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ALLELE SPECIFIC GENOTYPING ASSAY OF *FGR* GENE AND SEED QUALITY EVALUATION OF RICE VARIETIES OF PAKISTAN

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Abstract:

Pakistan is blessed with high quality rice having supreme attributes of physicochemical, cooking and eating properties and characteristic popcorn like aroma. Among all the quality traits, fragrance is the most valuable attribute with huge economic importance which is controlled by fragrant gene *(fgr)*. An 8-bp deletion and 3 SNPs in the exon 7 in fgr gene result in accumulation of 2-acetyl-1-pyrroline in aromatic rice conferring fragrance. The present study was conducted to evaluate the rice grain quality attributes (physical, chemical and cooking properties) along with organoleptic analysis. Rice aroma evaluation was done by sensory analysis (cooking / KOH test) and genotyping of *fgr* gene by Allele Specific Assay (ASA). Results showed that out of 35rice varieties tested, only 12 showed high score of aroma by cooking / KOH test. While these varieties had extra-long/long and slender grains with medium amylose content and soft texture. Allele Specific genotyping was carried out to validate the aroma by specifically amplifying of fgr gene (8bp deletion). The assay showed 255bp band for 12 varieties (Khushboo, Sughdaasi, Mehak, Basmati 385, GA-5015, Super Basmati, Shaheen Basmati, MG-Basmati, Basmati 2000, Basmati-198, Basmati 515 and Basmati 370) confirming their status of fragrance. The remaining 22 were found to be non-fragrant varieties giving 355bp PCR product which is particular for non- fragrant. This *fgr* allele specific markers / genotyping of fragrance allele can be utilized as a robust tool to discriminate between aromatic and non-aromatic rice varieties within local traditional varieties that possess *fgr* and facilitate in marker assisted selection in aromatic rice breeding programs.

Key Words: Aroma, Rice, Quality evaluation, Allele specific assay, Genotyping

Introduction

Aroma of rice is evaluated by three methods, cooking, chemical and genetic analysis related to fragrance of rice The limitations with sensory and chemical methods include large number of samples, tasting of individual grains (Reinke *et al.,* 1991), variability in smelling abilities of analysts and damaging effect to the nasal passage where chemicals are involved in the sensory tests. While the quantification of 2AP compound for fragrance is tedious with requirement of large tissue samples (Lorieux *et al.,* 1996; Widjaja *et al.,* 1996) and involvement of expensive and sophisticated techniques like gas chromatography and mass spectrometry. Many DNA based approaches are utilized with highest reliability (Vemireddy *et al.,* 2015; Primrose *et al.,* 2010) for the detection of aromatic trait, for both breeders and traders. DNA based marker system coupled with low price sequencing facilities has made easier the identification and authentication processes in food industry (Voorhuijzen *et al.,* 2012). Up till now, a variety of DNA based markers are available for the identification of Basmati rice but not predicting the 100% status of fragrance. Fortunately, the availability of the rice molecular maps and genome sequences, has provided an opportunity to discover the gene responsible for fragrance by the comparison of the sequences of fragrant and non-fragrant genotypes., suggesting *fgr* gene (badh2) on chromosome 8 were associated with the aromatic character in rice (Ahn *et al*., 1992; Lorieux *et al.,* 1996; Cordeiro *et al.,* 2002). Presence of a mutated portion (8-bp deletion and 3 SNPs in the exon 7) in this gene was reported to result in truncation of betaine aldehyde dehydrogenase enzyme whose loss-of-function lead to the accumulation of a major aromatic compound, 2-acetyl 1-pyrroline (2AP) in fragrant (Bradbury *et al.,* 2005b; Prathepha, 2008; Sakhtivel *et al*., 2009). A rapid and easy genotyping method of detecting the 8-bp deletion and 3 SNPs using Allele-Specific Amplification (ASA) was devised for distinguishing between homozygous and heterozygous fragrant and non-fragrant rice (Bradbury *et al.,* 2005b).

It is need of the day to characterize and screen the rice grain quality determinants in a wide range of genetic resources available along with authentic genetic status of fragrance to encounter the adulteration of aromatic and non-aromatic rice. Keeping in view above facts, present study was focused on evaluation of rice grain quality attributes (physical, chemical and cooking properties) along with organoleptic tests of rice germplasm of Pakistan to determine the general acceptability of rice among consumers. On the other hand, investigations were carried out for determination of fragrance of rice germplasm using inexpensive and robust method of Allele Specific Amplifications that will be very helpful in improving quality attributes and developing molecular-assisted breeding of aromatic rice of Pakistan.

Material and Methods

Plant material: A total of 35 rice varieties were collected from three Research Institutes of Pakistan namely National Agriculture Research Council (NARC) Islamabad, Nuclear Institute of Agriculture (NIA) TandoJam and IRRI Dokri Sindh, Pakistan.

Grain classification: To determine the physical characteristic (grain shape, size) procedures described by

Dela Cruz & Khush, 2000 were employed. Ten dehusked rice kernels of each variety were measured for Length (L) and Breadth (B). L/B ratio was calculated and grains were classified as Extra Long Slender (ELS), Long Slender (LS), Short Slender (SS), Medium Slender (MS), Long Bold (LB), Short Bold (SB).

Kernel length after cooking (KLAC): Length of five rice Kernels of each variety were measured in mm. The grains were then soaked with tap water for 30 minutes. Each test tube was then placed in water bath at 100^{o}C for 15 minutes. After boiling, the grains were placed in petri plates and the length of the cooked grains was measured. Elongation Ratio (ER) was calculated by dividing KLAC by initial length of uncooked grains.

Alkali spreading value (ASV): Six rice kernels were incubated for 23 hours at 27-30^{o}C with 10ml of 1.7% KOH solution. The alkali spreading value was then calculated as Low, Intermediate and Total dispersion. (Biswas & Juliano., 1988).

Gel consistency (GC): 100 mg rice flour was taken in a long test tube containing 2ml of 0.2N KOH, 0.2ml of ethanol containing 0.25% thymol blue and kept in water bath for 8 minutes. After removing from water bath, the test tubes were vortexed and kept on ice bath for 20 minutes. After 20 minutes incubation, the test tubes were kept horizontally undisturbed for 1 hour. The length of blue gel was measured using a graph paper (Cagampang *et al*., 1973).

Amylose content: For measurement of amylose content, 1ml of 95% ethanol and 9ml of 1N NaOH was added in 100mg rice flour in a flask. The mixture was mixed well and kept in boiling water bath for 10minutes. After removing the samples from water bath, the volume was made up to 100ml. From this mixture, 5ml of sample was taken in another flask and 1ml of acetic acid (57.75 ml /1L) and 1.5ml of iodine solution (0.2% iodine + 2% potassium iodide) was added and the volume was made up to 100ml with distilled water. The samples were kept for incubation at room temperature for 20 minutes. The absorbance of the samples was measured at 620nm. NaOH solution was used as control. The standard graph was made by using potato amylose, and the amylose content of the samples was calculated. (Perez & Juliano., 1978).

Aroma Sensory Evaluation

Cooked rice test: For cooked rice test, 5g of rice was soaked in 15ml of water for 15 minutes. The samples were cooked for 15 minutes; the samples were kept in refrigerator for cooling for 20 minutes. The cooked rice samples were smelled by 10 panelists and graded on four point scale as Very Strong scented (3), Strong scented (2), Mild Scented (1), Non Scented(0).

KOH test: For KOH test, 10ml of 1.7% KOH solution was added in 1gm rice flour of each sample in conical flasks. The flasks were covered and incubated for 1hr. The samples were smelled by 10 panelists and scored on four point scale as Very Strong scented (3), Strong scented (2), Mild Scented (1), Non Scented (0).

Organoleptic tests: For organoleptic test, 5g of rice samples were soaked in 15ml of water for 10 minutes. The samples were cooked for 15minutes and graded on appearance, cohesiveness, tenderness on touching and chewing, taste, aroma, elongation and overall acceptability by ten panelists.

DNA extraction, PCR amplification and electrophoresis: DNA of all the rice varieties was extracted at the seedling stage by CTAB method following the protocol of Doyle & Doyle, 1990. Allele specific PCR was performed by using 1ul of Taq Polymerase (Fermentas ®) 2.5 units, 3ul of genomic DNA 50ng/ul, 3ul of 25mM $MgCl_2$, 2.5ul of 10X buffer, 0.5ul of each allele specific primer 10mM, in a total volume of 25 ul per reaction as reported by Bradbury *et al*., 2005. External Sense Primer (ESP): TTGTTTGGAGCTTGCTGATG, External Antisense Primer (EAP): AGTGCTTTACAAGTCCCGC, Internal Fragrant Anti-sense Primer (IFAP): CATAGGAGCAGCTGAAATATATACC and Internal Non-fragrant Sense Primer (INSP): CTGGTAAAAAGATTATGGCTTCA. The 100kb DNA marker was used for gel analysis. (Gene Ruler, Thermo scientific®, USA. Cat no. SM0241)

The PCR cycling conditions were: 94^{0}C for 4 minutes of initial denaturation, 35 cycles of 94^{0}C for 30seconds, 56^{0}C annealing temperature for 35 seconds and 72^{0}C extension temperature for 30seconds which was followed by final extension for 7 minutes at 72^{0}C.

The PCR products were separated on by electrophoresis on 1.5% agarose gel.

Results and Discussion

Rice seed quality parameters including physical, chemical, cooking and sensory characteristics were analyzed for 37 varieties and genetic analysis of aroma was conducted on 34 varieties of basmati and non-basmati rice collected from different research institutes of Pakistan.

Physical attributes: Among physical attributes, GA-5015 exhibited maximum grain length (8.75mm) followed by Shaheen Basmati (8.1mm) and Bas-515 (7.98mm) whereas, the lowest length was recorded in rice variety JP-5 having kernels 5.5mm long. The lowest breadth was observed in basmati varieties such as Mehak with 1.71mm length, Super Basmati and Basmati-2000 with 1.81mm and Bas-370 with 1.87mm breadth. While JP-5 with 2.93 mm breadth was the variety with most broad kernels among non-basmati varieties. High length and small breadth of the kernels are two determinants of rice quality which are widely preferable for consumers (Shi *et al*., 2000; Iwata *et al*., 2010) that make up the overall shape of the grain which is determined by length and breadth ratio. The L/B ratio ranged from 4.51-1.89, which showed a remarkable diversity of all rice varieties. Maximum L/B ratio was observed by GA-5015 followed by Mehak, Bas-370 and Bas-385 with extra long grain size and slender shape. Regarding grain shape and grain size, 10 out of 37 varieties were extra long and slender, 2 Very long Slender, 17 Long slender, 5 Medium slender and only 1 Short Bold (Table 1) However, the results of physical attributes of few Pakistani varieties were found to be slightly deviating with previous reported results (Akram *et al.,* 2009), which may be due to the difference in growing conditions and availability of favorable environment to the plants.

Table 1. Physical properties of rice: Length, Breadth, Length and Breadth ratio, Grain size and shape

S.no	Varieties	Length	Breadth	L/B Ratio	Grain Size	Grain Shape
1	Shahkar	7.77±0.24	2.03±0.15	3.82	E.Long	Slender
2	Sada Hayat	7.24±0.22	2.25±0.15	3.21	Long	Slender
3	Kanwal	7.56±0.24	2.16±0.06	3.5	E.Long	Slender
4	IR-8	7.26±0.18	2.16±0.15	3.36	Long	Slender
5	IR-6	7.45±0.42	2.1±0.03	3.54	Long	Slender
6	DR-57	6.93±0.18	2.06±0.15	3.36	Long	Slender
7	DR-58	7.21±0.03	2.1±0.2	3.43	Long	Slender
8	DR-92	7.06±0.06	1.99±0.18	3.54	Long	Slender
9	DR-82	6.41±0.46	2.05±0.2	3.12	Medium	Slender
10	DR-83	7.35±0.23	2±0.66	3.67	Long	Slender
11	Khushboo	6.57±0.18	2±0.05	3.28	Medium	Slender
12	Shua	6.44±0.07	2.05±0.05	3.14	Medium	Slender
13	Shadaab	7.11±0.64	1.93±0.08	3.68	Long	Slender
14	Jajai77	6.3±0.58	1.92±0.14	3.27	Medium	Slender
15	Shandaar	6.89±0.11	2.17±0.19	3.17	Long	Slender
16	Sughdaasi	7.39±0.01	1.99±0.17	3.71	Long	Slender
17	Sarshar	7.6±0.62	2.02±0.63	3.76	E.Long	Slender
18	Mehak	7.76±0.29	1.71±0.16	4.5	E.Long	Slender
19	KangriTorr	6.72±0.23	2.1±0.17	3.2	Long	Slender
20	Swat-1	7.49±0.14	2.07±0.27	3.61	Long	Slender
21	Bas- 385	7.76±0.3	1.84±0.1	4.21	E.Long	Slender
22	KSK-133	7.65±0.18	2.09±0.14	3.66	E.Long	Slender
23	KSK-282	7.58±0.32	2.09±0.16	3.62	Long	Slender
24	GA-5015	8.75±0.29	1.94±0.12	4.51	E.Long	Slender
25	IR-9	7.69±0.09	1.79±0.01	4.29	E.Long	Slender
26	Super Basmati	7.65±0.25	1.81±0.02	4.22	E.Long	Slender
27	Shaheen Basmati	8.1±0.2	1.94±0.08	4.17	E.Long	Slender
28	JP-5	5.55±0.05	2.93±0.07	1.89	Short	Bold
29	MG Basmati	7.47±0.05	1.71±0.09	4.36	Long	Slender
30	Dilrosh 97	7.38±0.5	2.01±0.6	3.67	Long	Slender
31	Basmati 2000	6.8±0.05	1.81±0.0	3.75	Long	Slender
32	Basmati 198	7.05±0.05	1.89±0.0	3.73	Long	Slender
33	Bas-515	7.98±0.47	1.98±0.1	4.03	V.Long	Slender
34	Bas-370	7.97±0.4	1.87±0.1	4.26	V.Long	Slender
35	Fakhar-e-Malakand	6.58±0.05	2.71±0.04	2.42	Medium	Slender
36	NIA-102	7.69±0.02	2.07±0.03	3.71	E.Long	Slender
37	NIA-625	7.71±0.05	2.04±0.0	3.77	E.Long	Slender

*The values are mean± SD of three independent determinations

Chemical and cooking attributes: Most preferable chemical and cooking properties of rice are; intermediate amylose content, higher elongation after cooking and strong aroma. All the varieties exhibited variable response for these attributes; data revealed that Kernel Length after Cooking (KLAC) and Kernel Elongation Ratio (ER) ranged between 17-8.3mm and 2.05-1.14 respectively. Whereas highest KLAC was found in aromatic varieties GA-5015 (17mm) and Shaheen Basmati (16.3mm) with 1.94 and 2.05 Elongation Ratio respectively.

Among chemical characteristics, gel consistency (GC) was measured into soft, medium and hard. Gel Consistency of IR-8, KSK-282 and IR-9 varieties was medium to hard with length of blue gel ranging from 3.43-4.06cm while 20 varieties were soft in gel consistency (Table 2). The medium gel consistency was observed in 14 varieties which are mostly aromatic rice types. The varieties having soft to medium gel consistency tend to stay soft for a longer period of time after cooking making it more feasible for consumption (Champagne, 2010).

Gelatinization temperature was determined by the test of alkali spreading value which is the measure of dispersion of kernels in the presence of alkali solution. It was observed that some varieties showed almost very low or no dispersion of kernels with G.T more than 74°C (Table 2) while 10 varieties were intermediately dispersed having G.T 70-74°C. As the intermediate and low GT is considered one of the good rice quality parameter which exhibits capability of water absorption with little water uptake and fast cook (Stork *et al.,* 2005).

The GT of the rice varieties is known to vary between 50^0C to 79^0C and classified as low (55-69°C); intermediate (70-74 °C) and high 75-79°C (Juliano, 1979). Among aromatic varieties, only Khushboo, Sughdassi, Mehak, Super Basmati, Shaheen Basmati and Basmati 198 qualified the intermediate/low GT ranking. Cooking time of the rice depends on coarseness of the grain. The intermediate ASV indicated the medium disintegration and classified as intermediate GT which highly desirable for quality grain (Bansal *et al.,* 2006).

Another important quality index of cooking attributes is amylose content because it determines the texture of grains after cooking either soft or firm as well as also helps in maintaining low glycemic index (Foster *et al*., 2002) for diabetes. In this study, it showed a broad range of amylose contents ranged from 18.93 -32.7 (units) for all rice varieties. Based on standard classification, Swat-1 is categorized under those varieties with low amylose content (18.93) while all other varieties including aromatic and non-aromatic both have intermediate amylose content which is good quality indicator, except few varieties (Table 2). As the amylose content is directly related to the rice texture, the low the amylose content of rice, they will be soft and sticky after cooking. Rice with intermediate amylose content are preferred by majority of consumers, the preference may vary in different parts of the world (Shahidullah *et al.,* 2009; Lestari *et al*., 2011). In the current study, the amylose contents estimated in the rice varieties varied from the previous reported experiments (Akram *et al*., 2009). This lack of reproducibility in estimating amylose contents among different laboratories could be due the use of amylose from different sources for construction of standard curve and the iodine binding capacity of the chemical as discussed by Fitzgerald *et al*., 2009.

Table 2. Chemical and cooking properties of rice: Gel consistency (G.C), Alkali spreading value (ASV), Geltatinization temperature (G.T), Amylose content, Kernel length after cooking (KLAC) and Elongation Ratio (E.R)

S.no	Varieties	Length of gel	G.C	A.SV.	G.T	Amylose	KLAC	E.R
1	Shahkar	6.0±0.12	Medium	Intermediate	70-74	29.24±0.59	9±0.1	1.15
2	Sada Hayat	8.7±0.244	Soft	Intermediate	70-74	24.92±0.8	8.3±0.11	1.14
3	Kanwal	7.9±0.21	Soft	Low	<74	29.93±0.81	9.6±0.04	1.26
4	IR-8	4.06±0.16	Medium Hard	No./ Low	<74	30.3±0.43	11.6±0.45	1.59
5	IR-6	6.03±0.12	Medium	Low	<74	31±1.15	12.1±0.1	1.62
6	DR-57	9.13±0.26	Soft	Total	55-69	26.85±0.33	11±0.1	1.58
7	DR-58	7.8±0.16	Soft	Intermediate	70-74	28.58±0.36	10±0.30	1.38
8	DR-92	6.03±0.12	Medium	Intermediate	70-74	29.53±0.83	10±0.4	1.41
9	DR-82	8.26±0.20	Soft	Total	55-69	26.16±0.4	11±0.11	1.71
10	DR-83	6.96±0.12	Soft	No./ Low	<74	31.1±1.4	9.6±0.1	1.3
11	Khushboo	7.93±0.16	Soft	No./ Low	<74	25.46±0.47	10±0.1	1.52
12	Shua	8.33±1.24	Soft	Total	55-69	27.99±0.9	10.6±0.4	1.64
13	Shadaab	8.3±0.14	Soft	Low	<74	26.12±0.38	10.1±0.3	1.42
14	Jajai77	6.26±0.20	Soft	Intermediate	70-74	24.86±0.57	9.3±0.07	1.47
15	Shandaar	5.9±0.14	Medium	Low	<74	23.63±0.47	12.16±0.10	1.76
16	Sughdaasi	5.4±0.08	Medium	No./ Low	<74	26.76±0.41	10.6±0.35	1.43
17	Sarshar	7.93±0.16	Soft	Intermediate	70-74	29.7±0.53	8.8±0.3	1.15
18	Mehak	7.26±0.2	Soft	No./ Low	<74	25.63±0.55	9.5±0.1	1.22
19	KangriTorr	8.2±0.43	Soft	Low	<74	27.1±0.11	9.5±0.35	1.41
20	Swat-1	10.56±0.41	Soft	Total	55-69	18.93±0.31	13.1±0.1	1.74
21	B-385	5.53±0.16	Medium	Total	55-69	26.18±0.27	12.1±0.1	1.55
22	KSK-133	4.9±0.08	Medium	Total	55-69	31.76±0.66	13±0.05	1.69
23	KSK-282	3.9±0.14	Medium Hard	Total	55-69	32.24±0.43	12±0.04	1.58
24	GA-5015	4.9±0.08	Medium	Total	55-69	29.35±0.30	17±0.1	1.94
25	IR-9	3.43±0.09	Medium Hard	Total	55-69	32.7±2.35	11.5±0.1	1.49
26	Super Basmati	6.43±0.26	Soft	Intermediate	70-74	26.14±0.16	12.1±0.1	1.58
27	Shaheen Basmati	7.13±0.61	Soft	Intermediate	70-74	31.44±0.10	16.3±0.1	2.01
28	JP-5	7.13±0.26	Soft	Total	55-69	29.8±0.34	9.1±0.05	1.63
29	MG Basmati	7.73±0.2	Soft	Total	55-69	29.11±0.83	12.5±0.1	1.67
30	Dilrosh 97	7.86±0.12	Soft	Intermediate	70-74	28.03±0.97	10.3±0.25	1.39
31	Basmati 2000	4.7±0.29	Medium	Total	55-69	26.46±0.35	14±0.05	2.05
32	Basmati 198	5.6±0.29	Medium	Intermediate	70-74	27.16±0.20	12.1±0.1	1.71
33	Bas-515	4.56±0.32	Medium	Total	55-69	26.26±0.30	12.5±0.05	1.56
34	Bas-370	5.33±0.12	Medium	Total	55-69	24.33±0.30	9.3±0.15	1.16
35	Fakhar-e-Malakand	6.1±0.29	Soft	Total	55-69	27.59±0.69	9±0.43	1.36
36	NIA-102	7.86±0.18	Soft	Low	<74	28.29±0.25	9.5±0.05	1.23
37	NIA-625	6±0.08	Medium	No./ Low	<74	27.43±0.37	13.5±0.05	1.75

*The values are mean± SD of three independent determinations

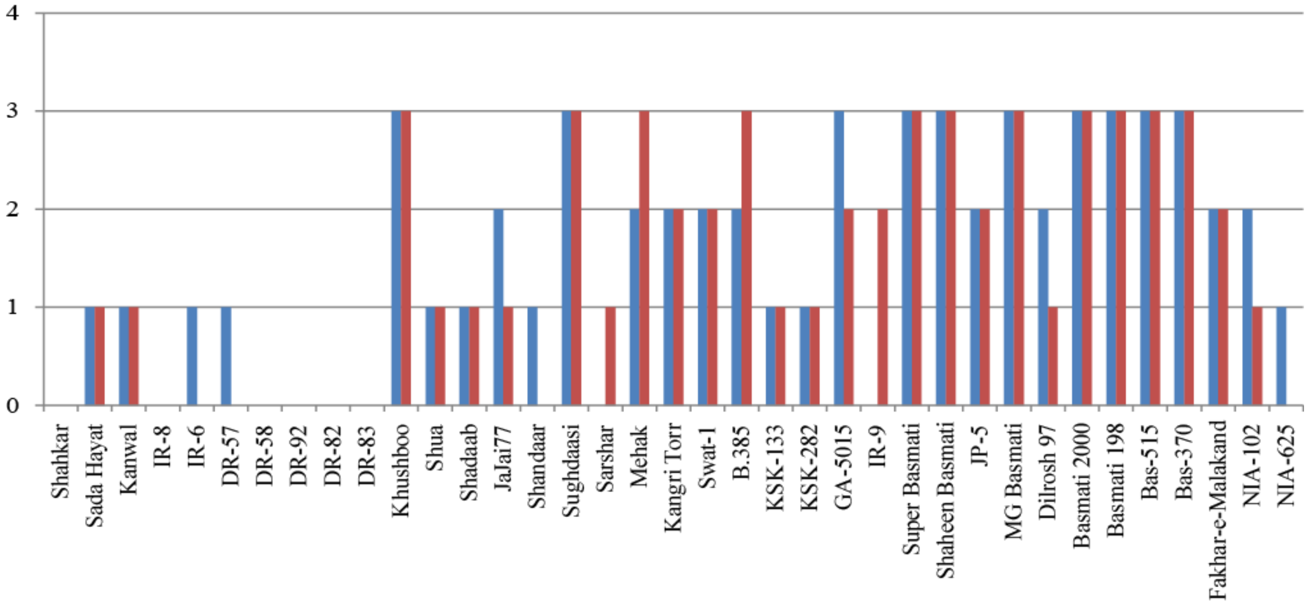

Fig.1 Aroma evaluation of rice varieties by Cook Aroma and KOH Aroma tests Aroma scale : Very Strong scented (3), Strong scented (2), Mild Scented (1), Non Scented(0)

Table 3. Organoleptic analysis of rice varieties of Pakistan

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Appearance																			
White																		+	+
Creamish white/brown	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+		
Red streaks									+										
White with brown streaks										+				+		+			
White with black streaks																			
Cohesiveness																			
Well separated	+	+			+	+	+	+		+									
Partially separated			+	+								+							+
Slightly separated									+		+		+	+	+	+	+		
Moderately separated																		+	
Very sticky																			
Tenderness on touching																			
soft			+										+						
Moderately soft									+		+	+		+		+		+	+
Moderately hard															+		+		
hard	+	+			+	+	+	+		+									
Very soft				+															
Tenderness on chewing																			
soft			+													+			
Moderately soft											+	+		+				+	+
Moderately hard									+						+		+		
hard	+	+			+	+	+	+		+									
Very soft													+						
Taste																			
Good										+	+	+					+	+	+
Desirable		+	+			+	+	+					+			+			
Tasteless	+			+	+				+					+	+				

1:Sada Hayat, 2:Shahkar, 3:Kanwal, 4:IR-6, 5:IR-8, 6: DR-57, 7:DR-58, 8:DR-92, 9:DR-82, 10:DR-83, 11:Mehak, 12:Khushboo, 13:Shandar, 14:Shua, 15:Shadab, 16:Sarshar, 17:Jajai 77, 18:Sughdasi, 19:Kangri Torr

Table 4. Organoleptic analysis of rice varieties of Pakistan

	Characteristics	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
A	**Appearance**																		
5	White	+	+	+	+		+	+	+	+				+	+	+	+		
4	Creamish white/brown										+	+	+					+	+
3	Red streaks																		
2	White with brown streaks					+													
1	White with black streaks																		
B	**Cohesiveness**																		
5	Well separated		+		+			+			+		+						
4	Partially separated	+		+			+		+					+	+		+	+	+
3	Slightly separated					+				+		+							
2	Moderately separated															+			
1	Very sticky																		
C	**Tenderness on touching**																		
5	soft	+				+			+	+			+	+	+		+		
4	Moderately soft		+	+			+					+				+		+	
3	Moderately hard				+			+			+								+
2	hard																		
1	Very soft																		
D	**Tenderness on chewing**																		
5	soft	+				+			+	+			+	+	+		+	+	
4	Moderately soft		+	+			+					+				+			
3	Moderately hard				+			+			+								+
2	hard																		
1	Very soft																		
E	**Taste**																		
4	Good	+							+	+			+		+		+		
3	Desirable		+	+			+				+	+		+					+
2	Tasteless				+	+		+								+			
1	undesirable																	+	
F	**Elongation**																		
4	Excellent	+											+						
3	Good		+		+	+						+		+	+		+		
2	Moderate			+			+	+	+	+	+					+			+
1	None																	+	
G	**Overall acceptability**																		
4	Excellent	+							+	+			+		+		+		
3	Good		+				+					+		+					
2	Acceptable			+	+	+		+			+					+			+
1	Undesirable																	+	

20:JP-5, 21:Shaheen Basmati, 22:Basmati 2000, 23: Swat-1, 24: IR-9, 25:KSK-133, 26: Fakhar-e-Malakand, 27:Basmati-385, 28:KSK-282, 29:MG-Basmati, 30:Basmati 370, 31:Dilrosh 97, 32: Basmati 198, 33: GA-5015, 34: Basmati 515, 35: Super Basmati, 36: NIA-102, 37: NIA-625

Sensory analysis of aroma: Aroma of all the varieties was evaluated by 10 panelists by using two types of sensory analysis i.e. aroma after cooking test and KOH test for aroma. It showed some inconsistency among results of both tests. According to KOH aroma test 10 varieties were graded with mild aroma, 6 with moderate aroma and 11 varieties were detected to have high aroma. The cooking aroma tests gave slightly deviating results from KOH test with 10 varieties detected with mild aroma, 10 with moderate aroma and 10 varieties were found to be strongly aromatic. Variation among results could be attributed because of the difference in signal perception of panel analysts due to their varying abilities. Comparatively, KOH test gave higher scores of aroma as compared to cooking aroma test for some varieties. It is reported that KOH aroma test is correlated with GC-MS method for quantification of aromatic compound (Widjaja *et al.* 1996) and helpful in early selecting the aromatic varieties in breeding programs. Consistent findings of both cooking aroma and KOH tests graded 9 varieties (Khushboo, Sughdassi, Super Basmati, Super Basmati, Shaheen Basmati, MG Basmati, Bas 2000, Bas 198, Bas 370 and Bas 515) as highly aromatic varieties, 4 varieties (Swat-1, JP-5, Fakhar e Malakand and KangriTorr) as moderately aromatic, 6 varieties (Sada Hayat, Kanwal, Shua, Shadab, KSK-133 and KSK-282) as slightly aromatic and 6 varieties (Shahkar, IR-8, DR-58, DR-92, DR-82, DR-83) as were non-aromatic (Fig. 1). Rest of 12 varieties exhibited some ambiguities regarding the ranking of aroma for these tests. It is evident from the results that Basmati varieties are ranked highly aromatic than other cultivars (Nadaf *et al*., 2006).

Table 5. Correlations among physical, chemical, cooking and sensory quality characteristics of 37 rice varieties

Correlations										
	Gel consistency	**Amylose**	**Alkali spreading**	**KOH Aroma**	**Elongation Ratio**	**KLAC**	**Breadth**	**Length**	**Cook Aroma**	**L/B Ratio**
GEL consistency	1									
Amylose	-.637**	1								
Alkali spreading	-.142	.172	1							
KOH Aroma	-.148	.076	.153	1						
Elongation Ratio	-.209*	.157	.313**	.163	1					
KLAC	-.318**	.322**	.272**	.242*	.866**	1				
Breadth	.133	-.101	.063	-.253**	-.088	-.322**	1			
Length	-.290**	.374**	-.016	.219*	-.032	.465**	-.530**	1		
Cook Aroma	-.078	.009	.190*	.940**	.286**	.304**	-.177	.112	1	
L/B Ratio	-.206*	.262**	.023	.323**	.046	.400**	-.762**	.750**	.219*	1

**. Correlation is significant at the 0.01 level (2 tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Organoleptic analysis: The organoleptic analysis was carried out by panelists using descriptive analysis for appearance, cohesiveness, tenderness on touching and chewing, taste, elongation and overall acceptability. The varieties ranked for excellent overall acceptability are KangoriTorr, Swat-1, Super Basmati, Shaheen Basmati, Basmati 2000, Bas-515 and Bas-370 among aromatic varieties while Shua and Sarshar among non-aromatic varieties. Good overall acceptability was found for Kanwal, Khushboo, Shadab, Mehak, Bas-385, IR-9, Dilrosh 97 and Bas-198. (Tables 4, 5) It is reported that organoleptic analysis provides the better comparative account of different rice varieties elaborating consumer preferences. In this regard, training and expertise of sensory analyst panels are very critical in the process of sensory analysis and organoleptic test (Lefebvre *et al.,* 2010).

Correlation of physiochemical attributes: The correlation coefficients for different physiochemical attributes were determined by Pearson's correlation presented in Table 4. Data revealed that gel consistency had highly significant and negative correlation with amylose contents ($r = -0.637$, $p<0.01$), KLAC ($r = -0.318$, $p<0.01$) and grain length ($r = -0.290$, $p<0.01$). While amylose content of grain showed positive and significant relationship with KLAC ($r = -0.322$, $p<0.01$), grain length ($r = 0.374$, $p<0.01$) and L/B ratio of kernels ($r = 0.262$, $p<0.01$). There was significant positive correlation found between some of the important quality parameters of rice such as KOH aroma test with Kernel length ($r = 0.219$, $p<0.05$), L/B Ratio ($r = 0.323$ $p<0.01$) and cook aroma ($r = 0.940$, $p<0.01$) while negative significant relationship with grain breadth ($r = -0.253$, $p<0.01$). Grain length was found to have highly significant and positive correlation with KLAC ($r = 0.465$, $p<0.01$) and negative correlation with breadth ($r = 0.530$, $p<0.01$). Here, cooking aroma test is highly correlated with elongation ratio ($r = 0.286$, $p<0.01$) and KLAC ($r = 0.304$, $p<0.01$) which is reported in several studies that the property of E.R is highly associated with aromatic varieties but in contrast KOH aroma test showed a non-signification correlation with ER.

KLAC has shown a positive and significant relationship with alkali spreading value ($r = 0.272$, $p<0.01$) and ER ($r = 0.866$, $p<0.01$). L/B ratio is positively significant with KLAC ($r = 0.400$, $p<0.01$) and length ($r = 0.750$, $p<0.01$) (Table 6) .

Allele Specific genotyping assay for fragrance: The external primers ESP and EAP used in the study amplified the fragment of approximately 580bp which served as a positive control. The internal primers IFAP and INSP were selective to amplify any of the two possible alleles, intact *fgr* gene (BADH2 allele) and truncated *fgr* gene (badh2 allele). The combination of IFAP and ESP primers generated the fragments of 255bp which indicates the presence of 8bp deletion hence badh2 allele. (Bradbury *et al.,* 2005) The fragment of 355bp was generated by INSP and EAP primers, which corresponds to the non-fragrant genotype. All the rice samples analyzed for fragrance shown either homozygous fragrant or homozygous non-fragrant genotype. As, it revealed from results, only 12 (Khushboo, Sughdaasi, Mehak, Basmati 385, GA-5015, Super Basmati, Shaheen Basmati, MG-Basmati, Basmati 2000, Basmati-198, Basmati 515, Basmati 370) were genotyped as fragrant by showing 255bp fragment while remaining 22 were found to be non-fragrant varieties giving 355bp PCR product (Fig. 2**).** It is evident that 8bp deletion and 3 SNPs in exon 7 of *fgr* gene lead to the non-functional BAD2 enzyme by premature stop codon only in aromatic varieties and accumulation of 2 AP compound (Vanavichit *et al.*, 2006). This polymorphism helped in construction of Allele Specific genotyping assay either homozygous fragrant, homozygous non-fragrant or heterozygous non-fragrant rice varieties (Bradbury *et al.,* 2005).

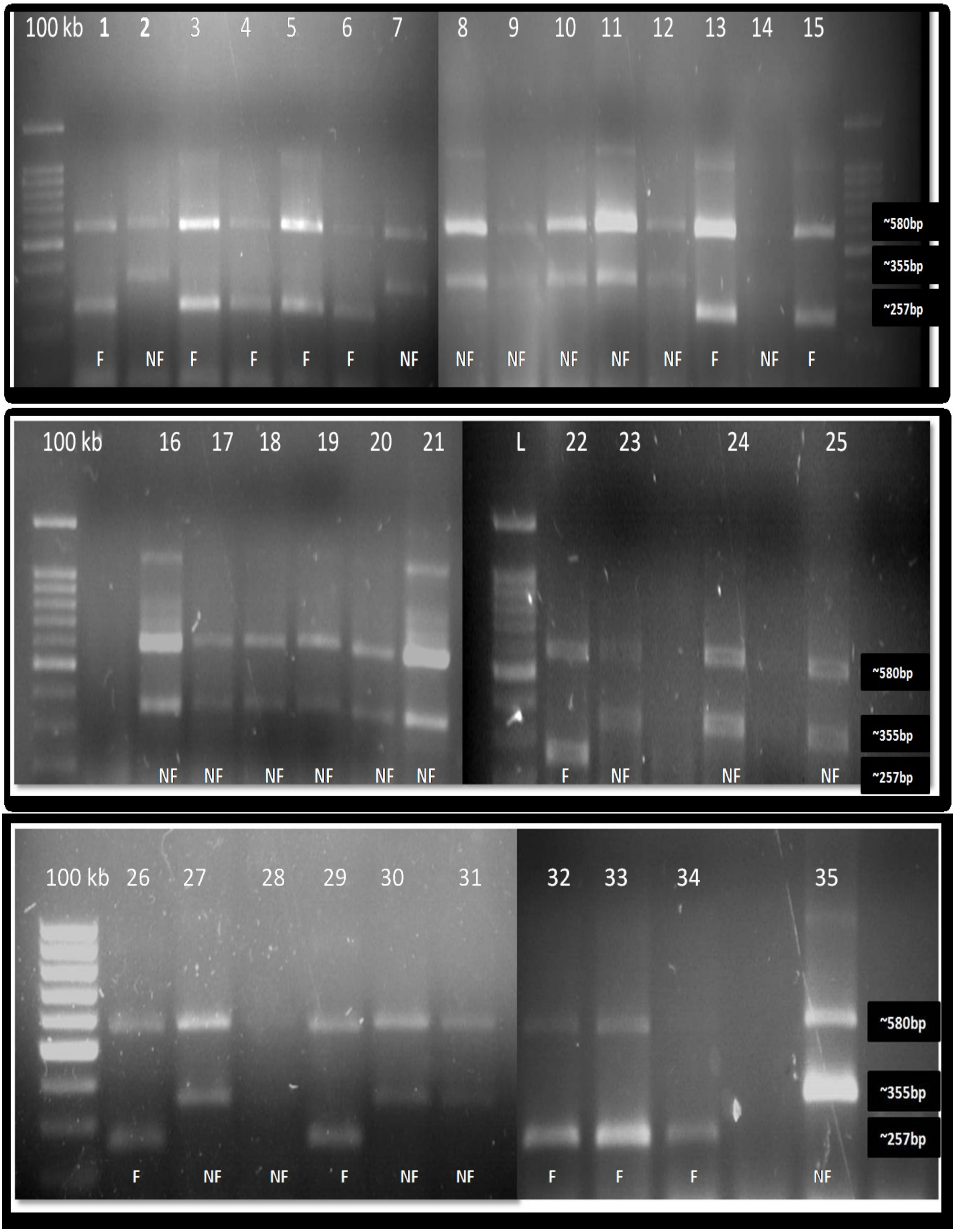

Fig.2 (a,b,c) Fragrance genotyping profile generated from several rice varieties by using *fgr*markers. Band of approximately 580bp corresponds to the positive control PCR product by primers ESP and EAP .The band of 355bp corresponds to non-fragrant allele amplified by primers INSP and EAP. The band of 255bp corresponds to fragrant allele amplified by using IFAP and ESP primers. (1:Khushboo, 2:Shahkar, 3:Sughdaasi, 4:Mehak, 5:Basmati 385, 6: GA-5015, 7:Sada Hayat, 8:Kanwal, 9:IR-8, 10:IR-6, 11:DR-57, 12:DR-58, 13:Super Basmati, 14:DR-92, 15:Shaheen Basmati, 16:DR-82, 17:DR-83, 18:Shua, 19:Shadab, 20:Shandar, 21:Sarshar, 22:MG-Basmati,23:NIA-102, 24: KSK-133, 25:Swat-1, 26:Basmati 2000, 27:IR-9, 28:JP-5, 29:Basmati-198, 30:DR-97, 31:Fakhar-e-Malakand, 32: Basmati 515, 33: Basmati 370, 33: Jajai 77, 35:NIA-625)

Table 6. Comparative profiling of all aroma evaluation tests for rice varieties used in this study

S.No	Varieties	Cook Aroma	KOH Aroma	Exon 7 (8bp del 3 SNPs)
1	Shahkar	-	-	-
2	Sada Hayat	+	+	-
3	Kanwal	+	+	-
4	IR-8	-	-	-
5	IR-6	+	-	-
6	DR-57	+	-	-
7	DR-58	-	-	-
8	DR-92	-	-	-
9	DR-82	-	-	-
10	DR-83	-	-	-
11	Khushboo	+++	+++	✓
12	Shua	+	+	-
13	Shadaab	+	+	-
14	Jajai77	++	+++	✓
15	Shandaar	+	-	-
16	Sughdaasi	+++	+++	✓
17	Sarshar	-	+	-
18	Mehak	++	+++	✓
19	KangriTorr	++	++	-
20	Swat-1	++	++	-
21	Bas-385	++	+++	✓
22	KSK-133	+	+	-
23	KSK-282	+	+	-
24	GA-5015	+++	++	✓
25	IR-9	-	++	-
26	Super Basmati	+++	+++	✓
27	Shaheen Basmati	+++	+++	✓
28	JP-5	++	++	-
29	MG Basmati	+++	+++	✓
30	Dilrosh 97	++	+	-
31	Basmati 2000	+++	+++	✓
32	Basmati 198	+++	+++	✓
33	Bas-515	+++	+++	✓
34	Bas-370	+++	+++	✓
35	Fakhar-e-Malakand	++	++	-
36	NIA-102	++	+	-
37	NIA-625	+	-	-

Conclusion

Present investigation reveals the physicochemical and cooking properties, organoleptic and genetic analysis of aromatic and non-aromatic rice varieties of Pakistan. Among aroma evaluation methods, KOH test gave more consistent and strong detection of aroma as compared to the cooked rice test. Although, organoleptic analysis is helpful in identifying best rice varieties with consumer preferences for their consumption but organoleptic analysis also revealed some difficulty and unreliability for panel analysts due to successive saturation of aroma through nasal passage and large sample size. Aroma is not only controlled by environmental factors but also genetic factors. An 8bpdeletion in *fgr* gene causes inactive BAD2 enzyme which is responsible for increased 2 AP aromatic compounds and conferring fragrance. This dependence on genetic cause helped to devise such strategies to discriminate aromatic and non-aromatic rice varieties with actual status of fragrance. It is noted that objective evaluation of fragrance is fairly possible by the allele specific assay of *fgr* gene which is very simple single tube allele specific PCR method to screen the either homozygous fragrant, homozygous non-fragrant or heterozygous non-fragrant, across a wide range of rice varieties. These studies are of paramount importance in terms of evaluation of grain quality features and could be used for breeding programs and biotechnological research for the improvement of aroma.

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