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Genetic Relatedness in Elite Cultivars of Moth Bean using Morpho-agronomic and Molecular Markers

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ABSTRACT

Background: Moth bean is a very nutritive and hardy crop, especially for resource poor-arid agriculture. The limited use of cultivars in moth bean breeding resulted into narrow genetic base. This study aimed to diversify the conical genetic base and developimproved cultivars, through molecular (RAPD) and morpho-agronomic characterization of commercially elite moth bean cultivars.

Methods: Elite moth bean cultivars (15) were evaluated for 11 morpho-agronomic traits. Eleven RAPD markers were used to amplify genomic DNA and perform molecular characterization.Clustering approach was used for grouping of the cultivars.

Result: RAPD markers revealed high polymorphism in 15 elite moth bean cultivars. The polymorphism information content (PIC) values varied within the range of 0.45 to 0.88, with an average of 0.77. The number of alleles at different loci ranged from 4 to 16, with an average of 10 alleles per locus. When employing UPGMA-based cluster analysis, utilizing 11 morpho-agronomic traits, the cultivars were grouped into four clusters. However, when RAPD markers were used, the cultivars were grouped into five clusters. The Jaccard's similarity coefficient and Manhattan dissimilarity coefficient fell within the ranges of 0.18 to 0.70 and 0.02 to 0.42, respectively. These values signify the degree of genetic variability within the cultivars. Furthermore, a Mantel test was conducted to examine the correlation between agronomic traits and the RAPD-based matrix. The results showed a negative correlation, but it was not statistically significant.The high PIC values and the successful amplification of multiple loci demonstrate the efficacy of RAPD markers in assessing genetic diversity in moth beans. The study revealed enormous genetic variation among cultivars and crosses can be attempted between cultivars of different groups to create better recombinants in moth bean breeding programmes.

Key words: Cataloguing, Cluster analysis, Diversity, Moth bean, RAPD.

INTRODUCTION

Moth bean, scientifically known as *Vigna aconitifolia* (Jacq.) Maréchal with a chromosome count of 2n=22, belongs to the Fabaceae family. It is widely recognized for its suitability as a crop in tropical arid regions due to its exceptional tolerance to drought and heat stress, surpassing other species within the *Ceratotropis* subgenus (Somta *et al*., 2018). Moth bean's adaptive genetic characteristics, which include a deep root system, a short life cycle, an expansive canopy, trailing growth habits and its ability to thrive in variable and unstable climatic conditions in arid zones, make it a resilient and cost-effective choice as an annual legume. In the context of mixed cropping systems, moth bean is often chosen as a key component alongside pearl millet (Kandpal *et al*., 2009), making it a vital resource for resource-poor agriculture in arid regions where drought and high temperatures are frequent occurrences, particularly in sand dunes, plainsand degraded lands.

Nonetheless, there has been a noticeable shortage of initiatives aimed at enhancing the genetic attributes of this legume (Kumar and Singh, 2002). Furthermore, the availability of high-yielding, superior moth bean varieties remains quite limited. The primary hindrances to boosting moth bean productivity in rainfed cultivation are the absence of high-yield and early-maturing varieties, along with the prevalence of biotic stresses. The foremost drawback in the moth bean breeding programme is the unceasing use of

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the same cultivars in the development of new varieties, which crafted the narrowness in the genetic base of moth bean germplasm ensuing in the paramount selection pressure towards compliance to harsh environments rather than keeping aim on breaking the yield plateau. Thus, cataloguing of the elite predominant cultivars and assessment of genetic diversity in these cultivars has become the first and foremost need in moth bean improvement programme owing to the crafted narrow genetic base of this crop.

For assessment of genetic diversity in elite cultivars, genotypes and breeding lines, various approaches are used based on morpho- agronomic traits and molecular markers. Morpho-agronomic portrayalin combination with molecular characterization remained a profound approach for assessment of genetic diversity prevalent in the germplasm of any crop (Choudhary *et al*., 2014; Choudhary *et al.,* 2016).

In moth bean, the commercially predominant elite cultivars have been identified and released on the basis of below threshold level of phenotypic variability, using different morpho-agronomic characters (Chaudhari *et al*., 2021). Nonetheless, the paramount genetic drift and unsustainability towards tolerance hugely demands the replacement in varieties, which could further be achieved by tailoring the breeding methods including creation of genetic variability and identify it.

Furthermore, within the realm of crop improvement programs, the utilization of DNA marker technology has advanced significantly over the past decade. The exploration and application of molecular markers, rooted in DNA distinctions, provide a substantial opportunity to comprehend and pinpoint the diverse genetic resources within crop species. Owing to detection of variability of the DNA sequences among the genotypes as well as bypassing the environmental effects, molecular markers became the indispensable tools for characterizing genetic resources Consequently, these advancements offer a multitude of valuable applications in the realm of crop improvement Bottom of Form (Choudhary *et al*., 2016). Among the several molecular markers, owing to its user-friendly nature and fastest detection technique, embarks RAPD as a successful technique for depiction of intra species genetic diversity in several grain legumes (Abbas *et al*., 2008). Though there are few reports of morpho-agronomic characterization in moth bean using different genotypes are available (Chaudhari *et al*., 2021; Kohakade *et al*., 2017; Vir and Singh, 2015), but meagre research efforts have been done formolecular characterization of elite cultivars in moth bean crop as there is no report available on the correlation between the molecular and morpho-agronomic matrices generated on the same elite cultivars. This correlation depicts the synergy between the genotypic and phenotypic diversity, pointing out the significance of environmental effects in the phenotypic variability.

Hence, the present study has been carried out in 2018 with the objective of cataloguing the predominant, commercial and elite cultivars of moth bean on the basis of morpho-agronomic and molecular markers.

MATERIALS AND METHODS

The experimental material for the present study comprised 15 elite moth bean varieties (Table 1) released for different eco-geographical regions of India, maintained at the field experimental area of Central Arid Zone Research Institute, Jodhpur.

A diverse set of germplasm lines was meticulously chosen for both morpho-agronomic and molecular diversity analysis, ensuring the generation of valuable data. The genomic DNA of these varieties was meticulously extracted from young leaves, following the established procedure outlined by Doyle and Doyle (1987). The quality of the extracted DNA was validated by comparing it with λ DNA and its quantity was precisely determined using a spectrophotometer. The DNA stocks were carefully maintained in TE buffer and the working DNA sample was appropriately diluted to a standardized concentration of 25 ng/µl.

For the DNA amplification, Polymerase Chain Reaction (PCR) was employed, utilizing RAPD primers. The PCR reactions were carried out in 20 µl volumes, which included 1X PCR reaction buffer, 50 ng of template DNA, 0.6 U of Taq DNA polymerase, a 0.2 mM dNTP mix and 0.5 pM of both forward and reverse primers. The amplification process was conducted using a thermocycler from G-Storm (UK), programmed with an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles comprising denaturation at 94°C for 1 minute, annealing for 1 minute, elongation at 72°C for 2 minutes and a final extension at 72°C for 7 minutes. After PCR amplification, the amplified products were subjected to electrophoresis by loading them onto a 6% polyacrylamide gel prepared in 0.5X TBE buffer. Subsequently, the gels were silver-stained and visualized.

In this analysis, a total of 11 RAPD markers (Table 2) from the OPD series (sourced from *Eurofins Genomics*) were employed. These markers, as documented in various studies conducted in our laboratory, were known to exhibit polymorphism. They were used to assess polymorphism in all 15 cultivars of moth beans.

The genotyping data for each RAPD marker was systematically recorded in a binary format, representing the presence (1) or absence (0) of bands. To compute the Polymorphism Information Content (PIC) values, the established methodology outlined by Botstein *et al*. in 1980 was employed as follows:

$$PIC= 1-\sum_{i=1}^{n}(P_{ij})^2-\{\sum_{i=1}^{n}(P_{ij})^2\}^2+\sum_{i=1}^{n}\{(P_{ij})^2\}^2$$

Where

P_{ij}= Frequency of j^{th} allele in i^{th} primer and summation extends over 'n' patterns.

The binary data derived from the analysis of 11 polymorphic markers across all moth bean cultivars were

employed to compute a distance matrix using the dissimilarity coefficient analysis, as outlined by (Rogers and Tanimoto, 1960). This distance matrix served as the basis for constructing a dendrogram through the unweighted pair group method with arithmetic average (UPGMA) utilizing NTSYS pc-2.02 software, as developed by (Rohlf, 1998).

Likewise, data collected from observations of eleven morpho-agronomic traits, encompassing parameters such as Days to flowering, days to maturity, plant height, plant spreading, number of branches, pod length, number of clusters, pod length, seeds per pod and pod yield, were employed in cluster analysis based on genetic distances. This analysis also employed the unweighted pair-group method with arithmetic averaging (UPGMA) to generate a dendrogram illustrating the relationships among the different varieties. This analysis was conducted using the same NTSYS pc-2.02 software developed by Rohlf (1998).

Additionally, the matrices containing genetic distances derived from both the morpho-agronomic analysis and the molecular diversity analysis, after conversion into dissimilarity matrices, were subjected to Mantel's test, originally proposed by Mantel (1967). This test aimed to explore the relationships between the two distinct analytical approaches.

RESULTS AND DISCUSSION

Polymorphism of the RAPD markers

In the present study, all 11 RAPD markers showed amplification in moth bean and were highly polymorphic (Fig 1). To highlight the potential utility of these RAPD markers in future molecular investigations related to moth bean, the Polymorphism Information Content (PIC) values were employed as a parameter. These values ranged from 0.45 to 0.88, with an average value of 0.77. Notably, more than 60% of the RAPD loci exhibited PIC values exceeding this average threshold (refer to Fig 2). Additionally, the number of bands or alleles varied between 4 and 16, with an average of 10 alleles per locus, as detailed in Table 3.

It is widely recognized that markers must possess a substantial number of bands or alleles to be deemed useful for the evaluation of genetic diversity, as articulated by (Ribeiro-Carvalho *et al*., 2004). The high PIC value polymorphic RAPD markers obtained in this study offer promising prospects for next-generation association mapping, gene tagging and the potential application of marker-assisted selection (MAS). These markers are poised to play a pivotal role in enhancing genetic studies and breeding efforts in moth bean.Total 109 bands/alleles were generated by these 11 primers. Accordingly, earlier studies

Table 1: List of 15 elite cultivars of moth bean used in the study.

Variety	Year	Adaptation	Salient features
Jawala	1985	Rajasthan	YMV resistance, fodder yield 17-18 q/ha, harvest index of 24-28%.
Maru Moth	1989	Rajasthan	Semi-spreading type, tolerant to *Cercospora* leaf spot disease, suited for intercropping.
CZM-1	1999	Rajasthan	Semi-erect type, profuse bearing, protein 24-26%, YMV resistance.
RMO-40	1994	Rajasthan and Gujarat	First early variety, erect synchronous growth habit, escape to drought and YMV, suitable for close spacing.
RMO-225	1999	Rajasthan	Semi-erect type, grain color light brown, escapes drought and YMV infection
RMO-435	2001	Rajasthan	Escapes drought, YMV resistance, semi spreading growth habit, 10-12% yield superiority over best check.
RMO-257	2005	Rajasthan, Gujarat, Haryana and Maharashtra	Semi-erect growth gives 18-20 q/ha fodder yield, bears 3-6 branches/plant, less YMV infection.
CAZRI Moth-2	2003	Rajasthan, Gujarat, Haryana and Maharashtra	First variety from hybridization, (CZM-2) darkgreen color
CAZRI Moth-3 (CZM-3)	2005	Rajasthan, Gujarat, Haryana and Maharashtra	Erect and synchronized growth, escapes YMV
GMO-1		Gujarat	Dual purpose for grain and fodder, chocolate color seeds, protein 19% and seed index is 2.5 g.
GMO-2	2004	Rajasthan and Gujarat	Erect and semi-determinate plant type, seed index is 2.9 g, escape YMV infection
RMO 423	2004	Rajasthan	Resistant to yellow mosaic virus and also has good fodder value.
RMB 25	2005	Rajasthan	Resistance towards YMV, bacterial leaf spot, root rot as well as insect resistance.
RMO-2251	2016	Rajasthan and Gujarat	Upright branching, mild resistance to YMV.
RMO-141	2004	Rajasthan	Resistant to yellow mosaic virus and also has good fodder value.

by (Moitra and Mandal, 2003) marked the initial effort to create a DNA fingerprint for seven moth bean accessions by utilizing the M13 forward primer as an RAPD marker. Their findings revealed limited yet discernible polymorphism among the various genotypes.

In a separate study by Saxena *et al.* (2006), the amplification of 72 RAPD primers in 12 moth bean genotypes, which encompassed released varieties, mutant lines and advanced breeding materials, was investigated. Among these 72 primers, 52 generated a total of 208 bands, with an average of 4.0 bands per primer. Out of these 208 bands, 171 were deemed scorable and repeatable and 79 of them exhibited polymorphism. These results collectively contribute to our understanding of the genetic diversity within moth bean genotypes.

Additionally, the product size was determined for each of the primers by calculating the average in the current study. The presence of multiple loci amplification and the high polymorphism information content (PIC) values collectively signify the effectiveness of these RAPD markers in the characterization of moth bean germplasm, evolutionary studies, breeding applications and phylogenetic investigations in the context of moth bean.

It is worth noting that several RAPD markers exhibited multiple banding patterns, some of which featured very faint bands. However, it's important to mention that these faint bands were not included in the analysis for the current study.

Diversity analysis based on morpho- agronomic characters

A dendrogram (Fig 3A) based on Manhattan dissimilarity coefficient analysis grouped 15 moth beancultivars intofour main clusters, among which RMB-25 deviates itself from all the three major clusters and formed a mono-genotypic cluster. Cluster IV was the largest cluster having 8 cultivars followed by cluster II with4 cultivars and Cluster I with 2

Table 2: RAPD primers used in the study.

Primer	Sequence (5′-3′)
OPD-03	GTCGCCGTCA
OPD-07	TTGGCACGGG
OPD-08	GTGTGCCCCA
OPD-09	CTCTGGAGAC
OPD-11	AGCGCCATTG
OPD-13	GGGGTGACGA
OPD-14	CTTCCCCAAG
OPD-16	AGGGCGTAAG
OPD-17	TTTCCCACGG
OPD-18	GAGAGCCAAC
OPD-19	CTGGGGACTT

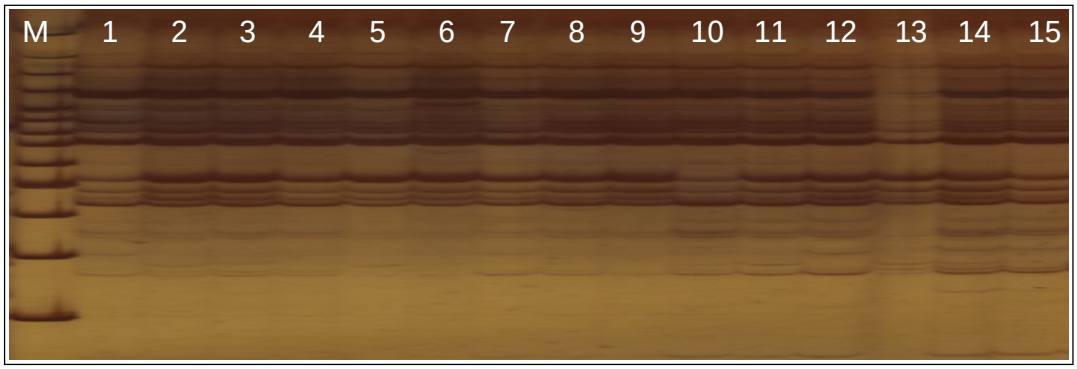

Fig 1: Genomic DNA amplification pattern of 15 cultivars with RAPD primer OPD-18. Genotype order 1-15: RMO-2251, RMO-141, RMO-40, RMO-225, RMO-435, RMO-257, CZM-2, RMO-423, RMB-25, Jwala, Maru moth, CZM-1, CZM-3, GMO-1 and GMO-2.

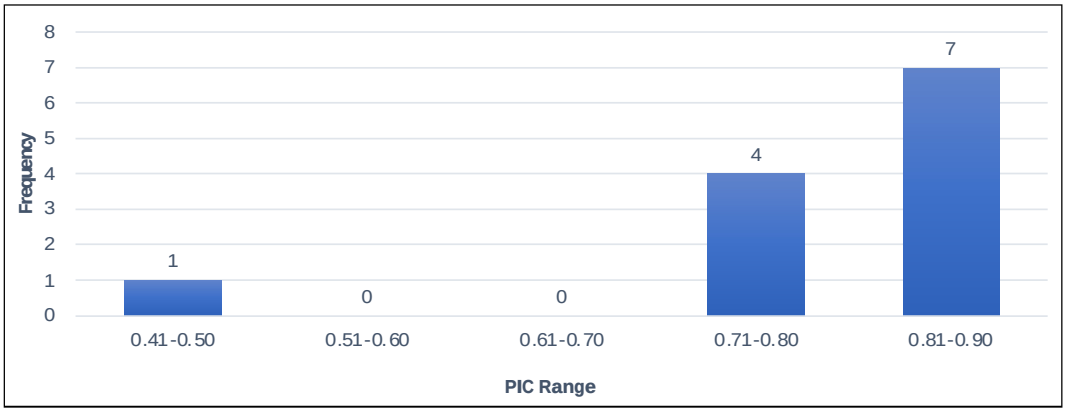

Fig 2: Frequency distribution of the range of PIC values among the polymorphic RAPD markers.

cultivars (Table 4). Upon careful examination of the cluster analysis, it became evident that individuals within the same cluster exhibit a closer degree of relatedness to each other than they do to individuals located in different clusters.

Thus, discriminating power and consistency for genetic diversity studies shown by the morpho-agronomic trait markers was endorsed by the attained clustering pattern despite the so called phenotypic itinerary.

Further sub clustering resulted the bifurcation of cluster II into two subclusters depicting the RMO-141's deviation from the other cultivars of cluster II. Similarly, sub clustering of cluster IV depicted the trifurcation and deviation of RMO-423 and CZM-3 from the remaining cultivars of cluster IV.NTSYS also analysed the Manhattan dissimilarity coefficient which was ranging from 0.02 to 0.42 depicting a transitional amount of diversity among the cultivars by visualizing the extent of genetic similarities among the testedcultivars. Minimum dissimilarity of 2% was found between RMO-435 and RMO-40. This observation suggests the possibility that the markers associated with the morphological traits employed in this study might be closely linked to specific genomic regions within these cultivars. While the maximum dissimilarity of about 42% was observed between RMO-257 and CZM-2. Hence, these cultivars are implicated to get minimum similarity, facilitating their use in developing mapping population for diverse traits and establishing the utility of morphological markers in identifying diverse pairs.

Diversity analysis based on RAPD markers

Without any prior sequence information, RAPD technique provides a sensitive and fast method which facilitates the identification of a large number of genotypes. Thus, based on the RAPD marker scoring, a dendrogram (Fig 3B) based

Table 3: Primer Sequences and characteristics of 11 RAPD markers.

Primers	Amplified product size (bp)	No. of bands	PIC value
OPD -07	440.00	8	0.77
OPD-08	324.14	14	0.87
OPD-09	439.67	15	0.84
OPD-11	962.50	4	0.45
OPD-13	561.50	10	0.83
OPD-16	504.69	16	0.88
OPD-18	528.00	10	0.81
OPD-17	357.50	10	0.82
OPD-14	471.00	5	0.73
OPD-3	270.17	6	0.71
OPD-19	517.73	11	0.80

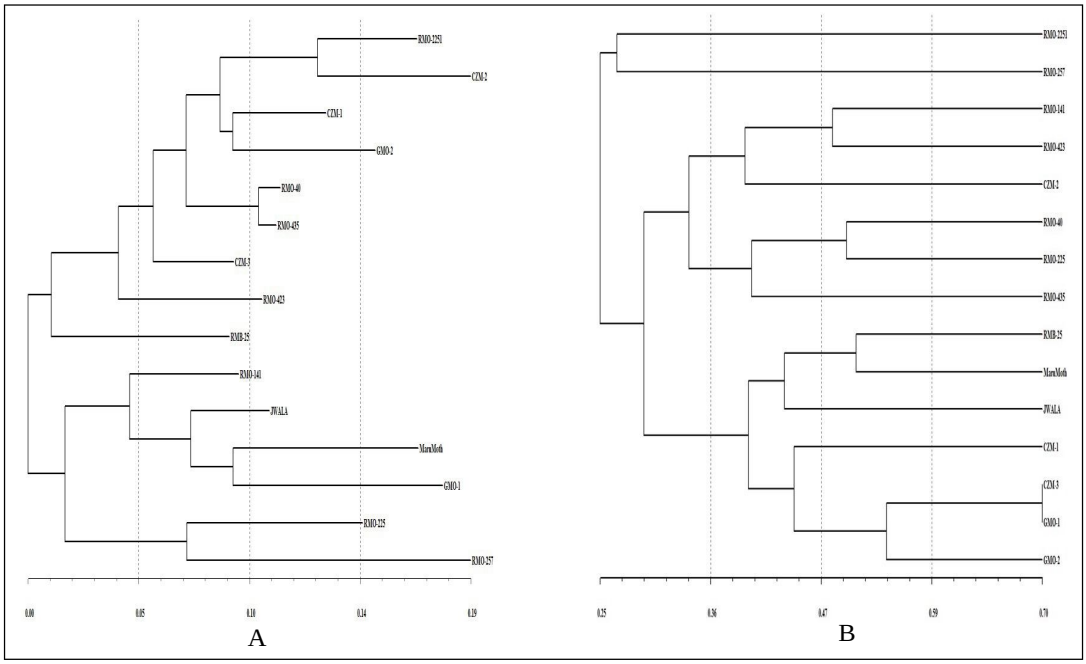

Fig 3: A) Morphological (Manhattan distance) and B) Molecular (Jaccard's similarity matrix) characterization of 15 elite cultivars of moth bean.

on the Jaccard's similarity coefficient analysis grouped 15 cultivars of moth bean in three main clusters, where RMO-257 and RMO-2251 deviates itself from all the three major clusters and formed two differentmono-genotypic cluster.

The perusal of the cluster analysis revealed that the individuals within any one cluster are more closely related than are individuals in different clusters. Cluster I was the largest cluster with 7 cultivars followed by cluster IIwith3 cultivars and Cluster III with 3 cultivars (Table 5).

Cluster II have all the three cultivars from same origin point. This showed the clustering pattern obtained from RAPD markers synchronized with the geographical diversity as well. Thus, discriminating power and consistency for genetic diversity studies shown by the RAPD markers was endorsed by the realized clustering pattern. Further sub clustering of cluster I resulted in four subclusters and depiction the deviation of primitive Jwala and CZM-1 from the rest of the cultivars of cluster I. Similarly, sub clustering of cluster II and cluster III bifurcated them into two sub clusters respectively. Among them RMO-435 and CZM-2 deviates themselves from the other cultivars of the main clusters.The range of Jaccard's similarity coefficient observed from 0.18 to 0.70, depicting a transitional amount of diversity among tested cultivars by visualizing the extent of genetic dissimilarities among them. Maximum similarity of 70% was found in the cultivar GMO-1 with CZM-3. GMO-1 also shown the considerable similarity with GMO-2. This mark a possibility that the RAPD markers used in the study may be linked to the genomic region in these cultivars. On the other hand,GMO-2 and RMO-257 showed the minimum similarity of 18% followed by GMO-2 and RMO-435. These pairs of cultivars are implicated to get maximum dissimilarity and hence can be used in mapping population development and establishing the utility of RAPD markers in identifying diverse pairs. Accordingly, results were reported by (Imran, 2011) subgrouping the 31 moth bean accessions in three subgroups using seven RAPD primers. Mamta (2012) also reported clustering of 10 moth bean cultivars into five clusters using 40 RAPD primers.

Correlation between morpho-agronomic and molecular matrices

Undoubtedly, both approaches represent complementary facets of the same coin. Molecular analysis, as employed in this study, offers a broader sampling of the genome compared to morphological analysis. Notably, it is rare to find a study that assesses the same or even a similar number of morphological and molecular markers.

However, it's important to acknowledge that a certain discrepancy emerged when examining the association between the diversity of morpho-agronomic traits and the variability observed in RAPD markers. This discrepancy is evident in the low and non-significant negative correlation (r= -0.00073, P= 0.05).

This observed lack of association may be influenced by the fact that a significant portion of the variation detected by molecular markers is non-adaptive and, therefore, not subject to either natural or artificial selection. On the other hand, phenotypic traits are subject to both natural and artificial selection, in addition to being highly dependent on environmental factors.

In conclusion, the genetic diversity assessment based on both morpho-agronomic characters and RAPD markers lays a solid foundation for future research endeavours.

Table 4: Clustering pattern generated by the markers related to morpho-agronomic traits.

Cluster	Genotype count	Genotype	No. of sub clusters	Cultivars in sub clusters
I	2	RMO-257 and RMO-225	0	
II	4	GMO-1, Maru moth, Jwala and RMO-141	-	-RMO-141 Remaining cultivars
III	1	RMB-25	0	
IV	8	RMO-423, CZM-3, RMO-435, RMO-40, GMO-2, CZM-1, CZM-2 and RMO-2251	3	CZM-2 RMO-423 Remaining cultivars

Table 5: Clustering pattern obtained by RAPD markers.

Cluster	No. of genotype	Genotype	No. of sub clusters	Cultivars in sub clusters
I	7	GMO-2, GMO-1, CZM-3, CZM-1, Jwala, Maru moth and RMB-25	4	CZM-1 Jwala GMO-2, GMO-1,CZM-3 Marumoth and RMB-25
II	3	RMO-435, RMO-225, RMO-40	2	RMO-435 RMO-225 and RMO-40
III	3	CZM-2, RMO-423 and RMO-141	2	CZM-2 RMO-423 and RMO-141
IV	1	RMO-257	0	-
V	1	RMO-2251	0	-

Preliminary studies like these are pivotal for the effective planning and execution of projects involving mapping populations and further genetic studies.

CONCLUSION

In conclusion, when it comes to a lesser-studied crop like moth bean, RAPD markers offer a convenient and practical choice compared to a range of other DNA markers such as AFLPs, SSRs and SNPs, which have been developed with advancements in marker technology. By introducing a diverse array of moth bean cultivars and incorporating them into a well-structured scientific moth bean hybridization program, we can enhance the broader moth bean cultivar development effort. To be more specific, the future strategy might prioritize molecular characterization using DNA markers, in conjunction with morpho-agronomic profiling, as a means to address phenotypic variations effectively. Among the various marker discrimination indices, PIC (Polymorphic Information Content) is deemed a more reliable indicator when it comes to selecting specific markers or combinations of markers for germplasm characterization. This comprehensive approach can be a promising avenue for advancing our understanding and improvement of moth bean crops.

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Conflicts of interest

The authors declare no any conflict of interest.

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