Theor Appl Genet (2003) 106:1122–1128
DOI 10.1007/s00122-002-1134-7

S. Panda · J. P. Martín · I. Aguinagalde

Chloroplast and nuclear DNA studies in a few members of the *Brassica oleracea* L. group using PCR-RFLP and ISSR-PCR markers: a population genetic analysis

Received: 17 June 2002 / Accepted: 14 August 2002 / Published online: 29 October 2002

Abstract A population genetic analysis of chloroplast and nuclear DNA was performed covering nine wild populations of *Brassica oleracea*. Three members of the n = 9 group, all close to *B. oleracea, Brassica alboglabra* Bailey, *Brassica bourgeaui* (Webb) O. Kuntze and *Brassica montana* Pourret, were also studied to better understand their relationship with *B. oleracea*. Chloroplast DNA was analysed using the PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) method. The ISSR-PCR (inter-simple sequence repeat – polymerase chain reaction) technique was adopted to study nuclear DNA. Twelve primer pairs of chloroplast DNA showed very good amplification. The amplified product of each primer pair, digested by three restriction enzymes, revealed no variation of cpDNA among the taxa studied. This indicates they may have the same chloroplast genotype. Seven selected ISSR primers have detected genetic variation, both within and among the populations/taxa surveyed. The information obtained on the intra- and inter-populational genetic diversity of wild populations of *B. oleracea* neatly defined the individual plants. It could provide important guidelines for backing management and conservation strategies in this species. The study confirms a close relationship between *B. alboglabra, B. bourgeaui* and *B. montana*, which is parallel to their morphological similitude.

Keywords *Brassica oleracea* · Chloroplast and nuclear DNA · ISSR-PCR · PCR-RFLP · Population genetics

Communicated by H.F. Linskens

S. Panda · J.P. Martín (✉) · I. Aguinagalde
Departamento de Biología Vegetal,
Escuela Técnica Superior de Ingenieros Agrónomos,
Ciudad Universitaria s/n, 28040-Madrid, Spain
e-mail: jpmartin@bio.etsia.upm.es
Tel.: +34-91-336-5662, Fax: +34-91-336-5656

Introduction

Brassica oleracea L. (*Brassicaceae*) is a suffrutescent perennial, usually up to 150-cm tall, native to west Atlantic Europe (Toxopeus 1974). The wild populations of *B. oleracea* are restricted to the "Coasts of N. Spain, W. and N. France, the British Isles and the isle of Helgoland (Germany)" (Snogerup et al. 1990). Wild *B. oleracea* is the progenitor species of *B. oleracea* cultivars (Snogerup 1980; Song et al. 1988) and may be an important reservoir of useful genes for future breeding work, as emphasised by Bothmer et al. (1995). Genetic diversity in *B. oleracea* has been studied using isozyme and molecular methods, such as traditional RFLPs, RAPDs, AFLPs and microsatellites (Song et al. 1990; Warwick et al. 1992; Lázaro and Aguinagalde 1998a,b; Sebastian et al. 2000; Plieske and Struss 2001; Saal et al. 2001). Intrapopulational genetic analyses of chloroplast DNA (cpDNA) in wild populations of *B. oleracea* are not yet available. Also there is no information on the nuclear DNA (nDNA) diversity in wild populations of the species based on ISSR markers.

Three members of the *B. oleracea*-group, namely *Brassica alboglabra* Bailey, *Brassica bourgeaui* (Webb) O. Kuntze and *Brassica montana* Pourret, have jointly or independently been considered by some authors as cospecific to *B. oleracea* (Snogerup 1980; Song et al. 1988; Snogerup et al. 1990; Tutin et al. 1993; Gómez-Campo 1999; Gladis and Hammer 2001), while others treated them as different species (Borgen et al. 1979; Song et al. 1990; Lázaro and Aguinagalde 1998a, b; Hansen et al. 2001). Therefore, their taxonomic status may still be considered uncertain. *B. bourgeaui* is an extremely rare, endemic taxon to the Canary Islands (Borgen et al. 1979); *B. montana* is distributed across N.W. Italy, S. France and N.E. Spain (Snogerup et al. 1990); and *B. alboglabra* is probably an old cultivar of *B. oleracea* (Gómez-Campo 1999), locally cultivated in S. Italy and China. These three taxa are also suffrutescent perennials, similar to *B. oleracea* in vegetative and floral characters, and exhibit the same chromosome

number, n = 9 (Gómez-Campo and Hinata 1980; Snogerup et al. 1990; Prakash et al. 1999). Since molecular markers reflect genotypes very closely, simultaneous use of cpDNA and nDNA markers was expected to provide a better understanding of the relationship of these taxa with *B. oleracea*.

In the present study, the cpDNA was assessed using the PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) technique. PCR-RFLP using universal primers (Taberlet et al. 1991; Demesure et al. 1995; Dumolin-Lapègue et al. 1997) for amplification plus restriction digestion, has often detected intra- and inter-specific cpDNA variations (Gielly and Taberlet 1994). Our study has analysed variation in the nDNA as well, employing ISSR (inter-simple sequence repeat) markers (Zietkiewicz et al. 1994). This method offers great potential to determine genetic diversity and phylogenetic relationships at intra- and inter-specific levels, and also for individual accessions/cultivars (Wolfe and Liston 1998; Joshi et al. 2000). They exhibit specificity of sequence-tagged-site markers, but need no sequence information for primer synthesis providing the advantage of random markers. This is the first report of PCR-RFLP and ISSR-PCR analyses with the wild populations of *B. oleracea* and its three above-mentioned relatives.

The main objectives of this study were to perform a population genetic analysis of cpDNA and nDNA in several wild populations of *B. oleracea* in Europe, and to clarify the status of *B. alboglabra, B. bourgeaui* and *B. montana* on the basis of such genetic assessment.

Materials and methods

Plant material

Nine wild populations comprising 80 individuals of *B. oleracea* were covered in this study, which included six populations from Northern Spain, two from the United Kingdom and one from France (Table 1). Ten individuals of one *B. oleracea* cultivar (Aubervilliers Sel, Melena) were also studied. One population (ten individuals) for each of *B. alboglabra, B. bourgeaui* and *B. montana* was also investigated. Three to four leaves were collected from each plant/seedling, grown in the glasshouse (seed source: Germplasm Bank, Departamento de Biologia Vegetal, E.T.S.I. Agrónomos, Universidad Politécnica, Madrid, Spain; see Gómez-Campo 1990; or collected from the natural habitats), and frozen at –80 °C until DNA extraction. Geographical origin of the materials together with the germplasm bank codes are shown in Table 1.

DNA extraction

Total DNA was extracted from frozen leaf material following the protocol supplied in the "NucleoSpin Plant Kit" (CLONTECH Laboratories, Inc.). Extracted DNA was quantified comparing band intensities with known standards of lambda DNA on 1.2% agarose gels. A working solution of DNA (4 ng/µl) was made using sterile de-ionised water.

PCR amplification and restriction digestion (PCR-RFLP)

Amplification of cpDNA was done using 14 universal primer pairs (HK, K1K2, QR, *rpo*CC, CD, DT, TC, CS, SfM, AS, ST, TF, FV and VL, see Table 2; all defined in Dumolin-Lapègue et al. 1997), and 4–8 ng of total DNA, following the conditions for amplification as in Mohanty et al. (2000). The PCR products were checked by electrophoresis on 1.2% agarose gels, and then by ethidium bromide staining. The approximate product size was calculated by comparison of the migration distance of the PCR product with a DNA marker (1-kb ladder, Pharmacia).

Three restriction enzymes (*Alu*I, *Hin*fI and *Taq*I; Amersham) were used for the digestion of the PCR-amplified cpDNA (8.5 µl of the PCR product/enzyme). Digestions were carried out with three units of *Alu*I, *Hin*fI or five units of *Taq*I, and incubated for 18 h at 37 °C (*Alu*I, *Hin*fI) or 65 °C (*Taq*I). Restriction fragments were separated on 2.6% agarose gels in Tris-borate-EDTA buffer (1×), run at 3 V/cm for 4 h, then stained with ethidium bromide, visualized under UV light and photographed. The size of the bands were analysed using Kodak Digital Science 1D Image Analysis Software. A 50-bp ladder (Pharmacia) was used as a molecular size marker.

ISSR-PCR amplification and electrophoresis

After an initial screening using 32 ISSR primers provided in primer set #9 of the University British Columbia Biotechnology Labo-

Table 1 Species, populations/origin and number of individuals studied

Species	Population/origin	Accession number[a]	Code	No. of individuals
B. oleracea	Camino Faro Sta. Catalina, Lekeitio, Vizcaya/Spain	(–)	Bo-A	10
	Playa de Barrika, Vizcaya/Spain	(–)	Bo-B	10
	Tunel de Laredo, Cantabria/Spain	(–)	Bo-C	10
	Cabo de Oyambre, Cantabria/Spain	7663	Bo-D	10
	San Juan de Gaztelugatxe, Baquio, Vizcaya/Spain	7665	Bo-E	10
	Monte Urgull, San Sebastian/Spain	7670	Bo-F	10
	Granville, Normandie/France	7672	Bo-G	5
	Swanage, Dorset/United Kingdom	2191	Bo-H	5
	Glamorgan, Wales/United Kingdom	2192	Bo-I	10
	Commercial Seed, Variety: Aubervilliers Sel, Melena (company: CIA, IB, Semillas, S.L., Spain)	–	Bo-J	10
B. alboglabra	Commercial Seed/China	5975	Ba	10
B. bourgeaui	Ladera Alta, Frontera, Hierro-Canary Islands/Spain	8856	Bb	10
B. montana	Palmaiola Island/Italy	6801	Bm	10

[a] At the germplasm bank of the 'Departamento de Biologia Vegetal, E.T.S.I. Agrónomos, Madrid, Spain'; (–) indicates plant material directly collected in the natural habitats

Table 2 PCR conditions, size of the amplified fragments and degree of amplification using 14 cpDNA universal primer pairs in the four *Brassica* taxa studied

Abbrev. of cpDNA primers[a]	PCR conditions		Amplified fragment (bp)	Degree of amplification[b]
	Annealing temperature	Extension time		
HK	62 °C	2 min 30 s	NA	NA
K1K2	53.5 °C	3 min	2,600	++
QR	56.2 °C	4 min	1,700	++
*rpo*CC	45 °C	5 min 30 s	5,000	+
CD	55 °C	4 min 30 s	2,400	++
DT	52 °C	2 min	1,200	++
TC	50 °C	4 min	3,100	++
CS	55 °C	3 min	1,600	++
SfM	62 °C	2 min	1,350	++
AS	55 °C	4 min 30 s	3,000	++
ST	50 °C	2 min	1,400	++
TF	50 °C	2 min 30 s	1,700	++
FV	50 °C	4 min 30 s	3,000	++
VL	55 °C	4 min 30 s	3,800	++

[a] Abbreviations are as in Dumolin-Lapègue et al. (1997)
[b] NA, no amplification; ++, good amplification; +, faint amplification

Table 3 ISSR primer sequences, annealing temperature, number of fragments scored and approximate size range (in base pairs) of the fragments resulted from each primer in the 120 individuals of the four *Brassica* taxa studied

Primer		Annealing temperature (°C)	No. of fragments scored	Fragment size range (bp)
Code	Sequence[a]			
UBC808	$(AG)_8C$	55	5	1,000–650
UBC835	$(AG)_8YC$	53	11	1,270–400
UBC841	$(GA)_8YC$	53	9	1,050–240
UBC868	$(GAA)_6$	53	7	1,200–400
UBC889	$DBD(AC)_7$	57	12	1,200–390
UBC890	$VHV(GT)_7$	55	15	1,700–330
UBC891	$HVH(TG)_7$	52	13	1,300–380

[a] Y stands for pyrimidine, B for non-A, D for non-C, H for non-G, V for non-T residues

ratory (UCB, Vancouver, Canada), seven UBC-primers were selected for this study (see Table 3). The ISSR-PCR amplification conditions were introduced by Martín and Sánchez-Yélamo (2000), with a little modification of the annealing temperature of the primers (see Table 3), using 16–20 ng of total DNA. Two percent of formamide in the PCR reaction was used only for the primer UBC891 for a better amplification. The ISSR-PCR products were resolved by electrophoresis on 2% agarose gels, followed by ethidium bromide staining, then visualised and photographed under UV light. The size of amplified bands was estimated with the help of a DNA marker (100-bp ladder, Pharmacia).

Data analysis

The PCR-RFLP bands were found to be monomorphic. The ISSR bands were analysed to estimate the genetic variations/relationships among the populations and the taxa studied. The ISSR bands were interpreted as dominant markers and were scored as diallelic characters either as 1 (present) or 0 (absent). A pair-wise similarity matrix was calculated using Dice's coefficient (Dice 1945). The similarity matrix was employed to construct a dendrogram by the unweighted pair group method with arithmetical averages (UPGMA), using the SAHN-clustering and TREE programs from the NTSYS-pc, version 2.02 package (Rohlf 1998). A cophenetic matrix was derived from the similarity matrix to test goodness of fit of the clusters, by comparing the two matrices using the Mantel matrix correspondence test in the MXCOMP program of the NTSYS-pc package.

In addition, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was applied to estimate variance components for ISSR phenotypes, partitioning the variation among populations and among individuals within populations of *B. oleracea*. For these analyses, the distance metric used was $D = 100\ (1 - S)$ for all pairs of wild individuals of *B. oleracea*, where S is Dice's estimator of similarity (Dice 1945). Levels of significance of variance-component estimates were computed by non-parametric permutational procedures (i.e. 2,000 random permutations). Homogeneity of molecular variance among populations was tested with Bartlett's statistics. Pairwise Phi_{st} distances (analogous to F-statistics at the molecular level; Excoffier et al. 1992) were calculated among populations, and their significance was also tested by a permutation procedure, using 2,000 permutations. All analyses were carried out with WINAMOVA version 1.55, provided by Laurent Excoffier (Genetics and Biometry Laboratory, University of Geneva, Switzerland).

Results

PCR-RFLP analysis

Fourteen universal primer pairs of cpDNA were initially screened to assess the degree of amplification and the size of the amplified fragment in *B. oleracea, B. alboglabra, B. bourgeaui* and *B. montana* (Table 2). The primers HK and *rpo*CC showed "no" and "faint" amplifications, respectively; the rest of the 12 primers showed good amplification and were chosen for this investigation, which represented about 25.15 kb of the chloroplast genome. The amplified product of each primer was of same size in these taxa. Each of the 12 amplified fragments was digested by three restriction enzymes (*Alu*I, *Hin*fI and *Taq*I). All the 36 primer-enzyme combinations revealed no variation of cpDNA, both within and among the populations and the taxa studied. *Taq*I could not cut the CS amplified fragment, while *Alu*I failed to digest the K1K2 and ST amplified fragments; the rest of the 33 restriction combinations resulted in a monomorphic banding profile for all the 120 individuals (see Table 1) of the four *Brassica* members investigated.

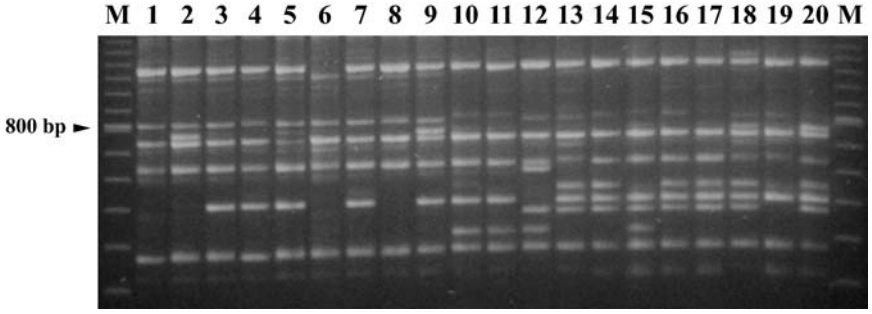

Fig. 1 Inter-simple sequence repeat (ISSR) patterns obtained on an agarose gel for two populations of *B. oleracea* (*lanes 1 to 10* = San Juan de Gaztelugatxe, Baquio, Vizcaya/Spain; *lanes 11 to 20* = Monte Urgull, San Sebastian/Spain) using the primer UBC889. M = molecular-size marker (100-base pair ladder, Pharmacia)

ISSR-PCR analysis

All seven ISSR primers have yielded very clearly identifiable bands and informative patterns in the relevant four *Brassica* members. ISSR-PCR amplifications using seven primers generated a total of 72 reliable fragments from the 120 individuals of the *Brassica* taxa studied (Table 3). The size of these fragments ranged between 240 to 1,700 bp (Table 3). Of the 72 ISSR markers scored, only two bands were present in all the individuals and the rest of the fragments were polymorphic within and among the *Brassica* members investigated. Considering the nine wild populations of *B. oleracea*, 68 ISSR bands were scored and four of them were monomorphic. Figure 1 shows the amplification patterns generated using the primer UBC889 in 20 individuals from two wild populations of *B. oleracea*.

The minimum and maximum number of fragments generated per primer were 5 (primer UBC808) and 15 (primer UBC890), respectively (Table 3), with an average of 10.3 fragments. The average number of fragments per primer was higher for the three 5′ triple-anchored primers (13.3) than for those three with 3′ double-anchored primers (8.3).

The UPGMA dendrogram obtained using the 72 ISSR bands scored in the 120 individuals showed well-defined groups for all the populations studied (Fig. 2). The individuals of *B. oleracea* formed different clusters for each wild population, with a minimum of 80% of their individuals, except for the Bo-C (Tunel de Laredo, Santander/Spain) and Bo-G (Granville, Normandie/France) populations which were genetically more diverse. The individuals of population Bo-C were grouped into two clusters, but the five individuals of population Bo-G were dispersed in the dendrogram (Fig. 2). The ten individuals of *B. alboglabra* formed a distinct group outside the principal cluster comprising 110 individuals. However, individuals of *B. bourgeaui* and *B. montana* were found inside, joined to the *B. oleracea* populations (Fig. 2). The Mantel test revealed a significant cophenetic correlation ($r = 0.77$; $P = 0.001$), which indicates that the dendrogram provides a good fit to Dice's similarity matrix.

Highly significant ($P < 0.0005$) genetic differences were detected in the analysis of molecular variance (AMOVA) among the wild populations of *B. oleracea*. Of the total molecular variance, 32% was attributable to

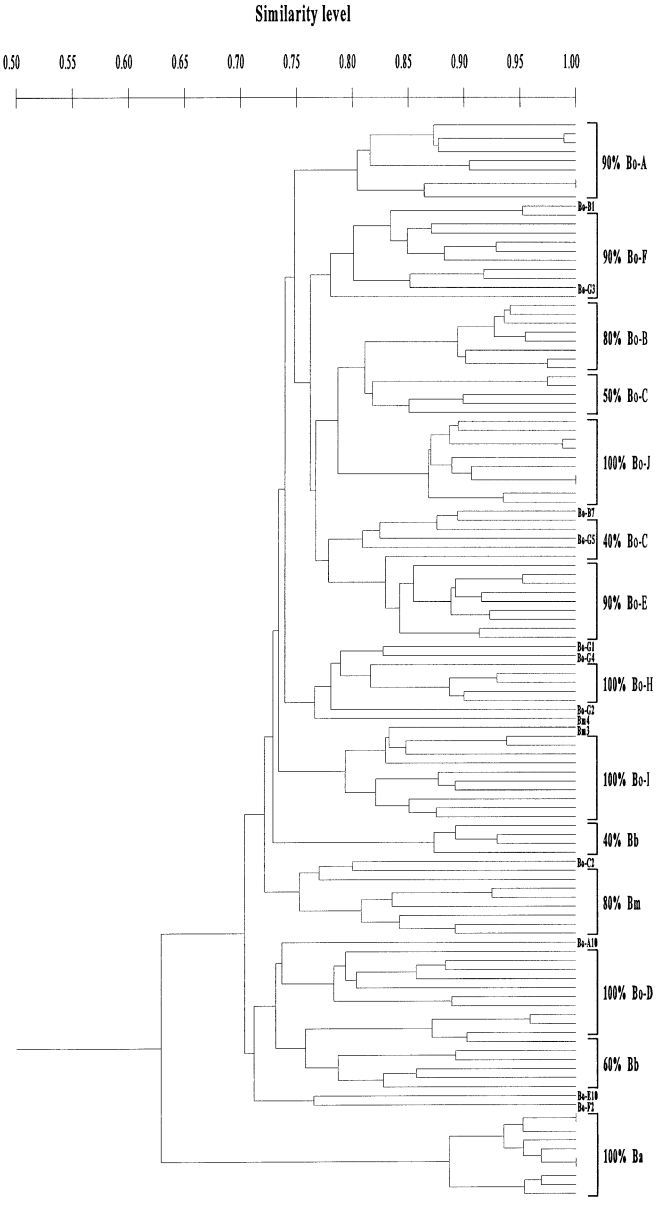

Fig. 2 Dendrogram of 120 individuals of the four *Brassica* taxa (*Bo* = *B. oleracea*; *Ba* = *B. alboglabra*; *Bb* = *B. bourgeaui*; *Bm* = *B. montana*) generated by UPGMA clustering analysis, based on 72 ISSR markers (see Table 1 for the codes of the individuals/populations/taxa)

population divergence and 68% to individual differences within populations. Bartlett's test for homogeneity of the ISSR variance for all pairwise population comparisons were highly significant ($P < 0.0005$). Genetic distances among the wild populations of *B. oleracea* obtained from AMOVA (Phi_{st} values between pairs of populations) revealed that all distances between pairs of populations were significantly different from zero.

Discussion

The PCR-RFLP analysis of 80 individuals of wild *B. oleracea* (nine wild populations; Table 1) revealed no variation of cpDNA in this species. This indicates they may have the same chloroplast lineage. In general, chloroplast DNA, at the intra-specific level, exhibits very low variation (Palmer 1987). However, significant variation (Mohanty et al. 2000) or no variation (Clegg et al. 1984; Gepts and Clegg 1989) has also been observed.

The ISSR-PCR analysis with the wild individuals/populations of *B. oleracea* has detected intra-specific polymorphism of nDNA. Using only seven ISSR primers almost every individual/population could be distinguished. It is noteworthy that the average number of fragments per primer was higher for the three 5′ triple-anchored primers (13.3) than for those three with 3′ double-anchored primers (8.3). Though we did not carry out an extensive comparison between 3′ double- and 5′ triple-anchored primers using the same core repeat, similar to Zietkiewicz et al. (1994), Moreno et al. (1998), Blair et al. (1999) and Martín and Sánchez-Yélamo (2000), our results indicate that 5′-anchored primers were more non-specific (lacking selective nucleotides at the critical 3′ end) than the others, thus generating a higher number of fragments.

Analyses of the ISSR markers using two different approaches (cluster analysis and AMOVA) demonstrated similar interpretations of the genetic diversity and structure of the wild populations of *B. oleracea*. The nine wild populations studied may be considered genetically to be significantly different from each other. In the case of the six Spanish populations, these results indicate high genetic isolation among populations. The Bo-D (Cabo de Oyambre, Cantabria/Spain), Bo-H (Swanage, Dorset/United Kingdom) and Bo-I (Glamorgan, Wales/United Kingdom) populations formed complete clusters with 100% of their individuals, and were genetically less diverse, whereas the Bo-C (Tunel de Laredo, Santander/Spain) and Bo-G (Granville, Normandie/France) populations were the most diverse. The dendrogram explains the similarity level of the different populations covered in this study.

However, a significant loss of diversity through genetic drift has not been detected in the present study (high diversity within populations has been found), probably because the size of the populations is not critically low, as shown by Ellstrand and Elam (1993). Due to their hostile ecological niches, on coastal cliffs, the geographical isolation may be an important factor influencing the genetic structure of wild *B. oleracea* populations. Similar observations were made in fragmented populations of *Caesalpinia echinata* (Cardoso et al. 1998) and *Rosmarinus tomentosus* (Martín and Hernández-Bermejo 2000).

B. alboglabra, B. bourgeaui and *B. montana* often showed variations in their taxonomic status; some authors assigned them to an infra-specific category under *B. oleracea* (Snogerup 1980; Song et al. 1988; Snogerup et al. 1990; Tutin et al. 1993; Gómez-Campo 1999; Gladis and Hammer 2001), while others cited them as distinct species (Borgen et al. 1979; Song et al. 1990; Lázaro and Aguinagalde 1998a,b; Hansen et al. 2001). The exomorphic differences among these taxa are very slight (discussed later), and they exhibit the same chromosome number, n = 9 (Prakash et al. 1999). The crossing data showed that these taxa (also most other members of the *B. oleracea*-group) were interfertile, and produced sufficient fertile hybrids; therefore, they all belong to one and the same "coenospecies" according to the biological species concept (Borgen et al. 1979). Such hybrids, in general, exhibited a high bivalent pairing at meiotic MI, indicating that these *B. oleracea*-group members have basically the same genome (Bothmer et al. 1995). Based on nuclear RFLP studies, Song et al. (1990) commented that the n = 9 chromosome wild brassicas related to *B. oleracea* were closely related to each other (and to the cultivated forms); therefore, they may belong to a single species.

B. alboglabra (Chinese kale) was considered to be an ancient form of *B. oleracea*, cultivated in South Italy and China, and probably reached China from the Mediterranean region (Gómez-Campo 1999). Morphologically, it mainly differs from *B. oleracea* showing white flowers (petals whitish yellow/pale yellow in *B. oleracea*; Snogerup et al. 1990). The crossing between wild *B. oleracea* × *alboglabra* produced sufficient fertile hybrids (64%), and *alboglabra* is suggested as conspecific with and an included taxon of *B. oleracea* (Bothmer et al. 1995). Isozyme and RAPD data showed *B. alboglabra* very close to *B. oleracea* in the dendrogram, and suggested it as a "subspecies" of *B. oleracea* (Lázaro and Aguinagalde 1998a,b). The nuclear RFLP studies found the wild *B. oleracea* and *B. alboglabra* clustered together (Song et al. 1990). The PCR-RFLP analysis demonstrated that *B. alboglabra* and *B. oleracea* have a similar cpDNA. The dendrogram based on the present ISSR study found *B. alboglabra* showing more than a 60% genetic similarity level with *B. oleracea*, and formed a distinct group with 100% of their individuals (outside the principal cluster comprising 110 individuals), which can be a result of its "selection of cultivation" since ancient times (Gómez-Campo 1999). Whereas, a recent cultivar of *B. oleracea* (Bo-J; Table 1) also formed a complete group with 100% of their individuals, and occupied an inside position in the principal cluster showing affinity with the wild *B. oleracea* populations. On the basis of such accumulated data, it could therefore be established

that *B. alboglabra* is a variety of *B. oleracea*, rather than a distinct species.

B. bourgeaui is an extremely rare plant, probably on its way to extinction and endemic to the Canary Islands (Borgen et al. 1979). It was assigned a subspecific status under *B. oleracea* (as *B. oleracea* ssp. *bourgeaui*; *cf.* Gladis and Hammer 2001). Morphologically, it differs from *B. oleracea* possessing basal leaves, which are simple, obovate, sharply dentate, subsessile, cauline leaves with very few but not amplexicaule (in *B. oleracea* basal leaves are pinnatipartite-lyrate, not bluntly dentate, petiolate, and cauline leaves abundant and amplexicaule; Snogerup et al. 1990). The morphological differences between *B. bourgeaui* and *B. oleracea* are so slight that the taxonomic rank of *B. bourgeaui* as a species of its own was questioned (Borgen et al. 1979). The crosses between wild *B. oleracea* × *bourgeaui* resulted in a high fertility and good seed set (Bothmer et al. 1995). The traditional RFLP investigation of cpDNA showed *B. bourgeaui* very similar to *B. oleracea*, and it was concluded that the taxon does not warrant specific ranking and should be included within *B. oleracea* (Warwick and Black 1993). The PCR-RFLP analysis found no variation of cpDNA between these taxa. ISSR patterns revealed nearly 75% genetic similarity between *B. bourgeaui* and *B. oleracea*. The sub-specific ranking of *B. bourgeaui* under *B. oleracea* could, therefore, be confirmed. *B. bourgeaui* formed two isolated clusters in the dendrogram, and this further demonstrated the genetic diversity of this population.

B. montana was included under *B. oleracea* ssp. *robertiana* (Gay) Bonnier & Layens (Tutin et al. 1993), and a later combination (illegitimate) was made by Gladis and Hammer (2001) as *B. oleracea* ssp. *robertiana* (Gay) Gl. et Hm. *comb. nov*., which included *B. montana* as a synonym. Morphologically, *B. montana* mainly differs from *B. oleracea* showing its leaves not or slightly greyish, pure to bluish green, and rostrum seeds are common (in *B. oleracea* leaves are greyish-glaucous, and rostrum seeds often absent; Snogerup et al. 1990). Isozyme and RAPD analyses found nearly 60% genetic similarity between *B. oleracea* and *B. montana* (Lázaro and Aguinagalde 1998a,b). The PCR-RFLP analysis found no variation of cpDNA between these members. ISSR results showed *B. montana* individuals inside, and joined to, the *B. oleracea* populations. *B. montana* is also interfertile with *B. oleracea* (Kinanian and Quiros 1992). The morphological, molecular and crossing data, therefore, could also suggest the sub-specific ranking of *B. montana* under *B. oleracea*.

Traditional RFLP analysis of cpDNA has provided information on the maternally inherited chloroplast genome in *Brassica* spp. (Erickson et al. 1983; Palmer et al. 1983). The present PCR-RFLP study recorded the cpDNA of the relevant four *Brassica* taxa as monomorphic, which indicates that these taxa have the same chloroplast (and maternal) lineage, further supporting the view that they are in the same cytodeme (Gómez-Campo and Hinata 1980; Warwick and Black 1993).

The study demonstrates the usefulness of ISSR markers in detecting genetic variation within and among the wild populations of *B. oleracea*, and provides a further understanding of species relationships in the *B. oleracea*-complex. Populations showing relatively high or low genetic diversity were identified that could facilitate management and conservation programs in this species. ISSR patterns uphold that *B. alboglabra, B. bourgeaui* and *B. montana* are co-specific (perhaps with a subspecies category) to *B. oleracea*. All these members have a common chloroplast lineage as reflected by their monomorphic PCR-RFLP pattern.

Acknowledgements S.P. is grateful for a Spanish Government Fellowship awarded to him. The research was supported by the Spanish project grant INIA-RF01-016. We thank Prof. C. Gómez-Campo for providing seed material, and for helpful suggestions and valuable comments on the manuscript.

References

Blair MW, Panaud O, McCouch SR (1999) Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oriza sativa* L.). Theor Appl Genet 98:780–792

Borgen L, Rustan OH, Elven R (1979) *Brassica bourgeaui* (Cruciferae) in the Canary Islands. Norwegian J Bot 26:255–264

Bothmer Rv, Gustafsson M, Snogerup S (1995) *Brassica* sect. *Brassica* (Brassicaceae). II. Inter- and intra-specific crosses with cultivars of *B. oleracea*. Genet Resour Crop Evol 42:165–178

Cardoso MA, Provan J, Powell W, Ferreiras PCG, De Oliveira DE (1998) High genetic differentiation among remnant populations of the endangered *Caesalpinia echinata* Lam. (Leguminosae-Caesalpinioideae). Mol Ecol 7:601–608

Clegg MT, Rawson JR, Thomas K (1984) Chloroplast DNA variation in pearl millet and related species. Genetics 106:449–461

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129–131

Dice LR (1945) Measures of the amount of ecologic association between species. Ecology 26:297–302

Dumolin-Lapègue S, Pemonge M-H, Petit RJ (1997) An enlarged set of consensus primers for the study of organelle DNA in plants. Mol Ecol 6:393–397

Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size: implications for plant conservation. Annu Rev Ecol Syst 24:217–242

Erickson LR, Straus NA, Beversdorf WD (1983) Restriction patterns reveal origins of chloroplast genomes in *Brassica* amphidiploids. Theor Appl Genet 65:201–206

Excoffier NC, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Gepts P, Clegg MT (1989) Genetic diversity in Pearl Millet *Pennisetum glaucum* (L.) R.Br. at the DNA sequence level. J Hered 80:203–208

Gielly L, Taberlet P (1994) The use of chloroplast DNA to resolve plant phylogenies: non-coding vs RbcL sequences. Mol Biol Evol 11:769–777

Gladis T, Hammer K (2001) Nomenclatural notes on the *Brassica oleracea*-group. Genet Resour Crop Evol 48:7–11

Gómez-Campo C (1990) A germplasm collection of crucifers. Catálogos INIA 22

Gómez-Campo C (ed) (1999) Biology of *Brassica* coenospecies. Elsevier Science, The Netherlands

Gómez-Campo C, Hinata K (1980) A check list of chromosome numbers in the tribe *Brassiceae*. In: Tsunoda S, Hinata K, Gómez-Campo C (eds) *Brassica* crops and wild allies: biology and breeding. Japan Scientific Societies Press, Tokyo, pp 51–63

Hansen LB, Siegismund HR, Jorgensen RB (2001) Introgression between oilseed rape (*Brassica napus* L.) and its weedy relative *B. rapa* L. in a natural population. Genet Resour Crop Evol 48:621–627

Joshi SP, Gupta VS, Aggarwal RK, Ranjekar PK, Brar DS (2000) Genetic diversity and phylogenetic relationship as revealed by inter-simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. Theor Appl Genet 100:1311–1320

Kinanian SF, Quiros CF (1992) Trait inheritance, fertility, and genomic relationships of some n = 9 *Brassica* species. Genet Resour Crop Evol 39:165–175

Lázaro A, Aguinagalde I (1998a) Genetic diversity in *Brassica oleracea* L. (Cruciferae) and wild relatives (2n = 18) using isozymes. Ann Bot 82:821–828

Lázaro A, Aguinagalde I (1998b) Genetic diversity in *Brassica oleracea* L. (Cruciferae) and wild relatives (2n = 18) using RAPD markers. Ann Bot 82:829–833

Martín JP, Hernández-Bermejo JE (2000) Genetic variation in the endemic and endangered *Rosmarinus tomentosus* Huber-Morath & Maire (*Labiatae*) using RAPD markers. Heredity 85:434–443

Martín JP, Sánchez-Yélamo MD (2000) Genetic relationships among species of the genus *Diplotaxis* (*Brassicaceae*) using inter-simple sequence repeat markers. Theor Appl Genet 101:1234–1241

Mohanty A, Martín JP, Aguinagalde I (2000) Chloroplast DNA diversity within and among populations of the allotetraploid *Prunus spinosa* L. Theor Appl Genet 100:1304–1310

Moreno S, Martín JP, Ortiz JM (1998) Inter-simple sequence repeats PCR for characterization of closely related grapevine germplasm. Euphytica 101:117–125

Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am Nat 130:S6–S29

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. Theor Appl Genet 65:181–189

Plieske J, Struss D (2001) Microsatellite markers for genome analysis in *Brassica*. I. Development in *Brassica napus* and abundance in *Brassicaceae* species. Theor Appl Genet 102:689–694

Prakash S, Takahata Y, Kirti PB, Chopra VL (1999) Cytogenetics. In: Gómez-Campo C (ed) Biology of *Brassica* coenospecies. Elsevier Science, The Netherlands, pp 59–106

Rohlf FJ (1998) NTSYS-PC: numerical taxonomy and multivariate analysis system. Version 2.0. Exeter Software, New York

Saal B, Plieske J, Hu J, Quiros CF, Struss D (2001) Microsatellite markers for genome analysis in *Brassica*. II. Assignment of rapeseed microsatellites to the A and C genomes and genetic mapping in *Brassica oleracea* L. Theor Appl Genet 102:695–699

Sebastian RL, Howell EC, King GJ, Marshall DF, Kearsey MJ (2000) An integrated AFLP and RFLP *Brassica oleracea* linkage map from two morphologically distinct doubled-haploid mapping populations. Theor Appl Genet 100:75–81

Snogerup S (1980) The wild forms of the *Brassica oleracea* group (2n = 18) and their possible relations to the cultivated ones. In: Tsunoda S, Hinata K, Gómez-Campo C (eds) *Brassica* crops and wild allies: biology and breeding. Japan Scientific Societies Press, Tokyo, pp 121–132

Snogerup S, Gustafsson M, Bothmer Rv (1990) *Brassica* sect. *Brassica* (*Brassicaceae*). I. Taxonomy and variation. Willdenowia 19:271–365

Song KM, Osborn TC, Williams PH (1988) *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). 2. Preliminary analysis of subspecies within *B. rapa* (syn. *campestris*) and *B. oleracea*. Theor Appl Genet 76:593–600

Song KM, Osborn TC, Williams PH (1990) *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). 3. Genome relationships in *Brassica* and related genera and the origin of *B. oleracea* and *B. rapa* (syn. *campestris*). Theor Appl Genet 79:497–506

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Toxopeus H (1974) Proceedings of Eucarpia meetings 1974, pp 1–7

Tutin TG, Burges, NA, Chater, AO, Edmondson JP, Heywood VH, Moore DM, Valentine DH, Walters SM, Webb DA (eds) (1993) Flora Europaea, volume 1, Psilotaceae to Platanaceae, 2nd edn. Cambridge University Press, UK

Warwick SI, Black LD (1993) Molecular relationships in subtribe Brassicinae (Cruciferae, tribe Brassiceae). Can J Bot 71:906–918

Warwick SI, Black LD, Aguinagalde I (1992) Molecular systematics of *Brassica* and allied genera (subtribe Brassicinae, Brassiceae) – chloroplast DNA variation in the genus *Diplotaxis*. Theor Appl Genet 83:839–850

Wolfe AD, Liston A (1998) Contributions of PCR-based methods to plant systematics and evolutionary biology. In: Soltis DE, Soltis PS, Doyle JJ (eds) Plant molecular systematics II. Chapman Hall, New York, pp 43–86

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183