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Genetic analysis of *japonica* x *indica* recombinant inbred lines and characterization of major fragrance gene by microsatellite markers

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Traditional basmati rice varieties are very low yielding due to their tendency to lodging and increasing susceptibility to diseases. To improve the characters of basmati rice variety and study the inheritance of various physio-morphological and quality characters, F_5 population comprising of 204 lines from the cross between NPT II (non-aromatic, *japonica*) and Taraori Basmati or HBC19 (aromatic, *indica*), were evaluated. Ample amount of genetic variability was observed for the characters plant height, tillers per plant, kernel length, kernel breath and L/B ratio. The grain yield/plant showed positive correlation with productive tiller/plant and test weight. Path coefficient analysis showed that the productive tiller/plant and test weight contribute to grain yield/ plant through direct effect. The parent off-spring regression was high for all the characters under study suggesting improvement of these characters by mere selection. Based on divergence study, 204 lines were categorized in seven clusters whereas parents were grouped in different clusters. Molecular restricted selection using specific SSR markers with depicting high correlation with aroma could offer great promise to select high yielding rice among high aroma lines. A total of 54 randomly selected F_5 plants were subjected to SSR marker analysis using SSR markers. The F_5 plants had an allele from either of the two parental lines (homozygous condition) or alleles from both the parental rice varieties (heterozygous condition). At some SSR loci, new/recombinant alleles were observed, which indicate the active recombination between genomes of two rice varieties and can be used for linkage mapping once complete homozygosity is achieved. SSR allelic profile based on two dimensional principal component analysis demonstrated high level of diversity among parents and F_5 plants spread between them.

Key words: *Oryza sativa* L., basmati, microsatellite, phenotyping, rice, recombinant inbred lines (RILs).

INTRODUCTION

Based on a number of morphological, physiological, biochemical and molecular traits, Asian cultivated rices are organized in two major subspecies, that is, *Oryza sativa japonica* and *Oryza sativa indica* (Oka, 1988). These two subspecies are commonly associated with differences in growth habitat and are the products of

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independent domestication events from ancestral *Oryza rufipogon* populations in different locations and at different times (Sang and Ge, 2007). Basmati rices are known to originate in undivided India and Pakistani Punjab. Traditional basmati varieties have unique quality in the world. However, these are tall, photoperiod sensitive low yielder, weak stemmed non-responsive to increased nitrogenous fertilizer dosages. Basmati rice varieties with exquisite aroma, superfine grain characteristics and excellent cooking qualities have gain greater attention due to greater preference in domestic and international markets. According to its grain shape, rice is primarily classified into long, medium and short categories. The cooking quality of rice grain is associated with grain shape (Yang et al., 2001; Luo et al., 2004). In most cases, long grain rice has a high grain amylose content and after cooking, it is often firm and fluffy (not sticky); medium grain rice has a low amylose content and after cooking, it is often soft, moist and sticky in texture. The cooking quality and amylose content in short grain rice are similar to those of rice in the medium grain category. Grain shape has attracted significant attention in rice breeding programs due to its contributions to rice yield and quality.

Grain shape has been widely accepted as a complex trait controlled by multiple genes with small effects. Grain size is usually evaluated by test weight, which is one of the three key components, along with grain length, grain width and panicle number per plant. The most important component of fragrance in basmati varieties is 2-acetyl-1-pyrroline (2-ACP) (Buttery et al., 1983; Paule and Powers, 1989; Petrov et al., 1996). The detailed biosynthesis pathway of this compound has not yet been completely elucidated (Lorieux et al., 1996; Bradbury et al., 2005). The studies have established that in most of the varieties, a single recessive gene is responsible for fragrance (Lorieux et al., 1996). Identification of ACP by smelling or chewing seeds is subjective and non-reliable as analyst sense become saturated or physical damage occurs from chewing hard grain. Further, identification of ACP using gas chromatography require large sample of tissue and also time consuming. Initial genetic studies performed by Tanksley's group (Ahn et al., 1992), localized a gene controlling aroma or fragrance (*frg* gene) in Della (Jasmine-derived aromatic variety) on the long arm of chromosome 8. Later, Lorieux et al. (1996) tagged this gene as a major and recessive quantitative trait locus (QTL) in the same region, but limited to a 12 cm genetic interval and in a (IR64 × Azucena) DH population where the traditional upland variety Azucena was the donor of aroma. Application of molecular marker technology in linkage mapping and molecular dissection of the complex traits such as fragrance can greatly enhance the efficiency and accuracy of breeding process. It is difficult to intogress desirable traits into basmati rice by conventional breeding methods due to complex nature of basmati rice grain quality and its poor combining ability with other rice genotypes (Singh et al., 2000). Glaszmann (1987) identified basmati rice as a genetically distinct cluster, distinct from that of typical *indica* and *japonica.* The genetics of basmati rice quality traits are not well understood, and finding desirable segregants possessing all the desirable basmati characteristics has been difficult.

In the present investigation, we describe the development and phenotyping of RIL's derived from cross NPT II and HBC 19 with respect to yield, yield attribute and quality and also determines association between Rg-28 locus and basmati rice aroma.

MATERIALS AND METHODS

Mapping populations of RILs were developed from a cross between NPT II (IR 68552-100-1-2-2) and HBC 19 (pureline selection from Taraori basmati). NPT-II is a *japonica*, non-scented high yielding plant type. It was crossed as female to HBC 19, a commercially important premium basmati variety having long slender grain, intermediate amylose content with high alkali spreading value and elongation ratio. The two parental rice varieties, NPT II and HBC 19 and 204 F_5 recombinant inbreed lines were grown in Augmented design at CCSHAU. Each progenies / lines was planted in a row of 2.5 m length.

Phenotyping of population

RILs in F_5 generation were evaluated for agronomic traits namely, plant height, effective tillers/plant, test weight and grain yield/plant. Mature F_6 grains of the RILs and parental lines were dried at 37°C for 15 days and hulled in laboratory mill. The 10 representative unbroken rice grains were measured for Kernel length and Kernel breath using photographic enlarger. The correlation coefficient was calculated according to the method suggested by Johnson et al. (1955) and path coefficients were obtained according to Dewey and Lu (1959). The degree of resemblance between relatives indicates the proportionate amount of additive genetic variance in the population. For measuring the resemblance between offspring and parents, the grouping of observation was done in a different way. The sum of cross products is used to calculate the covariance of offspring with parents and the regression of offspring on parents is calculated to measure the degree of resemblance. The regression of parents b_{op} was calculated following Falconer method (1983). In order to quantify the genetic diversity between parental lines and RILs, Euclidean Cluster Analysis was employed. Constellation of genotypes into cluster was done following Ward's minimum variance method (Ward, 1963). The aroma of F_6 grains was determined by placing ten milled rice grains in a Petri plate containing 1.7% KOH and incubated at ambient temperature for 10 min as suggested by Sood and Siddiq (1978) method. The RILs were scored as aromatic, moderately aromatic and non-aromatic.

Genotyping of RILs

A total of 54 plants were randomly selected from the NPT II × HBC 19 F_5 population for microsatellite marker analysis. Genomic DNA was isolated from one month-old plant leaf samples (~ 100 mg each) using CTAB method (22N). DNA was checked for its quality and quantity by 1% agarose gel electrophoresis using a standard containing 100 ng/µl genomic λ DNA. Thirty five (35) markers (obtained from Research Genetics, Huntsville, AL, U.S.A.) were evaluated for polymorphism between NPT II and HBC 19 (data not

Table 1. Mean, range, phenotypic and genotypic coefficients of variations, heritability and genetic advance for various traits in F_5 population derived from cross NPT II x HBC 19.

Character	Mean± SE(m)	Range	PCV	GCV	Heritability	Genetic advance (%mean)
Plant height (cm)	121.58 ± 0.81	90.00 - 157.00	10.32	9.59	86.3	23.51
Productive tillers/plant	8.29 ± 0.14	5.00 - 23.50	47.29	25.70	29.5	36.86
Yield/plant (g)	8.39 ± 0.25	1.90 - 32.76	46.53	44.52	92.3	112.9
1000 grain weight (g)	18.67 ± 0.19	12.10 - 28.50	18.17	15.09	69	33.08
Kernel length(mm)	7.09 ± 0.05	5.00 - 10.50	24.11	10.80	20.1	12.78
Kernel breath(mm)	1.95 ± 0.02	1.38 - 3.00	33.69	15.48	21.1	18.77
L/B ratio	3.71 ± 0.05	2.09 - 6.00	22.14	19.73	79.4	46.43

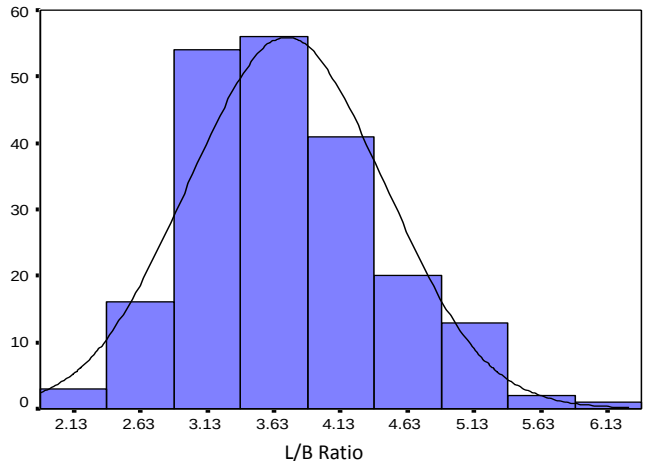

Figure 1. Frequency distribution graph of the RIL population for L/B ratio.

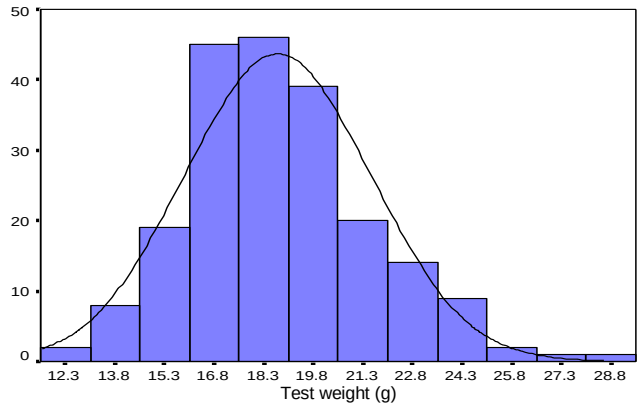

Figure 2. Frequency distribution graph of the RIL population for test weight (g).

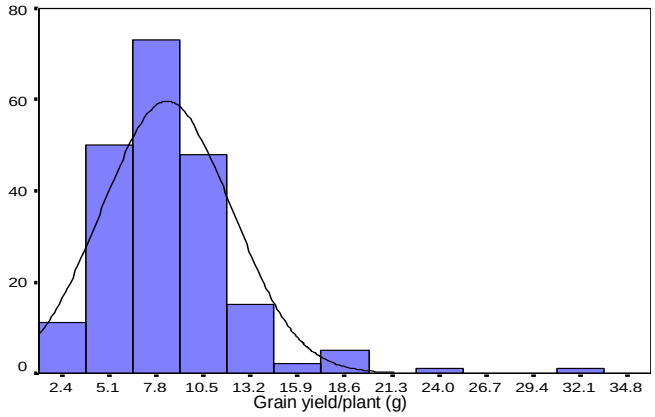

Figure 3. Frequency distribution graph of the RIL population for grain yield per plant (g).

shown) and six of these showing polymorphism and clear discrete banding profiles were used for analysis of NPT II x HBC 19 F_5 plants. The PCR reaction was conducted in a reaction volume of 20 µl containing 1x PCR buffer, 100 µM dNTPs, 0.4 µM of each primer, 1.2 mM $MgCL_2$, 1 unit Taq DNA polymerase and 50 ng template DNA. PCR amplification was performed with initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and final extension at 72°C for 7 min before cooling at 4°C. Amplification products were denatured and resolved at 4% denatured polyacrylamide gel, as described by Saini et al. (2004). The size (in nucleotides base pairs) of the most intensely amplified band of stutter for each microsatellite marker was determined based on its migration relative to molecular weight size marker (10 bp DNA ladder from Gibco BRL, Md.).

RESULTS AND DISCUSSION

Phenotyping variation among RILs

F_5 population showed significant variation among 204 RILs for all the characters. Ample amount of variation was observed for almost all the characters (Table 1) showing these traits are responsible for variation in grain yield in various lines. The coefficient of skewness for grain yield/plant, plant height, test weight and L/B ratio were 2.248, 0.297, 0.582 and 0.446; was less than the standard deviation 3.714, 11.66, 2.817 and 0.7338, respectively which indicates normal distribution of the population (Figures 1 to 3) and that these characters are governed by polygene. Long slender grain is a defining characteristic of basmati rice varieties. The grain length in

Table 2. Phenotypic correlation coefficient in F_5 population derived from cross NPT II x HBC 19.

Character	Plant height (cm)	Productive tillers/plant	Yield/plant (g)	1000 grain weight (g)	Kernel length (mm)	Kernel breath (mm)	L/B ratio
Plant height (cm)	-	0.0760	0.0054	0.0530	0.0541	0.0037	0.0148
Productive tillers/plant			0.3222**	0.0880	0.0778	0.0174	0.0658
Yield/plant (g)				0.2008**	-0.0886	0.0515	-0.0416
1000 grain weight (g)					0.0911	0.1387	-0.0537
Kernel length(mm)						-0.1052	0.6116**
Kernel breath(mm)							-0.8283**
L/B ratio							

**Significant at 1%; *Significant at 5%.

Table 3. Direct (diagonal) and indirect effects of component traits on grain yield/plant in F_5 population derived from cross NPT II x HBC 19.

Character	Plant height (cm)	Productive tillers/plant	1000 grain weight (g)	Kernel length (mm)	Kernel breath (mm)	r with grain yield/plant
Plant height (cm)	-0.0216	-0.0016	-0.0011	-0.0012	-0.0001	0.0054
Productive tillers/plant	0.0241	0.3175	0.0279	0.0247	0.0055	0.3222**
1000 grain weight (g)	0.0098	0.0163	0.1848	0.0168	0.0256	0.2008**
Kernel length (mm)	-0.0069	-0.0100	-0.0117	-0.1283	0.0135	-0.0886
Kernel breath (mm)	0.0000	0.0001	0.0010	-0.0007	0.0070	0.0515

RILs ranged from 5 to 10.50 mm. The two parental variety HBC 19 and NPT II are quiet similar in test weight measuring 24 and 24.5, respectively. However, test weight in the RILs ranged from 12 to 28.5 g with a population mean 18.67 g. The transgressive segregants were noticed for almost all the characters. High heritability together with genetic advance was recorded for grain yield/plant, plant height, test weight and L/B ratio indicate the presence of additive gene effect and ample scope of improving these characters through simple selection. High genotypic and phenotypic coefficient of variance for grain yield and its component were observed indicating that variability among the genotype is genetic and selection for these characters will be effective. Characters with low GCV such as plant height and test weight showed high heritability which indicate that variation for these characters is heritable.

Phenotypic correlation coefficient analysis was carried out to assess the association between various traits in NPT II x HBC19 F_5 population (Table 2). Yield per plant had positive correlation with productive tillers per plant and test weight (0.3222** and 0.2008**, respectively). Kernel length had positive correlation with L/B ratio (0.6116**) and negative correlation with kernel breath (-0.8283**). Correlation between the traits could be due to linkage or pleiotropy. Path coefficient analysis provides a clean and more realistic picture of a complex situation that exists at the correlation level. Portioning of genotypes correlation between yield/plant and its component characters indicated that all the characters contribute to grain yield /plant through direct effect, not by indirect effect (Table 3). Productive tillers/plant (0.3175) and test weight (0.1848) had high positive direct effect on grain yield/plant. The degree of resemblance between relative indicates the proportionate amount of additive genetic variance in the population. To phrase it otherwise, the resemblance between relatives which is one of the basic genetic properties of the population is determined by the relative magnitude of the additive and non-additive components of genetic variation. Since additive component is fixable part of the genetic variance, its relative magnitude plays a key role in deciding the breeding methodology for improving the character. More, the resemblance between the relatives, higher the magnitude of additive genetic variance and thus more easy is the improvement in the character.

The parent offspring regression (b) were 0.783, 0.553, 0.431, 0.328, 0.293, 0.246 and 0.213 for 1000 grain weight, grain yield per plant, plant height, kernel breath, L/B ratio, kernel length and tillers per plant, respectively. It was high for most of the characters suggesting that these characters can be improved mere by selection.

Cluster analysis

Based on divergence studies, parents and 204 RILs were categorized into nine clusters (Table 4) with highest

Table 4. Mean values of different clusters for 7 traits in F_5 population derived from cross NPT II x HBC 19.

Cluster	Number of RILs	Plant height (cm)	Productive tillers/plant	Yield/plant (g)	1000 grain weight (g)	Kernel length (mm)	Kernel breath (mm)	L/B ratio
1	43	120.23	8.15	8.77	17.32	3.91	1.60	4.36
2	42	111.91	8.11	7.66	18.85	7.11	2.13	3.37
3	33	120.45	7.01	8.28	17.00	6.50	2.08	3.17
4	32	127.06	8.06	9.13	22.77	7.15	1.96	3.70
5	22	129.65	10.86	9.10	18.35	6.91	2.30	3.04
6	18	130.48	7.83	5.87	18.98	7.69	1.91	4.04
7	14	123.80	7.95	6.54	16.14	8.46	1.80	4.74
8	HBC 19	136.00	23.50	24.69	24.00	8.25	1.38	6.00
9	NPT II	96.00	12.50	32.76	24.55	5.75	2.75	2.09

Table 5. Microsatellite alleles for the polymorohic marker on aromatic and non-aromatic rice.

S/N	Primer	HBC 19	NPT II
		Allele size (bp)	Allele size (bp)
1	RM 223	151	158
2	RM 149	240	259
3	RM 210	143	157
4	RM 42	159	165
5	RM 308	131	146
6	RM 195	302	308

number of genotypes in cluster I (43). Parents and RILs come in different group; this means that recombination occurred in segregating population and the RILs differ from parents. Test weight of cluster IV (22.77 g) is comparable to the parents whereas cluster VII has more kernel length (8.46 mm) than that of HBC 19(8.25).

Aroma

Aroma is one of the most important quality trait for basmati rice consumers. Petrov et al. (1996) reported more than 100 volatile compounds in the rice grain, of which 2-acetyl-1-pyrroline was the most predominant component. The two parental lines differ in grain aroma; HBC 19 highly aromatic whereas, NPT II non-aromatic. Out of 204 lines, very few lines have almost same aroma and yield (20 g) as comparable to HBC 19. Out of 54 agronomically superior lines which are subjected to SSR analysis, 32 lines are aromatic and 22 lines are non-aromatic. Among these RILs line, no. 111 and 143 has highest kernel length (10 and 9 mm) and L/B ratio (5.25 and 4.5) which is much higher than better Better parent HBC 19. No intra line variation for aroma was observed. These lines also differ for grain shades ranging from red to white (data not shown). Few RILs (5.55%) reconstitute the original aroma of HBC 19 suggesting involvement of 3 or more genes as their expected proportion in RILs will be ½ n with n number of genes (Loriex, 1996).

Microsatellite marker analysis

Micosatellite marker analysis was carried out on 54 (32 aromatic) randomly selected F_5 plants (NPT II × HBC 19) and two parental varieties using six polymorphic markers (Table 5) to assess allelic distribution and percent homozygosity achieved so far and association of molecular marker with aroma. Eleven (11) RILs (7, 8, 10, 12, 13, 16, 17, 24, 28 and 30) were homozygous for all the locus except RM 195; whereas nine (Line no. 23, 26, 34, 35, 39, 44, 46, 48 and 50) out of 54 RILs were giving banding pattern similar to HBC 19 except at locus RM 195 which shows NPT II type banding and biochemical assay showed the presence of aroma which might be due to recombination and consequently Rg 28 locus was transferred to NPT II background or vice-versa. RM 342 was significantly positively correlated to aroma while all other markers are positively correlated to aroma except RM 149 (Table 6). This may be due to the fact that aroma in generally is recessive or possibly because of failure to recognize the effect of xenia and the fact that aroma is an endosperm characteristic product of sexual fertilization is a one generation advance from that of the plant on which

Table 6. Correlation coefficient between molecular markers and aroma in F_5 population derived from NPT II x HBC 19.

Primer	RM 223	RM 149	RM 210	RM 308	RM 42	RM 195	Aroma
RM 223	--	0.399**	0.183	0.431**	0.094	0.048	0.235
RM 149			0.382**	0.486**	0.128	0.121	-0.100
RM 210				0.569**	0.543**	0.217	0.211
RM 308					0.389**	0.122	0.156
RM 42						0.205	0.283**
RM 195							0.174

**Significant at 1%; *significant at 5%.

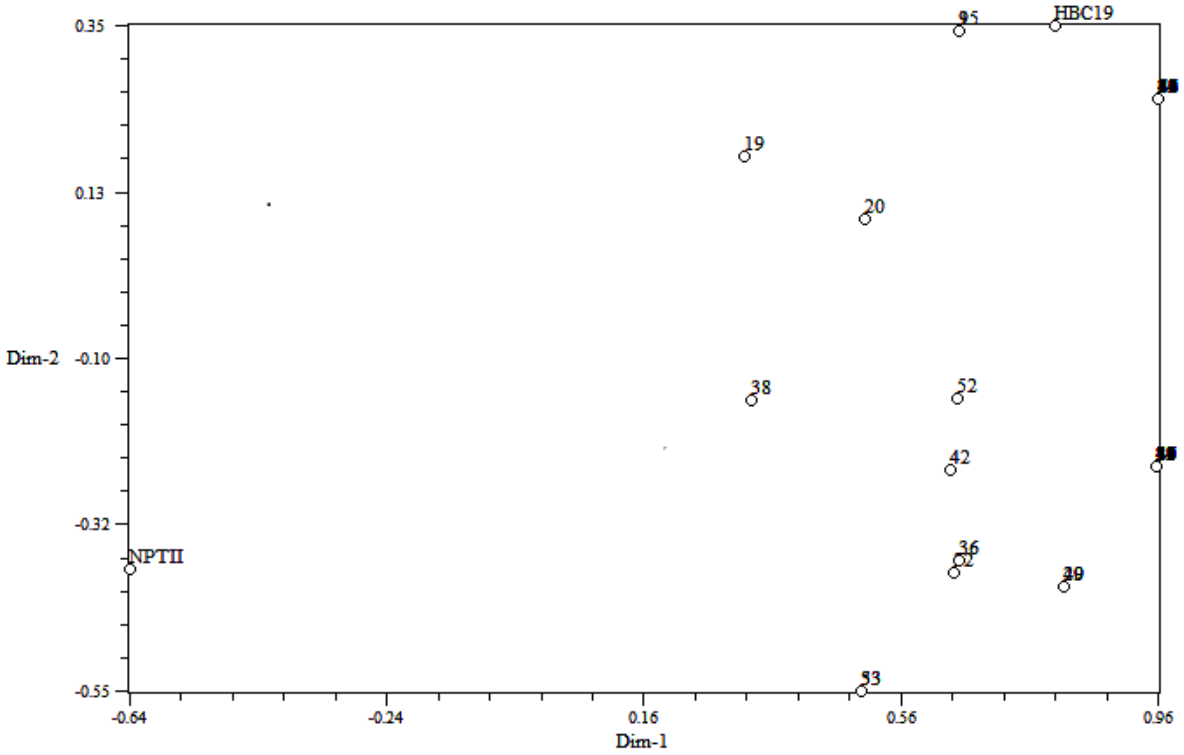

Figure 4. Two dimensional principal component analysis (PCA) scaling of 54 NPT II x HBC 19 F_5 genotypes using Jaccard similarity coefficient data of polymorphic markers.

it develops (Bollic, 1992).

Molecular analysis showed that there was still some residual heterozygosity in RILs probably due to insufficient number of selfing cycles at the F_5 generation. Percent homozygosity varied from 52 to 100% with an average of 72%. This value is lower than the expected value of 96.8% in F_5 generation. Deviation of observed frequency of the two segregating alleles of individual markers from the expected 1:1 Mendelian ratio may be due to segregating distortion which can seriously affect the QTL mapping results (Xu et al., 1997). At one loci, RM 210 one (line no. 19) out of 54 F_5 plants showed the presence of new or recombinant alleles different from those present in either of the two parental rice varieties. Occurrence of such alleles may result from crossing over, and also some microsatellite loci have been reported to be more hot spots, where mutation occurs up to 100 times more frequently than the normal mutation rate (Jain et al., 2004). The two-dimensional PCA (Rohlf, 1993) generated using SSR allelic diversity data showed interspersing of 54F_5 plants between the two diverse parental genotypes (Figure 4). Some of the aromatic F_5 plants were close to parents whereas some lines shows variability beyond the parents which may be consequence of recombination between the genomes of two parental lines and the subsequent segregation that leads to the occurrence of unique genotypes.

In self pollinated crops, the germplasm is available in

the form of multitude of pure lines. The breeding endeavor revolves around recombining the genes of interest spread over different pure lines in one or few backgrounds. By way of recombination breeding which involves crossing of pure lines followed by selection in segregating generations till the selected lines become homozygous. In this context, the gene constellation governing aroma, yield, kernel length, test weight can be standardized by crossing. Such an attempt was expected to generate a population with much expended variability toward concurrent tandem selection for yield and grain quality including aroma. Molecular restricted selection using specific SSR markers with depicting high correlation with aroma could offer great promise to select high yielding rice among high aroma lines. The present study thus can be rated as an integrated approach for rice improvement using conventional recombination breeding compliment by molecular marker assisted selection.

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