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Diversity analysis in snowball cauliflower (*Brassica oleracea* var. *botrytis*) using microsatellite markers

HANUMAN RAM[1], AMISH K SUREJA[2], S GOPALA KRISHNAN[3], S S DEY[4] and B S TOMAR[5]

ICAR-Indian Agricultural Research Institute, New Delhi 110 012

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ABSTRACT

A set of 50 microsatellite markers were used for analysis of molecular diversity in 12 parental lines consisting of five *Ogura* CMS lines and 7 testers of snowball cauliflower (*Brassica oleracea vas botrytis* L.). Twelve markers were found to be polymorphic and the polymorphic information content (PIC) of the simple sequence repeat (SSR) markers ranged from 0.15 to as high as 0.83. The genetic dissimilarity based on simple matching coefficient for 12 genotypes ranged from 0.06 to 0.61 with an average of 0.36. The principal coordinate analysis based on molecular polymorphism, delineated the genotypes into five different groups. Genotype specific alleles was identified, which can be used as a reference for varietal identification as well as to identify the purity of the hybrid seeds in snowball cauliflower. The present study demonstrates the utility of microsatellite markers for estimating molecular diversity as well as genotype identification in snowball cauliflower.

Key words: Cauliflower, Genetic diversity, Molecular characterization, SSR markers

Cauliflower (*Brassica oleracea* var. *botrytis* L.) is a popular cruciferous vegetable grown mostly as winter season crop in India. Snowball cauliflower displays a very low level of intra-specific polymorphism. Understanding of genetic diversity of the crop is of paramount importance for genetic improvement of the crop. The knowledge of genetic divergence of the parental lines is one of the key factors in tailoring an effective and successful hybrid breeding programme. Conventionally, genotype identification and genetic diversity in plants is based on phenotypic evaluation of morphological traits which are often influenced by the environment. The potential of molecular markers, which are independent of environment effects, in evaluating genetic diversity and its successful implementation in crop improvement has been well recognized.

Several studies was conducted for the assessment of genetic diversity in cauliflower using different molecular markers such as Restriction Fragment Length Polymorphism (RFLP) (Sebastian *et al*. 2000), Random amplified polymorphic DNA (RAPD) (Astarini *et al.* 2007), Amplified Fragment Length Polymorphism (AFLP) (Sebastian *et al.* 2000), SSR (Zhao *et al*. 2014) and Expressed Sequence Tags derived SSR (EST-SSR) (Vaidya *et al*. 2012). Microsatellites, also called simple sequence repeats (SSRs), are short tandem nucleotide repeats of 1–6 bp that can be repeated up to 100 times (Tautz and Schlotterer, 1994). Microsatellite loci are inherently unstable with high mutation rates, a phenomenon that is reported to be caused by DNA polymerase slippage and/or unequal recombination. Due to their high mutability, SSRs play a significant role as molecular markers for evolutionary and population genetic studies. Microsatellites offer several advantages compared to other molecular markers: they are co-dominant, highly reproducible, highly polymorphic, high level of variation, abundant information, ease of manipulation PCR-based and readily portable within a species; of these features, they are widely employed in many species. The sequencing of *Brassica oleracea* provides a wealth of information of the genome content based on which, a large number of microsatellite markers have been identified for genome analysis and genetic improvement in *Brassica* species (Li *et al*. 2011).

With the above view point, the present study was undertaken with the objective to assess the molecular diversity of the parental lines of snowball cauliflower using SSR markers.

MATERIALS AND METHODS

The experimental material for the present investigation comprised of 12 snowball cauliflower genotypes including 5 CMS lines and 7 testers. Molecular work was undertaken at the Division of Genetics, IARI, New Delhi. A set of 50 Simple Sequence Repeats (SSR) markers were identified from a set of 1 398 SSR markers identified in a earlier study (Li *et al*. 2011) based on the polymorphism reported,

[1](e mail: hramdhanari@gmail.com, [2](e mail: aksureja@gmail.com), [5]Head, Division of Vegetable Science, [3]Division of Genetics, IARI, New Delhi. [4]IARI, Regional Station, Katrain, Kullu Valley, Himachal Pradesh.

scorability, the annealing temperature (53-58°C) and product size (120-300 bp). The complete list of SSR markers employed in molecular diversity analysis along with sequences of the forward and reverse primers, motifs, GenBank ID, annealing temperature and expected product size is presented in Table 1.

Total genomic DNA was isolated from 5 g/m of fresh young leaf tissue, collected from hundred 15-20 days old bulked leaf samples following the cetyl tri-methyl ammonium bromide (CTAB) method. DNA was precipitated with chilled iso-propanol and DNA pellet was rinsed with 70% ethanol for 10-15 min to remove excess CTAB. The pellet was dried at room temperature overnight and dissolved in TE buffer (pH=8). The purified DNA was quantified on 3.0% agarose gel along with a 50 bp DNA ladder. The total genomic DNA was diluted to 10 ng/µl for use in PCR analysis.

PCR reactions were carried out in a Bioer XP cycler using PCR microplates. The total PCR reaction volume was 10 µl, composed of 1.0 µl of 10 ng genomic DNA, 1.0 µl 10X PCR buffer (Tris with 15 mM MgCl2, Conc. 10X), 0.8 µl deoxynucleotides (dNTPs), 1.0 µl primer, 0.2 µl Taq DNA polymerase (conc. 3 U/µl) and 6.0 µl sterile deionized water. PCR tubes containing the above components were capped and given a pulse spin to allow proper settling of reaction mixture. PCR amplification was performed with initial denaturation at 94ºC for 3 min followed by 35 cycles of 94ºC for 1 min, 50º, 55º or 60ºC (depending upon the primer) for 1 min, 72ºC for 2 min, and a final extension at 72ºC for 10 min before cooling at 4ºC. Amplification products were resolved on 3% metaphor agarose gel and DNA bands were visualized by staining with ethidium bromide.

Fragments amplified by microsatellite primers were scored as presence (1) or absence (0) relative to the molecular weight standard (50bp DNA ladder). The polymorphic information content (PIC) of each primer was determined according to Botstein *et al.* (1980). SSR allelic composition for each genotype at every marker locus was determined by

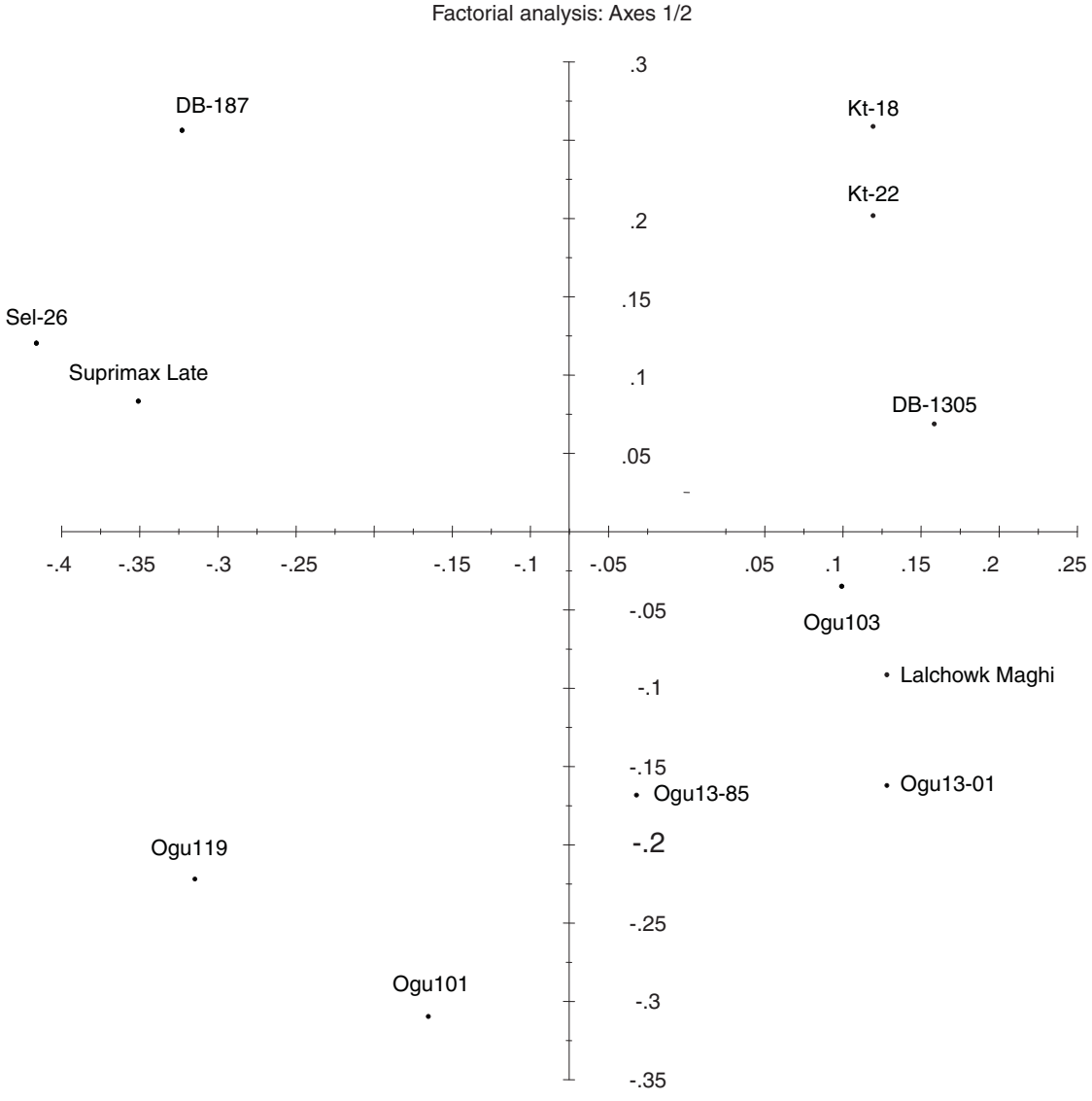

Fig 1 Dispersion of the 12 cauliflower genotypes in the principal coordinate analysis

Table 1 SSR markers used in the study for screening the cauliflower genotypes

Marker name	GenBank ID	Left primer (5'-3')	Right primer (5'-3')	Motif	Product size (bp)	Left primer TM	Right primer TM
BoGMS1186	17613545	GACTGGAACGACAACGACT	GCGGAGGTAGATTAGGGA	(CTC)9	174	56.109	55.215
BoGMS1474	17666366	ATGGAGTCACAATGGAAGAG	AAATCAAGGACACACAAACAC	(CT)13	282	55.013	55.036
BoGMS1441	17676832	CTCCGATTACCTCCTGAA	ACGGGTTTCTTTCTTTGAC	(GTTC)6	218	53.495	54.297
BoGMS0641	17691844	TCAAACAACCTTCAGAGAGAA	CAGCGGAGAACAACAATC	(TCA)13	147	55.144	55.054
BoGMS1498	17720659	TCAACAGAACACATCCACAG	TAGTGCCATAGAAACCATCTT	(CAA)8	281	55.372	54.112
BoGMS1403	17732264	CCATCTTCTTCGCTTACTACA	CGTCTGGTCTTATTGACTTCT	(TCT)8	151	54.912	53.69
BoGMS1461	17775368	CATCAAAGACAACAAACCAAG	CACAGATTCCACAGAGAGTGT	(GAA)8	274	55.815	55.188
BoGMS0468	17787617	TGACAGCAACCAATGATG	CTCTCTGGAACCTTTGAACT	(AT)25	259	54.549	53.575
BoGMS1042	17812094	ATAGTGAATAATGGAAGGCTG	GAGAGAGGAGAGAACAGAGGA	(TC)14	183	53.662	55.289
BoGMS1430	17846392	TGTCTCTACACCAAGTTCCC	TCCTAACACATCCTTCATCAC	(TA)13	182	55.103	55.045
BoGMS1114	17849298	CACCACTAACCTATGAACCTG	TGTCTCTTCTCCACTTTGTTG	(AG)14	274	54.774	55.52
BoGMS1460	17850678	CGAGAGGTGAAGAACAAGAG	AAATAAGAGAAGAGAAACCGTC	(GA)13	216	55.153	53.746
BoGMS0726	17857292	GTTCCGAGGGTTGTTCTT	CCATCAGGTTCAGCCATAC	(AT)18	215	54.975	56.423
BoGMS0877	17860145	AAACTCTATGCTCCACACTCA	TCCTCCTAATCCTAATGCTCT	(AT)16	190	55.036	54.844
BoGMS1059	17860168	TAAGCACGCAACTGTGGA	GGGTTATTTGAACGGAGTATG	(TA)14	213	57.393	56.17
BoGMS0338	17860907	CAAGATTCGCTTTAGGGTTT	AACGCACACACAGTTCCT	(TC)15	167	56.189	54.872
BoGMS0416	18709231	TTCACACCTAAGAATCATCAAG	AGACAACACAAAGCATCACA	(ATA)19	222	54.565	55.083
BoGMS0744	18718713	TTCTGTGTGTCTGCGTATTAG	GATTGAAGAGTATTTGAGAGCA	(TA)17	258	54.114	53.959
BoGMS1110	18718779	CAGCCTCCAATGCTACAA	AACAATGATAACGCTCTAAGTTC	(AGC)9	203	55.755	54.673
BoGMS0345	18720086	AACATCAGAAATGACGAGAAG	CTAAGAACGGAGTTGCGG	(GAG)10	251	54.505	56.991
BoGMS0556	18763489	AACAATGAGACAAAGCAACC	GTAAGACTGATGGACGAAGTG	(ATA)14	292	55.276	54.866
BoGMS0974	18764171	TCTGAAACCAAGAGAAAGTGA	TCATACCACCAGCAACAAC	(CTG)10	299	55.144	55.304
BoGMS0523	18805285	ACCCTCTTTCTCTCTCATTTC	TAGCCACCAAGTCCTGTTC	(TA)22	207	54.241	56.193
BoGMS0788	18810192	CGCATTACTATCTCCATTGTC	GGCTGTCACTCTCTTCGG	(AT)17	258	54.943	56.873
BoGMS0816	18834055	ATAAAGCCCAATAACCTCAAC	CGTTTGTCGGAAATAGCA	(TA)16	246	55.05	55.751
BoGMS0544	18878317	CCCAATAAGCAGCAATACTAA	CAAACAACAAAGAGATTCCAG	(ATG)14	179	54.909	55.031
BoGMS0658	23422958	TATTTCATTTGCCGCTTC	CATTCATAGGTTGGTCTGCT	(TA)19	264	54.84	55.24
BoGMS0851	23430643	TTGTATCATCACCACTGTAACC	TTCTTCTTCTCCCTCTTCTTC	(TGA)10	160	55.03	54.546
BoGMS0206	23432845	TACTCCACGGTCTTCTACTTG	GGGATAGTGATGTTGTTGATG	(CAT)16	287	54.645	55.348
BoGMS0987	23437503	AAACACGCATAGTCCAAGT	AGTCTTCCACCTACTCCAATC	(AT)15	271	53.147	54.88
BoGMS1505	23548960	GGTCTGTTTATCCATCTTCTTC	GCAAGTCATCCCTGTGTT	(AT)13	228	54.576	54.173
BoGMS0050	23573051	AGAGGAAGAGGAAGAGGAAGT	CAAACGCAACAAGTCATAAA	(GAA)24	295	55.41	54.969
BoGMS0600	23576841	ACTGTCTCATCGTCTTCTTTG	ACGCATAACATTCTTCACATT	(TA)20	236	54.56	54.852
BoGMS1334	23578670	GACATTGCTGTTCGTGCT	ATCGGTGTTGTTGCTTTCT	(ATT)9	203	55.641	55.731
BoGMS1401	23581985	ATGTTCCCATTTCAGAGTCA	TTATCCCAATCCTCATCATC	(TGA)8	256	55.463	54.793

Contd.

Table 1 (*Concluded*)

Marker name	GenBank ID	Left primer (5'-3')	Right primer (5'-3')	Motif	Product size (bp)	Left primer TM	Right primer TM
BoGMS1564	23642758	ATTATTCTTGCGGGTTACATC	CTCCCTCTTTCACTCCAAA	(AG)13	212	55.844	55.326
BoGMS0478	23666259	GTCACGAAGGAGGAGATTG	GACCGACAAGGCTGAAGA	(TAA)16	286	55.623	57.334
BoGMS1240	23698574	TCGCTTCTCTCTAATCTTCCT	TCTGGGTCTCGCTTTATCT	(AAGA)7	283	55.185	55.008
BoGMS1404	26678132	AGAGAGAGAGAGAGGACGA	GGTGAAAGACACATACAGAGAC	(TCT)8	189	55.018	53.812
BoGMS0952	26742067	CAGTGAGTAACATTTGGCTG	CGAGAGAGAAAGTGATGAGAG	(TCC)10	148	54.327	54.347
BoGMS0821	26767912	GTAGCCGAAGAAAGCGTC	ATTGACCGAAAGAAAGAAATG	(GA)16	215	56.144	56.02
BoGMS0906	26768487	TACCTCTCTGCTTCTCTCTTG	GGTGATTGCCAGTTTCTTT	(TA)15	202	54.124	55.179
BoGMS0929	26784913	TCAGACCCAAAGCCAGTT	TTGTGGAAGATGAAACCATT	(AAG)10	244	56.656	55.468
BoGMS1496	26785643	TTTCACAGCATTGGTAGATG	TGGCACTTCTCTCTTATGTCCT	(CA)13	202	54.683	54.937
BoGMS0260	27012242	AACGAACCTCTCTCTCTCTGT	TTTCCGACCAAACTTATTTCT	(GA)17	203	54.811	55.627
BoGMS1383	27022289	TTCCTGCTTACATTACTCACAG	GTTAGGTTTAGGGAGAGAGAAC	(AAGAA)5	199	54.846	53.194
BoGMS1467	27023419	ATGGCTTTGTTCTCTTTCTT	GACTTCAGCACGCCTTTC	(CTG)8	274	54.958	56.995
BoGMS0829	27034127	CACACTCATCTCTGTTTCAGTT	TTCACTTCCAGCATTACGA	(CT)16	274	54.49	55.277
BoGMS1068	27050318	GAAGATTTACAGGATTTGCTC	CTATCAGAACACCCAACGCA	(GTTA)7	275	53.317	55.676
BoGMS0666	33820553	ATTGGTCGTAAATCGTTGGT	CTCTTCGTCGGATGTTTG	(CT)19	261	56.94	55.068

Reference: Li *et al*. (2011)

counting the number of alleles per locus and the allele frequencies and polymorphism information content (PIC) was determined using the formula,

$$PIC = \left(1 - \sum_{i=1}^{k} \hat{p}_i^2\right) \frac{2n}{2n-1} - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2\hat{p}_i^2 \hat{p}_j^2$$

where p_i was the estimated allele frequencies of k alleles (i=1 to k) and n was the number of individuals sampled. The genetic dissimilarities were calculated for each pair of lines using the simple matching coefficient (Sokal and Michener 1958). Principal coordinate analysis was performed on a dissimilarity matrix of simple matching coefficients using DARwin version 5.0.158 (Perrier *et al*. 2003) with 30 000 permutations.

RESULTS AND DISCUSSION

A set of 50 SSR markers was used to assess genetic diversity in 12 snowball cauliflower genotypes. Of these, 32 markers producing amplification in snowball cauliflower but only 12 SSR markers were found to be polymorphic and the rest 20 showed monomorphism. The marker statistics of the polymorphic markers are presented in Table 2. The polymorphic information content (PIC) of the SSR markers ranged from 0.15 to as high as 0.83. BoGMS1110 amplified as many as 6 alleles with a PIC of 0.83 while BoGMS1441 and BoGMS0345 amplified only 2 alleles with a PIC as low as 0.15. The expected heterozygosity for the marker loci ranged from 0.16 to 0.86. Markers, BoGMS1441 and BoGMS0345 produced genotype specific alleles in Kt-18 (220bp) and DB-187 (230bp), respectively. BoGMS1041 could differentiate Sel-26 and Suprimax Late (265bp) from all other genotypes used in the study.

The genetic dissimilarity calculated based on simple matching coefficient for 12 genotypes ranged from 0.06 to 0.61 with an average of 0.36 (Table 3). The genotypes Ogu13-01 and DB-187 were found to be the most diverse showing maximum dissimilarity (0.61) while the genotypes Sel-26 and Suprimax Late were found to be the most similar (0.06)

Table 2 Marker statistics of the polymorphic microsatellite markers in cauliflower genotypes

Marker	Motif	No. of alleles amplified	Expected heterozygosity (H_u)	PIC
BoGMS0206	(CAT)16	3	0.59	0.50
BoGMS0345	(GAG)10	2	0.16	0.15
BoGMS0468	(AT)25	3	0.68	0.61
BoGMS0726	(AT)18	5	0.78	0.75
BoGMS0851	(TGA)10	3	0.62	0.55
BoGMS0952	(TCC)10	2	0.39	0.32
BoGMS0974	(CTG)10	2	0.52	0.40
BoGMS1110	(AGC)9	6	0.86	0.83
BoGMS1401	(TGA)8	2	0.31	0.27
BoGMS1441	(GTTC)6	2	0.16	0.15
BoGMS1460	(GA)13	2	0.5	0.39
BoGMS1498	(CAA)8	4	0.74	0.68

Table 3 Dissimilarity matrix of cauliflower genotypes based on simple matching coefficient of the molecular data

Genotypes	1	2	3	4	5	6	7	8	9	10	11
2	0.29										
3	0.24	0.31									
4	0.41	0.25	0.41								
5	0.36	0.26	0.39	0.26							
6	0.38	0.53	0.38	0.56	0.41						
7	0.30	0.45	0.26	0.52	0.33	0.21					
8	0.33	0.41	0.18	0.53	0.24	0.31	0.24				
9	**0.61**	0.53	0.47	0.35	0.42	0.50	0.36	0.44			
10	0.33	0.29	0.29	0.47	0.24	0.38	0.30	0.17	0.56		
11	0.53	0.38	0.47	0.35	0.45	0.50	0.52	0.47	0.29	0.47	
12	0.44	0.29	0.41	0.41	0.42	0.44	0.42	0.44	0.39	0.39	**0.06**

1, Ogu 13-01; 2, Ogu 101; 3, Ogu 103; 4, Ogu 119; 5, Ogu 13-85; 6, Kt-18; 7, Kt-22, 8 DB-1305; 9, DB-187; 10, Lalchowk Maghi; 11, Sel-26; 12, Suprimax Late. *Source:* IARI Regional Station, Katrain.

The principal coordinate analysis based on molecular polymorphism, delineated the all genotypes into five different groups with Sel-26, Suprimax Late and DB-187 forming a group and Kt-18 grouped with Kt-22 and DB-1305. The CMS lines Ogu 13-01 and Ogu 103 grouped with Lalchowk Maghi while Ogu 13-85 could be distinguished from all other groups. Ogu 119 and Ogu 101 was found to be distinct and diverse from all other accessions.

Molecular markers are useful in assessment of diversity in the crop germplasm. Microsatellite markers are reliable markers for assessment of genetic diversity among the germplasm in a crop owing to its co-dominant nature and reproducibility not only with different genotypes but also being robust enough to produce reproducible results.

Out of the 50 markers used in the present study as many 32 markers produced scorable and robust amplified fragments. Twelve markers were found to be polymorphic resulting in a substantiate polymorphism percentage of 37.5% as compared to 54.4% of polymorphism. The percent polymorphism for a diploid species such as snowball cauliflower is higher, establishing the robustness of the SSR markers identified for use in the present study. The markers which were found to be monomorphic in the present study could be polymorphic, if more diverse genotypes could be used in the study as reported by Li *et al*. (2011).

The markers such as BoGMS1110 with high PIC of 0.83 are ideal to study the extent of molecular diversity in the snowball cauliflower germplasm while markers producing genotype specific alleles such as BoGMS1441 in case of Kt-18 (220bp), BoGMS0345 in case of DB-187 (230bp) could be useful in identification of these genotypes inspite of having a very low PIC. The utility of microsatellite markers for cultivar differentiation and identification of vegetable brassicas have been well established (Louarn *et al*. 2007). Furthermore, since both these genotypes are male parents of the hybrids with good GCA (General Combining Ability), the marker BoGMS0345 could be useful for testing the genetic purity of hybrid seeds from a heterotic cross such as Ogu101X DB-187 while the marker, BoGMS1441 could be used for testing the genetic purity of hybrid seeds in the heterotic cross Ogu13-01X Kt-18. This would provide a quick and reliable alternative for the conventional grow out test (GOT) for testing the genetic purity of hybrid seeds thereby saving time and cost. The use of pollen parent specific markers has been successfully utilized for testing the genetic purity of hybrid seeds in many crops such as rice (Garg *et al*. 2006) and *Brassica* (Janeja *et al*. 2003), where hybrid seed production is based on CGMS system.

The assessment of molecular diversity based on SSR markers showed that the 12 parental genotypes of the snowball cauliflower used in the present study were diverse with dissimilarity as high as 0.61 between the genotypes. However, the average dissimilarity between the parental lines is only 0.36. This may be due to lesser genetic diversity in snowball cauliflower as most of the snowball cauliflower lines in India have derived from European materials. Lan *et al*. (2012) also reported higher genetic similarity in cauliflower as compared to broccoli.

Generally, in hybrid breeding, parental lines and superior cross combinations are identified by genetic divergence analysis based on observations on morphological traits and then performance of crosses between these diverse lines is assessed under extensive field trials. With the advent of molecular markers, the molecular diversity between parental lines based on markers has been viewed as a potential tool for predicting hybrid performance (Gopalakrishnan *et al*. 2013). The first report of molecular diversity and relation to prediction of hybrid performance came in maize (Lee *et al*. 1989) wherein a linear relationship has been observed between genetic distance and heterosis (Reif *et al*. 2003).

The marker BoGMS1110 was found suitable for analysing the genetic diversity in snowball cauliflower germplasm while genotype specific markers such as BoGMS1441 and BoGMS0345 were useful in cultivar identification. Additionally, these markers provide a reliable alternative for the conventional grow out test (GOT) for testing the genetic purity of hybrid seeds thereby saving time and cost.

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