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Chloroplast DNA analysis in oak stands (*Quercus robur* L.) in North Rhine-Westphalia with presumably Slavonian origin: Is there an association between geographic origin and bud phenology?

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Summary

Slavonian oaks (*Quercus robur* subsp. *slavonica*) have been introduced into Germany in the second half of the 19th century from the lowlands of the rivers Save and Drava in today's Croatia. If compared to indigenous oak stands, they are characterized by good growth, comparatively low seed production and a late bud burst. Based on the information of European-wide variation patterns at chloroplast DNA markers in oaks we adapted chloroplast microsatellites for the analysis of all oak stands of presumably Slavonian origin in the Münsterland and lower Rhine regions. We were able to distinguish between Slavonian haplotypes with no natural occurrence in the study area and indigenous types that do not occur in the Balkan region. A generally high differentiation among stands was observed at chloroplast markers (G_{ST} = 0.674). Based on the haplotype information and historic records we found that stands with Slavonian material have been established between the years 1878 and 1903. In a total of 910 analysed trees the Slavonian haplotypes 5, 2 or 17 were the most frequent ones but a considerable amount of samples with indigenous haplotype 1 or haplotype10 with presumed origin in Southwestern Europe was also present. A clear association between haplotype 2 and late bud burst was detected in adult stands and in a field trial established with seeds from Slavonian and indigenous oak stands.
The information about the haplotype composition in all Slavonian stands can be used as reference for the certification of reproductive material. The analysis of cpDNA haploytpes in old oak stands that had been established before the introduction of foreign seed material can give valuable information for the identification of indigenous oak stands.

Introduction

Slavonian pedunculate oaks had been introduced into Germany (Münsterland, North Rhine-Westphalia) in the second half of the 19th century when extensive seed trade by railway started. Historical documents and genetic marker analyses using chloroplast DNA indicated that Slavonian oaks originated from the lowlands of the rivers Save and Drava in today's Croatia (GAILING et al., 2007; GEHLE, 1999; HESMER, 1955; WACHTER, 2001). Slavonian oaks in Germany are characterized by late bud burst, fast growth and long clear boles when compared to indigenous oaks that had been planted at the same time in the same stand (GAILING et al., 2003; WACHTER, 2001).
Earlier studies indicated that stands established with Slavonian oaks flushed two or three weeks later than neighboring indigenous oak stands, possibly resulting in better resistance to late frosts and lower susceptibility to pests (e.g. feeding damage by the European oak leaf roller *Tortrix viridana*) (GAILING et al., 2003; WACHTER, 2001). However, the number of presumably Slavonian stands that have been characterized at chloroplast DNA markers and for bud phenology was rather limited.
Chloroplast DNA (cpDNA) markers can give valuable information about the geographic origin of oaks and other angiosperms. The chloroplast genome is uniparentally inherited in maternal lineages; usually, recombination does not occur. Thus, patterns of seed dispersal can be recognized at cpDNA markers (DUMOLIN-LAPÈGUE et al., 1998).
An European-wide inventory of more than 2600 populations of white oaks using PCR-RFLPs of four non-coding chloroplast regions (PETIT et al., 2002b) revealed a total of 45 haplotypes with a characteristic distribution in Europe (PETIT et al., 2002b).
In a former study we adapted chloroplast microsatellites (DEGUILLOUX et al., 2003; WEISING and GARDNER, 1999) for the analysis of oak stands in the Münsterland region. By applying these markers we were able to distinguish most chloroplast haplotypes and found complete congruence with the PCR-RFLP procedure (GAILING et al., 2007). These and an additional new marker were used in the present study in order to determine the haplotype composition of all putative Slavonian oak stands in North Rhine-Westphalia (Germany). Three control stands were included in the analysis that have been identified to be indigenous according to their growth habit, bud phenology and /or due to the early stand establishment before the introduction of Slavonian oaks into Germany. Here, we present the results of the cpDNA analysis of 33 newly investigated stands. Haplotype frequency and diversity within and among stands were analysed by including data from 17 formerly analysed stands (GAILING et al., 2003; GAILING et al., 2007).
The aims of the present study are to give an overview of the haplotype composition of all pedunculate oak stands with presumably Slavonian origin in North Rhine-Westphalia and to determine the time frame in which stands with different origins (haplotypes) have been established. More specific aims are the development of reliable and easy-to-use genetic markers as a basis for the certification of reproductive material of Slavonian oaks in Germany and to lay a basis to distinguish between indigenous and introduced plant material. Finally, we want to test for an association between geographic origin of oaks (identified by the chloroplast haplotype) and the putatively adaptive character bud burst.

Materials and methods

Plant material

A total of 50 stands (mean of 18.2 samples per population) of *Quercus robur* in the Münsterland and from the Lower Rhine region have been studied including all putative stands of Slavonian oak (*Quercus robur* ssp. *slavonica*) in North Rhine-Westphalia (Tab. 4). Most of them have been characterized as Slavonian oaks according to their growth habit, bud phenology (late flushing) and according to historical documents. Samples from one stand (Croatia) were collected directly in Croatia (Vinkovsi). Three of the stands were characterized to be indigenous *Q. robur* according to growth habit and historic documents and were used as references. Seventeen of the stands were included in earlier studies (GAILING et al., 2003; GAILING et al., 2007). Additionally, four provenances of presumably Slavonian origin grown in a field trial in Dormagen (Abt. 33C/F, Frh. v. Nagel-Doornick; Abt. 35C1; Abt. 161 A1, Frh. v. Boeselager; Abt. 343a3, FA Peine) and a reference stand (Frh. v. Vittinghoff-Schell) were characterized at chloroplast DNA markers and for bud phenology (20 trees per

provenance). The field trial was established in 1993 with seedlings grown from seed material that was collected in stands in 1988.

DNA isolation

Total genomic DNA was extracted from fresh leaves (a small slice of about 1 cm^2) or from buds with the DNeasy Plant Kit for 96 probes from Qiagen (Hilden, Germany). The amount of DNA was checked on 1% agarose gels after staining with ethidium bromide.

PCR-RFLP technique

The following non-coding regions of the chloroplast genome *trnD-trnT* with *Taq*I (*DT*), *trnC-trnD* with *Taq*I (*CD*), *psa-trnS* with *Hinf*I (*AS*) were studied with the PCR-RFLP technique (DEMESURE et al., 1995). The PCR profile was as described by DEMESURE et al. (1995). PCR amplification was performed in a 15 µl volume containing about 10 ng template DNA, 1x Qiagen PCR buffer; 1x Q-solution (Qiagen), 2 mM of $MgCl_2$, 0.20 mM each dNTP (Fermentas); 0.5 µM of each primer and 1U *Taq* DNA polymerase (Qiagen). The restriction reactions were performed by adding 5 µl of PCR product to a mix containing 3.5 µl H_2O, 1.0 µl 10x enzyme buffer (Roche) and 5 units of the enzyme. The reactions were incubated for 5h at 37°C (*Hinf*I) and at 65°C (*Taq*I). The restriction fragments were separated on a 8% polyacrylamid (PAA) gel, stained with SYBR Gold (Molecular probes) and visualized under UV light. The interpretation of the restriction pattern followed PETIT et al. (2002b). Control probes were used to assign the resulting patterns to haplotypes described in the European-wide inventory of oak species at cpDNA markers.

Chloroplast microsatellites

Thirteen chloroplast microsatellites developed for dicotyledonous angiosperms (WEISING and GARDNER, 1999) or specifically for oak (DEGUILLOUX et al., 2003) were tested for polymorphisms (GAILING et al., 2007). Additionally one cpSSR marker (*odt*) has been developed from sequencing the *trnD-trnT* region of the chloroplast genome. The sequencing reactions were carried out with the Big Dye terminator v. 1.1 Cycle sequencing kit (Applied Biosystems). Primers were designed using the software primer 3 (ROZEN and SKALETSKY, 2000). In total, six cpSSRs were polymorphic in our sample (Tab. 1). Two of these markers (*ucd4*, *udt4*) were sufficient to distinguish between all main haplotypes detected by the PCR-RFLP method (GAILING et al., 2007). Only H5 and H7-26 could not be distinguished by cpSSRs. However, they were easily distinguishable by amplification of the *trnD-trnT* region and restriction with *Taq*I. Differences in the restriction pattern were clearly recognized on a 2% agarose gel.

Data analysis

As in most angiosperms the chloroplast genome in oaks is maternally inherited reflecting the dispersal by seeds (DUMOLIN-LAPÈGUE et al., 1998). Since it is equivalent to a single haploid locus the following analyses are based on the haplotype. Haplotypes were determined on the basis of the combination of different chloroplast microsatellite markers (see Tab. 2). Absolute haplotype frequencies were determined in each stand. Haplotype frequencies were used as input to calculate within-population diversity (H_S) and total diversity of haplotypes (H_T) in the program RAREFAC (PETIT et al., 1998). Likewise, allelic richness as a measure adjusting for different sample sizes was calculated after rarefaction in the program RAREFAC (PETIT et al., 1998) with a rarefraction size equalling the smallest sample size. G_{ST} $((H_T\text{-}H_S)/H_T)$ was calculated for the partitioning of diversity among stands. R_{ST} was also calculated as a similar measure of population differentiation that is derived from estimates of allelic richness (PETIT et al., 1998).

Assessment of bud burst

Bud burst was scored on a scale ranging form 0 (buds closed) to 5 (leaves fully expanded). When samples were collected in the mid of May 2006, stands with extreme phenotypic values for bud burst were assessed (all trees of a given stand without leaves, or all leaves fully expanded).

The experimental plot in Dormagen has been established with seedlings from four different stands of presumably Slavonian origin and one reference stand (Vittinghoff-Schell) in year 1993. Pronounced differences in the dating of bud burst have been detected with late and early flushing trees growing side by side. Early and late flushing trees were randomly selected on April 30th in 2007. Bud phenology was assessed for individual trees using a scale from 0 (buds closed) to 5 (leaves fully expanded). The haplotype of each tree was determined as described above.

Results

PCR RFLPs

Combining the information from chloroplast regions *CD*, *DT* and *AS* a total of 7 haplotypes could be distinguished that corresponded

Tab. 1: Characterization of chloroplast microsatellites

locus name	primer sequences (5'-3')	repeat	size (bp)	n_a	T_a (°C)	location	author
udt4	FAM-GATAATATAAAGAGTCAAAT CCGAAAGGTCCTATACCTCG	$(A)_9$	144-145	2	touchdown	Intergenic trnE-trnT	Deguilloux et al. 2003
ucd4	FAM-TTATTTGTTTTTGGTTTCACC TTTCCCATAGAGAGTCTGTAT	$(T)_{12}$	93-96	4	touchdown	Intergenic ycf6-psbM	Deguilloux et al. 2003
ukk4	HEX-TTGTTTACCTATAATTGGAGC TAGCGGATCGGTTCAAAACTT	$(T)_9$	109-110	2	53	Intergenic matK-trnK	Deguilloux et al. 2003
ccmp2	FAM-GATCCCGGACGTAATCCTG ATCGTACCGAGGGGTTCGAAT	$(A)_{11}$	233-234	2	50	5'to trnS	Weising and Gardner 1999
ccmp10	HEX-TTTTTTTTTAGTGAACGTGTCA TTCGTCGDCGTAGTAAATAG	$(T)_{14}$	111-112	2	52.5	Intergenic rp12-rps19	Weising and Gardner 1999
odt	FAM-GAGCGTCTCGCAAATTGTTA TTACCGCTGTTCATTTGCTC	(A11)	228-229	2	51	Intergenic trnD-trnT	this study

Tab. 2: Characterization of chloroplast haplotypes with microsatellites. The size of the fragments is given in base pairs (bp)

Haplotype	*ucd4*	*udt4*	*ukk4*	*odt*	*ccmp2*	*ccmp10*
1	95	145	110	228	233	112
2	93	145	110	228	233	111
4	95	144	109	229	233	112
5a	94	144	109	229	233	112
5b	94	144	109	228	233	112
7-26	94	144	109	229	233	112
10-11-12	95	143	109	228	234	111
17	96	145	109	228	234	111

ccmp2, *ccmp10* (WEISING and GARDNER, 1999), *ucd4*, *udt4*, *ukk4* (DEGUILLOUX et al., 2003), *odt*. Haplotype 5 und haplotype 7-26 can be distinguished by the primer-enzyme combination *trnD-trnT Taq*I. No distinction was made between southwest European types H10, H11 and H12 (designated as H10* in the text). Haplotypes 7 and 26 (H7-26) are closely related most likely due to a post-colonisation mutation event (PETIT et al., 2002a) and can not be distinguished with chloroplast microsatellites.

to haplotypes H1, H2, H4, H5, H7-26, H10* and H17 of the European-wide inventory (PETIT et al., 2002b). Haplotypes 7 and 26 (H7-26) are closely related most likely due to a post-colonisation mutation event (PETIT et al., 2002a) and and are summarized as H7-26 in the following.

The comparison with the distribution of haplotypes in Europe showed that H1, H4 and H10* are described for the study area in Germany (Münsterland and Lower Rhine region) but are missing in the Balkan region (BORDÁCS et al., 2002; KÖNIG and STAUBER, 2004; KÖNIG et al., 2002; PETIT et al., 2002b). H1 is the most frequent type in the western part of Germany and dominant in central Europe. It had its glacial refugia most likely in southern Italy (PETIT et al., 2002a). H10* reveals a center of distribution in the southwest and west of Europe with presumed refugia on the Iberian Peninsula. H4 rarely occurs, mainly in Germany, Hungary, Poland and Romania (PETIT et al., 2002b).

H2 and H17 are frequent in Croatia but do not occur naturally in Germany. H5 has a center of distribution in the Balkan region but is comparatively rare in Germany. In western Germany natural populations with this haplotype are apparently missing (KÖNIG and STAUBER, 2004). H5 was also found in all samples of the reference stand from Croatia (Tab. 4)

H2, 5, 7-26, 17 have a center of distribution in the Balkan region (BORDÁCS et al., 2002; PETIT et al., 2002b) but none of the haplotypes is restricted to this region. While haplotype H7-26 is mainly concentrated in low mountain ranges in the western part of Croatia, haplotypes 2, 5, and 17 occur in the lowlands of the river Save between Zagreb and the Serbian border. H2 was also found in the Dinarides in Croatia. Only haplotype 7-26 occurs both in natural stands in the study area (KÖNIG and STAUBER, 2004), and in populations from the Balkan region, but it is comparatively rare (n= 20) in the studied stands.

Identification of haplotypes with cpSSRs

All but haplotypes H5 and H7-26 could be distinguished with the combination of chloroplast microsatellites *ucd4* and *udt4* (Tab. 2). These two variants can be separated by PCR-RFLPs of *trnD-trnT* with *TaqI*. The results of the cpSSR method were fully congruent with the results obtained from the PCR-RFLP procedure (see also GAILING et al., 2007).

Additionally, two variants of what was hitherto haplotype 5 were identified at chloroplast microsatellite *odt* (Tab. 2). Both types showed about the same frequency in the analysed stands. Most of the variation of those two types is distributed among populations (G_{ST} =0.72, calculated for populations where H5 is dominating). A higher frequency of one or the other type in stands with predominant haplotype 5 is indicated by asterisks in Tab. 4.

Variation within and among stands at cpDNA markers

Complete haplotype information has been obtained for a total of 910 adults trees in 50 stands. The most frequent haplotype was H5 (n = 405) followed by H2 (n=169), H1 (n=154), H10* (n=93), H17 (n = 54), H7-26 (n = 21) and H4 (n = 14). Most stands either showed only one haplotype (15 stands) or they are characterized by one predominant haplotype, a result that is reflected by high differentiation values among stands (G_{ST} = 0.674, R_{ST} = 0.734). The mean haplotype diversity and allelic richness within stands was 0.243 and 1.85, respectively. Thirteen stands had a haplotype diversity higher than 0.5 (probability to encounter two different haplotypes in a stand) due to a mixture of two or more (up to four) haplotypes per stand.

Stands with predominant haplotypes 2 or 5 show the lowest haplotype diversity and allelic richness when compared to stands with other dominating haplotypes. Haplotypes 2 and 5 were the predominant types in eight and 20 stands, respectively. The indigenous haplotype 1 was the most frequent variant in six stands, and three additional stands (controls) were fixed on this type. This indigenous haplotype was detected in several stands in low frequency (Tab. 4).

The oldest stand analysed established between 1826 and 1864 when seed transfer was less extensive revealed the indigenous haplotype 1. The Slavonian haplotypes 2 and 5 occurred in stands established between 1880 and 1895 as predominant types (Tab. 3). Another Slavonian type (H17) that is not indigenous to Germany occurred as the dominant type in six stands established between 1886 and 1912, the most recent one (Kottenforst 154B, 1912) was established from nursery-produced seedlings.

A long period of time during which a stand had been established was not always associated with high haplotype diversity. For example, stand 76A (Cappenberg) was fixed on the indigenous type 1 and had been established between 1826 and 1864.

However, while nine out of 20 stands established by direct sowing were fixed on only one haplotype, the five stands established from young nursery-produced seedlings showed a mixture of haplotypes reflected in higher haplotype diversity (H_S = 0.468 (0.279-0.611)) as compared to stands established from seeds (H_S = 0.232 (0 - 0.659)).

Association of cpDNA haplotypes with bud burst

Late bud burst was mainly observed in stands with predominant haplotype 2 (Tab. 4). In stand „Steprath“ trees with haplotypes 1, 10

Tab. 3: Estimate of haplotype diversity for stands with different dominating haplotypes

Dominant haplotype	year dominant type	Year	S	N	H_S average	H_S range	N_A	N_A range
H1	1826-1905	1826-1907	7	154	0.237	0 - 0.659	0.91	0 - 2.43
H2	1880-1894	1880-1894	8	166	0.175	0 - 0.363	0.78	0 - 1.76
H5	1880-1895	1878-1912	20	406	0.142	0 - 0.611	0.58	0 - 2.48
H7-26	1894	1881-1894	1	20	0.503		1.55	
H10*	1886-1907	1880-1907	6	93	0.321	0 - 0.753	1.03	0 - 2.74
H17	1886-1912	1880-1912	6	54	0.541	0.485 - 0.602	1.42	1 - 1.90

H_S: genetic diversity of haplotypes; N_A: allelic richness, S: number of stands where the haplotype is dominating, N: total number of samples with the respective haplotype. H4 occurs in 14 samples and dominates in none of the stands. H4 occurred at low frequency in stands established between 1886 and 1912.

and 7-26 (n=4) showed completely unfolded leaves in early May 2006, while buds of trees with haploytpe 2 were still mostly closed (n =16). The same observation had been made in stands with mixed haplotype composition of individual trees in the Münsterland region (GAILING et al., 2003). Also in the experimental plot of Dormagen where Slavonian and indigenous oaks grow in the same environment, a strong association of Slavonian haplotype 2 with late bud burst was detected in early May 2007 (bud stage 3.56 ± 1.64, n = 32). Plants with haplotype 2 originating from stand 161A1 (Freiherr von Boeselager) revealed an even later bud burst (bud stage 2.43 ± 1.87, n = 12) than plants from stand 343a3 (Forstamt Peine, bud stage = 4.35 ± 0.81, n = 20). A few plants with Slavonian haplotype 5 show a slightly earlier bud stage (bud stage 4.70 ± 0.47, n = 27) than plants with indigenous haplotype 1 (n = 22) or southwest European haplotype 10* (n = 9, all bud stage 5, leaves completely unfolded).

Discussion

Certification of reproductive material of Slavonian oaks

Stands that had been established in the late 19th century with plant material from Croatia (Slavonian stands) show several favourable characteristics as fast growth, a long clear bole, and late bud burst that may result in a lower susceptibility to late frost and pest damage (WACHTER, 2001). On the other hand, seed set and natural regeneration in Slavonian oaks in Germany is lower than in indigenous pedunculate oaks possibly due to the colder climate in the study area (lower annual mean temperatures) and/or restricted gene flow between indigenous and Slavonian oaks. For example, it was shown that flushing dates of indigenous oaks and Slavonian oaks grown side by side are nearly non-overlapping (GAILING et al., 2003). However, gene flow between Slavonian oaks and indigenous oaks and the effect on potentially adaptive characters as growth characteristics and seed production has not yet been studied.

The data on the haplotype composition available for all oak stands of Slavonian origin lays the basis for the certification of reproductive material from these stands. Since cpDNA markers are maternally inherited in angiosperms with all seeds and natural regeneration showing the same chloroplast information as their mother tree, they show high uniformity within but a clear differentiation between stands. This is especially true for barochorous, wind-pollinated tree species such as oaks where seed dispersal is quite restricted if compared to dispersal by pollen. Thus, chloroplast markers are specifically suited to test and falsify the origin of reproductive material of Slavonian and indigenous oaks. Since many stands of Slavonian oaks with a specific haplotype (H2) are uniform for favourable growth and stem characteristics and bud phenology (WACHTER, 2001), the analysis of seeds and young plants may allow to conclude on the phenotype of adult trees (see below experimental plot in Dormagen). However, gene flow by pollen from neighboring stands may affect potentially adaptive traits as bud burst (see below) or growth rate and other yield characteristics.

The analysis of the highly informative cpSSRs markers allows the identification of the chloroplast haplotype even in older material or wood samples with highly degraded DNA (see below). An accurate distinction of small informative size differences can be performed in high-resolution capillary electrophoresis. By using internal standards an unambiguous distinction between chloroplast haplotypes is easily achievable (GAILING et al., 2007).

Identification of Slavonian and indigenous oak stands

Evidence for the establishment of stands with non-indigenous material is unambiguous for stands showing predominant haplotypes 2 or 17 that do not occur naturally in Germany. For stands showing predominant haplotypes with a wider distribution range in Europe a clear distinction between indigenous and introduced material based on cpDNA alone is not possible. Especially in Germany the diversity of different haplotypes is comparatively high due to a mixing of cpDNA lineages from different glacial refugia (PETIT et al., 2003). For example, H10* is most abundant in southwestern and western Europe but is a rarer occurrence in the study area. However, for at least one stand with H10* the establishment with non-autochthonous material is well-documented (159A in Westtünnen). This stand is characterized by late bud burst and late leave fall, a combination of characters that is not found in indigenous and Slavonian stands in the same area and that may be interpreted as an adaptation to different climatic conditions in southwestern Europe (GAILING et al., 2003). Also H5 that was identified as the most common haplotype in the Slavonian stands of the study area does also naturally occur in Germany but according to historical documents and cpDNA analyses is most likely absent in natural stands of the study area (KÖNIG and STAUBER, 2004; KÖNIG et al., 2002). Since extensive seed transfer started with the expansion of railway connections in the second half of the 19th century, allochthonous oak populations are unlikely to be found among those established before 1860. The cpDNA analysis confirmed that seed material from Slavonian oaks was introduced only during the second half of the 19th century with the earliest occurrence of the Slavonian haplotypes H2 and H5 in 1880 and 1878, respectively. According to historical documents and cpDNA haplotype information Slavonian stands were established (from seeds) in Germany in the Münsterland and Lower Rhine region between 1878 and 1903. The further analysis of the haplotype composition of stands established before that time might help to identify cpDNA haplotypes that are characteristic for stands indigenous to the study area in the Münsterland and Lower Rhine regions.

Since cpSSR primers amplify short informative regions of the

Tab. 4: Haplotype frequencies in *Quercus robur* stands

Forest district	stand	year	area (ha)	planting/ sowing	collection	chloroplast haplotypes								
							H1	H2	H4	H5	H7	H10*	H17	
FA Bergisch Gladbach, Königsforst	76B	1891	5.0	seeds	buds closed	May-06	0	19	0	0	0	0	0	H2
FA Bergisch Gladbach, Königsforst	127C	1887	3.4	seeds	bud stage 5	May-06	0	0	0	20*	0	0	0	H5
FA Bonn, Kottenforst	10B2	1893	1.5	plants		May-06	2	0	0	16	1	1	0	H5
FA Bonn, Kottenforst	37C	1907	2.2	seeds		May-06	8	0	0	1	0	9	0	H10*
FA Bonn, Kottenforst	40B	1907	2.2	seeds	low growth	May-06	0	0	0	0	0	19	0	H10*
FA Bonn, Kottenforst	70D	1903	2.5	seeds		May-06	0	0	0	9*	0	0	10	H17
FA Bonn, Kottenforst	85A	1912	3.7	plants	bud stage 5	May-06	0	0	0	4	0	0	8	H17
FA Bonn, Kottenforst	85B	1905	4.1	seeds		May-06	10	0	4	0	0	2	0	H1
FA Bonn, Kottenforst	85D	1904	8.6	seeds		May-06	0	0	0	0	0	19	0	H10*
FA Bonn, Kottenforst	89A[a]	1904	8.4	seeds		May-06	7	0	0	1	0	1	5	H1
FA Bonn, Kottenforst	95B	1903	2.8	seeds		May-06	0	0	0	4	0	12	4	H10*
FA Bonn, Kottenforst	134A/C	1902/1900	6.1	plants/seeds	low growth	May-06	5	0	6	2	0	7	0	H10*
FA Bonn, Kottenforst	154B	1912	3.2	plants		May-06	0	0	3	5	0	0	11	H17
Freiherr von der Leyen	17C	1885	7.1	?	bud stage 5	May-06	1	0	0	15*	0	3	0	H5
Fürst zu Salm-Salm, Rhede	105/4A1/A7	?		?		May-06	17	0	0	0	0	3	0	H1
Gut Ulenburg	4C	?	1.6	?		May-06	17	0	0	0	1	1	0	H1
Stadt Viersen	36B/38	1886	16.7	seeds		May-06	0	0	1	18	0	0	0	H5
Steprath	3H	1881	0.5	?	buds mostly closed	May-06	1	16	0	0	1	2	0	H2
Letmathe, H. Blix, Cappenberg	Flur 2/155	1888	1.0	?		May-06	0	0	0	20**	0	0	0	H5
Letmathe, Estermann	116A1	1894	2.2	?		May-06	1	17	0	0	1	1	0	H2
Letmathe, Graf v. Kanitz	77C	1883	4.4	seeds		May-06	2	0	0	18*	0	0	0	H5
Letmathe, Graf v. Kanitz	76A	1826-1864	9.5	?		May-06	20	0	0	0	0	0	0	H1
Letmathe, Graf v. Kanitz	19A	1883	1.6	seeds		May-06	0	0	0	16*	0	0	0	H5
Letmathe, Graf v. Kanitz	32H/39A	?	2.0	?		May-06	0	0	0	20**	0	0	0	H5
Letmathe, Frhr. v. Boeselager	159A	1886	2.2		late bud burst, late leave fall	Apr-02	0	0	0	0	0	10	0	H10*
Letmathe, Frhr. v. Boeselager	159B	1887	3.3		early bud burst	Apr-02	9	1	0	0	0	0	0	H1
Letmathe, Frhr. v. Boeselager	160B	1887	2.0		early bud burst	Apr-02	10	0	0	0	0	0	0	H1
Letmathe, Frhr. v. Boeselager	161A1	1887	1.2	seeds	late bud burst	Apr-02	0	8	0	2	0	0	0	H2
Letmathe, Frhr. v. Boeselager	161A2	1879	1.0	seeds	intermediate bud burst	Apr-02	0	4	0	4	0	2	0	H2/H5
Letmathe, Schulze-Becking	54B1/B2	1880	1.5			May-06	0	6	0	13**	0	0	1	H5
Münsterland, BR Deutschland	1B1a	~1894	0.4	plants	late bud burst	Jul-05	0	17	0	1	2	0	0	H2
Münsterland, BR Deutschland	1B1b	~1890	0.5	plants	intermediate bud burst	Jul-05	2	4	0	12	0	0	2	H5
Münster, Ziebell, Lüdinghausen	Flur 1/299	1880	4.0	?		May-07	0	19	0	0	0	1	0	H2
Obereimer, Gr. von Plettenberg	14D1	1890?	1.0	?		May-07	10	0	0	9	1	0	0	H1
Obereimer, Gr. von Plettenberg	104A	1895	1.8	?		May-06	1	0	0	19**	0	0	0	H5
Obereimer, Gr. von Plettenberg	106G	1888	2.9	?		May-06	0	0	0	19**	0	0	0	H5
Obereimer, VEBA	43	~1894	0.7	?		Jul-05	0	18	0	1	1	0	0	H2
Warendorf, Frh. v. Nagel-Doornick	13A	1886	1.5	?		Jul-05	2	0	0	5	0	0	12	H17
Warendorf, Frh. v. Nagel-Doornick	24B	~1887	1.8	seeds		Jul-05	0	0	0	17	1	0	0	H5
Warendorf, Frh. v. Nagel-Doornick	33C	1878	1.3	seeds		Jul-05	0	0	0	20	0	0	0	H5
Warendorf, Frh. v. Nagel-Doornick	33D	1879	1.9	seeds		Jul-05	20	0	0	0	0	0	0	H1
Warendorf, Frh. v. Nagel-Doornick	33F	1880	0.8	seeds		Jul-05	0	0	0	20	0	0	0	H5
Warendorf, Frh. v. Nagel-Doornick	36E	~1882	1.5	seeds		Jul-05	0	20	0	0	0	0	0	H2
Warendorf, Frh. v. Nagel-Doornick	50B	~1883	1.6	seeds		Jul-05	2	0	0	17	0	0	0	H5
Warendorf, Schulze-Pellengahr	4H	1893	1.2	?		May-06	0	0	0	20**	0	0	0	H5
Warendorf, Schulze-Sutthoff	Flur 2/77	~1890	0.6	?		Jul-05	0	0	0	19	0	0	0	H5
Warendorf, Suttorp, Everswinkel	Flur 11/129	?	2.0	?		May-07	0	20	0	0	0	0	0	H2
Warstein-Rüthen, Kirche Anröchte	32C	1894	4.2	?		May-06	5	0	0	1	12	0	0	H7
Westkirchen, Quante	Flur 2/90-92	~1889-1890	2.2	seeds		Jul-05	2	0	0	17	0	0	1	H5
Croatia, Vinkovsi		2007				May-06	0	0	0	20**	0	0	0	H5
sum haplotypes							154	169	14	405	21	93	54	

[a]: samples were collected in the southwestern part of the stand. H7 is a shortcut for H7-26. Stand were either established by direct sowing (seeds) or from nursery-produced seedlings (plants).

*: higher frequency of H5a

**: higher frequency of H5b

chloroplast genome that are present in high copy numbers per cell in comparison with nuclear DNA markers, those markers can also be analysed in highly degraded samples (for example old wood samples) (DEGUILLOUX et al., 2004; RACHMAYANTI et al., 2006). The analysis of old and / or prehistorical wood samples at these markers might give additional information on the haplotype composition in oak stands before extensive long-distance seed transfer started. More recent studies indicate that authentic DNA can be retrieved from wood samples up to 1,000 years of age (LIEPELT et al., 2006).

Association between haplotype and phenotype

Slavonian provenances with haplotypes 2 and 5 of pedunculate oak showed an up to three week later bud burst than neighboring indigenous oak stands in the Münsterland region (GAILING et al., 2003). These differences were found in four consecutive years where trees with H2 showed an even later bud burst than those with H5 (GAILING et al., 2003). In the present study bud burst has been assessed on a fixed date (April 30th) in a field trial in Dormagen containing plants with Slavonian, indigenous and southwest European haplotypes. While a strong association of haplotype 2 with later bud burst was detected, only minor differences in flushing date were recorded between individuals with Slavonian haplotype 5 and non-Slavonian haplotypes, possibly due to the assessment of the trait relatively late in spring. Progenies from stand 161A1 (Freiherr von Boeselager) with uniform late bud burst (GAILING et al., 2003) also showed the latest bud burst in the field trial reflecting a putatively strong genetic component (heritability) of the trait. Since also other stands with Slavonian haplotypes show a uniformly later bud burst than neighboring indigenous oak stands (GAILING et al., 2003; GAILING et al., 2007; WACHTER, 2001), underlying genetic differences have possibly evolved in response to different environmental (climatic) conditions in the regions of origin. While chloroplast markers can indicate geographic origin, they are obviously not directly involved in the control of bud burst. Thus, due to the wide geographic distribution of most haplotypes, a prediction of the phenotype only from the haplotype information is in most cases not possible. Accordingly, KREMER et al. (2002) found in general no significant impact of chloroplast haplotype (maternal origin) on phenotypic traits such as bud burst in a test with 16 provenances of *Q. petraea*. The association between haplotype and late bud burst found for Slavonian stands in this study is most likely due to the fact that Slavonian oaks had been introduced into Germany from a restricted area in the lowlands of the rivers Save and Drava with similar environmental conditions. In order to resolve the genetic basis of adaptive differences in bud burst, an intraspecific crosses between adults trees of Slavonian and German origin with pronounced differences in flushing date have been produced. QTLs (Quantitative Trait Loci) will be located on genetic linkage maps calculated in a progeny of 192 full-sibs (GAILING, submitted).

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