Absence of geographical structure of chloroplast DNA variation in willow, *Salix caprea* L.

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In the present study, we have used PCR-RFLP markers to investigate the chloroplast DNA variation in 24 European populations of *Salix caprea* L. A subset of these populations has also been analysed with chloroplast microsatellites. The main feature of both markers is the absence of a clear geographic structure ($G_{ST(PCR-RFLP)} = 0.090$, $G_{ST(microsatellites)} = -0.017$) and high levels of variation within populations. This lack of phylogeographic structure in *S. caprea* is suggested to be the consequence of the joint action of several factors: (i) presence of intermediate latitude refugia with large population sizes during the last glacial maximum, (ii) a high speed of recolonisation and dispersal

ability, (iii) a high mutation rate and (iv) extensive hybridisation with other willow species. In addition to the *S. caprea* samples, a limited number of individuals from several other *Salix* species were also analysed with PCR-RFLP: *S. cinerea*, *S. aurita*, *S. purpurea*, *S. atrocinerea*, *S. appendiculata*, *S. elaeagnos*, *S. fragilis* and *S. alba*. Many of the haplotypes found in *Salix caprea* were also detected in *S. cinerea*, *S. aurita*, *S. purpurea*, *S. atrocinerea* and/or *S. appendiculata* but not in *S. alba*, *S. elaeagnos* or *S. fragilis*. Our data suggest that hybridisation and gene flow have occurred within these two groups but not between them.

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Introduction

Cytoplasmic markers have undoubtedly been useful in recovering post-glacial migration routes for a range of organisms (Taberlet *et al.*, 1998; Hewitt, 2000). In trees, recolonisation patterns have been unravelled in, among others, *Quercus* (Petit *et al.*, 2002), *Fagus* (Demesure *et al.*, 1996) and *Picea* (Vendramin *et al.*, 2000) by combining information from chloroplast DNA and fossil pollen maps. In most cases, the fixation index, a classical measure of the genetic differentiation among populations, had a very high value. A recent review (RJ Petit, unpublished) shows that the average fixation index for chloroplast DNA in the angiosperm species that have been studied so far is about 76%, the lowest value being 25%. These values are fairly high, although not completely unexpected. Chloroplast DNA is generally maternally inherited in angiosperms (Rajora and Dancik, 1992; Dumolin *et al.*, 1995) and has therefore a smaller effective population size than nuclear DNA (1/4 in dioecious species and 1/2 in monoecious ones). Maternal inheritance also means that chloroplast DNA is only dispersed through seeds. Smaller effective population sizes and restricted dispersal thus imply that genetic differentiation among populations is generally more pronounced for chloroplast DNA than nuclear DNA.

There are however reasons to believe that the clear geographic structure obtained, for example, in oaks or

beech, may not be found in other species, making it difficult to decipher their evolutionary history in relation to climatic changes affecting their distribution. First, oaks and beech are not cold tolerant and were therefore confined to refugia in Southern Europe during the last glacial maximum (LGM). These refugia were probably of limited size and isolated from each other, allowing for population differentiation to develop among refugia. Other species, such as birches, pines, spruces or larches, are cold tolerant and were apparently able to survive the LGM at higher latitudes (Willis *et al.*, 2000). Macroscopic charcoal remains of *Salix* dated to about 23 000 ¹⁴C years BP have been found in Hungary, indicating survival at these latitudes, but it has not been possible to determine the exact *Salix* species (Willis *et al.*, 2000). Second, the recolonisation process for the species studied so far, especially beech, was relatively slow when compared to the rate at which species such as birch (*Betula*) or hazel (*Corylus*) may have colonised northern Europe as the ice retreated (Huntley and Birks, 1983; Bennett, 1997). Third, the mutation rate must be sufficiently low so as not to erase the phylogeographic information. Chloroplast mutation rates are poorly known, but may vary among species (Bousquet *et al.*, 1992). Fourth, while hybridisation in oaks apparently occurred during the recolonisation process of northern Europe, it remained confined to a few species. A more extensive hybridisation pattern could potentially weaken the phylogeographic signal carried by a given species.

Sallow is a cold-tolerant pioneer species which occurs in a large range of habitats and is one of the few willow species able to grow in forest understories (Rameau *et al.*, 1989). Its natural range covers much of Europe except the

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most northern and southern parts (Tutin *et al*, 1964) and it also extends into Asia. Many members of the *Salix* genus are known to hybridise with each other, and *Salix caprea* in particular hybridises with a number of other *Salix* species (Tutin *et al*, 1964; Meikle, 1984). The extent of gene flow among natural populations remains, however, unknown. The fossil pollen record is difficult to interpret since the pollen of different *Salix* species cannot be distinguished (Huntley and Birks, 1983). Willow is certainly cold resistant enough to be able to survive at high latitudes during the LGM. Finally, willow is dioecious increasing the likelihood of detecting high levels of differentiation for chloroplast DNA markers.

The main objective of this study is to investigate the phylogeographic structure of genetic variation in *S. caprea* L. across Europe. To do so, chloroplast DNA variation was studied in 24 *S. caprea* populations distributed across a large part of the species' natural range. In addition, other willow species that could potentially hybridise with *S. caprea* were included since hybridisation was suspected to be important in *S. caprea*.

Material and methods

Sampling

S. caprea was sampled in 24 locations across Europe (Table 1 and Figure 1). Most samples consisted of leaves, although buds were collected from populations in Croatia, Romania, Slovakia and Greece. In each location samples from about 10 individuals were gathered, making a total sample of 240 *S. caprea* individuals. A

more limited number of samples were also collected in populations of *S. cinerea*, *S. aurita*, *S. purpurea*, *S. atrocinerea*, *S. appendiculata*, *S. elaeagnos*, *S. fragilis* and *S. alba* (31, 7, 5, 11, 5, 2, 1 and 21 individuals, respectively; Table 1). According to Skvortsov (1968) *S. caprea*, *S. cinerea*, *S. aurita*, *S. purpurea*, *S. atrocinerea* and *S. appendiculata* all belong to the subgenus *Vetrix*, subsection *Vetrix*, while *S. fragilis* and *S. alba* belong to subgenus *Salix*, subsection *Salix* (*S. elaeagnos* was not included in this study). Most of these species have a wide distribution across Europe but *S. atrocinerea* is restricted to western Europe, *S. appendiculata* to the Alpine region, Italy and the Balkans, and *S. elaeagnos* is only found in the southern half of Europe (Jalas *et al*, 1996). To avoid sampling clones or close relatives, sampled individuals of the same species were separated by at least 200 m.

The leaf samples were frozen in liquid nitrogen in the field or kept fresh until they could be frozen in a -80°C freezer. These samples, if we did not collect them ourselves, were sent to us by express mail frozen in dry ice. The buds were collected as whole branches, stored at about 4°C and then sent by express mail to our lab. They were then separated from the branches, frozen and subsequently stored at -80°C .

DNA extraction

DNA was extracted with a protocol adapted from Doyle and Doyle (1990). Leaf or bud tissue was ground in liquid nitrogen and about 10 mg of this powder was added to 800 µl of extraction buffer. The extraction buffer used consists of alkyltrimethylammonium bromide (20 g/l), EDTA (0.02 M), Tris HCl (0.1 M), NaCl (1.4 M)

Table 1 Sampling locations where *Salix caprea* and in some cases also other *Salix* species have been collected

Population	Country	Code	Longitude	Latitude	<i>Salix caprea</i>	SCI	SAU	SPU	SAT	SAP	SEL	SFR	SAL
Glen Affric	Great Britain	1	-4.83	57.32	8	—	—	—	—	—	—	—	—
Lake District	Great Britain	2	-3.00	54.27	10	—	—	—	—	—	—	—	—
Dean	Great Britain	3	-2.65	51.83	10	—	—	—	—	—	—	—	—
Tofta	Sweden	4	11.7	57.87	11	3	—	—	—	—	—	—	—
Stenshuvud	Sweden	5	14.25	55.65	11	—	—	—	—	—	—	—	—
Halltorps Hage	Sweden	6	16.53	56.75	9	3	3	—	—	—	—	—	—
Seebachtal	Germany	7	8.12	47.88	11	—	—	—	—	5	—	—	—
Bovenden	Germany	8	10.05	51.57	10	—	—	—	—	—	—	—	—
Kelheim	Germany	9	11.83	48.93	9	—	—	—	—	—	—	—	—
Fontainebleau	France	10	2.67	48.42	8	6	1	—	—	—	—	—	1
Chizé	France	11	-0.40	46.14	9	—	—	—	—	—	—	1	—
Seillon	France	12	5.00	46.00	10	3	3	—	—	—	—	—	3
Valbonne	France	13	4.55	44.24	10	—	—	1	—	—	1	—	5
Valdu Niellu, Aitone	Corsica, France	14	8.88	42.28	13	6	—	4	1	—	1	—	—
Devesa da Rogueira	Spain	15	-7.08	42.25	9	—	—	—	—	—	—	—	—
Valle de Salzar	Spain	16	-0.92	42.83	10	—	—	—	—	—	—	—	—
Montejo de la Sierra	Spain	17	-3.50	41.13	—	—	—	—	10	—	—	—	—
Casertinesi	Italy	18	11.8	43.78	10	10	—	—	—	—	—	—	9
Bresciano	Italy	19	10.88	45.8	10	—	—	—	—	—	—	—	—
Calabria	Italy	20	16.58	39.00	10	—	—	—	—	—	—	—	—
Mt Medvencia	Croatia	21	15.95	45.87	10	—	—	—	—	—	—	—	—
Savarsin	Romania	22	22.23	46.02	8	—	—	—	—	—	—	—	3
Boki	Slovakia	23	19.12	48.57	13	—	—	—	—	—	—	—	—
Mt Voras	Greece	24	23.73	40.56	11	—	—	—	—	—	—	—	—
Voronez reserve	Russia	25	39.5	51.83	10	—	—	—	—	—	—	—	—
Σ					240	31	7	5	11	5	2	1	21

The numbers of individuals analysed from each population and species are given. The species abbreviations represent the following species: SCI=*Salix cinerea*, SAU=*S. aurita*, SPU=*S. purpurea*, SAT=*S. atrocinerea*, SAP=*S. appendiculata*, SEL=*S. elaeagnos*, SFR=*S. fragilis*, SAL=*S. alba*.

Figure 1 Geographic distribution of chloroplast PCR-RFLP haplotypes in *S. caprea*.

and PVP (10 g/l), to which 1,4-dithiothreitol was added just before use.

PCR-RFLP analysis

PCR conditions: Primers from Dumolin-Lapégue *et al* (1997b) and Démesure *et al* (1995) (CS, fMA, AS, VL, HK, K1K2, CD, DT, TF) were used for the amplification of nine different parts of the chloroplast genome. The PCR was performed in 1 × PCR buffer (Fermentas), 1.8 mM MgCl₂, 0.2 mg/μl BSA (Fermentas), 0.1 mM dNTP (Roche), 0.2 μM of each primer and 0.016 U/μl *Taq* DNA polymerase (Fermentas). A touch-down PCR program was used for the amplification: 4 min at 94°C, 14–20 cycles of 45 s at 94°C, 45 s at 60–48°C decreasing 0.5°C with each cycle to 53–40°C, 3–6 min at 68–72°C, then 15–20 cycles of 45 s at 94°C, 45 s at 53–40°C, 3–6 min at 68–72°C and finally 10 min at 68–72°C (the exact temperatures and times depend on the fragment; see Palmé and Vendramin, 2002).

Restriction: Each PCR product was cut with two restriction enzymes: *TaqI* (Fermentas) and *HinfI* (Fermentas). The restriction reactions contained 15 μl PCR product, 2 μl H₂O, 1 × buffer (Fermentas) and 3 U enzyme. After mixing these components, the tubes were placed at a temperature optimal to each restriction enzyme, 37°C for *HinfI* and 65°C for *TaqI*, and left there over night. After the restriction 15 μl of stop-solution (formamide with 3 mg/ml xylencyanol, 3 mg/ml bromophenol blue and 10 mM EDTA pH 8) was added to each tube. Just before loading, the restriction mix was heated to 85°C for 4 min and then placed on ice. A volume of 2.4 μl of this mix was then loaded onto the gel.

Fragment analysis: The restriction fragments were analysed on a denaturing 6% acrylamide gel. Samples from the populations of Glen Affric, Halltorp, Kelheim, Chizé, Montejo de la Sierra and Casentinesi were analysed on small gels (18 cm × 16 cm × 1 mm). Variable fragments were reanalysed on larger but thinner gels (43 cm × 35 cm × 0.4 mm) together with individuals from the other populations. The buffer used in both cases was 1 × TBE. The small gels were run at 300 V for 4 h 30 min and were then stained in ethidiumbromide, visualised on a UV-table and photographed. The large gels were run at 2000 V for 5000 volt hours, silver-stained and then scanned.

Screening: An initial screening of variation was conducted on 49 *S. caprea* individuals collected in six different localities: Glen Affric, Halltorp, Kelheim, Chizé, Montejo de la Sierra (only two individuals that were not used in further analysis) and Casentinesi. To increase the chances of finding variation, the chosen populations were as geographically distant from each other as was possible at the time. The screening was conducted with nine different universal chloroplast DNA primer pairs (CS, fMA, AS, VL, HK, K1K2, CD, DT, TF from Démesure *et al*, 1995; Dumolin-Lapégue *et al*, 1997b) and each PCR product was cut with two restriction enzymes: *TaqI* and *HinfI*. Eight primer enzyme combinations proved to be variable in this sample: TF, K1K2, HK and CD cut with both *TaqI* and *HinfI*. The screening of the total sample of 240 *S. caprea* individuals, as well as the 83 individuals from the other *Salix* species, was conducted using only the variable primer enzyme combinations.

Microsatellites

PCR conditions: Microsatellites ccmp2, ccmp7 and ccmp10 from Weising and Gardner (1999) were amplified according to the following protocol: 5 min at 95°C, 5 min at 80°C during which the enzyme was added, 30 cycles of 1 min at 95°C, 1 min at 50°C, 1 min at 72°C, and finally 8 min at 72°C. The PCR was performed in 1 × PCR buffer (Pharmacia), 2.5 mM MgCl₂, 0.2 mM dNTP, 0.2 µM of each primer and 0.04 U/µl *Taq* DNA polymerase (Pharmacia).

Fragment analysis: The success of the amplification was confirmed on agarose gels and the amount of DNA was estimated. The PCR products were then diluted to the appropriate concentration and microsatellites ccmp2, ccmp7 and ccmp10 were mixed for each individual. Size-standards were added to each mix before loading onto ReproGel Long Read acrylamide gels, which were run on an automated sequencing machine (Alf Express, Pharmacia). The output was then analysed with Fragment Manager 1.2 (Pharmacia).

Screening: In *S. caprea* 51 individuals from Glen Affric, Halltorp, Kelheim, Chizé, Montejo de la Sierra (only two individuals) and Casentinesi were analysed with three different chloroplast microsatellite primer pairs, ccmp2, ccmp7, ccmp10 (Weising and Gardner, 1999). These primer pairs were chosen from a first screening on eight individuals with six microsatellite primer pairs from Weising and Gardner (1999): ccmp2, ccmp3, ccmp5, ccmp7, ccmp6 and ccmp10. The primers used for further analysis were chosen on the basis of variability and ability to amplify one single PCR product.

Data analysis

Distribution maps of haplotypes were drawn using *ArcView GIS 3.2* (Environmental Systems Research Institute, Inc.) and a reduced median haplotype network, with equal weighing of the characters, was constructed with *Network 3.1.1.1* (Bandelt *et al*, 1999). The *Network* package was also used to obtain the mismatch distribution. Under an infinite-site model and neutrality, a constant-size population will lead to an L-shaped distribution whereas a bell-shaped distribution will indicate an expanding population. The skew of the distribution will depend on the time at which the expansion occurred: an early expansion will give a left-skewed distribution and a late expansion a right-skewed one (Rogers and Harpending, 1992; Rogers, 1997).

Calculations of gene diversity within each population (equivalent to expected heterozygosity) were performed using the *Arlequin 2.000* package (Schneider *et al*, 2000). The regression analysis of diversity measures and latitude was performed in MINITAB (Minitab Inc.) where the correlation was tested with a *t*-test. The G_{ST} and N_{ST} were calculated with *HaPermut 2.1* following Pons and Petit (1996). The introgression ratio, IG, and the expected introgression ratio, IG^e, were calculated according to Belahbib *et al* (2001). IG reflects the amount of shared haplotypes between two species and is expected to be one when there is no difference between the species and zero when they are totally different. The IG^e is the expected value if the sharing of haplotypes is not geographically structured, that is, if the presence of a haplotype in one population does not increase the

probability of finding this haplotype in the related species in the same area.

Results

PCR-RFLP

Variability: The screening of the total sample of 240 *S. caprea* individuals, as well as the 83 individuals from the other *Salix* species, was conducted using only the variable primer enzyme combinations: TF, K1K2, HK and CD cut with *TaqI* and *HinfI*. The most variable fragment was CD, where seven variable bands were found when cut with *HinfI* and five when cut with *TaqI* (Table 2). The other fragments showed lower but still substantial variability. The variation found in all the investigated fragments combined into 35 haplotypes, of which 28 were found in *S. caprea* (Table 2).

Haplotype network: The haplotype network of the *S. caprea* haplotypes is centred around the two most frequent haplotypes H1 and H3 and has a somewhat star-like topology (Figure 2). The mismatch distribution, that is the distribution of pairwise differences, is bell-shaped and right-skewed, suggesting a growing population (Rogers and Harpending, 1992; Rogers, 1997) (Figure 3).

Haplotype distribution in *Salix caprea*: The main feature of the haplotype distribution is the absence of a clear geographic structuring of the haplotypes. The most common haplotype, H1, is found in 22 of the 24 populations and makes up 46% of the total sample. Haplotype H3 is also common and it is present in 20% of the individuals and in 75% of the populations (Figure 1). The other 26 haplotypes are rare or very rare as they occur in only 0.4–3.7% of the individuals, and 12 of these are represented by one single copy. All of the haplotypes found in more than one individual are, with the exception of haplotype H23, present in more than one population where they are often represented by a single individual. Many of these rare haplotypes are geographically widespread. Haplotype H5, for example, is present in Glen Affric, Lake District (Great Britain), Kelheim (Germany), Fontainebleau (France), Devesa da Rogueira, Valle de Salzar (Spain), Bresciano (Italy) and Boki (Slovakia), even though it is only observed in a total of nine individuals (Figure 1).

Haplotype distribution among species: Many of the haplotypes found in *S. caprea* were also detected in *S. cinerea*, *S. aurita*, *S. purpurea*, *S. atrocinerea* and/or *S. appendiculata* (Figure 5). In fact, 54% of the *S. caprea* haplotypes were shared by one or several of the other species and 75% of the haplotypes represented by more than one copy in *S. caprea* were found in at least one of the other species. All haplotypes of *S. cinerea*, *S. aurita* and *S. appendiculata* were present in *S. caprea*. In *S. purpurea*, on the other hand, all four individuals from population 14 have haplotype H22, which is absent in *S. caprea*. *S. atrocinerea* has a single haplotype, H27, which is only found in one individual in this species. All haplotypes found in this group of *Salix* species are very similar (Table 2) except for haplotype H2, which is separated from the rest by at least 19 mutational steps. The IG and the IG^e were calculated for *S. caprea* and

Table 2 PCR-RFLP haplotypes, designated by H1–H25, H27–H36, and the band pattern associated with each haplotype

Haplotype	TF <i>TaqI</i> band: 1234	TF <i>HinfI</i> band: 123	K1K2 <i>HinfI</i> band: 127	K1K2 <i>TaqI</i> band: 24	HK <i>TaqI</i> band: 23	HK <i>HinfI</i> bands: 15	CD <i>HinfI</i> band: 1234567	CD <i>TaqI</i> band: 12345
H1	1111	111	111	11	11	11	1111111	11111
H2	2221	443	122	11	12	11	4453122	25421
H3	1111	111	111	11	11	11	1111111	11211
H4	1111	111	111	11	11	11	2111111	12211
H5	1111	411	111	11	11	11	1111111	11111
H6	1111	111	211	31	11	11	1111111	11211
H7	1111	111	111	11	11	11	1112111	11111
H8	1111	111	111	11	11	11	1121111	11111
H9	1111	111	111	11	11	11	1211111	11311
H10	1112	111	111	11	11	11	1111111	11211
H11	1411	151	111	11	11	11	1111111	11211
H12	1411	151	111	11	11	11	1121111	11211
H13	1111	111	111	11	21	21	1111111	11211
H14	1111	111	111	11	11	11	1111211	11211
H15	1111	111	111	11	31	11	1111111	11211
H16	1111	111	111	12	21	21	1111111	11211
H17	1111	111	111	12	11	11	1111111	11211
H18	1111	111	211	21	11	11	1111111	11111
H19	1511	121	111	11	11	11	1111111	11211
H20	1111	111	111	11	11	11	1111111	12211
H21	1111	111	111	11	11	12	1111111	11111
H22	1111	511	111	11	11	11	1111111	11111
H23	1111	511	111	11	11	11	1111111	11211
H24	2221	442	122	11	12	11	3353122	23421
H25	1111	111	211	21	11	11	1112111	11211
H27	1111	111	111	11	11	11	1112111	11211
H28	1111	111	111	11	41	31	1111111	11211
H29	4111	211	111	11	11	11	1111111	11111
H30	1111	111	111	11	21	21	1112111	11311
H31	1411	151	111	11	11	11	1131111	11311
H32	1111	111	111	11	11	11	1141111	11311
H33	2221	442	122	11	12	11	3153122	23421
H34	2221	442	122	11	12	11	1353122	24421
H35	2221	442	122	11	12	11	4453122	25421
H36	3331	334	211	22	12	11	5514133	36532

In each column, the variable bands found in a certain fragment (TF, K1K2, HK or CD) cut with restriction enzymes *TaqI* or *HinfI* are given. The different states found for each band are represented by numbers (1–6).

Figure 2 Reduced median haplotype network based on the PCR-RFLP data. Only *S. caprea* haplotypes are represented and the most common haplotypes, H1 and H3, are in the centre of the network. The lengths of the branches are proportional to the number of changes between haplotypes. The sizes of the circles reflect, but are not totally proportional to, the frequency of the haplotypes.

Figure 3 Mismatch distribution (the distribution of pairwise differences) (Rogers and Harpending, 1992) for the PCR-RFLP haplotypes.

S. cinerea, but not for the other species pairs due to low sampling sizes. The resulting IG and IG^e were 1.05 and 0.50, respectively.

Haplotypes observed in *S. caprea* were absent in *S. alba*, *S. elaeagnos* and *S. fragilis*, but the most common haplotype in *S. alba* was also found in the few sampled individuals of the other two species (Figure 5). The haplotypes found in this group of *Salix* species are distinctly different from those found in the *S. caprea* group, except from *S. caprea* haplotype H2, which is separated by only one mutational step from haplotype H35 in *S. alba* (Table 2).

Distribution of variation in *S. caprea*: All populations have a high level of chloroplast DNA variation, and very little of the variation can be attributed to differences between populations ($G_{ST}=0.090$, $SE=0.023$, $N_{ST}=0.095$, $SE=0.028$). The least variable population has three haplotypes and a haplotype diversity of 0.50, while the most variable populations have up to eight haplotypes in 11 individuals (Tofta, Sweden) and a diversity of 0.91 (Lake District, Great Britain) (Table 3). There is a tendency for northern populations to be more variable than southern populations (Figure 6): there is a significant correlation between latitude and the number of haplotypes in a population ($P=0.026$), while the correlation between diversity and latitude is nonsignificant ($P=0.054$).

Microsatellites

Microsatellite ccmp7 was monomorphic (135 bp) in the analysed sample and microsatellite ccmp10 had only limited variation, with three size variants (107, 109, 110 bp) of which two were rare. Microsatellite ccmp2 was much more variable and had five different size variants (206, 208, 209, 210, 211 bp). The size variants of microsatellite ccmp2 and ccmp10 combine into eight different haplotypes. The haplotypes A and C are common, occurring in 31 and 43% of the individuals. They are also widely distributed geographically (Figure 4): A is present in all populations and C in all populations except one (Montejo de la Sierra), but that might simply be due to the low sample size in this population. Haplotypes B and D are less common but they are also geographically

Figure 4 Geographic distribution of chloroplast microsatellite haplotypes in *S. caprea*.

Figure 5 Sharing of haplotypes among the nine investigated *Salix* species. Each column represents one haplotype and its distribution among the species.

Table 3 Diversity and number of haplotypes in the investigated *S. caprea* populations

Population	Number of individuals	Number of haplotypes	Diversity
Glen Affric	8	4	0.75
Lake District	10	7	0.91
Dean	10	5	0.82
Tofta	11	8	0.89
Stenshuvud	11	5	0.78
Halltorps Hage	9	4	0.75
Seebachtal	11	3	0.35
Bovenden	10	4	0.64
Kelheim	9	3	0.67
Fontainbleau	8	5	0.89
Chizé	9	5	0.72
Seillon	10	5	0.82
Valbonne	10	3	0.51
Valdu Niellu, Aitone	13	3	0.50
Devesa da Rogueira	9	5	0.72
Valle de Salzar	11	5	0.84
Casentinesi	10	3	0.64
Bresciano	10	3	0.38
Calabria	10	3	0.51
Mt Medvencia	10	3	0.51
Savarsin	8	4	0.64
Boki	13	6	0.68
Mt Voras	11	4	0.75
Voronez reserve	10	3	0.51

widespread. The other haplotypes occurred only in single individuals.

Population differentiation was basically nil ($G_{ST} = -0.017$, $SE = 0.0187$; $N_{ST} = 0.005$, $SE = 0.0025$). The G_{ST} and N_{ST} values are based on only five populations as Montejo de la Sierra was excluded because of its limited sample size.

Discussion

In a recent review, entitled The genetic legacy of the Quaternary Ages, Hewitt (2000) describes the types of patterns unravelled by previous phylogeographic studies (his Figure 2) and shows three patterns of recolonisation from refugia in southern Europe. This picture certainly captures most of the patterns observed so far in both plants and animals. However, it fails to represent *S. caprea*, and probably would not do justice to other cold-tolerant pioneer species that simply may not have been confined to southern Europe during the LGM. This is, for example, the case for *Calluna vulgaris*, a cold-tolerant species with a similar distribution as *S. caprea* (Rendell and Ennos, 2002), and for Arctic species (Tollefsrud *et al*, 1998; Abbott *et al*, 2000). The lack of phylogeographic structure in *S. caprea* might be the consequence of one or more of several factors: (i) lack of well-defined southern refugia, (ii) rapid speed of recolonisation and high dispersal ability, (iii) high mutation rates and (iv) extensive hybridisation with other willow species.

Glacial refugia and dispersal

The occurrence of small, isolated populations in buffered microclimates close to the ice sheet during the LGM has been controversial but is now slowly gaining acceptance (Willis *et al*, 2000; Stewart and Lister, 2001; but see Carcaillet and Vernet, 2001). Evidence from macroscopic charcoal records confirms the presence of *Salix* sp and seven other woody species in Hungary during the last full-glaciation (Willis *et al*, 2000). This does not necessarily mean that *S. caprea* was present, since it is not possible to distinguish fossils of different *Salix* species and there is little hope that it will be possible in the near future, unless seed or leaf fossils are available (K Bennett,

Figure 6 Correlation between latitude and the number of haplotypes in a population as well as between latitude and diversity. The former correlation is significant ($P=0.026$) but the latter is not ($P=0.054$). The linear regression line and its coefficient of determination (R^2) are represented in both graphs.

personal communication). Unfortunately, the pollen fossil record is also not very informative since again *S. caprea* cannot be distinguished from other *Salix* species. Nonetheless, the presence of *Salix* sp., as well as *P. sylvestris*, *P. cembra*, *Betula* sp., *Picea* sp., *Juniperus* sp., *Larix* sp. and *Carpinus betulus* in Hungary during LGM, indicates that the climate, at least locally, was suitable for *S. caprea*. Important refugia at intermediate latitudes are thus possible.

Since *S. caprea* cannot be separated from other *Salix* species in the pollen fossil record, the speed of recolonisation is difficult to infer. However, even if we assume that the *Salix* genus spent the LGM in Central Europe, its presence 16 000 BP in southern Scandinavia (Brewer, 2002) implies rapid migration. Trees can indeed spread very fast; for instance, the white spruce in the Western Interior (Canada) spread across 2000 km during roughly 1000 years. In this case, strong surface winds and flat open terrain could have affected the rapid seed dispersal over large distances (Ritchie and McDonald, 1986). The seeds of *S. caprea* are dispersed by wind (Rameau et al, 1989) and have therefore a great dispersal potential, especially in the open landscape of recently deglaciated Europe, where strong winds may also have prevailed during the recolonisation. The high dispersal ability of willow might also have continued to affect its genetic structure since seed dispersal between populations should prevent population differentiation and cause a wider distribution of the haplotypes. The pattern found in *S. caprea*, where not only common haplotypes but also rare ones are widely distributed geographically, could thus be due, at least in part, to the high dispersal ability of the species.

In most species studied so far there is a tendency for southern populations to show more variation than northern populations (*Fagus sylvatica*: Demesure et al, 1996; *Alnus glutinosa*: King and Ferris, 1998; *Quercus*: Petit et al, 2002) but in *S. caprea* the opposite trend is observed and most variation is found in the north (Figure 6, Table 3). A similar trend could be observed in *C. vulgaris*, where haplotype diversity within populations was higher in the northern part of the range than in the southern ($h_s(\text{north})=0.576$, $h_s(\text{south})=0.322$) (Rendell and Ennos, 2002). In *Calluna* as well as in *Salix*, refugia outside the traditional southern refugia have been suggested and this would certainly affect the distribution of variation and increase the amount of variation at higher latitudes.

Genetic variation and mutation rates

Compared to most other tree species, *S. caprea* exhibits very high levels of chloroplast DNA variation. When similar sampling and molecular methods were used in many other tree species such as *Acer campestre*, *Acer pseudoplatanus*, *A. glutinosa*, *Betula pendula*, *C. betulus*, *F. sylvatica*, *Fraxinus excelsior*, *Quercus robur*, *Tilia cordata* and *Ulmus minor*, between 5 and 17 haplotypes were found at the European scale and the differentiation between populations was generally high ($0.40 \leq G_{ST} \leq 0.97$) in most of these species (Petit et al, 2001). In contrast, in *S. caprea*, 28 haplotypes were detected and very little of the variation is due to differentiation between populations ($G_{ST}=0.09$). A similar pattern can be found in another Salicaceae, *Populus tremula*, where 21 haplotypes were found with PCR-RFLP and the G_{ST} value is low (0.12) (Bittkau and Müller-Stark, in preparation). Interestingly enough it seems to be the same parts of the genome that are responsible for most of the variation in both species since the same PCR-RFLP marker (CD) and microsatellite marker (ccmp2) show the highest levels of variation in both species. High levels of chloroplast DNA variation have also been found in other *Salix* species such as *S. interior*, *S. melanopsis* and *S. exigua* (Brunsfield et al, 1992), and the comparison of nucleotide variation in the K1K2 region among 14 species (Grivet, 2002) showed a high level of variation in *S. caprea*. High rates of substitution can obscure geographical patterns since homoplasy can create haplotypes that are identical by state but not by descent. This could potentially also affect the geographical structure in *S. caprea*.

Hybridisation

The presence of shared haplotypes between *S. caprea* and *S. cinerea*, *S. aurita*, *S. purpurea*, *S. atrocinerea* and/or *S. appendiculata* together with their absence within *S. alba*, *S. elaeagnos* or *S. fragilis* suggests that hybridisation takes place between *S. caprea* and the former, but not with the latter. Alternative explanations could be lineage sorting or convergence, identical haplotypes in the different species having independent origin. Given the extent and pattern of haplotype sharing among the species, these explanations seem less likely than hybridisation. Non-molecular data support hybridisation between many pairs from the first group and also between *S. alba* and *S. fragilis* (Tutin et al, 1964; Meikle, 1992; Rechner, 1992).

Salix phylogenies based on ITS sequences (Leskinen and Alström-Rapaport, 1999) showed that, although the differentiation among *Salix* species was generally low, *S. alba* and *S. fragilis* belonged to a separate clade. Unfortunately, *S. caprea* was not included in this phylogeny but *S. purpurea*, with which *S. caprea* seems to hybridise, was included and it does not belong to the *S. alba* clade. The traditional *Salix* taxonomy also supports this division. Skvortsov (1968) puts *S. alba* and *S. fragilis* into subgenus *Salix*, subsection *Salix* and *S. caprea*, *S. cinerea*, *S. aurita*, *S. atrocinerea* and *S. appendiculata* into subgenus *Vetrix*, subsection *Vetrix*. The separation of the subgenus *Vetrix* from most of the species from subgenus *Salix* is also supported by phylogenies based on the chloroplastic *rbcl* gene (Azuma *et al*, 2000), and by our data. The PCR-RFLP data show that the haplotypes found in *S. alba*, *S. elaeagnos* and *S. fragilis* are very different from those found in the other species (Table 2). What the cause of this hybridisation barrier might be is difficult to say but the two subgenera are not separated by differences in polyploidy levels (Blackburn and Harrison, 1924; Tutin *et al*, 1964; Thibault, 1998) and the individuals studied have largely overlapping distributions (Jalas *et al*, 1996).

When species share haplotypes to the extent that *S. caprea*, *S. cinerea*, *S. aurita*, *S. atrocinerea* and *S. appendiculata* do, it is difficult to identify in which species each haplotype has its origin. It is very improbable, though, that all the haplotypes found in *S. caprea* were created there and it is safe to assume that many of these haplotypes have their origin in other species. This will of course significantly affect the genetic structure of a species and would have a somewhat similar effect to an increased population size or immigration. Repeated transfer of genes between species is expected to result in an increased number of haplotypes and therefore a high level of genetic variation, such as in *S. caprea*. Notably, the occurrence of some rare haplotypes over a large geographical range could be explained by occasional introgression in *S. caprea* of haplotypes that are in high frequency in related species.

The chloroplast genetic structure of many other species has also been influenced by hybridisation to various degrees (Wagner *et al*, 1987; Bobola *et al*, 1996; Dumolin-Lapégue *et al*, 1997a; Terry *et al*, 2000). In some cases only a limited number of individuals in a hybrid zone are affected, while in others hybridisation concerns the whole species. The IG, which can be used to compare different pairs of species, is an attempt to quantify hybridisation, or rather the extent of gene sharing (Belahbib *et al*, 2001). The IG ratio for *S. caprea* and *S. cinerea* is 1.05, which means that these two species are not differentiated with regard to chloroplast DNA haplotypes, suggesting high levels of introgression. This ratio is in fact higher than in any other species investigated so far. In different pairs of oak species, the ratio ranges from 0.63 to 0.97 (Belahbib *et al*, 2001) and in species pairs in *Alnus*, *Betula*, *Crataegus*, *Fraxinus*, *Rubus* and *Tilia* it ranges from 0.17 to 0.89.

In the case of the oak species complex, which exhibits high IGs, a large number of closely related species share the locally common chloroplast haplotype whenever they are sympatric (Petit *et al*, 1993; Dumolin-Lapégue *et al*, 1997a). This kind of pattern is much more difficult to detect in a species that shows little or no geographic

structure. To investigate whether there actually is an increased chance of locally finding an allele in *S. cinerea* if it is present in *S. caprea* (or *vice versa*), we compared the IG with the IG^c if there were no geographic concordance between the species. The IG (1.05) is in this case much higher than the expected value (0.50) indicating local hybridisation and introgression. Hybrids between these two species have also been identified with traditional methods (Meikle, 1984; Rejchinger, 1992) and there may be plenty of opportunity for hybridisation due to their largely overlapping distributions (Jalas *et al*, 1996).

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