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Assessing Genetic Diversity among Foxtail millet (*Setaria italica* (L.) P. Beauv.) Accessions Using RAPD and ISSR Markers

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Abstract

Foxtail millet [*Setaria italica* (L.) P. Beauv.], is an important grain crop in temperate, subtropical, tropical Asia and in parts of southern Europe. In India it is cultivated in Andhra Pradesh, Karnataka, Tamil Nadu, Rajasthan, Madhya Pradesh and some parts of Maharashtra. The present study was conducted to analyse the molecular diversity in the foxtail millet genotypes by using RAPD and ISSR markers and identify diverse accessions for use in varietal improvement programmes. A set of 44 foxtail millet accessions selected from Kolhapur (Western Maharashtra) region of India were analyzed using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. A total of 212 (135 RAPD and 77 ISSR) scoreable markers were generated with 29 RAPD and 19 ISSR primers. Average number of alleles generated by RAPD was 7.1 and ISSR was 6.41. Per cent polymorphism shown by RAPD primers varied from 62.50% to 100% and ISSR from 66.66% to 100%. The average PIC value for RAPD and ISSR was 0.74 and 0.73, respectively. The dendrogram generated using UPGMA revealed three major clusters, with 22 genotypes in one cluster and 20 genotypes in second cluster and two genotypes in third cluster. The genetic similarity matrices based on the Jaccard's coefficient ranged from 0.374 to 0.964 and 0.35 to 0.98 for RAPD and ISSR, respectively. The genotypes KOFM 1, KOFM 14, KOFM 36, KOFM 89, KOFM 90, KOFM 94 and KOFM 95 were found superior for creating more variability in foxtail millet.

Keywords: Genetic diversity, Land races, dendrogram, molecular markers

1. Introduction

Foxtail millet [*Setaria italica* (L.) P. Beauv.], is also known as Italian millet, *Kangu, Kangani, Kalakangani, Koni, Rala and Kaon* in different parts of India. It is the second most widely grown species amongst millets and important grain crop in temperate, subtropical, tropical Asia and in parts of southern Europe. China, India and Japan are the major foxtail millet growing countries in the world. According to Vavilov (1926), the principal centre of diversity for foxtail millet is East Asia, including China and Japan. In China, foxtail millet is the most common millet and one of the main food crops, especially among the low income groups in dry northern parts of the country. Foxtail millet is known for its drought tolerance in India and several countries. At present it is cultivated in Andhra Pradesh, Karnataka, Maharashtra, Tamil Nadu, Rajasthan, Madhya Pradesh, and some parts of Maharashtra. Foxtail millet is largely a self pollinated crop with cross pollination averaging about 4 per cent (Li et al., 1935). Foxtail millet is mixed with legumes to make porridge and also mixed with soybean to make mixed flour. Foxtail millet has low glycemic index (GI), used for preparation of low GI biscuits and burfi, a sweet product, and it is an ideal food for people suffering from diabetes (Thathola et al., 2010, Anju and Sarita, 2010, Wang et al., 2016). Foxtail millet is also fermented to make vinegar, yellow wine, maltose, beer and other related products. It is also used for feeding cage birds and by-product of the foxtail millet is used as animal feed. The foxtail millet grain is rich in protein (11.2 g 100 g^{-1}) and iron (2.8 mg) as compared to rice.

Morphological characterization does not reliably portray the genetic relationships among the genotypes because of environmental interactions, unknown genetic control of the traits and inadequate sampling of the genome in terms of phenotype. Thus, for genetic diversity assessment, molecular markers offer considerable advantages over the morphological markers (Reddy et al., 2006; Chander et al., 2017; Ramesh et al., 2017).

The diversity studies at molecular level in foxtail millet are very meagre and were mainly based on RFLP analysis (Fukunaga et al., 2002), using these markers they have concluded that the

foxtail millet landrace belonging to China were more variable. The RAPD markers used in some earlier studies (Li et al., 1998, Schontz and Rether 1999). Till recently the co-dominat marker systems like microsatellites were not available in foxtail millet. RatnaKumari et al., 2011, used inter simple sequence repeats (ISSR) for diversity analysis. During our investigation also the co-dominant markers were not available; the analysis was done using ISSR and RAPDs. In last 4-5 years number of studies have been performed using SSR and EST-SSRs for genetic diversity analysis in foxtail millet (Goron and Raizada, 2015, Ali et al., 2016, Wang et al., 2016, Andesen and Nepal, 2017, Chander et al., 2017).

2. Materials and Methods

2.1. Plant material, DNA extraction and purification

Forty-four genotypes for the present investigation were collected from the Millet Breeder, All India Coordinated Research Project on Small Millets (AICRP on SM), NARP, Shenda Park, Kolhapur. The list of genotypes used is given with their pedigree in Table 1. Seeds of 44 genotypes were sown in greenhouse and about 1–2 g of young healthy leaves per genotype were harvested from 15 to 20 days old seedlings for DNA isolation method given by Li et al. (1998) for RAPD and Reddy et al. (2009) for ISSR with some modifications.

After quantification, a working DNA stock of concentration of 5 ng μl^{-1} was prepared and stored at 4 °C until use. For PCR amplification 29 RAPD primers and 20 ISSR primers from the commercial kits obtained from Operon Technologies Inc., Alameda, USA were screened. Out of these 19 RAPD and 12 ISSR were found polymorphic (Table 2). PCR amplification was optimized and conducted in a reaction buffer of 25 µl containing 10X PCR buffer B for RAPD; 10X buffer F for ISSR; 1 Unit Taq DNA polymerase; 0.2 mM each of dATP, dGTP, dCTP and dTTP; 25 mM of MgCl2 (all these reagents from Life Technologies, India), 1 µM of respective primer and 20 ng of genomic DNA. PCR amplification was carried out in Thermal Cycler (Eppendorf, Master cycler gradient, Germany). The thermal cycling conditions for RAPD were as follows: initial denaturation at 94 °C for 5 min, followed by 44 cycles of denaturation (94 °C) for 1 min, annealing (37 °C) for 1 min, primer extension (72 °C) for 2 min, followed by an extension at 72 °C for 5 min. For ISSR the cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation (94 °C) for 30sec, annealing (45–55 °C) for 30 sec, primer extension (72 °C) for 30 sec, followed by an extension at 72 °C for 10 min. The amplification products of both RAPD and ISSR were analyzed by electrophoresis on 1.2 % agarose gel stained with ethidium bromide (10 mg/ml) and photographed under UV transilluminater in gel documentation system (Flour Chem. TM Alpha Innotech, USA). A 100 bp plus DNA ladder (MBI Fermentas, Germany) was used as standard.

2.2. Data analysis

Table 1: List of foxtail millet accessions with pedigree

Sr. No.	Genotype	Pedigree
1.	KOFM 1	Local collection from Western Maharashtra, India
2	KOFM 2	Local collection
3.	KOFM 6	Local collection
4.	KOFM 14	Local collection
5	KOFM 17	Local collection
6.	KOFM 18	Local collection
7.	KOFM 24	SIA 3043
8.	KOFM 25	Sel from SIA 326
9.	KOFM 28	SIA 3039
10.	KOFM 29	SIA 3044
11.	KOFM 33	GPUS 27
12.	KOFM 36	GPUS 30
13.	KOFM 37	CO 5×TNAU 200
14	KOFM 41	SIA 3035
15.	KOFM 42	Local collection
16.	KOFM 44	Local collection
17.	KOFM 46	Local collection
18.	KOFM 48	Local collection
19.	KOFM 51	Local collection
20.	KOFM 52	Local collection
21.	KOFM 53	Local collection
22.	KOFM 54	Local collection
23	KOFM 55	Local collection
24.	KOFM 58	Local collection
25.	KOFM 59	Local collection
26.	KOFM 61	Local collection
27.	KOFM 62	Local collection
28.	KOFM 64	Local collection
29.	KOFM 65	Local collection
30.	KOFM 66	Local collection
31.	KOFM 70	Local collection
32.	KOFM 73	Local collection
33.	KOFM 77	Local collection
34.	KOFM 79	Local collection
35.	KOFM 80	Local collection
36.	PS 4	Mutant of 543/ Sie 2616
37	GPUS 28	UAS Bangalore, India
38.	SIA 326	Pureline selection from Mandya
39.	KOFM 88	Sie 1472 UK

Continue...

Sr. No.	Genotype	Pedigree
40.	KOFM 89	Sie 1537
41.	KOFM 90	Sie 1539
42.	KOFM 93	Sie 1541
43.	KOFM 94	Sie 1598
44.	KOFM 95	Sie 1599

Table 2: List of RAPD primers used with their sequences

Sr. No.	Primer code	5| to 3|
1.	OPA 3	AGTCAGCCAC
2.	OPD 1	ACCGCGAAGG
3.	OPD 5	TGAGCGGACA
4.	OPD 11	GAGTCTCAGG
5.	OPD 18	GAGAGCCAAC
6.	OPE 3	CCAGATGCAC
7.	OPE 4	GTGACATGCC
8.	OPE 9	CTTCACCCGA
9.	OPE 12	TTATCGCCCC
10.	OPE 13	CCCGATTCGG
11.	OPE 15	ACGCACAACC
12.	OPE 17	CTACTGCCGT
13.	OPE 18	GGACTGCAGA
14.	OPE 19	ACGGCGTATG
15.	OPK 9	CCCTACCGAC
16.	OPL 2	TGGGCGTCAA
17.	OPL 11	ACGATGAGCC
18.	OPL 14	GTGACAGGCT
19.	OPL 15	AAGAGAGGGG
20.	OPL 16	AGGTTGCAGG
21.	OPL 18	ACCACCCACC
22.	OPM 5	GGGAACGTGT
23.	OPM 9	GTCTTGCGGA
24.	OPM 10	TCTGGCGCAC
25.	OPM 12	GGGACGTTGG
26.	OPM 14	AGGGTCGTTC
27.	OPM 17	TCAGTCCGGG
28.	OPM 18	CACCATCCGT
29.	OPM 20	AGGTCTTGGG

The RAPD and ISSR products were scored as presence (1) or absence of band (0) in all samples for each primer genotype combination. A binary data matrix based on presence/absence was used for analysis with NTSYSpc Software Package (Rohlf 1997).The polymorphism information content (PIC) value was calculated as $PIC=\Sigma (1 - Pi^2)/ n$ where, n is the number of band positions analyzed in the set of accessions and Pi is the frequency of i^{th} pattern. Jaccards similarity Coefficient was used for the construction of dendrogram by the Unweighted Pair Group Method using Arithmetic Mean (UPGMA).

3. Results and Discussion

In view of the several merits of this crop and very limited research undergone, there is a need for the study of genetic and molecular diversity for its effective utilization in development of improved cultivars. Therefore, the present investigation was formulated to study the molecular diversity present in the genotypes by using RAPD and ISSR markers in foxtail millet.

Out of 29 RAPD and 20 ISSR primers surveyed, 19 RAPD and 12 ISSR primers were selected for the present study based on the extent of polymorphism observed in the amplicons. The amplification obtained with RAPD and ISSR primers was good and consistent (Table 3 and 4). A total of 212 scoreable amplification products (135 RAPD and 77 ISSR)

Table 3: Sequences and fixed optimum annealing temperature for ISSR primers used in ISSR analysis

S r . No.	ISSR primers	Sequence of primers (5'-3')	OAT
1.	ISSR 807	AGAGAGAGAGAGAGAGT	42.4
2.	ISSR 808	AGAGAGAGAGAGAGAGC	46.8
3.	ISSR 809	AGAGAGAGAGAGAGAGG	46.3
4.	ISSR 810	GAGAGAGAGAGAGAGAT	42.8
5.	ISSR 811	GAGAGAGAGAGAGAGAC	43.2
6.	ISSR 816	CACACACACACACACAT	51.0
7.	ISSR 817	CACACACACACACACAA	52.7
8.	ISSR 819	GTGTGTGTGTGTGTGTA	47.8
9.	ISSR 820	GTGTGTGTGTGTGTGTC	50.5
10.	ISSR 822	TCTCTCTCTCTCTCTCA	45.7
11.	ISSR 823	TCTCTCTCTCTCTCTCC	47.3
12.	ISSR 826	ACACACACACACACACC	53.1
13.	ISSR 834	AGAGAGAGAGAGAGAGYT	45.1
14.	ISSR 835	AGAGAGAGAGAGAGAGYC	45.7
15.	ISSR 840	GAGAGAGAGAGAGAGAYT	45.7
16.	ISSR 841	GAGAGAGAGAGAGAGAYG	46.1
17.	ISSR 880	GGAGAGGAGAGGAGA	49.0
18.	ISSR 885	BHBGAGAGGAGAGAGAGA	46.4
19.	ISSR 890	VHVGTGTGTGTGTGTGT	51.0
20.	ISSR 891	HVHTGTGTGTGTGTGTG	51.9

OAT: Optimum annealing Temp. (°C); Single letter abbreviations for mixed base positions: Y: (C, T); B: (C, G, T i.e. not A); H: (A, C, T i.e. not G); V: (A, C, G i.e. not T)

Table 4: Per cent polymorphism shown by different RAPD primers

Sr. No.	Primer	TNBG	TNMB	TNPB	PP	PIC Values
1.	OPA 03	4	0	4	100.00	0.440
2.	OPD 05	8	1	7	87.50	0.789
3.	OPE 03	9	2	7	77.77	0.529
4.	OPE 04	7	0	7	100.00	0.701
5.	OPE 15	5	0	5	100.00	0.701
6.	OPE 18	5	0	5	100.00	0.754
7.	OPE 19	5	0	5	100.00	0.704
8.	OPK 09	10	1	9	90.00	0.882
9.	OPL 02	6	0	6	100.00	0.613
10.	OPL 14	7	0	7	100.00	0.797
11.	OPL 18	6	0	6	100.00	0.707
12.	OPM 05	8	3	5	62.50	0.849
13.	OPM 09	6	1	5	83.33	0.787
14.	OPM 10	11	0	11	100.00	0.878
15.	OPM 12	8	0	8	100.00	0.814
16.	OPM 14	7	1	6	85.71	0.651
17.	OPM 17	8	0	8	100.00	0.816
18.	OPM 18	7	0	7	100.00	0.779
19.	OPM 20	8	3	5	62.50	0.838
Total		135	12	123	91.11	--

TNBG: Total number of band generated; TNMB: Total number of monomorphic bands; TNPB: Total number of polymorphic bands; PP: Per cent Polymorphism (%)

were generated. The number of amplicons generated by each primer varied from four (OPA 03) to eleven (OPM 10) for RAPDs with an average of 7.1 amplicons per primer and four (ISSR 807, ISSR 809 and ISSR 811) to nine (ISSR 810 and ISSR 823) for ISSRs. Average number of alleles generated by ISSR was 6.41. The average number of polymorphic bands amplified for each primer (7.1) recorded in the present study were comparable with the earlier reports in foxtail millet by Li et al. (1998) (7.78), Schontz and Rether (1998) (6.25); Ratna Kumari et al. (2011) (7.18) in foxtail millet and Fakrudin et al., (2004) (6.86), Kumari and Pande, (2010) (6.64) in finger millet. While Gupta et al. (2010) reported 8.5 bands per primer in finger millet which were higher than that obtained in the present study.

Percent polymorphism shown by RAPD primers varied from 62.50 to 100%. It was found that, total 135 bands were generated by amplification out of which 123 were polymorphic with an average of 91.11% polymorphism. Similar results were recorded by Li et al., (1998) using 19 RAPD primers in 20 accession of foxtail millet with 72.80% polymorphism. Fakrudin et al. (2004) used RAPD primers among 12 selected finger millet accessions and reported 85.82% polymorphism. Similarly, KalyanBabu et al. (2006) have reported 91% polymorphism among 32 finger millet accessions using 50 RAPD primers. In ISSR analysis out of 77 bands, 72 were polymorphic with an average of 93.50% polymorphism. RatnaKumari et al. (2011) also reported similar values for percent polymorphism (37.5 to 100) using RAPD and ISSR markers in foxtail millet.

The polymorphic information content (PIC) value as a relative measure of polymorphism level ranged between 0.440 (OPA 03) to 0.882 (OPK 09) in RAPD and it was ranged between 0.466 (ISSR 807) to 0.847 (ISSR 810) for ISSR. The average PIC value for RAPD (0.74) and ISSR (0.73) was higher than that of Jia et al. (2009) (0.69 for SSR) in foxtail millet and Panwar et al. (2010) (0.35 and 0.505 for RAPD and SSR, respectively) and Gupta et al. (2010) (0.51 and 0.19 for RAPD and ISSR, respectively) in finger millet. It was similar to that of Liu et al. (2011) (0.72 for SSR) in foxtail millet.

The higher PIC value indicated the informativeness of the primer. Among the primers used in the study three primers each from RAPD (OPK 09, OPM 10 and OPM 05) and ISSR (ISSR 810, ISSR 823 and ISSR 808) exhibited the PIC values from 0.882 to 0.816. These primers can provide the basis for foxtail millet DNA profiling system. To examine the genetic relationship among the 44 foxtail millet genotypes under study based on the RAPD and ISSR results, the data scored from 19 RAPD and 12 ISSR primers were compiled and analyzed separately using NTSYS Pc programme (Rohlf,1997) (Table 5). The similarity matrix was computed using RAPD and ISSR markers based on Jaccard's coefficient. The genetic similarity

Table 5: Per cent polymorphism shown by different ISSR primers

Sr. No.	Primer	TNBG	TNMB	TNPB	PP	PIC values
1.	ISSR 807	5	1	4	80.00	0.466
2.	ISSR 808	8	2	6	75.00	0.816
3.	ISSR 809	6	2	4	66.66	0.725
4.	ISSR 810	9	0	9	100.00	0.847
5.	ISSR 811	4	0	4	100.00	0.702
6.	ISSR 817	5	0	5	100.00	0.749
7.	ISSR 820	5	0	5	100.00	0.703
8.	ISSR 823	9	0	9	100.00	0.839
9.	ISSR 826	6	0	6	100.00	0.732
10.	ISSR 834	8	0	8	100.00	0.745
11.	ISSR 880	7	0	7	100.00	0.779
12.	ISSR 885	5	0	5	100.00	0.703
Total		77	5	72	93.50	--

matrices based on the Jaccard's coefficient ranged from 0.374 to 0.964 and 0.35 to 0.98 for RAPD and ISSR, respectively. The genetic similarity matrix also revealed that the KOFM 95 and KOFM 14, KOFM 94and KOFM 37 and KOFM 41 were distantly related which was indicated from the lowest genetic similarity coefficient (0.374), while KOFM 95 and KOFM 94 were closely related with a genetic similarity coefficient of 0.964.

Interestingly, the dendrograms generated based on UPGMA method of cluster analysis using RAPD and ISSR marker data revealed exactly similar grouping of genotypes into three major clusters (Figure 1 and Figure 2, respectively). Cluster I comprised of two sub clusters of which KOFM 1, KOFM 28, KOFM 29, KOFM 36, KOFM 33, KOFM 48, KOFM 53, KOFM 54, KOFM 51 and KOFM 52 were in a separate sub cluster, while

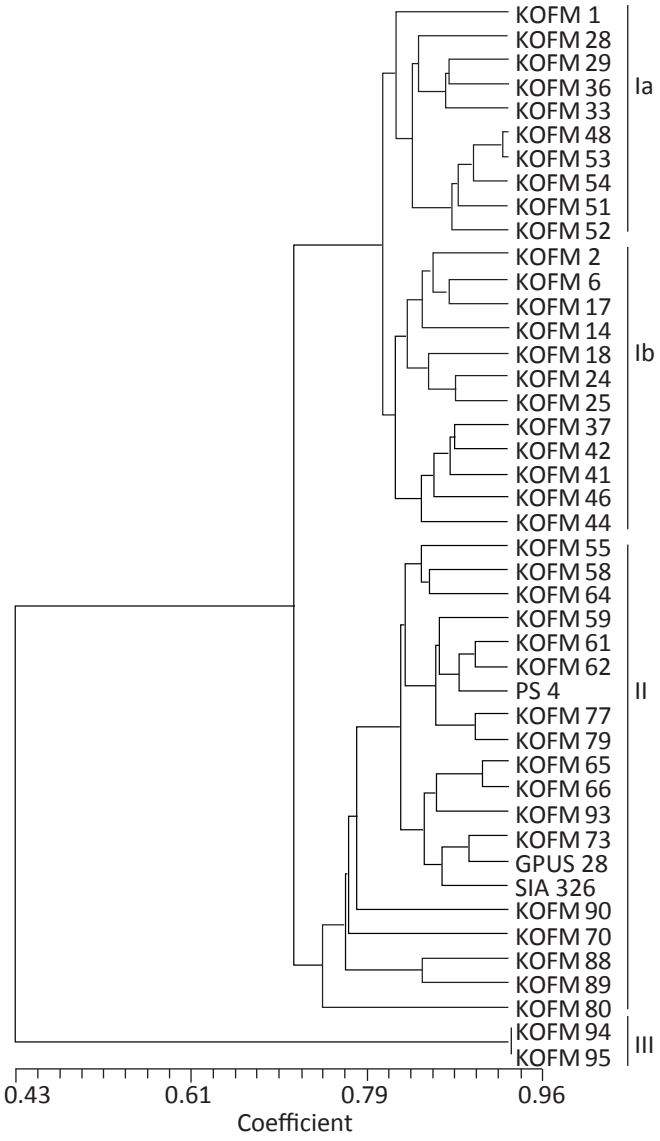

Figure 1: Dendrogram constructed with NTSYSpc ver.2.02 using UPGMA clustering algorithm from the pair-wise genetic similarity matrix to compare 44 foxtail millet genotypes based on allelic information from 19 RAPD markers

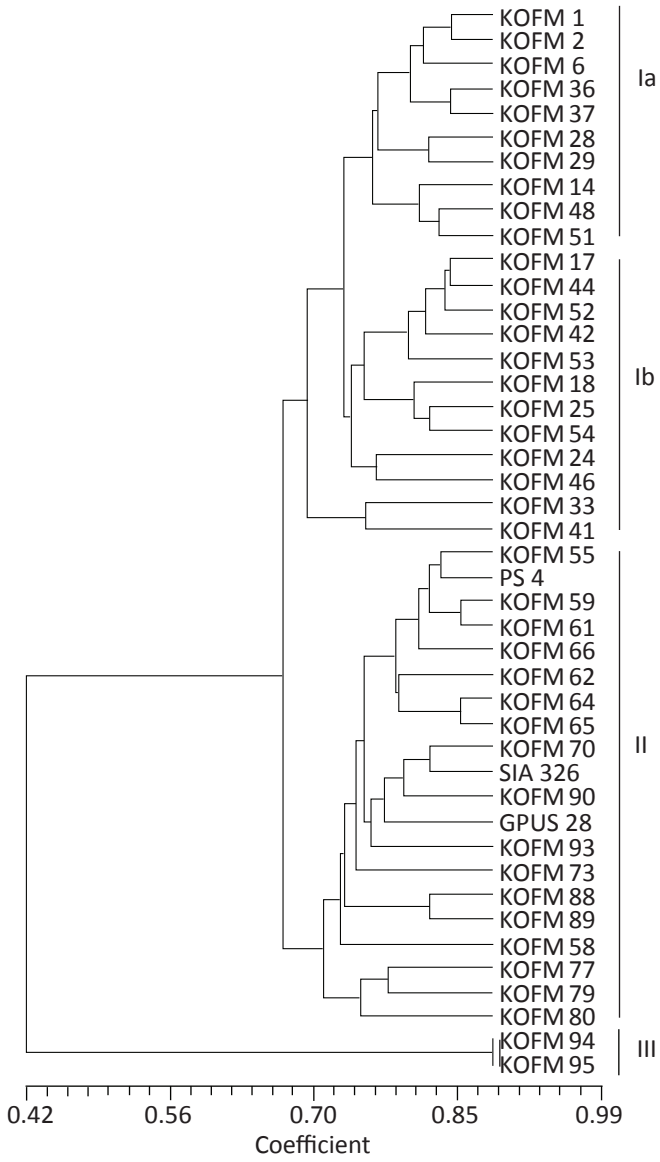

Figure 2: Dendrogram constructed with NTSYSpc ver.2.02 using UPGMA clustering algorithm from the pair-wise genetic similarity matrix to compare 44 foxtail millet genotypes based on allelic information from 12 ISSR markers

KOFM 2, KOFM 6, KOFM 17, KOFM 14, KOFM 18, KOFM 24, KOFM 25, KOFM 37, KOFM 42, KOFM 41, KOFM 46 and KOFM 44 were in another sub cluster of cluster I. The second cluster contains KOFM 55, KOFM 58, KOFM 64, KOFM 59, KOFM 61, KOFM 62, PS 4, KOFM 77, KOFM 79, KOFM 65, KOFM 66, KOFM 93, KOFM 73, GPUS 28, SIA 326, KOFM 90, KOFM 70, KOFM 88, KOFM 89 and KOFM 80 genotypes. KOFM 94 and KOFM 95 genotypes were in third cluster.

The distribution of the genotypes in the dendrogram was mostly consistent with the known pedigree information and the morphological attributes of the genotypes. The close grouping between KOFM 94 and KOFM 95 which may be attributed to the selections of these genotypes from the closely related parents Sie 1598 and Sie 1599. These two

genotypes exhibited high similarity value (0.967).

4. Conclusion

Considerable diversity existed among the foxtail millet accessions studied. This study identified diverse genotypes viz., KOFM 95, KOFM 14, KOFM 94, KOFM 37 and KOFM 41 for use in hybridization program for foxtail millet improvement.

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