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Chloroplast DNA variation of oaks in western Central Europe and genetic consequences of human influences

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Abstract

Oak chloroplast DNA (cpDNA) variation was studied in a grid-based inventory in western Central Europe, including Belgium, The Netherlands, Luxembourg, Germany, the Czech Republic, and the northern parts of Upper and Lower Austria. A total of 2155 trees representing 426 populations of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. were screened for polymorphism in up to four PCR-amplified cpDNA fragments. Eleven haplotypes belonging to four lineages were detected; these lineages were formerly restricted to glacial refugia in the Iberian Peninsula, the Apennine Peninsula and the Balkan Peninsula. The haplotypes originating from the Apennines are particularly well represented in the study region, but there is also a significant contribution from the other refugia, which explain the high overall level of cpDNA diversity. The strong human impact in western Central Europe during the past centuries, which has resulted in the clearance of most forests, was followed by reforestation, sometimes involving seed transfers. Despite this strong human impact, broad geographic patterns of lineages and haplotypes could still be detected. To evaluate further the consequences of the former human activities on the present day oak cpDNA genetic structure, four regions where increasingly strong human impact was anticipated (ranging from hilly regions in southern Germany to roadsides plantations in The Netherlands) were selected. There, a comparison of the levels of intrapopulation cpDNA diversity and spatial structuring was made. Over the whole area, within stand diversity was significantly higher in *Q. robur* than in *Q. petraea* ($h_S = 0.24$ vs. 0.16). Since total diversity is identical for both species, this results in a significantly lower level of fixation for *Q. robur* than for *Q. petraea* ($G_{ST} = 0.68$ vs. 0.79). The analyses also reveal a decrease of fixation with increasing human impact on oak populations. The Dutch roadside plantations (*Q. robur*) exhibit a very low level of fixation ($G_{ST} = 0.28$) as compared to *Q. petraea* in southern Germany ($G_{ST} = 0.91$). The significance of the spatial genetic structure was tested using geostatistical methods. For the complete data set, a strong spatial genetic structure is confirmed, with higher than average genetic similarities between populations distant from up to 270 km, whereas there is no spatial structure in the roadside plantations in The Netherlands or in the northern German lowlands. These results should help to differentiate introduced from autochthonous populations, and provide a framework for the identification of the geographic origin of seed lots. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Q. robur*; *Q. petraea*; PCR-RFLPs; Haplotypes; Spatial genetic patterns; Genetic differentiation; Deforestation; Seed transfer

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1. Introduction

The distribution of tree species in Europe during the last ice-age has been classically studied by mapping pollen frequencies obtained from sediment cores taken in peat bogs or lakes (e.g., Firbas, 1949; Huntley and Birks, 1983). Due to low temperatures and aridity during the ice-age, many trees could only survive in limited areas in the Mediterranean region, so-called refugia, where a favourable climate existed. Oaks, in particular, were confined to three main refugia in the Iberian Peninsula, the Apennine Peninsula and the Balkan Peninsula, from where re-colonisation took place (Huntley and Birks, 1983; Brewer et al., 2001).

Several investigations have also demonstrated the value of molecular genetic markers for the identification of post-glacial colonisation routes of oaks in Europe. In many countries in Europe, marked regional geographic patterns based on chloroplast DNA (cpDNA) polymorphisms, with a single haplotype or a mixture of related haplotypes predominating over large areas, have been described (e.g., Ferris et al., 1993; Petit et al., 1993, 2002a; Dumolin-Lapègue et al., 1997a, 1998; Cottrell et al., 2002; Olalde et al., 2002). On the other hand, in Central Europe, multiple colonisation from different refugia may have resulted in a mixture of haplotypes belonging to different lineages due to the geographic situation not far away from the Iberian Peninsula, the Apennines and the Balkans. In addition, a particularly strong human impact on oak forests may have further blurred the original genetic structure in this region.

The objective of this study is, therefore, to analyse cpDNA variation in *Q. robur* L. and *Q. petraea* (Matt.) Liebl. in western Central Europe including Belgium, Luxembourg, The Netherlands, Germany, the Czech Republic and parts of Austria, in order to try to reconstruct the post-glacial history of oaks, to identify the original re-migration routes and to evaluate the influence of human activities on the cpDNA structure within and among populations. This study greatly extends previous preliminary investigations in the region (Dumolin-Lapègue et al., 1997a; König et al., 1998).

Today's cultural landscape is the result of human impact upon natural ecosystems. The area of native oak stands has been reduced considerably in the region

studied, either as a consequence of deforestation, or through the transformation of former broadleaved forests into conifer forests. In addition, artificial seed transfers may have directly influenced the genetic structure of oak forests. For conservation purposes, and in order to achieve a sustainable management of forest genetic resources, it is necessary to distinguish between autochthonous and allochthonous populations. Until recently, the origin of planted forests could only be traced back on the basis of historic documentation but the advent of cpDNA techniques may help to distinguish allochthonous from autochthonous populations. To check this, spatial cpDNA variation in several smaller regions characterised by increasing degrees of human impact has been studied. In order to identify these regions, a review of the history of human activities in forests since the post-glacial in Central Europe is provided. It relies largely on the work of Firbas (1949), Krahl-Urban (1959) and Hasel (1985), as well as the more recent study of Küster (1998). It is followed by a description of the importance of oaks in the countries investigated, as well as by some preliminary indications on their degree of autochthony, thus providing a basis for the cpDNA investigations.

1.1. Overview of forest history in the study region

Optimal growing conditions for oaks prevailed between 7500 and 4500 before present (BP), given the higher temperatures present at that time. Mixed oak forests were the prevailing vegetation type in the lowlands between Lake Constance and the Harz Mountains as well as in the northern and eastern moraine landscape. Subsequently, the climate became more humid and colder, and oaks became confined to the slopes of the drier hills. Many mixed oak forests were replaced by beech, fir or spruce at higher elevations. This process was more pronounced in western Germany than in eastern Germany, where the sites are frequently drier and poorer. This natural evolution is considered to have come to an end between about 2500 and 1500 BP (Hasel, 1985).

Prior to the Neolithic (7000–4000 BP), it is unlikely that man had a significant impact on the oak woods. Then during this period, some forests growing on stoneless loess soils in the lowlands were converted into cultivated land. The natural openings in the light

oak forests were enlarged by cutting small trees and by removing emerging seedlings. The remaining oaks, however, provided an abundant supply of acorns for consumption by humans and domestic animals. Man would have started to make a significant impact during the Bronze and Iron Ages when oak was required for mining as pit timber, as a source of charcoal for use in smelters, or for the production of salt.

Additional oak forests were cut during Roman times when woods located along the Rhine and the Mosel rivers were cleared to release land for vineyards and orchards. The clearing continued during the Middle Ages to provide agricultural land and areas to settle. At that time it was begun to float wood on the rivers Rhine, Weser, Elbe and Oder from the forests in the south of Central Europe to the wood-demanding cities in the north. Oak was used for building houses, windmills, ships and harbours. Demand for oak was particularly high in the 17th and 18th centuries when intensive deforestation occurred, boosted by the development of the glass factories and the salines. By the end of the 18th century, large areas west of the river Weser had been converted to heathland, and only a few isolated regions remained wooded. Similarly, in The Netherlands, woodland covered only 1% of the country 150 years ago (de Vries, 1998a).

These large clearances resulted in a scarcity of wood. The wooded areas were too small to act as a source of regeneration for the large unwooded expanses, and artificial restoration techniques of forests had to be developed. Seeding and planting had been used since the 14th and 15th centuries (Hasel, 1985). Oaks were used for planting because of their importance as seed and timber trees. Planting of oak in coppice forests is probably the oldest method of artificial forest regeneration. The oldest record of this practice is from the region of Dortmund in 1343 and there are several records in subsequent centuries as well. In the 16th century, the forest regulations contained advice on how to carry out broadleaf seeding. In Prussia, a regulation from 1680 demands that each man has to plant six oaks before he gets married. Later, users of oak wood were obliged to participate in reforestation work, even though these constraints were probably not very effective in the restoration of the devastated forests. Sowing of coniferous species such as pine, spruce and fir proved much more successfully and considerably changed the aspect of the landscape and the original

composition of forests. In former broadleaf forest areas, such as the upper Rhine plane, pine replaced oak as the main timber species.

1.2. Present importance and status of oaks in the countries investigated

In Belgium, the total forest cover is approximately 600,000 ha, mainly concentrated in the southern region. There are 83,500 ha (18%) of oak forests in Wallony (both *Q. robur* and *Q. petraea*) and only 17,200 ha (8%) in Flanders (Jacques and de Cuyper, 1998). In Flanders, native populations are rare, but some (putative) autochthonous *Q. petraea* populations are recorded in the eastern region. In The Netherlands, oaks are dominant in 60,000 ha, i.e. 16% of the total forest area (=11%). Most of these forests are pure planted *Q. robur* stands. In addition, about 25% of line and roadside plantations are established with oak (de Vries, 1998b). In general, natural populations of oaks are very rare in The Netherlands (Maes, 1993) and even those that are described as native may be intermixed with introduced material. In Luxembourg, the total forest area is about 90,000 ha (33% of the territory), including 28% of oaks (Wagner et al., 1998).

In Germany, the proportion of oak amounts to 8.5% of the total forest area of 10.7 million hectares (BML, 1994). The main oak species are *Q. robur* and *Q. petraea* (Röhrig, 1980), but *Q. pubescens* also occurs on warm and dry sites in southwestern Germany. The western part of Germany is especially rich in oaks, the foothills of the Alps and all other regions of higher elevations in Germany have few oaks (Krahl-Urban, 1959). The presence of native and introduced oak populations in Germany is likely to be heterogeneous. In the Czech Republic, forest covers 2.63 million ha, corresponding to 33% of the national territory (Hynek, 1998). As in other Central European countries, oak is now underrepresented, covering only 6%, compared with an estimate of about 18% some centuries ago. Austria is the most densely forested country (46%) of the studied region (BMLF, 1995), but oak represents only 2.3% of standing timber volume (Schadauer, 1994). The majority of oaks are situated in southeastern and northeastern Austria and in the region north of the Alps, especially in the Danube valley where oaks represent up to 25% of the forest in east

Austria (Schume and Starlinger, 1996). In the Alpine river valleys oak is very scarce.

2. Material and methods

2.1. Sampling of plant material

In general, it was decided to use a grid system (50 km × 50 km) to select forests for sampling. To increase the probability of sampling autochthonous populations, older stands were preferentially chosen. As a rule, five trees per site were sampled at a distance of at least 50 m in order to reduce the likelihood of sampling closely related trees. In The Netherlands, 13 natural populations (five *Q. robur* and eight mixed *Q. robur*–*Q. petraea* stands) were selected, based on a survey for autochthonous oak populations (Maes, 1993). Additionally, four populations had been previously investigated. The Dutch investigations included also samples from a *Q. robur* provenance test at The Rips which comprised 34 seed sources from roadside plantations. These were not included in the general survey, but in the investigations on diversity and spatial genetic structures. In Flanders, four natural populations of *Q. petraea* and five of *Q. robur* were selected based on a similar survey (Maes and Rövenkamp, 1999). In Germany, additionally field or nursery trials with well documented provenances were used (127 provenances from provenance experiments, 84 from nurseries). In some areas (Lower Saxony and Northrhine-Westphalia), the sampling density exceeds the number of grid points, mainly as a consequence of the large representation of provenances from these regions in the field trials (the location of German Federal States is provided in Fig. 1). Also, in stands of Northrhine-Westphalia, where a high degree of human interference was anticipated, sampling density was increased and up to 10 trees per stand were sampled. In Austria, only those populations located north of the Alps were included; for sampling methods see Csaikl et al. (2002b). Initially, the species status was not checked further in the provenances sampled in the German field trials. In subsequent collections, the species was systematically determined on the basis of the sampled leaves, using a binocular and following several criteria detailed in Aas (1996) (petiole length, basal shape of lamina, number of intercalary veins and hairiness).

2.2. Laboratory methods

The analyses were made in five different laboratories: BFH in Germany (323 populations), Alterra in The Netherlands (13 natural populations and 34 roadside plantations), CLO-Gent in Flanders (Belgium) (9 populations), ARCS in Austria (41 populations) and INRA in France (for samples from Wallony (9 populations) and Luxembourg (2 populations)). Furthermore, 29 populations analysed by Dumolin-Lapègue et al. (1997a) were included.

The methods generally follow those described by Dumolin-Lapègue et al. (1997a,b), with some modifications. In Germany, minipreparations of total DNA were carried out from fresh leaves or peeled buds according to Dumolin et al. (1995), but with a final treatment with 0.5 µg RNase (Boehringer Mannheim, Germany). For DNA extraction of a subset of samples the DNeasy Plant Mini Kit (QIAGEN, GmbH Hilden, Germany) was used. For the investigations in The Netherlands DNA was extracted with the Puregene isolation kit (Gentra Systems, Minneapolis, USA), including 4% PVP-40 in the extraction buffer to remove phenolic compounds. The modifications applied in Austria are described in detail by Csaikl et al. (2002b). The primer pairs used were designed by Taberlet et al. (1991) and Demesure et al. (1995). In Germany and in France, the four primer–enzyme combinations used were DT–*TaqI*, CD–*TaqI*, AS–*HinfI* and TF–*HinfI*. In The Netherlands, Flanders and Austria the fragment TF was digested by *AluI* instead of *HinfI*, but the same insertion–deletion was scored to distinguish haplotype 10 from haplotype 11. Electrophoresis, visualisation and coding of the polymorphisms were done according to the revised description in annexes 1 and 2 of Petit et al. (2002b). As evident from earlier studies (Dumolin-Lapègue et al., 1997a), only a reduced number of haplotypes was expected in the study region. As a consequence, when diagnostic mutations were identified with two primer–enzyme-systems, the remaining fragments were not analysed in all samples.

2.3. Data analysis

Populations containing only one haplotype are referred to as monotypic, the remaining as polytypic. Genetic diversity and geostatistical analyses were

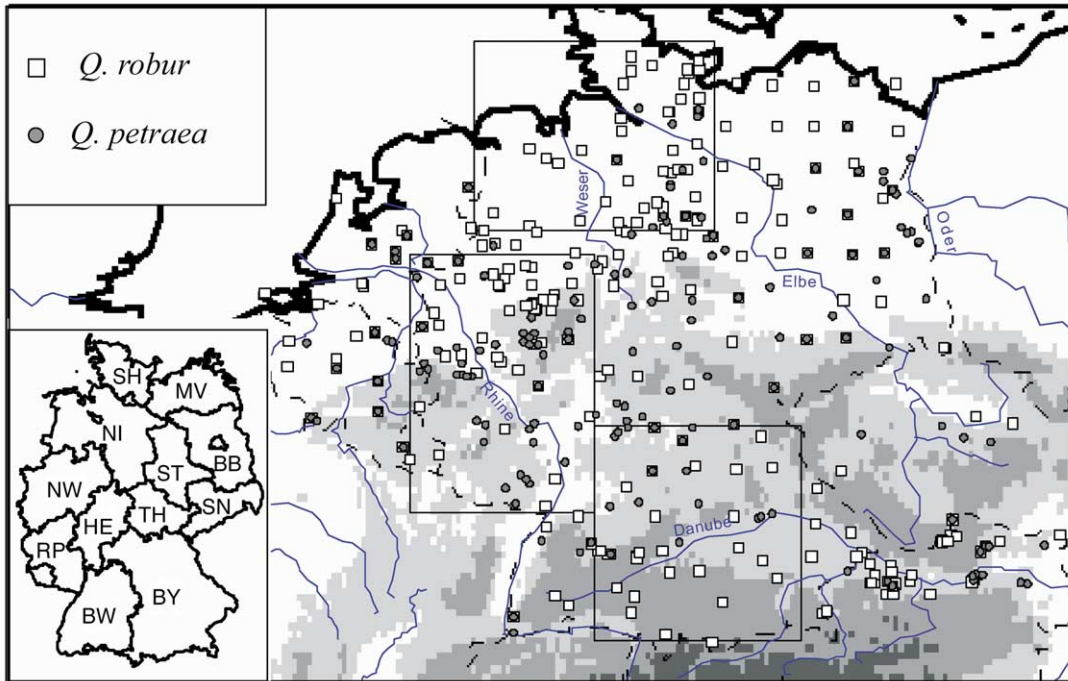


Fig. 1. The study region western Central Europe. The shaded areas indicate the hilly or mountainous regions at intervals of 250, 500 and 1000 m, respectively. Each symbol (square for *Q. robur* and circle for *Q. petraea*) marks the location of an analysed population. The three rectangles comprise selected sub-areas with different degrees of human impact (see text). The inset on the left side gives the abbreviations of German Federal States mentioned in the text—BW: Baden-Württemberg, BY: Bavaria, BB: Brandenburg, HE: Hesse; MV: Mecklenburg-Vorpommern, NI: Lower Saxony, NW: Northrhine-Westphalia, RP: Rhineland-Palatinate, SN: Saxony, ST: Saxony-Anhalt, SH: Schleswig-Holstein, TH: Thuringia.

performed and applied to the whole data set as well as to four subsets as described below. Only those populations represented by at least three trees were included in the analysis. The within-population genetic diversity h_S , the total diversity h_T , and the coefficient of genetic differentiation G_{ST} were calculated according to the method of Pons and Petit (1995). G_{ST} was estimated separately for *Q. robur* and *Q. petraea* both for the whole data set as well as for the different subsets. The difference between sets was tested against zero by the t -test, first within species and then among species of the same region. To test the existence of a spatial genetic structure, distograms were constructed. The procedure follows that described by Degen and Scholz (1998): mean pairwise genetic distances are plotted for different distance classes. The genetic distance (d) used is that proposed by Gregorius (1974), with the haplotype frequencies substituted directly into the formula. The permutation test is made according to Noreen (1989).

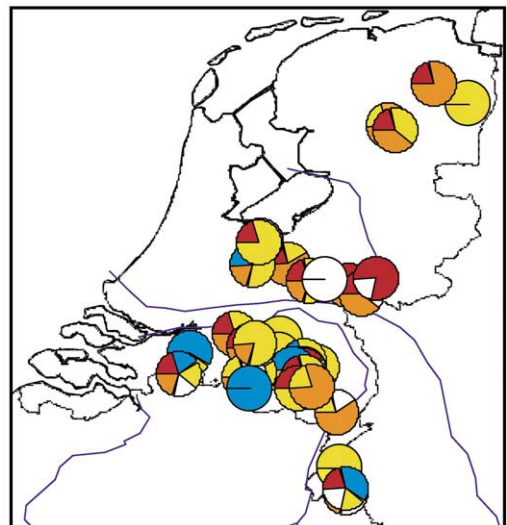


Fig. 3. Haplotype distribution in and among roadside plantations of *Q. robur* in The Netherlands. Assignment of haplotypes to colours see Fig. 4.

(For the software see: <http://kourou.cirad.fr/genetique/index.html>: Spatial Genetic Software by B. Degen.) To guarantee a sufficient number of populations in each distance class, and to enable a comparison between the four selected areas, the distogram analysis is restricted to *Q. robur* and distance classes of 30 km were selected. Confidence intervals of the genetic distances in each distance class were obtained by using 500 permutations. All maps were produced using the GIS-software Mapinfo Professional Version 3.5.

The first region selected to evaluate human impacts on the cpDNA genetic structure of *Q. robur* are The Netherlands. Only roadside plantations were included. Trees for line and roadside plantations have been subjected to mass selection for good performance and stem form for a long time, and mixtures of material originating from several nurseries is a common practice (de Vries and van Dam, 1998).

Furthermore, within Germany, three more or less even-sized areas of 60,000–70,000 km² were chosen for comparative genetic analyses (see the position of these regions in Fig. 1). The first region, the northern lowlands of Germany (52.3–54.5°N in latitude and 7.0–11.0°E in longitude) is characterised by long-term human impact. First, in this region, prehistoric activities have been documented since more than 10,000 years in the lower courses and the mouths of the rivers Weser and Elbe (Rust, 1972). In the past centuries, the large cities of Bremen and Hamburg had high demands for wood. Additionally, in Lüneburg, a salt processing plant existed which required a huge amount of wood. As a consequence, in the 18th century, the majority of the forests of this region was devastated, and many of the oak populations that grow there today are likely to have been established artificially.

The second area (49.0–52.0°N, 6.0–9.0°E) is crossed by the Rhine from the southeast to the northwest, and is also characterised by long-term human impact. The Rhine valley was settled early during prehistoric times; subsequently, the Romans cleared forests there, not only to extract wood, but also to establish vineyards or to plant chestnuts. In the lower course of the Rhine, oak plantations were established in order to produce firewood, bark for tannin extraction, and bowed stems, which were needed for the construction of the bulgy cogs in The Netherlands (Küster, 1998). In the hilly

region of Northrhine-Westphalia coppice systems have been applied, mainly with *Q. petraea*, since up to 2500 years ago (Hesmer, 1958).

The third region (47.5–50.0°N, 9.0–12.5°E) includes a large part of Bavaria and the eastern part of Baden-Württemberg. Oak populations in this region are scarce and often limited to wet sites (*Q. robur*); therefore a low degree of human interference is to be expected.

3. Results

3.1. Geographic distribution of species

In total, 2155 oak trees were sampled from 426 populations. These were collected either in provenance tests, in nursery beds, or directly in the forests (called “units” in Table 1). In this sample set 1214 trees (56%) turned out to be *Q. robur* and 879 (41%) turned out to be *Q. petraea*. The remaining 62 trees had morphology of an intermediate nature or the species was not determined. Fig. 1 gives an overview on the geographic distribution of the samples and the species. *Q. petraea* was found more frequently in the hilly region, with the exception of the region situated between the Alps and the Danube, where the occurrence of oak is rare in general. In the northern lowlands of Germany, and north of the Alps in South Germany and Austria, *Q. robur* is the dominant species.

3.2. cpDNA polymorphisms

In total, 11 haplotypes were detected (Table 1) corresponding to four lineages (A, B, C, E). The composition of these lineages is indicated in Figs. 1 and 2 of Petit et al. (2002b). However, only three lineages (A, B, C) and eight haplotypes are well represented. Lineage C (of inferred Apennines origin) is the most common (43% of the samples), followed by lineage A (of Apennines and/or Balkans origin) (30%) and by lineage B (from the Iberian refugia) (26%). The three most frequent haplotypes are haplotype 1 (40%), haplotype 7 (23%) and haplotype 10 (14%), which belong to the C, A and B lineages, respectively. All other haplotypes exhibit a frequency below 10%. Haplotypes 6 and 15 were only found in

Table 1
Overall statistics

Country	Haplotype (Lineage)											No. of trees	No. of units
	1 (C)	2 (C)	4 (A)	5 (A)	6 (A)	7 (A)	10 (B)	11 (B)	12 (B)	15 (E)	17 (E)		
<i>Number of trees per haplotype, total number of trees and units investigated</i>													
Belgium	57	0	0	0	– ^a	4	36	0	22	–	0	119	22
The Netherlands	22	0	0	0	–	12	14	4	34	–	–	86	17
Luxembourg	0	0	0	0	–	0	0	10	0	–	0	10	2
Germany	758	8	90	48	–	392	244	87	118	–	–	1745	337
Czech Republic	11	4	0	0	–	32	0	0	0	1	–	48	9
Austria	18	41	0	11	2	45	0	0	0	–	30	147	40
Sum	866	53	90	59	2	485	294	101	174	1	30	2155	426
Percentage	40.2	2.5	4.2	2.7	0.1	22.5	13.6	4.7	8.1	0.0	1.4		
Percentage/lineage	42.7 (C)		29.5 (A)			26.4 (B)			1.4 (C)				
<i>Separation by species</i>													
<i>Q. robur</i>													
Belgium, Luxembourg and													
The Netherlands	36	0	0	0	–	12	37	14	40	–	–	139	20
Germany	437	8	33	27	–	212	167	38	56	–	–	978	181
Czech Republic	6	0	0	0	–	19	0	0	0	1	0	26	4
Austria	14	15	0	0	0	29	0	0	0	0	13	71	17
Sum	493	23	33	27	0	272	204	52	96	1	13	1214	222
<i>Q. petraea</i>													
Belgium, Luxembourg and													
The Netherlands	43	0	0	0	–	4	13	0	16	–	–	76	8
Germany	300	0	57	21	–	177	73	49	56	–	–	733	132
Czech Republic	5	4	0	0	–	13	0	0	0	–	0	22	4
Austria	2	25	0	5	0	5	0	0	0	0	11	48	4
Sum	350	29	57	26	0	199	86	49	72	0	11	879	148

^a Haplotype was not scored in the investigating laboratory.

one population each, whereas haplotype 17 (of lineage E) is restricted to a part of Austria. Other polymorphisms had been detected in Austria (Csaihl and König, 2001), but are not discussed here as they could be distinguished only with more resolute separation methods and would have gone unrecognised with the experimental conditions used in France, Belgium, The Netherlands or Germany.

3.3. Geographic variation of cpDNA polymorphisms

Maps of the geographic structure are provided in Fig. 2a–d, separately for *Q. robur* (a) and *Q. petraea* (b) and for monotypic (c) and polytypic (d) stands. The frequency of the different haplotypes is similar for the two species and varies among regions. In the southeast haplotype 7 is dominant, in the west haplotype 1 is most

common, and haplotypes 10–12 are the most frequent ones in the northwest (Fig. 2a and b). Polytypic populations are much rarer in *Q. petraea* than in *Q. robur*. In Lower Saxony, *Q. robur* had a particularly high frequency of polytypic populations, whereas in the southeast of Germany many populations are fixed for the one haplotype. Fig. 3 illustrates the very special case of roadside plantations in The Netherlands, where most populations comprise several haplotypes.

The distribution of individual haplotypes is shown in Fig. 4. Haplotypes originating from the Iberian refugia (lineage B: haplotypes 10–12) are more common in the western part of the study region. Haplotype 10 (13.6%) was detected in Belgium, The Netherlands and from the lowlands of North Germany to the Baltic Sea, where many of the populations are fixed for this haplotype. Haplotype 10 also extends

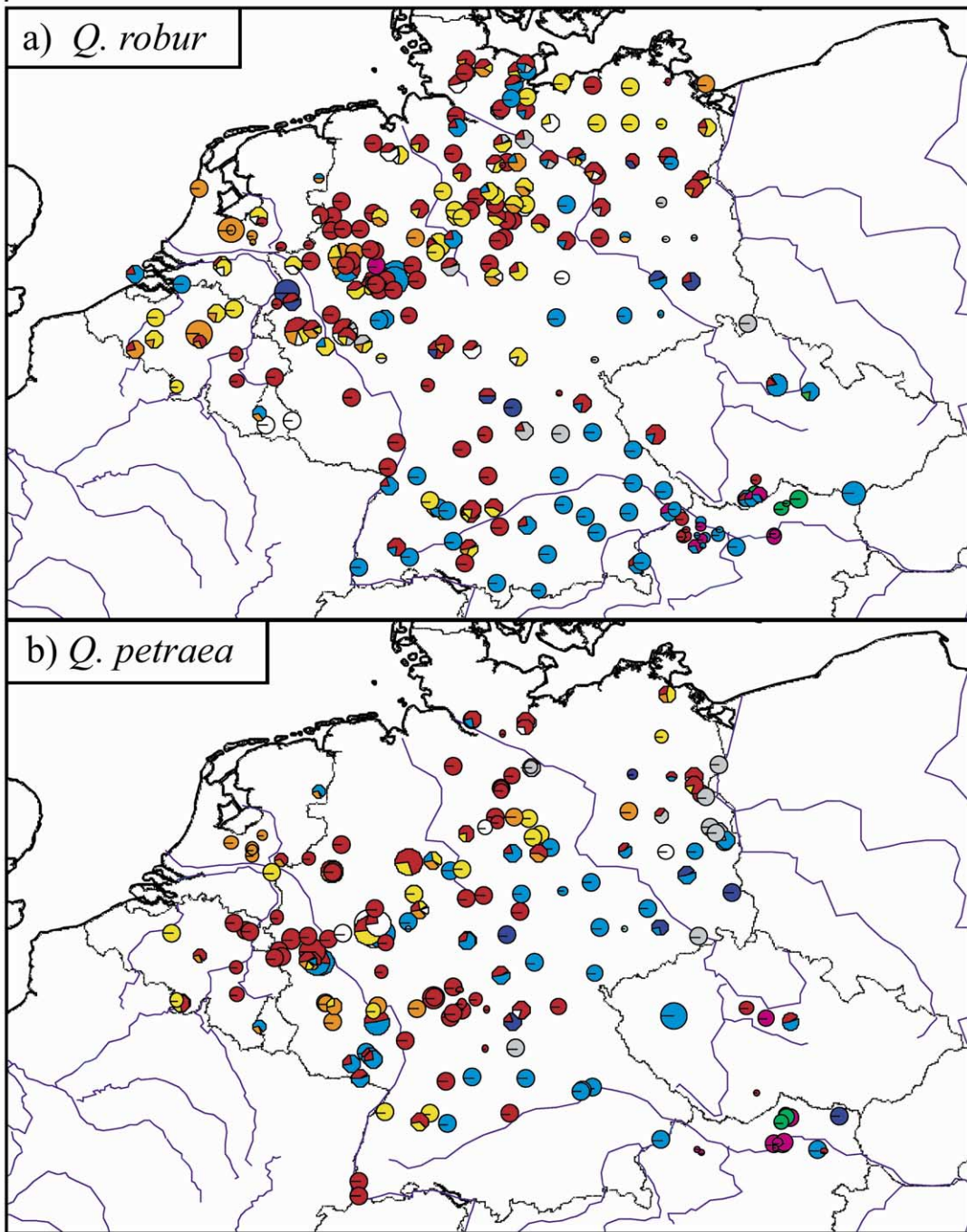


Fig. 2. Distribution of haplotypes in western Central Europe separated by species and by distribution within populations. Each colour represents a different haplotype. Assignment of haplotypes to colours see Fig. 4. (a) Distribution for *Q. robur* and (b) for *Q. petraea*. (c) Populations of *Q. robur* and *Q. petraea* fixed for one haplotype (monotypic populations). (d) Populations with more than one haplotype (polytypic populations).

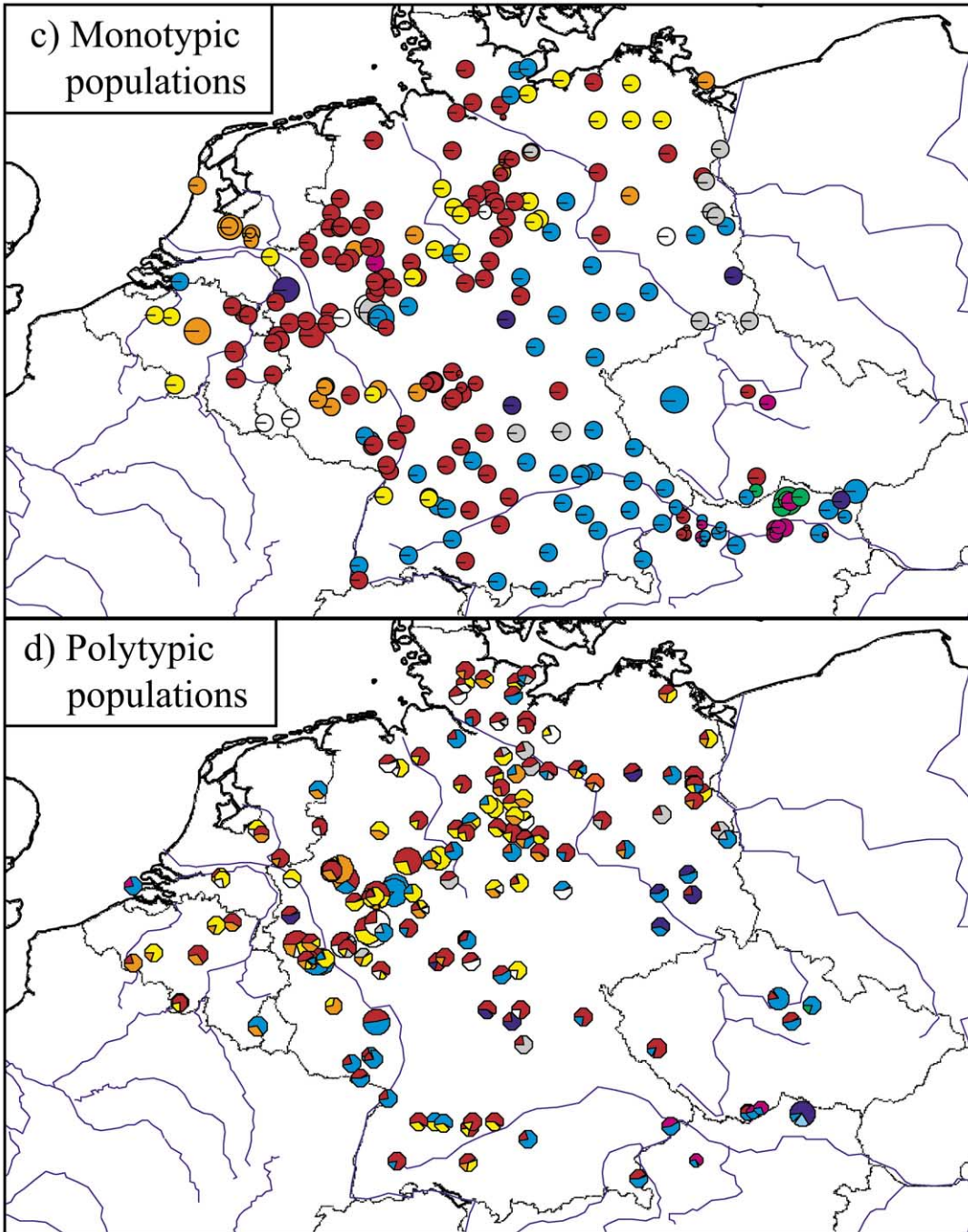


Fig. 2. (Continued).

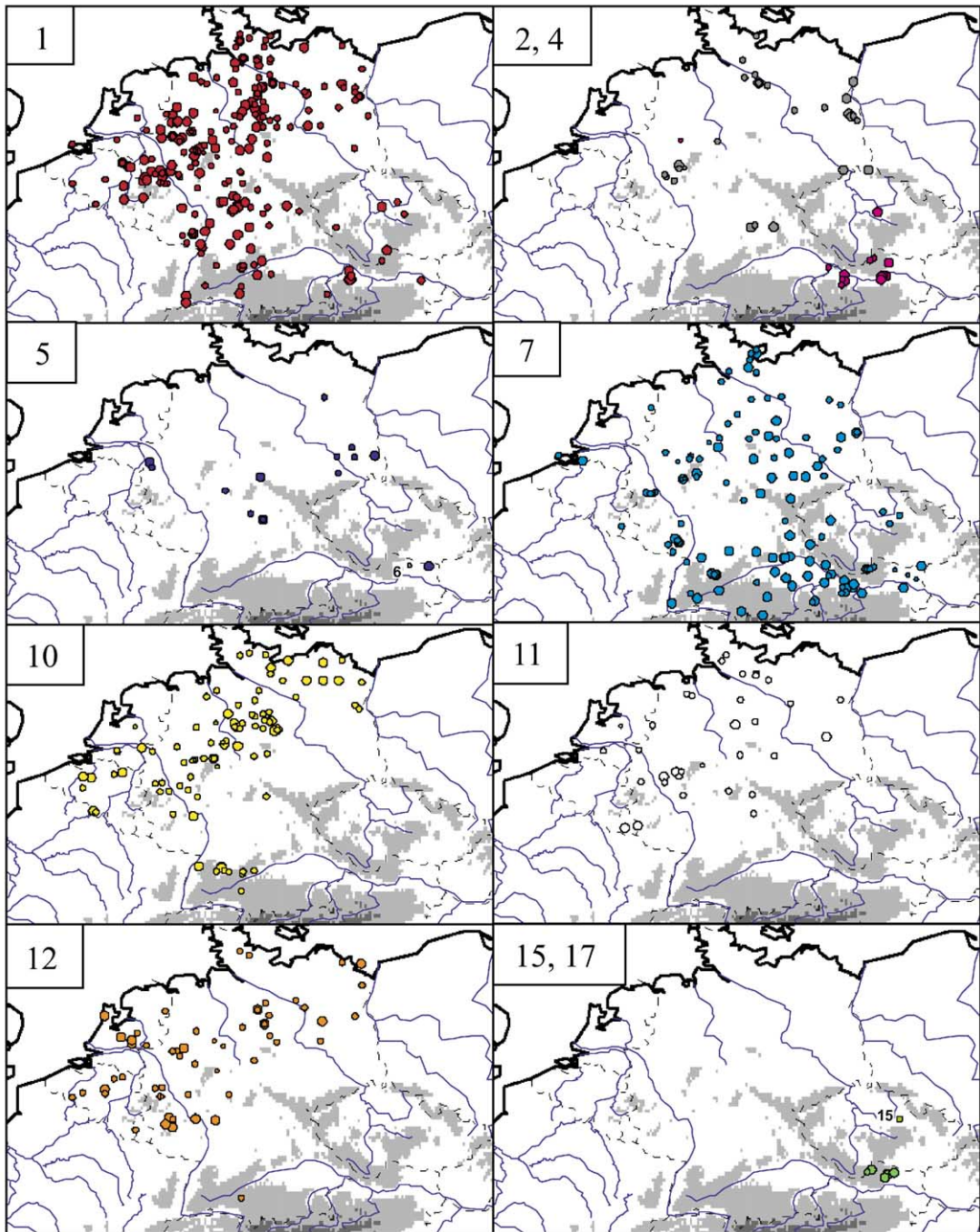


Fig. 4. Distribution of individual haplotypes in the study region (statistics see Table 1). Information is taken from both monotypic (bigger circles) and polytypic populations (smaller circles).

from Alsace into the hilly region of Baden-Württemberg. Haplotype 11 (4.7%) has a more scattered distribution throughout the northwestern part of the region. Only six monotypic populations were detected for this haplotype, four of which occur in Luxembourg and in Northrhine-Westphalia. Haplotype 12 (8.1%) has a distribution very similar to that of haplotype 10. There are clusters of this haplotype in The Netherlands, in Northrhine-Westphalia and in the Lüneburger Heide (northeastern Lower Saxony). The cluster in the Mosel-Hunsrück region extends to the east side of the Rhine. Only one tree with this type was found in the south.

Populations of lineage C are more or less equally distributed over the western and central part of the region investigated. This lineage is nearly exclusively represented by haplotype 1 (40.2%). Regional clusters of monotypic populations can be observed in Fig. 2c. Haplotype 2 (2.5%) was found rarely in western Germany and was limited to a monotypic stand in Northrhine-Westphalia and a single tree close to Hanover. In Austria, this haplotype is relatively frequent forming a cluster in the part of the Danube valley called Wachau (between the cities of Melk and Krems) and also west of that cluster in Upper Austria. Haplotype 2 occurs also in one population in the Czech Republic.

Haplotype 7 (lineage A) is most common in the eastern part of Germany, especially in the Federal States Saxony and Bavaria. It is also found in the Czech Republic and Austria. Two other haplotypes belonging to lineage A: haplotypes 4 and 5, are scattered at low frequencies throughout the study region (4.2 and 2.7%, respectively). Haplotype 4 was detected in Germany in populations of east Brandenburg (close to the border with Poland), in the forest district Göhrde and its surroundings (in the northeast of Lower Saxony, along the Elbe river) and in the Sauerland (Northrhine-Westphalia). This haplotype was not detected in Belgium, The Netherlands, Czech Republic and Austria. Haplotype 5 has a similar distribution in Germany, but it was also detected in one population in eastern Austria. Finally, haplotypes 6, 15 and 17 were found only in the eastern part of the study region. Two trees of type 6 were detected in the north of Austria, where there is also a cluster of haplotype 17, and one tree sampled in the Czech Republic was haplotype 15.

3.4. cpDNA diversity and differentiation

Estimates of genetic diversity and differentiation are given in Table 2 for each species and subset (study region). Diversity within stands (h_S) is significantly lower in *Q. petraea* than in *Q. robur* for the whole data set (0.16 vs. 0.24, $t = 2.72$, $P < 0.01$). The difference between the two species is also significant in the case of the north German lowlands ($t = 2.19$, $P < 0.05$). An exception from this general trend occurs in the Rhine region where both species have similar values for h_S . The Dutch roadside plantations (*Q. robur*) have the highest within-population genetic diversity ($h_S = 0.53$), more than twice the overall value for the complete data set. The lowest intrapopulation diversity values were estimated for populations in Bavaria, particularly for stands of *Q. petraea*. The G_{ST} estimates show overall values of 0.68 for *Q. robur* and 0.79 for *Q. petraea* (the difference is highly significant: $t = 2.97$, $P < 0.01$). In each of the three regions in which both species were compared, *Q. petraea* had the higher G_{ST} values; the difference is significant in the case of Bavaria ($t = 2.01$, $P < 0.05$), where *Q. petraea* has the highest G_{ST} value (0.91), compared to the extremely low G_{ST} value (0.28) for the *Q. robur* roadside plantations in The Netherlands. This latter value represents the lowest G_{ST} reported in the literature for oaks (Petit et al., 2002b and references therein). It differs significantly from all other values reported in this study, and in particular with the second lowest G_{ST} value (0.52), that of *Q. robur* in the northern lowlands ($t = 2.76$, $P < 0.01$). For both oak species, the ranking of the three regions studied is the same: G_{ST} is smallest in the northern lowlands, intermediate in the Rhine region, and largest in Bavaria.

3.5. Tests of spatial genetic structures

To support previous results based on the fixation index G_{ST} , the existence of a spatial genetic structure was tested in *Q. robur* in the same regions used in the previous analyses (Figs. 5 and 6). On the basis of the complete data set, a continuous increase of cpDNA genetic distance with geographic distance is observed in *Q. robur* (Fig. 5). Genetic distances are significantly lower than expected by chance in the first distance classes (up to 300 km) and significantly higher in the classes ranging from 420 to 600 km. In the four

Table 2
Levels of diversity and differentiation by region and species

Set	No. of populations (≥3 individuals)	Harmonic mean of individuals per population	No. of haplo-types	h_S (standard error)	h_T (standard error)	G_{ST} (standard error)	Test ^a
<i>Q. robur</i>							
(1) Roadside plantations (NL)	39	5.00	5	0.531 (0.049)	0.742 (0.029)	0.283 (0.062)	2, 3, 4
(2) North German lowlands	49	5.00	7	0.329 (0.044)	0.683 (0.041)	0.519 (0.059)	1, 4
(3) Rhine region	47	5.34	8	0.201 (0.043)	0.597 (0.070)	0.663 (0.061)	1
(4) Danube region	31	4.88	6	0.170 (0.047)	0.670 (0.044)	0.746 (0.064)	1, 2
(5) All populations	231	4.80	10	0.239 (0.019)	0.742 (0.016)	0.678 (0.025)	
<i>Q. petraea</i>							
(6) North German lowlands	23	4.31	6	0.178 (0.053)	0.611 (0.092)	0.708 (0.089)	
(7) Rhine region	49	5.24	6	0.199 (0.039)	0.744 (0.036)	0.732 (0.054)	
(8) Danube region	14	5.00	5	0.057 (0.039)	0.669 (0.064)	0.914 (0.054)	
(9) All populations	172	4.63	9	0.164 (0.020)	0.766 (0.021)	0.785 (0.026)	

^a The test indicates the set(s) to which the difference between the G_{ST} values is significant with $P < 0.05$. Additionally, the difference between the G_{ST} values of all populations of *Q. robur* vs. *Q. petraea* (set 5 vs. 9) is significant.

selected areas, the analysis was restricted to the first 150 km, given the small size of the regions investigated (Fig. 6a–d). No spatial structure was found among the roadside plantations in The Netherlands (Figs. 4 and 6a). Similarly, the distogram corresponding to the north German lowlands does not present any significant value (Fig. 6b). For the Rhine area, there is

a stronger increase of genetic distances over space, with the two distance classes 90–120 and 120–150 km showing higher than expected genetic distances (Fig. 6c). Finally, in the Danube region, there is a strong increase of genetic distances with geographic distances, with the lowest (and significant) value in the first distance class (Fig. 6d).

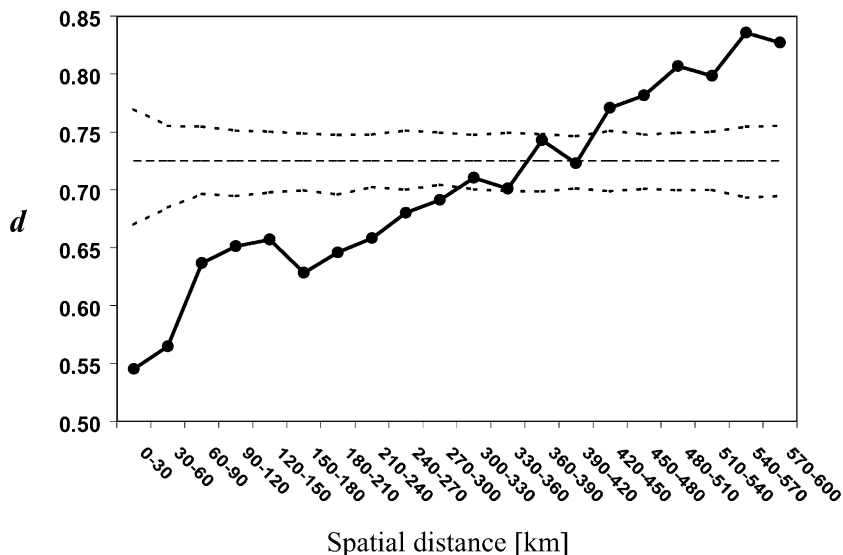
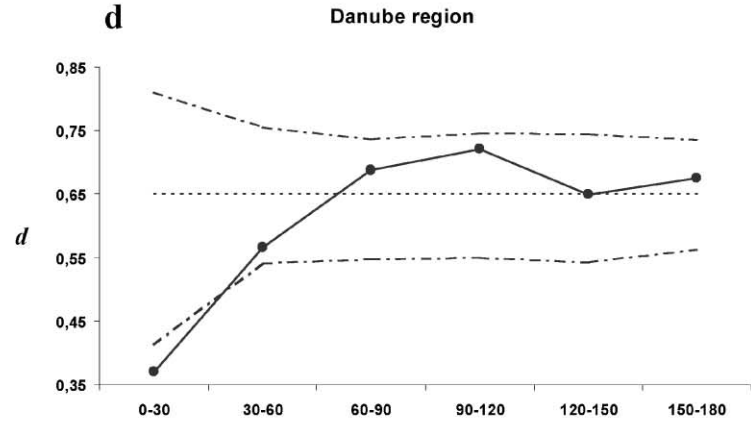
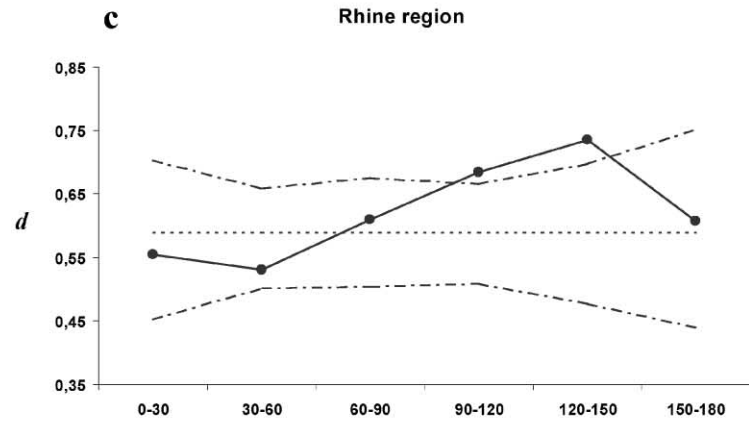
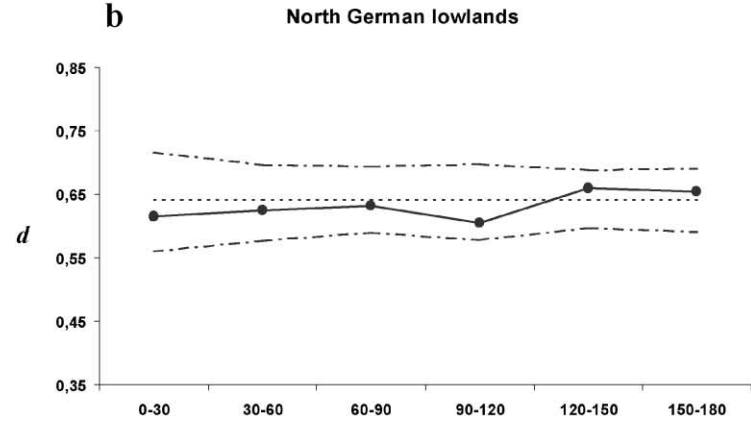
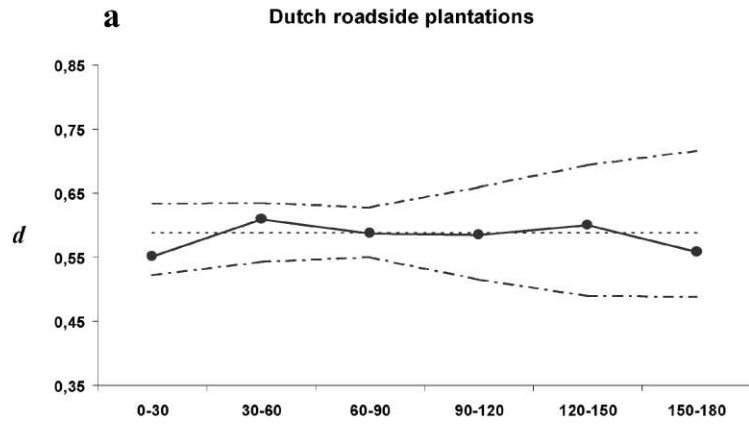


Fig. 5. Distogram of average genetic distance (d) in 20 spatial distance classes for all investigated *Q. robur* populations in western Central Europe. The dotted lines include the 95% confidence interval of 500 permutations.



Spatial distance [km]

Fig. 6. Distograms of average genetic distance (d) in six spatial distance classes for *Q. robur* populations in four selected sub-areas. The 95% confidence interval of 500 permutations is presented.

4. Discussion

4.1. Distribution of haplotypes and re-colonisation routes

Routes of re-colonisation can be inferred from the present geographic distribution of haplotypes. For example, haplotypes 10–12 of lineage B (Fig. 4), which originate from the Iberian refugia, appear to have moved north–northeast through France, into Luxembourg, Belgium and The Netherlands, and finally entered Germany in the northwest from France (in the Mosel region), Belgium and The Netherlands. The subsequent movements were northeastwards, up to the Baltic Sea. The native status of oak populations characterised by these haplotypes is supported by the presence of numerous stands fixed for haplotypes of lineage B in northeast Germany (Mecklenburg-Vorpommern, especially haplotype 10). The few populations from southwest Germany (Baden-Württemberg and West Bavaria) characterised by haplotypes 10 and 12 could indicate a separate colonisation route through the Porte de Bourgogne, between the Vosges mountains and the Swiss Jura, rather than from the north. This hypothesis is supported by the fact that haplotypes of lineage B are absent in the adjacent Palatinate hilly region. Since the populations characterised by this lineage did not penetrate further east than to the border region of Baden-Württemberg and Bavaria, they may have arrived there later, although the distance from the Iberian refugia to the Porte de Bourgogne is much shorter than to northern Germany. Another possibility is that they may have encountered populations coming from the east relatively early in their migration, which could have hindered a further extension.

Haplotype 1 is most likely of Italian origin (Dumolin-Lapègue et al., 1997a; Petit et al., 2002a). It is the most common haplotype detected in Germany (40%). The location where oaks carrying this haplotype may have passed the Alps, is detailed elsewhere (Mátyás, 1999; Csaikl et al., 2002b). The founder populations migrated to Germany in the southwestern corner of the territory from where they moved largely northwards. The distribution of this haplotype broadens as it moves northwards from Belgium and The Netherlands to northwest Poland (Csaikl et al., 2002a). In Saxony and Bavaria, haplotype 1 occurs with a low frequency. There is a gap between the populations characterised

by this haplotype found in the Czech Republic and in Austria, and those found in southwestern Germany. The migration of haplotype 1 into those countries can be explained either by long distance migration from West Germany, or by the existence of a more easterly migration route through northeast Italy and Austria. On the other hand it can also not be excluded that several trees are allochthonous, as for example that one found in the park of Schönbrunn castle (easternmost point in Fig. 4, 1). Lineage C is also represented in the study region by haplotype 2. Although the general distribution of this haplotype is centred further east of western Central Europe, it occurs in the extreme southeastern corner of the region considered here.

Lineage A, which likely originates from the Balkans, is represented by haplotypes 4, 5 and 7 in the study region. Haplotype 4 is more frequent in Hungary and in Slovakia (Bordács et al., 2002), but further investigation in the Czech Republic may allow to determine more precisely the distribution area of this haplotype. This would indicate whether acorns have floated during inundations or been shipped by man downstream along the rivers Elbe and Oder. Two clusters of haplotype 4 occur in northern Germany, on the lower courses of these rivers, but the possible source populations have not yet been identified. Shipments may have been made to feed cattle, or for reforestation purposes in the 19th century after the large forest fellings.

Similarly, for haplotype 5, disjunct stands have been identified in Bavaria and Northrhine-Westphalia. This haplotype is the most frequent in the area to the east of the study region, in Romania, Hungary, Slovenia and eastern Austria (Bordács et al., 2002). It may have migrated into Germany through south Poland and possibly through the Czech Republic since a cluster of haplotype 5 exists in the border region between Germany, Poland and the Czech Republic.

Finally, haplotype 7 has a broad distribution in the region investigated. In The Netherlands and in Belgium it is very rare (and probably introduced), and scarce in northwest Germany (near the Atlantic) as well as in Mecklenburg-Vorpommern (near the Baltic Sea), in south Hesse and in Frankonia. This would suggest a migration route northwards along the border with the Czech Republic. Although it may be autochthonous further north in pure haplotype 7-stands, its presence

elsewhere in polytypic woods would suggest that it has been planted by man (see Fig. 2).

4.2. Intra- and inter-specific diversity

The majority of the trees analysed belong to *Q. robur*, and this might partly be due to the sampling procedure. The large provenance trials in Lüchow and Freiburg (Germany), which have been used for sampling, consist entirely of *Q. robur*. The Dutch provenance trial also comprises only *Q. robur* but was not integrated in these statistics as all populations involved were known to be of artificial origin. A higher level of within-population diversity (h_S) and lower G_{ST} in *Q. robur* compared to *Q. petraea* is a general trend which has also been found elsewhere in Europe (Dumolin-Lapègue et al., 1999; Bordács et al., 2002; Petit et al., 2002b). This lends support to the idea that acorns of *Q. robur* have been more frequently transferred by man and planted than those of *Q. petraea*. In Germany, 84% of the selected *Q. petraea* seed stands are classified as autochthonous, compared to only 42% for *Q. robur* (BLE, 1999). Currently, acorns from *Q. robur* are more frequently imported into Germany than acorns from *Q. petraea*, as indicated by internal reports of the German Federal Office for Agriculture and Food. In The Netherlands, the oak line plantations along the roads are entirely established with *Q. robur*. In the present study, the diversity in these populations ($h_T = 0.74$) is equal to that of the complete *Q. robur* data set. Thus, as a consequence of the mixture between nurseries, and the introduction of foreign seed sources, the level of intrapopulation diversity has been increased and the G_{ST} has been decreased (0.28).

With the single exception of populations in North-rhine-Westphalia, there are generally higher levels of intrapopulation diversity in *Q. robur* than in *Q. petraea*. There oak coppice stands were made up mostly of *Q. petraea* that can be sometimes traced back to about 2500 years (Hesmer, 1958). Many of them have now been converted into high forests (Ziegenhagen, 1981). Today, these coppice stands are composed of a mixture of oaks and birch or hornbeam which had been established by replacing beech forests (Seibert, 1955), most likely using seeds collected locally. Generally, a coppice systems can be considered as a silvicultural treatment conserving the genetic constitutions of

populations, because regeneration takes place by resprouting of cut phenotypes. However, there are reports showing that the coppiced stands have been restocked by direct seeding, following death of the original trees by over-coppicing, and other seed sources may then have been used.

All major haplotypes are found in both species, with generally similar frequencies (Table 1 and Fig. 2). As discussed elsewhere (e.g., Petit et al., 1997), past and ongoing hybridisation and introgression can account for this observation. Controlled crosses carried out in northern Germany have proved that hybridisation between *Q. robur* and *Q. petraea* is possible under artificial conditions (Kleinschmit and Kleinschmit, 1996; Steinhoff, 1998).

4.3. Spatial distribution of haplotypes and fixation

Even in those regions where different post-glacial re-colonisation routes have met, clusters made up of a single haplotype can often be observed. In oaks, cpDNA variants can spread only via movements of acorns as they are maternally inherited (Dumolin et al., 1995). With the exception of the rare events of medium or long distance transfer by birds, man or water, most acorns tend to germinate in the surroundings of the mother tree, leading to strong founding events during re-colonisation. The founding haplotype is likely to form large patches of fixed haplotype descendants given that a single tree produces large quantities of seed. Therefore, naturally regenerating oak woods comprising one single haplotype may persist over many generations (Petit et al., 1997). The two processes: founding event followed by limited gene flow because of large population sizes, should lead to monotypic stands, with some exceptions at the junction between patches characterised by different haplotypes. This means that, in the case of mixed haplotype composition, human influence may be assumed, but only when the genetic composition does not fit with the overall geographic distribution pattern.

4.4. Consequences of human activities

Hausrath (cited in Hasel, 1985, p. 206) estimated that around 1300 A. D. broadleaved forests represented 75% of the total German forests. This proportion had decreased to only 28% by 1937.

This suggests that over a period of 600 years human activities have greatly modified the composition of the forests. Also the percentage of forests, where oak was the main species has decreased in some regions; for example in the forest district of the Bramwald from 82% in 1587 to only 16% in 1948 (Krahl-Urban, 1959). Exceptions occurred in regions with coppice systems.

However, it is not clear to what extent these human activities also affected intra-specific genetic variation. It is evident that the large conifer sowings have not only changed the structure of forest ecosystems, but also largely determined the genetic structure of conifer populations. Conifer seed could be collected far away, the seeds could be easily stored and transported over large distances, and with a few kilograms several tens of thousands of trees could be raised. This is quite different with oak. One kilogram of seeds consists on average of 350 acorns of *Q. petraea* or of 250 acorns of *Q. robur* (Krahl-Urban, 1959). For the establishment of an oak forest, a huge amount of acorns is therefore necessary, given the high density of acorns or plants used in artificial regeneration. For instance, in Lower Frankonia (BY), as much as 600–750 kg of acorns are sown per hectare (Grimm, 1995). If used for reforestation, acorns have to be protected against desiccation and deterioration. It is difficult to envisage that storage and transportation occurred on any appreciable scale in the Middle Ages and in the following centuries. An exception might be through transport by ships, along major rivers. Elsewhere, if acorns were needed for planting or sowing, they would have been of local origin. Even the vast areas of deforestation present in the 17th, 18th and first half of the 19th century were probably regenerated using seed from local remnant oaks near farmers' buildings.

The situation might have changed in the middle of the 19th century with the development of the railroad system. Big forest seed transfer occurred during the 19th and the beginning of the 20th century. There were considerable imports from Austria and Hungary as well as from Belgium and France. In the case of acorns, Lüdemann (1961) compiled the available statistics between 1888 and 1940. Before World War I, the annual imports amounted to about 500–1500 t per year, with a maximum of 3000 t in 1911. Between the two World Wars, imports were greatly reduced. These seed transfers, both within and between countries, must have

affected the cpDNA genetic structure, resulting, in many cases, in an increased cpDNA-diversity within-stands or within-forest-districts due to the mixing of seed sources.

4.5. Increasing genetic structure with decreasing human influence

Because of its greater propensity to be manipulated by man, *Q. robur* was chosen to test the assumption that an increased degree of human influence should result not only in increased levels of diversity within populations, but also in decreased levels of spatial genetic structure. This implies the assumption that for reforestations not only local but also non-indigenous material has been used. First, the distogram based on the overall set of *Q. robur* populations confirms the observation that the haplotypes are not randomly distributed throughout the study region. This means that even in a regionally intensively managed species, the original geographic structure that was created at the onset of post-glacial recolonisation may still be identified. Within the subsets (Fig. 6a–d) a differential human influence was assumed. Both for the Dutch roadside plantations as well as for the north German lowland the genetic distances do not deviate from the random expectations: all values remain within the confidence limits and these are rather narrow. But the conditions for both sets are quite different. In The Netherlands the populations included in this kind of investigation are entirely planted, using material from outside the country. In total five haplotypes were identified. But according to the general remigration patterns only haplotypes of lineage B (10–12) would be expected to have migrated naturally into The Netherlands. Haplotype 1 is considered to be autochthonous only in the southeastern corner of The Netherlands, and haplotype 7 seems to have been introduced. In North Germany, on the contrary, seven haplotypes from different lineages were found, and this area can be considered as a hybrid zone in the sense of Hewitt (1999). So Fig. 6b cannot be interpreted as being exclusively the result of human activity. Both mingling of lineages and mixing of seed from both local and non-local origins might have contributed to the random distribution pattern of haplotypes. *Q. petraea* has experienced less human interference

and therefore the current geographic distribution of cpDNA variation in this species is probably more representative of the original pattern. For the region of the Rhine valley and surrounding hills, some (limited) level of genetic structure remains, despite the presence of some polytypic forests of likely introduced origin. The *Q. robur* populations growing at intermediate elevation in Bavaria present a distinct spatial genetic structure in the lowest distance class. This is probably due to the big cluster of monotypic stands of haplotype 7 in and south of the Danube valley.

4.6. Indications for autochthony/allochthony

The overall spatial genetic structure that was established at the outset of post-glacial re-colonisation can be used to evaluate the autochthony of oak populations. The introduction of non-autochthonous material will be particularly evident where there is a large geographic distance between the stand and the region where the corresponding haplotype occurs at high frequency. This is true for the monotypic stand in Northrhine-Westphalia characterised by haplotype 2. This haplotype is more frequently distributed in Croatia, Hungary and East Austria from where it extends as far as the Austrian–German border region. This isolated stand is separated by 500 km from the nearest populations sharing the same haplotype.

More frequently, however, intermediate situations will occur. In the case of haplotype 5, there is one fixed haplotype stand of *Q. robur* in West Germany close to the border with The Netherlands (between the Meuse and the Rhine rivers). The distance between this stand and the closest monotypic stands in Thuringia and Bavaria is almost of about 270 and 300 km, respectively. Although it may be possible that this stand was established by a long-distance seed dispersal event during post-glacial re-colonisation, it seems more likely that the acorns were introduced to establish a coppice plantation in the river plain; this could be checked by analysing further samples in the vicinity of the isolated haplotype 5 western population.

The arguments concerning autochthony can be reinforced by examining the taxonomic status of the populations investigated. The clusters of haplotype 4 in the lower course of the rivers Elbe and Oder have already been interpreted as possibly introduced

populations. These trees were largely *Q. petraea*. Since *Q. robur* is more suited to the ecological conditions prevailing in these river plains, the hypothesis that the *Q. petraea* trees are allochthonous is supported. The presence of haplotype 4 in Northrhine-Westphalia and in Bavaria, in the form of two disjunct patches, could also indicate human transfer, especially in the westernmost location.

Another indicator for non-autochthony is the heterogeneity of a stand. Many polytypic woods exist in the densely sampled regions of Northrhine-Westphalia and Lower Saxony. The existence of polytypic stands which comprise haplotypes of all three main lineages or contain haplotypes which do not occur in the surrounding stands provides strong evidence that the basic material is (at least partly) introduced. The Dutch roadside plantations demonstrate this clearly. On the other hand, stands planted with introduced material may also be monotypic, as shown above in the case of some stands fixed for haplotypes 2, 4 or 5.

5. Conclusions

The results of this study have provided important information about the location of the post-glacial migration routes of oak in western Central Europe. Although humans have considerably altered the landscape in this part of Europe, it seems that the mapped patterns largely reflect the original post-glacial distribution of haplotypes. Because of the position of the region at the meeting-point of the three lineages from the Balkans, Apennines and Iberia, a comparatively high level of cpDNA diversity exists naturally. Nevertheless, in several regional clusters a uniform genetic composition exists, which points to the persistence of material of local origin. In regions where human influence has been particularly strong, an increased cpDNA diversity within populations and a decreased spatial genetic structure could be observed, but this might also be due to the mingling of lineages.

The use of molecular markers can assist in the identification of autochthonous material and indicate the amount of past seed transfer within a region. Stands not fitting into the general remigration pattern can be classified as having been established with introduced

material. The method can be used for the genetic characterisation of seed stands and, to some degree, for identity control of forest reproductive material moving in trade.

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