

The east-west-north colonization history of the Mediterranean and Europe by the coastal plant *Carex extensa* (Cyperaceae)

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Abstract

Coastal plants are ideal models for studying the colonization routes of species because of the simple linear distributions of these species. *Carex extensa* occurs mainly in salt marshes along the Mediterranean and European coasts. Variation in cpDNA sequences, amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs) of 24 populations were analysed to reconstruct its colonization history. Phylogenetic relationships indicate that *C. extensa* together with the South American *Carex vixdentata* and the southern African *Carex ecklonii* form a monophyletic group of halophilic species. Analyses of divergence times suggest that early lineage diversification may have occurred between the late Miocene and the late Pliocene (Messinian crisis). Phylogenetic and network analyses of cpDNA variation revealed the monophyly of the species and an ancestral haplotype contained in populations of the eastern Mediterranean. The AFLP and SSR analyses support a pattern of variation compatible with these two lineages. These analyses also show higher levels of genetic diversity and differentiation in the eastern population group, which underwent an east-to-west Mediterranean colonization. Quaternary climatic oscillations appear to have been responsible for the split between these two lineages. Secondary contacts may have taken place in areas near the Ligurian Sea in agreement with the gene flow detected in Corsican populations. The AFLP and SSR data accord with the 'tabula rasa' hypothesis in which a recent and rapid colonization of northern Europe took place from the western Mediterranean after the Last Glacial Maximum. The unbalanced west-east vs. west-north colonization may be as a result of 'high density blocking' effect.

Keywords: 5'trnK intron, AFLP, LGM, Messinian crisis, microsatellites, Pleistocene glaciations

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Introduction

Recently, there have been an increasing number of phylogeographical studies on coastal and halophilic plants in the Mediterranean Basin and European coasts (see Weising & Freitag 2007). Coastal plants with linear distribution ranges have been suggested as simple models that can be used to elucidate the evolutionary his-

tory of plant species (Kadereit *et al.* 2005). Multiple hypotheses and causes have been proposed to explain the present genetic structure and distribution of halophilic species (see Table 1). Primarily, researchers have concentrated on the climatic and geological history of halophilic plants' habitats, i.e. the geological events of the Tortonian-Messinian Miocene (Tremetsberger *et al.* 2004), Pleistocene glaciations (Clausing *et al.* 2000; Kadereit *et al.* 2005; Lambracht *et al.* 2007), habitat fragmentation by rivers (Ortiz *et al.* 2007) and the influence of sea straits (Westberg & Kadereit 2009) as barriers to

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Table 1 Phylogeographical patterns shown by plant species distributed in Mediterranean and European halophilic habitats (local studies were excluded)

Species	Distribution/Habitat	Molecular markers	Geographical and colonization patterns	References
<i>Armeria pungens</i>	Ibero-Corso-Sardinian/coasts	AFLP ^a nDNA ^b	Two main lineages: Gulf of Cadiz vs. Corso-Sardinian and southwest Portuguese populations. Colonization from southwest Portugal to Corsica and Sardinia	Piñeiro <i>et al.</i> (2007) ^a Piñeiro <i>et al.</i> (2009) ^b
<i>Atriplex halimus</i>	Mediterranean/coasts and inland	RAPD	Two main lineages: northern Mediterranean vs. southern Mediterranean populations	Ortiz-Dorda <i>et al.</i> (2005)
<i>Cakile maritima</i>	Mediterranean and European/coasts, very rarely inland	RAPD ^a ISSR ^a AFLP ^b	Several incipient geographical lineages in the Mediterranean and Europe but without significant support. Colonization of northern Europe after LGM	Clausing <i>et al.</i> (2000) ^a Kadereit <i>et al.</i> (2005) ^b
<i>Calystegia soldanella</i>	Worldwide, warm and temperate regions/coasts, occasionally inland	AFLP	Lack of phylogeographical structure. Multiple colonization of northern Europe	Arafeh & Kadereit (2006)
<i>Carex extensa</i>	Mediterranean and European/coasts, very rarely inland	cpDNA AFLP nSSR	Origin in the Messinian crisis. East-to-west colonization and subsequent differentiation during Pleistocene glaciations. Secondary contact nearby the Ligurian Sea. Colonizations of northern Europe from the western Mediterranean after LGM	This study
<i>Crithmum maritimum</i>	Mediterranean and European/coasts	AFLP	Several incipient geographical lineages in the Mediterranean and Europe but without significant support. Colonization of northern Europe after LGM	Kadereit <i>et al.</i> (2005)
<i>Eryngium maritimum</i>	Mediterranean and European/coasts	RAPD ^a ISSR ^a AFLP ^b	Two main lineages: Mediterranean vs. Atlantic populations. Incipient groups within the two main lineages. Colonization of northern Europe after LGM	Clausing <i>et al.</i> (2000) ^a Kadereit <i>et al.</i> (2005) ^b
<i>Halimione portulacoides</i>	Mediterranean and European/coasts, very rarely inland	AFLP	Two main lineages: Mediterranean vs. Atlantic populations. Incipient groups within the two main lineages. Colonization of northern Europe after LGM	Kadereit <i>et al.</i> (2005)
<i>Hordeum marinum</i>	Mediterranean and European/coasts and inland	cpDNA cpSSR	Subdivision between Iberian Peninsula and the remaining Mediterranean. Vicariance during Pleistocene glaciations	Jakob <i>et al.</i> (2007)
<i>Hypochaeris salzmanniana</i>	SW Spain and Atlantic Morocco/coasts	AFLP	Differentiation among Moroccan populations, but high gene flow between Moroccan and S Iberian populations. Northward migration during Pleistocene glaciations	Ortiz <i>et al.</i> (2007)
<i>Microcnemum coralloides</i>	Mediterranean/inland	rDNA cpDNA	Two main lineages: eastern vs. western Mediterranean. East-to-west colonization during Pleistocene glaciations	Kadereit & Yaprak (2008)
<i>Posidonia oceanica</i>	Mediterranean/submersed coasts	nSSR	Two main lineages: eastern vs. western Mediterranean. Vicariance during Pleistocene glaciations	Arnaud-Haond <i>et al.</i> (2007)

Table 1 Continued

Species	Distribution/Habitat	Molecular markers	Geographical and colonization patterns	References
<i>Salsola kali</i> s.l.	Mediterranean and European/coasts and inland	AFLP	Two main lineages: Mediterranean vs. Atlantic populations. Incipient groups within the two main lineages. Colonization of northern Europe after LGM	Kadereit <i>et al.</i> (2005)
<i>Suaeda maritima</i> complex	Mediterranean and Europe/coasts and inland	rDNA cpDNA	No clear taxonomic vs. genetic or geographical vs. genetic correlation. Northward migration	See Weising & Freitag (2007)
<i>Triglochin maritima</i>	Circumboreal/coasts and inland	AFLP	Two main lineages: western and northern Atlantic populations vs. northern European, central European and Mediterranean populations. Two independent colonization of northern Europe after LGM	Lambracht <i>et al.</i> (2007)
<i>Zostera marina</i>	Northern Hemisphere, temperate regions/submersed coasts	nSSR	Four main lineages: two New World lineages and two Old World lineages (Mediterranean, Black Sea and Portugal vs. the remaining Europe). Northward migration of Europe and eastern America after LGM	Olsen <i>et al.</i> (2004)

Superscripts indicate when the results from different molecular markers were published in different studies.

gene flow. By contrast, other authors have focused on the present requirements of plants such as ecological requirements in the context of seed germination, plant growth and survival conditions (Jakob *et al.* 2007; Piñeiro *et al.* 2007). Additional factors have also been proposed, such as sea currents, which can constitute a barrier or transport route for propagules (Kadereit *et al.* 2005; Gandour *et al.* 2008; Westberg & Kadereit 2009). Some authors even further interpreted the data and proposed specific properties of species, i.e. high dispersal capacity or prolonged clonal age, to account for colonization success (Arafeh & Kadereit 2006; Gandour *et al.* 2008).

These studies include the hypothesis that surviving populations will contain higher amounts of genetic variation than recently colonized areas (founder effect) (Taberlet *et al.* 1998; Hewitt 2000). Accordingly, geographical clines of decreasing genetic variation can help us to interpret migration routes and the direction of colonization (Ibrahim *et al.* 1996; Schaal & Olsen 2000). Unfortunately, misinterpretation of diversity patterns can occur when (i) the source populations experience posterior bottlenecks (Coyer *et al.* 2003; Chiang *et al.* 2006); (ii) founded populations are the result of multiple colonization events (Amsellen *et al.* 2000; Olsen *et al.* 2004); and (iii) higher levels of diversity and heterozygosity are observed in admixture populations from secondary contacts between previously differentiated lineages (Kropf *et al.* 2002). As a consequence, inferring the evolutionary history of populations that have

resulted from multiple events can become a very difficult task. Therefore, researchers should analyse different molecular markers from different genomes and with different shift rates to disentangle the different partial steps of the evolutionary history of a species (Templeton 2007).

With *c.* 2000 species worldwide (Reznicek 1990), *Carex* is one of the largest non-agamospermic angiosperm genus in the world and one that has undergone a relatively rapid radiation (Magallon & Sanderson 2001). It has been hypothesized that this broad distributional pattern and rapid radiation in the genus are related to successful colonization and to rapid chromosome evolution (Hipp 2007; Escudero *et al.* 2009). In addition, this genus has a high diversification in the Mediterranean and Europe with *c.* 200 species (Maire 1957; Chater 1980). Patterns of population diversification may help reconstruct the processes responsible for the colonization history of this successful genus of angiosperms. Among all *Carex* candidates, *C. extensa* is a convenient species to test early stages of rapid colonization because of: (i) its wide distributional range spanning Europe and the Mediterranean (Hultén 1962); (ii) a limited, linear distribution associated with salt marshes in coasts, very rarely inland (see map of European and Mediterranean salt marshes in Silliman *et al.* 2009); (iii) potentially active gene flow by pollen (anemophily) and seed (long dormancy; potential hydrochory and zoochory by waterbirds) (Ridley 1930; Schütz 1998; Escudero *et al.* 2008b); (iv) the distribution of the two species (*Carex*

ecklonii, southern Africa; *Carex vixdentata*, South America) most closely related, which are separated by thousands of kilometers (Escudero & Luceño 2009; Escudero *et al.* 2009). In addition, early stages of differentiation of *C. extensa* is manifested by previous studies in which low molecular variation (a unique ITS ribotype) and cytogenetic stability (always $2n = 30^{II}$) regarding to other species of sect. *Spirostachyae* (Luceño & Castroviejo 1993; Escudero *et al.* 2008a; Escudero & Luceño 2009).

The main objective of this study was to reconstruct the colonization history of *C. extensa* using plastid sequence, amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR) data. The specific aims were (i) to date the origin of the diversification of the closely related halophilic species of *C. extensa*; (ii) to infer the genetic structure of *C. extensa* to identify refugia and colonization routes; (iii) to elucidate whether climatic events are related to the current genetic structure of this species; and (iv) to compare our results with the distributional and dispersal patterns of other halophilic species.

Materials and methods

The study species

Carex extensa Good. (subsect. *Spirostachyae* of sect. *Spirostachyae*; Escudero & Luceño 2009) is a diploid ($2n = 60$), wind-pollinated, medium cold-tolerant perennial herb (minimum generation time is two years). This species is suspected to be inbred, which has been inferred in previous studies of some caespitose *Carex* species (see Arens *et al.* 2005). The taxon is well characterized within sect. *Spirostachyae* by its caespitose habit (without creeping rhizomes), canaliculated or rolled leaves that are longer than its stem, a single upper male spike and 3–5 lower female spikes (sometimes gynecandrous) at the top of the stems (or, at times, one relatively distant spike), and a lower bract much longer than the inflorescence (Luceño & Escudero 2008). This species occurs along the coasts of the Black Sea, the Mediterranean Basin, the northern African and European Atlantic coasts, the North Sea and the Baltic Sea coasts, with a few populations in the Azores and Madeira islands. *Carex extensa* has no continuous distribution along the coastal line because the species has high ecological requirements and grows only in coastal salt marshes, very rarely inland (see map in Silliman *et al.* 2009).

Sampling strategy for estimating diversification times

The ITS study to estimate diversification times included a total of 47 ITS sequences from 47 ingroup species.

Carex extensa and 20 more species of sect. *Spirostachyae* (including *Carex ecklonii* and *Carex vixdentata*) were analysed, together with 15 more species of subgenus *Carex* (Waterway & Starr 2007). Eleven species representing the diversification of the other three main clades of tribe *Cariceae* (*Schoenoxiphium*, unispicate *Carex* and *Vignea* clades; Waterway & Starr 2007) were also included in the ingroup. Two species were used as outgroup samples (*Eriophorum vaginatum* and *Scirpus polystachyus*). All sequences were taken from the GenBank data base (<http://www.ncbi.nlm.nih.gov/>, Material S1A).

In the *trnK* study to estimate diversification times, a total of 40 *trnK* intron accessions of 40 ingroup species were included. *Carex extensa* and 20 more species of sect. *Spirostachyae* (including *C. ecklonii* and *C. vixdentata*) were analysed, together with 14 more species of subgenus *Carex* (Waterway & Starr 2007; Escudero & Luceño 2009). Five species representing the *Schoenoxiphium*, unispicate *Carex* and *Vignea* clades (Waterway & Starr 2007) were also included in the ingroup. Two species were used as outgroup samples (*Cyperus alternifolius* and *Schoenoplectus supinus*). All sequences were gathered from the GenBank database Material S1B.

Sampling strategy for phylogeny and phylogeography

We sampled (5–) 8–10 individuals per population in 24 populations (224 individuals) of *C. extensa*, which were representative of its distribution: 10 populations from the Mediterranean Basin coasts (representing the Aegean Sea, the Ionic Sea and the western Mediterranean Basin), 10 populations from the Atlantic coasts, three populations from the North Sea, and one population from the Baltic Sea (see Table 2, Fig. 1).

For the sequencing study, the 5'*trnK* intron and the intergenic *ycf6-psbM* spacer were sequenced. The first was shown the most variable cpDNA region for *Carex* sect. *Spirostachyae* (Escudero & Luceño 2009) in comparison with *trnL-F*, *trnT-L* and *rps16*. The second was shown to be the most variable cpDNA region in other *Carex* species of subgenus *Carex* (P. Jiménez-Mejías pers. com.) in comparison with the cpDNA region tested in Shaw *et al.* (2005). Sixty-six individuals of *C. extensa* from the 24 populations (2–4 per population; Table 2) were included in the *trnK* (Material S1C) and *ycf6-psbM* (Material S1D) study. Three accessions of the most closely related species, *C. ecklonii* (two accessions) and *C. vixdentata* (one accession), from subsect. *Spirostachyae* were included in the *trnK* study. Two accessions representing the other lineages of subsect. *Spirostachyae* (one from *Carex distans* and the other from *Carex tasmanica*) and two accessions representing subsect. *Elatae* of sect. *Spirostachyae* (one from *Carex helodes* and the other from *Carex binervis*) were included as outgroup samples.

Table 2 List of studied populations of *Carex extensa*. Population coding (Pop.) indicating eastern group, southwestern subgroup, or northwestern subgroup; locality, longitude (Long.) and latitude (Lat.) (in decimal degrees); and the number of individuals in *trnK*, AFLP, and SSR (N) are also indicated. Capital letters A/B indicate the two haplotypes detected within *C. extensa*. The gene diversity index (H_S), observed heterozygosity index (H_O) and inbreeding index (F_{IS}) are also indicated. (* $P < 0.05$)

Pop.	Locality (Log/Lat)	Source	<i>trnK</i>		AFLP		SSR				
			N	Hap.	N	H_S	N	H_O	H_S	F_{IS}	
Eastern group	TUR	Turkey, Çanakhale, between Lapseki and Çanakhale, Strait of Dardanelos, 26.66240/40.33118	M. Escudero <i>et al.</i> , 75ME06	3	A	6	0.043	6	0.083	0.266	0.677*
	GRE1	Greece, Amondia, Aqueronte River, 20.45608/39.27977	P. Vargas and M. Luceño, 259PV04	2	A	5	0.018	5	0.000	0.000	NA
	GRE2	Greece, Fthiotida, Lamia, Paralia-Pelasgia, 22.83720/38.90620	M. Luceño <i>et al.</i> , 4ML08	3	A	7	0.045	—	—	—	—
	ITA1	Italy, Toscana, Orbetello, Natural Reserve Orbetello Lake, 11.21450/42.47006	M. Escudero and R. Piñeiro, 129ME07	3	A	10	0.047	10	0.000	0.133	1.000*
	ITA2	Italy, Lazio, Sabaudia, Circeo Natural Park, Cabrolage Lake, 13.01438/41.29880	M. Escudero and R. Piñeiro, 130ME07	3	A	10	0.033	10	0.000	0.000	NA
	FRA-COR1	France, Corsica, Biguglia, Biguglia Lake, 9.45992/42.64192	M. Escudero and M. Luceño, 69ME07	4	A	8	0.100	9	0.194	0.264	0.228
Southwestern subgroup	FRA-COR2	France, Corsica, Porto Vecchio, 9.32447/41.61822	M. Escudero and M. Luceño, 91ME07	4	B	10	0.064	10	0.050	0.088	0.419
	SPA-MED1	Spain, Barcelona, El Prat, between the beach and the airport, 2.09823/41.28540	M. Escudero, 128ME07	2	B	10	0.047	10	0.000	0.088	1.000*
	SPA-MED2	Spain, Valencia, El Saler, -0.32235/39.26858	E. Carrió <i>et al.</i> , s.n.	2	B	10	0.058	10	0.050	0.050	-0.059
	SPA-ATL1	Spain, Cádiz, Conil de la Frontera, Playa de Bateles, -6.09232/36.27202	P. Jiménez and R. Olmedo, 459PJM05	3	B	10	0.023	10	0.000	0.000	NA
	SPA-ATL2	Spain, Huelva, El Rompido, La Flecha del Rompido, -7.17691/37.21778	E. Sánchez Gullón, s.n.	3	B	10	0.047	10	0.250	0.237	-0.125
	MOR1	Morocco, near Larache, Moulay-Bousselhan, Merja-Zerja, -6.28289/34.87678	M. Escudero <i>et al.</i> , 115ME07	3	B	9	0.061	10	0.025	0.104	0.757*
	MOR2	Morocco, Martil, Martil River, -5.28178/35.63822	M. Escudero <i>et al.</i> , 120ME07	3	B	10	0.030	10	0.000	0.000	NA
	POR1	Portugal, Alto Alentejo, Sines, -8.80469/37.92297	M. Escudero <i>et al.</i> , 65ME07	3	B	10	0.017	10	0.000	0.088	1.000*
	POR2	Portugal, Aveiro, Vagos, -8.76044/40.55961	M. Escudero <i>et al.</i> , 58ME07	3	B	9	0.059	9	0.167	0.264	0.342
Northwestern subgroup	SPA-ATL3	Spain, Asturias, near Villaviciosa, Ría Rodiles, -5.38835/43.51795	P. Jiménez <i>et al.</i> , 434PJM04	2	B	10	0.027	10	0.000	0.117	1.000*
	SPA-ATL4	Spain, Burgos, Puérnigas, -3.47066/43.16561	J.A. Alejandro and M.J. Escalante, 1001/2007	3	B	9	0.021	9	0.028	0.231	0.879*
	SPA-ATL5	Spain, Gipuzkoa, Zarautz, Inurritza, -2.38833/43.28953	J. Garmendia and L. Oreja, s.n.	2	B	8	0.017	10	0.000	0.050	1.000*
	FRA-ATL1	France, Vannes, Belz, -3.17572/47.67846	M. Escudero and M. Luceño, 4ME08	3	B	10	0.045	—	—	—	—

Table 2 Continued

Pop.	Locality (Log/Lat)	Source	<i>trnK</i>		AFLP		SSR			
			N	Hap.	N	H _S	N	H _O	H _S	F _{IS}
FRA-ATL2	France, Haute-Normandie, Havre, 0.27178/49.43410	M. Escudero and M. Luceño, 7ME08	3	B	10	0.045	—	—	—	—
SCOT	Scotland, Dunbar, Barris Ness, -2.50952/56.00151	S. Martín-Bravo <i>et al.</i> , 130SMB07	2	B	8	0.037	10	0.000	0.000	NA
NET1	The Netherlands, Zuidland, Oostvoorne, Duinen van Voorne, 4.05531/51.91295	M. Escudero, 133ME07	2	B	8	0.008	10	0.025	0.127	0.800*
NET2	The Netherlands, Schiermonnikoog Island, 6.25718/53.49993	M. Escudero, 135ME07	2	B	9	0.031	10	0.000	0.133	1.000*
SWE	Sweden, Vellinge, Skanör, 12.84372/55.41220	S. Martín-Bravo <i>et al.</i> , 188SMB08	3	B	9	0.026	—	—	—	—

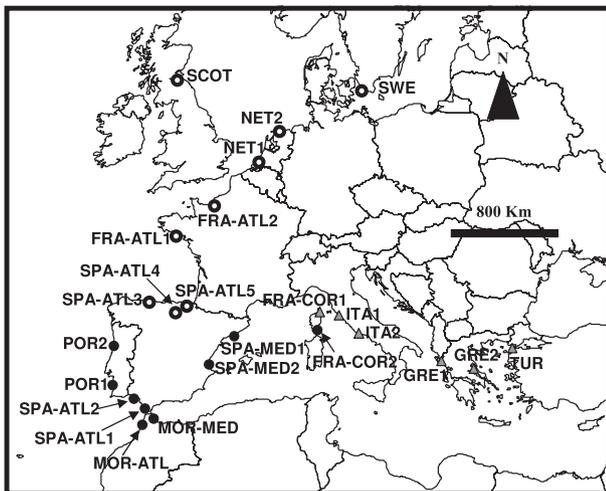


Fig. 1 Sample map of *Carex extensa* with population coding as in Table 2. Grey triangles indicate population locations of the eastern group with haplotype A. Circles indicate population locations of the western group with haplotype B (southwestern subgroup in black and northwestern subgroup in black and white).

These seven *trnK* additional accessions were gathered from the GenBank database (Material S1C) from the study by Escudero & Luceño (2009). In total, 73 samples were included in *trnK* study (Material S1C). Two hundred and fifteen individuals from the same 24 *C. extensa* populations [(5-) 8–10 per population] were included in the AFLP study (see Table 2). One hundred and eighty-eight individuals from 20 of the 24 populations [(5-) 9–10 individuals per population] were included in the SSR study. The represented populations included nine from the Mediterranean Basin coasts (representing the Aegean Sea and the western Mediterranean Basin), eight from the Atlantic coasts, and three from the North

Sea (see Table 2). The SSR and AFLP studies displayed homologous samplings. This approach implies that all individuals of the AFLP study from these 20 populations (179 individuals) were included in the SSR study (188 individuals in total).

DNA extraction, sequencing, AFLPs and SSRs

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen). The *trnK* intron region was amplified and sequenced as described by Escudero & Luceño (2009). The *ycf6-psbM* was amplified and sequenced as described by Shaw *et al.* (2005).

The AFLP procedure of Gaudeul *et al.* (2000) was followed. To compare the present study with a previous one on a closely related species, *C. helodes* (Escudero *et al.* 2008b), the same *EcoRI* AGA (6-FAM)–*MseI* CAC and *EcoRI* AGG (VIC)–*MseI* CAA primer combinations were chosen. For each individual, 0.5 µL of 6-FAM-labelled and 0.5 µL of VIC-labelled selective PCR products were combined with 0.5 µL of GeneScan 500 LIZ (Applied Biosystems) and 13.5 µL of formamide and run on a capillary sequencer (ABI Prism 3700 DNA analyzer; Applied Biosystems). One replicate per population (24) was used to test for reproducibility.

For the SSR study, the amplification conditions were 94 °C for 3 min.; 35 cycles of 94 °C for 30 s, ramp: 1 °C/s to 50 °C, 50 °C for 30 s, ramp: 1 °C/s to 72 °C, and 72 °C for 2 min.; and 72 °C for 10 min. PCR reactions were performed in a total volume of 20 µL per sample with a 1× PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 2 µM forward and reverse primers, and 0.015 U/µL of AmpliTaq Gold™ DNA Polymerase (Applied Biosystems). If amplification failed, we used the QIAGEN® Multiplex PCR (Applied Biosystems) for

optimization. The pilot proofs were performed using 20 microsatellite loci (CL88, CL100, CL101, CL102, CL113 and CL114 designed for *Carex limosa* and published here (Table S1); Cko1-9, Cko1-11, Cko1-12, Cko1-47, Cko1-68, Cko1-78, Cko1-80, Cko1-134, Cko2-56, Cko2-112, Cko2-113, Cko2-118, Cko2-135 and Cko2-139 designed for *Carex kobomugi* by Ohsako & Yamane (2007)) with the silver stain sequencing method (Promega Silver Sequence™ DNA Sequencing System). The complete data set was genotyped based on four microsatellite loci that displayed variability (CL101, CL113, Cko2-112 and Cko2-118). Forward primers were labelled (HEX-CL101F, 6-FAM-CL113F, NED-Cko2-112F and 6-FAM-Cko2-118F) for complete data set amplification. PCR products were run on a capillary sequencer (ABI Prism 3700 DNA analyzer; Applied Biosystems). To check the homology of the different observed alleles at each locus, for each locus we amplified (see conditions above) 18 homozygous (for the four microsatellite loci) individuals representing the geographical and allelic diversity (72 sequences in total). PCR products were sequenced using dye terminators (Big Dye Terminator version 2.0, Applied Biosystems) and run on a capillary sequencer (ABI Prism 3700 DNA analyzer, Applied Biosystems).

Data analysis to estimate diversification times

Absolute time estimations of the stem node of *C. extensa* were performed to provide a temporal framework for biogeographic inferences. Crown node estimation of *C. extensa* was not made because of the poor differentiation detected in ITS (a single ribotype, Escudero *et al.* 2008a) and *trnK* (present results).

For estimating diversification times, we used two methods: (i) the parametric uncorrelated log-normal relaxed clock method (Drummond *et al.* 2006); and (ii) the semiparametric penalized likelihood method (Sanderson 2002). In the first method, phylogenetic trees from the ITS matrix (SM2) with branches proportional to time were estimated using the uncorrelated log-normal relaxed clock model, as implemented in BEAST version 1.4.8 (Drummond & Rambaut 2007). The GTR + I + G substitution model was selected based on the Akaike Information Criterion (AIC) in MrModeltest 1.1b (Nylander 2002). The analysis was conducted using four independent MCMC runs of 10 000 000 generations each to assess convergence, assuming the Yule tree prior with mean substitution rate set at 1.0. Node ages were estimated in years, using previously reported ITS clock calibrations for herbaceous angiosperms (Kay *et al.* 2006). The ITS matrix (excluding 5' 8S and indels; SM2) was analysed as described above, with monophyly of *C. extensa*, *C. ecklonii* and *C. vixdentata*. Age

distributions were estimated based on 5000 subsamples drawn at random in R v 2.8.1 (R Development Core Team, 2008; code available from A. L. Hipp's website: http://redwood.mortonarb.org/lab_pages/hipp/).

For the second method, AIC as implemented in MrModeltest 1.1b (Nylander 2002) was used for selecting the simplest model sequence evolution that best fit the data, ITS and *trnK* matrices (SM3 and SM4 respectively). Bayesian analyses were performed under the selected model using MRBAYES 3.0b4 (Ronquist & Huelshenbeck 2003). Four Markov Chain Monte Carlo runs were performed simultaneously in the Bayesian analysis for 5 000 000 generations with an interval of 100 generations. Burn-in was evaluated over generations. After trees yielded before the likelihood values became stationary were discarded, the remaining trees were compiled in a majority rule consensus tree, using posterior probability as a measure of clade support (Alfaro *et al.* 2003). Two calibrating points for the *trnK* study and three calibrating points for the second ITS study were used for the estimations (because of a better resolution in the ITS tree): (i) the origin of *Cariceae* (fossil data: min. 59 Ma; for the ITS and *trnK* studies); (ii) the split between subgenera *Carex* and *Vignea* (43 ± 12 Ma from Escudero *et al.* 2009; only for the ITS study); and (iii) the diversification of subgenus *Carex* (36 ± 12 Ma from Escudero *et al.* 2009; for the ITS and *trnK* studies). We evaluated rate heterogeneity among lineages by means of the Langley and Fitch test (Langley & Fitch 1974). The null hypothesis of the molecular clock (constant rate) was rejected; therefore, divergence times were estimated by applying a penalized likelihood method (PL; Sanderson 2002) with the Truncated Newton algorithm, as implemented in the rate smoothing program r8s version 1.71 (Sanderson 2004). We obtained the smoothing parameter for these analyses using a cross-validation procedure, which involves pruning terminal branches and predicting the rate along that branch. The PL search parameters included five initial and five perturbed restarts. The best smoothing parameter resulting from the cross-validation was 3.2 in both studies. The standard errors (SD) of the divergence time estimates were obtained using a nonparametric bootstrap procedure (Baldwin & Sanderson 1998), which involves the generation of 1000 resampled data matrices with the SEQBOOT program, implemented in PHYLIP version 3.67 (Felsenstein 2007).

Data analysis for phylogeny and network reconstructions

The *ycf6-psbM* region did not render any variation in *C. extensa*; therefore, it was excluded of the analysis (see results). The 73 *trnK* accessions were manually aligned

in a matrix. Gaps were analysed as missing data and were coded as absence/presence (SM5). The resulting plastid haplotype matrix, excluding the two accessions of subsect. *Elatae* (*C. helodes* and *C. binervis*), was analysed under statistical parsimony, as implemented in the program *TCS* 1.20 (Templeton *et al.* 1992; Clement *et al.* 2000). The parsimony analyses were conducted under the Fitch parsimony, as implemented in TNT (Goloboff *et al.* 2003), with equal weighting of all characters and transitions:transversions. Heuristic searches were replicated 10 000 times with tree bisection–reconnection branch swapping, retaining a maximum of two trees in each replicate. A second heuristic search set was made based on the trees that were retained in RAM memory in the first heuristic search set. The strict consensus tree was performed from all trees obtained in the heuristic searches. Clade supports were assessed by bootstrapping with 10 000 resamplings based on the conditions of the first heuristic search set.

Data, excluding the indel coding, were analysed using *MRBAYES* 3.0b4 (Ronquist & Huelsenbeck 2003), as described in the phylogenetic analysis for the estimation of diversification times (see above).

AFLP and SSR analyses for phylogeography

For clarity, groupings of populations are defined prior to these phylogeographical analyses. The complete data set of *C. extensa* was organized in two groups: eastern vs. western. This split was based on the two haplotypes of *C. extensa* (see results). Subsequently, the western group was structured in two subgroups: southwestern vs. northwestern. This subgrouping was based on the inferred distribution of *C. extensa* in Last Glacial Maximum (LGM). Accordingly, we inferred the distribution in LGM from: (i) the cold-tolerance of species from the present distribution and the present temperature latitudinal distribution (Hann 1987); and (ii) the temperature latitudinal distribution in the LGM (van Andel 2002). *Carex extensa* is a medium cold-tolerant species because its present distribution occurs at higher latitudes than the current 14 °C mean July isotherm e.g. in northern Scotland. By contrast, no populations currently occur at higher latitudes than the present 12 °C mean July isotherm. Accordingly, we split the western group into northwestern and southwestern subgroups following the 12 °C mean June–July–August LGM isotherm. Specifically, northern populations of the 12 °C mean June–July–August LGM isotherm were termed the northwestern subgroup and southern populations were termed the southwestern subgroup.

In the AFLP study, GeneMapper Software v3.7 (Applied Biosystems) was used to visualize and score the DNA fragments. A present/absent (i.e. 1/0) matrix

(SM6) was elaborated to perform reproducibility tests and subsequent analyses (Meudt & Clarke 2007). Gene diversity indexes (H_S , H_T) and F_{ST} values (Nei 1972, 1987) were calculated using the program *POPGENE* version 1.31 (Yeh *et al.* 1999) and assuming dominant diploid markers at Hardy–Weinberg (HW) equilibrium. In species or populations with absence of HW equilibrium (see below), Nei genetic diversity does not provide a heterozygosity measurement, but displays a reliable estimate of genetic variability (Lowe *et al.* 2005). This index was calculated (i) at the population level (24 populations); (ii) at the group level based on the distribution of the two detected haplotypes (Table 2), where groups included the eastern group (six populations) and the western group (18 populations); and (iii) at the subgroup level within the western group, examining the southwestern subgroup (nine populations) and the northwestern subgroup (nine populations). Pairwise genetic distances between the 24 populations were calculated using the Nei distances [following Lynch & Milligan 1994 implemented in *AFLP-SURV* version 1.0 (Vekemans 2002)]. Branch reliability was assessed by bootstrapping (1000 replicates of the original matrix). Analysis of tree topology and support was performed using Neighbor Joining (NJ) of the *NEIGHBOR* and *CONSENSE* packages of *PHYLIP* version 3.7 (Felsenstein 2007). Pairwise genetic distances between all AFLP phenotypes were calculated using the Nei–Li distances (Nei & Li 1979) and implemented with the NJ using *PAUP** (Swofford 2002). Branch reliability was assessed by bootstrapping (10 000 replicates). Bayesian Analysis of Population Structure (*BAPS* version 4.14; Corander *et al.* 2006) was used to estimate the population structure by clustering individuals and populations into panmictic groups (mixture clustering). We ran 10 simulations from $K = 2$ to $K = 20$. An analysis of the molecular variance (AMOVA) was performed with Φ_{PT} implemented and recommended for multiple marker studies in the software *GENALEX* version 6 (Peakall & Smouse 2006). This AMOVA was performed (i) for the whole data set [(a) with two hierarchical levels and (b) with three hierarchical levels with two main groups (the eastern group vs. the western group)]; and (ii) with two hierarchical levels in four subsets of data [(a) the eastern group, (b) the western group, (c) the southwestern subgroup and (d) the northwestern subgroup]. The pairwise correlation between genetic (Nei 1972) and geographical distances (direct and coastline geographic distances) was estimated with a Mantel test (implemented in *Genalex*) for the whole data set and for the four data subsets described above.

In the SSR study, four microsatellites showed variation: CL101, CL113 (published here, see Table S1), Cko2-112 and Cko2-118 (Ohsako & Yamane 2007;

Table S1) (SM7). These microsatellites were then used to assess the population genetic structure in *C. extensa*. Genotyper v2.0 (Applied Biosystems) was used to visualize and score the DNA fragments. We used MICRO-CHECKER to identify the presence of null alleles at each locus for each population (van Oosterhout *et al.* 2004). For each population, we estimated genetic diversity across loci using the average observed heterozygosity (H_O), gene diversity (H_S ; Nei 1987) and inbreeding (F_{IS}) (including the significance levels in each population) (F_{STAT} , Goudet 2001; GENALEX version 6, Peakall & Smouse 2006). We analysed patterns of genetic diversity using F_{STAT} (Goudet 2001) and GENALEX (version 6, Peakall & Smouse 2006) by calculating global estimates of H_T , H_S , H_O , F_{ST} and F_{IS} and by testing differences between groups or subgroups (eastern vs. western group; southwestern vs. northwestern subgroup). We used 1000 permutations when the units of randomization were populations. We assessed the appropriateness of R_{ST} and R_{IS} vs. F_{ST} and F_{IS} for our data using SPAGeDI ver. 1.2d (Hardy & Vekemans 2002) through allele-size permutations using 1000 permutations (Hardy *et al.* 2003). Finally, we explored Hardy–Weinberg equilibrium of our data set over F_{IS} (F_{STAT} ; Goudet 2001). We performed the analyses with several data sets: (i) whole dataset (20 population); (ii) the eastern group (five populations); (iii) the western group (15 populations); (iv) the southwestern subgroup (nine populations); and (v) the northwestern subgroup (six populations). The software program BAPS, version 4.14 (Corander *et al.* 2006), was used to estimate the population structure by clustering individuals and groups into panmictic groups (mixture clustering). We ran 10 simulations from $K = 2$ to $K = 20$. We calculated DA genetic distances (Nei *et al.* 1983) from allelic frequencies and used the resulting distance matrix to create an NJ tree. We assessed the reliability of the tree using 1000 bootstraps replicates. These analyses were performed using MSA version 4.05 (Dieringer & Schlöterer 2003) and the NEIGHBOR and CONSENSE packages of PHYLIP version 3.7 (Felsenstein 2007). The AMOVA and Mantel tests were performed with the program GENALEX version 6 (Peakall & Smouse 2006), similar to the AFLP data analyses.

Results

Times of diversification

The parametric analysis of ITS indicates that the diversification of *Carex ecklonii*, *Carex vixdentata* and *Carex extensa* is dated between the end of the Miocene and the middle of the Pliocene (4.8 ± 2.2 Ma). The ITS and *trnK* phylogenies (results not shown) were congruent with

previously reported results (Waterway & Starr 2007). In contrast to the parametric ITS estimation, the semiparametric analysis of the ITS tree (result not shown) dated the diversification of this plant group between the middle and end of the Miocene (11.5 ± 3.1 Ma). The semiparametric analysis of the *trnK* tree (result not shown) estimated diversification times between the end of the Miocene and the end of the Pliocene (4.3 ± 3.6 Ma).

Sequence analysis

The *ycf6-psbM* region did not show any variation in *C. extensa* (SM1D). Nevertheless, two different *trnK* haplotypes were identified within *C. extensa* based on a 21 bp indel at position 115–136 of the aligned matrix (position 114 of the *C. extensa trnK* sequences). One haplotype (termed haplotype A) characterized populations from the eastern Mediterranean Basin (TUR, GRE1, GRE2), Italy (ITA1, ITA2) and northern Corsica (FRA-COR1) (eastern group), and a second haplotype (termed haplotype B) characterized populations from the rest of the sample (western group) (Tables 2, 3; Fig. 1). The statistical parsimony analysis of the *trnK* yielded a single network, which included six plastid haplotypes with one loop (Fig. 2A). Haplotype B of *C. extensa* was only connected to haplotype A of *C. extensa* (Fig. 2A), which was in turn connected to the rest of the haplotype network.

The characteristics of the MP analysis are also shown in Table 3. The parsimony and Bayesian phylogenetic reconstructions confirmed the previously reported monophyly of *C. extensa* (Escudero & Luceño 2009) and *C. ecklonii* or *C. vixdentata* as sister-group candidates.

Table 3 Summary of *trnK* sequence characteristics and major features obtained from the phylogenetic reconstructions

Data set	5' <i>trnK</i> intron
<i>Carex extensa</i>	
Length range (bp)	658–679
Number of variables vs. informative characters	0/0
Number of indels	1 (21 bp)
Sect. <i>Spirostachyae</i>	
Length range (bp)	577–679
Aligned length (bp)	690
Number of variables vs. informative characters	29/11
Number of indels	9
Number of variables vs. informative indels	9/3
CI	0.9744
RI	0.9667
Number of steps	39
Number of most parsimonious trees	1

Again, two groups of populations based on the two haplotypes were reported: the eastern group (haplotype A) vs. the western group (haplotype B) (Fig. 2B).



Fig. 2 (A) Statistical parsimony network of six cpDNA haplotypes, including the two haplotypes detected in *Carex extensa* and the four haplotypes detected in the remaining species. Species names indicate the six haplotypes, black dots refer to the haplotypes that were extinct or not found, and each line between haplotypes indicate sequence mutation steps. (B) Strict consensus of the three most parsimonious trees was found in the analysis of the 5' *trnK* intron. Numbers above branches represent bootstrap percentages >50% in the Maximum Parsimony analysis, and values below branches indicate posterior probability >0.90 in the Bayesian analysis.

Table 4 Results from the analyses of AFLPs and SSRs of *Carex extensa* indicating gene diversity (H_T , H_S , H_O), differentiation (F_{ST} , R_{ST}) and inbreeding (F_{IS} , R_{IS}) indices for five different data sets: the whole data set and four subsets (eastern group, western group, southwestern subgroup and northwestern subgroup)

Data set	AFLP			SSR						
	H_T	H_S	F_{ST}	H_T	H_S	H_O	F_{ST}	F_{IS}	R_{ST}	R_{IS}
<i>Carex extensa</i>	0.106	0.039	0.631	0.539	0.100	0.044	0.810	0.601 ^d	0.864	0.381 ^b
Eastern group	0.119	0.048	0.598	0.241	0.129	0.056	0.532	0.563 ^d	0.417	0.489
Western group	0.067	0.037	0.452	0.366	0.101	0.039	0.738	0.615 ^d	0.846	0.367 ^c
SW subgroup	0.078	0.045	0.427	0.376	0.097	0.059	0.764	0.392 ^{d a}	0.851	0.026 ^c
NW subgroup	0.045	0.029	0.360	0.258	0.107	0.008	0.632	0.921 ^{d a}	0.787	0.950

^a $P < 0.01$ for significance of differences between groups.

^b $P < 0.01$ and ^c $P < 0.05$ for significance of differences after allele-size permutations.

^d $P = 0.001$ for significance of inbreeding.

AFLP analysis

The reproducibility tests gave values of 95% when all scored markers and replicates were compared. Eighty AFLP loci were included in the complete analysis (32 from *EcoRI* AGG [VIC]–*MseI* CAA and 48 from *EcoRI* AGA [6-FAM]–*MseI* CAC). The average within-population expected heterozygosity (H_S) varied from 0.008 (in NET1) to 0.100 (FRA-COR1) (Table 2). The total expected heterozygosity (H_T) was 0.106 and the average within-population expected heterozygosity (H_S) was 0.039 (Table 4). The total expected heterozygosity, average within-population expected heterozygosity and fixation index were higher in the eastern group ($H_T = 0.119$, $H_S = 0.048$, $F_{ST} = 0.598$) than in the western group ($H_T = 0.067$, $H_S = 0.037$, $F_{ST} = 0.452$). Similarly, the total expected heterozygosity, average within population expected heterozygosity and fixation index were higher in the southwestern subgroup ($H_T = 0.078$, $H_S = 0.045$, $F_{ST} = 0.427$) than in the northwestern subgroup ($H_T = 0.045$, $H_S = 0.029$, $F_{ST} = 0.360$) (Table 4). No major well-supported groups were retrieved in the individual NJ tree (Fig. S1). However, the split between the eastern and western groups was well supported (71% bootstrap support) in the population NJ tree (Fig. 3). Individual clustering and group clustering BAPS analyses showed complementary results, and the best partitions were found at $K = 8$ (individual clustering; Fig. S2) and $K = 7$ [population clustering, Fig. 3 (topology and branch lengths from BAPS analysis not shown)]. BAPS results are congruent with the eastern and western groups. No clear geographical structure signal was reported within these two major groups. The AMOVA for the whole data set with two hierarchical levels [analysis (1a); Table 5] assigned 62% of the total genetic variance to variation among the 24 populations. However, AMOVA with three hierarchical levels [analysis (1b); Table 5] attributed a higher percentage of vari-

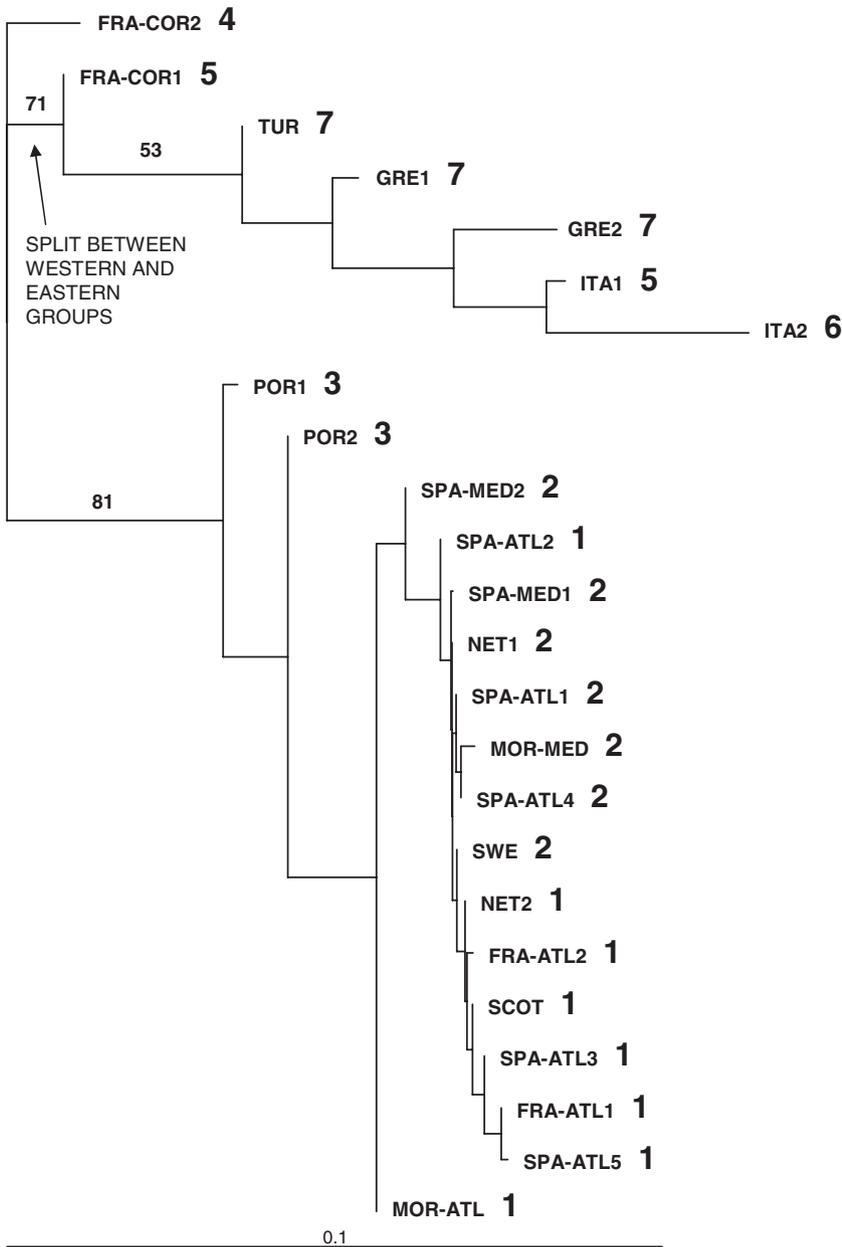


Fig. 3 Population neighbor-joining tree of the 215 AFLP phenotypes from the 24 populations of *Carex extensa* using the genetic distance coefficient of Lynch & Milligan (1994). Numbers above branches indicate bootstrap values >50% (1000 replicates). Clustering groups (numbers) from the BAPS analysis are shown after population coding (see Table 2).

ance to that among regions (49%) than to that among populations within regions (25%). When we performed an AMOVA on each data set, the percentage of overall variation attributed to variation among populations was higher within the eastern group [analysis (2a), 61%] than within the western group [analysis (2b), 44%]. Finally, when we split the western group set into the southwestern [analysis (2c), 43%] and northwestern [analysis (2d), 37%] subgroups, the percentage of overall variation attributed to variation among populations was higher in the southwestern subgroup. The Mantel test with direct distances detected isolation by distance when the whole data set ($R = 0.407$, $P = 0.010$) and the

southwestern subgroup [$R = 0.604$, $P = 0.051$ (only marginally significant)] were analysed, but the eastern group ($R = 0.554$, $P = 0.110$), the western group ($R = 0.068$, $P = 0.285$) and the northwestern subgroup ($R = -0.262$, $P = 0.040$) showed no isolation by distance. Similar levels of isolation by distance were detected when we entered the coastal distances instead of the direct distances.

SSR analysis

Four SSR loci were included in the complete analysis (CL101, CL113, Cko2-112, Cko2-118). The 72 SSR

Table 5 Analysis of molecular variance (AMOVA) of *Carex extensa* for AFLP and SSR fingerprints considering the whole data set (24 populations) with two and three hierarchical levels (eastern group vs. western group) or subsets with two hierarchical levels (eastern group, western group, southwestern subgroup, and northwestern subgroup)

Data set	Source of variation	AFLP		SSR	
		d.f.	Percentage (%) of variation	d.f.	Percentage (%) of variation
(1a) 24 populations	Among populations	23	62 ^a	19	84 ^b
	Within populations	191	38	168	16
(1b) 24 populations separated into two groups: eastern vs. western groups	Among groups	1	49 ^a	1	62 ^b
	Among populations (within groups)	22	25 ^a	18	29 ^b
	Within populations	191	26 ^a	168	9 ^b
(2a) Populations from the eastern group	Among populations	5	61 ^a	4	59 ^b
	Within populations	40	39	35	41
(2b) Populations from the western group	Among populations	17	44 ^a	14	78 ^b
	Within populations	151	56	133	22
(2c) Populations from the southwestern subgroup	Among populations	8	43 ^a	8	82 ^a
	Within populations	79	57	80	18
(2d) Populations from the northwestern subgroup	Among populations	8	37 ^a	5	64 ^a
	Within populations	72	63	53	36

^a $P = 0.001$; ^b $P = 0.010$.

sequences (18 accessions per locus; Table S1) allowed us to check for homology of the different alleles from the different populations for the four loci. The MICR-OCHEKER detected that supposed null alleles (resulting from excess of homozygotes) may be present only at locus CL101 in the populations TUR, SPA-MED1, SPA-ATL3, SPA-ATL4 and NET1, suggesting few modifications in the allelic frequencies. The analyses showed similar results for the real and estimated data. Nevertheless, we prefer to show the results from the real data because an excess of homozygotes could be a consequence of the breeding system of *C. extensa* or its evolutionary history. The total number of alleles per locus was two for CL113 and Cko2-118, four for CL101 and nine for Cko2-112 (a total of 17 alleles at the four loci) (see Fig. S3 for more details). The gene diversity (H_S) index and the observed heterozygosity (H_O) generally showed low values (Table 2). By contrast, the inbreeding coefficient (F_{IS}) generally displayed high values (Table 2). The expected total heterozygosity (H_T) was 0.539 for *C. extensa*, and the average expected within-population heterozygosity (H_S) was 0.100 (Table 4). The average observed within-population heterozygosity (H_O) was 0.044 (Table 4). Total expected heterozygosity, inbreeding and fixation indices were lower in the eastern group ($H_T = 0.241$; $F_{IS} = 0.563$; $F_{ST} = 0.532$) than in the western group ($H_T = 0.366$; $F_{IS} = 0.615$; $F_{ST} = 0.738$). However, average expected and observed heterozygosity were higher in the eastern group ($H_O = 0.056$; $H_S = 0.129$) than in the western group ($H_O = 0.039$; $H_S = 0.101$). In contrast to the AFLP results, these

results do not show a clear pattern in the levels of diversity and differentiation. Accordingly, no significant differences for these parameters were found when we compared both groups. Within the western group, the expected total heterozygosity, observed heterozygosity, and fixation index ($H_T = 0.376$; $H_O = 0.059$; $F_{ST} = 0.764$) were higher in the southwestern group than in the northwestern group ($H_T = 0.258$; $H_O = 0.008$; $F_{ST} = 0.632$). However, inbreeding and expected heterozygosity were higher in the northwestern ($F_{IS} = 0.921$; $H_S = 0.107$) than in the southwestern subgroup ($F_{IS} = 0.392$; $H_S = 0.097$). Similar to the AFLP results, these results display a clearer pattern in the levels of diversity and differentiation, and F_{IS} was significantly different in the southwestern and northwestern groups (^a $P = 0.009$; Table 4). The R_{IS} of the whole data set, the western group, and the southwestern subgroup showed significant differences after allele-size permutations (^b $P < 0.01$, ^c $P < 0.05$ and ^c $P < 0.05$ respectively; Table 4). Finally, significant departure of HWE (based on F_{IS}) was detected (^d $P = 0.001$), as has been previously estimated for some caespitose *Carex* species (see Arens *et al.* 2005). Major well-supported groups (eastern vs. western) were revealed in the populations NJ tree (Fig. 4). Individual clustering and group clustering BAPS analyses showed complementary results, and the best partitions were $K = 19$ (individual clustering; Fig. S4) and $K = 14$ [population clustering, Fig. 4 (topology and branch lengths from BAPS analysis not shown)]. In the clustering of populations and individual BAPS analyses, results were congruent within the two major groups.

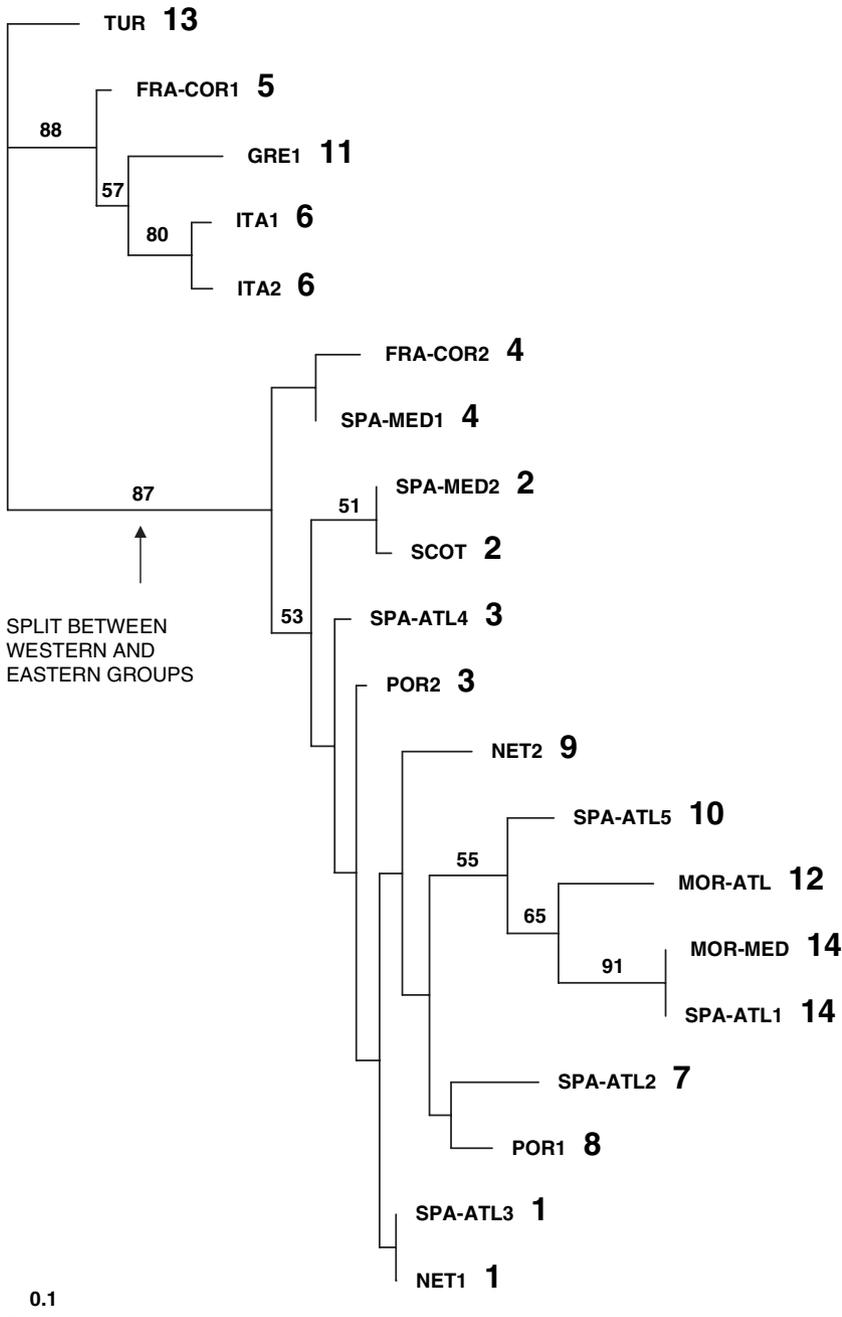


Fig. 4 Population neighbor-joining tree of the 188 SSR genotypes from the 20 populations of *Carex extensa* using the DA genetic distance coefficient (Nei *et al.* 1983). Numbers above branches indicate bootstrap values >50% (1000 replicates). Clustering groups (numbers) from the population BAPS analysis are shown population coding (see Table 2).

Nevertheless, no clear geographical structure signal was reported within the two major groups. In addition, NJ and BAPS analyses of SSR and AFLP data showed some incongruity in the clustering of populations and individuals. The AMOVA for the whole data set with two hierarchical levels [analysis (1a), Table 5] assigned 84% of the total genetic variance to variation among the 20 populations. However, similar to the AFLP results, the AMOVA with three hierarchical levels [analysis (1b), Table 5] attributed a higher percentage of the variance to that among regions (62%) than to that

among populations within regions (29%). When we applied AMOVA to each data set, the percentage of overall variation attributed to variation among populations was higher within the western group [analysis (2a), 78%] than within the eastern group [analysis (2b), 59%], which is incongruent with the AFLP results. Finally, when we split the western group into the southwestern [analysis (2c), 82%] and northwestern [analysis (2d), 64%] subgroups, the percentage of overall variation attributed to variation among populations was higher in the southwestern group, consistent with

AFLP results. The Mantel test with direct distances detected isolation by distance when the whole data set ($R = 0.291$, $P = 0.020$), eastern group ($R = 0.729$, $P = 0.010$), western group ($R = 0.205$, $P = 0.050$) and southwestern subgroup ($R = 0.325$, $P = 0.005$) were analysed. However, the northwestern subgroup showed nonsignificant results ($R = 0.312$, $P = 0.060$). Lower levels of isolation by distance were detected when we used the coastal distances instead of the direct distances (results not shown).

Discussion

The populations of *Carex extensa* were shown to be monophyletic, with the halophilic species *Carex ecklonii* (South America) and *Carex vixdentata* (southern Africa) most closely related (see also Escudero & Luceño 2009). The ancestor of this *Carex* group appears to have been specialized for saline environments; its ancestral area was Europe and Mediterranean Basin and colonized South America and southern Africa (Escudero *et al.* 2009). Our estimated times of diversification of this species group indicate an early split (semiparametric ITS: 11.5 ± 3.1 Ma; parametric ITS: 4.8 ± 2.2 Ma; semiparametric *trnK*: 4.3 ± 3.6 Ma) more related to the Messinian crisis – the desiccation of the Mediterranean Sea and the increase of salt deposition – (5.96 – 5.33 Ma; Krijgsman *et al.* 1999; Duggen *et al.* 2003; Thompson 2005) than to the Pleistocene glaciations (2.58 – 0.012 Ma; van Andel & Tzedakis 1996; Thompson 2005). Therefore, the end of the Messinian salinity crisis may have favoured early stages of differentiation of *C. extensa* in the Mediterranean region.

East-to-west Mediterranean colonization

Our phylogenetic and network reconstructions are consistent with an east-west Mediterranean differentiation of *C. extensa* (Fig. 2). Two groups of two exclusive haplotypes were inferred: the eastern group, including populations from the eastern Mediterranean; and the western group, which is composed of populations from the western Mediterranean and Atlantic coasts of northern Africa and Europe. In *C. extensa*, a strong process of east-west isolation may have been more dramatic than any other arrangement in its genetic structure (Figs 2–4). The AMOVA performed using AFLP and SSR data with three hierarchical levels indicated that a higher proportion of variation was attributable to differences among (AFLP: 49%; SSR: 62%; Table 4) rather than within groups of populations (AFLP: 25%; SSR: 29%; Table 4). An east-west pattern of differentiation has also been described in other angiosperms: the laurel tree (Rodríguez-Sánchez *et al.* 2009), the olive tree (Besnard *et al.* 2007), and the

holm oak (Lumaret *et al.* 2002). In addition, Mediterranean halophilic plants that occur inland (*Microcnemum coralloides*, Kadereit & Yaprak 2008) and in submersed coasts (*Posidonia oceanica*, Arnaud-Haond *et al.* 2007) display a remarkable east-west cleavage of genetic differentiation. However, this clear east-west Mediterranean pattern of differentiation was not provided in previous studies of coastal plants (Kadereit *et al.* 2005; Arafeh & Kadereit 2006; see also Table 1).

The Pleistocene glaciations appear to have been the most important paleoclimatic events in shaping the genetic structure of plant populations in the Mediterranean (van Andel & Tzedakis 1996; Thompson 2005). Previous phylogeographical results of halophilic species from submersed coasts (*Posidonia oceanica*, Arnaud-Haond *et al.* 2007) or inland salty environments (*Microcnemum coralloides*, Kadereit & Yaprak 2008) clearly interpret an east-west pattern of differentiation as a result of Quaternary climatic oscillations. The east-west *C. extensa* dissection may constitute the result of east-west isolation in the coldest periods of Pleistocene glaciations and maintained to this day by low gene flow despite of suspected high dispersal capacity (Escudero *et al.* 2009). The question remains as to whether this strong differentiation between eastern and western populations is the result of vicariance or colonization from one of the Mediterranean extremes.

Reconstruction of vicariance vs. colonization patterns to account for the current distribution of Mediterranean plants is not an easy task given the long period after the end of the Messinian crisis and the lack of continuous dramatic geological or climatic barriers since that time (van Andel & Tzedakis 1996; Krijgsman *et al.* 1999; Duggen *et al.* 2003; Thompson 2005). Both hypotheses, vicariance (*Posidonia oceanica*, Arnaud-Haond *et al.* 2007) and colonization (*Microcnemum coralloides*, Kadereit & Yaprak 2008), have already been reported for halophilic species. Nevertheless, the geographical and colonization patterns of the Mediterranean are poorly known for coastal plants (Table 1). Our network analysis shows that the tip position of the western haplotype is consistent with an east-to-west colonization (Fig. 2A). The genetic pattern of AFLP variation indicates higher genetic diversity and differentiation in the eastern than in the western group, despite the lower number of populations sampled (eastern: six populations, $H_T = 0.119$, $H_S = 0.048$, $F_{ST} = 0.598$; western: 18 populations, $H_T = 0.067$, $H_S = 0.037$, $F_{ST} = 0.452$; Table 4). Higher levels of diversity and differentiation of certain populations, as occurs in the eastern populations of *C. extensa*, are congruent with the canonical hypothesis of higher amounts of genetic variation in source areas than in newly colonized areas (Taberlet *et al.* 1998; Hewitt 2000; Schaal & Olsen 2000). Patterns of SSR variation show

balanced levels of genetic diversity and differentiation (Table 4), although nuclear microsatellites have been proven to be highly variable and more suitable to the study of recent gene flow between populations (Ouborg *et al.* 1999).

In contrast to the pattern of isolation between the eastern and western groups of *C. extensa*, the central Mediterranean area near the Ligurian Sea (Corsica) displayed populations with genetic admixture (AFLP: Fig. S2; SSR: Fig. S3), although each Corsican population retained a single haplotype. These results suggest gene flow between the two previously differentiated groups (eastern group vs. western group). Consequently, unexpectedly high levels of genetic diversity in Corsican populations have been observed (Table 2), which support the secondary contact hypothesis (Comes & Abbott 1999; Kropf *et al.* 2002). Moreover, significant differences of R_{IS} after allele-size permutations of the whole data set indicate 'recent admixture of populations having differentiated for a long time' (Hardy *et al.* 2003), which also supports this hypothesis (Table 4). As far as we are aware, the split into two main Mediterranean groups including a suture zone on the Ligurian Sea is first time hypothesized at the population level. It is difficult to find geographical, geological or climatic causes responsible for this recent pattern, a marked east-west differentiation with a moderated gene-flow only in a restricted suture zone.

South-to-north colonization of Europe

The LGM limited the distribution of plant species in northern areas of Europe (Hewitt 2000). As a consequence, current northern populations originated in the Holocene, when suitable habitats were available for colonization ('*tabula rasa*' hypothesis, Brochmann *et al.* 2003). As previously analysed, the influence of the Quaternary glaciations in species distributions has been postulated to depend strongly on the degree of cold tolerance (Schmitt 2007). In particular, cold-sensitive species may have retreated more from northern Europe, which may be observed in the levels of genetic variation and the geographical subdivision (Pinceel *et al.* 2005). In this context, three isotherms (12, 14 and 16 °C) of the warmest months (June–July–August) were considered critical to relate plant distributions to the cold tolerance of coastal plants (Kadereit *et al.* 2005).

The low levels of diversity and differentiation (Tables 4, 5) found in the northwestern subgroup of *C. extensa* (distributed between the current 12 °C mean isotherm of July and the 12 °C mean LGM isotherm of June–July–August) are interpreted as strong evidence of recent and rapid colonization of northern Europe from western Mediterranean after LGM. The same was

shown to be true when using the 14 and 16 °C isotherm thresholds; although the pattern was stronger using the 12 °C isotherm threshold (results not shown). In addition, significant increase in inbreeding and lack of isolation by distance in the northwestern subgroup, in comparison with the southwestern subgroup, strongly support a founder effect in the colonization of northern Europe by *C. extensa* (see Table 4). Moreover, the distribution of the genetic variation across populations reveals that the colonization processes of *C. extensa* may also have arisen from multiple source areas. Our clustering analyses of AFLP (Fig. 3) and SSR (Fig. 4) fingerprints depicted little association between northern European populations. Instead, northern populations share less genetic similarity among them than to certain Mediterranean populations (Figs 3, 4). Nevertheless, partial incongruence between AFLP and SSR data (Figs 3, 4) prevents us from describing in detail multiple colonization patterns. The lack of phylogeographical structure of the northern European populations and the phylogeographical relationships with the Mediterranean populations (multiple colonization hypothesis) have already been demonstrated for *Calystegia soldanella* and *Triglochin maritima* (Arafeh & Kadereit 2006; Lambrecht *et al.* 2007).

The northward migration after Pleistocene glaciations may be the most common pattern in European halophilic plants (Table 1) as well as for other kind of angiosperms (Schmitt 2007). By contrast, glacial survival hypothesis seems to have been more plausible in halophilic plants from western American coasts (*Carex macrocephala*, King *et al.* 2009; *Zostera marina*, Olsen *et al.* 2004). Dispersal mechanisms and the suitability of the postglacial environment (*tabula rasa*) may have been the primary determinants of northern European colonization. In this way, one remaining question is why the western Mediterranean lineage of *C. extensa* had a successful colonization of northern Europe after LGM, but failed in its expansion to the whole Mediterranean where the colonization was stopped in central Mediterranean. Such pattern may support the 'high density blocking' hypothesis (Hewitt 1993). Eastern and western lineages colonized the restricted specific habitat of this species in the Mediterranean until both lineages took contact in the cited suture zone, which could limit the colonization process of the two lineages.

Conclusions

The diversification times of the halophilic, natural group of *Carex extensa*, *Carex ecklonii* and *Carex vixdentata* herein estimated may be related to the Messinian crisis. Subsequently, *C. extensa* may have colonized the western Mediterranean from the eastern extreme, where

the highest levels of diversity and differentiation were found. Pleistocene climatic oscillations may have played an important role in the differentiation of eastern and western populations, although secondary contacts can be now detected in areas of the Ligurian Sea (central Mediterranean basin). This primarily east-west cleavage has been maintained to this day by low gene flow despite apparently high dispersal capacity of *C. extensa*. By contrast, recent and rapid colonization of northern Europe from the western Mediterranean after LGM is consistent with the present genetic structure in the northern distribution ('*tabula rasa*' hypothesis). Such unbalanced west-east vs. west-north colonization gives further support to the 'high density blocking' hypothesis (Hewitt 1993).

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References

- Alfaro ME, Zoller S, Lutzoni F (2003) Bayes or bootstrap. A simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling or bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution*, **20**, 255–266.
- Amsellen L, Noyer JL, Le Bourgeois T, Hossaert-McKey M (2000) Comparison of genetic diversity of the invasive weed *Rubus alceifolius* Poir. (Rosaceae) in its native range and in areas of introduction, using amplified fragment length polymorphism (AFLP) markers. *Molecular Ecology*, **9**, 443–455.
- van Andel TH (2002) The climate and landscape of the middle part of wide part of Weichselian glaciation in Europe: the stage 3 project. *Quaternary Science Reviews*, **57**, 2–8.
- van Andel TH, Tzedakis PC (1996) Palaeolithic landscapes of Europe and environs, 150,000–25,000 years ago: an overview. *Quaternary Science Reviews*, **15**, 481–500.
- Arafeh R, Kadereit JW (2006) Long-distance seed dispersal, clone longevity and lack of phylogeographical structure in the European distributional range of the coastal *Calystegia soldanella* (L.) R. Br. (Convolvulaceae). *Journal of Biogeography*, **33**, 1461–1469.
- Arens P, Bijlsma R-J, van't Westende W *et al.* (2005) Genetic structure in populations of an ancient woodland sedge, *Carex sylvatica* Hudson, at a regional and local scale. *Plant Biology*, **7**, 387–396.
- Arnaud-Haond S, Migliaccio M, Diaz-Almela E *et al.* (2007) Vicariance patterns in the Mediterranean Sea: east-west cleavage and low dispersal in the endemic seagrass *Posidonia oceanica*. *Journal of Biogeography*, **34**, 963–976.
- Baldwin BG, Sanderson MJ (1998) Age and rate of diversification of the Hawaiian silversword alliance (Compositae). *Proceedings of the National Academy of Sciences, USA*, **95**, 9402–9406.
- Besnard G, Rubio de Casas R, Vargas P (2007) Plastid and nuclear DNA polymorphism reveals historical processes of isolation and reticulation in the olive tree complex (*Olea europaea*). *Journal of Biogeography*, **34**, 736–752.
- Brochmann C, Gabrielsen TM, Nordal I, Landvik JY, Elven R (2003) Glacial survival or tabula rasa? The history of North Atlantic biota revisited *Taxon*, **52**, 417–450.
- Chater AO (1980) *Carex*. In: *Flora Europaea*, vol. 5. (eds Tutin TG, Heywood VH, Burges NA *et al.*), pp. 290–323. Cambridge University Press, Cambridge, UK.
- Chiang YC, Hung KH, Schaal BA *et al.* (2006) Contrasting phylogeographical patterns between mainland and island taxa of the *Pinus luchuensis* complex. *Molecular Ecology*, **15**, 765–779.
- Clausing G, Vickers K, Kadereit JW (2000) Historical biogeography in a linear system: genetic variation of Sea Rocket (*Cakile maritima*) and Sea Holly (*Eryngium maritimum*) along European coasts. *Molecular Ecology*, **9**, 1823–1833.
- Clement MD, Posada D, Crandall KA (2000) *TCs*: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.
- Comes HP, Abbott RJ (1999) Population genetic structure and gene flow across arid versus mesic environments: a comparative study of two parapatric *Senecio* species from the Near East. *Evolution*, **53**, 36–54.
- Corander J, Marttinen P, Sirén J, Tang J (2006) *BAPS v.4.14: Bayesian Analysis of Population Structure*. Available at: <http://www.rni.helsinki.fi/~jic/bapspage.html>.
- Coyer JA, Peters AF, Stam WT, Olsen JL (2003) Post-ice age recolonization and differentiation of *Fucus serratus* L. (Fucales: Phaeophyta) populations on Northern Europe. *Molecular Ecology*, **12**, 1817–1829.
- Dieringer D, Schlotterer C (2003) MICROSATELLITE ANALYZER (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes*, **3**, 167–169.
- Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.
- Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with confidence. *PLoS Biology*, **4**, 699–710.
- Duggen S, Hoernie K, van den Bogaard P *et al.* (2003) Deep roots of the Messinian salinity crisis. *Nature*, **422**, 602–606.
- Escudero M, Luceño M (2009) Systematics and evolution of *Carex* sects. Spirostachyae and Elatae (Cyperaceae). *Plant Systematics and Evolution*, **279**, 163–189.
- Escudero M, Valcárcel V, Vargas P, Luceño M (2008a) Evolution in *Carex* L. sect. Spirostachyae (Cyperaceae): a molecular and cytogenetic approach. *Organisms Diversity and Evolution*, **7**, 271–291.
- Escudero M, Vargas P, Valcárcel V, Luceño M (2008b) The Strait of Gibraltar: an effective gene flow barrier for the wind-pollinated *Carex helodes* Link (Cyperaceae), as revealed

- by DNA sequence, AFLP and cytogenetic variation. *American Journal of Botany*, **95**, 745–755.
- Escudero M, Valcárcel V, Vargas P, Luceño M (2009) Ecological vicariance and long distance dispersal significance in the diversification of *Carex* sect. *Spirostachyae* (Cyperaceae). *American Journal of Botany*, **96**, 2100–2114.
- Felsenstein J (2007) *PHYLIP: Phylogenetic Inference Package, Version 3.67*. Available from the author. Department of Genome Sciences and Department of Biology, University of Washington, Seattle, Washington, USA.
- Gandour M, Hessini K, Abdelly C (2008) Understanding the population genetic structure of coastal species (*Cakile maritima*): seed dispersal and the role of sea currents in determining population structure. *Genetics Research*, **90**, 167–178.
- Gaudeul M, Taberlet P, Till-Bottraud I (2000) Genetic diversity in an endangered alpine plant, *Eryngium alpinum* L. (Apiaceae), inferred from amplified fragment length polymorphism markers. *Molecular Ecology*, **9**, 1625–1637.
- Goloboff PA, Farris JS, Nixon K (2003) Tree analysis using New Technology (TNT) version 1.0. Available at: <http://www.zmuc.dk/public/phylogeny>.
- Goudet J (2001) *FSTAT: a program to estimate and test gene diversities and fixation indices, version 2.9.3*. Available at: www2.unil.ch/popgen/softwares/fstat.htm.
- Hann J (1987) Isothermen von Europe. In: *Berghaus' Physikalischer Atlas, Atlas der Meteorologie*, vol. 3 (ed. Berghaus HCW), pp. 30. Justus Perthes, Gotha, Germany.
- Hardy OJ, Vekemans X (2002) SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, **2**, 618–620.
- Hardy OJ, Charbonnel N, Fréville H, Heuertz M (2003) Microsatellite allele sizes: a simple test to assess their significance on genetic differentiation. *Genetics*, **163**, 1467–1482.
- Hewitt G (1993) Postglacial distribution and species substructure: lessons from pollen, insects and hybrid zones. In: *Evolutionary Patterns and Processes. Linnean Society Symposium Series 14* (eds Lees DR, Edwards D), pp. 97–123. Academic Press, London, UK.
- Hewitt G (2000) The genetic legacy of quaternary ice ages. *Nature*, **405**, 907–913.
- Hipp AL (2007) Nonuniform processes of chromosome evolution in sedges (*Carex*: Cyperaceae). *Evolution*, **61**, 2175–2194.
- Hultén E (1962) The Circumpolar Plants I. Vascular Cryptogams, Conifers, Monocotyledons. *K. Svenka Vet.-Akad. Handl. Ser. 4*, **8**, 1–275.
- Ibrahim KM, Nichols RA, Hewitt GM (1996) Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity*, **7**, 282–291.
- Jakob SS, Ihlow A, Blattner FR (2007) Combined ecological niche modelling and molecular phylogeography revealed the evolutionary history of *Hordeum marinum* (Poaceae) – niche differentiation, loss of genetic diversity, and speciation in Mediterranean Quaternary refugia. *Molecular Ecology*, **16**, 1713–1727.
- Kadereit G, Yaprak AE (2008) *Microcnemum coralloides* (Chenopodiaceae-Salicornioideae): an example of intraspecific East-West disjunctions in the Mediterranean region. *Anales del Jardín Botánico de Madrid*, **65**, 415–426.
- Kadereit JW, Arafeh R, Somogyi G, Westberg E (2005) Terrestrial growth and marine dispersal? Comparative phylogeography of five coastal plant species at a European scale *Taxon*, **54**, 861–876.
- Kay KM, Whittall JB, Hodges SA (2006) A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects. *BMC Evolutionary Biology*, **6**, Art. no. 36.
- King MG, Horning ME, Roalson EH (2009) Range persistence during the last glacial maximum: *Carex macrocephala* may have been unrestricted to glacial refugia. *Molecular Ecology*, **18**, 4256–4269.
- Krijgsman W, Hilgen FJ, Raffi I *et al.* (1999) Chronology, causes and progression of the Messinian salinity crisis. *Nature*, **400**, 652–655.
- Kropf M, Kadereit JW, Comes HP (2002) Late Quaternary distributional stasis in the submediterranean mountain plant *Anthyllis montana* L. (Fabaceae) inferred from ITS sequences and amplified fragment length polymorphism markers. *Molecular Ecology*, **11**, 447–463.
- Lambracht E, Westberg E, Kadereit JW (2007) Phylogeographic evidence for the postglacial colonization of the North and Baltic Sea coasts from inland glacial refugia by *Triglochin maritima* L. *Flora. Morphology, Distribution, Functional Ecology of Plants*, **202**, 79–88.
- Langley CL, Fitch WM (1974) An examination of the constancy of the rate of molecular evolution. *Journal of Molecular Evolution*, **3**, 161–177.
- Lowe L, Harris S, Ashton P (2005) *Ecological genetics. Design, analysis, and application*. Blackwell, Oxford, UK.
- Luceño M, Castroviejo S (1993) Cytotaxonomic studies in the sections *Spirostachyae* (Drejer) Bailey and *Ceratocystis* Dumort. of the genus *Carex* L. (Cyperaceae), with special reference to Iberian and North African taxa. *Botanical Journal of the Linnean Society*, **112**, 335–350.
- Luceño M, Escudero M (2008) *Carex* sect. *Spirostachyae*. In: *Flora Ibérica, vol XVIII* (eds Castroviejo S, Luceño M, Galán A, Jiménez-Mejías P, Cabezas F, Medina L), pp. 178–191. Real Jardín Botánico – CSIC, Madrid, Spain.
- Lumaret R, Mir C, Michaud H, Raynal V (2002) Phylogeographical variation of chloroplast DNA in holm oak (*Quercus ilex* L.). *Molecular ecology*, **11**, 2327–2336.
- Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, **3**, 91–99.
- Magallon S, Sanderson MJ (2001) Absolute diversification rates in angiosperm clades. *Evolution*, **55**, 1762–1780.
- Maire R (1957) *Flore de L'Afrique du Nord*. Éditions Paul Lechevalier, Paris, France.
- Meudt HM, Clarke AC (2007) Almost forgotten or latest practice? AFLP applications, analyses and advances *Trends in Plant Sciences*, **12**, 106–117.
- Nei M (1972) Genetic distance between populations. *American Naturalist*, **106**, 283–292.
- Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York, NY.
- Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences, USA*, **76**, 5269–5273.
- Nei M, Tajima F, Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. *Journal of Molecular Evolution*, **19**, 153–170.

- Nylander JAA (2002) *MrModeltest 1.0b*. Available from the author. Department of Systematic Zoology, Uppsala University, Uppsala.
- Ohsako T, Yamane K (2007) Isolation and characterization of polymorphic microsatellite loci in Asiatic sand sedge, *Carex kobomugi* Ohwi (Cyperaceae). *Molecular Ecology Notes*, **7**, 1023–1025.
- Olsen JL, Stam WT, Coyer JA *et al.* (2004) North Atlantic phylogeography and large-scale population differentiation of the seagrass *Zostera marina* L. *Molecular ecology*, **13**, 1923–1941.
- van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.
- Ortiz MA, Tremetsberg K, Talavera S *et al.* (2007) Population structure of *Hypochaeris salzmanniana* DC. (Asteraceae), an endemic species to the Atlantic coast on both sides of the Strait of Gibraltar, in relation to Quaternary sea level changes. *Molecular Ecology*, **16**, 541–552.
- Ortiz-Dorda J, Martínez-Mora C, Correal E, Simón B, Cenis JL (2005) Genetic structure of *Atriplex halimus* populations in the Mediterranean Basin. *Annals of Botany*, **95**, 827–834.
- Ouborg NJ, Piquot Y, van Groenendael JM (1999) Population genetics, molecular markers, and the study of dispersal in plants. *Journal of Ecology*, **87**, 551–569.
- Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.
- Pinceel J, Jordaens K, Pfenninger M, Bäckeljau T (2005) Rangeland phylogeography of a terrestrial slug in Europe: evidence for Alpine refugia and rapid colonization after the Pleistocene glaciations. *Molecular ecology*, **14**, 1133–1150.
- Piñeiro R, Aguilar JF, Munt DD, Nieto-Feliner G (2007) Ecology matters: Atlantic-Mediterranean disjunction in the sand-dune shrub *Armeria pungens* (Plumbaginaceae). *Molecular Ecology*, **16**, 2155–2171.
- Piñeiro R, Costa A, Aguilar JF, Nieto-Feliner G (2009) Overcoming paralogy and incomplete lineage sorting to detect a phylogeographic signal: a GapC study of *Armeria pungens*. *Journal of Botany*, **87**, 164–177.
- Reznicek AA (1990) Evolution in sedges (*Carex*, Cyperaceae). *Journal of Botany*, **68**, 1409–1432.
- Ridley HN (1930) *The dispersal of plants throughout the world*. Reeve, London, UK.
- Rodríguez-Sánchez F, Guzmán B, Valido A *et al.* (2009) Late Neogene history of the laurel tree (*Laurus* L., Lauraceae) based on phylogeographical analyses of Mediterranean and Macaronesian populations. *Journal of Biogeography*, **36**, 1270–1281.
- Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.
- Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molecular Biology and Evolution*, **19**, 101–109.
- Sanderson MJ (2004) r8s, version 1.70 user's manual. Available at: <http://loco.biosci.arizona.edu/r8s/>.
- Schaal BA, Olsen KM (2000) Gene genealogies and population variation in plants. *Proceedings of the National Academy of Sciences, USA*, **97**, 7024–7029.
- Schmitt T (2007) Molecular biogeography of Europe: pleistocene cycles and postglacial trends. *Frontiers in Zoology*, art. no. 11.
- Schütz W (1998) Seed dormancy cycles and germination phenologies in sedges (*Carex*) from various habitats. *Wetlands*, **18**, 288–297.
- Shaw J, Lickey EB, Beck JT *et al.* (2005) The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany*, **92**, 142–166.
- Silliman BR, Grosholz ED, Bertness MD (2009) *Human Impacts on Salt Marshes: A Global Perspective*. University of California Press, Berkeley and Los Angeles, CA.
- Swofford DL (2002) *PAUP*: Phylogenetic Analysis Using Parsimony Version 4.0b10*. Sinauer Associates, Publishers, Sunderland, MA, USA.
- Taberlet P, Fumagalli L, Wust-Saucy A-G, Cosson J-F (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7**, 453–464.
- Templeton AR (2007) Genetics and recent human evolution. *Evolution*, **61**, 1507–1519.
- Templeton AR, Crandall AK, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence mapping. III. Cladogram stimation. *Genetics*, **132**, 619–633.
- Thompson JD (2005) *Plant evolution in the Mediterranean*. Oxford University Press, Oxford, UK.
- Tremetsberger K, Talavera S, Stuessy TF *et al.* (2004) Relationship of *Hypochaeris salzmanniana* (Asteraceae, Lactuceae), an endangered species of the Iberian Peninsula, to *H. radicata* and *H. glabra* and biogeographical implications. *Botanical Journal of the Linnean Society*, **146**, 79–95.
- Vekemans X (2002) *AFLP-SURV version 1.0*. Available from the author. Laboratoire de Génétique et Ecologie Végétale, Université Libre de Bruxelles, Belgium.
- Waterway MJ, Starr JR (2007) Phylogenetics relationships in tribe *Cariceae* (Cyperaceae) based on nested analyses of four molecular data sets. *Aliso*, **23**, 165–192.
- Weising K, Freitag H (2007) Phylogeography of halophytes from European coastal and inland habitats. *Zoologischer Anzeiger*, **246**, 279–292.
- Westberg E, Kadereit JW (2009) The influence of sea currents, past disruption of gene flow and species biology on the phylogeographical structure of coastal flowering plants. *Journal of biogeography*, **36**, 1398–1410.
- Yeh FC, Yang RC, Boyle T (1999) *POPGENE (version 1.31)*. Microsoft window-bases freeware for population genetics analysis. University of Alberta and Centre for International Forestry Research. Available at: <http://www.ualberta.ca/~fyeh/>.

This study is part of Marcial Escudero's PhD thesis on molecular and cytogenetic analyses in *Carex* sects. *Spirostachyae* (Cyperaceae), including phylogeography of several species. Pablo Vargas investigates systematics and evolution of Mediterranean plants in the five Mediterranean floristic regions, particularly in snapdragons (*Antirrhinum* and relatives) and rockroses (*Cistus*). Paul Arens is interested in studies on genetic diversity, population genetics and conservation biology. Joop Ouborg is interested in molecular ecology of plants and animals and eco-dynamics of life-history traits. Modesto

Luceno's interest has been focused in the last two decades in cytogenetics and systematics of Cyperaceae, with special emphasis in the tribe Cariceae.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Individual neighbor-joining tree from the 215 AFLP phenotypes from the 24 populations of *Carex extensa* using Nei & Li (1979) genetic distance coefficient. Numbers above branches indicate bootstrap values >50% (10 000 replicates). Individual labels are only provided for the supported clades and for individuals from the Corsican populations. Population coding as in Table 2.

Fig. S2 NJ tree of clustering of individual BAPS analysis using the 215 AFLP phenotypes from the 24 populations of *Carex extensa* and the genetic distance coefficient of BAPS analysis. Number of individuals and populations of origin are shown. Population coding as in Table 2.

Fig. S3 Simple sequence repeats sampling map. For each locus, alleles have been colour coded. When an allele is fixed in a population, the population is colour coded as the allele.

When a population is polymorphic, allele frequency is shown and colour coded as the allele that it represents.

Fig. S4 Neighbor Joining tree of clustering of individual BAPS analysis using the 188 SSR genotypes from the 20 populations of *Carex extensa* and the genetic distance coefficient of BAPS analysis. Number of individuals and populations of precedence are shown. Population coding as in Table 2.

Table S1 *Carex extensa* specific sequences for the polymorphic loci CL101 (from *Carex limosa*), CL113 (from *C. limosa*), Cko2-112 and Cko2-118. For these loci 18 individuals have been sequenced. Primer sequences of loci CL88, CL100, CL102 and CL114 originating from *C. limosa* are given as well

Material S1 (A) List of materials for the ITS *Spirostachyae* molecular clock data set, indicating GenBank number. (B) List of materials for the *trnK-Spirostachyae* molecular clock data set, indicating GenBank number. (C) List of materials for the *trnK* data set for phylogenetic and network analysis, indicating GenBank number. (D) List of materials for the *ycf6-psbM* data set indicating GenBank number.

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