GENETIC DISTANCE AND TRANSMISSION OF GENETIC INFORMATION AT RAPD AND SSR LOCI IN REGULARLY AND LATE BUDDING PEDUNCULATE OAK (*QUERCUS ROBUR* L.)

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ABSTRACT

One late budding and four regularly budding stands of pedunculate oak (*Quercus robur* L.) which were planted in the 19th century in the south-west of Germany were investigated with respect to their genetic variabilities and genetic distances at 36 RAPD (random amplified DNA) and 28 SSR (simple sequence repeat) loci. The late budding stand and one of the regularly budding stands are licenced for seed harvesting. Offspring of these stands were included in the investigations.

Among the seven populations, subpopulation differentiation – based on the Tanimoto-distance – was largest for one of the regularly budding stands as was the expected heterozygosity. On the other hand, subpopulation differentiation and expected heterozygosity were lowest for offspring of the late budding stand.

At seven loci in offspring of the late budding stand and at four loci in offspring of the regularly budding stand, locus frequencies deviated from the corresponding parental population by more than 30 %. Subpopulation differentiation in offspring of the late budding population and the regularly budding population were respectively 3.51 % and 1.46 % larger than in the corresponding parental population. The mean expected heterozygosity in offspring of the late budding parent stand and the regularly budding stand decreased by 5.36 and 2.93 %. The different transmission of genetic information in the regularly and the late budding population is discussed in terms of different adaptational processes and seed management.

Key words: budding, heterozygosity, offspring, pedunculate oak, population genetics, *Quercus robur* L., Slovenian pedunculte oak, Tanimoto distance

INTRODUCTION

Since the Middle Ages pedunculate oak (*Quercus robur* L.) was often planted in Germany, regardless of its habitat demands (RÖHRIG & GUSSONE 1980). One of the reasons was that a considerable part of its natural habitat, especially river valley woodlands, had been destroyed. In the Southwest of Germany, in the state of Rhineland-Palatinate, pedunculate oak is therefore mostly found outside its original habitat. When these stands were created at the end of the 19th century they were mostly planted with seedlings of unknown origin. On the other hand, on some elevated areas of the Eifel hills, seeds of late budding varieties, imported from Slovenia, were used to establish pedunculate oak woods. These populations bud one to three weeks later than indigenous ones, which start budding at the beginning of May. The Slavic varieties were planted because they are resistent to late frosts and several parasites such as oak Tortrix species and winter moth species. Some of the regularly budding and the late budding stands are currently licensed for seed harvesting. In years of abundant fructification acorns are collected from these stands and seedlings raised for two to three years in a nursery before they are used for reforestations.

This type of forest management raises some questions about the genetics of these artificially established populations. For example: how large are their genetic variations and distances and how much genetic information is transmitted from parent to offspring populations?

We tried to answer these questions through the use of RAPD (random amplified polymorphic DNA) and SSR (simple sequence repeat) markers. RAPD markers have been applied to the study of several tree species (AIDE & RIVERA 1998, ALLNUTT *et al.* 1998, CHALMERS *et al.* 1994, GILLIES *et al.* 1999, YEH *et al.* 1995) including oak (PETIT *et al.* 1993). Nuclear microsatellite markers were studied by applying AMP (anchored microsatellite primed) PCR (polymerase chain reaction) technology (ZIETKIEWICZ *et al.* 1994). Both markers are dominant markers but they directly assess DNA variation without any need for DNA sequencing information (NEWTON *et al.* 1999, WINTER & KAHL 1995).

MATERIALS AND METHODS

Stand locations

Investigations were performed on pedunculate oak in the state of Rhineland-Palatinate (Rheinland-Pfalz), Southwest-Germany. Trees were planted in the 19th century and are growing in the forest districts of Hagenbach, Mayen, Simmern, Speyer and Trippstadt (Table 1, Figure 1). At each stand, 50 oaks which had been classified before according to their leaf and fruit traits as morphologically pure pedunculate oak (*Quercus robur* L.) (ROMMEL *et al.* 1995) were probed. In addition, 50 offspring, each at the age of eight years were studied, which originated from the investigated stands in the forest districts of Hagenbach and Mayen which are licenced for seed harvesting. The offspring are part of a provenance trial which had been established at forest district Neupfalz (Figure 1, Table 1).

Mode of sampling

Short twigs, with recently flushed leaves, were shot from the trees, transported to the lab and kept in water until extracted within the next two days. From the samplings young leaves were taken and stored between wet filter papers at 8 °C and extracted within three days.

DNA extraction

Leaves were washed with distilled water and dabbed between layers of filter paper. DNA was extracted by a modified version of DELLAPORTA *et al.* (1983) and Doyle (1991): Approximately 1 gram of fresh leaves was ground under liquid nitrogen in a mortar together with 100 mg of dry Polyclar AT (insoluble polyvinylpyrrolidone). The resulting leaf powder was mixed with 5 ml of extraction medium contained in a 12 ml polypropylene centrifuge tube and heated in a water bath for 1 h to 65 °C, inverting the tube from time to time. The extraction medium consisted of 2 % (w/v) hexadecyltrimethylammoniumbromide, 1.4 M NaCl, 20 mM EDTA-Na_2 and 1 % (w/v) PVP-40 in 100 mM Tris, adjusted to pH 8.0 with HCl; shortly before use, 0.2 % (v/v) 2-mercaptoethanol was added to the me-

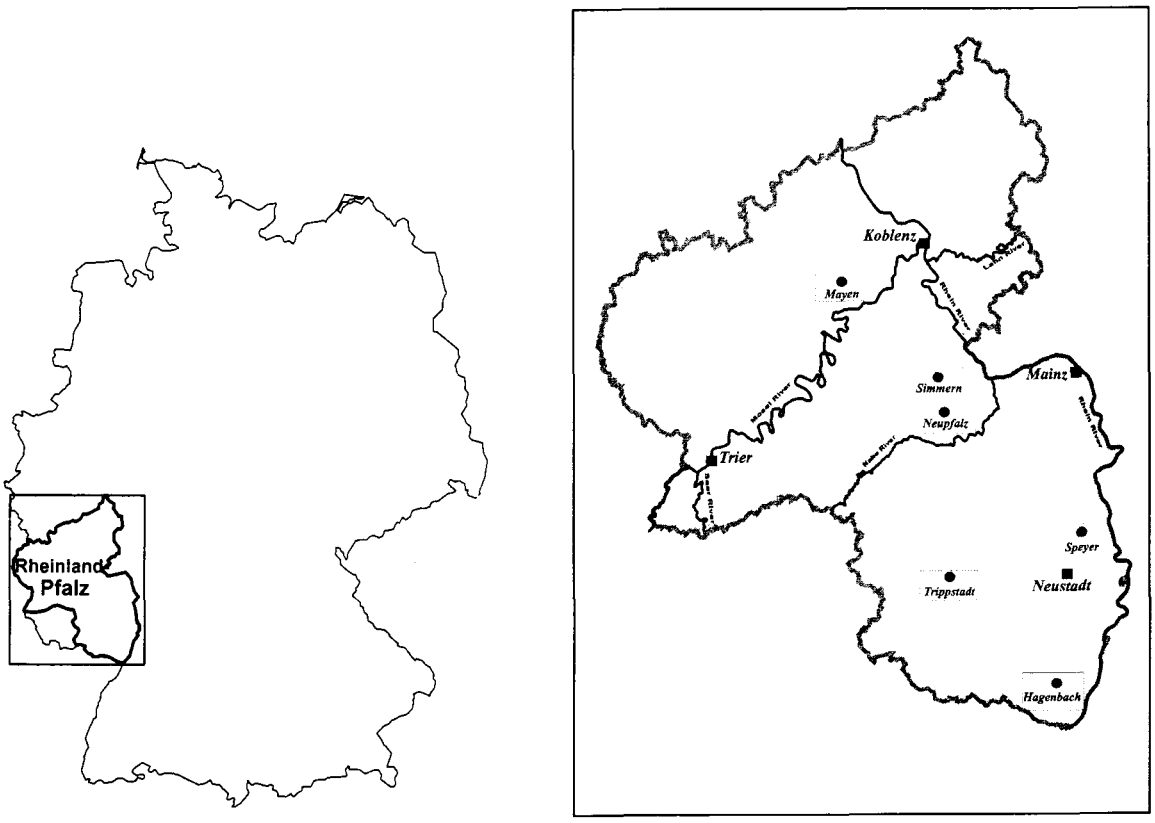

Figure 1. Upper part: Outline map of Germany including the borderlines of the state of Rhineland-Palatinate. Lower part: Outline map of Rhineland-Palatinate including the positions of the main rivers and the cities of Koblenz and Mainz. The locations of the pedunculate oak populations investigated are framed. The regularly budding stands are located in the forest districts Hagenbach, Simmern, Speyer and Trippstadt, the late budding stand is growing in the forest district Mayen. Saplings of the mother stands Simmern and Mayen are growing in the forest district Neupfalz (for further details see Table 1).

Table 1. Characterization of the seven pedunculate oak (*Quercus robur L.*) populations studied in the state of Rhineland-Palatinate, south west Germany.

Location					Age of trees (years 2001)
Forest region	Forest district	Sub-district	Stand	Altitude a.s.l. (m)	
Palatinate Rhine valley	Hagenbach [a]	Streitwald, south	VI 2 a [2]	120	177
Eifel	Mayen [b]	Graf von Eltz [d]	Abt. 28 C[1]	240–260	92
Hunsrück	Simmern [c]	Argenthal	13 a	430	196
Palatinate Rhine valley	Speyer [c]	Mutterstadt	17 [1]	100	123
Palatinate Forest	Trippstadt [a]	Langeck, Schmetzeck	XI 15 b [2] XI 6 a [3]	410	170
Hunsrück	Neupflaz [a]	Thiergarten	137 3 [e]	420	12 [g]
Hunsrück	Neupflaz [a]	Thiergarten	137, 7 [f]	420	12 [g]

[a] state forest, [b] private forest, [c] community forest, [d] late budding provenance imported as seeds from Slovenia, [e] offsprings of the stand investigated at forest district Simmern, [f] offsprings of the stand investigated at forest district Mayen, [g] the offsprings were 8 years old when investigated, they are part of a provenance trial established at forest district Neupfalz. In the sub-district Langeck 33 oak trees were probed while in the sub-district Schmetzeck short twigs with young leaves were sampled from 16 trees. At all other places 50 trees were probed.

dium. Afterwards, 1 ml of aqueous 5 M ammonium acetate solution and 6 ml of chloroform / isoamylalcohol (24 : 1 (v/v)) were added and the tube inverted several times. The mixture was centrifuged at 6 000 x g for 10 min (Sorvall RC-5B centrifuge) at RT. The upper aqueous phase, containing the DNA, was carefully taken off and transferred into another 12 ml centrifuge tube. Afterwards, 1 vol (v/v) of chloroform / isoamylalcohol (24:1 (v/v)) was added to further free the DNA from impurities, inverted several times and then centrifuged (as above). The aqueous phase was transferred to another 12 ml centrifuge tube, 2/3 vol (v/v) of cold (–20 °C) isopropanole added and the tube, carefully inverted, several times. Then it was stored for 30 min at –20 °C and centrifuged for 3 min at 10 000 x g and 4 °C. The supernatant was decanted, the pellet dried for 30 min in vacuum at RT and finally suspended in 1 ml of TE buffer (10 mM Tris, 1 mM EDTA-Na_2, adjusted to pH 8.0 with HCl). After addition of 3.3 µl RNase A (3 µg/µl, Sigma) the suspension was incubated for 60 min at 37 °C and 1 ml of 5 M ammonium acetate in distilled water and 2.5 vol of pre-chilled (–20 °C) ethanol added. The DNA was precipitated after several careful inversions of the centrifuge tube and sedimented by centrifugation at 10 000 × g for 10 min at 4 °C. The supernatant was decanted, the DNA precipitate washed with 3 ml of 80 % (v/v) aqueous ethanol, and centrifuged as before. The supernatant was decanted and the precipitate dried for 30 to 60 min in vacuum at RT. Finally the DNA was suspended in 500 µl TE-buffer and stored at 4 °C (or –20 °C) until used.

Estimation of the concentration and purity of DNA

The DNA concentration of leaf extracts was estimated photometrically according to the equation: c_{DNA} [µg/ml] = $(A_{260} - A_{320}) \times 50 \times$ diluting factor (SAMBROCK *et al.* 1989). DNA purity was determined from the ratio $A_{260} - A_{320}/A_{280} - A_{320}$ (SAMBROCK *et al.* 1989). The presence of high molecular weight DNA was determined by agarose electrophoresis and only extracts containing high molecular DNA were used for PCR reactions. Before electrophoresis DNA extracts were diluted to 1 µg/5 µl. To 5 µl extract 2.5 µl loading buffer (5 M urea and 0.02 % Xylene cyanole, BASSAM *et al.* 1991) was mixed and 5 µl of this mixture loaded per gel trough. Electrophoresis was performed for 90 min at RT and 100 V on 0.8 % agarose gels in TBE buffer (45 mM Tris, 40 mM boric acid, 1.25 mM EDTA-Na_2, pH 8.4). As size marker a Hind III restricted DNA of λ–phage (600 µg/ml, Eurogentec, Seraing, Belgium) was used. DNA extracts were stored at 4 °C, reagents at –20 °C.

PCR (polymerase chain reaction) procedure

The polymerase chain reaction (PCR) was performed in 15 ml reactions containing 7.5 µl of mastermix and 7.5 µl of DNA extract (10 ng/µl). Thermal cycling was done in a programmable heating block (MJ Research, type PTC-200, Watertown, Mass., USA). Reactions were started at 94 °C for 4 min, continued with 40 cycles at 94 °C for 45 sec, 36 °C for 45 sec (primer number 26, 28 and 29) or 46 °C for 45 sec (primer number 50 and 51) and 72 °C for 90 sec and terminated at 72 °C for 5 min.

The master mix consisted of 1.5 µl $MgCl_2$ (25 mM),

1.2 µl sterile double distilled water, 1.5 µl of dNTPs (2 mM each of dATP, dCTP, dGTP and dTTP), 1.5 µl Stoffel-buffer (100 mM KCl, 100 mM Tris, adjusted with HCl to pH 8.3), 0.3 µl DNA polymerase (10 U/µl, Stoffel fragment, Perkin Elmer/Cetus, Langen, Germany) and 1.5 µl primer (30 µM).

Primer sequences

Of several RAPD primers used to amplify oak DNA (MOREAU 1993, MOREAU *et al.* 1994) three were used in this study: primer number 26 (5´-ACGGATCCTG-3´), number 28 (5´-ACGGTACCAG-3´) and number 29 (5´-TGCTGCAGGT-3´) (WERNER *et al.* 1997). Of several anchored simple sequence repeat primers (ZIETKIEWICZ *et al.* 1994, WEISING *et al.* 1991) two brought interpretable DNA patterns: number 50 (5´-GTG(GATA)$_4$-3´) and number 51 (5´-GGA(GATA)$_4$-3´). Primers were obtained from GENterprise, University of Mainz.

Agarose electrophoresis

Amplified DNA fragments were separated and visualized by electrophoresis in 1.2 % agarose gels in TBE buffer (45 mM Tris, 40 mM boric acid, 1.25 mM EDTA-Na$_2$), pH 8.4. A sample of 7 µl amplified DNA was mixed with 3.5 µl of loading buffer consisting of 5 M urea and 0.02 % Xylene cyanole and 8 µl loaded per slot. As molecular marker a "Biomarker Low standards" comprising DNA fragments of 50, 100, 200, 300, 400, 500, 525, 700 and 1000 bp (600 µg/ml, BioVentures, Eurogentec, Darmstadt, Germany) was used. The marker was mixed with an equal volume of loading buffer and 8 µl applied per slot. Electrophoresis was performed for 4 h under a field strength of 100 V at RT. DNA bands were stained for 30 min in ethydiumbromide (5 µg/ml) and destained for 30 min in tap water. Gels were photographed through a yellow filter under UV light (302 nm) using a Polaroid 667 film.

Evaluation of DNA-patterns

Photographs were used for analysis of the amplification products. The length of a DNA-amplification product was calculated from its position in the agarose gel relative to the standardised scale of DNA-marker bands. Amplification was repeated at least twice and the coarseness of each band determined. Only the bands reproducible in multiple runs, regardless of their intensity, were utilized in the survey (Table 2).

Quantification of genetic variation

Once RAPD- and AMP-loci were noticed in all investigated individuals the presence (1) or absence (0) of phenotypes was entered into a 1/0-matrix. A RAPD-/AMP-locus was considered to be polymorphic if its phenotypes varied.

The genetic difference between two populations was taken as equivalent to the difference in their RAPD or AMP patterns and quantified by the Tanimoto distance, δ_T, using the computer programm „CoTrix, a computer

Table 2. Fragment length of 64 loci, amplified with three RAPD primers (number 26, 28 and 29) and two AMP primers (number 50 and 51).

Locus/fragment length (bp)				
no. 26	no. 28	no. 29	no. 50	no. 51
A (120±10)	A (120±10)	A (200±10)	A (220±10)	A (220±10)
B (180±10)	B (180±10)	B (270±10)	B (260±10)	B (260±10)
C (230±10)	C (220±10)	C (350±10)	C (350±10)	C (300±10)
D (300±10)	D (280±10)	D (400±15)	D (400±10)	D (350±10)
E (370±10)	E (330±10)	E (470±15)	E (430±10)	E (400±10)
F (400±10)	F (390±10)	F (600±15)	F (470±10)	F (430±10)
G (430±10)	G (440±10)	G (680±15)	G (500±10)	G (470±10)
H (470±10)	H (520±15)	H (830±20)	H (580±15)	H (500±10)
I (520±15)	I (600±15)	I (900±20)	I (630±15)	I (580±10)
J (620±15)	J (660±20)	J (950±20)	J (700±15)	J (630±10)
K (650±15)	K (740±20)	K (1050±20)	K (750±15)	K (680±10)
L (730±20)	L (850±20)		L (930±20)	L (700±10)
M (860±20)			M (1000±20)	M (750±15)
				N (930±15)
				O (1000±20)

programme for analysis of DNA banding patterns, version 2.0" (DEGEN 1996).

Relative frequencies of homozygous null genotypes (0/0) were calculated and then used to determine allele frequencies under the assumption that RAPD and AMP genotypes are two allelic systems and that genotypes are distributed according to Hardy-Weinbergs´ theorem (STEPHENS *et al.* 1992, LYNCH AND MILLIGAN 1994, DAWSON *et al.* 1995). Accordingly, frequencies of the null allele (p) resulted from $p = \sqrt{0/0}$ and those of the one allel (q) from $q = 1 - p$. Allel frequencies were calculated by use of the computer program „Population Genetic Analysis" (PopGene, Version 1.21, YEH *et al.* 1997).

Expected heterozygosities (H_I), (H_e) and (H_s) were calculated by use of the computer program PopGene, version 1.21 (YEH *et al.* 1997) as were Nei´s total genetic diversity (H_T), diversity within populations (H_s), diversity between populations (D_{ST}) and the coefficient of the genetic diversity (G_{ST}) (NEI 1973, 1987).

Cluster analyses were performed following the unweighted pair-group method with arithmetic mean (UPGMA) (SNEATH & SOKAL 1973, FERGUSON 1980).

RESULTS

Amplification success

On average 88 % (between 50 to 96 %) of all investigated pedunculate oaks resulted in clearly identifiable DNA patterns. Amplification of random DNA was primer dependent and population specific. The least amplification success was obtained with trees of the Trippstadt population (RAPD: 68 %, AMP-PCR: 52 %), the best success was obtained with individuals of the offspring of the stands Mayen and Simmern (RAPD: 93 %, AMP-PCR: 93 %).

Number and size of amplified DNA fragments

By use of primers number 26, 28 and 29, at maximum 13, 12 and 11 DNA bands could be accessed. Their sizes ranged from 1050 to 120 base pairs (Table 2). When using the anchoring microsatellite primers No. 50 and 51, a maximum of 13 and 15 DNA bands could be accessed, respectively. Their fragment length ranged from 1000 to 220 bp (Table 2).

Polymorphic loci

Of the 64 loci which were accessible on average 87 % were polymorphic. Of the RAPD primer loci 64 to 100 % and of the AMP primer loci 69 to 100 % proved to be polymorphic. The number of polymorphic loci decreased in the two offspring populations as compared to their parent populations. The decrease was 6 % in offspring of the regularly budding population Simmern while it was 8 % in offspring of the late budding population Mayen.

Tanimoto distances

The Tanimoto-distance, which can be applied to dominant DNA markers like RAPD and AMP markers, was used to calculate the sub-population differentiation which equals the genetic difference between a certain sub-population and the complement of the remaining sub-populations.

The average sub-opulation Tanimoto-distance was exclusively related to the five adult populations, δ_T = 46.18 %. The smallest distance was observed for the regularly budding stand Trippstadt (δ_T = 44.71 %), the largest distance was calculated for the late budding stand Mayen (δ_T = 47.42 %). (At a value of 100 % all individuals would be unequal.)

Table 3. Tanimoto-distances of the 7 pedunculate oak populations under study in relation to the remaining complement of the six populations (sub-population Tanimoto-distances).

Population	Average sub-population Tanimoto-distance at the gene cluster							
	no. 26	no. 28	no. 29	Mean	no. 50	no. 51	Mean	Grand mean
Hagenbach	0.5792	0.4097	0.3802	0.4564	0.5786	0.3629	0.4708	0.4621
Mayen	0.5071	0.4362	0.4928	0.4787	0.5187	0.4030	0.4609	0.4716
Mayen-offspring	0.5213	0.4436	0.4257	0.4635	0.4408	0.3510	0.3959	0.4365
Simmern	0.5040	0.4263	0.4385	0.4562	0.4612	0.3814	0.4213	0.4423
Simmern-offspring	0.5490	0.4785	0.4375	0.4883	0.4205	0.3990	0.4098	0.4569
Speyer	0.5420	0.3950	0.4836	0.4735	0.5492	0.4093	0.4793	0.4758
Trippstadt	0.5557	0.4442	0.4082	0.4694	0.4343	0.3800	0.4072	0.4445
Grand mean	0.5369	0.4334	0.4366	0.4694	0.4862	0.3839	0.4350	0.4554

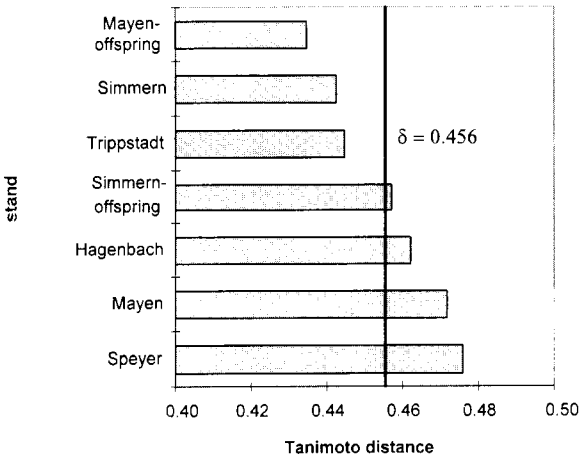

Figure 3. Tanimoto sub-population distances related to the 64 loci investigated. : average of all seven distances.

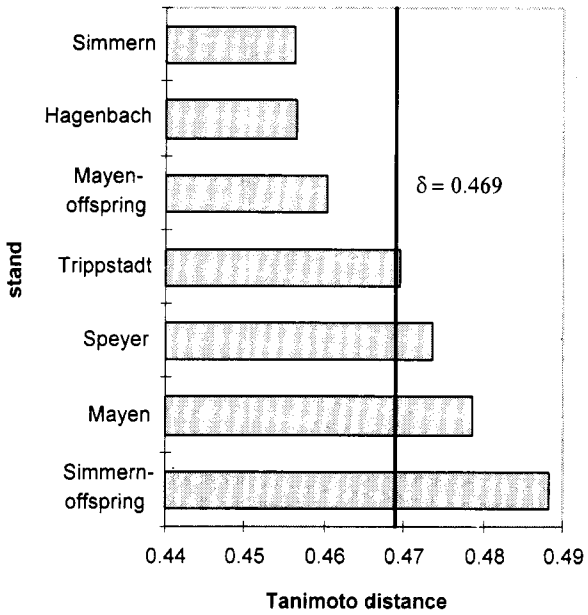

Figure 4. Tanimoto sub-population distances related to the loci amplified with primers number 26, 28 and 29. δ: average of the seven distances.

30.85 %) (Table 4).

Heterozygosity was reduced from the parent stands to the offspring populations. From the regularly budding parent stand Simmern to their offspring heterozygosity decreased by 2.93 % and in offspring from the late budding Mayen stand heterozygosity decreased by 5.36 % (Table 4). With RAPD loci, the heterozygosity of the Simmern offspring population decreased in relation to the parent population by 1.67 % and in the Mayen population it decreased in relation to the parent population by 5.58 %. With AMP amplification, the heterozygosity of the Simmern parent and offspring

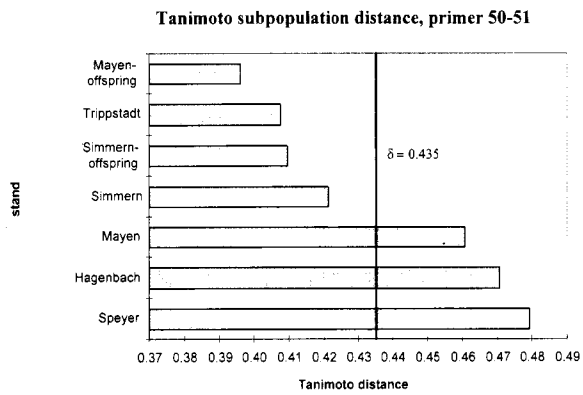

Figure 5. Tanimoto sub-population distances related to the loci amplified with primers number 50 and 51. : average of the seven distances.

populations decreased by 4.80 % and in the Mayen parent and offspring populations it decreased by 5.01 % (Table 4).

Genetic diversity

The genetic diversity H_T (NEI 1973) of the studied populations was primarily based upon the genetic diversity within the populations. The genetic diversity between populations (D_{ST}) in relationship to the total genetic diversity (H_T) was only 9.06 % (Table 5). As a result, the genetic diversity within populations was 90.94 %. The G_{ST} value related to the gene loci amplified with RAPD primers was a little smaller (G_{ST} = 8.94 %) than the G_{ST} value (G_{ST} = 9.25 %) of the samples analyzed with the AMP primers (Table 5).

DISCUSSION

RAPD markers have been increasingly applied to provide estimates of genetic diversity within and between tree populations (AIDE & RIVERA 1998, ALLNUTT *et al.* 1998, 1999, CHALMERS *et al.* 1992, GILLIES *et al.* 1997, NEWTON *et al.* 1999). Of the six decameric RAPD primers (MOREAU 1993, MOREAU *et al.* 1994) tried in this study to quantitate genetic variation in pedunculate oak, three (Nos. 26, 28 and 29) resulted in reproducible and interpretable DNA patterns.

SSR motives, such as $(CA/GT)_n$ and $(GA/CT)_n$ were investigated in five tropical tree species and found to be relatively abundant with 10^4 and 10^5 such sites in the genomes (CONDIT & HUBBELL 1991). In bur oak (*Q. macrocarpa*), the number of dinucleotide repeats per genome was estimated to be 3 x 10^5 (BARRENECHE *et al.* 1998). The $(GA/CT)_n$ repeats were sequenced and showed average repeat length of 16.2 (DOW *et al.* 1995). In *Quercus robur* and *Q. petraea* the genome

Table 5. Nei' s genetic diversities.

Diversity measure	Genetic diversity of primer loci					
	no. 26	no. 28	no. 29	no. 50	no. 51	Mean
H_T	0.3401	0.3643	0.3148	0.2831	0.2277	0.3060
H_S	0.3053	0.3367	0.2792	0.2419	0.2059	0.2738
G_{ST}	0.1014	0.0699	0.0970	0.1079	0.0770	0.0906
		0.0894			0.09245	

Table 6. Relative band-frequencies (p %) at RAPD and AMP loci which differ in the two parent stands and their offsprings by more than 30 %.

Locus	Mayen		Δ (%)	Locus	Simmern		Δ (%)
	parent stand p (%)	progeny p (%)			parent stand p (%)	progeny p (%)	
26-K	78	46	-32	26-K	81	40	-41
				26-L	67	27	-40
28-B	47	5	-42	28-B	67	16	-51
28-C	42	9	-33				
28-D	81	48	-33				
28-F	63	25	-38				
28-H	44	93	49				
29-I	41	72	31				
50-A	27	94	67				
50-E	18	60	42				
51-C	46	13	-33				
51-H	67	98	31				
				51-F	55	24	-1

Mayen: 7 RAPD and 4 AMP loci with band frequencies > 30 %. Simmern: 3 RAPD and 1 AMP locus with band freguencies > 30 %, Tanimoto sub population differences (δ_T): Mayen = 47.16 %, Mayen - offspring = 43.65 %, Simmern = 44.23 %, Simmern - offspring = 45.69 %. Pairwise Tanimoto distances (δ_T): Mayen / Simmern = 45.45 %, Mayen - offspring / Simmern - offspring = 40.31 %, Mayen / Mayen - offspring = 46.21 %, Simmern / Simmern offspring = 42. 90 %. Simmern: regularly flushing pedunculate oak, Mayen: late flushing pedunculate oak.

size is 0.86 x 10^9 bp in the haploid genome with about one $(GA)_n$ site every 120 kb (STEINKELLNER *et al.* 1997, BARRENECHE *et al.* 1998). Investigating genetic polymorphisms of 5 GA-microsatellite DNA loci 5 to 12 alleles per locus were observed in *Q. petraea* and *Q. robur* (STEINKELLNER *et al.* 1996). In both species, the same level of differentiation was detected and none was restricted to one species alone. In these studies microsatellite sequences were amplified by PCR using primers from the conserved flanking regions (STEINKELLNER *et al.* 1996).

Anchored microsatellite primed (AMP)-polymerase chain reaction (PCR), or as it is also named, simple sequence repeat anchored (SSRA)-PCR, amplifies genomic segments flanked by inversely oriented, closely spaced, repeated sequences (ZIETKIEWICZ *et al.* 1994). AMP-PCR depicts various repetitive sequences in eucariotic genomes with a length of two to six bases (ZIETKIEWICZ *et al.* 1994, WINTER & KAHL 1995). As primers, oligonucleotides are used that are composed of a complementary repetitive sequence and a random two to four nucleotide long sequence at the 5′ terminus. This end anchors outside the repetitive sequence within the genome (ZIETKIEWICZ *et al.* 1994). Polymorphisms evolve at RAPD and SSR loci either by mutation (insertion, deletion), within sequences between primer binding sites, or by alterations within the primer binding sites themselves (WINTER & KAHL 1995). AMP-PCR is similar to RAPD-PCR in that it also uses only one primer, needs no sequence information prior to analysis, and its amplified sequences are dominantly inherited (TROGGIO *et al.* 1996, ZIETKIEWICZ *et al.*

Table 7. Expected heterozygosities (H_e %) at RAPD and AMP loci which differ in the two studied pedunculate oak stands and their offsprings by more than 30 %.

Locus	Mayen		Δ (%)	Locus	Simmern		Δ (%)
	parent stand H_e (%)	offspring H_e (%)			parent stand H_e (%)	offspring H_e (%)	
26-E	0	49.92	49.92				
28-B	39.29	4.49	-34.80	28-B	48.80	14.90	-33.90
28-K	49.06	0	-49.06				
29-D	46.49	0	-46.49	29-D	47.46	0	-47.46
				29-F	0	32.75	32.75
				50-G	49.64	0	-49.64
51-A	45.97	0	-45.97				
51-D	35.03	0	-35.03				
				51-E	34.12	0	-34.12

Mayen: 4 RAPD and 2 AMP loci with expected heterozygosities > 30 %. Simmern: 3 RAPD and 2 AMP loci with expected heterozygosities > 30 %. Simmern: Regularly flushing oak. Mayen: Late flushing oak.

1994). Here we tried 24 anchored microsatellite primers of the types 5′-NNN(GTG)$_5$-3′ and 5′-NNN(GATA)$_4$-3′, which resulted in sharp and reproducible bands. Among these, the latter type revealed the greater differentiation.

The average number of polymorphic RAPD loci studied in this investigation was 89.57 % (64 to 100 %). In various tree species this number ranges from 35.7 % (*Pigerodendron uviferum* (ALLNUTT *et al.* 1999)) to 100 % (*Picea mariana* (ISABEL *et al.* 1995)) and on average (8 species) it is 71.34 % (ALLNUTT *et al.* 1999). The average number of polymorphic AMP loci which we investigated was 85.07 % (69 to 100 %). Thus the average number of polymorphic AMP loci was a little lower (–3.5 %) than the number of RAPD loci. The chance that RAPD primers amplify SSR loci is rather low and may be one in twenty, as estimated for Scots pine (LU *et al.* 1997). In RAPD, the absence of an amplification product can mostly be traced back to an alteration of the primer binding site, as shown in *Pinus sylvestris* (LU *et al.* 1997).

During the re-immigration of sessile oak and pedunculate oak from the Mediterranean area to Central Europe after the last ice age, several subpopulations evolved. They differ in a certain sequence of the plastidic t-RNA-gene (KREMER *et al.* 1991; PETIT *et al.* 1993). However, small area population differences cannot be elucidated with these sequences.

Although the genetic distances between the pedunculate oak populations which we investigated here are low ($G_{ST} \leq 10$ %) they can be differentiated according to frequency differences at RAPD and AMP primed loci. Frequencies of Tanimoto's sub-population distances depended on the DNA sequences investigated. Within RAPD loci amplified with primers No. 26, 28 and 29 sub-population distances increased as follows: Simmern (45.46 %), Hagenbach (45.53 %), Trippstadt (45.53 %), Speyer (46.62) and Mayen (47.25 %), with the late budding population Mayen having the largest distance. With the AMP loci amplified with primers No. 50 and 51 sub-population distances increased differently within the investigated stands: Trippstadt (43.59 %), Simmern (44.48 %), Hagenbach (47.59 %), Mayen (47.68 %) and Speyer (48.53 %). Here, the late budding stand came fourth before the reglarly budding population at Speyer. At all 64 loci together subpopulation differences came close to those obtained with the two AMP loci: Trippstadt (44.71 %), Simmern (45.07 %), Hagenbach (46.31 %), Speyer (47.38 %) and Mayen (47.42 %). Here again the late budding variety exhibited the largest distance towards the remaining complement of the four populations.

The investigated parent populations Mayen and Simmern are certified for seed harvesting according to the official directives for forest reproductive materials in Germany („Gesetz über forstliches Saat- und Pflanzgut"). The offspring of both stands were raised in a nursery for three years and then transplanted to a different forest district where they are part of a provenance trial. Sub-population distances in both offspring are different from those of the respective parental stands. In offspring of the Simmern stand, sub-population differentiation was 1.46 % larger than in the parent stand while in offspring of the late budding stand Mayen it was 3.71 % lower than in the parent stand. Moreover, at several single loci there are clear differences in both offspring (Table 6). In offspring of the

late budding Mayen stand at 11 different loci frequency differences of 31 % and more were observed in comparison to the parent trees. At six loci (26-K, 28-B, -C, -D, -F and 51-C), the frequencies were lower in the offspring than in the parent trees, while at five loci (28-H, 29-I, 50-A, 50-E and 51-H) they were larger (Table 6). These alterations may be considered as a selection towards the climatic and edaphic conditions at the Hunsrück hills as compared to the Slovenian habitat. In offspring of the regular budding stand Simmern, at four different loci (26-K, 26-L, 28-B and 51-F), frequencies decreased by more than 30 % as compared to the parent trees (Table 6). These alterations occurred predominantly at RAPD-loci (Mayen: 7 RAPD and 4 AMP loci, Simmern 3 RAPD and 1 AMP locus). That RAPD-loci may be subjected to selection has been demonstrated for Limber pine (*Pinus flexilis* James) (LATTA & MITTON 1997).

Different genetic developments in the two offspring populations investigated can also be recognized when expected heterozygosities are compared. The average expected heterozygosity calculated for stand Mayen was *He* = 29.24 % and that of its offspring *He* = 23.88 %. For the Simmern stand, the expected heterozygosity was *He* = 29.64 % and that of its offspring *He* = 26.71 %. BARRENECHE et al. (1998) found 18 % of RAPD markers to be heterozygous in two *Q. robur* trees. The reason for the reduction in the expected heterozygosities in offspring of the Mayen stand (-5.36 %) and in the Simmern stand (-2.93 %) may be that seeds were collected from a inadequate number of mother trees. A loss in genetic variation is generally thought to infer adaptive abilities of a population, but heterozygosity may increase during stand growth in case of selection against homozygotes.

Single locus events can also be recognized when expected heterozygosities are compared between the offspring and parental populations (Table 7). In the offspring of the late budding Mayen stand, expected heterozygosity decreased at 5 loci by more than 35 % (28-B, -K, 29-D, 51-A, 51-D) and increased by more than 35 % at one locus (26-E) as compared to the parental stand (Table 7). In the offspring of the regularly budding Simmern stand, the expected heterozygosity decreased at four loci by at least 33 % (28-B, 29-D, 50-G and 51-E), but increased at one locus by 33 % (29-F) as compared to the parental stand.

Altogether, clear variations in the two offspring generations are indicated. In the late budding Mayen population, frequency of alterations at 10 out of 64 loci (= 16 %) can be taken as significant, while in the regular budding Simmern population, serious frequency alterations affected only 4 out of 64 loci (= 6%). These, and lower frequency differences contribute to the unequal population differentiations. To avoid a further reduction in genetic variability, stands should be created with young plants from different sources but similar habitat.

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