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Genetic Diversity in *Centella asiatica* (L.) Urb., a Memory-Enhancing Neutraceutical Herb, using RAPD Markers

K. Padmalatha • M. N. V. Prasad*

Department of Plant Sciences, University of Hyderabad, Hyderabad, Andhra Pradesh, 500 046, India

Corresponding author: * **mnvsl@uohyd.ernet.in**

ABSTRACT

The present study is the first report of genetic diversity analysis of *Centella asiatica* (L.) Urb, a medicinal and aromatic plant collected from various locations of Andhra Pradesh, India. Though there are several reports on diversity analysis of *C. asiatica* using RAPD analysis the data presented in this article is the first report on diversity studied in accessions collected from Andhra Pradesh State. It addresses the determination of genetic variations using a few visual morphological parameters and by RAPD markers. We collected sixteen accessions which demonstrated clear morphological variations and used nine to test for molecular variation. Wide phenotypic variations were observed. Molecular analyses revealed that out of the 30 primers screened, the 16 that were selected for data analysis generated a total of 137 scorable polymorphic markers out of 156 total number of markers. An 87% polymorphism was observed. Cluster analysis based on Dice's coefficient showed two major groups indicating that in cross-pollinated plants, high levels of differentiation among accessions exists. The grouping of these accessions was independent of the geographical distance. Hence the results of the present study can be seen as a starting point for future research on the population and evolutionary genetics of this species and understanding such variation would facilitate their use in various conservation management practices, and for generating an elite variety from which the production of secondary metabolites can be enhanced.

Keywords: Asiatic pennyworth, conservation, genetic distance, morphological variation, Random Amplified Polymorphic DNA

INTRODUCTION

Centella asiatica (L.) Urb. (Apiaceae), commonly known as "Asiatic/Indian pennywort" or "Mandookaparni", is a medicinal, aromatic, and neutracuetical, stoloniferous perennial herb, native to parts of India, China, Indonesia, Sri Lanka, the Western South Sea Islands, Australia, Madagascar and Southern middle Africa (Bonati 1980). In Andhra Pradesh (AP) it is distributed in many districts in the temperate to tropical regions on moist, sandy or clayey-soils, forming a dense green carpet (Anonymous, 1992) at an elevation of approximately 700 m in India and Ceylon (Ohwi, 1965). It can be propagated by seeds and stem cuttings.

In the traditional system of Indian medicine, *C. asiatica* is used for the treatment of asthma, elephantiasis, peptic ulcers, gastric catarrh, kidney troubles, leprosy, tuberculosis, skin diseases, urethritis and is also antibacterial, antifeedant, antistress, wound healing, etc. (Chakraborty *et al*. 1996). It contains essential oils, triterpenoids and primary pharmacologically active constituents, i.e., saponins and three triterpenic acids and other compounds. Plants are reported to contain glycosides like indocentelloside, brahmoside, brahminoside, asiaticoside, theankuniside and isothankuniside. Leaves are rich in carotenoids, and vitamins B and C. It is one of the components of the drug "Geriforte" used for senile pruritis (Anonymous 1992). In 1990, the estimated annual requirement of *C. asiatica* was 12,700 tonnes of dry biomass valued at Rs. 1.5 billion (Ahmed 1993), has risen sharply in view of the popularity of the commercial drugs in the market. In Malaysia the value of herbal market is estimated to be at US$3.8 billion and is growing at 10 to 20% yearly. High-frequency somatic embryogenesis and plant regeneration was achieved on callus derived from leaf (petiole and lamina) and internode explants of *C. asiatica*. Calluses developed on MS medium fortified with 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 5.37 μM α-naphthaleneacetic acid (NAA), both with 2.32 μM kinetin (KN), were superior for somatic embryogenesis. Embryo-derived plantlets established in field conditions displayed morphological characters identical to those of the parent plant (Martin 2004).

Due to the unrestricted exploitation by the pharmaceutical industry and limited cultivation, it is listed as threatened species by the International Union of Conservation of Nature and Natural Resources (IUCN) (Pandey *et al*. 1993) and an endangered species (Sharma and Kumar 1998). It is reported that there are some genotypic differences in glycoside content and medicinal properties (Upadhyay *et al*. 1991). The localization was determined for the triterpenoids, asiaticoside and madecassoside, in different organs of glasshouse-grown plants and cultured material, including transformed roots, of two phenotypes of *C. asiatica*. Differences were observed in terpenoid content that were tissue specific and varied between glasshouse-grown plants and tissue culture-derived material when analysed through HPLC. However, asiaticoside and madecassoside were undetectable in transformed roots and undifferentiated callus (Aziz *et al*. 2007). The assessment of genetic variability in natural populations can provide new insights into the evolutionary history and phylogenetic relationships of *C. asiatica*, and can address the cultivation practices (Vaughan 1989). In addition, the development of appropriate strategies for conservation and exploitation of plant genetic resources requires a detailed knowledge of the amount and distribution of genetic variation within the species (Vaughan 1994). RAPD analysis is a popular method for estimating genetic diversity and relatedness in plant populations, cultivars and germplasm accessions (Huang *et al*. 2008; Ranade *et al*. 2008).

Ex situ conservation and cultivation of *C. asiatica* was initiated in 2000 at the University of Hyderabad campus and are being maintained in the field gene bank and seed bank (Prasad *et al*. 2007). Much phenotypic variation was

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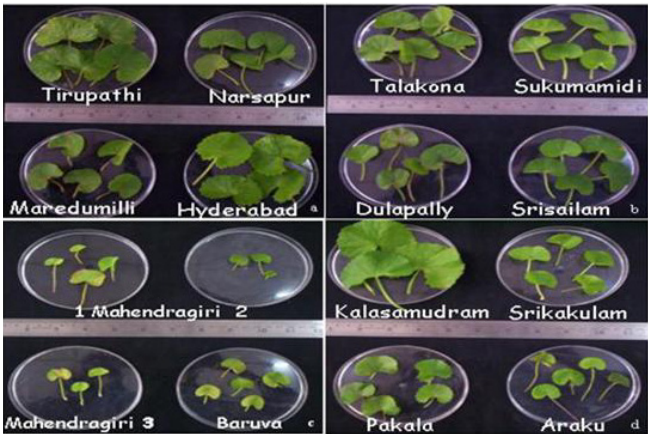

Fig. 1 Morphological variations in leaf characteristics of *Centella asiatica*. a) Tirupathi, Narsapur, Maredumilli, Hyderabad; b) Talakona, Sukumamidi, Dulapally, Srisailam; c) Mahendragiri 1, 2 and 3, Baruva; d) Kalasamudram, Srikakulam, Pakala, Araku.

Table 1 Distribution and physiological parameters of *C. asiatica* in various locations in India.

Accession №	Location	State/District	Latitude	Longitude
CAHC	Tarnaka*#	Hyderabad	27°20′N	78°20′E
CANA	Narsapur*#	Medak	18° 0′ N	78°14′E
CAPA	Pakala*#	Warangal	17°55′N	79°50′E
CASM	Srisailam*#	Kurnool	$16^0$05′N	78°52′E
CAM1	Mahendragiri1*#	Srikakulam	19°20′N	83°40′E
CAM2	Mahendragiri2*#	Srikakulam	19°05′N	84°15′E
CAM3	Mahendragiri3*#	Srikakulam	19°08′N	83°53′E
CABA	Baruva*#	Srikakulam	19°0′N	83°20′E
CAKA	Kalasamudram#	Ananthapur	15°05′N	78°15′E
CAAU	Araku#	Vishakapatnam	19°08′N	82°30′E
CASA	Srikakulam#	Srikakulam	19°04′N	83°18′E
CADY	Dulapally#	Medak	27°20′N	78°20′E
CASI	Sukumamidi#	Khammam	17°45′N	81°50′E
CAMI	Maredumilli#	East Godavari	19°35′N	80°48′E
CATA	Talakona#	Chittoor	13°30′N	79°08′E
CATI	Tirupathi#	Chittoor	13°10′N	77°20′E

* Accessions used for RAPD analysis.
\# Accessions used for morphological studies.

visually observed (**Fig. 1**). There has been no report to date on the diversity analysis of this species. Hence the objective of the present study was to assess the genetic diversity among the accessions of *C. asiatica* by morphological and molecular analysis using RAPD markers which might help to preserve the biodiversity of *C. asiatica* in AP and provide a framework for future efforts to incorporate wild germplasm into rootstock breeding and hybridization programmes.

MATERIALS AND METHODS

Plant source

Seedlings (30-40) were collected from various locations of AP, India and planted in concrete rings and on the ground with irrigation in a field gene bank (Prasad *et al*. 2007). All 16 accessions were used for morphological diversity studies and nine among them for molecular diversity analysis (**Table 1**).

Morphological diversity analysis using phenotypic parameters

A few morphological traits like leaf characteristics (length, width, margin, shape), and stalk length were studied in mature plants (5-months old) collected from 16 locations, namely Talakona, Tirupati (Chittoor), Sukumamidi (Khammam), Dulapally, Hyderabad (Hyderabad), Srisailam (Kurnool), Narsapur (Medak), Maredumilli (East Godavari), Mahendragiri 1, 2 and 3, Baruva, Srikakulam (Srikakulam), Kalasamudram (Ananthapur), Pakala (Warangal) and Araku (Vishakapatnam). All the parameters were observed visually. The district names of all the locations are mentioned in parentheses. Statistical analysis was done by applying ANOVA (Analysis of Variance). The experiments were carried out with 3 replicates of 20 samples each. The means of various morphological characters were differentiated based on LSD (Least Significant Difference). The level of probability was calculated at the 5% level. CD (Critical Difference) was used to interpret along with the treatment means to compare the data and interpret the significance of the phenotypic data of the accessions (**Tables 2, 3**).

Table 2 ANOVA table for morphological variations in leaf length, leaf width and stalk length in seedlings of *C. asiatica*

Source of variation	DF	SS (leaf length)	SS (leaf width)	SS (stalk length)
Treatments	16	851.2	256.94	8500.56
Replications	19	106.13	35.17	106.29
Error	304	233.6	86.16	964.2
Total	339	1191.01	378.28	9571.08
CD		0.54	0.329	1.103

DF: Degrees of freedom; SS: Sum of squares; CD: Critical Difference

Table 3 Least Significant Difference (LSD) table indicating the treatment means and significance for morphological variations in leaf length, leaf width and stalk length on seedlings of *C. asiatica*

Accession	Leaf length CD - 0.54		Leaf width CD - 0.329		Stalk length CD -1.103	
	Mean	Significance*	Mean	Significance*	Mean	Significance*
Talakona	0.25	0.15 a < CD	0.07	0.08 c < CD	0.22	0.43 c < CD
Tirupathi	0.40	0.17 a < CD	0.15	0.20 e < CD	0.65	0.00 h < CD
Sukumamidi	0.57	0.00 e < CD	0.35	0.10 d < CD	0.65	0.37 b < CD
Dulapally	0.57	0.13 a < CD	0.45	0.07 c < CD	1.02	0.05 a < CD
Hyderabad	0.70	0.65 c > CD	0.52	0.23 e < CD	1.07	0.10 b < CD
Srisailam	1.35	0.15 a < CD	0.75	0.05 b < CD	1.17	0.03 a < CD
Narsapur	1.50	0.27 b < CD	0.80	0.20 e < CD	1.20	0.60 d < CD
Maredumilli	1.77	0.68 c > CD	1.00	0.25 e < CD	1.80	0.27 b < CD
Mahendragiri 1	2.45	0.77 c > CD	1.25	0.07 c < CD	2.07	1.85 e > CD
Mahendragiri 2	3.22	0.18 a < CD	1.32	0.48 f > CD	3.92	1.03 e < CD
Mahendragiri 3	3.40	0.12 a < CD	1.80	0.12 d < CD	4.95	0.40 c < CD
Baruva	3.52	0.18 a < CD	1.92	0.03 a < CD	5.35	0.50 d < CD
Srikakulam	3.70	0.15 a < CD	1.95	0.07 c < CD	5.85	0.40 c < CD
Kalasaudram	3.85	0.37 b < CD	2.02	0.15 d < CD	6.25	0.00 h < CD
Pakala	4.22	1.65 d > CD	2.17	0.03 a < CD	6.25	2.80 f > CD
Araku	5.87	1.55 d > CD	2.20	1.10 g > CD	9.05	12.45 g > CD

CD: Critical Difference; *Different letters within a column denote statistical similarity among the accessions.

Isolation of genomic DNA

DNA was isolated by using CTAB (Sigma Aldrich, USA) protocol developed by Doyle and Doyle (1987) with slight modifications. Young leaf tissue (3 g) was ground into a fine powder in liquid nitrogen along with 0.1 g of polyvinyl polypyrrolidone (PVP) (Sigma-Aldrich, USA) and transferred to 10 ml of preheated (65°C) extraction buffer [2% CTAB, 100 mM Tris HCl (Sigma-Aldrich), 20 mM EDTA (Sigma-Aldrich), 1.4 M NaCl (Qualigens Fine Chemicals, India), pH 8.0] containing 10 mM β-mercaptoethanol (Sigma-Aldrich) per g of tissue. The slurry was incubated for 90 min in a 65°C water bath. An equal volume of chloroform: isoamylalcohol (24:1 v/v) (Qualigens Fine Chemicals) was added to the extract prior to centrifugation at 12,000 rpm for 15 min at room temperature (RT). To the supernatant equal volumes of ice-cold isopropanol (Qualigens Fine Chemicals) was added and incubated at –20°C for a minimum of 30 min followed by centrifugation at 12,000 rpm for 15 min at RT.

The pellet was washed with 70% ethanol (Sigma-Aldrich) by centrifuging at 10,000 rpm for 8 min, dried and dissolved in 100 µl of TE buffer and 5 µl of RNase A (10 µg/µl) (Sigma-Aldrich) was added and incubated at 37°C overnight. This was followed by phenol: chloroform: isoamylalcohol (25:24:1 v/v) extraction by centrifuging at 8000 rpm for 15 min. To the supernatant equal volumes of chloroform: isoamylalcohol was added and centrifuged at 12,000 rpm for 15 min. To the supernatant $1/10^{th}$ volume of 3 M sodium acetate and equal volumes of ice-cold isopropanol was added and left for 30 min for minimum at –20°C to precipitate DNA followed by centrifugation at 12000 rpm for 15 min. The DNA was precipitated by adding 70% ethanol and centrifuging at 10000 rpm for 10 min (Sambrook *et al*. 1989). The pellet was dissolved in TE buffer and stored at 4°C. DNA quality and quantity were evaluated spectrophotometrically at 260/280 nm and the DNA concentrations were rechecked by visual assessment of band intensities on 0.8% agarose gel in comparison to Lambda DNA marker. DNA samples were diluted with sterile Milli Q water to 50 ng/µl for further use in RAPDs.

RAPD analysis

In a preliminary study, 30 decamer primers of arbitrary sequence (Kits A and C provided by Operon Technologies Inc., Alameda, CA) were tested for PCR amplification out of which 16 primers were selected for further experiments. PCR reactions were carried out in a DNA Thermocycler (MJ Research Inc., USA). The concentrations of ingredients and the conditions for PCR were followed accordingly (Padmalatha and Prasad 2006a).

PCR products were electrophoresed on 2% (w/v) agarose gels, in 1X TBE buffer at 50 V for 3 h and then stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed under UV light. Lambda DNA *Eco*RI/*Hind*III double digest was used as the molecular size marker (Genetics Ltd., New Delhi, India). Screening of the entire set of samples was repeated in triplicate to assess repeatability of the RAPD profiles.

Data scoring and analysis

PCR reactions and electrophoresis were repeated at least twice to ascertain the reproducibility of the bands. For each accession, polymorphism was scored as 1 for the presence and 0 for the absence of a band. RAPD data generated with 16 primers was used to compile a binary matrix for cluster analysis based on Dice's similarity coefficient (Dice 1945) using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) version 2.02j package (Rohlf 1993). Based on the data the polymorphism level was observed which is shown in **Table 4**.

Table 4 RAPD analysis in percentage of polymorphism, total number of bands and polymorphic bands.

Primer code	Primer sequence	Total bands	Polymorphic bands	% polymorphism
OPA-01	CAGGCCCTTC	11	10	90
OPA-03	AGTCAGCCAC	4	4	100
OPA-05	AGGGGTCTTG	10	6	60
OPA-06	GGTCCCTGAC	8	8	100
OPA-09	GGGTAACGCC	6	6	60
OPA-12	TCGGCGATAG	4	4	100
OPC-02	GTGAGGCGTC	7	7	100
OPC-03	GGGGGTCTTT	10	10	100
OPC-04	CCGCATCTAC	14	8	57
OPC-09	CTCACCGTCC	8	7	87.5
OPC-10	TGTCTGGGTG	19	19	100
OPC-13	AAGCCTCGTC	11	11	100
OPC-15	GACGGATCAG	6	6	100
OPC-16	CACACTCCAG	8	8	100
OPC-18	TGAGTGGGTG	12	12	100
OPC-19	ACTTCGCCAC	18	11	61

TB: total bands
PB: polymorphic bands

RESULTS

Morphological diversity analysis

Among the 16 accessions of *Centella asiatica* collected from various locations of AP, field observations i.e., differences in the characteristic leaf shape i.e., broadly caudate, reniform, toothed, rooted nodes with long internodes and elongated petioles, in the natural conditions was observed (**Fig. 1**). Distinct variations were observed in leaf length, leaf width and stalk length. The differences in the mean values among the different accessions would be due to the differences in the edaphic and environment characteristics. According to the data analysed by ANOVA test there is also a statistically significant difference ($P \leq 0.001$) observed which may due to the external factors (**Table 2**). The mean differences were compared with the critical difference values among the accessions to check the significance of the results obtained. According to the LSD test all the phenotypic characters showed a wide range of variation among the accessions (**Table 3**).

RAPD analysis

Analysis of nine accessions of *C. asiatica* revealed 87% polymorphism (**Fig. 2A, 2B**). The number of scorable polymorphic markers generated were 137 out of 156 total markers (**Table 5**). The levels of genetic diversity within acces-

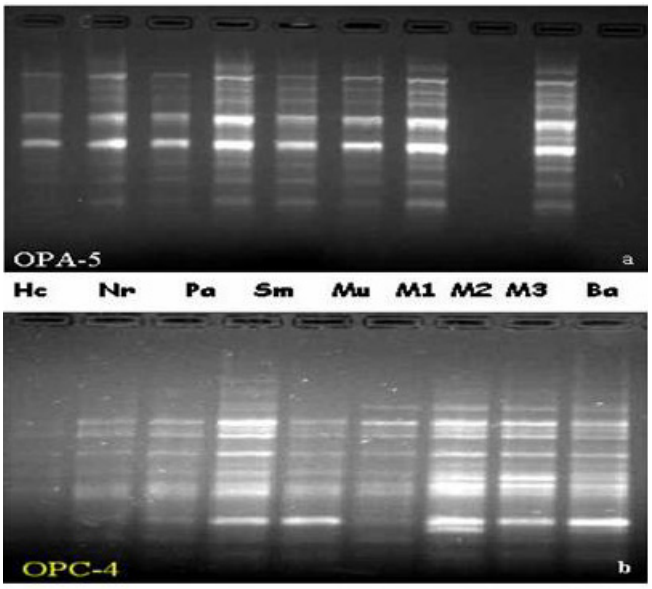

Fig. 2 (**A**) RAPD profile using primer OPA-05 (5′-AGGGGTCTTG-3′). Polymorphism is observed, as there is no amplification in lane M3. Monomorphic markers are generated among the other accessions of *C. asiatica.* (**B**) RAPD profile using primer OPC-04 (5′-CCGCATCTAC3-′). Polymorphic markers are generated in lane Sm whereas unique markers are generated in lanes M1, M2, M3 and BA accessions of *C. asiatica*.

Table 5 Polymorphism levels in individual accessions of *C. asiatica.*

Primer code	CA1		CA2		CA3		CA4		CA5		CA6		CA7		CA8		CA9	
	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPA-01	1	0	1	0	1	0	9	8	7	6	11	10	11	10	11	10	11	10
OPA-03	0	0	4	4	2	2	4	4	0	0	4	4	4	4	4	4	4	4
OPA-05	9	5	9	5	9	5	9	5	9	5	9	5	10	6	4	0	10	6
OPA-06	0	0	5	5	1	1	8	8	6	6	5	5	8	8	1	1	8	8
OPA-09	0	0	0	0	5	5	4	4	6	6	6	6	1	1	5	5	5	5
OPA-12	0	0	0	0	2	2	4	4	0	0	1	1	4	4	4	4	4	4
OPC-02	0	0	0	0	0	0	6	6	0	0	0	0	7	7	7	7	4	4
OPC-03	0	0	0	0	0	0	10	10	3	3	2	2	8	8	8	8	7	7
OPC-04	10	5	9	4	9	4	14	9	9	4	9	4	13	8	12	7	13	8
OPC-09	1	1	8	3	8	3	8	3	7	2	7	2	8	3	8	3	8	3
OPC-10	0	0	0	0	4	4	19	19	13	13	12	12	19	19	17	17	16	16
OPC-13	0	0	9	9	8	8	10	10	10	10	10	10	11	11	11	11	10	10
OPC-15	0	0	0	0	0	0	6	6	0	0	3	3	3	3	3	3	3	3
OPC-16	0	0	5	5	3	3	4	4	4	4	5	5	5	5	8	8	4	4
OPC-18	0	0	4	4	5	5	11	11	9	9	9	9	12	12	11	11	10	10
OPC-19	10	3	10	3	10	3	14	7	13	6	9	2	15	8	18	11	17	10

Table 6 Similarity matrix of *C. asiatica* generated from Dice estimate similarity based on the number of shared fragments.

	CAHC	CANA	CAPA	CASM	CAMU	CAM1	CAM2	CAM3	CABA
CAHC	1.000								
CANA	0.702	1.000							
CAPA	0.554	0.704	1.000						
CASM	0.320	0.547	0.564	1.000					
CAMU	0.392	0.604	0.710	0.676	1.000				
CAM1	0.336	0.571	0.653	0.794	0.822	1.000			
CAM2	0.342	0.551	0.579	0.837	0.734	0.826	1.000		
CAM3	0.209	0.400	0.528	0.742	0.656	0.777	0.872	1.000	
CABA	0.337	0.552	0.614	0.793	0.738	0.796	0.903	0.861	1.000

sions i.e., the genetic distance (GD) ranged from 0.209-0.903. The mean value of GD among the accessions was 2.622. The minimum GD of 0.209 was exhibited by accessions collected from Mahendragiri 3 and Hyderabad, a maximum GD of 0.903 by plants collected from Balpally and Mahendragiri 2.

The values of genetic distances calculated for the accessions collected from Maredumilli and Mulugu is 0.822, from Mahendragiri 2 and Balpally GD was 0.903 and from Narsapur to Pakala showed a GD of 0.704 (**Table 6**). The polymorphism exhibited by individual *C. asiatica* accessions is shown in **Table 3**. The percentage polymorphism for individual accessions were: 45% (Hyderabad), 65% (Narsapur), 67% (Pakala), 84% (Srikakulam), 77% (Mulugu), 78% (Mahendragiri 1), 82% (Mahendragiri 2), 83% (Mahendragiri 3), 86% (Balpally). The primer with maximum number of polymorphic bands was OPC-10 (19 bands) and the minimum with OPA-12 (4 bands). No primer exhibited complete monomorphism. Hence the range of the bands generated for all the primers fell between 4 and 19. The GC% of all the primers ranged from 60 to 70%, which did not show any effect on amplification. The average number of polymorphic bands per primer generated was 8.5 out of the total number of bands (9.7). The primers OPA-03, OPA-06, OPA-12, OPC-02, OPC-03, OPC-10, OPC-13, OPC-15, OPC-16 and OPC-18 exhibited 100% polymorphism with all the accessions. The size of the amplified fragments ranged from 300-3500 bp (**Fig. 2**).

DISCUSSION

Morphological variations

The morphological differences are not significantly correlated with the geographical distances. The large phenotypic variability observed in leaf characteristics facilitated a clear distinction among the 16 accessions. According to the statistical test for significance, there was a wide variation in leaf length in accessions collected from Talakona, Tirupathi, Dulapally, Srisailam, Mahendragiri 2, Mahendragiri 3, Baruva and Srikakulam, when compared to accessions from Pakala and Araku. When leaf width was considered wide variations were observed in accessions collected from Baruva and Pakala with that of accession collected from Araku location. When stalk length was considered wide variations were observed from accessions collected from Dulapally, Srisailam when compared with that of accession collected from Kalasamudram location (**Tables 2, 3**).

The leaf shape varied from round to caudate and reniform apart from a notch at the centre of the leaf which clearly indicates the existence of location specific adaptations due to the influence of environmental, edaphic and developmental changes (**Fig. 1**). Such phenotypic variations may be the result of phenotypic plasticity or genetic diversification.

Molecular variations by RAPD analysis

Similar studies were also reported in *Rauvolfia tetrahylla* L. (Padmalatha and Prasad 2006b) It is expected that obligate outcrossing species show high genetic variation at the population level (Apostol *et al*. 1996). In *C. asiatica* 87% polymorphism was observed (**Fig. 2A, 2B**). The differentiation of accessions could broadly be explained as a result of abiotic (geographical, e.g., hydrographic connections, or climactic differentiation. e.g., annual rainfall differences) and biotic (pollination between populations and seed dispersal, etc.) factors.

The range of genetic diversity calculated in terms of GD is 0.209-0.903 and the mean level of genetic diversity within 9 accessions is 2.622. From this it is evident that GD showed no correlation with geographical distances between the location sites of accessions, negating a simple isolation by distance mode (**Table 6**).

Cluster analysis based on Dice coefficient revealed two major groups. The significant degree of variation between the accessions collected from Mahendragiri 1 and Hyderabad reveals maximum diversity. There is a close similarity of 90% between the accessions from Mahendragiri 2 and Baruva, which clearly depicts that genetically they are more or less similar. There is also a similarity of 82% observed between the accessions from Mulugu and Mahendragiri 1

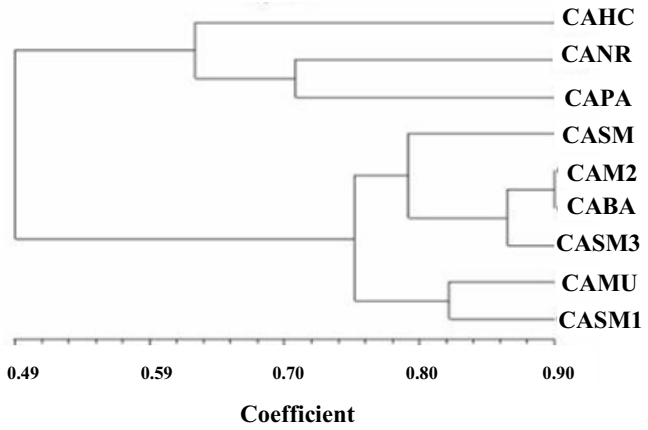

Fig. 3 Dendrogram of the nine accessions of *C. asiatica* germplasm obtained by UPGMA.

though geographically they are quite distant whereas accessions collected from Srisailam and Mahendragiri 3 though they are quite distant there is a genetic similarity of 78%, which clearly indicates that there is no correlation between genetic distances and geographical distances. It is also in correlation with that of the tested morphological parameters. But these correlations may not be completely in accordance, as it may not represent the exact parameter, as there may be many other phenotypic parameters, which may exhibit variation, which could be due to non-effective gene flow among the accessions (**Fig. 3**).

Therefore apart from diversity genetic homogeneity is also maintained since self-pollination may also occur apart from cross pollination. It can be inferred that in the accessions which are clustered in similar groups, there is effective gene flow confined to those particular locations but whereas in Hyderabad and Mahendragiri 1 accessions, gene flow may be less and hence they are highly divergent when compared to other accessions. This situation arises only in the case of natural populations where there is a free/random pollen flow and fertilization, as is the case in most of the cross-pollinated species. This is probably the reason that accessions, which are closely related at the genetic level, are geographically from distinct areas of A.P.

In *C. asiatica* gene flow homogenizes population structure and counteracts the effects of drift and diversifying selection. Thus populations can undergo genetic differentiation from one generation to another (Slatkin 1987). The differences found in the dendrogram could be partially explained by different number of PCR products analyzed for RAPDs 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 RAPDs (Karp *et al*. 1997). However, in our experiments, once the PCR conditions were well set up we obtained a high reproducibility for RAPDs.

Implications for *ex situ* conservation

The genetic analyses presented here could be used for the development of conservation and management strategies (Newton *et al*. 1999). The high levels of variations suggest that sampling from a few localities for either breeding or conservation could capture a large proportion of the variation within the species. Inadequately any one of the accessions that have been grouped together in cluster analysis has to be maintained, as all of them are genetically more or less similar. In doing so genetic variation among different accessions can be maintained without any loss and transfer of germplasm between different locations should be avoided, to ensure that the genetic material is adapted to local site conditions (Ennos 1998).Since the genetic variation is high, in order to maintain the diversity, appropriate techniques for seed sampling must be applied.

The results of the present study can be viewed as a starting point for future research for which a large number of natural populations collected from the whole distribution should be analyzed and additional primers should be tested. However detailed morphological study is also desirable in order to understand all aspects of variations. High priority genotypes i.e., elite varieties can also be known hypothetically.

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