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A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences

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Summary

- Here, palaeobotanical and genetic data for common beech (*Fagus sylvatica*) in Europe are used to evaluate the genetic consequences of long-term survival in refuge areas and postglacial spread.
- Four large datasets are presented, including over 400 fossil-pollen sites, 80 plant-macrofossil sites, and 450 and 600 modern beech populations for chloroplast and nuclear markers, respectively.
- The largely complementary palaeobotanical and genetic data indicate that: (i) beech survived the last glacial period in multiple refuge areas; (ii) the central European refugia were separated from the Mediterranean refugia; (iii) the Mediterranean refuges did not contribute to the colonization of central and northern Europe; (iv) some populations expanded considerably during the postglacial period, while others experienced only a limited expansion; (v) the mountain chains were not geographical barriers for beech but rather facilitated its diffusion; and (vi) the modern genetic diversity was shaped over multiple glacial–interglacial cycles.
- This scenario differs from many recent treatments of tree phylogeography in Europe that largely focus on the last ice age and the postglacial period to interpret genetic structure and argue that the southern peninsulas (Iberian, Italian and Balkan) were the main source areas for trees in central and northern Europe.

Key words: beech (*Fagus sylvatica*), chloroplast markers, fossil pollen, glacial refugia, nuclear markers, plant macrofossil, Quaternary.

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Introduction

European beech (*Fagus sylvatica* L.) is one of the most thoroughly investigated European tree species, having been the subject of scientific studies in diverse fields such as ecology, palaeoecology and genetics. Reasons for this interest include its wide distribution and great economic importance. As a consequence, a wealth of fossil data documenting its past distribution have accumulated, and many genetic surveys using different markers and statistical approaches have been carried out. There is therefore a great opportunity to link available genetic and fossil data. This should clarify some long-standing puzzles in palaeoecology and facilitate the conservation and sustainable management of the genetic resources of this emblematic tree species.

Coupling of modern DNA data with past distributions of plant populations has been increasingly used to evaluate the genetic consequences of postglacial spread and long-term survival in refuge areas (e.g. Comes & Kadereit, 1998; Taberlet *et al.*, 1998; Hewitt, 1999, 2000; Cruzan & Templeton, 2000; Willis & Whittaker, 2000; Stewart & Lister, 2001; Petit *et al.*, 2002; Taberlet & Cheddadi, 2003; Lascoux *et al.*, 2004; McLachlan *et al.*, 2005). The identification and characterization of areas where refugia were located during the last glacial maximum are of special interest as this information can help set priorities for conservation and management of genetic resources (e.g. Hampe & Petit, 2005).

Starting with a review of the fossil and genetic data available for *F. sylvatica* and also unpublished data and analyses, the aim of the present work was (i) to reconstruct the history of this species in Europe since the middle Pleistocene using pollen and macrofossil data, (ii) to describe its modern genetic structure using different markers (isozymes and chloroplast DNA) and analytical methods, and (iii) through the integration of fossil and genetic data, to find possible answers to the following questions.

Which type of data (fossil and genetic) is best suited to the detection of the glacial refugia? How do they complement each other? Do the genetic data confirm the palaeobotanical data or support alternative explanations?

Was the modern genetic diversity shaped during the last glacial period, or during multiple glacial–interglacial cycles?

Did beech survive the last glacial period in a single refuge area in Europe, or at multiple sites? In the latter case, how many glacial refugia can be distinguished? Where are they located? Can we identify any glacial refuge further north than previously hypothesized?

Did all beech populations respond to postglacial climate change simultaneously and at the same rate? Did they contribute equally to the postglacial spread of beech? Did the physiographic features of Europe influence the main migration routes of beech?

This study, which combines multiple extensive datasets, is unique in its scope and tackles questions that are of interest beyond the specific case of European beech.

The Palaeobotanical Data

European beech has been used as a model species in European palaeoecology, and its past distribution has been discussed in a number of papers (a review of the state of knowledge is reported in Supplementary Item S1, available as part of the online article). However, to link genetic and palaeobotanical data, a new and thorough synthesis of the published pollen data was required. For this purpose, we included regions that had been little investigated and integrated complementary macrofossil data to propose a complete revision of the history of beech in Europe since the middle Pleistocene.

Materials and methods

The datasets Two different datasets of fossil material have been used to produce maps of the past distribution of *Fagus* in Europe: those of pollen and macrofossils.

The pollen data in part were extracted from the European Pollen Database (EPD; http://www.ncdc.noaa.gov/paleo/epd) and thoroughly checked with respect to the chronology and some other potential sources of error (223 sites), and in part represent either unpublished data or data obtained from the published literature but not included in the EPD (185 sites). A complete list and a location map of the fossil pollen sites are presented in Supplementary Items S2 and S3, respectively (available online). As the pollen morphology of *F. sylvatica* cannot be distinguished from that of *Fagus orientalis* Lipsky (Praglowski, 1982), a number of pollen records in the present-day distribution of *F. orientalis* are also mapped. The two species have, however, separate ranges which help to distinguish their respective histories. In general, the chronologies of the records younger than 40 kyr before present (BP) are based on radiocarbon dates and on interpolations between dates. After consideration of European beech pollen productivity and dispersal, we chose to map only pollen values above 2%, as explained in Supplementary Item S4 (available online). This implies that the time of arrival of beech at a given location cannot be obtained, but possible inaccuracies, such as pollen contamination and long-distance transport, are minimized.

The *Fagus* macrofossil dataset is derived from the published literature (80 samples). A location map and a complete list of the macrofossil sites are presented in Supplementary Items S3 and S5. The chronological setting of the macrofossils is based on radiocarbon measurements (70 sites), on correlation with nearby dated records (seven sites) or on dendrochronology (three sites). The available macrofossil dataset contains mostly fragments of wood and charcoal as well as fruits, bud scales and leaves. Compared with the pollen records, the macrofossil finds are sparse, but they represent a very useful complement for assessing the existence of beech in a region (e.g. Jackson *et al.*, 1997, 2000; McLachlan & Clark, 2004). As might be expected, evidence for the arrival of beech in the macrofossil record often predates that in the pollen record. The presence of macrofossils is therefore particularly useful to identify putative refugia, along with the spatial patterns of establishment of beech as an element of the local vegetation.

Palaeobotanical results

Middle and Upper Pleistocene history of European beech

While the existence of beech in Europe is documented before the Quaternary (Mai, 1995; Denk *et al.*, 2002), the chronostratigraphical setting of the Quaternary fossil remains is often questionable and very imprecise, because of the fragmentary nature of most of the continental records. Tzedakis *et al.* (1997) have linked the four longest European pollen records to the deep-sea oxygen isotope record with the aim of developing a chronology for the terrestrial sequence of vegetation events over the last 500 kyr. In the present paper, the same four records, located in the Massif Central, France, in central Italy, in north-west Greece, and in eastern Macedonia, are used to describe the Middle and Late Pleistocene history of beech in southern Europe (Fig. 1).

The composite diagram in Fig. 2 shows that beech repeatedly appeared in Europe during most of the temperate periods of the last 500 kyr. In Italy it was the dominant tree during various forest phases and also reached appreciable pollen percentages during the glacial periods [marine isotope stages (MIS) 6 and 3]. In France it was especially abundant in the postglacial period, but was also diffuse in previous temperate phases (MIS 11c, 9e and 7c). Beech is less well represented in Greece: at Ioannina it showed a few important expansions (MIS 9e and 5c), but at Tenaghi Philippon it was always a minor component of the vegetation. On the whole, the behaviour of beech appears rather variable among sites and among temperate phases.

A key period for the history of beech in Europe is the last interglacial (the Eemian, *c.* 127–112 kyr BP). High percentages of beech during a forest phase assigned to the Eemian are found in eastern Bulgaria, in the modern range of *F. orientalis* (Bozilova & Djankova, 1976). Elsewhere in Europe, beech showed a moderate expansion only in central Italy (Fig. 2), while at Les Echets near Lyon (de Beaulieu & Reille, 1984) and on the western Swiss Prealps (Welten, 1982) only low percentages are recorded. Very sparse pollen finds in the rest of Switzerland (Wegmüller, 1992) and in Germany (Grüger & Schreiner, 1993) indicate that the French beech populations were not connected with the beech populations recorded in the eastern Alps (Šercelj, 1966; Drescher-Schneider, 2000). In central Europe an important find of *Fagus* charcoal in Slovakia (Hajnalová & Krippel, 1984) documents the local presence of beech in association with *Taxus*. Sporadic occurrences of beech pollen are recorded at several Eemian sites in Poland, indicating that small populations could be restricted towards the south-west of Poland (Mamakowa, 1989; Malkiewicz, 2002; Granoszewski, 2003). No pollen data are available for the Iberian Peninsula.

During the forest expansion following the Eemian, corresponding to MIS 5c (approx. 105–95 kyr BP; Fig. 2), *Fagus* expanded at many European sites, becoming quite abundant (Fig. 3). As expected, the highest percentages of *Fagus* are found close to the Eemian refuge areas: 30–50% in Italy, up to 30% in France and 5% in Slovenia. Beech is also well represented in the Balkans, reaching 20% in north-west Greece. In the central European sites, *Fagus* pollen, although present,

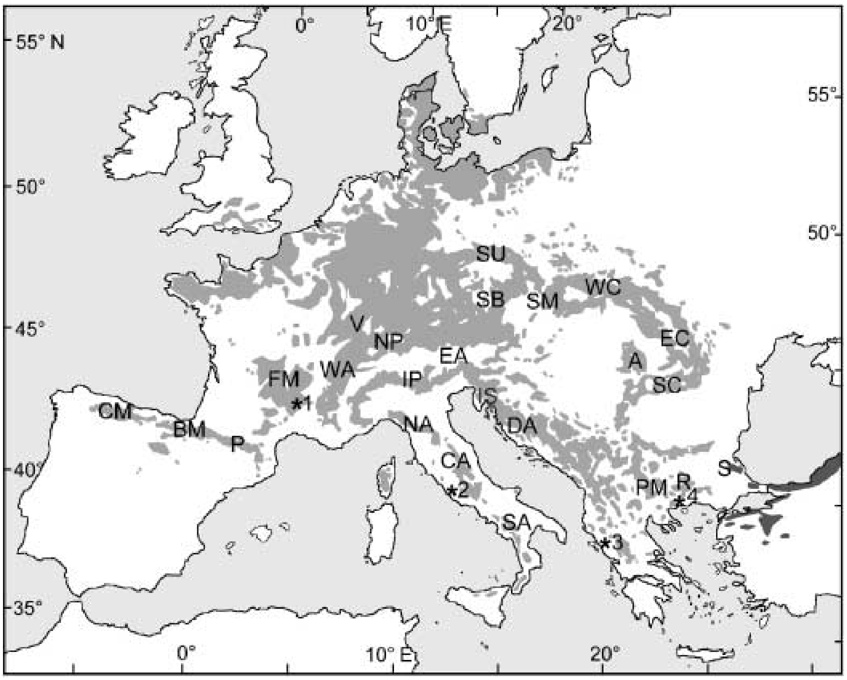

Fig. 1 Modern distribution of *Fagus sylvatica* (light grey; modified from Pott, 1997) and *Fagus orientalis* (dark grey; redrawn from Atalay, 1994) in Europe. Mountain ranges mentioned in the text: Cantabrian Mountains (CM), Basque Mountains (BM), Pyrenees (P), French Massif Central (FM), western Alps (WA), Vosges (V), northern Prealps (NP), Italian Prealps (IP), eastern Alps (EA), southern Bohemia (SB), southern Moravia (SM), Sudety (SU), western Carpathians (WC), eastern Carpathians (EC), Apuseni (A), southern Carpathians (SC), Dinaric Alps (DA), Pirin Mountains (PM), Rhodopes (R), Strandza Mountains (S), southern Apennines (SA), central Apennines (CA), and northern Apennines (NA). Asterisks indicate the location of the four longest European pollen records: (1) Bouchets/Praclaux, (2) Valle di Castiglione, (3) Ioannina and (4) Tenaghi Philippon.

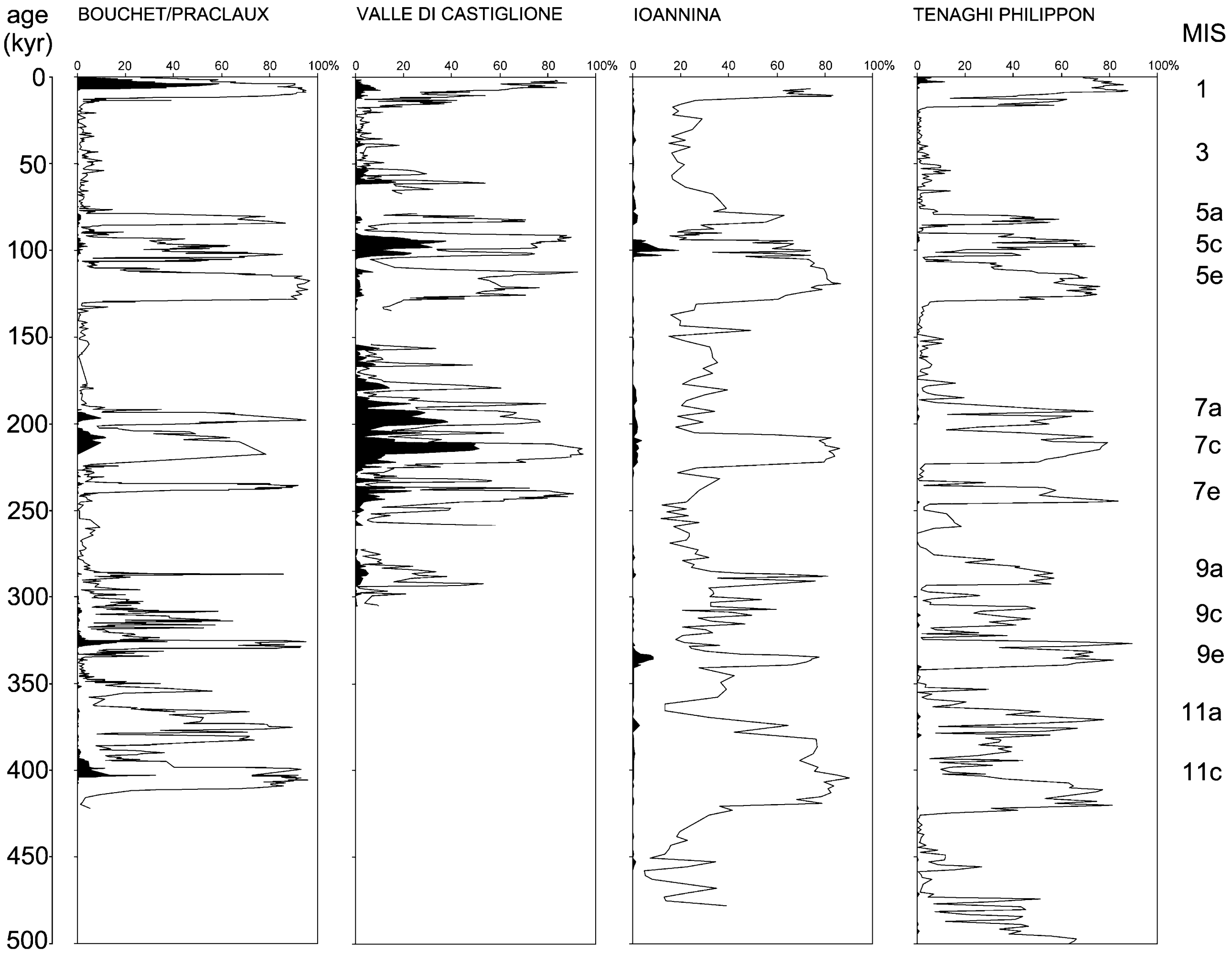

Fig. 2 Comparison of the *Fagus* record (black area) in the four longest European pollen records: Lac du Bouchet-Praclaux (Reille & de Beaulieu, 1990, 1995; Reille *et al*., 1998), Valle di Castiglione (Follieri *et al*., 1988), Ioannina (Tzedakis, 1993, 1994) and Tenaghi Philippon (Wijmstra, 1969; Wijmstra & Smit, 1976; Wijmstra & Groenhart, 1984). The solid line represents the percentages of arboreal pollen excluding *Pinus*. Chronology is according to Tzedakis *et al*. (1997).

never reaches the 2% value. Sporadic macrofossils of beech (charcoal) have been found at archaeological sites in Slovakia, suggesting persistence of local populations of Eemian age. These data indicate that at *c*. 100 kyr BP *Fagus* underwent a marked expansion in the Italian and Balkan peninsulas, as well as in south-eastern France, while in the southern part of central Europe it was only a minor component of the forest vegetation.

In the forest phase corresponding to MIS 5a (approx. 85–70 kyr BP; Figs 2, 3), beech was present at moderate percentages in France (de Beaulieu & Reille, 1984) and in the Balkan peninsula (Tzedakis, 1994), while it was still very abundant in peninsular Italy (Magri, 1999; Allen *et al.*, 2000) and in north-eastern Italy (Canali, 2005). In the Iberian Peninsula, the data available for this period indicate a considerable expansion both in the Pyrenees (Burjachs *et al.*, 1996) and along the coast of the Cantabrian Sea (Ramil-Rego *et al.*, 2000).

Locating the main diffusion areas of beech during the forest expansions immediately preceding the last glacial period is of fundamental importance to assess the refuge areas for beech between 70 and 10 kyr ^{14}C-BP (radiocarbon ages), when beech was greatly reduced everywhere in Europe. In Italy, significant pollen percentages witness the local presence of *Fagus* at a number of sites during both MIS 3 and the late glacial period (Follieri *et al.*, 1998; Allen *et al.*, 2000; Canali, 2005). In Spain, the presence of beech is demonstrated by pollen both near Barcelona (Burjachs & Julià, 1994) and in Galicia (Gómez-Orellana Rodríguez, 2002) during MIS 3, and by macrofossils in the Pyrenees (Vernet, 1980; Uzquiano, 1992) during the last glacial maximum and the late glacial period, and in the Cantabrian Range during the late glacial period (Uzquiano, 1992). In southern France the existence of beech is demonstrated by macrofossils during the last glacial maximum (Vernet, 1980; Bazile-Robert, 1982). In

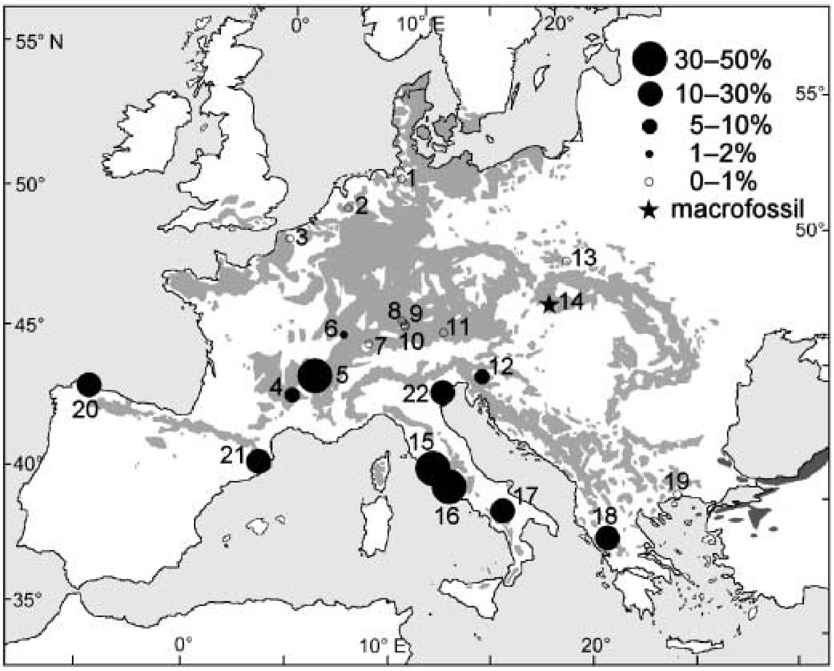

Fig. 3 Pollen percentages and macrofossils of *Fagus* at selected sites during the early interstadials of the last glacial period. St Germain I interstadial: (1) Oerel (Behre, 1989), (2) Amersfoort (Zagwijn, 1961), (3) Watten (Emontspohl, 1995), (4) Lac du Bouchet (Reille & de Beaulieu, 1990), (5) Les Echets (de Beaulieu & Reille, 1984), (6) La Grande Pile (de Beaulieu & Reille, 1992), (7) Gondiswill (Wegmüller, 1992), (8) Jammertal (Müller, 2000), (9) Füramoos (Müller *et al*., 2003), (10) Wurzach (Grüger & Schreiner, 1993), (11) Samerberg (Grüger, 1979), (12) Ljubljana (Šercelj, 1966), (13) Imbramowice (Mamakowa, 1989), (14) Bojnice-Kostnica (Hajnalová & Krippel, 1984), (15) Lagaccione (Magri, 1999), (16) Valle di Castiglione (Follieri *et al*., 1988), (17) Lago Grande di Monticchio (Allen *et al*., 2000), (18) Ioannina (Tzedakis, 1994) and (19) Tenaghi Philippon (Wijmstra, 1969). St Germain II interstadial: (20) Area Longa (Ramil-Rego *et al*., 2000), (21) Pla de l'Estany (Burjachs *et al*., 1996), and (22) Venice – BH2 (Canali, 2005).

Moravia, a find of *Fagus* charcoal dated *c.* 26 kyr ^{14}C-BP (Damblon & Haesaerts, 1997) is supported by pollen analysis (Svobodová, 1991), as well as by other sporadic finds in various glacial sites of Slovakia (Hajnalová & Krippel, 1984; Lityńska-Zając, 1995; Jankovská *et al.*, 2002). In Slovenia-Istria there are a few finds of beech macrofossils from over 38 kyr ^{14}C-BP to 12.5 kyr ^{14}C-BP (Osole, 1976, 1983; Šercelj & Culiberg, 1991; Culiberg & Šercelj, 1995; Šercelj, 1996; Turk *et al.*, 1989).

Holocene history of European beech At the beginning of the Holocene, between 10 and 9 kyr ^{14}C-BP (Fig. 4; 11.5–10.2 kyr cal-BP (calibrated ages)), *Fagus* pollen values increased rapidly in southern and central Italy and in the eastern Alps–Istria–western Hungary. This might have been an effect of migration of small populations present in southern Italy and Istria during the late glacial period (Fig. 4; 13–10 kyr ^{14}C-BP; 15.4–11.5 kyr cal-BP), or growth of populations already locally present but undetected by pollen. In northern Spain beech is present in the Basque mountains. In southern France, beech is found at low elevations, near the estuary of the Rhône river. In the Balkan Peninsula, beech is recorded in an area where populations of *F. orientalis* are now present (Filipova-Marinova, 2003).

By 8 kyr ^{14}C-BP (Fig. 4; 8.9 kyr cal-BP), *Fagus* was well established in southern and central Italy. Beech attains 2% in western Bulgaria, in the north-western Pirin Mountains, where the continuous presence of mesophilous trees is recorded since the early Holocene. Sparse occurrences of beech pollen in the Rila Mountains support the opinion that *Fagus* had not vanished from the south-west of the Balkan Peninsula (Bozilova & Tonkov, 2000). If the same evaluation standard is applied to other regions, then some areas of southern Moravia and southern Bohemia, where beech was already sporadically present *c.* 9 kyr ^{14}C-BP (10.2 kyr cal-BP) and attained values of 2% between 8.5 and 8 kyr ^{14}C-BP, might also have allowed survival of beech during the last glacial maximum. This hypothesis is supported by the fact that between Slovenia and Bohemia-Moravia, regions that are separated by the Danube Valley, there are a number of studied pollen sites with no beech; so no clear migration pattern can be recognized from the south. A fossil wood of *Fagus* near Lake Constance (8450 ± 80 BP; Billamboz, 1995), predating by thousands of years the 2% pollen threshold, is authenticated by an increasing number of similar cases in central-northern Europe. For example, the very first Holocene record of *Quercus* wood in Poland is dated to 9200 ± 70 and 9120 ± 50 ^{14}C-BP (Goslar & Pazdur, 1985), while in the regional diagram *Quercus* pollen just attains 1% at this time (Ralska-Jasiewiczowa *et al.*, 1998).

Between 8 and 7 kyr ^{14}C-BP (Fig. 4; 8.9–7.9 kyr cal-BP), beech reached 2% along the Dalmatian coast. As data from Croatia are very scarce, it is difficult to determine whether the colonization proceeded from the north or from the south. However, the presence of beech in northern Dalmatia already in the late glacial period and its absence in Albania until *c.* 6.5 kyr BP (7.4 kyr cal-BP) support the hypothesis of a migration route starting from the Slovenian–north Dalmatian population. This same population expanded northwards into Austria, where it arrived in the proximity of the rapidly increasing Bohemian populations. The presence of beech in the Swiss Prealps is confirmed by wood ^{14}C dated 7540 ± 75 BP, while pollen is still below the threshold value. In the Vosges, a 8-kyr-old (^{14}C) macrofossil demonstrates the local

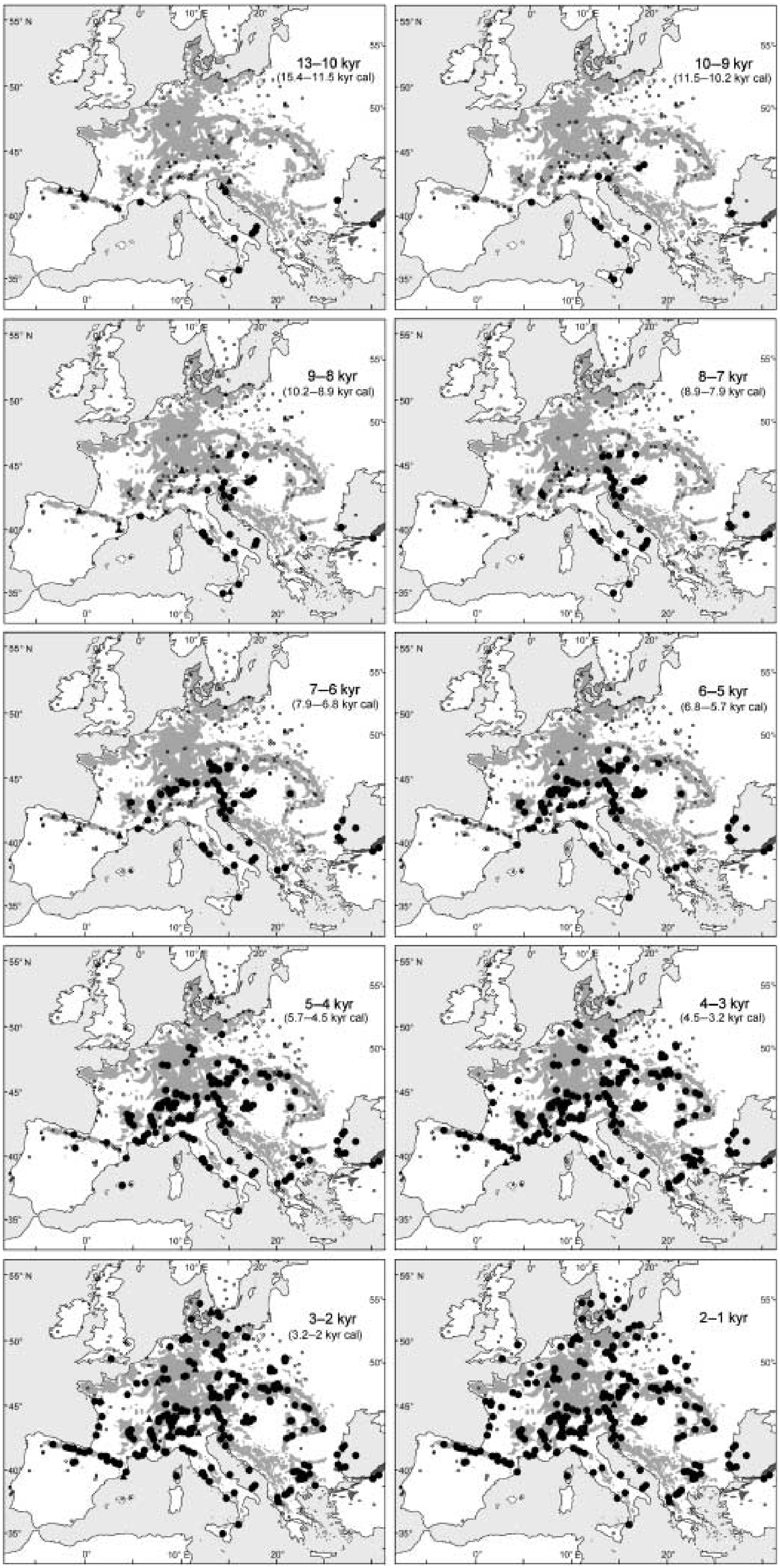

Fig. 4 Geographical distribution of the late glacial and postglacial records of beech in Europe. ○, *Fagus* pollen < 2% or absent; ●, *Fagus* pollen > 2%; ▲, *Fagus* macrofossil. The grey area corresponds to the modern beech distribution (light grey, *Fagus sylvatica*; dark grey, *Fagus orientalis*).

presence of beech, although the pollen records register *Fagus* only 1000 years later. Instead, there is pollen evidence for beech in the French Alps, at two sites facing the Rhône valley. The results of anthracological investigations along the Rhône valley indicate that beech could have survived the last glacial period both in the Mediterranean coastal area and in the eastern Alps (Delhon & Thiébault, 2005). In Spain, three finds of macrofossils in the Basque territory and the Cantabrian Mountains leave no doubt as to the local presence of beech.

The main feature of the expansion of *Fagus* between 7 and 6 kyr BP (Fig. 4; 7.9–6.8 kyr cal-BP) is the very rapid colonization of the outer Alpine chain: by 6 kyr ^{14}C-BP (6.8 kyr cal-BP) *Fagus* reaches 2% in most of Switzerland and the French Alps. However, it is not easy to distinguish from the palaeobotanical data whether these populations originated from Austria–Switzerland or from the sites near the Rhône valley, or even from the Vosges. Around 6.5 kyr ^{14}C-BP (7.4 kyr cal-BP), the French Massif Central was also colonized. Three records of macrofossils in northern Spain confirm that beech was continually present in the Iberian Peninsula, although very sparse. In Romania, in the Apuseni Mountains, *Fagus* reaches 2% at one site, where it was sporadically present from *c.* 8 kyr ^{14}C-BP (8.9 kyr cal-BP). Interpretation of this record is difficult, as the Carpathians were not yet colonized and the western Bulgarian sites have only sparse records. Beech is recorded in Albania and in Greece, where scattered occurrences have been recorded since the beginning of the postglacial period. The area along the Black Sea is largely colonized by beech, probably *F. orientalis.*

Between 6 and 5 kyr ^{14}C-BP (Fig. 4; and 6.8–5.7 kyr cal-BP) a rapid expansion of beech occurred along the inner-Alpine chain, after a few millennia of stasis. The northern Apennines were colonized from central Italy. A second pattern of rapid expansion of beech is found from Bohemia and Moravia towards the western border of the central Bohemian Basin and towards the western Carpathians, respectively. A macrofossil from Luxembourg (5750 ± 60 BP, charcoal) testifies to a northward migration pattern, probably from the Vosges, predating any pollen evidence. *Fagus* shows an important diffusion also in the French Massif Central.

The expansion of beech between 5 and 4 kyr ^{14}C-BP (Fig. 4; 5.7–4.5 kyr cal-BP) was very rapid in central Europe, especially in central Germany. It crossed the north Sudety Mountains and the Carpathians to enter Poland. One macrofossil in Denmark at 56° N latitude, predating pollen records by 1000 years, indicates that beech was moving northward very rapidly. At the same time there was a rapid migration eastwards along the Carpathian Mountains. In the Balkan Peninsula, beech has been found at many sites, including sites in Albania, northern Greece and the Pirin, Rila and Rhodopes mountains in Bulgaria. However, these Balkan populations were still completely separated from those in Romania. In Spain, although only two sites reach 2%, many diagrams show continuous presence of beech pollen at very low percentages.

Between 4 and 3 kyr ^{14}C-BP (Fig. 4; 4.5–3.2 kyr cal-BP) the Cantabrian Mountains as well as the Pyrenees were well colonized by beech. Both mountain ranges appear to have hosted beech populations throughout the last glacial maximum. However, other French populations might also in part have colonized the Pyrenees, as beech was abundant in the Massif Central already *c.* 5 kyr ^{14}C-BP. In western Europe, *Fagus* spread to northern France, whereas in central Europe it migrated into northern Germany. In Poland, beech migrated further to the north into the southern and eastern Poland uplands, while in Romania the eastern and southern Carpathians were colonized. In Bulgaria and Greece the number of sites with beech increased markedly.

Between 3 and 2 kyr ^{14}C-BP (Fig. 4; 3.2–2.0 kyr cal-BP), *Fagus* reached England, expanded in southern Scandinavia and rapidly spread in north-western Poland. The time-window of 2–1 kyr BP (Fig. 4) is characterized by a further rapid expansion towards the north (southern Sweden) and east (northern Poland). Around 1 kyr BP *Fagus* reached its maximum extension, very similar to that of today.

Discussion on the palaeobotanical data

Several recent studies in North America (e.g. Jackson & Booth, 2002; Parshall, 2002; Lyford *et al.*, 2003) show that landscape structure and climate variability play important roles in governing the pattern and pace of natural invasions, with cases of extremely rapid population expansions and cases of populations persisting for hundreds to thousands of years without expanding. These heterogeneous colonization patterns also characterize the postglacial expansion of European beech. In many European regions, pollen values below 2% occur throughout long sections of the postglacial profiles before actual expansion of beech takes place, suggesting that small populations of beech were unable to increase their role in forest communities for thousands of years. The macrofossil evidence strongly supports this conclusion. In several cases, wood of beech has been found at sites where its pollen representation was negligible (Fig. 4). The case of the Iberian Peninsula is particularly striking: fossil wood of beech is found in the Pyrenees, in the Basque territory and on the Cantabrian coast dating from the last glacial maximum, the late glacial period, and the first half of the postglacial period, at a time when the pollen record is lower than 2% and discontinuous. Although beech expanded in the region only after 4 kyr ^{14}C-BP, it appears to have been present in a number of refugia in northern Spain since at least the full glacial period.

The analysis of the regional patterns of beech spread indicates that the expansion of this species was not simultaneous, suggesting that local/regional factors controlled this process. For example, the data from Bohemia, Moravia and Slovakia show differences in beech expansion of up to several thousands of years for populations a few tens of kilometres apart (Rybničková & Rybniček, 1996; Svobodová *et al.*, 2001, 2002; Rybníček & Rybničková, 2002).

Similarly, in the Balkans, where no doubt exists as to the survival of beech during the last glacial period, the pollen data indicate a very late expansion of beech: in the north-western Pirin Mountains, low but appreciable frequencies of *Fagus* are recorded in the early Holocene (Tonkov, 2003a), at a time when other sites in the Pirin Mountains show only rare occurrences (Tonkov *et al.*, 2002; Stefanova & Ammann, 2003). Beech expansion out of these small refugia is observed only after 4 kyr ^{14}C-BP, and in the nearby Osogovo-Belasitsa mountain range only after 2.5 kyr ^{14}C-BP (Tonkov, 2003a,b). Similar patterns are found at the northern fringes of the range of this species, where human activity also played an important role (Aaby, 1986; Latałowa, 1992; Björkman, 1996; Latałowa *et al.*, 2004). In areas where low frequencies of beech pollen are recorded already at 5.5–5 kyr ^{14}C-BP, the expansion in forest communities took place not earlier than *c.* 3–2.8 kyr ^{14}C-BP (Latałowa, 1992; Jahns, 2000). According to Björkman (1996) beech still expands in southern Sweden, while in Poland, at its north-eastern range, it still moves east (Matuszkiewicz, 2002; Latałowa *et al.*, 2004).

These observations show that *Fagus* did not always start to expand from the refuge areas at the beginning of the postglacial period: in many cases, its population increase has been either very slow or very late. As a result, the different refuge areas have contributed to different extents to the colonization of Europe. The populations expanding early and quickly, such as those from Slovenia, played a much more important role than the populations spreading late and slowly, for example those from Greece.

Such delayed population growth considerably complicates the identification of beech refuge areas in Europe. Small refugia might have remained completely undetected, especially if they have been overrun by the migration of stronger populations. The case of the Vosges illustrates this problem well: a single 8 kyr ^{14}C-BP-old piece of wood suggests the existence of a refuge for beech (Fig. 4; 8.9–7.9 kyr cal-BP). The pollen records, indicating a local presence for beech 2000 years later, point instead to a migration pattern from the Swiss mountains into the Vosges.

Hence, beech appears to have survived in many regions of Europe during the last glacial period. The data collected in recent years confirm that the Italian and Balkan Peninsulas were refuge areas, as suggested by previous studies (Huntley & Birks, 1983; Huntley, 1988; Lang, 1992, 1994; Pott, 1997, 2000; Brewer, 2002). However, neither the Italian nor the Balkan Peninsulas were the source areas for the colonization of Europe by beech. In fact the Italian population migrating from the south reached the northern Apennines rather late (*c.* 5 kyr ^{14}C-BP; 5.7 kyr cal-BP), when beech was already present in most of central Europe. The Balkan populations underwent a real expansion even later, *c.* 4 kyr ^{14}C-BP (4.5 kyr cal-BP), and were distinctly separated from those in the Carpathians. The new evidence from the Iberian Peninsula demonstrates that there were refuge areas for beech, which expanded late in the Holocene and did not migrate northward. Contrary to what was generally believed, the palaeobotanical data show that *none of the three southern peninsulas* (Iberian, Italian and Balkan) was a source area for beech in central and northern Europe.

The macrofossil and pollen records from Slovenia and Istria indicate that beech survived there during the last glacial period. The diffusion of beech in the eastern Alps and in western Hungary already at the beginning of the postglacial period probably suggests that it survived in a rather large territory, from where it could spread rapidly into central Europe.

Pollen data also suggest the hypothesis of south Bohemia and south Moravia as refuge areas. These regions, separated from Slovenia by Austria and the Danube valley, were colonized by forest trees very early in the postglacial period. The modern vegetation of the Šumava Mountains and of the Bohemian Forest, which includes both relic boreal populations and species that presumably emigrated from the Alps, supports the hypothesis that these areas were the starting points for the vegetation development of central Europe during the postglacial period (Svobodová *et al.*, 2001, 2002).

Similarly, the data from the Rhône valley in France indicate that beech probably survived the last glacial period in various localities in the region. In the Alpine region, not far from the site of Les Echets, where *Fagus* was present in the last interglacial period, the 2% threshold was reached between 7.5 and 7 kyr ^{14}C-BP (8.4–7.9 kyr cal-BP).

In the Apuseni Mountains, beech is found before 6 kyr ^{14}C-BP (6.8 kyr cal-BP), at a time when *Fagus* is not yet recorded anywhere in the Carpathians. However, even if the Apuseni were a refuge area, it was not an important source area for the colonization of the Carpathians, which were colonized instead from central Europe.

According to the palaeobotanical data, Slovenia, the eastern Alps, the French Alps and possibly south Moravia should be considered as the main source areas from which *Fagus* colonized central and northern Europe. All these regions are located at a latitude (between 45° N and 49° N) much higher than expected from previous palaeobotanical studies. This casts new light on the recent debate on the existence of northern refugia of temperate trees in Europe (Willis *et al.*, 2000; Carcaillet & Vernet, 2001; Stewart & Lister, 2001; Tzedakis *et al.*, 2002, 2003; Stewart, 2003; Willis & van Andel, 2004).

A comparison between the distribution of beech during the last interglacial period (the Eemian, 127–112 kyr BP) and that during the last glacial period (70–15 kyr BP) highlights unexpected concordances: in both cases, the extension of beech was very reduced and central-southern Italy, south-eastern France, south Moravia and Slovenia were survival areas. The abundance of beech during the forest phases following the Eemian, both in the Balkan and in the Iberian Peninsulas, suggests that *Fagus* may also have been present in these regions during the Eemian at a level undetectable by pollen (Fig. 3). As beech had only a very moderate spread in Europe during the Eemian, we can assume that the Eemian populations largely reflect the distribution of refuge areas for beech during the previous glacial period, corresponding to MIS 6 (approx. 170–130 kyr in its most severe phase). If so, a number of beech populations should have persisted throughout at least two glacial–interglacial cycles in central-southern Italy, south-eastern France, southern Moravia, and possibly the Balkan and Iberian Peninsulas. The residual populations of the last pleniglacial period might therefore show genetic differentiation resulting from hundreds of thousands of years of survival in isolation.

The fossil data indicate that beech expanded along particular geographical courses. In the first half of the postglacial period, beech mainly spread along the hilly and mountainous regions of central-southern Europe and it was only after 4 kyr ^{14}C-BP (4.5 kyr cal-BP) that it started to colonize the lowlands of central-northern Europe. This behaviour indicates that the mountain chains were not geographical barriers for beech; on the contrary, in many cases they facilitated its survival and diffusion (cf. the Pyrenees, Cantabrian Mountains, Apennines, French Massif Central, Bohemian Mountains, Dinarian Range, Pirin Mountains and Rhodopes). Beech did not cross the highest chains (e.g. the Alps) but expanded along the slopes. In contrast, *F. sylvatica* appears to have been limited by large plains with continental climate and by important river valleys, for example the Hungarian plain, the Po valley and the lower Danube valley. This suggests that the common opinion that mountain ranges are important barriers causing the isolation of genetic lineages (Taberlet *et al.*, 1998) should be revised, at least for beech.

The fossil data represented on the maps in Fig. 4 clearly show that the postglacial spread of *Fagus* was irreversible, or at least did not include important retreats, so that the modern distribution of European beech roughly corresponds to its maximum extension. In other words, the modern populations are likely to be the direct descendants of the populations that first settled in that area.

The Genetic Data

Beech was first studied by means of chloroplast DNA (cpDNA) by Demesure *et al.* (1996). They showed that a single haplotype predominates across much of the range, suggesting a bottleneck at the time of postglacial recolonization. However, major parts of the range in the Apennines, Balkans and Asia Minor were not included in the study. *F. sylvatica* has also been the subject of intensive surveys at nuclear markers both on a regional and on a range-wide scale (Gömöry *et al.*, 1999; Comps *et al.*, 2001). In the southernmost Apennines and Balkan Peninsula, nuclear genetic markers (isozymes) show a high allelic richness steadily declining towards the north-west, while observed and expected heterozygosities show the opposite trend (Comps *et al.*, 2001). In the present work, chloroplast and nuclear markers were used to analyse a new set of populations sampled across the entire natural distribution range of the species.

Materials and methods: chloroplast markers

Sampled populations and DNA isolation The number of analysed populations ranged from 352 to 468, depending on the type of chloroplast marker (PCR-RFLP, Fig. 5a, and chloroplast microsatellites, Fig. 5b). Natural or naturally regenerated managed forests were selected. Individual samples were taken from trees > 50 m distant from each other. For the majority of the populations, beech buds were collected by the Institute of Forest Genetics, Großhansdorf (Germany) in various International Beech Provenance Trials in Germany. Some populations were sampled in native beech stands by selecting at random at least five nonadjacent adult trees. Crosses performed at the University of Parma (Parma, Italy) were used to investigate the chloroplast DNA (cpDNA) mode of inheritance (Scarfi *et al.*, 2004). The parents of four crosses were screened for length variation using four chloroplast simple sequence repeat (cpSSR) loci and the progeny of informative crosses were further characterized.

Chloroplast data originated from three laboratories: Institut National de la Recherche Agronomique (Cestas, France), Consiglio Nazionale delle Ricerche (Florence, Italy; Vettori *et al.*, 2004), and the Institute of Forest Genetics (Vienna, Austria). In all laboratories, DNA was extracted from peeled buds using the QIAamp Blood Kit (Qiagen, Alameda, CA, USA) and the Dneasy Plant Kit (Qiagen).

Screening for polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) A subset of the populations from the whole range were initially used to screen for PCR-RFLP. Forty-two primer-pair combinations were used: a list is available upon request (see also Grivet *et al.*, 2001; Heinze, 2001). Polymorphism was detected at ORF184-*pet*A, *trn*D-*trn*T, *trn*C-*trn*D, and *ndh*Af-*ndh*Ar; the first two were used for the complete screening (Supplementary Item S6; available online). Restriction digests were run either on 2% agarose gels visualized by ethidium bromide staining or on 8% polyacrylamide gels visualized by silver staining.

Screening for chloroplast microsatellites Six primer pairs were initially used for the amplification of chloroplast microsatellite markers [cp-microsatellite, also called simple sequence repeats (SSRs)]: ccmp2, ccmp3, ccmp4, ccmp6, ccmp7 and ccmp10 (Weising & Gardner, 1999). PCR amplifications were carried out as described in Heuertz *et al.* (2004a). After some initial tests, ccmp4, ccmp7 and ccmp10 were used for the complete screening.

Data analysis Total genetic diversity, average within-population diversity and differentiation among populations were estimated using PCR-RFLP and chloroplast microsatellite data. These estimates were obtained either by taking into consideration genetic distance between haplotypes (Pons & Petit, 1996; parameters are v_T (total diversity), v_S (average within-population diversity) and N_{ST} (genetic differentiation)) or by ignoring it

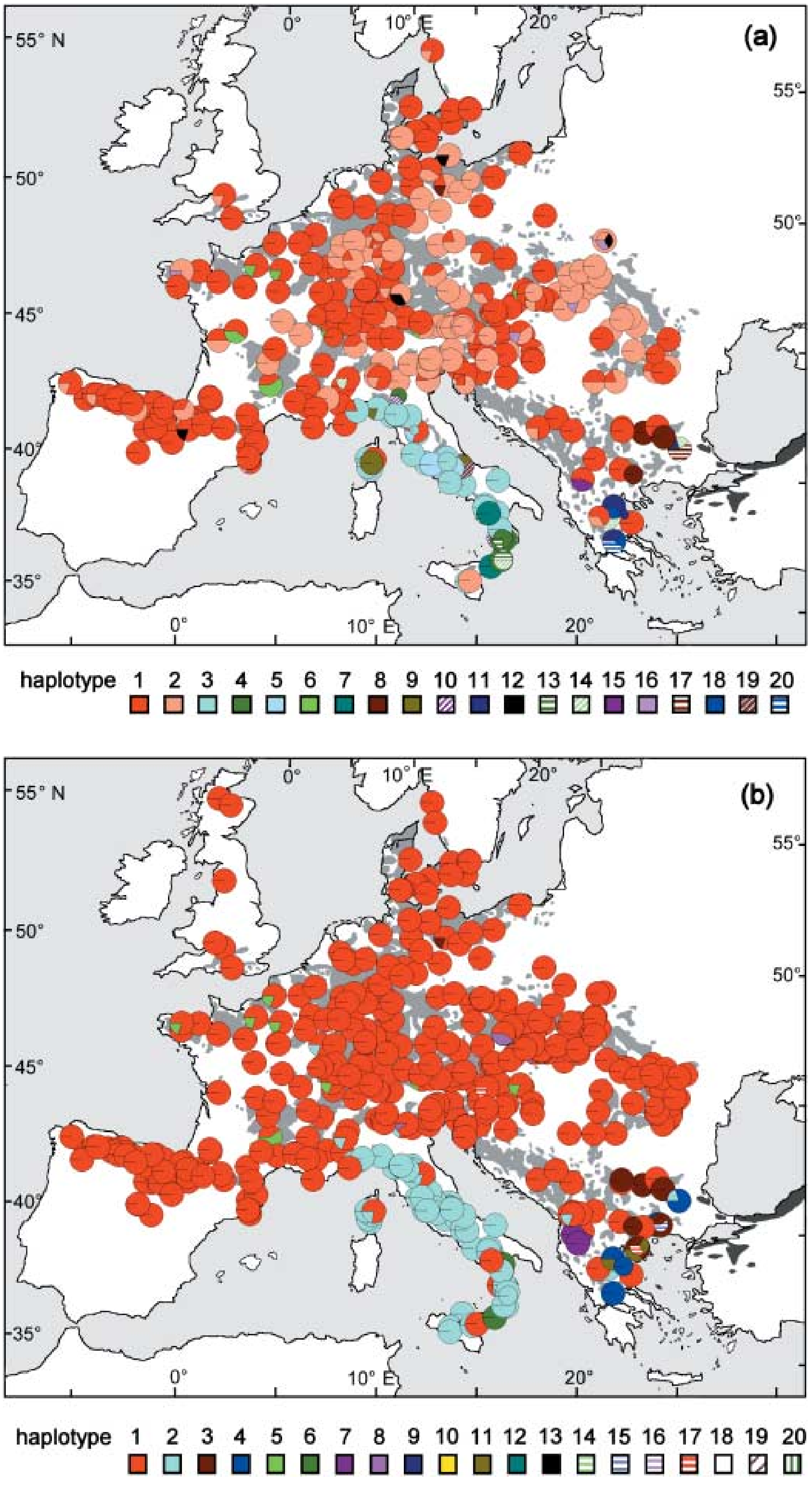

Fig. 5 Geographical distribution of (a) chloroplast haplotypes detected using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and (b) microsatellites (data for the Italian Peninsula were taken from Vettori *et al*., 2004).

(Pons & Petit, 1995; parameters h_T (total diversity) h_S (average within-population diversity) and G_{ST} (genetic differentiation)). A permutation approach was used to test the significance of the differences between G_{ST} and N_{ST} (Petit *et al*., 2002) with the program PERMUT available at http://www.pierroton.inra.fr/genetics/labo/software.

Isolation by distance according to Rousset (1997) was calculated with a Mantel test with 10 000 random permutations between the matrix of pairwise genetic differentiation between populations, using $G_{ST}/(1 - G_{ST})$, and the matrix of the natural logarithm of geographic distance, using the software SPAGeDi 1.2 (Hardy & Vekemans, 2002).

Results: chloroplast markers

Among all parental trees of the full-sib families investigated, only ccmp7 showed size variation between parental trees. One female parent had a size variant of 148 bp while the male parent had a variant of 149 bp; all 87 seedlings available for that cross had the 148-bp variant (identical to that of the mother), thus indicating at least predominant maternal inheritance of the chloroplast genome in beech, as in *Quercus robur* (Dumolin *et al.*, 1995), the only other Fagaceae where organelle inheritance has been investigated to date.

A total of 20 different haplotypes were detected using PCR-RFLPs in 1800 beech trees from 352 populations (Fig. 5a). Five length variants were detected at DT (trnD-trnT) and eight at OA (ORF184-petA). Three of the 20 haplotypes were found in more than 80% of the trees. Four, nine and four variants were detected in 2815 beech trees from 468 populations at ccmp4, ccmp7 and ccmp10, respectively, which combine into 26 chloroplast microsatellite haplotypes. Among the 1800 beech trees for which complete data are available (PCR-RFLP and cpSSRs), the 20 PCR-RFLP haplotypes and 24 (of 26 from the complete survey) SSR haplotypes combine into 58 haplotypes. The among-population coefficient G_{ST} was 0.78, significantly lower than the among-population coefficient N_{ST} (0.87) ($P < 0.01$).

A very similar pattern of geographic distribution was observed with the two types of chloroplast markers, i.e. PCR-RFLP and microsatellites (Fig. 5). PCR-RFLP haplotypes 3 and 4 (Fig. 5a) occur in the Italian peninsula, along the Apennine chain; the closely related haplotypes 1 and 2 are widespread over much of Europe; and haplotypes 17–20 occur in the southern part of the Balkan Peninsula and could reflect the presence of *F. orientalis* and/or hybridization between *F. sylvatica* and *F. orientalis.* The distribution of chloroplast microsatellites revealed the presence of a single haplotype widespread in Europe (haplotype 1; Fig. 5b), a typical Italian haplotype (cpSSR haplotype 2; Fig. 5b) and some divergent haplotypes in the southern part of the Balkan Peninsula (haplotypes 7–20; Fig. 5b).

The analysis of a larger set of populations using chloroplast microsatellites did not provide much additional information on the distribution of haplotypic diversity. The same geographic organization of genetic variation is evident from the haplotype frequency maps based on PCR-RFLP and microsatellite markers (Fig. 5). One haplotype occurs across Europe, except in the central and southern Italian Peninsula and the southern Balkan Peninsula. There is clear differentiation between the central-southern Italian populations and the northern Italian populations along the Alps where the typical 'European' haplotype was scored (Fig. 5b). The southern part of the Balkan Peninsula has a high number of divergent haplotypes.

Within-population variation at cpSSRs was relatively low, with an average of $N = 1.12 \pm 0.12$ (mean ± standard error) haplotypes per population. Differentiation among populations is high ($G_{ST} = 0.81$ and $N_{ST} = 0.88$). As for PCR-RFLP, the difference between G_{ST} and N_{ST} is statistically significant ($P < 0.01$).

The test of isolation by distance shows that among-population differentiation increases significantly with the logarithm of geographical distance (Mantel permutation test: $P < 0.001$), although linear regression explains only *c.* 9% of the total variance ($R^2 = 0.092$).

Materials and methods: nuclear markers

Sampled populations and allozyme analysis Isozyme data originate from two extensive surveys carried out during the 1990s at the University of Bordeaux I (Bordeaux, France) and Technical University of Zvolen (Slovakia). The two surveys focused on different regions. The 'western dataset' collected in Bordeaux comprises 379 populations mainly from western, central and southern Europe, and many regions in eastern Europe are also covered (see details in Comps *et al.*, 2001; in this paper populations from the Crimea were excluded because they do not belong to *F. sylvatica*, as were two populations for which data were not in the appropriate multilocus format). The 'eastern dataset' collected in Zvolen comprises 221 populations from central, eastern and south-eastern Europe. *F. orientalis* was also investigated intensively but results are not shown here. Together, the sampling covers the whole distribution range and the whole altitudinal span (Fig. 6a). Forty to fifty (but sometimes up to 280) nonadjacent trees were chosen at random over an area with supposedly homogeneous microclimate and soil conditions in each population.

Enzymes were extracted from buds and cortical tissues of each individual, and were separated by means of starch and polyacrylamide gel electrophoresis. Isozyme systems differed between the laboratories. In total, 12 isozyme systems coded by 16 loci were used, seven of which were the same in the two laboratories. Supplementary Item S7 (available online) gives an overview of the extraction and electrophoretic separation methods used. Staining procedures followed Merzeau *et al.* (1989) and Müller-Starck & Starke (1993). Alleles were designated by their relative electrophoretic mobilities (R_f). This designation, as well as personal experience gained through working in the partner laboratory, allowed the identification of alleles and thus the combination of the two datasets in the overlapping loci for a common interpretation.

Data analysis Spatial analysis of variance (SAMOVA) based on a simulated annealing procedure was used to define

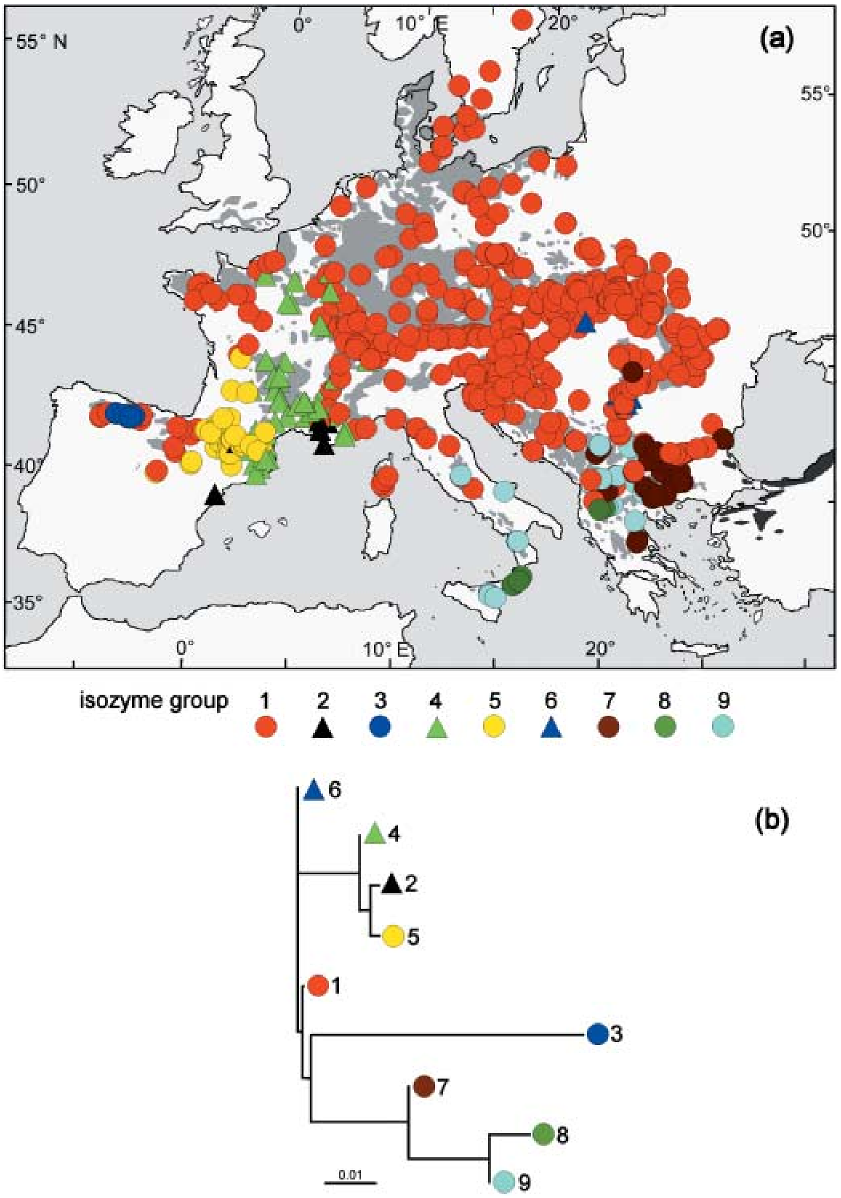

Fig. 6 Spatial analysis of variance (SAMOVA) on isozyme data. (a) geographical distribution of groups; (b) phylogenetic tree obtained using the neighbour-joining approach on the F_{CT} distance matrix for SAMOVA isozyme groups.

groups of populations that are geographically homogeneous and maximally differentiated from each other (Dupanloup *et al.*, 2002). The program (SAMOVA 1.0) iteratively seeks the composition of a user-defined number K of groups of geographically adjacent populations that maximizes F_{CT}, i.e. the proportion of total genetic variance resulting from differences among groups of populations. The program was run for 10 000 iterations for $K \in \{2, \dots, 13\}$ from each of 500 random initial conditions. For each K, the configuration with the largest F_{CT} values after the 500 independent simulated annealing processes was retained as the best grouping of populations. Input data consist of multilocus genotypes; the program (SAMOVA 1.0) is available at http://web.unife.it/progetti/genetica/Isabelle/samova.html.

Two criteria were considered to select the number of groups. First, the pattern of F_{CT} values as a function of K was examined. In particular, we checked the number of groups necessary for F_{CT} to reach a plateau. Secondly, we excluded configurations with one or more single-population groups, because this indicates that the group structure is disappearing. Two SAMOVA analyses were performed, one on the western subset using 12 enzyme systems, and the other on all populations using the seven isozymes in common between the two laboratories. Only the latter is reported here.

Similarities between groups were measured by performing phylogenetic analysis of the groups identified by SAMOVA. Pairwise F_{CT} values between groups were computed by creating an ARLEQUIN version 2.0 (Schneider *et al.*, 2000) input file for all possible pairs of groups and by running an analysis of molecular variance (AMOVA; Schneider *et al*., 2000). The F_{CT} distance matrix was then used to construct a tree representing the genetic relationships between the different groups using a neighbour-joining approach.

Results: nuclear markers

SAMOVA performed on isozyme markers did not unambiguously identify the number (K) of groups of populations that had the highest differentiation among groups, F_{CT}. This was because F_{CT} values increased with K, reaching a plateau at $K \approx 9$. We retained the configuration of $K = 9$, because for $K \geq 10$ one or more groups contained a single population, indicating that the group structure was disappearing (Fig. 6a).

The presence of a well-separated group in central-southern Italy was confirmed, in agreement with cpDNA data. A more complex pattern was found within Europe, in particular in the western part of the natural range of beech. SAMOVA revealed a well-separated group of populations in the Iberian Peninsula, namely in the Cantabrian Mountains. Distinct groups were also identified for the populations along the Pyrenees and in France. An additional subdivision was obtained within the French group, with some populations in the south-east being different from the others. Eastern European, central European and Balkan populations represent a single group, as already observed using chloroplast markers. Some populations of the southernmost Balkan Peninsula cluster together with the southernmost Italian populations: this similarity was also observed using chloroplast markers. A small group of differentiated populations from the Carpathians was also identified (Fig. 6a).

The neighbour-joining tree (Fig. 6b) based on the F_{CT} distance matrix between groups of populations obtained by SAMOVA revealed a clustering which reflects the geographic structure in beech: one cluster of the western groups (2, 4 and 5), a second cluster of the Balkan and Italian groups (7, 8 and 9), which includes Sicily, Calabria, southern Italy, Albania, Macedonia, southern Serbia, Greece, and Bulgaria, and a third cluster of central European groups (1 and 6). Spain and France harbour several distinct groups of populations. Group 3 in the Cantabrian Mountains clusters close to group 1 and is clearly separated from the other western groups. Additional partitioning was also observed in the Carpathians, with group 6 distinguished from the other Balkan groups (7, 8 and 9).

Discussion on the genetic data

Three main groups of haplotypes were detected using chloroplast markers, one in the Italian peninsula, the second all over the rest of Europe and the third in the southern Balkan Peninsula, as in Demesure *et al.* (1996), who interpreted this as the result of colonization from two main refugia, in Italy and in the Balkan Peninsula, and hypothesized that the Balkan refugium had expanded across the rest of Europe (excluding the central and southern Italian Peninsula), with a relatively rapid colonization to the north and west, which prevented northward diffusion from the Italian refugia.

The drastic reduction in population size of European beech documented for the last glacial period has been considered as one of the main factors affecting allelic richness, as measured by using isozyme markers, although heterozygosity remained high in the more recently established populations (Comps *et al*., 2001). For cpDNA markers, the maps in Fig. 5 show that the southernmost parts of the natural range of beech have higher diversity than the central and northern areas, the highest number of haplotypes being detected in the southern Balkans and in Italy. This supports the 'southern richness vs northern purity' paradigm (Hewitt, 2000), although no significant geographic pattern in within-population haplotypic diversity was observed.

As expected given their biparental inheritance, isozymes displayed less pronounced level of genetic structure than cpDNA, but showed a more complex pattern, allowing the detection of groups of populations undetected with chloroplast markers (Fig. 6). The SAMOVA analysis resulted in separate groups for the Iberian Peninsula, the south-east of France and the Carpathians.

Nuclear markers therefore provide additional insights, as in *Fraxinus excelsior*, where nuclear microsatellite markers have revealed the presence of distinct groups of populations that had remained undetected with chloroplast markers (Heuertz *et al*., 2004b). These results highlight the importance of the use of combined molecular approaches for historical reconstructions.

Combined Fossil and Genetic Data

Both the genetic and the fossil data demonstrate that beech populations of distinct origins occur in Europe at present. However, neither the palaeobotanical analyses nor the genetic data alone can provide a complete picture of the relationships between the location of the original populations at the beginning of the postglacial period and modern genetic diversity. Only DNA analyses of fossil plant remains would provide definitive data on those haplotypes present at a given location in the past, but this remains a challenging task (Gugerli *et al*., 2005).

The fossil data indicate that the postglacial spread of *Fagus* in Europe was continuous, without important retreats and readvances. Except in very sporadic cases, beech does not disappear from the record once it has reached the 2% threshold, supporting the assumption that modern beech populations are the direct descendants of the populations that first settled in the area. It is of course also possible that subsequent invasions extirpated the original populations, but no cases of multiple successive beech invasions are discussed in the palynological literature at any site. Based on the evidence of the persistence of beech populations in the colonized territories,

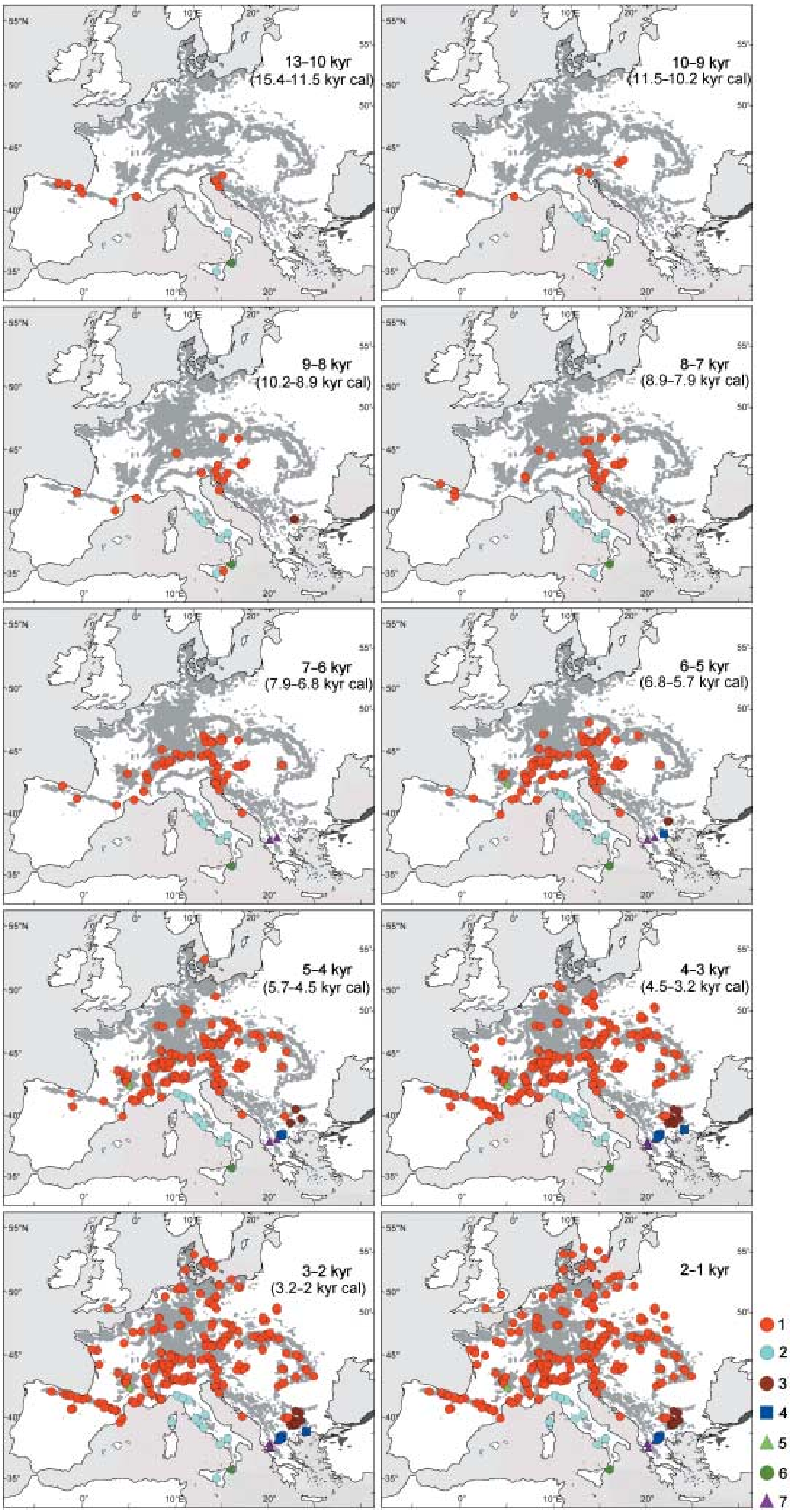

Fig. 7 The late glacial and postglacial records of beech in Europe assigned the colours of the cp-haplotypes shown in Fig. 5b.

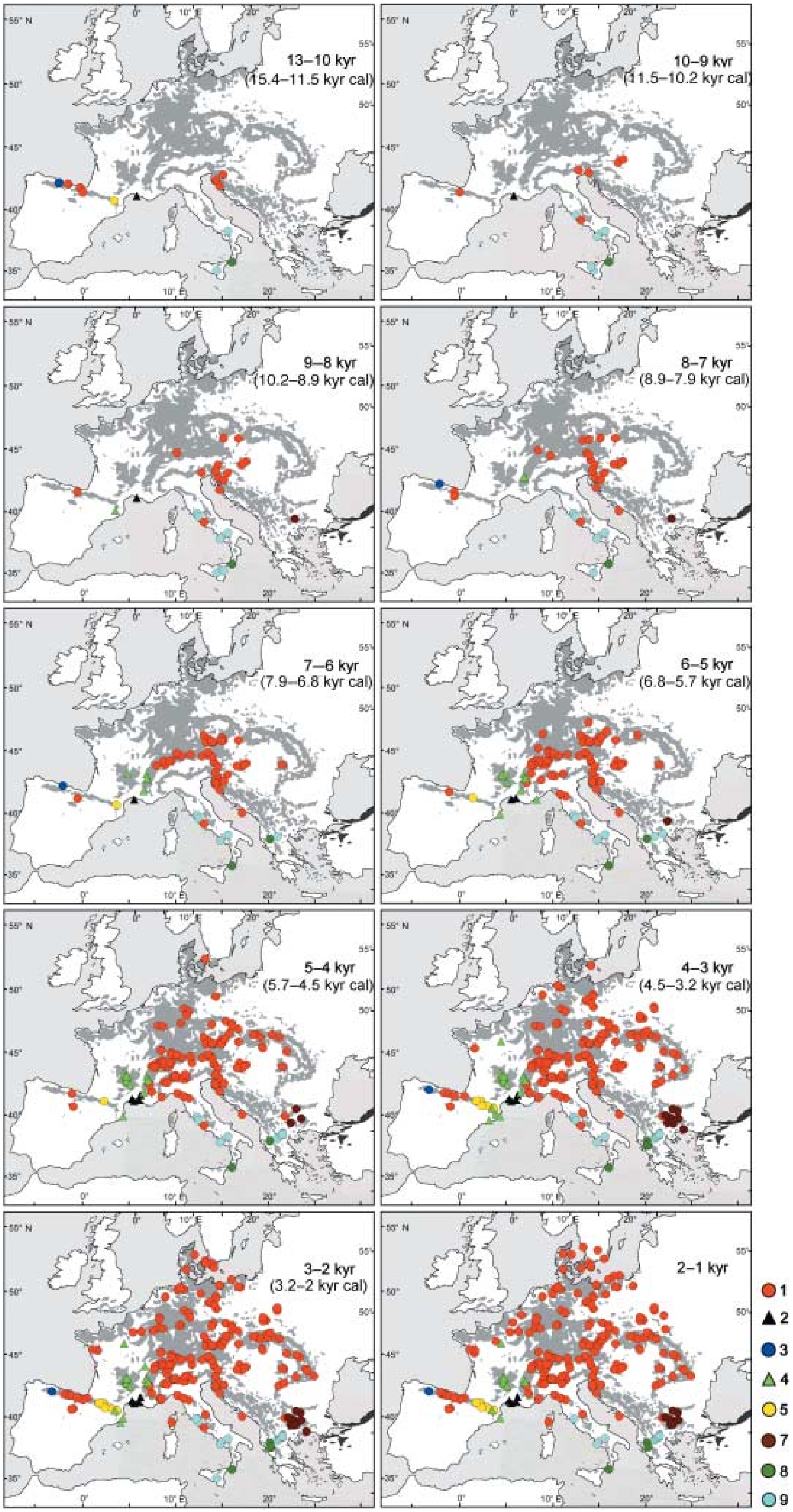

Fig. 8 The late glacial and postglacial records of beech in Europe assigned the colours of the isozyme groups shown in Fig. 6a (600 populations and seven loci).

we have combined the fossil maps of Fig. 4 with the genetic maps of Figs 5b and 6a, by assigning the colours of the modern genetic populations to the fossil records in the same geographical area (Figs 7, 8). In this way, it is possible to follow the origin of each genetic type and to locate its starting point.

Combined Fossil Data and Chloroplast Markers

In Fig. 7 the fossil data are combined with the results of the chloroplast markers (cpSSRs) with the aim of tracing back the origin of each haplotype. Only the seven dominant haplotypes (frequency > 5%) are shown, with haplotypes found in areas without palaeobotanical data being excluded.

Cp-haplotype 1, which is widespread in Europe except in the Italian Peninsula and the southern part of the Balkan Peninsula, appears to have spread from different refuge areas, including the northern Iberian Peninsula, the western Alps, eastern Alps–Slovenia, and possibly southern Bohemia–southern Moravia. The finding of a unique chloroplast haplotype in these populations (two closely related haplotypes with PCR-RFLP) suggests that they are remnants of a very important pre-Holocene diffusion of beech, characterized by cp-haplotype 1 and extending at least from the Cantabrian Mountains to the eastern Alps. As *Fagus* expanded during the interglacial phases, this observation leads to the hypothesis that the populations in Spain, France, Slovenia and Moravia were undifferentiated as to cpDNA already during the last glacial period, and probably also during the previous glacial episode, corresponding to MIS 6 (approx. 170–130 kyr in its most severe phase). In fact, during the forest expansions corresponding to MIS 5 (approx. 130–70 kyr BP) the populations in Spain and France were probably not connected with the Slovenian and Moravian populations (Fig. 3). The last widespread diffusion of beech over all of central Europe might date back to the Holsteinian in the Middle Pleistocene (Müller, 1974; Krupiński, 1995; de Beaulieu *et al.*, 2001).

Cp-haplotype 2, restricted to the Italian Peninsula, survived in southern–central Italy, from where it spread northward into the northern Apennines, which were completely colonized by 4 kyr ^{14}C-BP (4.5 kyr cal-BP). The distinct origins of beech from the Apennines and from the Alps suggested by the palaeobotanical data are fully confirmed by the genetic data which distinguish cp-haplotype 2 in the Apennines and cp-haplotype 1 in the Alps. A different cp-haplotype (6), which did not significantly expand during the postglacial period, is found in the southernmost part of the Italian Peninsula. The palaeobotanical data indicate that beech was present in southern Italy (Calabria) already in the Pliocene (Combourieu-Nebout, 1993), but they cannot indicate when cp-haplotype 2 and cp-haplotype 6 became genetically distinct.

In the Balkan Peninsula, four genetic groups (cp-haplotypes 1, 3, 4, and 7) were detected. Cp-haplotype 1 is found in Dalmatia. While the palaeobotanical data do not clearly reveal the origin of this population, because of the scantiness of the records, the genetic data suggest a connection with the Istrian population. The refuge area for cp-haplotype 3 could be located in southern Bulgaria, from where it would have spread early but slowly at the beginning of the postglacial period, colonizing the Bulgarian mountains. Cp-haplotype 4 is found in a more southern position, in Greece, where beech started its expansion rather late, between 6 and 5 ^{14}C-kyr BP (6.8–5.7 kyr cal-BP). Cp-haplotype 7 corresponds to Albania and north-western Greece, where beech pollen reached 2% between 7 and 6 kyr BP (7.9–6.8 kyr cal-BP). On the whole, the situation of beech in the Balkan Peninsula appears rather complex, with genetically different populations spreading at different times during the postglacial period, but never colonizing extensive areas. Although there are no macrofossil records from the Balkan Peninsula demonstrating the local persistence of beech, the existence of refugia in the Balkans has not been questioned (Bennett *et al.*, 1991; Tzedakis *et al.*, 2002, 2003). The diffusion of beech in north-western Greece *c.* 100, 210, 330 and 370 kyr BP supports this hypothesis (Fig. 2).

Considering that the Spanish and the Slovenian populations, which have probably been separated since at least the Middle Pleistocene, have maintained the same cpDNA variant, it seems that the differentiation within the Balkans may have taken place during earlier glacial periods. It is therefore remarkable that it could be maintained for such a long time in a relatively restricted area, without being wiped out during the phases of forest expansion (Bennett, 1997). The mosaic of climatic and environmental conditions in the Balkans may have favoured the maintenance of different genetic characters, as in the mountainous regions of southern Italy.

Combined Fossil Data and Nuclear Markers

In Fig. 8 the fossil data are combined with the results of the SAMOVA on isozyme data (600 populations and nine groups) represented in Fig. 6. The number of isozyme groups represented in Fig. 8 is reduced to eight, as no palaeobotanical analyses are available in the vicinity of the sites with isozyme-group 6.

The original area of isozyme-group 1 can be traced back to Slovenia–Istria and possibly southern Bohemia–southern Moravia (Fig. 8). From this figure, it appears that beech spread from these regions and reached central and northern Europe, extending from north-western France to the eastern Carpathians. The two populations in Slovenia–Istria and in southern Bohemia–southern Moravia were possibly connected during phases of the forest periods corresponding to MIS 5 (130–70 kyr BP; Fig. 3); their common genetic characters may therefore be attributed to that time.

The presence of isozyme-group 1 in the Iberian Peninsula (Fig. 6) is probably a result of a lack of resolution, as it was not observed in analyses based on 12 loci (results not shown).

Isozyme-group 3 is restricted to the Cantabrian Mountains, where it must have survived during the last glacial period, as macrofossils of beech are found in the region already during the late glacial period. Isozyme-group 5 is typical of the Pyrenees, where macrofossils dating from the late glacial period have been found. Isozyme-group 4 appears to be connected with southern French populations, either as a remnant of a former beech expansion including both southern France and the eastern Pyrenees, or as an immigrant from France in the course of the Holocene. A macrofossil find at the beginning of the postglacial period supports the first hypothesis. The presence of three different isozyme groups in such a small area as northern Spain confirms that a mountainous topography may constitute a particularly favourable environmental situation for the differentiation and maintenance of the genetic structure. A clear increase in the level of genetic differentiation (G_{ST}) with altitude was detected in *F. sylvatica* by Petit *et al.* (2001). Taken together, these results demonstrate the advantages of using genetic data to distinguish different populations in restricted geographical regions where the palaeobotanical data do not have sufficient resolution.

Isozyme-group 2 shows a modest diffusion in southern France. It appears to be related to the population that expanded already during the late glacial period in the Mediterranean coastal region.

Isozyme-group 4 appears to have expanded from the French Alps, one of the probable refuge areas for beech according to the fossil evidence. In fact, *Fagus* pollen is found in this region during both the Eemian interglacial (*c.* 127–112 kyr BP) and the subsequent forest phases before the last glacial period (105–70 kyr BP). Before the Eemian, a succession of several expansions and retreats has been documented in the French Massif Central over the last 450 kyr (Fig. 2). However, we cannot identify when the French populations corresponding to isozyme-group 4 separated from the populations that survived in central–eastern Europe (isozyme-group 1) and from the population that survived the last glacial period along the French Mediterranean coast (isozyme-group 2).

Isozyme-groups 8 and 9 are found both in central–southern Italy and in the western Balkan Peninsula, in contrast with the palaeobotanical information which indicates a long history of isolation in the two peninsulas for beech. While the lowering of the Adriatic Sea during the last glacial period may account for a connection of the northern parts of the two peninsulas, the southern regions were separated by the sea. It is, however, important to note that the nuclear markers separate the Italian and Balkan populations from the rest of Europe. A certain degree of similarity of isozymes between the Italian and Balkan populations may also be accounted for by the differential dispersion of nuclear and chloroplast markers, as pollen may easily move across the Adriatic Sea while seed dispersal is generally restricted to shorter distances, as inferred in conifer species from contrasting paternally inherited cpDNA markers and maternally inherited mitochondrial DNA markers (e.g. Liepelt *et al.*, 2002; Burban & Petit, 2003).

The eastern regions of the Balkan peninsula are populated by isozyme-group 7, which survived the last glacial period on the Bulgarian mountains, as indicated by pollen records between 9 and 8 kyr ^{14}C-BP (10.2–8.9 kyr cal-BP), and spread regionally rather late during the postglacial period.

Discussion on the Combined Fossil and Genetic Data

The combination of fossil and genetic data highlights new perspectives in the reconstruction of the history of European beech. The cpDNA markers (Fig. 7) revealed genetic differences in the Italian and Balkan Peninsulas, in agreement with the palaeobotanical results. The nuclear markers (Fig. 8) very effectively distinguished different populations in Spain and France, whose long-term persistence is confirmed by the macrofossil and pollen records.

The differences between the two combined fossil–genetic reconstructions (Figs 7, 8) may also be a source of information on how the genetic structure can develop. For example, in both sets of maps the Slovenian–Istrian and Moravian–Bohemian populations are genetically similar. The palaeobotanical records do not rule out the possibility that the two populations were connected during some of the forest expansions preceding the last glacial period (e.g. MIS 5: 130–70 kyr BP; Fig. 3). A time period of 100 000 years was therefore not sufficient to produce changes in the genetic constitution in either of the two populations. The French and Spanish populations showed the same cp-haplotype as the Slovenian–Moravian populations (Fig. 7), but are clearly distinct according to nuclear markers (Fig. 8). The fossil data suggest that the French and Slovenian populations were not connected during MIS 5 (Fig. 3), although it is not possible to determine with certainty the timing of the last event of connection; one possibility would be during MIS 11, *c.* 400 kyr BP (de Beaulieu *et al.*, 2001).

In some cases, the genetic data considerably refine inferences based on palaeobotanical data. For example, the distribution of isozyme-group 4 delimits the extent of the westward spread of the beech population that originated in the eastern Alps and migrated along the outer Alpine arch, and separates this population from that of French provenance also moving towards Switzerland (Fig. 8). It also appears that the population that expanded already in the late glacial period along the French Mediterranean coast can be distinguished from that on the French Alps by means of isozyme analysis. In the Balkans, the cpDNA markers point to differences in beech populations living in adjacent mountain ranges, possibly indicating a long-term persistence of beech in the area. The palynological data can hardly detect these populations which were very scanty and expanded late during the postglacial period. Similar results were obtained in northern Spain, where three different isozyme groups were found, but only

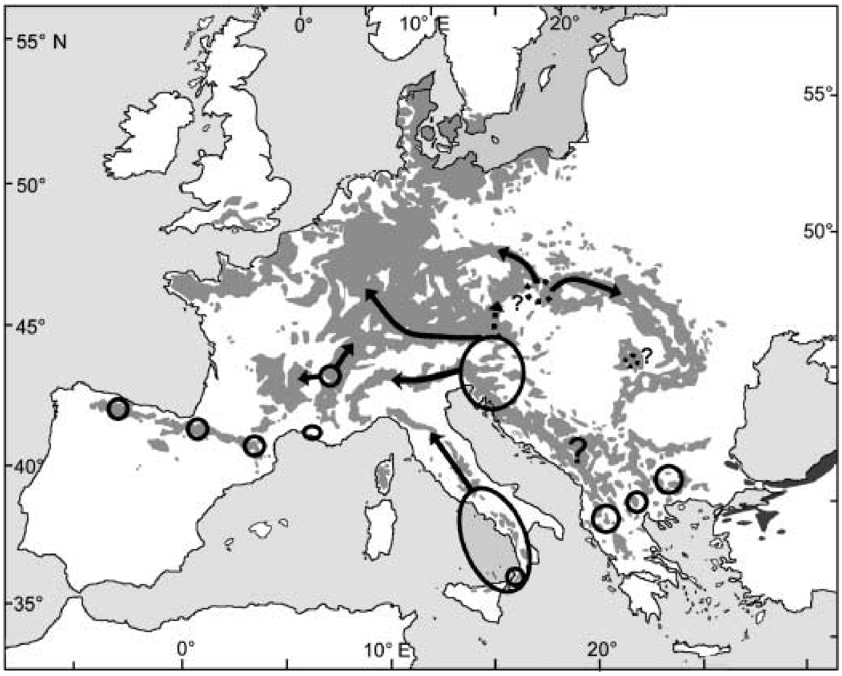

Fig. 9 Tentative location of refuge areas for *Fagus sylvatica* during the last glacial maximum and main colonization routes during the postglacial period.

macrofossils, not pollen, demonstrate the presence of very sparse beech populations until the mid-Holocene.

The fossil and genetic data show a general correspondence in the fundamental features of the distribution of beech populations in Europe. In particular, both datasets indicate that (i) beech survived the last glacial period in multiple refuge areas, (ii) the central European refugia were separated from the Mediterranean refugia, (iii) the Mediterranean refuge areas did not contribute to the colonization of central and northern Europe, (iv) some populations considerably expanded during the postglacial period, while other populations showed only moderate growth.

A possible scenario for the location of beech refugia during the last glacial period, based on both fossil and genetic data, is proposed (Fig. 9). Three separate areas are indicated in the Iberian Peninsula, where macrofossils confirm the existence of beech, and isozymes separate three groups in different mountain chains. These populations expanded only locally and did not contribute to the colonization of the rest of Europe. The long-term persistence of beech in the western Alps in France is indicated by the pollen data and supported by the isozyme data. This population would be responsible for the colonization of most of southern France. A genetically different population survived in the French coastal zone and expanded early but very slowly, occupying a relatively small area. The refuge areas of beech in southern Italy are indicated by the fossil data and confirmed by the existence of cp-haplotypes peculiar to this region. The southern Italian beech population spread northward, but never reached Italy north of the Po valley. In fact, the Italian Alps were colonized from a population in the eastern Alps–Slovenia–Istria, which expanded rapidly and vigorously at the beginning of the postglacial period, and extended westward along the outer Alpine chain to the Vosges, northern France and England. Although the data from Serbia and Croatia are very scanty, it seems possible that the Slovenian population also migrated southward along the Dinaric Alps. This population is genetically similar to the population in southern Moravia–southern Bohemia, considered to be a possible refuge area on the basis of fossil data. The hypothetical residual Moravian population would have been responsible for the colonization of the Carpathians. There is also the possibility of a refuge area in the Apuseni Mountains, where the pollen record indicates a rather early spread during the postglacial period at only one site. From the genetic point of view, the populations in the Apuseni Mountains are characterized by rather high allelic richness (Comps *et al.*, 2001; Gömöry *et al.*, 2003). The populations belonging to SAMOVA group 6 are also found close to the Apuseni Mountains. It is thus possible that a secondary refugium was located in this area, but did not contribute to the colonization and was overlain by immigrants from the Moravian refugium over the Carpathian arc. In any case, the Romanian populations are clearly distinct from the Balkan populations, where the fossil data document a long-term persistence of beech, confirmed by high genetic diversity, suggesting that at least three different genetic groups can be recognized in different mountain ranges. These Balkan groups did not spread northward to colonize the rest of Europe.

The pollen records indicate a rather early and vigorous expansion in Slovenia, the southern part of the Czech Republic and southern Italy. However, the Iberian and Balkan refugial populations remained restricted in size, as did those in

Calabria (southern Italy) and near the estuary of the Rhône River in France. These patterns are partially recognizable in the distribution of the genetic variation: the recent Iberian beechwoods are characterized by low allelic richness, whereas the Slovenian populations, which were probably quite extensive during the last glacial period, display high allelic richness (Comps *et al.*, 2001).

Other recent studies have compared surveys based on modern DNA markers and fossil-based inferences of past distributions of tree populations (e.g. Petit *et al.*, 2002; Palmé *et al.*, 2003; McLachlan *et al.*, 2005). However, none has directly combined genetic and fossil data across a species range as in the current study. In contrast to the situation for white oaks (*Quercus*) (Petit *et al.*, 2002) and *Betula* (Palmé *et al.*, 2003) in Europe, the history of beech in Europe involves a single taxon (*F. sylvatica*) except for a limited area in the south-east. In eastern North America, cpDNA surveys have shown that the geography of postglacial range expansion of *Fagus grandifolia* significantly differs from that expected from pollen-based reconstructions (McLachlan *et al.*, 2005). However, on the isopoll maps recently published by Williams *et al.* (2004), four distinct populations of beech are present in eastern North America by 9 kyr cal-BP, which may correspond to centres of diversity for different clades of cp-haplotypes, and so reconcile the patterns of pollen maps and molecular data. Our experience shows that a thorough synthesis of palaeobotanical data, including both pollen and macrofossil data, is a necessity in this kind of reconstruction.

Conclusions

Fossil and genetic data together reveal new, unexpected perspectives on the history of European beech, answering some questions concerning the relationships between the past geographic distribution of this species and its modern genetic distribution. Both nuclear and chloroplast markers indicate that most of the beech populations of central, eastern and northern Europe have a very homogeneous genetic structure. For southern and western Europe, the combined results for the two markers indicate a rather inhomogeneous genetic structure, with a number of separate populations in the Iberian, Italian and Balkan Peninsulas, as well as in southern France.

The palaeobotanical records provide information on the geographical origin of at least some of the existing beech populations, by identifying a number of refuge areas where beech could survive during the last glacial period and indicating possible colonization routes followed during the postglacial period. A detailed analysis of the fossil data highlights a much more complex situation than in the previously published reconstructions of the history of beech. Southern France, eastern Alps–Slovenia–Istria, and possibly southern Moravia–southern Bohemia should be considered as the main source areas from which *Fagus* colonized central and northern Europe, confirming the existence of relatively northern refugia of temperate trees in Europe. In contrast, the populations that survived the last glacial period in the Mediterranean regions did not spread into central Europe.

The location of the glacial refugia casts new light on the possible mechanisms of genetic divergence during the colonization phase. It appears that the French and Slovenian populations that colonized central Europe cannot be considered as the 'leading edge' of the southern populations, as they were geographically and genetically distinct from the populations in Spain and the Balkans. The beech populations that survived in northern Spain and in the Italian and Balkan Peninsulas could have migrated to the north, where they could find suitable environmental conditions, already at the beginning of the postglacial period, but they expanded late and did not move much to the north. For instance, the Balkan populations did not colonize the Carpathians, and the populations from central Italy reached the northern Apennines only in the second half of the Holocene. Most of the glacial refugia were located in mountainous territories, where the residual populations could find sheltered and humid places for survival. The spread of these populations took advantage of the mountain ranges that are found in southern and central Europe, but was hindered by large plains (e.g. the Hungarian plain) or valleys (e.g. the Danube valley) that became colonized by beech only rather late in the Holocene.

A comparison between the distribution of beech during the last interglacial period and that in the last pleniglacial period indicates that, at the time at which the distribution of beech was most reduced, the survival areas for *Fagus* were central–southern Italy, south-eastern France, Slovenia, and possibly southern Moravia, the Balkans and the northern Iberian Peninsula. Therefore, beech might have persisted in the same refuge areas for at least two interglacial–glacial cycles, providing an indication of the time-span (hundreds of thousands of years) necessary to produce the observed genetic divergence. It appears that the modern genetic diversity was not shaped by a single glacial episode but by multiple interglacial–glacial cycles, since at least the Middle Pleistocene.

Our conclusions contrast with those arrived at in previous studies on this species, and thus call into question earlier attempts (in this species as well as in other taxa) to infer glacial refugia on the basis of limited fossil (pollen) or genetic (organelle DNA) evidence.

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Supplementary Material

The following supplementary material is available for this article online:

Item S1 State of the art for the postglacial history of beech in Europe.

Item S2 Locations of and references for the late glacial and postglacial pollen sites in Europe reported in Figs 4, 7 and 8.

Item S3 Location map of the fossil records.

Item S4 Selection of the *Fagus* pollen threshold.

Item S5 Location of and references for the macrofossil sites reported in Figs 4, 7 and 8.

Item S6 List of primer pairs used for polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) resulting in polymorphism.

Item S7 Overview of methods of enzyme extraction and electrophoretic separation used within the isozyme studies in the laboratories in Bordeaux and Zvolen.

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