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NUCLEAR MICROSATELLITES REVEAL CONTRASTING PATTERNS OF GENETIC STRUCTURE BETWEEN WESTERN AND SOUTHEASTERN EUROPEAN POPULATIONS OF THE COMMON ASH (*FRAXINUS EXCELSIOR* L.)

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Abstract.—To determine extant patterns of population genetic structure in common ash and gain insight into postglacial recolonization processes, we applied multilocus-based Bayesian approaches to data from 36 European populations genotyped at five nuclear microsatellite loci. We identified two contrasting patterns in terms of population genetic structure: (1) a large area from the British Isles to Lithuania throughout central Europe constituted effectively a single deme, whereas (2) strong genetic differentiation occurred over short distances in Sweden and southeastern Europe. Concomitant geographical variation was observed in estimates of allelic richness and genetic diversity, which were lowest in populations from southeastern Europe, that is, in regions close to putative ice age refuges, but high in western and central Europe, that is, in more recently recolonized areas. We suggest that in southeastern Europe, restricted postglacial gene flow caused by a rapid expansion of refuge populations in a mountainous topography is responsible for the observed strong genetic structure. In contrast, admixture of previously differentiated gene pools and high gene flow at the onset of postglacial recolonization of western and central Europe would have homogenized the genetic structure and raised the levels of genetic diversity above values in the refuges.

Key words.—Admixture, Bayesian methods, microsatellites, population genetic structure, postglacial recolonization.

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Patterns of population genetic structure within plant species over a wide distribution range are shaped by the interaction of many factors. Some of these are intrinsic to the species life history (breeding system, modes of seed and pollen dispersal, life form, gregariousness), whereas others are perturbations induced by natural processes (ice ages, climatic stochasticity) or human impact (habitat fragmentation, global change). In particular, the climatic fluctuations during the Quaternary produced important changes in the distribution ranges of many plant species and thereby strongly affected their large-scale genetic structure (Comes and Kadereit 1998; Hewitt 2000). The signature of the Quaternary ice ages in present-day patterns of genetic structure may be especially important for species with long life cycles, such as temperate tree species. In these species, relatively few generations (100 to 1000 depending on the species, Kremer 1994) have elapsed since the last glacial maximum 15,000 radiocarbon (^{14}C) years ago (Walker 1995), allowing comparatively less impact of other factors on genetic structure than in more short-lived species.

For temperate tree species, a common observation is that they present very high genetic diversity at nuclear loci, but very little genetic differentiation among populations (Hamrick and Godt 1989; Hamrick et al. 1992). Biological characteristics of temperate tree species such as long generation time, woody life form, high fecundity, outcrossing mating system, and predominant wind pollination have been put forward to explain this general pattern (Hamrick and Holden 1979; Loveless and Hamrick 1984). Indeed, some of these features are expected to influence genetic variation and population genetic structure through their effects on the mutation-selection balance (Charlesworth et al. 1993), effective population size (Pollak 1987; Schoen and Brown 1991), and gene flow (Levin 1981).

During the last glacial period (115,000 to 15,000 ^{14}C years ago, Birks 1986), European temperate forests were much more restricted than today and occupied mainly refuges in the southern peninsulas (e.g., Huntley and Birks 1983; Bennett et al. 1991; Tzedakis et al. 2002). Long-term isolation led to genetic differentiation among refuge populations (e.g., Kremer et al. 2002). The recolonization of Europe began 15,000 to 10,000 ^{14}C years ago depending on the species (Brewer 2002), and has been suggested to involve long-distance seed dispersal accompanied by recurrent founder events, causing a decrease in genetic diversity within, and an increase in genetic structure among colonizing populations (Hewitt 1996). This view has been challenged by some authors suggesting that founder effects in tree species were probably attenuated by their particular demographic characteristics such as an extended juvenile phase that allows additional colonizers to arrive before the first founders reach sexual maturity (Austerlitz et al. 2000), or by a potential increase in gene flow through long-distance dispersal of pollen in situations of low density of adult trees (Comps et al.

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2001). Moreover, population admixture in areas where populations recolonizing from separate refuges are merging has also been suggested to overcome the influence of founder effects (Lagercrantz and Ryman 1990; Zanetto and Kremer 1995). Thus, contrasted predictions arise for levels of genetic diversity and more strikingly of population genetic structure in recently recolonized areas, depending on the relative importance of founder effects versus demographical and population admixture effects.

Inference of historical processes has been attempted by analyzing population samples over a wide geographical area with uniparentally or biparentally inherited markers. Phylogeographic patterns of haplotypes at uniparentally inherited markers were generally interpreted jointly with the spatial and temporal distribution of fossil pollen to infer patterns of postglacial recolonization (e.g., Demesure et al. 1996; King and Ferris 1998; Petit et al. 2002). However, each organelle genome represents only a single gene genealogy, and can therefore hardly capture all historical events the populations experienced (Nordborg 2001). Conversely, nuclear markers allow for recombination, integrating several genealogical processes. Most broad-scale nuclear marker studies hitherto were allozyme studies, mainly focusing on variation patterns in genetic diversity. In some species, postglacial recolonization lead to a loss of both allelic richness and gene diversity in agreement with the theory of founder events (Tomaru et al. 1997; Ally et al. 2000; Ledig 2000), whereas in others, allelic richness decreased, but gene diversity increased (e.g., in sessile oak, Zanetto and Kremer 1995; and in beech, Comps et al. 2001). However, patterns of genetic differentiation among populations at nuclear markers have received limited attention, partly because the overall differentiation in temperate tree species is low (mean $G_{ST} = 0.084$ for tree species, mean $G_{ST} = 0.228$ for plant species, Hamrick et al. 1992). No clear patterns are emerging from the studies in sessile oak or in beech: in oak the highest differentiation values were found among the most ancient populations, close to refuges, and among the most recently colonized populations from Scandinavia, but not among those from the British Isles (Zanetto and Kremer 1995); in beech, the statistic F_{ST} increased with increasing distance to refuges, but differentiation for allelic richness was highest among the most ancient populations (Comps et al. 2001). In spruce, strong differentiation was observed among populations from widely distant areas evolving from different glacial refuges (Lagercrantz and Ryman 1990).

The availability of highly polymorphic microsatellite markers, which identify multilocus genotypes with a better resolution than allozymes, and the development of Bayesian numerical procedures for the analysis of multilocus genotype structure (Pritchard et al. 2000; Dawson and Belkhir 2001; Anderson and Thompson 2002; Corander et al. 2003) provide new opportunities to test the effect of the recolonization process on patterns of genetic structure. Advantages of microsatellites over allozymes are that their high polymorphism ensures low standard errors in estimates of population genetic statistics (Goudet et al. 1996), and that they may contain phylogenetic information in the distribution of allelic sizes (Hardy et al. 2003). The power of the Bayesian approaches in determining patterns of population structure is partly due to the fact that they combine information from several loci into a single probability model, as opposed to the simple averaging used, for instance, in traditional F_{ST} analysis (Corander et al. 2003). It also arises from their ability to detect hidden substructure trough pooling of combinations of the sampled populations (approach implemented in BAPS, Corander et al. 2003) or pooling of sets of individuals independently of the actual sample structure (approach implemented in STRUCTURE, Pritchard et al. 2000). Moreover, some Bayesian methods allow estimation of the amount of genetic admixture at the population or individual level. Geographical information can either be incorporated as priors in the models, or be used a posteriori for visual inspection of its relationship with the inferred population genetic structure. Bayesian methods have, for instance, successfully identified genetic clusters in humans, where genetic differences among major groups constitute only 3–5% (Rosenberg et al. 2001) and they have identified hybrids between wild and domestic cats, which could not be achieved with distance-based or multivariate methods (Randi et al. 2001). In tree species, they have mostly been applied for the assessment of hybridization (Jones et al. 2002; Craft et al. 2002; Coart et al. 2003) and for the identification of hidden population substructure (Corander et al. 2003).

The general objective of our study was to use microsatellites and Bayesian methods to detect patterns of population genetic structure at the scale of Europe in common ash, a temperate wind-pollinated tree species (Tutin et al. 1972) with low among-population differentiation ($F_{ST} = 0.087$ among populations from Bulgaria; Heuertz et al. 2001), in order to gather information on population genetic processes associated to post-glacial recolonization. Due to technical difficulties with allozyme markers, no information on broad-scale patterns of genetic structure was previously available in this species. The common ash, *Fraxinus excelsior* L. (Oleaceae), occurs in mixed deciduous forests from nearly all of Europe, with the exclusion of the most southern and most northern parts. It exhibits intermediate properties between a pioneer species and a permanent forest component; its colonization capacity is strong, but competitive ability is high only when ecological requirements are met. 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; A. Lamb and D. Boshier, pers. comm.). The single-seeded fruits, the samaras, are wind dispersed. Fossil pollen records (Huntley and Birks 1983; Gliemeroth 1997; Brewer 2002) suggest glacial refuges for ash in the Balkan Peninsula and the Alps and probably Italy, whereas refuges in Iberia and north of the Black Sea are less strongly supported. Chloroplast haplotype data are consistent with postglacial recolonization of continental Europe from those refuges (G. G. Vendramin, pers. comm.). We sampled 36 populations of common ash in Europe and analyzed them at five nuclear microsatellite loci. Our specific objectives were (1) to identify geographical patterns of multilocus genetic structure using Bayesian approaches; (2) to investigate the relationship between these patterns and patterns of variation in classical population genetics statistics; and (3) to interprete the observed patterns in terms of population demography and

postglacial recolonization dynamics during the current glacial interstadial.

Material and Methods

Plant Material

Samples of *Fraxinus excelsior* consisting of buds or leaves were collected from an average of 30 nonadjacent trees in 36 putatively autochthonous forests in Europe ($n = 1069$; Figs. 1 and 2, Appendix). Buds were shipped to the laboratory on their twigs, wrapped in wet paper. They were dissected out, separated from their scales, and conserved at $-70°$; leaves were dried and kept at room temperature prior to DNA extraction.

DNA Extraction

Total DNA was extracted with the DNeasy Plant mini kit (Qiagen, Leusden, The Netherlands) or the CTAB procedure of the NucleoSpin Plant kit (Macherey Nagel, Düren, Germany.) from 60 to 90 mg of dry leaves or from 50 to 70 mg fresh weight of buds ground by hand or in the automatic grinding mill MM200 (Retsch, Haan, Germany). 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).

Microsatellite Analysis

Five highly polymorphic microsatellites (Table 1) were analyzed as previously described (Heuertz et al. 2001). Fluorescent labeling of the forward polymerase chain reaction primers allowed detection of amplification products on an automated DNA sequencer (ABI PRISM 377 DNA sequencer, Applied Biosystems, Foster City, CA). Sizing was performed with the programs Genescan 3.1 and Genotyper 2.5 from Applied Biosystems by comparison with an internal sizing standard (Genescan-350 Rox). Modifications to the previously published protocol include (1) reamplification with LA Taq polymerase (TaKaRa, Shiga, Japan) of samples which produced bad amplifications and (2) production of polyacrylamide gels with LongRanger gel solution (FMC Bioproducts, Rockland, ME).

Data Analysis

For each microsatellite locus, we recorded the total number of alleles K, the range of allele sizes and the total gene diversity H_T to assess overall polymorphisms. We computed Wright's inbreeding coefficient F_{IS} providing information on the cumulative action of inbreeding, population substructure, and potential null alleles. Differentiation between populations was analyzed based on allele identity with the statistic F_{ST}, or on allele size with R_{ST}. To characterize polymorphism and among-population differentiation for the species, multilocus estimates of these statistics were obtained, and the contribution of stepwise-like mutations to population differentiation, that is, whether $R_{ST} > F_{ST}$, was investigated by testing whether the observed R_{ST} was larger than its value obtained after permuting allele sizes among alleles within populations (Hardy et al. 2003). The software SPAGEDI (Hardy and Vekemans 2002, available at: http://www.ulb.ac.be/sciences/lagev/spagedi.html) was used for the computations.

Geographical trends in the distribution of genetic diversity at the continental scale were investigated with Bayesian methods for the analysis of population genetic structure (Pritchard et al. 2000; Corander et al. 2003). In these methods, the multilocus genotypic data X are considered to be random draws from a parametric model, which describes their organization into clusters. A joint probability distribution $P(X, \theta)$ is established over the data X and the model parameters and missing data θ. It consists of the prior distribution $P(\theta)$, where prior information such as the geographical location of samples can be incorporated, and the likelihood $P(X \mid \theta)$. The posterior distribution $P(\theta \mid X)$ of the model conditional on the data is then established, and it is used for inference of the model parameters, such as the joint estimation of cluster membership and allele frequencies in the different clusters. Because θ consists of several multidimensional variables, it is usually not possible to compute $P(\theta \mid X)$ directly, but an approximate sample from $P(\theta \mid X)$ can be obtained using a Markov chain Monte Carlo simulation approach. The methods of Pritchard et al. (2000) and Corander et al. (2003) require multilocus genotypes at unlinked genetic markers. Therefore, to verify the independence of our microsatellite loci, we analyzed linkage disequilibrium for all pairs of loci in each sampled population with exact tests with GENEPOP version 3.3 (Raymond and Rousset 1995) and applied a sequential Bonferroni correction (Rice 1989) to discard accidental correlations between loci.

The Bayesian Analysis of Population Structure (program BAPS, Corander et al. 2003, available at: http://www.rni.helsinki.fi/~mjs/) estimates hidden population substructure by clustering sampled populations into panmictic groups. The only prior information given was the population of origin of each sampled individual, other priors concerning the population allele frequencies and structure parameters were automatically set to be uninformative. A joint posterior distribution of partitions of the sampled populations into panmictic groups was produced along with their respective allele frequencies. BAPS was run ten times for 10^5 iterations after a burn-in period of 20,000, randomly mixing the order of populations in the input file. The resulting partitions were averaged based on their posterior probabilities and plotted onto the map of Europe. The inferred panmictic groups of populations will be referred to as ''demes.''

The model-based clustering method (program STRUCTURE vers. 2.1, Pritchard et al. 2000, available at: http://pritch.bsd.uchicago.edu/software/structure2_1.html) assigns individual multilocus genotypes probabilistically to a user-defined number K of clusters or gene pools, achieving linkage equilibrium within clusters. STRUCTURE was run three times for each $K \in \{1, \ldots, 20\}$ for 10^6 iterations after a burn-in period of 10^5 on the total dataset ($n = 1069$) without any prior information on the population of origin of each sampled individual. We used the admixture model in which the fraction of ancestry from each cluster is estimated for each individual. The parameter of individual admixture alpha was chosen to be the same for all clusters and it was given a uniform prior. The allele frequencies were kept independent

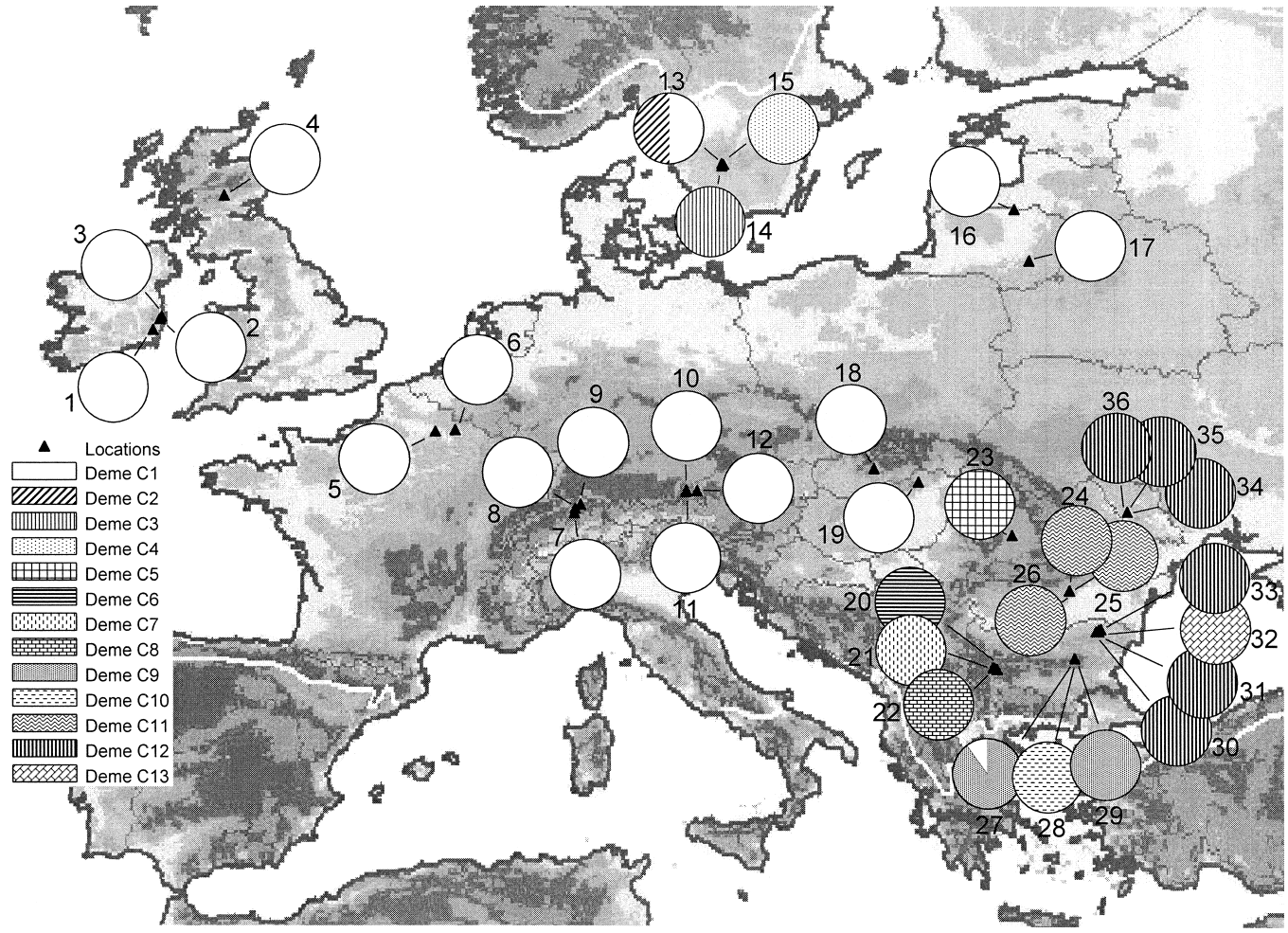

FIG. 1. Geographical distribution of the demes obtained with the Bayesian Analysis of Population Structure (BAPS) (Corander et al. 2003).

among clusters in order to avoid overestimating the number of clusters (Falush et al. 2003). The fractions of ancestry were averaged over individuals within each of the 36 population samples and the corresponding pie charts were plotted onto the map of Europe. The clusters will be referred to as ''gene pools.''

The Bayesian methods identified contrasting features in population genetic structure in different geographical regions of Europe (see Results). To help interpret these regional differences, we computed classical population genetics statistics for populations in these regions and tested for differences among them. We partitioned the populations into the following three regions according to results from the BAPS method (Fig. 1, Table 2): (1) western and central Europe except Sweden (population numbers 1–12 and 16–19); (2) southeastern Europe (numbers 20–36); (3) Sweden (numbers 13–15). We analyzed differentiation among populations within each group using F_{ST} and R_{ST} to test for homogenization of gene resources by postglacial gene flow and we assessed the contribution of stepwise-like mutations to differentiation with SPAGEDI. To detect the effects of founder events and/or population admixture, we looked at patterns for two measures of polymorphism, allelic richness, and gene diversity (expected heterozygosity), first separately, then jointly with the statistic T_2 from Cornuet and Luikart (1996). Recent founder events or population bottlenecks affect mainly rare alleles, producing transiently a stronger decrease in allelic richness than gene diversity, that is, an excess of gene diversity (Nei et al. 1975; Cornuet and Luikart 1996). Population admixture may have various effects on the diversity statistics depending on characteristics of the admixing populations. We used FSTAT version 2.9.3 (Goudet 1995, available at: http://www.unil.ch/izea/softwares/fstat.html) to compute an average estimate of allelic richness over loci within populations, A_S, and across populations from each group, A_T. To obtain estimates independent of sample size variation, we applied the rarefaction method according to El Mousadik and Petit (1996) with a standard sample size of $n = 36$ gene copies, or 18 diploid individual trees, which corresponds to the smallest population sample with a complete genotype at all five loci (in population 30). Multilocus estimates of gene diversity were obtained within populations, H_E, and across populations, H_T, from each group. The ''bottleneck'' statistic T_2 (Cornuet and Luikart 1996) was computed at the population level. It represents an average over loci of the deviation of the actual gene diversity H_E from the gene diversity expected

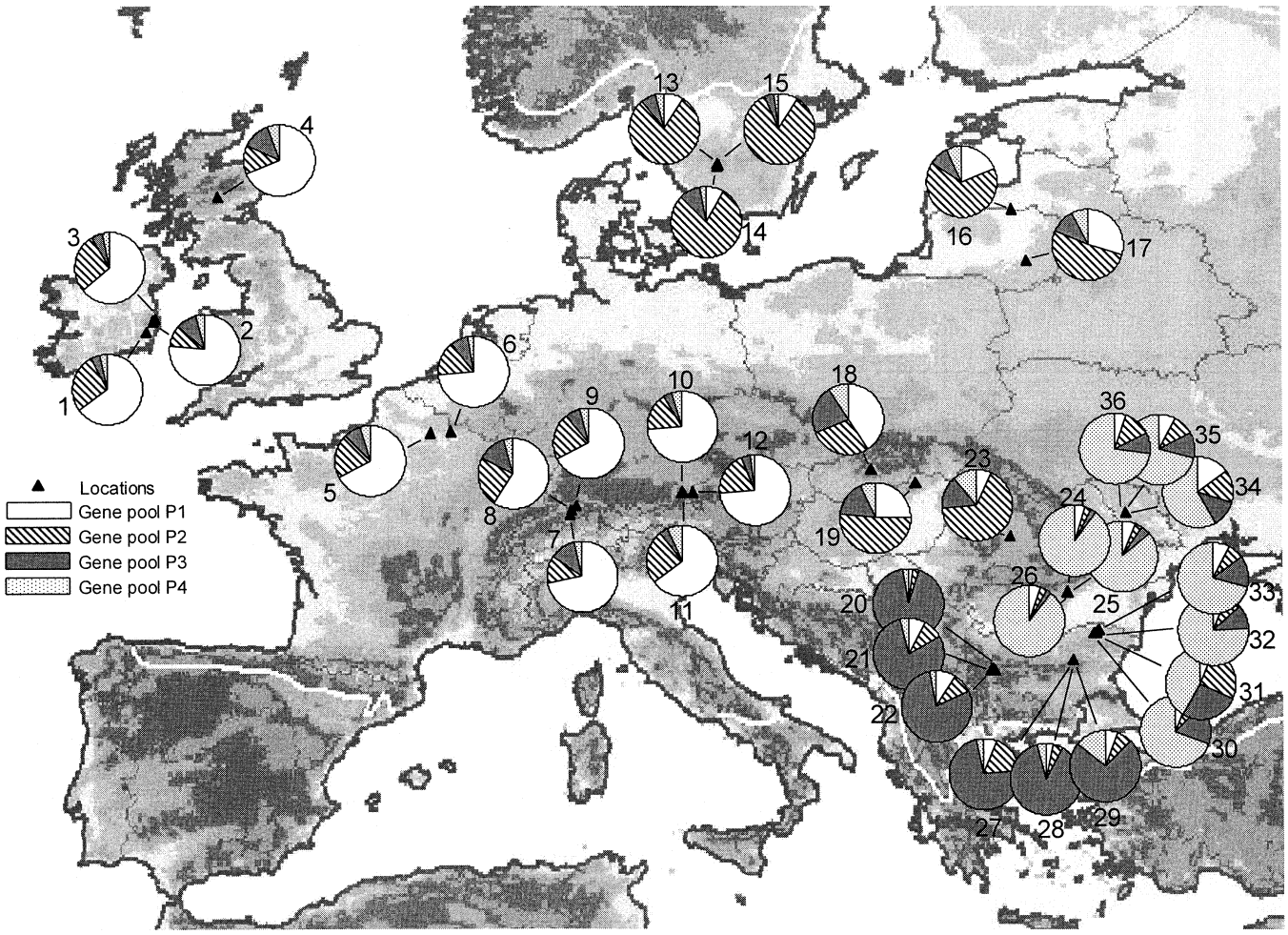

FIG. 2. Proportions of ancestry of each population in each of six gene pools defined with the model-based clustering method from Pritchard et al. (2000).

from the number of alleles in the population H_A assuming mutation-drift equilibrium. Positive values of T_2 reflect a gene diversity excess possibly caused by recent founder events, whereas negative values are consistent with recent population expansion without immigration. T_2 was computed with BOTTLENECK version 1.2.02 (Cornuet and Luikart 1996, available at: http://www.montpellier.inra.fr/URLB/bottleneck/bottleneck.html) according to the Infinite Alleles Model (T_2 IAM) and the Stepwise Mutation Model (T_2 SMM). These represent two extreme mutation models, as our loci do not follow a strict SMM (many alleles differed by one base pair, which is less than one repeat unit). Significant deviation from equilibrium gene diversity was determined with the Wilcoxon signed rank test, which is the most appropriate test when only few polymorphic loci are analyzed (Piry et al. 1999). Further, we computed Wright's inbreeding coefficient F_{IS} with FSTAT and tested deviation of genotypic frequencies from Hardy-Weinberg proportions with the program GENEPOP version 3.3 (Raymond and Rousset 1995) to check whether inbreeding occurred or microsatellites had null alleles. Differences between groups of populations in the mean values of A_S, H_E, F_{IS}, and F_{ST} were investigated with permutation tests with FSTAT; differences for R_{ST} were analyzed comparing confidence intervals based on standard errors of jackknifed estimators; and differences for T_2 IAM and T_2 SMM were analyzed with Mann-Whitney rank sum tests.

Spatial genetic structure was investigated according to Rousset (1997) by testing for a pattern of isolation by distance (IBD): a Mantel test with 10,000 random permutations was performed between the matrix of pairwise genetic differentiation between populations, using $F_{ST}/(1 - F_{ST})$, and the matrix of the natural logarithm of geographic distance. These

TABLE 1. Allelic diversity of the nuclear microsatellite loci scored in the common ash. Allele sizes (nt), range of sizes of PCR products, in nucleotides; K, total number of alleles; H_T, total gene diversity; F_{IS}, Wright's inbreeding coefficient; F_{ST}, relative differentiation based on allele identity; R_{ST}, relative differentiation based on allele size.

Locus	Allele sizes (nt)	K	H_T	F_{IS}	F_{ST}	R_{ST}
M2-30	176–294	83	0.979	0.018	0.059	0.150
FEMSATL4	155–298	71	0.898	0.062	0.066	0.066
FEMSATL11	176–249	48	0.904	−0.018	0.067	0.034
FEMSATL16	170–210	16	0.433	0.203	0.093	0.079
FEMSATL19	142–230	57	0.949	−0.001	0.106	0.350

TABLE 2. Genetic variation within and among common ash populations analyzed with nuclear microsatellite loci. Populations, population numbers included in each group; N, total number of populations; A_S, within-population allelic richness computed for standardized samples of 36 gene copies (SD); A_T, total allelic richness for standardized samples of 36 gene copies; H_E, within-population gene diversity (SD); H_T, total gene diversity; T_2, bottleneck statistic from Cornuet and Luikart (1996) computed on the basis of the infinite alleles model (T_2 IAM; SD) or the stepwise-mutation model (T_2 SMM; SD); F_{IS}, Wright's inbreeding coefficient (SD); F_{ST}, relative among-population differentiation based on allele identity; R_{ST}, relative among-population differentiation based on allele size; P (H1: $R_{ST} > R_{ST}$ perm.), one-sided test of information content of allele sizes from Hardy et al. (2003): ns, nonsignificant; *, $P \leq 0.05$; ***, $P \leq 0.001$. [a] This group does not contain populations from Sweden, [b] See Materials and Methods for the types of tests used; nd, not determined; ns, nonsignificant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

					Test[b]		
	Europe	Western and central Europe[a]	Southeastern Europe	Sweden	Western and central Europe[a] vs. southeastern Europe	Western and central Europe vs. Sweden	Southeastern Europe vs. Sweden
Populations	1–36	1–12, 16–19	20–36	13–15			
N	36	16	17	3			
A_S	11.329 (2.016)	12.355 (1.070)	10.836 (2.218)	8.650 (1.379)	*	**	ns
A_T	16.134	14.428	15.825	10.451	nd	nd	nd
H_E	0.770 (0.062)	0.814 (0.037)	0.736 (0.052)	0.684 (0.051)	***	***	ns
H_T	0.832	0.837	0.817	0.776	nd	nd	nd
T_2 IAM	−0.107 (1.181)	0.648 (0.598)	−0.589 (1.226)	−1.397 (0.844)	**	***	ns
T_2 SMM	−4.934 (2.937)	−3.055 (1.306)	−6.064 (3.048)	−8.553 (2.255)	**	***	ns
F_{IS}	0.033 (0.052)	0.064 (0.045)	0.012 (0.046)	−0.001 (0.033)	**	*	ns
F_{ST}	0.076	0.027	0.093	0.153	***	**	ns
R_{ST}	0.173	0.033	0.227	0.077	ns	ns	ns
P (H1: $R_{ST} > R_{ST}$ perm.)	***	ns	***	ns			

analyses were performed with SPAGEDI on the whole dataset and separately for populations from western and central Europe (populations 1–12 and 16–19), and southeastern Europe (numbers 20–36).

RESULTS

Overall Diversity

The total number of alleles observed per locus in the overall sample of 1069 individuals ranged from 16 to 83, with an overall total of 275 alleles scored over the five loci (Table 1). The size ranges of PCR products corresponding to these alleles were roughly comprised between 140 and 300 nucleotides (Table 1). For loci FEMSATL11 and FEMSATL16, most alleles differed by two nucleotides; FEMSATL4 displayed even and odd-sized alleles that were clearly distinguished whereas in FEMSATL19 and M2–30, the variation of allele sizes seemed almost continuous. For the latter loci, alleles were binned into allele classes that were approximately one nucleotide wide (Heuertz 2003). Overall gene diversities (H_T) were very similar for each locus with the exception of FEMSATL16 that showed less than half the polymorphism of the other loci. Moderate variation in F_{ST} (CV = 25.9%) but strong variation in F_{IS} (CV = 168.8%) and R_{ST} (CV = 93.5%) were observed among loci (Table 1).

There were on average 11.329 (±2.016 SD) alleles per locus and per population (standardized samples of 36 gene copies) and at the species level, the allelic richness based on the same sample size was 16.134 (Table 2). The average within-population gene diversity equaled $H_E = 0.770$ (±0.062 SD) and the total gene diversity $H_T = 0.832$ (Table 2). Populations were on average slightly inbred, with a mean inbreeding coefficient of $F_{IS} = 0.033$ (±0.052 SD). Differentiation among populations taking into account the allele sizes, $R_{ST} = 0.173$, was significantly larger than differentiation based on allele identities, $F_{ST} = 0.076$ ($P \leq 0.001$), indicating that stepwiselike mutations contributed to overall among-population differentiation.

Linkage Disequilibrium

Among a total of 344 tests for linkage disequilibrium between pairs of loci, six were significant ($P \leq 0.05$), three of which concerning population 12 from Germany. We concluded that the analyzed loci were sufficiently independent for the application of Bayesian methods for the analysis of population structure.

Patterns of Population Genetic Structure

In each of 10 independent runs of BAPS, the distribution among populations of most demes was the same, revealing a clear pattern of regional heterogeneity in population genetic structure of European populations of the common ash (Fig. 1). Differences among runs concerned only populations 13 and 27. Very homogeneous genetic structure was observed in western and central Europe, where populations 1–12 and 16–19 were assembled into the same deme (C1). In Sweden, population 13 grouped with this deme in half of the analyses, or it formed a deme on its own, whereas populations 14 and 15 always formed separate demes. In southeastern Europe on

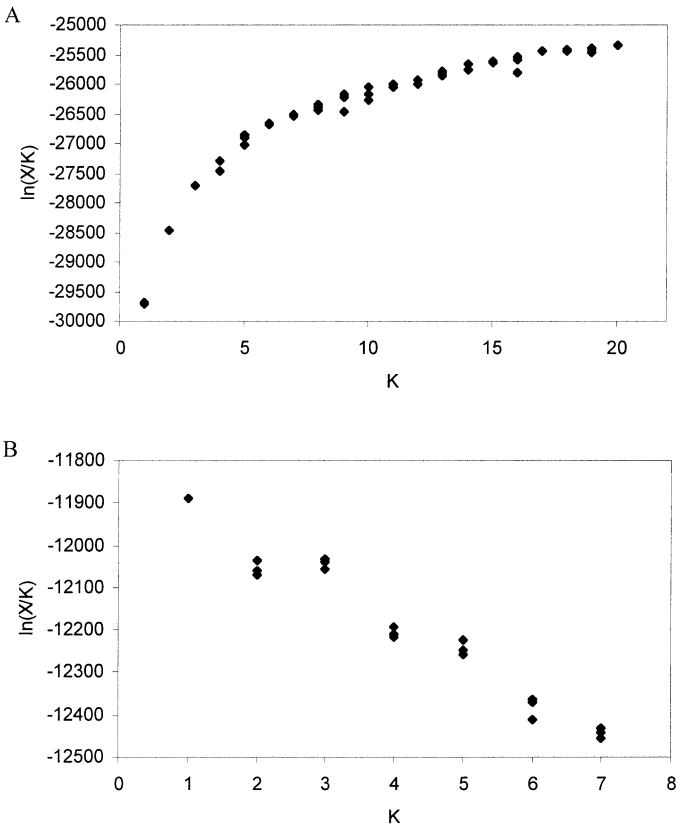

FIG 3. Values of log likelihood of the multilocus genotypic data, $\ln(X \mid K)$, as a function of the number of clusters, K, used with the STRUCTURE method (Pritchard et al. 2000) applied to (A) the entire common ash dataset, $n = 1069$ or to (B) individuals from populations 1 to 12 and 16 to 19 from western and central Europe, $n = 452$.

the other hand, genetic structure was much more pronounced, as a total of 10 distinct demes were observed.

The model-based clustering method (program STRUCTURE) of Pritchard et al. (2000) confirmed the observation of regional heterogeneity in patterns of population genetic structure in common ash across Europe (Fig. 2). However, the inference of the number of gene pools K was not straightforward since log-likelihood values for the data conditional on K, $\ln(X \mid K)$, increased progressively as K was increased (Fig. 3A). Pritchard and Wen (2003, software documentation available at http://pritch.bsd.uchicago.edu/software/structure2_1.html) state that in such a case it may not be possible to know the true value of K and they suggest choosing the smallest value that captures the major structure in the data. We inspected the geographical distribution of gene pools for different K and observed a biologically informative pattern from $K = 4$ upwards. For graphical representation, we chose the run with the highest $\ln(X \mid K)$ value for $K = 4$ for the sake of simplicity, because in $K = 5$, which is more likely, only minor changes occur. The geographical structure is the following (Fig. 2): gene pool P1 is predominant across western and central Europe; gene pool P2 is most abundant in Sweden and its frequency decreases from Lithuania over central to western Europe; gene pool P3 is predominantly found in southeastern Europe within the Balkan Mountains whereas gene pool P4 mainly occurs in southeastern Europe east of the Balkan and Carpathian Mountains. The average admixture of gene pools within sampled populations, expressed as the proportion of genes from a given sample assigned to the best-scoring gene pool P_{best} in the sample, was 71%. Admixture was highest in populations sampled from Lithuania, Slovakia, and Hungary ($P_{best} = 53\%$ for populations 16–19) and higher than average in western and central Europe ($P_{best} = 65\%$ for populations 1–12). As K was increased, the gene pools defined for $K = 4$ progressively split up, in the following order: (1) in P4, populations 24–26 were separated from the other populations east of the Carpathians and Balkans; (2) in P3, populations 22 and 28 split from the others; (3) in P2, populations 14 and 15 from Sweden were discriminated whereas 13 was admixed; (4) further splits occurred in southeastern Europe. Numerous splits of gene pools in southeastern Europe indicate heterogeneity of ash genetic resources in that region. On the other hand, the analysis with STRUCTURE of populations from western and central Europe only (1–12 and 16–19) unambiguously produced the highest log likelihood for a number of $K = 1$ gene pool (Fig. 3B); demonstrating that this group of populations forms a single deme in accordance with results obtained with BAPS.

Geographic variation in differentiation statistics confirmed these heterogeneous patterns of population genetic structure at the scale of Europe (Table 2): based on F_{ST}, populations from western and central Europe were less differentiated ($F_{ST} = 0.027$) than those from southeastern Europe ($F_{ST} = 0.093$; $P \leq 0.001$). R_{ST}-values were not significantly different ($R_{ST} = 0.033$ vs. $R_{ST} = 0.227$), but a significant contribution of stepwise-like mutations to population differentiation was observed in the latter ($P \leq 0.001$) but not in the former region. Populations from Sweden were significantly more differentiated than those from western and central Europe based on F_{ST} ($P \leq 0.01$), and resembled thereby the populations from southeastern Europe.

Spatial Organization of Genetic Diversity

Contrasting patterns between western and central Europe on the one hand and southeastern Europe on the other were observed for a number of diversity statistics. Populations from western and central Europe exhibited on average higher allelic richness, $A_S = 12.355$, and higher gene diversity, $H_E = 0.814$, than populations from southeastern Europe, where $A_S = 10.836$ and $H_E = 0.736$ ($P \leq 0.05$ and $P \leq 0.001$, respectively, Table 2). The geographical trend was similar for total gene diversity, with a higher value in western and central Europe, $H_T = 0.836$, than in the southeast, $H_T = 0.817$, but it was opposite for total allelic richness, which was higher in the southeast, $A_T = 15.825$, than in the west and the center of Europe, $A_T = 14.428$. Over the five microsatellite loci, 106 alleles occurred in both regions, whereas 77 occurred in the southeast only and 29 in the western and the central regions only. Further, compared to the southeast, populations from western and central Europe displayed higher values of T_2 IAM and T_2 SMM ($P \leq 0.01$ for both statistics, Table 2), indicating a relative excess of gene diversity over the value expected from the observed number of alleles. When applied to T_2 IAM, Wilcoxon's signed rank test ($\alpha =$

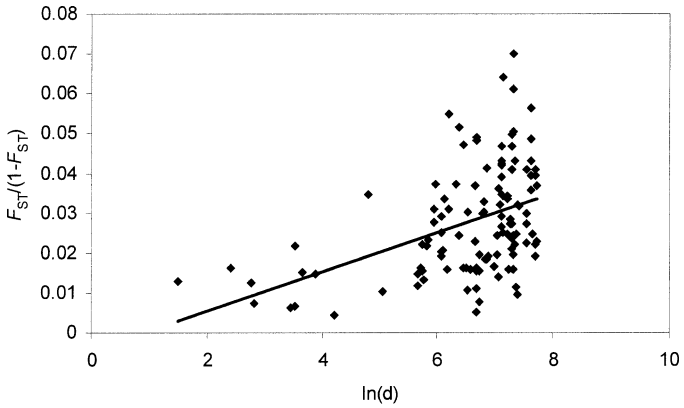

FIG. 4. Plot of pairwise $F_{ST}/(1 - F_{ST})$ ratios against the logarithm of distances (in km) between populations from western and central Europe, except Sweden. Under isolation by distance, values of the ratios are expected to increase linearly with the logarithm of distance (Rousset 1997).

0.05) identified a significant excess of gene diversity in seven populations, five of which were located in western and central Europe (2, 3, 5, 6, and 10) and two in southeastern Europe (20 and 22). Two populations from southeastern Europe (21 and 23) showed a significant deficiency of gene diversity. Values of T_2 SMM were consistently lower than those of T_2 IAM and all but one populations displayed negative values, which resulted in significant deficiency of gene diversity in 28 populations (Wilcoxon's signed rank test, $\alpha = 0.05$). A geographical pattern at the scale of Europe was also found for Wright's inbreeding coefficient, with populations from western and central Europe being on average more inbred (mean $F_{IS} = 0.064$, 11 of 16 populations with a significant heterozygote deficit at $\alpha = 0.05$) than populations from southeastern Europe (mean $F_{IS} = 0.012$, two of 17 populations with a significant heterozygote deficit and two with a significant heterozygote excess at $\alpha = 0.05$; $P \leq 0.01$). Populations from Sweden featured low allelic richness, low gene diversity, strongly negative average T_2 statistics, and no departure from Hardy-Weinberg equilibrium, differing thereby from the other western and central European populations, but resembling those from southeastern Europe (Table 2).

Patterns of Isolation by Distance

At the level of Europe, no significant pattern of IBD was observed. However, between-population differentiation measured as $F_{ST}/(1 - F_{ST})$ increased significantly with the logarithm of the geographical distance between populations in western and central Europe (with the exception of Sweden, $R^2 = 0.190$, $P \leq 0.001$; Fig. 4) and in southeastern Europe ($R^2 = 0.090$, $P \leq 0.01$).

DISCUSSION

The overall pattern of genetic variation at microsatellite loci that we observed for the common ash is typical for a long-lived outcrossing species with high genetic diversity, $H_E = 0.77$, a low level of inbreeding within populations, $F_{IS} = 0.03$, and low differentiation among populations $F_{ST} = 0.08$ (Hamrick et al. 1992). Nevertheless, over the distribution range, Bayesian analysis methods effectively identified two contrasting patterns of population genetic structure, paralleled with distinctive patterns of genetic diversity: (1) all populations from western and central Europe essentially formed a single deme with very low genetic differentiation among populations and high allelic richness and gene diversity within populations, whereas (2) several distinct demes occurred in southeastern Europe and in Sweden and genetic differentiation among populations was strong relatively to that in western and central Europe, but allelic richness and gene diversity within populations were relatively low. Concomitantly, a high degree of inbreeding and a tendency to excess gene diversity as revealed by Cornuet and Luikart's (1996) T_2 statistics were observed in western and central European populations, and the opposite pattern in regions of high among-population differentiation. We discuss below the application of Bayesian methods to identify population structure and provide possible causes for the clear contrasting patterns detected in common ash.

Use of Bayesian Methods for Inference of Population Genetic Structure

The Bayesian methods allowed detecting subtle patterns of population genetic structure of common ash in Europe, despite overall very low differentiation in this wind-pollinated tree species. In comparison, detection of geographic patterns of population structure with alternative methods was less satisfactory: for instance, (1) principal component analysis on gene frequency data detected no structure but a separation of Romanian populations 24–26 from all others (results not shown), and (2) distance-based clustering methods detected genetic differentiation between western and central European populations on the one hand, and southeastern European populations on the other hand, but internal branches of the trees were often short and had low bootstrap support, and the heterogeneity in population genetic structure between the two regions was not detected (Fig. 5). However, the stronger genetic structure in southeastern as compared to western Europe that was detected by Bayesian methods was confirmed a posteriori through a detailed analysis using classical F_{ST} statistics. With the BAPS method, the heterogeneous structure was detected in a single step. With the STUCTURE method, several runs with different numbers of gene pools K had to be compared, and precise inference of K was difficult with our dataset, but the overall pattern was confirmed. In addition, evidence for genetic admixture in the western and central European populations was suggested by this method, and the particularly high levels of admixture detected in Slovakia and Hungary suggested the occurrence of a suture zone among separately colonizing lineages. Because Bayesian clustering methods have not been fully explored in situations with known patterns of genetic structure, we suggest that results be compared from different Bayesian approaches and examined for consistent patterns that can be biologically explained.

Evolution of Population Genetic Structure during Postglacial Recolonization

In southeastern Europe, different gene pools in the east and the west, strong differentiation among common ash pop-

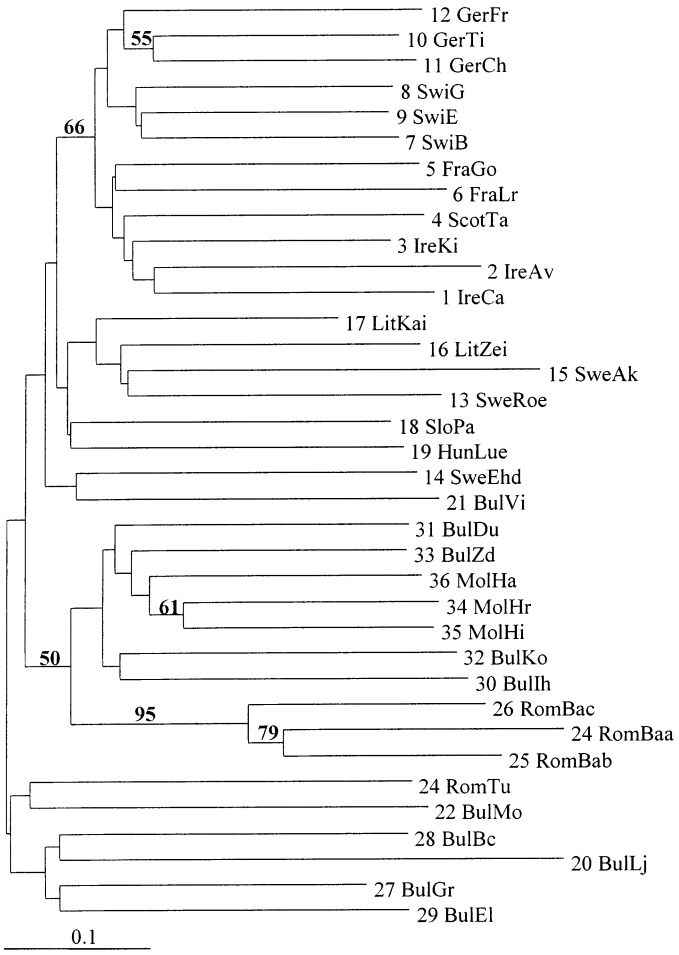

FIG. 5. Midpoint-rooted neighbor joining tree computed from Cavalli-Sforza and Edwards' (1967) chord distance among pairs of populations. The bootstrap support of the branches is given in bold numbers as the percentage out of 126 possible bootstraps.

ulations, and a contribution of stepwise-like mutations most probably reflect the maintenance of differentiation among genetic resources from distinct glacial refuges. This is in agreement with fossil pollen records, which have suggested glacial refuges for ash in both the eastern and the western Balkan Peninsula (Brewer 2002). A complex topography and a late, but extremely rapid postglacial spread (Huntley and Birks 1983; Gliemeroth 1997; Brewer 2002) with abundant well-adapted autochthonous colonizers outcompeting immigrants from other regions may have counteracted extensive postglacial gene flow. Previous investigations have indeed provided indirect evidence for relatively limited pollen and seed effective dispersal in common ash in southeastern Europe (Heuertz et al. 2001, 2003) and a pattern of population expansion with little immigration is also supported by low values of the bottleneck statistics in this area.

The recolonization of western and central Europe may have produced a reduction in among-population differentiation and an increase in diversity relative to southeastern Europe, an area close to refuges. This is probably due to an input and efficient mixture of diversity from several source populations, which may not all be of Balkan origin, considering the occurrence of 29 non-Balkan alleles and higher than average population admixture in western and central Europe as indicated by STRUCTURE. The genetic pattern of recolonization in ash agrees with theoretical expectations from a migrant pool model for metapopulation dynamics (Wade and McCauley 1988): if all colonizers originate from all preexisting populations with equal probability and colonizers are at least twice as numerous as migrants among populations, gene diversity, H, will increase and differentiation, F_{ST}, will decrease during colonization. The mixture of genetic resources in common ash must be due to efficient pollen flow since maternally inherited markers are strongly differentiated in western and central Europe in ash and in other tree species with similar life-history characteristics (Petit et al. 2003; G. G. Vendramin, pers. comm.). This conclusion is not at odds with the observed high levels of differentiation in southeastern Europe but seems to support the suggestion that during recolonization of a tree species, similar levels of dispersal of seed or pollen into stands with low effective population size result in higher effective gene flow than under stable demographic conditions (Austerlitz et al. 2000).

A reduction in among-population differentiation with recolonization was also observed in pedunculate oak with $G_{ST} \approx 0.06$ in southern central and southeastern Europe versus $G_{ST} \approx 0.02$ in western Europe (Zanetto and Kremer 1995). In beech, differentiation based on allelic richness decreased with recolonization ($A_{ST} \approx 0.16$ in southeastern vs. $A_{ST} \approx 0.05$ in central Europe), but this pattern was not paralleled in F_{ST} ($F_{ST} = 0.03$ in southeastern vs. $F_{ST} = 0.04$ in central Europe, Comps et al. 2001). In our study, broad-scale differentiation patterns were confirmed with A_{ST} ($A_{ST} = 0.34$ in southeastern Europe vs. $A_{ST} = 0.15$ in western and central Europe).

Population Admixture and Founder Events during Recolonization

Fossil pollen analyses identified postglacial spread of ash from putative refuges in the Balkan Peninsula and the Alps, and possibly in the Iberian Peninsula, in Italy, and in northeastern Europe (Huntley and Birks 1983; Gliemeroth 1997; Brewer 2002). Our limited sampling scheme did not allow reliable determination of the glacial origin of nuclear gene pools in western and central Europe. However the geographic distribution of gene pools P3 and P2 suggests a contribution of refuges from the western Balkan Peninsula and northeastern Europe, respectively, and a suture zone of strong admixture in Slovakia and Hungary. Chloroplast DNA data agree with colonization from Iberia, Italy, and/or the Alps and the Balkans (G. G. Vendramin, pers. comm.), but provide no evidence for a northeastern refuge. The existence of northern glacial refuges, that is, close to the ice sheets, for temperate tree species is controversial (Willis et al. 2000; Carcaillet and Vernet 2001; Stewart and Lister 2001) and unequivocal evidence for the presence of a taxon can only be provided by macrofossils (e.g., Birks and Birks 2000). However, Brewer (2002) reports proportions of ash pollen above one percent in southern Sweden as early as 12,000 ^{14}C bp (Brewer 2002) indicating local presence of ash following the criteria of Huntley and Birks (1983), and genetic character-

istics of Swedish common ash populations were similar to those from southeastern Europe.

A loss of alleles seems to have occurred during recolonization, because total allelic richness was lower in western and central than in southeastern Europe (A_T = 14.4 vs. A_T = 15.8), and 77 alleles remained private to the latter region. This pattern could be due to founder events as well as to an uneven contribution of different refuge populations to recolonization (Bennett et al. 1991; Davis and Shaw 2001). Evidence for founder events in recently recolonized ash populations was weak because gene diversity was high and bottleneck statistics were significant in few populations only. Conversely, an allozyme study in beech reported strong founder events with recolonization, the loss of alleles affecting even the within-population level (Comps et al. 2001). The markers used could partly explain this discrepancy: microsatellites used for ash may have recovered diversity more quickly than allozymes after founder events, because of their higher mutation rates (Jarne and Lagoda 1996), which are supported by the contribution of stepwise-like mutations to differentiation at the scale of Europe in ash. Further, high bottleneck statistics in recently recolonized areas may not only reflect signatures of recent population bottlenecks, but also population admixture. For instance, when two or more populations with identical allele composition but different allele frequencies are mixed, the number of alleles remains the same, but the distribution of allele frequencies becomes more even, producing a relative increase of gene diversity.

Isolation by Distance in the Common Ash

We observed no significant pattern of IBD at the continental scale, probably because strong differentiation at relatively short distances in southeastern Europe masked the IBD pattern with weak among-population differentiation in western and central Europe. The significant IBD patterns within southeastern Europe may reflect colonization from different glacial refuges. It is remarkable that common ash populations from western and central Europe show a significant trend of IBD and at the same time form essentially a single deme, as assessed with BAPS. This indicates that BAPS only recognizes discontinuities in genetic structure, which are apparently absent from this region.

Geographic Variation in Within-Population Level of Inbreeding

Populations from western and central Europe were on average more inbred than those from southeastern Europe (F_{IS} = 0.064 vs. F_{IS} = 0.012, $P \leq 0.001$), which confirms the observation of Morand et al. (2002) in common ash populations from France. Possible causes for this pattern are variation across Europe in the frequency of null alleles, in the mating system, and/or in the level of biparental inbreeding (mating among relatives). Controlled crosses reported in Morand et al. (2002) did not detect any null alleles for loci FEMSATL4, FEMSATL11, FEMSATL19 and M2-30, and we did not observe unreliable amplification reactions at any analyzed locus. However, when locus FEMSATL16 was removed from the data, the trend was much weaker (F_{IS} = 0.034 vs. F_{IS} = 0.006, P = 0.09), suggesting putative local null alleles at this locus. In a previous study on populations from Bulgaria, average values of the inbreeding coefficient (F_{IS} = 0.014) were lower than average kinship coefficients between neighbor trees ($F_{\text{short distance class}}$ = 0.021), indicating that most of the observed inbreeding was due to biparental inbreeding (Heuertz et al. 2001). In contrast, within a large French population, the average inbreeding coefficient ($F_{IS} \approx$ 0.082) was much higher than kinship coefficients between neighbor plants ($F_{\text{short distance class}} \approx$ 0.021, M.-E. Morand-Prieur, pers. comm.). This suggests that in this French population most inbreeding is due either (1) to self-fertilization, although no progenies derived from self-fertilization were found in a parentage analysis performed with genetic markers (M.-E. Morand-Prieur, pers. comm.), or (2) to nonspatially determined biparental inbreeding, such as assortative mating. Self-fertilization has been observed in controlled crosses in France (Morand-Prieur et al. 2003), and self-competent plants may have had a selective advantage over obligate outcrossers during colonization. Alternatively, if some reproductive barriers had evolved between refuge populations, assortative mating could have been enforced in the admixed recolonizing populations. Data on variation in morphology, phenology, and sexual phenotype within populations, as well as direct assessments of outcrossing rates are necessary to distinguish between these two hypotheses. For instance in *Fraxinus lanuginosa*, an androdioecious ash species from Japan, increasing levels of inbreeding attributed to self-fertilization were observed in seeds as the frequency of male individuals decreased within populations (Ishida and Hiura 2002).

Conclusions

Our study demonstrates that microsatellite markers and Bayesian methods are alternative tools to allozymes or chloroplast markers for the analysis of population genetic structure and the inference of population history at broad geographical scale. The highly differentiated common ash populations in southeastern Europe may have been coexisting for a long time without substantial genetic exchanges and are therefore important for conservation of genetic resources. Peculiar genetic structure patterns of yet unknown origin in Swedish common ash populations also suggest particular conservation efforts in this region. In contrast, central and western European populations harbor much more homogeneous resources which presumably result from the processes of recolonization and admixture of previously differentiated gene pools. It remains unclear whether this overall pattern is unique to the common ash or whether it is shared with other tree species yet to be identified using similar analyses of multilocus genetic structure.

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Appendix

Sampling locations and sample sizes for common ash; *n*, sample size per population.

Population	Acronym	Location	Latitude	Longitude	*n*
1 Camolin	IreCa	Ireland	52°36′N	6°28′W	30
2 Avoca	IreAv	Ireland	52°54′N	6°11′W	29
3 Kilmacurra	IreKi	Ireland	53°00′N	6°10′W	30
4 Loch Tay	ScotTa	Scotland	56°35′N	4°03′W	30
5 Saint Gobain	FraGo	France	49°35′N	3°22′E	20
6 La Romagne	FraLr	France	49°40′N	4°19′E	20
7 Bremgarten	SwiB	Switzerland	47°20′N	8°18′E	30
8 Ehrendingen	SwiG	Switzerland	47°29′N	8°21′E	30
9 Eglisau	SwiE	Switzerland	47°35′N	8°31′E	30
10 Tiroler Ache	GerTi	Germany	47°50′N	12°31′E	30
11 Chiemsee	GerCh	Germany	47°48′N	12°31′E	29
12 Freilassing	GerFr	Germany	47°50′N	12°59′E	30
13 Rödjan	SweRoe	Sweden	57°19′N	13°59′E	30
14 Ehd	SweEhd	Sweden	57°14′N	13°59′E	30
15 Akeras	SweAk	Sweden	57°17′N	14°04′E	29
16 Zeimelis	LitZei	Lithuania	56°16′N	24°03′E	30
17 Kaisiadorys	LitKai	Lithuania	54°53′N	24°22′E	30
18 Slovakia	SloPa	Slovakia	48°34′N	19°08′E	26
19 Lillafüred Lusta-Völgy	HunLue	Hungary	48°08′N	20°40′E	28
20 Ljulin monastir	BulLj	Bulgaria	42°39′N	23°11′E	29
21 Vitosha	BulVi	Bulgaria	42°38′N	23°14′E	32
22 Kokalyane monastir	BulMo	Bulgaria	42°33′N	23°26′E	37
23 Tutuleac	RomTu	Romania	46°13′N	24°48′E	31
24 Balota 1	RomBaa	Romania	44°50′N	26°04′E	30
25 Balota 2	RomBab	Romania	44°50′N	26°04′E	30
26 Balota 3	RomBac	Romania	44°50′N	26°03′E	30
27 Golyamoto ravnishte	BulGr	Bulgaria	42°50′N	26°03′E	36
28 Bukatchov chukar	BulBc	Bulgaria	42°50′N	26°03′E	30
29 Elena	BulEl	Bulgaria	42°50′N	26°04′E	30
30 Iri hissar	BulIh	Bulgaria	43°51′N	26°46′E	20
31 Dulovo	BulDu	Bulgaria	43°54′N	26°54′E	32
32 Kodga ormani	BulKo	Bulgaria	43°54′N	26°54′E	36
33 Zli dol	BulZd	Bulgaria	43°50′N	27°03′E	35
34 Hrjauca 1	MolHr	Moldavia	47°18′N	28°12′E	31
35 Hrjauca 2	MolHi	Moldavia	47°17′N	28°15′E	27
36 Hrjauca 3	MolHa	Moldavia	47°20′N	28°17′E	32