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Chloroplast DNA variation and postglacial recolonization of common ash (*Fraxinus excelsior* L.) in Europe

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Abstract

We used chloroplast polymerase chain reaction-restriction-fragment length polymorphism (PCR-RFLP) and chloroplast microsatellites to assess the structure of genetic variation and postglacial history across the entire natural range of the common ash (*Fraxinus excelsior* L.), a broad-leaved wind-pollinated and wind-dispersed European forest tree. A low level of polymorphism was observed, with only 12 haplotypes at four polymorphic microsatellites in 201 populations, and two PCR-RFLP haplotypes in a subset of 62 populations. The clear geographical pattern displayed by the five most common haplotypes was in agreement with glacial refugia for ash being located in Iberia, Italy, the eastern Alps and the Balkan Peninsula, as had been suggested from fossil pollen data. A low chloroplast DNA mutation rate, a low effective population size in glacial refugia related to ash's life history traits, as well as features of postglacial expansion were put forward to explain the low level of polymorphism. Differentiation among populations was high (G_{ST} = 0.89), reflecting poor mixing among recolonizing lineages. Therefore, the responsible factor for the highly homogeneous genetic pattern previously identified at nuclear microsatellites throughout western and central Europe (Heuertz *et al*. 2004) must have been efficient postglacial pollen flow. Further comparison of variation patterns at both marker systems revealed that nuclear microsatellites identified complex differentiation patterns in south-eastern Europe which remained undetected with chloroplast microsatellites. The results suggest that data from different markers should be combined in order to capture the most important genetic patterns in a species.

Keywords: chloroplast DNA, chloroplast microsatellite, *Fraxinus excelsior*, PCR-RFLP, phylogeography, population history

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Introduction

Fossil pollen records since the end of the last ice age, approximately 180 000 years ago, indicated that most temperate forest tree species spent the cold period in the southern European Peninsulas (Huntley & Birks 1983; Tzedakis *et al*. 2002), although more northern refugia in sheltered sites were also identified (Willis *et al*. 2000; Stewart & Lister 2001). The postglacial history of numerous European tree and shrub species has been investigated to date using genetic markers and the results have been interpreted together with fossil pollen data when available. Similar genetic patterns were identified in a number of species; for example the long-term isolation in glacial refugia resulted in strong

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genetic drift, which is reflected in high chloroplast DNA differentiation (G_{ST}) in extant populations located close to glacial refugia (Dumolin-Lapègue *et al*. 1997; Demesure *et al*. 1996; Petit *et al*. 2003). The expansion from refugia since about 12 000 years ago was predicted to result in a loss of variation owing to successive founder events (Hewitt 1996). However, many species displayed high diversity in recolonized forests (Petit *et al*. 2003). This apparent attenuation of founder events can be explained through the admixture of haplotypes from merging postglacial recolonization routes (Petit *et al*. 2003), possibly in combination with a prolonged juvenile phase in woody species, which allows for substantial immigration before the first colonizers begin to reproduce (Austerlitz *et al*. 2000). Another important feature of postglacial history is the individualistic behaviour of recolonizing species; for instance in Petit *et al*.'s (2003) multispecies chloroplast DNA study, about half of the 22 investigated European tree and shrub species showed poor or no agreement with the previously described patterns of diversity and differentiation, presumably owing to differences in their respective ecological ranges and life history traits (Petit *et al*. 2003). It is also well established that species co-occurring today may have undergone very different postglacial histories (Bennet 1997; Taberlet *et al*. 1998).

In this study, we examine in detail the phylogeography of common ash, *Fraxinus excelsior*, a wind-pollinated European forest tree species with heavy, wind-dispersed seeds, using a large data set represented by 201 populations sampled across the natural range of the species. Common ash prefers deep base-rich soils with good moisture availability, although it is also often found on dry sites (Wardle 1961). It has a strong colonization capacity but adult trees mostly have a scattered distribution, so that ash is rarely a dominant tree in mixed deciduous forests (Falinski & Pawlaczyk 1995). The mating system is polygamous: flowers are male, hermaphroditic or female and there is a continuum from pure male to pure female individuals with hermaphroditic intermediates (Picard 1982; Wallander 2001).

The representation of *F. excelsior*-type pollen in pollen records is generally weak (Huntley & Birks 1983). Fossil pollen data (Huntley & Birks 1983; Gliemeroth 1997; Brewer 2002) suggested glacial refugia for ash in the Balkans and the eastern Alps and possibly Italy, while refugia in Iberia and north of the Black Sea were less strongly supported. A nuclear microsatellite study (Heuertz *et al*. 2004) confirmed that postglacial recolonization of western and central Europe most likely occurred from several refugium populations, which were located possibly, but not exclusively, in the western Balkan Peninsula and in north-eastern Europe. A putative refugium in the eastern Balkan Peninsula would have contributed little to recolonization (Heuertz *et al*. 2004). Further, it was found that common ash populations from the British Isles to Lithuania throughout central Europe displayed virtually no differentiation, which was interpreted as the result of strong gene flow during postglacial recolonization (Heuertz *et al*. 2004). The limited sampling design in that study, however, did not allow making inference about refugia in Iberia, Italy or the Alps. Hence several questions about the postglacial history of common ash remain unanswered: (1) is there evidence for the occurrence of refugia in the Iberian and Italian Peninsulas and did they contribute to postglacial recolonization of western and central Europe? (2) Do molecular data support the refugium in the eastern Alps suggested by fossil pollen data? (3) How robust are the previously observed genetic structure patterns in eastern and south-eastern Europe across different categories of molecular markers?

Chloroplast DNA is a useful tool for the identification of postglacial colonization routes (King & Ferris 1998; Palmé & Vendramin 2002; Petit *et al*. 2002a,b) because (1) it is nonrecombining, therefore haplotypes remain mostly unchanged when passed to the next generation, and (2) in angiosperms it is generally transmitted through seeds only (Rajora & Dancik 1992; Dumolin *et al*. 1995), therefore colonization patterns which derive from seed dispersal are not blurred by pollen flow. The maternal inheritance of chloroplast DNA also implies that the effective population size is smaller for chloroplast DNA than for nuclear DNA (two times smaller for monoecious species and four times smaller for dioecious ones, with the polygamous ash being probably situated in between these two extremes). Therefore, all else being equal, levels of differentiation are expected to be higher for chloroplast DNA than for nuclear DNA markers. However, as recombination is absent, chloroplast DNA corresponds effectively to a single gene, featuring its idiosyncratic genealogical process. The genealogical process is highly variable among genes (Nordborg 2001) and genealogies are affected by demographic events, such as population expansion, bottlenecks, vicariance or migration, and by the accidental loss of lineages (Knowles & Maddison 2002). Therefore, a single gene will hardly capture all major events in a species history. Single-gene genealogies may, however, be relatively informative on species history in situations where genetic drift is comparatively unimportant to nonequilibrium factors such as colonization or population splitting (Wakeley 2004). In phylogeography, palaeoecological data can be used as an independent source of information to evaluate those conditions, which may for example be met in a phase of rapid colonization after refugia have been left by plant species (Lascoux *et al*. 2004). Another caveat associated with chloroplast DNA is that in most plant species mutation rates are low (Wolfe *et al*. 1987), so that the most common chloroplast DNA markers used in angiosperms to date, PCR-RFLPs (polymerase chain reaction–restriction fragment length polymorphisms; Demesure *et al*. 1996; King & Ferris 1998; Petit *et al*. 2002a,b), may display very low polymorphism

(Provan *et al*. 2001). The discovery of polymorphic microsatellites in chloroplast DNA, which feature variable numbers of mononucleotide repeats, provides new opportunities to analyse population genetic structure and address phylogeographical issues in plant species (Provan *et al*. 2001). Chloroplast microsatellites have extensively been applied to gymnosperms (Echt *et al*. 1998; Vendramin *et al*. 1999, 2000; Gómez *et al*. 2003) and to angiosperms (Drummond *et al*. 2000; Palmé & Vendramin 2002; Rendell & Ennos 2002; Lian *et al*. 2003; Lira *et al*. 2003; Palmé *et al*. 2003a,b; Rendell & Ennos 2003).

In this paper, we used PCR-RFLP and chloroplast microsatellites to detect geographical patterns of diversity and population genetic structure throughout the distribution range of common ash. The geographical patterns are interpreted jointly with previously available palynological and nuclear microsatellite data to infer patterns of glacial isolation and postglacial recolonization. Possible impacts of evolutionary processes and the species' life history traits are discussed to explain the observed levels of variation and differentiation.

Materials and methods

Sampling

Leaf or bud samples were collected from an average of 6.37 (± 2.57 SD) *F. excelsior* trees ($n = 1280$) in each of 201 putatively natural populations (see Appendix). Sampled trees were widely spaced in order to avoid collecting closely related individuals. Leaves and buds were dried or kept fresh until they could be frozen in a −80 °C freezer.

DNA extraction

Total DNA was extracted with the DNeasy Plant mini kit (Qiagen) or the CTAB procedure of the NucleoSpin Plant kit (Clontech) from approximately 50 mg of dry leaves, 100 mg of fresh leaves, or from 60 mg fresh weight of buds ground by hand or in the automatic grinding mill MM200 (Retsch). Alternatively, high throughput DNA extraction was performed simultaneously on 192 samples of about 20 mg of dry leaves with the DNeasy 96 Plant Kit (Qiagen).

PCR-RFLP analysis

Chloroplast DNA was amplified using six universal primer pairs amplifying the following fragments: CD, CS, DT, HK, K_1K_2 (Demesure *et al*. 1995) and VL (Dumolin-Lapègue *et al*. 1997). Amplification reactions, digestion with restriction enzymes (*Taq*I and *Hin*fI), and gel electrophoresis followed procedures described in Demesure *et al*. (1996) and Fineschi *et al*. (2003). The 12 primer–enzyme combinations were analysed on a subset of 62 populations (bold type in Appendix) evenly spread across the species distribution range.

Chloroplast microsatellite analysis

Chloroplast microsatellites corresponding to poly(A) or poly(T) repeats were amplified with six universal primer pairs for angiosperms (ccmp2, ccmp3, ccpm4, ccmp6, ccmp7, and ccmp10 from Weising & Gardner 1999). The reaction mix (25 μL) contained four dNTPs, each 0.2 mM, 2.5 mM of $MgCl_2$, 0.2 μM of each primer, 1% of BSA (bovine serum albumin), approximately 20 ng of template DNA and 1 U of *Taq* polymerase (Amersham) in Amersham PCR buffer. The PCR thermal profile was as follows: 5 min at 96 °C, 25 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C with a final extension step of 7 min at 72 °C. Amplification products were multiplexed by size (ccmp2, ccmp3 and ccmp10 on the one hand, and ccmp4, ccmp6 and ccmp7 on the other) and loaded onto Reprogel Long Read acrylamide gels (Amersham). Electrophoresis was run for about 70 min at 1500 V on an ALF Express automatic sequencer (Amersham) in TBE buffer. Fragment sizes were determined by comparison with internal and external size standards with the software FRAGMENT MANAGER version 1.2 (Amersham). At each chloroplast microsatellite locus, all different size variants were cloned into a plasmid vector (TA cloning kit; Invitrogen) and sequenced in both directions using an ALF Express automatic sequencer (Amersham).

In a first phase, 62 populations were screened using both PCR-RFLP and chloroplast microsatellite markers. Considering that PCR-RFLP produced only two haplotypes and additional haplotypes were detected with chloroplast microsatellites, the remaining set of 139 populations were analysed using only the six chloroplast microsatellites.

Genetic data analysis

The genetic analysis was carried out only on the chloroplast microsatellite data.

To determine phylogenetic relationships among chloroplast DNA haplotypes, a statistical parsimony network was constructed with the software TCS version 1.13 (Clement *et al*. 2000) using a distance matrix in which the distance between two haplotypes was the sum over the six chloroplast microsatellite loci of the absolute number of nucleotides distinguishing the haplotypes. Hence, in agreement with our sequencing results (see Results), each one-nucleotide difference between haplotypes was considered to result from a mutation, or slip-strand mispairing event, involving a single nucleotide (Levinson & Gutman 1987).

The level of polymorphism within populations was estimated using the number of haplotypes K and the haplotypic diversity based on unordered (h_S) or ordered

haplotypes (v_S) following Pons & Petit (1996), defining the distance between two haplotypes as above. In order to allow for straightforward comparison of haplotypic diversity statistics, the weights for v-type statistics were divided by a correction factor computed from the overall distance matrix between haplotypes according to Petit *et al*. (2002a). In the overall sample, total haplotypic diversity statistics based on unordered or ordered alleles (h_T and v_T, respectively) were calculated following Pons & Petit (1996) and Petit *et al*. (2002a) and differentiation among populations was computed from unordered and from ordered alleles (G_{ST} and R_{ST}, respectively). Geographic trends were investigated by computing diversity (h_S, v_S, h_T and v_T) and differentiation (G_{ST} and R_{ST}) statistics for three groups of populations: southern, central and northern Europe. The southern European group was chosen in a way to include potential refugium areas based on fossil pollen data; it comprised populations from within and south of the Pyrenees or Alps, and populations south of the rivers Save and Danube in the east. The northern and central European groups were delimited at 52° latitude (approx. latitude of the cities Den Haag and Warsaw), in order to cover sampling areas of similar size.

The geographical structure of genetic variation at chloroplast DNA in common ash was additionally investigated with four approaches. First, a haplotype frequency map was constructed using MAPINFO Professional Version 4.1 (MapInfo Corporation, New York, NY, USA). Second, we tested for the presence of phylogeographical structure by comparing R_{ST} estimates with values of R_{ST} computed after 10 000 random permutations of allele (haplotype) types among alleles (O. J. Hardy, unpublished) using the program SPAGEDI version 1.1 (Hardy & Vekemans 2002). If $R_{ST} > R_{ST}$ (permuted), there is phylogeographical structure, i.e. on average, phylogenetically similar haplotypes are found together in the same population more often than randomly chosen haplotypes. Third, we tested for a pattern of isolation by distance according to Rousset (1997): a Mantel test with 10 000 random permutations was performed between the matrix of pairwise genetic differentiation between populations, using $G_{ST}/(1 - G_{ST})$, and the matrix of the natural logarithm of geographical distance with the software SPAGEDI version 1.1 (Hardy & Vekemans 2002). Fourth, a simulated annealing procedure implemented in the SAMOVA algorithm (Dupanloup *et al*. 2002) was used to define groups of populations that are geographically homogeneous and maximally differentiated from each other. The program iteratively seeks the composition of a user-defined number K of groups of geographically adjacent populations that maximizes F_{CT}, the proportion of total genetic variance due to differences among groups of populations. In addition, SAMOVA identifies genetic barriers between these groups of populations. The program was run for 10 000 iterations for $K \in \{2, \ldots, 13\}$ 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.

Results

Variation identified with PCR-RFLP

A total number of 68 fragments were generated by PCR-RFLP. Only one combination (DT region digested with *Taq*I) displayed polymorphism: two haplotypes differing by one point mutation were detected in the subsample of 62 populations. The two PCR-RFLP haplotypes differentiated populations from Iberia and the British Isles (numbers 1, 2, 3, 14, 15, 16, 35 and 75 in the Appendix, carrying essentially chloroplast microsatellite haplotype H04; see Table 1) from all other populations.

Variation at chloroplast microsatellites and phylogenetic relationships among haplotypes

Two (ccmp2 and ccmp4) of the six chloroplast microsatellite loci analysed were monomorphic, displaying fragment lengths of 194 bp and 140 bp, respectively. The other loci showed low levels of polymorphism (Table 1): two distinct size variants separated by one nucleotide were observed at both ccmp3 and ccmp7, whereas ccmp6 and ccmp10 displayed three size variants each, with amplification fragment sizes of 97, 98 and 99 bp, and 103, 104 and 106 bp, respectively. Sequencing confirmed that the polymorphism was caused by variable numbers of microsatellite repeats (poly(A) or poly(T)) at all regions.

Table 1 Characteristics of the haplotypes detected with four polymorphic chloroplast microsatellites in the common ash

Haplotypes	Number of individuals	Size of amplified fragment (bp) at chloroplast microsatellites			
		ccmp3	ccmp6	ccmp7	ccmp10
H01	464	97	97	118	103
H02	403	97	99	117	104
H03	197	97	99	117	103
H04	149	97	98	118	104
H05	47	97	98	117	103
H06	5	97	97	117	103
H07	5	97	97	118	104
H08	6	97	98	117	106
H09	1	97	99	118	103
H10	1	97	98	117	104
H11	1	97	97	118	106
H12	1	96	97	118	103

Codes as in Weising & Gardner (1999).

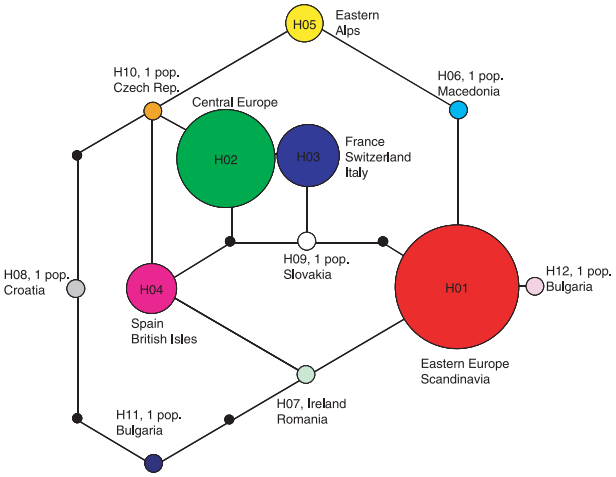

Fig. 1 Statistical parsimony network of chloroplast microsatellite haplotypes detected in the common ash.

Interestingly, the two monomorphic chloroplast microsatellites (ccmp2 and ccmp4) have a mononucleotide stretch shorter than 8 bp, while the two showing higher variation (ccmp6 and ccmp10) have a mononucloetide stretch longer than 10 bp, thus suggesting a relationship between length of the microsatellite stretch and level of variation. The size variants combined into a total of 12 haplotypes (Table 1), four of which were encountered in 94% of the individuals.

The haplotype network in Fig. 1 indicates the minimum numbers of evolutionary events separating the haplotypes. Most haplotypes are related to 1–3 others by one single microsatellite length mutation. Five putative haplotypes, corresponding to intermediate evolutionary steps, were not detected in our dataset (black circles in Fig. 1).

Chloroplast microsatellite haplotype H07 documents an event of homoplasy because it was found in individuals carrying the two different PCR-RFLP haplotypes: H07 individuals in populations 35 and 75 from Ireland bore the PCR-RFLP haplotype typical for Iberia and the British Isles, whereas H07 individuals in population 125 from Romania held the common PCR-RFLP haplotype found throughout the rest of Europe. Allelic associations at loci ccmp6, ccmp7 and ccmp10 (Table 1) contain additional evidence for homoplasy. For example, size variants 117 and 118 at ccmp7 and 103 and 104 at ccmp10 occurred in all four possible associations, which require the origin of at least one size variant to be homoplastic in a nonrecombinant molecule.

Population genetic analysis and geographical distribution of variation

Within-population variation at cpSSRs was low, with an average number (± SD) of $K = 1.19 \pm 0.39$ haplotypes per population and average haplotypic diversity values of $h_S = 0.081 \pm 0.188$ and $v_S = 0.064 \pm 0.184$ based on unordered or ordered alleles, respectively (Table 2). Both measures of within-population haplotypic diversity tended to decrease from southern to northern Europe (Table 2), but the trend was not significant (*t*-tests). Total haplotypic diversity in the overall sample was much higher than within populations, $h_T = v_T = 0.717$. When computed for the three regions, h_T based on unordered alleles was highest in southern and lowest in central Europe (Table 2). Conversely, total diversity for ordered alleles, v_T, displayed less variation among regions and was largest in central Europe. Differentiation among populations was high ($G_{ST} = 0.888$ and $R_{ST} = 0.911$) and tended to increase from south to north (Table 2).

A geographical organization of genetic variation is evident from the haplotype frequency map (Fig. 2): haplotype H01 occurs in south-eastern, eastern and northern Europe; H02 is widespread over central Europe, ranging from the Dinaric Alps to Denmark and eastern France, although it is also found in Italy; H03 occurs in Italy, Switzerland and France; H04 is only found in Spain and the British Isles; H05 occurs in several populations from the eastern Alps, whereas all other haplotypes are found in a total of only nine populations, seven of which are located in eastern or south-eastern Europe.

A phylogeographical signal was also detected with permutation procedures in the total sample ($R_{ST} = 0.911 > R_{ST}$ (permuted) = 0.887, $P = 0.043$; Table 2). The phylogeographical pattern was stronger in central Europe (R_{ST} =

Table 2 Chloroplast marker diversity (*K*, *h*, v) and differentiation (G_{ST}, R_{ST}) statistics from 201 common ash populations in Europe

	n	K_S	h_S	v_S	K_T	h_T	v_T	G_{ST}	R_{ST}	R_{ST} (perm)	H1: $R_{ST} > R_{ST}$ (perm.)
North	39	1.15 (0.37)	0.045 (0.111)	0.048 (0.136)	4	0.612	0.629	0.927	0.925	0.926	$P = 0.649$
Centre	100	1.16 (0.37)	0.070 (0.181)	0.055 (0.158)	8	0.572	0.664	0.879	0.918	0.876	$P = 0.006$
South	62	1.26 (0.44)	0.120 (0.228)	0.089 (0.243)	8	0.759	0.640	0.845	0.863	0.844	$P = 0.272$
Total	201	1.19 (0.39)	0.081 (0.188)	0.064 (0.184)	12	0.717	0.717	0.888	0.911	0.888	$P = 0.043$

n, number of populations. Subscripts: $_S$, within populations; $_T$, total population. Standard deviations over populations are given in parentheses. H1: $R_{ST} > R_{ST}$ (perm.): permutation test (10 000 permutations) for the presence of phylogeographical structure according to O. J. Hardy (unpublished).

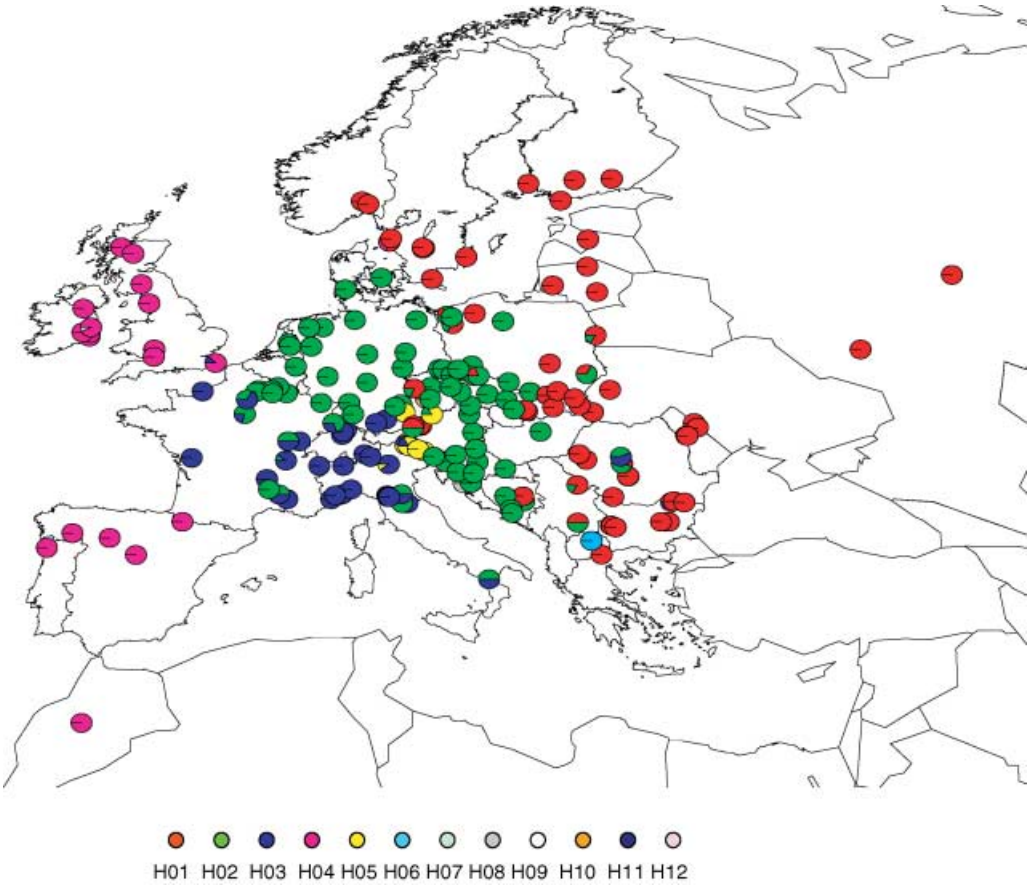

Fig. 2 Geographical distribution and frequency of chloroplast microsatellite haplotypes in the common ash (for the codes of haplotypes see Table 1).

$0.918 > R_{ST}$ (permuted) = 0.876, $P = 0.006$), but absent from the south and the north of Europe (Table 2).

The test of isolation by distance showed that among-population differentiation increased significantly with the logarithm of geographical distance (Mantel permutation test: $P < 0.001$), although the linear regression explained only 10% of the total variance ($R^2 = 0.102$).

The SAMOVA algorithm did not allow us to unambiguously identify the number K of groups of populations displaying the highest differentiation among groups, F_{CT}. This was because F_{CT} values increased progressively as K was increased, reaching a plateau at $K \approx 6$ (Fig. 3). We retained the configuration of $K = 6$, since for $K \geq 7$, at least one of the groups contained a single population, meaning that the group structure was disappearing. The composition of groups for $K = 6$ (Fig. 4) corresponded strongly to the geographical organization of haplotypes visually identified on the haplotype frequency map (Fig. 2). Four of these groups were composed of populations containing predominantly one haplotype, i.e. featuring on average 94% or more of haplotypes H01 to H04, respectively. A fifth group comprised Alpine populations containing mainly H05. The last group comprised only two populations from Romania, one of which contained H03 that otherwise occurs in western Europe. The populations that contained rare haplotypes clustered with those harbouring the major haplotype

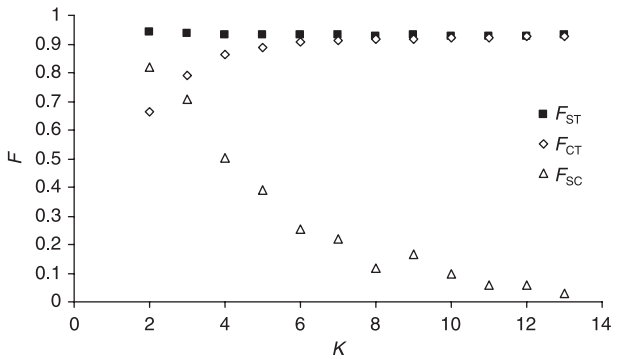

Fig. 3 Fixation indices F obtained with the SAMOVA program (Dupanloup *et al*. 2002) as a function of the user-defined number K of groups of populations. F_{ST}, differentiation among populations; F_{CT}, differentiation among groups of populations; F_{SC}, differentiation among populations within groups.

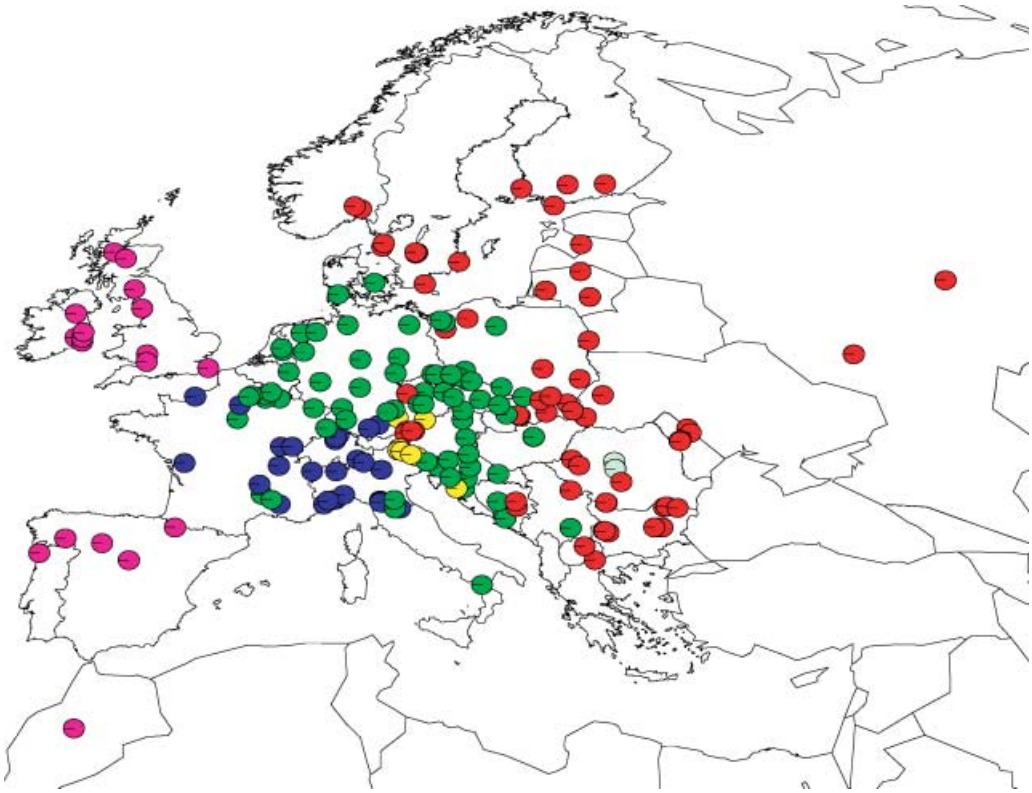

Fig. 4 Group structure defined by SAMOVA (Dupanloup *et al*. 2002) (colours indicate different groups of populations).

surrounding them: (1) population 73 from the Czech Republic containing H10 clustered in the central European group with mainly populations with H02; (ii) population 145 from Macedonia containing H06, population 119 from Slovakia containing H09, and populations 71 and 84 from Bulgaria containing H11 and H12, respectively, clustered in the northern and eastern European group with populations of mainly H01; and (iii) population 78 from Croatia containing H08 clustered in the Alpine group.

Discussion

Location of refugia and postglacial recolonization

Our study revealed a clear-cut geographical organization of chloroplast DNA diversity in common ash, with four major haplotypes that were geographically widespread and showed little overlap in their distribution areas. This picture is globally consistent with fossil pollen data (Huntley & Birks 1983; Gliemeroth 1997; Brewer 2002) and suggests that postglacial expansion occurred from several distinct glacial refugia probably located in all three southern European peninsulas and the Alps, as detailed below.

In Western Europe, a distinct chloroplast lineage was detected in Iberia and the British Isles by both PCR-RFLP and chloroplast microsatellites (haplotype H04), supporting the existence of an Iberian refugium, as suggested by Gliemeroth (1997) and Brewer (2002). H04 was not detected in France, hence, the Pyrenees and/or the possible earlier occurrence of H03 in France may have prevented north-eastward colonization. Therefore, northward colonization by H04 may have occurred along the shores of the Atlantic, by circumventing the Pyrenees in the west through the Bay of Biscay, in analogy with white oaks (Petit *et al*. 2002b). The occurrence of H04 in a common ash population from Morocco is possibly an artefact, since the natural distribution of the species is not known to reach such southern latitudes (Tutin *et al*. 1972). The Apennines seem to have held a refugium for H03, given the wide occurrence of this haplotype throughout Italy and the absence of clear evidence for a southward expansion from the Alps by fossil pollen data (Huntley & Birks 1983; Gliemeroth 1997; Brewer 2002). H03 was also found in France, but not north or north-east of the Alps, which suggests that H03 may have crossed and/or circumvented the mountain chain in its western part. H02 is widespread over central Europe and the sharp boundary with H03 along the northern flanks of the Alps suggests an earlier arrival of H02. This would agree with glacial isolation of H02 in an eastern Alpine refugium and westward expansion around 9500 years ago, as indicated from fossil pollen data (Huntley & Birks 1983; Brewer 2002). Alternatively, considering that

H02 occurs widely across former Yugoslavia, it is also reasonable that a refugium harbouring H02 has existed in the western part of south-eastern Europe (i.e. the Dinaric Alps). Fossil pollen records are scarce for this region, but it is known to have contained glacial refugia for tree species, for example for Norway spruce (Huntley & Birks 1983; Lagercrantz & Ryman 1990). Yet another scenario for the geographical patterns of H02 and H03 is suggested by their co-occurrence in population 79 from southern Italy: they may have shared one refugium and employed different recolonization routes, i.e. a western route for H03 and an eastern one for H02. It is even possible that either of them arose from the other, regarding their phylogenetic proximity. The distribution of H02 on both the western and eastern shores of the Adriatic Sea illustrates the connection between refugia in the Italian and the Balkan Peninsulas, which has been observed in oaks (Petit *et al*. 2002b), ivy (Grivet & Petit 2002), and the English holly (Rendell & Ennos 2003). More support for a glacial refugium for ash in the eastern Alps comes from the occurrence of a hotspot of genetic diversity (haplotypes H01, H02, H03 and H05 are found in this region) and the relatively local distribution of H05, in agreement with the view that refugium populations may accumulate higher diversity and/or unique haplotypes owing to their persistence and relative stability over glacial cycles (Hewitt 1996; Newton *et al*. 1999; Tzedakis *et al*. 2002). Chloroplast data also confirm the existence of a (south)-eastern European refugium (Huntley & Birks 1983; Gliemeroth 1997; Brewer 2002) through the wide distribution of H01 in south-eastern, eastern central and northern Europe. The finding of several rare haplotypes in different populations from south-eastern and eastern central Europe may suggest several eastern refuges, however, chloroplast DNA does not clearly identify separate colonizations from south-eastern and north-eastern Europe, as suggested from both fossil pollen (Huntley & Birks 1983; Gliemeroth 1997) and nuclear microsatellites (Heuertz *et al*. 2004).

Although it was shown from palaeoecological (Bennett 1997) as well as from molecular data (Taberlet *et al*. 1998; Petit *et al*. 2003) that every species has an individualistic behaviour in response to environmental changes, some postglacial expansion patterns may be shared to a certain extent (Taberlet *et al*. 1998; Hewitt 2000). The contribution of western, central and eastern European lineages to postglacial recolonization observed in the common ash was also documented in silver fir (*Abies alba*; Konnert & Bergmann 1995; Vendramin *et al*. 1999) and in white oaks (*Quercus* sp.; Dumolin-Lapègue *et al*. 1997; Petit *et al*. 2002b).

Genetic diversity and differentiation

Both PCR-RFLP and chloroplast microsatellites displayed a relatively low level of variation with, respectively, only two and 12 haplotypes detected in 62 and 201 populations, respectively. We observed that H08 and H11 carry size variant 106 at ccmp10, which is typical for *Fraxinus ornus* (G. G. Vendramin, unpublished); hence, these haplotypes may represent either sampling errors or cases of introgression, since they occur in south-eastern Europe where the distributions of *F. excelsior* and *F. ornus* overlap.

In comparison, a survey of similar scale in white oaks detected 23 PCR-RFLP haplotypes in 1412 individuals from 345 populations (Dumolin-Lapègue *et al*. 1997), and the average number of chloroplast DNA haplotypes in about 21 populations of European broadleaved species was 17, whereas the corresponding figure for ash was only seven (Petit *et al*. 2003).

The difference in polymorphism detected between the two marker systems in common ash is probably due to their different mutation rates; these are higher for single nucleotide slippage at chloroplast microsatellite than for point mutations (or indels) causing PCR-RFLP variation (Provan *et al*. 2001). The mutation mechanism at chloroplast microsatellites makes them predisposed to homoplasy, i.e. variants at chloroplast microsatellite loci can be identical in state without being identical by descent (Goldstein & Pollock 1997; Provan *et al*. 2001), which may represent a major drawback in phylogenetic applications. In our data set, we observed a clear example of homoplasy with chloroplast microsatellite haplotype H07, which probably evolved from two different PCR-RFLP backgrounds in Ireland and Romania. Evidence for homoplasy was also reflected in allelic associations. Nonetheless, a clear geographical pattern of chloroplast DNA variation was observed, which indicates that homoplasy does not override the biological signal. Considering the low overall level of variation and, in particular, the weak divergence between haplotypes from different refugia (they differ by 3 bp at most), this seems to point to a low mutation rate at our chloroplast microsatellites. A low chloroplast DNA mutation rate might be a feature of the Oleaceae; for example Besnard *et al*. (2002) found variation in only five out 40 primer-enzyme combinations (six point mutations and five indels) and in two out of eight chloroplast microsatellites (ccmp5 and ccmp7 from Weising & Gardner 1999; displaying six and three length variants, respectively) among 143 cultivated and 334 wild olive trees (*Olea europaea*) from 37 locations around the Mediterranean Basin.

In addition to a low mutation rate, a low level of variation in common ash populations may reflect a lack of variation in glacial refugium populations, especially since few rare haplotypes were detected in extant populations from these areas. A possible explanation is strong genetic drift due to a low effective population size, N_e, in refugium populations. For species with a scattered distribution such as common ash, N_e might indeed be much lower than for more social species, such as oaks. The polygamous mating

system in common ash further reduces N_e for chloroplast DNA compared with hermaphrodite species. The latter factors might also have led to fixation of haplotypes over large geographical areas before the last ice age, making it difficult to identify the precise location of refugia.

Diversity and differentiation in recolonized populations are affected by the variation in recolonizing lineages, as well as features of recolonization, such as founder events (Hewitt 1996), selection or lineage admixture. Palaeoecological studies reported that recolonization in ash started late at the beginning of the Holocene, when other species had already left the refugia (Huntley & Birks 1983; Brewer 2002). Colonization into already occupied areas may have had a stronger selection effect than into open lands and may have produced strong founder effects leading to loss of variation. For example, in ash, the Alpine haplotype H05 remained trapped in a relatively small area, and none of the rare haplotypes (except H07) was observed in more than one population. Strong among-population differentiation ($G_{ST} = 0.89$) reflects little mixing of recolonizing common ash lineages in Europe, apparently indicating that historical effective seed dispersal occurred mainly over short distances. Genetic studies at a local scale also suggested that the heavy winged ash seed mainly disperse at short distances (Heuertz *et al*. 2003, M. E. Morand-Prieur 2003, personal communication). In contrast, palaeoecological studies reported colonization speeds reaching up to 500 m/year in ash (Huntley & Birks 1983; Brewer 2002), which would require at least sporadic long-distance seed dispersal and establishment. Ash is well-known for its strong short-scale colonization behaviour and our study demonstrated that the Pyrenees and Alps did not constitute major obstacles to recolonization in ash, unlike in hornbeam (*Carpinus*; Grivet & Petit 2003) and beech (*Fagus*; Demesure *et al*. 1996). These features are best explained by efficient medium- to long-distance dispersal mediated by the wind, or even birds (Falinski & Pawlaczyk 1995; Wilkinson 1997).

Petit *et al*. (2003) found that among-population differentiation was strongly influenced by the mode of seed dispersal, although long-term range fragmentation also had an effect. Differentiation in ash was higher than in an average of seven species with winged wind-dispersed seeds (average $G_{ST} = 0.66 \pm 0.20$ SD; Petit *et al*. 2003) and was close to the estimate for species with heavy seeds dispersed by gravity and cached by animals (average $G_{ST} = 0.82 \pm 0.08$ SD; Petit *et al*. 2003) such as Quercus (Dumolin-Lapègue *et al*. 1997), *Fagus* (Demesure *et al*. 1996) and *Corylus* (Palmé & Vendramin 2002). The relatively high G_{ST} for chloroplast DNA in ash despite good colonization abilities may reflect (1) that colonization not always results in establishment or effective dispersal (e.g. colonizers die before reproduction) and/or (2) initially low variation (i.e. effective dispersal may not produce differentiation).

The geographical trends observed for unordered alleles in common ash were a decrease in diversity and an increase in differentiation from south to central Europe (i.e. accompanying assumed postglacial recolonization). This pattern can be explained by the occurrence of founder events and poor mixing among lineages, as outlined above. In comparison, the multispecies pattern in 25 populations of temperate broadleaf species revealed maximum differentiation among refugium populations, and intrapopulation diversity was highest at intermediate latitudes (Petit *et al*. 2003). In that study, the diversity pattern in common ash was correlated with the multispecies diversity pattern (Petit *et al*. 2003). The apparent opposition between the two studies may be explained by a sampling effect. The intermediate latitude common ash populations included in Petit *et al*. 2002) paper corresponded to 11 populations from the central European group in this study; five of them were polymorphic, four of which were located in France (45% polymorphic populations). In this study, all additional populations for the central European group were sampled at more eastern latitudes, and many of them were monomorphic, which resulted in 16 polymorphic populations out of 100.

Phylogeographical structure

A comparison of the diversity statistics based on unordered or ordered alleles in common ash revealed similar geographical patterns than in oaks (Dumolin-Lapègue *et al*. 1997; Petit *et al*. 2002a), in which the three southern peninsulas contributed to postglacial recolonization like in ash. In refugia (i.e. the southern European group), allelic richness was relatively high and $h_T > v_T$. This means that most variation was confined within lineages (the pattern was observed even though Iberian populations were pooled with those from Italy and the Balkan Peninsula because of low sample size). In central Europe, there were many variants from different lineages ($h_T \leq v_T$), whereas in Northern Europe, diversity was lower, but the contribution of different refugia was relatively balanced ($h_T \leq v_T$).

A phylogeographical pattern was revealed through permutation analysis in the overall data set and in the central European group. It is essentially explained by the frequent co-occurrence of the phylogenetically close haplotypes H02 and H03 in polymorphic populations (6 out of 16) from the central European group.

In order to investigate in more details the genetic structure of ash, we applied the SAMOVA algorithm to define groups and identify the most important genetic barriers. This approach confirmed what had already been inferred from the haplotype distribution about the history of this species. In addition, SAMOVA identified in Romania a group of two populations, of which one or both may have an artificial origin as a result of seed transfer that would have

taken place during historical time (occurrence of H03 that has a more westward distribution otherwise). The ability of SAMOVA to identify recent introduction events, particularly important for conservation purposes, has been demonstrated by Dupanloup *et al*. (2002). However, the clear congruence between geographical distribution of haplotypes and fossil pollen data seems to exclude a strong human impact on this species, as for example observed for deciduous oaks in central Europe (König *et al*. 2002).

Comparison of markers and conservation issues

Our chloroplast DNA data in *F. excelsior* suggest recolonization of Europe from refugia located in Iberia, Italy, the Alps and the Balkan Peninsula. This pattern remained essentially undetected with nuclear microsatellites (Heuertz *et al*. 2004), which identified a genetically homogeneous deme extending from the British Isles over central Europe to Lithuania. Since chloroplast DNA provides evidence for poor mixing of recolonizing maternal lineages, the homogenization of genetic variation at nuclear markers in this area must be essentially due to efficient postglacial pollen flow. The discrepancy in differentiation patterns in chloroplast and nuclear markers also points to the difference in N_e, which is much larger for nuclear markers. In eastern Europe, nuclear microsatellites provided better resolution of postglacial history in common ash than chloroplast markers. Nuclear microsatellites suggested a north-eastern European refugium, in agreement with fossil pollen data (Huntley & Birks 1983; Brewer 2002), which was not detected with chloroplast markers. Further, in the Balkans, chloroplast DNA identified limited areas where rare haplotypes survived without spreading northward or westward. In this region, nuclear microsatellite markers provided additional insights, demonstrating the occurrence of highly differentiated groups of populations that may have been coexisting for a long time without substantial genetic exchanges (Heuertz *et al*. 2004). The comparison of genetic patterns between markers in the common ash suggests that the identification of genetic resources for conservation should be based on data from differentially inherited genetic markers, possibly in combination with markers under selection.

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Appendix

Sampling locations and frequencies of the haplotypes encountered in the sampled populations

Population	Country	Longitude	Latitude	*n*	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
1. Glen Afric	**UK**	**−4.83**	**57.32**	**10**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
2. Lake District	**UK**	**−3**	**54.27**	**10**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
3. Dean	**UK**	**−2.65**	**51.83**	**10**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
4. Göteborg	**Sweden**	**11.93**	**57.6**	**10**	**0.8**	**0**	**0**	**0.2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
5. Stenshuvud	**Sweden**	**14.33**	**55.58**	**10**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
6. Haltorps Hage	**Sweden**	**16.62**	**56.78**	**10**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
7. Schönberg	**Germany**	**7.83**	**47.96**	**10**	**0**	**0.7**	**0.3**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
8. Bovenden	**Germany**	**10.05**	**51.57**	**10**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
9. Kelheim	**Germany**	**11.83**	**48.93**	**10**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
10. Fontainebleau	**France**	**2.67**	**48.42**	**9**	**0**	**0.78**	**0.22**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
11. Chizé	**France**	**−0.4**	**46.14**	**10**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
12. Seillon	**France**	**5**	**46**	**10**	**0**	**0.1**	**0.9**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
13. Valbonne	**France**	**4.55**	**44.24**	**6**	**0**	**0.5**	**0.5**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
14. Devesa da Rogueira	**Spain**	**−7.08**	**42.25**	**9**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
15. Valle de Salazar	**Spain**	**−0.92**	**42.83**	**10**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
16. Montejo de la Sierra	**Spain**	**−3.5**	**41.13**	**2**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
17. Casentinesi	**Italy**	**11.8**	**43.78**	**10**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
18. Garda Bresciano	**Italy**	**10.88**	**45.8**	**9**	**0**	**0**	**0.89**	**0**	**0.11**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
19. Akeras	**Sweden**	**14.06**	**57.29**	**6**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
20. Anterselva	Italy	12.08	46.83	6	0	0	0.67	0	0.33	0	0	0	0	0	0	0
21. Valle Aurina	Italy	12	46.92	6	0	0	0	0	1	0	0	0	0	0	0	0
22. Avoca	Ireland	−6.55	52.5	6	0	0	0	1	0	0	0	0	0	0	0	0
23. Bremgarten	**Switzerland**	**8.3**	**47.33**	**6**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
24. Karnerviertel	**Austria**	**15.88**	**47.46**	**6**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
25. Graffenwoerth	Austria	15.76	48.4	6	0	1	0	0	0	0	0	0	0	0	0	0
26. Weitwoerth	Austria	12.98	47.88	6	0	0.17	0	0	0.83	0	0	0	0	0	0	0
27. Balova	**Romania**	**24.51**	**45.16**	**12**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
28. Bukatchov chukar	**Bulgaria**	**26.3**	**42.83**	**8**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
29. Berchtesgaden	Germany	12.9	47.81	6	1	0	0	0	0	0	0	0	0	0	0	0
30. Borgo Sesia	Italy	8.28	45.72	6	0	0	1	0	0	0	0	0	0	0	0	0
31. Biokovo	Croatia	17.1	43.28	2	0	1	0	0	0	0	0	0	0	0	0	0
32. Brunico	**Italy**	**11.92**	**46.8**	**6**	**0**	**0**	**0.33**	**0**	**0.67**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
33. Bratovoiesti1	Romania	23.51	44.1	6	1	0	0	0	0	0	0	0	0	0	0	0
34. Bratovoiesti2	Romania	23.5	44.1	7	1	0	0	0	0	0	0	0	0	0	0	0
35. Camolin	Ireland	−6.55	52.7	8	0	0	0	0.88	0	0	0.13	0	0	0	0	0
36. Chiemsee	Germany	12.48	47.9	8	0.13	0	0	0	0.88	0	0	0	0	0	0	0
37. Křivoklátsko	Czech Republic	13.66	49.83	6	0	1	0	0	0	0	0	0	0	0	0	0
38. Climauti	**Moldavia**	**28.78**	**47.95**	**6**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
39. Bohemian Switzerland National Park	Czech Republic	14.25	50.78	6	0	1	0	0	0	0	0	0	0	0	0	0
40. Passo della Consuma	**Italy**	**11.5**	**43.8**	**6**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
41. Krasnochetajskij Leskhoz	Russia	46.4	55.8	6	1	0	0	0	0	0	0	0	0	0	0	0
42. Freilassing1	Germany	12.49	47.83	6	1	0	0	0	0	0	0	0	0	0	0	0
43. Delnice	Croatia	14.78	45.4	6	0	1	0	0	0	0	0	0	0	0	0	0
44. Dulovo	**Bulgaria**	**26.91**	**43.9**	**10**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
45. Dargov	Slovakia	21.55	48.73	6	0.83	0.17	0	0	0	0	0	0	0	0	0	0
46. Eglisau	Switzerland	8.51	47.58	6	0	0	1	0	0	0	0	0	0	0	0	0
47. Pernes les Fontaines	France	5	44	10	0	0.3	0.7	0	0	0	0	0	0	0	0	0
48. Ehd	Sweden	13.98	57.24	6	1	0	0	0	0	0	0	0	0	0	0	0
49. Elena	Bulgaria	26.5	42.83	8	1	0	0	0	0	0	0	0	0	0	0	0
50. Laneuville sur Meuse	France	5.17	49.5	2	0	1	0	0	0	0	0	0	0	0	0	0
51. Oermingen	**France**	**7.15**	**49**	**2**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
52. La Romagne1	France	4.67	49.83	2	0	1	0	0	0	0	0	0	0	0	0	0
53. Pérois les Gombries	France	2.83	49.17	3	0	0.33	0.67	0	0	0	0	0	0	0	0	0
54. Port Lesney	France	5.83	47	2	0	0	1	0	0	0	0	0	0	0	0	0

Appendix *Continued*

Population	Country	Longitude	Latitude	*n*	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
55. Freilassing2	Germany	12.48	47.83	8	1	0	0	0	0	0	0	0	0	0	0	0
56. St Gobin1	France	3.53	49.67	2	0	1	0	0	0	0	0	0	0	0	0	0
57. Acy Romance	France	4.33	49.5	3	0	1	0	0	0	0	0	0	0	0	0	0
58. Aurelle Verlac	France	3	44.5	2	0	1	0	0	0	0	0	0	0	0	0	0
59. Seurre	France	5.17	47	2	0	0.5	0.5	0	0	0	0	0	0	0	0	0
60. Ehrendingen	Switzerland	8.35	47.48	4	0	0	1	0	0	0	0	0	0	0	0	0
61. Canterbury	UK	1	51.1	8	0	0	0.13	0.88	0	0	0	0	0	0	0	0
62. Ghedus	Romania	21.86	46.33	8	1	0	0	0	0	0	0	0	0	0	0	0
63. Gornji Grad	**Slovenia**	**14.78**	**46.3**	**6**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
64. Golyamoto	Bulgaria	26.03	42.83	8	1	0	0	0	0	0	0	0	0	0	0	0
65. Garesio	Italy	8	44.22	8	0	0	1	0	0	0	0	0	0	0	0	0
66. Hrjauca	Moldavia	28.28	47.33	6	1	0	0	0	0	0	0	0	0	0	0	0
67. Hrjauca	Moldavia	28.2	47.3	6	1	0	0	0	0	0	0	0	0	0	0	0
68. Nyehi Hegy	**Hungary**	**19.81**	**47.5**	**6**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
69. Hrjauca	Moldavia	28.25	47.28	6	1	0	0	0	0	0	0	0	0	0	0	0
70. HT-Slovenia	Slovenia	14.13	45.93	6	0	1	0	0	0	0	0	0	0	0	0	0
71. Iri Chissar	Bulgaria	26.76	43.85	8	0.88	0	0	0	0	0	0	0	0	0	0.13	0
72. Canningstown	Ireland	−7	54	10	0	0	0	1	0	0	0	0	0	0	0	0
73. Javorina	Czech Republic	13.3	49.22	5	0	0.8	0	0	0	0	0	0	0	0.2	0	0
74. Kaisyadoris	Lithuania	24.33	54.88	7	1	0	0	0	0	0	0	0	0	0	0	0
75. Kilmacurra	**Ireland**	**−6.5**	**53**	**12**	**0**	**0**	**0**	**0.92**	**0**	**0**	**0.08**	**0**	**0**	**0**	**0**	**0**
76. Kodga ormani	Bulgaria	26.9	43.9	8	1	0	0	0	0	0	0	0	0	0	0	0
77. Königsee	Germany	12.94	47.49	6	0	0	0	0	1	0	0	0	0	0	0	0
78. Krasno Polje	Croatia	15.08	44.83	6	0	0	0	0	0	0	0	1	0	0	0	0
79. LagoNegro	Italy	16.08	39.88	6	0	0.5	0.5	0	0	0	0	0	0	0	0	0
80. Lato Hegy	Hungary	19.8	47.5	5	0	1	0	0	0	0	0	0	0	0	0	0
81. Tiglieto	**Italy**	**8.67**	**44.5**	**5**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
82. Ferrere	**Italy**	**8.17**	**44.25**	**5**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
83. Andagna	Italy	7.5	44	5	0	0	1	0	0	0	0	0	0	0	0	0
84. Ljulin monastery	Bulgaria	23.18	42.65	8	0.88	0	0	0	0	0	0	0	0	0	0	0.13
85. Limbazi	Latvia	24.41	57.75	12	1	0	0	0	0	0	0	0	0	0	0	0
86. Lillafüred	Hungary	19.8	47.51	6	1	0	0	0	0	0	0	0	0	0	0	0
87. Landsberg	Germany	10.87	48.13	6	0	0	1	0	0	0	0	0	0	0	0	0
88. Mtizi n'Ait Ouira	Morocco	−6.3	32.56	2	0	0	0	1	0	0	0	0	0	0	0	0
89. Medvednica	**Croatia**	**15.98**	**45.92**	**6**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
90. Kokalyane monastery	Bulgaria	23.43	42.55	8	1	0	0	0	0	0	0	0	0	0	0	0
91. Muráň	Slovakia	20.7	48.77	5	1	0	0	0	0	0	0	0	0	0	0	0
92. Murta	Romania	23.5	44.11	6	1	0	0	0	0	0	0	0	0	0	0	0
93. Negova	Slovenia	15.95	46.61	6	0	1	0	0	0	0	0	0	0	0	0	0
94. Podyjí National Park	Czech Republic	15.85	48.88	6	0	1	0	0	0	0	0	0	0	0	0	0
95. Šumava National Park	Czech Republic	13.55	49.2	6	0	1	0	0	0	0	0	0	0	0	0	0
96. Határ Nyereg	Hungary	19.81	47.51	6	0	1	0	0	0	0	0	0	0	0	0	0
97. Ottobeuren	**Germany**	**10.3**	**47.95**	**6**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
98. Vals Près le Puy	France	3.88	45.03	9	0	0	1	0	0	0	0	0	0	0	0	0
99. Červený Kláštor	**Slovakia**	**20.43**	**49.4**	**6**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
100. Pian di Novello	Italy	10.8	44.1	6	0	0	1	0	0	0	0	0	0	0	0	0
101. Villabaruz de Campos	Spain	−5	42.01	9	0	0	0	1	0	0	0	0	0	0	0	0
102. Pian degli Ontani	**Italy**	**10.7**	**44.2**	**4**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
103. Braga	Portugal	−8.5	41.5	5	0	0	0	1	0	0	0	0	0	0	0	0
104. Palota	Slovakia	22	49.25	6	1	0	0	0	0	0	0	0	0	0	0	0
105. Pulfero	**Italy**	**13.5**	**46.2**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
106. Passo Raticosa	Italy	11.5	44.25	4	0	0.5	0.5	0	0	0	0	0	0	0	0	0
107. Riscone	Italy	11.85	46.85	6	0	0	0.17	0	0.83	0	0	0	0	0	0	0
108. Saraj	Macedonia	21.33	42.8	2	0.5	0.5	0	0	0	0	0	0	0	0	0	0
109. Romanesti1	Romania	24.5	45.16	6	1	0	0	0	0	0	0	0	0	0	0	0
110. Hillerstorp	Sweden	13.98	57.32	6	1	0	0	0	0	0	0	0	0	0	0	0
111. Pucheni	**Romania**	**22.23**	**46.02**	**10**	**0.9**	**0.1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Appendix *Continued*

Population	Country	Longitude	Latitude	*n*	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
112. Romanesti2	Romania	24.5	45.17	6	1	0	0	0	0	0	0	0	0	0	0	0
113. Voronec	**Russia**	**39.5**	**51.83**	**9**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
114. Saharna	Moldavia	28.93	47.71	6	1	0	0	0	0	0	0	0	0	0	0	0
115. Sarajevo	Bosnia-Herzegovina	18.42	43.87	6	0.5	0.5	0	0	0	0	0	0	0	0	0	0
116. Moffat	**UK**	**−3.5**	**55.3**	**6**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
117. Sestaione	**Italy**	**10.75**	**44.15**	**6**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
118. Silute	Lithuania	21.7	55.23	6	1	0	0	0	0	0	0	0	0	0	0	0
119. Zvolen	Slovakia	19.12	48.57	10	0.9	0	0	0	0	0	0	0	0.1	0	0	0
120. Svaljava	Ukraine	22.97	48.52	6	1	0	0	0	0	0	0	0	0	0	0	0
121. Teisendorf	Germany	12.77	47.81	6	1	0	0	0	0	0	0	0	0	0	0	0
122. La Thuile	Italy	6.9	45.72	9	0	0	1	0	0	0	0	0	0	0	0	0
123. Tiroler Ache	Germany	12.4	47.7	8	0.5	0.5	0	0	0	0	0	0	0	0	0	0
124. Saint Martin du Bec	France	0.22	49.6	3	0	0	1	0	0	0	0	0	0	0	0	0
125. Tutuleac	**Romania**	**24.26**	**45.83**	**14**	**0.79**	**0**	**0**	**0**	**0**	**0**	**0.21**	**0**	**0**	**0**	**0**	**0**
126. Tovshiv	Ukraine	24.15	49.68	12	1	0	0	0	0	0	0	0	0	0	0	0
127. Val Bregaglia	**Switzerland**	**9.53**	**46.33**	**6**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
128. Val Casies	**Italy**	**12.1**	**46.75**	**6**	**0**	**0**	**0.17**	**0**	**0.83**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
129. Valea Dracului	**Romania**	**24.25**	**45.83**	**11**	**0.55**	**0.45**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
130. Velebit	Croatia	15.5	44.83	3	0	1	0	0	0	0	0	0	0	0	0	0
131. Villa Franca	**Romania**	**24.25**	**46.2**	**14**	**0**	**0.43**	**0.57**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
132. Vitosha	Bulgaria	23.23	42.63	8	1	0	0	0	0	0	0	0	0	0	0	0
133. Val Visdende	Italy	12.63	46.58	6	0	0	0	0	1	0	0	0	0	0	0	0
134. Vihorlat	Slovakia	22.25	48.87	6	1	0	0	0	0	0	0	0	0	0	0	0
135. Villa Nova Mondovì	Italy	7.75	44.2	6	0	0	1	0	0	0	0	0	0	0	0	0
136. Vocin	Croatia	17.53	45.57	6	0	1	0	0	0	0	0	0	0	0	0	0
137. Vrbovsko	Croatia	15.7	45.33	6	0	1	0	0	0	0	0	0	0	0	0	0
138. Zli dol	**Bulgaria**	**27.5**	**43.83**	**8**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
139. Zeimelis	Lithuania	24.03	56.27	12	1	0	0	0	0	0	0	0	0	0	0	0
140. Sielnica	Slovakia	19.05	48.65	5	1	0	0	0	0	0	0	0	0	0	0	0
141. Hanko	Finland	23.13	59.86	6	1	0	0	0	0	0	0	0	0	0	0	0
142. Hattula	**Finland**	**24.28**	**61.03**	**6**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
143. Konjic	Bosnia-Herzegovina	17.64	43.48	2	0	1	0	0	0	0	0	0	0	0	0	0
144. Kladanj	Bosnia-Herzegovina	18.38	44.15	4	1	0	0	0	0	0	0	0	0	0	0	0
145. Negorci1	Macedonia	22.49	41.15	5	0	0	0	0	0	1	0	0	0	0	0	0
146. Valkeala	Finland	26.7	61.083	6	1	0	0	0	0	0	0	0	0	0	0	0
147. Travnik	Bosnia-Herzegovina	17.37	44.15	6	0	1	0	0	0	0	0	0	0	0	0	0
148. Uusikaupunki	Finland	21.25	60.816	6	1	0	0	0	0	0	0	0	0	0	0	0
149. BabiaGora	Poland	19.46	49.57	6	0	1	0	0	0	0	0	0	0	0	0	0
150. Białowieża	Poland	23.82	52.57	6	0.83	0.17	0	0	0	0	0	0	0	0	0	0
151. Sondrio	**Italy**	**9.87**	**46.17**	**6**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
152. Gory Stołowe	Poland	16.28	50.42	6	0	1	0	0	0	0	0	0	0	0	0	0
153. Negorci2	Macedonia	22.48	41.15	5	1	0	0	0	0	0	0	0	0	0	0	0
154. Piano Sinatico	**Italy**	**10.72**	**44.11**	**6**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
155. Roztocze	Poland	22.92	50.52	6	0.33	0.67	0	0	0	0	0	0	0	0	0	0
156. Amerongen	**The Netherlands**	**5.45**	**52**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
157. Bunde	**The Netherlands**	**5.75**	**50.9**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
158. Choszczno	Poland	15.3	53.17	6	0.83	0.17	0	0	0	0	0	0	0	0	0	0
159. Jawor	Poland	16.2	50.97	6	0	1	0	0	0	0	0	0	0	0	0	0
160. Jamy-Białochiowo	Poland	15.3	53.53	5	0	1	0	0	0	0	0	0	0	0	0	0
161. Jamy-Chełmno	Poland	18.38	53.33	6	0	1	0	0	0	0	0	0	0	0	0	0
162. Nowogard	Poland	15.08	53.63	6	0.17	0.83	0	0	0	0	0	0	0	0	0	0
163. Rolde	The Netherlands	6.63	52.97	5	0	1	0	0	0	0	0	0	0	0	0	0
164. Szczecinek	**Poland**	**16.7**	**53.73**	**6**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Appendix *Continued*

Population	Country	Longitude	Latitude	*n*	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
165. Teckop	The Netherlands	5.38	52.15	5	0	1	0	0	0	0	0	0	0	0	0	0
166. Winterswijk	The Netherlands	6.72	51.97	5	0	1	0	0	0	0	0	0	0	0	0	0
167. Draved	Denmark	8.97	55.02	5	0	1	0	0	0	0	0	0	0	0	0	0
168. Asker	Norway	10.8	59.67	3	1	0	0	0	0	0	0	0	0	0	0	0
169. La Romagne2	France	4.3	49.67	6	0	1	0	0	0	0	0	0	0	0	0	0
170. St Gobain2	France	3.37	49.58	6	0	1	0	0	0	0	0	0	0	0	0	0
171. Loch Tay	UK	−4.08	56.97	23	0	0	0	1	0	0	0	0	0	0	0	0
172. Asker	Norway	10.42	59.83	3	1	0	0	0	0	0	0	0	0	0	0	0
173. Burlandingen	Germany	9	48.4	5	0	1	0	0	0	0	0	0	0	0	0	0
174. Drahany	Czech Republic	16.92	49.45	5	0	1	0	0	0	0	0	0	0	0	0	0
175. Esterwegen	Germany	7.5	53	5	0	1	0	0	0	0	0	0	0	0	0	0
176. Gera	Germany	12.08	50.83	5	0	1	0	0	0	0	0	0	0	0	0	0
177. Göteborg2	Sweden	12.05	57.8	5	1	0	0	0	0	0	0	0	0	0	0	0
178. Heralec	Czech Republic	15.37	49.55	5	0	1	0	0	0	0	0	0	0	0	0	0
179. Hradiště	Czech Republic	18.37	48.66	5	0	1	0	0	0	0	0	0	0	0	0	0
180. Ingolstadt	Germany	11.45	48.8	5	0	1	0	0	0	0	0	0	0	0	0	0
181. Jánské Lázně	Czech Republic	15.8	50.63	5	0	1	0	0	0	0	0	0	0	0	0	0
182. Kamenický Šenov	Czech Republic	14.47	50.77	5	0	1	0	0	0	0	0	0	0	0	0	0
183. Long Ashton	UK	−2.67	51.43	5	0	0	0	1	0	0	0	0	0	0	0	0
184. Koblenz	Germany	7.6	50.37	5	0	1	0	0	0	0	0	0	0	0	0	0
185. Kořenov	**Czech Republic**	**15.37**	**50.75**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
186. Leigh	Ireland	−6.97	52.73	5	0	0	0	1	0	0	0	0	0	0	0	0
187. Maulbronn	Germany	8.8	49	5	0	1	0	0	0	0	0	0	0	0	0	0
188. Mladá Boleslav	Czech Republic	14.93	50.43	5	0	1	0	0	0	0	0	0	0	0	0	0
189. Neusterlitz	Germany	13.15	53.4	5	0	1	0	0	0	0	0	0	0	0	0	0
190. Olszany	Poland	22.65	49.75	5	0.8	0.2	0	0	0	0	0	0	0	0	0	0
191. Polanica	Poland	16.52	50.4	5	0.2	0.8	0	0	0	0	0	0	0	0	0	0
192. Polubny	Czech Republic	15.38	50.77	5	0	1	0	0	0	0	0	0	0	0	0	0
193. Raciborz	Poland	18.25	50.08	5	0	1	0	0	0	0	0	0	0	0	0	0
194. Suchedniow	Poland	20.83	51.08	5	1	0	0	0	0	0	0	0	0	0	0	0
195. Szymbaric	Poland	21.07	49.62	5	1	0	0	0	0	0	0	0	0	0	0	0
196. Valašské Klobouky	Czech Republic	18	49.15	5	0	1	0	0	0	0	0	0	0	0	0	0
197. Forsinge	**Denmark**	**11.25**	**55.65**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
198. Vodslivy	Czech Republic	14.83	49.83	5	0	1	0	0	0	0	0	0	0	0	0	0
199. Oldendorf	**Germany**	**9.43**	**53.38**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
200. Vellberg	Germany	9.92	50.07	5	0	1	0	0	0	0	0	0	0	0	0	0
201. Wolfen	Germany	12.33	51.67	5	0	1	0	0	0	0	0	0	0	0	0	0

n, Sample size. Populations labelled with number 1 and 2 indicate two different sampled sites within the same forest. In some cases, population names refer to the closest village.
Bold type: indicates a subset of populations evenly spread across the species distribution range on which 12 primer–enzyme combinations were analysed (see text, PCR-RFLP analysis).