Research Note Electronic Journal of Plant Breeding, Vol.17(2):221-226

Molecular diversity analysis of rough lemon (*Citrus jambhiri* Lush.) in different agroclimatic zones of Assam

Purnima Pathak[1], Aradhana Phukan[1*], Bhupen Kumar Baishya[2] and Gayatree Hazarika[3]

https://doi.org/10.37992/2026.1702.028

Abstract

Rough lemon (*Citrus jambhiri* Lush.) is an important rootstock in Citrus group. Being indigenous to North-Eastern part of India, a wide variability has been observed in the region particularly in the state of Assam. However, not much evolved types have been reported in due course of time. SSR markers were used to find their molecular diversity and to document the different types. A total of fifty-four SSR markers were selected from different sources related to citrus species and analysed for eighteen rough lemon types collected from different agro-climatic zones of Assam. Forty-four markers showed polymorphism. Dendrogram showed clear evidence of two separate clusters at a level of approximately 60% dissimilarity. The similarity co-efficient was ranged from 0.04 to 0.58. This study embarked the possibility of a particular group of germplasm with similar genetic nature comparatively resistant towards water stressed condition, which could be further selected for grafting or conservation studies.

Keywords: Rough lemon, molecular diversity, SSR markers, rootstock

The genus citrus is diverse in species, cultivars and clones and the North-Eastern region is rich in diversity. Citrus fruits play an important role in human health as they are rich in plant compounds that have various health benefits, including anti-inflammatory and antioxidant effects (Barbora *et al.,* 2020). *Citrus jambhiri* belonging to the family Rutaceae is popularly known as Gul Nemu in Assam. The flavour provided by this crop is among the most preferred in the world. *Citrus jambhiri* is mostly known for rootstock. Therefore, their identification and conservation are necessary. However, the presence of nuclear embryony, long generation time and heterozygous nature, it is very difficult to develop or to identify cultivar by conventional breeding (Ahmad *et al*., 2003). The use of molecular markers has eased the cultivar identification. Microsatellite markers or simple sequence repeat (SSR) markers are one of the most important and reliable markers that are abundant throughout the nuclear genome of eukaryotes. These are proven markers for finger printing (Smith and Devey, 1994), cultivar identification and genetic mapping (Guilford *et al*., 1997; Kijas *et al*., 1997, Swain *et al*., 2025). In this view, the molecular characterization of rough lemon (*Citrus jambhiri* Lush.) was done to determine the closely related cultivars which may have evolved through mutation and their utilization in conservation studies.

The germplasm was collected from different locations across the state in the year 2022 and the study was done in the year 2022-23. The plant materials were selected and identified based on Citrus descriptors IPGRI (1999). The germplasm identification was also supported by the descriptions provided by Bhattacharya and Dutta (1956). This selection of germplasm was based on a primary work done under Department of Horticulture, Assam Agricultural University (Pathak, 2020). A total of eighteen genotypes were selected and named according to their locations (**Table 1**). Fifty-four SSR primers were selected from different sources related to citrus species (Kijas *et al.*,1995; Barkley *et al.,* 2006; Steve and Skalatsky, 2000).

[1]College of Horticulture and Farming System Research, Assam Agricultural University, Nalbari.
[2] Krishi Vigyan Kendra, Assam Agricultural University, Nalbari
[3]Department of Plant Breeding and Genetics, Assam Agricultural University, Jorhat.

Corresponding author : Purnima Pathak, College of Horticulture and Farming System Research, Assam Agricultural University, Nalbari. *E-Mail: aradhana.phukan@aau.ac.in

How to cite this artilce: Purnima Pathak, Aradhana Phukan, Bhupen Kumar Baishya and Gayatree Hazarika. 2026. Molecular diversity analysis of rough lemon (*Citrus jambhiri* Lush.) in different agroclimatic zones of Assam. *Electronic Journal of Plant Breeding.* 17(2): 221-226.
doi:10.37992/2026.1702.028

Received:06.02.2026 **Revised**:08.06.2026 **Accepted**: 16.06.2026

DNA isolation and purification

The total genomic DNA from each genotype was extracted following the protocol of Murray and Thompson (1980) with slight modifications. DNA was extracted from young leaves of eighteen samples. Leaf samples were chopped and crushed into fine powder using liquid nitrogen and placed into 1.5 ml eppendorf tube, 750 µl of CTAB buffer

Table 1. Details of rough lemon germplasm collected across the state

S.No.	Accession Number	Location/ Village	Name of the owner	Local name	Longitude (EW)	Latitude (NS)	Tree age	Uses/Other information	Genotype number (For characterization)
1	*UB1*	Panjan Gaon	Ranjit Saikia	Gol nemu	94.20716	26.49616	5	High yielder	7
2	*UB2*	Pokamura gaon	Subhalakshmi Borah	Gol nemu	94.16627	26.69679	10	Smooth skinned fruit	8
3	*UB3*	Tengajan Gaon	Prabin Saikia	Godha nemu	94.208335	26.49792	9	Bumpy textured fruit with nipple and areola	14
4.	*LB1*	Dwikhargurii	Lumnath Basumatary	Gol nemu	89.57809	26.29811	10	Pointed apex fruit	11
5.	*LB2*	Dwikharguri	Nami Basumatary	Gol nemu	89.57822	26.29817	9	High yielder	12
6.	*LB3*	Kandonapara	Tuleswar Roy	Gol nemu	90 .00916	26.30394	8	Rough skinned fruit	13
7.	*CB1*	Borkachari Gaon	Madhav Basumatary	Gul Nemu	26.1426	92.4647	10	Big sized, rough textured with prominent nipple and areola	3
8.	*CB2*	Gendhali Bebejia	Dipen Gogoi	Gul Nemu	26.2334	92.5043	9	Late bearer	1
9.	*CB3*	Samuguri Tinali	Monju Kalita	Gul Nemu	26.2530	92.5032	4	Small sized with smooth skin	2
10.	*NB1*	Morol Gaon	Raju Sarmah	Gul Nemu	26.4328	93.1116	10	High yielder	4
11.	*NB2*	Rangamuri,Pavoi	Punya Prasad Khound	Gul Nemu	26.4738	93.8500	3	Smooth skin and pointed apex	6
12.	*NB3*	BNCA campus	Annonymous	Gul Nemu	26.4330	93.7340	11	Rough textured	5
13.	*HZ1*	Rongkhelan	Ghanashyam Teron	Gul Nemu	25.5139	93.2750	5	Healthy and disease free, small sized fruits	9
14.	*HZ2*	Hidisagir	Bilison Rongpi	Gul Nemu	25.5329	93.2655	8	Bumpy skin texture	15
15.	*HZ3*	Birla	Jirjar Hanse	Gul Nemu	25.5252	93.2651	9	High yielding	10
16.	*BV1*	Brahmansashan	Pranab Mahanta	Gul lebu	24.46786	92.18787	9	Regular bearer	16
17.	*BV2*	Bagargool	Arati Nath	Gul lebu	24.45145	92.30170	14	Smooth skinned	17
18.	*BV3*	Bagargool	Niladri Nath	Gul lebu	26.45655	92.30001	5	Profuse bearer	18

was added, mixed well and incubated at 65 ˚C for 1 hr with occasional mixing. It was then brought to room temperature and centrifuged at 10,000 rpm for 12 minutes at 4 ˚C. Then, the supernatant was taken out in another microfuge tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed well. Samples were centrifuged in a bench top centrifuge at 12000 rpm for 15 minutes. The supernatant was carefully transferred into a new eppendorf tube; double volume of chilled ethanol was added and kept overnight at 4 ˚C. Samples were then spinned at 12,000 rpm for 12 minutes to pellet down the DNA, on next day. The DNA pellet was washed with 70 % ethanol (by centrifuging at 3,000 rpm for 3 minutes) and allowed to air dry. The DNA pellet was then dissolved in 50 µl of 1X buffer and stored at 4 ˚C for further use.

Quality evaluation and quantification of genomic DNA

To confirm the DNA extraction, samples were run on agarose gel electrophoresis and DNA quantification was done by using Nanodrop ND-1000. The isolated genomic DNA of each genotype was subjected to agarose gel electrophoresis. A photograph of the gel under the UV light was recorded digitally in a gel documentation system (UVP, UK) to enable estimation of quantity of each sample by LABWARE software. The DNA sample with strong band under the UV with minimum shearing was considered as good quality DNA for the present study.

PCR amplification and gel documentation

The isolated DNA was used for amplification by using the primers. The amplification was carried out for 35 cycles in a thermal cycler with each cycle consisting of denaturing at 94°C for 3 min., annealing at 55°C for 1 min. and extension at 72°C for 2min. This was followed by a single extension at 72°C for 7 min. For separation of PCR products with SSR marker, 2 % agarose gel electrophoresis was used.

Genomic data analysis

SSR data were analyzed with the software GenAlEx v. 6 (Peakall and Smouse 2006). Amplified fragment bands were scored as discrete variable '1' is present and '0' as absent. The diversity analysis was done on the basis of Jaccard's similarity coefficient. The dendrogram was also built based on the Jaccard's similarity coefficient using the UPGMA clustering method displaying genetic relationships between the eighteen germplasms. Similar methods were used by Saha *et al*. (2022) for construction of phylogenetic tree in various germplasm of watermelon.

Forty-four primers were found polymorphic that amplified for eighteen selected germplasm (**Plate 1).**The similarity coefficients were ranged from 0.04 to 0.58) (**Table 2)**. Dendrogram showed clear evidence of two separate clusters (I and II) at a level of approximately 60% dissimilarity (**Fig. 1**). Each major group again sub divided

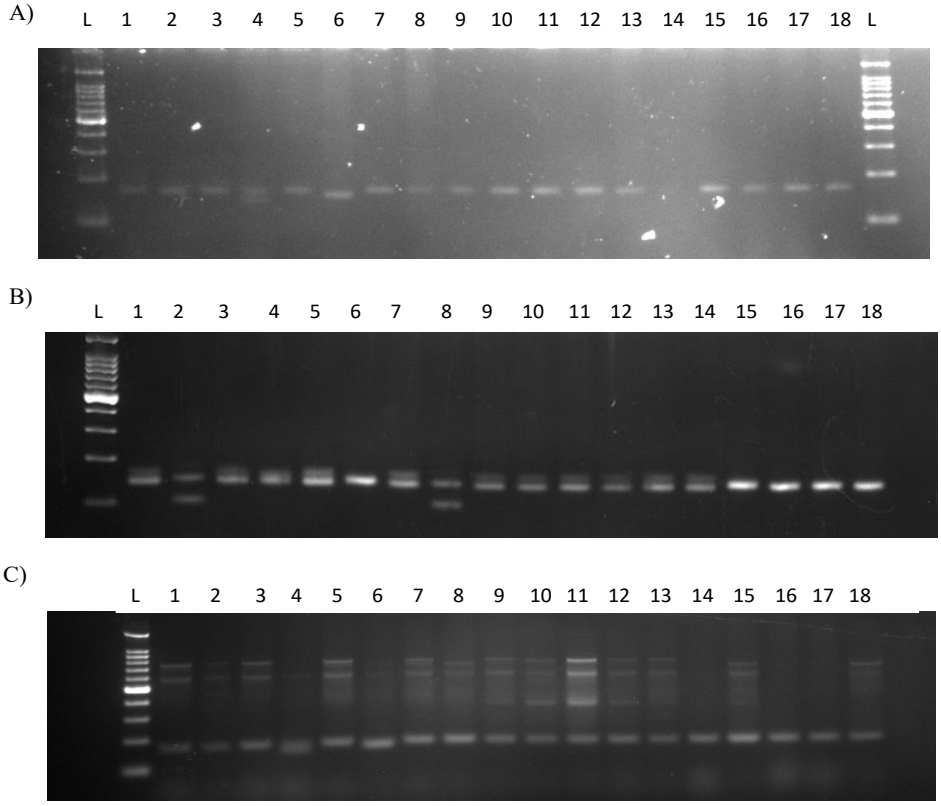

Plate 1. SSR gel image of various SSR markers. A). Org-1, B). CT02, C). AG14
For name of the genotypes refer Table 1

Table 2. Jaccard's similarity co-efficients

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1																		
2	0.16																	
3	0.04	0.13																
4	0.21	0.22	0.18															
5	0.39	0.43	0.39	0.44														
6	0.52	0.54	0.52	0.50	0.29													
7	0.36	0.45	0.39	0.45	0.17	0.30												
8	0.46	0.51	0.49	0.49	0.24	0.33	0.21											
9	0.18	0.29	0.21	0.33	0.41	0.56	0.43	0.46										
10	0.21	0.25	0.24	0.30	0.41	0.51	0.38	0.46	0.30									
11	0.30	0.39	0.32	0.39	0.33	0.47	0.33	0.39	0.29	0.34								
12	0.30	0.31	0.30	0.32	0.40	0.54	0.46	0.47	0.29	0.29	0.31							
13	0.27	0.32	0.27	0.32	0.31	0.46	0.35	0.44	0.30	0.32	0.18	0.29						
14	0.35	0.39	0.32	0.37	0.33	0.47	0.30	0.46	0.39	0.39	0.31	0.41	0.24					
15	0.39	0.45	0.39	0.47	0.25	0.39	0.23	0.31	0.45	0.43	0.29	0.38	0.29	0.25				
16	0.56	0.58	0.57	0.58	0.41	0.35	0.37	0.38	0.54	0.57	0.47	0.56	0.48	0.49	0.39			
17	0.54	0.58	0.56	0.58	0.40	0.36	0.40	0.42	0.58	0.54	0.40	0.53	0.45	0.44	0.33	0.25		
18	0.52	0.54	0.52	0.54	0.37	0.37	0.42	0.43	0.54	0.51	0.41	0.49	0.40	0.45	0.34	0.30	0.20	

For name of the genotypes refer Table 1

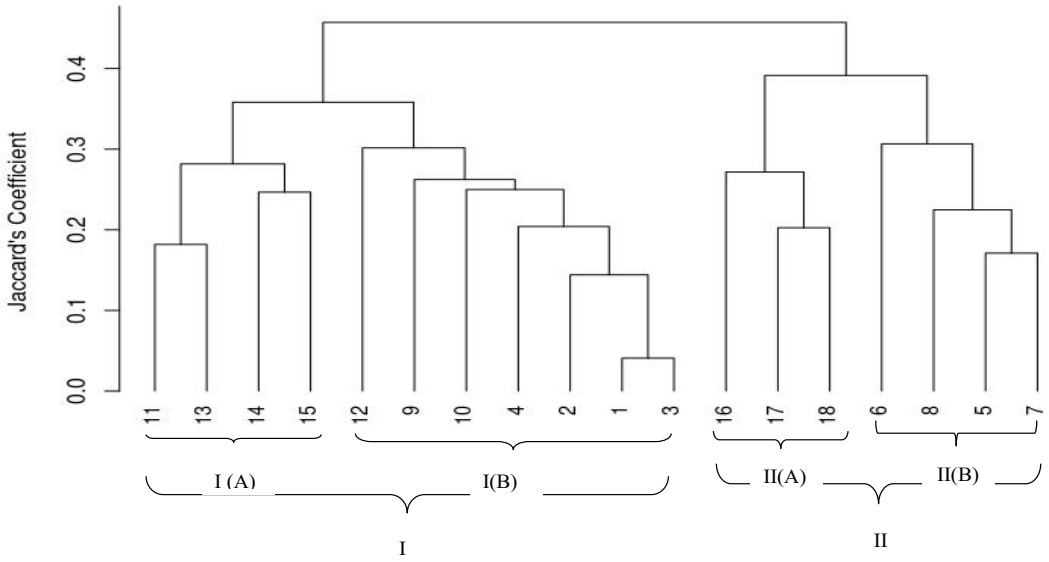

Fig. 1. Dendogram based on Jaccard's similarity co-efficients in 18 genotypes of rough lemon
For name of the genotypes refer Table 1

into two sub groups- sub group I (A), sub group I (B) and sub group II (A) and II (B). Sub group I (A) consists of varieties 11 (LB1), 13 (LB3), 14 (UB3) and 15 (HZ2); sub group I (B) consists of 12 (LB2), 9 (HZ1), 10 (HZ3), 4 (NB1), 2 (CB3), 1 (CB2) and 3 (CB1). Similarly sub group II (A) consists of 16 (BV1), 17 (BV2), 18 (BV3) and sub group consists of II (B) consists of 6 (NB2), 8 (UB2), 5 (NB3) and 7 (UB1). It was observed that the genotypes 1 (CB2) and 3 (CB1) are the most similar among all the genotypes. The close similarity may be because they are chance seedlings from the same tree and also may be because of nucellar embryony. The highest dissimilarity was observed between genotypes 16 (BV1) and 2(CB3), between 17 (BV2) and 2 (CB3), between 16 (BV1) and

4 (NB1), between 17 (BV2) and 4 (NB1) and between 17 (BV2) and 9 (HZ1). The minimum similarity may be explained on the basis of their geographical areas of occurrence and corresponding G x E interaction. Similar results were observed by Rohini *et al.* (2020) in the same crop across the country of India.

The knowledge on genetic diversity will provide an opportunity for plant breeders to develop improved cultivars with desirable characteristics (Saharia and Sarma, 2022). For any rootstock breeding programme, the primary objective is to accumulate the maximum available diversity which further can be screened or selected for crop improvement work. 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 Assam enabling it to be used for the improvement of Citrus industry in India and worldwide. It also endorsed the fact that North eastern region of India more particularly in Assam, harbour the diversity of *C. jambhiri* which needs to be conserved for further sustainable utilization.

As Assam faces recurrent waterlogged conditions due to heavy rain showers and flood every year, many of the rough lemon types shows decline in production and dies subsequently. Tamuli (2023) conducted an experiment to observe the response of rough lemon germplasm to water logging. It was observed that germplasm in I (A) category exhibited better tolerance to water logging including UB3 and HZ2. Similar research in this section of Citrus rootstock in future will be beneficial in unveiling some germplasm with unexplored qualities for conservation and quality improvement studies.

ACKNOWLEDGMENT

The authors would like to acknowledge Assam State Biodiversity Board for their financial assistance in this work.

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