Applied Biological Research 25(3): 311-318; (2023)
DOI: 10.5958/0974-4517.2023.00036.8

GENETIC RELATEDNESS ANALYSIS OF LITTLE MILLET (*Panicum sumatrense* Roth. Ex Roem & Schult.) USING RAPD MARKERS

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(Received 13 April, 2023; accepted 2 July, 2023)

ABSTRACT

Little millet, having a significant nutritional value, has not been exploited to its true potential so remains underutilized. The molecular relatedness analysis of available genotypes is a crucial step in crop improvement and germplasm conservation of millets. The present study evaluated genetic relatedness among 16 little millet genotypes, collected from Dang region of Gujarat (India), using random amplified polymorphic DNA (RAPD) markers. Initially, a total of 120 RAPD primers were screened, of which only 25 RAPD primers produced distinct bands so were used for further analysis. RAPD analysis with selected 25 decamer primers amplified a total of 193 bands with amplicon sizes in between 50-2392 bp. There were 126 polymorphic bands (64.25%) with mean polymorphic bands of 5.04 per primer. The maximum marker index of 41.25, maximum polymorphic information content of 0.59 and maximum RAPD primer index of 5.69 were recorded in primers OPC-13, OPC-17, and OPA-12, respectively. The RAPD primers OPA-12, OPC-13, and OPC-17 proved most efficient for genetic relatedness analysis in little millet. The Jaccard's similarity coefficient among the genotypes was in the range of 0.53 to 0.98 revealing a moderate genetic diversity among the available genotypes. The dendrogram divided 16 genotypes of little millet into two major clusters and further sub-clusters, demonstrating that RAPD technique was effective to distinguish the studied genotypes. The dendrogram and PCoA analysis revealed that genotypes WV-151 and WV-161 were highly diverse. These genotypes along with genetic relatedness established in this study can be exploited in future little millet improvement programs with conventional and molecular breeding methods.

Keywords: Genetic relatedness, little millet, molecular marker, RAPD

INTRODUCTION

Little millet (*Panicum sumatrense* Roth. Ex Roem & Schult.) is a self-pollinated tetraploid (2n = 4x = 36) that belongs to the family *Poaceae* and subfamily Panicoideae (Patel *et al*., 2018). It is an annual erect crop that was domesticated 5,000 years ago in the Eastern Ghats of India (De Wet, 1986). Little millet is a hardy crop that grows well in a variety of agro-ecological environments and variable soils with a wide range of temperatures, photoperiod, drought, and water logging conditions. Due to these unique qualities, little millet is now a preferred crop in rain-fed, tribal, and hilly agriculture. According to USDA, the global production of millets was reported to be 90.65 Mn MT during the year 2022

(APEDA, 2022). In India, minor millets are cultivated in a 4.23 lakh ha area with a production of 3.75 lakh tonnes (APEDA, 2022). The area, production, and productivity of little millets are not well documented and are mostly reported together as 'millets', unlike major crops. In Gujarat, little millet is cultivated in an area of 9430 ha with mean productivity of 1320 kg ha^{-1} grown in the mountainous region of Dangs and Valsad area and is known locally as "Vari" (Ladumore *et al.*, 2021). Apart from these, it is rich in minerals and nutrients which help to maintain human health and fight against several diseases.

The knowledge regarding the prevailing genetic relatedness among genotypes is a prerequisite for crop improvement (Laurentin, 2009; Dong *et al*., 2014; Yaman *et al*., 2014). Traditionally, the evaluation of genetic resources of millet species was done by descriptions of their morphological characteristics (Brink and Belay, 2006; Jansen, 2006; Kaume *et al*., 2006), health-impact traits (Kalinova and Moudry, 2006). The morphological markers used for the developmental assessment are not stable over time and are greatly influenced by environment and low levels of polymorphism are major issues (Nadeem *et al*., 2018). The DNA marker technology is not affected by environmental conditions and the developmental stage of plants, hence can be used to assess several novel evolutionary and taxonomy concerns. DNA markers are genes and specific DNA segments that function as an identification of variation at the DNA level. The assessment of diversity using the molecular markers technique would facilitate to eliminate delicacy within the germplasm assortment, as a result, it will save time, labour, and land. The genetic relatedness analysis in little millet using molecular markers such as RAPD and SSR is scanty and only a few reports are available (Fakrudin *et al.*, 2004; Tiwari *et al.*, 2018; Waghmode *et al*., 2018; Ghimire *et al*., 2019). The SSR markers are abundant, polymorphic, and multi-allelic and require sequencing data for the development of SSR markers, However, due to the limited information on sequencing in little millet the RAPD markers are of choice (Williams *et al*., 1990). In Gujarat, the mountainous region of Dangs and Valsad is traditionally occupied with local varieties of little millet and exhibits a rich diversity but is unexploited for breeding purposes. Compared to other cereal crops, little millet has gained less attention in varietal development, and only 20 varieties of little millet released across India (Nagraja *et al*., 2023). Thus, the present study aimed to evaluate genetic relatedness among little millet genotypes from the Dang region that can be exploited in future crop improvement programs.

MATERIALS AND METHODS

Seed material and DNA extraction

Sixteen little millet genotypes used in the present study were procured from the Hill Millet Research Station (HMRS), Navsari Agricultural University, Waghai, Dangs, Gujarat, India. This study was conducted during the years 2020-2021 at the Department of Plant Molecular Biology & Biotechnology, ASPEE College of Horticulture and Forestry, NAU, Navsari (India). The seeds were germinated in plastic bags (three replicates) in a greenhouse and ten-day-old seedlings were used for the extraction of DNA by CTAB-DNA extraction method (Rogers and Bendich, 1988). The germinated leaves (200 mg) were crushed to a fine powder using liquid nitrogen and added to 2 mL of pre-warmed CTAB buffer and incubated in a water bath at 65°C for 1 h. The mixture was extracted with 2 mL chloroform: isoamyl alcohol (24:1) twice and then the DNA was precipitated using chilled absolute alcohol followed by incubation overnight at -20°C followed by wash with 70% ethanol. The pellets were air-dried and dissolved in 50 µL tris–EDTA buffer. The quality of DNA was checked by agarose gel electrophoresis and quantification was done by nanodrop **(**Thermo Scientific, USA). The final DNA concentration was adjusted to 50 ng μL^{-1} with the appropriate amount of 0.1X TE buffer and used for further application.

RAPD analysis

The genetic relatedness analysis was carried out using the RAPD marker as explained by Tiwari *et al*. (2018). A total of 120 RAPD primers (Operon™ Technologies, Alameda, California, USA) from six different operon series were screened out of which only 25 primers were able to show clear distinct amplicon. from the initially screened 120 primers representing 6 operon series. The 25 µL reaction mixture for polymerase chain reaction (PCR) amplification contained 100 ng of each genomic DNA, *Taq* buffer (10x) - 2.5 µL, dNTPs mix (2.5 mM each) - 0.5 µL each, RAPD primer (10 pmoles) and *Taq* DNA polymerase (3U μL^{-1}) - 0.5 µL (TakaraBio, USA) sterilized Milli-Q water - 17.5 µL. Amplification was carried out in a thermal cycler (Agilent Technologies, India) with a heated lid to reduce evaporation. PCR was performed at an initial denaturation at 94ºC for 5 min, followed by 40 cycles of denaturation at 94ºC (1 min), annealing at 40ºC (1 min), extension at 72ºC (1 min), and final extension at 72ºC for 10 min. The PCR products (10 µL) were then subjected to electrophoresis with marker DNA of known molecular weight, in a 1.8% agarose gel at 60V for 1.45 h and photographed using a gel documentation system.

The bands were visually scored as 1 for presence and 0 for absence, using a gel documentation system (Bio-Rad, USA). For each primer, parameters including the total number of bands (TB), the number of polymorphic bands (PB), the number of monomorphic bands (MB), the percentage of polymorphism (PPB), and the range of fragment sizes were calculated. The polymorphism percentage, polymorphism information content (PIC), RAPD primer index (RPI), and marker index (MI) were calculated as per the following equation.

Polymorphism (%) = {(Total No. of bands – No. of monomorphic bands)/ Total number of bands} x 100

PIC = $1-\sum f^2$ where f is the frequency of the i^{th} allele

RPI = Submission of all PIC values for the concerned RAPD primer

MI = Polymorphism (%) × PIC.

Diploid data analysis for the dominant marker was performed with the assumption of Hardy-Weinberg equilibrium to calculate Jaccard's similarity, clustering, and principal coordinate analysis (PCoA) for assessing the genetic diversity and relationships among the genotypes (Jaccard, 1908). The dendrogram was constructed using the unweighted pair-grouped method arithmetic average (UPGMA) using Past software version 3.01 (Hammer *et al*., 2001).

RESULTS AND DISCUSSION

Molecular relatedness analysis helps in the categorization of genotypes and in determining the distinctness of parental sources, hybrids, and varieties. Molecular markers substantially aid breeders in selecting the superior and diverse parents for crop development (Sikdar *et al*., 2018). In present study, 120 RAPD primers were screened, of which only 25 RAPD primers gave distinct and clear bands so were used for the analysis of 16 little millet genotypes (Table I). The selected 25 decamer primers amplified a total of 193 bands with amplicon sizes in the range of 50-2392 bp and an average number of 7.72 bands per primer. The maximum number of bands (13) were scored by primer OPA-12, while the minimum number of bands (4) were scored by the primers OPC-13, OPC-20, and OPG-12 (Fig. 1). The average number of polymorphic bands per primer was 5.04. The polymorphism reported through RAPD markers was in the range of 11.11 to 100% with an average of 64.25%. The primers OPA-10, OPA-20, OPB-12 and OPB-18 showed 100% polymorphism among genotypes. The highest MI value (41.25) was recorded for primer OPC-13, and lowest for OPB-03 (1.22). The RPI values varied from 0.36 to 5.69, with an average of 2.66. The PIC value of a primer determines its suitability for genetic analysis. The efficiency of the corresponding RAPD markers in evaluating genetic relatedness can be indicated by high PIC values. The PIC values for primers were in the range of 0.04 (OPA-04) to 0.59 (OPC-17). In present study, the primers OPA-12, OPC-13 and OPC-17 were found the most efficient for genetic relatedness analysis in little millet genotypes.

Table I: RAPD primers amplification analysis among sixteen little millet genotypes

Primer	Total bands	Polymorphic bands	Polymorphism (%)	PIC	RPI	MI
OPA-01	8	5	62.50	0.31	2.47	19.29
OPA-03	6	3	50.00	0.34	2.06	17.15
OPA-04	9	3	33.33	0.04	0.36	1.35
OPA-05	7	6	85.71	0.34	2.40	29.14
OPA-08	7	1	14.29	0.14	1.00	2.03
OPA-10	10	10	100.00	0.33	3.29	32.93
OPA-12	13	9	69.23	0.44	5.69	30.31
OPA-18	6	5	83.33	0.31	1.88	26.15
OPA-20	11	11	100.00	0.31	3.39	30.82
OPB-03	9	1	11.11	0.11	0.98	1.22
OPB-04	8	6	75.00	0.41	3.25	30.47
OPB-07	10	7	70.00	0.46	4.57	31.96
OPB-10	12	6	50.00	0.43	5.10	21.26
OPB-12	6	6	100.00	0.27	1.61	26.82
OPB-18	11	11	100.00	0.39	4.32	39.24
OPC-07	7	5	71.43	0.55	3.84	39.22
OPC-13	4	3	75.00	0.55	2.21	41.25
OPC-14	7	4	57.14	0.24	1.68	13.71
OPC-17	5	3	60.00	0.59	2.93	35.16
OPC-20	4	3	75.00	0.49	1.95	36.62
OPG-08	5	2	40.00	0.25	1.23	9.84
OPG-10	11	7	63.64	0.49	5.34	30.87
OPG-12	4	2	50.00	0.26	1.02	12.79
OPH-14	6	4	66.67	0.32	1.93	21.44
OPH-19	7	3	42.86	0.27	1.87	11.43
Total	193	126	1606.24	8.61	66.38	592.48
Average	7.72	5.04	64.25	0.34	2.66	23.70

PIC - Polymorphic Information Content; RPI - RAPD Primer Index; MI - Marker Index

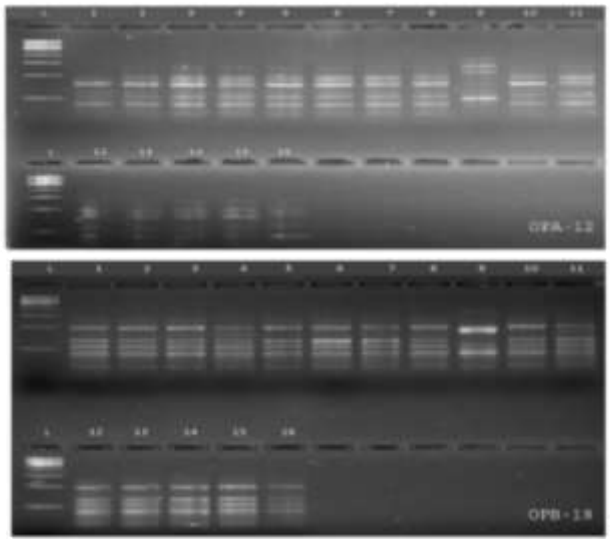

Fig. 1: RAPD profile of little millet genotypes with OPA-12 and OPB-18; **Lane: L**: DNA ladder, **1:** WV-151, **2:** WV-152, **3:** WV- 153, **4:** WV-154, **5:** WV-155, **6:** WV-156, **7:** WV-157, **8:** WV-158, **9:** WV-161, **10:** WV-162, **11:** WV-163, **12:** WV-166, **13:** WV-167, **14:** WV-168, **15:** GNV-3, **16:** JK-8

The Jaccard's similarity coefficient among the studied little millet genotypes ranged from 0.53 to 0.98, with the highest similarity in WV-166 and WV-168 (Table 2). The similarity coefficients showed moderate genetic diversity among genotypes. The dendrogram was constructed based on a similarity matrix using NTSYS 2.0 software grouped sixteen little millet genotypes into two major clusters: cluster-I and cluster-II. Cluster-I had only one genotype (WV-161), while cluster-II had two sub-clusters, i.e., II-A and II-B. The sub-group II-A was further divided into two, with one having 13 genotypes and the other one having only one genotype (WV-154). The sub-group II-B had one genotype (WV-151). The cluster pattern showed that WV-161 and WV-151 were the most diverse (Fig. 2).

Table 2: Jaccard's similarity matrix based on RAPD analysis among 16 little millet genotypes

Genotypes	WV-151	WV-152	WV-153	WV-154	WV-155	WV-156	WV-157	WV-158	WV-161	WV-162	WV-163	WV-166	WV-167	WV-168	GNV-3	JK-8
WV-151	1.00	0.81	0.80	0.73	0.77	0.78	0.77	0.75	0.53	0.76	0.78	0.77	0.78	0.78	0.77	0.76
WV-152	0.81	1.00	0.97	0.84	0.89	0.90	0.88	0.90	0.58	0.91	0.87	0.91	0.88	0.90	0.89	0.91
WV-153	0.80	0.97	1.00	0.86	0.90	0.91	0.91	0.88	0.57	0.89	0.87	0.91	0.89	0.91	0.89	0.90
WV-154	0.73	0.84	0.86	1.00	0.86	0.88	0.91	0.83	0.53	0.85	0.86	0.88	0.86	0.87	0.87	0.88
WV-155	0.77	0.89	0.90	0.86	1.00	0.96	0.93	0.88	0.54	0.88	0.90	0.90	0.89	0.91	0.93	0.90
WV-156	0.78	0.90	0.91	0.88	0.96	1.00	0.94	0.88	0.55	0.90	0.92	0.92	0.89	0.91	0.93	0.92
WV-157	0.77	0.88	0.91	0.91	0.93	0.94	1.00	0.87	0.54	0.89	0.90	0.92	0.91	0.91	0.93	0.92
WV-158	0.75	0.90	0.88	0.83	0.88	0.88	0.87	1.00	0.58	0.91	0.90	0.90	0.88	0.90	0.88	0.89
WV-161	0.53	0.58	0.57	0.53	0.54	0.55	0.54	0.58	1.00	0.56	0.54	0.57	0.56	0.56	0.55	0.56
WV-162	0.76	0.91	0.89	0.85	0.88	0.90	0.89	0.91	0.56	1.00	0.93	0.93	0.90	0.92	0.91	0.91
WV-163	0.78	0.87	0.87	0.86	0.90	0.92	0.90	0.90	0.54	0.93	1.00	0.93	0.89	0.91	0.94	0.91
WV-166	0.77	0.91	0.91	0.88	0.90	0.92	0.92	0.90	0.57	0.93	0.93	1.00	0.96	0.98	0.96	0.96
WV-167	0.78	0.88	0.89	0.86	0.89	0.89	0.91	0.88	0.56	0.90	0.89	0.96	1.00	0.97	0.92	0.93
WV-168	0.78	0.90	0.91	0.87	0.91	0.91	0.91	0.90	0.56	0.92	0.91	0.98	0.97	1.00	0.94	0.95
GNV-3	0.77	0.89	0.89	0.87	0.93	0.93	0.93	0.88	0.55	0.91	0.94	0.96	0.92	0.94	1.00	0.96
JK-8	0.76	0.91	0.90	0.88	0.90	0.92	0.92	0.89	0.56	0.91	0.91	0.96	0.93	0.95	0.96	1.00

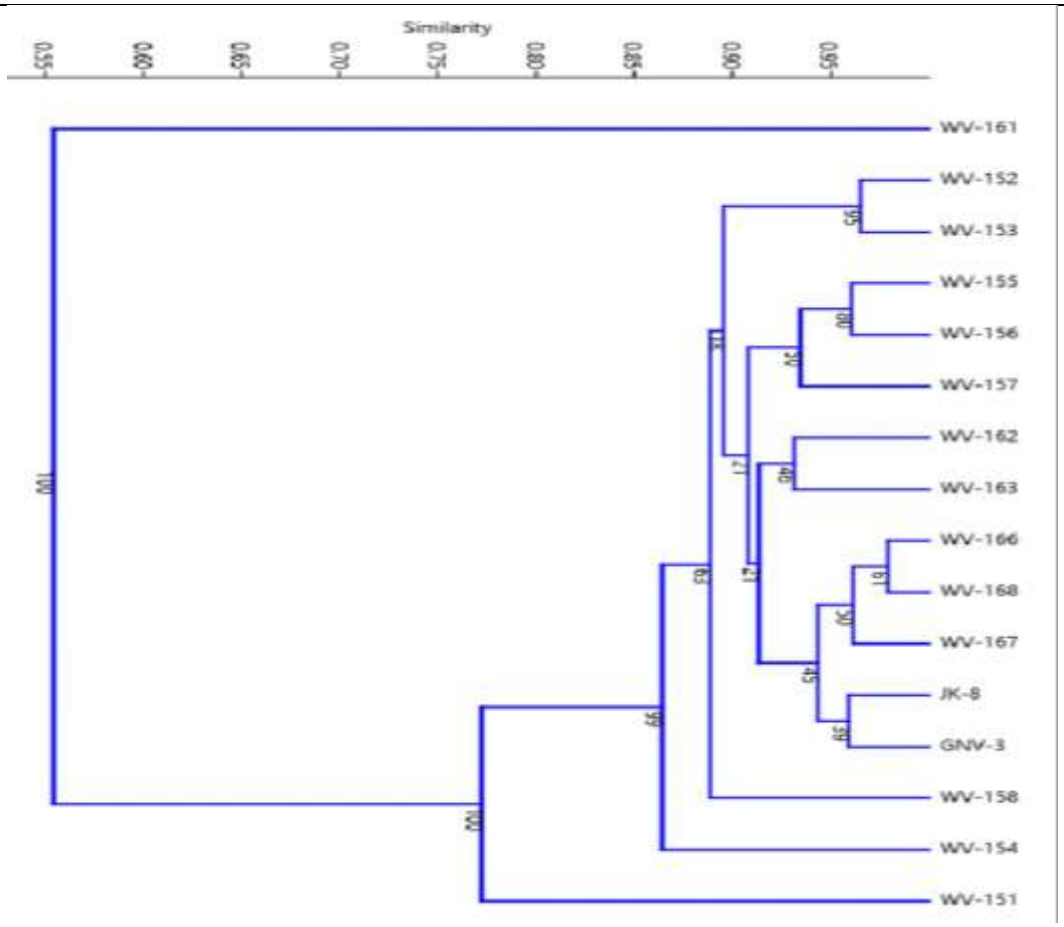

Fig. 2: Dendrogram showing genetic relatedness among 16 little millet genotypes based on RAPD analysis

The PCoA is widely used to identify the inter- and intra-population genetic similarities based on their geographic location and species identity. In the present study, the PCoA results revealed that genotypes were distributed in four clusters across the co-ordinates. As per RAPD data, the first two

axes explained 51.98% variation (Co-ord. 1 = 38.35% and Co-ord. 2= 13.63%). The outcome of PCoA analysis is in agreement with cluster analysis. The first group had two genotypes WV-152 and WV-153. The genotype WV-151 was included in 2nd group. The group-III had all the remaining 12 genotypes, except WV-161. Overall, PCoA results revealed genotypes WV-151 and WV-161 to be highly diverse from each other completely consistent with cluster analysis results (Fig. 3).

The analysis of genetic relatedness helps in understanding the molecular basis of variation, a biological phenomenon in plants. For the successful breeding of a new variety, the knowledge of genetically diverse parents from the available germplasm is essential. In the present study, RAPD was used as a genetic marker to evaluate the relatedness among 16 genotypes of little millet from the Dang district. The selected 25 primers amplified 193 bands, with an average of 7.72 bands per primer. Of these bands, 126 fragments (64.25%) were polymorphic across the whole set. The PIC values for primers ranged from 0.04 (OPA-04) to 0.59 (OPC-17). These findings are in agreement with Tiwari *et al*. (2018) who used 36 RAPD primers for 32 genotypes of little millet from different landraces of Madhya Pradesh and recorded amplified product with a size ranging from 100 to 1900 bp,175 bands (mean 4.86 bands per primer), 88.58% polymorphism and PIC value 0.102-0.517. M‟Ribu and Hilu (1994) used different OPA series RAPD primers and recorded diversity in *Panicum* millets with a product size ranging from 170 to 3790 bp.

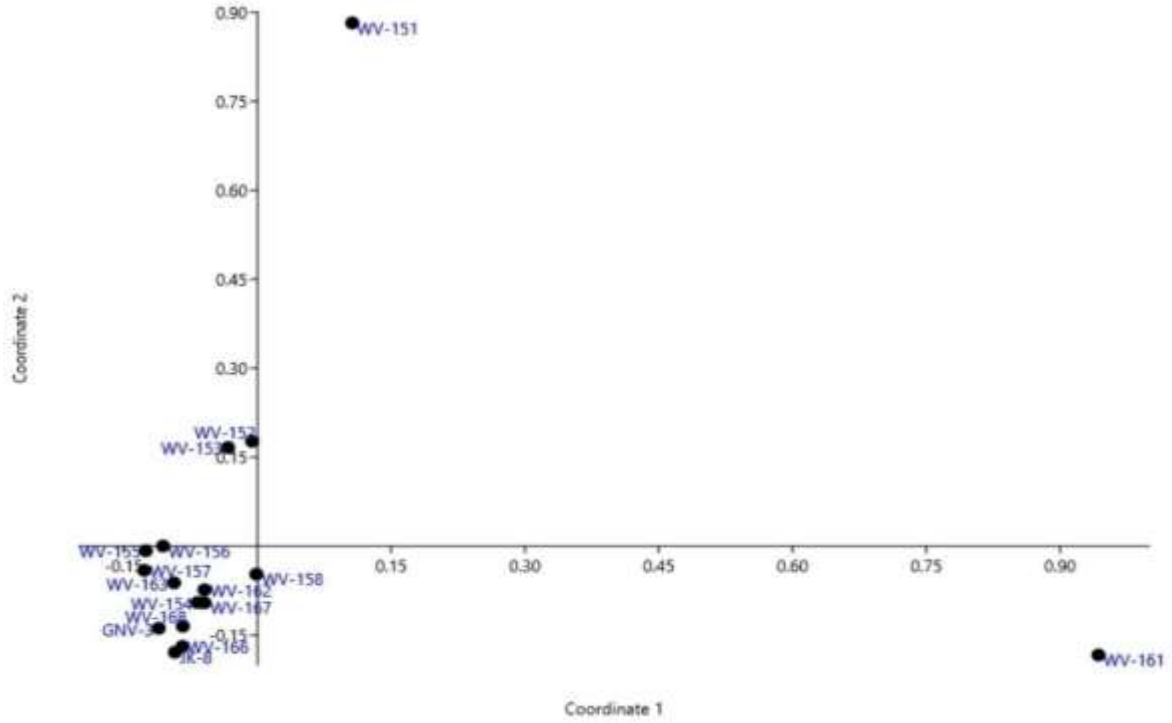

Fig. 3: Principal coordinate analysis plot showing clustering of sixteen little millet genotypes produced with RAPD analysis

The Jaccard's similarity coefficient which ranged from 0.53 to 0.98 revealed moderate genetic diversity, with minimum genetic similarity between WV-151 and WV-161, and WV-154 and WV-161; and greatest similarity between WV-166 and WV-168. Earlier studies demonstrated RAPD as an effective molecular tool for genetic diversity study in minor millets (Fakrudin *et al.,* 2004; Waghmode *et al*., 2018; Ghimire *et al*., 2019). RAPD markers can be used to analyze genetic relationships and distinguish the cultivars of little millet. The present study revealed a moderate level of genetic diversity among the available little millets genotypes. Molecular relatedness analysis is a crucial step for crop improvement and germplasm conservation. In this study, moderate genetic

diversity among available little millet genotypes was observed; WV-151 and WV-161 were highly diverged genotypes that can be exploited for further genetic improvement programs.

Acknowledgment: The authors are grateful to the Hill Millet Research Station, Navsari Agricultural University (NAU), Waghai, Gujarat, India for providing seed material; and the Department of Plant Molecular Biology and Biotechnology, ASPEE College of Horticulture and Forestry, NAU, Navsari for providing the facilities for research work.

Conflicts of Interest: The authors declare that they do not have any conflict of interest.

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