

BIOCHEMICAL AND MOLECULAR IDENTIFICATION OF BOTTLE GOURD (*Lagenaria siceraria*) CULTIVAR

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Bottle gourd is annual herbs (Edwin-Wosu and Ndukwu, 2008). The leaves of bottle gourd are alternate and variable. The flowers are unisexual and white; they are present on the same plant (monoecious). The fruit have bottle shape. It has white pulp, often is an indehiscent gourd (hard-shelled). Bottle gourd fruit is traditionally use for its cardio protective, cardio tonic, general tonic and aphrodisiac properties. It is also use in treatment of various allergic and inflammatory disorders like bronchial asthma, rhinitis, bronchitis and rheumatism (Gangwal et al., 2010).

There are about 18 genera and 75 species of this family in Southern Africa. Beside the range of species already cultivated, many species are known to occur in the wild (at least six of such species) with their nutritional potential and other uses apparently unknown. There is a need to develop accurate data, and to put in place more effort toward the cultivation, improvement and conservation of various cucurbits germplasm (Edwin-Wosu and Ndukwu, 2008)

Genetic diversity refers to the variation of genes within species, that is, the

heritable variation within and between populations. Other kinds of genetic diversity can also be identifying at all levels of organization in the nucleus, including the amount of DNA per cell, chromosome number and DNA structure (Tasrif *et al.*, 2004).

The advent of molecular biology techniques has provided another dimension to detect genetic polymorphism based on protein or DNA facilitating in biological research branches such as phylogenetic relationships, taxonomy and genetic diversity (Williams *et al.*, 1990; Saghai *et al.*, 1994). In this regards, proteins and enzymes polymorphism have been successfully used to identify cultivars in various fruit and crop species, including Bottle gourd (Upadhyay and Ram, 2006), sunflower (Raymond *et al.*, 1995), *Cucurbitaceae* species (Pasha and Sen, 2003), *Populus* spp. (Su and Su, 1999) and *Acacia* (Ahmed *et al.*, 2003). On the other hand, many investigators employed RAPD-PCR to assay genetic fingerprint and diversity in wide range of plants such as *Artemisia* (Sangwan *et al.*, 1999) and *Juniperus* (Ashworth and Obriedand, 1999). In addition, ISSR-PCR are used for the same purpose in plants such as

Cucurbita species (Heikal *et al.*, 2008), *Cauliflower* (Bornet *et al.*, 2002), *Plantago* (Wolf and Morgan, 1998) and *Almis* (King and Ferris, 2000).

The aim of this study is to determine the biochemical (protein SDS-PAGE and isozyme) and molecular (RAPD and ISSR-PCR) properties in different species of Bottle gourd

MATERIALS AND METHODS

Materials

Five cultivars of Bottle gourd, collected from different locations Egypt, Syria and India were used in this study as shown in Table (1).

Methods

The present study was carried out at Ain Shams Center of Genetic Engineering and Biotechnology, Fac. Agric., Ain Shams Univ. (ACGEB).

I. Biochemical genetic studies

a. SDS-PAGE for protein

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate SDS-seed storage protein according to the method of Laemmli (1970) and modified by Studier (1973).

b. Isozymes electrophoresis:

Bottle gourd seeds were soaked for 24 hours before extracting enzymes, Three isozyme systems, esterases include peroxidase and acid phosphatase, were per-

formed according to Stegemann *et al.* (1985) using 8% native-PAGE system.

II. Molecular genetic studies

Molecular characterization

Genomic DNA was extracted from leaf sample according to Junghans and Metzlatt (1990). The sequences of six oligonucleotide primers for RAPD are listed in Table (2), and for ISSR are listed in Table (3). The primers were synthesized by Genemed Synthesis Inc., USA.

PCR conditions

PCR conditions for RAPD analysis were applied as specified by Williams *et al.* (1990), an initial strand separation cycle at 94°C for 4 min, followed by 35 cycles including a denaturation step at 94°C for 1 min, an annealing step at 36°C for 1 min and polymerization step at 72°C for 2 min with a final extension step at 72°C for 10 min final at 4°C. The ISSR conditions were as following the initial strand separation cycle at 94°C for 4 min, followed by 35 cycles including a denaturation step at 94°C for 1 min then an annealing step at 57°C for 1 min and polymerization step at 72°C for 2 min with a final extension step at 72°C for 10 min. PCR was carried out in a 25 µl volume containing 50 p moles of each primer, 1.25 mM dNTPs, 50 ng of template DNA, and 1 U of *Taq* DNA polymerase. Supplied buffers with the enzyme were used according to the manufacturer's directions. After the reaction, 12 µl of amplified DNA was separated on 1.2% agarose gels (Sigma), which were

stained with ethidium bromide and were recorded with Gel Doc system (Bio-Rad). All PCR conditions were performed at least three independent times to ensure reproducibility.

Data analysis

The data obtained were exposed to proper statistical analysis according to statistical analyses system user's guide (SPSS, 1998). RAPD and ISSR profiles using Quantity One software (Bio-Rad) which identifies bands using an optimized set of parameters (as reported in Quantity One user guide for version 4.2 Windows Bio-Rad Laboratories) adjusted manually by visual inspection.

The results of bands identification were used to create a qualitative data matrix of presence (1) and absence (0) that using SPSS software, version 8. Pairwise similarities between cultivars were calculated using Jaccard's coefficient for qualitative data (Jaccard, 1908) according to the formula: Jaccard's coefficient = $a/(n-d)$, where n: is the total number of polymorphic bands, a: the bands present in both *cultivars* and d: the bands absent in both accessions. The resulting similarity matrix (calculated with the Jaccard coefficient) was used to construct a dendrogram by means of the UPGMA algorithm (unweighted pair-group method with arithmetical averages) (Sensi *et al.*, 2003).

RESULTS AND DISCUSSION

Table (4) illustrated the polymorphism and numbers of bands from bio-

chemical and molecular electrophoretic systems in the five cultivars of bottle gourd.

Biochemical genetic analysis for Bottle gourd

Biochemical genetic analysis has been conducted based on SDS-proteins and isozymes polymorphism to distinguish between the studied of bottle gourd cultivars.

a. SDS-Protein PAGE

Electrophoretic banding patterns of SDS-proteins for all the studied bottle gourd cultivars are illustrated in Fig. (1). The resulted profile of SDS-PAGE showed seventeen bands, the molecular weights of the recorded bands ranged from 28.5 to 107.6 KDa. Eleven monomorphic bands, three polymorphic bands and three unique bands (79.109, 62.239 and 41.103 KD) in cultivar Hama2 and LE were observed in electrophoresis gel. These results showed that SDS-protein electrophoresis harbored 35.3% polymorphism. The obtained results were in accordance with the results obtained by Megha and Ram (2006) in bottle gourd, Pasha and Sen (2003) in several taxa of Cucurbitaceae and Jagesh *et al.* (2006) in pumpkin

b. Isozymes variations

Isozyme electrophoresis analysis showed that eight different bands were observed in four enzyme systems. Only two unique bands in β -esterase was detected among five tested cultivars of Bot-

tle gourd which achieved polymorphism level in all enzymatic systems reach to 42% among the tested cultivars of bottle gourd. The highest percent of polymorphism was 50% in α -esterase, 67% in β -esterase (Fig. 2a) (Fig. 2b) and Acid phosphate (Fig. 2c). While there was no polymorphism in Peroxidase (Fig. 2d). One monomorphic band was observed in all enzymatic systems, and one polymorphic band observed in α -esterase and Acid phosphate. The obtained results were in accordance with the results obtained by Amaral-junior *et al.* (1994) in pumpkin to reveal esterase (EST), acid phosphatase (ACP), Amaral-Junior *et al.* (1996) studied seven pumpkin to reveal acid phosphatase and peroxidase Carvalho *et al.* (2003) in *Lagenaria siceraria* to reveal α - and β -esterases.

Molecular genetic analysis

a. RAPD-PCR fingerprinting

The genetic diversity was done using six primers within the cultivars of bottle gourd collection. They succeeded to amplify and produced strong, reproducible PCR products, (Fig. 3). The RAPD analysis of the six primers produced 103 different DNA fragments with wide molecular sizes. Primer OPA07 (Fig. 3a) produced 11 fragments with molecular sizes ranging from \approx 208 to 835bp, with 100% polymorphism. It showed eight polymorphic and three unique fragments. Primer OPA10 (Fig. 3b) gave 17 fragments. Their molecular sizes ranged from \approx 299 to 2930 bp. Two monomorphic bands, seven unique bands and seven polymorphic bands were

recorded with polymorphism degree reached to 82%. Primer OPB05 (Fig. 3c) exhibited 21 fragments with molecular sizes ranged from \approx 321 to 2181 bp. This primer revealed polymorphism percentage reach to (95%). One monomorphic band, 11 unique bands and 8 polymorphic bands were recorded. Primer OPB07 (Fig. 3d) gave 16 fragments with molecular sizes ranged from \approx 270 to 4183 bp. Three monomorphic bands, 4 unique and 9 polymorphic bands were recorded with 81% polymorphism. Primer OPB12 (Fig. 3e) produced 22 fragments with molecular size extended from \approx 233 to 2876bp. Three monomorphic bands, 9 unique bands and 10 polymorphic bands were observed. This primer revealed polymorphism percentage 86%. Primer OPB15 (Fig. 3f) gave 16 fragments, with molecular sizes ranged from \approx 221 to 1083 bp. Eight unique bands and eight polymorphic bands were recorded with 100% polymorphism. The obtained results were in accordance with the results obtained by Morimoto *et al.* (2006) investigate genetic diversity of Kenyan landraces of the white-flowered gourd (*Lagenaria siceraria*), Ram *et al.* (2006) investigate germplasm lines of bottle gourd (*Lagenaria siceraria*) with 19 primers.

b. Inter simple sequence repeat analysis (ISSR)

Initial screening of six ISSR primers with the five cultivars of bottle gourd, resulted with six ISSR primers can produce informative and polymorphic products resolvable by agarose gel elec-

trophoresis (Fig. 4). The ISSR electrophoretic analysis with six ISSR primers gave 102 different fragments with wide molecular size. Primer 814A (Fig. 4a) gave 23 fragments. Their molecular sizes ranged from 237 to 1096 bp. twelve unique bands and 11 polymorphic bands were recorded with 100% polymorphism. Primer 844A (Fig. 4b) gave 11 fragments. Their molecular sizes ranged from 148 to 476 bp. Two unique bands, six polymorphic bands and three monomorphic bands were recorded with 72% polymorphism. Primer 17899B (Fig. 4c) gave 13 fragments. Their molecular sizes ranged from 175 to 1047 bp. One monomorphic band, seven unique and 5 polymorphic bands were recorded with 92% polymorphism. Primer HB08 (Fig. 4d) gave 18 fragments. Their molecular sizes ranged from 143 to 1474 bp. Two monomorphic bands, 5 unique and 11 polymorphic bands were recorded with 89% polymorphism. Primer HB09 (Fig. 4e) gave 25 fragments. Their molecular sizes ranged from ≈ 177 to 3340 bp. Thirteen unique bands and 12 polymorphic bands were recorded with 100% polymorphism. Primer HB13 (Fig. 4f) gave 12 fragments. Their molecular sizes ranged from ≈ 240 to 1253 bp. One monomorphic band, seven unique bands and four polymorphic bands were recorded with 91.6% polymorphism. Heikal et al., (2008) using ISSR to identify the polymorphisms and the relationships between *Cucurbita* species with seven primers. Djè et al. (2010) used twenty primers ISSR to evaluate the genetic variability four accessions of edible seeded *Lagenaria siceraria*.

Phylogenetic tree

Variations in SDS-PAGE, isozymes, RAPD-PCR and ISSR-PCR bands were combined together to generate dendrogram using Dice equation as implemented in the computer program SPSS version-15 as shown in Table (5), the closest relationship was scored between cultivars Hama2 with Homs. The highest similarity value was 71.6% recorded between Hama2 and Homs, and the lowest similarity value was 44.2% recorded between LE and CB1.

The dendrogram illustrated in Fig. (5) separated the five cultivars into two main groups. The first main group divided into two sub groups. The first sub group includes Hama2 and Homs1. The second sub group includes CB1 and LI. the second main group included cultivar LE only.

Evaluation the efficiency of the used Biochemical and Molecular Genetic Techniques in Bottle gourd

Data in Table (4) summarize the obtained results of isozymes, SDS-protein, DNA electrophoresis RAPD and ISSR with the five tested cultivars of bottle gourd. The electrophoresis study for those different molecular systems revealed that 230 different bands pattern. It showed eleven bands in enzyme electrophoresis, 17 bands in total protein, 103 bands in RAPD-PCR and 102 bands in ISSR-PCR. In all used molecular system was detected, 32 of them were considered as common bands in all tested samples, while 92

bands were detected as unique, while 106 bands were observed as polymorphic. The electrophoresis studies in those different molecular systems reflected 65% polymorphism among tested cultivars.

SUMMARY

Five cultivars of bottle gourd (*Lagenaria siceraria*) were collected from different locations in Egypt, Syria and India to determine the biochemical genetics properties by SDS-PAGE, Isozymes and molecular genetics properties using RAPD and ISSR-PCR. Variations in these markers were combined together to generate dendrogram using Dice equation as implemented in the computer program SPSS version-15. The closest relationship was scored between cultivars Hama2 and Homs1. The highest similarity value was 71.6% and the lowest similarity value was 44.2% between LI with CB1. The dendrogram separated the five varieties into two main groups. The first main group divided into two sub groups. The first sub group includes Hama2 and Homs1. The second sub group includes CB1 and LI. the second main group included variety LE only.

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Table (1): Code number, species and source of five cultivars of Bottle gourd.

Code No.	Cultivars	Source
1	Local	Egypt, El Behaira
2	Hama2	Syria, (Ministry of Agricultural)
3	Homs1	Syria, (Ministry of Agricultural)
4	CB1	Syria, (Ministry of Agricultural)
5	Local	India, (Faculty of Agricultural Zagazig University)

Table (2): List of RAPD primers and their nucleotide sequence.

Primer name	Sequence	Primer name	Sequence
OP-A07	5' GGT GAC GCA G 3'	OP-B07	5' GGT GAC GCA G 3'
OP-A10	5' TCG GCC ATA G 3'	OP-B12	5' CCT TGA CGC A 3'
OP-B05	5' TGC GCC CTT C 3'	OP-B15	5' GGA GGG TGT T 3'

Table (3): List of ISSR primers and their nucleotide sequence.

Primer name	Sequence	Primer name	Sequence
814A	5' CTC TCT CTC TCT CTC TTG 3'	HB08	5' GAG AGA GAG AGA GG 3'
844A	5' CTC TCT CTC TCT CTC TAC 3'	HB09	5' GTG TGT GTG TGT GC 3'
17899B	5' CAC ACA CAC ACA GG 3'	HB13	5' GAG GAG GAG GC 3'

Table (4): Summary for polymorphisms and numbers of bands pattern resulted from different molecular systems electrophoretic of the five cultivars of Bottle gourd.

	Marker	Number of bands				polymorphism%
		polymorphic	Unique	Common	Total	
Total protein		3	3	11	17	35.3%
Isozymes	α -esterase	1	0	1	2	50%
	β -esterase	0	2	1	3	67%
	Acph	1	0	1	2	50%
	Peroxidase	0	0	1	1	0%
Total		2	2	4	8	42%
RAPD	OPA07	8	3	0	11	100%
	OPA10	7	7	3	17	82%
	OPB05	9	11	1	21	95%
	OPB07	9	4	3	16	81%
	OPB12	10	9	3	22	86%
	OPB15	8	8	0	16	100%
Total		51	42	10	103	91%
ISSR	A14	12	11	0	23	100%
	844A	6	2	3	11	72%
	17899B	5	7	1	13	92%
	HB08	11	5	2	18	89%
	HB09	12	13	0	25	100%
	HB13	4	7	1	12	91.6%
Total		50	45	7	102	91%
Total number of bands		106	93	32	230	65%

Table (5): Similarity matrix among the five varieties based on the combined data analysis.

	LE	Hama2	Homs1	CB1
Hama2	0.554			
Homs1	0.505	<u>0.716</u>		
CB1	<u>0.442</u>	0.632	0.587	
LI	0.479	0.606	0.622	0.634

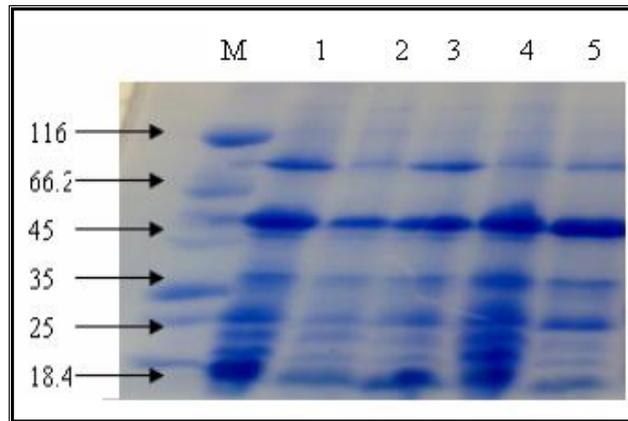


Fig. (1): SDS-PAGE of total protein banding pattern of bottle gourd. (1=LE, 2=Hama2, 3=Homs1, 4=CB1, 5=LI)

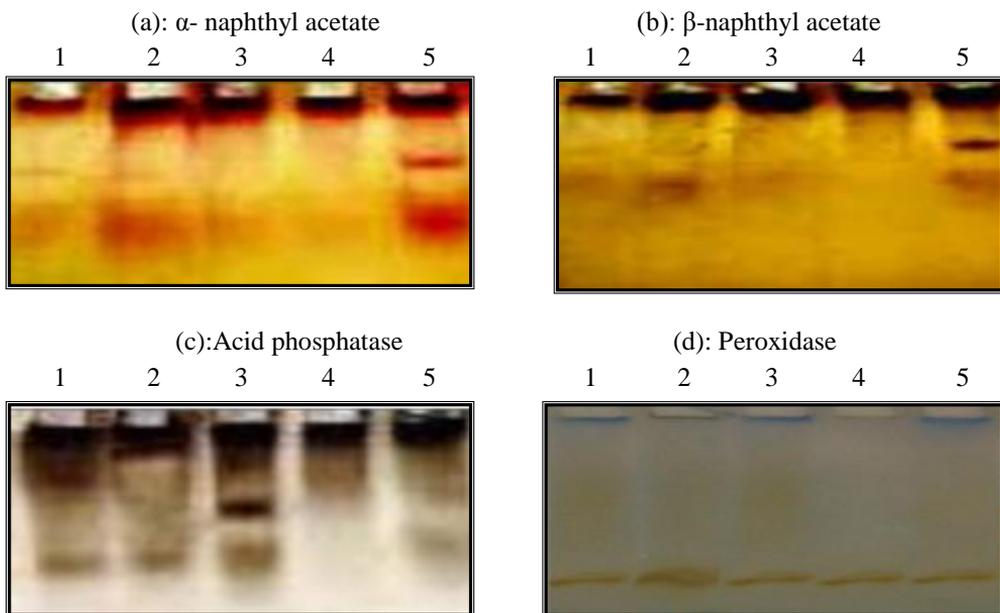


Fig. (2): Zymogram of the banding pattern among the five Bottle gourd cultivars (1=LE, 2=Hama2, 3=Homs1, 4=CB1, 5=LI).

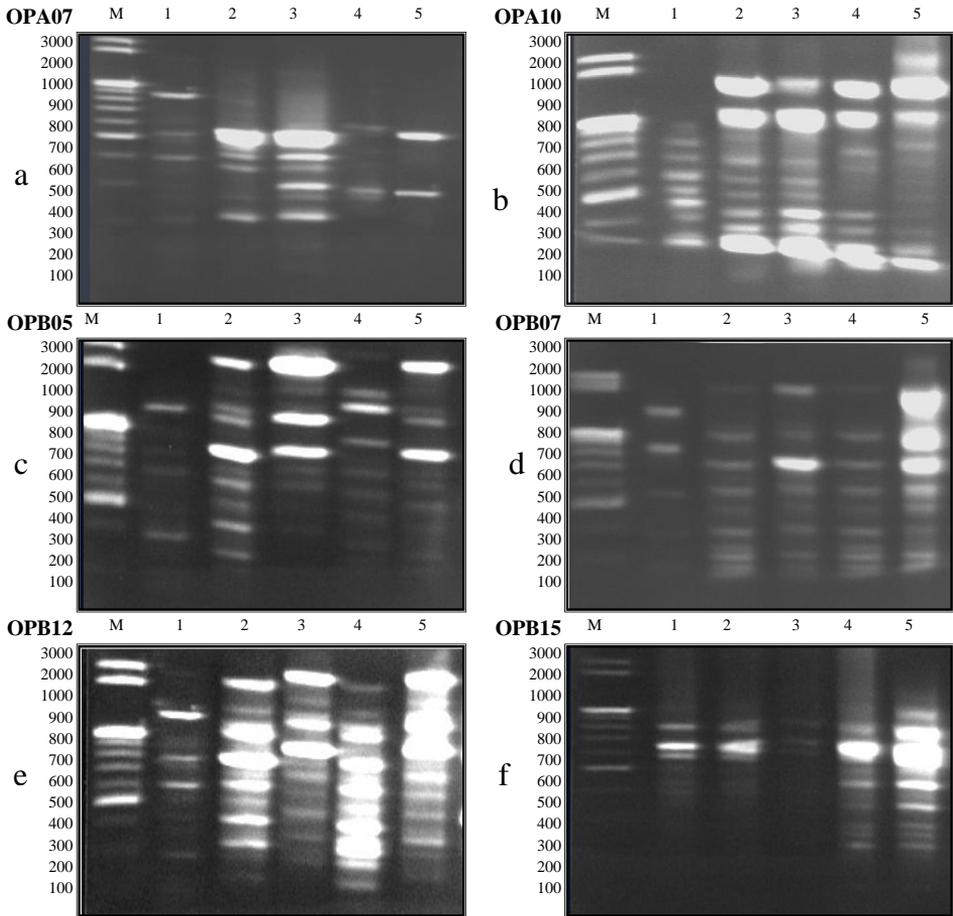


Fig. (3): DNA polymorphisms generated by six RAPD primers with the five cultivars (1=LE, 2=Hama2, 3=Homs1, 4=CB1, 5=LI)

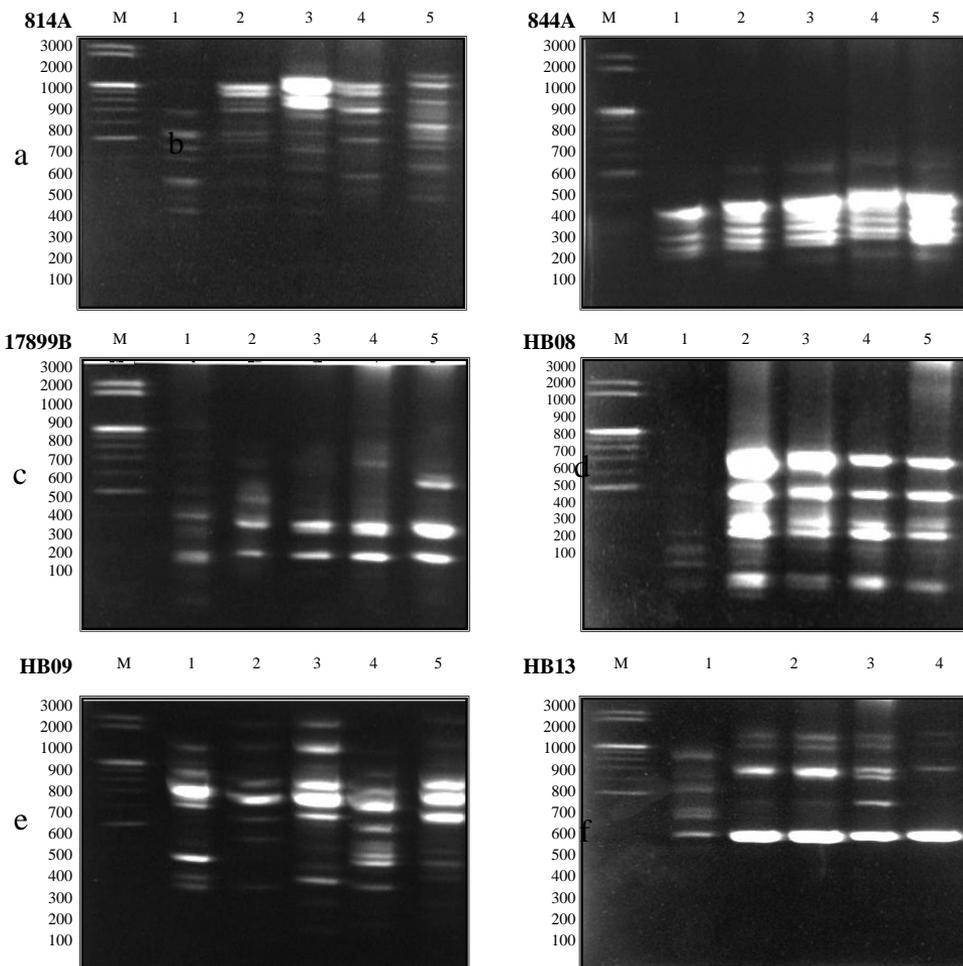


Fig. (4): DNA polymorphism generated by six ISSR primers with the five cultivars (1=LE, 2=Hama2, 3=Homs1, 4=CB1, 5=LI)

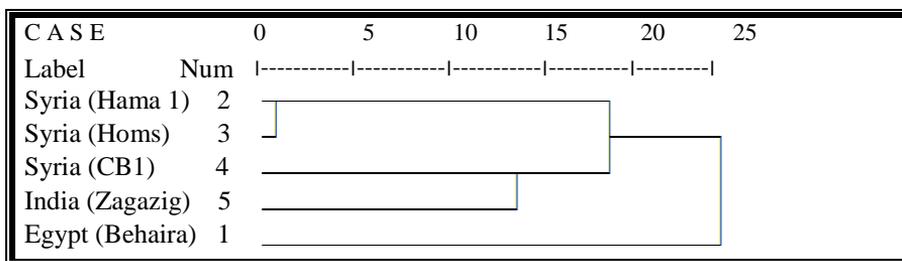


Fig. (5): Dendrogram of the five varieties based on the combined data analysis.