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Research Article

Growth, Essential Oil and Molecular Genetic Identification Studies Of Some *Eucalyptus* Species Cultivated Under Egyptian Conditions.

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Abstract

The present work was conducted to study the differences in growth characters, essential oil production as well as its chemical composition. Also, study the molecular genetics identification of eight species of genus *Eucalyptus* (*E. stricklandii, E. astringens, E. phaenophylla, E. leucoxylon, E. transcontinentalis, E. sargentii*) obtained from Forest and Water Ministry, Tunisia and the other two species,(*E. camaldulensis* and *C. citriodora* (botanists now use the name "Corymbia *Citriodora*" in referring to *Eucalyptus citriodora)*) obtained from The Forest and Timber Trees Research Department, Horticulture Research Institute, all of them cultivated under Egyptian conditions for breeding programs and as provenance. The volatile oil percent in the dry leaves ranged from (0.10% to 4.23 %) in the first year and from (0.10% to 4.44%) in the second year in *E. phaenophylla* and *E. astringens*, respectively. GC/MS analysis of the volatile oil of dry leaves was conducted for second year only. The main component of *E. stricklandii*, *E. astringns*, *E. transcontinentalis* and *E. camaldulensis* was α-Terpinene, *E. Phaenophylla* was Camphene, *E. Leucoxylon* was trans-4-Thujanol, while in *E. sargentii* was Myrtenol and in *C. citriodora* was α-Copaen-11-ol.

For molecular study Random Amplified Polymorphic DNA (RAPD) was performed and was efficient in detecting polymorphism and genetic variation within and between *Eucalyptus* species. In RAPD analysis, 5selected primers displayed a total of 85 amplified fragments, in which 68 (80%) were polymorphic fragments. Thirty-nine out of 85 RAPD-PCR fragments were found to be useful as cultivar specific markers. The largest number of RAPD-PCR markers was scored for *C. citriodora* (40 markers), while the lowest (26 markers) was scored for *E. leucoxylon*. In ISSR analysis, 5 of the tested ISSR primers generated variable banding patterns. A total of 55 out of 68 ISSR fragments were polymorphic. Thirty-nine DNA amplified fragments were considered as cultivar-specific markers. Genetic similarities among the *Eucalyptus* species were estimated according to the RAPD and ISSR data. In conclusion, RAPD and ISSR polymorphisms could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes, which could be useful in the breeding programs.

Key Words: *Eucalyptus* Species; Essential Oil Composition; GC/MS; DNA Fingerprinting; Genetic Relationship; Molecular Markers

Introduction

Eucalyptus is an evergreen, tall tree, or shrub, belonging to Myrtaceae family. The genus *Eucalyptus* comprises more than 600 species, but probably fewer than 10 species are represented in 90% of the area planted. Mostly found in tropical regions and is a native to Australia. *Eucalyptus* species grow under wide range of climatic and edaphic conditions in their natural habitat [1]. They are grown for their ornamental values, as windbreaks, for timber and fuel, and for oil, distilled from leaves, which is secondary compounds with pleasant aroma used as fragrance components in soap, detergents [2]. The essential oil of leaves from *Eucalyptus* species contains, in relatively high amounts, several monoterpene hydrocarbons, (α-pinene, limonene, p-cymene, β-pinene, α-phellandrene, camphene, γ-terpinene, etc., with the first three in major amounts) and in lower percentage several sesquiterpene hydrocarbons (aromadendrene, allo-aromadendrene, globulol, etc.), oxygenated mono terpenes (e.g. myrtenal, carvone and pinocarvone) and others [3,4].The composition of the essential oils from *E. camaldulensis*, especially from the leaves, has been widely studied. Thus, the first two main components were spathulenol and p-cymene detected in trees from Morocco [5], 1, 8-cineole and α-pinene from Mozambique [4], p-cymenen and spathulenol from Jerusalem [6] and 1,8-cineole and limonene from Burundi [7]. Essential oils of various *Eucalyptus* species are used in the pharmaceutical, toiletries, cosmetics, and food industries [8]. These broad applications are due to the antiseptic, anti hyperglycemic, anti-inflammatory, flavoring, and antioxidant

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properties of the molecules present in the oil [9]. Biochemical and molecular techniques now provide an alternative approach for evaluating genetic variation in a wide range of *Eucalyptus* [10-15]. Random amplified polymorphic DNA (RAPD) markers are easier and quicker to use and are preferred in application where the relationships between closely related breeding lines are of interest [16]. Inter-simple sequence repeats (ISSRs) have been shown to provide a powerful, rapid, simple, reproducible and inexpensive means to assess genetic diversity and identify closely related cultivars in many species [17]. Among the dominant markers, ISSRs are more reproducible than RAPDs and less expensive to use than amplified fragment length polymorphisms (AFLPs) for handling large numbers of samples [18]. ISSR-PCR has been used in genetic fingerprinting [19], gene tagging [20], detection of clonal variation [21], cultivar identification [22], phylogenetic analysis [23], detection of genomic instability [24], and assessment of hybridization [25] in many plant and animal species.

Here, for the first time, we describe the similarity and diversity in terms of RAPD and ISSR profiles of eight Eucalyptus species and investigate genetic diversity among them and determine whether secondary metabolites such as essential compounds would be used as taxonomic markers in these species and elucidate relationships between genetic and chemical diversity by comparing their hierarchical structures.

Materials and Methods

This study was conducted during extended season of 2012/2014, at the experimental farm of Medicinal and Aromatic Plants Research Department, El Kanater El Khairia, Kalubia Governorate, Biotechnology Lab. Horticulture Research Institute, Agriculture Research Centre, Egypt. Eight species of genus *Eucalyptus* were used to study the differences in growth characters, essential oil production as well as its chemical composition and molecular genetics identification between them under Egyptian conditions. Seeds of (*E. stricklandii, E. astringens, E. phaenophylla, E. leucoxylon, E. transcontinentalis, E. sargentii*) obtained from Forest and Water Ministry, Tunisia by professor Dr. Mahassen Abd EL-Ghanny Sidky and the other two species, (*E. camaldulensis* and *C. citriodora* obtained from Forest and Timber Trees Research Department, Horticulture Research Institute.

Experimental Procedure

(a) *Eucalyptus* species seeds were sown in cups contained mixed of peat-moss, sand and soil (1:1:1) on 15th October 2012 and the seedlings were transplanted to the field on 15th March 2013.

(b) In all experiments all plants received the recommended doses of fertilizers consisted of nitrogen as ammonium sulphate (20.5% N) at a rate of 100kg/fed; phosphorous fertilizer was added at 200kg/fed. as calcium super phosphate (15.5% P_2O_5) and potassium as potassium sulphate (48% K_2O) at the rate of 50 kg/fed. Fertilizers were added in equal two doses, the first one was added during soil preparation and the second was two months after transplanting in the first year and in the same time in the second year.

(c) Plants in all experiments received the recommended agricultural practices of irrigation and weeding.

The experiment was designed using a complete randomized blocks design with three replicates, every replicate contained all species and every experimental plot was 5 meters long and 4 meters width. Seedlings planted in lines 2 m between and plant distance was 2.5 m apart.

Data Recorded

The following data were recorded during the two years (in 15th July 2013 and 2014):

- Plant height (cm).
- Number of branches/plant
- Number of leaves/plant
- Leaf area (cm²).
- Leaves fresh and dry weights (g/plant).
- Essential oil percentage (determined in dry leaves according to the method described in the British Pharmacopoeia [26].
- Analysis of the essential oil by using GC/MS apparatus. (The chromatograph apparatus was fitted with capillary column BPX-5, 5% phenyle (equiv.) polysillphenylene-siloxane 30m X 0.25 mm ID X 0.25μm film).
- Molecular genetics identification using RAPD and ISSR methods.

RAPD -PCR Analysis

Polymerase Chain Reaction (PCR): In order to obtain clear reproducible amplification products, different preliminary experiments were carried out in which a number of factors were optimized. These factors included PCR temperature cycle profile and concentration of each of the template DNA, primer, $MgCl_2$ and Taq polymerase. A total of twenty random DNA oligonucleotide primers were independently used according to [27] in the PCR reaction. Only five primers succeeded to generate reproducible polymorphic DNA products. The PCR amplification was performed in a 25 μl reaction volume containing the following: 2.5 μl of dNTPs (2.5 mM), 1.5μl of Mg Cl2 (25 mM), 2.5 μl of 10x buffer, 2.0 μl of primer (2.5 μM), 2.0 μl of template DNA (50 ng/μl), 0.3 μl of Taq polymerase (5 U/μl) and 14.7 μl of sterile ddH2O. The reaction mixtures were overlaid with a drop of light mineral oil per sample. Amplification was carried out in Techni TC-512 PCR System. The reaction was subjected to one cycle at 95 ºC for 5 minutes, followed by 35 cycles at 96 ºC for 30 seconds, 37 ºC for 30 seconds, and 72 ºC for 30 seconds, then a final cycle of 72 ºC for 5 minutes. PCR products were run at 100 V for one hour on 1.5 % agarose gels to detect polymorphism between the *Eucalyptus* species under study. Only five primers succeeded to generate reproducible polymorphic DNA products. Table (a) lists the base sequences of these DNA primers that produced informative polymorphic bands. The PCR products were separated on a 1.5 % agarose gels and fragments sizes were estimated with 100bp ladder markers (3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400,300,200 and 100bp).

ISSR-PCR Analysis

Polymerase Chain Reaction (PCR): ISSR-PCR reactions were

conducted using five primers. Amplification was conducted in 25 µl reaction volume containing the following reagents: 2.5 µl of dNTPs (2.5 mM), 2.5 µl $MgCl_2$ (2.5 mM), and 2.5 µl of 10 x buffer, 3.0 µl of Primer (10 pmol), 3.0 µl of template DNA (25 ng/ µl), 1 µl of Taq polymerase (1U/ µl) and 12.5 µl of sterile dd H2O. the PCRs were programmed for one cycle at 94º C for 4 min. followed by 45 cycles of 1 min. at 94 ºC, 1 min. at 57 ºC, and 2 min at 72 ºC the reaction was finally stored at 72 ºC for 10 min. The PCR products were separated on a 1.5 % agarose gels and fragments sizes were estimated with the 100bp ladder marker. Only five primers succeeded to generate reproducible polymorphic DNA products. Table (a) lists the base sequences of these DNA primers that produced informative polymorphic bands.

Statistical Analysis

The experimental design was randomized block design with three replicates as described by [27] and L.S.D. at (5% level) for comparison the means of different treatments. The obtained PCR products were electrophoresed using agarose gel electrophoresis according to [28]. The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to calculate genetic similarity and to construct dendrogram tree among the *Eucalyptus* species under study. Calculation was achieved using Dice similarity coefficients [29] as implemented in the computer program SPSS-10.

Results and Discussion

Growth Parameters

Results of growth parameters including Plant height (cm), number of branches/plant, number of leaves/plant, leaf area (cm²), fresh and dry weights of leaves/plant (g), essential oil (%) in dry leaves and essential oil yield in dry leaves (ml)/plant of different *Eucalyptus* species in both years are shown in Table (1). Data generally, showed that, plant height ranged (51.67 – 123.33 cm) in the first year and (81.67 – 276.67 cm) in the second year, where the highest plant was always *E. camaldulensis*, while the shortest plant was *E. transcontinentalis* in both years respectively.

Regarding the number of branches it was clear from the data in Table (1) that the greatest branches numbers were recorded in *E. sargentii* (12.00) in the first year and were recorded in *E. camaldulensis* (34.33) in the second year, while the least branches number recorded in *E. stricklandii* (4.00) in the first year and in *E. transcontinentalis* (10.67) in the second year. Concerning the leaves number in Table (1) and Figure (1), it can be conducted that, the greatest leaves numbers were indicated in *E. sargentii* (2036.67 & 5210.67), while the least leaves number exhibited in *E. transcontinentalis* (87.00 & 220.67) in the two years respectively.

Data in Table (1) revealed that, the different *Eucalyptus species* showed significant differences in leaf area (cm²) in both two years, where the results showed that, *E. phaenophylla* produced the highest value (31.15 & 31.14 cm²), while the lowest value (4.56 & 4.43 cm²) recorded in *C. citriodora* in both two years respectively. The leaves fresh and dry weight (g) in Table (1) gave the same trend, the greatest in which fresh and dry leaves weight (g/plant) were recorded by *E. phaenophylla* (612.33 and 251.06 g/ plant) respectively in the first year, while those of *E. astringens* showed the greatest values (1616.67, 727.50 g/ plant) in the second year respectively. On the other hand, the least leaves weight recorded in *E. transcontinentalis* (35.00 and 88.33 g/ plant) for fresh weight and (14.35 and 36.66 g/ plant) for dry weight in the two years respectively.

Essential oil percent and yield (ml/plant)

According the essential oil data in Table (1) indicated that, essential oil percent in dry leaves ranged from (0.10% to 4.23 %) in the first year and from (0.10% to 4.44%) in the second year in *E. phaenophylla* and *E. astringens*, respectively. The maximum essential oil yield in dry leaves ml/plant was observed with *E. astringens* (9.51&32.30ml/plant), while the minimum was observed with *E. leucoxylon* (0.10&0.30 ml/plant) in the both years. The obtained results of this study confirmed that, *E. camaldulensis* was more suitable for Egyptian conditions than the others because it recorded the highest values in plant height and number of branches, despite the maximum essential oil yield in dry leaves ml/plant was observed with *E. astringens* (Table1). So it is favorable to introduce this species as exotic in Egypt.

The essential oil percent increased in the second year than the first one in all *Eucalyptus* species. This may be due to the changeful in environmental factors i.e. temperature (air and soil), light intensive, photo period and relative humidity. The synthesis of secondary metabolites has been related to the capture of light energy [30-33]. In this study, for *E. camaldulensis* produced higher oil percentage (1.48%) than those [34] who determined 1.34% oil in the same species. [35] Obtained (0.5-2%) oil from *C. citriodora*, in harmony with our results of oil from the same species (1.18%) where the values were the same limits. As for obtained here in results of the essential oil produced from the other *Eucalyptus* species it has not

Table (a): List of the used RAPD and ISSR primer names and their nucleotide sequences.

No	RAPD Primer code	Sequence	No	ISSR Primer code	Sequence
1	OP-A02	5´TGCCGAGCTG3´	1	14A	5`CTCTCTCTCTCTCTTG 3`
2	OP-A09	5´GGGTAACGCC3´	2	44B	5`CTCTCTCTCTCTCTGC 3`
3	OP-A10	5´GTGATCGCAG 3´	3	HB-8	5`GAG AGA GAG AGA GG 3`
4	OP-C04	5`CCGCATCTAC 3`	4	HB-10	5`GAG AGA GAG AGA CC 3`
5	OP-Q18	5`AGGCTGGGTG3`	5	HB-11	5`GTGTGTGTGTGTTGTCC 3`

Table (1): Growth parameters and essential oil (% and yield/ plant) of different Eucalyptus species cultivated under Egyptian conditions during extended season of 2012/2014.

Eucalyptus species	Plant height (cm)	No. of branches/plant	No. of leaves/plant	Leaf area (cm^2)	F.W of leaves/ plant (g)	D.W of leaves/plant (g)	Essential oil % in dry leaves	Essential oil yield in dry leaves (ml)/ plant
	First season (one year old)							
E.stricklandii	60.00	4.00	137.00	21.08	118.33	47.33	3.55	1.68
E.astringens	81.67	7.00	1079.67	23.03	500.00	225.00	4.23	9.51
E.phaenophylla	106.67	8.67	1187.67	31.15	612.33	251.06	0.10	0.25
E.leucoxylon	75.00	8.33	318.33	27.29	245.00	100.45	0.10	0.10
E.transcontinentalis	51.67	10.67	87.00	9.80	35.00	14.35	2.93	0.42
E.sargentii	90.00	12.00	2036.67	9.08	313.33	128.47	3.09	3.97
C. citriodora	63.33	8.33	990.00	4.56	90.00	40.50	1.18	0.48
E.camaldulensis	123.33	10.00	774.00	25.60	580.00	226.20	1.44	3.26
L.S.D at 5%	7.04	1.36	33.07	0.32	16.26	6.58	0.19	0.18
	Second season(two years old)							
E.stricklandii	110.00	12.33	349.67	21.90	304.67	118.80	3.64	4.33
E.astringens	170.00	26.00	3492.00	23.11	1616.67	727.50	4.44	32.30
E.phaenophylla	210.00	25.00	2208.67	31.14	1138.67	466.85	0.10	0.47
E.leucoxylon	176.67	19.33	849.33	27.08	653.33	267.80	0.11	0.30
E.transcontinentalis	81.67	10.67	220.67	10.38	88.33	36.66	3.32	1.22
E.sargentii	163.33	30.67	5210.67	9.81	801.67	328.68	3.25	10.68
C. citriodora	120.00	26.33	2288.00	4.43	208.00	91.27	1.26	1.15
E.camaldulensis	276.67	34.33	1619.33	25.36	1200.00	486.00	1.48	7.19
L.S.D at 5%	12.58	2.15	126.08	0.39	68.85	15.62	0.19	1.11

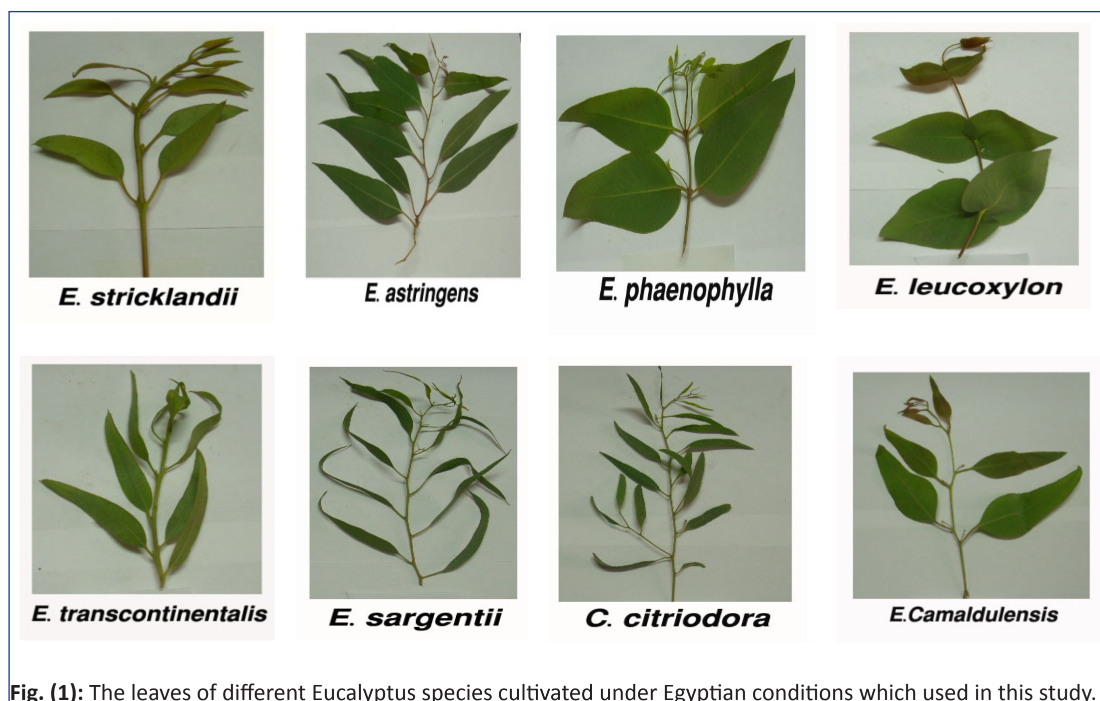

Fig. (1): The leaves of different Eucalyptus species cultivated under Egyptian conditions which used in this study.

available another literature.

Essential Oil Composition

The results of the GC/MS analysis of the volatile oils of the *Eucalyptus* species are shown in Table (2) and Table (3). The total identified compounds are ranged from 70.67% in *C. citriodora* to 96.54% in *E. transcontinentalis*. The majority of compounds (24 oxygenated monoterpenes) ranged from 20.21% in *C. citriodora* to 52.95% in *E. sargentii*, while the monoterpene hydrocarbons are represented (7 compounds) and ranged from 12.53% in *E. phaenophylla* to 44.36% in *E. stricklandii*. The sesquiterpene hydrocarbons (15 compounds) ranged from 8.73% in *E . stricklandii* to 28.77 % in *E. phaenophylla*, however oxygenated sesquiterpene (6 compounds) ranged from 5.41% in *E. sargentii* to 12.44% in *E. astringens*.

In addition, another compound carvacrol which is considered a phenol compound ranged from 0.45% in *E. sargentii* to 3.42% in *C. citriodora*. *Eucalyptus* species were differed in these contents of monoterpenes and sesquiterpenes, these plants were characterized by high contents of oxygenated monoterpenes, which ranged from 70.67% in *C. citriodora* to 96.54% in *E. transcontinentalis*. The content of volatile oils expressed in percentage was as follows: for *E. transcontinentalis* 96.54%, *E. Leucoxylon* 95.68%, *E. astringens* 91.4%, *E. camaldulensis* 91.22%, *E. Sargentii* 89.75%, *E .stricklandii* 83.34%, *E. Phaenophylla* 76.62% and *C. citriodora* 70.67%.

Table (2) cleared the chemical components of the volatile oils. The main constituents of *E. transcontinentalis* volatile oil were α-Terpinene (28.81%), β-cis-Ocimene (9.47%) and α-Guaiene (5.43%). Volatile oil of *E. Leucoxylon* major compounds were trans-4-Thujanol (14%), α-Terpinene (11.74%) and D-limonene (6.47%), whereas in *E. astringens*, α-Terpinene (24.17%), Camphene (8.64%), Terpinolene (8.2%) and Spathulenol (5.1%) were dominant. The most abundant components observed in *E. camaldulensis* were

Table (2): The volatile oil composition (%) of different Eucalyptus species under Egyptian conditions. (Second year).

Peak No.	components	Retention time (min)	*E. stricklandii*	*E. astringens*	*E. phaenophylla*	*E. leucoxylon*	*E. transcontinentalis*	*E. sargentii*	*C. citriodora*	*E. camaldulensis*
1	trans-4-Thujanol	5.74	-	-	-	14	-	-	-	-
2	D-limonene	6.28	-	-	-	6.47	-	-	-	-
3	Phenethyl alcohol, p,α,α-trimethyl	6.47	0.39	0.66	-	-	0.76	0.32	0.23	0.42
4	L-Pinocarveol	6.57	-	-	-	1.77	-	-	-	-
5	Terpinolene	7.29	0.63	8.2	1.44	1.81	0.39	0.28	2.94	0.38
6	α-Pinene	7.41	2.66	0.41	-	0.82	2.67	1.21	6.1	1.88
7	Camphene	7.84	13.4	8.64	9.61	3	-	9.48	0.3	8.5
8	β-cis-Ocimene	7.98	0.44	0.39	-	0.17	9.47	0.71	1.32	1.1
9	β-Pinene	8.19	0.63	0.34	0.28	0.62	1.19	0.56	0.8	0.48
10	8-Hydroxylinalool	8.59	-	-	0.99	-	-	-	-	-
11	α-Terpinene	9.42	26.6	24.17	1.2	11.74	28.81	0.34	1.82	26.33
12	Myrtenol	9.55	0.67	0.62	2.35	1.12	0.53	27.35	0.28	0.65
13	Linalool	9.77	1.47	1.25	2.43	0.34	1.4	0.4	1.07	1.54
14	cis-Verbenol	9.99	0.94	1.03	0.5	2.72	1.6	1.61	0.78	2.13
15	Terpineol, cis-β-	10.1	0.48	0.29	0.61	0.15	0.45	1.89	1.22	1.18
16	Carveol	10.26	0.55	0.46	1.33	0.49	0.74	0.86	1.07	-
17	Isopulegol	10.53	1.23	0.61	1.21	0.23	2.62	2.68	0.6	3.63
18	Terpinen-4-ol	10.8	1.45	2.6	2.3	1.5	1.34	2.78	2.29	2.83
19	cis-2-p-Menthen-1-ol	10.82	1.3	2.39	1.16	1.99	1.08	2.24	1.61	1.83
20	α-Terpineol	11.16	2.5	1.8	4.16	0.44	3.97	6.93	2.24	9.08
21	trans-Carveol	11.34	4	3.72	3.25	4.07	0.79	1.1	1.19	0.91
22	Isopinocarveol	11.44	0.69	1.04	1.57	2.85	1	1.07	0.83	1.41
23	cis-Sabinol	11.55	-	2.05	1.31	-	-	-	0.73	0.42
24	cis-Geraniol	11.65	1.38	0.35	0.56	3.64	2.23	2.22	0.4	2.03
25	Citronellal	11.87	3.44	0.9	0.29	1.39	0.69	0.31	3.24	0.36
26	Phellandral	12.06	-	0.34	0.44	1.69	0.58	-	0.53	0.36
27	Carvacrol	12.22	0.55	0.58	1.88	0.92	0.98	0.45	3.42	0.53

28	γ-Elemene	12.63	0.79	0.68	1.6	1.81	3.04	3.84	6.09	0.37
29	Cadina-3,9-diene	12.78	-	-	0.26	0.27	-	-	-	-
30	Geranyl acetate	12.97	-	0.65	0.82	3.21	0.65	0.66	-	-
31	γ-Muurolene	13.05	-	0.42	0.41	0.94	1.28	0.67	-	0.43
32	β-Elemen	13.17	-	0.73	2.24	2.07	1.6	0.57	2.07	0.76
33	β-Selinene	13.19	0.72	0.72	2.51	0.42	0.42	0.56	-	-
34	Longifolene	13.29	-	0.58	1.88	0.47	1.57	0.4	1.47	0.32
35	α-Gurjunene	13.44	-	0.3	-	-	0.94	0.53	0.52	0.35
36	Alloaromaden-drene	13.57	0.42	0.36	2.62	0.45	1.37	0.68	-	1.48
37	Leden	13.65	0.52	0.34	0.35	0.45	0.65	0.63	0.5	0.92
38	α-Guaiene	13.82	2.59	4.53	6.2	1.13	5.43	3.47	4.04	2.74
39	α-Copaen-11-ol	13.98	0.5	2.38	2.78	1.11	2.85	1.43	8.14	1.67
40	α-Selinene	14.1	0.46	1.01	1.29	1.17	1.51	1.75	-	1.62
41	Valencene	14.38	1.47	2.13	6.7	2.48	3.81	3.08	4.95	3.23
42	cubedol	14.5	-	0.43	1.57	0.75	0.83	0.56	-	0.61
43	β-Cadinene	14.6	0.74	0.59	1.24	0.73	1.41	-	1.66	0.6
44	γ-Selinene	14.73	-	0.45	0.23	2.15	1.43	1.06	0.98	1.4
45	Epiglobulol	15	1.95	3.97	0.55	1.71	1.64	1.52	-	1.11
46	γ-Eudesmol	15.1	1.02	1.92	1.19	0.69	-	1.12	-	0.89
47	Spathulenol	15.23	5.88	5.1	1.8	1.58	-	1.52	-	2.66
48	α-Eudesmol	15.85	-	-	0.56	1.53	-	-	-	-
49	trans-Farnesol	16.3	0.5	0.56	0.46	1.19	0.87	0.38	3.11	0.97
50	Tetradecanoic acid	16.61	0.38	0.71	0.44	0.61	1.95	0.53	1.39	0.65
51	Octadecanoic acid	17.65	-	-	-	2	-	-	-	-
52	Octadec-9-enoic acid	18.98	-	-	-	2.82	-	-	-	-
53	Z,E-3,13-Octadeca-dien-1-ol	19.03	-	-	-	-	-	-	0.74	0.46
	Total Identified		83.34	91.4	76.62	95.68	96.54	89.75	70.67	91.22
	Unidentified		16.66	8.6	23.38	4.32	3.46	10.25	29.33	8.78

Values are means of three replicates.

Table (3): The classification of volatile oil components of different Eucalyptus species under Egyptian conditions according to terpenoieds type. (Second **year).**

components	***E. stricklandii***	***E. astringens***	***E. phaenophylla***	***E. leucoxylon***	***E. transcontinentalis***	***E. sargentii***	***C. citriodora***	***E. camaldulensis***
Monoterpene hydrocarbons	44.36	42.15	12.53	24.63	42.53	12.58	13.28	38.67
Oxygenated monoterpenes	20.48	21.47	25.72	47.03	21.38	52.95	20.21	29.89
Sesquiterpene hydrocarbons	8.73	14.76	28.77	15.23	24.46	18.36	22.28	15.11
Oxygenated Sesquiterpene	8.83	12.44	7.72	7.87	6.19	5.41	11.25	7.02
Other compounds (phenol)	0.55	0.58	1.88	0.92	0.98	0.45	3.42	0.53

α-Terpinene (26.33%), α-Terpineol (9.08%) and Camphene (8.5%), while Myrtenol (27.35%) was the major component in *E. sargentii* followed by Camphene (9.48%) and α-Terpineol (6.93%).

Some of the previous results, contradicted with those reported by [36] who mentioned that the major constituents of the essential oil from *E. Camaldulensis* were ethanone (25.36%), eucalyptol (13.73%), β-caryophyllene (11.55) and carvacrol (9.05%). The main component of *E. stricklandii* was α-Terpinene (26.6%) followed by Camphene (13.4%) and Spathulenol (5.1%) was the least. According to *E. Phaenophylla,* the major volatile oil was Camphene (9.61%) followed by Valencene (6.75%) and α-Guaiene (6.2%), while in *C. citriodora,* α-Copaen-11-ol (8.14%), α-Pinene (6.1) and γ-Elemene (6.09) were the main components.

In this study, we will find reports for the first time of the analysis of the leaf volatile oil of these species which grown in Egypt. This study revealed that *E. sargentii* contents the highest value of Myrtenol (27.35%), whereas α-Terpinene was the major compound in *E. Transcontinentalis* (28.81), *Eu .stricklandii* (26.6%), *E. camaldulensis* (26.33%) *and E. sargentii* (24.17%). *E. Leucoxylon* volatile oil was unique because it contained some components which absent in the other species. These components are: trans-4-Thujanol (14%), D-limonene (6.47%), L-Pinocarveol (1.77%), Octadecanoic acid (2%) and Octadec-9-enoic acid (2.82%), other components occur in all studied species. Correlations between the constituents of volatile oil of *Eucalyptus* species and their taxonomic relationship, both within the genus and as a part of the Myrtaceae family, have been attempted. Hengnauer, for example, has addressed the issue in his *Chemotaxonomie der Pflanzen* series [37, 38].

As in natural stands, the oil of plantations varies greatly. Factors which affect these yields include: seed provenance and species differences, soil and nutrient properties, water supply, weather, weeds, pests and diseases [39]. In this respect, [40] reported that, the reason of this variation may be due to that the chemical compositions of volatile oils depends on climatic, seasonal and geographic conditions; harvest period and isolation technique.

Molecular Genetic Identification

Randomly Amplified Polymorphic DNA (RAPD) Markers: The five 10-mer arbitrary primers succeeded in amplifying DNA fragments for the eight genotypes of *Eucalyptus* species as illustrated in Table (4) and Figure (2). Polymorphism levels differed from one primer to another. OP-A10 primer exhibited low level of polymorphism (56.25%). On the other hand OP-Q18 (70.59%), OP-C04 (80.00%) and OP-A09 (88.89%) primers exhibited moderate levels of polymorphism. However OP-A02 (95.83%) primer exhibited high levels of polymorphism. The number of total amplified fragments (TAF), polymorphic fragments (PF), monomorphic fragments (MF) and specific markers (SM) for each sample using the five primers are shown in Table (4). OP-A02 primer produced twenty four fragments with molecular size ranging from108 to 629 bp (Figure1).

Twenty three fragments were polymorphic (95.83%) and seventeen of them were species - specific markers at (432,308,226) bp for *E. stricklandii*, (246,194)bp for *E. astringens*,(472,446,212)bp for *E. phaenophylla*,(188)bp for *E. leucoxylon,* (369,206,183)bp for *E. transcontinentalis,* (233,177)bp for *E. sargentii,* (419,112)bp for

Table (4): Species-specific RAPD and ISSR markers for Eucalyptus species genotypes

Primers code	Range of M.S.	TAF	MF	PF	SM	Polymorphism (%)
RAPD primers						
OP-A02	108-629	24	1	23	**17**(432,308,226)-(264,194)-(472,446,212)-(188)-(369,206,183)-(233,177)-(419,112)-(166,108)bp	95.83
OP-A09	177-785	18	2	16	**8**(267)-(159)-(424,253)-(246)-(0)-(448)-(380)-(360)bp	88.89
OP-A10	114-721	16	7	9	**6**(0)-(721)-(687,120)-(0)-(243)-(0)-(640,117)-(0)bp	56.25
OP-C04	159-618	10	2	8	**2** (0)-(0)-(0)-(335)-(367)-(0)-(0)-(0) bp	80.00
OP-Q18	121-1054	17	5	12	**6**(790,294,265)-(899)-(0)-(0)-(550)-(121)-(0)-(0) bp	70.59
Total RAPD primers		**85**	**17**	**68**	**39**	
ISSR primers						
14A	259-491	8	3	5	**2**(0)-(273)-(339)-(0)-(0)-(0)-(0)-(0))bp	62.50
44B	206-620	14	2	12	**8**(587,206)-(552)-(570)-(620)-(0)-(480)-(508)-(452)bp	85.71
HB-08	161-657	15	5	10	**9**(657,560)-(621,502)-(639)-(0)-(532)-(161)-(517,165) bp	66.67
HB-10	103-579	9	2	7	**3**(0)-(0)-(136)-(0)-(542)-(0)-(103)-(0) bp	77.78
HB-11	208-1081	22	1	21	**17**(519,277)-(925,308)-(351,456,203)-(237,507)-(400,479,208)-(1081,833,316,231)-(256)bp	95.46
Total ISSR primers		**68**	**13**	**55**	**39**	
Total		**153**	**30**	**123**	**78**	

TAF = Total Amplified Fragments, MF= Monomporphic Fragments, PF= Polymorphic Fragments,
SM= Specific Markers.

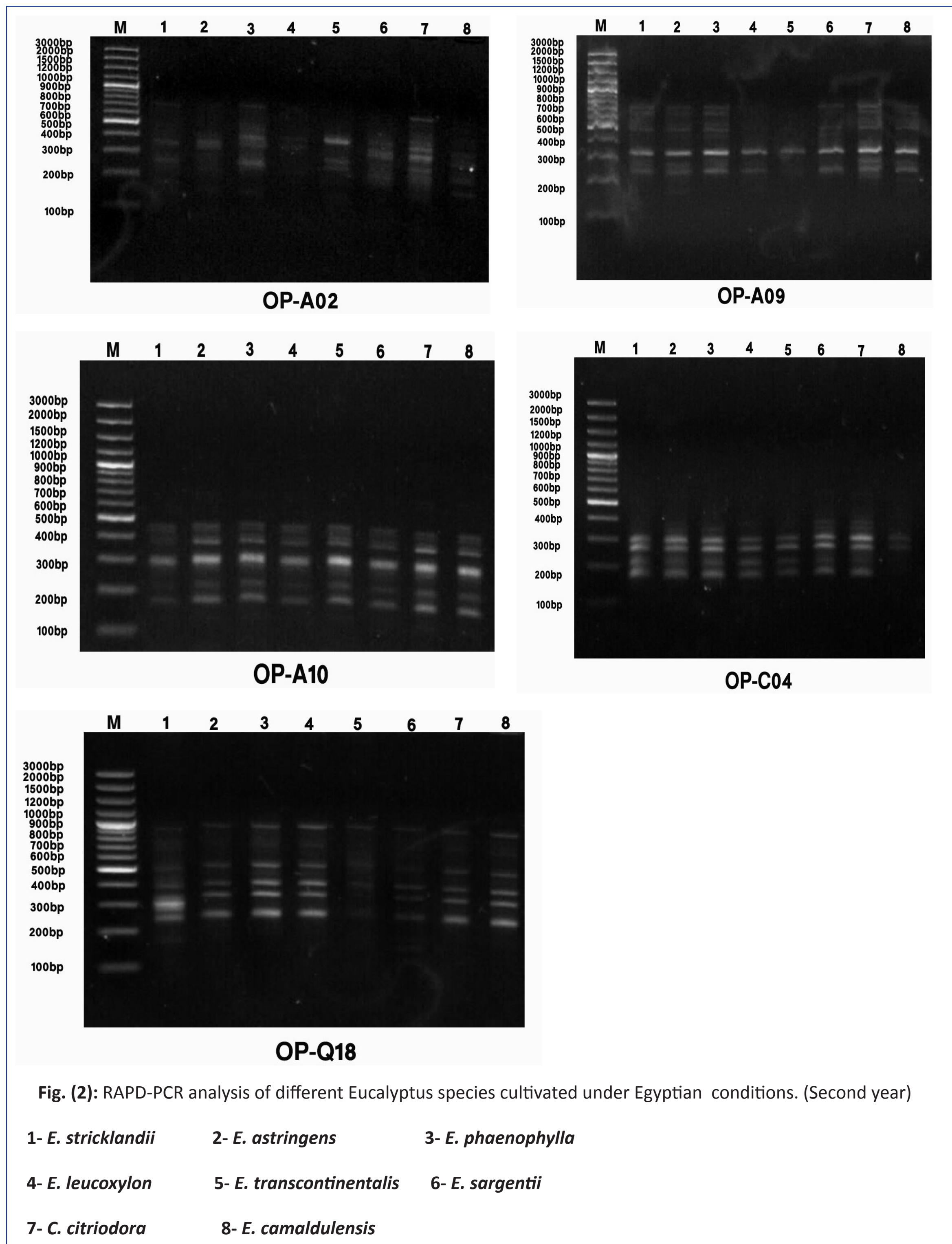

Fig. (2): RAPD-PCR analysis of different Eucalyptus species cultivated under Egyptian conditions. (Second year)

1- *E. stricklandii* **2- *E. astringens*** **3- *E. phaenophylla***

4- *E. leucoxylon* **5- *E. transcontinentalis*** **6- *E. sargentii***

7- *C. citriodora* **8- *E. camaldulensis***

C. citriodora and (166,108)bp for *E. camaldulensis*, while only one fragment was present in all genotypes which is considered as common fragment.

OP-A09 primer resulted in eighteen DNA fragments with molecular size ranging from 177 to 785bp, sixteen fragments were polymorphic (88.89%) in which eight of them were species- specific marker at (267)bp for *E. stricklandii*, (159)bp for *E. astringens*,(424,253)bp for *E. phaenophylla*, (246)bp for *E. leucoxylon*, (448)bp for *E. sargentii*, (380)bp for *C. citriodora* and(360)bp for *Eu .camaldulensis*, and the other two fragments were present in all genotypes which are considered as common fragments. OP-A10 primer resulted in sixteen DNA fragments with molecular size ranging from 114

to 721bp, nine fragments were polymorphic (56.25 %), and six of them were species- specific marker at (721)bp for *E. astringens*, (687,120)bp for *E. phaenophylla*, (243)bp for *E. transcontinentalis*, and (640,117)bp for *C. citriodora*, while the other seven fragments were presented in all genotypes which are considered as common fragments. OP-C04 primer resulted in ten DNA fragments with molecular size ranging from 159 to 618bp, in which eight fragments were polymorphic (80.00 %) and two of them were species - specific markers at (3354) bp for *E. leucoxylon* and (367)bp for *E. transcontinentalis* and the other two fragments were present in all genotypes which are considered as common fragments. OP-Q18 primer resulted in seventeen DNA fragments with molecular size ranging from 121 to 1054bp, twelve fragments were polymorphic (70.59 %) in which six of them were species - specific markers at (790,294,265)bp for *E. stricklandii*, (899)bp for *E. astringens*, (550) bp for *E. transcontinentalis* and (121)bp for *E. sargentii*, while the other five fragments were presented in all genotypes which are considered as common fragments.

Genetic similarity and cluster analysis based on RAPD markers: The RAPD data were used to estimate the genetic similarity values among the eight genotypes of *Eucalyptus* species by using UPGMA computer analysis (Table 5 and Figure. 2). The highest similarity value (1.0) was recorded between *E. transcontinentalis* and *E. camaldulensis* genotypes, while the lowest similarity value (0.012) was detected between *E. transcontinentalis* and *E. leucoxylon* genotypes. On the other hand there was no similarity between *E. sargentii* and *C. citriodora* genotype.

A dendrogram for the genetic relationship among the five genotypes of *Eucalyptus* species genotypes is exhibited in Figure (4), which separated them into two major groups. The first group included *E. leucoxylon* and *E. transcontinentalis* genotype, while the second group included two subgroups, the first subgroup involved *E. sargentii* and *C. citriodora* genotype. While, the other subgroup divided into two sub groups, the first one included *E. astringens* and *E. phaenophylla* genotypes and the other one involved *E. Stricklandii* and *E. camaldulensis* genotypes.

An earlier RAPD-based study in the temperate species *E. globulus* recorded 30.7% genetic diversity among its populations [13]. Similarly, a comparative estimation of hetero zygosity using SSR markers registered greater diversity in *E. tereticornis* (30.5%) than in *E. globulus* (22.4) [41]. As RAPD-based study on hybrid populations of *E. grandis* × *E. urophylla* and seedlings of *E. globulus* detected similar (63.8%) DNA polymorphism [13, 42]. In our study, polymorphism was higher (95.83%) than recorded in the previous studies.

Inter Simple Sequence Repeats (ISSRs) Markers: The five ISSR primers succeeded in amplifying DNA fragments for the five *Eucalyptus* species genotypes (Figure 3). Polymorphism levels differed from one primer to another, i.e. HB-10, 44B and HB-11 primers exhibited high levels of polymorphism (77.78%, 85.71% and 95.46%) respectively, while, (14A and HB-08) primers exhibited moderate level of polymorphism (62.50 and 66.67%) respectively as exhibited in Table (4). The number of total amplified fragments (TAF), polymorphic fragments (PF), monomorphic fragments (MF) and specific markers (SM) for each primer of the five primers are shown in Table (4). 14A Primer showed eight DNA fragments with molecular size ranging from 259 to 491bp (Figure 2 and Table 4), five fragments were polymorphic (62.50 %), and two of them were species- specific markers at (273)bp for *Eu astringens* and (339)bp for *E. phaenophylla* genotypes. 44B primer showed fourteen DNA fragments with molecular sizes ranging from 206 to 620bp, twelve fragments were polymorphic (85.71 %), and eight of them were species- specific markers at (587,206)bp for *E. Stricklandii* genotype, (552)bp for *E. astringens* genotype, (570)bp for *E. Phaenophylla* genotype, (620)bp for *E. Leucoxylon* genotype, (480)bp for *E. Sargentii* genotype, (508)bp for *C. citriodora* genotype and (452)bp for *E. camaldulensis* genotype. HB-08 primer showed fifteen DNA fragments with molecular size ranging from 161 to 657bp, ten fragments were polymorphic (66.67%), and nine of them were species- specific markers at (657,560)bp for *E. Stricklandii* genotype, (621,502)bp for *Eu astringens* genotype, (639) bp for *E. Phaenophylla* genotype, (532)bp for *E. Transcontinentalis* genotype, (161)bp for *C. citriodora* genotype and (517,165)bp for *E. camaldulensis* genotype.

HB-10 primer showed nine DNA fragments with molecular

Table (5): Similarity value (Pairwise comparison) of *Eucalyptus* species genotypes based on RAPD data

	E. stricklandii	*E. astringens*	*E. phaenophylla*	*E. leucoxylon*	*E. transcontinentalis*	*E. sargentii*	*C. citriodora*
E. stricklandii							
E. astringens	0.252						
E .phaenophylla	0.360	0.038					
E. leucoxylon	0.571	0.201	0.512				
E .transcontinentalis	0.803	0.450	0.571	0.012			
E. sargentii	0.512	0.512	0.778	0.512	0.760		
C. .citriodora	0.657	0.663	0.610	0.856	0.738	0.000	
E. camaldulensis	0.252	0.728	0.354	0.760	1.000	0.676	0.057

size ranging from 103 to 579bp, seven fragments of them were polymorphic (77.78 %), and three of them were species- specific markers at (136)bp for *E. phaenophylla* genotype, (542)bp for *E. Transcontinentalis* genotype and at (103)bp for *C. citriodora* genotype. HB-11 primer showed twenty two DNA fragments with molecular size ranging from 208 to1081bp, twenty one fragments were polymorphic (95.46 %) and seventeen of them were species-specific markers at (519,277)bp for *E. Stricklandii* genotype, (925,308)bp for *E. astringens* genotype, (351,456,203)bp for *E. phaenophylla* genotype, (237,507)bp for *E. Leucoxylon* genotype, (400,479,208)bp for *E. Transcontinentalis* genotype, (1081, 833, 316, 231)bp for *C. citriodora* genotype and (256)bp for *E. camaldulensis* genotype and the other one fragment was present in all genotypes which are considered as common fragments.

Genetic Similarity And Cluster Analysis Based on ISSR Markers: The ISSR data were used to estimate the genetic similarity values among the eight genotypes of *Eucalyptus* species by using UPGMA

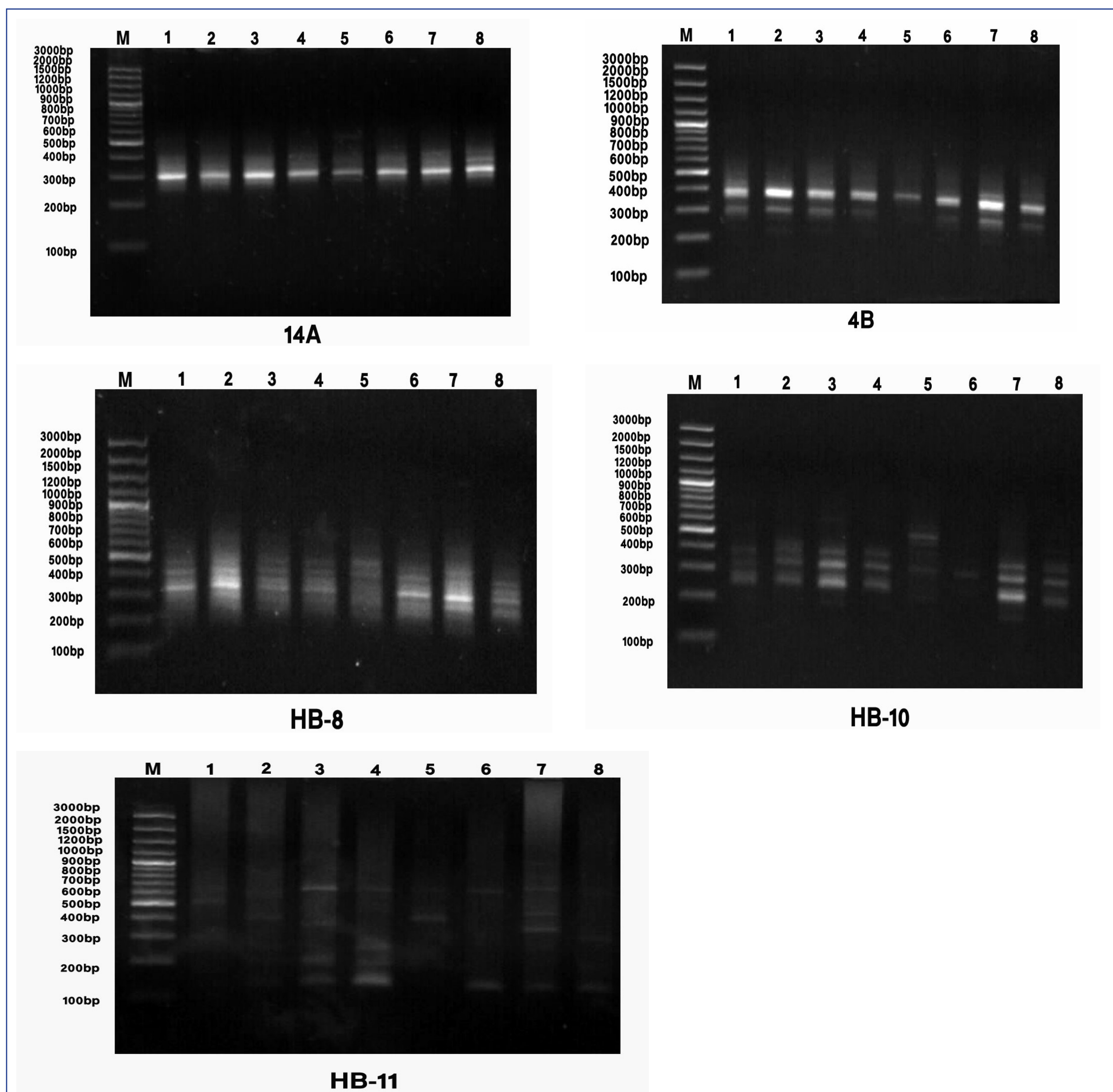

Fig. (3): ISSR-PCR analysis of different *Eucalyptus* species cultivated under Egyptian conditions. (Second year)

1- *E. stricklandii* **2- *E. astringens*** **3- *E. phaenophylla***

4- *E. leucoxylon* **5- *E. transcontinentalis*** **6- *E. sargentii***

7- *C. citriodora* **8- *E. camaldulensis***

computer analysis (Table 6 and Figure3). The highest similarity values were recorded (1.0) between *E. transcontinentalis* and *C. citriodora* genotypes, while the least similarity value (0.013) was recorded between *E. Sargentii* and *E. camaldulensis* genotypes and there was no similarity between *E. astringens* and *E. camaldulensis* genotypes.

A dendrogram for the genetic relationship among the eight genotypes of *Eucalyptus* species is illustrated in Figure (5), as they were separated into two major groups. The first group included only *E. transcontinentalis* genotype, while the second group was divided into two subgroups, The first subgroup included each of *E. astringens*, *E. camaldulensis* and *C. citriodora* genotypes and the other subgroup divided into two sub sub group, the first one included *E. Sargentii*, *E. Leucoxylon* and *E. Phaenophylla* genotypes and another one included only *E. Stricklandii* genotype. In our ISSR-based study, polymorphism was (95.46%), which higher (73.7%) than recorded in the study by [43] who reported that because the ISSRs indicated high polymorphism in natural populations of *Eucalyptus*, we anticipate that the results of ISSR-based studies will play a major role in the management, conservation and improvement of this tropical tree crop. This ISSR-based study has contributed to our understanding of the genetic status of eight *Eucalyptus* species. Based on this information, it will be useful to devise sampling strategies that efficiently capture genetic diversity for selection trials and subsequent distribution of clonal planting stock. As [44] stressed, high genetic variation is a safeguard against co-evolving biotic factors such as pests and diseases.

Hence, ISSR-based assessments will be helpful both in deciding how to conserve germ plasm and in planning crosses in breeding programs. Moreover, the assessment of genetic variation within species of *Eucalyptus* will assist in predicting achievable genetic gain in breeding programs and it may indicate the stability of progenies of interspecific crosses.

Combined identification based on RAPD and ISSR analyses: Varieties distribution on the consensus tree according to the banding patterns of RAPD differed from that based on ISSR banding patterns, which may be due to that each technique, amplified different parts of the genome. So, it is better to use the combination

Table (6): Similarity value (Pairwise comparison) of Eucalyptus species genotypes based on ISSR data

	E. stricklandii	*E. astringens*	*E. phaenophylla*	*E. leucoxylon*	*E. transcontinentalis*	*E. sargentii*	*C. citriodora*
E. stricklandii							
E. astringens	0.651						
E. phaenophylla	0.554	0.406					
E. leucoxylon	0.172	0.346	0.070				
E. transcontinentalis	0.623	0.954	0.855	0.631			
E. sargentii	0.263	0.447	0.327	0.055	0.573		
C. citriodora	0.552	0.411	0.598	0.401	1.000	0.337	
E. camaldulensis	0.290	0.000	0.346	0.109	0.748	0.013	0.058

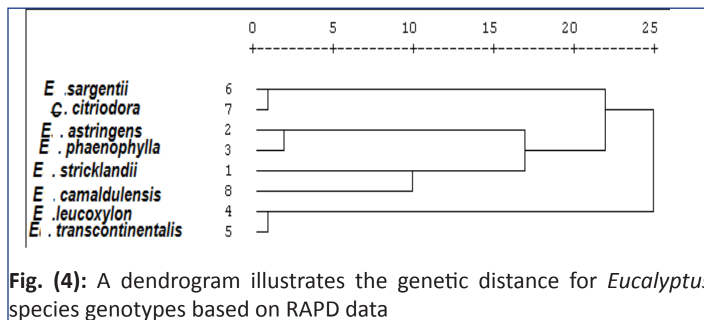

Fig. (4): A dendrogram illustrates the genetic distance for *Eucalyptus* species genotypes based on RAPD data

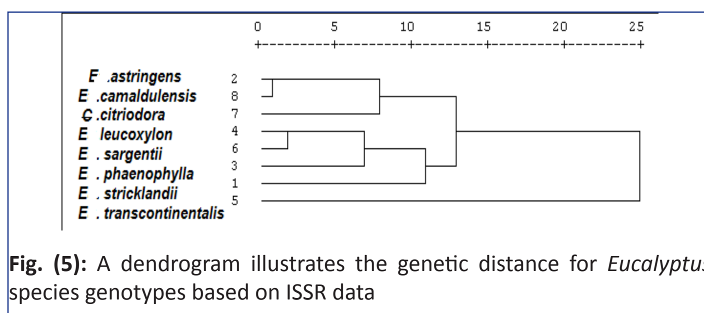

Fig. (5): A dendrogram illustrates the genetic distance for *Eucalyptus* species genotypes based on ISSR data

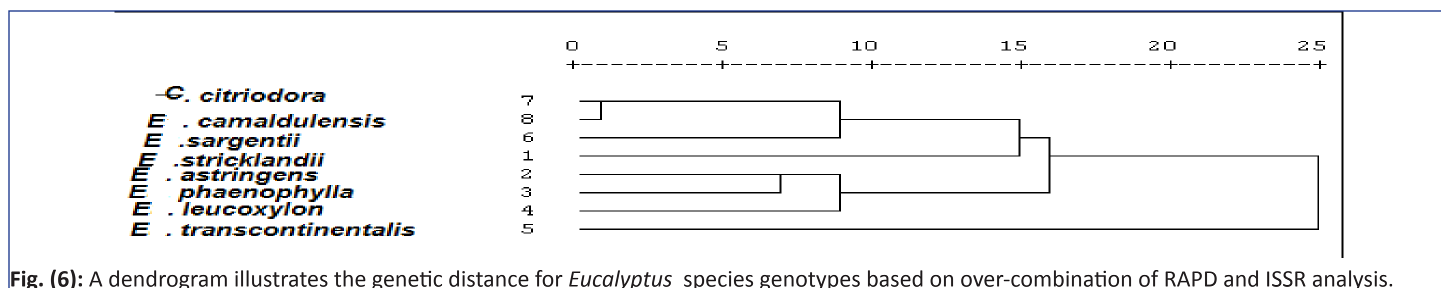

Fig. (6): A dendrogram illustrates the genetic distance for *Eucalyptus* species genotypes based on over-combination of RAPD and ISSR analysis.

of the banding patterns of the two techniques to use more segments of the genome that will increase the validity of the consensus tree. Genetic similarities and phylogenetic relationships among the eight Eucalyptus species genotypes based on a combined data of RAPD and ISSR-PCR markers (Table 7 and Figure 6) were determined using UPGMA computer program. The highest similarity values were recorded (1.0) between *C. citriodora* and *E. camaldulensis* genotypes, while the least similarity value (0.131) was recorded between *E. Sargentii* and *C. citriodora* genotypes and there was no similarity between *E. transcontinentalis* and *E. camaldulensis* genotypes. The dendrogram based on RAPD and ISSR-PCR markers (Figure 6) separated *E. Transcontinentalis* genotype from the other seven Eucalyptus species genotypes and divided them into two major groups. The first group divided into two sub group, the first one included *C. citriodora, E. Camaldulensis* and *E. Sargentii* genotypes, while the second sub group included only *E. Stricklandii* genotype. The second group included *E. astringens, E. Leucoxylon* and *E. Phaenophylla* genotypes. Molecular marker techniques such as AFLP, RAPD and SSR have been used for genetic linkage mapping and to assess genetic diversity and phylogeny of Eucalyptus species [11,45,46,47,42].

Considerable morphological and genetic variation was observed among Eucalyptus species also showed close affinities with each other which might be due to sharing of almost similar habitat and ecology (figure1). Further studies need to be done on different aspects including more species ecology, medicinal importance and further molecular studies. In this study, we used RAPD and ISSR markers to estimate the genetic relationships of eight Eucalyptus species. We found high inter-species diversity, but little intra-species diversity. A similar result was reported for the Populus species, P. tremula L. and P. alba L. [48].

Conclusion

-From previous results it can be concluded that, *E. camaldulensis* overpass of plant height (cm) and number of branches characters. Meanwhile, *E. astringens* overpass of fresh and dry leaves weight/ plant (g), essential oil % in dry leaves and essential oil yield/plant (ml).

- The obtained results of this study confirmed that, *E. camaldulensis* was more suitable for Egyptian conditions than the others because it recorded the highest values in plant height and number of branches, despite the maximum essential oil yield in dry leaves ml/ plant was observed with *E. astringens* (Table1). So it is favorable to introduce this species as exotic in Egypt.

- An adverse range of oil yield and chemical composition has been demonstrated due to the kind of volatile oils isolated from *Eucalyptus species*. The oil yield and its chemical compositions have been described above. Such wide differences and the pharmacological profiles provide a stimulus make an effort for further researches and liable to find more new compounds will be isolated from an increasing number of *Eucalyptus species* cultivated in the future.

- According to the obtained dendrogram of the combination between RAPD and ISSR results, *E. Transcontinentalis* existed alone in a group. This may be explain the growth results of this study where *E. Transcontinentalis* showed the lowest value in most growth parameter (plant height, No of branches/plant, No of leaves/plant, fresh and dry leaves weight/plant, essential oil (%) and essential oil yield per plant.

- This study provides evidence that RAPD and ISSR polymorphism could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes and this study revealed that the phenotypic was as a result to interaction between the genotypic and ectopic.

Acknowledgments

We are thankful to professor Dr. Mahassen Abd EL-Ghanny Sidky who obtained the seeds of six introduced *Eucalyptus* species under this study from Forest and Water Ministry, Tunisia and for her support in any respect during completion of the research.

Table (7): Similarity value (Pairwise comparison) of Eucalyptus species genotypes based on over-combination of RAPD and ISSR analysis.

	E. stricklandii	***E. astringens***	***E. phaenophylla***	***E. leucoxylon***	***E. transcontinentalis***	***E. sargentii***	***C. citriodora***
E.stricklandii							
E.astringens	0.489						
E.phaenophylla	0.489	0.209					
E.leucoxylon	0.375	0.270	0.270				
E.transcontinentalis	0.804	0.804	0.804	0.343			
E.sargentii	0.415	0.517	0.619	0.279	0.751		
C. citriodora	0.670	0.581	0.670	0.685	0.996	0.131	
E.camaldulensis	0.262	0.359	0.359	0.442	0.000	0.375	1.000

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