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Assessement of Genetic Diversity of Native Pumelo (*Citrus maxima* Merr) by Utilizing SSR Markers

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Abstract: ***Genetic diversity of 29 accessions included 10 native pumelo varieties in Vietnam was analyzed by using 35 SSR markers. The results showed that 3.29 alleles per SSR loci; PIC value range from 0.0 to 0.82, with average is 0.45. All grapefruit accessions showed heterozygosity from 35.26% to 51.61%. Genetic distance was determined using Jaccard's similarity coefficient and final dendrogram construction using a UPGMA which revealed that 29 accessions were divided into the 8 distinguished heterotic groups with genetic similarity from 0.32 to 1.0. The results also showed that: There were 2 varieties Dien and Bang Luan, in which, between accessions have had no difference on 35 loci. In other varieties, a narrow genetic different within and among accessions in a variety suggesting for germplasm fingerprinting, preeminent selection and proper utilization should be further done via breeding programs.***

Keywords: Citrus, grapefruit, native, genetic diversity, microsatellite

1. Introduction

Citrus, part of the Rutaceae family, is known as a major fruit crop in the world. Due to its tasty fruit, Citrus also has high potential in human health protection, especially antioxidant activity [1]. The antioxidants of citrus include polyphenolic compounds, antioxidant vitamins and flavonoids [2, 3, 4]. Moreover, Flavonoids contained in citrus have a wide range of biological activities, such as antibacterial effect and various clinical properties [5, 6, 7]. Pumelo (*Citrus maxima* Merr.) is one of the most abundant species of citrus with particular characteristics. Arccoding to the study of Li [8] and Ye [9], the origin of pumelo is in Southeast Asia. In Vietnam, Pumelo has been cultivated for many years and playing an important role in economy with very high rate of annual consumption. In addition, there are more than 70 varieties, which were grown throughout this country.

Scientists realized that Pumelo has high genetic diversity in compare with other kind of citrus due to a long history and diverse climates [9, 10, 11, 12, 13]. To date, many studies on genetic resources of pumelo were carried out [14, 12, 13]. In those studies, Simple sequence repeat (SSR) is the first choice of molecular markers. Barthe et al. [15] indicated that SSR is an effective marker and popularly applied in the research on genetic diversity of many species.

In Vietnam, the research on citrus using molecule markers has been carried out, such as Isozyme, RAPD and SSR [16, 17]. However, in case of pumelo, comprehensive study on genetic variation has not been conducted before. In addition, the knowledge about the evolutionary connections among pumelo germplasms and the phylogenetic relationships among varieties is limited. Therefore, in this study, SSRs were used to analyse the genetic diversity of 29 accessions of pumelo and its relatives belonging to 10 local commercial varieties which are native in Vietnam, to constitute a DNA finger map of excellent pumelo germplasms, and to analyse their genetic relationships. The purpose of the study was to provide molecular evidence for pumelo breeding, identification of excellent germplasms, conservation and further utilizing of Vietnamese native pumelos.

2. Materials and Methods

The origins of the 11 pumelo germplasms and their relatives used in this study were collected in collection farm of SOFRI and FAVRI and farmer growing orchards which are shown in Table 1.

Table 1: Vietnamese native pumelo varieties origin and collection places

No.	*Local name*	*Acessment No.*	*Place*	*Origin/ province*
1	Suu Chi Dam	S1, S2, S3	Phu Tho	Phu Tho
2	Bang Luan	BL1, BL2, BL3	Phu Tho	Phu Tho
3	Dien	D1, D2, D3	Fruit and Vegetable Research Institut **(Vietnam)**	Ha Noi
4	Do Me Linh	DML1, DML2, DML3	Fruit and Vegetable Research Institut **(Vietnam)**	Vinh Phuc
5	Phuc Trach	PT1, PT2, PT3	Fruit and Vegetable Research Institut **(Vietnam)**	Ha Tinh
6	Thanh Tra	TT1, TT2, TT3	Fruit and Vegetable Research Institut **(Vietnam)**	Hue
7	Pho Trach	PTr1, PTr2	Fruit and Vegetable Research Institut **(Vietnam)**	Hue
8	Nam Roi	NR1, NR2, NR3	Ben Tre	Vinh Long
9	Da Xanh	DX1, DX2, DX3	Southern horticultural research institute **(Vietnam)**	Ben Tre
10	Duong La Cam	DLC1, DLC 2, DLC3	Tan Trieu	Đong Nai

Total DNA of young leaves was extracted using the cetyl triethyl ammonium bromide (CTAB) modified method [18]. DNA concentration and quality were detected by an Ultrospec 2100 ultraviolet scanner (Biochrom Ltd., Cambridge, UK). The extracted DNA was diluted to 50 ng/ml and stored at -20^{0}C. SSR analysis was conducted as

described by Kijas et al [19] with some modifications. The total volume of the polymerase chain reaction (PCR) amplification reaction system was 15 µl, which contained 50 ng genomic DNA, 1.5mmol/l $MgCl_2$, 0.2mmol/l deoxynucleoside triphosphates (dNTP), 1.0U Taq DNA polymerase (TaKaRa Biotechnology (Dalian) Co. Ltd., China), and 0.1 mmol/l forward and reverse primers.

PCR amplifications were performed in a PTC-200 Peltier Thermal Cycle (MJ Research Inc., Massachusetts, USA) programmed for 94 ^{o}C for 5min, followed by 35 cycles of 94 ^{o}C for 60 s, 55 ^{o}C for 30 s, 72 ^{o}C for 60 s and a final elongation of 72 ^{o}C for 10min. Sampling buffer solution (3/4 volume) was added to the amplification products, which were denatured for 5min at 95 ^{o}C, then cooled immediately.

Amplification products were separated by denaturing 6% polyacrylamide gel electrophoresis (apparatus from Beijing Liuyi Instrument Factory). The electrophoresis time was 1–1.5 h and the voltage was 1800 V. After this, the gel was fixed with 0.5% acetic acid solution, then dyed with 0.2% silver nitrate solution. Bands were visualized in 1.5% sodium hydroxide solution, and development was stopped by sodium carbonate solution. Finally, bands were washed and air-dried. Bands of different samples were recorded. The SSR profiles were scored 1 (presence) and 0 (absence) and the original matrix was used to calculate parameters as follows. Polymorphism information content (PIC) =1-∑nj =1P2ij, where Pij indicates the frequency of the jth allele at ith locus and n the number of alleles [20]. The similarity coefficient was calculated using the Qualitative Data Program of NTSYS PC Version 2.10e software (State University of New York, Stony Brook, New York, USA), and a similarity coefficient matrix was obtained. The cluster analysis was performed with the SAHN program P.H.A. Sneath and R.R. Sokal, Freeman, San Francisco, USA and the unweighted pair-group arithmetic average (UPGMA) method. The Tree Plot program was used to create a cluster map.

3. Results and Discussion

3.1. PIC values and pomlelo SSR Marker

The value of allelic polymorphism information content (PIC) shown in Table 2 that PIC values of 35 primers ranged from 0 (only one band detected) to 0.82 (GT03 could detect 7 alleles), with an average of 0.45 per primer.

The analysis of 35 pairs of primers on 29 accessions from 10 Vietnamese native pumelo varieties showed that 4 primers gave one band/locus (Ci02B10, Ci07E06, mCrCIR06A03 and NTCP9) and 31 primers gave more than one bands per locus, all 35 primers generated total 115 alleles. The average number of alleles per locus was 3.29 (Table 2; Figure 1).

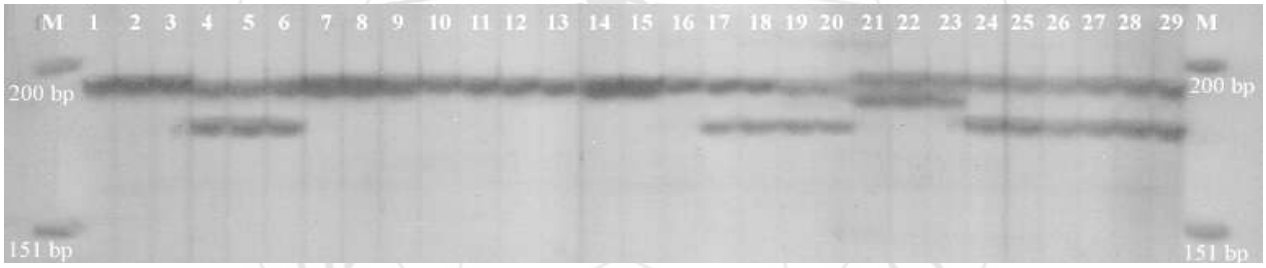

Figure 1: Electrophoretic patterns of 10 pumelo varieties with SSR primer m*CrCIR06A08. (M: PhiX174 DNA/HinfI Marker. Lanes 1-3 = Suu; 4-6 = Bang Luan; 7-9 = Dien; 10-12 = Do Me Linh; 13-15 = Phuc Trach; 16-18 = Thanh Tra; 19-20 = Pho Trach; 21-23 = Nam Roi; 24-26 = Da Xanh; 27-29 = Duong La Cam).*

Table 2: PIC values and number of alleles generated from SSR primers

No.	*SSR Primer*	*Number of alleles*	*Total alleles/primer*	*PIC*	*Sno.*	*SSR Primer*	*Number of alleles*	*Total alleles/primer*	*PIC*
1	Ci01A07	2	29	0.50	20	mCrCIR06A02	4	40	0.57
2	Ci01C07	4	52	0.65	21	mCrCIR06A03	1	29	0.00
3	Ci01H05	5	35	0.70	22	mCrCIR06A08	4	48	0.56
4	Ci02A04	2	33	0.12	23	P73	3	46	0.58
5	Ci01D11	2	57	0.50	24	P94	2	29	0.48
6	Ci02B07	4	34	0.40	25	CAC23	3	36	0.37
7	Ci02B10	1	29	0.00	26	NTCP9	1	28	0.00
8	Ci02F07	3	31	0.45	27	AC01	4	49	0.71
9	Ci06A05b	4	46	0.55	28	AG14	7	45	0.74
10	Ci07B09	2	27	0.20	29	CAG01	4	49	0.62
11	Ci07D10	2	31	0.07	30	CAT01	2	40	0.45
12	Ci07E06	1	29	0.00	31	CT02	2	55	0.49
13	Ci07G07	5	47	0.65	32	CT21	4	45	0.53
14	Ci08A10	2	28	0.07	33	CTT01	4	58	0.71
15	Ci08C05	2	44	0.48	34	GT03	7	41	0.82
16	mCrCIR01B02	4	37	0.35	35	CT19	2	32	0.50
17	mCrCIR01B10	3	36	0.46	**Total**		115	1395	15.83
18	mCrCIR01D06a	6	53	0.81	**Average**		3.29	39.86	0.45
19	mCrCIR01F04a	7	47	0.76					

3.2 Missing ratio and percentage of heterozygote

Highest Missing ratio per locus was found in two Thanh Tra pumelo (14.29%), 11 pumelo accessions have missing ratio ranged from 2.86-11.43% and 16 were zero. So, the evaluation of 35 locus from 29 pumelo accessions have meaning in statistical analysis (Table 3).

Table 3: Missing ratio (M%) and percentage of Heterozygote

No.	*Acc. No.*	*M%*	*H%*	*Sno.*	*Acc. No.*	*M%*	*H%*
1	S1	5.71	36.36	16	TT1	0.00	40.00
2	S2	0.00	40.00	17	TT2	14.29	40.00
3	S3	2.86	41.18	18	TT3	14.29	36.67
4	BL1	0.00	42.86	19	PTr1	2.86	41.18
5	BL2	0.00	42.86	20	PTr2	0.00	40.00
6	BL3	0.00	42.86	21	NR1	2.86	41.18
7	D1	5.71	39.39	22	NR2	2.86	41.18
8	D2	0.00	37.14	23	NR3	0.00	40.00
9	D3	0.00	37.14	24	DX1	0.00	45.71
10	DML1	2.86	41.18	25	DX2	8.57	46.88
11	DML2	0.00	40.00	26	DX3	11.43	51.61
12	DML3	0.00	42.86	27	DLC1	0.00	45.71
12	PT1	0.00	42.86	28	DLC2	0.00	42.86
14	PT2	0.00	37.14	29	DLC3	5.71	42.42
15	PT3	2.86	35.29				

The percentage of heterozygotes per marker detected in our native pumelo varieties ranged from 35.29% in case of Phuc Trach pumelo to 51.61% in case of Daxanh pumelo. The mean observed hetero-zygosity for pumelo varieties in the South was 44.17% higher than in the North (39.58%).Therefore, the analyse of 35 primers from 29 accessions of 10 pumelo varieties showed that there are all heterozygotic in all 35 loci. In the same variety, except for Bang Luan pumelo, they are all different in their reaction with each other in different locus. These results are very helpful for determining the genetic variation and can be used for detection of right commercial variety for further pumelo breeding program and propagation.

3.3. Cluster analysis of Vietnamese native pumelo germplasms

A similarity coefficient was calculated between each pair of accessions, using the original matrix composed of the data of 115 loci of 29 pumelo germplasms showed that the similarity coefficient level range from 0.32 to 1.0. The different of SC level between Suu and Pho trach was the highest (0.34) and then Do Me Linh and Nam Roi was 0.36. The dendrogram (Figure. 2) showed that at a similarity coefficient level of 0.57 all accessions could be divided into eight groups.

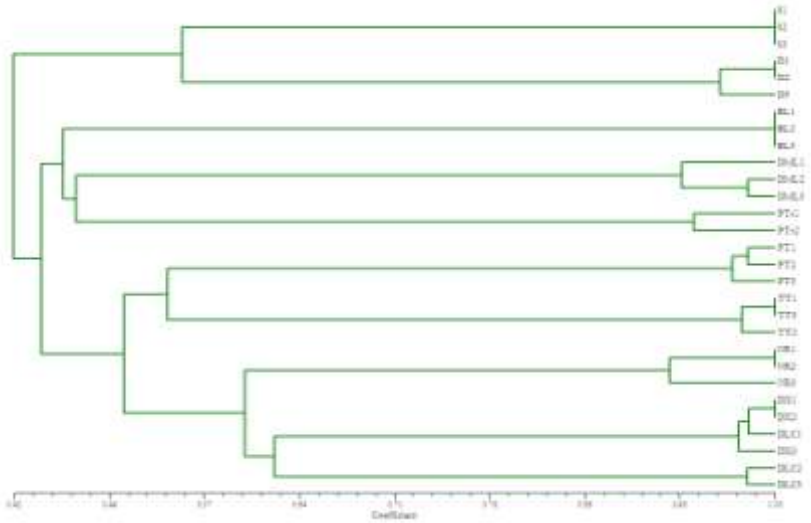

Figure 2: Dendrogram of cluster analysis for 10 pumelo varieties, based on SSR markers

Group 1 comprised the Suu pumelo group (3 accessions) which have same reaction with 35 SSR primers and SC was 1.0;

Group 2 was Dien pumelo (3 accessions) in which D1 and D1 have SC 1.0 and D3 have a different with D1 and D2 and SC is 0.96;

Group 3 comprised Bang Luan pumelo (3 accession) and their SC are 1.0;

Group 4 was formed by Do Melinh pumelo (3 accessions) in which the SC between pair of accessions DML1 - DML2, DML1 - DML3, DML2 - DML3 are 0.94, 0.92 and 0.98 respectively;
Group 5 was Pho Trach pumelo (2 accession) which has SC level 0.96 between the two accession;

Group 6 comprised the Phuc Trach pumelo in which the SC between pair of accessions PT1 - PT2, PT1 - PT3, PT2 - PT3 were 0.98, 0.98 và 0.96 respectively; Group 7 comprised Thanh Tra pumelo, in which TT1 and TT3 have same genotype in 35 loci and TT2 have the same SC level 0.98 as compare with TT1 and TT3;And group 8 comprised from the South pumelo varieties and can be divided into three subgroups: Subgroup 8.1 was Nam Roi pumelo which NR1 and NR2 have the same genotype and NR3 have SC of 0.92 with NR1 and NR2; Subgroup 8.2 comprised Da Xanh pumelo (3 accessions) and one accession of Duong La Cam (DLC1) in which DLC1 and DX1, DX2 and DX3 were clustered together at 0,98, 0,98 and 0,96 respectively, DX3 and DX1, DX2 were clustered together at 0.98; Subgroup 8.3 was Duong La Cam (2 accessions) and DLC 1 and DLC 2 was clustered together at a similarity coefficient level of 0.98.

In last some decades, numberous reports focused on genetic resources of pumelo were done by using SSR markers [14, 12, 13]. Vietnam is an agricultural country where is rich in tropical pumelos resources. This is the first report to assess genetic diversity of pumelo by SSR markers. This study provide useful information on genetic diversity, facilitates future genome research as well as breeding programs.

4. Conclusions

Vietnamese native pumelo varieties have a vast genetic diversity. The analysis from 35 SSR primers on 29 accessions of 10 pumelo varieties resulted in 115 alleles (average 3.29 alleles/primer) and PIC values ranged from 0.0 to 0.82 (0.45/locus) and H% of all accessions ranged from 35.29% to 51.66%.

The Vietnamese native pumelo varieties could be divided into 10 subgroups from 8 groups at the SC level of 0.57, and each variety belonged to each group or Subgroup and hence they could be distingushed with other variety. This can be used to define marker for identification of different pumelo varieties in Vietnam and for investigation on the evolution of species origin, new gene resource detection, and breeding improvement.

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