Comparison of Individual- and Bulk-Samples-Based Approaches Using RAPD and ISSR Markers for Identifying Genetic Relationships in *Brassica* Cultivars

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Summary Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) variation among 24 cultivars of four *Brassica* species (*B. rapa*, *B. juncea*, *B. napus*, and *B. oleracea*) was examined using individual- and bulk-samples-based approaches. In both the approaches, eight plants were sampled for each cultivar, and were amplified individually and as bulk DNA, respectively. In total, 305 and 422 bands were scored in the individual- and bulk-samples-based approaches, respectively, and almost all the bands (99.8–100%) were polymorphic. Based on the individual-samples dataset, the within cultivar variation was smaller than the among cultivars variation, leading to a distribution reflecting genome compositions of each cultivar with principal coordinates analysis (PCoA) and unweighted pair-group method with arithmetic average (UPGMA) analysis. Although PCoA and UPGMA analysis based on bulk-samples data clustered the cultivars into groups with more emphasized variance among species than the results obtained by the individual-based approach, both PCoA plots and dendrograms of the individual- and bulk-samples-based approaches, respectively, were highly correlated with each other. These findings indicate that the number of individuals used to profile each cultivar can be reduced to eight per cultivar for analysis of the genetic variability in *Brassica* using RAPD and ISSR polymorphism, and the bulk-samples-based approach can be used to describe the genetic relationship among *Brassica* cultivars.

Key words Random amplified polymorphic DNA, RAPD, Inter-simple sequence repeat, ISSR, Individual-based approach, Bulk-samples-based approach, *Brassica*.

Molecular markers have proved valuable in crop breeding, especially in studies on genetic diversity and for assessing relationships in a number of crop species. Among the different molecular genetic markers, RAPD and ISSR markers have been used to generate vast quantities of data, resulting in their rapid application for addressing a diverse range of biological questions (Semagn *et al.* 2006, Agarwal *et al.* 2008, Verma *et al.* 2016, Kalia *et al.* 2017). Because of their efficiency and convenience, these techniques have been applied to taxonomic comparisons of many plant species (Fernández *et al.* 2002, Sudupak 2004, Wu *et al.* 2004, Liu and Wang 2006, Han and Wang 2010, Sultana and Alam 2016, Dasgupta *et al.* 2018). To estimate the genetic

distance between cultivars or populations, analyzing a large number of accessions individually within a cultivar or population is desired. Especially, in the estimation of the genetic structure of allogamous species, the diversity existing among individuals within cultivars or populations should be taken into account (Ghérardi *et al.* 1998, Raimondo *et al.* 2012). However, these approaches are laborious and impractical if a large number of populations is to be characterized.

The bulk samples approach is based on mixing equal quantities of genomic DNA, and can reduce the experimental effort, as the large size of a collection makes its management and use difficult (Michelmoré *et al.* 1991, Kongkiatngam *et al.* 1996, Taški-Ajduković *et al.* 2014). Hence, this approach has been widely used for estimating genetic variation and relationships among species, cultivars, and populations, such as alfalfa (Yu and Pauls 1993, Segovia-Lerma *et al.* 2003, Taški-Ajduković *et al.* 2014), red clover (Kölliker *et al.* 2001, Dias *et al.* 2008), *Brumus catharticus* (Puecher *et al.* 2001), radish (Muminović *et al.* 2005, Wang *et al.* 2008),

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B. napus (Dulson *et al.* 1998), and *B. oleracea* (Lázaro and Aguinalde 1998, Divaret *et al.* 1999), and the efficacy of this strategy has been examined. The bulked samples approach simplifies the marker profile of an individual cultivar or population because it favors the detection of consensus fragments (*i.e.*, common alleles) in each population (Michelmore *et al.* 1991, Yu and Pauls 1993). Segovia-Lerma *et al.* (2003) reported that this approach may reflect a population's general genetic composition more accurately compared to the evaluation of fewer individual genotypes. Although an appropriate bulk size, reflecting genetic diversity existing between individuals within the population, depends on the degree of heterozygosity in species, the relationship between an appropriate bulk size and the degree of heterozygosity has not been fully examined for each plant species analyzed in this study.

The genus *Brassica* contains some of the most important arable and forage crop species, such as turnip (*B. rapa*), leaf mustard (*B. juncea*), oil seed rapa (*B. napus*), and cabbage (*B. oleracea*). Their genomes have been denoted as A, B, and C, respectively, and the hybrid taxa, *B. napus*, *B. juncea*, and *B. carinata*, are the known amphidiploids of *B. rapa*×*B. oleracea* (AC), *B. rapa*×*B. nigra* (AB), and *B. oleracea*×*B. nigra* (BC), respectively (U 1935). Among the *Brassica* species, *B. rapa* and *B. oleracea* are allogamous species, owing to their self-incompatible nature, and are expected to be genetically heterogeneous, especially in the indigenous

varieties. In a previous study, we reported the congruity between genetic similarities among cultivars, calculated from the data based on individual and bulked population samples, to assess genetic relationships between *Brassica* cultivars, including heterogeneous species (Wakui *et al.* 2009). In the above-mentioned study, we also determined that the number of plantlets that should be analyzed per cultivar using RAPD and ISSR analysis is 37, for each of the six cultivars of Japanese turnip (*B. rapa* ssp. *rapa*). In the present study, to apply the results described in the previous report and to evaluate the efficacy of the method proposed therein, we compared the genetic relationships among 24 *Brassica* cultivars and determined the genetic variability, inferred by individual- and bulked-samples-based approaches, using RAPD and ISSR markers.

Materials and methods

Plant materials and DNA preparation

Twenty-four cultivars, including 17 traditional and 7 commercial cultivars selected from four cultivated *Brassica* species, were used in this study (Table 1). Among them 17 cultivars were of *B. rapa*, two each were of *B. juncea* and *B. napus*, and three were of *B. oleracea*. *B. rapa* was represented by six subspecies, three of ssp. *pekinensis*, nine of ssp. *rapa*, one each of ssp. *chinensis*, *japonica* and *oleifera*, and two of ssp. *narinosa*. Eight seedlings per cultivar were randomly selected and the

Table 1. *Brassica* cultivars included in the present study.

Species	Genome	ID	Cultivars	Type	He±SD ^a
<i>B. rapa</i> ssp. <i>pekinensis</i>	AA	A	Matsusima-shin-nigouhakusai	Commercial	0.073±0.166
		B	Kanazawadaigo-kettukyuhakusai	Traditional	0.092±0.179
		C	Tokyo-bekana	Traditional	0.116±0.191
<i>B. rapa</i> ssp. <i>rapa</i>		D	Maruba-komatsuna	Traditional	0.117±0.185
		E	Kiso-benikabu	Traditional	0.131±0.196
		F	Kamo-sugukina	Traditional	0.102±0.177
		G	Hinoguchi-kabu	Traditional	0.091±0.176
		H	Hinona-kabu	Traditional	0.092±0.173
		I	Tennoji-kabu	Traditional	0.124±0.192
		J	Hakuba	Commercial	0.044±0.132
		K	Kuretsubo-kabu	Traditional	0.083±0.172
		L	Nozawana	Traditional	0.114±0.190
<i>B. rapa</i> ssp. <i>chinensis</i>		M	Yukishiro-taisai	Traditional	0.118±0.192
<i>B. rapa</i> ssp. <i>japonica</i>		N	Bansei-sensuji-mizuna	Traditional	0.106±0.185
<i>B. rapa</i> ssp. <i>narinosa</i>		O	Bitamin-na	Commercial	0.099±0.182
		P	Tijimi-na	Traditional	0.084±0.174
<i>B. rapa</i> ssp. <i>oleifera</i>		AABB	Q	Hayachine-na	Traditional
<i>B. juncea</i>	R		Kigarashi-na	Traditional	0.093±0.183
	S		Miike-takana	Traditional	0.033±0.117
<i>B. napus</i>	AACC	T	Senpousai-nigou	Commercial	0.055±0.145
		U	Miyauchi-na	Traditional	0.055±0.147
<i>B. oleracea</i>	CC	V	Delicious	Commercial	0.045±0.135
		W	Watanabeseikou	Commercial	0.088±0.173
		Z	Stick senyori	Commercial	0.042±0.129
Mean					0.088±0.170

^a The expected heterozygosity (He) was estimated by 305 loci of RAPD and ISSR using eight individuals per cultivar. SD means Standard deviation.

first leaf of each of the seedlings was sampled from each cultivar. These leaves were frozen in liquid nitrogen and stored at -80°C . DNA extraction was carried out by the cetyltrimethylammonium bromide method (Rogers and Bendich 1988). DNA concentration was determined using an UV-visible spectrophotometer (UV-1600, Shimadzu). In the individual population sample-based approach, DNA of each of the eight individuals per cultivar was used independently as a template for PCR. On the other hand, in the bulked-samples-based approach, DNA samples were prepared by mixing 500 ng of DNA from each of the eight individuals per cultivar, and 20 ng DNA was used for PCR.

DNA amplification and data analysis

RAPD and ISSR primers used in this study were the same as those reported by Wakui *et al.* (2009). RAPD and ISSR analyses were performed using ten 10-mer random oligonucleotides (Eurofins Genomics) and ten selected ISSR primers from primer set No. 9 (Biotechnology Laboratory of University of British Columbia, Vancouver, B.C., Canada) (Table 2). The RAPD and ISSR fragments were manually scored as present (1) or absent (0). Only the distinct major bands were chosen for this study. To estimate the variation within populations, expected heterozygosity was calculated based on allele frequencies of respective loci for each population with data from individual samples. Analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was performed using GenAlEx v.6 (Peakall and Smouse 2012) to assess RAPD and ISSR variations among and within species

for the bulked-samples-based approach, and among species as well as, among and within cultivars for the individual-samples approach. To visualize the relationship among the cultivars, PcoA (Gower 1966) was performed using GenAlEx v.6 (Peakall and Smouse 2012). Genetic relationships among the cultivars were investigated by cluster analysis using the UPGMA using MEGA ver. 7.0 (Tamura *et al.* 2007) based on genetic distances (Nei 1972) calculated by the genetic identity matrix for the individual method and by genetic distances calculated with 1-JACCARD formula for the bulked-samples-based approach.

Results

Ten RAPD and ISSR primers used in this study produced good amplification products. The exception was primer UBC-807, used in the bulked-samples-based approach, which did not produce any clear bands and whose data were, therefore, discarded. Details of the primers are listed in Table 2. In the individual-based approach, the amplification of eight individual DNA samples per primer revealed clear variations within each variety (Fig. 1a). The band pattern of each variety was relatively similar within and among the *B. rapa* subspecies, compared with that among different *Brassica* species. The differences in the band patterns among varieties were more simply shown in the bulked-samples-based approach (Fig. 1b). Although the band pattern was the sum of the variation within the variety most detected by the individual method in bulk, there was also a band that was not detected. The PCR products were scored manually and independently, and the faint bands were excluded. The total number of bands scored was 305 and 422 in the individual- and bulked-samples-based approaches, respectively.

Based on individual-samples data, the expected heterozygosity values within each cultivar were calculated, which ranged from 0.033 to 0.131, whereas the average value in all the cultivars was 0.088 (Table 1). The results of AMOVA also revealed that the variation among the individuals within a cultivar was relatively low (24%) and most of the variation was responsible for the genetic variance among cultivars (40%) in the individual-based approach (Table 3). On the other hand, the variation (53%) among species in the bulked-samples-based approach was higher than that in the individual-based approach (36%).

PCoA of the individual- and bulked-samples data clustered the 24 cultivars into roughly four groups, reflecting genome compositions and species taxa (Fig. 2a, b). Coordinate 1, which accounted for 45% and 41% in the individual- and bulked-samples-based approach, respectively, resulted in the separation of four species. The known allopolyploid, *B. napus*, was observed to be located midway between its putative parent species, *B.*

Table 2. Sequences of primers employed and the number of scored polymorphic bands based on RAPD and ISSR analysis.

Name of primers	Sequences (5'-3')	No. of polymorphic bands	
		Individual-based approach	Bulked-samples-based approach
OPA-1	CAGGCCCTTC	14	12
OPA-2	TGCCGAGCTG	15	9
OPA-3	AGTCAGCCAC	14	10
OPA-4	AATCGGGCTG	19	21
OPA-7	GAAACGGGTG	14	15
OPA-8	GTGACGTAGG	15	6
OPA-9	GGGTAACGCC	11	4
OPA-10	GTGATCGCAG	13	17
OPA-11	CAATCGCCGT	12	13
OPA-20	GTTGCGATCC	13	18
UBC-807	(AG) ₈ T	20	—
UBC-808	(AG) ₈ C	20	50
UBC-810	(GA) ₈ T	20	55
UBC-811	(GA) ₈ C	17	26
UBC-812	(GA) ₈ A	17	31
UBC-813	(CT) ₈ T	14	32
UBC-814	(CT) ₈ A	14	21
UBC-815	(CT) ₈ G	20	29
UBC-822	(TC) ₈ A	12	22
UBC-823	(TC) ₈ C	11	31
Total		305	422

Fig. 1. RAPD band patterns produced by primer OPA-2 for eight individual samples (a) and a bulked sample (b) of *Brassica* cultivars. (a) Band patterns shown for 12 cultivars containing six *B. rapa* subspecies (B, C, E, F, M, N, P, and Q) and *B. juncea* (S), *B. napus* (T), and *B. oleracea* (W and Z). The arrow (a; F: individuals) shows a rare band detected in the individual-based approach. M is a 500-bp ladder DNA.

Table 3. Analysis of molecular variance (AMOVA) for individual-based and bulked-samples-based approaches using RAPD and ISSR.

Source	Degree of freedom	Sum of squares	Mean of squares	Estimates of variance	Variation (%)	Probability
Individual-based approach						
Among species	3	2090.91	696.97	17.63	36	<0.01
Among cultivars	20	3364.10	168.20	19.53	40	<0.01
Within cultivars	168	2003.88	11.93	11.93	24	
Total	191	7458.88		49.09	100	
Bulked-samples-based approach						
Among species	3	383.37	127.79	27.58	53	<0.01
Within species	20	487.22	24.36	24.36	47	
Total	23	870.58		51.94	100

Fig. 2. Scatter diagrams of the first two coordinates from the principal coordinate analysis (PCoA) of 24 cultivars (a and b) and cultivars of *B. rapa* (c and d) using RAPD and ISSR. a and c: Individual-based approach, b and d: bulked-samples-based approach. Each cultivar is indicated by a plant ID (defined in Table 1).

rapa and *B. oleracea*. Coordinate 2 accounted for 14% and 12% in the individual- and bulked-samples-based approaches, respectively. *Brassica juncea*, which included unique B genome in our samples, was separated from all other species along the second axis. The genetic relationships among the four species fit closely between both the PCoA plots, although variance among the species rather than within the species was more emphasized in the bulked-samples-based approach. Closer examination of the PCoA analysis revealed the grouping of taxa within the *B. rapa* cluster containing six subspecies (Fig. 2c, d). Coordinates 1 and 2 accounted for 31% and 25% of the variation, respectively, in the individual-based approach. The *B. rapa* cultivars were largely separated into three groups consisting of ssp. *pekinensis*, ssp. *rapa*, and a group of ssp. *chinensis*, *japonica*, and *oleifera*. On the other hand, the coordinate 1 and 2 accounted for 24% and 18% of the variation in the bulked-sample-based approach, respectively (Fig. 2d). The cultivars of ssp. *chinensis*, *japonica*, and *oleifera* were plotted closely, apart from the cultivars of ssp. *pekinensis* and ssp. *rapa*. The cultivars in ssp. *rapa* were scattered along the first axis, compared to their position in the plots in the individual-sample-based approach. As a result, ‘Maruba-komatsuna’

Fig. 3. UPGMA trees based on genetic distances estimated using RAPD and ISSR among 24 cultivars. a: Individual-based approach, b: bulked-samples-based approach. Each cultivar is indicated by a plant ID (defined in Table 1). The scale bars represent genetic distances.

(E) and ‘Kiso-benikabu’ (D) were close to ‘Tokyo-bekana’ (C) and were plotted without a clear division between ssp. *pekinensis* and ssp. *rapa*.

The UPGMA tree, based on the individual- and bulked-samples data, resulted in a clear separation of the four *Brassica* species (Fig. 3). On the whole, the topologies of both the trees were similar to each other although some clustering in the dendrograms was not absolutely

identical between them. In the bulked-sample-based approach, 'Maruba-komatsuna' (D) and 'Kiso-benikabu' (E) in *ssp. rapa* were clustered with *ssp. pekinensis* cultivars although the cultivars in *ssp. pekinensis* and *ssp. rapa* formed their own cluster, in the individual-based approach. In addition, in the bulked-samples-based approach, *ssp. narinosa* (O and P) were clustered closer to each other compared to locations in the individual-based approach.

Consequently, although some disagreements were observed as mentioned above, the results of PCoA and UPGMA, obtained using the individual- and bulked-samples-based approaches, reflected the genome composition and had a relatively similar pattern.

Discussion

To assess the genetic relationships among *Brassica* cultivars, including those among the out-crossing species, the number of individuals sampled should be sufficiently large, so that they can capture the heterogeneity within a cultivar. In the bulk method, use of a small sample size produces distortion in the detection of rare alleles (Divaret *et al.* 1999, Mailer and May 1999, Fu *et al.* 2003). Moreover, if the sample size of the bulk becomes large, a large difference in banding pattern will no longer be found (Divaret *et al.* 1999). This shows the importance of evaluating the number of individuals, which includes the variance of the variety used for analysis (Yu and Pauls 1993). Our previous study (Wakui *et al.* 2009) with local varieties of turnip indicated that six to eight individuals per cultivar were enough to calculate an accurate estimate of similarity with the individual-based approach.

Based on the above-mentioned results, we used eight individuals per cultivar for the RAPD and ISSR analyses in this study. The expected heterozygosity values showed relatively low genetic variation within the cultivars, compared to that reported previously on other out-crossing species (Durka *et al.* 2005, Flajoulot *et al.* 2005). AMOVA also revealed similar a result for variation among individuals within a cultivar. The isozyme loci of *B. campestris* (*B. rapa*) and *B. oleracea* also revealed low genetic variance within the cultivars (Simonsen and Heneen 1995). On the other hand, most of the variation was contributed to the genetic variance among the cultivars, showing that the genetic differentiation of each cultivar is progressing. An almost similar distribution was obtained after PCoA, based on the genetic similarity matrix of individual- and bulked-samples data. *B. napus* were grouped closer to *B. oleracea* than to *B. rapa*; this distribution was identical to the results reported by Thormann *et al.* (1994) and Liu and Wang (2006). The UPGMA trees also revealed that both the clusters based on individual- and bulked-samples data had an almost similar pattern reflecting genome composition at

the species level. However, the grouping of taxa within the *B. rapa* cluster containing six subspecies indicated that 'Maruba-komatsuna' (D) and 'Kiso-benikabu' (E) were situated close to *ssp. pekinensis* in the bulked-samples-based approach in PCoA. Also, in UPGMA, this result was reflected by the dendrogram in the bulked-samples-based approach. In addition, 'Bitamin-na' (O) in *ssp. narinosa*, which formed a small cluster with 'Tijimi-na' (P) of same subspecies in the bulked-samples-based approach, was connected with a cluster consisting of both the cultivars of *ssp. chinensis* and *japonica* in the individual-based approach. It is inferred that these differences in the genetic structure between both the approaches are caused by the degree of genetic diversity existing among individuals within a cultivar. As the result presented in Fig. 1 shows, the bulked-samples-based approach simplifies the marker profile of an individual cultivar without rare fragments because it favors the detection of consensus fragments in each cultivar. Among the materials used in this study, some cultivars in which the heterozygosity was relatively high, changed the genetic distributions more or less, and the results of PCoA and UPGMA tree between both the approaches are similar but different in their details. Therefore, the bulked-samples-based approach is useful for showing the genetic relationship based on the detected fragments that take precedence over common alleles, within the genetic diversity among individuals within each variety.

These comparative findings indicate that each cultivar could be profiled by RAPD and ISSR markers and classified similarly in the individual- and bulked-samples-based approaches. This favors high correlation coefficients between the similarity measures for individual- and bulked-samples data, as shown in our previous study (Wakui *et al.* 2009). Especially, eight individuals within a cultivar, which were determined based on the results of a previous study (Wakui *et al.* 2009), appear to be appropriate and include the variation within a cultivar, because the expected heterozygosity value within each cultivar was low (Table 1). An advantage of a high-throughput system for accurately characterizing the genetic diversity among populations using bulked DNA as templates was described previously (Segovia-Lerma *et al.* 2003, Muminović *et al.* 2005, Wang *et al.* 2008, Keivani *et al.* 2010, Taški-Ajduković *et al.* 2014). However, the method for determining the number of individuals required for preparing the bulk DNA and the result of the comparison to the individual-based approach were not described. In the present study, we compared the individual- and bulked-samples-based approaches to characterize the genetic variability and relationships among the population of out-crossing species on the basis of the number of individuals previously determined with well-grounded approaches. This study empirically showed that a suitable number of individuals treated in the individual-based approach could be reduced to eight

individuals from 37 individuals per cultivar in *Brassica* species. Furthermore, in the bulked-based approach, the genetic variability and relationships among and within species can be elucidated with accuracy in *Brassica* species.

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