Development of Robust SCAR Markers that Distinguish the Six Cultivated *Brassica* Species and Subspecies of the U-triangle

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ABSTRACT. This research describes the development of robust molecular descriptors that are reliable and easy to use (PCR-based) for the proper classification of the six cultivated *Brassica* L. species and subspecies that make up the U triangle. Sequence characterized amplified regions (SCAR) were derived from DNA fragments generated by randomly amplified polymorphic DNA (RAPD) primers that distinguished the U-triangle *Brassica* species: *B. rapa* L. (A, n = 10), *B. nigra* (L.) Koch (B, n = 8), *B. oleracea* L. (C, n = 9), *B. juncea* (L.) Czern (AB, n = 18), *B. napus* L. (AC, n = 19), and *B. carinata* Braun (BC, n = 17). The SCAR descriptors developed in this study reveal the presence-absence of a DNA fragment and sequence length polymorphisms to distinguish the three genomes (A, B, and C) of the six cultivated *Brassica* species. These markers should prove to be useful in revealing misclassifications at the subspecies level. The amplification of a single DNA fragment and reduced sensitivity to reaction conditions make these SCAR descriptors ideal to accurately classify and organize large numbers of cultivated *Brassica* accessions typically present in large germplasm collections.

Plant germplasm organization and classification are important to the understanding, utilization, and conservation of plant genetic resources. Historically hybridization, cytogenetic studies, and morphological descriptors have contributed to the classification of accessions from the six cultivated *Brassica* species (Gomez-Campo, 1980, 1999; Prakash and Hinata, 1980; U, 1935). Understanding the relationships among the six cultivated *Brassica* genomes has played an important role in the development and improvement of new *Brassica* cultivars.

The degree of structural similarity among *Brassica* morphotypes provides important information about the relationships among the species (Warwick and Black, 1992). Morphological descriptors such as color, shape and size of vegetative and reproductive structures have been widely used in taxonomic studies of *Brassica* species (Gomez-Campo, 1980, 1999). However, species classification based exclusively on morphological features can

be difficult because exceptions to general taxonomic rules exist (Dass and Nybom, 1967; Rodriguez et al., 1999). In addition, classification based on phenotypic data may be misinterpreted due to environmental effects on expression of morphological characteristics. Moreover, the use of morphological descriptors to distinguish among the six cultivated *Brassica* species can be difficult and time consuming for the non-specialist (Gomez-Campo, 1980) due to the immense variation within this set of species.

DNA-based molecular descriptors are useful in the characterization of genetic diversity and are less likely to be affected by environmental variation. Molecular descriptors have been used to distinguish among *Capsicum* L. (Rodriguez et al., 1999) and *Picea* A. Dietr. species (Perron et al., 1995), and among species of the *Triticeae* (Wei and Wang, 1995). In *Brassica* crops, RFLPs and RAPDs have been the predominant descriptors used for the study of the phylogeny of the genus (Lazaro and Aguinalalde, 1996; Song et al., 1988a, 1988b, 1990), genome evolution-organization (dos Santos et al., 1994; Quiros et al., 1991; Song et al., 1995), and cultivar identification (Diers and Osborn, 1994). More recently, AFLPs and SSRs have also been used for similar studies (Negi et al., 2000; Plieske and Stuss, 2001; Suwabe et al., 2002).

Genome and species-specific molecular descriptors have been observed among *Brassica* species (Heneen et al., 1995; Quiros et al., 1991). Quiros et al. (1991), reported 65 RAPD descriptors that were specific to the A, B, and C genome. RAPD markers

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have been useful in discriminating among the six *Brassica* taxa. However, lack of uniformity, consistency, and repeatability in amplifying similar fragments across laboratories has precluded broad adaptation of this technique (Quiros et al., 1995). In addition, little is known about the physical characteristics of the amplified fragments (Thormann et al., 1994), limiting their application in the study of the genetic structure of the *Brassica* species. The conversion of RAPD fragments to SCAR, by generating sequence-specific PCR primers pairs (Paran and Michelmore, 1993) provides more robust molecular descriptors that can be reliably used over a wide range of amplification conditions, and hence, are more repeatable across laboratories (Villand, 1998).

The specific research objectives of this study were to 1) clone and sequence RAPD descriptors identified in our laboratory as *Brassica* genome-specific markers 2) to develop SCAR primers (≈ 21 mer) from the corresponding RAPD primers, and 3) to validate the usefulness of these SCAR primers across 104 random accessions representing the diploid and amphidiploid species of the U-triangle.

Materials and Methods

PLANT MATERIAL. A total of 104 *Brassica* accessions were randomly sampled from several germplasm banks and seed companies, including: two accessions (IMB 211 and R500) from T. Osborn (Dept. of Agronomy, Univ. of Wisconsin, Madison); six *Brassica* rapid-cycling populations from the Crucifer Genetics Cooperative [CrGC (Dept. of Plant Pathology, Univ. of Wisconsin, Madison)]; 11 accessions from the Asian Vegetable Research and Development Center (AVRDC), Tainan, Taiwan; 22 commercial cultivars (10 from W. Atlee Burpee and Co., Warminster, Pa.; 11 from Geo. W. Park Seed Co., Greenwood, S.C.; one from Ferry-Morse Seed Co., Fulton, Ky.); and 63 accessions from the U.S. Dept. of Agriculture, Agricultural Research Service, National Plant Germplasm System [NPGS (Ames, Iowa; Geneva, N.Y.; Fort Collins, Colo.)] (Table 1). The number of accessions sampled for each of the six cultivated *Brassica* species roughly corresponded to the number of accessions available in the NPGS collection; thus, for example more *B. rapa* (33 accessions) were sampled compared to *B. nigra* (eight accessions) (Table 1).

Six to 10 seeds of each subspecies were grown under fluorescent lights (150 μmol) with an equal photoperiod. Two and one-half weeks after germination 0.8–1 g of immature leaf tissue (first true leaf) was harvested for DNA extraction. DNA isolation for RAPD screening and SCAR evaluation followed Johns et al. (1997).

IDENTIFICATION OF POLYMORPHIC RAPD FRAGMENTS. RAPD primers were selected based on two previous studies (Camargo et al., 1997; dos Santos et al., 1994) and a RAPD primer screen (data not published) carried out on 44 different *Brassica* accessions (Table 1). These three studies showed high levels of polymorphism among and within the six cultivated *Brassica* species. Twenty-five RAPD fragments were selected as potential candidates for SCAR conversion, based on their degree of species specificity. For example, to distinguish *B. rapa* from *B. oleracea*, RAPD fragments were selected that were present in *B. rapa* and absent in *B. oleracea*. The same criterion was applied for developing SCAR markers that could distinguish among subspecies categories. For example, in *B. rapa* RAPD fragments were selected that were present in pak choi [*B. rapa* ssp. *chinensis* (L) Hanelt] and absent in turnip [*B. rapa* ssp. *rapifer* Metzger]. RAPD PCR

mixtures were assembled as described by Skroch and Nienhuis (1995) and amplified using a PTC100 Programmable Thermal Controller (MJ Research, Waltham, Mass.) using cycling conditions described by Johns et al. (1997). Amplification products were resolved electrophoretically in 1.5% agarose gels.

CONVERSION OF RAPD FRAGMENTS INTO SPECIES-SPECIFIC SCAR MARKERS. RAPD fragments were converted into species-specific SCAR markers as described in Villand (1998). RAPD fragments of interest were excised from the agarose gel and placed in a microfuge tube with 20 μL 0.1X TE buffer. Heating (60 $^{\circ}\text{C}$) and repipeting were used to dissolve the excised agarose gel containing the fragment of interest. PCR reactions were run similar to the initial RAPD reactions (Skroch and Nienhuis, 1995) using 5 μL of the dissolved agarose fragments as the templates. The same cycling conditions were followed with the addition of a 72 $^{\circ}\text{C}$ extension for 30 min at the completion of the run. Reamplified products were separated in 1.5% agarose gels. All of the reamplified RAPD fragments were excised and purified following the BIO 101 GeneClean II gel purification protocol (Qbiogene, a division of MP Biomedicals, Irvine, Calif.). Concentrations of the purified DNA fragments were checked against DNA concentration standard electrophoresis. Ligation and transformation reactions were carried out using the Original TA Cloning kit (Invitrogen Corp., Carlsbad, Calif.). White colonies were selected for cell culture growth and placed in 10 μL dH_2O . Colony-PCR was performed, using 5 μL (template) of the colony-water mixture and universal primers [M13 forward (–20) and M13 reverse], to confirm the correct insertion size. Cultures of clones with the correct insertion size were grown from the remaining 5 μL colony-water mixture. Plasmid purification followed the Qiagen (Valencia, Calif.) mini prep protocol. Cycle sequencing PCR reactions including BigDye Terminator mix from Perkin Elmer Applied Biosystems (Foster City, Calif.), universal forward and reverse primers, and 30–90 ng DNA were run in 20 μL total volume reactions. The PCR cycle sequencing program profile was set as follows; 1 min at 96 $^{\circ}\text{C}$, 25 cycles of 30 s at 96 $^{\circ}\text{C}$, 15 s at 40 $^{\circ}\text{C}$, and 4 min at 60 $^{\circ}\text{C}$ followed by a cool down at 4 $^{\circ}\text{C}$. Purification of the cycle sequencing products were carried out by adding 2.2 μL of 3 M sodium acetate pH 5.2 and 50 μL of 100% ethanol to each 20 μL PCR reaction. Cloned amplification products were sequenced using an ABI 377XL automated DNA Sequencer at the University of Wisconsin Biotechnology Center DNA Sequence Facility (Madison). The resulting forward and reverse sequences were crosschecked for matching sequences beyond the original 10-mer RAPD primer sequence. Based on the obtained sequence information, approximately 10 more bases were added to the original (5' to 3') 10-mer RAPD sequence. The ≈ 20 -mer forward and reverse SCAR primers were synthesized at Operon Biotechnologies (Huntsville, Ala.).

SCAR ANALYSIS. The 25 SCAR primer pairs developed in this study were tested across 104 accessions representing the six cultivated *Brassica* species (Table 1). Amplification reactions and gel separation were described above for RAPD reactions, except that the annealing temperature (50–60 $^{\circ}\text{C}$) was adjusted according to the specification of each set of SCAR primers (Table 2). The robustness of the developed SCAR markers was tested over a number of amplification conditions that included, 1) amplification in different thermal cyclers, 2) varied concentrations of template, 3) different concentrations of PCR components (magnesium chloride, PCR buffer and dNTPs), and 4) different PCR tubes and microtiter plates.

Table 1. List of the 104 accessions from six cultivated *Brassica* species accessions for which SCAR markers were analyzed.

Species and accession	Intra-specific name	Common name	Species and accession	Intra-specific name	Common name
<i>Brassica rapa</i> L. (n=10)					
CrGC1-1 ^z		rapid cycling	PI462224	var. <i>botrytis</i>	cauliflower
IMB 211 ^z		rapid cycling	Earlana ^z	var. <i>capitata</i> L.	cabbage
Bp 09	ssp. <i>chinensis</i> (L.) Hanelt	pak choi	Salad Delight ^z	var. <i>capitata</i>	cabbage
Bp 31	ssp. <i>chinensis</i>	pak choi	Everlast Hybrid ^z	var. <i>capitata</i>	cabbage
Joi Choi Hybrid ^z	ssp. <i>chinensis</i>	pak choi	PI261769	var. <i>capitata</i>	cabbage
Joi Choi Hybrid ^z	ssp. <i>chinensis</i>	pak choi	PI296133	var. <i>capitata</i>	cabbage
PI430484 ^z	ssp. <i>chinensis</i>	pak choi	Jade Cross Early Hybrid ^z	var. <i>gemmifera</i> Zenker	brussels sprouts
PI430485	ssp. <i>chinensis</i>	pak choi	Prince Marvel ^z	var. <i>gemmifera</i>	brussels sprouts
PI478324	ssp. <i>chinensis</i>	pak choi	PI261759	var. <i>gemmifera</i>	brussels sprouts
BC11 ^z	var. <i>parachinensis</i> (Bailey) Hanelt	choi sum	NSL34225	var. <i>gemmifera</i>	brussels sprouts
BC14 ^z	var. <i>parachinensis</i>	choi sum	Express Forcer ^z	var. <i>gongylodes</i> L.	kohlrabi
BCC12	ssp. <i>pekinensis</i> (Lour.) Hanelt	chinese cabbage	PI188611	var. <i>gongylodes</i>	kohlrabi
BCC23	ssp. <i>pekinensis</i>	chinese cabbage	NSL6269 ^z	var. <i>gongylodes</i>	kohlrabi
Orient Express ^z	ssp. <i>pekinensis</i>	chinese cabbage	NSL6270	var. <i>gongylodes</i>	kohlrabi
Flash Hybrid ^z	ssp. <i>pekinensis</i>	chinese cabbage	Green Goliath	var. <i>italica</i> Plenck.	broccoli
PI257237	ssp. <i>pekinensis</i>	chinese cabbage	Gallant Hybrid ^z	var. <i>italica</i>	broccoli
PI419127	ssp. <i>pekinensis</i>	chinese cabbage	PI507856	var. <i>sabauda</i> L.	savoy cabbage
PI48975 ^z	ssp. <i>pekinensis</i>	chinese cabbage	Champion Hybrid ^z	var. <i>sabellica</i>	collard
Ames23075	ssp. <i>pekinensis</i>	chinese cabbage	NSL6143	var. <i>sabellica</i>	collard
NSL6007	ssp. <i>pekinensis</i>	chinese cabbage	NSL6152	var. <i>sabellica</i>	collard
PI340202	ssp. <i>dichotoma</i> (Roxb.) Hanelt	indian rape	NSL6697	var. <i>sabellica</i>	collard
Ames23083 ^z	ssp. <i>oleifera</i> (DC.) Metzg	turnip rape	NSL6797	var. <i>sabellica</i>	collard
NSL165769 ^z	ssp. <i>oleifera</i>	turnip rape	<i>Brassica juncea</i> (L.) Czern. (n=18)		
Ames25111	ssp. <i>oleifera</i>	turnip rape	CrGC4-1	var. <i>oleifera</i> Prain	rapid cycling
PTWG 5469 ^z	ssp. <i>rapifera</i> Metzg	turnip	Bj03	var. <i>oleifera</i>	indian mustard
Ames19208	ssp. <i>rapifera</i>	turnip	Bj10	var. <i>oleifera</i>	indian mustard
R500 ^z	ssp. <i>trilocularis</i> (Roxb.) Hanelt	yellow sarson	Florida Broad Leaf ^z	var. <i>oleifera</i>	indian mustard
PI346882	ssp. <i>trilocularis</i>	yellow sarson	Florida Broad Leaf ^z	var. <i>oleifera</i>	indian mustard
PI347608 ^z	ssp. <i>trilocularis</i>	yellow sarson	PI120923	var. <i>oleifera</i>	indian musta
PI426421	ssp. <i>trilocularis</i>	yellow sarson	PI179859	var. <i>oleifera</i>	indian musta
PI459020	ssp. <i>trilocularis</i>	yellow sarson	PI288725	var. <i>oleifera</i>	indian musta
Spring ^z	var. <i>Utilis</i>	broccoli raab	PI426333	var. <i>oleifera</i>	indian musta
31BR	var. <i>Utilis</i>	broccoli raab	Ames23094 ^z	var. <i>oleifera</i>	indian musta
<i>Brassica nigra</i> (L.) Koch (n=8)			Ames725		oriental mus
CrGC2-1		rapid cycling	PI531272	var. <i>longidens</i> L. H. Bailey	

continued next page

Table 1. Continued.

Species and accession	Intra-specific name	Common name	Species and accession	Intra-specific name	Common name
PI254362		black mustard	<i>Brassica napus</i> L. (n=19)		
PI271444 ²		black mustard	CrGC5-1 ²	var. <i>oleifera</i> Delile	rapid cycling
PI273638 ²		black mustard	PI250135 ²	var. <i>oleifera</i>	colza
PI368378		black mustard	PI311728	var. <i>oleifera</i>	colza
PI592737		black mustard	PI535865	var. <i>oleifera</i>	colza
PI603018		black mustard	Ames22970 ²	var. <i>oleifera</i>	colza
Ames24227		black mustard	PI279688	var. <i>rapifera</i> (DC) Metzger	rutabaga
<i>Brassica oleracea</i> L. (n=9)			PI458941	var. <i>rapifera</i>	rutabaga
CrGC3-9 ²		rapid cycling	PI469737 ²	var. <i>rapifera</i>	rutabaga
Dwarf Blue ²	ssp. <i>acephala</i> (DC.) Schübler	kale	PI469856 ²	var. <i>rapifera</i>	rutabaga
Winterbor Hybrid ²	var. <i>Alboglabra</i> (Bailey) Musil	kale	PI469881	var. <i>rapifera</i>	rutabaga
Ba08 ²	var. <i>Alboglabra</i>	chinese kale	<i>Brassica carinata</i> Braun. (n=17)		
Ba24	var. <i>Alboglabra</i>	chinese kale	CrGC6-1 ²		rapid cycling
PI249556	var. <i>botrytis</i> L.	cauliflower	PI193459		ethiopian m
Green Goddess Hybrid ²	var. <i>botrytis</i>	cauliflower	PI193959		ethiopian m
Ravella Hybrid ²	var. <i>botrytis</i>	cauliflower	PI195921 ²		ethiopian m
Shannon ²	var. <i>botrytis</i>	cauliflower	PI331378		ethiopian m
PI208480	var. <i>botrytis</i>	cauliflower	PI390133		ethiopian m
PI241609 ²	var. <i>botrytis</i>	cauliflower	Ames19183		ethiopian m
PI264651	var. <i>botrytis</i>	cauliflower			

²Represents the 44 accessions used for the identification of polymorphic RAPD fragments. For company names and national and international germplasm repository centers please see the materials and method section.

Results

SCAR CONVERSION. Twenty-five RAPD fragments were converted to SCAR makers by developing sequence-specific primer pairs. Each of these primer pairs amplified prominent fragments that varied in size from 240 to 1100 bp (Table 2). Eighteen of the 25 (72% conversion rate) SCAR primer pairs produced a portion of the same fragment pattern that was observed for the corresponding RAPD reaction. For the remaining seven SCAR primer pairs, polymorphisms detected in the initial RAPD reaction were lost (e.g., they revealed monomorphic PCR products). These SCAR markers were discarded for further consideration.

SCAR ANALYSIS. In general, SCAR primer amplification patterns were identical for all accessions within each subspecies; therefore, the patterns of only representative accessions from Table 1 are presented (Table 3). The SCAR analysis revealed that 2 out of the 64 accessions tested deviated from their respective subspecies specific pattern (and in one case led to the detection of a misclassification). The SCAR reaction and analysis was repeated three times under different laboratory conditions (thermal cyclers, different PCR component concentrations, etc.) to test for procedure repeatability. Similar results in the PCR profile were obtained under the different laboratory conditions as previously described.

SPECIES-SPECIFIC SCAR MARKERS. Seven SCAR primer pairs (primers 1 to 7), out of the eighteen SCAR primer pairs developed in this study, differentiated the six cultivated *Brassica* species that form the U-triangle (Tables 1 and 3). SCAR primers 1 to 7 revealed two types of polymorphic patterns, 1) the presence or absence of PCR amplification fragments associated with one of the *Brassica* genomes (A, B, or C), or 2) sequence length polymorphism associated with specific *Brassica* genome (A, B, or C) fragment size (Fig. 1). The two types of polymorphisms were consistent in both diploid and amphidiploid species. Thus, for example, the SCAR amplification products that were observed in all accessions of the diploid species, *B. rapa* (A), were also observed in the amphidiploid species *B. juncea* (AB) and *B. napus* (AC) that also contain the A genome. In a similar manner, SCAR amplification products observed in the diploid B genome of *B. nigra* and the diploid C genome of *B. oleracea*, were also observed in accessions from their related amphidiploid species, *B. juncea* (AB), *B. napus* (AC), and *B. carinata* (BC), respectively (Table 3).

SCAR primers 1, 2, and 3 amplified a single DNA fragment at 860, 610, and 710 bp in length, respectively, in all accessions containing the A genome (Tables 2 and 3). SCAR primer 4 amplified a single DNA fragment of 655 bp in length in all accessions containing the A genome and two DNA fragments of 320/661 bp in length, in all accessions containing the B genome (Tables

Table 2. *Brassica* species-specific SCAR marker type, primer sequences, annealing temperatures, and expected amplicon size.

Species-specific SCAR primers	1	F: 5'-ACGGGACCTGATGATACAAT-3' R: 5'-ACGGGACCTGTTTGAGGGAA-3'	60.0	A:860
	2	F: 5'-GTCGCCGTCAAGAAATCCGC-3' R: 5'-GTCGCCGTACGTAGCATGC-3'	65.5	A:610
	3	F: 5'-CGTAACCTTGCTTATTCTTGC-3' R: 5'-CGTAACCTTGTCTATTATC-3'	54.6	A:710
	4	F: 5'-CCTGCTTACGATATGGCTTGG-3' R: 5'-CCTGCTTACGGTACCAAC-3'	61.2	A:655 B:320/661 ^y
	5	F: 5'-CTCACCCTCCAGCCCGTGC-3' R: 5'-CTCACCCTCCTCTAACAAA-3'	62.0	A:880 B:550/710 C:910
	6	F: 5'-GACCGCTTGTGAAGAGAG-3' R: 5'-GACCGCTTGTCTGAGGATGG-3'	63.4	A:680 B:680/720 C:700
	7	F: 5'-CGAGCTTTGAAGCAAGGAGG-3' R: 5'-CGAGCTTTGAGTTGACTGC-3'	61.4	A:680 B:710 C:1100
	8	F: 5'-GGGGTGACGACACTAATTAA-3' R: 5'-GGGGTGACGATCGATTTTGAA-3'	60.0	A:670 B:600
	9	F: 5'-GACCGCTTGCCGAACAGT-3' R: 5'-GACCGCTTGATCTATGTTTAGTC-3'	62.8	A:1100 C:600
	10	F: 5'-ACGGTACCAGTTGAGATA-3' R: 5'-ACGGTACCAGAAGCAAACCTA-3'	65.0	A:850
	11	F: 5'-GACCGCTTGTCGATTTAAAGG-3' R: 5'-GACCGCTTGATGCACATATG-3'	60.7	A:670 C:720
	12	F: 5'-GACCGCTTGTCGATTTAAGGA-3' R: 5'-GACCGCTTGATGCACATAT-3'	59.2	A:680 C:750
	13	F: 5'-TCTGGCGCACTTGACTGTGC-3' R: 5'-TCTGGCGCACCACTAACTCAGACG-3'	66.1	A:620 C:600
	14	F: 5'-GACCGCTTGTCGATTTTAAC-3' R: 5'-GACCGCTTGATGCACATAT-3'	58.3	A:680 A+:710 ^x
	15	F: 5'-CCCCTCGAATCAATCAATC-3' R: 5'-CCCCTCGAATATTCATTAC-3'	58.1	A:710 A+:800 ^x C:750
	16	F: 5'-GGGCGACTACAACAACAG-3' R: 5'-GGGCGACTACTTCAGGCGCG-3'	62.0	C:440 B:240
	17	F: 5'-GTGAGGCGTCGATAGGCGGC-3' R: 5'-GTGAGGCGTCTCTTGATGACATT-3'	65.5	A:600
	18	F: 5'-GGACCCAACCCTCTTTAGAA-3' R: 5'-GGACCCAACCTTAGACTACG-3'	62.0	A:840

^zA, B, and C (capital letters) indicate amplification of the primer sequence associated with the *B. rapa*, *B. oleracea*, and *B. nigra* genomes, respectively, in both diploid and amphidiploid species.

^yTwo molecular sizes separated by a slash bar indicate the present of two DNA fragments for that particular SCAR marker.

^xA+ indicates sequence length polymorphism for the fragment amplified in the A genome.

2 and 3). Primer 5 amplified a single DNA fragment (880 bp) in all accessions containing the A genome; two DNA fragments (550/710 bp) in all accessions containing the B genome; and a single fragment (910 bp) in all accessions containing the C genome. Primer 6 amplified a single DNA fragment of 680 bp in all accessions containing the A genome; two DNA fragments of 680/720 bp, in all accessions containing the B genome; and a single fragment of 700 bp in all accessions containing the C genome. Primer 7 amplified a single DNA fragment of 680 bp in all accessions containing the A genome; a single DNA fragment of 710 bp in all accessions containing the B genome; and a single DNA fragment of 1100 bp in all accessions containing the C genome (Tables 2 and 3). SCAR primers 6 and 7 did not reveal an amplification product for the B genome in the amphidiploid species *B. juncea* (Tables 2 and 3).

SUBSPECIES-SPECIFIC SCAR MARKERS. Nine SCAR primer pairs (primers 8 to 18), all revealed either presence-absence or sequence length polymorphism at the subspecies level. Song et al. (1988a, 1988b) divided *B. rapa* subspecies into a European group including, turnip, turnip rape and yellow sarson; and an east Asian group, including pak choi, choi sum, chinese cabbage, and broccoli raab. SCAR primers 8, 9, and 10 each amplified a single fragment of 670, 1100, and 850 bp, respectively, in all accessions of the east Asian group. In contrast, SCAR primers 11, 12, and 13 each amplified a single fragment of 670, 680, and 620 bp, respectively, in all accessions of the European group. SCAR primer pairs 14 and 15 revealed sequence length polymorphism among the European *B. rapa* subspecies. Primer 14 amplified two DNA fragments of 680 and 710 bp that distinguished turnip rapes from turnips, respectively. Primer 15 amplified two DNA fragments of 710 and 800 bp that distinguished yellow sarsons from turnips and turnip rapes, respectively (Tables 2 and 3).

Among *B. oleracea* subspecies, SCAR primer pairs 12 and 16 revealed presence-absence polymorphisms between, cauliflower and cabbage compared to chinese kale, cauliflower, kohlrabi, and broccoli. Primer 12 amplified a single DNA fragment of 750 bp in all *B. oleracea* accessions except for cauliflower and cabbage. Primer 16 amplified a single DNA fragment of 440 bp in all *B. oleracea* accessions except for chinese kale, cauliflower, kohlrabi, and broccoli (Tables 2 and 3).

Among *B. juncea* (AB) subspecies, SCAR primer pairs 9, 12, 17 and 18 revealed presence-absence polymorphisms between indian mustard and *B. juncea* var. *longidens* L.H. Bailey. Primer 9, 12, 17, and 18 each amplified a single DNA fragment of 1100, 680, 600, and 840 bp, respectively; corresponding to the A genome, in all indian mustard accessions (Table 2 and 3).

Among *B. napus* (AC) subspecies, SCAR primer pairs 8, 9, and 13 revealed presence-absence polymorphisms between colza and rutabaga. Primer 8 amplified a single DNA fragment of 670 bp corresponding to the A genome in all rutabaga accessions. Primer 9 amplified a single DNA fragments for each of the A and C genomes of 1100 and 600 bp, respectively, except for colza accessions where the A genome fragment was not amplified. Primer 13 amplified a single DNA fragment for each of the A and C genomes of 620 and 600 bp, respectively, with the exception of the rutabaga accessions where the C genome was not amplified (Tables 2 and 3).

POTENTIAL MISCLASSIFICATIONS. Results presented in Table 3 indicate that misclassification may have occurred in two of 64 randomly chosen accessions from the NPGS. Accession, NSL6007 is labeled as *B. rapa* ssp. *pekinensis* (Lour.) Hanelt (chinese cabbage); however, the banding patterns of this acces-

sion using primers 8, 9, 10, 11, 12, 13, 14, and 15 are not like other *B. rapa* ssp. *pekinensis* accessions, but are identical to *B. rapa* ssp. *oleifera* (DC.) Metzg accessions (Table 3). Accession NSL6007 was grown in the greenhouse and was confirmed to be a turnip (*B. rapa* ssp. *oleifera*) (data not shown). In addition, SCAR banding pattern using primer 5 revealed an inconsistency in the amplification pattern of accession PI340202. This accession is labeled as indian rape *B. rapa* ssp. *dichotoma* (Roxb.) Hanelt. However, SCAR primer 5 profiles revealed an amplification pattern similar to the amphidiploid *B. napus* species (Fig. 1, Table 3). DNA extraction and PCR amplification were repeated and the same PCR profile was obtained.

Discussion

Previous studies (Negi et al., 2000; Quiros et al., 1991; Song et al. 1988a) reported the use of various molecular markers (AFLP, RFLP, and RAPD) for the understanding of relationships among the six cultivated *Brassica* species. However, marker-types such as RFLP or AFLP are time-consuming and require the use of isotopes and/or specialized laboratory equipment. RAPD markers are easier to use; however, they may be unrepeatable across laboratories (Gomez-Campo, 1999). In this study, a set of 18 SCAR primers were developed as diagnostic descriptors for the discrimination among the six cultivated *Brassica* species and subspecies. SCAR markers are low-cost PCR-based molecular descriptors that are repeatable in their amplification pattern across laboratories (Negi et al., 2000; Paran and Michelmore, 1993). The 18 SCAR primer pairs developed in this study were robust under various laboratory conditions, suggesting that these SCAR primers produce repeatable polymorphic patterns. The speed and simplicity of DNA extraction coupled with the low cost and consistent amplification patterns of SCAR markers make them ideal molecular descriptors for sampling large numbers of accessions.

The cost to characterize large numbers of accessions at national and international germplasm banks often precludes the systematic characterization of all accessions (Villard, 1998). In the case of the six cultivated *Brassica* species (and subspecies) one technique that can be used to differentiate them, apart from molecular markers, has been the use of cytogenetics (U, 1935). This allows for the proper identification of chromosomes number and thus the differentiation of the species. However, these methodologies can become complex due to the small size and similar length of *Brassica* chromosomes (Hasterok and Maluszynska, 2000; Howell et al., 2002; Snowdon et al., 2002). In addition, the employment of cytometric techniques requires specialized equipment that increases the cost of species classification. In this framework, the availability of fast, low cost and reliable molecular markers allows for proper classification and conservation of plant genetic resources.

The two SCAR marker types developed in this study (presence/absence and sequence length polymorphism) can reliably discriminate among the six cultivated *Brassica* species. Nevertheless, characterization based solely on the presence/absence of a fragment may be subject to experimental errors. The failure to amplify a fragment could be attributed to errors in the preparation of the PCR reaction mixture. The most useful SCAR markers are those that revealed sequence length polymorphism among species because amplification products are always expected. Sequence length polymorphism does not necessarily imply that homologous DNA fragments are being amplified by a given SCAR primer pair in polymorphic accessions. This is due to the little physical

Table 3. Pattern of SCAR primers amplification among representative samples of six cultivated *Brassica* species.^z

			Polymorphism type and SCAR markers 1-18 amplification pattern																	
			Species-specific SCAR primers							Subspecies-specific SCAR primers										
Species and																				
Accession ID	Intra-specific Name	Common name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>B. rapa</i> L.																				
(n=10)																				
PI430484	subsp. <i>chinensis</i> (7) ^y	pak choi	A ^x	A	A	A	A	A	A	A	A	A	0	0	0	0	A	0	A	A
BC11	var. <i>parachinensis</i> (2)	choi sum	A	A	A	A	A	A	A	A	A	A	0	0	0	0	A	0	A	A
PI257237	subsp. <i>pekinensis</i> (8)	chinese cabbage	A	A	A	A	A	A	A	A	A	A	0	0	0	0	A	0	A	A
NSL6007	Misclassified ^u	chinese cabbage	A	A	A	A	A	A	A	0	0	0	A	A	A	A ⁺ w	A ⁺	0	A	A
PI340202	Misclassified ^u	indian rape	A	A	A	A	AC	A	A	0	A	A	A	A	A	A	A	0	A	A
NSL165769	subsp. <i>oleifera</i> (3)	turnip rape	A	A	A	A	A	A	A	0	0	0	A	A	A	A	A ⁺	0	A	A
Ames19208	subsp. <i>rapifera</i> (2)	turnip	A	A	A	A	A	A	A	0	0	0	A	A	A	A ⁺	A ⁺	0	A	A
PI346882	subsp. <i>trilocularis</i> (5)	yellow sarson	A	A	A	A	A	A	A	0	0	0	A	A	A	A	A	0	A	A
31BR	var. <i>utilis</i> (2)	broccoli raab	A	A	A	A	A	A	A	A	A	A	0	0	0	0	A	0	A	A
<i>B. nigra</i> (L.) Koch																				
(n=8)																				
PI254362	(8)	black mustard	0 ^v	0	0	B ^x	B	B	B	B	0	0	0	B	0	0	0	B	0	0
<i>B. oleracea</i> L.																				
(n=9)																				
Dwarf Blue	subsp. <i>acephala</i> (2)	kale	0	0	0	0	C ^x	C	C	0	C	0	C	C	C	0	C	C	0	0
Ba08	var. <i>alboglabra</i> (3)	chinese kale	0	0	0	0	C	C	C	0	C	0	C	C	C	0	C	0	0	0
PI208480	var. <i>botrytis</i> (7)	cauliflower	0	0	0	0	C	C	C	0	C	0	C	0	C	0	C	0	0	0
PI261769	var. <i>capitata</i> (5)	cabbage	0	0	0	0	C	C	C	0	C	0	C	0	C	0	C	C	0	0
PI261759	var. <i>gemmifera</i> (4)	brussels sprouts	0	0	0	0	C	C	C	0	C	0	C	C	C	0	C	C	0	0
PI188611	var. <i>gongylodes</i> (4)	kohlrabi	0	0	0	0	C	C	C	0	C	0	C	C	C	0	C	0	0	0
Green Goliath	var. <i>italica</i> (2)	broccoli	0	0	0	0	C	C	C	0	C	0	C	C	C	0	C	0	0	0
PI507856	var. <i>sabauda</i> (1)	savoy cabbage	0	0	0	0	C	C	C	0	C	0	C	C	C	0	C	C	0	0
NSL6143	var. <i>sabellica</i> (5)	collard	0	0	0	0	C	C	C	0	C	0	C	C	C	0	C	C	0	0
<i>B. juncea</i> (L.) Czern. (n=18)																				
PI120923	var. <i>oleifera</i> (10)	indian mustard	Ab ^x	Ab	Ab	AB	AB	Ab	aB ^x	AB	Ab	Ab	0	Ab	Ab	0	Ab	aB	Ab	Ab
PI531272	var. <i>longidens</i> (1)		Ab	Ab	Ab	AB	AB	Ab	Ab	AB	0	Ab	0	0	Ab	0	Ab	aB	0	0
<i>B. napus</i> L. (n=19)																				
PI535865	var. <i>oleifera</i> (5)	colza	Ac ^x	Ac	Ac	Ac	AC	AC	AC	0	aC ^x	Ac	AC	Ac	AC	Ac	AC	0	0	Ac
PI279688	var. <i>rapifera</i> (5)	rutabaga	Ac	Ac	Ac	Ac	AC	AC	AC	Ac	AC	Ac	AC	Ac	aC	Ac	AC	0	0	Ac
<i>B. carinata</i> Braun. (n=17)																				
PI193459	(7)	ethiopian mustard	0	0	0	Bc ^x	BC	BC	BC	Bc	bC	0	bC	bC	bC	0	bC	Bc	0	0

^zSCAR primer amplification patterns were identical for all accessions with each subspecies; therefore, the pattern of only a representative accession is presented.

^yNumbers in parenthesis indicate the total number of accessions for which the SCAR primer amplification patterns were identical (see Table 1 for complete list of accessions).

^xA, B, and C (capital letters) indicate amplification of the primer sequence associated with the *B. rapa*, *B. oleracea*, and *B. nigra* genomes in both diploid and amphidiploid species, respectively. Lowercase letters (a, b, and c) associated with amphidiploid species indicates which genome was not amplified by a specific primer.

^wA+ indicates length polymorphism for the fragment amplified in the A genome.

^v0 indicates that the SCAR primer did not amplify the genome associated with that specific diploid or amphidiploid species.

^uMisclassified indicates that SCAR marker amplification patterns are inconsistent with the species or subspecies designation previously assigned to a particular accession.

Fig. 1. SCAR marker profile for primer 1 corresponding to the presence-absence polymorphism (top gel) and primer 5 corresponding to sequence length polymorphism (bottom gel). Lanes 1–9 represent the A genome of *Brassica rapa* in the following order: rapid-cycling *B. rapa*, pak choi, choi sum, chinese cabbage, indian rape, turnip rape, turnip, yellow sarson, and broccoli raab. Lanes 10–11 represent the B genome of *B. nigra* in the following order: rapid-cycling *B. nigra* and black mustard. Lanes 12–20 represent the C genome of *B. oleracea* in the following order: rapid-cycling *B. oleracea*, kale, chinese kale, cauliflower, cabbage, brussels sprouts, kohlrabi, broccoli, and collard. Lanes 21–23 represent the AB genomes of *B. juncea* in the following order: rapid-cycling *B. juncea*, indian mustard, and *B. juncea* var. *longidens*. Lanes 24–26 represent the AC genomes of *B. napus* in the following order: rapid-cycling *B. napus*, colza, and rutabaga. Lanes 27–28 represent the BC genomes of *B. carinata* in the following order: rapid-cycling *B. carinata* and ethiopian mustard. Lane Cn represents the negative control of the PCR reaction (no template). Molecular marker sizes [M (100-bp ladder)] are shown at both flanking sides of the gel. Base pair length and specific genome amplification are given in the far left portion of the figure.

information revealed by RAPD markers (Thormann et al., 1994). Hence, the polymorphism is dependent on the ability of RAPD primers to recognize different priming sites in the genome. Priming sites can also be altered or exhibit differences between diploid and amphidiploid *Brassica* species as shown in Fig. 1 (primer bottom gel). For instance, accessions 10 and 11 are classified as belonging to the B genome and exhibit fragment length of 550 and 710 bp, respectively. Interestingly, accessions 21, 22, and 23, classified as AB genome, yielded only one of the two fragments (550 bp). On the contrary, accessions 27 and 28 classified as BC genome, have the 710 bp fragment and not the 550 bp fragment. It would be expected that the AB or BC species would share both the 550 and 710 bp fragments observed for the B genome. The absence of one fragment could result from preferential amplification of PCR fragments in different genetic background (AB and/or BC amphidiploids). Therefore, the presence of a homeologous genome affects the way PCR amplifies the specific B genome fragments. It is also possible that the target priming site has been lost due to a rearrangement such as a non-reciprocal translocation in the amphidiploid genomes (Lukens et al., 2005; Song et al., 1995; Udall et al., 2005). For example, in the AB genome, the 710-bp B fragment might have been lost by an exchanged with the A genome resulting in only two fragments, the one specific to the A genome and other to the B genome (550 bp). Further examination of the sequence of the amplified SCAR fragments might be necessary to access their physical nature.

Molecular DNA techniques will continue to facilitate germplasm characterization. The present research provides a set of 18 SCAR primers that can be used to discriminate among the six

cultivated *Brassica* species (U-triangle). Moreover, these SCAR markers were able to identify two potentially misclassified *B. rapa* subspecies. The ideal descriptors are those that simply discriminate among the six cultivated *Brassica* species by amplifying genome-specific PCR products based on different length polymorphisms of a major single fragment. This is of extreme importance for sampling a large number of accessions to verify correct species assignments, especially at international and national germplasm banks. The amplification of a single fragment and reduced sensitivity to reaction conditions make these SCAR markers ideal for this task.

Literature Cited

- Camargo, L.E.A., L. Savides, G. Jung, J. Nienhuis, and T.C. Osborn. 1997. Location of the self-incompatibility locus in an RFLP and RAPD map of *Brassica oleracea*. *J. Hered.* 88:57–60.
- Dass, H. and H. Nybom. 1967. The relationships between *Brassica nigra*, *B. campestris*, *B. oleracea*, and their amphidiploid hybrids studied by means of numerical chemotaxonomy. *Can. J. Genet. Cytol.* 9:880–890.
- Diers, B.W. and T.C. Osborn. 1994. Genetic diversity of oilseed *Brassica napus* germplasm based on nuclear restriction fragment length polymorphisms. *Theor. Appl. Genet.* 88:662–668.
- dos Santos, B.J., J. Nienhuis, P. Skroch, J. Ti-vang, and M.K. Slocum. 1994. Comparison of RAPD and RFLP genetic markers in determining genetic similarities among *Brassica oleracea* L. genotypes. *Theor. Appl. Genet.* 87:909–915.
- Gomez-Campo, C. 1980. Morphology and morphotaxonomy of the tribe Brassiceae, p. 3–31. In: S. Tsunoda, K. Hinata, and C. Gomez-Campo (eds.). *Brassica crops and wild allies: Biology and breeding*. Japan Scientific Societies Press, Tokyo.
- Gomez-Campo, C. 1999. *Biology of Brassica coenospecies*. Elsevier, Amsterdam, The Netherlands.
- Hasterok R. and J. Maluszynska. 2000. Cytogenetic analysis of diploid *Brassica* species. *Acta Biol. Cracoviensis Ser. Botanica* 42:145–153.
- Heneen, W.K., B.Y. Chen, B.F. Cheng, A. Jonsson, V. Simonsen, R.B. Jorgensen, and J. Davik. 1995. Characterization of the A and C genomes of *Brassica campestris* and *B. alboglabra*. *Hereditas* 123:251–267.
- Howell, E.C., G.C. Barker, G.H. Jones, M.J. Kearsey, G.J. King, E.P. Kop, C.D. Ryder, G.T. Teakle, J.G. Vicente, and S.J. Armstrong. 2002. Integration of the cytogenetic and genetic linkage maps of *Brassica oleracea*. *Genetics* 161:1225–1234.
- Johns, M.A., P.W. Skroch, J. Nienhuis, P. Hinrichsen, G. Bascur, and C. Munoz-Schick. 1997. Gene pool classification of common bean landraces from Chile based on RAPD and morphological data. *Crop Sci.* 37:605–613.
- Lazaro, A. and I. Aguinalgalde. 1996. Phylogenetic relationships between the wild taxa of the *Brassica oleracea* L. group (2n = 18) using the random amplified polymorphic DNA assay. *Scientia Hort.* 65:219–227.
- Lukens, L.N., J.C. Pires, E. Leon, R. Vogelzang, L. Oslach, and T.C. Osborn. 2005. Patterns of sequence loss and cytosine methylation within a population of newly resynthesized *Brassica napus* allopolyploids. *Plant Physiol.* 140:336–348.
- Negi, M.S., M. Devic, M. Delseny, and M. Lakshmikumaran. 2000. Identification of AFLP fragments linked to seed coat color in *Brassica juncea* and conversion to a SCAR marker for rapid selection. *Theor. Appl. Genet.* 101:146–152.

- Paran, I. and R.W. Michelmore. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85:985–993.
- Perron, M., A.G. Gordon, and J. Bousquet. 1995. Species-specific RAPD fingerprints for the closely related *Picea mariana* and *P. rubens*. *Theor. Appl. Genet.* 91:142–149.
- Plieske J. and D. Struss. 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. and K. Hinata. 1980. Taxonomy, cytogenetics and origin of crop *Brassicas*. A review. *Opera Botanica* 55:1–57.
- Quiros, C.F., J. Hul, P. This, A.M. Chevre, and M. Delseny. 1991. Development and chromosomal localization of genome-specific markers by polymerase chain reaction in *Brassica*. *Theor. Appl. Genet.* 82:627–632.
- Quiros, C.F., P. This, M. Laudie, A. Benet, A-M. Chevre, and M. Delseny. 1995. Analysis of a set of RAPD markers by hybridization and sequencing in *Brassica*: A note of caution. *Plant Cell Rpt.* 14:630–634.
- Rodriguez, J.M., T. Berke, L. Engle, and J. Nienhuis. 1999. Variation among and within *Capsicum* species revealed by RAPD makers. *Theor. Appl. Genet.* 99:147–156.
- Skroch, P.W. and J. Nienhuis. 1995. Qualitative and quantitative characterization of RAPD variation among snap beans (*Phaseolus vulgaris*) genotypes. *Theor. Appl. Genet.* 91:1078–1085.
- Snowdon, R.J., T. Friedrich, W. Friedt, and W. Köhler. 2002. Identifying the chromosomes of the A- and C-genome diploid *Brassica* species *B. rapa* (syn. *campestris*) and *B. oleracea* in their amphidiploid *B. napus*. *Theor. Appl. Genet.* 104:533–538.
- Song, K.M., T.C. Osborn, and P.H. Williams. 1988a. *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). 1. Genome evolution of diploid and amphidiploid species. *Theor. Appl. Genet.* 75:784–794.
- Song, K.M., T.C. Osborn, and P.H. Williams. 1988b. *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, K.M., T.C. Osborn, and P.H. Williams. 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.
- Song, K., P. Lu, K. Tang, and T.C. Osborn. 1995. Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. *Proc. Natl. Acad. Sci. USA* 92:7719–7723.
- Suwabe K., H. Iketani, T. Nunome, T. Kage, and M. Hirai. 2002. Isolation and characterization of microsatellites in *Brassica rapa* L. *Theor. Appl. Genet.* 104:1092–1098.
- Thormann, C.E., M.E. Ferreira, L.A.E. Camargo, J.G. Tivang, and T.C. Osborn. 1994. Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. *Theor. Appl. Genet.* 88:973–980.
- U, N. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Jpn. J. Bot.* 7:389–452.
- Udall, J.A., P.A. Quijada, and T.C. Osborn. 2005. Detection of chromosomal rearrangements derived from homeologous recombination in four mapping populations of *Brassica napus* L. *Genetics* 169:967–979.
- Villand, J.M. 1998. Molecular marker based characterization of *Capsicum* germplasm. PhD Diss., Univ. of Wisconsin, Madison.
- Warwick, S.I. and L.D. Black. 1992. Molecular relationships in subtribe Brassicinae (Cruciferae, tribe Brassiceae). *Can. J. Bot.* 71:906–918.
- Wei, J.Z. and R.R.C. Wang. 1995. Genome and species-specific markers and genome relationships of diploid perennial species in *Triticeae* based on RAPD analysis. *Genome* 38:1230–1236.