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Identification of polymorphic, conserved simple sequence repeats (SSRs) in cultivated *Brassica* species

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Abstract The application of simple sequence repeat (SSR) genotyping for the characterization of genetic variation in crop plants has been hindered by ready access to useful primer pairs and potentially limited conservation of the repeat sequences among related species. In this phase of work, we report on the identification and characterization of SSRs that are conserved in *Brassica napus* L. (rapeseed) and its putative progenitors, *B. oleracea* L. (cabbage, and related vegetable types) and *B. rapa* (vegetable and oil types). Approximately 140 clones from a size-fractionated genomic library of *B. napus* were sequenced, and primer pairs were designed for 21 dinucleotide SSRs. Seventeen primer pairs amplified products in the three species and, among these, 13 detected variation between and within species. Unlike findings on SSR information content in human, no relationship could be established between the number of tandem repeats within the target sequence and heterozygosity. All primer pairs have been designed to work under identical amplification conditions; therefore, single-reaction, multiplex polymerase chain reaction (PCR) with these SSRs is possible. Once moderate numbers of primer pairs are accessible to the user community, SSR genotyping may provide a useful method for the characterization, conservation, and utilization of agricultural crop diversity.

Key words DNA marker · Genetic analysis · Genetic diversity · Genotyping · Microsatellite

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Introduction

Effective conservation of crop genetic resources is dependent on the ability of a curator to characterize, quantify, and make available genetic diversity from his/her collections. Molecular diagnostic techniques presently are being developed and utilized to improve genebank operations by more accurately and efficiently establishing the following genetic parameters: (1) identity of a particular accession, (2) relationship of genotypes within an accession or among accessions in a collection, (3) structure and quantity of variation among accessions and collections, and (4) location of particular genes or DNA sequences in an individual genotype (Kresovich et al. 1993). This fundamental information will allow curators to better organize and manage collections, as well as provide genetic markers that ultimately will find additional applications in crop improvement programs.

The simple sequence repeat (SSR) meets many requirements of an ideal marker for assessing genetic variation. SSRs are dispersed throughout eukaryotic genomes (Hamada et al. 1982; Tautz and Renz 1984; Tautz 1989; Weber and May 1989) and have been shown to be highly polymorphic (Weber and May 1989; Beckman and Soller 1990). SSRs follow simple Mendelian inheritance (Queller et al. 1993) and are sequence-tagged sites (Olson et al. 1989; Thomas and Scott 1993). Because SSRs have unique flanking sequences for site-specific amplification, they can readily be disseminated by publishing primer pair sequences or by sharing oligonucleotides. Moreover, for high throughput, typing of alleles of SSRs can be automated (Rafalski and Tingey 1993).

High levels of polymorphism have been reported with SSRs in selected plant species (Condit and Hubbell 1991; Akkaya et al. 1992; Poulsen 1993; Senior and Heun 1993; Zhao and Kochert 1993). Initial studies have provided evidence that it may be possible to distinguish among closely related genotypes using only 10-20 loci because of the high levels of information manifested by each marker (Rongwen et al. 1995). Therefore, an effort has been initiated to iden-

tify and characterize 20-30 loci in *Brassica napus* L. which would be utilized in mass genotyping efforts to better describe the oilseed and vegetable *Brassica* spp. genetic resources collections and, ultimately, develop higher quality holdings. We previously reported on the abundance of $(GA)_n$, $(CA)_n$, and $(GATA)_n$ repeats in a size-fractionated *B. napus* L. (rapeseed) genomic library (Kresovich et al. 1995). Findings on the first 124 SSR-containing clones were consistent with abundance estimates for other crop species. In this report, we present information on an additional 17 SSR markers of the *B. napus* genome that will amplify target sequences in closely related species and can be analyzed using identical polymerase chain reaction (PCR) conditions.

Materials and methods

Construction of size-fractionated genomic library

Genomic DNA was isolated from leaf tissue from *B. napus* 'Jet Neuf' using a modified CTAB technique with subsequent cesium chloride gradient purification (Sambrook et al. 1989). The genomic DNA then was digested with *Taq*I and electrophoresed on a low-melting temperature agarose gel (NuSieve GTG, FMC). Fragments of 100–500 bp were isolated from the excised gel slice by agarose digestion (Dumais et al. 1987), ligated to *Cla*I-digested, dephosphorylated pGEM-7Zf(+) (Promega). Recombinant clones were transformed into *E. coli* JM109 cells (Promega) using the supplier's protocol.

Screening of the genomic library

Colonies were transferred onto Duralose UV filters (Stratagene). Surveys of databases for abundance and informativeness of simple sequence repeats in human and plant species, especially B. *napus*, served as the basis for the selection of the oligonucleotides used to probe the library. Two dimeric oligonucleotide repeats, $(GA)_{10}$ and $(CA)_{10}$, and a single tetrameric oligonucleotide repeat, $(GATA)_6$, (supplied by Applied Biosystems) were labeled with [^{32}P] by a 5′ Terminus Labeling System (Gibco, BRL). Each of these oligonucleotides was used to screen 15,000 clones from the rapeseed genomic library according to the protocol supplied by Stratagene. An additional 15,000 colonies were screened with a $(GA)_{10}$ oligonucleotide labeled at the 3′ end with alkaline phosphatase and detected using the Boeringer Mannheim Genius Chemiluminescent kit and the supplier's protocol. For both [^{32}P]-labeled and alkaline phosphatase-labeled probes, hybridization and subsequent washes were conducted at 550C under high stringency. Each filter was screened at least twice, and only positive colonies detected in both screens were sequenced.

Sequencing of positive clones

Plasmid DNA of GA-, CA-, or GATA- repeat containing clones was isolated using a modified mini alkaline-lysis with PEG precipitation protocol supplied by Applied Biosystems. The template DNA was sequenced from M13 priming sites of pGEM 7Zf(+) using either the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit or the Prism Sequenase Terminator Double-Stranded DNA Sequencing Kit on an upgraded model 370 DNA Sequencing System (Applied Biosystems.)

Primer design

Oligonucleotides complementary to the flanking regions of the repeats were designed using Designer PCR (version 1.03, Research Genetics). To reduce problems with spurious banding patterns generated during amplification and also to allow single-reaction multiplex PCR, we employed stringent criteria in primer design: (1) primer T_m of 60°–65°C; and (2) ≤3.0°C difference in T_m between primers in a pair. Another critical criterion in primer design included limiting the size of amplified DNA fragments to a range of 80 to 400 bp. Primers were synthesized by Operon Technologies or on a model 392 Nucleic Acids Synthesizer (Applied Biosystems) and were purified by either HPLC or by oligonucleotide purification cartridges (Applied Biosystems) respectively.

Table 1 *Brassica* ssp. test array

Taxon	Origin	Cultivar
napus var oleifera	Belgium	Jet Neuf
napus var oleifera	Canada	Westar
napus var rapifera	Canada	Laurentian
oleracea var capitata	USA	Wisconsin Golden Acre
oleracea var capitata	USA	Jersey Wakefield
olercea var costata	Portugal	Couve Nabica
oleracea var selenesia	Netherlands	Westland Winter
rapa var oleifera	Canada	Span
rapa var narinosa	Japan	Tatsoi
rapa var pekinenesis	USA	Michihili
rapa var rapifera	USA	Purple Top White Globe

Evaluation of SSRs for amplification and polymorphism in a *Brassica* test array

To represent a range of diversity among *B. napus*, *B. oleracea*, and *B. rapa* species, we selected a test array of 11 *Brassica* accessions (Table 1). Leaf tissue from a single plant of each accession was harvested and freeze dried. Genomic DNA from a single plant of each accession was extracted by a modified CTAB extraction protocol (Colosi and Schaal 1993). PCR products from reaction mixtures containing the original clone that was sequenced to design a specific primer pair and 'Jet Neuf' (source DNA for genomic library construction) were used as positive controls for the estimate of fragment size range. The negative control included a reaction mixture without any DNA. For each primer pair, 30 ng of DNA was amplified in a 20 µl solution containing 1.0 µ*M* of each primer, 0.25 m*M* each dNTP, 3.125 m*M* $MgCl_2$, 1×Perkin-Elmer Cetus reaction buffer and 1.0 units *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus). After one denaturing step of 2 min at 94°C, a "touchdown" amplification program was utilized (Mellersh et al. 1993). DNA was denatured for 60 s at 94°C, annealed for 30 s, and extended for 45 s at 72°C. The initial annealing temperature was 68°C for 2 cycles and subsequently was dropped 10°C every 2 cycles until a final temperature of 58°C was reached. For the last 20 cycles of the amplification, an annealing temperature of 58°C was employed. Amplification products were separated by electrophoresis on a 4% FMC NuSieve 3:1 agarose gel with 1×TBE buffer. After electrophoresis, the gel was stained with ethidium bromide and products were visualized with UV light (Fig. 1). Fragments were sized by measuring the distance migrated relative to a 100-bp ladder (Gibco, BRL).

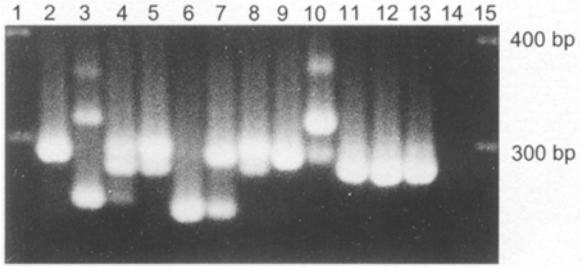

Fig. 1 Amplification products for marker B.n.12A. *Lanes 1* and *15* 100-bp ladders, *lane 2* DNA from clone 12A, *lanes 3–5*, *6–9*, and *10–13* accessions of *B. napus*, *B. oleracea*, and *B. rapa*, respectively, *lane 14* negative control

Table 2 Sequences of *Brassica* SSR primer pairs

SSR	5′ Primer sequence	Repeat motif	3′ Primer sequence
B.n.12A	5′ GCCGTTCTAGGGTTTGTGGGA	$(GA)_{11}(AAG)_4$	5′ GAGGAAGTGAGAGCGGGAAATCA
B.n.19A	5′ CACAGCTCACACCAAACAAACCTAC	$(GA)_8$	5′ CCCCGGGTTCGAAATCG
B.n.25C2	5′ AAACCTCCTCAAAAACCCCTAAACG	$(GA)_{10}$	5′ TCCCCTCTTTCCTCTCTCTCTAGGC
B.n.26A	5′ TAAACTTGTCAGACGCCGTTATC	$(GA)_{13}$	5′ CCCGTAAATCAAGCAAATGG
B.n.27B2	5′ CCGGATCCAAGCTTATCGACA	$(GA)_{12}$	5′ CCGAGAAGGGAAGCTAGAGAGGTC
B.n.34B1	5′ GACGAAAATCAATGGGAGGAAAAA	$(GT)_{11}(AAG)_3$	5′ TCCGGFATCCAAGCTTATCGAAA
B.n.35D	5′ GCAGAAGGAGGAGAAGAGTTGG	$(GA)_{13}$	5′ TTGAGCCGTAAAGTTGFTCACCT
B.n.38A	5′ TGGTAACTGGTAACCGACGAAAATC	$(TG)_{11}$	5′ ACGCTGTCTTCAGGTCCCACTC
B.n.40C1	5′ CCCCTCTTTGATTCTCCTCCGA	$(GA)_{10}$	5′ CGTGGTATGTTGGTATTGGGTCGT
B.n.50F	5′ CGTTGAAGCATCTCTGTATCTCTCC	$(GA)_{10}$	5′ TTTCTCTCCGCACCAAAACAC
B.n.59A1	5′ TGGCTCGAATCAACGGAC	$(CA)_{11}$	5′ TTGCACCAACAAGTCACTAAAGTT
B.n.68/1	5′ TCGCATGCTCCTCTAGACTCG	$(GA)_{34}(ACACA)_2$	5′ TTTAGCACGGGAATGTCAGG
B.n.72A	5′ GCCCACCCACCTTCTTGTCCT	$(TAA)_5(GA)_9$	5′ CCCTTCATCCAAACTCCTCCTCGT
B.n.75A	5′ ACCCCGGGTTCGAAATCG	$(GA)_{14}$	5′ CAATCCCACCAACCAGACAACC
B.n.80/3	5′ GACCCTTGTTTGAGCTGCCTCCTA	$(GA)_{15}(GGT)_2$	5′ ACAATCAATCCCACCAACCAGACA
B.n.83B1	5′ GCCTTTCTTCACAACTGATAGCTAA	$(GA)_{11}$	5′ TCAGGTGCCTCGTTGAGTTC
B.n.92A1	5′ ACCGCCCGTGACCAAA	$(A)_{28}$	5′ CCCACCCCGTTAACATATAAGTC

Results

In addition to the initial 124 clones sequenced in an earlier report (Kresovich et al. 1995), another 141 positive clones (102 GA- and 39 CA-positive) were characterized in this phase of the investigation. Among these clones, primer pairs could be designed for 21 to contain SSRs. This proportion (21/141, or approximately 15%) reflects that many clones had no SSR, that the SSR was too close to the restriction site for primers to be designed, that the high AT content of the insert precluded primer design, and the occasional unreconcilable sequence. In addition, approximately 5–10% of the clones sequenced were identical to previously characterized clones.

Of the 21 primer pairs synthesized for evaluation, 17 (Table 2) amplified a product or products in the expected size range (Table 3). Of these 17 primer pairs, 13 amplified polymorphisms in the *Brassica* spp. test array. Though the resolution could be improved with the use of polyacrylamide, the number of amplified fragments ranged from 1 to 8 per SSR (Table 3). Approximately 70 fragments in total were amplified by the 17 SSRs. If one excludes the monomorphic markers (4 pairs in this array), values for gene diversity of these SSRs (as calculated by the equation reported by Rongwen et al. 1995) were estimated to range from approximately 0.31 to 0.65. Because of the limitations associated with the detection methodology employed, the number of fragments reported represent a minimum value and the use of sequencing gels may yield more unique amplified fragments. Moreover, it is recognized that these values are subject to biases associated with the size and composition of the selected test array of *Brassica* spp. Overall, the number of amplified fragments within and between species were approximately equal.

On the basis of the amplification techniques optimized and employed, almost all primer pairs amplified products in the expected size range in all accessions of all *Brassica* spp. (*B. napus*, *B. oleracea*, and *B. rapa*) tested. Primer pair B.n.68/1, however, did not amplify a product in an accession of *B. oleracea* ('Wisconsin Golden Acre') and *B. rapa* ('Tatsoi'). Unlike the findings of Weber (1990) with human SSRs, we found no relationship between the size of the core tandem repeat and the information content of the SSR.

DNA sequences adjacent to the SSRs were searched against the National Center for Biotechnology Information (NCBI, Washington, D.C.) nonredundant database via the BLAST network server using the program BLASTN (Altschul et al. 1990). Significant matches were not obtained for any of the query sequences.

Table 3 Characteristics of *Brassica* SSR amplification products in the test array

SSR	Expected product size in 'Jet Neuf' (bp)	Size range of products (bp)	Number of amplified fragments per primer pair
B.n.12A	286	250–325	8
B.n.19A	197	175–225	4
B.n.25C2	142	130–175	4
B.n.26A	100	90–120	3
B.n.27B2	199	–	1
B.n.34B1	203	–	1
B.n.35D	221	210–270	6
B.n.38A	156	150–200	5
B.n.40C1	129	130–150	2
B.n.50F	100	–	1
B.n.59A1	430	425–480	6
B.n.68/1	276	200–400	7
B.n.72A	247	240–300	5
B.n.75A	254	–	1
B.n.80/3	111	100–145	3
B.n.83/1	194	190–240	4
B.n.92A1	113	110–145	3

Discussion

Our results corroborate with increasing evidence that SSRs are abundant in the *Brassica* spp. genome and are relatively easy to isolate and characterize (Kresovich et al. 1995; Lagercrantz et al. 1993). Moreover, these markers were conserved among closely related species.

The initial expense and time required to identify and characterize an individual SSR is a shortcoming of the marker (Cregan 1992). The number of false positive CA- and GATA-clones was the largest contributor to lost efficiency in screening of the *B. napus* library. Improved detection rates of SSR-positive clones may be obtained with more stringent hybridization conditions linked with the use of longer oligonucleotide probes or the development of genomic libraries enriched for specific nucleotide repeats. However, it must be emphasized that for SSR utilization in genetic resources conservation, less than 20 markers would be needed for most applications (Estoup et al. 1995). Furthermore, once developed and readily accessible, the utilization of SSR markers will be as cost effective as any other type of molecular assay.

Among the primer pairs evaluated, approximately 75% (13/17) detected variation in the *Brassica* spp. array. This proportion of informative markers is consistent with what might be expected with primarily allogamous, insect-pollinated species. Though the proportion was higher than what was observed with the first 7 characterized SSRs (Kresovich et al. 1995), the difference in results most likely reflects sample size differences rather than other factors. Among the total of 24 SSRs isolated and characterized in our laboratory (this effort and Kresovich et al. 1995), over 60% were polymorphic in the test array. Gene diversity values in the variant markers were estimated to range from 0.3 to 0.8 and were consistent with values reported in other plant species (Lagercrantz et al. 1992; Rongwen et al. 1995; Saghai Maroof et al. 1994).

When these optimized amplification protocols were employed as reported, all SSRs were conserved among the three *Brassica* spp. tested, though individual accessions within a species did not always produce a fragment. As noted previously (Kresovich et al. 1995), alterations in priming sites (as a result of changes in the SSR flanking sequences) may be responsible for the resultant non-amplification. However, before one can establish the population genetic or evolutionary implications and significance of these nucleotide divergences at priming sites, additional SSRs must be accessible for testing in a broader range of germplasm.

When the ultimate utility of the markers is taken into consideration, our results continue to be encouraging because of the high information content per marker and the high level of conservation of SSRs among the closely related *Brassica* spp. That is, we now have a total of 24 SSRs that amplify products in *B. napus*, *B. oleracea*, and *B. rapa*. Study of a broader range of diversity within each species might prove useful for the discrimination of putative species-specific alleles.

Among the 24 SSRs we have characterized in *Brassica* spp. thus far (this effort and Kresovich et al. 1995), the number of products detected by these primer pairs (or information content of the SSR) did not correlate with the repeat length within the target sequence. Other studies of human and plant SSRs (Weber 1990; Johansson et al. 1992; Saghai Maroof et al. 1994) noted a positive relationship between the number of tandem repeats and the degree of polymorphism of an SSR. Our results included that among 7 SSRs amplifying 5 or more fragments in the array, 5 contained 11–15 tandem repeats while 2 had a repeat lengths of 27 or greater. Again, further testing in more diverse germplasm is needed to clarify if a relationship does universally exist.

Because the characterization and quantification of genetic diversity (whether ex situ or in situ) will require the genotyping of large numbers of individuals and/or accessions, high throughput and low unit cost per assay must serve as the selection criteria for technique application. In response, primer pairs have been designed to function under identical, high stringency amplification conditions. This standardization not only eliminated the need to optimize amplification conditions for each primer pair but also has facilitated single-reaction multiplexing of a number of these SSRs. Conversely, this high level of stringency was the reason why primer pairs could not be designed for some sequences, i.e., positive clones were rejected because of high AT content and/or ambiguous sequence data.

We are currently multiplexing a number of these *Brassica* spp. SSRs in a single reaction mix using semi-automated, fluorescence-based detection techniques (unpublished data). This development will improve analytical resolution of amplified fragments, expedite data collection, and simultaneously reduce cost (Ziegle et al. 1992).

High-resolution, high-throughput genetic analysis (for establishing identity, relatedness, and structure in germplasm collections) will provide the framework for effective maintenance and utilization of crop plant diversity. Therefore, molecular genetic markers must be accessible, reliable, easy-to-use, and cost-effective. Because of these inherent attributes as well as their high information content, SSR markers will readily be employed for these purposes. This technological application ultimately will support the improved conservation and utilization of our ever-dwindling plant genetic resources.

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