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Assessment of genetic diversity of accessions in Brassicaceae genetic resources by frequency distribution analysis of *S* haplotypes

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Abstract Plant genetic resources are important sources of genetic variation for improving crop varieties as breeding materials. Conservation of such resources of allogamous species requires maintenance of the genetic diversity within each accession to avoid inbreeding depression and loss of rare alleles. For assessment of genetic diversity in the self-incompatibility locus (*S* locus), which is critically involved in the chance of mating, we developed a dot-blot genotyping method for self-incompatibility (*S*) haplotypes and applied it to indigenous, miscellaneous landraces of *Brassica rapa*, provided by the IPK Gene Bank (Gatersleben, Germany) and the Tohoku University Brassica Seed Bank (Sendai, Japan), in which landraces are maintained using different population sizes. This method effectively determined *S* genotypes of more than 500 individuals from the focal landraces. Although our results suggest that these landraces might possess sufficient numbers of *S* haplotypes, the strong reduction of frequencies of recessive *S* haplotypes occurred, probably owing to genetic drift. Based on these results, we herein discuss an appropriate way to conserve genetic diversity of allogamous plant resources in a gene bank.

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Introduction

Plant genetic resources are important sources of genetic variation for improving crop varieties to serve new breeding purposes, such as good quality of food products, disease resistance to new races of pathogens, tolerance to new environmental stresses, and enhancement or elimination of some chemical compounds. Genetic resources of many crop species and their close relatives are conserved in gene banks at many institutes and universities. Most plant species are stored as seeds under low temperature, and periodically rejuvenated. Rejuvenation and multiplication of autogamous plants are simple, but those of allogamous plants are problematical. Allogamous plants have genetic diversity in a population, and such diversity has to be conserved to avoid inbreeding depression. Furthermore, because of the small number of seeds collected and the small number of plants grown for the rejuvenation and multiplication of seeds, genetic diversity tends to be decreased and some rare alleles may be lost.

Self-incompatibility is a mechanism to promote outcrossing in allogamous species. The self-incompatibility locus (*S* locus) possesses a large amount of genetic variation (i.e., the number of *S* haplotypes) for increasing the chance of mating, because individuals exhibiting the same *S* phenotype cannot mate with each other (Wright 1939). Thus, a population with a scarcity of *S* haplotypes displays a critical reduction of fitness such that the chance of mating will be significantly reduced. Thus, assessment of an amount of genetic variation in the *S* locus is required for

each accession of genetic resources maintained in a gene bank.

Self-incompatibility systems can be classified into two types. One is gametophytic self-incompatibility (GSI), in which pollen-side recognition is dependent on an *S* haplotype of a pollen grain, and the other is sporophytic self-incompatibility (SSI), in which both pistil- and pollen-side recognitions rely on a parental diploid genotype. In the SSI system but not in the GSI system, there are dominance relationships between *S* haplotypes. The dominance relationships between *S* haplotypes in the SSI systems are expected to increase chances for mating by masking one of the recognition specificities of *S* heterozygotes (Vekemans et al. 1998). Thus, the most recessive *S* haplotypes would be the most frequent in a population, this effect being termed the "recessive effect" (Bateman 1952; Sampson 1974), and the maintained number of *S* haplotypes in a population would decrease (Schierup et al. 1997; Vekemans et al. 1998).

Diploid species in *Brassica*, including important vegetables such as cabbage, broccoli, cauliflower, Chinese cabbage, and turnip, have the SSI system. The SSI system is used for seed production of F_1 hybrid cultivars. The mechanisms of self-recognition and dominance relationships are well characterized in *Brassica* species. An *S* haplotype in *Brassica* is comprised of an allelic combination of *SRK* (a pistil-recognition gene; Stein et al. 1991), *SP11/SCR* (a pollen-recognition gene; Suzuki et al. 1999; Schopher et al. 1999), and *SLG*, whose function has been still under debate (Takasaki et al. 2000; Suzuki et al. 2000). The *S* haplotypes are classified into class I and class II at the sequence level (Nasrallah et al. 1991). The class-I *S* haplotypes are generally codominant, but some combinations of them exhibit complicated dominance relationships in the pistil and the pollen. In class II, a linear dominance relationship is observed in the pollen, while codominance is common in the pistil. Codominance is also generally observed in the pistil of heterozygotes of class-I and class-II *S* haplotypes, whereas class-I *S* haplotypes are dominant over class-II haplotypes in the pollen (Hatakeyama et al. 1998, 2001; Shiba et al. 2002; Kakizaki et al. 2003; Fig. 1).

In the present study, we investigated the number and frequency distribution of *S* haplotypes in *Brassica rapa* landraces (i.e., indigenous, miscellaneous cultivated types) using a newly developed *S* identification method. The landraces were obtained from the two distinct sources, the IPK Gene Bank (Gatersleben, Germany) and the Tohoku University Brassica Seed Bank (Sendai, Japan), which have been maintaining landraces using different population sizes. The genotyping data were used for assessment of the genetic diversity of *S* locus under complex dominance relationships in accessions of the genetic resources.

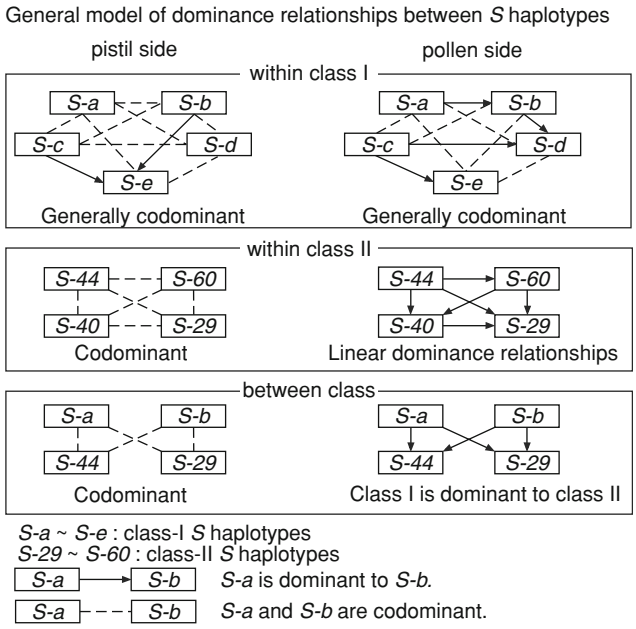

Fig. 1 Models of dominance relationships between *S* haplotypes in *B. rapa*. A model is based on experimental data on dominance relationships by Hatakeyama et al. (1998, 2001), Shiba et al. (2002) and Kakizaki et al. (2003)

Materials and methods

Plant materials and DNA extraction

Thirteen *S*-tester lines, i.e., *S* homozygotes in *Brassica rapa*, developed by Tohoku University (Nou et al. 1993; Nishio et al. 2006) and Kaneko Seed Co. LTD., were used (supplementary Table S1). Of these, the nucleotide sequences of eight *SP11* alleles of *BrS-25*, *-35*, *-53*, *-55*, *-56*, *-61*, *-62* and *-99* (supplementary Table S1) were determined according to Kimura et al. (2002) and were used for dot-blot genotyping of *S* haplotypes in *B. rapa* landraces.

Five *B. rapa* landraces obtained from the IPK Gene Bank were used for investigating genetic diversity of *S* haplotypes (Table 1). The ID symbols of the five landraces listed in Table 1 correspond to those in the previous study on inferring phylogenetic relationships among *B. rapa* landraces (Takuno et al. 2007). The landraces have been maintained as artificially isolated small populations after acquisition at the IPK Gene Bank (Table 1). For rejuvenation of landraces, about 50 individuals were grown under isolated conditions with bees for pollination. For harvesting seeds, all ripe siliques were collected as parental populations, from which 50 individuals were used for the next generation (Ulrike Lohwasser, personal communication). Genotyping of *S* haplotypes was carried out for more than 50 individuals per landrace as representatives of parental populations (Table 1). When using *S* genotypes of

Table 1 *B. rapa* landraces used and the number of *S* haplotypes

ID symbol	Source	ssp.	Origin	Acquisition year	Accession no.[a]	N[b]	No. of *S* haplotypes[c]
c_1^d	IPK	*chinensis*	China	1973	BRA462	64	9 (6, 3)
c_2^d	IPK	*chinensis*	Taiwan	1993	BRA1634	59	10 (8, 2)
o_2^d	IPK	*oleifera*	China	1991	BRA2813	54	7 (5, 2)
r_3^d	IPK	*rapa*	Sweden	1957	BRA329	51	8 (6, 2)
r_7^d	IPK	*rapa*	Tajikistan	1990	BRA2985	102	5 (2, 3)
c151-1978[e]	Tohoku University	*oleifera*	China	1978	c151	46	23 (20, 3)
c151-2008[e]				2008		48	14 (11, 3)
c475-1977[f]	Tohoku University	*rapa*	Japan	1977	c475	46	6 (3, 3)
c475-2006[f]				2006		45	6 (3, 3)

Unidentified *S* haplotypes are excluded

[a] Accession numbers in the IPK Gene Bank and the Tohoku University Brassica Seed Bank

[b] Number of individuals used

[c] Numbers in parentheses are the number of class-I and class-II *S* haplotypes, respectively

[d] Corresponding to Takuno et al. (2007)

[e] Original (c151-1978) and rejuvenated (c151-2008) population of the c151 landraces

[f] Original (c475-1977) and rejuvenated (c475-2006) population of the c475 landraces

randomly chosen sub-samples with $N = 50$, we obtained essentially the same results.

We also used two *B. rapa* landraces, maintained at the Tohoku University Brassica Seed Bank, in which about 20 individuals are usually used for rejuvenation of each accession (Table 1). The c151 (ssp. *oleifera*) was originally collected from China in 1978, while the c475 (ssp. *rapa*) was collected from Japan in 1977. Both landraces were once rejuvenated from the original population by random cross-pollination by hand in 2008 and 2006, respectively. We genotyped the *S* haplotypes in both the original (c151-1978, c475-1977) and the rejuvenated population (c151-2008, c475-2006) and compared the number and frequency distributions of *S* haplotypes.

Genomic DNA was prepared from young leaves by a modified CTAB method for all individuals (Escaravage et al. 1998).

Genotyping of class-I *S* haplotypes

Dot-blot analysis of *SP11* polymorphism was applied to identification of class-I *S* haplotypes with minor modifications (Fujimoto and Nishio 2003; Kitashiba et al. 2008). Data of 20 *SP11* alleles were obtained from the DDBJ database (e.g., Watanabe et al. 2000), and eight *SP11* sequences were newly determined. Allele-specific primer pairs 25 mer in length were designed for the 28 *SP11* alleles with ca. 40% GC content (supplementary Table S2), and multiplex PCR for synthesizing digoxigenin-labeled DNA fragments from each individual was performed using PCR DIG labeling mix (Roche Diagnostics, Switzerland), 14 mixed primer pairs (0.167 pmol/μl each) at most, and Ex Taq polymerase (Takara, Japan). Thermal cycler conditions were 28–30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 10 s. Two 25-mer allele-specific oligonucleotides with ca. 40% GC content for hybridization were designed for each *SP11* allele (supplementary Table S3) and were blotted onto a nylon membrane. Using the local BLASTN search, we confirmed that there were no sequences similar to those of the designed oligonucleotides in other *SP11* alleles, except for *BrS-32* and *BrS-36* (see below). Further procedures were carried out according to Fujimoto and Nishio (2003) and Kitashiba et al. (2008).

To confirm the results of *S* genotyping by dot-blot analysis, PCR-RFLP of *SLG* (Nishio et al. 1996) and Southern blot analysis of *SRK* and *SLG* (Sato et al. 2002) were carried out. PCR-RFLP was performed with primer pairs PS5—PS15 and HV-F—PS15 (supplementary Table S4). The thermal cycler conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. PCR products were digested by *Mbo*I. PCR with *S*-haplotype-specific primer pairs was also performed. The thermal cycler conditions were 28-30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 10 s. When PCR-RFLP and Southern blot analysis detected class-I *S* haplotypes with no dot-blot signal of class I, *SP11* alleles were cloned and sequenced according to Kimura et al. (2002).

BrS-32 and *BrS-36* were not distinguished from each other by dot-blot analysis because of the high sequence similarity of their *SP11* alleles. A PCR product was digested by *Alu*I, which cleaves *BrSP11-36* into two fragments, but does not cleave *BrSP11-32*. For identification of *BrS-22*, PCR-RFLP of *SLG* was carried out because *BrSP11-22* has not yet been cloned.

Genotyping of class-II *S* haplotypes

PCR analysis with SPII-F (as a forward primer) and four allele-specific primers (as reverse primers) for the class-II *SP11* alleles were performed for identification of class-II *S* haplotypes (supplementary Table S4). The thermal cycler conditions were 28–30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s. Although a previous extensive survey has revealed only four class-II *S* haplotypes in *B. rapa* (Sato et al. 2006), the existence of unknown class-II *S* haplotypes cannot be ruled out. Therefore, PCR products with SPII-F—40R (supplementary Table S4) were cloned and sequenced from randomly chosen individuals, at least five individuals per landrace.

Testing linkage of the newly identified *SP11* alleles to the *S* locus

We totally identified 18 putative *SP11* alleles in the present study. To test these alleles link to the *S* locus, linkage analysis was performed using F_2 populations, generated from heterozygotes of *S* haplotypes by bud pollination. The genotypes of *SLG* and *SP11* were determined by above methods for all the individuals of F_2 populations. The null hypothesis that *SLG* and *SP11* are inherited independently was rejected by *P* value calculated using χ^2 test.

Dominance relationships between class-I *S* haplotypes

For determining the dominance relationship between class-I *S* haplotypes in two landraces, i.e., c_2 and r_7 from the IPK, reciprocal crosses were performed between an *S* heterozygote and *S* homozygotes of the two *S* haplotypes. One day after pollination, pollen tubes were observed under UV fluorescence microscopy as described by Nakanishi and Hinata (1973). Three flowers were used for each crossing and the crossing experiment was repeated three times.

Results

Efficiency and reliability of the dot-blot method for identification of *B. rapa S* haplotypes

To evaluate genetic diversity of the self-incompatibility locus in *B. rapa* landraces, the dot-blot *S* genotyping method for class-I *S* haplotypes (Fujimoto and Nishio 2003) was improved. The data of nucleotide sequences of 28 class-I *SP11* alleles were collected, 20 of which were retrieved from the DDBJ database (e.g., Watanabe et al. 2000), and 8 of which (i.e., *BrS-25*, *-35*, *-53*, *-55*, *-56*, *-61*, *-62*, and *-99*) were newly cloned and sequenced from the *S*-tester lines (supplementary Table S1). *SP11*-allele-specific primers and oligonucleotides were designed from this data set (supplementary Tables S2, S3).

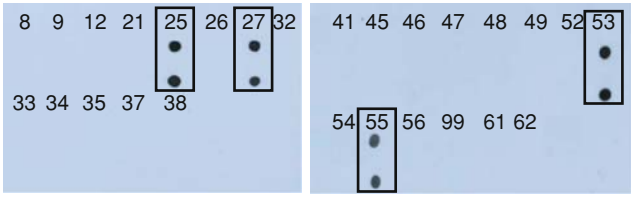

Fig. 2 Dot-blot analysis of *SP11* alleles. Genotypes of tested individuals were *BrS-25/BrS-27* and *BrS-53/BrS-55*. The *numbers* indicate the registered numbers of *S* haplotypes (i.e., *BrS-XX*)

This dot-blot method was highly reliable as an *S* genotyping technique. The results of dot-blot analysis using *BrS-25/BrS-27* and *BrS-53/BrS-55* heterozygotes are shown as examples. The specific signals corresponding to the respective *S* genotypes were clearly detected (Fig. 2) and specific signals for the other *S* haplotypes were also obtained. The results of *S* genotyping by dot-blot analysis were consistent with those by PCR-RFLP of *SLG* and Southern blot analysis of *SRK* and *SLG* (data not shown). The dot-blot method can also be applied to exploring an unidentified class-I *S* haplotype. Two and three *S*-tester lines from Tohoku University and Kaneko Seed Co. (supplementary Table S1) yielded no signal, indicating that they have unidentified class-I *S* haplotypes. The nucleotide sequences of *SP11* alleles of the five *S* haplotypes were sequenced and denoted as *BrS-63*, *-64*, *-70*, *-71*, and *-72* (supplementary Table S1). From above results, this method is expected to enable easier and more exact collection and management of *S*-tester lines, and to be applicable to other Brassicaceae species.

We cannot rule out the possibility that our newly identified *SP11* alleles will be located outside the *S* locus. Thus, we showed the three lines of evidence supporting that these *SP11* alleles link to the *S* locus. (a) Amino acid sequences of putative *SP11* alleles exhibited the typical features observed in previously identified *SP11* alleles (Watanabe et al. 2000; supplementary Fig. S1). (b) The results of the dot-blot *SP11* analysis and PCR-RFLP of *SLG* are completely consistent with each other (data not shown), indicating complete linkage disequilibrium between *SLG* and *SP11* at the population level. (c) Linkage analysis in the F_2 populations was performed for six newly identified *SP11* alleles. We found no recombinant and the null hypothesis that *SLG* and *SP11* are inherited independently was rejected by significant *P* values (supplementary Table S5).

Genotyping of *S* haplotypes in the *B. rapa* landraces from the IPK Gene Bank

Using the dot-blot method, we identified *S* haplotypes of more than 300 plants in the five *B. rapa* landraces provided

a

Pistil

Pollen (c2)	S-54	S-56	S-34	S-27	S-67	S-26	S-25	S-46	S-44	S-60
S-54	-						D		c	C[b]
S-56		-		C				D	c	c
S-34			-		C	C[a]	C	C[a]	C[a]	c
S-27		D		-			C	C	c	c
S-67			C		-				c	c
S-26			C[a]			-	C	C	C[a]	C[a]
S-25	C		C	C		C	-	C	C[a]	c
S-46		C	C[a]	C		C	C	-	C[a]	C[b]
S-44	r	r	R[a]	r	r	R[a]	R[a]	R[a]	-	c
S-60	r	r	r	r	r	R[a]	r	r	R[a]	-

b

Pistil

Pollen (r7)	S-66	S-65	S-60	S-40	S-29
S-66	-	C	c	c	c
S-65	R	-	c	c	c
S-60	r	r	-	c	c
S-40	r	r	R[a]	-	C[a]
S-29	r	r	R[a]	R[a]	-

Fig. 3 Dominance relationships between *S* haplotypes in pistil (*upper*) and pollen (*lower*) in c_2 (**a**) and in r_7 (**b**). The *underlined S* haplotypes are class II. *C* represents the codominance relationship between the *S* haplotypes listed *vertically* at the *left* and *horizontally* at the *top*. *D* and *R* indicate that the *S* haplotypes on the *left* are dominant or recessive to the ones at the *top*, respectively. The *gray-shaded boxes* indicate the dominance relationships determined in this study. The *white boxes* with *capital letters* indicate the dominance relationships determined by previous studies ([a]Hatakeyama et al. 1998, 2001; Shiba et al. 2002; Kakizaki et al. 2003; [b]Tetsu Sugimura unpublished results). The *white boxes* with *small letters* indicate that dominance relationships were assumed to be as shown in Fig. 1

by the IPK Gene Bank (Table 1). For genotyping of class-II *S* haplotypes, PCR-RFLP analysis with allele-specific primer pairs was performed. During this process, we found some individuals without any dot-blot signals, although PCR-RFLP and Southern blot analysis showed the band for class-I *S* haplotypes. Thus, we expected that such individuals would possess unidentified class-I *S* haplotypes and the five new *SP11* alleles, i.e., two alleles from the r_7 landrace and one each from the c_2, o_2, and r_3 landraces, were cloned and sequenced. They were designated as *BrSP11-65*, *66*, *67*, *68*, and *69*, respectively (supplementary Table S1). No novel class-II *S* haplotype was found.

Five to ten *S* haplotypes were found in each landrace (Table 1). All landraces had two or three class-II *S* haplotypes, whereas the numbers of class-I *S* haplotypes were variable among landraces (i.e., 2–8; Table 1). The numbers of *S* haplotypes were less than those in natural populations of Brassicaceae species (*e.g.*, Sampson 1967; Stevens and Kay 1989; Nou et al. 1993; Mable et al. 2003; Charlesworth et al. 2003; Glémin et al. 2005; Schierup et al. 2006), in which more than ten *S* haplotypes have been identified. Such a small number of *S* haplotypes could be due to the smaller population sizes ($N = 50$) than those of natural populations.

The number of *S* haplotypes influenced by their dominance relationships

For estimation of the expected number of *S* haplotypes within a population, information about dominance relationships between all combinations of *S* haplotypes is required. We investigated the dominance relationships among *S* haplotypes, which are important factors determining the number and frequency distribution of *S* haplotypes (Schierup et al. 1997, Vekemans et al. 1998). The c_2 landrace possessed the largest number of *S* haplotypes, and combinations of codominance between class-I *S* haplotypes both in the pistil and the pollen sides were frequent (Fig. 3a). On the other hand, the r_7 landrace possessed only five *S* haplotypes, including two class-I *S* haplotypes (*BrS-65* and *-66*). Since a dominance relationship on the pollen side was found (*BrS-66* > *BrS-65*), all the other combinations between class-I and class-II *S* haplotypes and between class-II *S* haplotypes are considered to have dominance relationships on the pollen side (Fig. 3b; Hatakeyama et al. 1998, 2001; Shiba et al. 2002; Kakizaki et al. 2003). In the other three landraces, codominance relationships were shown in at least one half of the class-I *S* haplotype combinations, though the number of examined combinations was small (data not shown).

The dominance relationships of *S* haplotypes in the r_7 landrace were consistent with the SSIdomcod model of Schierup et al. (1997), which presumes codominance in the pistil and linear dominance in the pollen (Fig. 3b). Their computer simulation showed 3.44 ± 0.57 *S* haplotypes under the equilibrium state of the SSIdomcod model with $N = 50$ (Schierup et al. 1997). The number of *S* haplotypes in r_7 is similar to this number. The large proportion of codominance was observed in the other landraces, and thus a greater number of *S* haplotypes in a population is expected (Schierup et al. 1997). Actually, the other landraces were found to possess more *S* haplotypes than r_7. Although the number of genotyped individuals exceeds 50 in r_7, no new *S* haplotype was found after genotyping 30 or 40 individuals.

Frequency distribution of *S* haplotypes at the IPK landraces

The frequency distribution of *S* haplotypes was investigated (Fig. 4). In general, the recessive effect is a major force for

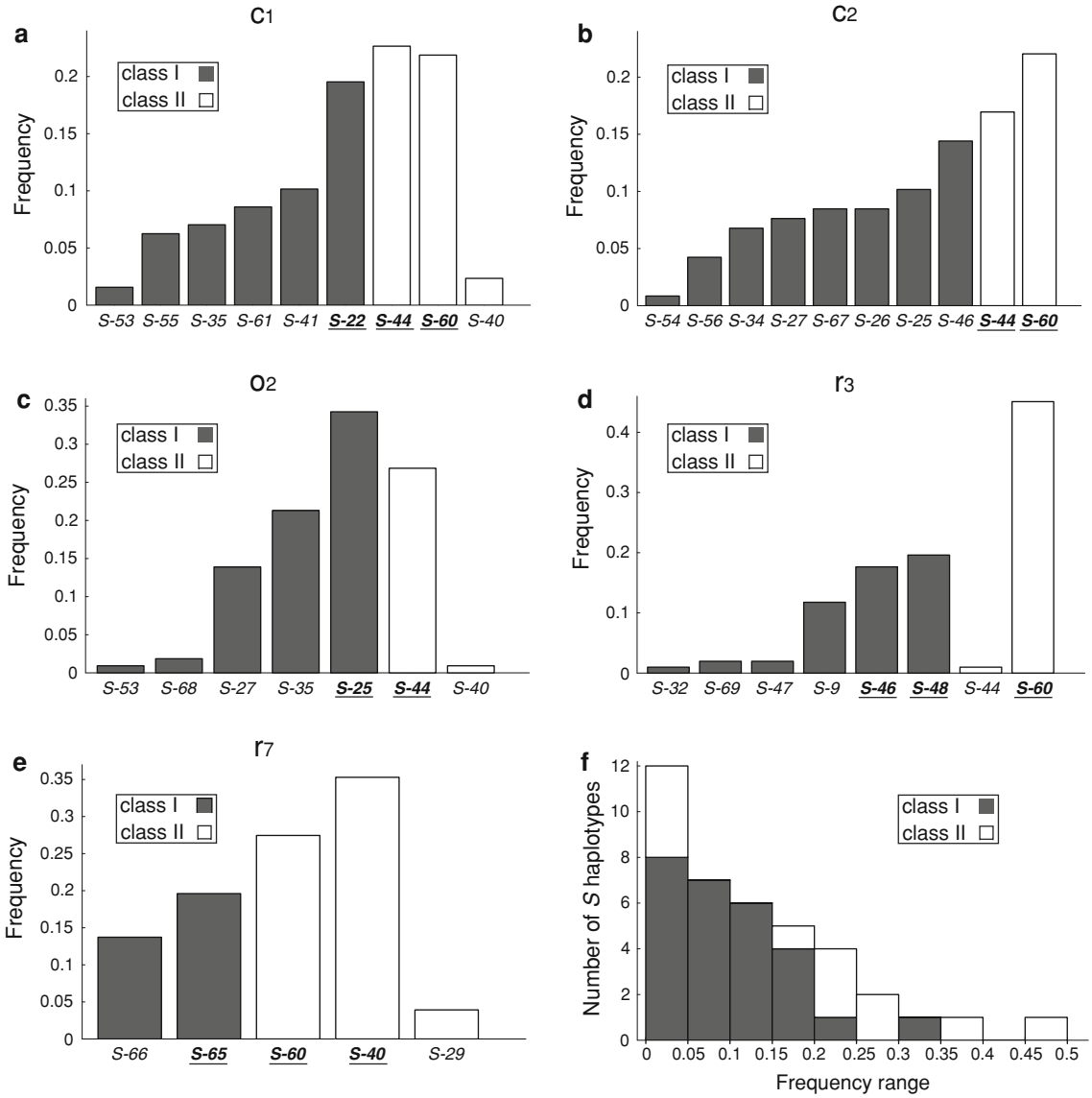

Fig. 4 Frequency distributions of *S* haplotypes in the five *B. rapa* landraces (**a**–**e**). The ID symbols of landraces are shown above the figures. The *black bars* and *white bars* represent class-I and class-II *S* haplotypes, respectively. The *underlined S* haplotypes indicate that the homozygotes of these *S* haplotypes were found. **f** Summary of frequency distribution within each landrace

shaping the frequency distribution of *S* haplotypes, which increases the frequencies of recessive *S* haplotypes (Bateman 1952; Sampson 1974). Contrary to theoretical expectations, however, in all the landraces except c_2, one class-II *S* haplotype with very low frequency was observed. Of these, in the three landraces c_1, o_2, and r_7, the most recessive *S* haplotypes within each landrace, *BrS-40* or *BrS-29*, showed low frequencies. Figure 4f summarizes the frequency distribution of *S* haplotypes in each landrace. The distribution of class-I frequencies is L-shaped, whereas that of the class-II frequencies is bimodal. Such low frequencies of recessive *S* haplotypes are unlike the case in natural populations of Brassicaceae species (Sampson 1967; Stevens and Kay 1989; Nou et al. 1993; Mable et al. 2003; Charlesworth et al. 2003; Glémin et al. 2005; Schierup et al. 2006). Such reduction of recessive *S* haplotypes may be caused by genetic drift.

Genetic variation of *S* haplotypes in the original and rejuvenated populations at the Tohoku University Brassica Seed Bank

The landraces c151 and c475 are maintained at the Tohoku University Brassica Seed Bank (Table 1). This gene bank has maintained an original cultivated population (c151-1978 and c475-1977) and a population rejuvenated once (c151-2008 and c475-2006) using 20 individuals (see "Materials and methods" for details). We performed *S* genotyping for

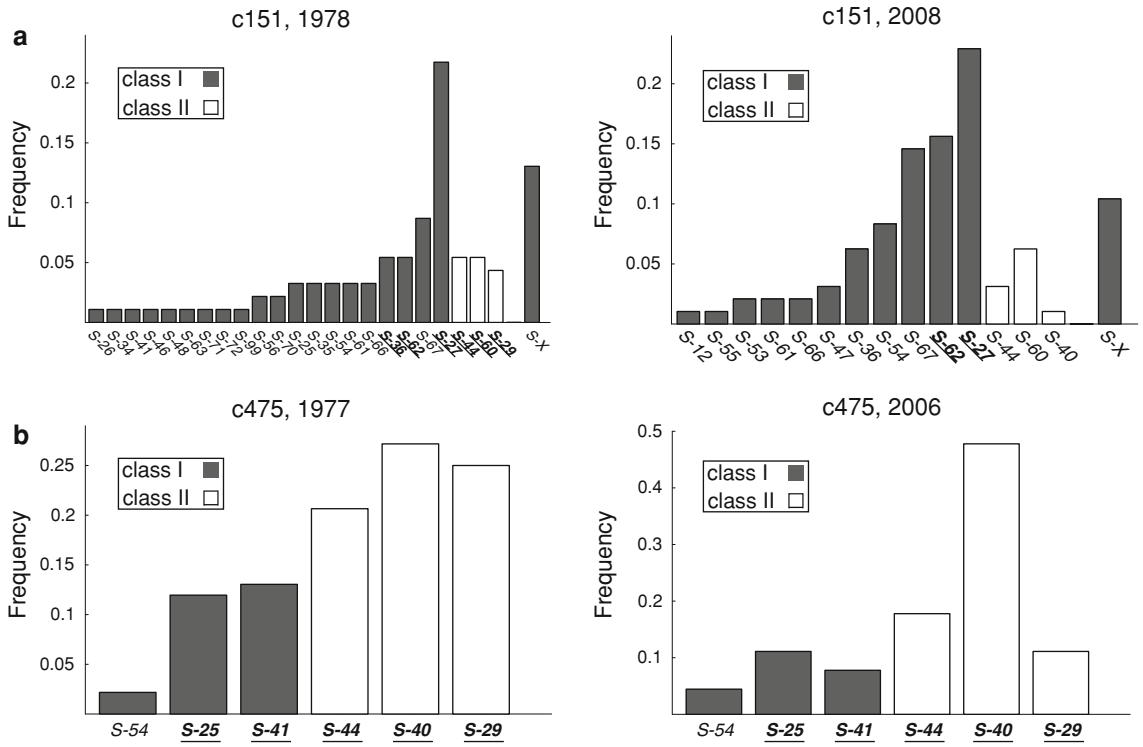

Fig. 5 Frequency distributions of *S* haplotypes in the original and regenerated population of **a** c151 and **b** c475 landraces in *B. rapa*. The *black bars* and *white bars* represent class-I and class-II *S* haplotypes, respectively. The *underlined S* haplotypes indicate that the homozygotes of these *S* haplotypes were found. *S-X* represents the unidentified class-I *S* haplotypes

original and rejuvenated populations to detect the effect of genetic drift throughout the rejuvenation process.

In the c151-1978, 23 *S* haplotypes were found, 20 of which were class I and 3 of which were class-II *S* haplotypes in about 50 individuals (Table 1). Some of the individuals possessed unidentified class-I *S* haplotypes. Frequency distributions of c151-1978 and c151-2008 are shown in Fig. 5a. Many class-I *S* haplotypes display very low frequency in c151-1978, nine of which were found only once. Such rare *S* haplotypes indicate that a greater number of cryptic *S* haplotypes may possibly be in the original population and that the original population size of c151 may be quite large. However, in c151-2008, we found only 11 and 3 *S* haplotypes of class I and class II, respectively. We directly observed a strong reduction of the number of *S* haplotypes, especially rare *S* haplotypes during rejuvenation. Frequencies of all the three class-II *S* haplotypes were found to be low in c151-1978, and further reduction of these *S* haplotypes was observed in c151-2008 (Fig. 5a).

On the other hand, we found six *S* haplotypes both in c475-1977 and c475-2006, of which three were class-I *S* haplotypes (Table 1). It is likely that the population size of c475-1997 may be lower than that of c151. Although we did not find the reduction of the number of *S* haplotypes, drastic change of frequency distribution of *S* haplotypes was observed during rejuvenation. The frequency of one of the class-II *S* haplotypes, *BrS-40*, approximately doubled and other two class-II *S* haplotypes showed very low frequencies (Fig. 5b). As a result, the c475 population lost a large amount of gene diversity. These results suggest that rejuvenation only once with a small population size reduces large amounts of genetic variation of *S* haplotypes, though the effect of genetic drift is usually weaker in the locus under balancing selection than in other neutral loci (Takahata 1990).

Testing the departure from the neutral expectation of frequency distribution of *S* haplotypes with dominance relationships

We tested the departure from the expected frequency distribution of *S* haplotypes employing the models of Uyenoyama (2000) and Billiard et al. (2007), introducing dominance relationships. The model of Uyenoyama (2000) assumes codominance between all *S* haplotypes in the pistil and dominance only between class I and class II in the pollen. Using this model, the bulked frequencies of class-I and class-II *S* haplotypes at an equilibrium state were calculated, conditional on the observed number of class-I and class-II *S* haplotypes (Table 1). The o_2 landrace showed a significant difference due to under-represented class-II *S*

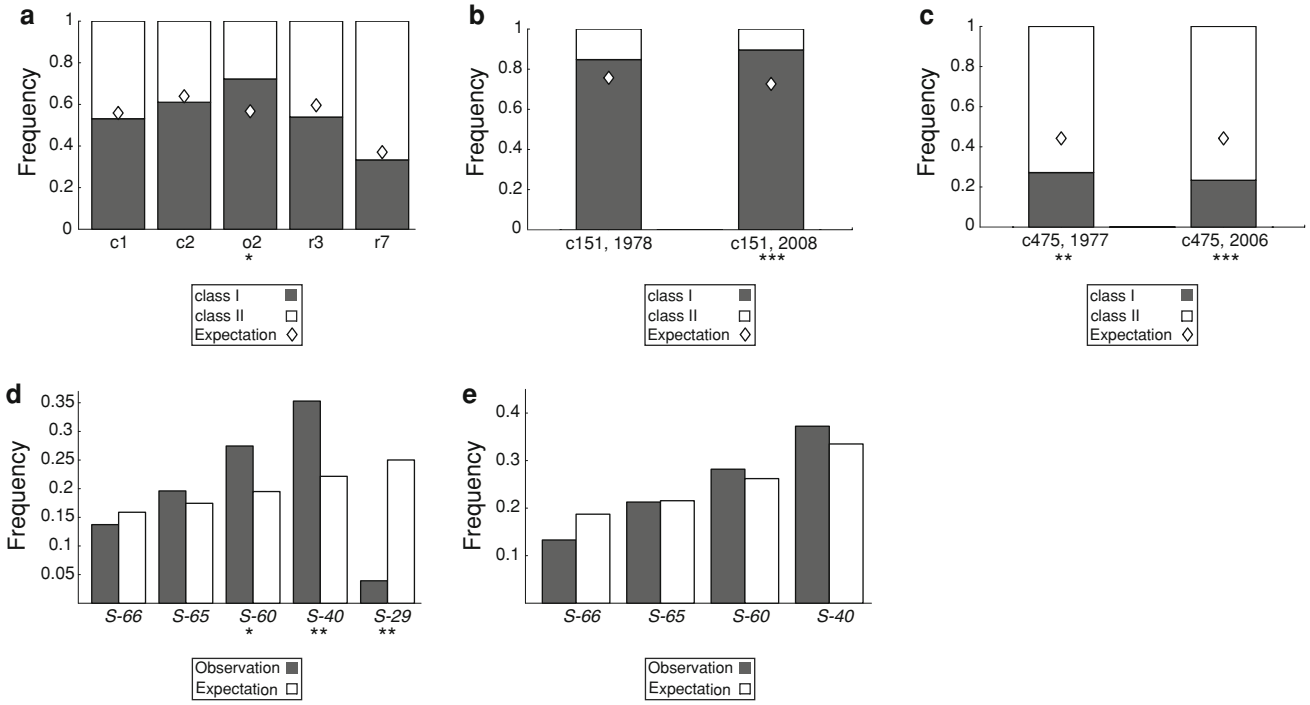

Fig. 6 Testing the difference between observed and expected *S*-haplotype frequencies. The *asterisks* indicate significant departures from expectation (*$P < 0.05$, **$P < 0.01$, ***$P < 10^{-3}$ with Bonferroni correction). **a**–**c** Comparison with expected frequencies by the model of Uyenoyama (2000). The *black bars* and *white bars* represent observed frequencies of class-I and class-II *S* haplotypes, respectively. The *white diamonds* indicate the expected frequencies of class-I *S* haplotypes. **a** Landraces from the IPK. **b** c151 from the Tohoku University Brassica Seed Bank. **c** c475 from the Tohoku University Brassica Seed Bank. **d**, **e** Observed frequencies of *S* haplotypes (*black bars*) and ones expected by the model of Billiard et al. (2007) (*white bars*) in r_7. **d** All individuals were used. **e** The individuals with *BrS-29* were excluded

haplotypes ($P < 0.05$, χ^2 test with Bonferroni correction; Fig. 6a). In the c151, the original population did not show a significant difference, but the regenerated population showed a strong significance due to the further reduction of class-II *S* haplotypes ($P < 10^{-3}$; Fig. 6b). On the other hand, in the c475, the original population (c475-1977) showed the low *P* value ($P < 0.01$) due to the excess of class-II *S* haplotypes. After rejuvenation, c475-2006 showed more severe *P* value ($P < 10^{-3}$) due to the excess of one class-II *S* haplotype (Fig. 6c). The c_1, r_3, and r_7 landraces, in which very low frequencies of one of the class-II *S* haplotypes were found, did not show significant differences. This is probably because the simplified model of Uyenoyama (2000) ignores dominance within each class.

Further detailed analysis on r_7 is shown using the model of Billiard et al. (2007), introducing within-class dominance relationships because dominance relationships between all pairs of *S* haplotypes were revealed only in r_7 (Fig. 3b). The observed frequency distribution of *S* haplotypes was significantly different from the expected one mainly due to the low frequency of *BrS-29* (Fig. 6d; $N = 102$, $P < 10^{-11}$, χ^2 test). On the other hand, when the frequency distribution of *S* haplotypes in r_7 was reanalyzed excluding the eight individuals with *BrS-29*, the fit between observation and expectation was very good (Fig. 6e; $N = 94$, $P > 0.5$, χ^2 test). Thus, the effect of genetic drift should more strongly work for the recessive class of *S* haplotypes than the dominant ones (Schierup et al. 1997).

Discussion

In the present study, we developed a method for *S* genotyping by dot-blot hybridization of PCR products with allele-specific oligonucleotide probes. This method was found to be useful in identifying 28 class-I *S* haplotypes in *B. rapa*. We carried out *S* genotyping of more than 50 individuals from one line of our seed stock and more than 300 individuals from landraces conserved in the IPK Gene Bank, and identified five new *S* haplotypes thanks to this distinct detection. PCR-RFLP analysis of *SLG* alleles has been commonly used for discrimination of *S* genotypes (Nishio et al. 1994, 1996; Sakamoto et al. 2000). However, this method is not useful for identification of *S* genotypes of *S* heterozygotes because of the presence of many *S* haplotypes in a species and complicated band patterns of the *S* heterozygotes. Furthermore, some *S* haplotypes lack the *SLG* gene (Sato et al. 2002) and some have sequence variation of *SLG* alleles (Kusaba et al. 2000). In those

cases, dot-blot analysis using *SP11* alleles enabled the distinct identification of *S* genotypes.

Fujimoto and Nishio (2003) have previously developed a dot-blot method for identification of *S* genotypes using polymorphisms of *SP11* alleles in *B. oleracea*, although the number of *S* haplotypes available was limited. In that method, PCR products from *S* homozygotes or cloned *SP11* sequences are required for dot-blotting on a membrane in advance. *S* homozygotes or *SP11* clones of various *S* haplotypes should be collected for this analysis and the preparation of probes becomes a limiting factor. In contrast, allele-specific primer pairs and allele-specific oligonucleotide probes that we designed in the present study are available for any researcher and breeder and allow them to identify *S* haplotypes easily. A multiplex primer set consisting of many allele-specific primer pairs, at least 14 pairs in *B. rapa*, was examined in PCR and showed satisfactory results in *S* genotyping by dot-blot analysis. This technique could contribute to saving time and labor for *S* genotyping. Since we have been cloning new *SP11* sequences from *B. rapa* and *B. oleracea* along with the accumulation of *S*-tester lines, the number of *S* haplotypes identified by this method will increase further.

The new dot-blot method was found to be applicable to the assessment of genetic diversity of the *S* locus in *B. rapa* landraces. For the landraces from the IPK Gene Bank, the results demonstrate that the numbers of possessed *S* haplotypes could be sufficient in a population with $N = 50$, but strong reduction of the frequency of one of the class-II *S* haplotypes occurred in four of the five landraces (Fig. 4), as opposed to the theoretical expectation (Bateman 1952; Sampson 1974). The frequency distributions at least in o_2 and r_7 showed statistically significant departure from neutral expectation (Fig. 6a, d). A similar result was obtained for the c151 landrace from the Tohoku University Brassica Seed Bank. In this landrace, frequencies of all the three class-II *S* haplotypes were reduced by one-time rejuvenation with $N = 20$ (Fig. 5a) and such a reduction could not happen only by chance (Fig. 6b). The c475 from the Tohoku University Brassica Seed Bank did not show the reduction of the number of *S* haplotypes during one-time rejuvenation, but gene diversity in the *S* locus was strongly decreased due to excess of class-II *S* haplotype, *BrS-40* (Figs. 5b, 6c).

The population sizes of the landraces at the IPK Gene Bank and Tohoku University Brassica Seed Bank are very small ($N = 50$ and $N = 20$), in which the effect of genetic drift is predicted to be very strong. Our observation may be consistent with this prediction and showed that the comprehensively recessive class of *S* haplotypes may be in an ongoing process of disappearance from the landraces as predicted by Schierup et al. (1997) and Uyenoyama (2000). A reduction of frequencies was observed in all the three class-II *S* haplotypes in the c151 and in the two class-II *S* haplotypes in the c475, whereas only in one of the class-II *S* haplotypes in the IPK landraces. The effect of genetic drift could be stronger in c151 and c475 than in the landraces from IPK because of the smaller population size as theoretically predicted (Schierup et al. 1997).

Using the dot-blot method, genetic variation of *S* haplotypes in the *B. rapa* landraces was assessed, and the results suggest that the effect of genetic drift is not ignorable in rejuvenation. The observed numbers of *S* haplotypes in the IPK Gene Bank seemed to be sufficient compared with the theoretical expectation (Schierup et al. 1997). However, analysis of frequency distribution suggests that genetic drift could accelerate the loss of the recessive *S* haplotypes. While the landraces from IPK have experienced rejuvenation multiple times with $N = 50$, c151 and c475 showed more significant reduction of all the three class-II *S* haplotypes by one-time rejuvenation with $N = 20$. Thus, the IPK's strategy of maintenance of allogamous genetic resources is considered to be more appropriate, although much larger population size is desirable. However, rejuvenation with a larger population size can only slow down the speed of loss of *S* haplotypes (Schierup et al. 1997). The rate of loss of alleles is lower at the locus under balancing selection, like *S* locus and major histocompatibility complex (MHC) locus, than that at neutral loci (Takahata 1990). In other words, the problematic effect of genetic drift on the neutral loci should be more severe, in which a large amount of useful genetic diversity can be lost. To overcome the undesirable effect of genetic drift, we should collect as many seeds as possible from a natural population or a landrace to avoid the founder effect, which enhances the effect of genetic drift by the strong reduction of population size, and limit the frequency of rejuvenation of the genetic resources to prevent the loss of genetic diversity. Therefore, the development of a method for long-term preservation of seeds is required, especially for allogamous plants.

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