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Characterization of '*Suduru samba*' rice (*Oryza sativa* L.) Accessions using genome wide SSR polymorphism and seed morphology

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Abstract

Molecular characterization of genotypes gives precise information about the extent of genetic diversity. Correct identification of the traditional rice varieties is a timely requirement as there are a number of accessions per variety available at the PGRC. The main objective of this study was to select a set of representative accessions of '*Suduru samba*' using molecular analysis inorder to solve variety identification problems while assessing the genetic diversity. A total of 13 accessions including eleven '*Suduru samba*', and controls (*Kaluheenati* and Bg360) were evaluated using 31 SSR markers distributed widely over the rice genome. DNA was extracted from tender leaves of 14 days old seedlings using modified CTAB method and PCR ampilificons were separated using 8% polyacrylamide gel electrophoresis. Average linkage clustering, Principle component analysis (PCA) and Polymorphic information content (PIC) analysis were performed to assess the genetic diversity. A total of 184 alleles were detected by 31 markers, all of which were (100%) polymorphic. Allele richness ranged from 3 to 9 per locus. The first two principal components cumulatively explained about 39.89% of the total variation among the 13 accessions. Polymorphic information content values varied from 0.8758 to 0.4230. Seed morphology analysis was done using 7 seed characters to compare the results. The first three principal components cumulatively explained over 85% of the variation and first two principal components explained about 74.79% of the variations. Molecular and seed morphology clusters delineated the 11 accessions effectively and highly related accessions AC2202, AC3333, AC3594, AC5402 and AC3671 were selected as the representative set of *Suduru samba*. These results will the pave way for establishing a core collection and identification of '*Suduru samba*' for evaluation of agronomic traits and seed paddy certification scheme to fulfill the current need.

Keywords: Core collection, Genetic diversity, SSR Polymorphism

Introduction

Rice (*Oryza sativa* L.) is an essential staple food of the inhabitants of Sri Lanka. More than 90% of the population in Asia considers rice as their main calorie source (Chuang *et al*., 2011) and it is the second most important cereal crop in the world. It is considered as the ideal model plant to study grass genetics and genome organization due to its diploid nature, comparatively small genome size 430Mb (Causse *et al*., 1994; Kurata *et al*., 1994), considerable level of genetic polymorphism (McCouch *et al*., 1998), availability of fully sequenced genome and large amount of well conserved genetically diverse material (Rabbani *et al*., 2010). In Sri Lanka, rice occupies one-third of the total cultivated land area of 0.77 million ha (34%) and currently produces 4.869 million tons annually. Sri Lankan rice germplasm exhibits a wide range of diversity. Sri Lanka is considered as one of the secondary diversity centers for rice genetic resources (Ikeda and Duncan, 1991). Rice cultivars grown in Sri Lanka from ancient times to the middle of the last century are known as traditional rice cultivars. Preference is higher towards traditional rice cultivars because of their high nutritional value, medicinal properties and adaptability to Sri Lankan soil types, climate, geography and harsh environmental conditions, diseases and pests (Efisue *et al*., 2008). With the introduction of high yielding new improved varieties, traditional rice cultivars were grown less and it will eventually become extinct from cultivation (Morishma and oka, 1995). However, most of the traditional varieties are conserved ex-situ as seeds in gene banks.

Around 500 traditional rice varieties were identified in 1924 (Molegode, 1924). Currently more than 1500 traditional rice accessions are available at the Plant Genetic Resources Center (PGRC) gene bank (Wasala *et al*., 2013). Correct identification of rice cultivars is difficult due to higher number of accessions for a certain local name and synonyms (Ebana *et al*., 2008). It is also difficult to know whether they are redundant or actually different accessions. Therefore, it is a requirement to recognize a representative diversity set of accessions of a variety for identification of each variety for commercial cultivation, evaluation for biotic and abiotic stresses and utilization for breeding of new varieties.

Varieties can be identified using morphological characters such as plant height, leaf shape, length, seed coat color, shape etc. Some of the morphological characters

can vary according to environmental conditions which lead to false justification. A comprehensive morphological characterization is laborious, time consuming and one has to wait until reproductive stage of a crop (Chuang *et al*., 2011). Morphological diversity analysis of most of the available accessions at PGRC has been already completed. However, no comprehensive studies have been performed on genotypic diversity analysis of the Sri Lankan traditional rice varieties. Genetic diversity assessment at the molecular level provides reliable information for selection of germplasm in the development of new rice varieties and in conservation of traditional rice genetic resources.

The application of SSR markers in rice include characterization of the genetic structure of the cultivated rice O. sativa at both inter and intra-varietal, genetic diversity and/or evolutionary analysis of landraces, weedy and wild rice germplasm, determination of the purity of breeding material or seed stocks (Alvarez *et al*., 2007). The SSR marker based molecular fingerprinting could serve as a sound basis in the identification of genetically distant accessions as well as in the duplicate sorting of the morphologically close accessions (Sajib *et al*., 2012).

The objective of this research was to characterize 11 accessions of *Suduru samba* stored in PGRC genebank, Sri Lanka to facilitate identification of duplicates, setting a core collection and variety identification using seed morphology and genome wide SSR markers.

Materials and Methods

Information on *Suduru samba* was collected by referring published and unpublished data such as PGRC passport data and Characterization Catalogue on Rice (*Oryza sativa*) Germplasm in Sri Lanka.

Plant material

Seeds of 11 available accessions of '*Suduru samba*' [Accession Nos 3572, 4362, 4479, 8545, 9318, 10731 and 2202 (originally received from Central Rice Breeding Station, Batalagoda), 3333, 3594, 3671 and 5402 (originally received from Agriculture Research Station Ambalantota)] *Kaluheenati* (Accession no 5484) and Bg360 (Accession no 8920) were obtained from the gene bank of Plant Genetic Resources Centre (PGRC).

Seed morphology analysis

Evaluation of rice accessions was carried out for different seed morphological parameters. Trait selection and measurement were based on IRRI standard evaluation system of rice (Anon., 1980). Grain shape (Shape of the grain 1-round, 2-semi-round and 3-half spindle shaped), Lemma and palea color (1-gold, 2-brown spots on straw and 3-brown furrows on straw), Seed coat color (1-white, 5-red), Sterile lemma color (1-pale, 2-gold), Pubescence on lemma (3-hairs on upper portion, 4-short hairs and 5-long hairs), Grain width (distance across the fertile lemma and the palea at the widest point) and Grain length (distance from the base of the lowermost sterile lemma to the tip (apiculus) of the fertile lemma or palea, whichever is longer) were measured using descriptor states explained in IRRI descriptors for *Oryza sativa* L. and photographs.

Molecular analysis

Seeds of each accession were soaked for 24 hours in water and were sown on tissue layered petri dishes. Next they were incubated in dark at room temperature to induce the germination of seeds. After germination, petri dishes were kept under greenhouse conditions without adding fertilizers.

Genomic DNA was extracted from the 2 weeks old immature tender leaves using the modified CTAB method described by Murray and Thompson (1980) with minor modifications. Presence of DNA was confirmed by using agarose gel (1%) electrophoresis. Quantification was done using spectrophotometer. DNA was amplified in a final volume of 15µl containing 2.5 ng μl^{-1} of template DNA, 2.5mM $MgCl_2$, 0.2mM each dNTPs, 0.4 mM μl^{-1} of each forward and reverse primers, 0.025 U μl^{-1} Taq DNA polymerase and 1X buffer (Promega, USA). The amplification program was as follows: initial denaturation of the template DNA at 94℃ for 5 minutes, followed by 35 cycles of denaturation at 95 ℃ for 1minute, annealing at 55℃/56.5℃/58℃ for 30 seconds and extension at 72℃ for 1 minute. The amplification was terminated by a final extension at 72℃ for 7 minutes. Annealing temperature was changed according to the primer. PCR products (2µl) were seperated on 8% polyacrylamide gel. Gels were stained using Ethidium bromide (0.5µg ml^{-1}) and detected by BIO-RAD gel documentation system. From previous SSR studies of rice (Akagi *et al*., 1997), 31 primers were selected for analysis (Table 1) to cover the genome with a small number of markers which could detect polymorphism in rice accessions.

Data analysis

Alleles of each SSR locus was scored for allele no. which ranged from1-9. All recorded morphological and molecular data were analyzed using two complementary procedures; cluster and Principal Component Analysis (PCA). The polymorphic information content (PIC) of each marker was calculated. Average linkage cluster analysis was performed to group the rice accessions and Principal Components (PCs) was performed to assess the pattern of variation among the parameters. Score plot of first three PCs were used to provide a graphical representation of the pattern of variation among the rice accessions. These tests were performed with the Statistical Analysis System software (SAS 9.3).

Results and Discussion

Most of the SSR markers amplified clear scorable alleles as shown in the Figure 1. The number of alleles ranged from 3 to 9 (mean 5.93) per locus and total number of alleles detected was 184 (Table 1). The highest polymorphism was observed in primers RM207 on chromosome 2 and RM440 on chromosome 5 yielding 9 alleles and the lowest polymorphism was observed in primers RM20B on chromosome 11 and RM270 on chromosome 12 detecting 3 alleles.

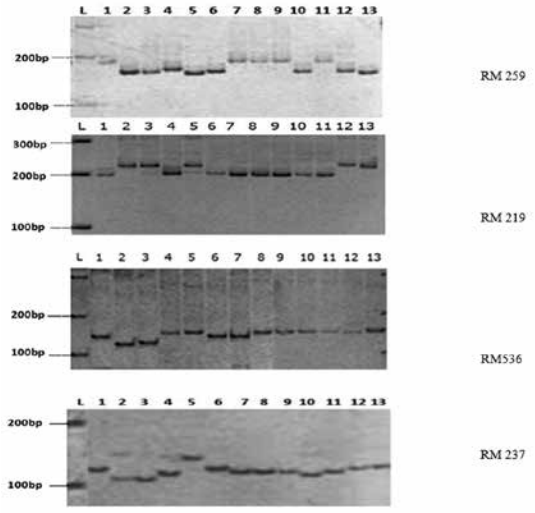

Figure 1. SSR banding pattern of rice accessions generated by primer pairs RM256, RM219, RM536 and RM237. The lanes represent L-100bp molecular size marker 1-3572,2-4362,3-4479,4-8545 ,5-9318,6-10731 ,7-2202,8-3333,9-3594,10-3671,11-5402,12 -Kaluheenati,13-Bg360

Table 1. Details of SSR markers used, their location on the rice chromosome, number of polymorphic alleles and polymorphism information content (PIC) values.

Primer	Chromosome No	Polymorphic alleles	PIC value
RM220	1	5	0.7337
RM259	1	8	0.7929
RM237	1	7	0.8195
RM84	1	4	0.6271
RM236	2	4	0.6035
RM208	2	4	0.6271
RM207	2	9	0.8758
RM213	2	6	0.7690
RM571	3	4	0.6627
RM518	4	8	0.8284
RM241	4	7	0.6745
RM255	4	6	0.7574
RM440	5	9	0.8047
RM480	5	5	0.6745
RM217	6	8	0.7456
RM539	6	7	0.7811
RM412	6	7	0.8284
RM418	7	7	0.7811
RM560	7	7	0.7831
RM25	8	5	0.6538
RM515	8	5	0.6508
RM219	9	5	0.6390
RM201	9	4	0.5561
RM215	9	7	0.7811
RM216	10	5	0.7455
RM228	10	6	0.6627
RM20B	11	3	0.5443
RM536	11	6	0.7100
RM202	11	7	0.7811
RM224	11	6	0.8040
RM270	12	3	0.4230

The Polymorphic Information Content (PIC) value, which measures the allelic diversity at a locus and reflects the allelic diversity and allelic frequency among the accessions, ranged from 0.8758 (RM207) to 0.4230 (RM270). The high PIC values found in these tested loci indicated the usefulness of these SSR markers in genetic characterization.

Cluster analysis based on the average distance was carried out based on molecular data to assess the genetic relationship of the tested accessions. All rice accessions were grouped into 3 clusters at 1.0 average distance between clusters (Figure 2) and Bg 360 a new improved variety which has a different genetic base was separated individually. Tested accessions, except 4362, amplified one band (homozygosity) proving the genetic purity of conserved '*Suduru samba*' germplsam. Accession 4362 amplified 2 bands (heterozygosity) for RM 25 and RM 237showing its genetic impurity. Eight accessions were clustered in the cluster 1 while 2 accessions were grouped in cluster 2 and the rest were clustered in cluster 3 in which '*Suduru samba*' accession and controls (*Kaluheenati* and Bg 360) were included. '*Suduru samba*' accessions 2202, 3333, 3594, 5402 and 3671 were grouped together at 0.7 average distances. These five accessions can be considered as the closely related accessions among the tested ones and also accessions 3333 and 3594 were first formed in to a group at 0.35 average distances between clusters. Therefore, these two accessions were the genetically closest accessions among the tested ones.

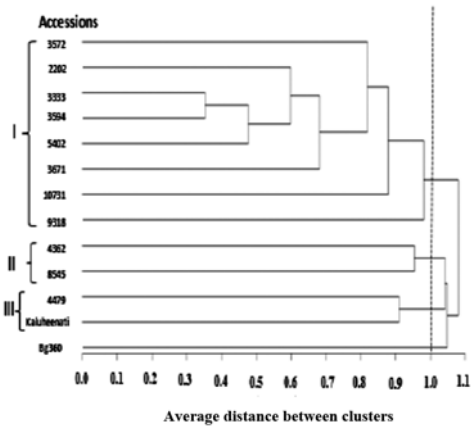

Figure 2. Dendrogram of rice accessions obtained through average linkage cluster analysis. Dashed line indicated the minimum distance between clusters. The clusters were identified as I, II and III.

Principal component analysis showed that the first three PCs having eigen values greater than 1 accounted for 50.72% of the total variation. The first principal component individually explained nearly one fourth of the total variation whereas the first two principal components cumulatively explained the 39.81% the total variation and the combination of the principal component 1 (PC_1) and principal component 3 (PC_3) explained the 38.82% of the total variation covered by the all the accessions (Table 2) Accessions no. 4479 and 'Kaluheenati' showed comparatively high positive PC_1 scores (Figure 3).

Table 2. Variation among rice accessions accounted for first three PCs.

Parameter	PC_1	PC_2	PC_3
Eigen value	6.158	2.780	2.548
Proportion of the variance %	27.91	11.90	10.91
Cumulative variance	27.91	39.81	50.72

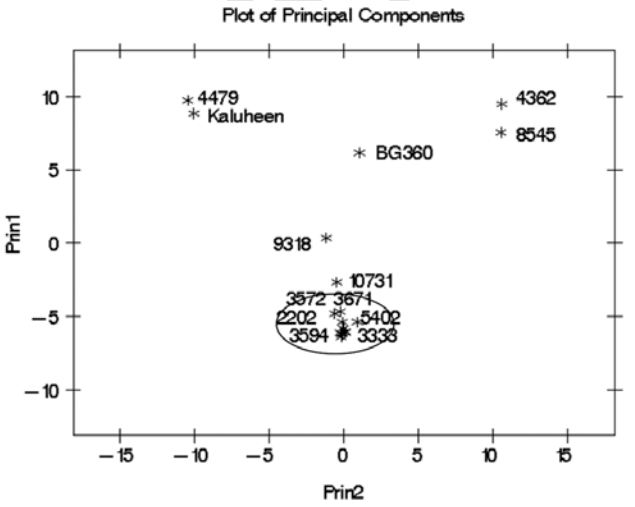

Figure 3. Score plot of PC_1 (Prin1) and PC_2 (Prin2) based on genetic difference of the accessions

Seed morphology analysis

The evaluation of morphological traits revealed important traits which are useful to characterize the rice germplasm accessions. Tested traits showed a considerable level of variability among the tested rice accessions.

'*Suduru samba*' accessions 3572, 2202, 3333, 3594, 3671, 5402 and Bg 360 had similarities in four seed characters, grain shape, lemma and palea colour, seed coat color and pubescence of lemma and having variation in sterile lemma color, grain width and length (Figure 4). Accession 4479 highly varied from other accessions due to presence of long hairs all over the lemma (velvety appearance). Accessions 9318 and 10731 had short hairs all over the lemma whereas other tested accessions having hair only in the upper portion of the lemma (Table 3).

Table 3. Results of Seed morphology analysis

Acc No.	Grain shape	Lemma and palea Color	Seed coat color	Sterile lemma color	Pubescence of lemma	Grain width (mm)	Grain length (mm)
3572	2	1	1	1	3	2.350	6.169
4362	1	1	5	1	3	3.104	5.932
4479	3	3	5	1	5	3.142	8.060
8545	1	3	5	1	3	2.788	5.975
9318	1	2	1	2	4	2.561	5.418
10731	2	1	1	1	4	2.607	6.704
2202	2	1	1	1	3	2.103	5.768
3333	2	1	1	1	3	2.076	5.930
3594	2	1	1	2	3	1.978	5.759
3671	2	1	1	1	3	2.356	6.020
5402	2	1	1	2	3	2.051	5.470
Kaluheenati	3	3	5	1	3	3.103	8.026
Bg 360	2	1	1	1	3	2.310	5.904

Accepted seed coat color of '*Suduru samba*' is white but accessions 4479, 8545 and 4362 showed red seed coat color (Figure 4) showing probable misidentification. Based on the cluster analysis all rice accessions were grouped into 2 clusters at 1.0 average distance between clusters (Figure 4) whereas '*Kaluheenati*' was separated individually. Nine '*Suduru samba*' accessions were grouped into the cluster I and other 3 accessions were grouped together into cluster II. Even though Bg 360 is a new improved variety

with a different genetic background (parents: 84-3346/IR36//Senerang), it was grouped together with '*Suduru samba*' accessions because of the similarities in seed morphology indicating a weakness in morphological data.

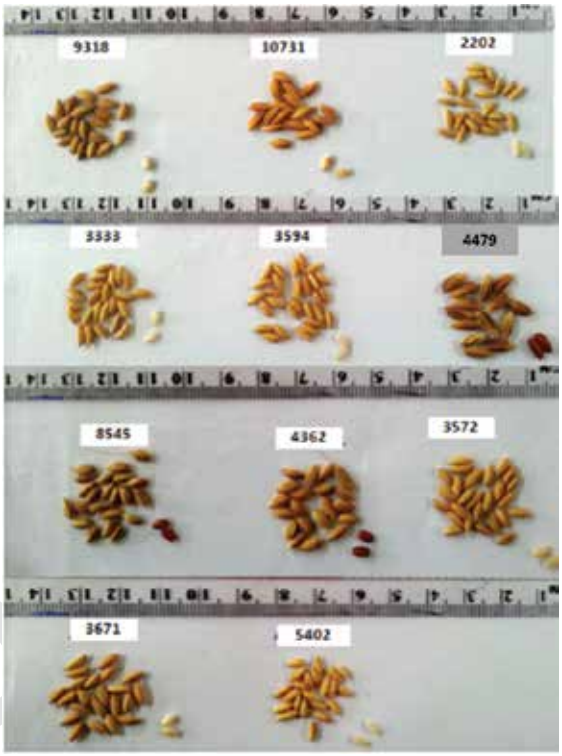

Figure 4. Seed morphology of *Suduru samba* accessions

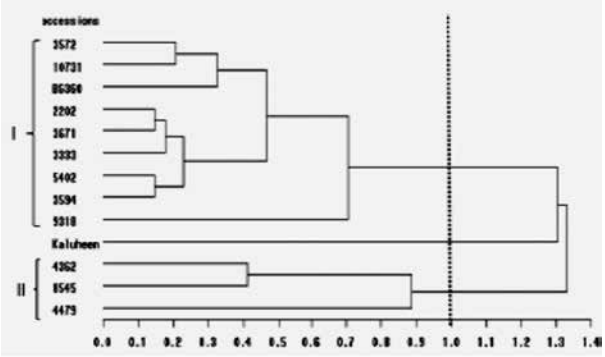

Figure 4. Dendrogram of rice accessions obtained through average linkage cluster analysis based on seed morphological characters. Dashed line indicated the minimum distance between clusters. The clusters were identified as I and II

'*Suduru samba*' accessions 2202, 3333, 3594, 5402 and 3671 were grouped together at 0.25 average distances and were also the genetically related accessions among the tested ones. The accessions 2202 and 3671 were included in to a group at 0.12 average distances between clusters. Therefore, these two accessions were the morphologically closest accessions among the tested ones.

Principal component analysis of seed morphological data showed that the first three PCs having eigen values greater than 1 accounted for 89.16% of the total variation. The first principal component individually explained nearly three fourth (3/4) of the total variation whereas the first two principal components cumulatively explained the 74.79% the total variation of the total variation covered by the all the accessions (Table 4).

Accessions 4479 showed the extremely high positive PC_1 scores, reflecting highest contribution from lemma and palea color, seed coat color and pubescence on lemma while accessions of 5402, 3594, 3671, 3333, 2202, Bg 360 and 3572 showed the comparatively low PC_1 scores (Figure 5). '*Kaluheenati*' showed comparatively high positive PC_2 scores, reflecting grain shape, sterile lemma color and pubescence on lemma.

Table 4. Variation among rice accessions accounted for first four principal components

Characters	PC_1	PC_2	PC_3	PC_4
Grain shape	0.1068	0.6861	-0.2831	-0.1657
Lemma and palea color	0.4811	-0.0349	0.2776	-0.1403
Seed coat color	0.4681	-0.2143	0.1411	-0.4862
Sterile lemma color	-0.0593	0.3295	0.8799	0.0312
Pubescence on lemma	0.4116	0.3295	-0.0171	0.7501
Grain width	0.3914	-0.4642	-0.0249	0.3038
Grain length	0.4601	0.2872	-0.2180	-0.2457
Eigen value	3.4600	1.17752	1.0057	0.4873
Proportion	49.43	25.36	14.37	0.0696
Cumulative	49.43	74.79	89.16	0.9612

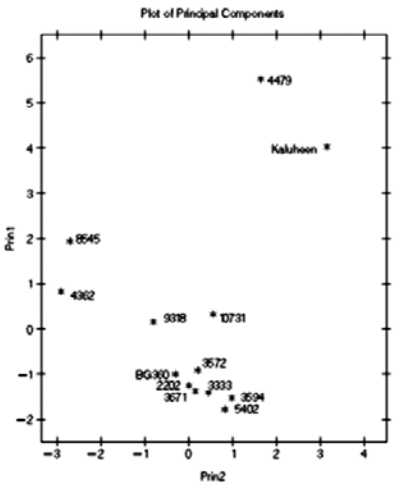

Figure 5. Score plot of PC_1 (Prin1) and PC_2 (Prin2) based on seed morphology

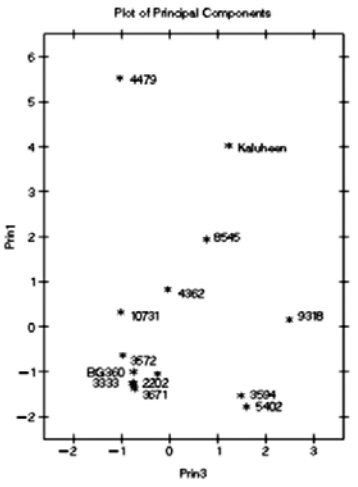

Figure 6. Score plot of PC_1 (Prin1) and PC_3 (Prin3) based on seed morphology

The PC_3 accounted for 14.37% of the total variation. Rice accession 009318 had the comparatively highest PC_3 than the other all accessions, indicating highest contribution from sterile lemma color, Figure 4.13 shows the plot obtained from the first and third eigenvectors of the PC analysis. Furthermore closely related *Suduru samba* accessions 2202, 3333 and 3671 having comparatively low PC_1 and PC_2 values while 3594 and 5402 having low PC_1 and PC_2 scores and high PC_3 scores.

The results of the SSR analysis cluster and the seed morphology analysis clearly grouped the same set of accessions despite few dissimilarities within the group. Comparison among accessions with the same variety name shows redundancy of the accessions but genetically they showed different SSR profiles. Molecular analysis and seed morphology analysis were compared and by combining average distance clustering and Principal Component Analysis resulted in five highly related accessions (2202, 3333, 3594, 5402 and 3671) (Figure 6). The genetic dissimilarity exhibited within this group depicts the existing valuable genetic diversity stored in the traditional variety '*Suduru samba*' useful for crop improvement.

Conclusion

The present study recorded a higher degree of genetic diversity among the accessions despite their morphological similarity. Molecular and seed morphology clusters delineated the 11 accessions effectively. Five closely related accessions 2202, 3333, 3594, 5402 and 3671 were selected as representative set of '*Suduru samba*'. Accessions 4362 is an out-crossed one and 8545 and 4479 are off types with red seed coat. Accessions 10731 and 3572 are also possible off types as they have larger seeds than representative set of '*Suduru samba*'. Seed morphological traits are also useful for preliminary characterization of accessions.

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