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Research Article

Diversity analysis of moringa (*Moringa oleifera* Lam.) mutant populations revealing extensive genetic variability for the morphological traits

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Abstract
Moringa is renowned as the miracle tree for its versatile applications in food, medicine, plant growth stimulation, animal feed, and its nutritional, pharmacological, and biotechnological potential. Initially, the mutation was induced by treating the seeds with gamma rays (100 Gy, 200Gy and 300 Gy) and Ethyl methane sulphonate (EMS) at 0.15%, 0.20% and 0.25%. Based on continuous evaluation of mutants from M_1 to M_3 generation, four selected mutant families: 15-1-09, 35-1-62, 35-1-63 and 35-1-68, with 15 mutants from each family were studied to identify high-performing mutants alongside PKM 1 Moringa. Principal component analysis (PCA) of 12 morphological traits for each mutant family revealed five principal components with eigenvalues exceeding one, collectively explaining 78.19, 74.88, 79.85 and 83.80% of the total variability from the mutant families 15-1-09, 35-1-63, 35-1-62, and 35-1-68 respectively. The analysis highlighted that foliage density, apex shape of the first leaf blade, leaf shape, and plant growth habit exhibited the highest variation, while the remaining traits showed lower variability. Through Agglomerative Hierarchical Clustering (AHC) and Principal Component Analysis (PCA), the genotypes 15-1-09-39, 35-1-62-72, 35-1-62-73, 35-1-68-79, and 35-1-63-06 emerged as the most diverse genotypes based on seedling hypocotyls colour, young shoot colour and petiole: anthocyanin colouration of the axis and branches. Hence, through this study, the identified diverse genotypes for specific traits of interest can be included in the moringa breeding programs for crop improvement.

Keywords: Clustering, Diversity, Moringa, Mutants, Variability

INTRODUCTION

Moringa oleifera Lam. is a deciduous tree highly esteemed worldwide for its multifaceted utility as a nutritious vegetable. Revered by various appellations, including "Miracle Tree", moringa is renowned for its extensive array of applications and medicinal attributes. Its diverse plant parts and derivatives serve as potent antioxidants, anti-cancer agents, ulcer remedies, antidiabetic aids, and antimicrobial substances. Furthermore, moringa is useful as animal fodder and a natural plant growth enhancer (Masih *et al.*, 2019). Tamil Nadu

leads in both area and production, followed by Andhra Pradesh and Karnataka. Specifically, Tamil Nadu encompasses an area of 20,684 hectares, of which Dindigul holds the largest area of 5,538 hectares, followed by Theni (2,951 hectares). Notably, 80 percent of the moringa leaves production contributes significantly to India's foreign exchange. The primary countries that import moringa leaves include China, the USA, Germany, Canada, South Korea, and various European countries (Samsai, 2023).

The primary challenge in this century revolves around augmenting food production in the foreseeable future amidst the constraint of limited agricultural land availability. Forecasts suggest that the escalating crop production rate through conventional means will not adequately match the increasing demand. Hence, alternative methodologies are sought to enhance both the yield and quality. Among the plant breeding strategies, induced mutagenesis has emerged as a viable approach to complement existing germplasm and to develop new varieties. Mutation induction can be achieved through chemical and physical mutagens or combinations (Kantoglu *et al*., 2014). Gamma rays, used as a physical mutagen, can induce mutations that may result in higher genetic alterations in plants (Vasudevan *et al*., 2023). Meanwhile, EMS is considered the most effective mutagen among the chemical mutagens used in plant breeding (Turkoglu *et al*., 2022).

Understanding this variability is crucial for effectively harnessing available genotypes in breeding initiatives and facilitating the introduction of commercially viable materials (Balaguru *et al*., 2020). The descriptors offered by the Protection of Plant Variety and Farmers' Rights Authority are instrumental in identifying, ensuring varietal purity and distinguishing the uniqueness of newly developed plant materials. The scoring system derived from DUS (Distinctness, Uniformity and Stability) descriptors aids in pinpointing the highly variable mutant. Thus, this study aimed to assess the variability among 60 mutants from four mutant families alongside the variety PKM1 Moringa by utilizing the scoring system derived from DUS descriptors. The study identified highly variable mutants based on the observed characters through DUS characterisation and multivariate analysis.

MATERIALS AND METHODS

Mutation treatment

The present study was conducted at the gamma chamber of Bhabha Atomic Research Centre (BARC), Mumbai in which the seeds were treated with gamma rays created by 60Co as the source of Gamma irradiation (Bharat *et al*., 2024). The doses applied consisted of 100 Gy, 200 Gy, and 300 Gy of gamma rays (400 seeds/dose). The duration of the exposure depends on the rate of dose from the source at the time of treatment. For chemical mutagen treatment, the pre-soaked seeds underwent treatment with various concentrations of ethyl methane sulphonate (EMS) (0.15%, 0.20%, and 0.25%) for 4 hours and washed to remove the residues. The seeds were raised as M_1 generation and evaluated. Best performing plants were selected based on leaf yield and yield-related characters and forwarded to M_2 generation (Udhayakumar *et al*., 2019).

Selection

Subsequently, the plants were selected from M_2 and forwarded to M_3 generation (Hari *et al*., 2022). From M_3 generation four *Moringa oleifera* Lam. mutant families *viz.,* 15-1-09, 35-1-68, 35-1-63 and 35-1-62 were selected and M_4 generation was raised, which was evaluated for leaf biomass yield during the year of 2023 in the Department of Vegetable Sciences, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Periyakulam, Tamil Nadu, India. A recommended package of practices was adopted to maintain healthy plants during the cropping period.

Observations

A total of 60 plants, comprising 15 mutants from each M_4 family and variety PKM 1 as control, were subjected to the study by employing the DUS descriptors of IPGRI, PPV & FRA (2001). Each mutant was visually scored by using one to nine scale for the 12 characters *viz*., basal shape of the first leaf blade (BSFLB), apex shape of the first leaf blade (ASFLB), leaf colour (LC), petiole colour (PC), leaf shape (LS), petiole: anthocyanin colour of the axis and branches (P: A), nature of branchlets (NB), young shoot colour (YSC), seedling hypocotyl colour (SHC), foliage density (FD), plant growth habit (PGH) and size of leaf in primary branch axis for length and breadth (SLPBLB).

Statistical analysis

No replication was followed as each mutant was considered an individual plant progeny. Uniformity was assessed based on a 5% population standard with an acceptable probability of at least 95%.

Principal Components (PCs) with Eigen values greater than one were selected (Jeffers, 1967) and standardized values were used for Principal Component Analysis (PCA) using the PAST4.03 application. The major components contributing to the total variation were determined visually using a scree plot. Rotated component matrix was used to determine the effect of different characters to the variability with the help of PAST4.03 application. Agglomerative Hierarchical Clustering (AHC) was conducted using dissimilarity matrix scores with Microsoft Excel and XLSTAT by

Addinsoft. Diversity index was also predicted for each character with the help of the Shannon Weaver diversity index by PAST4. 03 application.

RESULTS AND DISCUSSION

Cluster analysis of mutant family 15-1-09

The Agglomerative hierarchical cluster analysis of the moringa mutant family 15-1-09 consisting of 15 mutants and PKM 1Moringa as control falls in two clusters. Cluster I consists of 14 mutants along with PKM 1, whereas Cluster II consisted of only two mutants (15-1-09-39 and 15-1-09-53) that exhibited petiole anthocyanin colouration in axis and branches. The sub-cluster of Cluster I consisted of the mutants 15-1-09-44, 15-1-09-33, 15-1-09-25, 15-1-09-64, 15-1-09-21, 15-1-09-75 and PKM 1 that showed similarity in basal shape and apex shape of the first leaf blade, nature of branchlets and foliage density. Meanwhile the, another sub-cluster of Cluster I with the mutants 15-1-09-11, 15-1-09-31, 15-1-09-58, 15-1-09-16, 15-1-09-55, 15-1-09-70 and 15 -1-09-74 showed similarity in young shoot colour and seedling hypocotyl colour with absence of petiole anthocyanin colouration in axis and branches (Fig. 1). Similarly, a study conducted by Popoola *et al*. (2014) revealed that the morphological dendrogram effectively categorized the 13 accessions into three distinct groups, revealing the overall pattern of variation and genetic relatedness among them when genotypes from different geographical locations were studied. Formation of clusters with different mutants indicates diversity among the mutants. The grouping of mutants into different constellations did not follow any specific pattern and was found independent of the type of mutagen used in this study. Also, Cluster analysis with twenty accessions of moringa from different locations in Sri Lanka revealed that accessions M14 and M97 emerged as the most distinctive in the study. Conversely, M14, M94, and M97 were clustered together, indicating that the moringa leaf traits vary depending on the geographical location within the country (Kodikara *et al*., 2022).

Cluster analysis of mutant family 35-1-62

Three clusters were observed from the mutant family 35-1-62.Three clusters with 12 mutants, including control in Cluster I, three mutants *viz.*, 35-1-62-95, 35-1-62 -01 and 35-1-62-72 in cluster II and one mutant 35-1-62-73 in Cluster III were observed from the analysis. The mutant 35-1-62-73 showed the presence of petiole anthocyanin colouration in the axis and branches. The sub-cluster of Cluster 1 with the mutants 35-1-62-91, 35-1-62-15, 35-1-62-82, 35-1-62-69 and PKM 1 showed similarity in the traits of young shoot colour, seedling hypocotyl colour, apex shape of the first leaf blade, colour of leaf, colour of petiole and petiole anthocyanin colouration in axis and branches. From Cluster I, the mutants 35-1-62-27, 35-1-62- 85, 35-1-62-92, 35-1-62-07, 35-1-62-94, 35-1-62-14, 35-1-62-6 of another sub-cluster showed young shoot colour, apex shape of first leaf blade, plant growth habit and petiole anthocyanin colouration in axis and branches of similar nature. The mutants 35-1-62-95, 35-1-62-01 and 35-1-62-72 showed same petiole colour, seedling hypocotyl colour, leaf shape, basal shape of first leaf blade, apex shape of first leaf blade and petiole anthocyanin colouration of axis and branches (Fig.. 2).This type of variability and similarity was reported in cluster beans and the cluster analysis of cluster bean helps in categorizing the genotypes into two distinct clusters based on vegetable and gum type beans (Manivannan *et al*., 2016).

Cluster analysis of mutant family 35-1-63

The mutant family, 35-1-63 with PKM 1 Moringa, showed three clusters in which Cluster I consists of a maximum of 12 mutants, including control. Cluster II consisted of two mutants *viz.,* 35-1-63-01 and 35-1-63-12, whereas Cluster III consisted of only one mutant, 35-1-63-20. The single mutant of Cluster III had petiole anthocyanin colouration in the axis and branches, which was different from all the other mutants of this family. The mutants 35-1-63-01 and 35-1-63-12 from the sub cluster of Cluster II have similar young shoot colour, seedling hypocotyl colour, leaf colour, petiole colour, plant growth habit, size of leaf in primary branch axis for length and breadth and no petiole anthocyanin colouration. Absence of petiole anthocyanin colouration in axis and branches in all the other mutants from Cluster I was observed (Fig.. 3). Similar finding in moringa that clustering of 24 landraces of moringa based on 32 morphological characters was successfully done by Chan *et al*. (2018).

Cluster analysis of mutant family 35-1-68

Four clusters were obtained from the mutant family 35-1-68, among which the Cluster I is bigger with nine mutants and PKM 1 Moringa as control. Other three clusters have two, two and one mutant respectively in the Cluster II, III and IV. The mutants *viz.,*35-1-68-54, 35-1-68-07, 35-1-68-10, 35-1-68-56, 35-1-68-49 and PKM 1 from the sub-cluster of Cluster I resembled each other to the young shoot colour, seedling hypocotyl colour, colour of leaf and petiole, basal and apex shape of leaf, petiole: anthocyanin colouration of leaf axis and branches. The sub-cluster of Cluster I having the mutants *viz.,* 35-1-68-28, 35-1-68-43, 35-1-68-39 and 35-1-68-18 was similar in case of basal and apex shape of first leaf blade, nature of branchlets, colour of petiole, size of leaf in primary branch axis for length and breadth and petiole: anthocyanin colouration of the axis and branches. The mutants 35-1-68-41 and 35-1-68-71 had similar colour of the seedling hypocotyl and young shoot, petiole: anthocyanin colouration of the

Table 1. Principal component analysis of mutants from four mutant families of Moringa var. PKM 1

PC	Families							
	15-1-09		35-1-63		35-1-62		35-1-68	
	Eigenvalue	% variance	Eigenvalue	% variance	Eigenvalue	% variance	Eigenvalue	% variance
1	2.82	23.53	3.08	25.65	3.31	27.56	3.34	27.83
2	2.27	18.94	1.98	16.47	2.49	20.76	2.41	20.05
3	1.86	15.49	1.67	13.90	1.53	12.76	1.69	14.08
4	1.37	11.43	1.16	9.63	1.23	10.26	1.45	12.11
5	1.06	8.80	1.11	9.25	1.02	8.51	1.17	9.73

axis and branches, nature of branchlets, size of leaf in primary branch axis for length and breadth and foliage density are similar to each other which are in Cluster II. The Cluster III had three mutants *viz.,* 35-1-68-21, 35-1-68-35 and 35-1-68-04, which resembled each other for the traits *viz.,* young shoot colour, seedling hypocotyl colour, foliage density and axis, branches and petiole anthocyanin pigmentation of the same kind. The mutant 35-1-68-79 had different petiole anthocyanin colouration of the axis and branch from the other mutant was placed in Cluster IV (Fig. 4).Similarly, green fruit yield per plant and primary branches per plant in chilli showed maximum contribution to diversity when subjected to cluster analysis by Rahevar *et al*. (2021).

PCA of the M_4 generation of Moringa mutant families

Among the PCA of mutant family 15-1-09, five components were observed with eigen value of more than one. The total variability of the five Principal components was 78.19% (Fig. 5 (a)) and 23.53% was contributed by PC 1. In the mutant family 35-1-63 case, five components with more than one eigen value were observed, indicating wide variation among the mutants in this family (Table 1). Also, 25.65% variability was contributed by the first PC alone, with a total variability of 74.88% by the first five principal components (Fig.. 5 (b)). Principal component analysis in chilli with 58 genotypes indicates that 74.90 per cent of the total variation was given by the first five principal components (Rahevar *et al*., 2021).

Also, the other two mutant families, *viz.,* 35-1-62 and 35 -1-68 showed five PC's with eigenvalue of greater than one with 27.56% and 27.83% of variability represented by the first principal component of each mutant family, which showed 79.85% (Fig. 5 (b)) and 83.80% (Fig. 5 (d)) of total variability respectively (Table 1).

Whereas in moringa, 83.59% of total variability was observed in nine principal components with eigenvalue of higher than (>1) by Meena *et al*. (2021) and 88.1% in three principal components having more than 1 as an eigenvalue by Kurian *et al*. (2020). From the Principal Component Analysis, reproductive traits contributed about 60% of variations among the observed 40 accessions of moringa (Popoola *et al*., 2016).

Mutants with eigenvalue greater than one in different components

The mutant exhibiting the highest PC score within a specific principal component indicates the maximum values for the variables associated with that mutant. Mutants were chosen based on their PC scores within each component, ensuring positive values exceeding 1.0 in each PC (Table 2). Similarly, Karunakar *et al*. (2022) revealed that the moringa genotypes exhibited higher eigenvalue (greater than one) of the 20 genotypes evaluated for yield-related traits. Similarly, Chilli genotypes showed 79.45 per cent variation from five principal components with an eigenvalue greater than one out of the total variation (Belay *et al*., 2019).

Table 2. Mutants from various families with eigenvalue greater than one in different components

PC	Families			
	15-1-09	35-1-63	35-1-62	35-1-68
PC 1	15-1-09-39 (5.21)	35-1-63-06 (3.14) 35-1-63-37 (1.39)	35-1-62-72 (6.38)	35-1-68-28 (3.02)
	15-1-09-53 (1.54)	35-1-63-27 (2.46) 35-1-63-20 (2.14)		35-1-68-79 (5.01)
PC 2	15-1-09-39 (4.64)	35-1-63-27 (1.50)	35-1-62-07 (1.20)	35-1-68-35 (2.42)
	15-1-09-55 (1.10)	35-1-63-03 (2.63)	35-1-62-73 (5.11)	35-1-68-04 (3.76)
	15-1-09-25 (1.00)	35-1-63-28 (1.17)		35-1-68-79 (1.83)
PC 3	15-1-09-44 (1.10)	35-1-63-03 (2.39)	35-1-62-91 (2.01)	35-1-68-04 (1.64)
	15-1-09-16 (1.18)	35-1-63-20 (2.65)	35-1-62-07 (1.20)	35-1-68-54 (1.83)
	15-1-09-25 (2.01)	35-1-63-25 (1.30)	35-1-62-85 (2.83)	35-1-68-79 (1.92)

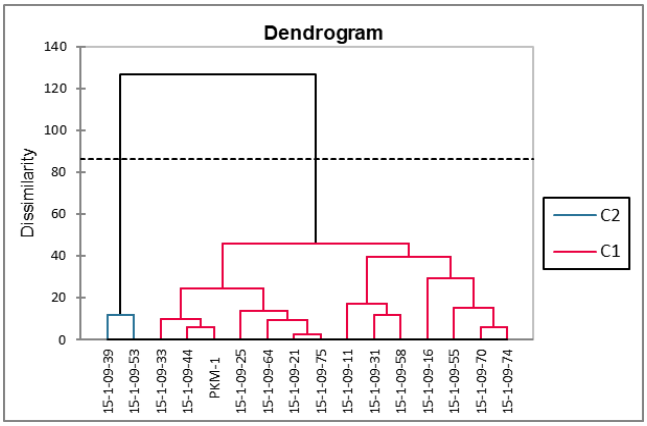

Fig. 1. *Cluster diagram of the mutant family 15-1-09 with PKM 1 moringa; C1 –Cluster I, C2- Cluster II*

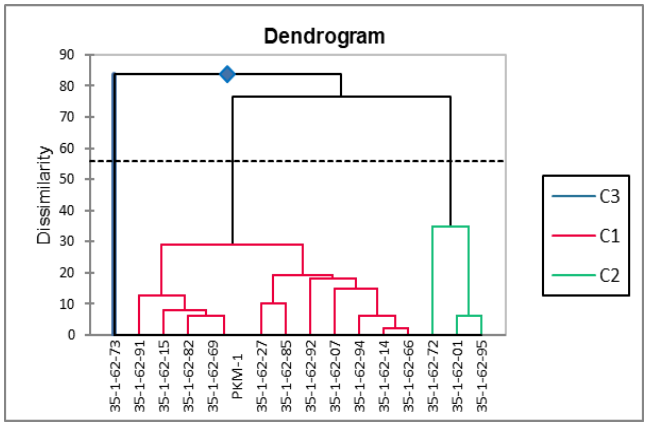

Fig. 2. *Cluster diagram of the mutant family 35-1-62 with PKM 1 moringa; C1 –Cluster I, C2- Cluster II, C3- Cluster III*

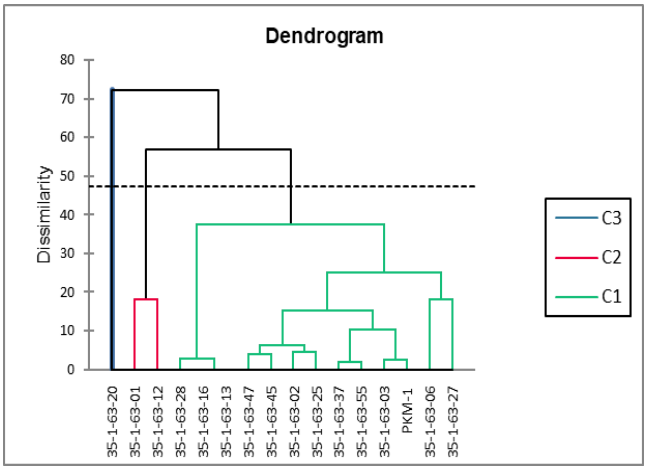

Fig. 3. *Cluster diagram of the mutant family 35-1-63 with PKM 1 moringa; C1 –Cluster I, C2- Cluster II, C3- Cluster III*

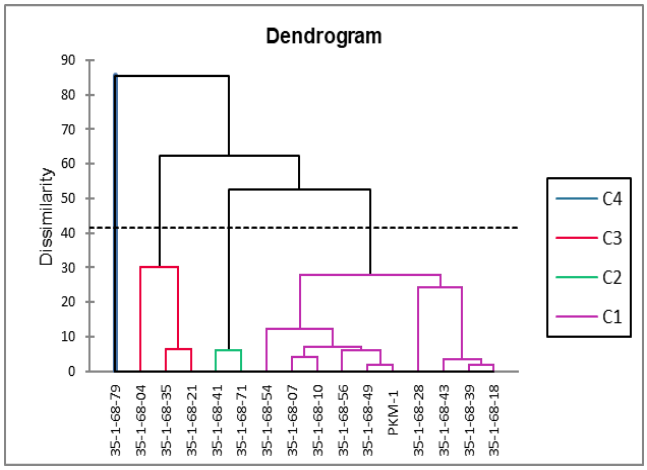

Fig. 4. *Cluster diagram of the mutant family 35-1-68 with PKM 1 moringa; C1 –Cluster I, C2- Cluster II, C3- Cluster III*

Rotated component matrix of Principal Components 1, 2 and 3 for the mutant families

Mutant family 15-1-09

The rotated component matrix of the mutant family 15-1-09 elucidated that the PC1 primarily captured traits such as young shoot colour, foliage density, petiole anthocyanin colour of branches and axis and seedling hypocotyl colour. PC2, on the other hand, predominantly reflected traits such as petiole colour and basal shape of first leaf blade. PC3 accounted for traits such as the apex shape of the first leaf blade, nature of branchlets and plant growth habits (Fig. 6). A similar study conducted by Karunakar *et al*. (2023) revealed that in moringa genotypes, PC 1 showed 52.53% variability with chlorophyll 'a' content, ascorbic acid, chlorophyll b, leaf soluble protein and crude protein as the highly loaded characters.

Mutant family 35-1-62

Nonetheless, within the mutant family 35-1-62, the rotated component matrix revealed that PC1 predominantly comprised traits like leaf colour, branchlet's nature, axis and branch's anthocyanin colour in petiole and petiole colour. In contrast, PC2 primarily encompassed features such as the basal shape of the leaf blade that initiates first, size of leaves in the primary branch for length and breadth and foliage density. Meanwhile, PC3 accounted for attributes, including young shoot colour and plant growth habit (Fig. 7). Principal component analysis with rotated component matrix and scree plot was carried out to find the variability of rice genotypes, which indicated yield and yield attributing traits contributed for some PC's, meanwhile for some PC's quality traits were responsible (Gour *et al*., 2017).

Mutant family 35-1-63

However, for the mutant family 35-1-63, the rotated component matrix revealed that PC1 mainly encompassed characteristics such as the nature of branchlets, plant growth habit, foliage density and petiole colour. Conversely, PC2 predominantly represented traits *viz.,* size of leaf in primary branch for length and breadth and young shoot colour. PC3, on the other hand, accounted for colour features such as colour of the young shoot, colour of leaf and the anthocyanin colour of the petiole in the axis and branches (Fig. 8). Similar to this, the rotated component matrix of chilli

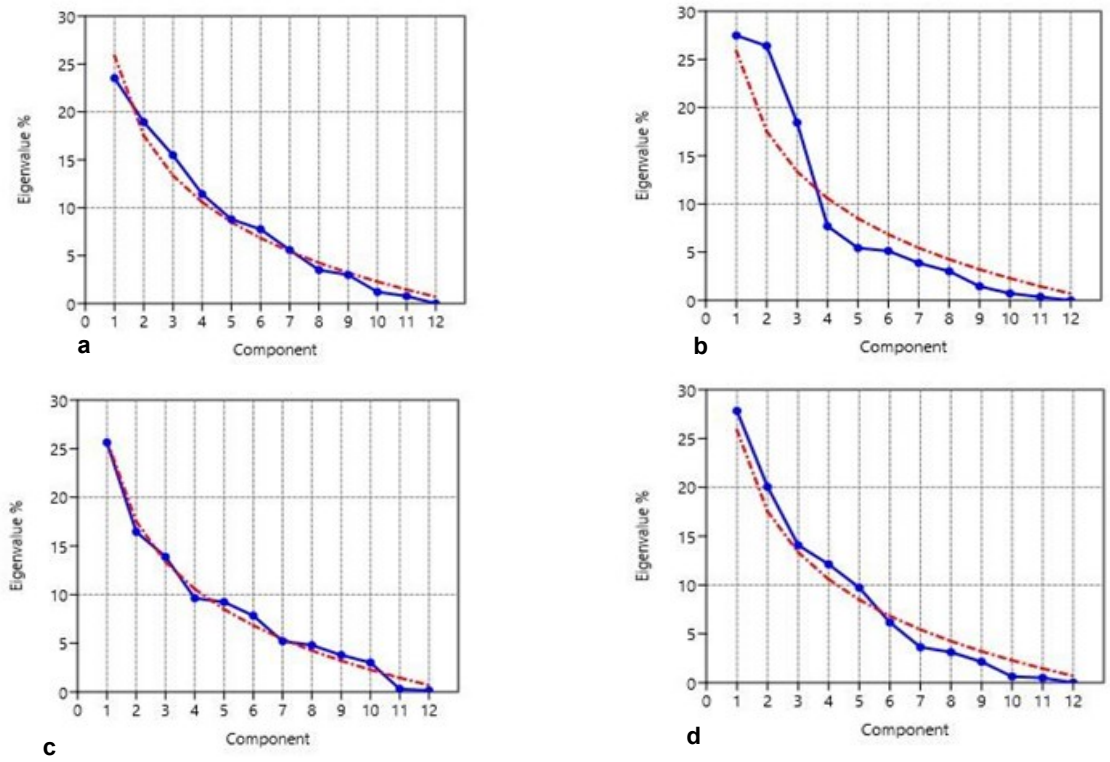

Fig. 5. *Scree plot explaining the percentage variation of characters of mutant family (a) 15-1-09, (b) 35-1-62, (c) 35-1-63 and (d) 35-1-68*

genotypes were assessed, which showed different characters dominating each Principal Component that helps in identifying genotypes based on desirable character (Singh *et al.,* 2020).

Mutant family 35-1-68

However, in the mutant family 35-1-68, the rotated component matrix unveiled that PC1 primarily includes colour traits such as colour of seedling hypocotyl as well as young shoot and petiole anthocyanin in axis and branches. Conversely, PC2 predominantly captured characteristics such as foliage density, colour of leaf as well as the basal shape of the first leaf blade. PC3, on the other hand, accounted for leaf attributes such as the leaf shape and first leaf blade's basal and apex shape (Fig. 9).

In relation to this, different characters were responsible for variation in moringa, especially anthocyanin pigmentation of petiole, axis and branches when the rotated component matrix of 32 moringa genotypes was studied for its diversity using DUS descriptors (Meena *et al*., 2021).

Shannon Weaver Diversity Index for mutant families

For the mutant family 15-1-09, foliage density showed more variability (2.76), whereas petiole anthocyanin colouration of branches and axis showed less variability (2.21) among the characters (Fig. 10 (a)). Similarly, for all the other mutant families *viz.,* 35-1-63, 35-1-68 and 35-1-62, variability was found to be minimum (2.35) with the parameter of petiole anthocyanin colouration of the axis and branches, respectively.

The apex shape of the first blade of the leaf showed the highest variability (2.76) among the characters in the case of the mutant family in 35-1-62 (Fig. 10 (b)), meanwhile Leaf shape (2.77) in the mutant family 35-1-63 (Fig. 10 (c)). In the mutant family 35-1-68, the highest diversity was seen in plant growth habit (2.49), colour of the petiole (2.48) and colour of the leaf (2.44) (Fig. 10 (d)). Similar results were obtained by Meena *et al*. (2021), where petiole anthocyanin colouration of the axis and branches showed less diversity among moringa genotypes. When the diversity of plant species in moringa based homestead system was studied, moringa contributed 19.1 per cent out of 26.3 per cent of vegetables (Prova *et al*., 2023) and 32 landraces of pumpkin analysed for diversity through Shannon Weaver Diversity Index indicated significant quantitative traits with an average value of 3.43 except for the characters such as number of vines and pods per plant (Aworunse *et al*., 2023).

Conclusion

The Shannon Weaver Diversity Indices (H') revealed that the foliage density (2.76), apex shape of first leaf blade (2.6), leaf shape (2.77) and plant growth habit

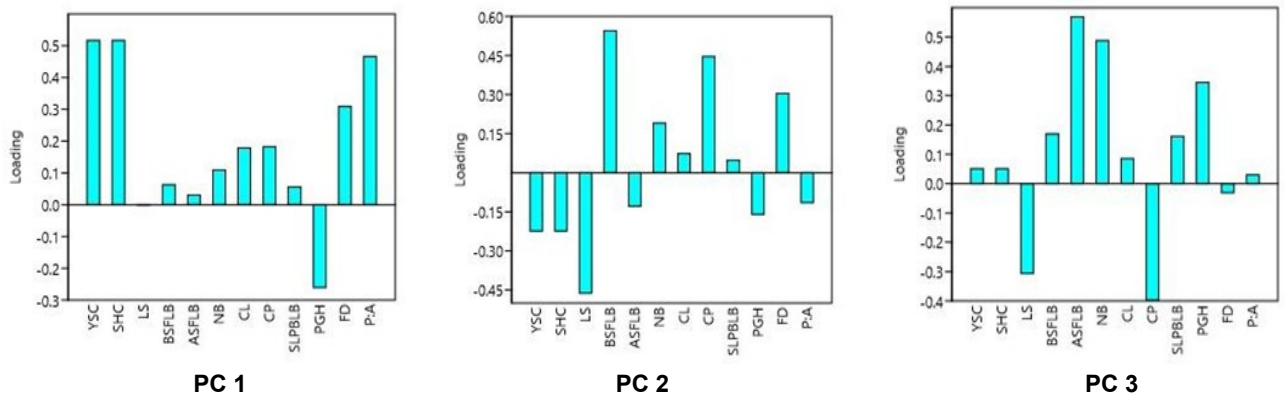

Fig. 6. *Rotated component matrix of PC 1, 2 and 3 for the mutant family 15-1-09*

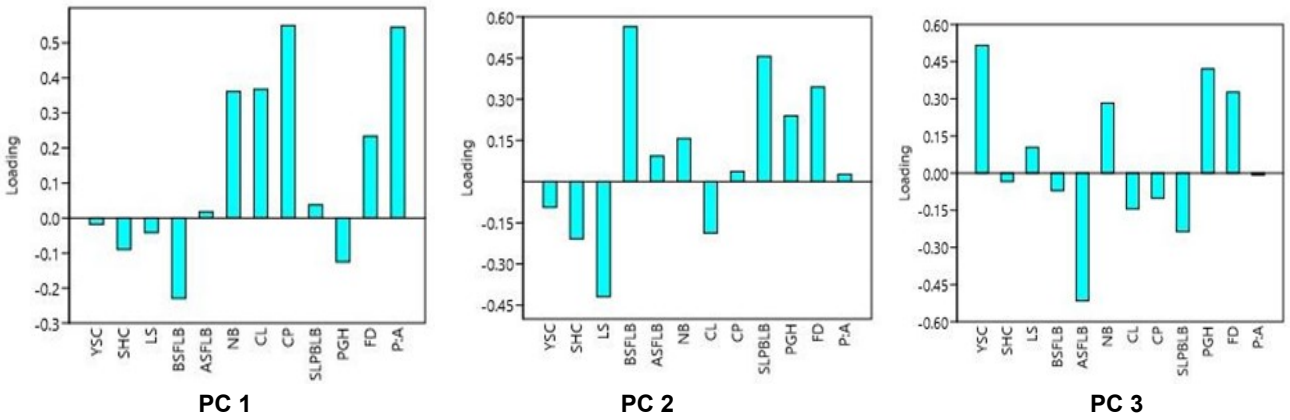

Fig. 7. *Rotated component matrix of PC 1, 2 and 3 for the mutant family 35-1-62*

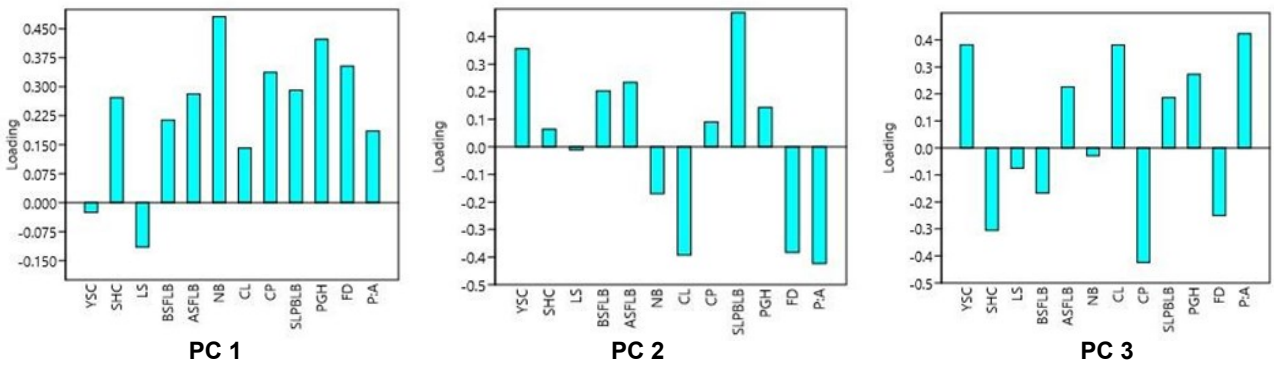

Fig. 8. *Rotated component matrix of PC 1, 2 and 3 for the mutant family 35-1-63*

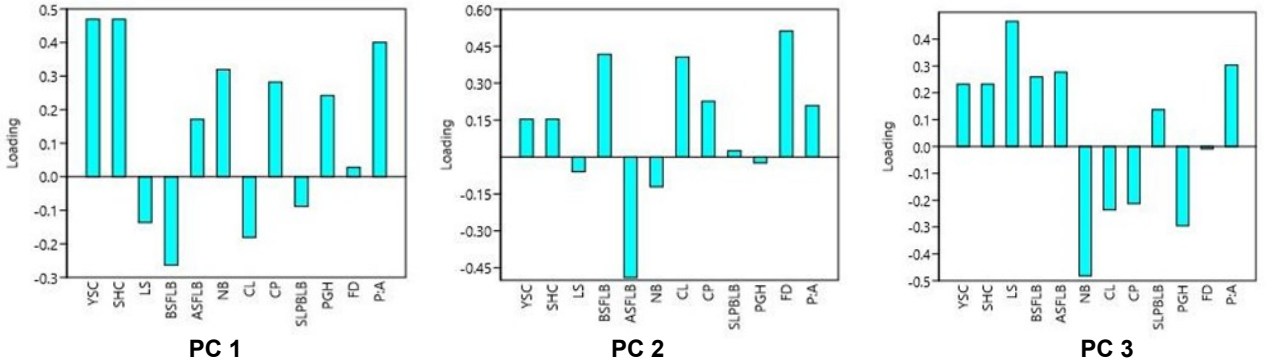

Fig. 9. *Rotated component matrix of PC 1, 2 and 3 for the mutant family 35-1-68*

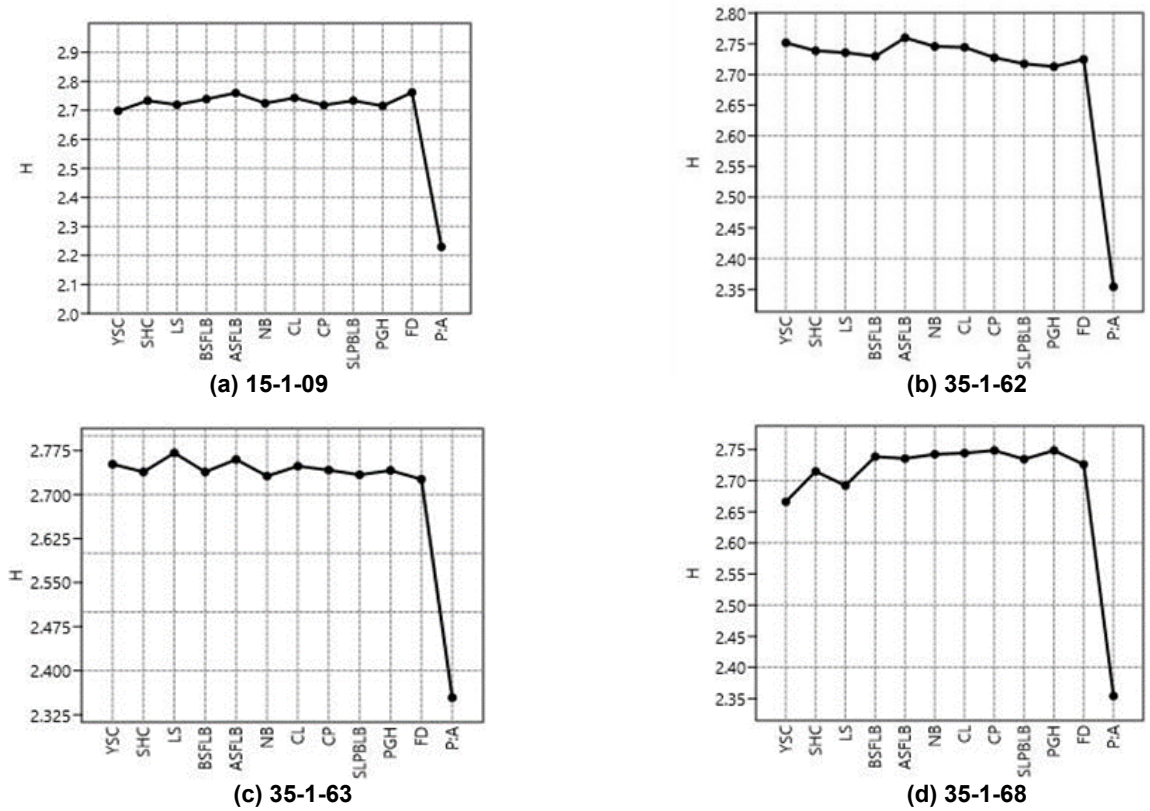

Fig.10. *Graphical representation of diversity index of four mutant families (a) 15-1-09, (b) 35-1-62, (c) 35-1-63, (d) 35-1-68*

(2.75) in the mutant families 15-1-09, 35-1-62, 35-1-63 and 35-1-68 respectively, accounted for the highest variation among the 60 mutants (15 mutants from each family) with PKM 1 of Moringa. Through variability analysis (PCA, AHC and H), the mutants *viz.,* 15-1-09-39, 35-1-62-72, 35-1-62-73, 35-1-68-79 and 35-1-63-06 were identified as highly variable, particularly in traits such as apex shape of the first leaf blade, seedling hypocotyl colour, young shoot colour and petiole: anthocyanin coloration of the axis and branches. Clustering analyses highlighted the significance of morphological features such as anthocyanin pigment in the petiole in elucidating the diversity among the genotypes. By exploring the mutants of high variability, a mutant with highly needed character can be selected and passed on to the next generation, which can be utilized to increase production.

Conflict of interest

The authors declare that they have no conflict of interest.

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