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Linking dendroecology and association genetics in natural populations: Stress responses archived in tree rings associate with SNP genotypes in silver fir (*Abies alba* Mill.)

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Running title**: Dendroecology in genetic association studies**

Abstract

Genetic association studies in forest trees would greatly benefit from information on the response of trees to environmental stressors over time, which can be provided by dendroecological analysis. Here, we jointly analyzed dendroecological and genetic data of surviving silver fir trees to explore the genetic basis of their response to the iconic stress episode of the 1970s and 80s that led to large-scale forest dieback in Central Europe and has been attributed to air pollution.

Specifically, we derived dendrophenotypic measures from 190 trees in the Bavarian Forest that characterize the resistance, resilience and recovery during this growth depression, and in the drought year in 1976. By focusing on relative growth changes of trees and by standardizing the dendrophenotypes within stands, we accounted for variation introduced by micro- and macroscale environmental differences. We associated the dendrophenotypes with single nucleotide polymorphisms (SNPs) in candidate genes using GLMs and the machine learning algorithm random forest with subsequent feature selection. Most trees at our study sites experienced a severe growth decline from 1974 until the mid-1980s with minimum values during the drought year. Fifteen genes were associated with the dendrophenotypes, including genes linked to photosynthesis and drought stress. With our study, we show that dendrophenotypes can be a powerful resource for genetic association studies that permit to account for micro- and macro-environmental variation when data is derived from natural populations. We call for a wider collaboration of dendroecologists and forest geneticists to integrate individual tree-level dendrophenotypes in genetic association studies.

Introduction

The response of forest trees to episodic stress, such as droughts, storms or pollution, is of particular interest in evolutionary forest ecology as the frequency of episodic stress is expected to increase significantly during the 21st century (IPCC, 2014). In this context, genotype-phenotype associations in natural populations are an important step towards the identification of genetic regions that are potentially involved in the stress response of trees (e.g. Budde et al., 2014; Parchman et al. 2012; Santiago C. González-Martínez, Ersoz, Brown, Wheeler, & Neale, 2006). However, recording phenotypic responses to episodic stress under natural conditions is notoriously difficult due to the unforeseeable timing of such episodes. Here we utilize the fact that trees archive their reaction to environmental conditions in their wood anatomical structure and annual growth rings (Fritts & Swetnam, 1989). This provides the unique opportunity to reconstruct and measure individual responses to stress in the past and we call such measures derived from the dated annual growth rings dendrophenotypes.

An iconic stress episode took place in Europe in the 1970s and 1980s (Kandler & Innes, 1995) which led to a large scale forest dieback and left a clear imprint in the growth pattern of surviving trees. This growth decline has been widely attributed to air pollution, particularly to SO_2 emissions in combination with a series of exceptionally dry years (Elling, Dittmar, Pfaffelmoser, & Rötzer, 2009). The conifer *Abies alba* Mill. (silver fir) was particularly affected with large scale dieback and severe growth decline as shown in previous dendroecological studies (Büntgen et al., 2014; Kandler & Innes, 1995). Since classical dendroecological studies discard the variation among individuals as noise and merely focus on reactions at the stand level (Buras et al., 2016), it is not yet clear to what extent individual trees differed in their responses to this stress episode. Consequently, it also remains unknown whether such putative different responses (including mortality) were merely stochastic, related to differences in microenvironment or caused by (pre-)adaptation.

In this study, we focused on individual level responses to the stress episode to determine the variation among trees. We then associated individual responses with candidate genes to determine whether trees that coped better with these conditions shared specific alleles in candidate genes. For this purpose, we characterized the growth decline of silver fir trees in the 1970s and 1980s by applying the resilience concept of Lloret *et al*. (2011). Specifically, we assessed the resistance, resilience and recovery for 193 silver fir trees from the Bavarian Forest National Park and associated them with SNPs at candidate genes mainly related to stress responses (Roschanski et al., 2016). Since growth as a quantitative trait is likely influenced by many genes, we not only applied a single-locus approach for the genetic association, but also used a random forest analysis to capture both the marginal effect of single SNPs, as well as the combined effect of multiple SNPs on a phenotype. As the stress episode caused significant dieback, we expect that a large majority of the surviving trees exhibit a marked growth decline in the 1970s and 1980s, but with a high level of variance among individuals regarding resistance, resilience and recovery. Further, we expect the variation in dendrophenotypes to be associated with genetic variation in a number of the targeted genes, and thus, to validate them as candidate genes for stress response. We discuss the potential of dendrophenotypic measures in genetic association studies for exploring the genetic basis of growth decline and resilience in episodic stress scenarios in the context of climate change.

Material and Methods

Study site

Silver fir trees were sampled and monitored in two sites in the Bavarian Forest National Park, Germany, which are situated at opposite ends of an elevational gradient but well within gene flow distance (as confirmed by our STRUCTURE and PCA results, see below) to maximize phenotypic variation and minimize drift effects at the same time (Lotterhos & Whitlock, 2015). Mean annual temperature varies between 3.8°C and 5.8°C with a mean annual precipitation from 1,200 to 1,800 mm in the National Park (Bässler, 2004). Silver fir grows in mixed mountain forests in combination with European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*), which is the natural vegetation at elevations below 1,150 m. Our sampling sites were located at 770 m a.s.l. (*Filzwald*; 48.929°N, 13.406°E) and 1.120 m a.s.l (*Rachelsee*; 48.975°N, 13.400°E) on the southern slope of Mt. Rachel (Fig. 1). At each site, 100 adult silver fir trees were georeferenced and permanently marked with numbered tags. Temperature and humidity were recorded at both sampling sites with data loggers (DK320 DM HumiLog, Drießen & Kern, Bad Bramstedt, Germany) starting in spring 2014. The lower elevation sampling site ('low site' hereafter) is characterized by flat terrain and subjected to accumulating cold air from higher elevations, which leads to frequent late and early frost events in spring and autumn, respectively. In contrast, the sampling site at higher elevation ('high site' hereafter) is located on a steep slope surrounding the Rachel lake. The lake influences the local climatic conditions by buffering cold temperatures. Therefore, early fall and late spring frost events are less frequent and maximum temperatures are lower than at the low site. Mean temperature, however, did not significantly differ between sites during our study period (Table S1).

Calculation of individual-level response parameters

To obtain data on tree-ring width (TRW), we extracted two wood cores per tree at breast height with a 5 mm increment borer (Haglöf Sweden AB, Långsele, Sweden). When trees grew on slopes, they were cored at a 90° angle to the slope to avoid compression wood. After drying, intact or slightly fractured wood cores were cut with a microtome (WSL, Birmensdorf, Switzerland) to obtain a smooth surface. The contrast between earlywood and latewood was enhanced with chalk. Cores that had several fractures (14 out of 375) were mounted on wooden holders and smoothed with sandpaper. For 11 trees, one of the cores could not be analyzed due to a high number of fractures or missing segments. TRW was measured with a precision of 0.01 mm using a LINTAB digital positioning table whose movements were transmitted to the TSAP-Win Scientific Software (Rinntech, Heidelberg). A master series for each site using COFECHA (Grissino-Mayer, 2001) was constructed and each series was cross-dated against this master series to avoid dating errors due to missing rings. In total, we obtained reliable data from 375 cores of 193 trees.

All tree ring time series were standardized to a mean value of one to obtain a dimensionless tree-ring index (TRI) using the *detrend* function in the R package dplR (Bunn, 2008; R Core Team, 2016). Based on the inspection of chronologies at the site level, we identified the years from 1974 to 1983 as the period where most trees exhibited the strongest growth decline. In the following, we refer to this period as "depression period" and to the ten years before and after as "reference periods" (Fig. 2). For the dendrophenotypic characterization of

individual trees, we determined their resistance, recovery and resilience (following Lloret et al., 2011) to possible effects of airborne SO_2 pollution and drought (Fig. 2). In this framework, resistance describes the ratio of TRI during vs. before the extreme event; recovery describes the ratio of TRI after vs. during the event; and resilience describes the ratio of TRI after vs. before the event.

Based on these definitions and rationale, we determined the following dendrophenotypes: (1) the steepness of the start of the depression period in 1974 was defined as the slope of the standardized TRI between the years 1973 and 1974; (2) the resistance in the depression period was defined as the ratio between the average TRI from 1964 to 1973 and the average TRI during the depression period (1974-1983); (3) the recovery after the depression period was calculated as the ratio between the average TRI in the ten year reference period after 1983 and the average TRI during the depression period, and (4) the end of the individual growth depression was defined as the year when growth surpassed the level during the pre-depression reference period. To calculate the latter, we compared mean TRI after 1973 in a moving window of three years to the mean growth in the period of 1964 to 1973 (Fig. 2) and determined the year when the mean of the moving window first surpassed the mean of this period.

In addition, we focused on the individual growth reaction in the year 1976, which has been identified as one of the driest summers in Europe (Briffa, van der Schrier, & Jones, 2009). We used the *res.comp* function in the R package *pointRes* (van der Maaten-Theunissen, van der Maaten, & Bouriaud, 2015) to calculate (5) the resistance , (6) resilience and (7) recovery of each tree towards the drought conditions in 1976. For the calculation, we considered a two-year window to take into account the already reduced growth in the two years prior to 1976 within the period of growth depression (Lloret et al., 2011). To determine whether dendrophenotypes differ between sites, we compared them with Welch's unequal variances *t*-tests.

Accounting for confounding environmental factors

Phenotype-genotype association studies in natural populations are generally challenged by the confounding effects of the environment. Using TRW time series in association studies offers a unique opportunity to account for these at different spatial scales. The rationale is that each tree remains its entire adult life in a relatively constant micro-environment (including elevation, slope, aspect, geology and edaphic conditions including relative water availability), while macro-scale environmental variation (including climate, biotic interactions or pollution) will act on the entire tree stand. Thus, by using dendrophenotypic measures that focus on relative changes in growth instead of absolute values, we derive an individual measure of a tree´s response largely independent of its micro-environment. This allows comparing individual tree responses within a stand, and associate it with the individual tree's genetic background.

Likewise, for comparisons among stands, macro-scale environmental differences were accounted for by focusing on the strength of the response of trees relative to trees from the same site. To achieve this, dendrophenotypes of all trees from a given site were centered and scaled by using the *scale* function in the R *base* package with default parameters before they were included in a single analysis. This permits to compare the relative responses of trees among sites.

Genotyping

For DNA extraction, fresh needles were collected from each tree, and immediately dried on silica gel. Needles were sent to LGC Genomics (Middlesex, United Kingdom) for genotyping. Using KASP (short for Kompetitive Allele Specific PCR) assays, we targeted 267 polymorphic and functionally annotated SNPs in candidate genes whose selection is described in detail by Roschans*ki et* al. (2013, 2016). Briefly, genes were selected based on a literature search that included the keywords adaptation, candidates, drought, evolution, RT-PCR and selection, or based on drought-related Gene Ontology terms. Out of these 267 SNPs, 241 could be successfully genotyped using the KASP technology in our samples.

The dataset was initially filtered by removing SNPs that were not called correctly for the majority of the data set (> 80 % missing data) because they could interfere with subsequent filtering steps. Subsequently, individuals and SNPs with more than 10 % missing data, as well as monomorphic SNPs, were removed. We then selected all SNPs with a minor allele frequency > 3 %. All SNPs were tested for pairwise linkage disequilibrium (LD) using the Genome Variation Server 147 v. 12.00 (National Heart, Lung, and Blood Institute, http://gvs.gs.washington.edu/GVS147/index.jsp). If pairs of SNPs were tightly linked ($r^2 = 1$) one of the SNPs was removed. This was only the case for SNPs located on the same contig of the transcriptome assembly (see Roschanski et al., 2013).

We imputed missing genotypes using Beagle 4.1 (Browning & Browning, 2016) without using a reference sequence. We acknowledge that this might introduce a certain error in the dataset but was unavoidable for our downstream data analysis as random forest does not tolerate missing data. After imputation, the dataset was cleaned again using the same filtering steps as described above. Finally, all SNPs with less than five individuals per allele combination were removed from the dataset to ensure sufficient replication, resulting in 193 individuals and 130 SNPs from 103 genes.

Population clustering

As the methods we used for phenotype-genotype association are sensitive to population structure, we applied two approaches to determine whether we could detect a genetic structure within or between sampling sites. First, we used the Bayesian clustering algorithm implemented in STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) based on the admixture model with correlated allele frequencies. We set the burn-in to 10^5 iterations followed by 5×10^5 Markov chain Monte Carlo (MCMC) repetitions. We conducted 10 runs for each K from 1 to 6. Second, we conducted a principal component analysis (PCA) as implemented in the R package *adegenet* (Jombart & Ahmed, 2011).

Genetic association analysis

For the association analysis of SNPs and dendrophenotypes, two approaches were used. First, we applied a frequently used univariate approach, namely general linear models (GLMs) as implemented in TASSEL v. 5.0 (Bradbury et al., 2007), with each SNP as the independent variable and each dendrophenotype as the response variable. For each dendrophenotype, we ran GLMs with 10,000 permutations to obtain *p*-values independent of the data distribution. The threshold for statistical significance at 5% was 0.007 after Bonferroni multiple test correction (0.05/7).

Univariate approaches only take into account a single SNP per test. However, most phenotypes are influenced by multiple genes with small effects (Pritchard, Pickrell, & Coop, 2010). Thus, we also applied the machine learning algorithm "random forest" which captures both marginal and interaction effects among SNPs. In this study, we used the feature selection procedures as implemented in the R packages Boruta (Kursa, Rudnicki, & others, 2010) and VSURF (Genuer, Poggi, & Tuleau-Malot, 2015). A detailed description of these methods is provided in the supplement (Methods S1).

Although SNPs were already annotated for a previous publication (Roschanski et al., 2016), we repeated this step, as the information in the NCBI database is constantly updated. All SNPs that could be associated with the dendrophenotypes with more than one of the above mentioned methods were compared to known sequences from NCBI's GenBank non-redundant protein database (NR) using the translated BLAST algorithm (blastx v. 2.6.1+, Altschul et al., 1997). The best hit, based on the Expect value that provided a functional annotation, was selected for each gene and the corresponding biological process keywords were retrieved from the Gene Ontology (GO) database ("Gene Ontology Consortium," 2015).

Results

Dendrophenotypes

We obtained data on dendrophenotypes for 98 and 95 individuals from the high and low site, respectively. Almost all trees from the high plot (97%) were affected by a growth decline, whereas only 80% of the low-site individuals showed such a response, as can be seen from the values for resistance in the depression period which are below 1 (Fig. 3B). For the end of the depression period, we determined mean values in 1986 and 1987 for the low and high site, respectively (Table 1). Individuals from the high site had significantly lower values for slope at the depression start and for the resistance in the depression period and, on average, significantly higher values for recovery after the depression when compared to the low site (Fig. 3, Table 1). Most trees on the high site showed a marked growth decline in 1976, while trees from the low site showed, on average, no further decline which resulted in a significantly higher value of resistance in 1976 (Fig. 3E). At the low site, trees already showed an increase in growth after 1976, which is reflected in the higher values for recovery and resilience, although only the latter differed significantly between sites (Fig 3F,G).

Population structure

Neither the STRUCTURE analysis, nor the PCA provided any indication for population substructure. The visual inspection of the bar plots in STRUCTURE clearly showed that almost all individuals were assigned to both clusters in a scenario with K = 2 without any apparent pattern (Fig. S1), and Ln P (D) declined steadily with increasing K (Fig. S2). In accordance, point clouds resulting from the PCA showed no apparent difference between the sampling sites (Fig. S3). Thus, population structure was ignored in the subsequent analysis.

Genetic association

The different association methods provided us with largely different numbers of SNPs associated to our dendrophenotypes (Table 2, Table S2). VSURF identified 10 to 22 SNPs for every dendrophenotypic trait with the exception of the recovery in 1976, for which VSURF only found one SNP. Boruta detected a lower number of SNPs, ranging from zero to six, which were also detected by VSURF in most cases. The GLM in TASSEL yielded no significant results (adjusted permutation p-value < 0.007).

In total, 15 SNPs were jointly identified by at least two approaches. Most of these SNPs are located in genes that code for membrane proteins related to transport and stress responses (Table 3).

Discussion

In our study, the large majority of the investigated silver fir trees in the Bavaria Forest showed a pronounced growth decline from 1974 until the mid-1980s. Growth of most trees was reduced even further during the drought year in 1976. When we jointly analyzed the dendrophenotypic and genetic data, and simultaneously considered the effects of multiple SNPs with a random forest approach, we found that the variation in five out of seven dendrophenotypic traits was associated to the allelic variation of SNPs in 15 candidate genes.

Dendrophenotypes

As expected, we found a pronounced population-level growth decline in the 1970s and 1980s and population level recovery thereafter, as described earlier for silver fir in southern Germany (Elling et al., 2009). Although 1976 was not detected in an analysis for so-called pointer years (defined as years were > 70% of all trees show a growth change >10 % compared to previous years, see Cropper, 1979), we found that the 1976 drought reduced silver fir growth, particularly at the high site. As the methods to determine pointer years take a number of prior years as reference they are likely to fail if the previous years are already marked by reduced growth. In contrast to 1976, dry years that did not coincide with the depression period (e.g. 1959, 1972, 1982 and 2003) did not have a strong effect on silver fir growth (Fig. 2). This is in line with Elling *et al*. (2009), who argued that SO_2 pollution not only causes direct harm to silver fir trees by impeding photosynthesis and leading to the shedding of needles, but that it also increases sensitivity to drought, which might be attributable to

damages of the fine-root system. While our dataset is limited to surviving trees, inventory data from the area showed that silver fir dieback was substantial in the 1970s and 1980s. For example, some forest stands in the Bavarian Forest lost more than 75% of 80 - 120 year-old silver firs (unpublished inventory data, National Park Plan 1992). We observed that trees at high elevations were affected more severely during the depression period, as reflected by the generally lower resistance and resilience. Two explanations are possible. Either, the trees from the two sites differ in their adaptation that our SNP data does not capture, or, more likely, environmental differences between the two sites, i.e. climate or SO_2 depositions, are responsible. That the existing climatic differences (Table S1) affect the trees can clearly be seen e.g. in differences of bud burst timing in spring (Figure S4) and in lower average growth rate on the high site (Table S3). Thus, it is plausible that additional stress under the less favorable growing conditions at the high site explain the observed differences. In addition, it is possible that SO_2 concentrations were higher at the high sites or that their effect was aggravated by site-specific conditions around the lake Rachel, e.g. higher air humidity. However, without on-site measurements of SO_2 concentrations this remains speculative.

It has also been proposed that tropospheric ozone (O_3) might have been a contributor to the observed forest decline (Krause et al., 1986; Schmieden & Wild, 1995). However, tropospheric O_3 concentrations have been increasing well beyond the timeperiod of the observed growth depression (de Vries et al. 2014), which makes it implausible that O_3 played a major role.

As shown for other Central European silver fir stands, growth rates after the depression reached an unprecedented high level, which is usually attributed to a combination of less dense forest structure after the dieback in the 1970s, as well as elevated nitrogen supply and increasing temperatures (Büntgen et al., 2014; Elling et al., 2009; Pinto, Gégout, Hervé, & Dhôte, 2007).

Dendrophenotype-genotype association

So far, a few studies have jointly analyzed genetic and dendroecological data in natural populations to explore the relationship between basic genetic parameters and growth traits at the level of individual trees (e.g. Pluess and Weber 2012; Babushkina et al. 2016). None of the studies found a strong genetic signal related to the investigated growth traits, which could either be attributed to stronger effects of the environmental signals compared to the genetic influence on growth processes, or to a lack of adequate genetic data (e.g. loci that are relevant for the phenotypic traits considered). To take this one step further, we correlated dendrophenotypes and variation at stress response candidate genes (Roschanski et al., 2016) using a genetic association approach.

To our knowledge, this is the first association study that links dendrophenotypes with SNPs in stress-related candidate genes in natural populations. At our study sites, SNPs in 15 of the 103 candidate genes associated significantly with individual dendrophenotypic traits. Many of the genes were membrane proteins of the chloroplast, mitochondria or tonoplast, and thus, tightly linked to photosynthesis or chloroplast development. For example, SNPs in contigs 716 and 14,580, which are associated to resistance and resilience in the depression period, respectively, encode for stromal 70 kDa heat shock-related protein and a proteolytic

subunit of the ATP-dependent Clp protease. Both are involved in protein folding with effects on chloroplast development and function (Latijnhouwers, Xu, & Møller, 2010; Sjögren, Stanne, Zheng, Sutinen, & Clarke, 2006). Since SO_2 pollution likely has negative effects on photosynthesis (Silvius, Ingle, & Baer, 1975), genes involved in these pathways could potentially determine how individual trees cope with conditions which were stressful to a degree that they caused the death of many individuals (unpublished inventory data, draft of the National Park Plan 1992). Two of the genes that were exclusively associated with resistance and resilience in 1976 can be directly related to drought stress response: aquaporin TIP2-1 and glucan-endo-1,3-beta-glucosidase. Aquaporins are regularly involved in drought response (Hamanishi & Campbell, 2011; Maurel, Verdoucq, Luu, & Santoni, 2008) and a similar aquaporin (TIP1-1) has already been identified as differentially expressed in response to drought stress in silver fir seedlings (Behringer, Zimmermann, Ziegenhagen, & Liepelt, 2015). Glucan-endo-1,3-beta-glucosidase was previously used as a drought stress candidate gene in *Pinus pinaster* (Eveno et al., 2008) and was also differentially expressed in response to drought stress in silver fir seedlings (Behringer et al., 2015).

Two previous studies investigated local adaptation in silver fir along altitudinal gradients in France (Roschanski et al., 2016) and Southern Europe (Brousseau et al., 2016) using the same candidate loci. Both studies identified a subset of SNPs that showed patterns of divergent selection or correlated with environmental variables. The SNPs that associated with the dendrophenotypes did not overlap with the SNPs that showed evidence of directional selection in France. Yet, two of the candidate genes that were associated with resistance and resilience in 1976 (contigs 04538 and 16332) in our study were among the SNPs that were considered to be under divergent selection in the study of Brousseau et al. (2016), which provides additional evidence that these genes play a role in adaptation to extreme environmental conditions and are thus validated as candidate genes. Eight of the SNPs found in associations are synonymous. These mutations might be in linkage with functional variants, or alternatively, might impact gene expression via codon bias, which has been shown to affect translational efficiency in conifers (Torre, Lin, Peer, & Ingvarsson, 2015). Naturally, our approach is limited to trees that have survived the stressful period in the 1970s and 1980s. Therefore, we are not able to detect genetic variants that might have contributed to the death vs. survival of silver fir, and thus, might miss a major effect of selection during this period.

One other current study (Housset et al., 2018) also uses dendophenotypes in a genetic association in *Pinus strobus*, yet, unlike our study, in a common garden setting. Similar to our results, they can confirm associations with candidate genes that were previously identified in a genotype-environment association study. As Housset and coworkers stated already in a study in 2016 (Housset et al., 2016), common gardens are the most direct way of accounting for environmental differences among populations, yet they are not available for most tree species. Furthermore, common gardens are usually even-aged, relatively young, and usually only represent a single and, more importantly, moderate environment. Thus, exposure to extreme stress in already established common gardens is extremely rare and responses to ecologically relevant stress can therefore hardly be studied. Studies in natural populations on the other hand, allow integrating the genetic diversity of populations that directly resulted from natural demographic and selection processes. They thus permit investigating phenotypic responses under realistic natural settings to which the trees are

adapted (Sork et al., 2013). Thus, while common gardens remain the most direct way to account for environmental differences, our new approach of using relative changes in annual growth to account for environmental differences among stands is a significant step forward in reducing false positives when working with natural populations in association studies. In conclusion, both approaches are relevant for unravelling the genetic basis of phenotypic traits and nicely complement each other.

Statistical analyses of dendrophenotypes in association studies

The dendrophenotypes in this study are complex quantitative traits, which are most likely influenced by many genes with small effects. Thus, classical single-locus approaches have limited power to detect the underlying genetic signal and it is key to utilize approaches that simultaneously consider the effects of multiple SNPs. Here, we applied a random forest based feature selection to identify SNPs that are likely associated with certain dendrophenotypes. The Boruta algorithm provides significant results by testing if the importance of a SNP for explaining a dendrophenotype is significantly ($\alpha = 5\ \%$) higher than the importance of the most important randomly permuted attribute, which, under the null hypothesis, is only associated by chance (Kursa et al., 2010). In contrast, VSURF does not incorporate any formal statistical hypothesis-test, but selects the most important SNPs regarding the association with a specific dendrophenotype (Genuer et al., 2015). However, this does not imply a statistically significant association. Both feature selection techniques are wrappers for the random forest algorithm and, as such, the importance value is a measure for the marginal effect of a SNP, as well as the interaction effect of all SNPs under consideration. It should be mentioned, however, that the relative contribution of marginal and interaction effect cannot be directly determined. Thus, SNPs identified by random forest procedures do not represent a network and have to be viewed independently. The benefit of such analyses is, however, that the influence of all other SNPs is incorporated in the importance of any given SNP, which provides a much better representation, given that in association studies of conifers, a single SNP never explained more than 5 % of the variation of a given trait (Eckert et al., 2009; S C González-Martínez, Huber, Ersoz, Davis, & Neale, 2008; e.g. Santiago C. González-Martínez et al., 2006). For our study, we acknowledge that only a minor part of the genetic variation is represented, and that future studies are needed to back up our findings incorporating exome capture or full genome data to represent the genetic variability underlying complex traits such as growth. For this purpose, the genome of silver fir is currently being sequenced (www.aforgen.org/sfgp/index). We are confident that random forest and subsequent feature selection are promising tools for association studies.

Conclusion and outlook

In our study, we have shown that the time series nature of dendrophenotypes permits to characterize past tree responses to extreme episodes in the past. Wood cores can be collected with an acceptable investment of time and money in the field and allow for diverse subsequent analyses of dendrophenotypes. Furthermore, we have shown that the availability of time series data permits to focus on relative measures and thus allows accounting for confounding variation of micro- and macro-scale environmental conditions in association-studies. While it will not be possible to fully eliminate environmental confounding factors, our approach should greatly reduce their influence on the analyses and thus

minimize the risk of false positives. This permits focusing on the genetic component of the phenotypic response to macro-scale environmental episodic stress also when comparing stands in contrasting environments. The effectiveness of this approach could further be validated in future studies by comparing the growth of clonal trees in common gardens in identical versus differing environments.

To facilitate this kind of research, dendroecologists should start to focus on individual tree signals and not discard them as noise per default. In that respect, available dendrocore repositories and databases could be a valuable resource for future genetic association studies. Likewise, while we focused on dendrophenotypes derived from tree-ring width, wood cores harbor many additional time-series traits that should be explored in future studies. These include anatomical features such as cell wall thickness or lumen area which are considered as proxy for physiological adaptations to external factors (Carrer, Brunetti, & Castagneri, 2016; Ziaco, Biondi, & Heinrich, 2016) as well as isotope measures, which, for example, can be used to characterize the water use efficiency of a tree (Seibt, Rajabi, Griffiths, & Berry, 2008). Microdensitometry can supplement ring width data with information of wood density, and thereby provide a more complete picture of growth, for example during extreme events (e.g. Martinez-Meier, Sanchez, Pastorino, Gallo, & Rozenberg, 2008). All these measures can be derived for long time series or with a focus on particular years of interest, providing exciting prospects for future studies. Forest geneticists on the other hand should focus on pathways and candidate genes that are potentially related to dendrophenotypes (e.g. genes related to wood formation).

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writing the manuscript: AP, ANP, BF, BZ, CB, DB, GGV, KH, LO, PA, RB, SVL, SL, UB

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Data accessibility

The SNP data used in the final analysis is available in the Table S4. This data set comprises the data as used, after cleaning, imputation, and removal of SNPs with a minor allele frequency < 3 %. Further, we provide the raw dendrophenotypes (Table S5) as calculated according to Lloret et al. (2011). This data has been standardized as described in the methods section prior to using it in the association analysis.

Figures

Figure 1. Study site in the Bavarian Forest National Park with the sampling sites Rachelsee ('high site', 1120 m a.s.l.) and Filzwald ('low site', 770 m a.s.l.).

Figure 2. Mean tree-ring index (TRI) for the low and high site for each year in the period from 1950 to 2013. The shaded reference and depression periods are the basis for the calculation of the response of individual trees to air pollution. The insets (modified after Lloret et al. 2011) are simplified graphical representations of the dendrophenotypic measures resistance (Rt), recovery (Rc), as well as the start (Slope) and end (End) of the growth depression (solid box) and resistance (Rt), resilience (Rs), recovery (Rc) for the drought year 1976 (dashed box). For details regarding the calculation of the indices, see material and methods.

Figure 3. Dendrophenotypes for trees from the low and high sampling site. Depicted dendrophenotypes describe the slope of the regression at the start of the depression period (A), the trees' resistance to (B) and recovery from (C) the conditions during the depression, as well as the end of the depression period (D). In the second row, dendrophenotypes are depicted that characterize the resistance (E), resilience to (F) and recovery from (G) the drought year 1976. For a better visualization of the data distributions, two extreme values for recovery from the drought year 1976 were removed.

Tables

Table 1. Summary statistics for the comparison of dendrophenotypes between the two sites. Test results from a Welch's *t*-tests are provided.

Site	***Slope 1973 – 74***	***Resistance DP***	***Recovery DP***	***End of depression***	***Resilience 1976***	***Resistance 1976***	***Recovery 1976***
Low site (mean ± SD)	-0.15 ± 0.13	0.82 ± 0.67	1.66 ± 1.11	1986.49 ± 11.21	1.37 ± 0.41	0.97 ± 0.30	1.59 ± 1.76
High site (mean ± SD)	-0.30 ± 0.20	0.58 ± 0.21	1.96 ± 0.89	1987.48 ± 6.57	0.95 ± 0.30	0.76 ±0.22	1.41 ± 1.37
t	-5.983	-3.3264	2.0079	0.72087	-7.9174	-5.4393	-0.81124
Df	169	109	179	146	171	173	176
p-value	**<0.001**	**0.0012**	**0.046**	0.47	**<0.001**	**<0.001**	0.48

SD: standard deviation, *t*: *t*-statistic, df: degrees of freedom. DP = depression period

Table 2. Overview of the results of the different association methods (Tassel GLM, Boruta and VSURF) for each dendrophenotype. Values in cells indicate the number of SNPs identified by each method for a given dendrophenotypes. DP = depression period.

	Slope 1973 – 74	***Resistance DP***	***Recovery DP***	***End of depression***	***Resilience 1976***	***Resistance 1976***	***Recovery 1976***
GLM	0	0	0	0	0	0	0
Boruta	1	4	6	1	3	6	0
VSURF	22	10	18	11	17	13	1
Boruta + VSURF	1	3	5	0	3	6	0

Table 3. SNPs associated with scaled dendrophenotypes using Boruta and VSURF. Only SNPs that were associated with a given dendrophenotype with both methods are shown. A full table with all detected associations is provided in the supplementary material (Table S2). SNP IDs refer to the contigs and position of the SNP within the respective contig in the assembly of Roschanski et al. (2013). SNP type indicates whether a SNP is synonymous (syn), non-synonymous (non-syn), or located in intergenic regions. For non-synonymous SNPs, the Amino acid change (AAC) is indicated. DP = depression period.

SNP ID	Protein	Biological Process (with capital letter: GO keyword)	SNP type	AAC	Associated dendrophenotype
00628.351	mitochondrial arginine transporter BAC2	Stress response, transport	intergenic		Resistance DP
00716.144	heat shock 70 kDa protein 7, chloroplastic	Protein transport, stress response, transport	syn		Resistance DP
02190.265	ATP-dependent Clp protease proteolytic subunit 4, chloroplastic-like	Chloroplast organization, regulation of timing of transition from vegetative to reproductive phase	Non-syn	A >T	Slope 1973 - 74
04538.470	60S ribosomal protein L7-2-like	Cytoplasmic translation, maturation of LSU-rRNA from tricistronic rRNA transcript	intergenic		Resistance 1976
08092.366	T-complex protein 1 subunit epsilon	protein folding	syn		Recovery DP
08855.137	Lhca4 protein, Type 4 protein of light-harvesting complex of photosystem I	Photosynthesis	syn		Recovery DP
09197.63	aquaporin TIP2-1	Transport	syn		Resistance 1976
10568.484	GDP-mannose pyrophosphorylase	Cellulose biosynthetic process, defense response to bacterium, GDP-mannose biosynthetic process, L-ascorbic acid biosynthetic process, response to ammonium ion, response to heat, response to jasmonic acid, response to ozone, response to salt stress	syn		Recovery DP
12178.301	glucan-endo-1,3-beta-glucosidase	Plant defense	syn		Resilience 1976
14580.627	ATP-dependent Clp protease proteolytic subunit-related protein 4, chloroplastic-like	Regulation of gene expression, response to reactive oxygen species	syn		Recovery DP
15256.604	Ferredoxin-NADP reductase, leaf-type isozyme, chloroplastic, partial	Electron transport, photosynthesis, transport	syn		Resistance DP, Resilience 1976, Resistance 1976
16332.419	proteasome subunit beta type-6	Proteolysis involved in cellular protein catabolic process	non-syn	D > E	Resilience 1976
16411.197	Mitochondrial carnitine/acylcarnitine carrier-like protein	Transport	intergenic		Resistance 1976
16430.504	NADH dehydrogenase (ubiquinone) iron-sulfur protein 7, mitochondrial	Electron transport, Respiratory chain, Transport	intergenic		Recovery DP, Resistance 1976
24318.117	LIM domain-containing protein WLIM2b isoform X1	-	intergenic		Resistance 1976