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Occurrence of metaxenia and false hybrids in *Brassica juncea* L. cv. Kikarashina × *B. napus*

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Imported genetically modified (GM) canola (*Brassica napus*) is approved by Japanese law. Some GM canola varieties have been found around importation sites, and there is public concern that these may have any harmful effects on related species such as reduction of wild relatives. Because *B. juncea* is distributed throughout Japan and is known to be high crossability with *B. napus*, it is assumed to be a recipient of *B. napus*. However, there are few reports for introgression of cross-combination in *B. juncea* × *B. napus*. To assess crossability, we artificially pollinated *B. juncea* with *B. napus*. After harvesting a large number of progeny seeds, we observed false hybrids and metaxenia of seed coats. Seed coat color was classified into four categories and false hybrids were confirmed by morphological characteristics and random amplified polymorphic DNA (RAPD) markers. Furthermore, the occurrence of false hybrids was affected by varietal differences in *B. napus*, whereas that of metaxenia was related to hybridity. Therefore, we suggest that metaxenia can be used as a marker for hybrid identification in *B. juncea* L. cv. Kikarashina × *B. napus*. Our results suggest that hybrid productivity in *B. juncea* × *B. napus* should not be evaluated by only seed productivity, crossability ought to be assessed the detection of true hybrids.

Key Words: *Brassica juncea*, *B. napus*, GM canola, gene flow, metaxenia, false hybrid.

Introduction

Cultivated *Brassica* consists of six species (U 1935) of which *B. napus* (2n = 38, AACC) and *B. rapa* (2n = 20, AA) are cultivated for oil and vegetables throughout the world. In 2009, of the 30 million hectares of cultivated canola, genetically modified (GM) canola was estimated to cover 6.4 million hectares. In Canada by 2009, approximately 93% of the total cultivated area of canola had been replaced by GM canola (James 2009).

Japan imports large quantities of rapeseed, approximately 2.3 million tons in 2010 (Japan Ministry of Finance 2011), including non-GM and GM canola from Canada and several other countries. Imported and commercialized GM canola has completed all regulations imposed by the Japanese government, such as their influence on biodiversity and food and feed safety. Several GM canola plants have been found along the roads or in vacant areas near the road because a few seeds may have been spilled along the road during transportation from the port to the oil industries (Nishizawa *et al.* 2009). All authorized GM canola lines belong to *B. napus*; however, some relative species, such as *B. rapa*, *B. juncea* (2n = 36, AABB), *B. nigra* (2n = 16, BB), *B. oleracea* (2n = 18, CC) and *R. sativus* (2n = 18, RR) are escaped plants from cultivated crops and *Raphanus raphanistrum* (2n = 18, RrRr) is wild species in Japan (Levy 1995, Matsuo 1989, Shimizu 2003, Shimizu *et al*. 2003). Because several species may cross with GM canola and produce hybrids, there is public concern about the possible harmful impacts on biodiversity such as reduction of wild relatives and/or dominance of GM canola in Japan.

No or very few seeds were obtained when *R. raphanistrum* and *B. napus* were crossed by open or artificial pollination (Chèvre *et al*. 2007, Halfhill *et al*. 2004, Lefol *et al*. 1997, Warwick *et al*. 2003). Furthermore, Takeshita *et al*. (1980) were unable to obtain progeny seeds by artificial pollination between *R. sativus* and *B. napus*, and Bing *et al*. (1996) could not obtain progeny seeds by open pollination between *B. nigra* and *B. napus*.

Hybrids between *B. napus* × *B. oleracea* and *B. napus* × *B. nigra* are especially difficult to obtain by artificial pollination (Scheffler and Dale 1994). In addition, the distribution regions of *B. nigra*, *R. raphanistrum* and *R. sativus* are limited compared to those of other related species in

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Japan (Shimizu 2003). From these reports, it appeared that *Raphanus*, *B. nigra* and *B. oleracea* could not be recipient candidates for *B. napus*.

On the other hand, both *B. rapa* and *B. juncea* have high crossability with *B. napus* and can be hybridized by both artificial and open pollination (Bing *et al*. 1991, Jørgensen *et al*. 1998, Roy 1980, Scheffler and Dale 1994). *B. juncea* is more widely distributed in Japan than *B. rapa*, and therefore, has a higher possibility of being a recipient of *B. napus* pollens.

Therefore, we have started investigation to clarify introgression from *B. napus* to *B. juncea*. We evaluated the crossability and efficiency of hybrid production in *B. juncea* × *B. napus*, and we also observed metaxenia in seed coat and that the occurrence of metaxenia and false hybrids was affected by varietal differences in *B. napus*.

Some studies have reported metaxenia in date palm (Swingle 1928), apple (Nebel and Trump 1932), corn (Pinter *et al*. 1987), cotton (Harrison 1931), common bean (Freytag 1979), and poppy (Bernáth *et al*. 2003); however, the occurrence of metaxenia in *Brassica* species has not yet been reported.

The occurrence of false hybrids (sometimes referred to as exhibiting "matromorphy" or called a matromorphic plant) in *Brassica* species was initially confirmed by observation of same morphological characteristics with maternal plant such as the width and thickness of the leaves and the flower and capsule size (Kakizaki 1925). Subsequently, Terao (1934) and Nishi *et al*. (1964) also identified false hybrids in many cross combinations by observing morphological characteristics (number, length, width, and shape of leaves; length of petiole; and root color). Nishi and Hiraoka (1962) estimated that a kind of apomixsis caused false hybrid. Although Mohammad and Sikka (1940) obtained 44 hybrids from 57 pollinated flowers of *B. juncea* × *B. napus*, identical to our cross combination, they did not detect any false hybrids. Ammitzbøll and Jørgensen (2006) evaluated the hybridity of progeny from *B. napus* × *R. raphanistrum* using inter-simple sequence repeat (ISSR) analysis and any genetic region derived from *R. raphanistrum* were not detected from some progenies, then these progenies were concluded *B. napus*-like plant, and these were false hybrid.

Here we report the crossability of *B. juncea* × *B. napus* to reveal the possibility of introgression from *B. napus* to *B. juncea*. This is the first report to reveal metaxenia and false hybrids in progeny of *B. juncea* × *B. napus*. We also confirmed that the differences in paternal varieties affected the rate of hybrid occurrence.

Materials and Methods

Plant materials

B. juncea L. cv. Kikarashina (Takii & Co., Ltd., Kyoto, Japan) with a yellow seed coat was used as the maternal parent. *B. napus* L. cv. Isuzu-natane (provided by Dr. Yasunobu Ohkawa of the National Institute of Agrobiological Sciences [NIAS]), Norin 16 (Kaneko Seeds Co., Ltd., Gunma, Japan) and Westar (Genebank of NIAS, JP No. 40734) were used as the paternal parents. Isuzu-natane and Norin 16 are Japanese winter-type varieties and Westar is a Canadian spring-type variety, used as a transgenic host. The seed coat color of *B. napus* varieties was dark brown. Seeds were germinated in Petri dishes on filter paper moistened with sterile distilled water (25°C, 48 h). Germinated seeds were incubated at 4°C for 2 weeks for vernalization. They were then transplanted into 15-cm plastic pots and grown in a glass greenhouse programmed at day/night temperatures of 25°C/22°C.

Interspecific hybridization

Artificial bud pollination in *B. juncea* L. cv. Kikarashina × *B. napus* was performed to evaluate interspecific crossability. And each selfing of parent species were also performed by artificial bud pollination as same as interspecific hybridization. Kikarashina plants were grown in the same greenhouse but in another room to avoid cross pollination. Blooming Kikarashina flowers were emasculated and covered with a parchment bag. Paternal plants were also covered with a parchment bag before blooming to avoid contamination with pollen from other varieties. After 1 day, parchment bags were removed and pollen of the paternal parent was applied to the stigma of the maternal parent. After pollination, plants were again covered with parchment bags. About 10 days after pollination, parchment bags were removed, then, the mature pods were harvested ca. 30 days after pollination. Forty buds per plant were crossed and five plants were used to examine crossability. Seed productivity by artificial pollination was reported as the number of seeds per pollinated bud.

Classification of seeds according to seed coat color

The progeny seeds from *B. juncea* L. cv. Kikarashina × *B. napus* were classified into four groups (Fig. 1) on the basis of seed coat color: BJ, level 1 (Lv 1), level 2 (Lv 2) and level 3 (Lv 3). Seeds in the BJ group had yellow (Kikarashina) seed coats, those in the Lv 1 group had less than one-third brown area, those in the Lv 2 group had more than one-third brown area with an area that was nearly yellow ocher, and those in the Lv 3 group had completely brown but not as dark as that of *B. napus* (designated BN). The funiculus of the seed was not included as part of the brown area in the seed groups.

Observation of brown area on seed coat

To confirm the occurrence of a brown area in the tissue of seed coat and mature embryo, cross sections of the seed, seed coat and embryo were observed. Before preparing the cross sections, seeds were soaked in water in a Petri dish for 6 to 8 h and cut in half before germination. Seeds were then soaked again in water with for 1 to 2 days, after which the embryo was separated from the seed coat. All samples were observed using a stereoscopic microscope (Leica MZ16FA, Leica Microsystems K. K., Tokyo, Japan).

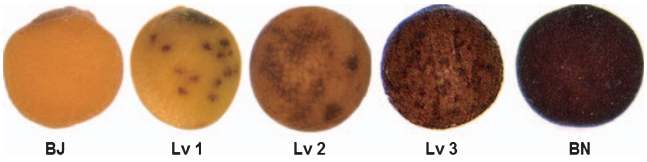


Fig. 1. The seed coat color in each seed group. This figure shows an example using Kikarashina (BJ), Isuzu-natane (BN) and progeny seeds from Kikarashina × Isuzu-natane (Lv 1, Lv 2 and Lv 3). Seeds were classified into BJ, BN, Lv 1, Lv 2 and Lv 3 groups according to the extent of brown area excluding the funiculus. BJ has seed coat color identical to Kikarashina and BN has seed coat color identical to each *B. napus* variety. Lv l has less than one-third brown area and Lv 2 has more than one-third brown area with a slightly yellow area that is nearly yellow ocher. Lv 3 is completely brown but not as dark brown as BN. Brown funiculi were confirmed (shown at the top of the seeds) in all seeds, but they were not included in seed coat color.

Confirmation of hybridity

Hybridity of progeny from *B. juncea* L. cv. Kikarashina × *B. napus* was evaluated by observing their morphological characteristics and then confirmed by random amplified polymorphic DNA (RAPD) analysis.

Morphological characteristics observed included flower organ size, the shape of the leaf margin, the leaf rugose, the leaf fairness, waxy leaf and the difference in flowering time.

RAPD analysis was performed according to the following method. Total DNA was extracted from young leaves using ISOPLANT II (NIPPON GENE Co., Ltd., Toyama, Japan) and used as the template for polymerase chain reaction (PCR). Nineteen primers from RAPD 10-mer kits A and B (Operon Technologies, Inc., Alameda, CA, USA) were used (Table 1). PCR fragments were amplified using Gene Taq (NIPPON GENE CO., Ltd.). The PCR reaction mixture consisted of 2.5 µl of 10× Gene Taq Universal Buffer, 4 µl of dNTP mixture (2.5 mM each), 1.25 µl of primer (20 µM), 0.25 µl of Gene Taq (5 U/µl), 0.5 µl of template DNA and 16.5 µl of water. PCR was performed at 94°C for 5 min; 35 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min; 72°C for 2 min. PCR was performed using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The amplified PCR products were mixed with loading dye and loaded on 2.0% agarose gel prepared in 1× TAE buffer. After electrophoresis, the gel was stained with ethidium bromide and bands were observed using an ultraviolet illuminator. RAPD analysis was performed in duplicate.

Results

Seed productivity in interspecific hybridization of B. juncea × B. napus

The number of seeds per pollinated bud (seeds/pollination) was calculated as an indicator of seed productivity in interspecific hybridization of *B. juncea* × *B. napus* (Table 2). The seeds/pollination obtained due to self-pollination of Kikarashina, Isuzu-natane, Norin 16, and Westar was 10.4, 14.2, 15.6 and 18.9, respectively, whereas seeds/pollination in the interspecific cross combination of Kikarashina × Isuzu-natane, Kikarashina × Norin 16 and Kikarashina × Westar was reduced to 3.9, 4.0 and 5.8, respectively. Although the seed productivity in all interspecific cross combinations decreased compared to that in self-pollination of parent varieties, progeny seeds were still obtained.

Table 1. A list of primer sequence, the number and band size of each marker used in RAPD analysis

primer name	5′ to 3′	*B. juncea* specific marker		*B. napus* specific marker	
		number	band size	number	band size
OPA 01	CAGGCCCTTC	2	300, 600	2	250, 400
OPA 05	AGGGGTCTTG	1	550	2	350, 750
OPA 08	GTGACGTAGG	1	600	1	450
OPA 10	GTGATCGCAG	3	500, 700, 800	1	600
OPA 12	TCGGCGATAG	1	400	—[a]	—
OPA 13	CAGCACCCAC	—	—	2	750, 900
OPA 14	TCTGTGCTGG	2	800, 1500	1	250
OPA 15	TTCCGAACCC	2	400, 1000	1	900
OPA 17	GACCGCTTGT	—	—	1	800
OPA 19	CAAACGTCGG	3	400, 600, 1000	1	200
OPA 20	GTTGCGATCC	—	—	2	500, 800
OPB 03	CATCCCCCTG	2	200, 600	2	450, 700
OPB 05	TGCGCCCTTC	4	200, 300, 450, 700	4	500, 900, 1100, 1200
OPB 08	GTCCACACGG	1	750	1	450
OPB 10	CTGCTGGGAC	1	550	—	—
OPB 11	GTAGACCCGT	1	400	2	950, 1300
OPB 12	CCTTGACGCA	2	250, 650	3	750, 1000, 1200
OPB 17	AGGGAACGAG	—	—	1	500
OPB 19	ACCCCCGAAG	1	1000	4	650, 800, 1300, 1600
Total		27		31	

[a] No specific bands were detected.

Table 2. Cross combinations and their seed productivity in *B. juncea* × *B. napus* by artificial pollination

Cross combination (♀ × ♂)	Pollinated flowers	Seeds per pollination[a]
B. juncea × *B. napus*		
Kikarashina × Isuzu-natane	193	3.9 ± 2.3
Kikarashina × Norin 16	181	4.0 ± 3.0
Kikarashina × Westar	194	5.8 ± 3.4
Mean of seeds per pollination	—	4.5 ± 1.1
B. juncea selfing		
Kikarashina	204	10.4 ± 3.6
B. napus selfing		
Isuzu-natane	100	14.2 ± 2.8
Norin 16	100	15.6 ± 3.5
Westar	100	18.9 ± 3.2
Mean of seeds per pollination	—	16.2 ± 2.4

[a] Seeds per pollination shows the the number of obtained seeds per pollinated flowers and the standard deviation for seeds per pollination in each pollinated plant.

Occurrence of metaxenia in seed coats of progeny seeds

Seed coat colors of *B. juncea* and *B. napus* were yellow and dark brown, respectively (Fig. 1). Because the seed coat is derived from mother tissue, the seed coat color of progeny seeds obtained from *B. juncea* L. cv. Kikarashina × *B. napus* was predicted to be yellow; however, the seed coat color of progeny seeds varied from yellow to brown (Fig. 1), with mottled brown areas on yellow seed coats (Fig. 2E). The brown areas were not observed in the mature embryo (Fig. 2B, 2H) and were limited to the seed coats (Fig. 2B, 2E). Winburne (1962) and Soule (1985) defined this phenomenon as metaxenia.

All seeds were classified into four groups according to the extent of metaxenia (Fig. 1 and Table 3). The occurrence rates of seed color type were as follows: BJ: 37%, Lv 1: 28%, Lv 2: 24%, Lv 3: 11% in Kikarashina × Isuzu-natane; BJ: 32%, Lv 1: 24%, Lv 2: 30%, Lv 3: 13% in Kikarashina × Norin 16; and BJ: 55%, Lv 1: 33%, Lv 2: 11%, Lv 3: 1% in Kikarashina × Westar. Metaxenia occurred in the seed coats of all tested interspecific cross combinations, and most seeds were categorized in BJ seeds, while Lv 3 seed was lowest appearance frequency.

Occurrence of false hybrid plants

Some progeny obtained from Kikarashina × Isuzu-natane and Kikarashina × Norin 16 were confirmed hybrids; however, the plant type of numerous progeny closely resembled *B. juncea*. This plant was named "*B. juncea*-like plant." Because the occurrence of hybrids and false hybrids was closely related to that of metaxenia, we investigated the relationship between metaxenia and hybridity in detail.

First, hybridity of the progeny was evaluated by the representative morphological characteristics and differences in flowering times (Fig. 3). Most characteristics indicated intermediate between *B. juncea* and *B. napus*, however, flowering time was controlled by the dominant trait derived from

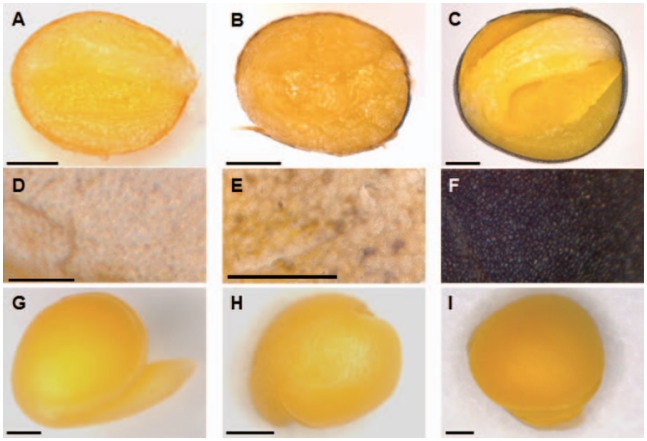


Fig. 2. Cross section of seed, seed coat and embryo of *B. juncea* L. cv. Kikarashina, metaxenia-containing seeds (Lv 2) and Westar. (A, D and G) Kikarashina; (B, E and H) metaxenia-containing progeny seeds from Kikarashina × Westar (Lv 2); (C, F and I) Westar. A, B and C are cross sections of seeds. D, E and F are seed coats that detached from the embryo after stimulation of germination. G, H and I are embryos. em = embryo and sc = seed coat. Bars = 0.5 mm.

Table 3. Seed number and occurrence rate of each metaxenia level in interspecific hybridization and self pollination of parent species

Cross combination (seed coat color)	Total seeds	Seed number and occurrence rate of each metaxenia level		
		Metaxenia level	Seed number	Occurrence rate[a] (%)
B. juncea × *B. napus* (Yellow × Dark brown)				
Kikarashina	748	BJ	295	37 ± 23
× Isuzu-natane		Lv 1	230	28 ± 8
		Lv 2	164	24 ± 11
		Lv 3	59	11 ± 11
Kikarashina × Norin 16	846	BJ	274	32 ± 26
		Lv 1	204	24 ± 22
		Lv 2	255	30 ± 14
		Lv 3	113	13 ± 9
Kikarashina × Westar	1116	BJ	613	55 ± 21
		Lv 1	364	33 ± 14
		Lv 2	126	11 ± 3
		Lv 3	13	1 ± 1
B. juncea selfing (Yellow × Yellow)				
Kikarashina × Kikarashina	2117	BJ	2117	100
B. napus selfing (Dark brown × Dark brown)				
Isuzu-natane	694	BN	694	100
Norin 16	582	BN	582	100
Westar	824	BN	824	100

[a] Occurrence rate shows the average and the standard deviation for number of seed in each metaxenia level.

B. napus. Flower organ size of *B. napus* was larger than that of *B. juncea*, and hybrid plants showed an intermediate size (Fig. 3A). For leaf characteristics, *B. napus* had undulate margins, *B. juncea* was highly rugose with incised margins, and hybrid plants were less rugose with toothed margins (Fig. 3B). *B. napus* had waxy leaves, while *B. juncea* had

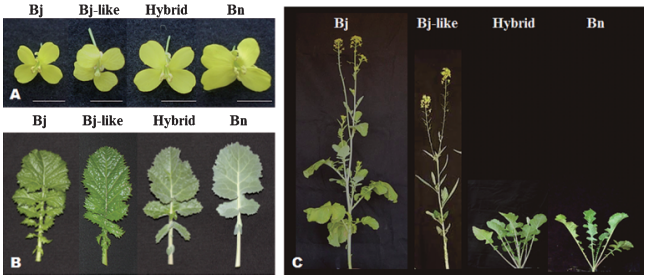
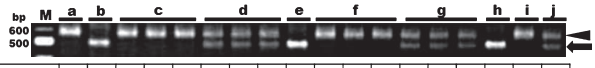


Fig. 3. Comparison of morphological characteristics and difference in flowering times among *B. juncea*, *B. napus*, and their progeny. (A) Flower organ size, (B) Leaf shape, (C) Plant type. Bj: *B. juncea* L. cv. Kikarashina, Bn: *B. napus* L. cv. Isuzu-natane (representative of *B. napus*), Bj-like: *B. juncea*-like plant that closely resembles Kikarashina from Kikarashina × *B. napus*, Hybrid: Hybrid plant from Kikarashina × Isuzu-natane. All plant types were observed at 50 days after sowing. Bars = 1.0 cm.



B. juncea specific band	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	–	+	+
B. napus specific band	–	+	–	–	–	+	+	+	+	–	–	–	+	+	+	+	–	+
Hybridity by RAPD analysis	J	N	J	J	J	H	H	H	N	J	J	J	H	H	H	N	J	H
Plant type by morphological characteristic	J	N	J	J	J	H	H	H	N	J	J	J	H	H	H	N	J	H

Fig. 4. Confirmation of hybridity in false hybrid and hybrid from *B. juncea* L. cv. Kikarashina × *B. napus* by RAPD analysis as an example of using OPB03 primer, and consistency of hybridity from morphological characteristics and RAPD analysis. (a) *B. juncea* L. cv. Kikarashina, (b) *B. napus* L. cv. Isuzu-natane, (c) False hybrids from Kikarashina × Isuzu-natane, (d) Hybrids from Kikarashina × Isuzu-natane, (e) Norin 16, (f) False hybrids from Kikarashina × Norin 16, (g) Hybrids from Kikarashina × Norin 16, (h) Westar, (i) False hybrid from Kikarashina × Westar, (j) Hybrid from Kikarashina × Westar. Arrow indicates *B. napus*-specific band and arrowhead indicates *B. juncea*-specific band. Different plants are indicated in each lane of e, d, f and g. J, N, and H is *B. juncea*-type plant, *B. napus* plant and hybrid-type plant, respectively.

abundant leaf fairness; hybrid plants expressed slight wax and had modest fairness. In addition, delay in flowering time was a typical trait for identifying hybridity.

On the other hand, the *B. juncea*-like plants did not have any characteristics of either hybrid plants or *B. napus* (Fig. 3). The *B. juncea*-like plants were evaluated to be putative false hybrids, and hybridity was confirmed by identifying molecular characteristics using RAPD markers.

We selected 19 random primers for detection of hybrid plants by RAPD analysis (Table 1). *B. juncea*- and/or *B. napus*-specific PCR fragments could be detected with these selected primers, and therefore, 27 *B. juncea* species-specific markers and 31 *B. napus* species-specific markers were used (Table 1). All species-specific markers were detected from hybrid plants (Fig. 4d, 4g, 4j and Table 4); whereas, all *B. juncea*-like plants had only *B. juncea*-specific markers; no *B. napus*-specific bands were detected (Fig. 4c, 4f, 4i).

The hybridity identified using morphological characteristics completely matched the results of RAPD analysis. These results showed that hybridity can be determined using morphological characteristics, and we concluded that the *B. juncea*-like plants were false hybrids.

Table 4. Identification of hybridity (false hybrid or hybrid) in progeny plant by morphological characteristic and RAPD analysis

Cross combination		Metaxenia level	Number of observation	Plant type		
Species	Variety			*B. juncea*	Hybrid	*B. napus*
B. juncea × *B. napus*	Kikarashina × Isuzu-natane	BJ	70	70	0	0
		Lv 1	60	56	4	0
		Lv 2	93	0	93	0
		Lv 3	22	0	22	0
	Total		245	126	119	0
	Kikarashina × Norin 16	BJ	48	48	0	0
		Lv 1	46	40	6	0
		Lv 2	80	0	80	0
		Lv 3	39	0	39	0
	Total		213	88	125	0
	Kikarashina × Westar	BJ	72	2	70	0
		Lv 1	76	1	75	0
		Lv 2	85	0	85	0
		Lv 3	7	0	7	0
	Total		240	3	237	0
B. juncea selfing	Kikarashina	BJ	30	30[a]	0	0
B. napus selfing	Isuzu-natane	BN	30	0	0	30
	Norin 16	BN	30	0	0	30
	Westar	BN	30	0	0	30

[a] These plants were not false hybrids, and they were obtained from selfing of Kikarashina.

Relationship between metaxenia and hybridity

Progeny seeds were categorized into four groups according to the extent of metaxenia (Fig. 1 and Table 3). The false hybrids or hybrids of progeny were identified. The relationship between metaxenia and hybridity is summarized in Table 4. All seeds categorized in Lv 2 and Lv 3 groups were hybrids. Furthermore, most progeny from Kikarashina × Westar in of BJ and Lv 1 groups were hybrids; however, most progeny from Kikarashina × Isuzu-natane and Kikarashina × Norin 16 in of BJ and Lv 1 groups were false hybrids as identified by morphological characteristics and RAPD analysis. When Westar was used as the paternal parent, 2 out of 72 plants in the BJ group and 1 out of 76 plants in the Lv 1 group were false hybrids. The occurrence rate of false hybrids was extremely low, and we found no relationship between the occurrence of metaxenia and hybridity.

On the other hand, in both Kikarashina × Isuzu-natane and Kikarashina × Norin 16, all progeny of BJ were false hybrids. Although the progeny of Lv 1 were nearly classified as false hybrids, 4 out of 60 and 6 out of 46 from Kikarashina × Isuzu-natane and Kikarashina × Norin 16 were hybrids. Consequently, hybrid formation efficiencies were 49% and 59% in Kikarashina × Isuzu-natane and Kikarashina × Norin 16, respectively.

The occurrence of metaxenia and hybridity was related and the occurrence of false hybrids was affected by varietal differences.

Discussion

Seed coat develops from integument palisade and pigment cells of maternal parents. Although the maternal parent *B. juncea* L. cv. Kikarashina has a yellow seed coat, seed coats of the progeny seeds from *B. juncea* L. cv. Kikarashina × *B. napus* varied from yellow to brown (Fig. 1, 2). The expression of brown area was affected by paternal varieties; this phenomenon is called metaxenia. Winburne (1962) and Soule (1985) provided a similar definition of metaxenia, a direct effect of the paternal parent on the seed and fruit outside the embryo, endosperm and embryo sac. Metaxenia has been reported in several plants such as seed weight and date of fruit ripening in the date palm (Swingle 1928), pH value and acidity in apples (Nebel and Trump 1932), grain weight of corn (Pinter *et al*. 1987), pod size of the common bean (Freytag 1979), and morphine content of the capsule of the poppy (Bernáth *et al*. 2003). For metaxenia, length of the lint and quantity of fuzz were reported in seed coat of cotton (Harrison 1931) and Radics (1977) reported the color and surface of seed coats in interspecific hybridization among six *Rorippa* species belonging to the Brassicaceae family.

Although numerous interspecific hybrids have been produced during *Brassica* breeding programs, metaxenia has not been reported in this genus. We speculated that because the seed coats of most *B. juncea* are brown or dark brown, it is very difficult to detect metaxenia by seed coat color change in this species. Since the maternal parent *B. juncea* L. cv. Kikarashina has a yellow seed coat, it is easy to detect seed coat color change from yellow to brown. We also speculate that maternal varieties also affect the occurrence of metaxenia, however, we could not use other maternal varieties with yellow seed coat in this experiment. Subsequently, maternal varietal difference should be evaluated.

According to some previous reports on seed coat color, brown is dominant over yellow (Liu *et al.* 2005, Mingli *et al*. 2009, Vera *et al*. 1979). Liu *et al.* (2005) reported that the occurrence of brown seed coats in *B. juncea* is controlled by two dominant genes. We observed that seed coats of all F_2 seeds (total 14 seeds) obtained from three cross combinations were brown, and this brown color is thought to be dominance over yellow. However, it is not clear that relation between brown color in F_2 seeds and these dominant genes.

We obtained many progeny seeds from artificial interspecific hybridization (Table 2). Our results of seed productivity were similar to those of previous reports (Bing *et al*. 1991, Frello *et al*. 1995); however, 126 out of 245 seeds (51%) and 88 out of 213 seeds (41%) were false hybrids when Isuzu-natane and Norin 16 were used as the paternal parent, respectively (Table 4). When Westar was used as the paternal parent, only three out of 240 seeds (1.3%) were false hybrids (Table 4). This result showed that the occurrence of false hybrids was significantly affected by varietal differences. Nishi and Hiraoka (1962) observed false hybrids generated from unfertilized egg cells and they thought that false hybrids were a form of apomixis. Moreover, the occurrence of false hybrids is defined as apomixsis (Solntseva 2003). However, the mechanism of false hybrids remains to be clarified.

False hybrids have been reported in various hybridizations in Brassicaceae (Ammitzbøll and Jørgensen 2006, Banga 1986, Ito *et al*. 1948, Kakizaki 1925, Mohammad and Sikka 1940, Nishi and Hiraoka 1962, Nishi *et al*. 1964, Noguchi 1935, Terao 1934). Most false hybrids mentioned in previous reports had been confirmed by observation of morphological characteristics. As the *B. napus*-like plants from *B. napus* × *R. raphanistrum* did not have any ISSR marker of *Raphanus* genome (Ammitzbøll and Jørgensen 2006), the *B. napus*-like plant is assumed to be a false hybrid. Nishi and Hiraoka (1962) observed that the egg cell began embryonic development after stimulation by pollination without fertilization. They estimated that the false hybrid was generated by pseudogamy, a type of apomixis; however, the mechanism for the occurrence of false hybrids has never been completely analyzed. We considered that the *B. juncea*-like plants in this study are false hybrids, generated by pseudogamy because these plants were confirmed to be related to *B. juncea* using morphological characteristics and RAPD analysis (Table 4 and Figs. 3, 4).

Because *B. juncea* has a greater ability to form hybrid progeny with *B. napus*, *B. juncea* is ranked second to *B. rapa* among the recipient species when crossed with *B. napus* (Scheffler and Dale 1994). The gene flow in *B. juncea* × *B. napus* has been investigated by several groups (Bing *et al*. 1991, 1996, Choudhary and Joshi 2001, Frello *et al*. 1995,

Huiming *et al*. 2007, Liu *et al*. 2010, Mohammad and Sikka 1940, Song *et al*. 2010), but false hybrids in *B. juncea* × *B. napus* have not been reported. We obtained numerous false hybrids when Isuzu-natane and Norin 16 were used as paternal parents. Thus, our results suggest the possibility that introgression cannot be evaluated by only seed productivity.

In cross combination of Kikarashina × Isuzu-natane and Kikarashina × Norin 16, a relationship between metaxenia and hybridity was observed. Most progeny in the BJ and Lv 1 groups were false hybrids and all progeny in Lv 2 and Lv 3 groups were hybrids. In these combinations, the occurrence of metaxenia appeared to be a useful visual marker for estimating hybridity in progeny.

If we reveal the possibility of introgression and the effect of the biosafety from *B. napus*, we will have to understand the productivity and fitness of the true hybrid. The data of only seed number by interspecific hybridization will lead to overestimate the introgression possibility. The accurate possibility for introgression must be evaluated by recognition of false hybrid. We assure that introgression from *B. napus* to *B. juncea* should be assessed by not only true hybrid productivity in progeny but also fitness of hybrid progeny for further study. The next step would be to investigate the fitness of hybrids from *B. juncea* × *B. napus.*

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