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SSR BASED MOLECULAR CHARACTERIZATION AND DIVERSITY ANALYSIS OF COWPEA

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Abstract

Twenty simple sequence repeat markers were used to analysis of genetic diversity in 20 cowpea accessions. Out of 20 SSR markers, 13 markers were shown polymorphism and 7 markers were shown monomorphic nature for molecular diversity. A total of 70 alleles were detected with a range of 1 to 10 alleles per marker and an average of 3.5 alleles per loci. Polymorphic information content (PIC) value ranged from 0.0 (VM10, VM27, VM36, VM38, VM40, VM68, VM70) to 0.833 (VM39) with an average of 0.342 indicating a considerable efficiency of markers for studying the polymorphism level available in the cowpea accessions. The genetic similarity coefficients for the 20 cowpea accessions based on 20 SSR markers ranged from 0.559 to 0.971. The highest similarity coefficient value occurred between IC-3004 vs IC-201081 was 97% and the lowest similarity coefficient value occurred between EC-472272 vs EC-9738 was 55%. Clustering classified the 20 cowpea accessions into 5 different clusters *i.e.* cluster A, B, C, D, E comprising of 5, 2, 4, 4, 5 cowpea accessions, respectively. In this study, presence of high level of diversity among the 20 cowpea accessions with SSR markers indicated their suitability for breeding program.

***Key words*:** Cowpea, SSR, PIC, Allele, cluster analysis and Genetic diversity.

Introduction

Cowpea is one of the most important pulse crops of significant economic importance worldwide, belongs to family *Fabaceae*. It is mainly grown in tropical and sub-tropical regions in the world for vegetable, seed purpose and to lesser extent as a fodder crop. Cowpea is consumed in many forms: the young leaves, green pods, and green seeds are used as vegetables; dry seeds are used in various food preparations; and the haulms are fed to livestock as nutritious supplement to cereal fodder. It can be cultivated both as pure crop and as mixed crop in association with cereals, other pulses and oilseeds. In the semi-arid and humid tropical regions of Africa, cowpea is a major source of protein and of considerable importance for human nutrition. It is called as vegetable meat owing to its high protein content (25%). The protein in cowpea seed is rich in the amino acids such as lysine and tryptophan compared to other legumes; hence, cowpea seed is valued as a nutritional supplement to cereals and as a protein source.

Knowledge of the genetic diversity available within the local and regional germplasm collections can enhance the overall effectiveness of cowpea improvement programs (Hegde and Mishra, 2009). Molecular markers are quicker and far more precise in species and genotypes identification. The advancements of molecular markers technology have broadened the area of genotyping and genetic diversity analysis by potentially revealing a large amount of genetic variation even between closely related taxa (Melchinger *et al.,* 1994). SSR markers have proved to be polymorphic but require nucleotide information for primer design (Sun *et al*., 1998). Simple sequence repeat markers are one of the most frequently used markers in the genetic diversity analysis of cowpea (Li *et al*., 2001; Ogunkanmi *et al*., 2008; Lee *et al*., 2009; Asare *et al*., 2010; Badiane *et al*., 2012). SSRs have also been extensively used in genotype identification, seed purity evaluation and variety protection (Brown *et al*., 1996; Senior *et al*., 1998), pedigree analysis (Ayres *et al*., 1997; Bowers *et al*., 1999), and genetic mapping of simple and quantitative traits and MAS (Blair and McCouch, 1997; Chen *et al*., 1997; Weising *et al*., 1998). So, the present study was undertaken to assess the genetic variation and relationship among different accessions for genetic improvement of cowpea using SSR markers.

Material and Methods

Seeds of 20 cowpea accessions were obtained from Scientific and Applied Research Centre (SARC), Meerut (UP) (Table-1). The genetic material was grown in plot for germination and growth at Scientific and Applied Research Centre, Meerut with standard agronomical practices. Young new leaves of every one cowpea genotypes were collected separately and packed into polybags. Afterward, polybags for every one accessions were freezing in liquid nitrogen and store up in deep freeze (-80°C) used for the separation of genomic DNA. Genomic DNA was isolated from young fresh leaves of 20 cowpea accessions using the CTAB

extraction method of Doyle and Doyle (1987) with minor modifications. Leaf material was ground to powder in liquid nitrogen and was then transferred to eppendorf tubes. 100mg leaf tissue was ground in 1ml CTAB extraction buffer (100mM Tris pH 8.0; 1.4M NaCl; 20mM EDTA pH 8.0; 0.2% (v/v) β-mercaptoethanol; 2 %(v/v) CTAB) and heated at 60°C for 30 min. DNA was isolated with one volume of a chloroform: isoamyl alcohol mix (24:1) and then centrifuged at 12000 rpm for 15 min at 4°C. Supernatant was taken and further mixed with one volume of chloroform: isoamyl. This was again centrifuged at 12000 rpm for 15 min at 4°C and then precipitated with isopropanol to 40% v/v final concentration. The DNA pellet was washed with 5mM ammonium acetate and 70% ethanol, dried and dissolved in 100µl of TE buffer (19mM Tris-HCl pH 8.0; 1mM EDTA pH 8.0). Purification of DNA was done to remove RNA, proteins and polysaccharides which were the major contaminants. RNA was removed by RNase treatment. RNase was added to the DNA sample @100 µg ml^{-1} and incubated at 37°C for 1 hour.

20 SSR primers were purchased from Integrated DNA Technology (IDT, USA) (Table-2). All SSR primers used in this study were dissolved in sterile TE buffer at a concentration of 15µg/ml. A 20 µL reaction volume containing 2.0 µL 10X Taq buffer, 2.0 µL of 25 ng/µL template DNA, 1.5 µL $MgCl_2$, 1.5 µL of 10mM dNTP mixture (dATP, dCTP, dGTP and dTTP), 1.0 µL of each (forward and reverse) primer, 0.4 µL Taq DNA polymerase enzyme (Bangalore GeNei, India) was loaded in Longe Gene thermal cycler for DNA amplification. Amplification were carried out in a thermal cycler programmed for 35 cycles with an initial melting at 94°C for 4 minute, followed by denaturation at 94°C for 1 minute. The annealing was performed at 37°C for 1 minute, which was then followed by polymerization at 72°C for 2 minute. Final extension step was at 72°C for 7 minute. Amplification products were subjected to horizontal electrophoresis unit on 1.8% agrose gel run in 1X TBE buffer at 50 volt for 2 hours and detected by staining using Ethidium Bromide. Standard molecular weight markers were used to determine the approximate size of amplification products. DNA bands were visualized on transilluminator and photographed by Gel documentation system.

Scoring was done manually for each of the gel sections allele was determined base on the positions of the bands. Band pattern for each of the microsatellite marker were recorded for each accession by assigning a letter to each band. Alleles were numbered as 'a_1', 'a_2' etc. In the data matrix presence of a band was represented by '1' and '0' for absence of a band is given. The marker data were entered directly into an excel spreadsheet with the microsatellite allele under rows and genotype under columns. PIC is equal to 1-∑ (P^2ij), where Pij is the frequency of Jth allele for i^{th} locus summed across all allele in the locus (Senior *et al*., 1998). The PIC valve ranging from '0' (Monomorphic) to '1' (highly discriminative with many alleles in equal frequency) is an indication of discriminitative power of marker, not only for number of alleles at a loci but also for relative frequencies of those allele in the accessions under study. SSR data has been used for cluster analysis. Genetic similarity based on SSR data can be calculated for all possible pairs of genotype using various coefficients. In the present study Jaccard´s coefficients (J), based on the formula, $J = N_{ij} / (N_{ij} + N_i + N_j)$ was used to estimate genetic similarities, where N_{ij} is the number of bands present in the both individual, N_i represents the total number of bands in the individual i and N_j is the total number of bands in the individual j. The similarity matrix was further analyzed by using NTSYS-pc version 2.2 to produce an agglomerative hierarchical classification (Rohlf, 2000), by employing UPGMA (Unweighted Paired Group method using Arithmetic Mean) with average linkage (Sneath and Sokal, 1973).

Results and Discussion

To measure the informativeness of SSR marker, Polymorphic Information Content (PIC) was determined for each of 20 SSR loci using 20 cowpea accessions. Out of 20 SSR markers, 13 SSR were shown high polymorphism and 7 SSR were shown monomorphic nature. In each of these SSR primers the PIC value ranged from 0.0 to 0.833 with an average of 0.342 (Table- 2). A total of 70 alleles were produced at the 20 SSR loci with an average of 3.5 alleles per locus with 1 allele in 7 primers, 2 alleles in 2 primers, 3 alleles in 2 primers, 4 alleles in 2 primers, 5 alleles in 2 primers and 6 alleles in 3 primers, 7 alleles in 1 primer and 10 alleles in 1 primer. The highest number of alleles (10 alleles) was observed in primer VM39 and the lowest number of allele (1 allele) was observed in primers *viz*; VM10, VM27, VM36, VM38, VM40, VM68, VM70. Table- 3 provides the summarized data regarding the allele number, allele distribution and PIC value for various primers studied on 20 cowpea accessions. Out of 20 SSR primers, only one SSR primer (VM39) were showing high polymorphic information content (83%) are shown in figure-1. SSR profiles showed a high level of genetic diversity among the cowpea accessions.

The PIC (polymorphism Information Content) values were estimated for different SSR loci based on the number of alleles and allele distribution. In each of these SSR primers the PIC value ranged from 0.0 to

0.833 with an average of 0.342. The PIC value ranging from ‘0’ (Monomorphic) to ‘1’ (highly discriminative with many alleles in equal frequency) is an indication of discriminative power of marker not only for number of alleles at a locus but also for relative frequencies of those alleles in the genotypes under study. On the basis of alleles number of sharing alleles and their frequencies, the highest value of PIC (0.833) was found for the primer VM39 because of well distributed presence of ten alleles across the accessions of cowpea followed by 0.796 for primer VM37, 0.775 in primer VM26, 0.772 with primer VM9, 0.729 for primer VM28 and 0.727 with primer VM11. The low PIC value was observed probably due to poor distribution of alleles in the genome. Various workers have already reported similar results in various crops like maize (Nikolić *et al*., 2015; Sharma *et al*., 2018), chickpea (Ahmad *et al*., 2014), Cowpea (Pradeep Kumar *et al*., 2017), Moongbean (Markam *et al*., 2018). The low level of polymorphism detected in this study (4-8 alleles) is in agreement with Asare *et al*. (2010) reported 4 to 13 alleles in cowpea collected from Ghana, while Sawadogo et al. (2010) reported 5 to 12 alleles in cowpea collected from Burkina This result agrees with Li *et al*., 2001 who find out that fifteen polymorphic microsatellites were able to distinguish 88 of the 90 breeding lines of cowpea accessions based SSR markers (Sarikamis *et al*., 2010). However, PIC values indicating a considerable efficiency of markers for studying the polymorphism level available in the cowpea accessions.

SSR data were used to construct pairwise grouping of the cowpea accessions by using software NTSYS-pc. Genetic relationship among 20 cowpea accessions was evaluated by similarity matrix based on Jaccard’s coefficient (Jaccard, 1908). The genetic similarity coefficients for the 20 cowpea accessions based on 20 SSR markers ranged from 0.559 to 0.971 (Table-3). The highest similarity coefficient value occurred between IC-3004 *vs* IC-201081 was 97% and the lowest similarity coefficient value occurred between EC-472272 *vs* EC-9738 was 55%. SSR based characterization, the genetic similarity value ranging from 0.559 to 0.971 which indicate the significant diversity (55% to 97%) among the accessions used for this study. A dendrogram was constructed by clustering of 16 cowpea genotypes are shown in Figure-2. The resulting dendrogram classified the 20 cowpea accessions into 5 different clusters *i.e*. Cluster A, B, C, D and E comprising of 5, 2, 4, 4 and 5 cowpea accessions, respectively (Table-4).

Cluster A grouped into two subclusters *viz*; A1 and A2. Subcluster A1 consisted of 3 cowpea accessions namely IC-3009, IC-3004 and IC-201081. In Subcluster A1, the maximum similarity coefficient occurred between IC-3004 *vs* IC-201081 with a value of 0.971 and the minimum similarity coefficient occurred between IC-3004 *vs* IC-3009 and IC-3009 *vs* IC-201081 with a value of 0.956. Subcluster A2 consisted of only 2 cowpea accessions namely IC-58905 and IC-249105A, which showed the similarity coefficient value of 0.897. Cluster B consisted of only 2 cowpea accessions namely IC-15665 and IC-4506, which showed the similarity coefficient value of 0.882. Cluster C grouped into two subclusters *viz*; C1 and C2. Subcluster C1 consisted of only 2 cowpea accessions namely EC-472283 and EC-390223, which showed the similarity coefficient value of 0.809. Subcluster C2 consisted of only 2 cowpea accessions namely IC-39095 and IC-51154, which showed the similarity coefficient value of 0.853. Cluster D grouped into two subclusters *viz*; D1 and D2. Subcluster D1 consisted of only 2 cowpea accessions namely IC-402106 and EC-97306, which showed the similarity coefficient value of 0.897. Subcluster D2 consisted of only 2 cowpea accessions namely IC-202280 and EC-91171A, which showed the similarity coefficient value of 0.750. Cluster E grouped into two subclusters *viz*; E1 and E2. Subcluster E1 consisted of only 2 cowpea accessions namely EC-911718 and EC-9738, which showed the similarity coefficient value of 0.632.Subcluster E2 consisted of 3 cowpea accessions namely IC-33932, IC-202826 and EC-472272. In Subcluster E2, the maximum similarity coefficient occurred between IC-33932 *vs* IC-202826 with a value of 0.882 and the minimum similarity coefficient occurred between IC-202826 *vs* EC-472272 with a value of 0.676.

SSR markers have been used to investigate genetic diversity and relationships in many important crops including maize (Senior *et al*., 1998), rice (Saini *et al*., 2004), wheat (Plaschke *et al*., 1995), soybean (Rongwen *et al*., 1995), rice bean (Muthusamy *et al*., 2008) and cowpea (Xu *et al*., 2008). SSR markers have been used to evaluate genetic diversity and phylogenetic relationships of cowpea genotypes (Choumane *et al*., 2000, Badiane *et al*., 2012). According to Diouf and Hilu (2005), the number of alleles ranged from 1 to 9 per SSR primer combination in cowpea germlasm from Senegal. Sixteen SSR primers generated a range of allele between 5 and 12 fragments with an average of 8.2 bands per primer combination among cowpea genotypes (Sawadogo *et al*., 2010). In this study, high level of genetic diversity was detected among the cowpea accessions using SSR markers. However, it is an effective approach for assessing the levels of genetic variability at the population level because of its broader genome coverage.

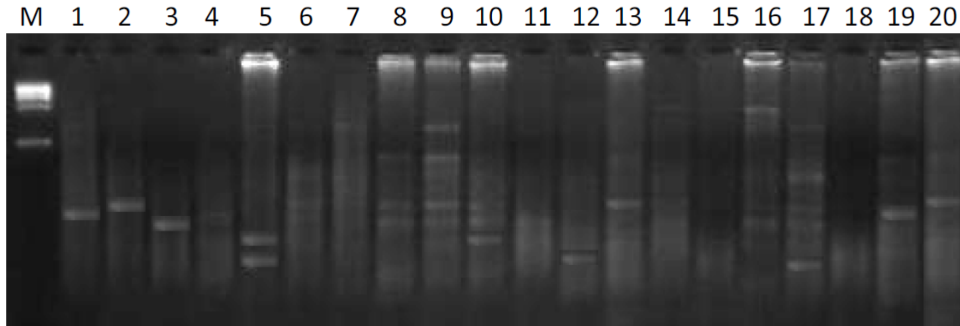

Fig. 1: SSR profile of 20 cowpea accessions with Primer VM39

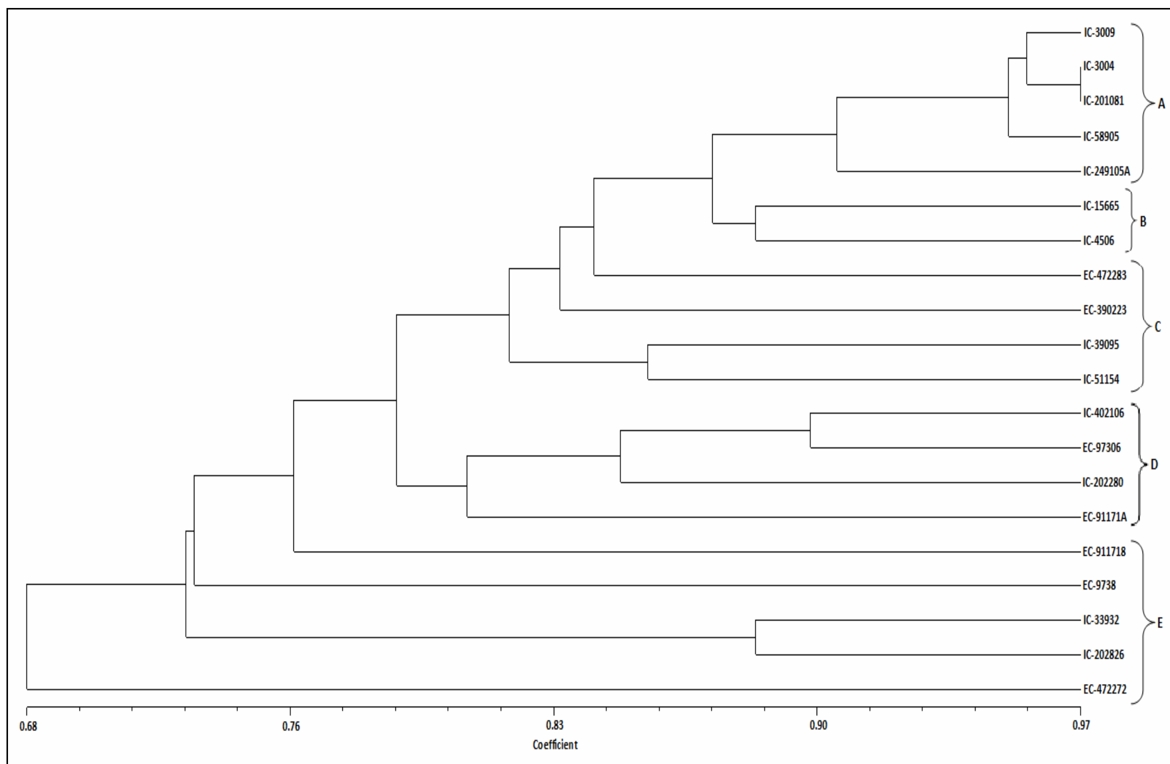

Fig. 2: UPGMA based cluster analysis of 20 cowpea accessions using SSR markers

Table 1: List of 20 Cowpea accessions

S.No.	Accessions	S.No.	Accessions
1	IC-3009	11	IC-51154
2	IC-15665	12	IC-202280
3	IC-39095	13	EC-390223
4	IC-402106	14	IC-201081
5	IC-58905	15	EC-97306
6	EC-472283	16	EC-911718
7	IC-3004	17	EC-472272
8	IC-33932	18	IC-249105A
9	IC-202826	19	EC-91171A
10	IC-4506	20	EC-9738

Table 2: PIC Values of SSR loci across 20 cowpea accessions

S. No.	Primer	Sequence (Forward and Reverse)	No. of Alleles	PIC
1	VM9	**F:** ACCGCACCCGATTTATTTCAT **R:** ATCAGCAGACAGGCAAGACCA	6	0.772
2	VM10	**F:** TCCCACTCACTAAAATAACCAACC **R:** GGATGCTGGCGGCGGAAGG	1	0.0
3	VM11	**F:** CGGGAATTAACGGAGTCACC **R:** CCCAGAGGCCGCTATTACAC	6	0.727
4	VM16	**F:** TCCTCGTCCATCTTCACCTCA **R:** CAAGCACCGCATTAAAGTCAAG	3	0.427
5	VM 17	**F:** GGCCTATAAATTAACCCAGTCT **R:** TGTGTCTTTGAGTTTTTGTTCTAC	2	0.104
6	VM 19	**F:** TATTCATGCGCCGTGACACTA **R:** TCGTGGCACCCCCCTATC	2	0.163
7	VM 22	**F:** GCGGGTAGTGTATACAATTTG **R:** GTACTGTTCCATGGAAGATCT	4	0.684
8	VM 26	**F:** GCCATCAGACACATATCACTG **R:** TGTGGCATTGAGGGTAGC	6	0.775
9	VM 27	**F:** GTCCAAAGCAAATGAGTCAA **R:** TGAATGACAATGAGGGTGC	1	0.0
10	VM 28	**F:** GAATGAGAGAAGTTACGGTG **R:** GAGCACGATAATATTTGGAG	5	0.729
11	VM 30	**F:** CTCTTTCGCGTTCCACACTT **R:** GCAATGGGTTGTGGTCTGTG	4	0.280
12	VM 31	**F:** CGCTCTTCGTTGATGGTTATG **R:** GTGTTCTAGAGGGTGTGATGGTA	5	0.345
13	VM 35	**F:** GGTCAATAGAATAATGGAAAGTGT **R:** ATGGCTGAAATAGGTGTCTGA	3	0.220
14	VM 36	**F:** ACTTTCTGTTTTACTCGACAACTC **R:** GTCGCTGGGGGTGGCTTATT	1	0.0
15	VM 37	**F:** TGTCCGCGTTCTATAAATCAGC **R:** CGAGGATGAAGTAACAGATGATC	7	0.796
16	VM 38	**F:** AATGGGAAAAGAAAGGGAAGC **R:** TCGTGGCATGCAGTGTCAG	1	0.0
17	VM 39	**F:** GATGGTTGTAATGGGAGAGTC **R:** AAAAGGATGAAATTAGGAGAGCA	10	0.833
18	VM 40	**F:** TATTACGAGAGGCTATTTATTGCA **R:** CTCTAACACCTCAAGTTAGTGATC	1	0.0
19	VM 68	**F:** CAAGGCATGGAAAGAAGTAAGAT **R:** TCGAAGCAACAAATGGTCACAC	1	0.0
20	VM 70	**F:** AAAATCGGGGAAGGAAACC **R:** GAAGGCAAAATACATGGAGTCAC	1	0.0
Total			70	6.855
Average			3.5	0.342

Table 4: Distribution of 20 cowpea accessions into 5 different clusters

S. No.	Cluster No.	No. of Cowpea accessions	Accessions
1.	A	05	IC-3009, IC-3004, IC-201081, IC-58905, IC-249105A
2.	B	02	IC-15665, IC-4506
3.	C	04	EC-472283, EC-390223, IC-39095, IC-51154
4.	D	04	IC-402106, EC-97306, IC-202280, EC-91171A
5.	E	05	EC-911718, EC-9738, IC-33932, IC-202826, EC-472272

Table 3: Genetic similarity coefficient of 20 cowpea accessions derived from SSR markers

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.000																			
2	0.868	1.000																		
3	0.809	0.853	1.000																	
4	0.809	0.765	0.765	1.000																
5	0.941	0.868	0.809	0.809	1.000															
6	0.853	0.809	0.721	0.750	0.853	1.000														
7	0.956	0.882	0.853	0.853	0.956	0.868	1.000													
8	0.809	0.824	0.735	0.706	0.779	0.721	0.824	1.000												
9	0.750	0.794	0.735	0.618	0.721	0.662	0.765	0.882	1.000											
10	0.897	0.882	0.794	0.735	0.897	0.809	0.882	0.853	0.794	1.000										
11	0.838	0.882	0.853	0.735	0.838	0.750	0.882	0.765	0.765	0.824	1.000									
12	0.853	0.809	0.750	0.868	0.882	0.824	0.897	0.750	0.662	0.779	0.809	1.000								
13	0.838	0.824	0.765	0.794	0.838	0.809	0.853	0.765	0.706	0.824	0.765	0.779	1.000							
14	0.956	0.882	0.824	0.824	0.956	0.868	0.971	0.794	0.735	0.882	0.853	0.868	0.853	1.000						
15	0.794	0.721	0.750	0.897	0.794	0.824	0.809	0.632	0.574	0.721	0.691	0.824	0.809	0.809	1.000					
16	0.794	0.750	0.750	0.750	0.794	0.706	0.809	0.662	0.632	0.750	0.721	0.765	0.721	0.809	0.765	1.000				
17	0.750	0.735	0.706	0.647	0.750	0.691	0.765	0.618	0.676	0.706	0.735	0.662	0.647	0.765	0.603	0.691	1.000			
18	0.897	0.824	0.794	0.765	0.897	0.809	0.912	0.735	0.676	0.824	0.824	0.809	0.794	0.912	0.779	0.779	0.706	1.000		
19	0.838	0.706	0.765	0.853	0.779	0.691	0.824	0.735	0.676	0.765	0.706	0.750	0.765	0.794	0.809	0.691	0.588	0.735	1.000	
20	0.721	0.706	0.706	0.794	0.721	0.662	0.765	0.706	0.676	0.706	0.706	0.721	0.824	0.735	0.750	0.632	0.559	0.765	0.765	1.000

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