Identification of species in tribe Brassiceae by dot-blot hybridization using species-specific ITS1 probes

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Identification of species in tribe *Brassiceae* by dot-blot hybridization using species-specific ITS1 probes

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Abstract

Simple, reliable methods for identification of species are required for management of many species and lines in a plant gene bank. Species-specific probes were designed from published sequences of the ITS1 region in rDNA of 16 species in *Brassica* and its related genera, and used as probes for dot-blot hybridization with plant genomic DNA. All the probes detected species-specific signals at dot blots of genomic DNAs of the sixteen species in *Brassica, Diplotaxis, Eruca*, and *Raphanus*. Signals of the *Brassica* digenomic species in the U's triangle, i.e., *B. napus, B. juncea*, and *B. carinata*, were detected by the probes of their parental monogenomic species, i.e., *B. rapa, B. nigra*, and *B. oleracea*. The probe for *B. oleracea* showed signals of *B. balearica, B. cretica, B. incana, B. insularis*, and *B. macrocarpa*, which have the C genome as *B. oleracea*. *Eruca vesicaria* DNA was detected by the probe for *E. sativa*, which has been classified as a subspecies of *E. vescaria*. DNA of leaf tissue extracted by an alkaline solution and seed DNA prepared by the NaI method can be used directly for dot-blotting. Misidentification of species was revealed in 20 accessions in the Tohoku University *Brassica* Seed Bank. These results indicate dot-blot hybridization to be a simple and efficient technique for identification of plant species in a gene bank.

Keywords: **species identification, ITS1, rDNA, dot-blot hybridization, *Brassiceae***

Introduction

Landraces of crops and wild species closely related to crop species are important genetic resources for crop improvement, and are therefore maintained at many gene banks in the world. In these gene banks, seeds of thousands of lines are stored, and they are propagated and rejuvenated once in about every ten years. Since many lines are handled at once, there is a risk of intermingling of lines. In species which have been seldom studied, misidentification of a species may also be a problem. Simple techniques for identification of lines or species are required for their maintenance without confusion.

In the tribe *Brassiceae*, there are many species closely related to *Brassica* crops. Identification of species is not easy even for a specialist of *Brassica* genetics. These species are generally identified by the morphology of seeds, flowers, and siliques, but some species are not easily distinguished from each other. For example, *Brassica juncea* is morphologically similar to *Brassica rapa*. Although ploidy can be estimated easily by flow cytometry, the most reliable method for identification of species in *Brassica* is counting the number of chromosomes. However, the chromosome count of *Brassiceae* species, which have relatively small chromosomes, requires skill and is laborious. An easier and less laborious technique is required for management of a large number of *Brassiceae* genetic resources.

Various methods for DNA polymorphism analysis can be applied to identification of plant species. Amplification of species-specific DNA fragments by polymerase chain reaction (PCR) using primers having species-specific nucleotide sequences is usable for this purpose, but success of DNA amplification by PCR depends on the purity of the template DNA, DNA polymerase, and even the PCR apparatus. Analyses of the simple sequence repeat (SSR) (Li et al. 2008) and the cleaved amplified polymorphic sequence (CAPS) (Song et al. 2001) can also be used for species identification. However, in these analyses, it is difficult to distinguish interspecific polymorphism from intraspecific polymorphism. DNA markers that have no polymorphism within species are required for this purpose.

rDNA has many copies in tandem in a plant genome, and the sequences of rDNA are conserved among plant species. On the other hand, the spacer sequences between rDNA sequences, i.e., ITS1 and ITS2, are variable between species. Southern-blot analysis of rDNA after digestion with a restriction endonuclease has been commonly used for identification of somatic hybrids (Cluster et al. 1996; Yamanaka et al. 1992). Although this method can be used for distinguishing plant species, estimation of DNA fragment sizes for identification of species by electrophoresis is not easy. Since each plant species has its specific nucleotide sequence in the ITS1 and ITS2 regions of rDNA, species-specific hybridization can be applied to identification of plant species. The ITS1 sequences have been used for identification of species of plant pathogens (Bailey et al. 2002; Lievens et al. 2006), and dot-blot hybridization with species-specific probes having spacer sequences between rDNA sequences has been used for identification of bacterial species (Park and Itoh 2005; Xiao et al. 2010). In the present study, we designed species-specific oligonucleotide probes for *Brassica* species and related genera in tribe *Brassiceae*, and used them in dot-blot hybridization. Contamination of different species in accessions in the *Brassica* Seed Bank of Tohoku University was investigated.

Materials and Methods

Plant materials

Fifteen species in genus *Brassica*, five species in *Diplotaxis*, two species in *Eruca*, two species in *Sinapis*, and one species in *Raphanus* maintained in the *Brassica* Seed Bank of Tohoku University, listed in Supplemental Table 1, were used as materials for examining a dot-blot method using species-specific probes

designed from published sequences of rDNA. The method developed in this study was applied to species identification in 115 accessions of *Brassica rapa*, 28 accessions in *Brassica oleracea*, 31 accessions in *Brassica nigra*, 37 accessions in *Brassica juncea*, 55 accessions in *Brassica napus*, and 17 accessions in *Brassica carinata*.

Preparation of genomic DNA

Genomic DNAs were prepared from 10 mg of leaf tissues by the method of Edwards et al. (1991) and from seeds by the NaI method (Sakamoto et al. 2000). The genomic DNA, 1 - 40 ng/µl, was mixed with an equal volume of alkaline denaturation solution (5 N NaOH, 0.5 M EDTA). To save labor in DNA preparation, a 1-cm^2 piece of a young, freshly-expanded leaf was macerated in 50 µl alkaline denaturation solution, and the suspension was used for dot-blotting without centrifugation.

Preparation of oligonucleotide probes

Comparing ca. 100 – 150 bp sequences of the internal transcribed spacer 1 (ITS1) region in rDNA sequences (Fig. 1), published by the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), between different plant species, we selected 17 – 25 bp sequences for preparation of species-specific oligonucleotide probes (Table 1). The species-specific oliginucleotide probe was prepared as a bridge probe for indirect hybridization with a digoxygenin-labeled probe according to Shiokai et al. (2010). The bridge probe was composed of a species-specific sequence, a 6-bp spacer sequence of TATATT, and a sequence (5'-TACATTCGCAATTGAGGCTTCGT-3') complementary to the sequence of a digoxygenin-labeled oligonucleotide probe (5'-TATATTTACATTCGCAATTGAGGCTTCGT-3').

Dot-blot hybridization

Genomic DNA in the alkaline solution, 0.3 – 0.5 µl, was dot-blotted on a membrane (Hybond-N, GE Healthcare UK), using a Multi-pin blotter (Atto, Japan), and hybridized with the

Fig. 1. A region used for designing the species-specific probes. The probe sequences were taken from a region of 100 – 150 bp from the 5' end of the ITS1 sequences of rDNA.

species-specific oligonucleotide probe and the digoxygenin-labeled oligonucleotide probe for more than four hours at the temperature shown in Table 1. After hybridization, the membrane was washed by 2 x SSC with 0.1% SDS at room temperature for 5 min. twice, and again washed under the same temperature for hybridization with the solution listed in Table 1. The hybridized digoxygenin-labeled probe was detected by an anti-digoxygenin IgG Fab fragment conjugated with alkaline phosphatase (Roche, Germany) followed by chemiluminescent reaction of CSPD (Roche). Chemiluminescence was exposed to X-ray film (Fuji, Japan).

PCR-RFLP analysis

To confirm species identified by dot-blot analysis of the rDNA ITS1 sequence, PCR-RFLP analysis of *SLR1*, sequences of which have been determined in many species in tribe *Brassiceae* (Inaba and Nishio 2002), was carried out. Genomic DNA of *SLR1* was (5'-ATGAGAGGTGTAATACCAAAC-3') and PS25 (5'-GAGATAAAGATCTTGACCTC-3') (Inaba and Nishio 2002) under 30 cycles of 1 min. at 94˚C, 2 min. at 55˚C, and 3 min. at 72˚C, followed by final extension at 72˚C for 5 min. PCR products were digested with *Mbo*I, and separated by electrophoresis in a 5% polyacrylamide gel.

Observation of root-tip chromosomes

amplified by PCR using primers PS24 Chromosomes in root-tip cells were observed according to Fukui et al. (1986). Root tips were treated in 0.05% colchicine for 1 hour, and fixed in

Table 1 Sequences of ITS1 oligonucleotide probes and conditions for hybridization and washing

Species name	Accession number of the rDNA sequence	Probe name	Probe sequence (5'-3')	Hybridization and washing temperature (˚C)	Second washing buffer
Brassica rapa	AF531563	BrITS1	TTGGCCAAGACTTCAGTTTTG	60	1×SSC/0.1%SDS
Brassica nigra	GQ268059	BniITS1	TACTTAGGTCTCGGTCGG	50	1×SSC/0.1%SDS
Brassica oleraca	GQ891879	BoITS1	CGTCACCGGCCCAGTTTCGG	50	0.5×SSC/0.1%SDS
Brassica barrelieri	GQ268071	BbrITS1	TCTCGGTTGGGTCATGCAC	50	1×SSC/0.1%SDS
Brassica elongata	GQ268078	BeITS1	GTGTTTTTGCGCGGAAG	50	1×SSC/0.1%SDS
Brassica oxyrrhina	AY722424	BoxITS1	GTGTTTCGTCCTTGGTC	45	1×SSC/0.1%SDS
Brassica villosa	GQ268064	BvITS1	GCTAAGTTTCGGTTAGATTATACG	45	1×SSC/0.1%SDS
Diplotaxis assurgens	Ay722443	DasITS1	ATGACTTCGGTCTTGAA	45	1×SSC/0.1%SDS
Diplotaxis catholica	AY722446	DcITS1	GAAGTCTTGAGATGGTC	35	1×SSC/0.1%SDS
Diplotaxis muralis	DQ983972	DmITS1	TCCTCAGCCAAGTTTATCTTGG	50	1×SSC/0.1%SDS
Diplotaxis siifolia	AY722453	DsfITS1	GTTAGCTTCCGGATTTCCGT	50	1×SSC/0.1%SDS
Diplotaxis siettiana	AY722451	DstITS1	TATTGTCTCGGTCAGGA	50	1×SSC/0.1%SDS
Eruca sativa	DQ249821	EsITS1	TTGTGCGTTCCGTCCCCGGCCAAGA	60	1×SSC/0.1%SDS
Sinapis alba	AY722486	SalbITS1	AAGTTCCCAGCCAGTAC	50	1×SSC/0.1%SDS
Sinapis arvensis	AY722487	SarvITS1	CTTCTGTCTCGGTCGGA	50	1×SSC/0.1%SDS
Raphanus sativus	AY722480	RsITS1	GGTCAAGGCTGGGTCGT	60	1×SSC/0.1%SDS

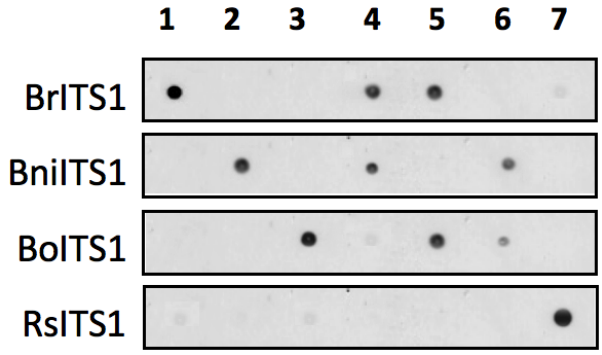

Fig. 2. Species-specific signal detection by dot-blot hybridization using the ITS1 probes.
Species names of genomic DNA dot-blotted on a membrane are 1: *B. rapa*, 2: *B. nigra*, 3: *B. oleracea*, 4: *B. juncea*, 5. *B. napus*, 6: *B. carinata*, and 7: *R. sativus*.

ethanol/acetic acid (3:1) for 1 hour. They were washed for 5 min. by 50%, 25%, 15%, 8% ethanol sequencially, and finally by water twice, and incubated in enzyme solution (4% Cellulase Onozuka RS, 1% Pectolyase, 75 mM KCl, 7.5 mM Na_2EDTA, pH 4.0). Chromosomes were stained for 30 min. by Giemza Stain Solution (079-04391, Wako, Japan) diluted to 4% with 1/15 M phosphate buffer, pH 6.8, and observed under a microscope.

Results

Development of a dot-blot hybridization method for identification of *Brassiceae* species

Genomic DNA of six species of the A, B, C, AB, AC, and BC genomes in the genus *Brassica*, i.e., *B. rapa, B. nigra, B. oleracea, B. juncea, B. napus,* and *B. carinata*, and *Raphanus sativus* extracted from leaves, were hybridized with species-specific probes for *B. rapa, B. nigra, B. oleracea*, and *R. sativus*. In all the species, species-specific signals were detected (Fig. 2). The digenomic species, i.e., *B. juncea, B. napus*, and *B. carinata*, showed signals for their constitutive monogenomic species, but with lower intensities.

Since non-specific signals were sometimes detected, the optimum concentration of genomic DNA for species-specific signal detection was investigated. The probes for *B. rapa* and *R. sativus* detected species-specific signals in dot-blots of 0.5 ng/µl genomic DNA after mixing with the alkaline solution, but detected non-specific signals in 10 ng/µl genomic DNA (Table 2). Signals of the A genome in *B. juncea* and *B. napus* were detected in 2 ng/µl genomic DNA. Non-specific signals were not detected in these seven species by the *B. nigra* probe. The *B. oleracea* probe detected C-genome signals in 2 ng/µl genomic DNA of *B. oleracea* and *B. napus* and in 5 ng/µl genomic DNA of *B. carinata*, but detected non-specific signals in 10 ng/µl genomic DNA of *B. rapa* and *R. sativus*. Although signal intensities were different depending on the probes, 5 ng/µl genomic DNA was found to be optimum for species-specific signal detection.

Sixteen species-specific ITS1 probes were used for analysis of 25 species in the tribe *Brassiceae*, i.e., 15 species in *Brassica*, five species in *Diplotaxis*, two species in *Eruca*, two species in *Sinapis,* and one species in *R. sativus*. Genomic DNAs of *B. barrelieri, B.*

Table 2 Intensities of species-specific signals detected in dot-blots of different DNA concentrations

Species name	DNA concentration (ng/µl)					
	20	10	5	2	1	0.5
BrITS1 probe						
B. rapa	++*	++	++	+	±	±
B. nigra	−	−	−	−	−	−
B. oleracea	−	−	−	−	−	−
B. juncea	++	++	+	±	−	−
B. napus	++	+	+	±	−	−
B. carinata	−	−	−	−	−	−
R. sativus	+	+	−	−	−	−
BniITS1 probe						
B. rapa	−	−	−	−	−	−
B. nigra	+	±	±	−	−	−
B. oleracea	−	−	−	−	−	−
B. juncea	++	+	±	−	−	−
B. napus	−	−	−	−	−	−
B. carinata	+	±	±	−	−	−
R. sativus	−	−	−	−	−	−
BoITS1 probe						
B. rapa	±	±	−	−	−	−
B. nigra	±	−	−	−	−	−
B. oleracea	++	++	+	±	−	−
B. juncea	±		−	−	−	−
B. napus	++	+	+	±	−	−
B. carinata	+	+	±	−	−	−
R. sativus	±	±	−	−	−	−
RsITS1 probe						
B. rapa	±	−	−	−	−	−
B. nigra	±	−	−	−	−	−
B. oleracea	±	−	−	−	−	−
B. juncea	±	±	−	−	−	−
B. napus	±	±	−	−	−	−
B. carinata	±	±	−	−	−	−
R. sativus	++	++	++	+	+	+

*: Signal peak area more than 1/2 (++), more than 1/4 (+), more than 1/10 (±), and less than 1/10 (-) of the maximum peak area obtained in this analysis.

elongata, B. oxyrrhina, B. villosa, D. assurgens, D. catholica, D. muralis, D. siifolia, D. siettiana, S. alba, *S. arvensis* and *R. sativus* were detected specifically by the species-specific ITS1 probes. However, the *B. oleracea*-specific probe detected positive signals in *B. balearica, B. cretica, B. incana, B. insularis*, and *B. macrocarpa* (Table 3). Both *E. sativa* and *E. vesicaria* showed positive signals by the *E. sativa* probe.

DNA preparation methods for species identification using dot-blot analysis

DNA preparation from mature leaves by the method of Edwards et al. (1991) is a reliable method for DNA isolation from *Brassica* species, but it requires considerable labor and time. Testing various methods, we found that DNA solution extracted with alkaline solution (5 N NaOH, 0.5 M EDTA) can be used directly for dot-blot analysis. Intensities of signals detected in the dot-blots of the DNA extracted with the alkaline solution were comparable to those prepared by the method of Edward et al. (1991). With alkaline-extraction, a slurry of leaf tissues without centrifugation can also be used with successful results. In this extraction method, the ratio of weights of leaf tissues and volume of alkaline solution was important due to the absence of ethanol

Table 3 Detection of species-specific signals in 25 species in *Brassiceae* by ITS1 dot-blot hybridization

Species name	Probe Name															
	BrITS1	BniITS1	BoITS1	BbITS1	BeITS1	BoxITS1	BvITS1	DasITS1	DcITS1	DmITS1	DsfITS1	DstITS1	EsITS1	SaITS1	SarITS1	RsITS1
B. rapa	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. nigra	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. oleracea	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−
B. juncea	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. napus	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. carinata	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. balearica	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. cretica	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. incana	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−
B. insularis	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. macrocarpa	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−
B. barrelieri	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
B. elongata	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
B. oxyrrhina	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
B. villosa	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
D. assurgens	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
D. catholica	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
D. muralis	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
D. siifolia	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
D. siettiana	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
E. sativa	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−	−
E. vesicaria	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
S. alba	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−
S. arvensis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
R. sativus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

precipitation for concentrating DNA. For the 50 µl alkaline solution, a 1 cm^2 piece of leaf was required.

Brassiceae germplasm is usually stored as seeds, and some seeds have tough dormancy. Use of seeds as materials of DNA is desirable for saving labor in the growth of plants. The NaI method has been found to be suitable for extraction of DNA from *Brassica* seeds (Sakamoto et al. 2000). DNA extracted from a single seed by the NaI method, concentrations of which were 2.5 - 5 ng in 1 µl solution in *Brassica* and 5 – 7.5 ng in *Raphanus*, was not sufficient for species-specific signal detection of the ITS1 sequences by dot-blot hybridization in *Brassica* species, while it was sufficient in *Raphanus* and *Eruca*, which have seeds larger than 2 mm in diameter. Two seeds were required in *Brassica* species, and four seeds were required in *Diplotaxis*. A slurry of seeds in alkaline solution was tested, but clear species-specific signals were not detected.

Use of ITS1 dot-blot hybridization in the screening of misidentified species

The dot-blot hybridization of species-specific ITS1 probes was used for screening of misidentified species in accessions of the Tohoku University *Brassica* Seed Bank. All the accessions in the six species in the U's triangle, i.e., *B. rapa, B. nigra, B. oleracea, B. juncea, B. napus*, and *B. carinata*, were tested using the ITS1 probes for *B. rapa, B. nigra*, and *B. oleracea*. Species-specific signals for different species were detected in 11 among 115 *B. rapa* accessions, two among 31 *B. nigra* accessions, one among 28 *B. oleracea* accessions, one among 37 *B. juncea* accessions, and five among 55 *B. napus* accessions (Table 4). C-463 and C-467 in the *B. rapa* accessions were identified as *B. napus* by dot-blot hybridization, and the other nine lines were shown to be *B. juncea*. Ni-135 and Ni-150 in *B. nigra* accessions were identified as *B. juncea* and *B. rapa*, respectively. O-142 in *B. oleracea* accessions and J-434 in *B. juncea* accessions were revealed to be *B. napus* and *B. rapa*, respectively. N-103 in *B. napus* accessions was shown to be *B. rapa*, and the other four lines were identified as *B. juncea*.

DNAs of the accessions, in which signals of different species were detected, were analyzed by PCR-RFLP using the primers for *SLR1*. Band patterns of *B. rapa* and *B. oleracea* were similar to each other and that of *B. napus* was not distinguished from that of *B. oleracea* by PCR-RFLP. *B. nigra* showed a clearly different band pattern from those of *B. rapa* and *B. oleracea*, but showed the same band pattern as *B. juncea*. The B genome-specific band pattern was detected in all the accessions that showed the B genome-specific signals in dot-blot analysis. Chromosome numbers of C-451, C-638, C-639, C-640, C-641, C-643, C-644, C-645, and C-650 in *B. rapa* accessions were 36. J-474 in *B. juncea* accessions and N-103 in *B. napus* accessions were found to be monogenomic species, and Ni-135 in *B. nigra* accessions and O-142 in *B. oleracea* accessions were found to be digenomic species. These results suggest that the accessions showing signals of different species in dot-blot analysis are misidentified species.

Discussion

Dot-blot hybridization using oligonucleotides having sequences of the ITS1 region of rDNA was found to be usable for identification

Table 4 List of misidentified species revealed by ITS1 dot-blot hybridization

Species name (Number of accessions tested)	Chromosome number (2n=)	Number of misidentified accessions	Code number of misidentification	Techniques for species identification		
				ITS1 dot-blot hybridization	PCR-RFLP	Chromosome number (2n=)
B. rapa (115)	20	11	C-451	*B. juncea*	*B. nigra or B. juncea*	36
			C-463	*B. napus*	*B. oleracea or B. napus*	38
			C-467	*B. napus*	*B. oleracea or B. napus*	38
			C-638	*B. juncea*	*B. nigra or B. juncea*	36
			C-639	*B. juncea*	*B. nigra or B. juncea*	36
			C-640	*B. juncea*	*B. nigra or B. juncea*	36
			C-641	*B. juncea*	*B. nigra or B. juncea*	36
			C-643	*B. juncea*	*B. nigra or B. juncea*	36
			C-644	*B. juncea*	*B. nigra or B. juncea*	36
			C-645	*B. juncea*	*B. nigra or B. juncea*	36
			C-650	*B. juncea*	*B. nigra or B. juncea*	36
B. nigra (31)	16	2	Ni-135	*B. juncea*	*B. nigra or B. juncea*	36
			Ni-150	*B. rapa*	*B. rapa*	20
B. oleracea (28)	18	1	O-142	*B. napus*	*B. oleracea or B. napus*	38
B. juncea (37)	36	1	J-474	*B. rapa*	*B. rapa*	20
B. napus (55)	38	5	N-103	*B. rapa*	*B. rapa*	20
			N-107	*B. juncea*	*B. nigra or B. juncea*	36
			N-265	*B. juncea*	*B. nigra or B. juncea*	36
			N-473	*B. juncea*	*B. nigra or B. juncea*	36
			N-474	*B. juncea*	*B. nigra or B. juncea*	36

of *Brassiceae* species. Sixteen species-specific probes were designed, and they detected species-specific signals in the 16 species tested. The *B. oleracea*-specific probe detected signals in *B. balearica, B. cretica, B. incana, B. insularis*, and *B. macrocarpa*, which have been considered to have the C genome as in the case of *B. oleracea*. Between *B. oleracea, B. cretica, B. insularis*, and *B. macrocarpa*, nucleotide sequence identities of the ITS1 region are from 93% to 99%. The probe for *Eruca sativa* detected DNA of *Eruca vesicaria*. *E. sativa* has been classified as a subspecies of *E. vesicaria*. (Gomez-Campo 1999), and nucleotide sequence identity in the ITS1 region between *E. sativa* and *E. vesicaria* is 97%. DNA sequences having such a high similarity can be detected by hybridization, indicating that differences of subspecies level cannot be distinguished by ITS1 dot-blot hybridization without strict control of hybridization conditions. However, the inability to distinguish different subspecies, in which gene exchange may occur, is considered to be an important factor of DNA markers for species identification. Such markers are better than the markers that detect intraspecific variation, e.g., SSR markers. These results suggest that the oligonucleotide probes produced in this study are suitable markers for identification of the 25 species used here.

Contamination of different species in accessions of the Tohoku University Brassica Seed Bank was revealed by dot-blot hybridization with the species-specific oligonucleotides. Repeated analysis using different seeds again yielded the same results, indicating that these accessions are not mixtures of seeds of different species, but uniform seeds of different species that were misidentified. Misidentification of species in these accessions was confirmed by detection of species-specific bands using PCR-RFLP analysis of *SLR1* and by chromosome count. Dot-blot hybridization using oligonucleotides of the ITS1 region was more reliable than the PCR-RFLP analysis of *SLR1* because some digenomic species were not distinguished from one of parental monogenomic species by PCR-RFLP of *SLR1*. In PCR-RFLP analysis of *S* heterozygotes using *SLG*-specific primers, preferential amplification of one of the *SLG* alleles has been reported (Brace et al. 1993). Preferential amplification of one of the *SLR1* sequences in digenomic species is considered to be the cause of the inability of PCR-RFLP to distinguish digenomic species from monogenomic species. It can be inferred that such preferential amplification is not limited to the *SLR1* gene when using a common primer pair for amplification because there are sequence variations in orthologous genes of different species in *Brassiceae*.

The most notable characteristic of dot-blot analysis using the ITS1 oligonucleotide probes is its suitability for analysis of a large number of samples. Without PCR, DNA simply extracted from a leaf by alkaline solution can be analyzed. DNA prepared from seeds can also be used for dot-blot analysis. Although procedures for hybridization, washing, and signal detection take time, a large number of samples can be analyzed at the same time. Accessions in the Tohoku University Brassica Seed Bank were successfully examined, and misidentification of species was revealed.

Since plant species with published ITS1 sequences are limited, determination of sequences in other species is required. Other genes with multiple copies per cell, such as chloroplast genes and mitochondrial genes, can also be used as species-specific probes. Sequences of *RbcL* in many species have been published and used for phylogenetic analysis (Chase et al. 1993; Davis et al. 2004). However, DNA polymorphism in *RbcL* between different species in *Brassica* is much lower than that in the ITS1 region of rDNA, suggesting difficulty of using the *RbcL* sequences for species-specific markers in *Brassica*. There are not so many published sequence data in other genes as there are in *RbcL* or the ITS1 sequences.

This method of dot-blot analysis using the ITS1 oligonucleotide probes is considered to be useful not only for management of *Brassiceae* germplasm but also for testing hybridity of plants obtained by interspecific crossing. In intergeneric crossing between *B. rapa* and *R. sativus*, many progeny similar to the maternal plants are obtained. We are successfully using this method for identification of intergeneric hybrids among plants obtained by the intergeneric crossing (Tonosaki et al. unpub.). A

concern is that a gene flow of transgenes from genetically engineered cultivars of *B. napus* to *Brassica* weeds, e.g., *B. juncea* and *B. rapa,* may occur (Song et al. 2010). Rapid identification of species names of *Brassica* plants having the transgene is required to evaluate the possibility of gene flow. This method may also be useful for such a purpose.

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Supplemental Data

Supplemental Table 1. List of accessions used in this study

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Supplemental Table 1 List of accessions used in this study

Species name	Code number in seed bank	Cultivar name
Brassica rapa	C-101	-
Brassica nigra	Ni-109	-
Brassica oleraca	O-121	-
Brassica juncea	J-103	-
Brassica napus	N-102	-
Brassica carinata	Ca-104	-
Brassica balearica	O-302	-
Brassica cretica	Cr-1	-
Brassica incana	Inc-1	-
Brassica insularis	Ins-1	-
Brassica macrocarpa	O-503	-
Brassica barrelieri	Ba-109	-
Brassica elongata	El-102	-
Brassica oxyrrhina	Ox-101	-
Brassica villosa	Vill-523	-
Diplotaxis assurgens	DIP-ASS-2	-
Diplotaxis catholica	DIP-CAT-1	-
Diplotaxis muralis	DIP-MUR-3	-
Diplotaxis siifolia	DIP-SII-2	-
Diplotaxis siettiana	DIP-SIE-1	-
Eruca sativa	ERU-SAT-1	-
Eruca vesicaria	ERU-VES-1	-
Sinapis alba	SIN-ALB-9	-
Sinapis arvensis	SIN-ARV-6	-
Raphanus sativus	-	Shogoin