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Morphological, Phytochemical and Molecular Characterization on Some Jatropha Species Cultivated in Egypt

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Abstract

This study was to screen the morphological, phytochemical attributes and describe the similarity and diversity in terms of RAPD and ISSR profiles of four *Jatropha* species (*J. multifida, J. gossypifolia, J. integrrima* and *J. curcas*). As well, investigate genetic diversity among them for using in conserving at Gene-Banks and genetic improvement. It appeared that show the variation in twenty-three quantitative and qualitative attributes among Jatropha species under Egyptian condition. The results of Pearson correlation among twelve quantitative revealed high significant positive correlations. Negative correlation observed between petiole length and other traits except seed breadth, whereas it was -0.97 when be compared with pod length. In PCA studied, it noted that leaf length, leaf width and petiole length were contributing most to the separation among species. The result showed that all secondary metabolites analyzed were present in all leaves of Jatropha species studied but at different concentrations and it obtained confirm the relatedness of these species and spotlight these important phytochemicals in the species. At molecular study, four RAPD-primers displayed a total of 66 amplified fragments, in which 50 (75.76%) were polymorphic fragments. The number of total amplified fragments scored per primer ranged from 10 (primer OP-D09) to 24 (primer OP-Z03). Thirty-six out of 66 RAPD-PCR fragments were found to be useful as cultivar specific markers. In ISSR analysis, 5 of the ISSR primers generated variable banding patterns. A total of 63 out of 90 ISSR fragments were polymorphic. 36 amplified fragments were considered as cultivar-specific markers. Results of the combination of the banding patterns of both techniques, data exhibited that the most two closely related species were *multifida* and *gossypifolia* with the highest similarity index (1.00). On the other hand, the two most distantly related species were *curcas* and *integerrima* with no similarity index (0.00). The 9 primers of RAPD and ISSR yielded 113 polymorphic markers that unambiguously discriminated 4 genotypes into three clusters. In conclusion, polymorphisms of both 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: Jatropha Species, Fingerprinting, Genetic Relationship, Molecular Markers.

Introduction:

Genus Jatropha belongs to the family Euphorbeaceae and contains approximately 170 known species. It widely distributed tropical and sub-tropical areas (Li et al., 2009). However, this crop is known to respond well to climatic conditions and exhibits phenotypic plasticity. The crop has acclimatized well in diverse agro-ecological and has accumulated variation over the years. Jatropha has a multipurpose with significant economic importance and having the capabilities to rehabilitate the degraded lands. Nowadays, the most of Jatropha's research mostly focused on the distribution areas, cultivation and nursery (Wenjun et al., 2008). Oil from seed is important as it used as bio-fuel (Banerji et al., 1985 and Takeda, 1982). Molecular studies around the world have reported, as not extensive, the existence of diversity in Jatropha. Jatropha plants widely used due to seed oil content as high as 40-

50% and environmental protection so become a high quality woody biomass energy materials species, in recent years, caused extensive attention all over the world and set off a wave of development and utilization (Basha and Sujatha, 2007). Regarding research has made certain progress, but the basic biological research of seed Jatropha curcas and its wild relatives are very limited, that makes big blindness in seed transfer aspect which seriously influence the scale cultivation and development (Sudheer et al., 2009).

The variation in morphological traits is necessary documented to tap effectively the available diversity in the crop improvement programs. The consequent descriptor and descriptor states is an initial step and not an exhaustive one as there exists still an unexplored and untapped genetic potential for the crop at large. The descriptor list helps in characterization of germplasm for development of a dataset of the collected material for utilization in the Jatropha improvement programs. Jatropha germplasm collection has its own set of challenges, lack of information and experience in identification of the areas of its availability are the major limitations. However, this crop characterized by variable and unpredictable yield for reasons that have not been identified, which limit the large-scale cultivation and warrant the need for genetic improvement of the species. Establishing genetic distances through DNA fingerprinting methods and the information generated can used for genetic improvement of the species (Ginwal et al., 2004).

Secondary metabolites are phytochemicals produced as by-products of primary metabolism (Bako and Aguh, 2007) and are less widespread in plants. It is of course this restricted occurrence among plants that renders them valuable and useful in taxonomic delimitation of species.

In addition to morphological traits, molecular evidences have been effectively used to assess genetic variation and phylogenetic relationships within and among populations of the same species, as well as between closely related species (Gottlieb, 1977; Crawford, 1985; Hamrick and Godt, 1997). Random Amplified Polymorphism DNA (RAPD) markers are a modification of Polymerase Chain Reaction (PCR) used in the late 1980 (Williams et al., 1990). Among PCR based molecular markers RAPD is a widely used technique in different plants (Nazar and Mahmood, 2011; Mahmood et al., 2011 and 2010 a and b). PCR technique is one of the best available DNA-based tools for scoring variations between cultivars within species (Lakshmikumaran and Bhatia, 1998). This technique widely used for the estimation of genetic variability. The cultivar identification and differentiation in various species including rice (Mackill, 1995), broccoli and cauliflower (Hu and Quiros, 1991), jews mallow (Mahdy, 2012), banana (Howel et al., 1994), Brassica (Jain et al., 1994), wheat (Chandrashekhar and Nguyen, 1993), alfalfa (Yu and Pauls, 1993), coffea (Orozco-Castillo et al., 1994), lettuce (Mahdy, 2012) and tomato (Williams and St Clair, 1993).

In the present paper, it was to screen the Morphological attributes and describe the similarity and diversity in terms of RAPD and ISSR profiles of four Jatropha species and investigate Genetic diversity among them for using in conserving at gene-banks and genetic improvement.

Materials and Methods:

Four species of Jatropha were J. multifida, J. gossypifolia, J. integrrima and J. curcas respectively used in this study. The Jatropha germplasm cultivated at the experimental Garden of the National Gene Bank, Giza, Egypt, under uniform soil conditions and subjected to uniform package of agriculture practices.

Morphological Attributes:

The germplasm morphologically established in characterization and pre-evaluation according to Sunil et al. (2010 and 2013). The comparison of four species conducted into eleven quantitative traits, as well as to twelve qualitative attributes as shown in Tables 2 and 3.

Sample preparation and extraction for phytochemical study

Chlorophyll (a and b) and carotenoids determination

The protocol devised by (Nagata and Yamashta, 1992) was followed to determine chlorophyll a, b and carotenoids contents. 0.2 gram Jatropha leaf sample was ground in 10 mL of 80% acetone and filtered through Whatman No. 1 filter papers. The filtered extract was transferred in cuvette and absorbance was noted at 662, 644 and 440 nm by using UV-spectrophotometer. Following formulae were used to calculate chlorophyll a, chlorophyll b and carotene contents.

Chlorophyll a = 0.999 A662 – 0.0989 A644

Chlorophyll b = -0.328 A662 + 1.77 A644

Carotenoids = 4.695 X A 440 – 0.268 X A664 + A662

For methanolic extraction: grounding (2 g) green leaves in a pestle with 20 ml of 80% methanol. The homogenate was filtered to obtain methanolic extraction colorless.

Spectrophotometric measurements:

The spectrophotometric measurements were performed using an ultraviolet-visible spectrophotometer (model MA9523-SPEKOL 211, ISKRA, Horjul, Slovenia).

Total phenols

The total phenolics content of methanolic extract was determined according to the method described by (Singleton et al., 1999) by folin-ciocalteu reagent. The absorbance was recorded at 725nm.

Total flavonoids

Total flavonoids were estimated using method of (Woisky and Salation, 1998) using aluminum chloride; the absorbance was measured at 420 nm.

Total antioxidant capacity:

The total antioxidant capacity of moringa leaves extracts was evaluated by the phosphomolybdenum method by (Prieto et al., 1999). The absorbance of the solution was measured at 695 nm with a spectrophotometer against methanol as the blank. Ascorbic acid (AA) was used as the standard.

Characterization of Jatropha at Molecular Level

Random Amplified Fragment DNA (RAPD-PCR) Analysis

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, MgCl2 and Taq polymerase. A total of twenty random DNA oligonucleotide primers were independently used according to Williams et al. (1990) in the PCR reaction. Only four 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 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 Jatropha species under study. Only four primers succeeded to generate reproducible polymorphic DNA products. Table (1) 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 the 100bp ladder marker (1500, 1000, 900, 800, 700, 600, 500, 400,300,200 and 100 bp).

Inter Simple Sequence Repeat (ISSR-PCR) Analysis:

ISSR-PCR reactions were conducted by 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 MgCl2 (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 separated on a 1.5 % agarose gels and fragments sizes estimated with the 100bp ladder marker.

Only five primers succeeded to generate reproducible polymorphic DNA products. Table (1) lists the base sequences of these DNA primers that produced informative polymorphic bands.

ISSR Primer		RAPD Primer	
Primer Name	**Sequence**	**Primer Name**	**Sequence**
HB-8	5`GAG AGA GAG AGA GG 3`	OP-D01	5´ACCGCGAAGG3´
HB-9	5` GTG TGT GTG TGT GC 3`	OP-D03	5´GTCGCCGTCA3´
HB-12	5` CAC CAC CAC GC 3`	OP-D09	5´CTCTGGAGAC 3´
HB-13	5` GAG GAG GAG GC 3`	OP-Z03	5`CAGCACCGCA 3`
HB-15	5` GTG GTG GTG GC 3`		

Table (1): List of the primer names and their nucleotide sequences of RAPD and ISSR procedures used in the study.

Statistical analysis:

The measured average values of morphological attributes carried out to evaluate the principal component analysis (PCA). The all data set was calculated using Pearson Correlation matrix. Phytochemical data were subjected analysis according to Snedecor and Cochran (1980). Mean separation was done using least significant difference (LSD) test at 1 and 5% level. All data represented as means of triplicate ± standard error. The DNA bands generated by each primer counted and their molecular sizes 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 studied Jatropha species. Calculation achieved using Dice similarity coefficients (Dice, 1945). All data set performed by SPSS (version. 14.0) Program.

Results:

Morphological characterization

The twenty-three characteristics of Jatropha species (Plate 1) examined. The morphological parameters of Jatropha species presented in Table (2). The average of the quantitative morphological traits employed presented in Table (3). It appeared that show the variation among Jatropha species under Egyptian condition

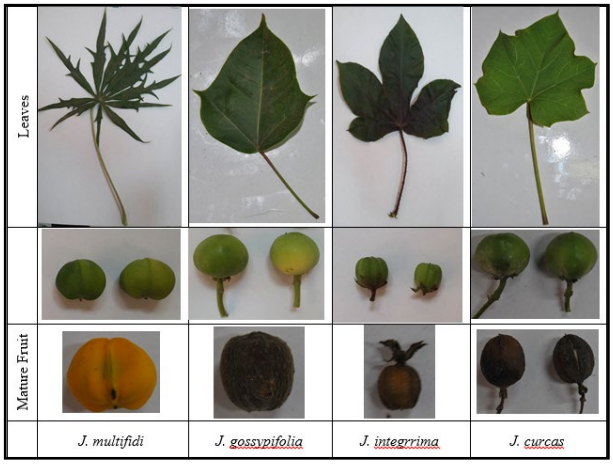


Plate (1): Shape of leaves and fruits for four Jatropha species. (Referred by Therese Labib, consultant of plant taxonomy).

No.	Characteristics	*J. multifida*	*J. gossypifolia*	*J. integrrima*	*J. curcas*
1	**Plant growth** (**1.**Shrub= <5m; **2.**Tree= >5m.)	2	1	1	2
2	**Plant canopy** (1.Narrow; 2.Intermediate; 3.spreading)	2	1	1	1
3	**Leafiness** (1.abundant; 2.moderate; 3.scanty)	3	3	1	1
4	**Branching pattern** (1.basal; 2.Intermediate; 3.top; 4.entire)	2	4	3	2
5	**Stem color** (1.green; 2.grey)	2	2	2	1
6	**Emerging leaves pigmentation** (1.green; 2.green-greyed purple; 3.yellow-green; 4.greyed-purple; 5.dark greyed purple; 6.red)	1	5	4	1
7	**Petiole base pigmentation** (0.Absent; 1.present)	0	1	0	0
8	**Leaf Color** (1.Light Green; 2.Green; 3.Dark Green)	2	2	3	1
9	**No. of leaf lobes** (1. 0 – 2; 2. 3 – 5; 3. >5)	3	2	2	2
10	**Inflorescence** (1.axillary; 2.terminal)	2	2	2	1
11	**Flower color** (1.Cream-yellow; 2.white; 3.red)	3	3	3	1

Table (2): The Morphological characteristics of Jatropha species.

No.	Characteristics	*J. multifida*	*J. gossypifolia*	*J. integrrima*	*J. curcas*
1	**Leaf blade length** (cm)	19.1	10.0	12.1	14.0
2	**Leaf blade width** (cm)	20.1	11.3	11.0	13.5
3	**Leaf Length/Width Ratio** (cm)	0.95	0.89	1.10	1.04
4	**Petiole length** (cm)	5.90	9.50	7.40	6.30
5	**Length of Fruit Stalk** (cm)	2.13	.95	0.51	3.82
6	**Width of Fruit Stalk** (cm)	0.86	0.52	0.20	0.31
7	**Pod Length** (cm)	2.92	1.41	1.88	2.88
8	**Pod Width** (cm)	1.82	1.46	1.81	2.60
9	**Pod Breadth** (cm)	1.61	0.97	1.04	1.11
10	**Seed Length** (cm)	1.83	0.66	0.64	1.63
11	**Seed Width** (cm)	1.33	0.47	0.41	1.18
12	**Seed Breadth** (cm)	1.38	1.40	1.56	1.38

Table (3): Quantitative Traits of Jatropha species.

Traits	Leaf blade length	Leaf blade width	Leaf Length/ Width Ratio	Petiole length	Length of Fruit Stalk	Width of Fruit Stalk	Pod Length	Pod Width	Pod Breadth	Seed Length	Seed Width
Leaf blade width	0.9628										
Leaf Length/ Width Ratio	-0.0083	-0.2774									
Petiole length	-0.8572	-0.6972	-0.4486								
Length of Fruit Stalk	0.4385	0.3819	0.0721	-0.6252							
Width of Fruit Stalk	0.6793	0.8498	-0.7180	-0.2154	0.0859						
Pod Length	0.8564	0.7513	0.2267	**-0.9510**	0.8056	0.3340					
Pod Width	0.2739	0.1017	0.5331	-0.6704	0.8762	-0.3362	0.7276				
Pod Breadth	**0.9724**	**0.9876**	-0.1863	-0.7164	0.2784	0.8140	0.7219	0.0509			
Seed Length	0.8753	0.8471	-0.0631	-0.8401	0.8097	0.5496	**0.9577**	0.5936	0.7890		
Seed Width	0.8654	0.8467	-0.0982	-0.8185	0.8139	0.5658	0.9467	0.5820	0.7837	**0.9993**	
Seed Breadth	-0.3752	-0.5321	0.6887	0.1588	-0.6713	-0.6332	-0.4483	-0.2342	-0.3933	-0.6663	-0.6945

Table (4): Correlation matrix basing on quantitative traits of Jatropha species.

Component matrix	PC 1	PC 2	PC 3	PC 4
Leaf blade length	**3.8652**	-0.3119	-0.0097	0.2038
Leaf blade width	**3.9062**	-0.1956	-0.0660	-0.2120
Leaf Length/Width Ratio	-1.1565	0.0469	-0.0745	0.0498
Petiole length	**1.4964**	1.0138	0.0853	-0.0074
Length of Fruit Stalk	-0.8410	-0.2805	0.3337	-0.0722
Width of Fruit Stalk	-1.3889	0.0007	-0.1270	-0.0772
Pod Length	-0.6690	-0.1249	0.0260	0.0565
Pod Width	-0.7797	-0.0097	0.0710	0.0929
Pod Breadth	-1.0996	-0.0018	-0.1082	-0.0053
Seed Length	-1.1121	-0.1317	-0.0159	-0.0419
Seed Width	-1.2446	-0.1008	-0.0372	-0.0422
Seed Breadth	-0.9764	0.0954	-0.0773	0.0550
Eigenvalue	3.8559	0.1179	0.0154	0.0108
Variability (%)	96.40	2.95	0.39	0.27
Cumulative (%)	96.40	99.34	99.73	100.00

Table (5): Factor loading of Jatropha species quantitative Traits.

Similarities matrix on correlation of Jatropha species (Table 4) showed that close resemblance of species could observed when certain characters employed. For instance, when leaf length was correlated with leaf width, the degree of affinity was 0.963 and 0.9724 when correlated with pod breadth. Similarly, when leaf width was correlated with pod breadth, the degree of resemblance was 0.9724, when pod length was correlated with seed length; the degree of affinity was 0.9577. Whereas, it was almost 0.9993 when the seed length compared with seed width. The results revealed highly significant positive correlations among almost all the analyzed traits. Negative correlation observed between petiole length and other traits except seed breadth, whereas it was -0.9724 when compared with pod length.

Table (5) showed the factor loading, the variability and cumulative Principal Component Analysis (PCA) of the twelve quantitative morphological characters, as well as it reveals that some traits are more valuable comparing with others in the genus variation. It was noted that leaf length, leaf width and petiole length were contributing most to the separation among species.

ii. Phytochemical Study:

The present study was carried on methanolic extracts of four jatropha species leaves to investigate the presence of medicinally important phytochemicals in the leaves of different Jatropha species. Data presented in Table (6) showed that the leaves of all studied species contain a significant amount of chlorophyll a, chlorophyll b, carotenoids, phenols, flavonoids and antioxidant content. The highest chlorophyll a and chlorophyll b content were found in J. integerrima (282.75 and 182.91 mg/100g) followed by J. multifida (258.35 and 102.98 mg/100g) respectively. While the lowest chlorophyll a content recorded by J. gossypifolia (89.31 mg/100g) and the lowest chlorophyll b content recorded by J. curcas (46.21mg/100g). The same trend was observed in carotenoids content; it represented (171.99, 153.93, 94.68 and 47.12mg /100g) for J. multifida, J. integerrima, J. curcas and J. gossypifolia respectively. The phenols content was quantitatively estimated and was starting from 17.09 mg/100g for J. multifida and 21.20mg/100g for J. gossypifolia, reaching up to 40.30mg/100g for J. curcas and 43.80 mg/100g for J. integerrima. Flavonoids content were observed to be much closed between the four Jatropha species. The highest flavonoids content (38.22 mg/100g) was observed in the leaves of J. multifida Table (6). Flavonoids was 37.16 mg/100g in J. curcas leaves, 36.15 mg/100g in J. gossypifolia and the least flavonoids content 33.13 mg/100g was observed in the leaves of J. integerrima. Similarly, all the species exhibited good quantity of total antioxidant capacity, the minimum content (193.26 mg/100g) was found in J. multifida, 300.96 mg/100g was in J. gossypifolia and the maximum content (302.33 and 302.77 mg/100g) were found in J. integerrima and J. curcas respectively.

Species	Chlorophyll a (mg/100g)	Chlorophyll b (mg/100g)	Carotenoids (mg/100g)	Total Phenols (mg/100g)	Total Flavonoids (mg/100g)	Total Antioxidant Capacity (mg/100g)
J. multifida	258.35±0.657	102.98±0.277	171.99±11.593	17.09±0.010	38.22±3.26	193.26±15.73
J. gossypifolia	89.31±0.277	76.65±0.557	47.12±0.098	21.20±0.018	36.15±2.11	300.96±0.03
J. integerrima	282.75±1.260	182.91±0.309	153.93±6.547	43.80±0.166	33.13±2.12	302.33±0.12
J. curcas	132.12±0.113	46.21±0.040	94.68±0.603	40.30±0.130	37.16±1.57	302.77±0.08
$LSD_{0.05}$	2.22	1.09	18.98	0.295	2.57	27.32
$LSD_{0.01}$	3.36	1.65	28.75	0.45	3.897	41.38

Table (6): Average mean and standard error of phytochemical contents for leaves of Jatropha species under study.

Molecular Genetic Identification:

Randomly amplified polymorphic DNA (RAPD) markers:

Table (7) and Figures (1 and 2) show the results of total amplified fragments (TAF), amplified fragments (AF) and specific markers (SM) for each species of Jatropha using RAPD-PCR analysis with four random primers. A total number of 66 DNA fragments were detected, in which 50 (75.76%) were polymorphic fragments. However, 16 bands were common (monomorphic) for all cultivars. Polymorphism levels differed from one primer to another, i.e. The results found that (OP-Z03 and OP-D09) primers exhibited high levels of polymorphism (91.67% and 80.00%) respectively. While, (OP-D03) primer exhibited moderate level of polymorphism (73.33%) and primer (OP-D01) represented the lowest level 52.94% as exhibited in Table (7). The lowest number of polymorphic fragments was detected for primer OP-D09 and OP-D01 (8 out of 10 amplified bands and 9 out of 17 amplified bands) respectively, while the highest number of polymorphic fragments was detected for primer OP-Z03 (22 out of 24 amplified bands). Cultivar-specific markers generated from RAPD-PCR analysis are shown in Table (7). Thirty-six out of 66 RAPD-PCR fragments were found to be useful as cultivar-specific markers. The largest number of RAPD-PCR markers was scored for gossypifolia (42 markers), while the lowest (25 markers) was scored for multifida. In the meantime, the highest number of RAPD-PCR cultivar-specific markers was generated by primer OP-Z03 (16 markers), while the lowest number (6 markers) was generated by primer OP-D03.

In conclusion, all of the four primers used allowed enough distinction among the species under study. These cultivar-specific markers can be used in subsequent

Table (7): Species-specific RAPD and ISSR markers for Jatropha species genotypes.

Primer Name	Range of MS*	TAF*	MF*	PF*	SM* (bp)	Polymorphism (%)
RAPD primers						
OP-D01	285-1641	17	8	9	7 (612, 356, 318) - (579, 285) - (0) - (425, 319)	52.94
OP-D03	186-1762	15	4	11	6 (0) - (553, 487) - (0) - (385, 363, 214, 186)	73.33
OP-D09	408-1234	10	2	8	7 (0) - (0) - (1117) - (1234, 1194, 899, 830, 683, 515)	80.00
OP-Z03	106-1449	24	2	22	16 (0) - (1449, 1074, 1016, 873, 737, 704, 627, 546, 439, 357, 308, 197) - (620, 449) - (680, 325)	91.67
Total RAPD		**66**	**16**	**50**	**36**	
ISSR primers						
HB-08	178-921	19	6	13	8 (453, 337) - (510) - (717 ,685, 349, 193, 178) - (0)	68.42
HB-09	248-1052	22	6	16	11 (1052, 859, 556, 465) - (470) - (600, 364, 333) - (1027, 444, 347)	72.73
HB-12	243-1524	19	8	11	4 (0) - (1497) - (426, 307) - (288)	57.90
HB-13	221-1653	16	2	14	5 (221) - (0) - (571) - (1653, 1168, 1075)	87.50
HB-15	215-1063	14	6	8	8 (0) - (0) - (949, 804, 478, 313, 288, 254, 215) - (380)	57.14
Total ISSR		**90**	**28**	**63**	**36**	
Total		**156**	**44**	**113**	**72**	

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

experiments to detect molecular markers for polymorphic genes with economic importance among these and other species.

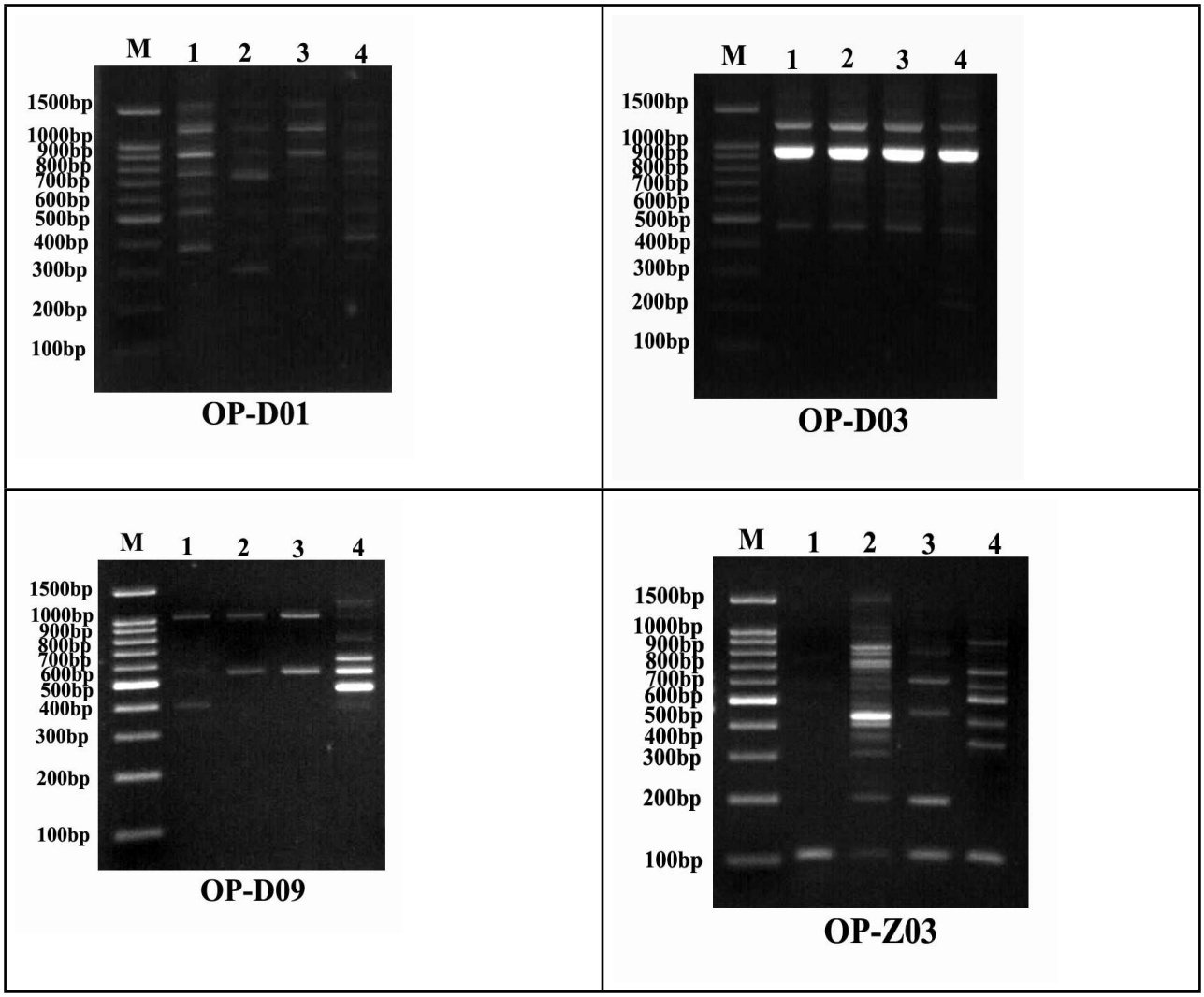
Genetic similarity and cluster analysis based on RAPD markers

Genetic similarities among the four Jatropha species were estimated according to the RAPD data by using UPGMA computer analysis (Table 7 and Fig. 1). Table (7) showed that the most two closely related species were multifida and integerrima with the highest similarity index (1.000). On the other hand, the results indicated that the two most distantly related species were integerrima and gossypifolia with low similarity index (0.609). The results showed that there was no similarity between gossypifolia and curcas species. A dendrogram for the genetic relationship among the four genotypes of Jatropha species genotypes is exhibited in Fig. (3), which separated them into two major groups. The first group included curcas only, while the second group included two subgroups, the first subgroup involved gossypifolia only and the other subgroup included integerrima and multifida genotypes.

Inter Simple Sequence Repeats (ISSR) markers:

The five ISSR primers succeeded in amplifying DNA fragments for the four Jatropha species genotypes (Fig. 2). Polymorphism levels differed from one primer to another, i.e. HB-13 primer exhibited high level of polymorphism (87.50%), while, HB-12 and HB-15 primers exhibited low levels of polymorphism (57.90% and 57.14%) respectively as exhibited in Table (7). The number of total amplified fragments (TAF), polymorphic fragments (PF), monomorphic fragments (MF) and specific markers (SM) for each primer of the four primers are shown in Table 6. HB-08 Primer showed nineteen DNA fragments with molecular size ranging from 178 to 921 bp (Fig. 2 and Table 7), thirteen fragments were polymorphic (68.42 %), and eight of them were positive species-specific markers at (453 and 337 bp) for multifida genotype, 510 bp for gossypifolia and (717, 685, 349, 193 and 178) for integerrima genotype. However, HB-09 primer showed twenty-two DNA fragments with molecular sizes ranging from 248 to 1052 bp, sixteen fragments were polymorphic (72.73 %) and eleven of them were positive species- specific markers at (1052, 859, 556 and 465 bp) for multifida genotype, 470 bp for gossypifolia, (600, 364 and 333 bp) for integerrima and (1027, 444 and 347 bp) for curcas. HB-12 primer showed nineteen DNA fragments with molecular size ranging from 243 to 1524 bp, eleven fragments were polymorphic

Figure (1): RAPD-PCR analysis of different Jatropha species cultivated under Egyptian conditions.



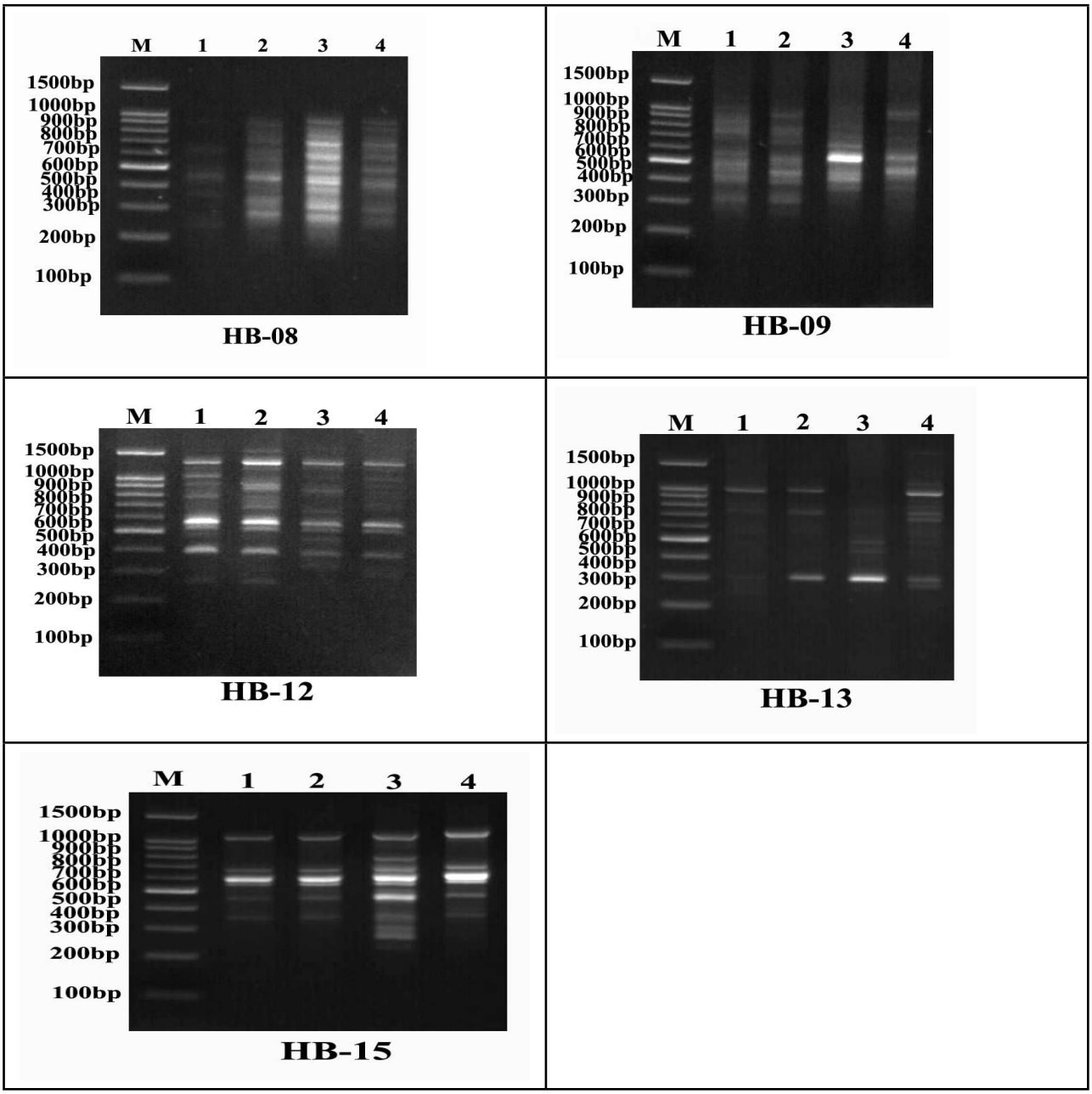


Figure (2): ISSR-PCR analysis of different Jatropha species cultivated under Egyptian conditions.
1- J. *multifida* 2-J. *gossypifolial* 3- J. *integerrima* 4-J. *curcas L.*

(57.90 %), and four of them were positive species- specific markers at (1497 bp) for gossypifolia, (426 and 307 bp) for integerrima genotype and at 288 bp for curcas. HB-13 primer showed sixteen DNA fragments with molecular size ranging from 221 to 1653 bp, fourteen fragments of them were polymorphic (87.50 %), and five of them were positive species- specific markers at (221 bp) for multifida genotype, (571 bp) for integerrima genotype and (1653, 1168 and 1075 bp) for curcas. Meanwhile, HB-15 primer showed fourteen DNA fragments with molecular size ranging from 215 to 1063 bp, eight fragments of them were polymorphic (57.14 %), and eight of them were positive species- specific markers at (949, 804, 478, 313, 288, 254 and 215 bp) for integerrima genotype and (380 bp) for curcas.

Genetic similarity and cluster analysis based on ISSR markers: According to ISSR results, the most two closely related species were gossypifolia and curcas (Table 9) with the highest similarity index (1.000). On the other hand, the most two distantly related species were multifida and gossypifolia with low similarity index (0.015) and the two varieties located very far were multifida and integerrima species with similarity index (0.000). Figure (4) indicated that the dendrogram revealed one main group of three species including two subgroups. Subgroup 1 included both gossypifolia and curcas and subgroup (2) included multifida species only. The remaining species integerrima represented distant sequences.

Species	*J. multifida*	*J. gossypifolia*	*J. integerrima*
J. gossypifolia	0.957		
J. integerrima	1.000	0.609	
J. curcas L.	0.837	0.000	0.689

Table (8): Similarity value (Pairwise comparison) of Jatropha species genotypes based on RAPD data.

Species	*J. multifida*	*J. gossypifolia*	*J. integerrima*
J. gossypifolia	0.015		
J. integerrima	0.000	0.702	
J. curcas L.	0.151	1.000	0.716

Table (9): Similarity value (Pairwise comparison) of Jatropha species genotypes based on ISSR data.

Species	*J. multifida*	*J. gossypifolia*	*J. integerrima*
J. gossypifolia	1.000		
J. integerrima	0.300	0.150	
J. curcas L.	0.850	0.600	0.000

Table (10): Similarity value (Pairwise comparison) of Jatropha species genotypes based on over-combination of RAPD and ISSR analysis.

Combined identification based on RAPD and ISSR analysis:

Species 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.

Therefore, it is better to use the combination of the banding patterns of the two techniques to use more segments of the genome that will increase the validity of the consensus tree. Results of the combined data as shown in Fig. (5) & Table (10) exhibited that the most closely related species were multifida and gossypifolia with the highest similarity index (1.000). On the other hand, the two most distantly related species were integerrima and gossypifolia with low similarity index (0.150) as well as the two species located very far were integerrima and curcas with similarity index (0.000)

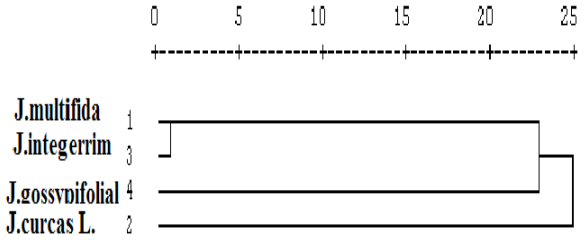


Figure (3): A dendrogram illustrates the genetic distance for Jatropha species genotypes based on RAPD data.

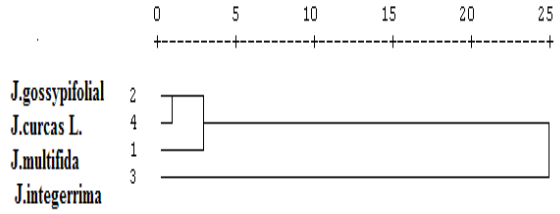


Figure (4): A dendrogram illustrates the genetic distance for Jatropha species genotypes based on ISSR data.

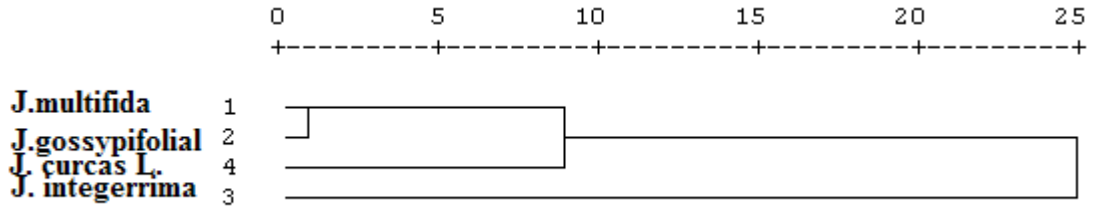


Figure (5): A dendrogram illustrates the genetic distance for Jatropha species genotypes based on over combination of RAPD and ISSR analysis.

The results of the consensus tree indicated that the tree divided the species into two main clusters, the first included integerrima species only. The second one divided into two subgroups, the first one included multifida and gossypifolia species and the other included curcas. This study provides evidence that RAPD and ISSR polymorphisms could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes.

Discussion:

Generally, quantitative traits add a quantitative element to species descriptions allowing more rigorous comparisons within a genus. In the numerical analysis of Jatropha species using twelve quantitative morphological traits, the results revealed that variations in the vegetative parts and fruit traits are important. Of the quantitative characteristics used, leaf length, leaf width and petiole length had the highest values compared with the others traits, confirming their usefulness for species identification purposes. Same trends had been observed by previous studies on Jatropha (Kolawole et al., 2016), Ficus species (Sonibare et al., 2004). The studied Jatropha species exhibited variations based on samples collected from different locations. The size of the fruit and bud length was dependent on the age of plants as earlier confirmed by other reports (Burkill, 1995). Leaf shape and size may vary within the same plant. Previous studies suggested that light intensity maybe affect the carbohydrate balance, which could affect the length of the cells in the direction of the long axis, thereby leading to differences in the length, shapes and width of the leaves (Soladoye et al., 2010). Such variations observed may be due to environmental, as well as genetic factors, and the interaction among them (Nwachukwu and Mbagwu, 2006).

This study reveals the presence of the subject secondary metabolites in the different Jatropha species to varying concentrations which is taxonomically useful; it also brings to bare the fact that the species are potential sources of these important phytochemicals. For instance, flavonoids are one of the most popular secondary metabolites possessing a variety of biological activities at nontoxic concentrations (Irshad et al., 2010). Dietary flavonoids are noted to play effective roles in cancer prevention (Ren et al., 2003; Aggarwal and Shishodia, 2006). Flavonoids together with the other secondary metabolites identified in Jatropha species have been severally reported in other plants to show curative activity against diverse pathogens, used traditionally as analgesic, antimicrobial and soothing herbs (Singh et al., 2009; Thirunavukkarasu et al., 2010; Ganesh and Vennila, 2011).

The RAPD polymorphism among Jatropha species in the present study ranged from 52.94 to 91.67 %. These results agree with the findings of Sudheer et al. (2008). Highest genetic similarity and minimum polymorphism was found in Jatropha curcas by RAPD analysis. Basha and Sujatha (2007) and Ram et al. (2007). In this work, we compared the applicability of ISSRs and RAPDs as genetic markers to characterize the four Jatropha species genotypes. The results found that RAPD markers were more efficient than the ISSR assay with regard to polymorphism detection, as they detected 75.76 % as compared to 70.00 % for ISSR markers. This is in contrast to the results as obtained for several other plant species like in wheat (Nagaoka and Ogihara, 1997) and Vigna (Ajibade et al., 2000).

The number of total polymorphic fragments is higher for ISSR than RAPDs. In fact, the ISSRs have a high capacity to reveal polymerphism and offer great potential to determine intra and inter genomic diversity as compared to other arbitrary primers like RAPDs (Zietkiewicz et al., 1994). A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two-marker techniques target different portions of the genome. The ability to resolve genetic variation among different genotype maybe more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed. Gupta et al. (2008) on Jatropha curcas and Mahdy (2012) on (Corchorus olitorius L. and Lactuca sativa L.) obtained the same conclusion. The differences found among the dendrogram generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (735 for RAPD and 646 for ISSR) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among barley cultivars. Loarce et al. (1996) have observed similar results in barley. Another explanation could be the low reproducibility of RAPDs (Karp et al., 1997). The differences in clustering pattern of genotypes using RAPD and ISSR markers may be attributed to marker sampling error and/or the level of polymorphism detected, reinforcing again the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among cultivars (Loarce et al., 1996).

Conclusion:

Conclusively, morphological parameters provided a greater discrimination along the spectrum of taxonomic differences among Jatropha species, as well as were more sensitive in the delimitation of the studied taxa. As well, this species has immense potential to be used in the area of pharmacology and as a prospective source of valuable drugs. In addition, the study hereby revealed more detailed information on the level of relationship within the genus Jatropha. As well as, it can conclude that the molecular analyses of RAPD and ISSR markers were extremely useful for studying the genetic relationships among Jatropha species, providing both markers a powerful tool for the generation of potential fingerprinting diagnostic markers.

It can be observed from RAPD dendrogram that J. curcas species is in a group alone and all the other species in another group. It may explain that the curcas species has some different morphological characteristics such as stem color, leaf color, flower color and inflorescence. It is observed from ISSR dendrogram that J. integerrima is distinguished from the rest of species. It is in agreement with the morphological characters. It is delimited from the rest of species morphologically by length & width of fruit stalk, seed breadth, branching pattern and leaf color. Consequently, it is simply to distinguish the J. integerrima & J. curcas morphologically as well as by ISSR and RAPDs respectively.

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