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Suitability of RAPD and ISSR to complement agro-morphological DUS descriptors for establishing distinctiveness in indigenous local strains of Kalanamak rice (*Oryza sativa*)

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ABSTRACT

Fortyfour indigenous local strains of Kalanamak rice (*Oryza sativa* L.) were characterized with 60 morphological DUS descriptors, RAPD and ISSR markers. On the basis of grouping characteristics unique morphological profiles were obtained for three Kalanamak strains, viz 3125 SN, 3122 SN and 3319 SN and rest of the 41 Kalanamak strains remained in five groups. The RAPD marker analysis was able to discriminate 33 (72%) kalanamak strains. Two out of a total of 15 random primers were able to establish unique molecular identification profiles (MIPs) for Kalanamak strains, viz 3126-P and 3129-SN. The UPGMA cluster analysis reveled that the ISSR loci enabled identification of 42 strains (95%). Two ISSR primers, viz LC 61 and LC 67 produced genotype specific loci in Kalanamak strains 3131-1P and 3119-SN which were able to discriminate them from rest of the strains. Higher number of average bands (8.2), number of average polymorphic bands (6.6), percentage of polymorphic bands (78.9%), average polymorphic information content (0.33), average resolving power (10.85), average effective multiplex ratio (5.6) and marker index (2.08) for ISSR marker as compared to RAPD reflected that ISSR marker is more efficient tool to establish distinctiveness amongst the present set of experimental material.

Key words: Indigenous local strains, Kalanamak rice, Molecular markers, Morphological descriptors

In India, among small and medium grain aromatic rice (*Oryza sativa* L.) , Kalanamak is one of the finest quality rice varietiy. Till a decade ago Kalanamak was popular in Himalayan *tarai* adjoining Nepal, particularly in districts Siddharthanagar, Sant Kabir Nagar, Gorakhpur, Maharajganj, Gonda and Basti of Uttar Pradesh and in small pockets at West Champaran in Bihar (Singh *et al.* 2005).

Since the fragrant indigenous rices are in great demand in the global market, it is extremely necessary to establish their identity and distinctiveness by various approaches. As per the DUS guidelines only morpho-physiological descriptors are used for establishing distinctiveness of the varieties (Anonymous 2007). However, serious problem may arise for establishing distinctiveness of varieties only on the basis of morpho-physiological descriptors as the number of candidate varieties are increasing with the decreased variability as well as expansion of reference collections (McGregor *et al*. 2000).

The working group on Biochemical and Molecular Techniques (BMT) of the UPOV has in fact identified isozyme protein markers that could be used as complementary descriptors in wheat (anonymous 1994a), barley (anonymous 1994b) and soybean (anonymous 1998). But results obtained from these may also be biased by the general consideration that only a minor portion of the genome is represented by these markers.

In line with the popularity and proven utility of molecular markers there is a clear tendency to introduce and use them as characters for distinctiveness (Law *et al*. 1998). The speed of analysis, independence from the growth stage, season, location and agronomic practices, give them a high value.

The objective of this study was to compare the morphological approach, currently in use with a molecular approach, based on RAPD and ISSR markers in view of its possible application as an analytical tool to supplement the current procedure for the assessment of distinctiveness in

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indigenous local strains of Kalanamak rice.

MATERIALS AND METHODS

A total of 44 indigenous local strains of Kalanamak rice (Table 1) were studied for 60 morpho-physiological descriptors as notified by Plant Variety Protection and Farmers Right (PPV & FR) Authority (Anonymous 2007). The experiments were conducted during the two rainy seasons of 2008 and 2009 in randomized complete block design with three replications. Each replication consists of three rows of 6m length with 30 cm × 20 cm spacing. Among the 60 morphological descriptors studied 46 were visually assessed and 14 were measurable characters. Data were recorded on 10 randomly selected plants from each replication. Grouping of strains was done by the eight grouping characters mentioned in the DUS test guidelines.

Table 1 Kalanamak rice strains used in the study

Strain	Strain
3114-SN	3130-SN
3114-2-P	3130-P
3117-SN	3131-SN
3119-SN	3131-1-P
3119-P	3131-2-SN
3119-1-SN	3131-2-P
3119-2-P	3212-P
3119-2-SN	3213-SN
3120-SN	3214-SN
3120-1-P	3215-P
3120-2-SN	3216-SN
3120-2-P	3216-1-P
3121-P	3219-P
3122-SN	3219-SN
3122-P	3222-SN
3124-P	3224-P
3124-SN	3229-SN
3125-SN	3256-P
3126-P	3256-1-SN
3128-P	3257-P
3128-SN	3319-SN
3129-SN	3322-P

The genomic DNA was extracted from 14-day-old etiolated seedlings .The quantification of DNA in RNA-free sample was done using a UV visible spectrophotometer.

PCR reactions were performed in 25 µl volume containing 10 X Assay Buffer, 0.5 unit of *Taq* DNA polymerase, 200 µm each of dNTPs, 50 ng/µl reaction of random primers and 50 ng of template DNA. Initially 70 random primers were used in the study. After preliminary testing on a few samples, 15 primers that gave clear and reproducible banding patterns were selected for the final study. Each PCR reaction was repeated twice to confirm the reproducibility.

PCR was performed in 'Eppendorf Thermocycler' by initial denaturation at 94^0C for 5 min., followed by 43 cycles of denaturation at 94^0C for one min, annealing at 39^0C for one min, extension at 72^0C for 2 min. and final elongation at 72^0C for 7 min. The PCR products were electrophoresed on 1.5% agarose gel, prepared in 1X TBE buffer containing 0.5 µg/ml of the ethidium bromide at 80V for 3hr with cooling. The gel was photographed under UV transilluminator.

ISSR amplification reactions were carried out in 25 µl volume containing 50ng template DNA, 0.5 U *Taq* DNA polymerase, 10 mM dNTP mix, 50 ng/µl primer in 1X reaction buffer is containing 10 mM Tris.HCl (*p*H 8.0), 50 mM NaCl and 2.5 mM $MgCl_2$. Initially a combination of 50 anchored and non-anchored primers were used in the study. Out of the 50 chosen primers, 13 were finally selected for further distinctiveness analysis based on clarity, scorability and reproducibility of banding patterns. Each PCR reaction was repeated twice to confirm the reproducibility.

Amplification was performed in an 'Eppendorf Thermocycler' by initial denaturation at 94^0C for 4 min., followed by 45 cycles of denaturation at 94^0C, annealing temperature was maintained at 52^0C for 1 min. extension at 72^0C for 2 min. and final elongation at 72^0C for 5 min. The amplification products were loaded on 2% agarose gel and separated in 1X TBE buffer at 75 V. The gels were visualized under UV after staining with ethidium bromide and documented using a gel documentation and image analysis system.

The standardized data matrix of measurable descriptors was used to generate dissimilarity indices based on Euclidean distances NTSYS-pc package (Rohlf 2005).

Molecular marker data were recorded from gels upon band size classification and expressed in binary code. Cluster analysis was conducted on similarity estimates, by using unweighted pair group method on arithmetic averages (UPGMA). The comparison of similarity/dissimilarity matrices of different markers was made by MAXCOMP module of NTSYSpc. Polymorphic information content (PIC) was computed for each marker from the formula:

$$PIC_i = 1 - \Sigma P_{ij}^2$$

where P_{ij} is the frequency of i^{th} allele in the j^{th} primer.

Different marker attributes like effective multiplex ratio (EMR), marker index (MI) and resolving power (Rp) were calculated to assess the discriminatory power or informativeness of the marker systems utilized according to the Prevost and Wilkinson (1999).

RESULTS AND DISCUSSION

Out of total 46 visually assessed DUS descriptors studied, 45 were found to be monomorphic and one character was dimorphic and none of the character was found to be polymorphic. This low polymorphism level of visually assessed characters among the Kalanamak strains might be attributed to the fact that all the local strains were domesticated

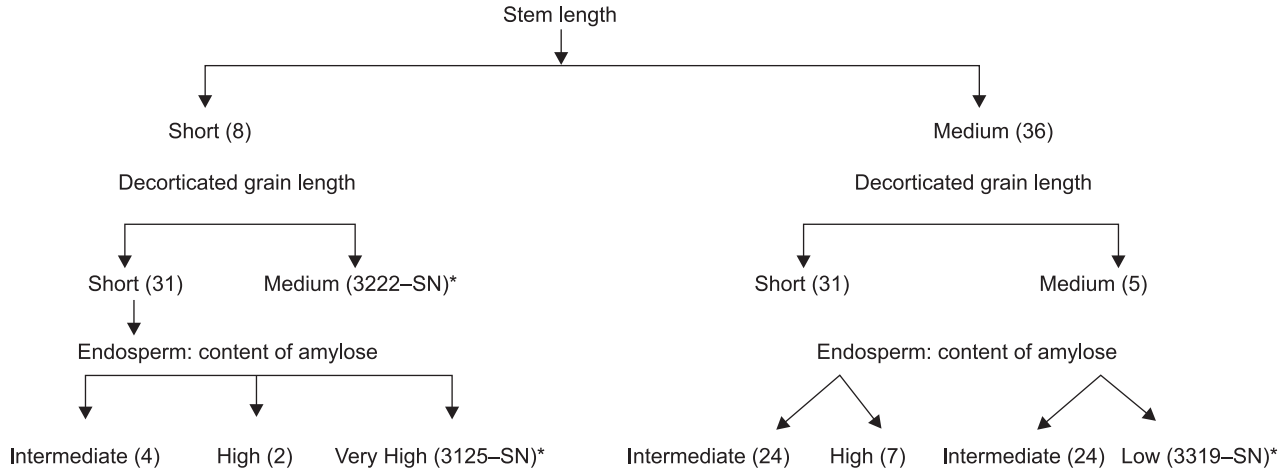

Fig 1 Dendrogram depicting the classification of 44 Kalanamak strains on the basis of grouping characteristics. Asterisk sign indicates the unique morphological profiles obtained for three Kalanamak strains.

to their respective agro-ecological zones with a similar and narrow genetic base. Out of eight grouping characters five characteristics, viz basal leaf: sheath colour, time of heading (50% of plants with panicles), decorticated grain: shape (in lateral view), decorticated grain: colour and decorticated grain: aroma, were monomorphic among the strains. Thus, grouping of varieties was based on three characters, viz stem length, decorticated grain length and endosperm content of amylose. On the basis of grouping characteristics unique morphological profiles were obtained for three Kalanamak strains, viz 3125 SN, 3222 SN and 3319 SN (Fig 1).

Morphological characters have been predominant tools used by breeders for distinguishing lines or cultivars in public domain. Characterization of genotypes using important characters has its own merits, since it provides relevant information about important agronomic characters in genotypes. However, Patra *et al*. (2010) have established that methods that relay on simple combination of morphological traits are neither consistent nor effective in unambiguous differentiation of farmer's varieties and elite breeding materials. Hence, molecular markers were considered for establishing the distinctiveness of Kalanamak rice strains under the study.

RAPD analysis

RAPD marker analysis conducted on DNA extracted from each of the 44 strains using 15 oligonucleotide primers. The total number of bands (TNB), number of polymorphic

Table 2 Details of RAPD primers used for the molecular characterization of 44 Kalanamak rice strains

Primer code	Primer sequence (5' to 3')	TNB	NPB	P%	PIC	(Rp)	NEB	EMR	MI
LC 71	TGCCGAGCTG	5	3	60	0.24	7.64		1.8	0.43
LC 73	AATCGGGCTG	9	7	77.7	0.23	13.96		5.5	1.23
LC 77	GGGTAACGCC	9	6	66.6	0.64	6.64		4	2.53
LC 78	GTGATCGCAG	10	7	70	0.24	15.28		4.9	1.16
LC 89	AGTCAGCCAC	10	7	70	0.04	3.45		4.9	0.23
LC 90	GTGAGGCGTC	8	8	100	0.12	14.09		8	0.96
LC 93	GGACCCAACC	10	6	60	0.03	6.44		3.6	0.14
LC 95	TGAGCGGACA	9	7	77.7	0.35	11.78	1	5.5	1.89
LC 96	TTGGCACGGG	5	4	80	0.40	6.00	1	3.2	1.28
LC 97	GTGTGCCCCA	8	6	75	0.27	11.78		4.5	1.19
LC 101	GGGGTGACGA	6	5	83.3	0.08	11.00		4.2	0.35
LC 102	CATCCGTGCT	5	5	100	0.45	5.59		5	2.21
LC 106	GTGACATGCC	6	4	66.6	0.19	9.64		2.7	0.53
LC 109	ACGCACAACC	6	4	66.6	0.35	7.90		2.7	0.9
LC 118	TCAGAGCGCC	5	2	40	0.10	7.19		0.8	0.08
Total		118	81						
Average		7.37	5.06	68.34	0.23	9.23		5.57	1.00

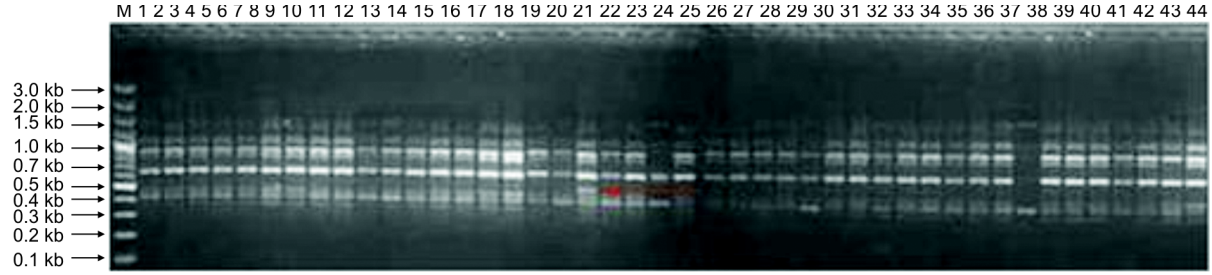

Fig 2 Molecular diversity generated among the Kalanamak rice strains by RAPD primer LC-95. Aroww indicates the genotype-specific band of approximately 500 bp amplified in strain 3126-P. The numbers corresponds to ones listed in Table 1.

bands (NPB), percentage of polymorphic bands (P%), polymorphic information content (PIC), Resolving power (Rp), number of exclusive bands (NEB), effective multiplex ratio (EMR) and marker index (MI) obtained from each primer are shown in Table 2.

All the selected markers amplified 118 RAPD loci (average of 7.37 bands/marker) across the 44 genotypes studied, out of which 81 loci were polymorphic. Two markers, viz LC 90 and LC 102 produced 100% polymorphism, which together accounted for moderate (68.34%) level of polymorphism. One of the most important applications of molecular fingerprinting studies is to identify a marker which can differentiate a genotype from the remaining genotypes but this is difficult to achieve in a closely related set of genotypes. Interestingly, two of the Kalanamak strains, viz 3 126-P and 3 129-SN could be discriminated by RAPD markers LC 95 (Fig 2) and LC 96 respectively. These unique molecular fingerprints generated in different varieties could be incorporated as additional or complementary descriptors to the standard morphological DUS descriptors for their registration and protection.

Marker index (MI) is the marker attribute used to calculate the overall utility of a marker system and is the product of PIC and effective multiplex ratio. The MI ranged from 0.08 (LC 118) to 2.53 (LC 77) with an average of 1.0/ primer. The PIC ranged from 0.03 (LC 93) to 0.64 (LC 77) with an average of 0.23/primer.

The probability of identical match by chance was found to be 3.2×10^{-5} based on these 15random primers. However, in a previous study on rice conducted by Ray Choudhary *et al* (2001) probability of identical match by chance was found to be 1.6×10^{-41}among the 48 aromatic rice varieties. This could be due to the higher number of RAPD markers used in the study (58 markers) as compared to the present study (15 markers).

Prevost and Wilkinson (1999) reported the Rp as the capacity of a given primer to discriminate among different genotypes. RAPD primers, viz LC 78, LC 90 and LC 73 having high resolving power of 15.28, 14.09 and 13.96 respectively were able to discriminate majority of the varieties.

The dendrogram based on UPGMA analysis grouped the 44 Kalanamak strains into two major clusters. It was interesting to note that by an analysis of RAPD loci, discrimination of 33 Kalanamak strains (75%) was achieved. The non-discriminated strains with 100 % genetic similarity were 3114-SN to 3114-2-P, 3119-1-SN to 3119-2-P, 3119-2-SN, 3122-SN to 3122 P, 3131-SN to 3131-2-P and 3131-1-P to 3131-2- SN and formed five clusters (Fig 3). Thus the 11 strains displaying a similarity value of 1.0 to at least one another accession, indicated that they are possibily the selections made by farmers from the single landrace. Consequently, the RAPD marker, providing fast varietal fingerprinting is limited in precise discrimination of closely related strains.

ISSR analysis

The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), polymorphic information content (PIC), resolving power (Rp), number of exclusive bands (NEB), effective multiplex ratio (EMR) and marker index (MI) obtained from each primer are shown in Table 3.

The PCR amplification using a combination of 13 anchored and non-anchored dinucleotide repeat primers gave rise to reproducible amplification products. The 13 primers on an average produced 107 bands across 44 strains of which 86 were polymorphic, accounting for a high level (78.9 %) polymorphism. Four primers, viz LC 49, LC 59, LC 62 and LC 67 revealed 100% polymorphism showing their ability to discriminate among the varieties (Table 3). The high level of polymorphism produced by ISSR as compared to RAPD is not unexpected because the ISSR technique amplifies at least two microsatellite regions (regarded as highly polymorphic) as well as unique regions in between.

The PIC value ranged from 0.15 for primer LC 47 and LC 69 to 0.68 for primer LC 67. Marker index ranged from 0.24 to 6.0 for primer LC 46 and LC 47 respectively with an average of 2.08 per primer. The Rp of ISSR primers ranged from 5.87 for primer LC 67 to 15.40 for primer LC 47 with an average of 10.85 for all the primers. Furthermore, three primers, viz LC 47, LC 66 and LC 49 having resolving power of 15.40, 13.09 and 12.87 respectively were able to distinguish most of the 44 Kalanamak rice strains under study. A strong and linear relationship between the ability of

a primer combination to distinguish accessions and Rp was observed in previous studies (Powell *et al*.1996).

Although a positive correlation was observed between MI and PIC value ($r^2 = 0.79$; $P < 0.005$), therefore confirming results of Prevost and Wilkinson (1999), but no specific relation was observed among the PIC, EMR and RP. This indicates that probably a single parameter is not a good indicator to assess the informativeness of a particular marker system.

The UPGMA cluster analysis reveled that the ISSR loci

Table 3 Details of ISSR primers used for the molecular characterization of 44 Kalanamak rice genotypes

Primer code	Primer sequence (5' to 3')	TNB	NPB	P%	PIC	(Rp)	NEB	EMR	MI
LC 46	AGAGAGAGAGAGAGAGT	6	3	50	0.16	10.09		1.5	0.24
LC 47	AGAGAGAGAGAGAGAGC	9	7	77.7	0.15	15.40		5.5	0.79
LC 49	GAGAGAGAGAGAGAGAT	8	8	100	0.19	12.87		8	1.6
LC 52	CTCTCTCTCTCTCTCTG	10	8	80	0.49	10.23		6.4	3.2
LC 59	GAGAGAGAGAGAGAGAYT	8	8	100	0.28	11.55		8	2.3
LC 61	ACACACACACACACACYG	7	6	85.7	0.18	9.87	1	5.2	0.9
LC 62	HBHAGAGAGAGAGAGAG	9	9	100	0.49	9.14		9	4.5
LC 63	BHBGAGAGAGAGAGAGA	7	3	42.8	0.22	10.96		1.3	0.28
LC 64	VHVGTGTGTGTGTGTGT	9	7	77.7	0.49	10.95		5.5	2.7
LC 65	HVHTGTGTGTGTGTGTG	9	7	77.7	0.28	9.32		5.5	1.5
LC 66	CATGGTGTTGGTCATTGTTCCA	9	7	77.7	0.48	13.09		5.5	2.7
LC 67	ATGATGATGATGATGATG	9	9	100	0.68	5.87	1	9	6
LC 69	GGGTGGGGTGGGGTG	7	4	57.1	0.15	11.69		2.3	0.35
Total	107	86							
Average	8.2	6.6	78.9	0.33	10.85		5.6	2.08	

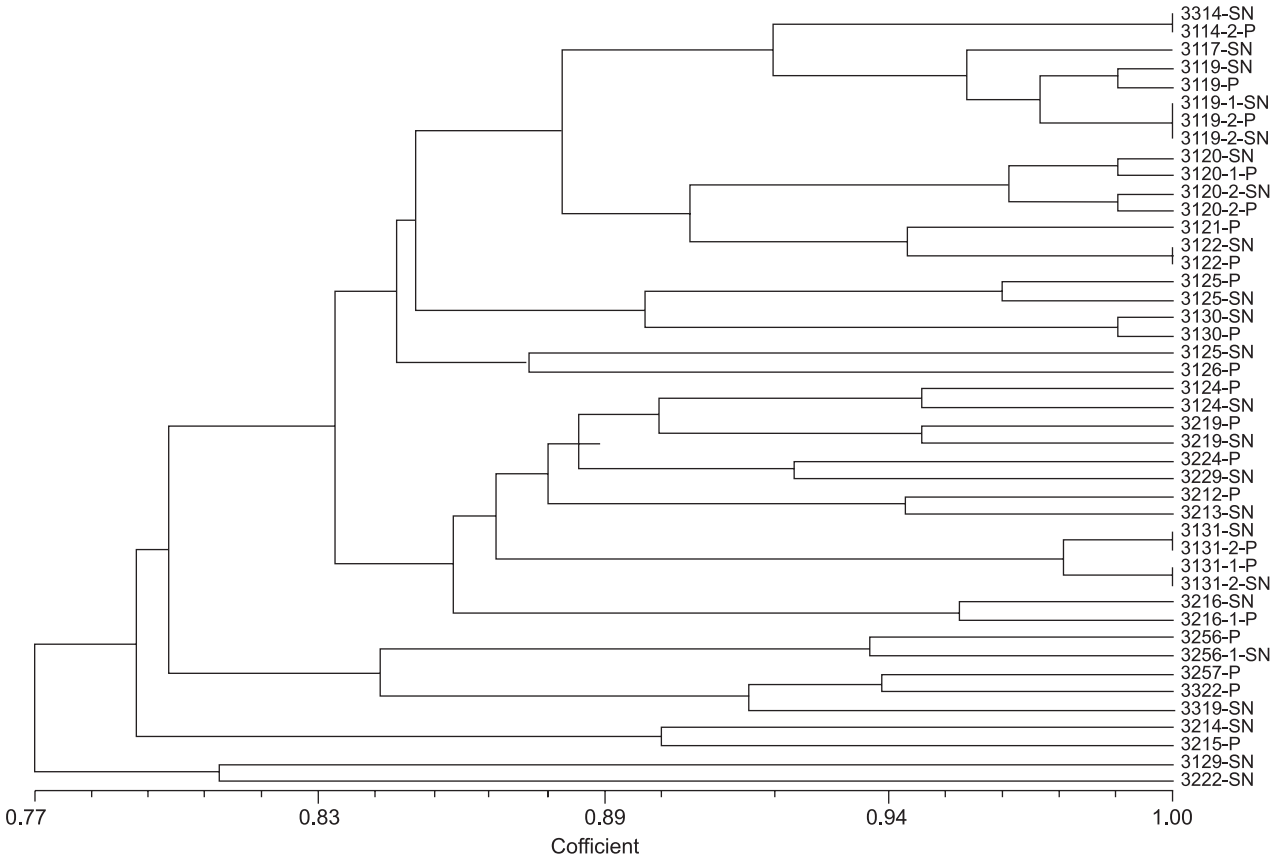

Fig 3 Dendrogram depicting the classification of 44 Kalanamak rice strains constructed through UPGMA method and based RAPD marker. The scale at the bottom is Jaccard's cofficient of genetic similarity

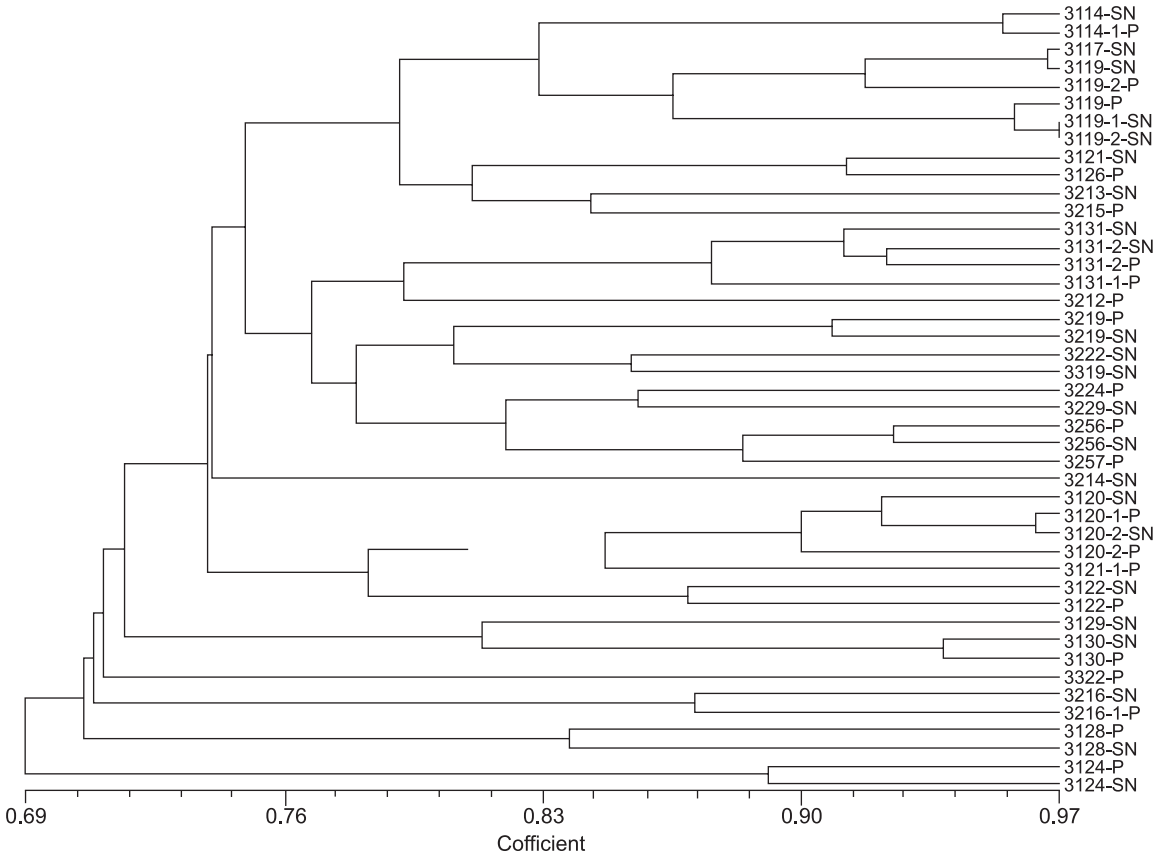

Fig 4 Dendrogram depicting the classification of 44 Kalanamak rice strains constructed through UPGMA method and based ISSR marker. The scale at the bottom is Jaccard's cofficient of genetic similarity

enabled discrimination of 42 strains (95%). The non-resolved strains with 100% genetic similarities were 3119-1SN and 3119-2 SN (Fig 4). Two ISSR primers, viz LC 61 and LC 67 produced genotype specific loci in Kalanamak strains 3131-1P (Fig 5) and 3119-SN, which were able to discriminate them from rest of the strains. These genotype-specific bands could be used as additional descriptors for plant variety protection and can also be converted into STS markers of great value to detect any mix up between the cultivars and as DNA fingerprints.

Probability of identical match by chance is an important calculation when the purpose of fingerprinting is to identify genotypes for checking identity and purity of a cultivar. The low probability (2.1×10^{-11}) of identical match by chance estimated from ISSR profiles as compared to RAPD markers (3.2×10^{-6}) further revealed its high resolving power. These results support our view that the identified informative set of ISSR markers would be more useful in establishing distinctiveness of kalanamak strains and provided unique identity to most of the strains under the study.

Exploiting the current molecular marker technologies in Kalanamak rice strains it was possible to develop a DNA fingerprinting system, which can effectively complement the widely used morpho- physiological DUS descriptors.

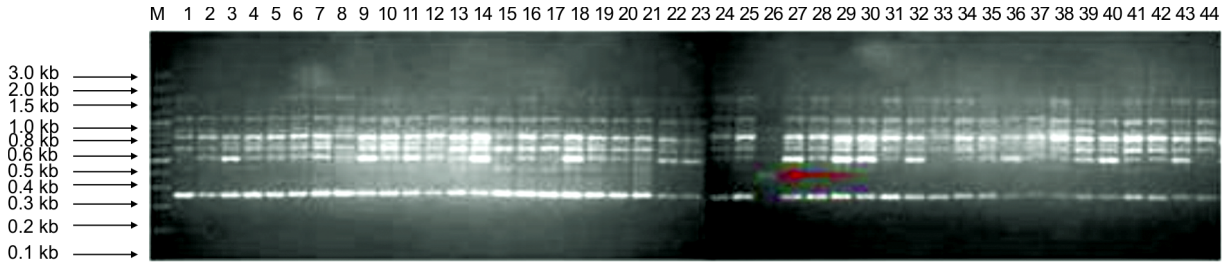

Fig 5 Molecular diversity generated among the Kalanamak rice strains by ISSR primer LC-61. The arrow indicates the genotype-specific band of approximately 400 bp amplified in strain 3131-1-P. The numbers corresponds to ones listed in Table 1

This would be equally applicable for conservation and protection of indigenous local strains (genetic resources) when it is important to know whether two strains phenotypically similar display a similar gene combination.

Results of this study provide sufficient evidence that molecular markers would increase the standards of DUS testing if they are included as additional descriptors. Besides, their introduction could offer several advantages. Further advantage of molecular markers is their relatively higher discrimination power generated by more balanced distribution of allele frequencies. This could indicate that erosion of variability introduced through continuous selection pressure is expressed with higher intensity on morphological than on molecular level. These attributes which have been tested and confirmed, call for the consideration that molecular markers are ideal additional descriptors for establishing distinctiveness of these indigenous local Kalanamak strains.

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