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Augmenting Genetic Diversity in *Brassica juncea* through its Resynthesis using Purposely Selected Diploid Progenitors

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ABSTRACT

Resynthesis of novel *B. juncea* (AABB, 2n=36) following hybridization between genetically diverse germplasm of progenitor species (*B. rapa* and *B. nigra*) was explored to augment genetic diversity in cultivated *B. juncea*. Seventy two reciprocal crosses were attempted between *B. rapa* (10) and *B. nigra* (9), of which only fifteen were successful and these were subjected to colchiploidy. The resultant amphiploids (11) were confirmed morphologically and cytologically to be resynthesized *B. juncea* (AABB; 2n=36). Stable A_3 resynthesized *B. juncea* (11) as well as the diploid parents (19) and natural *B. juncea* (4) genotypes were assayed for genetic diversity based on DNA polymorphism generated by SSR primers. Neighbour-joining analysis revealed a clear distinction between *nigra*, *rapa*, resynthesized *B. juncea* and natural *B. juncea*. As a group resynthesized *B. juncea* genotypes were closer to *B. rapa* and natural *B. juncea* as compared to *B. nigra*. The inherent genetic diversity in the germplasm of progenitor diploid species was apparent from SSR based diversity assays. Resynthesized genotypes also possessed a higher frequency of unique alleles. That the resynthesis route generated significant variability was evident from dissimilarity coefficients ranging from 0.13 to 0.74.

Keywords: cytology, polyploidy, SSR

INTRODUCTION

Cultivar development programmes in *Brassica juncea* have traditionally relied primarily on selections from segregating progenies of crosses between the genotypes having narrow genetic base (Srivastava *et al*. 2001). This is now believed to be the primary reason for relatively a slower pace of productivity upgradation. To realize continued gains in productivity, it is imperative that newer sources of variation are mobilized for broadening the genetic base of source germplasm. Resynthesis of *B. juncea* has been suggested (Olsson 1960; Prakash 1973; Song *et al.* 1995 and others) to be one such option. However, most of the past studies focused primarily on demonstration of the concept, and involved sampling of rather a small proportion of genetic diversity available in the basic diploid species, especially *B. nigra*. Resynthesis and assessment of eight *B. juncea* combinations were reported by Prakash (1973) wherein variability for different phenotypic traits was reported but no attempt was made to establish genetic distinctiveness of resynthesized genotypes using available diversity analysis protocols. Further, only one *B. nigra* genotype seemed to have been utilized for resynthesis. Srivastava *et al*. (2001, 2004) found newly resynthesised *B. juncea* genotypes (6) to be distinct from natural *B. juncea* on the basis of DNA polymorphism generated by AFLP primer. The extent of genetic diversity inherent in the source diploid progenitor species was, however, not reported.

In the present investigations, we report *B. juncea* resynthesis through hybridization between genetically and geographically diverse *B. nigra* and *B. rapa* progenitors. Genetic distinctness of resynthesized *B. juncea* as well as the diversity intrinsic in diploid progenitor germplasm base is also reported. Of the 11 synthetic *B.juncea* genotypes, two had *B. nigra* as the cytoplasm donor parent. Natural *B. juncea* is known to carry cytoplasm from *B. rapa* parent (Erickson *et al*. 1983; Banga *et al*. 1983).

MATERIALS AND METHODS

Resynthesis of *B. juncea* and morphological evaluation

Plants from the identified inbred lines (I_5) of *B. nigra* (9) and *B. rapa* (10) genotypes were used to carry out 72 two way (*rapa* × *nigra*, *nigra* × *rapa*) interspecific hybridizations. *In vitro* sequential ovary culture technique (Garg *et al.* 2007) was used to obtain hybrid seeds/seedlings as *in vivo* pollinations failed to set seed. Hybrid seedlings were raised on Murashige and Skoog's medium (Hi Media) supplemented with benzyl amino purine (BAP, 0.5 mg/l) for further multiplication. Young plantlets were transferred to the field after hardening and their axial buds treated with colchicine (0.2%) to induce chromosome doubling. Meiotic studies were conducted to confirm chromosome number in the interspecific hybrids (2n=18) and induced amphiploids (2n=36). Young inflorescences were fixed in Carnoy's solution (ethanol: choloform: acetic acid, 6: 3: 1). Anthers having pollen mother cells were squashed in 2% acetocarmine and viewed under the microscope for meiotic configurations. Male fertility status of the interspecific F_1's and the consequent amphiploids was inferred from pollen grain stainability in 2% acetocarmine. For morphological assessments, resynthesized genotypes (A_3) were evaluated along with natural *B. juncea* and the diploid progenitor species. Experiment was conducted in a split plot design with three replications.

Genetic diversity analysis

Test germplasm comprising resynthesized *B. juncea* (11), diploid progenitor genotypes (19) and natural *B. juncea* (4) was assayed for molecular diversity using A- and B-genome specific SSR primers (Suwabe *et al.* 2002; Lowe *et al.* 2004; Choi *et al.* 2007). Nuclear DNA was extracted from the young leaves (Doyle and Doyle 1990) of the single representative plant of each test germplasm and subjected to amplification using SSR primers following the protocol as suggested by Shokeen *et al.* (2007). Polymorphic information content (PIC) value for each assayed primer was cal-

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culated as:

$$PIC = 1 - \sum_{j=1} P_{ij}$$

where P_{ij} is the frequency of j^{th} allele in the i^{th} primer.

The allelic polymorphism data from all the primers were used to estimate the genetic diversity. The SSR profile of each genotype was scored for the presence (1) or absence (0) of alleles (fragments). The proportion of shared fragments was calculated (Nei and Li 1979) for all pairwise comparisons as:

F = 2 (number of shared fragments/number of total fragments)

Dissimilarity index D was calculated as D = 1-F to obtain the D matrix between test genotypes. A weighted neighbour-joining (NJ) analysis was performed on the dissimilarity matrix to determine clustering behaviour. Support for clustering was determined by a bootstrap procedure applied on 198 SSR bands. DARwin software was used for statistical analysis (Perrier *et al.* 2003). Chemicals for tissue culture and molecular investigations were sourced from Hi Media and Promega chemicals respectively.

RESULTS

Seventy two reciprocal combinations between *B. rapa* (10) and *B. nigra* (9) were attempted, of which only fifteen were successful (**Table 1**). Chromosome doubling was achieved only in 11 combinations. The majority of the F_1 hybrids resembled natural *B. juncea* in morphology. The flowers of the F_1 hybrids had small anthers having sterile pollen grains. In contrast, the colchicine-induced amphiploid shoots (resynthesized *B. juncea*) possessed large buds and flowers with normal anthers and fertile pollen grains (60-95% pollen grain stainability). Out of the eleven resynthesized *B. juncea* genotypes, nine had *B. rapa* cytoplasm while remaining two (SJN8, SJN14) carried *B. nigra* cytoplasm. The meiotic analysis of resynthesized *B. juncea* indicated expected euploid chromosome complement of 2n=36 with 18II as the predominant (.58) meiotic configuration at diakinensis/metaphase. First generation amphiploid plants showed aberrant meiosis as well as lower seed set which improved significantly following two generations of selfing. Normal 18II and 18-18 separation at anaphase I (**Fig. 1**) was apparent only by the A_3 generation of amphiploidy.

Table 1 Pedigree record of resynthesized *B. juncea* alongwith source of the parental germplasm.

Genotype code*	Parental combination			
B. juncea	*B. rapa*		*B. nigra*	
	Genotype	Source	Genotype	Source
SJR 2	Sunford	USA	PBn14	Pakistan
SJR 6	Sunbean	Norway	PBn13	India
SJR 17	TCN-01	India	PBn16	India
SJR 23	Sonja	Finland	PBn16	India
SJR 57	Mitra	India	PBn13	India
SJR 59	EC 513427	Canada	PBn22	Pakistan
SJR 63	CH 1	India	PBn7	Germany
SJR 76	EC 513427	Canada	PBn19	India
SJR 113	EC 3390-101	Sweden	PBn20	Canada
SJN 8	EC 513426	Canada	PBn17	India
SJN 14	Torch	Canada	PBn12	India

* SJR denotes combinations with *B. rapa* as female parent whereas SJN denotes genotypes with *B. nigra* as female parent

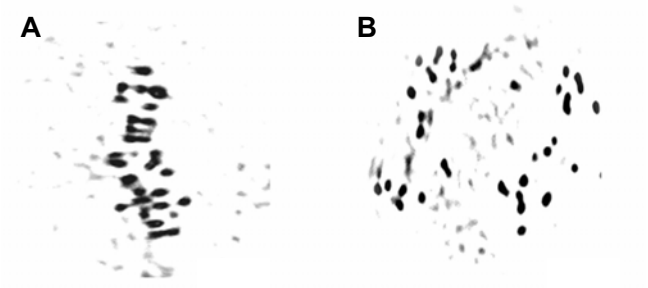

Fig. 1 Meiotic configurations in PMC are of resynthesized *B. juncea* amphiploids. (**A**) 18II, (**B**) 18-18 distribution at anaphase in A_3 amphiploids.

Genetic diversity

SSR profile of the 34 test genotypes was scored for allele-specific polymorphism against 46 primers. The number of amplified alleles per genotype varied from 2-12 and the PIC values ranged from 0.20 to 0.89, suggesting high informative content of the primers assessed. Most of the primers had PIC value in excess of 0.60. A total of 198 alleles were scored. The dissimarity coefficients calculated between all pair-wise comparisons ranged from 0.13 to 0.74. SSR polymorphism profile was screened for unique alleles (uncommon) in natural *vs.* resynthesized *juncea* types. It was observed that resynthesized genotypes had more unique alleles (**Table 2**) as evident from the allele richness index (4.34 *vs*. 3.25). The weighted neighbour-joining (NJ) analysis (**Fig. 3**) clearly differentiated the 34 genotypes into four species-specific clusters at bootstrap values varying from 55 to 100. High bootstrap values (>50) were indicative of consistency of the clusters. *B. nigra* genotypes appeared most distinct and were grouped together at a bootstrap value of 100. The strain PBn12 from India, appeared most diverse among the *B. nigra* genotypes. In the *B. rapa* group, Sunford and EC 3390-101 were most distinct as was apparent from longer branch lengths. The *B. rapa* cluster was clearly separated from the *B. nigra* cluster. The resynthesized *B. juncea* group was placed between *B. rapa* and natural *B. juncea*. It comprised two major nodes, suggesting significant dissimilarity within the group. Node 1 included five genotypes of which SJR17, SJR23 and SJR59 were genetically more akin, while SJR6 and SJR2 appeared relatively diverse. The second node in resynthesized *B. juncea* cluster was further divided into two groups, each having three genotypes. The fourth group comprising natural *B. juncea* appeared to be quite heterogeneous. Of the four genotypes evaluated, Indian genotype (JC Bold) was separated out on one side whereas Australian (JN-004, JR-049) and Chinese (XINYOU 4) were grouped together on the other side of the tree. Natural *B. juncea* types were intermediate to the progenitor species, whereas the resynthesized ones were closer to *B. rapa* and natural *B. juncea*.

Table 2 Unique alleles identified in resynthesized *B. juncea*

Group	Unique alleles		Specificity	
	№	Richness	*B. rapa*	*B. nigra*
Natural	13	3.25	2	7
Resynthesized	48	4.36	4	18

Agronomic assessment

The data for agronomic attributes of resynthesized *B. juncea* is presented in **Table 3**. As is apparent, the resynthesized *B. juncea* was generally lower yielding than the natural *B. juncea*. Further, there appeared to be no correlation between the performance of the resynthesized digenomic genotypes and their monogenomic parents. Multiple correlation analysis indicated that the diploid parent species explained only 10.3% of variability observed in the resynthesized *B. juncea*. All the resynthesized A_3 *B. juncea* genotypes revealed a high degree of self fertility.

Inbreeding in *Brassica* polyploids is a natural outcome of amphiploidy.

DISCUSSION

Digenomic *B. juncea* has evolved following multiple natural interspecific hybridizations between *B. rapa* and *B. nigra* (Prakash and Hinata 1980), *B. rapa* being the cytoplasm donor parent during evolution (Banga *et al.* 1983;

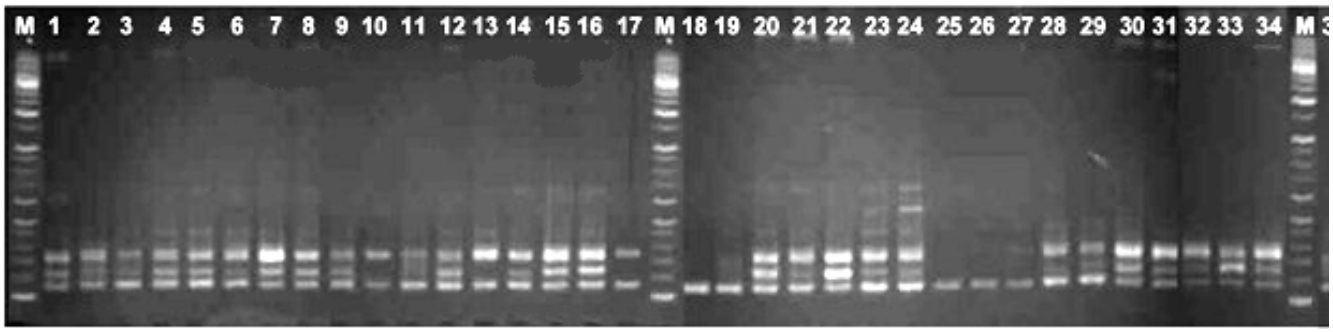

Fig. 2 Polymorphism in resynthesized *B. juncea* (11-21) and its progenitor species, *B. rapa* (1-10) and *B. nigra* (22-30) along with natural *B. juncea* (31-34) as generated by SSR primer BRMS 034. The test genotypes are Sanya (1), EC 3390-101 (2), EC 513426 (3), Mitra (4), EC 513427 (5), Torch (6), TCN 01 (7), Sunbean (8), CH I (9), Sunford (10), SJR 2 (11), SJR 6 (12), SJR 59 (13), SJR 23 (14), SJR 17 (15), SJR 76 (16), SJR 113 (17), SJN 8 (18), SJN 14 (19), SJR 57 (20), SJR 63 (21), PBn12 (22), PBn17 (23), PBn7 (24), PBn22 (25), PBn20 (26), PBn14 (27), PBn16 (28), PBn19 (29), PBn13 (30), JN 004 (31), JR 049 (32), XINYOU 4 (33) and JC Bold (34). M indicates molecular marker.

Table 3 Morphological characterization of the test germplasm.

	Plant height (cm)	Primary branches	Secondary branches	Main shoot length (cm)	Pods on main shoot	Pod length (cm)	Seed size (g)	Seed yield* (g)
B. rapa								
Sunford	81.6	3.5	5.0	39.7	18.5	4.0	3.0	19.2
EC 513427	88.6	3.0	2.5	35.9	22.5	4.4	3.3	20.1
EC 513426	81.9	3.0	4.0	35.9	20.0	3.6	2.8	26.0
Sunbean	82.8	3.0	3.2	35.6	21.0	4.2	3.1	16.3
Sonja	79.6	5.0	5.6	39.4	26.5	4.1	3.4	42.2
Torch	77.3	3.0	3.0	37.9	20.5	3.9	3.3	15.6
EC3390-101	91.1	4.0	7.0	42.1	23.0	4.0	2.8	19.7
Mitra	86.7	3.0	4.0	38.5	23.0	4.2	3.4	22.7
CH I	80.2	3.5	4.5	35.5	23.0	4.9	3.7	27.1
TCN 01	70.4	3.5	3.5	31.2	19.0	3.8	2.8	33.0
B. nigra								
PBn14	64.5	4.2	4.2	29.2	23.0	2.1	1.4	22.4
PBn22	86.4	3.0	3.2	29.6	25.2	1.9	1.1	11.9
PBn17	78.4	4.8	6.6	32.4	30.0	1.9	1.2	13.1
PBn13	93.6	3.6	4.2	37.0	30.0	1.9	1.3	17.3
PBn16	71.7	4.1	4.2	35.3	29.0	2.0	1.3	12.0
PBn12	121.0	5.0	7.8	51.0	36.4	2.0	1.8	17.5
PBn19	99.6	5.8	7.2	39.6	35.2	2.0	1.6	25.9
PBn20	105.2	4.4	6.2	39.4	34.8	2.0	1.4	21.3
PBn7	106.6	4.6	6.6	39.6	34.2	2.2	1.5	17.6
Resynthesized *B. juncea*								
SJR2	121.2	5.0	7.6	29.4	25.2	3.7	3.9	24.1
SJR6	126.7	4.9	8.6	35.3	26.3	3.9	3.5	15.2
SJR17	125.7	5.0	8.1	32.4	23.8	3.7	4.0	18.7
SJR23	96.7	4.6	8.4	33.8	24.4	3.6	2.8	15.4
SJR57	123.6	5.4	8.6	29.1	23.9	4.0	3.1	21.2
SJR59	108.9	5.7	7.7	29.2	18.0	4.1	3.3	17.3
SJR63	109.0	4.7	8.0	35.2	27.0	3.9	4.2	28.6
SJR76	138.7	6.0	9.9	36.8	28.2	4.0	3.4	22.3
SJR113	129.1	5.1	8.8	34.0	26.5	4.3	3.1	16.1
SJN8	116.9	5.5	9.7	27.2	25.2	4.2	2.5	25.7
SJN14	129.2	5.6	9.2	28.2	24.6	3.6	2.0	13.0
Natural *B. juncea*								
JN 004	134.8	6.0	15.5	35.6	25.3	3.6	3.0	44.4
JR 049	102.2	4.3	11.0	40.7	31.1	3.6	2.7	31.0
XINYOU 4	147.7	6.6	17.6	50.3	48.6	4.0	3.2	83.7
JC Bold	105.0	4.2	14.8	33.6	29.1	4.1	5.1	60.8
CD (5%)	17.00	1.42	2.54	8.90	6.80	0.51	0.83	15.00

* Mean yield of 10 plants

Erickson *et al.* 1983; Pradhan *et al.* 1992). Of the two progenitor species, *B. rapa* occurs naturally from the west Mediterranean region to central Asia. Its wider adaptability and earlier domestication has allowed the generation of tremendous morphotype variation. Natural occurrence of the second progenitor species, *B. nigra* is from *circum* Mediterranean to central Asia and the Middle East. At present, it is a minor crop in Ethiopia, Asia and a widespread weed in temperate regions (Sauer 1993). Due to its little importance as a commercial crop and major occurrence as a weed, the extent of variability in *B. nigra* for agronomically important traits is poor, although it is a known source of genes for resistance to biotic stresses. Resynthesis of *B. juncea* has been carried out in the past by utilizing a limited number of *B. nigra* accessions in combination with only a few *B. rapa* genotypes (Prakash 1973; Srivastava *et al.* 2001). As per the published evidence, no attempt has been made to appropriately characterize and then utilize the variability available in *B. nigra* for resynthesis of *B. juncea.* Present studies were successful in developing eleven resynthesized *B. jun-*

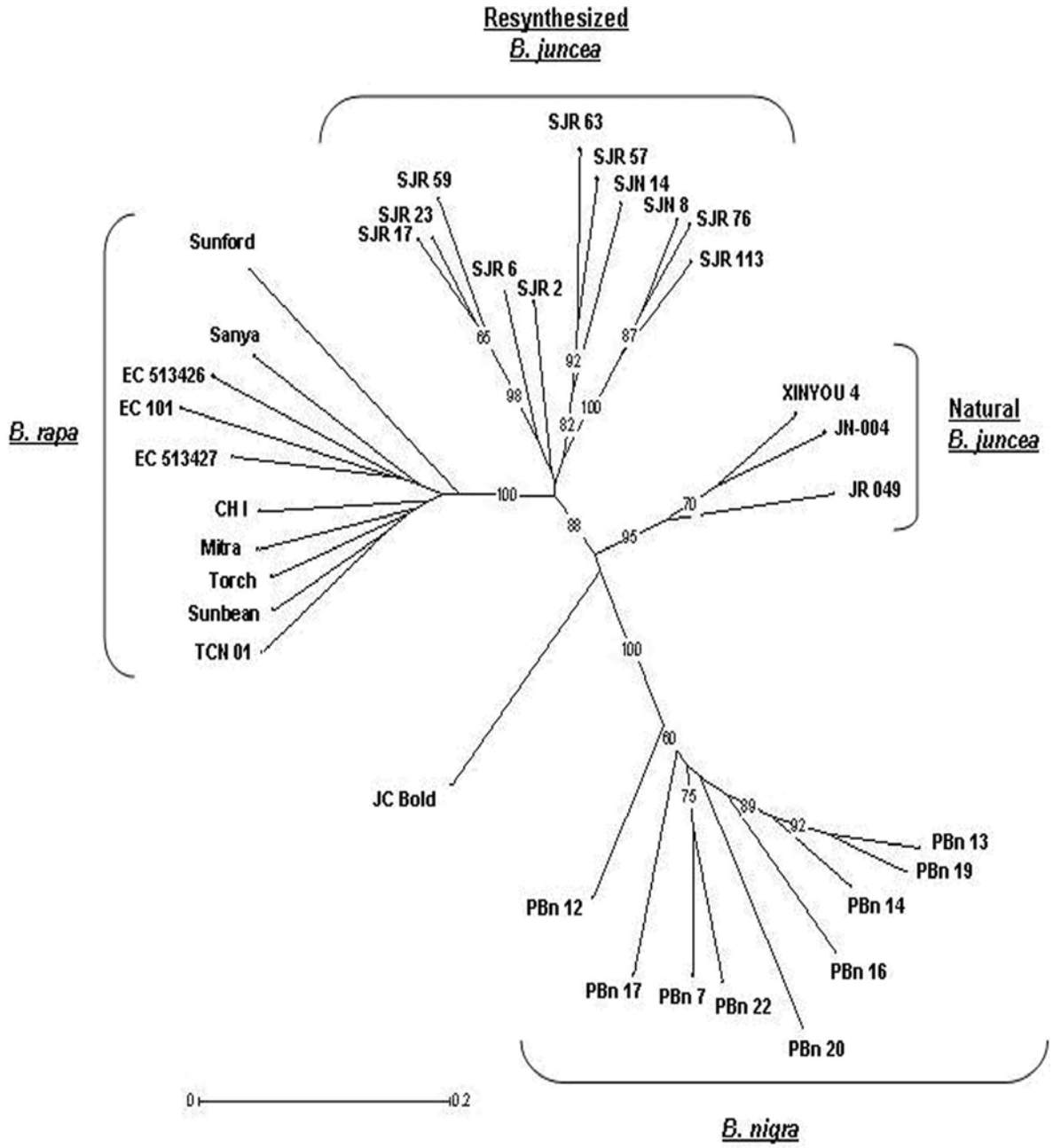

Fig. 3 Tree showing molecular diversity in resynthesized *B. juncea*, parents and natural *B. juncea*. Boot-strap values less than 50 not shown.

cea genotypes involving 19 parents. The stability of resynthesized A_3 *B. juncea* was apparent from normal bivalent formation and expected 18-18 disjunction during microspore formation (**Fig. 1**).

SSR-based clustering analysis indicated uniqueness of the resynthesized types (**Figs. 2, 3**) as these were grouped separately from the natural *B. juncea*. The resynthesized *B. juncea* was closer to *B. rapa* than *B. nigra*. Interestingly genotypes having one common diploid parent or similar origin were not always assigned to the same subgroup. For example, out of the eleven resynthesized *B. juncea* types, six shared three common diploid parents viz. PBn16 (common pollen parent in SJR 17 and SJR 23), PBn13 (common pollen parent in SJR 6 and SJR 57) and EC 513427 (common female parent in SJR 59 and SJR 57). At the molecular level, only SJR 17 and SJR 23 were placed in the same subcluster indicating similarity, whereas no such grouping was manifested in the other combinations. Similar inferences could be drawn from agronomic evaluation which clearly indicated a lack of association between performance at the diploid and amphiploid levels. There appeared to be no influence of cytoplasm on the grouping of genotypes based on genetic similarities. For example, SJN 8 and SJN 14, the resynthesised amphiploids with *B. nigra* cytoplasm were grouped along with SJR 63, SJR 57, SJR 76 and SJR 113, the resynthesized *B. juncea* carrying *B. rapa* cytoplasm. Assigning of genotypes to different sub-groups was, in general, independent of geographic diversity. In the *B. rapa* cluster, Indian genotypes CH I, Mitra and TCN 01 appeared in the sub-cluster having exotic Torch and Sunbean. In the *B. nigra* cluster, the exotic genotype, PBn7 was similar to PBn 22, a genotype from Pakistan. In the resynthesized group also, genotypes with exotic and Indian parents were placed in the same sub-clusters. This absence of correspondence between diploid progenitors and amphiploids species can accrue from the fact that *Brassica* genomes are highly plastic, and that polyploidy generates novel genetic variation through gene duplication, intergenomic heterozygosity, and perhaps even epigenetic changes (Luckens *et al*. 2004).

The resynthesized *B. juncea* appeared to possess a greater degree of unique alleles than those recorded for natural *B. juncea*. The majority of the unique alleles, both in natural and resynthesized *B. juncea* were *B. nigra*-specific (**Table 2**). This could be attributed to genetic and epigenetic changes as a consequence of polyploidy. The general clustering of resynthesized *B. juncea* irrespective of cytoplasmic

diversity (*rapa* vs. *nigra*) indicated a lack of influence of cytoplasm on genetic diversity of resynthesized *B. juncea* during early generations following amphiploidy. This is in contrast to the findings of Song *et al.* (1995) who reported the influence of cytoplasm on genomic changes during early generations following amphiploidy. However, not all *Brassica* allopolyploids are characterized by rapid genome changes following amphiploidy (Axelsson *et al.* 2000). The genetic divergence observed for resynthesized genotypes is understandable given the varied evolutionary pathways and response to selection in the two parental diploid species evolving under diverse ecological niches. The presence of a larger number of unique alleles in resynthesized *B. juncea* was also suggestive of genetic distinctness and possibly the breeding value of the resynthesized *B. juncea* which are now being evaluated for their use in Indian mustard breeding programmes, especially heterosis breeding.

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