

Comparative study of genetic variation and differentiation of two pedunculate oak (*Quercus robur*) stands using microsatellite and allozyme loci

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In a comparative study four codominant microsatellite loci and seven allozyme gene loci have been used to investigate the genetic variation and differentiation of two pedunculate oak stands in North Germany. Both number and effective number of alleles were five to six times higher and the observed heterozygosity was three times higher for the microsatellite than for the allozyme loci. One stand showed an overall excess of homozygotes. In general the microsatellites were closer to Hardy–Weinberg expectation. The genetic distances between the two stands were distinctly higher for microsatellites. For most parameters microsatellites exhibited smaller interlocus variation than the allozymes. The different impact of population genetic processes on the genetic structure as assessed by microsatellites or allozymes is discussed.

Keywords: allozymes, forest trees, genetic distance, heterozygosity, permutation test, simple sequence repeats.

Introduction

Genetic variation and differentiation of the pedunculate oak (*Quercus robur* L.) have been analysed using different gene markers. In many cases allozymes have been applied (Müller-Starck *et al.*, 1993; Bacilieri *et al.*, 1994; Zanetto *et al.*, 1994; Herzog, 1996). As is the case for many other tree species, allozyme studies reveal a low level of genetic differentiation between populations (Müller-Starck & Ziehe, 1991). In contrast, genetic inventories using maternally inherited chloroplast DNA markers have shown distinct genetic differentiation and an obvious spatial genetic pattern throughout European white oaks (Dumolin-Lapègue *et al.*, 1997; Johnk & Siegismund, 1997; Ferris *et al.*, 1998). Recently, codominant nuclear microsatellites have been developed and characterized in the genus *Quercus* (Isagi & Suhandono, 1997; Steinkellner *et al.*, 1997; Kampfer *et al.*, 1998). These markers have been used to analyse spatial genetic patterns within populations, to perform parentage

analysis and to reconstruct maternal relatedness within small seedlot samples (Dow & Ashley, 1996; Streiff *et al.*, 1998; Lexer *et al.*, in press). It has been commonly observed in broad-leaved as well as in coniferous tree species that microsatellites are highly polymorphic markers, thus exhibiting a higher level of genetic variation compared to allozymes (e.g. in oaks: Dow & Ashley, 1996; Streiff *et al.*, 1998; in pine and Norway spruce: Echt *et al.*, 1996; Pfeiffer *et al.*, 1997). A comparative genetic analysis was conducted for microsatellite and allozyme loci within a mixed stand of *Quercus robur* and *Q. petraea* (Streiff *et al.*, 1998), revealing a high potential for spatial genetic differentiation at this scale. However, to our knowledge, nothing is known about the genetic differentiation among oak populations using microsatellite and other markers simultaneously.

In this paper we report on the combined application of allozymes and microsatellites in two different populations of pedunculate oak. The aim was to estimate genetic variation and differentiation and to compare the results obtained using these two different types of gene markers. Both markers are codominant and biparentally inherited (Zanetto *et al.*, 1996; Steinkellner *et al.*, 1997). However, as opposed to allozyme markers, microsatellite

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variation originates from variable numbers of noncoding simple sequence repeat units (Tautz, 1989). Population genetic processes governed by mating system, genetic drift and gene flow are expected to affect both types of markers in the same way leading to similar genetic structures. However, the impact of selection may be different. Hence a comparative analysis may allow different population genetic processes to be distinguished. As an illustration, random mating and absence of genetic drift will lead to Hardy–Weinberg equilibrium for neutral markers whereas selective markers may deviate from Hardy–Weinberg expectations.

Materials and methods

Study stands

The two stands are located in North Germany ≈40 km south-west of Lübeck. According to the names of the nearby villages they are called ‘Behlendorf’ and ‘Steinhorst’, respectively. The distance between the stands is about 15 km. Both sites represent mixed stands of *Fagus sylvatica*, *Quercus robur* and *Carpinus betulus*. The oaks were artificially regenerated using seeds and saplings of local origin. All adult oaks in both stands (‘Behlendorf’: $N = 228$, ‘Steinhorst’: $N = 85$) were sampled. According to the recorded history, the sampled trees in ‘Behlendorf’ are between 180 and 210 years old and the trees in ‘Steinhorst’ between 150 and 259 years old. For both microsatellite and allozyme analysis, winter buds were collected.

Microsatellites

DNA extraction Total DNA was isolated from the buds after removing the scales. Five to six buds were prefrozen in liquid nitrogen and the DNA extracted according to the minipreparation of Dumolin *et al.* (1995) with slight modifications, including a final treatment with 0.5 µg RNaseA (Boehringer Mannheim, Germany) at 37°C for 30 min.

Microsatellite analysis Four microsatellite loci were analysed and the sequence information of the relevant primer pairs was taken from Steinkellner *et al.* (1997). According to Steinkellner *et al.* (1997) the loci are coded ssrQpZAG36, ssrQpZAG1/5, ssrQpZAG9 and ssrQpZAG104. All four are characterized by variable numbers of (AG)-repeats. Furthermore they follow simple codominant Mendelian inheritance (Steinkellner *et al.*, 1997).

PCR amplification PCR amplification was carried out in a total volume of 25 µL containing about 20 ng of template DNA, 2.5 mM MgCl₂, 100 µM of each dNTP,

0.2 µM of each primer and 0.25 U of Taq polymerase with the respective 1× PCR buffer (Taq polymerase and 10× PCR buffer were purchased from Eurogentec, Ougree, Belgium), following the cycle profile described by Streiff *et al.* (1998). PCR was run in the Touch-Down™ Thermal System (Hybaid Limited, Teddington, UK).

Separation and staining of PCR products The PCR products were pretreated according to Streiff *et al.* (1998) and run in a 6% denaturing polyacrylamide gel (Rotiphor 40, 38:2 acrylamide:bisacrylamide, Pharmacia, Freiburg, Germany), using sequencing gel apparatus (S2 Gibco BRL, Life Technologies, Eggenstein, Germany). The gels were run in 1× TBE buffer adjusted to pH 8.3 at 2000 V for 2.5–3 h. Silver staining of the gels was performed according to Streiff *et al.* (1998). A standard was constructed using the DNA of probes with different alleles. This standard was run every 5 or 10 lanes on each gel. The allelic variation of each sample was assessed by comparison to this standard.

Allozymes

For allozyme analysis, crude proteins were extracted from winter buds. Extraction procedures and the composition of the electrode and gel buffers followed those of Hertel & Zaspel (1996). Stains were adapted from Yeh & O’Malley (1980), Vallejos (1983) and Cheliak & Pitel (1984).

From the enzyme systems studied seven polymorphic loci that showed simple Mendelian inheritance in controlled crosses (Zanetto *et al.*, 1996) were used: *Aap-b* (alanine aminopeptidase), *Pgi-b* (phosphoglucose isomerase), *Mnr* (menadione reductase), *6pgdh-b* (6-phosphoglucose dehydrogenase), *Idh-b* (isocitrate dehydrogenase), *Pgm* (phosphoglucose mutase) and *Acp-c* (acid phosphatase). *Aap-b*, *Acp-c* and *Pgm* were monomeric, *6pgdh-b*, *Idh-b* and *Pgi-b* were dimeric and *Mnr* was tetrameric.

Data analysis

For each locus, allele frequencies (p_{ij}), the number of different alleles (A), observed heterozygosity (H_o), expected heterozygosity (H_e), the effective number of alleles ($A_e = 1/(1 - H_e)$), and the fixation index ($F = 1 - (H_o/H_e)$) were calculated as described by Weir (1990).

Two different genetic distances were applied to quantify genetic differentiation: D_G (Gregorius, 1978) and D_N (Nei, 1972):

$$D_G(i, j) = \frac{1}{2} \cdot \sum_{k=1}^n |p_{ik} - p_{jk}| \quad \text{and}$$

$$D_N(i, j) = -\ln \left(\frac{\sum_{k=1}^n (p_{ik} \cdot p_{jk})}{\sqrt{\sum_{k=1}^n p_{ik}^2 \cdot \sum_{k=1}^n p_{jk}^2}} \right),$$

where i and j represent two populations, n is the number of alleles and p_{ik} is the relative frequency of the k th allele in the i th population. Additional information is obtained by the measure D_G , as it may be used to compute the genetic distance between the genotype frequencies of two populations.

To estimate the significance of F , A_e , D_G and D_N , numerical tests were performed on the basis of Monte Carlo methods (Manly, 1991). To test the significance of the F -values for each population and each locus, 1000 permutations of the genotypes were run. Each permutation leads to a new random association of the alleles of the respective population (resampling without replacement). After each permutation the F -values were recalculated and compared to the observed values. The relative frequency of those cases leading to less extreme F -values than the observed values were used to estimate the probability of significant deviation from the Hardy–Weinberg expectation.

One-thousand permutations, with random resampling of single-locus genotypes from both populations, were carried out to test for significant differences of A_e between the two populations. After permutation the genotypic structure of each population represents a random mixture of genotypes from both original populations. The A_e values were calculated for both resampled populations (i and j). Within each permutation the mathematical difference (Dif) between the A_e values from the two resampled populations was computed ($Dif_{ij} = A_e(i) - A_e(j)$). The permutations led to a certain distribution of the pairwise differences (Dif). The relative frequency of those cases with a less extreme difference (Dif) than that observed was used to estimate the probability of a significant difference of the A_e values between the two stands. In the same way the significance of the genetic distances, D_G and D_N , was tested. After each permutation D_G and D_N were recalculated and compared to the original values. The relative frequency of those cases with distances smaller than the observed was used to estimate the probability of significance.

The significance of mean parameters (difference A_e , F , D_G and D_N) was determined according to the testing procedure for the single loci. For each permutation the means were recalculated and compared to the mean of the original data. The relative frequency of those cases with means smaller than the observed was used to estimate the probability of significance.

The coefficient of variation (CV = standard deviation/arithmetic mean) was calculated to quantify the interlocus variation of the parameters.

Results

Genetic variation and differentiation results are summarized in Tables 1 and 2. As shown by the sample sizes in Table 1, not all of the sampled individuals provided microsatellite or allozyme data.

Genetic variation

On average the microsatellite loci had five to six times more alleles (A), as well as effective number of alleles (A_e), than the allozyme loci (Table 1). The observed heterozygosity (H_o) was three times greater for microsatellites than for allozymes. For both categories of markers, the 'Behlendorf' stand was characterized by higher values of genetic variation (A and A_e). At locus *Pgi-b* the difference in the effective number of alleles (A_e) between the two stands was significant. In general the observed heterozygosity (H_o) was similar between the two populations for both categories of markers. The mean observed heterozygosity (H_o) for the allozymes was slightly greater in the 'Steinhorst' stand than in the 'Behlendorf' stand. In 'Behlendorf' the mean fixation index (F) was significantly positive for the allozymes and significantly negative for the microsatellites. In 'Steinhorst' the mean fixation indices (F) for allozymes and microsatellites were not significantly different from Hardy–Weinberg expectation. However, two single-locus F -values showed deviations from Hardy–Weinberg expectation: the fixation index for the locus *ssrQpZAG1/5* was highly significantly negative, whereas it was significantly positive for the locus *Aap-b*. The overall means including both categories of markers revealed a significant positive deviation from Hardy–Weinberg expectation for 'Behlendorf', whereas 'Steinhorst' was in equilibrium. As revealed by the mean fixation index (F), the microsatellites were closer to Hardy–Weinberg expectation than the allozymes. The coefficients of variation (CV) showed smaller interlocus variation of A_e , H_o and F for the microsatellites than for the allozymes.

Genetic differentiation

At allozyme locus *Pgm* and at the microsatellite locus *ssrQpZAG9* the genetic distance D_G , based on the genotype distribution between 'Behlendorf' and 'Steinhorst', was statistically significant (Table 2). There were no significant genetic distances between the allelic distributions. The gene pool distance D_G was 4.4 and

Table 1 Sample size (N), number of alleles (A), effective number of alleles (A_e), observed heterozygosity (H_o) and fixation index (F) for the oak stands 'Behlendorf' (Behl.) and 'Steinhorst' (Stein.) at 11 *allozyme* (*italic*) and microsatellite loci; probability of significant difference in A_e (Prob. *Dif*) between the two stands; probability for test of significance for deviation from Hardy–Weinberg expectation (Prob. F); coefficient of variation (CV = standard deviation/ arithmetic mean)

Locus	N		A		A_e			H_o		F			
	Behl.	Stein.	Behl.	Stein.	Behl.	Stein.	Prob. <i>Dif</i>	Behl.	Stein.	Behl.	Prob. F	Stein.	Prob. F
<i>Aap-b</i>	223	83	4	4	2.85	2.98	0.876	0.574	0.566	+0.117	0.987	+0.149	0.966
<i>Pgi-b</i>	228	84	4	2	1.28	1.15	0.971	0.206	0.119	+0.081	0.899	+0.098	0.790
<i>Mnr</i>	228	85	4	4	1.21	1.18	0.650	0.180	0.165	-0.049	0.851	-0.061	0.825
<i>6pgdh-b</i>	224	85	3	2	1.07	1.07	0.448	0.063	0.071	+0.095	0.870	-0.037	0.738
<i>Idh-b</i>	222	85	2	2	1.64	1.63	0.530	0.338	0.412	+0.139	0.967	-0.058	0.704
<i>Pgm</i>	207	83	3	4	2.08	2.03	0.673	0.319	0.506	+0.387	1.000	+0.004	0.515
<i>Acp-c</i>	201	85	3	2	1.54	1.54	0.528	0.313	0.341	+0.108	0.893	+0.035	0.655
ssrQpZAG36	213	85	17	13	7.90	7.85	0.313	0.878	0.882	-0.005	0.634	-0.011	0.664
ssrQpZAG1/5	219	80	15	12	7.25	6.57	0.675	0.895	0.938	-0.038	0.948	-0.106	0.995
ssrQpZAG9	210	83	14	11	7.62	6.65	0.797	0.876	0.843	-0.009	0.681	+0.007	0.507
ssrQpZAG104	214	81	33	28	13.40	12.64	0.394	0.944	0.921	-0.020	0.906	+0.008	0.523
Mean (allozymes)			3.28	2.85	1.66	1.65	0.880	0.285	0.311	+0.125	1.000	+0.019	0.677
Mean (microsatellites)			19.75	16	9.04	8.42	0.836	0.898	0.894	-0.018	0.960	-0.025	0.941
Mean (total)			9.25	7.63	2.16	2.10	0.814	0.509	0.523	+0.073	1.000	+0.003	0.483
CV (allozymes)			0.21	0.34	0.34	0.37		0.52	0.58	0.96		4.02	
CV (microsatellites)			0.39	0.43	0.27	0.29		0.03	0.04	0.71		1.84	

Table 2 Genetic distance D_G (Gregorius, 1978) and genetic distance D_N (Nei, 1972) between the distributions of genotypes and allele frequencies of the oak stands 'Steinhorst' and 'Behlendorf' for 11 *allozyme* (*italic*) and microsatellite loci; CV, coefficient of variation (CV = standard deviation/arithmetic mean)

Locus	Distribution of genotypes		Distribution of alleles			
	D_G	Probability	D_G	Probability	D_N	Probability
<i>Aap-b</i>	0.093	0.187	0.055	0.489	0.006171	0.505
<i>Pgi-b</i>	0.097	0.899	0.054	0.915	0.000897	0.753
<i>Mnr</i>	0.030	0.122	0.015	0.174	0.000086	0.113
<i>6pgdh-b</i>	0.013	0.203	0.002	0.040	0.000004	0.040
<i>Idh-b</i>	0.074	0.653	0.003	0.083	0.000015	0.083
<i>Pgm</i>	0.232	0.998	0.105	0.933	0.018098	0.915
<i>Acp-c</i>	0.033	0.178	0.006	0.053	0.000034	0.065
ssrQpZAG36	0.366	0.277	0.150	0.842	0.035364	0.746
ssrQpZAG1/5	0.410	0.774	0.141	0.847	0.031069	0.779
ssrQpZAG9	0.433	0.961	0.150	0.895	0.053617	0.944
ssrQpZAG104	0.616	0.203	0.166	0.304	0.034050	0.202
Gene pool (<i>allozymes</i>)	—	—	0.034	0.493	0.002748	0.700
Gene pool (microsatellites)	—	—	0.151	0.906	0.039396	0.880
Gene pool (total)	—	—	0.077	0.793	0.006197	0.808
CV (<i>allozymes</i>)	0.83		1.04		1.73	
CV (microsatellites)	0.20		0.05		0.22	

the gene pool distance D_N was 14.3 times greater for microsatellites than for allozymes. As indicated by the coefficients of variation (CV), the interlocus variation of the genetic distances was clearly higher for the allozymes than for the microsatellites. For the loci *Pgi-b* and *ssrQpZAG104*, and for the gene pool of allozymes, the calculated probabilities of the two distance measures (D_G and D_N) differed by more than 10%. In all other cases the differences of the probabilities were less than 10%. Both distance measures seem to be equally sensitive for detecting genetic differences.

Discussion

DNA polymorphisms have increasingly become validated as markers in the population genetics of forest trees, and thus comparative analyses allow the application of different categories of markers to the same populations. To evaluate different population genetic processes, different genetic markers may be useful, depending on their origin in coding or noncoding DNA regions, on differences in the mutation rates and processes, on their mode of transmission, and on the degree of resolution of the detection technique. In our study, we simultaneously applied two different categories of biparentally inherited codominant genetic markers, one originating from coding sequences (allozymes) and the other from sequences of the nuclear genome that, as is common for microsatellites, are of a noncoding nature (Tautz, 1989). In this study, as well as in previous studies, null alleles have so far not been observed at any of the four

microsatellite loci here analysed (H. Steinkellner, pers. comm.).

In accordance with a previous study (Streiff *et al.*, 1998) we found a higher level of variation for microsatellite than for allozyme gene loci. For a stand of *Quercus robur* in north-western France, Streiff *et al.* (1998) found a mean number of alleles $A = 19.75$ and a mean effective number of alleles $A_e = 9.95$, analysing the same microsatellite loci as in the present study. The values assessed in the French stand were four to six times higher for the microsatellite loci than for the allozyme loci. The genetic variation of the French stand, based on microsatellite loci, is very similar to that for the 'Behlendorf' stand.

The ranking of genetic variation (A and A_e) between the two stands, 'Behlendorf' and 'Steinhorst', is the same for both categories of gene markers; the differences were greater for microsatellites. Only for one allozyme locus was the difference of A_e between the stands statistically significant. The lower sample size in the 'Steinhorst' stand may be a simple explanation for the lower values of genetic variation compared to 'Behlendorf'. Because of the greater number of low frequency alleles, microsatellites are expected to be more sensitive to differences in sample size; sampling error is assumed to be higher (Gregorius, 1980; Krusche & Geburek, 1991). Hence genetic variation differences between populations based on microsatellites may fail to be statistically significant. However, bottleneck effects in population history may be easier to detect for highly variable loci (Aldrich *et al.*, 1998).

The mean fixation index (F) for all 11 loci was significantly positive in the 'Behlendorf' stand. In contrast, the mean value of 'Steinhorst' did not show significant deviation from Hardy–Weinberg expectation. Inbreeding and the Wahlund effect could have caused the significant excess of homozygotes in 'Steinhorst'. In both stands the mean F -values of the allozymes were positive (excess of homozygotes) and the values for the microsatellites were slightly negative (excess of heterozygotes). If the mating system were the only process responsible for deviation from Hardy–Weinberg equilibrium, we would expect F -values for all biparentally inherited codominant genetic markers to show the same trends (Wright, 1965). In some cases heterozygosity may be slightly overestimated for microsatellites, because of uncertainties in assigning the status of homozygotes when slippage bands interfere with the main 'allelic' bands. Problems arising from misinterpretation of slippage bands have been reported in the literature several times (e.g. Perlin *et al.*, 1995). This misinterpretation could explain the negative F -values of microsatellites. Streiff *et al.* (1998) also report higher F -values for allozymes than for microsatellites in an oak population.

The genetic distances, D_G and D_N , between both populations are clearly higher for microsatellites than for allozymes, but the higher distances did not include more significant differences. As for most of the other parameters (see A_e , H_o) the interlocus variation of the distances D_G and D_N is smaller for the microsatellites than for allozymes. Therefore, allozymes are more likely to reveal the impact of different and contrasting population genetic processes, whereas microsatellite variation reflects less interaction of population processes. The main difference may be the impact of selection, which could be stronger for allozymes. For tree populations, many studies have described the effect of selection on allozymes (e.g. Kim, 1985; Müller-Starck, 1985; for review see also Mitton, 1995). In contrast, microsatellites are supposed to be unaffected by selection, as long as they are not 'hitchhiking' with, or in sequences that are subjected to selection (e.g. Jarne & Lagoda, 1996).

The combined application of different gene markers, such as allozymes and microsatellites, is helpful to distinguish the impact of the various population genetic processes. In particular, a combination of 'neutral' and 'selective' markers helps to understand better the role of the mating system, selection and bottleneck effects in determining genetic structure. As demonstrated by our study, numerical tests using Monte Carlo methods provide powerful statistical tests for highly variable markers. In contrast to other statistics like the G -test or χ^2 -test, no distributional assumptions are required for these numerical tests.

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