RAPD and morphological analysis of bryophytes - *Thuidium tamariscinum* (Hedw.) Schimp. and *Hyophyla comosa* Dixon in P. de la Varde

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Abstract: DNA isolates of two moss species *Thuidium tamariscinum* (Hedw.) Schimp., and *Hyophyla comosa* Dixon in P. de la Varde, belong to morphologically distant families were analyzed using Random Amplified Polymorphic DNA (RAPD) to find out the genomic relationship. The outcome of this preliminary work revealed that the taxonomy based on morphological characteristics is in accordance at molecular level. It strengthens the use of morphological parameters is still an important tool in current taxonomy.
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1. Introduction

Taxonomy on the basis of morpho-anatomical characteristics has an old account. With increasing knowledge in molecular techniques now the taxonomy has been done at molecular level worldwide[1-3]. Bryophyta constitute an important component of plant diversity. They consist of three separate divisions, the Marchantiophyta (liverworts), Anthocerotophyta (hornworts), and Bryophyta (mosses). They are to be found in all ecosystems, from desert to alpine, with the exception of marine. The ecological role of bryophytes in any ecosystem is significant[4-9]. Bryophytes are a very interesting group in botany but few studies have successfully investigated their chemistry, especially at molecular level. Generally, bryophytes are known to possess extremely high amounts of terpenoids, phenolics (flavonoids and bibenzyl derivatives), glycosides, fatty acids, as well as some rare aromatic[8-9].

In addition to elucidating early patterns of morphological diversification in embryophytes, bryophytes are crucial to understanding plant genome evolution. Approximately 66% of genes identified from expressed sequence tag analyses of gene expression in gametophytes of *Physcomitrella patens*[10] have homologues in the *Arabidopsis* genome, consistent with the hypothesis that genes expressed in the diploid plant body of angiosperms were expressed in the gametophytes of early land plants and were recruited for sporophytic morphogenesis later in plant phylogeny[11-13]. Phylogenetic and functional analyses of genes expressed in *Physcomitrella* sp. gametophytes have clarified the phylogenetic history of several important gene families, including MIKC-type MADS-box genes and homeobox genes[11]. Phylogenetic analyses of the KNOX (homeobox) gene family across the land plant tree of life have provided insights into the history of gene duplication and functional divergence during embryophytic history[10]. Because KNOX genes are involved in expression of meristematic activity in vascular plant sporophytes, functional analyses of KNOX genes in mosses, liverworts, and hornworts are central to understanding evolution of plant development in embryophytes. Comparable studies of genes involved in flower development are underway, and, in the context of phylogenetic analyses of bryophytes, the early evolution of these genes is now a tractable problem for investigation [14-18].

In recent past bryophytes have been reclassified on the basis of their genome [19-26]. Mosses are studied for their genome due to their greater biomass than Hepaticae and Anthocerotae[27-36]. They are small, soft plants that are typically 1–10 cm (0.4–4 in) tall, though some species are much larger. They do not have flowers or seeds and their simple leaves cover the thin wiry stems. Bryophyta is the largest division of Bryophytes (84% of families and ~98% of species) [38]. Based on the morphological characters (branching patterns and location of sexual organs), the Bryophyta has been divided into two major groups as acrocarpous mosses and pleurocarpous mosses [38]. Several mosses have been studied for RADP analysis and polymorphism [39-44]. But in India these type of studies are still of rare occurrence. Only few attempts have been reported[45-47]

In present study an attempt has been made to find out the polymorphism between two moss species *Thuidium tamariscinum* and *Hyophyla comosa* belongs to two distinct orders Hypnales and Pottiales respectively using RAPD profiling. Mosses have delicate hair like projections called rhizoids which are the sources of contamination by means of

microorganism like algae, bacteria and fungi. During extraction process, DNA of these microscopic organisms interferes pure DNA isolation and responsible for undesirable amplication. Extraction of DNA from bryophytes is very difficult due to the presence of polyphenols, terpenoids and some other binding substance.

In this study the main objective is optimization and extraction of DNA isolation of moss samples which were collected from two bio-geographical distinct regions Ranthambore National Park and Mt. Abu of Rajasthan and Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu. It is a preliminary attempt to elucidate a correlation between morphological and genomic parameters used for their taxonomic account.

Both the orders are morphologically distinct as order Pottiales have plants in loose or dense, dark green to red-brown or blackish tufts, dull or occasionally with a metallic sheen. Stem densely foliate, 5-10(-20) mm, central strand strong. Leaves concave when moist, to 1.5-2(-2.5) mm, nonvascular. The order Hypnales has bright, yellowish-green or dark green shoots. They are usually tripinnately branched, 5–25 cm long, and form loose mats. The green or red-brown stems are covered with a felt of tiny, branched filaments and very broad, heart-shaped or triangular, opaque, longitudinally ridged, acute-tipped leaves, about 1.25 mm long.

2. Material and Methods

2.1 Plant material:

The plant samples were collected from biogeographically distinct regions-Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu, which is wide spread more than 6,000 km², ca. 2100 m, Latitude: 11° 08' to 11° 37' N by 185 km Longitude: 76° 27' E to 77° 4' E, Ranthambore National Park which covers an area of 392 km², which is 26°01′02″N 76°30′09″E , India. and Mt. Abu , located in Sirohi District, Rajasthan, 1722m above the sea level, 24°35′33″N 72°42′30″E ,India . Plants of *Thuidium tamriscinum* were collected from Coonoor, Nilgiri hills, Tamil Nadu (South India; alt. ca. 2100m; leg. & det. Afroz Alam & P.K. Verma; LWU12874/2008. The plants of *Hyophylla Comosa* were collected from Mount Abu, Sirohi, Rajasthan; alt. ca. 700 m; leg.: Afroz Alam & S. C. Sharma; det. Afroz Alam; BVH7860032.

2.2 Sample preparation and preservation

The sample which was collected from the above mentioned places were identified properly with the particular genera and species. .The sample was deposited in Banasthali Vidyapith Herbarium (BVH). For the isolation of DNA from the sample first the moss samples were soaked in the Luke warm distilled water and then it was properly washed with the help of brush so as to remove the mud and dirt, microscopic organism like algae, fungi and foreign plant tissues which is attached with the roots and the leaves of the moss. Then after washing it is stored at the room temperature and whenever it is required for the DNA extraction the sample is soaked in distilled water, 4 hrs before the DNA extraction.

2.3 Chemicals used

The chemicals which were used in the DNA extraction and the PCR were CTAB (Cetyl trimethyle ammonium bromide), Chloroform (A.R), PVP, Isopropanol, Bromophenol Blue, Agarose (low EEO), Tris-Buffer , EDTA , Taq polymerase with $Mgcl_2$ (3U/µl)+10X taq buffer, dNTPs 10Mm, DNA Rulers: 100bp-3kb(low range) and lamda Hind III double digest.

2.4 Methods

2.4.1 Isolation of genomic DNA

The total genomic DNA was isolated from various dry leaf samples of *Thuidium tamriscinum* and *Hyophylla Comosa* by a cetyl trimethyl-ammonium bromide (CTAB) method of Weising *et al.* (1995)[48] with minor modifications *viz.* extracted aqueous layer was treated with 5M NaCl to remove polysaccharide contamination, incubation for precipitating DNA after adding isopropanol was carried out overnight and washing steps were repeated twice. The extraction buffer contained 100 mM Tris-HCl (pH 8), 1.5 M NaCl, 25 mM EDTA, 2.5 % CTAB (w/v), 2 % β-mercaptoethanol and 1 % polyvinylpyrrolidone. The purified pellet was dissolved in a minimal amount of Tris EDTA (TE) buffer (Tris-Cl 0.05 M, EDTA 0.01 M).

2.4.2 Quantification of genomic DNA

Since the isolated DNA was to be used for the PCR-based study, its suitability for PCR along with its size heterogeneity was the most important criterion for purity .As a matter of general practice , all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

2.4.3 Quantification by UV spectrophotometry

The isolated genomic DNAs were tested for the purity and yield bye measuring their absorbance at 260 nm and 280 nm wavelength using a UV – visible spectrophotometer (ELCO SL 196 spectropharm). A DNA preparation was considered to be good if it had $A_{260\ nm}/A_{280\ nm}$ ratio as approximately 1.8. One of the quartz cuvettes contained 100µl of diluted DNA sample and estimation of OD was carried out with reference to the other cuvette containing equal volume of sterile water.

The concentration of DNA in µg/ml was calculated using following formula (Sambrook *et al.,* 1989)[49]. 1 OD unit at 260 nm is equivalent to 50 µg /ml of double stranded DNA.

2.4.4 Qualitative estimation of DNA by agarose gel electrophoresis

Agarose gel electrophoresis of the isolated genomic DNA was performed to carry out quantitative as well as qualitative analysis of DNA. The isolated DNA was checked on 0.8 % agarose gel by staining with ethidium bromide (0.5 g/ml) and visualized under mini-trans-illuminator (Bio-Rad, USA). The gel was then photographed and analysed by a Kodak gel documentation system (Model EDAS 290) using lamda DNA double digest (Bangalore Genei Pvt. Ltd., India) as standard.

2.4.5 RAPD-PCR reactions

A total of 10 random decamer primers (custom synthesized by Bangalore Genei Pvt. Ltd., India) were employed in the experiments for genome screening. The primers with more than 50 % GC content were used for RAPD analysis and of these 3 primers were finally selected for data analysis (Table 2). The RAPD analysis on each primer was repeated twice to check the reproducibility. Moreover, in all the PCR reactions, a negative control is included to avoid the erroneous interpretations. Only those gels which showed consistent amplification were considered in this study. The polymerase chain reaction (PCR) reactions were performed in a final volume of 25 µl containing genomic DNA (25 ng), primer (0.2 µM), Taq DNA polymerase (1.5 U/µl), dNTPs (2.5 mM), Taq polymerase buffer 1 x (10 x buffer contained 100 mM Tris-Cl, 500 mM KCl, 15 mM $MgCl_2$ and 0.1 % gelatin). The reaction mixture was overlaid with 40 µl of sterile mineral oil to prevent fluid evaporation. DNA amplification was carried out in a Gene Cycler (Bio-Rad, USA) with following thermal profile: 4 min at 94 ºC (initial denaturation) followed by 45 cycles of 15 sec at 94 ºC (denaturation), 45 sec at 40 ºC (primer annealing) and 90 sec at 72 ºC (primer elongation) and a final step of 4 min at 72 ºC (final extension). After completion of the cycle, PCR products were stored at –20 ºC until further use. Amplification products were separated on 1.5 % (w/v) agarose gels (migration distance: 10 cm) with 1× tris borate EDTA (TBE) buffer. Electrophoresis was performed as 100 V for 2 hrs. 10 µl of low range DNA ruler (Bangalore Genei, India) was run simultaneously, loaded on gel as a molecular standard, and then the gel was visualized, photographed and analyzed .

2.4.6 RAPD - data analysis

Statistical analysis for band was done only for primers that resulted in reproducible and consistent profiles. The analysis involved: scoring of the bands, identification of monomorphic and polymorphic bands, calculation of percent polymorphism.

2.4.7 Scoring of bands

For each gel, the molecular sizes of the fragments were estimated on the basis of the corresponding marker lane. Clear and well- separated bands were coded in a binary form by denoting '0' and '1' for absence and presence of bands (respectively) in each genotype .These data were then used to calculate the percentage polymorphism so as to study the genetic diversity among the two moss samples.

Percent Polymorphism % =No. of polymorphic Bands / Total no. of Bands

3. Results

3.1 Morphological distinguishing parameters Observed

Hyophila comosa

Plants grow in loose or dense, dark green to red-brown or blackish tufts, dull or occasionally with a metallic sheen. Stem densely foliate, 5-10(-20) mm, central strand strong. Leaves concave when moist, to 1.5-2(-2.5) mm, oblong-spatulate to obovate, occasionally with multicellular teeth in distal 1/4, rounded to rounded obtuse at the apex, sometimes apiculate ; costa stout, prominent abaxially, smooth on the abaxial surface to occasionally roughened at the apex, hydroids absent; laminal cells near insertion short-rectangular, 2-4:1, firm-walled, pale and brownish or hyaline , cells 8-10(-12) µm wide, in longitudinal and oblique rows , thin to thick-walled, bulging-mammillose on the adaxial surface, plane on the abaxial. Dioecious. Seta 6-7 mm in length, turns reddish to yellow-brown with age. Capsule erect, 1.5-3 mm, narrowly cylindrical from an indistinct neck, annulus well. Differentiated, red-brown, of vesiculose cells, persistent or deciduous; operculum erect, conical, 0.6-0.8 mm. Cells covering the adaxial costal of *Hyophila* are rather different from the laminal cells, being somewhat larger and quadrate to short-rectangular.

Thuidium tamariscinum

The bright, yellowish-green or dark green shoots of *T. tamariscinum* are amongst the most distinctive of among pleurocarpous mosses. The plants are usually tripinnately branched, 5–25 cm long, and form loose mats. The green or red-brown stems are covered with a felt of tiny, branched filaments and very broad, heart-shaped or triangular, opaque, longitudinally ridged, acute-tipped leaves, about 1.25 mm long. The nerve is wide and almost reaches the leaf tip. Branch leaves are narrower and shorter (up to 0.5 mm). The branches are arranged more or less in one plane and become shorter towards the end of the shoot. Capsules are uncommon, and form in autumn and winter. They are large and curved, and held on a long (2–4 cm), purple-red seta.

Table 1: Quantification of the moss DNA samples

Sample	Absorbance at A_{260}/A_{280}	Conc. $\mu g.\mu l^{-1}$	Yield $\mu g.fdw^{-1}$
Thuidium tamriscinum	1.7	0.703	351.80
Hyophylla comosa	1.8	0.302	151.25

3.2 DNA extraction

DNA was extracted from the six samples of bryophytes using CTAB method of Weising *et al.* (1995)[4.8], with some optimization in the protocol. The extracted DNA was analyzed by agarose gel electrophoresis and one major band was observed without smearing and shuttering in all samples. This indicated that a large part of the DNA did not break into smaller fragments during the process of extraction (Fig. 1). The purity of DNA samples is usually assessed by A_{260} and A_{280} ratio and pure DNA has an $A_{260/}$ A_{280} ratio of above 1.8 (Sambrook *et al.,* 1989)[46]. Therefore, a higher value of $A_{260/}$ A_{280} implies purer DNA. Most of the DNA samples in this study had an $A_{260/\ 280}$ around or above 1.8 which indicates that a high degree of purity of DNA was attained and preparations were of high quality and free of proteins and polysaccharides. Concentration of DNA ranges from (0.703-0.302) µg/µl. DNA yield was 351.80 µg/gm and151.25 µg/gm for *Thuidium tamriscinum* and *Hyophylla comosa* respectively. (Table 1).

Variation in the DNA yield pattern among the plant species is understandable as it mainly depends on the type of species, varieties, plant tissue or developmental stage of plant. Further, differences in yield are also attributable to secondary metabolites such as polyphenols and polysaccharides and their relative levels might interfere with the extraction (Palomera-Avalos *et al.,* 2008)[50].

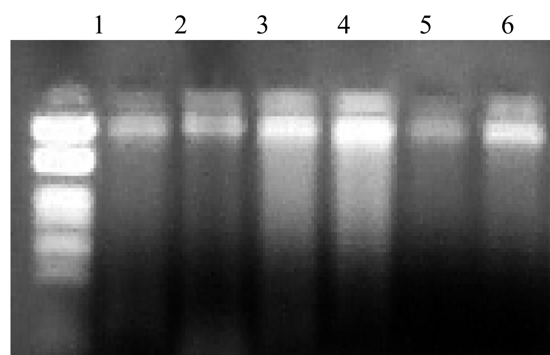

Figure 1: Gel picture of 6 samples of bryophytes on 0.8% agarose gel. Lane 1 Lamda Hind III double digest, Lane 2-4 *Thuidium tamariscinum;* Lane 5-6 *Hyophylla comosa*

3.3 PCR Amplification

Levels of polymorphism

RAPD marker system being employed to assess the genetic variation of *Thuidium tamariscinum* and *Hyophylla comosa* germplasm and found to be informative as it was able to to generate adequate polymorphism and unique DNA fingerprints. Overall, 8 random primers were used to examine the genetic variation pattern. Of the 8 primers, 3 primers (37.5 %) resulted in amplification, of which 3 primers gave reproducible and scorable results. The percent polymorphism varies from 40%-80% in this case. (Table:2). Out of these three primers used for the PCR amplification primer no.12 and 9 gave maximum number of bands i.e. 5 and primer 18 gave the minimum number of bands i.e. 3. Primer 12 showed 80% polymorphism, primer 9 showed 40% polymorphism, Primer 18 showed 66.6% polymorphism, primer 12 showed 80% polymorphism. Therefore, it can be said that primer number 12 showed maximum polymorphism. Percent polymorphism varies and number of bands varies significantly in different plant species. This is understandable as product amplified depends upon the sequence of random primers and their compatibility within genomic DNA. The number of markers detected by each primer depends on primer sequence and the extent of genetic variation, which is genotype specific (Upadhyay *et al.*, 2004)[51].

Table:2. RAPD primers employed for the genetic variation study in *Thuidium tamariscinum* and *Hyophylla comosa*

S.No.	Primer	No. of Total Bands	No. of Polymorphic bands	No. of monomorphic bands	Polymorphism %
1.	GGCTTGACCT	5	2	3	40%
2.	TCGGCGATAG	5	4	1	80%
3.	GTTGCGATCC	3	2	1	66.6%

4. Discussions

Understanding population genetic variation together with the relationship of morphological parameter is the key in molecular systematics. DNA extraction is the bottleneck for all downstream applications of molecular biology. Our results suggested that modified and tested DNA extraction protocol works very well with *Thuidium tamariscinum* and *Hyophylla comosa.* Primer 12 showed 80% polymorphism. Therefore, it can be said that primer number 12 showed maximum polymorphism The (RAPD) primer which shows higher polymorphism will be further used for the study of genetic diversity analysis. Although the

unique DNA fingerprint pattern polymorphism of two moss sample i.e. *Thuidium tamariscinum* and *Hyophylla comosa* clearly showed that these two species not only morphologically distant but also they are genomically distant too and exhibit 62.22% diversity at genomic level. Tested primers could be further used for genetic diversity analysis in selected plant species. Therefore it can be evidentially concluded on the basis of the present study by the help of RAPD markers that morphological parameters are still valid to classify the mosses as they have strong correlation with their genomes.

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