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Post-glacial history of the dominant alpine sedge *Carex curvula* in the European Alpine System inferred from nuclear and chloroplast markers

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Abstract

The alpine sedge *Carex curvula* ssp. *curvula* is a clonal, dominant graminoid found in the European Alps, the Carpathians, the Pyrenees and in some of the Balkan Mountains. It is a late-successional species of acidophilous alpine meadows that occurs on sites that were covered by ice during the last glacial maximum (LGM). By applying the amplified fragment length polymorphism (AFLP) fingerprinting and chloroplast DNA (cpDNA) sequencing, we attempted to identify the recolonization routes followed by the species after the last ice retreat. We relied on the genetic diversity of 37 populations covering the entire distributional range of the species. As a wind-pollinated species, *C. curvula* is characterized by a low level of population genetic differentiation. Nuclear and chloroplast data both support the hypothesis of a long-term separation of Eastern (Balkans and Carpathians) and Western (Alps and Pyrenees) lineages. In the Alps, a continuum of genetic depauperation from the east to the west may be related to a recolonization wave originating in the eastern-most parts of the chain, where the main glacial refugium was likely located. The Pyrenean populations are nested within the western Alps group and show a low level of genetic diversity, probably due to recent long-distance colonization. In contrast to the Alps, we found no phylogeographical structure in the Carpathians. The combination of reduced ice extension during the Würm period and the presence of large areas of siliceous substrate at suitable elevation suggest that in contrast to populations in the Alps, the species in the Carpathians underwent a local vertical migration rather than extinction and recolonization over long distance.

Keywords: AFLPs, alpine plants, *Carex curvula*, cpDNA, European Alpine System, phylogeography, Pleistocene refugia

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Introduction

Biogeographers have long observed marked similarities in the floristic, vegetational and historical features of the main temperate mountain ranges in Europe. This so-called European Alpine System (EAS) is a biogeographical entity that includes the Pyrenees, the Alps, the Apennines, the Carpathians and the mountains of the Central and North Balkans (Ozenda 1985). The last major events that severely influenced the species composition of the EAS were the repeated glacial crises that occurred during the Pleistocene. Although the importance of Quaternary glaciations for the origin and diversification of European high mountain taxa is still debated (Comes & Kadereit 2003), the major consequences on the geographical distribution of high-elevation taxa have been clearly demonstrated (Comes &

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Kadereit 1998; Hewitt 2000). The location of glacial refugia and the recolonization routes followed by species after deglaciation greatly impacted their present distribution as well as their genetic and phylogeographical structure.

Molecular studies examining a set of species in the Alps made it possible to generate the first scenarios of the Quaternary history of high-elevation taxa (Schönswetter *et al*. 2005). Refugia were located mainly on the eastern, southern, and southwestern portions of the Alpine chain border, and there was also some evidence for survival on nunataks (i.e. ice-free mountain tops or steep slopes within the glaciated areas). Other species survived the glaciations in areas outside of the Alps, notably in surrounding lowland tundra (Alsos *et al*. 2005; Schönswetter *et al*. 2005; Schönswetter *et al*. 2006a, b; Skrede *et al*. 2006). It is therefore probable that the postglacial recolonization patterns of high Alpine summits occurred in different ways for different species.

One might expect differences in Quaternary history of high mountain species in the eastern part of the EAS, because these areas (especially the Balkans) were much less glaciated than the Alps or Pyrenees (Charlesworth 1957; Frenzel *et al*. 1992; Vuia 2005). While the Alps were almost entirely covered by a large ice sheet, only localized glaciers were present on the highest summits of the Carpathian and Balkan mountains. However, phylogeographical studies for alpine or arctic-alpine plants have thus far neglected the eastern area of the EAS. The few published studies that also included the Balkans or the Carpathians (Després *et al*. 2002; Schönswetter *et al*. 2004b; Alsos *et al*. 2005; Koch *et al*. 2006; Skrede *et al*. 2006) considered only a small number of populations, which were insufficient to describe the regional particularities of the impact of the Ice Age on alpine floras (but see Mráz *et al*. 2007). For the Alps, the recolonization of large, unoccupied territories after deglaciation most likely arose from the nearby refugia (see the review in Schönswetter *et al*. 2005). This situation is unlikely for the Carpathians and Balkans, in which large areas in all the massifs remained ice-free, even during the last glacial maximum (LGM). These possible differences in the postglacial recolonization may be reflected in the actual genetic structure of the alpine plants that inhabit these areas. The colonization of the new territories is generally linked with loss of alleles, whereas long-term persistence in one area preserves a higher genetic diversity (Hewitt 1996). Indeed, two different scenarios have been proposed for the widespread subalpine plant *Hypochaeris uniflora* in the Alps and Carpathians. The higher stability has prevented the genetic depauperization of the Carpathian populations, whilst the multiple founder events have induced gradual loss of genetic variation during the colonization of the Alpine chain (Mráz *et al*. 2007).

It has been suggested that the ecological and life history characteristics of species in a given area induce differences in the population genetic structure that have consequences for evolutionary processes (Whiteley *et al*. 2004). Moreover, the species' position along a successional gradient (early- vs. late-successional) and geographical range distribution are also key determinants of genetic structure (Loveless & Hamrick 1984; Hamrick & Godt 1989). It is predicted that long-lived, late-succesional taxa that exhibit large population sizes have higher genetic variation within their populations and reduced genetic differentiation among populations; a reverse trend should be found for early-successional, narrowly distributed species with small populations. Other characteristics, like the breeding system, seed and pollen dispersal, population structure, or historical factors may also heavily influence the genetic structure of plants (Loveless & Hamrick 1984). The dominant late-successional alpine species of the EAS are still under-represented in phylogeographical studies, because their Quaternary history is still largely unknown. Dominant species, however, are the major contributors to the biomass of the plant community and are known to strongly impact many ecosystem processes (Grime 1998). Only a few studies have dealt with taxa of this type, such as the shrubby *Vaccinium uliginosum* (Alsos *et al*. 2005; Eidesen *et al*. 2007a) or *Dryas octopetala* (Skrede *et al*. 2006). Nevertheless, the alpine belt of the EAS is characterized by herbaceous communities dominated by graminoids (Körner 1998), and the phylogeographical patterns of these communities are poorly understood.

We used the crooked sedge *Carex curvula* All. ssp. *curvula* (*Cyperaceae*) as a model to study the phylogeography of a widespread, dominant alpine species at the European scale. This species is an endemic taxon to the entire EAS, excluding the Apennines. *Carex curvula* is a dominant species of acidophilous alpine grasslands. In this study, our main objectives were (i) to characterize the phylogeographical patterns of a dominant, wind-pollinated alpine graminoid of the EAS; (ii) to compare the recent Quaternary history of this species in the western and eastern parts of its range; and (iii) to compare the genetic structure of the species in the central and peripheral part of its distribution range.

Materials and methods

The study species

Carex curvula All. is found predominantly in the Alps, Pyrenees, and the Southeastern Carpathians, mainly between ~2200 and 2700 m altitude (Fig. 1). In the Balkans, the species has a fragmented distribution in Stara Planina, Rila, Pirin in Bulgaria and some of the highest peaks of the Dinarides (Puşcaş 2006). The local abundance of *C. curvula* throughout its distribution range varies. The species is more frequent and abundant in the eastern and central parts of the Alps and in the Carpathians, whereas it occurs in more scattered and scarce populations in the peripheral

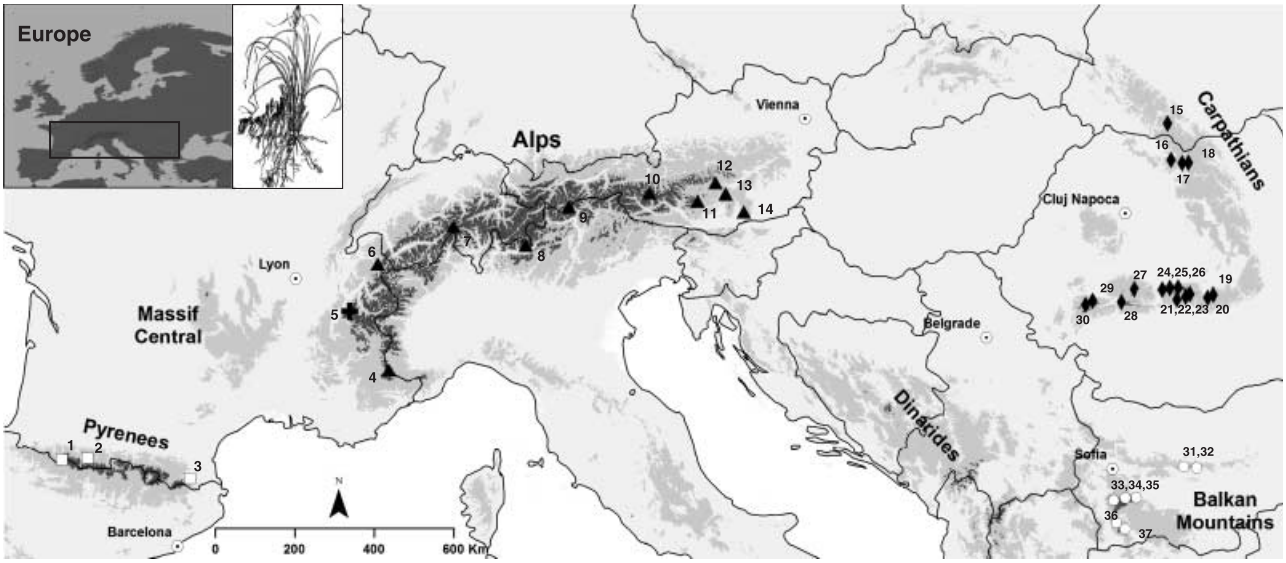

Fig. 1 Overall distribution range, location of the populations studied with AFLP and growth habit of *Carex curvula* subsp. *curvula*. Distribution of the species is shown in dark grey (shaded areas: altitude > 800 m). Left insert indicates the location of the mountain areas with *Carex curvula* in Europe and its habitus (adapted from Reisigl & Keller 1987). Sampled populations' numbers refer to Table 1. Symbols represent groups detected in the Neighbour-joining analysis of population: □ – Pyrenees, + – Dauphiné Alps, ▲ – Alps, ○ – Balkans, ◆ – Carpathians.

southern parts of its distribution area in the Balkans and especially the Pyrenees (P. Choler and M. Puşcaş, personal observations). An obvious gradient of decreased presence and abundance of the crooked sedge is also noticeable from the northern part of the French Alps to the southeastern part of the chain. *Carex curvula* is a clonal, long-lived species (Steinger *et al*. 1996) that forms extensive alpine swards (*Caricetum curvulae*), that were described as alpine climax vegetation on siliceous substrate over its entire distribution (Braun-Blanquet & Jenny 1926; Negre 1969; Coldea 1997; Roussakova 2000). The species is wind-pollinated, protogynous, and primarily outcrossing, and it produces heavy fruits with limited dispersal capacity (Erschbamer & Winkler 1995). *Carex curvula* s. l. has been divided into two distinct taxa (Gilomen 1938): *C. curvula* ssp. *curvula*, which is primarily calcifuge and found throughout the range of the species, and *C. curvula* ssp. *rosae*, which is primarily calcicole and found only in the Alps and Pyrenees (Chater 1980). The two taxa are genetically distinct (Choler *et al*. 2004), although some introgressive forms occur very locally in the Alps. Only *C. curvula* ssp. *curvula* is considered in this study.

Sampling strategy

During the summers of 1999–2004, 37 populations of *C. curvula* ssp. *curvula* were sampled throughout the entire distribution (Table 1, Fig. 1). Young, green leaves of five random individuals separated by 10 metres were collected for each population. The only exception was population 28 (Parâng Mts. from Carpathians), for which only four individuals were sampled. Vegetative tissues were stored in tubes with silica gel until DNA extraction.

DNA extraction and AFLP Protocol

Total DNA was extracted from roughly 10 mg of dried leaf tissue using the DNeasy 96 Plant Kit (Qiagen) according to the manufacturer's protocol. Six random individuals from the total sample set were extracted twice as blind samples (Bonin *et al*. 2004). Double digestion of genomic DNA was performed for two hours at 37 °C in a 20 µL mix using 2 units (U) of *Mse*I and 5 U of *Eco*RI (New England Biolabs). Following this, adapters were ligated to DNA in a 40 µL volume for 2 h at 37 °C using 1 U of T4 DNA Ligase (New England Biolabs). Diluted 10 X, digested, and ligated DNA was subjected to a preselective amplification using primers *EA* (5′-GACTGCGTACCAATTCA-3′) and *MC* (5′-GATGAGT CCTGAGTAAC-3′) in a 25 µL volume containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.25 µM of each primer, and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems). Diluted 20 X preselective products underwent selective polymerase chain reaction (PCR) with the following primer combinations: *EATC-MCAC*, *EATC-MCAT*, and *EATC-MCTG*. Selective amplifications were run in a 25 µL volume containing 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1.25 µM of each primer, and 1 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems). Following this step,

Table 1 Population location (country, coordinates), elevation and genetic diversity parameters of the 37 investigated populations of *Carex curvula* All. subsp. *curvula*. Means and standard errors with the same letters are not significantly different (ANOVA, post-hoc Tukey test, all $P < 0.03$).

Pop. no.	Sampling locality	Mountain range	Country	Coordinates (E/N)	Elevation (m)	Fragm.*	HJ**
1	Pic du Midi, Massif d'Ossau	Pyrenees	France	−0.45°/42.85°	2200	36	0.28
2	Pic de Neouvielle, Massif de Neouvielle		France	0.14°/42.86°	2500	40	0.40
3	Pic du Canigou, Massif de Canigou		France	2.45°/42.52°	2450	39	0.37
					Mean	**38.33 ± 2.08 a**	**0.34 ± 0.06 a**
4	Lac Fourchas, Massif de Mercantour	Alps	France	7.28°/44.11°	2300	62	0.61
5	Col de Sept Laux, Chaîne de Belledonne		France	6.08°/45.23°	2500	56	0.56
6	Flaine, Chaîne des Aravis		France	6.43°/46.01°	2400	57	0.61
7	Furka Pass, Berner Alpen		Switzerland	8.24°/46.35°	2400	57	0.64
8	Berninapass		Switzerland	10.09°/46.28°	2300	70	0.68
9	Obergurgl, Ötzaler Alpen		Austria	11.02°/46.51°	2400	61	0.60
10	Grossglockner Hochalpenstrasse, Hohe Tauern		Austria	12.5°/47.06°	2560	63	0.68
11	Dieslingsee, Gurktaler Alpen		Austria	13.93°/46.95°	2150	56	0.67
12	Klosterneuburger Hüte, Wölzer Tauern		Austria	14.33°/47.23°	2100	60	0.61
13	Lindersee – Zirbitzkogel, Seetaler Alpen		Austria	14.56°/47.06°	2250	66	0.66
14	Großes Kar, Koralpe		Austria	14.97°/46.79°	2000	65	0.67
					Mean	**61.18 ± 4.57 b,c**	**0.63 ± 0.03 b**
15	Shpytsi Peak, Chornohora Mts.	Carpathians	Ukraine	24.55°/48.11°	1904	54	0.51
16	Buhăiescu Mic, Rodna Mts.		Romania	24.59°/47.57°	2103	62	0.60
17	Iezerul, Rodna Mts.		Romania	24.64°/47.59°	1960	69	0.63
18	Ineu Peak, Rodna Mts.		Romania	24.88°/47.52°	2164	65	0.59
19	Omul Peak, Bucegi Mts.		Romania	25.45°/45.44°	2405	62	0.51
20	Coştila Peak, Bucegi Mts.		Romania	25.48°/45.41°	2475	56	0.54
21	Iezerul Peak, Iezer-Păpuşa Mts.		Romania	24.95°/45.45°	2280	66	0.61
22	Păpuşa Peak, Iezer-Păpuşa Mts.		Romania	25.06°/45.5°	2360	69	0.62
23	Grădişteanu Peak, Iezer-Păpuşa Mts.		Romania	25.07°/45.49°	2100	64	0.54
24	Laiţa Peak, Făgăraş Mts.		Romania	24.59°/45.6°	2327	73	0.67
25	Paltinul Peak, Făgăraş Mts.		Romania	24.6°/45.59°	2359	68	0.61
26	Fereastra Mare a Sâmbetei, Făgăraş Mts.		Romania	24.79°/45.6°	2270	67	0.60
27	Cindrelul Mare Peak, Cindrel Mts.		Romania	23.8°/45.57°	2244	56	0.59
28	Cârja Peak, Parâng Mts.		Romania	23.51°/45.37°	2129	59	0.59
29	Retezat Peak, Retezat Mts.		Romania	22.84°/45.38°	2485	65	0.61
30	Culmea Lolaia, Retezat Mts.		Romania	22.86°/45.37°	2200	58	0.55
					Mean	**63.31 ± 5.48 b**	**0.58 ± 0.04 c**
31	Botev Peak, Stara Planina	Balkan Mountains	Bulgaria	24.91°/42.71°	2370	55	0.56
32	Paradzika Peak, Stara Planina		Bulgaria	24.95°/42.71°	2300	58	0.62
33	Deno Peak, Rila		Bulgaria	23.57°/42.21°	2700	57	0.56
34	Musala Peak, Rila		Bulgaria	23.58°/42.18°	2900	55	0.52
35	Malyovica, Rila		Bulgaria	23.36°/42.17°	2600	61	0.56
36	Vihren Peak, Pirin		Bulgaria	23.30°/41.83°	2350	52	0.52
37	Todorka Peak, Pirin		Bulgaria	23.40°/41.78°	2400	56	0.61
					Mean	**56.28 ± 2.81 c**	**0.56 ± 0.03 c**

*Total number of AFLP fragments/population.
**Mean Jaccard Index/population.

Table 2 Characteristics of the primers developed for SNP detection on *Carex curvula*. Two combinations are expected after the multiplex PCR: one 133 bp product (if blue = haplotype w1, if green = haplotype e1)/and one 122 bp product (if blue = haplotype w2, if green = haplotype e2)

Location of the amplified fragment and the base substitution on the SNP point	Primers (5′–3′)		Haplotype	Size (bp)
trnG intron (**G/A**)	Forward: **trnG-F** TTTTGAATAATAATCGTGATAATG	Reverse (blue): **trnG-R-G** GTTAAGGGATCCTTCAGTTT**C**	w1	133
		Reverse (green): **trnG-R-A** GTTAAGGGATCCTTCAGTTT**T**	e1	
trnD(GUC)-trnE spacer (**A/G**)	Forward: **trnDE-F** CATATAAATACATGAAGTCTTCCC	Reverse (blue): **trnDE-R-A** GTTATATATGAATGATATAATAGAATTG**T**	w2	112
		Reverse (green): **trnDE-R-G** GTTATATATGAATGATATAATAGAATTG**C**	e2	

excess labelled primers were removed by spin-column purification. Finally, 1.5 µL of diluted (10 X) purification was added to a 10 µL mixture (10 : 0.1) of HiDi formamide and GeneScan-500 ROX (Applied Biosystems). Amplified frgament length polymorphism (AFLP) reactions were electrophoresed for 41 min on an ABI PRISM®3100 Genetic Analyser (Applied Biosystems) using 36 cm capillaries & POP-4™ polymer. The raw data were size-called using GENESCAN Analysis software, version 3.7 (Applied Biosystems). The size-calibrated GENESCAN files were imported into GENEOGRAPHER (version 1.6.0, James J. Benham, Montana State University, 2001; http://hordeum.oscs.montana.edu/genographer) for scoring. The thumbnail option was used to evaluate a given marker for all individuals at the same time. Fragments within the 50–500 bp range were scored to produce a present/absence matrix (individuals × markers).

cpDNA analysis

Eight chloroplast DNA (cpDNA) regions were initially amplified and sequenced for 12 individuals from the Pyrenees, Alps, Carpathians and Balkans, in order to look for nucleotide variation. Variation was found in two regions (*trnG* intron and *trnD*^{GUC}*-trnE* spacer) that were selected for subsequent analyses. The following primers were used: 5′trnG2G and 5′trnG2S (Shaw *et al*. 2005), which were used to amplify the *trnG* intron and trnD^{GUC}F (Demesure *et al*. 1995), and trnE^{UUC} (Shaw *et al*. 2005), which were used for the *trnD*^{GUC}*-trnE* spacer. Amplifications were performed in a 25-µL total reaction volume with 2.5 µL buffer (10 × Buffer II Applied Biosystems), 1.5 mM (for the *trnG* intron) or 3 mM (for the *trnD*^{GUC}*-trnE* spacer) $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer, 0.25 µL BSA, 0.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems), and 2 µL (15–40 ng) of genomic DNA. The cycling profile involved 10 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 45 s at 58 °C (*trnG*) or 53 °C (*trnD-trnE*), and 60–120 s at 70 °C. The protocol ended with 5 min at 70 °C of final elongation. PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio, USA). Sequencing was performed in both directions using BigDye Terminator V 3.1 (Applied Biosystems). The samples were run on an ABI PRISM®3100 Genetic Analyser. Sequences were assembled and edited using SEQSCAPE 2.5.0 (Applied Biosystems).

Two loci with single nucleotide polymorphisms (SNP) were detected: one in the *trnG* intron and one in the *trnD*^{GUC}*-trn*E spacer. We developed an SNP genotyping method using multiplex competitive PCR to find the haplotype combination for each individual in a single PCR run. Briefly, we amplified the two target regions with a mix of labelled fluorescent primers. The primers were designed to detect a length polymorphism and a specific fluorescence (blue or green) corresponding to a specific combination of haplotypes. The reverse primers were 3′ nucleotides specific to the mutation points (see Table 2). The obtained PCR products had different lengths (133 bp for the SNP locus in the *trnG* intron and 112 bp for the *trnD*^{GUC}*-trnE* spacer). The multiplex PCR conditions were: 25 µL total reaction volume with 2.5 µL buffer (10 × Buffer II Applied Biosystems), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM for trnG-F and trnG-R-A primers/0.2 µM for trnG-R-G, trnD-F and trnD-R-G primers/0.1 µM for trnD-R-A primer, 0.2 µL BSA, 0.5 U of AmpliTaq Gold® DNA polymerase, and 2 µL of genomic DNA. The PCR parameters included 95 °C for 10 min; 35 X (95 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min); 72 °C, 7 min PCR-products were diluted 10 times and electrophoresed on an ABI PRISM®3100 Genetic Analyser using POP-7™ polymer, and GeneScan-350 ROX standard was used for fragment size estimation. The fragments were scored using GENEMAPPER® 3.7 (Applied Biosystems). This procedure was first validated on 16 individuals with known sequences for the *trnG* intron and the *trnD-E* region before screening all the individuals.

Data analysis

For each population, we calculated the total number of AFLP markers present, and the intrapopulation diversity was evaluated as the mean pairwise distance between individuals (based on the Jaccard similarity index, Jaccard 1901). We performed one-way analyses of variance and multiple comparisons of means with posthoc Tukey tests to test for a geographical effect on genetic diversity indices. Populations were pooled into four main geographical regions (Alps, Balkans, Pyrenees, and Carpathians). To cope with possible biases due to the unbalanced sampling effort, we used re-sampling procedures to generate a large number of datasets consisting of an equal number of populations per region. The regional effect was found to be the same in these analyses (data not shown). These analyses were first performed by including the four regions and re-sampling three populations per region. The diversity indices of the Pyrenees were always significantly lower ($P < 0.001$). Another set of analyses was done for the Alps, Carpathians and Balkans with six populations randomly chosen for each region. The results were qualitatively similar to the posthoc Tukey tests for the Jaccard index. For the total number of fragments, the results were also similar except that we found a significant difference between the Alps and the Balkans.

The AFLP phenotypes were used to construct a neighbour-joining (NJ) tree, based on pairwise Nei's genetic distance between populations (Lynch & Milligan 1994). Support for resulting groups was calculated from 1000 bootstrap replicates. Principal Co-ordinate Analyses (PCoAs) were computed based on between-individual Jaccard similarities. For the analyses, we used the packages ADE4 (Chessel *et al*. 2004) and APE (Paradis *et al*. 2004), on R (R Development Core Team 2006, www.R-project.org).

We examined whether the frequency of each polymorphic marker was independent of the geographical regions. The analysis was based on presence/absence data at the population level. A given marker is considered present in a population if at least one individual exhibits that marker. For each contingency table crossing regions and presence/absence data, we generated an exact distribution for the χ^2 statistic using randomization tests (1000 re-samplings) and compared the observed value with this exact distribution. In cases lacking independence in the contingency table, we computed the standardized residual of each cell using the formula $(Obs - Exp)/(Exp)^{1/2}$, where 'Obs' and 'Exp' stand for observed and expected frequencies, respectively. This allowed us to estimate the contribution of each region to the significance of the test (at alpha = 0.05). Positive (or negative) residuals indicate more (or fewer) observed occurrences of a given marker than expected under the null hypothesis. These markers are called geographical diagnostic markers. Diagnostic markers may be found only in one region – in that case, the marker is specific or alternatively may be shared by several regions (see Results).

All computations and statistical analyses were performed with the software R (R Development Core Team 2006, www.R-project.org).

Analyses of molecular variance (AMOVA), with a partition of the total genetic variance into different levels (within populations, among populations, and among groups of populations), were carried out using ARLEQUIN version 2.000 (Schneider *et al*. 2000). The clustering of the populations followed the structures identified by the PCoA and NJ tree.

Results

AFLP

Using the three pairs of primers, 115 polymorphic fragments of lengths ranging from 51 to 487 bp were identified. The repeatability of the AFLP results was very high (99.3% for the overall test). Three AFLP markers were removed after checking the similarities between duplicates and the original individuals. Two identical genotypes were identified in one population (population 1, from the Pyrenees); one of them was excluded from the subsequent analyses to avoid redundant data. The final matrix consisted of 183 individuals and 112 unambiguous polymorphic markers.

The highest genetic diversity was found in populations from the Alps and the Carpathians (Table 1). While the Carpathians were the region that contained the richest populations in terms of the number of AFLP markers, populations from the Alps were the most polymorphic. The Pyrenean populations were the poorest in numbers of AFLP markers. In addition, these markers showed little variation. The highest number of geographical diagnostic markers was recorded for the Alps, and the second highest was recorded for the Carpathians (Fig. 4b). The Balkans and the Pyrenees showed few specific markers. The highest amount of genetic variation in *Carex curvula* was due to intrapopulation variation (AMOVA, 62% overall populations) (Table 3). Each mountain system is characterized by a weak differentiation between populations. Genetic variation among populations explained 17–21% of the total genetic variation in the Alps, Carpathians and Balkans and 30% in the Pyrenees.

NJ analysis of the 37 populations (Fig. 2) revealed a highly supported division between two major groups: a Western lineage grouping the Alps and the Pyrenees and an Eastern one with the Carpathians and the Balkans.

Within the Western lineage, the populations from the Pyrenees formed a distinct group. A group including the Dauphiné Alps and Pyrenees was also strongly supported and clearly nested within the larger group of the Alps.

Table 3 Analyses of molecular variance (AMOVA) based on 112 AFLP markers for 37 populations of *Carex curvula*

Grouping*	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
a: All populations	Among populations	36	1278.59	5.42	37.96
	Within populations	145	1284.45	8.85	62.04
b: Pyrenees, Alps, Carpathians, Balkans	Among groups	3	637.87	4.68	29.89
	Among populations within groups	33	640.72	2.14	13.67
	Within populations	145	1284.45	8.85	56.45
c: Pyrenees, Alps, Dauphiné Alps, Balkans, Carpathians	Among groups	4	678.64	4.77	30.53
	Among populations within groups	32	599.95	2.01	12.85
	Within populations	145	1284.45	8.85	56.62
d: Pyrenees	Among populations	2	14.82	1.07	30.60
	Within populations	11	26.75	2.43	69.40
e: Alps	Among populations	10	240.43	2.75	21.17
	Within populations	44	451.60	10.26	78.83
f: Carpathians	Among populations	15	290.16	2.00	17.52
	Within populations	63	594.85	9.44	82.48
g: Balkans	Among populations	6	95.30	1.66	17.51
	Within populations	27	211.25	7.82	82.49

*For characteristics of populations see Table 1.

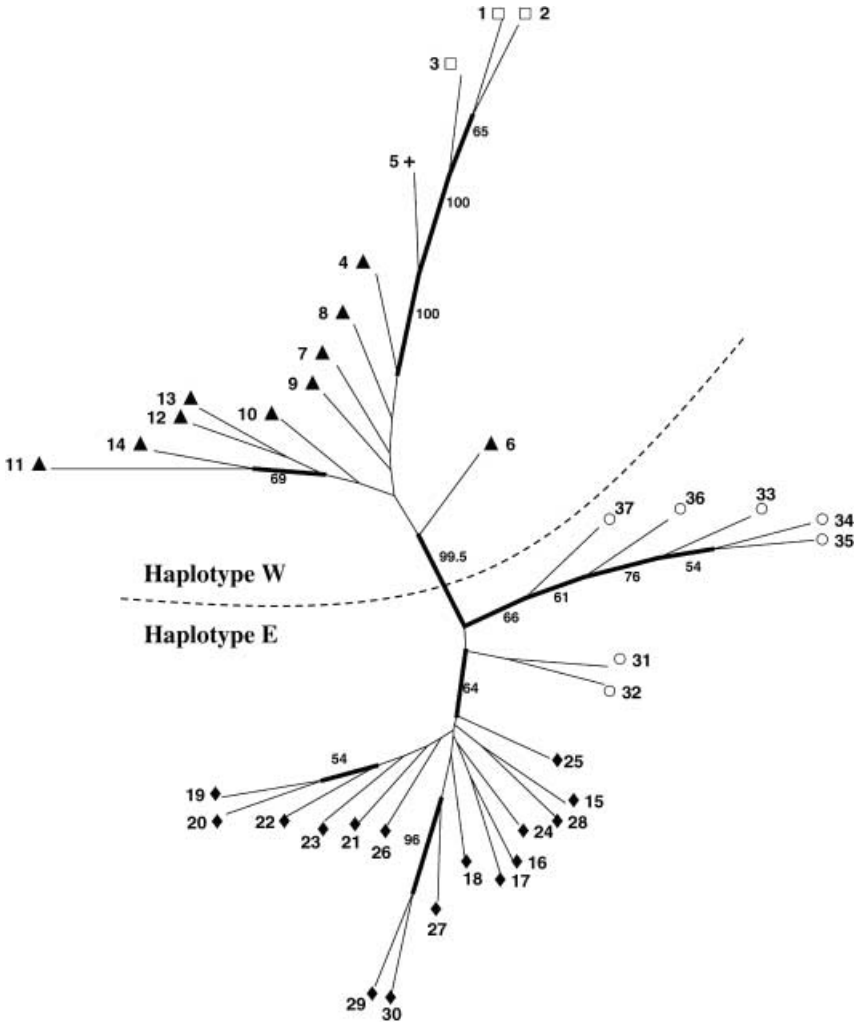

Fig. 2 Neighbour-joining analysis of the 37 populations of *Carex curvula* based on Nei's genetic distance of AFLP phenotypes. The numbers above the branches are bootstrap values (% of 1000 replicates). Numbers at the tips of branches are population numbers (Table 1) and the symbols are the same as in Fig. 1. The dashed line separates the distribution of the two identified cpDNA haplotypes (W and E).

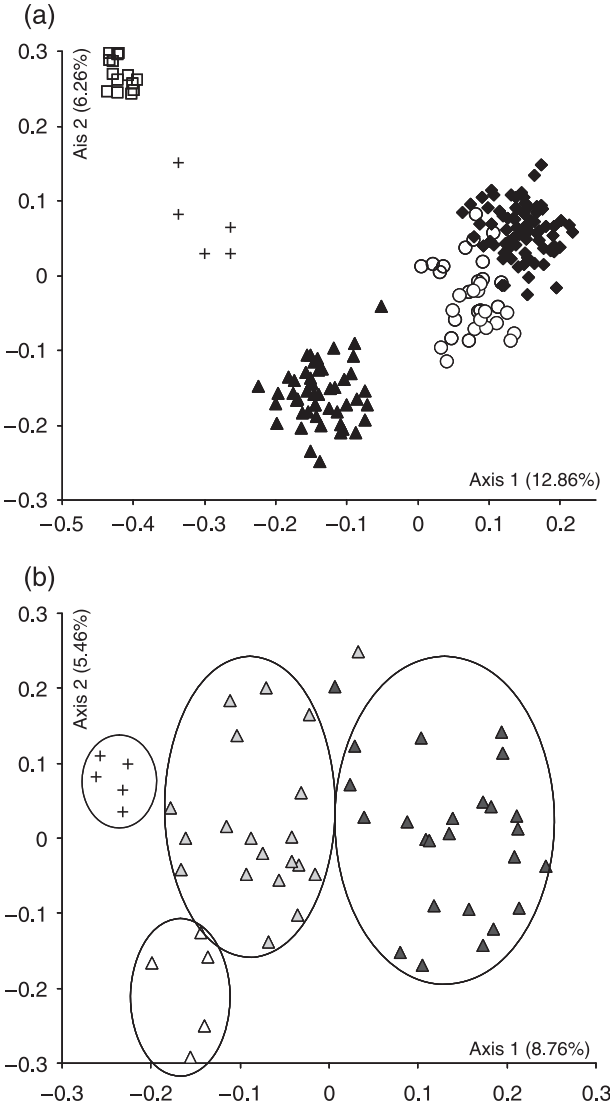

Fig. 3 Principal Coordinate Analysis (PCoA) based on AFLP data of *Carex curvula* individuals. (a) PCoA performed for all the analysed individuals (symbols as in Fig. 1). (b) Separate PCoA of individuals from the Alps; ovals separate the Dauphiné Alps (+ – pop 5), Maritime Alps (△ – pop 4), Central Alps (▲ – pop 6, 7, 8, 9) and eastern Alps (▲ – pop 10, 11, 12, 13, 14).

Within the Eastern lineage, all the Carpathian populations formed a distinct branch. Further resolution inside this group was not possible because of the very low bootstrap values (except for Retezat, populations 29–30). Some Balkan groups, in particular Rila and Pirin, were better supported.

The PCoA of the AFLP profiles of all the individuals (Fig. 3a) confirmed the same general patterns revealed by the NJ analysis. Interestingly, the PCoA carried out exclusively on individuals from the Alps showed an east–west genetic continuum within the chain (Fig. 3b). A significant decline in the mean Jaccard index from eastern to western Alps was detected along this longitudinal gradient (Fig. 4a). The cline was also supported by the other estimators of genetic diversity, such as the total numbers of polymorphic markers and the percentage of polymorphic markers per population (data not shown). On the contrary, no such geographical patterns were detected in the Carpathians or Balkans.

Further insights into the genetic structure in the Alps were brought about by the geographical distribution of regional diagnostic markers (Fig. 4b). Populations from the eastern Alps exhibited seven specific markers linked exclusively to this group, whereas populations from the southwestern Alps only had one or no such markers. One diagnostic marker was shared between the Pyrenean populations and the population from Dauphiné. Therefore, the overall decline in intrapopulation diversity along the longitudinal gradient is associated with a decrease in the number of specific markers. On the other hand, we found no trend in the distribution of regional markers within the Carpathians.

cpDNA analyses

The aligned sequences of the *trnG* intron for the 12 analysed individuals comprised 720 nucleotides and showed one substitution (G/A, position 684) determining two haplotypes. Haplotype w1 was present in individuals from the Alps and Pyrenees, and haplotype e1 was present in individuals from the Carpathians and Balkans. The sequences for the *trnD-E* spacer were 671 nucleotides long and showed one substitution (A/G, position 393) that determined two haplotypes. Haplotype w2 was found in the Alps and Pyrenees, whereas haplotype e2 was found in the Carpathians and Balkans.

Based on the preliminary test with 16 individuals, we found a perfect agreement between SNP test and cpDNA sequence. We found only two combined haplotypes after screening all the individuals: a western haplotype W (= w1 + w2) for the Alps and the Pyrenees and an eastern haplotype E (= e1 + e2) for the Carpathians and the Balkans (Fig. 2). Individuals from Vranica Planina (the most remote northwestern population from Dinarides – Fig. 1, subsequently added for only the SNP test) also possessed haplotype E.

Discussion

Two main lineages in *Carex curvula* ssp. *curvula* have been clearly distinguished by both cpDNA sequences and AFLP markers: the Western (including the Alps and Pyrenees) and the Eastern (including the Carpathians and Balkans) lineages. The geographical distribution of cpDNA haplotypes is of large significance, because it points out a major split between the Eastern and the Western populations

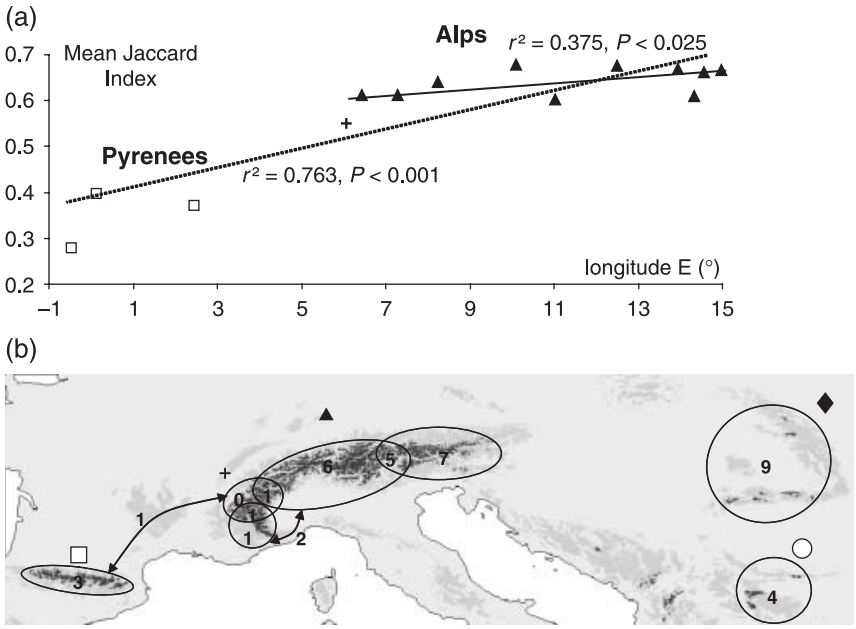

Fig. 4 Geographical patterns of genetic diversity in *Carex curvula* in Europe. (a) Correlation between geographical location (longitude) and the mean Jaccard index/population in the Western lineage (Alps and Pyrenees); linear regressions were calculated for both mountain ranges (dashed line) or only for Alpine populations (black line). (b) Regional distribution of the diagnostic markers. The arrows and intersections of the ovals indicate the diagnostic markers shared between adjacent subgroups in the Alps and the Pyrenees. Symbols are as in Fig. 2.

within the EAS. Other studies on arctic-alpine plants combining nuclear and plastid markers revealed small but informative variation of the chloroplast markers (Albach *et al*. 2006; Schönswetter *et al*. 2006a, b; Eidesen *et al*. 2007a). This variation was assumed to reflect ancient differentiation within the species (Eidesen *et al*. 2007b). These two geographical groups appear to be the result of an old disjunction within the distributional area of *C. curvula*, which most likely occurred well before the last glaciation. Each of the groups experienced an independent history after this separation. There are similar cases documented for other mountain species of plants (Ronikier *et al*. 2008), invertebrates (Pauls *et al*. 2006) and small mammals (Kotlík *et al*. 2006) that suggest a very old differentiation of populations from the Alps and Carpathians, without subsequent interferences between them.

The Western lineage

Within the Western lineage, one main result was the significant decrease of within-population genetic diversity and the number of regional diagnostic AFLP markers from east to west (i.e. from the Austrian Alps to the Maritime Alps, Fig. 4). This result supports the hypothesis of a single glacial refugium for *C. curvula* located in the eastern Alps. While the majority of the Alpine chain was completely glaciated, the eastern part of the Austrian Alps remained virtually ice-free even during the LGM (Voges 1995). Moreover, this part of the Alps is the only one out from the Alpine ice sheet exhibiting large surfaces of siliceous bedrock between 1000 and 1600 m (Puşcaş *et al.* unpublished manuscript), i.e. the range corresponding to the putative alpine belt during the Ice Age. Therefore, the eastern Austrian Alps were likely the main refugium for several alpine acidophilous plants (Schönswetter *et al*. 2005). The gradual genetic impoverishment may reflect a postglacial recolonization route followed by the species, in accordance with a *tabula rasa* scenario (Dahl 1987; Stehlik 2000). The rapid westward migration after deglaciation induced multiple founder events with a stepwise reduction in genetic diversity (Hewitt 1996; Després *et al*. 2002; Petit *et al*. 2002; Stehlik 2002; Mráz *et al*. 2007). Subsequently to the colonization, high levels of gene flow via pollen have prevented the genetic divergence of populations from different geographical areas and maintained a genetic continuum along the Alpine chain. The present distribution of the diagnostic markers within the Alpine groups was also congruent to this hypothesis; all the adjacent groups shared some of these markers without evident barriers between them (Fig. 4b). The same pattern of a low between-population differentiation and a lack of abrupt geographical changes along the distribution range has been documented for other wind-pollinated tree species (Demesure *et al*. 1996; Kremer *et al*. 2002; Burban & Petit 2003; Coart *et al*. 2005), and certain dominant and/or widespread graminoids (O'Brien & Freshwater 1999; Jonsson & Prentice 2000; Stenström *et al*. 2001).

Pyrenean populations of *C. curvula* are closely related to the southwestern Alps populations. They are nested within the Alpine group (Fig. 2) and characterized by a substantial loss of genetic diversity (Table 1). One diagnostic marker is shared between these two separate groups. It is more likely that this pattern reflects a recent colonization of the Pyrenees from the Dauphiné area than an old vicariance

between the two mountain ranges, as was proposed for other alpine species (Kropf *et al*. 2006). The French Massif Central has long been considered as a key area in the colonization of the Pyrenees by Alpine elements (Braun-Blanquet 1923), and it may be a likely stepping-stone for *C. curvula* as well. Colonization by long-distance dispersal was a common phenomenon after glaciations (Cain *et al*. 1998), and it has also been described for alpine or arctic-alpine plants (Gabrielsen *et al*. 1997; Tollefsrud *et al*. 1998; Schönswetter *et al*. 2002; Tribsch *et al*. 2002). These long-distance dispersal events have created a marked bottleneck effect (Table 1; Fig. 4). Despite such low diversity, the populations from the Pyrenees had a relatively high number of diagnostic markers. This fact could be the result of genetic drift that randomly fixed some previously existing alleles.

The Eastern lineage

One characteristic of the Carpathian group is its low level of between-population differentiation (17.5%, Table 3); indeed, this is one of the lowest reported for an alpine plant within a whole mountain range (see Stehlik 2002; Schönswetter *et al*. 2003; Schönswetter *et al*. 2004a; Ehrich *et al*. 2007; Mráz *et al*. 2007, etc.). This is surprising if one considers the current distribution of *C. curvula* in the mountain range (Fig. 1), which shows several very disjunct areas ('island' distribution). Even the most isolated populations (from Rodna and Chornohora, no. 15–18; Fig. 1) are genetically indistinct, because they group together with the populations from the southern chain of the Carpathians. Interestingly, the floristic composition of alpine meadows dominated by *C. curvula* in the Carpathians shows the same remarkable homogeneity without any significant geographical differences between regions (Puşcaş *et al*. 2005). The weak geographical genetic structure in the Carpathians, as well as its increased genetic diversity (Table 1, Fig. 4), may be explained by the particularities of the vegetation history during the last period of glaciation in this area. The Carpathians were only locally glaciated, and the snowline during the Würm glaciation was estimated near 1700–1800 m altitude (Wachner 1929; Niculescu 1969; Sârcu 1978; Reuther *et al*. 2004; Vuia 2005, etc.). Below this altitude, large areas were suitable for the alpine plants. Because of the widespread acidic bedrock in the entire Southeastern Carpathians (Coldea 1991), this area may be regarded as one large potential refugium for *C. curvula* and perhaps other acidiphilous alpine species during glaciations. Palynological studies also confirmed the existence of large refugia during the LGM for subalpine trees along the lower part of the Romanian Carpathians that were followed in higher elevations by a graminoid-type vegetation (Feurdean *et al*. 2007). Carpathian populations of *C. curvula* probably did not experience regional extinctions during the glaciations, but on the contrary, they may have occupied much larger areas than today (Puşcaş *et al.* unpublished manuscript). Gene flow among populations was likely intensified at this time and may have resulted in a large common genetic pool. We thus suggest that vertical local migration from a large pan-Carpathian refugium is the most likely scenario for the recolonization of high summits in the Southeastern Carpathians. Similar mechanisms were also proposed for other alpine plants locally in the eastern Alps (Tribsch *et al*. 2002; Tribsch & Schönswetter 2003).

Despite the weak overall genetic differentiation in the Balkans demonstrated by AMOVA (Table 3), the NJ tree revealed that the most pronounced individualization of a population occurred in this area (especially in Rila and Pirin – Fig. 2). Biogeographers have already observed a high level of endemism in the different mountain ranges of the Balkans (Stojanov & Kitanov 1926). It has also been shown that these mountains were less affected by glacial cooling than any other northern mountain ranges (Bennet *et al*. 1991). The snow line during the last glaciation was estimated at 2200–2300 m altitude (Bazilova & Tonkov 2000), which is much higher than that assumed for the Alps, Pyrenees, or Carpathians. As a possible consequence, the vertical migrations of *C. curvula* during glaciations may have been less marked than for the Carpathians. Even during that period, the species kept an island distribution in this region. Therefore, the opportunities for gene flow among Balkan populations have remained reduced, preventing the overall genetic homogenization as described for the Carpathians.

Peripheral vs. core populations and genetic diversity

The genetic diversity (marker richness and marker polymorphism) of Pyrenean and to a lesser extent Balkan populations is lower than that of populations from the Alps or Carpathians. Furthermore, few specific markers were found in these regions. This is unexpected for the Balkans, one of the important European glacial refugia (Bennet *et al*. 1991; Taberlet *et al*. 1998; Hewitt 1999; Hewitt 2000). These southern Balkan mountains are all under Mediterranean climate influences (Bazilova & Tonkov 2000; Roussakova 2000; Stefanova & Ammann 2003). Field observations show that *C. curvula* does not form extensive swards in these areas, probably because of the dry summer. A similar trend has been documented for the southwestern Alps, where populations of *C. curvula* are very patchy (Choler & Michalet 2002). It is therefore proposed that these peripheral areas also constitute marginal habitats for the species. The peripheral range of a species is often characterized by ecologically marginal environments (Brown *et al*. 1996; Johannesson & André 2006). Several authors have reported a trend towards genetic depauperation in peripheral and/or marginal populations as compared with the core populations (Durka 1999; Lammi

et al. 1999; Lönn & Prentice 2002; Johannesson & André 2006). The combination of harsh environmental conditions and fragmented populations may also promote a higher genetic divergence among marginal populations (Gapare & Aitken 2005; Arnaud-Haond *et al*. 2006). On the other hand, Choler *et al*. (2004) reported evidence for genetic introgression between the two ecotypes of *C. curvula* in marginal habitats of the Alps. The molecular signatures found here for southwestern Alps and Pyrenean populations, however, do not correspond to introgressed forms.

Conclusions

This study is the first to investigate the phylogeographical structure of a dominant alpine graminoid of the European Alpine System. Nuclear and cpDNA data support an old divergence between the populations of *Carex curvula* from the Alps-Pyrenees and Carpathians-Balkans. We propose two different postglacial histories for the recolonization of high summits in the Alps and the Carpathians, two mountain ranges representing the core part of the current distribution of the species, by drawing inferences from the AFLP data. These two mechanisms include a large east–west recolonization wave in the Alps and a local vertical upward migration in the Carpathians. These two scenarios concur with the contrasted glacial histories of these two ranges. The peripheral regions (the Pyrenees and the Balkans), which are located in southern Europe, do not seem to correspond to major glacial refugia for this cold-tolerant species. Long-distance dispersal (for the Pyrenees) or long-term persistence on isolated mountains (for the Balkans) are the most likely explanations for the present reduced level of genetic diversity in these areas.

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