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Genetic diversity analysis of *Centella asiatica* L. Urban in Vietnam by RAPD marker

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Abstract. *Centella asiatica* (L.) Urb., belonging to the Apiaceae family, grows widely in shady and moist or marshy places. *C. asiatica* has been used in traditional medical systems in Asia for over 100 years. A better understanding of genetic diversity is essential for its conservation and use. In this study, the genetic diversity analysis was performed in 24 cultivars of *Centella asiatica* using 24 selected RAPD markers. There are genetic differences among the studied samples. However, the molecular screening through RAPD markers showed that genetic differences (from 0.0025 to 0.2194) and genetic diversity indices (%P = 42.02%, h = 0.1272, I = 0.1938) are relatively low. Besides, the results of phylogenetic tree also indicated that HUIB_CA06 and HUIB_CA26 (from Thua Thien Hue province) have genetic differences compared to the remaining varieties, which warrant further analysis on these varieties.

Keywords: *Centella asiatica*, Apiaceae family, RAPD, genetic diversity, Vietnam

1 Introduction

Centella asiatica (L.) is a tropical herbaceous plant in the family Apiaceae. The origin of this plant is from South Asian countries such as India, Sri Lanka, China and some Southeast Asian countries such as Thailand [1]. *C. asiatica* has been used for hundreds of years as a traditional medicine to treat ailments such as headaches, body aches, dementia, asthma, leprosy, ulcers, eczemas, wound healing, tumor and cancer, diabetes and dementia [2]. In addition, it has been used as an important ingredient for cosmetic formulations [3] and even as an antibacterial agent [4].

The chemical composition analysis showed that *C. asiatica* contains 88.2% water, 3.2% protein, 1.8% carbohydrates, 4.5% cellulose and 2.3% total minerals [5]. Previous studies have shown that *C. asiatica* contains a large number of compounds belonging to different chemical groups such as triterpene saponins, phenol compounds, vitamins, minerals, free amino acids and polyacetylenic compounds [6]. In particular, triterpene saponins include triterpene acids and triterpene glycosides [7]. However, they are easily lost due to thermal decomposition and oxidation reactions during processing [8].

In Vietnam, *C. asiatica* is a familiar plant that distributes widely, from islands and coastal areas to mountainous areas with altitudes below 1800 m above sea level. This is a moisture-loving, slightly shade-tolerant plant, that typically grows in clusters in gardens, rivers, streams, upland fields, fields and forest edges [9]. Because of the diversity of biological functions and great influence of *C. asiatica* on human health, the demand for this plant is increasing day by day. At present, more and more specialized growing areas of *C. asiatica* have been formed to meet the needs of the market. In particular, the most prominent

models are in Thanh Hoa province and Thua Thien Hue province.

Although there have been many studies on chemical composition, biological activities, distribution characteristics, morphology..., there has not have a study on genetic differences between *C. asiatica* varieties in Vietnam yet. To study genetic diversity, people can use many different methodswhich using morphological markers, isozyme markers or molecular markers (restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR, microsatellites) [10]). Depending on the objectives, conditions and purpose of the study, people choose the most suitable method. In particular, RAPD is a dominant molecular marker widely used in genetic diversity research with many advantages such as fast, simple, effective; requiring only a small amount of DNA template; pre-knowledge of the genome sequence and primer sequence is not required. In addition, the application of RAPD technique has been used for many different animals, plants and microorganisms [11]. Therefore, we have studied the genetic diversity of *C. asiatica* varieties by RAPD technique to evaluate the genetic differences between varieties. The results obtained from this study can lay the basis for the development of conservation and management strategies [12].

2 Materials and methods

2.1 Material

This study used a total of 24 cultivars of *Centella asiatica* from 17 different localities in Vietnam. The characteristics of name, code, location of collection and coordinates are shown in Table 1. In addition, 200 UBC RAPD primers (University of British Columbia) synthesized by Bioneer Company (Korea) were used to genetic diversity survey of *C. asiatica* cultivars.

Table 1. List of 24 cultivars of *C. asiatica* in this study

No.	Material symbol	Code	Location of collection	Coordinates
1	HUIB_CA01 (HA01)	NA1	Hung Nguyen, Nghe An	18°40'35.4"N 105°36'50.9"E
2	HUIB_CA02 (HA02)	QT	Gio Linh, Quang Tri	16°59'39.1"N 107°03'14.7"E
3	HUIB_CA03 (HA03)	QN	Phu Ninh, Quang Nam	15°32'29.2"N 108°27'26.3"E
4	HUIB_CA04 (HA04)	TN	Song Kong, Thai Nguyen	21°27'25.5"N 105°50'35.8"E
5	HUIB_CA05 (HA05)	HT1	Vu Quang, Ha Tinh	18°28'17.8"N 105°31'56.9"E
6	HUIB_CA06 (HA06)	TTH1	Quang Dien, Thua Thien Hue	16°31'59.2"N 107°31'34.1"E
7	HUIB_CA07 (HA07)	HT2	Thach Ha, Ha Tinh	18°20'22.4"N 105°50'46.6"E
8	HUIB_CA08 (HA08)	GL	Kông Chro, Gia Lai	13°37'55.4"N 108°43'03.5"E
9	HUIB_CA09 (HA09)	TTH2	Phu Vang, Thua Thien Hue	16°29'42.8"N 107°36'24.2"E
10	HUIB_CA10 (HA10)	DL	Buon Ma Thuot, Dak Lak	12°40'33.5"N 108°02'57.8"E
11	HUIB_CA11 (HA11)	NB	Yen Mo, Ninh Binh	20°08'21.5"N 106°00'21.5"E
12	HUIB_CA12 (HA12)	TH1	Quang Hoa, Thanh Hoa	20°23'23.3"N 105°05'51.2"E
13	HUIB_CA13 (HA13)	QB	Le Thuy, Quang Binh	17°11'46.2"N 106°49'46.0"E
14	HUIB_CA15 (HA15)	NA2	Hung Nguyen, Nghe An	18°39'57.8"N 105°38'53.4"E

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No.	Material symbol	Code	Location of collection	Coordinates
15	HUIB_CA16 (HA16)	KT3	Ngo May, Kon Tum	14°21'25.4"N 107°59'59.5"E
16	HUIB_CA18 (HA18)	QNi1	Nguyen Hue, Dong Trieu, Quang Ninh	21°05'23.9"N 106°27'38.1"E
17	HUIB_CA19 (HA19)	QNi2	Nguyen Hue, Dong Trieu, Quang Ninh	21°05'23.9"N 106°27'38.1"E
18	HUIB_CA20 (HA20)	PY	Tuy Hoa, Phu Yen	13°04'36.4"N 109°18'18.0"E
19	HUIB_CA21 (HA21)	NA3	Hung Nguyen, Nghe An	18°40'14.9"N 105°36'42.8 "E
20	HUIB_CA25 (HA25)	CT	O Mon, Can Tho	10°07'54.9"N 105°37'16.1"E
21	HUIB_CA26 (HA26)	TTH3	Quang Tho, Quang Dien, Thua Thien Hue	16°31'58.4"N 107°31'35.9"E
22	HUIB_CA27 (HA27)	TTH4	Quang Phu, Quang Dien, Thua Thien Hue	16°32'39.3"N 107°30'36.5"E
23	HUIB_CA28 (HA28)	TG	Long Hung, Chau Thanh, Tien Giang	10°23'29.1"N 106°16'30.9"E
24	HUIB_CA29 (HA29)	HCM	Tan An Hoi, Cu Chi, Ho Chi Minh	10°57'30.7"N 106°28'48.5"E

2.2 Methods

Genomic DNA isolation

Genomic DNA was extracted from fresh leaves by the CTAB procedure (cetyl-trimethylammonium bromide) of Doyle và Doyle (1987) [13]. Then, extracted DNA was incubated with the SYBR Green I nucleic acid gel stain (Invitrogen, USA) for 10-20 min, separated on 1% agarose gels for 30 min at 120 V and photographed under UV light (HyperLadder™ 100 bp (Meridian Bioscience) was used as a molecular weight marker). DNA containing many impurities were purified using a QIAquick gel extraction kit (Qiagen, Germany) and measured DNA concentration by Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) with ratio A260/A280. Normally, a value of the A260/A280 ratio between 1.8 and 2.0 is considered as a pure DNA sample [14]. Great quality DNA extracts will be used for RAPD amplification.

PCR amplification

Firstly, 4 out of 24 cultivars were randomly selected to screen 200 RAPD primers to select the primers yielding the highest polymorphism. The highest polymorphic primers were then used to amplify 24 cultivars to assess genetic diversity.

PCR reactions were carried out according to the protocol of Truong et al. (2013) with RAPD primers. The 15 μL reaction volumes contained 25 mM $MgCl_2$ (Bioline-Meridian, UK), 200 μM deoxyribonucleotide triphosphate mix (Bioline-Meridian, UK), 5X PCR buffer, 1 U of Taq DNA polymerase (Bioline-Meridian, UK), 10 pmol of RAPD primer, 5-10 ng of total DNA and added sterile distilled water up to 15 μL. The amplification was performed in a Thermal Cycler with a total of 40 cycles: 1 min at 94°C, 1 min at 37°C and 2 min at 72°C. The total volume of the PCR products was evaluated in 1% agarose gels and visualized by a 1:10,000 dilution of the SYBR Green I nucleic acid gel stain (Invitrogen, USA). After electrophoresis, DNA bands profiling was observed under UV light and the images were analyzed using a Gel imaging system [15].

Data analysis

Based on the results of electrophoresis, bands that were present (clear, undistorted) would be assigned "1", absent (or are too faint) would be assigned "0". The size of each PCR product band was estimated based on the standard marker. The genetic diversity within populations were quantified using POPGENE programs [16] and estimated by calculating percentage of polymorphic loci (%P), number of expected alleles

(na), effective allele number (ne), Nei's gene diversity (Nei 1973) (h), Shannon's information index (I).

Besides, the data in the form of a logical matrix was fed into the NTSYSpc 2.1 program to build a phylogenetic tree using the UPGMA algorithm, with a distance matrix established based on SM similarity coefficient (Simple Matching, M Є [0;1]) [17].

$$SM = \frac{a+b}{a+b+c+d}$$

where, a: number of DNA segments present in both i and j cultivar; b: number of DNA segments absent in both i and j cultivarl c: number of bands appearing in i cultivar but not in j cultivar; d: number of bands appearing in j cultivar but not in i cultivar

3 Results

3.1 DNA isolation result

The results showed that the genomic DNA of all *C. asiatica* varieties had a good quality with a single, clean, unsmeared, clear band (Fig. 1). Besides, the 260/280 ratio of extracted DNA was ~1.8. Therefore, it could be used for further experiments.

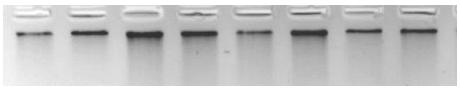

Fig. 1. Results of genomic DNA extraction

3.2 PCR results with RAPD primers

Primer screening result

Only 24 out of 200 primers surveyed with 4 random varieties were selected (Table 2, Fig. 2). These primers could produce the most conspicuous, bold and polymorphic bands. They were then used for genetic diversity assessment by RAPD for 24 cultivars of *C. asiatica*.

Table 2. List of selected polymorphic primers

No.	Primer	Sequence (5′-3′)
1	UBC#302	ACTTCCTCCA
2	UBC#312	ACAGGGAACG
3	UBC#316	ATGGCCTTAC
4	UBC#317	GCGAACCTCC
5	UBC#323	TGGACCACCC
6	UBC#330	GAGATCCCTC
7	UBC#341	GGTCTCCTAG
8	UBC#342	CCTCACCTGT
9	UBC#345	GTGGCCGCGC
10	UBC#354	CTAGAGGCCG
11	UBC#356	GCGGCCCTCT
12	UBC#362	CCGCCTTACA
13	UBC#365	TAGACAGAGG
14	UBC#376	CAGGACATCG
15	UBC#384	TGCGCCGCTA
16	UBC#408	CCGTCTCTTT
17	UBC#415	GTTCCAGCAG
18	UBC#420	GCAGGGTTCG
19	UBC#434	TCGCTAGTCC
20	UBC#476	TTGAGGCCCT
21	UBC#485	AGAATAGGGC
22	UBC#492	GTGACTGCTC
23	UBC#494	TGATGCTGTC
24	UBC#498	GACAGTCCTG

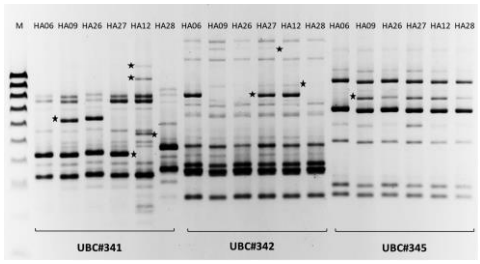

Fig. 2. PCR products of RAPD primers (UBC#341, UBC#342, UBC#345) showed polymorphisms among HUIB_CA06, HUIB_CA09, HUIB_CA26, HUIB_CA27, HUIB_CA12, HUIB_CA28; stars are markers of polymorphic expression bands; M: 100 bp Ladder

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Electrophoresis results of PCR products amplified by selected polymorphic RAPD primers

The results of electrophoresis of PCR products with 24 selected RAPD primers were presented in Table 3 and Table 4. In particular, 396 amplification bands were generated with 166 polymorphic bands (accounting for 42.02% of the total amplification bands) and the band sizes ranged from 210-1740 bp. Primer UBC#434 had the highest rate of polymorphism with 81.81% (Fig. 3). Meanwhile, UBC#302 and UBC#354 showed the lowest rate of polymorphic bands at 18.75% and 18.18%, respectively.

Table 3. Electrophoresis results of PCR products using 24 selected primers

Primer	Total of bands	Polymorphic bands number	Polymorphic bands rate (%P)	Size (bp)	Characteristic bands (bp)	Samples containing characteristic band
UBC#302	16	3	18.75	450-1520		
UBC#312	9	5	55.55	380-1520	480	HA13
UBC#316	15	2	13.33	230-940		
UBC#317	21	7	33.33	380-1250	900 740 650, 630 400	HA16 HA09, HA26 HA16 HA09
UBC#323	18	10	55.55	500-1410	1020	HA08
UBC#330	16	5	31.25	240-1050		
UBC#341	15	9	60.00	220-1500		
UBC#342	23	9	39.13	350-1400		
UBC#345	11	2	18.18	350-1580		
UBC#354	28	11	39.28	300-1740	1470	HA05
UBC#356	24	6	25.00	350-1550	1100	HA19
UBC#362	14	4	28.57	370-1140		
UBC#365	24	6	25.00	210-1520		
UBC#376	22	9	40.90	300-1690		
UBC#384	16	11	68.75	320-1600	1600	HA19
UBC#408	16	8	50.00	300-1270		
UBC#415	21	10	47.61	220-1440	1100 560	HA18 HA12
UBC#420	14	10	71.43	280-1340	900, 700	HA19
UBC#434	11	9	**81.81**	500-1310	900	HA16
UBC#476	14	5	35.71	420-1050		
UBC#485	4	3	75.00	400-1000	800	HA10
UBC#492	13	5	41.67	370-1360	530, 370	HA12
UBC#494	19	13	68.42	350-1470	950	HA06

Primer	Total of bands	Polymorphic bands number	Polymorphic bands rate (%P)	Size (bp)	Characteristic bands (bp)	Samples containing characteristic band
					450	HA10
UBC#498	12	4	33.33	630-1380		
Total	396	166	42.02	210-1740	20 bands	

Only 9/24 primers could produce polymorphic bands with the rate over 50%, namely UBC#312, UBC#323, UBC#341, UBC#384, UBC#408, UBC#420, UBC#434, UBC#485, UBC# 494. In addition, 20 characteristic bands were found. UBC#317 produced the largest number of characteristic bands (5 bands) with 2 bands of 740 bpand 400 bp typical for *C. asiatica* in Thua Thien Hue (HUIB_CA09, HUIB_CA26) (Table 3).

Out of a total of 24 studied varieties of *C. asiatica*, HUIB_CA10 was the individual with the most amplified bands (328 DNA bands), followed by HUIB_CA09 with 327 bands. *C. asiatica* with the least number of DNA bands formed was HUIB_CA26 (289 DNA bands). Thus, it could be seen that the number of amplification bands in different cultivars using the 24 studied primers was very large (the lowest was 289 and the highest was 328) (Table 4).

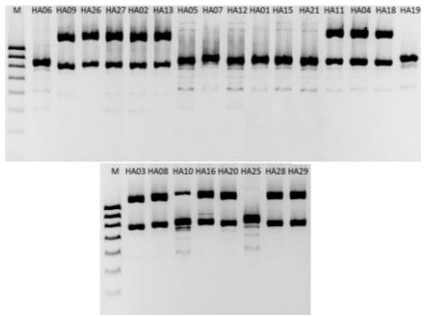

Fig. 3. PCR products of UBC#434 primer in all 24 varieties with the highest polymorphic band rate (81.81%); M: 100 bp Ladder

3.3 Results of genetic diversity analysis and building phylogenetic tree

Based on the presence or absence of DNA bands in the electrophoresis of PCR-RAPD products from 24 varieties, we have built a binary matrix for all analyzes using POPGENE software (version 1.32) to calculate genetic diversity indices and NTSYS software (version 2.1) to construct the phylogenetic tree.

Calculation results of genetic diversity indices from POPGENE 1.32 software

The level of genetic diversity within *C. asiatica* populations was quite low. In particular, the indices (na, ne, h, I) of the population were 1.4192, 1.2133, 0.1272, 0.1938, respectively (Table 5). In addition, Table 6 also showed that the genetic distance or genetic difference between individuals was low, with the largest difference of 0.2194 (21.94%) between HUIB_CA19 and HUIB_CA26, the smallest difference was 0.0025% between HUIB_CA28 and HUIB_CA29. Varieties obtained from the same locality such as HUIB_CA01, HUIB_CA15 and HUIB_CA21 (Nghe An); HUIB_CA05 and HUIB_CA07 (Ha Tinh); HUIB_CA06, HUIB_CA09, HUIB_CA26 and HUIB_CA27 (Thua Thien Hue) also had little genetic difference.

Table 4. Genetic diversity indices of *C. asiatica* populations

Indices	na*	ne*	h*	I*	N	Np	%P
	1.4192	1.2133	0.1272	0.1938	396	166	41.92
SE	0.4941	0.3297	0.1814	0.2626			

Nei (1987), Molecular Evolutionary Genetics [18]

**Note: Calculating percentage of polymorphic loci (%P), number of expected alleles (na), effective allele number (ne), Nei's gene diversity (Nei 1973) (h), Shannon's information index (I), total of bands (N), Polymorphic bands number (Np)*

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Table 5. Number of amplified DNA bands of each cultivar with each primer

No.	Material	UBC #302	UBC #312	UBC #316	UBC #317	UBC #323	UBC #330	UBC #341	UBC #342	UBC #345	UBC #354	UBC #356	UBC #362	UBC #365	UBC #376	UBC #384	UBC #408	UBC #415	UBC #420	UBC #434	UBC #476	UBC #485	UBC #492	UBC #494	UBC #498	Total
1	HA01	15	5	13	14	15	14	15	19	11	18	20	12	20	19	13	10	15	7	6	14	1	10	16	11	313
2	HA02	14	7	13	15	11	15	15	18	10	18	21	10	21	19	13	12	14	6	5	14	3	11	14	11	310
3	HA03	13	7	13	14	13	14	15	17	10	21	20	11	20	21	11	11	16	8	6	12	1	11	16	11	312
4	HA04	13	6	15	15	11	14	15	19	10	20	19	11	21	18	12	11	14	10	4	12	3	11	16	11	311
5	HA05	13	7	13	14	14	15	15	20	11	25	20	11	21	21	12	11	13	9	8	13	2	11	15	11	325
6	HA06	13	6	15	14	12	14	10	17	9	19	19	10	22	16	11	13	14	6	8	13	2	11	17	11	302
7	HA07	13	7	13	14	13	14	14	18	10	18	20	11	21	20	12	11	12	7	7	14	2	9	9	11	300
8	HA08	13	8	13	14	14	15	14	18	10	21	21	11	21	20	12	14	16	10	4	12	2	11	13	12	319
9	HA09	13	6	13	16	14	15	15	20	11	25	21	11	22	21	13	12	15	7	4	13	2	11	15	12	327
10	HA10	13	7	13	14	13	14	15	19	10	24	20	11	22	18	14	14	16	11	6	14	4	11	14	11	328
11	HA11	13	7	13	14	13	14	13	18	11	21	19	12	22	20	13	12	14	6	5	12	3	11	16	11	313
12	HA12	13	7	13	15	13	14	15	20	10	19	20	11	23	18	13	10	15	7	6	14	3	12	16	11	318
13	HA13	13	8	13	15	10	15	14	17	10	20	19	11	22	18	13	14	13	7	4	13	2	11	15	11	308
14	HA15	13	5	13	14	12	14	15	18	11	19	20	11	20	18	12	12	17	6	6	12	2	9	16	10	305
15	HA16	13	6	13	17	13	15	14	18	10	21	19	11	21	22	13	13	16	8	5	14	2	11	10	10	315
16	HA18	13	7	13	15	14	14	14	17	10	21	20	11	22	20	14	12	15	9	4	12	2	11	10	11	311
17	HA19	14	6	13	15	13	15	14	19	9	22	22	11	21	20	14	12	14	11	7	13	2	11	10	11	319
18	HA20	13	6	13	14	12	14	15	18	10	24	19	11	22	22	12	13	16	11	4	14	2	11	15	11	322
19	HA21	15	5	13	14	13	14	15	19	10	20	19	11	19	20	12	11	14	5	6	13	1	11	16	11	307
20	HA25	13	8	13	14	13	11	14	17	9	20	19	12	19	19	10	14	14	7	5	14	2	10	16	11	304
21	HA26	13	7	13	16	13	15	9	16	10	20	19	11	21	19	7	12	15	6	4	11	1	9	12	10	289
22	HA27	14	6	15	14	13	15	15	19	10	20	19	10	22	15	13	13	14	6	5	13	2	11	13	11	308
23	HA28	15	8	13	14	12	11	12	17	9	20	19	11	20	20	10	14	13	8	5	9	3	10	15	12	300
24	HA29	14	8	13	14	12	11	12	17	9	20	19	11	20	20	10	14	13	8	5	9	3	10	15	12	300
Total		322	160	318	349	306	336	334	435	240	496	473	264	505	464	289	295	348	186	129	304	52	255	340	265	7465

Table 6. Genetic distance between 24 individuals of *C. asiatica* from POPGENE 1.32 software

pop ID	HA01	HA15	HA21	HA02	HA03	HA04	HA05	HA07	HA06	HA09	HA26	HA27	HA08	HA10	HA16	HA11	HA12	HA13	HA18	HA19	HA20	HA25	HA28	HA29
HA01	****	0.9141	0.9293	0.8864	0.8864	0.8889	0.9040	0.9066	0.8561	0.8939	0.8485	0.8763	0.8737	0.8813	0.8687	0.8889	0.8965	0.8763	0.8586	0.8636	0.8712	0.8712	0.8359	0.8333
HA15	0.0898	****	0.9242	0.8965	0.9015	0.8838	0.8990	0.9015	0.8611	0.8737	0.8434	0.8813	0.9040	0.8611	0.8586	0.8889	0.9015	0.8965	0.8788	0.8535	0.8914	0.8763	0.8561	0.8586
HA21	0.0733	0.0788	****	0.9015	0.9268	0.9141	0.9192	0.9116	0.9015	0.8889	0.8636	0.9015	0.8939	0.8763	0.8788	0.8990	0.8914	0.8813	0.8687	0.8636	0.9066	0.9116	0.8712	0.8687
HA02	0.1206	0.1093	0.1037	****	0.9040	0.9116	0.8813	0.9040	0.8586	0.8914	0.8561	0.9293	0.8965	0.8737	0.8813	0.8813	0.8838	0.9091	0.8712	0.8359	0.8990	0.8889	0.8687	0.8712
HA03	0.1206	0.1037	0.0761	0.1009	****	0.9268	0.8965	0.8990	0.8636	0.8864	0.8662	0.8838	0.9217	0.8838	0.9066	0.8914	0.8586	0.8889	0.8864	0.8561	0.9242	0.9141	0.8838	0.8788
HA04	0.1178	0.1235	0.0898	0.0925	0.0761	****	0.8990	0.8914	0.8813	0.8939	0.8636	0.9116	0.9091	0.9015	0.8788	0.8939	0.8763	0.8965	0.8889	0.8535	0.9015	0.8965	0.8763	0.8535
HA05	0.1009	0.1065	0.0843	0.1263	0.1093	0.1065	****	0.9066	0.8662	0.9040	0.8333	0.8813	0.8939	0.9015	0.8687	0.8838	0.8914	0.8813	0.8788	0.8889	0.9066	0.8763	0.8510	0.8460
HA07	0.0981	0.1037	0.0925	0.1009	0.1065	0.1149	0.0981	****	0.8687	0.8712	0.8662	0.8838	0.8864	0.8788	0.8914	0.8864	0.9040	0.8990	0.8763	0.8914	0.8939	0.8838	0.8434	0.8561
HA06	0.1554	0.1495	0.1037	0.1525	0.1466	0.1263	0.1437	0.1408	****	0.8611	0.8813	0.9040	0.8460	0.8485	0.8258	0.8510	0.8586	0.8636	0.8207	0.8207	0.8687	0.8788	0.8535	0.8485
HA09	0.1121	0.1350	0.1178	0.1149	0.1206	0.1121	0.1009	0.1379	0.1495	****	0.8687	0.9015	0.9040	0.9015	0.8889	0.8889	0.8611	0.8914	0.8838	0.8434	0.9015	0.8662	0.8460	0.8586
HA26	0.1643	0.1703	0.1466	0.1554	0.1437	0.1466	0.1823	0.1437	0.1263	0.1408	****	0.8864	0.8485	0.8258	0.8384	0.8535	0.8207	0.8510	0.8434	0.8030	0.8510	0.8510	0.8561	0.8561
HA27	0.1321	0.1263	0.1037	0.0733	0.1235	0.0925	0.1263	0.1235	0.1009	0.1037	0.1206	****	0.8813	0.8788	0.8763	0.8611	0.8687	0.8939	0.8712	0.8359	0.8939	0.8737	0.8535	0.8788
HA08	0.1350	0.1009	0.1121	0.1093	0.0815	0.0953	0.1121	0.1206	0.1673	0.1009	0.1643	0.1263	****	0.9217	0.9091	0.8939	0.8662	0.8965	0.9040	0.8636	0.9116	0.8914	0.8763	0.8409
HA10	0.1263	0.1495	0.1321	0.1350	0.1235	0.1037	0.1037	0.1292	0.1643	0.1037	0.1915	0.1292	0.0815	****	0.8813	0.8864	0.8687	0.8586	0.8813	0.8813	0.9192	0.8737	0.8384	0.8333
HA16	0.1408	0.1525	0.1292	0.1263	0.0981	0.1292	0.1408	0.1149	0.1915	0.1178	0.1763	0.1321	0.0953	0.1263	****	0.8838	0.8561	0.8864	0.8889	0.8535	0.9066	0.8763	0.8308	0.8535
HA11	0.1178	0.1178	0.1065	0.1263	0.1149	0.1121	0.1235	0.1206	0.1613	0.1178	0.1584	0.1495	0.1121	0.1206	0.1235	****	0.8763	0.8864	0.8788	0.8586	0.8864	0.8763	0.8510	0.8359
HA12	0.1093	0.1037	0.1149	0.1235	0.1525	0.1321	0.1149	0.1009	0.1525	0.1495	0.1976	0.1408	0.1437	0.1408	0.1554	0.1321	****	0.8788	0.8813	0.8813	0.8788	0.8586	0.8333	0.8561
HA13	0.1321	0.1093	0.1263	0.0953	0.1178	0.1093	0.1263	0.1065	0.1466	0.1149	0.1613	0.1121	0.1093	0.1525	0.1206	0.1206	0.1292	****	0.8813	0.8359	0.8889	0.8636	0.8535	0.8535
HA18	0.1525	0.1292	0.1408	0.1379	0.1206	0.1178	0.1292	0.1321	0.1976	0.1235	0.1703	0.1379	0.1009	0.1263	0.1178	0.1292	0.1263	0.1263	****	0.8838	0.8763	0.8662	0.8611	0.8636
HA19	0.1466	0.1584	0.1466	0.1793	0.1554	0.1584	0.1178	0.1149	0.1976	0.1703	**0.2194**	0.1793	0.1466	0.1263	0.1584	0.1525	0.1263	0.1793	0.1235	****	0.8763	0.8460	0.8207	0.8232
HA20	0.1379	0.1149	0.0981	0.1065	0.0788	0.1037	0.0981	0.1121	0.1408	0.1037	0.1613	0.1121	0.0925	0.0843	0.0981	0.1206	0.1292	0.1178	0.1321	0.1321	****	0.9040	0.8636	0.8662
HA25	0.1379	0.1321	0.0925	0.1178	0.0898	0.1093	0.1321	0.1235	0.1292	0.1437	0.1613	0.1350	0.1149	0.1350	0.1321	0.1321	0.1525	0.1466	0.1437	0.1673	0.1009	****	0.9192	0.9217
HA28	0.1793	0.1554	0.1379	0.1408	0.1235	0.1321	0.1613	0.1703	0.1584	0.1673	0.1554	0.1584	0.1321	0.1763	0.1854	0.1613	0.1823	0.1584	0.1495	0.1976	0.1466	0.0843	****	0.9975
HA29	0.1823	0.1525	0.1408	0.1379	0.1206	0.1292	0.1584	0.1673	0.1554	0.1643	0.1525	0.1554	0.1292	0.1733	0.1823	0.1584	0.1793	0.1554	0.1466	0.1945	0.1437	0.0815	**0.0025**	****

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Phylogenetic tree

Phylogenetic analysis showed that there was genetic diversity among individuals of *C. asiatica*. With 24 research primers, the genetic similarity coefficient among individuals of *C. asiatica* varied from 0.80303 to 0.997475. Based on the genetic similarity coefficient, the studied populations were divided into 2 main clades (Clade I and Clade II) (Fig. 4).

Clade I consisted of 2 operational taxonomic unit (OTU), HUIB_CA06 and HUIB_CA26 with a similarity coefficient of 0.881313. These were 2 varieties collected from Quang Dien district, Thua Thien Hue province. From here, it could be seen that these two varieties had genetic differences compared to the remaining varieties.

Clade II included the remaining 22 OTUs of *C. asiatica*. In this clade, varieties could be separated into 2 subgroups, subgroup IIa (including 19 individuals) and subgroup IIb (including 3 individuals, HUIB_CA25, HUIB_CA28, HUIB_CA29). In particular, 2 individuals HUIB_CA28, HUIB_CA29 had the highest similarity coefficient (0.997475) and 2 individuals HUIB_CA18, HUIB_CA19 showed the lowest similarity coefficient (0.883838).

In subgroup IIa, 19 individuals of *C. asiatica* continued to be divided into two subgroups. The first subgroup included 7 individuals HUIB_CA01, HUIB_CA21, HUIB_CA15, HUIB_CA05, HUIB_CA07, HUIB_CA12 and HUIB_CA11; the second subgroup consisted of 10 individuals and further divided into 2 smaller groups. Namely, HUIB_CA02, HUIB_CA27, and HUIB_CA13 were in the same group, HUIB_CA03, HUIB_CA04, HUIB_CA08, HUIB_CA10, HUIB_CA20, HUIB_CA09 and HUIB_CA16 were in the other.

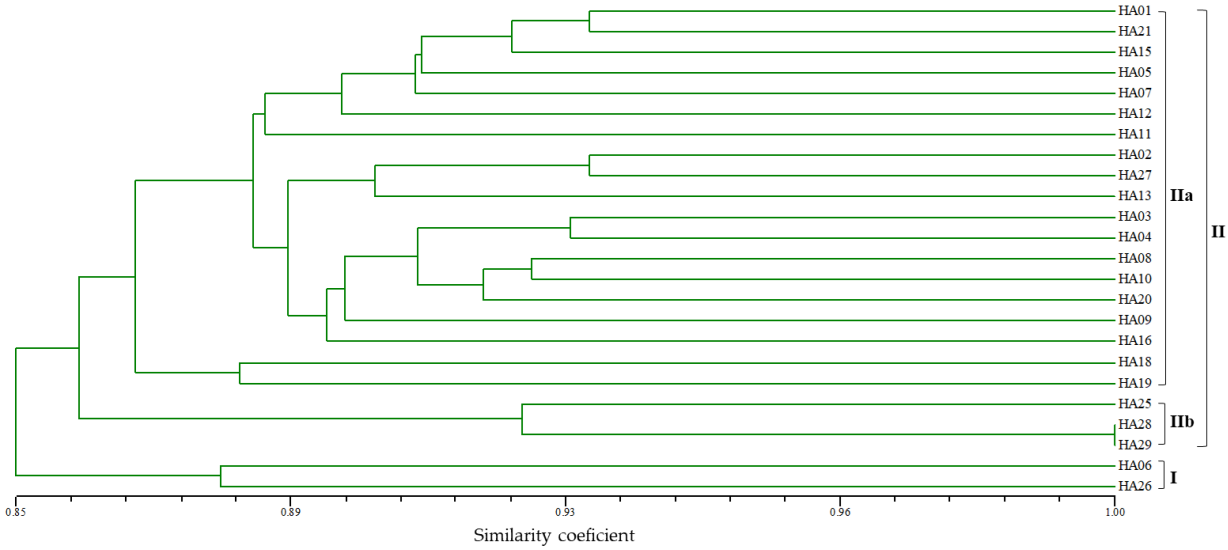

Fig. 4. Dendrogram of the 24 cultivars of *C. asiatica* obtained by UPGMA

4 Discussion

RAPD marker was used in order to analysis the genetic diversity and differentiation in 24 cultivars of *C. asiatica*. This technique was found to be effective in revealing polymorphisms in this species. However, the major limitations of this technique are its lack of reproducibility [19, 20] and its sensitivity to small variations in PCR conditions. In order to overcome these limitations, DNA of high quality was used.

Genetic diversity within populations is of great concern to ecologists and geneticists. A reduced genetic variation is thought to affect the ability of populations to adapt to changing environments, thereby increasing their probability of extinction [21]. Waugh and Powell (1992) stated that the analysis of polymorphism by molecular markers could help to select priority areas for conservation and provide vital information for the development of genetic sampling and improvement [22].

In this study, we obtained 166 polymorphic DNA bands out of a total of 396 amplified bands (accounting for 42.02%) from 24 RAPD primers. There were only 9/24 primers for polymorphic bands over 50% and the genetic diversity indices (na, ne, h, I), the genetic distance between individuals were low. This may be because gene flow in *C. asiatica* homogenizes population structure and counteracts the effects of drift and diversifying selection. Thus, populations can undergo genetic differentiation from one generation to another [23]. The results are similar to those of Sakthipriya (2018). Specifically, genetic diversity analysis was performed by the authors on 30 species of *Centella asiatica* (L.) Urb. with 10 SSR primers. Out of the 10 primers, only 2 primers were polymorphic which indicated low level of genetic variation between the samples. It could be due to the narrow genetic basis or inbreeding, genetic drift, restricted gene flow, and small population size [24].

Based on the value of genetic similarity coefficient between individuals when compared with each other, NTSYSpc 2.1 software automatically arranged individuals with high genetic similarity coefficient into a group. The more genetically similar individuals are, the greater the similarity coefficient is and vice versa. The closer the genetic similarity coefficient is to 0, the greater the genetic difference is and the higher the value used in breeding. A similarity coefficient closer to 1 is genetically closer. In our study, genetic difference values ranged from 0.0025 to 0.2194 and similarity coefficient among individuals of population varied from 0.80303 to 0.997475 though geographically distances of these varieties are quite large. Therefore, there is no correlation between genetic distances and geographical distances. However, two varieties, HUIB_CA06 and HUIB_CA26 (from Thua Thien Hue province) had genetic differences compared to the remaining varieties in phylogenetic tree. Differences could be partially explained by different number of PCR products analyzed for RAPD reinforcing the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among the plant species of *C. asiatica*. Another explanation could be low reproducibility of RAPD [25]. However, in our experiments, once the PCR conditions were well set up, a high reproducibility for RAPD were obtained.

5 Conclusion

DNA markers (molecular markers) are a potent tool to analyze the genetic diversity in various organisms based on the polymorphism found in the DNA sequences. The present study using RAPD markers revealed pretty low levels of genetic variations among the studied *C. asiatica* varieties, which might be due to the effect of environmental factors; however, further studies are required to verify this assumption. In particular, this study shows that HUIB_CA06 and HUIB_CA26 (from Thua Thien Hue province) have genetic differences compared to the remaining varieties. The results of the present study can be viewed as a starting point for next research on genetic differences of *C. asiatica* varieties obtained from Thua Thien Hue province.

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Authors' contributions

Ho NTH, Rasphone S, Hatsadong C, Nguyen BLQ and Le HMD conducted the experiments, participated in interpretation of results and manuscript preparation. Truong HTH participated in the design of studies and the discussion for preparing the manuscript and last edited. All authors read and approved this manuscript.

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