ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Research Article

Analysis of Genetic Diversity of *Centella asiatica* Using SSR Markers

M. Sakthipriya[1], S.S. Vishnu[2], S. Sujith[2], P. Rajesh Kumar[2], K.K. Sabu[1*]

[1]Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram 695562, India
[2]Udaya School of Engineering, Vellamodi, Tamil Nadu 629204, India

Abstract

Genetic diversity represents the heritable variation within and between populations of organisms. A better understanding of genetic diversity and its distribution is essential for its conservation and use. In this study the genetic diversity analysis was performed in 30 accessions of *Centella asiatica* (L.) Urb. using 10 SSR markers. *C. asiatica* is a tropical medicinal plant from Apiaceae family native to Southeast Asian countries. It is among the top herbs in the category of anti-aging and CNS drugs used worldwide. The whole plant and aerial parts are used widely in traditional and alternative medicines due to its ample pharmacological activities. The molecular screening through microsatellite markers showed low polymorphism (0.019) between the samples analyzed. Further studies, including the effect of environmental factors, genetic composition or possibility of inbreeding, are required to analyze the probable reason for the low variability exists in the species.

Keywords: *Centella asiatica*; genetic diversity; SSR; microsatellites; medicinal plant

Introduction

The genus *Centella* belongs to family Apiaceae and inhabits tropical and subtropical regions of world in wet places. *Centella asiatica*, commonly known as Indian pennywort is one of the species belonging to the *Centella* genus. It is a perennial herb with long slender, horizontal stolons characterized by long interrnodes from which cluster of ascending petiolate leaves arise at each node. Being an important herbal medicinal plant, it has been used in Indian Ayurvedic medicine as a nerve tonic. Its utilization has been known for many years in treating all kind of diseases such as gastrointestinal disease, gastric ulcer, asthma, wound healing and eczema (Hamid *et al*., 2002; Subathra *et al*., 2005; Kimura *et al*., 2008; Singh *et al*., 2008; Ullah *et al*., 2009; Pittella *et al*., 2009). Apart from the medicinal uses, its utilisation as food and beverage has increased over the years due to its antioxidant, anti-inflammatory, wound healing and memory enhancing properties (Hashim, 2011). The use of *Centella* as an alternative natural antioxidant especially against age-related changes in the brain and defense system, has notably increased in recent years (Subathra *et al*., 2005).

Genetic diversity is the variation at the level of individual genes. In a population, genetic diversity means that the

Cite this article as:

M Sakthipriya et al. (2018) Int. J. Appl. Sci. Biotechnol. Vol 6(2): 103-109. DOI: 10.3126/ijasbt.v6i2.19583

***Corresponding author**
K K Sabu,
Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram 695562, India
Email: sabu@jntbgri.res.in

Peer reviewed under authority of IJASBT

population contains most of the possible alleles for each particular gene locus and the total number of genetic characteristics in the genetic makeup of a species. Each species is made up of individuals that have their own particular genetic composition. Within a species there may also be discrete populations in which distinctive genes are found, DNA markers or molecular markers that are based on DNA sequence polymorphisms have been proved to be valuable and powerful tools for the analysis of genetic diversity and gene mapping. DNA sequences decide the diversity of organisms, so the techniques used to assess DNA polymorphisms directly measure the genetic diversity. Because molecular markers show Mendelian inheritance, it is possible to predict the fingerprint of each organism and determine the evolutionary history of the species by phylogenetic analysis, studies of genetic relationship, population genetic structures and genetic mapping (Hoshino *et al*., 2012).

The differences that discriminate one plant from another are encoded in the plant's genetic material (Semagn *et al*., 2006). Simple sequence repeat (SSR) markers were first developed for genetic mapping in humans (Litt and Luty, 1989) and they are the most promising PCR-based markers. Microsatellites are tandem repeated motifs of variable lengths that are distributed throughout the eukaryotic nuclear genome in both coding and non-coding regions (Miah *et al*., 2013). They also appear in prokaryotic and eukaryotic organellar genomes, e.g., chloroplast (Powell *et al*., 1995) and mitochondria (Wang *et al*., 2009). In this study EST-SSR markers were used to analyze the genetic diversity of *C. asiatica*. In addition to being highly informative, EST-SSR markers are an easy, low-cost PCR detection method, when compared to other molecular markers.

Materials and Methods

Plant sample collection

Thirty accessions of *Centella asiatica* were collected from different locations of Tamil Nadu and Kerala (Table 1). The plant samples collected were grown in the JNTBGRI campus under uniform environmental conditions for diversity analysis (Fig. 1).

Fig. 1: View of the germplasm collection of *C. asiatica* used in the study

Table 1: Details of *Centella asiatica* accessions used in the study

Pop	Code	Place	Latitude	Longitude	Altitude
1	CAP01	Charode, Thukalay	8°15'25.82"N	77°19'6.68"E	134m
2	CAP02	Krishnapuram, Thukalay	8°15'9.07"N	77°19'21.73"E	144m
3	CAP03	Kumaracoil	8°13'52.13"N	77°20'47.69"E	119m
4	CAP04	Thottiyod	8°12'45.31"N	77°21'43.14"E	121m
5	CAP05	Thottiyod	8°12'16.27"N	77°22'11`58"E	136m
6	CAP06	Kanyakulam	8°12'24.07"N	77°24'9.5"E	144m
7	CAP07	Kanyakulam	8°12'48.43"N	77°24'27.84"E	147m
8	CAP08	Erachakulam	8°13'30.68"N	77°25'31.37"E	136m
9	CAP09	Navalkadu	8°14'55.53"N	77°26'26.57"E	112m
10	CAP10	Karuparai	8°10'30.76"N	77°19'35.91"E	122m
11	CAP11	Karuparai	8°10'28.61"N	77°19'39.37"E	18m
12	CAP12	Mandaikadu	8°9'52.25"N	77°16'34.35"E	32m
13	CAP13	Colachel Rd	8°9'29.58"N	77°17'6.8"E	24m
14	CAP14	Karuparai	8°10'29.13"N	77°19'42.68"E	112m
15	CAP15	Karuparai	8°10'39`32"N	77°19'36.95"E	45m
16	CAP16	Karuparai	8°10'43.82"N	77°19'31.67"E	18m
17	CAP17	Kunnathukulam	8°10'51.11'N	77°19'28.04"E	14m
18	CAP18	Kunnathukulam	8°10'54.75"N	77°19'29.12"E	45m
19	CAP19	Kunnathukulam	8°10'37.92"N	77°19'34.76"E	28m
20	CAP20	Karuparai	8°10'35`65"N	77°19'37.82"E	48m
21	CAP21	Alummoodu	8°25'6.55"N	77°3'42.91"E	130m
22	CAP22	Kodangavila	8°25'7.66"N	77°3'49.61"E	124m
23	CAP23	Pallichal	8°25'18.25"N	77°1'59.47"E	152m
24	CAP24	Pallichal	8°25'25.84"N	77°2'1.78"E	133m
25	CAP25	Balaramapuram	8°25'27.09"N	77°2'19.18"E	142m
26	CAP26	Balaramapuram	8°25'48.77"N	77°2'20.51"E	154m
27	CAP27	Thannivila	8°26'21`01"N	77°1'59.45"E	153m
28	CAP28	Balaramapuram	8°25'53`33"N	77°2'11.52"E	159m
29	CAP29	Pallichal	8°25'0.89"N	77°2'1.39"E	147m
30	CAP30	Balaramapuram	8°25'27.76"N	77°2'1.26"E	171m

SSR analysis

Plant Genomic DNA isolation Kit (Origin Labs, Kerala) was used to isolate total genomic DNA from the collected *C. asiatica* leaf samples. Extracted DNA's integrity and quality were checked by Agarose gel electrophoresis (0.8%). The SSR PCR analysis was carried out in a 25 µl of PCR reaction containing about 20 ng of DNA, 1x Taq buffer (Origin, Kerala) and 0.5 unit Taq polymerase enzyme (Origin, Kerala), 200 µM of each of the four dNTPs, 15 picomole of EST-SSR primers (19-22 mer; Eurofins genomics India Pvt Ltd) (Table 2) (Thomas, 2016). The PCR program was as: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, specific annealing temperature (48°C to 56°C) for 1 min, 72°C for 2 min, and 72°C for 7 min for the final extension (Veriti® Applied Biosystems, USA). The final PCR products were separated on 3% agarose gel containing 0.5 µg mL^{-1} ethidium bromide in 1 X TBE buffer and was documented in Gel documentation system (UVP, UK). The bands were scored as loci and alleles, these scored separated products were analyzed using POPGENE ver. 3.2 (Yeh *et al.*, 1999) and PowerMarker ver. 3.25 (Liu and Muse, 2005) software to find out the genetic distance and diversity between the collected *Centella* accessions. The dendrogram were constructed using Tree View software 1.6.6 (Page, 1996).

Results and Discussion

Genetic diversity of a wide variety of plants has been estimated using SSR markers (Garcia *et al*., 2004; Acquadro *et al*., 2005; Stafne *et al*., 2005; Hasan *et al*., 2006; Sun *et al*., 2008; Cavagnaro *et al*., 2011; Sajib *et al*., 2012; Matin *et al*., 2012; Wangari *et al*., 2013; Rodier-Goud *et al*., 2013; Piya *et al*., 2014; Tan *et al*., 2014; Olango *et al*., 2015). However studies in C. *asiatica* using SSR marker are few. Due to multi-allelic nature, reproducibility, co-dominant inheritance, high abundance and extensive genome coverage SSR are regarded as an ideal molecular marker to investigate genetic diversity (Hoshino *et al*., 2012). In this study ten EST-SSR primers were used to study the genetic diversity of Tamil Nadu and Kerala accessions of *C. asiatica* (Table 2). The level of polymorphism among the collected samples was estimated by determining the allelic number and PIC values for each of the ten SSR loci evaluated. The amplified products using the EST-SSR primers in our study were in the range of 180 bp to 1.5 kb. A total of 12 alleles were identified with an average of 1.2 alleles per locus (Table 3). The maximum number of alleles per locus was found to be 2. The polymorphism information content (PIC) which is the expression of allele diversity frequency among the varieties, ranged from 0.0000 to 0.1239, with an average of 0.0195 which is very low when compared to the previous reports (Rodier-Goud *et al*., 2013). Out of the 10 primers, only 2 primers were polymorphic which indicates 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 (Furlan *et al*., 2012). Nei's method measures the genetic similarity and genetic distance (Nei, 1972) which was predicted between the samples by means of POPGENE**.** The highest genetic identity value was found to be 1.00 and the lowest value was 0.40. Genetic distance between each accession ranged from 0.00 to 0.91 (Table 4). Based on the genetic distance a dendrogram was constructed which grouped the populations into many subgroups (Fig. 2). In general, the sub-grouping was based on geographic distances as they often comprise of accessions from the adjacent localities.

Table 2. Details of EST-SSR primer sequences used in the genetic diversity analysis

S. N.	Primer Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')
1	TBG-Centa F1	AGGACTTGACACTGCTTTTGCT	TGCCTTCTCCTTCTTCATCTTC
2	TBG-Centa F2	CTACTCTATCCCGCAAATCCTT	CTCTCTCTCGTTTCTCGCC
3	TBG-Centa F3	AGTGTTGATGATGATGACGAGG	CAGACTCATTTGCTTTTGCTTG
4	TBG-Centa F8	AGAATCAATACATACAGCCCCG	AAACGAAAGATTGTGAGAAGGG
5	TBG-Centa F10	CCAAAACCATTCTCTCCACTTC	CTCTTCTTTGTCGCCATCTTCT
6	TBG-Centa F14	TCCTCCAAAATACCACCATACC	GACCAATGAGTGCCAAAAGAAT
7	TBG-Centa F15	GAACTTTCGCCTCTTCTCTTGA	TCCTCATTTATCTCCCTCGGTA
8	TBG-Centa F19	TTAGCATTTAGAAGGTCAGGGC	ATTTACAGCAATCAGAGACGCA
9	TBG-Centa F26	ATGGGAGAGAAATAAAGGAGCC	GAAACGATAGTCAGGGATTGGA
10	TBG-Centa F31	AGAGCACACCTTTATCCCTTTG	AGAAGAAGAAGGAGGATTTGGG

Table 3. Summary of Genetic Variation Statistics for 30 *Centella asiatica* accessions

Marker	Major Allele Frequency	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
Centa1	1.0000	1.0000	30.0000	21.0000	1.0000	0.7000	0.0000	0.0000	0.0000
Centa2	1.0000	1.0000	30.0000	29.0000	1.0000	0.9667	0.0000	0.0000	0.0000
Centa3	1.0000	1.0000	30.0000	23.0000	1.0000	0.7667	0.0000	0.0000	0.0000
Centa8	1.0000	1.0000	30.0000	24.0000	1.0000	0.8000	0.0000	0.0000	0.0000
Centa10	1.0000	1.0000	30.0000	30.0000	1.0000	1.0000	0.0000	0.0000	0.0000
Centa14	1.0000	1.0000	30.0000	28.0000	1.0000	0.9333	0.0000	0.0000	0.0000
Centa15	0.9615	2.0000	30.0000	26.0000	2.0000	0.8667	0.0740	0.0000	0.0712
Centa19	0.9286	2.0000	30.0000	28.0000	2.0000	0.9333	0.1327	0.0000	0.1239
Centa26	1.0000	1.0000	30.0000	24.0000	1.0000	0.8000	0.0000	0.0000	0.0000
Centa31	1.0000	1.0000	30.0000	25.0000	1.0000	0.8333	0.0000	0.0000	0.0000
Mean	0.9890	1.2000	30.0000	25.8000	1.2000	0.8600	0.0207	0.0000	0.0195

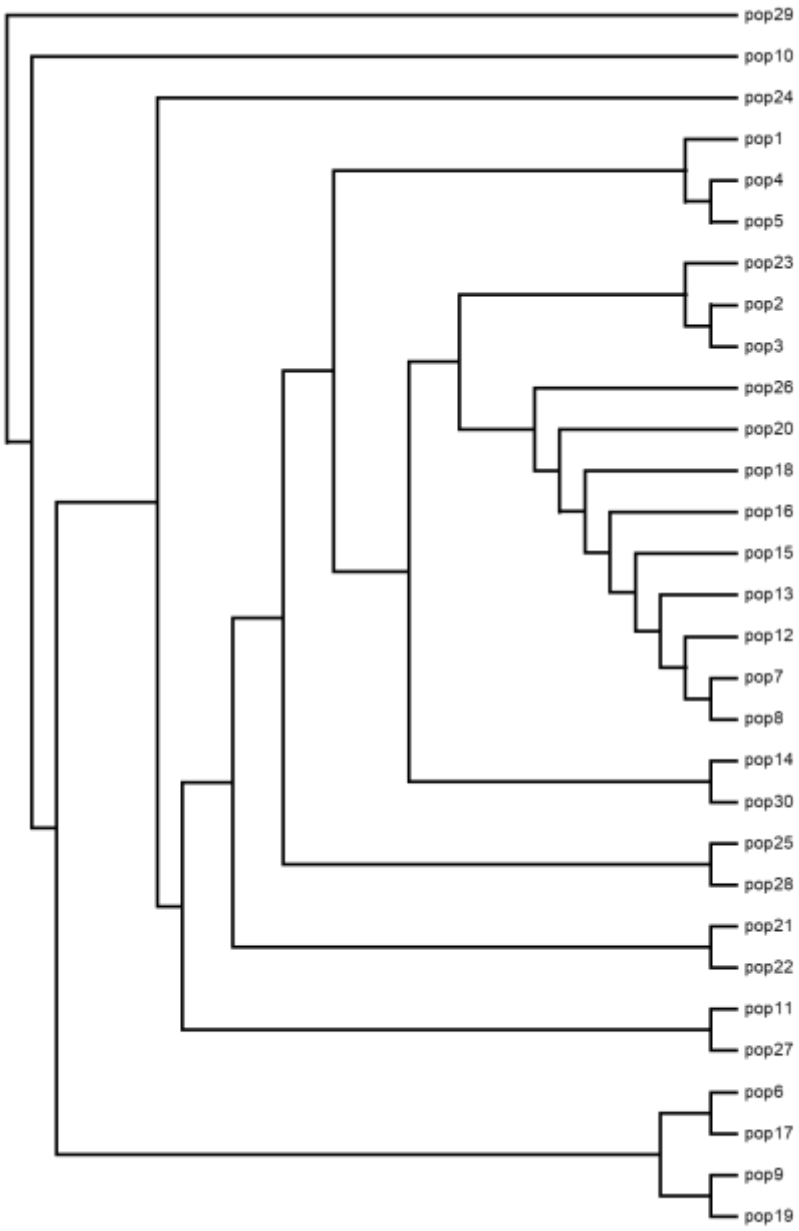

Fig. 2: Dendrogram showing genetic relationships of the collected *C. asiatica* accessions (see Table 1 for details) inferred from the microsatellite marker data

Table 4: Nei's original measures of genetic diversity and genetic distance (Nei, 1972) estimated from the SSR analysis

pop	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	****	0.83	0.83	0.94	0.94	0.94	0.89	0.89	0.75	0.58	0.67	0.89	0.89	0.83	0.89	0.89	0.79	0.89	0.80	0.89	0.75	0.83	0.83	0.80	0.83	0.89	0.75	0.78	0.67	0.75
2	0.19	****	1.00	0.89	0.89	0.76	0.95	0.95	0.71	0.82	0.88	0.95	0.95	0.89	0.95	0.95	0.60	0.95	0.76	0.95	0.83	0.89	1.00	0.88	0.89	0.95	0.94	0.84	0.76	0.94
3	0.19	0.00	****	0.89	0.89	0.76	0.95	0.95	0.71	0.82	0.88	0.95	0.95	0.89	0.95	0.95	0.60	0.95	0.76	0.95	0.83	0.89	1.00	0.88	0.89	0.95	0.94	0.84	0.76	0.94
4	0.06	0.12	0.12	****	1.00	0.88	0.95	0.95	0.71	0.68	0.76	0.95	0.95	0.89	0.95	0.95	0.75	0.95	0.76	0.95	0.83	0.89	0.89	0.76	0.89	0.95	0.83	0.84	0.76	0.83
5	0.06	0.12	0.12	0.00	****	0.88	0.95	0.95	0.71	0.68	0.76	0.95	0.95	0.89	0.95	0.95	0.75	0.95	0.76	0.95	0.83	0.89	0.89	0.76	0.89	0.95	0.83	0.84	0.76	0.83
6	0.07	0.28	0.28	0.13	0.13	****	0.84	0.84	0.80	0.62	0.71	0.84	0.84	0.76	0.84	0.84	0.85	0.84	0.86	0.84	0.80	0.76	0.76	0.71	0.76	0.84	0.67	0.72	0.57	0.67
7	0.11	0.05	0.05	0.05	0.05	0.18	****	1.00	0.78	0.77	0.84	1.00	1.00	0.95	1.00	1.00	0.71	1.00	0.84	1.00	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
8	0.11	0.05	0.05	0.05	0.05	0.18	0.00	****	0.78	0.77	0.84	1.00	1.00	0.95	1.00	1.00	0.71	1.00	0.84	1.00	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
9	0.29	0.35	0.35	0.35	0.35	0.22	0.25	0.25	****	0.58	0.80	0.78	0.78	0.71	0.78	0.78	0.79	0.78	0.94	0.78	0.75	0.71	0.71	0.67	0.71	0.78	0.75	0.67	0.40	0.63
10	0.55	0.20	0.20	0.39	0.39	0.48	0.26	0.26	0.55	****	0.77	0.77	0.77	0.68	0.77	0.77	0.55	0.77	0.62	0.77	0.87	0.82	0.82	0.62	0.68	0.77	0.72	0.65	0.62	0.72
11	0.40	0.13	0.13	0.28	0.28	0.34	0.18	0.18	0.22	0.26	****	0.84	0.84	0.76	0.84	0.84	0.68	0.84	0.86	0.84	0.80	0.76	0.88	0.71	0.76	0.84	0.94	0.72	0.57	0.80
12	0.11	0.05	0.05	0.05	0.05	0.18	0.00	0.00	0.25	0.26	0.18	****	1.00	0.95	1.00	1.00	0.71	1.00	0.84	1.00	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
13	0.11	0.05	0.05	0.05	0.05	0.18	0.00	0.00	0.25	0.26	0.18	0.00	****	0.95	1.00	1.00	0.71	1.00	0.84	1.00	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
14	0.19	0.12	0.12	0.12	0.12	0.28	0.05	0.05	0.35	0.39	0.28	0.05	0.05	****	0.95	0.95	0.60	0.95	0.76	0.95	0.83	0.89	0.89	0.76	0.89	0.95	0.83	0.84	0.63	0.94
15	0.11	0.05	0.05	0.05	0.05	0.18	0.00	0.00	0.25	0.26	0.18	0.00	0.00	0.05	****	1.00	0.71	1.00	0.84	1.00	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
16	0.11	0.05	0.05	0.05	0.05	0.18	0.00	0.00	0.25	0.26	0.18	0.00	0.00	0.05	0.00	****	0.71	1.00	0.84	1.00	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
17	0.24	0.52	0.52	0.29	0.29	0.17	0.35	0.35	0.24	0.60	0.39	0.35	0.35	0.52	0.35	0.35	****	0.71	0.85	0.71	0.79	0.75	0.60	0.51	0.60	0.71	0.63	0.57	0.51	0.47
18	0.11	0.05	0.05	0.05	0.05	0.18	0.00	0.00	0.25	0.26	0.18	0.00	0.00	0.05	0.00	0.00	0.35	****	0.84	1.00	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
19	0.22	0.28	0.28	0.28	0.28	0.15	0.18	0.18	0.07	0.48	0.15	0.18	0.18	0.28	0.18	0.18	0.17	0.18	****	0.84	0.80	0.76	0.76	0.71	0.76	0.84	0.80	0.72	0.43	0.67
20	0.11	0.05	0.05	0.05	0.05	0.18	0.00	0.00	0.25	0.26	0.18	0.00	0.00	0.05	0.00	0.00	0.35	0.00	0.18	****	0.89	0.95	0.95	0.84	0.95	1.00	0.89	0.90	0.72	0.89
21	0.29	0.19	0.19	0.19	0.19	0.22	0.11	0.11	0.29	0.14	0.22	0.11	0.11	0.19	0.11	0.11	0.24	0.11	0.22	0.11	****	0.94	0.83	0.67	0.83	0.89	0.75	0.78	0.67	0.75
22	0.19	0.12	0.12	0.12	0.12	0.28	0.05	0.05	0.35	0.20	0.28	0.05	0.05	0.12	0.05	0.05	0.29	0.05	0.28	0.05	0.06	****	0.89	0.76	0.89	0.95	0.83	0.84	0.76	0.83
23	0.19	0.00	0.00	0.12	0.12	0.28	0.05	0.05	0.35	0.20	0.13	0.05	0.05	0.12	0.05	0.05	0.52	0.05	0.28	0.05	0.19	0.12	****	0.88	0.89	0.95	0.94	0.84	0.76	0.94
24	0.22	0.13	0.13	0.28	0.28	0.34	0.18	0.18	0.40	0.48	0.34	0.18	0.18	0.28	0.18	0.18	0.68	0.18	0.34	0.18	0.40	0.28	0.13	****	0.88	0.84	0.80	0.84	0.71	0.80
25	0.19	0.12	0.12	0.12	0.12	0.28	0.05	0.05	0.35	0.39	0.28	0.05	0.05	0.12	0.05	0.05	0.52	0.05	0.28	0.05	0.19	0.12	0.12	0.13	****	0.95	0.83	0.95	0.76	0.83
26	0.11	0.05	0.05	0.05	0.05	0.18	0.00	0.00	0.25	0.26	0.18	0.00	0.00	0.05	0.00	0.00	0.35	0.00	0.18	0.00	0.11	0.05	0.05	0.18	0.05	****	0.89	0.90	0.72	0.89
27	0.29	0.06	0.06	0.19	0.19	0.40	0.11	0.11	0.29	0.33	0.07	0.11	0.11	0.19	0.11	0.11	0.46	0.11	0.22	0.11	0.29	0.19	0.06	0.22	0.19	0.11	****	0.78	0.67	0.88
28	0.25	0.17	0.17	0.17	0.17	0.33	0.11	0.11	0.40	0.44	0.33	0.11	0.11	0.17	0.11	0.11	0.57	0.11	0.33	0.11	0.25	0.17	0.17	0.18	0.05	0.11	0.25	****	0.84	0.78
29	0.40	0.28	0.28	0.28	0.28	0.56	0.33	0.33	0.91	0.48	0.56	0.33	0.33	0.46	0.33	0.33	0.68	0.33	0.85	0.33	0.40	0.28	0.28	0.34	0.28	0.33	0.40	0.18	****	0.67
30	0.29	0.06	0.06	0.19	0.19	0.40	0.11	0.11	0.47	0.33	0.22	0.11	0.11	0.06	0.11	0.11	0.75	0.11	0.40	0.11	0.29	0.19	0.06	0.22	0.19	0.11	0.13	0.25	0.40	****

Nei's genetic distance (below diagonal) and genetic identity (above diagonal)

Conclusion

Centella asiatica (L.) Urb. (gotu kola, hydrocotyle or Indian pennywort), belongs to the Apiaceae family, grows widely in shady and moist or marshy places. *Centella* has been used in traditional medical systems in Asia for over 100 years. DNA markers (molecular markers) are a potent tool to analysis the genetic diversity in various organisms based on the polymorphism found in the DNA sequence. The present study using microsatellite markers revealed very low levels of genetic variations among the studied *C. asiatica* accessions, which may be due to the propagation methods of the species or due to the effect of environmental factors, however further studies are required to verify this assumption.

Acknowledgements

The authors thank the Director, JNTBGRI for providing necessary facilities to carry out the study. MS thank the financial support by Department of Science and Technology-INSPIRE Fellowship (IF 150469).

References

Acquadro A, Portis E, Lee D, Donini P, Lanteri S (2005) Development and characterization of microsatellite markers in *Cynara cardunculus* L. *Genome* 48(2): 217–225. DOI: 10.1139/g04-111

Cavagnaro PF, Chung S-M, Manin S, Yildiz M, Ali A, Alessandro MS, et al. (2011) Microsatellite isolation and marker development in carrot-genomic distribution, linkage mapping, genetic diversity analysis and marker transferability across Apiaceae. *BMC Genomics* 12(1):386. DOI: 10.1186/1471-2164-12-386

Furlan E, Stoklosa J, Griffiths J, Gust N, Ellis R, Huggins RM, et al. (2012) Small population size and extremely low levels of genetic diversity in island populations of the platypus, *Ornithorhynchus anatinus*. *Ecol Evol*. 2(4):844–857. DOI: 10.1002/ece3.195

Garcia AAF, Benchimol LL, Barbosa AMM, Geraldi IO, Souza Jr CL, Souza AP de (2004) Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical

Olango TM, Tesfaye B, Pagnotta MA, Pè ME, Catellani M (2015) Development of SSR markers and genetic diversity analysis in enset (*Ensete ventricosum* (Welw.) Cheesman), an orphan food security crop from Southern Ethiopia. *BMC Genet* **16**(1): 98. DOI: 10.1186/s12863-015-0250-8

Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**(4):357–358.

Pittella F, Dutra RC, Junior DD, Lopes MTP, Barbosa NR (2009) Antioxidant and cytotoxic activities of *Centella asiatica* (L). Urb *Int J Mol Sci* **10**(9):3713–3721. DOI: 10.3390/ijms10093713

Piya S, Nepal MP, Butler JL, Larson GE, Neupane A (2014) Genetic diversity and population structure of sickleweed (*Falcaria vulgaris*; Apiaceae) in the upper Midwest USA. *Biol Invasions* **16**(10): 2115–2125. DOI: 10.1007/s10530-014-0651-z

Powell W, Morgante M, Andre C, McNicol JW, Machray GC, Doyle JJ, et al. (1995) Hypervariable microsatellites provide a general source of polymorphic DNA markers for the chloroplast genome. *Curr Biol* **5**(9):1023–1029. DOI: 10.1016/S0960-9822(95)00206-5

Rodier-Goud M, Rivallan R, Lussert A, Danthu P, de Lamotte F, Ralambofetra E, et al. (2013) Insight into the biology, genetics and evolution of the *Centella asiatica* polyploid complex in Madagascar. *Ind Crops Prod* **47**: 118–125. DOI: 10.1016/j.indcrop.2013.02.022

Sajib AM, Hossain M, Mosnaz A, Hossain H, Islam M, Ali M, et al. (2012) SSR marker-based molecular characterization and genetic diversity analysis of aromatic landreces of rice maize inbred lines. *Genet Mol Biol.* 27(4):579–588. DOI: 10.1590/S1415-47572004000400019

Hamid AA, Shah ZM, Muse R, Mohamed S (2002) Characterisation of antioxidative activities of various extracts of *Centella asiatica* (L) Urban. *Food Chem* **77**(4):465–469. DOI: 10.1016/S0308-8146(01)00384-3

Hasan M, Seyis F, Badani AG, Pons-Kühnemann J, Friedt W, Lühs W, et al. (2006) Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. *Genet Resour Crop Evol.* **53**(4): 793–802. DOI: 10.1007/s10722-004-5541-2

Hashim P (2011) *Centella asiatica* in food and beverage applications and its potential antioxidant and neuroprotective effect. *Int Food Res J.* **18**(4).

Hoshino AA, Bravo JP, Nobile PM, Morelli KA (2012) Microsatellites as tools for genetic diversity analysis. In: *Genetic diversity in microorganisms*. InTech. DOI: DOI: 10.5772/35363

Kimura Y, Sumiyoshi M, Samukawa K, Satake N, Sakanaka M (2008) Facilitating action of asiaticoside at low doses on burn wound repair and its mechanism. *Eur J Pharmacol* **584**(2–3): 415–23. DOI: 10.1016/j.ejphar.2008.02.036

Litt M, Luty JA (1989) A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am J Hum Genet*. **44**(3): 397.

Liu K, Muse S V (2005) PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics* **21**(9): 2128–2129. DOI: 10.1093/bioinformatics/bti282

Matin S, Ashrafuzzaman M, Islam MM, Sikdar SU, Zobayer N (2012) Molecular marker based (SSR) genetic diversity analysis in deep water rice germplasms of Bangladesh. *Int J Biosci* **2**(10): 64–72.

Miah G, Rafii MY, Ismail MR, Puteh AB, Rahim HA, Islam KN, et al. (2013) A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance. *Int J Mol Sci.* **14**(11): 22499–22528. DOI: 10.3390/ijms141122499

Nei M (1972) Genetic distance between populations. *Am Nat* **106**(949): 283–292. DOI: 10.1086/282771

(*Oryza sativa* L.). *J Biosci Biotechnol.* 1(2).

Semagn K, Bjørnstad Å, Ndjiondjop MN (2006) An overview of molecular marker methods for plants. *African J Biotechnol.* 5(25).

Singh RH, Narsimhamurthy K, Singh G (2008) Neuronutrient impact of Ayurvedic Rasayana therapy in brain aging. *Biogerontology* **9**(6): 369–374. DOI: 10.1007/s10522-008-9185-z

Stafne ET, Clark JR, Weber CA, Graham J, Lewers KS (2005) Simple sequence repeat (SSR) markers for genetic mapping of raspberry and blackberry. *J Am Soc Hortic Sci* **130**(5): 722–728.

Subathra M, Shila S, Devi MA, Panneerselvam C (2005) Emerging role of *Centella asiatica* in improving age-related neurological antioxidant status. *Exp Gerontol* **40**(8–9): 707–715. DOI: 10.1016/j.exger.2005.06.001

Sun Q-B, Li L-F, Li Y, Wu G-J, Ge X-J (2012) SSR and AFLP markers reveal low genetic diversity in the biofuel plant *Jatropha curcas* in China. *Crop Sci* **48**(5):1865–1871. DOI: 10.2135/cropsci2008.02.0074

Tan C, Wu Y, Taliaferro CM, Bell GE, Martin DL, Smith MW .

(1014) Development and characterization of genomic SSR markers in *Cynodon transvaalensis* Burtt-Davy. *Mol Genet genomics* **289**(4): 523–531. DOI: 10.1007/s00438-014-0829-1

Thomas J, Sabulal B, Dan M, Sabu KK (2016) Development and validation of novel EST-SSR markers in *Centella asiatica. Proc of 26th Swadeshi Science Congress*, Kochi.

Ullah MO, Sultana S, Haque A, Tasmin S (2009) Antimicrobial, cytotoxic and antioxidant activity of *Centella asiatica. Eur J Sci Res* 30(2): 260–264.

Wang ML, Barkley NA, Jenkins TM (2009) Main content area Microsatellite Markers in Plants and Insects. *Part I: Applications of Biotechnology. Genes, genomes and genomics* **3**(1): 54–67.

Wangari NP, Gacheri KM, Theophilus MM, Lucas N (2013) Use of SSR markers for genetic diversity studies in mulberry accessions grown in Kenya. *Int J Biotechnol Mol Biol Res* **4**(3):38–44. DOI: 10.5897/IJBMBR11.057

Yeh FC, Yang R, Boyle T (1999) Microsoft Windows-based free ware for population genetic analysis (Release 1.31). Univ Alberta, Edmont