

Genetic Diversity Analysis of Five Different Species of *Riccia*

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Abstract

Five species of the *Riccia* genus have been investigated by Rapid Amplified Polymorphic DNA (RAPD) analysis. The species is explained and demonstrated with its genetic diversity based on morphological variations. Samples were collected from different parts of the University of Dhaka growing in different habitats around the University campus area. After the study of its morphology, it has been observed that the population of this taxon shows significant variation in plant size, shape, colour, ventral scales, appendages of scales, rhizoids, and the position of male and female receptacles, etc. The RAPD marker system was employed to estimate the genetic diversity within and between the populations based on such morphological variations. Approximately 82% of the variations have been observed within and between genotypes of *Riccia* as revealed with both phenotypic and genotypic data. The RAPD markers are being used increasingly to analyse the phylogenetic relationship among the liverworts to give the exact framework of taxonomic identification of naturally occurring liverwort *Riccia*.

Introduction

Genus *Riccia* is one of the most abundant liverwort observed in Bangladesh. This liverwort belongs to the order Marchantiales from the family Ricciaceae under the class Hepaticopsida. The name was coined by the Swiss Albrecht von Haller (1708-1777), and the Italian Pier Antonio Micheli (1679-1737), who in his book *Nova Plantarum genera* coined the name *Riccia*, named after Pietro Francisco Ricci, a Florentine politician and botanist (<https://sussexbryophytes.wordpress.com/2017/09/19/riccia-fluitans/>).

Riccia L. (Ricciaceae) is the largest genus among the complex thalloid liverworts, with 152 species worldwide constituting 24% of the global species diversity (Söderström et al. 2016). However, in Bangladesh, 45 species have been recorded so far from different regions (Siddiqui et al. 2007). The plants possess simple linear or oblong gametophytic thalli differentiated into the upper photosynthetic zone and lower storage tissue, with or

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without simple pores similar to other thallose members. It is unique in having fertile plants with the anacrogynous condition, sporophytic sporogonia or capsules embedded singly within the thalli, absence of pseudoperianth, and cleistocarpous capsules without elaters. It differs from *Ricciocarpos Corda*, the other twin genus of Ricciaceae in lacking idioblastic oil cells, which are present in the latter (Crandall-Stotler et al. 2009). The majority are terrestrial mesophytes that grow on exposed or shaded mixed alluvial sandy soil with few aquatic and semiaquatic forms (Singh et al. 2019). All species are terrestrial and prefer to grow in moist and shady places except *Riccia fluitans*, an aquatic species that occur floating in still stagnant water or submerged below the surface of standing water. A basal land plant species with the capacity to live in freshwater and terrestrial conditions would be the ideal model for research on molecular pathways (Zachgo 2020). The accessibility of molecular tools, specifically various genome editing strategies for sporeling, gemmae and thallus transformations has increased interest in using *M. polymorpha* for molecular investigations (Ishizaki et al. 2008). Since the *Riccia* L. genus, which comprises over 200 species, dominates the complex thalloid liverworts under Class Marchantiopsida and is the most diversified, developing molecular tools for this species would help us better understand the land plants' evolutionary history more precisely (Gratani 2014).

Genetic diversity of all five *Riccia* samples were examined. Using Random Amplified Polymorphic DNA (RAPD) markers. RAPD is a powerful approach for systematic research and analyzing polymorphism in and among taxa (Adams and demeke 1993, Alam et al. 2012). Boisselier-Dubayle and Bischler (1994) investigated the phylogeny of liverworts and used genetic analysis to resolve taxonomical issues. The genetic variation in different liverworts has been reported using isozymes, RFLP, and RAPD markers (Boisselier-Dubayle and Bischler 1995). No study on any bryophytes from Bangladesh has yet been published to characterize using the RAPD markers. This would be the first work on a molecular marker investigation of *Riccia* from Bangladesh, the most prevalent genus of liverworts.

Materials and Methods

Five species of *Riccia*, namely *Riccia dhakensis*, *Riccia discolor*, *Riccia bengalensis*, *Riccia gangetica*, and *Riccia compaginata* were collected for RAPD analysis (Fig. 1 A-E). Template DNA used in the RAPD experiment was isolated from the thallus of the plants. Delicate thallus was used to extract total genomic DNA by using the modified CTAB method (Doyle and Doyle 1987). DNA concentration was quantified through a spectrophotometer (Analytikjena, Specord 50, Germany). The A260/280 readings for DNA samples were 1.6- 1.8. Fifteen random primers were used in the present study- OPA-1 (CAG GCC CTTC), OPA-3 (AGT CAG CCA C), OPA-4 (AAT CGG GCT G), OPA-6 (GGT CCC TGA C), OPA-7 (GAA ACG GGT G), OPA-8 (GTG ACG TAGG), OPA-9 (GGGTAA CGC C), OPA-10 (GTC ATC GCA G), OPB-2 (TGATCCCTGG), OPB-3 (CATCCCCCTG),

OPB-5 (TGCGCCCTTC), OPB-6 (TGCTCTGCCC), OPB-7 (GGTGACGCAG), OPB-8 (GGTGACGCAG) and OPB-9 (TGGGGGACTC) (Table 1).

The PCR reaction mixture for 25 μ l containing template DNA (20 ng) 2 μ l, de-ionized distilled water 16.3 μ l, and Taq buffer A 10 \times (10 mM Tris-HCl) 2.5 μ l, 1.5 mM MgCl₂ 2.5 μ l, primer (10 μ M) 1.0 μ l, dNTP mix (10 mM) 0.5 μ l, Taq DNA polymerase (5U/ μ l) 0.2 μ l. PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 35 cycles after initial denaturation at 94°C for 5 min and at 94°C for 45 seconds, annealing at 34°C for 30 sec, extension at 72°C for 3 min and final extension at 72°C for 7 min.

Amplified products from the RAPD reactions were separated by horizontal gel electrophoresis using 1.2% agarose gel containing 0.5 μ g/ml ethidium bromide in TAE buffer at 50 volts for 1.5 hrs. The gel was visualized by a UV trans illuminator to examine the banding patterns and photographed by a gel documentation system (CSL-Microdoc System, Cleaver Scientific Ltd., USA). Each PCR reaction was repeated twice to ensure the consistency of RAPD banding patterns and only reproducible stable products were scored. The photographs of the RAPD gel analysis were critically discussed on the basis of presence (1) or absence (0) of bands, size of bands, and overall polymorphism of bands. The scores obtained using all RAPD analysis primers were then pooled to construct a single data matrix. This was used for estimating polymorphic loci, genetic diversity, genetic distance (D) and constructing an Unweighted pair group method of arithmetic means (UPGMA) dendrogram among the studied samples using the computer program "POPGENE" (Version 1.32).

Results and Discussion

In terms of abundance, five different species of *Riccia* were collected from the University of Dhaka Campus area. Their characteristic features have identified each population through literature and online database consultation. The dichotomously branched liverworts can be both monoecious and dioecious. The dorsal surface generally is green, with a pronounced median furrow. In some instances, cilia can be seen in the margin. *Riccia*'s chlorophyll-bearing layer might be made up of small, deep air spaces or enormous chambers that open all the way across. Two rows of well-developed ventral scales can be detected in maturity. Smooth and tuberculated rhizoids can be seen in *Riccia*. Antheridia and archegonia are found singly on the dorsal surface, are submerged at maturity, have no involucre, and the antheridial chambers' ostioles are usually below the epidermis, but they can be long and conspicuous in some circumstances. Archegonia is usually purple at the tip, with a protruding neck occasionally. Capsule sessile, submerged, without a foot or a seta. The calyptra is persistent. Spores are tetrahedral, isobilateral, or rhomboidal in rare occurrences. A taxonomic account of these five species collected for investigation is provided below including revised nomenclature for each species and a succinct description with photographs.

R. bengalensis Khan, Bryologist, 60:28. f. 6-9 (1957) is a monoecious species. The rosette-forming thallus is compact and thick, with a smooth edge and distinct median, a broad, flat anteriorly that is flattened and broad where it narrows posteriorly, and airpores that are distinctly visible from the dorsal surface (Fig. 1A). In Bangladesh, it was reported from Balda garden, Dhaka, and Faridpur (Kamruzzaman 1995, Siddiqui et al. 2007).

R. dhakenis Zaman et Syed, Kamruzzaman, Ph.D. thesis Univ. Dhaka, p. 193-197 (1995), a monoecious species, up to four times dichotomously branched thallus, linear, slender, margin more or less smooth, apex somewhat round, dorsal furrow distinct (Fig. 1B). In Bangladesh, it has been reported from Mirpur, Dhaka, Sripur, Gazipur, and Munshiganj districts (Kamruzzaman 1995, Siddiqui et al. 2007)

R. discolor L. et L., Pugil. 4:1 (1832) is a monoecious species, forms compact rosette or irregular colony, distinct dorsal furrow, and thallus with margin wavy. Male plants are smaller than female ones (Fig. 1C). In Bangladesh, it has been collected from Dhaka, Jamalpur, Dinajpur, and Rajbari districts (Kamruzzaman 1995, Siddiqui et al. 2007)

R. compamginata Zaman et Syed, Kamruzzaman, Ph.D. thesis Univ. Dhaka, p. 89-92 (1995) is a dioecious species, thallus straight, flat, unbranched or branched once or twice, irregular overlapping colony (Fig. 1D). It was reported in Bangladesh from Rajshahi city and Nawanganj district (Kamruzzaman 1995, Siddiqui et al. 2007).

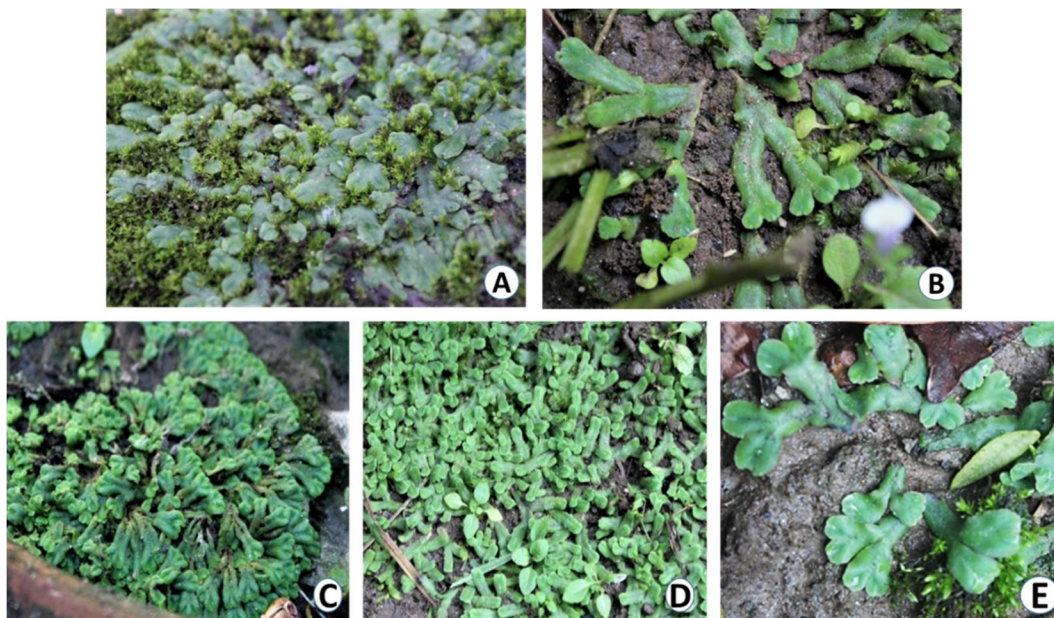


Fig. 1. Fives species of *Riccia* collected from University of Dhaka campus area A. *Riccia bengalensis*, B. *Riccia dhakenis*, C. *Riccia discolor*, D. *Riccia compamginata* and E. *Riccia gangetica* (Habitat).

R. gangetica Ahmad, Curr. Sci. 11: 433 (1942) is monoecious, blue-green plant that grows single or in uneven patches, and only very rarely forms a rosette. The thallus is succulent, with a broad anterior border and a shorter posterior border, a conspicuous dorsal furrow that runs the length of the thallus and a brown edge (Fig. 1E). It has been recorded in Rajshahi, Balda Garden, and Dhaka, Bangladesh (Ahmad 1942, Kamruzzaman 1995, Siddiqui et al. 2007).

The genomic DNA of five *Riccia* species was amplified using 15 random primers (Table 1) that produced distinct polymorphic bands in each DNA sample. RAPD studies yielded 106 bands, 86 of which were polymorphic. The proportion of polymorphic loci was (82%) indicating a higher degree of divergence. A diverse level of polymorphism in different liverworts has been reported in monoecious thalloid liverwort *Plagiochasma appendiculatum* (Kumar et al. 2009, Kumar et al. 2014), Leafy Liverwort *Porella canariensis* (Freitas and Brehm 2001), *Pellia epiphylla* a (Szweykowska-Kulinska et al. 1998) and three subspecies of *Marchantia polymorpha* (Boisselier-Dubayle et al. 1995).

Table 1. No. of PCR amplification products and level of polymorphism generated with RAPD markers.

Primer Code	Sequence	Total Bands	bp size range	No. of Unique bands with variety and bp size	Polymorphic bands	% of average Polymorphisms
OPA-1	CAG GCC CTTC	13	3000-250	1	12	82.07%
OPA-3	AGT CAG CCA C	12	4000-100	-	12	
OPA-4	AAT CGG GCT G	02	3000-250	2	0	
OPA-6	GGT CCC TGA C	0	-	-	0	
OPA-7	GAA ACG GGT G	13	3000-250	1	12	
OPA-8	GTG ACG TAGG	07	3000-100	4	3	
OPA-9	GGGTAA CGC C	15	3000-100	4	11	
OPA-10	GTC ATC GCA G	11	4000-750	1	10	
OPB-2	TGATCCCTGG	0	-	-	0	
OPB-3	CATCCCCCTG	02	3000-1000	2	03	
OPB-5	TGCGCCCTTC	03	4000-1000	1	02	
OPB-6	TGCTCTGCCC	03	2500-250	1	02	
OPB-7	GGTGACGCAG	11	4000-250	1	10	
OPB-8	GGTGACGCAG	14	3000-250	1	13	
OPB-9	TGGGGGACTC	0	-	-	0	

Fig. 2A displays the RAPD profiles of the amplified products of two representative primers. The data set generated during this experiment was adequate to classify all 5 samples (Table 1). The band size had a range of 100-4000 bp. In contrast to the reported 11.57 bands/primer employing seven different primers, the average number of bands per

primer was 7.07 (El-Hady et al. 2010). The primer OPB 8 generated the highest number of polymorphic bands (13) whereas other primers failed to produce any bands at all.

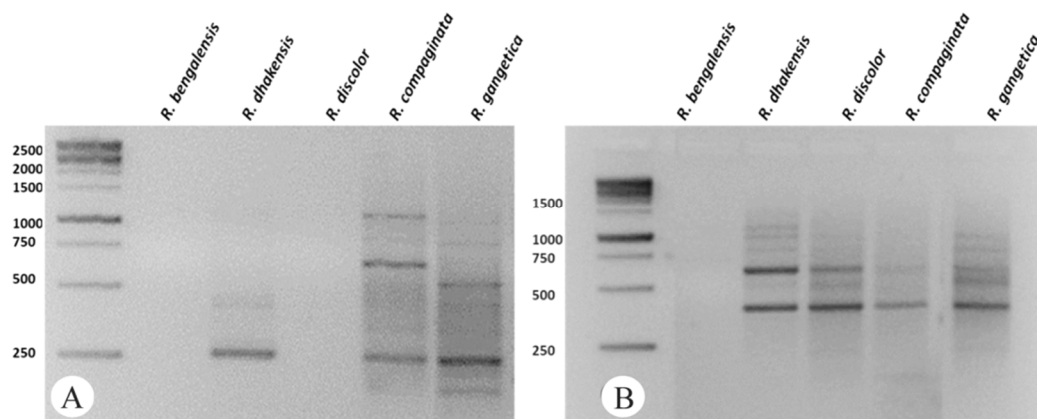


Fig. 2. (A-B): RAPD profile of five collected *Riccia* species. (A) Amplifications of bands with OPA-08 primer. Lane L: DNA ladder (1kb) and Lanes 1-5: amplified DNA of five *Riccia*, Same as (a) but with OPA-09.

The majority of the variants had one or more unique bands that were not seen in other types. These bands can be used successfully as genetic markers to identify these varieties. Except for primers OPA-3, OPA-6, OPB-2, and OPB-9, which were not successful in producing any bands, the primers OPA-8 and OPA-9 were determined to be the most effective in generating at least one distinct band. Eleven primers resulted in a total of 19 distinct bands across five species. (Table 1). These distinct bands are variety specific and so useful for distinguishing one species from another.

The dendrogram constructed based on Nei's (1972) genetic distance segregated the five *Riccia* species into two major clusters (Fig. 3). *R. bengalensis* and *R. gangetica* formed cluster 1 on the other hand, *R. dhakensis*, *R. discolor* and *R. compaginata* formed cluster 2. In cluster 1, *R. bengalensis* and *R. gangetica* formed group 1. Cluster 2 was divided into two sub-clusters. *R. compaginata* alone formed a sub-cluster 1 and group 2. *R. dhakensis* and *R. discolor* were included in sub-cluster 2 and group 3.

The RAPD analysis revealed substantial diversity among the five species of *Riccia* gathered from various regions of the Dhaka University campus. The RAPD analysis revealed that the genotypes of cluster 1 (*R. bengalensis* and *R. gangetica*) are closely linked and cluster 2 (*R. discolor* and *R. compaginata*) are closely related. However, *R. compaginata* is in a distinct cluster, which is farther distant than the other four species. In spite of their fairly similar morphological traits, the five species of *Riccia* differ genetically more than one may anticipate.

Since the morphologies of the species categorised in *Riccia* are so similar, it is challenging to distinguish between them.

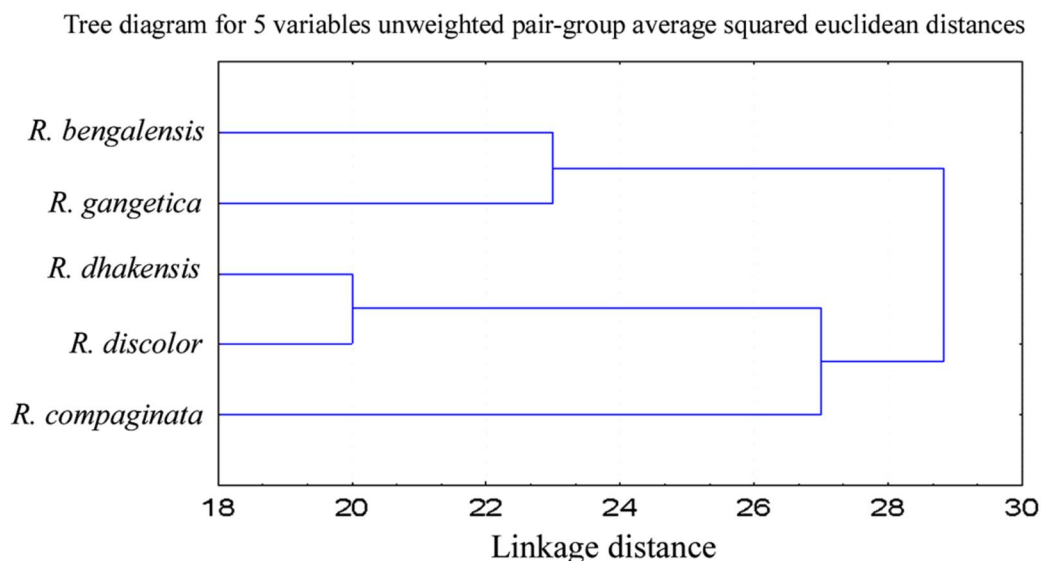


Fig. 3. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiating among five species of *Riccia* by RAPD analysis.

To develop molecular markers, the RAPD approach was employed. RAPD markers were generated by amplifying genomic DNA with a single or many short primers of any nucleotide sequence. Some of the amplified genomic segments are species-specific, and hence can be used as taxonomic identifiers to help assign unidentified individuals to certain species. Furthermore, the presence of a high number of DNA fragments of genus and species specificity allows for the investigation of species phylogenetic connections. This study discusses the potential taxonomic utility of employing the RAPD approach to detect and categorise *Riccia* species.

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