Population genetics of silver fir (*Abies alba* Mill.) in the Northern Black Forest – preconditions for the recolonization of windthrow areas and associated ectomycorrhizal communities

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Population genetics of silver fir (*Abies alba* Mill.) in the Northern Black Forest – preconditions for the recolonization of windthrow areas and associated ectomycorrhizal communities

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Ein Land ohne Wälder ist kein Heimatland.

Atatürk

Preface

This dissertation was carried out at the Department of Conservation Biology at the Philipps-University of Marburg from January 2005 to February 2009 under the supervision of Prof. Dr. Birgit Ziegenhagen. It was accomplished within the interdisciplinary project ‚Untersuchungen zur Co-Dynamik der genetischen Diversität von Tanne (*Abies alba* Mill.) und mit ihr vergesellschafteter Ektomykorrhiza-Pilze nach Großstörungsereignissen (Süddeutsche Windwurfflächen)', funded by the ‚Deutsche Forschungsgesellschaft' (DFG; project: Zi698 5/1-2).

Within this project, two PhD positions were appointed focusing on the ectomycorrhizal fungi of silver fir (department 'Mycology') and focusing on the population genetics of silver fir (department 'Conservation Biology'). Combined manuscripts and publications were developed according to manifold interferences of the common subject. This challenged us (my dear colleague Kathrin Donges and me) not to exploit the data received by the other and to focus largely on the respective single topic within our dissertations ('population genetics of silver fir' and 'ectomycorrhizal fungi of silver fir', respectively). I therefore tried to discuss discretely on silver fir as main focus integrating the ectomycorrhizal aspect only additionally. An extensive discussion of the ectomycorrhizal fungi is given within the dissertation of Kathrin Donges.

Marburg, in February 2009

This thesis is based on the following publications and manuscripts. They will be referred to in the text by the term ‘paper’ and their roman numerals.

I. **Identification and characterization of nuclear microsatellite loci in *Abies alba* Mill.**

E. Cremer, S. Liepelt, F. Sebastiani, A. Buonamici, I.M. Michalczyk, B. Ziegenhagen, G.G. Vendramin

Molecular Ecology Notes (2006) 6: 374-376

II. **Estimating local seed dispersal in silver fir (*Abies alba* Mill.)**

E. Cremer, K. Donges, B. Ziegenhagen, C. Mengel, R. Bialozyt, K. Schulerowitz, E. Hussendörfer, S. Liepelt

Manuscript

III. **Ontogenetic and genotypic effects of silver fir (*Abies alba* Mill.) on associated ectomycorrhizal communities**

E. Cremer, K. Donges (both authors contributed equally), S. Liepelt, K.-H. Rexer, G.G. Vendramin, I. Leyer, G. Kost, B. Ziegenhagen

Manuscript

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Abstract

Facing climate change, we expect an increasing frequency of extreme weather events such as storms that affect forest ecosystems. In the last decades, several storm events in Central Europe have damaged huge areas of forest stands that have to be recolonized. Symbiotic systems between trees and ectomycorrhizal (ECM) fungi play a decisive role for the stability and the vitality of trees. In the context of promoting the rare, but 'stabilizing' tree species silver fir in mountain forests and facing the recolonization of windthrow areas, three fir populations were genetically investigated in the Northern Black Forest, Germany. For this purpose, in a first step nuclear microsatellite (nSSR) markers were developed for silver fir. Fir trees of different ontogenetic stages (adults, saplings, seedlings) were genotyped at six nSSR loci and analysed in terms of diversity and abundance of the associated ECM fungi.

The results demonstrate that silver fir populations in the Black Forest maintain a suitable genetic potential with high diversity within and less differentiation among populations. The remaining natural fir regeneration on the windthrow area did not show a reduced genetic diversity in comparison to the adjacent forest stands which include different generations. In addition, dispersal characteristics (gene flow) of firs revealed a sufficient seed and pollen flow of at least a few hundred meters from the mother trees. A high number of mother trees contributed to the seed dispersal and led to a multifaceted seed entry, even into the windthrow areas. Beyond, the analysis of the associated ECM fungi exhibited an identical spectrum of ECM fungi on the windthrow area and in the forest stand. We did not find evidence that the age of the trees can be regarded as driving factor for associated ECM communities on the population level. Based on the individual tree, adults host a higher number of ECM fungi than juveniles. Since the pre-windthrow offspring exhibited a well-balanced ECM profile they serve as 'reservoir hosts' for post-windthrow offspring promoting their vitality. Finally, we examined the data with respect to a possible correlation between host genotypes and associated ECM fungi. It became evident that the genomic background of silver fir as represented by single-locus variation has an effect on the composition of the associated ECM community. Consequently, ECM communities may be considered as extended phenotypes of the host populations. Protecting silver fir as a means of forest gene conservation therefore implies not only the tree species, but as well the interacting ECM community as part of the ecosystem.

Based on the overall findings including tree genetic, dispersal and mycological aspects, silver fir populations in the Black Forest provide an appropriate basis for natural regeneration processes within the forest stand as well as for the recolonization of windthrow areas. Natural regeneration is an appropriate method for the reintroduction of larger proportions of silver fir in the Black Forest.

Zusammenfassung

Im Zuge des Klimawandels ist von einer zunehmenden Häufigkeit extremer Wetterlagen auszugehen, die die Waldökosysteme beeinflussen. In den letzten Jahrzehnten haben zahlreiche Sturmereignisse mitteleuropäische Waldbestände großflächig geworfen. Die entstandenen Windwurfflächen müssen nun wiederverjüngt werden. Lebensgemeinschaften von Bäumen mit Ektomykorrhizapilzen (ECM) spielen hierbei eine entscheidende Rolle für die Stabilität und Vitalität von Waldökosystemen.

Da die Weißtanne (*Abies alba* Mill.) eine starke Reduktion in ihrem natürlichen Verbreitungsgebiet in Deutschland erfahren hat, ist eine Erhöhung ihres Anteils aufgrund der stabilisierenden Eigenschaften waldbaulich vorgesehen. Angesichts der Zunahme von Sturmereignissen wurden drei Tannenbestände im Nordschwarzwald genetisch und mykologisch untersucht, um das Wiederbesiedlungspotential der Tanne abzuschätzen. Zu diesem Zweck wurden in einem ersten Schritt Kern-Mikrosatellitenmarker (nSSR) für die Tanne entwickelt. Anschließend wurden Tannenkollektive verschiedener Altersklassen (Altbäume, Jungbäume, Sämlinge) an sechs nSSR-Orten genotypisiert sowie hinsichtlich Vorkommen und Diversität der mit ihr vergesellschafteten ECM untersucht.

Die Ergebnisse zeigen, dass die Weißtannen über ein ausreichendes genetisches Potential mit einer hohen Diversität innerhalb und einer geringen Differenzierung zwischen den Beständen verfügen. Die Naturverjüngung der Tanne auf den angrenzenden Freiflächen wies keine reduzierte genetische Diversität auf im Vergleich zu den ungleichaltrigen Beständen. Die Untersuchungen zum Genfluss bei der Tanne lassen zudem auf eine ausreichende Samen- und Pollenausbreitung schließen, zumindest im Abstand von wenigen hundert Metern vom Mutterbaum. Eine Vielzahl von Mutterbäumen trug zur Samenverbreitung bei und führte zu einem vielfältigen Sameneintrag sowohl innerhalb der Bestände als auch in die Windwurfflächen hinein. Die Analyse der ECM zeigte im Altbestand und auf der Freifläche ein vergleichbares Spektrum hinsichtlich Artenzahl und Häufigkeit. Es gab keine Anhaltspunkte dafür, dass das Alter der Bäume in einer Population die Artzusammensetzung der ECM beeinflusst. Auf der Ebene des Einzelbaumes zeigten jedoch ältere Tannen eine höhere Anzahl verschiedener ECM als jüngere. Die auf den Windwurfflächen bereits vorhandene Verjüngung zeichnet sich durch ein ausgewogenes ECM-Profil aus. Sie besitzt damit eine ‚Reservoir-Funktion' für die später ankommende Tannen-Verjüngung. Darüber hinaus wurde geprüft, ob das Tannen-Genom, d.h. die nSSR-Genotypen, einen Einfluss auf die Besiedlung mit ECM hat. Es zeigte sich, dass bestimmte nSSR-Genotypen mit der ECM-Artzusammensetzung signifikant korreliert sind. Daher kann

die ECM-Gesellschaft als so genannter ‚erweiterter Phänotyp' (extended phenotype) der Tannen betrachtet werden.

Der Schutz und die Förderung der Weißtanne als Maßnahme im Rahmen der forstlichen Generhaltung bezieht daher nicht nur die Baumart selbst ein, sondern ebenfalls die mit ihr assoziierten ECM-Gesellschaften als Teil des Ökosystems. Aus genetischer, verbreitungsbiologischer und mykologischer Sicht bieten Weißtannenpopulationen im Nordschwarzwald geeignete Voraussetzungen für eine natürliche Verjüngung sowohl im Bestand als auch zur Wiederbesiedlung von Freiflächen.

1. Introduction

Forest ecosystems occupy about 30 % of the Earth's surface (FAO, 2007). They are characterized by a predominance of trees and are more productive and have a greater biodiversity than other types of terrestrial vegetation forms. There is a variety of forest types resulting from a complex of factors, including species, frequency and type of disturbances, seed sources, soils, slope and aspect, climate as well as history of human influence. Thereby, indigenous, site-adapted and adaptable species and communities are decisive basic requirements for the performance, stability and productivity of forest ecosystems. This means, in turn, that any changes of the genetically determined adaptedness and any restriction of the adaptability increase the risk of destabilization. Like all natural systems, forests are vulnerable to the impact of climate change that is expressed among other things in global warming (IPCC, 2007). This may lead to changes in tree species ranges, in forest growth, in phenology (e.g. leaf unfolding), and/or in increasing extreme events like the occurrence of fire and storms. Extreme climate events such as the storms in 1999 and 2002 dramatically affect forest ecosystems, especially those where the management practice does not facilitate rapid repair. Although there is converging evidence that climate change is increasing the frequency and severity of storm events (Tebaldi *et al.,* 2006), their quantitative impacts and their long-term effects are not well understood. Storm events resulting in windthrow of forest trees can cause a decline in population size of forest tree populations and a reduction in gene flow through fragmentation, respectively. This, again, can lead to a reduced genetic diversity (bottleneck) especially in the new regeneration within the disturbed habitats (windthrow areas). Seed dispersal as part of gene flow plays an essential role in the recolonization of habitats and must be sufficient to maintain the level of diversity in the future generations.

Besides, forest tree species are an example of foundation species for various associated organisms stabilizing fundamental forest ecosystem processes. In forests, mutualistic systems with fungi play a decisive role for the stability and the viability of the trees. About one third of the fungi that are associated with forest trees are mycorrhizal symbionts (Egli and Brunner, 2002), most of them are ectomycorrhizal (ECM) fungi. Both, fungal and plant partners, can benefit from this association (Smith and Read, 1997). A detailed knowledge of ecosystem processes and community structure becomes more and more important, since stabilizing mutualistic systems might be especially advantageous in terms of climate change.

A frequent species composition in the mixed mountain forests of Central Europe consists of beech (*Fagus sylvatica* L.), spruce (*Picea abies* (L.) H. Karst) and silver fir (*Abies alba* Mill.) as the natural components of the forest ecosystem. Such forests are generally characterized

by high stability and productivity. Thereby, silver fir exhibits a specific role as a stabilizing element due to its pronounced and deep seated root system and due to its ability to regenerate and survive long periods (up to 100 years) under shade (Schütt, 1994). It belongs to the family *Pinaceae* and is a characteristic tree species of the montane and submontane regions. Its natural range extends from 52 °N in northern Germany to 38 °N in the southern part of Italy and from 22 °E in eastern Romania to 03 °W in the western Pyrenees in France (Liu, 1971). In Germany, silver fir occurs with a proportion of 2 % of the total forest area (Schütt, 1994). It is a monoecious and wind-pollinated conifer species and its seeds are dispersed mainly through wind.

Silver fir has suffered serious reduction in its range over the last centuries as a consequence of silvicultural preferences for monocultures of Norway spruce and environmental stress factors within the natural distribution in Germany. For instance, the proportion of silver fir was reduced by one-half in the Black Forest within the last 100 years (Horndasch, 1993). As a consequence and due to its important ecological and economic role, a reintroduction of larger proportions of silver fir into the mountainous forests has been promoted by forest management plans. The genetic status in silver fir is thereby a decisive aspect since it forms the basics for all processes of life as well as for adaptability and adaptedness. Generally, genetic diversity as one of the three fundamentals of biodiversity has gained new importance through the Convention on Biological Diversity (CBD) that has been prepared within the Conference on Environment and Development in Rio de Janeiro (UNCED, 1992).

Genetic desoxyribonucleic acid (DNA) markers enable us to determine genetic structures and its underlying processes in tree populations such as silver fir populations. Currently, neutral genetic markers predominate in conservation and management applications of population genetics in forest trees. According to their origin (nuclear or organelle) different types of information can be supplied with DNA markers. While nuclear markers are codominant and thus more informative, organelle markers are uniparental and reflects paternal (chloroplast) or maternal (mitochondria) structures in conifers. Especially microsatellite markers (also known as simple sequence repeats (SSRs)), both organelle and nuclear, are the markers of choice for diversity and differentiation studies as well as for studies of contemporary gene flow as mediated by pollen and seeds (Gomez *et al.,* 2004).

Against the background of promoting silver fir in the mountain forests and facing the increase of storm events, fir populations of the Northern Black Forest were studied with SSR markers to gain information about the genetic status of silver fir in this region. Genetic structures of the fir trees are considered in relation to associated ECM fungi and furthermore are used for the study of seed dispersal characteristics.

On that account, the following main objectives have been focused on in this thesis:

I) *Genetic diversity and differentiation*

In order to genetically characterize three silver fir populations (in terms of diversity and differentiation), a comprehensive nuclear microsatellite (nSSR) analysis was conducted. For this, appropriate and specific nSSR markers had to be developed and validated in a first step, since they were not available. The genetic diversity and differentiation analysis on the regional and population scale was necessary to answer the questions: Is the extent of genetic variation of silver fir in the Northern Black Forest sufficient for the natural regeneration especially on disturbed sites caused by windthrow? Are there any indications of drift or fragmentation effects on the genetic structure of the fir populations? And can the three different fir populations (study sites) be treated as one reproductive community for true repetitions with regard to the following analysis with associated ECM fungi?

II) *Gene flow processes*

Gene flow is a key determinant of genetic structures and patterns within forest populations. Thereby seed dispersal plays a decisive role for recolonization of disturbed habitats since seed is the only movable stage within the life cycle of trees. Silver fir proved to be an interesting model species for dispersal characteristics as it is characterized by one of the largest pollen and seed grain among indigenous species. Thus, direct estimates of seed and pollen dispersal processes were derived from genetic analysis of adults and progeny in silver fir. The analysis was conducted to answer the questions: Are the dispersal qualities in silver fir sufficient for recolonization purposes? Can a genetic bottleneck be expected in the natural regeneration of the recolonized site? And can morphological features of the seeds be regarded as driving factors of seed dispersal?

III) A*ssociated ectomycorrhizal communities*

Mutualistic interactions play a decisive role for the stabilization and functioning of forest ecosystems. So far, little is known about the impacts of intraspecific variation of the host on the associated fungal community. However, recent analyses demonstrate first insights of the impact of host or foundation species such as forest trees through their `extended phenotype´ (Whitham *et al.,* 2003; Bailey *et al.,* 2004). Thus, the relationship between silver fir trees and the associated ECM community is of high interest in the scope of ‘community genetics’.

First, we re-visited the early-late-stage hypothesis within ECM fungi (Izzo *et al.,* 2005) and analysed the effects of ontogenetic stages of the host trees (adults, saplings) on the ECM community including site effects (windthrow area vs. closed forest stand). Thereby, the following question was focused on: Can the age of the firs be regarded as driving factor for colonization of ECM fungi?
In a final step we analysed whether the ECM community could be considered as the extended phenotype of the host using an individual-based genetic approach to answer the question: Is there a verifiable relationship between distinct single-locus genotypes in the firs as characterized by neutral DNA markers and the associated ECM fungi?

Subsequently, these objectives are comprehensively discussed with regard to the questions ‘Which preconditions does silver fir bring along for natural regeneration processes and recolonization of windthrow areas in the Black Forest?’

2. Population genetic analysis

So far, levels of diversity in forest trees and especially in the genus *Abies* have been assessed using different kind of genetic markers such as isozymes (e.g. Hussendörfer *et al.,* 1995), amplified fragment length polymorphisms (AFLPs) (Tang *et al.,* 2008) and chloroplast microsatellites (cpSSR) (Vendramin and Ziegenhagen, 1997; Ziegenhagen *et al*., 1998). Although most cpDNA regions exhibit low within-population polymorphism, cpSSR markers often have sufficient polymorphisms for paternal lineage identification within a population in conifers (Ziegenhagen *et al*., 1998). They are useful for paternity analysis and were applied for estimating pollen flow within the present study (see chapter 3).

Due to their high levels of polymorphism and co-dominant inheritance, nSSR markers provide a powerful tool for addressing genetic questions such as genetic diversity within populations and differentiation among populations. They have become the genetic markers of choice in forest trees (Vendramin *et al.,* 2004). Since nSSR markers were not available for the species *Abies alba* they have been developed in order to investigate genetic structures (paper III), to conduct identity assignment of mother trees (paper II) and parentage analysis (chapter 3) as well as to perform association genetics (paper III) within the fir population in the present study.

2.1. Nuclear microsatellite markers

Development of nuclear microsatellites for Abies alba

Microsatellites or SSRs are tandem repeats of short sequence motifs with a repeat unit of one to six nucleotides and are distributed across the nuclear and organelle genome. They typically show a high number of alleles per locus while the alleles differ in the number of repetitions and thus in length (Tautz, 1989).Their high degree of variability is due to a high mutation rate caused by the repetitive structure. Thus, they usually exhibit a high degree of discrimination and can be used to determine genetic differences between individuals (Vendramin *et al.,* 2004). Generally, SSRs are species specific markers and must therefore be developed for each species separately. In some cases, however, they can be transferred to other species within the genus (Hansen *et al.,* 2005).

For the development of nSSR markers in *Abies alba*, an enriched genomic library for di- (GA, GT, AT, GC), tri- (CAA, ATT, GCC) and tetranucleotide (GATA, CATA, ATAG) motifs was constructed according to Edwards *et al.* (1996). Afterwards these fragments were cloned into a common plasmid vector. A total of 170 randomly chosen clones were sequenced and more than 90 % of the clones contained a SSR stretch.

Since in several cases the stretches were too close to the vector, too long or compound-interrupted, they were excluded. However, for 44 clones it was possible to design primer pairs for polymerase chain reaction (PCR) amplification of the loci. Fourteen of the 44 nSSR loci yielded distinct and analysable PCR products. The variability test including 17 to 24 fir individuals from Bulgaria, France, Germany and Switzerland resulted in eleven polymorphic loci that are useful for population genetic analysis (paper I).

The nSSR locus SF78 exhibiting 35 alleles (within 1200 fir samples) reveals a long range of fragment sizes from 158 bp to 276 bp and was thus investigated in more detail. A short and a long allele were sequenced to guarantee that it is from the same locus. The alignment of both sequences shows that the two fragments originate from the same locus, because the flanking regions are largely identical (Figure 1). The large allele contains two compounding SSRs ((CAG)(CA)) and the short allele only one SSR (CA). It is difficult to explain the development of this compound SSR fragment. Possible explanations are a slippage event at the flanking region of the first SSR or a recombination event at the level of the SSR, but the latter is less probable. As a consequence of the extreme allele size range, this nSSR locus should not be applied in software analysis that is based on step-wise mutation models.

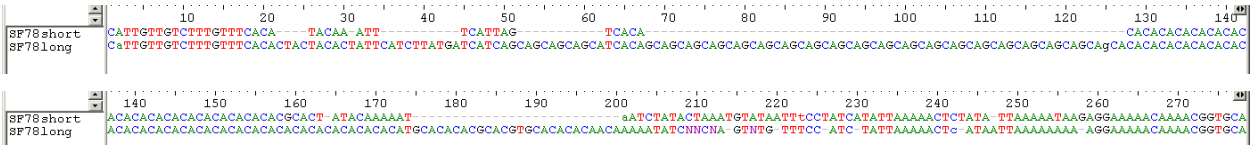


Figure 1 DNA sequence of two variants within the region of the nSSR locus SF78. The longer fragment consists of 245 bp and the shorter fragment of 158 bp (due to the fragment length analysis using the Amersham MegaBACE1000).

Application of nuclear microsatellite loci

For the further analysis of the fir populations and individuals those nSSR loci were chosen that consist of dinucleotide repeats (SFb4, SFb5, SF331, SF333), have many alleles (SF78) and / or show very clear banding patterns (SF1), respectively. Using 360 fir samples of the study site EY, the SSR data were analyzed by the software Micro-Checker (Van Oosterhout *et al.,* 2004) for detecting null alleles, scoring error due to stuttering and large allele dropout. Neither evidence for scoring error due to stuttering nor evidence for large allele dropout was detected for the six nSSR loci. Evidence for null alleles that is based on the excess of homozygotes (as deviation from Hardy-Weinberg-equilibrium) could be found for the locus SFb4.

Such deviation can either be a result of population genetic processes (genetic drift, inbreeding, absence of natural outcrossing mating system), a small sample size effect or a subpopulation structure in the sample design (Wahlund effect) and can thus be misinterpreted as null alleles (Chakraborty *et al.,* 1992). Since microsatellite null alleles might introduce biases on average exclusion probabilities within parentage analysis (Dakin and Avise, 2004) the locus SFb4 was excluded for the pollen and seed dispersal analysis. However, the population genetic analysis was calculated using both, the six-loci combination in comparison to the five-loci combination excluding the locus SFb4 in a first approach. Since, adding the locus SFb4 did not change the interpretation of the results (concerning mean number of alleles per locus, heterozygosity, differentiation and genetic distance) and, yet, increases the diversity parameters, it was included for the further population genetic analysis. Moreover, the probability of identity (P_{ID}) (Paetkau *et al.,* 1998) was obviously decreased for the six-locus combination with $P_{ID} = 4 \times 10^{-5}$ in comparison to the $P_{ID} = 7 \times 10^{-4}$ for the five-loci combination. According to Waits *et al.* (2001) a P_{ID} of less than 1×10^{-3} is acceptable low and sufficient for forensic applications in natural populations. Therefore, the six-locus combination is appropriate to distinguish individuals accurately and to answer population genetic questions properly.

The newly developed nSSR markers have been proved to be an appropriate tool for diverse applications in the present study that allowed us to analyse the fir trees under different aspects. They facilitate an analysis on different scales and could be successfully applied for following purposes:

i) diversity and differentiation analysis of silver fir populations on the regional and on the population scale (paper III),

ii) discrimination and / or identification analysis of single fir individuals to detect seed source trees (‘mothers’) (paper II),

iii) comparative analysis between fir single-locus genotypes and ECM diversity on the individual scale of the fir trees (paper III).

Actually, markers such as nSSRs are supposed to reveal neutral genetic variation and are useful for characterizing patterns of variation, but are generally not instructive for adaptive patterns of genetic variation (Avise, 1994; Porcher *et al.,* 2006). However, we used them for association genetics since candidate genes governing symbiotic interactions are not yet identified or described for silver fir. With that approach, a unique opportunity arose, as it was possible to screen for single-locus variations of the fir which could be linked to certain genomic regions that are relevant for interactions with symbiotic partners (paper III).

Several nSSR loci offer evidence to be located close to regulatory DNA-regions with functional relevance related to the production of molecules that are controlling the interaction between symbionts. Congruently, Neale and Ingvarsson (2008) analysed natural selection processes and its effects on the genome of cottonwood and assumed that it should be possible to move away from using strictly neutral models as sequence data sets of tree species are becoming more and more available.

2.2. Genetic diversity and differentiation

Genetic differentiation analysis on the regional scale as initial step for further analysis

In previous studies using different genetic markers such as isozymes (Konnert and Bergmann, 1995), cpSSRs (Vendramin *et al.,* 1999) and mtDNA markers (Liepelt *et al.,* 2002; Gomöry *et al*., 2004) silver fir has revealed highly differentiated genetic patterns on a large geographical scale throughout Europe depending on glacial refugia and postglacial migration pathways (Liepelt *et al.*, 2008). In comparison to other European tree species silver fir reveals some specific characteristics such as geographical clines in allele frequencies at several allozyme gene-loci, area-specific alleles and a clinal variation in population diversity over the whole distribution range (Konnert and Bergmann, 1995). The genetic structure and differentiation on a regional scale was analysed by Sagnard *et al.* (2002). Neither on the basis of allozyme data nor on the basis of quantitative traits could the different silver fir populations from the south-western Alps (France) be grouped geographically.

Using the newly developed nSSR markers, the differentiation of silver fir populations on the regional scale was analysed in the present study as an initial step (paper III). For this, three fir populations were genetically investigated which are located on the same geological substrate (middle red sandstone) in the Black Forest. Each of the study sites includes adult fir trees as well as juveniles trees (seedlings and saplings) and furthermore consists of a closed forest stand with an adjacent open area as a result of windthrow caused by the storm event ‘Lothar’ in 1999. Sampled trees were evenly distributed within the stand and along four transects (50 m and 100 m, respectively) into the adjacent windthrow areas (Figure 2). Ontogenetic stages of the trees were defined as follows: seedlings = 1 to 3 year old trees assumed to be younger than the storm event; saplings = juvenile trees up to a height of 1.5 m and adult trees = firs in the fructification age (> 60 years).

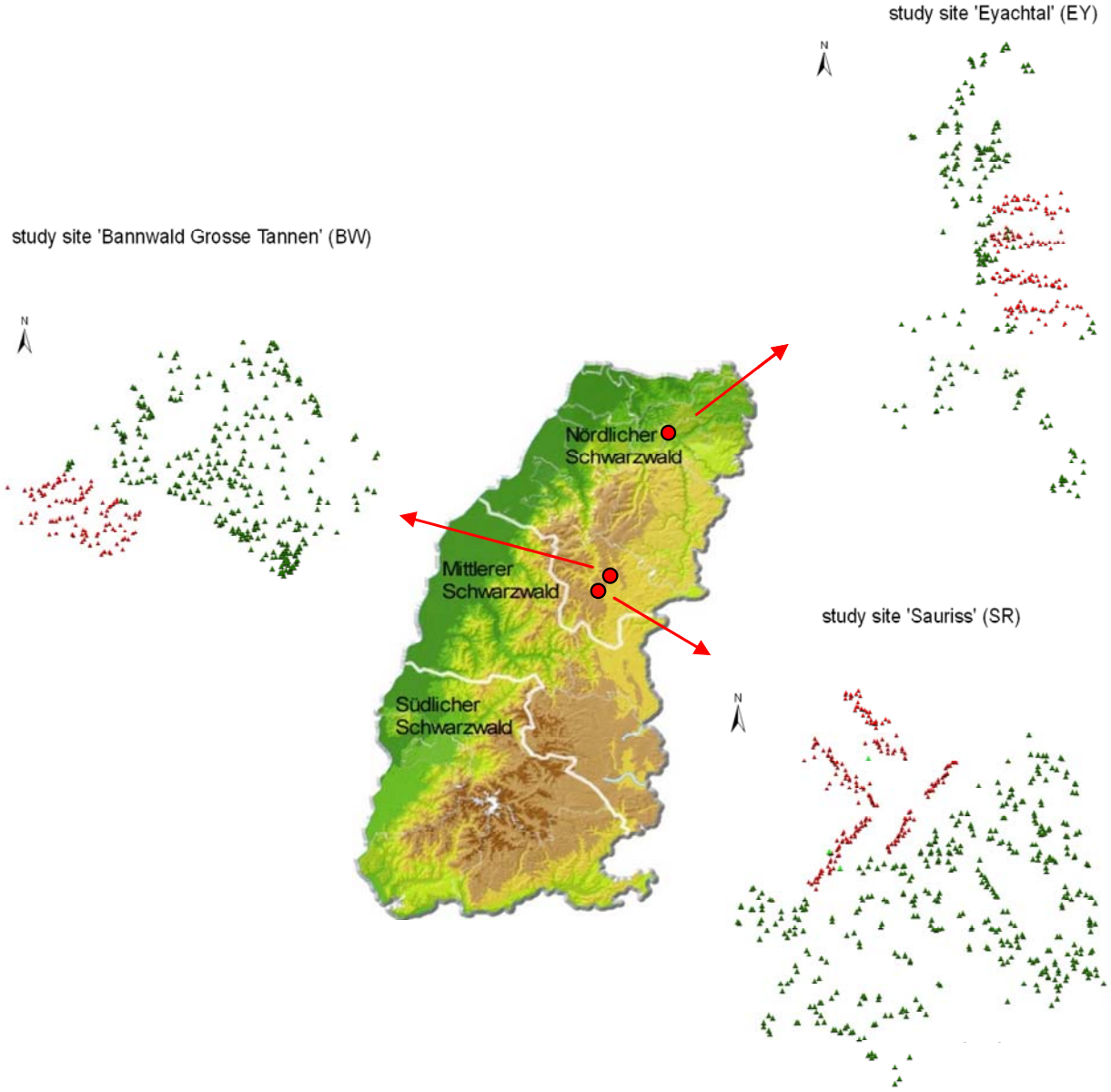


Figure 2 The location of the three silver fir study sites (BW, EY, SR) in the Black Forest, Germany, and sample design of each study plot, showing the sampled fir trees within the forest stand (marked in green) and on the windthrow area (marked on red).

Based on the six nSSR markers no distinct differentiation among the three fir populations could be detected as indicated by global F_{ST} values close to zero (F_{ST} = 0.008). A Bayesian model assigned them to a most likely number of 'one' group. Moreover, the 'Analysis of Molecular Variance' (AMOVA) that allows the partitioning of variation among and within populations indicated that variance was much lower among populations (1 %) than within (99 %) (Figure 3). Influences such as isolation or strong fragmentation events that can affect the balance between drift and gene flow and can increase genetic differentiation due to a loss of gene flow (Templeton *et al.*, 2001) can thus be excluded.

Interestingly, the reduction of silver firs in the Black Forest in the last decades, i.e. the current fragmentation, is not imprinted in the genetic structure. Thus, silver fir is characterized as having uniform genetic structures within the research area of the Black Forest with a spatial maximum distance of about 100 km. These results verify the starting hypothesis that the fir populations within the Black Forest behave genetically like a single population or a reproductive community, respectively. They can therefore be treated as independent replicates in this study and are suitable for further interaction analysis with the associated ECM fungi (chapter 4 and paper III). A bias caused by genetic differentiation on the population scale can be eliminated.

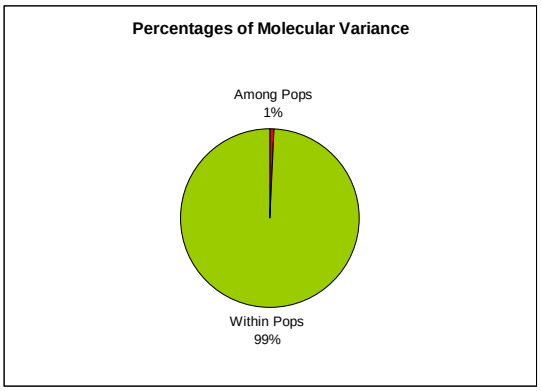


Figure 3 Results of AMOVA considering the fir trees within and among the three fir population in the Black Forest.

Using the same nSSR markers Donges *et al.* (unpublished) have observed clear differentiations between divers silver fir provenances (from Germany, Macedonia and Romania) evidencing the potential of the used marker system for detecting genetic differentiation or unity, respectively (Figure 4).

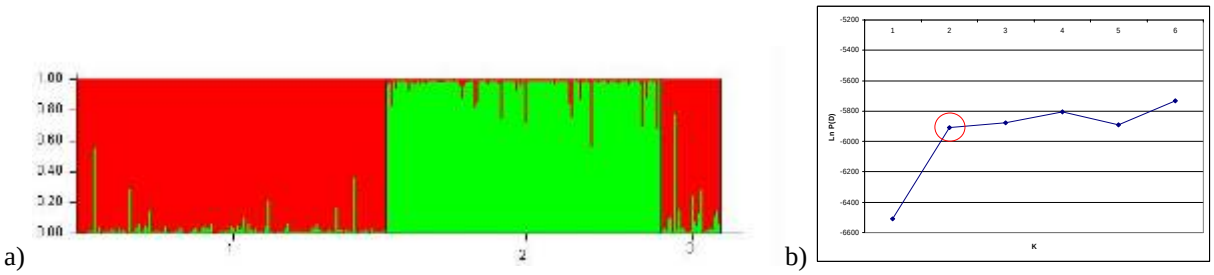


Figure 4 Genetic assignments of silver firs to populations, based on Bayesian modelling. a) Bar plot, illustrating the allocation of individual fir samples to the provenances (1) Macedonia, (2) Black Forest and (3) Romania. b) Simulation of most probable number of populations (K), in a range of K = 1 to K = 6. Red circle indicates the most probable result (out of Donges *et al.*, unpublished).

In summary, our findings agree with those of Sagnard *et al.* (2002) showing less variation within silver fir populations on a regional scale which originate from the same glacial refugia (Liepelt *et al.,* 2008). At the same time it is possible to detect pronounced genetic differentiation between Black Forest silver fir provenances and Romanian and Macedonian provenances (Donges *et al*., unpublished) using the same markers. This, in turn, reflects the findings of Konnert und Bergmann (1995), Vendramin *et al.* (1999), Liepelt *et al.* (2002), Gomöry *et al.* (2004) using other genetic markers (see above). Generally and as demonstrated in the present study, conifers are featured by higher levels of genetic variation within populations and lower levels of genetic differentiation among populations (Hamrick *et al.,* 1992; Tang *et al.,* 2008). Thus, the extinction of a relatively large proportion of a conifer species' population would result in relatively little overall loss of genetic diversity. Due to their life-history strategies such as wind pollination, efficient seed dispersal via wind and/or animals and the longevity of conifers they are thought to be less vulnerable to landscape fragmentation (Williams *et al*., 2007).

Characterization of genetic diversity on the population scale

The genetic variation within each of the three analysed fir populations can be assessed as relatively high with a total mean heterozygosity (H_e) of 57.1 % in study site BW, of 56.0 % in study site EY and of 57.4 % in study site SR, respectively (Table 1). So far, most genetic studies in conifers, especially in *Abies alba* have not been conducted using nSSR loci, but they are mostly based on isozyme or cpSSR markers. Thus, there are only a few comparative studies. However, in a recent study the mean genetic diversity of H_e = 33.7 % was detected in an A*bies ziyuanensis* population with eight nSSR loci which is comparatively low (Tang *et al*., 2008). In comparison, a H_e-value of 85.5 % could be detected in an *Abies sachalinensis* population analyzed with five nSSR loci (Lian *et al*., 2008). In the latter case, the variability of the used nSSR loci was very high with an average number of alleles per locus of 19.5. Compared with these studies and with population genetic analyses of related conifer species analysed with nSSR markers (e.g. *Picea abies* (Achere *et al.,* 2005), *Pinus pinaster* (Mariette *et al.,* 2001)) it can be assumed that the genetic diversity parameters found for the silver fir populations within the present study are comparatively high.

Focusing on the subpopulation in dependence of the ontogenetic stage (adults, saplings and seedlings), genetic diversity parameters were compared in order to assess the transfer of genetic information over various generations. Genetic diversity statistics averaged over the six nSSR loci in the different ontogenetic stages are shown in Table 1.

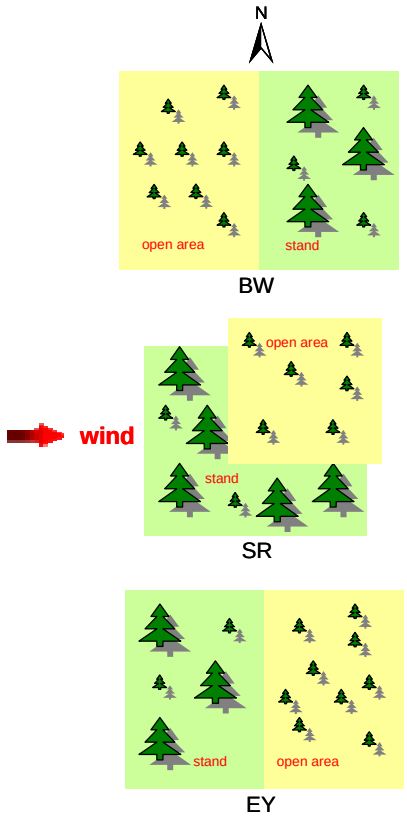
The genetic parameters describing genetic variation (A, A_r, N_e, H_o, H_e) were similar in all stages and although they vary slightly there was no consistent trend detectable over all three populations. That means, small differences within the genetic structure could not be explained by ontogenetic stages and genetic information is transferred over generations. Congruently, by means of 12 isozyme markers no significant differences in the genetic structure of the fir trees in the study site BW could be found by comparing fir individuals of four different ontogenetic stages (over-, middle-, under-storey and regeneration) (Ernst, 2006).

Table 1 Genetic parameters for the subpopulations adults, saplings and seedlings, each in the forest stand and on the windthrow area, of the three study sites in the Black Forest (EY; BW SR) analysed with six nSSR loci: mean number of alleles (A), number of effective alleles (N_e), allelic richness (A_r), observed heterozygoty (H_o), expected heterozygoty (H_e).

Population	**N**	**A**	**A_r**	**N_e**	**H_o**	**H_e**
EY – adult *stand*	54	6.7	27.9	2.97	0.440	0.558
EY – saplings *stand*	104	7.7	28.8	3.20	0.480	0.586
EY – saplings *windthrow area*	100	7.5	25.8	3.11	0.466	0.548
EY – seedlings stand	53	6.3	25.3	3.10	0.492	0.558
EY – seedlings *windthrow area*	50	6.0	22.9	3.07	0.466	0.548
BW - adults *stand*	200	10	32.5	2.99	0.474	0.565
BW – saplings *stand*	100	7.8	30.7	3.32	0.459	0.577
BW - saplings *windthrow area*	100	8.8	33.7	2.80	0.474	0.571
SR -adults *stand*	200	9.2	31.2	3.24	0.492	0.578
SR - saplings *stand*	200	9.2	30.7	3.3	0.482	0.578
SR - saplings *windthrow area*	200	10.2	31.8	3.1	0.459	0.565

In order to check for a possible genetic difference between fir saplings that grow within the forest stand and those growing on the windthrow area, Nei's genetic distance was calculated for the respective pairs (Figure 5). For this, only the saplings as fir regeneration were considered, since seedlings could not be found for the study sites BW and SR in a statistical usable sample size. With this approach, only those juvenile fir individuals were included in the calculations that were regenerated before the storm in 1999 and thus were established under forest cover.

As displayed in Figure 5, the genetic distances between fir saplings of the windthrow area and saplings as well as adults of the forest stand were small in the study sites EY and SR with values up to 0.014 and were not significantly different (Fisher's exact test, $p > 0.001$). The adults and the saplings of the forest stand in the study site BW also revealed small genetic distances of 0.008 while the distance increased up to 0.031 when the adults and saplings of the stands were compared to the saplings of the windthrow area. In the latter case the allelic and genotypic structure between the subpopulations were statistically significant for two nSSR loci (Fisher's exact test, $p < 0.001$). This slight increase of the genetic distance can be explained by the differences in the allele structure. This, in turn, might be a result of the location of the respective windthrow area in relation to the forest stand (Figure 5). Considering that the main wind direction in the Black Forest is from west to east, less seeds are probably dispersed against the wind into the western positioned windthrow area of the study site BW. Different situations are given in the case of study site EY and SR. Here, the windthrow areas are located eastward of the forest stand leading to higher seed dispersal into the open area. Thus, these findings verify the role that wind plays in the dispersal of seeds and subsequently in the genetic structure of the established regeneration.



BW	adults *stand*	saplings *stand*
saplings - *stand*	0.008	
saplings - *windthrow*	0.031	0.029

SR	adults *stand*	saplings *stand*
saplings - *stand*	0.005	
saplings - *windthrow*	0.006	0.005

EY	adults *stand*	saplings *stand*
saplings - *stand*	0.014	
saplings - *windthrow*	0.014	0.012

Figure 5 Schematical illustration of the three study sites showing the location of the windthrow area in comparison to the forest stand and results of pairwise Nei's genetic distances.

The genetic diversity in all three fir populations is comparatively high, combined with the fact that there is no reduction of allelic richness or diversity, respectively, in the juvenile generations. This leads to the conclusion that the genetic diversity has so far not been reduced and that there are no genetic bottleneck effects within the analysed fir trees in the Black Forest. Thus, the analysed fir stands are an appropriate basis for future natural regeneration from the genetic point of view and in terms of recolonization of disturbed habitats. Our findings verify forest management practices in terms of natural regeneration methods which consider respective wind situations as well as the location of the open area in relation to the forest stand including the seed source trees.

3. Gene flow – dispersal of seeds and pollen

Gene flow is defined as the proportion of immigrant genes that either move into a given population (interpopulation gene flow) or move within a given population (intrapopulation gene flow) (Endler, 1977). It can take place in two ways. The first involves the dispersal of pollen, successful fertilization of an ovule by this pollen, and finally establishment of the resulting seed. Gene flow can also occur by dispersal of seed, and the successful establishment of the dispersed seed within a new habitat or population. Studying gene flow implies the detection of sources and sinks for pollen and seeds. Thereby, the incorporation of landscape information can help to understand whether populations are sources or sinks. Here, we focus on forest windthrow areas that display a sink for dispersed seeds while the surrounding reproductive trees might be a source. Since forest trees such as silver fir are sedentary organisms, the dispersal of diasporas states the only movable part and is thus, essential for the genetic structure of future generations. New habitats can only be colonized by diasporas. Thereby, an essential differentiation between seed and pollen movement is that only seed flow can colonize open habitats and provide a biological foundation for subsequent pollen flow.

In two different case studies we analysed seed dispersal and pollen dispersal, respectively. Since repetitions are missing they cannot be used for generalised statements about gene flow in silver fir. Rather, these analyses provide initial insights mainly into local seed and pollen movement for the estimation of the recolonization potential of silver fir within a few hundred meters from the forest margin. They also offer valuable perspectives for continuative studies.

Estimating seed dispersal

Knowledge on seed dispersal distances of firs plays a major role in assessing the potential for natural regeneration in fir populations – in forest stands as well as on open areas as exemplified here. Seed dispersal is also essential for migration processes as response to climate change. Despite the importance of seed dispersal, quantitative information on seed dispersal distances has been scarce. This has been mainly due to methodological difficulties in quantifying seed dispersal. The recent innovation of using genotypes derived from purely maternal tissue of seeds made it possible to identify the source or mother tree of the dispersed seeds directly and thus, efficiently (Godoy and Jordano, 2001; Grivet *et al.,* 2005). Using this method and applying nSSR markers, seed dispersal in silver fir was exemplarily analysed in the study site SR. Here, seed traps were set up in the forest stand as well as on the adjacent windthrow area to collect the fir seeds.

The analysis gave the following results (paper II):

In total, 674 seeds were found in the seed traps. Seed entry into the windthrow area was reduced in comparison to the forest stand but nevertheless was sufficient within short distances of a few hundred meters from the source trees.

The morphological features of the fir seeds (seed weight and length of wing) as well as the condition of the seeds (empty or filled) do not appear to have a significant influence on the dispersal distance.

Fourteen percent of the fir seeds could be assigned to a mother tree within the sampled trees of the forest stand. The remaining 86 % could not be identified because their maternal tree had not been genotyped or were outside the sampled area.

A mean dispersal distance of 98 m and a maximum dispersal of 275 m were detected. Shorter dispersal distances were observed for the seeds collected in the stands than for those collected in the windthrow area due to fewer barriers and higher wind velocity.

The long dispersal period – from September to the end of the collection period in January – led to seed dispersal under various weather conditions and wind directions and therewith, promotes multifaceted dissemination.

Longer distance dispersal events of more than 150 m, detected by the exclusion of all reproductive trees in the sampled population, accounted for up to 31 % of the seeds trapped. Besides, 477 different multilocus genotypes among the total of the 661 genotyped seeds show the high number of contributing mother trees. This implies a high level of genetic diversity in the seed population.

Summing up, our results indicate sufficient local-distance seed delivery combined with some long distance dispersal events and a marked mosaic of multiple mother trees for the seeds found. Seed dispersal appears to be independent of seed morphology and is efficient within the closed forest stand as well as in the windthrow area close to the forest margin. These dispersal characteristics should enable the fir population to maintain genetic diversity from the dispersal point of view. This leads to the final conclusion that the available potential of seed for dispersal should not result in any genetic bottleneck of fir regeneration even within windthrow areas if the distance does not exceed a few hundred meters from the source trees.

In addition to dispersal, realized gene flow requires successful fertilization, germination and survival from competition (Savolainen *et al.,* 2007). The method used considers all seeds that are dispersed – even those that have not led to successful establishment of propagules. Factors influencing the establishment of seeds are not considered here and the actual success of fir establishment can hardly be estimated. However, very few seedlings that have established themselves after the storm event could be found on the windthrow areas in general (e.g. Budde, unpublished). This indicates that environmental site conditions of the windthrow area may negatively influence the establishment of seeds even though enough seeds seem to be dispersed.

Estimating pollen flow

Pollen dispersal is a major component of gene flow (Ennos, 1994; Oddou-Muratorio *et al*., 2001). It is an essential factor influencing genetic structure of wind pollinated forest trees facilitating interconnection between individuals or populations. Although it lacks the colonization function of seeds, the potential for long-distance transport of male gametes greatly influences genetic processes that have central effects, such as gene exchange among spatially isolated populations (Ennos, 1994). Assessing gene flow by indirect methods such as models of population differentiation with data on population genetic structure has the drawback that they do not readily distinguish between seed and pollen flow (Slatkin, 1985). A more direct approach for estimating pollen dispersal is provided by paternity analysis using uniparental cytoplasmatic markers. Paternity analysis methods (Marshall *et al.,* 1998) attempt to detect, for each offspring, if paternity can be attributed to one of the firs present in the study site. Generally, parentage analysis is an appropriate tool for the assignment of parent trees to offspring, especially if the maternal side has to be determined prior to paternity analysis. Here, a combined approach of parentage and paternity analysis was applied in order to detect mothers and fathers out of the sampled adult trees for the fir saplings of the stand and on the windthrow area of the study site BW (Figure 6) (for detailed approach and methods, see appendix). Using the highly variable nSSR markers in combination with isozyme markers enables us to specify the parent pairs effectively. In addition, paternally inherited chloroplast DNA markers (cpDNA) provide direct information for the determination of the gender in parental analysis (Latta *et al*., 1998; Lian *et al.,* 2003; Ziegenhagen *et al.,* 1998). Subsequently, for the subset of saplings for which a parent pair has been clearly found, including information about the gender, the spatial position of mothers and fathers could be used to assess pollen dispersal distances.

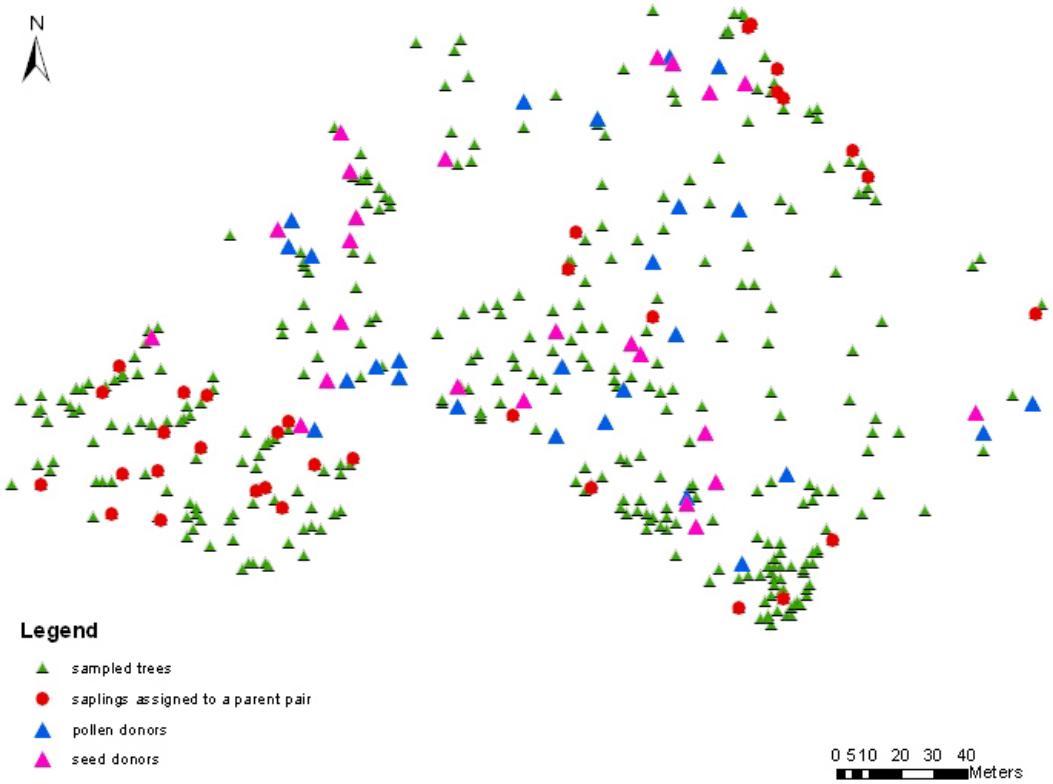


Figure 6 Spatial locations of the 400 silver fir individuals in the study site BW, given are pollen and seed donors for the 34 assigned saplings.

Thirty-four fir saplings could be assigned to a parent pair with a clear identification of father and mother, respectively. For these data, the average effective pollen dispersal distance was calculated and a pollen dispersal function was generated (Figure 7 and appendix, Table A). Unexpectedly, the estimated rates of pollen dispersal with a mean distance of 90 m and a maximum dispersal of 233 m were smaller than the rates of seed dispersal that were detected by means of 94 dispersed seeds in the study site SR (paper II). Indeed, 30 % of the effective pollen was dispersed less than 40 m (and 62 % of the pollen less than 100 m) with scattered longer distance dispersal events up to 230 m. Thus, pollen dispersal was partly restricted favouring mating with neighbouring individuals. These findings are congruent to the general assumption that *Abies* species are featured by limited pollen dispersal ability (Arista and Talavera, 1996). Based on their findings, Koenig and Ashley (2003) stated the hypotheses that, contrary to previous assumptions, dispersal of pollen in wind-pollinated trees might be very short and even less than dispersal distances of larger seeds.

Similar findings have been reported frequently for other wind-pollinated tree species (*Quercus robur* and *Quercus petraea* (Streiff *et al.,* 1999); *Pinus densiflora* (Lian *et al.,* 2001)). Especially in small and isolated populations, the occurrence of pollen sources impacts the effective pollen movement significantly (Robledo-Arnuncio and Gil, 2005; Burczyk *et al.,* 2004).

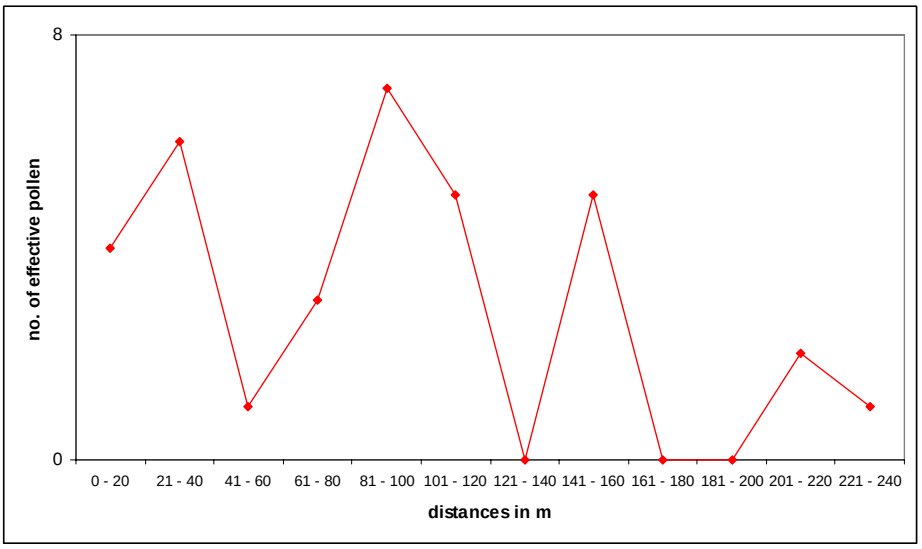


Figure 7 Pollen dispersal function based on 34 mating events of firs within the study site BW.

Long-distance pollen dispersal can be estimated for 18 % of the saplings for which no father was assigned based on the cpSSR haplotypes within the 200 adult firs. Thus, pollen entry from outside the stand with a dispersal of more than a few hundred meters is most likely for the non-assigned saplings since nearly all adult fir trees within the stand were sampled.

Generally, several factors may affect the pollen flow estimation process such as the degree of isolation, the number of trees within the population and the degree of self-fertilization (selfing). Our method, which does not account for the effect of selfing, would thus provide an increased biased estimate of the pollen dispersal distance. Such processes should therefore be considered in future studies which rely best on sampling of mother trees along with a sample of their offspring.

The combined approach of parentage and paternity analysis using biparental and paternal inherited markers offers a unique possibility to detect the male and the female parent of saplings in monoecious species. It can be applied to individuals of any ontogenetic stage and

is thus, independent of maternal tissue that is only existent in the seed stage and is analysed within maternal tissue analysis (Grivet *et al.,* 2005). Of course, the accuracy of the estimates will increase with the amount of data available. Since the present data is limited, consisting of 34 detected mating events, we must be cautious with generalized interpretations. Due to the storm event in 1999, larger parts of the adult trees within the study site were blow down after the regeneration process and could not be sampled any more. This might be a reason for the low amount of assigned parents even though most adult firs within the remaining forest stand were sampled.

Contemporary versus past gene flow

By using the direct approach to monitor gene flow as conducted in the present study for pollen and seed dispersal it is possible to estimate ongoing or contemporary gene movement. Indirect methods enable the estimation of gene movement that is averaged over time and space (past or historical gene flow). Levels of gene flow have traditionally been assessed through indirect methods that infer average historical values from the distribution of genetic variation within adult populations (Sork *et al.,* 1999). In particular, seed dispersal patterns should directly impact the spatial genetic structure (SGS) of populations. Species whose seeds are dispersed near the mother plant should have more obvious fine-scale genetic structure than species whose seeds are dispersed in a large spatial scale by animals or wind (Hamrick *et al*., 1993). Thus, indirect methods of assessing gene flow, in particular seed dispersal, use the observed spatial genetic structure within adult populations as for example was conducted in studies of Aldrich *et al.* (1998) or Ueno *et al.* (2000). An essential feature is thereby a significant autocorrelation value over short distances caused by a strong clumping of dispersed seeds.

Since SGS reflects past gene flow processes, an analysis of fine-scale genetic structure was conducted to compare past to contemporary gene flow. The SGS analysis was based on a correlation coefficient that is closely related to Moran's Index (Smouse and Peakall, 1999) and was performed within the adult trees of the three silver fir populations. As demonstrated in Figure 8, no significant SGS at any distances was found within the three adult fir populations. Positive autocorrelation indicates that genetically similar individuals cluster together spatially, for which one explanation is limited gene flow. In contrast, our findings let us assume a high and balanced level of past gene flow that have led to the present, non-significant SGS. Although past gene flow events include complex influencing parameters such as selection these results are in agreement with the results of the direct measurement revealing sufficient seed dispersal.

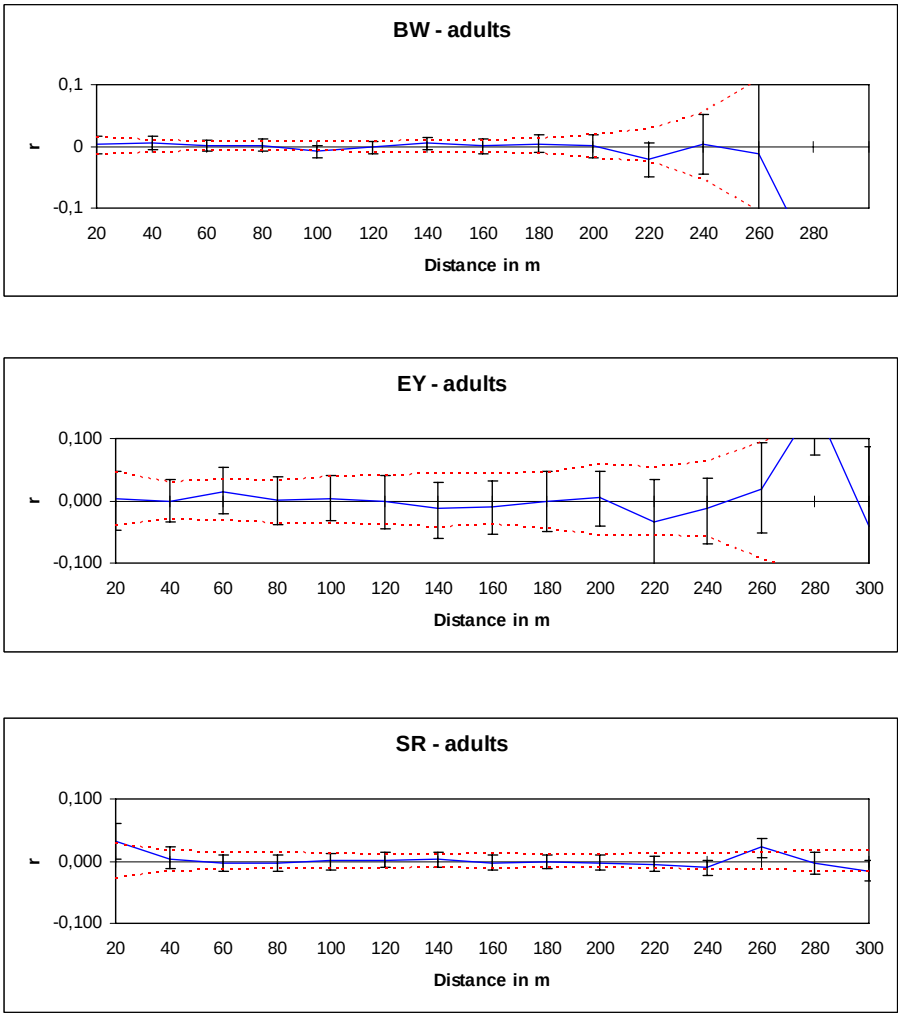


Figure 8 Correlograms of the genetic correlation coefficient (*r*) plotted by spatial distance classes (in m) for adult trees in the three study sites BW, EY and SR. A significant (95 %) observed correlation is indicated if the blue line is located above or below the confidence limits.

The extent of geographic variation results from a balance of forces tending to produce local genetic differentiation (such as selection and fragmentation) and forces tending to produce genetic homogeneity such as gene flow (Slatkin, 1987). Thus, genetic differentiation among populations offers additional insights of past gene flow processes on a larger spatial scale resulting from possibly restricted gene flow by pollen and seeds. The three analysed fir populations reveal hardly any genetic differentiation as demonstrated in chapter 2. This fact provides additional evidence that silver populations in the Northern Black Forest are characterized by balanced gene flow processes.

As consequence, silver fir trees and populations appear to be characterized by non-restricted, past and contemporary gene flow processes and do not seem to be influenced by strong historical events, e.g. major bottleneck (cp. chapter 2). As in the present study for silver fir, seed and pollen movement has been analysed in several tree species, but little is known about the combined effect. If seed dispersal creates bottlenecks at the time of colonization, subsequent high pollen flow might eventually mitigate the low genetic diversity, if the number of available pollen sources is not constrained. If pollen flow is extensive and results in seed and seedlings, then seed dispersal from only a few source trees will not create a genetic bottleneck in the seedling pool (Sork and Smouse, 2006).

4. Associated ectomycorrhizal communities

Ectomycorrhizal diversity in relation to the age of the fir and to the site type

Mutualistic interactions such as mycorrhizal symbioses are important for the stability and the viability of forest ecosystems. Mycorrhizae are fungus-root associations that comprise the fine roots of most forest trees. Mycorrhizal fungi provide the plant with soil nutrients and water and, in turn, receive photosynthetically derived plant carbohydrate. About one third of the fungi that are associated with forest trees in Central Europe are mycorrhizal fungi (Egli and Brunner, 2002). Most of them are ectomycorrhizal (ECM) fungi. They usually form a mantle enclosing the tree rootlet from which hyphae radiate outward into the soil as well as inward between the root cells to form a hyphal network called the 'Hartig net' (Wiensczyk *et al.,* 2002). ECM fungi play a fundamental role in temperate forest ecosystems as they can improve the uptake of nutrients from the soil, enabling better growth of the forest trees under non-optimal environmental conditions. Both fungal and plant partners benefit from this association (Smith and Read, 1997).

Colonization and species composition of ECM communities can be strongly influenced by various aspects (Koide *et al*., 2005). Distinct ECM fungi are known as early- or late-stage ECM depending on the age of the host (introduced by Mason *et al.,* 1982; Izzo *et al.,* 2005; Redecker *et al.,* 2001; Smith and Read, 1997). For silver fir as host, we re-visited this phenomenon and analysed the effects of ontogenetic stages of the host on the ECM community (paper III). In addition, the diversity of distinct ECM fungi was considered. Focusing on ten ECM fungi that could be clearly determined on the species or genus level (= 'operational taxonomic units', OTUs), the ECM profile was examined for 753 firs within the two study sites EY and SR. These ten ECM OTUs associated with silver fir roots belong to the basidiomycetes, being members of the family *Russulaceae* (comprising the genera *Lactarius* and *Russula*), of the genera *Laccaria*, *Tomentella* and *Cortinarius* as well as including distinct species *Amphinema byssoides* (Pers.) J. Erikss., *Clavulina cristata* (Holmsk.) J. Schrot., *Xerocomus pruinatus* (Fr. and Hok) Quel. and the asexual ascomycete *Cenococcum geophilum* Fr. All of them are generalists in that they are associated with several host species including conifers (Krieglsteiner, 1977; Rexer *et al.,* 1995; Kõljalg, 1996; Dahlberg *et al.,* 1997; Fiore-Donno and Martin, 2001; Redecker *et al.,* 2001; Koide *et al.,* 2005; di Pietro *et al.,* 2007; Peter *et al.,* 2008). Generally, the ten OTUs were found within both study sites, in the stand as well on the windthrow area and throughout all ontogenetic stages of the firs except for three OTUs that were absent in the seedlings (*Laccaria amethystine*, *Russula species* II and *Lactarius spp*.). Thus, the former hypothesis of a clear distinction between early- and late-stage ECM fungi could not be verified by our results.

Shannon-Index and evenness revealed similar diversity values for the respective subsets of the OTUs due to the age of the host and due to the site type. Summing up, there was not a large decrease of ECM species richness on the windthrow areas compared to the forest stands at the fungal population scale. Additionally, Donges *et al.* (unpublished) did not find an abnormally high abundance of single species in the disturbed windthrow areas within the Black Forest. However, the number of OTUs was significantly dependent on the ontogenetic stage of the firs based on the individual tree. Adult fir trees on average host a higher amount of different ECM fungi than juveniles indicating a significant increase of the ECM richness on the individual scale over time. Other observations confirm that ECM species are usually added to the fungal community, but that they do not necessarily replace the earlier ones (Visser, 1995; Bradburry *et al.,* 1998). It appears that the individual tree with increasing age increases its ECM community by allowing for multi-mycorrhization of the expanding root systems. Interestingly, focusing on the population scale the ECM diversity is not so much dependent on the ontogenetic stage of the host as driving factor or on effects of site type like closed forest stands or windthrow areas. Similarly to the fir offspring within the forest stand, the pre-windthrow offspring exhibit a well-balanced ECM profile and thus, serves as an adequate inoculum (i.e. material that is the source of fungal cells) for the post-windthrow offspring. This emphasises the reservoir function of existing fir saplings on the windthrow area for newly arising fir seedlings.

A high diversity of ECM species with balanced abundance is desirable in order to stabilize the individual tree and therewith the forest ecosystem. Egli *et al.* (2002) have shown that a windthrow event can reduce the number of ECM fungi significantly if no ‘reservoir trees’ are left. The number of ECM species should, be kept as high as possible after a windthrow event. A feasible way to achieve this goal is to protect as well as possible those young trees and seedlings that have survived a windthrow. Rexer *et al.* (1998) showed that tree seedlings on windthrow areas had obtained their mycorrhizal symbionts from the species spectrum present on the roots of the surviving young trees. Hagerman *et al.* (2001) have detected that even ECM plant hosts that persist following disturbances can successfully serve as sources for ECM fungal inocula for regenerating tree species as in the case of bearberry (A*rctostaphylos uva-ursi*) and Douglas fir (*Pseudotsuga menziesii*). ECM fungi generally cannot survive in the soil for long periods without a host, so hyphae are typically attached to living roots and the recovery of ECM fungi following a disturbance takes time, usually decades (Visser, 1995). In case of a ‘total loss’ situation, when no fir tree exists on the open area which is to be colonized, other tree species that are available on the open area might serve as sources for ECM hyphae.

This appears feasible for beech and spruce that often coexists with silver fir in the Black Forest region, since the ECM profile of these three tree species within European forests show a broad overlap (Donges *et al*., unpublished). Moreover, the majority of the ECM fungi associated with the analysed fir trees was represented by unspecific generalists.

Genetic structure of the firs in correlation to the associated ectomycorrhizal community

If it is just the number of OTUs which is driven by ontogenesis of the host what about drivers that select for distinct OTUs or OTU communities? Is there evidence for driving factors which are to be found in the genetic background of tree individuals?

Genetic structures of the foundation species such as forest trees may affect levels higher than the population and therewith, may influence community structure (Witham *et al*., 2003). Recent studies in the field of 'community genetics' introduced the importance of the 'extended phenotype' of foundation species and 'interspecific indirect genetic effects' (IIGEs) that affect a multitude of associated organisms and, thus, species communities (Witham *et al*., 2003; 2006; Shuster *et al.,* 2006).

With silver fir as a model we analysed the tree-fungus relationship in order to obtain deeper insights into the symbiotic interaction with its associated ECM fungi community (paper III). Focusing on nSSR genotypes of the firs in association with the ten analysed OTUs, a significant relationship between a distinct single-locus genotype of the host and the ECM OTUs could be revealed. Thereby, the observed frequencies of 38 % of the single-locus fir genotypes deviated significantly in association with the OTUs. Thus, the genetic structure of silver fir seems to have an effect on the composition of the associated ECM community. In a previous transplant experiment Donges *et al.* (unpublished) found evidence for an interspecific indirect genetic effect between firs and associated ECM species acting at the provenance level and using the same genetic marker system. A direct gene-to-gene correlation between nSSR loci of the firs and ECM associations cannot be expected since nuclear SSR markers are considered to mark neutral genetic variation without coding for any phenotype (Porcher *et al.,* 2006). Rather a `chromosomal vicinity´ between the nSSR loci and genomic regions that have an influence on the mycorrhization can be assumed. SSR loci are dispersed throughout the genome, and thus might be located close to regulatory DNA-loci with functional relevance related to ECM colonization. Two of the six SSR loci do not exhibit significant relationships to the ECM OTUs analysed indicating that not all of the analysed loci seems to be located close to regulatory regions.

Several studies demonstrated that host plants produce essential metabolites – as a product of genetic information - which are able to affect fungal partners (Fries *et al.,* 1987; Horan and Chilvers, 1990; Ditengou and Lapeyrie, 2000; Martin *et al.,* 2001; Langenheim, 1994).

Using selectively neutral markers we cannot provide direct information about candidate genes that might have an impact on the mycorrhization. Nonetheless, our data show that there is strong evidence for variable genomic regions of the host which can be regarded as driving factors of community structure and dynamics of its associated ECM fungi. Sequencing programmes in forest trees (such as http://dendrome.ucdavis.edu/crsp) should be enlarged to provide more detailed insights into species genetic diversity in relation to gene-expressive DNA-loci. This leads to a deeper understanding of the genetic basis of phenotypic differentiation such as ECM diversity that can be considered as 'extended phenotype'.

In conclusion, the diversity of community structure appears to be dependent on the genetic diversity of the foundation species such as forest tree species. Against this background, the principle of genetic sustainability within forest ecosystems gains even more significance. The conservation of a high genetic variation within tree populations is not only of high importance in terms of adaptability to environmental changes, but also to ensure generally a multiple community structure, e.g. with associated ECM fungi, that support the vitality of forest trees. Especially under non-optimal environmental conditions such as on windthrow areas, a well-balanced mycorrhization is desired to stabilize individual trees. This indirectly contributes to the ecological and economic success of forest stands and should be considered within sustainable forest management plans.

5. Conclusion and perspective

In the Black Forest, a decline of silver fir has occurred in the last decades as a consequence of environmental stress factors and silvicultural preferences for other conifers. Due to its important ecological and economic role, a reintroduction of greater proportions of silver fir into the mountain forests of the Black Forest has been promoted by forest management plans. The following general conclusions are derived from the present findings in consideration of the question 'Which preconditions does silver fir bring along for natural regeneration and recolonization of windthrow areas in the Black Forest?'

Silver fir populations in the Northern Black Forest maintain a suitable genetic potential with high diversity within and less differentiation among populations. The decrease of silver fir within the Black Forest in the last decades does not seem to have influenced the genetic structures seriously. Strong fragmentation effects such as an interruption of gene flow leading to drift and increasing genetic differentiation among populations could not be detected. Thus, the silver fir populations provide an appropriate basis for natural regeneration processes within the forest stand as well as in terms of recolonizing open habitats due to windthrow.

Fir seedlings representing the post-windthrow offspring could largely not be found on the windthrow areas. The actual recolonization potential of silver fir and the preliminary hypothesis of a possible reduced genetic diversity within the post-windthrow offspring could, thus, not be sufficiently incorporated within the present study. However, based on the remaining silver fir regeneration on the windthrow area that was already established before the storm event, a reduced genetic diversity was not visible in comparison to the adjacent forest stand including different generations. Additionally, fir saplings in the forest stand and on the windthrow area reveal an identical spectrum of associated ECM fungi that is comparable to the ECM profile of the adult firs on the population scale. Thus, the pre-windthrow fir saplings can be an effective source of inocula and can serve as important reservoir hosts for subsequent fir regeneration on the windthrow area – similar to remaining mature trees. Both, from a tree genetic and from a mycological point of view, windthrow areas should be left as untouched as possible if pre-windthrow offspring is available in order to guarantee a well-balanced diversity of ECM fungi and a sufficient genetic diversity within post-windthrow fir seedlings. Furthermore, pre-windthrow fir regeneration on the windthrow area supports the establishment of incoming seedlings due to the characteristics of silver fir as a climax tree species.

Additionally, first hints are given that the dispersal characteristics of silver fir are sufficiently high to colonize at least those windthrow areas that do not exceed a few hundred meters from the remaining stand. From the dispersal point of view, it can therefore be assumed that fir populations in the Black Forest maintain genetic diversity in recolonized habitats such as windthrow areas.

As part of global efforts to preserve biodiversity and to assure sustainability, the conservation of genetic resources within forest management plans integrating intraspecific genetic diversity are of important interest. Generally, knowledge of genetic diversity maintained in natural forest populations plays a central role in conservation programmes, particularly in threatened or disturbed habitats. So far, this has been considered mainly on the single-species level. As a consequence of ECM associations that can be regarded as extended phenotypes of the host trees, conservation programmes of forest genetic resources should consider the impact of genetic diversity of host species on associated species communities within forest ecosystems. Thus, the 'minimum viable interacting population size' (MVIP) better reflects the goals to conserve genetic diversity at levels required by interacting species instead of the conventional used 'minimum viable populations size' (MVP) (Whitham *et al.*, 2003).

Based on the overall findings of the present study, natural regeneration within forest stands as well as recolonization of windthrow areas in silver fir can be assumed to be an appropriate and promising method for the reintroduction of larger proportions of silver fir in the Black Forest. Our results are preliminarily data for the estimation of the regeneration potential of windthrow areas in silver fir. They mainly focus on the close-up-range of the forest stand as well as on short distance dispersal and represent only a short view. Long-term and large-scale studies that may lead to insights into colonization processes on a larger temporal and spatial scale will be necessary, particularly in terms of climate change. As gene flow might be the key to maintaining genetic diversity and adaptability of forest trees, the combined knowledge of short- and long-distance gene flow events could provide more information about the effects of 'total loss' scenarios on recolonization processes. This would contribute to a better reconstruction of colonization history of tree species after the last glaciation and to predict future colonization processes of new sites due to climatic shifts. As the Black Forest represents the northern edge of the natural distribution area of silver fir, these fir populations could possibly form the future colonization front expanding towards the north.

6. References

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Publications and Manuscripts

Paper I

Identification and characterization of nuclear microsatellite loci in *Abies alba* Mill.

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PRIMER NOTE

Identification and characterization of nuclear microsatellite loci in *Abies alba* Mill.

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Abstract

Eleven polymorphic nuclear microsatellite markers for *Abies alba* Mill. were developed from an enriched genomic library. An average of 5.2 alleles per locus and a mean expected heterozygosity of 0.532 were found in a sample of 24 *Abies alba* individuals from different populations within Europe. These loci can be used in studies of genetic diversity for parentage analysis and for estimation of gene flow in silver fir populations. Moreover, successful amplifications were obtained for eight other Mediterranean *Abies* species, suggesting that these loci may be useful for similar applications in other fir species.

Keywords: *Abies*, cross-species amplification, SSR

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Abies alba Mill., a conifer species belonging to the family of Pinaceae, is a key species of the mountainous forest ecosystems in Europe. In Central Europe, a decline of *A. alba* has occurred as a consequence of environmental stress factors and silvicultural preferences for other conifers, mostly Norway spruce. Due to its important ecological role as a 'stabilizing' tree species, a reintroduction of larger proportions of silver fir into the mountainous forests has been promoted by forest managements. For this, it is necessary to have a more detailed knowledge about the dynamics of genetic diversity within and among silver fir populations. Population genetic studies so far relied on isozyme gene markers (e.g. Hussendörfer *et al*. 1995) and chloroplast microsatellite markers (e.g. Ziegenhagen *et al*. 1998; Vendramin *et al*. 1999). Nuclear microsatellites as highly polymorphic, selectively neutral and codominant markers are best suited for the analysis of small-scale genetic diversity. So far, they were not available for silver fir. Here, we present a set of novel nuclear microsatellite markers developed in *A. alba* and demonstrate the transferability to other *Abies* species.

An enriched genomic library for di- (GA, GT, AT, GC), tri- (CAA, ATT, GCC) and tetranucleotide (GATA, CATA, ATAG) was constructed and screened for microsatellite sequences, following the protocol of Edwards *et al*. (1996). The plasmids containing the microsatellite candidates were screened by a polymerase chain reaction (PCR). DNA fragments larger than 250 bp were sequenced with an Amersham MegaBACE 1000 automated sequencer using the DYEnamic ET Terminator Sequencing Kit (Amersham Biosciences). For sequencing, primers flanking the polycloning site of the pBlueScriptII vector (Stratagene) were designed external to the M13 universal primers.

A total of about 170 clones were sequenced. More than 90% of the clones contained the microsatellite stretches; however, it was possible to design primers, using PRIMER 3 software (Rozen & Skaletsky 2000), only in 44 cases (about 30%) because in several cases the stretches were too close to the end of the clone and/or too long. Fourteen primers (about 32%) yielded distinct PCR products of the expected size.

The 14 loci were checked for variability analysing needles from 17 to 24 *A. alba* individuals collected in Bulgaria, France, Germany and Switzerland. Total DNA was extracted as described by Dumolin *et al*. (1995). PCRs were performed in a 25 μL reaction volume containing 30 ng of DNA, 1× PCR buffer (Promega), 2 μM of each primer (forward primer fluorescence labelled), 5 mM of each dNTP, 2.5 mM $MgCl_2$ (A) or 1.5 mM $MgCl_2$ (B), 1 U Go *Taq* polymerase (Promega) and 0.8% BSA. The amplification was carried

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2 PRIMER NOTE

Table 1 Characteristics of 14 nuclear microsatellite loci developed for *Abies alba*

Locus name (Accession no.)	Primer sequence (5′–3′)	Repeat motif	PCR product size (bp)	PCR profile	*N*	*A*	H_O	H_E
SF 1 (DQ218453)	F: HEX-TTGACGTGATTAACAATCCA R: AAGAACGACACCATTCTCAC	$(CCG)_9$	221–226	A	24	3	0.333	0.598*
SF b4 (DQ218454)	F: FAM-GCCTTTGCAACATAATTGG R: TCACAATTGTTATGTGTGTGG	$(GT)_{16}$	166–186	A	17	5	0.294	0.599**
SF b5 (DQ218455)	F: FAM-AAAAAGCATCACTTTTCTCG R: AAGAGGAGGGGAGTTACAAG	$(CT)_{15}$	143–155	A	24	5	0.625	0.593n.s.
SF g6 (DQ218456)	F: FAM-GTAACAATAAAAGGAAGCTACG R: TGTGACACATTGGACACC	$(AC)_9$	107–113	A	22	3	0.136	0.129n.s.
SF 50 (DQ218457)	F: FAM-CATTTGGTGCGGTTCATTTC R: AGTGGCATTTCACTTATTGG	$(GT)_{11}(GC)_{10}$	96–102	B	23	4	0.087	0.475***
SF 78 (DQ218458)	F: FAM-CATTGTTGTCTTTGTTTCACA R: TGCACCGTTTTGTTTTTCC	$(CGCA)_8(CA)_{15G}(_{CA})_8$	158–244	B	22	15	0.682	0.883***
SF 83 (DQ218459)	F: FAM-AGCAGCATAACCAAGGGTCAA R: TCTGAATTTCTAAAGGCGGC	$(CTT)_3\ldots(GCC)_5$	197–206	A	24	2	0.042	0.041n.s.
SF 239 (DQ218460)	F: HEX-GCTCTGTGCACTGCCTGT R: TTCGGAGACTAACGCATCTCA	$(TG)_{11}$	108–122	B	21	6	0.286	0.642**
SF 324 (DQ218461)	F: FAM-TTTGAACGGAAATCAAATTCC R: AAGAACGACACCATTCTCAC	$(CCG)_8$	110–116	A	24	3	0.333	0.348n.s
SF 331 (DQ218462)	F: FAM-TGTAATGCTTTTCATGGGCAA R: TTACATGGGAAAACCATCCA	$(GT)_{11}$	106–116	C†	19	5	0.211	0.747***
SF 333 (DQ218463)	F: FAM-ATTTGTTCATTTTGGTCCTG R: ACACAGGAAAAAGTCGGTAA	$(CA)_{12}(TA)_4$	168–178	A	23	6	0.391	0.792**
SF 2 (DQ218464)	F: FAM-TGTTGGATTTATGGTTACCTC R: GACAAAACCCTTTTGCAAAAC	$(CA)_4GA(CA)_4GA(CA)_3$	166	A	24	1	—	—
SF 8 (DQ218465)	F: FAM-TACAGCAGCCTGTAGGTATG R: GAGTGGTCGATACACACAAA	$(GCC)_6$	132	A	24	1	—	—
SF g36 (DQ218466)	F: FAM-CACAAGAAAAAGCTGGTAAA R: TAGGAGTTTGGGACTTCAGA	$(TG)_9(CG)_6\ldots(GT)_5$	99	A	19	1	—	—

†C: 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 30 s at 72 °C and a final extension at 72 °C for 7 min.
N, sample size; *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.
*, ** and ***: significant departure from Hardy–Weinberg equilibrium at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. n.s. = not significant.

out in a thermal cycler (T1, Biometra) with following conditions: 5 min at 94 °C followed by 10 touchdown cycles of 30 s at 94 °C, 30 s at 60 °C (A) or at 65 °C (B) (1 °C lower per cycle), 40 s at 72 °C and 25 cycles of 30 s at 94 °C, 50 s at 50 °C (A) or 55 °C (B), 40 s at 72 °C with a final extension time of 7 min at 72 °C. The PCR products were separated by capillary electrophoresis using the Amersham MegaBACE automated sequencer (Amersham Biosciences). Alleles were sized using the size standard MegaBACE ET400-R (Amersham Biosciences) and the MEGABACE FRAGMENT PROFILER version 1.2 (Amersham Biosciences).

Genetic diversity parameters were estimated using GENALEX version 6 (Peakall & Smouse 2005). Eleven loci were polymorphic, and three loci were monomorphic (Table 1). For the polymorphic loci, between two and 15 alleles were detected with an average of 5.2 alleles per locus. The expected heterozygosity ranged from 0.041 to 0.883 with a mean value of 0.532. Significant deviation from expected heterozygote frequencies at Hardy–Weinberg equilibrium was observed for SF 1 ($P < 0.05$), SF b4, SF 239, SF 333 ($P < 0.01$) and SF 50, SF 78, SF 331 ($P < 0.001$). These loci showed deficiencies of heterozygotes, which might be due to a sampling effect (small sample size, different population genetic structures (Wahlund effect), absence of natural out-crossing mating system). We cannot exclude the presence of null alleles, either. Those alleles, however, that could be checked in controlled crosses followed Mendelian segregation (data not shown). All loci were tested for linkage disequilibrium (LD) using the software GENEPOP (Raymond & Rousset 1995). This test did not reveal any significant cases of LD.

Cross-species amplification of the 14 primer pairs was tested in eight other Mediterranean *Abies* species with two to five individuals each (Table 2). One hundred and seven of 112 combinations of loci and species (96%) were able to amplify products. All amplification products obtained from the eight species ranged within the sizes of the respective *A. alba* microsatellite loci. Most of the loci were polymorphic,

Table 2 Transferability test to additional *Abies* species

Species	Locus name													
	SF 1	SF b4	SF b5	SF g6	SF 50	SF 78	SF 83	SF 239	SF 324	SF 331	SF 333	SF 2	SF 8	SF g36
Abies borisii regis	+ m	(+)	+	+	+	+	+	+	+	+	+	–	+	+
Abies bornmuelleriana	+ m	+	+	+	+	+	+	+ m	+	+	+	+	(+)	+
Abies cephalonica	+ m	–	+	+	+	+	+	+ m	+	+	+	+	+	+
Abies cilicica	+ m	+	+	+	+	+	+	+ m	+	+	+	+	(+)	+
Abies equitrojani	+ m	+	+	+	+	+	+	+ m	+	+	+	+	+	+
Abies marocana	+ m	+	+	+	+	+	+	+	+	–	+	+	–	+
Abies numidica	+ m	–	+	+	+	+	+	+ m	+	+	+	+	+	+
Abies pinsapo	+ m	+	+	+	+	+	+	+ m	+	+	+	+	(+)	+

+, PCR amplification; –, no PCR amplification; m, multibanding pattern; (+), weak amplification.

suggesting that these markers are also useful for similar applications in other *Abies* species.

Overall, these results illustrate the utility of the newly developed microsatellite loci for assessing spatial patterns of genetic diversity for parentage analysis, for gene flow studies and for individual identification in *A. alba* populations. Using the newly developed markers in ongoing studies, we are comparing the dynamics of genetic diversity in silver fir between closed stands and large-sized forest gaps.

Acknowledgements

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Paper II

Estimating local seed dispersal in silver fir (*Abies alba* Mill.)

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Manuscript

Estimating local seed dispersal in silver fir (*Abies alba* Mill.)

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In memory of Günther Groß

Abstract

Seed dispersal as part of gene flow is one of the most important factors influencing the genetic structure of forest trees. It plays an essential role in recolonizing habitats and must be sufficient to maintain the level of genetic diversity in future generations. We investigated local seed dispersal by estimating the spatial dispersal distances of silver fir seeds (*Abies alba* Mill.). Furthermore, the effect of morphological seed parameters on the dispersal was analysed and the level of immigration seeds from longer distances was estimated. For this, a grid of 37 seed traps was set up within a forest area in the Black Forest, Germany, on a windthrow area as well as within the adjacent forest stand. By comparing the microsatellite genotypes of the maternal tissue of the fir seeds with the genotypes of reproductive fir trees in the forest stand, we aimed to identify source trees ('mothers') of the seeds.

Ninety-four out of 674 trapped seeds could be assigned to a mother tree. For those, a mean dispersal distance of 98 m was calculated. Shorter dispersal distances were observed for the seeds collected in the stands than for those collected in the windthrow area. There was no significant impact of morphological parameters of fir seeds on the dispersal distances. Seed entry into the windthrow area was slightly reduced in comparison to the forest stand but nevertheless sufficient within short distances of approximately 150 m. The high number of contributing mother trees implies a high level of genetic diversity in the seed population. The implications of local seed dispersal events are briefly discussed with regard to recolonization of open habitats and silvicultural management practices.

Keywords: *Abies alba* Mill., seed movement, dispersal distances, recolonization, windthrow areas, nuclear microsatellite markers, source trees

Introduction

Forest ecosystems are characterized by high levels of genetic diversity within the tree species. Due to their long generation times the mechanisms for maintaining genetic diversity are difficult to study. In natural plant populations such as forest tree populations, the spatial-temporal dynamics of genetic diversity are shaped by gene flow as a key determinant (Loveless and Hamrick, 1984). Gene flow, in turn, depends on the seed and pollen dispersal capacity of the tree species. Explicit information about seed dispersal is scarce for the majority of the species. Indeed it is a driving factor for a variety of characteristics in plant populations and their genetic structure (Hamrick *et al.,* 1992). Seeds provide the vital genetic link and dispersal agent between successive generations of plants. In tree species, seeds are the only mobile stage within the life-cycle of plants. Their dispersal determines the chances of establishment and survival away from the parent trees and thus, the distribution of the next generation and displays an essential aspect for the colonization of new sites.

The state of the art on seed-mediated gene movement lags far behind that for pollen movement, because it has been more difficult to study. Using parentage analysis it is possible to obtain information about the parent pairs, but the mother and the father cannot be distinguished (analysing monoecious species with codominant markers and without using uniparentally inherited cytoplasmatic markers). However, the recent innovation of using genotypes derived from purely maternal tissue of seeds made it possible to identify the source or mother tree of the dispersed seeds directly (Godoy and Jordano, 2001; Ziegenhagen *et al.,* 2003) and thus, overcame the problem of parentage analysis. Thereby, molecular markers offer a useful tool to effectively study seed movement (Ouborg *et al.,* 1999; Sork *et al*., 1999; Grivet *et al.,* 2005). Especially nuclear microsatellites (simple sequence repeats = SSR) have proved to be the marker of choice for identification and parentage analysis (reviewed in: Jones and Arden, 2003; Luikart and England, 1999).

The dispersal of seeds plays a decisive role in seedling recruitment, migration among populations and colonization of new sites (Sork and Smouse, 2006). Thus, in terms of increasing disturbance events as a consequence of climate change, understanding the process of dispersal in natural regeneration of tree species is an essential factor for achieving sustainable forest management. Storm events may lead to extensive windthrow areas in forest ecosystems that should be reforested under natural and stand stabilizing aspects using either natural or planted regeneration.

Natural forest regeneration might be critical due to the lack of a sufficient number of parent trees in the adjacent forest stands and limited seed movement when windthrow areas are large.

However, the dispersal of seeds generally depends on different factors such as the height of the trees which release the seeds and the wind speed as well as morphological features of the seeds, like weight, shape and size (Rohmeder, 1972). Moreover, the morphological features of silver fir cones and seeds exhibit substantial heritable variation (Tracz and Barzdajn, 2007). The traits of fir seeds (e.g. cone size, seed length and length of seed wings) are therefore used for provenance identification (Ballian and Cabaravdic, 2005) and can characterize single trees and populations significantly (Tracz and Barzdajn, 2007).

In a case study, we estimated local seed dispersal in a forest stand and in an adjacent open area due to windthrow in the tree species *Abies alba* (Mill.) – a wind dispersed conifer. Its comparatively large seeds are winged and wind is the main agent for dispersal. By comparing the SSR genotypes of the maternal tissue of the fir seeds (seed coat and wing) with the SSR genotypes of reproductive fir trees in the forest stand, we aimed to identify source trees ('mothers') of the seeds that were collected in the seed traps. Besides measuring explicit seed dispersal, morphological parameters of the seeds were considered as driving factors of dispersal processes. Thereby we focused on following questions:

(i) Do morphological properties of fir seeds and temporal aspects of seed movement influence the process of dispersal?

(ii) How far are fir seeds dispersed within a closed forest stand and an adjacent windthrow area?

(iii) Based on the genetic findings, what can we infer about the number of contributing mothers?

Materials and Methods

Study Site

This work was conducted on a study site in the Black Forest, a low mountain range in the south-western part of Germany. This forest region is characterized by a mixture of silver fir (*Abiea alba* Mill.) with Norway spruce (*Picea abies* (L.) H. Karst) and beech (*Fagus sylvatica* L.) – a typical forest composition in the submontane regions. The study site containing silver fir as the main tree species (with an amount of 50 %) is characterized by a natural forest stand and an adjacent windthrow area with a size of about 45 hectares due to the storm event 'Lothar' in 1999.

Study species

For the present study silver fir has been chosen as a model species. It is suitable as a model species for seed dispersal in wind-pollinated trees since its life history traits do not support very high rates of gene flow. For instance, silver fir is characterized by having one of the largest pollen grains of wind-pollinated trees (Stanley and Linskens, 1974) and comparatively large seeds, as well as a long generation time and a long life period. Silver fir has suffered serious range reduction over the last centuries as a consequence of environmental stress factors and silvicultural preferences for other conifers, mostly Norway spruce. Due to its important ecological role as a 'stabilizing' tree species (Schütt, 1994), a reintroduction of larger proportions of silver fir into the mountain forests has been promoted by forest management plans.

Like most conifers, silver fir produces monoecious flowers that are wind pollinated in the spring (May to June) and seeds maturing in fall (September). Fruit ripening and seed dispersal occur in the same fall and involves separation of cone scales and seeds, leaving only the cone spindle on the tree. Wind is the major agent for the dispersal of the winged fir seeds. Although most seed is usually disseminated in fall (September – October) seedfall may continue into winter (Young and Young, 1992).

Seed collection and sampling of plant material

A grid of 37 seed traps with a size of 1 m² each was set up within an area of about one hectare. Twenty-five seed traps were located on the windthrow area (up to a distance of 100 m into the open area from the forest margin) and twelve seed traps were positioned within the adjacent forest stand (Figure 1a and 1b). Over a period of 14 weeks (from September 2006 to January 2007), 674 seeds were collected between September 2006 and January 2007 in two week intervals. The seeds were recorded and removed during each visit. They were stored at 4°C until analysed.

In the forest stand needles of 203 adult trees were randomly sampled within a distinct area of about six hectare which represent about 50 % of the entire adult trees. Sampled firs were geo-referenced by GPS (using a GS5, Leica Geosystems, Heerbrugg, Switzerland) as indicated in Figure 1a. The needles of the adult trees were kept in cold storage (-20°C) until analysed.

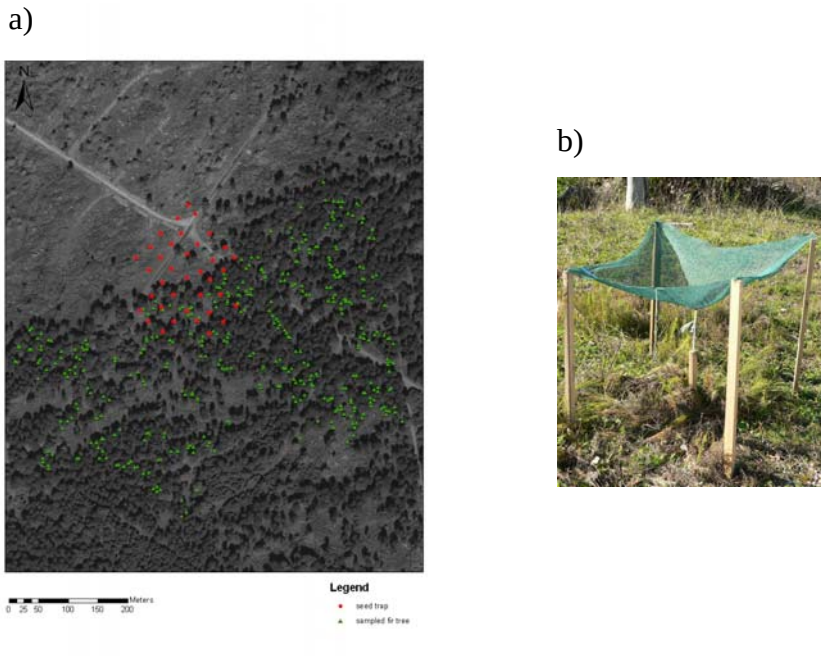


Figure 1 Aerial view of the study plot 'Sauriss' in the Black Forest with sampled fir trees marked in green and seed traps marked in red (a) having a size of 1m^2 (b).

Seed characterization and microsatellite genotyping

After collecting the seeds, morphological and quantitative parameters of each seed were recorded including the weight of the seeds and the length of the seed wings. The seeds were cut with a scalpel to determine the condition of the seeds, i.e. whether they contained a viable embryo (filled) or not (empty).

Total DNA was extracted from frozen needles as well as from the seed coats and wings adopting an alkyltrimethyl ammonium bromide (ATMAB) method described by Dumolin *et al*. (1995). All samples were genotyped with five nuclear microsatellite (nSSR) loci (SF1i, SFb5, SF78, SF331, SF333) using PCR conditions as described in Cremer *et al. (*2006). For the nSSR locus SF333 the PCR program had to be optimized for the amplification of the seed coats and wings as follows: 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 54 °C, 30 s at 72 °C and a final extension at 72 °C for 7 min. Forward primers carried a fluorescent label (Hex, Fam or Tamra). The DNA was amplified in a thermal cycler (T1, Biometra) and the PCR products were separated by capillary electrophoresis using the MegaBACE automated sequencer (GE Healthcare, Freiburg, Germany).

Alleles were sized using the size standard MegaBACE ET400-R (GE Healthcare) and the MegaBACE fragment profiler version 1.2 (GE Healthcare). A cross-validation check for reproducibility was conducted by re-analysing 20 randomly chosen samples.

Statistical analysis

A linear regression analysis was performed using the statistical software SPSS 14 (SPSS Inc., Chicago, Il, USA) to analyse whether the morphological parameters seed weight and length of seed wings are correlated to each other. A further question was, whether these parameters influence the dispersal distance of the seeds.

Using a one-way ANOVA it was tested if the condition of the seeds (filled or empty) have an impact on the parameters seed weight, length of seed wings and dispersal distance by means of the statistical software SPSS 14 (SPSS Inc., Chicago, Il, USA). With the condition of seeds as explanatory factor containing the levels 'filled' and 'empty' (k=2) we have tested the following null hypothesis: the mean of seed weight, the mean of length of wings and the mean of dispersal distances, respectively, is the same for both conditions of seeds. Thereby, the morphological parameters are the response variables.

The software GENEPOP (Raymond and Rousset, 1995) was used to test for linkage disequilibrium between the analysed microsatellite loci.

In order to get statistical confidence for the differentiation potential of individual trees and the potential of individual assignment with the used marker system, the probability of identity (P_{ID} and P_{IDsibs}) was calculated using GenAlEx (Peakall and Smouse, 2006). P_{ID} is the probability that two randomly drawn (unrelated) individuals exhibit by chance identical multilocus genotypes (Paetkau *et al.,* 1998). Thereby, P_{IDsibs} takes into account the genetic similarity among siblings.

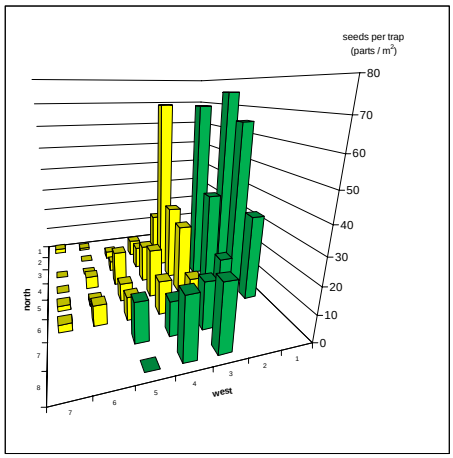
Since the pericarp and the wing of the fir seeds is maternal tissue (Liu, 1971; Strasburger *et al.,* 1998), a direct identification of the mother trees is possible by means of molecular markers. On the basis of the multilocus genotypes of the seeds and those of the adult trees as characterized at the five high variable nSSR loci, an identity analysis with matching multilocus genotypes was conducted using the software CERVUS 3.0.3 (Marshall *et al.,* 1998; Kalinowski *et al.,* 2007). Within this procedure mismatch was excluded and a minimum number of four matching loci was chosen as settings. In case that a seed was assigned to two adult trees based on matching multilocus genotypes, the average distance between the seed and each of the two possible mother trees was calculated and considered for further analysis.

Results

In total, 674 seeds were collected in the 37 seed traps within a time period of 14 weeks. Thereby, 395 seeds were found in the twelve traps which were located within the stand and 279 seeds were found in the 25 traps on the windthrow area. With increasing spatial distance from the forest stand less seeds were dispersed into the windthrow area (Figure 2a). Those seed traps that contained most of the seeds (with 65 to 75 each) were located within the stand or at the forest margin, respectively. Three of the four seed traps that did not contain any seeds during the collection period were located on the windthrow area. These findings indicate that obviously more seeds are dispersed locally within the forest stand than into the windthrow area.

Considering the temporal aspect, more than 50 % of the seeds (358 seeds) were distributed during the last two weeks of October (Figure 2b). Thus, the main seed dispersal took place two to six weeks after the fruit ripening in September and beginning of October. The seed dispersal shows a main peak around the end of October, but slight dispersal continues until the end of the collection period. As displayed in Figure 2b the proportion of filled seeds ranges from 100 % (September) to 27 % (October) and decreases even more in the seeds that are dispersed later in fall and winter.

a)



b)

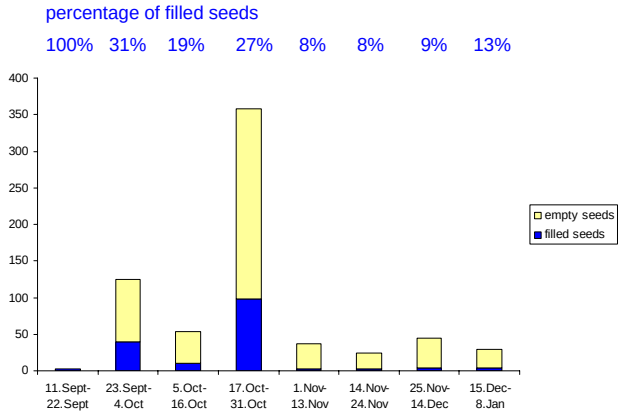


Figure 2 Spatial distribution of the 674 seeds within the 37 seed traps and number of seeds found in each trap (traps located within the forest stand are coloured green, traps in the windthrow area are coloured yellow) (a) and temporal distribution of seeds, divided into number of filled seeds (blue) and number of empty seeds (yellow) (b).

Morphological seed parameters and their effects on seed dispersal

By means of a regression analysis we tested whether there is a linear relation (i) between the weight of the seeds and the length of the seed wings, (ii) between the dispersal distance and the length of the seed wings and (iii) between the dispersal distance and the weight of the seeds. As expected, the weight of the seeds was significantly correlated to the length of the seed wings with a correlation coefficient of R^2 = 0.597 (Figure 3a). In contrast, the parameters length of seed wings and weight of the seeds were not linearly correlated to the seed dispersal distance (Figure 3b and 3c).

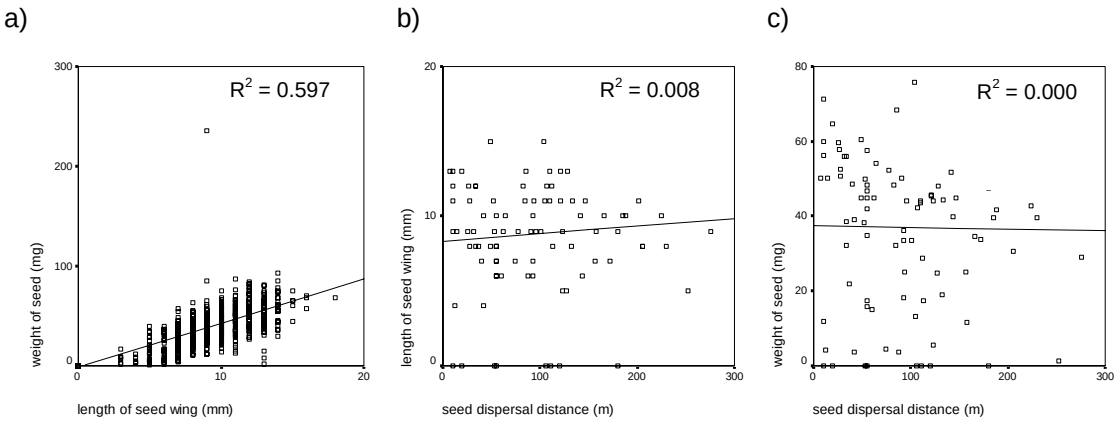


Figure 3 Scatter plot containing a linear regression line displaying accordance between length of seed wings and weight of seeds (a), length of seed wings and seed dispersal distance (b) and weight of seeds and seed dispersal distance (c).

The results of the ANOVA revealed that the seed weight and the length of the seeds were significantly dependent on whether they were filled or empty (Figure 4). Thereby, filled seeds were heavier and featured longer wings than empty seeds. Against that, the condition of seeds had statistically no significant effect on the flight distance of the seeds. Generally, the total amount of empty seeds was quite high with 75.8 % (510 seeds).

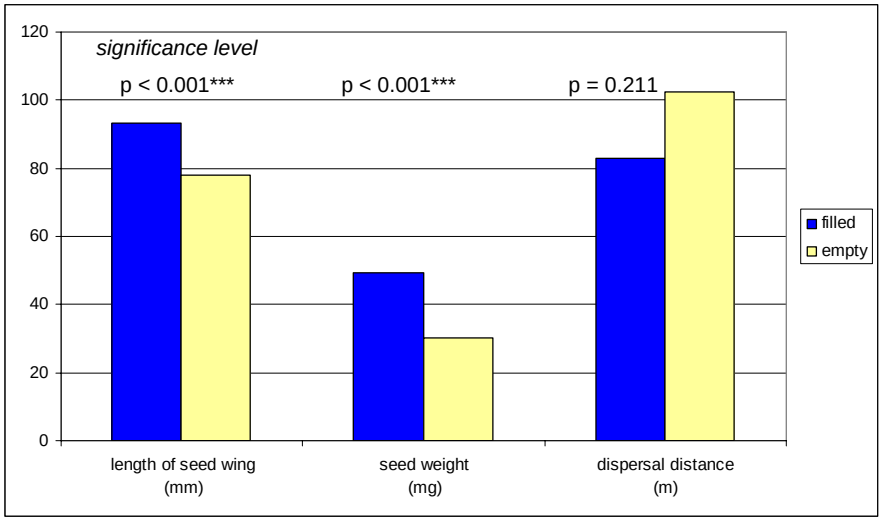


Figure 4 Mean values of length of seed wings (in mm), seed weight (in mg) and dispersal distance (in m) for the two seed conditions 'filled' (blue) and 'empty' (yellow); given are the significance levels as results of the ANOVA.

Molecular analysis for assigning seeds to mother trees

No significant linkage disequilibrium was detected among the five analysed nSSR loci. On average 10.8 alleles per locus were observed in the 674 seed wings and in the 203 adult trees with the number of alleles per locus ranging from seven (SF1 and SF333) to 23 (SF78). In order to estimate the identification potential of the marker system used, the P_{ID} was computed. Based on the five nSSR loci the P_{ID} for the adult trees and the seeds revealed a value of 4.1×10^{-4} ($P_{IDsibs} = 4{,}1 \times 10^{-2}$) indicating that in four of 10,000 cases two samples drawn at random from a population cannot be distinguished from each other. Alternatively, this value shows sufficient identification potential based on the multilocus genotype for the assignment of seeds to mother trees.

Out of the 674 seeds that were collected in the seed traps, 661 seeds could be genetically characterized. Thereby, we detected 477 different multilocus genotypes among the total sample of 661 seeds. Comparing the multilocus genotypes of the seeds that are derived from the maternal tissue with those of the 203 adult trees, 61 seeds could be clearly assigned to a mother tree based on four and five matching loci. Thirty-three seeds could be assigned to two possible mother trees. For those, the average dispersal distance was calculated as the distance between the seed trap and the two possible mother trees. Thus, a total of 94 seeds (that means 14.2 %) could be included for the estimation of seed dispersal which originates from one of the 203 adult fir trees within the stand. Only 23 seeds originate from traps of the windthrow area, mostly from traps located close to the forest margin.

The 61 seeds that could clearly be assigned to a mother tree originated from 28 different mother trees (Figure 5). The allocation of the identified mother trees shows that most of them are located close to the forest margin and therewith, close to the seed traps. Unexpectedly, none of the assigned seeds could be identified as offspring from the three remaining adult trees on the windthrow area (hold-over trees) although cones were visible.

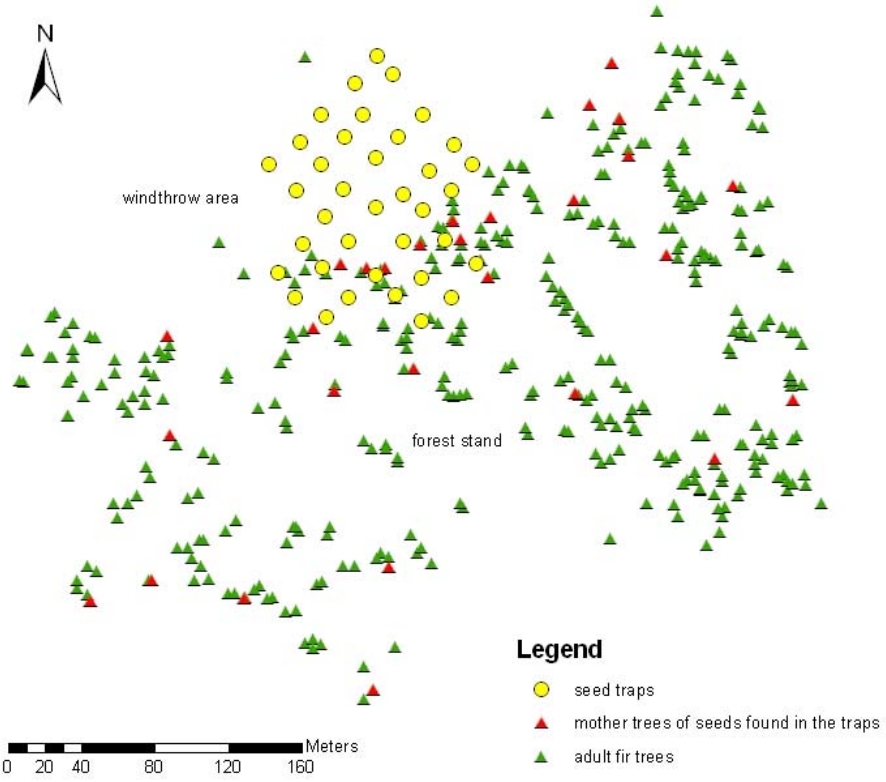


Figure 5 Position of genotyped silver fir trees and of the seed traps within the study site; successfully assigned mother trees of the trapped seeds are indicated with red symbols.

Additional twelve seeds could be assigned to more than *two* mother trees. They had to be excluded for the further analysis due to the multiple assignments. Using four to five SSR loci we detected 477 different multilocus-genotypes among the total sample of the genotyped fir seeds. The 94 assigned seeds revealed 37 different multilocus genotypes while the twelve seeds that resulted in a multiple assignment show an additional multilocus-genotype. That means we found 439 different multilocus-genotypes within the maternal tissue of the seeds that do not match with any multilocus-genotype of the sampled adult fir trees. Thus, at least 439 trees that were not sampled dispersed seeds into the traps.

By quantifying the spatial distances between the traps of the 94 assigned seeds and their identified mother trees, it was possible to determine the seed dispersal distance of the fir seeds and to construct a seed dispersal curve (Figure 6). A minimum of 7 m and a maximum dispersal distance of 275 m were observed with a mean dispersal distance of 98 m (median: 92 m). Sixty-five percent of the fir seeds dispersed within 120 m of their mother trees. Focusing on the seeds found in the stand and those found in the open area separately, shorter dispersal distances were observed for the seeds collected in the stands with a mean dispersal distance of 96 m and a maximum of 252 m. In contrast, seeds trapped in the windthrow area exhibited an average dispersal distance of 103 m with a maximum distance of 276 m.

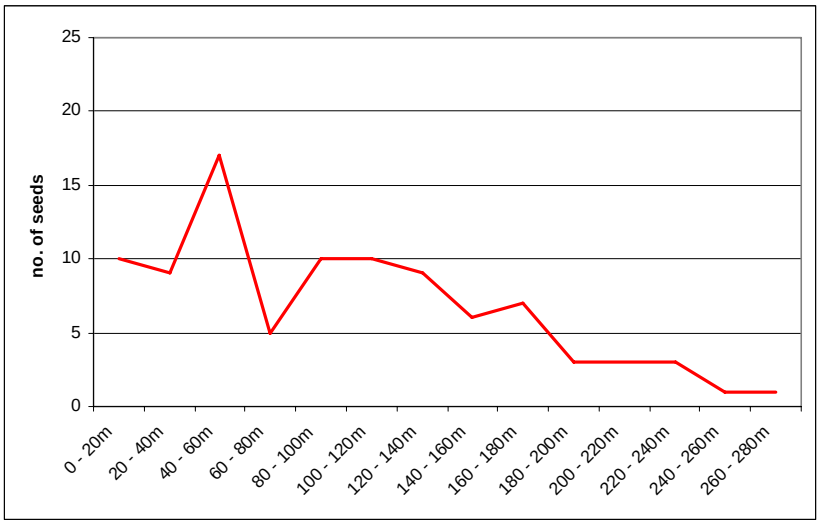


Figure 6 Seed dispersal function of silver fir seeds based on 94 seeds.

Discussion

This study contributes to a better understanding of local seed movement in wind-mediated tree species silver fir under the spatial aspect and the impact of morphological characteristics of seeds.

The present case study displays the seed dispersal with an average cone crop in silver fir showing a comparatively low yield of filled seeds as occurred for the Black Forest in 2006 (T. Ebinger, pers. communication). Larger crops usually occur at a 2- to 4-year interval (Young and Young, 1992). In another study the genetic structure of the adult trees within the study plot had been intensively analysed in comparison to two other fir stands in the Black Forest (Cremer *et al.*, unpublished). The results demonstrate that the adult tree genotypes (including the mother trees) are representative for the Black Forest region concerning genetic diversity and allelic structure.

Morphological characteristics of seeds and temporal aspects of seed dispersal

The morphological parameters seed weight and length of the wings do not appear to have an influence on the dispersal distance of the seed while both parameters are not mutually independent. These findings are congruent with those of Tracz and Barzdajn (2007) who also detected a significant correlation between length of the wings and other main features of seeds and cones such as length of seed and scale length of cone. Empty seeds are significantly lighter and have smaller wings than filled ones. Focusing on the mean value of the dispersal distance they disperse over larger distances whereas this result was statistically not significant. Since lighter seeds should disperse over larger distances and in contrast smaller wings might lead to less dispersal, both observations partly counterbalance each other. These considerations might result in a non-significant relationship between the condition of seeds and the dispersal distance. Due to the reduced weight and length of the wing of empty seeds, a deficit within development of the seeds can be assumed. However, many agents can cause a formation of empty seeds and with it a reduced seed yield. Typically, a large percentage of mature *Abies* seeds are empty, between 40 % and 70 % (Rohmeder, 1972; T. Ebinger, pers. communication). The responsible factors have not been completely identified yet, but lack of pollination, selfing or genetic irregularities have been suggested (Young and Young, 1992). The reproductive success is also reduced when the flowering periods of the individuals do not overlap (Austerlitz *et al.,* 2004) or by adverse weather conditions, e.g. by late spring frost, rain and summer drought.

Eighty percent of the seeds collected in the traps were dispersed by the end of October. Dispersal events occur slightly delayed after fruit ripening, usually September and October in silver fir (Young and Young, 1992) due to the drying processes of the cones before releasing the seeds. Although most fir seeds are usually disseminated in fall, seed fall sometimes continues well into the winter (Young and Young, 1992). Congruently, a long dispersal period of the fir seeds until the end of the collection period in January was observed. The long dispersal period leads to a seed dispersal under various weather conditions and wind directions and therewith, promotes multifaceted dissemination. Overall the amount of empty seeds was comparably high with 75 %, but especially within the second half of the dispersal period an even higher amount of empty seeds of up to 90 % was observed. This can be a result of the location of the empty seeds within the cone since they are mainly located at the bottom of the cone (Young and Young, 1992). Therefore, those seeds might be loosened from the disintegrating cone at a later date.

Spatial scale of seed movement

The accumulation of the seed entry was especially high close to the forest margin and within the forest stand. However, seeds were also found in the seed traps of the windthrow area with the largest distance from the forest margin. Congruently, Kohlermann (1950), detected that 50 % of spruce and pine seeds that would have been deposited within the stand are dispersed in the open area within a distance that correspond to the double adult tree height. In a distance that corresponds to the fourfold tree height, only 10 % of the seed density in the forest stand was estimated.

Analysing maternal tissue of seeds such as coats and wings offers the unique possibility to identify mother trees of dispersed seeds when their genotypes are compared with those of the adult trees in a population. Conducting the direct maternity approach such as Godoy and Jordano (2001) and Grivet *et al.* (2005) we could localize maternal trees for 14 % of the fir seeds found in the traps. The remaining 86 % could not be identified because their maternal tree had not been genotyped or was outside the sampled area. Most seeds were dispersed at a distance of 40 m to 60 m from the maternal tree which was probably due to the fact that winged seeds are blown with the wind and usually do not fall directly to the ground. Similar results were found in the Bavarian Forest by analysing seed dispersal in spruce where the maximum number of seeds was found at a distance of 45 m to 60 m (Schirmer and Konnert, unpublished).

As the seed traps were set up to an extent of about 100 m into the windthrow area, a maximum dispersal range between mother tree and seed deposition of 350 m was covered in the study site. Thus, we mainly focused on local seed dispersal. Seeds trapped in the windthrow area exhibited a higher dispersal distance probably due to fewer barriers during seed fall and higher wind velocity. Lian *et al.* (2008) detected distances between *Abies sachalinensis* recruits and their presumed mother trees ranging from 1.7 m to 236.9 m which is congruent with our findings in silver fir. Indeed, the mean distribution of the A. *sachalinensis* was smaller with 23.7 m and showed that 81.6 % of the recruits established within 30 m of their mother trees. Godoy and Jordano (2001) found evidence for a distribution of seeds primarily in the neighbourhood of the maternal tree for the animal-dispersed species *Prunus mahaleb,* a Mediterranean shrub. Our findings could not completely support the previous results in A. *sachalinensis and P. mahaleb* since only 20 % of the assigned seeds were dispersed within a 40 m radius of the mother tree.

Contributing mother trees

Our estimates of dispersal distances do not include the long seed dispersal from other parts of the forest area (immigrant seeds). When potential mother trees are too widely distributed to locate, maternity analysis is also not practical, despite good genetic resolution of the markers used. Nonetheless, a genetic analysis can provide valuable information about the genetic consequences of seed dispersal, even when a maternity analysis is not feasible (Grivet *et al.* 2005). About 86 % of the seeds were not assignable to any analysed adult tree. However, the estimation of the fraction of immigrant seeds within the seed deposition can be derived as follows.

According to the yield table for silver fir of Hausser (1956), a quantity of 243 firs per hectare is assumed for a fir stand at the age of 130 years. Thus, a total number of 437 adult firs can be expected within the investigated stand that is characterized by a size of about six hectare, an amount of 50 % firs and a stocking density of 0.6. Subtracting the 203 sampled adult fir trees, there are about 234 adult firs left within the investigated stand that has not been sampled and thus, not been genotyped. Taking into account that 439 different multilocus genotypes of the maternal tissue of the seeds could not be matched to any sampled adult fir, at least 205 fir trees which provide seeds to the seed traps must be located outside of the study plot. Consequently, these trees dispersed their seeds at least 150 m which is in accordance to the minimum distance between adult firs located outside of the sampled area and the nearest seed trap. However, 205 seeds with unique multilocus genotypes in the maternal tissue correspond to 205 different mother trees that must be located outside of the sampled area due to the described considerations above. Thus, 205 of the 661 genotyped seeds found in the traps (31 %) must have been dispersed from mother trees outside the sampled area with a minimum dispersal distance of 150 m. These findings let us assume moderate to high levels of seed inflow. As a consequence of the high number of contributing mother trees, a recolonization of disturbed areas is unlikely to cause genetic drift.

Although seeds can fly over few hundred meters, most actually fall within one or two tree heights of the mother trees. Similar findings were detected for noble fir (Franklin, 1983). In the present case study, we detected efficient seed dispersal within the closed forest stand as well as in the windthrow area close to the forest margin enabling the tree population to maintain genetic diversity. In particular, seed dispersal patterns should directly impact the genetic structure of populations. Species whose seeds are dispersed near the mother plant should have more obvious fine-scale genetic structure than species whose seeds are dispersed singly by animals or wind (Hamrick *et al*., 1993).

The present dispersal distance estimations support evidence to our previous findings of less spatial autocorrelations among adults and saplings in the investigated stand and in two other fir populations which are not included in this study (Cremer *et al.,* unpublished).

Beside tree- and seed-specific features, the dispersal of seeds is heavily dependent on the wind as a decisive determinant, especially on the wind speed. The efficiency of the wind for seed dispersal is thereby correlated to the sinking rate of the seeds. Based on a starting height of 14 m (= tree height) Kohlermann (1950) exemplarily calculated an increased mean dispersal distance for increasing wind speed in silver fir: 26 m at a wind speed of 1.7 m/sec, 34 m at a wind speed of 2.7 m/sec and 72 m at a wind of 5.9 m/sec. Hence, in the present study the dispersal probably occurred largely under moderate wind conditions since seeds dispersed on average 98 m while most seeds dispersed within the distance class of 40 m to 60 m. The study of Kohlermann (1950) also shows that fir seeds are blown some distances even at lower wind velocities. This might explain the course of the dispersal function for fir seeds revealing that only few seeds were dispersed underneath the mother tree.

Forest management practices

Generally, conifers that grow at a high density and show wind-mediated seed dispersal are expected to extensively disperse seeds and have overlapping seed fall (Knowles, 1991). Focusing on the question ‘How much of the seeds do actually disperse into a windthrow area in terms of recolonization or reforestation purposes?’ we detected efficient local seed dispersal within the forest stand as well as in the windthrow area within a 100 m range from the forest margin. As a consequence for silvicultural management an additional planting of silver firs within the local neighbourhood of the forest stand on the windthrow area does not seem to be necessary from the dispersal point of view. Factors such as increased game and frost damage on open areas that might lead to a hindered growth of fir saplings are not considered here, but should be also taken into account for silvicultural management activities. Moreover, it must be considered that the amount of empty seeds trapped (75 %) was comparatively high. Most of the seeds would not have resulted in seedling establishment. The amount of empty seeds varies considerably from year to year (Kormutak and Lindgren, 1996). Therefore the present result of only one-year mast can not be generalized.

For silvicultural purposes in terms of natural regeneration methods it is important to know at which distance a sufficient seed deposition can be expected based on the seed bearing adult trees.

With the present results of the genetic investigations of seed dispersal into open areas, the most acceptable size of regenerative cuttings (e.g. strip system) can be derived to guarantee sufficient seed deposition. Consequently to our findings, regenerative cuttings of up to 150 m in size should not lead to a genetic bottleneck effect within the natural regeneration of silver fir. This verifies the long term practice of strip cutting in width of one to two tree lengths (Smith, 1962).

Conclusion

This study presents a genetic approach to estimate local seed dispersal in silver fir. The analysis of maternal seed tissue using nSSRs provides a suitable tool for seed dispersal studies. The results demonstrate that there is no significant impact of morphological parameters of fir seeds on the dispersal distance. Seed entry on the adjacent open area is slightly lower than in the forest stand but sufficient within shorter distances of a few hundred meters. The high number of contributing mother trees let us assume a high level of genetic diversity in the seed population. Thus, for small windthrow areas no genetic risk can be expected due to the efficient and multifaceted seed distribution, especially against the background of continuous seed entry over multiple generations.

Our results give only first insights into the seed movement of silver fir. As our sample size was limited, further research is necessary in order to get more detailed information about the natural recolonization potential of silver fir for large windthrow areas.

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Paper III

Ontogenetic and genotypic effects of silver fir (*Abies alba* Mill.) on associated ectomycorrhizal communities

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Manuscript

Ontogenetic and genotypic effects of silver fir (*Abies alba* Mill.) on associated ectomycorrhizal communities

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Abstract

Tree - mycorrhiza interactions are essential components for forest ecosystem functioning. Using silver fir (*Abies alba* MILL.) as a model species, we revisited an old question whether the age of the host drives the community structure of its ectomycorrhizal (ECM) fungi. In a second step we asked if the ECM community could be regarded as the `extended phenotype´ of the host population. Silver fir trees and their associated ECM fungi were studied in three forest stands in the Black Forest. While the host populations were analyzed at DNA microsatellite loci, the associated ECM community was characterized by operational taxonomic units (OTUs). Morphotyping and internal transcribed spacer region (ITS) analyses revealed 33 different OTUs with ten of them identified to the species or genus level.
No differentiation could be detected for the silver fir populations and a Bayesian model assigned them to a most likely number of just one group. Thus, the studied stands of *A. alba* can be considered as parts of one population most probably connected through extensive gene flow. The hypothesis of early *vs*. late stage fungi could not be supported by our study. Instead, as revealed by ANOVA, adult trees hosted a higher number of different ECM OTUs than juveniles. The correlation between fir genotypes and associated ECM fungi was displayed by a PCA biplot. It became evident that the genomic background of silver fir as represented by single-locus variation has an effect on the composition of the associated ECM. ECM communities therefore may be considered as extended phenotypes of the host populations which has inferences for conservation genetic measures.

Keywords: silver fir (*Abies alba* Mill.), ectomycorrhizal fungi, nuclear microsatellite markers, ITS, single-locus-genotype-effect, extended phenotype

Introduction

Organismic interactions are pre-requisites for ecosystem functioning since species do not evolve in isolation, but rather live and co-evolve with other species in the respective environments. It is generally assumed that relatively few species are responsible for community structure within ecosystems. Such species are called foundation species (Whitham *et al.,* 2006). They structure a community by creating adequate conditions for other species. In case their impact on the ecosystem community is disproportionally large those species are also termed keystone species (Power *et al.,* 1996). More recent analyses demonstrate the impact of keystone species through their `extended phenotype´ (Whitham *et al.,* 2003; Bailey *et al.,* 2004). `Extended phenotype´ is defined by Whitham *et al.* (2003) as an effect of genes at levels higher than the population which can determine species interactions. The genetic composition of the foundation species may influence the community structure leading to 'interspecific indirect genetic effects' (Shuster *et al.,* 2006). Such hypotheses are baselines of the new discipline of `Community Genetics´ which is investigating 'the role of intraspecific variation in affecting community organization and ecosystem dynamics' (Whitham *et al.,* 2003). Facing global change it has become evident that knowledge about possible impacts of intraspecific variation of the foundation species on community properties, e.g. community composition or multitrophic interactions (Agrawal, 2003) is still scarce and should be urgently enlarged. This may hold true for trees in particular.

Large parts of terrestric ecosystems consist of tree species, many of them being foundation species for various associated organisms. In forests, symbiotic interactions with fungi play a decisive role for tree vitality in general and particularly for the viability of trees under suboptimal conditions. About one third of the fungi in our forests are mycorrhizal fungi (Egli and Brunner, 2002). In temperate forests ectomycorrhizal (ECM) fungi play a major role as compared to other mycorrhizal life forms (Smith and Read, 1997).

The present study focuses on the tree species silver fir (*Abies alba* Mill.). It is known for its important ecological function in montane European forest ecosystems as a so-called 'stabilizing' tree species (Pfeil, 1842; Gayer, 1898; Ellenberg, 1996). Thus, it can be considered as a keystone or foundation species. Here, a novel approach was used which is based on an individual-oriented investigation of fir as a foundation species and its associated ECM fungi. Each of the studied firs has been genotyped using nuclear microsatellites (or simple sequence repeats (SSRs)) and at the same time diversity and abundance of the associated ECM were studied.

Three natural forest sites were investigated which are located on the same geological substrate (middle-red sandstone) in the Black Forest, Germany. Each of the study sites includes adult firs as well as juvenile firs and, furthermore consists of a closed forest stand with an adjacent open area as a result of windthrow. In order to use these sites as replicates from a genetic point of view we investigated the genetic structure. Thus, we attempted to verify that the studied silver fir populations are not completely different with regard to genotypic composition and distribution. Subsequently, we elaborated on putative drivers of the associated ECM community:

i) We re-visited the early-late-stage ECM hypothesis (Izzo *et al.,* 2005; Redecker *et al.,* 2001; Smith and Read, 1997) and analysed the effects of ontogenetic stages of the host trees (adults, saplings) on the ECM community.

ii) Next, we explored the data for a possible correlation between fir genotypes and associated ECM taxa which may indicate the existence of an extended phenotype. Since candidate genes governing symbiotic interactions are not yet identified or described, we used the same microsatellite markers as applied for population genetic purposes. The idea was that the variation at certain microsatellite marker loci could be linked to certain genomic regions of the host which are involved in such symbiotic interactions.

The present study aims at deepening our understanding of tree – ECM interaction in temperate forest ecosystems. It is a comprehensive analysis of fir genotypes and associated ECM which furthermore provides a data base for future modelling community responses under disturbance regimes.

Materials and Methods

Sites and sampling

This work was conducted at three study sites in the Black Forest, a low mountain range in the south-western part of Germany. Two research sites are located in the middle part of the Black Forest (‘Bannwald Grosse Tannen’ (BW) and ‘Sauriss’ (SR)) while the third site is located in the northern part of the Black Forest (‘Eyachtal’ (EY)). The elevation of the three sites is quite similar ranging from 700 m to 780 m. All three study sites are naturally mixed forest stands with silver fir as the main tree species located on the geological substrate ‘middle-red sandstone’ and are characterized by an adjacent open area due to the windthrow in 1999. The research area, i.e. the Black Forest, is located at the north-western border of silver fir’s natural range.

We sampled 1360 adult and juvenile silver fir trees from all three sites for genotyping. From these, a total of 753 provided mycorrhizal samples. One site, BW, completely failed for mycorrhizal analysis (see Table 1). At this site the rust fungus *Pucciniastrum epilobii* and the aphid D*reyfusia nordmannianae* caused an infection of the firs which led to a reduced and deficient development of the ECM. Needles of the trees were sampled during winter and spring 2005 while the ECM samples were collected in the summer 2005 from the same trees. We needed to wait until the ECM hyphae were well developed (Buée *et al.,* 2005). For sampling of ECM on adult silver firs, the upper soil layer was broken up around the stem and lateral roots were cut off (as described by Ritter, 1990). Several roots per tree were collected and in case of seedlings the entire plants were extracted.

The position of each tree was mapped by attributing geo-coordinates (using GS5, Leica Geosystems, Heerbrugg, Switzerland). Within the stands, sample trees were evenly distributed and in the adjacent windthrow areas saplings were sampled along four transects of 50 m or 100 m length. We defined two site types, 'closed forest stand' and 'windthrow area'. Ontogenetic stages of the trees were defined as follows: seedlings = 1 to 3 year old trees assumed to be younger than the storm event; saplings = juvenile trees up to a height of 1.5 m and adult trees = firs at fructification age (> 60 years). The saplings in the windthrow areas regenerated before the storm event. This is important to know for a correct interpretation of the data.

Table 1 Sample statistics related to study sites, ontogenetic stages and site type.

Study sites		**Closed forest stand**						**Windthrow area**			
Codes	Full names, Geocoordinates	**Adults**		**Saplings**		**Seedlings**		**Saplings**		**Seedlings**	
		fir	ECM	fir	ECM	fir	ECM	fir	ECM	fir	ECM
BW	Bannwald 48°46'41.75''N 8°30'56.46''E	200	-	100	-	-	-	100	-	-	-
EY	Eyachtal 48°46'41.75''N 8°29'34.02''E	54	**54**	104	**98**	51	50	100	**89**	52	51
SR	Sauriss 48°31'16.14''N 8°28'56.31''E	200	**100**	200	**124**	-	-	200	**187**	-	-

'-' no samples available, in bold types: samples used for ANOVA

Microsatellite genotyping and genetic data analysis of silver fir

Total DNA was extracted from frozen needles or dried wood adopting an alkyltrimethyl ammonium bromide (ATMAB) method described by Dumolin *et al*. (1995). The DNA was stored at -30°C until use. All samples were genotyped using six nuclear microsatellites (SF1i, SFb4, SFb5, SF78, SF331, SF333) and polymerase chain reaction (PCR) conditions were applied as described in Cremer *et al.* (2006). Forward primers carried a fluorescent label (Hex, Fam or Tamra). The DNA was amplified in a thermal cycler (T1, Biometra, Göttingen, Germany) and the PCR products were separated by capillary electrophoresis using the MegaBACE automated sequencer (GE Healthcare, Freiburg, Germany). Alleles were sized using the size standard MegaBACE ET400-R (GE Healthcare) and the MegaBACE fragment profiler version 1.2 (GE Healthcare). After all runs were completed a reproducibility check was conducted by re-analysing 20 to 50 randomly chosen samples.

The software GENEPOP (Raymond and Rousset, 1995) was used to test for linkage disequilibrium. Genetic diversity within and among populations or subsets, respectively, was estimated by mean number (A) and effective number of alleles per locus (N_e), allelic richness (A_r), and heterozygosity (observed H_o and expected H_e). Global F_{ST} according to Weir and Cockerham (1984) was calculated. Genetic distance according to Nei (1972) was computed between pairs of populations and ontogenetic stages. Genetic diversity and genetic distances were computed with the help of GenAlEx 6.0 (Peakall and Smouse, 2006). Population differentiation occurs when a large proportion of the total genetic variation is found among populations. To detect and test for such a differentiation, we used an Analysis of Molecular Variance (AMOVA) with 9,999 random permutations (Excoffier *et al.*, 1992; Peakall *et al.*, 1995; Michalakis and Excoffier, 1996). Additionally, an *a posteriori* assignment of genotypes to a maximum number of possible populations was conducted with the help of Bayesian modelling. This procedure is implemented in the computer program Structure 2.2 (Pritchard *et al.,* 2000). We computed such an assignment to a possible number of K=1 to K= 6 populations (at least twice as much as the number of sampled populations) with 10,000 simulations while the ‘admixture model’ and the ‘correlated frequency model’ were chosen as settings. For investigating fine-scale spatial genetic patterns of each ontogenetic stage within the three fir populations spatial autocorrelation analysis was performed using the spatial autocorrelation option in GenAlex (Smouse and Peakall, 1999; Peakall and Smouse, 2006) for even distance classes of 20 m. Statistical significance was tested by 1,000 random permutations with a 95 % confidence level.

Morphotyping and molecular taxonomic diagnosis of ectomycorrhizal fungi

The morphotyping of the mycorrhizal samples of the 753 silver fir trees was based on macroscopical features in a first step. Mycorrhized root tips were washed, analysed under a dissecting microscope and classified into morphotypes following Agerer (1987-98). The ECM tips which had been subjected to morphotyping before were stored in a fixation solution containing NaCl and ATMAB at room temperature until DNA extraction. Immediately before DNA extraction the samples were frozen and ground with a shaking mill. Extraction procedure followed the protocol of Dumolin *et al*. (1995).

For species identification the fungal internal transcribed spacer region (ITS) was amplified, using the primer pairs ITS1 and ITS4, ITS1F and ITS4B (Gardes and Bruns, 1993) or Nsa3 and Nlc2 (Martin and Rygiewicz, 2005), respectively. ITS analysis was generally based on the primer pair ITS1/ITS4. If this did not result in PCR products, in a second step the primer pair Nsa3/Nlc2 and in a third step ITS1F/ITS4B was applied. The PCR was carried out in a 20 µl standard volume containing 1.5 mM $MgCl_2$, 6 % DMSO, 0.2 mM dNTP-Mix, 1x NH_4-PCR buffer, 0.8 U Mango*Taq*^{TM} (Bioline, London, UK), 0.6 pM of each primer and 20-40 ng template DNA. The DNA was amplified in a Hybaid Px2 Gradient Cycler (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) under the following general framework: initial denaturation for 5 min at 95°C, followed by 30-35 cycles with 30 sec at 95°C (denaturation) 30 sec at the primer dependent annealing temperature (55 or 58°C), 45-60 sec at 72°C (elongation), and final elongation for 10 min at 72°C.

A representative number of ECM morphotype samples were sequenced in both directions. For this, the ITS region of the respective sample was amplified in a 50 µl volume, purified with QIAGEN MinElute 96 UF PCR Purification Kit (QIAGEN GmbH, Hilden, Germany), applied to the Amersham Dye Terminator Sequencing Kit (Amersham Biosciences, Piscataway, New Jersey, USA) and sequenced using the MegaBACE automated sequencer (GE Healthcare). The obtained sequences were edited with the software BioEdit (Ibis Biosciences, Carlsbad, California, USA). Aligned sequences then were subjected to a blast analysis in the data bases UNITE (Kõljalg *et al.,* 2005) and NCBI Blast (NCBI, Bethesda, Maryland, USA). The results of the diagnostic procedure were treated as OTUs (operational taxonomic units). These included four categories. Following a decreasing degree of specificity there are i) unambiguously identified ECM species, ii) genus-specific OTUs in the cases where different con-genus species matched as a result of the BLAST procedure, iii) OTUs which were macroscopically identified by morphological features in case of sequence failure and finally iv) ‘unidentified’ OTUs if morphological features of ECM root tips were not assignable.

Analysis of ECM diversity

In order to quantify the diversity of the ECM partners found at the fir trees, Shannon's Diversity Index and evenness were calculated within each ontogenetic stage of the two study sites. Only for OTUs of the categories `i´ and `ii´, were frequencies scored and a presence-absence matrix was composed. The latter served as an input matrix for univariate statistics. The effects of different ontogenetic stages of the firs on the number of OTUs that colonize a fir tree were analysed by ANOVA using the software SPSS 14 (SPSS Inc., Chicago, Il, USA). Data was previously inspected for homogeneity of variance and normal distribution. We could only include two sites due to missing ECM data at the site BW. The ontogenetic stage `seedling´ could not be analysed within the ANOVA since data was only obtained for just one of the three study sites (Table 1).

Correlation between fir genotypes and ECM

In order to analyse the correlation between single-locus genotypes of firs and the occurrence of associated OTUs, a Principle Component Analysis (PCA) was conducted using the biplot-analysis option of the software CANOCO version 4.5 (Ter Braak and Smilauer, 2002). The analysis was based on frequencies of fir single-locus-genotypes occurring for each of the ten OTUs (appendix Table A-1, A-2). Only genotypes with a frequency > 2 % were included. Genotypes with less frequency provoke a complex biplot with irresolvable background noise. Subsequently, a Chi^2-test was performed in order to discover those genotypes that are significantly related to OTUs in general, but not to specific single OTUs. This procedure checks for deviations between observed and expected frequencies and was conducted for those single-locus-genotypes that revealed PCA vector values of > 0.5 or < -0.5, respectively.

Results

Genetic diversity and differentiation of silver fir

Mistyping errors of microsatellites were neglectable and we did not find any significant linkage disequilibrium among the six microsatellite loci. A total of 81 alleles was observed with a number of alleles per locus ranging from 6 (SF1 and SF333) to 35 (SF78). Genetic distances between the three fir populations were small. They ranged from 0.7 % between SR and BW to 1.9 % between EY and BW (Table 2). According to AMOVA only 1 % of the total variation was distributed among the populations, and thus 99 % within the populations. Additionally, global F_{ST} revealed low differentiation among the three fir populations (F_{ST} = 0.008).

Genetic assignment based on a Bayesian approach did not illustrate any grouping of the three different fir stands within the Black Forest. Simulations of the most probable number of populations resulted in K = 1 (Figure 1). Thus, the three study sites are not represented by genetically distinct populations and behave rather like a single population which constitutes a reproductive community.

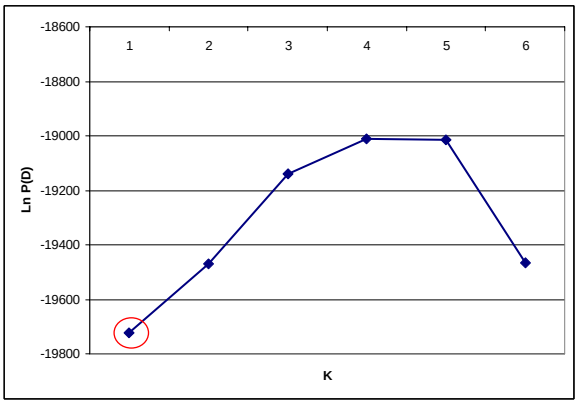

Figure 1 Simulation of the most probable number of populations (K), within a range of K = 1 to K = 6. Red circle indicates the most probable number of K = 1.

Focusing on the within-population scale, genetic diversity parameters were estimated for each ontogenetic stage (adults, saplings and seedlings). Mean number of alleles (*A*) ranged as follows: from 6.7 (adults) to 9.0 (saplings) at the site EY, from 10 (adults) to 10.2 (saplings) at the site BW and from 9.2 (adults) to 11.0 (saplings) at the site SR. Mean effective number of alleles (N_e) ranged from 2.9 to 3.2 for EY, from 3.0 to 3.1 for BW and was consistently with 3.2 for SR. Mean observed heterozygosity (H_o) varied from 44.0 % to 47.9 % at site EY, from 46.7 % to 47.4 % at site BW and from 47.1 % to 49.2 % at site SR.

Although heterozygosity was varying there was no consistent trend, meaning that differences within the sites could not be explained by ontogenetic stages. Nei's pairwise genetic distance between ontogenetic stages were low in general and varied from 0.4 % between adults and saplings at site SR and to 2.0 % between adults and seedlings at site EY (Table 3).

Table 2 Pairwise Nei's genetic distance between the three fir populations.

Pairs of populations	Genetic distance
EY - SR	0.009
EY - BW	0.017
SR - BW	0.007

Table 3 Pairwise Nei's genetic distances between ontogenetic stages.

Pairs of ontogenetic stages	Genetic distance		
	EY	SR	BW
adult – sapling*	0.011	0.004	0.013
adult – seedling*	0.020	-	-
sapling – seedling*	0.011	-	-

* fir individuals of windthrow area and forest stand are combined for the saplings and seedlings

Absence of genetic differentiation and genetic distances at a regional scale does not necessarily imply the absence of genetic structure at the smaller scale within the populations. According to spatial autocorrelation no spatial genetic structure was detected for the adult trees at sites BW and EY, nor for the seedlings at site EY (data not shown). Only marginal positive autocorrelation at very short distances was found for the adult trees and for the saplings at the site SR (distance class of 0 – 20 m). The same holds true for the saplings at sites BW and EY (data not shown).

Associated ECM fungi

By morphotyping and sequence analysis of the ECM fungi we obtained a total number of 33 OTUs of different taxonomic specificity. Considering all 33 OTUs, Shannon's diversity index revealed similar values for the different ontogenetic stages, while the highest diversity was obtained for the saplings in the windthrow area at site SR (Table 4). Similar results were obtained for evenness, the values ranging from 1.12 to 1.45 with the highest value of 1.84 in the saplings at site SR. Thus, the frequency distributions of the OTUs seem to be similar.

Table 4 ECM species diversity (Shannon's diversity index and evenness) within the ontogenetic stages of each study site.

	Shannon-Index	**evenness**
Study site EY		
adults	4.72	1.45
saplings (stand)	3.86	1.12
saplings (windthrow area)	4.16	1.26
seedlings (stand)	4.56	1.40
seedlings (windthrow area)	4.16	1.33
Study site SR		
adults	5.70	1.22
saplings (stand)	4.94	1.44
saplings (windthrow area)	6.43	1.84

Ten of the 33 OTUs could be clearly identified at the species and genus level (categories i and ii). These ten OTUs are response variables in the ANOVA and in the PCA (see below). The frequencies of these OTUs are given in Table 5. Generally these fungi were detected within both study sites, in the stand as well as in the windthrow area and throughout all ontogenetic stages of the firs except for *Laccaria amethystina, Russula species II* and *Lactarius spp.* which are absent in the seedlings. There is a clear differentiation between common and less abundant OTUs, with *Tomentella stuposa* and *Cenococcum geophillum* as the most frequent ones followed by *Russula ochroleuca* and *Lactarius* spp.

Table 5 Frequencies of the 10 identified ECM OTUs [%].
1: *Tomentella stuposa,* 2: *Cenococcum geophilum,* 3: *Laccaria amethystine,* 4: *Russula species II,* 5: *Russula ochroleuca,* 6: *Clavulina cristata,* 7: *Xerocomus pruinatus,* 8: *Cortinarius spp.,* 9: *Amphinema byssoides,* 10: *Lactarius spp*

	OTU									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Total	24.2	22.1	7.1	1.5	11.9	5.3	2.9	4.9	3.4	16.6
Study site										
SR	23.0	20.3	10.1	1.2	11.4	5.4	1.5	5.1	4.2	17.9
EY	26.0	25.1	2.3	2.1	12.9	5.2	5.2	4.7	2.1	14.5
Ontogenetic stage										
adults	24.2	19.1	5.6	1.3	14.9	6.9	2.0	6.6	2.3	16.8
saplings	23.5	20.9	8.7	1.8	10.9	4.3	3.5	4.1	3.9	18.5
seedlings	28.7	42.5	0.0	0.0	10.3	8.0	1.1	5.7	3.4	0.0
Site type										
stand	27.6	20.5	6.6	1.9	10.7	7.7	4.4	3.6	1.6	15.6
windthrow area	21.2	25.5	8.6	1.4	11.0	2.3	2.3	5.0	5.6	17.3

ANOVA revealed that the number of OTUs was significantly dependent on the ontogenetic stage of the firs (Table 6). The highest number of OTUs was detected on the roots of adult trees (Figure 2) documenting an increase of the ECM diversity due to the age of the host.

Table 6 Results of ANOVA showing the effect of ontogenetic stage on the number of OTUs colonizing a fir tree; *** = p < 0.001.

Source of variation	d.f.	MS	Number of OTUs *F*
ontogenetic stage	1	9.61	11.23***

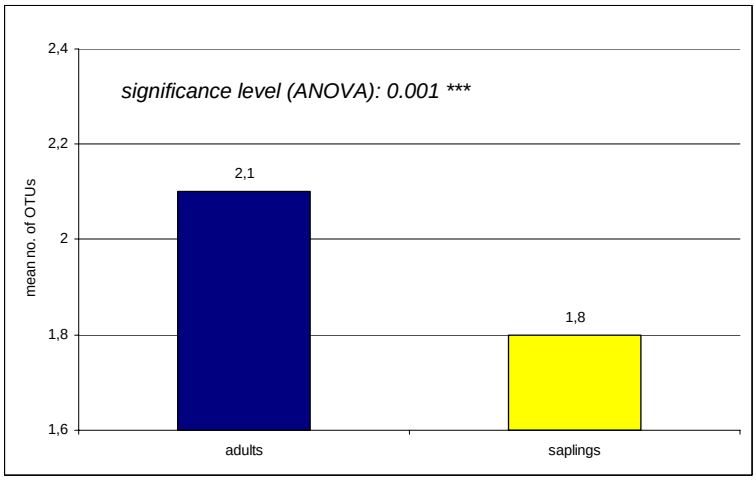

Figure 2 Mean values of the number of OTUs for the two ontogenetic stages 'adult' and 'saplings'.

Correlation between single-locus-genotypes and ECM fungi

With the help of the PCA, a biplot was obtained in which the first coordinate explains 44.3 % and the second coordinate 20.9 % of the total variation (Figure 3). Overall, 60 single-locus genotypes were included in the analysis with frequencies > 2 %. Forty of these single-locus genotypes are displayed whose vector load was > 0.5 or < -0.5, respectively. Thus, they were included in a Chi^2-test for statistic validation (appendix, Table B-1, B-2). The observed frequencies of 15 single-locus-genotypes in association with the respective OTUs deviated significantly from the expected (Figure 3, genotypes in red circles). These single-locus genotypes are affiliated to four of the six SSR loci (SFb4, SF 78, SF 331, SF333). The locus SFb4 exhibited a maximum number of eight single-locus-genotypes significantly correlated with OTUs.

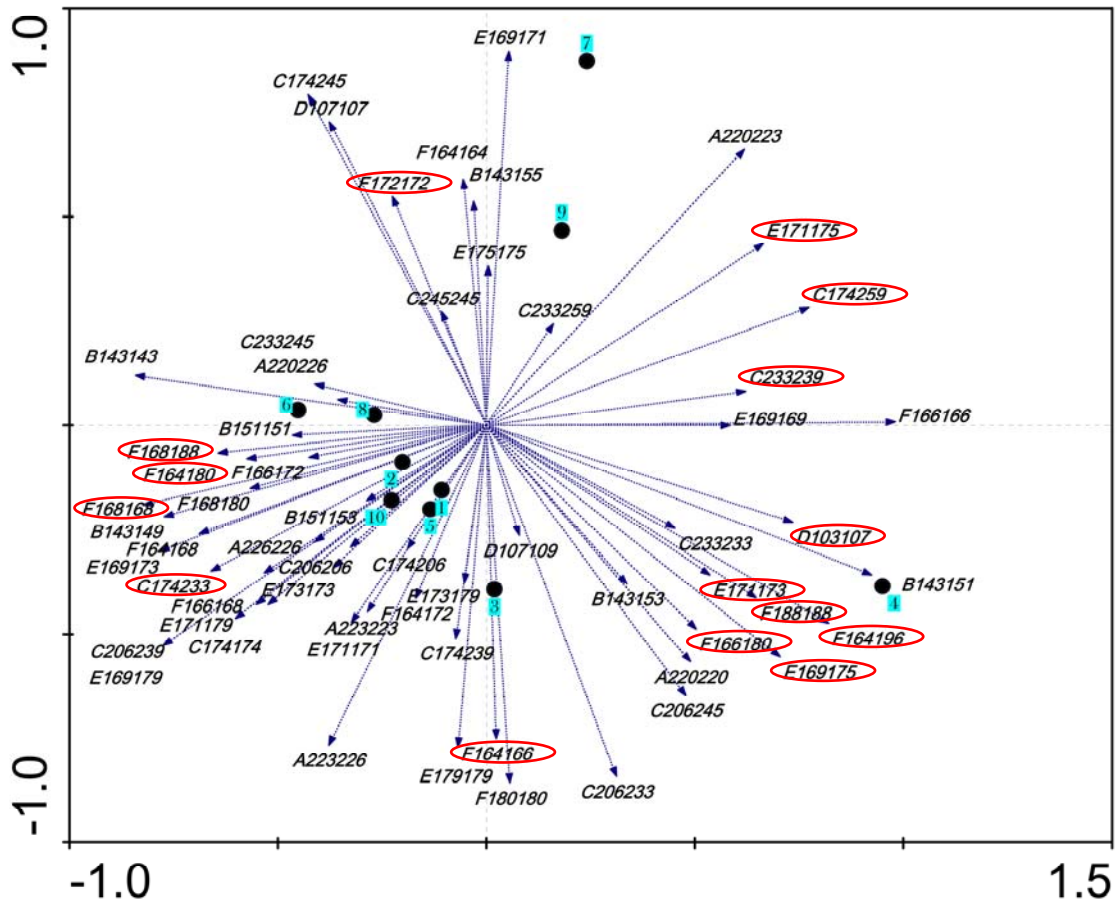

Figure 3 Biplot of Principal Component Analysis (PCA). Single-locus-genotypes of firs as explanatory variables are displayed as vectors while the 10 OTUs as response variables are displayed as points (1: *Tomentella Stuposa,* 2: *Cenococcum geophilum,* 3: *Laccaria amethystine,* 4: *Russula species II,* 5: *Russula ochroleuca,* 6: *Clavulina cristata,* 7: *Xerocomus pruinatus,* 8: *Cortinarius spp.,* 9: *Amphinema byssoides,* 10: *Lactarius spp.).* The six numbers characterising the single-locus-genotypes reflect the sizes of the SSR alleles, e.g. 164180 codes two alleles, one with 164 bp and the other with 180 bp in length; the letter correspond to the SSR locus (A: SF1, B: SFb5, C: SF78, D: SF331, E: SF333, F: SFb4). Significant single-locus-genotypes are circled with a red line.

Discussion

This study contributes to a better understanding of the relationship between trees and ECM fungi as symbiotic partners in temperate forest ecosystems. With silver fir as model species, it revisits an old question as to whether there is an age-dependent structure of the associated ECM community. Moreover, the study profits from a comprehensive individual-orientated tree by fungi approach in which each host individual is genotyped and at the same time analysed for its ECM community. Here, a unique opportunity arose by the microsatellite DNA markers used. On the one hand it could be assured that the populations under study did not differ in their genetic structure. On the other hand, the association of single-locus genotypes and certain ECM provided first evidence for the ECM community representing an extended phenotype of the fir population.

Silver fir of the Black Forest had been suffering from severe decrease since the beginning of the 19th century when the formerly mixed forest stands with beech, Norway spruce and silver fir had largely been replaced by monocultures of Norway spruce (Günther Groß, pers. communication). As a consequence we may expect fragmentation effects such as interruption of gene flow leading to drift and increasing genetic differentiation (Templeton *et al.,* 2001). If so we would risk introducing a `population genetic bias´ into our ecological genetic investigation which was based on three fir sites. These were meant as independent replicates for statistical tests. A different level of fir genetic diversity could also -*per se*- drive the diversity of the associated ECM community. Schweitzer *et al*. (2008) demonstrated that different levels of host tree genetic diversity can be positively correlated with the diversity of the associated species community. Based on the six nuclear SSR markers of our study neither different levels of diversity nor distinct differentiation among the three populations and between the ontogenetic stages could be detected. Interestingly, the current fragmentation of silver fir populations is not imprinted in the genetic structure. In this respect, our study supports conclusions that in long-lived plants with effective gene flow through wind-dispersed pollen, genetic effects of fragmentation can be less pronounced than in others with contrasting life history traits (e.g. Young et *al.,* 1996; Williams *et al.,* 2007). Is there a possibility that the markers used, mask differentiation which is really present? In another study using exactly the same six markers we found a pronounced genetic differentiation between Black Forest silver fir provenances on the one hand and Romanian and Macedonian provenances on the other hand (unpublished data). Here, the differentiation at the nuclear microsatellite loci reflected the distinct differentiation at a mitochondrial DNA locus (Liepelt *et al.,* 2002; 2008).

Besides, among-population differences, within-populations genetic diversity may not be distributed homogeneously resulting in spatial genetic structures (SGS, Jump and Penuelas, 2007; Vekemanns and Hardy, 2004). In the present study, slight positive spatial-genetic correlations at very short distances could be observed in only a sub-set of the sample populations. Conifers which grow at high density and show wind-mediated seed dispersal are generally expected to extensively disperse seeds and have overlapping seed shadows, eliminating obvious genetic structure within populations (Knowles, 1991; Lian *et al.,* 2008). Congruently, from a population genetic point of view, the three study sites were considered to be legitimate replicates.

We therefore explored the ECM fungal communities for statistical significance of the postulated drivers such as ontogenetic stages and single-locus-genotypes.

ECM communities are known to be highly diverse, even in stands dominated by a single plant species (Visser, 1995; Dahlberg *et al.,* 1997; Horton *et al.,* 1999; Jonsson *et al.,* 1999a, b; Byrd *et al.,* 2000). We found a total of 33 ECM OTUs. With all 33 OTUs included, the observed differences of Shannon´s diversity indices and evenness could not be explained by the different sites and/or ontogenetic stages. Thus, we decided to only include those ten OTUs in our statistical tests which had been clearly identified as species or genera, respectively. By including all OTU categories the analyses could suffer from taxonomic uncertainties, e.g. redundancy.

All ten OTU species or genera are supposed to be generalists in the way that they are associated with numerous host species including conifers. They are indicators for acidic and nutrient poor soils (Krieglsteiner, 1977; Rexer *et al.,* 1995; Kõljalg, 1996; Dahlberg *et al.,* 1997; Fiore-Donno and Martin, 2001; Redecker *et al.,* 2001; Koide *et al.,* 2005; di Pietro *et al.,* 2007; Peter *et al.,* 2008). At both study sites a clear differentiation between common and less frequent OTUs occurred. Besides the most frequent species *T. stuposa* and *C. geophilum* well known as common species in various forests (Horton and Burns, 2001), *Russula ochroleuca* and members of the genus *Lactarius* were frequently found as well. Wiensczyk *et al.* (2002) state that different ECM fungal communities generally change with different ages of trees. More specifically, *L. amethystina* is known as early stage ECM with transient mycelia, high fructification rates and dispersal by meiospores (Deacon and Fleming, 1992; Smith and Read, 1997). In contrast, the genus *Xerocomus* is classified as late stage ECM, with widespread, long-lasting mycelia and low fructification ratios (Fiore-Donno and Martin, 2001). However, we did not find any such distinct differentiation. Rather, a significant difference in the number of OTUs between the ontogenetic stages was found. Adult trees are colonized by a higher number of different OTUs than younger trees indicating a significant increase of the ECM diversity on the individual level. One may argue that this could be due to a sampling effect. However, the same sample size per tree was taken independently from the ontogenetic stage, meaning from the size of the root system. ECM richness obviously increases over time with increasing ages of trees. Other observations confirm that ECM species are usually added to the fungal community, but that they do not necessarily replace the earlier ones (Visser, 1995; Bradburry *et al.,* 1998). Horton and Bruns (2001) concluded on a higher ECM diversity in elder forest stands than in younger ones by compiling the results of different studies. If it is just the number of OTUs which is driven by ontogenesis what about drivers that select for distinct OTUs or OTU communities? Is there evidence for drivers which are to be found in the genetic background of tree individuals?

It is generally well known that the genetic composition of a host species can have impacts on the colonization of distinct pathogens like pathogenic fungi.

For example, in crop cultures there is a risk of monocultures or clones, respectively, provoking severe pests due to gene-to-gene interactions (Bishop and Cook, 1981; Oldfield, 1984). Recent studies have shown that there are genetic components within the host plants that may determine the structure of the dependent community (Bailey *et al.,* 2006; Wimp *et al.,* 2004; Johnson and Agrawal, 2005). Bailey *et al.* (2006) demonstrated on cottonwood that direct and indirect interaction among species result in distinct community composition that is predictable by genotype. Effects of genetically based interactions between organisms are currently analysed within the new and emerging field of community genetics. Such studies refer to plant-plant species communities (Booth and Grime, 2003; Vellend, 2006), plant-insect communities (Crutsinger *et al.,* 2006; Johnson, 2006; Johnson and Agrawal, 2005) and plant-microbial communities (Schweitzer *et al.*, 2008).

We have chosen *Abies alba* as model for obtaining deeper insights into the symbiotic interaction with its ECM fungi. In a previous transplant experiment of ours we found evidence for an interspecific indirect genetic effect (IIGE; Whitham *et al.,* 2003, 2006; Wilson and Swenson, 2003; Shuster *et al.,* 2006) acting at the provenance level. We found that local silver fir provenance performed better than far-distant provenances originating from a different refugial gene pool. After correlating genetic variability and ECM variability we concluded that the local provenance made better use of the local ECM variability (unpublished data).

The results of the present study reveal a significant relationship between single-locus genotypes of the host and ECM OTUs as associated organisms. The Chi^2-test showed that distinct genotypes determine parts of the OTU community, but it is still to be proven if they determine the association of explicit OTUs.

Two loci of the six microsatellite loci do not exhibit any significant relationship with OTUs (SFb5, SF1), while one locus is disproportionately frequently involved in significant correlations. Since this locus, SFb4, revealed tendencies of harbouring null alleles we were concerned about a possible statistical bias. However, we can exclude a bias due to the high number of five heterozygote genotypes at this locus that reveal significant correlations.

Since the fir trees have been characterized at nuclear SSR loci which are considered to be neutral without coding for any phenotype (Avise, 1994; Porcher *et al.,* 2006), a direct gene-to-gene correlation between SSR loci and ECM associations are excluded. Rather a `chromosomal vicinity´ between the SSR loci and genomic regions that have an influence on mycorrhization can be assumed. Since SSR loci are dispersed throughout the genome, they might be located close to gene-expressive and/or regulatory DNA-regions with functional relevance. In the case of trees and mycorrhiza this may relate to the expression of proteins which control the interaction between the symbionts. Several studies demonstrate that host plants produce essential metabolites which are able to affect fungal partners.

These metabolites are released into the rhizosphere to cause basidiospore germination (Fries *et al.,* 1987), to affect the growth of hyphae towards the root (Horan and Chilvers, 1990) or to influence the early developmental steps of mycorrhiza formation (Ditengou and Lapeyrie, 2000; Martin *et al.,* 2001; Langenheim, 1994). Using selectively neutral markers we cannot provide direct information about candidate genes that might have an impact on mycorrhization. Candidate genes controlling interactive processes between mycorrhizal fungi and host root are therefore of high interest (Martin *et al.,* 2001).

Symbiotic interactions are assumed to be controlled by the genomes of both partners. In the present study, we focused on the tree as driver for the ECM abundance and diversity. In contrast, Martin *et al.* (2001) describes a first analysis of the nature of signals released by the ECM symbionts, how these signals are transduced within the partners, and the impact on the formation of symbiotic tissues. The feedback effect by the fungi should not be disregarded. Trees forming a diverse array of ectomycorrhizae are thought to be better suited to survive under variable climatic conditions than trees forming ECM with only few fungal species (Hagermann *et al.,* 1999b).

Conclusion and perspectives

Our data demonstrate that ECM diversity is not so much dependent on different ages of the host or on site conditions like closed forest stands or windthrow areas. However, it seems that the individual tree with increasing age increases its ECM community.

Our study also reveals that there is strong evidence for variable genomic regions of the host which can be regarded as driving factors of community structure and dynamics of its associated ECM fungi. Overall these findings support the general hypothesis that plant genomic traits can have strong organizational effects on the community level.

As part of global efforts to preserve biodiversity and to assure sustainability, the conservation of genetic resources of forest trees has implemented *in situ* management of genetic diversity (Cavers *et al.,* 2005; Lowe *et al.,* 2005). As a consequence of the extended phenotype, conservation programmes of forest genetic resources must consider the impact of genetic diversity of host species on associated species within forest ecosystems. Therefore, Whitham et al. (2003) suggest a minimum viable interacting population size (MVIP) that better reflects the goals to conserve genetic diversity at levels required by interacting species instead of minimum viable populations size (MVP).

To come up with distinct conservation measures, further research is necessary in order to understand the cross-talking between signalling networks of the symbiotic partners by expression of symbiosis-regulated genes. First steps which are directly related to our results could be i) analysis of the DNA adjacent to the mentioned SSR loci by large-fragment-sequencing, ii) looking for genes that are functionally related to the loci that influence the mycorrhization of a tree by checking common gene banks, and iii) conducting association genomics to verify the functional relationship between genotype and phenotype.

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Appendix

Table A-1 Genotype frequencies within the different OTU groups at the nSSR loci SF1, SFb5, SF78, SF331 and SF333 serving as basic matrix for the PCA (1: *Tomentella stuposa,* 2:*Cenococcum geophilum,* 3: *Laccaria amethystine,* 4: *Russula species II,* 5: *Russula ochroleuca,* 6: *Clavulina cristata,* 7: *Xerocomus pruinatus,* 8: *Cortinarius spp.,* 9: *Amphinema byssoides,* 10: *Lactarius spp).*

nSSR locus	genotype	OTU									
		1	2	3	4	5	6	7	8	9	10
SF1	**220220**	3,30	3,23	0,00	7,69	4,50	0,00	0,00	0,00	0,00	4,35
	220223	25,94	25,80	21,31	38,46	29,73	30,61	42,31	27,91	35,71	26,81
	223223	43,87	41,93	44,26	38,46	34,23	40,82	34,62	46,51	32,14	34,78
	220226	6,13	4,84	6,56	0,00	6,31	4,08	0,00	9,30	14,29	5,07
	223226	18,87	21,50	21,31	15,38	22,52	18,37	7,69	11,63	10,71	24,64
	226226	0,00	0,00	3,28	0,00	0,00	4,08	0,00	0,00	0,00	2,90
SFb5	**143143**	44,81	48,92	50,82	30,77	52,25	53,06	50,00	48,84	39,29	48,55
	143149	4,25	5,38	0,00	0,00	7,21	10,20	0,00	9,30	0,00	6,52
	143151	19,34	16,13	24,59	46,15	19,82	6,12	19,23	13,95	17,86	16,67
	151151	0,00	0,00	0,00	0,00	0,00	6,12	0,00	0,00	0,00	2,17
	143153	8,96	6,45	11,48	15,38	8,11	12,24	7,69	13,95	7,14	6,52
	151153	0,00	2,69	0,00	0,00	0,00	0,00	0,00	0,00	0,00	2,90
	143155	10,85	8,06	4,92	7,69	7,21	6,12	11,54	13,95	10,71	9,42
SF78	**174174**	3,77	4,84	6,56	0,00	6,31	4,08	0,00	6,98	0,00	0,00
	174206	4,25	9,14	8,20	7,69	7,21	10,20	7,69	6,98	0,00	6,52
	206206	4,25	3,76	3,28	0,00	0,00	0,00	0,00	4,65	0,00	0,00
	174233	10,38	7,53	0,00	0,00	13,51	8,16	0,00	6,98	0,00	13,04
	206233	8,02	8,06	16,39	15,38	7,21	8,16	0,00	6,98	7,14	9,42
	233233	9,43	9,14	9,84	15,38	7,21	14,29	7,69	6,98	10,71	7,97
	174239	3,77	6,99	0,00	7,69	6,31	6,12	0,00	6,98	0,00	3,62
	206239	3,30	2,69	4,92	0,00	2,70	6,12	0,00	0,00	0,00	4,35
	233239	6,13	8,06	9,84	15,38	3,60	4,08	15,38	6,98	0,00	0,00
	174245	2,83	4,30	3,28	0,00	3,60	10,20	11,54	9,30	7,14	4,35
	206245	3,30	0,00	3,28	7,69	2,70	0,00	0,00	4,65	0,00	4,35
	233245	7,08	9,68	3,28	0,00	6,31	0,00	7,69	11,63	0,00	5,80
	245245	0,00	2,69	0,00	0,00	3,60	0,00	0,00	4,65	7,14	0,00
	174259	0,00	0,00	0,00	7,69	2,70	0,00	7,69	0,00	0,00	0,00
	233259	0,00	0,00	3,28	0,00	0,00	0,00	0,00	0,00	7,14	0,00
SF331	**103107**	2,83	0,00	4,92	7,69	2,70	0,00	0,00	0,00	7,14	0,00
	107107	87,74	91,39	86,88	84,62	87,39	93,88	92,31	88,37	92,86	86,23
	107109	7,08	3,23	6,56	7,69	5,41	6,12	7,69	6,98	0,00	7,97
SF333	**169169**	21,70	20,97	31,15	30,77	23,42	26,53	26,92	13,95	28,57	23,19
	169171	18,40	18,82	16,39	15,38	16,22	18,37	38,46	23,26	21,43	18,84
	171171	18,40	17,74	13,11	15,38	17,12	16,33	11,54	16,28	14,29	13,77
	169173	5,19	4,84	3,28	0,00	7,21	10,20	0,00	9,30	0,00	3,62
	171173	0,00	0,00	0,00	7,69	0,00	0,00	0,00	4,65	0,00	3,62
	173173	3,30	2,69	0,00	0,00	2,70	0,00	0,00	0,00	0,00	3,62
	169175	2,83	0,00	6,56	15,38	3,60	4,08	0,00	0,00	0,00	0,00
	171175	2,36	0,00	0,00	7,69	2,70	0,00	11,54	0,00	0,00	0,00
	175175	0,00	0,00	0,00	0,00	0,00	0,00	0,00	4,65	7,14	0,00
	169179	8,49	9,68	6,56	0,00	9,91	8,16	0,00	6,98	0,00	11,59
	171179	7,55	8,60	4,92	0,00	5,41	0,00	0,00	9,30	0,00	6,52
	173179	2,83	0,00	3,28	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	179179	4,72	3,76	3,28	7,69	7,21	6,12	0,00	4,65	0,00	7,97

Table A-2 Genotype frequencies within the different OTU groups at the nSSR locus SFb4 serving as basic matrix for the PCA (1: *Tomentella stuposa,* 2:*Cenococcum geophilum,* 3: *Laccaria amethystine,* 4: *Russula species II,* 5: *Russula ochroleuca,* 6: *Clavulina cristata,* 7: *Xerocomus pruinatus,* 8: *Cortinarius spp.,* 9: *Amphinema byssoides,* 10: *Lactarius spp).*

SSR locus	genotype	OTU									
		1	2	3	4	5	6	7	8	9	10
SFb4	**164164**	15,09	16,67	13,11	7,69	10,81	8,16	19,23	9,30	17,86	15,94
	164166	7,08	4,84	18,03	7,69	8,11	0,00	0,00	6,98	0,00	10,87
	166166	21,70	19,89	21,31	53,85	23,42	10,20	30,77	20,93	32,14	19,56
	164168	3,30	5,91	0,00	0,00	6,31	4,08	0,00	6,98	0,00	3,62
	166168	2,36	0,00	4,92	0,00	0,00	4,08	0,00	4,65	0,00	4,35
	168168	8,02	6,99	6,56	0,00	10,81	20,41	0,00	13,95	7,14	10,87
	164172	0,00	2,69	3,28	0,00	2,70	0,00	0,00	0,00	0,00	0,00
	166172	0,00	0,00	0,00	0,00	2,70	4,08	0,00	0,00	0,00	0,00
	172172	2,83	0,00	0,00	0,00	2,70	6,12	7,69	0,00	0,00	2,90
	164180	2,83	0,00	0,00	0,00	2,70	6,12	0,00	4,65	0,00	0,00
	166180	4,25	3,23	0,00	7,69	6,31	0,00	0,00	0,00	0,00	0,00
	168180	3,77	4,30	0,00	0,00	0,00	6,12	0,00	0,00	0,00	2,90
	180180	4,72	5,38	8,20	7,69	3,60	6,12	0,00	4,65	0,00	3,62
	168188	0,00	2,69	0,00	0,00	0,00	6,12	0,00	4,65	0,00	3,62
	188188	0,00	4,30	0,00	7,69	2,70	0,00	0,00	0,00	0,00	0,00
	164196	0,00	0,00	3,28	7,69	0,00	0,00	0,00	0,00	0,00	0,00

Table B-1 Results of the Chi^2-test conducted subsequent to PCA to test for statistic significance between distinct fir genotypes and ECM OTUs; considered are those fir genotypes that are related to the first coordinate of the PCA biplot; (for OTU designation see Table A-1).

fir genotype		***OTUs*** **1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**		**Chi-Quadrat**
		N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	N_o **Chi^2**	N_e $N_o - N_e$	
first coordinate of PCA																						
SF 78 - 174233	no. of OTUs	22.00	18.69	14	16,4	0	5,34	0	1,14	15	9,77	4	4,35	0	2,29	3	3,81	0	2,44	18	12,13	**19,82***
f: 12,5 %	present	**0,59**	3.31	**0,35**	-2,4	**5,34**	-5,34	**1,14**	-1,14	**2,8**	5,23	**0,03**	-0,35	**2,29**	-2,29	**0,17**	-0,81	**2,44**	-2,44	**2,84**	5,87	
vl -0.6932	no. of OTUs	190.00	193.72	172	170	61	55,34	13	11,86	96	101,21	45	45,07	26	23,72	40	39,54	28	25,3	120	125,72	
	not present	**0.07**	-3.72	**0,02**	2	**0,58**	5,66	**0,11**	1,14	**0,27**	-5,21	**0,0001**	-0,07	**0,22**	2,28	**0,01**	0,46	**0,29**	2,7	**0,26**	-5,7	
SFb4 - 168168	no. of OTUs	17	18,9	13	16,59	4	5,4	0	1,16	12	9,88	10	4,4	0	2,31	6	3,86	0	2,47	15	12,27	**18,44***
f: 9,6 %	present	**0,19**	-1,9	**0,78**	-3,59	**0,36**	-1,4	**1,16**	-1,16	**0,45**	2,12	**7,13**	5,6	**2,31**	2,31	**1,19**	2,14	**2,47**	-2,47	**0,61**	2,73	
vl -0.8420	no. of OTUs	195	193,51	173	169,81	57	55,29	13	11,85	99	101,1	39	45,02	26	23,7	37	39,49	28	25,27	123	125,58	
	not present	**0,01**	1,49	**0,06**	3,19	**0,05**	1,71	**0,11**	1,15	**0,04**	-2,1	**0,8**	-6,02	**0,22**	2,3	**0,16**	-2,49	**0,29**	2,73	**0,05**	-2,6	
SFb4 -164180	no. of OTUs	6	3,4	0	2,98	0	0,97	0	0,21	3	1,78	3	0,79	0	0,42	2	0,69	0	0,44	0	2,21	**19,25***
f: 1,7 %	present	**2,14**	2,7	**2,98**	-2,98	**0,97**	-0,97	**0,21**	-0,21	**0,82**	1,21	**6,18**	2,21	**0,42**	-0,42	**2,49**	1,31	**0,44**	-0,44	**2,21**	-2,21	
vl -0.6387	no. of OTUs	206	209,02	186	183,42	61	59,72	13	12,8	108	109,2	46	48,63	26	25,59	41	42,66	28	27,3	138	135,65	
	not present	**0,04**	-3,02	**0,04**	2,58	**0,03**	1,28	**0,003**	0,2	**0,01**	-1,2	**0,14**	-2,63	**0,01**	0,41	**0,06**	-1,66	**0,02**	0,7	**0,04**	2,3	
SFb4 - 168188	no. of OTUs	0	3,19	5	2,8	0	0,91	0	0,2	0	1,66	3	0,74	0	0,39	0	0,65	0	0,42	5	2,07	**20,58***
f: 2,5 %	present	**3,19**	-3,19	**1,73**	2,2	**0,91**	-0,91	**0,2**	-0,2	**1,66**	-1,66	**6,9**	2,26	**0,39**	-0,39	**0,65**	-0,65	**0,42**	-0,42	**4,15**	2,93	
vl -0.6912	no. of OTUs	212	209,23	181	183,61	61	59,78	13	12,81	111	109,31	46	48,68	26	25,62	43	42,7	28	27,33	133	135,79	
	not present	**0,04**	2,77	**0,04**	-2,61	**0,02**	1,22	**0,003**	0,19	**0,03**	1,69	**0,15**	-2,68	**0,01**	0,38	**0,002**	0,3	**0,02**	0,67	**0,06**	-2,8	
SF78 - 233 239	no. of OTUs	13	12,11	15	10,63	6	3,46	2	0,74	4	6,33	2	2,82	4	1,48	3	2,47	0	1,58	0	7,86	**22,13****
f: 5,1 %	present	**0,07**	0,89	**1,8**	4,37	**1,86**	2,54	**2,15**	1,26	**0,86**	-2,33	**0,24**	-0,82	**4,29**	2,52	**0,11**	0,53	**1,58**	-1,58	**7,86**	-7,86	
vl +0.6309	no. of OTUs	199	200,31	171	175,78	55	57,23	11	12,26	107	104,65	47	46,6	22	24,53	40	40,88	28	26,16	138	130	
	not present	**0,01**	-1,31	**0,13**	-4,78	**0,09**	-2,23	**0,13**	-1,26	**0,05**	2,35	**0,003**	0,4	**0,26**	-2,53	**0,02**	-0,88	**0,13**	1,84	**0,49**	-8	
SF78 - 174 259	no. of OTUs	0	1,49	0	1,3	0	0,42	1	0,09	3	0,78	0	0,35	2	0,18	0	0,3	0	0,19	0	0,96	**39,21*****
f: 1,9 %	present	**1,49**	-1,49	**1,3**	-1,3	**0,42**	-0,42	**9,2**	0,91	**6,32**	2,22	**0,35**	0,35	**18,4**	1,82	**0,3**	-0,3	**0,19**	-0,19	**0,96**	-0,96	
vl +0.7639	no. of OTUs	212	210,93	186	185,1	61	60,27	12	12,91	108	110,2	49	49,07	24	25,83	43	43,05	28	27,55	138	136,89	
	not present	**0,01**	1,07	**0,01**	-1,1	**0,01**	0,73	**0,06**	-0,91	**0,04**	-2,2	**0**	-0,07	**0,13**	1,83	**0**	-0,05	**0,01**	0,45	**0,01**	1,11	
SF331 - 103 107	no. of OTUs	6	3,61	0	3,17	3	1,03	1	0,22	3	1,89	0	0,84	0	0,44	0	0,74	2	0,47	0	2,34	**21,58***
f: 1,3 %	present	**1,58**	2,39	**3,17**	-3,17	**3,77**	1,97	**2,77**	0,78	**0,65**	1,11	**0,84**	-0,84	**0,44**	-0,44	**0,74**	-0,74	**4,98**	1,53	**2,34**	-2,34	
vl +0.6937	no. of OTUs	206	208,8	186	183,24	58	59,66	12	12,78	108	109,09	49	48,58	26	25,57	43	42,61	26	27,27	138	135,51	
	not present	**0,02**	-2,2	**0,04**	2,76	**0,05**	-1,66	**0,05**	-0,78	**0,01**	-1,09	**0,004**	0,42	**0,01**	0,43	**0,004**	0,39	**0,06**	-1,27	**0,05**	2,49	
SF333 - 171 173	no. of OTUs	0	1,91	0	1,68	0	0,55	1	0,12	0	1	0	0,44	0	0,23	2	0,39	0	0,25	5	1,24	**25,16****
f: 1,8 %	present	**1,91**	-1,91	**1,68**	-1,68	**0,55**	-0,55	**6,45**	0,88	**1**	-1	**0,44**	-0,44	**0,23**	-0,23	**0,95**	0,61	**0,25**	-0,25	**11,4**	3,76	
vl + 0.5758	no. of OTUs	212	210,5	186	184,73	61	60,14	12	12,89	111	109,98	49	48,97	26	25,78	41	42,96	28	27,49	133	136,61	
	not present	**0,01**	1,5	**0,01**	1,27	**0,01**	0,86	**0,06**	-0,89	**0,01**	1,02	**0**	0,03	**0,002**	0,22	**0,09**	-1,96	**0,01**	0,51	**0,1**	-3,61	

f: frequency, vl: vector loading, N_o: number of observed OTU, N_e: number of expected OTU, N_o-N_e: difference of observed and expected number of OTU

Table B-2 Results of the Chi²-test conducted subsequent to PCA to test for statistic significance between distinct fir genotypes and ECM OTUs; considered are those fir genotypes that are related to the first and the second coordinate of the PCA biplot; (for OTU designation see Table A-1).

fir genotype		OTUs 1 N_o / **Chi²**	1 N_e / $N_o - N_e$	2 N_o / **Chi²**	2 N_e / $N_o - N_e$	3 N_o / **Chi²**	3 N_e / $N_o - N_e$	4 N_o / **Chi²**	4 N_e / $N_o - N_e$	5 N_o / **Chi²**	5 N_e / $N_o - N_e$	6 N_o / **Chi²**	6 N_e / $N_o - N_e$	7 N_o / **Chi²**	7 N_e / $N_o - N_e$	8 N_o / **Chi²**	8 N_e / $N_o - N_e$	9 N_o / **Chi²**	9 N_e / $N_o - N_e$	10 N_o / **Chi²**	10 N_e / $N_o - N_e$	**Chi-Quadrat**
first coordinate of PCA																						
SF333 - 169 175	no. of OTUspres	6	4,46	0	3,91	4	1,27	2	0,27	4	2,33	2	1,04	0	0,55	0	0,91	0	0,58	0	2,89	**29,01*** **
f: 2,5 %	ent	**0,53**	1,54	**3,91**	-3,91	**5,87**	2,73	**11,08**	1,73	**1,2**	1,67	**0,89**	0,96	**0,55**	-0,55	**0,91**	-0,91	**0,58**	-0,58	**2,89**	-2,89	
vl +0.7470	no. of OTUs	206	207,74	186	182,3	57	59,35	11	12,72	107	108,53	47	48,33	26	25,44	43	42,4	28	27,13	138	134,82	
	not present	**0,01**	-1,74	**0,08**	3,7	**0,09**	-2,35	**0,23**	-1,72	**0,02**	-1,53	**0,04**	-1,33	**0,01**	0,56	**0,01**	0,6	**0,03**	0,87	**0,08**	3,18	
SF333 - 171 175	no. of OTUs	5	2,97	0	2,61	0	0,85	1	0,18	3	1,55	0	0,69	3	0,36	0	0,61	0	0,39	0	1,93	**33,39*** **
f: 1,7 %	present	**1,39**	2,03	**2,61**	-2,61	**0,85**	-0,85	**3,74**	0,82	**1,36**	1,45	**0,69**	-0,69	**19,36**	2,64	**0,61**	-0,61	**0,39**	-0,39	**1,93**	-1,93	
vl +0.6439	no. of OTUs	207	209,44	186	183,8	61	59,84	12	12,82	108	109,42	49	48,73	23	25,65	43	42,74	28	27,36	138	135,92	
	not present	**0,03**	-2,44	**0,03**	2,2	**0,02**	1,16	**0,05**	-0,82	**0,02**	-1,42	**0,001**	0,27	**0,27**	-2,65	**0,002**	0,26	**0,01**	0,64	**0,03**	2,08	
SFb4 - 166 180	no. of OTUs	9	5,74	6	5,03	0	1,64	1	0,35	7	3	0	1,33	0	0,7	0	1,17	0	0,75	0	3,72	**18,42***
f: 3,6%	present	**1,85**	3,26	**0,19**	0,97	**1,64**	-1,64	**1,21**	0,65	**5,33**	4	**1,33**	-1,33	**0,7**	-0,7	**1,17**	-1,17	**0,75**	-0,75	**3,72**	-3,72	
vl +0.5337	no. of OTUs	203	206,68	180	181,37	61	59,05	12	12,65	104	107,98	49	48,08	26	25,31	43	42,18	28	26,99	138	134,13	
	not present	**0,07**	-3,68	**0,01**	-1,37	**0,06**	1,95	**0,03**	-0,65	**0,15**	-3,98	**0,02**	0,92	**0,02**	0,69	**0,02**	0,82	**0,04**	1,01	**0,11**	3,87	
SFb4 - 188 188	no. of OTUs	0	2,97	8	2,61	0	0,85	1	0,18	3	1,55	0	0,69	0	0,36	0	0,61	0	0,39	0	1,93	**24,29****
f: 1,8 %	present	**2,97**	-2,97	**11,13**	5,39	**0,85**	-0,85	**3,65**	0,81	**1,36**	1,45	**0,69**	-0,69	**0,36**	-0,36	**0,61**	-0,61	**0,39**	-0,39	**1,93**	-1,93	
vl +0.6969	no. of OTUs	212	209,44	178	183,8	61	59,84	12	12,82	108	109,42	49	48,73	26	25,65	43	42,74	28	27,36	138	135,92	
	not present	**0,03**	2,56	**0,18**	-5,8	**0,02**	1,16	**0,05**	-0,82	**0,02**	-1,42	**0,001**	0,27	**0,005**	0,35	**0,002**	0,26	**0,01**	0,64	**0,03**	2,08	
SFb4 - 164 196	no. of OTUs	0	0,64	0	0,56	2	0,18	1	0,04	0	0,33	0	0,15	0	0,08	0	0,13	0	0,08	0	0,41	**44,11*** **
f: 1,1 %	present	**0,64**	-0,64	**0,56**	-0,56	**18,4**	1,82	**23,04**	0,96	**0,33**	-0,33	**0,15**	-0,15	**0,08**	-0,08	**0,13**	-0,13	**0,08**	-0,08	**0,41**	-0,41	
vl +0.8904	no. of OTUs	212	206,04	186	185,85	59	60,51	12	12,97	111	110,64	49	49,27	26	25,93	43	43,22	28	27,66	138	137,44	
	not present	**0,17**	5,96	**0**	0,15	**0,04**	-1,51	**0,07**	-0,97	**0,001**	0,36	**0,001**	-0,27	**0**	0,07	**0,001**	-0,22	**0,004**	0,34	**0,002**	0,56	
second coordinate of PCA																						
SFb4 - 172 172	no. of OTUs	6	4,25	0	3,73	0	1,21	0	0,26	3	2,22	3	0,99	2	0,52	0	0,87	0	0,55	4	2,58	**23,26****
f: 2,4 %	present	**0,72**	1,75	**3,73**	-3,73	**1,21**	-1,21	**0,26**	-0,26	**0,27**	0,78	**4,08**	2,01	**4,21**	1,48	**0,87**	-0,87	**0,55**	-0,55	**0,78**	1,42	
vl +0.6993	no. of OTUs	206	208,17	186	182,68	61	59,48	13	12,74	108	108,76	46	48,43	24	25,49	43	42,48	28	27,19	134	135,1	
	not present	**0,02**	-2,17	**0,06**	3,32	**0,04**	1,52	**0,01**	0,26	**0,01**	-0,76	**0,12**	-2,43	**0,09**	-1,49	**0,01**	0,52	**0,02**	0,81	**0,01**	-1,1	
SFb4 - 164 166	no. of OTUs	15	14,87	9	13,05	11	4,25	1	0,91	9	7,77	0	3,46	0	1,82	3	3,03	0	1,94	15	9,65	**23,94****
f: 6,8 %	present	**0,001**	0,13	**1,26**	-4,05	**10,72**	6,75	**0,01**	0,09	**0,19**	1,23	**3,46**	-3,46	**1,82**	-1,82	**0**	-0,03	**1,94**	-1,94	**2,97**	5,35	
vl -0.7189	no. of OTUs	197	197,55	177	173,36	50	56,44	12	12,09	102	103,21	49	45,96	26	24,19	40	40,32	**28**	25,8	123	128,2	
	not present	**0,002**	-0,55	**0,08**	3,64	**0,73**	-6,44	**0,001**	-0,09	**0,01**	-1,21	**0,2**	3,04	**0,14**	1,81	**0,003**	-0,32	**0,19**	2,2	**0,21**	-5,2	

f: frequency, vl: vector loading, N_o: number of observed OTU, N_e: number of expected OTU, N_o-N_e: difference of observed and expected number of OTU

Appendix

Methods of assessing pollen dispersal

Using the parentage model in combination with the paternity analysis, we assessed the pollinating males and the respective females as parent pair for fir saplings in the study site BW out of 200 sampled adult trees.

A total exclusion battery comprising the five nSSRs SF1i, SFb5, SF78, SF331, SF333 described in Cremer *et al.* (2006) and twelve isozyme loci (AAT-A, -B, -C, IDH-A, -B, MDH-A, MNR-B, NDH-A, PGDH-A, PGDH-B, PGM-A, -B) according to Hussendörfer *et al.* (1995) were used to assign parentage to the 200 fir saplings. The genetic marker system used provides an exclusionary power of 98.4 % (paternity exclusion) and 99.9 % (pair parent exclusion). Additionally, four paternally inherited cpSSRs were applied for paternity analysis of the saplings in order to detect the male of the parent pair (Pt30141, Pt30249 (Liepelt *et al.,* 2001), Pt71936 (Vendramin *et al.,* 1996) and aacptrnCD (Mayland-Quellhorst, pers. communication)).

The combined procedure of parentage and paternity analysis for the estimation of pollen dispersal distances included following steps:

I. Parentage analysis on the basis of five nSSR and twelve isozyme loci

Parentage analysis was applied by a maximum-likelihood assignment using the software Cervus 3.0 (Kalinowski *et al.,* 2007) in a first step. Proceeding the parentage analysis, the computer simulations were based on 'logarithm of the odds (LOD) score' and were conducted 10,000 times with 1 % as mistyped rate, 95 as strict and 80 % as relaxed confidence level and 220 individuals as probable candidate parents. Only those adult trees have been taken into account as 'true' parents that were assigned to a sapling with a positive LOD score and showing no mismatch. In doing so, possible complementary parent pairs were chosen for the further analysis. Non-complementary parent candidates were excluded. Besides, a clear assignment of saplings to only one parent tree was not detected and, thus, self-fertilization could not be considered.

II. Paternity analysis on the basis of the four cpSSR loci

Paternity assignment was conducted by a simple identity matching procedure based on the cpSSR multilocus haplotype profiles. By means of the 'identity analysis function' of the software Cervus 3.0 (Kalinowski *et al.,* 2007) cpSSR haplotypes of possible parent pairs were compared with those of the given offspring (saplings) assigned by parentage analysis. For the identity analysis the 'minimum number of matching loci' of four and 'no fuzzy match' were chosen as settings.

The gender of each parent could not be identified if both adults had the same cpSSR haplotype profile as that of the sapling or if both adults had different cpSSR haplotype profiles from that of the sapling. However, parent pairs were considered for further analysis if one adult had the same cpSSR haplotype profile as the sapling (pollen parent = father) and the other one had a different cpSSR haplotype profile (seed parent = mother).

III. Measurement of spatial inter-parent distances

For those saplings for which a credible parent pair was found and the pollen parent could be distinguished from the mother, the position of the fathers and mothers were used to calculated the pollen dispersal distances. In total, 34 of the 200 saplings could be clearly assigned to a parent pair as well as to a male parent (Table A).

Table A Results of pollen dispersal estimations (in m): fir saplings that could be assigned to a male and a female parent by the combined approach of identity matching (using cpSSR haplotype profiles) and parentage analysis; given are also the geographical coordinates of the assigned parent trees.

sapling (tree no.)	potential male parent (tree no.)	Gauß-Krüger coordinates of male parent		potential female parent (tree no.)	Gauß-Krüger coordinates of female parent		pollen dispersal distance (in m)
		X	Y		x	Y	
V100	199	3462600.5	5378319.78	130	3462695.79	5378216.66	140.41
V101	163	3462485.54	5378224.01	167	3462480,38	5378223,85	5.16
V106	168	3462476.01	5378209,28	23	3462678.79	5378213.75	202.82
V113	71	3462588,33	5378276,6	67	3462579.77	5378259.64	19.00
V126	180	3462468,31	5378265,36	109	3462489,05	5378274,24	22.56
V128	42	3462621,49	5378194,87	177	3462425,63	5378236,58	200.25
V136	49	3462606,17	5378275,93	169	3462472,07	5378210,01	149.43
V137	107	3462469,25	5378272,52	26	3462593,14	5378178,79	155.35
V138	131	3462606,62	5378167,95	200	3462608,48	5378314,84	146.90
V141	189	3462540,25	5378308,51	165	3462484,39	5378241,69	87.10
V150	151	3462551,92	5378227,54	75	3462581,36	5378322,98	99.88
V153	107	3462469,25	5378272.52	137	3462598.56	5378193.29	151.65
V155	94	3462502.1	5378229.72	169	3462472.07	5378210.01	35.93
V161	134	3462590.42	5378188.44	153	3462575.52	5378232.02	46.05
V17	57	3462564.92	5378211.45	22	3462680.7	5378207.74	115.84
V171	189	3462540.25	5378308.51	106	3462465.11	5378270.41	84.26
V175	107	3462469.25	5378272.52	188	3462524.72	5378296.02	60.24
V179	67	3462579.77	5378259.64	93	3462520.45	5378221.51	70.52
V184	105	3462475.21	5378262.39	107	3462469.25	5378272.52	11.75
V20	161	3462501.75	5378224.88	67	3462579.77	5378259.64	85.42
V200	42	3462621.49	5378194.87	133	3462590.19	5378185.98	32.54
V28	42	3462621.49	5378194.87	92	3462540.41	5378217.72	84.25
V30	66	3462587.42	5378237.95	139	3462596.12	5378208.35	30.86
V39	22	3462680.7	5378207.74	65	3462573.1	5378235.18	111.04
V46	155	3462571.21	5378221.44	67	3462579.77	5378259.64	39.15
V48	151	3462551.92	5378227.54	119	3462486.93	5378288.12	88.85
V53	149	3462550.47	5378206.99	74	3462586.08	5378321.23	119.66
V54	194	3462584.8	5378322.65	108	3462487.03	5378267.2	112.40
V59	194	3462584.8	5378322.65	198	3462596.78	5378311.78	16.18
V62	160	3462519.81	5378216.17	84	3462549.97	5378239.23	37.97
V69	95	3462495.44	5378227.91	185	3462515.98	5378292.05	67.35
V90	130	3462695.79	5378216.66	180	3462468.31	5378265.36	232.64
V91	191	3462563.37	5378304.16	149	3462550.47	5378206.99	98.026
V93	149	3462550.47	5378206.99	120	3462483.93	5378300.18	114.51
Mean							**90.5**
Median							**86.3**
Min							**5.2**
Max							**232.6**

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Erklärung

Ich versichere, dass ich meine Dissertation

‘Population genetics of silver fir (*Abies alba* Mill.) in the Northern Black Forest – preconditions for the recolonization of windthrow areas and associated ectomycorrhizal communities’

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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