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Morphological and Physiochemical Characterization of Ten Lime and Lemon Accessions and the Assessment of their Genetic Diversity Maintained at ISSR Marker

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Abstract: Among the national objectives of the citriculture scientists in Egypt are the collection, characterization, evaluation and conservation of the Citurs genetic resources along with enhancing the productivity characteristics in both quantity and quality attributes. The present study aims to investigate the morphological and physiochemical characterization as well as estimating the genetic polymorphism and relationships among 10 Citrus cultivars (4 Lime and 6 Lemon) accessions based on ISSR markers. 31 morphological characteristics were studied to describe leaves, inflorescences, fruits and seeds for each of the 10 cultivars. The morphological characterization (mainly leaf lamina and fruit shape as well as flavedo color) showed a wide range of differences among Lime and Lemon accessions. Similarly; the Chemical analysis of lime and lemon fruit juice exerted significant differences between Lime and Lemon accessions. Data revealed that pH range from 6.20 in Succari lime to 2.12 in ponderosa cultivar. However, titratable acidity ranged from 6.45 % in Eurek-1 lemon to 0.40 % in Helou lime. The highest total soluble solids (TSS) content (10.50) °Brix was determined in Sweet lime, Sweet lemon and ponderosa, while the lowest TSS content (5.80) °Brix was detected in Succari lime. The ascorbic acid (Vitamin C) content ranged from 48.39 mg/100 ml in Eurek-1 lemon to 15.33 mg/100 ml in Sweet lemon. Inter-simple sequence repeats (ISSR) marker was used to study the genetic diversity and phylogenetic relationships among four lime included one (*Citrus aurantifolia*), three (*Citrus limetta*) and six lemon included one (*Citrus medica*), one (*Citrus jambhiri*) and four (*Citrus limon*) accessions. Thirteen ISSR primers produced the total number of amplified amplicons among tested primers ranged from 9 to 19 fragments. P_2 primer amplified the highest number of fragments 19 bands while; H_{14} and P_{11} primers generated the lowest number of amplicons (9 bands). The average number of fragments/primer was (12.8) and the size of these fragment ranged from 75-2530 bp. The percent of polymorphism revealed by different primers ranged from 33 to 89 % with average of 66.2%.

Key words: *Citrus medica* · *Citrus aurantifolia* · Germplasm characterization · ISSR marker.

INTRODUCTION

Citrus production occupies an important share in the total area and fruit production in Egypt which grown on 541,723 feddans with production of 4,098,590 tons in year 2013 [1]. Besides, citrus is an extremely important crop on a world-wide basis and is grown wherever the climate is suitable. It is widely grown in most areas with suitable climates tropical, subtropical and borderline subtropical/temperate [2]. Egypt is one of the top 10 producers of orange in the world. Acid lime (*Citrus aurantifolia*) belongs to the family Rutaceae and sub family Aurantiodae. It is one of the important commercial fruits, production and productivity of acid lime in Egypt is low 8.85 ton per fed., as compared to other countries like Argrntina,19 ton per ha. and India, 12.2 ton per ha. Low productivity is due to the limitation of high yielding variety [3]. High level of genetic erosion was observed in acid lime landraces, with narrow genetic base [4]. Assessment of genetic diversity within the acid lime landraces is the basis for breeding, conservation of genetic resources and variety development work.

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The estimation technique of genetic diversity in the plant species is different. Traditionally, evaluation of germplasm has been carried out on the basis of morphological traits [5]. Morphological markers are widely used for estimation of diversity and characterization in sweet potato evaluation although it is affected by environment [6]. In citrus, morphological analysis was used to study variation between kinnow mandarin and rough lemon [7,8]. In Himalayan citrus, morphological marker was used for study of diversity [9].The morphological marker is known for its coverage in study of agronomic traits in addition to convenience. Further the technique is relatively cheaper and easier to conduct. Many previous authors reported that molecular and morphological diversity is independent and rather complementary to genetic diversity in citrus[10,11].

In recent years, a number of polymerase chain reaction (PCR) based and DNA based molecular marker technology have been developed for the effective quantification of genetic variation and cultivar identity [12]. Maximum utilization of any germplasm for breeding can be achieved by understanding the level of genetic diversity it contains [13]. Genetic diversity estimates are also important to understand its adaptive potential in different environments [14]. Evaluation of genetic divergence and relatedness among breeding materials has significant implications for crop improvements. And knowledge on genetic diversity in Lime and Lemon accessions could help breeders and geneticists to understand the structure of germplasm and to predict which Combination would produce best offspring and facilitate in widening up the genetic basis of breeding material for selection [15]. Therefore, this study aimed at morphological characterization and assessment of genetic diversity for Lime and lemon accessions maintained at ISSR marker.

MATERIAL AND METHODS

Plant Materials: Plant materials used for this study were collected from Qalyubia governorate during 2014 and 2015 years, four lime and six Lemon accessions aged from 18-20 years old trees planted at 3×5m in clay soil under flood irrigation and grown in Moshtohor Faculty of Agriculture Research Farm, Benha University, Egypt. (Table 1).

Table 1: List of plant materials *Citrus* species and cultivars used in this study

Accession number	Accession name	Scientific name
12180	Balady lime	*Citrus aurantifolia* L.
12181	Helou lime	*Citrus limetta* L.
12182	Sweet lime	*Citrus limetta* L.
12183	Succari lime	*Citrus limetta* L.
12184	Rough lemon	*Citrus jambhiri* L.
12185	Sweet lemon	*Citrus limon* L.
12186	Eureka lemon-1	*Citrus limon* L.
12187	Pink variegated	*Citrus limon* L.
12188	Eureka lemon-2	*Citrus limon* L.
12189	Ponderosa	*Citrus medica* L.

Morphological Characterization: The morphological characteristics used to characterize and discriminate the 10 Lime and Lemon cultivars were based on those previously prescribed for Citrus by the International plant Genetic Resources Institute [16] taking into consideration all the precautions reported. In this respect, 17 quantitative and 11 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 2). Presents traits 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.

Table 2: 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)Pyriform;(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	
3. chemical analysis		
1.Total soluble solids (T.S.S)	2. pH and total acidity	3. Vitamin C content

Determination of pH and Total Acidity: Total acidity of the juice was determined by titration method as reported by Rekha *et al.* [17]. Fruit juice was diluted to 10% with distilled water and then titrated against 0.1N NaOH (standardized using standard Oxalic acid) using Phenolphthalein indicator. The end point was noted when the colour changed from colorless to pale pink. All measures were done in triplicate and dilution factor was considered; total acidity was calculated in terms of citric acid using the following formula, Acidity (g/100ml)= Normality of the juice x Equivalent weight of citric acid. The pH of citrus juice was determined using pH meter (Thermo©, USA).

Determination of Total Soluble Solids (TSS): Total soluble solids (TSS) were measured using digital refractmeter (Atago Co., Ltd., Tokyo, Japan). All measures were done in triplicate; the TSS results were reported as (°Brix).

Estimation of Ascorbic Acid (Vitamin C) Content: Ascorbic acid content in fruit juice was determined by the 2,6 dichlorophenol-indophenol titrimetric method according to AOAC method No. 967.21 [18]. All measures were done in triplicate; the vitamin C content was expressed as mg/100ml.

Molecular Characterization: Young leaves samples of four limes and six lemon 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© Germany) according to the manual procedures. A total of 13 primers (Table 3) were used to amplify DNA fragments; these primers were selected after screening 30 primers. PCR reaction was performed in 25 µl reaction mix containing 1 X PCR buffer, 2 mM $MgC1_2$, 0.2 mM of each dNTPs, 1 µM oligonucleotide primer, 25 ng genomic DNA and 1 unit of Taq DNA polymerase (Promega©, USA). Amplification was performed in a 96-well BioRad© 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.

Table 3: List of primer names, sequences and annealing temperatures used in this study

Primer Name	Sequence	Annealing Temp. °C
5'Anchored repeats		
P_{16}	$ACG(GT)_7$	50.0
3'Anchored repeats		
H_{12}	$(GA)_8YT$	41.0
H_{13}	$(GA)_8YC$	42.5
H_{14}	$(GA)_8YG$	44.0
H_{15}	$AG)_8YT$	52.0
H_{16}	$(AG)_8YC$	56.5
H_{17}	$(AG)_8YG$	59.5
H_{21}	$(GT)_8YC$	60.5
H_{29}	$(GACA)_4AT$	41.5
P_2	$(CA)_6GG$	48.0
P_3	$(CA)_6AC$	42.5
P_4	$(GTG)_3GC$	52.5
P_{11}	$(GAG)_3GC$	45.0

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

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 according to the procedures reported by Snedecor and Cochran [19] and means were compared by Duncan's test at $P<0.05$ was considered statistically significant. The banding patterns generated by ISSR primers were analyzed and compared to determine the genetic relatedness among different Citrus cultivars. The amplified fragments were scored either as present (1) or absent (0). The genetic similarity and similarity matrix among cultivars were estimated according to Dice coefficient [20]. Dendrograms showing the genetic relationships were constructed using the Un-weighted Pair Group Method with Arithmetic Averages (UPGMA) by Phoretix 1D software (Total Lab, UK).

RESULTS AND DISCUSSIONS

Morphological Characterization

Quantitative Characteristics: Table (4) presents leaves and flowers quantitative traits of the Lime and Lemon cultivars under the present investigation. Results showed that Rough Lemon cultivar exhibited the highest leaf length (9.77 cm), followed by Lemon (Pink variegated,Sweet, Eureka-2 and Eureka-1) 8.40, 8.25, 8.18 and 8.10 cm respectively. The lowest leaf length was presented by Sweet Lime (6.10 cm). The rest of the cultivars showed intermediate values of leaf length. On the other hand, there was no significant difference between Lime and Lemon cultivars of leaf width. The uppermost ratio of leaf lamina shape (length/ width ratio) was 2.39 cm for Rough Lemon followed by Succari Lime 2.31 cm. The lowest ratio was characterized by Ponderosa Lemon 1.47 cm. The rest of the Lime and Lemon cultivars showed intermediate values of leaf length/ width ratio. The Sweet Lime showed the highest value of flower pedicle length 0.5 cm. The lowest ones (0.32 cm) were exhibited by Helou Lime, Lemon cultivars (Sweet, Eureka-1, Pink variegated and Eureka-2), respectively. Whereas the rest of the cultivars illustrated intermediate values. Balady and Sweet Lime, Rough, Sweet and Pink variegated Lemon cultivars showed five petals per flower. Whereas, the rest of the cultivars showed four petals per flower. All cultivars of Lemon (Eureka-1, Eureka-2, Rough, Sweet, Ponderosa and Pink variegated) gave the highest petal length (cm) 1.72, 1.71, 1.70, 1.69, 1.68 and 1.67 respectively, without differences among them. The lowest petal length was observed with Sweet Lime (0.8 cm). Whereas, the rest of Lime cultivars illustrated intermediate values. The petal width was different among the cultivars; where Pink variegated and Ponderosa Lemon gave (0.82 and 0.81 cm) presented the highest measurements. Followed by Sweet and Eureka-2 Lemon as it was (0.79 and 0.77 cm). The lowest petal width was presented by Lime cultivars (Sweet and Succari) as they gave (0.36 and 0.35 cm), respectively. Whereas, the rest of the Lime and Lemon cultivars illustrated intermediate values.

The quantitative characteristics of fruits and seeds are demonstrated in (Table 5). Ponderosa Lemon showed the highest significant fruit weight (295.80 g) followed by Rough Lemon (152.76 g). On the other hand, Sweet Lime had the lowest significant fruit weight (15.26 g). The rest of the cultivars gave intermediate fruit weights. Also, Ponderosa Lemon exhibited the greatest significant fruit diameter (8.25 cm), followed by Rough Lemon which was (7.46 cm). Sweet Lime displayed the lowest significant fruit diameter (3.00 cm). The remaining cultivars had intermediate values ranged from (5.00 – 4.00 cm). The highest significant fruit length was demonstrated by Ponderosa Lemon (8.36 cm), followed by Rough Lemon which was (6.83 cm). However, no significant differences were obtained as compared with the rest Lemon cultivars (Eureka-1 and Eureka-2) which showed the same fruit length (2.28 cm) and (Sweet and Pink variegated) which showed 6.27 and 6.25 cm, respectively. Meanwhile, Sweet Lime showed the lowest significant value of fruit length (3.66 cm). The rest of the Lime cultivars gave an intermediate fruit length with significant differences among them.

Table 4: Morphology quantitative characteristics of leaves and flowers of ten Lime and Lemon cultivars grown in Moshtohor Faculty of Agriculture Research Farm, Benha University (average of two seasons 2014-2015).

Cultivars		Leaf length (cm)	Leaf width (cm)	Leaf ratio L/w	Flower pedicel length (cm)	Petal number	Petal length (cm)	Petal width (cm)
Lime	Balady	7.20bc	3.40A	2.11c	0.45ab	5a	1.20c	0.40de
	Helou	7.10bc	3.50A	2.02d	0.32d	4b	1.30b	0.45d
	Sweet	6.10c	3.60A	1.69g	0.50a	5a	0.8e	0.36e
	Succari	7.40bc	3.20A	2.31b	0.35cd	4b	1.10d	0.35e
Lemon	Rough	9.77a	4.08A	2.39a	0.40bc	5a	1.70a	0.60c
	Sweet	8.25ab	4.25A	1.94e	0.32d	5a	1.69a	0.79ab
	Eureka-1	8.10ab	4.40A	1.84f	0.32d	4b	1.72a	0.75b
	Pink variegated	8.40ab	4.10A	2.04d	0.32d	5a	1.67a	0.82a
	Eureka-2	8.18ab	4.33A	1.89ef	0.32d	4b	1.71a	0.77ab
	Ponderosa	7.50bc	5.10A	1.47h	0.42b	4b	1.68a	0.81a

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

Table 5: Morphology quantitative characteristics of fruit and seed of ten lime and lemon cultivars grown in Moshtohor Faculty of Agriculture Research Farm, Benha University (average of two seasons 2014-2015).

Cultivars		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)
Lime	Balady	43.50h	5.00c	6.00d	0.10e	17.50e	0.97de	0.62d	0.10b
	Helou	35.44i	4.00g	3.88f	0.20d	16.33e	0.92e	0.42f	0.10b
	Sweet	15.26j	3.00h	3.66g	0.10e	8.50f	1.00cd	0.50e	0.18a
	Succari	66.46f	4.88de	4.75e	0.20d	20.0d	0.85f	0.44f	0.13ab
Lemon	Rough	152.76b	7.46b	6.83b	0.50a	50.0b	0.98d	0.52e	0.13ab
	Sweet	68.25e	4.84e	6.27c	0.28c	21.50cd	1.09ab	0.74c	0.13ab
	Eureka-1	75.28c	5.00c	6.28c	0.25cd	22.00c	1.13a	0.96a	0.15ab
	Pink variegated	61.22g	4.68f	6.25c	0.30c	21.0cd	1.04bc	0.52e	0.10b
	Eureka-2	71.77d	4.92d	6.28c	0.27c	21.75cd	1.11a	0.85b	0.14ab
	Ponderosa	295.8a	8.25a	8.36a	0.40b	107.0a	0.00g	0.00g	0.00c

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

The fruit thickness indicates that the Rough Lemon had the greatest significant fruit rind thickness (0.50 cm), followed by Ponderosa Lemon (0.40 cm). No significant differences were obtained as compared with the rest Lemon cultivars (Pink variegated, Sweet, Eureka-2 and Eureka-1) 0.30, 0.28, 0.27 and 0.25 cm respectively. The lime cultivars (Helou and Succari) gave the same (0.20 cm) fruit rind thickness. Meanwhile, Balady Lime and Sweet Lime showed the lowest value of fruit rind thickness (0.10 cm).

The amount of juice content (ml/fruit), the greatest was 107.0 ml in the Ponderosa Lemon, followed by Rough Lemon (50.0 ml). The least juice content was obtained from Sweet Lime (8.50 ml) whereas, the rest of the cultivars showed intermediate juice amount.

The Lemon (Eureka-1, Eureka-2 and Sweet) showed the highest significant seed length value (1.13, 1.11 and 1.09 cm) respectively, followed by Pink variegated Lemon (1.04). The least seed length was obtained in Succari Lime (0.85 cm). Whereas, Ponderosa Lemon had none seeds. The remaining of the cultivars observed intermediate seed length (Table 5). Also, Eureka-1 Lemon showed the greatest seed width (0.96 cm), followed by Eureka-2 Lemon (0.85). The lowest width was observed in Succari and Helou Lime (0.44 and 0.42 cm) respectively. The rest of the cultivars showed intermediate seed width. Whereas, Ponderosa Lemon had none seeds. Seed weight from cultivars Lime (Sweet) and Lemon (Eureka-1 and Eureka-2) had the greatest seed weight (0.18, 0.15 and 0.14 g) and cultivars lime (Succari) and lemon (Rough and Sweet) which showed the same value (0.13 g) had the greatest significant seed weight. The lowest seed width (0.10 g) was obtained in Balady Lime, Helou Lime and Pink variegated Lemon.

It is concluded that Ponderosa Lemon has the best quantitative traits as proved by the highest fruit weight, diameter, length and juice content. Whereas, Eureka-1 gave high seed length, width and weight.

Qualitative Characteristics: Tables 6 and 7 present the qualitative traits of fruit, seed and leaf of the ten Lime and Lemon cultivars under the present study. In this regard, only the Rough Lemon showed obloid fruit shape. While, the cultivars (Balady, Helou and Succari) showed spheriod fruit shape. Sweet Lime, cultivars Lemon (Sweet, Eureka-1 and Eureka-2) showed ellipsoid fruit shape. The rest of the cultivars (Pink variegated and ponderosa) showed ovoid fruit shape (Table 6).

The fruit skin colour included 12 colour ranged from green to red-orange. The cultivars Lime (Balady and Succari), Lemon (Sweet and Eureka-2) had yellow fruit skin colour. Only Eureka-1 Lemon had light yellow fruit skin colour. While, Helou Lime, Rough Lemon and Ponderosa Lemon were dark yellow. The rest of the cultivars (Sweet lime and pink variegated lemon) showed green yellow fruit skin colour. Pink variegated was the only one showed grooved texture of skin surface. whereas, the only one showed Rough texture of skin surface was Helou Lime. The cultivars Lime (Balady, Sweet and Succari), Lemon (Eureka-2 and Ponderosa) proved smooth skin surface texture. Whereas, Lemon (Rough, Sweet and Eureka-1) showed papillate surface skin texture (Table 6).

The flesh colour presented in (Fig.1 and Table 6) orange colour of fruit flesh was clear in the Rough Lemon, where as it was pink in Pink variegated Lemon. Meanwhile, all Lime cultivars showed yellow fruit flesh and the rest of the cultivars of Lemon. All cultivars of Lime and Lemon demonstrated solid except Rough Lemon indicated hollow fruit axis. (Table 6). Only Helou Lime showed truncate shape of fruit base. While, Pink variegated Lemon showed necked shape of fruit base.

The cultivars Lime (Balady or Succari) and Ponderosa Lemon revealed convex fruit base shape. On the other hand, Sweet Lime showed concave of fruit base. The rest of the cultivars showed concave collared of fruit base (Table 6). Regarding fruit apex shape, all Lime and Lemon cultivars showed mammiform fruit apex shape.

Table 6: Performance of different lime and lemon cultivars regarding fruit shape, fruit skin colour, fruit axis, fruit shape of base and fruit shape of apex.

cultivars		Fruit shape	Fruit skin colour	Skin texture	Flesh colour	Fruit axis	Fruit base shape	Fruit apex shape
lime	Balady	Spheroid	Yellow	Smooth	Yellow	Solid	Convex	Mammiform
	Helou	Spheroid	Dark yellow	Rough	Yellow	Solid	Truncate	Mammiform
	Sweet	Ellipsoid	Green-yellow	Smooth	Yellow	Solid	Concave	Mammiform
	Succari	Spheroid	Yellow	Smooth	Yellow	Solid	Convex	Mammiform
lemon	Rough	Obloid	Dark yellow	Papillate	Orange	Hollow	Concave collared	Mammiform
	Sweet	Ellipsoid	Yellow	Papillate	Yellow	Solid	Concave collared	Mammiform
	Eureka-1	Ellipsoid	Light yellow	Papillate	Yellow	Solid	Concave collared	Mammiform
	Pink variegated	Ovoid	Green-yellow	Grooved	Pink	Solid	Necked	Mammiform
	Eureka-2	Ellipsoid	Yellow	Smooth	Yellow	Solid	Concave collared	Mammiform
	Ponderosa	Ovoid	Dark yellow	Smooth	Yellow	Solid	Convex	Mammiform

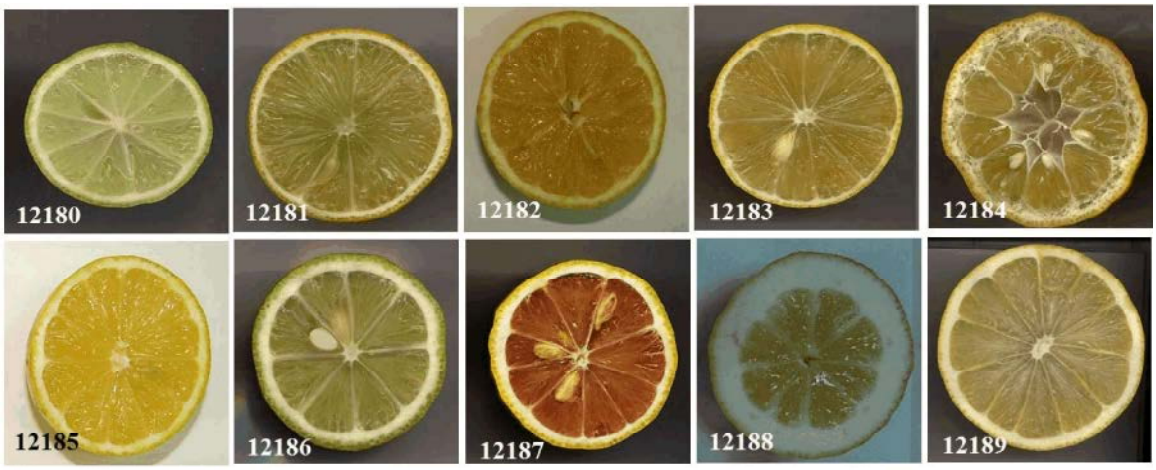

Fig. 1: Fruits cross section of four Lime accessions number from (12180) to (12183) namely, Balady; Helou; Sweet and Succari and six lemon accessions number from (12184) to (12189) namely, Rough; sweet ; Eureka-1; Pink variegated ; Eureka-2 and Ponderosa collected from Moshtohor Faculty of Agriculture Research Farm, Benha University.

Table 7: Quantitative characteristics of Lime and Lemon cultivars.

Cultivar		No. of segment	No. of seed/fruit	Seed shape	Seed surface	Leaf lamina shape	Petiole wings shape
lime	Balady	10-14	5-9	Spheroid	Smooth	Obovate	obdeltate
	Helou	10-14	1-4	Ovoid	Smooth	Ovate	Obovate
	Sweet	5-9	1-4	Clavate	Wrinkled	Obovate	absent
	Succari	10-14	1-4	Cuneiform	Smooth	Ovate	Obovate
lemon	Rough	5-9	5-9	Cuneiform	Wrinkled	Elliptic	absent
	Sweet	5-9	1-4	Ovoid	Smooth	Ovate	obdeltate
	Eureka-1	5-9	1-4	Ovoid	Wrinkled	Ovate	obdeltate
	Pink variegated	5-9	5-9	Ovoid	Smooth	Ovate	obdeltate
	Eureka-2	5-9	5-9	Ovoid	Smooth	Ovate	obdeltate
	Ponderosa	5-9	None	None	None	Elliptic	absent

The qualitative characteristics of no. of seed/fruit, seed shape, seed surface, leaf lamina shape and petiole wings shape are presented in (Table 7). The Lime cultivars (Balady, Helou and Succari) illustrates (10-14) segments per fruit. While, the rest of the Lemon cultivars and Sweet Lime had (5-9) segment/fruit.

The average no. of seed/fruit was 5-9 in Balady Lime and Lemon cultivars (Rough, Pink variegated and Eureka-2). Only Ponderosa Lemon had none seeds. The rest of the cultivars had (1-4) seeds/fruit.

Regarding the seed shape, Balady Lime was presented spheroid seed shape. one cultivar of Lime (Sweet) showed clavate seed shape. Also, Succari Lime and Rough Lemon demonstrated cuneiform seed shape. Meanwhile, Ponderosa Lemon had none seeds. Seed shape of the rest of the cultivars was ovoid seed shape (Table 7). Among the studied Lime and Lemon cultivars, three cultivars of Lime (Balady, Helou and Succari) and three cultivars of Lemon (Sweet, Pink variegated and Eureka-2) were demonstrated smooth seed surface. Ponderosa Lemon had none seeds. The residues of the cultivars 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, Lanceolate or obcordate or orbicular leaf lamina shape. Tow only of Lime cultivars (Balady and Sweet) exhibited obovate leaf shape. However, Rough Lemon and Ponderosa Lemon cultivars showed elliptic leaf shape. The rest of the cultivars displayed ovate leaf lamina shape (Table 7). The absence or presence of petiole wings were evaluated for the different germplasm. The Sweet Lime, Rough and Ponderosa Lemon demonstrated absent petiole wings. While, two only cultivars Lime had Obovate petiole wings shape (Helou and Succari). On the other hand, the rest of the cultivars showed obdeltate petiole wings shape.

Morphological characterization of Lime and Lemon was studied by Hana *et al.,* [21] they found that in general, lime fruits are smaller than lemon fruits and have a thinner rind. The main differences between lemon and lime fruits observed in length to width ratio, shape index, seed and rind thickness. Lemon fruits often have a high length to width ratio. The highest percentage of fruit juice was measured in M5 (42.86%) while, the least juice was observed in R4 (42.93%). Description of morphological characters is a usual method accepted for evaluation and registration of varieties.

Chemical Analysis of Lime and Lemon Fruit Juice: The TSS content for the analyzed accessions varied significantly. The TSS is known to increase as and when the fruit matures while total acidity remain constant. The decrease in total acidity was due to dilution effect as a result of increase in fruit size and increase in TSS content [22].The marketability of citrus is determined by the ratio of TSS to total acidity. The significant variation among the accessions for this ratio also supplement to existence of diversity. Data in (Table 8) shown that, pH ranged from 6.20 in Succari lime to 2.12 in ponderosa Varity. However, titratable acidity ranged from 6.45 % in Eurek-1 lemon to 0.40 % in Helou lime. The highest total soluble solids (TSS) content (10.50) °Brix was determined in Sweet lime, Sweet lemon and ponderosa, while the lowest TSS content (5.80) °Brix was detected in Succari lime. The ascorbic acid (Vitamin C) content ranged from 48.39 mg/100 ml in Eurek-1 lemon to 15.33 mg/100 ml in Sweet lemon. On the other hand, ranking the best source of Vitamin C according to Levin *et al.* [23]. include four categories Fairly good (more than 6 mg of Vitamin C), good 5 mg of Vitamin C, very good 15 mg to 30 mg of Vitamin C and excellent more than 30 mg of Vitamin C. the results in (Table 8) shown that Eureka- lemon had excellent source of vitamin C and the Sweet lemon had good source of Vitamin C While, the rest of accessions showed very good source of Vitamin C.

Table 8: Some physicochemical characteristics of lime and lemon cultivars collected form Moshtohor region, Faculty of Agriculture Research Farm, Benha University (average of two seasons 2014-2015).

Accessions		pH	Acidity (%)	T.S.S (°Brix)	Vitamin C mg /100ml	*Ranking
lime	Balady	2.50F	4.96C	6.10D	33.6B	Very good
	Helou	5.94B	0.40G	6.93C	24.30De	Very good
	Sweet	2.68E	1.18F	10.50A	26.33Cd	Very good
	Succari	6.20A	1.13F	5.80D	24.30De	Very good
lemon	Rough	2.40G	4.50D	6.47Cd	20.63Fg	Very good
	Sweet	5.91B	1.84E	10.07A	15.33H	good
	Eureka -1	3.23C	5.56B	7.00C	48.39A	excellent
	Pink variegated	2.50F	4.90C	5.90D	22.74Ef	Very good
	Eureka -2	2.78D	6.45A	8.10B	28.76C	Very good
	Ponderosa	2.12H	1.76E	10.23A	18.01Gh	Very good

Means followed by the same letter within the same column are not significantly different (P= 0.05) *ranking the best source of vitamin C according to Levine *et al.,* 1999 (Fairly good more than 6 mg of vitamin, good 15 mg of vitamin C, very good 15 mg to 30 mg of vitamin C and excellent more than 30 mg of vitamin C).

Chemical composition of genetic resources is an essential identification process in monitoring of the genetic quality during improvement and conservation [16]. Citrus is a good source of vitamin C, which is the most important nutrient component in Citrus fruit juice [24]. Our study showed that lime and lemon fruits are very good sources of vitamin C. These findings are compatible with other results published by other workers [25,26]. On the other hand, lemon and lime varieties showed a moderate ascorbic acid content (15-48 mg/100ml),which is in agreement with results reported by Rekha *et al.* [17].

Molecular Characterization

Polymorphism Detected by ISSR Primers: ISSR amplification from all DNA samples of 10 lime and lemon accessions (collected from Moshtohor region) generated productive banding figures for all 13 primers (Fig. 2). The total number of amplified amplicons among tested primers ranged from 9 to 19 fragments. 3'anchored P_2 primer amplified the highest number of fragments (19 bands) while, H_{14} and P_{11} generated the lowest number of amplicons (9 bands).The average number of fragments/ primer was (12.8) and the size of these fragments ranged from 75-2530 bp. All the used primers produced polymorphic bands (Table 9). Of the total 167 scorable fragments, 117 were polymorphic among the accessions (Fig. 1). The number of polymorphic bands ranged from 3 to 17 resulting in an average of polymorphism/ primer of (9). Primers P_2 revealed the highest number of polymorphic bands (17) however; the lowest number of polymorphic amplicons (3) was generated by primer P_{11}.The percent of polymorphism revealed by different primers ranged from 33 to 89% with an average of 66.2%. These results agreement with Gulsen [27] reported that similarity level of citron and lemon-rough lemon group was 0.65 based on their ISSR data. On the other hand, according to Uzun [28], genetic similarity among lemons and rough lemon *C. volkameriana* group was 0.80. Rough lemons and *C.volkameriana* were closely related. At the same way, *C. volkameriana* was clustered with rough lemon as in the RAPD [29] and SCAR [30] based studies. It is reported there was low level of polymorphism among most of lemons derived via clonal selection whereas higher genetic diversity was found in lemons which had hybrid origin [28].

Table 9: Total number of amplicons, monomorphic amplicons, polymorphic amplicons and polymorphism percentages as revealed by ISSR marker among the 10 lime and lemon accessions collected from Moshtohor region.

Primer	Total amplicons	monomorphic amplicons	polymorphic amplicons	polymorphism (%)
P_2	19	2	17	89
P_3	12	4	8	67
P_4	13	4	9	69
P_{11}	9	6	3	33
P_{16}	13	2	11	85
H_{12}	11	7	4	36
H_{13}	13	3	10	77
H_{14}	9	5	4	44
H_{15}	12	4	8	67
H_{16}	15	3	12	80
H_{17}	18	2	16	89
H_{21}	13	2	11	85
H_{29}	10	6	4	40
Total	167	50	117	
Average	12.8	3.8	9.0	66.2

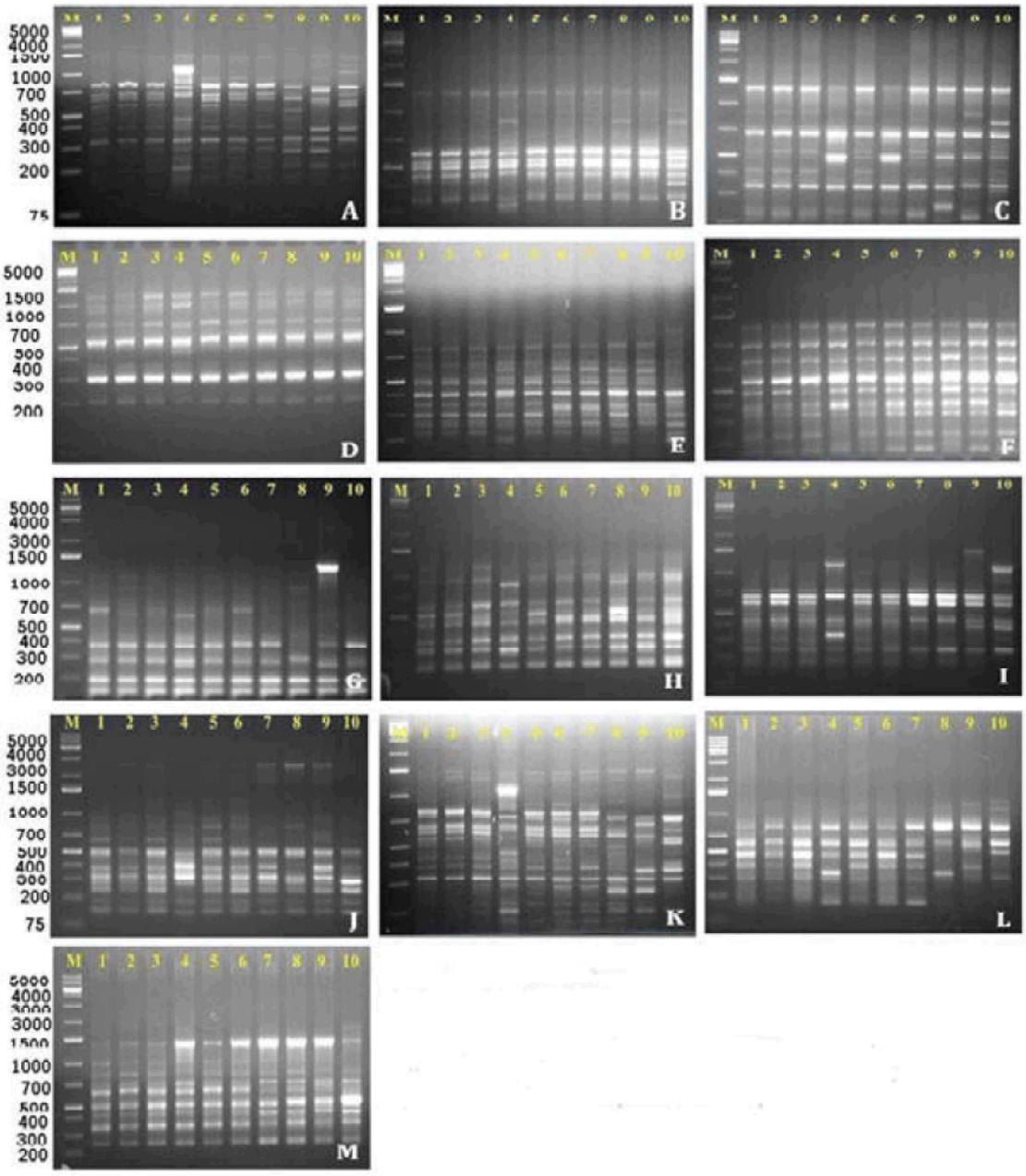

Fig. 2: Electrophoretic separation patterns of ISSR-PCR products (as revealed on 1.8 % agarose gel) using primers P_2 (A),P_3(B), P_4(C), P_{11}(D), P_{16}(E), H_{12}(F), H_{13} (G), H_{14}(H), H_{15}(I), H_{16} (J), H_{17}(K), H_{21} (L) and H_{29}(M). Lane M: 1Kb plus DNA ladder. Lanes 1 to 10 represented Moshtohor Faculty of Agriculture Research Farm lime and lemon cultivars: Eureka-1, Eureka-2, Pink variegated, Ponderosa, Sweet lemon, Sweet lime, Sweet lime, Succari lime, Rough lemon and Balady lime, respectively.

Clustering Analysis: The UPGMA cluster analysis of genetic distance among the 10 Lime and lemon accessions is shown in Fig (2). Phylogenetic analysis showed a high degree of genetic variation among tested genotypes, though the interrelated accessions of the same genotype; Sweet lime and Eureka lemon were grouped in the same lineage. On the other hand, other related accessions of the same genotype; Pink Variegated lemon were grouped in separate clusters. The dendrogram ranked lime and lemon accessions into two major clusters at 73% level of similarity, the first included one genotype Ponderosa *(Citrus medica* L.) however; the second included

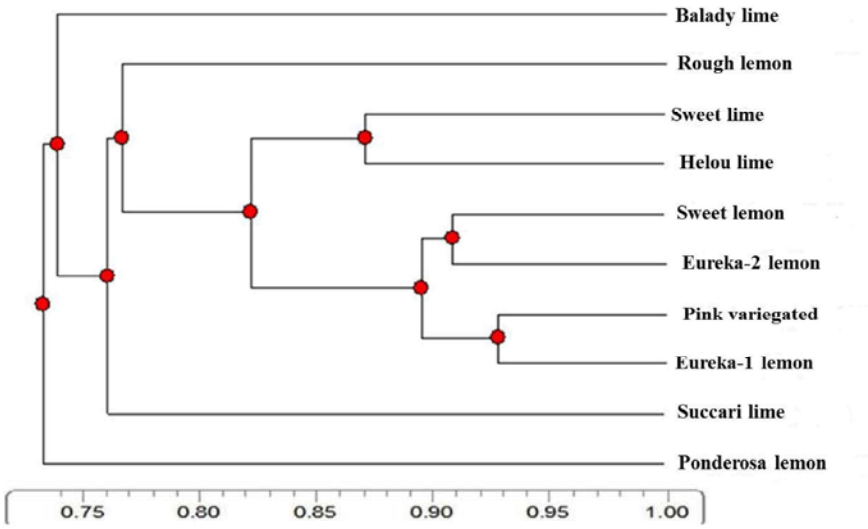

Fig. 3: Dendrogram for the 10 lime and lemon genotype accessions (collected from Moshtohor region) constructed from the ISSR generated data using UPGMA method and similarity matrices computed according to Dice's similarity coefficient.

Table 10: Lime and Lemon 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

		Unique positive		Unique negative		
Genotype	Primer	Size in bp	Total	Size in bp	Total	Total
Eureka-2	P_2	-	-	1511	1	2
	H_{21}	831	1	-	-	
Eureka -1	H_{13}	334	1	-	-	2
	H_{16}	441	1	-	-	
Pink variegated	H_{12}	542	1	-	-	2
	H_{21}	231	1	-	-	
Ponderosa lemon	P_2	469	1	-	-	8
	P_3	318	1	-	-	
	P_{11}	445	1	-	-	
	P_{16}	421	1	-	-	
	H_{12}	-	-	241	1	
	H_{15}	329	1	-	-	
	H_{16}	-	-	242	1	
	H_{17}	457	1	-	-	
Succari lime	P_2	-	-	386	1	5
	H_{13}	445, 910	2	-	-	
	H_{17}	-	-	384, 664	2	
Rough lemon	P_2	-	-	674	1	12
	P_3	682	1	-	-	
	P_{16}					
	-	-	298	1		
	H_{13}	1024, 1302	2	288	1	
	H_{15}	1500	1	-	-	
	H_{16}	1699	1	-	-	
	H_{17}	1203	1	-	-	
	H_{21}	564, 665	2	480	1	
Balady lime	P_3	412	1	1281	1	9
	P_4	312, 368	2	-	-	
	P_{16}	-	-	812	1	
	H_{16}	-	-	317	1	
	H_{21}	1128	1	-	-	
	H_{29}	1684	1	628	1	

the other 9 lime and lemon accessions. The highest genetic similarity was detected between Eureka-1 and Pink Variegated genotypes with 93% level of similarity; nevertheless the highest genetic difference was identified between the Balady lime and Rough lemon as well as Pink Variegated and Rough lemon genotypes with 70% level of similarity. In respect of lime Egyptian genotypes, a high degree of genetic overlapping was detected among the local genotypes (Sweet lemon, Sweet lime, Succari lime and Balady lime) with other exotic lemon varieties. According to ISSR-phylogenetic analysis, Succari lime and Balady lime showed the highest genetic diversity among all tested Egyptian local genotypes. Molecular markers show diversity among lemons [31,32]. but genetic diversity of a large sample of lemon cultivars from a wide range of geographic locations has not been reported. The high genetic similarity found amongst lemons and limes was previously reported by other workers they found high similarity values, ranging between 0.82 and 0.88 were found among lemons, Sweet lemons (*Citrus limon* L.) and Rough lemons (*Citrus jambhiri*) and Bergamot orange (*Citrus bergamia*) while, similarity value were lower (0.64 to 0.72) among lemons, Mexican lime (*Citrus aurantifolia*) and Sweet limes (*Citrus limetta*) [27,30].

Genotype Identification by Unique ISSR Markers: Genotype- specific ISSR unique markers were able to differentiate seven lime and lemon genotype accessions, Eureka -1, Eureka-2, Pink variegated, Ponderosa lemon, Succari lime, Rough lemon and Balady lime. The ISSR markers generating primers and the positive and/or negative markers approximate size are shown in Table (10). Out of all tested ISSR primers, 12 primers were able to generate unique markers (positive and/ or negative), that could differentiate lime and lemon accessions. However, primer H_{14} only was failed to produce any unique marker. The number of generated unique markers ranged from 2 to 12 markers. The maximum number of unique markers was identified with the accession Rough lemon by 12 markers. On the other hand, Eureka-1, Eureka -2, Pink variegated, accessions were characterized by two unique markers.

In conclusion, Morphological and molecular characterization will facilitate the identification of duplicate germplasm and in selecting the core collection for long term conservation. On the other hand, the phylogenetic tree based on ISSR markers, separated the Citrus varieties into discrete clusters according to their respective group. Meanwhile, wide variation existed among the accessions with respect to quantitative characters. On the other hand qualitative characters did not vary much among the analyzed accessions.

Recommendation: Understanding of genetic diversity in Citrus is essential for planning and application of breeding programs, establishing germplasm collection and carrying out molecular studies. It is also important for Citrus researcher and breeders to arrange their future studies.

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