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Molecular characterization and DNA fingerprinting of *Centella asiatica* using SSR markers

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Abstract
Centella asiatica (L.) Urban is an important medicinal herb used both in the traditional and modern system of medicine. Owing to its growing demand, consumption by Indian herbal industry is increasing and has got great export demand. Being a clonally propagated species, it is very difficult to differentiate the genotypes morphologically and to identify the superior cultivars. Thus, the present study was carried out to genetically characterize and develop DNA fingerprint of seventeen genotypes using SSR markers. Results showed that selected SSR markers could distinguish the collections and provide clonal identity. Both, normal and fluorescence based PCR technique was employed. The total number of alleles detected ranged from 1 to 8 with an average of 3.100 alleles per locus. The polymorphism percentage ranged from 85.71 to 100 with an average of 92.96% polymorphism across all the 17 accessions. The mean expected heterozygosity obtained was 0.456 and average PIC value of the marker obtained was 0.390. This study enabled to develop DNA barcode of the studied genotypes which would further help in establishing clonal identity, avoiding duplicate accessions, maintaining purity of elite lines and assuring quality of raw drug.

Keywords: Centella asiatica, DNA fingerprint, SSR marker, polymorphism, heterozygosity

Introduction
Centella asiatica (L.) Urban belonging to the family Apiaceae is an important medicinal herb used both in the traditional and modern system of medicine. It is known for its memory boosting ability and is being used from time immemorial as a memory or nerve tonic for children. The herb is perennial, prostrate and stoloniferous in habit, growing commonly in the damp areas in different tropical countries. The plant is known by different names in different regions of the country *viz*., Mandukaparni, Indian pennywort, Gotu kola, Ondelega, Kudangal etc. It is extensively used for enhancing memory, longevity, in the treatment of leprosy, tuberculosis and skin diseases, healing of skin wounds and burns. It is also used as an antidote to cholera and as an external application in rheumatism and elephantiasis. The main active principles of *C. asiatica* are Asiaticoside, Madecassoside, Asiatic acid, Madecassic acid, Asiaticoside B and Terminolic acid. *C. asiatica* grows widely and plant is massively collected from wild for local use and export. Consumption by Indian herbal industry is about 980 MT and by rural households is 638 MT (Ved and Goraya, 2007) [14]. It has great export potential and is one of the top eight best selling herbs in USA and Europe.

Under the WTO (World Trade Organization) regime, medicinal plants exported should have precise passport data in the way of their authentic characterization that is supported by DNA fingerprinting. The use of DNA fingerprinting for germplasm characterization is also useful in establishment of sovereignty rights over indigenous plant genetic resources for ensuring fair benefit sharing that is called for under the Benefit Sharing Regime of the Convention of Biological Diversity (CBD), to which India is a signatory. In view of the future crop improvement programmes, for avoiding ownership conflicts and in the present era of farmers and plant breeders rights etc. there is a need to unambiguously identify improved genotypes using DNA Fingerprinting. Moreover, there has been a growing threat to the existence of this herb under the natural condition due to its unscrupulous harvest to meet the export demand. This situation has urged the drug industry to go for adulteration and substitution of this plant which is always at the cost of quality of the herbal drug. Thus, developing DNA Fingerprinting will assure the quality of the herbal produce without doubt.

SSR markers are useful for a variety of applications in plant genetics and breeding because of their reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and good genome coverage. These markers are popular tools in genetics and breeding and considered the most reliable markers for fingerprinting owing to their relative abundance compared to other molecular marker types, high degree of polymorphism (number of variants), and easy assaying by PCR (Zhu *et al*., 2012) [16]. Thus, the study was carried out to test the functionality of SSR markers derived from enriched genomic library for DNA fingerprinting of *Centella asiatica* genotypes collected from different agroecological zones of India

Materials and Methods

The study was carried out at the Division of Floriculture and Medicinal crops, ICAR-IIHR, Bengaluru, Karnataka during the year 2016-2017.

Plant Material

Seventeen genotypes of *Centella asiatica* collected from different regions of India were used for the study (Table1). The genotypes included Vallabh Medha variety released for commercial cultivation and also two varieties Arka Prabhavi and Arka Divya identified for release at ICAR-IIHR.

Table 1: List of seventeen genotypes used in the experimental study

IIHR accession number	Place of collection	Latitude N	Longitude E	Altitude (m above msl)
IIHR CA-1(Arka Divya)	Maharashtra	19.45	75.42	491
IIHR CA-2	Bangalore	12.52	77.35	914
IIHR CA-3(VallabhMedha)	DMAPR(Gujarat)	22.5	73.0	39
IIHR CA-4	Maharashtra	19.45	75.42	491
IIHR CA-5	Mangalore	12.55	74.51	48
IIHR CA-6	Jabalpur	23.1	79.59	421
IIHR CA-7	Jabalpur	23.1	79.59	421
IIHR CA-8	Meghalaya	25.29	91.21	951
IIHR CA-9	Khanapur	15.38	74.31	652
IIHR CA-10	Khanapur	15.38	74.31	652
IIHR CA-11	Honnavar	14.16	74.26	14
IIHR CA-12	Gonikoppal	12.11	75.55	850
IIHR CA-13(Arka Prabhavi)	Shivamoga	13.55	75.34	591
IIHR CA-14	Induced polyploid	12.52	77.35	914
IIHR CA-15	Induced polyploid	12.52	77.35	914
IIHR CA-16	Coorg	12.33	75.80	1,150
IIHR CA-17	Bangalore	12.52	77.35	914

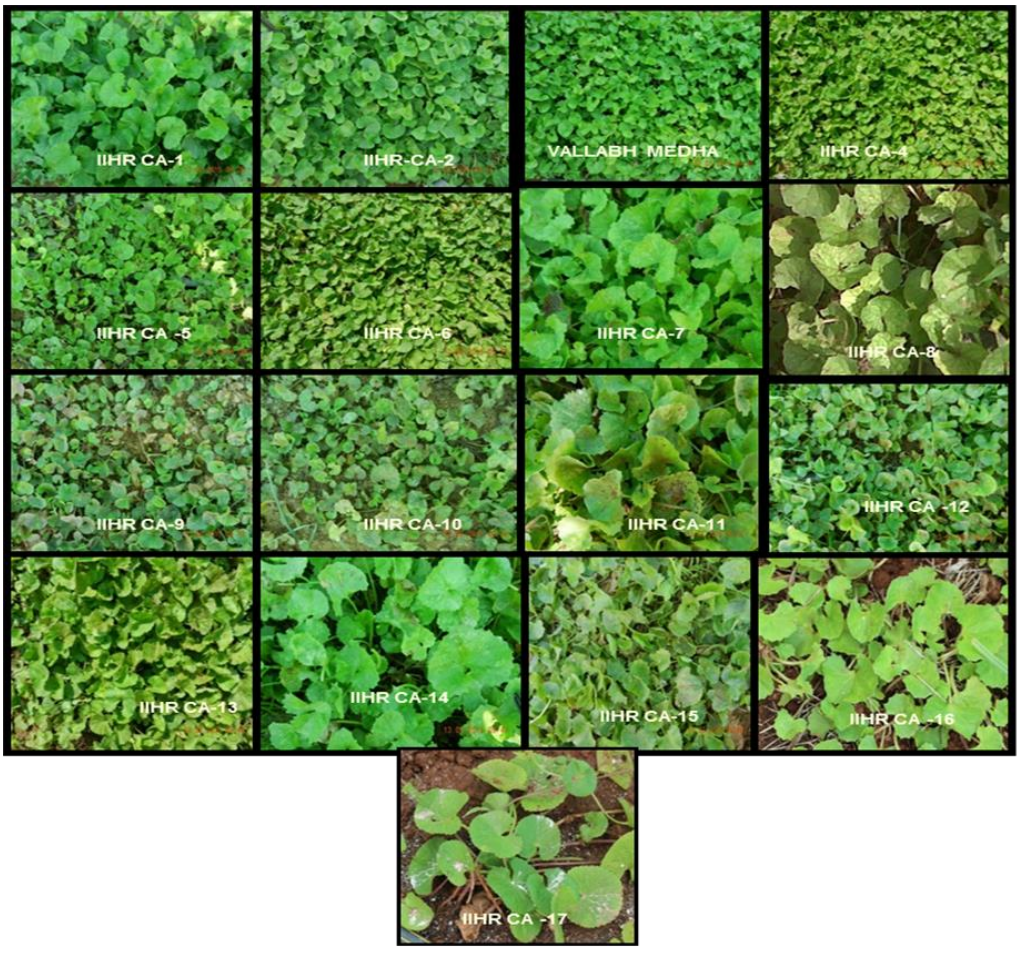

Plate 1: *C. asiatica* genotypes used in the study (Courtesy: Dr. D. H. Sukanya)

DNA Isolation

DNA was isolated from the young leaves using modified CTAB (Cetyltrimethyl ammonium bromide) protocol (Doyle and Doyle, 1990) [2]. A total of 1.0g leaf material of young leaf from the genotype was used for DNA extraction. DNA concentrations were estimated by mass spectrophotometry at 260 nm and working dilutions of concentration 30 ng/ µl were prepared for the study. Quality of genomic DNA was determined through electrophoresis on 0.8% agarose gel.

Selection of SSRs and primer design

Twenty SSR primers derived from the GA/GT-enriched genomic library of *Centella asiatica* accession from Madagascar was used for the study (Rakotondralambo *et al*., 2012) [8] (Table 2). Two methodologies were followed for molecular characterization and DNA fingerprinting of the genotypes. First method used the normal PCR and gel electrophoresis with the SSR markers. The second method used Fluorescence-based PCR (Schuelke, 2000) [13] using M13 tailed primers. The primer sets were M13 tailed by adding a sequence, that is, 'GTAAAACGACGGCCAGT' (17mer) at their 5'end for all the forward primers and a pig tail of 'GTTTCTT' sequence at the 5' end of all the reverse primers. Additionally, two M13 sequences (17mers) were labelled with two different dyes namely FAM and HEX at their 5' end for using as probes. (Schuelke, 2000) [13].

Table 2: Details of 20 SSR primers used in the study

Sl. No	Primer Name	Primer sequence (5'- 3')	*T* a (° C)(Annealing temp)
1	mCaCIR002	F:GTAAAACGACGGCCAGT CCACAGGTAACACCGAAT R:GTTTCTTGCACTTGCACTATCTGGAA	55 ºC
2	mCaCIR004	F:GTAAAACGACGGCCAGTGGGTGGTCTGCCTAAAGA R:GTTTCTTTGGAGATCAAGTTTCATGC	59 ºC
3	mCaCIR005	F:GTAAAACGACGGCCAGT GGCCTTCAATGTATGCTG R:GTTTCTTTTTGATTTGTTGGGTCTTG	55 ºC
4	mCaCIR006	F:GTAAAACGACGGCCAGT ACGGGCATTTATTCCATT R:GTTTCTT GCAAACCACCACAACTTC	59 ºC
5	mCaCIR007	F:GTAAAACGACGGCCAGT TGGAGGTGGTGTAACTGG R:GTTTCTT AGGGGATCAAACCTCATC	55 ºC
6	mCaCIR009	F:GTAAAACGACGGCCAGT TGCCTATCCTTTGAATGC R:GTTTCTTCAAACATGACATTCTTAAAACA	55 ºC
7	mCaCIR010	F:GTAAAACGACGGCCAGT AATGTAAAATTCCCGGTGT R:GTTTCTTTAAACAGGCGTTCCAAGT	55 ºC
8	mCaCIR011	F:GTAAAACGACGGCCAGT TTCATAAAAGTCCTTCCACA R:GTTTCTTTAGGTTGATGTGGCCTCT	55 ºC
9	mCaCIR012	F:GTAAAACGACGGCCAGT CACGAAAATTGGAAACAA R:GTTTCTT CATGTGAGTTTATGAGTTTCTATG	55 ºC
10	mCaCIR013	F:GTAAAACGACGGCCAGT CAAGTTCCTCCCACGAAT R:GTTTCTTGCCGAAATAATCGAAATATAAG	55 ºC
11	mCaCIR018	F:GTAAAACGACGGCCAGTTTGAGTTTAAGAAGTCCCAAAT R:GTTTCTTAATCCTTCACACTCCTAAAGC	55 ºC
12	mCaCIR019	F:GTAAAACGACGGCCAGT TTTCTTGTTAAATGCGATGA R:GTTTCTT AATGACATCACTGCTATGGA	55 ºC
13	mCaCIR020	F:GTAAAACGACGGCCAGT TTTAGGAAGTTGGATTTTGC R:GTTTCTTGGTTTAATTCAGGACGCTTA	53 ºC
14	mCaCIR021	F:GTAAAACGACGGCCAGTTGCCTAGATTTTGGGTTTT R:GTTTCTTTCTTACAATGCAATCAACCT	55 ºC
15	mCaCIR022	F:GTAAAACGACGGCCAGT AGGAGTATTGACAAGAGGTGA R:GTTTCTTGGATGGCAGTCCATTTTA	55 ºC
16	mCaCIR024	F:GTAAAACGACGGCCAGT TCTTTCGTTGATACATGCAC R:GTTTCTTAAAACTTAAAGAAGATACAAACTCC	55 ºC
17	mCaCIR027	F:GTAAAACGACGGCCAGT ACCCCAAGACCTTCAGTT R:GTTTCTTCCTTCTGCTTTCCCTTTT	55 ºC
18	mCaCIR028	F:GTAAAACGACGGCCAGT CAGAGTTTGGGCAGAAAA R:GTTTCTTGACGAGTGGAGGATAAGAAA	55 ºC
19	mCaCIR029	F:GTAAAACGACGGCCAGT GGTCTGAGGTCTGTTGAGG R:GTTTCTTCGCATTGACAGAACAAAA	55 ºC
20	mCaCIR030	F:GTAAAACGACGGCCAGT GGCAAATCGAGAGCAATA R:GTTTCTT ACGGAAAAGCCTAACAGC	55 ºC

PCR and Polymorphism Analysis

PCR amplification was performed in a 20µl volume containing 50 ng DNA, Taq Buffer10X (Tris with 15 mM $MgCl_2$, pH 9) , 10 mM dNTPs, 0.2 p mol μl^{-1}forward primer, 0.8 p mol μl^{-1} reverse primer, and 1 U μl^{-1}unit of Taq DNA polymerase (Bangalore Genei, India). PCR was performed in Corbett Palm Cycler with the following temperature profile: 94 °C for 1 min followed by 30 cycles of annealing at 94 °C for 1 min, optimal annealing temperature of for 1 min, and 72 °C for 2 min. A final extension reaction was performed at 72 °C for 10 min. The resultant amplified products were run on 3% agarose gel for confirmation of the amplification and scoring.

In Fluorescence-based PCR method 5 µM probe was also added along with other components as mentioned above in normal PCR. Two different probes (FAM and HEX) were used alternatively with the markers for all the genotypes. In order to reduce the time and cost of genotyping, the PCR products were mixed by combining two PCR products, labelled with different probes (FAM and HEX) into a single sample. These samples were separated on the automatic 96 capillary-automated DNA Sequencer.

Data Analysis

Amplification profiles of 20 primers in 17 genotypes of *Centella asiatica* were scored for presence (1) or absence (0) manually and the binary data are converted to binary matrix for analysis using Numerical Taxonomy and Multivariate Analysis System (NTSYS pc, ver. 2.02). A pairwise genetic similarity matrix between accessions was estimated using Jaccard's similarity coefficient (Jaccard, 1908) [5]. Dendrogram were constructed using UPGMA of NTSYS-pc version 2.10e (Rohlf, 2000) [11]. Principal component analysis (PCA) was also carried out following the NTSYS pc software package (Rohlf, 1993) [10]. PCA was used for identification of number of groups based on eigenvectors. This analysis was carried out by calculating the eigenvectors and eigen values from Eigen programme in the NTSYS software, resulted in a two and three dimensional plot.

The raw data generated using Fluorescence-based PCR were analysed and compiled using Peak Scanner V1.0 software (Applied Biosystems) for detecting the alleles. The Peak Scanner software produces a size curve based on the known size standard fragments, with the help of which the unknown fragment sizes are determined. The results obtained were used for genetic analysis using Cervus 3.0 software (Kalinowski *et al*., 2007) [6]. for determining the number of alleles, observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC) and Probability of Identity value (PI).

Result and Discussion

PCR amplification of the genomic DNA with 20 SSR markers yielded a total of 60 alleles, of which 50 were polymorphic (Table 3).The number of alleles detected ranged from 1 to 8, with an average of 3.0 alleles per primer pair. The maximum number of polymorphic alleles obtained was 8. The polymorphism percentage ranged from 50 to 100% with an average of 72.91% polymorphism across all the 17 accessions. This shows that there is a moderate amount of genetic variation between the accessions.

Table 3. Details of amplified bands generated based on 20 SSR primers in 17 accessions of *C. asiatica*

Sl. No	Primer	No: of alleles detected	No: of polymorphic alleles	Polymorphism %
1	mCaCIR002	3	2	66.67
2	mCaCIR004	4	3	75
3	mCaCIR005	1	0	0
4	mCaCIR006	2	1	50
5	mCaCIR007	4	2	50
6	mCaCIR009	3	2	66.67
7	mCaCIR010	1	0	0
8	mCaCIR011	3	3	100
9	mCaCIR012	4	4	100
10	mCaCIR013	1	0	0
11	mCaCIR018	2	2	100
12	mCaCIR019	2	1	50
13	mCaCIR020	5	5	100
14	mCaCIR021	3	3	100
15	mCaCIR022	2	2	100
16	mCaCIR024	4	4	100
17	mCaCIR027	1	1	100
18	mCaCIR028	3	3	100
19	mCaCIR029	4	4	100
20	mCaCIR030	8	8	100
Total		60	50	
Mean		3.0	2.5	72.91

Among the seventeen genotypes of *C. asiatica*, genetic similarity coefficient ranged from 0.78 to 0.92 with an average of 0.85 indicating the presence of relatively low amount of genetic diversity among the genotypes. Low level of genetic variation between *C. asiatica* genotypes was obtained by the study conducted by Sakthipriya (2018) [12] where out of the 10 SSR primers, only 2 primers were polymorphic. In a highly cross pollinated clonally propagated species, low genetic diversity may be due to the effect of inbreeding, genetic drift, or restricted gene flow and small population size (Furlan *et al*., 2012) [3]. Dendrogram based on UPGMA method from SSR analysis is illustrated in Figure 2. At 0.85 similarity coefficient, genotypes were grouped into 6 clusters. The cluster analysis revealed that IHR CA-5, IHRCA-6, IIHRCA-17, IIHRCA-7 and IIHRCA-2 were distinct from rest of the genotypes. Among this, IHRCA-5 and IHRCA-6 were the most distinct. IIHRCA-1 which is an identified variety for release was found to have maximum similarity with IIHRCA-9. IIHR CA-12 and IIHR CA-13 both collected from Karnataka were also genetically similar to the above genotypes. Released variety, Vallabh Medha (IIHRCA-3) clustered with IIHRCA-8 collected from Meghalaya. Three dimensional plot generated by PCA based on eigen vectors also supported the UPGMA grouping of the genotypes (Figure 2). 3-D plot clearly shows the accessions IIHRCA-5, IIHRCA-6, IIHRCA-17, IIHRCA-7 and IIHRCA-2 standing out distinctly from the rest of the accessions (Figure 1). Though there was an evident difference between the accessions morphologically, selected SSR markers could genetically distinguish the collections and provide clonal identity, which is very important in *Centella.* Irrespective of the morphological variations like large or small sized leaves, there is a general tendency that accessions from same geographical location are similar. Similar results were obtained by Rakotondralambo *et al*., (2013) [9] in their studies on genetic diversity in *C. asiatica* accessions collected from Indian and South African continent. Cluster analysis indicated three separated groups, clearly distinct from Indian and South African genotypes. Since there is only low level of genetic diversity observed between the genotypes, the chemical characterization is important to be coupled with molecular characterization for identifying superior genotypes with high

active ingredient content. However the study carried out by Prasad *et al*., (2014) [7] shows that the extent of genetic diversity studied in four germplasm accessions was not correlated with their chemical diversity in terms of centellosides content and yield.

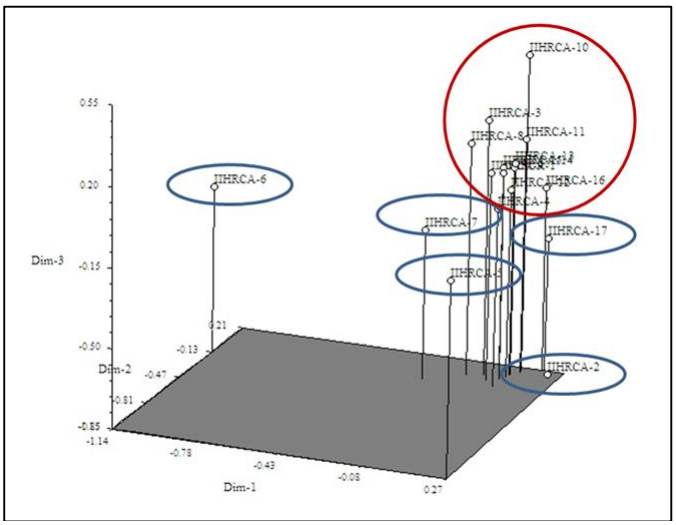

Fig 1: 3-D plot derived based on PCA analysis of 17 accessions of *C. asiatica* using SSR markers

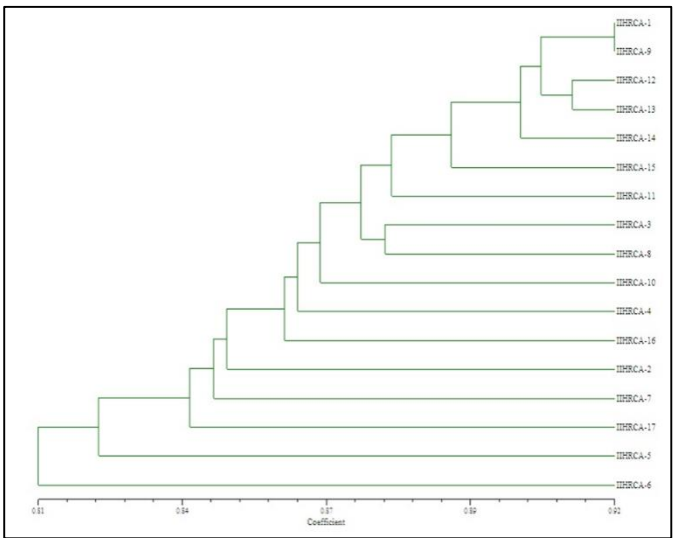

Fig 2: UPGMA Dendrogram of 17 *C. asiatica* accessions generated based on SSR data

Out of the 20 SSR markers used, 10 SSR's which were 100% polymorphic were used for DNA fingerprinting. DNA fingerprinting of the genotypes using Fluorescence-based PCR detected the diversity parameters using peak scanner and Cervus software.The total number of alleles detected ranged from 1 to 8 with an average of 3.100 alleles per locus. Results obtained from the Cervus analysis of the genetic data is depicted in Table 4. The mean expected heterozygosity obtained is 0.4567. Similar results were obtained by Rakotondralambo *et al*., (2012) [8] when 17 *C. asiatica* genotypes were tested using 20 SSR markers out of which seventeen markers were polymorphic and the remaining three were monomorphic. A total of 73 alleles were produced at the 17 polymorphic loci, with an average of 4.3 alleles per locus. Our results showed that average PIC value obtained is 0.3905. The primer mCaCIR030 was the most efficient in detecting maximum number of alleles with a PIC value of 0.791. Experiment showed that similar results were obtained irrespective of the different methods of PCR used. Efficiency of SSR markers in establishing distinctness in clonally propagated grapevine cultivars was reported by Ibanez *et al*., (2009) [4]. Probability of identity values for individual markers indicated the efficiency of each marker in individual genotype identification. The lower the PID value, higher will be its ability to discriminate individuals. Thus, based on the results, 8 primers with PID value less than 1.00 could be reliably used for DNA Fingerprinting of the genotypes. Primer, mCaCIR030 with the lowest PID value of 0.0582 is the most efficient in genotyping. For purposes of individual identification, the least amount of the most polymorphic and heterozygous microsatellite loci that give the lowest probability of identity (PID) value must be identified (Waits *et al*., 2001)[15]. However, as more markers are added, more genotyping errors are inadvertently introduced in the study (Dewoody *et al*., 2006) [1]. Therefore, utmost care must be observed in detecting and minimizing genotyping errors. DNA barcode developed using Cervus software for the 17 genotypes of *C. asiatica* with 10 primers is illustrated in the figure 3.

Table 4: Genetic diversity parameters analysed for different loci in *C. asiatica* accessions using Cervus software

Sl. No.	Locus	HObs	HExp	PIC	PI value(Probability of identity)
1	mCaCIR011	0.250	0.433	0.371	0.3882
2	mCaCIR012	0.000	0.615	0.501	0.2536
3	mCaCIR018	0.000	0.000	0.000	1.0000
4	mCaCIR020	0.667	0.727	0.593	0.1852
5	mCaCIR021	1.000	0.645	0.555	0.2109
6	mCaCIR022	0.143	0.143	0.124	0.7611
7	mCaCIR024	0.333	0.660	0.557	0.2086
8	mCaCIR027	0.000	0.000	0.000	1.0000
9	mCaCIR028	0.688	0.502	0.414	0.3360
10	mCaCIR030	1.000	0.841	0.791	0.0582
	Mean		0.4567	0.3909	

Fig 3: DNA barcode of 17 genotypes developed using SSR markers

Variety	1A	6B	8A	8B	9A	9B	10A	10B	1B	2A	2B	4A	4B	5A	5B	6A
IIHR-CA-1	|		|	|	|	|	|	|	|	|	|	|	|			
IIHR-CA-2	|		|	|	|	|	|	|	|	|	|	|	|			
IIHR-CA-3	|	|	|	|	|	|	|	|	|					|	|	|
IIHR-CA-4	|		|	|	|	|	|	|	|			|	|	|	|	
IIHR-CA-5	|		|	|	|	|	|	|	|	|				|	|	
IIHR-CA-6	|		|	|	|	|	|	|	|			|	|			
IIHR-CA-7		|	|	|	|	|	|	|	|					|	|	|
IIHR-CA-8	|	|	|	|	|	|	|	|	|	|	|			|	|	|
IIHR-CA-9		|	|	|	|	|	|	|	|					|	|	|
IIHR-CA-10	|		|	|	|	|	|	|	|	|	|						
IIHR-CA-11			|	|	|	|	|	|	|	|	|	|	|			
IIHR-CA-12					|				|					|			
IIHR-CA-13		|	|	|		|	|	|				|	|		|	|
IIHR-CA-14			|	|	|	|	|	|	|	|							
IIHR-CA-15			|	|	|	|	|	|			|	|	|	|		
IIHR-CA-16		|	|	|	|	|	|	|	|					|	|	|
IIHR-CA-17	|	|	|	|	|	|	|	|					|	|	|	|

Conclusion

Characterization of germplasm is very important in highly demanded medicinal crop such as *C. asiatica* in view of future crop improvement programmes. Analysis of the amount and extent of genetic diversity present will aid in the selection of suitable genotypes for cultivation. DNA fingerprinting and development of DNA barcode of the accessions will unambiguously identify improved genotypes

which will further help in avoiding ownership conflicts in the present era of farmers and plant breeders rights. The finding will also help in assuring the quality and purity of the herbal produce.

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