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A MULTIPLEX MICROSATELLITE PCR METHOD FOR DETECTION OF *Brassica* L. A, B AND C GENOME FRAGMENT INTROGRESSIONS UPON INTERSPECIFIC HYBRIDIZATION

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Abstract

The genus *Brassica* L. is a source of oilseeds, vegetables, spices, fodder and ornamental crops widely cultivated around the world. The six most cultivated species of the genus *Brassica* comprise allotetraploid species *B. juncea* (L.) Czern. (2*n* = 36, genome AABB), *B. napus* L. (2*n* = 38, genome AACC) and *B. carinata* A. Braun (2*n* = 34, genome BBCC), which are natural hybrids of corresponding diploid species *B. rapa* L. (2*n* = 20, genome AA), *B. nigra* L. (2*n* = 16, genome BB), and *B. oleracea* L. (2*n* = 18, genome CC). An effective way to increase the genetic diversity and improve the agronomic traits of *Brassica* crops, such as high yields, resistance to diseases, and abiotic stresses is to introduce traits of interest by the interspecific hybridization. To control the introgression of genomic material upon the hybridization, the development and implementation of genetic markers are necessary. This paper proposes an effective approach for controlling the introgression of A, B, and C genomes of *Brassica* in intraspecific hybrids. The investigation aimed to develop a high-throughput technology based on multiplex PCR analysis of genome-specific microsatellite markers for controlling the introgression of A-, B-, and C-genomes in *Brassica* intraspecific hybrids. Control samples were obtained from the Center for Genetic Resources CGN (Netherlands) and the All-Russian Institute of Plant Genetic Resources N.I. Vavilov (VIR, St. Petersburg). Plant material for the genomic material introgression study were obtained from the Timofeev Breeding Station (Moscow). Genomic DNA was extracted by sorbent method. PCR was run with specific primers for the Na10-D09, Na12-A02, Na12-F12, Ni2-B02, Ni2-F02, Ni3-G04B, Ol12-A04, Ra2-E12, BRMS-043, BN6A2 loci. Fluorescently labelled PCR products were analyzed by high-resolution electrophoresis using a Nanofor-05 genetic analyzer (Syntol — The Institute for Analytical Instrumentation, Russia). The length of the amplified DNA fragments was determined using the DNA Fragment Analysis software (The Institute for Analytical Instrumentation, Russia). A multiplex PCR technique was developed based on the six microsatellite loci Na12-A02, BRMS-043, Na10-D09, Ol12-A04, Ni2-F02, BN6A2, allowing us to determine the markers of three *Brassica* genomes in one run. A, B, and C genome-specific markers were identified during multiplex PCR analysis of control samples of six *Brassica* species with known taxonomic attributions and genome compositions: *B. rapa* (AA), *B. nigra* (BB), *B. oleracea* (CC), *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC). The length of marker fragments was determined by high resolution electrophoresis using a genetic analyzer with an accuracy of one nucleotide. A-genome specific markers were identified at the loci Na12-A02 (178 bp, 180 bp, 182 bp), BRMS-043 (303 bp, 307 bp, 313 bp), and Na10-D09 (283 bp, 285 bp, 291 bp, 293 bp, 299 bp). B-genome specific markers were detected at the loci Na12-A02 (196 bp, 198 bp, 200 bp, 202 bp, 204 bp, 212 bp, 214 bp, 216 bp), Ol12-A04 (125 bp, 127 bp, 129 bp), Ni2-F02 (198 bp, 200 bp ., 202 bp, 204 bp, 208 bp), and BN6A2 (222 bp). C-genome specific markers were detected at the loci Na12-A02 (164 bp, 168 bp, 170 bp) and Ni2-F02 (164 bp, 166 bp, 168 bp, 186 bp). The developed multiplex PCR system reveals introgressions of fragments of genomes A, B and C in the genetic profiles of interspecific hybrids

(Et2 × KK)2 × Tsv9, (Et2 × KK)1, Green × FBLM(1), JR × Agr2ki, BK × ZM1-1(6), BK × ZM1-1(8), BK, and KB. The method also confirmed the presence of the corresponding genomes in the studied samples with a known breeding history. Due to the automation, analysis allows the large-scale screening of plant samples. The proposed technology can be used in breeding practice as a tool for controlling the introgression of A, B and C genome material upon the interspecific hybridization, as well as controlling its inheritance in subsequent generations.

Keywords: *Brassica*, U triangle, *Brassica* genomes, interspecific hybridization, introgression, microsatellites, genome-specific markers

Among *Brassicaceae* family, the genus *Brassica* L. is of the greatest economic value as a source of oilseeds, vegetables, spices, fodder and ornamental crops widely cultivated all over the world. The genus *Brassica* includes 39 species [1]. Most of the cultivated plants belong to *B. napus* L. (oilseed rape, rutabaga), *B. rapa* L. (Asian collard and head cabbage, turnip and turnip), *B. oleracea* L. (head, Brussels sprouts and cauliflower, broccoli, kohlrabi), *B. juncea* Czern. (Sarepta mustard), *B. carinata* A. Braun (Ethiopian mustard), and *B. nigra* (L.) K. Koch (black mustard).

Morphological, cytogenetic and molecular relationships between cultivated *Brassica* species have been extensively studied. There are six cytogenetic groups of *Brassica* [2]. According to the U's evolutionary model [3], natural hybridization of three diploid species, the *B. rapa* ($2n = 20$, AA genome), *B. nigra* ($2n = 16$, BB), and *B. oleracea* ($2n = 18$, CC), resulted in appearance of amphidiploid species *B. juncea* ($2n = 36$, AABB), *B. napus* (2n = 38, AACC), and *B. carinata* (2n = 34, BBCC). A comparative analysis of the three *Brassica* genomes reveals significant conservatism, which indicates that all three genomes completely inherited from the common ancestor, were significantly rearranged [4]. The difference in the number of chromosomes presumably arose as a result of fusion/fragmentation of chromosomes during species divergence [5]. Analysis of chloroplast, mitochondrial, and nuclear genomes shows two evolutionary pathways of *Brassica* species, the *B. nigra* (B genome) and *B. rapa*/*B. oleracea* (A/C genomes) [6]. According to genetic mapping data, genomes A and C, despite the difference in the number of chromosomes, are highly colinear [7]. Divergence between *B. nigra* and *B. rapa*/*B. oleracea* presumably occurred 7.9 million years ago, *B. rapa* and *B. oleracea* diverged about 4 million years ago. The hybridization of the species that gave rise to *B. napus* apparently took place 10 thousand years ago [6]. Also, some differences were revealed in the structure of subgenomes of allotetraploid species as compared to the corresponding genomes of diploid species [8, 9]. Differentiation of subgenomes during interspecific hybridization and long-term domestication of polyploid *Brassica* species could occur due to translocation, inversion, deletion, duplication, and homeologous recombination [8].

Distant hybridization is an effective way to improve the agronomic traits of *Brassica* crops, such as high yield, resistance to diseases and adverse environmental conditions [10]. In crosses of *Brassica* species with each other and with other related members of the *Brassicaceae* family, the barrier of interspecies or intergeneric incompatibility is successfully overcome by embryo rescue or somatic hybridization techniques [11, 12].

Wild members of *Brasicaceae* family can be donors of potentially useful agronomic traits for crop improvement [12, 13], e.g. *Brassica maurorum* Durieu and *Eruca versicaria* ssp. *sativa* (Mill.) Thell. resistant to white rust *Albugo candida* Pers.) [14], *Raphanus sativus* L. resistant to nematode *Heterodera schachtii* Schmidt [15], Sinapis alba L. resistant to nematode *H. schachtii* and blackleg *Leptosphaeria maculans* (Sowerby) P. Karst. [16], *Sinapis arvensis* L. resistant to blackleg *L. maculans*) [17], *Sinapis incana* L. and *Diplotaxis* L. as sources of cytoplasmic male sterility [18].

Cultivated *Brassica* species also possess useful agronomic traits, for

example, *B. oleracea* shows resistance to powdery mildew caused by *Hyaloperonospora parasitica* (Pers.) Constant [12], *B. rapa*, *B. oleracea* and *B. napus* are resistant to clubroot disease (*Plasmodiophora brassicae* Woronin) [19], *B. carinata* to vascular disease caused by *Xanthomonas campestris* (Pammel) Dowson [20], and *B. juncea* possesses tolerance to heavy metals [21]. Brassica species with B genome are genetic donors of resistance to blackleg and unfavorable environmental conditions [22, 23].

Interspecific hybridization is a natural phenomenon which results in appearance of new species or introgression of adaptive traits. In *Brassica* allotetraploids resynthesized from diploid parental species the genetic diversity increases. Allotetraploids *B. juncea*, *B. napus*, and *B. carinata* were de novo assembled in order to expand their genetic basis [24-26]. The *Raphanus* genome was used to produce synthetic nematode-resistant allotetraploids [12]. *Brassica* allohexaploids ($2n = 54$, AABBCC) derived from crossing *B. napus* × *B. nigra* and *B. carinata* × *B. rapa* [27]. In hybridization of *B. carinata* and *B. rapa*, meiotically stable allohexaploids were obtained [28-31]. For a long time, meiotic aberrations due to uncontrolled pairing between three genomes hindered creation of stable allohexaploids with the expected number of chromosomes [32]. Synthetic polyploids are involved into crosses as sources of new agronomic traits

Mechanisms underlying relationships between genomes A, B and C are of practical importance. A capability of economically important *Brassica* crops to acquire traits from *B. nigra* and related members of genera *Sinapis* and *Raphanus* may depend on the degree of genomic collinearity between *B. nigra* and widely cultivated *B. oleracea* and *B. rapa* species. Detection of homologous regions in genomes will improve the methodology of genetic determinant transfer into resynthesized hybrids via homeologous recombination followed by marker-assisted selection of forms with the desired hybrid chromosomes.

Modern breeding technologies that allow distant hybridization required effective breeding control based on genetic analysis. Along with cytogenetic methods, such as fluorescence in situ hybridization technique (FISH) [31] and genomic in situ hybridization (GISH) [32, 33] techniques, DNA markers, e.g. RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) [34], SSR (simple sequence repeats) [35-37], and SNP (single nucleotide polymorphism) [38], are widely used to study genetic relationships between *Brassica* species, evolutionary changes in genomes and to control chromosome inheritance when creating digenomic and trigenomic hybrids.

Microsatellite markers are effective for studying introgressions in interspecific hybrids. Due to uniform distribution over the genome, codominant inheritance and high polymorphism, the microsatellite markers are a good tool to estimate homozygosity or heterozygosity of loci. Since the genomes of related *Brassica* species are highly linear, they are characterized by homology of flanking sequences of microsatellite loci. Due to conservativeness of flanking sequences, loci found in one species can in most cases be used to study related species.

For genus *Brassica*, microsatellite markers were developed independently by several research teams [39-41]. Linkage groups and locations on the genetic map have been established for many of these loci. A number of works have shown advantages of microsatellite loci in assessment of interspecific and intraspecific diversity of *Brassica* [42, 43], distinctness, uniformity, and stability of cultivars [44, 45], and in use as markers of diseases resistance genes, for example, upon clubroot [46, 47] and vascular disease [48].

In this research study, we have identified effective A-, B-, and C-genome specific microsatellite markers of *Brassica* and determined their length by a high-resolution electrophoresis with an accuracy of one nucleotide. The obtained results show the possibility of using these markers for the analysis of breeding samples upon interspecific hybridization.

Our goal was to develop an efficient methodology based on multiplex micro-satellite PCR analysis of genome-specific microsatellite markers, which would be suitable for monitoring the introgression of the A, B and C genomes in *Brassica* plants during distant hybridization.

Materials and methods. The control plants were obtained from the CGN Center for Genetic Resources (the Netherlands) and Vavilov All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg). Plants for the introgression study were provided by the LLC. Timofeev Breeding Station (Moscow).

Genomic DNA was extracted by adsorption method on a sorbent in accordance with instructions for the Fitosorb kit (OOO NPF Sintol, Russia). Five plants of each denomination were used. Microvolumes of solutions were dispensed automatically (Lenpipet, Russia), the sedimentation was carried out using a Centrifuge 5415D (Eppendorf, Germany). The plant biomass was lysed (a Termit thermostat, NPO DNA-Tekhnologiya, Russia). A Microspin FV-2400 mini-vortex centrifuge (SIA Biosan, Latvia) was used to mix and sediment DNA samples.

Plant DNA was amplified by PCR (a CFX-96 thermal cycler, Bio-Rad, USA) in a 25 μl reaction mixture of 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 2.5 mM MgCl2; 5 units/μl of Taq-DNA polymerase (NPO DNA-Technology LLC, Russia), 25 mM dNTP (Medigen LLC, Russia), 5-20 pmol of each primer, depending on the fluorescence intensity (LLC NPF Syntol, Russia), and 10 ng of DNA template. The primers were specific to the loci Na10-D09, Na12-A02, Na12-F12, Ni2-B02, Ni2-F02, Ni3-G04B, Ol12-A04, Ra2-E12 [39], BRMS-043 [40], and BN6A2 [41]. The PCR protocol was as follows: 5 min at 95 °C; 30 s at 94 °C, 30 s at 48 °C, 30 s at 72 °C (30 cycles); 5 min at 72 °C. To confirm amplification, the products were electrophoresed on 2% agarose gel stained with ethidium bromide.

Fluorescently labeled PCR fragments were analyzed by capillary electrophoresis under denaturing conditions in a Nanofor-05 genetic analyzer (OOO NPF Sintol, FGBNU Institute for Analytical Instrumentation RAS — IAI RAS, Russia) according to the instructions for the instrument (Shared-Use Equipment Center Biotechnology, All-Russian Research Institute of Agricultural Biotechnology). For fragment analysis, 1 μl of the PCR product mixed with 1 μl of molecular weight marker S-450 (OOO NPF Syntol, Russia) and 8 μl of Super DI formamide (MCLab, United States), were denatured for 5 min at 95 °C.

The PCR fragment length was determined using a DNA Fragment Analysis software tool (FGBNU IAI RAS, Russia).

Results. In previous study, using an 8% polyacrylamide gel electorophoresis, we revealed the most polymorphic loci suitable for *B. rapa*, *B. nigra*, *B. oleracea*, *B. napus*, *B. juncea*, and B. *carinata* species differentiation [49-51]. Since most of these loci have conserved flanking sequences in the A, B, and C genomes, their amplification occurs in all six *Brassica* species, which allows comparative analysis and identification of genome-specific markers. As a result, 10 microsatellite loci were selected. Length polymorphism of the microsatellite fragments was assessed in the control samples of six species, the *B rapa* (AA), *B. nigra* (BB), *B. oleracea* (CC), *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC), with known species attribution and genomic composition (Table 1).

1. Members of six *Brassica* L. species and interspecies hybrids with studied microsatellite fragment length polymorphism

Name	Description
Control samples	
1-02 (VIR), Ural (VIR), VikRos (VIR)	*B. napus* L. ($2n$ = 38, genome AACC)
CGN06832, CGN06818, 114 (VIR), 107 (VIR)	*B. rapa* L. ($2n$ = 20, genome AA)
CGN06619, CGN006634, CGN02656	*B. nigra* L. ($2n$ = 16, genome BB)
CGN03950, CGN03952	*B. carinata* A. Braun ($2n$ = 34, genome BBCC)
CGN15778, CGN06998, CGN07004, CGN07022	*B. oleracea* L. ($2n$ = 18, genome CC)
CGN04588, CGN04594, CGN015193	*B. juncea* (L.) Czern. ($2n$ = 36, genome AABB)
Breeding samples	
Nos. 63, 69, 70,]97,]98,]106	Regenerants derived from in vitro microspore culture of BC_2 plants upon interspecific hybridization *B. oleracea* and *B. carinata*: {[(*B. oleracea* × *B. carinata*) × *B. oleracea*] × *B. oleracea*}
(Et2 × KK)2 × Tsv9	Progeny BC_1 from interspecific hybridization (*B. oleracea* × *B. rapa*) × *B. oleracea*
Tsv9	Inbred line of *B. oleracea*
KK	Inbred line of *B. rapa*
(Et2 × KK)1	Interspecific hybrid F_1 (*B. oleracea* × *B. rapa*)
Grin × FBLM(1)	Interspecific hybrid F_1 (*B. oleracea* × *B. juncea*)
FBLM	Inbred line of *B. juncea*
JR	Inbred line of *B. rapa*
JR × Agr2ki	Interspecific hybrid F_1 (*B. rapa* × *B. oleracea*)
PR3	Inbred line of *B. oleracea*
ZM tetr	Tetraplois line of *B. oleracea*
BK × ZM1-1(6), BK × ZM1-1(8)	Progeny BC_1 from interspecific hybridization *B. oleracea* and *B. carinata*: [(*B. oleracea* × *B. carinata*) × *B. oleracea*)]
B. carinata 1	Inbred line of *B. carinata*
BK	Interspecific hybrid F_1 (*B. oleracea* × *B. carinata*)
B. carinata 2	Inbred line of *B. carinata*
KB	Interspecific hybrid F_1 (*B. carinata* × *B. oleracea*)

Note. CGN — Centre for Genetic Resources (the Netherland), VIR — Vavilov All-Russian Institute of Genetic Resources (St. Petersburg). The breeding samples are produced by OOO Timofeev Breeding Station (Moscow).

Fragments of a certain length identified in plants of the species *B. rapa* (AA) and absent in control samples of *B. nigra* (BB) and *B. oleracea* (CC) were taken as markers of the A genome material (A-genome-specific). Fragments identified in *B. nigra* (BB) but not in *B. rapa* (AA) and *B. oleracea* (CC) were deemed markers of the B genome (B-genome-specific). Fragments identified in B. oleracea (CC) and not found in *B. rapa* (AA) and *B. nigra* (BB) were markers of the C genome (C-genome-specific).

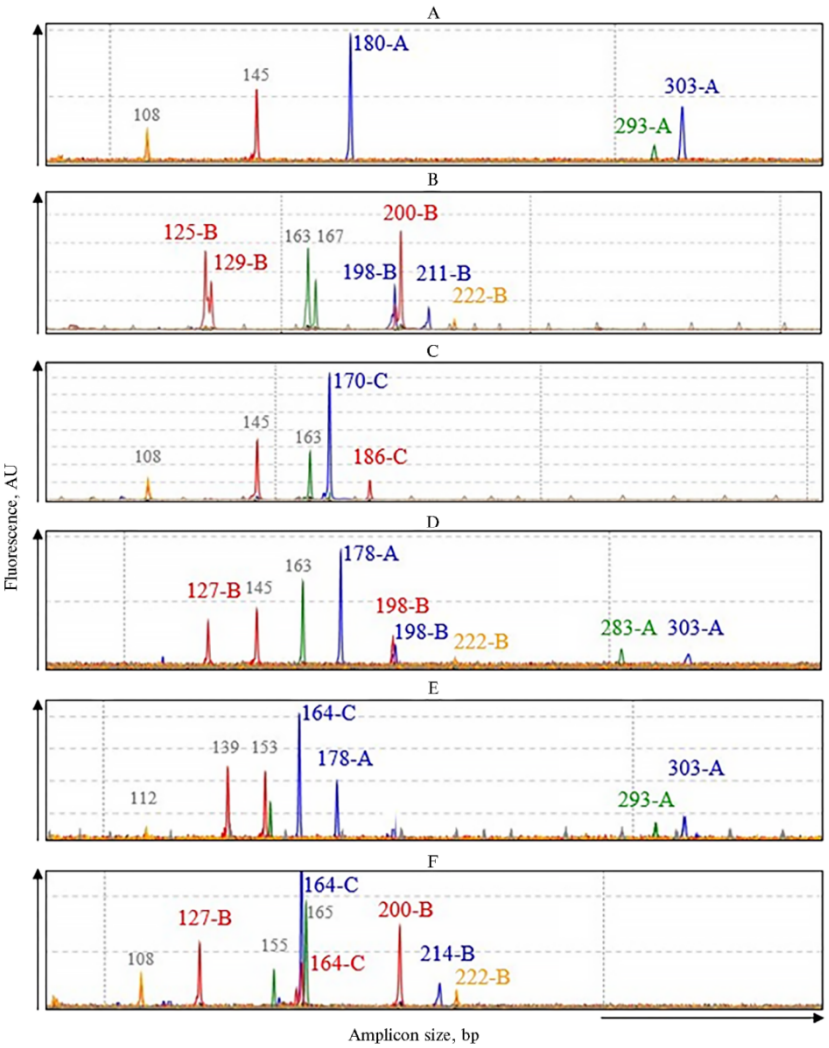
PCR analysis of Na12-A02 locus in *B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC) plants revealed the A-, B-, and C-genome-specific fragments. Also, a codominant combination of the corresponding fragments was identified in amphidiploid species *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC). Locus BRMS-043 was amplified only in plants with A genome, i.e. *B. rapa* (AA), *B. juncea* (AABB), and *B. napus* (AACC), which allows us to distinguish them from plants with B and C genomes. For Na10-D09 locus, A-genome-specific fragments were found. PCR products were synthesized in the studied samples of all six species. However, only the DNA fragments found in *B. rapa* (AA) plants significantly differed in length from those in *B. nigra* (BB) and *B. oleracea* (CC) plants. B-genome-specific fragments were identified for loci Ol12-A04 and BN6A2. PCR analysis of Ni2-F02 locus revealed B- and C-genome-specific fragments. In loci NI2-B02, RA2-E12, NA12-F12, fragments of similar length were found in control samples of *B. rapa*, *B. nigra*, and *B. oleracea*. That is, these loci were unsuitable for studying the A-, B-, and C-genome introgressions and not further used.

Based on investigation of the control samples, six microsatellite loci (Na12-A02, BRMS-043, Na10-D09, Ol12-A04, Ni2-F02, and BN6A2) were selected to develop a multiplex PCR system that allows assay for all loci simultaneously (Table 2). For amplification of microsatellite loci in one PCR run, a

single optimal annealing temperature for primer pairs was selected (T_m = 48 °C). In the multiplex system, of each microsatellite locus was amplified with a pair of specific primers, one of which was labeled with a certain fluorescent dye (FAM, R6G, ROX, and Sy630). This allows for separate assay of PCR fragments for each locus through the corresponding detection channel (see Table 2). The loci were selected in such a way that the ranges of the lengths of their fragments did not overlap when detected through the same channel. The PCR fragment length was measured with a single nucleotide accuracy due to use of high-resolution capillary electrophoresis with fluorescence detection technique (an automatic genetic analyzer Nanofor-05).

2. Parameters of the multiplex PCR system for analysis of A, B and C genome introgressions in *Brassica* L. species based on microsatellite loci

Locus	Genome specificity	Stain	Amplicon size, bp
Na12-A02	ABC	FAM	164-216
BRMS-043	A	FAM	303-313
Na10-D09	A	R6G	155-299
OI12-A04	B	ROX	125-153
Ni2-F02	BC	ROX	164-200
BN6A2	B	Sy630	108-222



Genetic profiling of control plants of six *Brassica* L. species by a multiplex PCR analysis of microsatellite

loci: A — CGN06832 (*B. rapa*, genome AA), B — CGN006634 (*B. nigra*, genome BB), C — CGN15778 (*B. oleracea*, genome CC), D — CGN015193 (*B. juncea*, genome AABB), E — ВИР ВикРос (*B. napus*, genome AACC), F — CGN03950 (*B. carinata*, genome BBCC). The color of the peak (fragment) corresponds to the detection channel on the Nanofor-05 device (OOO NPF Syntol —FGBNU Institute for Analytical Instrumentation RAS, Russia) and indicates the locus the fragment belongs to, i.e. blue (FAM) for Na12-A02 and BRMS-043, green (R6G) for NA10-D09, red (ROX) for Ol12-A04 and Ni2-F02, and orange (SY630) for BN6A2. Non-discriminatory fragments amplified simultaneously in two or three genomes are shown in gray. For a description of the samples, see Table 1.

The developed multiplex system generates output data as digitized genetic profiles of *Brassica* samples, in which each peak corresponds to a PCR fragment of a certain length (Fig.). Microsatellite analysis of the control plants identified fragments specific for genomes A, B, and C. In the genetic profile of the control sample CGN06832 (*B. rapa*) there were 180 bp (locus Na12-A02), 303 bp (locus BRMS-043), and 293 bp (locus Na10-D09) fragments characteristic of the genome A (see Fig., A). The profile of the sample CGN006634 (*B. nigra*) contained distinctive markers of the B genome with a length of 198 bp and 211 bp (locus Na12-A02), 125 bp and 129 bp (locus OL12-A04), 200 bp (locus NI2-F02), and 222 bp (locus BN6A2) (see Fig., B). In sample CGN15778 (*B. oleracea*), the 170 bp (locus Na12-A02) and 186 bp (locus Ni2-F02) C-genome-specific fragments were found (see fig., B). Genetic profiles of the allotetraploid species showed a codominant combination of fragments of the corresponding diploid genomes (see Fig. D-F).

From the profiling data, we identified the allele composition of each microsatellite locus and the markers specific for genomes A, B and C (Table 3).

3. A-, B- and C-genome-specific markers revealed in six *Brassica* L. species by a multiplex PCR analysis of microsatellite loci

Locus	Genome	Microsatellite fragment length, bp		
		A-specific	B-specific	C-specific
Na12-A02	ABC	178, 180, 182	196, 198, 200, 202, 204, 212, 214, 216	164,168,170
BRMS-043	A	303, 307, 313	–	–
Na10-D09	A	283, 285, 287, 291, 293, 299	–	–
Ol12-A04	B	–	125, 127, 129	–
Ni2-F02	BC	–	198, 200, 202, 204, 208	164, 166, 168, 186
BN6A2	B	–	222	–

N o t e. Dashes mean the absence of a corresponding genome-specific marker in the loci.

4. Genome-specific markers (bp) **revealed in lines and interspecific hybrids of *Brassica* L. by a multiplex PCR analysis of microsatellite loci:**

Sample	Locus						Genome
	Na12-A02	BRMS-043	Na10-D09	Ol12-A04	Ni2-F02	BN6A2	
KK	180^{A}	303^{A}	293^{A}	145	–	108	AA
JR	180^{A}	311^{A}	285^{A} 341^{A}	143	–	114	AA
No. 63	164^{C}	–	155	–	166^{C}	108	CC
No. 69	164^{C}	–	155	–	166^{C}	108	CC
No. 70	164^{C}	–	155	–	166^{C}	108	CC
No. 97	164^{C}	–	155	–	166^{C}	108	CC
No. 98	164^{C}	–	155	–	166^{C}	108	CC
No. 106	164^{C}	–	155	–	166^{C}	108	CC
Tsv9	164^{C}	–	155	–	166^{C}	108	CC
PR3	164^{C}	–	–	–	166^{C} 186^{C}	108	CC
ZM tetr	164^{C}	–	–	–	166^{C} 186^{C}	108	CC
BK × ZM1-1(6)	164^{C} 168^{C}	–	167	–	–	108	CC
BK × ZM1-1(8)	164^{C} 168^{C}	–	155	–	166^{C}	108	CC
(Et2 × KK)2 × Tsv9	170^{C} 180^{A}	303^{A}	155 293^{A}	145	166^{C}	108	AACC
(Et2 × KK)1	170^{C} 180^{A}	303^{A}	293^{A}	145	166^{C}	108	AACC

Continued Table 4

JR × Agr2ki	164^{C}	311^{A}	287^{A}	145	166^{C}	108	AACC
	180^{A}					114	
FBLM	178^{A}	313^{A}	163	127^{B}	198^{B}	222^{B}	AABB
	198^{B}		285^{A}	145			
Grin × FBLM(1)	164^{C}	313^{A}	163	127^{B}	166^{C}	108	AABBCC
	178^{A}		287^{A}	145	198^{B}	222^{B}	
	198^{B}						
B. carinata 1	16	–	155	127^{B}	164^{C}	108	BBCC
			167		200^{B}		
BK	164^{C}	–	155	127^{B}	166^{C}	108	BBCC
	214^{B}		167		200^{B}	222^{B}	
B. carinata 2	164^{C}	–	155	127^{B}	166^{C}	108	BBCC
	214^{B}		167		200^{B}	222^{B}	
KB	164^{C}	–	155	127^{B}	166^{C}	108	BBCC
	168^{C}		167		200^{B}	222^{B}	
	214^{B}						

N o t e. Superscripts (^{A}, ^{B}, ^{C}) indicate the genomic specificity of the marker fragment. Dashes indicate the absence of the corresponding genome-specific marker of the indicated locus. For a description of the samples, see Table 1.

We also used the developed PCR system to identify A-, B-, and C-genome-specific fragments in breeding samples (see Table 1), obtained their genetic profiles and found genome-specific fragments (Table 4). Consequently, the multiplex system used makes it possible to reliably detect the of A, B, and C genome fragment introgressions in species of the genus *Brassica*. Codominant combinations of genomic fragments in the genetic profiles of breeding samples (Et2 × KK)2 × Tsv9 obtained from hybridization (*B. oleracea* × *B. rapa*) × *B. oleracea*, (Et2 × KK)1 (F_1 *B. oleracea* × *B. rapa*), Grin × FBLM (1) (F_1 *B. oleracea* × *B. juncea*), JR × Agr2ki (F_1 *B. rapa* × *B. oleracea*), BK (F_1 *B. oleracea* × *B. carinata*) and KB (F_1 *B. carinata* × *B. oleracea*) confirmed introgressions as a result of interspecific hybridization. The data obtained by microsatellite analysis correspond to the breeding history of the samples.

The developed system for multiplex analysis based on six microsatellite loci specific for genus Brassica A, B, and C genomes reliably differentiates plants of six species of the U triangle and also can evaluate genetic diversity, since each genomic marker possesses several allelic variants. Each genome is defined by at least two markers, which serve as internal controls for each other.

This multiplex system also provides tracing an introgression of certain regions of the *Brassica* genome, since linkage groups for the loci used in it have been determined (http://www.brassica.info/resource/markers/ssr-exchange.php). For example, it was shown that the BRMS-043 locus is associated with resistance to vascular diseases of *B. rapa* (48).

The effectiveness of DNA markers in genotyping new forms and detection of genomic material transfer during plant breeding has been demonstrated in a number of works. The analysis of potential genetic changes in 25 synthesized allohexaploids (H1 *B. rapa* × *B. carinata*, AABBCC, $2n = 54$) was carried out using 162 combinations of A-, B-, and C-genome-specific SSR primers [31]. To assess the genetic variability of the new form of *B. napus* obtained by crossing *B. rapa* with a hexaploid (*B. napus* × *B. oleracea*, AACCCC), 153 combinations of primers for microsatellite loci were used [32]. With 34 SSR markers, homeological recombination of chromosomes was detected in the offspring of *B. napus* × *B. carinata* (ABCC) trigenomic hybrids derived from microspores [37].

The Illumina Infinium *B. napus* 60K SNP (Illumina, Inc., USA) array of SNP markers for *B. napus* allotetraploid was developed to identify *Brassica* species and assess genetic diversity [52]. Also, a multiplex PCR analysis (MPCR) of A-, B-, and C-genome-specific sequences using five combinations of primers was proposed for rapid identification of *Brassica* species of the U triangle [35]. The MPCR

results were validated on 120 genetically characterized *Brassica* samples. Due to the direct detection of specific fragments in a 2% agarose gel, the MPCR assay is useful as an affordable and rapid diagnostic technique that can be easily applied in a conventional laboratory. However, as noted by the authors themselves, this method has a rather low throughput and should be adapted for high throughput real-time screening.

In contrast to expensive and more complicated methods that require special data processing (for example, when using the Illumina Infinium *B. napus* 60K SNP DNA chip), the technology we propose is more accessible and convenient to use. Electrophoretic analysis is carried out automatically using high-precision equipment. This significantly increases the reliability of the obtained data and thir interpretation (un-like the empirical assessment in the gel without precise determination of the lengths of the analyzed DNA fragments) [35, 45, 48]. Due to the automation of all stages in a 96-well plate format, the proposed approach allows large-scale screening of selection samples. The multiplex system can be used to assess the introgression of *Brassica* genomes during interspecific hybridization and control the inheritance of genomic material in subsequent generations.

Thus, we have developed a system for multiplex PCR analysis of six *Brassica* microsatellite loci (Na12-A02, BRMS-043, Na10-D09, Ol12-A04, Ni2-F02, BN6A2) for detecting A, B, and C genome fragments. The reliability of the system is confirmed with the control samples of known genomic composition and taxonomic attribution. The developed markers allowed us to identified the A-, B- and C-genome-specific fragments and to determine the genomic composition of a number of breeding samples. The results of the investigation can be used to detect sequences specific for genomes A, B, C in *Brassica* plants and to control the inheritance of genetic material during distant hybridization.

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