Research Article

Morphology and Genetic Diversity of *Garcinia cowa* (Roxb.) in Upper Brahmaputra Valley of Assam

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ABSTRACT

Background: *Garcinia cowa* is a lesser-known species and it is therefore the limelight of this study to bring to notice its morphological and genetic diversity. **Materials and Methods:** The morphological diversity was studied using morphological markers and the genetic diversity was analyzed using 30 different primers for ISSR analysis. The DNA isolation in 2X and 4X buffer for PCR was done using the method given by Doyle and Doyle. The method was slightly modified. **Results:** Tinsukia district showed the highest leaf length, leaf breadth and leaf vein. Dibrugarh district showed the highest leaf petiole. 4X buffer proved to be more effective lysis buffer in comparision to 2X buffer. *Garcinia cowa* showed 100% polymorphism with primers UBC-41 and UBC-42. **Conclusion:** *Garcinia cowa* was found to have considerable morphological diversity. 4X buffer proved to be more effective. The accessions of *Garcinia cowa* showed polymorphism only with primers UBC-40, UBC-41 and UBC-42 and the polymorphism percentage of *Garcinia cowa* was calculated which was found to be 100% with primers UBC-41 and UBC-42.

Keywords: *Garcinia cowa,* ISSR, Morphological diversity, PCR.

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INTRODUCTION

The Brahmaputra valley is the heart of the state of Assam, harbouring a rich diversity. The Brahmaputra valley of Assam lies between 25-44°N latitude and 89.4-96.02°E. This region is regarded as a hotspot for the evolution of world flora due to its endemicity, also the temperature and climate of the area support many life forms. Since this region is a biological hotspot there is an urgent need to explore the important resources of the area. One important forest resource of high utilitarian value in Assam is *Garcinia* species which are well known for their edible value and medicinal effects.

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Around the world there are about 200 species of *Garcinia*. About 35 species with immense medicinal properties are found to occur in India.[1] *Garcinia* has many beneficiary effects and additional studies will help develop new therapeutic products from this plant.[2] *Garcinia cowa* is a lesser-known species of this genus with high utilitarian value. Exploring this species is the objective of the study. The sundried fruit slices of most of the *Garcinia* speciesare used in folk medicines.[3] A popular organic acid -Hydroxycitric acid (HCA) inhibits the synthesis of fatty acid and lipids.[4] *Garcinia cowa* contains Hydrocitric acid in its leaves and fruits.[5,6] Studies suggest the ethno botanical aspects of *Garcinia cowa* among tribal and allied peoples.[7] *Garcinia* sp. are a treasure house of xanthones- A secondary compound possessing antimalarial and antibacterial properties.[8,9] Chromatographic studies reveal a collection of Xanthones A-E from *Garcinia cowa.*[10,11] *Garcinia cowa* is a highly valued medicinal plant[12] used in herbal treatment for many ailments.Fruit rinds of

Garcinia cowa bear antibacterial properties effective against many food-borne pathogens and spoilage bacteria.[9] The twigs of *Garcinia cowa* is a treasure house of garcicowin A(1), and garcicowin B-D(2-4) which show their selective toxicity towards cancer cells.[13] The bark extracts of *Garcinia cowa* is seen to exhibit antiplatelet aggregation property.[14]

Studies suggest that *Garcinia cowa* also bear antioxidant and antiaflatoxigenic properties.[15] *Garcinia cowa* is found to occur in most parts of Northeast India, Bangladesh, Malaysia, Vietnam etc. Inadequate knowledge, destruction of habitat erodes this important resource and a large amount of species are threatened.[16] Therefore the present investigation leads to the study of the morphological and genetic variability among the collected genotypes of *Garcinia cowa*.

MATERIALS AND METHODS

Study area

In order to carry out the study, six districts of the upper Brahmaputra valley of Assam was selected and field study was carried out from December 2015 to June 2016. The districts of the study were Tinsukia, Dibrugarh, Jorhat, Sivsagar, Lakhimpur and Dhemaji.

Materials

For the study of Morphological diversity, 12 accessions of the species under study *Garcinia cowa* Roxb. were collected from six districts of Assam valley. The collection from each district included three to four different places within a 25-35 km radius. The morphological studies were carried out taking into consideration the measurement of the following: Leaf size, Leaf petiole size, Leaf length, Leaf breadth and Fruit size (if available).

For isolation of genomic DNA i.e Deoxyribonucleic Acid and PCR i.e Polymerase chain reaction young leaves were collected. After the collection the leaves were kept at -20°C and the further experiment was performed, only more morphological higher diversity i.e. 3 genotypes were further put for the genetic study.

Preparation of chemicals and reagents

Cetyltrimethyl ammonium bromide (CTAB) buffer preparation

For the preparation of 4% CTAB buffer, 100 mL tris HCl was dissolved in nuclease-free water. For the study the pH to be adjusted to 8.0. Then 30 mL of Ethylenediamine Tetraacetic Acid (EDTA) was added, and after dissolving the EDTA, 1.4 molar NaCl was added. Then 4 g CTAB and 15% of PVP were added and autoclaved. 0.3 mercaptoethanol was freshly added before the use of CTAB buffer.

5X Tris-Borate EDTA (TBE) buffer

5X TBE buffer was prepared with 54 g Tris base, 27.5 g boric acid plus 20 mL EDTA of pH 8.0 and finally volume was adjusted to 1:1 with water. A 1X working solution from this 5X buffer was made. To make 1% concentration, 1 g of agarose powder was diluted to 100 mL of 1X TBE buffer.

0.8% agarose gel

0.8g agarose dissolved in 100 mL TBE buffer. This preparation gives us 0.8% agarose gel.

Tris-EDTA (T.E) buffer preparation

To make 100 mL of T.E buffer solution, 1 mL of 1 M trisbase, 0.2 mL of 0.5 M EDTA and 20 mL of 5M NaCl were added. Addition of distilled water made the final volume to 100 mL.

Collection of sample

The sample collections were direct from fresh living plants. The collected materials included tender leaves, old leaves, and fruits (if found). For the DNA extraction, bags with zip locks were taken and fresh leaves were collected. Further stored at -20°C. This was done to prevent the degradation of DNA by chemicals and enzymes during the process.

Identification of plant

The collected specimen was identified in the department of Lifesciences, Dibrugarh University using a few referral data.[17,18]

Study of morphological diversity

A morphological study was carried out using various morphological markers like leaf length, Leaf breadth, Leaf mid vein and Leaf pedicel size.

DNA extraction method

One of the most effective methods for the isolation of DNA is the CTAB method.[19] 1 g of the fresh leaf was crushed with liquid nitrogen and made into powder with the help of mortar pastel. 10 mL of 4% CTAB buffer was added and mixed gently. 1 mL of CTAB mixed plant tissue was taken in a 2 µL centrifuge tube. 4µL of mercaptoethanol was added in each 2µL centrifuge vial. Then incubation was carried out in a water bath for 1 hr at 60°C with occasional shaking. The next incubation was done at 37°C by adding 6 µL of RNase in each tube for about 30 min. The next step was addition of chloroform isoamyl alcohol (24:1) and centrifugation was carried out for 15 min at 12000rpm

at room temperature. The supernatant was separated. Further 2M NaCl and chilled isopropanol was added in the volume 0.1 and 0.7 respectively in the supernatant by mixing gently and kept at -20°C for 1 hr. Centrifugation of the sample was carried out at 12000 rpm. After centrifugation, the DNA sediments at the bottom of the tube and the aqueous phase were removed. The DNA pellets were collected. The pellets obtained were washed with the help of wash buffer and centrifuged at 11000 rpm for 3-5 min. The pellets were then air dried and dissolved in 500 µL of T.E buffer and stored at 4°C for future use.

Gel electrophoresis

To separate DNA fragments according to their size and charge, gel electrophoresis was done. The separated molecules form a band by reducing convection currents and diffusion.

Method of preparation of gel electrophoresis

The open end of the tray in which the gel was cast supplied with the electrophoresis apparatus was sealed with autoclave tapes so as to form mould. 0.8g agarose was taken and added in preheated (at boiling temperature) 1 X tris-acetate EDTA (TAE) buffer. The agarose solution was allowed to cool followed by the addition of 3µL ethidium bromide mixed thoroughly. The agarose solution was then poured into the electrophoresis tray till it solidifies. A comb was poured carefully into the gel to form pores such that air bubbles doesn't exist. After solidification of the gel, the comb was removed with care. In the electrophoresis tank the gel was taken for mounting. Electrophoresis buffer was then added to cover the gel upto 1mm. 2µL loading dye was added to the 5µL DNA and using a micropipette this was loaded in the slots of the submerged gel. The lid of the gel tank was closed and allowed to run with 80V for 40 min. DNA was allowed to migrate towards the anode by attaching the electrical leads. After the migration of the dye to the desired distance the electric current was switched off, the leads were removed and the pre-stained gel was examined by ultraviolet light in the gel documentation system.

Isolation and quantification

For quantifying DNA, DNA base pair ladder was compared by the method of spectrophotometer. Concentration of DNA upon extraction was determined by the following formula:

Genomic DNA (µg/µL) = 50µg/mL × measured at 260 wavelength × dilution factor

PCR reaction

The PCR analysis was carried out in *Garcinia cowa* Roxb. by almost 30 primers (Table 1). The PCR amplification required for Inter Simple Sequence Repeats (ISSR) analysis was performed on more morphological diverse genotypes.

For PCR analysis the master mix was made by taking some chemicals in definite quantities.

Thermo profile for PCR

PCR thermocycler programme consisting of 1 initiating cycle for 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 50°C and 1 min at 72°C along with the final extension cycle for 10 min at 72°C were performed. The reaction product was then stored at 4°C for electrophoretic analysis.

The first PCR cycle is the initiation cycle. The second is the denaturation cycle in which the denaturation of DNA takes place. In the third PCR cycle, the primers anneal to the template DNA strand. The next step is the extension step where complementary strands gets synthesized by DNA polymerase enzyme from the 3'OH of the primers. The next step is the final extension step. Thus, 1 cycle of PCR ends.

RESULTS

Collection and documentation

The samples of *Garcinia cowa* Roxb. ex DC were studied from 18 accessions of six districts. The samples were studied per district at a distance of 20-35 km apart.

Morphological observation

Three accessions were taken from each district total of 18 accessions are under observation. Mature leaves were choosen and collected. Further observations were done by morphological markers such as leaf size, petiole size, fruit size (if found) etc. The mean of the morphological characters of each district was calculated and the standard deviation was tabulated (Table 2). *Garcinia cowa* showed a considerable variation in morphological characters of different places. The collections of Tinsukia district showed the highest leaf length. Similarly, the leaf breadth of samples collected from 3 different places in Dhemaji district showed the highest variation and Dibrugarh district showed the least. The leaf vein of the different samples collected from Tinsukia district shows more variation as compared to other districts. The leaf petiole of collections from

Table 1: Primers used for carrying out the PCR reactions are listed in the table below.

Primer name	Tm (°C)	Sequence
PKBT-2	51.4	ACACACACACACACTT
PKBT-3	50.4	AGAGAGAGAGAGAGT
PKBT-4	51.4	AGAGAGAGAGAGAGAA
PKBT-5	50.4	AGAGAGAGAGAGAGA
PKBT-6	51.4	AGAGAGAGAGAGAGTT
PKBT-7	54.5	GAGAGAGAGAGAGAGAA
PKBT-8	56.7	GAGAGAGAGAGAGAGAC
PKBT-10	54.5	GTGTGTGTGTGTGTGTA
PKBT-11	56.7	GTGTGTGTGTGTGTGTCGT
PKBT-12	54.5	GTGTGTGTGTGTGTGTT
PKBT-14	69.5	CCCGGATCCAGAGAGAGAGAGAGA
ISSRED-14	49.2	GACAGACAGACAGACA
UBC857a	56.0	ACACACACACACACCG
UBC840b	51.4	GAGAGAGAGAGAGATT
UBC835b	53.7	AGAGAGAGAGAGAGTC
UBC835a	56.0	AGAGAGAGAGAGAGCC
UBC812	50.00	GAGAGAGAGAGAGAA
UBC818	52.00	CACACACACACACAG
UBC830	52.00	TGTGTGTGTGTGTGG
UBC836	52.00-54.00	AGAGAGAGAGAGAGYA
UBC838	36.00-40.00	TATATATATATATARC
UBC840	52.00-54.00	GAGAGAGAGAGAGAYT
UBC841	54.00-56.00	GAGAGAGAGAGAGAYC
UBC842	54.00-56.00	GAGAGAGAGAGAGAYG
UBC843	52.00-54.00	CTCTCTCTCTCTCTRA
UBC848	54.00-56.00	CACACACACACACARG
UBC850	54.00-56.00	GTGTGTGTGTGTGTYC
UBC852	52.00-54.00	TCTCTCTCTCTCTCRA
UBC860	52.00-54.00	TGTGTGTGTGTGTGRA
UBC868	48.00	GAAGAAGAAGAAGAAGAA

Table 2: Variation in morphological characters at different locations of the 6 districts under study by comparing the standard deviation (mean±sd, n=3).

Characters	Tinsukia district	Dibrugarh district	Sivsagar district	Jorhat district	Lakhimpur district	Dhemaji district
leaf length	16.78±2.40	13±1.92	15.63±1.11	12.97±1.31	15.04±1.57	14.77±0.97
leaf breadth	7.09±0.24	6.03±0.03	7.86±0.58	6.7±0.26	7.5±1.74	7.87±0.56
leaf vein	16.02±2.36	11.96±2.05	14.88±1.36	12.40±1.28	14.08±1.47	13.73±0.76
leaf petiole	0.7±0.65	0.92±0.37	0.76±0.25	0.53±0.05	0.9±0.2	1.04±0.27

Dhemaji district showed more variation in comparison. The results obtained were tabulated (Table 2).

Standardization of protocol

DNA was isolated by CTAB method[19] (Doyle and Doyle method, 1990) with few modifications:

A comparative study of Doyle and Doyle (1990) method and modifications done in this process is stated.

Quantification of genomic DNA

Agarose gel electrophoresis results show the band intensities of DNA isolated by the modified method in comparison with 5000 bp (Figure 1).

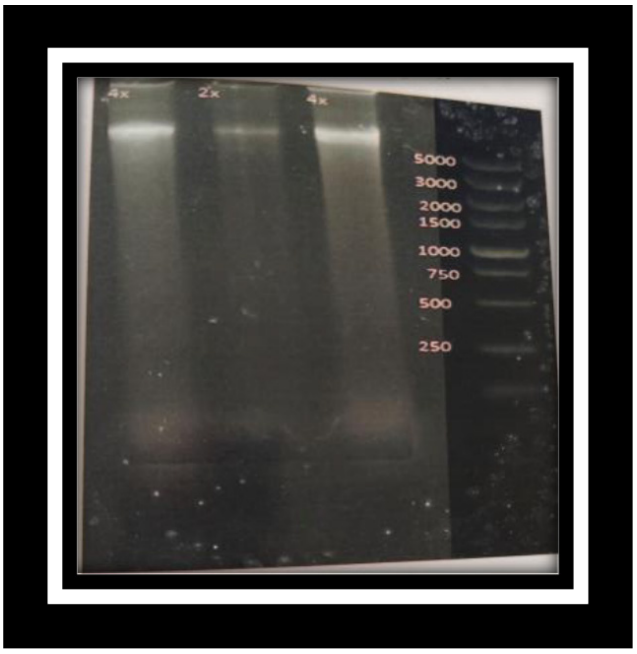

Figure 1: Genomic DNA extracted with 2X and 4X buffer.

Table 3: Quantification of genomic DNA isolated in 2x and 4x buffer.

Buffer	Concentration(ng/µL)	Purity
2X CTAB	53.4	1.067
4X CTAB	69.2	1.384

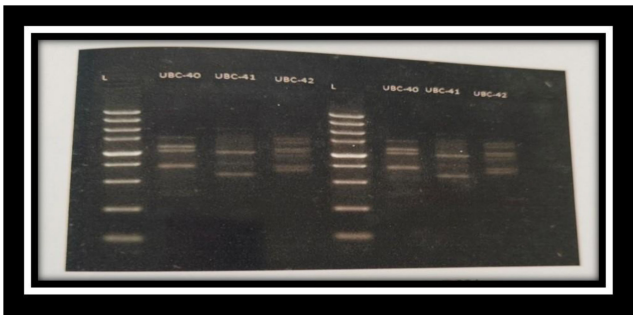

Figure 2: Polymorphism detection of *Garcinia cowa*.

The result shows that the base pair of the isolated genomic DNA is more than 5000 bp. The concentration of isolated genomic DNA in both 2X and 4X CTAB buffer is recorded (Table 3).

The study reveals that the concentration of isolated genomic DNA from *Garcinia cowa* Roxb. was more in 4X buffer than in 2X buffer. The result shows 4X CTAB buffer to be a more effective lyses buffer than 2X CTAB buffer.

PCR analysis

The result of PCR analysis using the ISSR marker was predicted (Figure 2).

The gel shows that the smaller and larger accessions of *Garcinia cowa* show polymorphism only with UBC-40, UBC-41 and UBC-42 primers. The polymorphic and monomorphic percentage with these three primers was calculated using the formula below:

Polymorphic percentage with UBC-40; the number of polymorphic bands formed/total no. of bands X 100(Total no. Of bands formed= 8) In the case of UBC-40, FOUR (4) polymorphic bands were formed= $4/8 \times 100 = 50\%$

Monomorphic percentage with UBC-40; In case of UBC-40, 2 monomorphic bands were formed = $2/8 \times 100 = 25\%$

Polymorphic percentage with UBC-41; (Total no. Of bands formed = 3) In the case of UBC-41, three (3) polymorphic bands were formed. = 3/3 X 100= 100%

Monomorphic percentage with UBC-41; 0

Polymorphic percentage with UBC-42; (Total no. Of bands formed = 7) = 7/7 X 100= 100%

Monomorphic percentage with UBC-42: = 0

Hence, the results showed that polymorphism of the accessions of *Garcinia cowa* Roxb. showed 100% with primers UBC-41 and UBC-42.

DISCUSSION

The procedure for isolating DNA from plant sources is different for different species due to the presence of different amounts of secondary metabolites in the plant species. Usually, for a higher concentration of polyphenols, it is necessary to use a higher concentration of extracted buffer. In this present method of study, Doyle and Doyle protocol was used with slight modifications. Instead of only 2X buffer, the experiment was carried out in 2X and 4X buffer. A high amount of DNA isolation in 4X buffer proved it efficient for the isolation of quality DNA. β – mercaptoethanol was not used in DNA isolation by Doyle and Doyle method, which marked a dissimilarity with the modified protocol. Doyle and Doyle's method added RNase after adding chloroform isoamyl alcohol but in this study, RNase is added before adding chloroform isoamyl alcohol. 50µL 2M NaCl was added extra which leads to the accumulation of DNA. For the DNA pellets, centrifugation was performed at 11000 rpm for 10 min instead of 5000 rpm for 10 min as in Doyle and Doyle's (1990) method. The DNA pellets were dissolved in 500µL of T.E buffer rather than dissolving in 50-100 µL of T.E buffer as in Doyle and Doyle protocol. A number of earlier workers[20,21] also recommended the use of PVP with the molecular weight of 10,000 at 2% (w/v) to address the problem of phenolics.

PVP with low molecular weight has less tendency of precipitating with the nucleic acids as compared to PVP with the high molecular weight.[22] Also, Sahasrabudhe *et al*.[23] worked on standardization of DNA extraction and optimization of RAPD-PCR conditions of *Garcinia indica*. They worked on modified CTAB protocol adding Polyvinylpyrrolidone (PVP) in separate tubes and precipitating with 5M NaCl along with chilled alcohol to increase the solubility of polysaccharides.They could isolate pure and sufficient amount of DNA and optimize RAPD conditions.

The experimental observations showed that the average petiole length of the leaves collected from Dhemaji district showed the highest leaf petiole length and the average petiole length of the Jorhat district showed the lowest petiole length. The average mid-vein of the leaves collected from Tinsukia showed the highest mid-vein length and the average mid-vein length of the Jorhat district showed the lowest mid-vein length. Similar observation was observed on diversity of *Garcinia* species in the Western ghats, that *Garcinia* species shows varying degree of diversity in floral morphology, fruit morphology and leaf morphology.[24]

The ladder in this experiment was more than 5000 bp and its comparison with the extracted DNA revealed that the genomic DNA of *Garcinia cowa* was more than 5000 bp in gel electrophoresis. Spectrophotometry revealed the concentration of isolated DNA to be more in 4X buffer than in 2X buffer.

PCR analysis showed polymorphism only with UBC-40, UBC-41 and UBC-42 primers out of 30 taken for the study. Genetic diversity was found to be present among larger accession from Khonamukh (Sivsagar) and the smallest accession from Jokai (Dibrugarh) with a polymorphic percentage of 50% with UBC- 40 and 100% with UBC- 41 and UBC-42. Similar study was carried out by Gogoi *et al.,*[25] out of 42 genotypes of *Garcinia* species, *G. dulcis* and *G. xanthochymus* to be the closet species genetically which were established by both ISSR molecular marker and ITSR region sequence analysis.

CONCLUSION

The highest number of *Garcinia cowa* was found in Tinsukia district. Morphological diversity was observed in both parts of the Brahmaputra valley (both in the northern and southern banks). 4X buffer is provided to be more efficient than 2X buffer in case of DNA isolation. Polymorphism percentage in *Garcinia cowa* was seen to be 100% in the case of ISSR primer UBC-41 and UBC-42. It can be concluded that the diversity of *Garcinia cowa* is not only affected by the environment but also this species is genetically diverse. This is the first genetically analysed report in North East India, which revealed the genetic diversity among the different genotypes of *G. cowa*. This study will help and encourage other researchers and plant breeders to understand the molecular diversity and identification of *Garcinia* species. Such approaches will not only help in the management of germplasm collections by avoiding redundancy but also in authentication of the genus *Garcinia*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DNA: Deoxyribonucleic Acid; **PCR:** Polymerase chain reaction; **CTAB:** Cetyltrimethyl ammonium bromide; **TBE:** tris Borate EDTA; **T.E:** Tris-EDTA; **ISSR:** Inter Simple Sequence Repeats; **EDTA:** Ethylenediamine tetraacetic acid.

SUMMARY

Garcinia cowa is an economically beneficial yet lesser known species. North east India is blessed with diverse forms of *Garcinia* and one among them is *Garcinia cowa*. Field study was carried out to study the morphological diversity of the species. Genomic DNA was extracted using 2X and 4X buffer and genetic polymorphism was detected using PCR analysis. The polymorphic percentage was also calculated. Further research on this species can reveal many unknown facts. Hence, this research is an initial step in the line.

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