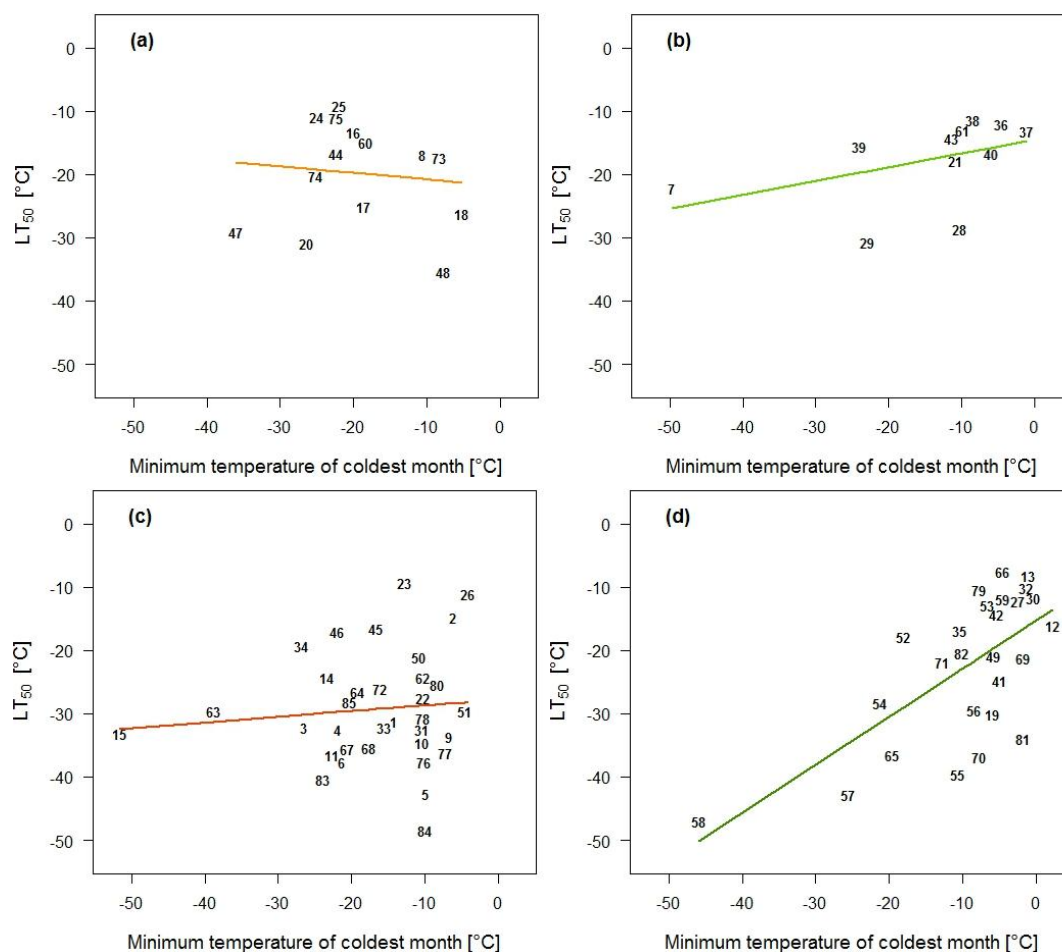


Figure 2.4 Correlation between frost resistance expressed as LT_{50} -values and minimum temperature of the coldest month (MTCM) for deciduous herbaceous species (a), evergreen herbaceous species (b), deciduous woody species (c) and evergreen woody species (d) Species are coded by numbers (see Tab. 2.1).

Table 2.3 Phylogenetic signal (Abouheif/Moran's I , Pagel's λ , Blomberg's K) in each of the five species' characteristics: species frost resistance (expressed as LT_{50} -value), minimum temperature of the coldest month (MTCM), annual mean temperature (MAT), life form (herbaceous vs. woody) and leaf habit (deciduous vs. evergreen).

	LT_{50} -value	MTCM	MAT	Life form	Leaf habit
Abouheif/Moran's I	0.306	0.058	0.262	0.666	0.377
p-value	0.001	0.2	0.001	0.001	0.001
Pagel's λ	0.636	0	0.648	1.005	0.704
p-value	0.001	1	0.003	< 0.001	< 0.001
Blomberg's K	0.181	0.039	0.11	1.414	0.2
p-value	0.004	0.984	0.185	< 0.001	0.002

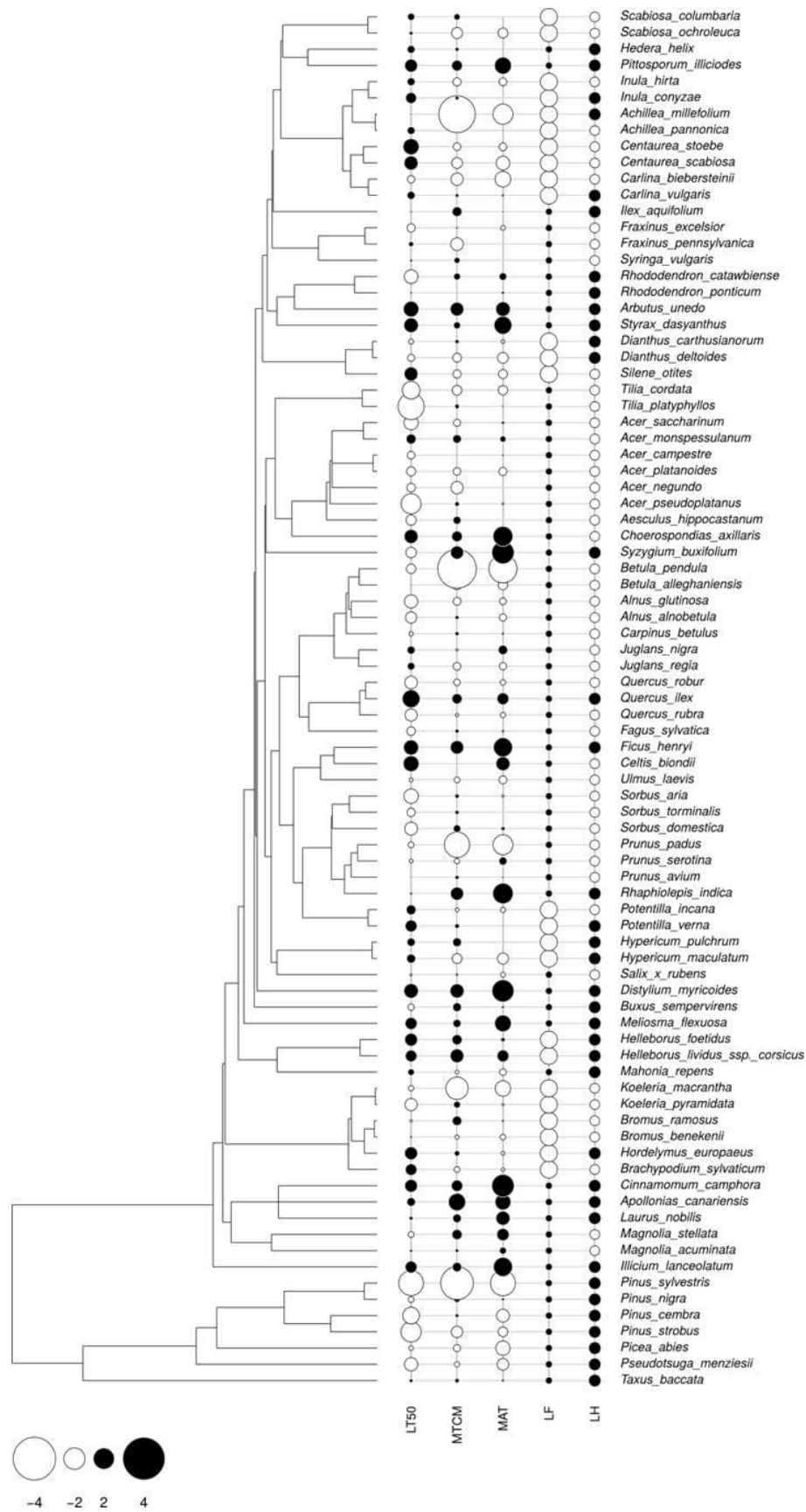


Figure 2.1 Distribution of species' frost resistance (expressed as LT_{50} -value), minimum temperature of coldest month (MTCM), annual mean temperature (MAT), life form (LF, herbaceous vs. woody) and leaf habit (LH, deciduous vs. evergreen) across the phylogeny of the 85 species. Trait values are symbolized by circles: large circle = large value, small circle = low value, white circle = negative value, black circle = positive value.

2.5 Discussion

Frost resistance and climate

In contrast to expectations, the relationships between physiological frost resistance as component of the fundamental niche and macroclimatic niche characteristics describing the realized niche, were rather weak. Thus, we found only limited support for the first hypothesis that frost resistance is related to minimum temperature of the coldest month in the species' distribution range. The relationships found were mainly driven by evergreen woody species, and disappeared when needle-leaved conifers were excluded.

The regression tree analysis indicated that life form and leaf habit seems to have a greater impact on frost resistance than macroclimatic variables. Thus, the regression tree as well as the analysis of the different subset provided evidence to support the second hypotheses that the relationship between frost resistance and distribution range differs between the different types of life form and leaf habit. In particular, herbaceous species did not show any relationship between LT_{50} -values and MTCM or MAT, which might be explained by the fact that some herbaceous species might tolerate foliage loss by freezing. Similar to tolerance to grazing or other types of disturbance they might resprout from buds near to the soil surface or belowground organs. It might also be that herbaceous plants, at the time when lowest temperatures occur, are covered by snow. Snow results in effective insulation, and prevents temperatures to fall below freezing levels (Sakai & Larcher 1987, Bruelheide & Heinemeyer 2002, Rixen et al. 2010). In consequence, there would be no or less selection pressure on frost resistance in herbaceous species as compared to tree species, which have buds, leaves or needles that are exposed to air temperature. If frost was not an important selective force, it would probably also not be a range-limiting factor in herbaceous species. Then, reasons other than frost have to be invoked for range boundaries of herbaceous species. However, support for this interpretation is equivocal at best. For example, when comparing eight pairs of congeneric species that differed in their macroclimatic niche, Hofmann et al. (2013) did not find any relationship to physiological drought tolerance.

Similar to the herbaceous species, frost resistance within the groups of all deciduous species or of all deciduous tree species was also unrelated to macroclimatic range characteristics. This lack of relationship points to a general ability to withstand frost, irrespective of the absolute value of frost temperatures. Although there was considerable variation in bud frost hardiness of deciduous tree species, this variation seems not to be relevant for the species' geographic range boundaries. Again, other factors than freezing temperatures in winter might then be important for range limits. For example, Kollas et al. (2014) suggested that late spring frost

events are a stronger constraint for deciduous tree species than minimum temperatures in winter. In contrast to deciduous species, frost resistance was correlated with macroclimatic variables in the subset of evergreen species. However, this relationship resulted from the fact that the broad-leaved evergreens were clearly more frost sensitive than the investigated conifers, which showed the highest frost resistance in this study.

In summary, the observed mismatch between fundamental and realized niches for the whole dataset of 85 species was mainly caused by strong differences among life forms and leaf habits and by absent relationships within groups of species with the same life form and leaf habit.

Assuming that the LT_{50} -values determined by us and the 1%-percentiles of MTCM are suitable predictors for the species' fundamental and realized niches, respectively, most species would not fill the range of minimum temperatures they could physiologically tolerate. This would point to incomplete range filling (Anderson and Raza 2010), which was also supported by the study of Humphreys & Linder (2013) on nine species of Danthonioideae. The authors found that the realized temperature niches as predicted from their native distribution ranges underestimated the ability to survive winter temperatures in a common garden trial in seven out of the nine species tested. Thus, most species would be physiologically able to grow in colder regions beyond their current range boundaries. Incomplete range filling would also imply that range boundaries are not in equilibrium with current climatic conditions.

We also have to consider that methodological inadequacies are responsible for the poor match between fundamental and realized niches. First, frost resistance shows intra-specific variation, e.g. between organs, tissues, phenological stage and developmental stages (e.g. Bigras et al. 2004, Augspurger 2009, Hofmann et al. 2015, Hofmann & Bruelheide 2015). Thus, the measured LT_{50} -values might not be good indicator for survival. For tree seedlings, Hofmann et al. (2014) have demonstrated a close link between LT_{50} - values and survival, but adult trees have multiple strategies to compensate for bud or leaf losses (Kreyling et al. 2012). Second, all plants were sampled in central parts of the species' distribution ranges and not at the species' range boundaries. This might have resulted in incomplete maximum acclimatization of the tested species (Nielsen & Rasmussen 2009). Frost resistance increases with decreasing temperatures during the winter season (Sutinen et al. 1992, Poirier et al. 2010), and therefore, some of the investigated species might do not have reached their maximum frost resistance under the given climatic conditions in the study region. If some species were fully acclimatized while others were not, additional random variation would have been added to the measured LT_{50} -values. However, this would also imply that we underestimated LT_{50} ,

which would result in even more incomplete niche filling than discussed above. Third, freezing temperatures might have more indirect effects rather than cell death which we assessed with electrolyte leakage. Higher temperatures than the measured LT_{50} -values might influence species performance, which can result in a higher susceptibility for subsequent frost events or other detrimental factors (Sakai & Larcher 1987, Repo et al. 2001). For example, non-lethal freezing temperatures led to a reduced growth and survival of seedlings of *Digitalis purpurea* (Bruehlheide and Heinemeyer 2002) and *Quercus robur* (Repo et al. 2008). In addition, freeze–thaw events can result in increased xylem embolism in trees (Sperry et al. 1994, Lens et al. 2013) and therefore, the number of freeze-thaw cycles might be more important than absolute minimum temperatures.

Frost resistance and phylogeny

We found support for the third hypothesis that frost resistance showed stronger phylogenetic conservatism than macroclimatic characteristics derived from the species' distribution range. Thus, niche characteristics might have evolved more readily than physiological frost resistance. This contrasts findings on strong differences in frost resistance within species, as in many plant species locally adapted ecotypes have been described (Visnjic & Dohrenbusch 2004, Alberto et al. 2013, Kreyling et al. 2014). In consequence, at the level of provenances, a close relationship between frost resistance and macroclimatic conditions at the provenances' origin has been reported (Jensen & Deans 2004, Leiblein-Wild et al. 2013). If our finding is correct that relationships between frost resistance and macroclimate do not apply to the species level any more, different evolutionary mechanisms might work on the intra- and interspecific level of differentiation. However, testing this would require extensive sampling of numerous species as well as populations across the whole distribution range of the same species.

Conclusion

In contrary to expectations, physiological frost resistance does not seem to provide a general explanation for the distribution limit of the studied species into cold regions. The mismatch between fundamental and realized niche characteristics probably results from a different evolutionary history. While physiological frost resistance was found to be more phylogenetically conserved, macroclimatic range characteristics evolved more recently. Consequently, both types of niche only match poorly. The disagreement between both types of niches will have consequences on species distribution modelling. First, if the species' distribution data underestimate the species' fundamental niches,

the species potential ranges would be much larger than anticipated (Anderson & Raza 2010) and species might not readily respond to climate change as is generally assumed (Thomas et al. 2004, Pompe et al. 2010). Second, the differences between life forms and leaf habits have demonstrated that some relationships are only valid within certain groups of species that share the same trait combinations. Thus, future species distribution models should combine realized niche data from species occurrences with either experimental studies on species fundamental niches (e.g. Kearney 2006, Humphreys & Linder 2013) or on species traits (e.g. Pompe et al. 2014).

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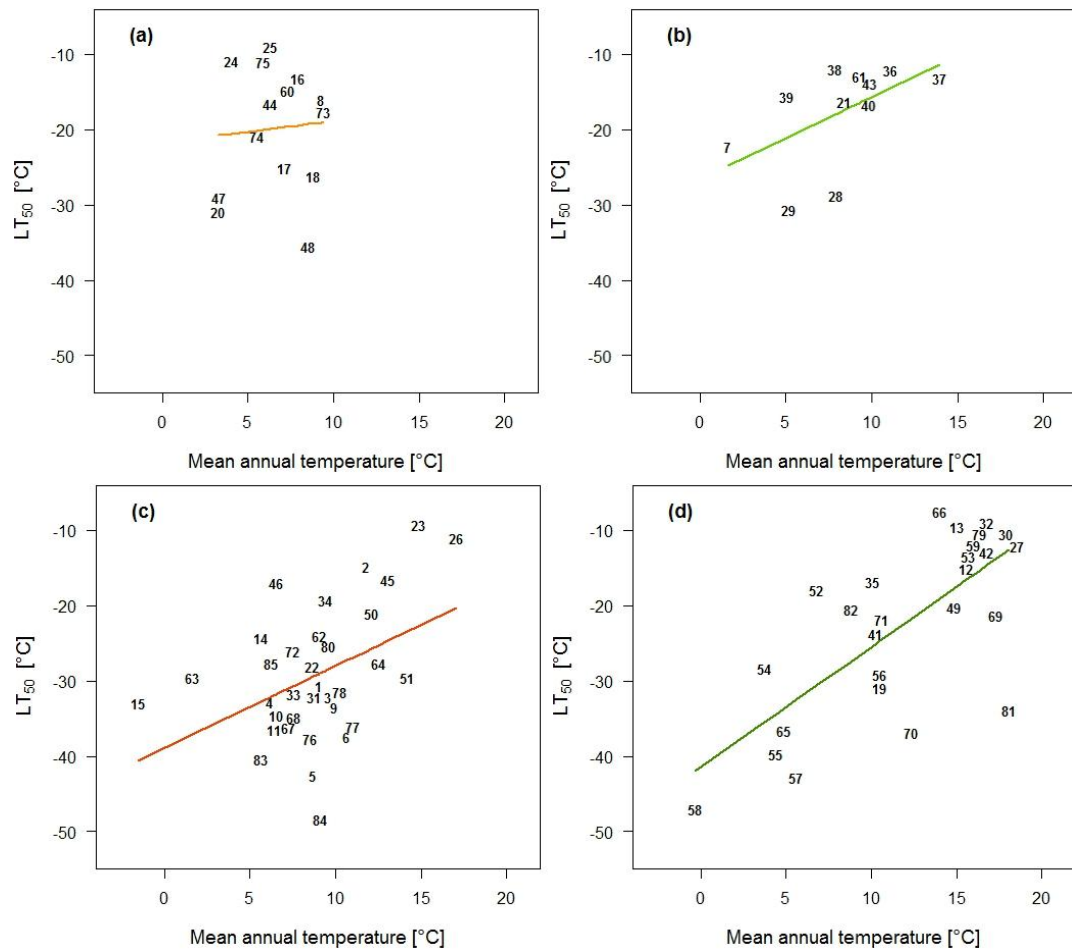
2.8 Appendix

Appendix 2.1 Sampling year, frost levels and sampled tissue of the investigated species. B = buds, L = leaves, N = needles.

Species	Year	Frost levels	Organ
<i>Acer campestre</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Acer monspessulanum</i> L.	2009	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Acer negundo</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Acer platanoides</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Acer pseudoplatanus</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Acer saccharinum</i> L.	2009	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Achillea millefolium</i> L.	2009	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	L
<i>Achillea pannonica</i> SCHEELE	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Aesculus hippocastanum</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Alnus alnobetula</i> (EHRH.) K. KOCH	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Alnus glutinosa</i> (L.) P. GAERTN.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Apollonias canariensis</i> (WILLD.) NESS	2009	-10, -18, -80	L
<i>Arbutus unedo</i> L.	2006	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Betula alleghaniensis</i> BRITT	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Betula pendula</i> ROTH	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Brachypodium sylvaticum</i> (HUDS.) P. BEAUV.	2005	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Bromus benekenii</i> (LANGE) TRIEMEN	2009	-8, -12, -16, -20, -24, -28, -32, -36, -40, -80	L
<i>Bromus ramosus</i> HUDS. S. STR.	2009	-8, -12, -16, -20, -24, -28, -32, -36, -40, -80	L
<i>Buxus sempervirens</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	L
<i>Carlina biebersteinii</i> BERNH. EX HORNEM.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Carlina vulgaris</i> L. S. STR.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Carpinus betulus</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Celtis biondii</i> PAMP.	2009	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Centaurea scabiosa</i> L. S. L.	2007	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Centaurea stoebe</i> L. S. L.	2007	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Choerospondias axillaris</i> (ROXB.) B.L.BURTT & A.W.HILL	2008	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Cinnamomum camphora</i> L.	2009	-4, -8, -12, -16, -20, -24, -28, -32, -36, -40	L
<i>Dianthus carthusianorum</i> L.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Dianthus deltooides</i> L.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Distylium myricoides</i> HEMSL.	2009	-4, -8, -12, -16, -20, -24, -28, -32, -36, -40	L
<i>Fagus sylvatica</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Ficus henryi</i> WARBURG EX DIELS	2013	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	L
<i>Fraxinus excelsior</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Fraxinus pennsylvanica</i> MARSHALL	2012	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Hedera helix</i> L.	2005	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Helleborus foetidus</i> L.	2007	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Helleborus lividus</i> subsp. <i>corsicus</i> (BRIQ.) YEO	2007	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Hordelymus europaeus</i> (L.) JESSEN EX HARZ	2005	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Hypericum maculatum</i> CRANTZ S. L.	2009	-8, -12, -16, -20, -24, -28, -32, -36, -40, -80	L
<i>Hypericum pulchrum</i> L.	2009	-8, -12, -16, -20, -24, -28, -32, -36, -40, -80	L
<i>Ilex aquifolium</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	L
<i>Illicium lanceolatum</i> A.C.SM.	2009	-4, -8, -12, -16, -20, -24, -28, -32, -36, -40	L
<i>Inula conyzae</i> (GRIESS.) MEIKLE	2009	-8, -12, -16, -20, -24, -28, -32, -36, -40, -80	L
<i>Inula hirta</i> L.	2009	-8, -12, -16, -20, -24, -28, -32, -36, -40, -80	L

Appendix 2.1 continued.

Species	Year	Frost levels	Organ
<i>Juglans nigra</i> L.	2012	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Juglans regia</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Koeleria macrantha</i> (LEDEB.) SCHULT.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Koeleria pyramidata</i> (LAM.) P. BEAUV.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Laurus nobilis</i> L.	2013	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	L
<i>Magnolia acuminata</i> L.	2012	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Magnolia stellata</i> (SIEB. & ZUCC.) MAXIM.	2012	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Mahonia repens</i> (LINDL.) G. DON	2005	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Meliosma flexuosa</i> PAMPANINI	2009	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Picea abies</i> (L.) H. KARST.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	N
<i>Pinus cembra</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	N
<i>Pinus nigra</i> J. F. ARNOLD	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	N
<i>Pinus strobes</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	N
<i>Pinus sylvestris</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	N
<i>Pittosporum illiciodes</i> MAKINO	2009	-4, -8, -12, -16, -20, -24, -28, -32, -36, -40	L
<i>Potentilla incana</i> P. GAERTN., B. MEY. & SCHERB.	2007	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Potentilla tabernaemontani</i> ASCH.	2007	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Prunus avium</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Prunus padus</i> L.	2012	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Prunus serotina</i> EHRH.	2012	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Pseudotsuga menziesii</i> (MIRB.) FRANCO	2012	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	N
<i>Quercus ilex</i> L.	2006	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Quercus robur</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Quercus rubra</i> L.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Rhaphiolepis indica</i> (L.) LINDL.	2009	-4, -8, -12, -16, -20, -24, -28, -32, -36, -40	L
<i>Rhododendron catawbiense</i> MICHX.	2002	-6, -9, -12, -15, -18, -21, -24, -27, -30, -196	L
<i>Rhododendron ponticum</i> L.	2002	-6, -9, -12, -15, -18, -21, -24, -27, -30, -196	L
<i>Salix x rubens</i> SCHRANK	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Scabiosa columbaria</i> L.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Scabiosa ochroleuca</i> L.	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Silene otites</i> (L.) WIBEL	2009	-6, -9, -12, -15, -18, -21, -24, -27, -30, -40	L
<i>Sorbus aria</i> (L.) CRANTZ S. STR.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Sorbus domestica</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Sorbus torminalis</i> (L.) CRANTZ	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Styrax dasyanthus</i> PERKINS	2009	-3, -6, -9, -12, -15, -18, -21, -24, -27, -30	L
<i>Syringa vulgaris</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Syzygium buxifolium</i> HOOKER & ARNOTT	2013	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	L
<i>Taxus baccata</i> L.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	N
<i>Tilia cordata</i> MILL.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Tilia platyphyllos</i> SCOP.	2010	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B
<i>Ulmus laevis</i> PALL.	2011	-4, -8, -12, -16, -20, -24, -28, -32, -40, -80	B



Appendix 2.2 Correlation between frost resistance expressed as LT_{50} -values and MAT for deciduous herbaceous species (a), evergreen herbaceous species (b), deciduous woody species (c) and evergreen woody species (d). Species are coded by numbers (see Tab. 2.1).

3 Intraspecific variability in frost hardiness of *Fagus sylvatica* L.

Maria Hofmann, Walter Durka, Mirco Liesebach & Helge Bruelheide

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3.1 Abstract

This study investigated the intraspecific variability of frost hardiness of *Fagus sylvatica*. We tested for local adaptation by relating the frost hardiness of different provenances to the climatic conditions at the populations' origin and searched for genetic markers that coincided with frost hardiness. Twenty provenances of *F. sylvatica* were selected covering the major part of the climatic gradient within the species' range. Frost hardiness was assessed in winter and tested in a climate test chamber by exposing buds to different freezing temperatures and estimating LT_{50} -values by the electrolyte leakage method. Additionally, the genotypes of all investigated provenances were analyzed using amplified fragment length polymorphism (AFLP) fingerprinting. The frost hardiness differed up to 10.3 K between provenances. In contrast to our expectation, we did not find any relationship between LT_{50} and climate variables. Although the populations were not well differentiated by AFLP markers, the first PCoA axis of all loci of seven different primers was strongly related to LT_{50} -values. Linear regressions showed that frost hardiness could be predicted from presence/absence of 12 loci. The high intraspecific variation in frost hardiness revealed a high potential of this species to different climates. The ability to withstand low temperatures was neither related to the species' phylogeography, nor to the current climatic conditions of provenances. This points to a more recent evolution of frost hardiness and points to a link of frost hardiness to other characteristics (e.g., drought tolerance), which might have been subjected to other selection pressures than low temperatures.

Keywords: AFLP, beech, geographic distribution, electrolyte leakage, LT_{50} -value, provenance trial

3.2 Introduction

Variation in local environmental conditions across the whole range of a species with a large geographical distribution can lead to locally adapted ecotypes (e.g., Deans & Harvey 1996, Repo et al. 2001, Jensen & Deans 2004, Visnjic & Dohrenbusch 2004). Such intraspecific adaptive variation in response to different climatic conditions can be larger than interspecific variability. For instance, the frost hardiness of different *Quercus* species in Europe showed higher intraspecific than interspecific variation (Morin et al. 2007). Provenance trials can be used to assess intra-specific variation as different provenances are subjected to the same growing conditions (e.g., Varelides et al. 2001, König 2005). As low temperatures are considered the main driver of plant species distribution worldwide (e.g., Woodward 1987), common garden trials have often been used to test for intraspecific differences in susceptibility to low temperatures (Lawes et al. 1995). Thus, many field and common garden studies have demonstrated a relationship between frost hardiness and the climatic conditions of the populations' geographic origins, with species from northern provenances or higher elevation being more frost tolerant than species from southern provenances or low elevation (e.g., Beuker et al. 1998, Jensen & Deans 2004, Aldrete et al. 2008, Kathke & Bruelheide 2011, Kreyling et al. 2012a).

Intraspecific adaptation becomes the more important, the wider a species is distributed in climate space. Thus, widespread tree species such as *Fagus sylvatica* with a range of about 20 K in annual mean temperature (MAT -2.8–18.0°C) and 1500 mm in mean annual precipitation (MAP 416–2030 mm) should display a strong intraspecific variation in frost hardiness. Common beech is excluded from regions with extreme winter frost (MAT below -35 °C according to Bolte et al. (2007)), which probably exceed the bud's frost tolerance (Huntley et al. 1989). Thus, damage to beech trees has been reported after exceptional frost events (Szafer 1932). Intraspecific variation has already been demonstrated in *F. sylvatica*, e.g., with respect to specific leaf area and growth rates (Hjelmqvist 1940, Kriebitzsch et al. 1999), development of forked trunks (Turok 1996, Hosius et al. 2003, Dounavi et al. 2010), resistance to ozone (Paludan-Müller et al. 1999) or to drought (Schraml & Rennenberg 2002, Bilela et al. 2012), but studies on frost hardiness are still rare. Using provenances only from a sub-region of the distribution range of *F. sylvatica*, Visnjic & Dohrenbusch (2004) demonstrated local adaptation to winter temperature for saplings. Similarly, Kreyling et al. (2012a) showed that 3-yr old saplings varied in their response to late spring frosts according to the climate at the populations' origin. However, a test of older trees for local adaptation to frost is lacking so far.

As all phenotypic characteristics of a plant individual, frost hardiness is both influenced by the species' genome as well as by the environment. On the one hand, frost hardiness has been demonstrated to have a clear genetic basis. For example, in *Arabidopsis thaliana*, several hundreds of genes have been shown to be affected by low temperatures and different origins differ in expression of these genes (Fowler & Thomashow 2002, Hannah et al. 2006). Similar patterns are to be expected for temperate deciduous trees, where, in addition, the genetic responses might even differ between different organs. For example, inducing frost hardiness in overwintering buds will involve many different genes, affecting membrane stability, accumulation of carbohydrates, and the tolerance to tissue dehydration (Beck et al. 2007). For *Pseudotsuga menziesii*, high genetic correlations across different tissues have been described (Aitken & Adams 1997). On the other hand, frost hardiness is affected by acclimatization, where low and high temperatures induce hardening and dehardening (e.g., Beck et al. 2004). Thus, the detection of genetic differences requires strongly standardized timing of sampling for frost hardiness, when samples are taken from the same common garden. However, the change of hardening and dehardening was also found to depend on genotype. For example, Charrier et al. (2011) found that frost acclimation changes differed significantly between different cultivars of walnuts. Furthermore, hardening patterns and absolute frost hardiness were found to be related (Aitken & Adams 1997, Kathke & Bruelheide 2011). Finally, frost hardiness might also be coupled to other characteristics of a plant, as timber-oriented walnut genotypes were found to be significantly more frost-resistant than fruit-oriented genotypes (Charrier et al. 2011). This makes it difficult to focus on single candidate genes and justifies the use of neutral markers to test for genetic differentiation. Nevertheless, neutral markers such as amplified fragment length polymorphism (AFLP) have also been used to identify putatively adaptive loci. For example, Jump et al. (2006) encountered clear changes in allele frequencies of *F. sylvatica* in one particular AFLP locus along an altitudinal gradient in Catalonia.

In this study, we investigated 20 provenances of *F. sylvatica*, covering the whole distribution range of the species, planted in 1995 in a common garden trial (von Wühlisch et al. 1998, Liesebach 2012b). We hypothesised that (1) frost hardiness differs between provenances, (2) frost hardiness corresponds to the climate of the seed origin, and thus, shows local adaptation, resulting in higher frost resistance of provenances with lower winter temperatures than provenances with higher winter temperatures, and (3) phenotypic variation in frost hardiness is reflected in molecular genetic variation, thus providing indications for the molecular genetic basis of frost hardiness.

3.3 Material and methods

Experimental design

We used 20 provenances of *F. sylvatica* from a provenance trial near to Kiel (latitude: N 54.296694°, longitude: E 10.268855°). We determined the minimum temperature in the coldest month at the geographic origin of all available provenances in the trial ($n = 141$) planted in this trial and selected those provenances that covered most of the climatic gradient of the species geographical range (Tab. 3.1, Fig. 3.1). On each of four sampling dates (January 19, 25, 31, and February 6, 2011), buds from ten individuals per provenance were sampled and pooled. From each batch, we took two replicates. We had to repeat the analysis on four dates, which were 1 week apart, because the subsequent analysis of frost damage was too time consuming to be carried out in a single run. Thus, in total, there were eight replicates per provenance (2 replicates per date x 4 dates), each of them including buds from the same ten trees per provenance. On 9–10 August 2011, we sampled leaves from the same 20 provenances and (if possible) from the same individuals used for the frost hardiness analysis to carry out molecular genetic analyses ($n = 129$ individuals). Plant material for genetic analysis was dried on silica gel.

Frost experiment

Frost hardiness was assessed on freshly harvested buds in a climate test chamber (SANYO Atmos Chamber MTH-4400) according to Hofmann et al. (2013). The buds were exposed to 11 temperature levels successively (+4, -4, -8, -12, -16, -20, -24, -28, -32, -40, -80 °C), with two replicates per provenance. At the end of each temperature level, one sample batch was removed from the climate test chamber and stored at +4 °C. On the next day after frost exposure, the buds were transferred into test tubes with 3%-isopropanol solution and tested for electrolyte leakage according to Murray et al. (1989). The electric conductivity in the solution was measured six times: first immediately after preparing the buds to define a baseline for electrical conductivity, followed by four measurements after 4, 24, 48, and 72 h after the transfer into test tubes. A final measurement was conducted after boiling the samples for 20 min, which resulted in a complete destruction of the tissue and gave the maximum electrical conductivity of the bud tissue. Based on this Relative Conductivity (RC) was calculated according to formula 1 (Murray et al. 1989).

$$RC = \frac{C_t - C_0}{C_b - C_0} = 1 - e^{-k*t} \quad (1)$$

The rate of electrolyte leakage (k values) of every replicate per provenance ($n = 4 \times 2 = 8$ per provenance in total) was calculated by a 4-parametric sigmoid regression according to formula 2.

$$k = f(T) = c + \frac{a}{1 + e^{-\left(\frac{T-LT_{50}}{b}\right)}} \quad (2)$$

The regression parameter LT_{50} describes the point of inflection of the resulting curve and is the temperature at which 50% of the maximum electrolyte leakage was reached. The eight replicates per provenance were pooled, obtaining one LT_{50} -value and standard error based on 88 RC measurements per provenance. Thus, a total of 20 LT_{50} -values was calculated, with one LT_{50} -value per provenance.

Table 3.1 Geographic origin (country, latitude, longitude and altitude), minimum temperature of the coldest month (BIO 6) and LT_{50} -value (\pm SE) of the 20 selected provenances of *F. sylvatica*.

Provenance	Country	Latitude	Longitude	Altitude	BIO 6	LT_{50}
		[decimal degrees]		[m]	[°C]	[°C]
2	Spain	N 42.784	W 2.253	950	-0.2	-20.21 \pm 1.36
4	Spain	N 41.793	E 2.462	1100	2.6	-26.78 \pm 1.47
9	France	N 48.397	W 1.167	180	1.5	-26.96 \pm 3.07
10	France	N 49.282	E 2.625	160	-0.3	-25.05 \pm 1.15
14	France	N 44.138	E 2.640	850	-1.1	-20.43 \pm 1.16
18	France	N 48.661	E 5.273	350	-2.2	-22.08 \pm 0.94
20	France	N 47.211	E 6.264	600	-2.9	-21.53 \pm 1.46
24	Denmark	N 55.289	E 10.265	20	-2.0	-23.74 \pm 2.10
46	Germany	N 52.989	E 13.120	70	-3.2	-20.51 \pm 0.80
61	Germany	N 51.547	E 9.050	305	-2.6	-23.04 \pm 1.26
94	Germany	N 48.211	E 7.910	445	-2.1	-21.25 \pm 0.93
107	Italy	N 44.143	E 10.674	1300	-3.1	-21.43 \pm 1.26
112	Czech Rep.	N 49.090	E 14.443	520	-5.5	-19.27 \pm 1.05
114	Poland	N 49.414	E 20.994	850	-8.9	-21.49 \pm 1.21
117	Poland	N 50.360	E 16.853	440	-6.2	-21.68 \pm 0.89
125	Slovakia	N 49.092	E 18.291	430	-6.6	-19.22 \pm 1.31
139	Croatia	N 45.348	E 14.295	400	2.1	-23.71 \pm 1.23
143	Ukraine	N 44.514	E 21.964	400	-4.1	-26.26 \pm 1.05
144	Ukraine	N 48.059	E 24.206	500	-8.6	-29.56 \pm 1.59
150	Romania	N 46.603	E 24.997	900	-9.6	-21.47 \pm 1.07

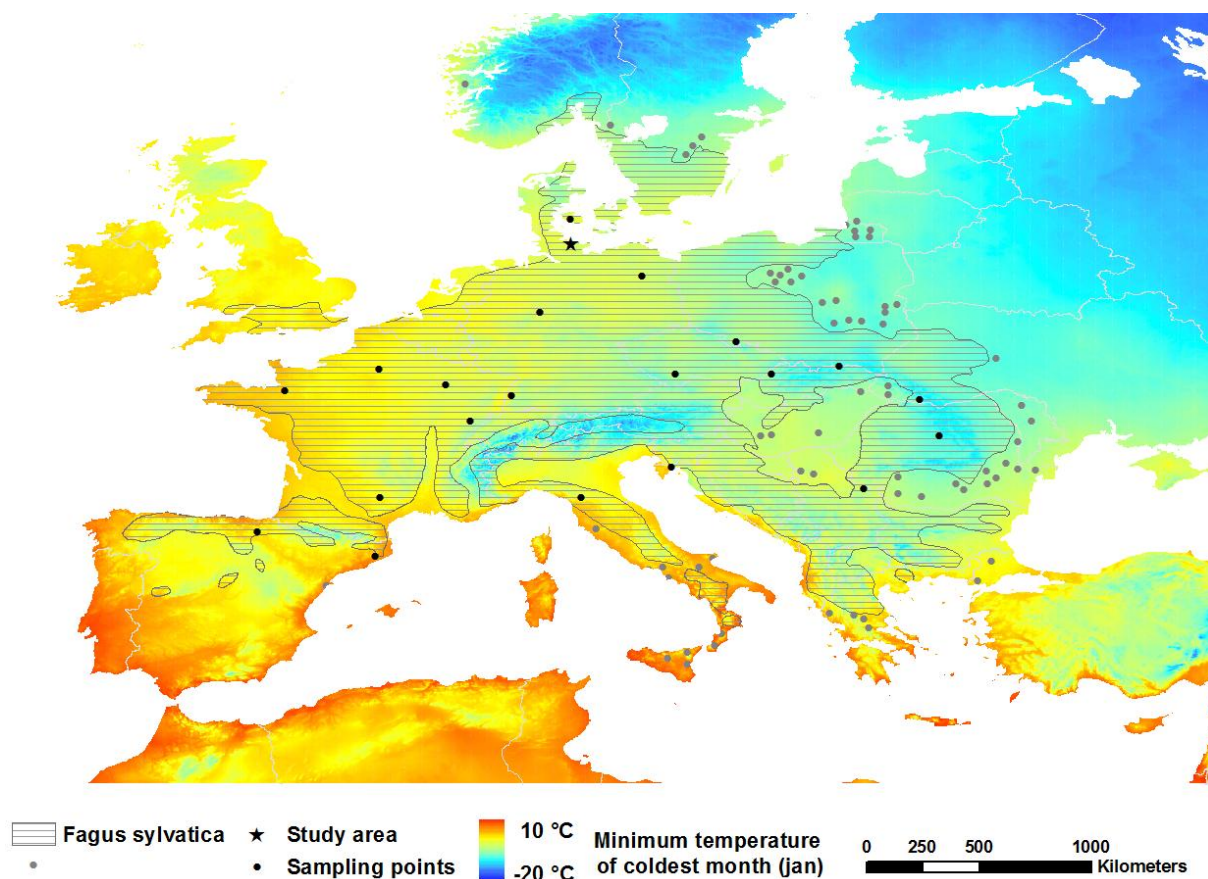


Figure 3.1 Distribution map of *F. sylvatica*. Gray distribution range, black dots selected provenances, black asterisk study site.

Genetic analyses

We extracted DNA from leaves according to the ATMAB (Alkyltrimethylammonium bromide) protocol Dumolin et al. (1995). DNA concentration was measured with the NanoDrop 1000 spectrometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). We conducted the AFLP method following Kloss et al. (2011, see Appendix) using seven primer combinations (Mse1-CTC/EcoR1-ACT [FAM], Mse1-CAG/EcoR1-AAG [NED], Mse1-CTC/EcoR1-AAG [NED], Mse1-CTC/EcoR1-AGC [PET], Mse1-CAC/EcoR1-AGC [PET], Mse1-CAC/EcoR1-ACA [VIC], Mse1-CAG/EcoR1-ACA [VIC]). GeneMapper (Version 3.7, Applied Biosystems) was used for manual genotyping which resulted in 278 polymorphic dominant loci which were used as a 0/1 matrix indicating peak absence/presence of a peak. Genetic relationships among individuals were visualised by Principal Coordinate Analysis (PCoA) based on 129 individuals and 278 loci. In GenAlEx (version 6.2, Peakall & Smouse 2006) genetic distance (based on Euclidian distances) between the provenances were calculated based on 999 permutations. For regression analyses, band frequencies were calculated for each locus and provenance.

Statistical analysis

Frost hardiness of 20 provenances as described by LT_{50} was related to the respective climatic conditions drawn from the Worldclim dataset (Hijmans et al. 2005). We extracted the following BIOCLIM variables: annual mean temperature (BIO 1), maximum temperature of the warmest month (BIO 5), minimum temperature of the coldest month (BIO 6), temperature annual range (BIO 7), annual precipitation (BIO 12), precipitation of the coldest quarter (BIO 19), minimum, maximum, and mean temperature per month from September to February, number of months with minimum, maximum, and mean temperature below 0 °C and below +4 °C. We used three approaches to predict LT_{50} -values because we had more predictor variables than provenances. Regression tree analysis and multiple linear regression with a stepwise forward selection based on AIC were used to predict LT_{50} -values from 31 climatic variables. We run these models both without and with including the reciprocal standard errors of LT_{50} -values as weights in the regressions. Additionally, we used multiple linear regression with a stepwise forward selection based on Bayesian Information Criterion (BIC) to predict LT_{50} -values from band frequencies at all 278 AFLP loci. Mantel tests were used to test for correlations between genetic distances, geographic distances, and differences between LT_{50} -values of pairs of provenances based on 999 permutations. In these analyses, genetic distance was based both on all loci and exclusively on those loci that were significantly related to frost hardiness.

The sigmoid regressions were calculated with Sigmaplot 11.0 (Systat Software 2008), whereas all the other statistical analyses were conducted using R 3.0.3 (R Development Core Team, 2014).

3.4 Results

Frost hardiness between provenances

The mean LT_{50} -value at which 50% of maximum rate of electrolyte leakage was reached was -22.8 °C across all provenances of *F. sylvatica* investigated. Frost hardiness differed by about 10.34 K among the provenances ranging from -19.22 to -29.56 °C (see Tab. 3.1). The most frost-sensitive individuals originated from Slovakian (provenance 125, LT_{50} -value = -19.22 °C) and Czech populations (provenance 112, LT_{50} -value = -19.27 °C). The most frost-resistant individuals belonged to an Ukrainian population (provenance 144, LT_{50} -value = -29.56 °C). As the standard errors in LT_{50} -values were much smaller than the differences in LT_{50} -values in most pairs of provenances, many (but not all) provenances differed significantly from each other. LT_{50} -values did not reflect the mean temperatures at the populations' origins. Individuals from Romania and Poland, the provenances with the lowest minimum temperature in the coldest month (provenance 150, BIO 6 = -9.6 °C and provenance 114, BIO 6 = -8.9 °C, respectively), showed relative high LT_{50} -values (-21.47 and -21.49 °C, respectively, see Fig. 3.2). In contrast, individuals from Spain, the provenance with the highest minimum temperature in the coldest month (provenance 4, BIO 6 = 2.6 °C), showed a relative low LT_{50} -values (-26.78 °C, see Fig. 3.2). Overall, there was a slight tendency of increasing LT_{50} -values, thus decreasing frost hardiness, with decreasing winter minimum temperatures.

The best linear model was: $LT_{50} \sim$ number of months with minimum temperature below $+4$ °C with AIC= 40.55 and Δ AIC= 1.97 to the intercept-only model. As for the relationship between LT_{50} -values and winter minimum temperatures, the relationship contrasts the expectations, as LT_{50} -values increased with increasing number of months with minimum temperature below $+4$ °C (see Fig. 3.3). Including reciprocal standard errors as weights in the analysis improved the predictions but resulted in the same model ($p = 0.062$, $R^2 = 0.180$, without weights as compared to $p = 0.059$, $R^2 = 0.184$ including the reciprocal standard errors as weights). Including weights gave generally slightly better predictions but essentially the same models; thus, in the following we present only regressions without including weights.

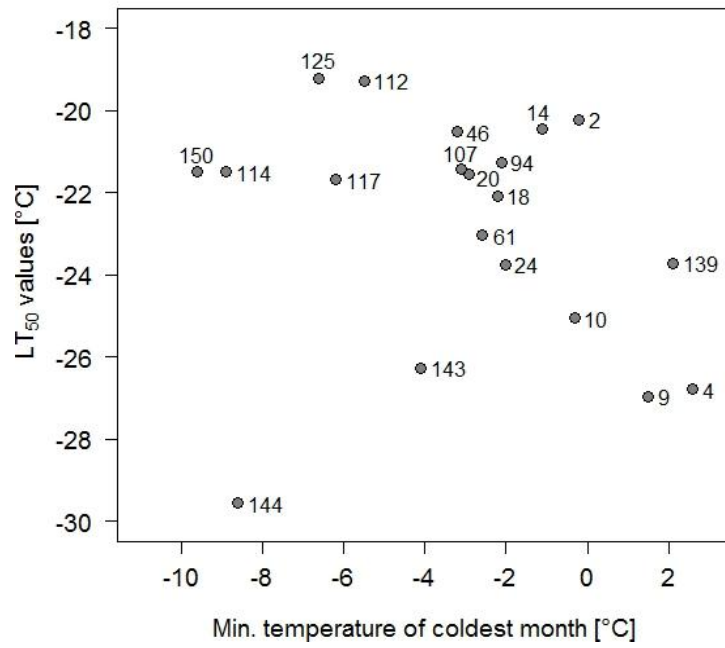


Figure 3.2 Frost hardiness of all 20 beech provenances expressed as LT_{50} -values as a function of minimum temperature of the coldest month, $p = 0.379$, $R^2 = 0.043$. Sample numbers refer to provenances (see Tab. 3.1).

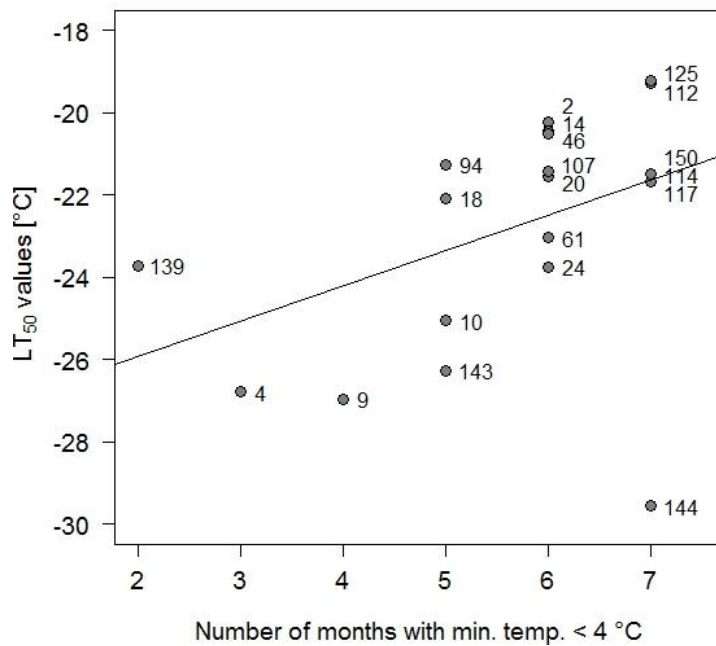


Figure 3.3 Frost hardiness of all 20 beech provenances expressed as LT_{50} -values as a function of the number of months with a minimum temperature below +4 °C, $p = 0.062$, $R^2 = 0.180$. Sample numbers refer to provenances (see Tab. 3.1).

Frost hardiness and climate

To determine whether relationships between provenances and LT_{50} -values might only apply to subgroups, we subjected the whole data set to a regression tree analysis to determine environmental key variables for differences LT_{50} -values between different groups of populations. The regression tree showed a first split into provenances with a minimum temperature in October at $+6.45\text{ }^{\circ}\text{C}$ (Fig. 3.4). Interestingly, the group of provenances with a high October minimum temperature ($>6.45\text{ }^{\circ}\text{C}$) showed the lowest LT_{50} -values (on average $-25.25\text{ }^{\circ}\text{C}$). The group of provenances with a minimum temperature in October below $+6.45\text{ }^{\circ}\text{C}$ showed a second split into provenances with a temperature annual range (BIO 7) below and above $+29.15\text{ }^{\circ}\text{C}$. The group of provenances with a high annual temperature annual ($>29.15\text{ }^{\circ}\text{C}$) exhibited lower LT_{50} -values ($-23.60\text{ }^{\circ}\text{C}$) as compared to a more even temperature distribution. The group of provenances with a temperature annual range below $+29.15\text{ }^{\circ}\text{C}$ showed a third split according to the maximum temperature of the warmest month (BIO 5). No climate variable that minimized the within-group variation in the regression tree analysis was related to winter minimum temperatures.

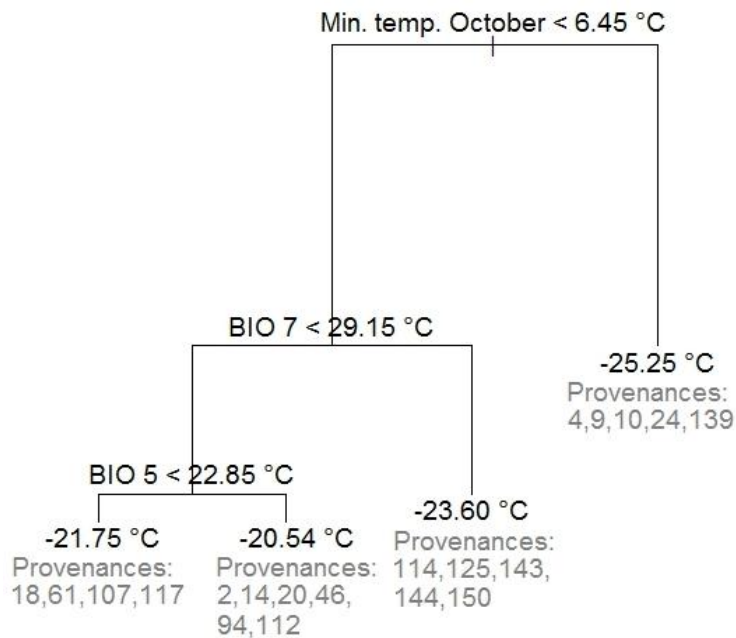


Figure 3.4 Regression tree for predicting LT_{50} -values, min. temp. October: minimum temperature in October, BIO 7: temperature annual range, BIO 5: maximum temperature of the warmest month. *Black values* at the *tree tips* are mean LT_{50} -values across the populations indicated.

Genetic variation

The PCoA of AFLP genotypes showed no clustering of provenances (Fig. 3.5). The AMOVA revealed that 5 and 95 % of molecular variance was encountered among and within populations, respectively. However, there was a marginally significant correlation between LT_{50} -values of provenances and the scores of the first PCoA axis. Without provenance 4 (Spain) this correlation was significant ($p = 0.048$). We tested which loci were responsible for these encountered patterns by stepwise forward regression. The best linear model between LT_{50} -values involved 12 loci with AIC= 211.92 (see Appendix 3.2). Genetic distance and differences in LT_{50} -values of pairs of provenances were not correlated according to a Mantel test ($r = -0.0083$, $p = 0.52$). In contrast, geographic distances were significantly and positively related to genetic distances ($r = 0.3668$, $p = 0.001$), while differences in LT_{50} -values were not ($r = 0.1196$, $p = 0.118$). When genetic distances were based only on the 12 loci that had a significant relationship to frost hardiness, there was a significant correlation to LT_{50} -values ($r = 0.4902$, $p = 0.001$), while the correlation strength between genetic and geographic distances decreased ($r = 0.2085$, $p = 0.047$).

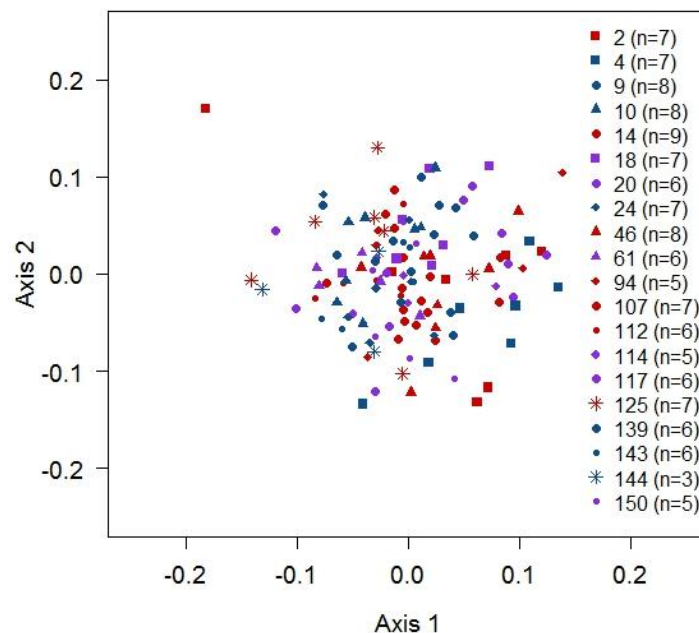


Figure 3.5 Principal Coordinate Analysis (PCoA) of all 20 beech provenances. Frost-sensitive provenances are shown with *red symbols* (LT_{50} -values from -19.22 to -21.43 °C), moderately frost-resistant provenances with *magenta symbols* (LT_{50} -values from -21.47 to -23.04 °C) and frost-resistant provenances with *blue symbols* (LT_{50} -values from -23.71 to -29.56 °C). Sample numbers refer to provenances (see Tab. 3.1).

3.5 Discussion

Frost hardiness between provenances

The variation of intraspecific frost hardiness in this study was about 10.4 K ranking from -19.2 to -29.6 °C. Thus, our first hypothesis of distinct differences in frost hardiness between provenances was confirmed. This finding is also in accordance with other study investigating varying frost hardiness between provenances of different tree species. For example, Kreyling et al. (2012b) detected a intraspecific variation of frost hardiness about 10 K between different European provenances of *Pinus nigra*, ranking from -21.2 as well as -23.2 to -32.1 °C as well as -33.1 °C in two subsequent years. Furthermore, northern provenances of *Pinus greggii* in Mexico reached on average 6 K lower LT_{50} -values in February than southern provenances (Aldrete et al. 2008). Additionally, the frost hardiness in January between lowland and montane provenances of *Picea abies* in Germany varied from -28.8 and -52.3 °C, respectively (Kathke & Bruelheide 2011).

Frost hardiness and climate

The most frost-sensitive individuals originated from the Slovakian provenance, which is one of the regions with the coldest winter temperatures in the sample set. However, the most frost-resistant individuals originated from the Ukraine provenance, which also showed the coldest winter temperatures of the investigated provenances. Conversely, the individuals from the provenance in Romania, which was the coldest origin in the sample set, exhibited only low frost hardiness and the individuals from the warmest provenance in Spain were among those with highest frost hardiness. The most plausible explanation for the lack of a significant relationship of frost hardiness to temperatures and the counterintuitive relationship between frost hardiness and the number of months with minimum temperature below +4 °C is that the populations sampled had not been subjected to an on-site frost hardiness selection regime. Such a lack of local adaptation to winter temperatures might be the result of a comparably recent and rapid migration of *F. sylvatica*. In their reconstruction of the migration history of beech, Magri et al. (2006) pointed out that some populations considerably expanded during the postglacial period, while other populations showed only moderate expansion. In consequence, the degree of adaptation might vary considerably. The rapid colonization of central and northern Europe from populations in southern France and eastern Alps-Slovenia-Istria (Magri et al. 2006) might have led to a spread of genotypes that do not show local adaptation to frost hardiness. The absence of a differentiation in frost hardiness is supported by our genetic analyses (see below), but does not account for the observed population differences in frost hardiness. Under a scenario of generally fast migration a more uniform frost hardiness of populations would be expected. Our observed differences in frost

hardiness of populations from similar climatic regions point to different origins and/or different time to adapt to the local climates. Another possible cause of our findings might be a human impact in the species' distribution pattern. Possibly, beech stands in some of the regions sampled have been founded from populations with unknown origin of the seed material. Similar mismatches have also been described for other species (Hosius et al. 2006). For example, no relationship between frost hardiness and climatic conditions at the populations' origin has also been reported for the invasive shrub *Buddleja davidii* (Ebeling et al. 2008). The authors attributed this lack of local adaptation to the species' invasion history in Europe. Most *B. davidii* populations in Europe might originate only from a single region of the native distribution range, and therefore, might show no adaptation to the range of minimum temperatures encountered in the invaded range. This explanation might be also possible for the target species *F. sylvatica*.

Another possible reason for the lack of local adaptation of *F. sylvatica* might be that we tested frost hardiness in the wrong season. Early and late frost events may have stronger effects on species survival, while mid-winter frost hardiness may not be under strong selection pressure. All investigated individuals showed considerably lower LT_{50} -values than minimum temperatures in the coldest month, indicating that all provenances were adapted to the prevalent winter climatic conditions accordingly. The sensitivity of *F. sylvatica* to late frost events after leaf flushing has been pointed out before (Dittmar et al. 2006, Ningre & Colin 2007, Kreyling et al. 2012a). Similarly, Beuker et al. (1998) found clear differences in autumn frost hardiness of different provenances of *Pinus sylvestris* and *P. abies*, but not in mid-winter. For *Quercus petraea*, differences in frost hardiness between provenances were also much greater in autumn and spring than in winter (Deans & Harvey 1996). Thus, differentiation between provenances of *F. sylvatica* may occur with respect to autumn and spring frosts, which we did not test.

Finally, any phenotypic trait (including also LT_{50} -values) is not only dependent on genotype but also on the interaction of genotype and environment. It is possible that potential frost hardiness at the location of the common garden has not been expressed to the same extent that might be seen at the geographic origin. In consequence, we might have failed to measure maximum frost hardiness of some provenances. To exclude this possibility, multiple common gardens would be required, covering the temperature gradient across the geographic origins of the provenances included in the study (e.g., see Pérez et al. 2014). As several provenance trials of *F. sylvatica* have been established in Central Europe, such comparisons would be highly valuable. However, sampling twigs from different countries and bringing them to the same laboratory involves a logistic challenge that could not be mastered in this study.

Genetic differences

The lack of a clear spatial population structure across all provenances supports earlier findings on six provenances from the same experiment (Liesebach 2012a). A major finding of Liesebach (2012a) was that the Spanish samples differed from the other provenances from Romania, Austria, Germany, and Czechia. This peculiarity of one of the Spanish populations was also encountered by us. In contrast to the microsatellites used by Liesebach (2012a), AFLP provides a much higher number of markers and thus has a higher power to detect population differentiations (Jump & Peñuelas 2007). Nevertheless, only 5% of genetic variation was attributable to among population variation. The lack of a clear population structure is typical for species with large range expansion (Müller-Starck et al. 1992). In addition, the mating system of *F. sylvatica* strongly contributes to blurring any population structure. Being a wind-pollinated self-incompatible species (Fryxell 1957, Bengt & Karlsson 2000), the high gene flow through pollen can be expected to override frost selection pressure. Accordingly, our findings showed that the intraspecific differences in frost hardiness were not in accordance with their genetic distances, when based on all AFLP loci. Therefore, we have to reject our third hypothesis that the phenotypic variation in frost hardiness is reflected in molecular genetic variation. Conversely, we can also conclude that the selection pressure by mid-winter minimum temperatures is not strong enough to maintain population differentiation at such high levels of gene flow. Our results correspond to those reported by Kreyling et al. (2012a), who also detected intraspecific differences in frost hardiness but found no explicit genetic differentiation between German and Bulgarian provenances of *F. sylvatica*. However, these conclusions do not imply that frost hardiness is not genetically fixed. First, we found clear differences in frost hardiness between the different individuals analysed, and second, we encountered a tight relationship of frost hardiness to certain AFLP loci, which was also reflected in a significant Mantel correlation between genetic distances based on these specific loci and LT_{50} -values. From annotated genomes, such as *A. thaliana*, it is known that frost hardiness involves genes of more than 200 metabolites (Hannah et al. 2006). Thus, it is not surprising that we found 12 loci to be strongly related to frost hardiness. The next step would be to compare DNA sequences of specific candidate genes to identify the genes identified by our AFLP markers.

3.6 Acknowledgements

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3.8 Appendix

AFLP-protocol according to Kloss et al. (2011) with few modifications

For restriction and ligation we used 6 µl of the DNA-extract and 5 µl reaction mix (containing 0.55 µl BSA [1 mg/ml], 1.1 µl 10x T4 DNA Ligase buffer, 1.1 µl NaCl [0.5 M], 0.1 µl Eco RI [10 u/µl], 0.1 µl Mse I [10,000 u/ml], 0.05 µl T4 DNA Ligase [2,000,000 u/ml], 1 µl Mse I adapter [50 pmol/ µl] and 1 µl Eco RI adapter [5 pmol/µl]) and incubated this reaction overnight (respectively 2 h at 37 °C). Afterwards 5 µl of the products were diluted with 10 µl H₂O.

In the next step, the preselective amplification, we used 4 µl of diluted restriction / ligation product and 16 µl of the PCR I mix (containing 9.84 µl H₂O, 1 µl Eco RI preselective primer [30 ng/ µl], 1 µl Mse I preselective primer [30 ng/ µl], 2 µl 10x dNTPs [2 mM], 2 µl 10x Dream Taq buffer and 0.16 µl Dream Taq polymerase [5 u/µl]). The preselective amplification had the following PCR conditions: 20 cycles of 20 sec at 94 °C, 30 sec at 56 °C and 2 min at 72 °C, and finally 30 min at 60 °C. Afterwards 2 µl of the products were diluted with 18 µl H₂O.

In the next step, the selective amplification, we used 2 µl of diluted preselective amplification product and 6.8 µl of the PCR II mix (containing 5 µl of 2x Multiplex PCR Master Mix (QIAGEN), 1 µl forward primer and 1 µl reverse primer). In the selective amplification, the PCR protocol was: 15 min at 95 °C, 10 cycles of 20 sec at 94 °C, 30 sec at 66 °C, with a temperature decrease of 1 °C in every cycle, and 2 min at 72 °C. Afterwards, 20 cycles with 20 sec at 94 °C, 30 sec at 56 °C, and 2 min at 72 °C were passed, and finally 30 min at 60 °C. Afterwards 2 µl of the products of Mse1- CAC/EcoR1-ACA [VIC] and Mse1- CAG/EcoR1-ACA [VIC] were diluted with 8 µl H₂O.

We used seven primer combinations (Mse1- CTC/EcoR1-ACT [FAM], Mse1- CAC/EcoR1-ACA [VIC], Mse1- CAG/EcoR1-ACA [VIC], Mse1- CAG/EcoR1-AAG [NED], Mse1- CTC/EcoR1-AAG [NED], Mse1- CTC/EcoR1-AGC [PET], Mse1- CAC/EcoR1-AGC [PET]). These products were analysed with a 3130 xl Genetic Analyzer (Applied Biosystems). The reaction mix for the sequencer contained 2 µl of the product of the selective amplifications, 2.55 µl GeneScan-500 LIZ size standard and 7.45 µl deionized formamid.

Appendix 3.1 Loci which entered the best linear model with AIC= 211.92.

Loci	Estimate	p-value
Mse1- CAC/EcoR1-ACA 94	3.735	0.001
Mse1- CAC/EcoR1-ACA 224	-1.794	0.003
Mse1- CAC/EcoR1-ACA 319	-5.848	0.002
Mse1- CAC/EcoR1-AGC 126	-1.752	< 0.001
Mse1- CAC/EcoR1-AGC 167	-3.181	0.002
Mse1- CAC/EcoR1-AGC 310	-3.345	< 0.001
Mse1- CAC/EcoR1-AGC 398	4.118	< 0.001
Mse1- CTC/EcoR1-AAG 140	3.498	< 0.001
Mse1- CTC/EcoR1-AAG 227	3.214	< 0.001
Mse1- CTC/EcoR1-AAG 353	2.505	< 0.001
Mse1- CTC/EcoR1-AGC 285	-3.416	< 0.001
Mse1- CTC/EcoR1-ACT 171	1.257	< 0.001

4 Frost hardiness of tree species is independent of phenology and macroclimatic niche

Maria Hofmann & Helge Bruelheide

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4.1 Abstract

The differences in timing in bud burst between species have been interpreted as an adaptation to late frost events in spring. Thus, it has been suggested that the degree of frost susceptibility of leaves is species-specific and depends on the species' phenology and geographic distribution range. To test for relationships between frost tolerance and phenology as well as between frost tolerance and distribution range across Central European tree species, we studied the frost hardiness of closed buds before bud burst and of freshly opened buds at the time of bud burst. We hypothesized that species with early bud burst and species distributed in eastern and northern areas were more frost tolerant than species with late bud burst and species distributed in western and southern areas. Frost hardiness was estimated by exposing twigs to 11 frost temperatures between -4 °C and -80 °C and by assessing tissue damage by the electrolyte leakage method. In contrast to our hypotheses, neither frost hardiness of closed buds nor frost hardiness of freshly opened buds were related to any variable describing species' macroclimatic niche. Furthermore, frost hardiness of freshly opened buds did not differ among species. Thus, the investigated species with early bud burst take higher risks of frost damage than the species with late bud burst. These findings indicate that frost hardiness might not play the key role in limiting the geographic distribution ranges previously anticipated.

Keywords: biogeography, bud burst, LT_{50} , macroclimatic niche, spring frost

4.2 Introduction

In particular for trees, low temperatures are supposed to determine northern and eastern range boundaries in the boreal and temperate zone of the northern hemisphere (Sakai & Larcher 1987, Pither 2003, Kreyling 2010). Thus, clear relationships have been described between plant distribution ranges and macroclimatic variables such as minimum temperature (Sakai & Larcher 1987, Huntley 1990, Woodward 1997). For example, *Fagus sylvatica* is supposed to be excluded from regions with a mean temperature in January below -3 °C (Bolte et al. 2007). However, mechanistic evidence for these claims is virtually lacking (Hofmann et al. 2013).

Stress from freezing temperatures results in damages at different levels, from the cell to the whole organism (Weiser 1970, Pearce 2001), either directly by ice formation or indirectly by freeze dehydration (Pearce 2001, Beck et al. 2004). In general, the degree of frost damage depends on (i) the duration and (ii) the intensity of frost stress, as well as on (iii) the rates of cooling (and rewarming) and (iv) the locality of ice formation (Beck et al. 2004). While intracellular ice crystals cause the disintegration of cell membranes and result in plasma efflux, with inevitably lethal effects for the cell, extracellular ice formation might dehydrate cells but not necessarily result in lethal injuries (Beck et al. 2004).

In temperate regions, frost is an annually recurring stressor; thus, species develop hardening mechanisms in autumn to impede intracellular and to tolerate extracellular ice formation (Beck et al. 2007). Because of the high energetic costs imposed by frost hardiness, the hardening level is only sustained when environmentally required (Huner et al. 1998). Therefore, plants modulate their frost resistance mechanisms in relation to environmental conditions (Weiser 1970). In addition, frost tolerance varies between organs, tissues, phenological stage and age of the species (Sakai & Larcher 1987, Calmé et al. 1994, Lennartsson & Ögren 2003, Bigras et al. 2004, Taschler et al. 2004, Augspurger 2009). In the case of deciduous trees, buds are the most important hibernation tissue and bud hardening occurs gradually by accumulation of carbohydrates and progressive dehydration of the buds (Siminovitch et al. 1953, Sakai & Larcher 1987, Améglio et al. 2004, Morin et al. 2007, Callister et al. 2008).

Bud burst is a crucial phenological stage for trees (Pop et al. 2000) because increasing temperatures in early spring initiate bud dehardening (Leinonen et al. 1997, Beck et al. 2007, Hänninen et al. 2007) and freshly opened buds with young unfolded leaves are highly sensitive to frost damage (Cannell & Smith 1984, Dittmar et al. 2006, Augspurger 2009). Thus, earlier bud burst increases the risk of late frost damage on freshly opened buds and young leaves (Maxime & Hendrik 2011). As a result, trees face a trade-off between

maximizing the duration of the photosynthetically active period and minimizing the risk of frost damage (Lockhart 1983, Saxe et al. 2001, Leinonen & Hänninen 2002, Kramer et al. 2010). For example, comparing juvenile trees of different deciduous species in the US, Augspurger & Bartlett (2003) found a significant correlation between time of bud burst and carbon gain. In contrast, early dehardening in spring bears the high risk of injuries by late spring frost events (e.g. Cannell & Smith 1986, Prozherina et al. 2003). This provides an explanation why northern populations of *Quercus petraea* displayed latest bud burst and highest late spring frost tolerance (Ducousso et al. 1996). Even if the frost damage at this stage was not lethal, it would ensue a reduced assimilation and reduced growth in that year (Dittmar & Elling 1999, Schweingruber & Nogler 2003, Clark 2010).

When comparing among-species differences in responses to environmental factors, within-species variation might be taken into account, as within-species can be higher than among-species variation at a particular site (Clark 2010). With respect to frost, testing this assumption would require to take samples from different climatic zones for different species, preferably also including the range boundaries of the different species. However, simultaneous sampling from different regions would involve the risk of including plants with a different degree of acclimatization, and thus, of comparing hardened with dehardened plants (Kathke & Bruelheide 2011). An alternative would be provenance trials of different tree species grown under the same common garden conditions, which, to our knowledge, do not exist. In any case, we would not expect that intra-specific adaptation overrules inter-specific differences in frost tolerance, as geographic range limits have been successfully interpreted by the species ecophysiological properties (Sakai & Larcher 1987, Jäger 1990, Milnes et al. 1998). This view is also supported by the findings that the potential geographic range of a species can be predicted from ecophysiological functions by mechanistic models that apply to the species as a whole (Pearson & Dawson 2003, Hijmans & Graham 2006).

The present study analyse frost damage of winter buds and freshly developed leaves of different deciduous tree species from Central Europe. We hypothesized that early-budding species show a higher frost resistance of freshly developed leaves after bud burst than species with a bud burst later in spring. Thus, late-budding species would escape the risk of frost damage by late frosts. Secondly, we hypothesized that the degree of frost hardiness of winter buds as well as freshly opened buds of a species is reflected in the species' geographic range, with species reaching farther into cold regions in winter and spring displaying higher frost tolerances. Hence, differences in frost hardiness among plant species should reflect the species' physiological potential to tolerate the minimum temperatures experienced over the whole geographical range where the species occurs.

4.3 Material and methods

Study species and sampling design

We investigated frost hardiness of closed buds and freshly opened buds with developed leaves of eight Central European deciduous tree species (Tab. 4.1). The buds were taken from adult trees growing outside, thus acclimation followed outdoor conditions (Fig. 4.1). Frost hardiness was determined twice in 2010: first in March before bud burst and, depending on the species, in March or April exactly two days after bud burst (for the exact dates see Tab. 4.1). The study species all sampled in near vicinity to each other across the city of Halle (Saale), thus avoiding differences in climatic conditions that might affect hardening and dehardening. From the available trees at a site, individuals of a species were selected randomly. The measurements before bud burst represent frost hardiness close to the maximum, as the species had been exposed to frost temperatures throughout the winter and dehardening did not started yet. The measurements after bud burst represent the same phenological phase for all species, irrespective of the actual date of bud burst. The sampling directly after bud burst made sure that all species had the same degree of dehardening. In the following, we refer to the developing new leaves including the bud scales as freshly opened buds (Fig. 4.2).

Table 4.1 Locality and sampling date before and after bud burst of the eight investigated tree species.

Species	Locality (decimal degrees)		Sampling date	
	Latitude (N)	Longitude (E)	before bud burst	after bud burst
<i>Betula pendula</i> Roth	51.492903	11.935010	08.03.2010	06.04.2010
<i>Carpinus betulus</i> L.	51.489379	11.962099	02.03.2010	31.03.2010
<i>Fagus sylvatica</i> L.	51.507332	11.925347	01.03.2010	25.04.2010
<i>Fraxinus excelsior</i> L.	51.488725	11.944768	01.03.2010	28.04.2010
	51.510041	11.954827		
<i>Juglans regia</i> L.	51.489339	11.958316	02.03.2010	19.04.2010
	51.494918	11.953218		
<i>Salix x rubens</i> Schrank	51.493494	11.955545	16.03.2010	28.03.2010
	51.493848	11.948703		
<i>Tilia cordata</i> Mill.	51.493047	11.939792	09.03.2010	15.04.2010
<i>Ulmus laevis</i> Pall.	51.502245	11.947365	16.03.2010	11.04.2010

Note that *Fraxinus excelsior* and *Salix x rubens* have been collected at different, but proximate locations.

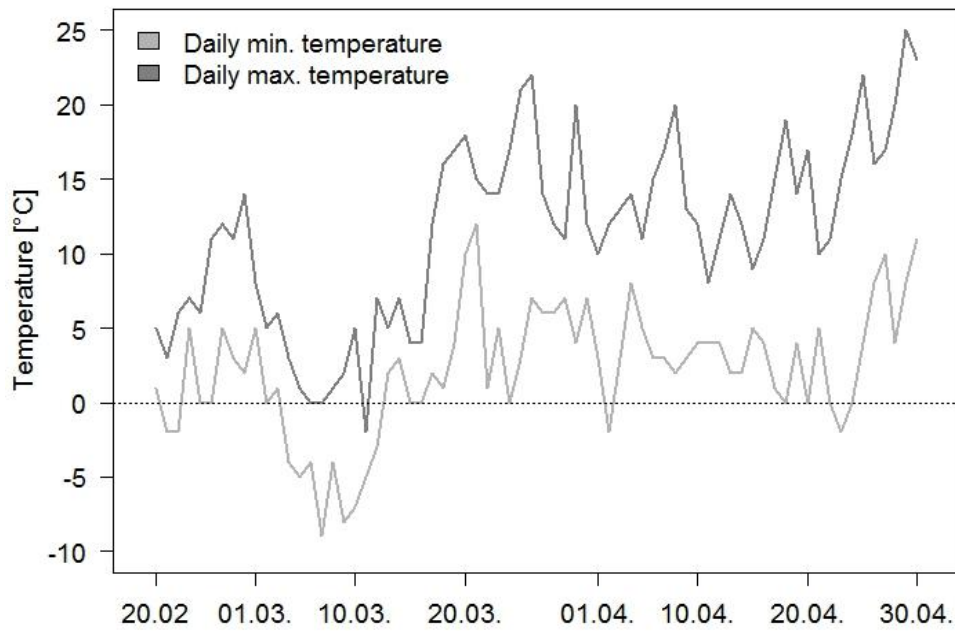


Figure 4.1 Climatic conditions during the study period in winter and spring 2010 of the sample according to www.wetter-online.de (weather station: Halle (Saale)).



Figure 4.2 State of bud development defined as freshly opened buds of *Betula pendula* (A), *Carpinus betulus* (B), *Fagus sylvatica* (C), *Fraxinus excelsior* (D), *Juglans regia* (E), *Salix x rubens* (F), *Tilia cordata* (G) and *Ulmus laevis* (H).

Frost measurements

We measured frost hardiness in a climate test chamber (SANYO Atmos Chamber MTH-4400). Freshly harvested buds and young not yet unfolded leaves were exposed stepwise to 11 temperature levels (+4 °C, -4 °C, -8 °C, -12 °C, -16 °C, -20 °C, -24 °C, -28 °C, -32 °C, -40 °C, -80 °C) with eight replicates per level. Each temperature level took 45 min (resulting in 8.15 hours in total) and samples were freeze with a cooling rate of 0.13 °C / min. At the end of one temperature level, one sample batch with buds was removed and stored at +4°C to the next day (see Hofmann et al. 2013). On the next day after frost exposure, the buds were transferred into test tubes with isopropanol solution and tested for electrolyte leakage according to Murray et al. (1989). The electric conductivity in the solution was measured six times: first immediately after preparing the whole buds (C_0), followed by four measurements after 4 h, 24 h, 48 h and 72 h after the transfer into test tubes (C_t), and a final measurement after boiling the samples for 20 minutes (C_b), which results in a complete destruction of the tissues and maximum electric conductivity. The first measurement was needed to define the baseline for electric conductivity, the final measurement served to scale the response to the maximum potential electric conductivity of that particular plant organ. We calculated the Relative Conductivity (RC) using formula 1 (Murray et al. 1989).

$$RC_t = \frac{C_t - C_0}{C_b - C_0} = 1 - e^{-k*t} \quad (1)$$

The rate of electrolyte leakage (k values) of every species was used to calculate a 4-parametric sigmoid regression according to formula 2.

$$k = f(T) = c + \frac{a}{1 + e^{-\left(\frac{T-LT_{50}}{b}\right)}} \quad (2)$$

The regression parameter LT_{50} describes the point of inflection of the resulting curve and is the temperature at which 50% of the maximum electrolyte leakage was reached.

Statistics

Frost hardiness as described by LT_{50} was related to the species' macroclimatic niches. This was achieved by compiling the global distribution for every species. The distribution range of the study species was derived from floristic atlases and online published databases. As the distribution data of *Salix x rubens* Schrank were not reliable, we combined the range data from the two parent species *Salix fragilis* L. and *Salix alba* L.. All distribution data were georeferenced and digitized using ArcMap (ESRI) and then used for extracting climatic data for all occurrence points from the Worldclim dataset (Hijmans et al. 2005).

To determine species macroclimatic niche we extracted the lower 1 %-percentile of annual mean temperature, annual precipitation, temperature annual range, minimum temperature of the coldest month and minimum temperature per month for November until April as well as the upper 1 %-percentile of annual precipitation, temperature annual range, precipitation of the coldest quarter and precipitation per month for November until April. A total of 22 macroclimatic variables was compiled. The relationships of both LT_{50} values of closed buds and freshly opened buds to all macroclimatic variables were tested by linear regression models. With multiple testing and assuming random distribution of predictor variables, we would expect 5% of the 22 models (i.e. 1.1 models) to be significant by chance. Thus, for assuming a significant relationship between frost hardiness and macroclimatic niche, more than one significant regression of LT_{50} values to macroclimatic variables would be required. Incremental model improvement by forward selection was employed to identify the variables with the highest absolute correlation coefficients.

The sigmoid regressions were calculated with Sigmaplot 11.0 (Systat Software 2008), whereas all the other statistical analyses were conducted using R 2.12.0 (R Development Core Team 2010).

4.4 Results

All species showed a higher LT_{50} value of closed buds than of freshly opened buds. While frost hardiness of closed buds differed between -17.1 °C and -40.5 °C, the freshly opened buds displayed a lower range from -8.5 °C to -14.8 °C (Tab. 4.2). In addition, frost hardiness of closed buds was not significantly related to frost hardiness of freshly opened buds ($p = 0.943$, Fig. 4.3).

Table 4.2 Minimum temperature of the coldest month (lower 1%-percentile) as derived from the climate envelope of the study species and LT_{50} values from the 4-parametric sigmoid regression of the buds (\pm standard error) before and after bud burst.

Species	Min. temp. of coldest month	LT_{50} of closed buds	LT_{50} of freshly opened buds
<i>Betula pendula</i>	-50.8 °C	-32.3 °C \pm 2.46 °C	-13.5 °C \pm 2.15 °C
<i>Carpinus betulus</i>	-10.3 °C	-28.2 °C \pm 0.63 °C	-10.5 °C \pm 1.12 °C
<i>Fagus sylvatica</i>	-10.1 °C	-30.4 °C \pm 0.88 °C	-10.2 °C \pm 2.35 °C
<i>Fraxinus excelsior</i>	-15.4 °C	-29.7 °C \pm 1.32 °C	-8.5 °C \pm 1.17 °C
<i>Juglans regia</i>	-23.7 °C	-17.1 °C \pm 0.67 °C	-10.7 °C \pm 1.41 °C
<i>Salix x rubens</i>	-16.1 °C	-26.1 °C \pm 1.52 °C	-10.2 °C \pm 1.02 °C
<i>Tilia cordata</i>	-23.5 °C	-40.5 °C \pm 1.28 °C	-10.6 °C \pm 0.95 °C
<i>Ulmus laevis</i>	-21 °C	-27.9 °C \pm 1.60 °C	-14.8 °C \pm 1.15 °C

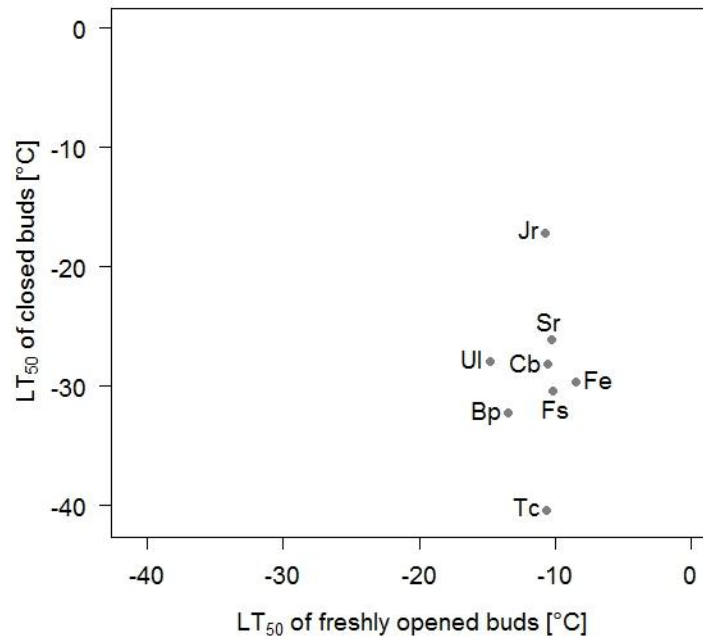


Figure 4.3 Frost hardiness of the closed buds expressed as LT_{50} values and frost hardiness of the freshly opened buds ($p = 0.943$). Bp = *Betula pendula*, Cb = *Carpinus betulus*, Fe = *Fraxinus excelsior*, Fs = *Fagus sylvatica*, Jr = *Juglans regia*, Sr = *Salix x rubens*, Tc = *Tilia cordata*, Ul = *Ulmus laevis*.

The date of bud burst was neither related to the frost hardiness of the closed buds ($p = 0.950$) nor the freshly opened buds ($p = 0.390$, Fig. 4.4). While the species with earliest bud burst showed only moderate frost resistance (*Salix x rubens*, -10.2 °C), the most frost tolerant species (*Ulmus laevis*, 14.8 °C) had an intermediate bud burst phenology.

Among all macroclimatic variables tested, there was no significant correlation to the LT_{50} -value of the closed buds (Tab. 4.3). The species with the highest and the lowest levels of frost hardiness before bud burst (-40.5 °C and -17.1 °C for *Tilia cordata* and *Juglans regia*, respectively) had distribution ranges that extended into regions with similarly extreme frosts; thus both species displayed about -24 °C as minimum temperatures of the coldest month ($p = 0.695$, Fig. 4.5). Conversely, the species with lowest and highest minimum temperature in the coldest month (-50.8 °C and -10.1 °C for *Betula pendula* and *Fagus sylvatica*, respectively; for distribution maps see Appendix) did not differ in their frost hardiness of the closed buds (about -30 °C).

There were only two significant correlations between the LT_{50} values of the freshly opened buds and the macroclimatic variables (Tab. 4.3). The LT_{50} values of the freshly opened buds were significantly correlated with precipitation of the coldest quarter and precipitation in December in the species' distribution range ($p = 0.024$, Fig. 4.6). The species with the lowest precipitation in the coldest quarter as well as lowest precipitation in December (*Ulmus laevis*) showed the highest frost hardiness, whereas the species with the highest precipitation values (*Fraxinus excelsior*) displayed the lowest frost hardiness.

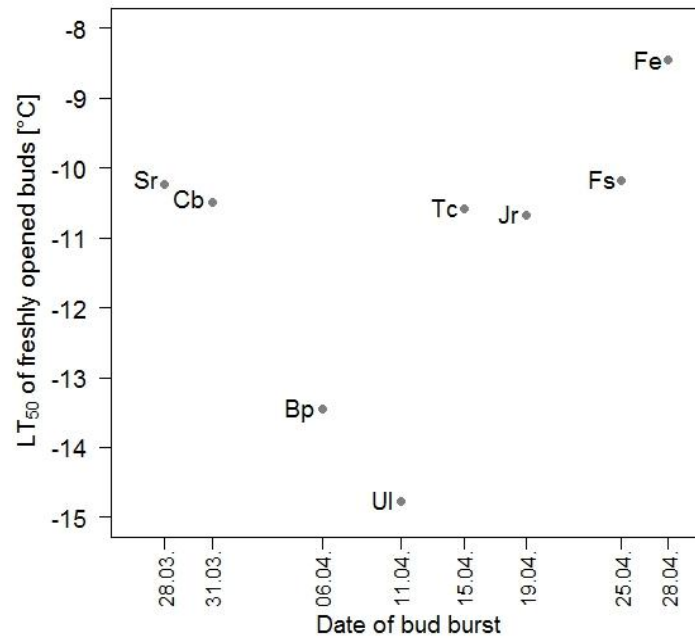


Figure 4.4 Frost hardiness of the freshly opened buds expressed as LT_{50} values as a function of the date of bud burst ($p = 0.390$). Bp = *Betula pendula*, Cb = *Carpinus betulus*, Fe = *Fraxinus excelsior*, Fs = *Fagus sylvatica*, Jr = *Juglans regia*, Sr = *Salix x rubens*, Tc = *Tilia cordata*, Ul = *Ulmus laevis*.

Table 4.3 Summary of correlation coefficients (R) and p -values (p) of Pearson's rank correlation testing for relationships of LT_{50} values as obtained from the 4-parametric sigmoid regression against macroclimatic variables (from the Worldclim dataset).

	LT_{50} value of buds			
	before bud burst		after bud burst	
	R	p	R	p
LT_{50} values of buds after bud burst [°C]	0.031	0.943	-	-
Date of bud burst	-0.027	0.950	0.354	0.390
Sampling date	-0.141	0.740	0.335	0.388
Annual mean temp.[°C]	0.063	0.883	0.471	0.239
Annual precipitation [mm] LP	-0.450	0.263	0.444	0.271
Annual precipitation [mm] UP	-0.396	0.331	0.705	0.051
Temp. annual range [°C] LP	0.581	0.130	-0.503	0.204
Temp. annual range [°C] UP	-0.210	0.618	-0.601	0.115
Min. temp. of coldest month [°C] LP	0.166	0.695	0.562	0.147
Precipitation of coldest quarter [mm]	-0.002	0.996	0.734	0.038
Min. temp. November [°C]	0.181	0.668	0.540	0.168
Min. temp. December [°C]	0.198	0.639	0.559	0.149
Min. temp. January [°C]	0.166	0.695	0.562	0.147
Min. temp. February [°C]	0.180	0.670	0.570	0.140
Min. temp. March [°C]	0.174	0.680	0.557	0.152
Min. temp. April [°C]	0.113	0.790	0.427	0.291
Precipitation November [mm]	-0.668	0.070	0.673	0.067
Precipitation December [mm]	-0.295	0.478	0.776	0.024
Precipitation January [mm]	0.032	0.941	0.698	0.054
Precipitation February [mm]	0.264	0.527	0.620	0.101
Precipitation March [mm]	0.342	0.407	0.562	0.148
Precipitation April [mm]	0.568	0.142	0.409	0.314

In all regressions, degrees of freedom = 6. LP = lower 1%-percentile, UP = upper 1%-percentile. Bold fonts indicate statistically significant differences.

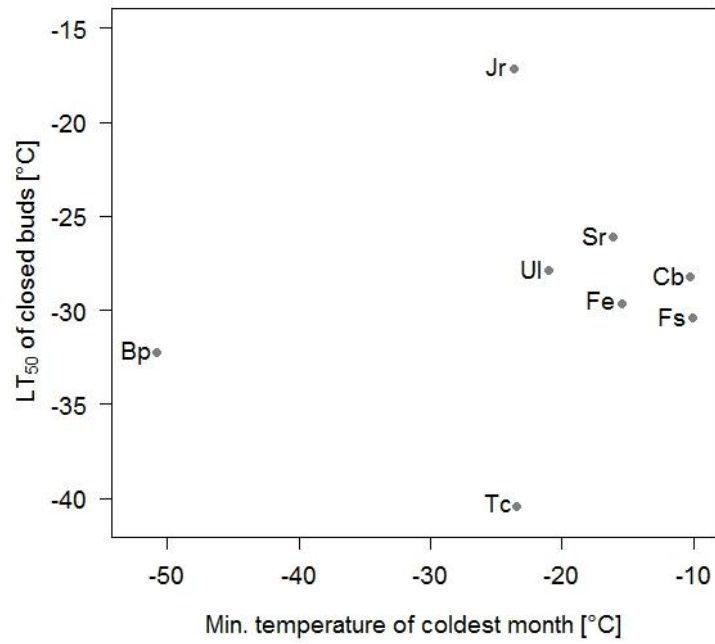


Figure 4.5 Frost hardiness of the closed buds expressed as LT_{50} values as a function of minimum temperature of the coldest month ($p = 0.695$). Bp = *Betula pendula*, Cb = *Carpinus betulus*, Fe = *Fraxinus excelsior*, Fs = *Fagus sylvatica*, Jr = *Juglans regia*, Sr = *Salix x rubens*, Tc = *Tilia cordata*, Ul = *Ulmus laevis*.

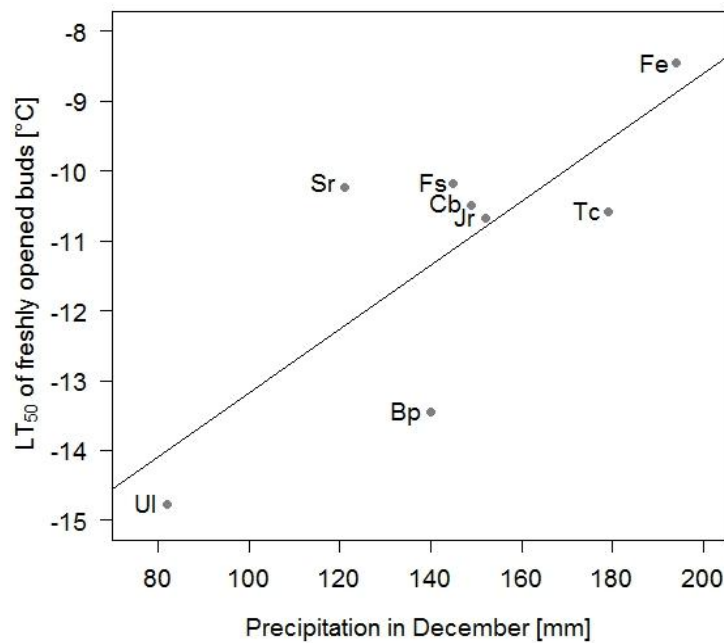


Figure 4.6 Frost hardiness of the freshly opened buds expressed as LT_{50} values as a function of the precipitation in December (upper 1%-percentile) ($p = 0.024$, $R^2 = 0.602$). Bp = *Betula pendula*, Cb = *Carpinus betulus*, Fe = *Fraxinus excelsior*, Fs = *Fagus sylvatica*, Jr = *Juglans regia*, Sr = *Salix x rubens*, Tc = *Tilia cordata*, Ul = *Ulmus laevis*.

4.5 Discussion

Frost hardiness and date of budburst

Freshly opened buds, i.e. the young, yet unfolded leaves, of all species tested were more or less frost-resistant to the same degree. In addition, frost hardiness of the different species showed no correlation with the date of bud burst. Thus, our first hypothesis has to be rejected. The absence of a relationship between frost hardiness before and after bud burst can be explained by the limited number of ways growing tissues can cope with frost stress. The main strategy is the decrease in osmotic potential, either by accumulating sugar, organic acids or compatible solutes (e.g. Améglio et al. 2004, Morin et al. 2007). Nevertheless, there are osmotic constraints to lowering freezing temperatures, obviously confining this mechanism to frosts of -8 to -14°C (Sakai & Larcher 1987). The significant relationship between precipitation in winter (precipitation of the coldest quarter as well as precipitation in December) and frost hardiness of the freshly opened buds has probably been caused by the tissue's water contents. Species usually experiencing dry winters might produce new developing shoots and leaves with lower water content, a hypothesis that has not been tested yet. Then, lower water contents of the buds would have indirectly resulted in a higher frost resistance (e.g. Améglio et al. 2004, Morin et al. 2007). Whatever the mechanism, there is probably no ecological relevance because the range of frost hardiness encountered was not large, and no relationship was encountered to the minimum temperatures to which the plants in the species range are exposed at the time of bud burst.

However, the degree of frost hardiness displayed by the different species was sufficient to withstand frost temperatures at the study site that occurred in the whole span of bud burst. Thus, the investigated species with early bud burst are not better protected but have strategy of taking higher risks of frost damage than the late budding species. Frost damage should occur more frequently in early budding species and it can be assumed that early budding species are able to compensate the loss of injured tissues. Although late frost damages have been found to reduce net primary productivity (Awaya et al. 2009) and to reduce tree-ring growth (Dittmar et al. 2006), many species are able to flush a second time. Although this involves high costs (Augspurger 2009), the second flush leaves can reach even higher rates of photosynthesis (St. Clair et al. 2009).

Frost hardiness and species' distribution

Frost hardiness of the closed buds was not related to any variable that described the species' macroclimatic niches, although at least one significant correlation would have to be expected. Similarly, frost hardiness of the freshly opened buds was only related to the precipitation in the coldest month and to that of December. However, we have to consider that these correlations were false positives, and simply the result of multiple hypothesis testing. This interpretation is supported by the fact that there were no significant relationships to winter temperatures. Thus, we have to reject our second hypothesis, because the degree of frost tolerance of a species was not reflected by the temperatures in the species' geographic range.

There are several possible explanations for this outcome. Relationships between frost hardiness and biogeography might exist but we might have focused on the wrong life stage (e.g. Cannell & Smith 1984, Sakai & Larcher 1987, Repo et al. 2001) or measuring the wrong plant organ. It is well-known that frost hardiness varies between the ages of plants (e.g. Bigras et al. 2004, Taschler et al. 2004), with seedlings exhibiting a lower frost hardiness than adults. For example, Morin et al. (2007) showed for different European *Quercus* species that frost hardiness of adults was significantly higher than that of seedlings. Furthermore, it might be that frost affects adult trees more through xylem embolism caused by freeze–thaw events. In this case, frost hardiness of adult trees would be not determined by bud frost resistance but by wood properties. This would explain the relationship tracheid lumen diameter and altitudinal distribution of *Pinus sylvestris* provenances in Spain (Martín et al. 2010). Alternatively, relationships between frost hardiness and the temperatures tolerated in the species' geographical distribution range (i.e. the species' macroclimatic niches) do not exist across different species, which would have far-reaching implications for climate niche models and future climate predictions (see below). The frost experienced at the experimental site for all species was certainly well above the minimum temperatures that the species are able to tolerate in other parts of the distribution range. Thus, the comparably mild winter period in the study area might have allowed the species to stay above their physiological limits. Differences in frost hardiness might only become apparent when plants are exposed to more extreme conditions (Nielsen & Rasmussen 2009). More extreme frost temperatures might also result in a higher degree of hardening. Support for this idea comes from the observation that frost hardiness increases in the course of the cold season (Sutinen et al. 1992, Martz et al. 2006, Morin et al. 2007, Nielsen & Rasmussen 2009, Poirier et al. 2010, Kathke & Bruelheide 2011). In deciduous tree species, an increase in frost hardiness is brought about by increasing dehydration of the buds (e.g. Sakai & Larcher 1987, Beck et al. 2007). Furthermore,

adult trees might suffer much less from frost damage compared to seedlings and juveniles (e.g. Cannell & Smith 1984, Sakai & Larcher 1987, Woodward 1987, Repo et al. 2001, Bigras et al. 2004). Frost damage to seedlings has been often described as potential cause of distribution range boundaries. For example, the northern distribution limit of *Pinus sylvestris* has been attributed to the seedlings' resistance to withstand low temperatures (Repo et al. 2001).

The alternative explanation would be that the investigated species actually do not differ in frost hardiness. In fact, although the correspondence of physiological performance and macroclimatic niche of different species is commonly assumed in biogeography (Jäger 1992), there are not many experimental studies supporting this idea. Such rare examples for a relationship of frost tolerance to the macroclimatic niche across different species are provided by different ericoid species in the British Isles (Bannister & Polwart 2001) and by eight temperate and subtropical tree species in Australia (Cunningham & Read 2006). At first sight, the absence of a relationship between macroclimate niche and frost hardiness across different species might be counter-intuitive but might be explained by a strong intra-specific differentiation. Differences in frost hardiness between different provenances has been described for many tree species (Sakai & Weiser 1973, Lawes et al. 1995, Beuker et al. 1998, Leinonen & Hänninen 2002, Kathke & Bruelheide 2011). These differences in frost hardiness among provenances have often resulted in significant relationships to the climatic conditions at the origin localities. For the example of *Fagus sylvatica*, Visnjic & Dohrenbusch (2004) found a tight relationship between winter frost hardiness and the mean annual minimum temperature at the origin sites. Similarly, Kathke & Bruelheide (2011) detected a much lower LT_{50} in montane compared to lowland morphotypes of *Picea abies*. Repo et al. (2001) were able to relate frost hardiness of *Pinus sylvestris* provenances to latitude of origin. Morin et al. (2007) as well as Jensen & Deans (2004) described differences in frost hardiness of different provenances of *Quercus* species in Europe. However, there are also studies that failed to detect differences in winter frost hardiness among provenances (Deans & Harvey 1996, Beuker et al. 1998). Irrespective of the existence of a correlation between frost hardiness and temperatures at the populations' origins, it might well be that the variation in frost hardiness of different species growing at the same locality is lower than that of different provenances of the same species.

Conclusion

In summary, we have to conclude that the frost hardiness of the study species assessed at our site both in winter and at the time of bud burst did not correspond to the species' macroclimatic niche, as derived from the species' distribution ranges. This might have important implications for the current use of climate envelopes, which are much *en vogue* for predicting the climate change impacts on species distribution ranges (e.g. Iversen & Prasad 1998, Thomas et al. 2004, Thuiller et al. 2005). In almost all models, winter minimum temperatures are important predictors as they coincide, for example, with northern and eastern distribution boundaries in Europe (Huntley et al. 1995, Pompe et al. 2008, 2010). However, if these distribution boundaries are not mechanistically linked to frost hardiness, changing winter temperatures might not result in the foreseen range shifts of species in a changing climate. Given the high intra-specific differentiation in frost hardiness observed in many species (e.g. Lawes et al. 1995, Deans & Harvey 1996, Beuker et al. 1998, Leinonen & Hänninen 2002), species might rather respond with rapid evolution rather than with migration. Such evolutionary responses have been described for phenological shifts in response to periods of drought (Franks et al. 2007), and might also apply to adaptive responses to frost.

4.6 Acknowledgements

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4.7 References

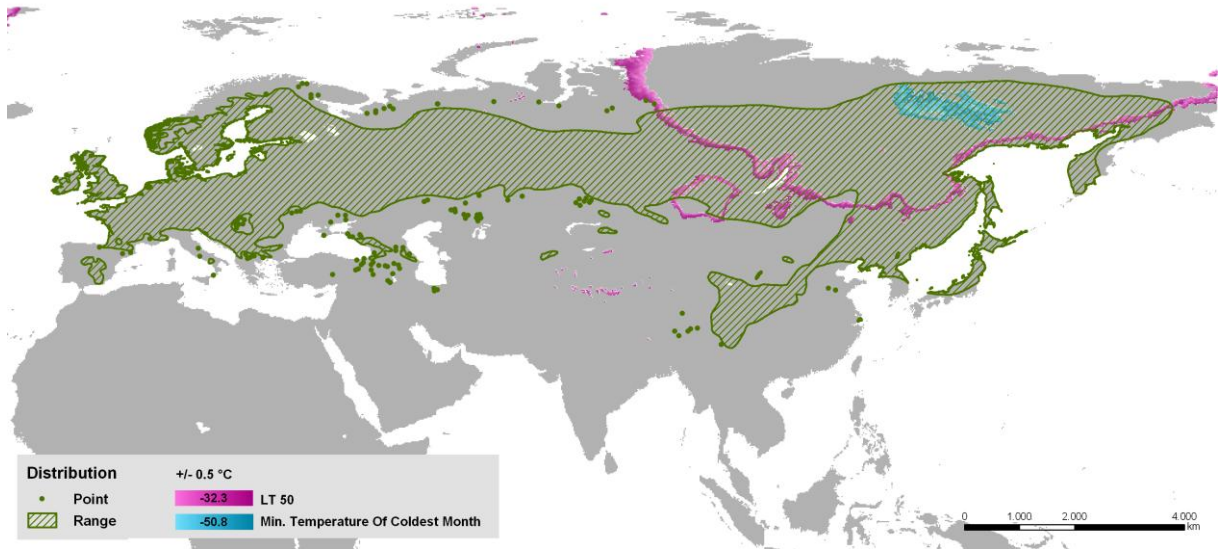
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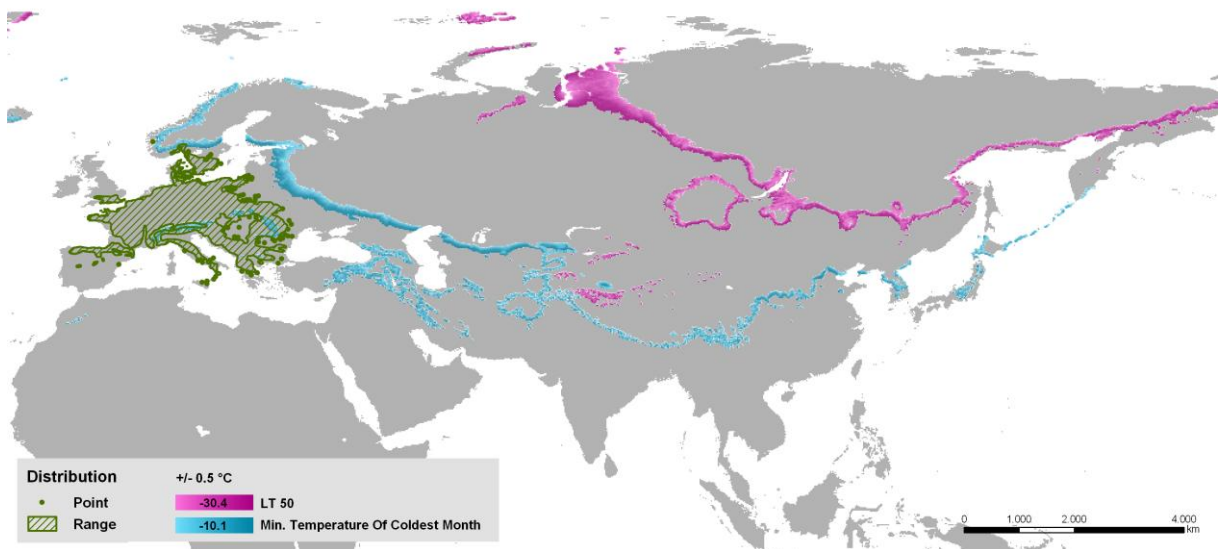
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4.8 Appendix



Appendix 4.1 Distribution map of *Betula pendula*. Green = distribution range. The region limited in magenta colours shows minimum temperatures of the coldest month that were lower than the LT_{50} value of closed buds measured in this study (-32.3 °C). The region limited in blue colour shows the lowest 1%-percentile of the minimum temperature of the coldest month (-50.8 °C).



Appendix 4.2 Distribution map of *Fagus sylvatica*. Green = distribution range. The region limited in magenta colours shows minimum temperatures of the coldest month that were lower than the LT_{50} value of closed buds measured in this study (-30.4 °C). The region limited in blue colour shows the lowest 1%-percentile of the minimum temperature of the coldest month (-10.1 °C).

5 Relationship between frost hardiness of adults and seedlings of different tree species

Maria Hofmann, Michael Jager & Helge Bruelheide

iForest 7: 282-288

5.1 Abstract

The aim of this study was to investigate the relationship between frost hardiness and the macroclimatic niche of adult individuals and seedlings of eight temperate tree species. Frost damage was investigated on the winter buds and needles of adult individuals and on the freshly germinated seedlings. We hypothesized that frost hardiness of adult individuals and seedlings is in accordance with their macroclimatic niche and that frost hardiness of seedlings increases with increasing plant age. Frost hardiness was tested in a climate chamber by exposing the plant material to different freezing temperatures and was assessed by LT_{50} -values. In contrast to our expectations, we did not find any relationship between LT_{50} -values and the macroclimatic niche variables, neither for adults nor for seedlings. There was a positive trend between seedlings development and frost hardiness, although average frost hardiness of all species differed only between -7.5 and -9 °C for one-week old and two- or four-weeks old seedlings respectively. We have to conclude that frost hardiness of adult individuals as well as seedlings at our study site does not reflect the species' geographic distribution range, and therefore, it seems not be possible to predict the geographical distribution ranges of tree species from their frost tolerance.

Keywords: developmental stage; electrolyte leakage; LT_{50} ; spring frost

5.2 Introduction

Out of all factors worldwide that potentially limit geographical distribution ranges of plants, minimum temperatures are considered the most important (Woodward 1987, Dahl 1998, Holten 1998). In particular, frost is thought to be the cause of northern and eastern distribution range boundaries of plant species in Europe (Sakai & Larcher 1987, Pither 2003, Kreyling 2010). The coincidence between species distribution boundaries and freezing temperature thresholds has been already demonstrated for various plant species (Sakai & Larcher 1987, Huntley 1990, Woodward 1997). For example, *Fagus sylvatica* L. does not occur in regions with a mean temperature in January below -3 °C (Bolte et al. 2007). However, these observations are purely based on correlations and knowledge on which plant organs are affected by frost in which phenological state is virtually absent. Low temperatures affect individuals directly or indirectly by frost dehydration (Pearce 2001, Beck et al. 2004), whereby single cells up to the whole organism can be damaged (Weiser 1970, Pearce 2001). Thus, tree species in the northern hemisphere have evolved a certain frost hardiness despite the high energetic costs involved (Huner et al. 1998). Frost hardiness of European plant species shows a seasonal pattern and at the end of the growing season it is induced by decreasing temperatures (e.g. Xin & Browse 2000, Repo et al. 2001, Beck et al. 2004) and is brought about by accumulation of carbohydrates and dehydration of the cells, tissues and organs (Siminovitch & Briggs 1953, Sakai & Larcher 1987, Améglio et al. 2004, Thomas et al. 2004, Morin et al. 2007, Callister et al. 2008).

Long-lived plant species go through various development stages with different environmental condition requirements. It is well-known that frost hardiness varies between the plant life cycle (Sakai & Larcher 1987, Bigras et al. 2004, Taschler et al. 2004), with seedlings exhibiting a lower frost hardiness than adults. For example, Morin et al. (2007) showed for different European *Quercus* species that adults were significantly more frost-tolerant than seedlings. However, despite such differences in frost tolerance between adults and juveniles, one would expect that frost tolerance mechanisms of different ontogenetic stages are similar for given species. Moreover, seedlings and adults might not share the same micro-environment but are exposed to the same macro-climate at any growth location. In consequence, the survival and growth of seedlings of the tree species *Acer pictum* subsp. *mono* (MAXIMOWICZ) H. OHASHI and *Fagus crenata* BLUME were found to be in accordance with the distribution patterns of the adult individuals of these species in Japan (Masaki et al. 2005).

Periods of low temperatures are critical events in the development of tree seedlings. While seeds of temperate tree species are generally frost-tolerant down to $-196\text{ }^{\circ}\text{C}$ (Sakai & Larcher 1987), they lose frost hardiness with the onset of germination (Marcante et al. 2012). As most temperate and boreal tree species germinate at the beginning of the growing season in spring, they are often exposed to late frost events which frequently occur in the temperate and boreal zone (Bigras et al. 2004), and therefore, frost events can severely affect seedling growth and survival (Bigras et al. 2004, Kreyling et al. 2012). For plant species in an alpine glacier foreland, Marcante et al. (2012) showed that freezing temperatures in the growing season are potentially severe enough to kill plants at early stages of their development. Similarly, Funkenberg et al. (2012) demonstrated the importance of late frosts for establishment of *Fallopia japonica* (HOUTT.) RONSE DECR. seedlings in Germany, whereby the freezing sensitivity depended on the timing, intensity and duration of late frost events. The fact that trees often can be grown in Botanical Gardens outside the species' native distribution range when planted as saplings, indicates that tree species distribution ranges might depend more on the susceptibility of seedlings rather than that of adults. Furthermore, frost hardiness of seedlings depends on their developmental stage. There is evidence that frost tolerance decreases with increasing seedling age as demonstrated by Bigras et al. (2004) for seedlings of *Picea glauca* (MOENCH) VOSS.

In this study, we asked to which degree frost tolerance of adult individuals and seedlings of eight temperate tree species common to Central Europe corresponds to their geographical distribution range. We tested the hypothesis that the more tolerant to frost is a species the further its distribution range extends to regions with low winter temperatures.

In particular, we expected the species' geographical distribution is better explained by the frost tolerance of seedlings than that of adult individuals. Furthermore, we tested the hypothesis that the frost hardiness of seedlings increases with increasing developmental stage.

5.3 Material and methods

Experimental design

We investigated individuals of the following eight tree species: *Acer negundo* L., *Acer platanoides* L., *Alnus glutinosa* (L.) P. GAERTN., *Betula pendula* ROTH, *Fagus sylvatica* L., *Picea abies* (L.) H. KARST., *Pseudotsuga menziesii* (MIRB.) FRANCO and *Tilia cordata* MILL. For all species we investigated four different development stages: adult trees, one-week, two-weeks and four-weeks old seedlings. While in the one-week old seedlings the first leaves (either the cotyledons or the primary leaves) just unfolded, they were developed in two-weeks old seedlings and subsequent leaves already emerged in the four-week old seedlings (Fig. 5.1).

Frost hardiness of the adult individuals was analyzed on winter buds (*A. negundo*, *A. platanoides*, *A. glutinosa*, *B. pendula*, *F. sylvatica* and *T. cordata*) or needles (*P. abies* and *P. menziesii*). Except for *A. negundo*, which is highly invasive in Central Germany, and *P. menziesii*, which is an important plantation species, all species were native to the study region in the surrounding of Halle (Saale), Germany (latitude: N 51.497°, longitude E 11.969°, elevation 87 m a.s.l., annual mean temp: 9.0 °C, annual precipitation 490 mm). Plant material was sampled about 12 hours before the start of the frost experiment and stored at +4 °C.

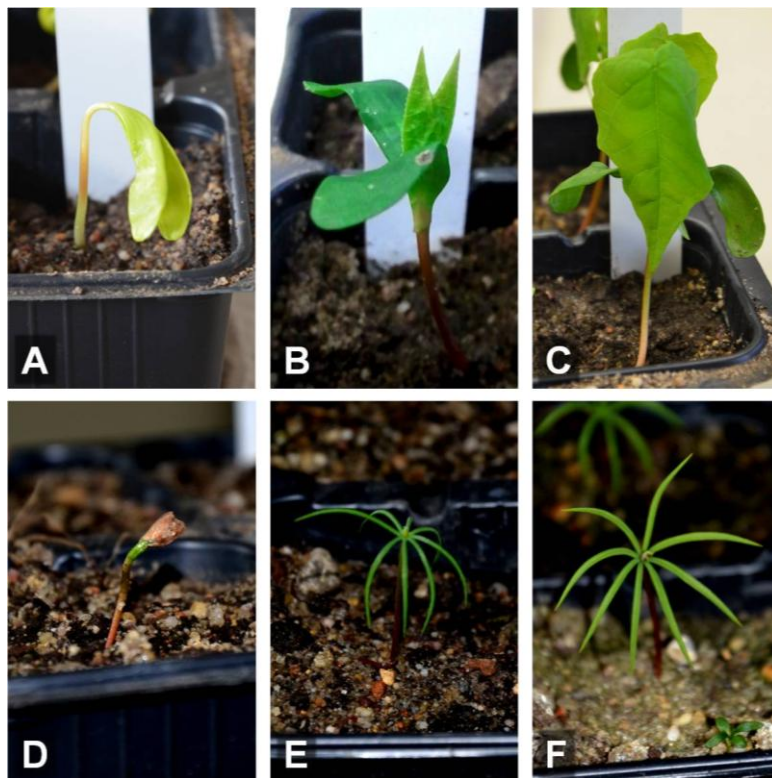


Figure 5.1 Developmental stages of one-week (A, D), two-weeks (B, E) and four-weeks old seedlings (C, F) of *Acer platanoides* (A-C) and *Pseudotsuga menziesii* (D-F).

Frost hardiness of seedlings was analyzed on whole individuals. Seedlings derived from seeds collected from natural stands in Saxony-Anhalt (Landesdarre Saxony-Anhalt, Annaburg, Tab. 5.1). In early spring 2012 the seeds were sown in pots containing compost and sand (2:1 v / v) in a glasshouse chamber (day / night cycle 12 h, 20 °C / 10 °C). Because of the low germination rates of *F. sylvatica* and *T. cordata*, further seedlings were collected in the field in April 2012. The seedlings of every development stage were acclimated in a climate chamber (RUMED, day / night cycle 12 h, 8 °C / 4 °C ± 0.6 °C temperature, lamps from ISTA, 35000 Lumen) one week prior to the frost experiment to simulate outdoor conditions.

Frost experiment

Frost hardiness was tested in a climate test chamber (SANYO Atmos Chamber MTH-4400), using the procedure described in Hofmann et al. (2013). Frost hardiness of the adult species was determined in winter 2010 / 2011 (*A. negundo*, *A. platanoides*, *A. glutinosa*, *F. sylvatica*, *T. cordata*) and 2011 / 2012 (*B. pendula*, *P. abies*, *P. menziesii*), using one to seven individuals per species (*A. negundo*: n = 6, *A. platanoides*: n =5, *A. glutinosa*: n = 1, *B. pendula*: n = 1, *F. sylvatica*: n = 3, *P. abies*: n =1, *P. menziesii*: n = 1, *T. cordata*: n = 7). Frost hardiness of the seedlings was determined in spring 2012. Briefly, the plant material was exposed stepwise to 11 temperature levels for buds of adult individuals: +4 °C, -4 °C, -8 °C, -12 °C, -16 °C, -20 °C, -24 °C, -28 °C, -32 °C, -40 °C and -80 °C; for seedlings: +4 °C, 0 °C, -2 °C, -4 °C, -6 °C, -8 °C, -10 °C, -14 °C, -18 °C, -22 °C and -40 °C with a cooling rate of 0.13 and 0.07 °C / min between frost levels for adults and seedlings, respectively. Each temperature level lasted for 45 minutes (resulting in 8.15 hours in total). At the end of each level, one batch of winter buds from adult trees and eight pots with seedlings were removed from the climate test chamber and transferred to a refrigerator at +4 °C to allow for controlled thawing and stored there until the next day. Eight replicates per treatment, resulting in a total of 88 replicates per developmental stage and species were considered.

Table 5.1 Geographic origin (in decimal degrees) of the seed material of the investigated species.

Species	Latitude	Longitude
<i>Acer negundo</i>	N 51.4970	E 11.9688
<i>Acer platanoides</i>	N 51.4970	E 11.9688
<i>Alnus glutinosa</i>	N 51.6833	E 11.4667
<i>Betula pendula</i>	N 52.4465	E 11.4862
<i>Fagus sylvatica</i>	N 51.4347	E 12.0379
<i>Picea abies</i>	N 51.6993	E 10.7219
<i>Pseudotsuga menziesii</i>	N 52.4325	E 11.7698
<i>Tilia cordata</i>	N 51.9411	E 11.4281

Assessment of frost hardiness of adult individuals

Frost hardiness of the adult individuals was measured as the rate of electrolyte leakage of buds. On the next day after frost exposure, the buds were transferred into test tubes with 3%-isopropanol solution and tested for electrolyte leakage according to Murray et al. (1989). Intracellular ice formation results in cell membrane disruption, whereby protoplasm escapes, resulting in an increase of the electrolyte conductivity in the surrounding solution (Ashworth & Pearce 2002). The electrical conductivity in the solution was measured six times: first immediately after preparing the buds to define a baseline for electrical conductivity (C_0), followed by four measurements after 4 h, 24 h, 48 h and 72 h (C_t) after the initial measurement. A final measurement was conducted after boiling the samples for 20 minutes (C_b), which resulted in a complete destruction of the tissue and served as the maximum electrical conductivity of the plant material. Relative Conductivity (RC) was calculated according to eqn. 1 (Murray et al. 1989):

$$RC = \frac{C_t - C_0}{C_b - C_0} = 1 - e^{-k*t}$$

The rate of electrolyte leakage (k -values) was calculated for all samples ($n = 88$) per species and development stage, by a 4-parametric sigmoid regression according to eqn. 2:

$$k = f(T) = c + \frac{a}{1 + e^{-\left(\frac{T-LT_{50}}{b}\right)}}$$

where a is the range of k -values, b is the steepness of the curve, c is the minimum k -values, T is the temperature. The regression parameter LT_{50} describes the point of inflection of the resulting curve and indicates the temperature at which 50% of maximum electrolyte leakage was reached.

Assessment of frost hardiness of seedlings

Frost hardiness of the seedlings was measured by visual inspection of the seedlings' survival. On the following day of the frost experiment the seedlings were transferred into a-glasshouse chamber (day/night cycle 12 h, 20 °C / 10 °C). Two days later the survival of every individual seedling was assessed. Survival was then regressed on the temperature levels applied in a logistic model and LT_{50} was obtained from eqn. 3:

$$\ln\left(\frac{p}{1-p}\right) = f(T) = aT + b$$

where p is the proportion of seedlings that survived, a and b are regression parameters, T is the temperature. The point of inflection of the above function is the LT_{50} , i.e. the temperature at which 50% of the seedlings survived, and is given by $-b/a$. A pre-study demonstrated that the LT_{50} -values obtained by electrolyte leakage were comparable to the LT_{50} -values calculated by survival rates. These results show that frost hardiness of seedlings assessed by visual inspection and electrolyte leakage was closely related (Fig. 5.2).

Statistic analysis

Frost hardiness of the different development stages described by the LT_{50} -values was related to the species' macroclimatic niches. Therefore, global occurrence data on the investigated species, based on floristic atlases and online published databases, were georeferenced and digitized using ArcMap (ESRI). Climatic data were extracted from the Worldclim dataset (Hijmans et al. 2005) in a 2.5 arc minutes grid across the whole distribution range. For the following analyses we used the lower 1st percentile of all macroclimatic variables that described frost conditions (Tab. 5.2). To test the first hypothesis, *i.e.*, the relationship between frost tolerance and the climatic conditions in winter, linear regression models were applied using LT_{50} -values and the minimum temperature of the coldest month as well as the minimum temperatures of the single months November to May. As for the second hypothesis, differences between adults and seedlings and between different seedling ages were verified using paired *t*-tests. Additionally, we related the LT_{50} -values of the adult individuals to the LT_{50} -values of all three development stages of the seedlings, using linear regression models. Differences in seedlings frost tolerance among species was tested using a generalized linear models with binomial error distribution, including temperature level and species identity as fixed factors. These two-factorial models were subjected to Tukey's *post-hoc* tests to determine which species differed from each other. Similarly, we tested for differences in frost tolerance among the different seedlings development stages, using temperature level and seedling age as fixed factors.

The sigmoid regressions were calculated with Sigmaplot 11.0 (Systat Software[®], v. 2008), whereas all the other statistical analyses were conducted by using R 2.12.0 (R Development Core Team 2010).

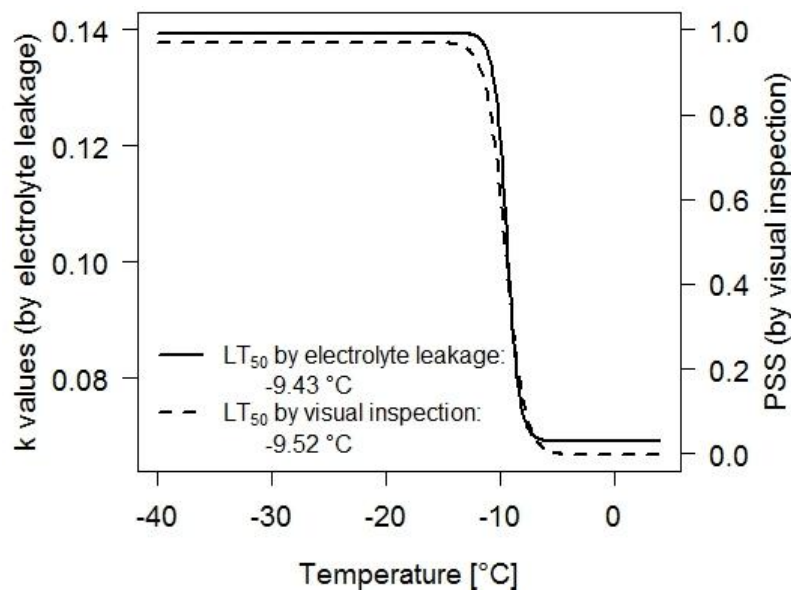


Figure 5.2 Comparison of the frost hardiness measurements for four-weeks old seedlings of *Tilia cordata*. Solid line: *k* values assessed by electrolyte leakage; dashed line: proportion of seedlings survival (PSS) assessed by visual inspection.

5.4 Results

Frost hardiness of the adult individuals, expressed as LT_{50} -values, ranged from -45.29 (*T. cordata*) to -22.55 °C (*A. glutinosa* - Tab. 5.2). No significant relationships were found between the LT_{50} -value of the adult individuals and any of the macroclimatic variables tested (Tab. 5.3, Fig. 5.3). Moreover, no significant correlations were detected between the frost hardiness of seedlings and any climatic variable (Tab. 5.4). There was one marginally significant effect between frost hardiness of four-weeks old seedlings and the minimum temperature of April, but the direction of the effect was opposite to the expectation, with the tendency of species with lower frost hardiness of seedlings to occur at higher minimum temperature. In addition, no significant correlations were found between the frost hardiness of any development stage of the seedlings and the frost hardiness of the adult individuals (Fig. 5.4).

Frost hardiness of the seedlings ranged from -12.08 to -6.01 °C, whereby *A. glutinosa* and *T. cordata* exhibited the lowest frost hardiness (one-week old seedlings) and *P. menziesii* (two-weeks old seedlings) exhibited the highest frost hardiness (Tab. 5.2). Species only differed in the age class of one-week and two-weeks old seedlings, while there was no statistically significant difference in frost tolerance among species in four-weeks old seedlings. One-week and four-week old seedlings of *T. cordata* and *A. glutinosa* were significantly more sensitive to frost than those of *F. sylvatica*, *P. abies* and *P. menziesii* (Tab. 5.2). Across all species, frost hardiness of seedlings was significantly lower than that of adults ($p < 0.001$ for adults versus one-, two- and four-weeks old seedlings in paired t -tests). While frost tolerance across all species differed between one- and two-weeks old seedlings ($p = 0.0028$), there was no difference between two- and four-weeks old seedlings ($p = 0.883$), according to paired t -tests.

The seedlings of *A. negundo*, *A. platanooides*, *A. glutinosa*, *F. sylvatica* and *T. cordata* showed an increase in frost hardiness with increasing age with a maximal difference of 3.9 °C between the one-week-old and the four-weeks-old developmental stage (*T. cordata* - Tab. 5.2). In contrast, the seedlings of *B. pendula*, *P. abies* and *P. menziesii* displayed the highest frost hardiness at the two-weeks-old developmental stage.

Table 5.2 Minimum temperature of the coldest month (the lower 1st percentile - °C) and frost tolerance expressed as LT_{50} -values (°C) of the seedlings (one-week, two-weeks and four-weeks old) and the adult individuals for all eight species studied. Different letters in the seedlings columns indicate statistically significant differences between species according to Tukey's *post-hoc* tests.

Species	Min. temp. coldest month	Seedlings						Adult individuals
		One-week		Two-weeks		Four-weeks		
<i>Acer negundo</i>	-26.4	-6.26	ab	-9.11	bc	-9.35	a	-32.17
<i>Acer platanoides</i>	-21.1	-8.32	abc	-9.39	cd	-9.35	a	-32.56
<i>Alnus glutinosa</i>	-22	-6.01	a	-6.76	a	-8.28	a	-22.55
<i>Betula pendula</i>	-21.8	-6.26	ab	-8.83	abc	-7.77	a	-23.41
<i>Fagus sylvatica</i>	-10.2	-8.59	bc	-9.39	cd	-9.63	a	-32.18
<i>Picea abies</i>	-21.1	-10.04	c	-10.69	cd	-9.35	a	-29.2
<i>Pseudotsuga menziesii</i>	-20	-9.42	c	-12.08	d	-9.54	a	-36.61
<i>Tilia cordata</i>	-23.9	-6.01	a	-7.52	ab	-9.93	a	-45.29

Table 5.3 Summary of R^2 and p-values of the linear regression models testing for relationships between LT_{50} -values of the buds of adult individuals and the macroclimatic variables (from the Worldclim dataset). In all regressions, degrees of freedom = 6.

Variable	Adult individuals	
	R^2	p-value
Minimum temperature in the coldest month	0.145	0.353
Minimum temperature in November	0.152	0.340
Minimum temperature in December	0.146	0.350
Minimum temperature in January	0.146	0.351
Minimum temperature in February	0.158	0.330

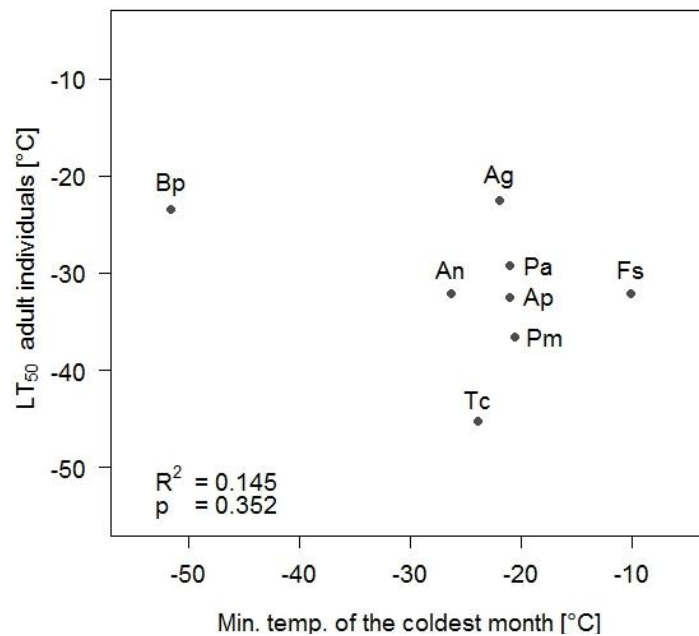


Figure 5.3 Correlation between frost hardiness of adult individuals expressed as LT_{50} -values and minimum temperature of the coldest month. (Ag): *Alnus glutinosa*; (An): *Acer negundo*; (Ap): *Acer platanoides*; (Bp): *Betula pendula*; (Fs): *Fagus sylvatica*; (Pa): *Picea abies*; (Pm): *Pseudotsuga menziesii*; (Tc): *Tilia cordata*.

Table 5.4 Summary of R^2 and p-values of the linear regression models tested for relationships between LT_{50} -values of all three development stages of the seedlings (one-week, two-weeks and four-weeks old), as obtained from the 4-parametric sigmoid regression, and LT_{50} -values of the adult individuals, as well as the macroclimatic variables (from the Worldclim dataset). In all regressions, degrees of freedom = 6.

Variable	One-week		Two-weeks		Four-weeks	
	R^2	p-value	R^2	p-value	R^2	p-value
LT_{50} adult individuals	<0.001	0.946	0.029	0.685	0.270	0.200
Min. temp. in March	0.070	0.528	0.002	0.909	0.478	0.057
Min. temp. in April	0.001	0.929	0.034	0.664	0.491	0.053
Min. temp. in May	0.015	0.772	0.177	0.299	0.458	0.065

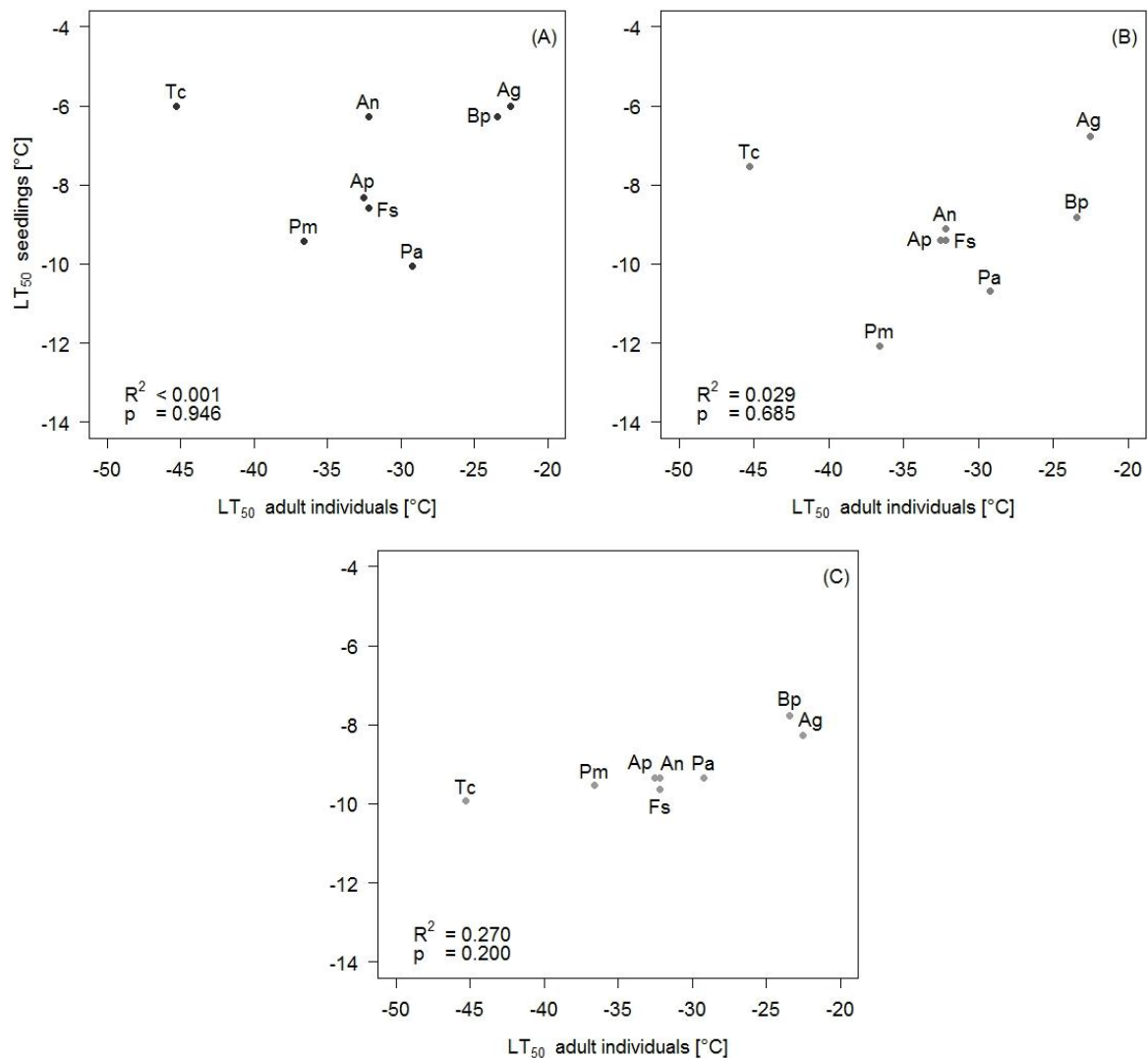


Figure 5.4 Frost hardiness of one-week (A), two-weeks (B) as well as four-weeks old seedlings (C) and frost hardiness of the adult individuals expressed as LT_{50} -values. (Ag): *Alnus glutinosa*; (An): *Acer negundo*; (Ap): *Acer platanoides*; (Bp): *Betula pendula*; (Fs): *Fagus sylvatica*; (Pa): *Picea abies*; (Pm): *Pseudotsuga menziesii*; (Tc): *Tilia cordata*.

5.5 Discussion

We found no relationship between the frost tolerance of the eight tree species and the macroclimatic variables derived from their geographical distribution range, neither for adult winter buds or needles, nor for seedlings at three different ages. Thus, the hypothesis of a relationship between species' frost hardiness and the extension of their distribution range to regions with low winter temperatures (our first hypothesis) has to be rejected. As this result is exactly the opposite of the current biogeographical understanding, we have to consider that there might be several methodological reasons. A major drawback of our approach adopted in this study was that all plant material subjected to frost hardiness measurements was taken from the same geographical location, *i.e.* Halle (Saale). This may preclude the possibility of detecting any effects of intra-specific adaptation. Indeed, it might be that the study species were adapted to the much more moderate frost conditions at the study site than to those encountered at the geographical distribution limits, where the climatic variables had been drawn from. Even the lowest measured frost hardiness of any adult tree (*i.e.*, *A. glutinosa* with a frost hardiness of -22.6 °C) was high enough to safely survive under the climatic winter conditions in the study region. Many studies have investigated local adaptation to frost, demonstrating a relationship between frost hardiness and the climatic conditions of the populations' geographic origins, with species from northern provenances or higher elevation being more frost tolerant than species from southern provenances or lower elevation (Beuker et al. 1998, Jensen & Deans 2004, Aldrete et al. 2008, Kathke & Bruelheide 2011, Kreyling et al. 2012). In particular, species with a wide distribution range such as *Quercus robur* L. have developed ecotypes with respect to frost hardiness (Deans & Harvey 1996). However, if intra-specific adaptation had blurred inter-specific differences in frost tolerance between species, this would indicate that intra-specific variation in frost hardiness might be higher than variation among species growing in the same area. Testing this assumption would require to take samples of both adults and seedlings from different climatic zones for different species. Preferably, the different provenances would also include the range boundaries of the different species. For adult trees, there are not only logistic obstacles with such a sampling scheme, as simultaneous sampling from different regions involves the risk to include plants with a different degree of acclimatization. It is well known that winter frost hardiness is acquired gradually and the degree of hardening depends on outdoor temperatures (Sakai & Larcher 1987, Beck et al. 2004). While we could control for differences in the degree of hardening by taking all bud and needle samples of adult trees at the time of maximum hardening, this will probably not be possible when sampling over a large

geographical area. A solution to this challenge would be provenance trials of different tree species grown under the same common garden conditions. For seedlings, simultaneous testing of provenances is simpler as the seeds can be germinated at the same time.

Our study is not the only one that failed to detect differences in frost tolerance between different species. Comparing eight congeneric pairs of grassland species with contrasting distribution ranges, Hofmann et al. (2013) also failed to confirm the correspondence between frost resistance and macroclimatic niche variables. Similar to our results, Baltzer et al. (2007) detected no relationship between the fundamental and realized niches with respect to the seasonality of the rainfall for different tropical tree species.

In contrast to our expectation, seedlings frost tolerance did not reflect the species' macroclimatic niche better than that of adults. Since long, frost hardiness of seedlings has been claimed to be an important limiting factor of the distribution ranges of tree species. This statement is mainly based on cursory observations on lethal effects of exceptional frost events on seedlings, as reported by Szafer (1932) for *F. sylvatica*. Our results did reveal only moderate differences in frost hardiness of seedlings of the different species studied. Thus, seedlings of different tree species would be affected quite similarly by exceptional late frost events. As a consequence, seedling frost hardiness can hardly be a good predictor for the different distribution ranges of tree species. Similar doubts on the relevance of late frost events have also been expressed by other authors, such as by Funkenberg et al. (2012) for the establishment of *Fa. japonica* seedlings.

We have to consider that our study focused only on seedling frost lethality and not on the effects of non-lethal freezing temperatures on seedling performance. It is possible, that non-lethal freezing temperatures higher than the measured LT_{50} -values result in a weakening of plants, making them more vulnerable to subsequent frost events or to other types of biotic or abiotic stress (Repo et al. 2008). For example, these non-lethal effects led to a reduced growth of *Q. robur* seedlings and the ensuing growth reduction has been interpreted as a limiting factor for the northern distribution boundary of this species (Repo et al. 2008). Similarly, comparing the growing rates of *F. sylvatica* seedlings, Kreyling et al. (2012) reported a reduced growth as a consequence of spring frost events in the Bulgarian but not in the German provenance. Non-lethal freezing temperatures also resulted in reduced growth and survival of *Digitalis purpurea* L. seedlings at the altitudinal distribution limit (Bruehlheide & Heinemeyer 2002).

We found support for our second hypothesis as the investigated seedlings showed increasing frost hardiness with increasing age for five of eight species. With the exception of the two evergreen species, *P. abies* and *P. menziesii*, the four-weeks old seedling were more frost tolerant than the one-week old seedlings. This might point to fundamental differences in the sensitivity of cotyledons and primary leaves between angiosperm and gymnosperm seedlings. While cotyledons seem to be more susceptible to frosts than primary leaves in angiosperms, while the opposite might be the case for gymnosperms. However, organ-specific tests would be required to test this idea.

As frost events become rarer with the progress of the growing season and frost hardiness of our six angiosperm seedlings increases with seedling age, the risk of being damaged by late frost events in spring decreases disproportionally with time. This underlines the role of germination timing. A later germination has been often interpreted as acclimation to late frost events. For example, Chuine & Cour (1999) postulated that the timing of germination is adaptive to climate conditions of the species' geographical distribution range. Our results show that this might not be the case, as frost hardiness of one-week old seedlings of different species is rather similar, which has the consequence that early germinating tree species take higher risks, irrespective of their macroclimatic niche.

Conclusion

Based on our experimental results, predictions on the geographical distribution ranges of the eight tree species considered based on their frost tolerance seems to be unfeasible. Thus, the general assumption that minimum temperatures below a species-specific threshold simply kill needles, buds or seedlings has to be reconsidered. Nevertheless, frost might operate through a multitude of other mechanisms, affecting organs different from needles or buds, or biotic interactions such as pollination, thus, contributing to limit the distribution ranges of European tree species.

5.6 Acknowledgements

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6 Synthesis

6.1 Main results

This thesis focuses on the relationship between the fundamental niche, with respect to frost hardiness expressed as LT_{50} -value, and the realized niche, with respect to the geographical distribution range expressed as macroclimatic variables. The hypothesis tested was whether species with a distribution range stretching into colder regions (i.e. northern or eastern regions in the northern hemisphere and higher altitudes) are more frost resistant than species with a distribution range limited to warmer regions (i.e. southern or western regions and lower altitudes). Results from all four studies demonstrate that frost hardiness is not correlated with macroclimate in the geographical distribution range and thus fundamental and realized niches are not closely related to each other with respect to frost hardiness.

Frost hardiness of the 85 species studied in chapter 2 differed strongly between the different types of life form and leaf habit (Fig. 2.3). Only a small group of evergreen species, including needle-leaved conifers ($n = 7$), showed a significant relationship between the LT_{50} -values and minimum temperature of the coldest month (Tab. 2.2). In addition, more than 80 % of the investigated species showed lower LT_{50} -values than expected from the species' macroclimatic niche (Fig. 2.2). The phylogenetic analysis indicated that frost hardiness was highly phylogenetically conserved. In contrast, macroclimatic variables did not show a phylogenetic signal (Tab. 2.3, Fig. 2.5).

In chapter 3, frost hardiness of *Fagus sylvatica* showed large variation (up to 10.3 K) among the 20 provenances (Tab. 3.1). However, frost hardiness of the investigated provenances was unrelated to the climatic conditions at the populations' geographic origins.

Frost hardiness of the different early-budding and late-budding species in chapter 4 did not correlate with the species' macroclimatic niche, neither before nor after budburst (Tab. 4.3). While frost hardiness of closed buds differed between the investigated species, shortly after budburst, frost hardiness decreased and freshly opened buds of all investigated species were found to be frost-resistant, nearly to the same degree (Tab. 4.2, Fig. 4.3). In addition, frost hardiness after budburst was unrelated to the date of budburst (Fig. 4.4).

Frost hardiness of both seedlings and adults in chapter 5 was unrelated to macroclimatic variables (Tab. 5.3, Tab. 5.4, Fig. 5.3). As expected, seedlings were clearly more frost sensitive than adult individuals in all investigated species (Tab. 5.2). However, frost hardiness did not differ between the three different seedling stages, and seedling frost hardiness was unrelated to the particular frost hardiness of adult individuals (Fig. 5.4).

6.2 Discussion

The expected pattern that species with a high physiological frost hardiness are distributed further into colder regions than species with lower frost hardiness (e.g. Bannister & Polwart 2001, Pither 2003, Cunningham & Read 2006) was not confirmed in any of the four studies included in this thesis. Thus, in the context of Hutchinson's niche concept, the species' fundamental niches were not related to their realized niches. There may be several explanations for the detected mismatch. First, the variability in frost hardiness between different phenological and developmental stages of the individuals indicates that the species' frost hardiness is not reflected in a single species-specific value. Hence, using one frost hardiness value might be inadequate to describe a species' fundamental niche. Second, the temperatures that actually act on the plants in the field can differ from the macroclimatic variables attributed to the species' distribution range. Thus, it may be questionable to use such macroclimatic variables to describe a species' realized niche. Third, the lack of correlation between frost hardiness and the distribution range at both the interspecific and the intraspecific level may suggest that the generally assumed match between both types of niche does not exist for frost hardiness.

Complexity of frost hardiness

Frost hardiness strategies are species-specific, which is demonstrated by the different studies included in the thesis. This variability indicates that frost hardiness is not reflected in a single species-specific value, but it is a complex plant trait which is influenced by numerous components, such as the prevailing phenological or developmental stage.

The results of chapter 4 demonstrate that frost hardiness differs between phenological stages, indicating that the onset of the growing season is the most sensible phase for deciduous species (e.g. Kang et al. 1998, Inouye 2008, Lenz et al. 2013). While frost hardiness before budburst showed strong interspecific variability, frost hardiness after budburst in all species

decreased rapidly to nearly the same degree (Tab. 4.2, Fig. 4.3). Moreover, frost hardiness after budburst was unrelated to the date of budburst or to the species' frost hardiness before budburst (Tab. 4.2, Fig. 4.4). In consequence, early-budding species face a greater risk of frost damage caused by the earlier onset of the vegetation period. In contrast, previous studies demonstrated a correlation between frost hardiness and the date of budburst (e.g. Kang et al. 1998, Stevenson et al. 1999, Leinonen & Hänninen 2002). However, the results of these studies can hardly be compared with the result of this thesis because they only investigated different provenances of a single species, especially *Fagus sylvatica* (e.g. Visnjic & Dohrenbusch 2004, Gömöry & Paule 2011, Kreyling et al. 2012b). One reason for the lack of relationship between spring frost hardiness and the onset of the growing season might be that vegetative organs can better tolerate lower freezing temperatures than reproductive organs (Hänninen 2006, Neuner et al. 2013). It may be that the frost hardiness of flowering buds differs between early-budding and late-budding species, this possibility was not investigated in this thesis. Another reason might be that early-budding and late-budding species might differ in their ability to compensate for the loss of leaves caused by late frost events. This ability of refoliation involves high costs for the individual plant (Augspurger 2009, Vitasse et al. 2014b) and can result in reduced productivity and growth (Sakai & Larcher 1987, Dittmar et al. 2006, Awaya et al. 2009). For example, a late frost event in Germany in May 2011 led to notable non-lethal frost damage in several tree species, whereby the resulting refoliation shortened the vegetation period by about eight weeks (Kreyling et al. 2012a). Therefore, it might be possible that early-budding species are no more frost resistant than late-budding species, but early-budding species may compensate for late frost damage better than late-budding species. Thus, the consequences of refoliation on the individuals should be analysed in further studies.

In addition to differences between phenological stages, frost hardiness also differed between developmental stages, as demonstrated in chapter 5, where frost hardiness was shown to increase with plant age (Tab. 5.2). However, the seedlings' frost hardiness was unrelated to the frost hardiness of the adult individuals (Fig. 5.4). Since later germination represents an effective strategy to avoid freezing injuries (Chuine & Cour 1999, Leiblein-Wild et al. 2013), it might be assumed that the investigated young seedlings germinated when the risk of frost events was very low. As such, greater frost hardiness in one-year old individuals than in seedlings might be more important for establishment, and seedlings may differ in their strategy to avoid frost damage than adults (Alberto et al. 2013, Vitasse et al. 2014a).

Woodward & Williams (1987) already summarized that frost events may only affect species at one particular developmental stage, and many studies have demonstrated that winter survival of seedlings in particular seems to be important for species distribution (Repo et al. 2001, Bruelheide & Heinemeyer 2002, Lim et al. 2014).

In this thesis one aspect of species' fundamental niches was determined by frost hardiness, whereby the level of frost hardiness is defined as the temperature at which 50 % of the investigated plant material is destroyed, i.e. the LT_{50} -value (e.g. Visnjic & Dohrenbusch 2004, Azzarello et al. 2009, Sjøgaard et al. 2009). The LT_{50} -value can be assessed *in situ* or *ex situ* using numerous methods (see review by Pearce 2001). In all four studies included in the thesis, frost hardiness was assessed using the electrolyte leakage method, according to Murray et al. (1989), and the method-specific LT_{50} -values represent thresholds that can be used as an indicator of relative frost tolerance under natural conditions (Woodward 1997, Morin et al. 2007, Alberto et al. 2013). However, the interpretation of frost hardiness using LT_{50} -values requires caution as there are several constraints (Sutinen et al. 1992).

First, species tested *in situ* seem to be more frost resistant than species tested *ex situ* (Prozherina et al. 2003, Marcante et al. 2012). For example, 32 alpine plant species tested in the field were, on average, 1.1 K more frost resistant than those tested in the laboratory (Taschler & Neuner 2004). In addition, the freezing of a small number of buds, leaves or needles in the laboratory does not necessarily represent the actual freezing process in the intact whole individual under natural climatic conditions (Sakai & Larcher 1987, Räisänen et al. 2006, Neuner 2014). However, an additional comparison of frost hardiness of four week old seedlings of *Fagus sylvatica* and *Tilia cordata* (which is not included in chapter 5), indicates that frost hardiness estimated using the electrolyte leakage method is comparable to the frost hardiness method of visual estimation with reference to the degree of damage observed (Jager 2012). Furthermore, *ex situ* measurements provide a direct link between frost damage and freezing temperatures (Larcher et al. 2010). As such, laboratory freezing tests represent a powerful tool in determining species' frost hardiness.

Second, the measured LT_{50} -values focus only on the lethal effect of freezing temperatures and do not provide any information on non-lethal damage and the consequences to the individual. Temperatures that are higher than the measured LT_{50} -values can increase the susceptibility for later frost events or other stress factors (Sakai & Larcher 1987, Repo et al. 2001, Bremer & Jongejans 2010). For example, frost damage in *Fraxinus excelsior* supported fungal colonization of the damaged organs and led to a dieback of these trees in Poland

(Pukacki & Przybyl 2005). However, non-lethal damage and consequences to the individual are difficult to measure. One reason is that the analysis of the non-lethal effect of freezing events requires long-term and cost-intensive field studies (Kreyling et al. 2012a).

The measurement of species' physiological frost hardiness using LT_{50} -values estimated by the electrolyte leakage method is state-of-the-art, despite the several constraints. Similarly, LT_{50} -values can be used as a good indicator for species' frost hardiness, although they do not reflect absolute values. This thesis is one of only few studies which used the electrolyte leakage method for determining frost hardiness of a large species pools and therefore, which allows the comparison of frost hardiness among numerous species.

The demonstrated considerable influence of phenology and development on the frost hardiness of individual species goes in line with previous studies (e.g. Bruehlheide & Heinemeyer 2002, Morin et al. 2007, Aldrete et al. 2008, Kreyling et al. 2012c). The variability in frost hardiness within a species indicated that it is questionable whether the determination of a species' frost hardiness using one single LT_{50} -value can support any conclusion on its fundamental niche. Hence, the possible limitation in determining a species' fundamental niche might have consequences in terms of the relationship between both types of niche.

Complexity of distribution range

Much like frost hardiness, a species' distribution range depends on numerous factors (Brown et al. 1996, Holt & Keitt 2005, Holt et al. 2005, Alexander & Edwards 2010). Under natural conditions, other abiotic environmental factors, such as precipitation, mean annual temperature or length of the growing season, can occur simultaneously with freezing temperatures, which makes it difficult to disentangle the relative importance of individual factors (Parmesan et al. 2005, Sexton et al. 2009). As such, a particular combination of several environmental factors can exclude a species from regions where it might otherwise be able to occur due to its frost hardiness. As with abiotic factors, biotic factors, e.g. competition and predation, may prevent species from occupying macroclimatically suitable habitats (e.g. Holt & Keitt 2005, Colwell & Rangel 2009, Anderson & Raza 2010). All these factors might influence the relationship between frost hardiness and distribution range. However, the finding of all four studies included in this thesis, i.e. that a species' fundamental and realized niche are unrelated. Furthermore, natural limits, e.g. oceans, mountains and deserts (Jeffree & Jeffree 1994, Wiens & Graham 2005), as well as human impact, e.g. land use and

land management (Brown et al. 1996, Peñuelas et al. 2007, Normand et al. 2011), can lead to discontinuous distribution ranges. Moreover, historical factors have to be considered, in particular the last glaciation in Europe (21,000 years ago), as they may have constrained species migration (Guisan & Zimmermann 2000, Svenning & Skov 2005, Soberón & Nakamura 2009). It can however be assumed that under natural conditions, numerous species do not occupy all habitats in which they might potentially occur in relation to their physiological ability, i.e. such species exhibit unfilled ranges (e.g. Colwell & Fuentes 1975, Anderson & Raza 2010, Guisan et al. 2014). The different causes for the unfilled range might lead to a false interpretation of the actual distribution boundaries and may therefore be one reason for the lack of correlation between macroclimatic variables and species' frost hardiness. For example, in chapter 2, more than 80 % of the investigated species were able to tolerate lower temperatures in the experiment than that expected from the minimum temperature of the coldest month derived from the species' distribution ranges (Fig. 2.2), which is consistent with other studies (e.g. Humphreys & Linder 2013, Lenz et al. 2013, Kollas et al. 2014). This clearly demonstrates that such species are able to colonize a larger environmental space beyond the geographical range limit represented by their realized niche (Wiens & Graham 2005, Holt 2009).

As with the measurement of frost hardiness, there are some constraints in the analysis of species distribution range boundaries. In all four studies of this thesis, the species realized niche was determined by macroclimatic variables (Bioclim), which were extracted from the Worldclim dataset based on each species' occurrence record (Hijmans et al. 2005). Environmental conditions can vary substantially, even within small areas (Müller-Starck et al. 1992, Kreyling et al. 2012a), but they are characterized by only a single climatic value (2.5 arc minutes grid, corresponds to about 10 km). Hence, microclimatic differences such as mountain microclimate, e.g. slope aspect (Scherrer et al. 2010, Körner 2011, Guisan et al. 2014, Briceño et al. 2014), are neglected. As such, this method may overestimate a species' climatic niche, because some species are able to occur in only small micro-habitats representing exceptional climatic conditions within such wide climatic zones (Brown et al. 1996, Scherrer et al. 2010). In addition, the macroclimatic variables employed do not consider the effect of snow (see below). As with frost hardiness, the analysis of species' macroclimatic niche using Bioclim variables derived from species distribution range is state-of-the-art. However, the estimated macroclimatic values extracted from species distribution data reflect only possible abiotic thresholds and do not reflect absolute values (Larcher et al. 2010,

Offord 2011). Conclusions on a species' climatic requirements should not be derived directly from its distribution range boundaries (Jäger 1975, Bruelheide 2003, Araújo et al. 2013). Therefore, this thesis is one of only few studies which connect macroclimatic values with experimentally determined frost hardiness for a large species pool.

Match between fundamental and realized niche

The analysis of frost hardiness of 85 different plant species in chapter 2 indicated that macroclimatic range characteristics seem to have a minor impact on frost resistance compared to life form and leaf habit (Fig. 2.3). As expected, the relationship between frost resistance and distribution range differs between the different types of life form. In particular, the frost hardiness of herbaceous species was unrelated to the macroclimatic variables, which might be explained by the effect of snow cover. Under snow, temperatures remain relatively constant at between 0 and -5°C and rarely drop below -10°C (e.g. Sakai & Larcher 1987). Herbaceous species that are usually covered by snow at the time when the lowest temperatures occur are protected from extreme freezing temperatures (e.g. Ungar 1975, Rixen et al. 2010, Neuner 2014) and relatively low frost hardiness may suffice to prevent frost damage. For example, Larcher et al. (2010) demonstrated that alpine species that were snow-protected showed clearly lower frost hardiness (-20 to -25°C) than alpine species that were not covered by snow (more than -80°C). In contrast to herbaceous species, the buds, leaves or needles of woody species are directly exposed to freezing temperatures. As air temperatures at the height of the canopy can be much lower than temperatures near the soil surface (Körner & Paulsen 2004), woody species make a greater investment in frost hardiness (Larcher 2001, Zanne et al. 2014). As such, frost does not seem to be an important range-limiting factor in herbaceous species.

As with life form, the relationship between frost resistance and distribution range differs between the different types of leaf habit. In the subset containing all deciduous species as well as the subset containing deciduous tree species, frost hardiness was unrelated to macroclimatic range characteristics. This lack of relationship indicates that frost hardiness does not seem to be related to a species' geographic range boundary. Factors other than winter frost hardiness of dormant buds may be important to the distribution of such species. For example, Kollas et al. (2014) suggested that late spring frost events had a stronger limiting effect on deciduous tree species than minimum temperatures in winter. In the subset of evergreen species, frost hardiness was correlated with macroclimatic variables, which was caused by the fact that the needle-leaved evergreen species were clearly more frost resistant than the broad-leaved evergreens.

The demonstrated mismatch between fundamental and realized niche within the different subsets of species indicates that frost hardiness is not an important range limiting factor for many of the investigated species. Comparing this result with previous studies is difficult because only a small number of studies investigated this relationship using a large species pool. For example, for a large set of species worldwide, Araújo et al (2013) assumed that species' climatic distribution limits were in accordance with their cold tolerance. However, the authors compared very frost sensitive tropical species with extremely frost tolerant boreal species, which may have produced the match between cold tolerance and distribution. As with the lack of correlation found between fundamental and realized niche at the interspecific level, frost hardiness was not correlated with climatic variables at the intraspecific level. The frost hardiness of 20 provenances of *Fagus sylvatica* in chapter 3 differed by up to 10.3 K (Tab. 3.1). This amount of variability in frost hardiness is comparable to other species such as *Picea abies* (Kathke & Bruelheide 2011), *Pinus greggii* (Aldrete et al. 2008) or *Pinus nigra* (Kreyling et al. 2012c). Species with large distribution ranges, such as *Fagus sylvatica*, are known to develop ecotypes, which might differ in their response to environmental factors (Clark 2010, Kreyling et al. 2014). However, frost hardiness was unrelated to macroclimatic variables at the provenances' geographic origin, which was in contrast to previous studies (e.g. Bannister & Polwart 2001, Jensen & Deans 2004, Leiblein-Wild et al. 2013). One reason for this mismatch might be the prevailing climatic conditions at the study site, which have to be considered when interpreting a species' winter survival. For example, in chapter 5, even the most frost sensitive species exhibiting the lowest measured LT_{50} -value were resistant enough to survive without damage under the prevailing climatic winter conditions. It might be possible that the investigated individuals were suitably acclimated to the prevailing climatic conditions, whereby the temperatures, especially in winter, were too mild for some individuals to develop their maximum potential frost hardiness (Loehle 1998, Nielsen & Rasmussen 2009, Kreyling et al. 2015). As such, one potential research approach is to use multiple-site provenance trials to disentangle the interaction of genotype and environment (Holt 2009, Alberto et al. 2013) and use experimental sites that cover a broad environmental gradient of the geographic range (Pérez et al. 2014). However, such provenance trials can be conducted only for a small set of species.

Phylogenetical conservatism of frost hardiness

In chapter 2, frost hardiness was found to be phylogenetically conserved, i.e. closely related species showed similar frost hardiness than more distantly related species, while macroclimatic niche characteristics did not show such patterns (Tab. 2.3, Fig. 2.5). This conservatism of the fundamental niche is defined as the tendency of a lineage to retain niche-related trait values over time (e.g. Huntley et al. 1989, Wiens et al. 2010, Crisp & Cook 2012). While the species' fundamental niche is conserved, the species' realized niche might have evolved more readily. The shift of realized niche was mainly observed for biological invasions (Wiens & Graham 2005, Pearman et al. 2008a) and can lead to a lack of correlation between physiological frost hardiness and the distribution range. For example, the North American herbaceous species *Ambrosia artemisiifolia* showed a correlation between frost hardiness and distribution in its native range, but not in the invasive range of Europe (Leiblein-Wild et al. 2013). In contrast to that observed at the interspecific level, species' frost hardiness at the intraspecific level clearly varied among individuals and provenances (chapter 3). It might be assumed that many species show locally adapted ecotypes, with correlations being evident between frost hardiness and climatic conditions at the geographic origin of the provenances (e.g. Bannister & Polwart 2001, Jensen & Deans 2004, Leiblein-Wild et al. 2013). However, this relationship could not be demonstrated for the investigated provenances of *Fagus sylvatica* in this thesis. While species' frost hardiness is conserved at the interspecific level, the large variability in frost hardiness at the intraspecific level might be primarily caused by a rapid evolution within species due to the different environmental conditions within a species geographical range, i.e. among provenances (e.g. Bilton et al. 2010, Albert et al. 2010, 2011). Testing any assumption of different evolutionary mechanisms on the intra- and interspecific level would require long-term common garden experiments using provenances from the whole distribution range and for a large set of species.

6.3 Conclusion

Based on a large species pool, all four studies included in this thesis demonstrated that the species' frost hardiness was not related to their geographical distribution range. As such, the overarching hypothesis has to be rejected. In contrast, all hypotheses regarding interspecific and intraspecific frost hardiness were confirmed: frost hardiness differed between life forms and leaf habits as well as among provenances, and also between the phenological and developmental stages, while the hypothesis that frost hardiness is phylogenetically conserved was confirmed (Fig. 6.1).

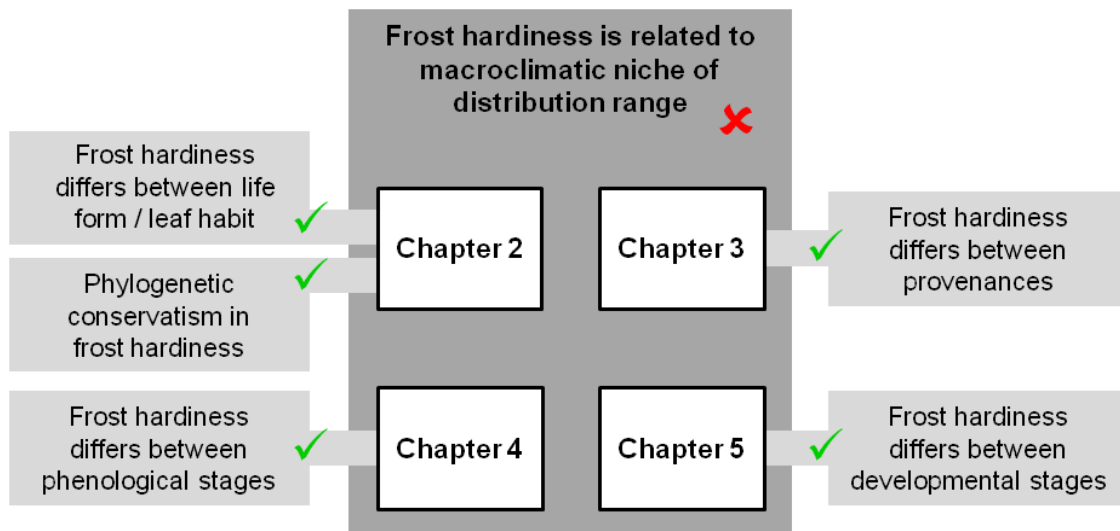


Figure 6.1 The major hypothesis (dark grey) and the specific hypotheses (light grey) tested in the present thesis and their affiliation to the different studies. The red cross indicates that the hypothesis was not confirmed, the green tick indicates that the hypothesis was confirmed.

On the one hand, the lack of relationship between a species' frost hardness and distribution range might be due to the fact that both frost hardness and macroclimatic niche are not “simple traits” but characteristics that depend on a multitude of variables. On the other hand, it might be possible that the supposed relationship between the fundamental and realized niche with respect to freezing temperatures does not exist. In common with this thesis, several other studies also failed to detect a relationship between both types of niche (e.g. Read & Hill 1989, Ebeling et al. 2008, Hofmann et al. 2013). This repeatedly demonstrated lack of correlation might imply, that the approach of future studies dealing with the relationship between the fundamental and realized niche should be modified. Such studies should disentangle underlying mechanisms and investigate additional co-influencing factors of frost hardness to determine possible reasons for the lack of correlation between both niches. Furthermore, future experiments should include individuals from the centre and the boundaries of species' distribution ranges (Holt & Keitt 2005, Kreyling et al. 2014) and seek to include different developmental stages (Alberto et al. 2013). Such experiments will be necessary to improve our understanding on the relationship between species' fundamental and realized niches.

The disagreement between both types of niche regarding frost hardness (D'Andrea et al. 2009, Helaouët & Beaugrand 2009) might have important implications on species distribution modelling. Such models relate occurrence records to environmental and, particularly, climatic variables to model a species' realized niche (Broennimann et al. 2006, Pearman et al. 2008b,

Elith & Leathwick 2009, Anderson & Raza 2010). The mismatch between both types of niches identified in this thesis indicates that distribution range boundaries are not in equilibrium with prevailing climatic conditions, which is a basic requirement for the use of climate envelopes (Guisan & Zimmermann 2000, Dormann et al. 2012, Kramer et al. 2012). Thus, distribution models based exclusively on a species' actual occurrence data will most likely underestimate the species' fundamental niche (Rouget et al. 2001, Araújo & Luoto 2007). Such models may also lead to inaccurate predictions of potential range shifts in response to climate change (Iverson & Prasad 1998, Thomas et al. 2004, Thuiller et al. 2005, Holt 2009). Biogeographical models should therefore be directly linked to the experimental quantification of a species' physiological climatic requirements, i.e. the fundamental niche (e.g. Kearney 2006, Pearson et al. 2007, Godsoe 2010, Humphreys & Linder 2013), or to species traits (e.g. Pompe et al. 2014), and they should account for the effects of biotic interactions (Pearman et al. 2008b, Elith & Leathwick 2009, Kramer et al. 2012).

As this thesis focuses on one part of the study species' niches, the findings contribute substantially to a major research area in ecology: the analysis of species–environment relationships in understanding the patterns and limits of a species' distribution range (Guisan & Zimmermann 2000, Orsenigo et al. 2014, Welk et al. 2014). In addition to frost hardiness, there is a strong requirement for further studies on other potentially limiting factors to species' distribution ranges, e.g. drought, soil conditions or mean annual temperature. Since the study species' frost hardiness was phylogenetically conserved in spite of the macroclimatic niche, it is possible that the species' resistance to other environmental factors may also have been phylogenetically conserved. As such, other potentially limiting factors should be investigated in further laboratory experiments to define any thresholds of the physiological limits. However, the complete characterization of the niche would require knowledge on a large number of environmental factors (Godsoe 2010). Therefore, the relationship between both types of niche should be investigated with a holistic approach. Ideally, laboratory experiments should be combined with field experiments to allow conclusions to be drawn on the interactions between species and their environments (Pearman et al. 2008b, Orsenigo et al. 2014).

6.4 References

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Appendix

Curriculum vitae

Contact information

Name: Maria Hofmann (née Auerswald)
Address: Coburger Straße 47, 14612 Falkensee

Personal details

Date of birth: 21.06.1985
Place of birth: Schlema
Nationality: German

Work experience

04/2012 – 03/2013 Scientific assistant, Martin Luther University Halle Wittenberg
04/2010 – 03/2012 Scholarship student of the graduate scholarship of Saxony-Anhalt,
Martin Luther University Halle Wittenberg

Education

Present – 04/2010 PhD-thesis, Martin Luther University Halle Wittenberg
Topic: Relationship between fundamental and realized niche in plant
species frost hardiness.
03/2010 – 10/2004 Studies of Biology, Martin Luther University Halle Wittenberg
Major subject: geobotany
Minor subjects: plant physiology, behaviour ecology and soil science
Diploma thesis: Frost hardiness and drought tolerance as range limiting
factors of oceanic grassland species.
08/2004 Abitur, Matthes-Enderlein Gymnasium, Zwönitz

Halle (Saale), den 28.05.2015

Maria Hofmann

List of publications

Publications in peer review journals

- Hofmann M, Bütöf A, Welk E & Bruelheide H (2013) Relationship between fundamental and realized niches with respect to frost and drought resistance. *Preslia* **85**: 1-17.
- Hofmann M, Jäger M & Bruelheide H (2014) Relationship between frost hardiness of adults and seedlings of different tree species. *iForest* **7**: 282-288.
- Hofmann M & Bruelheide H (2015) Frost hardiness of tree species is independent of phenology and macroclimatic niche. *Journal of Biosciences* **40**: 147-157.
- Hofmann M, Durka W, Liesebach M & Bruelheide H (2015) Intraspecific variability in frost hardiness of *Fagus sylvatica* L.. *European Journal of Forest Research* **134**: 433-441.
- Hofmann M, Purschke O, Seidler G & Bruelheide H (under review) Can frost hardiness explain plant species' distribution ranges? *Frontiers of Biogeography*

Symposia contributions

- Hofmann M, Liesebach M & Bruelheide H (2011) Intraspecific variability in frost hardiness of *Fagus sylvatica*. *Verhandlungen der Gesellschaft für Ökologie* **41**. Talk
- Auerswald M & Bruelheide H (2010) Phenological differences in frost hardiness of different Central European tree species. *Verhandlungen der Gesellschaft für Ökologie* **40**. Talk
- Auerswald M, Bütöf A & Bruelheide H (2009) Frost hardiness and drought tolerance as range limiting factors of oceanic and continental grassland species. *Verhandlungen der Gesellschaft für Ökologie* **39**. Poster

Erklärung über den persönlichen Anteil an den Publikationen

1. Publikation (Kapitel 2)

Hofmann M, Purschke O, Seidler G & Bruelheide H (under review) Can frost hardiness explain plant species' distribution ranges? *Frontiers of Biogeography*

Laborarbeit: **M. Hofmann** (50%), andere (50%)
 Datenanalyse: **M. Hofmann** (60%), H. Bruelheide (30%),
 O. Purschke (10%)
 Schreiben: **M. Hofmann** (60%), H. Bruelheide (40%),
 Korrekturen von O. Purschke

2. Publikation (Kapitel 3)

Hofmann M, Durka W, Liesebach M & Bruelheide H (2015) Intraspecific variability in frost hardiness of *Fagus sylvatica* L.. *European Journal of Forest Research* **134**: 433-441.

Laborarbeit: **M. Hofmann** (100%)
 Datenanalyse: **M. Hofmann** (60%), H. Bruelheide (30%), W. Durka (10%)
 Schreiben: **M. Hofmann** (60%), H. Bruelheide (40%),
 Korrekturen von W. Durka und M. Liesebach

3. Publikation (Kapitel 4)

Hofmann M & Bruelheide H (2015) Frost hardiness of tree species is independent of phenology and macroclimatic niche. *Journal of Biosciences* **40**: 147-157.

Laborarbeit: **M. Hofmann** (100%)
 Datenanalyse: **M. Hofmann** (70%), H. Bruelheide (30%)
 Schreiben: **M. Hofmann** (60%), H. Bruelheide (40%)

4. Publikation (Kapitel 5)

Hofmann M, Jager M & Bruelheide H (2014) Relationship between frost hardiness of adults and seedlings of different tree species. *iForest* **7**: 282-288.

Laborarbeit: **M. Hofmann** (60%), M. Jager (40%)
 Datenanalyse: **M. Hofmann** (70%), H. Bruelheide (30%)
 Schreiben: **M. Hofmann** (60%), H. Bruelheide (40%)

Halle (Saale), den 28.05.2015

Maria Hofmann

Eigenständigkeitserklärung

Hiermit erkläre ich, dass die Arbeit mit dem Titel **“The relationship between fundamental and realized niche in plant species’ frost hardiness“** bisher weder der Naturwissenschaftlichen Fakultät I der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion vorgelegt wurde.

Ferner erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst sowie keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen wurden als solche von mir kenntlich gemacht.

Ich erkläre weiterhin, dass ich mich bisher noch nie um einen Doktorgrad beworben habe.

Halle (Saale), den 28.05.2015

Maria Hofmann