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Characterisation of *Brassica oleracea* L. by microsatellite primers

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Abstract. The recent development of molecular marker technology is revolutionising the study of plant populations, providing opportunities to address questions requiring a precise knowledge of pedigrees. We applied Inter-Simple Sequence Repeat (ISSR) PCR to several *Brassica oleracea* accessions and to *Brassica napus*. Four microsatellite-primers were screened and, among the 136 reproducible fragments recorded, 25 (18.4%) fragments were common for all *Brassica*, 27 (19.9%) were unique and 84 (61.7%) were phylogenetically informative. Each individual test sample exhibited a unique molecular genotype. ISSR markers provided a rapid approach to analyse genetic diversity and reflected the known genetic relationships among selected entries. ISSR markers appeared of great value in gene bank management and the establishment of genetic similarity, and can be applied to allogamous (autumn and winter cauliflower) crops.

Key words: *Brassica oleracea* L., cauliflower, diversity, germplasm, inter-simple sequence repeats, microsatellites.

All investigated eukaryotic genomes have microsatellites or Simple Sequence Repeats (SSRs). Microsatellites have been shown to be useful as genetic markers (Litt and Luty 1989, Weber and May 1989). Akkaya et al. (1992) successfully used SSRs for comparative analysis and mapping of plant genomes. However, this technique is limited by the following stages which can be exceptionally time consuming and expensive: construction of a genomic library, screening of the library with repeat sequences, sequencing positive clones, synthesising specific primers from the flanking sequences for each cloned SSR locus, and screening for polymorphisms. Inter-Simple Sequence Repeat (ISSR) PCR amplification has been developed to circumvent these limitations (Zietkiewicz et al. 1994). The primers are particular SSR sequences and generate products when microsatellites are identical, closely spaced and inversely orientated. The primers used in ISSR analysis can be based on any of the microsatellite motifs (simple perfect, simple imperfect or compound), giving an enriched polymorphic banding pattern relative to single-locus SSR. Microsatellite-primers have been applied to cultivar identification (Charters et al. 1996, Fang and Roose 1997), to the assessment of genetic diversity (Kantety et al. 1995), to detect polymorphism among accessions (Nagaoka and Ogihara 1997), for genome stability in in vitro culture (Leroy et al. in press) and have been mapped on to plant chromosomes (Kojima et al. 1998).

Diploid *Brassica* species are ancient polyploids that have evolved through frequent rearrangements and chromosomal fusion.

Several species of *Brassica* are of considerable economic importance, including a wide variety of horticultural crops of the *B. oleracea* group (broccoli, Brussels sprouts, cabbage, cauliflower, kale). In the present communication, genetic variation among *Brassica oleracea* L. accessions and the fingerprint of individual genotypes using microsatellite-primers are reported.

Materials and methods

Plant materials. Plant materials included *B.o.* var. *gemmifera* (Brussels sprouts; cv. Sanda), *B.o.* var. *capitata capitata* (cabbage; cv. Précoce de Louviers), *B.o.* var. *capitata sabauda* (cabbage; cv. De Pontoise 2), *B.o.* var. *acephala* (kale; cv. Proteor), *B. napus* (turnip; cv. Wilhemsburger) and *B.o.* var. *botrytis* (cauliflower; cv. Meurz, DH_1 to DH_6). The six doubled haploid (DH_{1-6}) cauliflower lines were supplied by Dr. Tim Lunn (Organisation Bretonne de Sélection, Plougoulm, France), whereas the other *B. oleracea* were obtained on the market place. Plants were raised in a growth chamber (14-h photo- and thermo-period (light and T = 22 ± 1 °C with hygrometry = 75%; dark and T = 18 ± 1 °C with hygrometry = 80%)) and DNA was extracted from young leaf tissue (100 mg) using a CTAB (CetylTrimethylAmmonium Bromide) protocol (Saghai-Maroof et al. 1984).

Inter-SSR PCR amplification. The primers used in this study were purchased from Cybergene, Saint Malo, France. PCR amplifications were performed in a Perkin Elmer GeneAmp PCR 2400 Thermo-cycler. For each ISSR reaction, 15 ng of DNA was used in 25 µl of a reaction mixture containing 75 mM Tris-HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.001% (w/v) Tween 20, 2.5 mM $MgCl_2$, 200 µm of each dNTP (Eurogentec) with 0.1 nM primer, and 1.25 unit of *Taq* DNA polymerase (Goldstar 'red', Eurogentec). Amplifications were carried as follows: an initial denaturation step of 1 min at 94 °C; 1 min at 94 °C, 1 min at specific annealing temperature, 4 min at 72 °C for 27 cycles, followed by 7 min at 74 °C. 2 µl of each PCR amplification product were mixed with 0.5 µl of loading buffer (10 mM EDTA, 0.1% (w/v) bromophenol blue and 0.1% (w/v) xylene cyanol), loaded on 5% non-denaturing polyacrylamide gel and silver stained (Gootlieb and Chavko 1987). Gel images were acquired and analysed with Gel Doc 1000 (Bio-Rad).

Data analysis. The PHYLogeny Inference Package (PHYLIP 3.5c 1993) was used in our study (Felsenstein 1993). The distance coefficient used for analysis was the Nei and Li (1979) coefficient. The matrix was formatted with bands represented as 1 and the absence of bands is represented as 0. Cluster analysis was performed using an unweighted pair-group method, arithmetic average (UPGMA).

Results and discussion

Four primers which proved useful in somaclonal studies were used to generate ISSR patterns (Table 1). These primers can vary in length from 15 to 16 nucleotides, with a [G + C] content from 25% to 66.6%. All of the amplified scorable fragments were between 250 bp and 2000 bp or 3000 bp, according to the primer used. One hundred and thirty-six scorable fragments were amplified and their numbers ranged from 29 to 45 per microsatellite primer.

Table 1. Number of *Brassica* loci generated by the four microsatellite primers. $(CAG)_5$: CAGCAGCAGCAGCAG. $(CAA)_5$: CAACAACAACAACAA. $(GACA)_4$: GACAGACAGACAGACA. $(GATA)_4$: GATAGATAGATAGATA

Primer	*B.o. gemmifera*	*B.o. acephala*	*B.o. cap. cap*	*B.o. cap. sab.*	*B.o. botrytis*	*B. napus*	No. fragments
$(CAA)_5$	18	19	15	20	20	13	29
$(CAG)_5$	17	16	17	22	20	18	31
$(GATA)_4$	19	21	18	17	10	18	31
$(GACA)_4$	28	22	25	33	22	29	45
Total	82	78	75	92	72	78	136

For the PHYLIP program, all of the fragments were used in constructing an unrooted network.

Reliability and repeatability of primer amplication are essential for fingerprinting. Differences in amplification observed by Blair et al. (1999) might be due to the annealing conditions and to the component concentrations of the PCR mixture. For each of these primers, an optimal hybridisation temperature was determined: 42 °C, 52 °C, 54 °C, and 62 °C for $(GATA)_4$, $(GACA)_4$, $(CAA)_5$ and $(CAG)_5$, respectively. All other PCR parameters were tested using the $(GACA)_4$ primer and its optimal hybridisation temperature (52 °C). $MgCl_2$ and dNTP concentration strongly affected the yield of the reaction, as well as its specificity. Quantity of *Taq* DNA polymerase added to the reaction mix had relatively few consequences, and amplification profiles obtained with 1 to 2 units of red goldstar DNA polymerase were clearly similar. Between 50 and 200 pmoles/reaction, primer concentration only affected the yield of the amplification reaction, but for all these concentrations, all the amplified ISSRs are detectable The number of PCR cycles used proved to be important for obtaining clear amplification profiles. Although 22 cycles were sufficient to amplify some ISSRs to a detectable level, complete profiles were only obtained after 27 cycles. However, after 30 cycles, smears were observed upon electrophoresis of the product, reflecting a loss of specificity for the reaction.

Applying the improved ISSR conditions, the four primers amplified products for all accessions of *Brassica oleracea* (*gemmifera*, *acephala*, *cap. capitata.*, *cap. sabauda.*, *botrytis*) and *Brassica napus*. These primers generated complete patterns with an average of 94, 82, 78, 75 and 72 markers for *B. o. cap. sabauda*, *B. o. gemmifera*, *B. o. acephala*, *B. o. cap. capitata* and *B. o. botrytis*, respectively, and 78 markers for *B. napus* (Table 1).

Variations between *Brassica* species were observed with few bands common among the six species and 111 polymorphic amplification products (81.6%). Using primers $(GACA)_4$, $(CAG)_5$ and $(CAA)_5$, we identified respectively 36, 23 and 23 polymorphic bands. DNA fingerprinting based on PCR with the microsatellite $(GATA)_4$ showed 93.5% of polymorphism in 31 scored markers. This marker exhibited two monomorphic bands and one specific marker for *B. o. acephala*, *B. o. cap. capitata* and *B. napus* (Table 2). The most genome specific markers were obtained by $(GACA)_4$ with 9 markers, the least by $(GATA)_4$ with 3 markers. Twenty seven bands had species-specific variants and 17 were specific to *B. napus*.

The effects of using trinucleotides, tetranucleotides or combining all four primers were investigated. PCR using trinucleotides gave a total of 60 markers; of these primers 15 markers were species specific and 14 were monomorphic. PCR using the two tetranucleotide primers gave a total of 76 ISSRs markers among the accessions, with an average of 12 species specific bands and of 11 monomorphic bands. Among the primers evaluated, approximately 81.6% (111/136) detected variation in the *Brassica* array. The *B. oleracea* accessions evaluated were readily fingerprinted using microsatellite primers, with any of the four primers being sufficient to generate distinct

Table 2. Number of genome specific markers generated and polymorphism exhibited

Primer	*B.o. gemmifera*	*B.o. acephala*	*B.o. cap. cap*	*B.o. cap. sab.*	*B.o. botrytis*	*B. napus*	No. specific markers (%)
$(CAA)_5$	1		1		1	4	7 (24.1)
$(CAG)_5$				1		7	8 (25.8)
$(GATA)_4$		1	1			1	3 (9.7)
$(GACA)_4$	1			2	1	5	9 (20)
Total	2	1	2	3	2	17	27 (19.9)

fingerprints for all the accessions. These four primers generated 119 markers. This is in contrast to the use of RAPDs to fingerprint *B. oleracea* L. genotypes, where a total of 60 primers was required to amplify 62, 100 and 89 markers in cauliflower, cabbage and kale, respectively (Margalé et al. 1995). Combined with the separation of amplification products on polyacrylamide gels, ISSR amplification can reveal much larger numbers of polymorphic fragments per primer than RAPD analysis (Wolff et al. 1995).

Based on ISSR fragments, a similarity matrix was generated using the coefficient of

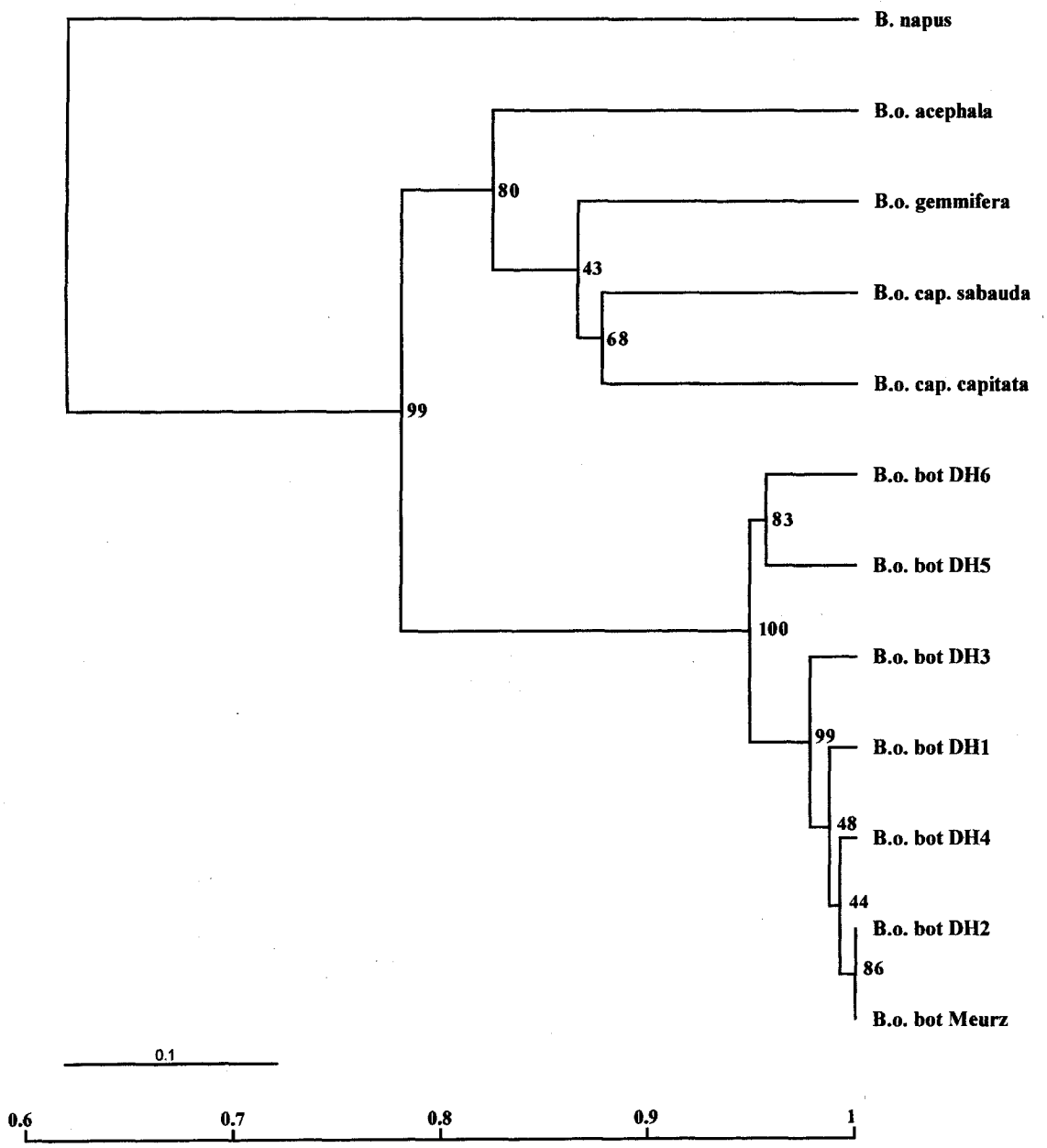

Fig. 1. Dendrogram illustrating the genetic relationships between *Brassica* accessions. The dendrogram was drawn based on UPGMA cluster analysis, using the similarity matrix derived from 136 ISSR fragments. The scale represents 0.1 unit of the Nei and Li distance. The significance of branching nodes was tested by boostrap resampling (100 samples) using the programs SEQBOOT and CONSENSE of PHYLIP

Nei and Li (1979). Figure 1 is the dendrogram constructed by UPGMA cluster analysis. From this dendrogram, all accessions can be separated into three major groups. The *B. napus* sample gave a very distinct banding pattern and was clearly separated from the *B. oleracea* accessions. Between *B. oleracea* and *B. napus*, 17 fragments were useful for discrimination (Table 2). The second group contained the cauliflowers. The dendrogram separated the cauliflower cultivars into two distinct populations. DH_5 and DH_6 joined together closely at the 83% level, whereas another subgroup consisted of Meurz and DH_1 to DH_4. Those two subgroups corresponded to the seasonally sampled groups: winter and autumn. Undoubtedly, seasonal adaptation has been the main selection factor for cauliflower, with few cross pollination leading to genetic exchanges, due to temporal isolation between the groups. The separation of cauliflower from kale and cabbage, illustrated by the separate line of evolution of cauliflower in the eastern Mediterranean from other Kale in western Europe, was similar to the grouping observed by Dias (1995). The kale-cabbage-Brussels sprouts group was less structured, but the two varieties of conv. *capitata* (*sabauda* and *capitata*) were clustered separately from Proteor and Sanda. Whereas in previous studies Brussels sprouts appeared to be related to cauliflower (Song et al. 1988, 1990), in our study this accession is separated from the cauliflower group and is closer to the cabbage one.

The variation identified in this study indicated that $(CAA)_5$, $(CAG)_5$, $(GATA)_4$ and $(GACA)_4$ were common in the *Brassica* genome and, if single locus microsatellites were to be developed, $(GATA)_4$ repeats should provide a useful source of markers (Leroy et al. in preparation). ISSRs markers offered great potential for differentiating closely related cauliflower cultivars and it would be interesting to investigate the molecular basis of the differences between them by sequencing the polymorphic markers. In preliminary results, modifications in the microsatellite repetition number were shown up, with an increase of an internal $(CT)_7$ for one of the DH lines as well as mutation of a microsatellite for a cauliflower somaclonal variant (data not shown). The presence of additional internal microsatellites was of great interest, implying that there may be clustering of microsatellites in some genomic regions (Fisher et al. 1996). Two major advantages of such a technique were that the construction of an enriched-library was technically simple and fast and that once microsatellite loci were identified, most loci required the design and synthesis of only one additional PCR primer, reducing the cost per locus. At the same time, internal microsatellites, were amplified by two specific primers, as normal, and could be used for detecting polymorphism between closely related material.

On the other hand, a certain degree of pattern similarity observed in related species could serve to infer evolutionary relationships. This method greatly facilitated the simultaneous identification of multiple genomic polymorphisms and offered a great advantage by identifying polymorphic markers linked to genetic traits of interest. Microsatellite-primers appeared as an efficient and generic technique for genome fingerprinting, germplasm evaluation and whole-genome comparisons. The first results provided evidence of the usefulness of ISSRs as genetic markers to clarify the relationships among closely related species and will allow us to complete the study with the available seed bank accessions in order to establish the more accurate phylogenetic relationship among Brassicaceae and to evaluate core collections.

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