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Morphological and Issr Polymorphisms in Some Egyptian Grapes (*Vitis vinefera* L.) Collection

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Abstract: Grape (*Vitis vinefera* L.) is one of the main crops in Egypt. Grape culture consists of ecotypes and local cultivars called varieties. These are clonally propagated and widely extended throughout all the country. However, for several decades Egyptian grapes are seriously threatened by severe genetic erosion due to biotic and abiotic stresses. In order to preserve local genotypes and to improve grape culture in Egypt, we become interested in the genetic diversity analysis in this important crop. Hence, a study was carried out using three cultivars and 20 morphological characters. The genetic analysis through molecular markers was performed using ten primer of Inter Simple Sequence Repeat (ISSR). Here we report how the mentioned analysis could provide us markers suitable for the evaluation of grape genetic diversity and as criteria in the cultivar identification and study the phylogenetic relationships between cultivars. These traits were also useful for the genetic preservation in this crop.

Key words: Grape · Cultivar · Characterization · DNA fingerprint · Dendrogram

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important crop plants of the world. In the last decades it has become imperative to handle the large germplasm of grapevine as well as identify the different cultivars. The domestication of grapes and wine making began in 3200 BC in Egypt [1]. Almost 90% of grapes produced in Egypt are consumed as fresh grapes and the remaining used for raisins and juice. Egypt is one of the top 13 producers of grape in the world, which the total production was 1.531418 tones [2]. The characterization of cultivars is an essential stage in the certification program, improvement and conservation of germplasm and monitoring of the genetic quality [3]. In this regard, characterization descriptors are comprised of highly heritable qualitative traits that can be equally expressed in all conditions [3]. It may also include a limited number of additional traits thought desirable by a consensus of users. The limitation related to the characters that have additive inheritance, which are highly influenced by the environment to cultivate germplasm with great phenotypic similarity [4]. PCR based DNA markers, provide powerful tools for genetic analysis because of their simplicity and ease of handling. Inter-Simple Sequence Repeat Polymorphic DNA (ISSR) is a fast technique for generating genetic markers [5]. ISSR are applied in many aspect of genetic research such as cultivar identification [6, 7] determination of genetic relationship [8], analysis of genetic diversity, construction of linkage genetic maps and others [9-10]. In grapes, ISSR approach has been applied so far to analysis a limited number of varieties [11, 12].

This study aimed to study the morphological and molecular characteristics of three Egyptian grape varieties (*Vitis spp*.) using ISSR techniques.

MATERIALS AND METHODS

Morphological and molecular characterization of three Egyptian grape cultivars under the present investigation was carried out on 10-15 years old grapevines grown at El-Monofyia, Assiut and Matrouh Governorates.

Morphological Characterization: The morphological characteristic used to characterize and discriminate the three grape cultivars were based on descriptor prescribed for grape by the International Plant Genetic Resources

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Institute [3]. The study was performed using three vines for each cultivar; each vine was considered a replicate. Young shoots were characterized for shoot tip form and anthocyanin coloration of tip and shoots were characterized for length of tendril. Young leaf were collected and characterized for color of upper surface, density of prostrate hairs between veins and density of prostrate hairs on main veins. Thirty mature and fully developed leaves per vines (mature leaves from one year old branches) were collected and characterized for shape of blade, numbers of lobes and anthocyanin coloration of main veins on upper side of blade, general shape of petiole sinus, shape of upper lateral sinus and depth of upper lateral sinus. For Inflorescence only data were recorded for sex of flower. All observations on bunches and its related parts were made at the optimum maturity stage according to IPGRI [3]. Data were documented for bunch density, single bunch weight, berry shape, presence of seeds, skin color and single weight of berry. Fully developed seeds were extracted from fully ripened berries taken from each vine of three replicates to estimate the seed weight.

Statistical Analysis: The obtained data were statistically analyzed according to Sendecor and Cochran [13]. Least significant difference (LSD) at $p \leq 0.05$ was employed to estimate the significant of differences between the treatment means.

Molecular Marker

Isolation of Plant Genomic DNA: DNA extraction was carried out using leaf materials collected from each cultivar. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (QIAGEN, Chatsworth, CA).

Scoring of Data: Scoring of ISSR data was performed using 1% agarose gel electrophoresis profile, as clear and distinct fragment were scored as (1) for presence and (0) for absence.

Molecular Fingerprinting of Grape Varieties Based on (Inter Simple Sequence Repeats (ISSRs): Inter Simple Sequence Repeats (ISSRs) was carried out according to the procedure given by Sharma *et al.* [14]. ISSR analysis was carried out in a total volume of 50 µl containing 5 µl of 10x buffer, 10 µl Q solution,5 µl of 2 mM dNTPs, 80 pmol primer, 0.5 µl hot start taq polymerase and 25 ng DNA. The temperature profile composed of initial denaturating cycle at 95°C for 25 min followed by 10 touch down cycles of 95°C/30 sec, 65-55°C/1 min, 72°C 90 sec. This was followed by 30 cycles of 95°C/ 0 sec, 55°C/1min, 72°C/90 sec and then a final extension cycle at 72°C for 7 min. The sequences of the ten ISSR primers (17899-B, 17898-A, 17899-A, 807, AW-3, TE, BEC and HAD were synthesized by Metabion while BC 827 and BC 848 were synthesized by Pioneer) are presented in Table 1.

Table 1: Name and sequence of the primers used in ISSR analysis.

Primer code	Nucleotide sequences 5`_____3`
17899-B	(CA)6 GG
17898-A	(CA)6 AC
17899-A	(CA)6 AG
807	(AG)8 T
AW-3	(GT)7 AG
TE	GT (GGT)3 GAC
BEC	(CA)7 TC
HAD	CT (CCT)3 CAC
BC 827	(AC)8 G
BC 848	(CA)8 AG

RESULTS AND DISCUSSION

Morphological Characterization: The young shoot and mature leaf characters of the grape cultivars are presented in Table 2.The results illustrated that the cultivars Bez El-Anza and Red Romy have half open form of tip, whereas Matrouh has fully open. The degree of anthocyanin coloration of tip was weak for Bez El-Anza and Red Romy cultivars, while it was absent in Matrouh cultivar. For shoot characterization, Bez El-Anza exhibited the highest length of tendril length 19.33 cm (medium). Whereas, The lowest length of tendril was presented by Matrouh 10.04 cm (very short) and Red Romy showed intermediate value of tendril length 16.48 cm (short). Upper surface color of young leaf in Bez El-Anza was copper-yellow and in Matrouh showed green with bronze while in Red Romy was reddish. In young leaf, Bez El-Anza and Red Romy demonstrated absence of both density of prostrate hairs between veins and main veins. But, Matrouh had very spare prostrate hairs between veins and sparse prostrate hairs on main veins, respectively (Table 2 and Fig.1). The blade shape of mature leaf the three cultivars was recorded as pentagonal shape. Bez El-Anza and Matrouh showed 5 lobes of mature leaf, whereas Red Romy cultivar exhibited 3 lobes. Anthocyanin coloration of main veins on upper side of blade showed very weak, absent and medium for Bez El-Anza, Red Romy and Matrouh, respectively. Also, the general shape of petiole sinus

Table 2: Young shoot, shoot, young leaf and mature leaf characteristics.

		Varieties		
Characteristics		Bez El-anza	Red Romy	Matrouh
Young shoot	Form of tip	Half open	Half open	Fully open
	Anthocyanin coloration of tip	Weak	weak	Absent
Shoot	Length of tendril (cm)	19.33 a	16.48 b	10.04 c
Young leaf	Color of upper surface of young leaf	Copper yellow	Reddish	Green with bronze
	Density of prostrate hairs between veins	Absent	Absent	Very sparse
	Density of prostrate hair on main veins	Absent	Absent	Sparse
Mature leaf	Shape of leaf blade	Pentagonal	Pentagonal	Pentagonal
	Number of lobes	5 a	3 b	5 a
	Anthocyanin coloration of main veins on upper side of blade	Very weak	Absent	Medium
	Shape of petiole sinus	Wide open	Half open	Slightly open
	Shape of upper lateral sinus	Open	Closed	Lobes slightly overlapping
	Depth of upper lateral sinus	Medium	Shallow	Deep

Table 3: Inflorescence and fruit characteristics.

	Varieties		
Characteristics	Bez El-anza	Red Romy	Matrouh
Sex of flower	Male and female	Male and female fully	Male and female fully developed
Bunch density	Dense	Medium	Dense
Single bunch weight (g)	675.3 a	516.7 ab	475.2 a
Berry shape	Oblong	Round	Round
Presence of seed	Well developed	Well developed	Well developed
Berry Skin color	Green - yellow	Red	Blue - black
Berry single weight (g)	4.71 a	5.10 a	5.32 a
100 seed weight (g)	3.98 a	2.48 a	3.71 a

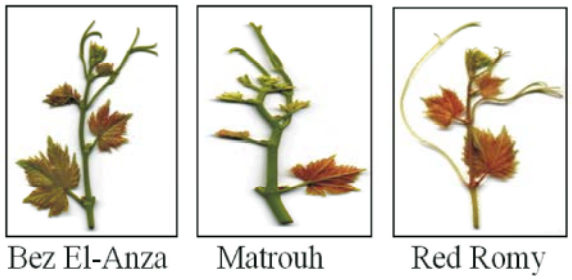

Fig. 1: Shoot tip

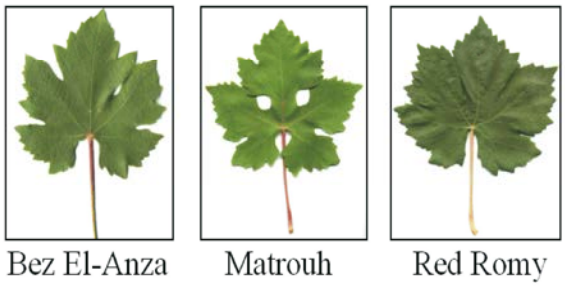

Fig. 2: Mature leaf

(degree of the opening of the petiole sinus) was recorded wide open, half open and slightly open in Bez El-Anza, Red Romy and Matrouh, respectively. The shape of upper lateral sinus showed open, closed and slightly overlapping lobes and medium, shallow and deep depth of upper lateral sinus for Bez El-Anza, Red Romy and Matrouh, respectively. The depth of upper lateral sinus of mature leaf was medium, shallow and deep in Bez El-Anza, Red Romy and Matrouh, respectively (Table 2 and Fig.2).

Data presented in Table 3 illustrated that the sex of flower and fruit characteristics, of three grape cultivars under study. In this regard, all cultivars showed male and female fully development flower. For fruit characteristics Bez El-Anza and Matrouh were dense bunch although, Red Romy showed medium bunch. Also, Bez El-Anza showed the highest fruit weight 675.3 g (high) followed by Red Romy 516.7 g (medium) and Matrouh 475.2 g (medium), whereas no significant differences were found between them. Berry shape of Red Romy and Matrouh showed round shape while; Bez El-Anza was oblong (Table 3 and Fig.3). All cultivars gave well developed seeds. The berry skin color was green-yellow in Bez El-Anza, red color in Red Romy and Matrouh recorded blue - black color. There was no significant difference among the three cultivars in berry single weight 4.71, 5.10

Fig. 3: Fruit

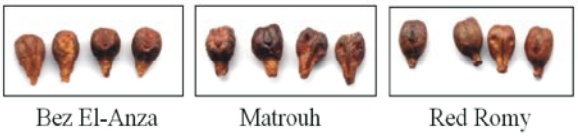

Fig.4: Seeds

and 5.32 g (medium) for Bez El-Anza, Red Romy and Matrouh, respectively. For 100 seed weight, Bez El-Anza and Matrouh recorded 3.98 and 3.71g (medium). While, Red Romy recorded 2.48 g (low) as shown in Table 3 and Fig.4.

Molecular Fingerprinting of Grape Varieties on Inter Simple Sequence Repeats ISSRs: Initially, 15 ISSR primers were screened for polymorphism and based on the clear scorable band pattern, 10 primers were selected for DNA analysis of the cultivars and were of good quality (Fig. 5). The size of the amplification products ranged from 213 to 1046 bp. The total numbers of scored bands were 89. The number of bands per primer varied from 7 to 14 with an average of 7 bands /template. The highest number of polymorphic bands was obtained with primers, 17899-B, 17898-A, BEC, BC 827, 807 and AW-3 (Table 4).

Table 4: Unique ISSR markers showing polymorphic bands among the three grape cultivars (*Vitis vinifera* L.).

Primer	Band size in bp	Bez El-anza	Matrouh	Red Romy
17899-B	722.855	0	1	1
	673.124	0	1	1
	644.551	0	1	1
	642.493	1	1	0
	589.81	1	1	0
	258.368	0	1	0
17898-A	678.278	0	0	1
	532.381	1	0	1
	496.06	0	0	1
17899-A	663.519	1	0	0
	556.767	1	0	1
807	600.046	1	1	0
	538.674	0	1	0
	469.83	1	0	1
	412.652	1	0	1
	369.072	0	1	1
	335.041	1	0	0
AW-3	637.987	0	1	1
	595.046	1	0	1
	522.352	1	0	0
	456.6	0	0	1
	425.016	0	1	0
	386.305	1	0	1
TE	447.219	0	1	1
	408.897	1	0	1
	395.866	0	1	1
	165.222	1	0	0
BEC	943.642	0	1	0
	723.701	1	0	0
	600	0	1	1
	581.151	1	0	1
	560.371	0	1	1
	544.216	0	1	0
	478.209	0	1	1
	431.477	1	0	0
	311.328	1	0	0
	281.137	0	1	0

Table 4: Continued

Primer	Band size in bp	Bez El-anza	Matrouh	Red Romy
BC 827	568.839	1	0	0
	455.061	0	1	1
	268.12	0	1	1
HAD	944.618	1	0	1
	846.28	1	1	0
	668.32	0	1	1
	635.342	1	0	1
	521.032	1	0	1
	470.021	1	0	1
BC 848	357.465	0	1	0
	324.218	1	1	1
	252.248	1	1	0

Notes: (1) means presence band, (0) means absent band.

Table 5: Statistics of the ISSR fragments for the three grape (*Vitis vinifera* L.) cultivars based on the ten ISSR primers.

AF SSR primers	Total amplified fragments	Polymorphic fragments	Percentage of polymorphism (%)
17899-B	12	6	50
17898-A	7	3	42.9
809	7	2	28.6
AW-3	7	6	85.7
17899-A	13	6	46.2
TE	7	4	57.1
BEC	14	10	71.4
HAD	7	6	85.7
BC-827	7	3	42.9
BC-848	8	2	25.0
Total	89	48	53.93

Total number of polymorphic bands was 48 with an average of 4.8 polymorphic fragments/primer. This represents an average of polymorphism 53.93 % (Table 6). The number of polymorphic markers varied among the different primers. Primers BEC generated 10 polymorphic bands with 71.4 % polymorphism. While primer BC848 showed low level of polymorphism (25 %) (Table 5). Eighteen out of eighty nine ISSR (about 20 %) were found to be useful as cultivar-specific markers (Table 5 and Fig.5) which some of them present in one cultivar and absent in the others cultivars in this study.

Number of ISSR-PCR fragments generated by using the ten primers and could be used as cultivar-specific markers, were arranged descending as primer BEC (six markers), primer AW-3 (three markers), Primers 17898-A and 807 (two markers), primers 17899-B, 17899-A, TE, BC827and BC848 (one marker). On the other hand, the primer HAD didn't show no cultivar-specific markers (Table 4 and Fig.5). Several authors reported on the usefulness of ISSR for cultivar identifications. The ISSR technique, isn't much more difficult for marker development than RAPD and also, requiring a small amount of DNA for amplification, enables the detection of the genome [5]. ISSRs are ideal as markers for genetic mapping and population studies because of their abundance and the high degree of polymorphism between individuals within a population of closely related genotypes [15]. ISSR analysis was used for the DNA profiling and characterization of total 143 Indian Grape varieties and rootstocks [16]. Genetic relationships were detected among the 15 cultivars of *Vitis spp.* materials using ISSRs markers [17]. Morphological and microsatellites analysis were the most useful techniques in the differentiation of the studied cultivars and accessions, though the latter one was definitive for the correct identification of cultivars.

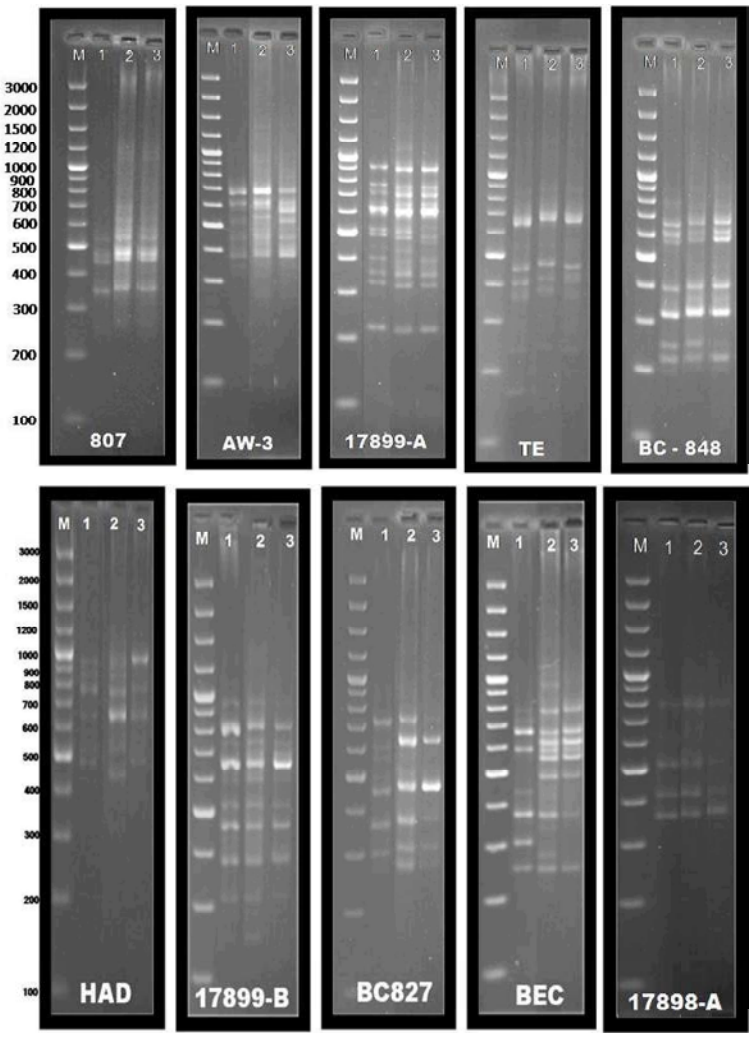

Fig. 5: ISSR profiles as detected by different ISSR primers for three Grape cultivars, Bez El-anza, Matrouh and Red Romy. M = 100 bp marker.

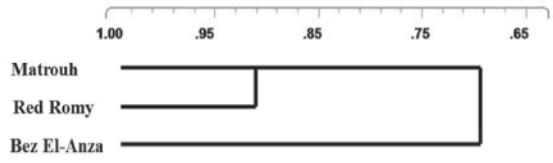

Fig. 6: Dendrogram showing the genetic relationships among the three grape cultivars (*Vitis vinifera* L.) based on the analysis of ISSR data.

Cluster Analysis as Revealed by ISSR: ISSR dendrogram obtained from UPGMA cluster analysis of genetic distances is presented in Fig.6. The genetic relationship linkage between the examined three varieties of *Vitis vinifera* L. is illustrated in dendrogram using the linkage between varieties. It is clear that the two varieties Red Romy and Matrouh are closer related than the Bez El-anza.

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