Genetic variation assessment of acid lime accessions collected from south of Iran using SSR and ISSR molecular markers

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Abstract Iran has a long history of acid lime cultivation and propagation. In this study, genetic variation in 28 acid lime accessions from five regions of south of Iran, and their relatedness with other 19 citrus cultivars were analyzed using Simple Sequence Repeat (SSR) and Inter-Simple Sequence Repeat (ISSR) molecular markers. Nine primers for SSR and nine ISSR primers were used for allele scoring. In total, 49 SSR and 131 ISSR polymorphic alleles were detected. Cluster analysis of SSR and ISSR data showed that most of the acid lime accessions (19 genotypes) have hybrid origin and genetically distance with nucellar of Mexican lime (9 genotypes). As nucellar of Mexican lime are susceptible to phytoplasma, these acid lime genotypes can be used to evaluate their tolerance against biotic constricts like lime “witches’ broom disease”.

Keywords *Citrus aurantifolia* · Genetic variation · Iran · Lime

Introduction

Acid citrus are used for culinary and non-culinary purposes. Juice, pickles, whole fresh or dry fruit and slices of acid citrus are widely used in Iranian dishes. Among lemons, limes and other acid citrus species, acid lime which also known as Omani, Indian, Mexican or Key lime [*Citrus aurantifolia* (Christm) Swingle] is well adapted to dry conditions of south citrus growing regions of Iran, especially coastal regions of Persian Gulf. Acid lime has economic importance in provinces such as Hormozgan, Sistan and Baluchestan, Kerman, Fars, and Bushehr. In recent years, due to a lethal disease [Witches’ Broom Disease of Lime (WBDL), “*Candidatus* Phytoplasma aurantifolia”], cultivation and yield of acid lime have dramatically decreased (Salehi et al. 2008). For example just in Hormozgan province, from the first emergence of this disease in the 1990s (Bove et al. 2000) the cultivation area of Mexican lime has decreased from 20,000 ha to 10,000 ha. Not only in Iran but also in many other countries such as India, Nepal, Saudi Arabia and Oman, the cultivation of lime faces a lot of biotic and abiotic restricts. For this reason in recent years, study on genetic diversity of acid citrus, limes and lemons for selection of new variants, breeding and cultivar improvement have been interested (Al-Sadi et al. 2012).

Like other phytoplasmas, WBDL is normally controlled by planting of disease tolerant cultivars provided that control of insect vector, quarantine rules, control of weed and other hosts to be accomplished. Tolerant cultivar may be obtained in different approaches such as screening of germplasm collection, mutation breeding and selection among plants survived in natural infected field populations (Roose 2000). On the other hand, availability of germplasm having high genetic variation is necessary for any desirable selection or to do any breeding program. The more genetic variation is available in a particular fruit tree the more desirable selection will be accomplished.

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Because in south citrus growing regions of Iran for many decades the acid lime has only been propagated by seed, it is anticipated that many of its variants have been emerged. However, no information about molecular variation of this natural gene pool is available. This subject needs to be clarified.

Among many molecular markers, Simple Sequence Repeat (SSR) and Inter-Simple Sequence Repeat (ISSR) markers have been used in citrus in a wide range of applications including cultivar identification (Fang and Roose 1997; Novelli et al. 2000; Shahsavari et al. 2007; Biswas et al. 2010; Uzun et al. 2010), phylogenetics (Pang et al. 2003; Marak and Laskar 2010), zygotic and nucellar seedlings differentiation (Ruiz et al. 2000; Oliveira et al. 2002; Krueger and Roose 2003) and the construction of linkage maps for marker assisted breeding and map-based cloning of genes (Kijas et al. 1997; Sanker and Moore 2001).

In the present study, we used both SSR and ISSR molecular markers to evaluate molecular variation and genetic relatedness among those acid lime accessions showing distinct morphological characteristics in south of Iran, Hormozgan province (Bahrami et al. 2011). The results of this study will be

useful for any conservation, selection and breeding program to deal with WBDL.

Materials and methods

Plant material and DNA isolation

In this study, 28 accessions of acid lime found in south of Iran and 19 accessions of other citrus species/or cultivars for comparison were used. The acid lime accessions were different in morphological characteristics and collected from five WBDL-infected areas of Hormozgan province including Minab, Rudan, Hashtbandi, Sandarak and Joghini (Bahrami, et al. 2011) (Table 1 and Fig. 1). Total genomic DNA was isolated from fresh leaves following the procedure described by Murray and Thompson (1980) with some modification. After quantity determination of the DNA using a spectrophotometer (Nano Drop 2000, Thermo Scientific), the templates were diluted to 25 ng/μl for using in PCR reactions.

Table 1 List of plant materials used in this study

No.	Common or Local name	Scientific name	Location of sampling	Accession's code (or name in figures)
1	Balang	<i>Citrus medica</i> L.	Kotra collection*	Citron
2	Clementine	<i>C. reticulata</i> Blanco	Kotra collection	Clementine
3	Darabi	A local <i>Citrus</i> cultivar	Kotra collection	Darabi
4	Sweet lime	<i>C. limettoides</i> Tan.	Kotra collection	Sweet Lime
5	Bakraii	A local lime-like cultivar	Kotra collection	Bakraii
6	Off-type of Mexican lime	<i>C. aurantifolia</i> Swingle	Kotra collection	N6
7	Mineola tangelo	<i>C. paradisi</i> × <i>C. reticulata</i>	Kotra collection	Mineola Tangelo
8	Eureka lemon	<i>C. limon</i> [L.] Burm. F	Kotra collection	Eureka Lemon
9	Siavaraz	A local orange cultivar	Kotra collection	Siavaraz
10	Lisbon lemon	<i>C. limon</i> [L.] Burm. F	Kotra collection	Lisbon Lemon
11	Orlando tangelo	<i>C. paradisi</i> × <i>C. reticulata</i>	Kotra collection	Orlando Tangelo
12	Frost Valencia orange	<i>C. sinensis</i> (L.) Osb.	Kotra collection	Valencia Orange
13	Washington Navel Orange	<i>C. sinensis</i> (L.) Osb.	Kotra collection	Washington
14	A local lemon-like cultivar	<i>Citrus</i> spp.	Kotra collection	N14
15	A local citrus cultivar	<i>Citrus</i> spp.	Kotra collection	N15
16	Persian lime	<i>C. latifolia</i> Tan.	Ramsar collection*	Persian Lime
17	Duncan grapefruit	<i>C. paradisi</i> Macf.	Ramsar collection	Duncan
18	Sour orange	<i>C. aurantium</i> L.	Ramsar collection	Sour Orange
19	Mexican lime	<i>C. aurantifolia</i> Swingle	Kotra collection	Mexican Lime
20 to 30	Acid lime accessions**	<i>Citrus</i> spp.	Minab (M)	M1, M2, M3, M4, M5, M6, M7, M11, M12, M13, M14
31 to 38	Acid lime accessions	<i>Citrus</i> spp.	Rudan (R)	R1, R2, R3, R5, R6, R7, R8, R10
39 to 42	Acid lime accessions	<i>Citrus</i> spp.	Hashtbandi (H)	H2, H3, H4, H5
43 to 45	Acid lime accessions	<i>Citrus</i> spp.	Sandarak (S)	S1, S2, S5
46 to 47	Acid lime accessions	<i>Citrus</i> spp.	Joghini (J)	J1, J2

* Both Kotra and Ramsar collections are located in north of Iran, Mazandaran province

** Acid lime accessions were collected from south of Iran, Hormozgan province

Fig. 1 Morphological polymorphism aspects in nine different acid limes collected from south of Iran. Each accession code is same as that of Table 1

Molecular marker analyses

SSR

SSR assays were performed by using 22 primers from the 26 sets described by Ahmad et al. (2003). They were: CMS-2, CMS-4, CMS-6, CMS-7, CMS-14, CMS-16, CMS-17, CMS-18, CMS-19, CMS-20, CMS-21, CMS-23, CMS-24, CMS-26, CMS-30, CMS-31, CMS-34, CMS-39, CMS-45, CMS-46, CMS-47, and CMS-48. Three other primers described by Kijas et al. (1997) (TAA15, TAA 41 and CAC19) were also tested. The PCR amplifications were conducted in a total volume of 15 μ l [PCR buffer (1 $\times$), 50 ng of genomic DNA, 0.2 mM of each dNTP, 0.1 mM of each forward and reverse

primer, 1 unit of DNA *Taq* polymerase (Fermentas) and 2 mM of $MgCl_2$]. The PCR condition was: 94 $^{\circ}C$ for 5 min, 35 cycles of 94 $^{\circ}C$ for 30 s, 55 $^{\circ}C$ for 30 s and 72 $^{\circ}C$ for 30 s, ending with 72 $^{\circ}C$ for 5 min. The PCR products were separated on 6 % denaturing polyacrylamide gel in TBE buffer (1 $\times$) (45 mM Tris- Boric, 1 mM EDTA, pH 8.0). The gels were stained by silver nitrate (Fig. 2).

ISSR

A total of nine ISSR primers were used for PCR amplifications (Table 3) (Awasthi et al. 2008). The concentration of PCR reagents was almost same as that of SSR excepting 0.3 mM of ISSR primer and 2.5 mM of $MgCl_2$ were used.

Fig. 2 Polymorphism of SSR molecular marker (locus CMS-19) in the citrus accessions of this study. M: Size marker. Three alleles in No. 16 are related to 'Persian' lime, a triploid citrus species. The numbers are related to the accessions of Table 1

Fig. 3 ISSR polymorphism resulted from (GA)8C primer. The numbers are related to the accessions of Table 1

The PCR condition was: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 2 min, ending with 72 °C for 10 min. The PCR products were separated on 1.5 % agarose gels (Top Vision) in TBE buffer (1×). The gels were stained by ethidium bromide (Fig. 3).

Data analysis

The presence of an amplified product (fragment) was identified as “1” and the absence was nominated as “0” and a similarity matrix was constructed based on Jaccard’s coefficient. The accessions were grouped by cluster analysis using the unweighted-pair group method with arithmetic averages (UPGMA). The computer program used was NTSYS-PC, version 2.011 (Rohlf 2000). The cophenetic correlation coefficient, r , was calculated based on Mantel’s test (Mantel 1967) to check the goodness of fit of the cluster analysis to the matrix on which it was based. Total number of effective alleles (N_e), expected heterozygosity (H_e), observed heterozygosity (H_o),

and PIC (polymorphic information content) was calculated using Gen AlEx (Ver. 6.5) (Peakall and Smouse 2012) and Mstools computer programs. PIC provides an estimate of the discriminatory power of a locus. PIC values for codominant markers like SSR range from 0 (monomorphic) to 1 (very highly discriminative). It refers to the value of a marker for detecting polymorphism within a population and is equivalent to gene diversity. PIC for dominant markers like ISSR is a maximum of 0.5 (De Riek et al. 2001).

Results and discussion

SSR polymorphism and genetic relationships

After screening of the 25 SSR primers, seven primers from Ahmad et al. (2003) and two primers from Kijas et al. (1997) were selected for final PCR amplification and data scoring. Using these nine selected primers, in total 49 polymorphic

Table 2 Number of total and effective alleles (N_e), H_e , H_o and PIC for nine SSR loci

Loci	Total alleles	Polymorphic alleles	SSR marker Polymorphism range (%)	N_e	H_e	H_o	PIC
CMS-4	8	8	100	4.53	0.760	0.531	0.720
CMS-7	6	6	100	4.16	0.767	0.893	0.729
CMS-16	3	3	100	2.6	0.379	0.195	0.305
CMS-19	6	6	100	4.94	0.806	0.787	0.766
CMS-24	6	6	100	4.79	0.745	0.511	0.718
CMS-45	4	4	100	2.51	0.609	0.588	0.527
CMS-46	3	3	100	2.99	0.676	0.735	0.592
TAA-15	5	5	100	1.77	0.442	0.422	0.412
TAA-41	8	8	100	5.36	0.779	0.673	0.742
Total	49	49	—	—	—	—	—
Mean	5.45	5.54	100	3.69	0.663	0.537	0.612

Table 3 Number of total and Polymorphic alleles and PIC for nine ISSR loci

Primer	Total alleles	ISSR marker Polymorphic alleles	Polymorphism range (%)	PIC
(AG) ₈ G	18	18	100	0.39
(GA) ₈ C	17	15	88.2	0.38
(GA) ₈ A	18	18	100	0.33
(CT) ₈ T	12	8	66.6	0.32
(AC) ₈ Y*T	17	16	94.1	0.32
(GT) ₈ AGTCY	12	12	100	0.34
(GACA) ₄	20	15	75	0.37
(AG) ₈ T	18	16	88.8	0.35
(AC) ₈ G	16	13	81.2	0.34
Total	148	131	88.2**	0.35**

*Y: C/T

**Mean

alleles were obtained. PIC values ranged from 0.305 for CMS-16 to 0.766 for CMS-19. The mean value of PIC (0.612) was relatively high which confirmed the high polymorphism among the genotypes. This high level of polymorphism again could be detected from the values of H_e (also called diversity index) (Table 2 and 3). Cophenetic correlation was found to be high ($r = 0.937$) suggesting that 94 % of the similarity matrix

was represented by the clustering analysis. In the dendrogram (Fig. 4), two main clusters, A and B, with a similarity value of 0.19 were observed. The first cluster (A) was consisted of two sub-clusters, A_I and A_{II}, with a similarity value of 0.25. In the sub-cluster A_I, Citron ('Balang'), 'Darabi' (a local citrus cultivar), Sweet lime, M12, 'Bakraii' (a local acid lime cultivar), M5, N6, 'Eureka' and 'Lisbon' lemons (identical), N14 (a

Fig. 4 Dendrogram of 47 citrus accessions obtained from SSR markers using Jaccard's coefficient and UPGMA clustering method

lemon cultivar) and R10 were grouped. In the sub-cluster A_{II}; ‘Clementine’ mandarin, ‘Siavaraz’ (a local sweet orange); ‘Frost Valencia’ and ‘Washington Navel’ oranges (without separation), ‘Minneola’ and ‘Orlando’ tangelos, N15, ‘Duncan’ grapefruit; sour orange and R8 (identical), M2; M6 and H5 (without separation) were grouped. The second cluster (B) was mostly consisted of acid lime accessions of south of Iran. In this cluster, Mexican lime was not distinguished from the accessions M3, M4, M7, M14, R2, H2, S2, S5 and J1. They were very close to R5, M11, M1 and R1. Other accessions including R3, R6, R7, H3, M13, S1, ‘Persian’ lime, H4 and J2 were distributed in this cluster.

ISSR polymorphism and genetic relationships

All of the nine ISSR primers produced easy to score fragments. The PIC values of the ISSR primers were less than 0.5 as it was expected. Cophenetic correlation was found to be high ($r = 0.932$) suggesting that 93 % of the similarity matrix was represented by the clustering analysis. The ISSR dendrogram (Fig. 5) was very similar to that obtained by SSR markers. The differences were; A_{II} sub-cluster of SSR was changed to B cluster of ISSR Fig. 6. Two accessions, R10

and M2, were grouped with Mexican lime and citron, respectively, using ISSR markers (Figs. 4 and 5).

SSR unique alleles

A number of unique SSR alleles were found for two accessions, H4 and Persian lime. H4 showed four specific SSR fragments using CMS-19, CMS-24, CMS-45 and TAA15 primers (one for each). Persian lime is a triploid species (Hodgson 1967). Congruent with this characteristic it showed three alleles using five primers, CMS-7, CMS-19 (Fig. 2, No. 16), CMS-24, CMS-45 and TAA41. In other fruit trees like loquat (*Eriobotrya japonica* Lindl.) triploidy of a few varieties has been also confirmed using 3-allele producing SSR primers (Watanabe et al. 2008).

Both SSR and ISSR markers found no differentiation between ‘Eureka’ and ‘Lisbon’ lemons, and between ‘Frost Valencia’ and ‘Washington Navel’ sweet oranges in agreement with results of previous studies on citrus cultivar identification (Ahmad et al. 2003; Uzun et al. 2009). These markers could not differentiate those cultivars of sweet orange and grapefruit originated through mutation too (Fang and Roose 1997; Uzun et al. 2010).

Fig. 5 Dendrogram of 46 citrus accessions obtained from ISSR markers using Jaccard's coefficient and UPGMA clustering method

Fig. 6 Dendrogram of 47 citrus accessions obtained from morphological markers using Jaccard's coefficient and UPGMA clustering method

'Orlando' is a sibling of 'Minneola' tangelo, having 'Dancy' mandarin and 'Duncan' grapefruit as its parents. Remaining closely related to mandarin and grapefruit, these two tangelos were well separated from each other. The similarity value between them was 0.77.

'Siavaraz' is a local cultivar of sweet orange with a long history of cultivation in northern part of Iran, Mazandaran and Guilan provinces. It is a highly seedy and juicy cultivar with unknown origin. In the present study, with a slight divergence this cultivar was found very closely related to sweet oranges.

The origin of 'Bakraii' is un-known. This cultivar is usually used as a rootstock for citrus cultivars especially Mexican lime in south of Iran. Its fruits are consumed occasionally despite their bitterness (Shahsavari et al. 2007). Recently through SSR and PCR-RFLP markers, Golein et al. (2012) found that 'Bakraii' is a hybrid between rough lemon (*C. jambhiri* Lush.), as maternal parent, and sweet lime (*C. limettioides* Tan.). Shahsavari et al. (2007) also found a close affinity between 'Bakraii' and sweet lime using ISSR markers. Our results also support a close relationship between 'Bakraii' and sweet lime. The only accession that showed close affinity to 'Bakraii' was M5 here. We suggest that it can be a hybrid of 'Bakraii'.

'Persian' lime is a unique and new cultivar to citrus industry of Iran and is not being cultivated widespread. The flowers

of 'Persian' lime are devoid of viable pollen also contain exceedingly few functional ovules (Hodgson 1967). These characteristics prevent its natural hybridization with other citrus species. Probably for these reasons, no accession was closely clustered with 'Persian' lime in this study, but it was located in acid lime cluster.

Acid lime has a long history of cultivation in the south regions of Iran such as Fars, Hormozgan, Kerman, Bushehr and Sistan-Baluchestan provinces. Too many hectares of acid lime orchards have been established in these provinces. Trees of these orchards have been mostly originated by seed propagation of Mexican lime (Faghihi et al. 2011). The seeds of Mexican lime are two types; mono-embryonic (20–25 %; with only one zygotic embryo) and poly-embryonic (75–80 %, containing one zygotic and one or more nucellar embryos) (Frost and Soost 1968). Through seed propagation both zygotic and nucellar seedlings could be produced. The nucellar seedlings are genetically identical to their source or mother tree (true to type) whereas zygotic seedlings are not. Krueger and Roose (2003) found that in many seed sources, nucellars of all citrus species had identical ISSR finger printings with their seed parent. Cluster analyses in the present study proved that only nine acid lime accessions of Iran, M3, M4, M7, M14, R2, H2, S2, S5 and J1, had both SSR and ISSR banding

patterns identical to Mexican lime which give evidence of their origin either as its nucellar or somatic mutants. Their nucellar origin is strongly supported here for above reason. Furthermore, until recently, there is no report on use of natural mutants for cultivar improvement in south citrus growing regions of Iran. Mexican lime is the most sensitive citrus species to WBDL. The above nine accessions will certainly react to WBDL as same as Mexican lime due to being its nucellar. Different reaction to WBDL is expected from other accessions rather than these nine ones.

Recently, Bahrami et al. (2011) reported that the accessions M11, R2, R3, R6, R8, R10, H4, H5 and J2 were tolerant to WBDL. All of these accessions excepting R2 and M11 (identical and very close to Mexican lime, respectively) were located far from Mexican lime in the SSR and ISSR dendrograms of this study. We suggest that the main reason for their different reaction to WBDL can be related to their difference in genetic basis comparing with Mexican lime.

In fruit trees, existence of genetic diversity is essential for any breeding program especially for disease resistance improvement. In the present study, both SSR and ISSR markers indicated that the acid lime accessions could be distinguished from one another excluding identical accessions to Mexican, therefor considerable diversity could be seen among them. This variation will be useful for different purposes such as breeding, tolerance to biotic (WBDL) and abiotic restricts.

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