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Unraveling the Genetic Diversity of Bitter Gourd (*Momordica charantia* L.) Using IRAP and REMAP Retrotransposon Markers

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ABSTRACT

The dispersion and abundance of mobile genetic elements in plant genomes have made them valuable molecular markers. Understanding genetic diversity is crucial for the organization and conservation of plant materials. This study aimed to investigate the genetic diversity of the economically and medicinally significant plant *Momordica charantia* L. (bitter gourd) using IRAP and REMAP markers. The identification of retrotransposon insertions was performed on seeds of 11 bitter gourd accessions collected from different regions. The results showed that most of the retrotransposons were active in the bitter gourd genome, and 71 polymorphic loci were generated using 6 IRAP primers, and 103 polymorphic loci were generated using 12 REMAP primers. The expected mean heterozygosity (He) was 0.24 for IRAP and 0.31 for REMAP. Cluster analysis based on REMAP and IRAP data, using the Dice similarity coefficient and the complete linkage algorithm, grouped the 11 genotypes into 5 major clusters. The genetic diversity obtained from the IRAP marker was 66% within populations and 34% between populations, while the REMAP marker showed 61% within populations and 39% between populations. The lowest genetic similarity was observed in the IRAP data between the Durga seeds from Hong Kong and the Kanarkee accession. In REMAP analysis, the least similarity occurred between the Long Green and Durga India baby accessions, as in REMAP-IRAP, it was noted between the Kanarkee and Durga Indian baby accessions. Based on these findings, it is recommended that the Kanarkee and Durga India baby accessions be utilized as parent lines in breeding programs.

Keywords: Karela, Genetic diversity, Retrotransposons, Molecular markers, IRAP, REMAP

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INTRODUCTION

Medicinal plants are of special value and importance in providing health and well-being to communities, both in terms of treatment and prevention of diseases [1, 2]. One notable medicinal plant is Karela, or bitter gourd (*Momordica charantia* L.), which belongs to the Cucurbitaceae family. The green and unripe fruit of bitter gourd has a desirable sour taste and is typically consumed in this immature stage. However, when fully ripe, the fruit turns yellow and red, and its flavor becomes bitter due to the presence of quinine. Bitter gourd fruits are rich in Vitamin A, Vitamin C, and iron, and they also contain a significant amount of insulin, which is effective in reducing blood sugar levels. Consequently, people in India use these fruits for traditional and local treatments of diabetes [3]. Bitter gourd fruit also exhibits anti-cancer properties, particularly against blood cancer, and has been the subject of extensive research for its potential therapeutic applications [4].

Bitter gourd is believed to have originated in India and possibly China, and its production is now widespread in the tropical regions of Iran, such as Sistan and Baluchestan, and Hormozgan [3]. There are numerous hybrid and conventional varieties of bitter gourd available. The production of hybrid seeds aims to enhance productivity and economic significance while contributing to health and disease management in plants like bitter gourd. This focus has led to extensive research into genetic diversity [7, 8].

The study of genetic diversity is of utmost importance for the organization and creation of diverse plant collections, as well as the conservation of plant materials. Reduction in genetic diversity can lead to a decrease in fitness and adaptability to environmental changes [9]. Evaluation of genetic diversity through the assessment of morphological traits for a large number of samples requires a lot of time and cost, and due to the environmental effect on gene expression, may not be a reliable method for determining genetic differences [3, 10, 11]. Therefore, the use of various molecular markers as complementary and efficient tools for investigating genetic diversity and relationships between individuals has significantly increased following advancements in the field of molecular genetics and molecular marker technology [12, 13].

Nowadays, techniques based on retrotransposons have become suitable and ideal tools for the molecular analysis of diversity among different plant species, thanks to the distribution and abundance of retrotransposons in plant genomes [14, 15]. Some of the most important retrotransposon-based markers are IRAP and REMAP, which are particularly useful for genetic mapping, genetic fingerprinting, pedigree analysis, and assessing the level of genetic changes and diversity in plant germplasms due to their high genome coverage, reproducibility, and polymorphism [16-18].

While previous studies have investigated the genetic diversity of bitter gourd using RAPD, ISSR, and AFLP markers [3, 19, 20], the genetic diversity of this plant species has not yet been studied using

the more informative IRAP and REMAP retrotransposon-based markers. There is no direct evidence that IRAP (Inter-Retrotransposon Amplified Polymorphism) and REMAP (Retrotransposon-Microsatellite Amplified Polymorphism) markers have been specifically employed to investigate the genetic diversity of bitter gourd (*M. charantia* L.). However, these markers have proven effective in studying genetic diversity in other plant species. For instance, IRAP and REMAP markers were successfully used to assess genetic diversity among Lallemantia iberica varieties, demonstrating high polymorphism and effectiveness in estimating genetic diversity [21]. Additionally, IRAP and REMAP markers are widely recognized for analyzing the genetic diversity and structure of plant populations [22]. In the case of bitter gourd (*M. charantia* L.), other molecular marker techniques have been utilized to explore genetic diversity. Specifically, Simple Sequence Repeat (SSR) markers have been developed and employed to analyze genetic diversity in bitter gourd [23, 24]. Random Amplified Polymorphic DNA (RAPD) markers have also been used to reveal genetic diversity among bitter gourd genotypes [25]. Furthermore, whole-genome sequencing and resequencing approaches have been applied to investigate genetic diversity and domestication in bitter gourd [26]. These markers have the potential to provide a more comprehensive understanding of the genetic diversity within bitter gourd populations, which is crucial for the development of breeding programs and the conservation of this economically and medicinally important crop. Therefore, the present study aims to investigate the genetic diversity among different bitter gourd populations and identify diversity in different varieties using these advanced molecular techniques.

MATERIALS AND METHODS

Samples Preparation

For this research, 11 varieties of *M. charantia* L. (bitter gourd) seeds were obtained and cultivated under controlled laboratory conditions (Table 1). DNA was extracted from 100 mg of leaves from plants that were 12 to 18 days old using the Dellaporta method [27]. To address the issues of genetic purity and uniformity among the seeds, DNA was extracted from two seeds individually from each genotype, to ensure proper representation. The quality and quantity of the extracted DNA were assessed using a spectrophotometer and 1% agarose gel electrophoresis.

Table 1 Bitter gourd seed samples used in the research and corresponding analysis codes

Seed name	Abbreviation for experiment	Country of origin
Jaunpuri	A	India
Jason F1	B	Pakistan
Long green	C	India
Durga seeds	D	Hong Kong
Durga seeds small	E	India
Durga larj tup	F	India
Durga bankok larj	G	Thailand
Durga India baby	H	India
Durga jomboty	I	India
Palee F1	J	Thailand
Konarak Sample	K	Collected

Primer Preparation and PCR Analysis

In this study, the primers were designed using the sequences of IRAP and REMAP retrotransposons. The primers were obtained from Macrogen, a company in South Korea, and were used for the polymerase chain reaction (PCR) amplification.

The optimization of the primer annealing temperature was performed using a temperature gradient PCR. Each PCR reaction was conducted in a final volume of 15 µL, containing 1 µL of genomic DNA (5 ng/µL), 7 µL of Master Mix, 0.5 µL of each primer, and 6 µL of distilled water. The thermal cycling conditions included an initial denaturation step at 94 ℃ for 4 minutes, followed by 35 cycles of 94 ℃ for 45 seconds, 53-65.6 ℃ (depending on the primer) for 40 seconds, and 72 ℃ for 2 minutes, with a final extension step at 72 ℃ for 5 minutes.

The amplified products were separated on a 1.5% agarose gel using a BioRAD electrophoresis system in 0.5X TBE buffer at 65 V for approximately 3 hours. The gels were then stained with an appropriate dye and visualized under UV light using a gel documentation system.

Statistical Analysis

In this analysis, scoring for each marker was based on the presence (1) or absence (0) of the amplified bands. We calculated the total number of amplified loci and the percentage of polymorphic loci for each primer. Additionally, we determined several metrics for each genotype using GenAlEx software version 6.5, including the number of loci with a frequency of 5% or greater, the number of population-specific loci, the number of loci with a frequency of 25% or less, the number of loci with a frequency of 50% or less, the mean heterozygosity, gene flow, and the standard error of the mean heterozygosity.

The genetic similarity matrix of the genotypes was obtained using the NTSYS software version 2.1 [28]. The similarity between the cultivars was based on the Dice genetic similarity coefficient (1978), and the Complete Linkage algorithm was used to construct the dendrogram. The cophenetic correlation coefficient (r) was calculated to evaluate the goodness of fit between the dendrogram matrix (cophenetic matrix) and the genetic distance matrix [28].

To analyze the relationships between the distance matrices derived from the REMAP, IRAP, and IRAP+REMAP data, we conducted a Mantel test with 100 permutations using NTSYS software version 2.1. Additionally, we performed a molecular analysis of variance (AMOVA) using GenAlEx software version 6.5 to assess the genetic diversity among and within the studied cultivars and individuals. The effective number of alleles (Ne), Shannon's information index (I), and expected heterozygosity (He) were calculated using the GenAlEx software version 6.5.

RESULTS

IRAP Markers and the Distribution Pattern of Retrotransposon Families

Six individual IRAP primers and 12 IRAP primer combinations from the retrotransposon families of bitter gourd were used to analyze 11 cultivars and 22 individuals (2 samples per cultivar). Ultimately, 8 primers produced polymorphic and scorable banding patterns [29-31] (Fig. 1 and Table 2).

This study on bitter gourd employed Inter-Retrotransposon Amplified Polymorphism (IRAP) markers to assess genetic diversity among 11 cultivars. It has utilized six individual IRAP primers and 12 IRAP primer combinations derived from the bitter gourd retrotransposon family, analyzing 22 individual samples (2 per cultivar). Eight primers successfully produced polymorphic and scorable banding patterns. The analysis revealed a total of 71 alleles across all IRAP primer combinations, with LTR2452 (IRAP1) and IRAP1+IRAP4 primers generating the highest number of alleles

(11 each). The polymorphism index ranged from 0.21 (LTR2467 - IRAP3) to 0. 57 (LTR2473 - IRAP60), with an overall average of 0.37. In total, 71 alleles were identified for the IRAP primer combinations, and the LTR2452 (IRAP1) and IRAP1+IRAP4 primers had the highest number of 11 alleles among the alleles generated by the used primers. The highest level of polymorphism index was 0.57, which was related to the LTR2473 (IRAP60) primer, and the lowest level of this index was 0.21 for the LTR2467 (IRAP3) primer. An overall average of 0.37 was observed for the polymorphism index (Table 3). The mean heterozygosity in the genotypes ranged from 0.14 to 0.38, with an average of 0.24. Cluster analysis using the Complete Linkage algorithm and the Dice genetic similarity coefficient based on the IRAP markers, as well as principal coordinate analysis (PCoA), were performed as complementary clustering methods (Fig. 2). The relationships in the two-dimensional plot also confirmed the clustering method and overall grouped the studied genotypes into five main clusters (Fig. 3). Among them, cultivars like Kanarake (k) and Palee F1 Thailand (j) were in one group, while the Durga jamboty India (i) cultivar was in a separate group, and on average, each cultivar was placed in different groups, indicating that the genetic diversity within cultivars was greater than the diversity between cultivars (Fig. 4). Based on the results of the analysis of molecular variance (AMOVA), 66% of the genetic variation was found within populations, and 34% was found between populations.

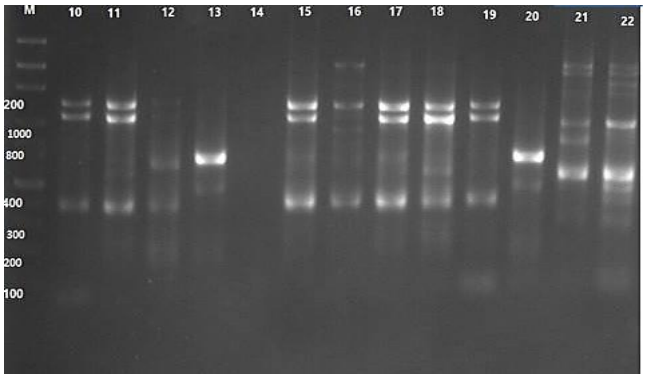

Fig. 1 Band formation pattern for the IRAP2+IRAP4 primer, with DNA M marker. The bands are arranged from left to right as follows: The numbers 10 to 22 represent the second repeat of the experiment, corresponding to the genotypes A, B, C, D, E, F, G, H, I, J, and K, respectively (based on Table 1).

Table 2 Characteristics of the primers used

Primer name	Primer sequence (5'-3')	Primer source
LTR2452(IRAP1)	TCCTGGTAACACTATGGATACGAC	*M. charantia* L. retrotransposon
LTR2453(IRAP2)	CTTATACGTCTGAAGGACAGGGTTTC	*M. charantia* L. retrotransposon
LTR2467(IRAP3)	ACGGTTACGGGCGTGTTCCTCTTCCA	*M. charantia* L. retrotransposon
LTR2476(IRAP4)	GACTTCAAGCTACTTCGAATGGGTTGTC	*M. charantia* L. retrotransposon
U		retrotransposon
[[		
LTR2469(IRAP5)	AGAGAGAGAGAGAGAGAGAGT	*M. charantia* L. retrotransposon
LTR2473(IRAP6)	CGCATCCATCTAGCACGAGG	*M. charantia* L. retrotransposon
UBC808(ISSR1)	AGAGAGAGAGAGAGAGC	ISSR
UBC811(ISSR2)	GAGAGAGAGAGAGAGAAC	ISSR
UBC816(ISSR3)	CACACACACACACACAT	ISSR
UBC825(ISSR4)	ACACACACACACACACT	ISSR
UBC826(ISSR5)	ACACACACACACACACC	ISSR
UBC834(ISSR6)	AGAGAGAGAGAGAGAGY*T	ISSR
UBC840(ISSR7)	GAGAGAGAGAGAGAGAY*T	ISSR
UBC855(ISSR8)	ACACACACACACACACYT	ISSR
UBC-818(ISSR9)	CACACACACACACACAG	ISSR
UBC-857(ISSR10)	ACACACACACACACACT	ISSR
UBC-815(ISSR11)	CTCTCTCTCTCTCTCTG	ISSR
UBC880(ISSR12)	GGAGAGGAGAGGAGA	ISSR

Table 3 IRAP primer combinations and their characteristics

Primer name	Annealing temperature (°C)	Number of bands	Number of polymorphic bands	Percentage of polymorphism	Polymorphism index	Effective alleles number	Expected heterozygosity
LTR2452(IRAP1)	63.5	11	8	72.72	0.31	1.22	0.15
LTR2467(IRAP3)	66.3	8	4	50	0.21	1.23	0.14
LTR2476(IRAP4)	68.7	9	7	77.77	0.34	1.30	0.21
LTR2473(IRAP6)	65.2	8	8	100	0.57	1.67	0.38
IRAP1+IRAP4	61.9	11	7	63.63	0.36	1.44	0.23
IRAP2+IRAP3	65.2	8	7	87.5	0.49	1.56	0.33
IRAP2+IRAP4	58.8	8	6	75	0.39	1.43	0.26
IRAP5+IRAP6	62.6	8	5	62.5	0.30	1.32	0.19
Total		71	45				
Average		8.8	6.4	73.64	0.37	1.39	0.24

From the combination of the REMAP primer, a total of 21 combinations were tested on 11 digits of bitter gourd, of which 12 primer combinations amplified the multi-shaped and scorable bands (Fig. 5 and Table 4).

12 primer combinations amplified a total of 103 markers, of which 86 markers (84%) were polymorphic. The average number of polymorphic bands amplified per primer was 8.5. The range of the amplified bands varied from 80 to 2000 base pairs. The primer pattern related to REMAP6 is shown in Fig. 5. Among the REMAP primer combinations, REPAP4, REPAP6, and REPAP11 showed the highest number of bands and the highest number of polymorphic bands, indicating the presence of a large number of LTR-containing retrotransposons near the microsatellite motifs related to the ISSR9, ISSR29, and ISSR10 primers. The average heterozygosity was 0.31 (Table 4).

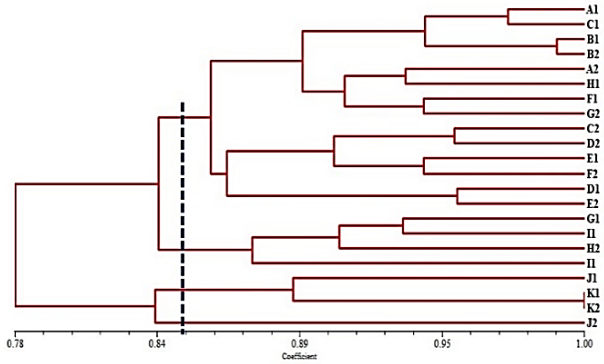

Fig. 2 Complete Linkage dendrogram for 22 genotypes based on IRAP markers and Dice similarity coefficient. The genotypes, labeled A, B, C, D, E, F, G, H, I, J, and K, are based on Table 1, with two replicates for each genotype.

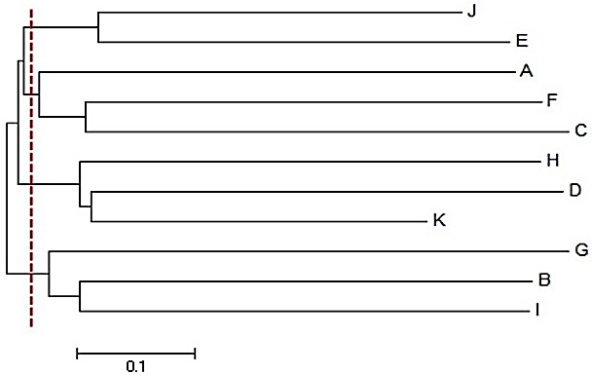

Fig. 3 Complete Linkage dendrogram for 11 cultivars based on IRAP markers and Dice similarity coefficient. The genotypes are labeled A, B, C, D, E, F, G, H, I, J, and K, based on Table 1.

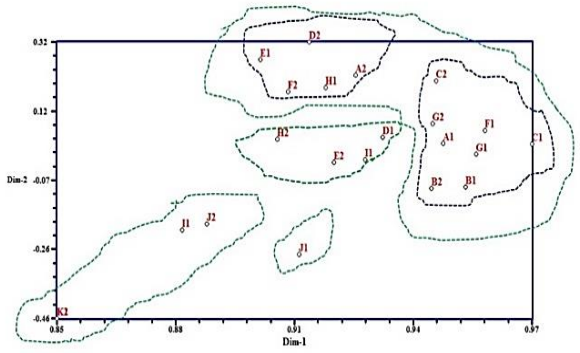

Fig. 4 Two-dimensional plot of the genetic relationships among 22 bitter gourd genotypes based on Principal Coordinate Analysis (PCoA) using IRAP markers. The genotypes, labeled A, B, C, D, E, F, G, H, I, J, and K, are based on Table 1, with two replicates for each genotype.

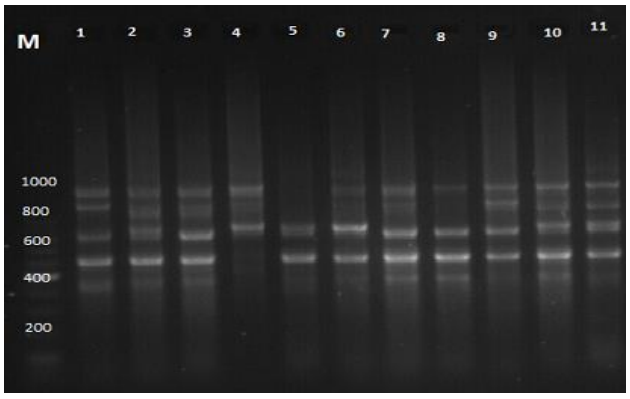

Fig. 5 Banding pattern for the REMAP6 primer, with DNA M marker. The bands are arranged from left to right as follows: The numbers 1 to 11 represent the first repeat of the experiment, corresponding to the genotypes A, B, C, D, E, F, G, H, I, J, and K, respectively (based on Table 1).

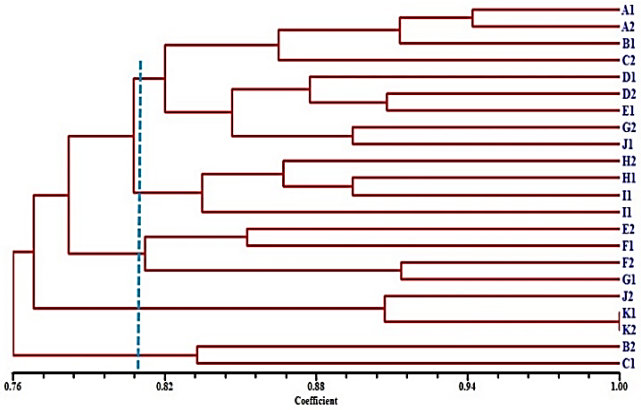

Fig. 6 Complete linkage dendrogram for 22 genotypes based on REMAP markers and Dice similarity coefficient. The genotypes are labeled A, B, C, D, E, F, G, H, I, J, and K, as shown in Table 1, with two replicates for each genotype.

Cluster analysis using the Complete Linkage algorithm (Fig. 6) and the Dice genetic similarity coefficient, along with the dendrogram resulting from the data analysis (Fig. 7) based on the REMAP markers, grouped the cultivars into five main clusters. The Kanareh (k) cultivar was placed in a separate group and, on average, was more distantly related to the other cultivars in the remaining groups, indicating that the genetic diversity within cultivars was greater than the diversity between cultivars.

Table 4 REMAP primer combinations and their characteristics

Primer name	Annealing temperature (°C)	Number of bands	Number of polymorphic bands	Percentage of polymorphism	Polymorphism index	Effective alleles number	Expected heterozygosity
REMAP1=IRAP3+ISSR9	68	9	7	0.25	77.77	1.42	0.24
REMAP2=IRAP2+ISSR5	68	8	7	0.30	87.5	1.70	0.38
REPAP3=IRAP3+ISSR6	68	8	8	0.38	100	1.75	0.41
REMAP4=IRAP6+ISSR9	66	9	9	0.27	100	1.54	0.31
REMAP5=IRAP1+ISSR4	64	7	6	0.24	85.71	1.61	0.35
REMAP6=IRAP5+ISSR2	62	9	9	0.32	100	1.79	0.43
REMAP7=IRAP1+ISSR8	62	8	8	0.16	100	1.41	0.27
REMAP8=IRAP2+ISSR8	60	9	4	0.28	44.44	1.35	0.19
REMAP9=IRAP3+ISSR11	60.5	9	7	0.38	77.77	1.43	0.27
REMAP10=IRAP2+ISSR8	59	9	5	0.30	55.55	1.37	0.21
REMAP11=IRAP4+ISSR10	61	9	9	0.27	100	1.58	0.36
REMAP12=IRAP5+ISSR12	64	9	7	0.35	77.77	1.43	0.26
Total		103	86				
Average		8.5	7.1	0.29	83.87	1.53	0.31

REMAP Markers and the Distribution Pattern of Retrotransposons

Combined Analysis of REMAP and IRAP Markers

The Mantel test to examine the correlation between the two cophenetic similarity matrices derived from the REMAP and IRAP markers showed a significant correlation (r=0.73) between the IRAP and REMAP matrices. To increase confidence in the clustering results, the binary matrices of IRAP and REMAP markers were combined to form a single genetic similarity matrix, which was then used for cluster analysis.

The overall mean genetic similarity among the cultivars was 0.8. The dendrogram produced from the Complete Linkage cluster analysis classified the cultivars into five and four main groups, respectively, in Figures 8 and 9, a distinction that is also evident in the two-dimensional plot (Fig. 10). Notably, the Kanareh cultivar was assigned to a separate group, while the other cultivars were grouped in the remaining categories.

The strong correlation between the IRAP and REMAP similarity matrices indicates that both marker systems offered consistent and complementary insights regarding the genetic relationships among the studied cultivars. The combined analysis of IRAP and REMAP markers enhanced the clustering results and highlighted the distinct genetic profile of the Kanareh cultivar in comparison to the others.

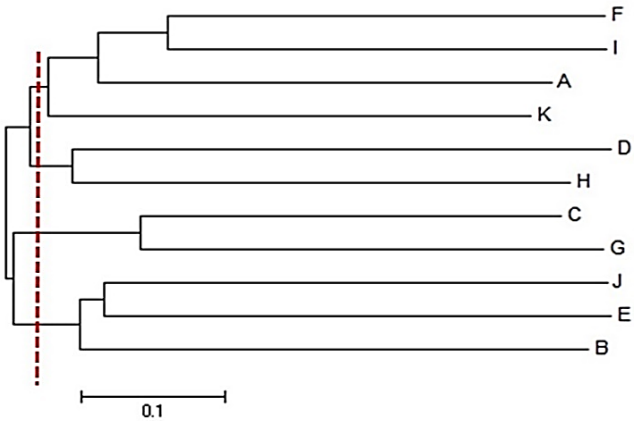

Fig. 7 Complete linkage dendrogram for 11 cultivars based on REMAP markers and Dice similarity coefficient. The genotypes are labeled A, B, C, D, E, F, G, H, I, J, and K, as shown in Table 1.

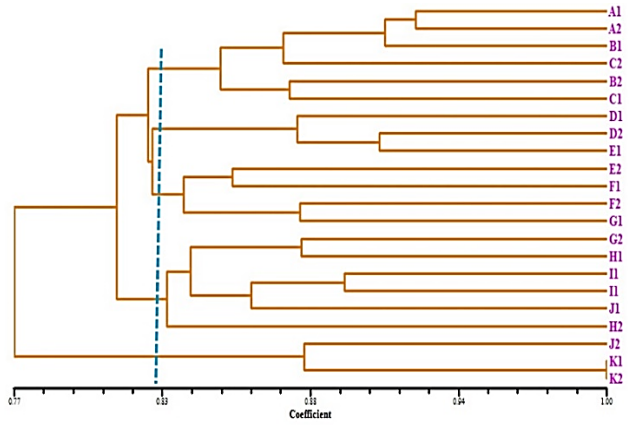

Fig. 8 Complete Linkage dendrogram for 22 cultivars based on REMAP+IRAP markers and Dice similarity coefficient. The genotypes are labeled A, B, C, D, E, F, G, H, I, J, and K, as shown in Table 1, with two replicates for each genotype.

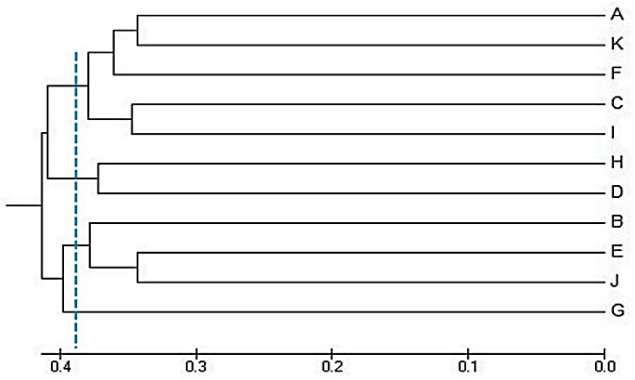

Fig. 9 Complete linkage dendrogram for 11 cultivars based on REMAP+IRAP markers and Dice similarity coefficient. The alphabetic of A, B, C, D, E, F, G, H, I, J, and K are the genotypes name based on Table 1.

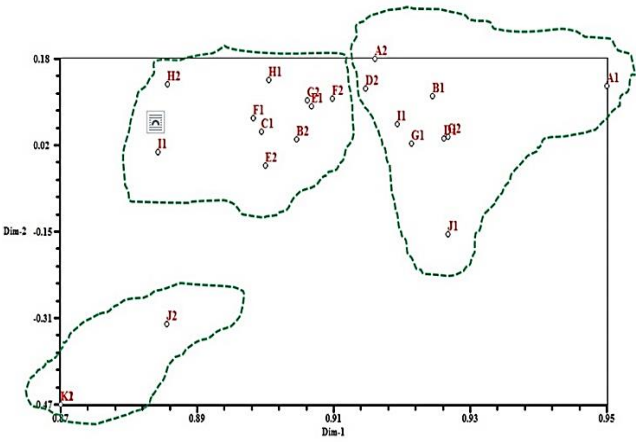

Fig. 10 Two-dimensional plot of the genetic relationships among 22 bitter gourd genotypes based on Principal Coordinate Analysis (PCoA) using REMAP+IRAP markers. The genotypes are labeled A, B, C, D, E, F, G, H, I, J, and K, based on Table 1, with two replicates for each genotype.

DISCUSSION

The results of this study on Bitter Gourd (*M. charantia* L.) using IRAP markers reveal both similarities and differences when compared to other published research. Specifically, 8 out of 18 IRAP primers (44.4%) produced polymorphic bands. In a study of *Lallemantia iberica*, IRAP markers exhibited high polymorphism [21]. This study identified a total of 71 alleles across all IRAP primer combinations. In contrast, in bitter gourd, 33 out of 82 SSR markers were polymorphic, yielding 2-7 alleles per locus, with an average of 3.46 [25]. For apple, IRAP primers generated 6-15 informative fragments per primer [32]. In this study, the PIC values ranged from 0.21 to 0.57, with an average of 0.37. Bitter gourd studies using SSR markers reported an average PIC of 0.38 [25]. The *Lallemantia iberica* study indicated that IRAP markers with high PIC values were considered more informative [21]. Overall, the IRAP marker results for bitter gourd align closely with findings from various plant species, demonstrating the effectiveness of IRAP markers in assessing genetic diversity.

The use of retrotransposon-based molecular markers, such as IRAP and REMAP, has proven to be a powerful approach for assessing the genetic diversity of various plant species, as demonstrated in the current study on *M. charantia* (bitter gourd). The high levels of genetic diversity observed within and between the bitter gourd accessions are consistent with the known characteristics of retrotransposons in plant genomes. Plant genomes are known to harbor a diverse and abundant array of retrotransposon families, which can contribute significantly to intraspecific genetic variation

[33, 34]. The random integration and variable copy number of retrotransposons across the genome facilitate their utility as informative molecular markers, as evidenced by the high number of polymorphic loci generated in this study using the IRAP and REMAP techniques [34].

The observed higher genetic diversity within populations compared to between populations aligns with the dynamic and transposable nature of retrotransposons. The ongoing activity and mobilization of retrotransposon elements can lead to the accumulation of unique insertions within individual genotypes, contributing to elevated intrapopulation diversity [14, 35, 36]. This finding suggests that the bitter gourd accessions may have experienced differential retrotransposon activity and proliferation, leading to the observed genetic heterogeneity within the populations.

Furthermore, the widespread distribution of retrotransposons in euchromatic regions and around genes [37, 38] increases the likelihood of detecting markers linked to important agronomic traits. This has important implications for the utilization of these markers in marker-assisted breeding and the identification of genomic regions harboring genes of interest in bitter gourd [14, 36]. The distinct clustering of the Kanarkee and Durga Indian baby accessions, as revealed by the combined REMAP-IRAP analysis, suggests potential genetic divergence among these genotypes. This information could be valuable for selecting diverse parental lines in breeding programs aimed at broadening the genetic base and improving the agronomic performance of bitter gourd cultivars [14, 36].

In recent years, there has been a growing interest in the genetic improvement and cultivation of *M. charantia*, driven by its nutritional and medicinal properties [39, 40]. Researchers have explored various aspects of bitter gourd, including the identification of novel germplasm resources, the development of improved cultivars, and the understanding of the genetic basis of important traits [5, 20, 41]. These advancements in bitter gourd research have the potential to enhance the crop's productivity and utilization in various applications.

CONCLUSION

In conclusion, this study highlights the effectiveness of retrotransposon-based markers in revealing the genetic diversity of *M. charantia*. The findings can aid in the conservation, management, and genetic enhancement of this economically and medicinally significant crop. Additionally, an ongoing exploration of retrotransposon diversity and its applications in plant breeding and genomics, alongside recent advancements in bitter gourd research, offers promising opportunities to deepen our understanding of this valuable crop and support the development of improved varieties.

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