Journal of Oilseed Brassica, 15 (2) : 199-210, July 2024

Characterization of F_1 hybrids developed from the crosses between *Brassica rapa* and *Brassica fruticulosa*: A step towards the development of synthetic transient amphidiploids

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(Received: 20 January 2024; Revised: 28 June 2024; Accepted: 30 June 2024)

Abstract

In the present study, the F_1 hybrids were developed from the crosses between *Brassica rapa* and *Brassica fruticulosa* through wide hybridization. Morphological, molecular, cytological and fertility analyses were done to identify the true hybrids. Further amphidiplodization can be done for the development of synthetic amphidiploid. Most of the F_1 progenies were sterile when analyzed for pollen viability, and the pollen was not globular. The mitotic analysis of hybrids showed 2n=18 chromosomes. MS-70 SSR primer was identified as the polymorphic marker among the parents from a set of fifty-three SSR primers used for hybridity confirmation. Also, the morphometric characters of the F_1 hybrids were analyzed, and some showed the intermediate plant type from both the parents, viz. type of petiole, leaf edge, leaf waxiness, leaf colour, leaf hairiness, flower colour, and the anther type in the flowers are rudimentary which are visible very easily. On the basis of these analyses total of 34 F_1 plants out of 35 plants were identified as true hybrids. These true hybrids will be further amphidiplodized using colchicine and used for the alien introgression line development program.

Keywords: *Amphidiploid, Brassica fruticulosa, crop wild relatives, hybrid confirmation, wide hybridization*

Introduction

The *Brassica* is a genus of plant under the Brassicaceae family, includes rapeseed-mustard, oilseed crops (Burel *et al.,* 2000) and cruciferous vegetables like cabbage, kohlrabi, kale, turnip, broccoli, cauliflower, *etc.* (Singh, 2015). The family Brassicaceae includes more than 372 genera and 4060 species (The Plant List, 2010), comprising crops, weeds and ornamental plants (Love, 2004). In the Brassicaceae family, a vast diversity exists, which are the major sources of edible and non-edible oil, vegetables, fodder and forage and condiments. In India, rapeseed-mustard (*Brassica* species) is grown during the winter season under water limited conditions or rainfed conditions in North-Western and Central India. In *Brassica*, six species include three diploid species viz., *Brassica rapa* (AA, 2n=20), *B. nigra* (BB, 2n=16), *B. oleracea* (CC, 2n=18); and three amphidiploid species derived from the diploid species viz., *B. juncea* (AABB, 2n=36), *B. carinata* (BBCC, 2n=34), and *B. napus* (AACC, 2n=38) as depicted by the Triangle of U (Nagaharu and Nagaharu, 1935) which described the interrelationship among the cultivated diploid and amphidiploid species. Crop wild relatives (CWRs) of crop plants have possess extensive genetic diversity and are rich in the source of the different biotic as well as abiotic stresses (Kashyap *et al.,* 2023a), exploring the wild species is one of the major objectives for the plant researchers which can facilitate them to use these wild species traits for the improvement program (Kashyap *et al.,* 2022). The transfer of these gene(s)/traits from CWR to cultivated species is the major concern for the researchers. Wide or distant hybridization involves mating individuals from different species or genera, enabling the integration of highly divergent genomes into a single nucleus. This process effectively transcends the species barrier, facilitating gene transfer between genetically distant organisms. As a result, wide hybridization can induce significant changes in the genotypes and phenotypes of offspring (Kumar, 2015). This process involves the exchange or modification of genes through crossing between species from distant gene pools. This approach serves as a valuable tool for introducing beneficial traits in various agricultural contexts, particularly advantageous agronomic traits and play a crucial role in expanding the genetic diversity of crop species (Yadav *et al.,* 2018). Also, it is used as a tool for the transfer of gene(s) conferring tolerance of major abiotic and biotic stresses. It improves the quality characteristics of the cultivated species from wild species (Prakash and Chopra, 1988). Interspecific hybridization can dramatically widen the available genetic pools so that novel genetic variation can be utilized by plant breeders (Visarada and Venkateswaran, 2019).

In rapeseed-mustard, interspecific hybridization serves as a pivotal technique for investigating intergenomic relationships, facilitating the transfer of desirable traits between species, and for the development of synthetic amphidiploids, among other applications. The applications of wide hybridization for improving different agronomic traits, viz., pest and/or disease resistance or abiotic stress tolerance, increasing yield, or improving quality traits of the crop plants (Kumar, 2015; Kashyap *et al.,* 2022). Novel genetic variation for male sterility, drought, and pest and disease resistance has been frequently identified in related species and genera, and somatic hybrids provide an opportunity to exploit this expanded gene pool for improvements of *Brassica* crops in the future**.** This interspecific hybridization or crossing among species may result in the formation of an F_1 hybrid. F_1 hybrid is a term used in genetics and selective breeding. F_1 stands for Filial 1; the first filial generation plant offspring resulting from a cross-mating of distinctly different parental types (Khan, 2017). The primary breeding objective in Indian mustard and other crop improvement programs is to enhance seed yield while maintaining acceptable seed quality and ensuring stable performance across varying environmental conditions. To achieve stability in the production and development of introgression lines of *Brassica*, researchers reported some genotypes that exhibit resistance or tolerance to significant biotic stresses such as *Alternaria brassicae* and *Alternaria citri*, white rust (*Albugo candida*), powdery mildew (*Erysiphe cruciferarum*), and Sclerotinia rot (*Sclerotinia sclerotiorum*) (Sharma, 2015; Singh, 2015; Mehta *et al.,* 2023b, 2023a; Meena *et al.,* 2024) and resilience against abiotic stresses like heat and drought (Kashyap *et al.,* 2023b). By transferring the genes or genomic regions responsible for tolerance from these genotypes into elite cultivars of *Brassica*, will make aim to enhance the cultivars' resilience and durability under challenging environmental conditions. This approach ensures that the introgression lines maintain consistent performance and adaptability in agricultural settings.

In this study, a wild species of the genus *Brassica* is *B. fruticulosa* (FF, 2n=16) was used as the male parent species. A mediterranean wild cabbage, or twiggy turnip, is a member of the agriculturally significant genus *Brassica* described by Domenico Maria Leone Cirillo in 1792 and originated from southern Europe and North Africa (Tropicos, 2024). Therefore, in the present study, the true F_1 hybrids plant developed from a cross between *B. rapa* and *B. fruticulosa* were checked, and ultimately, the true hybrids will be further used for the pre-breeding program.

Materials and Methods

Development of plant materials

In the present research work, one accession of *Brassica rapa* (L.) (AA, 2n=20), *i.e.*, NRCPB *rapa* 8 (National Identity IC0623820 and Registration no. INGR17050) and one wild species *B. fruticulosa Cirillo*. (FF, 2n=16), their F_1 progenies were used which is from ICAR-NIPB, Delhi. Interspecific hybridization experiments were performed in 2019-20 by using *B. rapa* ssp. as a maternal parent (♀) and *B. fruticulosa* as a paternal parent (♂). The obtained seeds were sown in the year 2020-21 (October 2020) in the net-house facility at ICAR-NIPB, New Delhi.

Morphometric characterization

The phenotype/ morphometric traits of the parents and F_1 were observed for the deviation from the parental phenotype in hybrid plants (Kumar, 2015).

Colchicine treatment

The synthetic amphihaploid plantlets, derived from made crosses, were raised in pots at net house facility of ICAR-NIPB, Delhi. To induce amphidiploidization, the axillary buds and apical meristem of these hybrid plantlets were subjected to treatment with 0.2% colchicine, applied via non-absorbent cotton, administered every alternate day over a period of 5-7 days, specifically between 3-5 PM.

Pollen viability

A pollen viability test was performed to identify whether the pollen was sterile or fertile. In this study, acetocarmine solution (1% w/v) was used as a dye for staining the pollen(s). An unopened bud was dissected, and anther(s) was placed on a glass slide. A drop of acetocarmine stain was added on top of anther. The anther was then mashed using a needle. A smear was made, removing all cell debris. Then, the cover slip was carefully put on the stain. Lastly, the slide was then heat-fixed and placed under a microscope with 100X magnification for observation.

DNA isolation

A leaf sample of 300 mg was used for the extraction of total DNA. Then it was diluted in sterile distilled water, and the concentration of the working solution was made as 25ng/µl. For genomic DNA isolation, cetyl trimethyl ammonium bromide (CTAB) method (Doyle, 1990) was used.

Purification of genomic DNA

Five microliters of *RNase* solution (10 mg/ml) was added to 100 µl of DNA solution and incubated at 37°C for 1 hour. An equal volume of chloroform: Isoamyl alcohol (24:1) was then added to the sample and gently mixed by invert mixing. The mixture was then centrifuged at 13,000 rpm for 1 minute at room temperature to separate the aqueous layer, which was carefully collected. Subsequently, 1 ml of 100% chilled isopropanol was added to the aqueous phase and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the DNA pellet was washed by adding 500 µl of 70% ethanol, gently mixed, and centrifuged again at 13,000 rpm for 10 minutes at 4°C. After removing the supernatant, the DNA pellet was air-dried at 37°C, then dissolved in 100 µl of TE buffer and stored at 4°C for further use.

SSR primers

The 5′ 3′ anchored SSR primers were synthesized from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore and GCC Biotech Pvt. Ltd., India. The primers were diluted in nuclease free water according to their specified concentrations. Subsequently, they were diluted with nuclease free water to achieve a final working concentration of 10 µM. The primer sequences are detailed in Table 1.

Table 1: List of primers and their sequences used for the parental polymorphism and hybrid confirmation

S. No.	Primers ID	Forward primer	Reverse primer
Brassica rapa specific SSR primers			
1	R13	GTCGATGATCACGGAAGAGG	CTTGACAGCTACGGTTTGTCC
2	R5	ACCAAAATGTGTGAAGCCAC	CTTGTGGCCAGATTCATCAC
3	R3	AACTCGCTTTTACCGTCGTC	CAAGACGTGGAGCTGAAGTG
4	R17	TTCAAAGGATAAGGGCATCG	CTCAAAGGATAAGGGCATCG
5	R9	ATCGACATCGAACGAAAAGC	TCACCCTCTACCTCCACCAC
6	MS_65	AAGGCTGTCGTTTCGTCTCT	GTGACGACGATCTCCAAGAA
7	MS_66	GTACTACGAAGACGAGCGTGG	TCCAAAGCGGCCATGCA
8	MS_67	TGCCAACAAATCAAGGATGC	CCGAAGTTCACTTGTTATTCCAAC
9	MS_68	TTCCAGGTTGGAACCACTTC	CTGCATCCACATGAAACCAC
10	MS_69	AAGAAGCAAAACTGGTTCGTTTCTT	CAGTAAGTAACCACGTTGACCTCG
11	MS_70	GAGCTATCTATTCTTTGTGACATTGA	AATCCTTGCTTGAGTACTCAAGTC
12	MS_71	CTCTCGTCTCGGAGGATCTAAA	GTGAGAGTGGTTGCTGAGTGAG
13	MS_72	AGACTGCTGGGGAAGGTGCA	ACAACGGAAATGAGCTTCGTTCA
14	MS_73	GGGCCAAGTTACATGGAAAA	GAAGGAGGATGAGAGCCGTT
15	MS_74	TGTGTCGCTCGTCTACGTCT	ACCATCGACTTCGTGGAAAC
16	MS_75	TTTCAGCCATGGAGGACGTT	AGCATTGCACCAGTCTCAAAA
17	MS_76	AGGGAGTTGAAACGAAAGCA	CAATACAAAAACCGGGCAAG
18	MS_77	AACAAAAACCTTGGCTCCCC	ACGCTCCACTGAAAGACGTTAC
19	MS_78	GTCGAACATCCCTCGCTAGT	AGTAGATGTGGTCGCCTCCT
20	MS_79	GCTTTCCCTTCACTGCTCTCT	GGGTGAGATTGTTGGTGAGTCT
21	MS_80	AACAGTTTCTACGCATCGTGT	GCGTTTTCGATTTTAAATTTTG
22	MS_81	AGACGAGACCTTCGATCCTTC	GCCTTCTTCAGAACTTCCTC
23	MS_82	GCTGGTTCCAGCTAATGGTTAC	TGAATTTATGAGATTCGGATTGG
24	MS_83	GCATCTTCGGCTTGAGTTG	TGAGGAGAAGTAACCGAGCA
25	MS_84	GTAGATGTGTGCCGTGGATATG	GTGCGTCGTACCAAGTCTGTCTA
26	MS_85	TTGGAGAACATGAAGAAGCA	GGGACTTTTATGACAAAACCG
27	MS_86	CGAACTGCTATCATCAGAATTGG	CCGTTTATCAAGGCCTCGTC
28	MS_87	TTCACTTCTGCGTTCGTTGCG	ACAATGAGGAGCTGCACAGATG
Brassica napus specific SSR primers			
29	A13	TCTTGAGCAAAGAAACTTGG	CAAACTGAGCCATACACAAAGG
30	A27	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC
31	A41	AAGAACGTCAAGATCCTCTGC	ACCACCACGGTAGTAGAGCG

32	A19	GCCTTAGATTAGATGGTCGCC	ACTTCAGCTCCGATTTGCC
Brassica juncea specific SSR primers			
33	UGM624	ATACCTGGAGGGTACCTTCC	TTGGTTCGAAATTGAATCG
34	UGM625	GCAATGGGTATGCCTGTTG	GAACTTGAGCACATTTCAACTCG
35	UGM626	GTGCTCCAAATGGGAAG	CGCTTCATTACAGGGAGC
36	UGM627	CATCGTCAAATCATTCCTCG	AACGTTGACCATTGTTCATCTG
37	UGM628	GATTCGTGATTGGCCAAG	GATTCGTGATTGGCCAAG
38	UGM629	TCAGAAGCTTTAGTGTAGCGTC	CTTTCTTTCTTCTTATCCAGAGAAC
39	UGM630	GAAGATCTCTCGGATCTTCG	GTGGTTGTCACCGGTG
40	UGM631	AGAGATTTGCTGCAGAACGAG	TGCCATGAACTGCATCTG
41	UGM632	ACGACCCTTCACCTTCCTC	ACACATTCCCGTCCTGC
42	UGM633	CCAGATGCCTCCAGAGC	GAGACTGAGGTTGCTGTGC
43	UGM658	ACTTGCTCAATGAGATGGCA	CGACAAAGGCCTCTCTCTCTT
44	UGM704	TCATGCCACTAACCGGTTG	ACCACAGCCAACGTATTCAGG
45	UGM804	TAGGAGAGATAGTGCATAGCAAGTC	GAGGAGGAAGATTTTGAACTGC
46	UGM808	GAGGCTGAGTTGGTTGAGG	CTCGTGGCTCAGTCAAGC
47	UGM816	TCATCCCATGGCTTAACTTC	TGGAAAAGCCGTTGGAG
Diplotaxis erucoides specific SSR primers			
48	DE 47	AAGAAGGTGCAAAAGGCAGA	TCCCAAGGAAGCTGAGAGAA
49	DE 48	CGAGGAACATGAATCCGTCT	AAGCGAGAGATCAGGAACCA
50	DE 49	TTATCACAGGGCCAAAAAGC	TGTATTGCGCCAAGTTGAAG
51	DE 51	AAGACAACTGGCTCCGAAGA	TTATGCACCATTCTCCACCA
52	DE 87	TAGTGAATTGCACCGTCAGC	AGCGAAAGCTTGGAGAAACA
53	DE 100	GACACAATGGGAATGCAGTG	CAAGGAGAAGCTTGGTGGAG

Hybrid confirmation

Using Parental polymorphism

Parental polymorphism was carried out with parental genotypes *B. rapa* and *B. fruticulosa*. The identified polymorphic primers for the respective parents, *B. rapa* and *B. fruticulosa,* which showed the best amplification among various primers, were then further selected to confirm the true F_1 hybrids. The gradient amplification cycle comprised of initial denaturation for 5 minutes at 95^{0}C, 35 cycles of 95^{0}C for 1 minute, annealing ranging from 56^{0}C to 60^{0}C for 45 minutes and extension at 72^{0}C for 2 minutes, followed by a final extension at 72^{0}C for 10 minutes. The best amplification was observed at temperature 56^{0}C, at which further PCR reactions were carried out.

Cytogenetic analysis

Mitosis

Root tips were collected early in the morning and immersed in a 0.002M solution of 8-hydroxyquinoline (8-HQ) for preservation. They were subsequently fixed in freshly prepared Carnoy's solution (Ethanol: Glacial acetic acid, 3:1 v/v ratio). After 4 hours of incubation at 4ºC, the roots were transferred to 70% ethanol. The chromosome number was assessed following the protocol described by Snowdon *et al.* (1997). For slide preparation, root tips were treated with a combined enzyme solution of 5% cellulase and 1% pectinase at 37°C for 45 minutes, and excess enzyme solution was removed with filter paper. The tissue was fragmented with a needle to release cells, and a coverslip was applied to spread cells, pressed gently under filter paper. Slides were then fixed in liquid nitrogen, coverslips removed, air-dried, stained with DAPI (4,6-diamidino-2-phenylindole), and re-covered. Chromosomes were visualized using a fluorescent light microscope (Carl Zeiss Axiolab 5)(Rao *et al.,* 2024).

Results and Discussion

Morphometric observation of plantlets

The phenotypes of both the parents and 35 progenies of *Brassica* were observed. All the progenies have shown to be intermediate for most of the morphological characters respective to the parents. Various morphological observations were shown in F_1 hybrid plants in comparison to parent plants (Fig. 1-3 and Table 2).

Colchicine treatment and pollen viability

The chromosome number was doubled with the action of colchicine treatment and the amphidiploid seeds of *B.*

rapa and *B. fruticulosa* were obtained.

The pollen viability test was performed to analyze the fertility of the pollens in percent and was performed for both the parents and F_1 hybrids. Acetocarmine dye was used for staining the pollens. This process takes up the fact that fertile pollen takes up dye acetocarmine while sterile pollens do not. The results were observed under a light microscope which showed fertile pollens appearing red. In contrast, sterile pollen appeared to be yellow or colourless (Fig. 4). As the F_1 generation is amphihaploids, the hybrids obtained were sterile. The parents, *B. rapa* and *B. fruticulosa* showed 95.50 % and 92.61% pollen fertility, respectively (Table 3). All of the F_1 progenies showed 0 % pollen fertility. Out of 35 F_1 progenies analyzed, 34 progenies produced sterile pollens, and one progeny (i.e., 5-3) produced fertile pollens (Table 5). In the case of 5-3 progenies, the pollen fertility is 10.71% observed (Table 4).

Morphometric characterization

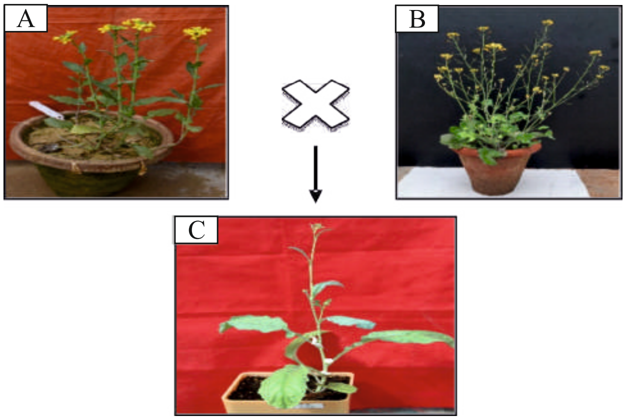

Fig. 1: Phenotypes of the parental and hybrid progenies of *Brassica.* The diagram represent; (A) *B. rapa* (genome: AA; 2n=20); (B) *B. fruticulosa* (genome: FF; 2n=16); (C) F_1 hybrid plant (genome: AF; n=18)

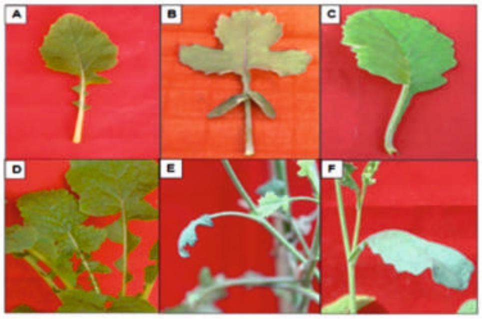

Fig. 2: Leaf morphology of the parental and hybrid genotypes of *Brassica.* Photos of leaf: (A) *B. rapa*; (B) *B. fruticulosa*; (C) F_1 hybrid plant; and Hairiness on leaf: (D) *B. rapa*; (E) *B. fruticulosa*; (F) F_1 hybrid plant

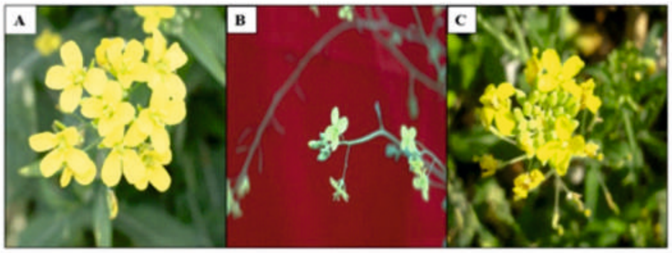

Fig. 3: Morphological traits of the inflorescence of the parental and hybrid genotypes of *Brassica*. Photos of flower buds: (A) *B. rapa*; (B) *B. fruticulosa*; (C) F_1 hybrid plant

Table 2: Comparison of morphological characters of parents and their F_1 hybrids (*B. rapa* × *B. fruticulosa*)

Characters/ descriptors	*Brassica rapa* Female parent (♀)	*Brassica fruticulosa* Male parent (♂)	F_1 hybrids
Type of petiole	Sessile	Petiolated	Petiolated
Leaf edge	Serrated	Serrated	Slightly serrated
Leaf waxiness	Present	Present	Present
Leaf colour	Medium green	Dark green	Light green
Leaf hairiness	Less dense	Dense	Less dense
Anther type	Mature anther	Mature anther	Rudimentary anther
Flower colour	Dark yellow	Light yellow	Light yellow

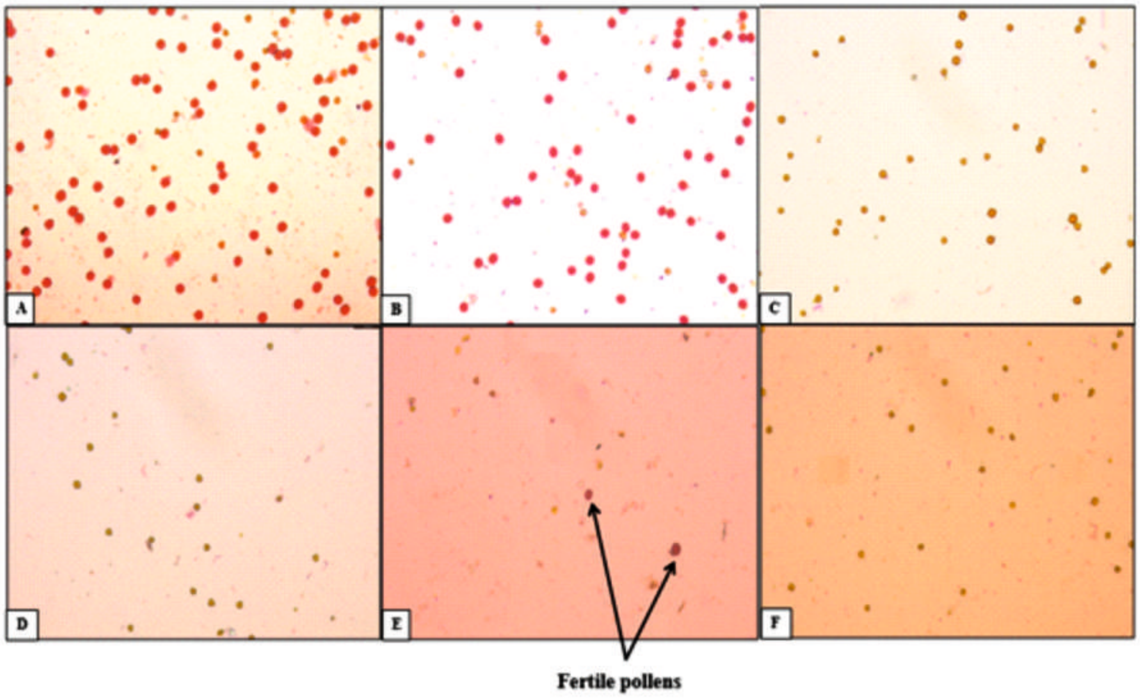

Fig. 4: Analysis of pollen fertility of the parents and F_1 hybrids of *Brassica*. Photos of pollens: (A) *B. rapa*; (B) *B. fruticulosa*; and F_1 hybrids plants (*B. rapa* × *B. fruticulosa*); (C) 5-1; (D) 5-2; (E) 5-3; (F) 5-4. Magnification 100x

Table 3: Fertility percentage of parental lines of *Brassica*

Cross plant	Total pollens	Fertile pollens	Sterile pollens	Percent fertility (%)
B. rapa	178	170	8	95.50
B. fruticulosa	149	138	11	92.61

Table 4: List of sterile F_1 hybrid plants of *Brassica*

Cross	No. of hybrids	No. of fertile hybrids	No. of sterile hybrids
B. rapa × *B. fruticulosa*	35	1	34

Table 5: Fertility percentage (%) of F_1 progenies of *Brassica*

No. of F_1 progeny	Total pollens	Fertile pollens	Sterile pollens	Percent fertility (%)
5-1	27	0	27	0
5-2	22	0	22	0
5-3	28	3	25	10.71
5-4	46	0	46	0
5-5	4	0	4	0
5-6	23	0	23	0
5-7	44	0	44	0
5-8	30	0	30	0
5-9	50	0	50	0
5-10	43	0	43	0
5-11	18	0	18	0
5-12	5	0	5	0
5-13	13	0	13	0
5-14	3	0	3	0
5-15	3	0	3	0
5-16	38	0	38	0
5-17	33	0	33	0
5-18	61	0	61	0
5-19	3	0	3	0
5-20	10	0	10	0
5-21	25	0	25	0
5-22	86	0	86	0
5-23	83	0	83	0
5-24	51	0	51	0
5-25	22	0	22	0
5-26	157	0	157	0
5-27	13	0	13	0
5-28	123	0	123	0
5-29	21	0	21	0
5-30	5	0	5	0
5-31	27	0	27	0
5-32	2	0	2	0
5-33	7	0	7	0
5-34	12	0	12	0
5-35	33	0	33	0

DNA quantification

The DNA isolated from the parental lines and the F_1 progenies of *Brassica* produced discrete bands on 0.8 % agarose gel when subjected to electrophoresis (Fig. 5).

Parental polymorphism

Parental polymorphism was done to identify the markers which can be used for the confirmation of F_1 hybrids generated from the specific crosses. For the identification of polymorphic SSR primers, parental DNA, *i.e*., *B. rapa* and *B. fruticulosa,* was used as a template and different primers were used for the amplification. Among all the primers used only those which showed different amplification for both the parents were chosen further for hybridity confirmation. Various primers used for the parental polymorphisms are shown in Table 1. Among Fifty-three SSR primers, MS-70 primer shows the best amplification in both parental genotypes (Fig. 6A and B).

Parental polymorphism through gradient PCR

The gradient PCR was carried out with the MS-70 primer as per the above-stated thermal cycle regime. As the intensity of the amplified product with this primer was not so high, the primer was amplified at different temperatures to determine the specific temperature for annealing further in the hybrids. For this PCR with 5 temperature gradients *i.e*., 56 to 60°C, was used with parental DNA only. The best and most intense amplification was observed at 56°C at which amplification was observed (Fig. 6 C and D).

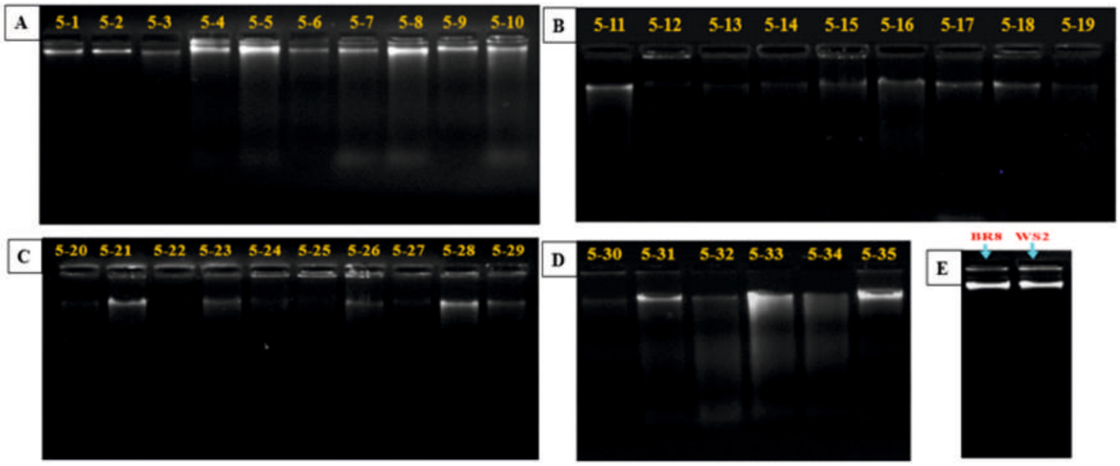

Fig. 5: DNA quantification of parents and F_1 hybrids plant using 0.8% agarose gel; (A) Progenies 5-1 to 5-10; (B) Progenies 5-11 to 5-19; (C) Progenies 5-20 to 5-29; (D) Progenies 5-30 to 5-35; (E) (BR8; *B. rapa* 8 and WS2; *B. fruticulosa*)

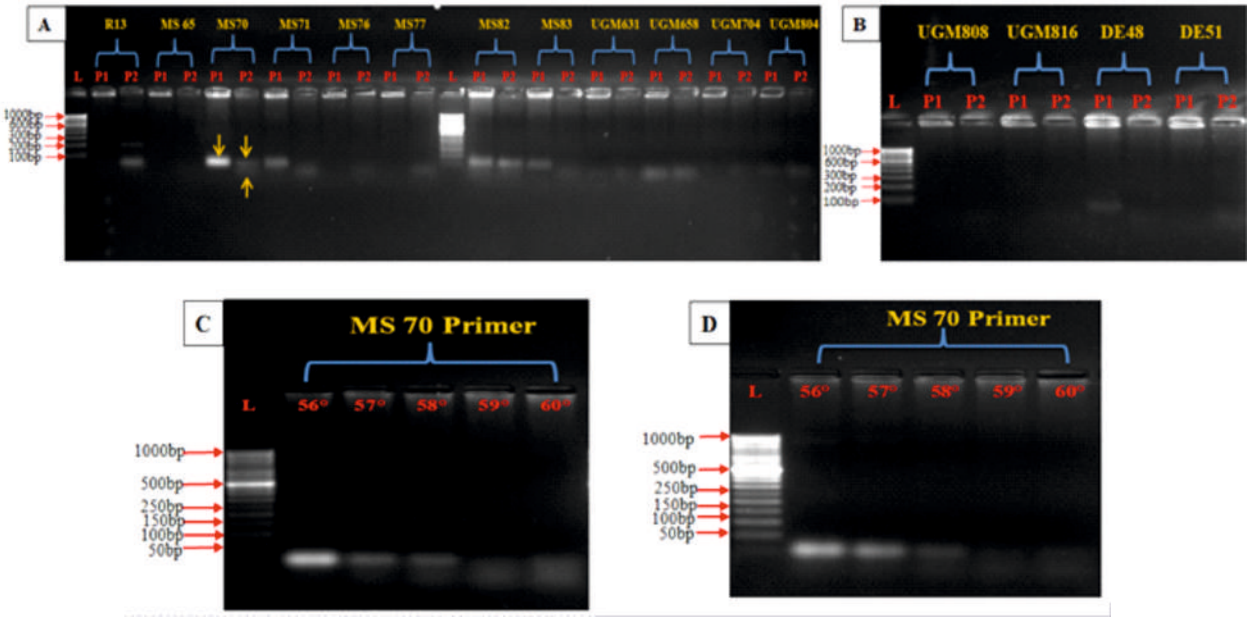

Fig. 6: Analysis of DNA polymorphism in the parental genotypes of *Brassica*.(A) and (B) Amplification with various sets of primers; Gel description: Ladder: 100 bp; P1: *B. rapa*; P2: *B. fruticulosa* and amplification with MS_70 Primer at different Tm; Amplification by gradient PCR; (C) Ladder: 50 bp; *B. rapa*; (D) Ladder: 50 bp; *B. fruticulosa*

Hybrid confirmation

Confirmation of F_1 hybrids using SSR molecular markers

For confirmation of the hybridity status of various plantlets generated from different crosses, the SSR primers were used. Firstly, the DNA from all the parents and F_1 plants were isolated using the CTAB method. The parental polymorphisms were checked using SSR primers between the parents in combination. The MS-70 primers, which showed the polymorphism between the parental genotypes of the particular crosses, were used to confirm the hybridity in the respective F_1 plantlets. The polymorphic primer was selected, which differentiates both the parents and since the *B. rapa* was used as the female parent, the presence of any amplification from the male parent was accessed. The markers available from the cultivated genome and a few from the *Diplotaxis erucoides* were used; these primers are not much amplified in the *B. fruticulosa*, which was used as a female parent. A total of 35 F_1 plants were analyzed for hybridity using MS-70 primer, and among them, a total of 16 progenies, which are 5-3, 5-5, 5-8, 5-14, 5-18, 5-21, 5-22, 5-24, 5-25, 5-27, 5-28, 5-30, 5-32, 5-33, 5-34, 5-35 shows specific amplification of both the parents which was confirmed the hybridity, while total 19 progenies which are 5-1, 5-2, 5-4, 5-6, 5-7, 5-9, 5-10, 5-11, 5-12, 5-13, 5-15, 5-16, 5-17, 5-19, 5-20, 5-23, 5-26, 5-29, 5-31 having amplification of maternal genome or non-specific amplification (Fig.7; Table 6).

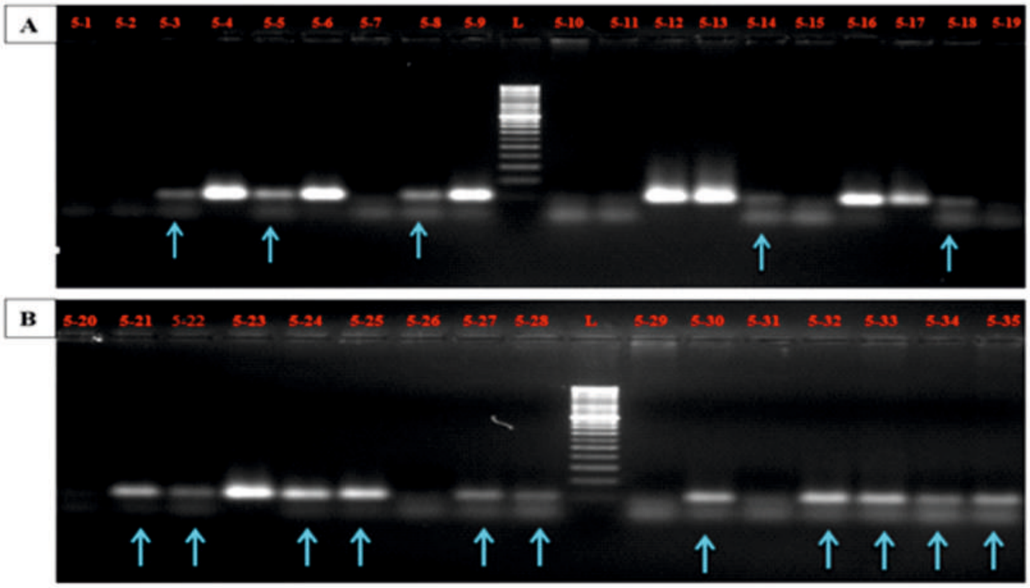

Fig.7: Confirmation of F_1 hybrids progenies through PCR amplification with SSR primer MS_70; (A), 5-1 to 5-19 F_1 progenies; (B), 5-20 to 5-35 F_1 progenies; L=Ladder 50 bp; (Blue arrow shown for the confirmed hybrids)

Table 6: List of confirmed or not confirmed F_1 hybrids

Cross	No. of hybrids	No. of confirmed hybrids on the basis of SSR amplification	No. of not confirmed hybrids
B. rapa × *B. fruticulosa*	35	16	19

Confirmation of F_1 hybrids using cytogenetic analysis

The mitotic analysis for F_1 hybrids were determined for their chromosome numbers. The diploid (2n) parental species, *B. rapa* and *B. fruticulosa*, have 2n=20 and 2n=16 chromosomes, respectively. In contrast, the synthetic F_1 amphihaploid hybrids (AB) exhibited 2n=18 chromosomes. This chromosome count reflects the presence of one haploid set of chromosomes derived from each parent, *B. rapa* and *B. fruticulosa*, determined using DAPI staining (Fig. 8).

Observation of plant phenotypes

Weerakoon *et al.* (2009) also observed the morphology of 70 resulting F_1 hybrid plants between *B. juncea* and *B. oleracea*. They evaluated the morphological characterization or morphological traits such as leaf/petiole/stem colour and hairiness, flower colour *etc*., were observed for each plant. A similar result was reported by Garg *et al.* (2007), that the F_1 hybrids between *B. rapa* and wild crucifers *D. erucoides* and *B. maurorum* in each combination were morphologically intermediate to the respective parents. Kumar *et al.* (2018), also reported the morphological observation of F_1 plants obtained through sexual hybridization between *B. juncea and B. fruticulosa*. The F_1 plants (2n=34) were intermediate for most of the morphological attributes, including leaf colour, leaf hairiness, petal colour, *etc*. Similar morphological observation of F_1 plants was also reported by others (Chandra *et al.,* 2004; Chen, 2011; Choudhary and Joshi, 2012; Tu *et al.,* 2020).

Pollen viability test of parents and F_1 hybrid plants of *Brassica*

Garg *et al.* (2007), also reported that the F_1 hybrids developed by hybridizing *B. rapa* with wild crucifers *D. erucoides* and *B. maurorum* in each combination were completely male sterile. Morphologically, both the F_1 hybrids were intermediate to the parents. Male fertility, as indicated by pollen stainability in 2% acetocarmine, was very low or less than 2%.) reported the true hybrid

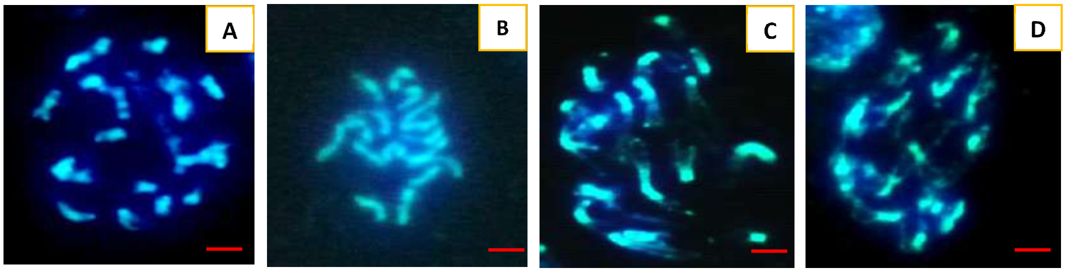

Fig. 8: Confirmation of mitotic chromosome analysis in parents and F_1 hybrids: Parents: A) *B. rapa* (♀, 2n=20); B) *B. fruticulosa* (♂, 2n=16); F_1 hybrids: (C-D) *B. rapa* ×*B. fruticulosa* (2n= 18) Scale bar: 500µm

nature of the offspring through morphological analysis. They assayed the pollen grain fertility of F_1 from pollen grain stainability with acetocarmine solution (2%); F_1 plants were highly pollen-sterile.

Similarly,), observed that diploid parental species, *B. rapa* and *B. fruticulosa*, exhibited high pollen fertility rates of 91.12% and 83.23%, respectively. In contrast, F_1 hybrids displayed a significant reduction in pollen fertility, with only 8.60% observed. This reduction indicates a substantial level of pollen sterility in the hybrids, likely attributed to meiotic irregularities and segregational anomalies.), made natural crosses among the cultivated *Brassica* species *B. napus, B. rapa,* and *B. juncea* and their two weedy relatives *B. nigra* and *S. arvensis*. At flowering, pollen viability and fertility of these plants were examined to differentiate the true interspecific or intergeneric hybrids from selfed progenies of parental plants. Plants with non-viable pollen grains and poor female fertility were considered interspecific and/or intergeneric hybrids, whereas the plants that had normal viable pollen grains and normal female fertility were classified as selfed progeny of parental species.

Confirmation of F_1 hybrids using SSR molecular markers and cytogenetics analysis

Weerakoon *et al.* (2009), confirmed the F_1 hybrid which was derived from *B. juncea* and *B. oleracea* genotypes in which 70 resulted F_1 hybrid plants were evaluated. They confirmed the true hybrid nature of the putative F_1 hybrid plant by using SSR molecular markers and confirmed four true hybrids. Similarly,), confirmed the hybridity of *B. juncea* L. based on the complementary banding patterns between the hybrid plants and parents; the polymorphic SSR markers were identified as the specific markers which enable to distinguish and identify hybrid from their parental lines. This study suggests that the identification and use of SSR markers can effectively reduce the cost and simplify the procedure of hybridity and purity testing. Molecular confirmations out of 70 hybrids, four F_1 hybrids were confirmed to be true hybrids with the SSR marker (Kumar *et al.*, 2018).

Conclusion

Based on the present study, it was concluded that the phenotype of F_1 hybrid plants, which were developed from the crosses between *B. rapa* and *B. fruticulosa,* were intermediate to their respective parents for most of the morphological traits. Almost all of the F_1 hybrid plants have sterile pollen or reduced pollen fertility. It is required to check in progeny 5-3, which have 10.71 % of pollen fertility and are confirmed as fertile hybrids. The complementary banding patterns generated through the MS-70 SSR marker, as well as the diploid chromosome observed in F_1 hybrids, enabled us to confirm the true hybrid nature of F_1 plants from their parental lines. However, codominance could not be observed using this primer. Further molecular and advanced cytological analysis is required by the screening of more numbers of SSR primers to detect codominance conditions.

Acknowledgement

Funding: This research was funded under the collaborative project with the Alliance of Bioversity International and CIAT under sub-agreement LOA No. L20INDIA155.

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