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Association analysis between agronomic traits and known BPH resistance markers in germplasm of rice (*Oryza sativa* L.)

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Abstract
Rice (*Oryza sativa* L.), the world's most important cereal crop, is the primary source of food and calories for about half of the human population. Finding association between molecular markers and agronomic traits provide an excellent tool for indirect selection of a trait of interest in the population. A total of 10 SSR markers were used for this study which are reported linked markers to nine BPH resistance genes viz., *Bph2, Bph3, Bph6, Bph9, Bph10, Bph15, Bph18, Bph21 and Bph26* of which 5 primers were polymorphic. The association between trait and markers were calculated using single marker analysis (SMA) in Microsoft Excel program. The significant marker trait associations were indicated by a P-value (<0.05). We detected a total of 16 significant marker-trait association (P<0.05). All of the significant SSR loci were identified for the agronomic traits. The P-value ranges from 0.003 to 0.049.

Keywords: Association, Analysis, Agronomic Traits, BPH, Resistance Markers, Germplasm, Rice

Introduction
Rice (*Oryza sativa* L.), the world's most important cereal crop, is the primary source of food and calories for about half of the human population. The brown plant hopper (BPH), *Nilaparvata lugens* Stal. (Homoptera: *Delphacidae*), is a destructive and wide spread insect pest throughout the rice areas in Asia. The DNA-based markers are promising and effective tools for measuring genetic diversity in plants germplasm and elucidating their evolutionary relationships.

They are more reliable and remain unaffected across different growth stages, seasons, locations and agronomic practices. Amongst the polymerase chain reaction (PCR) based markers, the microsatellites are useful as genetic markers because they detect high levels of allelic diversity. Alam and Cohen (1998) [1] and Soundararajan *et al.* (2004) [2] mapped several QTL associated with resistance to BPH in rice. As such, this study has used these tools to make the study an exhaustive one and to provide the base material for the rice breeders for exploitation of land races possessing one or more desirable characters.

Materials and Methods
Generation and maintenance of rice brown plant hopper breeding materials along with molecular work were done at the research farm and molecular biology laboratory of Department of Genetics and Plant Breeding. The studies extended over a period of one cropping wet seasons (*Kharif*) 2016.

The experimental material consisted of 24 elite rice genotypes are IC75889, IC75844,

IC75767, IC217492, IC75964, IC76046, IC216609, IC75832, IC217509, IC216606, IC75845, IC75829, IC 540584, IC 216579, IC75795, IC75839, IC218650, IC75874, IC216612, T-12, IC216563, Swarna, ARC-10550 and IC218607, where T- 12 and ARC 10550 were used as resistant check and Swarna used as susceptible check, respectively.
Molecular work was carried out in Plant Molecular Biology Laboratory, Department of Genetics and Plant Breeding, IGKV, Raipur (C.G.) India.

DNA Extraction
Total rice genomic DNA was extracted from young succulent, disease and insect free seedlings by mini prep method. The DNA was extracted from 24 lines out of which 13 were resistant and 11 were susceptible. The DNA was isolated from the leaves of 3 weeks old plants. Mini prep method was followed for the isolation of DNA.

PCR amplification using SSR primers
PCR analysis was done using a set of ten random SSR (simple sequence repeat) markers (Table 1) to identify the parental polymorphism between 24 elite lines.

Visualization of amplified products in Polyacrylamide gel electrophoresis
Five per cent polyacrylamide gels (vertical) were used for better separation and visualization of PCR amplified microsatellite products, since polyacrylamide gels have better resolution for amplified products.

Microsatellite marker analysis
A total of 24 rice genotypes were used in the SSR analysis. The primer sequences and chromosomal positions for primer pairs were downloaded from genome database, Rice Genome Microsatellite Markers (http:/www.gramene.org/db/markers.html). Ten primers were chosen randomly covering three the chromosomes or genomic regions. four primers exhibited monomorphic fragments and were therefore excluded from further analysis.

Scoring and Data Analysis
All the genotypes were scored for the presence and absence of the SSR bands. Ethidium bromide staining of gels generally showed several bands. The size of the most intensively amplified band for each microsatellite marker was determined based on its electrophoretic mobility relative to molecular weight markers (increments of 50). Clearly resolved unambiguous bands were scored visually for their presence or absence with each primer. The score were obtained in the form of matrix with "1" and "0", which indicate the presence and absence of bands in each variety respectively.

Results and Discussion-
Graphical genotyping of rice germplasm lines using SSR maker data
A graphical representation of molecular marker data can be an important tool in the process of selection and evaluation of plant material. A computer program was developed that enables representation of molecular marker data by simple chromosome drawings in several ways. Commonly used marker file types that contain marker information serve as input for this program, which was named "GGT" (an acronym of Graphical Genotypes) (GGT user manual 2007). Graphical outputs of genotyping data in this study were generated using GGT version 2.0 tool.

The study showed genomic constitution analysis (marker allele contribution) of rice germplasm lines and varieties are based on chromosome-wise distribution of polymorphic SSR loci. The chromosome wise allelic contribution of marker alleles A and B is calculated for 24 rice germplasm lines selected in the study. Marker alleles for each locus were marked in different colours and incorporated in ascending order of position of markers (in cM.) on the chromosomes. The analysis revealed that the two alleles more or less contributed equally in case of markers located on chromosome 6 rice germplasm lines where as in case of chromosome 12 showed maximum contribution of allele A and minimum contribution of allele B.
Out of 10 markers 5 were polymorphic and 5 were mono morphic. Five polymorphic markers were RM261, RM589, RM3331, RM260 and RM6869 and remaining five monomorphic markers are RM119, RM313, RM5479, RM463 and RM6217. Marker RM261 and RM119 found in chromosome no.4, RM589 found in chromosome no.6 and marker RM6217, RM3331, RM3331, RM260, RM313, RM463, RM5479 and RM6869 found in chromosome no.12.

Genotyping of rice genotypes using known BPH resistance markers associated to other agronomic characters
Other markers are also associated with different traits like marker RM261is significantly associated with the trait leaf length present on chromosome no. 4 at 35.4cM show the p-value 0.033 and %R^2 is 18.98%, indicates the presence of gene *Bph15*.
Marker RM261 is highly significantly associated with the trait plant height present on chromosome no. 4 at 35.4cM show the p-value 0.0033 and %R^2 is 32.91%, indicates the presence of gene *Bph15*.
Marker RM261 is highly significantly associated with the trait panicle length present on chromosome no. 4 at 35.4cM show the p-value 0.0032 and % R^2 is 33.08%, indicates the presence of gene *Bph15*.
Marker RM261 is significantly associated with the trait total tiller present on chromosome no. 4 at 35.4cM show the p-value 0.0187 and % R^2 is 22.66%, indicates the presence of gene *Bph15*.
Marker RM261 is significantly associated with the trait effective tiller present on chromosome no. 4 at 35.4cM show the p-value 0.0249 and % R^2 is 20.83%, indicates the presence of gene *Bph15*.
Marker RM589 is significantly associated with the trait plant height present on chromosome no. 6 at 3.2 cM show the p-value 0.0293 and % R^2 is19.81%, indicates the presence of gene *Bph3*.
Marker RM589 is significantly associated with the trait panicle length present on chromosome no. 6 at 3.2 cM show the p-value 0.0392 and % R^2 is 17.93%, indicates the presence of gene *Bph3*.
Marker RM589 is significantly associated with the trait total tiller present on chromosome no. 6 at 3.2 cM show the p-value 0.0164 and % R^2 is 23.45%, indicates the presence of gene *Bph3*.
Marker RM589 is significantly associated with the trait no. of effective tiller per plant present on chromosome no. 6 at 3.2 cM show the p-value 0.0327 and % R^2 is 19.10%, indicates the presence of gene *Bph3*.
Marker RM589 is significantly associated with the trait grain yield present on chromosome no. 6 at 3.2 cM show the p-value 0.0458 and % R^2 is 16.91%, indicates the presence of gene *Bph3*.

Marker RM589 is significantly associated with the trait unfilled spikelets per panicle present on chromosome no. 6 at 3.2 cM show the p-value 0.0360 and % R^2 is 18.47%, indicates the presence of gene *Bph3*.
Marker RM3331 is significantly associated with the trait grain yield present on chromosome no. 12 at 23.49 cM show the p-value 0.0355 and % R^2 is 18.57%, indicates the presence of gene *Bph18*.
Marker RM3331 is significantly associated with the trait harvest index present on chromosome no. 12 at 23.49 cM show the p-value 0.0394 and % R^2 is 17.90%, indicates the presence of gene *Bph18*.
Marker RM260 is significantly associated with the trait grain width present on chromosome no. 12 at 61.7 cM show the p-value 0.0387 and % R^2 is 20.04%, indicates the presence of gene *Bph10*.
Marker RM260 is significantly associated with the trait grain yield present on chromosome no. 12 at 61.7 cM show the p-value 0.0169 and % R^2 is 9.73%, indicates the presence of gene *Bph18*.

Association analysis between traits and molecular markers

Table 1: Association between SSR markers, agronomic traits and BPH resistance $P<0.05$

Trait	Chromosome No.	Position	Marker	Gene	P-Value	%R2
Leaf length	4	35.4	RM261	*Bph15*	0.033*	18.98
Plant height	4	35.4	RM261	*Bph15*	0.0033**	32.91
Panicle length	4	35.4	RM261	*Bph15*	0.0032**	33.08
No. of tillers per plant	4	35.4	RM261	*Bph15*	0.0187*	22.66
No. of effective tillers per plant	4	35.4	RM261	*Bph15*	0.0249*	20.83
Plant height	6	3.2	RM589	*Bph3*	0.0293*	19.81
Panicle length	6	3.2	RM589	*Bph3*	0.0392*	17.93
No. of tillers per plant	6	3.2	RM589	*Bph3*	0.0164*	23.45
No. of total tillers per plant	6	3.2	RM589	*Bph3*	0.0327*	19.10
Biological yield	6	3.2	RM589	*Bph3*	0.0458*	16.91
Unfilled spikelets per panicle	6	3.2	RM589	*Bph3*	0.0360*	18.47
Grain yield	12	23.49	RM3331	*Bph18*	0.0355*	18.57
Harvest index	12	23.49	RM3331	*Bph18*	0.0394*	17.90
Grain width	12	61.7	RM260	*Bph10*	0.0387*	20.04
Grain length	12	61.7	RM260	*Bph10*	0.0169*	9.73
Susceptibility/ resistance index	12	105.5	RM6869	*Bph2, Bph18*	0.041*	35.5

* = Significant at 5% and ** = Significant at 1%

A total of 10 SSR markers were used for this study which are reported linked markers to nine BPH resistance genes viz., *Bph2, Bph3, Bph6, Bph9, Bph10, Bph15, Bph18, Bph21* and *Bph26* of which 5 primers were polymorphic.
Coleoptile length (CL): There are 3 markers locus significantly associated with (CL) RM261 on chromosome 4 with p-value 0.017 with distance of 35.4cM, RM119 on chromosome 4 (p-value=0.021 and 0.049) with distance of 76.1cM and RM463 on chromosome 12 (p-value=0.017) with distance of 75.5cM.
Harvest index (HI): There are 4 markers locus significantly associated with (HI) RM261 on chromosome 4 with p-value 0.026 with distance of 35.4cM, RM260 on chromosome 12 (p-value = 0.049) with distance of 61.7cM, RM313 onchromosome 12 (p-value = 0.041) with distance of 65.5cM and RM6869 onchromosome 12 (p-value = 0.049) with distance of 105.5cM.
Unfilled spikelets per panicle (USPP): There are five markers loci significantly associated with (USPP) RM261 on chromosome 4 with p-value 0.027 with distance of 35.4cM, RM119 on chromosome 4 (p-value=0.028) with distance of 76.1cM, RM463 on chromosome 12 (p-value=0.005) with distance of 75.5cM, RM5479 on chromosome 12 (p-value=0.005) with distance of 93.3cM and RM589 on chromosome 6 (p-value=0.036 and 0.036) with distance of 3.2cM.
Leaf length (LL): There are 3 markers locus significantly associated with (LL) RM261 on chromosome 4 with p-value 0.033 with distance of 35.4cM, RM119 on chromosome 4 (p-value=0.021) with distance of 76.1cM and RM463 on chromosome 12 (p-value=0.004) with distance of 75.5cM.
Plant height (PH): There are 3 markers locus significantly associated with (PH) RM261 on chromosome 4 with p-value 0.003 with distance of 35.4cM, RM463 on chromosome 12 (p-value=0.025 and 0.011) with distance of 75.5cM and RM589 on chromosome 6 (p-value=0.030 and 0.030) with distance of 3.2cM.
Panicle length (PL): There are 5 markers locus significantly associated with (PL) RM261 on chromosome 4 with p-value 0.003 with distance of 35.4cM, RM119 onchromosome 4 (p-value=0.047) with distance of 76.1cM, RM313 on chromosome12 (p-value=0.030 and 0.021) with distance of 65.5Cm, RM5479 on chromosome 12 (p-value=0.048) with distance of 93.3cM and RM589 on chromosome 6 (p-value =0.038 and 0.038) with distance of 3.2cM.
No. of tellers per panicle (NTPP): There are four markers locus significantly associated with (NTPP) RM261 on chromosome 4 with p-value 0.022 with distance of 35.4cM, RM463 on chromosome 12 (p-value=0.010) with distance of 75.5cM, RM260 on chromosome 12 (p-value=0.045) with distance of 61.7cM and RM589 on chromosome 12 (p-value =0.027 and 0.027) with distance of 3.2cM.
No. of effective tillers per panicle (NETPP): There are 4 markers locus significantly associated with (NETPP) RM261 on chromosome 4 with p-value0.025 with distance of 35.4cM, RM260 on chromosome 12 (p-value=0.034) with distance of 61.7cM and RM463 on chromosome 12 (p-value=0.008) with distance of 75.5cM and RM589 on chromosome 6 (p-value =0.033 and 0.033) with distance of 3.2cM.
Filled spikelet's per plant (FSPP): There are two markers locus significantly associated with (FSPP) RM119 on chromosome 4 with p-value 0.048 with distance of 76.1cM, RM3331 on chromosome 12 (p-value=0.035 and 0.025) with distance of 23.49cM.
Leaf width (LW): There are 3 markers locus significantly associated with (LW) RM119 on chromosome 4 with p-value

0.049 with distance of 76.1cM, RM3331on chromosome 12 (p value=0.045 and0.000) with distance of 23.49cM and RM313 on chromosome 12 (p value=0.022) with distance of 65.5cM.

Grain yield (GY): There are 3 markers locus significantly associated with (GY) RM119 on chromosome 4 with p-value 0.046 with distance of 76.1cM, RM3331on chromosome 12 (p-value=0.033) with distance of 23.49cM and RM6869 on chromosome 12 (p-value=0.046) with distance of 105.5cM.

Grain width (GW): There are 2 markers locus significantly associated with (GW) RM6217 on chromosome 12 with p-value 0.017 with distance of 22.13cM, RM6869 on chromosome 12 (p-value=0.029) with distance of 105.5cM.

100 Grains Weight (100 GWt): There are 2 markers locus significantly associated with (100 GWt) RM6217 on chromosome 12 with p-value 0.016 with distance of 22.13cM and RM260 on chromosome 12 (p-value=0.028) with distance of 61.7cM.

The association between trait and markers were calculated using single marker analysis (SMA) in Microsoft Excel program. The significant marker trait associations were indicated by a P-value (<0.05). We detected a total of 16 significant marker-trait association (P<0.05).

All of the significant SSR loci were identified for the agronomic traits. The P-value ranges from 0.003 to 0.049. Markers like RM261, RM589, RM3331, RM260 and RM6869 were also significantly associated with agronomic traits like Leaf length, Plant height, Panicle length, No. of tillers per plant, Grain yield, No. of effective tillers per plant, Unfilled spikelet's per panicle, Grain yield, Harvest index, Grain width and Grain length with BPH resistance.

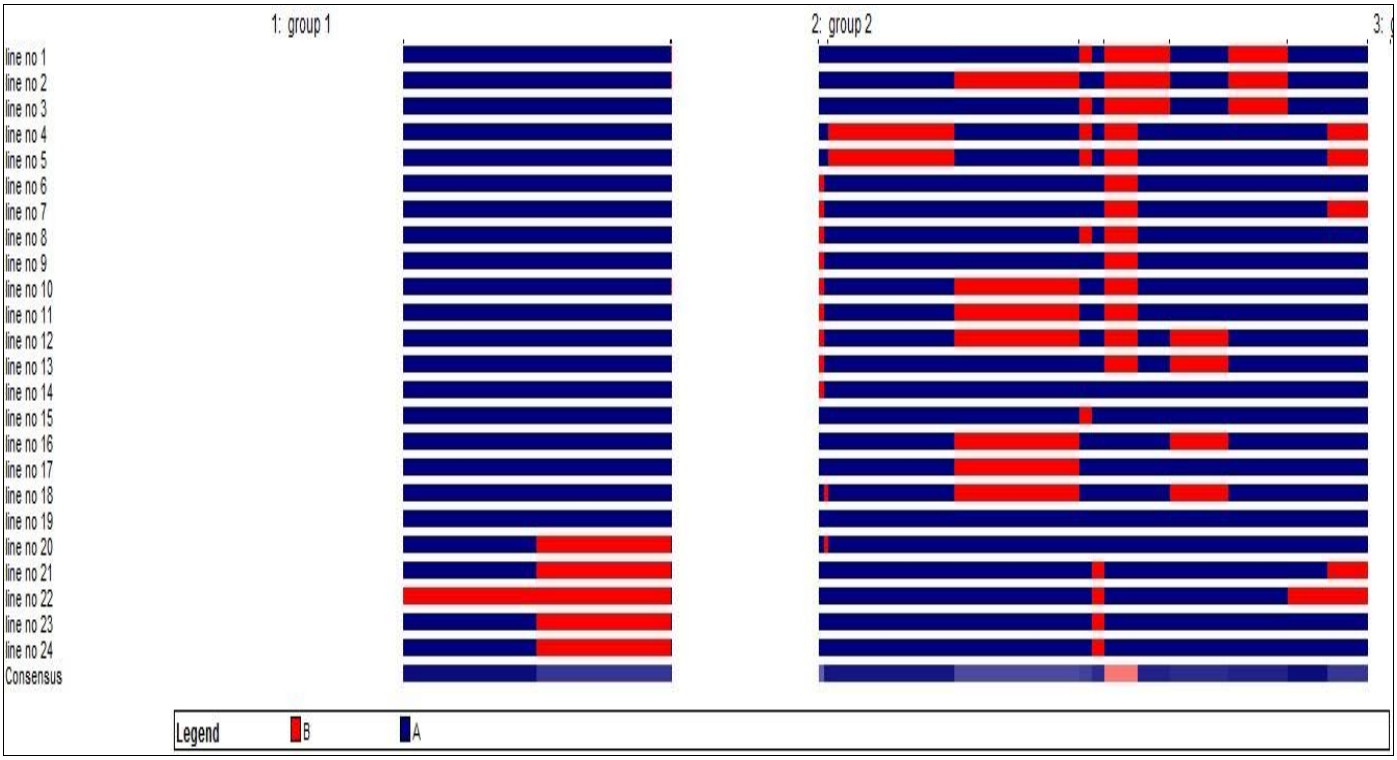

Fig 1: Genomic constitution of 24 rice germplasm lines based on position of SSR alleles generated using 10 SSR markers. The discriminated allele's types A & B are marked in blue & red colors respectively. Graphical outputs of genotyping data were generated using GGT version 2.0 tool. Group 1 represent the chromosome no. 4 related markers and group 2 represent the chromosome 12 related markers

Table 2: Association analysis between traits and molecular markers

Trait	Chromosome	Position (cm)	Marker	Gene	P-value
CL	4	35.4	RM261	*Bph15*	0.017*
HI	4	35.4	RM261	*Bph15*	0.026*
USPP	4	35.4	RM261	*Bph15*	0.027*
LL	4	35.4	RM261	*Bph15*	0.033*
PH	4	35.4	RM261	*Bph15*	0.003**
PL	4	35.4	RM261	*Bph15*	0.003**
NTPP	4	35.4	RM261	*Bph15*	0.022*
NETPP	4	35.4	RM261	*Bph15*	0.025*
FSPP	4	35.4	RM261	*Bph15*	0.048*
CL	4	76.1	RM119	*Bph6*	0.021*
CL	4	76.1	RM119	*Bph6*	0.049*
LL	4	76.1	RM119	*Bph6*	0.021*
LW	4	76.1	RM119	*Bph6*	0.049*
PL	4	76.1	RM119	*Bph6*	0.047*
GY	4	76.1	RM119	*Bph6*	0.046*

USPP	4	76.1	RM119	*Bph6*	0.028*
100GWt	12	22.13	RM6217	*Bph9*	0.016*
GW	12	22.13	RM6217	*Bph9*	0.017*
LW	12	23.49	RM3331	*Bph18*	0.049*
LW	12	23.49	RM3331	*Bph18*	0.000
GY	12	23.49	RM3331	*Bph18*	0.033*
FSPP	12	23.49	RM3331	*Bph18*	0.035*
FSPP	12	23.49	RM3331	*Bph18*	0.025*
NTPP	12	61.7	RM260	*Bph10*	0.045*
NETPP	12	61.7	RM260	*Bph10*	0.034*
HI	12	61.7	RM260	*Bph10*	0.049*
100GWt	12	61.7	RM260	*Bph10*	0.028*
LW	12	65.5	RM313	*Bph10*	0.022*
PL	12	65.5	RM313	*Bph10*	0.030*
PL	12	65.5	RM313	*Bph10*	0.021*
HI	12	65.5	RM313	*Bph10*	0.041*
LL	12	75.5	RM463	*Bph2*	0.004**
CL	12	75.5	RM463	*Bph2*	0.017*
PH	12	75.5	RM463	*Bph2*	0.025*
USPP	12	75.5	RM463	*Bph2*	0.005**
PH	12	75.5	RM463	*Bph 2*	0.011*
NTPP	12	75.5	RM463	*Bph 2*	0.010*
NETPP	12	75.5	RM463	*Bph2*	0.008**
PL	12	93.3	RM5479	*Bph21, Bph26*	0.048*
USPP	12	93.3	RM5479	*Bph21, Bph26*	0.005**
GY	12	105.5	RM6869	*Bph2, Bph18*	0.046*
HI	12	105.5	RM6869	*Bph2, Bph18*	0.049*
GW	12	105.5	RM6869	*Bph2, Bph18*	0.029*
PH	6	3.2	RM589	*Bph3*	0.030*
PH	6	3.2	RM589	*Bph3*	0.030*
PL	6	3.2	RM589	*Bph3*	0.038*
PL	6	3.2	RM589	*Bph3*	0.038*
NTPP	6	3.2	RM589	*Bph3*	0.027*
NTPP	6	3.2	RM589	*Bph3*	0.027*
NETPP	6	3.2	RM589	*Bph3*	0.033*
NETPP	6	3.2	RM589	*Bph3*	0.033*
USPP	6	3.2	RM589	*Bph3*	0.036*
USPP	6	3.2	RM589	*Bph3*	0.036*

(CL = Coleoptile Length, BY= Biological Yield, FSPP = Filled Spikelets Per Panicle, GL = Grain Length, GY = Grain Yield, HI= Harvest Index, LL = Leaf Length, LW = Leaf Width, NTPP = No. of Tillers Per Panicle, NETPP = No. of Effective Tillers Per Panicle, PH = Plant Height, PL = Panicle Length, SL = Seedling Length, USPP = Unfilled Spikelets Per Panicle, 100GWt = 100 Grains Weight)

Table 3: List of microsatellite (SSR) markers used for molecular studies

S. No.	SSR markers	Chromosome number	Forward 5'→3'	Reverse 5'→3'
1.	RM119	4	CATCCCCCTGCTGCTGCTGCTG	CGCCGGATGTGTGGGACTAGCG
2.	RM261	4	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC
3.	RM313	12	TGCTACAAGTGTTCTTCAGGAC	GCTCACCTTTTGTGTTCCAC
4.	RM463	12	TTCCCCTCCTTTTATGGTGC	TGTTCTCCTCAGTCACTGCG
5.	RM589	6	ATCATGGTCGGTGGCTTAAC	CAGGTTCCAACCAGACACTG
6.	RM5479	12	AACTCCTGATGCCTCCTAAG	TCCATAGAAACAATTTGTGC
7.	RM6217	12	CGCAGATGGAGATTCTTGAAGG	ACAGCAGCAAGAGCAAGAAATCC
8.	RM260	12	ACTCCACTATGACCCAGAG	GAACAATCCCTTCTACGATCG
9.	RM3331	12	CCTCCTCCATGAGCTAATGC	AGGAGGAGCGGATTTCTCTC
10.	RM6869	12	GAGCTCCTTGTAGTGACCCG	ATCAGCCTCGCCAGCTTC

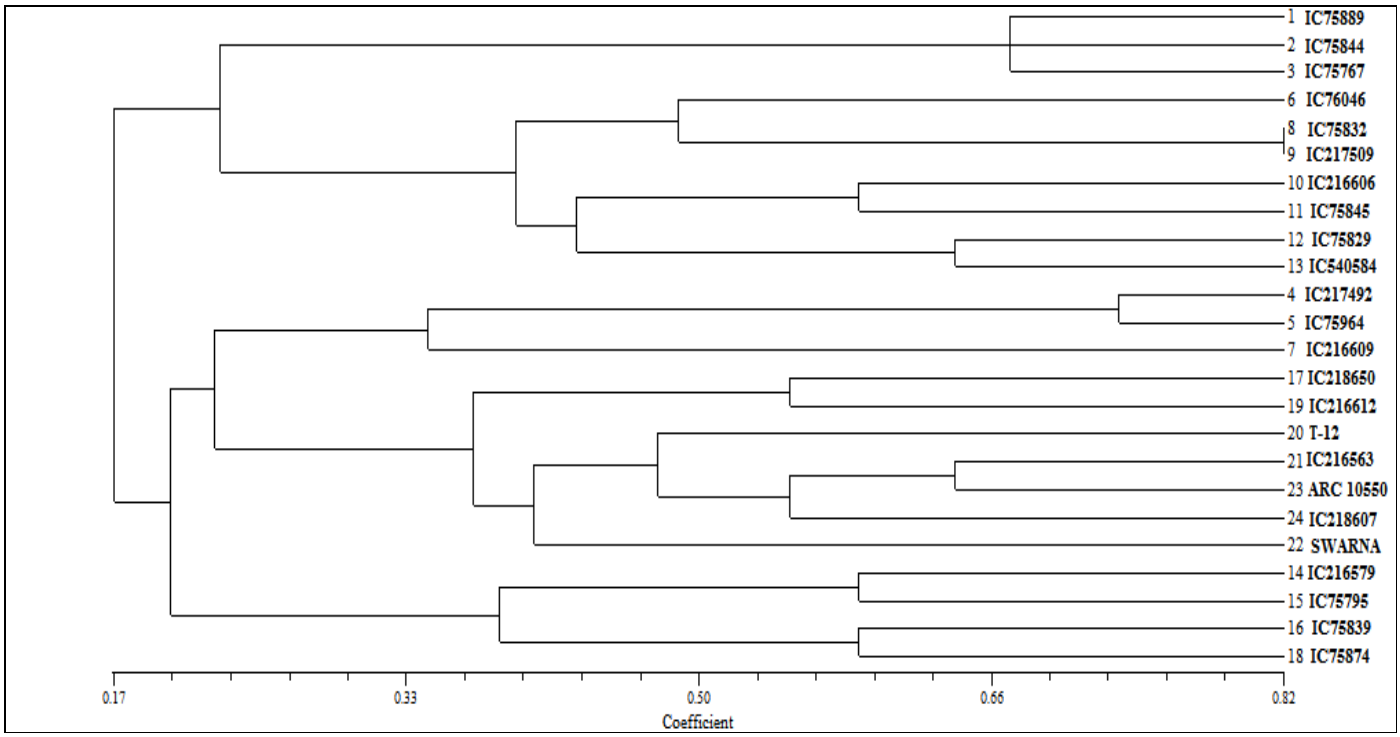

Fig. 2: Dendogram derived from microsatellite (SSR) analysis of 24 rice genotypes

A dendogram (Fig. 2) based on UPGMA analysis shows the genetic diversity among the 24 germplasm of rice. The 24 collections was divided in to two main clusters at 17% similarity. The cluster I was found to ten genotypes i.e. IC75889, IC75844, IC75767, IC76046, IC75832, IC217509, IC216606, IC75845, IC75829 and IC540584. Cluster II contain 14 genotypes i.e. IC217492, IC75964, IC216609, IC218650, IC216612, T-12, IC216563, ARC 10550, IC218607, Swarna, IC216579, IC75795, IC75839 and IC75874. The cluster I and cluster II were further divided in to sub clusters i.e. I1, I2 and II1, II2 at 23% and 20% similarity respectively. Cluster I1 contained three genotypes and cluster I2 contained seven genotypes. Cluster II1 contained ten genotypes and cluster II2 contained four genotypes. (Fig. 2) Maximum similarity i.e. 82% was found between IC75832 and IC217509 showing that these two germplasm lines are relatively closely related.

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