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AFLP FINGERPRINTING ANALYSIS OF *CITRUS* CULTIVARS AND WILD ACCESSIONS FROM OMAN SUGGESTS THE PRESENCE OF SIX DISTINCT CULTIVARS

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A study was conducted to evaluate genetic relatedness of 27 citrus cultivars and 6 wild citrus accessions using AFLP fingerprinting. The 27 citrus cultivars belonged to *Citrus sinensis, C. aurantifolia, C. aurantium, C. paradise, C. reticulata, C. limon, C. latifolia, C. maxima, C. limettoides, C. limetta, C. medica* and *C. Jambhiri*. The wild cultivars were obtained from Oman while the other cultivars originated from Oman and other countries. AFLP analysis using 4 primer pair combinations resolved 910 polymorphic alleles. All citrus cultivars and accessions had low genetic diversity (H = 0.0281 to 0.1300), with the percent polymorphic loci ranging from 8 to 35%. Populations of the six wild citrus accessions showed a very low level of genetic diversity (< 0.0700). Cluster analysis of the 33 cultivars and accessions showed that they share a high level of genetic similarity (81–99%; mean = 92%). The six wild accessions clustered into two main clusters, with the analysis indicating that the six wild accessions may make up six distinct cultivars. The study provides information on the phylogeny of citrus cultivars and citrus diversity in Oman, a country through which citrus moved in the past from Asia to different African and European countries. In addition, it shows that some distinct citrus cultivars are present in this part of the world.

Key words: phylogenetic analysis, genetic diversity, lime, orange, mandarin, lemon

Globally, citrus is the second largest fruit crop being grown in 49 countries which are having plentiful water supply, endurable cold span in winter and tropical to subtropical climates (Singh *et al.* 2002; FAO 2015; Spiers *et al.* 2017). A total of six genera comprising of 29 species and 11 varieties are grouped together as true citrus fruit trees. Three of these six genera (*Clymenia*, *Eremocitrus*, and *Poncirus*) are monotypic; whereas, *Fortunella*, *Microcitrus* and *Citrus* possess 4, 6 and 16 species respectively (Swingle & Reece 1967).

The taxonomy of the genus *Citrus* has been complex, confusing, debatable and its intra as well as inter generic hybrid-ability makes it even more intricate and confusing (Singh *et al.* 2002; Sun *et al.* 2016; Wang *et al.* 2017). Swingle and Reece (1967) subdivided the genus *Citrus* into two sub-genera i.e, Papeda and Eucitrus with 6 and 10 species in each, respectively. Tanaka (1977) divided the genus in 162 species, whereas, Hodgson (1967) presented a better compromising classification of the genus proposing 36 species in all.

Most of the scientists follow Swingle and Reece (1967) classification for its simplicity and clarity. In this classification the sub-genus *Eucitrus* comprising 10 species covers all of major citrus varieties of commercial importance. Among these 10 major citrus species, Citron (*Citrus medica* L.) probably

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originated in south China to India and lime (*Citrus aurantifolia* Swingle) in East Indian archipelago. Grapefruit (*Citrus paradisi* Macf.) is thought to be originated in West Indies as a mutant or natural hybrid of pummelo, whereas, mandarins (*Citrus reticulata* Blanco) in Indo-china to south China (Davies & Albrigo 1994). Lemon (*Citrus limon* Burmann) is thought to be a hybrid of citron and lime with unknown origin (Barrett & Rhodes 1976). Sour orange (*Citrus aurantium* L.) originated in Southeast Asia or India, whereas, sweet orange (*Citrus sinensis* [L.] Osbeck) is thought to be originated in Southern China or Indonesia (Webber 1967). Malaysia and Indian archipelagos are believed to be center of origin of pummelo (*Citrus grandis* [L.] Osbeck). *Citrus Limettioides* Tan. is thought to be originated in north-east India.

Recognizing the constraints in describing plant morphological differences, isozyme has been used effectively in citrus genetic variability studies (Ashari *et al.* 1989; Fang *et al.* 1997; Elisiário *et al.* 1999). However, it may give little false impressions due to environmental and morphogenesis impacts. Molecular marker-based techniques have been used frequently to differentiate genetic variability because of its reproducibility and discrimination ability. Random amplified polymorphic DNA (RAPD) markers procedure is also used in identifying citrus species (Dehesdtani *et al.* 2007; Shahzadi *et al.* 2016). However, this procedure may not recognize intraspecific differences and its reproducibility precision is minimal. Other markers, for example simple sequence repeats (SSR) are largely locus specific technique and have shown their usefulness in recognizing close genetic relatedness, but this is labor intensive and costly too (Jannati *et al.* 2009; Yang *et al.* 2010). The AFLP markers are described as a strong molecular apparatus extensively exercised in genetic mapping, population genetics, phylogenetics or plant cultivar documentation. The AFLP procedure is extremely steady and has the capacity of reproducibility without depending on earlier sequence information (Liang *et al.* 2007; Al-Sadi *et al.* 2012; Dorji & Yapwattanaphun 2015). It has been used in phylogenetic studies of citrus and related genera (Chao *et al.* 2005; Althoff *et al.* 2007; Pang *et al.* 2007; Dorji & Yapwattanaphun 2015). Therefore, the procedural and reproducibility advantage of AFLP marker in citrus and other related crops have made it a useful technique to study the genetic variability and cultivars description.

Oman is on the southern-eastern part of the Arabian peninsula, acing the Indian Ocean. It has been active in trade and in the transfer of crops from the Indian subcontinent to other parts of the world (Grey 1911; Davies & Albrigo 1994). This makes Oman likely a potential place for the presence of several citrus cultivars.

The main objective of study was to examine phylogenetic relationship among 33 citrus cultivars and accessions based on AFLP fingerprinting, specifically:

1. To examine the diversity and genetic relatedness of the known and unknown citrus cultivars and accessions.
2. To characterize relationship of six wild citrus accessions from Oman to 27 citrus cultivars.

This will help understand the diversity of citrus cultivars present in this part of the world. In addition, it would also help geneticists and plant breeders in Oman to isolate germplasms and thereafter parents for initiating novel breeding / hybridization programs not undertaken so far systematically.

MATERIAL AND METHODS

Sample collection

A total of 27 citrus cultivars were included in this study. The cultivars, which belong to *Citrus sinensis, C. aurantifolia, C. aurantium, C. paradise, C. reticulata, C. limon, C. latifolia, C. maxima, C. limettoides, C. limetta, C. medica* and *C. Jambhiri,* were obtained from governmental experimental stations in Oman. In addition, samples from 6 unknown citrus accessions that were obtained from growers were included in the study (Table 1). Young leaves (approx. 10 leaves) were collected from each cultivar, 2–4 replicate seedlings or trees per cultivar (Al-Sadi *et al.* 2012). The leaves were kept at –80°C and processed within one week of collection.

DNA Extraction

DNA was extracted from citrus leaves as explained by Doyle and Doyle (1990). Leaves were

grinded in liquid nitrogen using mortar and pestle. This was followed by addition of 500 µl of pre warmed 2x CTAB extraction buffer and incubation at 65°C for 30 min. The remaining steps were as explained by Al-Abadi *et al.* (2016).

AFLP Fingerprinting

AFLP fingerprinting was used to examine the relationship among 33 citrus cultivars and wild accessions as described by Al-Sadi *et al.* (2012) using *Eco*RI (NEB, Frankfurt, Germany) and *Mse*I (NEB, Frankfurt, Germany) enzymes. In the beginning, the efficiency of the 12 AFLP selective primers in analyzing genetic diversity of 8 citrus cultivars was studied (2 *Eco*RI+3 × 6 *Mse*I+3). The 4 best primers with the highest polymorphic alleles were included in further analysis of the 33 citrus

T a b l e 1

Citrus cultivars and accessions included in the study

	Scientific name	Cultivar
1	*Citrus sinensis*	Delta Valencia
2	*Citrus sinensis*	Navelate Orange
3	*Citrus sinensis*	Salustiana Orange
4	*Citrus sinensis*	Succari Orange
5	*Citrus sinensis*	Hamllin Orange
6	*Citrus latifolia*	Tahiti lime
7	*Citrus limon*	Eurek lemon
8	*Citrus maxima*	Wahaq
9	*Citrus aurantifolia*	Citrus aurantifolia
10	*Citrus reticulata*	Kinnow mandarin
11	*Citrus reticulata*	Fortune Blanco
12	*Citrus limettoides*	Sweet lime (Bu ruqab)
13	*Citrus reticulata*	Ortanic tangor
14	*Citrus sinensis*	Turocruza
15	*citrus medica*	Citron
16	*Citrus limetta*	Mediterranean sweet lime
17	*Citrus aurantifolia*	Kagzi lime – Bermasi lime
18	*Citrus jambhiri*	Rough lemon
19	*Citrus limettoides*	Sweet lime (Daeeri)
20	*Citrus paradise*	Marsh grapefruit
21	*Citrus paradise*	Star ruby
22	*Citrus sinensis*	Jaffa orange
23	*Citrus sinensis*	Temple orange
24	*citrus limon*	Fino lemon
25	*Citrus aurantium*	Sour orange
26	*Citrus limon*	Berma lemon
27	*Citrus limon*	Kishmiri lemon
28	Unknown 1	OM 1
29	Unknown 2	OM 2
30	Unknown 3	OM 3
31	Unknown 4	OM 4
32	Unknown 5	OM 5
33	Unknown 6	OM 6

cultivars and wild accessions.

The AFLP protocol included digestion, annealing, pre-selective amplification and selective amplification as explained by Al-Sadi *et al.* (2012). FAM-6 labeled *Eco*RI-AGA selective primer and *Mse*I-xxx primers were used in the selective amplification reaction (Table 2). Fragment analysis was carried out at Macrogen Inc. (Korea).

Analysis of AFLP data

Data generated from the fragment analysis were subjected to analysis to estimate the levels of diversity and relatedness among citrus cultivars and accessions. Nei's gene diversity (Nei 1973) and genetic distance (Nei 1978) were calculated using POPGENE (v 1.32) (Yeh & Boyle 1997). Genetic distance data were used to construct dendrograms to show the levels of relatedness among different cultivars and accessions. Genetic differentiation among cultivars and accessions was analyzed using the analysis of molecular variance (AMOVA), which was done using the program Arlequim v.3.1 (Excoffier *et al.* 2005).

RESULTS

AFLP primer pair combinations

Analysis of 12 AFLP primer pair combinations showed that there were 41 to 234 polymorphic loci.

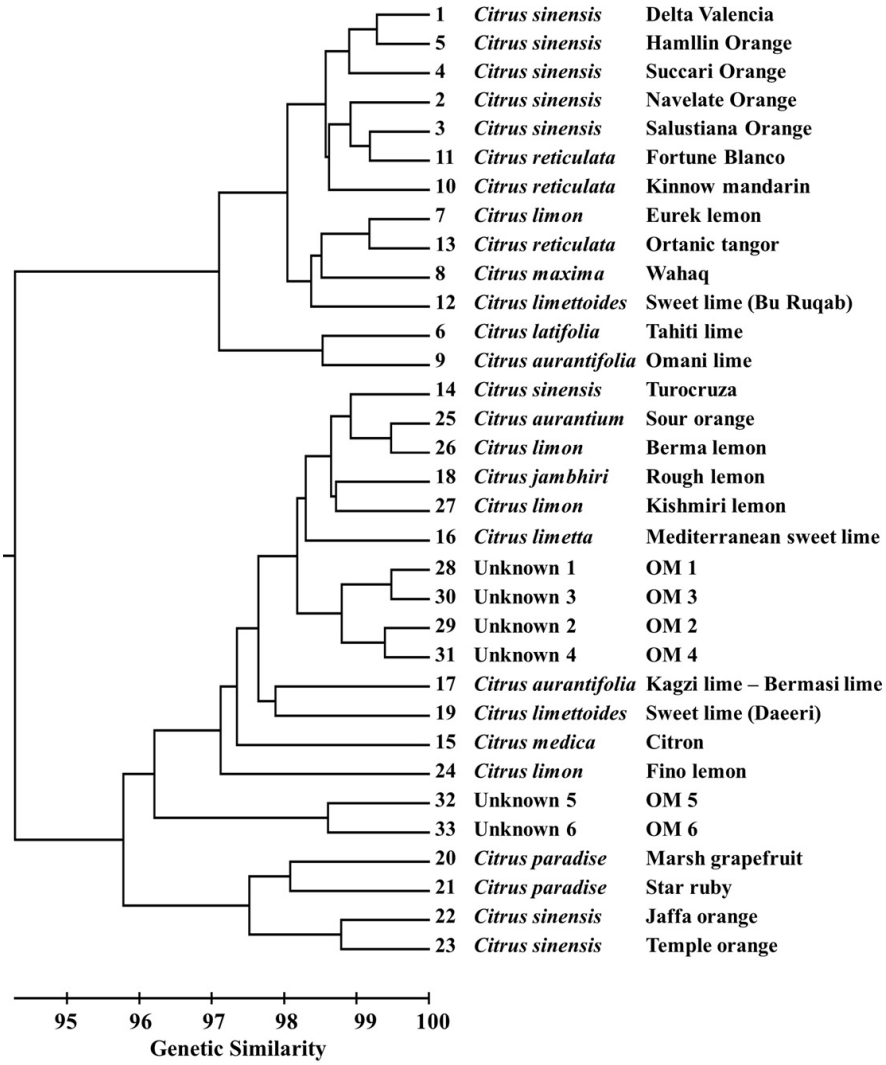

Figure 1. UPGMA dendrogram illustrating genetic distances (Nei 1978) of 33 citrus cultivars and accessions based on AFLP fingerprinting analysis using 910 polymorphic loci

The percent polymorphic loci (91–100%) produced by these primer-pair combinations were also high. Four primer pairs, E-AAC+M-CAG, E-ACC + M-CAG, E-AAG + M-CTC, and E-ACA + M-CAA, produced 137–234 polymorphic alleles (Table 2) and were used in further experiments.

Genetic diversity in citrus cultivars

Analysis of genetic diversity in the 33 citrus cultivars and accessions using 4 primer combinations resulted in 910 polymorphic alleles. Citrus populations (cultivars and accessions) were found to have low genetic diversity (H = 0.0281 to 0.1300). Polymorphism in the loci in all populations ranged from 8 to 35%. The wild citrus accessions had low levels of genetic diversity (<0.0700). Unique alleles in populations ranged from 19–38 (Table 3).

Genetic similarity and cluster analysis

Cluster analysis of the 33 cultivars and accessions showed that they shared high genetic similarity (81–99%; mean = 92%) (Figure 1). The cultivars and accessions made two separate clades. Five *C. sinensis* cultivars clustered in clade 1, while 3 were in clade 2. The six unknown accessions grouped in clade 2 and formed two separate sub-clusters. Analysis based on citrus accessions showed that unknown accessions are distinct from any other citrus cultivars tested (Figure 2).

Partition of genetic variation

AMOVA analysis indicated the existence of moderate and significant levels of genetic differentiation among all citrus cultivars and accessions (F_{ST} = 0.14572) (Table 4). However, most of the variation was found to be within citrus populations.

DISCUSSION

Citrus is an important genus in Oman and elsewhere and it has several industrial and health benefits (Ishisono *et al.* 2017; M'hiri *et al.* 2017; Zhu *et al.* 2017; Ben Abdelaali *et al.* 2018). Several cultivars of citrus are available worldwide and new cultivars are evolving rapidly ever. Despite the presence of citrus in the Arabian Peninsula over hundreds of years, little is known about the diversity of citrus in this part of the world. This study is the first to characterize phylogenetic relatedness among citrus cultivars present in the southern-eastern part of the Arabian Peninsula.

AFLP fingerprinting proved to be powerful in grouping citrus cultivars according to their level of relatedness to each other. AFLP is a widely used technique in the population genetic and phylogenetic studies of plants and microorganisms (Keivani *et al.* 2010; Al-Sadi *et al.* 2012; Kumar *et al.* 2012;

T a b l e 2

Evaluation of 12 primer pair combinations for use in studying phylogenetic relationship among 33 citrus cultivars and accessions

	Primer combination	Polymorphisms	% of polymorphisms
1	E-AAC + M-CAG	234	96.69421
2	E-AAC + M-CAT	90	98.9011
3	E-AGA + M- CAG	114	99.13043
4	E-AGA + M-CTG	73	100.00
5	E-ACC + M-CAG	190	100.00
6	E-AGA + MCGT	119	98.34711
7	E-AAC + M-CAG	91	97.84946
8	E-AAG + M-CTC	166	100.00
9	E-AGT + M-CTC	95	100.00
10	E-ACA + M-CTC	93	100.00
11	E-AAG + M-CAA	41	91.11111
12	E-ACA + M-CAA	137	100.00

Casasnovas *et al.* 2013; Al-Maamari *et al.* 2014; Al-Sadi *et al.* 2015). AFLP is also commonly used for studying relationship and genetic diversity in citrus species (Nartvaranant & Nartvaranant 2011; Khiavi *et al.* 2015; Zhou *et al.* 2017).

We examined known and unknown citrus genera and its relatives, including 10 species belonging to Oranges, Lime, Lemon, mandarin, Citron and Grapefruit and six unidentified using AFLP analysis. A low level (H = 0.0281 to 0.1300) of genetic diversity was found in the 33 citrus cultivars. This is in agreement with the fact that citrus is not native to this part of the world, but has been introduced to it, mainly from the Indian subcontinent and some other places (Gmitter & Hu 1990; Davies & Albrigo 1994).

The data also suggest that outcrossing, which can induce variation in citrus, is limited in the fields from which the samples were collected. This is also supported by the moderate levels of genetic differentiation among the 33 cultivars and accessions, indicating that the level of gene flow is low among the cultivars. Previous studies have shown that populations with limited outcrossing tend to having a low level of genetic diversity and a moderate to high level of genetic differentiation (Al-Sadi *et al.* 2012; Al-Maamari *et al.* 2014; Gannibal *et al.* 2014; Al-Sadi *et al.* 2015). However, it has been reported that there was a range of polymorphism (41–70%) in mandarin analyzed by AFLP which suggested the possibility of having diverse type of mandarins within the accessions examined (Dorji & Yapwattanaphun 2015). Genetic variation and their relatedness studied in pummelo cultivars with AFLP markers indicated that the similarity was high in samples even collected from different locations. It shows that AFLP primer combinations have high reproducibility to exhibit greater polymorphic groups in mandarins and other related citrus species (Nartvaranant & Nartvaranant 2011).

The results showed that the citrus species examined from Oman can be stacked into 5 clusters e.g. Oranges, mandarin, pummelo, lime and lemon clusters largely. While clustering results revealed

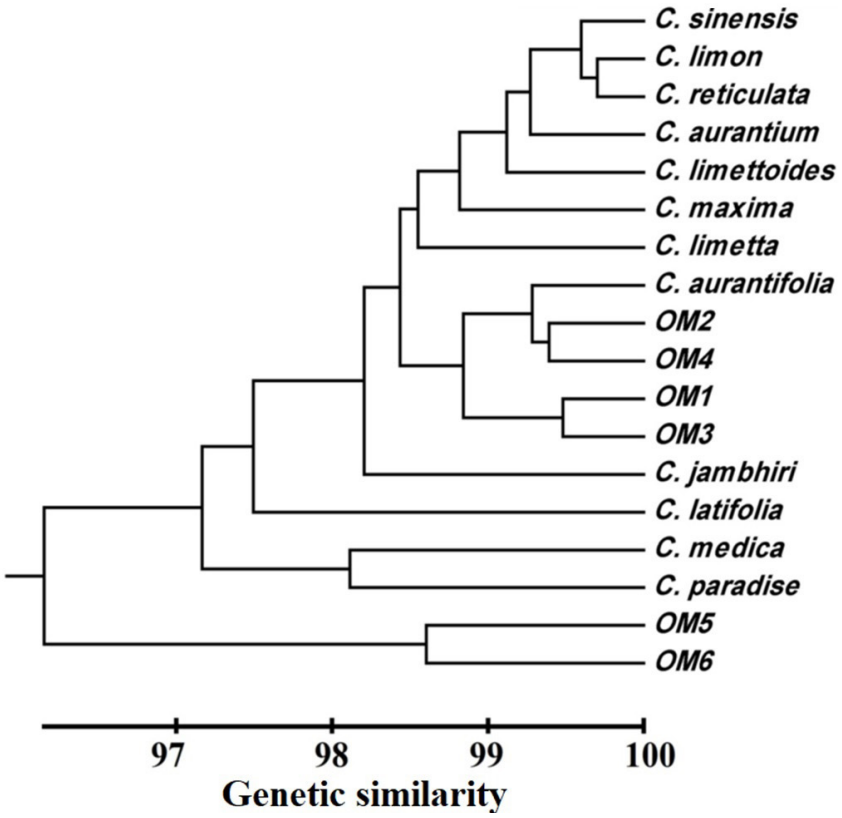

Figure 2. UPGMA dendrogram illustrating genetic distances (Nei 1978) of 12 citrus species and 6 accessions based on AFLP fingerprinting analysis using 910 polymorphic loci

T a b l e 3

Population genetic analysis of citrus cultivars and accessions

	Citrus cultivar	N	NPL	PPL [%]	g	H
1	Delta Valencia	3	273	30	3	0.1145
2	Navelate Orange	3	205	23	3	0.0814
3	Salustiana Orange	3	308	34	3	0.1269
4	Succari Orange	3	299	33	3	0.1208
5	Hamllin Orange	3	211	23	3	0.0751
6	Tahiti lime	2	96	11	2	0.0437
7	Eurek lemon	2	191	21	2	0.0869
8	Wahaq	2	144	16	2	0.0655
9	Citrus aurantifolia	4	217	24	4	0.0805
10	Kinnow mandarin	3	281	31	3	0.1157
11	Fortune Blanco	3	293	32	3	0.1133
12	Sweet lime (Bu ruqab)	2	244	27	2	0.1111
13	Ortanic tangor	3	253	28	3	0.0914
14	Turocruza	3	323	35	3	0.1300
15	Citron	3	200	22	3	0.0828
16	Mediterranean sweet lime	3	308	34	3	0.1213
17	Kagzi lime – Bermasi lime	3	149	16	3	0.0631
18	Rough lemon	3	252	28	3	0.1014
19	Sweet lime (Daeeri)	3	169	18	3	0.0713
20	Marsh grapefruit	2	184	20	2	0.0838
21	Star ruby	2	125	14	2	0.0569
22	Jaffa orange	2	150	16	2	0.0683
23	Temple orange	2	220	24	2	0.1001
24	Fino lemon	3	193	21	3	0.0785
25	Sour orange	3	199	22	3	0.0740
26	Berma lemon	3	184	20	3	0.0722
27	Kishmiri lemon	3	94	10	3	0.0430
28	OM1	3	111	12	3	0.0492
29	OM2	3	172	19	3	0.0699
30	OM3	3	69	8	3	0.0281
31	OM4	3	130	14	3	0.0585
32	OM5	3	206	23	3	0.0778
33	OM6	3	114	13	3	0.0473

OM1, OM2, OM3, OM4, OM5 and OM6 are the wild (unknown) accessions

T a b l e 4

Variation as measured using AFLPs among and within citrus cultivars and accessions based on hierarchical analysis of molecular variance (AMOVA)

Source of variation	d.f.	Sum of squares	Variance component	Percent variation	Fst	*P*
Among populations	32	3338.467	12.06413	14.57	0.14572	<0.00001
Within populations	59	4172.750	70.72458	85.43	0.14572	

that most of exclusive citrus species were grouped together, there was an exceptional grouping of unknown citrus species too. That shows theses are distinct type of plants clustered with *citrus limetteiods* and *citrus aurantifolia* (sweet and sour lime), it is worthy to note that both citrus species are grown widely in Oman. Lime is generally called as a chance seedling, having possible of parentage of *C. medica* and *papeda* (Bayer *et al*. 2009). Furthermore, citrus species have polyembryonic characters, e.g. lime and some others can be reproduced asexually from nucellar embryos. Asexual propagation, practiced through grafting, budding, and layering, is another method to desired characters of a cultivar. Therefore, limes have been mentioned in maintaining the heterozygous state over the course of its diversification. The most intriguing finding citrus genetic analyses was to observe the 6 unknown accessions clustered separately from all other citrus cultivars. The level of diversion from each other may indicate that the six accessions would be six distinct cultivars and apparently looks like the offspring of sour or sweet lime. A future study on these accessions using morphological and growth parameters is required in order to understand their characteristics and yield potential.

CONCLUSIONS

This study is the first to examine phylogenetic relationship of citrus cultivars in Oman. It showed that citrus cultivars have a low level of genetic diversity. In addition, it indicates that some distinct accessions/cultivars are present in this part of the world that would lead further investigation in future studies. Genetic variability observed in this study could provide an opportunity for further citrus germplasm collection, identification and integration in crop improvement programs at larger.

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