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Evaluation of Genetic Diversity and Phylogeny in Cultivars of Citrus Species by RAPD Markers and Rbcl Gene Sequencing

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ABSTRACT:
All marker techniques effectively indicated the degree of polymorphism and genetic variability in citrus, proving their utility in the investigation of germplasm accession. RAPD markers, as a quick and simple method, can identify sufficient polymorphism, allowing for the differentiation between different Citrus species and cultivars as well as an understanding of their interrelationships. Since they can be produced and utilized for building quickly and in sufficient quantities, RAPD markers are useful for producing a linkage map. Recent studies using the rbcL gene and matK sequences as the primary plant DNA barcoding markers have examined plant variety underground. The focus of the current research is on using a variety of marker systems to look at genetic diversity at the DNA level in the genus Citrus. Numerous accessions in our study have not before been the subject of earlier studies. Clauseneae and Citreae are the two tribes that make up the subfamily Aurantioideae. Using just one chloroplast DNA sequence, our current molecular research convincingly distinguishes Citreae from Clauseneae. In many ways, our analysis supports earlier findings regarding the molecular phylogeny of the Aurantioideae. On the other hand, we have offered fresh knowledge regarding these genetic connections.

KEYWORDS: Phylogeny, citrus species, RAPD, Rbcl, molecular markers.

I INTRODUCTION

Mandarin, orange, lemon, grapefruit, and lime are all members of the genus Citrus, which also has considerable economic and nutritional importance. One of the seven subfamilies of the family Rutaceae is the subfamily Aurantioideae, to which this genus belongs. In light of this, a phylogenetic analysis of the genus Citrus and the family Aurantioideae is significant. There are 33 genera in 2 tribes in the Aurantioideae family [1]. The tribes Citreae, which includes Triphasiinae, Citrinae, and Balsamocitrinae, and Clauseneae, which includes Micromelinae, Clauseninae, and Merrillinae, each have three subtribes. None of the species of Clauseneae produce axillary leaflets that are connected alternately, and odd-pinnate leaves have spines. Except for Merrillia, the fruits are often tiny and contain semi-dry or juicy berries. In the Citreae, on the other hand, almost all species grow axillary spines. Simple leaves can easily be differentiated from those of the Clauseneae tribe. Due to the presence of pulp vesicles in the fruit, the subtribe Citrinae of the tribe Citreae stands out from all other subtribes in the Aurantioideae. "True citrus fruit trees" are regarded as the best developed taxa in this subtribe based on morphological characteristics [1]. "True citrus fruit trees" are those of the genus Citrus. Asexual reproduction, a high frequency of mutations, and interspecies compatibility are traits of citrus species. Due to these traits, there is significant morphological and among the species of citrus, ecological diversity. In order to clarify the phylogeny of Aurantioideae, particularly that of Citrus and its close relatives, morphological [2-4] and biochemical research [5–9] have been carried out since the 1970s. These connections have undergone substantial research thanks to advancements in DNA analysis. Several methods, including sequence-related DNA analysis (SRA), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), restriction fragment length polymorphism (RFLP), and Studies on taxonomic diversity frequently make use of amplified polymorphism (SRAP) [10–14]. The significant use of small DNA fragments, particularly those of the Phylogenetic analyses based on the various areas of the chloroplast genome, has been made possible by recent advancements in DNA sequencing techniques. These analyses have been carried out in the family Rutaceae and the subfamily Aurantioideae [15–24].
On the basis of rbcL gene sequences, we have also previously revealed the evolutionary connections among the Aurantioideae, including Citrus and its relatives [25]. The large subunit of ribulose 1, 5-bisphosphate

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Vol. 10, Issue 7 , July 2023

carboxylase/oxygenase, an enzyme that catalyzes carbon fixation in photosynthesis, is encoded by the rbcL gene, which is found on the chloroplast DNA (cpDNA). The nucleotide substitution rate of the rbcL gene is relatively modest when compared to most genes contained in the cpDNA. Our earlier study [25] stood out for its inclusion of a number of accessions that had not been looked at in other studies [15–24]. However, the discrimination's power in our earlier investigation was not that strong. Phylogenetic relationships are studied using the chloroplast genome.

II MATERIALS AND METHODS

(A)Plant Material

Citrus species with known wild populations provided plant material for the isolation of genomic DNA. In the experiment, leaves were transported in liquid nitrogen and kept in zip-top bags at -20°C. By making a few small adjustments to the CTAB method21, the leaves were treated to the extraction of genomic DNA.

(B)Protocol for DNA Isolation

Using liquid N2, fresh green leaves weighing 0.5 g were de-veined and ground into a fine powder in a mortar and pestle. By mixing 30 ml of DDW with 9 ml of 150 mM Tris-Cl, 3 ml of 25 mM EDTA, and 18 ml of 1.5 M NaCl (all at pH 8.0) and warming it to 65 oC, a 60 ml homogenization buffer stock was created. The pre-warmed solution received 2.1g CTAB and 1.8g PVP additions, and 180 l Beta Mercaptoethanol was added before homogenization. Then, 3 cc of previously warmed CTAB solution was added to the fine leaf powder. 20 l of RNase were added to the 3 ml suspension after it had been transferred to a sterile centrifuge tube. With mild inversions, the solution was incubated for 45 minutes at 65 °C. After the tube had reached room temperature, 3 ml of chloroform in a 24:1 IAA ratio was added to it. To create an emulsion, the tube was gently flipped upside down 20–25 times. Centrifuging the emulsion at room temperature for 10 minutes at 10,000 rpm. Without disrupting the interphase, the upper aqueous layer was pipetted out and put into sterile centrifuge tubes. The aqueous phase was then given a 3 ml addition of 3M NaCl before being centrifuged one more at RT at 10,000 rpm. Isopropyl alcohol was added to the aqueous phase in 0.6 volumes (1.8 ml), thoroughly mixed, and incubated for 30 minutes at room temperature. Centrifuging the solution at RT for 15 minutes at 10,000 rpm. The obtained supernatant was gently drained off. The resulting pellets were thoroughly cleaned with 750 ml of 70% ethanol and spun for 5 minutes at 10,000 rpm. The resultant white pellet was air dried (for about 45 minutes) and then re-suspended in 30 l of TE (10 mM Tris HCl+ 0.1 mM EDTA; pH 8.0) at 4oC. The supernatant was discarded. The T.E. Buffer + DNA solution received another addition of 3M sodium chloride solution, and ethanol reprecipitation was carried out. After 15 minutes of centrifugation at 10,000 rpm, the sample's pellet was redissolved in TE buffer. Three times the procedure was carried out. In contrast to other approaches, this one enabled the recovery of DNA of high quality, appropriate for full restriction digestion and amplifiable by PCR.

(C)Quantification of DNA

The spectrophotometric approach (UV-Vis Spectrophotometer, Pharmaspec UV-1700, Shimadzu, Japan) was used to measure the content of DNA. At 260 nm and 280 nm, the solution's absorbance was measured. Calculated is the OD260/OD280 ratio. All of the samples' quantification followed the same process. For use in PCR analysis, DNA samples were diluted to final concentrations of 5 and 10ng/l. For additional DNA quantification, DNA samples with the highest quality, as shown on an agarose gel, and an OD260/OD280 ratio closer to 1.7-1.8 were used.[26-28]

(D) Conditions for PCR using RAPD Primers

Stock solutions of 100 pmol l-1 for the RAPD primers, which were purchased as dry desalted powder in vials, were made using MQ water. Before opening, the vials were spun at 1000 rpm for 5 min to make sure the oligonucleotides were deposited in the bottom of the tube. The necessary volume of MQ water was then added. The stock solutions were carefully vortexed to ensure adequate mixing.
The working solutions of 10 pmol/l for all the primers were made from the stock solutions of 100 pmol/l each. All of the primer stocks were kept in the dark at -20°C. By PCR amplification, RAPD primers were assessed by hybridizing them with genomic DNA samples of different citrus species. To begin with, 35 random decamer primers from the MAP, OPA, OPN, OPJ, OPH, OPU, and OPG series (produced by Eurofins Genomics, India) were evaluated for their suitability for PCR amplification of the whole genomic DNA of citrus species. Twenty random decamer primers out of

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Vol. 10, Issue 7 , July 2023

35 passed the preliminary assay, and 11 of these 20 random decamer primers produced amplified products that were repeatable and clear under similar circumstances.

(E)PCR amplification conditions optimization

In order to produce the most amplicons feasible, each unique primer was amplified under a range of annealing temperatures. The annealing temperature for RAPDs ranged from 30 to 40 degrees Celsius. Magnesium ions are necessary for Taq polymerase to bind to the DNA template, hence the concentration of magnesium ions in the reaction mixture plays a key role in regulating the production of amplicons. MgCl2 serves as the source of these ions. For both the ISSR and RAPD primers, different MgCl2 concentrations (2.0 to 3.5 mM) were utilized, and the effect on band formation was investigated.

•Concentration of dNTPs - The quantity of dNTPs in the reaction mix was changed in order to assess the impact of dNTP concentration on DNA amplification during PCR. We evaluated the dNTPs at doses of 0.20, 0.25, 0.30, 0.35, and 0.40 mM. All the variations shared a different set of conditions.

•Thermo cycles - The amplification reactions using RAPD primers were carried out for 40–55 cycles in order to observe the impact of the number of repeated cycles on DNA amplification. On the other hand, 30-35 cycles of DNA amplification employing ISSR primers were carried out.

•Template DNA - 25, 50, 75, and 100 ng of template DNA were employed individually to produce amplification products in order to examine the impact of template DNA concentration on DNA amplification.

All amplifications used Taq DNA polymerase, which was isolated from E.coli and expressed a cloned Thermus aquaticus DNA polymerase (In vitrogen (platinum) & Bangalore Genei, India). Both 3'---5' exonuclease and 5'--- 3' DNA polymerase activity are present in this enzyme. A single polypeptide with a molecular weight of roughly 94 KDa makes up the enzyme.

Different concentrations or units (0.5, 1.0, 1.5, 2.0, and 2.5 U) of Taq polymerase were utilized for the 25mgl reaction mixture in order to assess the impact of Taq DNA polymerase concentration on DNA amplification.

III RESULT AND DISCUSSION

(A)Assessment of Genetic Diversity based on RAPD primers

The row dendrogram in Fig. 1 for RAPD primer OPU 02 data have been constructed using UPGMA with a SAHN module of NTSYS.

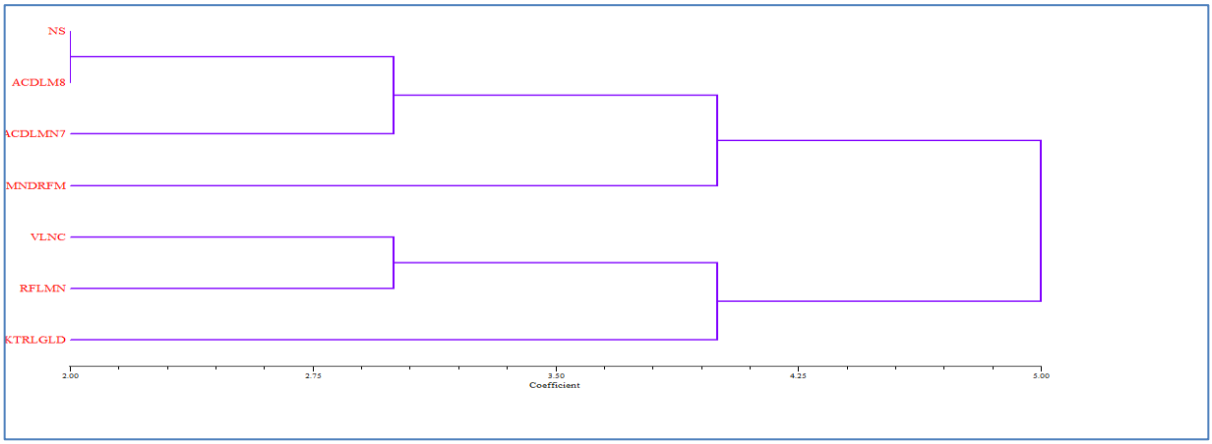

Fig.1 : Dendrogram generated using un-weighted pair of group method with arithmetic average analysis (UPGMA), showing relationships between different populations of *citrus species* using RAPD primer OPU 02 data.

ISSN: 2350-0328
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The dendrogram reveals 2 clusters comprising of 7 and 5 sample populations respectively. The larger cluster includes- NS, RL, KG, RFL and VO; while the second smaller cluster comprises of- ALN8, MF, ALN7, ALT and VLNC.

(B) Similarity Coefficient Analysis of RAPD primer OPU 02

Jaccard's pair-wise similarity coefficient values among all the populations of *Citrus species* using RAPD primer OPU 02 vary between 0.32 and 1.00 as shown in table1.

Table 1Jaccard's similarity matrix between different populations of *Citrus species by* RAPD primer OPU 02

Sample	NS	ALN7	ALN8	MF	RL	KG	VO	RFL	ZA	ALT	BR	VLNC
NS	1.00											
ALN7	0.32	1.00										
ALN8	0.32	0. 98	1.00									
MF	0.32	0. 98	0. 98	1.00								
RL	1.00	0.32	0.32	0.32	1.00							
KG	1.00	0.32	0.32	0.32	1.00	1.00						
VO	1.00	0.32	0.32	0.32	1.00	1.00	1.00					
RFL	1.00	0.32	0.32	0.32	1.00	1.00	1.00	1.00				
ZA	1.00	0.32	0.32	0.32	1.00	1.00	1.00	1.00	1.00			
ALT	0.32	0. 98	0. 98	0.98	0.32	0.32	0.32	0.32	0.32	1.00		
BR	1.00	0.32	0.32	0.32	1.00	1.00	1.00	1.00	1.00	0.32	1.00	
VLNC	0.32	0. 98	0. 98	0.98	0.32	0.32	0.32	0.32	0.32	0. 98	1.00	1.00

32% similarity has been observed between: NS-ALN7, MF-NS, ALN8-RL, VO-ALN7, RL-ALT, RFL-ALN7, KG-VLNC and some other pairs. A similarity coefficient of 0.98 or 98% similarity has been observed between: MF-ALN7, ALN8-MF, ALT-ALN8, ALN7-ALT, VLNC-ALT, MF-VLNC and a few others. The highest similarity of 100% has been recorded in sample populations like NS-KG, RL-RFL, VO-RFL.

(C) Assessmentof Phylogeny by Rbcl Genes

On the basis of rbcL gene sequences, we have also previously described the evolutionary connections among the Aurantioideae, which include Citrus and its relatives. The large subunit of ribulose 1, 5-bisphosphate carboxylase/oxygenase, an enzyme that catalyzes carbon fixation in photosynthesis, is encoded by the rbcL gene, which is found on the chloroplast DNA (cpDNA). The nucleotide substitution rate of the rbcL gene is relatively modest when compared to the majority of cpDNA-encoded genes. Our prior study stood out for its inclusion of a number of accessions that were not subjected to other analyses. However, the discrimination's power in our earlier investigation was not that strong.

(D) Amplification of Rbcl Genes

Purification of amplified samples of Rbcl Citrus species nrDNA was followed by their amplification using ddNTPs with individual forward and reverse primers. The ABI sequences were visualized in Chromas LITE 2.1.1(fig 2-4) with peaks of individual nucleotides.

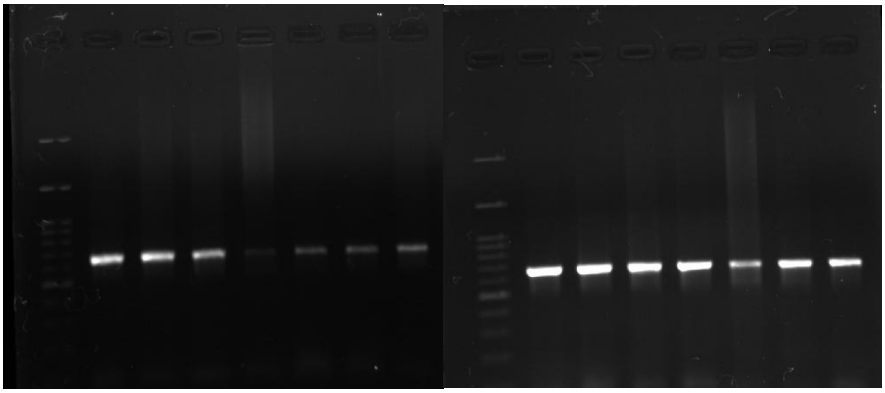

Figure-2 Electrophoretic profile of amplified gene Rbcl

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Vol. 10, Issue 7 , July 2023

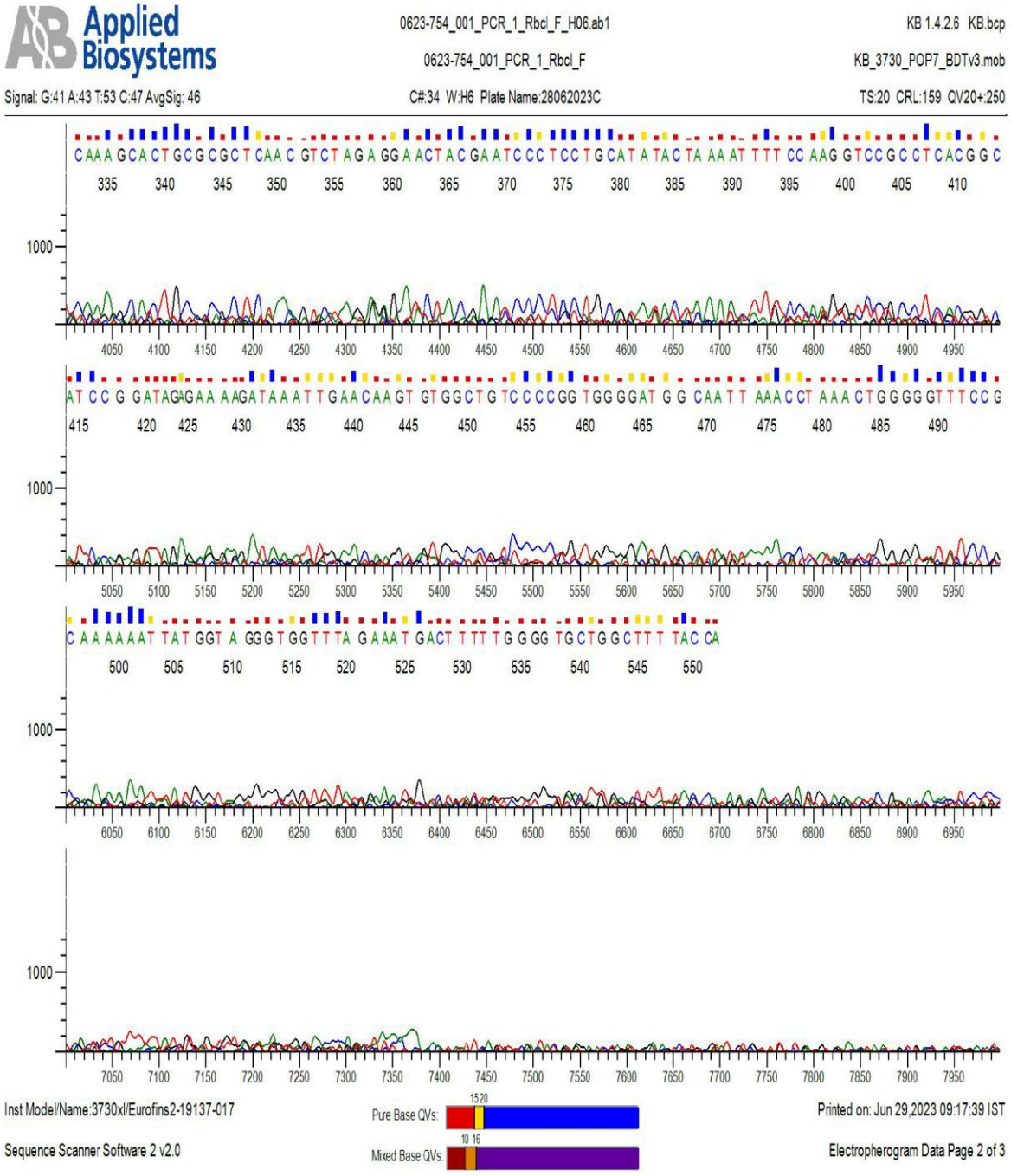

Fig-3 Rbcl-F primer gene sequence

ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 10, Issue 7 , July 2023

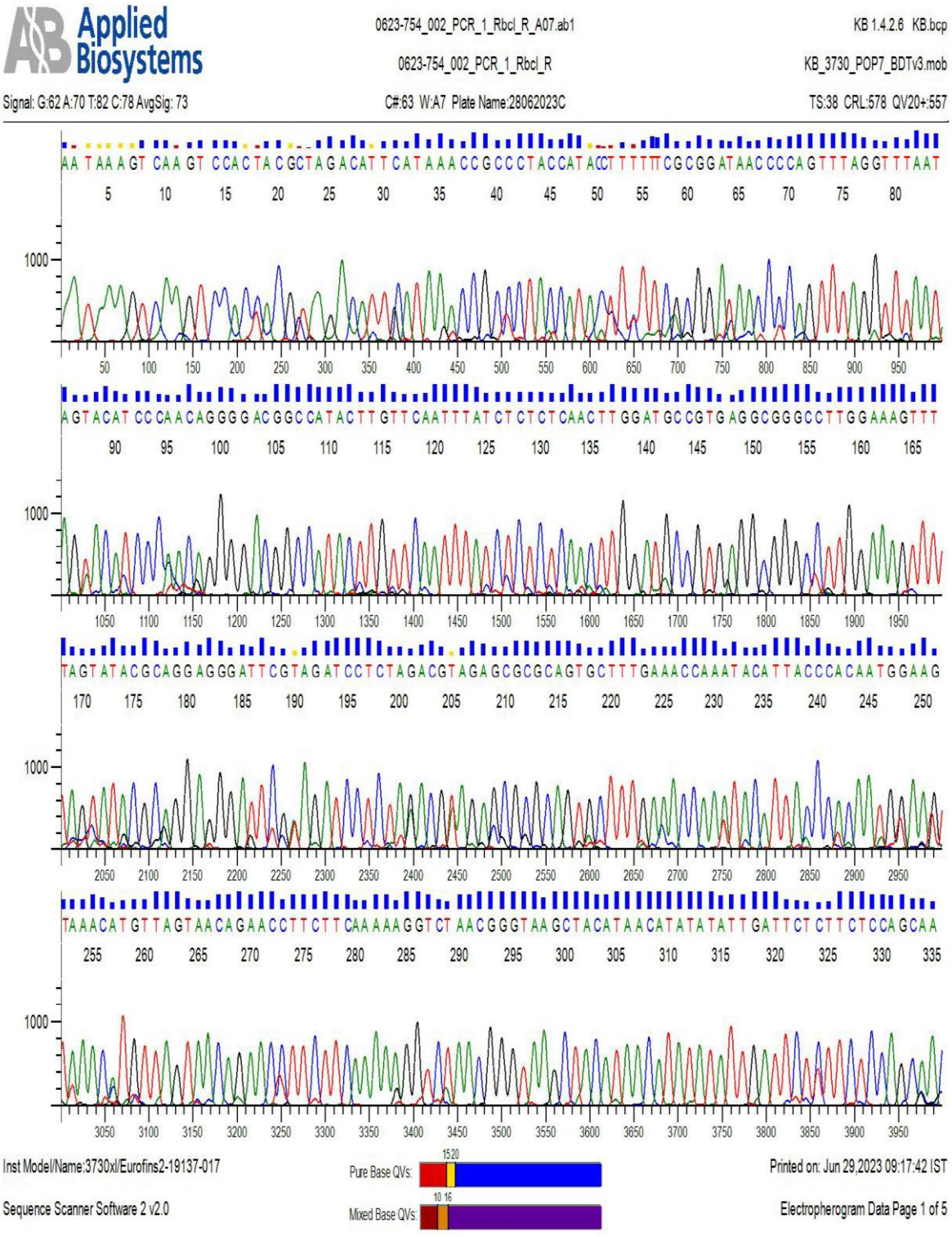

Fig-4 Rbcl-Rprimer gene sequence

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Vol. 10, Issue 7 , July 2023

According to Swingle's methodology, Kumar et al. (2012) was unable to distinguish clearly between the subgenera Citrus and Papeda. This confirms prior findings by Pang et al. (2007) and Nicolosi et al. (2000). the trees created by both methods produced very similar topologies and supported Swingle and Reece's (1967) classification of the subfamily Aurantioideae as monophyletic In the Citrinae, our analysis did not clearly support the distinction between "primitive citrus fruit trees" and "near citrus fruit trees" by Swingle and Reece (1967). Both trees strongly supported the polytomous clade containing H. crenulata and three Citropsis spp., as similarly. The rbcL topology generally agreed with various previous phylogenetic studies of Citrus, although some of the branches received low bootstrap support. According to those previous studies, the genus Citrus has only three true species—C. medica (citron), C. reticulata (mandarin), and C. maxima (pummelo)—and the other species, such as C. sinensis (sweet orange), C. paradisi (grapefruit), and C. limon (lemon), are derived from hybridization.[Silva et.al, Stace HM et,al, Suyama Y, Tamura K and Tanaka T et.al].The present study provides important information to resolve the genetic relationships and evolution within the tribes and subtribes of the Rutaceae subfamily Aurantioideae. Both dendrograms support the monophyletic nature of the subfamily.

IV CONCLUSION

In the present study the dendrogramfor the all the 12 sample populations using RAPD markersshowed an intriguing pattern formation of clusters. The present study provides novel information that resolves the genetic relationshipsamong members of the Aurantioideae, especially of the genus Citrus, and confirms previous observations.

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International Journal of Advanced Research in Science, Engineering and Technology

Vol. 10, Issue 7 , July 2023

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