Received: 03 Aug 2023 | Revised: 24 Aug 2023 | Accepted: 29 Sep 2023

Electronic Journal of Plant Breeding

Research Note

Assessment of Quality Protein Maize (QPM) inbreds for genetic diversity using morphological characters and simple sequence repeats markers

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Abstract
An investigation was performed to characterize 25 indigenous Quality Protein Maize (QPM) inbred lines for several morphological traits to study the level of genetic diversity present in the inbreds by utilizing morphological characters and microsatellite markers. On the basis of morphological traits by adopting D^2 statistics and Tocher's method of clustering all the studied inbred lines were clustered into seven clusters and by using molecular markers, based on Jaccard's similarity coefficient, these were clustered into five clusters. The inbred lines MTCA 1, MTCA 2, MTCA 8, MTCA 6, and MTCA 11, were grouped together, indicating that these inbred lines of maize were closely related to one another in terms of morphological and molecular characterization. Out of the total primers studied, five were observed with more than 0.5 PIC value. SSR primers utilized for the molecular characterization of inbred lines with the mean PIC value of 0.543, were quite informative. A total of 41 alleles were observed with an allele count ranging from 2-5 per locus. The 13 SSR primer pairs used for estimation yielded a total of four unique alleles, with a mean of 0.31 unique alleles per primer. The similarity coefficient estimates for pairs of individuals varied from 0.000 to 1.00. Among the pair-wise combinations of entries evaluated, the magnitude of the similarity coefficient between MTCA 11 and MTCA 10 (1.00) was the highest. However, the molecular data and the cluster diagram based on morphological data were at odds which may be due to the enormous genome size of a cross-pollinated crop like maize.

Keywords: QPM, SSR, maize, cluster, polymorphic, molecular

Amongst cereals, next to wheat and rice maize is considered a principal source of food all around the world. It is known as the "Queen of cereal crops" and is used as a model crop owing to its high production potential (Stanley *et al.* 2020). It is predicted that by the year 2050, the population of humans will reach up to 9.7 billion from

current level of approximately 8 billion (UN Department of Economic and Social Affairs, 2023). To handle this challenge increasing the production of maize in addition to wheat and rice needs to be considered as a possible solution. The food grain production of 2022-23 shows an estimated maize production of 23.1 million tones. The maize crop is grown in an area of 22.67 lakh ha in India 20223. The state of Bihar (6.53 lakh ha) covers the maximum area followed by Maharashtra (3.88 lakh ha), Telangana (2.26 lakh ha) and Tamil Nadu (1.96 lakh ha). Thus, Bihar state holds about 28.8% of total Indian maze production (Maize Outlook 2023).

Malnutrition remains the biggest problem in South Asian countries where the poor communities are devoid of balanced nutrition. The affordability of quality protein in these poor communities is a matter of concern. Protein deficiency causes nutritional disorders like kwashiorkor, which in turn increases the susceptibility to some other diseases (Rolfes *et al.* 2009). Breeding for nutritionally rich crops can solve this deficiency when consumed in recommended quantities (Groote *et al.* 2010). Thus, QPM can elevate the nutritional status especially quality protein deficiency of the poor communities which are especially dependent on maize as the source of food. QPM contains two times the enhanced quantity of the amino acids *i.e.,* lysine and tryptophan (Krivanek *et al.* 2007) as compared to the normal maize. The presence of Opaque-2 gene in homozygous conditions is responsible for the enhanced content of lysine and tryptophan in them. Lack of good maize inbreds is a major problem in increasing maize production, especially in Indian conditions (Singh *et al.* 2021). Thus, the maize inbred lines can be characterized by various morphological traits which will in turn provide in-depth knowledge of the diversity present among the lines. It will also help in broadening the genetic base of maize and the improving the sustainability of maize breeding program (Swapnil *et al.* 2021). It can be utilized for selection of superior parents for the development of new maize hybrids (Ihsan *et al.* 2005). Simple Sequence Repeats (SSR) are highly polymorphic and informative markers due to their codominant nature and hence are widely used in plant breeding to study the diversity among the genotypes. In this experiment, the morphological and molecular characterization of 25 different maize genotypes was done to evaluate the potential of inbred lines, to determine the diversity for agro-morphological traits and to check the presence of opaque-2 gene.

Table 1. Details of experimental materials (Procured from AICRP, TCA Dholi)

Inbred	Pedigree
MTCA 1	[CL-G2501*CML170]-B-2-2-2-B-1-1-BBB-#
MTCA 2	CML161*165-18-2-1-2-BBB-#
MTCA 3	CML161*165-50-1-3-B*4-#
MTCA 4	(CML161*165)-F2-21-3-1-B*5-#
MTCA 5	(CML176*CLG2501)-B-55-1-5-2-BBB-#
MTCA 6	(CML165*CL-02843)-B-12-2-4-B-3-BBB-#
MTCA 7	(CLQ-6601*CL-02843)-B-23-2-1-B-1-BBB-#
MTCA 8	(CLQ-6601*CL-02843)-B-26-1-1-BB-1-B*6-#
MTCA 9	P70C0-BBB-6-B*6-#
MTCA 10	CLQ-RCYQ28-B-3-B*6-#
MTCA 11	CLQ-RCYQ41-BB-2-B*6-#
MTCA 12	CLQ-RCYQ035-B*11-#
MTCA 13	CLQ-RCYQ12-B-1-B*6-#
MTCA 14	CML161*165-3-2-3-B*4-#-B1
MTCA 15	G34QC24-BBB-16-B*8-#-B
MTCA 16	POO117C8(TEYFQPM)-B-117-B*10
MTCA 17	CML161*165-16-2-1-B*10
MTCA 18	G33QMH103-3-1-5-1-B*14
MTCA 19	(CML176*CLG2501)-B-55-1-2-B*4
MTCA 20	CLQRCYQ44-B*4-1-#-B
MTCA 21	CML161-1-B*8-#-B
MTCA 22	CML451Q-B*8
MTCA 23	CML165-B*9-#
MTCA 24	CML193-B*6-#
MTCA 25	(CML161*CLQ-RCYQ31)-B-22-2-B*5

A total of 25 QPM inbred lines were used for evaluation in *kharif*, 2018 in RBD with three replications at TCA campus, Dholi and the plot size adopted was 6 m^2 (1.5m x 4.0m). All the recommended package of practices were followed for better plant growth. All the genotypes were studied for 11 quantitative traits *viz.* plant height, cob length, tassel length, cob diameter, ear height, days to 75% brown husk, days to 75%tasseling, days to 75% silking, number of kernel rows per cob, number of kernels per row, and grain yield.

Genomic DNA extraction and PCR amplification: The leaves of 10-15 days old maize plants were used for DNA extraction using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990). The extracted DNA were analysed for quality and quantity using 0.8% agarose gel electrophoresis and then diluted to a consistent concentration of 50 ng/µl.

The selected SSR primers were used for the amplification of DNA in thermal cycler to conduct PCR. 2µL (20 ng) of DNA, 3µL of 5X PCR buffer [$MgCl_2$], 3µL of 1mM dNTPs, 1.2µL each of forward and reverse primers with concentrations of 6mM, 0.5µL of Taq DNA Polymerase enzyme (1U/µL), and 2.8µL of autoclaved MB grade water were included in the 15 µL. The reaction was set up to have an initial hold at 94°C for 4 min, subsequently followed by 35 cycles of primer annealing at 56°C for 40 s, denaturation at 94°C for 30 s, and extension for 1 min at 72° C. The samples were held for 2 minutes at 4°C after 10 minutes of further extension at 72° C. The SSR amplified product with an anticipated product size of greater than 100 bp was electrophoresed on a 2% agarose gel at 110 volts for 1.5 hours before being visualized and recorded using a gel documentation system (Biorad Inc., USA). Thermo-Scientific's 50 bp DNA ladder was used to assess the size of the amplified fragment.

Polymorphism among the primer pairs and the allelic diversity were evaluated by comparing the polymorphism information content (PIC) value of the primer pairs to further detect the polymorphic and informative markers that assist in further characterization and to distinguish the indigenous maize inbred lines. In order to determine the

Table 2. List of 13 primers utilized for amplification of maize genomic DNA

S. No.	Primer	Primer Sequence 5'-3'	Annealing temperature (°C)
1	Phi036	**(F)** CCGTGGAGAGACGTTTGACGT **(R)** TCCATCACCACTCAGAATGTCAGTGA	60
2	umc1545	**(F)** GAAAACTGCATCAACAACAAGCTG **(R)** ATTGGTTGGTTCTTGCTTCCATTA	57
3	Phi059	**(F)** AAGCTAATTAAGGCCGGTCATCCC **(R)** TCCGTGTACTCGGCGGACTC	62
4	umc1265	**(F)** GCCTAGTCGCCTACCCTACCAAT **(R)** TGTGTTCTTGATTGGGTGAGACAT	60
5	umc1963	**(F)** CTCGTTCGAGGGGATGTACAAG **(R)** CTTGCACTGGCACAGAGACG	61
6	phi083	**(F)** CAAACATCAGCCAGAGACAAGGAC **(R)** ATTCATCGACGCGTCACAGTCTACT	62
7	umc1304	**(F)** CATGCAGCTCTCCAAATTAAATCC **(R)** GCCAACTAGAACTACTGCTGCTCC	64
8	umc 1161	**(F)** GGTACCGCTACTGCTTGTTACTGC **(R)** GCTCGCTGTTGGTAGCAAGTTTTA	57
9	Umc1403	**(F)**GTACAACGGAGGCATTCTCAAGTT **(R)**TGTACATGGTGGTCTTGTTGAGGT	61
10	phi029	**(F)**TTGTCTTTCTTCCTCCACAAGCAGCGAA **(R)**ATTTCCAGTTGCCACCGACGAAGAACTT	57
11	Umc1165	**(F)**TATCTTCAGACCCAAACATCGTCC **(R)**GTCGATTGATTTCCCGATGTTAAA	55
12	umc 1367	**(F)** TGGACGATCTGCTTCTTCAGG **(R)**GAAGGCTTCTTCCTCGAGTAGGTC	62
13	Phi453121	**(F)** ACCTTGCCTGTCCTTCTTTCT **(R)**GGATTCCTTTATGACGGGGT	55

PIC value of the SSR markers, the value was estimated using the formula provided by Aderson *et al.* (1993). The following formula was used to determine the PIC of the SSR primer pairs-

$$\mathbf{PIC}i = 1 - \sum_{j=1}^{k} P^2 ij1 - \sum_{j=1}^{k} P^2 ij$$

Where, k is the total number of alleles detected for a particular locus of a marker, Pij equals j^{th} allele frequency for i^{th} marker.

The presence or absence of the SSR bands were assessed for each inbred line. Based on this pattern of SSR bands, polymorphism in the various inbred lines were recorded. Discrete variables were used to enter the data into a binary matrix, which undergo further analysis. The presence or absence of common bands was used to determine the genetic similarity between inbred lines. Genetic relationships among the studied inbred lines were assessed, by computing the Jaccard's similarity coefficient (Jaccard, 1908) for pair-wise comparisons based on the percentages of common bands generated by primers.

Similarity coefficient = a/(a+b+c)

Where, a is the bands present between j^{th} and k^{th} inbred lines, b is the bands present in j^{th} inbred lines but absent in k^{th} inbred lines, c is the bands absent in j^{th} inbred lines but present in k^{th} inbred lines.

Clustering of the inbred lines was done on the basis of similarity coefficients. The morphological data sets were analyzed for genetic diversity by Wards minimum distance in INDOSTAT software and the molecular analysis was done using Sequential Agglomerative Hierarchical Non-overlapping (SAHN) clustering method based on similarity coefficients. The un-weighted Pair Group Method using Arithmetic Mean (UPGMA) was used for development of dendrograms. NTSYS-pc software version 2.0 was used for the analysis (Rohlf, 2000). The genetic variation could be fully exploited using morphological and molecular characterization (Arzhang *et al.* 2022, Andorf *et al.* 2019). Based on the morphological traits under consideration 25 maize genotypes were grouped into seven clusters (**Table 3 and Fig. 1**). Cluster one comprised of highest no. of inbred lines (15) *viz*. MTCA 5, MTCA 6, MTCA 11, MTCA 4, MTCA 19, MTCA 21, MTCA 8, MTCA 16, MTCA 2, MTCA 1, MTCA 25, MTCA 14, MTCA 24, MTCA 18 and MTCA 15. Cluster three included two inbred lines *viz.,* MTCA 20 and MTCA 17. Cluster four included four inbred lines *viz.,* MTCA 7, MTCA 23, MTCA 13 and MTCA 9 whereas mono-genotypic clusters *i.e.* cluster two had inbred line namely MTCA 3, cluster five included inbred *viz.* MTCA 10, cluster six included MTCA 13 inbred and cluster seven included MTCA 22 inbred. Similar results were reported by Joshi *et al.* (2020), Sathua *et al.* (2018), Alam (2013), Gupta and Singh (2011), Ganesan *et al.* (2010), Jabeen *et al. (*2007), and Bhoite and Dumbre (2007).

The morphological traits are under the influence of the environment which creates a disadvantage in better analysis of genetic diversity. Hence, the use of molecular markers is beneficial as they have discriminatory power and are repeatable in nature. In this study, 25 QPM inbred lines are studied with 13 diverse SSR markers. Out of the different markers which are available, the microsatellites are highly polymorphic and specific in nature. With the help of a single SSR marker, the results are not authentic. Hence, 13 SSR markers were used for authenticity of the results (**Fig. 2**). Twenty-six loci were assigned to all thirteen SSR marker/primer pairs. By studying 25 QPM inbred lines, a total of 41 different alleles were found which had 3.15 alleles (on average) per loci. The number of alleles/loci varied from two in the cases umc1367, phi059, umc1161, umc1161 to five in the cases of phi036. These differences in number of alleles depict the presence of large amount of genetic diversity in the experimental materials used in this study. Four unique alleles (A unique allele is the allele which is present in a single inbred line) were reported in the study with 13 SSR markers which concluded 0.31 unique alleles (on average) per primer. Due to the presence of unique alleles, these inbred lines could be utilized by plant breeders as a genetically rich and diverse source in the maize breeding program. Similar results were reported by

Table 3. Clustering pattern of twenty-five inbred lines of QPM inbreds on the basis of D^2 statistics

Clusters	Number of genotypes within clusters	Genotypes in cluster
I	15	MTCA 5, MTCA 6, MTCA 11, MTCA 4, MTCA 19, MTCA 21, MTCA 8, MTCA 16, MTCA 2, MTCA 1, MTCA 25, MTCA 14, MTCA 24, MTCA 18 and MTCA 15
II	1	MTCA 3
III	2	MTCA 20 and MTCA 17
IV	4	MTCA 7, MTCA 23, MTCA 13 and MTCA 9
V	1	MTCA 10
VI	1	MTCA 13
VII	1	MTCA 22

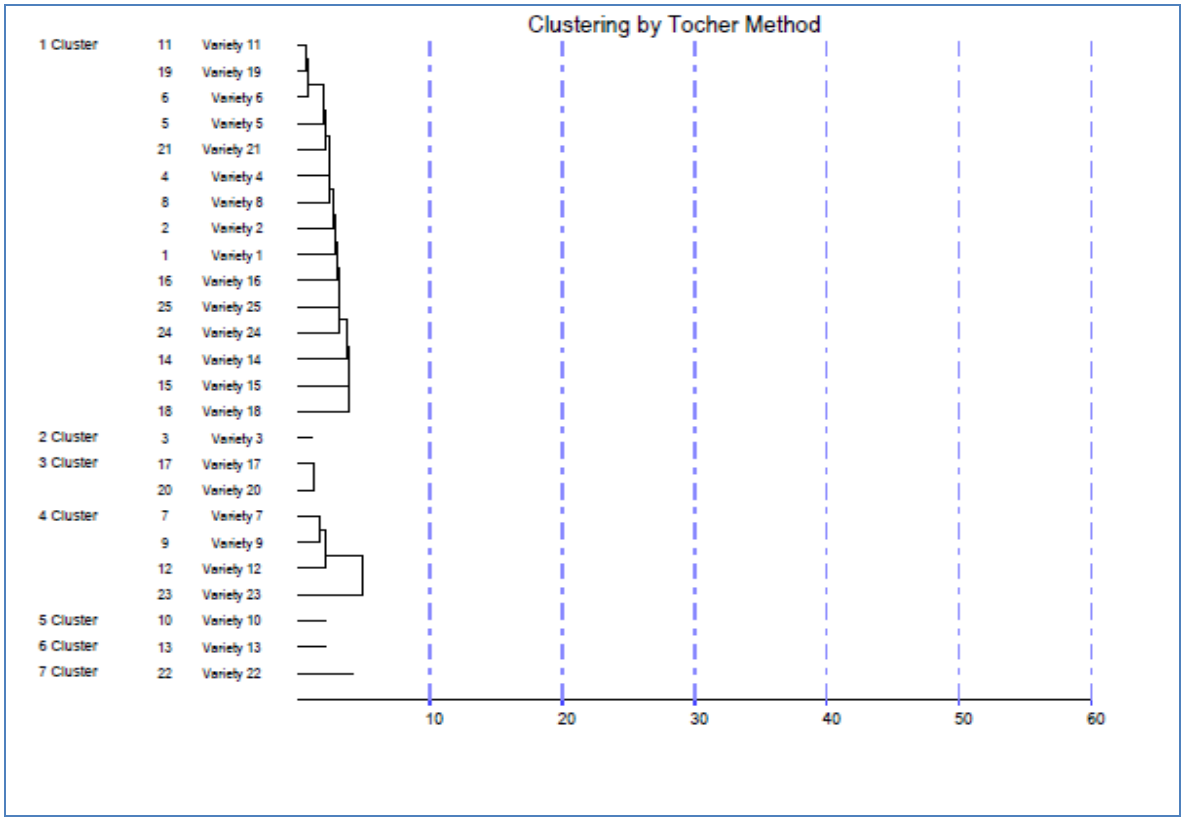

Fig. 1. Ward Minimum Variance Dendrogram for distribution of 25 QPM inbred lines in seven clusters based on non-hierarchical Euclidean Cluster analysis

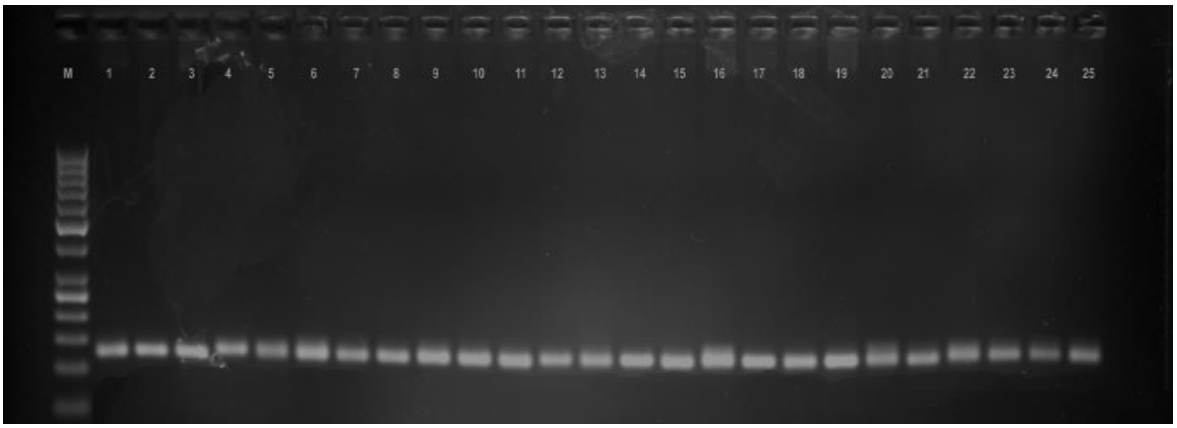

Fig. 2. Representative PCR amplification product of 25 genotypes of Quality Protein Maize inbreds with umc1403 primer

Adeyemo *et al.* (2019), Sathua *et al.* (2018), Khandakar *et al.* (2014), Sivaranjani *et al.* (2014), Kanagarasu *et al.* (2013) and Babu *et al.* (2012). The polymorphism information content (PIC) value varied from 0.339 (for umc1265) to 0.678 (for phi083 and phi029). In this study, an average PIC value of 0.683 indicates that the SSR markers used in the experiments were very informative. The previous study conducted by Sharma *et al.* (2019), Mukesh *et al.* (2016), Nikolic *et al.* (2015), Sivaranjani *et al.* (2014), Kanagarasu *et al.* (2013) and Babu *et al.* (2012) depicted similar PIC values in the study of maize genotypes with the SSR primers. The similarity coefficients values ranged from 0.000 to 1.00 in the pair-wise estimation study

Table 4. Primer pairs used for the amplification of genomic DNA extracted from 25 QPM inbred lines

Primers	Size of allels (bp)	No. of alleles	No. of unique alleles	% of unique alleles	PIC	No. of entries having null alleles
Umc1545	64.458-82.905	3	1	33.33	0.458	0
Umc1304	67.356-88.096	3	0	0	0.394	0
Phi083	129.088-153.777	4	1	25	0.678	0
Phi453121	482.955-511.340	3	0	0	0.547	0
Umc1165	106.194-133.046	3	0	0	0.342	0
Umc1403	110.464-132.946	3	0	0	0.442	0
Umc1161	138.715-154.207	2	0	0	0.365	0
Phi029	155.469-196.478	4	1	25	0.678	0
Umc1265	107.578-137.231	3	0	0	0.339	0
Phi036	53.972-89.205	5	1	20	0.554	0
Umc1367	168.391-198.553	2	0	0	0.480	0
Phi059	98.815-118.705	2	0	0	0.403	0
Umc1963	99.370-134.169	4	0	0	0.643	0

Table 5. Clustering of inbred lines based on Jaccard's similarity coefficients for 13 primer pairs used to amplify twenty-five QPM inbred lines of maize

Cluster	Number of entries	Composition of clusters
A	11	MTCA 1, MTCA 2, MTCA 8, MTCA 6, MTCA 3, MTCA 7, MTCA 9, MTCA 10, MTCA 11, MTCA 12 and MTCA 13
B	2	MTCA 4 and MTCA 5
C	6	MTCA 14, MTCA 15, MTCA 16, MTCA 17, MTCA 18 and MTCA 19
D	1	MTCA 20
E	5	MTCA 21, MTCA 22, MTCA 23, MTCA 24 and MTCA 25

(**Table 4**). Among all the pair-wise combinations used in the study, the maximum similarity coefficient (1) was reported between MTCA 10 and MTCA 11 genotypes.

For the assessment of genetic diversity at the molecular level, the 25 genotypes of maize were divided into five clusters with the help of a phenon line drawn at 0.54 similarity units in the dendrogram (**Table 5**). Out of the five clusters, cluster D is monogenotypic, comprising MTCA 20. Whereas di-genotypic clusters, namely cluster B consisting of MTCA 4 and MTCA 5, the cluster A and E are multi-genotypic in nature. Cluster E consisted of five inbred lines namely MTCA 21, MTCA 22, MTCA 23, MTCA 24 and MTCA 25. Cluster A consisted of the maximum number of inbred lines *i.e.* 11 namely MTCA 1, MTCA 2, MTCA 8, MTCA 6, MTCA 3, MTCA 7, MTCA 9, MTCA 10, MTCA 11, MTCA 12, MTCA 13 and the remaining six inbreds in cluster C.

The study revealed substantial genetic variations among all the 25 QPM inbred lines and all the inbred were grouped into five and seven clusters respectively based on SSR markers and Tocher's method. Cluster analysis showed there was enough diversity among the inbred lines The pair-wise genetic dissimilarity coefficients calculated on the basis of 13 SSR markers showed that a few of the inbred pairs (MTA 10 and MTA 11) had maximal genetic distance values. This study also highlighted the coherence of genetic relationships with pedigree data. Thus, SSR markers could be used to characterize lines precisely at the molecular level and help maize breeders efficiently assign lines to heterotic groups and guide them in the choice of parents for the development of new hybrids. At the same time, SSR markers provide a means for distinctly identifying individual genotypes with their unique allelic pattern, an application that is becoming important in varietal protection. Further studies by increasing the number of markers and correlating the current data with agro-morphological traits may help in better selection strategies to come out with the genotypes with durable promising outcomes.

Table 6. Estimates of thirteen SSR primer pairs on the basis of Jaccard's similarity coefficients among 25 QPM inbred lines

Inbreds	In-1	In-2	In-3	In-4	In-5	In-6	In-7	In-8	In-9	In-10	In-11	In-12	In-13	In-14	In-15	In-16	In-17	In-18	In-19	In-20	In-21	In-22	In-23	In-24
In-2	0.923																							
In-3	0.720	0.640																						
In-4	0.480	0.400	0.500																					
In-5	0.480	0.560	0.417	0.833																				
In-6	0.769	0.692	0.640	0.720	0.720																			
In-7	0.769	0.692	0.560	0.480	0.320	0.615																		
In-8	0.769	0.846	0.640	0.480	0.640	0.769	0.692																	
In-9	0.769	0.692	0.640	0.480	0.480	0.769	0.846	0.846																
In-10	0.615	0.539	0.640	0.560	0.400	0.615	0.846	0.692	0.846															
In-11	0.615	0.5385	0.640	0.560	0.400	0.615	0.846	0.692	0.846	1.00														
In-12	0.461	0.385	0.560	0.400	0.320	0.461	0.539	0.539	0.615	0.692	0.692													
In-13	0.538	0.462	0.640	0.400	0.320	0.539	0.615	0.539	0.692	0.692	0.692	0.615												
In-14	0.462	0.539	0.480	0.160	0.240	0.307	0.539	0.462	0.462	0.539	0.539	0.462	0.615											
In-15	0.385	0.307	0.480	0.320	0.160	0.385	0.461	0.385	0.462	0.615	0.615	0.539	0.615	0.615										
In-16	0.539	0.461	0.400	0.160	0.160	0.385	0.462	0.385	0.462	0.462	0.462	0.462	0.539	0.692	0.846									
In-17	0.462	0.385	0.560	0.320	0.240	0.462	0.385	0.462	0.462	0.539	0.539	0.539	0.692	0.615	0.846	0.769								
In-18	0.462	0.385	0.560	0.320	0.240	0.462	0.385	0.462	0.462	0.385	0.385	0.462	0.462	0.462	0.462	0.539	0.692							
In-19	0.539	0.462	0.480	0.240	0.160	0.385	0.462	0.385	0.385	0.307	0.307	0.231	0.385	0.539	0.539	0.615	0.615	0.769						
In-20	0.307	0.385	0.080	0.000	0.160	0.154	0.230	0.307	0.231	0.077	0.077	0.231	0.231	0.462	0.462	0.615	0.385	0.461	0.539					
In-21	0.480	0.480	0.417	0.167	0.250	0.480	0.400	0.560	0.560	0.400	0.400	0.320	0.480	0.480	0.560	0.560	0.480	0.480	0.400	0.560				
In-22	0.385	0.462	0.320	0.160	0.320	0.462	0.307	0.539	0.462	0.307	0.307	0.231	0.385	0.462	0.462	0.462	0.385	0.462	0.385	0.539	0.800			
In-23	0.308	0.385	0.320	0.240	0.240	0.385	0.385	0.462	0.385	0.385	0.385	0.231	0.385	0.308	0.539	0.385	0.385	0.462	0.385	0.385	0.640	0.846		
In-24	0.308	0.0.308	0.400	0.240	0.240	0.385	0.308	0.385	0.385	0.308	0.308	0.231	0.385	0.308	0.308	0.231	0.231	0.385	0.462	0.308	0.560	0.615	0.692	
In-25	0.385	0.385	0.400	0.320	0.240	0.462	0.462	0.462	0.462	0.462	0.462	0.308	0.462	0.308	0.462	0.308	0.308	0.385	0.308	0.231	0.640	0.692	0.846	0.846

https://doi.org/10.37992/2023.1403.145

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