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ASSESSMENT OF GENETIC DIVERSITY IN PEARL MILLET (*PENNISETUM GLAUCUM* L.) GENOTYPES USING MORPHOLOGICAL AND MOLECULAR MARKERS

Bahy R. Bakheit[1], Asmaa A. Mohamed[1], Atif Abo-Elwafa[1], Bahaa E.S. Abd El-Fatah[2,3], Mohamed A. Abdein[4]*, Amany M. Mousa[5], Noha A. Sukar[6], Samah A. Sabry[7], Salwa E. Mohamed[8], Mohamed Z. Diyasty[9], Mai M. Wahba[10], Sahar A. El-Sanusy[2]

[1]Department of Agronomy, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt
[2]Department of Genetics, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt
[3]Faculty of Biotechnology, Badr University in Cairo (BUC), Cairo, 11829, Egypt
[4]Seeds Development Department, El-Nada Misr Scientific Research and Development Projects, Turrell, Mansoura, 35511, Egypt
[5]Department of Botany and Microbiology, Faculty of Science (Girls), Al-Azhar University, Cairo 11884, Egypt
[6]Agricultural Botany (Genetics) Department, Faculty of Agriculture (Girls), Al-Azhar University, Cairo, Egypt
[7]Department of Agricultural Botany, Faculty of Agriculture, Suez Canal University, Ismailia 41522, Egypt
[8]Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Sadat City 32897, Egypt
[9]Genetic Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt
[10]Botany Department, Faculty of Science, Mansoura University, 35516, Mansoura, Egypt
***Correspondence author:** Mohamed A. Abdein: abdeingene@yahoo.com

ABSTRACT

A strategy for any crop development effort is the effective and methodical use of diversity at the morphological and DNA levels. Three methods for molecular markers (SSR, ISSR and SRAP) and morphological descriptors were utilized to assess the genetic variance between 21 pearl millet genotypes. Genotype samples were collected from seven regions: the United States, Nebraska, Egypt, Niger, Oman, India, Burkina Faso, and Yemen. Significant differences between the genotypes for the characteristics under investigation were found using analysis of variance. The PCV was greater than the GCV for each character in the research. For fresh forage productivity, substantial genetic progress as a ratio of mean and strong heritability were observed. Three major clusters may be formed from the 21 pearl millet genotypes using the Euclidean distance method. The dendrogram, which combined three molecular marker systems with agro-morphological characteristics, separated the 21 Pearl millet genotypes into four main groups. Both sets' genotypes showed a weak except substantial positive correlation ($r = 0.303$, $P < 0.001$) between simple matching distance (molecular distance, SM) and Euclidean distance (phenotypic distance, ED). Only the genotypes with the highest fresh forage yield/plant, tillers/plant, and leaves/stem values possessed DNA fragments measuring 249 bp (HB13), 356 bp and 260 bp (HB15), 289 bp (SRAP-6), and 195 bp (SSR-3). Distinct bands were seen among genotypes taken from the same location. Combining agro-morphological characteristics with molecular markers may facilitate differentiation between these genotypes. Pearl millet's agro-morphological traits may also be enhanced via breeding programs that use a range of genotypes.

KEYWORDS: *Pennisetum glaucum* L.**,** genetic diversity, morphological markers, geographic regions, molecular markers

INTRODUCTION

Pearl millet (*Pennisetum glaucum* L.) (2n=2x=14) is considered a highly versatile crop in many regions of the world. Behind sorghum, barley, maize, rice and wheat, it is the sixth furthermost significant cereal globally **Singh and Perz-Maldonado (2003**). Pearl millet is cultivated for food and drink by resource-poor farmers in the Indian subcontinent and sub-Saharan Africa (**Abubakar et al. 2019**). Pearl millet is especially important in semi-arid dryland areas of Southeast Asia and Africa **Baltensperger (2002).** It is considered a superior fodder crop in the USA and Australia, outperforming other fodder grasses. During the summer months, pearl millet is frequently used in Egypt to feed animals.

Natural selection drives survival through genetic diversity, which is the range of genes within a species and is essential for adaptability and the preservation of favorable features **Holden and Peacock (1993**). This variation can manifest itself in several ways, including variations in DNA sequences, chromosome numbers and structures, and cell DNA amounts. Selection, migration, genetic drift, recombination, and all contribute to genetic diversity. It is essential for investigating plant genetic resources, which have shown promise in agricultural development and conservation genetics **Sharma (2001**).

Knowledge of the gene pool of a crop is critical for the success of crop improvement programs, as both the environment and genetics contribute significantly to an organism's phenotype **Sharma (2001)**. Argo-morphological traits were initially used by researchers to assess genetic diversity in many crops, such as sorghum (**Beta and Cork, 2001;Gelata and Labuschagne, 2005;Barnaud et al. 2007**), barn swallows **Cadee (2000**), and spring wheat **Briggs (1991**). However, several investigations have demonstrated that environmental influences can significantly affect morphological markers, making them insufficient for reliably classifying accessions (**Redfearn et al. 1999; Cadee, 2000; Bahram et al. 2014**). Characterizing genetic distances across genotype groups and differentiating germplasm accessions are made more accurate by molecular markers (**Antunes et al. 1997**).

Recently, a variety of molecular markers, both dominant and co-dominant, have been employed to define the genetic distinction of plants. Among these, Simple Sequence Repeat (SSR) markers are found randomly in both non-transcribed and transcribed sections across the genome and exhibit substantial polymorphism within populations. According to **(Sargai-Maroof et al. 1994; Manzelli et al. 2007),** SSR markers are designed to detect tandem duplicates of di- or tetra-nucleotide DNA templates. SSR markers have been effectively used to assess genetic variation in a variety of crops, such as *Pennisetum purpureum* (**Azevedo et al. 2012**), sorghum (**Cuevas et al. 2014**), maize (**Salami et al. 2016**), *Vicia faba* **EL-Shaer** and **Helal (2020)** and rice (**Salem et al. 2024).** A few SSR markers have been used for pearl millet. Several SSR markers designed to evaluate genetic diversity have been applied to pearl millet (**Stich et al. 2010; Nepolean et al. 2012; Gupta et al. 2015**). Furthermore, evaluations of genetic diversity have made use of dominant Sequence-Related Amplified Polymorphism (SRAP) markers that target genomic coding regions **Li and Quiros (2001**). SRAP has been effectively utilized for the creation of linkage maps in pearl millet, despite the paucity of reports on its application for genetic diversity **Pedraza-Garcia et al. (2010**). Many studies have shown the efficiency of SRAP for founding genetic variation between genotypes (**Ammar et al. 2015; Youssef et al. 2019; Alharbi et al. 2024**; **Jadhav et al. 2025).** Recently, **Abdein et al. 2026** studied SRAP Markers in Sixteen Rutaceae Taxa. Higher polymorphism frequency, speed, technical simplicity, low DNA requirement (just a few nanograms), no requirement for prior DNA sequence knowledge, and automation are some of the benefits of RAPD and ISSR markers (**Fahima et al. 1999**). ISSR markers can be amplified using primers that are 18–25 bp long and target areas of the genome that are bordered by microsatellite sequences. These are dominant, multi-locus markers that amplify unknown sequences using a single, arbitrary primer **Ng and Tan (2015**). Genome mapping, gene tagging, phylogenetic studies, genetic diversity analysis, and evolutionary biology have all made substantial use of RAPD and ISSR markers across a broad range of crops. These markers have been remarkably effective at revealing genetic variation at the genome level RAPD (**El-Adl et al. 2012; Tomar et al. 2014; Mohamed et al. 2018; Hussein et al. 2019; Osman & Abdein 2019**, **Mostafa et al. 2021** and **Ibrahim et al. 2022**). ISSR markers were utilized in numerous research to evaluate genetic diversity such as Pumpkin accessions **(Abdein 2018**), Tomato genotypes **(Abdein et al. 2018** & El-Mansy **et al. 2021),** *Thymus decussatus* **(Mahgoub et al. 2020)**, Thymus species **(Alqahtani et al. 2020**), genus Tephrosia Pers. **(Abdein et al. 2020),** Summer Squash genotypes **(Abdein et al. 2021),** Wheat genotypes **(Shaban et al. 2022),** Family Asteraceae **Alhaithloul et al. 2023,** Soybean genotypes **(Khalifa et al. 2023),** Bottle gourd **(Ibrahim et al. 2024)**, Rosemary (**Alqahtani et al. 2024),** Olive **(Alharbi et al. 2024** & **Alhaithloul et al. 2024)** and *Clitoria ternatea* **(Yusuf et al. 2025).** Applying molecular markers and agronomic performance measures, this study intends to assess the field performance of 21 *Pennisetum glaucum* genotypes by examining their genetic diversity variety.

MATERIALS AND METHODS

For this investigation, twenty pearl millet genotypes were used, in addition to the Egyptian variety Shandaweel-1. As indicated in **Table 1,** the initial authors' prior accessions were acquired from the US USDA ARS Plant Genetic Resources Unit. These genotypes were cultivated at EL-Ghorieb, Faculty of Agriculture, Assiut University, over the two growing summer seasons of 2022 and 2023 (92.1 sand %, 4.3 silt %, 3.7% clay, Ph 8.2 total nitrogen 0.02%, total $CaCo_3$ 15.2%, Field capacity 16.2). Three replications of these twenty-one genotypes were set up in R.C.B.D. One row, three meters long and spaced 60 centimeters apart, made up the plot. The hills were separated by 10 cm. When the seedlings fully emerged, there was just one plant per hill. For optimal pearl millet yield, all cultivation techniques were kept at their best. Every season, three cuts were made. At the time of each cut for every season, ten randomly selected plants from the middle of each row were measured. The characteristics, such as the total fresh forage yield per plant, mean leaves/stems ratio, and number of tillers/plants, were examined. Two seasons were used as the means for the investigation of differences, and the means were compared at the 5% level of probability using the R.L.S.D. analysis **Gomez and Gomez, (1984**). The following is an estimate of the wide definition of heritability based on the phenotypic (PCV) and genotypic (GCV) the phenotypic and genotypic coefficient of variability calculated using **Burton (1952)**: Heritability (H) $=\frac{\sigma_g^2}{\sigma_p^2}\ x\ 100$

Molecular analysis

DNA isolation

DNA was extracted from the young leaf tissues of the 21 genotypes of pearl millet using the CTAB technique established by **Doyle and Doyle (1987).** Ethidium bromide staining and 0.8% agarose gels were used to assess the isolated DNA's purity. At 260 nm, the amount was determined by a UV spectrophotometer.

SRAP, ISSR and SSR detection and analyses

The template DNA was amplified using six SSR primer combinations, five SRAP primers, and five ISSR primers (**Table 2**) that were purchased from Metabolon International AG Company (Germany). The PCR reaction contained the following components: 11.7 µL of dH2O, 3.0 µL of 10X reaction buffer, 3.0 µL of dNTP mix (2.5 mM each dNTP; Promega), 2.0 µL of primer (2.5 µM for ISSR), 1.0 µL of forward primer, 1.0 µL of reverse primer for SRAP and SSR, 4.0 µL of MgCl2 (25 mM), 0.3 µL of Taq DNA polymerase (5 U per µL; Promega), and 1.2 µL of template DNA (50 ng per µL). For PCR activities, a Lab Cycler (Model Sens Quest, GmbH, Germany) was utilized. The following were the ISSR marker PCR amplification parameters: One minute at 92°C, one minute of annealing at 38°C to 44°C, two minutes at 72°C, ten minutes at 72°C, and finally a final hold at 4°C make up the 45 cycles. This is how the PCR program for SRAP marker amplification appears: Ten cycles of 92°C, 35°C, and 72°C for one minute each; thirty-five cycles of 92°C, annealing primer, and 72°C for one minute each; ten cycles of 10 minutes each at 72°C; and a final hold at 4°C. The SSR markers were as follows: 45 cycles of 1.5 minutes at 92°C, 1.5 minutes of primer pair annealing, and 2 minutes at 72°C, followed by 10 minutes at 72°C, five minutes at 94°C, and a final hold at 4°C. PCR yields were split on 1.4%, 2%, and 2.5% agarose gels for ISSR, SRAP, and SSR, respectively. A GelDoc-It®2 Imager was used to view the DN A bands after the gels were stained with 0.5 µg/mL ethidium bromide (EB). Visual grading of each primer's presence (1) or absence (0) of DNA bands in each genotype produced a binary matrix. The genetic similarity (Gs) amongst genotypes of pearl millet was determined using **Dice's approach (1945).** Similar estimates were used to generate a dendrogram with Rohlf's NTSYS-pc version 2.11T (2000). We calculated the associations among the DNA markers and agro-morphological parameters using the **Mantel** (**1967**) test. The three parameters, marker index (MI), polymorphic information content (PIC), and resolving power (Rp) were calculated using the following formulas: MI = PIC x ηβ **(Powell et al.1996)**, PIC = 1- [(p)2 + (q)2] (**Ghislain et al. 1999**) and PR = ∑Ib **Prevost and Wilkinson (1999)**.

RESULTS

The results obtained from this study are as follows:

Performance of pearl millet genotypes

The results of this study demonstrated that the number of tillers per plant, the ratio of leaves to stems, and the amount of fodder produced varied significantly from genotype to genotype. PCV for each trait under investigation being higher than the GCV. The number of tillers and fresh forage yield demonstrated moderate PCV and GCV, the leaves/stem ratio demonstrated low PCV and GCV. The number of tillers had a moderate genetic advance, the leaves/stem ratio had a low genetic advance, and the fresh forage yield had a high genetic improvement as a ratio of mean. In this study, fresh forage productivity showed considerable genetic progress and strong heritability. There was moderate heritability in the quantity of tillers and the leaves/stem ratio **(Table 3).**

For 21 genotypes of pearl millet, the average fresh forage yield, leaves/stems ratio, and number of tillers/plants during two seasons are displayed in Table 4. For forage crops, the number of tillers or plants is one of the most crucial criteria in determining the forage production. There were 5.4 tillers/plants representing genotype No. 9 and 7.3 used for genotype No. 16, with an average of 6.17.

The four genotypes, G6, G11, G16, and G20, produced much better results than the control cultivar, shandaweal-1, and the average overall genotypes. Moreover, the ratio of leaves to stems is one of the most crucial influences in defining the feed crops' quality and palatability. For genotype No. 18, the leaves/stems ratio was 0.51, whereas for genotype No. 11, it was 0.66, with an average of 0.57%. Outperforming the check cultivar and the average leaves/stems ratio for all genotypes were five of them: G3, G8, G11, G16, and G20.

These superior genotypes could improve pearl millet breeding by increasing the ratio of leaves to stems. Genotype No. 15 produced an average total fresh forage yield of 770 g, while genotype No. 20 produced an average of 1539 g per plant. Check variety shandaweel-1 and average fresh forage production were significantly surpassed by four G6, G11, G16, and G20 genotypes and three G6, G16, and G20 genotypes, respectively. The amount of fresh feed produced per plant could be increased by using these genotypes.

Cluster Analysis based on agro-morphological data

The Euclidean distances for 21 pearl millet genotypes were computed using standardized agro-morphological data, and the results were used to create a UPGMA dendrogram. Two major groupings were identified from a multivariate analysis of 21 pearl millet genotypes (**Fig. 1**).

Cluster I included the most genotypes (15), accounting for 71.45% of all genotypes. Next in line was Cluster II, which comprised six genotypes and represented 28.57% of all genotypes. The greatest mean values in Cluster II were found in the fresh forage yield (1435.55), number of tillers (7.03), and leaves/stem ratio (0.63).

Molecular marker analysis

ISSR analysis

Sixty-three recorded bands were obtained from five ISSR primers that were screened across 21 genotypes of pearl millet (**Table 5**). The bands differed in size from 170 bp (ISSR-844) to 972 bp (HB13), and all genotypes underwent amplification (**Fig. 2**). **Table 5** shows that the average number of amplified bands per primer was 12.6 and ranged from 10 (HB15) to 15 (ISSR-844). With an average of 6.8 polymorphic bands each primer, 34 (53.97%) of the sixty-three amplified bands were polymorphic. There were five bands (HB15) to nine (ISSR-844), with an average polymorphism of 53.97%. Additionally, the percentage of polymorphic bands showed a mean of 0.17 and ranged from 50.0% (HB12 and HB15) to 60.0% (ISS-844) **(Table 5).** According to Table 5, the PIC values ranged from 0.14 (primer HB15) to 0.21 (primer ISSR-814). The five ISSR primers had a mean Rp of 3.01 and varied from 1.81 for primer HB15 to 4.19 for primer ISSR-814. (**Table 5**). Using two ISSR primers (ISSR-844 and ISSR-814), which had the highest Rp values (4.0 and 4.19, respectively), all 21 Pearl millet genotypes could be identified.

SRAP analysis

Fifty-five scored bands were obtained from the five SRAP primer combinations that were tested across the 21 pearl millet genotypes **(Table 5)**. All genotypes' amplicons were between 1478 bp (SRAP-1) and 184 bp (SRAP-5) in size **(Fig. 3).** There were six amplified (SRAP-2) and fourteen amplified (SRAP-3). Of the 55 amplified bands, 34 (61.82%) had polymorphic bands, and an average of 6.8 polymorphic bands/primer, compared to an average of 11 bands/primer (**Table 5**). The range of these bands was 4 (SRAP-2) to 10 (SRAP-3). The average of 61.82% was shown by the proportion of polymorphic bands exerted among the primers, which ranged from 54.55% (SRAP-1 and SRAP-5) to 71.43% (SRAP-3). Primers SRAP-1, SRAP-NM 2, and SRAP-3 had PIC values ranging from 0.15 to 0.22, with a mean of 0.19 **(Table 5).** Additionally, the five SRAP primers had an average Rp of 2.72, ranging from 1.71 (SRAP-2) to 4.38 (SRAP-3) **(Table 5)**. The SRAP-3 primer, with the highest Rp value of 4.38, was used to identify the genotypes of pearl millet.

SSR analysis

Six SSR primer pairs, presenting 4 (SSR-3 and SSR-10) to 11 (SSR-4) bands with a mean of 7 bands/primer, were used to evaluate 42 bands with high intensity. Twenty four of the 42 amplified bands recorded polymorphic, with each primer containing an average of 6.0 polymorphic fragments (**Table 5 and Fig. 4**). Polymorphism extended from 45.45% (SSR-4) to the highest of 75.0% (SSR-3 and SSR-10) with an average of 57.14% for full genotypes (**Table 5**). With a mean of 0.16, the PIC values extended from 0.13 (SSR-4) to 0.19 (SSR-8), as shown in **Table 5**. Additionally, the Rp averaged 1.4, ranging from 0.86 for primers SSR-3 and (SSR-10) to 2 (SSR-8) (**Table 5**). Two SSR primers (SSR-4 and SSR-8) with the greatest Rp values (1.7 and 2.0, respectively) were used to differentiate the pearl millet genotypes (**Table 5**).

Combined analysis of molecular data

A combined examination of three distinct molecular marker techniques (ISSR, SRAP, and SSR) yielded more precise genetic estimations. One hundred sixty bands were obtained by all 16 primers using the ISSR, SRAP, and SSR molecular marker systems. with an average of 10 bands for each primer or primer pair, and the average frequency of polymorphisms was 58.89% (**Table 5).** The genotype G8 had the most bands (126) of all the DNA pieces, followed by G3 and G11 (125); the genotype G4 had the fewest bands (110). The GS coefficients (G4 and G17, G6 and G17, G4 and G18) ranged from 0.79 to 0.98 (G1 and G2, G12 and G14) according to the **Dice coefficient (1945)**. According to the dendrogram, the 21 Pearl millet genotypes were separated into four main groups **(Fig. 5).** Cluster I, the largest cluster with 11 genotypes, was further divided into two smaller clusters **(Fig. 5)**. Cluster I-1 contained the following five genotypes: G1, G2, G4, G5, and G7. Cluster I-2 had six genotypes: G10, G12, G13, G14, G19, and G21 **(Fig. 5)**. The genotypes exhibited moderate values for fresh forage yield/plant, leaves/stem, and tillers/plant. Cluster II consisted of six genotypes: G3, G6, G8, G11, and G20. With a similarity of 0.855, G16 is isolated from the other genotypes in a single branch and displays distinct performance. It was found in Cluster III. The genotypes in clusters II and III displayed the highest values for each trait. Cluster IV consisted of four genotypes; the lowest values for each trait were found in G9, G15, G17, and G18. The current study's findings demonstrated that the dendrograms' cophenetic correlation values were as follows: SRAP + SSR (r = 0.688), ISSR + SRAP (r = 0.668), ISSR + SSR (r = 0.471), and SSR + (SRAP + ISSR) (r = 0.537). Furthermore, the Mantel test showed a small but significant correlation between simple matching distance (molecular distance, SM) and Euclidean distance (phenotypic distance, ED) for pearl millet genotypes of both sets ($r = 0.303$, $P < 0.001$).

The genotypes that were investigated can be applied in direct breeding programs to develop the agro-morphological characteristics of pearl millet, based on these overall results. Agro-morphological characteristics and variety based on DNA markers may also help with genotype differentiation. Only the six genotypes G3, G6, G8, G11, G16, and G20 produced DNA fragments by molecular sizes of 249 bp (HB13), 356 bp and 260 bp (HB15), 289 bp (SRAP-2), and 195 bp (SSR-2), according to the study's final results. These genotypes also had the maximum values of tillers/plant, leaves/stem, and fresh forage yield/plant. A DNA fragment with a molecular size of 855 bp (ISS-814) for the 11 genotypes—G1, G2, G4, G5, G7, G10, G12, G13, G14, G19, and G21—was found. These genotypes had fresh forage yield/plant, tillers/plant, and leaves/stem at intermediate levels. Twelve DNA fragments were identified in genotypes that had high and moderate levels of fresh forage

yield/plant, tillers/plant, and leaves/stem (i.e., 308, 489 bp (HB12), 344 bp (HB13), 375 bp (SRAP-1), 280, 407, 634 bp (SRAP-4), 558 bp (SRAP-5), 183, 607 bp (SSR-1), 361 bp (SSR-4), and 499 bp (SSR-5). For all traits, the genotypes with the lowest values did not have these fragments. According to the results, only the six genotypes—G1, G8, G10, G12, G14, and G15—obtained from Yemen included 176 bp DNA fragments (SRAP-2). The genotypes collected from Yemen might be unique to this band. The two genotypes G2 and G9 collected from Burkina Faso were the only ones with noteworthy DNA segments of 489 bp (SRAP-3) and 367 bp (ISSR-844). Only DNA fragments that have molecular sizes of 964 bp (SRAP-3) and 212 bp (SRAP-5) were found in the five Indian genotypes G3, G7, G11, G17, and G20). Only the two genotypes collected from Niger, G4 and G16, possessed DNA fragments including a molecular size of 231 bp (HB12). A 543 bp DNA fragment was only present in the genotype G5 Oman (SRAP-3). Only the four genotypes G6, G13, G18, and G20 collected from Nebraska, USA, included DNA fragments having molecular sizes of 1056 bp, 699 bp (SRAP-3), 283 bp (SRAP-5), and 202 bp (SSR-6). DNA segments having molecular weights of 184 bp (SRAP-5), 388 bp (ISSR-844), and 390 bp (HB13) were amplified only in the genotype G21 that was acquired from Egypt.

DISCUSSION

Plant species are essential in scientific research of plant genetic distinction and evolutionary traits. Agronomic and molecular estimates are utilized to investigate genetic diversity among landraces and genotypes. According to **Farooq et al. (2017)**, these are the initial steps in improving advantages through plant breeding initiatives. Genetic investigation of numerical traits is essential for plant breeding systems because it enables a systematic approach to strategic planning and breeding method design**.**

The analyzed pearl millet genotypes shown very significant differences based on the analysis of variance for agronomical variables as an average of two seasons for the number of tillers/plants, leaves/stem, and fresh forage yield/plant, as shown in **Table 3.** various findings support those of **(Yadav and Bhatnagar 2001; Yadav et al. 2014; Bakheit et al. 2021).** The study's findings showed that various genotypes had different genetic compositions. The results of the current study showed how the environment affects the expression of traits, with the PCV for each trait under investigation being higher than the GCV (**Table 3**). Research by **Rachel (2014) and Osman et al. (2012)** demonstrated that the degree of variation among the genotypic and phenotypic coefficients of variation can be used to quantify the change of the environment on a trait. This outcome is consistent with their findings. A larger difference indicates a stronger environmental effect, while a smaller difference suggests a greater genetic influence.

For each variable in this study, the slight differences amongst the genotypic and phenotypic coefficients of variation imply that the environment influences these traits' manifestation to some extent. This suggests that future breeding efforts would benefit from selection based on these features **Falconer (1960)**. **Lush (1949)** defined heritability as the proportion of genetic variety passed down from parents to children. In this case, the estimated heritability of fresh forage production was high. The leaves/stem ratio showed little genetic advancement and moderate heritability. The characters' moderate to high heritability recommended that the choice would be more useful. These traits, which are mostly controlled by additive gene activity, can be enhanced by individual plant selection **Panse (1958)**. As demonstrated by **Johnson et al. (1955)**, a trait with great heritability does not constantly correspond to great genetic advancement. According to **Dudley and Moll (1969); Johnson et al. (1955**), estimation of heritability combined with genetic progress provides more useful selection criteria than heredity alone.

Selection mechanisms are therefore thought to benefit from a trait that has both strong genetic progress and high heritability. Pearl millet will therefore profit from selection for this trait. According to this, pearl millet will profit from selection for this trait since additive gene activity is thought to be a key influence in it. Similar findings were obtained by **Shanmugapackiam and Raguchander (2018); Gulnaz et al. (2011); Ajmal et al. (2009),** who revealed a moderate intensity of genetic progress and heredity for the quantity of tillers.

Overall, the findings demonstrate the genetic variety of the genotypes studied that were the subject of the investigation. These results are consistent with those of **Yadav and Kumar (2013) and Bakhat et al. (2021**).

Cluster I illustrated the lowest mean values for each feature. Similar results agreed with studies (**Subhash et al. 2020; Abubakar et al. 2019).** Previous studies by **(Ramya et al. 2017; Singh et al. 2017; Kaushik et al. 2018; Abubakar et al. 2019; Fasoula et al. 2020; Kumar et al. 2020 ;Darshanaben et al. 2023)** have identified possible parents for heterotic expression of yield components in pearl millet by clustering germplasm accessions based on quantitative data. As potential breeding material, the parents selected from cluster II will be employed to improve the traits that contribute to yield in the hybridization effort.

Pedigree and origin of genotypes were not discovered in the genotype distribution, despite the fact that genotypes were suspiciously sorted and compared by classifying according to phenotypic data in phenotypic diversity analysis. Similar results were also detected on pearl millet by **(Yadav et al. 1994; Darshanaben et al. 2023; Kumar et al. 2020).**

Molecular marker analysis

Molecular marker technology is a valuable tool for researching genetic variability. Traditional DNA markers are broadly used to survey the diversity of plants. Some examples of these markers are SSR, SRAP and ISSR (**Yan et al. 2010; Zhang et al. 2022).** DNA assessment with ISSR and SRAP markers has been shown to be a cost-effective and effective method of providing molecular data for assessing genetic diversity **Li and Ge (2001)**.

Microsatellite markers are highly reproducible, have multiple alleles, abundance, high levels of informative, polymorphism, repeatable and co-dominant, they are widely used for genetic diversity, molecular mapping, cultivar identification and germplasm characterization, (**Singh et al. 2018; Irshad et al. 2022; Khaled et al. 2022 ; Junaid et al. 2023)**.

Because of their high allelic difference and ability to characterize co-sites, SSRs markers are a great method for genetic research and marker-supported differentiation in breeding **(Gupta and Varshney 2000; Hernandez et al. 2002)**.

Our results indicate that the total bands for three markers under study recorded (160 bands). ISSR markers produced the grates number of bands (63 bands) as compared with SRAP (55 bands) and SSR (42 bands). According to **Aziz and Tahir (2023**), PIC and MI are useful tools for determining genetic differences within and between populations.

In this study the PIC average recorded the highest value by SRAP marker (0.19) followed by ISSR (0.17) and finally, SSR marker (0.16). Furthermore, the three molecular markers showed effectiveness in assessing genetic diversity across genotypes. All 21 Pearl millet genotypes could be identified according to **(Shital et al. 2023 and David et al. 2015).** Previous study has also investigated genetic variation on pearl millet genotypes using SSR marker **(Chandra et al. 2007; Kapila et al. 2008; Gupta et al. 2018).** The remarkable genetic diversity and featured pearl millet and other genotypes were found by **(Cadee, 2000; Jaya Prakash et al. 2006; Tomar et al. 2014)**. These results in agreement with studies (**Liu et al. 2008; Bhatt et al. 2017; Zala et al. 2017; Darshanaben et al. 2023).**

To examine the genetic relationships amongst 21 Pearl millet genotypes, a dendrogram was created utilizing the combined data from 3 molecular marker techniques (ISSR, SRAP, and SSR). Twenty-one pearl millet genotypes were divided into 4 major groups. Clusters I, II, III and IV had 11,5, one and 4, eight genotypes respectively. Forty-nine pearl millet accessions were grouped into 8 clusters using SSR markers**(Nehra et al.2017)**.In pearl millet, **Kumar et al. (2020)** used 74 SSRs to divide 18 genotypes into three clusters. The dendrogram, which was made using combined data from SSR and SRAP markers separated forty-eight pearl millet genotypes into 7 primary groups **(Gunguniya et al. 2023).**

CONCLUSION

We used two distinct marker methods morphological traits and molecular markers (ISSR, SRAP, and SSR) to produce valuable pre-breeding information. This information can help select suitable parents to boost genetic variety in pearl millet breeding operations and ultimately help breeders develop new strains. Significant genetic variation was discovered at together molecular and geomorphological levels. Furthermore, the three molecular markers showed effectiveness in assessing genetic diversity across pearl millet genotypes. We were able to produce a more comprehensive and perceptive profile of the germplasm by combining morphological and molecular characterization, which can significantly improve the efficacy and success of breeding programs.

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Data Availability Statement: The data presented in this study is available on request from the corresponding author.

Declare no conflict of interest: The authors declare that they have no known competing financial interests or personal relationships.

Table 1. Name and origin of the studied pearl millet genotypes

Item	Accessions	Plant Name	Country
G1	P1 587007	1092	Yemen
G2	P1 536416	Yiro	Burkina Faso
G3	P1 661270	Griflbbog	India
G4	P1 537066	Ennele	Niger
G5	P1 532178	7168	Oman
G6	P1 613107	NM-3A1	United States Nebraska
G7	P1 661277	Griflbblb	India
G8	P1 587000	1060	Yemen
G9	P1 536377	Nato	Burkina Faso
G10	P1 587015	1197	Yemen
G11	P1 661270	Grif lbbog	India
G12	P1 586999	1049	Yemen
G13	P1 613104	NM-2A1	United States Nebraska
G14	P1 587005	1086	Yemen
G15	P1 586995	1026	Yemen
G16	P1 537069	Baqudarache	Niger
G17	P1 661273	Griflbb12	India

G18	P1 613104	NM-2A1	United States Nebraska
G19	P1 613108	NM-3A4	United States Nebraska
G20	P1 661276	Grig lbb15	India
G21	Shandaweel-1	-	Egypt

Note: G = Genotype code; Pl = Plant number.

Table 2. ISSR, SRAP and SSR primer sequences used in the study

	Sequence (5′ to 3′)	
	ISSR	
HB12	5'-GTGGTGGTGGC -3'	
HB13	5'-CTCTCTCTCTCTCTCTAG -3'	
844	5'-GAGGAGGAGGC -3'	
814	5'-CTCTCTCTCTCTCTCTTG -3'	
HB15	5'-CACCACCACGC-3'	
	SSR	
SSR-1	5'-CGTCGAAGGATTTCACTATC-3'	5'-CAATTGCACATCAGATTCAC-3'
SSR-2	5'-TGTTTTACCCCTAACCGTCCC-3'	5'-TACCCGTACCCGTCCGTA-3'
SSR-3	5'-TAGCAAAAGCGATGATTGTCTG-3'	5'-CCCCTAAACCCTAATCCTGACT-3'
SSR-4	5'-TCAAGACAAGGATAAAGTCGCAC-3'	5'-TAATGCAGGGTGATTTAGATGG-3'
SSR-5	5'-TAACGCAAACTGAACTCTCGTTA-3'	5'-ACCTAACCCTAACCCTAAACCG-3'
SSR-6	5'-CGTCGAAGGATTTCACTATC-3'	5'-CAATTGCACATCAGATTCAC-3'
	SRAP	
SRAP-1	Me-2 5′-TGAGTCCAAACCGGAGC-3′	Em-2 5′-GACTGCGTACGAATTTGC-3′
SRAP-2	Me-3 5′-TGAGTCCAAACCGGAAT-3′	Em-3 5′-GACTGCGTACGAATTGAC-3′
SRAP-3	Me-4 5′-TGAGTCCAAACCGGACC-3′	Em-4 5′-GACTGCGTACGAATTTGA-3′
SRAP-4	Me-5 5′-TGAGTCCAAACCGGAAG-3′	Em-5 5′-GACTGCGTACGAATTAAC-3′
SRAP-5	Me-6 5′-TGAGTCCAAACCGGACA-3′	Em-6 5′-GACTGCGTACGAATTGCA-3′

Note: ISSR = Inter Simple Sequence Repeat; SRAP = Sequence-Related Amplified Polymorphism; SSR = Simple Sequence Repeat.

Table 3. Analysis of variance for agronomical traits of 21pearl millet genotypes (average of two seasons)

Source of Variance	D.F	Means square		
		Number of tiller/plants	Leaves/stem Ratio	Fresh Forage Yield
Replication	2	0.26	0.001	6278.96
Genotype	20	1.43**	0.005**	113836.35**
Error	40	0.27	0.001	10651.47
GCV		10.08	6.36	15.08
PCV		13.13	8.42	17.26
H (heritability)		58.9	57.1	76.4
GG		15.93	9.91	27.15

Note: GCV = Genotypic Coefficient of Variation; PCV = Phenotypic Coefficient of Variation; H = Heritability; ** = significant at $P < 0.01$.

Table 4. Means of number of tillers per plant, leaves/stem ratio and fresh forage yield of 21 pearl millet genotype (of two seasons).

Genotypes	Number of tiller/plants	Leaves/stem ratio	Fresh Forage yield
G1	5.8	0.56	1180
G2	6.1	0.55	1035
G3	6.8	0.64	1380
G4	5.9	0.56	1305
G5	5.9	0.56	1290
G6	7.1	0.61	1420
G7	5.8	0.56	1150
G8	6.6	0.62	1380

G9	5.4	0.54	915
G10	6.0	0.56	1330
G11	7.2	0.66	1383.3
G12	6.0	0.55	1250
G13	6.1	0.57	1150
G14	6.6	0.57	1335
G15	5.0	0.53	770
G16	7.3	0.63	1520
G17	5.4	0.52	1080
G18	5.0	0.51	980
G19	6.2	0.54	1230
G20	7.2	0.64	1530
G21	6.2	0.57	1210
Mean	6.17	0.57	1229.68
R.L.S.D (0.05)	0.852	0.054	170.31

Note: Data represent mean values of two growing seasons. RLSD = Revised Least Significant Difference at 0.05 probability level.

Table 5. Summary of ISSR, SRAP, SSR and primer combination characteristics

ISSR	TB	PB	PPB	PIC	MI	RP
HB12	14	7	50.00	0.16	1.12	2.86
HB13	11	6	54.55	0.15	0.90	2.19
ISSR-844	15	9	60.00	0.18	1.64	4.00
ISSR-814	13	7	53.85	0.21	1.47	4.19
HB15	10	5	50.00	0.14	0.70	1.81
Total	63	34				
Average	12.6	6.8	53.97	0.17	1.17	3.01
SRAP-1	11	6	54.55	0.15	0.88	2.10
SRAP-2	6	4	66.67	0.22	0.86	1.71
SRAP-3	14	10	71.43	0.22	2.22	4.38
SRAP-4	13	8	61.54	0.16	1.26	2.57
SRAP-5	11	6	54.55	0.18	1.10	2.86
Total	55	34				
Average	11	6.8	61.82	0.19	1.26	2.72
SSR-2	8	4	50.00	0.14	0.58	1.43
SSR-3	4	3	75.00	0.17	0.50	0.86
SSR-4	11	5	45.45	0.13	0.63	1.71
SSR-5	8	5	62.50	0.15	0.77	1.52
SSR-8	7	4	57.14	0.19	0.75	2.00
SSR-10	4	3	75.00	0.18	0.54	0.86
TOT	42	24				
AVR	7	4	57.14	0.16	0.63	1.40
Total	160.00	92.00				
Average			58.89	0.17	1.00	2.32

Note: TB = Total Bands; PB = Polymorphic Bands; PPB = Percentage of Polymorphic Bands; PIC = Polymorphic Information Content; MI = Marker Index; RP = Resolving Power.

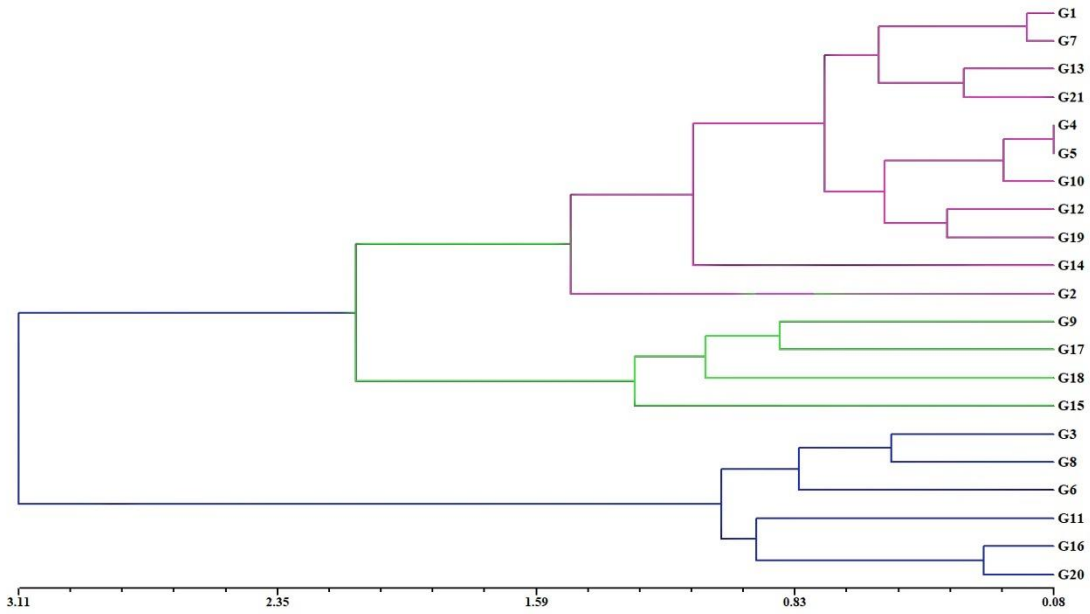

Fig. 1. A dendrogram based on the Euclidean distance coefficient for 21 genotypes of pearl millet, derived from agro-morphological traits

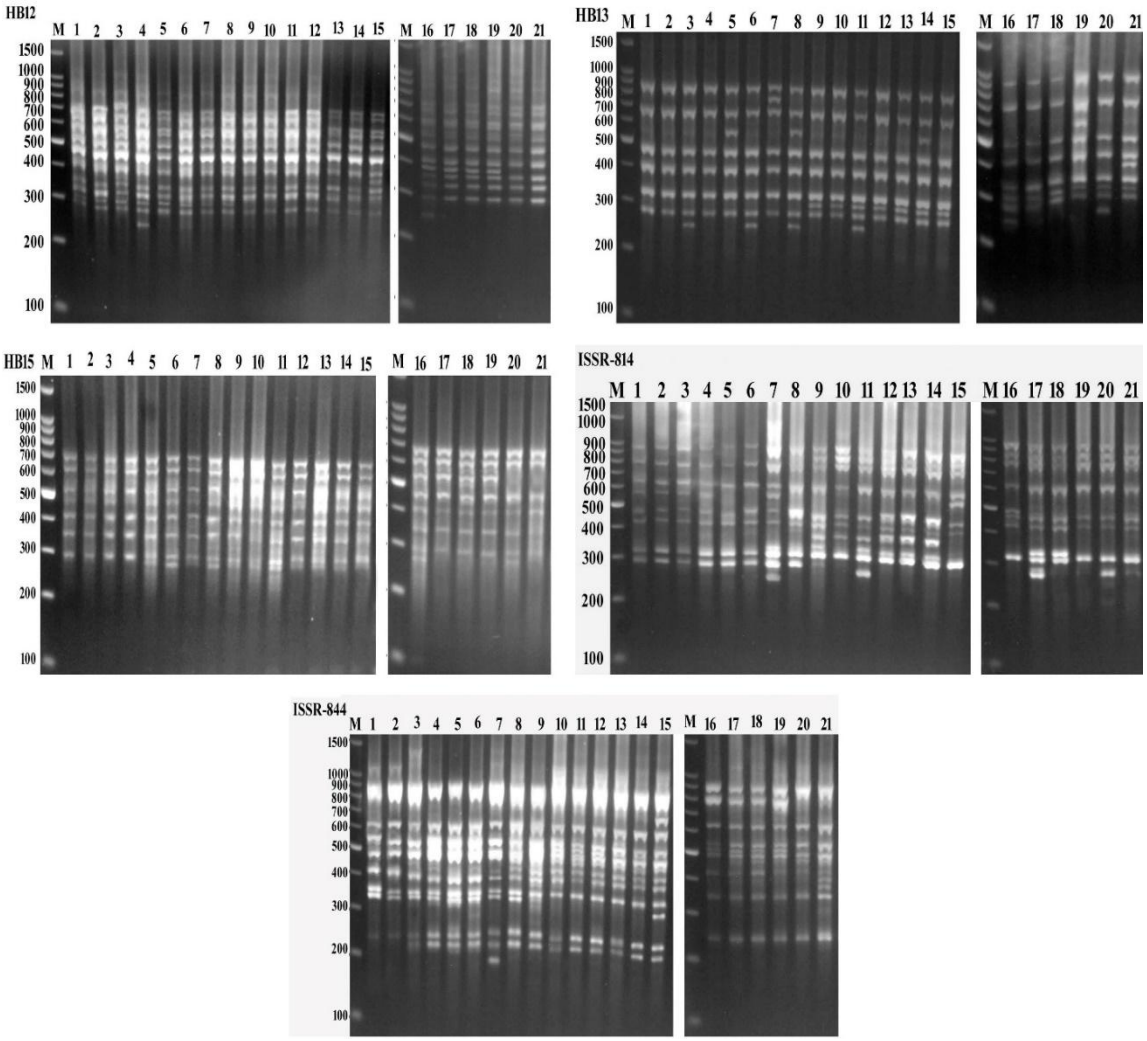

Fig. 2. The gel electrophoresis patterns of the five ISSR markers across 21 pearl millet genotypes

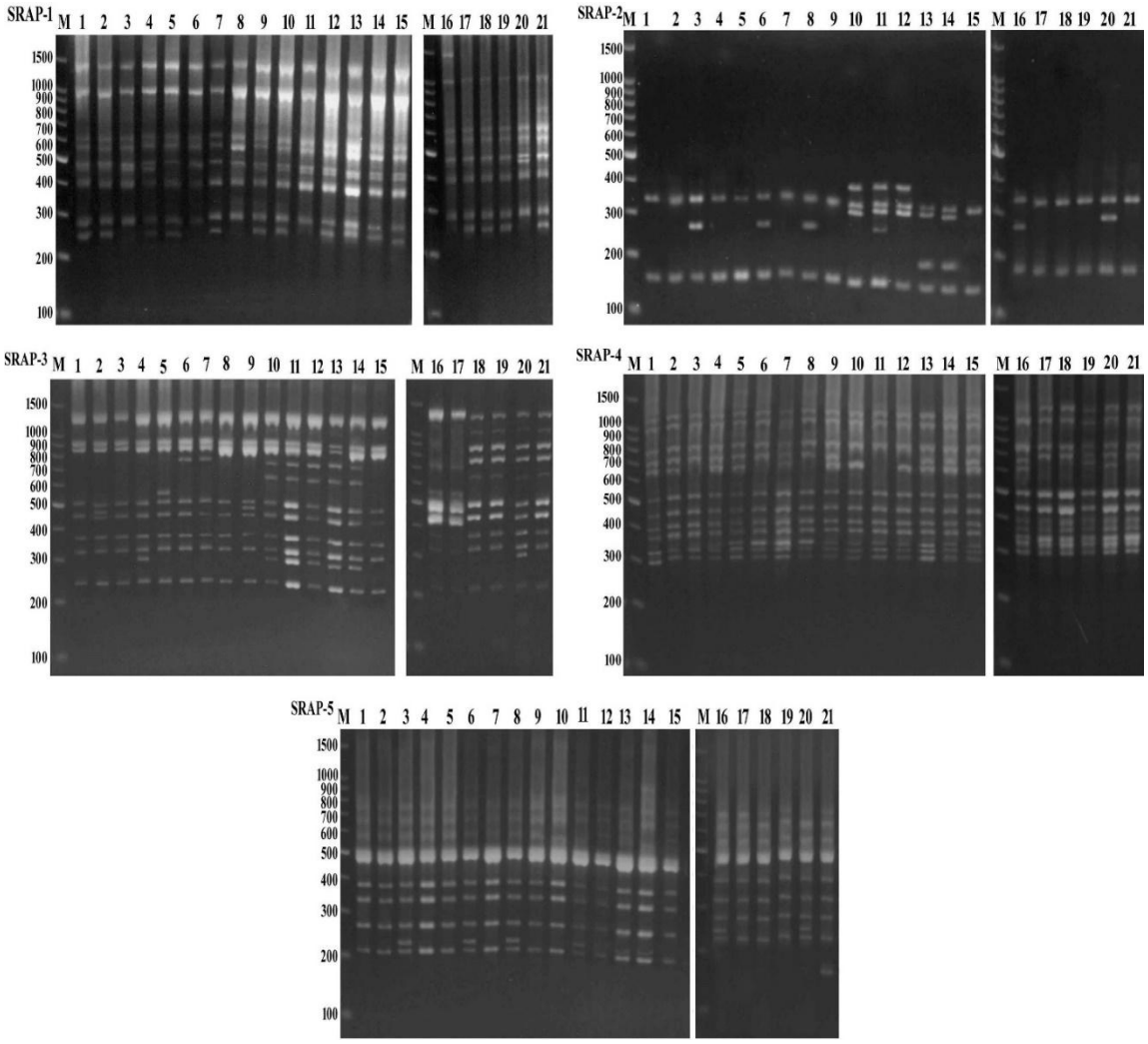

Fig. 3. The gel electrophoresis patterns of the five SRAP markers across 21 pearl millet genotypes

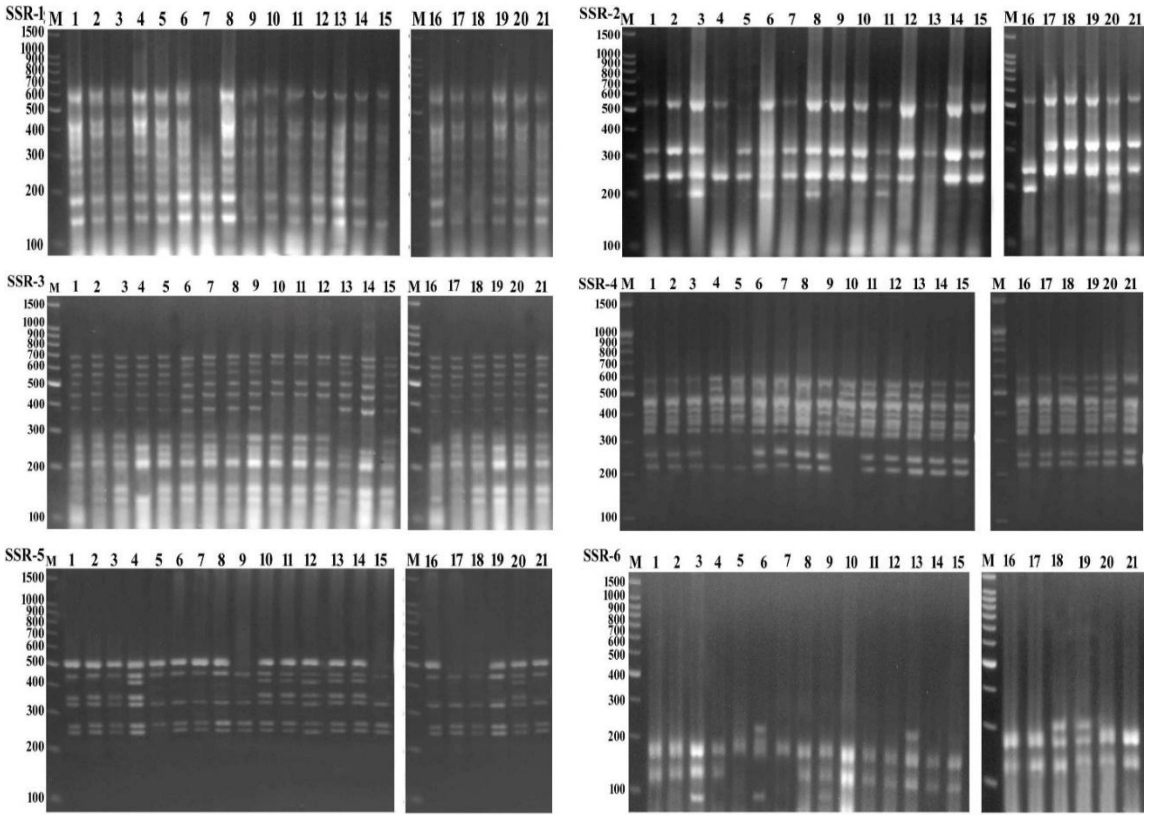

Fig. 4. The gel electrophoresis patterns of the five ISSR markers across 21 pearl millet genotypes

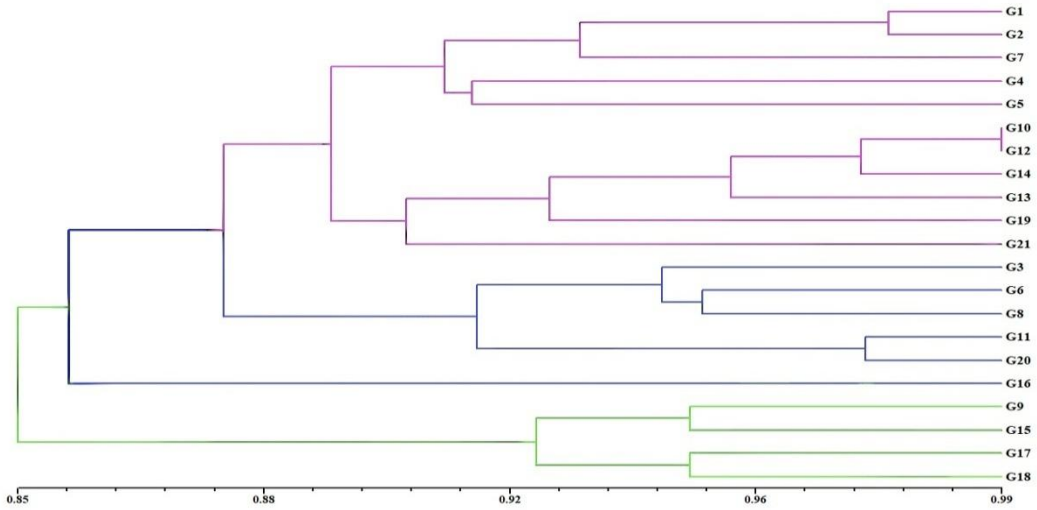

Fig. 5. A dendrogram illustrating 21 genotypes of Pearl millet was constructed utilizing ISSR, SRAP, and SSR data through UPGMA analysis. The scale employed is derived from the Dice coefficients of similarity**.**

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