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GENETIC RELATIONSHIPS OF SEVERAL LOCAL *MELIENTHA SUAVIS* PIERRE IN VIETNAM'S NORTHERN MOUNTAINOUS AREA BY SIMPLE SEQUENCE REPEAT MARKERS

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Abstract:

The study aims to evaluate the genomic diversity of some local *melientha suavis* Pierre SSR technique. By using 10 primer pairs to analyze 20 *melientha suavis* Pierre lines shows that the number of Alleles were from 2 to 4 alleles and the polymorphic information contents ranged from 0.05 to 0.15. Forty-one alleles were identified with avarage of 0.15 alleles. The SSR technique shows that the differences among the varieties genes based on the number of alleles and the polymorphic information contents. It means that the gene of the local melientha suavis Pierre has been divided into 5 groups. The genetic variation coefficience among largest genetic differences is obtained approximately 3%.

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NGHIÊN CỨU ĐA DẠNG DI TRUYỀN HỆ GEN TẬP ĐOÀN RAU NGÓT RỪNG BẢN ĐỊA (*MELIENTHA SUAVIS* PIERRE) KHU VỰC MIỀN NÚI PHÍA BẮC VIỆT NAM

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Melientha suavis Pierre, đa dạng di truyền, đa dạng sinh học, hệ gen, cây bản địa.

Tóm tắt

Nghiên cứu này mong muốn tìm ra sự khác biệt về vật chất di truyền học của tập đoàn cây Rau ngót rừng bản địa lâu năm, trồng từ hạt thu thập tại khu vực Miền núi phía Bắc Việt Nam. Qua kết quả nghiên cứu đã chỉ ra về sự đa dạng di truyền về hệ gen ở tập đoàn *Melientha suavis* Pierre bằng kỹ thuật SSR sử dụng thống kê các phân đoạn DNA, minh chứng sự sai khác đó qua nội dung triển khai ở 10 cặp chỉ thị và trên 20 mẫu giống ngót rừng bản địa đã ghi nhận; số alen giao động từ 2 đến 4 alen và chỉ số đa dạng giao động từ 0,05 đến 0,15, đã phát hiện được 41 alen, số alen đạt trung bình là 0,15 alen. Thông qua một số kỹ thuật trong sinh học phân tử đã cho thấy hệ gen giữa một mẫu giống có xuất hiện sự sai khác thông qua ở số lượng các alen và chỉ số đa dạng. ***Điều đó chỉ ra rằng, hệ gen của tập đoàn cây ngót rừng bản địa miền núi phía Bắc Việt Nam trồng từ hạt đã có sự phân ly***, phân bố thành 5 nhóm chính. Bằng minh chứng hệ gen khoảng cách di truyền khác xa nhất khi so sánh một sô giống trong nghiên cứu với hệ số khác biệt là 3,0% và phân loại các giống theo sơ đồ về mối quan hệ di truyền.

1. Introduction

Melientha suavis Pierre (other names: cassava plant) is a rare and special forest vegetable with high commercial value on the market listed in the Vietnam Red Book. This is an endemic vegetable in the limestone mountains, having the scientific name Melientha Suavis Pierre, belonging to the family Opiliaceae, order Santalales. The northern mountainous ecological region, where the research samples were collected, has a tropical monsoon climate and complex topography, so a number of different climate sub-regions have been formed.

The diversity of climate and changes in living environment is one of the causes leading to the diversity of biological characteristics to adapt to external conditions. This is considered to be one of the reasons for the diversity of the genome of the native plant species population, and is the basis for research, evaluation, and proof of the genetic diversity of the genome by molecular biomarkers.

This study is applied from the basic field of life sciences and meeting current practice to bring data of indigenous genetic resources to the public, contributing to the embellishment and preservation of human genomes, information data to shed more light on biodiversity and especially conservation of indigenous genetic resources in the Northern Mountains region of Vietnam.

2. Materials and Methods

2.1. Plant samples

Twenty indigenous citrus grown in the mountainous region of Northern Vietnam were collected for this study studied. Sample symbols and collection locations are shown in Table 1.

Table 1. Location of citrus types used in the study

Sr.no	Sampling location	Code nam	Sr.no	Sampling location	Code name
1	Vo Nhai, Thai Nguyen	01VN-TN	11	Bac Son, Lang Son	11BS-LS
2	Dinh Hoa, Thai Nguyen	02DH-TN	12	Huu Lung, Lang Son	12HL-LS
3	Dong Hy, Thai Nguyên	03DH-TN	13	Tan Trao, Tuyên Quang	13TT-TQ
4	Na Ri, Bac Kan	04NR-BK	14	Luc Yen, Yen Bai	14LY-YB
5	Phu Thong, Bac Kan	05PT-BK	15	Van Chan, Yen Bai	15VC-YB
6	Ba Be, Bac Kan	06BB-BK	16	Nho Quan, Ninh Binh	16NQ-NB
7	Hoa An, Cao Băng	07HA-CB	17	Thanh Son, Phu Tho	17TS-PT
8	Nguyen Binh, Cao Băng	08NB-CB	18	My Duc, Ha Noi	18MD-HN
9	Bao Lac, Cao Bang	09BL-CB	19	Bac Quang, Ha Giang	19BQ-HG
10	Luc Nam, Bac Giang	10LN-BG	20	Binh Lieu, Quang Ninh	20BL-QN

2.2. Methods

Experiment collection: Samples used for DNA collection were taken from young shoots and leaves, and stored for no more than 1 week at -20 ^{o}C before doing the experiment. Evaluation of genomic diversity was performed by SSR technique. The experiment was conducted with 10 pairs of SSR primers, the nucleotide sequences of primer pairs for PCR-SSR reaction, as proclaimed by Goh Pik Seah ELCY (2011). Primers were synthesized by Genotech, Korea Advanced Institute of Science and Technology - KAIST (South Korea), and the order of primers presented in Table 2.

Table 2: SSR primers used in this study

Primer name	Forward primer (F) & Reverse primer (R) from 5' to 3'		Dimension (bp))	Base type	Primer name	Forward primer (F) & Reverse primer (R) from 5' to 3'		Dimension (bp)	Primer name
SSR-001	F	GTCAATACGATCCAC-GGG	231-259	(TC)5 CT (TC)5	**SSR-006**	F	ATAAAATGAGGGCG-CCAG	203-207	(CT)8(G) (CT) 6(TG)8
	R	TTGAGCCAAAGAAC-GGTG				R	GCATTTTCA-CAGTCTCGCA		
SSR-002	F	CAGCTGCTGAAGAA-CAACA	214–218	(AGC)6	**SSR-007**	F	TTTGCAAAGTTGG-GAGGA	268–282	(CAG)4
	R	GTTGCT-GAACTTGTCCGC				R	TAAAAATCCCGTCAC-CGC		
SSR-003	F	ATCTAGGGTTTTGC-CGGA	218–228	CAG)5	**SSR-008**	F	AAATAGAGCACGGG-CCAT	278–312	(ACC)3 (GCT) (ACC)3
	R	ATCCGTACACGCTG-CACT				R	GCATCGCTATTGC-CGTTA		
SSR-004	F	CCACGT-GCTTTCAACCAT	171–176	(CCG)4	**SSR-009**	F	TTAGCCCAACAGTG-CCC	280–300	(TGC)5
	R	AGGGAAGGGAGTG-CAATG				R	GGAAGCGCTT-GAACCTTT		
SSR-005	F	AGATTGCAGACTGG-CGAA	204–268	(TG)2 (T)2 (TG)4	**SSR-010**	F	GAGATGCAGACGGCT-CAC	253–281	(GT)9

DNA extraction protocol

DNA is extracted from the young leaves of each sample. Using 300 mg of young leaves and grind in liquid nitrogen into a fine powder, then add 1ml of wash buffer (Tris-HCl 1M pH 8, EDTA 0,5M pH 8, Sorbitol 0,35M, Na_2HPO_4 0,4%), shaking the test tube for 40 seconds, then centrifuge (12000 rpm, 4^{o}C, 12 mins), remove the floating part of solution, repeat this step 1-2 times. Add 800µl of extraction buffer (Tris HCl 0,1M pH 8; EDTA 0,5M pH 8; NaCl 6M; -Mecaptoethanol 0,14M; CTAB 4%), incubate at 65^{o}C for 90 minutes to extract DNA. Store the sample at room temperature for 10 minutes, add 0.8 ml of Chloroform/Isoamyl alcohol (24:1), gently shake the tube

for 15 minutes. Then centrifuge 12,000 rpm for 15 minutes at 4°C and use the pipette to suck the upper layer into new eppendorf 2ml tube. Add equal Isopropanol volume (cool) and gently shake, store the sample at 4°C for 30 minutes, centrifuge at 12000 rpm for 10-15 minutes at 4°C. Remove the floating solution, wash the DNA precipitate with 500µl alcohol 70%, centrifuge 12,000rpm for 4 minutes at 4°C, repeat this step 2 times. Then remove the floating solution and keep only the precipitate of DNA. Dry the DNA in the ventilated cabinet and then add 50µl of deionized water and store it at -20°C before conducting other tests. Total DNA was determined by spectroscopic method.

The principle of the method is based on the absorption of light at the wavelenght of 260nm and 280nm purine and pyrimidine bases. One unit of OD_{260nm}(Optical Density 260 nm) is equal to a concentration of 50 µg/ml for the double-stranded DNA solution which is calculated by the formula: C_{DNA} (µg/ml) = OD_{260nm} x 50 x dilution coefficient The DNA solution was considered to be clean (without protein) when the ration OD_{260nm}/OD_{280nm} is between 1.8 - 2.0 [8].

PCR – SSR reaction

The PCR - SSR reaction is based on PCR technique, which allows rapid cloning of a DNA sequence many times in a few hours. PCR is performed inside the thermal cycler where DNA template, Taq-polymerase, specialized primers and four type of dNTPs were included [8]. The PCR reaction performs the following steps: Mix the above-mentioned components in 2ml eppendorp tube and transfer the mixture to a 25 µl PCR tube.

Table 3: PCR-SSR Reaction component

Sr.no	Component	Concentration	Volume (*µl*)
1	10x Buffer		2.5
2	MgCl2	25mM	1.5
3	Forward primer	10 pmol	1.0
4	Reverse primer	10 pmol	1.0
5	dNTPs	100 µM	1.5
6	AND taq polymerase	200 ηg/µl	0.2
7	AND structure	200 ηg/µl	2.0
8	Deionized water		15.3
Total volume			**25**

The mixture is centrifuged at 3000 rpm, so that the above components settled to the bottom of the PCR tube and then PCR reaction is about to happen. The heat cycle for the reaction is 95°C for 4 minutes; repeat 33 cycles with 95 °C/45 seconds, 47°C to 59°C (depending on primer)/45 seconds, 72°C/1 min; 72 °C/9 minutes; storage of product at 4°C.

Evaluation of PCR-labeled probes was conducted by agarose gel electrophoresis

Agarose gel electrophoresis

The product obtained from PCR-SSR reaction is electrophoresed on 0.8% agarose gel, in buffer TAE 1X and run electrophoresis at 110 volts for 1 hour. After that, imbue gel with 0.5% EtBr solution, which is capable of intermingling with the nucleic acid bases that illuminate them under ultraviolet (UV) with wavelength of $\lambda \approx 300$ nm in the form of orange red lines, easy to observe or capture to evaluate the results of the experiment [8].

Data processing and building of genetic correlation tree diagram

Based on the image results of electrophoresis of PCR products and the emergence of SSR bands of Citrus for each pair of primers as the basis for data analysis. Data analysis on digitalization convention: Number (1) appearance of SSR band. Number (0) does not appear SSR band.

The digitized data is processed by computer to analysis data. Of which, the H - genetic variation index for each molecular marker is determined by the Microsoft Office Excel 2007 with the formula.

$$H = 1 - \sum Pi^2$$

(Pi is the allele repeat frequency of i^{th} of each molecular marker).

The tree diagram was built to determine the genetic distance of the crop varieties using NTSYS - version running on personal computer 2.0 [8].

3. Results and discussion

3.1. The Polymorphic of the SSR markers of melientha suavis *Pierre samples*

Results of total DNA electrophoresis on 0.8% agarose gel; 60 minutes; the Intron 1000bp marker showed that all 20 samples were suitable for conducting subsequent use (Figure 1), according to the simultaneous use of methods to determine concentration and DNA by spectroscopy.

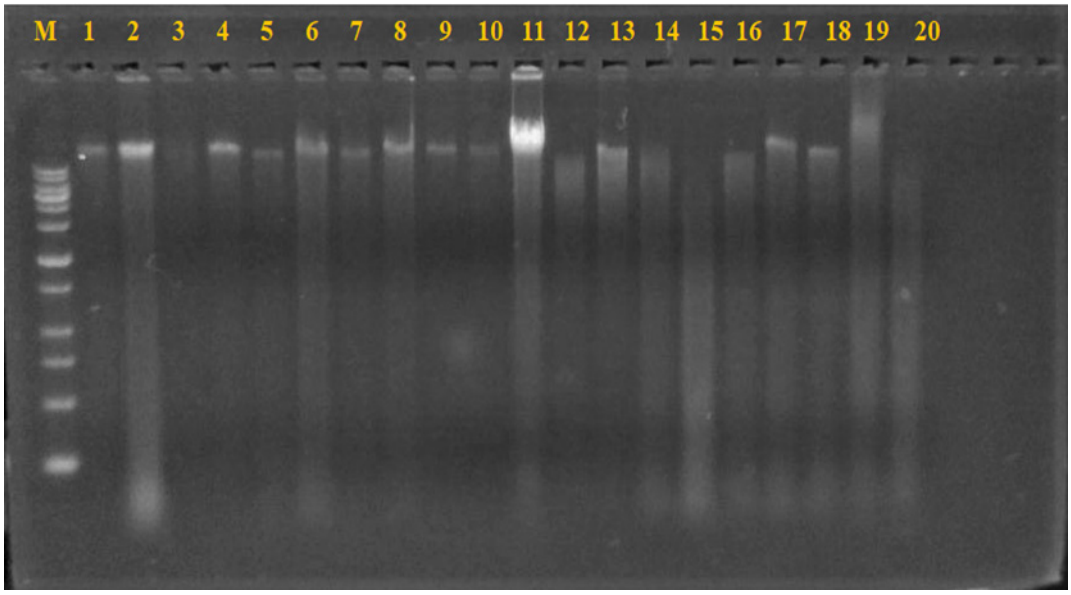

Figure 1. Total DNA electrophoresis spectrum obtained from *melientha suavis* Pierre samples

The results are based on the analysis of 10 *Melientha suavis* Pierre samples using 20 primer pairs of SSR marker. Size of alleles, number of alleles and variation index of the primers are presented in Table 4.

Table 4: Number of alleles and variation index of SSR primer pairs

Sr.No	Name of SSR primer pair	Allele size (bp)	Number of allele	Variation index	Sr.No	Name of SSR primer pair	Allele size (bp)	Number of allele	Variation index
1	SSR 001	209–217	3	0,392	6	SSR 006	278–312	6	0,211
2	SSR 002	214–218	2	0,059	7	SSR 007	280–300	5	0,391
3	SSR 003	218–228	4	0,095	8	SSR 008	256–281	2	0,059
4	SSR 004	171–176	2	0,050	9	SSR 009	204–268	6	0,466
5	SSR 004	268–282	4	0,072	10	SSR 010	253–281	6	0,430
Average			**4.15**	**0.55**	**Average**			**4.15**	**0.55**

The variation index H is evaluated as the degree of appearance of the primer pair in each sample. In the experiment, the variation index is calculated on the basis of the presence or absence of SSR band in each primer, sample/kind of *melientha suavis* Pierre to determine the genetic variation index H for each molecular marker. Overall evaluation in 10 pairs of results marker shows that; allele number is ranging from 2 to 8 alleles and the variation index is ranging from the lowest 0.15 to the highest 0.466 [4] [10].

Table 4 shows that the SSR 006, SSR 009 and SSR 010 primer pairs show the highest variation with 8 alleles and indicated the lowest variation of SSR 002, SSR 004 and SSR 008 with 2 alleles. The average value is 4.15 allele per molecular marker. This result shows that the number of alleles is equivalent to evaluations of Goh Pik Seah ELCY (2011) or Behrouz Golein (2012) and many geneticists interested in *melientha suavis* Pierre. The number of alleles is normally having from 2 to 12 alleles per marker

In terms of the index variation (H) value, the index is reflected as the markers on the DNA sequence in the genome. The presence of markers and the relative distance between them reflect the degree of variability among individuals, crops, or species in the population. Creatures have the ability to duplicate their DNA with high accuracy, but many mechanisms can modify DNA structure, as simple as base pairs or more complicated as inversion, repetition, or segmentation, etc. so, molecular marker is considered as an effective tool for evaluating genetic variation for crop selection.

The experiment results show that the variance index of the SSR index is varied from 0.050 to 00.466, the average value for the SSR marker is 0.15. This marker is asymptotic compared to the research conducted by Hidaka T (2012) on 24 kinds of Citrus in Northern Japan (average variation index of 0.56) [9]. Results taken from PCR-SSR reaction, products are checked on agarose gel 0.8%. Results show that, among 10/10 SSR markers which are used for the analysis of genetic variation, all 20 SSR markers are polymorphic, electrophoresis of 22 lines/crops have SSR bands with size of 100 bp to 300 bps (figure 2

and 3). This is the database used for the NTSYS - version running on personal computer 2.0. to determine coefficients which are the same or different of the *melientha suavis* Pierre by tree diagram.

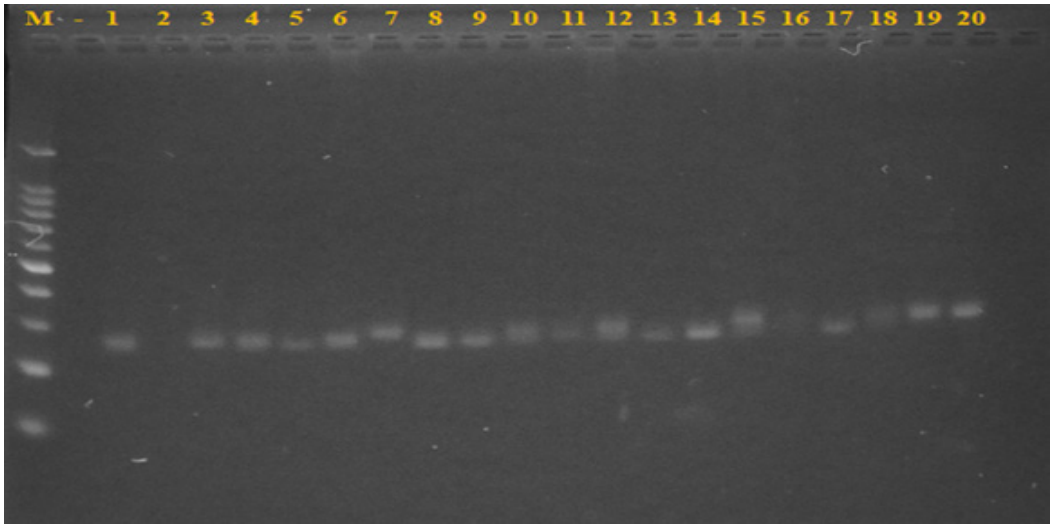

Figure 2. PCR electrophoresis spectrum of SSR 001 primer pair

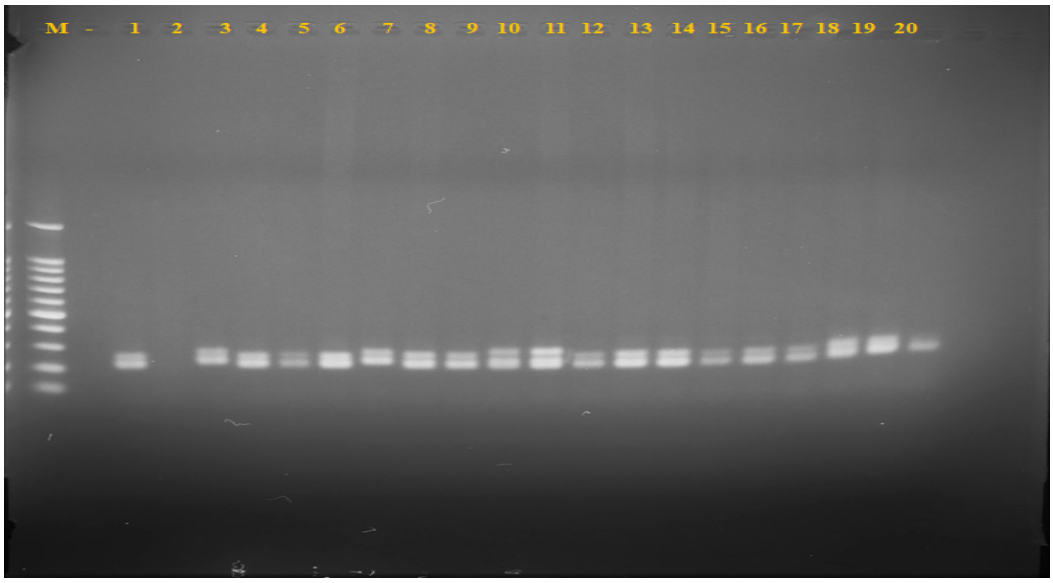

Figure 3. PCR electrophoresis spectrum of SSR 009 primer pair

Results of PCR electrophoresis analysis of 10 pairs of primers for 20 indigenous *Melientha suavis* Pierre cultivars, PCR clones specific for each SSR molecular marker and SSR fragment size close to corresponding size of each SSR marker.

Analysis of DNA fragments which are cloned showed 41 alleles, with the average of 4.15 alleles. This indicates that the genomes of indigenous *Melientha suavis* Pierre growing from seeds have a significant separation. SSR technique revealed the different of genome between cultivars in the number of alleles and variation indexes. The result of this experiment is lower than in previous experiments on citrus genetic variation as proclaimed by Kinley Dorji et. al (2015) on *Melientha suavis* Pierre cultivars in 50 Asian countries with average allele of 7.82 [3], or in comparison with the Hidaka T (2012) on 24 citrus varieties in Northern Japan (average variation index of 0.56 and had an average of 6.45 alleles of total 30 SSR markers [9]

The average of total alleles in the experiment were lower than those reported, here it is assumed that the native *Melientha suavis* Pierre consortium is genetically more conservative than that reported by previous studies in the consortium of native trees. This indicated that *Melientha suavis* Pierre in the Northern Mountains of Vietnam has high genetics but still still exhibits diversity [7].

The genetic differences of cultivated variety

The genetic variance of cultivated variety in the research was analyzed based on SSR molecu-

lar markers with NTSYS 2.0 software. From that we can determine the genetic difference coefficient and mapping of the relationship between the varieties of *Melientha suavis* Pierre (Figure 4). The result showedgenetic variation ranging from 0.0% to 3.0%. This difference has proved that the plant grows from seeds naturally and has cross-pollination leading to the segregation into many different lines/varieties. Therefore, this is a rich source of materials to hybridize, select and conserve indigenous genetic resources.

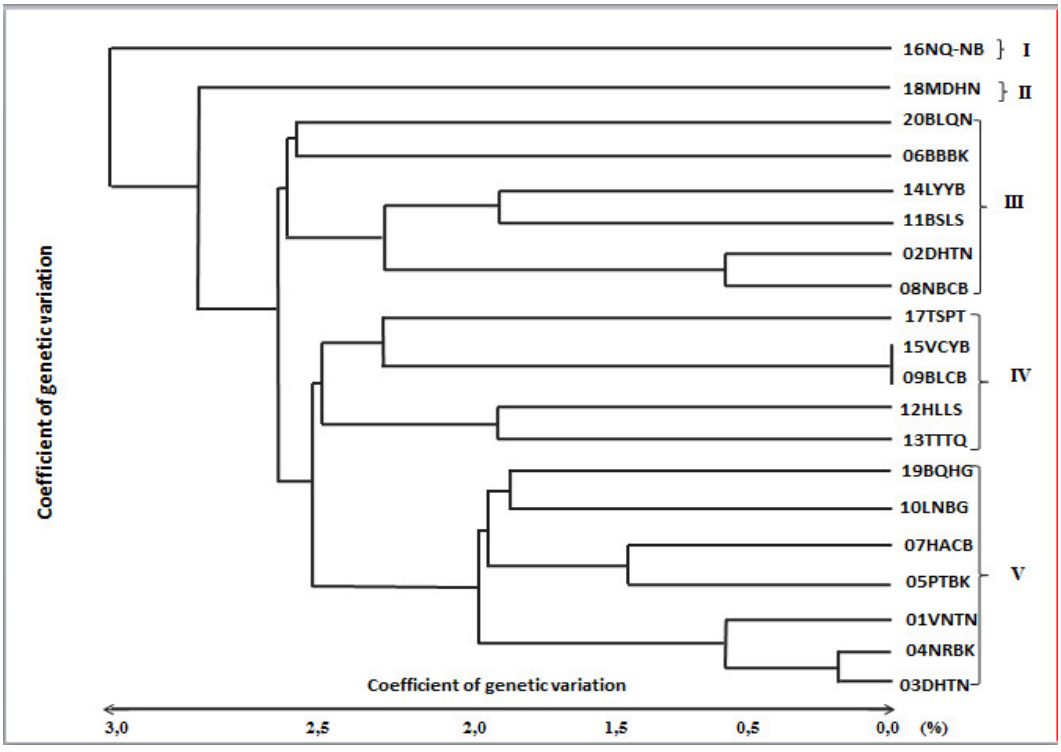

Figure 4. Diagram of genetic relationship

of *Melientha suavis* Pierre cultivars based on SSR analysis

The tree diagram shows that 20 cultivars which are divided into 5 main branches. The first one is only Nho Quan *Melientha suavis* Pierre. The second branch is only My Duc *Melientha suavis* Pierre. The third branch includes 6 varieties: Binh Lieu (Quang Ninh), Ba Be (Bac Kan), Luc Yen (Yen Bai), Bac Son (Lang Son), Dinh Hoa (Thai Nguyen), and Nguyen Binh (Cao Bang). The fourth branch consists of five varieties: Thanh Son (Phu Tho), Van Chan (Yen Bai), Huu Lung (Lang Son), Tan Trao (Tuyen Quang). The fifth branch includes the remaining seven varieties: Bac Quang (Ha Giang), Luc Nam (Bac Giang), Hoa An (Cao Bang), Phu Thong (Bac Kan), Vo Nhai (Thai Nguyen), Na Ri (Bac Kan), and Dinh Hoa (Thai Nguyen). Here, The first (16 NQ–NB) and second (18MD-HN) groups are not located in the center of the northern mountainous area in terms of geography and topography.

The analysis of genetic differences by the program NTSYS pc version 2.0 also showed that group I and group II had a genetic distance of 0.3% compared to the remaining groups in the experiment.

In the present study, a genetic difference from 0.0% to 3.0% showed that this distance is closer to the same evaluation on citrus in the Southern Vietnam, Nguyen Huu Hiep et al. (2004) published 68 cultivar specimens including citrus and lemon, the genealogy diagram is divided into four main groups and the genetic variation ranging from 0.0 to 4.3% [1]. The results also show that the genetic variation in citrus in Vietnam is lower than that publication of Sadaf Altaf (2014), Goh Pik Seah ELCY (2011) or Xiao-Yan Yang (2012) in Asian countries with the value varies from 0.0 – 10.0% [10],[11].

The general classification based on genomics in this study showed that the genetic distance in *Melientha suavis* Pierre group consisted of 5 groups (I, II, III, IV and V – figure 3). This correlation, we continue to evaluate on a broader geographical scale larger enough for genomic related analysis.

4. Conclusion

The assessment of genetic diversity on *Melientha suavis* Pierre group by SSR technique using statistical analysis of DNA segments were conducted in 10 pairs

of markers and over 20 samples of indigenous forest jasmine varieties were recorded; the results showed the number of alleles ranges from 2 to 4 alleles and the diversity index ranges from 0.05 to 0.15, 41 alleles have been detected, the average number of alleles is 0.15 alleles.

The experiments on the SSR technique have shown that the genomes between varieties have differences through the number of alleles and diversity index. This indicates that the genome indigenous Melientha suavis Pierre grown from seeds in the Northern mountainous region of Vietnam has been segregated and distributed into 5 main groups. The genetic evidence showed that the genetic distance is the furthest when compared to the varieties with a coefficient of difference of 3.0%.

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