Seed Res., Vol. 43(1): 34-40, June 2015

Molecular characterization of some scented rice varieties

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ABSTRACT Locally popular forty one scented land races were collected from northern Karnataka. These genotypes were analyzed by SSR markers. Of the 10 SSR markers used in the study only 9 markers were able to distinguish the aromatic rice genotypes. A set of 4 informative SSR markers (CHR-4-8, CHR-8-34, CHR-8-29 and CHR-8-6) could analyze closely related genotypes. These primers amplified a total of 24 profiles across 32 scented rice genotypes. Cluster analysis based on Unweighted Pair Group Method Average Analysis (UPGMA) led to recognition of 4 clusters. Cluster I was the largest with 26 genotypes followed by cluster III. Genetic diversity among some of these cultivars indicated that the cultivars can effectively contribute to the gene pool of aromatic rice cultivars.

Key Words: scented rice, SSR markers, aroma, characterization, genomic DNA,

INTRODUCTION

The aroma of rice plays a role in its consumer acceptability. More than 100 compounds that contribute to the aroma of rice have been identified [1]. Some of these volatile compounds contribute to consumer's acceptance of certain types of rice, whereas other compounds contribute to consumer rejection. The popcorn-like smell of aromatic rice stemming primarily from its 2-acetyl-1-pyrroline (2-AP) content is preferred by many consumers. Several methods are used to detect aroma like smelling vegetative tissue after warming or soaking in KOH, tasting raw kernels and conclusively, cooking [2]. In recent years, actual quantification of 2-AP has been done using different distillation and extraction procedures. However, these methods are laborious, expensive, time consuming and require lot of time for each sample for estimation. Conventional selection procedures take long time for developing lines with suitable genes. DNA markers help to identify the desirable genes controlling the trait. With the help of the DNA markers we can select lines with desirable genes more rapidly. Identification of DNA markers linked to aroma will solve most of the problems linked to its quantification [3]. The wild relatives and landraces that possess tremendous genetic polymorphism are at the verge of extinction. Characterization of varieties at phenotypic level based on morphological characters supplemented with molecular characterization at DNA level is the first step in the course of conserving the existing genetic diversity. Though range of plant characters are currently available for distinguishing between closely related individuals, their sensitivity to environment and less genome coverage hinders their usage.

Molecular markers like RAPD, RFLP *etc.* clearly allow the comparison of the genetic material. The DNA markers have been utilized for many purposes including genome mapping, gene tagging, estimation of genetic diversity, varietal differentiation, resolution of uncertain parentage and purity testing. Among Polymerase Chain Reaction (PCR) based markers, microsatellite markers are highly polymorphic, more reproducible, co-dominant and distributed throughout the genome. More than 2200 microsatellite markers have been mapped to specific locations in rice genome. A random set of these mapped markers providing genome-wide coverage should facilitate an unbiased assay of genetic diversity and thus provide a robust, unambiguous molecular description of rice cultivars.

Considering various methods of cultivar identification a wholistic approach on molecular tests are highly essential. In the light of above facts, present study was conducted in rice varieties with the objective to characterize scented rice using DNA markers for the purpose of identification and documentation.

MATERIALS AND METHODS

A set of SSR markers located across 3 chromosomes were used to assess the genetic variation among the 32 selected genotypes of scented rice. Seeds of all genotypes were sown in earthen pots containing peat: vermicompost:soil in 1:1:1 proportion, under green house condition, at Department of Biotechnology,

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University of Agricultural Sciences, Dharwad. The seedlings were allowed to grow for 20 days in order to get enough leaf material for extraction of DNA.

A total of 10 SSR primers were used, out of which only nine SSR primers produced repeatable and consistent amplicon patterns across all the scented rice genotypes.

DNA extraction

Genomic DNA was isolated from young leaf tissues of 32 rice genotypes by following a modified CTAB method [4]. 2 g of leaf samples were cut into small bits and ground in a pestle and mortar using liquid nitrogen. The ground powder was allowed to soak in 10 ml of pre-heated extraction buffer in polypropylene centrifuge tubes and incubated for 30 minutes at 65°C in water bath with occasional mixing. The tubes were removed from the water bath and equal volume of chloroform:Isoamyl alcohol mixture (24:1 v/v) was added and mixed by inversion for 15 minutes. It was centrifuged at 10,000 rpm for 20 minutes at 4°C. The clear aqueous phase was transferred to a new sterile centrifuge tube. Equal volume of ice cold isopropanol was added and mixed gently by inversion and then kept in the freezer until DNA was precipitated out. Then it was centrifuged and the supernatant was discarded. The pellet was washed with 70% alcohol by centrifugation. The alcohol was discarded and DNA was completely air-dried. Then the DNA pellet was dissolved in 150 - 250 µl of TE and then 1 ml of RNase was added and incubated at 37°C for 30 minutes. Quality of DNA isolated from rice leaf tissues was checked by running it on 0.8 % agarose gel and visualizing gel under UV gel documentation unit. The genomic DNA was quantified using nanodrop instrument. The gel was loaded with 1 ml of DNA sample dissolved in TE and was run at 60 V for 1-1.5 hours. Bands were visualized and documented using a gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., USA).

SSR analysis

The forward and reverse primers were developed using the unique sequences that flank microsatellites [5]. Primer pairs were derived from sequence information obtained from DNA libraries and published sequence data [6]. A total of 10 hyper variable SSR primer pairs distributed across the different chromosomes were used for PCR amplification. The sequence details of the primer pairs and their optimum annealing temperature (T_m) are given in Table 1.

PCR amplification

The reaction in thermal cycler (Eppendorf, Germany) was programmed as follows:

Profile 1:	95°C for 5 minutes	Initial denaturation
Profile 2:	94°C for 1 minute	Denaturation
Profile 3:	55°C for 1 minute	Annealing
Profile 4:	72°C for 1 minute	Extension
Profile 5:	72°C for 5 minutes	Final extension
Profile 6:	4°C	Hold the samples

Profiles 2, 3 and 4 were programmed to run for 30 cycles.

Table 1. Nucleotide sequence of the 10 polymorphic SSR primers, optimum annealing temperature used for the experiment

Oligo Name	Forward primer	Reverse primer	Annealing temperature (Tm) in °C
CHR-3-21	GGAGACAGCACGAAATCC	AGGAGCAAGAGAACGACC	61.8
CHR-3-22	GGGTATTTGTAAGGTGAGGTG	AACCGAGAGAGAGATGTGTG	61.8
CHR-4-8	GTCAACTGGCACTGAATGT	AGCACGAATCCTAAAGCC	53.4
CHR-4-13	CACAGACACAGAGAGTGAGGT	TAGGATTGAGGTGGGTTACT	61.8
CHR-8-3	TACCACCGATTATTGTCGTAT	GAAGTGAGGAAGGGAAGAATA	46.7
CHR-8-29	GAGAGAGAAGAGAAGCTCCAG	CAGCTACATTCTGCAACAAAT	50.5
CHR-8-6	CGTACATACTTGGGATGAGC	GCCATGATCAAAGCATTATT	45.0
CHR-8-34	ACTGTTCCTCTCCTCCTGTT	AGTGCTCAATGGTTGGTTAG	53.4
CHR-8-10	ACATCACCCTCATCACTGTT	AGGCTAGATGATCGAAACAA	56.7
CHR-8-5	CACACAGATGAACTGCACAT	GGTACGTACGTGTGTGAATG	61.8

Agarose gel electrophoresis of SSR-PCR products

After PCR amplification, the PCR products were separated by 2 % Agarose gel electrophoresis.

Scoring and analysis of the profile of the amplified fragments

Distinct amplicon were scored to get a binary data (0 or 1) matrix based on the absence or presence of the amplicon band in each lane at equidistance from the loading well. This binary data matrix was subjected to cluster analysis [7]. Sequential agglomerative hierarchial non overlapping (SAHN) clustering was performed. The programme used was cluster analysis joining (tree clustering) with row input data of each population separately. The main parameter that guided in joining the process linkage rule is Unweighted Pair Grouping Method Using Arithmetic averages (UPGMA) and the distance was computed from the raw data using Euclidean distance. SSR raw data was used for plotting the dendrogram.

RESULT AND DISCUSSION

The result showed 100 per cent polymorphism among the scented rice genotypes. The primer CHR-8-29 generated highest number of profiles (10) followed by primer CHR-4-8 (6), where as, CHR-3-21 generated least number of profiles (1). The number of amplicons generated by any single primer varied from 1 to 10 within a range of 120 bp to 950 bp. An average of 3.89 amplicons per primer was recorded. DNA polymorphism as revealed by 4 of the highly polymorphic primers is depicted in Plate 1. Based on the level of polymorphism detected by individual primers, the most informative primers (CHR-4-8, CHR-8-34, CHR-8-29 and CHR-8-6) were identified. These primers amplified a total of 24 profiles across 32 scented rice genotypes. The percentage of polymorphism with these 4 primers was 68.6 per cent

Except for the primer CHR-8-10, which amplified the bands in genotype Ambemore, all other primers did not show any amplification of bands. In genotype Deharadhun basmati, only CHR-3-22 and CHR-8-5 showed amplification. Similarly in genotype Jeeregisanna, amplification of alleles was observed only for the primers CHR-8-34, CHR-3-22 and CHR-8-6. Primer CHR-3-21 amplified the bands in genotypes Parimalakalavi, Beeraga, Vasanebhatta, Local ambemore and Pusa sugandha-2. Rest of genotypes did not show any amplification. PCR product size base pairs (BP) on respective chromosome and polymorphic bands produced is listed in Table 2. Summary statistics of SSR analysis is presented in Table 3.

Based on the simple matching coefficients, a genetic similarity matrix was constructed by using SSR markers data to assess the genetic relatedness among the 32 genotypes. Similarity co-efficient estimated on the basis of all the 9 SSR primers ranged from 0.11 to 1.00 indicating varied genetic relationship among the 32 genotypes. Minimum genetic relatedness was noticed for genotype Ambemore with genotype Karigajivile, Pusa-44 and Pusa basmati-1 (about 11 per cent each).

Genetic similarity of 100 per cent was noticed between the genotypes of Beeraga and Pusa sugandha-2; between Kumada, Karigajivile, Delhi basmati, Pusa-44 and Pusa basmati-1; between Yalakkisali, Mugadsugandha, Badshabhog, Huggibhatta and Kagisanna; between Mugadsugandha, Badshabhog, Huggibhatta and Kagisanna; between Badshabhog,

Table 2. List of 9 polymorphic SSR marker used for molecular characterization of scented rice genotypes along with PCR product size (bp) on respective chromosome and polymorphic bands produced

Sl. No.	Primers	Chromo some	Expected product size (bp)	Total number of profiles/ amplicons	Number of polymorphic bands	% polymorphism
1	CHR-8-34	8	386	4	4	100
2	CHR-3-22	3	365	2	2	100
3	CHR-8-10	8	333	3	3	100
4	CHR-3-21	3	339	1	1	100
5	CHR-4-8	4	376	6	6	100
6	CHR-8-29	8	325	10	10	100
7	CHR-8-3	8	346	3	3	100
8	CHR-8-6	8	399	4	4	100
9	CHR-8-5	8	378	2	2	100

Table 3. Summary statistics of SSR analysis in scented rice genotypes

Sl. No.	Particulars	
1.	Total number of polymorphic profiles	35
2	Primers used	10
3	Primers finally studied for amplification	9
4	Maximum number of levels observed	10
5	Minimum number of levels observed	1
6	Average number of polymorpic bands/primer	3.89

Belgaum basmati, Malgudisanna, Kari basmati, Andra basmati, Pusa 1460 and Pusa sugandha-5; between Badshabhog, Huggibhatta, Kagisanna, between Belgaum basmati, Malgudisanna, Kari basmati, Andra basmati, Pusa 1460, Pusa sugandha-5; between Huggibhatta and Kagisanna; between Karigajivile, Delhi basmati, Pusa-44 and Pusa basmati-1; between Malgudisanna, Kari basmati, Andra basmati, Pusa 1460 and Pusa sugandha-5; between Pusa sugandha-4 and Kagisali, between Pusa 1460 and Pusa sugandha-5; between Pusa-44 and Pusa basmati-1; between Kari basmati, Andra basmati, Pusa 1460 and Pusa sugandha-5; between Andra basmati, Pusa 1460 and Pusa sugandha-5; between Delhi basmati, Pusa basmati-1 and Pusa-44. Genetic similarity coefficients indicating the extent of relatedness among accessions are furnished in Table 5. A cluster analysis using UPAGMA, based on similarity coefficients was done to resolve the phylogenetic relationships among the different aromatic rice genotypes, considered for the present study. Similarity coefficient ranged from 0.34 and 1.00 for all accessions and minimum genetic relatedness was 34 per cent between Ambemore and Deharadhun basmati, with rest of the genotypes (Fig. 1) under the study. The highest similarity observed between genotypes was 100 per cent. All the selected accessions were grouped into four clusters (Table 4). Cluster-I is the largest cluster with 26 genotypes, followed by cluster-III (3 genotypes), cluster-IV (2 genotypes) and cluster-II (1 genotype). All the seven pusa genotypes grouped under the cluster-I. However, cluster-II consisted of only one rice genotype, Parimala Kalavi from Uttarakannada district which is 56 per cent similar to all other genotypes of clusters-I. The cluster-I further divided to 3 sub clusters. Sub cluster-I (SC-I) consists of 10 genotypes, sub cluster-II (SC-II) had 7 genotypes and sub cluster-III (SC-III) had 9 genotypes. These three sub groups were similar to each other by 81 per cent.

Popular genotypes like Pusa basmati-1 and Karigajivile were found in sub cluster-II whereas, Kalanamak and Gandhasali were grouped in sub cluster-I. Another popular genotype Mugadsugandh was grouped in sub cluster-III along with Yalakkisali. The classification of scented rice genotypes based on four most informative SSR primers was in conformation with cluster analysis similarity.

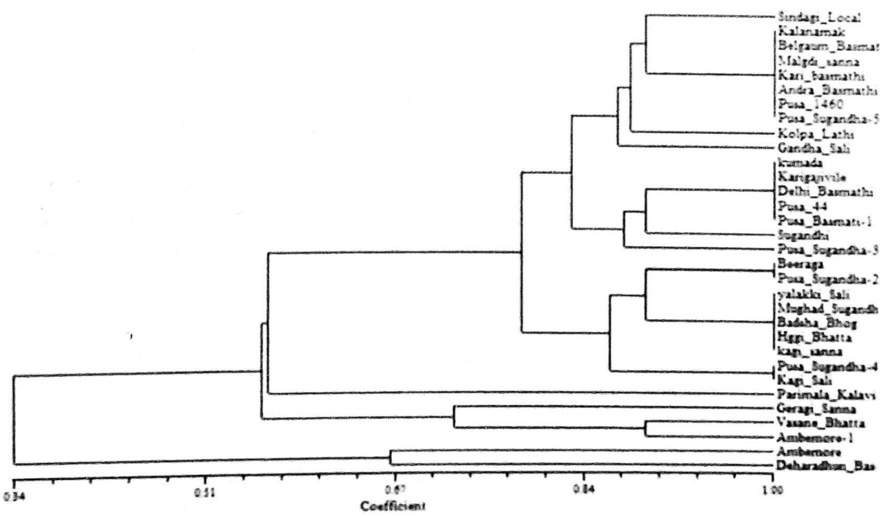
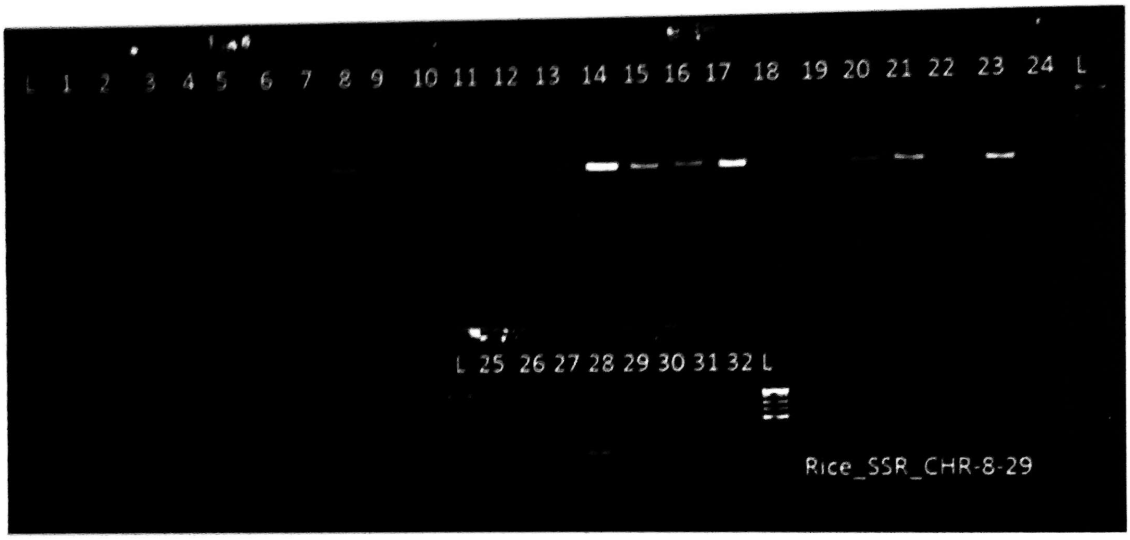


Fig. 1. Dendrogram showing similarity between selected scented rice genotypes

Table 4. Distribution of rice genotypes in different clusters based on analysis of SSR data

Cluster	No. of genotypes	Genotypes
I	26	Sindagi local, Kalanamak, Belgaum basmati, Malgudisanna, Andra basmati, Kari basmati, Pusa 1460, Pusa sugandhha-5, Gandhasali, Karigajivile, Kolpalathi, Kumada, Delhi basmati, Pusa 44, Pusa basmati-1, Pusa sugandhha-2, Pusa sugandhha-3, Pusa sugandhha-4, Sugandhi, Beeraga, Yalakkisali, Mugadsugandha, Badshabhog, Huggibhatta, Kagisali, Kagisanna,
II	1	Parimala kalavi,
III	3	Jeeregi sanna, Local ambemore, Vasane bhatta,
IV	2	Ambemore, Deharadhun basmati,

Plate 1. SSR profile of aromatic rice obtained with primer CHR8-29,



Genetic diversity is a pre requisite for obtaining variation for crop improvement. Though, a range of plant morphological traits is used for distinguishing the genotypes, environment play an important role in influencing their expression. DNA polymorphism would act as an extremely powerful tool for variety characterization and identification. The most effective approach for the assessment of genetic diversity is based on molecular markers. Among the various molecular markers available, microsatellites or Simple Sequence Repeat (SSR) markers are widely used for diversity analysis [8, 9, 10]. DNA markers have been employed for genetic analysis in rice [11, 12]. Prabakaran *et al.* [13], evaluated the genetic divergence of 12 rice land races using five SSR markers. The present study provided a detailed analysis and quantification of genetic diversity in selected genotypes of Western Ghats and Semi Transitional belt of Northern Karnataka. The data also reaffirm the powers of SSR markers to distinctly group closely related landraces.

CONCLUSION

The use of SSR marker for characterizing different genotypes is a simple and less time consuming method of generating molecular data. For this reason, this technique has been used in many taxonomic, phylogenetic and diversity related studies. This SSR marker fingerprinting makes identification and characterization of genotypes very easy and further it will be of greater help in assessing genetic purity. Based on the observed results, it can be concluded that in situations where the morphological DUS descriptors fail to establish distinctiveness of a variety, then molecular markers may be used as additional descriptors for resolving distinctiveness of indigenous aromatic rice varieties for granting plant variety protection under Protection of Plant Varieties and Farmers' Rights Act.

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Table 5. Similarity matrix of 34 different scented rice genotypes based on SSR profile

	2	3	5	6	7	8	16	17	18	27	28	29	30	31	32	33	34	35	36	37	38	40	41	42	19	20	21	22	23	24	25	26
2	1.00																															
3	0.67	1.00																														
5	0.67	0.78	1.00																													
6	0.78	0.44	0.67	1.00																												
7	0.78	0.67	0.89	0.78	1.00																											
8	0.33	0.44	0.22	0.11	0.33	1.00																										
16	0.78	0.67	0.89	0.78	1.00	0.33	1.00																									
17	0.89	0.56	0.78	0.89	0.89	0.22	0.89	1.00																								
18	0.78	0.67	0.89	0.78	1.00	0.33	1.00	0.89	1.00																							
27	0.89	0.56	0.78	0.89	0.89	0.22	0.89	1.00	0.89	1.00																						
28	0.78	0.67	0.89	0.78	1.00	0.33	1.00	0.89	1.00	0.89	1.00																					
29	0.78	0.44	0.67	1.00	0.78	0.11	0.78	0.89	0.78	0.89	0.78	1.00																				
30	0.33	0.44	0.67	0.56	0.56	0.33	0.56	0.44	0.56	0.44	0.56	0.56	1.00																			
31	0.89	0.56	0.78	0.89	0.89	0.22	0.89	1.00	0.89	1.00	0.89	0.89	0.44	1.00																		
32	0.89	0.56	0.78	0.89	0.89	0.22	0.89	1.00	0.89	1.00	0.89	0.89	0.44	1.00	1.00																	
33	0.89	0.56	0.78	0.89	0.89	0.22	0.89	1.00	0.89	1.00	0.89	0.89	0.44	1.00	1.00	1.00																
34	0.78	0.44	0.67	0.78	0.78	0.33	0.78	0.89	0.78	0.89	0.78	0.78	0.33	0.89	0.89	0.89	1.00															
35	0.44	0.56	0.33	0.44	0.44	0.67	0.44	0.33	0.44	0.33	0.44	0.44	0.44	0.33	0.33	0.33	0.22	1.00														
36	0.67	0.56	0.56	0.89	0.67	0.22	0.67	0.78	0.67	0.78	0.67	0.89	0.44	0.78	0.78	0.78	0.67	0.56	1.00													
37	0.78	0.44	0.67	1.00	0.78	0.11	0.78	0.89	0.78	0.89	0.78	1.00	0.56	0.89	0.89	0.89	0.78	0.44	0.89	1.00												
38	0.78	0.67	0.89	0.78	1.00	0.33	1.00	0.89	1.00	0.89	1.00	0.78	0.56	0.89	0.89	0.89	0.78	0.44	0.67	0.78	1.00											
40	0.56	0.44	0.44	0.56	0.56	0.56	0.56	0.44	0.56	0.44	0.56	0.56	0.78	0.44	0.44	0.44	0.33	0.67	0.44	0.56	0.56	1.00										
41	0.78	0.44	0.67	0.78	0.78	0.33	0.78	0.89	0.78	0.89	0.78	0.78	0.56	0.89	0.89	0.89	0.78	0.44	0.67	0.78	0.78	0.56	1.00									
42	0.44	0.56	0.78	0.67	0.67	0.22	0.67	0.56	0.67	0.56	0.67	0.67	0.89	0.56	0.56	0.56	0.44	0.56	0.56	0.67	0.67	0.67	0.67	1.00								
19	0.67	0.33	0.56	0.89	0.67	0.22	0.67	0.78	0.67	0.78	0.67	0.89	0.67	0.78	0.78	0.78	0.67	0.33	0.78	0.89	0.67	0.67	0.67	0.56	1.00							
20	0.67	0.78	1.00	0.67	0.89	0.22	0.89	0.78	0.89	0.78	0.89	0.67	0.67	0.78	0.78	0.78	0.67	0.33	0.56	0.67	0.89	0.44	0.67	0.78	0.56	1.00						
21	0.67	0.56	0.78	0.89	0.89	0.22	0.89	0.78	0.89	0.78	0.89	0.89	0.67	0.78	0.78	0.78	0.67	0.56	0.78	0.89	0.89	0.67	0.67	0.78	0.78	0.78	1.00					
22	0.89	0.56	0.78	0.89	0.89	0.22	0.89	1.00	0.89	1.00	0.89	0.89	0.44	1.00	1.00	1.00	0.89	0.33	0.78	0.89	0.89	0.44	0.89	0.56	0.78	0.78	0.78	1.00				
23	0.78	0.44	0.67	1.00	0.78	0.11	0.78	0.89	0.78	0.89	0.78	1.00	0.56	0.89	0.89	0.89	0.78	0.44	0.89	1.00	0.78	0.56	0.78	0.67	0.89	0.67	0.89	0.89	1.00			
24	0.89	0.56	0.78	0.89	0.89	0.22	0.89	1.00	0.89	1.00	0.89	0.89	0.44	1.00	1.00	1.00	0.89	0.33	0.78	0.89	0.89	0.44	0.89	0.56	0.78	0.78	0.78	1.00	0.89	1.00		
25	0.78	0.44	0.67	1.00	0.78	0.11	0.78	0.89	0.78	0.89	0.78	1.00	0.56	0.89	0.89	0.89	0.78	0.44	0.89	1.00	0.78	0.56	0.78	0.67	0.89	0.67	0.89	0.89	1.00	0.89	1.00	
26	0.67	0.56	0.78	0.89	0.89	0.22	0.89	0.78	0.89	0.78	0.89	0.89	0.67	0.78	0.78	0.78	0.67	0.56	0.78	0.89	0.89	0.67	0.67	0.78	0.78	0.78	1.00	0.78	0.89	0.78	0.89	1.00

LEGEND

2	3	5	6	7	8	16	17	18	19	20
Sindagi local,	Parimala kalavi,	Beeraga,	Kumada,	Yalakki sali,	Ambemore,	Mugad sugandha,	Kalanamak,	Badsha bhog,	Pusa, sugandha-3	Pusa sugandha-2,
21	22	23	24	25	26	27	28	29	30	31
Pusa sugandha-4,	Pusa 1460,	Pusa 44,	Pusa sugandha-5,	Pusa basmati-1,	Kagi sali, basmati,	Belgaum bhatta,	Huggi jivile,	Kariga bhatta,	Vasane sanna,	Malgudi
32	33	34	35	36	37	38	40	41	42	
Kari basmati,	Andra basmati,	Kolpa lathi,	Deharadhun basmati,	Sugandhi,	Delhi basmati,	Kagi sanna,	Jeeragi sanna,	Gandha sali,	Local ambemore,	

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