Australian Journal of Crop Science AJCS
AJCS 6(2):261-267 (2012) ISSN:1835-2707

Morphological and molecular analyses define the genetic diversity of Asian bitter gourd (*Momordica charantia* L.)

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Abstract

Bitter gourd (*Momordica charantia* L.) is a nutritious vegetable crop of Asian origin. Fifty indigenous and exotic bitter gourd genotypes were characterized using 12 quantitatively inherited traits and genotypic variation was analysed using RAPD and ISSR markers. Based on the morphological traits, the first female flower appeared at lowest node (9th node) in gynoecious genotypes DBGy201 and DBGy202. Sel-2 possessed the highest yield per hectare (10,866 kg). Seventeen RAPD markers produced 84 amplicons in 50 accessions, of which 33 (41.34 %) were found polymorphic. Joint comparisons among the 50 accessions using Jaccard's similarity coefficient indicated that genetic distances (GD) ranged from 0.03 to 0.28. Eleven ISSR primers provided a total of 58 amplicons of which 41 (70.0 %) were polymorphic in 50 accessions. The Jaccard's similarity coefficient matrix values ranged from 0.50 to 0.95 based on molecular analysis. The polymorphic information content (PIC), resolving power (RP) and marker index (MI) were 0.17, 1.14 and 0.82, respectively for RAPD markers, whereas ISSR markers showed comparatively high polymorphic information content (0.40), resolving power (1.87), and marker index (2.11). The combined data analysis of RAPD and ISSR markers indicated that the relative polymorphism among accessions was 52.6 % with 2.64 polymorphic amplicons per primer. The value of average polymorphic information content, resolving power and marker index were 0.26, 1.42, and 1.33, respectively. These data demonstrate a large genetic variability among the Asian bitter gourd genotypes examined, which indicates that they should be considered as a valuable gene pool for bitter gourd breeding programs.

Keywords: *Momordica charantia*, molecular markers, morphological markers.
Abbreviations: CTAB: Cetyl Trimethyl Ammonium Bromide, PIC: Polymorphic Information Content, RP: Resolving Power, MI: Marker Index, RAPD: Random Amplified Polymorphic DNA, ISSR: Inter Simple Sequence Repeat

Introduction

Bitter gourd (*Momordica charantia* L.; 2n= 2x=22), which belongs to family Cucurbitaceae, is an important vegetable mainly valued for its nutritional and medicinal properties. The origin of this crop is probably India with secondary centre of diversity in China (Grubben, 1977). It is widely cultivated in India, China, Malaysia, Africa, and South America (Minraj et al., 1993; Singh, 1990). The total production of pumpkin, squashes and gourds in the world is 22.14 million tons from an area of 16.7 lakhs hectare and India produces about 4.11 million tons from an area of 4.6 lakhs hectares (FAOSTAT, 2010). Bitter gourd has been used for centuries in the ancient traditional medicine of India, China, Africa, and Latin America. Bitter gourd fruits also possess anti-oxidant, anti-microbial, anti-viral, anti-diabetic activities (Welihinda et al., 1986; Raman and Lau, 1996). Based on historical literature (Miniraj et al., 1993; Chakravarty, 1990; Walters and Decker-Walters, 1988), and recent RAPD (Dey et al., 2006), ISSR (Singh et al., 2007) and AFLP (Gaikwad et al., 2008) molecular analyses, eastern India may be considered as a probable primary center of diversity of bitter gourd. The diverse morphological characters (i.e., sex expression, growth habit, maturity, and fruit shape, size, colour and surface texture; Robinson and Decker-Walters, 1997; Behera et al., 2007) of *M. charantia* in India provide for its relatively broad phenotypic species variation. Assessment of genetic diversity based on phenotype has limitations, since most of the morphological characters of economic importance are dramatically influenced by environmental factors and plant developmental stage. In contrast, molecular markers based on DNA sequence polymorphisms are independent of environmental conditions and show a higher level of polymorphism. Morphological markers reflect variation of expressed regions of genome while molecular markers indicate variation of all genome including expressed and non-expressed regions. Molecular markers provide a quick and reliable method for estimating genetic relationships among plant genotypes (Thormann et al., 1994). Among the different types of molecular markers, randomly amplified polymorphic DNAs (RAPDs) are useful for the assessment of genetic diversity (Williams et al., 1990) owing to their simplicity, speed, and relatively low-cost (Rafalski and Tingey, 1993) when compared to other types of molecular markers. ISSR markers have also proven useful for detecting genetic polymorphisms among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the

genome. Also, the utility costs of ISSRs are lower that than of RAPD (i.e., differences in identifying reproducible polymorphisms) (Yang et al., 1996). Our previous works on genetic diversity assessment were based on the local land races and varieties {RAPD (Dey et al., 2006), ISSR (Singh et al., 2007) and AFLP (Gaikwad et al., 2008)}. The current study was designed to assess the genetic diversity among 50 diverse bitter gourd accessions of Asia using morphological and molecular markers (RAPD and ISSR) to 1) discriminate among accessions of diverse origin; 2) characterize genetic relationships among and between accessions, and; 3) to determine the relative efficacy of these marker systems for population analysis.

Results

Variation in morphological characteristics among the genotypes

There were significant differences among the genotypes for the traits examined. The mean performance, range, standard error of difference (SE) and Least Significant Difference (LSD) values are presented in Table 2 (Supplementary). A wide range of variation was observed for all characters except leaf L: D ratio and seed L: D ratio, in which the variation was comparatively narrow. The widest range was recorded for yield per hectare (3629.55- 10866.89 kg) followed by vine length, fruit weight, number of fruits per plant, number of seeds per fruit, 100 seed weight, node bearing first female flower, fruit L: D ratio, internode length, and ovary length (Supplementary Table 2). The wide variation in these yield related traits will be useful for selecting best genotypes with high yield.

Genetic variability on the basis of RAPD analysis

Out of 84 reproducible amplicons generated by 17 RAPD primers, 33 were polymorphic. The size of the amplified products varied from approximately 200 bp to 1700 bp. The number of amplicons per primer ranged from 3 (OPC-16, OPW-8, OPF-12 and OPW-18) to 7 (OPW-20 and OPD-15), with an average of 4.9 amplicons per primer. The average number of polymorphic amplicons per primer was 1.9. The percentage of polymorphism ranged from 14.28% (OPW-20) to 66.66 % (OPC-16 and OPW-8) with an average of 41.34 %. The maximum number of polymorphic amplicons (3) was obtained with the primers OPW-7, OPW-6, OPX-1, and OPW-19 with an average of 1.9 per primer. The average polymorphic information content (PIC) value was 0.17 and ranged from 0.06 (OPD-15 and OPE-19) to 0.26 (OPX-1). The resolving power ranged from 0.36 (OPE-19) to 2.16 (OPW-13) with average of 1.14. The marker index ranged from 0.30 (OPE-19) to 1.42 (OPW-13) with an average of 0.82. The Jaccard's similarity coefficients ranged from 0.72 to 0.97, with an average of 0.86 (Supplementary Table 3).

Genetic variability based on ISSR analysis

Out of 58 reproducible amplicons generated by 11 ISSR primers, 41 were polymorphic. The size of the amplified products varied from approximately 200 bp to 1550 bp. The number of amplicons per primer ranged from 4 (UBC-861and UBC-888) to 8 (UBC-848), with an average of 5.3 amplicons per primer. The average number of polymorphic amplicons per primer was 3.7. The percentage of polymorphism ranged from 40% (UBC-825) to 83% (UBC-854), with an average of 70%. The maximum number of polymorphic amplicons (6) was obtained with the primers UBC-848 with an average of 3.7 per primer. The average polymorphic information content (PIC) value was 0.40 and ranged from 0.19 (UBC-854) to 0.58 (UBC-855). The primers, UBC-890, UBC-880, UBC-841 and UBC-856 have the higher PIC values. The resolving power ranged from 0.36 (UBC-855) to 4.60 (UBC-848) with average of 1.87. The marker index ranged from 1.11 (UBC-854) to 3.29 (UBC-841), with an average of 2.11. The other primers recorded higher marker index values were UBC-848, UBC-855, UBC-890, UBC-880 and UBC-856. The Jaccard's similarity coefficients ranged from 0.50 to 0.95, with an average of 0.71(Supplementary Table 3).

Genetic variability based on RAPD plus ISSR analysis

Of the 142 reproducible amplicons generated by 28 RAPD and ISSR primers in combination, 74 were polymorphic. The average amplicons per primer was 5 of which 2.7 were polymorphic. The average percentage of polymorphism was 52.6%. The average value of polymorphic information content (PIC), resolving power and marker index were 0.26, 1.42 and 1.33, respectively(Supplementary Table 3). The Jaccard's similarity coefficients ranged from 0.65 (DBG-35 vs DBGy201) to 0.97 (Pusa Do Mausami vs Pusa Vishesh) with an average of 0.80 (data not presented).

Cluster analysis based on quantitative traits

Cluster analysis of 50 genotypes based on 12 quantitative traits was performed by UPGMA method and a dendrogram was constructed as depicted in Figure 1. It was observed that all the genotypes were resolved into three major clusters. In cluster III, the genotype Sel-2 was quite diverse from rest of the 8 indigenous lines genotypes whereas in the cluster-II, 4 exotic lines were grouped together with one indigenous line. It was revealed from the dendrogram that the gynoecious line DBGy-201 is very close to a commercial variety, Pusa Do Mausami. The parental lines from these distant clusters may be used in hybridization programmes to produce populations with wide variability with transgressive segregants possessing high heterotic effects. These data suggest that the entries included in Cluster III possess a close resemblance in character expression of 9 indigenous materials whereas genotypes in Cluster II possess a close resemblance in character expression of 4 exotic materials and one land race DBG-49.

Cluster analysis based on RAPD analysis

The phylogenetic tree of the relationship among the 50 bitter gourd genotypes using 17 RAPD primers is shown in Figure 2. All genotypes were grouped in seven clusters at Node I (similarity coefficient= 0.78), Node II (similarity coefficient= 0.82), and Node III (similarity coefficient=0.85). The maximum number of genotypes (35) was present in cluster I, the gynoecious line (DBGy202) was a outlier. Although Cluster I contained mixed genotypes, it could be further sub-divided into four sub-clusters, sub-cluster Ia comprised of indigenous genotypes, sub-cluster Ib contained exotic genotypes, sub-cluster Ic contained indigenous germplasm and sub-cluster Id composed of exotic lines. The sub-clustering pattern was in consonance with the geographical distribution and within the cluster exotic lines grouped separately from the indigenous lines. Cluster II had commercial varieties and land races mostly cultivated in India where as Cluster III contained exotic lines. The association amongst different genotypes is presented in the

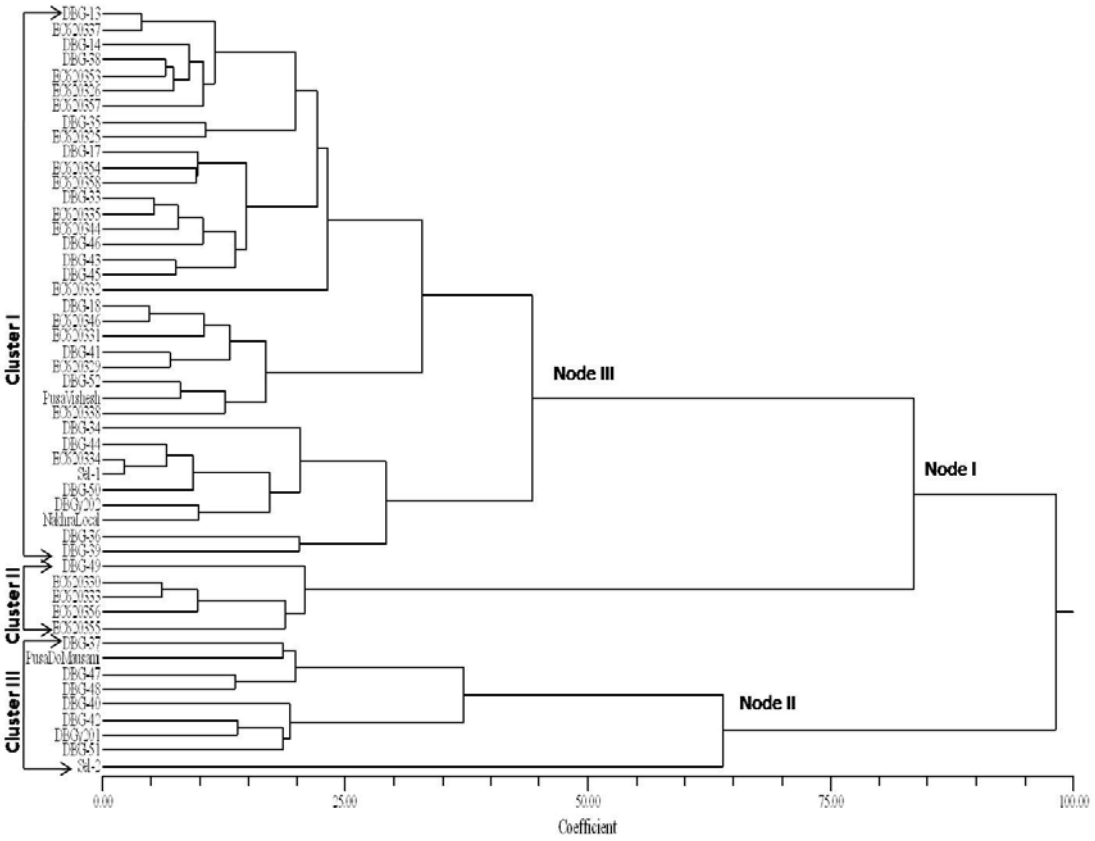

Fig 1. Genetic relationship among 50 genotypes of bitter gourd based on 12 quantitative traits by using UPGMA cluster analysis of the distance matrix.

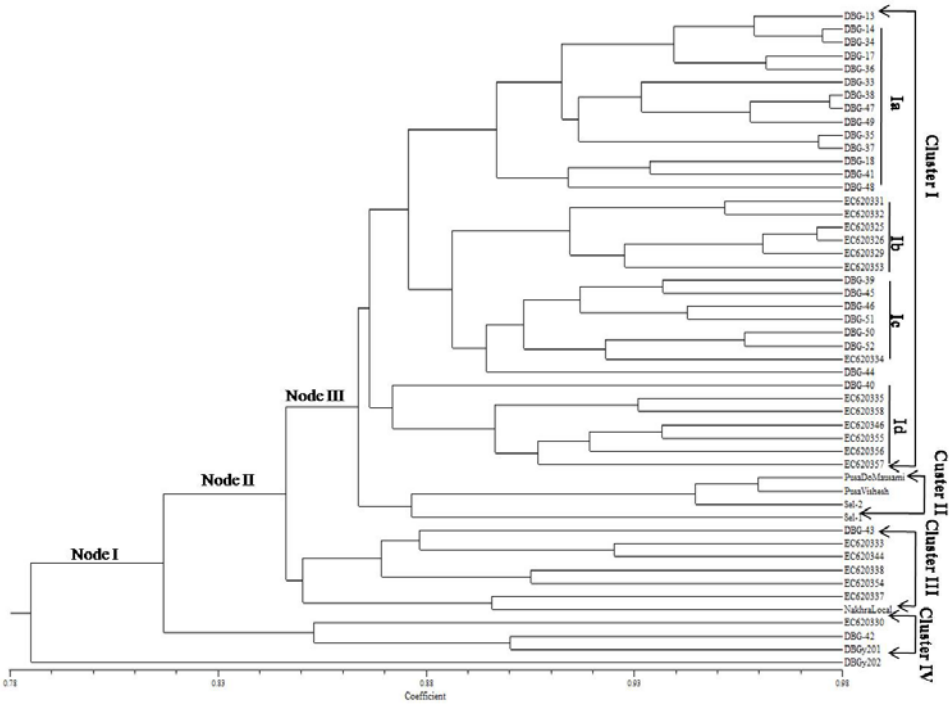

Fig 2. Consensus cluster analysis of the 50 genotypes of bitter gourd using RAPD analysis data from a Jaccard's similarity matrix and the UPGMA method.

form of dendrogram prepared using rescaled distances (Fig. 2). The resemblance coefficient between the two genotypes is the value at which their branches were joined. The dendrogram also showed the relative magnitude of resemblance among different clusters.

Cluster analysis based on ISSR analysis

The dendrogram generated from the Jaccard's similarity values using NTSYS software based on 11 ISSR primers is presented in Figure 3. The genotypes were grouped in three clusters at Node I (similarity coefficient=0.66) and Node II (similarity coefficient=0.68). The dendrogram also revealed the relative magnitude of resemblance among different clusters. All the indigenous genotypes were grouped in one cluster (Cluster I), except the commercial cultivars and advanced breeding lines like Pusa Do Mausami, Pusa Vishesh, Sel-1, Sel-2, Nakhra Local, DGBy 201, and DGBy 202.

Cluster analysis based on combined RAPD and ISSR analysis

The dendrogram generated by combined 17 RAPD and 11 ISSR primers analysis is presented in Figure 4. The combined analysis produced similar dendrogram to that produced using RAPD marker analysis. The genotypes were grouped in four clusters at Node I (similarity coefficient=0.75), Node II (similarity coefficient=0.77), and Node III (similarity coefficient=0.80). The maximum number of genotypes (23) were present in cluster I and all were indigenous genotypes. Although Cluster II contained mixed genotypes, it could be further sub-divided into four sub-clusters, sub-cluster IIa comprised of exotic genotypes, sub-cluster IIb contained only commercial varieties, sub-clusters IIc and IId contained exotic lines. The sub-clustering pattern was in consonance with the geographical distribution and within the cluster II exotic lines grouped separately from the indigenous lines. Cluster III contained exotic lines where as Cluster IV comprised of two gynoecious lines (DBGy201 and DBGy202).

Discussion

Based on field evaluation over two years, wide range of phenotypic variation exists for all the traits in the genotypes examined, except leaf L: D ratio and seed L: D ratio that showed narrow range of variation. Similar results were obtained by Dey et al. (2007) in bitter gourd. The earliness in bitter gourd and cucumber is judged through appearance of female flower at lower node and days required for first picking. It favors early fruit harvest. Two gynoecious accessions DBGy201 and DBGy202 bore a first female flower at lowest node number ($\approx 9^{th}$ node). Early appearance of female flower in gynoecious lines was also observed in the earlier experiments (Dey et al., 2006). Line Sel-2 was observed to be the highest yielding genotype. Moreover, it had longest ovary and as expected the maximum fruit L: D ratio was also recorded in this genotype. The highest fruit weight and lowest number of fruits per plant was recorded in genotype EC620332. In the present study, the average percentage of polymorphism obtained by RAPD markers was 41.34 % which was higher than 36.5% in bitter gourd lines (Dey et al. 2006). However, percent polymorphism of 70 as detected by ISSR markers was similar to that obtained by Dey et al. (2006) in bitter gourd (74.7%). The average number of polymorphic amplicons per RAPD primer was 1.9 and 3.7 in ISSR primers. However, Behera et al. (2008b) obtained higher number of average polymorphic amplicons per primer, 2.6 amplicons per RAPD primer and 6.3 amplicons per ISSR primer. It is concluded in this study that ISSR markers were more effective (i.e., more banding morphotypes identified) than RAPD analysis for genotyping the accessions examined. Similar results were obtained when RAPD and ISSR marker analysis was applied to relationship analyses in bitter gourd (Behera et al., 2008a). These studies and the data presented herein indicate that the bitter gourd accessions analysed are genotypically diverse, since they were drawn from distinctly different ecosystems and are morphologically dissimilar (Behera et al., 2008a). The relative efficiency of marker types for genetic analysis varies among crop species. The greater discriminatory power of ISSR markers when compared to RAPD markers may be due to comparatively higher values of average polymorphic information content (PIC; 0.40 versus 0.17), resolving power (RP; 1.87 versus 1.14) and marker index (MI; 2.11 versus 0.82) as well as the diverse nature of the genotypes. In bitter gourd, greater genetic diversity has been detected by AFLPs when compared with RAPD and ISSR analyses using indigenous germplasm array [RAPD-36.5% polymorphism; Dey et al. (2006) and ISSR-74.5% polymorphism; Singh et al. (2007)]. In the present study, the average number of polymorphic bands was 1.9 per RAPD primer and 3.7 per ISSR primer. The polymorphism per primer in RAPD (2.6) and ISSR (6.3) analyses was similar to that found by Dey et al. (2006) and Singh et al. (2007) respectively, in separate studies of Indian germplasm. The increased level of discrimination witnessed herein was likely due in part to the number and comparatively high discriminatory power of ISSR markers employed, as well as the diverse nature of the germplasm examined. The wide range of Jaccard's similarity coefficient values (0.72 to 0.97) in RAPD markers, (0.50 to 0.95) in ISSR markers and (0.65 to 0.97) in combined data analysis suggests that the germplasm collection represents a genetically diverse population. These results are in conformity with our earlier results and similar range of Jaccard's similarity coefficient (0.57 - 0.93) in RAPD, (0.48 - 0.91) in ISSR and (0.57-0.87) in bitter gourd accessions (Behera et al., 2008b). The dendrogram and genetic similarity matrix produced from both RAPD and ISSR data were compared and revealed similar but not identical phylogenetic relationships (Figures 2 and 3). The result obtained from these two marker systems was highly correlated. Similar magnitudes of correlation coefficients were also found among AFLP, ISSR and RAPD markers by Behera et al. (2008a) in bitter gourd. When compared to other arbitrary primers like RAPDs, ISSRs offer enormous potential for resolving intra- and intergenomic relationships (Zietkiewicz et al., 194). The clustering based on morphological characters does not match that of groupings derived through molecular analysis, except clustering of two gynoecious genotypes (Figures 1 and 4). The main reason of mismatch between clustering based on molecular markers and quantitative traits may be that most of the quantitative traits are controlled by a large number of genes (polygenes) and these traits are highly influenced by environment. Besides, markers like RAPD are randomly distributed throughout the genome and in majority of cases most regions of the genome (nearly 90%) are not expressed at phenotypic level (Dey et al., 2006). So, it is very difficult to find out similarity between groupings based on molecular and quantitative traits. The non-coding regions (un-expressed) which constitute the major portion of genome (nearly 90%) is not accessible to phenotypic expression and as marker system like RAPDs which randomly assay the

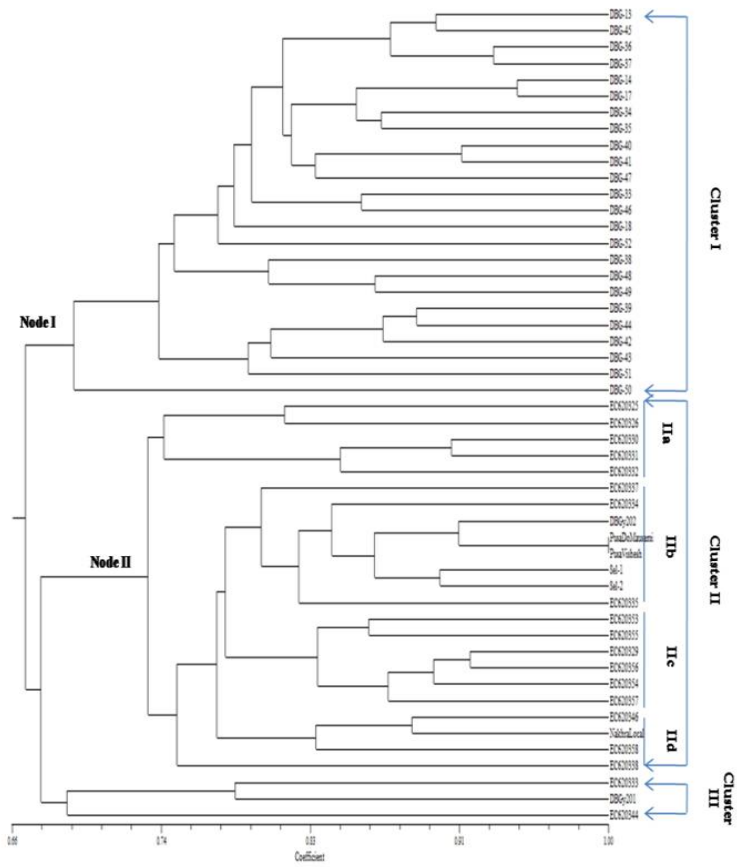

Fig 3. Consensus cluster analysis of the 50 genotypes of bitter gourd using ISSR analysis data from a Jaccard's similarity matrix and the UPGMA method

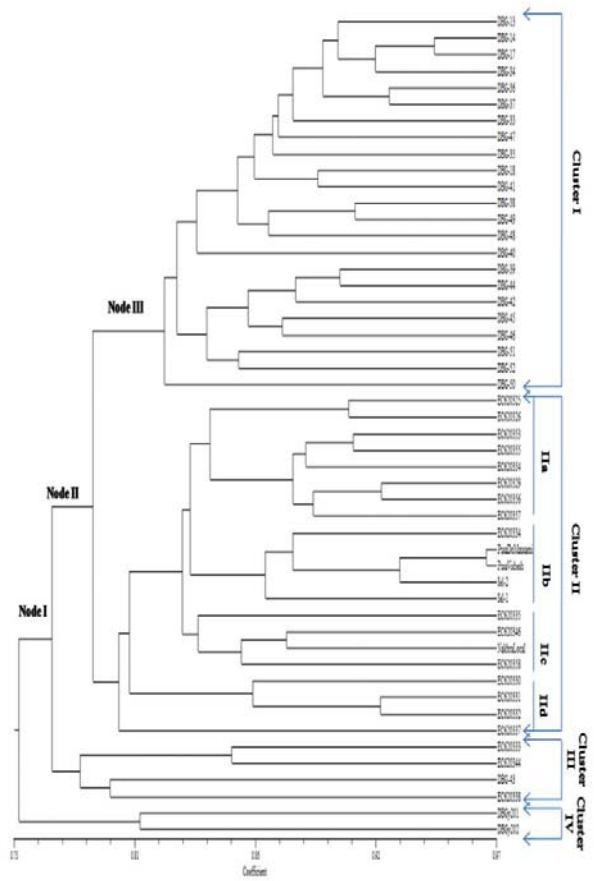

Fig 4. Consensus cluster analysis of the 50 genotypes of bitter gourd using RAPD and ISSR analysis data from a Jaccard's similarity matrix and the UPGMA method

genome result in disagreement between the phenotypic and molecular diversity. The quantitative traits which were selected to evaluate the genetic diversity might not explain the genetic variation completely; there could be other traits physiologically and biochemically more important which might explain molecular genetic diversity more precisely (Dey et al., 2006). The exotic collections grouped in cluster IV recorded highest fruit weight (cluster mean-88g) but lowest fruit number (cluster mean-8.6) per plant (data not presented). Hence, by increasing the number of fruits in these lines through recurrent selection, yield might be increased. The small-fruited genotypes examined herein were grouped together those recorded highest number of fruits (cluster mean-40) per plant (data not presented). When RAPD, ISSR, and RAPD + ISSR derived dendrograms were compared, the discrimination among genotypes within these clusters was more effective with the combined analysis (Figures 2, 3 and 4). Most of the accessions studied in the present experiment differed based on their geographic origin. For instance, genotypes DBG-13, DBG-14, DBG-17, DBG-34, DBG-36, DBG-37, DBG-33, DBG-47, DBG-35, DBG-18, DBG-41, DBG-38, DBG-49, DBG-48, DBG-40, DBG-39, DBG-44, DBG-42, DBG-45, DBG-46, DBG-51, DBG-52, and DBG-50 originating from West Bengal and Orissa (i.e., East India) were genetically distinct from the other bitter gourd accessions examined. Given that Eastern India has been proposed as one of the centers for domestication of bitter gourd (Behera et al., 2008a; Yang and Walters, 1992), these accessions are of considerable interest for genetic studies and plant improvement. Similarly the exotic lines originated from Thailand and P.R.China such as EC620325, EC620326, EC620355, EC620354, EC620356 and EC620357 were also grouped in a single sub cluster IIa (Figure 4). The Indian cultivars and promising lines (Pusa Do Mausami, 'Pusa Vishesh, Sel-2 and Sel-1) were remained in one sub-cluster IIb (Figure 3) based on combined analysis of RAPD and ISSR markers. Using the combined analysis of RAPD and ISSR loci used in our study, it was observed that monoecious bitter gourd is genetically distant to gynoecious germplasm (DBGy 201 and DBGy 202) (Figure 4). These two horticultural promising gynoecious lines (DBGy-201 and DBGy-202) also could be considered as potential parents for use in map construction (Behera et al., 2008b). Based on morphological analysis, these two lines (DBGy201 and DBGy202) were also grouped in separate cluster (Cluster IV; Figure 1). These lines are considered early genotypes with short vine length (≈143 cm) and yield was at par with Pusa Do Mausami, a commercial cultivar of India (Supplementary Table 2). As we know, in most living beings, only 5% or lower of genome genes are expressed and most of the genome is silent. Therefore, it is desirable to choose extreme parent according to molecular markers besides morphological markers. ISSR and RAPD markers strengthened the evaluation of Asian bitter gourd genotypes which might be a rich source of genetic diversity for plant improvement. The genetic variation among these genotypes was found to be

relatively high. The genetic variation among modern cultivars has been decreasing most probably due to monoculture practices (Behera et al., 2010). The assessment of genetic variation among the bitter gourd genotypes provided herein defines a marker array (combined ISSR and RAPD) for improved germplasm curation, allows for the development of a standard accession reference array for further genetic analyses, and provides for the selection of potential parents for genome mapping and breeding (Behera et al., 2008b). The establishment of a discriminatory marker array is the first step to broader assessments of bitter gourd germplasm for their genetic characterization, and the eventual development of a core collection in this species (Lo´pez-Sese´ et al., 2003; Staub et al., 2002).

Material and methods

Genotypes

The experimental materials comprised of 50 indigenous and exotic accessions of bitter gourd (Supplementary Table 1). The indigenous Indian genotypes were maintained at Division of Vegetable Science, Indian Agricultural Research Institute (IARI), New Delhi (India), whereas the exotic lines were obtained from Asian Vegetable Research and Development (AVRDC)-World Vegetable Centre, Taiwan and maintained at IARI, New Delhi (India).

Field evaluation and data collection

The experiment was laid out in a randomized complete block design (RCBD) with three replications for phenotypic evaluation at the Research Farm, Division of Vegetable Science (India), during spring–summer (dry) seasons of 2009 and 2010. The field was irrigated and the recommended dose of fertilizer and appropriate agronomic practices were performed to raise a successful crop during the spring-summer seasons of both years. Seeds were sown on both sides of the channel with a spacing of 2 m between channels and 60 cm between hills. The fruits were harvested at marketable stage. Five plants were selected for the measurements after discarding the border plants at both ends. These plants were examined for 12 traits including: leaf length and width (L: D) ratio, internode length, node bearing first female flower, ovary length, fruit L: D ratio, fruit weight, number of fruits per plant, yield per hectare, vine length, seed L: D ratio, number of seeds per fruit, and 100 seed weight. Means across three replications were calculated for each character.

DNA extraction and PCR amplification

The total genomic DNA from young leaves was extracted by CTAB (Cetyl Trimethyl Ammonium Bromide) method given by Murray and Thomson (1980) and Saghai- Maroof et al. (1984). DNA quantification and quality analysis was performed using an agarose gel electrophoresis (0.8 % agarose) and banding morphotypes were compared with uncut lambda DNA of known quantity (100 ng) as a standard for comparative analyses. The genomic DNA (25ng/µl-final concentration) was subjected to PCR amplification using 27 RAPD and 15 ISSR primers (15-23 decameric oligonucleotides) selected based on previous study on *Momordica charantia* accessions (Behera et al., 2008a).

A total of 27 RAPD and 15 ISSR primers were used to amplify two genotypes of bitter gourd in order to optimize PCR using different annealing temperature, template DNA and primer concentration. Seventeen RAPD and 11 ISSR primers produced reproducible, bright, clear bands (amplification products) and were selected in the present study for genetic diversity analysis among the bitter gourd genotypes. Various gel concentrations (2 to 2.5%) were also optimized depending on the size of amplified products for their better resolution.

Data analysis

The data were subjected to analysis of variance according to Panse and Sukhatme (1967). The genetic diversity was analyzed by Mahalanobis D^2 analysis and the genotypes were grouped into various cluster groupings following Torcher's method. NTSYS-pc Version 2.02 (Numerical Taxonomic System) software was used to calculate Jaccard's similarity coefficients between genotypes. The resulting similarity matrix was subjected to the cluster analysis using the Unweighted Paired Group method using Arithmetic Averages (UPGMA) approach and dendrograms were generated based on genotypic differences. The Polymorphic information content (PIC) was calculated for each primer as: PIC = 1-$\sum pi^2$, where pi is the frequency of the ith alleles or PIC estimated by using the formula PIC = 2 F (1-F), where F is the frequency of band present (Lynch and Walsh, 1998). The resolving power (*R*p) of a primer was calculated as $Rp = \sum I_b$, where I_b describes relative band informativeness and takes the value 1-{2 (0.5-p)}, with p being the proportion of the 50 genotypes containing the band (Prevost and Wilkinson, 1999). The marker index (MI) as proposed by Powell et al. (1996) and used by Milbourne et al. (1997) was also calculated. MI is the product between diversity indexes (equivalent to PIC). This parameter was calculated for each primer.

Acknowledgment

I am thankful to Dr Jack E. Staub, USDA-ARS, Forage & Range Research Laboratory, Utah State University, Logan, USA for his kind help in checking the language of this manuscript.

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