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Molecular characterization of rice (*Oryza sativa* L.) genotypes using target region amplification polymorphism (TRAP) markers in relation to grain iron content

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Abstract

In the present investigation, based on the seven rice putative candidate iron transporter genes, novel TRAP markers were developed. These markers were successfully employed in the molecular diversity study among 30 rice genotypes representing improved rice cultivars and land races with varied grain iron content (7.38 - 30.58 ppm). Totally, thirty TRAP primer combinations were screened, which generated 703 bands out of which 654 were polymorphic (93%) with an average of 21.8 bands per primer combination. The average polymorphic information content (PIC) values ranged from 0.09 (Osysl4b+ME05) to 0.25 (Osnramp5c+ME05, Osnramp1b+ME02 and Osysl4a +ME02). Gene diversity ($\hat{H}$) ranged from 0.10 (Osysl4b+ME05) to 0.31 (Osnramp1b+ME02 and Osysl4a +ME02). The Jaccard dissimilarity ranged from 0.15 to 0.52, explaining 37% of genetic variation (Table 4). Grouping of genotypes based on UPGMA and principal coordinate analysis (PCoA) were found comparable and grouping of genotypes into a different cluster was found mainly on the basis of pedigree relationships. TRAP markers revealed well resolved relationships among rice genotypes. The information generated from this study will helps to select parental combinations for breeding high iron content rice varieties.

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Rice (*Oryza sativa* L.) is a one of the staple cereal food crops and 50% of world population depends on rice as a main source of nutrition (White, 1994). Despite of main nutritive source, it is a poor source of micronutrients such as Fe and Zn (Bouis and Welch, 2010). Micronutrients deficiency is a global problem contributing to world's malnutrition. This in turn leads to high rate of mortality in women and children (WHO, 1996). Since, rice is known to be a staple food crop and having lowest Fe concentration in grains is the major nutritional concern (Senadhira *et al.,* 1999). Therefore, biofortification through conventional, molecular breeding and genetic engineering is an excellent method to combating the problem of malnutrition.

Among the various methods, biofortification through molecular breeding based on QTLs/candidate gene for grain iron content is most important. Candidate genes are sequenced genes of known biological function putatively involved in the expression of a given trait or trait variation, based on its map localization (Pflieger *et al.,* 2001). These gene based marker association studies have proven extremely powerful in studying the genetic architecture of complex traits (Patel and Patel, 2013).

Genetic diversity study is a prerequisite step for selection of diverse parents for developing improved rice cultivars. In these lines, previously various DNA markers techniques have been employed *viz.,* Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs) and Amplified Fragment Length Polymorphism (AFLP) to assess genetic diversity of various rice cultivars throughout the world (Joshi *et al.,* 2000).

But the polymorphism of such marker systems detected is random across the genome and may not reflect functional polymorphisms of functionally characterized genes or targeted genes. Therefore, one such marker system namely targeted region amplification polymorphism (TRAP) (Hu and Vick, 2003) generates markers around targeted genes. TRAP is a rapid and an efficient PCR-based technique, which uses known target regions in genome as fixed primers in combination with arbitrary primers to generate polymorphism around target gene sequences (Hu and Vick, 2003). As the TRAP technique can be used to generate markers for specific gene sequences, it is useful for genotyping germplasm and generating markers associated with desirable traits in crop plants for marker-assisted breeding (Hu *et al.,* 2005). This technique has been successfully applied in other crops such as sugarcane and sunflower (Alwala *et al.,* 2006; Yue *et al.,* 2009) and it was found very effective tool in germplasm evaluation, genetic linkage mapping for traits of interest (Chen *et al.,* 2011).

In order to demonstrate applicability of TRAP markers in characterization of rice genotypes in relation to grain iron content, we developed TRAP markers based on the information of candidate gene specific primers reported by Soman *et al.* (2014). This study represents the first attempt to use TRAP markers in rice for genetic diversity study. The markers data obtained were analyzed with appropriate statistical tools and discussed in relation to grain iron content.

Materials and methods

Plant material

Thirty rice genotypes representing released varieties and local land races were grown under aerobic condition (Shashidhar *et al.*, 2007) (Table 1). Plants were raised by direct seeding with a spacing of 30 cm between the rows and 15 cm between the plants. The field management followed the recommended package of practices of UAS, Bengaluru.

Estimation of Iron

Dehusked rice washed immediately with 0.1N HCl and followed by double distilled water to avoid surface contamination. Washed samples were dried in an oven for 70^{o}C for 48-72h. After drying the samples were ready for iron content estimation was done using (XRF) (OXFORD Instruments X-Supreme 8000) (Paltridge *et al.*, 2012), at M. S. Swaminathan Research Foundation, Chennai. These genotypes were

grouped in to three different phenotypic groups based on grain iron content as high (>20 ppm), medium (15-20 ppm) and low (<15 ppm).

DNA isolation and TRAP marker analysis

Genomic DNA was extracted from the young leaves of 15 day old seedlings using CTAB (cetyl trimethyl ammonium bromide) extraction method as described by Doyle and Doyle (1990). The purity and concentration of the isolated genomic DNA sample was quantified using Nanodrop (Biotech Epoch). Finally all the DNA samples were diluted to working concentration of 25 ng/µl for TRAP analysis.

TRAP analysis was performed on 30 rice genotypes based on seven putative iron transporter candidate genes (Soman *et al.*, 2014). Total 30 primer combinations involving 30 gene specific fixed and two arbitrary primers were used (Table 2). DNA amplification was carried out in a 10 µl reaction volume constituting of 25 ng template DNA, 2.5mM of each dNTP, 1x PCR buffer (10mM Tris-HCl, 50mM KCl, 1.5mM MgCl2 pH 8.8), 10 pico molars each of forward fixed primer and reverse arbitrary primer and 1 unit of *Taq* polymerase was used. The PCR amplification was performed: 1 cycle of denaturation at 94◦C for 5 min, followed by 5 cycles of 94◦C for 45 s, 35◦C for 45 s, 72◦C for 1 min, then 30 cycles of 94◦C for 45 s, 55◦C for 45 s, and 72◦C for 1 min, with a final extension step of 7 min at 72◦C. The final PCR products were separated on 6% (w/v) polyacrylamide gel for 2- 3 hrs at 150V and visualized by fast silver staining method as described by Benbouza *et al.* (2006).

Statistical analyses

TRAP bands were scored manually for presence (1) or absence (0), to obtain 0, 1 binary data. The pooled marker data were analyzed using DARWIN program *ver* 5.0. Jaccard dissimilarity matrix was calculated to construct UPGMA (Unweighted Pair Group Method with Arithmetic Average) dendrogram and Principal coordinate analysis (PCoA) graph. Polymorphism information content (PIC) and average gene diversity ($\hat{H}$) of a given primer were calculated using POWERMARKER program *ver* 3.0.

Results and discussion

Molecular characterization of rice genotypes in relation to grain iron content is very important pre-requisite step for breeding high iron lines. This can aid in biofortification of rice through conventional and molecular breeding approaches. Therefore, in this study we attempted to develop TRAP markers around putative candidate iron transporter genes specifically expressed in rice grains (Fig. 1). Similarly, TRAP markers were deployed effectively in common beans (Miklas *et al.,* 2006), sugarcane (Creste *et al.,* 2010), gerbera (Song *et al.,* 2012), radish (Cheng *et al.,* 2013) and chickpea (Kumar *et al.,* 2014). These studies revealed good utilities of the TRAP marker system for developing molecular markers from target functional genes.

In rice, previously various DNA markers techniques *viz.,* RAPD, SSR and AFLP, were employed for diversity study (Joshi *et al.,* 2000). But, these techniques revealed polymorphism arising from non-coding region of genome. In order to understand trait based functional polymorphism in rice use of candidate gene based markers is very informative. In this context, various candidate genes for grain iron content accumulation were reported. Chandel *et al.* (2011) characterized two QTLs, qFE-1 and qFE-9 governing iron content and identified eight candidate genes (OsYSL1, OsYSL4, OsMTP1, OsNAS3, APRT1, OsNRAMP1, OsNRAMP5 and OsZIP8) involving in uptake, transport and accumulation of iron. Similarly, Anuradha *et al.* (2012) reported two candidate genes OsYSL1 and OsMTP1 underlying QTLs for high iron accumulation in rice seeds. However, developing markers based on candidate genes is a tedious process. But, here we report development of TRAP markers around the iron transporter genes that are more information.

In the present study, TRAP amplification of 30 rice genotypes was carried out with 30 primer combinations which successfully amplified a total of 703 recordable bands. Of the 703 amplicons scored,

654 (93 %) were polymorphic and 49 (7 %) were monomorphic among the genotypes. The number of bands amplified by each primer combination ranged from 15 (Osysl4b+ME05) to 30 (Osnas3a+ME02). For each primer combination, an average of 23.4 total bands was scored and an average of 21.8 was polymorphic (Table 3). Similarly in chickpea 14 - 30 bands were reported for TRAP analysis (Kumar *et al.,* 2014). The average polymorphic information content (PIC) values ranged from 0.09 (Osysl4b+ME05) to 0.25 (Osnramp5c+ME05, Osnramp1b+ME02 and Osysl4a +ME02). Gene diversity (H^) ranged from 0.10 (Osysl4b+ME05) to 0.31 (Osnramp1b+ME02 and Osysl4a +ME02). Similarly, Alwala *et al.* (2006) in sugarcane reported TRAP markers with average of 20 polymorphic bands per primer combination and average PIC of 0.36 which are comparable with present study.

Table 1. List of genotypes and brown rice iron content.

Sl.No	Genotype	Grain Iron content (ppm)	Iron content group
1	Alursanna	16.6	Medium
2	AM65	27.4	High
3	Amrutha	14.7	Low
4	Antrasali	12.7	Low
5	Azucena	21.17	High
6	Bangara kovi	21.6	High
7	Bangaru sanna	17.7	Medium
8	BI33	16.73	Medium
9	BI43	23.2	High
10	Bidarlocal-1	20.2	High
11	Bile kalavi	19.7	Medium
12	BJ21	30.58	High
13	Burmablack	13.63	Low
14	Champakali	19.6	Medium
15	Chippiga	17.5	Medium
16	Dambersali	14.7	Low
17	Devamallige	20.88	Medium
18	Doddamullare	19.6	Medium
19	Doddiga	17.7	Medium
20	Farm valya	20.7	High
21	Gandhasali	21.2	High
22	Gopaldoddiga	14.1	Low
23	Gujrat Buddha	13.1	Low
24	Holesali chipiga	19.2	Medium
25	Honasu	16.2	Medium
26	Honnekattu	22.7	High
27	HY 258-1	16.1	Medium
28	IR20	10.25	Low
29	J-192	19.2	Medium
30	Jeerige sanna	7.38	Low

Based on the grain Fe content, 30 rice genotypes were divided into three groups. The grain Fe content ranged from 7.38 to 30.58 ppm representing a diverse phenotypic variation. The UPGMA dendrogram based on TRAP markers depicted well resolved relationships among rice genotypes with high bootstrap values (Fig. 2). All the 29 rice genotypes were grouped into two major clusters (CLI & CLII) and one genotypes Bangara kovi formed a separate node. Cluster I constituted 8 rice genotypes representing three high (BJ21, Azucena and Am65), 2 medium (BI33 and Devamallige) and three low (IR20, Burmablack and Jeerige sanna) iron content genotypes. Cluster II comprised of 21 rice genotypes (with 5 high, 9 medium and 5 low iron lines), which are distributed into three sub-cluters SC1 (with 3 genotypes), SC2 (9 genotypes) and SC3 (8 genotypes). One rice genotype Doddiga formed a separate node. In the dendrogram even though high iron lines distributed into different cluster, they tend to group together as distinct subgroups indicating common pedigraal sharing of candidate gene for high iron transporters. But for medium and low iron content genotypes were erratically grouped into different clusters. Similarly, correlations between morphological characteristics and molecular markers have been reported in tomato (Berloo. V *et al.,* 2008) and *Auricularia auricula* (Li *et al.,* 2011).

Table 2. Details of primer sequences used for TRAP analysis.

Sl. no	*Primer Name*	*Sequences (5'-3')*
	Fixed Primers	
1	Aprt1a	CTCTCTCCCGCTTTTTGCTT
2	Aprt1b	GTTGTGAAATATCAGGTGTTGAAGC
3	Aprt1c	GGAAGTTTCCTTTTGGCTGT
4	GrmM 1-1	GTCGTCATGATCTGGGACT
5	Osmtp1a	TCTCTCCTCCCCATCTCCAA
6	Osmtp1b	GGGGACCTTCTTGTTGTTGG
7	Osmtp1d	TGCCATGTGACAATCACTCA
8	Osnas3a	TCACCAGTTGGAGCTAATCG
9	Osnramp1b	TTGTACGTCTTTGAGACTTTGACTG
10	Osnramp5c	GCTTTGCTGATCGGGATTAGTT
11	Osnramp5d	TAATTTCGGGCTCCAGTACC
12	Osnramp5f	GATTAGCAAAATGTCACTGACTAGC
13	Osnramp5g	GATCATTACGTATGTCGTTGTATCTC
14	Osnrmp1c	GGGTTCTCATTGCTGGCTCT
15	Osysl1a	TAAACCAAAGATGGCAGACC
16	Osysl4a	GCAAACACTACCCCAAAAGC
17	Osysl4b	AGCTCTGCGGGTGAACATAC
18	Osysl4c	TATTAGTGAGGGCGATCCAC
19	Osysl4d	TCTAGAACGGTCACACAGAAAA
20	Osysl4e	TATGCATGCGGTGGATGA
21	Osysl4f	CACCTCTACATCCCGAGCAT
22	Oszip8a	ATGAGGACGAACACCACCAC
	Arbitory primers	
1	ME02	TGAGTCCAAACCGGAGC
2	ME05	TGAGTCCAAACCGGAAG

Table 3. Degree of polymorphism as revealed by TRAP primer combinations in rice.

SL.NO	*Primer combinations*	*TNB*	*NPB*	*%P*	*PIC*	*Average gene diversity (H^)*
1	Aprt1a + ME02	27	26	96.2	0.21	0.25
2	Aprt1a + ME05	20	15	75	0.17	0.21
3	Aprt1b + ME02	26	25	96.1	0.24	0.29
4	Aprt1c + ME02	28	28	100	0.24	0.3
5	Grm M 1-1 + ME02	25	23	92	0.21	0.25
6	Osmtp1a + ME02	24	23	95.8	0.22	0.26
7	Osmtp1a + ME05	19	17	89.4	0.23	0.28
8	Osmtp1b + ME05	27	27	100	0.23	0.29
9	Osmtp1d + ME05	16	12	75	0.18	0.22
10	Osmtp1d + ME02	20	20	100	0.24	0.29
11	Osnas3a + ME02	30	29	96.6	0.21	0.25
12	Osnramp1b + ME02	25	25	100	0.25	0.31
13	Osnramp5c + ME02	27	27	100	0.21	0.25
14	Osnramp5c + ME05	21	21	100	0.25	0.3
15	Osnramp5d + ME02	26	26	100	0.23	0.28
16	Osnramp5f + ME02	22	19	86.3	0.21	0.26
17	Osnramp5g + ME02	20	18	90	0.24	0.3
18	Osnramp5g + ME05	14	13	92.8	0.16	0.18
19	Osnrmp1c + ME02	28	27	96.4	0.23	0.28
20	Osnrmp1c + ME05	24	23	95.8	0.24	0.29
21	Osysl1a + ME02	20	20	100	0.23	0.27
22	Osysl4a + ME02	24	24	100	0.25	0.31
23	Osysl4b + ME02	27	23	85.1	0.21	0.26
24	Osysl4b + ME05	15	9	60	0.09	0.1
25	Osysl4c + ME02	24	22	91.6	0.22	0.28
26	Osysl4d + ME05	17	15	88.23	0.24	0.3
27	Osysl4e + ME02	26	23	88.4	0.21	0.25
28	Osysl4e + ME05	27	21	77.7	0.2	0.25
29	Osysl4f + ME05	25	25	100	0.24	0.3
30	Oszip8a + ME02	29	28	96.5	0.21	0.25
	Average	23.4	21.8	92.2	0.2	0.3

Note* TNB-Total number of bands; NPB- Number of polymorphic bands; % P-Percent polymorphism; PIC-Polymorphism information content.

Genetic distance in terms of Jaccard dissimilarity coefficients ranged from 0.15 to 0.52, explaining 37% of genetic variation across 30 genotypes. In the dendrogram two high iron content genotypes namely Bidar local 1 (20.2 ppm) and BI 43 (23.2) were placed at one extreme end and Jeerige sanna a very low iron content (7.38 ppm) genotype at other end. Selection of such contrasting parents for crossing program can help in developing elite rice cultivars with improved traits.

Table 4. Jacccard dissimilarity matrix as obtained based on TRAP marker data.

Am65	0.00													
Azucena	0.16	0.00												
BI33	0.29	0.27	0.00											
BJ21	0.31	0.30	0.26	0.00										
Devamallige	0.33	0.34	0.30	0.32	0.00									
Burmablack	0.33	0.33	0.29	0.31	0.26	0.00								
IR20	0.36	0.38	0.36	0.34	0.29	0.26	0.00							
Jeerigesanna	0.37	0.38	0.38	0.35	0.38	0.36	0.34	0.00						
Alursanna	0.47	0.46	0.47	0.49	0.44	0.42	0.45	0.44	0.00					
Amrutha	0.43	0.42	0.41	0.43	0.40	0.39	0.41	0.42	0.31	0.00				
Antrasali	0.49	0.50	0.50	0.51	0.49	0.47	0.51	0.49	0.35	0.34	0.00			
Bangara kovi	0.48	0.48	0.52	0.51	0.50	0.49	0.51	0.47	0.46	0.44	0.41	0.00		
Bangaru sanna	0.43	0.43	0.41	0.44	0.40	0.40	0.42	0.42	0.36	0.34	0.38	0.42	0.00	
BI43	0.41	0.40	0.39	0.40	0.38	0.38	0.41	0.39	0.37	0.32	0.42	0.43	0.29	0.00
Bidarlocal-1	0.40	0.38	0.35	0.40	0.39	0.37	0.40	0.40	0.37	0.29	0.40	0.43	0.29	0.20
Bile kalavi	0.44	0.43	0.44	0.45	0.44	0.42	0.44	0.42	0.37	0.35	0.38	0.42	0.33	0.28
Champakali	0.50	0.50	0.50	0.51	0.48	0.49	0.51	0.50	0.40	0.39	0.38	0.43	0.38	0.37
Chippiga	0.45	0.44	0.44	0.47	0.45	0.43	0.48	0.47	0.41	0.38	0.41	0.46	0.36	0.35
Dambersali	0.44	0.43	0.45	0.46	0.44	0.42	0.45	0.44	0.36	0.31	0.37	0.42	0.30	0.33
Doddamullare	0.42	0.42	0.40	0.44	0.41	0.39	0.42	0.42	0.37	0.33	0.37	0.42	0.32	0.30
Doddiga	0.48	0.48	0.51	0.52	0.45	0.47	0.47	0.49	0.42	0.39	0.39	0.45	0.41	0.40
Farm valya	0.44	0.43	0.44	0.46	0.40	0.41	0.43	0.46	0.40	0.37	0.38	0.43	0.35	0.37
Gandhasali	0.48	0.50	0.49	0.49	0.46	0.45	0.45	0.48	0.41	0.42	0.41	0.47	0.37	0.41
Gopaldoddiga	0.46	0.47	0.45	0.47	0.45	0.42	0.45	0.45	0.42	0.39	0.40	0.45	0.35	0.38
Gujrat buddha	0.44	0.46	0.45	0.47	0.45	0.41	0.44	0.46	0.40	0.37	0.40	0.46	0.34	0.37
Holesali chipiga	0.44	0.45	0.42	0.45	0.41	0.38	0.41	0.44	0.41	0.36	0.38	0.47	0.33	0.35
Honasu	0.44	0.44	0.43	0.46	0.43	0.41	0.43	0.44	0.41	0.37	0.39	0.46	0.33	0.32
Honnekattu	0.47	0.48	0.47	0.50	0.47	0.45	0.48	0.48	0.40	0.39	0.40	0.46	0.39	0.38
HY 258-1	0.46	0.48	0.46	0.48	0.45	0.43	0.45	0.48	0.43	0.38	0.42	0.50	0.38	0.39
J-192	0.45	0.46	0.45	0.45	0.43	0.41	0.44	0.47	0.43	0.36	0.41	0.48	0.37	0.37

Table 4. Continue.

Am65																
Azucena																
BI33																
BJ21																
Devamallige																
Burmablack																
IR20																
Jeerigesanna																
Alursanna																
Amrutha																
Antrasali																
Bangara kovi																
Bangaru sanna																
BI43																
Bidarlocal-1	0.00															
Bile kalavi	0.25	0.00														
Champakali	0.34	0.33	0.00													
Chippiga	0.33	0.34	0.38	0.00												
Dambersali	0.31	0.30	0.33	0.31	0.00											
Doddamullare	0.29	0.30	0.36	0.32	0.27	0.00										
Doddiga	0.39	0.39	0.43	0.43	0.31	0.32	0.00									
Farm valya	0.33	0.36	0.40	0.34	0.34	0.30	0.33	0.00								
Gandhasali	0.38	0.40	0.45	0.43	0.37	0.34	0.39	0.30	0.00							
Gopaldoddiga	0.35	0.35	0.43	0.41	0.35	0.33	0.40	0.35	0.27	0.00						
Gujrat buddha	0.36	0.38	0.42	0.41	0.33	0.32	0.37	0.35	0.35	0.32	0.00					
Holesali chipiga	0.33	0.37	0.41	0.39	0.33	0.29	0.37	0.32	0.30	0.27	0.25	0.00				
Honasu	0.31	0.35	0.42	0.36	0.34	0.30	0.37	0.32	0.32	0.27	0.28	0.23	0.00			
Honnekattu	0.37	0.39	0.38	0.38	0.36	0.35	0.41	0.35	0.37	0.34	0.34	0.34	0.32	0.00		
HY 258-1	0.37	0.40	0.43	0.37	0.39	0.35	0.44	0.34	0.37	0.35	0.33	0.27	0.29	0.28	0.00	
J-192	0.34	0.38	0.42	0.36	0.37	0.34	0.41	0.32	0.34	0.32	0.31	0.25	0.28	0.31	0.15	0.00

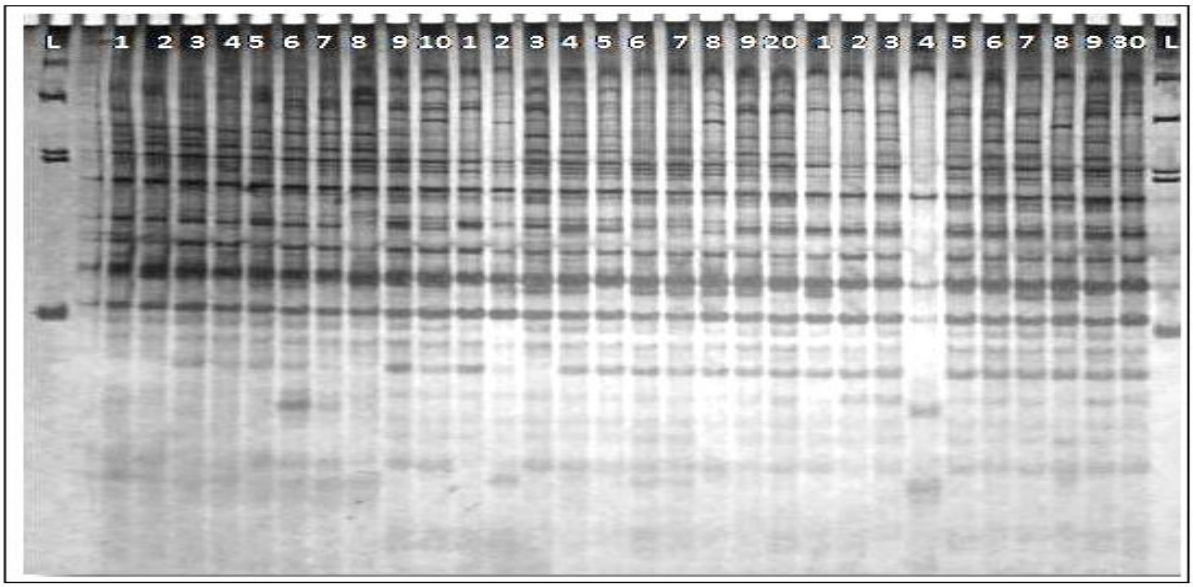

Fig. 1. TRAP marker pattern from the primer combination OSNRMP1c and ME02.

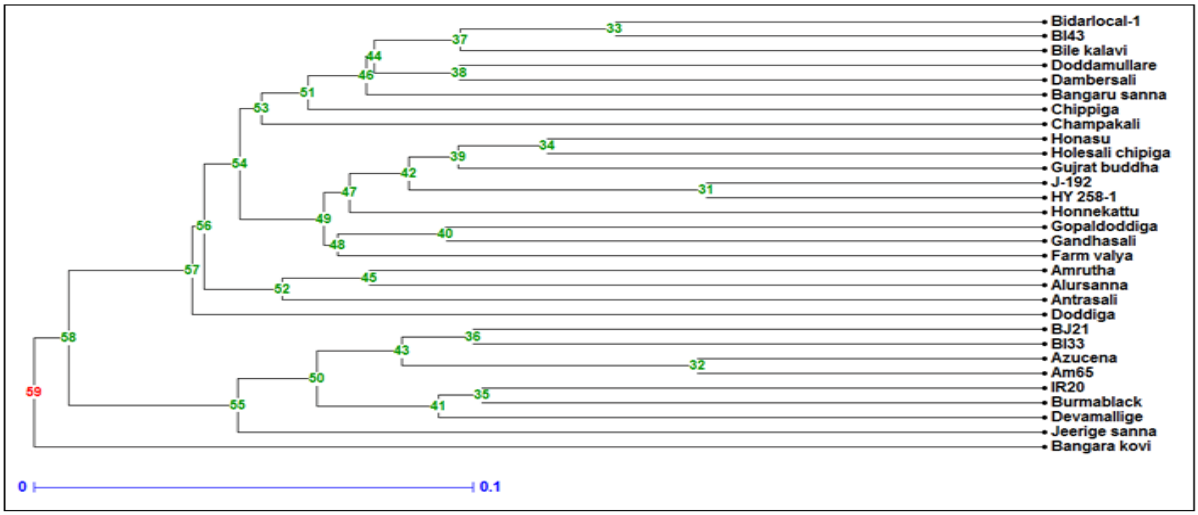

Fig. 2. UPGMA-dendrogram showing clustering pattern for 30 genotypes of rice.

In summary, we have successfully demonstrated the development of TRAP markers in rice. The Fe transporter based TRAP markers could be best markers of choice for association studies that can provide useful information for biofortification for Fe in rice.

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