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Genetic Identification and Estimation of Genetic Relationships among Some Onion Cultivars and Lines using RAPD Analysis and Morphological Characters

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ABSTRACT

Genetic differentiation of two lines and three cultivars of onion were studied by RAPD analysis. RAPD analysis revealed that 67 bands with an average of 6.7 amplicons per primer were generated by 10 decamer primers. However, the total number of polymorphic bands was 57, which represents an 83.9% level of polymorphism. RAPD-based Dice similarity coefficient analysis showed that the highest genetic similarity value was observed between cvs. 'T.E.Y.G' and 'Giza 20' (96.8% similarity), while the lowest (61.5%) genetic similarity value was between 'Puss' and 'T.E.Y.G'. A dendrogram based on genetic similarities separated the two lines and the three cultivars of onion into two main groups. Using OPC-08, OPC-14 and OPD-11 primers to compare A- or B-line with other cultivars, a high level of polymorphism was shown. Primer OPD-01 was a unique marker that could characterize A- and B-lines from other cultivars. In general, RAPD can be effectively used to assess genetic variation and identification among onion lines and cultivars and can thus assist onion breeders to predict sterile and fertile onion plants.

Keywords: *Allium cepa*, genetic similarity, genetic diversity, phylogenetic analysis, polymorphism, random amplified polymorphic DNA (RAPD)
Abbreviations: EDTA, ethylenediaminetetraacetic acid, **PCR**, polymerase chain reaction, **RAPD**, random amplified polymorphic DNA, **TBE**, tris-borate-EDTA

INTRODUCTION

Onion (*Allium cepa* L.; Alliaceae) is one of the most important vegetable crops all over the world. Onion breeding programs are mostly based on the release of hybrid strains which depend on the use of male sterility. However, the identification of parental lines that form superior hybrids is the most costly and time-consuming phase in onion hybrid development. The production of hybrid onion seed became economically feasible with the discovery of cytoplasmic gene male sterility (CMS) (Jones and Emsweller 1936; Berninger 1965; Cho *et al*. 2005, 2006). To increase onion productivity and bulb quality, more effort must be directed to production of high-yielding and better quality varieties. The discovery of molecular marker technologies offers simple, reliable and effective tools and lead to detailed genetic analysis, which will lead to the improvement of crop plants and assist breeders in their programs.

Many standard molecular biological techniques with modifications of the original PCR procedure were designed to suit a range of needs. One such technique is randomly amplified polymorphic DNA (RAPD), which was developed by Welsh and McCallum (1990) and Williams *et al*. (1990). DNA-based markers can be extremely useful in studying genetic diversity and taxonomy and as a tool in plant breeding and selection programs (Bebeli *et al.* 1997). These markers can be used to identify any DNA sequence polymorphism between individuals.

Many authors used RAPD analysis for genetic analysis in *Allium.* Wilkie *et al*. (1993) used RAPD to assess the degree of polymorphism within the genus. Twenty–two onion cultivars were examined by RAPD (Tanikawa *et al*. 2002). Seventeen of the 100 primers screened produced clear, reproducible polymorphic banding profiles. A total of 88 fragments were produced by 17 primers of which 35 were polymorphic among all cultivars. All 22 cultivars could be distinguished by a combination of polymorphic bands generated by various primers. Kuttym *et al*. (2006) also used RAPD markers to estimate genetic diversity among 24 cultivars of short-day onions. Total genomic DNA was extracted and subjected to RAPD analysis using 90 arbitrary 10-mer primers. The results indicated that 15 primers were selected which yielded 137 bands, 91.24% of which were polymorphic.

Phuong *et al*. (2009) evaluated wakegi onion (*Alllium* x *wakegi* Araki) strains as a genetic resource for breeding onions in Vietnam. Genetic variation among and between wakegi onions collected from the North, Central and South regions in Vietnam and Japanese bunching onion were assessed, and additional shallot strains from Japan and Thailand were also studied based on morphological, physiological characters and polymorphisms of nuclear, chloroplast and mitochondrial DNAs. The results demonstrated that the intraspecific analysis among wakegi onion showed that 16 out of 40 primers used in RAPD analysis produced a total of 160 distinct bands in all the strains examined; 135 bands were polymorphic. Ebrahimi *et al*. (2009) studied the diversity of 17 wild Persian shallot populations collected from different parts of Iran by morphological and RAPD markers. Fifteen random decamer primers could amplify DNA well and produced polymorphic bands among populations. The results showed that the highest number of fragments ampli-

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fied by one primer was 27 out of which 15 fragments were polymorphic and the least was 11 with 6 polymorphic bands.

In this study we have evaluated the use of RAPD for detecting polymorphism in different onion cultivars and lines for effective genotype identification.

MATERIALS AND METHODS

Plant materials

This study was carried out at the Agricultural Research Centre (ARC), Field Crops Research Institute (FCRI), Giza Station, Onion Research Department, Giza, Egypt. Three certified onion (*Allium cepa* L.) cultivars ('Puss', 'Giza 20' and 'T.E.Y.G.') and two lines (A-line and B-line) were used.

Cultivation of cultivars and lines in the field

Seeds of different cultivars and lines were sown during the 2007-2008 season. Seeds were grown in a nursery of the Department in October, 2006 then transferred to an open field in January, 2007 and cultivated in rows and irrigated every 2 weeks. Common agricultural practice was performed during the growth of onion plants. Two months later, 10 random young leaf samples were taken from each cultivar and one sample was taken from each line. DNA was extracted from all samples for RAPD analysis.

Genomic DNA extraction and RAPD analysis

Leaves from all cultivars and lines were extracted using the DNeasy plant mini kit (Quiagen Inc., Hildin, Germany, imported from Valenica, USA). RAPD-PCR reactions were conducted using 10 arbitrary 10-mer primers with the 5'→3' sequences as shown in **Table 1**. The reaction conditions were optimized and mixtures were prepared (30 µl total volume). Amplification was carried out in a PTC-200 thermal cycler (MJ Research, Watertown, USA) as follows: Denaturation, 94°C for 2 min then for 40 cycles. Each cycle consisted of 1 min at 94°C, 1 min at 37°C, 150 sec at 72°C, followed by a final extension time of 12 min at 72°C and 4°C (indefinitely). The amplification products were analyzed by electrophoresis in a 1.2% agarose gel in TBE (Tris-borate-EDTA) buffer (pH 8.0), stained with 0.2 µg/ml ethidium bromide. Bands were detected on a UV-transilluminator and photographed by a Gel documentation system (2000, Bio-Rad). DNA ladder (Sibenzyme, Novosibirsk, Russsia) was used to determine band size.

Data analysis

Only reproducible banding patterns generated by RAPD were considered. Each band scored was considered as an independent attribute and was accounted qualitatively by its presence (1) or absence (0). Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between the two lines and the three cultivars was estimated according to Dice's coefficient (Sneath and Sokal 1973) using Dice's formula:

$$GS(ij) = 2a / (2a + b + c)$$

where GS (ij) is the measure of genetic similarity between individuals i and j, (a) is the number of bands absent in i and present in j. Cluster analysis were based on a similarity matrix obtained with the unweighted pair group method using arithmetic average (UPGMA), and relationships between lines and cultivars were illustrated as dendrograms.

Morphological data

During the vegetative stage, different morphological data were recorded for all lines and cultivars tested. The data were: number of sprouts; number, length and diameter of seed stalks; diameter of umbels; number of seeds; weight of seed/plant; weight of 1000-seed.

Table 1 Sequences of random primers for RAPD-PCR analysis.

Primer	Primer Sequence (5'→3')
OPC02	GTGAGGCGTC
OPC07	GTCCCGACGA
OPC08	TGGACCGGTG
OPC09	CTCACCGTCC
OPC14	TGCGTGCTTG
OPC15	GACGGATCAG
OPD01	ACCGCGAAGG
OPD08	GTGTGCCCCA
OPD11	AGCGCCATTG
OPD16	AGGGCGTAAG

Table 2 Number of amplicons, monomorphic and polymorphic amplicons and the percentage of polymorphism as revealed by RAPD markers.

Primer	Total No. of amplicons	Monomorphic amplicons	Polymorphic amplicons	Polymorphism (%)*
OPC02	5	2	3	60
OPC07	6	1	5	83
OPC08	3	0	3	100
OPC09	6	1	5	83
OPC14	9	0	9	100
OPC15	9	2	7	78
OPD01	7	1	6	86
OPD08	6	2	4	67
OPD11	9	0	9	100
OPD16	7	1	6	86
Total	67	10	57	86
Average	6.7	1.0	5.7	83.9

* % of Polymorphism = (No. of polymorphic bands ÷ Total No. of amplicons) × 100

Table 3 Unique line or cultivar-specific bands based on RAPD.

Line and cultivar	Primer	UPM (bp)	UNM (bp)	Total for Primer	Total for Line or cultivar
A-line	OPD01	982	---	1	2
		2451	---	1	
B-line	OPD01	---	677	1	4
		982	---	1	
		2451	---	1	
	OPD08	---	881	1	
Giza 20	OPC08	---	1958	1	5
	OPC15	---	954	1	
	OPD11	---	998, 1247	2	
	OPD16	---	396	1	
T.E.Y.G.	OPC07	163	---	1	2
	OPC09	---	323	1	
Total		5	8		13

UPM = Unique positive marker; UNM = Unique negative marker

Statistical analysis

Experiments were set up in a completely randomized design and the collected data from field experiments were statistically analyzed according to Gomez and Gomez (1984). The mean value of treatments was compared according to Duncan's multiple range test (Duncan 1955). All statistical analysis was performed using analysis of variance techniques by means of SPSS Ver.13 software.

RESULTS AND DISCUSSION

RAPD analysis, polymorphism and unique markers

Thirty five 10-mer random primers were first screened with all cultivars and lines used in this study, 10 of which generated reproducible bands. Results are presented in **Table 2** and **Figs. 1A-E** for the following primers: OPC-07 primer (**Fig. 1A**), OPC-08 (**Fig. 1B**), OPC-14 (**Fig. 1C**), OPD-01 (**Fig. 1D**) and OPD-11 (**Fig. 1E**).

The 10 primers amplified 67 bands with an average of 6.7 amplicons/primer (**Table 2**) while the total number of monomorphic amplicons was 10 with an average of 1.0 am-

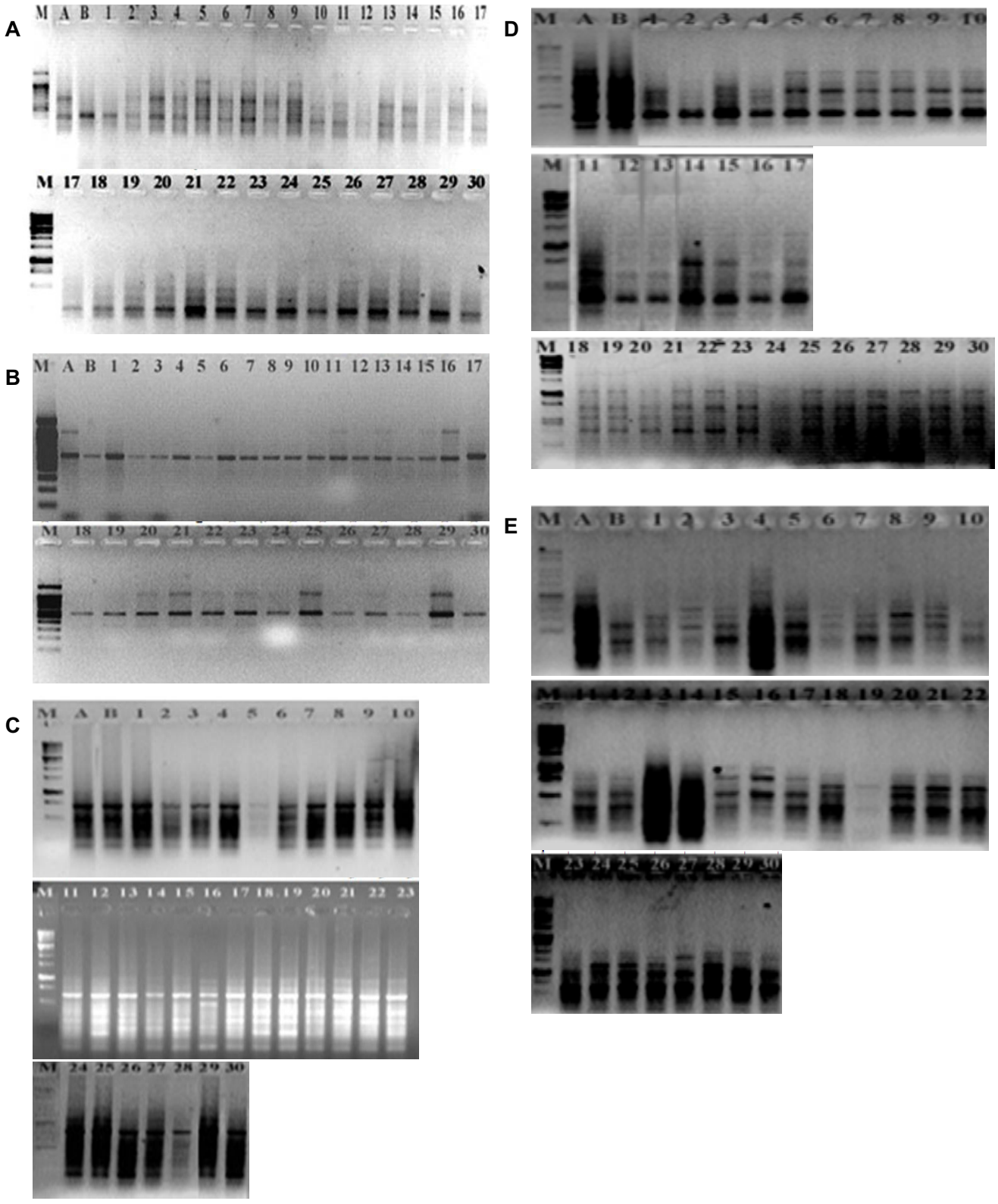

Fig. 1 DNA polymorphism of the two onion lines and three cultivars using randomly amplified polymorphic DNA with five primers. (**A**) OPC07; (**B**) OPC08; (**C**) OPC14; (**D**) OPD01; (**E**) OPD11. In (**A-E**), A = Line A, B = Line B, Lanes 1-10 = cv. 'Puss', 11-20 = cv. 'Giza 20', 21-30 = cv. 'T.E.Y.G.'; M = DNA ladder size (250-10,000 bp).

plicons/primer. The total number of polymorphic bands was 57 representing 83.9% polymorphism. The number of polymorphic amplicons ranged from 3 to 9 with an average of 5.7 amplicons/primer. Different primers expressed different levels of polymorphism (**Table 2**), ranging from 60% with primer OPC-02 to 100% with primers OPC-08, OPC-14 and OPD-11.

Table 3 shows the onion lines or cultivars characterized by unique positive and/or negative RAPD markers and total number of markers identifying each onion line or cultivar.

RAPD in onion

RAPD polymorphism was reported in many studies on onion, the earliest being that by Wilkie *et al.* (1993). In that study, RAPD was used to assess the degree of polymorphism within the genus *Allium*. Seven out of 20 primers revealed scorable polymorphisms between several *A. cepa* cultivars. Moreover, wide variations in banding profiles between species were observed with nearby every primer tested: approx. 91 bands were scored for all cultivars. Assessment of gene-

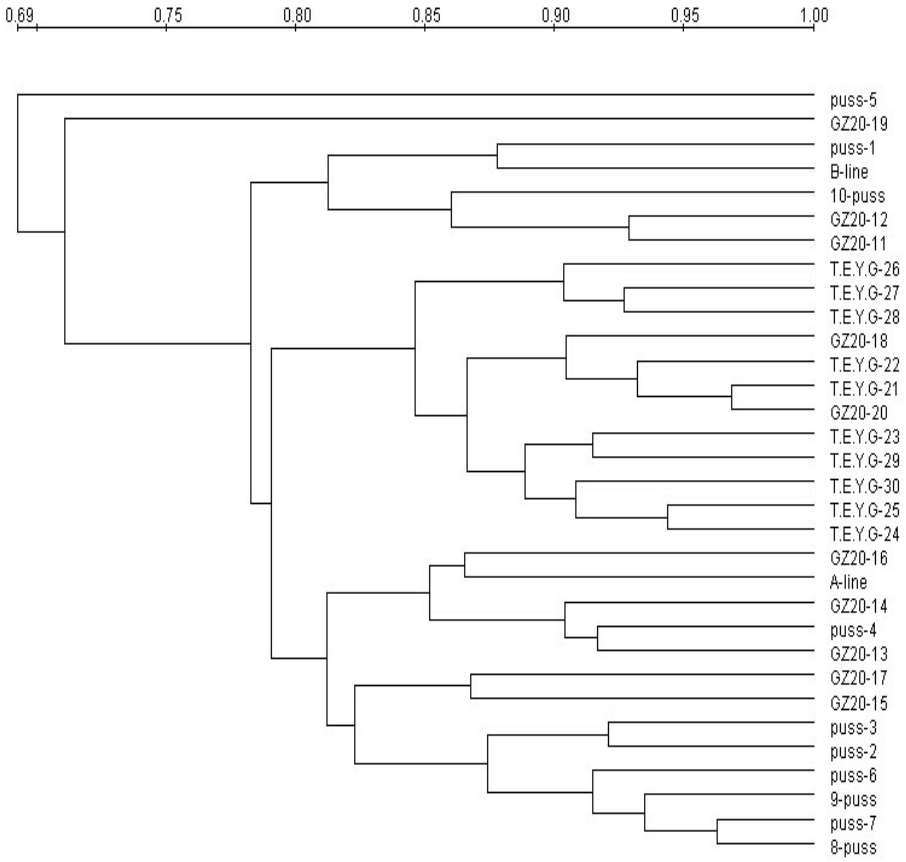

Fig. 2 Dendrogram for two onion lines and three cultivars generated UPGMA cluster analysis of the similarity values.

tic diversity and crop classification among *A. cepa* genotypes was studied by Dennequin *et al*. (1997). Four primers generated 24 reproducibly scorable DNA bands which gave individual fingerprinting for representative accessions of both onions and shallots. The results indicated that the seed-propagated shallot is more closely related to onions than to vegetatively propagated shallots and, moreover, reveal a geographical structure of genetic diversity.

In another study, 22 *Allium cepa* cultivars were examined using RAPD markers (Tanikawa *et al*. 2002): 17/100 primers screened produced clear polymorphic banding profiles and a total of 88 fragments were produced by 17 primers of which 35 were polymorphic. All 22 cultivars could be distinguished by a combination of polymorphic bands generated by various primers. Kuttym *et al*. (2006) estimated genetic diversity among 24 cultivars of short-day onions by RAPD analysis. They tested 90 arbitrary 10-mer primers, 15 of which were selected yielding 137 bands, 91.24% of which were polymorphic. None of the primers produced a unique banding pattern for each cultivar. They concluded that there was high diversity among the onion cultivars selected indicating the potential of RAPD markers for identification and maintenance of onion germplasm for crop improvement purposes. More recently, Ebrahimi *et al*. (2009) evaluated the diversity of 17 wild Persian shallot populations collected from different parts of Iran across the Zagross Mountains by morphological and RAPD markers. Fifteen 10-mer primers could amplify DNA and produce polymorphic bands among populations. Most number of fragments/primer was 27, 15 of which were polymorphic.

Recently, Maniruzzaman *et al*. (2010) applied RAPD analysis to l0 varieties of onion to assess the degree of polymorphism within the genes and to investigate if this approach was suitable for genetic studies of onion. For this study, 10 cultivars of onion were evaluated for variability using a set of 15 random l0-mer primers. They found that among them, 12 primers revealed scorable (168 bands) polymorphisms between cultivars of *A. cepa* and the rest did not show polymorphism at the genetic level. In this study, 'Bermis' and 'India-2' were more dissimilar while, 'Faridpuri' and 'Bhati' were the most similar at the genetic level.

Genetic relationship and cluster analysis of onion lines and cultivars

The dendrogram constructed from UPGMA cluster analysis of the Dice similarity coefficients calculated from RAPD data is shown in **Fig. 2**.

Morphological characters

The oldest and most widely used genetic markers are morphological traits, which are still useful for management of certain germplasm and cultivars. The main advantages of morphological markers are their simple processing, inexpensive assays; disadvantages are that they may vary with environmental conditions, and judgment is subjective. Due to these disadvantages, molecular markers (RAPD) were developed in this study. Different morphological characters were recorded (**Table 4**), as described below in more detail.

Number of sprouts: The most sprouts (6.75) were recorded in 'Puss', fewest (5.60) in 'Giza' 20. There were no significant differences between the onion lines and cultivars.

Number of seed stalk: 'Giza' 20 had the highest value (6.4), 'B-line' the lowest (6.0). There were no significant differences between the onion lines and cultivars.

Length of seed stalk: Longest seed stalks (82.6 cm) were observed in 'Giza 20', shortest in 'Puss' (72.25 cm). There were no significant differences between the onion lines and cultivars.

Diameter of seed stalk: 'A-line' had the thickest (diameter) seed stalks (3.02 cm), thinnest for 'T.E.Y.G.' (1.54 cm). There were highly significant differences between the onion lines

Table 4 Morphological characters of onion lines and cultivars.

Lines and cultivars / Morphological characters	A-line	B-line	Puss	Giza 20	T.E.Y.G.	F. Value	P. Value
Number of sprout	6.6250 a	6.6250 a	6.7500 a	5.6000 a	6.2000 a	0.285	0.884
Number of seed stalk	6.1250 a	6.0000 a	6.2500 a	6.4000 a	6.2000 a	0.22	0.999
Length of seed stalk (cm)	81.8750 a	77.0000 a	72.2500 a	82.6000 a	81.0000 a	0.854	0.507
Diameter of seed stalk (cm)	3.0250 c	2.7500 c	1.6400 b	1.6400 ab	1.5400 a	21.272	0.0000
Number of umbels	7.4250 a	7.4500 a	4.1250 c	6.5000 b	6.9600 ab	37.822	0.000
Number of seeds	449.0000 ab	222.0000 ab	95.0000 b	741.2000 a	359.0000 ab	2.008	0.130
Weight of seed/plant (g)	1.3119 ab	0.7450 b	0.4275	3.3200 a	2.0020 ab	2.348	0.88
Weight of 1000-seed (g)	3.6888 b	3.2875 b	3.6275 b	5.2900 a	5.4260 a	6.942	0.001

Means ± SE, n = 5. Means sharing the same letter in the same column is not significantly different ($P < 0.05$).

and cultivars.

Number of umbels: 'B-line' had the most umbels (7.45), but not significantly different to 'A-line' and 'T.E.Y.G.' 'Puss' had fewest umbels (4.12) and was highly significantly different to all lines and cultivars.

Number of seeds: 'A-line' produced most seeds/plant (449.000) followed by 'T.E.Y.G.' (359.000). 'Puss' produced fewest seeds/plant (95.000). There were no significant differences between 'A-line', 'B-line' and 'T.E.Y.G.'

Weight of seed/plant: 'Giza 20' had the highest seed weight/ plant (3.32) and was significantly different to 'B-line' and 'Puss', which had the lowest seed weight (0.42); 'Puss' was insignificantly different to all lines and cultivars, except for 'Giza'.

Weight of 1000-seed: 'T.E.Y.G' had the highest 1000-seed weight (5.42), lowest in 'B-line' (3.28). There were no significant differences between the onion lines and cultivars.

'Puss' had the highest mean values for number of sprouts only and the lowest mean values for seed stalk length, number of umbels, number of seeds and seed weight. These morphological results were confirmed by RAPD analysis where this cultivar was grouped in the first cluster alone, indicating that this cultivar was very different from other lines and cultivars. Since there were no significant differences between most of the cultivars and lines, morphological characters alone should not be used to identify them; molecular marker analysis is required for correct identification.

Morphological characters have been used in many studies to identify species, families and cultivars. Bendegumbal *et al*. (2008) studied seed production in onion and found that the higher seed yield in T_{10} may be attributed to increased seed weight per plant (15-339), seed yield per plant (1.96) umbel diameter (15.0 cm) and seed weight per umbels (5.10 g). However, plant height, number of leaves per plant-leaf area, bulb length diameter and weight yield onion increased significantly with increasing levels of vermicompost and fertilizer. Recently, Ebrahimi *et al*. (2009) Evaluated 17 wild Persian shallot populations from Iran by morphological and RAPD markers. They reported that cluster analysis based on RAPD data did not show any correlation with morphological characters.

Many investigators used RAPD as a tool for determining the extent of genetic diversity among onion genotypes. In this respect, the degrees of polymorphism within the genus of onion species were studied using RAPD analysis. Genetic distance between each of the cultivars were calculated and cluster analysis was used to generate a dendrogram which showing phylogenetic relationships between them (Wilkie *et al*. 1993). RAPD data of 24 cultivars of short-day onions were used to calculate a Squared-Euclidian Distance matrix (Kuttym *et al*. 2006). The results revealed a minimum genetic distance between cultivars 'AFLR-722' and 'PBR-140', and a maximum genetic distance between cultivars 'PBR-139' and 'A.Kalyan', and 'MS-48' and 'A.Kalyan'. Ebrahimi *et al*. (2009) used RAPD analysis to evaluate the diversity of 17 wild Persian shallot populations collected from different parts of Iran across the Zagross Mountains. At the similarity of 0.60 on dendrogram constructed on the base of RAPD data, the samples were divided into eight sub-clusters. The highest and lowest detected similarities were 0.73 and 0.49, respectively.

Recently, Phuong *et al*. (2009) evaluated wakegi onion (*Alllium* x *wakegi* Araki) strains as genetic resource for breeding of onions in Vietnam in comparison with Japanese bunching onion and shallot strains from Japan, Thailand. In the dendrogram of RAPD data, variation was detected between wakegi onion strains. There were many sub- groups however all the strains from the same region were not always formed into one sub-group. Three distinct groups were found as shallot group, wakegi onion group and Japanese bunching onion group. According to these results, RAPD markers can be used as a tool for grouping genotypes in heterotic groups in accordance with their pedigrees. Maniruzzaman *et al*. (2010) examined RAPD analysis to l0 varieties of onion. The polymorphisms in PCR amplification products were subjected to the unweighted pair group method for arithmetic averages (UPGMA) and plotted in a phenogram. They reported that the dendogram constructed from the similarity data showed that all the cultivars analyzed were related.

CONCLUSIONS

In conclusion, results demonstrated that RAPD analysis may serve as a major source of information for separation of closely related lines and cultivars of onion, especially when integrated with morphological measures. Generally, RAPD can be a very useful tool for evaluating existing onion germplasm collections and for assigning newly acquired onion genotypes to previously determined onion groups. This assignment will provide quick information about the genetic relationships between recently acquired onion genotypes and previously characterized onion clones, and allows germplasm collectors to predict plant characteristics of newly acquired onion genotypes. In this way, fertile and sterile plants of onion can be characterized efficiently and confirmed by RAPD analysis and morphological data.

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