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Research Article

Genetic Diversity Assessment of 'Njavara' Rice Germplasm Using ISSR based Molecular Markers

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Abstract

'Njavara' is an endemic rice variety of Kerala which is widely used in Ayurvedic treatments. Njavara is highly popular due to its wide applications and is considered to possess unadultered gene pool. Inter simple sequence repeat (ISSR) polymorphism was used to determine the genetic diversity and phylogenetic relationship among 24 'Njavara' cultivars collected from different parts of Kerala and 6 out groups (Improved rice varieties). Seven ISSR primers representing di-nucleotide repeats were selected for amplification which produced a total of 45 banding patterns of which 33 were polymorphic. All the markers displayed polymorphic amplicons. The number of amplicons produced by each primer varied from 5 to 7. The percentage of polymorphism varied with each primer ranging from 57.1% to 100% (100% polymorphism was shown by the primer UBC 840). The UPGMA based clustering analysis grouped 'Njavara' accessions in a major node with four sub clusters and the out groups in a minor node. The molecular screening ISSR markers showed low polymorphism (73.3%) between the samples analyzed. The findings from the present study indicates that ISSR fingerprints are highly efficient in the detection of genetic diversity between the 'Njavara' cultivars. The study might be a significant framework for the genetic structure of 'Njavara' which could be considered as a basis for future research initiatives in 'Njavara'. Furthermore, the data we submitted during the study will help this medicinal rice landrace to be better known to the world of rice research to explore out its various genetic attributes for rice improvement.

Keywords

'Njavara' rice, ISSR, Genetic Diversity, Medicinal rice, Polymerase Chain Reaction.

Declaration of Conflicting Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Rice is one among the most significant food crops in the world consumed as a basic food by half of the world's population which provides about 50 to 80% of their daily calories. Apart from using rice as a staple food, some varieties of the rice are aromatic and few are medicinal especially used in

the Ayurvedic system of medicine. As one of the primary centres of origin of Oryza sativa, India has rich and diverse genetic wealth of rice. In India, the Malabar Coast of Kerala region has been considered as one of the hot centres of rich genetic diversity in rice germplasm. Even though the cultivable area in the highly populated state of Kerala is diminishing at an alarming rate, the state harbours rich diversity in cultivars, release varieties and traditional landraces of O. sativa. 'Njavara' rice also coined as 'the rice that cures' and its medicinal property has been explained by various authors such as Charaka, Susruta, Kautilya, Varahamihira, and Panini in their respective ancient compilations. Rice has got more attention in similar treaties than any other cereal such as wheat (Nene, 2005). In the array of land races and improved varieties of rice, 'Njavara' variety has a special status, it being predominantly medicinal rice with equal importance in food and curative purposes. This rice variety is endemic to Kerala where it is regarded to be originated. Considering its importance, the rice landrace 'Njavara' was given Geographical Indication (GI) Registry of Intellectual Property Rights under the Geographical Indication of Goods (Registration and Protection Act, 1999). This was the first time that a rice variety of Kerala received Geographical Indication Registry. It was noted that 'Njavara' has been under cultivation in Kerala for about 2500 years. According to the Indian indigenous system of medicine or Ayurveda, it is regarded as a special rice variety having beneficial properties for the circulatory, respiratory, digestive and nervous systems. Two major treatments in Ayurveda for arthritis, paralysis, neurological disorders, degeneration of muscles and tuberculosis include the Njavarakizhi and Njavaratheppu. In addition to various medicinal properties, 'Njavara' gruel is also included in the diet for developing immunity and is also considered a safe food for diabetics. This indigenous race of rice exist in two forms, black and golden yellow glumed. Among that black glumed 'Njavara' rice seems to be healthier than the golden one but is more prone to lodging and drought.

In order to exploit the plant genetic resources in depth knowledge on the rate of genetic variation exists within the species is a mandate (Vaughan, 1994). In order to develop elite varieties bearing desirable characteristics genetic diversity study is considered as the basic requirement. Molecular markers offers consistent analysis of variation in the genetic information bearing the different individuals which are significant to detect and explore the genetic variability for crop improvement programmes. To assess the genetic variation within the population, molecular markers act as a valuable tool which gives information on both varietal classification, and germplasm identification of rice (Virk et al., 1995; Masataka et al., 2003; Singh et al., 2006; Rabbani et al., 2008). Among the widely used markers, inter simple sequence repeat (ISSR) marker which is a PCR based molecular marker in which a DNA region situated between two similar microsatellite motifs aligned in opposite directions got amplified. ISSR marker based polymorphism has been widely accepted all over the world to designate genetic variations in plant species. Because they exhibit specificity of sequence-tagged-site markers, without prior sequence information for the synthesis utilizing the advantage of random markers (Zietkiewicz et al., 1994; Goodwin et al., 1997). The ISSR primers used to determine the polymorphism is based on any of the microsatellite (SSR) motifs (di-, tri-, tetra- or penta-nucleotides), which could amplify wide range of amplified products (Zietkiewicz et al., 1994). Moreover, the ISSR method was reported to be successful in detection isogenic lines of the Poaceae family (Akagi et al., 1996) and the diversity analysis of many rice varieties (Parsons et al., 1997).

In the present study, the genetic diversity of medicinal rice variety 'Njavara' (both black and golden yellow glumed) rice genotypes was determined by using ISSR markers. This information would be critical to detect the potential diverse genotypes which can be used as a parent in 'Njavara' rice breeding program in the future.

2. Materials and Methods

2.1 Plant material

Twenty four Njavara ecotypes viz., 3 from Rajiv Gandhi Centre for Biotechnology (RGCB, Govt of India) and twenty one collected from different locations of Kerala along with seven check varieties (Ezhome 1, Ezhome 2, Jyothi, Kuthir, Kuttoosan, Pokka V1) formed the material for this study. The details of rice varieties used for the study are given in **Table 1** and **2**.

2.2 DNA Extraction and ISSR Analysis

Rice seedlings of all rice varieties were developed in the greenhouse of Inter University Centre for Genomics and Gene Technology, University of Kerala. DNA extraction was carried out from the tender leaves of each variety following cetyltrimethyl ammonium bromide (CTAB) method (Murray & Thomson, 1980) with some modifications by adding 2% (v/v) ß-mercaptoethanol (added just before use) and 2% (w/v) polyvinylpyrrolidone. Integrity of extracted DNA was determined by running to 1% agarose gel electrophoresis, and the quality and concentration of DNA was determined with UV-visible spectrophotometer (Eppendorf, Germany). ISSR profiling was performed using 8 primers (UBC-807, UBC-810, UBC-818, UBC-820, UBC-840, UBC-842, UBC-845) (**Table 3**) purchased from Integrated DNA Technologies Inc, USA.

The PCR reaction mixture was prepared with a total reaction volume of 25 µl containing 10 pmol of each primer, 200µM of deoxynucleotides, 5X buffer with MgCl2, 1 unit Taq polymerase and 100ng of DNA template. Amplification was carried out in a thermal cycler with an initial strand separation at 94°C for 5min (initial denaturation), followed by 32 cycles of 95°C for 30sec (denaturation), 55°C for 1min (annealing), and 72°C for 1min (polymerisation). After 32 cycles, there was a final extension step at 72°C for 5 minutes.

Negative control without the rice genomic DNA was also maintained in the PCR amplification along with all primer to check primer quality and presence of nonspecific amplifications contaminations and primer dimers. The amplified products (15µl) were loaded to 1.2% agarose gel stained with ethidium bromide in 1X tris-base boric acid-EDTA buffer and run at 100V. The electropherograms were documented using Chemi Doc image documentation analysis system (Bio Rad, US). The DNA bands obtained were compared with 100bp DNA ladder (New England Biolabs, USA).

2.3 Data Analysis

The ISSR primers are dominant markers and the amplified bands were scored as (1) for presence and (0) for absence of bands. Genetic diversity parameters such as percentage polymorphic loci (PPL), effective number of alleles (Ne), gene diversity (h), Shannon's information index (I), gene frequency, and gene flow (Nm) were computed (Nei, 1972). Genetic differentiation of population (GST), is the measure of the proportional amount of dissimilarity within subpopulation as compared with the total population, was enumerated. When GST is equal to "0," this implies that the subpopulations are identical; when the value is "1," they are completely different. The scored separated products were

Sl.No.	Accessions	Glume colour	Location
1	N1	Black	Kalpetta
2	N2	Golden yellow	Mulackamthuruthy
3	N3	Golden yellow	Puthukkad
4	N4	Black	Wayanad
5	N5	Black	Wayanad
6	N6	Black	Malappuram
7	N7	Black	Malappuram
8	N8	Black	Palakkad
9	N9	Black	Palakkad
10	N10	Golden yellow	Palakkad
11	N11	Golden yellow	Palakkad
12	N12	Golden yellow	Palakkad
13	N13	Black	Puthukkad
14	N14	Black	Puthukkad
15	N15	Golden yellow	Puthukkad
16	N16	Golden yellow	Kannur
17	N17	Golden yellow	Kannur
18	N18	Black	Cheruthuruthy
19	N19	Black	Cheruthuruthy
20	N20	Golden yellow	Payannur
21	N21	Black	Pattambi
22	N22	Golden yellow	Pattambi
23	N23	Black	Pattambi
24	N24	Black	Pattambi

Table 1 Details of Njavara accessions used for the study

Sl no.	Name of variety	Accessions	Location/ Source	Type
1	Ezhome 1	O1 (25)	Farmer's plot, Kannur	Improved variety, designed for saline - prone Kaipad rice fields
2	Ezhome 2	O2 (26)	Farmer's plot, Kannur	Improved variety, designed for saline - prone Kaipad rice fields
3	Jyothi	O3 (27)		RRI, Vytila
4	Kuthir	O4 (28)	Farmer's plot, Kannur	Kaipad rice variety
5	Kuttoosan	O5 (29)	Farmer's plot, Kannur	Kaipad rice variety
6	Pokka V1	O6 (30)	RRI, Vytila	Pokkali rice variety

Table 2 Details of check varieties used for the study

Sl no.	ISSR primer	Primer sequence (5'-3')	T_a(°c)
1	UBC-807	AGAGAGAGAGAGAGAGT	45
2	UBC-810	GAGAGAGAGAGAGAGAT	45
3	UBC-818	CACACACACACACACAG	47
4	UBC-820	GTGTGTGTGTGTGTC	47
5	UBC-840	GAGAGAGAGAGAGA(CT)A	38
6	UBC842	GAGAGAGAGAGAGAYG	50```
7	UBC-845	CTCTCTCTCTCTCTRG	48

Table 3 List of ISSR primers used for the study

Primers	No. of Alleles	Total fragments	Polymorphic bands	Polymorphism (%)
UBC-807	10	6	4	66.6
UBC-810	8	5	3	60
UBC-818	11	6	5	83.3
UBC-820	12	7	5	71.4
UBC-840	14	7	7	100
UBC-842	11	7	4	57.1
UBC-845	12	7	5	71.4
Total	78	45	33	73.3

Table 4 List of primers, number of amplified fragments and number of polymorphic bands generated by PCR using a panel of seven ISSR primers

3. Results and Discussions

The ISSR amplification with 24 'Njavara' cultivars collected from different parts of Kerala generated 45 fragments that could be scored, of which 33 were polymorphic while the remaining were monomorphic in nature (**Figure. 2** and **3**). Diversity analysis showed that the total number of amplified fragments ranged from 05 (UBC 810) to 7 (UBC 820, UBC 840, UBC842 and UBC 845) which varied in size from 180 bp to 1200bp. Of the 45 amplified bands, 33 bands (73.3%) were polymorphic with an average of 6.42 polymorphic fragments per primer (**Table 4**).

The total number of alleles produced by the seven primers is 78 including 45 polymorphic markers and only 33 monomorphic markers. A 100% polymorphism was scored for UBC-840 which produced the maximum number of alleles compared to other primers. (**Table 3**). Similarly low polymorphism of 57.1%, 60.0% and 66.6% also was scored for primer UBC-842, UBC-810 and UBC-818. The total percentage of polymorphic markers for all primers in the examined 24 accessions is 73.3%%, which indicated low level of genetic diversity among the tested 'Njavara' cultivars.

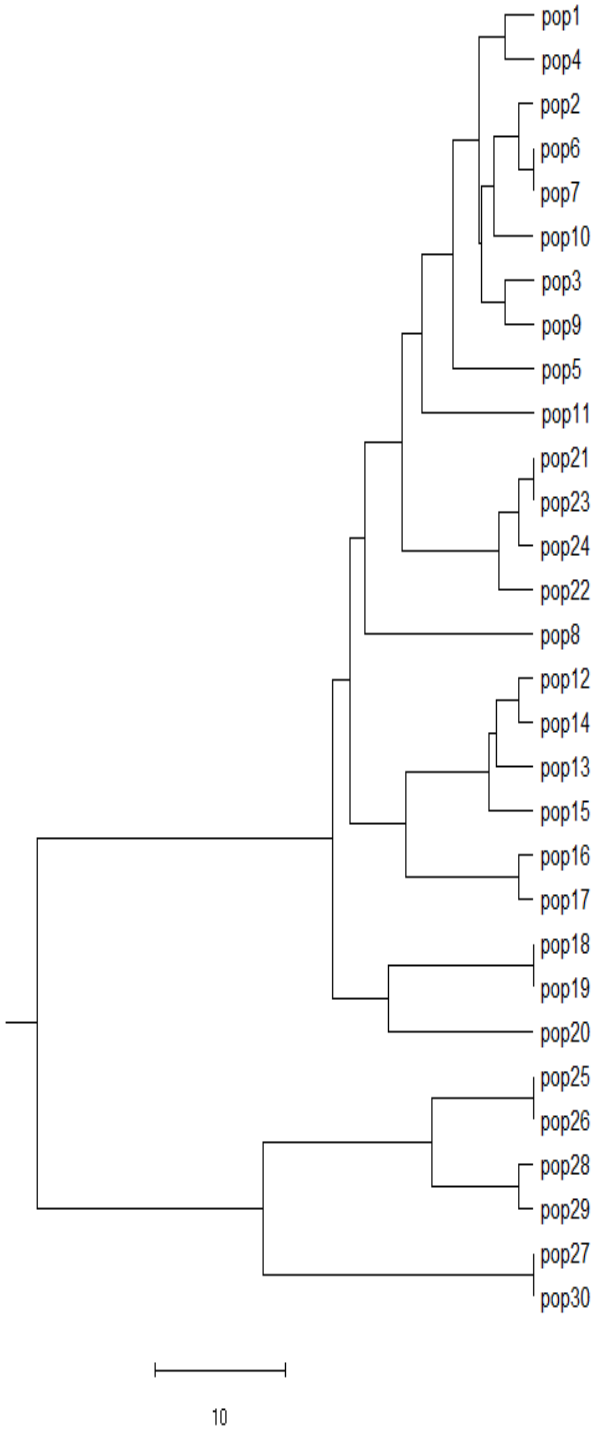

Figure 1 Dendrogram showing genetic relationships of the Njavara accessions inferred from the ISSR polymorphism data (Pop1 to Pop24 represents Njavara accessions and Pop25 to Pop30 represents check varieties used for comparison)

analyzed using POPGENE ver. 3.2 (Yeh et al., 1999) software check the genetic distance and diversity between the 'Njavara' rice verities collected from different parts of Kerala. Similarity matrix was computed between the selected 24 'Njavara' accessions based on Nei's genetic distance as executed in the NTSYS-pc and the genetic distance between the accessions and the out groups used for the study was indicated as a distance tree by using the NTSYS-pc software using un-weighted pair-group method with arithmetic averages (UPGMA) and simple matching coefficient (Rohlf, 2002).

The genetic similarity and genetic distance between the populations was measured using Nei's method (Nei, 1972) and was predicted through POPGENE. The highest genetic identity value was found to be 1.00 and the lowest value was 0.75. Genetic distance between each accession ranged from 0.00 to 0.54 (Table 5). The average number of polymorphic loci per primer was found to be 4.7. Shannon's Information Index (I) differ from 0 to 0.4965 with an average of 0.1934. The effective number of alleles (ne) among the sample ranges from 1 to 1.9862 and Nei's (h) genetic diversity value ranged from 0 to 0.6897 (Table 6). A dendrogram was constructed based on the genetic distance and genetic identity which grouped the populations into many subgroups and clusters (**Figure 1**). Based on the data, dendrogram was constructed by grouping the populations into several subpopulations. The UPGMA dendrogram showed two distinct nodes, the major node represents 24 'Njavara' accessions used for the study separated in sub clusters and the six out groups taken for the study was grouped in the minor node. The major nodes divides the 'Njavara' accessions further into four clusters.

Accessions grouped under each clusters includes both golden yellow and black 'Njavara' varieties. The accessions, N21 (B), N22 (Y). N23 (B), N24 (G) were grouped in same cluster were collected from Pattambi of Palakkad district. All accessions collected from Kannur were grouped in one cluster (N16 and N17 (Y)) whereas 'Njavara' varieties such as N1 (B) and N4 (B) collected from two different places (Kalpetta and Wayanad) also showed genetic relatedness by residing in same sub cluster. Similarly N4 (B) and N5 (B) are collected from Wayanad district, Kerala, but showed variability and was placed separately in a branch. When considering each clusters and sub-clusters it can be assumed that glume colour doesn't possess a greater influence over genetic variability within the accessions. All the out groups used in the study were grouped under the minor node as a single cluster, among them Ezhome 1 and 2, Kuthir and Kuttoosan, the kaipad rice varieties were more genetically similar. From the analysis it is to be noted that even though all the accessions showed low level of genetic variability, the

Pop id	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	****	0.95	0.93	0.95	0.86	0.93	0.93	0.82	0.93	0.95	0.86	0.77	0.73	0.80	0.77	0.75	0.73	0.73	0.73	0.71	0.82	0.82	0.82
2	0.04	****	0.93	0.91	0.91	0.97	0.97	077	093	0.95	0.86	0.77	0.73	0.80	0.77	0.75	0.73	0.77	0.77	0.75	0.86	0.82	0.86
3	0.06	0.06	****	0.88	0.84	0.91	0.91	0.80	0.95	0.93	0.84	0.80	0.75	0.77	0.80	0.77	0.75	0.75	0.75	0.68	0.80	0.75	0.80
4	0.04	0.09	0.11	****	0.82	0.88	0.88	0.82	0.88	0.91	0.82	0.77	0.73	0.80	0.77	0.71	0.68	0.68	0.68	0.66	0.77	0.82	0.77
5	0.14	0.09	0.16	0.19	****	0.93	0.93	0.77	0.84	0.91	0.77	0.73	0.68	0.75	0.73	0.71	0.68	0.77	0.77	0.75	0.82	0.77	0.82
6	0.06	0.02	0.09	0.11	0.06	****	1.00	0.75	0.91	0.93	0.84	0.75	0.71	0.77	0.75	0.73	0.71	0.80	0.80	0.77	0.88	0.84	0.88
7	0.06	0.02	0.09	0.11	0.06	0.00	****	0.75	0.91	0.93	0.84	0.75	0.71	0.77	0.75	0.73	0.71	0.80	0.80	0.77	0.88	0.84	0.88
8	0.19	0.25	0.22	0.19	0.25	0.28	0.28	****	0.80	0.82	0.73	0.73	0.68	0.71	0.73	0.75	0.73	0.60	0.60	0.57	0.73	0.77	0.73
9	0.06	0.06	0.04	0.11	0.16	0.09	0.09	0.22	****	0.93	0.84	0.80	0.75	0.77	0.80	0.77	0.75	0.71	0.71	0.68	0.80	0.75	0.80
10	0.04	0.04	0.06	0.89	0.09	0.06	0.06	0.19	0.06	****	0.86	0.82	0.77	0.84	0.77	0.80	0.77	0.77	0.77	0.75	0.82	0.77	0.82
11	0.14	0.14	0.16	0.19	0.25	0.16	0.16	0.31	0.16	0.14	****	0.82	0.77	0.84	0.82	0.84	0.82	0.73	0.73	0.66	0.82	0.77	0.82
12	0.25	0.25	0.22	0.25	0.31	0.28	0.28	0.31	0.22	0.19	0.19	****	0.95	0.97	0.95	0.84	0.82	0.73	0.73	0.66	0.73	0.73	0.73
13	0.31	0.31	0.28	0.31	0.37	0.34	0.34	0.37	0.28	0.25	0.25	0.04	****	0.93	0.91	0.84	0.86	0.77	0.77	0.71	0.68	0.68	0.68
14	0.22	0.22	0.25	0.22	0.28	0.25	0.25	0.34	0.25	0.16	0.16	0.02	0.06	****	0.93	0.82	0.80	0.75	0.75	0.68	0.75	0.75	0.75
15	0.25	0.25	0.22	0.25	0.31	0.28	0.28	0.31	0.22	0.25	0.19	0.04	0.09	0.06	****	0.80	0.77	0.68	0.68	0.62	0.73	0.73	0.73
16	0.28	0.28	0.25	0.34	0.34	0.31	0.31	0.28	0.25	0.22	0.16	0.16	0.16	0.19	0.22	****	0.97	0.80	0.80	0.68	0.80	0.75	0.80
17	0.31	0.31	0.28	0.37	0.37	0.34	0.34	0.28	0.28	0.25	0.19	0.19	0.14	0.22	0.25	0.02	****	0.82	0.82	0.71	0.77	0.73	0.77
18	0.31	0.25	0.28	0.37	0.25	0.22	0.22	0.51	0.34	0.25	0.31	0.31	0.25	0.28	0.37	0.22	0.19	****	1.00	0.80	0.77	0.73	0.77
19	0.31	0.25	0.28	0.37	0.25	0.22	0.22	0.51	0.34	0.25	0.31	0.31	0.25	0.28	0.37	0.22	0.19	0.00	****	0.80	0.77	0.73	0.77
20	0.34	0.28	0.37	0.40	0.28	0.25	0.25	0.54	0.37	0.28	0.40	0.40	0.34	0.37	0.47	0.37	0.34	0.22	0.22	****	0.75	0.71	0.75
21	0.19	0.14	0.22	0.25	0.19	0.11	0.11	0.31	0.22	0.19	0.19	0.31	0.37	0.28	0.31	0.22	0.25	0.25	0.25	0.28	****	0.95	1.00
22	0.19	0.19	0.28	0.19	0.25	0.16	0.16	0.25	0.28	0.25	0.25	0.31	0.37	0.28	0.31	0.28	0.31	0.31	0.31	0.34	0.04	****	0.95
23	0.19	0.14	0.22	0.25	0.19	0.11	0.11	0.31	0.22	0.19	0.19	0.31	0.37	0.28	0.31	0.22	0.25	0.25	0.25	0.28	0.00	0.04	****
24	0.22	0,16	0.25	0.28	0.22	0.14	0.14	0.34	0.25	0.22	0.22	0.34	0.40	0.31	0.34	0.25	0.28	0.28	0.28	0.31	0.02	0.06	0.02

Table 5 Nei's Original Measures of Genetic Identity and Genetic distance estimated from ISSR amplification with 24 Njavara Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Marker	Locus	na*	ne*	h*	I*
UBC 807	1	2.0000	1.1803	0.1528	0.2868
	2	2.0000	1.2800	0.2188	0.3768
	3	1.0000	1.0000	0.0000	0.0000
	4	2.0000	1.0868	0.0799	0.1732
	5	2.0000	1.0868	0.0799	0.1732
	6	2.0000	1.9862	0.4965	0.6897
UBC 810	1	1.0000	1.0000	0.0000	0.0000
	2	1.0000	1.0000	0.0000	0.0000
	3	2.0000	1.2800	0.2188	0.3768
	4	2.0000	1.0868	0.0799	0.1732
	5	2.0000	1.0868	0.0799	0.1732
UBC 818	1	1.0000	1.0000	0.0000	0.0000
	2	2.0000	1.3846	0.2778	0.4506
	3	2.0000	1.3846	0.2778	0.4506
	4	2.0000	1.7041	0.4132	0.6036
	5	2.0000	1.3846	0.2778	0.4506
	6	2.0000	1.0868	0.0799	0.1732
UBC 820	1	1.0000	1.0000	0.0000	0.0000
	2	2.0000	1.0868	0.0799	0.1732
	3	2.0000	1.0868	0.0799	0.1732
	4	2.0000	1.7041	0.4132	0.6036
	5	2.0000	1.8000	0.4444	0.6365
	6	1.0000	1.0000	0.0000	0.0000
	7	1.0000	1.0000	0.0000	0.0000
UBC 840	1	2.0000	1.0868	0.0799	0.1732
	2	2.0000	1.0868	0.0799	0.1732
	3	2.0000	1.0868	0.0799	0.1732
	4	2.0000	1.9862	0.4965	0.6897
	5	2.0000	1.9862	0.4965	0.6897
	6	2.0000	1.9459	0.4861	0.6792
	7	2.0000	1.3846	0.2778	0.4506
UBC 842	1	2.0000	1.0868	0.0799	0.1732
	2	1.0000	1.0000	0.0000	0.0000
	3	2.0000	1.0868	0.0799	0.1732
	4	1.0000	1.0000	0.0000	0.0000
	5	1.0000	1.0000	0.0000	0.0000
	6	1.0000	1.0000	0.0000	0.0000
	7	2.0000	1.8000	0.4444	0.6365
UBC 845	1	2.0000	1.4922	0.3299	0.5117
	2	1.0000	1.0000	0.0000	0.0000
	3	2.0000	1.6000	0.3750	0.5623
	4	2.0000	1.3846	0.2778	0.4506
	5	2.0000	1.9459	0.4861	0.6792
	6	2.0000	1.7041	0.4132	0.6036
	7	2.0000	1.8824	0.4688	0.6616
Mean		1.7333	1.3165	0.1934	0.3026
Standard Deviation		0.4472	0.3493	0.1839	0.2562

Table 6 Genic Variation Statistics for All Loci in 24 Njavara accesions

* na = Observed number of alleles, * ne = Effective number of alleles [Kimura and Crow (1964)], * h = Nei's (1973) gene diversity, * I = Shannon's Information index [Lewontin (1972)]

4.Conclusion

'Njavara' (Oryza sativa L.) is the medicinal rice of Kerala, India, which is extensively used in Ayurvedic system of medicines. ISSR based markers are proved as an efficient tool to detect the genetic diversity in numerous species based on the polymorphism relies in the DNA sequence. Seven ISSR markers have been successfully used to estimate the extent of genetic diversity in 24 'Njavara' accessions (including both golden yellow (Y) and black (B) glumed) along with 6 out groups (other improved rice verities). The dendrogram produced by scoring the ISSR amplified product clearly depicted that 'Njavara' accessions used for the study are highly similar in its genetic level and grouped as clusters in the major node. However, the six out groups kept for comparisons were included another node which shows their polymorphism level in the genetic level. 'Njavara' germplasm retains a composite of extremely homozygous genetically isolated units. This distinctness of 'Njavara' accessions was evident from the ISSR dendrogram with 24 'Njavara' accessions collected from different parts of Kerala when compared with few improved rice strains. This low level of genetic mixing and the unadulterated gene pool must be due to selection process traditionally practiced by farmers.

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