

Genetic Diversity and Dendrogram of *Cardiospermum Halicacabum*- An In vitro Study

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Cardiospermum halicacabum commonly known as Balloon plant or Love in a puff, a climbing plant often found in tropical and subtropical Africa and Asia. It is widely used in homeopathic medicine for the treatment of arthritis, purulent discharge and others. This particular plant species is becoming endangered due to their extensive use for commercial purposes and genetic diversity at inter-species level arises due to geographical distribution, climate changes and environmental stress. As an initiative for conservation of this species, the present contemplation was done by collecting DNA samples from leaves of the above mentioned plant which were grown in five different locations in and around Chennai and the investigation of their diversity was done using genetically reliable DNA markers like OPA 5, OPA 9, OPA 10, OPB 18, OPC 7, OPN 7 and OPN 12. RAPD PCR was performed. It provided providing information at molecular level and the total number of bands formed, designate the presence of genetic variations among species in different location. Dendrogram was constructed using scoring methods found the differences among the same species arising from evolution, it could be environmental change and technological developmental impacts.

Keywords: *C.halicacabum*, Arthritis, Genetic diversity, PCR, Dendrogram.

Plants have been used since ancient times for the treatment of various ailments. Out of about 15,000 species of higher plants in India, medicinal uses have been attributed to 1500 species (Handa, 1998). Due to the technological advancements, the natural genetic setup of the plants experiences a drastic change. Therefore, we decided to work on genetic diversity in *Cardiospermum halicacabum*. Different parts of this plant are used in traditional medicine for the treatment of rheumatism, lumbago, cough, hyperthermia, nervous diseases, stiffness of limbs and snake bite (Chopra, 1980). According to research work done by Krishnamoorthy Ganesan *et al.*, 2011, *C. halicacabum* cures inflammation and collagen degradation. The alcoholic extract

of *C. halicacabum* when administered orally produced significant anti-inflammatory effect in rats as tested by the granuloma pouch and cotton pellet implantation methods (Gopalakrishnan *et al.*, 1976). The ethanol and hexane extracts of *C.halicacabum* found to act against pyrexia in rats (Asha *et al.*, 1999). This plant is also used against throat infection and headache (Chellaiah Muthu *et al.*, 2006). The ethanol extract of *C. halicacabum* proved to have anti-ulcer activity against ethanol induced gastric ulcer in rats (Sheeba *et al.*, 2006) and anti-hyperglycaemic effect against streptozotocin (STZ) induced diabetic male albino Wistar rats (Veeramani *et al.*, 2008). The ethanol extract of the *C. halicacabum* suppresses the

production of TNF-alpha and nitric oxide in human peripheral blood mononuclear cells (Venkatesh Babu *et al.*, 2006) This particular plant species is becoming endangered due to their extensive use for commercial purposes. This study would help in their conservation and use. Genetic diversity at inter-species level arises due to geographical distribution, climate changes and environmental stress. Genetic diversity is the reason for inter-species differences. This diversity is also observed within species, and in plants this variation can be a result of difference in climatic conditions, geographical distribution and environmental stress (Enelge Gildenhuys, 2013). The genetic diversity may not result in morphological changes to a great extent but this genetic diversity may lead to the change or disappearance in the medicinal properties present in the plant. Genetic diversity may also result in the extinction of the plant or the adaptation of the plant to existing environment which may result in the plant to produce some toxic components instead of the medicinal components so the study of the genetic diversity of the plant is very important to preserve the plants and its medicinal values and prevent them from being endangered. Genetic diversity help detect the phylogeny of a particular plant species, and experiments such as RAPD (Random Amplified Polymorphic DNA) show the extent of genetic polymorphism observed within a particular species, despite morphological similarities (Annamalai *et al.*, 2012).

MATERIALS AND METHODS

Plant material

Young Tender leaves of *Cardiospermum halicacabum* were collected from Ponmar, K.K.Nagar, Ottiyambaakaam, Vengaivaasal and Pallikaranai locality and authenticated by Botanist and permitted for DNA isolation after washing with water and saline.

DNA Isolation

The procedure for DNA isolation using CTAB is a modification of Saghai-Marouf *et al.*, (1984) method. We further slightly modified this method by adding 0.2% PVP to the CTAB extraction buffer to prevent co-isolation of phenolics and polysaccharides. 0.5g of plant material was weighed and frozen in liquid nitrogen.

The frozen tissue was ground in a clean motor and pestle. To this extract 1.5ml (w/v) of preheated 4X CTAB buffer was added and incubated at 65°C for about 60 minutes. The buffer was mixed well by inversion for every ten minutes and equal volume of ice-cold Chloroform: isoamylalcohol (24:1) was added. After mixing thoroughly, the tubes were centrifuged at 10,000rpm for 15minutes. Using a wide bore tip the top aqueous phase was transferred to a new sterile microfuge tube. The volume was measured and one-tenth the volume of 3M Sodium acetate and 0.6 volume of cold isopropanol was added and mixed gently to precipitate the nucleic acids. The tubes were stored at -20 for about one hour or left overnight (optional). The nucleic acid pellet was recovered by centrifuging at 10,000rpm for 15 minutes. The pellet was washed with 70% ethanol twice and was air-dried briefly at room temperature. The pellet was dissolved in T.E buffer and stored under -20°C.

Gel Electrophoresis

DNA quality was assessed on a 0.8% Agarose Gel (Tanksle *et al.*, 1989) (in Tris Acetate EDTA buffer) electrophoresis at 30 Volts. DNA was stained with Ethidium Bromide visualized on a UV transilluminator. Gel electrophoresis of the nucleic acids suspended in T.E. showed the presence of RNA, as well as, high molecular weight DNA. So they were subjected to RNase treatment.

DNA Estimation

A double beam Spectrophotometric (2203) quantification of DNA was done by the procedures described by Zachleder (1984) Danovaro *et al.*(1993) and Danovaro (1996) with modification. 3µl of the DNA sample was diluted to 3ml with T.E buffer pH 8.0 (Dilution factor is 1000 times). Concentration was calculated using the formula

$$\text{Concentration of DNA} = \frac{\text{O.D at } 260\text{nm} \times 50}{\text{Dilution factor}}$$

The purity of the DNA samples was checked by the ratio of absorbance 260nm to 280nm. (The ratio approximately 1.8 indicates that the samples are free from protein and RNA contamination). This concentration of the DNA samples and their ratio are listed.

RNase Treatment

DNA extracts were treated with RNase in order to remove Ribonucleic Acids (Fara *et*

PCR Standardization

PCR Parameter	Tested range	Optimum conditions	Remarks
DNA concentration (ng)	25ng & 50ng	50ng	Absence of amplification with lower concentration and presence of smear at higher concentration Affected the repeatability.
Magnesium chloride (mM)	2, 2.5 & 3	2.5mM	Excess/lower concentration increases the non specificity and yield of the product.
Primer concentration (iM)	0.3, 0.4 & 0.5	0.4µM	Lower and higher concentrations lead to absence of amplification and Primer dimer formation, respectively.
Taq polymerase (units)	2.5, 2 & 1	2.5U	Lower concentration did not show Proper amplification. High concentration showed decreased Specificity.

al., 1996). RNaseA in the concentration of 10µg/ml was added to the diluted DNA sample to final concentration of 10µg/ml and incubated at 37°C for 1 hour. Equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed well by inversion (equilibrated Phenol was used). The aqueous phase was recovered by centrifugation at 10,000rpm, 4°C for 2-5 minutes. Aqueous layer was transferred to a new microfuge along

with one-tenth volume of 3M Sodium acetate and 2.5 volumes of ice-cold 99% ethanol. The tubes were then incubated at -20°C for about 1 hour and centrifuged at 12,000rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. Ethanol was then aspirated by air-drying the samples. The pellet was then redissolved in 50 microlitre of 1X T.E buffer and stored under -20°C.

Table 1&2. PCR Standardization

Milli Q water	10.83µl
10X Buffer	2.0µl (1X)
50mM Magnesium chloride	1.0µl (2.5mM)
2mM DNTP's	2.0µl 0.2mM)
DNA (50ng/µl)	1.0µl (50ng)
Taq polymerase (5U/µl)	0.5 (2.5U)
3µM Primer	2.67(0.4µM)

Table 3. Primer screening for *Cardiospermum halicacabum*

S.no	Primer	No. of Bands
1.	OPC7	5
2.	OPC10	4
3.	OPB 18	NIL
4.	OPB 19	NIL
5.	OPB 20	NIL
6.	OPA 18	NIL
7.	OPA 19	NIL
8.	OPA 20	NIL
9.	OPA 11	NIL
10.	OPA 5	3

PCR-Polymerase Chain Reaction & Visualisation Of Products

PCR involves multiple cycles of denaturation, renaturation and polynucleotide synthesis that amplifies a particular DNA sequence. DNA samples were diluted to 50ng/µl concentration.

PCR conditions were standardized by varying the concentration of its components. The

Table 4. Primer under study

S.no	Primer	Sequence 5'-3'	GC%
1.	OPC7	GTCCCGACGA	70
2.	OPC10	TGTCTGGGTG	60
3.	OPA5	AGGGGTCTTG	60

Table 5. Scoring for Primer OPC7

LOCI	C1	C2	C3	C4	C5
250	1	1	1	1	1
500	1	1	0	1	1
850	1	0	0	1	1
1300	1	1	1	1	1
1400	1	1	1	1	1

variations and the remarks were tabulated (Table No.1 and Table No.2).

RAPD – PCR

Primer Screening

Ten Random primers were used to screen the genomic DNA samples. Ponmar sample was used for screening.

Analysis On Agarose GEL

PCR product 25µl as loaded into 1.5% agarose gel along with a 1kb DNA ladder (Biotools, as a reference). Samples were run at 75volts for 30-60 minutes hours till bromophenol blue present in the gel loading dye reaches 3/4th of the length of the gel. Gel was visualized on a UV transilluminator/gel documentation unit and the bands were scored.



Fig. 1. Banding pattern for OPC7



Fig. 2. Banding Patterns For OPC 10

Table 6. Scoring for Primer OPC 10

LOCI	C1	C2	C3	C4	C5
450	1	1	1	1	1
500	0	1	0	0	0
600	1	0	0	1	1
1000	1	1	1	1	1
1500	1	0	1	1	1
1700	0	1	1	0	1

Construction of dendrogram

The presence and absence of bands between samples was scored and data was transcribed into binary format. That is, a score of 1 was given for the presence of the particular band and 0 given for its absence. From this data, a statistical analysis was done using commercial software SPSSv16. Difference matrix was calculated using UPGMA based analysis. The obtained values were used to construct the dendrogram.

RESULT AND DISCUSSION

From the RAPD PCR primer screening, 3 primers were selected to develop a fingerprint of *Cardiospermum* samples.

Dendrogram

The banding pattern for OPC7 and OPC10 appeared smeared as shown in the figure 1 and figure 2 whereas the banding pattern for OPA 5, appeared convex shaped and smeared. These banding pattern was found similar to the work done by Annamalai *et al.*, 2012. The researcher had chosen random primers and the banding pattern observed was smeared. However the dendrogram constructed showed genetic distances proving the fact that the environment/conditions in which the plant grows, show genetic level differences. In

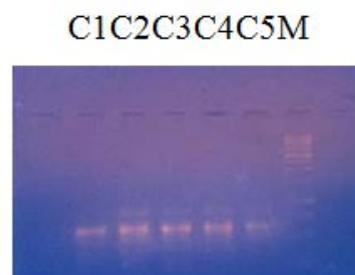
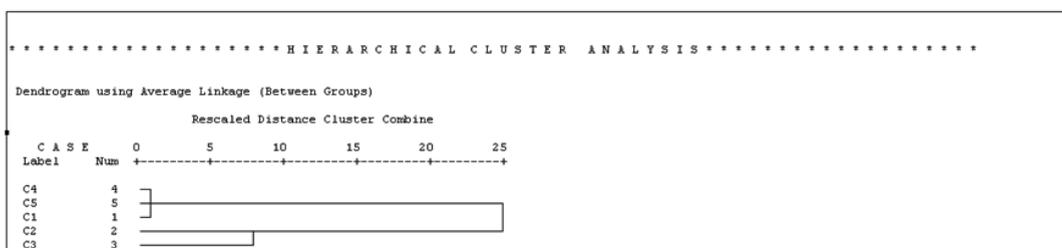


Fig. 3. Banding Pattern For OPA 5

Table 7. Scoring for Primer OPA5

LOCI	C1	C2	C3	C4	C5
250	0	1	1	1	0
350	1	1	1	1	1
500	0	1	1	1	1

Dendrogram

other another work carried out in shodhganga, the *C.halicacabum* collected from the western ghats and forest regions of India showed less genetic variation. The UPGMA dendrogram for five selected populations of *C. halicacabum* collected from five different regions of kerala showed two clusters. Accessions from Trivandrum, Kanyakumari and Kollam were grouped in one cluster whereas populations from Alappuzha and Palakkad in the other cluster (M.S. Sheeba *et al.*, 2014).

CONCLUSION

Environmental pollution leads to change in the environment in which the plant survives so it adapts itself to the existing environment which is caused due the mutation of genes which causes decrease in the medicinal quality of plant so its a call of the hour to protect and preserve these medicinal plants from the environmental changes

REFERENCES

1. Asha VV and Pushpangadan P. Antipyretic activity of *Cardiospermum halicacabum*. *Indian J Exp Biol.*; **37**:411–4 (1999).
2. Annamalai A, Christina V.L and Lakshmi P.T.V. Evaluation of Genetic Diversity in *Cardiospermum halicacabum* based on Geographical Variation Using RAPD Markers, *Pharmacognosy Communications* ; **2**(1) :62-65 (2012).
3. Chellaiah Muthu, Muniappan Ayyanar, Nagappan Raja, and Savarimuthu Ignacimuthu. Medicinal plants used by traditional healers in Kancheepuram District of Tamil Nadu, *India J EthnobiolEthnomed.*; **2**: 43-53 (2006).
4. Chopra R.N , Nayar S.L and Chopra I.C. Glossary of Indian Medicinal plants. New Delhi (1956).
5. Danovaro R. Detritus-bacteria-meiofauna interactions in a seagrass bed (*Posidonia oceanica*) of the NW Mediterranean. *Mar. Biol.*, **127**: 1–13 (1996).
6. Danovaro R, Fabiano M and Della Croce N. Labile organic matter and microbial biomasses in deep-sea sediments (Eastern Mediterranean Sea). *Deep-Sea Res.*; **40**: 953–965 (1993).
7. Enelge Gildenhuis , Allan G. Ellis , Scott P. Carroll and Johannes J. Le Roux. The ecology, biogeography, history and future of two globally important weeds: *Cardiospermum halicacabum* Linn. and *C. grandiflorum* Sw. *NeoBiota.*; **19**: 45–65 (2013).
8. Fara A, Berdalet , and Arin L. Determination of RNA and DNA concentrations in natural plankton samples using Thiazole Orange in combination with DNase and RNase digestions. *J. Phycol.*; **32**: 1074–1083 (1996).
9. Gopalakrishnan C, Dhananjayan R and Kameswaran L. Studies on the pharmacological actions of *Cardiospermum halicacabum*. *Indian J PhysiolPharmacol.*; **20**: 203–6 (1976).
10. Handa, S.S. Indian efforts on standardization and quality control of medicinal plants using scientific parameters. *Amruth (The Traditional Healthcare Magazine)*, **2**: 10 (1998).
11. Krishnamoorthy Ganesan, Praveen Kumar Sehga, Asit Baran Mandal and Sadulla Sayeed. Protective Effect of *Withania somnifera* and *Cardiospermum halicacabum* Extracts Against Collagenolytic Degradation of Collagen. *Applied Biochemistry and Biotechnology.*; **165** (3–4), 1075–1091 (2011).
12. Sheeba MS and Asha VV. Effect of *Cardiospermum halicacabum* on ethanol-induced gastric ulcers in rats. *J.Ethnopharmacol.*; **106**: 105–10 (2006).
13. Tanksley S.D ,Young N.D ,Paterson.A.H and Bonierbale,M.W. RFLP mapping in plant breeding:New tools for an old sciences,*Biotechnology.*; **7**: 257-264 (1989).
14. Veeramani C, Pushpavalli G and Pugalendi KV. Antihyperglycaemic effect of *Cardiospermum halicacabum* Linn.leaf extract on STZ induced diabetic rats. *J Appl Biomed.*; **6**: 19–26 (2008).

15. VenkateshBabu KC and Krishnakumari S. *Cardiospermum halicacabum* suppresses the production of TNF-alpha and nitric oxide Anti-arthritis activity of the Indian leafy vegetable *Cardiospermum halicacabum* by human peripheral blood mononuclear cells. *Afr.J.Biomed Research.*; **9**: 95–99 (2006).
16. Zachleder V. Optimization of nucleic acids assay in green and blue green algae: extraction procedures and the light-activated diphenylamine reaction for DNA. *Arch. Hydrobiol.*; **67**: 313–328 (1984).