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GENETIC DIVERGENCE STUDIES IN KODO MILLET (*PASPALUN SCROBICULATUM* L.) USING D² STATISTICS AND MOLECULAR MARKER

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ABSTRACT

Kodo millet *Paspalum scrobiculatum* (2n= 4x=40) is important nutria-cereal with high nutritive value. It is hardy, drought tolerance crop cultivated in rainfed area. 49 genotypes of kodo millet were evaluated and characterized for 19 quantitative and 18 qualitative traits during *kharif* 2021 and genetic diversity studied using Mahalanobis' D^2 statistics and molecular marker. D^2 analysis grouped the 49 genotypes into eight clusters. Cluster I with 37 genotypes emerged as the largest cluster. Maximum inter cluster distance was observed between cluster V and VI followed by cluster IV and VII. It was observed that total phenol content contributed maximum (19.40%) with 228 first ranks, towards total genetic divergence, Assessment of genetic divergence was also carried out through 34 SSR markers, of which 11 markers were found polymorphic. PIC value ranged from 0.469 (LM_GE_10) to 0.739 (UMC1690) with an average of 0.538. These 11 SSR primers generated 25 alleles with band size ranging from 52bp (UMC2252) to 625bp (BM_GE_10).

Keywords **:** Kodo millet, Genetic Divergence, D^2 statistics, Molecular markers.

Introduction

Kodo millet *(Paspalum scrobiculatum* L.) is a tetraploid (2n=4x=40) cereal belonging to the family poaceae (gramineae). It is self-pollinated for cleistogamous condition with rare exceptions.

The crop originates in tropical Africa, and it is estimated to have been domesticated in India 3000 years ago (Upadhyaya *et al*., 2016). It is widely cultivated in India, Pakistan, Phillippines, Indonesia, Vietnam, Thailand and West Africa (https://www.fao.org/). It also known as cow grass, rice grass, ditch millet, native paspalum, or Indian crown grass.

Kodomillet is an excellent source of micro-nutrients (iron, magnesium, calcium and some others), fibre and vitamin (Yadav and Jain, 2006) with high anti-oxidants, which is comparable to that of other small millets. It is rich in vitamin B complex, especially niacin, B6 and folic acid (Muthamilarasan *et al*., 2015). Hence it is a good substitute to rice and wheat for its low glycemic index. It is consumed traditionally as health and vitality foods in rural India. The seeds have an excellent storage life hence the crop can be used as famine reserve offering food security (Prajapati, 2016).

It is propagated through seed, preferably in fertile, clay-based soil, var. *scrobiculatum* is better suited to drier conditions than its wild counterpart, which is suited to sub- humid, arid conditions (https://www.agrifarming.in).

Rice and wheat had replaced this traditional crop, but nowadays intensified drive to increase millets cultivation is taken as millets offer regional food security in the dry and marginal lands, as well as for

nutritional superiority and stress tolerance, compared to the major cereals (Kumar *et al*., 2017). United Nation (UN) declared 2023 as "International Year of Millets" and aim behind this is to create awareness towards improving production and productivity of nutri-cereals.

India is the largest producer of small millets with a 41.0% global market share (https://www.fao.org/). Kodo millet has highest productivity among the small millets in India. Due to favorable agro-climatic conditions and farmers' interest in millet farming, Due to favourable agro-climatic condition (arid to semi-arid), Gujarat has potential in augmenting kodo millet production in the country**.** Precise breeding strategy could augment the Production of kodo millet and could fetch good economic return to farmers at marginal levels.

Mahalanobis' D^2 statistics (1936) is a useful technique to assess genetic diversity. Clustering of genotypes based on diversity for different desirable traits and assessing relative contribution of different components to total diversity helps in selecting parents for hybridization (Rao, 1952).

Molecular markers or DNA markers are efficient for exploiting variations in genotypes as they are least influenced by environmental factors. Among the different DNA marker systems, Simple Sequence Repeats (SSRs) are most suitable due to their high level of polymorphism, locus specificity, multi allelic, high reproducibility and high abundance, wide distributed throughout a genome, codominant nature and transferable among related species (Babu *et al*., 2018).

In minor millets genome specific SSR markers are rare. So there is urgent need to sequence its genome and to develop SSR markers. Application of SSR markers developed for one species and applied to another, called "transferability" has been successfully demonstrated in many crops including millets. Cross genome transferability is quick and efficient method to develop SSR markers in crops with little genomic information, as in minor millets. Transferability of SSR markers was first reported from finger millet and pearl millet to kodo and barnyard millet, increasing the prospect of breeding efficiency of kodo and barnyard millet crop improvement programmes. It also helps in revealing information on genome organization, genetic diversity, mapping population and identification of novel genes for important agronomic traits. Transfer rates of 35% to 90% from other millets and potential polymorphism of 58% to 78% has been reported in kodo millet (Arya *et al*., 2014).

Keeping the above fact under consideration an experiment entitled "Assessment of genetic diversity and character association in kodo millet (*Paspalum scrobiculatum* L.)" was conducted.

Materials and Methods

Detail of the 49 genotypes considered for the present study is provided in Table 1.

Table 1 : List of genotypes used in the present study

Sr. No	Genotypes	Sr. No	Genotypes	Sr. No	Genotypes	Sr. No	Genotypes
1	ERP-78	**14**	RPS-661	**27**	ELB-56	**40**	RPS-510
2	RPS-712	**15**	RPS-584	**28**	RPS-310	**41**	GAK-3
3	RPS-754	**16**	ELB-61	**29**	RPS-771	**42**	RPS-533
4	RPS-589	**17**	ELB-89	**30**	RPS-802	**43**	ERP-55
5	RPS-745	**18**	RPS-811	**31**	RPS-794	**44**	ELB-103
6	ERP-49	**19**	RPS-824	**32**	ELSB-77	**45**	ELB-76
7	RPS-560	**20**	RPS-648	**33**	RPS-612	**46**	ELB-109
8	RPS-903	**21**	RPS-882	**34**	RPS-877	**47**	RPS-784
9	RPS-685	**22**	RPS-974	**35**	RPS-697	**48**	RPS-599
10	RPS-1004	**23**	RPS-727	**36**	RPS-925	**49**	ELSG-22
11	RPS-981	**24**	ERP-77	**37**	ELB-80		
12	ELB-77	**25**	RPS-689	**38**	GK-4		
13	RPS-623	**26**	RPS-866	**39**	RPS-861		

The material was planted in randomized complete block design with three replications at the Experimental Farm of Department of Genetics & Plant Breeding, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat (22^o 35' N, 72^o 55' E, 45.01 meters above mean sea level), during *kharif*, 2021 with a spacing of 30 × 10 cm. All the recommended package of practices was followed to raise a good crop.

D^2 Statistics

After testing the difference in regard to individual characters through Analysis of Variance (ANOVA), a simultaneous test of significance for difference of mean values in regard to the pooled effect of 19

characters was carried out using Wilk's criterion (Wilk, 1932; Rao, 1952).The genetic divergence among the genotypes was computed by means of Mahalanobis' D^2 statistics. Grouping of genotypes in different clusters was carried out using Tocher's method (Rao, 1952). The criterion used in clustering was that any two genotypes belonging to a same cluster should on an average show a smaller D^2 value than those belonging to different clusters. After the formation of clusters, average intra cluster distance (D^2) and inter-cluster distance was calculated by measuring the distance between different clusters. Intra and inter cluster distance, cluster means and contribution of each trait to the divergence were estimated as suggested by Singh and Chaudhary (1985).

Genetic Diversity Using Molecular Markers

Glassware and plasticware used were of Borosilicate (Schott Duran, Germany) and Eppendorf (Germany), respectively. All chemicals and reagents used were of extra pure or molecular biology grade quality obtained from various companies in India and abroad. Glassware, plasticware and reagents were sterilized through double autoclaving before use and stored as per their requirements.

Genomic DNA extraction from leaf samples of all 49 genotypes of kodo millet under study was carried out at the Department of Genetics and Plant Breeding, B. A. College of Agriculture, Anand Agricultural University, Anand. Leaves were collected from young seedling and DNA was isolated using modified Cetyl Trimethyl Ammonium Bromide (CTAB) protocol (Doyle and Doyle, 1990).

Quality assessment of DNA was carried out through agarose gel electrophoresis (1%) with 100bp DNA ladder (Fermentas, USA).

Quantification of DNA through spectrophotometry Quantification of isolated genomic DNA was estimated by spectrophotometer instrument (NanoDrop-1000, Software V.3.3.0). Total 1µl of isolated DNA was loaded into the well of NanoDrop and the concentrations at A_{260}/A_{280} were measured.

A total of 34 Simple Sequence Repeats (SSR) primer pairs were used to screen the extracted DNA samples, the list is provided in Table 2. 15 of those markers were specific to maize, 11 were specific to little millet, whereas six were for barnyard millet and two were for finger millet. All the markers were procured from the Department of Agricultural Biotechnology, Anand Agricultural University, Anand.

PCR amplification was carried out using Emerald 2x PCR master mix (Takara). PCR reaction consisted of 5µl of PCR master mix, 1µl of 100ng DNA, 2µl of 10 picomole forward and reverse primers in a total volume reaction of 10µl. The volume was adjusted with nuclease free water.

Table 2 : List of primers

Sr. No.	Primer	Primer name	F/R	Sequence (5′ - 3′)	Tm
1	3L	Lm_Ge_3	F	TTCGAATCTCACTTTAAGACG	58.60
			R	CTATGATCCCGGTAAAGAGTT	
2	6L	Lm_Ge_6	F	TCTTTTTGCCATTGTACTTTC	58.45
			R	GTTATGGCTTGGACCTTTTAT	
3	10L	Lm_Ge_10	F	AAGACAAACTTTCACTGACGA	58.75
			R	CTGGAACTTGCTTTTGAATAA	
4	5B	BM_GE_5	F	CTATAGCACGAAAAACCATTC	58.40
			R	AAAGAGAGAGCTTTGCATTCT	
5	6B	BM_GE_6	F	TGCTTTCTCAATCTTTCTCTG	58.90
			R	TACTGTTGCGAGACTGGTACT	
6	8B	BM_GE_8	F	ATTCATTCTTGGAAAAAGAGG	58.55
			R	TTATCTCCTTCTACGACATGC	
7	10B	BM_GE_10	F	GGCTAGAGATTTCTTGGAAAA	58.60
			R	TTTAGTTGTATCGACCCAGTC	
8	11B	BM_GE_11	F	CAACGAATCTCTTGACCTAAA	58.65
			R	TAGACACGATGTCGCTTTATT	
9	8M	UMC2252	F	CACTGCACTGCAAGGTACATACG	62.40
			R	GTCTTTGACCCCTTCCTCTTCTTG	

10	28M	UMC1136	F	CTGCATACAGACATCCAACCAAAG	61.70
			R	CTCTCGTCTCATCACCTTTCCCT	
11	39M	UMC1690	F	ACCTTAGTTACACAGGCACACGGT	60.60
			R	GGTGATGGGATTTTCGCATTATTA	

F: Forward; R: Reverse T_m: Melting temperature (ºC) L= Little millet B= Barnyard mille F= Finger millet M= Maize

Genomic DNA extracted from 49 genotypes were subjected to PCR amplification using SSR primers. PCR amplification was carried out in a 200µl thin walled PCR tube containing 10µl reaction mix in Applied Biosystem Thermocycler (Veriti 96 well). The amplified fragments were resolved using gel electrophoresis.

Coefficients of similarity were calculated using Jaccard's similarity coefficient and cluster analysis was performed by agglomerative technique using UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) method by SIMQUAL function and SAHN clustering function of NTSYS version 2.02 (Rohlf, 1998), respectively.

Polymorphism information content (PIC) was estimated as per Botstein *et al.* (1980).

Results and Discussion

D^2 Statistics

The 49 genotypes under investigation were distributed over eight clusters. Cluster I with 37 genotypes emerged as the largest cluster (Table 3). Clustering pattern clearly indicated common ancestral origin of most of the RPS genotypes along with ELB, ERP and ELSG genotypes, as those appeared together in the clusters I, II and III. Similarly, single genotype forming separate clusters (cluster IV, V, VI, VII, VIII) indicated unique genetic constitution of the genotypes.

Table 3 : Clustering of 49 genotypes of kodo millet based on D^2 statistics

Sr. No.	Clusters	No. of genotypes	Name of genotypes
1	I	37	ERP-78, RPS-712, RPS-754, RPS-589, RPS-745, RPS-903, RPS-685, RPS-1004, RPS-981, ELB-77, RPS-661, ELB-61, ELB-89, RPS-811, RPS-648, RPS-882, RPS-727, ERP-77, RPS-689, RPS-866, ELB-56, RPS-310, RPS-771, RPS-802, RPS-794, ELSB-77, RPS-612, RPS-877, RPS-925, ELB-80, GK-4, RPS-861, RPS-510, RPS-533, ERP-55, ELB-76, ELB-109
2	II	2	ERP-49, RPS-584
3	III	5	RPS-623, RPS-974, RPS-784, RPS-599, ELSG-22
4	IV	1	RPS-560
5	V	1	RPS-824
6	VI	1	RPS-697
7	VII	1	GAK-3
8	VIII	1	ELB-103

Inter cluster and intra cluster distances are presented in Table 4. Intra cluster distance ranged from 0.00 (cluster IV, V, VI, VII, VIII) to 958.50 (cluster III). High intra cluster distance in cluster I (831.48) and cluster III (958.50) with only five and 37 genotypes are indicative of diverse genetic makeup of the genotypes from the rest of the collection.

Maximum inter cluster distance was observed between cluster V and VI (3985.31) followed by cluster IV and VII (3616.82) and cluster IV and VIII (3436.50) indicating wide divergence among these clusters.

Cluster I had highest distance with cluster IV, cluster II with VIII, IV with VII, whereas cluster III and cluster V were most distant from cluster VI and *vice versa.*

Least inter cluster distance was recorded for cluster I and cluster VIII. Cluster II and V were closet to cluster I. Cluster III and IV were closet to each other, so was for cluster VI and VII. This indicated genetic proximity of the genotypes from the said clusters.

Table 4 : Average intra and inter cluster D^2 values

Cluster	I	II	III	IV	V	VI	VII	VIII
I	**831.48**							
II	1597.19	**291.68**						
III	1896.53	1680.38	**958.50**					
IV	2611.16	3003.82	1369.41	**0.00**				
V	1434.08	1694.17	1631.65	3059.77	**0.00**			
VI	2021.61	2355.19	3281.10	2907.18	3985.31	**0.00**		
VII	2052.53	2688.06	2805.37	3616.82	2803.94	1993.56	**0.00**	
VIII	1288.15	3049.95	2510.82	3436.50	1924.94	3086.70	1532.59	**0.00**

Characters Contribution towards Genetic Divergence

Contribution of individual characters towards total genetic divergence is given in Table 4. It is observed that total phenol content contributed maximum (19.40%) with 228 first ranks, towards total genetic divergence, followed by flag leaf blade length (15.17%), peduncle length (13.16%), 1000 seed weight (9.05%) and total carbohydrate content (8.20%), plant height (7.62%), raceme length (4.63%) and grain yield per plant (3.90%). These eight characters together contributed to >>80.00% of genetic divergence, whereas other 11 characters contributed only ~19.00% cumulatively. Lowest contribution was made by culm branching.

This result is contradictory with the studies in kodo millet by Sreeja *et al*. (2015), Sao *et al*. (2016), Nirubana *et al*. (2017b), Yadav (2017) and Jyoti *et al*. (2020), where D^2 technique revealed that days 50% flowering, days to maturity, grain yield and fodder yield contributed maximum to the genetic divergence.

Qualitative and Quantitative Analysis of Genomic DNA

Quality and integrity of total genomic DNA, isolated through CTAB method described by Doyle and Doyle (1990), was verified by visualization of DNA on agarose gel (1%) with standard DNA ladder (100bp). A single sharp band was observed for isolated DNA for all 49 genotypes, indicating no contamination of RNA or protein.

Table 5 : Contribution of various traits towards total genetic divergence

Sr. No.	Characters	Time ranked first	Contribution (%)	Cumulative (%)
1	Total phenol	228	19.40	19.40
2	Flag leaf blade length	178	15.17	34.57
3	Peduncle length	155	13.16	47.73
4	1000 seed weight	106	9.05	56.78
5	Total carbohydrate	96	8.20	64.98
6	Plant height	90	7.62	72.60
7	Raceme length	54	4.63	77.23
8	Grain yield per plant	46	3.90	81.13
9	Panicle length	46	3.87	85.00
10	Crude protein	39	3.28	88.28
11	Fodder yield per plant	36	3.07	91.35
12	Days to maturity	30	2.51	93.86
13	Harvest index	16	1.35	95.21
14	Flag leaf blade width	14	1.23	96.44
15	Days to 50% flowering	11	0.92	97.36
16	Number of raceme per tiller	9	0.79	98.15
17	Number of productive tillers per plant	9	0.78	98.93
18	Number of basal tillers per plant	7	0.55	99.48
19	Culm branching	6	0.52	100.00

Genetic Diversity Using Molecular Markers

A set of 34 SSR primers were screened, out of which 11 (32.35%) primers were found to be polymorphic. The primer specific information is presented in Table 6.

Table 6 : Result of SSR marker analysis

Sr. No.	Locus name	Amplicon size (bp)	Number of alleles	Percent polymorphism	PIC
1	LM_GE_3	316 - 447	2	100%	0.494
2	LM_GE_6	323 - 420	2	100%	0.493
3	LM_GE_10	455 - 611	2	100%	0.469
4	BM_GE_5	457 - 530	2	100%	0.500
5	BM_GE_6	462 - 536	2	100%	0.483
6	BM_GE_8	378 - 470	2	100%	0.489
7	BM_GE_10	524 - 625	2	100%	0.500
8	BM_GE_11	396 -530	2	100%	0.496
9	UMC2252	52 – 209	3	100%	0.597
10	UMC1136	113 - 314	3	100%	0.659
11	UMC1690	107 - 254	3	100%	0.739
	Total	**-**	25	-	
	Average	**-**	2.27	100%	0.538

PIC = Polymorphism Information Content

These 11 SSR primers generated 25 alleles with band size ranging from 52bp (UMC2252) to 625bp (BM_GE_10). Yadav (2017) reported bands ranging from 190bp to 1450bp in kodo millet using ISSR markers.

The number of total bands, as well as polymorphic bands ranged from two to three bands per primer with an average of 2.27 bands per primer (Plate 1). Markers, UMC2252, UMC1136 and UMC1690 were found to produce three polymorphic loci each, hence can be exploited further in molecular study on kodo millet involving large and diverse population.

Cidade *et al*. (2013) reported an average of 7.12 bands per locus in *Paspalum* spp. with SSR markers. The mean PIC was 0.65, and an average of 15.58 bands per locus was found.

Percentage of polymorphism was found to be 100% for all 11 polymorphic primers which was higher than that reported in kodo millet by Cicade *et al*. (2013) using EST-SSR markers (52%), and by Rajput *et al*. (2019) using ISSR markers (88.88%).

In the present study, PIC value ranged from 0.469 (LM_GE_10) to 0.739 (UMC1690) with an average of 0.538. It was higher than the reports (0.276 to 0.652) of Manimekalai *et al*. (2018) in barnyard millet.

Result indicated the present of variability among different genotype of kodo millet Variation in DNA sequence leads to polymorphism. More PIC value is indicative of more diversity and use of diversity in broad genetic base of hybrid development also.

Genetic Relationship among Kodo Millet Genotypes and Cluster Composition

Genetic similarity between genotypes as calculated based on Jaccard's similarity coefficient. Genetic coefficient of similarity among the genotypes ranged from 0.07 to 0.92 and the average similarity coefficient was (0.54). The lowest (0.07) genetic similarity was observed between RPS-712 and RPS-811, which indicated that this pair of genotypes differed much from each other at genomic level and can be exploited to develop mapping populations. The highest (0.92) genetic similarity was observed between ELB-89 and RPS-612 indicating that those genotypes might have common ancestral origin.

A dendrogram was constructed from above similarity coefficients of 49 kodo millet genotypes, which were grouped into four main clusters, *viz*., Cluster A, B, C and D with 31, 2, 5 and 11 genotypes, respectively (Fig. 1). Maximum number of genotypes (31) were grouped in cluster A, indicating high genetic similarity among the grouped genotypes. Cluster A, B, C and D was further divided into sub-clusters *viz*., A_1 (Three ELB, Two ERP and 12 RPS genotypes) and A_2 (Nine RPS, Two ERP and ELSG-22, ELB-109 and GAK-3). Cluster B was sub divided into two sub-clusters with one genotype each, *viz*., B_1 (RPS-584) and B_2 (RPS-511). Cluster C, also had two sub-clusters, *viz*., C_1 (four RPS genotypes) and C_2 (RPS-974 genotype). Cluster D was further divided into two sub-clusters, *viz.,* D_1 (Two RPS and One GK-4 genotypes) and D_2 (four ELB, One ELSB and Three RPS genotypes).

Table 7 : Cluster composition based on Jaccard's similarity coefficients

Sr. No.	Main cluster	Sub cluster	Genotypes
1	A	A_1	ERP-78, RPS-560, RPS-661, RPS-599, ELB-77, ELB-89, RPS-612, ELB-61, ERP-77, RPS-689, RPS-925, RPS-784, RPS-745, RPS-1004, RPS-727, RPS-824, RPS-877
		A_2	RPS-589, RPS-981, ERP-49, RPS-310, ERP-55, RPS-903, RPS-685, GAK-3, RPS-794, RPS-623, ELSG-22, RPS-648, RPS-861, ELB- 109
2	B	B_1	RPS-584
		B_2	RPS-811
3	C	C_1	RPS-712, RPS-510, RPS-882, RPS-866
		C_2	RPS-974
4	D	D_1	RPS-754, GK-4, RPS-533
		D_2	ELB-56, ELB-80, ELSB-77, RPS-802, ELB-103, ELB-76, RPS-771, RPS-697

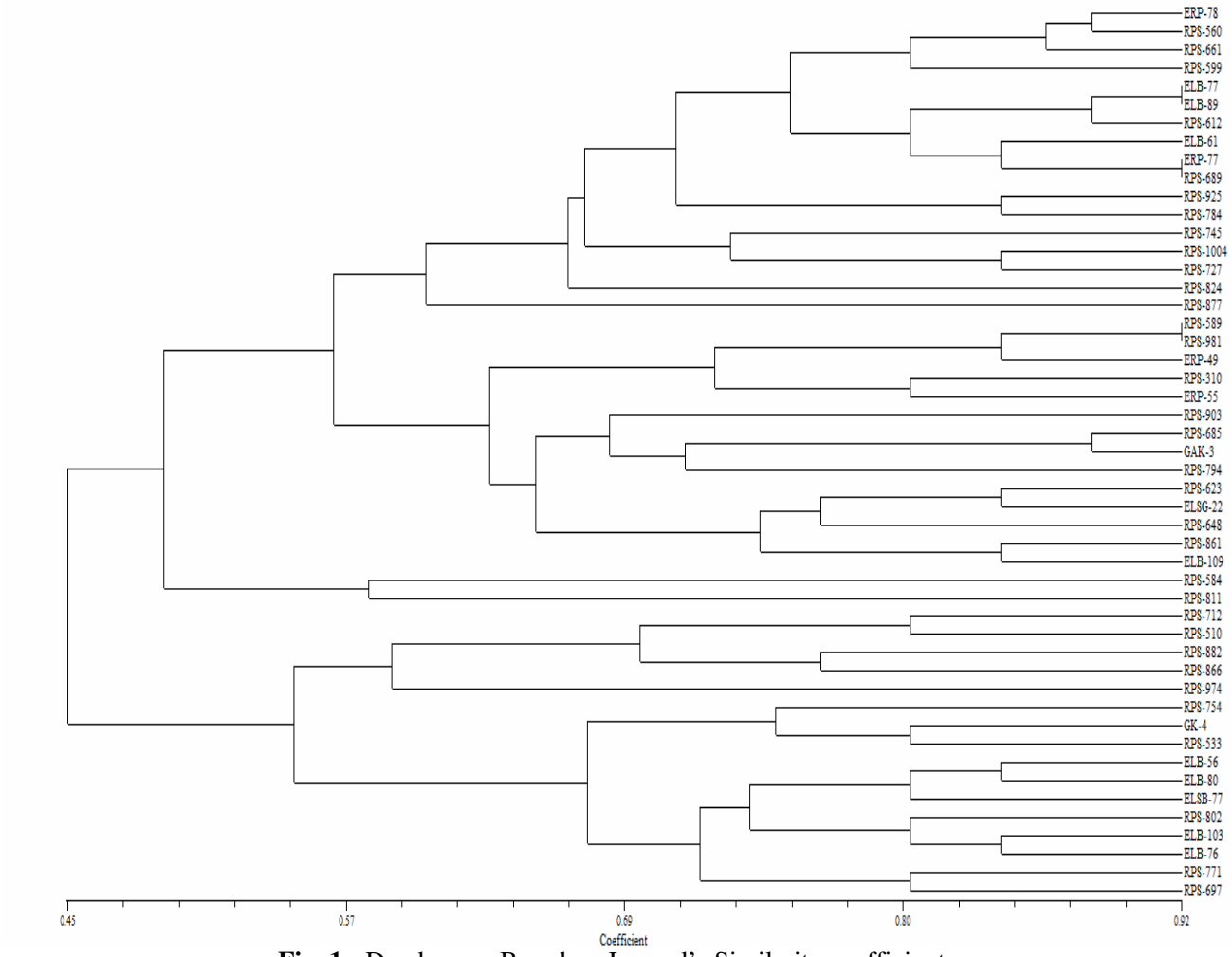

Fig. 1 : Dendogram Based on Jaccard's Similarity coefficients

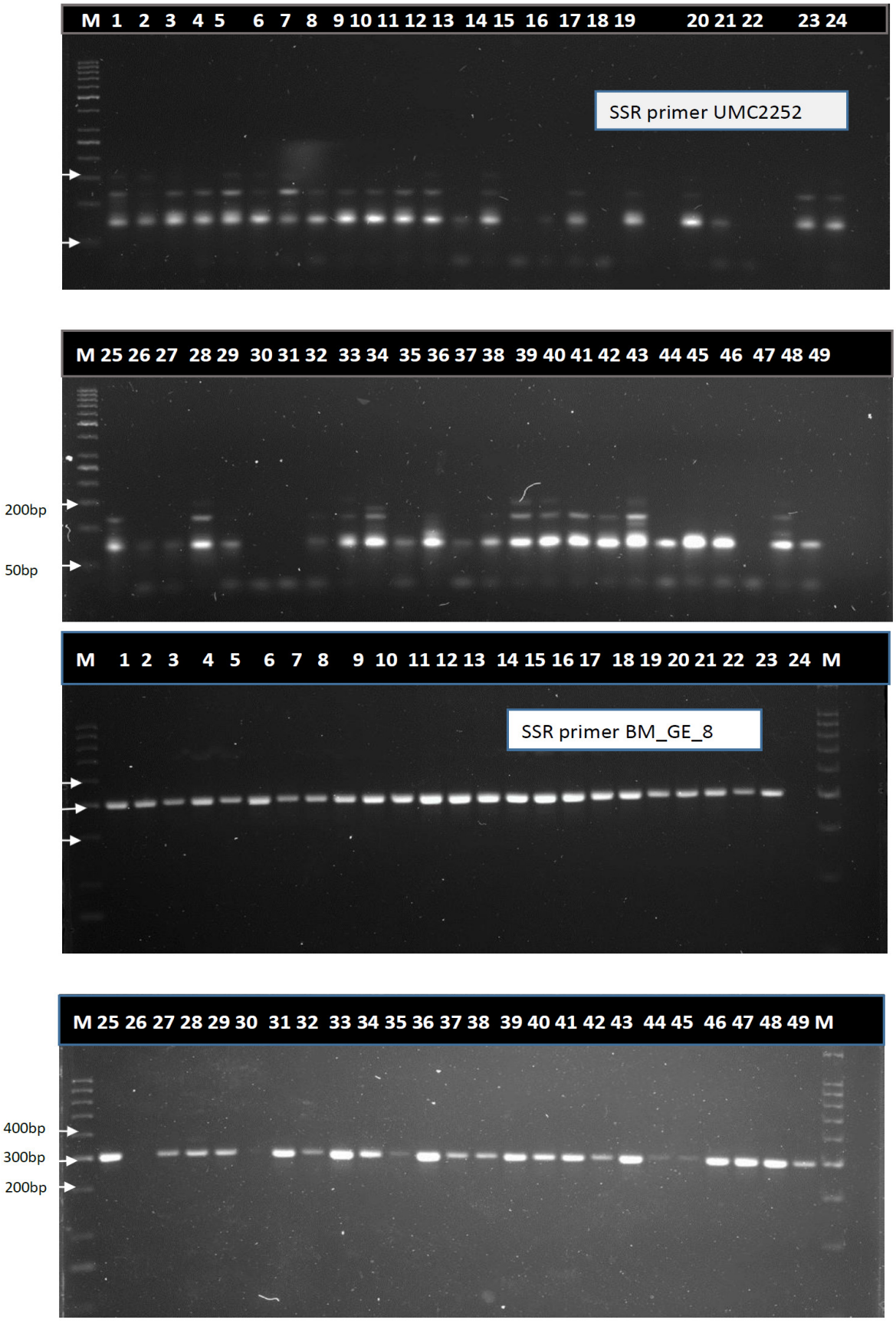

Plate 1 : Banding pattern generated for 49 genotypes for SSR Primers (SSR primer UMC2252 First and second from top SSR primer BM_GE_8 third and fourth from top)

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