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DNA fingerprinting of root-knot nematode resistant rice genotypes

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ABSTRACT

Forty seven microsatellite markers were used for fingerprinting and assessing genetic diversity of eight rice genotypes differing in resistance to root-knot nematode. Forty five marker loci revealed polymorphism among these genotypes. A total of 104 alleles were amplified, of which 97 were polymorphic. The number of alleles detected per locus ranged from two to five with an average of 2.21. The polymorphism information content (PIC) ranged between 0 and 0.941 with an average of 0.64 per locus, indicating the suitability of the markers for the detection of genetic diversity. Genetic similarities among the genotypes varied from 0.367 to 0.792 with an average of 0.56. The UPGMA cluster analysis grouped the rice genotypes into two major clusters at 49% level of genetic similarity. Twelve unique alleles were identified which could be useful for developing diagnostic markers.

Key words: *DNA fingerprinting, genetic diversity, rice, root-knot nematode*

Rice is cultivated on almost all the continents, over an area of more than 150 million/hectare with production of nearly 600 million tons under three major ecosystems namely, rain-fed upland, irrigated medium land and rainfed lowland. Nearly 52% of the global production of rice is lost annually owing to the damage caused by biotic stress (Bharathi *et al*., 2008). Among the biotic stress, the root-knot nematode (RKN), *Meloidegune graminicola* is the major parasite which causes significant yield losses in all rice ecosystems (Prasad *et al*., 1985; 1886; Rao *et al.,* 1986; Mac Gowan, 1989; Bridge *et al.,* 1990; Jairajpuri and Baqri, 1991). It causes swelling and formation of hooked like galls throughout the root system, a symptom characteristic of root-knot nematode disease. Nematode feeds on the cells and interferes with water uptake, nutrient uptake and translocation resulting in stunting and chlorosis of young plants. The options to control RKN are still limited. Development and use of resistant cultivars offers the best management tool for controlling root-knot nematode. Lack of resistance to the nematode has been a major factor hindering the genetic improvement of cultivated rice. In order to find out a good source of resistance several high-yielding rice cultivars have been screened, but not enough genetic variability has been found for resistance to the rice root-knot nematode. (Soriano, 1995; Tandingan *et al.,* 1996).

Genetic uniformity among new rice varieties is an alarming situation, as it has increased the vulnerability of the rice crop to disease epidemics and insect infestation. Durability of resistance depends on the knowledge of genetics of resistance and diversity present in the rice cultivars. A high level of genetic diversity reduces the risk of widespread epidemics of pest and diseases (Zhu *et al*., 2000; Newton *et al*., 2009). In order to determine the level of risk, it is the first step to identify and evaluate the genetic diversity of tolerant genotypes.

In the past, the characterization of germplasm diversity was carried out by means of morphological and biochemical markers, which in many cases, did not have the resolution power for revealing polymorphism and/or differentiating closely related genotypes. Molecular markers have demonstrated a potential to detect genetic diversity and to aid the management of plant genetic resources. Several molecular markers like RFLP, RAPD, SSR, ISSR, AFLP and SNPs are presently available to assess the variability and diversity at molecular level (Joshi *et al*., 2000). Single sequence

repeats markers (microsatellites) are co-dominant hyper variable, abundant and well distributed throughout the rice genome (Temnykh *et al.*, 2001). These molecular markers have been extensively used for identification and assessment of genetic diversity of varieties, gene and genome mapping, prediction of hybrid performance, management of genetic resources; phylogenetic analysis, marker-assisted selection breeding and map based cloning of useful genes in rice.

The present study was under taken to fingerprint and assess the genetic diversity of rice cultivars having different degrees of resistance to RKN, to identify genotype specific markers and to develop DNA fingerprint/ profile data set using molecular markers.

MATERIALS AND METHODS

Six rice genotypes and two breeding lines, CR 3003-184 and CR 3003-11-186 from the cross Annapurna / Ramakrishna having different degree of resistance to rice root-knot nematode (RRKN) were selected for DNA fingerprinting (Table 1). Seeds of these genotypes were collected from Gene Bank of Central Rice Research Institute, Cuttack and fingerprinting was done in the pest genomics laboratory of crop protection division. Fifteen seeds per genotypes were germinated in petridishes and seven days old seedlings were harvested for genomic DNA isolation. Ten to twelve seedlings per genotype were bulked and genomic DNA was isolated from bulked leaf samples following Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thomson, 1984). The quantity and quality was estimated using spectrophotometer and agarose gel electrophoresis using known concentration of Lambda DNA. The samples were diluted in $T_{10}E_1$ buffer to get final concentration of 20ng/µl for amplification.

A set of 47 primers specific for microsatellite loci distributed over 12 chromosomes of rice were used for PCR amplification. The primer sequences were downloaded from Gramene database (http://www.gramene.org) and custom synthesized by Qiagen Operon Technologies, Almeda, California, USA. These primers were selected on the basis of their uniform distribution and PIC value. The sequences and details of the primers used are available in the above mentioned database. The PCR amplification was performed in a 20 l reaction mixture volume containing 20 ng of DNA, 1X PCR buffer {75 mM Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$}, 200 µM dNTP mix (MBI Fermantas, Lithuania, USA), 5 pmol of primer, 2 mM of magnesium chloride and 1 U of *Taq* (*Thermus aquaticus*) DNA polymerase enzyme (Biotools, Spain). The PCR was performed in a thermal cycler (Thermal Cycler, Perkin Elmer, Cetus) as per following cycling parameters: initial denaturation at 94^0C for 3 min followed by 40 cycles of denaturation at 94^0C for 30 sec, annealing at 55-65^0C (depending upon primer) for 1 min and extension at 72^0C for 1.5 min and final extension at 72^0C for 7 min. Four micro liter of loading buffer was added to amplified products and mixed well. Ten micro liters of amplified products were loaded on 2.5% agarose gel containing ethidium bromide. The electrophoresis was done for about 4 hours at 80 volts using 1X TBE buffer to separate the amplified fragments. After electrophoresis the gel was visualized under UV trans-illumination and was photographed

Table 1. List of root-knot nematode resistant rice genotypes used in the study

Genotypes	Parentage	Reaction to root-knot nematode*	Other importantfeatures/ attributes**
Ramakrishna	IR8 /TKM6	MR	Resistant to BB
Annapurna	PTB10 / TN1	S	Dwarf grain with high yield,40-45h/ha
Tetep	Vietnam released variety	R	Resistant to blast
Tadukan	Philippines released variety	HR	Resistant to SB
TKM6	GEB24/Co 18	MR	Resistant to BB, SB, RTV and BPH
CR3003-11-186	Annapurna/ Ramakrishna	HR	-
CR3003-184	Annapurna/ Ramakrishna	HR	-
Zenith	USA landrace	HR	Moderately resistant to blast and BB

*HR-Highly resistant, R-Resistant, Moderately resistant- MR, S-Susceptible (Ref: Jena *et al.*, 2012)
** BB-Bacterial blight, SB-Stem borer, BLB-Bacterial leaf blight, RTV-Rice tungro virus, BPH-Brown plant hopper

using Gel-Doc system (Fluor Chem™ 5500, Alpha Innotech, USA). DNA fragment sizing and matching was done by scoring photographs directly. The molecular weight marker (50 bp plus DNA ladder, MBI Fermentas, Lithuania) was used to compare the molecular weights of amplified products. Amplified products were stored at -20°C until further use.

The amplified bands were scored as present (1) or absent (0) for each primer genotype combination. The data was entered into a binary matrix (0/1) and subsequently analyzed using the computer package, NTSYS-pc (Version 2.02) (Rolf, 1998). The total number of bands/alleles per marker locus, percentage of polymorphic alleles, common alleles and unique alleles were calculated to assess diversity of alleles of marker locus. The Polymorphic Information Content (PIC) value of each marker loci was calculated by $PIC = 1-\sum Pij^2$, where Pij is the frequency of i^{th} allele for the j^{th} locus and summation extends over n alleles (Anderson *et al*., 1993). Dice similarity coefficients were calculated using SIMQUAL subroutine and used to assess the genetic relationship among eight rice genotypes (Nei and Li, 1979). The resultant similarity matrix was employed to construct dendrograms using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic Means (UPGMA) to infer genetic relationships among cultivars.

In order to find the efficiency of microsatellite markers for differentiation of genotypes, the discriminating power (D) of each marker loci was calculated following formula,

$$Dj = 1-Cj = 1-\sum Pi\ (NPi-1)/(N-2),$$

where Dj is discriminating power of j^{th} locus, Pi is frequency of i^{th} allele, Cj confusion probability of jth locus [21]. Further, in order to know minimum number of marker loci required to identify and differentiate genotypes from each other, total number of non-differentiated pairs(Xj) of genotypes were calculated for the j^{th} locus using formula, $Xj = \{N(N-1)/2\}Cj$.

RESULTS AND DISCUSSION

Microsatellites are considered to be appropriate for assessment of genetic diversity, fingerprinting for variety identification and assessment of seed purity because of their ability to detect large numbers of discrete alleles accurately and efficiently. Here, we have used 47 microsatellite marker specific primers to assess genetic diversity and develop DNA fingerprint data set for rice genotypes having different degrees of resistance to RKN.

Forty-three out of forty-seven microsatellite revealed polymorphism between genotypes. A total of 104 reproducible bands/alleles were amplified, of which 97 (93.27%) were polymorphic. The number of alleles per locus ranged from two to four with an average of 2.21. Four primers RM 20, RM 281, RM 333 and RM 335 amplified highest number alleles (i.e. 4). The overall size of bands/alleles varied from 50bp (RM335) to 540bp (RM333). The size difference between the smallest and largest allele at a given microsatellite locus varied from 10bp to 390bp. The number of alleles per cultivar varied from 48 (Tadukan) to 59 (Tetep) with an average on 51.75 alleles per genotype. The amplification pattern with microsatellite primers RM224, RM231 and RM14149 is shown in figure 1. The number of alleles per microsatellite locus detected in the present study corresponded well with earlier reports (Ram *et al*., 2007; Juneja *et al*., 2006 Joshi *et al*., 2010; Behera *et al*.,2012 a,b). However, Jain *et al*. (2004) obtained higher number of alleles (3 to 22) as compared to present study, because of inclusion of *Basmati as* well as non-*Basmati* varieties and use of fluorescent techniques in their study.

The polymorphism information content (PIC) value provides an estimate of discriminating power of a marker based on the number of alleles at a locus and relative frequencies of these alleles. The PIC values for 47 SSR loci in our study varied from 0 (RM189, RM216, RM253, RM 342A) to 0.941(RM 333) with an average of 0.64 (Table 2). Thirty nine loci showed high PIC values (>0.500). Eight loci RM263, RM426, RM334, RM413, RM264, RM281, RM333, RM19, and RM 337 showed the higher PIC values of > 0.8. Polymorphic information content value depends upon the genetic diversity among the accessions. In our study, the presence of relatively higher PIC values might be due to higher genetic diversity present in selected rice genotypes. Similar to our findings higher PIC values for SSRs were also reported in the literature (Ram *et al*., 2007; Joshi *et al*., 2010; Upadhyaya *et al*., 2011, Behera *et al*., 2012a,b).

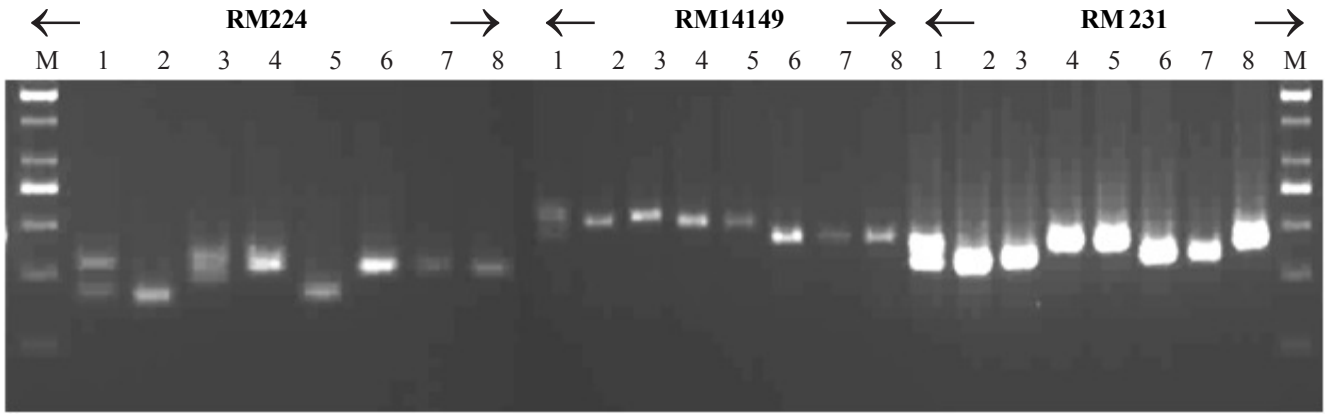

Fig. 1. **DNA amplification pattern of RKN resistant rice genotypes obtained with Microsatellite locus, RM 224, RM 14149 and RM 231**

The lane number corresponds to a rice genotype 1-Tetep,2-Tadukan,3-TKM6,4-Zenith, 5-Ramakrishna, 6-Annapurna, 7- CR 3003-184, 8-CR 3003-11-186, M-50bp DNA ladder. Microsatellite loci are shown on the top of the gel.

Genetic similarity coefficients varied from 0.367 to 0.792 with an average of 0.56, indicating a wide range of genetic variation present in rice genotypes having different degrees of resistance to BPH. Tadukan showed the highest genetic similarity with Tetep (*i.e.* 0.792) while Annapurna showed the least similarity with Tadukan (*i.e.* 0.367). CR3003-11-186 showed 0.653 genetic similarities with CR3003-184. Both CR3003-11-186 and CR3003-184 are recombinant inbreed lines (F_9) developed from the cross Ramakrishna and

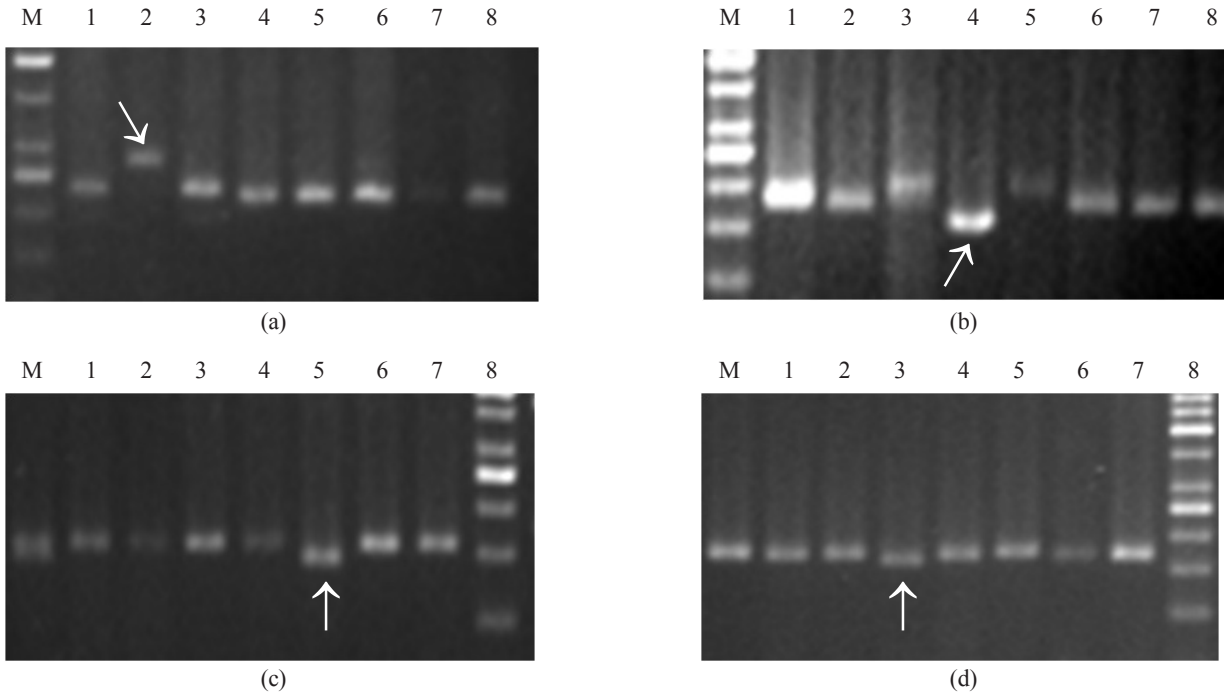

Fig. 2. **Amplification of unique alleles in rice genotypes obtained with microsatellite locus, A) RM 401, B) RM 263, C) RM495 and D) RM 530**

The lane number corresponds to a rice genotype 1-Tetep, 2-Tadukan, 3-TKM6, 4-Zenith, 5 -Ramakrishna, 6-Annapurna, 7-AR184, 8-AR-186, M-50bp DNA ladder. Arrows indicate the unique band/allele.

Table 2. Data on chromosome number, number of total alleles, number of polymorphic alleles, polymorphism information content (PIC) and discriminating power obtained using 47 microsatellite primers in 8 rice genotypes

Microsatellite locus (RM)	Chromosome #	TA	PA	Size of allele (bp)	Size difference(bp)	PIC	DP
RM 212	1	2	2	120-150	30	0.687	0.714
RM 488	1	2	2	180-210	30	0.773	0.804
RM 495	1	2	2	140-160	20	0.609	0.625
RM 10022	1	2	2	180-360	180	0.585	0.607
RM 233A	2	3	3	140-160	20	0.692	0.726
RM 263	2	3	3	150-200	50	0.843	0.869
RM475	2	2	2	200-210	10	0.770	0.804
RM530	2	2	2	160-170	10	0.609	0.625
RM14149	2	2	2	190-200	10	0.73	0.768
RM218	3	2	2	140-160	20	0.73	0.768
RM 231	3	2	2	180-190	10	0.67	0.714
RM232	3	2	2	150-160	10	0.64	0.679
RM 251	3	3	3	140-450	10	0.802	0.833
RM426	3	2	2	220-250	30	0.859	0.893
RM 570	3	2	1	240-260	20	0.49	0.5
RM335	4	4	4	50-160	110	0.671	0.705
RM 401	4	2	2	240-290	50	0.6	0.625
RM470	4	2	1	170-200	30	0.42	0.446
RM 169	5	2	2	180-210	30	0.546	0.571
RM 334	5	3	3	170-200	30	0.864	0.893
RM 413	5	3	3	80-110	30	0.88	0.917
RM 469	5	2	2	100-110	10	0.71	0.732
RM 225	6	2	2	140-150	10	0.73	0.768
RM 253	6	1	0	140	-	0	0
RM 11	7	2	2	140-160	20	0.73	0.768
RM18	7	2	2	150-160	10	0.73	0.768
RM234	7	2	2	150-160	10	0.73	0.768
RM 560	7	2	2	240-270	30	0.77	0.804
RM264	8	2	2	150-170	20	0.85	0.893
RM342A	8	1	0	140	-	0	0
RM 281	8	4	4	140-170	30	0.835	0.866
RM433	8	2	2	220-230	10	0.75	0.786
RM 8271	8	2	2	170-230	60	0.734	0.768
RM285	9	2	2	210-260	50	0.54	0.571
RM 189	9	1	0	400	-	0	0
RM333	10	4	4	150-540	390	0.941	0.964
RM 330	10	2	1	200-240	40	0.429	0.446
RM269	10	2	2	190-300	110	0.335	0.357
RM 216	10	1	0	150	-	0	0
RM 21	11	2	2	140-160	20	0.69	0.679
RM 144	11	2	2	230-240	10	0.71	0.732
RM202	11	2	2	170-190	20	0.67	0.714
RM224	11	2	2	140-150	10	0.64	0.679
RM17	12	2	2	180-200	20	0.67	0.714
RM 19	12	3	3	230-250	20	0.84	0.869
RM 20	12	4	4	240-300	60	0.74	0.768

TA = Total number of alleles, PA = Polymorphic alleles, PIC= polymorphism information content, DP= Discriminating power

Annapurna and highly tolerant to RKN. Similar to our observations, other studies using SSR markers revealed varying degrees of genetic similarity among the accessions of cultivated and wild species of rice (Juneja *et al*., 2006; Jayamani *et al*., 2007; Joshi *et al*.; Behera *et al*., 2012a, b).

UPGMA cluster analysis based on genetic similarity values provided a clear resolution of relationships among all the 8 rice genotypes. Two major clusters were observed at 49% of genetic similarity coefficient (Fig. 3). First major cluster contained six rice genotypes with an average similarity index of 0.616 and further sub-grouped into three sub-clusters, IA, IB and IC. Two genotypes Tetep and Tadukan, were included in IA while Ramakrishna and its derivatives CR3003-11-186 and CR3003-184 were included in IB. TKM6 was included in IC. Zenith and Annapurna were included in the second major group II (Table 3).

Earlier, we have considered the number of eggs produced after completion of second life cycle by the root-knot nematode as a parameter to classify cultivars as resistant/ susceptible (Nanda *et al*., 2011). Annapurna showed susceptible reaction while other genotypes Ramakrishna, Tetep, Zenith, TKM6, CR3003-11-186, CR3003-184 and Tadukan showed

Table 3. Unique alleles amplified by microsatellite loci in different rice genotypes

Microsatellite Locus	Chromosome #	Size of unique allele (bp)	Genotype
RM495	1	140	Annapurna
RM263	2	150	Zenith
RM530	2	160	Zenith
RM570	3	240	Tetep
RM401	4	290	Tadukan
RM334	5	170	Tetep
RM469	5	110	Annapurna
RM281	8	160	TKM6
RM333	10	530	Annapurna
RM333	10	540	Zenith
RM144	11	240	Ramakrishna
RM19	12	230	Zenith

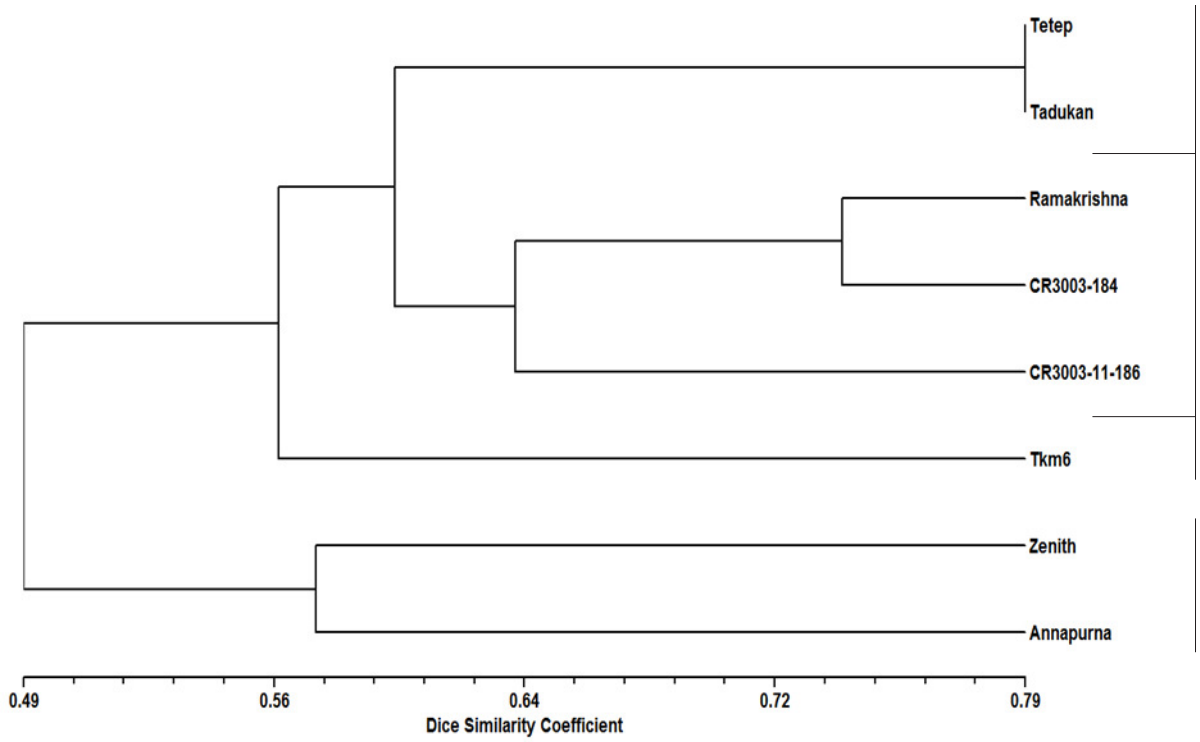

Fig. 3. Dendrogram showing genetic relationships between rice genotypes differing in resistance to root-knot nematode

Scale on the bottom indicates the Dice Similarity Coefficient. The major clusters and sub-clusters are indicated on right margin.

different degrees of resistance to root-knot nematode (Table 1). Cultivars Tetep and Tadukan showed resistant and highly resistant reaction to nematode population of CRRI farm, respectively. In Philippines, many high yielding blast resistant and hybrid varieties have been developed s using these two varieties (Shu-Huang *et al*., 1975). CR3003-11-186 and CR3003-184 breeding lines showed a good amount of diversity at molecular level even if they have been developed from same parents Annapurna and Ramakrishna. These lines showed significantly fewer galls and females on the roots than all other varieties (11.7 and 16.8 % of the number of galls on the susceptible *cv*. Annapurna, respectively), thus confirming their resistance to the nematode (Jena *et al*., 2012). These lines can be used as good source of resistance to nematode in breeding programs after confirming their other quality attributes. We could observe clear genetic diversity among 8 genotypes and different intensities of reaction pattern to the pest root-knot nematode in spite of using a limited number of genotypes. Based on the above clustering pattern, breeding experiments may be initiated between diverse cultivars with resistance to RKN for getting more genetic variation, transgressive segregants and with genes/QTLs of diverse mechanisms of resistance to RKN.

Twelve genotype specific (unique) alleles were identified in six genotypes, Annapurna, Zenith, Tetep, Tadukan, TKM6, and Ramakrishna (Table 3). RM 333 amplified two unique alleles of 530bp in Annapurna and 540bp in Zenith. Four unique alleles were amplified in Zenith and three in Annapurna and two in Tetep, while Tadukan, TKM6 and Ramakrishna each amplified one unique alleles (Fig. 3). These unique alleles would be useful for developing molecular tags. However, the relationship of these alleles with RKN resistance has to be assessed using mapping populations. Behera *et al*. (2012b) identified seven unique alleles among BPH resistant cultivars using microsatellite markers. Similarly, others also detected unique alleles both in cultivated and wild rices (Saini *et al*., 2004; Kaushik *et al*., 2011; Upadhyay *et al*., 2011; Behera *et al*., 2012a, b).

All the 8 rice genotypes used in the present study could be distinguished precisely from each other. None of the microsatellite locus could differentiate all the 8 genotypes. The discriminating power of microsatellite loci varied from 0 (RM189, RM216, RM253, RM 342A) to 0.964(RM333) (Table 2). On the basis of discriminating power, the minimum number of microsatellite loci required to differentiate between genotypes in the present study was found to be three (*i.e.* RM333, RM334 and RM 413). These three SSR loci amplified a total of 10 alleles, all being polymorphic. The frequency of these alleles ranged from 1/8 to 4/8. Similar results were obtained by others (Olufowote *et al*., 1997; Rahman *et al*., 2009, Behera *et al.,* 2012 a, b). Behera *et al*. (2012b) were able to discriminate 19 BPH resistant rice cultivars using only four microsatellite markers. The combination of all the polymorphic and non-polymorphic alleles obtained with all the 47 microsatellite marker loci enabled development of DNA fingerprint/ profile data set for 8 rice genotypes differing in resistance to RKN (data not shown), which would be very useful for future reference. The probability of identical match was found to be 1.8 X 10^{-2} based on the DNA fingerprints/ profiles generated by the three most discriminating microsatellite loci (*i.e.* RM333, RM334 and RM 413), suggesting that 55 rice cultivars can be distinguished by using these three loci. Inclusion of all the 47 microsatellite loci provided a very high resolution power enabling nearly 10.75X10^{12} cultivars to be precisely identified (Table 5). Several workers have been demonstrated the utility and power of microsatellite markers in establishing distinction of rice varieties (Santhy *et al*., 2000; Behera *et al.,* 2012a, b).

Table 4. Clustering pattern of root-knot nematode resistant rice genotypes

Major clusters	Sub clusters	Genotypes*
I	IA	Tetep (R), Tadukan (HR)
	IB	Ramakrishna (MR), CR 3003-184 (HR), CR 3003-11-186 (HR)
	IC	TKM6 (MR)
II	-	Zenith (HR), Annapurna (HS)

* Parenthesis shows the reactions of genotypes to root-knot nematode, R= Resistant, HR- Highly resistant, MR- Moderately resistant, HR-Highly susceptible

The present study indicated that microsatellite markers are efficient in identification and assessment of genetic diversity present in rice genotypes having

Table 5. Analysis of DNA fingerprints using different sets of microsatellite loci

Items	3 microsatellite loci	47 microsatellite loci
Average no. of alleles for each variety(n) + SD	2.88 + 0.35	51.75+ 4.77
Average similarity ($\overline{X}_D$)+SD	0.25 + 0.20	0.56 +0.09
Probability of identical match by chance $(\overline{X}_D)^n$	1.8 X 10^{-2}	9.3 X 10^{-14}

different degrees of resistance to RKN. A basic molecular data set was created for these rice genotypes which can be used for variety registration and protection of the plant breeders as well as farmers' rights. The genotypes included in our study are known to possess desirable characters like high resistance to root-knot nematode, high yield potential, abiotic stress tolerance, and multiple resistance genes. Evaluation of genetic diversity and their host reaction towards pathogen gives clear information, so these varieties qualify as suitable parental choice for varietal development in rice.

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