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Microsatellite analysis of maternal half-sib families of *Quercus robur*, pedunculate oak: detection of seed contaminations and inference of the seed parents from the offspring

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Abstract Microsatellites were used for genetic analysis of maternal half-sib families of *Quercus robur*, pedunculate oak, a highly outcrossing tree species. A model half-sib family including the mother tree and 28 offspring individuals as well as samples from six single tree harvests from a forestry company, 4–8 individuals each, were genotyped at 9 microsatellite loci. No prior information about the genotypes of the mother trees were available for these six seedlot samples. Analysis of the model half-sib family revealed that the maternal genotypes can be inferred from the offspring genotypes due to codominant Mendelian inheritance of the microsatellites. Analysis of the single tree harvests, supplied as six maternal half-sib families, revealed contaminations with unrelated seedlings in four out of six cases. Average relatedness between the remaining individuals indicated that they were indeed half-sibs, probably with a proportion of full-sibs among them. For five samples the genotypes of the mother trees were partially inferred from the offspring genotypes. The supposed number of five different mother trees was confirmed by direct comparison of the maternal genotypes and by pairwise F_{ST} calculations between families. We show that correct genotype reconstruction can be confirmed by monitoring recombination events between linked markers. Our results demonstrate that microsatellite analysis is a suitable means to approach two key problems of legal regulations on the marketing of seed material from pedunculate oak: the number of trees included in seed harvests and the detection of seed contaminations.

Key words Oak · *Quercus* · Half-sibs · Microsatellites · Simple-sequence repeats

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Introduction

The sustainable conservation of genetic resources in forestry is of great concern to the governments in Europe (Anonymus 1990 – Strasbourg resolution). Several countries have made provisions in their legislation regulating the marketing or use of reproductive material (seeds and plants). Among them, Austria's laws and bye laws for forest reproductive material, in force since August 1996, are among the more advanced, demanding commercial seed harvests from stands of *Q. robur* and *Q. petraea* to include at least 20 different trees (Anonymus 1996a, b; Geburek und Heinze 1998). The number of trees included in seed harvests predetermines the level of genetic diversity among the seedlings. Therefore, to demand a minimum number of trees is a measure taken to guarantee that a minimum level of genetic diversity to be passed on to the following generations. However, these measures run contrary to economical considerations during seed harvest, as gathering seed from only a few trees bearing abundant fruit is less costly.

For any marketable oak seedlot in Austria, at least five acorns from each single tree need to be shipped to the Federal Forest Research Centre (FBVA) for genetic analysis, with the aim to confirm half-sib family structure within the samples and to infer the number of

different maternal oak trees (seed parents) involved. Microsatellites are the ideal genetic markers for this purpose because they are highly polymorphic and codominantly inherited and therefore have a high potential to resolve genetic relatedness (Blouin et al. 1996).

Microsatellite markers have been developed for the closely related species *Q. petraea* (Steinkellner et al. 1997) and *Q. robur* (Kampfer et al. 1998), all of the loci being conserved in both species. Four of these markers have been used to study within-population genetic structure in a mixed stand of *Q. robur* and *Q. petraea*, revealing remarkable expected heterozygosity values of 0.85 to 0.94 (Streiff et al. 1998). So far 14 of the markers have been located on a genetic map of *Q. robur* (Barreneche et al. 1998). Although the 14 microsatellites were located on seven different linkage groups, 5 of them formed a cluster of linked markers. These results provide the possibility to select unlinked or linked markers as the situation demands.

In the North American species *Q. macrocarpa* microsatellites have also been isolated (Dow et al. 1995) as a tool to study parentage and seed dispersal (Dow and Ashley 1996, 1998). In those studies most of the putative parent trees were genotyped in addition to the saplings in the stand, making it possible to find matches between saplings and parents. In contrast, in our situation there is no prior information available on the parental genotypes, neither on those of the seed parents nor on those of the pollen donors. No material from the mother trees is available for commercial oak seedlots. Furthermore, the sample sizes demanded by the Austrian forest seed law are extremely small (five to eight acorns per mother tree). Therefore, the objective of our investigation was to validate methods of data analysis that are applicable to small sample sizes and that require no prior knowledge of the possible parental genotypes. In order to approach these problems we designed our experiments as follows: in the first step we made use of a maternal model half-sib family including the genotype of the mother tree. In the second step our experiences with this model half-sib family were used to examine six commercial seedlot samples. We present a pilot study to confirm whether or not the legal rules were adhered to.

Materials and methods

Plant materials and DNA isolation

DNA of a model half-sib family was obtained by the Institute of Forest Genetics and Forest Tree Breeding, Grosshansdorf, Germany. This DNA had been extracted from leaves of 28 germinated seedlings and the mother tree using a method modified from Dumolin et al. (1995). The model half-sib family originates from the arboretum of the institute in Grosshansdorf, where about 40 different trees of *Q. robur* and *Q. petraea* served as potential pollen donors.

Since the autumn of 1996 acorn samples from seeds harvested in Austria are shipped to the Federal Forest Research Centre (FBVA) in Vienna, Austria. Sample sizes usually range between five and eight acorns per mother tree, acorns collected from different trees being supplied separately. The acorns were sown in the nursery of the FBVA, and DNA was isolated from leaves of the germinated seedlings using a protocol slightly modified from Edwards et al. (1991) by the addition of charcoal before grinding (Vroh et al. 1996). The DNA was further purified over a BioRad P60 gel (Sambrook et al. 1989).

The samples from the six single tree harvests were labelled harvest 3 (4 seelings, 1 out of 5 seedlings did not germinate), harvest 6 (5 seedlings), harvest 9 (6 seedlings), harvest 13 (8 seedlings), harvest 15 (8 seedlings) and harvest 16 (5 seedlings).

Microsatellite markers

Eight $(GA)_n$ microsatellites isolated from *Q. petraea* (Steinkellner et al. 1997) and 1 $(GA)_n$ microsatellite isolated from *Q. robur* (Kampfer et al. 1998) were used for genetic analysis. Mendelian inheritance for all microsatellites was confirmed using controlled crosses (Steinkellner et al. 1997, Lexer et al. unpublished). No null-alleles were observed for any of the markers. Assignments to different linkage groups on the genetic map of *Q. robur* (Barreneche et al. 1998) are listed in Table 2. Markers ssrQpZAG 1/5 and ssrQpZAG 9 are separated by 24 cM (male map) and 37 cM (female map), respectively, on linkage group 7. Marker ssrQrZAG 112 is not yet integrated into the genetic map, but linkage analysis in the same mapping population revealed no significant linkage to any of the other microsatellites (Lexer et al. unpublished). Markers ssrQpZAG 36, ssrQpZAG 46 and ssrQpZAG 104 are linked within linkage group 2, with recombination frequencies ranging from 1.1% to 5.6%, when computed from both parents of the mapping cross.

Microsatellite amplification and electrophoresis

Markers ssrQpZAG 15, ssrQpZAG 3/64 and ssrQrZAG 112 were amplified using a two-step polymerase chain reaction (PCR) program that minimizes PCR artefacts introduced by the nontemplated addition of nucleotides by *Taq* polymerase (Smith et al. 1995). All other markers were amplified as described previously (Steinkellner et al. 1996). PCR primers for ssrQrZAG 112 were labelled with the fluorescent dye HEX (Perkin Elmer ABI), primers for the 8 other markers were labelled with Cy-5 (Pharmacia Biotech). PCR products from ssrQrZAG 112 were analyzed by capillary gel electrophoresis on an ABI 310 automated sequencer according to the manufacturer's instructions. For all other markers, PCR products were analyzed on 6% denaturing polyacrylamide gels using an ALF Express automated sequencer (Pharmacia Biotech). Allele sizes were determined using internal size standards.

Data analysis

General estimates of diversity were calculated for each locus in terms of expected heterozygosity $H_E = 1 - \Sigma p_i^2$, where p_i is the relative frequency of the i^{th} allele, and in terms of the effective number of alleles $A_E = 1/(1 - H_E)$, according to Nei (1987). Both measures of polymorphism were calculated from the six seedlot samples studied here (Table 2). For determination of half-sibling relationships and reconstruction of the maternal genotypes we made use of a color code that assigns a specific color to each allele within a sample. Both features were first studied for the model half-sib family, and correct genotype reconstruction was confirmed for each locus by comparison to the genotype of the mother tree. Then analysis was extended to the seedlot samples, where no information on the mother trees was available. Unrelated individuals within single tree harvests were

detected using two methods, one based on private alleles, present only in 1 individual within a sample, the other one based on the proportion of shared alleles among pairs of seedlings within a sample. Private alleles were identified using color coding as described above. The proportion of shared alleles (Ps) for pairs of individuals was calculated as the number of shared alleles summed over loci divided by twice the number of loci, as in Bowcock et al. (1994). A genetic distance between pairs of individuals was obtained by -ln (Ps) using the computerprogram MICROSAT (Minch 1997). UPGMA cluster analysis was conducted using the PHYLIP software package (Felsenstein 1989).

Average relatedness (Queller and Goodnight 1989) within single tree harvests was computed using the FSTAT software (Goudet 1995). Seven unlinked loci were used for the computations, including ssrQpZAG 104 from linkage group 2 (Table 2). Means and standard deviations for average relatedness were obtained by jackkniving over loci in FSTAT. Differentiation between single tree harvests was estimated using F_{ST} according to Weir and Cockerham (1984). Again 7 unlinked loci were selected as described above. The significance of F_{ST} was tested using permutations, a procedure that is not dependent on Hardy Weinberg equilibrium. Both, F_{ST} and tests of significance, were calculated using FSTAT. A principal component analysis based on pairwise F_{ST} values was conducted using the SPSS software package (SPSS, Chicago).

Results and discussion

Genetic analysis of a model half-sib family

Twenty-eight offspring of a maternal half-sib family of *Quercus robur* were genotyped at 9 microsatellite loci. Between 6 and 12 alleles per locus were found among the offspring. The genotype data of the offspring individuals were used in identifying reliable and simple methods (1) to confirm or reject the hypothesis of half-sibling relationship among seedlings from acorns supplied as single tree harvests; (2) to reconstruct the maternal genotypes of single tree harvests.

At 2 loci, ssrQpZAG 36 and ssrQpZAG 46, 1 allele was present in each of the offspring individuals, suggesting that the mother tree was homozygous at both loci (Table 1). All other alleles at these loci occurred at much lower frequencies and would therefore not fit a segregation ratio of 1 : 1 as expected for the maternal alleles under a simple codominant inheritance hypothesis. Therefore, all other alleles could be unequivocally assigned to the pollen contribution.

At 7 loci, no single allele was found to be present in each offspring individual, indicating that the mother tree was heterozygous at these loci (Table 1). In each case only one possible pair of alleles constituting the maternal genotype was identified. All other alleles could be excluded on the basis of codominant Mendelian inheritance: (1) an allele can only be of maternal origin if there is a second allele that is present whenever the first one is absent; (2) an allele that is present in homozygous state in at least 1 offspring individual *must* be present in the mother tree; (3) if two different homozygotes are found among the offspring then no other allele can be of maternal origin.

Successful reconstruction of the maternal genotype was confirmed for each locus by comparison to the microsatellite profiles of the mother tree. Homozygous offspring genotypes were only observed for alleles of true maternal origin, strongly suggesting that false homozygotes carrying a null-allele were not present among the offspring. The absence of null-alleles (Pemberton et al. 1995) is an essential prerequisite and is also very likely for the loci under study, since none of them showed null-alleles in studies of controlled crosses (Steinkellner et al. 1997; Lexer et al. unpublished).

The highest number of different paternal alleles observed among the offspring was 10 (locus ssrQrZAG 112). At all loci, the maternal alleles were more frequent than any of the paternal alleles. Furthermore at 7 loci the maternal alleles segregated 1 : 1 according to Mendelian expectations. These observations are consistent with a half-sibling relationship (Table 1). Similar observations were made by Dow and Ashley (1996) in parentage studies *of Q. macrocarpa*. In that study a dead tree in the stand was inferred to be the probable mother

Table 1 Genetic analysis of a maternal half-sib family

Locus	Maternal genotype	Frequencies of maternal alleles[a]	Frequency of most frequent paternal allele[a]	Maternal alleles: observed segregation ratio[b]	Chi-square for segregation 1 : 1 (1 *df*)
ssrQpZAG 1/5	165/168	15/22	9	11 : 14	0.36
ssrQpZAG 9	185/193	18/17	8	12 : 12	0.00
ssrQpZAG 15	109/143	18/13	8	15 : 10	1.00
ssrQpZAG 3/64	155/157	16/19	4	11 : 14	0.36
ssrQpZAG 110	205/211	28/13	10	16 : 6	4.54[c]
ssrQpZAG 36	213/213	31	7	–	–
ssrQpZAG 46	205/205	32	7	–	–
ssrQpZAG 104	198/208	15/17	8	12 : 15	0.34
ssrQrZAG 112	86/94	19/18	5	10 : 14	0.66

[a] Absolute frequencies
[b] Individuals bearing paternal alleles identical to 1 of the maternal alleles were not counted
[c] Significant deviation at $P = 0.05$ but not at $P = 0.01$

of a cluster of genotyped seedlings, although in this particular case they were not able to confirm the result by genotyping the mother tree.

We conclude that in principle the maternal alleles can be inferred from maternal half-sib families of *Quercus robur* using highly polymorphic microsatellites. No prior information about the mother tree is necessary. The paternal alleles can be assigned to the pollen donor on the basis of codominant Mendelian inheritance. Consequently, if all of the alleles present are assigned to the pollen contribution, or if more than two homozygotes are found, then the group under study can not be a pure half-sib family. Hence, this method has the potential to determine the purity of single tree harvests and to infer their maternal genotypes.

Detection of unrelated individuals within single tree harvests

Samples of six single tree harvests from a natural *Quercus robur* population, 36 trees in total (see Materials and methods), were genotyped at 9 microsatellite loci. Between 8 and 18 alleles per locus were found. The effective number of alleles ranged from 2.5 to 10; expected heterozygosity values ranged between 0.60 and 0.90 (Table 2). For two of the samples, harvest 3 and harvest 6, the hypothesis of half-sibling relationship was confirmed using genotype data from 9 loci. For four samples, the hypothesis was rejected at 1 or more loci, because all occurring alleles were excluded as being of maternal origin. Unrelated individuals within each of the four samples were detected using the proportion of private alleles, present only in 1 individual within a sample, as well as the proportion of shared alleles within a sample and subsequent UPGMA cluster analysis. The last individuals to join the UPGMA phenograms, located in distal positions on the phenograms, also displayed the largest number of private alleles. These seedlings were removed from the dataset. For harvests 9, 13 and 16, 1 unrelated individual each was detected among the half-sibs (Fig. 1). After removal of individuals 9b, 13f and 16c, allelic segregation at 9 loci was consistent with a half-sibling relationship. For harvest 15, the hypothesis of half-sibling relationship was still rejected after removal of 3 out of 8 individuals (not shown). The group was not included in further studies. In order to check if mistakes had been made in the nursery, we added each of the 3 unrelated seedlings, 9b, 13f and 16c, to each of the five pure families and repeated genetic distance calculations. Within each of the families these seedlings had higher mean genetic distance values than any of the other seedlings. This suggests that contaminations were not due to mistakes among the five half-sib families (not shown).

The detection of unrelated individuals within single tree harvests may be explained by the fact that the

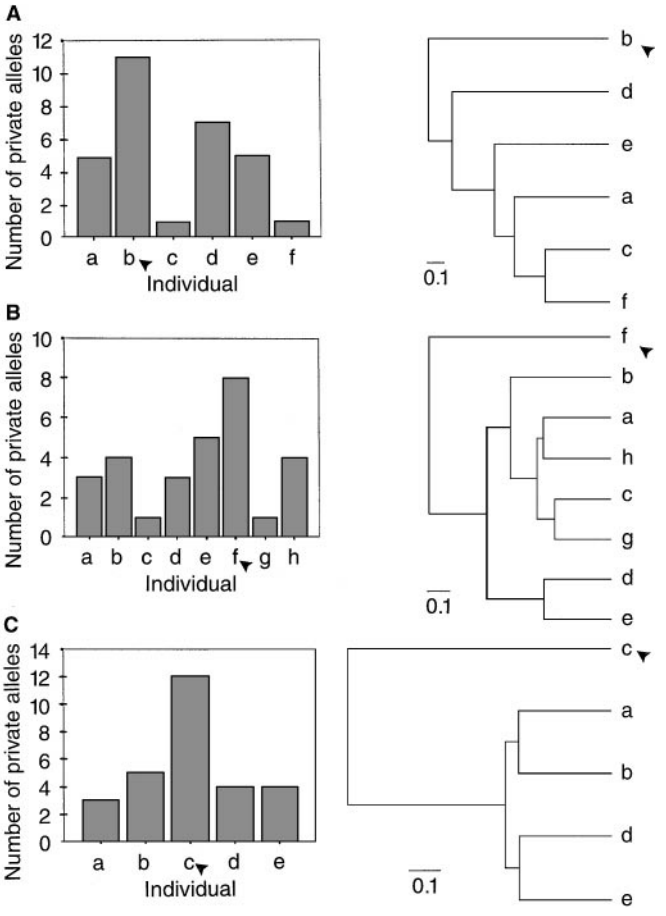

Fig. 1A–C Two methods for detecting unrelated individuals within single tree harvests. *Left* The number of private alleles—alleles that are present only in 1 individual within a sample. *Right* UPGMA phenograms using the proportion of shared alleles. **A** Harvest 9, **B** harvest 13, **C** harvest 16. Unrelated individuals are indicated by *black arrowheads*

Table 2 Microsatellite markers used in this study, their observed number of alleles (A_O), effective number of alleles (A_E) and expected heterozygosity (H_E) calculated from 36 seedlings from six different acorn samples originating from one population. Assignments to linkage groups refer to the genetic map of *Q. robur*

Locus	A_O	A_E	H_E	Linkage
ssrQpZAG 1/5	9	5.6	0.82	Lg 7[a]
ssrQpZAG 9	10	6.7	0.85	Lg 7[a]
ssrQpZAG 15	9	4.0	0.75	Lg 9
ssrQpZAG 3/64	18	10.0	0.90	Lg 6
ssrQpZAG 110	8	2.5	0.60	Lg 8
ssrQrZAG 112	15	7.1	0.86	–[b]
ssrQpZAG 36	9	5.6	0.82	Lg 2[c]
ssrQpZAG 46	17	9.1	0.89	Lg 2[c]
ssrQpZAG 104	15	9.1	0.89	Lg 2[c]

[a] Positioned on the same linkage group, but separated by 24 cM (male map) and 37.5 cM (female map), respectively
[b] Not yet positioned on the map, but unlinked to all other markers
[c] Closely linked (see Materials and methods)

acorns were collected from the ground, as is usually done for commercial acorn seedlots. This opens the possibility of dispersal by animals such as squirrels or jays (Bossema 1979). Furthermore, the harvest site was located on a steep slope, making it likely that acorns were dispersed by gravitation. Our results show that such contaminations, whatever the cause may be, can be detected with microsatellites.

Relatedness within single tree harvests

Average relatedness according to Queller and Goodnight (1989) and its standard deviation were estimated within five single tree harvests. Average relatedness was below 0.25, the value for a half-sibling relationship, when individuals 9b, 13f and 16c were included in the calculations. After 9b, 13f and 16c were omitted one by one, average relatedness increased to 0.32, suggesting half-sibling and full-sibling relationships among the remaining individuals (Fig. 2). Such family relationships were expected as seed parents may be pollinated by the same pollen donor more than once.

A random distribution was obtained by omitting 1 individual from the dataset randomly 100 times and recalculating average relatedness each time. The upper 95% confidence limit of the resulting distribution was at 0.24, while the values after omitting 9b, 13f or 16c were between 0.26 and 0.27. This suggests that the increase in average relatedness after omitting each of the 3 seedlings was not due to chance alone and that we had identified truly unrelated individuals using the methods described above.

Differentiation between single tree harvests

Differentiation between the five pure single tree harvests, after removal of the 3 unrelated seedlings, was estimated using F_{ST} according to Weir and Cockerham (1984). F_{ST} between the five harvests was 0.16 ($P < 0.005$), indicating significant differentiation. This F_{ST} value is higher than previously observed intra-population values of *Quercus robur* obtained with microsatellites (Streiff et al. 1998). As the five seedlot samples originate from one population, strong genetic differentiation suggests that they indeed represent distinct family structures. It may be expected, however, that differentiation decreases as more half-sib families of one population are analysed because this would result in an increased probability for some of the seed parents to act also as pollen donors.

In order to determine the number of different mother trees present in the samples we calculated pairwise F_{ST} between the five single tree harvests. Additionally, the maternal model half-sib family from the arboretum was divided into five groups of 5 seedlings each in order to simulate five single tree harvests from the same mother tree. F_{ST} was calculated between the ten samples to obtain a pairwise F_{ST} matrix. A principal component analysis (PCA) was conducted on the pairwise F_{ST} matrix, and the first three principal components were plotted (Fig. 3). Differentiation between the five commercial single tree harvests appeared to be pronounced, while differentiation between the five samples from the same mother tree was extremely weak. Separation of the latter samples was particularly weak on the first principal component which accounted for 56% of the total variance.

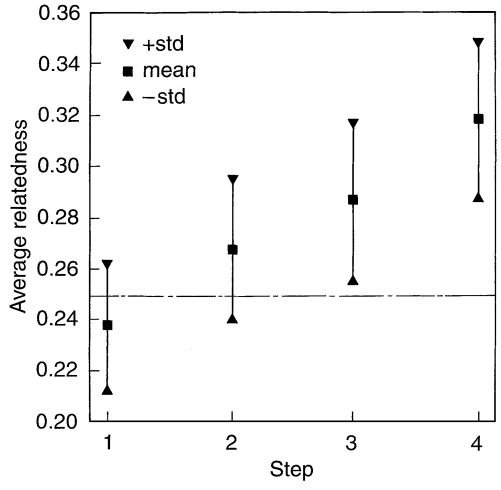

Fig. 2 Average relatedness within five samples from single tree harvests, calculated from 7 independent loci. Means and standard deviations were obtained by jackknifing over loci. *Step 1* Initial dataset, *step 2* after omitting 9b, *step 3* after omitting 9b and 13f, *step 4* after omitting 9b, 13*f*, and 16c. The *horizontal line* marks 0.25, the value for a half-sibling relationship

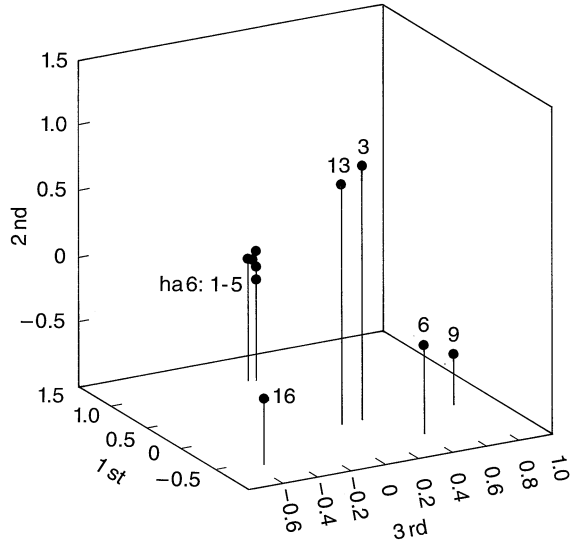

Fig. 3 Plot of the first three principal components of pairwise F_{ST} between ten samples. *3, 6, 9, 13* and *16* indicate samples from five different commercial single tree harvests, *ha6: 1–5* indicates five samples from the same mother tree, obtained by dividing the model half-sib family ha6. The first three principal components accounted for 56%, 24% and 11% of the total variance, respectively

The maternal genotypes have a strong effect on differentiation because the maternal alleles are present in each of the offspring individuals. Identical mother trees create low pairwise F_{ST} values, while different mother trees cause higher pairwise F_{ST} values. Our results suggest that pairwise F_{ST} may be a suitable first check for the number of different mother trees included in commercial seed harvests.

Reconstruction of the maternal genotypes

Applying the rules of codominant Mendelian inheritance as described above, we unequivocally identified both maternal alleles in 13 out of 45 locus/family combinations (9 loci, five families) and at least 1 maternal allele in 40 out of 45 locus/family combinations. Combining information from 9 loci we obtained a unique genotype for each mother tree. As expected there was a clear relationship between the polymorphism of a marker and its ability to infer the maternal alleles; the 2 less polymorphic markers ssrQpZAG 15 and ssrQpZAG 110 were the only markers that did not identify any complete maternal genotype. In general, the potential to reconstruct the maternal genotypes was smaller for the seedlot samples than for the model half-sib family, obviously due to sample size.

Correct genotype reconstruction was confirmed using markers that are closely linked on the genetic map of *Q. robur* (Barreneche et al. 1998). No recombinant genotype was observed between markers ssrQpZAG 46 and ssrQpZAG 36 for harvests 13 and 9 (Fig. 4A, B). Recombination frequency between these markers was 1.1% in the *Q. robur* mapping population (Lexer et al. unpublished). Only one recombinant was observed between ssrQpZAG 36 and ssrQpZAG 104 for harvest 13 (Fig. 4A), the recombination frequency in the mapping population being 4.4%. As these 3 markers are highly polymorphic, it is improbable that such combinations of alleles were obtained by chance alone. If an unexpectedly high number of recombinants is observed within a family, then a mistake during identification of the maternal alleles is probable. However, more data have to be collected on controlled crosses or open-pollinated families of *Q. robur* to cofirm the degree of conservation of the linkages.

Practical considerations

The use of polymorphic microsatellites has great potential for genetic analysis of seedlots from pedunculate oak. We have detected unrelated individuals within samples from single tree harvests, we have rejected or confirmed half-sibling relationships within the samples and we have shown that five samples indeed originated from five different mother trees. Pairwise F_{ST} and subsequent PCA may in practice serve as a quick check for the number of mother trees included in seed harvest. For those families that are not clearly differentiated the maternal genotypes may be reconstructed and directly compared. In cases where both maternal alleles have been identified, correct genotype reconstruction may be confirmed using linked microsatellites.

The methods outlined above proved to be suitable for small sample sizes. Still, five acorns may not be sufficient for genetic analysis, because single tree harvests may get contaminated with unrelated acorns. Furthermore, some acorns may not produce seedlings, thereby decreasing the number of available individuals

A

Individual	*QpZAG 46*		*QpZAG 36*		*QpZAG 104*		*Recombination*
a	203	206	219	221	202	206	0
b	186	203	217	219	204	206	0
c	203	223	219	221	206	222	0
d	194	214	213	215	200	202	0
e	191	194	213	223	198	200	0
g	203	223	219	221	200	222	1
h	203	203	219	221	206	222	0

B

Individual	*QpZAG 46*		*QpZAG 36*		*Recombination*
a	202	202	221	225	0
c	194	202	217	221	0
d	198	211	215	215	0
e	202	223	221	221	0
f	194	211	215	217	0

Fig. 4A, B Recombination between closely linked markers in two samples: **A** harvest 13, **B** harvest 9. Offspring individuals are arranged in rows, the genotypes at 3 loci (**A**) and 2 loci (**B**) are listed in columns (allele sizes in basepairs). Maternal alleles as inferred from the offspring genotypes are shaded *bright* and *dark*. Only one recombinant was observed between ssrQpZAG 36 and ssrQpZAG 104 in harvest 13. For harvest 9, locus ssrQpZAG 104 was not listed because the maternal alleles were not identified unequivocally. For details see text

further. The latter problem may be overcome by extracting DNA directly from the acorns as they are shipped. To overcome the first problem we suggest that in future the possibility of seed contaminations due to seed dispersing animals or other natural factors should be considered when legal regulations on sample size are worked out. Larger sample sizes would increase the chance that enough individuals are left for genetic analysis after removal of the unrelated seedlings. Besides, increasing the sample size would increase the potential to identify both maternal alleles and would therefore decrease the number of loci required.

In the present pilot study we have shown that the number of different seed parents can be inferred from a structured sample of seeds using microsatellite markers.

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