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Genetic Variation of Lime (*Citrus sp.*) Accessions Using Flow Cytometry Technique, Morphological Characteristics and Molecular Markers

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Abstract
Because of sexual propagation of Mexican lime in southern regions of Iran, there are many lime accessions (lime biotypes) in Mexican lime gardens in which appear some variation in fruit and tree shape. However, most of these accessions are susceptible to witches broom disease of lime (WBDL). Persian lime (*C. latifolia* Tanaka) is a triploid WBDL-tolerant species. Considerable number of un-described lime biotypes have been cultivated by farmers as Persian lime with no documented evidence related to their origin and genetic background. To unveil systematic modality of the lime accessions, we investigated 18 lime accessions collected from Fars, Hormozgan and Mazandaran provinces (Iran) using flow cytometry (FCM) approach, morphological characteristics, ISSR and SSR markers in 2016. The results of FCM revealed that 14 out of 18 lime accessions were triploid. Triploid accessions showed higher leaf dimension values together with lower stomata and secretory sacs density compared with diploid accessions. Application of SSR markers confirmed the obtained results of ploidy level determination using FCM and morphological analysis. Results of morphological and ISSR markers demonstrated that four out of 18 accessions were different from others and it was in correspondence with the results of FCM. SSR results also grouped the accessions in line of FCM. In conclusion, it has been shown that IFJKh, Cucumber-shaped lime and IFJKMes accessions, are diploid and their cultivation is not recommended in the infected regions to WBDL phytoplasma.

Keywords: DNA index, ISSR, Secretory sacs, Tahitian lime, Triploid.

Introduction

Persian lime (PL) (*Citrus latifolia* Tanaka) is a triploid and seedless hybrid of citrus widely cultivated in India, Mexico, China, Argentina, and Brazil (Cantuarias-Aviles et al., 2012). Persian lime has been specifically considered in the recent years due to its tolerant response to witches broom disease of lime (WBDL) phytoplasma (Salehi et al., 2005). Curk et al. (2016) manifested that Persian lime accessions originated from

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fertilization of a haploid ovule of *C. limon* by a diploid gamete of *C. aurantifolia*. Polyploid plants often display significant characteristics like drought tolerance, apomixis, pest resistance, and biomass production (Shahriari-Ahmadi et al., 2008).

From the practical aspects, the analysis of genome content and chromosome karyotyping provide additional information that support better understanding of the genomic evolution in polyploid plants. Flow cytometry (FCM) method has been used for analysis of DNA contents of genomes and specific chromosomes and chromosomal karyotyping (Lee et al., 2004). FCM is considered as a valuable and rapid method for determination of the DNA content and ploidy levels of the plants in which measurement of the statistical distribution of DNA content in a large population of nuclei is possible (Seker et al., 2003). Investigation of the stomatal and secretory sac frequency is an alternative approach which has been used in ploidy determination (Padoan et al., 2013; Afshar-Mohammadian et al., 2013). In addition, SSR markers have been introduced as a useful approach to confirm triploidy of some citrus species and other fruit trees such as Persian lime (Sharafi et al., 2016), Oroblanco hybrid (Ahmad et al., 2003) and Loquat (Watanabe et al., 2008).

DNA markers have been extensively used to study phylogenetic relationships in many plants (Golein et al., 2012). The inter-simple sequence repeat (ISSR) markers are dominant markers which have been used profusely in citrus identification. ISSRs have been used substantially in citrus studies (Sharma et al., 2015). It has been also applied for discrimination among very genetically close cultivars (Tripolitsiotis et al., 2013).

Competitive potential of Persian lime with Mexican lime and it's tolerance to WBDL phytoplasma, is the most important reason for tendency toward increasing Persian lime cultivation in south of Iran. According to the triploid nature of Persian lime, determination of ploidy levels of the similar accessions to this citrus species can be used as an approach to determine their originality. In the current study we aimed to study the ploidy levels and genetic diversity of various accessions of lime species which are morphologically similar to Persian lime and determine their originality.

Table 1. The evaluated diploid and triploid citrus accessions.

Code	Accession	Genotype name	Abbreviation	Location	Latitude (N)	Longitude (E)
1	IFDF	*Citrus* sp.	IFDF	Fars-Darab	28°19′32′′	55°11′38′′
2	IFD	*Citrus* sp.	IFD	Fars-Darab	28°44′58′′	54°32′59′′
3	IFJAb	*Citrus* sp.	IFJAb	Fars-Jahrom	28°31′14′′	53°40′40′′
4	IHRA1	*Citrus* sp.	IHRA1	Hormozgan-Rudan	27°37′06′′	57°11′30′′
5	IHRA2	*Citrus latifolia*	IHRA2	Hormozgan-Rudan	27°37′04′′	57°11′30′′
6	IHRA3	*Citrus* sp.	IHRA3	Hormozgan-Rudan	27°37′02′′	57°11′30′′
7	IFJAm	*Citrus* sp.	IFJAm	Fars-Jahrom	28°32′32′′	53°36′30′′
8	IFJK	*Citrus* sp.	IFJK	Fars-Jahrom	28°29′13′′	53°34′48′′
9	Tahiti lime (standard triploid)	*Citrus latifolia*	TL	Hormozgan-Minab	27°06′27′′	57°05′39′′
10	Mexican lime (standard diploid)	*Citrus aurantifolia*	MX	Hormozgan-Rudan	27°37′02′′	57°11′29′′
11	Deperse lime	*Citrus latifolia*	DepL	Hormozgan-Minab	27°06′27′′	57°05′39′′
12	IFJKMes	*Citrus* sp.	IFJKMes	Fars-Jahrom	28°29′13′′	53°34′46′′
13	IFJKh	*Citrus* sp.	IFJKh	Fars-Jahrom	28°32′32′′	53°36′32′′
14	Cucumber-shaped lime	*Citrus* sp.	CuL	Fars-Darab	28°44′58′′	54°32′59′′
15	IAC	*Citrus latifolia*	IAC	Mazandaran-Ramsar	36°54′26′′	50°39′22′′
16	IFJAn1	*Citrus* sp.	IFJAn1	Fars-Jahrom	28°27′32′′	53°31′13′′
17	IFJAn2	*Citrus* sp.	IFJAn2	Fars-Jahrom	28°27′32′′	53°31′12′′
18	Bears lime	*Citrus latifolia*	BL	Mazandaran-Sari	36°38′09′′	53°11′48′′

Materials and Methods

This study was performed in completely randomized design with 18 accessions including 14 lime accessions similar to Persian lime (*C. latifolia* Tanaka), Mexican lime (*C. aurantifolia* Swingle) (as diploid standard plant), Cucumber-shaped lime (*C.* sp.) and two unknown genotypes namely IFJKh and IFJKMes, in Agricultural and Natural Resources Research and Education Center of Hormozgan in 2016 (Table 1). Young and mature stages of the leaf samples were collected from Hormozgan, Fars and Mazandaran provinces of Iran.

Stomata and secretory number density were evaluated in twelve leaves of each accession, according to the mentioned procedures by Golein et al. (2015) and Afshar-Mohammadian et al. (2013), respectively.

In order to perform FCM analysis FCM device Partec PA, Germany equipped with an arc-UV lamp based on Gu et al. (2005) procedure with partial changes was used. Three replications were applied for FCM analysis. Ploidy levels were determined using DNA index (DI), which calculated according to the following formula (Hosseini et al., 2015):

$$DI = \frac{\text{Mode of the G1 DNA peak of a sample}}{\text{Mode of the G1 DNA peak of a diploid standard}}$$

On this basis, DNA index of the diploid samples should be ≤1.000. According to the known DNA content of Tahiti lime (1.170 pg) as an evident sample (Seker et al., 2003), DNA content was calculated for each sample in conformity with the below formula:

$$\text{DNA content}(\text{pg}) = \text{triploid standard DNA content} * \left(\frac{\text{unknown sample G1 peak mean}}{\text{triploid standard G1 peak mean}}\right)$$

where, triploid standard DNA content and G_1 peak were 1.170 pg and 66.91, respectively. Based on DNA content of triploid standard sample/samples DNA content (DNA content ratio), the accessions were classified into the diploid and triploid group. In this manner, the samples in which DNA content ratio was equal to 1.396≤ were grouped as diploid and the others were categorized as triploid samples (Seker et al., 2003). Statistical analysis was carried out using SAS 9.1 software and the means were compared using the protected LSD test ($p < 0.01$).

Morphological attributes study was done by using IPGRI (1999) descriptor list for five trees of each accession. The morphological attributes were including branch angle, density of branches, tree shape, shoot tip color, spine density, spine shape, leaf lamina shape, leaf lamina margin, leaf apex, leaf length, leaf width, leaf length/width ratio, petiole length, petiole wing situation, petiole wing width, petiole wing shape, color of flower, number of petal, petal length, petal width, pedicle length, calyx diameter, color of anther, anther/stigma ratio. Morphological dendrogram constructed using NTSYS software based on UPGMA algorithm.

Total genomic DNA was isolated from the young leaves using the modified EDWARD method (Edward et al., 1991). A total of 14 ISSR primers previously evaluated by other researchers (Tripolitsiotis et al., 2013) were used. ISSR amplification reactions were prepared to a final volume of 10 µl [25 ng of template DNA, 0.2 mM dNTPs, 0.5 µM primer 1.0 µl of 10× PCR buffer, 1.5 mM of $MgCl_2$ and one unit of DNA Taq polymerase (Cinnagen, Iran)]. The amplifications were performed on a PEQStar 96 Universal Gradient 96 wells thermal cycler with reaction conditions programmed as initial pre-denaturation at 95°C for 5 min followed by 39 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 2 min. A final 7 min extension at 72°C followed the completion of 39 cycles. After amplification, the DNA fragments were separated by electrophoresis in 1.5% Agarose gel. The DNA stained using fluoroDye. DNA fragments were

visualized and documented with the help of Uvitec Geldoc system. The relative contribution of each attribute to the diversity among accessions was assessed by NTSYS software ver. 2.02. By scoring amplified fragments, ISSR products were translated to numerical data as either 1 (present) or 0 (absent) of a band. A pair-wise similarity matrix was constructed using Dice similarity. Dendrogram constructed using NTSYS software based on complete algorithm. Polymorphism information content (PIC) was calculated using the formula: PIC= 2*fi*(1-*fi*), where *fi* is the frequency of the amplified allele (present band), and (1-*fi*) is the frequency of the null allele (absent band).

SSR appraisal were done using two primers GT03 (F: GCCTTCTTGATTTACCGGAC, R: TGCTCCGAACTTCATCATTG) and AG14 (F: AAAGGGAAAGCCCTAATCTCA, R: CTTCCTCTTGCGGAGTGTTC). The PCR amplifications were performed in a total volume of 10 µl [PCR buffer (1×), 50 ng of genomic DNA, 0.2 mM of each dNTP, 0.5 µM of each forward and reverse primer, 0.2 unit of DNA Taq polymerase (Cinnagen, Iran) and 1.5 mM of $MgCl_2$]. The PCR program was: 94 °C for 5 min, 35 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min, ending with 72 °C for 7 min. The PCR products were separated on 6% denaturing poly acryl amide gel in TBE buffer (1×) (45 mM Tris- Boric, 1 mM EDTA, pH 8.0). The gels were stained with silver nitrate.

Results

The results of FCM analysis revealed that 14 lime accessions were triploid and the rest were diploid. Mean of histogram mode for standard diploid accession (Mexican lime) and triploid standard accession (Tahiti lime) were 47.94 and 66.91, respectively. The results of DNA content ratio, showed only IFJKMes, IFJKh and Cucumber-shaped lime accessions were diploid and the others studied accessions were triploid. In addition, diploid nature of the mentioned accessions was confirmed based on DNA index parameter (Table 2).

Table 2. Results of FCM analysis in the evaluated accessions including DNA content, DNA content ratio, DNA index, ploidy levels and FCM mode and mean outputs

Genotype	Mode	Mean	DNA content (pg)	DNA content ratio †	DNA index ††	Ploidy level
IFDF	69	68.75	1.202	0.854	1.438	3x
IFD	69	69.40	1.214	0.964	1.438	3x
IFJAb	73	73.78	1.290	0.907	1.521	3x
IHRA1	64	62.63	1.090	1.073	1.333	3x
IHRA2	65	63.89	1.117	1.047	1.354	3x
IHRA3	63	62.54	1.094	1.070	1.313	3x
IFJAm	74	74.19	1.297	0.902	1.542	3x
IFJK	74	72.79	1.273	0.919	1.542	3x
TL	67	66.91	1.170	1.000	1.396	3x
MX	48	47.94	0.838	1.396	1.000	2x
DepL	65	64.55	1.129	1.037	1.354	3x
IFJKMes	48	46.30	0.810	1.445	1.000	2x
IFJKh	43	43.45	0.760	1.540	0.896	2x
CuL	44	43.68	0.764	1.532	0.917	2x
IAC	66	65.72	1.149	1.018	1.375	3x
IFJAn1	71	70.59	1.234	0.948	1.479	3x
IFJAn2	75	73.69	1.289	0.908	1.563	3x
BL	64	59.00	1.032	1.134	1.333	3x

†DNA content ratio: proportion of triploid accession (Tahiti lime) DNA content to the studied accessions.
††DNA index: proportion of the studied accession mode to diploid accessions (Mexican lime) mode.

Table 3. Mean comparison of leaf dimensions, stomatal and secretory sacs density in the studies accessions

Accessions	Leaf length (mm)	Leaf width (mm)	Stomatal density (100x)	Secretory sacs density (4x)
IFDF	94.4^{def}	54.1^{c-f}	6.8^{gh}	32.3^{ef}
IFD	83.3^{f-i}	50.1^{efg}	7.7^{fgh}	33.7^{de}
IFJAb	90.1^{def}	56.1^{cde}	8.3^{fg}	23.0^{jkl}
IHRA1	111.0^{ab}	56.6^{b-e}	6.3^{h}	21.0^{l}
IHRA2	87.1^{d-g}	56.1^{cde}	7.7^{fgh}	21.7^{kl}
IHRA3	81.7^{f-j}	51.0^{d-g}	8.2^{fg}	28.0^{gh}
IFJAm	99.9^{bcd}	63.7^{ab}	8.2^{fg}	32.0^{ef}
IFJK	96.6^{cde}	59.7^{abc}	8.8^{ef}	24.3^{i-l}
TL	85.3^{e-h}	46.7^{g-j}	10.3^{de}	25.7^{g-j}
MX	73.7^{h-k}	39.7^{jk}	12.3^{bc}	47.0^{b}
DepL	83.1^{f-i}	47.1^{f-i}	9.2^{ef}	27.0^{ghi}
IFJKMes	58.6^{l}	39.0^{k}	13.0^{ab}	64.0^{a}
IFJKh	74.4^{g-k}	45.9^{g-k}	14.0^{a}	64.3^{a}
CuL	68.1^{kl}	40.0^{ijk}	12.0^{bc}	65.0^{a}
IAC	113.3^{a}	67.0^{a}	7.8^{fgh}	37.0^{d}
IFJAn1	100.0^{bcd}	57.3^{b-e}	9.2^{ef}	25.0^{h-k}
IFJAn2	109.0^{abc}	58.3^{bcd}	8.3^{fg}	29.0^{fg}
BL	119.7^{a}	67.0^{a}	7.7^{fgh}	29.0^{fg}
LSD	12.94	7.35	1.65	3.43

Means followed by different letters in columns are significantly different according to LSD test (P<0.01).

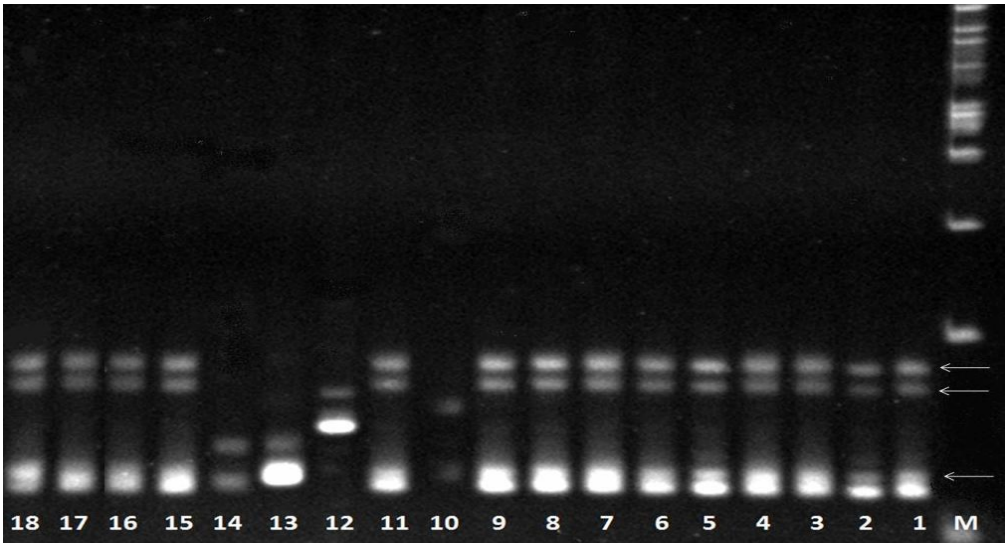

Fig. 1. SSR profiles amplified from DNA of 18 citrus accessions using primer GT03M. DNA size marker (100-3000 bp), 1: IFDF, 2: IFD, 3: IFJAb, 4:IHRA1, 5: IHRA2, 6: IHRA3, 7: IFJAm, 8: IFJK, 9: Tahiti lime, 10: Mexican lime, 11: Deperse lime, 12: IFJKMes, 13: IFJKh, 14: Cucumber-shaped lime, 15: IAC, 16: IFJAn1, 17: IFJAn2, 18: Bearss lime.

Based on the mean comparison results, the lowest leaf length and width were observed in IFJKMes, Cucumber-shaped lime, Mexican lime and IFJKh accessions. The triploid accessions showed the highest leaf length and width (Table 3). The maximum number of stomata secretory sac per unit leaf sample was observed in IFJKh, IFJKMes, Mexican lime and Cucumber-shaped lime (Table 3). Lime accessions similar to Persian lime significantly had less secretory sacs than the diploid accessions (Table 3).

According to the observed banding patterns of two SSR primers, three alleles were observed in triploid accessions (Fig. 1). These observations were accordance with determined ploidy level and grouping the accessions by FCM and morphological attributes.

Among the studied morphological

attributes, the accessions could categorize properly according to the leaf and flower traits. All accessions divided into two main groups based on Morphological-derived phylogenetic tree. So that, Mexican lime and IFJKMes clustered in the same group. The evaluated accessions were grouped into six clades, according to the Figure 2.

ISSR data showed that eight out of 14 primers were polymorphic (Fig 3). In total, 100 bands were produced in which 84 bands were polymorphic (Table 4). Molecular analysis clustered all accessions in two main groups: the first group including Mexican lime, IFJKMes, IFJKh, CuL, IFJAn1 and IFJAn2 and the known accessions of Persian lime including Deperse lime, Tahitian lime, Bearss lime and IAC and the second group contained all accessions propagated in south of Iran as Persian lime. According to the reference line, all accessions divided into six clades as shown in Fig. 4. Deperse lime, Bearss lime, Tahiti lime and IAC are well-known accessions among the studied PL accessions which have been introduced to Iran from other countries. The other PL accessions in the present study have been propagated by budding method using the collected scions from genetically-unknown mother plants cultivated in Fars province.

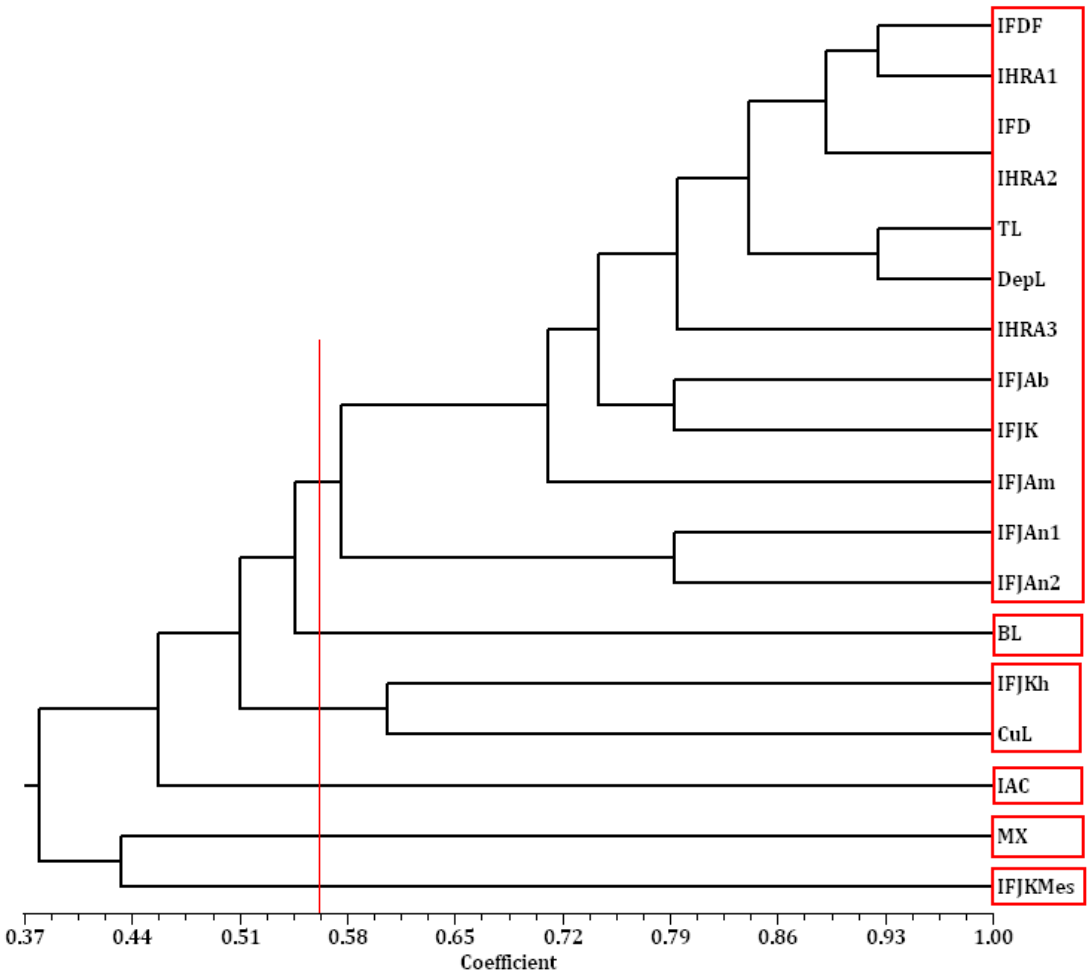

Fig. 2. Genetic similarity among 18 citrus accessions. The dendrogram generated using UPGMA clustering method based on Jaccard similarity matrix containing 26 morphological markers.

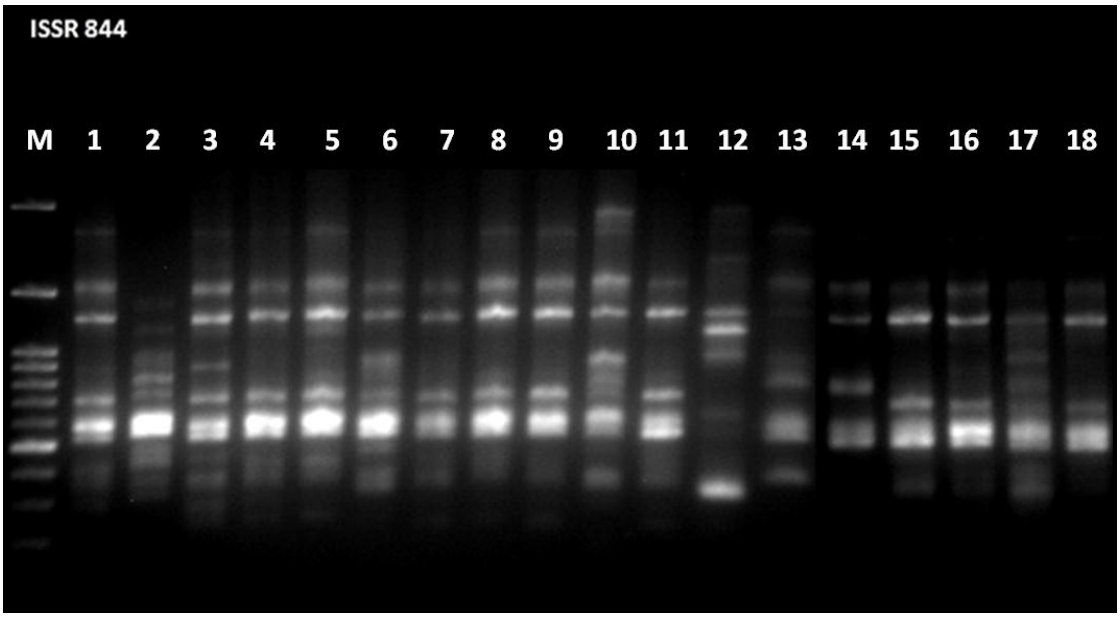

Fig. 3. ISSR profiles amplified from DNA of Citrus accessions using primer ISSR 844. M: DNA size marker (100-3000 bp), 1: IFDF, 2: IFD, 3: IFJAb, 4: IHRA1, 5: IHRA2, 6: IHRA3, 7: IFJAm, 8: IFJK, 9: Tahiti lime, 10: Mexican lime, 11: Deperse lime, 12: IFJKMes, 13: IFJKh, 14: Cucumber-shaped lime, 15: IAC, 16: IFJAn1, 17: IFJAn2, 18: Bearss lime.

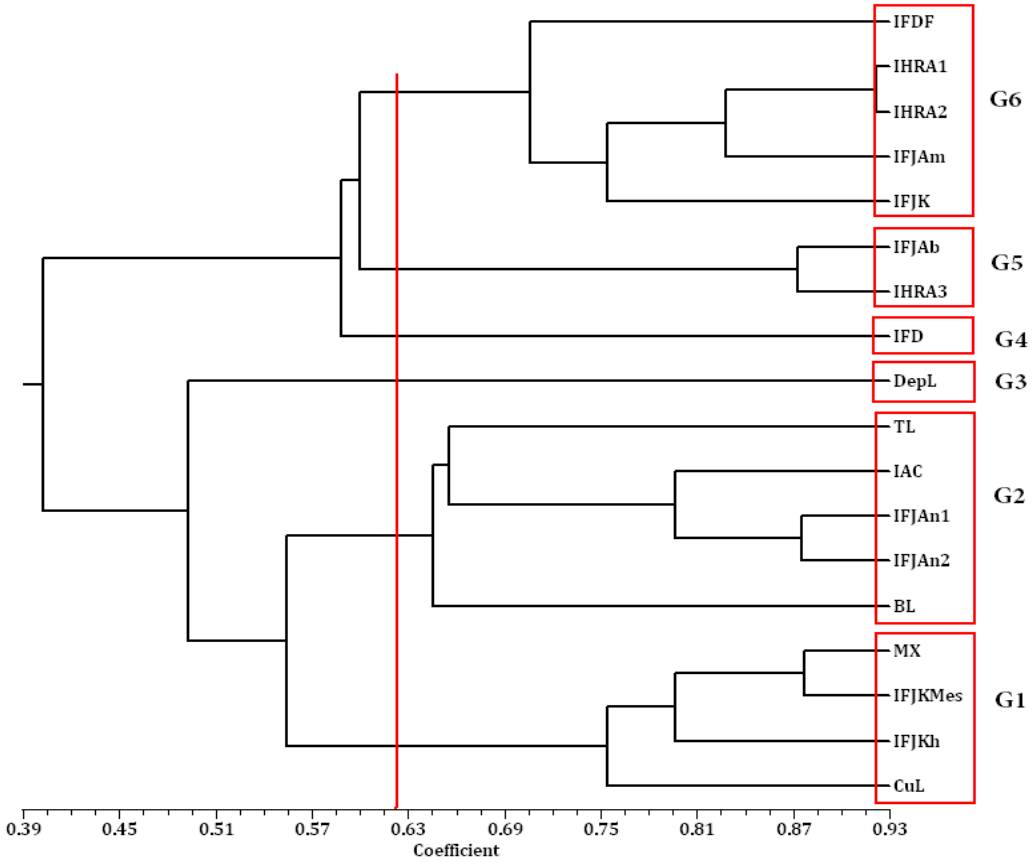

Fig. 4. Genetic similarity among 18 citrus accessions. The dendrogram generated using COMPLETE clustering method based on Dice similarity matrix containing 8 ISSR markers.

Table 4. List of the primers used in ISSR analyses

Primer	Sequence (5′–3′)	TBN	PBN	P%	PIC	MI
ISSR 1	$BDB(TCC)_5$	11	3	27	0.477	1.431
N3	$DBD(AC)_7A$	19	19	100	0.467	8.873
N5	$(AG)_8YT$	15	14	93	0.466	6.524
N7	$(AC)_8YG$	12	12	100	0.456	5.472
809	$(AG)_8G$	6	1	17	0.018	0.018
810	$(GA)_8T$	13	12	92	0.496	5.952
814	$(CT)_8A$	6	6	100	0.489	2.934
844	$(CT)_8AGC$	18	17	94	0.498	8.466
Mean		12.5	10.5	77.9	0.421	4.959

Y: Pyrimidine, B: non-A, D: non-C, H: non-G, V: non-T, R: Purin. TBN : Total band number, PBN : Polymorphism band number, P% : Polymorphism percent, PIC: Polymorphism information content, MI: Marker index.

Discussion

In the present study, DNA content ratio was 1.462 in triploid compared to the diploid accessions, in which the results of diploid and triploid groupings can be regarded as reliable value. Seker et al. (2003) with availability of DNA content of triploid standard plant (Tahiti lime), reported that all evaluated rootstocks were diploid. Zhu et al. (2009) used Bendizao tangerine (*C. reticulata*) as a diploid control and evaluated ploidy level of the triploid BHR progenies by FCM analysis. In the present study, the results of leaf dimension parameters revealed that the triploid accessions had higher leaf length and width when compared to the leaf dimension parameters of other accessions. It has been reported that the polyploidization increases leaf size and area (Ye et al., 2010; Hosseini et al., 2015). In the current study, it was revealed that triploid accessions had fewer stomata per unit leaf surface compared with diploid accessions, which is consistence with Padoan et al. (2013), Ye et al. (2010) and Hosseini et al. (2015) findings. The coordination of the size and frequency of stomata is an important feature for maximizing water use efficiency against environmental conditions (Padoan et al., 2013). In average, triploid lime accessions had 21.0-37.0 secretory sacs per unit leaf surface which was significantly less than the diploid accessions. These results are in consistent with Afshar-Mohammadian et al. (2013) findings in which number of secretory sacs in tetraploid Mexican lime was significantly less than diploids ones. It seems the number of secretory sacs is decreased by increasing ploidy level and leaf surface. Generally, results of the current study cleared that IFJKh, Cucumber-shaped lime and IFJKMes (the named Mesri, which are erroneously introduced as Persian lime accessions in south of Iran) accessions, are diploid. Due to susceptibility of accessions resulted from Mexican lime to WBDL; their cultivation in the infected regions to WBDL phytoplasma is not recommendable.

Application of SSR markers confirmed the obtained results of FCM and morphological analysis about grouping the accessions to diploid and triploid groups. SSR markers capability to recognize triploidy level has been reported by other scholars (Sharafi et al., 2016; Ahmad et al., 2003; Watanabe et al., 2008), which is in agreement with our findings. Comparison of morphological and molecular outcomes is a key factor to conclude the extent of genetic diversity present in the set of cultivars (Sharma et al., 2015). In our study, there was no correlation between both morphological and ISSR markers (r=0.060).The evaluated accessions showed morphological similarity however they had genetic dissimilarity. Our results also revealed close relation between PL accessions and Mexican lime. It has been showed that there was no difference among selections of PL such as IAC-5, Persian 58 and Bearss lime (Santos et al., 2013), which is not according to our findings. The similarity coefficient from ISSR analyses were 0.75-0.88 between Mexican lime and

three unknown lime accessions *i.e.* IFJKMes, IFJKh and Cucumber-shaped lime. The mentioned accessions are similar to Mexican lime and they have been possibly derived from sexual propagation of Mexican lime. Mexican lime was propagated by seed in Iran and its seeds are poly-embryonic; and one of the embryos has sexual origin and the others result from nuclear tissue of embryo sac. Accordingly, high variation has been shown among these seed born accessions and it has been used as a potential tool in WBDL resistance trials.

After WBDL occurrence in southern regions of Iran in decade of 1990, a significant part of the Mexican lime producing regions, as a main host of WBDL, were devastated by this phytoplasmal disease. However, no symptoms of WBDL have been observed on Persian lime (*C. latifolia* Tanaka) as another lime species by now and it is considered as a tolerant lime to WBDL. In recent years, with regards to requests of lime producer in the south of Iran, cultivation of Persian lime has been increasingly developed and nurserymen are producing Persian lime on different rootstocks. In some cases, in Fars and Hormozgan provinces, Persian lime is known as Limoo Mesri (Egyptian lime).In fact, Egyptian lime is a derived seedling from Mexican lime (*C. aurantifolia* Swingle) (USDA, 2002). Based on the obtained results of the present study, IFJKMes, IFJKh (Short thorn lime) and Cucumber-shaped lime accessions are diploid and their cultivation is not recommended as Egyptian (Mesri) or Persian lime in the infected regions with WBDL. As we expected, Persian and Mexican lime accessions showed close genetic relation.

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