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BRIEF COMMUNICATION

Genetic diversity in important members of *Cucurbitaceae* using isozyme, RAPD and ISSR markers

B. SIKDAR[1], M. BHATTACHARYA[2], A. MUKHERJEE[3], A. BANERJEE[2], E. GHOSH[2], B. GHOSH[2] and S.C. ROY[2]*

Department of Genetic Engineering and Biotechnology, University of Rajsahi, Bangladesh[1]
Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata-700019, West Bengal, India[2]
Department of Botany, Dinabandhu Mahavidyalaya, Bongaon-743235, West Bengal, India[3]

Abstract

Biochemical and molecular markers have been used on eleven species of *Cucurbitaceae* collected from lower Gangetic plains. Six enzyme systems were selected. Among 40 primers examined, 14 random amplified polymorphic DNA (RAPD) and 10 inter-simple sequence repeat (ISSR) primers were selected for the analysis. Generated RAPD (100) and ISSR (100) fragments showed high variations among the species. Jaccard similarity coefficients were used for the evaluation of pairwise genetic divergence; cluster analysis of the similarity matrices was performed to estimate interspecific diversity. Further, principal coordinate analysis was performed to evaluate the resolving power of the three marker systems to differenciate among the species.

Additional key words: cluster analysis, Jaccard similarity coefficient, molecular marker, primer.

Cucurbitaceae family contains about 90 genera and over 700 species of economic importance. The family is distinct morphologically and biochemically from other families and is therefore considered monophyletic. As the plants of this family produce unisexual flowers, cross-pollination is a regular feature. Few studies have been reported on the phylogeny of *Cucurbitaceae* (Helm and Hemleben 1997, Sanjur *et al*. 2002, Levi *et al*. 2005). The evaluation of genetic diversity and phylogenetic relationships among the members would promote the efficient use of genetic variations in the breeding programme (Paterson *et al.* 1991). DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann 1983) Information on genetic diversity and relationships within and among crop species and their wild relatives is essential for the efficient utilization of plant genetic resource collections. Isozyme profiles were used as co-dominant markers for phylogenetic analysis in plants. It has been used for this purpose in several plants (Booy and Van Raamsdonk 1998, Aradhya *et al*. 1998) due to its reproducibility. With the advent of polymerase chain reaction (PCR) technique, two quick and simple methods called random amplified polymorphic DNA, RAPD (Williams *et al*. 1990) and inter-simple sequence repeat, ISSR (Zietkiewicz *et al*. 1994) are now widely used for the study of phylogeny and genetic diversity. RAPD markers have been used for the identification of cultivars and for assessing genetic diversity among cultivars of several crops like bean (Skroch *et al*. 1992), pea (Hoey *et al*. 1996), soybean (Brown-Guedira *et al*. 2000), *Ceratotropis* (Kaga *et al*. 1996), mungbean (Santalla *et al*. 1998, Lakhanpaul *et al*. 2003), *etc*. ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Like RAPDs, ISSRs markers are also quick and easy to handle and have been successfully utilized for the phylogenetic analysis of *Oryza* (Joshi *et al*. 2000), genetic relationships in the genus *Vigna (*Ajibade *et al*. 2000) and cultivar identification in strawberry (Arnau *et al*. 2002). The potential supply of ISSR markers depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Depeiges *et al*. 1995).

The objectives of the present study are to determine the

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Abbreviations: ISSR - inter-simple sequence repeat; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA.
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* Corresponding author; e-mail: scroyind@yahoo.com

genetic diversity and phylogenetic relationships among some economically important members of *Cucurbitaceae* and to compare the resolving power of isozymes, RAPD and ISSR for their applicability in the phylogenetic studies.

Eleven species from eight genera of the family *Cucurbitaceae* were selected from the lower Gangetic plains of West Bengal. These are *Lagenaria siceraria*, *Cucurbita maxima*, *Cucumis sativus*, *Benincasa hispida*, *Luffa acutangula*, *Luffa cylindrica*, *Trichosanthes cucumerina*, *Trichosanthes dioica*, *Momordica charantia*, *Momordica dioica* and *Coccinia cordifolia*.

For isozyme analysis, young leaves were homogenized with double volumes of cold extraction buffer (0.1 M Tris-HCl pH 8.0, 1 mM EDTA, 0.5 % (m/v), polyvinylpyrrolidone, 2 % polyvinylpolypyrrolidone, 2 mM dithiotreitol, 10 mM 2-mercaptoethanol). The samples were then centrifuged at 10 000 *g* for 30 min at 4 °C. The amount of protein in the sample was estimated according to Lowry (1953) with bovine serum albumin as standard. About 30 µg of the total protein per sample was applied to a native discontinuous polyacrylamide gel (4 % stacking gel, 10 % resolving gel) and run in Tris-glycine buffer (pH 6.8) at 15 mA until the blue front reached the bottom of the gel. Acid phosphatase (ACP, E.C. 3.1.3.2), malate dehydrogenase (MDH, E.C. 1.1.1.37), superoxide dismutase (SOD E.C. 1.15.1.1), carbonic anhydrase (CAR, E.C. 4.2.1.1), esterase (EST E.C. 3.1.1.6) and peroxidase (POX E.C. 1.11.1.7) were examined in this study. The staining was done following Apavatjrut *et al*. (1999). Reproducibility of isozyme banding patterns was tested at least three times.

Plant genomic DNA was extracted by a CTAB protocol (Doyle and Doyle 1987) with some modifications. Leaf tissues (100 mg) were ground in 1 cm^3 of CTAB extraction buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA [pH 8.0], 0.2 % β-mercapto-ethanol, 2 % CTAB) and heated at 60 °C for 30 min. DNA was extracted with one volume of a chloroform: isoamyl alcohol mix (24:1) and precipitated in presence of isopropanol (40 %, final concentration). The DNA pellet was washed with 5 mM ammonium acetate and 70 % ethanol, dried, and dissolved in 0.1cm^3 of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After addition of 0.001 cm^3 of RNase (10 mg cm^{-3}), the concentration was measured with spectrophotometer and absorbance ratio $A_{260/280}$ was calculated. The dissolved DNA pellet was stored at -20°C until further use.

RAPD amplification was performed with random decamer primers obtained from *Operon Technologies* (Alameda, CA, USA). Amplifications were performed in a 0.025 cm^3 reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 20 pmol RAPD primers, 50 mg of genomic DNA, and 0.5 U Taq DNA polymerase (*Bangalore Genei*, Bangalore, India). Amplifications were performed in a *Perkin-Elmer GeneAmp 2400* PCR system. Amplification conditions were an initial denaturation at 94 ºC for 4 min and 45 cycles at 94 ºC for 1 min, 37 ºC for 1 min, 72 ºC for 2 min, followed by 5 min at 72 ºC. Amplified products were separated on 1.5 % agarose gel in 0.5× TBE buffer (100 mM Tris HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA) at 50V. The gels were stained with 0.5 µg cm^{-3} ethidium bromide solution and visualized under UV radiation. The sizes of the amplification products were determined by comparison of λDNA digested with EcoR1 and Hind Ш.

For ISSR amplification, some anchored microsatellite primers from University of British Columbia, Canada, and some primers designed in our laboratory (Ray and Roy 2007) were randomly selected and used. ISSR amplification were carried out in 0.025 cm^3 volume containing 50 ng template DNA, 0.5 U Taq DNA polymerase, 10 mM dNTP, 10 mM primer in 1× reaction buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$. Amplification was performed in *Perkin-Elmer Gene Amp 2400* PCR system. Amplification conditions were as follows: an initial denaturation at 94 ºC for 4 min followed by 40 amplification cycles each at 94 ºC for 1 min, 1 min in annealing temperature, 72 ºC for 2 min, followed by a final extension for 5 min at 72 ºC. Amplified products were separated on 1.5 % agarose gel in 0.5× TBE buffer at 50V. The gels were stained with ethidium bromide solution and visualized under UV radiation. The sizes of the amplification products were determined by comparison λDNA digested with EcoR1 and Hind Ш.

After visualizations of gel, data analyses were done for all the three marker types. For isozyme analysis, the relative mobility (Rm) of each band was calculated as the ratio of distance traveled by the band to the tracking dye. Bands were numbered on the basis of increasing Rm values. The taxa were identified based on presence or absence of specific bands. For RAPD and ISSR profiles, each amplified fragment was treated as a unit character regardless of its intensity and scored in terms of a binary code (presence - 1, absence - 0). Only clear and reproducible bands were considered for scoring. To analyze data obtained from the zymograms and DNA profiles, the *NTSYS-pc version 2.1* statistical package (Rohlf 2000) was used. Among the various similarity matrices, primarily Jaccard and Dice similarity coefficients were chosen since they do not attribute the coincidence of band absence.

The similarity matrices then were used to construct dendrograms using unweighted pair group method with arithmetic average (UPGMA) and sequential agglomerative hierarical nested (SAHN) cluster analysis. Cophenetic matrices were derived from the dendrograms using the COPH (cophenetic values) program, and the goodness-of-fit of the clustering to the two data matrices were calculated by comparing the original similarity matrices with the cophenetic value matrices using the Mantel matrix correspondence test (Mantel 1967) in the MXCOMP program. Principal co-ordinate analysis (PCOORDA) was performed based on the similarity coefficients using DCENTER module to transform the symmetric similarity matrix to scalar product form and then EIGEN module was used to extract eigenvectors

resulting into a PCOORDA. This is a multivariate approach which is more informative regarding distances among major groups (Hauser and Crovello 1982). This can complement the cluster analysis and identify patterns of association among taxa in a three dimensional space.

Zymograms obtained from six enzyme systems showed good resolution. Staining and banding patterns were reproducible for each enzyme system among all the taxa. Of the total 60 primers used, 12 RAPD and 8 ISSR primers gave reproducible results, which were further considered for data analysis. Total number of bands and fragment size ranges were calculated (Table 1). For both isozyme and DNA based markers, cophenetic correlations were higher in case of Jaccard coefficient than Dice coefficient. Thus, all the phylogenetic relationships will be discussed on the basis of the dendrograms obtained with Jaccard coefficient. Fig. 2 shows the dendrograms obtained from different marker types based on the similarity matrices calculated with Jaccard coefficient. Finally, a consensus tree based on the two DNA based markers was shown.

A total of 66 highly reproducible bands were obtained (12 from both malate dehydrogenase and SOD, 11 from peroxidase, 8 from acid phosphatase, 16 from carbonic anhydrase and 7 from esterase). Genetic similarities among all taxa ranged from 0.08 to 0.64, with a mean similarity of 0.36. For the isozyme based dendrogram, cophenetic correlation using Jaccard coefficient was estimated as $r = 0.917$, corresponding to a very good fit. UPGMA dendrogram shows four different subclusters (Fig. 2*A*). The first subcluster consists of *Lagenaria siceraria*, *Momordica dioica* and *Momordica charantia*. The second subcluster was made by *Cucurbita maxima*, *Trichosanthes dioica* and *Trichosanthes cucumerina*. The two species of *Luffa* made a separate clade and the fourth clade was made up of *Benincasa hispida* and *Coccinia cordifolia*. Species of *Momordica* and *Luffa* showed identical banding patterns and could not be differentiated by the cluster analysis. However, the three dimensional ordination could differentiate between the species of these two genera. The first three most informative PC components explained 53.73 of the total variation.

Out of the sixty random decamer primers, twelve produced informative data for the phylogenetic analysis. These 12 primers produced 228 RAPD fragments, an average of 19 bands per primer. Highest number of bands was produced by OPA 11 (28) and lowest was produced by OPD 02 (10 bands). Amplified product sizes ranged from 259 bp to 2644 bp (Table 1). Fig. 1*A* shows the RAPD profile obtained with the primer OPA 18.

Genetic similarities among all taxa ranged from 0.05 to 0.75, with a mean similarity of 0.40. For the RAPD based dendrogram, cophenetic correlation using Jaccard coefficient was estimated as $r = 0.925$, corresponding to a very good fit. The UPGMA dendrogram divided the taxa into 3 main groups (Fig. 2*B*). The first subcluster was formed by *Lagenaria siceraria*, *Momordica dioica*, *Momordica charantia* and *Cucumis sativus*. The second

Table 1. Description of RAPD (OPA, OPD) and ISSR (UBC, ISSRCR) fragments generated in all the species surveyed.

Primer	Sequence (5'-3')	Size [bp]	Total number
OPA 19	CAAACGTCGG	1061-2644	22
OPA 11	CAATCGCCGT	664-1870	28
OPA 12	TCGGCGATAG	617-1927	23
OPA 06	GGTCCCTGAC	776-1865	18
OPA 17	GACCGCTTGT	753-1603	16
OPA 07	GATGACCGCC	773-1732	27
OPA 18	GAACGGACTC	530-1831	23
OPB 11	GTAGACCCGT	774-1630	18
OPB 12	GGCACTGAGG	573-1375	17
OPD 01	GTCAGGGCAA	827-1559	14
OPD 02	GGTCGGAGAA	549-2067	10
OPD 04	GGTGAGGTCA	259-2497	12
UBC 825	$(AC)_8T$	826-2232	21
UBC 864	$(ATG)_6$	807-2399	14
UBC 816	$(CA)_6GG$	818-1411	10
UBC 846	$(CA)_8RT$	599-1797	18
UBC 865	$(CCG)_6$	801-3450	17
UBC 866	$(CTC)_6$	778-1968	24
ISSRCR-8	$(GTG)_5GC$	818-1873	13
ISSRCR-2	$(CA)_8AG$	744-1607	22

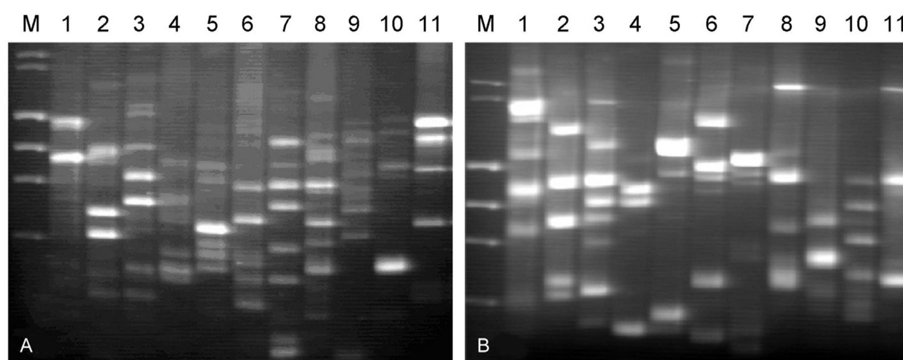

Fig. 1. DNA fingerprinting patterns of the 11 species of *Cucurbitaceae*. *A* - RAPD profile obtained with primer OPA 18. *B* - ISSR profile obtained with primer UBC 866. *Lane* M - molecular mass marker, *lanes* 1 - *Lagenaria siceraria,* 2 - *Cucurbita maxima,* 3 - *Cucumis sativus,* 4 - *Benincasa hispida,* 5 - *Luffa acutangula,* 6 - *Trichosanthes cucumerina,* 7 - *Luffa cylindrica,* 8 - *Trichosanthes dioica,* 9 - *Momordica dioica,*10 - *Coccinia cordifolia,* 11 - *Momordica charantia*.

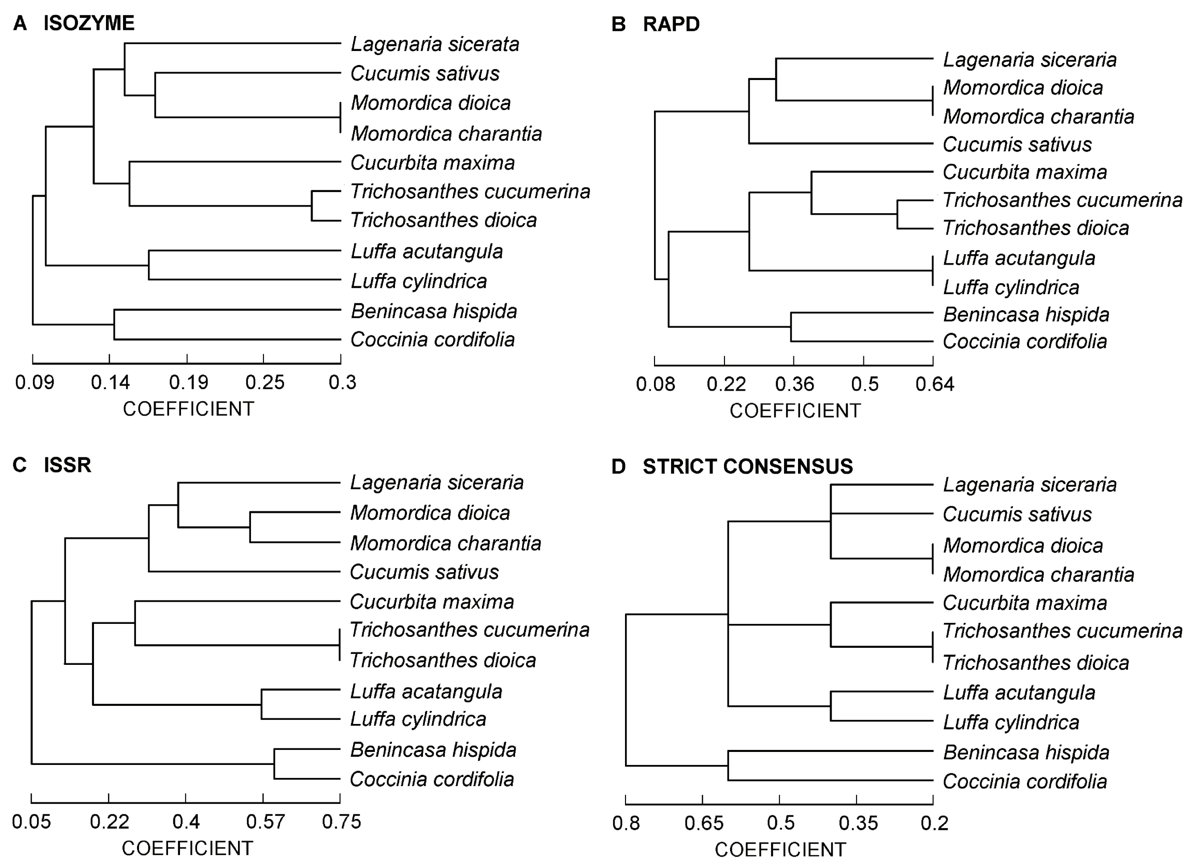

Fig. 2. Dendrograms generated using unweighted pair group method with arithmetic average (UPGMA) analysis, showing relationships between different members of *Cucurbitaceae*, using isozyme (*A*), RAPD (*B*), ISSR (*C*) data and a strict consensus tree based on both RAPD and ISSR data (*D*).

subcluster was formed by *Cucurbita maxima*, *Trichosanthes dioica*, *Trichosanthes cucumerina*, *Luffa acutangula* and *Luffa cylindrica*. The third subcluster was formed by *Benincasa hispida* and *Coccinia cordifolia*. At the interspecific level, a total of 17 (10 %) bands were shared between *Momordica charentia* and *M. dioca*, 10 (12 %) fragments between *Trichosanthes dioica* and *T. cucumerina* and 20 (10 %) between *Luffa reticulata* and *L. cylindrica.* The three dimensional ordination is compatible with the cluster analysis. The first three most informative PC components explained 56.25 of the total variation. Species of the genera *Trichosanthes*, *Luffa* and *Momordica* are closely spaced and are sharply separated from each other.

Out of the ten (ISSR) primers, eight produced informative data for the phylogenetic analysis. These 8 primers produced 139 ISSR fragments, an average of 17.37 bands per primer. Amplified product sizes ranged from 599 to 2399 bp (Table 1). Highest number of bands was produced by UBC 866 (24 bands) and lowest was produced by UBC 816 (10). Fig. 1*B* shows the banding pattern obtained with the primer UBC 866.

Genetic similarities among all taxa ranged from 0.09 to 0.30, with a mean similarity of 0.19. For the ISSR based dendrogram, cophenetic correlation using Jaccard coefficient was estimated as $r = 0.767$, corresponding to a moderate fit. The UPGMA dendrogram divided the taxa into 4 main groups (Fig. 2*C*). The first subcluster was formed by *Lagenaria siceraria*, *Cucumis sativus, Momordica dioica* and *M. charantia*. The second subcluster was formed by *Cucurbita maxima*, *Trichosanthes dioica* and *T. cucumerina*. *Luffa acutangula* and *L. cylindrica* formed the third subcluster. The fourth subcluster was formed by *Benincasa hispida* and *Coccinia cordifolia*. At the interspecific level, a total of 13 (36 %) bands were shared between *Momordica charentia* and *M. dioca*, 14 (29 %) fragments between *Trichosanthes dioica* and *T. cucumerina* and 4 (16 %) between *Luffa reticulata* and *L. cylindrica.* The three dimensional ordination is compatible with the cluster analysis. The first three most informative PC components explained 36.23 of the total variation. Species of the genera *Trichosanthes*, *Luffa* and *Momordica* are closely spaced and are sharply separated from each other.

A strict consensus tree based on the RAPD and ISSR data were constructed. This dendrogram shows more similarity to ISSR based dendrogram with some uniqueness (Fig. 2*D*). Jaccard similarity coefficient ranges from 0.2 to 0.80 with a mean similarity of 0.5. The dendrogram divided the taxa into 4 main groups. The first subcluster was formed by *Lagenaria siceraria*, *Cucumis sativus Momordica dioica* and *M. charantia*. The second

subcluster was formed by *Cucurbita maxima*, *Trichosanthes dioica* and *T. cucumerina*. *Luffa acutangula* and *L. cylindrica* formed the third subcluster. The fourth subcluster was formed by *Benincasa hispida* and *Coccinia cordifolia*. The matrices for RAPD and ISSR markers were also compared using Mantel's test (Mantel 1967) for matrix correspondence. The correlation between the matrices of cophenetic correlation values for the dendrogram based on RAPD and ISSR data was moderate (r = 0.58).

RAPD and ISSR studies have been widely used for population genetic studies in both wild (Khasa and Dancik 1996, Nebauer *et al*. 1999, Das *et al*. 2007, Dikshit *et al*. 2007, Yang *et al*. 2008, Yao *et al.* 2008) and cultivated plants (Souframanien and Gopalakrishna 2004). By contrast, previous reports on ISSR analysis mainly focused on cultivated species (Moreno *et al*. 1998, Blair *et al*. 1999). Isozyme analysis, however, has long been used in the phylogenetic studies of various plant species like *Trifolium* (Lange *et al.* 2000), *Curcuma* (Das and Mukherjee 1997), *etc*. Here, we have compared the discriminating power of the three marker systems: isozymes, RAPD and ISSR. Our results suggest that all three markers have the similar potential for phylogenetic relationships in eleven *Cucurbita* species. However, moderate amount of similarity exists among the three marker systems. In all the UPGMA based dendrograms, we obtained the species under the same genus clustered together. However, relationships among the different genera differ to some extent with the marker type. For RAPD and ISSR markers, a high reproducibility in dendrogram topologies was obtained, with a few differences. Also, both marker types showed a good power of discrimination between the taxa. However, isozymes could not distinguish between the species of *Momordica* and *Luffa*, whereas RAPD failed to distinguish between *Tricosanthes* species. ISSR, on the other hand, could not distinguish between *Momordica* species. This shows the importance of use of several marker systems like isozyme, RAPD and ISSR in the phylogenetic analysis. Genetic variation among cucurbits based on RAPD and ISSR analysis could be useful to select parents to be crossed for generating appropriate populations which may be useful for genome mapping and breeding purposes. The correlation between Jaccard's similarity coefficient values of RAPD and ISSR was moderate in magnitude. A possible explanation for this difference is that the two-marker techniques target different portions of the genome. The ability to resolve genetic variation among different genotype may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed. Nei (1978) reported that a relatively reliable estimate of average heterozygosity could also be obtained from a small number of individuals if a large number of loci are examined. The correlation between Jaccard's similarity values generated from the two different marker techniques was moderate (*r* = 0.58), and there was some consensus between the RAPD- and ISSR-based groupings of the 11 taxa. Clustering of taxa within groups was not exactly similar when RAPD- and ISSR-derived dendrograms were compared. These differences can be attributed to marker sampling error or the level of polymorphism detected, reinforcing the importance of the number of loci and their coverage of the overall genome to obtain reliable estimates of genetic relationships among taxa (Loarce *et al*. 1996). The putatively similar bands originating for RAPDs in different taxa may not be homologous, although they may be of same size in base pairs. When calculating genetic relationships, this may lead to erroneous results (Fernandez *et al*. 2002). The level of observed polymorphism is very high, and the ability of the ISSR technique to effectively distinguish species in the genus *Vigna* was reported by Ajibade *et al*. (2000). Our results indicate the presence of great genetic variability among cucurbit species and are useful in the assessment of cucurbit diversity, and the selection of a core collection to enhance the efficiency of germplasm management for use in cucurbit breeding and conservation.

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