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Garcinia species are tropical, endemic, potential under-exploited, wild and semi-domesticated crops. It has also been considered to make foods more filling and satisfying and has been used routinely for many centuries with no known toxicity. This herb has been used historically in India to support the treatment of various health conditions. This under-exploited crop Garcinia requires a detailed study with respect to its biochemical and molecular properties and also its relation with geographical distribution. The species of Garcinia incorporated in the present study are Garcinia gummigutta, Garcinia indica, Garcinia tinctoria and Garcinia cowa.

4.1. MATERIALS, REAGENTS AND INSTRUMENTS FOR THE STUDY

4.1.1. Sources of Chemicals and other materials for the study

Chemicals for the preparations of solutions, buffer, organic solvents and reagents were purchased from the following companies and stored according to the manufacturer’s recommendation:

1. Bangalore Genei Pvt Ltd
2. Hi-Media laboratories
3. Merck Ltd India
4. Qualigen Fine chemicals, India
5. Sigma chemicals
6. SISCO Research Laboratory Pvt Ltd
7. Fluka Laboratory chemicals and Analytical reagents
4.1.2 Instruments used in the present study

1. UV 160A spectrophotometer
2. HPLC system with Shimadzu SPD-10A VP, UV-VIS spectrophotometric detector
3. HPLC system with Shimadzu RID-10A refractive index detector
4. Perkin-Elmer Gas Chromatography with FID detector
5. Shimadzu Gas chromatography (GC-2010) with mass spectrum detector
6. Eppendorf PCR machine
7. Agarose Gel electrophoretic system
8. Alpha imager Gel documentation system
9. Hai com blue tooth GPS
10. HP IPAQ hx 2400 series palmtop with blue tooth technology
11. PC with DIVA- GIS software as a tool

4.1.3 Accessions to enable the study

Four fruiting *Garcinia* species viz. *Garcinia gummigutta*, *Garcinia indica*, *Garcinia tinctoria* and *Garcinia cowa* were collected by

- Conducting surveys through Western Ghats with the help of GPS and Palmtop where maximum species richness and diversity is available
- Germplasm accessions from different locations maintained at NBPGR, Thrissur
- Repository germplasm of IISR Peruvannamuzhy and Calicut
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4.1.3.1. Collection of *Garcinia* species from natural forests using GPS and Palmtop.

The longitude, latitude and altitude of each *Garcinia* collection spots in Western Ghats were recorded by Hai- com blue tooth GPS connected with HP iPAQ hx 2400, blue tooth series palmtop as points in Arc-map software. These points were used in GIS studies for preparing maps.

4.1.3.1.1. GPS and Palmtop - its use in collection

The Global Positioning System (GPS) is a satellite based navigation system that sends and receives radio signals. Using GPS technology, it is possible to determine location, velocity, time for 24 hrs in a day at any weather condition anywhere in the world. In this study, the corresponding latitude and longitude were scored as points (X, Y) in Arc-Map software which was already installed in HP palmtop. These points were taken again in the lab and converted to corresponding latitude and longitude by the below formulae,

To add the X coordinate of points

```vba
Dim Output As Double
Dim pPoint As IPoint
Set pPoint = [Shape], Output = pPoint X
```

To add the Y coordinate of points

```vba
Dim Output As Double
Dim pPoint As IPoint
Set pPoint = [Shape], Output = pPoint Y
```
4.2. BIOCHEMICAL ANALYSIS IN *Garcinia* Species

Biochemical constituents such as (-) hydroxycitric acid, dextrose and fructose were quantified from this species with the help of HPLC techniques. The leaf volatile oil constituents and free fatty acids from seed kernel were identified by Gas chromatography (GC).Total Carbohydrate, Starch, Reducing Sugars and Total phenols were quantified from the leaf, fresh and dry rinds of this species by spectrophotometric method.

4.2.1. Estimation of (-) Hydroxycitric Acid and Sugars in *Garcinia* Spp. by HPLC

Detector used for the quantification of (-) hydroxycitric acid was UV-VIS spectrophotometric, but for sugars it was RI detector with mobile phase of water and acetonitrile in the ratio 75:25.

4.2.1.1. Estimation of (-) Hydroxycitric Acid in *Garcinia* species.

4.2.1.1.1. Preparation of standards

A standard solution for HPLC was prepared with 0.1 g HCA calcium salt and 0.03 g fumaric acid. These standards were dissolved in deionized water (100 ml) of pH 2 separately. They were mixed in the proportion of 4:1, which contained 0.8 g/lit of (-) HCA calcium salt and 0.06 g/lit of fumaric acid respectively. Here fumaric acid was used to get the ratio of peaks, to quantify the exact amount of HCA in the sample.

\[ \text{DO234} \]
4.2.1.1.2. Extraction and preparation of sample

1g of the tissue (leaf/ind) was extracted with deionized water (pH 2). The filtrate was made up to 100 ml. 20 ml of the sample was taken and was made up to 25 ml with fumarc acid.

4.2.1.1.3. HPLC analysis of standards and samples

25 microlitre of standard solution was injected into HPLC. From the chromatogram, the retention time and area under the (-) HCA calcium salt and that of Fumaric acid were determined. This was repeated three times. Similarly, 25 microlitre of sample solution was injected into the column and the area under (-) HCA and Fumaric acid were determined based on the earlier information from the standard. As the standard used was fumaric acid, the calculation was done against fumaric acid.

4.2.1.1.4. Chromatographic condition

Shimadzu LC-10AT VP pump was connected to Shimadzu SPD-10A VP, UV-VIS spectrophotometric detector and integrated C-18 reversed phased column was used for the isocratic elution and the absorbance was noted at 203 nm. Mobile phase was 0.1 M sodium sulphate solution with a pH 2 and the flow rate was maintained at 1 ml/minute. (Asish et al, 2007)
4.2.1.5. Calculation

Since HCA calcium salt contains only 80.5% available HCA

\[
\frac{\text{Area of Fumaric Acid} \times 80.5}{\text{Area of HCA calcium salt}} = R
\]

% of free HCA in the sample = \[
\frac{\text{Area of HCA in the sample} \times R \times 100}{\text{Area of Fumaric Acid}}
\]

4.2.1.2. Estimation of (-) Sugars in *Garcinia* species

4.2.1.2.1. Preparation of standards

A standard solution (10 mg/ml) for HPLC was prepared with distilled water by using glucose and fructose as standards

4.2.1.2.2. Extraction and Preparation of Samples

0.25 g of the tissue (leaf/rind) was homogenized and digested directly with 2.5 N HCl and it was neutralized with sodium carbonate and finally made to 25 ml with distilled water. This solution was used for HPLC quantification

4.2.1.2.3. HPLC analysis of Standards and Samples

25 microlitre of standard solution was injected into HPLC. With the help of chromatogram, the corresponding retention time and area of standards were determined. Similarly 25 microlitre of sample solution was injected into the column and the area based on the earlier information was noted and used for calculation
4.2.1.2.4. Chromatographic condition

Shimadzu LC-20A D pump was connected to Shimadzu RID-10A refractive index detector and integrated with Lab solution software. C-18 reverse phased column was used for the isocratic elution. Mobile phase used for the analysis was water and acetonitrile in the ratio 75:25.

4.2.1.2.5. Basic formulae for Calculation

\[
\text{Area of sample} \times \text{Purity Factor} \times 100 = \% \text{ of compound} / \text{Area of standard}
\]

Where concentration of the sample and standard are same.

4.2.2. Identification of volatile constituents and Fatty acid from *Garcinia* Spp. by Gas Chromatography (GC).

Essential oil was extracted by hydro distillation. The oil was further subjected to GC-MS and corresponding constituents were identified by matching the mass spectral data with those stored in NIST and Wiley libraries. Similarly, the free fatty acids present in the seed kernel of *G. gummigutta* and *G. indica* were converted to fatty acid methyl esters (FAME) and were identified by GC-FID using authentic standards.

4.2.2.1. Identification of volatile constituents from the leaves of *Garcinia* Spp. by GC-MS.

4.2.2.1.2. Extraction of volatile oil from leaves of *Garcinia* Spp.

About 300-400 gms of dried leaves of *Garcinia* species collected from different locations of Western Ghats were extracted by hydro distillation using...
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clevenger trap method (ASTA, 1968) for essential volatile oil. The oil yield was noted after the extraction. The oil was further analyzed using a Shimadzu GC-2010 gas chromatograph equipped with QP 2010 mass spectrometer.

\[
\text{Volatile oil (\%)} = \frac{\text{Volume of oil (ml)}}{\text{Weight of sample (g)}} \times 100
\]

4.2.2.1.3. Chromatographic condition for GC-MS and Identification

The carrier gas used here was helium with a flow rate of 1.0 ml/min. The injection port was maintained at 250°C, the detector temperature was 220°C, the oven was programmed as follows: 70°C for 5 minutes and then increased to 110°C at the rate of 5°C/ min, then up to 200°C at the rate of 3°C/ min again up to 220°C at the rate of 5°C/min, at which the column was maintained for 5 minutes. The constituents of the oil were identified by matching the mass spectral data with those stored in NIST and Wiley libraries and wherever possible, by co-injection with authentic standards. Among the compounds, which showed maximum similarity with NIST and Wiley libraries only was taken and others which showed lesser similarity was not taken for consideration.

4.2.2.2. Identification of free fatty acids from the seeds of *Garcinia gummigutta* and *G. indica* by GC-FID

4.2.2.2.1. Extraction of butter from the seed kernels of *G. gummigutta* and *G. indica*

Fresh fruits were cut into halves and separated seeds were dried for 15-20 days. The dried seeds were rubbed on hard surface with a wooden plank to
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separate the kernels from the seed coat. About 100 gm's of kernel paste was boiled in 500 ml of water for 4-6 hrs in an open pan. Later the pan was covered with cloth and butter was separated by decantation and yield of butter was noted (ASTA, 1968).

4.2.2.2. Conversion of free fatty acid to fatty acid methyl esters (FAME)

About 500 mg of butter was incubated (70°C) with 5 ml of 3N of NaOH and methanol in the ratio 1:9 for 2 hrs. It was separated with 15 ml of hexane for removing steroids from free fatty acids. Lower fraction was taken from the separating funnel and it was acidified with 0.8 ml of 6 N HCl. The acidified fraction was further separated with 15 ml of hexane and upper fraction was taken for treatment. The upper fraction separated was treated with methanol and HCl in the ratio 5:1 and incubated at 70°C for 5 hrs. Again it was separated with 15 ml of hexane and upper fraction was taken for further analysis. Thus the converted FAME was used for identification of free fatty acids with the help of GC-FID (Hennessey et al., 1983).

4.2.2.2.3. Chromatographic condition for fatty acid standards and samples

The carrier gas used was Nitrogen. The injection port was maintained at 240°C, the detector temperature was 240°C, the oven was programmed as follows, 150°C for 3 minutes and then increased to 200°C at the rate of 5°C/ min at which the column was maintained for 4 minutes. The constituents of the hexane extract were identified by Perkin Elmer Gas chromatographic system with authentic standards of Stearic, Palmitic and Arachidic acid methyl esters.
4.2.3. Estimations of primary metabolites in *Garcinia* Spp. using Spectrophotometer.

Estimations of Total Carbohydrate, Starch, Reducing Sugars and Total Phenols from *Garcinia* leaves and rinds were quantified using UV 160A spectrophotometer with wavelength varying from 190-630 nm.

**4.2.3.1. Estimation of Total Carbohydrate - Phenol Sulphuric acid method**

In hot acid medium, glucose is dehydrated to hydroxy methyl furfural. This forms a green colored product with 5% phenol, and has an absorption maximum at 490nm.

To estimate total carbohydrate from *Garcinia*, young matured leaves, dried rinds and fresh fruits were homogenized and digested directly with 25N HCl. The amount of total carbohydrate present in the *Garcinia* Spp. is dehydrated to hydroxyl methyl furfural and the colour developed by phenol was estimated at 490 nm as described by Sadasivam and Manickam (1992).

**4.2.3.2. Estimation of Starch - Anthrone reagent method**

To remove sugars, the sample is treated with 80% alcohol. Then starch is extracted with perchloric acid. In hot acid medium, starch is hydrolyzed to glucose and dehydrated to hydroxy methyl furfural. This compound forms a green coloured product with Anthrone and has the maximum absorption at 630nm. Starch from youngest matured fresh leaves and homogenized fruit
samples were estimated by the Anthrone method as described by Sadasivam and Manickam (1992)

4.2.3.3. Estimation of Reducing Sugars - Nelson-Somogyi method

The reducing sugar from leaf and fruit samples of *Garcinia* was estimated by the Nelson-Somogyi method as described by Sadasivam and Manickam (1992). The blue colour developed is read at 620 nm against glucose standard.

4.2.3.4. Estimation of Total Phenol Content - Folin - Ciocalteue method

Phenols react with Phosphomolybdic acid in Folin – Ciocalteue reagent in alkaline medium and produce blue colored compound. Phenol was extracted from *Garcinia* leaves and fruits by 80% alcohol. The extract was treated with Folin’s reagent and sodium carbonate. The developed blue colour was read at 660 nm as described by Sadasivam and Manickam (1992).

4.2.3.5. Calculation for Spectrophotometric method

\[
\text{Percentage of the sample} = \frac{\text{Vol of sample} \times \text{Conc of aliquot}}{\text{Wt of sample} \times \text{aliquot taken}} \times 100
\]

4.3. MOLECULAR STUDIES IN *Garcinia* Species

A modified Doyle and Doyle (1990) protocol for the isolation of DNA from *Garcinia* species were standardized. In addition to this, an optimum concentration and condition for RAPD profiling in this species was also normalized.
4.3.1. Isolation of DNA

Successful isolation of genomic DNA is a pre-requisite to any work in plant molecular biology. Several methods were used to isolate genomic DNA from the selected species of *Garcinia*. Since satisfactory results were not obtained with the protocols already reported for other plant species, a modified protocol was standardized for DNA isolation of *Garcinia* by repeated trial and error method.

4.3.1.1. Reagents and Preparation

1. Cetyl Trimethyl Ammonium Bromide (CTAB Buffer)
   (a) 1M Tris (pH 8.0)
   (b) 0.5 M EDTA
   (c) 5M NaCl
   (d) 4% CTAB
   (e) 2% β-mercaptoethanol

(a) 1M Tris

121 g of Tris base was dissolved in 800ml of water. The pH was adjusted to 8.0 by adding conc HCl.

(b) 0.5M EDTA

186.1 g of disodium ethylene diamine tetra acetate 2H2O was added to oneL of distilled water. Stir vigorously on a magnetic stirrer. The pH was
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adjusted to 8.0 with NaOH Dispensed in aliquots and sterilized by autoclaving

c) 5M NaCl

292 g of sodium chloride was dissolved in 800ml of water. The volume was adjusted to one litre. Dispensed in aliquots and sterilized by autoclaving.

d) β-mercaptoethanol

Usually obtained as 14 4M solution stored in dark bottle at 4°C

2. RNase

Pancreatic RNase was dissolved at a concentration of 5mg/ml in distilled water. It was heated for 15min and allowed to cool slowly to room temperature. pH was adjusted to 5.2 by adding 0.1 volume of Tris HCl (pH 7.4) dispensed into aliquots and stored at -20°C.

4.3.1.2. Protocol for DNA isolation from Garcinia leaves and rinds

- 100 mg of the tissue was ground in 500μl of 4% / 2% CTAB buffer using a chilled mortar and pestle and transferred to 1.5 ml centrifuge tubes.
- The tubes were incubated at 65°C for 30 minutes.
- The tubes were taken out and an equal volume of phenol chloroform isoamyl alcohol (25:24:1) was added.
- The tubes were centrifuged at 8,000 rpm for 10 minutes at 4°C.
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- The supernatant was transferred to a new centrifuge tube and 200 µl of 10% CTAB was added and mixed. After mixing, 200 µl of chloroform isoamyl alcohol (24:1) was added and mixed well.
- The tubes were centrifuged at 8,000 rpm for 10 minutes at 4°C.
- The supernatant was collected and the DNA was precipitated by adding an equal volume of ice cold isopropanol. It was incubated at room temperature for one hour.
- The tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C.
- The DNA pellet was washed with 70% ethanol, air dried and dissolved in 100 µl of nuclease free water and transferred to 1.5 ml microfuge tubes.
- 2 µl of RNase (10 mg/ml) was added to the DNA solution and was kept at 37°C for one hour.
- An equal quantity of phenol chloroform isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at 10,000 rpm for 15 minutes at 4°C.
- The supernatant was transferred to a new tube, an equal volume of chloroform isoamyl alcohol (24:1) was added, mixed well and was centrifuged at 10,000 rpm for 15 minutes at 4°C.
- To the supernatant, 100% alcohol was added so as to precipitate the DNA.
- DNA pellet was collected by centrifugation at 10,000 rpm for 10 minutes at 4°C and washed with 70% ethanol and air dried.
- Re-dissolve the pellet in appropriate volume of nuclease free water and was stored at -20°C for future use.
4.3.2. DNA quantification

The isolated DNA has to be quantified in order to perform PCR reaction. The most common methods of DNA quantification are agarose gel electrophoresis and spectrophotometric method.

4.3.2.1. Agarose Gel Electrophoresis

4.3.2.1.1 Preparation of Reagents

(a) Tris Acetate EDTA (TAE) Buffer- 50 X

242 g of Tris base was dissolved in water, 57 ml of glacial acetic acid, and 100 ml of 0.5 M EDTA (pH 8.0) were added. Volume was made up to 1 litre. Dispensed into aliquots and sterilized by autoclaving.

(b) 6X Loading Dye

Dissolve 0.25 g of Bromophenol Blue in 99 ml of 30% glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and keep in 4°C.

(c) Ethidium Bromide

1 gram of ethidium bromide was added to 100 ml water. It was stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The container was wrapped in aluminium foil or transferred to a dark bottle and stored at 4°C.
4.3.2.1.2. Protocol for Electrophoresis

- Fix the tapes to ends of gel tray. Keep the combs and place the gel tray horizontally.
- Prepare 0.8% agarose in 1X TAE (0.8 g agarose in 100 ml 1X TAE). Boil the solution in a microwave oven until all the agarose particles are completely dissolved. Allow to cool.
- Add 2 μl of ethidium bromide and pour to gel tray.
- After 20 minutes, remove the tape and comb, place the gel into the tank and pour 1X TAE until the gel is fully immersed.
- Load the DNA samples with loading dye (6X) into the wells with a standard marker DNA.
- Run gel at 50-60 volts.
- Visualize the DNA bands on a UV transilluminator and document gel using a Gel documentation system (Alpha Imager).

4.3.2.2. Quantification - using UV Spectrometer

1 μl of all the DNA samples were diluted to 50 μl using nuclease free ultra pure water and absorbance at 260 and 280 nm were measured. Absorbance reading of 1 at 260 nm is assumed to contain 50 μg of double stranded DNA per ml free of proteins, RNA and polysaccharide contaminants.

The quantity of DNA present in the undiluted sample was calculated as per the following formula.

\[
\text{Amount of DNA (μg/ml)} = A_{260} \times 50 \times \text{dilution factor}
\]
4.3.3. Standardisation of RAPD-PCR reaction in *Garcinia* species

RAPD-PCR parameters were optimized in *Garcinia* species with random primers. The optimum DNA concentration and annealing temperature were also noted. The concentrations for dNTPs, Taq DNA polymerase and MgCl₂ were also standardized and confirmed by trial and error method.

4.3.3.1. PCR components

4.3.3.1.1. Template DNA

Diluted DNA sample was taken for 25 µl reaction.

4.3.3.1.2. Taq DNA polymerase

An optimum enzyme concentration of Taq DNA polymerase was used in 25 µl reaction mixture was used for the analysis' (Bangalore Genei, India).

4.3.3.1.3. dNTPs

Concentration of dNTP's were also standardized in *Garcinia* species for RAPD-PCR reaction (Bangalore Genei, India).

4.3.3.1.4. MgCl₂ and Assay buffer

A proper concentration of MgCl₂ (Bangalore Genei, India) was used for amplification along with buffer having a final concentration of one 'X' (Bangalore Genei, India).
4.3.3.1.5. Primers
About 30 random primers were screened for RAPD and 12 primers were found for suitable amplification. 10 pico moles of each primer were used in 25 μl reaction volume. The reaction mixture for PCR reaction was also standardized.

4.3.3.1.6. Amplification Condition

The PCR amplification condition for RAPD analysis is standardized in selected species of Garcinia. Several conditions were used to amplify genomic DNA from Garcinia spp. Since satisfactory results were not obtained with the conditions already reported, a modified condition with a suitable annealing temperature was standardized by repeated trial and error method.

4.3.4. Distribution of Garcinia species based on RAPD analysis

The amplified products of RAPD were resolved by electrophoresis using 1.5% agarose gel. The DNA bands were visualized on a UV transilluminator and the gel was resolved using a gel documentation system (Alpha Imager2220). The products were scored and data analyzed statistically using appropriate software package.

The electrophoretic patterns were visually analysed and DNA bands were scored as present (1) or absent (0). The matrix obtained was entered into NTSys-pc program (Rohlf, 1993) and Dice similarity index was calculated for each pair of samples. A UPGMA dendrogram was constructed.
4.3.5 Heterogeneity index in *Garcinia gummigutta* and *G. indica*

Heterogeneity index for the collected accessions of Garcinia species *viz.* *G. gummigutta* and *G. indica* were noted. The molecular diversity or heterogeneity was calculated according to the formula by Nei (1975) and is as follows:

\[
\text{Heterogeneity index} = 1 - \frac{\sum X^2}{N}
\]

Where \(X\) = No of occurrence of a particular band

\[N\] = Total number of samples

\[N\] = Total Number of bands with all the primers

4.4 MOLECULAR AND BIOCHEMICAL DIVERSITY IN *Garcinia* spp. USING GIS

GIS is becoming an important tool in plant genetic resources conservation, including mapping the eco geographical distribution and predicting potential distribution of species using geo-referenced data from diverse sources. The approach of using GIS is to combine different kinds of data in some sort of mathematical model. DIVA – GIS is user-friendly GIS software and handles most of the tasks in plant biodiversity mapping. Analysis of such eco-geographic patterns can make considerable contributions to several plant genetic resources research activities, including planning collecting programs, targeting genetic resources for breeding programs, developing core conditions, selecting and designing sites for *in situ* conservation and assessing the potential impact of the products arising from the use of plant genetic resources.
4.4.1. About DiVA-GIS

The DIVA-GIS software allows analysis of gene bank and herbarium database to elucidate genetic, ecological and geographic pattern in the distribution of crops and wild species. DIVA-GIS help to improve data quality by assigning coordinates, using a large gazetteer. DIVA can also be used to check existing coordinates using overlays of the collecting site and administrative boundary databases and maps can then be made of the collection sites. Analytical functions implemented in DIVA include mapping of richness and diversity, distribution of useful traits and location of areas with complementary diversity. DIVA can also extract climate data for all terrestrial locations, which can be used to describe the environment of collection sites.

The most popular diversity index is Shannon’s diversity index (SHDI) based on information theory (Shannon and Weaver 1949). The value of this index represents the amount of "information" per individual (or patch). The absolute magnitude of Shannon’s diversity index is not particularly meaningful, therefore, it is used as a relative index for comparing different landscapes or the same landscape at different times.

Shannon’s diversity index

$$H' = - \sum n_i \ln p_i$$

$$n_i = \text{number of individual in the } i\text{-th class}$$

$$p_i = \text{proportional abundance of the } i\text{-th class} = \frac{n_i}{N}$$
4.4.1.1. Relation of Ecological Variables in *Garcinia* species diversity using GIS

Ecological factors such as temperature, altitude, and rainfall maps (BIOCLIM) were prepared by DIVA - GIS, to study the reaction of these micro environmental variables with respect to *Garcinia* diversity and find any possible effect of these variables in clusters. (Hijmans et al., 2003)

4.4.1.2. Species Diversity studies using GIS technology

The biochemical and molecular data were analyzed with DIVA-GIS to identify the geographical areas which showing maximum diversity grids (Grid of 50 - 50 km cells/10 - 10 km) for simple and neighborhood diversity were prepared for a detailed analysis to isolate the geographical areas which were showing a very high diversity of this species (Hijmans et al., 2003)