MICROSATELLITE AND ISOZYME MARKERS FOR SEED SOURCE IDENTIFICATION IN SILVER FIR

E. Cremer [1,5*], S. Liepelt [2,3*], B. Ziegenhagen [3] & E. Hussendörfer [4]

[1)] Forest Research Institute of Baden-Wuerttemberg, Wonnhaldestrasse 4, D-79100 Freiburg, Germany, Email: evacremer@gmx.de
[2)] Federal Research Centre for Forestry and Forest Products, Institute for Forest Genetics and Forest Tree Breeding, Sieker Landstrasse 2, D-22927 Grosshansdorf, Germany
[3)] Philipps-University of Marburg, Faculty of Biology, Nature Conservation Division, Karl-von-Frisch-Strasse, D-35032 Marburg, Germany
[4)] FH Weihenstephan, FB Wald und Forstwirtschaft, Am Hochanger 5, 85354 Freising, Germany
[5)] New address: Forest Research Institute of Lower Saxony, Forstamtstrasse 6, D-34355 Staufenberg, Germany
* both authors contributed equally

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ABSTRACT

The present paper reports on the genetic differentiation of single trees in silver fir (*Abies alba* Mill.) using a combined approach of isozyme gene markers with cpDNA-microsatellite markers. The question was whether it is possible to distinguish single trees by analysing a sample of their seeds. For this purpose needles or buds and seed samples from 20 silver fir individuals of a seed source population were analysed at 11 isozyme gene loci and three chloroplast microsatellite loci.

The results of the investigation showed that neither by using only isozyme gene markers nor by using only cpDNA-microsatellites was it possible to distinguish all trees genetically. However, the combined use of both marker types made the differentiation of all analysed silver firs possible. Moreover, the probabilities of identity (P_{ID}) calculated for both marker types exhibited lower values than the P_{ID} calculated for each single marker system.

The use of both markers can be helpful for controlling forest reproductive material and seed source-identification.

Keywords: allozymes, cpSSR, *Abies alba*, individual differentiation, certification.

INTRODUCTION

In many genetic studies it is necessary to distinguish single trees within populations. This holds especially true for controlling the chain of custody of forest reproductive material which is strictly regulated. For example, the *German Law on Forest Reproductive Material* states that seeds must be collected from at least 20 trees of a seed source population. In Austria the same regulation is in force. In order to ensure this, the State Administrations for Forest of Bavaria and Baden-Wuerttemberg as well as private nurseries established a voluntary control system depending on so-called "reference samples". Within this system it was determined that the number of harvested trees, in addition to the provenances, should be controlled in a better way. For silver fir, a reference sample of about 20 cones must be taken from each seed tree and clearly identifiably stored (KONNERT & HUSSENDÖRFER 2002). Thus, in the case of suspicion the reference samples may serve as a control. This only works reliably if the single seed donor trees can be unambiguously differentiated from each other.

The initiative of the control system has intended that the genetic investigations for proving the number of harvested trees follow two steps. In the first step, isozymes are used for genotyping the mother trees. If not all trees can be distinguished using this method, DNA-markers can be applied additionally in a second step to differentiate the trees which could not be distinguished by isozymes.

Nuclear microsatellites (simple sequence repeats = SSR) have proved to be the marker of choice for identification and parentage analysis. For example, DOW & ASHLEY (1996) and STREIFF *et al.* (1999) used nuclear microsatellites for parentage analysis in oak. In cherry, these markers were used for individual identification (SCHUELER *et al.* 2003). Using nuclear and chloroplast microsatellite markers, individuals of oak and silver fir could be identified using maternal tissue

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of their fruits and seeds (ZIEGENHAGEN *et al.* 2003).

It was decided by the South-German control initiative to use mainly isozyme gene markers since microsatellite markers or other DNA-markers are not yet available for all tree species under control. For *Pinaceae*, it is well known that the multilocus-genotype of a seed tree can be determined by analysing 6 to 12 megagametophytes from its seeds using isozymes (BARTELS 1971, HATTEMER *et al.* 1993, BREITENBACH-DORFER & GEBUREK 1995). Thus, if each seed tree within a population shows a different multilocus-genotype, it can easily be controlled whether the seeds of the single-tree reference samples originate from a minimum number of 20 trees or not.

If seeds of two or more single-tree reference samples show identical multilocus-genotypes after analysing endosperms, at least two interpretations are possible. On the one hand, it could be possible that two or more reference samples were taken from the same seed tree, indicating that fewer seed trees than legally required were harvested (BREITENBACH *et al.* 2002). On the other hand, the power of discrimination of the marker could be too poor to discriminate among all reference samples.

In order to test the power of discrimination of a combined approach, a case study on silver fir (*Abies alba* Mill.) was conducted. In addition to 11 polymorphic isozyme gene markers, three chloroplast DNA (cpDNA) microsatellites were used that had previously been described as highly polymorphic in this species (ZIEGENHAGEN *et al.* 1998, VENDRAMIN *et al.* 1999, LIEPELT *et al.* 2001). Using buds or needles and a sample of the seeds of single-trees, it was tested whether all seed trees could be genetically distinguished using each marker type separately. In a second step, it was tested if a combined use of both marker types would increase the number of trees that could be distinguished.

The results are discussed for their combined usage in the control system of silver fir starting with isozyme markers and then incorporating a second test using cpDNA microsatellites if further discrimination is necessary.

MATERIAL AND METHODS

The following samples for the genetic investigation were taken from a seed source population of silver fir located in the Southern Black Forest, Baden-Wuerttemberg, Germany, forest district "Spaichingen":

- Single-tree seed samples of 20 silver firs consisting of about 20 cones per tree. Fig.1 shows endosperm and embryo in a seed of *Abies alba.*

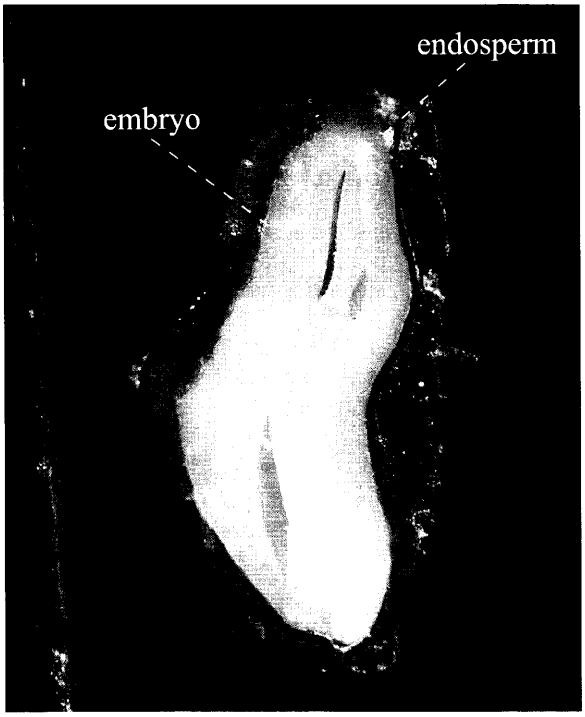

Figure 1. Longitudinal section of an *Abies alba* seed.

- Buds from each of the 20 seed-trees for isozymic investigation.
- Needles of 17 seed-trees (no.2 – no.8 and no.10 – no.19 in table 4) for the investigation by means of cpDNA-microsatellites. Needle samples of the trees nos. 1, 9 and 20 were not available.

Isozyme gene analysis

Using endosperms of seeds and bud tissue, the genotype of each of the 20 trees was determined by isozyme gene markers at 11 polymorphic gene loci. The analysis was carried out according to the method of HUSSENDÖRFER *et al.* (1995). The designation of alleles and genotypes in Table 1 follows the suggestions of HUSSENDÖRFER *et al.* (1995).

By analysing several endosperms from a single tree seed sample, the diploid isozyme genotype of the mother tree (predicted or putative genotyping) was compared to that obtained from buds (actual genotyping).

CpDNA marker analysis

The haplotypes of 17 silver firs were determined at three highly variable chloroplast-DNA-microsatellite loci, Pt 30141, Pt 30249 and Pt 71936 (primer se-

quences in VENDRAMIN *et al.* 1996 and LIEPELT *et al.* 2001). For this, genomic DNA was extracted from needles according to the method of DUMOLIN *et al.* (1995). Then the fragments that had to be analysed were amplified through polymerase chain reaction (PCR). A 25µl PCR reaction cocktail contained 1x PCR-buffer, 1.6 mM $MgCl_2$, 0.2 mM dNTPs, 0.2µM each of forward and reverse primer, 20 ng genomic DNA, 1U Taq Polymerase. The conditions for the PCR were: 5 min denaturation at 95 °C followed by 25 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a final step of 10 min at 72 °C.

The PCR products were subjected to fragment length analysis using an automated sequencer according to the method of VENDRAMIN & ZIEGENHAGEN (1997).

Statistical analysis

In order to get a statistical confidence for the differentiation of individual trees, the probability of identity (P_{ID}) was computed. P_{ID} is the probability that two randomly drawn individuals exhibit identical genotypes. For the isozyme data this was computed using the following equation (PAETKAU *et al.* 1998):

$$P_{ID} = \sum_i p_i^4 + \sum_i \sum_{j>i} (2p_i p_j)^2$$

where p_i and p_j are the frequencies of the *i*th and *j*th alleles. P_{ID} was calculated for each locus and then multiplied across loci.

For the chloroplast microsatellite data the equation was adapted to haploid data:

$$P_{ID} = \sum_i p_i^2$$

where p_i is the frequency of the *i*th haplotype. Because the chloroplast genome is uniparentally inherited in firs and thus not recombining, it was treated as one locus and the different haplotypes were treated as alleles.

RESULTS

The percentage of hollow seeds in the seeds that were available was quite high, with an average of 72 %. Therefore, in some cases only few endosperms per silver fir could be investigated using isozymes. For silver firs nos. 1, 14 and 20, no genetic data could be scored by endosperm investigation because all seeds were hollow. For these trees, only "actual" genotypes are given.

The multilocus-genotype of each of the seed-trees is presented in Table 1. Since the three gene loci *Aat-C*, *6pgdh-B* and *Pgm-A* showed no variation they are not listed in this table.

Table 1 shows that in most cases the "actual" genotype was correctly predicted by the "putative" genotype. Exceptions are the trees no.9 (gene locus *Mdh-A*), no.18 (gene loci *Idh-A* and *-B*) and no.19 (gene loci *Aat-B* and *Idh-B*). In these cases the number of analysed endosperms was too small to determine the actual genotype of the trees because there were only four or less filled seeds available per mother tree.

Nineteen of the twenty trees showed a different multilocus-genotype. Two trees (no.2 and no.15) had the same multilocus-genotype.

CpDNA-microsatellites were used as a second marker system. Table 1 also shows the fragment lengths in base pairs (bp) at the three analysed microsatellite loci. In the 17 analysed trees 11 haplotypes could be distinguished. Trees no.2 and no.15 showing the same isozyme multilocus-genotype could clearly be differentiated using cpDNA-microsatellites.

Therefore, by the combined use of isozyme-markers and DNA-markers, it was possible to differentiate all 20 fir trees.

The probabilities of identity (P_{ID}) were calculated for the isozyme gene markers (11 gene loci), the cpDNA-microsatellites (3 microsatellite loci) and for the combination of both marker systems. P_{ID} was 0.04339 for the isozymes, 0.11419 for the cpDNA-microsatellites and 0.00496 for the combination of both.

DISCUSSION

In the present study two types of genetic markers were tested regarding the possibility to distinguish individuals genetically and regarding their practical and scientific use.

The results of the investigations showed that neither by using only isozyme gene markers (11 gene loci) nor by using only cpDNA-microsatellites (3 microsatellite loci) was it possible to distinguish all 20 trees. However, the combined use of both marker types allowed the differentiation of all analysed trees.

To our knowledge, this is the first time that a combination of these markers was applied to differentiate single trees in silver fir.

The results of the probability of identity (P_{ID}) calculated for the isozyme gene markers (0.04339) and for the cpDNA-microsatellites (0.11419) reveal, that neither of the probabilities is sufficiently low. Only the P_{ID} calculated for both marker types shows an essentially lower P_{ID} of 0.00496. That means, in about 995 of 1000 cases two fir trees drawn at random from a

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Table 1. List of the putative (analysing endosperm) and the actual (analysing buds) genotypes of the 20 silver firs and results of the fragment length analysis at three cpDNA microsatellite loci (in bold type: the trees with the same isozyme multilocus-genotype; printed in italic and bold type: those putative and actual genotypes which are not identical).

Tree	Genotype	*Ap-A*	*Aat-A*	*Aat-B*	*Idh-A*	*Idh-B*	*Mdh-A*	*Mnr-B*	*6pgdh-A*	Pt 30141	Pt 30249	Pt 71936	Haplo-type
1	actual	35	22	22	13	33	11	33	23		n.a.		
2	putative	33	22	22	13	35	12	33	33				
	actual	**33**	**22**	**22**	**13**	**35**	**12**	**33**	**33**	**147**	**100**	**150**	**1**
3	putative	35	22	22	11	55	12	33	33				
	actual	35	22	22	11	55	12	33	33	147	100	150	1
4	putative	35	22	23	33	55	11	33	33				
	actual	35	22	23	33	55	11	33	33	141	99	152	2
5	putative	35	22	23	33	33	11	33	33				
	actual	35	22	23	33	33	11	33	33	139	100	152	3
6	putative	55	22	22	13	33	12	33	23				
	actual	55	22	22	13	33	12	33	23	147	100	150	1
7	putative	55	22	23	33	35	12	13	23				
	actual	55	22	23	33	35	12	13	23	139	99	152	4
8	putative	33	12	22	13	55	12	33	23				
	actual	33	12	22	13	55	12	33	23	148	99	150	5
9	putative	33	22	22	13	55	**22**	33	23				
	actual	33	22	22	13	55	**12**	33	23		n.a.		
10	putative	55	22	22	13	35	11	33	23				
	actual	55	22	22	13	35	11	33	23	139	99	152	4
11	putative	35	22	23	33	55	11	33	23				
	actual	35	22	23	33	55	11	33	23	148	99	150	5
12	putative	35	22	23	13	55	11	33	33				
	actual	35	22	23	13	55	11	33	33	141	98	155	6
13	putative	35	22	22	11	33	11	33	23				
	actual	35	22	22	11	33	11	33	23	139	99	152	4
14	actual	33	22	22	11	55	11	33	23	144	99	150	7
15	putative	33	22	22	13	35	12	33	33				
	actual	**33**	**22**	**22**	**13**	**35**	**12**	**33**	**33**	**136**	**98**	**156**	**8**
16	putative	33	22	22	13	55	11	33	33				
	actual	33	22	22	13	55	11	33	33	146	99	151	9
17	putative	33	22	23	33	55	11	33	23				
	actual	33	22	23	33	55	11	33	23	141	98	155	6
18	putative	33	22	22	**11**	**33**	11	33	33				
	actual	33	22	22	**13**	**35**	11	33	33	136	98	155	10
19	putative	33	22	**33**	33	**55**	22	33	23				
	actual	33	22	**23**	33	**35**	22	33	23	142	99	155	11
20	actual	35	22	22	33	35	11	33	33		n.a.		

population can be distinguished from each other. For discussion purposes, WAITS *et al.* (2001) chose a P_{ID} between 0.01 (1 in 100) and 0.0001 (1 in 10000) as an acceptable low P_{ID}. Thus, the P_{ID} for the combination of isozyme gene markers and cpDNA-microsatellites can be considered to be in an acceptable range for differentiating purposes. According to WAITS *et al.* (2001) a P_{ID} of less than 0.001 is necessary for most law enforcement forensic applications in natural populations.

It turned out that a sufficient amount of seeds was necessary to reconstruct the diploid maternal genotype using the endosperms of single tree seed samples. If the number of available endosperms is too small, the risk of a misinterpretation is quite high. Usually, at least six endosperms per tree are investigated for an inventory of genotypes (HATTEMER *et al.*, 1993), whereas BREITENBACH-DORFER & GEBUREK (1995) recommend to use at least 13 endosperms per tree to reach a probability of misclassification < 5%.

Within the scope of the South-German control system, only seeds (no needles) can be scored to make the control possible. Secondly, the seed samples are used for further controlling aspects (KONNERT & HUSSENDÖRFER 2002). Therefore, in this case, the genotypes of the mother trees can only be obtained indirectly by endosperm analyses.

In this study the cpDNA-microsatellite analysis was carried out using needles of the respective mother trees, but the same investigation would also have been possible using just one endosperm per mother tree (VENDRAMIN *et al.* 1999). VENDRAMIN & ZIEGENHAGEN (1997) have shown that the endosperm of fir seeds has the same chloroplast haplotype as the mother tree and can be analysed easily.

A similar question was investigated in ZIEGENHAGEN *et al.* (2003). From their study it was concluded that the identity and number of mother trees (*Abies alba*) in a seed lot sample can be exactly determined from a reference sample by simply haplotyping maternal tissue of the seeds using cpDNA-microsatellites.

The investigated population has an average diversity level compared to other silver fir populations (allelic isozyme-diversity: 1.299 and Nei's diversity index h = 0.886). For example, 20 analysed fir stands in Switzerland showed an average diversity of 1.26 for isozymes (HUSSENDÖRFER 1997). For cpDNA-microsatellites, VENDRAMIN & ZIEGENHAGEN (1997) calculated an average level of Nei's diversity index of h = 0.849. Starting from the point that most seed-source-populations have a high or an average diversity, respectively, isozyme gene markers in combination with cpDNA-microsatellites seem to be an adequate system for tree differentiation in silver fir due to a high discrimination power among genotypes and haplotypes, respectively. Hence, using genetic markers within the control system for seed collecting and for source identification, the combined use of both marker types is an appropriate method. Specifically, the real number of seed-trees can be controlled on the basis of reference samples, using first isozyme gene markers because of a lower P_{ID} and then cpSSR markers in a second step if necessary.

In the second step, we used highly polymorphic chloroplast microsatellites because nuclear microsatellites with a higher exclusion percentage are not yet available. In the future, nuclear microsatellites could be applied instead of chloroplast microsatellites.

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