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RESEARCH ARTICLE

Morphological characterization and analysis of genetic diversity and population structure in *Citrus* × *jambhiri* Lush. using SSR markers

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Abstract Rough lemon (*Citrus* × *jambhiri* Lush.) is one of the important species largely used as a rootstock for commercial plantations of Citrus across the world. In the present study, thirty-eight accessions of *C. jambhiri* were characterized using morphological and SSR markers for diversity analysis and population structure studies. Morphological characterization of 27 qualitative and 14 quantitative characters indicated the existence of moderate to sufficiently high amount of variability as revealed from the pair-wise similarity analysis value of 0.36. Molecular diversity analysis using 17 SSR primers detected 85.29% polymorphism indicating existence of moderately high amount of variability between the accessions in terms of studied loci. A total of 60 bands were generated, of which all the 60 were polymorphic (100%). The total number of alleles produced varied from 1 to 5 alleles with an average of 3.52 alleles per locus. Although the correlation between the morphological and molecular data was low in the analysed accessions of *C. jambhiri*, both methods allowed the clustering of accessions based on the analysed traits. Population genetic analysis by SSR markers revealed that accessions collected from North Eastern India were most diverse in terms of genetic diversity parameters and genetic distance analysis of populations showed that the accessions from North East and Himachal Pradesh were most similar genetically while population collected from Himachal Pradesh and Karnataka were the most distinct genetically. Structure analysis of different populations revealed that there is no genetic differentiation happening between the populations.

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Keywords *Citrus jambhiri* · Characterization · SSR marker · Population · Genetic differentiation

Introduction

Citrus is one of the major fruit crops grown in tropical and sub tropical countries of the world. It belongs to the sub- family Aurantioidae of the family Rutaceae. It is one of the largest grown fruit in India occupying the second position in terms of area (1.003 million ha) and third position in terms of production (12.54 million tonnes) (NHB database 2017–2018). South East Asia, particularly North East India is considered as one of the centres of origin of Citrus as it harbours rich diversity of several *Citrus* species (Tanaka 1954; Webber et al. 1967). In India, North Eastern region, North Western region, foothills of Himalayas and a few parts of South India are potentially rich areas of citrus genetic diversity. *Citrus* × *jambhiri* Lush. commonly known as rough lemon is one of the native species which is largely been used as a rootstock for commercial plantations of Citrus across the world. In India, rough lemon is still the most widely used rootstock in majority of the Citrus growing belts of North, North East, Central and South India owing to its superior effect on vigour and yield of scion, good adaptability for sandy soils, tolerance to drought and salinity, good growth under deep soil conditions and resistance to Tristeza virus, Exocortis and Xyloporosis viroids. *C. jambhiri* is believed to be a hybrid between Citron and Mandarin based on the earlier morphological and biochemical studies (Scora 1975). Hybrid origin was also supported by molecular markers like RAPD, SSR, cpDNA etc. (Barkley et al. 2006; Jena et al. 2009). Characterization and assessment of diversity is essential for the identification of distinct genotypes, for deciphering genetic relationships including parentages and for efficient management and utilization of germplasm. Morphological characterization is still the basic and initial step for diversity assessment before employing any other advanced methods. Although the Citrus genus is very complex involving multitude of species and their hybrids, but still the morphological characters especially the fruit and leaf characters are useful for visual scoring and distinguishing accessions within a species. It allows simple grouping of accessions, development of core collections, identification of gaps in collection, identifying specific germplasm for breeding programmes etc. On the other hand, molecular markers are of utmost importance for characterization studies in *Citrus* sp. because of its complex taxonomy and phylogeny. Studies at molecular level including the use of markers (RAPD, ISSR, AFLP, SSR's) and sequence analyses of rbcL and matK gene region of chloroplast DNA (Uchoi et al. 2016) has been undertaken to infer the phylogenetic relationships between different species. DNA studies using as well as SSR's have been considered as almost ideal markers for genetic diversity analysis because of their reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and good genome coverage. These markers are popular tools in genetics and breeding because of their relative abundance compared to other molecular marker types, high degree of polymorphism (number of variants), and easy assaying by PCR (Zhu et al. 2012). Thus, in the present study an attempt has been made to assess the genetic diversity present in *Citrus jambhiri* accessions available throughout India using both morphological and SSR markers and also to study the extent of differentiation occurring between the different populations.

Materials and methods

Plant material

Thirty-eight accessions of *Citrus jambhiri* collected from different agro ecological zones of India were taken for the study. Accessions were collected from wild habitats in North East India (19 accessions), Himachal Pradesh (6 accessions), field gene bank of Regional Research Station, Abohar under Punjab Agricultural University (5 accessions) and field gene bank of ICAR-IIHR, Central Horticultural Experimental Station (CHES), Chettali, Karnataka (8 accessions) as given in Table 1 and Fig. 1. The sample size was selected in such a manner that it comprised of maximum available allelic diversity present in the species. Unlike in annuals, this is a highly heterozygous and heterogenous group wherein each tree itself has more number of diverse alleles and therefore each tree was considered as a replicate and samples were collected from different ecological regions which

sufficiently incorporated the diversity required for characterization studies. Leaf and fruit samples of each accession were collected for confirmation of taxonomic identity, characterization and DNA extraction. Individual accessions collected from single plant were given an indigenous collection number (IC number).

Morphological characterization

The morphological characterization was done based on the Citrus descriptors developed by IPGRI (presently Bioversity International). Altogether, characterization data of 28 qualitative and 14 quantitative (fruit, leaf and seed) characters were recorded for the collected germplasm of *C. jambhiri* (Tables 2 and 3).

The study was performed using three trees for each accession, each tree was considered a replicate. Five mature and fully developed leaves per replication were characterized for leaf characters. Fruit characteristics were observed on 3 typical fruits per accession. Fully developed seeds were also extracted from fully ripened fruits and seed characters were recorded. Quantitative data was analysed using mean, range, standard error and coefficient of variation and qualitative characters were converted into multi-state code and a pair-wise similarity matrix was generated based on simple matching coefficient method using software NTSYS ver. 2.10e. (Rohlf 2000). For grouping of the accessions based on morphological traits, dendrogram was generated using the Unweighted Pair Group Method with Arithmetic average (UPGMA) based on simple matching coefficient in NTSYS software. Principal Component Analysis (PCA) was also carried out to study correlations among the variables and establish relationships among accessions using the same software.

DNA extraction and SSR-PCR amplification

DNA was extracted from the young leaves using modified CTAB (Cetyl trimethyl ammonium bromide) protocol (Doyle and Doyle 1990). A total of 1.0 g leaf material was used for DNA extraction. DNA concentrations were estimated by mass spectrophotometry at 260 nm and working dilutions of concentration 10 ng/µl were prepared for the study.

Screening of 40 SSR primers were done to select 17 polymorphic SSR's for the analysis of 38 DNA samples. The PCR-amplification was carried out in 25 µl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.0–2.5 mM MgCl2, 0.2 mM dNTP each, 1.0 U Taq DNA polymerase(G-Biosciences, India), 0.2 mM primer and 20–25 ng genomic DNA. PCR was performed in a BioeR Xp thermocycler with reaction conditions programmed as initial pre-denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °Cfor 1 min, annealing at optimized temperature for 1 min, and extension at 72 °C for 1 min and a final 7 min extension at 72 °C. PCR products containing 3 µl loading dye were separated by electrophoresis on 3% agarose gel. 1 kb Gene ruler (Fermentas, International, Inc) was loaded in the first lane of each gel to determine the size of amplified bands. Electrophoresis was carried out at 75 V for 2 h and photographed using UV transilluminator (Mega Biosystematica, UK).

Data analysis

SSR markers were scored on the basis of allele sizes and allelic data was analysed using PowerMarker V3.25 (Liu and Muse 2005) and DARwin V.5.0.158 software (Perrier and Jacquemoud-Collet 2006). Using DARwin, a pair-wise dissimilarity matrix is obtained for constructing dendrogram using unweighted neighbour-joining method. Polymorphism information content (PIC) of each primer was determined as described by Smith et al. (1997) as $PIC = 1 - \Sigma fi2$, where *fi* is the frequency of the *i*th allele. These 38 accessions were grouped into four populations on the basis of their source, viz. North Eastern India (19), Himachal Pradesh (6), Punjab (5), Karnataka (8). Genetic variation within the four populations was analysed on the basis of the banding profile using various parameters such as percentage polymorphism, observed no: of alleles (n_a), effective no: of alleles (n_e), the total heterozygosity (Ht), Nei's gene diversity (h), estimation of geneflow (N_m) and Shannon's information index (I) using the software GenAlEx 6.5 (Peakall and Smouse 2012). Genetic relationship between the populations was drawn from the estimates of Nei's (1972) measure of genetic identity and genetic distance.

Table 1 *Citrus jambhiri* accessions used for the study

Sl. no.	Accession name	IC number	Common name	Region of collection	In situ/Ex situ collection
1	MD-8	IC395343	Jamir	Sikkim	In situ
2	MD-21	IC395354	Naity Jamir	Sikkim	In situ
3	MD-24	IC395357	Jamir	Sikkim	In situ
4	MD-34	IC395367	Kachai Lemon	Sikkim	In situ
5	MD-37	IC395369	Kachai Lemon	Tinsukia, Assam	In situ
6	MD-40	IC395372	Gol Nimbu	Tinsukia, Assam	In situ
7	MD-41	IC395373	Hathi Nimbu	Tinsukia, Assam	In situ
8	MD-53	IC395384	Champra	Tinsukia, Assam	In situ
9	MD-54	IC395385	Sinduri Lemon	Tinsukia, Assam	In situ
10	MD-57	IC395388	Rough Lemon	Tinsukia, Assam	In situ
11	MD-67	IC591426	Soh Bitter	Tinsukia, Assam	In situ
12	MD-69	IC395398	Adha Jamir	Tinsukia, Assam	In situ
13	MD-76	IC395404	Nimbu Tenga	Tinsukia, Assam	In situ
14	MD-81	IC395409	Gol Nimbu	Tinsukia, Assam	In situ
15	MD-92	IC591451	Hasu	Momokchung, Nagaland	In situ
16	MD-96	IC591455	Gangen	Wokha, Nagaland	In situ
17	MD-100	IC591459	Rough Lemon	Kohima, Nagaland	In situ
18	MD-2	IC395337	Rough Lemon	Arunachal Pradesh	In situ
19	MD-7	IC395342	Rough Lemon	Arunachal Pradesh	In situ
20	MSA-34	IC593866	Rough Lemon	Kangra, Himachal Pradesh	In situ
21	MSA-41	IC593879	Rough Lemon	Kangra Himachal Pradesh	In situ
22	MR-01	IC395376	Rough Lemon	Kangra Himachal Pradesh	In situ
23	MR-02	IC470336	Rough Lemon	Kangra, Himachal Pradesh	In situ
24	MR-03	IC470338	Rough Lemon	Kangra, Himachal Pradesh	In situ
25	MR-17	IC395358	Jamir	Hamirpur, Himachal Pradesh	In situ
26	MR-12	IC395392	Jatti Khatti	Abohar,Punjab	Ex situ
27	MR-13	IC395393	Jullandir Khatti	Abohar, Punjab	Ex situ
28	MR-14	IC395394	Florida Rough	Abohar, Punjab	Ex situ
29	MR-15	IC395411	Italian Rough	Abohar, Punjab	Ex situ
30	MR-16	IC395412	Jatti Khatti	Abohar, Punjab	Ex situ
31	MR-04	–	Jatti Khatti	Karnataka	Ex situ
32	MR-05	–	Karna Khatta	Karnataka	Ex situ
33	MR-06	–	Jambhiri kodur sri	Karnataka	Ex situ
34	MR-07	–	Poona Jambhiri	Karnataka	Ex situ
35	MR-08	–	Moogu Nimbu	Karnataka	Ex situ
36	MR-09	–	Jatti Khatti	Karnataka	Ex situ
37	MR-10	–	Jullandir Khatti	Karnataka	Ex situ
38	MR-11	–	Jambir Kodur	Karnataka	Ex situ

Fig. 1 Phenotypic variability collected in *C. jambhiri* accessions

Results

Morphological characterization

C. jambhiri trees were 10–15 m tall with spreading canopy and brevipetiolate (petiole shorter than leaf lamina) leaves with very narrow petiole wings. Evident variations were observed in the leaf lamina shape between the accessions ranging from elliptic to ovate with acute, acuminate or sometimes obtuse apex (Table 4). Leaf lamina length ranged from 39.7 to 94 mm and lamina width ranged from 16 to 49 mm. The coefficient of variation was the lowest for leaf lamina width (2.88) among all other quantitative traits

Table 2 Qualitative leaf, fruit and seed characters measured

Leaf character	Fruit character	Seed character
Leaf division	Fruit shape	Seed shape
Intensity of green colour	Fruit base	Seed surface
Leaf lamina attachment	Fruit apex	Seed colour
Leaf lamina shape	Fruit skin colour	Cotyledon colour
Leaf lamina margin	Fruit surface texture	Chalazal spot colour
Leaf apex	Adherence of albedo to pulp	Seed embryony
Absence/presence of petiole wings	Nature of oil glands	
Petiole wing width	Density of il glands	
Petiole wing shape	Oil gland size	
	Albedo colour,	
	Pulp colour,	
	Pulp firmness	

Table 3 Quantitative leaf, fruit and seed characters measured

Leaf character	Fruit character	Seed character
Leaf lamina length (mm)	Fruit weight (g)	No. of seeds/fruit
Leaf lamina width (mm)	Fruit diameter (mm)	Seed weight (g)
Ratio leaf lamina	Fruit length (mm)	Seed moisture (%)
Leaf thickness (mm)	Width of epicarp (mm)	
	Fruit rind thickness (mm)	
	No: of segments per fruit	
	Total soluble solids (TSS)	

(Table 5). Fruit characteristics of rough lemon accessions showed wide variation between themselves. Fruit shape varied from spheroid to ellipsoid and obloid but fruit base was observed to be mostly of mammiform type which is the key morphological trait of *C. jambhiri* fruits. Skin colour of young fruits was greenish yellow which at maturity turned to dark yellowish or orange. Fruits mostly had pitted surface texture with conspicuous oil glands. Fruit size also showed wide variation with fruit length varying from 36.2 to 94.52 mm, fruit diameter from 33.83 to 75.09 mm, epicarp width from 1.05 to 4.56 mm and fruit weight from 50 to 273.33 gm. Most of the accessions were with high juice content and TSS value ranged from 1 to 10 with an average of 6.64. Rough lemon accessions were all seeded having number of seeds in the range 6.67–38.67 with an average of 16 seeds per fruit. Fresh seeds of *C. jambhiri* showed high moisture content ranging from 33.17 to 51.9% with an average of (Tables 4 and 5).

Pair-wise similarity analysis of 42 morphological characters in the 38 accessions of *C. jambhiri* revealed that maximum similarity (0.82) occurred between the accessions MR-02 and MD-37 and between MD-21 and MD-34. MR-02 collected from Himachal Pradesh and MD-37 collected from Assam was most similar in terms of qualitative characters of fruit, leaf and seed. Accessions MD-21 and MD-34 both collected from Sikkim were similar mainly in terms of the fruit characters. The average similarity value of 0.36 indicated that accessions showed moderate to significant variability with respect to morphological traits. Dendrogram generated based on UPGMA method grouped all the 38 accessions of *C. jambhiri* into four major clusters (Fig. 2). Accessions MD-40 and MR-04 formed the most diverse cluster with similarity value of 0.27. Second cluster comprised of five accessions namely MD-57, MR-02, MD-92, MD-7 and MD-96. Within this cluster the accessions MD-21 and MD-34 were most similar morphologically showing a similarity value of 1.00. Principal Component Analysis (PCA) gave first 10 principal components, which contributed 76% of the total variability of the

Table 4 Variations in qualitative leaf, fruit and seed characters obtained in 38 accessions of *Citrus jambhiri*

Character	Variables
Fruit	
Fruit shape	Spheroid, ellipsoid, obloid
Fruit base	Convex, truncate, concave, collard neck
Fruit apex	Mammiform, rounded, truncate, depressed
Fruit skin colour	Green-yellow, yellow, dark yellow, orange, dark orange, light orange
Fruit surface texture	Pitted, smooth, rough, papillate
Nature of oil glands	Conspicuous
Density of oil glands	Intermediate, high, low
Oil gland size	Small, large
Albedo colour	Yellow, green, orange, pink, white
Adherence of albedo to pulp	Strong, medium, weak
Pulp firmness	Soft, intermediate, firm
Pulp colour	Yellow, orange, pink
Juice content	High, medium, low
Seed	
Seed shape	Clavate, ovoid, semideltoid
Seed surface	Smooth
Seed colour	Brown, Yellow, Cream, Green
Cotyledon colour	Light yellow-cream, light green, green, pink, white
Chalazal spot colour	Brown, light brown, purple, reddish
Seed embryony	Polyembryony
Leaf	
Leaf division	Simple
Intensity of green leaf colour	Dark
Leaf lamina attachment	Brevipetiolate
Leaf lamina margin	Entire, sinuate, crenate, dentate
Leaf lamina shape	Elliptic, ovate, obovate, lanceolate
Leaf apex	Obtuse, acute, emarginate
Petiole wing shape	Obovate, obdeltate
Petiole wing width	Narrow
Absence/presence of petiole wings	Present

collected accessions among which fruit and leaf characters were predominantly variable.

SSR analysis

After screening of forty primers, seventeen SSR primers were selected for the analysis based on the reproducibility and banding patterns. A total of 60 bands were generated, of which all the 60 were polymorphic (100%). The total number of alleles produced varied from 1 to 5 alleles with an average of 3.52 alleles per locus. PIC values were calculated for each primer, highest PIC value of 0.6433 was obtained for the primes ATC09 followed by 0.615 for the primer CTT01. Average PIC value obtained for SSR markers is 0.410. Major allele frequencies for each locus ranged from 0.381 to 1.00 with an average of 0.646. Gene diversity ranged from 0 to 0.697 with an average of 0.460 and heterozygosity ranged from 0 to 1 with an average of 0.506 (Table 6).

The dissimilarity co-efficient among 38 accessions of *C. jambhiri* based on SSR markers ranged from 0.03

Table 5 Quantitative leaf, fruit and seed characters of 38 accessions of *Citrus jambhiri*

Character	Mean	Range	SD	SE (±)	CV (%)
Leaf					
Leaf lamina length (mm)	61.5	39.7–94.0	1.24	0.20	2.016
Leaf lamina width (mm)	27.7	16.0–49.0	0.80	0.13	2.88
Leaf lamina ratio	2.30	1.79–2.81	0.30	0.04	13.04
Leaf thickness (mm)	0.21	0.14–0.44	0.06	0.009	28.57
Fruit					
Fruit weight (g)	115.89	50–273.33	43.97	7.13	37.94
Fruit diameter (mm)	54.87	33.83–75.09	10.93	1.77	19.91
Fruit length (mm)	59.47	36.2–94.52	4.32	0.7	7.26
Epicarp width (mm)	2.19	1.05–4.56	0.92	0.14	32.00
Fruit rind thickness (mm)	4.32	1.13–8.78	1.96	0.32	35.37
No. of segments/fruit	9.42	7.33–11	0.89	0.14	9.44
TSS	6.64	1.00–10.00	2.18	0.35	32.83
Seed					
No. of seeds/fruit	16.73	6.67–38.67	7.14	1.15	6.87
Ten seed weight (g)	0.92	0.26–1.94	0.36	0.06	39.13
Seed moisture (%)	40.87	33.17–51.90	4.46	0.72	10.91

SD—standard deviation
SE—standard error
CV (%)—coefficient of variation percentage

to 1 with an average of 0.53. Dendrogram generated based on SSR method grouped all the 38 accessions into two major clusters (Fig. 3). First cluster comprised of twelve accessions, 11 of them were from North East and one from Karnataka. Second cluster comprised of 26 accessions in which MR-02 and MD-2 were genetically most similar. Majority of the accessions from Punjab clustered together with accessions from Karnataka. MD-2, MD-7, MD-81 and MD-76 from North East India were also grouped with accessions from Punjab, Karnataka and Himachal Pradesh in the second cluster.

Population genetic analysis

Population genetic analysis of SSR data was undertaken to study the extent of diversity present in *C. jambhiri* accessions over different regions of collection (Table 7). The analysis showed that the accessions collected from North East were more diverse in terms of number of observed alleles (3.29), number of effective alleles (2.17), high Shannon diversity index (0.871). The markers revealed comparatively high observed heterozygosity (0.511) and high expected heterozygosity (0.497) in this collection and accounted for 94.12% polymorphic loci. The accessions collected from Karnataka were the second most diverse set. This collection depicted a value of (2.17) for the observed number of alleles, (1.81) for effective number of alleles and Shannon index of (0.606). The observed heterozygosity was (0.581) and expected heterozygosity was (0.415) with 92.12% polymorphic loci. The accessions collected from Himachal Pradesh represented the third diverse collection with respect to observed number of alleles (1.94), effective number of alleles (1.55), Shannon index (0.470), observed heterozygosity (0.363) and expected heterozygosity (0.306). 82.35% of polymorphic loci were observed from the collections. The accessions collected from Punjab represented the least diverse population among the four. They depicted very low values for all the diversity parameters viz., observed number of alleles (1.88), effective number of alleles (1.59), Shannon index (0.452), observed heterozygosity (0.412), expected heterozygosity (0.291) and 72.59% polymorphism loci. Overall, at the species level SSR markers could detect observed number of alleles (2.32), effective number of alleles (1.78), Shannon index (0.606), observed heterozygosity (0.467) and expected heterozygosity (0.377) over all the loci. Low Fst value (0.231) and high gene flow value (4.69) obtained indicated low level of genetic differentiation between the populations (Fig. 4).

Nei's (1978) unbiased measures of genetic identity and genetic distance among 4 populations of *C. jambhiri* generated by SSR markers revealed that the

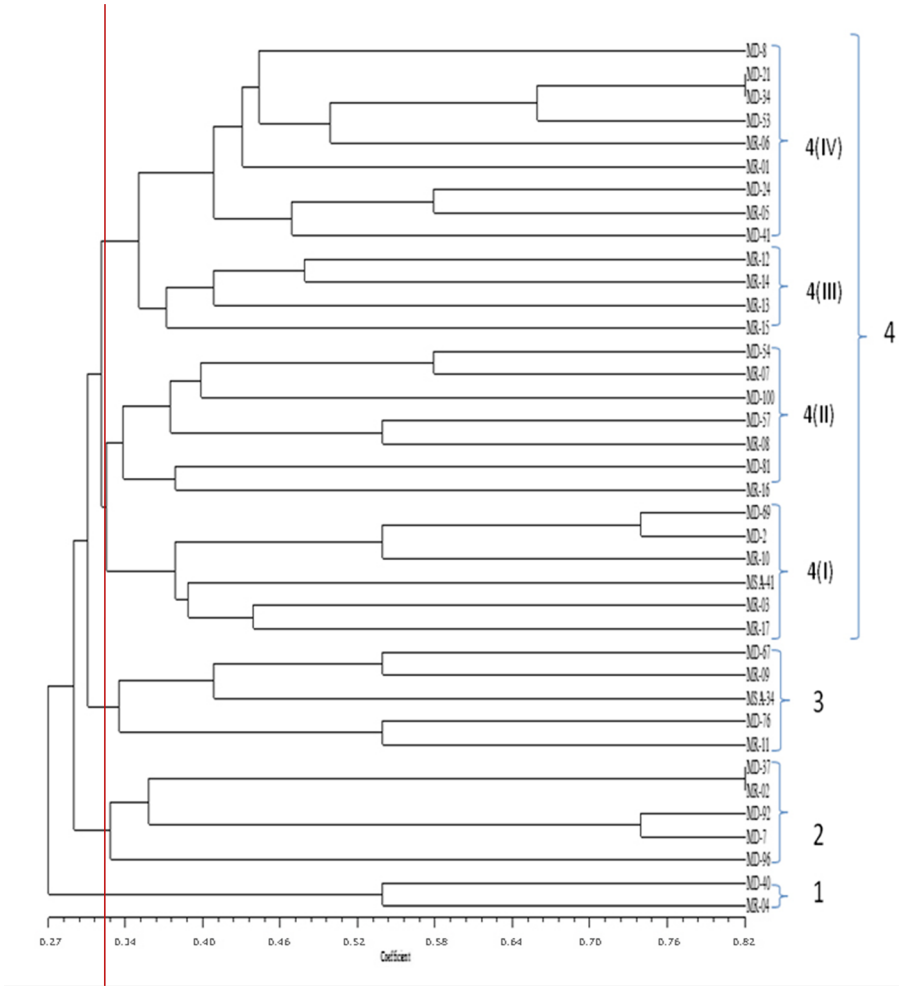

Fig. 2 UPGMA Dendrogram generated based on morphological data of 38 *C. jambhiri* accessions

maximum genetic identity (0.822) occurred between populations from North East India and Himachal Pradesh showing that these populations were genetically more similar and the maximum genetic distance (0.310) occurred between populations from Himachal Pradesh and Karnataka indicating their differences in genetic makeup (Table 8).

The AMOVA analysis showed that only 27% of the total variation was attributed to the variability among the populations, whereas 72% variability was observed within the individuals among the population and 1% was observed among the individuals within the populations (Fig. 5). Principal Coordinate Analysis (PCoA) of 38 accessions with 17 SSR markers showed that the first three axes explained 61.91% of cumulative variation in which first component contributed maximum variation of 28.98% followed by the second component with 20.91%. In principal coordinate analysis (PCoA), all the accessions were grouped according to their geographical region of collection separately (Fig. 6). Intermixing of some accessions across the coordinates indicates the migration of genotypes across borders.

Population structure analysis

SSR allelic data was analysed using the model based STRUCTURE software to start a new project by setting various parameters such as burn in period as 10,000; number of repeats as 100,000; number of iteration as 3; assumed K value as 7 and the K is tested for 1–10. The structure obtained by Evanno method

Table 6 Details of amplified bands generated based on 17 SSR primers in 38 accessions of *Citrus jambhiri*

Sl. no.	SSR loci	Repeat motif	No: of alleles detected	Polymorphic alleles	Major allele frequency	Gene diversity	Heterozygosity	PIC
1	AG14	GA	3	3	0.8889	0.2030	0.1481	0.1922
2	AC01	CA/TA	4	4	0.4167	0.6771	0.9167	0.6141
3	ATC09	TCA	5	5	0.3810	0.6973	0.6667	0.6435
4	CT02	CT	4	4	0.6833	0.4678	0.5000	0.4065
5	CT19	TC	5	5	0.5000	0.6451	0.6190	0.5888
6	CT21	TC	1	1	1.0000	0.0000	0.0000	0.0000
7	CAT01	CAT/CTT	3	3	0.5000	0.5938	1.0000	0.5112
8	CCT01	CCT	4	4	0.5263	0.6440	0.6316	0.5965
9	CAG01	AGC	5	5	0.6481	0.5384	0.3333	0.5026
10	CTT01	CTT	4	4	0.5000	0.6633	1.0000	0.6153
11	GT03	GT	4	4	0.6842	0.4723	0.2105	0.4161
12	UCM05	CT	3	3	0.8333	0.2882	0.1667	0.2640
13	UCM06	TC/GT	3	3	0.8333	0.2882	0.1667	0.2640
14	UCM08	TA/CA	2	2	0.9500	0.0950	0.1000	0.0905
15	UCM17	CTC	2	2	0.5000	0.5000	1.0000	0.3750
16	UCM20	GT/GC	5	5	0.6481	0.5384	0.3333	0.5026
17	UCM21	TA/CA	3.	3.	0.5000	0.5147	0.8182	0.3969
Total			60	60				
Mean			3.52	3.52	0.6466	0.4603	0.5065	0.4105

resulted in dividing 38 accessions of *C jambhiri* into two subpopulations (Fig. 7a) where the highest peak for K was obtained at two. This showed that *C. jambhiri* accessions are divided into only two sub-populations, and thus population structure is not significant. Further, summary statistics obtained from STRUCTURE software in the form of colour chart indicated that there was varying degree of genetic intermixing between the accessions from different regions. The accession having more than 20% of the genetic background of other species was considered as intermixed genotypes. Among the 38 accessions, MD-34, MD-69, MSA-34, MR-15, MR-05 and MR-06 were found as intermixed genotypes (Fig. 7b).

Discussion

In the present study, *Citrus jambhiri* accessions were characterized and grouped on the basis of leaf, fruit and seed characters and moderate to sufficiently high amount of variation in morphological features was observed for the accessions collected from different geographical regions of India. Leaf characters showed variation between the accessions for leaf lamina shape which varied from elliptic to ovate with acute, acuminate or sometimes obtuse apex. Since majority of the accessions were characterized by ovate lamina with obtuse leaf apex and sinuate margin, the selection of genotypes for breeding may make use of these quality characters. Similar result was obtained by Singh and Singh (2006) in rough lemon accessions which showed wide variation in leaf lamina shape like obcordate, elliptic, ovate, obovate, lanceolate and orbicular. Fruit characters of *C. jambhiri* accessions showed significant variation among themselves with respect to both qualitative and quantitative traits. These results were in accordance with the findings of Gaikwad et al. (2018) in rough lemon rootstocks. Rough lemon accessions are highly polyembryonic in nature with very little variation in seed characters except number of seeds. Number of seeds per fruit varied in the range of 6.67–38.67. Thus, results of the

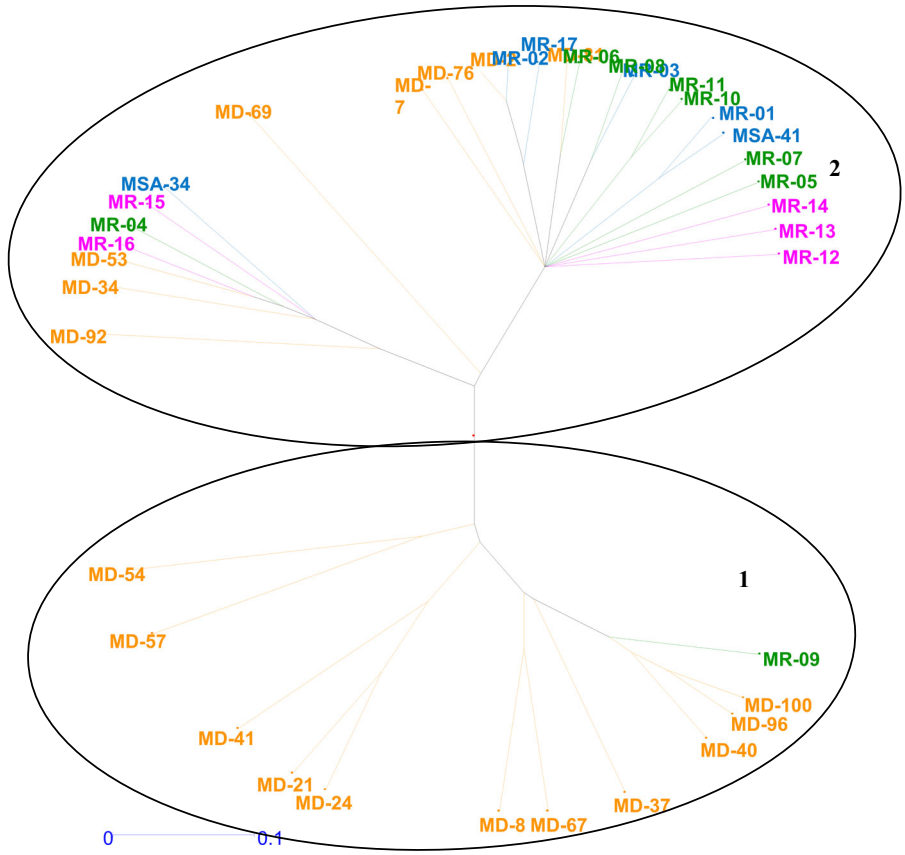

Fig. 3 Dendrogram generated based on SSR data of 38 accessions of *C. jambhiri*

present study were similar to the findings of Paudyal and Haq (2008) which revealed significance of fruit shape, pulp colour, seed number, leaf shape and petiole wing shape in germplasm characterization as they are mostly genetically controlled and less dependent on the environmental response. Quantitative characters are influenced by external environmental conditions, but still these characters are important in analyzing genetic diversity as explained by Yao et al. (2007). Principal component analysis undertaken using morphological traits expressed the traits which contributed maximum to the variability. The fruit characters like fruit weight, fruit diameter, TSS, fruit length, fruit shape, leaf characters like leaf lamina shape, leaf apex and petiole wing width significantly contributed to the variability of *C. jambhiri* accessions.

In the present study, pair wise similarity analysis of 38 accessions of *C. jambhiri* based on morphological traits showed that the accessions collected from the same geographical region or with the same agro-climatic conditions were more similar to each other. Maximum similarity (0.82) occurred between the accessions MR-02 and MD-37 and between MD-21 and MD-34. MR-02 collected from Himachal Pradesh and MD-37 collected from Assam was most similar in terms of qualitative characters of fruit, leaf and seed. Accessions MD-21 and MD-34 both collected from Sikkim were similar mainly in terms of the fruit characters. The close similarity in morphological characters may be because they are chance seedlings from the same tree and also may be because of nucellar embryony. The minimum similarity between the accessions like MD-40 (Assam) and MR-12 (Punjab) may be explained on the basis of their geographical areas of occurrence and corresponding G X E interaction. Less similarity between the accessions occurring in the same region like MD-67(Assam) and MD-40(Assam) may be because they arose from zygotic seedlings. The average similarity value of 0.36

Table 7 Genetic data for four populations of *Citrus jambhiri* derived from SSR markers

Population	Sample size	Mean n_a (SD)	Mean n_e (SD)	Mean I (SD)	Mean H_o (SD)	Mean H_e (SD)	PPL (%)
North East India	19	3.2941 ± 0.4258	2.171 ± 0.3809	0.871 ± 0.2749	0.511 ± 0.0388	0.497 ± 0.0388	94.12
Himachal Pradesh	6	1.9411 ± 0.5189	1.559 ± 0.3241	0.470 ± 0.2666	0.363 ± 0.0331	0.306 ± 0.0331	82.35
Punjab	5	1.8823 ± 0.3631	1.591 ± 0.3360	0.452 ± 0.2443	0.412 ± 0.0304	0.291 ± 0.0304	72.59
Karnataka	8	2.1764 ± 0.5136	1.814 ± 0.2981	0.631 ± 0.2411	0.581 ± 0.0271	0.415 ± 0.0271	92.12
Mean		2.324 ± 0.117	1.784 ± 0.070	0.606 ± 0.042	0.467 ± 0.046	0.377 ± 0.025	85.29 ± 5.63

F_{st}—0.231 ± 0.033

N_m—4.693 ± 3.620

n_a—Observed number of alleles

n_e—Effective number of alleles

I—Shannon's Information index

H_O—Observed Heterozygosity = No. of Hets/N

H_e—Expected Heterozygosity = 1 − Sum pi^2

Gst—Coefficient of gene differentiation, calculated as Gst = Ht − Hs/Ht (Nei 1978)

N_m—Estimate of gene flow, calculated as $N_m = 0.5(1 - Gst)/Gst$

PPL—Percentage of polymorphic loci

indicated that the cultivars showed moderate to significant variability among themselves with respect to morphological traits. Cluster analysis of *C. jambhiri* cultivars based on morphological traits divided the accessions with similar morphological traits in the same cluster irrespective of the region of collection. Few accessions collected from same region, however were grouped in different clusters and vice versa. Cluster analysis helped to identify the most distinct and most unique accession amongst all. In this study, MD-40 (Assam) and MR-04 (Karnataka) were the most distinct in term of fruit shape, fruit base, apex, colour etc. from the rest of the accessions. Such unique genotypes from individual cluster can be used in breeding programs after assessing their rootstock performance.

Seventeen SSR markers could detect 100% polymorphic alleles over all the loci. Average heterozygosity value of 0.506 and gene diversity value of 0.460 indicated the presence of sufficient genetic variation among the collected accessions. High PIC value of 0.410 indicated that SSR are efficient marker for assessing genetic diversity in *C. jambhiri*. Zerihun et al. (2009) also used SSR markers to study the genetic diversity between different species of Citrus and found that SSRs were powerful in differentiating closely related Citrus cultivars. The dendrogram generated using SSR has clearly demarcated most of the accessions from North East India into a separate cluster indicating that the diversity is still conserved in the region. Accessions from Punjab, Karnataka, Himachal Pradesh and eight from North East like (MD-34, MD-53, MD-69, MD-81, MD-76, MD-2, MD-7, MD-92) have mingled up due to the migration factors.

Population genetic analysis is very useful and informative in the differentiation and estimation of genetic variability parameters among and within crop populations to understand the occurrence of genetic differentiation which can further throw light on the phylogenetic relationships. Several studies aimed to understand the genetic structure and genetic relationship of Citrus populations existing in different geographical regions. This kind of analysis is important in Citrus as it is one of the diverse crop genus with numerous species and with sexual compatibility within and among the genus. Barkley et al. (2006) studied genetic diversity and population structure in Citrus germplasm collection maintained at University of California, Riverside using microsatellites. They

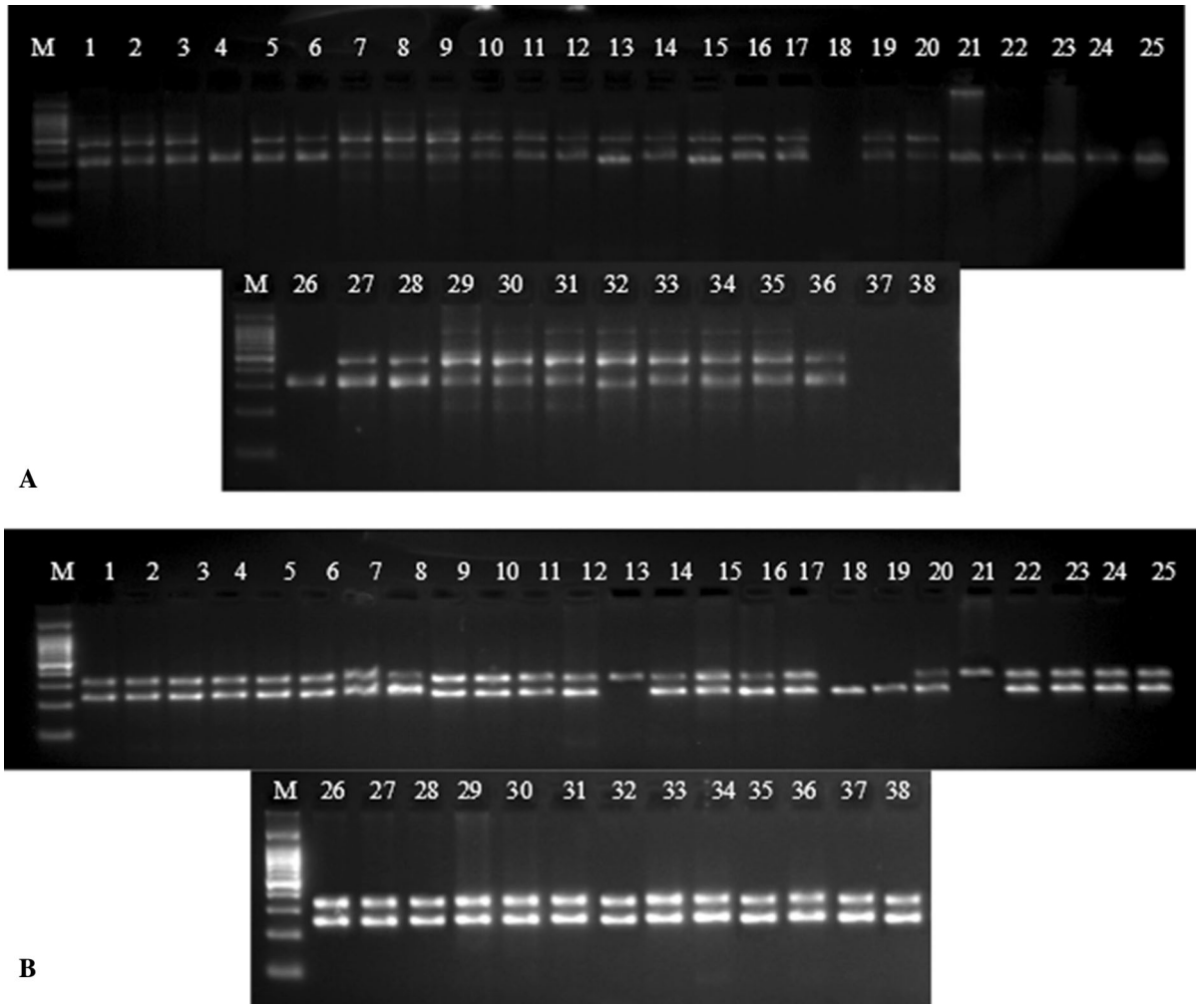

Fig. 4 Representative gel profiles of 38 accessions of *Citrus jambhiri* based on SSR primers **a** UCM 08 and **b** UCM 17. M represents 100 bp DNA ladder. (Note: Numbers are equivalent to those listed in Table 1)

Table 8 Nei's (1978) unbiased measures of genetic identity and genetic distance among 4 populations of *Citrus jambhiri* generated by SSR markers

Pop ID	North East India	Himachal Pradesh	Punjab	Karnataka
North East India	****	**0.822**	0.737	0.824
Himachal Pradesh	0.196	****	0.765	0.733
Punjab	0.306	0.268	****	0.779
Karnataka	0.194	**0.310**	0.249	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Bold indicates the maximum genetic identity (0.822) and maximum genetic distance (0.310) values

****—Nil, indicates the genetic distance between the same population

could illustrate that *Fortunella* clusters within the genus Citrus but *Poncirus* is a sister genus to Citrus. Similar studies were carried out by Barbhuiya et al. (2016) to analyse genetic structure and diversity

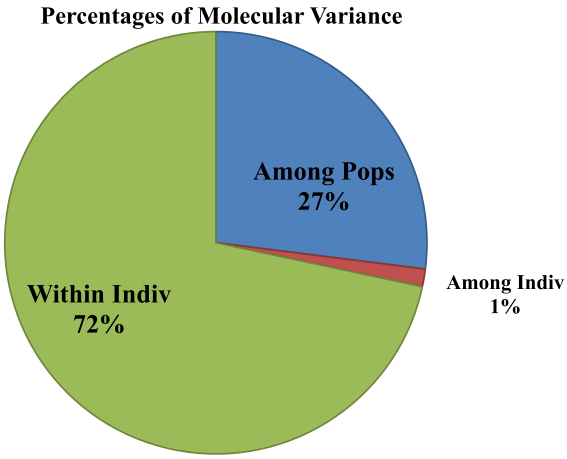

Fig. 5 Analysis of molecular variance of 38 accessions of *C. jambhiri* based on SSR markers

of natural and domesticated populations of *Citrus medica* L. in the North East India using SSR markers. The study showed that domesticated population were genetically more similar compared to its wild population. In the present study, SSRs were employed to analyse the genetic relationship between the accessions collected from different geographical regions treated as different populations. The results indicated that all the diversity was highest for the collections from North East India. This complements the fact that North Eastern India is the centre of origin of many *Citrus* species including *C. jambhiri* (Scora 1975). Most of the accessions are found in the wild state in this region which conserves the genetic diversity in its natural habitat as such. High gene flow and low Fst values obtained with SSRs indicated comparatively less genetic differentiation among the four regions of collection for *C. jambhiri*. This indicates that sufficient outbreeding has been occurring between the individuals which is maintaining the gene flow and genetic diversity among and between the geographical areas. Clustering of some accessions with the accessions of different areas indicate that all accessions originated from a single large population and diverged to different geographical areas. This finding supports the hypothesis by Barkley et al. (2006) that there are only a few naturally occurring species of Citrus and most other types of Citrus arose through various hybridization events between these naturally occurring forms.

Nei's (1978) unbiased measures of genetic identity and genetic distance among 4 areas of *C. jambhiri* generated by SSR markers revealed that the genotypes collected from North East and Himachal Pradesh were most similar genetically owing to their similar ecological conditions of adaptation and genotypes collected from Himachal Pradesh and Karnataka were the most distinct genetically owing to entirely different ecological conditions. Principal coordinate analysis of the accessions based on first and second coordinates using SSR markers have clearly divided the collections into 4 coordinates according to their geographical origin which again supports the dendrogram. High percentage of variability within the individuals among the populations estimated by

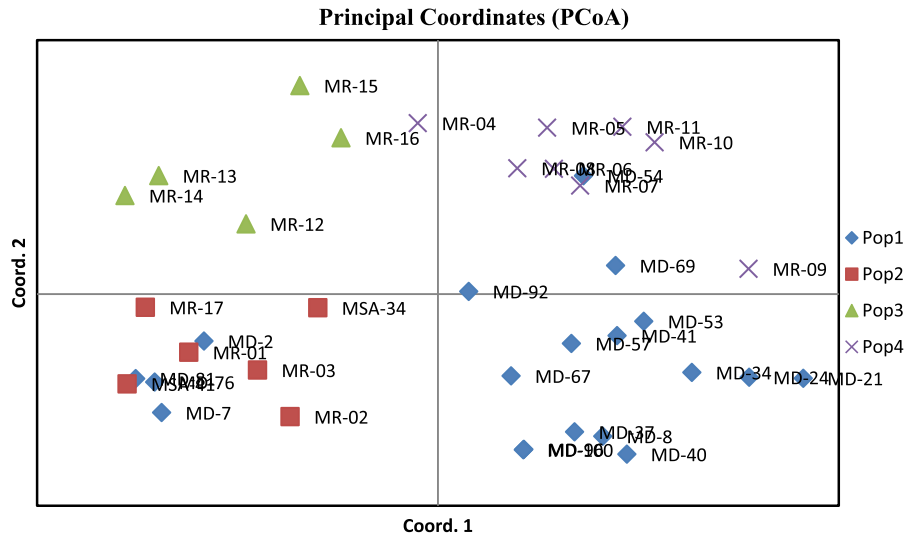

Fig. 6 Principal coordinate analysis (PCoA) of *C. jambhiri* accessions using SSR markers

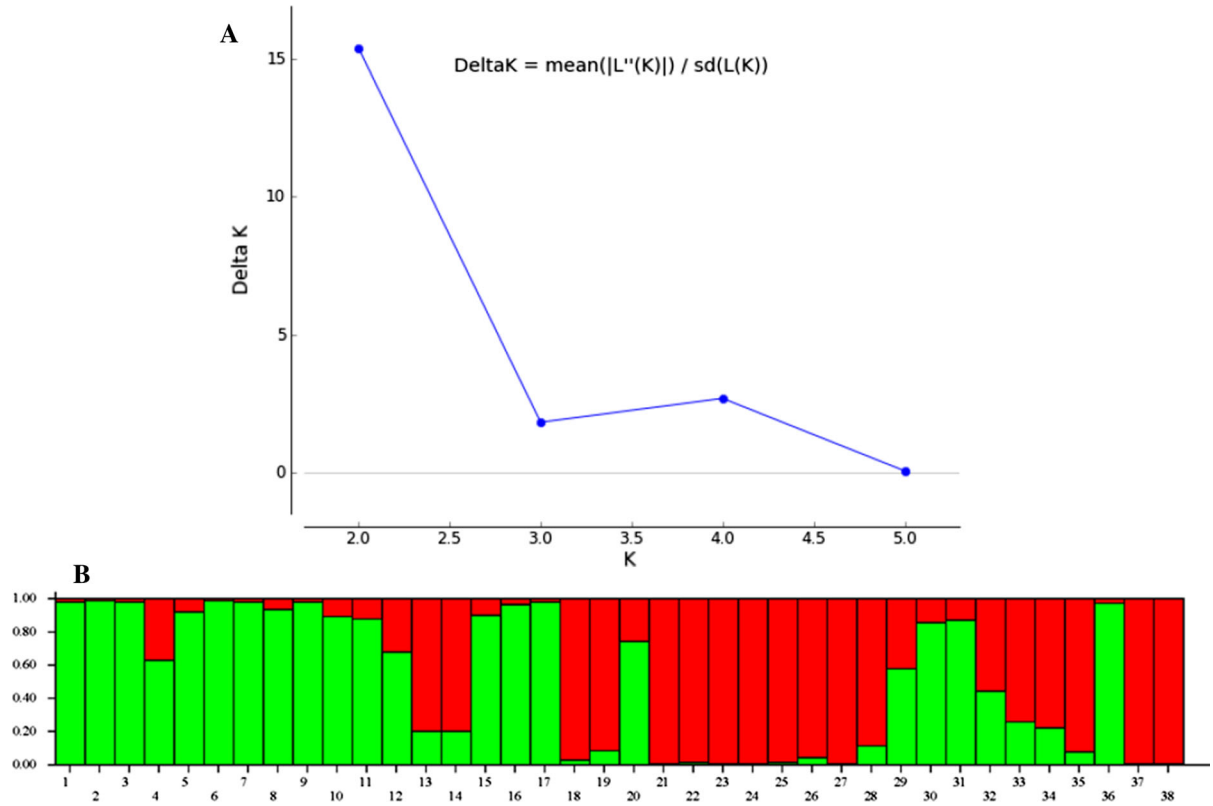

Fig. 7 Population structure analysis of *C. jambhiri* using SSR markers

analysis of molecular variance indicated that the structuring of the population is insignificant and there is continuous gene flow occurring between populations. Population structure studies are useful for distinguishing number of population or sub-populations based on the distribution (commonality or difference) of allele frequency among accessions. This could further be used in identifying pure genotypes from the admixtures in the gene banks and in breeding programs (Chen et al. 2017).

Thus, it was found that based on morphological and SSR marker analysis a moderate to sufficient amount of variability was deciphered among the collected accessions of *C. jambhiri,* which is in accordance with earlier studies conducted by Akhter et al. (2009) in Jamir accessions of Bangladesh and by Maya et al. (2012). Moreover, there is very less genetic differentiation observed between different areas of collection indicating it to be originated from a single large population and spread vegetatively without much introgression.

Morphological characterization is essential in a tree species like Citrus because it is a vast genera comprising of many species each with a unique attribute. Authentic identification of the species itself is the first and foremost step for undertaking any study in this genus. Morphological markers serves as the first inevitable tool for genotype identification and thus forms the basic and initial step for diversity studies before employing any other advanced methods. At the same time, owing to the complexity of the Citrus genus and its phylogenetic relationship, morphological data alone is not sufficient to derive any conclusion with respect to extent of diversity or variation present unless it is complemented by molecular studies. *Citrus jambhiri* being a highly polyembryonic species offers very limited diversity unless it is geographically differentiated. Thus, the study has attempted to make use of both morphological and molecular markers for diversity and population structure studies. Although the study revealed sufficient genetic variation in *C. jambhiri* accessions both from the morphological as well as SSR analysis but there was no correlation

observed between the morphologically and genetically distinct accessions. Morphologically similar accessions had a different genetic profile indicating the role of particular area of adaptation in modulating the expression of morpho traits or may be due to some spontaneous mutations or further interspecific hybridizations occurring in nature. On the other hand, genetically similar accessions had a different morphology which again supplements the fact that phenotype is the result of interaction between the genotype and its environment. Distinctness in genetic composition coupled with morphology can be attributed to their hybrid nature. These distinct accessions can be utilized as suitable material for rootstock breeding for favourable agronomic characters mainly the abiotic stress tolerant traits. Based on the rootstock performance they can be utilized in breeding programmes for increasing fruit production and development of species specific markers for this important rootstock would help in improvement of planting material for commercial utilization thus helping in avoiding the use of unwanted rootstocks.

Many studies have been conducted for assessing the genetic diversity in different Citrus rootstock species across the world. Mouei et al. (2011) reported characterization of 31 genotypes representing ten rootstocks of *Citrus* species using 10 microsatellite and 17 operon primers and identified specific markers differentiating the rootstocks which could be used for marker assisted selection in breeding programs. Similarly, Lamine and Mliki (2015) used RAPD and SSR markers to analyse their comparative efficiency in assessing the genetic diversity among sour orange rootstocks. Singh et al. (2017) studied six citrus rootstocks, viz., rough lemon, trifoliate orange, Swingle citrumel, Rangpur lime and Gou Tou to assess the morphological and genetic variabilityand reaction against Phytophthora. Fifty-five SSR markers were used for evaluation of genetic diversity amongst the six rootstocks. Gaikwad et al. (2018) analysed thirty citrus rootstock genotypes representing four species (Rough Lemon *C. jambhiri*, Rangpur lime *C. limonia*, Galgal *C. pseudolimon* and Alemow *C. macrophylla*) using 79 morphological characters. The analysis of variance for the thirty-nine quantitative traits revealed statistically significant differences for all the characters studied among tested genotypes. When India is concerned, particularly North and Western parts of India, *C. jambhiri* is the most widely used rootstock and no genetic diversity studies has been taken up exclusively in this species so far. For any rootstock breeding programme, the primary objective is to accumulate the maximum available diversity which further can be screened or selected for improvement work. As part of this study we were able to collect many *C. jambhiri* accessions from the North Eastern India which is home to many Citrus species including *C. jambhiri*, thus providing an apparent source of diversity which can be utilized in crop improvement programmes. Thus, the above study has been successfully conducted to analyse the extent of diversity present exclusively in *C. jambhiri* across different agro ecological regions of India enabling it to be used for the improvement of Citrus industry in India and worldwide and also endorsed the fact that North eastern regions of India still harbours the maximum allelic diversity of *C. jambhiri* which needs to be conserved for further sustainable utilization.

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