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Evaluation of genetic diversity in cowpea, *Vigna unguiculata* (L.) Walp gentotypes using Random Amplified Polymorphic DNA (RAPD)

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ABSTRACT

Random amplified polymorphic DNA markers were used to evaluate the genetic diversity in a representative population of cowpea (*Vigna unguiculata* (L.) Walp) from different eco-geographical regions of India. 30 primers could generate a total of 120 RAPD fragments, of which 109 bands (90%) were polymorphic. The polymorphism was scored and used in band sharing analysis to identify genetic relationship. Cluster analysis based on Jaccard's coefficient using UPGMA grouped all the 30 genotypes into three groups at a similarity coefficient 25. Similarity indices ranged from 0.463 to 0.784. The highest similarity coefficient was observed between genotypes TPTC-24 and TPTC-22 indicating the less divergence between them and the lowest was observed between genotypes HC-3-8 and CPD-108 indicating more divergence. Distinct phenotypes identified using RAPD markers could be potential sources of germplasm for cowpea improvement in breeding program.

KEY WORDS: Cowpea, genetic diversity, polymorphism, RAPD

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp], is an essential food crop in less-developed countries of the tropics and subtropics, especially in sub-Saharan Africa, Asia, and Central and South America (Singh *et al*., 1997).

Of the 12.5 million tons of cowpea grains produced worldwide, over 64% takes place on low-input, subsistence farms (FAO 2009) in West and Central Africa. Due to its good protein quality and high nutritional value, cowpea is often referred to as the "poorman's meat". It can grow in soil of low fertility due to its high rates of nitrogen fixation effective symbiosis with mycorhizae and low levels in phosphorous (Singh, 2003) and ability to withstand acid and alkaline soil condition as well as considerable drought condition.

In addition to its use as human food, cowpea hay is an important source of animal fodder during the dry season in many parts of West Africa and India. The plant is generally drought tolerant and when used in rotation with cereals, its ability to fix nitrogen helps restore soil fertility. Despite its importance, the production of cowpea which is about 1000 kg/ha in Sub-saharian regions, does not meet the need of consumers

Knowledge about genetic diversity in available germplasm is very useful for plant breeders. It supports their decision on the

selection of cross combinations from large sets of parent genotypes and is also helpful when they want to widen the genetic basis of a breeding program. Molecular markers based on differences in DNA sequences between individuals generally detect more polymorphism than morphological and protein-based markers and constitute a new generation of genetic markers (Tanksley *et al.,* 1989). Molecular genetic techniques using DNA polymorphism have been increasingly used to characterize and identify a novel germplasm/genetic diversity within the available germplasm collections for use in the crop breeding process (O'Neill *et al*., 2003).

DNA markers survey both functional as well as neutral genetic variation. Alternative molecular markers showing higher levels of polymorphism among closely related genotypes include RAPDs, microsatellites and AFLPs. RAPD markers have been shown to be useful in assessing intraspecific or interspecific genetic variability in many crops species (Diouf and Hilu, 2005). The present study has been taken up to study the RAPD analysis of cowpea genotypes to utilise this technique in assessing genetic diversity in this pulse crop.

MATERIALS AND METHODS

Plant material

The plant material for the study comprised of 30 cowpea genotypes. All genotypes were obtained from different eco-geographical regions of India (Table 1) and used for assessment of diversity. Entries were sown during *kharif*, 2011 at Regional Agricultural Research Station, Tirupati which is situated at an altitude of 182.90m above mean sea level,13^0N latitude and 79^0E longitude. Young and healthy leaves were collected separately from all 30 genotypes of 20 to 25 days old plant, washed and quickly frozen and powdered using liquid nitrogen.

DNA extraction

DNA was extracted from young leaves by using a modified CTAB (cetyl tri methyl ammonium bromide) method (Murray and Thompson, 1986). Fresh young leaves were collected and ground to a fine powder in liquid nitrogen using mortar and pestle and immediately transferred to 1.5ml centrifuge tube containing 700 µl of extraction buffer (0.1M Tris-HCl at pH 8.0, 1.4M NaCl, 0.02 M EDTA at pH 8.0, 2% CTAB, 1% PVP, 0.1% β mercaptoethanol). The suspension was mixed well and incubated for 60 min at 60°C. The tube was centrifuged at 10,000 rpm for 10 min at room temperature. The solution was emulsified with an equal volume of a mixture of chloroform-iso amyl alcohol (24:1) for 5 minutes and repeated twice. Following centrifugation, the upper aqueous phase was collected and transferred into centrifuge tube and DNA was precipitated in presence of 0.6v of ice cold isopropanol and 0.1v of sodium acetate by quick inversion. Precipitated DNA was collected by centrifugation at 13,000 rpm for 10' at 4^0C, rinsed with 70% ice cold ethanol and dried before redissolving in TE buffer. The RNA contamination was removed by giving RNase treatment at 37^0C for 1hr. The purified DNA was quantified by using Nanodrop spectrophotometer (ND-1000 spectrophotometer). The quality of genomic DNA was checked by using 1% agarose in presence of EtBr. DNA samples were stored at -20^0C until further analysis.

RAPD analysis

PCR amplification reactions (Williams *et al*., 1990) were performed with

decamer primers obtained from Operon Technology (Almeda, Calif., USA). Total of 30 decamer primers were screened by polymerase chain reaction. PCR amplification reaction were carried out in a total reaction volume of 20 µl containing 1XAssay buffer (50Mm KCl, 10Mm Tris-HCl, 0.01% gelatin) 2mM MgCl2, 0.2mM dNTP, 1 picomole primer, 25-30ng of genomic DNA and 1U Taq DNA polymerase (Fermentas). Amplification was performed in 0.2ml thin walled tubes using a thermocycler (Eppendorf, Germany) programmed for initial denaturation at 94^0C for 2 min followed by 40 cycles of denaturation at 94^0C for 1min, 37^0C for 1 min and 72^0C for 2 min. The amplification was completed with 7 min final extension for 72^0C. The amplification products were subjected to electrophoresis on 1% agarose gels with 1XTBE buffer stained with EtBr. The 1 Kb DNA ladder plus molecular weight marker was used to compare the molecular weight of amplified products. The DNA bands was then visualized under UV light and photographs were taken by using Gel Documentation system (Alpha Infotech, Alpha Imager). To test the reproducibility of RAPD markers, the reactions were repeated at least twice. The list of primers, their sequences and characteristics of the amplification products obtained from 30 genotypes (Table 2).

Data analysis

The amplified products for RAPD analysis were scored visually based on the presence (taken as '1') or absence (taken as '0') of band for each primer. Each RAPD fragment was treated as a unit character and only clear and unambiguous bands were scored. The data was used to generate Jaccard's coefficients for expressed RAPD bands (Table 3). The Jaccard's coefficients were used to construct a dendrogram using the unweighted pair group method with Arithmetic averages (UPGMA). The computer package NTSYS-PC was used for cluster analysis.

RESULTS AND DISCUSSION

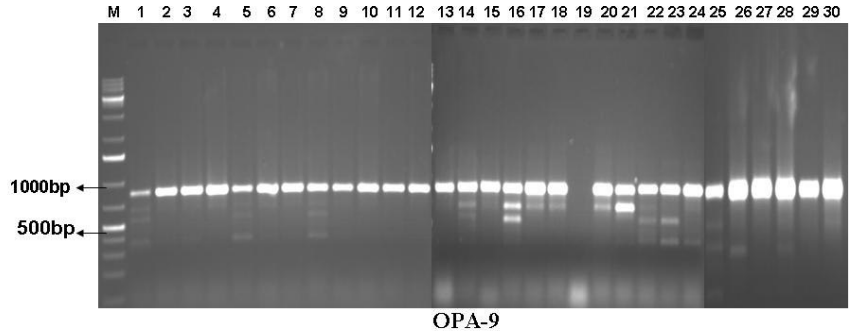
The objective of the present study was to assess the extent of genetic diversity based on DNA bands data in 30 cowpea genotypes. Morphological characteristics provide the basic information about the magnitude of genetic variability in seed coat color, 100 seed weight and pod length in cowpea of various varieties. Table 1 revealed a wide variability in seed size; seed is influenced by natural and artificial selection, socio economic conditions and consumers preferences within localities. RAPD technique is a simpler and quicker method for characterization and analysis of genetic diversity among cowpea genotypes. Analysis of the relationship is based on number of the DNA fragments. 30 decamer primers used to detect RAPD markers among the 30 cowpea genotypes. A total of 120 bands were scored of which 90% exhibited polymorphism. Out of 30 primers, 14 primers show 100% polymorphism and 6 primers show more than 80% polymorphism while rest of the primers resulted in no amplification. The number of bands ranged between 2 to 15 (Table 2). The number of RAPD loci generated was higher for the primer OPB-10 which amplified 15 fragments followed by OPB-1(10 fragments) and OPA-1(8 fragments). The lowest number of fragments was generated by the primer OPA-14 and OPA-16 (1 fragment). A representative profile of RAPD products (amplified with primer OPA-9 and OPB-10) from all 30 cowpea genotypes (Fig.1). The ability to resolve genetic variation may be more directly related to the number of polymorphism detected by the marker techniques and the percentage of

polymorphic RAPDs.Genetic relationships between wild and cultivated Vigna species were studied by cluster analysis and genetic distance determination by using RAPD markers (Samarajeewa *et al*.,2002)

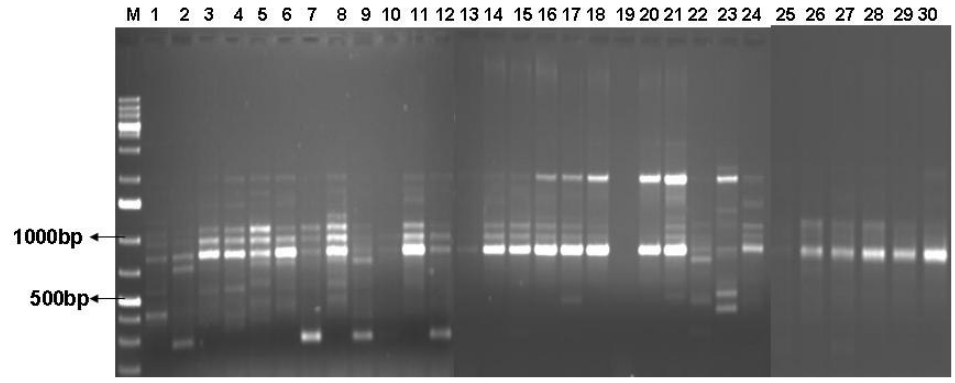
Simple matching coefficient was used to assess genetic similarities, divergence and relations among 30 cowpea genotypes. A dendrogram based on simple matching coefficient analysis grouped 30 genotypes into 3 clusters (Fig. 2), with similarity indices ranging from 0.463 to 0.784 (Table 3) at a similarity coefficient of 25. Cluster I was comprises of GC-817, GC-815, and SUBHRA. Cluster II comprised of HC-3-8, PTB-1, KBC-4, DC-15. Cluster III comprised of 23 genotypes. Cluster-III was further divided into two sub clusters among GC-810, CPD-119, VS-15-3-1, GC-3, CPD-108, PGCP-12, RC-101-5, TPTC-21, TPTC-28, TPTC-31 showed independent positions and rest of the genotypes are enclosed in it. The highest value of similarity coefficient (0.784) was detected between genotypes TPTC-24 and TPTC-22 indicates the less divergence., and could not be used in hybridization programme .These results are in accordance to the studies of phylogenetic diversity and relation ship in cowpea by using RAPD polymorphic DNA marker (Karuppanapandian *et al*.,2006). The lowest value of similarity coefficient (0.463) was evident between genotypes HC-3-8 and CPD-108 indicates more divergence respectively and superior lines could be developed by using these parents in breeding programme. The dendrogram and simple matching coefficient values give an idea about the nature of the individual sample in the whole sample set. All cowpea samples could be distinguished from one another based on these polymorphic bands. These genotypes could be useful in breeding programmes.

CONCLUSION

The results of the present study showed that cowpea genotypes with in India constitute a broad genetic base. From clustering pattern and genetic relationship obtained using RAPD markers, breeders can identify the diverse genotypes from different clusters and employ them in their future breeding programmes. Further, a perusal on the clustering pattern based on phenotypic traits of seed size, pod length, seed colour vis-à-vis the one based on RAPD markers reflected that there was no clear similarity between the two. The selection is to be based on either on the phenotypic traits or on the molecular markers separately. Hence, studies on morphological markers are quite useful in analyzing the genetic differences in plant population at DNA level (Yoon *et al*.,). The genetic diversity obtained in this study might be useful in selection of superior parents for evolution of desired genotypes.



OPA-9



OPB -10

Fig. 1: **DNA fragments of 30 genotypes of cowpea using RAPD primers: OPA-9 and OPB-10**

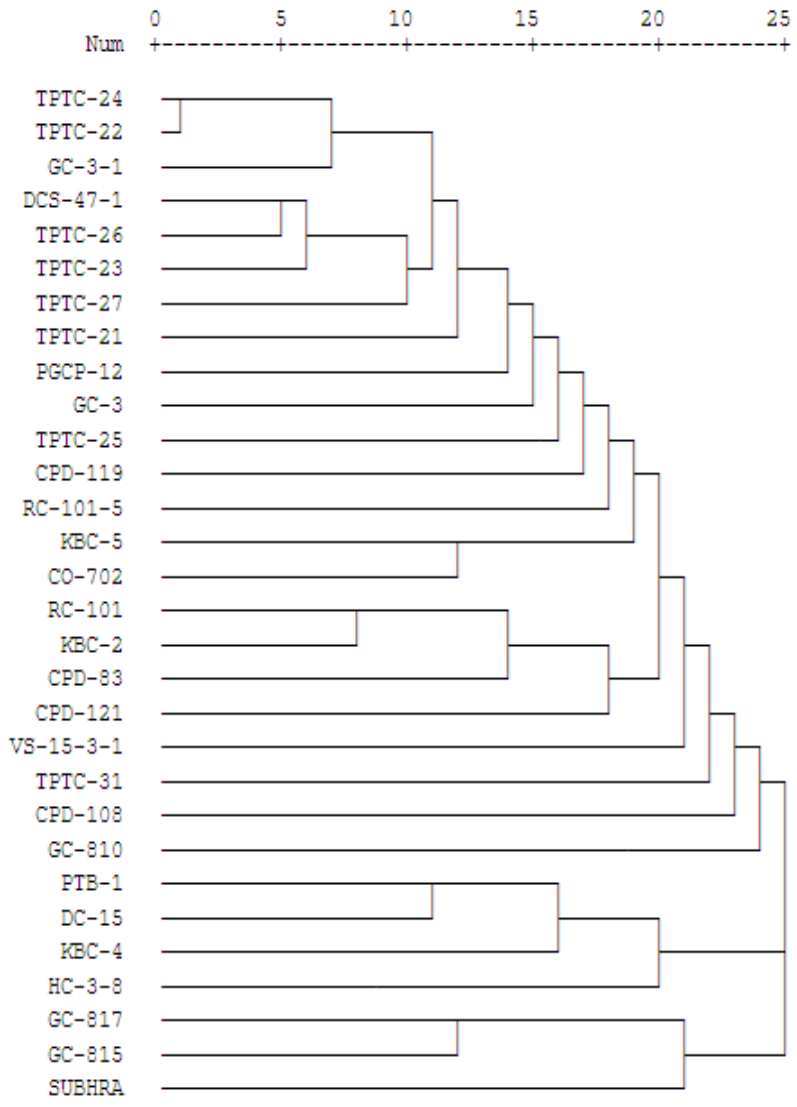


Fig. 2: Dendrogram showing various genetic relationship among various cowpea genotypes

Table 1 : Morphological characteristics of cowpea genotypes used in diversity analysis

S. No	Genotypes	Seed colour	100 seed weight (gm)	Pod length (cm)	Source
1	GC-810	White	17	14	SK.Nagar
2	GC-817	Brown	16	16.2	SK.Nagar
3	GC-815	Brown	16	17	SK.Nagar
4	CPD-83	White with black eye	15	14.4	Durga pura
5	CPD-119	White with brown eye	12	13.8	Durga pura
6	CPD-121	White	14	13.4	Durga pura
7	HC-3-8	White	19	16	Hisar
8	VS-15-3-1	White	16	16.4	Pattambi
9	PTB-1	White	11	14.6	Pattambi
10	KBC-5	Brown	14	17.4	Banglore
11	KBC-4	Brown	16	14	Banglore
12	DC-15	White with Brown eye	14	13	Darwad
13	RC-101	Purple red	16	17	Rajasthan
14	GC-3	White with red eye	12	14.6	Gujarat
15	KBC-2	Brown	20	24.6	Karnataka
16	CPD-108	Brown	21	12.8	Durga pura
17	DCS-47-1	Variegated seed	12	10	Darwad
18	SUBHRA	Light Brown	14	13.8	Pattambi
19	PGCP-12	Dark Brown	16	10.8	Panth Nagar
20	RC-101-5	white	15	10.4	Rajasthan
21	GC-3-1	White with red eye	12	11.8	Gujarat
22	CO-702	Brown	12	12.2	Coimbatore
23	TPTC-26	White with Brown eye	14	11	Andhra Pradesh
24	TPTC-21	Brown	17	13.6	Andhra Pradesh
25	TPTC-23	Dirty white	11	7.6	Andhra Pradesh
26	TPTC-27	Brown	18	9.8	Andhra Pradesh
27	TPTC-24	Dark Brown	9	11.8	Andhra Pradesh
28	TPTC-28	Brown	14	13.8	Andhra Pradesh
29	TPTC-22	White with Brown eye	12	12.8	Andhra Pradesh
30	TPTC-31	Brown	20	17.5	Andhra Pradesh

Table 2 : Amplified DNA bands and polymorphism generated in 30 cowpea genotoypes using 30 RAPD primers.

S.No	Primer	Sequence	TNB	NPB	% p
1	OPA 1	CAGGCCCTTC	8	8	100
2	OPA 2	TGCCGAGCTG	6	6	100
3	OPA 3	AGTCAGCCAC	7	6	85.7
4	OPA 4	AATCGGGCTG	6	5	83.3
5	OPA 5	AGGGGTCTTG	5	4	80
6	OPA 6	GGTCCCTGAC	2	0	0
7	OPA 7	GAAACGGGTG	0	0	0
8	OPA 8	GTGACGTAGG	0	0	0
9	OPA 9	GGGTAACGCC	4	4	100
10	OPA10	GTGATCGCAG	0	0	0
11	OPA 12	TCGGCGATAG	0	0	0
12	OPA 13	CAGCACCCAC	6	6	100
13	OPA 14	TCTGTGCTGG	1	0	0
14	OPA 16	AGCCAGCGAA	1	1	100
15	OPA 17	GACCGCTTGT	4	4	100
16	OPA 18	AGGTGACCGT	0	0	0
17	OPA 19	CAAACGTCGG	0	0	0
18	OPA 20	GTTGCGATCC	0	0	0
19	OPB 1	GTTTCGCTCC	10	9	90
20	OPB 3	CATCCCCCTG	8	7	87.5
21	OPB 6	TGCTCTGCCC	6	5	83.3
22	OPB 7	GGTGACGCAG	4	4	100
23	OPB 9	TGGGGGACTC	2	2	100
24	OPB 10	CTGCTGGGAC	15	15	100
25	OPB 12	CCTTGACGCA	5	3	60
26	OPB 13	TTCCCCCGCT	3	3	100
27	OPB 15	GGAGGGTGTT	4	4	100
28	OPB 17	AGGGAACGAG	4	4	100
29	OPB 19	ACCCCCGAAG	2	2	100
30	OPB 20	GGACCCTTAC	7	7	100

Table 3 : Average similarity coefficient values calculated on the basis of similarity matrices of 30 genotypes of cowpea.

S No	1	2	**3**	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	1.00																													
2	0.52	1.00																												
3	0.551	0.7	1.00																											
4	0.54	0.567	0.581	1.00																										
5	0.558	0.568	0.567	0.653	1.00																									
6	0.52	0.63	0.577	0.634	0.649	1.00																								
7	0.484	0.56	0.526	0.565	0.551	0.56	1.00																							
8	0.591	0.524	0.553	0.606	0.653	0.618	0.49	1.00																						
9	0.485	0.54	0.539	0.56	0.607	0.638	0.586	0.563	1.00																					
10	0.505	0.612	0.627	0.551	0.553	0.546	0.595	0.57	0.587	1.00																				
11	0.55	0.56	0.574	0.612	0.627	0.608	0.591	0.582	0.649	0.608	1.00																			
12	0.475	0.593	0.575	0.597	0.582	0.61	0.647	0.509	0.706	0.561	0.656	1.00																		
13	0.525	0.567	0.581	0.655	0.62	0.634	0.6	0.59	0.591	0.67	0.612	0.597	1.00																	
14	0.529	0.539	0.568	0.606	0.67	0.602	0.568	0.561	0.659	0.652	0.646	0.616	0.622	1.00																
15	0.561	0.555	0.602	0.659	0.592	0.638	0.586	0.578	0.645	0.655	0.649	0.688	0.733	0.659	1.00															
16	0.505	0.485	0.514	0.49	0.538	0.485	0.463	0.509	0.524	0.5	0.608	0.5	0.49	0.539	0.509	1.00														
17	0.56	0.6	0.613	0.634	0.589	0.6	0.523	0.605	0.638	0.6	0.673	0.66	0.65	0.666	0.702	0.663	1.00													
18	0.547	0.591	0.606	0.515	0.564	0.525	0.521	0.519	0.567	0.626	0.604	0.606	0.612	0.581	0.634	0.494	0.676	1.00												
19	0.509	0.581	0.549	0.602	0.586	0.597	0.563	0.62	0.639	0.631	0.642	0.612	0.618	0.557	0.622	0.534	0.632	0.53	1.00											
20	0.79	0.515	0.5	0.617	0.553	0.578	0.494	0.495	0.571	0.546	0.591	0.545	0.634	0.539	0.604	0.53	0.696	0.574	0.614	1.00										
21	0.584	0.625	0.622	0.66	0.672	0.64	0.61	0.6	0.647	0.656	0.699	0.669	0.693	0.66	0.663	0.64	0.747	0.637	0.689	0.673	1.00									
22	0.515	0.556	0.604	0.545	0.563	0.51	0.588	0.612	0.534	0.696	0.57	0.54	0.681	0.564	0.614	0.51	0.594	0.568	0.642	0.51	0.666	1.00								
23	0.532	0.584	0.613	0.574	0.618	0.57	0.509	0.605	0.563	0.647	0.626	0.644	0.683	0.666	0.622	0.57	0.754	0.58	0.647	0.584	0.715	0.656	1.00							
24	0.612	0.606	0.588	0.642	0.625	0.656	0.572	0.611	0.598	0.691	0.666	0.636	0.594	0.66	0.68	0.574	0.701	0.585	0.656	0.59	0.711	0.616	0.685	1.00						
25	0.553	0.594	0.576	0.646	0.613	0.594	0.545	0.555	0.542	0.61	0.621	0.592	0.715	0.647	0.65	0.548	0.737	0.656	0.676	0.712	0.698	0.636	0.737	0.634	1.00					
26	0.616	0.578	0.607	0.613	0.628	0.61	0.545	0.631	0.601	0.61	0.605	0.576	0.663	0.663	0.633	0.594	0.704	0.59	0.66	0.61	0.682	0.636	0.704	0.683	0.66	1.00				
27	0.509	0.614	0.645	0.585	0.601	0.565	0.58	0.528	0.59	0.631	0.594	0.58	0.688	0.62	0.622	0.581	0.679	0.53	0.649	0.519	0.689	0.642	0.679	0.623	0.611	0.66	1.00			
28	0.514	0.54	0.57	0.575	0.592	0.587	0.569	0.548	0.628	0.621	0.649	0.585	0.591	0.61	0.595	0.587	0.653	0.535	0.673	0.587	0.679	0.581	0.638	0.598	0.617	0.666	0.606	1.00		
29	0.538	0.594	0.623	0.613	0.644	0.61	0.577	0.57	0.633	0.61	0.605	0.656	0.715	0.696	0.633	0.578	0.721	0.543	0.676	0.61	0.73	0.653	0.721	0.666	0.686	0.686	0.784	0.65	1.00	
30	0.531	0.526	0.541	0.515	0.534	0.464	0.473	0.504	0.49	0.559	0.54	0.494	0.597	0.567	0.52	0.559	0.552	0.554	0.578	0.559	0.656	0.622	0.567	0.571	0.591	0.642	0.578	0.568	0.642	1.00

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