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Original Article

Genetic diversity and SSR marker assisted salt screening of rice (*Oryza sativa* L.)

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Abstract

Assessment of genetic diversity of rice (*Oryza sativa* L.) is an important tool for rice breeding and an essential component in germplasm characterization and conservation. Phenotypic and genotypic evaluation for salinity tolerance was done at the seedling stage. Phenotyping was done in hydroponic system using salinized (EC 12 dS/m) nutrient solution following International Rice Research Institute (IRRI) standard protocol. Among them, 7 germplasms were found to be saline tolerant viz. Horkuch, STL20, PBSAL730, PBSAL655, FL378, FL478 and PBRC37. According to SES scoring, 24 rice germplasms were selected to identify the level of salt tolerance and fingerprinted by using nine simple sequence repeat (SSR) primers. In DNA profiling, a total number of 76 alleles were detected. The number of allele per locus ranged from 6 to 15, with an average of 8.44 and the Polymorphism Information Content (PIC) value ranged from 0.5956 to 0.7725 with an average of 0.6847. The average gene diversity over all SSR loci for the 24 genotypes was 0.7140, ranging from 0.6188 to 0.7908. Positive correlations were found between gene diversity, PIC value and number of allele. The "Unweighted Pair Group Method of Arithmetic Means (UPGMA)" dendrogram constructed from Nei's (1983) genetic distance produced two main distinct clusters of the 24 rice genotypes. A cluster was consisted with a highly susceptible germplasms, while the second cluster comprises of mostly salt tolerant and moderately tolerant germplasms because of lower genetic distance between the varieties. These findings can have the potential role for further improvement of salinity tolerance rice genotypes through marker assisted breeding.

Keywords: Genetic diversity, rice, salinity tolerance, SSR primers, DNA profiling, UPGMA.

1. Introduction

Rice (*Oryza sativa* L.) is the most important food crop and the staple food for more than 50 percent of the world population (Aggarwal *et al.* **2002**). More than 90 percent of rice is produced and consumed in Asia (FAO **2004**). This staple food ranked first position by production was estimated 34.8 million metric tonnes in the year 2014 among all cereals in Bangladesh (FAO **2014**).

Salt stress is a major constraint to cereal production worldwide (Tuteja **2012**). In Bangladesh, about 2.8 million hectares of coastal soil has become saline due to heavy withdrawal of surface and

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groundwater for irrigation and intrusion of seawater. The total saline area forms a third of the 9 million hectares of total cultivated area in Bangladesh (ABSPII **2006**). The coastal saline soils are distributed unevenly in 64 thanas of 13 districts, covering portions of eight agro-ecological zones of the country (Seraj and Salam **2000**). Agriculture is the major sector for Bangladesh's economy and the coastal area of Bangladesh is very fertile for growing rice. Increase in salinity intrusion and increase in soil salinity will have serious negative impacts on agriculture. The food production seems to face a great concern in near future due to climate change. In Bangladesh, rice production may fall by 10 % and wheat by 30 % by 2050 (IPCC **2007**).

Deoxyribonucleic acid (DNA) is the genetic material that contains the clue to identify the potentiality and diversity among different plant germplasms (Semagn *et al.* **2006**). Though, rice genome sequence is available (The Rice Genome Mapping Project **2005**), most researchers are trying to identify particular segment of DNA or gene in a specific chromosome (Semagn *et al.* **2006**). Molecular markers are the molecules that can trace a desired gene in examined genotypes. Molecular markers provide information that can help to define the distinctiveness of germplasms and their ranking according to the number of close relative and their phylogenetic position.

DNA-based marker such as SSR markers (or microsatellites), were codominant and highly polymorphic; offer an easy, accurate, and quantifiable measure of the genetic variation within crop plants (Sunnucks **2000**; Collard **2008**). SSR or microsatellite markers have been proved to be ideal for making genetic maps (Islam **2004**; Niones **2004**), assisting selection (Bhuiyan **2005**) and studying genetic diversity in germplasms. SSR markers play an important role while identifying gene for salt tolerance or in introgressing the genes to develop new cultivars. For rice, there are nearly about 15,000 SSRs now available (www.gramene.org) and are currently being used to develop high density genetic maps, genotype rice accessions, determine the genetic structure and diversity patterns, optimize the assembly of core collections, and for marker-assisted breeding (McCouch *et al.* **2002**; Yu *et al.* **2003**; Garris *et al.* **2005**). Microsatellites, referred to as simple sequence repeats (SSR), are a useful marker for genome analysis, because of the significant level of allelic diversity that may be revealed (Ishii *et al.* **2001**). Polymorphisms in the microsatellite region are considered to result from misreplication of repeated sequences (Richards and Sutherland **1994**), and the polymorphisms detected by PCR, using pairs of primers specific to the sequences flanking the microsatellite repeats.

Therefore, the objective of the present study was undertaken to screen rice germplasms under salinized conditions at the seedling stage and to identify salt stress tolerent rice genotypes using microsatellite markers at seedling stage.

2. Materials and methods

2.1. Plant material

The experiments were done at Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh in the early year of 2010. Twenty four rice germplasm accessions, with diverse genetic background, were used in this study (**Table 1**).

Table 1. List of plant material used in the study

SL.	Variety	Source
1.	Iratom-24	BINA
2.	PBSAL-613	BINA
3.	PBSAL-614	BINA
4.	PBSAL-655	BINA
5.	Binadhan-7	BINA
6.	PBSAL-656	BINA
7.	PBSAL-730	BINA
8.	Binadhan-4	BINA
9	STL-15	BINA
10.	STL-20	BINA
11.	FL-378	BINA
12.	FL-478	BINA
13.	KASHRAIL	BINA
14.	HORKUCH	BINA
15.	ASHFAL	BINA
16	PBRC-37	BINA
17.	PBRC-83	BINA
18	PBRC-90	BINA
19	PBRC-110	BINA
20.	IR84645	BINA
21.	IR45427	BINA
22.	IR68144	BINA
23.	IR72593	BINA
24.	IR63731	BINA

2.2. Phenotypic evaluation of salinity tolerance

The genotypes were screened for salt tolerance at seedling stage in hydroponic scheme using IRRI standard protocol (Gregorio **1997**). Salinized and non-salinized setups with three replications were maintained. The evaluation was done using Yoshida *et al*. (**1976**) nutrient solution at the glasshouse. The nutrient solution was salinized by adding crude salt to obtain desired EC (12 dS/m). The modified standard evaluation system was used in rating the visual symptoms of salt toxicity (IRRI **1997**). Visual rating of salinity tolerance was done according to **Table 2**. This scoring discriminated

the susceptible from the tolerant and the moderately tolerant genotypes. Initial and final scoring was done at 13 days and 22 days after salinization.

Table 2. Modified standard evaluation score (SES) of visual salt injury at seedling stage

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips of few leaves whitish and rolled	Tolerant
5	Growth severely retarded, most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

2.3. CTAB mini preparation DNA extraction

DNA isolation was done from fresh leaf tissues of 14-day old seedlings. DNA was extracted using the mini preparation CTAB method. Grinding of leaf sample with extraction buffer and SDS was followed by incubating the leaf sap at 65 °C for 10 min. 100 µl NaCl and 100 µl CTAB were added sequentially and mixed well; and incubated again at 65 °C for 10 minutes. After that the suspensions were transferred to a new plate. 900 µl chloroform: isoamyl (24:1) was added and mixed well by a shaker. The sample was then centrifuged at 11,000 rcf/g for 10 minutes. After that the supernatant were transferred into new eppendorf tubes. Then 600 µl ice-cold isopropanol was added into the new eppendorf tubes and shaken slowly and then centrifuged at 11,000 rcf/g for 15 minutes. The supernatant was decanted and air dried for at least one hour. Pelletes were washed with 70 % ethanol (200 µl), spinned for 15 minutes at 11,000 rcf/g and then air-dried for 30-60 mins. Then the ethanol was removed and air-dried. The pelletes were resuspended in 40.0 µl X TE buffer.

2.4. Primer designing, DNA amplification and SSR

A total of nine SSR primers were screened (**Table 3**) to yield amplification products on the total DNA obtained from the leaf tissues. The optimal reaction for SSR analysis was set up under the following conditions: 1.5 µl 10X PCR buffer, 0.5 µl Taq DNA polymerase, 1 µl forward primer, 1 µl reverse primer, 0.75 µl dNTPs, 8.25 µl sterilized ddH_2O and 1 µl template DNA for total volume of 14 µl. The amplification products were separated on 1.5 % agarose gels in 0.5X TBE buffer. The DNA band patterns were visualized under UV light and photographed using a polaroid camera.

Table 3. Summary of microsatellite (SSR) markers used for diversity study

Primer name	Expected product size (bp)	Sequence	Annealing Temp. (°C)
RM10720	204	**F:**GCAAACGTCTACG TGAGAAACAAGC	55
		R:GCATGTGGTGCCT TAACATTTGG	
RM510	122	**F:**AACCGGATTAGTT TCTCGCC	55
		R:TGAGGACGACGA GCAGATTC	
RM585	233	**F:**CAGTCTTGCTCCG TTTGTTG	55
		R:CTGTGACTGACTT GGTCATAGG	
RM336	154	**F:**CTTACAGAGAAAC GGCATCG	55
		R:GCTGGTTTGTTTC AGGTTCG	
RM152	151	**F:**GAAACCACCACAC CTCACCG	55
		R:CCGTAGACCTTCT TGAAGTAG	
RM310	105	F:CCAAAACATTTAA AATATCATG	55
		R:GCTTGTTGGTCAT TACCATTC	
RM21	157	**F:**ACAGTATTCCGTA GGCACGG	55
		R:GCTCCATGAGGGT GGTAGAG	
RM28102	168	F:CACTAATTCTTCG GCTCCACTTTAGG	55
		R:GTGGAAGCTCCGA GAAAGTGC	
RM28502	155	**F:**CGAGCAGATCTGA TGTCGTCTTCC	55
		R:CTTTGCTTTGCATG CCTCACG	

2.5. Analysis of SSR Data

The summary statistics including the number of alleles per locus, major allele frequency, gene diversity and PIC values were determined using POWER MARKER version 3.25 (North Carolina, USA) (Liu and Muse **2005**), genetic analysis software. Molecular weights for microsatellite products, in base-pairs, were estimated with Alpha Ease FC 4.0 software. The individual fragments were assigned as alleles of the appropriate microsatellite loci.The PIC value described by Botstein *et al*. (**1980**) and modified by Anderson *et al*. (1993) for self-pollinated species was calculated as follows

$$PIC_i = 1-\sum_{J=1}^{n} P^2_{ij}$$

Where, P*ij* is the frequency of allele for the *i*th marker, and summed over *n* a

Twenty four germplasms were clustered based on the matrix of genetic similarities using the unweighted pair group method with arithmetic averages (UPGMA). The cluster analysis and dendrogram construction were performed with NTSYS-PC (version 2.1) (Setauket, NY, USA). Genetic variation was measured in terms of genetic diversity and was computed by averaging PIC estimates over all loci (Weir **1996**). Number of alleles, average PIC values, and average GS were computed on the basis of different rice gene pools according to the results from cluster analysis and origin of the accessions. Differences in average PIC values between the three groups were evaluated by analysis of variance (SAS Institute, Inc. **1998**). PIC values were calculated for the accessions grouped in each gene pool at each locus. Loci were used as blocks to separate the variation among loci from the error term and increase the sensitivity of the statistical analysis. Heterogeneity (HG) by accession and by marker was calculated as percentage of heterogeneous loci per accession across all accessions and loci, respectively.

3. Results

3.1. Analysis of salinity screening and genetic diversity

SSR markers have been used to characterize the genetic diversity of the germplasms (Emanuelli *et al*. **2013**). 24 germplasms of rice were analyzed using nine SSR markers and all markers are polymorphic with 76 polymorphic fragments ranging in size from 54 to 385 bp. The number of alleles detected by a single marker ranged from 6 (RM152) to 15 (RM510) with an average of 8.44 alleles (**Table 4**). The PIC value denotes the allelic diversity and frequency among genotypes. The PIC was calculated for each marker as a relative measure of informativeness and ranged between 0.5956 (RM152) to 0.7725 (RM510) with an average value of 0.6847 (Table 4). The average gene diversity over all SSR loci for the 24 genotypes was 0.7140, ranging from 0.6188 to 0.7908 (Table 4).

Table 4. Data on repeat motif, number of alleles, number of rare alleles, PIC value and gene diversity (GD) found among 24 rice germplasms for 9 SSR markers

Locus	*Repeat Motif	No. of alleles	*Rare alleles	Allele Size ranges (bp)	Difference (bp)	PIC	Gene Diversity
RM10720	(TA)34	9	5	50-315	265	0.7232	0.7552
RM510	(GA)15	15	11	37-422	385	0.7725	0.7908
RM585	(TC)45	7	2	64-212	148	0.7479	0.7804
RM336	(CTT)18	8	3	22-211	189	0.7534	0.7831
RM152	(GGC)10	6	3	56-110	54	0.5956	0.6188
RM310	(GT)19	7	2	39-159	120	0.5975	0.6233
RM21	(GA)18	9	4	55-176	121	0.7114	0.7361
RM28102	(TATC)10	8	4	50-176	126	0.6379	0.6710
RM28502	(GA)26	7	2	98-274	176	0.6226	0.6675
Mean		8.44	4			0.6847	0.7140

However, significant positive correlations were found between gene diversity, PIC value and number of allele.

3.2 Cluster Analysis

The using of the unweighted pair group method with arithmetic mean (UPGMA) cluster tree analysis led to the grouping of 24 germplasms in two major clusters (**Fig 1**). Iratom24 alone belonged to cluster 1 and this genotype is separated from other 23 genotypes. Under salt stress, this line was highly susceptible and shown higher genetic distance (0.8750) (**Table 5 S1**). Among the lines of sub-cluster 2, Horkuch along in sub-sub cluster 2(a) and PBRC83, Ashfal, PBRC37, PBSAL655, PBSAL613, PBSAL614, Binadhan4, Binadhan7, STL15, FL378, STL20, PBSAL730, FL478, PBSAL656 and Kashrail belonged to sub-sub cluster 2(b).

In sub-sub cluster 2 (b), PBSAL730 and FL478; PBSAL613 and PBSAL614 both showed lowest similarity (0.0881) (**Table 5 S1**). Therefore, at seedling stage these germplasms were observed moderately tolerance under salt stress respectively. Based on UPGMA analysis and average gene diversity, Horkuch, STL20, PBSAL730, PBSAL655, FL378, FL478 and PBRC37 were concluded to be the salt tolerant lines among the 24 germplasms.

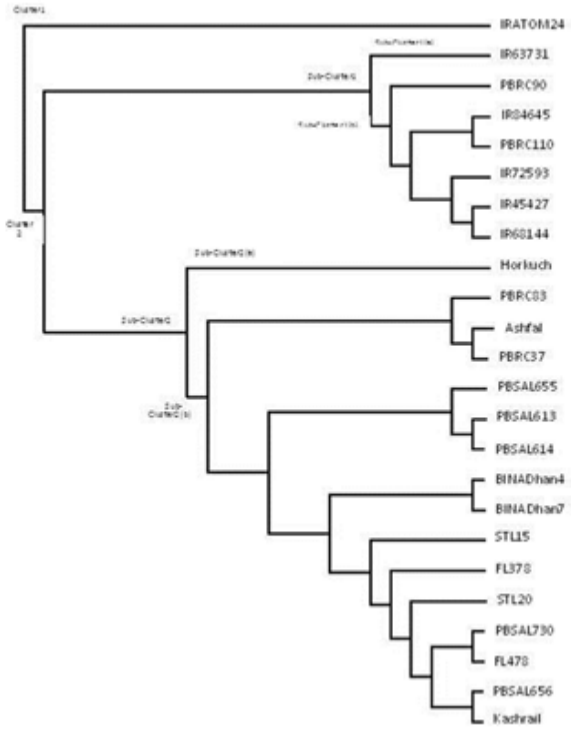

Fig 1. Genetic similarity among 24 rice genotypes based on simple sequence repeat (SSR) marker data.

4. Discussion

High salinity has detrimental effects on plant growth and development in the form of impaired metabolism, necrosis and limited protein synthesis (Astorga and Meléndez **2010**). Salinity affects yield and yield components in rice germplasms. In the present study, a total of 7 germplasms designated as Horkuch, STL20, PBSAL730, PBSAL655, FL378, FL478 and PBRC37 were confirmed as the most salt tolerant lines, besides 16 moderately salt tolerant lines and a single highly susceptible line. Mapping of salt tolerant genes can be performed using SSR markers (Nguyen 2001). In our study, markers RM1072, RM510, RM585, RM336, RM152, RM310, RM21, RM28102 and RM28502 showed significant association with one or more of the studied traits at salt stress. Therefore, these markers may be further tested either on segregating populations or recombinant inbred populations derived from tolerant and sensitive rice varieties for their potential in marker-assisted selection for salt tolerance. Pervaiz *et al*. (**2009**) assessed genetic variability in 35 Asian rice cultivars using 32 SSR markers. They found considerable polymorphism between Basmati and coarse rice varieties as indicated by the amplification of 144 alleles in 35 rice cultivars. Number of alleles in their study ranged from 2 (for markers RM10, RM13, RM19) to 13 (for marker RM70) with an average of 4.5 alleles per locus. In our study, PIC values showed a significant positive linear correlation with the number of alleles at SSR locus. Rahman *et al*. (**2010**) screened 28 local rice varieties with 7 primer pairs and found 82 alleles. Marker RM335 produced the maximum number of alleles (15) and had the highest PIC value (0.91). Kanawapee *et al*. (**2011**) investigated genetic diversity in 30 rice genotypes using RAPD and SSR markers and found higher level of polymorphism in SSR than in RAPD markers. Eight SSR markers were employed by Niones *et al*. (**2006**) to develop a high resolution map of salt tolerant germplasms in rice. Identification of molecular markers tightly linked to salt tolerant genes can serve as landmarks for the physical localization of such genes facilitating marker assisted selection (MAS). Al-Amin *et al*. (**2013**) used twenty eight rice germplasms for identification of salt tolerant rice lines both phenotypically (salinity screening at the seedling stage) and genotypically using SSR markers. The tested markers they employed were RM127, RM140 and RM443 to identify salt tolerant lines in rice and can also be used in marker-assisted selection (MAS) for breeding, quantitative trait loci (QTL) mapping, study of genetic diversity in germplasms in rice salinity breeding program.

Clustering based on SSR marker grouped the selected salt-tolerant/moderately tolerant and salt-sensitive/moderately sensitive genotypes into two clusters. Several additional sub-clusters were observed within the main clusters. The dendrogram revealed that the genotypes that derivatives of genetically similar type clustered together. Chakravarti *et al*. (**2006**) classified the rice genotypes into 11 distinct groups. Cluster analysis of the 193 accessions parental lines of rice showed 3 major groups and nine subgroups (Yu *et al*. **2003**). Based on above result, it was concluded that, the maximum tolerant and moderately tolerant germplasms were grouped in same cluster due to the lower genetic distance of the gene types and higher similarity.

5. Conclusion

The SSR markers used in this study could not classify the selected genotypes into different salt-tolerant categories. However, we observed an association between a representative set of the studied genotypes with SSR markers. These markers may be useful in screening for salt tolerance in rice germplasm. However, the association of these markers with salt tolerant genes/quantitative trait loci needs to be confirmed in segregating populations or any other suitable mapping populations so that the potential of these markers in marker- assisted selection schemes can be determined. Therefore, SSR marker analysis is capable to detect major gene locus for plant breeders to develop new cultivars. The identified salt tolerant rice germplasms of the present study could be further tested in saline areas

to observe yield potentiality for developing high yielding salt tolerant varieties suitable for saline areas.

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Conflict of interest statement

We declare that we have no conflict of interest.

Abbreviations

IRRI: International Rice Research Institute, SSR: Simple Sequence Repeat, PIC: Polymorphism information content, UPGMA: Unweighted Pair Group Method of Arithmetic Means, DNA: Deoxyribonucleic acid, MAS: Marker-assisted Selection, QTL: Quantitative Trait Loci.

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Table 5 S1: Summury of Nei's(1983) genetic distance (below diagonal)values for various cultivar pairs of rice .

OUT	Ashfal	Bina dhan4	PBSAL730	IRATOM24	FL378	FL478	Horkuch	IR45427	IR63731	IR68144	IR72593	IR84645	PBSAL656	Kashrail	PBRC110	PBRC37	PBRC83	PBRC90	PBSAL613	PBSAL614	PBSAL655	Bina Dhan7	STL15	STL20
Ashfal	….																							
Bina dhan4	0.7222	….																						
PBSAL730	0.5191	0.5651	….																					
IRATOM24	0.7222	0.7778	0.7873	….																				
FL378	0.4635	0.5325	0.2318	0.8103	….																			
FL478	0.4405	0.5651	**0.0881**	0.7873	0.1992	….																		
Horkuch	0.6206	0.7778	0.7318	0.7222	0.6437	0.7318	….																	
IR45427	0.4540	0.7222	0.4310	0.8333	0.5325	0.3429	0.8333	….																
IR63731	0.5325	0.8333	0.6762	0.8103	0.7318	0.5976	0.8333	0.5651	….															
IR68144	0.5556	0.5556	0.6762	0.8333	0.6762	0.5976	0.8333	0.3103	0.5881	….														
IR72593	0.6875	0.8125	0.7241	0.8750	0.8232	0.6982	0.7866	0.3491	0.6616	0.5000	….													
IR84645	0.6206	0.8889	0.6762	0.8103	0.7548	0.6532	0.8103	0.3984	0.6992	0.6111	0.4741	….												
PBSAL656	0.4175	0.5421	0.1762	0.6857	0.1762	0.1437	0.7318	0.5095	0.6762	0.6532	0.8232	0.6762	….											
Kashrail	0.3524	0.5881	0.2318	0.6992	0.2318	0.1437	0.7548	0.4770	0.6206	0.6206	0.8491	0.6532	0.0976	….										
PBRC110	0.6206	0.7222	0.6532	0.6206	0.7548	0.6532	0.7222	0.3659	0.7222	0.5000	0.4116	0.3103	0.6762	0.6762	….									
PBRC37	0.3990	0.6429	0.5541	0.6847	0.5837	0.5541	0.6133	0.6847	0.5714	0.7857	0.8276	0.8571	0.5837	0.5837	0.7143	….								
PBRC83	0.5732	0.7500	0.5366	0.7500	0.6616	0.5366	0.7241	0.5625	0.7866	0.7500	0.6133	0.7241	0.6616	0.6616	0.5991	0.4704	….							
PBRC90	0.6206	0.8333	0.8103	0.8333	0.8333	0.7318	0.7318	0.5881	0.7873	0.6437	0.5991	0.5556	0.8103	0.7318	0.4770	0.8571	0.5991	….						
PBSAL613	0.6111	0.5325	0.5516	0.7222	0.5191	0.4961	0.6437	0.7548	0.6667	0.7222	**0.9375**	0.6762	0.5286	0.5191	0.6992	0.6429	0.7866	0.5976	….					
PBSAL614	0.7222	0.5325	0.6302	0.7222	0.5421	0.6302	0.5881	0.8889	0.6992	0.7778	**0.9375**	0.7548	0.6072	0.6532	0.7778	0.6429	0.8750	0.7548	**0.0881**	….				
PBSAL655	0.6111	0.5556	0.5976	0.5556	0.5095	0.5976	0.6437	0.8889	0.8103	0.8889	**0.9375**	0.8659	0.4961	0.5095	0.7548	0.5418	0.8125	0.8103	0.5325	0.4770	….			
Bina Dhan7	0.6667	0.1214	0.4635	0.6667	0.5421	0.5191	0.6437	0.7222	0.8333	0.6667	0.8125	0.8889	0.4961	0.5421	0.7222	0.5714	0.5625	0.7778	0.6667	0.6667	0.5881	….		
STL15	0.5976	0.7548	0.5746	0.7873	0.5095	0.5421	0.8103	0.7222	0.8659	0.8659	0.7866	0.6992	0.3984	0.3849	0.7778	0.7857	0.8125	0.7548	0.6762	0.7548	0.6206	0.8103	….	
STL20	0.4310	0.4540	0.2548	0.8103	0.1992	0.1762	0.6992	0.5095	0.6992	0.5651	0.8491	0.7778	0.1532	0.2087	0.7548	0.5837	0.6616	0.7548	0.3849	0.5191	0.5651	0.5421	0.4865	….

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