ASSESMENT OF GENETIC DIVERSITY IN GARLIC CLONES USING SSR AND ISSR MARKERS

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Garlic (*Allium sativum* L.) is one of the most important bulb crops which has been known since at least 5000 years (Panthee *et al*., 2006) and cultivated 3000 years ago in Egypt (Ipek and Simon, 2001). It belongs to family *Liliaceae* and genus *Allium*, which contains more than 600 species (Osman *et al*., 2007). It is a perennial plant whose bulb is economically important as a food additive. Garlic bulbs and leaves contain very useful compounds for our health such as oligosaccharides, steroidal glycosides, essential oil, flavonoids, anthocyanins, lectins, prostaglandins, fructan, pectin, adenosine, and vitamins (Arzanlou and Bohlooli, 2010). The organosulfur compound (Allicin) is responsible for the medicinal properties of garlic (Arzanlou and Bohlooli, 2010). Moreover, the regular consumption of garlic prevents cardiovascular diseases, diabetes, asthma, and cancer (Rana *et al*., 2011). Etoh and Simon (2002) summarized the current classification of the *Allium sativum* species based on morphological, isozymes and molecular markers into four informal subspecies: the rather diverse *longicuspis* group including most garlic from Central Asia; the *subtropical* group which developed under climatic conditions of South, South East and East Asia; the *Ophioscorodon* group from Central and East Europe; and the Mediterranean s*ativum* group. Garlic has a large and complex genome with two pairs of satellite chromosomes in the basic karyotype (Lee *et al*., 2003). It is an unusual crop in that, despite being exclusively propagated asexually over centuries, it maintains a diverse phenotype amongst different clones. This makes garlic an ideal species for investigating heritage and diversity.

Traditional methods for evaluating garlic diversity rely on resolving differences in morphological characters. However, the information provided by this approach is limited since the expression of such characters may differ under varying environmental conditions. Because of phenotypic flexibility and the occurrence of mutations, the identification and systematic classification of garlic is difficult. DNA polymorphisms are the markers of choice for identification and characterization of plants. They may be representative of the whole genome and they are not subject to environmental modification (Bachmann *et al.*, 2001). Different molecular techniques have been developed to

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study garlic diversity, mostly Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP). Simple sequence repeats (SSRs) or microsatellites are a small array of tandem arranged bases (one to six) dispersed throughout the genome. They have been recognized as useful molecular markers in marker-assisted selection, analysis of genetic diversity and population genetic analysis in various species (Li *et al*., 2003; Agrama *et al*., 2007; Ma *et al*., 2009; Moe *et al*., 2010). SSRs are reported to be more variable than restriction fragment length polymorphism (RFLP) or RAPD, and they have been widely utilized in plant genomic studies (He *et al*., 2003). Therefore, these markers would be useful for assessing genetic variation among asexually propagated garlic clones. Today, SSRs are the marker of choice for a broad number of genetic studies because of their high polymorphism, co-dominance, genomic abundance, and facility in laboratory usage. The number of available polymorphic SSR markers for garlic in literatures is still few compared to other minor crops.

Inter Simple Sequence Repeat (ISSR) primers target simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and evolve rapidly (Ipek *et al*., 2003; Volk *et al*., 2004; Xu *et al*., 2005). It is a PCR-based method developed by Zietkiewicz *et al*. (1994). This method has been used to fingerprint the different plant species and cultivars (Nagaraju *et al*., 2002; Al-Humaid *et al.*, 2004) and successfully to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species. It can rapidly differentiate closely related individuals and have been successfully used to assess genetic diversity among closely related cultivars which were difficult to distinguish with other molecular markers (Dagani *et al.*, 2003; Salhi-Hannachi *et al*., 2004; Okpul *et al.*, 2005; Salhi-Hannachi *et al*., 2005).

The present work aimed to assessment of the genetic relationships of the most common garlic clones cultivated nowadays in Egypt in comparison with some of the foreign garlic genotypes using SSR and ISSR markers.

MATERIALS AND METHODS

Plant materials

Twenty one garlic clones were used in the present work. Eight of them called; Balady, EGA 4, EGA 5 (white color), Egaseed 1, EGA 1, Egaseed 2, EGA 2 and EGA 3 (Purple color) were kindly provided by Egyptian Agricultural Company for Seed Production (EGAS) while clones named AZO 1 through AZO 5 (white color) and Sids 40 (purple color) were obtained from Sids Research Station, ARC, Giza, Egypt. Plants derived from bulbils (topsets) of Egassed 2 clone which abbreviated as Egaseed 2 (ft) and Growers clone (purple color) were also included. In addition, five foreign garlic genotypes (California Late, Early Red Italian, Lorz Italian, Mexican and White Brazilian) which were friendly imported to Egypt

from Italy, Brazil, Mexico and the United States of America by MUCIA (Midwest Universities Consortium for International Activities) office (Giza, Egypt) were also incorporated. These entries were classified to the Artichoke garlic group, which belongs to *Allium sativum* subsp. *Sativum*.

Extraction of DNA

DNA was extracted in 600 µl of Cornel extraction buffer (500 mM NaCl; 100 mM Tris-HCl, pH 8.0; 50 mM EDTA and 0.84% SDS, equilibrated to 65°C, mixed with 0.38 g sodium bisulfite/100 ml buffer, and then pH of the warm buffers was adjusted to 7.8-8.0 with NaOH). DNA concentration and purity were spectrophotometrically estimated according to Sambrook *et al.* (1989).

SSR and ISSR analyses

PCR conditions for SSR analysis

SSR analysis was performed using 16 microsatellite primers developed for *Allium sativum* (Cunha *et al*., 2012). The amplification program used is described by Ma *et al.* (2009), consisting of an initial denaturing step for 3 min/94°C; followed by 30 cycles of denaturation (30s/94°C), annealing (45 s/the specific annealing temperature of each pair of primers as shown in Table (1), and extension (1 min/72°C) afterward; 10 cycles of denaturation (30 s/94°C), annealing (45 s/2°C below the specific annealing temperature of each pair of primers) and extension (1 min/72°C); and a final elongation step for 10 min/72°C.

PCR conditions for ISSR analysis

ISSR analysis was performed using D24, HB13 and HB15 primers (Al-Otayk *et al*., 2008) which include di- and tri-nucleotide repeat motifs as shown in Table (2). Amplification was performed in a thermal cycler (Thermo Hybaid) programmed for one cycle of pre-denaturation at 94°C for 2 min; and 35 cycles at 94°C for 30 s, 44°C for 45 s, and 72°C for 1.5 min; followed by 20 min of post extension at 72°C.

Amplification products of SSR and ISSR reactions were confirmed by electrophoresis in 2% agarose gels stained in ethidium bromide. Sizes of the amplified fragments were estimated according to the standard ladder of 100 bp.

Data analysis

Data of SSRs and ISSRs were scored for computer analysis on the basis of the presence (1) or absence (0) of the amplified products for each sample using GelAnalyzer3 (http://www.geocities.com /egygene, Gel Analyzer Version three, 2007). Data of the similarity matrix were used for cluster analysis by using SPSS Ver. 11 software.

RESULTS AND DISCUSSION

EGA 3 clone was discarded from the results because of its unclear results of amplification and electrophoresis. Total number of amplified fragments, number of monomorphic, polymorphic, unique bands and percentage of polymorphism obtained

by using 16 SSR primers and three ISSR primers are shown in Table (3). In SSR analysis, the number of amplified bands per primer varied between 1 and 7. All of the studied ISSR primers were polymorphic conferring a 100% of polymorphism. The availability of a relatively high number of polymorphic ISSR markers reflects the heterozygosity of the genome. These results reveal the ability of ISSR technique to detect as much polymorphism in a vegetative as in sexually propagated species. However, the percentage of polymorphism which identified by SSR primers were varied between 33.3 and 100% as shown in Table (3). The results demonstrated that Asa14, Asa17, Asa18 and Asa59 primers generated one monomorphic band of 77, 120, 102 and 113 bp, respectively, which are plain in all studied clones (Fig. 1 a, b, c, and d, respectively). Two monomorphic bands of 104 and 177 bp were generated by using primer Asa24 (Fig. 1e). Asa17 and Asa59 SSR primers produced only one unique band of 154 and 646 bp, respectively, (Fig. 1b and d). Two unique bands of 225 and 250 bp were detected from Egaseed 2(ft) by using HB 13 ISSR primer (Fig. 1g).

Cunha *et al.* (2012) used the same 16 SSR markers that were used in the present work, to aid studies of genetic diversity and to define efficient strategies for germplasm conservation of 75 garlic accessions. A total of 44 alleles were identified, using the polymorphic SSR loci, ranging from two to eight alleles per loci with an average of 4.4 alleles per loci. However, using the same 16 SSR markers, the twenty genotypes studied in the present work revealed a total number of 55 alleles, ranging from one to eight alleles per loci with an average of 3.4 alleles. These results reveal the highest effect of the environmental growth on the genetic variability of garlic clones. Therefore, more studies using different molecular markers might be required to determine the polymorphic relationship of garlic clones cultivated in each environmental area.

Cluster analysis of studied garlic clones on the basis of polymorphisms

The main objective of the study was to analyze the genetic similarity/distance between the studied clones of garlic. Similarity coefficient values among these garlic clones based on band polymorphisms generated by using SSR and ISSR primers are illustrated in Table (4). The highest similarity value (0.969) was found between AZO 2 and AZO 3, while the lowest value (0.482) was found between AZO 4 and EGA 5 clones. Table (4) showed that the level of similarity among AZO clones (AZO 1 to AZO 5) ranged from 0.556 to 0.969. The highest similarity value (0.969) among these clones was found between AZO 2 and AZO 3 while the lowest value (0.556) was found between AZO 3 and AZO 4. The results in Table (4) also indicated that similarity among EGA clones (EGA 1, EGA 2, EGA 4 and EGA5) was 88%. The level of similarity among the foreign genotypes (California Late, Early Red Italian, Lorz Italian, Mexican and White Brazil-

ian) ranged from 0.917 to 0.835 (Table 4). Foreign genotypes showing about 85% and 90% similarity with Balady clone and EGA 2 clone, respectively. Low level of similarity was observed among AZO 4 clone and other tested clones except Egaseed 1, Egaseed 2 (ft), California Late and White Brazilian (Table 4). Data in Table (4) indicated that Balady clone has about 93% similarity with AZO 1, AZO 2, AZO 3, AZO 5 and EGA 4.3

Dendrogram of genetic distances among all twenty tested clones (Fig. 2) showed two distinct major clusters with overlapping. The first major cluster was separated into two sub-clusters, one of them included most of the foreign clones (Early Red Italian, Lorz Italian and Mexican) in addition to Sids 40 and Growers clone. Balady, AZO 1, AZO 2, AZO 3, EGA 1, EGA 4 and EGA 5 formed the second sub-cluster. Likewise the second major cluster was consisted of two sub-clusters. The first one includes Egaseed 2, EGA 2, AZO 5 and California Late, while Egaseed 2 (ft) and White Brazilian formed the second one (Fig. 2).

The high level of genetic variation observed in this study is consistent with the results from previous studies of garlic carried out using different molecular markers (Ipek *et al.*, 2003, 2005 and 2008; Lampasona *et al*., 2003; Volk *et al.*, 2004), thereby confirming the great diversity among garlic accessions.

SUMMARY

Sixteen Simple Sequence Repeats (SSR) and three Inter Simple Sequence Repeats (ISSR) primers were used to estimate the genetic diversity and its distribution in twenty garlic clones. A high level of polymorphism amongst studied clones was found with both SSR and ISSR markers. The total number of bands that were detected by all used primers was 75 including 6 monomorphic, 5 unique and 64 polymorphic. The percentage of polymorphism identified by SSR primers were varied between 33.3 and 100. However, all of the studied ISSR primers were polymorphic conferring a 100% of polymorphism. Results showed that each of the Asa14, Asa17, Asa18 and Asa59 primers generated one monomorphic band of 77, 120, 102 and 113 bp, respectively, in all of the studded garlic clones. Two monomorphic bands of 104 and 177 bp were generated by using Asa24 primer. Asa17 and Asa59 SSR primers produced only one unique band of 154 (Egaseed 2) and 646 bp (EGA 1), respectively. Two unique bands of 225 and 250 bp were detected for Egaseed 2 (ft) by using HB 13 ISSR primer. The highest similarity value (0.969) was found between AZO 2 and AZO 3, while the lowest value (0.482) was found between AZO 4 and EGA 5 clones. Dendrogram of genetic distances amongst all tested clones showed two distinct major clusters with overlapping. In general, the present results reveal the importance of using molecular markers to assess genetic diversity among such closely related

genotypes which were difficult to distinguish with other markers.

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(SSR)-anchored polymerase chain reaction amplification. Genomics, 20: 176-18.

Table (1): Characteristics of the 16 microsatellite primers developed for *Allium sativum.* Shown for each primer pair are the forward and reverse sequence, repeat type, annealing temperature when run individually, size of the original fragment, and GenBank accession number (Cunha *et al.*, 2012).

Loci	Primer sequence (5′ – 3′)	Repeat motif	*T* a (°C)	Allele size (bp)	GenBank Accession No.
Asa04	F: AGACTTTTGGAGGCTAGGGC R: CCCTGGTCTCTTTCAACCAA	(TCC) 5 (TCC) 4 (TCC) 5	54	264	JN084085
Asa06	F: GGGGTGTTACATTCTCCCCT R: ACCGCCTGATTTTGCATTAG	(TG) 5	57	192	JN084086
Asa07	F: CTCGGAACCAACCAGCATA R: CCCAAACAAGGTAGGTCAGC	(TG) 7	58	229 – 235	JN084087
Asa08	F: TGATTGAAACGAATCCCACA R: GGGGGTTACCTGAACCTGTTA	(GT) 8	56	209 – 257	JN084088
Asa10	F: TTGTTGTTCTGCCATTTT R: GATCTAAGCCGAGAGAAA	(AC) 7	48	225 – 239	JN084089
Asa14	F: TCTATCTCGCTTCTCAGGGG R: GCTGACAGAAGTAGTCTTTCC	(GT) 7	48	220 – 234	JN084090
Asa16	F: CACGACTTTTCCTCCCATTT R: GCTAATGTTCATGTCCCCAGT	(TG) 5 C(GT) 6	48	148 – 154	JN084091
Asa17	F: TCCACGACACACACACACAC R: ATGCAGAGAATTTGGCATCC	(CA) 12 (CT) 28	56	126 – 196	JN084092
Asa18	F: TCAAGCTCCTCCAAGTGTCC R: TCGGGATATGACAGCATTTG	(TG) 8	45	254 – 264	JN084093
Asa20	F: GAAGCAGCAAAGATCCAAGC R: CGTGCAGAACTTAACCTT	(G) 12	48	260	JN084094
Asa23	F: TGGAGGGGGAAAAAGGATAG R: TGTGAAGCAAGTGGGATCAA	(GA) 5	55	271	JN084095
Asa24	F: TTGTTGTGCCGAGTTCCATA R: CAGCAATTTACCAAAGCCAAG	(GT) 4 (GT) 3 (GT) 5	48	149 – 161	JN084096
Asa25	F: GCACTTCACTTTCCCCATTC R: GGCGACGGTGAAGAGAGAG	(CT) 3 (CT) 27	51	117 – 127	JN084097
Asa27	F: GGGAGAGAATGGCTTGATTG R: GGACAGCATCATCACCAC	(TC) 17 (TC) 5	55	127	N084098
Asa31	F: CAGAGACTAGGGCGAATGG R: ATGATGATGACGACGACGAG	(CTT) 7	50	237 – 243	JN084099
Asa59	F: CGCTTACTATGGGTGTGTGTC R: CAAGTGGGAGACTGTTGGAG	(ATCA) 3	50	290	JN084100

Note: T a = annealing temperature.

Table (2): ISSR primers characteristics (Al-Otayk *et al*., 2008).

Primers	Sequence 5` to 3`	Annealing temperature (°C)
D24	(CA)6CG	44
HB 13	(CTC)3GC	44
HB 15	(GTG)3GC	44

Table (3): Total number of monomorphic, polymorphic and unique PCR fragments and percentage of polymorphism obtained by using 16 SSR and 3 ISSR primers for studied garlic clones.

Primers	Monomorphic bands	Unique bands	Polymorphic bands		Total number of bands	Polymorphism (%)
			without Unique	with Unique		
Asa04	0	0	3	3	3	100.0
Asa06	0	0	3	3	3	100.0
Asa07	0	0	4	4	4	100.0
Asa08	0	1	5	6	6	100.0
Asa10	0	0	4	4	4	100.0
Asa14	1	0	2	2	3	66.7
Asa16	0	0	2	2	2	100.0
Asa17	1	1	0	1	2	50.0
Asa18	1	0	2	2	3	66.7
Asa20	0	0	4	4	4	100.0
Asa23	0	0	3	3	3	100.0
Asa24	2	0	1	1	3	33.3
Asa25	0	0	1	1	1	100.0
Asa27	0	0	3	3	3	100.0
Asa31	0	0	3	3	3	100.0
Asa59	1	1	6	7	8	87.5
D 24	0	0	8	8	8	100.0
HB 13	0	2	5	7	7	100.0
HB 15	0	0	5	5	5	100.0

Table (4): Similarity coefficient values among studied garlic clones based on bands polymorphism generated by SSR and ISSR-PCR primers.

Garlic Clones	Egaseed1	Sids 40	Egaseed 2	Egaseed 2 (ft)	Growers clone	Balady	AZO1	AZO2	AZO3	AZO4	AZO5	EGA1	EGA2	EGA4	EGA5	California Late	Early Red Italian	Lorz Italian	Mexican
Sids 40	.733																		
Egaseed 2	.713	.851																	
Egaseed 2 (t)	.720	.652	.744																
Growers clone	.689	.865	.871	.652															
Balady	.653	.783	.839	.700	.852														
AZO1	.617	.796	.857	.710	.852	.924													
AZO2	.713	.835	.839	.700	.904	.952	.908												
AZO3	.718	.838	.825	.667	.889	.953	.909	***.969***											
AZO4	.730	.545	.622	.645	.623	.568	.568	.568	.556										
AZO5	.729	.808	.896	.738	.848	.836	.874	.836	.821	.694									
EGA1	.707	.832	.836	.694	.885	.919	.889	.952	.937	.581	.852								
EGA2	.744	.880	.866	.706	.900	.811	.827	.847	.850	.685	.884	.862							
EGA4	.722	.847	.833	.625	.937	.902	.852	.934	.952	.595	.830	.917	.879						
EGA5	.667	.782	.785	.695	.836	.893	.842	.926	.927	***.482***	.800	.908	.811	.906					
California Late	.675	.845	.915	.732	.887	.833	.851	.833	.818	.714	.891	.830	.925	.846	.777				
Early Red Italian	.761	.868	.874	.681	.925	.872	.836	.906	.908	.633	.871	.922	.941	.938	.875	.909			
Lorz Italian	.651	.880	.845	.659	.880	.847	.808	.847	.850	.603	.821	.826	.896	.879	.811	.903	.902		
Mexican	.710	.822	.827	.674	.897	.881	.811	.915	.883	.600	.824	.897	.874	.895	.885	.840	.917	.835	
White Brazilian	.593	.779	.848	.725	.842	.811	.808	.811	.778	.618	.844	.827	.879	.804	.832	.886	.866	.835	.857

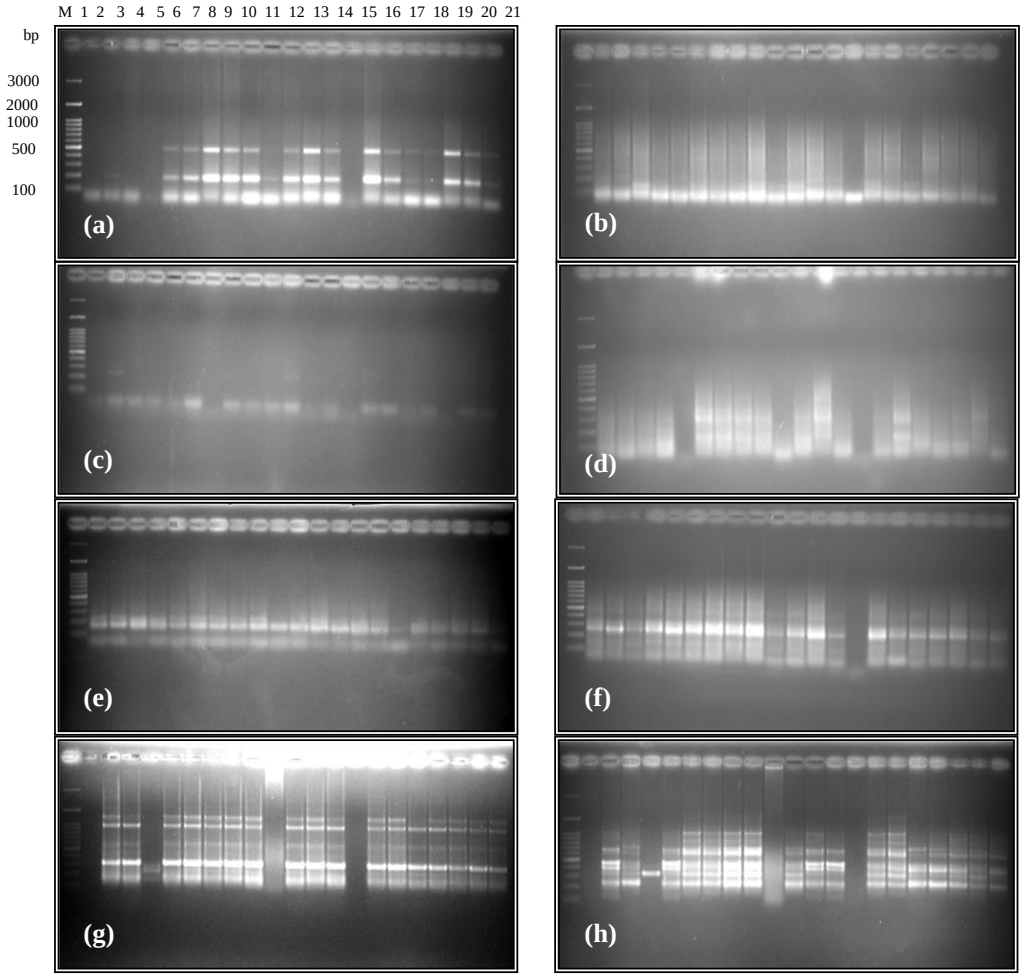

Fig. (1): Electrophoretic paterns of some SSR and ISSR primers used in the present work (a: Asa18, b: Asa17, C: Asa14, d: Asa59, e: Asa24, f: Asa08, g: HB 13, h: D 24). M: 100 bp ladder marker and lanes 1 through 21 refer to: Egaseed 1, Sids 40, Egaseed 2, Egaseed 2(ft), Growers clone, Balady, AZO 1, AZO 2, AZO 3, AZO 4, AZO 5, EGA 1, EGA 2, EGA 3, EGA 4, EGA 5, California Late, Early Red Italian, Lorz Italian, Mexican and White Brazilian, respectively.

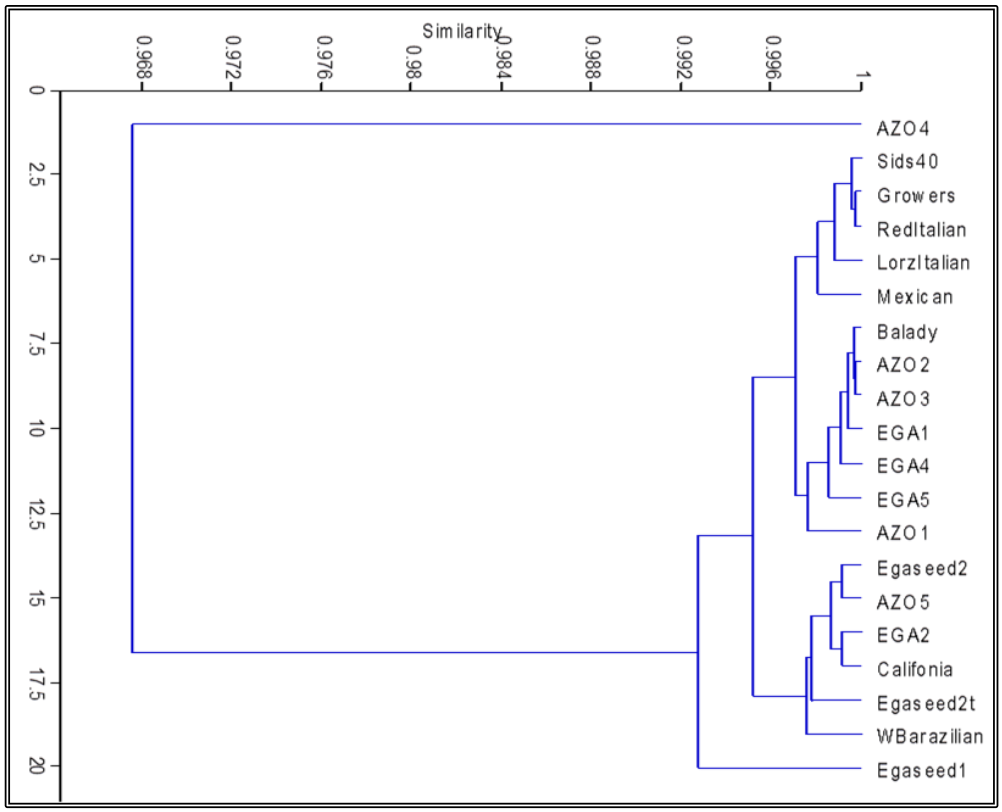

Fig (2): Dendrogram of the genetic distances among studied garlic clones based on the bands polymorphism generated by SSR and ISSR-PCR primers.