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INTEGRATION OF CYTOLOGICAL AND MOLECULAR ANALYSIS TO CONFIRM A HYBRIDITY IN F_1 *BRASSICA* PROGENY

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Abstract

In *Brassica*, interspecific hybridization is a potential and useful method for transferring valuable traits between species of commercial interest. In the previous experiments successful interspecific hybrids were obtained through hybridization between chosen rapeseed cultivars (*Brassica napus* L.) and *Brassica rapa* genotypes for transferring clubroot resistance from wild species into cultivated background. In the presented research morphological, cytological and simple sequence repeats (SSR)-based molecular analyses were carried out to confirm the hybrid nature of the F_1 plants. This paper evidences for a successful creation of twenty three F_1 interspecific hybrids where six out of eleven cross combinations were confirmed as fertile.

The obtained results have shown that analyzed morphological characters of F_1 hybrids were intermediate as compared to those of both parental genotypes. Furthermore, in pollen mother cells (PMCs) of the F_1 hybrids abnormalities like univalent, lagging chromosomes and disorders in synchronization were observed in meiosis. Generally, the abnormalities in meiosis occurred in most of the tested genotypes with the mean frequency of 63.8%. Among tested SSR primers, Na10-A08 was found to reveal highly polymorphic bands in hybrids. As a result, 41.1% of the investigated plants were confirmed as true hybrids.

Key words: Brassicas; Interspecific hybrids; Meiosis abnormalities; SSR markers.

Introduction

Rapeseed (*Brassica napus* AACC=2*n*=38) is one of the most important oilseed crops after oil palm and soybean used for human consumption worldwide (Zhang *et al.*, 2015). *Brassica* coenospecies, in particular, have been bestowed with nearly 100 species and genera of wild and weedy relatives, serving as a rich reservoir of genes conferring many agriculturally and economically important traits. These species can be effectively utilized to introduce the important traits to cultivated species as well as development of potential wide hybrids (Kumar *et al.*, 2015). *Brassica rapa* L., is one of the most economically important oil crops. Chinese cabbage (*Brassica rapa* AA, 2n=20) possesses many important resistance genes, which can be used to increase the genetic variation of rapeseed and to produce viable interspecies hybrids (Jesske *et al.*, 2013). This is possible because of a high crossability index between *B. napus* and *B. rapa* and low aneuploidy of their interspecific hybrids. In general, crosses between wild *B. rapa* and *B. napus* produces AAC hybrids with 2n = 29. To genome A belongs ten pairs of chromosomes and to the C genome - nine unpaired chromosomes. These univalent C-chromosomes are passed on in crosses, resulting in plants with a number of various chromosomes. In meiosis in the F_1 hybrid homologous recombination between A- and C-chromosomes can occur, however its frequency is still a matter of debate (Jong & Hesse 2012). According to the literature, F_1 hybrids identity could be confirmed by chromosome analysis and characterized by simple sequence repeats (SSR) analysis (Sun *et al.*, 2014). SSR markers are distributed throughout the genome and thus become the preferred markers for many applications in genetics (Abbas *et al*., 2009, Barchi *et al.*, 2011) e.g. to identify potential alleles affecting important traits for agronomy. Currently, with the development of next-generation sequencing, it is feasible to develop a large number of SSR markers. It will facilitate the fine mapping of QTLs, improve the identification and exploitation of genes affecting important traits, and enable selective breeding through genomic selection (Song *et al.*, 2015). Previously, we have reported that there was unilateral interspecific compatibility in crosses where *B. rapa* ssp. *chinensis* was used as a maternal form. Moreover from *B. rapa* ssp. *chinensis* x *B. napus* crosses, the highest number of F_1 hybrid plants was obtained (Niemann *et al*., 2015). This study seeks to confirm the generation of true interspecific hybrids carrying clubroot resistance genes.

Material and Methods

Plant material: For this study, fifty six F_1 hybrids obtained from interspecific crosses between collected *Brassica napus* cultivars i.e. Jet Neuf, Skrzeszowicki, Californium and Zhongshuang9 and three *Brassica rapa* accessions i.e. A - PI430485 98CI, B - Pak Choi 08 007569, C - Chinese Cabbage 08 006169 were selected.

Evaluation of morphotypes: Morphotypes of plants of the F_1 generation were studied and compared with the parental plants, as proposed by Wojciechowski (1985). To determine whether obtained plants were of the *B. napus* or *B. rapa* type, analysis of some of the selected morphological traits was performed, based on: a) leaf color (green or light-green), b) presence of trichomes on the lower side of the leaf blade (yes or no), c) type of inflorescence, f) flower character (sterile or fertile).

Observation of microsporogenesis: Flower buds of F_1 *Brassica* hybrids at different developmental stages were fixed in a mixture of ethyl alcohol and acetic acid (3:1 v/v) approximately for 1 to 24 h, then transferred to 70%

alcohol and stored at -20°C until use. Individual anthers were squashed and stained with 1% orceine using a smear method. For each plant, the mean percentage of abnormalities in meiosis was estimated for at least 100 PMCs. Observations and photographic documentation was performed under a Nikon light microscope.

Pollen viability test: Pollen viability (fertility) of 56 flowering F_1 *Brassica* hybrids was estimated by mounting mature pollen grains in glycero–acetocarmine (1:1) mixture (Belling, 1921). The pollen grains were immersed in the Belling fluid and wet microscopic preparations were made. Determination of viability was performed on the basis of counting the grains in 10 fields of the preparation, each containing a minimum of 30 pollen grains. Round/ complete pollens, which were stained and turned pink (viable ones) were taken as fertile, while incomplete/ shrunken and unstained pollens were considered as sterile (Sheidai *et al.,* 2003). Pollen grain measurements were made from Canada Balsam preparations with an ocular micrometer at 400× under a compound light microscope. The diameter of each pollen grain was measured. Statistical analyses were carried out considering plants per population using the Rstudio software package (Version 0.99.489 – © 2009-2015 Rstudio, Inc.).

Molecular confirmation: Simple sequence repeat (SSR) markers were used to confirm the paternity of the putative hybrids. DNA from young leaves of parental genotypes and putative F_1 hybrids was extracted with the Genomic Mini AX Plant kit. Polymerase chain reactions (PCR) were carried out in 12,5 µl reaction mixtures containing 6,25 µl DreamTaq PCR Master Mix (DreamTaq DNA Polymerase, 2X DreamTaq buffer, dNTPs, and 4 mM MgCl2), 20 pmol of each primer, 5 µl of nuclease-free water and 25 ng of genomic DNA. The five chosen primer sets i.e. Na10-A08, Ol10-D08, Na12-D04, Ol10-H02, BRMS043 were collected based on the published data by Ford *et al.,* (2006) and synthesized by Symbios, Poland.

PCR was run on Biometra thermal cycler according to the conditions described by Ford *et al*., (2006). Electrophoresis was carried out in 1x TBE buffer (10 mM Tris-Borate, 1 mM EDTA) at 80-90 V for 2-3 hours depending on allele sizes. PCR products were separated by electrophoresis using 2% agarose gel and were visualized under UV light after staining with Ethidium bromide.

Results

Morphology of hybrid plants: Generally, the resulting plants in the F_1 generation combined the characteristics of the genotypes involved in their generation. The F_1 plants were medium in height, profusely branched and intermediate to their parents for most of the morphological and florescence attributes (Fig. 1b). The majority of hybrid plants has large number of branches (from *B. napus*) (Fig. 1a), dark green leaves and a smooth leaf edge or the presence of trichomes on the lower side of the leaf blade (from *B. rapa*) (Fig. 1c). In addition, flower character was studied and six cross combinations were confirmed as fertile (Table 1). In crosses between *B. rapa* x *B. napus* there was a higher percent of total fertility than in *B. napus* x *B. rapa* (52.43% and 35.92%, respectively) (Table 1).

Observation of microsporogenesis: For the purpose of the study, 632 preparations were carried out from 56 hybrids, an average of 11.3 preparations per plant. The studies of PMCs of the analyzed F_1 hybrids revealed that abnormalities in meiosis occurred in most of tested genotypes with the mean frequency of 63.8% (Table 1). Laggard and eliminated chromosomes as well as a large number of nonsynchronous divisions were observed. The highest number of abnormalities (all types mentioned above) was observed in hybrids with the paternal form of *B. rapa* B (97.2%). Moreover, univalents have appeared more often in these hybrids types in which *B. rapa* C was a parental form (Fig. 1d). However, perfectly appropriate divisions had occurred in F_1 hybrids obtained from reciprocal crosses between *B. napus* cv. Jet Neuf and *B. rapa* A. Meiotic analysis of parents and their F_1 hybrids showing chromosome associations at diakinesis/ metaphase I, and chromosome distribution at anaphase I and II.

Pollen viability test: The mean percent of pollen fertility in F_1 hybrids was lower than in parental forms and ranged from 50.0% (*B. rapa* C x *B. napus* cv. Jet neuf) to 80.6 % (*B. rapa* B x *B. napus* cv. Zhongshuang 9). On the other hand, pollen fertility of both tested parental genotypes (*B. napus* and *B. rapa* C) was as high as 95.2% and 95.4% respectively (Table 2). Considerable variability in hybrids pollen size was also observed. Pollen grain diameters were measured and a few types of pollen sizes were identified in the F_1 hybrid (Table 2). Viable pollen with a length of 27.2–37.4 µm and width of 15.6-23.2 µm were observed. The hybrid pollen size was different from the size of their progenitors and the largest pollen grains belong to *B. napus* cv. Californium. Generally, *Brassica* pollen shape is similar to the coffee beans (oval) with more or less 2.0 length-width ratio., However, other shapes like circular (*B. napus* cv. Californium x *B. rapa* C - 1.18 length-width ratio) or irregular (*B. rapa* A x *B. napus* cv. Californium – 1.29 length-width ratio) were observed in F_1 plants as well (Fig. 1e).

Molecular confirmation: Based on the SSR analysis, 23 out of 56 putative F_1 hybrids were proved to be true hybrids (41.1%), indicating that whenever *B. napus* was used as a female parent and *B. rapa* as the male, the crosses were complete failure, producing no true hybrids (Table 3). Although from crosses between *B. napus* cv. Jet Neuf and *B. rapa* C, six seedlings were evaluated, only one true hybrid was confirmed (16.7%). From reciprocal crosses, 36 seedlings were obtained, with only 22 *B. rapa* x *B. napus* hybrids identified as true. The highest percentage of hybrid formation was observed in the crosses *B. rapa* x *B. napus* with 61.1% true hybrids (Table 3). Furthermore, the greatest polymorphism was found by the primer Na10-A08 (Fig. 1f).

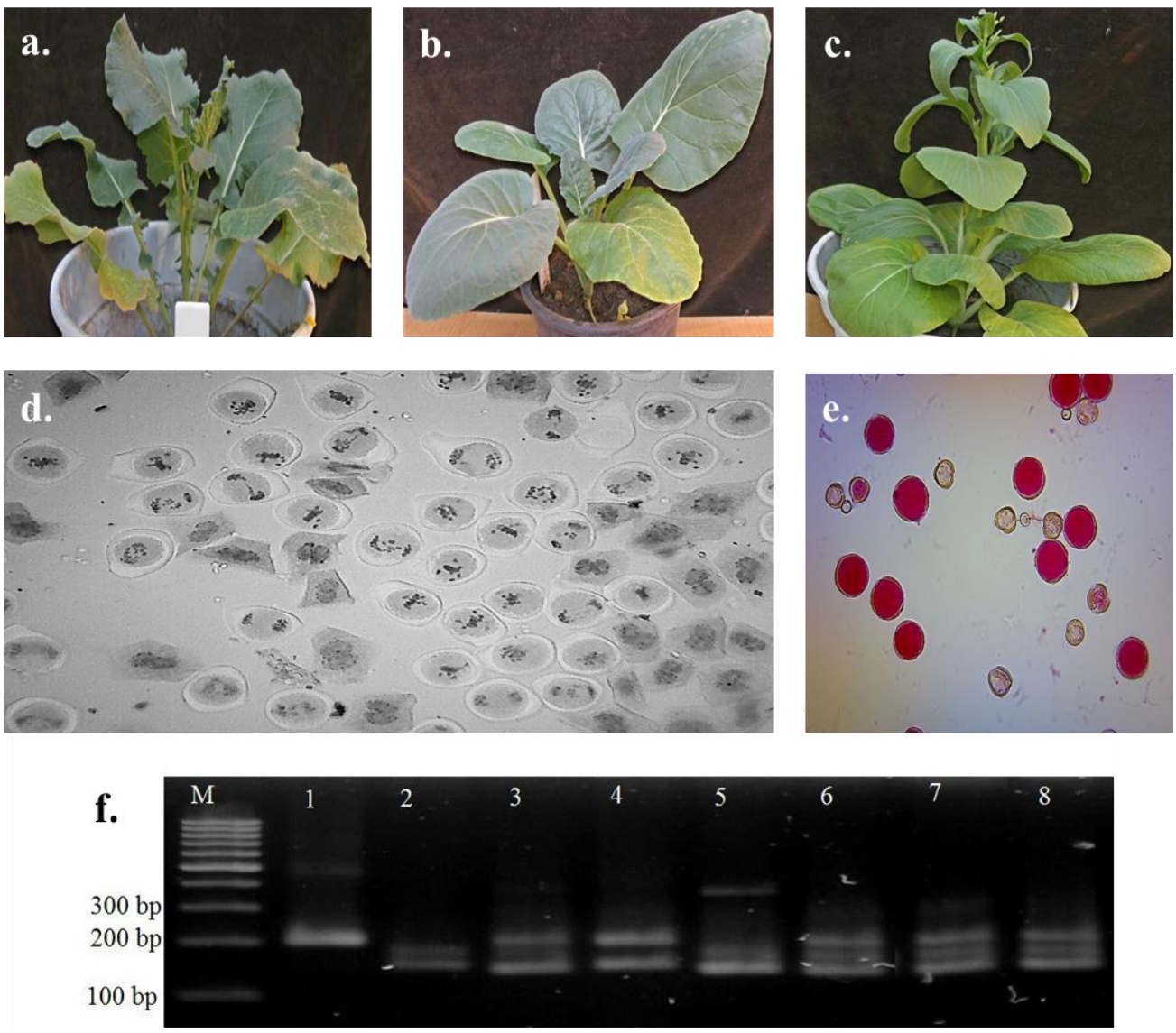

Fig. 1. Cytological and molecular analysis of F_1 hybrids: (a) Morphology of parental and hybrid plants: *B. napus* cv. Jet Neuf - the female parent; (b) F_1 hybrid plant; (c) *B. rapa* ssp. *chinensis* - the male parent; (d) *B. rapa* C x *B. napus* cv. Jet Neuf – anaphase and tetrads in one view indicates non synchronous division; (e) Viability of pollen grains *B. napus* cv. Californium x *B. rapa* ssp. *chinensis* A hybrid plant - the red one represents viable ones with different sizes; (f) DNA profile of parents and hybrids using SSR marker Na10-A08; M-100 bp DNA lagger, (1) female component *B. rapa* A; (2) male component *B. napus* cv. Californium; (3) F_1 hybrid; (4) female component *B. rapa* B; (5) male component *B. napus* cv. Zhongshuang9; (6-8) F_1 hybrids.

Table 1. The frequency of abnormal meiosis in interspecific F_1 *Brassica* hybrids.

Parental components				Abnormalities (%) Mean ± SD	Synchronization in meiosis[1]	Lagging chromosomes	Univalents	No. of chromosomes
♀		♂						
B. napus	Jet Neuf	*B. rapa* A		0	+	-	-	24
	Californium	*B. rapa* B		97.2 (±4.9)	+	+	+	23-24
	Skrzeszowicki			54.4 (±2.7)	+	-	-	22-23
	Jet Neuf			32.0 (±1.6)	+	+	-	21-27
	Californium	*B. rapa* C		93.5 (±4.7)	+/-	-	+	29-32
	Zhongshuang9			81.9 (±4.1)	-	+	+	23-29
			Total	**59.8 (±3.0)**				
B. rapa A		*B. napus*	Jet Neuf	0	-	-	-	20
			Californium	75.6 (±3.8)	+	+	-	22-29
B. rapa B			Zhongshuang 9	89.3 (±4.5)	-	+	+	21-25
B. rapa C			Jet Neuf	92.0 (±4.6)	-	+	+	20-27
			Skrzeszowicki	85.3 (±4.3)	-	-	+	23-28
			Total	**68.4 (±3.4)**				
			General	**63.8 (±3.2)**				

[1]+ occurs, - absent, +/- partial

Table 2. The pollen characteristics of the *Brassica* interspecific hybrids and their parents *a* significant at α=0,05, *b* significant at α=0,01 (Tukey's test).

Parental components			Pollen fertility (%) Mean ± SD	Length [µm]	Width [µm]	Length: width ratio	Pollen grains shape
B. napus cv. Californium			95.2 (± 4.8)	39.0 (± 2.0)	18.6 (± 0.9)	2.10	oval
B. rapa C			95.4 (± 4.8)	34.1 (± 1.7)	17.2 (± 0.9)	1,98	oval
♀	♂						
B. napus	Jet Neuf	*B. rapa* A	60.2 (± 3.0)^a	35.2 (± 1.8)^a	18.4 (± 0.9)^a	1.91	oval
	Californium		74.6 (± 3.7)^b	33.6 (± 1.7)^a	18.5 (± 0.9)^a	1.82	oval
	Skrzeszowicki	*B. rapa* B	67.5 (± 3.4)^a	37.4 (± 1.9)^b	20.5 (± 1.0)^a	1.82	oval
	Jet Neuf		54.5 (± 2.7)^b	31.9 (± 1.6)^a	16.5 (± 0.8)^b	1.93	oval
	Californium	*B. rapa* C	65.6 (± 3.3)^a	27.3 (± 1.4)^b	23.2 (± 1.2)^b	1.18	circular
	Zhongshuang9		72.7 (± 3.6)^b	34.2 (± 1.7)^a	22.1 (± 1.1)^b	1.55	circular
Total			**66.2** (± 3.3)	**33.0** (± 1.7)	**20.1** (± 1.0)	**1.64**	
B. rapa A			75.7 (± 3.8)^a	34.2 (± 1.7)^a	19.7 (± 1.0)^a	1.74	oval
	Californium		56.9 (± 2.8)^a	29.3 (± 1.5)^a	22.7 (± 1.1)^a	1.29	circular
B. rapa B	*B. napus*	Zhongshuang 9	80.6 (± 4.0)^a	35.9 (± 1.8)^a	19.4 (± 1.0)^a	1.85	oval
		Jet Neuf	50.0 (± 2.5)^a	29.3 (± 1.5)^a	15.6 (± 0.8)^a	1.88	oval
B. rapa C		Skrzeszowicki	65.7 (± 3.3)^a	36.8 (± 1.8)^a	19.2 (± 1.0)^a	1.92	oval
Total			**67.8** (± 3.4)	**33.5** (± 1.7)	**19.7** (± 1.0)	**1.70**	
General			**69.0** (± 3.5)	**33.9** (± 1.7)	**19.4** (± 1.0)	**1.75**	

Discussion

It is known that *B. napus* (AACC genome) can hybridize with related species, especially with its congener *B. rapa* (AA genome) (Warwick *et al.*, 2003), which facilitates interspecific gene flow between the two species. According to Hauser *et al.,* (2003) rapeseed (*B. napus* L.) genome enrichment of useful traits have a direct impact on the economic aspect. Because of the fact that within the genus *Brassica* several diploid and tetraploid cultivated species share closely related genomes (U 1935), the introgression of genes could be more frequent. In *Brassica* a close genomic similarity occurs especially between A and C genomes. High level of homology between these genomes allows recombination that leads in obtaining a functional hybrid (Cui *et al.,* 2012). Leflon *et al.,* (2006) reported that, the efficiency of interspecific crosses depended on the similarity between the implicated genomes as high levels of genome similarity was required to ensure appropriate chromosome pairing and genetic recombination. In this research, the lower hybridization success when *B. napus* was a maternal plant is in contrast to the relative ease on hybridization with *B. napus* (Ammitzboll *et al.,* 2005). Our previous results demonstrated that hybridization rates were significantly higher if *B. rapa* ssp. *chinensis* was a female parent, rather than vice versa, due to the self-incompatibility of *B. rapa.* Consequently, in crosses between *B. rapa* x *B. napus* there was a higher percent of total fertility than in *B. napus* x *B. rapa* (52.43% and 35.92%, respectively). Moreover, the intermediate appearance of many morphological characteristics of F_1 hybrids is in close agreement with the reports of Choudhary & Joshi (2001). Generally, cross-pollination between *B. rapa* (AA) and *B. napus* (AACC) gives rise to a triploid F_1 hybrids (AAC, 2n=29).

This F_1 hybrid has 20 A-chromosomes and 9 C-chromosomes. Because all C chromosomes are unpaired, problems may arise at gamete production during meiosis. Mikołajczyk (2008) reported that the frequency of crossing-over in triploids AAC is 1.6-3.2 higher than in tetraploids AACC. However, Leflon *et al.,* (2006) confirms that during meiosis, hybrid chromosomes derived from both A genomes, i.e. from *B. rapa* and *B. napus*, are similar enough to conjugate. Furthermore, Mason *et al.,* (2014) states that this is the result of the presence of entire karyotypes which are orientated and terminated at the same place on particular chromosomes in both genomes and derived from *Brassica* ancestors. Despite strong conjugation in some of the chromosomal regions, the formation of multivalents and univalent should not be excluded. In our studies meiotic abnormalities like multivalents, univalents and chromosomal lagging were observed as well, especially when *B. rapa* was a maternal component (68.44% cells with meiotic disorders). Hybrids combinations varied in the frequency of abnormal cells, making it similar to the results obtained by Mason *et al.,* (2011). Additionally, less synchronized divisions were observed in putative F_1 hybrids obtained from the crosses between *B. rapa* x *B. napus*. However, variations in the number of

crossovers essential for meiosis depend on many factors, such as genomic and genetic (Dooner 2002) but also environmental and developmental factors (Francis *et al.,* 2007). Carlton *et al.,* (2006) has demonstrated that even when some chromosomes in nucleus do not form cross-overs, this may lead to a compensatory increase in cross-overs on other chromosomes. On the other hand Cui *et al.,* (2012) pointed out inheritance outside the nucleus. Uptill to now it is not clear which of the *B. napus* ancestors is a cytoplasmic donor. Allender & King (2010) suggest that plastid donor in the *B. napus* genome comes from *B. rapa*. In this research, no relationship between characteristics of the pollen grains and meiosis or molecular results was observed. Similarly to other experiments, pollen viability was much lower in F_1 hybrids than in the two parental species (Pertl *et al.,* 2002, Choudhary & Joshi 2012). However, different shape of pollen grains did not affect the grains' viability. Occurrence of different sized pollen grains in obtained hybrids may result from unequal cell division during tetrad formation (Choudhary & Joshi 2012). Currently, expressions patterns and contents of proteins and carbohydrates are tested for understanding the specific mechanisms of fertilization on the side of pollen grain (Lyu *et al*., 2015, Leroux *et al.,* 2015). Due to the high crossability between *B. napus* and *B. rapa* novel agronomic traits from *B. rapa* have been successfully transmitted into commercial *B. napus* varieties (Zhang *et al.,* 2015). In this study, meiotic synchronization was remarkable when *B. napus* was used as a maternal component. This could be attributed to the crosses genotypes selection (Qian *et al.,* 2014). Cui *et al.,* (2013) has researched cytogenetic and genomic effects of genetic changes within the *Brassica* hybrids. The loss of parental bands were observed on the gel resulting from the connection of genomes due to their similarity, rather than the DNA elimination, Such changes arise as a result of polyploidization, inheritance outside the nucleus and the interactions between such similar genomes in *Brassica* species. The authors also show the hierarchy of changes in the genomes and conclude that A and C genomes have the most conservative character, potentially resulting from a methylation of some parts of the DNA. In the presented research, 41.1% of obtained F_1 hybrids were confirmed as true hybrids by molecular analysis using SSR markers. By a way of contrast, Hooftman *et al.,* (2015) confirmed hybridity between *B. napus* and *B. rapa* at the level of 65%.

Table 3. Percentage of interspecific *Brassica* hybrids confirmed by SSR marker (Na10-A08).

Parental components				No. of plants evaluated	No. of true hybrids	True hybrids (%)
♀		♂				
B. napus	Jet Neuf	*B. rapa* A		2	0	0
	Californium	*B. rapa* B		1	0	0
	Skrzeszowicki			2	0	0
	Jet Neuf			6	1	16.7
	Californium	*B. rapa* C		4	0	0
	Zhongshuang 9			5	0	0
			Total	**20**	**1**	**5.0**
B. rapa A		*B. napus*	Jet Neuf	10	5	50.0
			Californium	1	1	100.0
B. rapa B			Zhongshuang9	14	8	57.1
B. rapa C			Jet Neuf	2	1	50.0
			Skrzeszowicki	9	7	77.8
			Total	**36**	**22**	**61.1**
			General	**56**	**23**	**41.1**

Conclusions

As shown that the analyzed F_1 hybrids were intermediate in terms of morphological features as compared to those of both parental genotypes. Furthermore, in pollen mother cells (PMCs) of F_1 hybrids abnormalities like univalent, lagging chromosomes and disorders in synchronization were observed during meiosis.

The findings of this study allow to select *Brassica* true hybrids for use in the future experiments related to clubroot resistance.

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