CHAPTER - 4

METHODOLOGY

The bryophytes are a group of plants that have fewer species, are of a small size, have subdued colours and also have less ecological significance (Goffinet and Shaw, 2008) and hence they had have received lesser attention by plant researchers. This trend has changed in the past two decades and the group is now receiving attention of a diversity of plant researchers (Vanderpoorten *et al*., 2010). The objectives of the study in a very large way decide on the methodology to be followed. As enumerated in the objectives earlier, the current study focuses on determining the diversity and distribution of bryophytes for the Eastern Central Gujarat Region for which except for one locality, no information existed till 2013 (Shah and Gujar, 2016). The following figure (Fig 4.1) depicts the broad outline of the methodology that was followed to achieve the objectives of the current study. Detailed methodology followed for each of the objectives have been described under their specific headings.

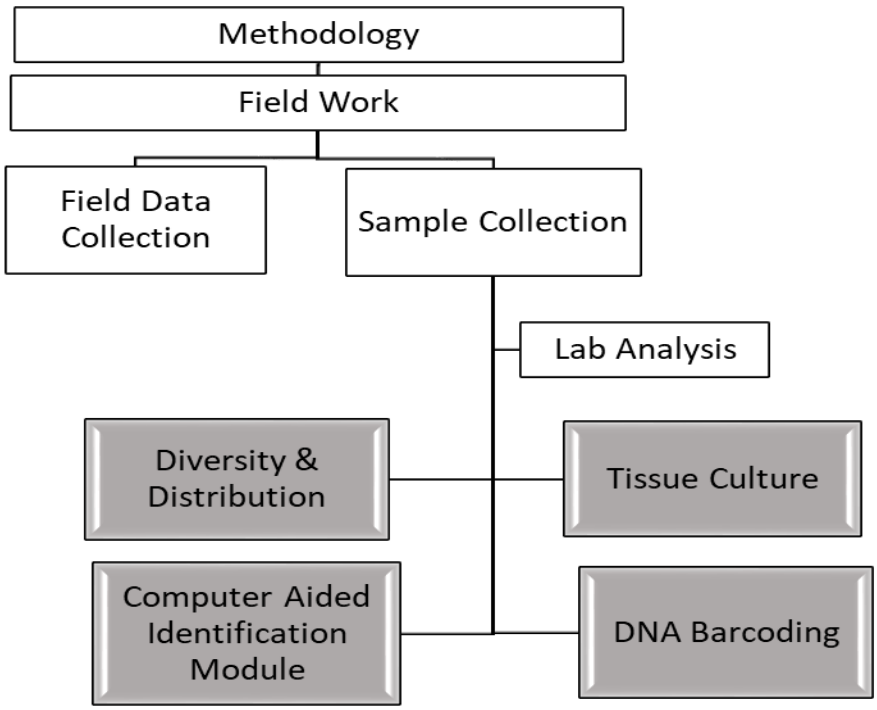

Fig. 4.1 Major objectives of the current study.

4.1 Diversity and distribution studies:

The review of literature had revealed that except for the Pavagadh hills, information about bryophyte diversity was lacking for the whole study area. To accomplish this objective of the study the methodology involved extensive field work as well as laboratory work. The outline of the methodology followed for this objective has been depicted in the figure below.

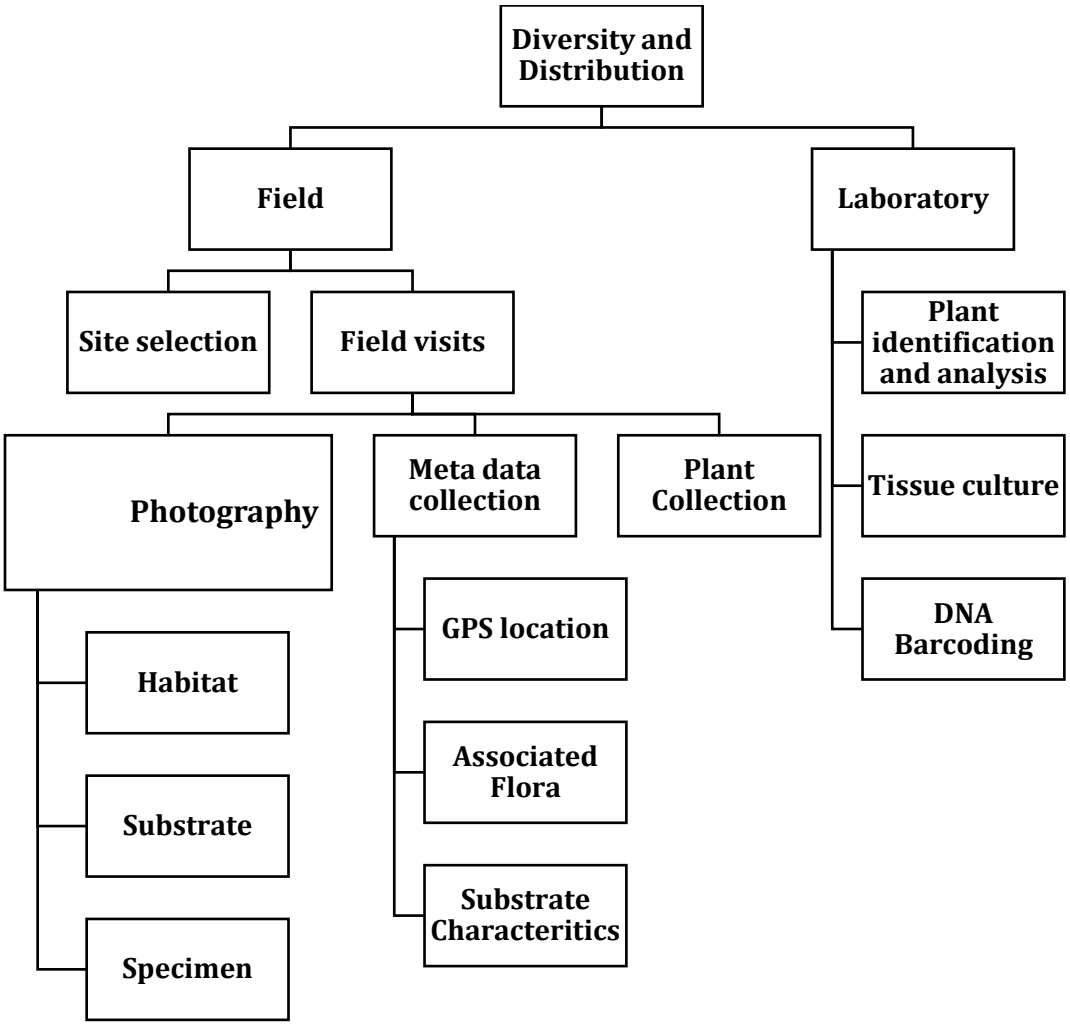

Fig 4.2: Outline methodology of diversity and distribution study

4.1.1: Permissions:

The study area comprised of wildlife sanctuaries, reserve forest areas and agricultural areas in addition to other land use classes. Permission to visit the Reserve Forest Areas was sought from the Forest Department - Vadodara Circle (Letter no.: T.21/B/337 of 2016-17 dated 25/05/2016). Permission for sample collection from the Wildlife Sanctuaries was sought from the PCCF-Wildlife (Letter no.: WLP/28/C/294-295/2016-17 dated 01/08/2016). This permission to work in the Sanctuaries took a long time to get by the time which the funding for field visits was over. After the permissions were received each of the sanctuaries were visited two times using own vehicle. However a detailed survey of the area could not be accomplished in the WLS areas.

4.1.2: Site Selection:

Bryophytes occur on a wide variety of substrates ranging from moist soils, old brick walls, building facades and are also found growing epiphytically on the bark and leaves of other plants (Berg *et al.*, 2002, Gabriel and Bates, 2005). As described earlier, the study area had a diversity of habitat types ranging from the agricultural plans in Vadodara District to the deciduous forests on the hilly terrain in Narmada and Dahod districts. The Pavagadh Hill had emerged as a center of bryophyte diversity during the work of Chavan *et al*. (1961) and Chaudhary *et al*. (2006) and a detailed field study was carried out on the pavagadh hills. Bryophytes have also been reported from old buildings by several workers in Gujarat and surrounding areas (Chavan, 1937; Chavan and Mahabale, 1945; Chavan *et al.*, 1961,Chaudhary and Sharma, 2002, Chaudhary *et al.*, 2006, Alam, 2011). Old monuments were hence, also prioritized for the study. About 8-12 sampling sites were finalized in each district of the study area after preliminary visits. Details of the sampling sites visited have been given in the results. Glimpses of sites are given below in fig. 4.3

4.1.3: Seasonality and duration of Field visits:

The study area shows a distinct dry and wet climate phase and hence except for some very restricted pockets, bryophyte growth is observed for a few months starting with the onset of the monsoon. Most of the field work was carried out during and after the monsoon

season (July – December). However a few sites were visited even till February-March due to delay in permissions. For sites which were located near Vadodara, the sites were visited for a day, while for sites in Narmada and Dahod districts the duration of the field visits would be 3-4 days. Glimpses of sites visited is shown in Plate1

4.1.4: Field data Collection:

The outline diagram given above show the different tasks involved in field data collection. After reaching the sites, a quick survey of the surrounding area was done and the locations where bryophyte species occurred were identified. Each location was then visited again for sample collection, field photography and collection of ancillary data.

4.1.4.1: Field Photography

Field photography was undertaken using a Canon Powershot A700 camera and a Celestron hand held field microscope camera. The Canon camera had a super macro mode which allowed photography of the specimens from a distance of less than 1 cm. This enabled close-up photography of the specimens. Photographs of the general habitat, the specimen and the substrate were taken. For close up photography a standard scale or a coin was used to get information about the size of the specimen and its different parts. Photographs of the surrounding plants and the substratum were also taken to study associations. Paper tags with details were also photographed so that which photographs are associated with each sample could be ascertained later on. Rainfall during the field visits made the photography during the field work challenging.

4.1.4.2: Sample Collection

The bryophytes were first observed using a hand lens or a 20x loupe before the samples were collected. The samples were scraped from the substratum with the help of knife or a scalpel. Care was taken that some amount of substratum was present along with sample. The guidelines given by Glime (2017) with necessary modifications were followed during sample collection. Several times during field work, rainfall was encountered and hence the collected samples were first stored in small rectangular plastic boxes along with a small amount of tissue paper. These samples at the end of the day were transferred to brown

paper bags and then allowed to air dry under a fan. Enough sample to analyze the specimen and prepare two herbarium packets was collected. Care was taken to collect the reproductive structures specifically the sporophyte, if present. Generally 5-10 gm. of sample was collected while in case of less availability of material, less amount of sample was collected. Care was taken so that the location was left with enough amount of plant material so it can continue growing. All the brown paper bags containing the specimens were kept in the open under a fan in the guest house during the duration of the field visit. They were then packed into cardboard cartons and then brought to the laboratory where they were again subjected to air drying under a fan.

4.1.4.3: Other data collection

In addition to the samples and the photographs, additional information regarding the substrate type, the associated plants species, the GPS coordinates and the altitude of the locality was collected initially in a field diary and as soon as possible they were transferred to a sample sticker which was then stuck to the collected bryophyte sample. The GPS coordinates and the altitude was collected using a GARMIN e-Trex 10 hand held GPS device. Care was taken that the GPS device connected to at least 5 satellites before the positions was established. For epiphytic specimens, the host plant was recorded and where it was not possible to identify it, twigs of the plant were collected to facilitate identification.

4.1.5 Laboratory Analysis:

The samples collected in the field were processed in different ways depending on the objective to be achieved.The below task were carried in the laboratory.

4.1.5.1 Plant Identification:

The collected samples were analyzed as soon as possible, however in situations where field work was carried out for several days the lab analysis would be delayed. Such samples would first be hydrated in a large watch glass or petri-plate. Sample analysis guidelines given in Glime and Wagner (2013) were followed. They were observed under LEICA M80 Stereo-binocular microscope. Characteristic features of the species present in the sample were photographed using either a 3.2 MP Tucsen Camera or a 1.2 MP Celestron camera

which could be attached to one of the eye pieces of the microscope. The sample was checked for the presence of sexual reproductive bodies and sporophytes. For each sample, a few individual plants of different species were segregated and separated out into separate slides or watch glasses and then photographed along with a standard scale.

Based on the type of bryophyte (liverwort, hornwort or moss), the specimens were subjected to detailed observations under stereo-binocular microscope and compound microscope. The different parts of the specimens were subjected to hand sectioning and the characteristic macro-morphological, micro-morphological and anatomical features were observed and photographs were taken. Stage and ocular micrometers were used to obtain the sizes of different cell types and spores. Some specimens were immersed in dilute HCl or KOH (Zander, 1993) to clear the specimens. The sections were not stained. Data sheets of each of the sample were prepared and filed. These characters and the photographs were then used to obtain preliminary identification of the species. Some families have characteristic micromorphological characters like papillose or mamillose leaf cells in Pottiaceae (Zander, 1993; Chaudhary *et al.,* 2006) which helped in the identification of species even in the absence of reproductive structures.

Several standard regional, national and international monographs and floras were used for the identification of specimens They include, Srivastava (1964), Gangulee (1969-1980), Asthana and Srivastava (1991), Zander (1993), Bapna and Kachroo (2000), Singh (2002), Chaudhary *et al.*(2006) Aziz and Vohra (2008) and Chaudhary *et al.*(2008). The herbarium and bryology laboratory of the National Botanical Research Insititute (NBRI), Lucknow was visited twice along with the preliminarily identified herbarium packets for confirmation of identification. The senior principal scientist and the technical assistant in the bryology lab have immensely helped in confirming the identity of the specimens as well as identifying the unknown specimens.

4.1.5.2 SEM Analysis:

Selected members of Bryaceae and Pottiaceae were subjected to scanning electron microscopy (SEM) analysis. The on-payment based facility at Gujarat Ecolgical and Environmental Research (GEER) foundation was utilized for SEM analysis. The samples

were analysed on the Zeiss EVO-18 model and photographs were obtained in digital format (400 dpi).

4.1.5.3 Plant Distribution Analysis:

The distribution information of the bryophytes obtained during the field work were input into an excel sheet. The elevation data was also stored in one of the columns. To prepare the distribution maps the open source GIS software QGIS version 3.4 was used on a desktop PC. QGIS is one of the most used open source software which has a graphic user interphase (Leutner *et al.*, 2016) and is platform independent. It has a large and strong discussion group where major queries regarding map preparation and analysis can be solved. The administrative boundary maps for India were obtained from DIVA-GIS site (diva-gis.org) using the download data by country option. The administrative boundaries of the study area were then extracted from the data in QGIS. The digital elevation data used during the analysis was the Shuttle Radar Topography Mission (SRTM) Void-filled, 30 m spatial resolution data. This was browsed and downloaded from Earth explorer website (https://earthexplorer.usgs.gov).The Table 4.1below shows the data product specification of the SRTM data used.

Table 4.1. Data specifications of SRTM Void filled data

Sr. No.	Parameter	Specification
1	Projection	Geographic
2	Horizontal datum	WGS84
3	Spatial resolution	30 m
4	Vertical units	Meters

4.2 Tissue Culture:

The preliminary objective of the study was to evaluate the protocol for micro-propagation of *Funaria hygrometrica*. Plants were collected from different localities within forested and non-forested areas. The specimens were collected with mature sporophytes which had not dehisced. This was during the period from August – September. As discussed earlier *Funaria hygrometrica* was selected in the present study due to its wide use as a model specimen for teaching of mosses at school, undergraduate and post graduate levels and the

irregular and wrong supply of specimens by plant suppliers. It is also known to play a role as a pollution indicator (Adie *et al.,* 2014). The samples collected from the field were mixed with other species of bryophytes and had to be segregated and cleared of mud and debris. The outline of the different aspects of the tissue culture work have been graphically depicted belowm fig. 4.4

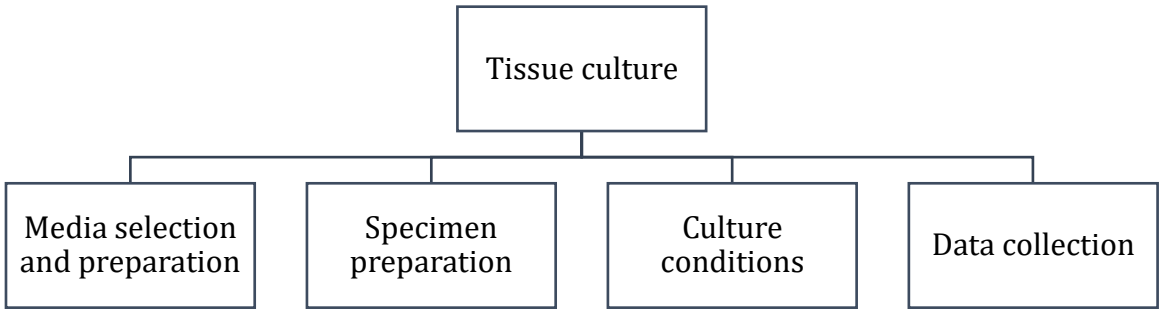

Fig 4.4: Outline methodology of Culturing of bryophytes.

4.2.1 Selection of appropriate media and its preparation:

The literature survey revealed that different researchers have used different media compositions for tissue culture studies. The common media composition used included the Knop media (Cano *et al.*, 1996; Chen *et al.*, 2009; Liang *et al.*, 2010), MS media (Post *et al.*, 2016, Sabovljevic *et al.*, 2002, 2010, Bijelovic *et al.*, 2004), Hoagland media (Asthana *et al.*, 2018), Parkers nutrient media (Pressel *et al.*, 2007), BCD (Liang *et al.*, 2010), Beneke media (Liang *et al.*, 2010) etc. Knop media is one the most preferred and widely used media by different bryologist for the culturing of mosses (Cano *et al.*, 1996; Chen *et al.*, 2009; Liang *et al.*, 2010, Sahu and Asthana, 2013, Silva-e-Costa *et al.*, 2016). Therefore for the present study, different concentrations of Knop media were used. The Table 4.2 shows the composition of the stock solution. All the chemicals were analytical grade and procured from SRL.

Table 4.2: Composition of stock solution for the above medium is given below (Reski & Abel, 1985):

Sr. No.	Chemical	Amount (g/l)
1	Potassium dihydrogen phosphate	25
2	Potassium chloride	25
3	Magnesium sulphate	25
4	Calcium nitrate	100

- For preparation of the stock solution, the above chemicals were dissolved in one litre of distilled water.
- The media was prepared from stock solution whenever needed. For 1 litre of Knop solution, 10 ml of above stock solution was taken and 12.5 mg ferrous sulphate was added. The pH was adjusted to 5.8 m using KoH/HCl.
- For Solid medium use 1.2% agar. Sterilize the medium. The media was stored in the refrigerator at a temperature of about 4 °C.
- The different combinations of the stock solution have been given in detail in the result section along with sucrose (2%).

4.2.2 Specimen preparation:

The pure *Funaria hygrometrica* patches which were segregated earlier were put in a beaker and washed in running tap water for about 1 hour by covering the mouth of flask with cheese cloth. (Flask was used as the narrow mouth of flask prevents the sample from moving out of the flask). A drop of liquid soap labolene was then added to the flask and the explants were agitated in the soap solution for a minute and solution was poured off. The explants were again washed in running tap water for 30 minutes.

Unopened capsule was cut open using sterile blade and inoculated in petriplate. For the surface sterilization, the sporophytes were kept in 1% sodium hypochlorite for few seconds. Then again they were rinsed with sterile distilled water.

4.2.3 Sterilization and Inoculation on Media:

Apparatus and materials such as the media to be used for the inoculation flasks, instruments used for aseptic transfer work such as inoculation forceps, scalpel, coupling jar, etc. were placed in laminar air flow chamber. The laminar hood stage was washed with Dettol's first then with rectified spirit. The ultra violet (UV) lamp was switched on to sterilize the working space in the laminar flow. After 20 minutes the airflow was switched on along with the UV lamp to blow away all the dust, spores, mycelia, etc. so as to make the inside of the chamber sterile as aseptic conditions were a pre-requisite for the culture work. After 20 minutes the UV lamp was switched off and the fluorescent lamp switched on.

Before starting the inoculation, the hands were swiped with 70% alcohol. The Petri plate containing the sterilized the media was brought near the flame for sterilization. The forceps were kept in the rectified spirit in a coupling jar and was flamed over a spirit flame. For the inoculation, operculum was removed, the sporophytes were split open with the help of sterilized forceps, and it was inoculated in the petri plate. For each treatment the inoculation was done in triplicate. Ṭhen the petriplates were kept in culture room under controlled conditions of temperature (25°C) and photoperiod. (8/16 Light/dark).

4.2.4 Data collection:

The progress of growth and the germination of the spores were documented. Micrometry of the protonema was carried out and the length and breath of Caulonema and Chloronema grown with/without sugar was measured.

4.3 DNA Barcoding

DNA barcoding was emerging as a new tool in the molecular identification of the specimens. The DNA barcoding of a few selected specimens was attempted to generate the barcodes for the specimens of the study area. The detailed methodology followed to accomplish this objective has been graphically in Fig. 4.5 below.

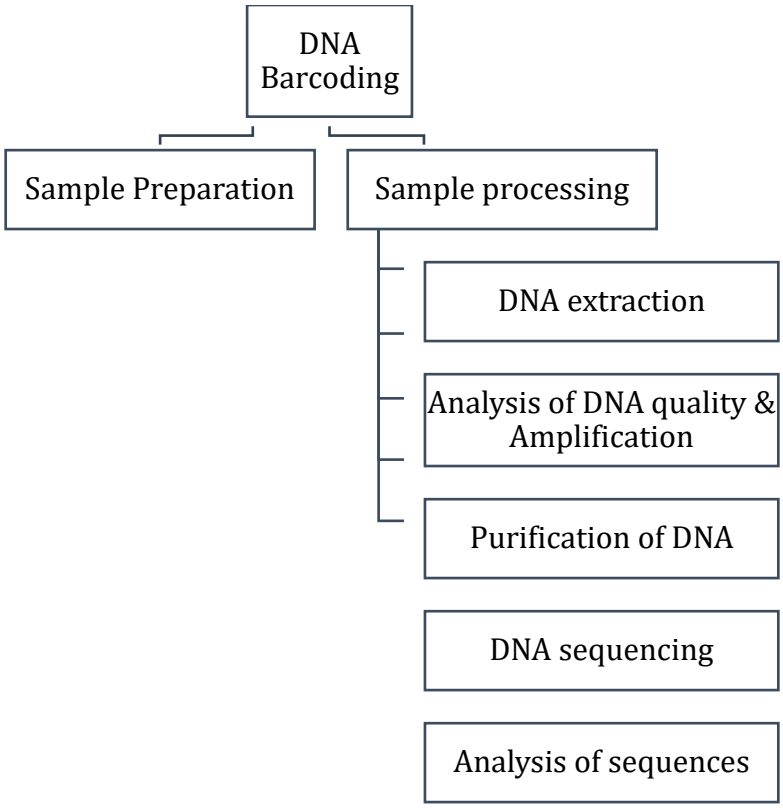

Fig. 4.5Outline methodology of barcoding of bryophytes

4.3.1 Sample selection and preparation:

Fresh samples were preferably used for DNA barcoding. The samples collected during the field work were observed under the microscope and all additional species were removed. The sample was thoroughly washed with tap water and then with distilled water. The lower part of the samples, i.e. the rhizoids was removed as it could have the presence of entangled algae within it. This sample was then washed with 70% ethyl alcohol to sterilize it.

4.3.2 Sample processing:

The samples were then taken to the molecular biology Laboratory of the Gujarat State Biotechnology Mission (GSBTM), Gandhinagar for further processing.

4.3.2.1 DNA extraction and amplification:

The 100 mg of plant sample (thallus or gametophytic plant body) was crushed using liquid nitrogen in the mortal pastal to make it into powder form. Genomic DNA was extracted from the powdered form of the species using Plant/Fungi isolation kit (Sigma-

Aldrich, USA). For the extraction of the genomic DNA, the standard protocol given in the kit was followed.

DNA amplification using PCR: PCR was carried out by taking 10µl (1X) of ReadyMix™ Taq PCR Reaction Mix (Sigma), 100 mg/ of template DNA and 10 mole of each primer. The reaction was carried out in Thermal cycler (Applied BiosystemsVeriti®). Final concentration contained 20 µl reaction mixture. The amplification was carried out for 35 cycles (Levin *et al.*, 2003; Kress and Erickson, 2007) with the parameters as specified in table 4.3 below

Table 4.3: DNA amplification parameters

Sr. No.	Process parameter	Duration	Temperature
1	Initial denaturing	300 seconds	95°C
2	Denaturing	30 seconds	95°C
3	Annealing	40 seconds	55°C
4	Extension	120 seconds	72°C
5	Final extension	420 seconds	72°C

DNA barcoding in plants has been considered to be more difficult in comparison to animals (Chase *et al*., 2005; Fazekas *et al*., 2009; Pennisi, 2010). Multigene tiered approached had showed that rbcL serves as core region with sufficient variation that can help in discrimination of species (Newmaster *et al*., 2006). It was one of the most widely used primer for the molecular studies of bryophytes (Cox *et al*., 2000; Forrest *et al*., 2005; Hentschel *et al*., 2007; Wahrmund *et al.,* 2009; Liu *et al*., 2010). rbcL was thus used in the current study and the sequence is given in the table 4.4 below.

Table 4.4: Primers used in the study and their sequences

Sr. No.	Primer	Sequence
1	rbcLaF	ATGTCACCACAAACAGAGACTAAAGC
2	rbcLaR	GTAAAATCAAGTCCACCRCG

4.3.2.2 Analysis of quality of DNA and its amplification:

Gel electrophoresis was carried out to study the quality of DNA. For Gel electrophoresis DNA mixed with loading dye was loaded in the well using gel-loading tips. The gel images were recorded in JPEG format using gel documentation system (Biorad, USA). The gel was analysed by using the software Image lab version 3.0.

4.3.2.3 Purification of DNA:

Purification of rbcL gene amplified products were done using a Purelink® Quick PCR purification kit (Thermo Fisher Scientific, USA) following protocol given in the kit.

4.3.2.4 Sequencing:

The University/Department did not have the facility for the sequencing of PCR product. Hence, the sequence of the PCR product was obtained from Eurrofins Genomics India Pvt. Ltd., Bangalore. The sequence was obtained in *Fasta* format.

4.3.2.5 Analysis of DNA sequences: The obtained sequence was matched with available sequences in the NCBI database with the help of The Basic Alignment Search Tool (BLAST) The ident percentage was used for the identification of the species. Then, the nucleotide sequences were submitted to the Barcode of life system (BOLD) for generating the DNA barcodes.

4.4 Identification software Preparation:

The use of computer aided software and image analysis software in taxonomic identification has increased with the increase in digital technology. Image analysis software use a database of standard images in the identification of plants. However, character based identification software makes use of botanical characters to facilitate identification of specimens. Fig. 4.6 below shows the different components of the software preparation.

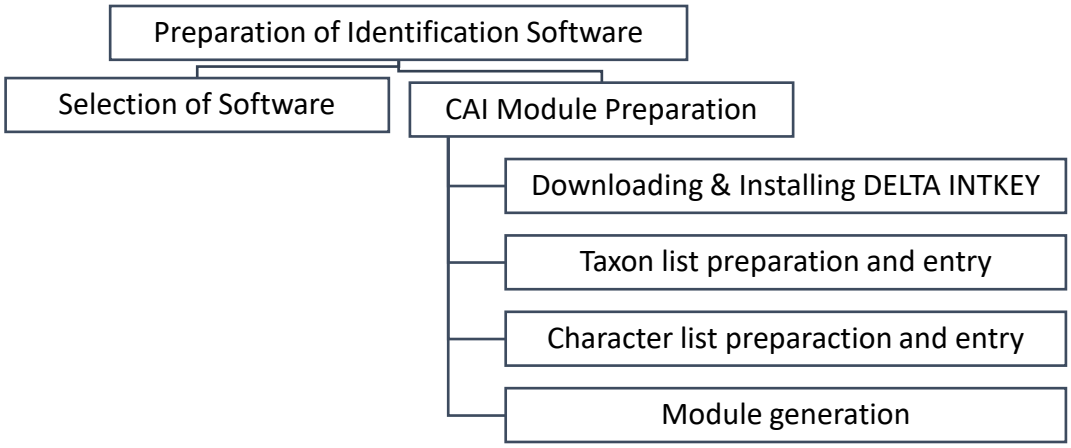

Fig 4.6 Outline methodology of Computer aided identication study

4.4.1 Selection of software:

There are several softwares available for preparation of computer aided identification module of which many are paid software's and few are free. These includes LucID (http://www.lucidcentral.org), Meka (http://ucjeps.berkeley.edu /meacham/meka/index03.html), Navikey (http://www.huh.harvard.edu/databases/legacy/navikey), XID (weed-id.com/), Delta Intkey (Dallwitz *et al.*,1993) etc. Softwares like LucID, XID are commercial softwares whereas software's like Meka, Navykey etc are free but not very user friendly. While DELTA INTkey is an open source and free software and it has been extensively used for development of character based identification software for plants, animals and insects as well. This software was hence selected for the preparation of the module.

4.4.2 CAI Module preparation:

The Delta INTKEY software was developed by Commonwealth Scientific and Industrial Research Organization's (CSIRO) entomology division in Australia developed the software from 1971-2000.

4.4.2.1 Downloading and Installing the DELTA INTKEY set up:

DELTA INTKEY is free and open source software and it can be downloaded from the https://www.deltaintkey.com/www/programs.htm website in Zip format. The

downloaded zip format is extracted and by double clicking *intkey5.exe*is installed in C drive. It runs in on most versions of Windows viz. Windows XP, Windows 7, Windows 8, Windows 8.1 and Windows 10.

4,4,2,2 Preparing Taxon list and entering it in module: As the three groups of bryophytes have a different set of distinguishing characters, three different modules were prepared for liverworts, hornworts and mosses respectively. The list of 24 moss species to be included in the software was prepared. The taxon name is added in the taxon editor dialog box. Information like Name of taxon and images were entered into it.

4.4.2.3 Character list preparation and character entry:

List of important identifying characters of Liverworts, Hornworts and Mosses to be included were selected. For liverworts hornworts and mosses 36, 19 and 36 characters respectively were selected for database preparation. They have been depicted in tables 4.5, 4.6, & 4.7 below. All the characters and list of species of the Liverworts, Hornworts and Mosses were entered in the Editor module. The Characters of species are added in "Character Edit" dialog box. Information like description of characters, character type, its units, images of character (if required), notes on character (if required), controlling characters etc. are entered in to the character edit dialog box. List of characters (liverworts, hornworts, Mosses) used in the preparation of INTKEY are given below at the end of methodology.

Table 4.5: Characters of Liverworts used in preparation of DELTA INTKEY

Liverworts					
Sr. No	**Characters**	**Sr. No**	**Characters**	**Sr. No**	**Characters**
1	Habit Type	13	Rhizoids Type	25	Female receptacle shaped
2	Plant length (mm)	14	Scales Presence	26	Sporophyte Presence
3	Plant width (mm)	15	Scale width	27	Sporophyte Position
4	Plant Colour	16	No of Row of Scales	28	Sporophyte Condition
5	Thallus articulation	17	Scale colour	29	Sporophyte Size
6	Thallus Rosette Presence	18	Appendages Presence	30	Capsule wall thickening Presence
7	Thallus rosette type	19	Air Chambers Presence	31	Elaters Presence
8	Branching Presence	20	Photosythetic filament Presence	32	Elaters branching Type (If present)
9	Braching type	21	Thallus Type	33	Elaters thickening
10	Dorsal median sulcus Presence	22	Male receptacle Presence	34	Spore colour
11	Dorsal median sulcus Visibility	23	Male receptacle shape	35	Spore size (micron)
12	Air pore Presence	24	Female receptacle Presence	36	Sex Type

Table 4.6: Characters of Hornworts used in preparation of DELTA INTKEY

Hornworts					
Sr. No	**Characters**	**Sr. No**	**Characters**	**Sr. No**	**Characters**
1	Thallus arrangement	8	Collumella Presence	15	Pseudoelaters Presence
2	Thallus nature	9	Line of dehiscence on columella	16	Stomata on capsule Presence
3	Thallus surface	10	Involucre Presence	17	Spore Size
4	Nature of Lamellate thallus (if lamellate)	11	Involucre Nature	18	Spore wall Nature
5	Thallus dorsal Ridge	12	Involucre Shape	19	Pseudoelater cell Number
6	Schizogenous cavities	13	Capsule Orientation		
7	Shape of sporophyte	14	Spores colour		

Table 4.7: Characters of Mosses used in preparation of DELTA INTKEY

Mosses					
Sr. No	**Characters**	**Sr. No**	**Characters**	**Sr. No**	**Characters**
1	Moss Type	13	Vaginant Lamina Presence	25	Sporophyte Presence
2	Substratum	14	Costa length	26	Sporophyte Position
3	Stem Length	15	Costa Type	27	Capsule Condition
4	Stem Colour	16	Leaf Margin-apex	28	Capsule Shape
5	Leaves Colour	17	Leaf Margin-middle	29	Persitome teeth Presence
6	Branching Presence	18	Leaf Margin-basal	30	Calyptra Type
7	Crowded at apex	19	Limbidium Presence	31	Calyptra Shape
8	Change in Plant Ondrying	20	Leaf cells shape	32	Seta length
9	Leaf Arrangement	21	Leaf cells Transparency	33	Spore Color
10	Leaf Length	22	Leaf Cells Type	34	Spore Size
11	Leaf Breath	23	Position, Papillose/Mamillose		
12	Apex Type	24	Alar cells Presence		

4.4.2.4 Character data entry:

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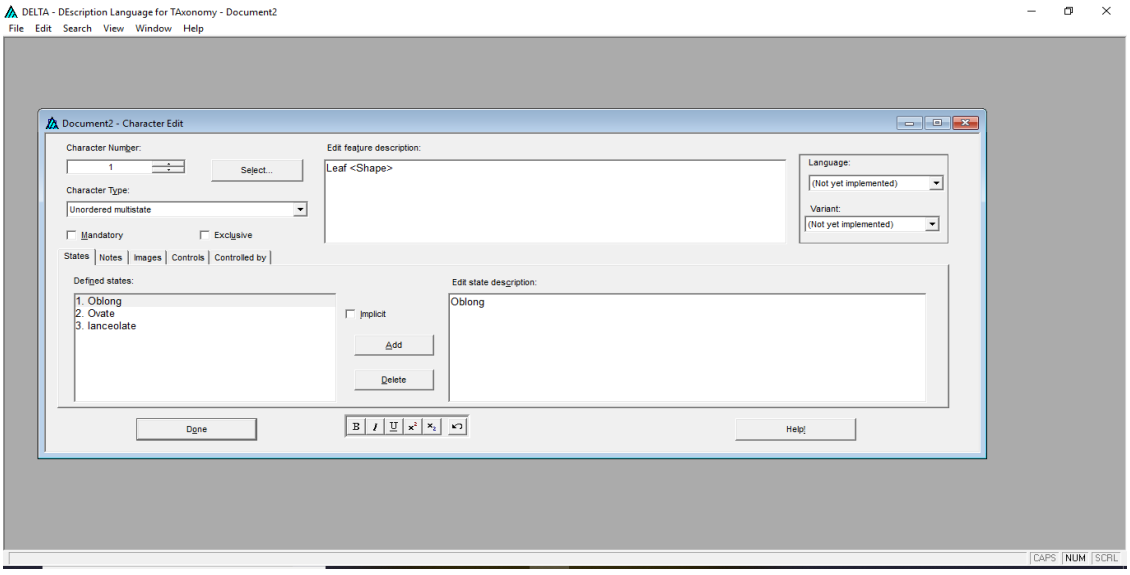

Fig 4.7: Dialog box for entering the Taxon characters

4.4.2.4 Module generation:

To obtain the data in the natural language it is necessary to run the editor programme and generate the necessary INTKEY module for each of the bryophyte groups. A indicated in the table below, the key is generated by clicking Action sets>Confor tab> INTKEY More details are given in Dallwitz (2016). The output of the analysis was viewed in INT key. Based on the available characters the species was identified.

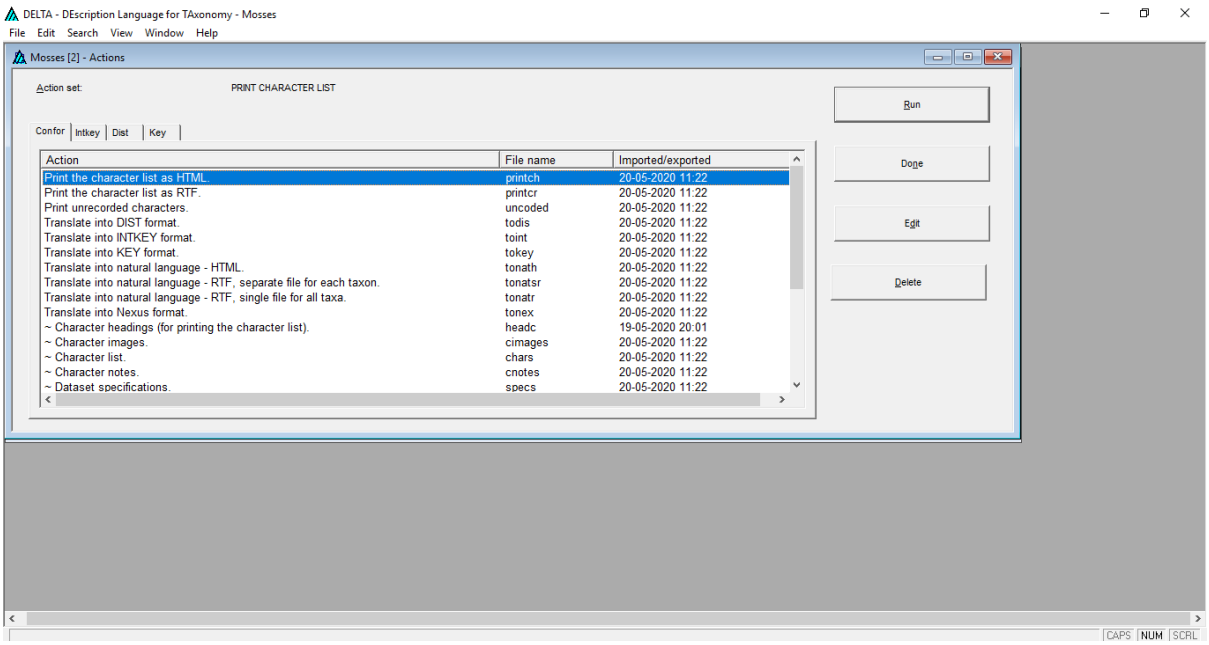

Fig 4.8: Method of running the confor files for final ouput