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# Assessment of Genetic Relationships among and Within *cucurbita* Species Using RAPD and ISSR markers

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**Abstract:** Two PCR molecular marker techniques; random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) were employed to identify the polymorphisms and the relationships between 14 genotypes, which belong to three different *Cucurbita* species (*C. pepo*, *C. moschata* and *C. maxima*). In RAPD analysis, six random primers revealed a total of 463 fragments, in which 405 (87.5%) were polymorphic. Thirty-one out of 463 RAPD-PCR fragments were found to be useful as genotype-specific markers. The highest number of RAPD markers was scored for Cm5 genotype (5 markers), while there was no specific markers scored for the genotypes, Cp5 and Cp9, which belong to *C. pepo*. In ISSR analysis, seven ISSR primers gave a total of 263 ISSR amplified fragments, in which 243 (92.4%) were polylmorphic. As a comparison between the three species, 155(92.3%), 18 (100%) and 70 (90.9) out of 168, 18 and 77 reproducible fragments were polymorphic with *C. pepo*, *C. moshata* and *C. maxima*, respectively. Ten genotypes out of 14 were identified by a total of 21 unique markers with the seven ISSR primers, which identified individual genotype from each other. Genotype Cm5 was distinguished from other genotypes by the presence of one unique fragment of 750 bp for primer S1, while two ISSR primers (S3 and S6) identified the genotypes belonging to *C. pepo* from genotypes belonging to the other two species. The RAPD, ISSR data and their combination revealed that the highest similarity indices (83.3%, 82.2% and 79.5%) were observed between the Cp5 and Cp6, Cp7 and Cp9 and Cp8, Cp7 and Cp9 genotypes, respectively. The lowest similarity indices (52%, 52.1% and 54%) were observed between the Cp1 and Cp9, Cp5 and Cm5 and Cp1 and Cp9 genotypes, respectively. The consensus tree indicated that the nine genotypes of *C. pepo* were not clustered together, even though the origin was the same for all genotypes. This implied that the genomic sequences of the nine summer squash genotypes varied at the genetic level. The genotypes Cp5, Cp7, Cp9, Cm1 and Cm2 were the most genetically related and therefore, it could be used in the breeding programs. In conclusion, the information on polymorphism using RAPD and ISSR in a set of genotypes is useful in the assessment of genetic diversity and genetic relationships and could be useful in the breeding programs.

**Key words:** *Cucurbita* spp., molecular markers, RAPD, ISSR, phylogentic relationships

## INTRODUCTION

*Cucurbita* genus is a member of the economically important Cucurbitaceae family. It containes five domesticated species; *C. pepo* (Summer squash and Zucchini), which is the most common commercial species, *C. maxima* (Pumpkin), *C. moschata* (Butternut), *C. mixta* (Cushaw) and *C. ficifolia* (Landrace cultigen), all native to the American origin[23]. In Egypt, summer squash and pumpkin are familiar; whereas, butternut squash starts to appear in the market during the last decade. They are economically important vegetables, consumed as patient food, cooking, stuffing, boiled, dessert, seed entertainment and for decoration purposes (pumpkin and butternut fruits). For an effective breeding program, information concerning the extent and nature of genetic diversity within and between species is a prerequisite for any crop improvement. It is particularly useful for organizing germplasm, characterizing individual

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accessions and genotypes, identification of cultivars as well as a general guide in the selection of the parents for hybridization to maximize the expression of heterosis[20,15,17,16].

Morphological features are traditionally used to assess genetic variation in *Cucurbita* species. However, many cases are controlled by quantitative factors and/or affected by environmental modification[4]. In recent years molecular markers were considered as powerful tools in the assessment of genetic diversity within and between plant populations[12,20,19,10,8].

Among the different molecular markers, some are relatively cheaper, faster, reliable and simple to utilize in variety of applications. Two of such useful markers are random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR), which dependend on polymerase chain reaction (PCR). They can rapidly differentiate closely related individuals[26,6,16]. RAPD marker[21] is highly suitable for quick fingerprinting, analyzing genetic relationships, tagging traits for use in marker-assisted selection, and for the rapid construction of a genetic linkage map[18,10,16]. The method called inter simple sequence repeat (ISSR) is based on the amplification of DNA region located between two microsatellites locus. In practice, when the primer successfully locates two microsatellite regions within an amplifiable distance away on the DNA strands, the PCR reaction generates a band of a particular size for that locus.

The objectives of this study aimed to identify the polymorphisms and determine genetic relationships among and within three of the most common cultivated *Cucurbita* species (*Cucurbita pepo*, *Cucurbita moschata* and *Cucurbita maxima*), using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers.

## MATERIALS AND METHODS

### Materials:

**Plant Material:** Fourteen pure lines of cucurbits genotypes; representing the three common cultivated *Cucurbita* species; namely *C. pepo* (summer squash), *C. moschata* (butternut) and *C. maxima* (pumpkin) were selected for this investigation. All genotypes exhibit morphological variation in some characters, such as fruit shape, fruit size and fruit color (Table 1). Cucurbits plants were grown inside greenhouse and maintained during winter season of 2006 in the Experimental Station Farm (Abies region), Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

### Methods:

### Molecular Genetic Studies:

**Genomic DNA Extraction:** DNA was extracted from young leaves (three to four weeks old) of each of the genotype. Leaves were homogenized using liquid nitrogen and genomic DNA was extracted using the QTA gene Kit.

**RAPD-PCR Analysis:** Six single arbitrary (18-30-base) random primers were used for RAPD-PCR amplification; their names and sequences are summarized in Table (2).

Each 25 µl PCR reaction mixture consisted of; 50 ng of genomic DNA, 3.0µl dNTPs, 7.0µl of random primer, 2.5µl 10x Taq DNA polymerase buffer and 0.8 unit of Taq DNA polymerase (Fanzyme). The reaction volume was completed by sterile $H_2O$. Reaction conditions were optimized according to Ahmed *et al*. (2006). The amplification was performed using a thermocycler (Perkin Elmer 9700) which was programmed as follows; initial denaturation step 95°C for 5 min followed by 35 cycles with 95°C for 1 min for DNA denaturation, annealing at 45 ˚C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min, followed by cooling to 4°C. Visualization of amplified DNA fragments was carried out on 2.0 % agarose gel in a 0.5X TAE buffer, stained with ethidium bromide and photographed using a gel documentation system (Alpha Imager 1220, Canada).

**ISSR-PCR analysis:** Seven primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were used in ISSR analysis (Table, 3).

The PCR reaction mixture consisted of 20ng genomic DNA, 5X PCR buffer (Promega), 25mM/L $MgCl_2$ (Promega), 100µM/L of each dNTP (Promega), 66ng/µl primer and 5 U/µl *Taq* polymerase in a 25µl volume. The amplification protocol was carried out according to Yao *et al*. [25]. The pre-reaction began with an initial denaturation at 94°C for 2 min, followed by 40 cycles of 10 s at 94°C, 30 s at 36°C, and 65 s at 72°C. The reactions were followed by a 10-min extension at 72°C and eventually stored at 4 ºC.

The amplified ISSR products were separated by electrophoresis on 1% agarose gel with 0.5x TBE buffer. After staining with ethidium bromide, banding patterns were visualized with a UV transilluminator.

**Data Analysis:** Patterns of the studied genotypes using six RAPDs and seven ISSRs primers were scored as presence (1), or absence (0) bands by using of the Phoretix 1D image analysis system (Phoretix International, London) to integrate the data. Similarity indices were calculated and consensus tree was developed based on the RAPD and ISSR banding patterns of the 14 *Cucurbita* genotypes using SPSS statistical analysis program (Version 10). The genetic relationships among the 14 genotypes, at the molecular level, were determined.

**Table 1:** List of the fourteen pure lines of Cucurbits genotypes belonging to three different species; their common name, scientific name and some fruit characteristics.

| No. | Code | Common name | Scientific name | Fruit characteristics | | |
|---|---|---|---|---|---|---|
| | | | | Fruit shape | Fruit size | Fruit color |
| 1 | Cp1 | Summer squash (El-Eskandarani cultivar) | *Cucurbita pepo* | Long slender | Small | green |
| 2 | Cp2 | | | | | |
| 3 | Cp3 | | | | | |
| 4 | Cp4 | | | | | |
| 5 | Cp5 | | | | | |
| 6 | Cp6 | | | | | |
| 7 | Cp7 | | | | | |
| 8 | Cp8 | | | | | |
| 9 | Cp9 | | | | | |
| 10 | Cm1 | Winter squash or Butternut (Waltham cv.) | *Cucurbita moschata* | Crookneck with short neck* | Medium | Deep orange |
| 11 | Cm2 | Pumpkin (Local cv.) | *Cucurbita maxima* | Long crookneck | Big | orange |
| 12 | Cm3 | | | | | |
| 13 | Cm4 | | | | | |
| 14 | Cm5 | | | | | |

* Mutschler and Pearson (1987).

**Table 2:** Code and sequence of the six different random primers

| No. | Oligo Name | SEQUENCE |
|---|---|---|
| 1 | A2 | 5'- GAA ACG GGT GGT GAT CGC -3' |
| 2 | ACTR | 5'- AAC TGG AGG AAG GTG GGG -3' |
| 3 | Chi35 | 5'- TTR GAT TGG GAA TAY CC -3' |
| 4 | EZ351 | 5'- AGG AGG TGA TCC AAC CGC -3' |
| 5 | NAH | 5'- GTT TGC AGC TAT GAC GGC TGG GGG TTC GCC -3' |
| 6 | NS2 | 5'- GGC TGC TGG CAC CAG ACT TGC -3' |

**Table 3:** Code and sequence of the thirteen different ISSR primers

| No. | OligoName | Code | SEQUENCE |
|---|---|---|---|
| 1 | ISSR 814A | S1 | 5'-CTC TCT CTC TCT CTC TTG-3' |
| 2 | ISSR 844A | S2 | 5'-CTC TCT CTC TCT CTC TAC-3' |
| 3 | ISSR 844B | S3 | 5'-CTC TCT CTC TCT CTC TGC-3' |
| 4 | ISSR 17898A | S4 | 5'- CAC ACA CAC ACA AC -3' |
| 5 | ISSR 17898B | S5 | 5'- CAC ACA CAC ACA GT -3' |
| 6 | ISSR 17899A | S6 | 5'- CAC ACA CAC ACA AG-3' |
| 7 | ISSR 17899B | S7 | 5'- CAC ACA CAC ACA GG-3' |

## RESULTS AND DISCUSSION

**RAPD-PCR Analysis:**

**Genotype-specific markers based on RAPD analysis:** Fig. (1) shows the polymorphisms among and within the fourteen *Cucurbita* genotypes using six RAPD primers. Table (4) illustrated that the total number of reproducible fragments reached by the six primers reached 463, out of them 405 (87.5%) were polymorphic fragments. The levels of polymorphisms among the fourteen genotypes of *Cucurbita* species are relatively high (69.8% - 97.0%), indicating a wide gene pool existence in the fourteen genotypes under study. Table (5) indicated that the amplification product of *Cucurbita pepo* genotypes produced 309 reproducible fragments, from which 275 (88.9%) were polymorphic. Furthermore, the amplification product of *Cucurbita moschata* genotype produced 31 reproducible fragments, from which 27 (87.1%) were polymorphic and the amplification product of *Cucurbita maxima* genotypes produced 123 reproducible fragments, from which 103 (83.7) were polymorphic. These results indicated that the three *Cucurbita* species exhibited high levels of polymorphism.

This high level of RAPD markers polymorphism in *Cucurbita* species genotypes is in accordance with the results of Ferriol *et al.*[7] and Ferriol *et al.*[8], who reported that *Cucurbita pepo* and *C. maxima* are highly polymorphic species. However, DNA polymorphism has been reported as moderate to high in other allogamous species such as *Brassica*[12,20], *Allium*[22,3], *Asparagus*[11] in addition to *Cucumis* species[14,9,15].

Overall, 31 specific markers were generated among the three *Cucurbita* species, using RAPD-PCR analysis (Table, 6). The number of specific bands per primer varied (2-8) as with size range of the fragments (133-870 bp). The highest numbers of specific markers were obtained with primers Chi35 and NS2 (8 and 7 fragments, respectively). The primer EZ351 produced the lowest number of specific bands (2 fragments). Primer A2 was identified that could discriminate the genotypes belonging to *Cucurbita maxima* from genotypes belonging to the other two species. The results indicated that, RAPD variation may allow identifying sufficient variation in *C. pepo* or *C. maxima* for further phylogenetic research and for other breeding studies. Furthermore, these findings confirmed that RAPD markers can be used to detect genetic variation at the intraspecific and interspecific levels between closely related species [12,20,22,1,17].

***Genetic Similarity and Cluster Analysis Based on RAPD Markers*:** The RAPD data were used to estimate the genetic similarities and the phylogentic relationships among the 14 *Cucurbita* species genotypes. The highest similarity index (88.0%) was observed between the Cp6 and Cp9 genotypes followed by between Cp5 and Cp6, and Cp6 and Cp8 genotypes (84.0%), which belong to the same species (*C. pepo*), while the lowest similarity index (53.0%) was recorded between Cp1 and Cp9 genotypes, which also belong to the same species (*C. pepo*) and between Cm1 and Cm5 (53.0%) which belong to other two species (*C. moschata* and *C. maxima*, respectively) as shown in Table (7). This result clearly supports the utility of RAPD markers for *Cucurbita* species genome analysis. The dendrogram for the phylogenetic relationships among the three species separated the 14 genotypes into two main clusters (Fig., 2). Cluster one included three genotypes belonging to the *C. pepo* (Cp1) and *C. maxima* (Cm3 and Cm5). Cluster two divided into two sub-groups; the first one contained the genotypes belonging to the three species; *C. pepo* (Cp2, Cp3 and Cp4), *C. moschata* (Cm1) and *C. maxima* (Cm2 and Cm4). The second sub-group combined the reminder genotypes of *C. pepo* (Cp5, Clp6, Cp8, Cp9 and Cp7). The results indicated that the nine genotypes of *C. pepo* and the four genotypes of *C. maxima* were not clustered together, even though the origin was the same for all genotypes. This implied that the genomic sequences of the nine summer squash and the four pumpkin genotypes genotypes varied at the genetic level.

The obtained results reflected that assessment of RAPD variation in the examined fourteen Cucurbits genotypes can allow for identifying sufficient variation in *Cucurbita* species for further phylogenetic research and for other breeding studies.

**ISSR Analysis:**

**Genotype-specific Markers Based on ISSR Primers:** PCR-based ISSR was carried out using seven primers with the genomic DNA from 14 *Cucurbita* genotypes. Amplification products of the three species with these primers produced a total of 263 reproducible fragments, from which 243 (92.4%) were polymorphic (Table, 8 and Fig 3). However, Table (9) indicated that the amplification products of *Cucurbita pepo* genotypes produced 168 reproducible fragments, from which 155 (92.3%) were polymorphic. Furthermore, the amplification products of *Cucurbita maxima* genotypes produced 77 reproducible fragments, from which 70 (90.9%) were polymorphic and the amplification product of *Cucurbita moschata* genotype produced 18 reproducible fragments, which were polymorphic (100%). These results confirmed that the three *Cucurbita* species exhibited very high level of polymorphism.

The seven primers in the ISSR analysis gave unique markers (Table, 10) which identified individual genotypes from each other. Out of the seven primers, two detected 4 unique markers, while 4 primers yielded three unique markers, and only one primer gave one unique marker. Only ten genotypes out of 14 could be identified by those unique markers. Genotype Cm5 could be distinguished from other genotypes by the

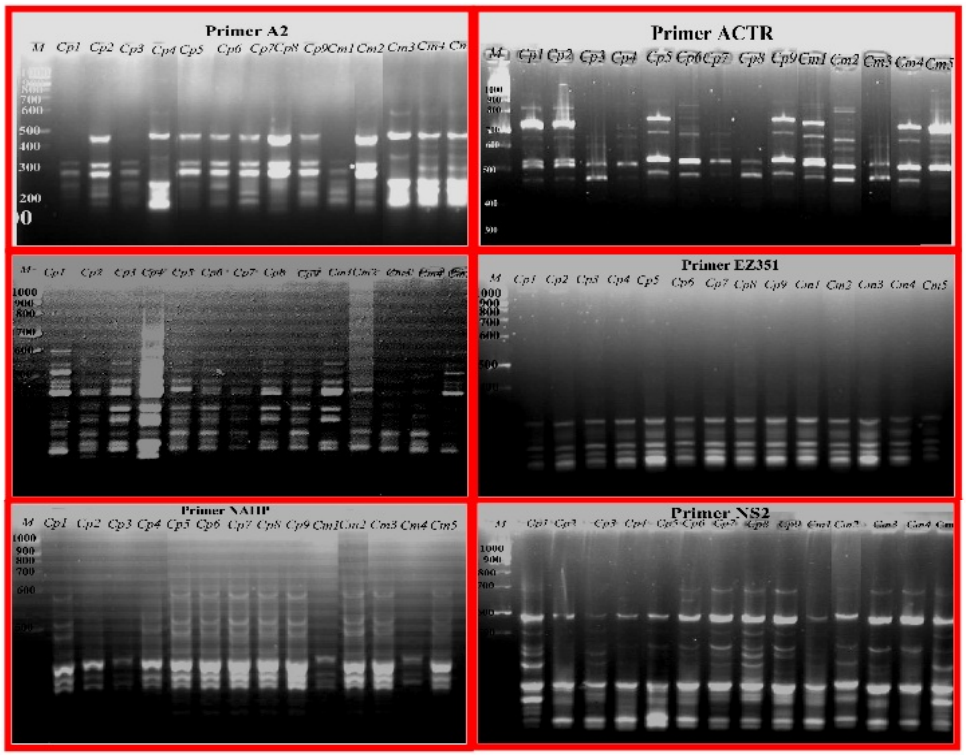


**Fig. 1:** RAPD-PCR banding patterns of DNA for fourteen *Cucurbita* genotypes using six RAPD primers. M refers to 100 bp Ladder.

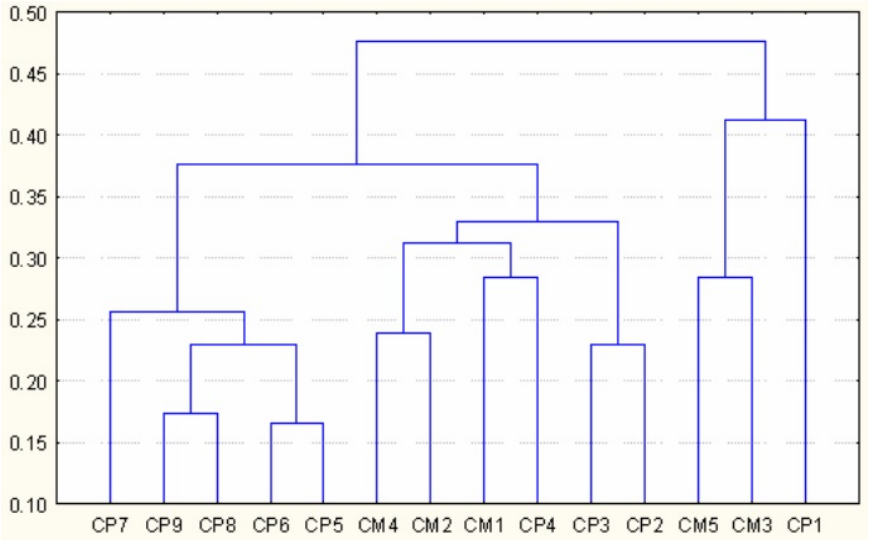


**Fig. 2:** Consensus tree for fourteen *Cucurbita* genotypes developed on the basis of their banding patterns with six RAPD primers.

presence of one unique fragment of 750 bp for primer S1. Two ISSR primers (S3 and S6) were identified that could discriminate the genotypes belonging to *Cucurbita pepo* from genotypes belonging to the other two species. These results agreed with Katzir *et al*., (1996), who reported that some primers detected polymorphism between *C. pepo* and *C. maxima*, whereas no specific signal was produced from *C. moschata*.

Thus, it could be concluded that fingerprinting of *Cucurbita* genotypes for identification purposes could be easily achieved by the ISSR technique. The same conclusion was reported by Wu[24] and Sun *et al*.[19]. Dje *et al*.[5] who indicated that ISSR markers could be

**Table 4:** The number of bands generated and polymorphism percentage as revealed by RAPD among the 14 cucurbits genotypes.

| Primer | TAF | PB | % PB | Fragment size range (bp) |
|---|---|---|---|---|
| A2 | 65 | 61 | 93.9 | 340-577 |
| ACTR | 55 | 49 | 89.1 | 458-870 |
| Chi35 | 89 | 82 | 92.1 | 140-682 |
| EZ351 | 67 | 65 | 97.0 | 133-230 |
| NAH | 71 | 67 | 94.4 | 320-500 |
| NS2 | 116 | 81 | 69.8 | 200-486 |
| Total | 463 | 405 | 87.5 | 133-870 |

TAF= Total amplified fragments, PB= Polymorphic bands

**Table 5:** The summation of the total number of polymporphic using six RAPD primers with the three species of *Cucurbita*.

| Primer | Species | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Cucurbita pepo* | | | *Cucurbita moschata* | | | *Cucurbia maxima* | | |
| | TAF | PB | % PB | TAF | PB | % PB | TAF | PB | % PB |
| A2 | 39 | 39 | 100 | 4 | 4 | 100 | 22 | 18 | 81.8 |
| ACTR | 33 | 32 | 96.9 | 5 | 4 | 80 | 17 | 13 | 76.5 |
| Chi35 | 67 | 61 | 91.0 | 8 | 7 | 78.5 | 14 | 14 | 100 |
| EZ351 | 43 | 42 | 97.7 | 5 | 5 | 100 | 19 | 18 | 94.7 |
| NAH | 47 | 44 | 93.6 | 3 | 3 | 100 | 21 | 20 | 95.2 |
| NS2 | 80 | 57 | 71.3 | 6 | 4 | 66.7 | 30 | 20 | 66.7 |
| Total | 309 | 275 | 88.9 | 31 | 27 | 87.1 | 123 | 103 | 83.7 |

TAF= Total amplified fragments, PB= Polymorphic bands

**Table 6:** The molecular weight and total number of specific polymorphic bands, using six RAPD primers with the three *Cucurbita* species.

| Pr. | Molecular weight of Specific bands (bp) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Cucurbita pepo* | | | | | | | | | *Cucurbita Moschata* | *Cucurbita maxima* | | | | Total specific bands in each primer |
| | Cp1 | Cp2 | Cp3 | Cp4 | Cp5 | Cp6 | Cp7 | Cp8 | Cp9 | Cm1 | Cm2 | Cm3 | Cm4 | Cm5 | |
| A2 | - | - | - | - | - | - | - | - | - | - | - | 577<br>340 | 405 | 358 | 4 |
| ACTR | - | 870 | - | - | - | - | - | - | - | 603 | 564 | 473 | 458 | 835 | 6 |
| Chi35 | 605 | 377 | 484 | 682<br>582<br>140 | - | - | - | 432 | - | 497 | - | - | - | - | 8 |
| EZ351 | 133 | - | - | - | - | - | - | - | - | - | - | - | - | 230 | 2 |
| NAHP | - | 378 | 392 | - | - | - | 500 | - | - | - | - | - | 320 | - | 4 |
| NS2 | 231<br>200<br>374 | -<br>7 | - | 210 | - | 406 | - | 412 | - | - | - | - | - | 486 | |
| Total | 4 | 3 | 2 | 4 | - | 1 | 1 | 2 | - | 2 | 1 | 3 | 3 | 5 | 31 |

**Table 7:** Similarity indices for the Forteen cucurbita sp. on the base of their banding patterns with RAPD.

| Case | Matrix File Input | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cp1 | Cp2 | Cp3 | Cp4 | Cp5 | Cp6 | Cp7 | Cp8 | Cp9 | Cm1 | Cm2 | Cm3 | Cm4 |
| Cp2 | .65 | | | | | | | | | | | | |
| Cp3 | .66 | .78 | | | | | | | | | | | |
| Cp4 | .56 | .68 | .67 | | | | | | | | | | |

**Table 7:** Continued.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cp5 | .63 | .71 | .65 | .67 | | | | | | | | | |
| Cp6 | .61 | .71 | .65 | .67 | .84 | | | | | | | | |
| Cp7 | .61 | .77 | .73 | .63 | .75 | .75 | | | | | | | |
| Cp8 | .63 | .69 | .68 | .70 | .80 | .84 | .80 | | | | | | |
| Cp9 | .53 | .68 | .67 | .78 | .79 | .88 | .78 | .83 | | | | | |
| Cm1 | .67 | .69 | .76 | .72 | .64 | .67 | .66 | .73 | .72 | | | | |
| Cm2 | .64 | .78 | .71 | .69 | .72 | .78 | .74 | .78 | .75 | .72 | | | |
| Cm3 | .60 | .63 | .60 | .66 | .67 | .70 | .68 | .73 | .71 | .63 | .71 | | |
| Cm4 | .62 | .72 | .67 | .71 | .67 | .68 | .65 | .70 | .69 | .70 | .73 | .75 | |
| Cm5 | .69 | .69 | .61 | .61 | .67 | .58 | .66 | .60 | .65 | .53 | .65 | .82 | .72 |

**Table 8:** The total number of polymporphic using seven ISSR primers.

| Pr. | Cp1 | Cp2 | Cp3 | Cp4 | Cp5 | Cp6 | Cp7 | Cp8 | Cp9 | Cm1 | Cm2 | Cm3 | Cm4 | Cm5 | TAF | PB | %PB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | 2 | 3 | 1 | 1 | 3 | 2 | 1 | 2 | 3 | 2 | 1 | 3 | 1 | 3 | 28 | 27 | 96.4 |
| S2 | 2 | 6 | 3 | 2 | 6 | 2 | 2 | 6 | 1 | 5 | 2 | 2 | 5 | 2 | 46 | 43 | 93.5 |
| S3 | 3 | 5 | 1 | 4 | 5 | 2 | 1 | 3 | 2 | 2 | 2 | 1 | 1 | 5 | 37 | 34 | 91.9 |
| S4 | 1 | 3 | 1 | 1 | 3 | 1 | 2 | 3 | 1 | 2 | 1 | 3 | 3 | 5 | 30 | 26 | 86.7 |
| S5 | 3 | 3 | 3 | 1 | 3 | 2 | 1 | 6 | 4 | 2 | 1 | 3 | 4 | 4 | 40 | 37 | 92.5 |
| S6 | 4 | 4 | 2 | 5 | 4 | 2 | 4 | 4 | 4 | 2 | 3 | 3 | 2 | 4 | 47 | 44 | 93.6 |
| S7 | 3 | 2 | 2 | 2 | 2 | 2 | 1 | 3 | 2 | 3 | 3 | 3 | 3 | 4 | 35 | 32 | 91.4 |
| Total | 18 | 26 | 13 | 16 | 26 | 13 | 12 | 27 | 17 | 18 | 13 | 18 | 19 | 27 | 263 | 243 | 92.4 |

TAF= Total amplified fragments, PB= Polymorphic bands

**Table 9:** The summation of the total number of polymporphic using seven ISSR primers with the three species of *Cucurbita*.

| Primer | Species | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Cucurbita pepo* | | | *Cucurbita moschata* | | | *Cucurbia maxima* | | |
| | TAF | PB | % PB | TAF | PB | % PB | TAF | PB | % PB |
| S1 | 18 | 18 | 100 | 2 | 2 | 100 | 8 | 7 | 87.5 |
| S2 | 30 | 28 | 93.3 | 5 | 5 | 100 | 11 | 10 | 90.9 |
| S3 | 26 | 23 | 88.5 | 2 | 2 | 100 | 9 | 9 | 100 |
| S4 | 16 | 15 | 93.8 | 2 | 2 | 100 | 12 | 9 | 75.0 |
| S5 | 26 | 24 | 92.3 | 2 | 2 | 100 | 12 | 11 | 91.7 |
| S6 | 33 | 30 | 90.9 | 2 | 2 | 100 | 12 | 12 | 100 |
| S7 | 19 | 17 | 89.5 | 3 | 3 | 100 | 13 | 12 | 92.3 |
| Total | 168 | 155 | 92.3 | 18 | 18 | 100 | 77 | 70 | 90.9 |

TAF= Total amplified fragments, PB= Polymorphic bands

**Table 10:** The molecular weight and total number of specific polymorphic bands, using seven ISSR primers with the three *Cucurbita* species.

| Pr. | Molecular weight of Specific bands (bp) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Cucurbita pepo* | | | | | | | | | *Cucurbita Moschata* | *Cucurbita maxima* | | | | Total specific bands in each primer |
| | Cp1 | Cp2 | Cp3 | Cp4 | Cp5 | Cp6 | Cp7 | Cp8 | Cp9 | Cm1 | Cm2 | Cm3 | Cm4 | Cm5 | |
| S1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 750 | 1 |

**Table 10:** Continued.

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S2 | 0 | 0 | 410 | 0 | 0 | 0 | 0 | 192 | 0 | 0 | 0 | 0 | 222 | 0 | 3 |
| S3 | 0 | 362 | 0 | 0 | 1233 | 982 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| S4 | 0 | 0 | 0 | 493 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 740 | 811 68 | 4 |
| S5 | 987 | 0 | 0 | 0 | 0 | 0 | 0 | 1137 | 0 | 0 | 0 | 231 | 0 | 0 | 3 |
| S6 | 0 | 1268 104 | 0 | 0 | 0 | 1105 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 648 | 4 |
| S7 | 757 | 0 | 989 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 527 | 3 |
| Total | 2 | 3 | 2 | 1 | 1 | 2 | 0 | 2 | 0 | 0 | 0 | 1 | 2 | 5 | 21 |

**Table 11:** Similarity indices for the Fourteen *Cucurbita* sp. on the base of their banding patterns with ISSR.

| Case | Matrix File Input | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cp1 | Cp2 | Cp3 | Cp4 | Cp5 | Cp6 | Cp7 | Cp8 | Cp9 | Cm1 | Cm2 | Cm3 | Cm4 |
| Cp2 | 0.71 | | | | | | | | | | | | |
| Cp3 | 0.63 | 0.56 | | | | | | | | | | | |
| Cp4 | 0.73 | 0.71 | 0.61 | | | | | | | | | | |
| Cp5 | 0.6 | 0.67 | 0.61 | 0.65 | | | | | | | | | |
| Cp6 | 0.67 | 0.64 | 0.68 | 0.72 | 0.67 | | | | | | | | |
| Cp7 | 0.73 | 0.65 | 0.69 | 0.73 | 0.68 | 0.72 | | | | | | | |
| Cp8 | 0.63 | 0.65 | 0.64 | 0.65 | 0.65 | 0.61 | 0.73 | | | | | | |
| Cp9 | 0.61 | 0.67 | 0.63 | 0.72 | 0.67 | 0.71 | 0.83 | 0.75 | | | | | |
| Cm1 | 0.68 | 0.73 | 0.63 | 0.73 | 0.72 | 0.69 | 0.76 | 0.71 | 0.75 | | | | |
| Cm2 | 0.69 | 0.69 | 0.68 | 0.75 | 0.72 | 0.73 | 0.78 | 0.64 | 0.79 | 0.75 | | | |
| Cm3 | 0.68 | 0.63 | 0.61 | 0.65 | 0.68 | 0.64 | 0.82 | 0.71 | 0.72 | 0.76 | 0.72 | | |
| Cm4 | 0.64 | 0.67 | 0.68 | 0.64 | 0.56 | 0.65 | 0.72 | 0.69 | 0.71 | 0.72 | 0.71 | 0.75 | |
| Cm5 | 0.64 | 0.61 | 0.55 | 0.64 | 0.53 | 0.57 | 0.64 | 0.59 | 0.65 | 0.61 | 0.65 | 0.61 | 0.71 |

**Table (12):** Similarity indices for the Fourteen Cucurbita sp. on the base of their banding patterns with RAPD and ISSR.

| Case | Matrix File Input | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cp1 | Cp2 | Cp3 | Cp4 | Cp5 | Cp6 | Cp7 | Cp8 | Cp9 | Cm1 | Cm2 | Cm3 | Cm4 |
| Cp2 | .68 | | | | | | | | | | | | |
| Cp3 | .65 | .65 | | | | | | | | | | | |
| Cp4 | .61 | .68 | .64 | | | | | | | | | | |
| Cp5 | .61 | .65 | .61 | .64 | | | | | | | | | |
| Cp6 | .61 | .64 | .63 | .69 | .74 | | | | | | | | |
| Cp7 | .68 | .73 | .69 | .69 | .74 | .75 | | | | | | | |
| Cp8 | .64 | .66 | .66 | .71 | .73 | .73 | .79 | | | | | | |
| Cp9 | .56 | .66 | .63 | .77 | .73 | .74 | .81 | .80 | | | | | |
| Cm1 | .68 | .69 | .69 | .74 | .63 | .66 | .70 | .72 | .73 | | | | |
| Cm2 | .65 | .73 | .71 | .71 | .72 | .74 | .72 | .71 | .76 | .73 | | | |
| Cm3 | .64 | .63 | .57 | .65 | .72 | .66 | .75 | .72 | .71 | .67 | .71 | | |
| Cm4 | .61 | .69 | .67 | .70 | .60 | .64 | .68 | .61 | .70 | .71 | .73 | .72 | |
| Cm5 | .61 | .66 | .60 | .60 | .62 | .56 | .66 | .60 | .65 | .67 | .64 | .66 | .68 |

powerful tools to study genetic diversity among African edible cucurbits.

**Genetic Similarity and Cluster Analysis Based on ISSR Markers:** Similarity indices and consensus tree were developed on the basis of the banding patterns of the 14 *Cucurbita* species genotypes using 7 ISSR primers as shown in Table (10) and Fig. (4). According to ISSR results, the most closely related genotypes were Cp7 and Cp9, which belong to *C. pepo*, with the highest similarity index (83.0%). On the other hand, the most two distantly related genotypes were Cp5 and Cm5, which belong to two different species (*C. pepo* and *C. maxima*, respectively) with the lowest similarity index (53.0%). The results of the consensus tree indicated that the tree was divided into two main clusters. Cluster one included four genotypes belonging to the *C. pepo* (Cp8, Cp5, Cp6 and Cp3). Cluster two divided into three sub-groups; the first one contained one genotype (Cm5), which belong to *C. maxima*, while the second sub-group combined the genotypes belonging to the three species; *C. maxima* (Cm4, Cm3 and Cm2), *C. moschata* (Cm1) and *C. pepo* (Cp9 and Cp7). The third sub-group contained the reminder genotypes of *C. pepo* (Cp2, Cp4 and Cp1). The results indicated that the nine genotypes of *C. pepo* distributed in the all clusters and these support the results which obtained from the consensus tree based on RAPD analysis.

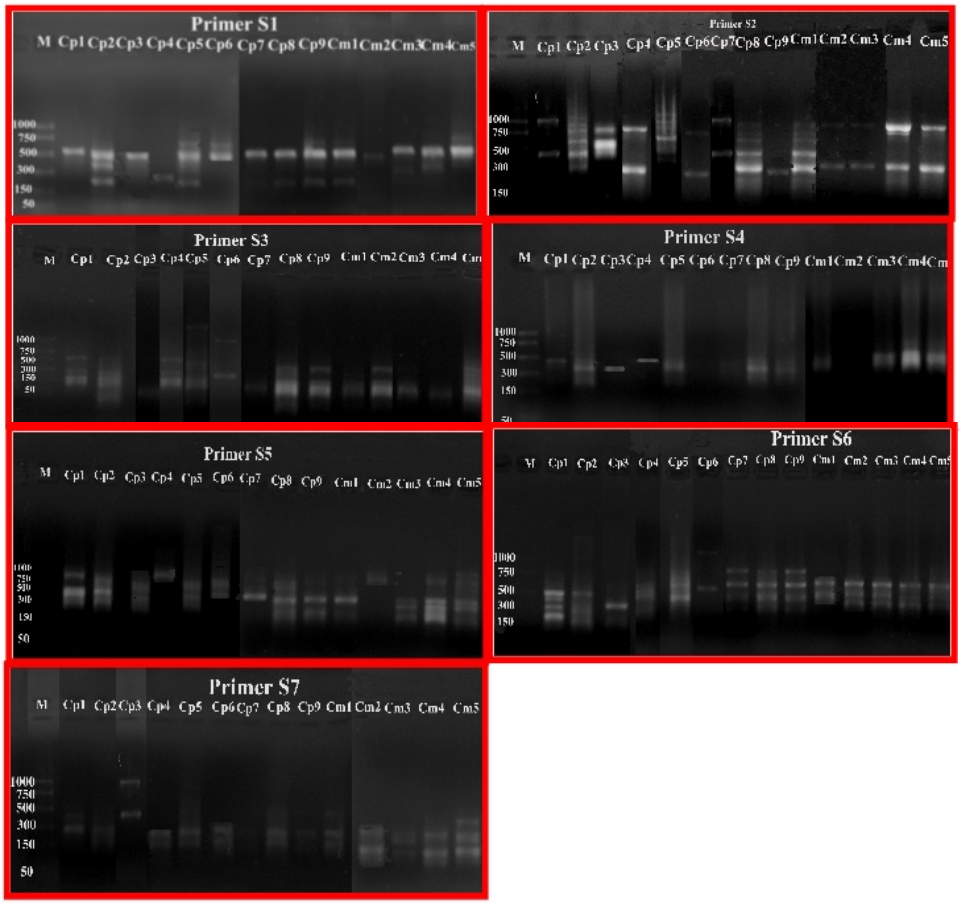


**Fig. 3:** ISSR-PCR banding patterns of DNA for fourteen *Cucurbita* genotypes using Seven ISSR primers. M refers to 250 bp Ladder.

**Combined Identification Based on RAPD-PCR and ISSR-PCR analysis:** Genotypes distribution on the consensus tree according to the banding patterns of RAPD differed from that based on ISSR banding patterns. This may be due to the fact that each technique amplified different segments of the genome. Therefore, it is better to use the combination of the banding patterns of the two technique to use more segments of the genome that will increase the validity of the consensus tree. Results of the combined data were shown in Fig. (5) and Table (11) exhibited that the most three closely related genotypes were betweenCp7, Cp9 and Cp8, which belong to *C. pepo*, with the highest similarity index (81% and 80%, respectively). On the other hand, the most two distantly related genotypes were between Cp1 and

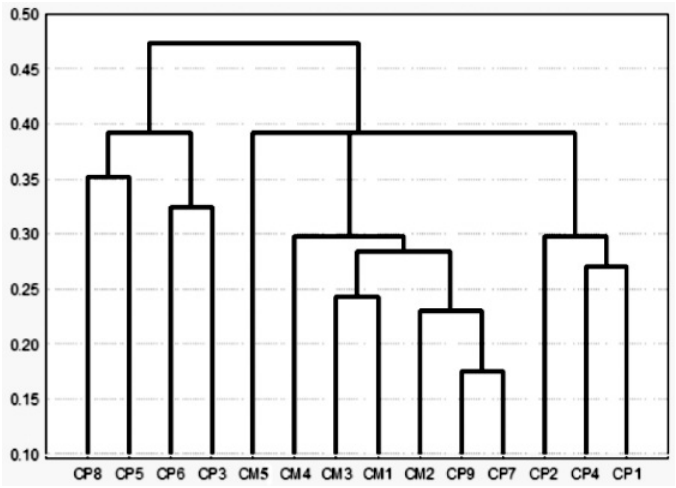


**Fig. 4:** Consensus tree for fourteen *Cucurbita* genotypes developed on the basis of their banding patterns with seven ISSR primers.

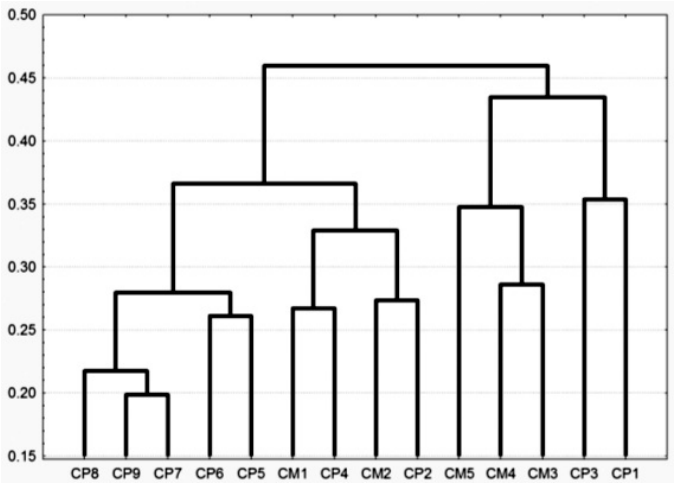


**Fig. 5:** Consensus tree for fourteen *Cucurbita* genotypes developed on the basis of their banding patterns with combination of RAPD and ISSR.

Cp9 and between Cp6 and Cm5 with the lowest similarity index (56% and 56%, respectively). The results of the consensus tree (Fig.,5) indicated that the tree was divided into two main clusters; the first included genotypes Cp1, Cp3 (*C. pepo*), Cm3, Cm4 and Cm5 (*C. maxima*).

The second main cluster was divided into two sub-clusters. The first sub-cluster included genotypes Cp2 and Cp4 (*C. pepo*), Cm2 (*C. maxima*) and Cm1 (*C. moschata*). The second one included Cp8, Cp9, Cp7, Cp6, Cp5 (*C. pepo*). On the other hand, the genotypes that could be used most successfully in the breeding programs were Cp8, Cp9, Cp7, Cp6. The results illustrated that the nine genotypes of *C. pepo* were not clustered together.

In conclusion, the consensus obtained trees based on RAPD, ISSR and the combination between them indicated that the nine genotypes belonging to *C. pepo* and four genotypes of *C. maxima* were not clustered together. These results indicated that the genomic sequences of the nine summer squash and four pumpkin genotypes varied at the genetic level. The information on polymorphism using RAPD and ISSR in a set of genotypes is useful in the assessment of genetic diversity and genetic relationships and could be useful in the breeding programs. Analysis of ISSR can be accomplished with the ease of analysis of RAPD, for studying the genetic relationships among and within genotypes of agronomic importance such as *Cucurbita* species to facilitate the prediction of crosses that will produce hybrids with higher performance in future breeding studies.

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## REFERENCES


1. Abdel-Razzak, H.S., M.M. Ahmed and A.A. El-mezawy, 2005. Genetic analysis of some pepper (*Capsicum annuum* L.) genotypes using three DNA-based markers. The Egyptian Science Magazine, 2(3): 51-58.
2. Ahmed, M.M., S.S. Soliman and E.H. Elsayed, 2006. Molecular identification of some Egyptian date palm males by females varieties (*Phoenix dactylifera* L.) using DNA markers. J. Appl. Sci. Res., 2(5): 270-275.
3. Al-Zahim, M., H.J. Newbury and B.V. Ford-Lloyd 1997. Classification of genetic variation in garlic (*Allium sativum* L.) revealed by RAPD. HortScience, 32(6): 1102-1104.
4. Dijkhuizen, A., W.C. Kennard, M.J. Havey and J.E. Staub, 1996. RFLP variation and genetic relationships in cultivated cucumber. Euphytica, 90: 79-87.
5. Dje, Y., GC. Tahi, Bi. IA. Zoro, M. Malice, JP. Baudoin and P. Bertin, 2006. Optimization of ISSR marker for African edible-seeded *Cucurbitaceae* species' genetic diversity analysis. African Journal of Biotechnology, 5(2): 83-87.
6. Fang, D.Q. and M.L. Roose, 1997. Identification of closely relative Citrus cultivars with inter simple sequence repeat markers. Theor. Appl. Genet., 95: 408-417.
7. Ferriol, M., M.P. Belén and F. Nuez, 2003. Genetic Resources and Crop Evolution. Springer., 50(3): 227-238.
8. Ferriol, M., B. Pico and F. Nuez, 2004. Genetic diversity of a germplasm collection of cucurbita pepo using SRAP and AFLP markers. Theor. Appl. Genet., 107: 271-282.
9. Garcia, E., M. Jamilena, J.I. Alvarez, T. Arnedo, J.L. Oliver and R. Lozano, 1998. Genetic relationships among melon breeding lines revealed by RAPD markers and agronomic traits. (Theor. Appl. Genet.) 96: 878-885.
10. Hossain, M.B., A. Awal, M.A. Rahman, S. Haque and H. Khan, 2003. Distinction between cold-sensitive and -tolerant jute by DNA polymorphisms. J. Bio. Mol. Bio., 36(5): 427-432.
11. Khandka, D.K., A. Nejidat and A. Golan-Goldhirsh, 1996. Polymorphism and DNA markers for asparagus cultivars identified by random amplified polymorphic DNA. Euphytica 87: 39-44.
12. Kresovich, S., J.G.K. Williams, J.R. McFerson, E.J. Routman and B.A. Schaal, 1992. Characterization of genetic identities and relationships of *Brassica oleraceae* L. via a random amplified polymorphic DNA assay. Theor. Appl. Genet., 85: 190-196.
13. Mutschler, M.A. and O.H. Pearson, 1987. The origin, inheritance, and instability of butternut squash (*Cucurbita moschata* Duchesne). HortScience, 22(4): 535-539.
14. Neuhausen, S.L., 1992. Evaluation of restriction fragment length polymorphism in *Cucumis melo*. Theor. Appl. Genet., 83: 379-384.
15. Oliver, M., J. Garcia-Mas, M. Cardus, N. Pueyo, A.I. Lopez-Sese, M. Arroyo, H. Gomez-Paniagua, P. Arus and M.C. de Vicente, 2001. Construction of a reference linkage map for melon. Genome 44: 836-845.
16. Patil, M.D., D.P. Biradar, V.C. Patil, B.S. Janagoudar and H.L. Nadaf, 2007. Analysis of genetic diversity of cotton genotypes using RAPD PCR technique. Karnataka J. Agric. Sci., 20(2): 215-217.
17. Singh, A.K., M. Singh, A.K. Singh, R. Singh, S. Kumar and G. Kalloo, 2006. Genetic diversity within the genus *Solanum* (Solanaceae) as revealed by RAPD markers. Current Science, 90(5): 711-716.
18. Stift, G., M. Pachner and T. Lelley, 2003. Comparison of RAPD fragment separation in agarose and polyacrylamid gel by studying *Cucurbita* species. Cucurbita Genetics Cooperative Report, 26: 62-65.
19. Sun, G.L., M. Wiliam, J. Liu, K.J. Kasha and K.P. Pauls, 2001. Microsatellite and RAPD polymorphisms Ontario corn hybrids related to the commercial sources and maturity ratings. Mol. Breeding, 7: 13-24.
20. Thormann, C.E., M.E. Ferreira, L. E.A. Camargo, J.G. Tivang and T.C. Osborn, 1994. Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. Theor. Appl. Genet., 88: 973-980.
21. Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalsky and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res., 18: 6531-6535.
22. Wilkie, S.E., P.G. Isaac and R.J. Slater, 1993. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor. Appl. Genet., 86: 497-504.
23. Wilson, H.D., J. Doebley and M. Duvall, 1992. Chloroplast DNA diversity among wild and cultivated members of *Cucurbita* (Cucurbitaceae), Theor Appl. Genet., 84: 859-865.
24. Wu, M., 2000. Genetic diversity and its relationship heterosis in maize as revealed by AFLPs and RAPDs. Maize Genet. Newslet., 74: 43-44.
25. Yao, Y., M. Ban and J. Brandle, 1999. A genetic linkage map for *Stevia rebaudiana*. Genome 42: 657-661.
26. Zietkiewicz E, A. Rafalski and D. Labuda, 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, 20: 176-183.