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Genetic variability of "Thanh tra" pummelo (*Citrus grandis* (L.) Osbeck) at Thua Thien Hue, Vietnam

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ABSTRACT

"Thanh tra" pummelo (Citrus grandis (L.) Osbeck) is a kind of speciality fruit tree in Thua Thien Hue province, Vietnam, they have significant differences in most of tree and fruit morphology characteristics such as tree and leaf size, fruit shape and size. The genetic variability of 42 "Thanh tra" pummelo trees was evaluated using random amplified polymorphic DNA (RAPD) analyses. All of the 68 amplified fragments revealed polymorphism among the trees, with the average number of polymorphic markers per primer being 7.56. The number of amplified fragments per primer varied from 5 to 11, with their sizes ranging from approximately 305 to 2,354 bp. Jaccard's similarity coefficient values changed from 0.00 to 1.00 with a mean of 0.26. An unweighted pair group method with arithmetic (UPGMA) phenetic tree was constructed and three "Thanh tra" pummelo were identified. Group A contains 31 trees with Jaccard's coefficients of genetic similarity (GS) approximately of 0.23 and subdivided into four subgroups, most of all were planted at the south of the Duong Hoa and Khuc Ly. Group B consist of 5 trees from all of collected sample regions except Duong Hoa (8TB, 11TB, 11TB, 8PS, 5PS and 27KL). Group C includes 6 genotypes (2TB, 3TB, 8PA, 11KL, 13KL and 26KL), explants in this group have less than 10 PCR products and genetic similarity less than 50%. "Thanh tra" pummelos in Thua Thien Hue have high level of genetic diversity.

Keywords: *Citrus*, genetic variability, polymorphism, RAPD, "Thanh tra" pummelo.

INTRODUCTION

Citrus is one of the world's most important fruit crops due to its wide distribution (throughout the tropical and subtropical regions) and large-scale production [13]. Pummelo (*C. grandis* (L.) Osbeck) has been regarded as one of the ancestral species as well as important commercial fruit tree under the genus *Citrus* [26]. The world production of grapefruits and pummelo is 4 million

tons and is grown in 74 countries on about 264,000 ha [13]. In Vietnam, citrus is one of most popular fruits and as a consequence they are grown widely from the North to the South, leading to a high abundance of genetic resources [16].

Thua Thien Hue is a province in the North Central Coast of Vietnam, approximately in the center of the country. The climate is tropical monsoon and the annual precipitation in the province is 3,200 mm. This climate is very suitable for tropical fruits production, including pummelo. "Thanh tra" pummelo (*C. grandis* (L.) Osbeck) is a kind of speciality fruit tree in Thua Thien Hue province and had a specific taste; it planted more than 200 years ago on about 1.000 ha in diffirent regions. "Thanh tra" grown scattered in Vietnam but the best quality was found in Thua Thien Hue. However, the quality of "Thanh tra" pummelo is not homogeneous among different regions, evenly in small area, the morphological characteristics are very diffirent and the genetic variability of them also was not fully reported [2].

Since the introduction of RAPD markers in 1990 [27], application in plant genetic analysis has increased in an exponential manner. In *Citrus*, RAPD analysis has been used mostly for genetic mapping [6] and to study genetic relationships between species and cultivars [3, 4, 5, 17, 18]. The present studies were undertaken to find out the genetic variability of "Thanh tra" pummelo in Thua Thien Hue and provide the information of its origin for breeding programs in the future.

MATERIALS AND METHODS

"Thanh tra" pummelo selection and morphological characteristics

A "Thanh tra" pummelo trees selection at Thua Thien Hue province, Vietnam (Fig. 1) were evaluated for morphological characteristics of tree, fruit and seed. The survey questionnaires were designed to collect information from farmers to identify their preferred phenotypes, and above all to obtain pointers to the genetic diversity. Traits, such as tree age, fruit bearing frequency, flowering, maturity period and fruit yield, were assessed from grower's information. Other parameters, such as tree height, canopy spread and trunk diameter (at 30 cm above the soil surface) were measured during the survey. Pulp colour, fruit shape and leaf shape were determined by visual observation following the "Descriptors for *Citrus*" of the International Plant Genetic Resources Institute [14]. Three ripe fruits were randomly used from each of the selected trees for quality assessment in the laboratory. Traits related to fruit quality, such as, percent of pulp, number of segment and seed, were determined on the basis of the fruit.

DNA isolation

Total DNA was isolated as described by Ahmed et al (2009) with slight modifications [1]. Leaf pieces were washed with tap water, cut into small piece (2×2 mm, 200 mg) and homogenize with 500 µL DNA extraction buffer (100 mM Tris.HCl, 100 mM ethylenediaminetetraacetic acid and 250 mM NaCl, pH 8) in a 1.5 mL eppendorf tube. Twenty-five microliters of 20% sodium dodecyl sulphate added, vortex for 30 sec and incubated at 65^{o}C for 60 min. Genomic DNA was purified twice by one volume of phenol:chloroform:isoamine alcohol solution (25:24:1), one volume of hydrated ether and precipitated with two volume of cold ethanol 100%. DNA pellets were dried for overnight at room temperature and resuspend in 20 µL double distilled water (DDW). Total DNA concentration and quality were determined by using NanoDrop ND-1000 (Thermo, USA) at 260/280 nm. After dilution with sterile DDW to a final concentration of 25 ng/µL, the DNA solution was used directly for PCR amplification.

RAPD analysis

Forty-two of genomic DNA explants (Table 1) and nine 10-mer primers based on Operon

Technologies sequences (Table 3) were used for RAPD analysis (samples with same origin were discarded). PCR reactions were carried out according to Coletta-Filho et al (1998), 25 µL final volume of a reaction mixture containing: 0.625 unit of Taq DNA polymerase in reaction buffer, 2 mM $MgCl_2$, 0.2 mM each of dNTP (PCR Master Mix, Fermentas), 20 pmol primer and 25 ng genomic DNA. Amplification reactions were performed in a thermocycle (iCycle, Bio-rad, USA) under the following conditions: 2 min at 92^{o}C; followed by 42 cycles for 1 min of denaturing at 92^{o}C; 1 min of annealing at 36^{o}C and a 2 min extension at 72^{o}C; a final extension for 10 min at 72^{o}C [9]. Amplification products were separated on a 1.4% agarose gel containing 1× TAE for 6 h at 40 V, and visualized under UV light (Gel documentation, Bio-rad, USA).

The molecular sizes of polymorphic markers ranged from 0.564 kb to 21.226 kb (λDNA/*Hin*dIII, Fermentas). RAPD markers on agarose gel were scored as presence (1) or absence (0) of a band in each "Thanh tra" pummelo tree. These RAPD data were used to compile a binary matrix for cluster analysis using the NTSYS-pc (numerical taxonomy system, Exeter Software, USA) version 2.1. Genetic similarity among trees was calculated according to Jaccard's similarity coefficient [15] using the SIMQUAL (similarity for qualitative data) routine. The similarity coefficients were then used to construct a dendrogram using the UPGMA (unweighted pair-group method with arithmetical averages) through the SAHN (sequential, agglomerative, hierarchical and nested clustering) routine of the NTSYS-pc package.

Statistical analysis

The data of tree, fruit and seed characteristics were presented as mean (n = 30), and the means were compared using a one-way analysis of variance followed by Duncan's test. A $p < 0.05$ was considered statistically significant.

RESULTS

Morphology characteristics

A total of more than 100 randomly selected "Thanh tra" pummelo trees at Thua Thien Hue, Vietnam were evaluated for morphological characteristics in the year of 2009. The results show that "Thanh tra" pummelo trees of Thua Thien Hue have significant differences in most of morphology characteristics such as tree morphology (tree height, canopy spread and trunk perimeter), leaf shape (lamina length and width) (Fig. 2), fruit morphology (fruit height, width, weight, percent of pulp and fruit rind thickness). "Thanh tra" fruits of Duong Hoa, Thuy Bang and Khuc Ly regions are bigger and more popular than that of Phong An and Phong Son regions.

RAPD analysis

Nine primers were selected based on previous reports [8, 20, 21, 24, 25] and used for genetic variation analysis. The total number of scorable bands amplified was 68, ranging in size from approximately 305 (AA10) to 2,354 bp (A18) (Table 3), and the number of fragments for each primers varied from 5 to 11 with a mean of 7.56 fragments per primer. All of bands were polymorphic and 8 were unique bands (presented in only one genotype).

A2 is the best primer for genomic DNA amplification of "Thanh tra" pummelo in this study with 38/42 trees amplified and 8 PCR-RAPD products produced (A2-478, A2-675, A2-786, A2-940, A2-1089, A2-1493, A2-1777, and A2-2140) (RAPD markers are defined as primer name and band size) (Fig. 3), these are also main bands of "Thanh tra" RAPD profile. Primer C2 and AA10 produced 11 bands from genomic DNA with high level of diversity. Seven of 68 bands are unique RAPD markers, including A18-1733 (9KL), AA10-1584 (25KL), AA10-799 (7KL),

AA10-677 (28KL), AA10-428 (1KL), B10-660 (3KL), AD10-940 (9KL). The unique bands presented only in Khuc Ly, this indicated that Khuc Ly is the most diversity region in our study.

Cluster analysis

The data obtained from RAPD (presence or absence of the band) were combined and phenetically analysed to determine the genetic similarity among the trees. The Jaccard's coefficient of genetic similarity (GS) were calculated using 68 RAPD combinations for the 42 genotypes. Figure 4 shows a UPGMA dendrogram produced from the Jaccard's coefficients. The 42 trees fell into three main groups (A, B and C), the genetic similarity between group A, B and C was approximately 0.12. In general, the genetic relationship of "Thanh tra" pummelo has a large distance, the genetic diversity level is high.

Group A contains 31 "Thanh tra" pummelo trees with GS approximately of 0.23 and subdivided into four subgroups. Subgroup A1 has 5 trees (1DH, 3PA, 10KL, 12KL and 20KL), GS value is approximately 0.55. Subgroup A2 has 18 genotypes from Duong Hoa (10) and Khuc Ly (8) regions (GS approximately 0.30), this is the largest subgroup. Subgroup A3 has 7 genotypes from Thuy Bang (6TB, 7TB, 9TB and 12TB), Phong An (1PA) and Phong Son (7PS and 9PS) while subgroup A4 has only one sample (5TB) with 14 DNA bands amplified by 5 primers (A2, A18, B5, C2 and C4). Group B consist of 5 trees from all of collected sample regions except Duong Hoa (8TB, 11TB, 11TB, 8PS, 5PS and 27KL). In this group, 8TB and 11TB have 100% genetic homology, they have 4 DNA bands amplified from A2 primer. Group C includes 6 genotypes (2TB, 3TB, 8PA, 11KL, 13KL and 26KL), explants in this group have less than 10 PCR products and genetic similarity less than 50%.

DISCUSSION

Using morphological traits is difficult to distinguish between many *Citrus* cultivars because some cultivars are distinguishable only by fruit traits and *Citrus* tree usually do not bear fruits until 3-4 years after planting. Phenotypic diversity, hybridization and mutation have prevented consensus on systematic classification of *Citrus* [9]. Various types of molecular markers have been used to characterize *Citrus* cultivars and germplasm accessions, beginning with isozyme studies in the late 1970s, followed by RFLPs and RAPDs in the 1980s and early 1990s, and more recently AFLPs (amplified fragment length polymorphism), SSRs (simple sequence repeat), ISSRs (intersimple sequence repeats), IRAPs (inter-retrotransposon amplified polymorphisms), and others [23]. The RAPD technique is less expensive per data point than others [12] so it was used in our study.

Our results show a mean of 7.56 polymorphic bands per primer in "Thanh tra" pummelo, higher than that of other reports (4.6 found at mandarin and pummelo, 2.2 or 1.95 among mandarin accessions) [9, 10, 18]. Primer C2 can produced 11 bands, suitable for "Thanh tra" genetic analysis but lower than 14 amplified bands from *Citrus* hybrid population [8]. Primer A2 is good for "Nam roi" pummelo (Vinh Long province, Vietnam) genetic analysis which 14 amplified fragments (12 polymorphic bands) [21], higher than our result (8 bands), this result shows that "Nam roi" and "Thanh tra" has a large distance in genetic relationship like the previous report [16, 25]. PCR amplification with primer C4 has 6 bands from 22 "Thanh tra" genotypes, lower than amplified bands number of *Citrus* hybrid population with this primer (14 bands in which four are polymorphic) [8]. The 5 other primers (A4, A18, B5, B10, and AD10) have limited results for "Thanh tra" genomic amplification (5-7 bands per primers) (Table 3). The number of PCR products of these primers different from other reports such as 50 bands from orange with primer A18 [24], 10 bands with B5 and 7 bands with B10 from 30 *Citrus* trees [8], 19 bands

from “Indian red” pummelo with AA10 [7], about 16 bands with AA10 and 11 bands with AD10 from introgression crosses of mandarin and pummelo germplasm [22] or 12 bands from mandarin Carvalhais [11].

In general, the higher the number of amplified products, the better the discrimination of trees, a minimum number of 50 different loci should be used for estimating genetic distances [19]. Hence the number of primers used is critical in a phenetic analysis. The present study, which used 68 polymorphic RAPD bands (loci) to determine the genetic relatedness among 42 “Thanh tra” pummelo, is adequate. The high level of genetic diversity shown that the diffirences in morphological characteristics and fruit quality of “Thanh tra” pummelo in Thua Thien Hue may be result from variation in genetic materials.

Table 1. The genotype code and locality

Locality	Code	Samples
Duong Hoa	DH	1, 13, 15, 16,17, 18, 19, 20, 21, 23 and 24
Thuy Bang	TB	2, 3, 5, 6, 7, 8, 9, 11 and 12
Phong An	PA	1, 3, 5 and 8
Phong Son	PS	7, 8 and 9
Khuc Ly	KL	1, 2, 3, 7, 9, 10, 11, 12, 13, 20, 25, 26, 27, 28 and 30

Table 2. Morphology characteristics of “Thanh tra” pummel

Characteristics	Duong Hoa	Thuy Bang	Phong An	Phong Son	Khuc Ly
Tree height (m)	6.85^{a}	5.81^{a}	4.28^{b}	3.89^{b}	4.75^{b}
Canopy spread (m)	7.48^{a}	6.30^{ba}	4.73^{c}	4.86^{c}	5.70^{b}
Trunk perimeter (cm)	87.37^{a}	84.67^{a}	63.11^{b}	58.22^{b}	75.28^{ba}
Leaf lamina length (cm)	11.43^{ba}	11.59^{ba}	10.99^{b}	12.06^{a}	11.99^{a}
Leaf lamina width (cm)	6.05^{b}	6.20^{b}	6.37^{b}	6.84^{a}	6.23^{b}
Petiole wing length (mm)	2.65^{a}	2.67^{a}	2.56^{a}	2.58^{a}	2.79^{a}
Petiole wing width (mm)	2.14^{a}	2.07^{a}	1.98^{a}	1.92^{a}	1.77^{a}
Number of fruits per tree	47.67^{b}	90.62^{ba}	76.67^{ba}	127.89^{ba}	133.83^{a}
Fruit height (cm)	13.32^{a}	12.81^{a}	9.50^{b}	9.62^{b}	13.43^{a}
Fruit diameter (cm)	12.10^{ba}	11.11^{b}	8.23^{c}	8.41^{c}	12.53^{a}
Fruit weight (g)	770.20^{a}	667.30^{a}	291.70^{b}	302.50^{b}	782.30^{a}
percent of pulp (%)	57.25^{b}	66.16^{b}	76.06^{a}	74.15^{a}	62.86^{b}
Fruit rind thickness (mm)	3.27^{a}	1.61^{b}	1.53^{b}	1.51^{b}	2.73^{a}
Number of segments per fruit	12.81^{a}	13.33^{a}	12.94^{a}	12.86^{a}	13.48^{a}
Number of seeds per fruit	123.26^{a}	135.98^{a}	128.56^{a}	129.50^{a}	147.21^{a}
Pulp colour	yellow	yellow	yellow	yellow	

Different letters in the row indicate significantly different means using Duncan’s test ($p<0.05$).

Table 3. Number of RAPD marker of “Thanh tra” pummel

Primer	Sequence (5’-3’)	Number of amplification genotypes	Polymorphic bands	Band size variations (bp)	Unique bands
A2	TGCCGAGCTG	38	8	478-2140	0
A4	AATCGGGCTG	25	7	431-1247	0
A18	AGGTGACCGT	19	7	331-2354	1
B5	TGCGCCCTTC	11	6	474-1524	0
B10	CTGCTGGGAC	14	7	485-1336	1
C2	GTGAGGCGTC	23	11	420-2223	0
C4	CCGCATCTAC	22	6	332-1247	0
AA10	TGGTCGGGTG	19	11	305-1584	4
AD10	AAGAGGCCAG	23	5	416-2322	1
Total		194	68	305-2354	7

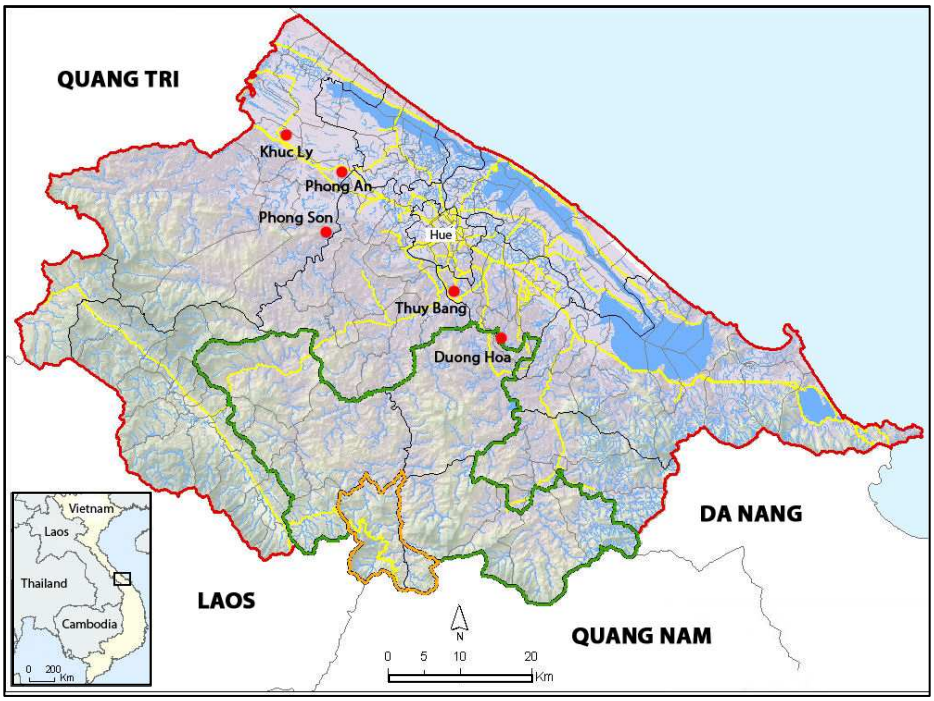

Figure 1. Sample collection regions at Thua Thien Hue.

Figure 2. Leaf and fruit shapes of "Thanh tra" pummelo in various areas of Thua Thien Hue. PA: Phong An, PS: Phong Son, TB: Thuy Bang, DH: Duong Hoa, and KL: Khuc Ly.

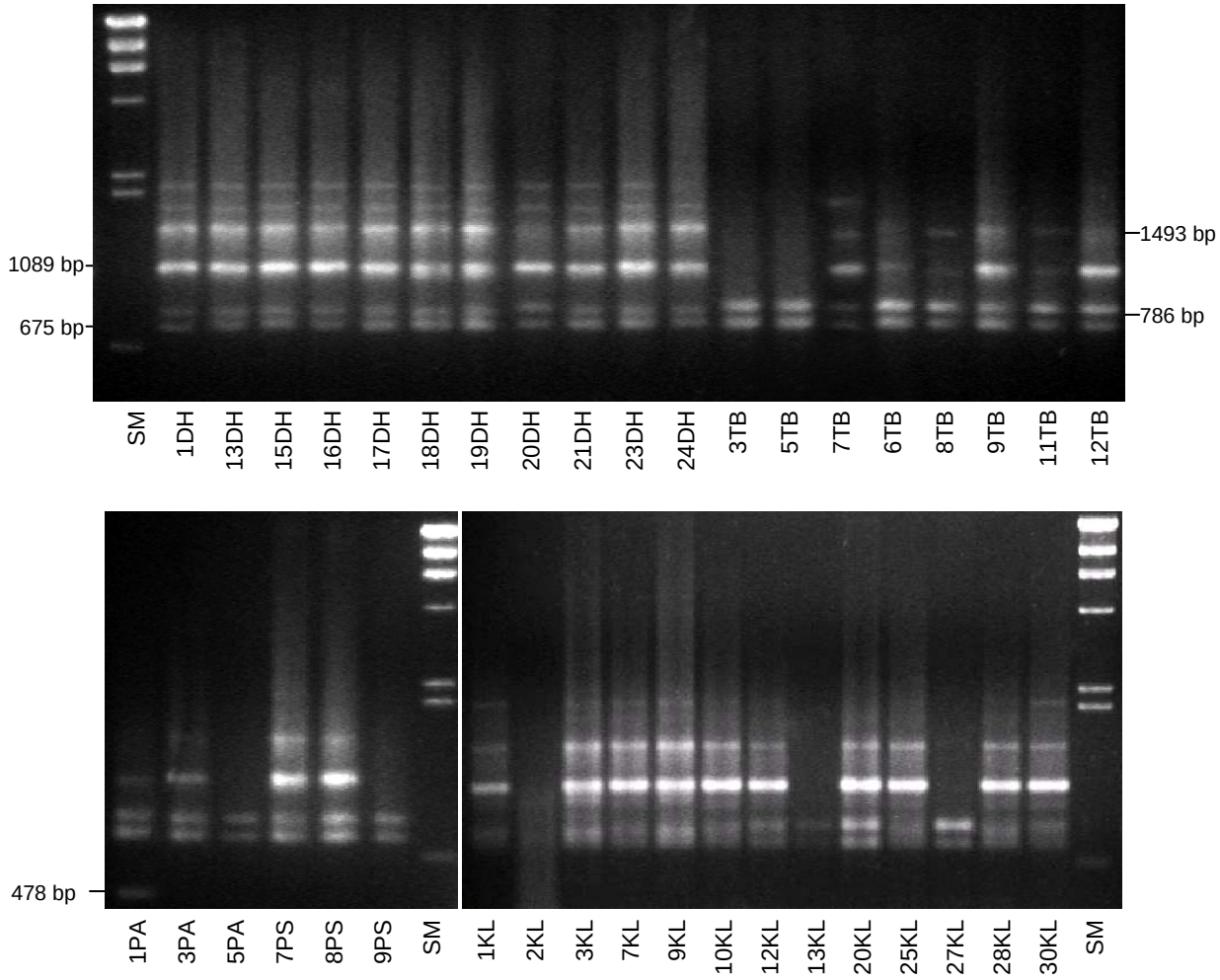

Figure 3. PCR products of A2 primer in various areas. SM: DNA size marker (Lamda DNA/*Hind*III). PA: Phong An, PS: Phong Son, TB: Thuy Bang, DH: Duong Hoa, and KL: Khuc Ly.

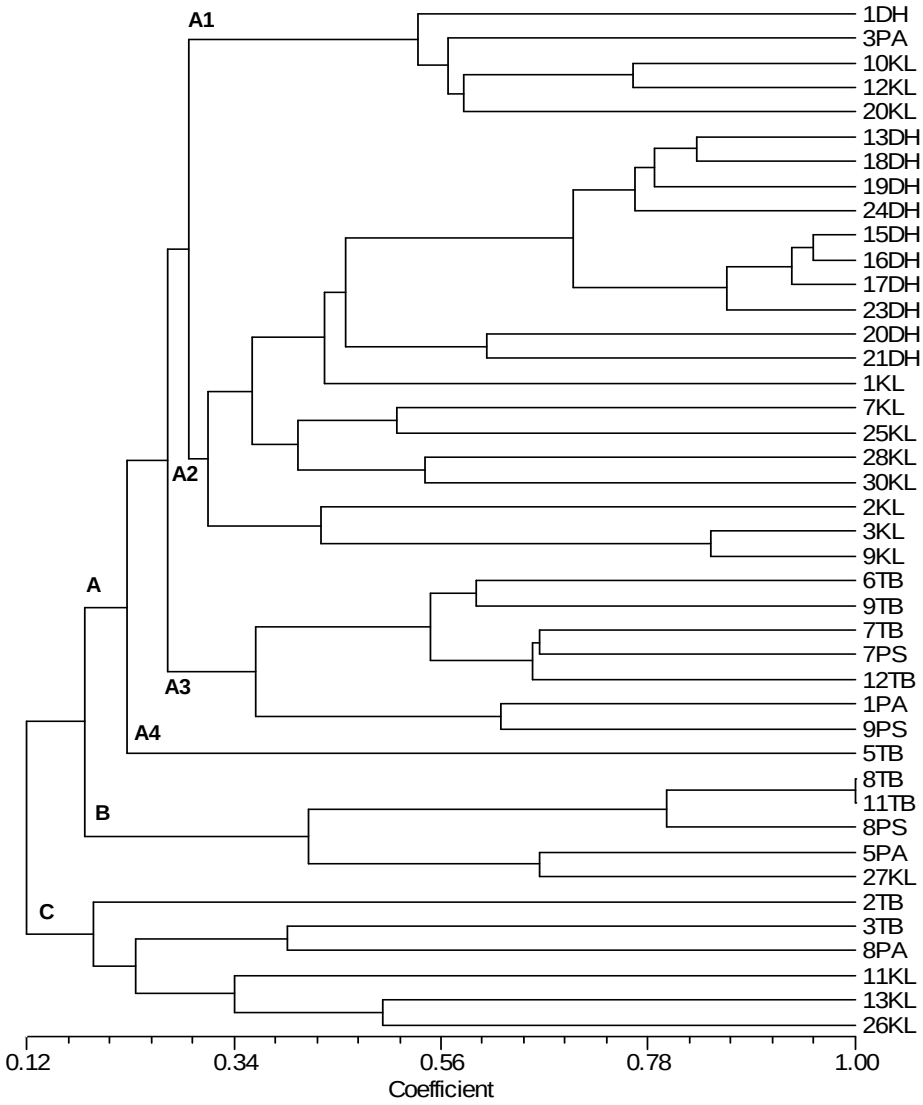

Figure 4. Phenogram of 42 "Thanh tra" pummelo trees derived using the UPGMA grouping method based on Jaccard coefficients obtained from RAPD analyses.

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