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ORIGINAL PAPER

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

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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 the 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

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Introduction

Variation in local environmental conditions across the whole range of a species with a large geographic distribution can lead to locally adapted ecotypes (e.g., Deans and Harvey 1996; Repo et al. 2001; Jensen and Deans 2004; Visnjic and 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 intraspecific 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 and Deans 2004; Aldrete et al. 2008; Kathke and 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 1,500 mm in mean annual precipitation (MAP 416–2,030 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 and 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 and Dohrenbusch (2004) demonstrated local adaptation to winter temperature for saplings. Similarly, Kreyling et al. (2012a) showed that 3-year-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 and 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 and 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 and Adams 1997; Kathke and 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 hypothesized 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.

Materials 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 geographic range (Table 1; Fig. 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 × 4 dates), each of them including buds from the same ten trees per provenance. On August 9–10, 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).

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

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

Table 1 Geographic origin (country, latitude, longitude and altitude), minimum temperature of the coldest month (BIO 6) and LT_{50}-value (±SE) of the 20 selected provenances of *F. sylvatica*

Provenance	Country	Latitude (decimal degrees)	Longitude (decimal degrees)	Altitude (m)	BIO 6 (°C)	LT_{50}-value (°C)
2	Spain	N 42.784	W 2.253	950	−0.2	−20.21 ± 1.36
4	Spain	N 41.793	E 2.462	1,100	2.6	−26.78 ± 1.47
9	France	N 48.397	W 1.167	180	1.5	−26.96 ± 3.07
10	France	N 49.282	E 2.625	160	−0.3	−25.05 ± 1.15
14	France	N 44.138	E 2.640	850	−1.1	−20.43 ± 1.16
18	France	N 48.661	E 5.273	350	−2.2	−22.08 ± 0.94
20	France	N 47.211	E 6.264	600	−2.9	−21.53 ± 1.46
24	Denmark	N 55.289	E 10.265	20	−2.0	−23.74 ± 2.10
46	Germany	N 52.989	E 13.120	70	−3.2	−20.51 ± 0.80
61	Germany	N 51.547	E 9.050	305	−2.6	−23.04 ± 1.26
94	Germany	N 48.211	E 7.910	445	−2.1	−21.25 ± 0.93
107	Italy	N 44.143	E 10.674	1,300	−3.1	−21.43 ± 1.26
112	Czech Rep.	N 49.090	E 14.443	520	−5.5	−19.27 ± 1.05
114	Poland	N 49.414	E 20.994	850	−8.9	−21.49 ± 1.21
117	Poland	N 50.360	E 16.853	440	−6.2	−21.68 ± 0.89
125	Slovakia	N 49.092	E 18.291	430	−6.6	−19.22 ± 1.31
139	Croatia	N 45.348	E 14.295	400	2.1	−23.71 ± 1.23
143	Ukraine	N 44.514	E 21.964	400	−4.1	−26.26 ± 1.05
144	Ukraine	N 48.059	E 24.206	500	−8.6	−29.56 ± 1.59
150	Romania	N 46.603	E 24.997	900	−9.6	−21.47 ± 1.07

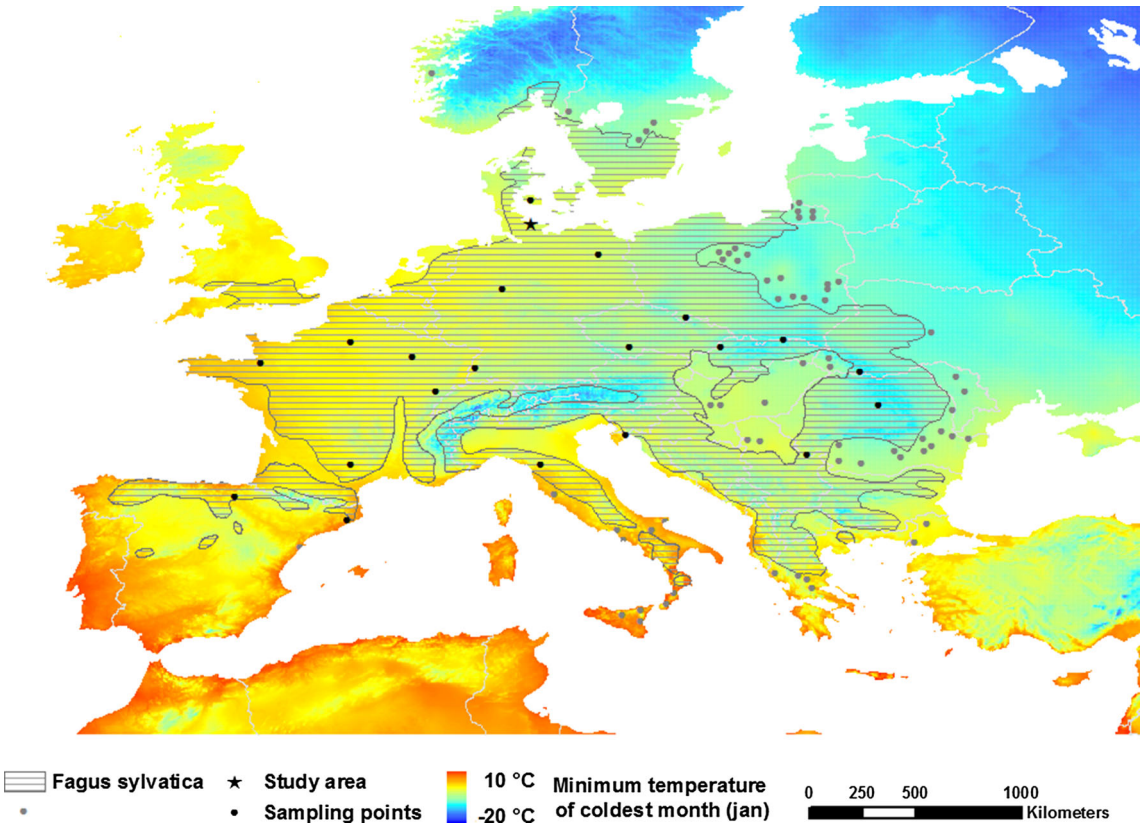

Fig. 1 Distribution map of *F. sylvatica*. *Gray* distribution range, *black dots* selected provenances, *black asterisk* study site

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.

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 of ESM) 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 visualized by principal coordinate analysis (PCoA) based on 129 individuals and 278 loci. In GenAlEx (version 6.2, Peakall and 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).

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 Table 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. 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. 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 ΔAIC = 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

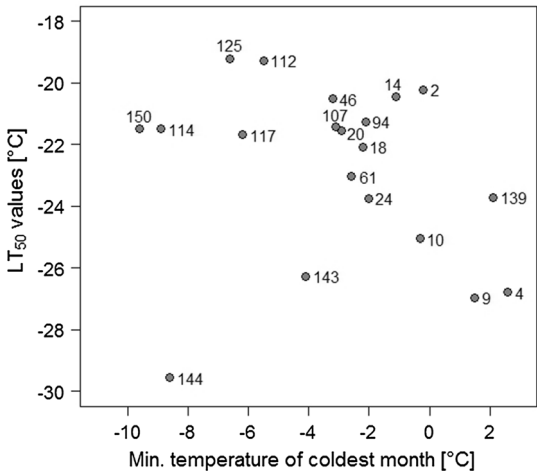

Fig. 2 Frost hardiness of all 20 beech provenances expressed as LT_{50}-values as a function of minimum temperature of the coldest month, $p = 0.379$, $R^2 = 0.043$. Sample numbers refer to provenances (see Table 1)

with minimum temperature below +4 °C (see Fig. 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.

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

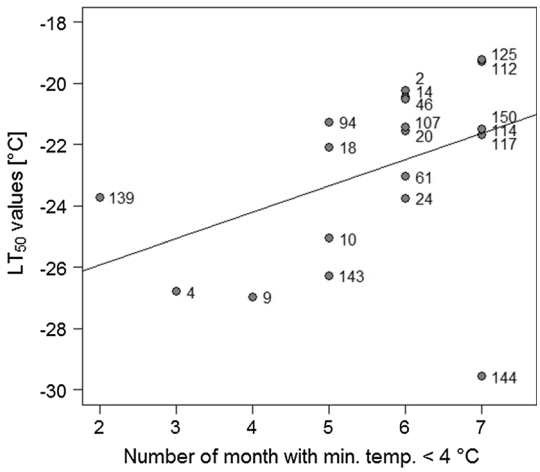

Fig. 3 Frost hardiness of all 20 beech provenances expressed as LT_{50}-values as a function of the number of months with a minimum temperature below +4 °C, $p = 0.062$, $R^2 = 0.180$. Sample numbers refer to provenances (see Table 1)

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 °C (Fig. 4). Interestingly, the group of provenances with a high October minimum temperature (>6.45 °C) showed the lowest LT_{50}-values (on average −25.25 °C). The group of provenances with a minimum temperature in October below +6.45 °C showed a second split into provenances with a temperature annual range (BIO 7) below and above +29.15 °C. The group of provenances with a high annual temperature (>29.15 °C) exhibited lower LT_{50}-values (−23.60 °C) as compared to a more even temperature distribution. The group of provenances with a temperature annual range below +29.15 °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.

Genetic variation

The PCoA of AFLP genotypes showed no clustering of provenances (Fig. 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 Table A1 of ESM). 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 ($r = 0.2085$, $p = 0.047$).

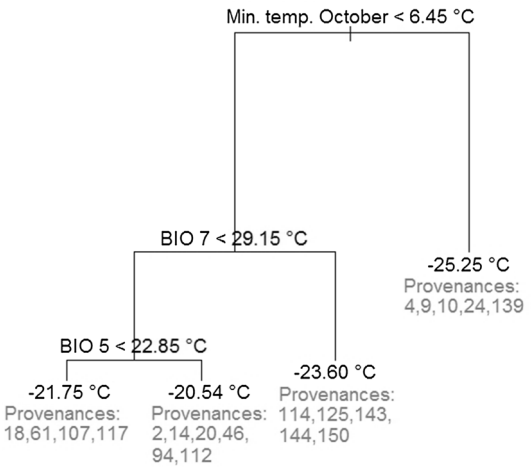

Fig. 4 Regression tree for predicting LT_{50}-values, min. temp. October: minimum temperature in October; BIO 7: temperature annual range; BIO 5: maximum temperature of the warmest month. *Black values* at the *tree tips* are mean LT_{50}-values across the populations indicated

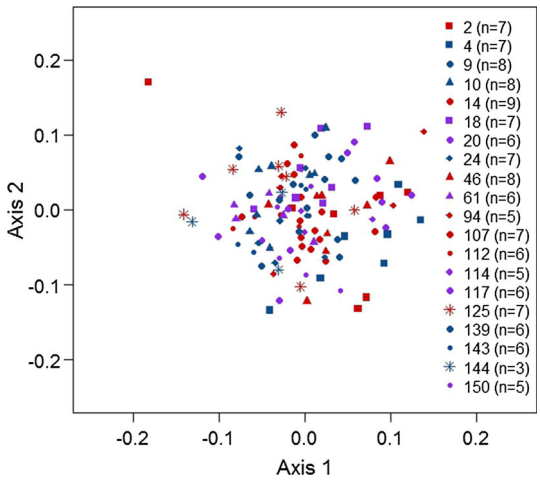

Fig. 5 Principal Coordinate Analysis (PCoA) of all 20 beech provenances. Frost-sensitive provenances are shown with *red symbols* (LT_{50}-values from −19.22 to −21.43 °C), moderately frost-resistant provenances with *magenta symbols* (LT_{50}-values from −21.47 to −23.04 °C) and frost-resistant provenances with *blue symbols* (LT_{50}-values from −23.71 to −29.56 °C). Sample numbers refer to provenances (see Table 1)

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 °C 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 and 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 post-glacial 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 and 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 and 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 and 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 and 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 analyzed, 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.

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