

Assessing morpho-chemical and genetic diversity in acid limes germplasm using microsatellite markers

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Abstract

Acid limes are highly nutraceutical and have great market demand. In Pakistan, two lime varieties are cultivated including 'Mexican lime' and 'Eustis limequat'. Hence, other potential varieties were characterized for morphogenetic variability. Leaf shape was elliptic in 'Mexican lime' and 'Eustis limequat' and obcordate in 'Tahiti lime', 'Seedless lime' and 'Tavares limequat' while the leaf area was greater in 'Seedless lime'. 'Tavares limequat' developed the heaviest fruit (74.55 g) with greater fruit length while fruit diameter was higher in 'Seedless lime'. Juice weight and juice percentage were greater in 'Tavares limequat' and 'Seedless lime' respectively. 'Tahiti lime', 'Seedless lime' and 'Tavares limequat' were found as a seedless variety. Titratable acidity was higher in 'Eustis limequat' while sugar to acid ratio was lower in 'Eustis limequat', 'Mexican lime' and 'Seedless lime' varieties. Simple sequence repeat (SSR) markers analysis revealed great genetic diversity among populations. Maximum alleles (9-11) were amplified by using CAC23 and CTT01 markers which showed higher discrimination and heterozygosity. CAC23 and CTT01 markers effectively amplified three alleles in triploids ('Tahiti lime' and 'Seedless lime'), a single allele in diploids ('Mexican lime', 'Eustis limequat') and two alleles, including a unique allele in diploid 'Tavares limequat'. Cluster analysis bunched closely related genotypes into two groups, STRUCTURE analysis confirmed the admixture nature of all genotypes and 'Tavares limequat' was found to be the most divergent. Conclusively, selected SSR markers effectively discriminated among diploid and polyploid acid limes and may be extended to other citrus species as well. These findings could be helpful for the selection and conservation of heterozygous material and utilization in breeding and germplasm enhancement programs.

Keywords: fruit breeding; germplasm; kagzi lime; molecular markers; ploidy; SSR

Introduction

Among commercial *Citrus* species, limes are one of the most popular and widely cultivated species from 40°N to 40°S around the globe (Mukhopadhyay, 2010). Limes originated from the Indo-Malayan region and

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are an extremely sensitive species to freezing temperatures. Tropical, warm, and humid subtropical regions with average daily temperatures above 4 °C are favorable for lime cultivation. There are a few cultivars that have an expanded harvest window, like 'Eustis limequat' 'EL' (Chinese Lime) and have higher demand in the local market. Acid limes hold great nutraceutical significance with distinct aroma, unique flavor, more minerals, high vitamin C and citric acid contents leading to their wide cultivation (Dubey *et al.*, 2016).

Limes and lemons are ranked as the third most important crops with a 13% share in global citrus production (Kaur *et al.*, 2023). Limes and lemons are largely produced in India, Mexico, China, Türkiye, and Argentina. Globally, 23.64 million tons of lemons and limes have been produced with an average yield of 17 tons ha⁻¹ (FAOSTAT, 2023). Global lime and lemon production is expected to fall from 0.65 to 1.60 million tons in 2024/25 with lower production in the EU and Türkiye due to unfavorable climatic conditions and lower yield (USDAFAS, 2025).

Pakistan has produced 97.57 thousand tons of fresh limes from an area of 12 thousand ha in 2023. During the last five years (2019 to 2023), the area under lime cultivation has declined (-1500 hectares), despite there is a net increase in fruit production (19976 tons), yield (2.41 tons' ha⁻¹) and share in total citrus production (1.5%) (FAOSTAT, 2023). The increase in production could be attributed to improved planting density. Mainly desi lime (seedlings of ML) and 'Eustis limequat' (Chinese Lime) cultivars are being produced while recently Seedless lime 'SL' has also gained some popularity. Hence, it is a dire need to introduce and screen new potential varieties to support germplasm enhancement program by making collections, characterization, conservation and domestication. Proper utilization and conservation of unique genetic resources is critical for increasing quality production for domestic and export markets (Elameen *et al.*, 2008). Phenotypic characterization is prerequisite in describing and classifying germplasm (Fatahi *et al.*, 2004).

Limes were believed to be related to lemon and citron (Barkley *et al.*, 2006); however, they are more complex involving four ancestral species (mandarin, pummelo, citron and papeda) and having diverse ploidy status (Garcia-Lor *et al.*, 2013). 'Mexican lime' 'ML' is diploid and a direct hybrid of citron and papeda (Curk *et al.*, 2015; Wang *et al.*, 2017) whereas Kagzi and West Indian limes are clonal selections of ML. Similarly, many diverse land races of ML are available in the Indo-Pak region, which shall be collected and characterized (Munankarmi *et al.*, 2014). Persian or 'Tahiti lime' (TL) is a spontaneous triploid and seedless variety, and its variants are also available in the market. It is believed to be developed from an unreduced gamete of ML with a haploid ovule of lemon (Wang *et al.*, 2017). A SL variety that resembles TL is also available in the market; however, it has not been characterized yet. Despite the above-discussed findings, there is still controversy in the origin of the different lime varieties (Usman *et al.*, 2019). Among limequats, 'Eustis limequat' 'EL' is commonly cultivated in Pakistan as an alternate lime fruit crop to ML as EL fruit bearing habit and market availability is better than ML. Another limequat Tavaras is also available which also needs to be characterized for fruit quality and genetic variability.

Fruit physical (size and weight) and chemical traits (juiciness, TSS, TA, and vitamin C) are the key factors for selection of elite genotypes in acid limes (Paudyal and Haq, 2008). Germplasm screening shall be done by following standard morphological (IPGRI, 1999) and biochemical markers. However, these markers are variable under diverse climatic conditions and are greatly influenced by environmental factors. Marker-Assisted Selection (MAS) is a useful tool to characterize fruit crops. Simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP), and inter-simple sequence repeats (ISSR) are commonly used genetic markers in citrus and lime species (Fatima *et al.*, 2015; Munankarmi *et al.*, 2018; Gill *et al.*, 2024). SSRs consist of tandem repeats with short nucleotide (1 to 6) sequences which are very useful for genetic characterization. SSRs are abundantly dispersed in mitochondria, chloroplasts, and nuclear genomes. Important genetic characteristics of SSRs include co-dominance, high polymorphism, repeatability, PCR-based, multi-allelic, and ease of access provided by published primer sequences. SSR markers are widely used in many plant species, especially to identify horticultural crops and varieties (Pardo *et al.*, 2023; Doyğacı and Atak, 2024). Moreover, these are also used to determine different traits, such as disease resistance or sex, in the early stages (Atak *et al.*,

2014; Amom *et al.*, 2017; Shidfar *et al.*, 2019; Yildirim *et al.*, 2019). SSR markers are also useful for linkage mapping and genetic characterization. For instance, SSRs have been used for the identification and characterization of nucellar and zygotic seedlings (Yildiz *et al.*, 2013) and polyploid germplasm (Fatima *et al.*, 2015) in *Citrus*. Hence, the potential of available acid lime germplasm, including commercial varieties and bigeneric hybrids was studied for estimation of physico-chemical and genetic variability using SSR markers.

Materials and Methods

Plant materials and physical characterization

Three acid lime genotypes ‘Mexican lime’ (ML), ‘Tahiti lime’ (TL), ‘Seedless lime’ (SL) and two acid lime bigeneric hybrids of *Citrus* and *Fortunella* viz. ‘Eustis limequat’ (EL), and ‘Tavares limequat’ (Tav. L), grafted on rough lemon rootstock, were used to measure physico-chemical and genetic variability in leaf and fruit traits. Young leaves and fresh fruits of these varieties were obtained from 6-7 years old healthy and vigorous plants maintained under standard cultural practices. The mature leaves (05) and fruit (10) samples were collected from Experimental Fruit Garden (EFG), Institute of Horticultural Sciences (IHS), University of Agriculture, Faisalabad (UAF) located at 31.4278°N and 73.0758°E and Citrus Research Institute (CRI), Sargodha located at 32.0740°N and 72.6861°E. Data was recorded for different physical (Table 1) parameters according to citrus descriptors (IPGRI, 1999).

Table 1. Diversity in leaf size, leaf thickness, fruit weight, fruit size and seed traits in acid lime varieties and hybrids

Genotypes	Leaf traits				Fruit and seed traits								
	LL (cm)	LW (cm)	Ratio (LL:LW)	LT (mm)	FW (g)	FL (mm)	FD (mm)	FL:FD	FRT (mm)	NSg	NS	SW (g)	NE
Mexican lime	5.44 ± 0.040 ^d	3.60 ± 0.045 ^b	1.51 ± 0.019 ^c	0.42 ± 0.019 ^c	45.49 ± 0.35 ^c	44.72 ± 0.67 ^b	42.64 ± 0.83 ^a	1.02 ± 0.020 ^c	1.44 ± 0.060 ^b	11.80 ± 0.58 ^a	7.80 ± 0.37 ^b	1.15 ± 0.04 ^a	2.20 ± 0.20 ^a
Tahiti lime	6.54 ± 0.051 ^c	3.26 ± 0.024 ^d	2.01 ± 0.011 ^a	0.74 ± 0.024 ^b	49.24 ± 0.43 ^{bc}	44.36 ± 0.34 ^b	42.96 ± 0.21 ^a	1.03 ± 0.012 ^c	1.84 ± 0.040 ^a	8.80 ± 0.20 ^{cd}	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	0.00 ± 0.00 ^b
‘Seedless lime’	9.88 ± 0.066 ^a	5.68 ± 0.037 ^a	1.74 ± 0.012 ^b	0.33 ± 0.017 ^d	52.44 ± 0.72 ^b	46.80 ± 0.64 ^b	43.84 ± 0.29 ^a	1.11 ± 0.019 ^b	1.98 ± 0.037 ^a	9.40 ± 0.24 ^{bc}	0.40 ± 0.24 ^c	0.04 ± 0.02 ^c	0.60 ± 0.40 ^b
Eustis limequat	4.92 ± 0.037 ^c	2.36 ± 0.051 ^d	2.09 ± 0.039 ^a	1.34 ± 0.024 ^a	28.46 ± 0.33 ^d	36.12 ± 0.24 ^c	35.04 ± 0.08 ^b	1.03 ± 0.007 ^c	1.60 ± 0.032 ^b	7.40 ± 0.24 ^d	9.00 ± 0.31 ^a	0.79 ± 0.03 ^b	1.60 ± 0.24 ^a
‘Tavares limequat’	7.20 ± 0.063 ^b	3.64 ± 0.068 ^{bc}	1.97 ± 0.039 ^a	0.34 ± 0.017 ^{cd}	74.55 ± 2.33 ^a	63.40 ± 1.61 ^a	33.37 ± 1.08 ^b	1.90 ± 0.016 ^a	0.94 ± 0.049 ^c	10.80 ± 0.37 ^{ab}	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	0.00 ± 0.00 ^b

Means sharing similar letters in a column are statistically non-significant (P>0.05)

Abbreviations: Leaf Length (LL), Leaf Width (LW), Leaf Ratio (LL: LW), Leaf Thickness (LT), Fruit Weight (FW), Fruit length (FL), Fruit Diameter (FD), Fruit ratio (FL:FD), Fruit Rind Thickness (FRT), No. of seeds (NS), No. of Segments (NSg), Seed weight (SW), No. of Embryo/seed (NE)

Biochemical characterization

Fruit samples were characterized by biochemical traits. Juice was extracted from freshly harvested fruits using a sharp knife, homogenized, and further utilized in aliquot preparation to study biochemical parameters. Total soluble solids (TSS) were recorded in °Brix by using a digital Refractometer (RX 5000, ATAGO, Japan). Standard titration method using alkali (1N NaOH) and 1% phenolphthalein (dye) was performed to calculate titratable acidity (TA) as described by Hortwitz (1960). Sugar acid ratio (TSS: TA) was calculated by dividing the values of TSS by TA. The protocol described by Ruck (1963) was utilized for the detection of ascorbic acid (AA) content and absorbance was taken at 520 nm. Ten ml of juice was diluted with a 0.4% solution of oxalic acid, and a 5 ml filtered aliquot was prepared. The developed solution was titrated against 2,6-dichlorophenol indophenol dye, which has a blue color. The light pink color was taken as the titration end point. Total anthocyanins (AN) were determined using the standard pH-differential method and absorbance was taken at 510 nm and 700 nm (Giusti and Wrolstad, 2001) using spectrophotometer (UV-1601 Shimadzu, Japan).

Total AN was calculated following standard procedures (Canan *et al.*, 2016). Total sugars (TS), reducing (RS) and non-reducing sugars (NRS) were determined by following the standard protocol of Sadasivam and Manickam (1992).

Experimental layout and data analysis

Mature leaf and fruit samples were collected for physico-chemical characterization of acid limes genotypes planted under a randomized complete block design (RCBD). The collected data were statistically analyzed by the Least Significant Difference test (LSD) for mean comparison (Steel *et al.*, 1997). Multivariate analysis was performed using XLSTAT 2014 software. Relatedness among genotypes was studied by PCA analysis. A correlation matrix among traits was developed by using mean values. Scattered plots were developed by following PCA analysis using XLSTAT software. Dendrograms were constructed according to the UPGMA method as described by Sokal and Michener (1958).

DNA isolation and molecular marker analysis

Total genomic DNA was isolated from young leaves using the procedure described by Khan *et al.* (2004) with minor modifications. After quality determination of the extracted DNA using a spectrophotometer (NanoDrop 2000, Thermo Scientific), the templates were diluted to 50 ng μL^{-1} for the polymerase chain reactions (PCR). SSR assays were performed using citrus-specific 20 SSR primers described by Kijas *et al.* (1997) and Barkley *et al.* (2006) to estimate genetic diversity in lime varieties. They were TAA3, TAA15, TAA27, TAA33, TAA41, CAC15, CAC23, CAC33, CAC39, cAGG9, CCT01, CTT01, GT03, AC01, CAG01, CAT01, ATC09, AG14, CT02 and CT19. The PCR reaction for each primer was carried out in a volume of 25 μL containing 50 ng/ μL template DNA, PCR buffer (10X), 2 mM MgCl_2 , dNTPs 200 μL each, 15 ng of each primer and 1.5 units of Taq DNA polymerase (Fermentas, UK). PCR was carried out on a T-100 Thermocycler (BioRad, USA) on the optimized conditions for a profile of 35 cycles: 95 °C for 1 min, different annealing temperatures (45-65 °C) for 30 s, 72 °C for 1 min and final extension at 72 °C for 7 min. The PCR products were separated on 3% denaturing polyacrylamide gel electrophoresis (PAGE) in TBE buffer (1x). The gels were stained using silver nitrate.

Molecular data analysis

Scoring of amplified products (fragment) was carried out by following binary matrix in which the presence of a band was denoted by '11' and absence of a band was denoted by '00' and a similarity matrix was constructed using Jacard's coefficient. Total number of alleles amplified per primer were counted. The accessions were grouped by cluster analysis using UPGMA (unweighted-pair group method with arithmetic averages) using popGen32 software. Major allelic frequency (MAF), gene diversity (He), observed heterozygosity (Ho) and polymorphism information content (PIC) was calculated using GenAlEx (Ver 6.5.1) (Peakall and Smouse 2012) and Microsoft tools programs. PIC values for codominant SSR markers ranged from monomorphic (0) to highly discriminative (1). The value of a marker refers to detected polymorphism (gene diversity) within a population. The dendrograms were constructed by Dendroscope (Ver 3.7.5).

Results

Phenotypic diversity

Foliage traits

Trees of all acid lime varieties and hybrids were evergreen having simple leaf division (only one leaf attached to a stalk). Leaf color intensity varied from dark green (ML, TL), medium green (EL and SL) to light green (Tav. L) while leaf color variegation was absent in all genotypes. Leaf lamina shaped elliptic (ML and EL) to obcordate (TL, SL and Tav. L). Leaf margins were entire (ML, EL and TL) and crenate (SL and Tav. L). All

leaves had brevipediculate (petiole shorter than leaf lamina) type of leaf lamina attachment while leaf apex was acuminate.

Maximum leaf size (length 9.88 cm and width 5.68 cm) and leaf area was measured (0.33 cm²) in SL whereas minimum values for these traits were noted in EL (Figure 1, Table 1).

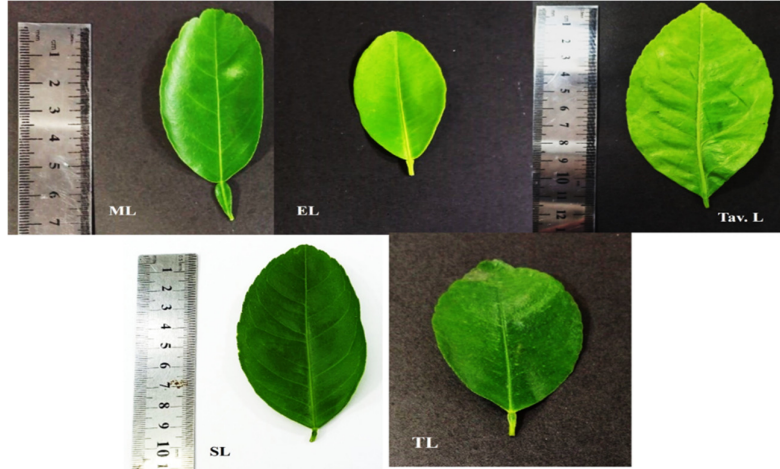


Figure 1. Foliage diversity in acid lime varieties and hybrids: ML ('Mexican lime'), EL ('Eustis limequat'), TL (Tahiti lime), SL ('Seedless lime') and Tav. L ('Tavares limequat')

Fruit traits

Fruit weight and fruit size

Significant genotypic differences were noted in all acid lime varieties. Fruit weight and fruit length was greater in TL (74.55 g, 63.40 mm) while the least values of fruit weight and length were noted in EL (28.46 g, 36.12 mm). Fruit diameter was maximum in SL (43.84 mm) while minimum values were found in TL (33.37 mm). Fruit ratio (FL: FD) was more in TL (1.90) while minimum ratio was found in ML (1.02) (Figure 2, Table 1).

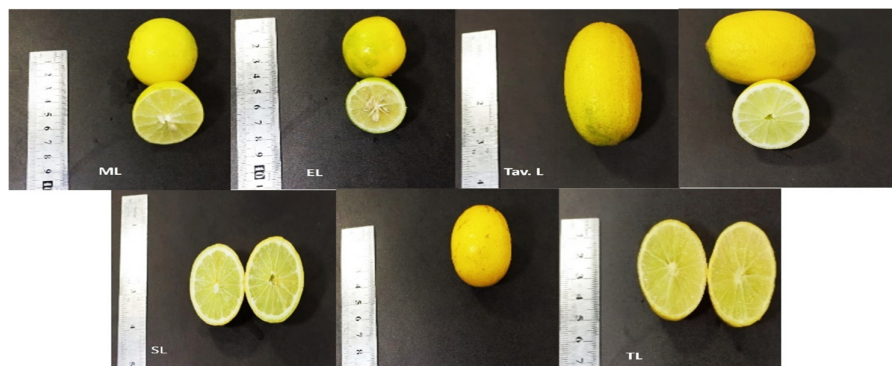


Figure 2. Morphological diversity in fruit of diploid and triploid acid lime varieties and hybrids. ML ('Mexican lime'), EL ('Eustis limequat'), TL ('Tahiti lime'), SL ('Seedless lime') and Tav. L ('Tavares limequat')

Rind thickness, number of segments and number of seeds and embryo number

Maximum fruit rind thickness was observed in SL (1.98 mm) while rind thickness was minimum in Tav. L (0.94 mm). The number of segments was higher in ML (11.80) while the lowest number was observed in EL (7.40). TL and Tav. L were seedless with no seeds while EL was noted as a highly seedy variety (9.00). Polyembryony was noted in ML with a maximum of 2.20 embryos per seed (Table 1).

Fruit biochemical diversity

Juice pH, juice weight and juice percentage

Juice was more acidic in EL (1.80) whereas acidity was low in genotypes like SL (2.54) and Tav. L (3.4). Juice weight (39.61 g) was more in Tav. L while juice % was higher in SL (54.19 %) (Table 1).

Total soluble solids, titratable acidity and ascorbic acid

Among acid lime genotypes, total soluble solids (TSS) ranged from 9.25 °Brix in TL to 7.90 °Brix in EL. Titratable acidity was higher in EL (6.54 %) while it was lower in Tav. L (3.13 %). Higher sugar to acid ratio (TSS: TA) was noted in Tav. L (2.82) and it was lower in EL (1.21). ML had maximum ascorbic acid (38.31 mg/100 ml) while TL had minimum ascorbic acid contents (29.38 mg/100 ml) (Table 2).

Anthocyanins and soluble sugars

Anthocyanin contents were higher in SL (0.39 mg/ 100 ml) while minimum anthocyanin contents were noted in EL (0.21). Higher total soluble sugars (%) were found in TL (6.08 %) while minimum sugars were found in EL (4.88 %). Reducing sugars % were more in Tav. L and ML (3.06 %) while minimum values were found in EL (2.63 %). Similarly, non-reducing sugars were more in SL (2.98 %) (Table 2).

Table 2. Diversity in fruit juice (%), juice weight (g) and other juice quality attributes in Acid lime varieties and hybrids

Genotypes	J (%)	JW (g)	J pH	TSS (°Brix)	TA (%)	TSS:TA	AA (mg/100 ml)	AN (mg/100 ml)	TS (%)	RS (%)	NRS (%)
Mexican lime	51.45 ± 1.54 ^a	23.40 ± 0.73 ^d	2.28 ± 0.02 ^c	8.34 ± 0.15 ^b	5.73 ± 0.09 ^b	1.46 ± 0.03 ^c	31.31 ± 0.35 ^b	0.30 ± 0.01 ^b	5.67 ± 0.15 ^b	3.06 ± 0.06 ^a	2.48 ± 0.19 ^{bc}
Tahiti lime	52.22 ± 1.38 ^a	25.69 ± 0.49 ^c	2.48 ± 0.01 ^{bc}	9.25 ± 0.03 ^a	4.44 ± 0.13 ^c	2.09 ± 0.06 ^b	29.38 ± 0.47 ^c	0.31 ± 0.01 ^b	6.08 ± 0.06 ^a	2.97 ± 0.05 ^{ab}	2.95 ± 0.09 ^{ab}
'Seedless lime'	54.19 ± 0.40 ^a	28.41 ± 0.36 ^b	2.54 ± 0.01 ^b	8.93 ± 0.02 ^a	5.76 ± 0.04 ^b	1.55 ± 0.01 ^c	34.51 ± 0.38 ^a	0.39 ± 0.01 ^a	5.81 ± 0.08 ^{ab}	2.67 ± 0.12 ^b	2.98 ± 0.06 ^a
'Eustis limequat'	31.33 ± 0.75 ^b	8.91 ± 0.21 ^c	1.80 ± 0.10 ^d	7.90 ± 0.01 ^b	6.54 ± 0.17 ^a	1.21 ± 0.03 ^d	32.85 ± 0.29 ^b	0.21 ± 0.01 ^c	4.88 ± 0.01 ^c	2.63 ± 0.12 ^b	2.13 ± 0.11 ^c
'Tavares limequat'	53.30 ± 1.32 ^a	39.61 ± 0.32 ^a	3.42 ± 0.03 ^a	8.83 ± 0.17 ^a	3.13 ± 0.04 ^d	2.82 ± 0.02 ^a	38.89 ± 0.49 ^a	0.34 ± 0.02 ^{ab}	5.93 ± 0.05 ^{ab}	3.06 ± 0.05 ^a	2.73 ± 0.03 ^{ab}

Means sharing similar letters in a column are statistically non-significant (P>0.05)

Abbreviations: Juice pH (J pH), Juice Weight (JW), Juice % (J%), Total soluble solids (TSS), Titratable Acidity (TA), Fruit to acid ratio (TSS:TA), Ascorbic acid (AA), Anthocyanins (AN), Total soluble sugars (TS), Reducing sugars (RS), Non-reducing sugars (NRS)

Correlations for physico-chemical parameters

Strong positive correlations were observed among most of the physical and chemical traits. Fruit weight was positively correlated with fruit length (0.98), juice pH (0.995), juice weight (0.995) and ratio TSS: TA (0.906) and negatively correlated with TA (-0.90). Fruit length was positively correlated with ratio FL: FD (0.927), juice pH (0.987) and ratio TSS: TA (0.904) whereas FL was negatively correlated with TA (0.890). Ratio FL: FD was positively correlated with juice pH (0.888). Number of segments was positively correlated with embryos (0.929) while it was negatively correlated with TSS (-0.938) and non-reducing sugars (-0.919). Seed weight was positively correlated with number of embryos (0.976). Juice pH was positively correlated with

JW (0.969) and TSS: TA (0.933) while it was negatively correlated with TA (-0.923). Juice % was positively correlated with total sugars (0.946) and anthocyanins (0.886). TSS was positively correlated with non-reducing sugars (0.966) and total sugars (0.927). Titratable acidity was negatively correlated with ration TSS: TA (-0.995). Total sugars were positively correlated (0.900) with non-reducing sugars (Table 3).

Table 3. Correlation coefficients among 20 quantitative traits in 05 acid lime varieties and hybrids

	FW	FL	FD	FL:FD	FRT	NS	Seg	SW	E	JpH	JW	JP	TSS	TA	TSS:TA	AA	AN	TS	RS
FL	0.982**	-																	
FD	-0.215	-0.365																	
FL:FD	0.851	0.927*	-0.687																
FRT	-0.549	-0.688	0.663	-0.815															
Seg	0.590	0.593	0.257	0.368	0.505														
NS	-0.760	-0.644	-0.060	-0.469	-0.057	-0.109													
Seg	-0.623	-0.529	0.145	-0.459	-0.104	0.188	0.947*												
E	-0.661	-0.581	0.210	-0.530	0.030	0.151	0.929*	0.976**											
JpH	0.995**	0.987**	-0.296	0.888*	-0.583	0.532	-0.756	-0.645	-0.692										
JW	0.987**	0.943*	-0.058	0.757	-0.440	0.635	-0.794	-0.624	-0.646	0.969**									
JP	0.760	0.642	0.470	0.310	-0.036	0.692	-0.735	-0.480	-0.461	0.702	0.854								
TSS	0.632	0.480	0.360	0.224	0.214	0.176	-0.938*	-0.821	-0.819	0.610	0.707	0.812							
TA	-0.902*	-0.890*	0.301	-0.821	0.593	-0.407	0.729	0.640	0.755	-0.923*	-0.864	-0.600	-0.645						
TSS:TA	0.906*	0.904*	-0.382	0.862	-0.611	0.357	-0.731	-0.667	-0.775	0.933*	0.857	0.552	0.613	-0.995**					
AA	0.430	0.529	-0.200	0.496	-0.676	0.792	0.198	0.362	0.367	0.401	0.406	0.258	-0.304	-0.164	0.169				
AN	0.737	0.628	0.299	0.356	0.086	0.475	-0.794	-0.630	-0.530	0.688	0.816	0.886*	0.747	-0.461	0.454	0.252			
TS	0.758	0.630	0.402	0.330	-0.027	0.525	-0.837	-0.625	-0.651	0.720	0.837	0.946*	0.927*	-0.730	0.680	0.013	0.780		
RS	0.602	0.599	0.117	0.438	-0.601	0.772	-0.237	0.002	-0.153	0.582	0.612	0.589	0.355	-0.707	0.644	0.389	0.201	0.635	
NRS	0.615	0.455	0.440	0.168	0.306	0.226	-0.919*	-0.787	-0.732	0.578	0.708	0.859	0.966**	-0.519	0.492	-0.201	0.870	0.900*	0.234

Values with * = Significant (P<0.05); ** = Highly significant (P<0.01)

Abbreviations: Fruit Weight (FW), Fruit length (FL), Fruit Diameter (FD), FL: FD, Fruit Rind Thickness (FRT), No. of seeds (NS), No. of Segments (NSg), No. of Embryo/seed (E), Juice pH (JpH), Juice Weight (JW), Juice % (J %), Total soluble solids (TSS), Titratable Acidity (TA %), Fruit to acid ratio (TSS:TA), Ascorbic acid (AA), Anthocyanins (AN), Total soluble sugars (TS), Reducing sugars (RS), Non-reducing sugars (NRS)

Principle component analysis (PCA) and Cluster analysis for fruit physico-chemical traits

A PCA plot was constructed for physico-chemical traits of acid lime varieties which depicted a total variation of 75.05% based on two component factors F1 (54.25%) and F2 (20.82%). Genotypes near the centre of an axis showed less variation compared with the scattered ones. ML and EL were clustered together at the lower right plane of the plot and illustrated less diversity in physico-chemical traits. Similarly, two genotypes (SL and TL) were grouped at the upper left plane of the plot due to greater similarity in fruit size, shape and seedlessness. Whereas Tav. L was found to be more divergent from other acid lime genotypes and was an outlier in the population due to its distinct fruit shape, size and physico-chemical traits (Figure 3). Cluster analysis was performed to assess the similarity matrix among different genotypes and a dendrogram was constructed based on variance data using agglomerative hierarchical clustering. Genotypes were partitioned into three major clusters including C1, C2 and C3 and sub-groups. Cluster 1 was separated with a similarity matrix of 0.99 and further divided into sub-group A having two genotypes (TL and SL). Cluster 2 was separated, having similarity matrix of 0.94 and was sub-divided into two groups B (ML) and C (EL). Cluster 3 contained only one genotype with the least similarity matrix of 0.88 indicating that the Tav. L was the most divergent genotype which is also aligned with the PCA plot. Cluster analysis revealed significant diversity among all acid lime varieties (Figure 3).

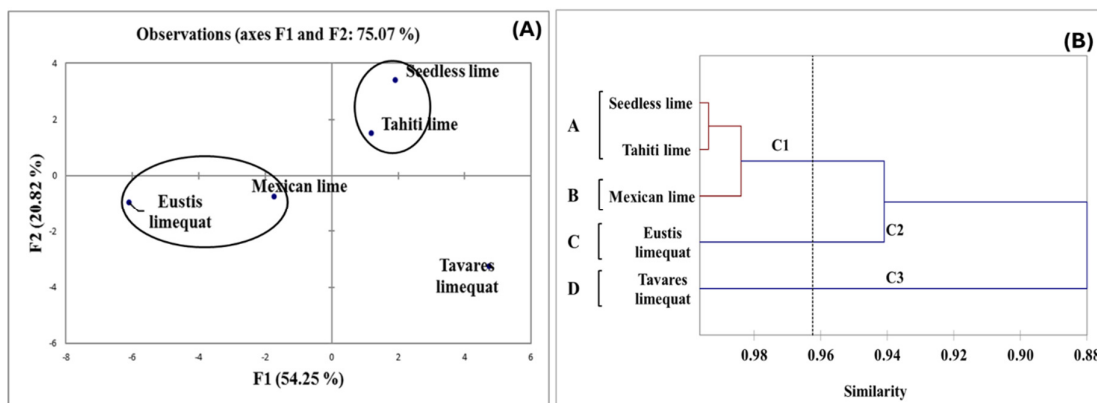


Figure 3. Two dimensional PCA plots (A) based on first two components for physico-chemical quantitative traits of 05 genotypes of acid limes, (B) cluster analysis developed from similarity matrix based on Ward’s (1963) method

Genetic diversity

A total of twenty SSR primers (P₁-P₂₀) were used to evaluate genetic polymorphism in acid lime varieties. All primers showed the range of allelic length of resolution of amplified DNA fragments on a PAGE gel from 100 to 300 bp in acid lime varieties (Figure 4). Among all varieties, average value for major allelic frequency (MAF), genetic diversity (GD), heterozygosity (Hz) and Polymorphism Information Content (PIC) was 0.65, 0.39, 0.37 and 0.57, respectively. Maximum MAF (0.82) was showed by primer P₇ followed by P₁₂ (0.8) and P₄ (0.74) while minimum MAF (0.51) was observed in P₁₉. Similarly, P₇ showed the maximum genetic diversity (0.47) followed by P₁₂ (0.45) while least genetic diversity (0.31) was noted in P₁₉ and P₂₀. Maximum PIC value was 0.76 in P₇ followed by P₁₂ (0.71) while minimum value of PIC was 0.43 in P₁₉ (Table 4).

Table 4. Characteristics of 20 SSR markers and summary of genetic diversity in acid lime varieties and hybrids.

Markers	Loci	Primer Sequence		Size of amplified bands (bp)	Number of bands amplified/primer	Major Allelic Frequency (MAF)	Gene Diversity (GD)	Heterozygosity (HZ)	Polymorphism Information Contents (PIC)
		Forward	Reverse						
P1	TAA3	AGAGAAGAACATT TGCGGAGC	GAGATGGGACTT GGTTCATCACG	200-300	10	0.62	0.33	0.37	0.59
P2	TAA15	GAAAGGGTTACTT GACCAGGC	CTTCCCAGCTGC ACAAGC	200-250	8	0.52	0.35	0.29	0.51
P3	TAA27	GGATGAAAAATGCT CAAAATG	TAGTACCCACAG GGAAGAGAGC	150-250	9	0.65	0.41	0.47	0.53
P4	TAA33	GGTACTGATAGTA CTGCGGCG	GCTAATCGCTAC GTCTTCGC	250-300	10	0.74	0.28	0.35	0.54
P5	TAA41	AGGTCTACATTGG CATTGTC	ACATGCAGTGCT ATAATGAATG	200-250	10	0.7	0.43	0.44	0.52
P6	CAC15	TAAATCTCCACTCT GCAAAAGC	GATAGGAAGCGT CGTAGACCC	250-300	9	0.62	0.29	0.33	0.66
P7	CAC23	ATCACAATTACTAG CAGCGCC	TTGCCATTGTAG CATGTTGG	250-300	11	0.82	0.47	0.48	0.76
P8	CAC33	GGTGATGCTGCTA CTGATGC	CAATTGTGAATT TGTGATTCCG	150-250	9	0.76	0.38	0.36	0.57
P9	CAC39	AGAAGCCATCTCT CTGCTGC	AATTCAGTCCCA TTCCATTCC	200-250	10	0.78	0.4	0.36	0.62
P10	cAGG9	AATGCTGAGATAA TCCGCG	TGCCTTGCTCTC CACTCC	250-300	9	0.67	0.47	0.46	0.54
P11	CCT01	TCAACACCTCGAA CAGAAGG	CCCACATGCTAG CACAAAGA	250-300	10	0.58	0.32	0.34	0.64
P12	CTT01	TCAGACATTGAGTT GCTCG	TAACCACTTAGG CTTCGGCA	200-250	9	0.8	0.45	0.46	0.71

P13	GT03	GCCTTCTTGATTTA CCGGAC	TGCTCCGAACTT CATCATTG	250-300	9	0.72	0.34	0.39	0.53
P14	AC01	TTTGACATCAACAT AAAACAAGAAA	TTTAAAAATCCC TGACCAGA	200-300	8	0.59	0.45	0.4	0.59
P15	CAG01	AACACTCGCACCA AATCCTC	TAAATGGCAACC CCAGCTTTG	250-300	10	0.68	0.44	0.39	0.61
P16	CAT01	GCTTTCGATCCCT CCACATA	GATCCCTACAAT CCTTGGTCC	200-250	10	0.6	0.47	0.34	0.53
P17	ATC09	TTCCTTATGTAATT GCTCTTTG	TGTGAGTGTTTG TGCGTGTG	250-300	9	0.62	0.46	0.36	0.52
P18	AG14	AAAGGGAAAGCCC TAATCTCA	CTTCCTCTTGCG GAGTGTTC	150-250	9	0.54	0.46	0.29	0.56
P19	CT02	ACGGTGCGTTTTG AGGTAAG	TGACTGTTGGAT TTGGGATG	200-250	8	0.51	0.31	0.23	0.43
P20	CT19	CGCCAAGCTTACC ACTCACTAC	GCCACGATTTGT AGGGGATAG	250-300	8	0.55	0.45	0.33	0.48
Average				150-300	9.25	0.65	0.39	0.37	0.572

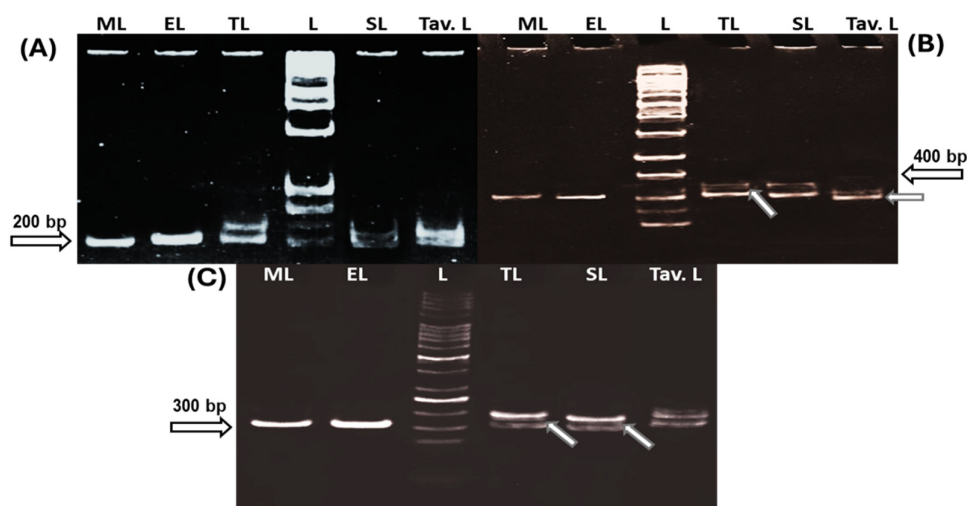


Figure 4. SSR marker based genetic diversity analysis in acid lime germplasm on PAGE Gel using CAC39 (A), CAC23 (B) and CTT01 (C) markers where L stands for 100 bp DNA marker. Arrows with black outline show ladder band size whereas arrows with grey outline show unique bands amplified in different acid limes genotypes. Where ML stands for ‘Mexican lime’, EL for ‘Eustis limequat’, TL for ‘Tahiti Lime’, SL for ‘Seedless lime’ and Tav. L. for ‘Tavaras Limequat’

PCoA and cluster analysis

Principal coordinate analysis (PCoA) based on genotyping by using 20 SSR primers revealed that two genotypes including SL and TL were assembled in lower quadrant having low genetic distance and higher resemblance. Similarly, ML and EL were put in the positive quadrant due to less diversity and significant genetic distance. However, Tav. L was placed in the left positive quadrant with maximum genetic distance exhibiting it as the most divergent genotype (Figure 5). Cluster analysis and dendrogram construction based on binary data of genetics for the acid lime genotypes developed three major clusters (C1-C3). Cluster 1 contained two closely related genotypes including TL and SL; Cluster 2 included EL and ML and Cluster 3 separated with the maximum genetic distance on Wards scale and had single genotype Tav. L which was referred to as the most divergent genotype (Figure 5).

Analysis of molecular variance (AMOVA) and population structure analysis

Analysis of molecular variance based on SSR genotyping data showed 88% genetic variation among populations while only 12% variation was found within populations indicating the existence of a considerable

genetic diversity in acid lime varieties (Figure 6). Population structure analysis was performed by using the STRUCTURE software to assess genetic diversity. Twenty SSR primers were employed to estimate the sub-population number (K) of acid lime varieties. Results were further analyzed by STRUCTURE harvester, and it was evident that the maximum peak of the delta K plot was observed at K=3 (Figure 6). A graphical illustration showed variation in the coloring pattern of bars. All five acid lime varieties and hybrids were admixtures and none of them were found to be pure lines due to their hybrid nature. Whereas vertical bars of P₃ (TL) and P₄ (SL) showed similar banding patterns exhibiting their close association with each other. Whereas P₅ (Tav. L) was found to be most divergent depicting clearer variations than other varieties (Figure 6).

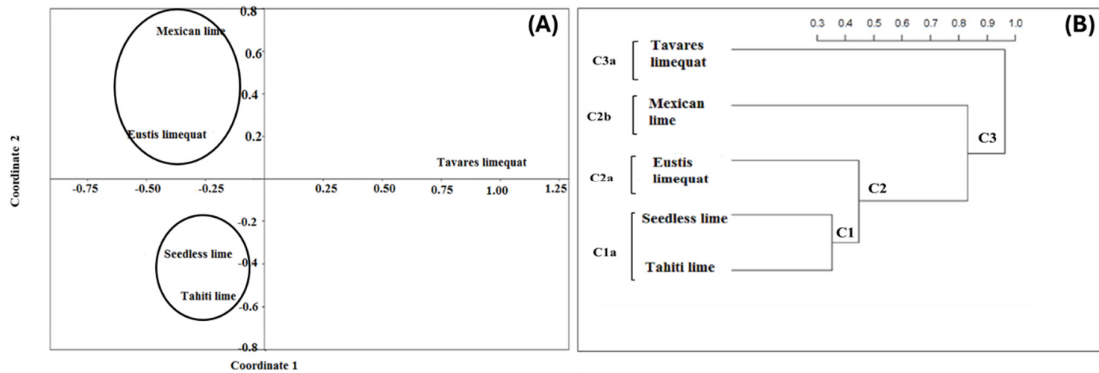


Figure 5. Principal coordinate (A) and cluster analysis (B) for 05 acid lime varieties and hybrids based on SSR markers genetic diversity analysis

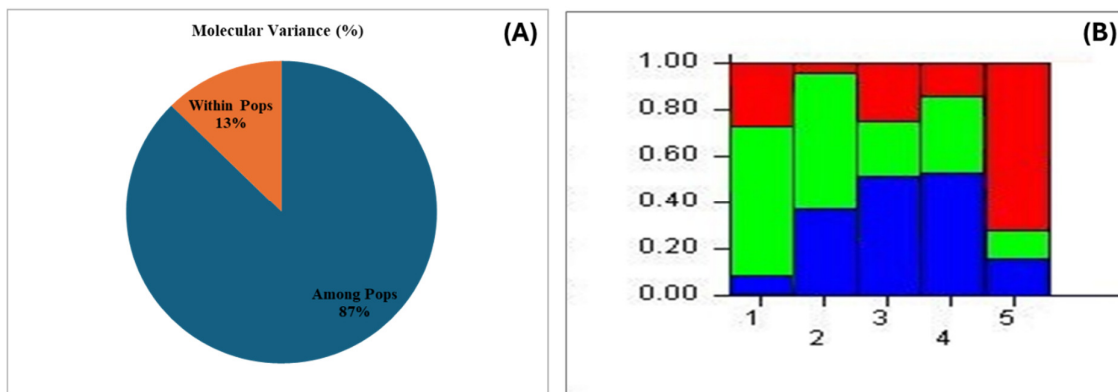


Figure 6. (A) Analysis of Molecular Variance (B) proportion of acid lime varieties and intergeneric hybrids with Model-Based Bayesian Structure Analysis

Note: Vertical line indicates an individual genotype, and each color represents different cluster where 1 ('Mexican lime'), 2 ('Eustis limequat'), 3 ('Tahiti Lime'), 4 ('Seedless lime') and 5 ('Tavaras Limequat')

Discussion

Global consumption of acid limes has increased in the last few decades leading to enhanced cultivation across tropical and subtropical regions. In USA, per capita consumption of limes is greater than oranges, lemon and grapefruit. Consumption of fresh lime is also increasing in Pakistan whereas there are only two varieties being commercially cultivated viz. ML and EL. Hence, there is need to screen the available germplasm resources, introduce exotic germplasm for diversification, identification of new candidate varieties and selection of

parents for crop improvement programs (Rouiss *et al.*, 2018; Rana *et al.*, 2020). Morpho-chemical characterization is utilized for selection of varieties with better fruit size and quality. Amongst qualitative and quantitative traits, quantitative traits are less influenced by the environment and are more reliable (Zamani *et al.*, 2013; Lado *et al.*, 2014). Frequent bud mutation, widespread sexual compatibility between related species, apomixes, and a long history of cultivation are the key factors causing higher genetic diversity in Citrus (Breto *et al.*, 2001). Moreover, varietal variability, agroclimatic conditions, ripening time and time of harvest may also affect citrus fruit quality (Lux *et al.*, 2019; Usman *et al.*, 2020a; Vincent *et al.*, 2020). The current study was conducted to characterize the available acid lime germplasm for morphogenetic diversity, to develop novel genetic markers for identification of diploids and polyploids, to broaden the varietal base and the market window, and in crop improvement programs.

Wide variation has been reported in foliage and fruit quality of different lime varieties (Prasanna *et al.*, 2023; Kaur *et al.*, 2023). In current study, remarkable variability was noticed in leaf size, fruit size, fruit shape, number of seeds and peel thickness in the lime germplasm. The largest leaves were noted in SL while smallest leaves were found in EL. Similarly, Tav. L had the longest and heavier fruit while fruit diameter was larger in SL. Large fruit and foliage size in Tav. L (2N) could be attributed to its hybrid nature (West Indian lime x oval kumquat) compared with EL (West Indian lime x round kumquat) and ML varieties. Limes have natural diploids and polyploids including triploid 'Tahiti lime' (TL) and tetraploid 'Giant Key' lime (Curk *et al.*, 2016). SL variety is presumed to be a closely related variant of TL which was verified by its matching morpho-chemical data of fruit and leaves with TL in the current study. Fruit weight, juice percentage and juice weight of SL was greater than TL and ML varieties. In limes, higher TA, thin fruit peel and more juiciness are important traits of economic interest and for breeding programs. The seeds and juice acidity was greater in EL and SL whereas juice weight was maximum in Tav. Greater fruit size and weight of SL led to more juice development compared with smaller fruit of ML and TL. Similarly, fruit of Tav. L was larger and heavier than SL and produced more juice (%) and juice weight. Higher juice content was reported in the heavier citrus fruit including sweet orange (Ketsa, 1988). Similar variability in fruit quantitative traits was reported by Zandkarimi *et al.* (2011) in different lime and lemon accessions of Iran. Like other citrus, seedlessness is also a desired trait in limes. In citrus, fruit having less than 5 seeds is considered as seedless (Khan and Kender, 2007). Hence, Tav. L, TL and SL varieties having less than five seeds are referred to as seedless genotypes while ML and EL are considered as low seeded genotypes. These findings indicate the potential of both SL and Tav. L as candidate seedless varieties for large and heavier fruit with more juice content compared with other acid lime varieties.

The association studies of different traits revealed a positive correlation between FL and FD with the NS in acid lime genotypes. Similar correlation was narrated by Andrade-Rodriguez *et al.* (2004) in 'Volkamerian' lemon. A negative correlation was observed in ratio TSS: TA and TA which was in accordance with Zandkarimi *et al.* (2011). A negative correlation between TA and fruit size was observed in this study as reported by Ketsa (1988) in other citrus cultivars. Ratio FL: FD was directly correlated to juice pH indicating more acidity in smaller sized fruit as noted in EL fruit (small size) compared with other lime varieties having large fruit size. Gorinstein *et al.* (2001) also studied the similar relations among biochemical parameters of different citrus fruits.

Morphological traits have been widely used to identify phenotypic similarities in different varieties (Moukoumbi *et al.*, 2011). however, morphological markers are epistatic- influenced by environment and do not reveal the exact genetic relationship among different varieties (Chesnokov *et al.*, 2020) Conversely, molecular markers are an ideal tool to assess the genetic relationship among different species (Vershany *et al.*, 2005; Yildirim *et al.*, 2019; Usman *et al.*, 2020b). Among PCR-based markers, the SSR markers have been widely used to elucidate genetic diversity in different citrus crops including limes (Golein *et al.*, 2006), grapefruit hybrids (Yildiz *et al.*, 2013), mandarins and sweet oranges (Fatima *et al.* 2015; Jabeen *et al.*, 2023). In the current study, twenty SSR markers were used to assess genetic diversity which amplified 185 putative

alleles varying from 8 (TAA15, AC01, CT02 and CT19) to 11 (CAC23) with an average of 9.5 alleles per locus which highlights existence of great polymorphism. The amplified fragments ranged from 150-300 bp and the bands (alleles) amplified by SSR primer sets showed great polymorphism. Most of the primer pairs have amplified bands ranging from 200-250 bp and 250-300 bp whereas a few primer pairs amplified bands ranging from 150-250 (TAA27, CAC33, AG14) and 200-300 bp (TAA3, AC01). These findings imply that 15 primer pairs that amplified 200-300 bp are more common in the genomes of acid limes species and hybrids whereas 05 primers pairs that amplified 150-250 and 200-300 bp are relatively less common. Comparison of SSR markers revealed a maximum number of alleles amplified at CAC23 loci (11) and CTT01 (9) loci, hence these loci are the most informative and common loci in the acid limes. In contrast, SSR markers CT02 and CT19 amplified the lowest number of alleles (8) and were less common. Similar findings of most informative (TAA41 and TAA52) and least informative (TAA27) SSR markers were reported in citrus species including unknown local varieties of acid limes (Munankarmi *et al.*, 2023). Discriminatory power of the loci could be estimated by the PIC values calculated by considering the number of alleles and their relative frequencies (Ogunkanmi *et al.*, 2008). The average PIC value was noted to be 0.572 in the current study whereas average PIC value was 0.625 in research focused on sweet orange and mandarins (Barkley *et al.*, 2006) and the highest PIC values were noted for TAA1 in Iranian acid limes (Raheb *et al.*, 2019). Higher discriminatory power of the SSR markers is essentially required for variation analysis in the gene pool and to identify the closely related individuals (Ogunkanmi *et al.*, 2008). Heterozygosity is also an important measure of genetic variability (Ellegren, 2004). In the current study, the PIC values of the two SSR markers CAC23 and CTT01 were considerably higher (0.71-0.76) along with higher heterozygosity and MAF compared with other SSR markers and are considered as highly polymorphic loci. In contrast, only one allele (176-205 bp) was amplified at locus CAC23 in navel oranges and mandarins (Hussein *et al.* 2003) whereas Kijas *et al.* (1997) have reported up to six bands at CAC33 locus. CAC23, TAA41 and GT03 were also reported as markers with the highest number of accession specific alleles. and CAA23 amplified 9 alleles with PIC value 0.600 in citrus germplasm (Barkley *et al.*, 2006). In contrast to our findings, another recent study on kagzi and ornamental limes revealed that the alleles amplified by CTT01 and CAC39 markers ranged from 100-200 bp (Gill *et al.*, 2024). Thus, the above discussed markers CAC23 and CTT01 could be more useful in discrimination of acid lime genotypes for genetic variability estimation.

TL is a known natural triploid which was remarkably revealed by CAC23 and CTT01 SSR markers amplifying three alleles with the highest PIC values compared with diploid genotypes (ML and EL) which were monomorphic at CAC23, CAC39 and CTT01 loci highlighting greater polymorphism in TL and SL varieties. CAC23 marker amplified two alleles in Tav. L including a unique allele. SL is considered as a closely related variant of TL which was also confirmed by amplification of three similar alleles when using CAC23 and CTT01 markers. TL and SL had least genetic distance in the PCoA, the dendrogram and were clustered together highlighting the possibility of these two genotypes to be clonal selection of Persian lime. Sharafi *et al.* (2016) agreed with our findings as they also reported few unique alleles in Persian lime (3N) cultivar through SSR marker analysis. Similarly, SSR markers detected genetic polymorphism and amplified unique alleles in the polyploid germplasm of Kinnow mandarin and Succari sweet orange (Fatima *et al.*, 2015). Watanabe *et al.* (2008) has also confirmed the triploid nature of the pear varieties by exhibiting 3-alleles produced by SSR markers. Grouping of different lime varieties and hybrids using UPGMA based cluster analysis revealed close association of TL and SL varieties, ML and EL varieties and Tav. L was found distantly related to all the four genotypes indicating greater diversity in Tav. L. Genetic diversity is mandatory in fruit crops for successful breeding programs and developing resistance against biotic and abiotic stresses. In acid lime germplasm, considerable genetic diversity was noted, and the most related species (SL and TL) were aligned together, indicating the higher efficiency of the used SSR markers in discriminating genetic relatedness in acid lime species.

Conclusions

Characterization of acid limes revealed the existence of great morphogenetic diversity among five genotypes which could be useful for germplasm enhancement. Fruit size, weight, ascorbic acid and anthocyanin contents were better in Tav. L and SL. Ratio sugar: acid was the lowest in EL, ML and SL varieties. Varieties (TL, SL and Tav. L) having less than five seeds were categorized as seedless varieties. SL fruit had maximum juice and could be utilized by the processing industry. The SSR markers used in this study effectively separated closely related (SL and TL; ML and EL) and distantly related (Tav. L) acid lime genotypes. SSR markers CAC23 and CTT01 amplified maximum (9-11) alleles with higher discriminatory powers (0.71-0.76), greater heterozygosity and MAF and revealed greater genetic variability among populations. Interestingly, CAC23 and CTT01 markers amplified three alleles in the triploids (TL and SL), a single allele in diploid varieties (ML, EL) and two alleles including a unique allele in other diploid variety (Tav. L). Hence, these markers could be effectively used to discriminate diploid and polyploid acid lime populations and may be extended to other citrus species as well. These findings will be helpful for selection and conservation of highly heterozygous material and utilize it in breeding programs for germplasm enhancement.

Authors' Contributions

Conceptualization MU, BF; Data curation AF; Formal analysis AF; Funding acquisition; BF, MU; Investigation; AF, MU, BF; Methodology; MU, AF, IAR, Project administration; BF, MU Resources; BF, MU; Software; MU, IAR, Supervision; MU Validation; MU, BF Visualization; MU, BF, Writing - original draft; AF, MU; Writing - review and editing MU, BF. Please note:

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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