Inheritance of allozymes and hybridization in two European *Tilia* species

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Inheritance analysis of seven enzyme systems in hexaploid *Tilia cordata* Mill. was performed utilizing single trees and their open-pollinated progenies. Genetic analyses of 12 polymorphic gene loci showed that *T. cordata* had disomic inheritance despite being an allopolyploid. *T. platyphyllos* Scop. was also used in the investigations although no genetic inheritance analysis was carried out. In comparison

with *T. cordata*, zymograms of 10 spontaneous *T. cordata* × *T. platyphyllos* hybrids showed markedly different banding patterns with species-specific alleles at 13 of the 14 described gene loci. Hence, differentiation between both species and their naturally occurring hybrid (*T. × europaea*) is easily feasible with allozyme studies.

Heredity (2003) 91, 337–344. doi:10.1038/sj.hdy.6800325

Keywords: *T. cordata*; *T. platyphyllos*; *T. × europaea* hybrids; gene markers; allozymes; inheritance

Introduction

Few investigations of allozyme gene loci have been carried out in *Tilia cordata* (Maurer and Tabel, 1995) and no inheritance analyses have been undertaken. Thus, the first step towards an analysis of its reproduction system was the development of electrophoretic methods, the clarification of allozyme inheritance in hexaploid *T. cordata* and the identification of polymorphic allozyme gene markers.

In regions where both *T. cordata* ($2n = 6 \times = 82$) and *T. platyphyllos* ($2n = 6 \times = 82$) occur sympatrically, spontaneous hybridization occurs (Keiper, 1916; Pigott, 1991). The pendulous inflorescence of *T. platyphyllos* is a dominant trait, and the inflorescences of pure *T. cordata* are erect (Pigott, 1991). A further distinguishing trait between the two species is the reddish-brown stellate hairs in the vein-axils on the underside of *T. cordata* leaves (Pigott, 1991). The hybrids between the two species show degrees of intermediacy for most other traits (Pigott, 1991); it is often not possible to differentiate between *T. cordata*, *T. platyphyllos* and their hybrid (*T. × europaea*) on the basis of morphological traits, especially in winter when the trees do not bear fruits (Scheller, 1972). Thus markers that discriminate between the species are also reported in this paper.

Material and methods

Material

A total of 138 dominant or codominant *T. cordata* and three *T. cordata* × *T. platyphyllos* hybrids grow in a 40 ha mixed forest near Schwiegershausen (10° 11' 25" E, 51° 40' 45" N), a small village west of the Harz Mountains;

the dominant species is *Fagus sylvatica*. The forest is surrounded by extensive agricultural land and may, therefore, be regarded as more or less isolated from other forests. For inheritance analysis, buds and more than 3700 seeds from 49 *T. cordata* were collected in the 1995 full mast year and investigated genetically. Additionally, buds of 91 *T. cordata*, 67 *T. platyphyllos* and seven hybrid individuals from other parts of Germany were used to identify species-specific alleles (Table 1). All trees were more than 120 years old.

The fruits were stored at –20°C. In winter, branches were cut from the trees and kept in climate chambers at 20°C until bud burst and leaves from the bursting buds were used for electrophoresis.

Electrophoretic methods

Extraction of enzymes

Leaf and bud tissues were homogenized in approximately 100 µl extraction buffer (97 mg Tris pH 7.5, 1 g saccharose, 300 mg PVP, 15 mg Na₂EDTA, 10 mg BSA and 30 mg DTT dissolved in 10 ml distilled water). For binding phenols and heavy metals, approximately 2 mg Polyclar was added to the extraction buffer before homogenizing the samples; addition of β-mercaptoethanol shows only a slight improvement in activity and the clarity of banding patterns for a few enzyme systems.

Separation and staining of enzymes

In order to investigate a large number of samples, a compromise was made between optimal results and the cost and effort, for example, for some gene loci involved (Table 2) better results were attained, although there was no significant improvement in the interpretation of the loci but a reduction in practicability. Recipes for enzyme staining were modified according to Maurer and Tabel (1995; details in Fromm (2001)); substrate specificity of the enzymes was tested for all gene loci.

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Received 27 March 2003

Genetic analysis

As no controlled crosses were carried out, the inheritance analysis method of Gillet and Hattermer (1989) was applied. This method has been used successfully for tree species, including *Castanea sativa* (Fineschi *et al.*, 1989), *Cupressus sempervirens* L. (Papageorgiou *et al.*, 1993), *Alnus acuminata* (Murillo and Hattermer, 1997), *Acer campestre* (Leinemann and Bendixen, 1999) and *Acer pseudoplatanus* (Konnert *et al.*, 2001). This method allows one to test the "one gene-one gene marker hypothesis" and of codominant inheritance on the basis of open-pollinated progenies of known seed trees.

Under the assumption of regular meiotic segregation and random fertilization of ovules, as well as the absence of viability selection, the following quantitative and qualitative relations in the investigated progenies must be found:

In the case of a homozygous seed tree (eg A_iA_i):

(a) All progeny must possess the allele of the maternal tree (A_i).

In the case of a heterozygous seed tree (eg A_iA_j , $i \neq j$):

(b) Each individual among the offspring must contain one of the maternal alleles, A_i or A_j .

(c) The number, N_{ij} , of heterozygous progenies, A_iA_j , is expected to be equal to the sum of the $N_{ii}+N_{jj}$ homozygous progenies, A_iA_i and A_jA_j , that is, $[N_{ij} = N_{ii}+N_{jj}]$.

(d) The number, N_{ik} , of the heterozygous progenies, A_iA_k , is expected to be equal to the number of the heterozygous progeny, A_jA_k , that is, $[N_{ik} = N_{jk} \text{ (} k \neq i, j \text{)}]$.

Table 1 Material collected in the Schwiegershausen population for genetic analyses and in southern Germany for species distinction

	Number of sampled trees		
	<i>T. cordata</i>	<i>T. × europaea</i>	<i>T. platyphyllos</i>
Schwiegershausen	138 and fruits of 49 trees	3	
Southern Germany (Bavaria, Baden-Württemberg and Rhineland-Palatinate)	91	7	67

Table 2 Separation systems for starch gel electrophoresis of *Tilia* allozymes. Running conditions valid for 27 × 11 cm gels

Buffer system/electrode buffer	Composition of gels	Running conditions			Enzyme system
		<i>I</i> const.	<i>U</i> var.	Time	
Ashton system 8.1 0.03 M LiOH/0.2 M H ₃ BO ₃	0.05 M Tris-citrate pH 8.1 plus 10% electrode buffer 10% starch 1% urea 0.07% Na ₂ EDTA	40 mA 80 mA	max. 500 V or max. 500 V	17 h 6 h	FDH AP PGM PGI
Histidine-citrate system pH 6.2 0.06 M Histidine-citrate	Electrode buffer plus 75% dist. H ₂ O 10.5% starch 2% sucrose 1% urea 0.07% Na ₂ EDTA	40 mA 80 mA	max. 250 V or max. 250 V	17 h 6 h	AP MDH MNR SKDH (IDH/ACO) ^a

^aOnly needed for the differentiation of *Tilia* species.

If statistical tests show a significant deviation between observed and expected frequencies, at least one of the above-mentioned relations or assumptions is violated and the gene locus concerned is unsuitable as a gene marker. If no significant deviation is detected, the gene locus can be regarded as a gene marker. However, it is possible that significant deviations can have methodological causes (eg sampling, data input).

Deviations between observed and expected frequencies were tested with χ^2 goodness-of-fit tests. Their results are presented in Tables 3–6 in terms of probabilities of greater values of χ^2 .

A value of 1.00 means that the observed and expected frequencies are in complete agreement, below 0.05 deviations are significant.

Results

For the enzyme system 6-PGDH (EC 1.1.1.44), two or more overlapping zones were found and, therefore, no interpretation of these complex banding patterns was possible. For the enzyme system ACO (EC 4.2.1.3), clear results were obtained for leaf material, but in seeds enzyme activities were too weak for routine investigation. For enzyme systems EST (EC 3.1.1.1), GDH (EC 1.4.1.3), G-6-PGDH (EC 1.1.1.49), NDH (EC 1.6.99.3) and SAP (EC 3.1.3.2), only weak and/or blurred patterns were obtained and no analysis was carried out. No IDH (EC 1.1.1.42) variation was detected in *T. cordata*, although variation was found in *T. platyphyllos*. Inheritance analysis was performed for gene loci coding for the following enzyme systems: AP (EC 3.4.11.1), FDH (EC 1.2.1.2), MDH (EC 1.1.1.37), MNR (EC 1.6.99.2), PGI (EC 5.3.1.9), PGM (EC 2.7.5.1) and SKDH (EC 1.1.1.25).

AP – aminopeptidase

Four zones of activity stain for the monomeric enzyme system AP using both leucine and alanine. Zones *Ap-A* and *Ap-C* usually show only faint activity, therefore their analysis was abandoned. Ashton and Braden's (1961) separation system was preferred for the analysis of locus *Ap-B*, while with the histidine-citrate system better separation of the enzyme variants was achieved. However, in the latter system cobands appeared at locus *Ap-B*,

Table 3 Inheritance analysis of *Ap-A*, *Ap-D* and *Fdh-A* in *T. cordata*

Type	Tree #	Progeny									N	χ^2 -test		
												a	b	
<i>Ap-B</i>		N_{11}	N_{22}	N_{33}	N_{44}	N_{12}	N_{13}	N_{14}	N_{23}	N_{24}	N_{34}			
B_1B_2	129	17	2			12	5		2			38	0.209 n.s.	0.257 n.s.
B_1B_3	130	2		12		2	12	1	8			37	0.695 n.s.	0.132 n.s.
B_1B_4	98				4	18	11	13		1	15	62	0.170 n.s.	0.450 n.s.
B_2B_3	15		31	43					76			150	0.870 n.s.	NA
	68		41	21			1		66	4	2	135	0.724 n.s.	0.705 n.s.
	112		6	6		3	1		23	1		40	0.063 n.s.	0.180 n.s.
	113		11	10					23	2	1	47	0.763 n.s.	0.564 n.s.
		and for B_2B_3 moreover trees # 3, 10, 14, 22, 23, 59, 71, 97, 104, 107, 111, 133, F3										607	All n.s.	All n.s.
B_2B_4	72		28		26			2	12	64	16	148	0.357 n.s.	0.273 n.s.
	134		8		5			2	7	15	7	44	0.705 n.s.	0.617 n.s.
B_3B_4	98a			11	5				2	4	17	39	0.862 n.s.	0.414 n.s.
<i>Ap-D</i>		N_{11}	N_{22}	N_{12}										
D_1D_2	10	7	29	31								67	0.541 n.s.	NA
	98	6	27	29								62	0.611 n.s.	NA
	F1	9	27	39								75	0.729 n.s.	NA
		and for D_1D_2 moreover trees # 8, 32, 107										85	all n.s.	
<i>Fdh-A</i>		N_{33}	N_{55}	N_{34}	N_{35}									
A_3A_5	2	50	24		76							150	0.870 n.s.	NA
	82	48	26		76							150	0.870 n.s.	NA
	114	50	14	2	82							148	0.185 n.s.	0.157 n.s.
	F1	26	7		42							75	0.299 n.s.	NA
		and for A_3A_5 moreover trees # 1, 23, 32, 33, 94a, 97, 98a, 112, 113, 123, 129, 130, 134										469	All n.s.	

NA: not applicable. N is the sample size, a refers to the test of the hypothesis $N_{ij}=N_{ii}+N_{jj}$, and b to the test of the hypothesis $N_{ik}=N_{jk}$ ($k \neq i, j$).

Table 4 Inheritance analysis of *Mdh-D*, *Mnr-A*, *Pgi-B* and *Pgi-C* in *T. cordata*

Type	Tree #	Progeny						N	χ^2 -test	
									a	b
<i>Mdh-D</i>		N_{11}	N_{22}	N_{12}						
D_1D_2	42	20	52	60				132	0.296 n.s.	NA
	68	4	45	56				105	0.495 n.s.	NA
	98a	3	16	20				39	0.873 n.s.	NA
	120	12	69	69				150	0.327 n.s.	NA
	and for D_1D_2 moreover trees # 23, 25, 115, 122, F1, F3							255	All n.s.	
<i>Mnr-A</i>		N_{33}	N_{44}	N_{34}						
A_3A_4	28	45	15	67				127	0.535 n.s.	NA
	130	8	11	18				37	0.869 n.s.	NA
	134	19	6	19				44	0.228 n.s.	NA
	F2	59	32	80				171	0.251 n.s.	NA
	and for A_3A_4 moreover trees # 27, 55, 98a, 104, 122, F1							247	All n.s.	
<i>Pgi-B</i>		N_{11}	N_{22}	N_{33}	N_{12}	N_{13}	N_{23}			
B_1B_2	36	20	17		31	43	43	154	0.467 n.s.	1.000 n.s.
	82	20	20		49	34	27	150	0.340 n.s.	0.370 n.s.
B_1B_3	38	2		10	2	12	6	32	1.000 n.s.	0.157 n.s.
B_2B_3	28		4	21			22	47	0.662 n.s.	NA
	59		4	12			14	30	0.715 n.s.	NA
	111		1	18			18	37	0.869 n.s.	NA
	113		1	24			22	47	0.662 n.s.	NA
	and for B_2B_3 moreover trees # 8, 27, 94a, 107, 123							191	All n.s.	
<i>Pgi-C</i>		N_{11}	N_{22}	N_{12}						
C_1C_2	92	69	5	66				140	0.499 n.s.	NA
	F3	12	7	20				39	0.873 n.s.	NA
	F4	28	21	54				103	0.622 n.s.	NA
	and for C_1C_2 moreover trees # 1, 129, 134							102	All n.s.	

NA: not applicable. N is the sample size, a refers to the test of the hypothesis $N_{ij}=N_{ii}+N_{jj}$, and b to the test of the hypothesis $N_{ik}=N_{jk}$ ($k \neq i, j$).

Table 5 Inheritance analysis of *Pgm-A*, *Pgm-B*, *Pgm-C* and *Pgm-D* in *T. cordata*

Type	Tree #	Progeny						N	χ^2 -test	
									<i>a</i>	<i>b</i>
<i>Pgm-A</i> <i>A</i> ₁ <i>A</i> ₂	1	<i>N</i> ₁₁ 5	<i>N</i> ₂₂ 4	<i>N</i> ₁₂ 11				20	0.655 n.s.	NA
<i>Pgm-B</i> <i>B</i> ₁ <i>B</i> ₂ <i>B</i> ₂ <i>B</i> ₃	F1 8 71 107 115 129	<i>N</i> ₁₁ 14	<i>N</i> ₂₂ 41 7 91 10 11 8	<i>N</i> ₃₃ 2 6 3 3 5	<i>N</i> ₁₂ 20	<i>N</i> ₁₃	<i>N</i> ₂₃ 11 77 17 15 25	75 20 174 30 29 38	<0.001*** 0.655 n.s. 0.129 n.s. 0.465 n.s. 0.853 n.s. 0.105 n.s.	NA NA NA NA NA NA
<i>Pgm-C</i> <i>C</i> ₁ <i>C</i> ₂	33 92 118 129 and for <i>C</i> ₁ <i>C</i> ₂ 17 further trees	<i>N</i> ₁₁ 6 25 48 11	<i>N</i> ₂₂ 14 39 33 8	<i>N</i> ₁₂ 19 76 69 19				39 140 150 38 1132	0.873 n.s. 0.310 n.s. 0.327 n.s. 1.000 n.s. All n.s.	NA NA NA NA NA
<i>Pgm-D</i> <i>D</i> ₂ <i>D</i> ₃	15 69a 109 F2 and for <i>D</i> ₂ <i>D</i> ₃ 13 further trees	<i>N</i> ₂₂ 46 30 23 46	<i>N</i> ₃₃ 34 48 19 36	<i>N</i> ₂₃ 70 72 43 89				150 150 85 171 798	0.414 n.s. 0.624 n.s. 0.914 n.s. 0.592 n.s. All n.s.	NA NA NA NA NA

NA: not applicable. N is the sample size, a refers to the test of the hypothesis $N_{ij}=N_{ii}+N_{jj}$, and b to the test of the hypothesis $N_{ik}=N_{jk}$ ($k \neq i, j$).

Table 6 Inheritance analysis of *Skdh-B* in *T. cordata*

Type	Tree #	Progeny										N	χ^2 -test	
													<i>a</i>	<i>b</i>
<i>Skdh-B</i>		N_{11}	N_{22}	N_{33}	N_{44}	N_{12}	N_{13}	N_{14}	N_{23}	N_{24}	N_{34}			
B_1B_2	118	1	1			1	1	2	3	1		10	0.564 n.s.	0.705 n.s.
B_1B_3	23	3		11		1	11	5	2		2	35	0.549 n.s.	0.527 n.s.
	120	34		13		10	35	30	4		24	150	0.185 n.s.	0.146 n.s.
B_1B_4	1	4			3		3	8			2	20	0.796 n.s.	0.655 n.s.
	2	18			27	4	17	64		3	17	150	0.069 n.s.	0.876 n.s.
	33	5			8	3	2	16		2	3	39	0.577 n.s.	1.000 n.s.
	55	2			9	1	3	12		3	1	31	0.835 n.s.	1.000 n.s.
	109	10			22		6	39		1	7	85	0.406 n.s.	0.593 n.s.
B_2B_3	111		2	1		1			5	13	15	37	0.480 n.s.	0.853 n.s.
	F3		12	3					16	1		32	0.857 n.s.	0.317 n.s.
B_2B_4	27		5		8	1		2	3	13	2	34	1.000 n.s.	1.000 n.s.
	42		20		26			2	10	58	13	129	0.239 n.s.	0.317 n.s.
	59		6		7			1	3	8	3	28	0.275 n.s.	0.705 n.s.
	123		5		9			1	4	9	5	33	0.297 n.s.	0.527 n.s.
	and for B_2B_4 moreover trees # 8, 94a, 97, 104, 113											139	All n.s.	All n.s.
B_3B_4	11			14	20		1		1	4	29	69	0.529 n.s.	0.414 n.s.
	14			12	16		2		1	3	4	35	0.378 n.s.	1.000 n.s.
	72			24	32		10	4	12	14	53	149	0.774 n.s.	0.527 n.s.
	82			36	36			2	5	4	67	150	0.671 n.s.	0.763 n.s.
	and for B_3B_4 moreover trees # 3, 15, 130, 133											236	All n.s.	All n.s.

N is the sample size, a refers to the test of the hypothesis $N_{ij}=N_{ii}+N_{jj}$, and b to the test of the hypothesis $N_{ik}=N_{jk}$ ($k \neq i, j$).

which made definite identification of the allozymes difficult (Figure 1). In *T. cordata*, variants at locus *Ap-D* could only be separated using histidine-citrate system; with the Ashton and Braden (1961) system this locus appeared monomorphic.

For *Ap-B*, four enzyme variants were found, none of which was specific for any of the *Tilia* species. Likewise, locus *Ap-D* has four variants, while variants *Ap-D*₁ and *Ap-D*₂ were observed in *T. cordata*, and variants *Ap-D*₃ and *Ap-D*₄ occurred only in *T. platyphyllos*. For hybrids of

Figure 1 Zymograms of *Tilia* for the enzyme systems AP, MDH and MNR, with schematic illustrations and genotype designation. Genotypes, *Ap-D₃D₄*, *Ap-D₄D₄*, *Mnr-A₁A₁*, *Mnr-A₁A₂* and *Mnr-A₂A₂* are from *T. platyphyllos*; genotype *Mnr-A₁A₃* is *T. cordata* × *T. platyphyllos* hybrid; all others are *T. cordata*.

T. cordata and *T. platyphyllos*, both of these species-specific enzyme variants were found. All segregation data were fully compatible with their expectations (Table 3).

MDH – malate dehydrogenase

There are at least four loci for the dimeric enzyme MDH in *Tilia*. Zone A (above the dashed line in Figure 1) showed no variation, although there appeared to be at least two gene loci which formed heterodimers. Inheritance analyses were successful only for locus *Mdh-D*, because the loci in zones *Mdh-B* and *Mdh-C* overlap, and the alleles *Mdh-B₁* and *Mdh-C₁* have almost identical positions on the zymograms. Differentiation between the genotypes *Mdh-B₁B₂* and *Mdh-B₂B₂*, *Mdh-C₁C₂* and *Mdh-C₂C₂*, respectively, was only possible through interpretation of slight differences in band staining intensity (lane 2 for *C₂C₂* and lane 3 for *C₁C₂* in Figure 1). Such differences

Figure 2 Zymograms of *Tilia* for the enzyme systems FDH and PGM with schematic illustrations and genotype designation. At the gene locus *Fdh-A*, genotypes *A₁A₁* and *A₁A₂* are *T. platyphyllos*, while *A₁A₃* and *A₂A₃* denote *T. cordata* × *T. platyphyllos* hybrids. At the gene locus *Pgm-D*, all genotypes containing *D₁* are *T. cordata* × *T. platyphyllos* hybrids. All other zymograms show *T. cordata*.

were too small for a reliable discrimination, especially in the seeds. Despite the missing inheritance analyses, a preliminary nomenclature for loci *Mdh-B* and *Mdh-C* is presented in Figure 1. The zymograms of adult trees could sometimes be interpreted if zymograms of their seeds were also available. For all three loci, only two variants were observed per locus, and there were no species-specific variants. All segregation data were fully compatible with their expectations (Table 4).

MNR – menadione reductase

For the tetrameric enzyme MNR, one zone of activity with four enzyme variants was visible (Figure 1). Allozymes *Mnr-A₃* and *Mnr-A₄* are *T. cordata*-specific and *Mnr-A₁* and *Mnr-A₂* are *T. platyphyllos*-specific; therefore *Mnr-A₁A₃* is a *T. cordata* × *T. platyphyllos* hybrid genotype. All segregation data were fully compatible with their expectations (Table 4).

FDH – formate dehydrogenase

One zone of weak activity was obtained for the dimeric enzyme FDH, for which six enzyme variants were detected (Figure 2). For genetic interpretation, the variants *Fdh-A₅* and *Fdh-A₆* were pooled, since differentiation of these allozymes was not possible in all cases.

The variants *Fdh-A₅* and *Fdh-A₆* were *T. cordata*-specific, variants *Fdh-A₁*, *Fdh-A₂* and *Fdh-A₄* were *T. platyphyllos*-specific, while variant *Fdh-A₃* occurred in both species. All segregation data were fully compatible with their expectations (Table 3).

PGM – phosphoglucosmutase

In *T. cordata*, four loci coding for the monomeric enzyme PGM were analysed (Figure 2). The zones *Pgm-A* and *Pgm-C* showed two variants, while zones *Pgm-B* and *Pgm-D* had three enzyme variants. Variant *Pgm-D₁* is *T. platyphyllos*-specific. PGM zymograms of *T. platyphyllos* show complex banding patterns that were very different from those of *T. cordata*. Apart from variant *Pgm-D₁*, an

identification of other *T. platyphyllos*-specific variants was not possible; for this, inheritance analyses for *T. platyphyllos* were needed.

With one exception, segregation results (Table 5) of all zones were compatible with their expectations; among the progenies of tree F1 with genotype B_1B_2 , by far too many homozygotes B_2B_2 were observed in comparison to heterozygotes B_1B_2 . The highly significant deviation between the observed and expected frequencies in the progeny of this tree F1 seems to be caused by the variant *Pgm-B₁*, since the inheritance mode for the allele *Pgm-B₂* is supported by the findings in several other single tree progenies. One of the above-mentioned assumptions must be violated and, therefore, the existence of the allele *Pgm-B₁* has to be questioned until it is clarified by further investigations.

PGI – phosphoglucose isomerase

Zone A of the dimeric enzyme PGI showed variation, but because of the blurred patterns the locus could not be analysed. However, the patterns of loci *Pgi-B* and *Pgi-C* were clear and could be analysed by using the heterodimers as visual keys. A double band in zone C indicates the presence of C_2 . This interpretation is supported by an additional band in the heterodimer region of loci *Pgi-B* and *Pgi-C*. Three allozymes at locus *Pgi-B* and two allozymes at *Pgi-C* were found in *T. cordata*. The enzyme variants of *T. platyphyllos* were different and no preliminary nomenclature of zones and alleles was possible. All segregation data were fully compatible with their expectations (Table 4).

SKDH – shikimate dehydrogenase

Zone A of the monomeric enzyme SKDH was variable but could not be resolved. However, zone A was useful for discriminating both *Tilia* species. Locus *Skdh-B* shows variation for seven allozymes, of which *Skdh-B₁–Skdh-B₄* are *T. cordata*-specific and variants *Skdh-B₅–Skdh-B₇* are *T. platyphyllos*-specific (genotypes B_4B_7 and B_1B_6 in Figure 3 are *T. × europaea*). Locus *Skdh-B* was difficult to interpret since homozygote variants appear as double bands, and in heterozygotes, three or four bands were visible depending on allele migration. Additional difficulties were caused by differing staining intensities of the double bands for leaf and seed material. In leaf material, the intensity of the lower band was highest, while for seeds it was reversed. Segregations of all genotypes carrying alleles $B_1–B_4$ were fully compatible with their expected frequencies.

IDH – isocitrate dehydrogenase—and ACO—aconitase

No inheritance analyses were carried out for these enzyme systems, although species-specific bands could still be utilized to distinguish *T. cordata*, *T. platyphyllos* and their hybrid.

Discussion

Species of the genus *Tilia* possess a chromosome set of either $n = 6 \times = 41$ or $n = 12 \times = 82$ (Seitz, 1951; Darlington and Wylie, 1955). *T. cordata* is assumed to be an allohexaploid and to originate through hybridization of an allotetraploid and a diploid species. The phenotypic expression of the enzymes corresponds to the banding

Figure 3 Zymograms of *Tilia* for the enzyme systems PGI and SKDH with schematic illustrations and genotype designation. For PGI, only *T. cordata* zymograms are shown. At the gene locus *Skdh-B*, genotypes B_4B_7 and B_1B_6 are *T. cordata* × *T. platyphyllos* hybrids. Note that SKDH zymograms contain only double bands.

patterns of diploid organisms; homozygotes have one band for monomeric and multimeric enzyme systems, while for heterozygotes, there are two bands for monomeric enzymes, three for dimeric and five for tetrameric enzymes. In spite of being polyploid, *T. cordata* had disomic inheritance, just as tetraploid *Acer pseudo-platanus* (Konnert *et al*, 2001). In *Tilia*, for example, for PGI and PGM, allopolyploidy is not expressed as greater numbers of intralocus bands but as an increased number of loci compared to diploid organisms, for example, *Fagus sylvatica* (Müller-Starck and Starke, 1993), *Quercus robur* (Zanetto *et al*, 1996), *Q. petraea*. (Müller-Starck *et al*, 1996) and *Acer campestre* (Leinemann and Bendixen, 1999).

No cytological observations of meiosis were made, and as the phenotypes and their frequencies in single tree progenies were compatible with disomic inheritance,

only tests based on the expectations of disomic segregation were made (Gillet and Hattermer, 1989). In case of autopolyploidy, the corresponding expectations were very different from those under disomic segregation, as shown for autotetraploid *Prunus spinosa* (Leinemann, 2000). Disomic segregation in *T. cordata* was of confirmed through statistical tests for almost all investigated loci and enzyme variants.

SKDH zymograms for homozygotes have only double bands. Corresponding findings have been made in other enzyme systems in forest trees: Bergmann (1974) found that two alleles at the gene locus coding for acid phosphatases in dormant seeds of *Picea abies* produce single bands while the other two alleles produce double bands even in haploid megagametophyte tissue. This author reports something similar on the gene locus *Lap-A*. In *Fagus sylvatica* double bands occur at *Aat-B* (aspartate aminotransferase, EC 2.6.1.1; Müller-Starck *et al*, 2001), and in *Abies alba* at *Idh-A* (Hussendörfer *et al*, 1995). It is unknown whether the two bands visible *in vitro* possess markedly different structure or whether they are only modified during electrophoresis.

T. platyphyllos genotypes were only inferred from the *T. cordata* results. Zymograms of *T. platyphyllos* showed different banding patterns indicating specific alleles at several gene loci. Since the two species also have alleles in common, the (minimum) proportion of hybrid F_1 seeds among the progeny of *T. cordata* in the Schwiegershausen forest can only be detected by considering all gene loci (Fromm, 1999). Although the differentiation of tree populations of the same species at allozyme gene loci is generally low and private alleles have rarely been found (Loveless, 1992; Müller-Starck *et al*, 1992), only more extensive surveys will confirm the species specificity of the alleles.

The high proportion of species-specific enzyme variants in both *Tilia* species leads to the assumption that interspecific hybridization and introgression are restricted. Introgression between the two *Tilia* species appears to take place only to a minor degree, in contrast to the genus *Quercus* (Rushton, 1993; Bacilieri *et al*, 1996; Finkeldey, 2001).

Restricted introgression may have several reasons. Different growing site demands for *T. cordata* and *T. platyphyllos* mean that the species are sympatric only at a few sites. Additionally, mating between the two species is restricted by different flowering periods; the flowering period of *T. platyphyllos* generally starts 2 weeks earlier than that of *T. cordata*. Furthermore, introgression of the two *Tilia* species is hindered through the low production of fertile seeds produced by the few hybrids. The fruit appendage of the hybrids is often large but most of the fruits contain only empty seeds (Scheller, 1972; Fromm, 2001). On the other side, the flowering periods of *T. cordata* and the hybrids overlap (Pigott, 1991; Fromm, 2001) and 20 of the 3864 fertile seeds collected from *T. cordata* in the Schwiegershausen forest carried *T. platyphyllos*-specific alleles and were inferred to be the result of fertilization by one of the three hybrids in the population. In the Schwiegershausen population, hybrid pollen were as successful as *T. cordata* pollen in fertilizing *T. cordata* ovules (Fromm, 2001). The 20 seeds were not included in segregation analyses. It is not known if these seeds are as viable as pure *T. cordata* seeds in later ontogenetic stages.

Acknowledgements

This research was supported by a grant from Deutsche Forschungsgemeinschaft. The authors are greatly obliged to two anonymous reviewers for their helpful comments.

References

- Ashton GC, Braden AWH (1961). Serum β -globulin polymorphism in mice. *Austr J Biol Sci* 14: 248–253.
- Bacilieri R, Ducousso A, Petit R, Kremer A (1996). Mating system and asymmetric hybridisation in a mixed stand of European oaks. *Evolution* 50: 900–908.
- Bergmann F (1974). The genetics of some allozyme systems in spruce endosperm (*Picea abies*). *Genetika* 6: 353–360.
- Darlington CD, Wylie AP (1955). *Chromosome Atlas of Flowering Plants*. George Allen and Unwin Ltd: London.
- Fineschi S, Gillet E, Malvolti E (1989). Genetics of sweet chestnut (*Castanea sativa* Mill.). III. Genetic analysis of zymograms of single tree offspring. *Silvae Genet* 39: 188–194.
- Finkeldey R. (2001). Genetic variation of oaks (*Quercus* spp.) in Switzerland. 2. Genetic structures on pure and mixed forests of pedunculate oak (*Quercus robur* L.) and sessile oak (*Q. petraea* (Matt.) Liebl.). *Silvae Genet* 50: 22–30.
- Fromm M (1999). Artbestimmung von Winterlinde, Sommerlinde und der Arthybride. *AFZ/Der Wald* 5: 244–246.
- Fromm M (2001). *Reproduktion einer entomophilen Baumart in geringer Populationsdichte—Das Beispiel der Winterlinde (Tilia cordata Mill.)*. PhD Thesis, Universität Göttingen, 239pp. <http://webdoc.sub.gwdg.de/diss/2001/fromm/index.html>
- Gillet E, Hattermer HH (1989). Genetic analysis of isoenzyme phenotypes using single tree progenies. *Heredity* 63: 135–141.
- Hussendörfer E, Konnert M, Bergmann F (1995). Inheritance and linkage of isozyme variants of silver fir (*Abies alba* Mill.). *For Genet* 2: 29–40.
- Keiper J (1916). Die Linde im Pfälzerwald und in den übrigen Waldgebieten der Pfalz. *Forstwiss Centralbl* 38: 223–369.
- Konnert M, Ruetz W, Fromm M (2001). Genetic variation in *Acer pseudoplatanus* L. I. Inheritance of allozyme variants. *For Genet* 8: 25–37.
- Leinemann L (2000). Inheritance analysis of allozyme phenotypes in tetraploid species using single plant progenies. An example in black thorn (*Prunus spinosa* L.). *For Genet* 7: 205–209.
- Leinemann L, Bendixen K (1999). Inheritance of allozyme variants in field maple (*Acer campestre* L.). *For Genet* 6: 73–79.
- Loveless MD (1992). Isozyme variation in tropical trees: patterns of genetic organization. *New Forests* 6: 67–94.
- Maurer WD, Tabel U (1995). A methodical study to improve the allozyme analysis for identification of clones of *Tilia*. *Silvae Genet* 44: 351–356.
- Müller-Starck G, Baradat PH, Bergmann F (1992). Genetic variation within European tree species. *New Forests* 6: 23–47.
- Müller-Starck G, Konnert M, von Wühlisch G (2001). Labormanual zur Durchführung von Isoenzymanalysen bei der Buche (*Fagus sylvatica* L.). Freising, Bund-Länder Arbeitsgemeinschaft 'Forstliche Genressourcen und Forstsatzgutrecht'. <http://www.ncbi.nlm.nih.gov>.
- Müller-Starck G, Starke R (1993). Inheritance of isoenzymes in European beech (*Fagus sylvatica* L.). *J Hered* 84: 291–296.
- Müller-Starck G, Zanetto A, Kremer A, Herzog S (1996). Inheritance of isozymes in the genus *Quercus*: II. Sessile oak (*Quercus petraea* (Matt.) Liebl.) and offspring from interspecific crosses. *For Genet* 3: 1–12.
- Murillo O, Hattermer HH (1997). Inheritance of allozyme variants of *Alnus acuminata* ssp. *arguta* (Schlectendal) Furlow. *Silvae Genet* 46: 51–55.

- Papageorgiou AC, Bergmann F, Gillet E, Hattermer HH (1993). Genetic analysis of isoenzyme variation in Mediterranean cypress (*Cupressus sempervirens* L.). *Silvae Genet* **42**: 109–111.
- Pigott CD (1991). Biological flora of the British Isles. *Tilia cordata* Miller. *J Eco* **79**: 1147–1207.
- Rushton BS (1993). Natural hybridisation within the genus *Quercus* L. *Ann Sci For* **50**: 73–90.
- Scheller H (1972). Die Linden in Gärten und Parks des unteren Maingebietes. *Mitt Dtsch Dendrol Ges* **65**: 7–42.
- Seitz FW (1951). Chromosomenzahlverhältnisse bei Holzpflanzen. *Z Forstgenetik* **1**: 22–32.
- Zanetto A, Kremer A, Müller-Starck G, Hattermer HH (1996) Inheritance of isozymes in pedunculate oak (*Quercus robur* L.). *Heredity* **87**: 364–370.