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Genetic Assessment of Some Egyptian Cultivated Citrus and its Relatives Using Retrotransposons and Microsatellite

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CITRUS a member of the Rutaceae family, is one of the commonly cultivated fruit trees in temperate regions. The taxonomy and systematics of the genus *Citrus* are very complicated, and the precise number of natural species is unclear. Towards this effort, here the retrotransposable and microsatellite represent one of the most effective aspects for determination the genetic diversity in the plant genome. In the present investigation, a detailed overview of evaluating 21 LTR-REMAP and 27 SSR markers in the discriminating capacity, efficiency and ability of the genetic variability among 20 genotypes in the genus *Citrus*. The LTR-REMAP profile displayed 1.28 fold higher value of marker index (MI) compare to the SSR profile, highlights the distinctive nature of LTR-REMAP assay. This marker was more sensitive and provided much more evidence to discriminate at low taxonomic levels, especially for Egyptian acidic, whereas, SSR can differentiate within the group level only. Intriguingly, the heatmap cluster analysis (HCA) and principal component analysis (PCA) data drew an accurate monophyletic annotations cluster in Citreneae species, offer unambiguous identification without overlapping groups between true species and related hybrids of citrus, resulted in their placement in different clades. The use of molecular technologies will help to overcome the obstacles of cultivars identification, genetic variability in the Egyptian citrus breeding.

Keywords: Citrus, SSR, LTR-REMAP, MI, cultivated citrus, Heatmap, Egypt.

Introduction

The genus *Citrus* belongs to the family Rutaceae, tribe Citreae. it is an economically important fruit crop, which widely grown all over the tropical and sub-tropical regions of the world (Talon & Gmitter, 2008). Brazil, China, the USA, Mexico, and India are the leading five major citrus producing countries globally (FAOSTAT, 2017). Egypt is the sixth-largest orange producer and the first or the second-largest exporter of fresh oranges in the world. *Citrus* taxonomy and phylogeny are complex, controversial and confusing (Nicolosi et al., 2000) by numerous attributes such as the long history of cultivation, high frequency of bud mutation, nuclear embryony, complicated genetic background, wide cross-compatibility between species and invasive species. Therefore, the number of Citrus species to be identified is the most important challenge in citrus breeding and systematic (Uzun &Yesiloglu, 2012). Swingle, (1943) and Tanaka, (1977) are the most two accepted taxonomic systems for citrus, recognized 16 and 162 species, respectively. Worldwide, there are many potential true species in the genus Citrus such as Lemon, lime, mandarin, sweet orange, grapefruit, pummelo and the associated hybrids (Gmitter et al., 2012).

For Egypt, the performance of the new species should be considered before cultivating to preserve the citrus genetic resources against the invasive citrus species. Under this scenario, molecular identifying represents an effective

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instrument for genome analysis and enables the linkage of heritable traits attached to genomic divergence. Presently, the next-generation sequencing technology (NGS) has been applied to create various genomic resources, such as the first announced genome sequence of citrus (Xu et al., 2013), and freely available EST and BAC-end sequences of citrus (Biswas et al., 2012). These genomic tools are valuable basics for knowledge and improving resource for understanding and developing various frequent sequences approaches as microsatellites and retro-transposons loci. It is well known that, microsatellite frequency diverges significantly among species and along with various genomic regions.

Thus, the SSR loci present a standard technique for improving a wide number of beneficial molecular markers in citrus (Palmieri et al., 2007). Over the past decade, SSR loci have shown to be the powerful marker of selection, because it is an inexpensive methodology, efficient, and can discover the highly diverse region in plant breeding and genetics (Amar et al., 2011 and Biswas et al., 2014). Recently, there are several models of molecular survey applying SSR in many plant species, including Citrus (Biswas et al. 2012 and Snoussi et al., 2012).

While retrotransposons are the common types of transposable elements (TE) in the eukaryotic genomes, and they predominate the number of the gene. Ty-like retrotransposons are a common class of transposable elements in the plant genome, including citrus. In their form, is transcribed the individual LTR of a joint element to generate almost complete template RNA copy, include a single copy of the LTR divided within its two sides (Kalendar et al., 2011). Long terminal repeats (LTRs) are features of LTR retrotransposons represent a major component of the structural DNA, approximately 40–70% of the total plant genome with 300-500 bp long direct repeats at both ends of the element (Habibollahi et al., 2017). These LTR are highly conserved in the structural evolution of plant genomes and are exploited for primer design in the development of retrotransposons-based markers.

A wide variety of retrotransposon-based marker systems use PCR primers, like Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) and Inter Retrotransposon Amplified Polymorphism (IRAP) designed to discover the performance of Transposable elements (TE) in the genetic diversity (Antonius et al., 2006). The LTR-REMAP is like to LTR-IRAP technique with the difference in one primer tied to the SSR locus. Therefore, the application of LTR-REMAP gets a position within the SSR region and the LTR elements (Kalendar et al., 1999 and Kalendar et al., 2011).

It seems that in most genomes, SSR linked to retrotransposons with a high proportion of mutation owing to the polymerase slippage, therefore, they may exhibit significant diversity at the sub-species level (Grandi & An, 2013). Accordingly, these unique properties of retrotransposons have been utilized as genetic implements for plant genotyping (Mansour, 2008), intraspecific relationships, functional analyses of genes and the genetic diversity (Kalendar & Schulman, 2014). Unfortunately, LTR-REMAP based marker is still less studied in citrus research (Du et al., 2018).

The objective of the current investigation is to infer parentage among several citrus varieties, with special emphasis to the cultivated Egyptian citrus LTR-REMAP and SSR analysis. In detail, to confirm the inferred of variability obtained with each marker system for estimating genetic diversity among the cultivated Egyptian citrus and related species.

Materials and Methods

Twenty genotypes belonging to the genus Citrus and its related species, comprising the following major groups of Citrus as listed in Table 1. Four Egyptian genotypes were collected from Agriculture Research Center (ARC), Nubaria Research Station, Egypt (latitude 30°30’1.4”N and longitude 30°19’10.9”E), while the remain genotypes were collected from National Center of Citrus Breeding, Huazhong Agricultural University (NCCB,HZAU), Wuhan, China (latitude 30°28’30.4”N longitude 114°21’10.8”E). For easy description, these species were divided into five groups according to the morphological description and fruit characterization as following, mandarins compress three species, oranges compress six species, pummelos and grapefruits compress four species, acidic and Egyptian acidic compress six species and kumquat has only one species.

Genomic DNA Isolation (gDNA)

This study was carried out in the biotechnology laboratories of the Egyptian Deserts Gene Bank (EDGB), Desert Research Center (DRC). Genomic DNA was extracted using Gene JET™ Plant Genomic DNA Purification Kit (#K0791,

TABLE 1. The list of 20 citrus genotypes and its relatives grouped according to morphological description.

Sl. No.	Scientific name	CV.	Group	Location	Morphological fruit
1	*C. reticulata* Blanco	Ponkan	Mandarin	NCCB,HZAU, China	Mandarins
2	*C. unshiu* Marc	Guoqing	Mandarin		
3	*C. reticulata* Blanco x *C. sinensis* (L.) Osbeck	Murcott	Mandarin		
4	*C. sinensis* Osbeck	Jincheng	Sweet Orange	NCCB,HZAU, China	Oranges
5	*C. sinensis* Osbeck	Valencia	Sweet Orange		
6	*C. sinensis* Osbeck	Anliu	Sweet Orange		
7	*C. sinensis* Osbeck	Cara Cara	Navel Orange		
8	*C. aurantium* (L.)	Daidai	Sour Orange		
9	*C. aurantium* (L.)	Bitter orange	Sour orange		
10	*C. grandis* (L.) Osbeck *x C. paradisi*	HB pummelo	Pummelo	NCCB,HZAU, China	Pummelos and Grapefruit
11	*C. grandis* (L.) Osbeck	Shatian pummelo	Pummelo		
12	*C. grandis* (L.) Osbeck	Guan Xi Miyon pummelo	Pummelo		
13	*C. paradisi* Macf.	Red Marsh grapefruit	Grapefruit		
14	*C. medica* var *sarcodactylis*	Fingered Citron	Citron	NCCB,HZAU, China	Acidic
15	*C. limon* (L.) Burm. f.	Eureka Lemon	Lemon		
16	*C. jambhiri* (L.) Burm. f.	Rough lemon	Lemon	ARC Egypt	Egyptian Acidic
17	*C. limon* (L.) Burm. f.	Egyptian Eureka lemon	Lemon		
18	*C. volkameriana* Ten. & Pasq	Volkamer lemon	Volkamer		
19	*C. aurantifolia* (Christm.) Swingle	Egyptian lime	Sour lime		
20	*Fortunella crassifolia* Swingle	Meiwa Kumquat	Kumquat	NCCB,HZAU, China	Kumquat

Thermo Scientific, Lithuania). The quality and concentration of the DNA samples were checked in a Quawell Q5000 UV-Vis spectrophotometer (V2.1.4, USA). A portion of the DNA was diluted to 50 ng/µl for use in SSR and LTR-REMAP profiles. Both the stock and diluted portions were kept at -20°C.

SSR profile

SSR primer pairs were designed from the flanking sequences, using SSRLocatorI V1.1 software (Da Maia et al., 2008) according to the draft genome reference of Citrus sinensis (Xu et al., 2013). A set of SSR markers were selected based on literature data and on previous experiences (Biswas et al., 2010 a, and Amar et al., 2011). The SSR repeats were selected with a set of 100–300 bp, the optimal primer length as 20 bp and the GC content had to be 40–60%, while the optimal primer annealing temperature (TM) was adjusted using the gradient program of Thermal Cycler PCR (Agilent SureCycler 8800, USA) to determine the optimum annealing temperature. Out of 88 SSR primers, a subset of 27 SSR markers displaying the clearer and more polymorphic profiles is given in Table 2. SSR amplification products were separated by 6% poly acrylamide gel electrophoresis (PAGE) and finally visualized by a simplified silver staining method previously described by Amar et al. (2011) and Xu et al. (2002).

TABLE 2. List of simple sequence repeat (SSR) primers information obtained from the current investigation.

SSR Primer name	Forward primer	Reverse primer	TM
AM-SSR1	ACACAAATCTGCCCACATCA	GTGTGTGCATGGATGAGGAG	55
AM-SSR2	AGTGTCTCGCACTCTCGGTT	GTGGCACGAGGGTAGTGAAT	56
AM-SSR3	GAAAGACCAAGCGAGTGAGC	TGAAACCTGGCGGTAACTCT	60
AM-SSR4	AACACTCGATCACCGAAACC	ACCGAAATTTCCGTGCTCCT	59
AM-SSR5	TTGGTCTTTTAGAGCGGACC	TAAACCTGCAACCTCCTGCT	57
AM-SSR6	ATCCCACTTGTGGCTCAAAC	GCTTAGCATCTTCAGGTGGC	54
AM-SSR7	GATCACCACAAGCAGCACAC	TCTCAAGAGCCCAGTTCGAT	56
AM-SSR8	ATGGCCCTTCTTTACAGGCT	TAAACCCATTGGTCCCTTTG	68
AM-SSR9	AACAAAAGCACCGGTTTGTC	GGCTCTGATAGGCTGTGGAG	59
AM-SSR10	GCTCGCAAACACTCTCTGAA	CAAGAAAGGGCAAGAAAACG	55
AM-SSR11	TCGGGTAAGAGGCAAAAATG	ATAATCGGAAAATCGGGGTC	56
AM-SSR12	AATTTGTTGCTGTGCTTCCC	GATCTGGGTTGGATCCTTGA	57
AM-SSR13	ATGCTAAGTGGGATGTTGGC	CAAAGCAATGACTTGACCTCC	59
AM-SSR14	CTGTTGCTGCTCTTGGATCA	GTTCCGGATTGAACCATGTC	60
AM-SSR15	GTAGCCATCTCAGCCACCAT	TTTGTTCCATCAGCATCCAA	56
AM-SSR16	GGCTTCGATTCTGGATGCTA	GCAACCTTTTCTTCTTGTTGG	58
AM-SSR17	TCCAATCCCATTGTTTGTGTT	TTAACTGGGGTGGTGGTGAT	55
AM-SSR18	TTGAAGACGTGCATTTAGGC	TCAAAGGCTAGGCTCCAACT	57
AM-SSR19	CAACCGTTCCTGACTCCATT	AAGTGTTTTCGAGGTGGGTG	54
AM-SSR20	CCTTCAATCCGTACGCTCTC	GTGTGGAGGTCTTCGGGTAA	56
AM-SSR21	CAACTCTTCAAGCAGCCCTC	AAGGGCAAAGGAATGTTGTG	57
AM-SSR22	TGCATGCCATTTTGTTCATT	CCCTGTTCGCTAAGAGTTGC	55
AM-SSR23	TAGAAGAAGGTGAGAGAGATTCCT	TAAGACGGTCAGTGTGGCTG	54
AM-SSR24	TCACAAATTTATGCCTTGCG	TCGATAGTGCACCACGACAT	56
AM-SSR25	AGCCTTGGCTGAGCTGTAAA	GGGTGCCATTTAAAAACCCT	55
AM-SSR26	CACTTCTAAACCCGAACCCA	TTGGAGGAATCAAGAGGGTG	58
AM-SSR27	GAGAGAGGTGGCAATTGAGC	TTGCCTCACAACAAACAAAGA	59

LTR-REMP profile

Sixteen primers synthesized from Ty-1/copia and Ty3-gypsy like sequences (Kalendar and Schulman, 2014) (Table 3), were combined with ten citrus EST-SSR primers performing twenty-one with highly efficiency EST-LTR primer combinations. The LTR primers were designed following the protocols published by Schulman AH (Kalendar et al., 1999). LTR-REMAP amplifications were performed in a final volume of 20µl, containing 30 ng DNA, 0.3 pmol of LTR primer, 0.2 pmol of SSR primer, 2.5 mM MgCl2, 2 mM dNTPs, 10X Taq buffer and 0.2 U Taq DNA polymerase (Thermo Scientific). The PCR amplification and visualized were performed according to Kalendar et al., (1999).

Amplicon scoring and data analysis

All clearly detectable of LTR-REMAP and SSR products were scored as band presence (1) and absence (0) using the Bio-Rad Gel Doc™ XR+ imaging analysis system with Image Lab™ (USA), and adjusted manually as necessary and assembled onto a data matrix. While the genetic characterization of the 27 codominant polymorphic genic-SSR markers were performed using GenAlEx software (Peakall and Smouse 2012). However, the comparisons of the

TABLE 3. List of retrotransposons (LTR) primers information obtained from the current investigation.

LTR Primer name	Forward primer	Reverse primer	TM
AM-LTR 1	TGCCACGATCAGCAAGAATCA	TCTCTTGACAATTCACGTGGCT	57
AM-LTR 2	AGTAACTGTAAGCTGACGTGGCT	GGTGTTGTAGAATCTTCCAGACT	53
AM-LTR 3	CCGTTTTGCCGTCTGATCTCT	AATCCACCTCCTCGTGGGAT	58
AM -LTR 4	TGTGGTGCAGTGAACCATTCA	TCGGCTGGAAACCCGAGCTTGC	59
AM -LTR 5	GCTCTCTGGCTGTTATCGGTT	AGGTTGGCCGAACCACGTAA	54
AM- LTR 6	TGCGAATCCACATGGTGATCACA	GGATCGTGATCTAGGAGCCTA	56
AM -LTR 7	TCGTCAATCCGCATGGCTTCCA	GACGTAGGCTAAAAGCCGAACCA	57
AM -LTR 8	GATACCAGGCTCTTACGGGACAC	CAACCGGCGTGCTCTGACTTGT	53
AM -LTR 9	GACTTCGCCCAAACTTTGTGA	GTAGGCGGGGATTGCCGAACCA	55
AM-LTR 10	TCGCCGTTGTTCGTTGAGTGTCT	CGAACCACGTAAAAATCCGCGTG	56
AM LTR 11	CAGCAACTGCACTGTTCCAGA	TCACTGTGGAGACGATCTTGA	55
AM LTR 12	TGCGAATCCACATGGTGATCACA	TAGGAGCCTAAATCACTTCA	53
AM LTR 13	CTCCTAATGGTTCCTAATACCAGACAA	ACCTCTCGAATTGTAGGTCAGG	56
AM LTR 14	GCAAACCAAGATTGGTGAGGGCA	GCAACCCGTTTTCGTCCAGA	57
AM LTR 15	TCGTTAGCTGCATCTGACTGGGA	TCATACATGGCTTGCATGGGAGT	58
AM LTR 16	ACCGACGAGAACTCAAACGCA	GAGTTGGAGAAGGATTAAGAGTA	56

discriminating capacity, level of polymorphism and informativeness of LTR-REMAP and SSR were calculated according to the indices of Powell et al., (1996). To compare the efficiency of the two markers in Citrus species, we estimated the following parameters for each assay unit according to Smith et al. (1997), using the following formula: PIC =1-$\sum f_i^2$, as follows:

• Number of monomorphic amplicons (n_{np})

• Number of polymorphic amplicons (n_p)

• Average number of polymorphic amplicons per assay unit (n_p/U)

• Number of allele (*L*)

• Number of allele /assay unit (n_u)

• Total banding pattern (*Bp*)

• Effective number of patterns/ assay unit (*p*)

• Total number of effective alleles (*Ne*)

• Polymorphism information content (*PIC*)

• Expected heterozygosity of the polymorphic loci (*He*)

• Fraction of polymorphic loci (β)

• Assay efficiency index (A_i)

• Effective multiplex ratio (*EMR*)

• *Marker index (MI)*

Diversity analyses

To gain accurate perspectives on the genetic diversity among the citrus germplasm, a graphic demonstration of principal coordinates analysis (PCA) and the heatmap cluster analysis (HCA) was carried out to display the multidimensional genetic relationship and its partition among varieties using ClustVis web tool for visualizing clustering of multivariate data (Metsalu & Vilo, 2015).

Results and Discussion

Level of polymorphism

In the present investigation the genetic diversity was assessed among twenty genotypes of citrus species using retrotransposable and microsatellite markers. About 56 LTR-REPAP and 88 SSR primers were initially tested the polymorphism among the 20 citrus germplasm. Among all primers, 21 LTR-REMAP and 27 SSR primers provided a high level of polymorphism as summarized in Tables 4 and 5 as well as Fig. 1a and b.

TABLE 4. Genetic characterization of 27 polymorphic genic-SSR markers for Citrus germplasm,

Locus	*Na*	*Ne*	*I*	*Ho*	*He*	*uHe*	*F*	*Fst*
AM-SSR1	5	2.88	1.25	0.70	0.65	0.67	1.000	-0.073
AM-SSR2	2	1.88	0.66	0.65	0.47	0.48	0.999	-0.387
AM-SSR3	6	5.00	1.68	0.70	0.80	0.82	0.999	0.125
AM-SSR4	5	2.75	1.27	0.70	0.64	0.65	0.978	-0.100
AM-SSR5	5	3.43	1.35	0.55	0.71	0.73	0.999	0.224
AM-SSR6	3	2.74	1.05	0.65	0.64	0.65	1.000	-0.024
AM-SSR7	4	2.81	1.13	0.85	0.64	0.66	0.998	-0.320
AM-SSR8	5	2.05	0.96	0.25	0.51	0.52	0.985	0.511
AM-SSR9	3	2.47	1.00	0.55	0.60	0.61	0.989	0.076
AM-SSR10	4	2.57	1.08	0.45	0.61	0.63	0.947	0.264
AM-SSR11	3	2.37	0.98	0.35	0.58	0.59	0.994	0.395
AMSSR12	7	4.57	1.62	0.90	0.78	0.80	0.976	-0.152
AMSSR13	5	2.17	1.08	0.60	0.54	0.55	1.000	-0.114
AM-SSR14	2	1.83	0.65	0.60	0.46	0.47	0.831	-0.319
AM-SSR15	3	1.63	0.64	0.35	0.39	0.40	0.997	0.094
AM-SSR16	4	3.28	1.24	0.35	0.70	0.71	0.977	0.496
AM-SSR17	5	4.17	1.51	0.90	0.76	0.78	1.000	-0.184
AM-SSR18	7	5.00	1.73	0.55	0.80	0.82	0.984	0.313
AM-SSR19	5	3.77	1.41	0.55	0.74	0.75	1.000	0.252
AM-SSR20	5	3.52	1.40	0.75	0.72	0.73	0.795	-0.047
AM-SSR21	7	3.94	1.59	0.80	0.75	0.77	0.959	-0.072
AM-SSR22	4	2.92	1.19	0.45	0.66	0.67	0.777	0.316
AM-SSR23	4	2.64	1.10	0.35	0.62	0.64	0.861	0.437
AM-SSR24	6	2.68	1.34	0.40	0.63	0.64	0.921	0.363
AM-SSR25	5	2.23	1.12	0.60	0.52	0.54	1.000	-0.154
AM-SSR26	3	2.30	0.99	0.54	0.59	0.62	0.997	0.085
AM-SSR27	4	2.80	1.12	0.85	0.63	0.67	0.996	-0.349

Where: (Na) No. of Different Alleles, (Ne) revealing No. Effective Alleles, (I) Shannon's Information Index, (Ho) Observed Heterozygosity, (He) Expected Heterozygosity, (uHe) Unbiased Expected Heterozygosity, while (F) Inbreeding coefficient and (Fst) against Fixation Index.

In Table 4 we evaluated the genetic characterization of the 27 polymorphic genic-SSR markers. Among them, eleven SSR markers were the most significant polymorphism markers with a high value of the fixation index (*Fst*). Highly informative of fixation index value (*Fst*) was showed with SSR markers such as AM-SSR 8, AM-SSR 16 and AM-SSR 23 (≥ 0.4), followed by AM-SSR 11, AM-SSR 23, AM-SSR 24, AM-SSR 22 and AM-SSR 18 (≥0.3), these markers are suitable for species identification and genetic evaluation of citrus germplasms. It should be noted that, the high value of the fixation index because of the significant values of the three-parameters, heterozygosity (*Ho*), expected heterozygosity (*He*) and unbiased expected heterozygosity (*uHe*), respectively. The overall number of amplicons shown for LTR-REMAP and SSR was quite high, being 300 and 218, respectively. Among them, 297 and 217 polymorphic amplicons were verified within LTR-REMAP and SSR markers, respectively. As a result, the total number of effective alleles (*Ne*) was correlate positively with the average number of polymorphic amplicons per assay unit (*np/U*), the number of allele /assay unit (*nu*) and the effective number of patterns

TABLE 5. Levels of polymorphism and PIC value ingenerated by LTR-REMAP and SSR assays for 20 citrus genotypes.

Index with their abbreviations		Marker systems	
		LTR-REMAP	SSR
Number of assay units	*U*	21	27
Number of monomorphic amplicons	*nnp*	3	1
Number of polymorphic amplicons	*np*	297	217
Average number of polymorphic amplicons /assay unit	*np/U*	10.24	8.03
Number of alleles	*L*	300	218
Number of allele/assay unit	*nu*	10.34	8.07
Total Banding pattern	*Bp*	293	203
Effective number of patterns/ assay unit	*p*	10.15	7.51
Total number of effective alleles	*Ne*	2677.50	1534.50
Minimum Range of PIC value	*Min. PIC*	0.770	0.845
Maximum Range of PIC value	*Max. PIC*	0.990	0.995
Average of PIC value	*Avg. PIC*	0.976	0.965

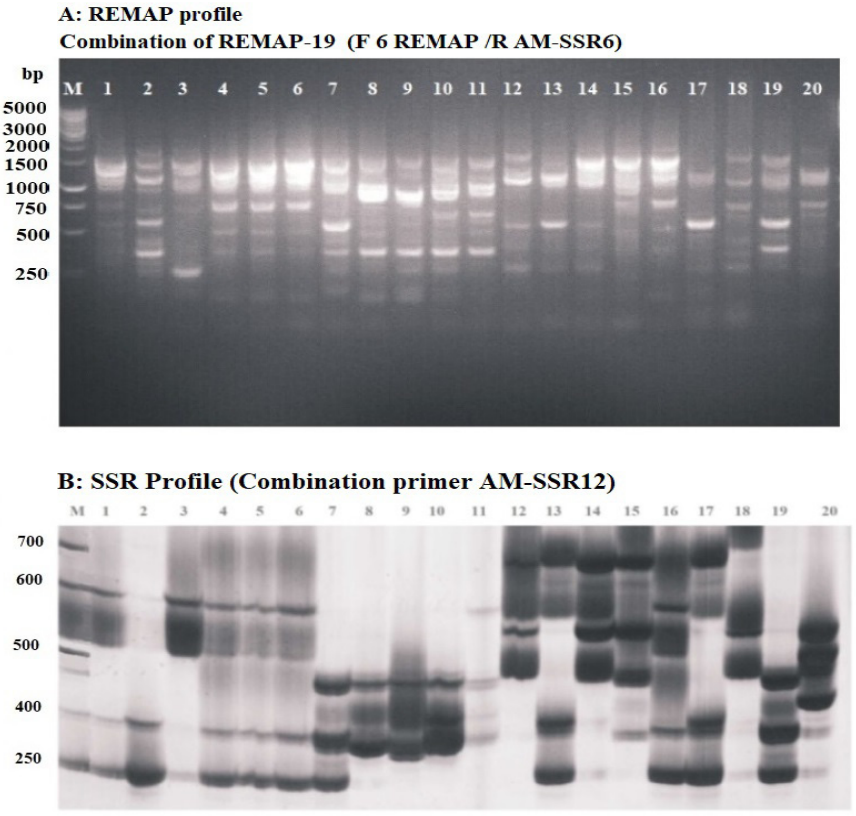

Fig. 1. Observed the LTR-REMAP (A) and SSR (B) profiles of Citrus species and its relatives.

assay unit (*p*-value). All these parameters counted high values of (*Ne*), (*np/U*) (n_u) and (*P*) were recorded within LTR-REMAP than SSR markers, as presented in Table 5. Contrasting to the PIC value, LTR-REMAP and SSR were relatively high, being 0.97 and 0.96, respectively.

Discriminating capacity of LTR-REMAP and SSR markers

A comparison summary of the discriminating capacity of LTR-REMAP and SSR markers are presented in Table 6 and Fig.2. Expected heterozygosity (*He*) of the polymorphic loci for LTR-REMAP and SSR was relatively high, 0.97 and 0.96, respectively. In contrast, the highest effective number of alleles per locus was 43.47 for LTR-REMAP, while SSR accounted the lower value being of 29.41. This certainly due to the higher values of the (*He*) for LTR-REMAP and SSR markers. Meanwhile, the exceptionally high values of *Ai* (92.32), *EMR* (10.24) and *MI* (9.99) for LTR-REMAP offers the notable characteristics of this marker. This is as a result of the concurrent discovery of multiple polymorphic markers per single reaction.

In this revised, the relatively high values of the effective number of patterns per assay units for the two markers used give confirmation of their discrimination capacity when handling a large number of samples. This trend is necessary for the germplasm banks certification, when many species require to be correctly characterized and identified (Belaj et al., 2003). In this study, the effective number of patterns per assay tracking the method: LTR-REMAP > SSR. This result inferred that the LTR-REMAP is more helpful evidence for *Citrus* species identification and certification compare to the SSR. This is seems caused by the distinction of the LTR-REMAP marker system. In subsequent studies, (Biswas et al., 2010a: Du et al., 2018) described that LTR-REMAP markers exhibited higher levels of heterozygosity due to the high copy number of effective alleles and its widespread distribution of LTR in citrus. The moderate value of the effective number of alleles per locus for SSR markers in comparison to LTR-REMAP may suggest the presence of many unique or less frequent alleles (Biswas et al., 2010a,b and Amar et al., 2011). Mainly, the marker index (MI) is a convenient value for marker efficiency (Belaj et al., 2003 and Amar et al., 2011). By this criterion, arithmetically 1.28 fold greater MI estimated for LTR-REMAP against to SSR, remarkable the unique character of the LTR-REMAP assay. This is certainly owing to the superior value of assay efficiency index (*Ai*) and effective multiples ratio (*EMR*) (Belaj et al., 2003 and Biswas et al., 2010a). Many studies confirmed that the LTR-REMAP marker had a superior discrimination capacity and have ability to discover more polymorphic locus per individual reaction (Biswas et al., 2010b). Recently, (Du et al., 2018) recommended that LTR-RTs occupied 28.1 Mb of the genome sequence, accounting for 9.74% of the whole genome. These results suggested that LTR-REMAP had an abundant presence of Ty-1 copia retrotransposons, which allow obtaining advantageous polymorphism between the tested genotypes of citrus germplasm. Our finding revealed that LTR-REMAP markers had several unique private loci that would enable differentiation within the sub-species of citrus and its relatives, which is in concurrence with earlier reports of citrus germplasm.

TABLE 6. Summary statistics of the information obtained and discriminating capacity of LTR-REMAP and SSR markers in 20 Citrus genotypes.

Index with their abbreviations		LTR-REMAP	SSR
Average of the allele frequency	*pi2*	0.023	0.034
Effective number of alleles per locus	*ne*	43.47	29.41
Expected heterozygosity of the polymorphic loci	*He*	0.977	0.966
Fraction of polymorphic loci	*β*	0.990	0.995
Expected heterozygosity	*Hep*	0.003	0.004
Assay efficiency index	*Ai*	92.32	56.83
Effective multiples ratio	*EMR*	10.24	8.03
Marker Index	*MI*	9.99	7.75

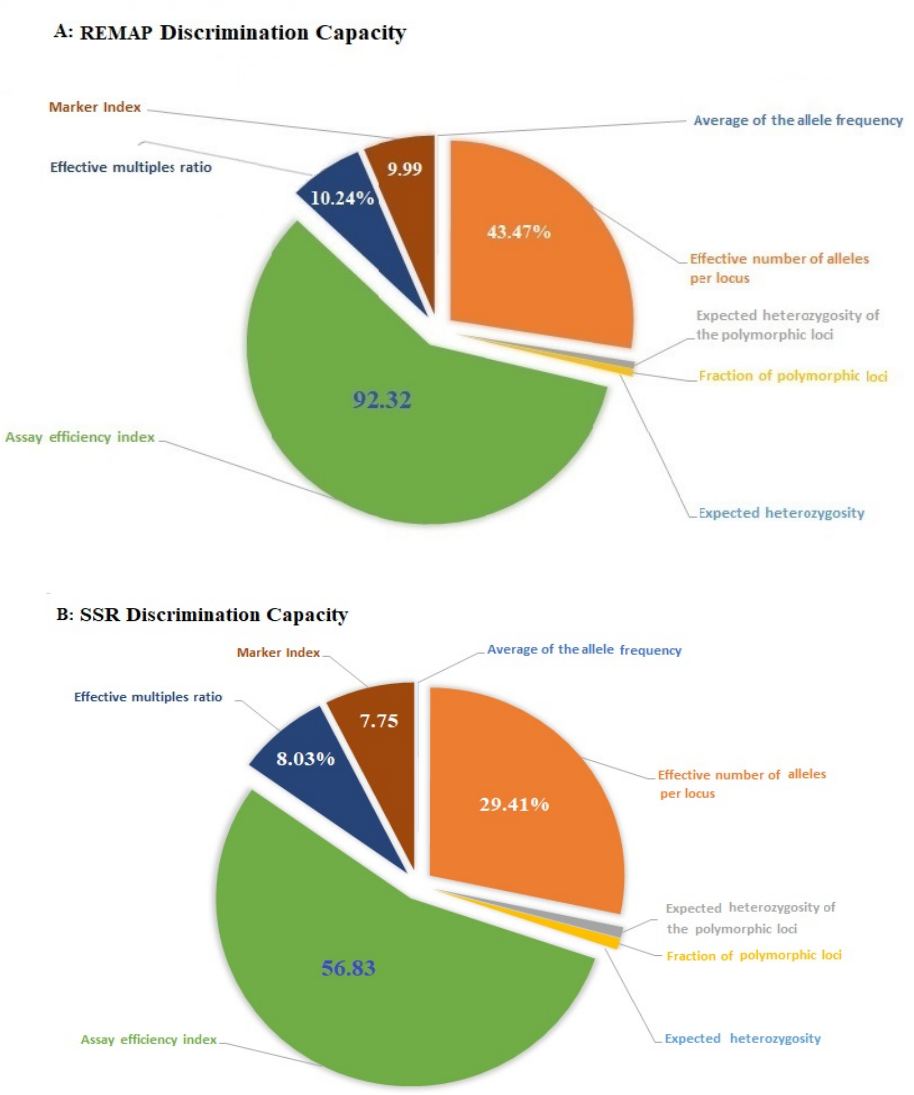

Fig. 2. Observed the comparison information obtained and the discrimination capacity of (A) LTR-REMAP profile and (B) SSR profile among Citrus species and its relatives.

Diversity analyses and phylogenetic heatmap

LTR-REMAP and SSR markers generated a comparable average of genetic distances. However, all genotypes could be differentiated by each of the molecular markers. Here, we present a graphic demonstration of both PCA and HCA, applying variable information matrix as input, wherever various dimensions of LTR-REMAP and SSR markers data are measured in several observations. The PCA-LTR-REMAP plot data as presented in (Fig. 3a) formed four relatively clustered groups, with a total of 60% of the molecular variance (PC1 - 36.6 %, PC2- 23.4 %).

Cluster I compressed all species of the acidic group besides to meiwa kumquat (*Fortunella crassifolia*), while cluster II involved mandarin and orange species with a closer relationship than other groups. Moreover, pummelos & grapefruits species were places jointly as cluster III, meanwhile, the sour orange species (daidai and bitter orange) were separated individually near the zero values of the axis. As for showing in Figure (3b), the PCA-SSR plot formed three relatively clustered groups, respectively, account for 60% of the total molecular variance in the data set (PC1 - 38.8 %, PC2- 21.2 %). Cluster

I compressed all species of the acidic group with a closer relationship than other groups, while cluster II assembled all mandarin and orange with sour orange species in a particular group. Meanwhile, pummelos & grapefruits species were places jointly as cluster III. The out-group species, kumquat (*Fortunella crassifolia*) was separated individually near the zero values of the axis.

Overall, the combined PCA data (Fig. 3c) was most compatible with the PCA-LTR-REMAP with the exception of the kumquat position which was independent alone near the axis of the zero values. Herein, each species was clustered based on their groups formed four strongly supported groups, which were distinguishable among the sub-tribe of Citreneae based on the combined data of LTR-REMAP and SSR.

To further determine the genetic diversity, HCA displays the abundance of the relationships between the 20 genotypes of citrus and its congener species. The distribution of hot points suggests significant differences between the major groups of the Rutaceae and able to cluster in a sub-clade. As a result, the two HCA tree was constructed based on data obtained from the LTR-REMAP and SSR data. The results were similar to each other with a little modification in the positioning of some genotypes, e.g., Egyptian acidic, sour orange and kumquat.

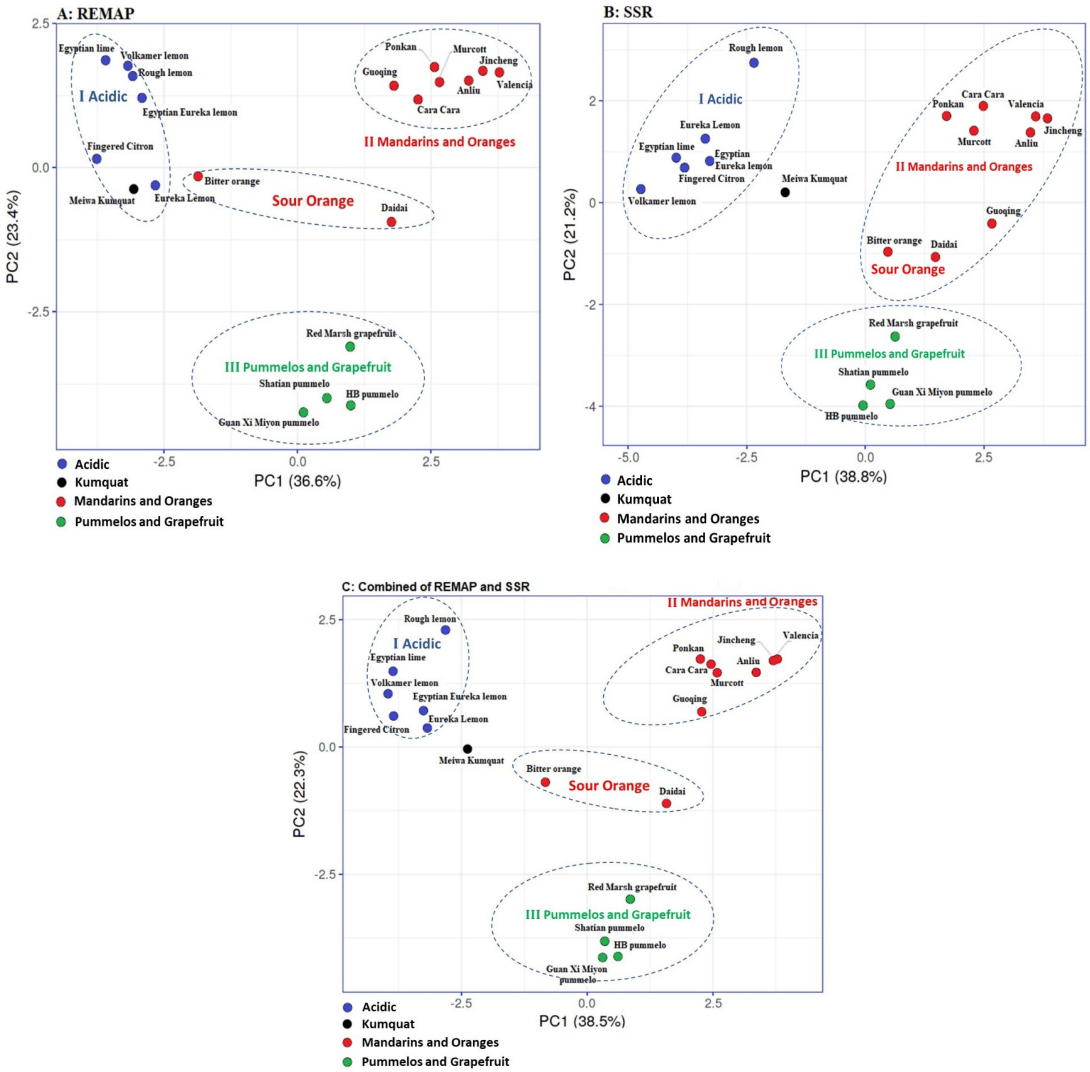

Fig. 3. Schematic representation the principal coordinates analysis of 20 *Citrus* and its relatives. (A) correspond to LTR-REMAP profile, (B) correspond to SSR profile and (C) referred to combined analysis of LTR-REMAP and SSR, while PC1 and PC2 refer to the first and second principal component, respectively.

A combined HCA was constructed based on the collective data of the two sets of LTR-REMAP and SSR markers (Fig. 4). Overall, six confirmed clades were identified, which have the ability to clearly differentiate among the twenty citrus species.The acidic species were formed into two monophyletic clades, not based on their type but on their sampling origin, where Egyptian acidic are separated with high portions of the other acidic species. In details, the first clade assembled *C. limon* (Rough Lemon and Eureka Lemon), and *C. medica* (Fingered Citron). While the second clade occupies all Egyptian acidic species, ***C. volkameriana*** (Volkamer Lemon), C. aurantifolia (Egyptian Lime) and C. lemon (Egyptian Eureka lemon) with a high proportion of close relationships. Whereas, *Fortunella crassifolia* (Meiwa Kumquat) was separated individually as an out-group of the all acidic species. Furthermore, two monophyletic clades shared all orange and mandarin species, where, *C. sinensis* (Cara Cara, Anliu, Jincheng, and Valencia) and *C. reticulata* (Ponkan, Murcott and Guoqing) are placed jointly in the third and fourth clades, respectively. However, *C. paradisi* (Red Marsh grapefruit) and *C. grandis* (HB pummelo, Shatian pummelo and Guan Xi Miyon pummelo) were excluded together in the fifth clade. Meanwhile, the two sour orange species of *C. aurantium* (Bitter orange and Daidai) were located at the basal position in the last clade showed a close genetic relationship to pummelo and grapefruit.

Overall, the above result revealed that the two markers had different discrimination power, a co-dominant SSR marker can differentiate within the group level only, while a dominant marker LTR-REMAP have demonstrated the inherent efficiency to discriminate within the group level in addition to species level with particular emphasis on the Egyptian citrus. Collectively, we found both PCA and HCA of the combined data have drawn a successful annotation relationship in Citreneae species and related genera to evaluate whether the specific cluster sort separate or overlap groups.

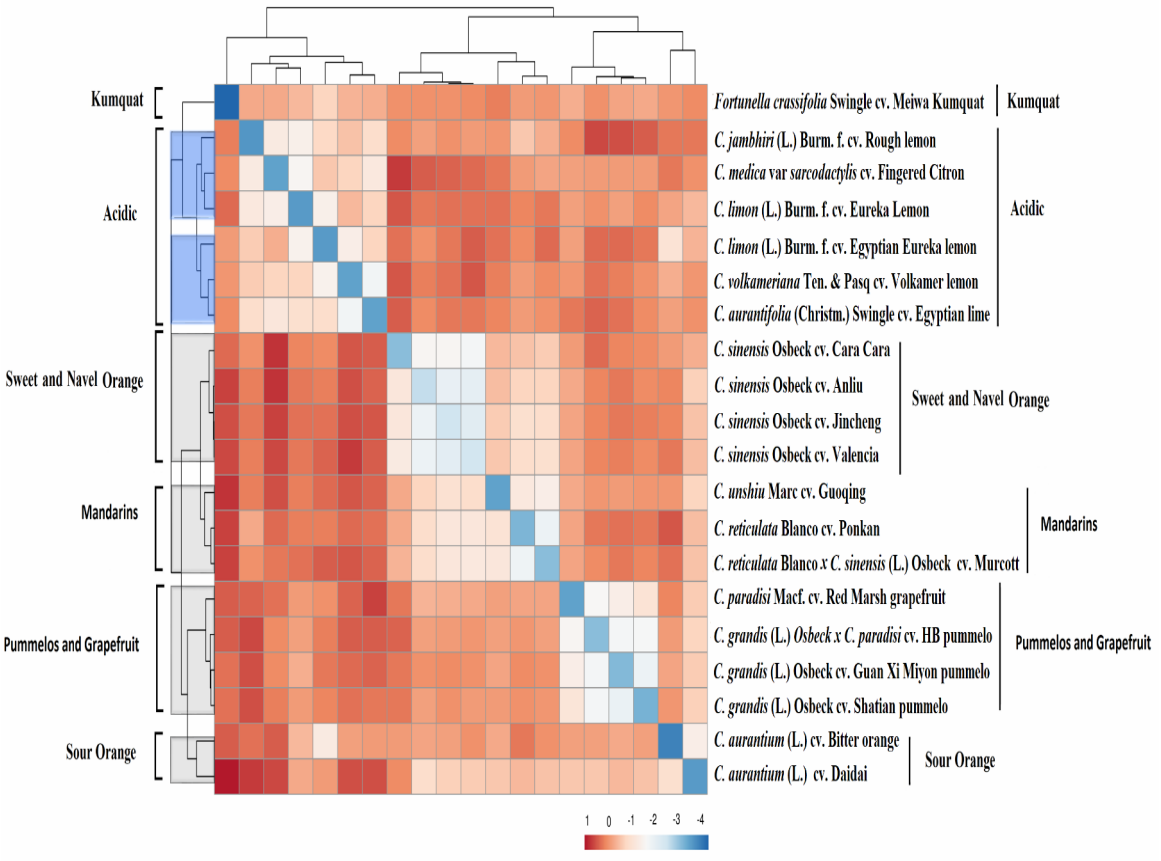

Fig. 4. Heatmap cluster analysis (HCA) signatures among 20 genotypes of Citrus and its relative's species using the combined data of LTR-REMAP and SSR profiles. Subclades are highlights by a colorful background, the scale bar showed on the bottom illustrates the relative genetic variability from 1 to -4.

Classification of the citrus germplasm in Egypt is critical to the helpful application of innovative citrus development approaches to this product. In the last decade, several new species of citrus imported from China without any genetic history or phylogenetic and conservation. This study was performed for identification of a few unique Egyptian citrus species (Acidic), was not tested anywhere else before, along with to investigate the comparative phylogenetic relationship with several important Chinese citrus species. Towards this effort, the implementation of retro-transposons and microsatellite techniques in plant genomic would permit the breeding programs to achieve unique molecular markers and are effective in the taxonomy and phylogeny of the genus Citrus. It is well known that, citrus phylogeny and taxonomy are often the subjects of controversy and the main problem in citrus breeding, due to their long history of cultivation, the high diversity of phenotypic variation and weak information of the genealogy of complicated admixture in reproduction system and cultivated citrus (Wu et al., 2018). In view of the performance of our results, the separation of the three true *C. reticulata*, *C. grandis* (*C. maxima*) and *C. medica* in distinct clades or subclades in our LTR-REMAP and SSR analyses supports their superiority as the real or primary cultivated species of citrus. This theory achieved more acceptance through previous molecular studies (Uzun & Yesiloglu, 2012, Amar et al., 2014, Shimizu et al., 2016 and Curk et al., 2016).

Previously, evidence suggested *C. lemon* is a complicated hybrid parent sharing citron and lime (Swingle 1943, Malik et al., 1974 and Scora, 1975), or sour orange and citron (Nicolosi et al., 2000), or sour orange and lime (Torres et al., 1978). The recent article of cytoplasmic and nuclear data of Curk et al., (2016), point to *C. medica* seem to be the directly male parent of lemon and lime, this due to the shared genomic structure. In our results, *C. limon* (Eureka lemon) classified with *C. medica* (Fingered citron) and *C. jambhiri* (Rough lemon), proves their potential mixture origin, as previously recommended by many phylogenetic studies (Amar et al., 2014 and Wu et al., 2018).

Volkamer lemon is one of the most promising rootstocks in the Egyptian acidic, due to their tolerance to many biotic and abiotic stresses. Apparently, it is a more controversial origin (Curk et al., 2016). Earlier authors considered that the ancestors of Volkamer lemon are thought to be the sour orange and lemon (Nicolosi et al., 2000). A possible originated from mandarin x sour orange (Barrett & Rhodes 1976), or mandarin x citron origin was also suggested (Carvalho et al., 2005). Evidence suggested *C. medica* was the male parent of *C. volkameriana, C. aurantifolia, C. jambhiri,* and Palestine lime (Ollitrault et al. 2003 and Curk et al., 2016). Based on our PCA data, we propose that Volkamer lemon is classed with the Egyptian *C. limon* and *C. aurantifolia* as the taxon with which it appears to be most closely affiliated. Indeed, our findings were parallel with the recent hypothesis of nuclear and cytoplasmic data indicate quite distant between volkamer' lemon, citron, 'rough' lemon and 'rangpur' lime (Snoussi et al., 2012, Penjor et al., 2013 and Curk et al., 2016).

Similarly, it is worth noting that sour orange (*C. aurantium*) was the main standard rootstock for citrus germplasm (Scora, 1975 and Barrett & Rhodes, 1976) and thought an offspring of *C. grandis*, *C. reticulata* and *C. medica* (Shimizu et al., 2016). In this study, the bitter orange and daidai which are all considered to be sour orange (*C. aurantium*), all clustered together with lightly associated with grapefruit and pummelo. These results in accordance with SSR, InDel (Insertion and Deletion) markers and the recent genomic data, boosted the hypothesis that sour oranges are natural hybrids of a mandarin and a pummel (Barkley et al., 2006, Shimizu et al., 2016 and Wu et al., 2018). Another striking characteristic, the PCA-LTR data provides convincing evidence in supporting that pummelo appeared as the female parent of sour orange, and acidic being the paternal parent. This view was supported by the hypothesis that sour orange showed the contribution of bitter orange and acidic, like the results by Barkley et al. (2006) and Bayer et al. (2009).

Our data assume a close genetic relationship between *C. reticulata* (mandarin) and *C. sinensis* (sweet orange) highlight that *C. reticulata* was sharing between sweet orange and mandarins, concordant with the view of Barrett & Rhodes, (1976). Several earlier reports support this opinion as *C. reticulata* was evolutionarily close with *C. sinensis* and suggested that sweet orange and citron as may be female and male parents, respectively (Nicolosi et al., 2000). Parallel results were also found in the recent reclassification of citrus origin (Wu et al., 2018), confirming that among mandarins and sweet orange, they found abroad association of relatedness that explains the domestication of these groups.

The grapefruit (*C. paradisi*) was recommended as a natural hybrid between pummelo and sweet orange (Gmitter et al., 2012). It appears that it has more resemblance with pomelo compared to sweet orange in phenotyping and biochemical formation, which reveals a hybrid with pummelo in one of its parents. Our study was evolutionarily close grapefruit with pummelo with a small apportion of sweet orange, supporting the view of a backcross to pummelo with sweet orange. Our data confirm this hypothesis since the grapefruit genotypes show identity with all pummelo species, concordant with the recent nuclear genome sequence (Shimizu et al., 2016), supporting the perspective that origins of grapefruit (a pummelo–sweet orange hybrid).

C. grandis, usually known as pummelo, is considered to be a real *Citrus* species (Scora, 1975 and Barrett & Rhodes, 1976), which provided rise to grapefruits and sour oranges via hybridization. According to the interpretation of the recent genomic data (Wu et al., 2018), the initial pummelo introgression within the mandarin gene pool, then the influence of which was reduced by repeated backcrosses with mandarins. Later, further pummelo introgressions did rise to sweet orange and mandarins. Indeed, the pummelo genome (*C.grandis*) has been a part of the parentage of several citrus cultivar's (Barkley et al., 2006). This outcome proves that *C. grandis was* the maternal parent of *C. lemon, C. aurantium*, *C. sinensis* and *C. paradisi.* This is consistent with the previous finding of the phylogeny evolutionary studies of pummelo (Tanaka, 1977, Nicolosi et al., 2000 and Penjor et al., 2013) and the chemotypes diversity (Dugrand-Judek et al., 2015), highly supported that grapefruits and sour oranges are direct descendants of pummelos.

Kumquats belong to the genus *Fortunella,* it highly looks like *Citrus* species, although their phenotype is extremely diverse. Evidence suggests that fortunella was the primary genus, while citrus seem to be the top phase of evolution (Bayer et al., 2009 and Amar et al., 2014). In our results, it is worth notice that *Fortunella* was nested within the citrus clade. This is in concurrence with earlier reports proposed that *Fortunella* has mixture origin involved in the genus *Citrus* and separate exclusive as a genus (Penjor et al., 2013, Amar et al., 2014 and Wu et al., 2018).

Conclusion

Microsatellite and retrotransposons represent a major component of the structural evolution, varying greatly in copy number within the plant genome. To facilitate such purposes, here we report a detailed overview of the ability, effectiveness and discriminating power of SSR and LTR-REMAP markers approach in the genus *Citrus* and related species. A dominant marker LTR-REMAP was more sensitive and could discriminate at low taxonomic levels, especially for Egyptian acidic, while a co-dominant SSR marker can differentiate within the group level of citrus.

The PCA and the HCA have drawn a successful annotations relationship in Citreneae species, support the monophyletic nature and provide unambiguous identification or overlapping clusters of real species and related hybrids like lime, lemon, citron, sour orange, grapefruit, mandarin, sweet orange, pummelo and fortunella, resulted in their placement in different clades. This article may be will offer a useful and potential additional knowledge for breeding programs and conservation approaches in the genus *Citrus* and its relative species with particular emphasis on the Egyptian citrus.

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Conflicts of interest

No potential conflict of interest was reported by the authors.

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التقييم الوراثى لبعض الموالح المصرية المنزرعة والانواع ذات الصلة باستخدام المايكروستلايت ، الرتيروترانسبوزون

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تنتمي الموالح الى عائلة Rutaceae ، وهي واحدة من أهم محاصيل أشجار الفاكهة في المناطق المعتدلة. إن التصنيف والتقسيم النباتى في جنس الحمضيات معقد للغاية ، والعدد الدقيق للأنواع الطبيعية غير واضح. لتحقيق هذا الجهد ، هنا تمثل المايكروستلايت ، الرتيروترانسبوزون أحد أكثر الجوانب فعالية لتقييم التنوع الوراثي في الجينوم النباتي. في هذا البحث نقدم نظرة عامة ومفصلة باستخدام LTR-REMAP 21، SSR 27 من حيث القدرة التمييزية والكفاءة والقدرة على التباين الوراثي بين عشرون نمط وراثي في جنس الحمضيات. لقد سجل LTR-REMAP 1.28 أضعاف قيمة مؤشر كفاءة الماركر (MI) مقارنة مع SSR، مما يسلط الضوء على الطبيعة المميزة لتقنية LTR-REMAP ،هذا الماركر أكثر حساسية وقدم مزيدًا من الأدلة للتمييز عند مستويات التصنيف المنخفضة ، خاصة بالنسبة للحمضيات المصرية ، في حين أن SSR يمكنها التفريق على مستوى المجموعات فقط. ومن المثير للاهتمام أن بيانات تحليل (HCA) وتحليل (PCA) رسمت مجموعة توضيحية دقيقة بين أنواع الموالح ، وتقدم تحديدًا لا التباس فيه دون تداخل ما بين المجموعات وبين الأنواع الحقيقية والانواع الهجينة ذات الصلة ، مما أدى إلى وضعها في أطوار تصنيفية محددة. لتسليط الضوء على الحمضيات المصرية ، كان هذا أول تقرير مفصل يمثل دورًا أساسيًا في شرح أداء التنوع الجيني إستنادا إلى المايكروستلايت، الرتيروترانسبوزون. تحقيقًا لهذه الغاية ، سيساعد إستخدام التقنيات الجزيئية في التغلب على معوقات تحديد الأصناف والتنوع الوراثي في تربية الحمضيات