GENETIC DIVERSITY AMONG SOME SPECIES OF THE GENUS *ALLIUM* L. USING SSR AND ISSR MARKERS

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Genus *Allium* L. comprises more than 800 species (Fritsch *et al.*, 2010), making it one of the largest monocotyledonous genera. It is a variable genus that is spread widely across the Holarctic region from the dry subtropics to the boreal zone. *Allium* includes some economically important species like common onion, garlic, chives, and leek under worldwide cultivation, and also species with medicinal properties and others of horticultural merit (Fritsch and Friesen, 2002). Several morphological and anatomical studies on *Allium* were carried out, and numerous data have been helpful in establishing and assessing the evolutionary lineages in the genus and dealing with newly described taxa (Cheremushkina, 1992; Hanelt and Fritsch, 1994; Friesen, 1995; Mathew, 1996; Khassanov, 1997; Xu and Kamelin, 2000; Dale *et al.*, 2002; Fritsch, 2009; Fritsch and Friesen, 2009). Cytological diversification is also well known in species belonging to genus *Allium* (El-Mamlouk *et al.*, 2002; Ata, 2005; Osman *et al.*, 2007; Ata and Osman, 2009; Ata *et al.*, 2010). The most common basic chromosome number is x = 8, but other numbers (x = 7, 9, 10, 11) and variation in ploidy also occurs (Xu *et al.*, 1998; Zhou *et al.*, 2007). DNA-based molecular markers were used in studies of genetic diversity and phylogenetic analysis in plants (Savolainen and Chase, 2003; Nybom, 2004). A first approach to characterize the genus *Allium* by molecular markers was published by Linne von Berg *et al.* (1996) who conducted a chloroplast DNA RFLP analysis. Among the different molecular markers, Inter-simple sequence repeats (ISSRs) is a PCR-based method developed by Zietkiewicz *et al.* (1994). ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific levels in a wide range of crop species (Ajibade *et al.*, 2000; Huang and Sun, 2000). ISSRs would be a better tool than RAPD for phylogenetic studies (Nagaoka and Ogihara 1997). For instance, ISSR primers produce more reliable and reproducible bands than RAPD primers. Few works have been done in different species, varieties and cultivars of *Allium* with these markers (Hao *et al.*, 2002; Son *et al.*, 2012). In the genus *Allium*, Fischer and Bachmann (2000) reported the development of 30 SSR markers on onion bulbs. Otherwise, Wako *et al.* (2002) and Song *et al.* (2004) also succeeded in isolating 118 SSRs from a genomic library of bunching onion. Conversely, for the garlic genome, a limited

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number of SSR markers have been developed and few are available to generate high density genetic maps in a crop with such a large genome size (Cunha *et al.*, 2012; Ipek *et al.*, 2012). However, there are still many gaps in our knowledge of infra-generic taxonomy, differentiation and evolution in the genus *Allium*. Thus, the goal of the present study was to study and clarify the diversity and phylogenetic relationships among some species of the genus *Allium* (*Allium sativum* L., *Allium cepa* L. and *Allium currat* L.) cultivated in Egypt, using SSR and ISSR markers.

MATERIALS AND METHODS

Plant materials

Two garlic (*Allium sativum* L.) clones called Balady or Egyptian (white bulbs) and EGA 3 (purple bulbs), one Egyptian leak (*Allium currat* L.) cultivar and two onion (*Allium cepa* L.) cultivars called Giza Red and Giza 6 Mohassan were used in the present study. All of these cultivars were kindly provided by the vegetable Branch, Horticulture Department, Faculty of Agriculture, Minia University.

Extraction of DNA

Total genomic DNA was extracted from young leaves of the aforementioned genotypes following the procedure described by Anwar *et al.* (2016). The concentrations and purities were spectrophotometrically estimated according to the procedure described by Sambrook *et al.* (1989).

PCR conditions for SSR analyses

Sixteen microsatellite primers (Table 1) developed in garlic *(Allium sativum* L.*)* by Cunha *et al.* (2012) were used in the present work. The PCR program was as follows: 3 min/94°C; followed by 30 cycles of denaturation (30s/94°C), annealing (45s/the specific annealing temperature of each pair of primers), and extension (1 min/72°C) afterward; 10 cycles of denaturation (30 s/94°C), annealing (45s/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 in accordance with the protocol of Ma *et al.* (2009).

PCR conditions for ISSR analyses

Three ISSR primers (D24, HB13 and HB15) were designed by Al-Otayk *et al.* (2008). The complete list of the primers and their sequences are shown in Table (2). Amplification was performed in a thermal cycler (Thermo Hybaid) programmed following the protocol of Al-Otayk *et al.* (2008). PCR products of SSR and ISSR reactions were separated using 2% (w/v) agarose stained in ethidium bromide. Sizes of the amplified fragments were estimated according to the standard ladder of 100 bp.

Data analyses

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)**.**

Sequence comparisons

Purified PCR product of SSR primer Asa20 (one monomorphic fragment) was sequenced directly in both directions by Lab. Technology laboratory as sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems). In order to find homologous sequences, the sequences earlier obtained were used to retrieve the sequences belonging to different genera from the BLAST database on NCBI (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequences were aligned with *Allium sativum* microsatellite Asa20 sequence (Cunha *et al*., 2012), that was retrieved from the NCBI Database (accession no. JN084094) using the Clustal Omega (Multiple Sequence Alignment) program (http://www.ebi.ac.uk/Tools/msa/clustalo) . Gaps were positioned to minimize nucleotide mismatches and treated as missing data in phylogenetic analysis.

RESULTS AND DISCUSSION

In this study, genetic diversity of some cultivars of the genus *Allium* L. using two different marker systems (SSR and ISSR markers) was investigated. Total numbers and profiles of monomorphic, polymorphic and unique bands along with the percentage of polymorphism in plants of Balady and EGA3 garlic (*Allium sativum* L) clones, Egyptian leak (*Allium currat* L.) cultivar and Giza Red and Giza 6 Mohassan onion (*Allium cepa* L.) cultivars using sixteen SSR and three ISSR primers are shown in Table (3). The used 19 primers generated a total of 100 fragments, distributed as, 39 monomorphic (39%) and 33 polymorphic (33%). The remaining 28 bands were identified as unique, 27 of them from SSR and one from ISSR. However, it may be converted into cultivar-specific markers following further studies based on the present observations (Table 3). The highest number of polymorphic fragments was detected by Asa10 (92.9%), then HB13 (85.7%), Asa59 (83.3%), Asa31 (77.8%) and HB15 (75%) primers. However, the percentage of polymorphism (66.7%) was observed by applying Asa16, Asa18, Asa24, Asa25 and Asa27 primers (Table 3). The lowest percentage of polymorphism (25%) was detected by Asa07 SSR primer.

Out of the 16 SSR primers applied with all studied *Allium* taxa samples, three primers (Asa14, Asa17 and Asa20) generated only one monomorphic band (0.0% polymorphism) of 88, 119 and 247 bp, respectively. Likewise, Asa04 primer showed only five common fragments of 98, 200, 283, 434 and 587 bp, while each of the Asa06 and Asa23 primers produced four common monomorphic bands in all clones and cultivars at molecular size of (95, 210, 366 and 507 bp) and (263, 428, 609 and 724 bp), respectively. Three

common fragments (108, 221 and 326 bp) were detected in all studied materials by using primer Asa07 (Fig. 1).

In addition to the polymorphic bands, each of the Asa08, Asa16, Asa18, Asa27 and Asa31 primers showed two monomorphic bands of (107 and 213 bp), (122 and 188 bp), (86 and 166 bp), (91 and 118 bp) and (92 and 832 bp), respectively (Fig. 1). Similarly, four SSR primers (Asa10, Asa24, Asa25 and Asa59) amplified one monomorphic band (Fig. 1) of 100, 102, 104 and 89 bp, respectively. In the present study, ISSR markers provided six monomorphic bands. Of them, one common band (288 bp) produced by using HB13, two (131 and 173 bp) with HB15 and three (203, 277 and 363 bp) with D24 ISSR primers (Fig. 1). According to the highest variability known for SSR and ISSR regions, these results of similarity demonstrate the presence of a concealable relationship amongst *Allium* species.

A total of 33 polymorphic bands without unique have been detected amongst the studied clones and cultivars (Table 3). Fourteen bands of 441 and 1186 bp (Asa18), 162 and 222 bp (Asa24), 490 bp (Asa27), 140 and 580 bp (Asa31), 253, 357 and 433 bp (Asa59), 1104 and 1439 bp (HB13 ISSR) and 311 and 1254 bp (HB15 ISSR) were observed in the two garlic (*Allium sativum* L.) clones namely Balady and EGA 3 (Fig. 1) and absent in the remaining cultivars. Such observations might suggest the use of these primers as molecular genetic marker for identifying garlic clones. Eight polymorphic bands (228 and 300bp by using Asa31; 208, 489 and 649 bp by using HB13 ISSR primer and 328, 415 and 524 bp using HB15 ISSR primer) could distinguish the two onion (*Allium cepa* L.) cultivars (Giza Red and Giza 6 Mohassan). Data analysis using primer HB15 illustrated one polymorphic band of 240 bp presented in Egyptian Leek and the two onion cultivars (*Allium cepa* L.) (Fig. 1). These results display the significant degree of similarity among clones belonging to each species of the genus *Allium*.

From the 28 unique fragments, data in Fig. (1) showed the highest numbers of unique bands in the cultivar Egyptian Leek (17 bands for SSR primers). Only one unique band of 578 bp was appeared in onion Giza 6 Mohassan cultivar by using Asa10 SSR marker, while three unique bands were detected in onion Giza Red cultivar. Two (806 and 1210 bp) and five (226, 293, 296, 984 and 1226 bp) unique bands were generated in Balady and EGA3 clones, respectively (Fig. 1).

The used 16 SSR primers in the present work may support the conclusion of Cunha *et al.* (2012) who developed these primers. The polymorphic markers developed in their study were useful for further studies of genetic diversity, allele mining, mapping and associative studies, in the management and conservation of garlic collections, and potentially for interspecific genetic studies within the genus *Allium*. The genetic variability within germplasm collections, assessed by those

SSR markers, was a source of information for breeding programs to obtain new varieties with higher nutritional value. Anwar *et al.* (2016) used the same 16 SSR and 3 ISSR markers that were used in the present work, to aid studies of genetic diversity among garlic clones. In agreement with our present data, they found a high level of polymorphism among the studied clones 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 was varied between 33.3 and 100. However, all of the studied ISSR primers were polymorphic conferring a 100% of polymorphism.

Sequence comparisons

DNA sequence of the monomorphic band generated from the five clones and cultivars used in the present work (2 garlic clones; 1-Egyptian leak and 2-onion cultivars) by SSR primer Asa20 were aligned together and with the earlier obtained sequences belonging to different genera and species of the BLAST database on NCBI. Although all of these sequences were obtained from one monomorphic band using the same primer, the pairwise alignment of the two garlic clones showed only 73.76% of homology (Table 4 & Fig. 2a). Furthermore, nearly the same percentage of homology (75.49%) was obtained between the sequences generated from the two onion cultivars (Table 4 & Fig. 2b). In general, the multiple alignments of the five sequences did not reveal a considerable degree of homology (Table 4). The dendrogram based on genetic similarities among cultivars showed that there are three major clusters (Fig. 3). Cluster 1 included only the Egyptian leek cultivar; cluster 2 comprised the two garlic clones and cluster 3 composed of the two onion cultivars. Data demonstrate the significant differences of the monomorphic SSR bands amongst the three *Allium* species and even between the cultivars belonging to the same species. Therefore, for classification and studying the phylogenetic relationships amongst and between species, much more primers and/or molecular markers should be carried out. Thus, construction of a phylogenetic tree according to the banding pattern of the PCR products using a limited number of primers might produce misleading data.

All of these sequences were aligned with *Allium sativum* microsatellite Asa20 sequence (Cunha *et al.*, 2012), that was retrieved from the NCBI Database (accession no. JN084094). According to the percentages of homology, garlic (*Allium sativum* L.) clones called Balady was the closest one to *Allium sativum* microsatellite Asa20 with 85% of homology. Likewise, the second garlic clone (EGA3) showed a considerable degree (81%) of homology to that reference sequence. However, Egyptian Leek as well as the two onion cultivars (Giza Red and Giza 6 Mohassan) showed low significant degree of homology with *Allium sativum* microsatellite Asa20 (Table 4). These results, also, represent the highest variability of

SSR regions between species although the significant conservation of the outside regions used for designing the used primers.

SUMMARY

Genus *Allium* includes some economically important species like common onion, garlic, chives, and leek under worldwide cultivation. In this study, genetic diversity of some cultivars belonging to three species (*Allium sativum* L., *Allium cepa* L. and *Allium currat* L.) of this genus was investigated using sixteen SSR and three ISSR primers. All primers generated a total of 100 fragments, distributed as, 39% monomorphic, 33% polymorphic and 28% unique. The percentage of polymorphism identified by SSR and ISSR primers varied between 25 and 92.9%. With the highest variability detected for SSR and ISSR regions, the results of similarity demonstrated the presence of a concealable relationship amongst *Allium* species with a significant degree of similarity among clones belonging to the same species of this genus. DNA sequence of the monomorphic band generated from the five clones and cultivars used in the present work by one of the SSR primers (Asa20) were aligned together and with the earlier obtained sequences belonging to different genera and species. Although all of these sequences were obtained from one monomorphic band using the same primer, the pairwise alignment of the two onion clones showed only 75.49% of homology and nearly the same percentage of homology (73.76%) was obtained between the sequences generated from the two garlic clones. The dendrogram based on genetic similarities between cultivars showed three major clusters. Cluster 1 included only the Egyptian Leek cultivar; cluster 2 comprised the two garlic clones and cluster 3 composed of the two onion cultivars. Generally, the present results corroborate the idea that SSR and ISSR techniques seems to be a convenient tool for genetic diversity of the genus *Allium*.

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Table (1): Characteristics of the 16 SSR microsatellite primers developed for *Allium sativum,* 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): Characteristics of the ISSR primers used in the present work (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 as well as percentage of polymorphism obtained from garlic (Balady and EGA3) clones, Egyptian leak cultivar and Giza Red and Giza 6 Mohassan onion cultivars by using 16 SSR and three ISSR primers.

Primers	Monomorphic bands	Unique bands	Polymorphic bands		Total number of bands	Polymorphism (%)
			without Unique	with Unique		
Asa04	5	0	0	0	5	0.00
Asa06	4	0	0	0	4	0.00
Asa07	3	1	0	1	4	25.0
Asa08	2	2	1	3	5	60.0
Asa10	1	11	2	13	14	92.9
Asa14	1	0	0	0	1	0.00
Asa16	2	3	1	4	6	66.7
Asa17	1	0	0	0	1	0.00
Asa18	2	2	2	4	6	66.7
Asa20	1	0	0	0	1	0.00
Asa23	4	2	0	2	6	33.3
Asa24	1	0	2	2	3	66.7
Asa25	1	2	0	2	3	66.7
Asa27	2	1	3	4	6	66.7
Asa31	2	3	4	7	9	77.8
Asa59	1	0	5	5	6	83.3
D 24	3	1	1	2	5	40.0
HB 13	1	0	6	6	7	85.7
HB 15	2	0	6	6	8	75.0
	39	**28**	**33**		**100**	

Table (4): Percent Identity Matrix - created by Clustal2.1 from monomorphic fragment (247 bp) data.

Clones and cultivars	*Allium sativum* microsatellite As20	Balady	EGA 3	Egyptian Leek	Giza Red
Balady	85.00				
EGA 3	81.00	73.76			
Egyptian Leek	55.40	54.50	56.52		
Giza Red	45.81	61.31	69.71	51.83	
Giza 6 Mohassan	51.93	65.13	73.40	57.67	75.49

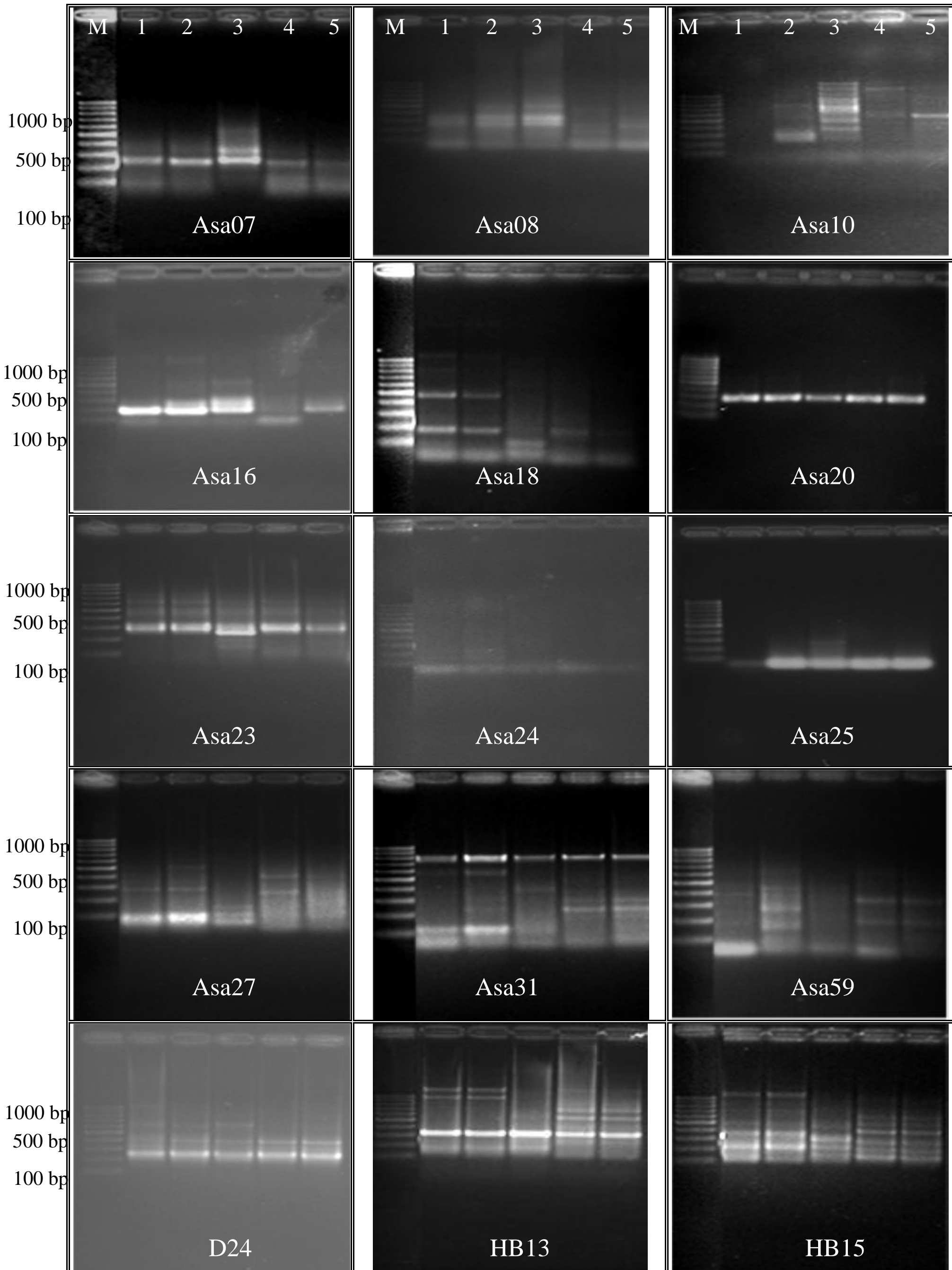

Fig. (1): Electrophoretic patterns of PCR product of 12 representative SSR and three ISSR primers. (M: 100 bp ladder marker and lanes 1 through 5 refer to: garlic clones (Balady, EGA 3), Egyptian Leak cultivar and onion cultivars (Giza Red and Giza 6 Mohassan), respectively. The used primer names are shown below their corresponding gel.

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Balady      -------TCGGCATGCCAGTATGTTGTTATCCATATGTTCATCTCATC-TTCTCATGCCG   52
EGA3        AGTGGCAGTAGCAAGCCAATAAAAAGATAACAATAAAACCACCTCAACAAACACAAGCCA   60
                        .***:****.**:.::*:**:*.***:..: ** ****:*::*:**:***

Balady      GTTAACTTTTCTAGTTCATAGACGGATAGAAGTAGGGCTTACCCGTCTTTTTGACTGAAC   112
EGA3        GTTAACTTTACTATTTCATAAACAAATAAAAAAAGGGCTTACCAGTCTTTTTAAAAGAAC   120
            *********:*** ******.**..***.**.:**********.********.*.:****

Balady      T-GTTTTCTTTCGCCTCTTTTCTTCCGTTTCCCCCCCCCACAAAAAAAGGAGGGGGGGGT   171
EGA3        TAGTTTTCTTTCCCCCCTTTTCTTCCGTTTCCCCCCCCCCCAAAAAAAAAAGGGGGGGAT   180
            * ********** ** **********************.********..********.*

Balady      CCCCCACCCAAGGGTGGGTTTTTCTCCCCCCA                               203
EGA3        CCCAAAAAAA-ATTTTTTTTTTTTTCCCCCCA                               211
            ***..*...* .  *   ***** ********
```

Fig. (2 a): Schematic diagram of the pairwise alignment of the two garlic clones (Balady and EGA3).

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Giza Red       TTT----AACTAAGACACAGTAATAAGTATAGATAAAACCACAATAAATTCCTTCCCCTA  56
G.6 Mohassan   GTTGACGAGAAGGGCCACAGAATA-AGT--AGA----TAAACAAAAAG-TTCTT--CCAA  50
                **    *..:..*.*****:*:: ***  ***    :..****:**. * ***  **:*

Giza Red       AAACTCAAGCCAATCCCCTTTTCTATTTTTATAAAAAAAAAAAAAATAATTACCCCATCT  116
G.6 Mohassan   AAAATCAAGGCGGTTACCTTTTTTATTTTTATAAAGAAAAAAAAATGAATTACTCAATCT  110
               ***.***** *..* .****** ************.*********: ****** *.****

Giza Red       TTTTTAAAAAACCCTTTTCTTTTCCCTCCTTTCCTCCCTTTCCCCCCCCCCCCAAAAAAA  176
G.6 Mohassan   TTTTAAAAGAAACATTTTTTTTTCCCTTCTTTTTTTCCGTTTCCCCCCCCCCCAAAAAAA  170
               ****:***.**.*.**** ******** ****  * ** ** *****************

Giza Red       AAGGGGGACCCCCCCCACAAAATTTTTTTTTTTCCCCCCCA                     217
G.6 Mohassan   AAGGGGGAATCCCAAAAAAAATTTTTTTTTTTTTGCCCA---                    208
               ********. ***...*.**::*********** ***.
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Fig. (2 b): Schematic diagram of the pairwise alignment of the two onion cultivars (Giza Red and Giza 6 Mohassan).

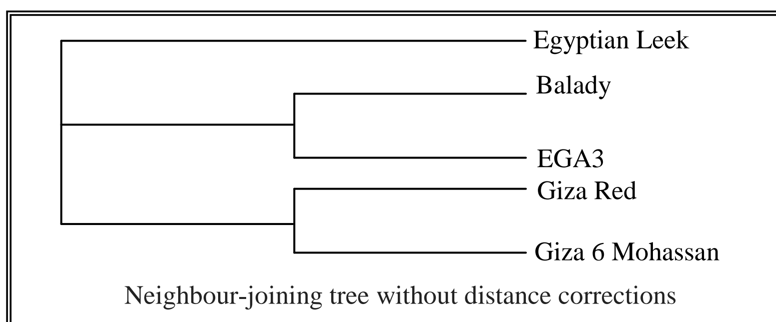

Fig.(3): Cluster analysis of the five studied *Allium* genotypes (Balady, EGA 3 of garlic, Egyptian Leak cultivar and onion cultivars Giza Red and Giza 6 Mohassan) based on the multiple alignments of their five sequences generated by Asa20 primer.