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Contribution to the Postglacial History at the Western Margin of *Picea abies*' Natural Area Using RAPD Markers

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RAPD analysis was used (1) to assess the diversity of indigenous *Picea abies* within the French massifs (Alps, Jura and Vosges) in comparison with the Hercyno-Carpathian and Alpine domains (central Europe); and (2) to examine the molecular relationships among provenances of these massifs and domains. One hundred and thirty-seven polymorphic RAPD fragments were screened. Results showed that the phenotypic diversity estimates within the different massifs and domains analysed were similarly high. Factorial correspondence and cluster analyses revealed geographical structuring along a latitudinal gradient among the French massifs and a longitudinal gradient from eastern Europe to France. Provenances from the southern French Alps appeared to be genetically distinct from the others. Hypothetical postglacial pathways into the French massifs are proposed and the putative implications of an additional refuge located in the Tuscan Apennines is discussed.

Key words: *Picea abies*, Norway spruce, phylogeography, phenotypic diversity, correspondence analysis, RAPD.

INTRODUCTION

In both animal and plant species, the genetic variability observed over the natural distribution area is structured by geographical conditions, ecological factors, mating system and also by historical events such as glacial periods (Dumolin-Lapègue *et al.*, 1997). Usually, the observed variability is lower in marginal populations compared to populations in more central positions or located in the vicinity of ancient glacial refuges. Indeed, populations growing at the limits of the natural range generally originate from a few founding individuals and thus have only received a subset of the diversity present in central populations. In addition, they often suffer from a reduced and continuous unidirectional gene flow (Mayr, 1970; Tigerstedt, 1973; Taberlet *et al.*, 1998).

In forest trees, such a decrease in genetic variability towards recently colonized regions (species margins) has been reported for the angiosperms *Fagus sylvatica* (Demesure *et al.*, 1996), *F. crenata* (Tomaru *et al.*, 1997) and *Quercus* sp. (Dumolin-Lapègue *et al.*, 1997), as well as for the gymnosperms *Picea mariana* (Yeh *et al.*, 1986), *Pinus kesiya* (Myburg and Harris, 1997), *P. monticola* (Steinhoff *et al.*, 1983), *P. rigida* (Guries and Ledig, 1982) and *Pseudotsuga menziesii* (Li and Adams, 1989). However, in other gymnosperm species such as *Picea glauca* (Tremblay and Simon, 1989), *P. sitchensis* (Yeh and El-Kassaby, 1980), *Pinus contorta* (Wheeler and Guries, 1982), *P. jeffreyi* (Furnier and Adams, 1986), *P. nigra* (Scaltsoyiannes *et al.*, 1994) and *P. sylvestris* (Prus-Glowacki and Stephan, 1994), marginal and central populations were found to maintain similar levels of genetic diversity, possibly in relation to the ecological amplitude and mating system of these species, the lack of effective barriers to prevent gene flow and the small number of generations since the last deglaciation.

The present investigation is concerned with one of the most common forest trees in Europe, *Picea abies* L. Karst. (Norway spruce), at the western limit of its distribution area. Norway spruce occurs throughout Fennoscandia and European Russia (extreme north excluded), as well as along the major mountains of eastern and central Europe.

The natural range of *Picea abies* is usually divided into three major domains, i.e. the Baltico-Nordic, Hercyno-Carpathian and Alpine domains, which are generally considered to be the result of postglacial recolonization from three putative refuges located in the Moscow area, in the Carpathians and in the Dinaric Alps, respectively (Huntley and Birks, 1983; Schmidt-Vogt, 1986). Morphological and allozyme variations across the whole range have been intensively investigated using IUFRO (International Union of Forestry Research Organizations) provenance-testing programmes (Lagercrantz and Ryman, 1990; Krutzsch, 1992). A clear geographical pattern of genetic variation along a northeastern–southwestern transect was revealed, probably reflecting relatively recent historical events related to the last glaciation in Europe. Moreover, the amount of genetic variation was considerably reduced in central European populations, possibly due to drastic restriction of population size (bottleneck effect) during this glacial period (Lagercrantz and Ryman, 1990; Goncharenko *et al.*, 1995; Lewandowski *et al.*, 1997). In contrast, only a few studies focused on the marginal populations. In the Baltico-Nordic domain, Tigerstedt (1973) found that genetic

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diversity was similar in marginal and central populations. In contrast, Italian populations at the southern limit of the natural distribution area appeared to be more variable than populations in central Europe (Lagercrantz and Ryman, 1990; Giannini *et al.*, 1991; Bucci and Menozzi, 1995). In addition, some populations exhibited specific allelic compositions suggesting a supplementary glacial refuge in the Tuscan Apennines.

The westernmost autochtonous populations studied to date are situated in Switzerland (20 populations: Müller-Starck, 1995), Germany (one population in the Black Forest: Konnert, 1991) and France (one population: Lagercrantz and Ryman, 1990). As in southern Europe, high genetic diversity was observed and several populations appeared to contain specific alleles.

French autochtonous populations of *Picea abies* are located at the western limit of the natural distribution area and are restricted to mountainous regions such as Jura, Alps and Vosges. In the northern Alps and Jura, vast populations are structured around geomorphologic traits such as valleys or plateaux. Conversely, the species is distributed in discontinous fragmented populations in the southern Alps and occurs in disseminated small stands in Vosges.

Little is known about these French provenances, and further investigation is required. In this paper we initiated a comprehensive study of molecular variation among French provenances, using RAPD markers. We first estimated the levels of phenotypic diversity within the French massifs (Alps, Jura and Vosges) and compared them with the two central European domains (Hercyno-Carpathian and Alpine). We then examined the relationships among the French provenances and between the French and central European provenances in order to clarify the postglacial history at the western margin of *Picea abies*' distribution.

MATERIALS AND METHODS

Plant material

RAPD analysis was performed on 90 adult *P. abies* trees distributed throughout their natural range in France. Thirty trees (15 populations) were sampled in an effort to cover the three French massifs: the Alps, Jura and Vosges (Fig. 1). Material from Jura and the Alps was collected in clonal-tests (INRA Champenoux, France), while material from Vosges was collected in the forest. These French trees were compared to 62 trees from both Hercyno-Carpathian (33 trees) and Alpine (29 trees) domains (Fig. 1) previously analysed (Collignon *et al.*, unpubl. res.). These trees were sampled in the international IUFRO provenance test 1964/1968 (Krutzsch, 1992) located in Amance forest (north-east France). The geographical parameters of sampled trees are given in Table 1.

DNA extraction

DNA was isolated from 1 g of needles using the Doyle and Doyle (1987) protocol with 1% 2-mercaptoethanol (v/v) and 1% PVP 40 000 (w/v) added to the CTAB extraction buffer. The extractions were performed using chloroform–octanol (24 : 1) instead of chloroform–isoamyl alcohol (24 : 1) and the resulting DNA pellet was washed with 70% ethanol before the final suspension step. DNA concentrations were determined by comparison with serial dilution of standard lambda DNA.

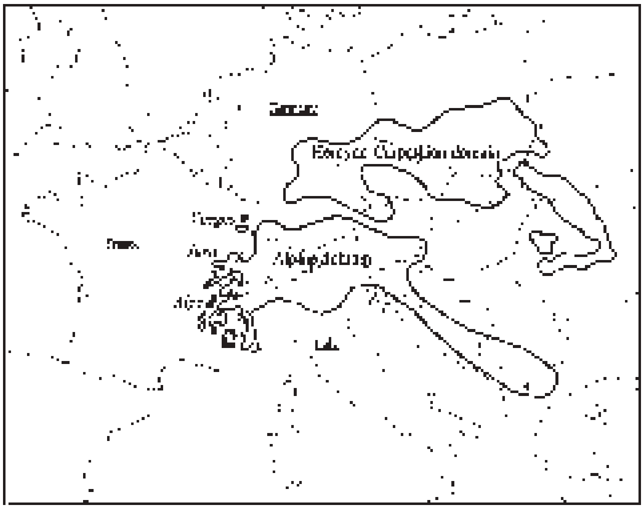

FIG. 1. Geographical location of the three French massifs and the two central Europe domains.

RAPD analysis

DNA amplification was performed according to Williams *et al.* (1990). Thirty-one random decamer primers obtained from Operon Technologies (Alameda, USA) and Genosys Biotechnologies (Cambridge, UK) were selected from the 200 originally tested for clarity and reproducibility of pattern produced (Table 2). Each amplification reaction contained 1× reaction buffer (Gibco, BRL), 200 µM each of dNTPs, 3 mM of $MgCl_2$, 0·4 to 3 µM of primer (Table 2), 12·5 ng of genomic DNA template and 0·4 units of *Taq* DNA polymerase (Gibco, BRL), in a total volume of 15 µl. The reaction mixtures were overlaid with mineral oil. For each primer, amplification of the 152 DNA samples was carried out in 96-well plates using an MJ Research thermal cycler (PTC-100) with the following programme: 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and 10 min at 72°C. Reaction products were checked onto 1·3% agarose gels (two gels per primer), 20 cm wide and 40 cm long (OWL apparatus). 10 µl of each reaction product were mixed with 1 µl loading buffer (50% glycerol, 0·4% bromophenol blue, 0·4% xylene cyanol) and rapidly applied to the mid-part (to avoid smiling) of three 42 well-lines (spaced at regular 13 cm intervals) using a multi-channel pipette. Two wells per line included a 100 bp DNA ladder (Gibco, BRL) as a size marker. The electrophoresis gel was run at 5 V cm^{-1} for 4 h in 0·5 × TBE buffer (45 mM Tris base, 45 mM boric acid and 1mM EDTA, pH 8·0). The three parts of each gel were stained for 30 min in ethidium bromide (0·5 µg ml^{-1}) and photographed under UV light.

TABLE 1. *Geographical parameters of sampled individuals of* Picea abies

Tree code	Location	Latitude (N)	Longitude (W)	Tree code	Location	Latitude (N)	Longitude (W)
Vosges (△)							
1 2	Gérardmer (FC)	48°06	6°53	17 18	Gérardmer (FD)	48°06	6°52
3 4	Gérardmer (FD)	48°03	6°52	19 20	Gérardmer (FD)	48°04	6°47
5 6	Gérardmer (FD)	48°04	6°58	21 22	Soultzeren (FC)	48°03	7°04
7 8	Gérardmer (FD)	48°05	6°48	23 24	Tholy (FC)	48°03	6°46
9 10	Jurassupt (FC)	48°06	6°48	25 26	Réhaupal (FC)	48°06	6°45
11 12	La Bresse (FC)	48°03	6°57	27 28	Vologne (FD)	48°07	6°51
13 14	Val de Senones (FD)	48°30	7°00	29 30	Haute-Meurthe (FD)	48°07	6°57
15 16	Stosswhir (FC)	48°03	7°02				
Alps (■)							
31 32	Chartreuse (FD)	45°22	5°45	47 48	Chamrousse (FC)	45°06	5°53
33 34	Aussois (FC)	45°45	6°44	49 50	Autrans (FC)	45°10	5°30
35 36	Lantosque (FD)	44°00	7°19	51 52	Verdache (FC)	44°16	6°20
37 38	Morzine (FC)	46°11	6°45	53 54	St-Et.-en-Dévoluy (FC)	44°42	5°56
39 40	Montriond (FC)	46°16	6°44	55 56	Chamonix (FD)	46°00	6°52
41 42	Névache (FC)	45°01	6°34	57 58	Lully (FC)	46°20	6°23
43 44	St Martin Vésubie (FC)	44°04	7°15	59 60	Châtel (FC)	46°16	6°50
45 46	Séez (FC)	45°37	6°49				
Jura (▲)							
61 62	Bonnétage (FDC	47°11	6°45	79 80	Champfromier (FC)	46°13	5°52
63 64	Esserval-Tartre (FC)	46°50	6°05	81 82	Massacre (FIC)	46°25	6°03
65 66	Grande-Côte (FC)	46°47	6°15	83 84	Bois D'Amont (FC)	46°29	6°05
67 68	FD de Levier (FD)	46°57	6°04	85 86	Cuvier (FC)	46°50	6°04
69 70	La Fuvelle (FD)	46°27	6°19	87 88	Massif du Risoux	46°29	6°05
71 72	Massacre (FIC)	46°24	6°02	89 90	Risol (FD)	46°39	6°13
73 74	Mignovillard (FC)	46°45	6°12				
75 76	Plan des Cosaques (FC)	46°29	6°06				
77 78	La Joux (FD)	46°49	6°00				
Hercyno-Carpathian domain (○)							
91	Poland	49°5	19°1	108	Slovakia	49°0	19°9
92	Poland	50°5	21°3	109	Slovakia	49°0	18°9
93	Romania	46°7	22°7	110	Slovakia	49°6	18°8
94	Romania	46°9	25°4	111	Slovakia	49°0	20°2
95	Romania	47°8	25°5	112	Slovakia	49°1	19°3
96	Czech Republic	49°8	17°3	113	Germany	50°7	10°7
97	Czech Republic	49°5	13°0	114	Germany	48°9	13°3
98	Czech Republic	50°2	16°5	115	Germany	49°5	12°3
99	Czech Republic	50°3	17°3	116	Germany	48°9	13°3
100	Czech Republic	48°9	13°8	117	Germany	49°1	10°1
101	Czech Republic	49°5	13°6	118	Germany	50°7	13°3
102	Czech Republic	49°5	17°1	119	Germany	48°6	13°4
103	Czech Republic	48°8	14°8	120	Germany	49°1	13°2
104	Czech Republic	50°1	12°3	121	Germany	49°9	8°9
105	Czech Republic	49°2	17°8	122	Germany	51°8	10°8
106	Czech Republic	49°3	18°3	123	Austria	48°6	15°5
107	Czech Republic	49°2	17°8				
Alpine domain (●)							
124	Germany	48°1	10°5	139	Serbia	43°9	19°5
125	Germany	48°0	9°7	140	Austria	46°8	14°1
126	Germany	47°8	8°5	141	Austria	47°6	15°8
127	Germany	47°7	12°9	142	Austria	47°0	12°5
128	Germany	47°5	11°1	143	Austria	47°2	10°1
129	Germany	48°1	12°2	144	Austria	47°3	13°2
130	Germany	48°0	8°5	145	Austria	47°1	13°8
131	Germany	48°0	10°2	146	Austria	47°6	15°3
132	Germany	48°3	8°9	147	Austria	47°4	15°2
133	Germany	48°7	10°3	148	Austria	47°5	13°9
134	Germany	48°1	10°6	149	Austria	46°8	14°1
135	Germany	48°0	9°2	150	Austria	47°0	14°5
136	Germany	48°1	10°9	151	France	46°8	6°1
137	Switzerland	46°5	8°5	152	France	44°0	7°3
138	Switzerland	46°5	8°8				

TABLE 2. *Nucleotide sequences of the 31 selected primers, concentration used in the PCR reaction (C) and number of polymorphic fragments scored (Nf)*

Primers	5′→3′	C	Nf	Primers	5′→3′	C	Nf
OPA02	TGCCGAGCTG	0·4	3	OPD12	CACCGTATCC	0·6	2
OPA05	AGGGGTCTTG	0·4	1	OPD20	ACCCGGTCAC	0·6	4
OPA10	GTGATCGCAG	0·4	4	OPE10	CACCAGGTGA	1·0	5
OPA11	CAATCGCCGT	0·4	7	OPH10	CCTACGTCAG	3·0	6
OPB04	GGACTGGAGT	0·4	6	DECA0502	AGCCGCTATC	0·4	4
OPB06	TGCTCTGCCC	0·4	1	DECA0509	AGGAGTAGGC	0·4	1
OPB08	GTCCACACGG	0·4	7	DECA0514	AGTCACCGCT	0·4	7
OPB14	TCCGCTCTGG	0·6	3	DECA0519	ATCAACCGGC	1·0	7
OPC02	GTGAGGCGTC	0·4	3	DECA0521	ATCCGGACCT	0·6	7
OPC05	GATGACCGCC	0·4	5	DECA0522	ATCGCCCTGT	0·4	3
OPC06	GAACGGACTC	0·4	3	DECA0549	CACTCGTCGT	1·0	3
OPC11	AAAGCTGCGG	0·4	7	DECA0550	CAGAAACCGC	0·4	3
OPC15	GACGGATCAG	0·4	2	DECA0561	CATACGGCCT	0·4	9
OPD01	ACCGCGAAGG	0·4	3	DECA0569	CCACGAATGC	0·4	5
OPD03	GTCGCCGTCA	0·4	8	DECA0573	CCAGCCGAAT	0·4	7
OPD05	TGAGCGGACA	0·4	1				

Data analysis

Only clear and polymorphic fragments were used for data analysis. These fragments were scored independently as present (1) or absent (0) and a binary data matrix was constructed. Fragment frequencies were calculated for each of the massifs and domains studied, and a Fisher exact test was applied. Factorial correspondence analysis (FCA) based on the χ^2 distances and especially adapted for qualitative data (Thioulouse *et al*., 1997), was used to obtain synthetic pictures of phenotypic molecular variation. An ascendant hierarchical classification (AHC) using the variance aggregation of Ward (1963) and Jaccard's distances (Jaccard, 1908) was also performed to aggregate individuals into major clusters. FCA and AHC analyses were performed using the ADE-4 programme (Thioulouse *et al*., 1997).

An analysis of molecular variance (AMOVA: Excoffier *et al*., 1992) was performed on a matrix of squared standard euclidean distances. Differentiation among massifs and/or domains and among the French massifs only, were inferred from Φst value. Significance values were obtained using resampling techniques with 500 permutations.

Phenotypic diversity within each massif and/or domain was quantified by the Shannon's index (King and Schaal, 1989). Estimates of diversity were calculated (1) for each locus *i*: $h'_i = -\Sigma\pi \log_2 \pi$, where π is the phenotypic frequencies; and (2) averaged across loci: $H' = (1/L) * \Sigma h'_i$, where L is the number of loci. Diversity was also partitioned within (Hpop/Hsp) and between [Ist = (Hpop − Hsp)/Hsp] components. Hpop provides a measure of the average diversity within massifs and/or domains and Hsp a measure of the diversity within species.

RESULTS

RAPD variation

A total of 137 reproducible and polymorphic fragments was produced by the 31 primers selected (Table 2), 130 being polymorphic among French provenances; none were found to be massif- or domain-specific. However, most of the fragments exhibited significant frequency variations ($P < 0·05$) between massif(s) and/or domain(s). The number of these informative fragments ranged from a minimum of 16 between the Hercyno-Carpathian and Alpine domains to a maximum of 49 between the Hercyno-Carpathian domain and the French Alps massif (Table 3). Considering the French massifs exclusively, 48 informative fragments were detected, enabling discrimination between the Alps and Vosges (29/48 fragments), the Alps and Jura (35/48), and Vosges and Jura (36/48) massifs.

Factorial correspondence analysis (FCA) and cluster analysis

A FCA involving all provenances is shown in Fig. 2. The first two axes explained 13% of the total variation. Clear discrimination between the central Europe and French provenances was observed along axis 1, which was found to be highly correlated with longitude ($r = 0·629$, $P < 0·001$).

The French provenances located in the right side of the plane were organized along axis 2. The cluster of Jura provenances was fairly well separated from the more

TABLE 3. *Number of fragments with significant frequency differences between each pairwise combination of domains (1,2) and/or French massifs (3,4,5)*

	1	2	3	4	5
1 Hercyno-Carpathian Domain	—				
2 Alpine Domain	16 (6)	—			
3 Vosges	41 (11)	38 (12)	—		
4 French Alps	49 (19)	42 (17)	29 (11)	—	
5 Jura	43 (12)	33 (11)	36 (10)	35 (12)	—

Fisher exact tests were performed on fragment frequencies with either $P < 0·05$ or $P < 0·01$ (in parentheses).

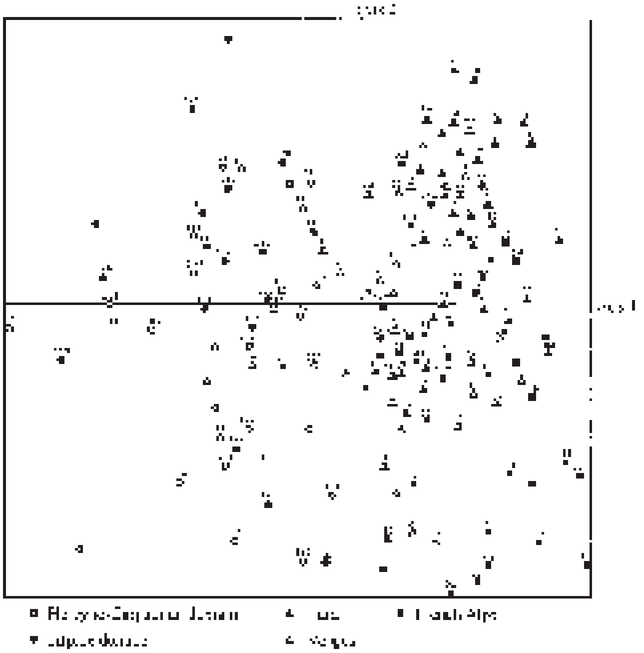

FIG. 2. Distribution of 152 spruces in the plane (1,2) of the factorial correspondence analysis (FCA) computed from 137 polymorphic RAPDs. Tree codes are indicated in Table 1.

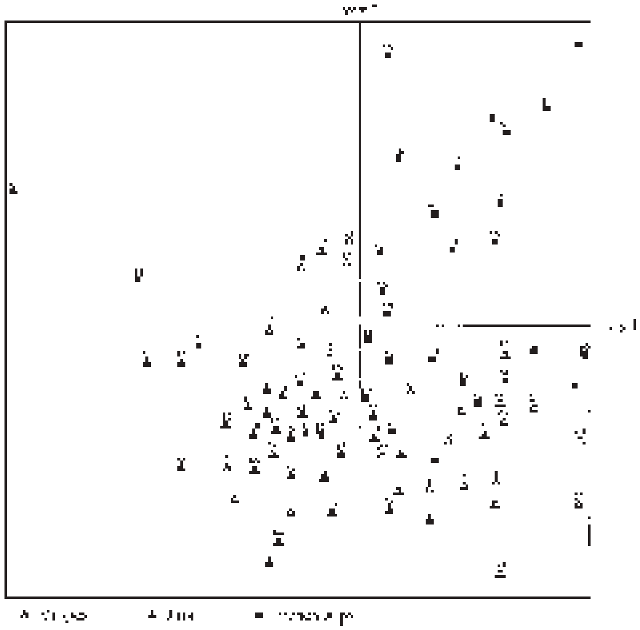

FIG. 3. Distribution of 90 French spruces in the plane (1,2) of the factorial correspondence analysis (FCA) computed from 130 polymorphic RAPDs. Tree codes are indicated in Table 1.

scattered French Alpine provenances. In contrast, the Vosges provenances were interspersed among the other massifs and domains, especially the French Alps and, to some extent, central Europe.

Correlation analysis of French tree co-ordinates on axis 2 with geographical parameters revealed variation along a latitudinal gradient ($r = 0{\cdot}360$, $P < 0{\cdot}05$). However, no geographical structure was detected within each individual massif.

Similar results were obtained using different hierarchical grouping methods such as UPGMA and Ward clustering based on Nei (1987) or Jaccard's distances (data not shown).

A more precise investigation of the relationships between the French Alps, Jura and Vosges massifs was performed in two additional FCA using either the 130 polymorphic fragments (Fig. 3) or the 48 informative fragments (Fig. 4). The first two axes explained 8·0 and 20·5% of the total variation in each FCA, respectively. As found previously, the discrimination between the Jura and Alps massifs was obvious and mainly obtained along axis 1. Concerning the Vosges massif, many provenances (60%) clustered apart from the Jura and, to a certain extent, from the Alpine provenances in the right lower side of the planes. This separation is particularly evident in Fig. 4.

Correlation analysis between tree co-ordinates either on the first or the second axis of the FCA involving the 130 polymorphic fragments (Fig. 3) and geographical parameters was performed. Latitudinal structure along axis 2 was detected when all provenances from the three French massifs were pooled ($r = 0{\cdot}513$, $P < 0{\cdot}001$). Remarkably, this latitudinal gradient was also detected within the French Alps massif ($r = -0{\cdot}480$, $P < 0{\cdot}01$). The westernmost provenances (Grande Chartreuse, Autrans and Chamrousse) appeared to be close to the Jura massif cluster, the northern provenances (Chamonix, Châtel, Montriond and Lully) were close to the Vosges massif, while the southern provenances (Saint-Martin-Vésubie, Névache, Verdache, Saint-Etienne-en-Dévoluy and Lantosque) clustered separately in the right top of the plane with the exception of tree number 36.

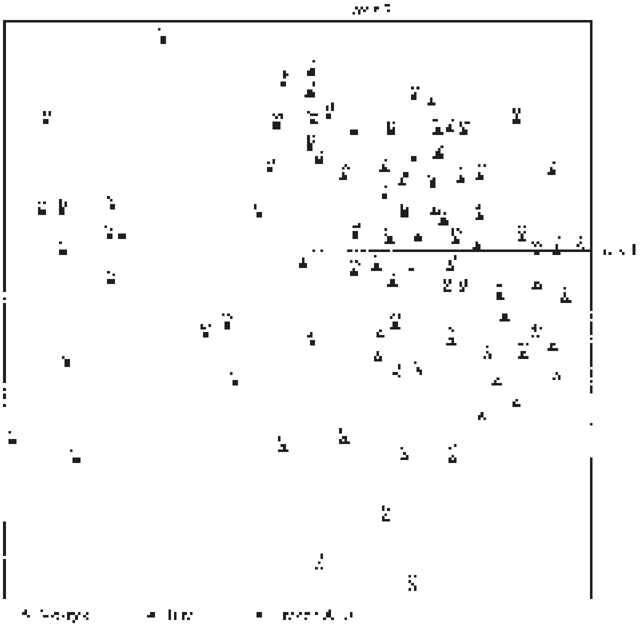

FIG. 4. Distribution of 90 French spruces in the plane (1,2) of the factorial correspondence analysis (FCA) computed from 48 informative RAPDs. Tree codes are indicated in Table 1.

Hierarchical grouping based on the Jaccard's distances resolved the individuals into four main clusters (Fig. 5). The first contained all the southern provenances from the French Alps, the second contained trees from the Jura provenances, the third was composed of a mix of the northern Alps and Jura provenances and the last consisted of almost all the Vosges provenances.

Phenotypic diversity and differentiation

The 137 polymorphic fragments were used to calculate the phenotypic diversity and differentiation estimates (Tables 4 and 5). Shannon's diversity index (H′) within each massif and domain showed a high level of diversity (Table 4). H′ ranged from 0·675 to 0·718. The observed between-massif and/or -domain differences were not significant.

Low levels of differentiation among massifs and/or domains were revealed either with AMOVA (Φst = 0·063) or Shannon's index (Ist = 0·069). These values were lower when the analysis was restricted to the three French massifs (Table 5).

Table 4. *Shannon's diversity index within massifs and domains calculated from 137 RAPD markers*

Domains or massifs	H′ ± s.e.
Hercyno-Carpathian domain	0·705 ± 0·009
Alpine domain	0·711 ± 0·009
French Alps	0·718 ± 0·010
Jura	0·675 ± 0·010
Vosges	0·696 ± 0·009

Table 5. *Diversity partitioning estimated from variance analysis and Shannon's index*

	AMOVA	Shannon's index		
	Φst	Hpop	Hsp	Ist
Among massifs and/or domains	0·063	0·701	0·753	0·069
Among French massifs	0·042	0·696	0·736	0·054

DISCUSSION

Diversity and differentiation

In this RAPD investigation, *Picea abies* appears to maintain a high level of diversity as observed in most woody plants (Hamrick *et al*., 1992). Coniferous species in particular often combine characteristics such as large and continuous populations, long life cycles, outcrossing breeding systems, and relatively long distance pollen and seed movement that ensure the preservation of diversity. In addition, the level of diversity should largely rely on the evolutionary history of the species. Species expanding from large populations are indeed expected to maintain more variation than species originating from a limited number of individuals (Hamrick *et al*., 1992).

The values of diversity indices obtained in the five large areas sampled (Hercyno-Carpathian and Alpine domains, Vosges, Jura and French Alps) are very similar (0·675 to 0·718), and, of the same magnitude as those estimated from RAPDs within several French populations (Collignon *et al*., unpubl. res.). Theoretically, genetic variation is supposed to be lower in marginal populations than in more central populations (Levin, 1970; Mayr, 1970; Lawton, 1993) because of genetic drift promoted by reduced gene flow and founder effects (Nei *et al*., 1975; McCommas and Bryant, 1990). However, efficient wind pollen- and seed-dispersal associated with a high level of outcrossing in conifers can maintain intense gene flow in marginal populations (Tigerstedt, 1973) resulting in similar levels of diversity in both marginal and central populations (Yeh and El-Kassaby, 1980; Wheeler and Guries, 1982; Furnier and Adams, 1986; Tremblay and Simon, 1989; Scaltsoyiannes *et al*., 1994; Prus-Glowacki and Stephan, 1994). In addition, there has probably not been enough time since the recent expansion of *Picea abies* in the French area (4500 years ago: de Beaulieu, 1977; Wegmüller, 1977; Kalis, 1984; de Beaulieu *et al*., 1994) to allow effective divergence of marginal populations by selection or drift.

The partitioning of diversity revealed that 93·1% of the variation was maintained within the central Europe domains and/or French massifs. These results are consistent with the general observation that woody species and especially conifers maintain most of their variation within populations (Giannini *et al*., 1991; Stoehr and El-Kassaby, 1991; Hamrick *et al*., 1992; Müller-Starck *et al*., 1992; Krutovskii and Bergmann, 1995). Φst (AMOVA), considered to be the less unbiased differentiation coefficient for RAPD analysis (Isabel *et al*., 1995, 1999), is of the same magnitude as the other phenotypic coefficient (Shannon's index). Although large areas (domains and massifs) have been considered as populations, the differentiation coefficient for the total sampled area (6·3%) is in the same order as that generally expected from single populations of conifers and outbreeding species such as *Picea abies* (Hamrick *et al*., 1992). Lagercrantz and Ryman (1990) calculated the genetic differentiation of 70 populations of *Picea abies* distributed over the whole European range to be 5%, while Giannini *et al*. (1991) investigated nine Italian populations and found a Gst value of 4·4%. More recently, Müller-Starck (1995) estimated the genetic differentiation of 20 Swiss populations to be 4·3%.

Geographical pattern of molecular variation in relation to the presumed postglacial re-immigration routes

RAPD data have been treated in a qualitative fashion and subjected to descriptive analyses such as cluster or factorial analysis. This approach is particularly interesting to correlate molecular variations with geographical parameters such as latitude, longitude or altitude in order to reveal possible clinal structuring. The two principal

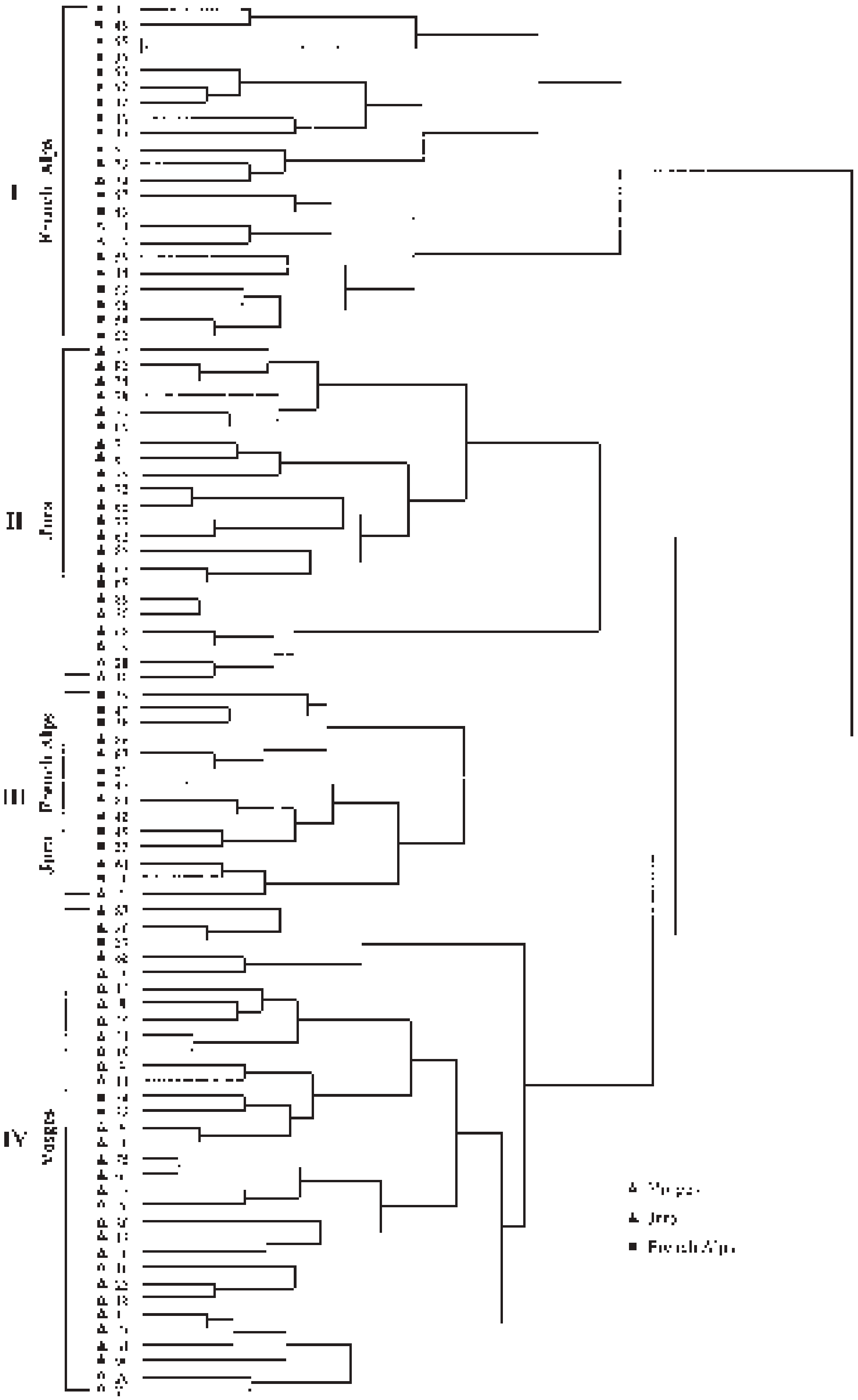

FIG. 5. Ward hierarchical aggregation of 90 French spruces based on Jaccard's distances inferred from 130 RAPD markers. Tree codes are indicated in Table 1.

synthetic variables stand for a low proportion of the total variation and the inertia rate of the principal axes is lower than 10%. This situation contrasts markedly with other molecular studies using RAPDs to evaluate genetic variation between species (Chalmers *et al.*, 1994) or between herbaceous plants accessions (Heun *et al.*, 1994; Orozco-Castillo *et al.*, 1994) where the two first axes account for more than 45% of the variation. This could be related in part to the large number of random markers analysed and the genomic characteristics of the species studied. Conifers do have large genomes (29 pg/2C in *Picea abies*: Dhillon, 1987) which are mainly composed of repetitive and potentially variable non-encoding DNA (75%). Thus, the total molecular variation detected is high and difficult to summarize on a factorial plane. Given the large number of loci analysed (137), it can be assumed that the variation represented by the two first axes of the FCA is significant (Lebart *et al*., 1997).

On the basis of both FCA and clusters analyses, two main groups can be distinguished, one including the central Europe provenances and the second the French provenances. This pattern reveals a clear longitudinal gradient across the large area sampled and supports the hypothesis of re-immigration in an east–west direction from eastern Europe to France. This major migration path is commonly accepted from pollen data analyses (Huntley and Birks, 1983) and from studies of morphological and isozyme variations as well (Lagercrantz and Ryman, 1990). During the Holocene, the re-invasion of Europe by *Picea abies* probably occurred from glacial refuge zones located in the Moscow area and in the eastern part of the major European mountains such as the Carpathians and the Dinaric Alps. Spruce expanded steadily, but relatively slowly, along the European mountains at rates of 80–240 m year^{-1}, reaching eastern Switzerland 6000–4800 years ago and the region around Lake Geneva, as well as the South Swiss Jura, 4800–2800 years ago (Firtion, 1950; Wegmüller, 1977; Burga, 1988). Thus, the spread of *Picea abies* within France probably started in the Jura mountains and then continued towards the Alps in a southerly direction and the Vosges in a northerly direction.

Remarkably, we found evidence of latitudinal gradients at the western limit of the natural range of *Picea abies* as well as clear molecular relationships between Vosges and Jura spruces and between Jura and northern alpine spruces, supporting, for the first time, the main re-immigration paths from the Jura mountains to the French Alps and Vosges using molecular markers (route 1, Fig. 6). However, the high molecular variability observed in Vosges and, to an even greater extent in the French Alps compared with the Jura mountains, suggested additional recolonization pathways in these massifs. Concerning the Vosges, two dates of possible expansion were proposed from pollen data, either 3500 years BP from the Jura mountains or 2500 years BP from the Black Forest and/or Swiss plain (Kalis, 1984). Some Vosges trees were interspersed with central European provenances on the factorial plane. This strongly suggests possible migratory paths straight from central Switzerland and/or the Black Forest, which are only 75 km away from the Vosges (route 2, Fig. 6).

The high level of diversity observed in the Vosges could also be explained by anthropogenic actions. Indeed, the small and disseminated indigenous stands in this massif are exposed to introgression from surrounding artificial populations which consist mainly of allochtonous material.

In the French Alps, latitudinal structuring is evident and three regions could be discerned. The first one consists of the westernmost region (Grande Chartreuse) which is genetically close to the Jura, the second consists of the northern provenances (Chamonix region) and the third of the southernmost provenances. These southern French Alps differed remarkably from the others in their molecular pattern and are known to be especially adapted to Mediterranean climate (Bouvarel, 1961). Such adaptive differentiation cannot be simply explained by genetic drift in a recolonization scheme initiated from the Jura mountains because the number of generations is generally considered too small (about 50 generations) for new mutations to have a noticeable impact (Maruyama and Fuerst, 1985; Leisica and Allendorf, 1995).

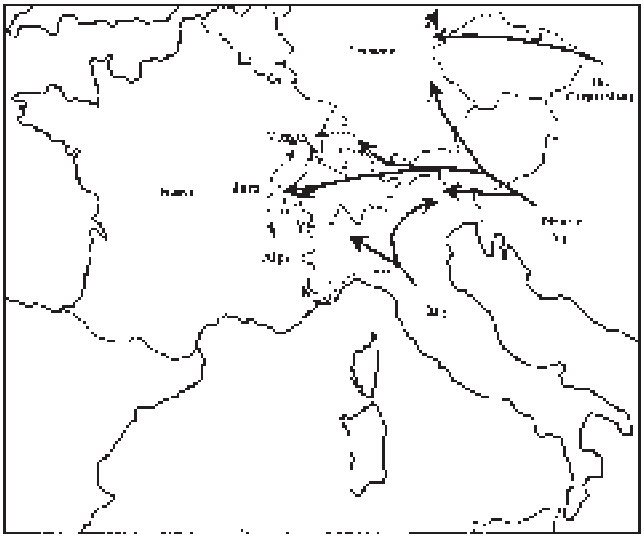

FIG. 6. Presumed postglacial re-immigration paths of *Picea abies* in central Europe. Black arrows represent the pathways reported by Schmidt-Vogt (1976, 1986), Giannini *et al.* (1991) and Burga (1988) studies. The numbered black arrows represent the paths deduced from our RAPD data (see Discussion).

Interestingly, the genetic differences between some Italian (Borghetti *et al*., 1988; Giannini *et al*., 1991) and Swiss populations (Müller-Starck, 1995) in their allelic composition suggested a postglacial re-immigration route from a putative additional refuge located in the Apennines (Italy) (Giacomini, 1958; Ferrarini, 1977; Magini *et al*., 1980) and mentioned by Krutzsch (1992). Like many other species, such as *Abies alba* and *Quercus* sp. (Konnert and Bergmann, 1995; Dumolin-Lapègue *et al*., 1997), *Picea abies* could have skirted the Mediterranean sea from the Tuscan Apennines and recolonized the southernmost French Alps before the dates generally proposed (3000 years BP) (route 3, Fig. 6). This hypothesis is clearly supported by the palynological observations of de Beaulieu (1977) who indicated the presence of *Picea abies* in the Mercantour (southeastern Alps) 4000–4500 years ago. Interestingly, this author further invoked a relictual glacial refuge in the southern French Alps.

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