

## Morphological Characterization of Grapefruit and Pummelo Accessions Using the ISSR Molecular Markers

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AMONG the national objectives of the National Gene Bank and Genetic Resources (NGBGR) in Egypt are the collection, characterization, evaluation and conservation of agricultural genetic resources. On the other hand the main goal of Horticulture Research Institute is to enhance the productivity of horticultural crops in quantity and quality. The present study investigates the morphological characterization and estimates the genetic polymorphism and relationships among 9 citrus cultivars (4 grapefruit and 5 pummelo) accessions based on ISSR markers. Twenty- eight morphological characteristics were studied to describe the tree, leaf, inflorescence, fruits and seeds. The morphological characterization showed wide range of differences among grapefruit and pummelo accessions. Characterization of leaf lamina shape resulted in five accessions having ovate shape, three were Elliptic and one was orbicular. Fruit shape studies indicated that three accessions (Ruby red, Moneybi and Egyptian) were obloid, five (Marsh, White, Rabehe, Gizawe and Broad leaf) were spheroid and one (Duncan) was pyriform. Shape of fruit apex demonstrated that four accessions were truncate, four were depressed, and one was rounded. Differences in flavido (rind skin) colour among accessions revealed that one cultivar was pink – yellow, two were green-yellow, one was dark yellow and the rest of the accessions were yellow. Meanwhile, flesh colour was white in 12169, 12171 and 12173, light yellow in 12172, yellow in 12167 and 12168, pink in 12170, light red in 12166 and red in 12174 flesh.

Inter-simple sequence repeats (ISSR) markers were used to study the genetic diversity and phylogenetic relationships among four grapefruit (*Citrus paradise*), five pummelo (*Citrus maxima*) accessions. Thirteen ISSR primers produced the total number of amplified amplicons among tested primers ranging from 7 to 18 fragments. The highest number of fragments was 18 bands for (AG)<sub>8</sub> YC primer. While, (GT)<sub>8</sub> YC primer generated the lowest number of amplicons (7 bands). The average number of fragments/primer was (11.5) and the size of these fragment ranged from 98-1500 bps. The percentage of polymorphism revealed by the different primers ranged from 29 to 83 % with average of 54.9%.

**Keywords:** *Citrus maxima*, *Citrus paradise*, Germplasm characterization, ISSR markers.

Citrus production occupies an important share in the total area and fruit production in Egypt which grown on 541,723 feddans with the production of 4,098,590 tons in the year 2013 (Ministry of Agriculture, 2013). Besides, citrus is an extremely important crop world-wide. It is widely grown in most areas with suitable climates *i.e* tropical, subtropical and borderline subtropical/temperate (Kahn *et al.*, 2001). Egypt is one of the top 10 producers of orange in the world (FAO, 2012). The grapefruit (*C. paradisi Macf.*) was notified as a natural hybrid between pummelo (*Citrus maxima* (Burm.) Merr.) and sweet orange (*C. sinensis L. Osb*). It originates from Barbados in the Caribbean islands and was first named as *Citrus paradisi Macf.* by James Macfedyan in 1837 (Scora *et al.*, 1982 and Scora, 1988). Grapefruits are highly polyembryonic. Therefore, they are of a nuclear and mutation origin. Genetic variation among common grapefruit cultivars was reported to be very low due to their mutation origin (Fang *et al.*, 1997 and Corazza-Nunes *et al.*, 2002). The pummelo is native to tropical and subtropical regions in Asia and has been cultivated in China for over 2000 years (Corazza-Nunes *et al.*, 2002 and Yong *et al.*, 2006). Pummelo was reported as one of the three true citrus species by Barrett and Rhodes (1976) and most of the subsequent studies were in agreement with this statement (Federici *et al.*, 1998, Nicolosi *et al.*, 2000, Barkley *et al.*, 2006 and Uzun *et al.*, 2009). Pummelo (*C. maxima*, or formerly known as *C. grandis*) is a tropical fruit plant species originated from South East Asia, which in the western world is called as shaddock (Uzun and Yesiloglu, 2012).

Utilization of the morphological characteristics may be useful however regularly lacking in separation of nearly related cultivars. Then again, certain morphologically diverse variations may be phylogenetic nearly related. Likewise morphological characteristics are exceptionally affected by nature (Fang *et al.*, 1998). Accordingly, utilizing morphological attributes, it can be hard to recognize numerous Citrus cultivars (Fang *et al.*, 1997). Since morphological characters are just of restricted utilization and cytogenetically parameters are lengthy, exchange methodologies, including application of molecular markers, have now been progressively received to address the issues in Citrus scientific categorization (Kumar *et al.*, 2013). Contrasted with the morphological information, sub-atomic apparatuses give inexhaustible data are exceedingly proficient and are coldhearted to ecological components.

Molecular markers has provided an ideal means for identifying genotypes, estimation of relatedness between different accessions and following inheritance of economically important characters.

Maximum utilization of any germplasm for breeding can be achieved by understanding the level of genetic diversity it contains (Vinu *et al.*, 2013). Genetic diversity estimates are also important to understand its adaptive potential in different environments (Lowe *et al.*, 2004). Evaluation of genetic divergence and relatedness among breeding materials has significant implications for crop improvements. The knowledge on genetic diversity in grapefruit and pummelo accessions could help breeders and geneticists to

understand the structure of germplasm and to predict which combination would produce the best offspring and facilitate in widening up the genetic basis of breeding material for selection (Singh, 2005). The present study investigates the morphological characterization, the genetic polymorphism and the relationships among grapefruit and pummelo accessions based on ISSR markers.

### Materials and Methods

#### *Plant materials*

Plant materials used for this study were collected from Qalyubia governorate during 2012 and 2013 years. Four grapefruit and five pummelo accessions aged from 18-20 years old trees planted at 3×5 m in clay soil under flood irrigation and grown in Moshtohor Fac. of Agric. Res. Farm, Benha Univ., Egypt. (Table 1).

**TABLE 1. List of plant materials Citrus species and cultivars used in this study .**

Accession number	Accession name	Scientific name
12166	Ruby red	<i>Citrus paradisi</i> L.
12167	Duncan	<i>Citrus paradisi</i> L.
12168	Marsh	<i>Citrus paradisi</i> L.
12169	White	<i>Citrus paradisi</i> L.
12170	Moneybi	<i>Citrus grandis</i> ,Osbeck
12171	Rabehe	<i>Citrus grandis</i> ,Osbeck
12172	Gizawe	<i>Citrus grandis</i> ,Osbeck
12173	Egyptian	<i>Citrus grandis</i> ,Osbeck
12174	Broad leaf	<i>Citrus grandis</i> ,Osbeck

#### *Molecular characterization*

Young leaves samples of four grapefruit and five pummelo accessions (Table 1) were used for this study.

#### *DNA extraction and ISSR-PCR amplification conditions*

Total genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen<sup>®</sup> Germany) according to the manual procedures. A total of 13 primers (Table 2) were used to amplify DNA fragments. These primers were selected after screening 30 different primers. PCR reaction was performed in 25 µl reaction mix containing 1 X PCR buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM of each dNTPs, 1 µM oligonucleotide primer, 25 ng genomic DNA and 1 unit of Taq DNA polymerase (Promega<sup>®</sup>, USA). Amplification was performed in a 96-well BioRad<sup>®</sup> Thermal cycler (USA) under the following conditions: 3 min at 94°C for 1 cycle, followed by 1 min at 94°C, (1 min at annealing temperature), and 2 min at 72°C for 35 cycles, and 7 min at 72°C for a final extension stage.

#### *Data analysis*

The banding patterns generated by ISSR primers were analyzed and compared to determine the genetic relatedness among different Citrus genotypes. The amplified fragments were scored either as present (1) or absent (0). The genetic

similarity and similarity matrix among genotypes were estimated according to Dice coefficient (Sneath and Sokal, 1973). Dendrograms showing the genetic relationships were constructed using the Un-weighted Pair Group Method with Arithmetic Averages (UPGMA) by Phoretix 1D software (TotalLab, UK).

**TABLE 2. List of primer names, sequences and annealing temperatures used in this study.**

Primer Name	Sequence	Annealing Temp. °C
<b>5' Anchored repeats</b>		
<b>P<sub>16</sub></b>	<b>ACG(GT)<sub>7</sub></b>	<b>50.0</b>
<b>3' Anchored repeats</b>		
H <sub>12</sub>	(GA) <sub>8</sub> YT	41.0
H <sub>13</sub>	(GA) <sub>8</sub> YC	42.5
H <sub>14</sub>	(GA) <sub>8</sub> YG	44.0
H <sub>15</sub>	AG) <sub>8</sub> YT	52.0
H <sub>16</sub>	(AG) <sub>8</sub> YC	56.5
H <sub>17</sub>	(AG) <sub>8</sub> YG	59.5
H <sub>21</sub>	(GT) <sub>8</sub> YC	60.5
H <sub>29</sub>	(GACA) <sub>4</sub> AT	41.5
P <sub>2</sub>	(CA) <sub>6</sub> GG	48.0
P <sub>3</sub>	(CA) <sub>6</sub> AC	42.5
P <sub>4</sub>	(GTG) <sub>3</sub> GC	52.5

R=purine, Y=pyrimidine (C or T), B=non-A, D=non-C, H=non-G, V=non-T

#### *Morphological characterization*

The morphological characteristics used to characterize and discriminate the 9 grapefruit and pummelo cultivars were based on those previously prescribed for Citrus by the International plant Genetic Resources Institute (IPGRI, 1999) taking into consideration all the precautions reported. In this respect, 17 quantitative and 13 qualitative morphological characteristics were selected for the present investigation. The study was performed using three trees for each cultivar, each tree was considered a replicate (Table 3). The present's traits were used for morphological characterization. Thirty mature and fully developed leaves per tree (mature leaves from one year old branches) were collected and characterized for leaf lamina length and width, ratio of leaf lamina length/width (leaf lamina shape), and petiole wings shape.

Data were recorded for flower pedicel length, number of petals per flower, petal length and width. All observations on the fruit and its related parts were made at the optimum maturity stage according to IPGRI, 1999. Fruit characteristics were observed on 10 typical fruits per each tree of the three replication trees. Data were documented for fruit weight, diameter, length and

shape. Records also included shape of fruit base and fruit apex, fruit rind skin colour, texture of skin surface and fruit rind thickness. The study comprised also number of segments per fruit, flesh colour, fruit axis and juice content in endocarp. Fully developed seeds were extracted from 10 fully ripened fruits taken from each tree of the three replications. In this respect, average number of seeds per fruit, seed shape, seed surface, seed length, seed width and seed weight were assessed.

**TABLE 3. Code of morphological traits used in citrus accessions characterization.**

Code	Characters	Character states	
1. Qualitative Traits			
L01	Leaf lamina shape	(1)Ovate,(2)Elliptic,(3)Orbicular	
L02	Petiole wings shape	(1)Obdeltate,(2)Absent,(3)Obcordate	
Fr03	Fruit shape	(1)Oboid,(2)Pyriiform,(3)Spheroid	
Fr04	Fruit skin colour	(1)Pink-yellow,(2)Green-yellow,(3)Yellow,(4)dark yellow	
Fr05	Fruit skin texture	(1)Rough,(2)Smooth,(3)Pitted	
Fr06	Fruit flesh colour	(1)light red,(2)White,(3)Pink,(4)Yellow	
Fr07	Fruit axis	(1)Solid,(2)Semi-hollow,(3)Hollow	
Fr08	Fruit shape of base	(1)Concave,(2)Necked,(3)Convex,(4)Truncate	
Fr09	Fruit shape of apex	(1)Truncate,(2)depressed,(3)Rounded	
Fr010	Number of segment/fruit	(1)[10-14],[2)[15-18]	
S011	Number of seed/fruit	(1)[5-9],[2)>50,(3)[20-50],[4)[10-19],[5)[1-4]	
2. Quantitative Traits			
L01	Leaf lamina length	Fr011	Fruit rind thickness
L02	Leaf lamina width	Fr012	Juice content/fruit
L03	Leaf ratio(L/W)	S013	Seed Shape
FL04	Flower pedicel length	S014	Seed surface
FL05	Number of petals/flower	S015	Seed length
FL06	Petal length	S016	Seed width
FL07	Petal width	S017	Seed weight
Fr08	Fruit weight		
Fr09	Fruit diameter		
Fr010	Fruit length		

#### Statistical analysis

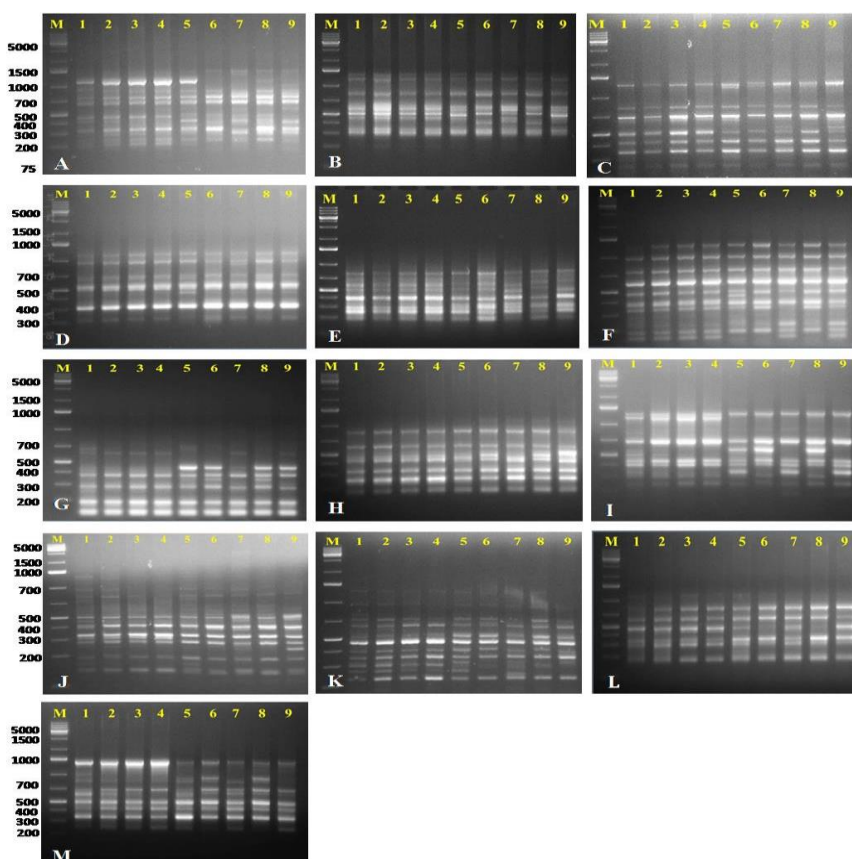
The data of leaf, flower, fruit and seed characteristics were presented as mean (n=30), and the means were compared using a one-way analysis of variance followed by Duncan's test at P 0.05 was considered statistically significant.

## Results and Discussion

### *Molecular characterization*

#### *Polymorphism detected by ISSR primers*

ISSR amplification from all DNA samples of 4 grapefruit and 5 pummelo accessions (collected from Moshtohor region) produced prolific banding profiles for all 13 primers (Fig. 1). The total number of amplified amplicons among tested primers ranged from 7 to 18 fragments. 3'anchored H<sub>16</sub> primer amplified the highest number of fragments (18 bands). While, H<sub>21</sub> primer generated the lowest number of amplicons (7 bands). The average number of fragments/ primer was (11.5), and the size of these ranged from 98-1500 bps.



**Fig. 1.** Electrophoresis separation patterns of ISSR-PCR products (as revealed on 1.8% agarose gel) using primers P2 (A), P3 (B), P4 (C), P11 (D), P16 (E), H12 (F), H13 (G), H14 (H), H15 (I), H16 (J), H17 (K), H21 (L) and H29 (M). Lane M: 1Kb plus DNA ladder marker. Lanes 1 to 9 represented Moshtohor grapefruit and pummelo genotypes: Ruby red, White, Duncan, Marsh, Broad leaf, Egyptian, Rabehe, Gizawy and Moneybi, respectively.

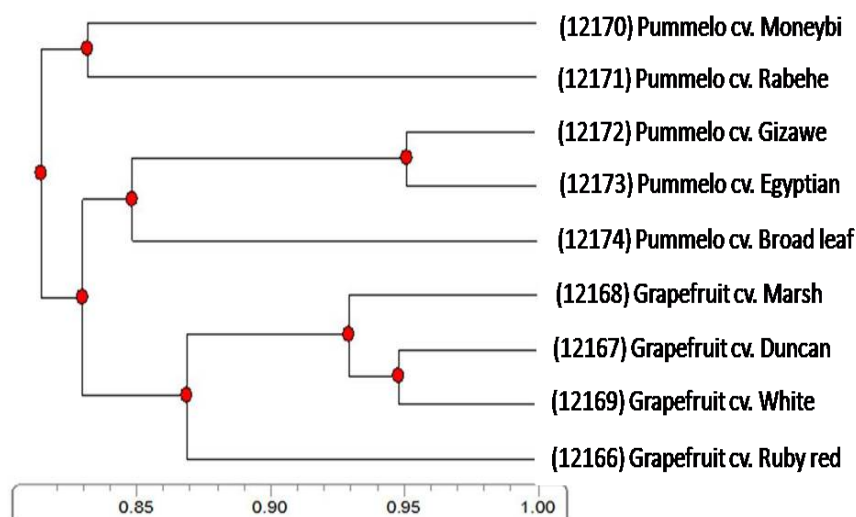
All the tested primers produced polymorphic bands (Table 4) of the total 150 scorable fragments, 86 were polymorphic among the accessions (Fig. 1). The number of polymorphic bands ranged from 2 to 13 fragments resulting in an average of polymorphism/ primer (6.6). Primer H<sub>16</sub> revealed the highest number of polymorphic bands (13). However, the lowest number of polymorphic amplicons (2) was generated by the H<sub>21</sub> primer. The percentage of polymorphism revealed by different primers ranged from 29 to 83% with an average of 54.9%.

**TABLE 4. Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism as revealed by ISSR markers among the 9 grape fruit and pummelo accessions collected from Moshtohor region.**

Primer	Total amplicons	Monomorphic amplicons	Polymorphic amplicons	Percentages of polymorphism
P <sub>2</sub>	12	2	10	83
P <sub>3</sub>	11	4	7	64
P <sub>4</sub>	12	6	6	50
P <sub>11</sub>	9	4	5	56
P <sub>16</sub>	9	6	3	33
H <sub>12</sub>	11	7	4	36
H <sub>13</sub>	10	5	5	50
H <sub>14</sub>	14	5	9	64
H <sub>15</sub>	13	5	8	62
H <sub>16</sub>	18	5	13	72
H <sub>17</sub>	14	5	9	64
H <sub>21</sub>	7	5	2	29
H <sub>29</sub>	10	5	5	50
Total	150	64	86	
Average	11.5	4.9	6.6	54.9

#### Clustering analysis

The UPGMA cluster analysis declared the degree of phylogenies among the different local pummelo accessions in comparison with exotic grapefruit genotypes (Fig. 2). Phylogenetic analysis showed a high degree of genetic overlapping among the tested accessions, which reflected the intensive genetic relationships between different grapefruit and pummelo germplasm. The dendrogram analysis ranked Egyptian local pummelo accessions into two separated clusters at 81% level of similarity, the first included Moneybi and Rabehe genotypes however, other pummelo accessions were grouped with introduced grapefruit genotypes in the second cluster. Gizawe, Egyptian and Broad leaf pummelo accessions were grouped in one lineage at 85 % level of similarity. Conversely, different genotypes of grapefruit were gathered together in one cluster at 87% level of similarity. The highest genetic similarity was detected between Gizawe and Egyptian genotypes of pummelo germplasm as well as Duncan and White genotypes of grapefruit germplasm with 95% level of similarity. On the other hand, the highest genetic difference was identified between the accession grapefruit Ruby red and Moneybi pummelo genotype with a 78% level of similarity.



**Fig. 2.** Dendrogram for the 9 Grape fruit and Pummelo genotype accessions (collected from Moshtohor region) constructed from the ISSR generated data using the UPGMA method and similarity matrices computed according to the Dice's similarity coefficient.

#### *Genotype identification by unique ISSR markers*

Despite the high genetic similarities among pummelo and grapefruit genotypes, genotype-specific ISSR unique markers were able to differentiate among 3 pummelo accessions (Broad leaf, Rabehe and Moneybi) as well as 3 grapefruit accessions (Ruby red, White and Marsh) out of the 9 tested accessions. The ISSR markers generated by primers and the positive and/or negative markers approximate size are shown in Table 5. Out of all tested ISSR primers, 11 primers were able to generate unique markers (positive and/or negative) that could differentiate pummelo and grapefruit accessions. However, 2 primers ( $H_{14}$  and  $H_{21}$ ) were failed to produce any unique markers. The number of generated unique markers in Broad leaf, Rabehe and Moneybi genotype's of pummelo were 4, 6 and 7, respectively. However, the number of generated unique markers in Ruby red, White and Marsh grapefruit were 9, 1 and 2, respectively.

#### *Intra-variation within each citrus group*

Grapefruit is the fourth economically most important Citrus fruit in the world (Uzun *et al.*, 2010). A high level of genetic similarity was detected among grapefruit accessions ranging from 0.85 to 0.95, this narrow genetic base among the grapefruit cultivars has been reported in previous publications using different molecular markers (Fang *et al.*, 1997, Corazza-Nunes *et al.*, 2002). The high level of similarity within the grapefruit group supported the hypothesis that the majority



of grapefruit cultivars were derived from the same ancestral tree by mutations (Gmitter, 1995). Pummelo has played an important role as a parent of many Citrus fruits, such as lemons, oranges and grapefruits. Among the five pummelo accessions, the genetic similarity ranged from a 0.85 to 0.95 this is in line with other published reports (Corazza-Nunes *et al.*, 2002 and Uzun *et al.*, 2010).

**TABLE 5. Grapefruit and pummelo genotypes (collected from Moshtohor region) characterized by unique positive and/or negative ISSR markers, marker size and total number of ISSR markers identifying each genotype.**

Accessions	Primer	Unique positive		Unique negative		Total
		Size in bp	Total	Size in bp	Total	
12166 (Ruby red)	H13	254	1	-	-	9
	H16	164,1330	2	419	1	
	H17	793	1	-	-	
	H29	1000	1	-	-	
	P2	-	-	257	1	
	P4	-	-	715	1	
	P11	706	1	-	-	
12169 (White)	P3	195	1	-	-	1
12168 (Marsh)	H16	-	-	852	1	2
	P3	-	-	616	1	
12174 (Broad leaf)	H12	448	1	-	-	4
	H16	781	1	-	-	
	P4	1266	1	-	-	
	P11	239	1	-	-	
12171 (Rabehe)	H12	-	-	333	1	6
	H15	177	1	-	-	
	H17	-	-	742	1	
	P3	1361	1	-	-	
	P11	886	1	-	-	
	P16	326	1	-	-	
12170 (Moneybi)	H15	-	-	294	1	7
	H17	-	-	973,1328	2	
	P2	588	1	482	1	
	P4	463	1	-	-	
	P16	-	-	586	1	

#### *Morphological characterization*

##### *Quantitative characteristics*

Table 6 presents leaves and flowers quantitative traits of the grapefruits and pummelo germplasm under the present investigation. Results showed that [12166, 12168 and 12170] accessions exhibited the highest leaf lamina length (cm) of 12.80, 12.70 and 13.00 cm, respectively. The lowest leaf length was presented by grapefruit accessions number 12169 (10.28 cm). The rest of the germplasm showed intermediate values of leaf length. Accession number 12166 showed the greatest leaf lamina width (6.43 cm). However, there was no significant difference among the accessions number [12167, 12168, 12170, 12171, 1272, 12173 and 12174], respectively. The lowest leaf width value obtained by accession number 12169 was (4.28). The uppermost ratio of

leaf lamina shape (length/ width ratio) was 2.52, 2.50 and 2.40 cm for the accession numbers [12167, 12168 and 12169], respectively. The pummelo accession number 12171 was [2.18], accession number 12170 gave (2.16) and accession number 12174 gave (2.21). The lowest ratio was characterized by grapefruit accession number 12166 was 1.99, Pummelo accessions number 12172 and 12173 (Table 6). The accession numbers 12166, 12167, 12172, 12173 and 12174 showed the highest value of flower pedicel length i.e. 1.81, 2.10, 2.05, 2.18 and 2.00 cm, respectively. The lowest ones (0.90 and 1.00 cm) were exhibited by the accession numbers 12168 and 12171, respectively. Whereas the rest of the germplasm illustrated intermediate values. Accessions number 12166 grapefruit, 12170 and 12171 pummelo germplasm showed five petals per flower whereas, the rest of the germplasm showed four petals per flower (Table 6). The petal length was different among the cultivars, where accessions number (12174) Pummelo gave (2.64 cm) presented the highest measurements followed by grapefruit accession number 12166 as it was (2.45 cm). Concerning Pummelo accession numbers [12170, 12171 and 12173] there were no significant difference among them. The lowest petal length was presented by grapefruits accessions number [12168 and 12169] as they gave (1.75 cm and 1.69 cm), respectively. On the other hand, there was no significant difference between grapefruit and pummelo germplasm of petal width (Table 6).

**TABLE 6. Morphology quantitative characteristics of leaves and flowers of nine grapefruits and pummelo germplasm grown in Egypt.**

Accessions	Leaf length (cm)		Leaf width (cm)		Leaf ratio L/w		Flower pedicel length (cm)		Number of petals		Petal length (cm)		Petal width (cm)	
<b>Grapefruit</b>														
12166	12.80	a	6.43	a	1.99	b	1.81	a	5	A	2.45	ab	1.10	a
12167	12.00	ab	4.75	ab	2.52	a	2.10	a	4	B	2.22	b	0.84	a
12168	12.70	a	5.08	ab	2.50	a	0.90	c	4	B	1.75	c	0.75	a
12169	10.28	b	4.28	b	2.40	a	1.40	b	4	B	1.69	c	0.77	a
<b>Pummelo</b>														
12170	13.00	a	6.00	ab	2.16	ab	1.20	bc	5	A	2.40	ab	0.74	a
12171	12.14	ab	5.57	ab	2.18	ab	1.00	c	5	A	2.60	ab	0.74	a
12172	11.70	ab	6.10	ab	1.92	b	2.05	a	4	B	2.24	b	0.97	a
12173	11.75	ab	5.88	ab	1.99	b	2.18	a	4	B	2.33	ab	0.92	a
12174	11.13	ab	5.03	ab	2.21	ab	2.00	a	4	B	2.64	a	0.97	a
C.V %	8.37		18.32		9.06		12.30		10.00		8.86		12.08	

Values have the same letter(s) in the same column are not significantly different at  $LSD \leq 0.05$  level

The quantitative characteristics of fruits and seeds are demonstrated in (Table 7). Pummelo accession number 12170 showed the highest significant fruit weight (1140.09 g) followed by pummelo accession number 12171 (996.1 g).

On the other hand, grapefruit accession number 12168 had the lowest fruit weight (351.5 g). The rest of the germplasm gave intermediate fruit weights (Table 7). Although pummelo accession number 12170 exhibited the greatest fruit diameter (14.25 cm), followed by pummelo accession number 12173 which was (13.20 cm). The grapefruit accessions number (12166, 12168 and 12169) displayed the lowest fruit diameter (9.00, 9.00 and 9.33 cm), respectively. The remaining germplasm had intermediate values ranged from (10.40 – 12.7 cm). The last fruit length was demonstrated by the grapefruit accession number 12167 (13.20 cm). However, no significant differences were obtained as compared with the pummelo accessions number 12170 (11.50 cm), accession number 12171 (11.25 cm) and accession number 12174 (11.21 cm). Meanwhile, grapefruit accession number 12166 showed the lowest value of fruit length (7.20 cm). The rest of the germplasm gave an intermediate fruit length (Table 7).

The fruit thickness indicates that the grapefruits accessions number 12167 and pummelo accessions number 12170 had the greatest fruit rind thickness (1.85 and 2.00 cm). Conversely, the grapefruit accession number 12166 and 12168 had intermediate fruit rind thickness. However, no significant differences obtained as compared with the germplasm grapefruit accession number 12169 (0.50 cm), pummelo accession number (12171, 12172, 12173 and 12174) (Table 7). The amount of juice content (ml/fruit) was different among germplasm. The greatest was 415.0 ml in the pummelo accession number 12171 followed by the accession number 12172 (383.0 ml). The least juice content was obtained from the grapefruit 12166 (135.0 ml) whereas, the rest of the germplasm showed intermediate juice amount (Table 7).

The pummelo accession number 12170 proved the highest significant seed length value ranging (2.25 cm), whereas the pummelo accession number 12173 was the least seed length (1.08 cm). Although the accessions number 12171, 12172 and 12174 showed intermediate seed length values as it was no significant differences compared with the pummelo accession number 12170. Similarly, grapefruit accessions number 12166, 12167, 12168 and 12169 showed no significant differences in comparison with the pummelo accession number 12170 (Table 7). Seed width from all accessions had no significant differences between grapefruits and pummelo germplasm.

Table 7 indicates that the pummelo accession number 12170 had the greatest average seed weight (0.65 g) followed by the grapefruit accession number 12169 (0.31 g). The rest of the germplasm exhibited intermediate average seed weight values. It had no significant differences in comparison with pummelo accession number 12170.

It is concluded that pummelo accession number 12170 has the best quantitative traits as proved by the highest fruit weight, diameter, length, rind thickness, and juice content, as well as the high seed length, width and weight.

**TABLE 7. Morphology quantitative characteristics of fruit and seed of nine grapefruits and pummelo germplasm grown in Egypt.**

Accession	Fruit weight (g)	Fruit diameter (cm)	Fruit length (cm)	Fruit rind thickness (cm)	Juice content ml/fruit	Seed length (cm)	Seed Width (cm)	Seed Weight (g)
<b>Grapefruit</b>								
12166	401.22 f	9.00 f	8.20 d	0.90 b	135.0 h	1.30 bc	1.00 a	0.22 b
12167	380.6 g	8.80 g	8.50 d	1.85 a	145.0 g	1.48 b	0.74 a	0.28 b
12168	351.5 h	9.00 f	8.33 d	0.60 bc	165.0 f	1.33 bc	0.87 a	0.21 b
12169	414.0 f	9.33 f	8.06 d	0.50 c	152.0 fg	1.36 bc	0.90 a	0.31 ab
<b>Pummelo</b>								
12170	1140.0 a	14.25 a	13.20 a	2.00 a	415.0 a	2.25 a	1.02 a	0.65 a
12171	996.1 b	12.25 c	11.25 b	0.50 c	264.0 d	1.40 bc	0.75 a	0.22 b
12172	862.5 e	11.40 d	10.60 c	0.50 c	383.0 b	1.30 bc	0.80 a	0.25 b
12173	977.5 c	13.20 b	10.80 c	0.50 c	313.0 c	1.08 c	0.80 a	0.24 b
12174	904.0 d	10.40 e	11.21 b	0.40 c	227.0 e	1.52 b	0.77 a	0.22 B
C.V %	1.40	1.77	1.95	13.23	4.29	13.82	13.53	12.23

Values have the same letter(s) in the same column are not significantly different at  $LSD \leq 0.05$  level .

#### *Qualitative characteristics*

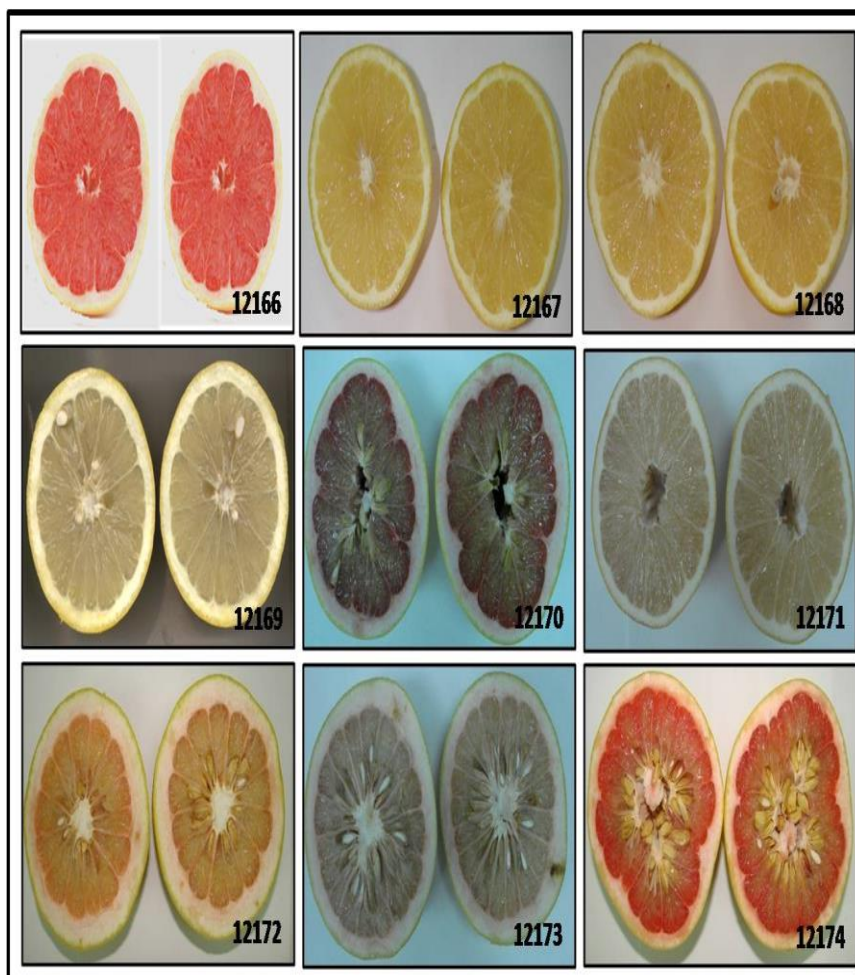
Table 8 presents the qualitative traits of fruit, seed and leaf of the nine grapefruits and pummelo germplasm under the present study. In this regard, only the grapefruit accession number 12167 showed pyriform fruit shape. While, the accessions number 12166, 12170, and 12173 showed obloid fruit shape. The rest of the germplasm showed spheroid fruit shape.

The fruit skin colour included 12 colour ranged from green to red-orange. The grapefruit accession number 12166 had pink-yellow fruit skin colour. While accessions number 12167 and 12170 were green-yellow. Only the pummelo accession number 12172 had dark-yellow. The rest of the germplasm showed yellow fruit skin colour. The pummelo accession no. 12173 was the only one showed pitted texture of skin surface. The cultivars Ruby red, White, Moneybi, Gizawe and Broad leaf proved rough skin surface texture, whereas grapefruits Duncan cv., Marsh and pummelo Rabehe CV. Showed smooth surface skin texture (Table 8).

The flesh colour presented in (Fig. 3 and Table 8) red colour of fruit flesh was clear in the grapefruit Ruby red cultivar and pummelo broad leaf cultivar only, where as it was yellow in grapefruit Duncan, Marsh and pummelo Gizawe cultivars. Meanwhile, pummelo Moneybi showed pink fruit flesh while the rest of the germplasm showed white colour of fruit flesh. The classifications, under which products of the fruit hub of the diverse cultivars were assessed were solid, semi-hollow and hollow fruit axis. The cultivars grapefruit Ruby red and pummelo Broad leaf demonstrated semi-hollow while, pummelo Moneybi and Rabehe cultivars indicated hollow fruit axis. Whatever is left of the germplasm indicated solid fruit axis (Table 8). Only the Ruby red cultivar showed concave shape of fruit base. The germplasm Duncan and Gizawe revealed necked fruit base shape. On the other hand, pummelo Moneybi and Rabehe cultivars showed hollow fruit axis. The rest of the germplasm showed solid fruit axis (Table 8). The Ruby red cultivar fruit base was concave in shape, but Duncan and Gizawe germplasm were necked fruit base shape. On the other hand, pummelo Moneybi and Rabehe accessions showed convex fruit shape of base. The accessions exhibited truncate fruit base shape (Table 8). Regarding fruit apex shape, 12168 CV. showed rounded fruit apex shape whereas, Ruby red, Duncan and pummelo broad leaf accessions demonstrated truncate. The remaining of the accessions displayed depressed shape of fruit apex.

**TABLE 8. Performance of different grapefruit and pummelo germplasm regarding fruit shape, fruit skin colour, fruit axis, fruit shape of base and fruit shape of apex.**

Accession	Fruit shape	Fruit skin colour	Skin texture	Flesh colour	Fruit axis	Fruit shape of base	Fruit shape of apex
<b>Grapefruit</b>							
12166	Obloid	Pink-yellow	Rough	Light red	Semi-hollow	Concave	Truncate
12167	Pyriiform	Green-yellow	Smooth	Yellow	Solid	Necked	Truncate
12168	spheroid	Yellow	Smooth	Yellow	Solid	Truncate	Rounded
12169	spheroid	yellow	Rough	White	Solid	Truncate	Truncate
<b>Pummelo</b>							
12170	Obloid	Green-yellow	Rough	Pink	Hollow	Convex	Depressed
12171	spheroid	Yellow	Smooth	White	Hollow	Convex	Depressed
12172	spheroid	Dark-yellow	Rough	Light Yellow	Solid	Necked	Depressed
12173	obloid	Yellow	Pitted	White	Solid	Truncate	Depressed
12174	spheroid	Yellow	Rough	Red	Semi-hollow	Truncate	Truncate



**Fig. 3.** Fruits cross section of four grapefruit accessions number from (12166) to (12169) and five pummelo accessions number from (12170) to (12174) collected from Egypt.

Table 9 illustrates the no. of segment per fruit (10-14) for accessions no. (12166, 12167, 12168, 12169 and 12171). While, the rest of the accessions had 15-18 segment/fruit. The qualitative characteristics of no. of seed/fruit, seed shape, seed surface, leaf lamina shape and petiole wings shape are presented in Table 9. The average no. of seed/fruit was generally high in most of the germplasm tested. The highest counted number of seeds/fruit (>50) was evident in the accessions no. 12170 and 12171. The accessions no. 12172 and 12173 showed (20-50) seeds/fruit. While, pummelo accession no. 12174 had (10-19) seeds/fruit. Only grapefruit accession no. 12169 had (1-4) seeds/fruit. The rest of the accessions had (5-9) seeds/fruit.

TABLE 9. Quantitative characteristics of grapefruit and pummelo germplasm.

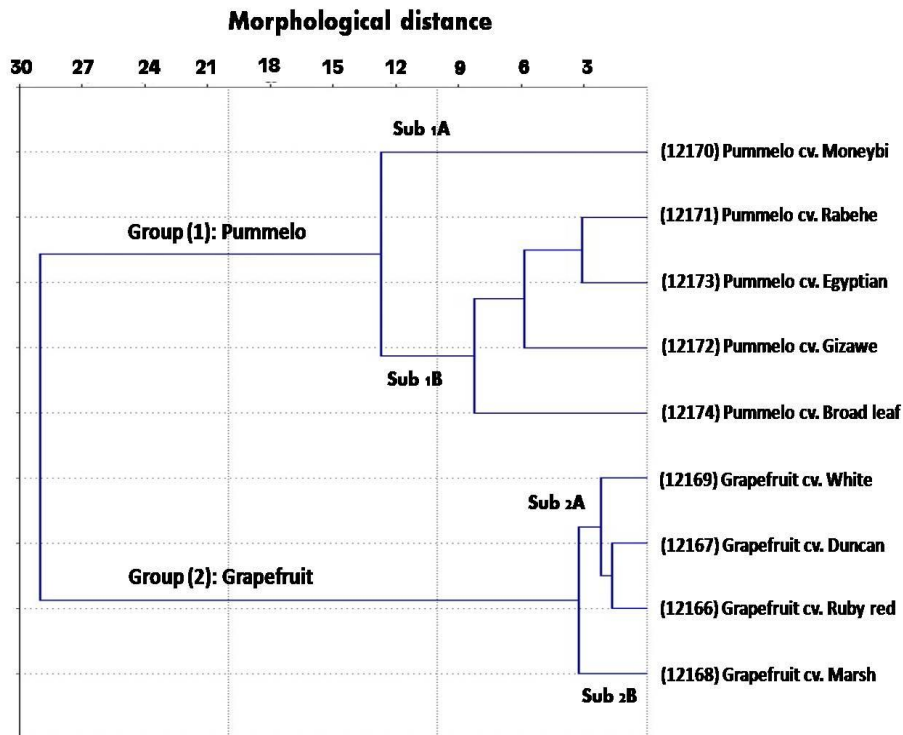
Accession	No. of segment	No. of seed/fruit	Seed shape	Seed surface	Leaf lamina shape	Petiole wings shape
<b>Grapefruit</b>						
12166	10-14	5-9	ovoid	Smooth	Ovate	Obdeltate
12167	10-14	5-9	Semi-deltoid	wrinkled	Elliptic	Absent
12168	10-14	5-9	Semi-deltoid	wrinkled	Ovate	obcordate
12169	10-14	1-4	Semi-deltoid	wrinkled	Elliptic	Obdeltate
<b>Pummelo</b>						
12170	15-18	>50	Semi-spheroid	wrinkled	Elliptic	obcordate
12171	10-14	>50	ovoid	Smooth	Orbicular	obcordate
12172	15-18	20-50	Semi-spheroid	wrinkled	Ovate	obcordate
12173	15-18	20-50	Semi-deltoid	Smooth	Ovate	obcordate
12174	15-18	10-19	Semi-deltoid	Smooth	Ovate	obovate

The grapefruit accession no. 12166 and pummelo accession no. 12171 were presented ovoid seed shapes. Just two germplasm specifically Moneybi and Gizawe showed semi-spheroid seed shape (Table 9). Seed shape of the rest of the cultivars (56 %) was semi-deltoid. Among the studied cultivars, the Ruby red, Rabehe, Egyptian and Broad leaf demonstrated smooth seed surface the residues of the germplasm had wrinkled seed shape. The investigation of leaf lamina shape included elliptic, ovate, obovate, lanceolate, orbicular and obcordate. None of the investigated cultivars under the present study indicated obovate, lanceolate or obcordate leaf lamina shape. Three only accessions number (12167, 12169 and 12170) exhibited elliptic leaf shape. However, pummelo accession no. 12171 showed orbicular leaf shape. The rest of the germplasm displayed ovate leaf lamina shape (Table 9). The absence or presence of petiole wings were evaluated for the different cultivars (Table 9). The grapefruit accession no. 12167 demonstrated absent petiole wings whereas those of the rest of the germplasm were present. One only accession had obovate petiole wings shape while, the grapefruit accession no. 12166 and 12169 exhibited obdeltate petiole wings shape. On the other hand, the rest of the germplasm showed obcordate.

Morphological characterization of grapefruit and pummelo was studied by several researchers (Uzun *et al.*, 2010) they found that the variation of fruit weight was more or less similar to those studied by (Ara *et al.*, 2008 and Samarasinghe, 2005). In contrast (Mitra *et al.*, 2011) obtained more wide range of fruit weight (570-2010 g). Although number of seeds per fruit is almost similar to that of present findings. Present finding showed significant variation of number of seeds per fruit (20-50) to (> 50). With less number of seeds was obtained in Type-9 and Type-12 but Hazarika (2013) obtained less number of seeds (12.67-37.50) among all the twelve collections of pummelo at Mizoram.

*Dendrogram of pomological relationship (cluster analysis).*

The UPGMA Dendrogram obtained with 28 qualitative and quantitative measurements for the 9 grapefruit and pummelo accessions are shown in Fig 4. The accessions were clustered into two main groups. Group 1 included five accessions that could be divided into two subgroups (1A and 1B). Generally, the accessions in this group had large fruit size. Only pummelo cultivar "Moneybi" with pink flesh colour and higher fruit weight (1140 g) was in subgroup 1B. The subgroup 1A consisted of four accessions of which "Rabehe" and "Egyptian" had similar fruit size and flesh colour and the genetic distance was (5-8). On the other hand, Group 2 consisted of 4 accessions and separated into two subgroups (2A and 2B) based on different morphological characteristics such as fruit weight, diameter, length, rind thickness, juice content, seed length, seed width and seed weight. Subgroup 2B which included one cultivar namely "Marsh" had rounded fruit shape of apex while, subgroup 2A consisted 3 accessions of which "Ruby red", "White" and "Duncan". But, "Duncan" and "White" cultivars showed very similar fruit characters the genetic distance was (3-6). The accessions in this group had small fruit weigh.



**Fig. 4.** UPGMA Dendrogram using the Manhattan distances based on morpho-pomological distances of grapefruits and pummelo accessions sampled from qalyubia governorate, Egypt.



In conclusion, in order to preserve the property rights of the genetic diversity of local citrus varieties in Egypt, there is an urgent need to collect additional samples of all local citrus varieties. In our study grapefruit and pummel varieties from other governorates and locations are to be targeted by conducting collection missions. In this connection, Citrus germplasm in all of Egypt regions are to be focused on to ensure that most of the genetic diversity has been captured and conserved *ex situ* and should go towards local varieties registration. Morphological and molecular characterization will facilitate the identification of duplicate germplasm and in selecting the core collection for long term conservation.

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## التوصيف المورفولوجي و الجزيئي باستخدام تقنية ISSR في الجريب فروت والشادوك

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تتضمن المهام القومية للبنك القومي للجينات والموارد الوراثية بجمهورية مصر العربية عمليات جمع وحفظ وتوصيف وتقييم الموارد الوراثية الزراعية، على الجانب الآخر فإن معهد بحوث البساتين هدفه الرئيسي هو زيادة إنتاجية الحاصلات البستانيه من حيث الكم و الجوده العاليه . لذلك تهدف هذه الدراسه إلى التوصيف الظاهري وتقدير التباين الوراثي والعلاقة بين ٩ أصناف من الموالح (٤ أصناف من الجريب فروت و ٥ أصناف من الشادوك) باستخدام تقنية التفاعل المتسلسل البلمرة (ISSR) حيث تم دراسة ٢٨ صفة مورفولوجية لتوصيف الشجرة منها الأوراق - الأزهار - الثمار - البذور ، حيث أظهرت الخصائص المورفولوجية مجموعه كبيره من الإختلافات بين الجريب فروت و الشادوك . و أدت نتائج توصيف شكل الورقه إلى وجود ٥ أصناف ذات أوراق بيضاويه الشكل ، و ثلاثة أصناف أوراقها قلبية الشكل ، و صنف واحد أوراقه مستديرة الشكل . أما عن شكل الثمار فأوضحت النتائج أن ثلاثة أصناف (روبي ردي- منيبي - مصري) كانت ثمارها مستطيله (بيضاويه ) الشكل و خمسة أصناف ( مارش - ابيض - جيزاوى - ربيعي - شادوك عريض) كانت ذات ثمار كروية الشكل بينما الصنف دنكان ذو ثمار كثرية الشكل. و من حيث شكل قمة الثمرة فكان هناك أربعة أصناف قمة الثمره فيها منبعجه و أربعة أصناف ذات قمة ثمره مبططه بينما الصنف الاخير تميز بقمة ثمره منخفضه . ومن حيث لون القشرة الخارجيه للثمره كانت طبقة الفلافيدو ذات لون أصفر - وردي في صنف ١٢١٦٦ وأخضر مصفر في الصنفين ١٢١٧٠ و ١٢١٦٧ و أصفر داكن في صنف ١٢١٧٢ بينما باقي الاصناف كان لونها أصفر. و كان لون اللحم (لب الثمرة) أبيض في الاصناف ١٢١٧١ و ١٢١٧٣ و ١٢١٦٩ و كان اصفر فاتح في الصنف ١٢١٧٢ و أصفر في ١٢١٦٧ و ١٢١٦٨ بينما كان لون اللب وردي في الصنف ١٢١٧٠ و أحمر في ١٢١٧٤.

تم استخدام التقنية الجزيئية (ISSR) لدراسة التباين الوراثي ودرجة القرابة الوراثية بين أربعة موارد وراثية من الجريب فروت وخمسة موارد وراثية من الشادوك حيث تم اختبار عدد ثلاثة عشر بادئ ISSR وأعطت أفضل نتائج المتحصل عليها من البادئات المختبرة تفريد (١٣) بادئ حيث تراوح ما بين ٧-١٨ حزمة ، وقد أعطي البادئ  $(AG)_8YC$  أعلى حزم وراثية بلغت ١٨ حزمة/بادئ بينما أظهر البادئ  $(GT)_8YC$  أقل عدد من الحزم الوراثية بلغت ٧ حزمة/بادئ بمتوسط ١١,٥ حزمة لكل بادئ وقد تراوحت أوزانها الجزيئية ما بين ٩٨-١٥٠٠٠ قاعدة وقد أعطت البادئات المختلفة نسبة تباين تراوحت ما بين ٢٩ إلى ٨٣ % كما كان متوسط عدد الحزم المتباينة ٥٤,٩ % لكل بادئ.