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Within-line Genetic Variation for Quantitative Characters and SSRs in Long-time Maintained Inbreds in Pearl Millet [*Pennisetum glaucum* (L.) R. Br.]

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ABSTRACT

Six maintainer (B-) and restorer (R-) lines each from ICRISAT's pearl millet hybrid parental line breeding program were investigated for within-line genetic variation for quantitative characters and simple sequence repeats (SSRs). Thirty two progenies of each inbred line were evaluated under two contrasting seasons and observed for 5 quantitative characters, and for 20 SSRs. Some B- and R- lines had small but significant within-line genetic variation for certain traits. Higher number of significant differences for within-line variation observed among progenies for B-lines (48%) than for R-lines (33%) indicated that ear-to-row procedure of line maintenance was more effective than bulking the phenotypically similar plants, for maintaining the genetic uniformity in inbreds. Amongst B-lines, 'ICMB 89111' had the maximum within-line variation for both quantitative characters and SSR markers. Amongst R-lines, 'IPC 802' and 'IPC 909' had within-line variation for maximum of 4 quantitative characters. Wide range of variability was observed for all the characters in both B- and R- lines, but genotypic coefficient of variation (GCV) was very low. SSRs were able to detect low level of residual heterozygosity in some of the inbreds.

Keywords: ear-to-row method, genetic diversity, genotypic coefficient of variation, residual heterozygosity, selfing, within-line variation
Abbreviations: DArT, diversity array technology; **GBS**, genotyping-by-sequencing; **GCV**, genotypic coefficient of variation; **IPR**, intellectual property right; **PCR**, polymerase chain reaction; **SNP**, single nucleotide polymorphism; **SSR**, simple sequence repeat

INTRODUCTION

A pearl millet [*Pennisetum glaucum* (L.) R. Br.] hybrid program is based on the development and identification of the best heterotic male and female pairs for high yield and desirable characters. Once the hybrid is released, the time for which it remains in the seed market depends upon its competitive performance, but the stable performance of the hybrid is dependent upon the genetic stability of the inbreds constituting this hybrid. Genetic variability, if present within the inbred, could affect the yield of hybrid combinations after several cycles of regeneration. Thus, it is important to monitor the genetic variation within inbreds involved in the hybrid program. In pearl millet, inbred lines once developed, are maintained by different procedures like ear-to-row method, sibbing or bulk-pedigree, depending upon the resources and the objectives of the breeder. Although inbreds are presumed to be homozygous and homogeneous after 8-10 cycles of inbreeding, large genetic variability for quantitative traits in inbred lines has been detected in several maize studies (Russell *et al*. 1963; Fleming *et al*. 1964; Russell and Vega 1973). These investigations on maize identified method of maintenance, residual heterozygosity and mutations to be the sources of such genetic variations (Busch and Russell 1964; Fleming *et al.* 1964; Higgs and Russell 1968; Bogenschutze and Russell 1986). Gethi *et al.* (2002) also detected small but significant variation within inbred lines using SSR markers in maize (*Zea mays* L.) while Zhang *et al.* (1995) detected within-line variability in 3 of the 4 sunflower (*Helianthus annuus* L.) inbred lines using 30 probe-enzyme combinations.

ICRISAT's pearl millet hybrid parental line breeding program at Patancheru develops, designates, and disseminates new parental lines every year, which are further utilized by public and private sector components of National Agricultural Research Systems (NARS). The impact of this program is reflected in that out of about 90 hybrids cultivated on around 4.5 million ha in India, at least 70 hybrids are directly or indirectly based on ICRISAT-bred parental lines (Mula *et al*. 2007). Thus, the extent of their utilization makes it very important to assess the level of existing genetic variation within seed parent inbreds (B-lines) and restorer parent inbreds (R-lines) developed from this program. Keeping this in view, the present study was conducted to estimate the level of genetic variability within pearl millet inbred lines from this breeding program on the basis of quantitative traits and SSR markers.

MATERIALS AND METHODS

Plant materials

Pearl millet crop improvement program at ICRISAT has designnated about 150 seed parent inbreds (A/B- pairs) and about 1800 pollinator inbreds (R-lines) through 2009. Both B- and R-lines were generated by using a range of diverse germplasm/improved breeding lines and differ from each other for numerous agronomic traits such as days to flowering, plant height, panicle exertion, panicle length, panicle diameter, panicle shape, panicle density, seed size and shape, and seed color (Talukdar *et al.* 1995; Rai *et al.* 2009; Gupta *et al*. 2011). On the basis of their wide usage in hybrid programs, phenotypic differences and diverse parentage, six long-time maintained B-lines ('843B', 'ICMB 89111', 'ICMB 93333', 'ICMB 97111', 'ICMB 01222' and 'ICMB 02111') and

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six long-time maintained R-lines ('IPC 422', 'IPC 715', 'IPC 795', 'IPC 802', 'IPC 828' and 'IPC 909') were selected. These B- and R-lines had passed through more than 10 generations of inbreeding by self-pollination at the time of their designation and release. Afterwards, they were maintained by selfing and bulking equal quantities of seed from 8-10 plants per season at Patancheru. From each of 12 inbreds (6 B- and 6 R-lines), panicles of 32 random plants were bagged for self-pollination in the post rainy season of 2007. The selfed seed from each of these 32 plants of each inbred was multiplied following ear-to-row method in the rainy season of 2007 to produce seed for these investigations.

Quantitative characters

The 32 progenies generated for each of the 12 inbreds were evaluated in separate trials for the B- and R-lines. A split-plot design with two replications was used in which main plots were inbreds and their subplots were the progenies. The trial was sown in post rainy (dry) season (March-June) of 2008 and then again in rainy season (July-October) of 2008. Each progeny was machine-sown in single row of 4 m with 60 cm spacing between the rows in post rainy season and 75 cm in the rainy season, and then thinned to 15 cm spacing between the plants in both seasons. The experiment was conducted in Alfisols with applied fertilizer levels of 80 kg ha^{-1} N (18% basal and balance as top dressing), and 46 kg ha^{-1} P (basal dose). Standard cultural and agronomic practices were followed including thinning and manual weeding at 15 days after sowing. Data were collected for days to 50% flowering on plot basis, while 5 random plants from each plot were used to record plant height (cm), panicle length (cm), panicle diameter (mm) and 1000-grain weight (g) in each replication. The mean plot values of the quantitative traits measured were subjected to analysis of variance for each season following the split-plot design using Genstat 10.1 software.

SSRs

Thirty-two progenies from each of these 12 inbreds were sown in pots in the green house. Leaf samples of 2-week old plants were collected from each of the 32 × 12 = 384 individual progenies in pre-chilled DNA extraction boxes. Tissue samples were bulked after removing the midribs and leaf tips without any cross-sample contamination. Genomic DNA was isolated following a standard DNA isolation protocol (Sharp *et al*. 1988). Quantification of genomic DNA was done using the TECAN liquid handling robot (Tecan Group Ltd., Mannedorf, Switzerland). T10E1 buffer was prepared from the stock solutions of 1M Tris (Biomatik, Delware, USA) and 0.5M EDTA (Sisco Research Laboratories Pvt. Ltd. (SRL), Mumbai, India) in the ratio of 10:1. T10E1 buffer and Picogreen® (Invitrogen, Carlsbad, CA, USA) were mixed together and the blank reading (i.e., without genomic DNA) was taken. One µl of genomic DNA was then added in the premix containing buffer and Picogreen® and the reading was taken for sample. DNA concentration was worked out by comparing the blank and sample readings, and using a standard calibration curve. The concentration of each DNA sample (each of the 384 genotypes) varied from 100 to 200 ng/µl. Normalization of individual DNA samples was done manually by adding double distilled water according to their original concentration. The concentration of normalized DNA was checked again using the TECAN liquid handling robot in order to ensure uniform working stocks of 10 ng/µl across all genotypes. The final concentration of DNA was uniformly maintained (~10 ng/µl) in all working samples to reduce allelic competition during polymerase chain reactions (PCRs) using DNA pools. For convenience in conducting the experiment, for the 32 representative progenies of a given inbred, DNA from first four progenies (1 to 4) were taken to form a single DNA pool. DNA from next four progenies (5 to 8) constituted the second DNA pool and so on. Thus for a single inbred, eight DNA pools were created (DNA pool 1-4, DNA pool 5-8, DNA pool 9-12, DNA pool 13-16, DNA pool 17-20, DNA pool 21-24, DNA pool 25-28 and DNA pool 29-32). Twenty-five µl of normalized DNA with a concentration of 10 ng/µl was taken from each of the four progenies and mixed together to get a final volume of 100 µl/pool. These DNA pools were further used for PCRs.

Table 1 List of polymorphic markers used and summary of their ability to detect heterozygosity across DNA pools of pearl millet inbred lines.

SSR marker loci	Linkage group	Presence of residual heterozygosity	Inbred*
Xpsmp2045	Not yet mapped	-	
Xpsmp2068	Not yet mapped	-	
Xpsmp2077	2	-	
Xpsmp2079	7	-	
Xpsmp2084	4	-	
Xpsmp2203	7	-	
Xpsmp2207	Not yet mapped	-	
Xpsmp2209	Not yet mapped	-	
Xpsmp2212	Not yet mapped	Yes	IPC 422 (3)
Xpsmp2214	3	-	
Xpsmp2218	Not yet mapped	-	
Xpsmp2227	3	-	
Xpsmp2237	2	-	
Xpsmp2273	1	-	
Xicmp3002	6	Yes	ICMB 89111 (1)
Xicmp3032	1	Yes	ICMB 89111 (7)
Xicmp3080	1	Yes	ICMB 89111 (7)
Xctm08	7	-	
Xctm10	3	-	
Xctm12	1	-	

* In parenthesis: Heterozygosity found in number of pools (out of 8)

Twenty pearl millet SSR (synthesized by Bangalore Genei, Bengaluru, India) markers (**Table 1**) providing reasonable genome coverage were taken to assess residual heterozygosity in each of the 12 inbred lines. PCRs were carried out in 5-µl volumes in 384-well PCR plates. Each PCR reaction mixture containing 5-10 ng of genomic DNA, 2 pmol/µl of each primer, 25 mM $MgCl_2$ (Applied Biosystems, India), 2 mM of each dNTP (Bioline, India), 10X reaction buffer (Applied Biosystems, India), and 0.2 U Amplitaq Gold Polymerase (Applied Biosystems, India). After one denaturing step of 15 min at 94°C, a touchdown amplification program was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, USA). This PCR profile consisted of a denaturing step of 25 sec at 94°C and an extension step of 30 sec at 72°C. The initial annealing step was 20 sec at 64°C for one cycle and subsequently the annealing temperature was reduced by 1°C for every cycle until a final annealing temperature of 55°C was reached. The annealing temperature of 55°C was maintained for the last 35 cycles of the amplification, followed by the final extension of 72°C for 7 min. PCR products were pooled post-PCR. Each PCR product pool contained 1 µl of each of dye-labeled (FAM, VIC, NED and PET) product (Applied Biosystems, India), 7 µl of formamide, 0.3 µl of the LIZ-labeled (500-250) size standard (Applied Biosystems, India), and 4.2 µl of double distilled water. DNA fragments were size-separated on an ABI3700 automatic DNA fragment analyser (Applied Biosystems, India) GeneScan 3.1 software (Applied Biosystems, India) was used to size peak patterns, using the internal LIZ (500-250) size standard and Genotyper 3.1 (Applied Biosystems, India) was used for allele calling. Peak sizes were converted to alleles by creating categories in Genotyper, which combines peak sizes within a predetermined range into the same allele, and thus takes into account small errors during size calling.

RESULTS

Quantitative characters

The interaction between lines and seasons was found significant for most of the traits in pooled analysis, resulting in carrying out the analysis for individual seasons. The differences among the 6 B-lines and 6 R-lines (**Table 2**) for all the characters were found significant in both the seasons, which validated the diversity in B- and R-lines under study. This was also evidenced from the character mean values which were significantly different for both B- (**Table 3**) and R- (**Table 4**) lines. For instance, the overall seasonal mean for days to 50% flowering varied from 41 to 56 days and

Table 2 Mean squares of within - progeny of B and R lines.

Source	df	Days to 50% flowering		Plant height (cm)		Panicle length (cm)		Panicle diameter (mm)		1000-grain weight (g)	
		E1	E2	E1	E2	E1	E2	E1	E2	E1	E2
B-LINES											
Replication	1	18.38	199.8	3.8	216	9.31	4.5	15.7	57.67	17.36	0.09
Inbreds	5	2045.9**	1354.7**	53525.5**	38401.7**	453.6**	639.4**	773.4**	477.15**	313.75**	539.3**
Error (a)	5	21.5	29.6	523.7	201.4	10.43	1.09	6.37	15.98	2.76	3.22
Within inbred 843B	31	0.81	2.95	11.9	28.2	0.69**	0.94	0.87	NA	0.79	1.49
Within inbred ICMB 89111	31	6.13**	3.96**	67.3**	81.8**	1.20**	1.54**	4.27**	NA	1.28**	1.51
Within inbred ICMB 93333	31	0.92	3.1*	59.83**	16.1	0.62**	1.04*	0.68	1.03	0.38	0.72
Within inbred ICMB 97111	31	1.42	3.12**	35.83**	30.6	0.83**	0.77	1.52*	1.51	1.02*	1.58
Within inbred ICMB 01222	31	2.67**	3.73**	19.01	17.6	0.45**	1.25**	1.7**	2.02	0.52	1.7
Within inbred ICMB 02111	31	2.5**	0.89	21.47	22.2	0.73**	0.28	1.48*	4.49**	1.28**	4.77**
Error (b)	186	1.28	1.96	23.09	21.2	0.37	0.65	0.84	2.09	0.62	1.9
R-LINES											
Replication	1	21.57	115.8	73.9	783.2	2.56	0.19	1.3	48.28	1.23	31.63
Inbred	5	1656.3**	1483.6*	9542.7**	7405.8**	1297.05**	1732.02**	1077.5**	1254.3**	353.6**	392.39**
Error (a)	5	4.71	293.1	387.2	345.8	2.46	7.82	2.58	9.16	1.1	5.83
Within inbred IPC 422	31	3.94	1.47	42.02	45.3	0.57	1.23**	0.95	2.53**	0.38	0.97
Within inbred IPC 715	31	1.18	4.91	28.09	41.7	0.32	0.26	0.83	0.79	0.73	0.37
Within inbred IPC 795	31	4.3	1.52	53.77**	48.7	1.45**	1.91**	0.67	1.76*	0.74	1.42
Within inbred IPC 802	31	7.42**	1.73	104.7**	86.5**	0.64	0.46	3.24**	1.14	2.39**	0.49
Within inbred IPC 828	31	1.38	3.66	31.3	52.9	0.55	0.72	2.90**	2.13**	1.01**	1.67*
Within inbred IPC 909	31	3.73	5.08	3.73	100.2**	0.88**	2.39**	0.34	2.74**	0.53	2.20**
Error (b)	186	2.57	4.48	29.28	39.5	0.43	0.52	1.17	1.1	0.48	1

*,** significant at p< 0.05 and 0.01 levels respectively: E1: Post rainy season 2008: E2: Rainy season 2008: NA : Not Available

Table 3 Genetic parameters for six B-lines of pearl millet evaluated in post rainy (E1) and rainy (E2) season of 2008, Patancheru.

Inbred	Parameter	Days to 50% flowering (days)		Plant height (cm)		Panicle length (cm)		Panicle diameter (mm)		1000-grain weight (g)	
		E1	E2	E1	E2	E1	E2	E1	E2	E1	E2
843B	Mean	40.1	42.6	78.1	73.0	15.27	12.79	22.28	NA	13.55	8.29
	Range	39-41	41-46	73-83	66-80	13.9-16.4	11.7-14.5	20.8-23.5	NA	10-15	5.4-9.4
	GCV* (%)	0.95	2.02	1.92	3.29	2.66	3.83	0.84	NA	2.30	4.60
ICMB 89111	Mean	46.4	47.2	93.0	93.7	16.81	15.89	22.65	NA	9.76	8.09
	Range	43-49	44-50	84-107	79-107	15.2-18.5	13.3-17.2	18.5-25.1	NA	8-11.1	6-9.5
	GCV (%)	3.27	2.20	5.41	6.07	3.49	4.03	5.70	NA	6.06	6.59
ICMB 93333	Mean	56.0	56.6	160.1	142.0	18.82	18.52	26.62	24.85	8.89	5.53
	Range	55-57	55-60	150-171	136-149	17.4-20.3	17.4-21.0	25.4-27.9	23.9-26.4	7.8-9.7	4.5-6.9
	GCV (%)	0.69	0.45	2.54	0.73	2.38	2.56	0.84	1.87	2.51	4.61
ICMB 97111	Mean	45.1	49.8	121.2	122.0	17.18	16.31	27.81	27.59	13.33	11.17
	Range	44-47	48-53	115-131	113-130	16.2-19.8	15-17.3	26.2-29.9	26.0-30.7	12-15.1	8.7-12.8
	GCV (%)	0.50	1.26	1.01	1.69	3.19	2.12	2.54	1.66	3.83	4.37
ICMB 01222	Mean	52.3	49.9	114.4	108.0	22.05	21.48	30.85	27.87	10.77	7.75
	Range	51-56	48-54	102-119	103-119	21.1-23.1	20-23.3	28.4-32.9	25.3-30.3	9.7-12	5.7-11
	GCV (%)	1.60	1.49	1.60	1.18	0.67	0.47	1.03	1.64	2.84	1.38
ICMB 02111	Mean	50.0	47.7	131.8	124.0	14.78	13.86	29.18	26.98	14.14	13.76
	Range	48-53	47-50	126-139	118-132	13.5-16.2	13.2-14.6	27.6-31.1	23.0-30.4	12.4-15.9	9.2-15.6
	GCV (%)	0.63	0.84	2.47	1.52	3.38	1.71	2.17	2.94	2.24	1.07

* Genotypic Coefficient of Variation; NA: Not Available

plant height from 75 to 121 cm among B-lines, while R-lines showed variation of 47-59 days for days to 50% flowering and 120-147 cm for plant height. Although wide ranges were observed for most of the characters, the contribution of genotypes in this observed variation within individual inbred lines was very small as genotypic coefficients of variation (GCVs) had very low values for all the characters in each of the lines.

The variance among the progenies was tested against the pooled error term (error b) combined for six inbreds in which homozygous error terms were present. The differences among progenies within B-lines were significant for 48% of cases i.e. 28 out of 58 comparisons (6 B-lines × 5 characters × 2 seasons; data of 2 comparisons were not available). Seasonal comparisons revealed that 18 cases of differences out of 30 were significant in the post rainy season while 10 cases out of 28 had significant differences in the rainy season. In the post rainy season, differences among progenies of 'ICMB 89111' were significant for all the characters, while difference within 'ICMB 97111' and 'ICMB 02111' were significant for four characters (plant height, panicle length, panicle diameter and 1000-grain weight) each. In the rainy season, 'ICMB 89111' had the maximum number of significant differences for 3 characters (days to 50% flowering, plant height and panicle length) followed by 'ICMB 93333', 'ICMB 01222' and 'ICMB 02111' (for 2 characters each). The differences among '843B' progenies were non-significant in both the seasons, except for panicle length in the post rainy season. All 6 B-lines had significant differences among progenies for panicle length in the post rainy season while 3 of them had significant differences in the rainy season. When compared amongst characters, a maximum of 4 B-lines had significant differences for days to 50% flowering in the post rainy season.

Among R-lines, 20 cases out of 60 (6 inbreds × 5 characters × 2 seasons) i.e. about 33% of cases had significant differences among progenies in both seasons. Eleven cases in the rainy season and 9 cases in the post rainy had significant differences among progenies of the 6 R-lines. The inbred 'IPC 715' showed non-significant differences among its progenies for all the characters in both seasons while

Table 4 Genetic parameters for six R-lines of pearl millet evaluated in post rainy (E1) and rainy (E2) season of 2008, Patancheru.

Inbred	Parameter	Days to 50% flowering (days)		Plant height (cm)		Panicle length (cm)		Panicle diameter (mm)		1000-grain weight (g)	
		E1	E2	E1	E2	E1	E2	E1	E2	E1	E2
IPC 422	Mean	49.9	48.8	134.5	116.3	16.51	15.69	22.19	22.12	9.90	8.47
	Range	47-53	48-52	124-144	106-125	15.4-17.6	14.4-17.3	20.7-23.6	19.2-23.7	8.5-11	7-10
	GCV* (%)	1.74	0.66	2.45	2.93	2.10	3.90	1.80	3.94	0.71	5.05
IPC 715	Mean	48.5	45.6	134.3	107.5	11.02	10.22	18.97	18.91	6.63	6.92
	Range	47-50	43-50	125-141	99-115	10.2-12.1	9.4-11.1	18.0-20.5	17.6-20.2	5.4-9.3	6-7.6
	GCV (%)	1.32	0.76	0.67	3.11	0.10	0.97	2.58	2.01	7.39	4.70
IPC 795	Mean	60.9	59.6	160.6	134.4	23.96	25.03	24.01	24.4	9.15	9.77
	Range	58-63	58-61	150-171	122-144	22-26	23.2-27.2	22.5-25.0	22.2-26.4	8-10.3	8.2-11.2
	GCV (%)	1.56	0.17	1.37	1.91	2.64	3.21	0.83	1.59	4.88	5.79
IPC 802	Mean	49.3	50.3	134.0	109.7	15.21	15.26	16.36	16.37	6.98	6.67
	Range	46-53	50-53	127-154	88-119	13.5-16.2	13.7-16.4	14.3-19.2	14.3-17.5	5.5-8.1	5.4-8.4
	GCV (%)	1.98	0.66	4.71	4.45	1.74	1.79	4.24	2.80	3.11	2.12
IPC 828	Mean	47.2	51.2	143.0	123.6	12.39	11.46	27.69	28.46	13.1	13.38
	Range	46-49	49-54	136-156	106-133	11.3-13.6	9.8-12.5	22.4-29.5	25.7-30.3	10.2-14.1	10-15
	GCV (%)	1.13	1.80	2.10	2.44	2.21	4.00	1.17	2.22	1.71	4.48
IPC 909	Mean	54.1	54.1	157.6	107.4	17.05	15.73	18.91	18.51	8.51	8.09
	Range	51-58	52-59	148-167	89-118	15.6-18.4	13.2-17.7	18.2-20.0	15.3-20.8	7.6-9.5	5.6-9.1
	GCV (%)	1.60	2.76	1.71	3.94	2.93	2.46	0.99	2.50	1.86	4.01

* Genotypic Coefficient of Variation

inbred lines 'IPC 802' and 'IPC 909' had significant differences for four characters in each season. Significant differences among progenies were found for panicle diameter in 4 R-lines in the rainy season, of which 2 also had significant differences in post rainy season. In most of other R-lines, significant differences were observed for two characters in the post rainy season.

SSRs

Three SSR marker loci (*Xicmp3002*, *Xicmp3032* and *Xicmp3080*) exhibited heterozygosity in only one of the six B-lines studied (ICMB 89111) (**Table 1**). Two loci (*Xicmp3032* and *Xicmp3080*) mapping to pearl millet linkage group 1 showed heterozygosity in seven of eight DNA pools of ICMB '89111' and a third locus (*Xicmp3002*) revealed heterozygosity in only one ICMB '89111' DNA pool (DNA pool 13-16). None of the other five B-inbreds exhibited clear-cut SSR marker heterozygosity for the 20 markers studied in this experiment.

In a similar way, only one out of six R-inbreds studied ('IPC 422') exhibited marker heterozygosity. Among 8 DNA pools of 'IPC 422', three of them (DNA pools 5-8, 9-12 and 13-16) showed heterozygosity for the SSR marker locus *Xpsmp2212* and the other 5 pools were homozygous for this locus. The rest of the 19 loci appeared to be homozygous for the 8 DNA pools of all 6 R-inbreds. Two DNA pools in two marker loci produced non-specific PCR product peak patterns, which were not considered in these results.

DISCUSSION

Significant differences for all the morphological characters amongst B- and R-lines in both seasons validated the genetic diversity amongst the selected inbred lines. Higher number of significant differences was observed among progenies for B-lines (48%) than for R-lines (33%). This is probably due to the B-lines developed by a selfing procedure where 10-15 phenotypically superior ears were bulked in each season while self pollination with ear-to-row method had been used during the course of development and maintenance of the R-lines. In breeding programs, inbred lines are variously maintained through self-pollination with or without bulking (pooling seed from different plants) and sibbing. Consequently, bulking of seed from many self pollinated ears in B-lines may have resulted in a heterogeneous mixture of homozygous genotypes resulting into the presence of small genetic variability, whereas the method of self pollination in ear-to-row progenies preferred by R-line program resulted in lesser within-line genetic variation where variants could be easily recognized and eliminated over successive generations. Higgs and Russell (1968) also showed that maize inbred lines from six different sources had significant differences in traits such as plant height, silking date, ear height and grain yield and attributed this to method of maintenance, residual heterozygosity, or mutation; and found self-pollination in ear-to-row progenies resulted in less genetic change compared with other methods of maintenance such as selfing and bulking and then sibmating. Bogenschutz and Russell (1986) reported significant variation over successive generations in the long-time maize inbreds maintained by selfing than for lines maintained under full-sib mating and recommended sibmating method of line maintenance for reducing residual heterozygosity in the inbred lines. Variation for several traits, including productivity, within long-term inbred lines of maize was also reported by others (Russell *et al*. 1963; Fleming *et al*. 1964; Fleming 1971; Grogan and Francis 1972). Tokatlidis (2000) reported intra-line variation for plant and ear traits in long-term maintained maize inbreds and also found this variation to be transmissible to the hybrids. Although elite cultivars are considered fairly homogeneous, genetic variation has been reported among single plants of cultivars. Such intra-cultivar variation has been reported in crops such as wheat (*Triticum aestivum* L.), cotton (*Gossypium hirsutum* L.), maize, and soybean (*Glycine max* (L.) Merr.) (Fasoula 1990; Fasoulas 2000; Fasoula and Fasoula 2000; Tokatlidis 2000; Tokatlidis *et al*. 2004; Fasoula and Boerma 2005; Tokatlidis *et al*. 2006).

The progenies of inbred 'ICMB 89111' displayed the largest number of observed significant differences for both quantitative characters and SSR markers. Earlier also, 'ICMB 89111' has recorded residual heterozygosity for downy mildew resistance (Hash *et al.* 2006). The observed genetic variation in inbred lines could be due to many reasons like residual heterozygosity, mutations (including those induced by retrotransposons), and perhaps due to the introgression of genes from other sources. Results from earlier conducted molecular studies have indicated that such variations are caused due to the constant remodeling and restructuring of genome (McClintock 1984; Zhang *et al*. 1995; Olufowote *et al*. 1997; Rasmusson and Phillips 1997; Gethi *et al*. 2002; Cullis 2005; Morgante *et al.* 2005). McClintock (1984) suggested that the genome is dynamic and it can modify itself in response to environmental stresses. However, in this set of inbreds the primary reason seems to be the method of inbred maintenance, which appears to have left a small but significant amount variability in the lines. As evidenced, a wide range of variability for most of

the observed characters in the progenies of these elite inbreds is present but the contribution of genotype in the observed within-line variation is quite small as evidenced from the low GCV values for all observed characters in both seasons. Thus, this residual within-line variation is too small to have any significant impact on the performance of their hybrids in the succeeding years after their release. This cryptic variability within designated B- and R-lines provides opportunity to seed producers to select variants and develop new materials for utilization in their breeding programs, while simultaneously taking into account the intellectual property rights (IPRs) of the originating breeding station. For instance, mean for plant height for 'ICMB 89111' was 93 cm in the post rainy season, but one progeny recorded 84 cm of plant height which can be selected to develop a more dwarf version of 'ICMB 89111' (if this was found desirable in particular target environments or hybrid combinations). One progeny of 'ICMB 02111' had panicle diameter of 30 mm, although the mean of line progenies was 27 mm; this thick-panicled progeny can be utilized to develop a large panicle volume version of 'ICMB 02111'. Similarly, mean days for 50% flowering in 'IPC 802' was 49 days in the post rainy season but one progeny that flowered in 46 days provides an opportunity to develop a more photoperiod sensitive version of 'IPC 802'.

In this study modest numbers of polymorphic SSR markers used were able to detect only a low level of residual heterozygosity in some of the inbreds. It seems that these SSR markers owing to their less number and therefore their limited coverage across the genome were not able to capture the observed phenotypic variation at DNA level. While, Gethi *et al.* (2002) on the basis of study on 6 long-term maize inbreds using 44 SSR loci reported 4.6% of the total variation due to intra-line variation; and accounted this to method of seed maintenance, and no evidence was found for mutation or outcrossing. Also, Heckenberger *et al.* (2002) reported residual heterozygosity in nine inbreds and five doubled haploids of maize using 100 SSR markers, and recommended genotyping of individual plants rather than of bulk sample derived from several plants, which may become source of variation due to remnant segregation in some regions of the genome. Heckenberger *et al.* (2003) reported bulking method used during maintenance breeding as the main source of high level of heterogeneity in highly inbred lines, rather than lab errors or foreign pollen. Thus, irrespective of sources, genetic variation should be assessed utilizing both quantitative characters and large number of SSR markers (or other high-throughput markers such as DArT, SNP or GBS) to reveal the hidden residual heterozygosity in inbreds. The estimation of residual heterozygosity in inbreds ensures the uniformity and stability of inbreds developed from the breeding programmes. Moreover, on a cautious side, the seed producers should consider comparing their most widely used inbreds periodically with stock culture maintained in cold storage to monitor whether genetic drift, as a result of within-line variability in elite inbreds, might be reducing the efficiency of their breeding programs. The availability of such information would also help in resolving concerns related to IPRs, mapping studies, marker development, and long-term recombinant inbred line development, especially when high resolution is required.

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