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Research Article

INTRA-SPECIES COMPARISON OF *MARSILEA MINUTA* LINN. AND *MARSILEA QUADRIFOLIA* LINN. USING RAPD MARKERS TO ANALYZE THE GENETIC VARIATIONS

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ABSTRACT

Objective: *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn. are considered to be the important plant species in Ayurvedic system of Medicine with high medicinal values. Since thousands of years the use of plants as complementary medicine is in existence. These plants have medicinal properties useful to treat hepatitis, diabetes, snake bite, inflammation, and infertility. As part of characterization in modern terms, we need to explore and study them on the basis of genetic polymorphism and evolutionary aspects.

Methods: In molecular evolutionary genetics, gene mapping, and plant breeding; RAPD markers have a wide range of applications. In this study a set of 25 plants RAPD universal primers (RPI 1 – RPI 25) were used for amplification. The amplification products were compared and the phylogenetic tree was drawn with the help of bioinformatics tool UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

Results: The RAPD PCR (Random Amplification of Polymorphic DNA- Polymerase chain reaction) amplification resulted in different successive polymorphic banding patterns with primers in both the plants. The amplification products were compared and the phylogenetic tree was drawn with the help of bioinformatics tool UPGMA.

Conclusion: The RAPD PCR polymorphism and UPGMA study of these plants have not been reported yet. Hence RAPD PCR patterns of both these important species would help in relating them at the genetic and evolutionary level.

Keywords: *Marsilea minuta*. L., *Marsilea quadrifolia.* L., RAPD PCR, Plant DNA Fingerprinting, UPGMA, Phylogenetics.

INTRODUCTION

The genus *Marsilea* is the common aquatic fern belonging to family Marsileaceae, distributed worldwide, and having structure like four clover leaf. It is an aquatic fern; the leaves float in deep water or grow erect in shallow water or on the land. The genus consists of 53 well defined living and 10 fossil species [1]. Out of these species nine are found in India [2].

The herbal medicines are believed to be much safer for use and proved as elixir in the treatment on various illness and diseases [3]. The *Marsilea minuta* Linn (Marsileaceae) is usually found near the ponds edges and channels and as a weed in a wet rice fields. It is found throughout the India [4]. *Marsilea minuta* Linn. has great traditional medicinal value. *Marsilea minuta* Linn. is reported to be possessing anti-infertility [5], antibacterial [6], anxiolytic [7], anticonvulsant and sedative [8], analgesic and anti-inflammatory [9], antidepressant [10], adaptogenic and antistress activity [11], hypocholesterolemic [12] and hepatoprotective [13] activities.

Marsilea quadrifolia Linn. also possess great medicinal value. The juice made from its leaves has medicinal properties like diuretic and febrifuge and is widely used to treat snakebite [14]. The phytochemical analysis has shown the presence of potent phytochemical substances such as tannins, flavonoids, alkaloids, phenols, glycosides, terpenoids, saponins, sugars and steroids [15]. Considering the medicinal value of these medicinal plants, it is essential to conserve genetic diversity of plant species. RAPD PCR (Random Amplification of Polymorphic DNA-(Random Amplification of Polymorphic DNA- Polymerase chain reaction) is used to analyze the genetic diversity of an individual species by using the random primers. In RAPD, segments of DNA in very small amounts like in nano-grams get randomly amplified at low annealing temperature [16]. Thus, the present study was aimed to explore the inter-species genetic variations among these *Marselia* species using RAPD Markers.

MATERIALS AND METHODS

The leaves of *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn. were collected from the Nagpur and Satara District of Maharashtra, India, respectively. These aquatic plant materials were verified by Mrs. A. G. Mhase, Botanist, at National Research Institute of Basic Ayurvedic Sciences (NRIBAS), Pune, India and the specimens were preserved in herbarium for future reference.

DNA Extraction

The leaves were washed and wiped with 70% alcohol. Then, the leaves were crushed in mortar and pestle with the help of liquid nitrogen and turned into a fine powder. Approximately, 100-120 mg of powdered sample was taken and the genomic DNA extractions of both plants were done by using 3B BlackBio Biotech kit. The DNA of both plants was quantified using UV Vis Spectrophotometer at absorbance A_{260} nm. The DNA was then subjected to electrophoresis in 1% agarose gel and bands were observed. DNA was then kept at 4°C until further use.

RAPD- PCR

In RAPD PCR, the reaction mixture was standardized to total volume of 20µl. The RAPD-PCR components used were MilliQ water (13µl), 10X PCR Buffer (2 µl), $MgCl_2$ (1.5 µl), dNTP (1 µl), Primers (1 µl), Template DNA (1 µl), and Taq polymerase (0.5 µl).

The amplification conditions used for RAPD were 94°C for 3 min, 94°C for 45 sec, 44°C for 30 sec, 72°C for 1 min, 72°C for 5min, and 4°C for hold. After the amplification step, the samples were analyzed by Gel Electrophoresis in 2% of Agarose gel. The PCR products were loaded in the wells with gel loading dye. The Marker (DNA Ladder) 100-1000 bp was also added. The electrophoresis was carried out at 75 Volts for approximately 60 minutes.

Statistical Analysis

The banding patterns of *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn. were compared by using Gelquest® and Clustervis® softwares to construct dendrograms with the help of bioinformatics tool UPGMA (Unweighted Pair Group Method with Arithmetic Mean). The UPGMA is one of the most popular methods for the classification of sampling units on the basis of their pairwise similarities in relevant variables [17]

Table 1: RAPD primer sets (3B BlackBio Biotech India)

S. No.	Name of Primer	Accession Numbers	Sl. No.	Name of Primer	Accession Numbers
1	RPl 1	AM765819	14	RPl 14	AM773774
2	RPl 2	AM750044	15	RPl 15	AM773775
3	RPl 3	AM773310	16	RPl 16	AM773776
4	RPl 4	AM773769	17	RPl 17	AM911710
5	RPl 5	AM773770	18	RPl 18	AM765830
6	RPl 6	AM773771	19	RPl 19	AM773777
7	RPl 7	AM773312	20	RPl 20	AM773317
8	RPl 8	AM773773	21	RPl 21	AM765820
9	RPl 9	AM773315	22	RPl 22	AM911711
10	RPl 10	AM750045	23	RPl 23	AM911712
11	RPl 11	AM911709	24	RPl 24	AM765821
12	RPl 12	AM773316	25	RPl 25	AM750054
13	RPl 13	AM750046			

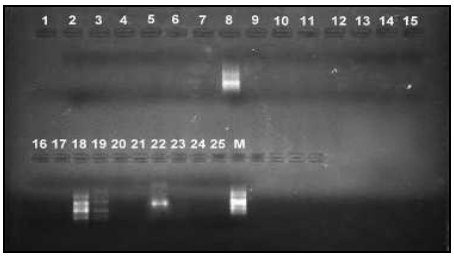

Fig. 1: RAPD PCR profiling of *Marsilea minuta* Linn.

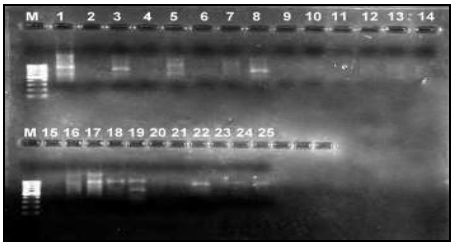

Fig. 2: RAPD PCR profiling of *Marsilea quadrifolia* Linn.

Fig. 3: Common RAPD PCR Polymerization bands of *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn. with marker ladder (100-1000 bp)

*M- *Marsilea minuta* Linn.

*Q- *Marsilea quadrifolia* Linn.

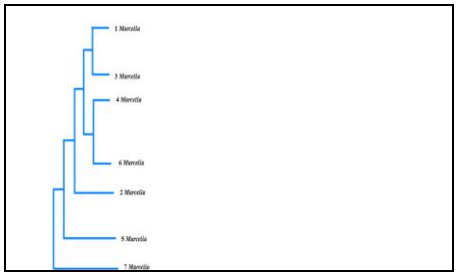

Fig. 4: The phylogenetic tree showing common ancestral linkage between *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn.

RESULTS AND DISCUSSION

Genomic Extraction

The extracted plant DNA was analyzed in UV Vis Spectrophotometer. DNA concentration obtained from *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn was 15 ng/µl and 17 ng/µl, respectively. Hence, due to good concentrations of genomic DNA in both the plants they were used as a DNA template for RAPD PCR for further studies successfully.

RAPD PCR Profiling

Twenty five random RAPD primers were used for amplification of *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn. The RAPD PCR amplification resulted in different successive polymorphic banding patterns with primers in both the plants. The different polymorphic bands were detected with all RAPD Primers. The *Marsilea minuta* Linn. species had successive bands with primers in lane Number 8, 17, 19 and 22 (fig. 1). In *Marsilea quadrifolia* Linn. species polymorphic bands were detected with primers in lane Number 1, 3, 5, 8, 16, 17, 18,19, 22, 24 and 25 (fig. 2). The names of all plant RAPD primers and their accession numbers have been denoted in Table 1. The only primers which had been resulted in both the species were considered for comparing them on the basis of UPGMA bioinformatics tool as shown in fig. 3. The marker ladder (100-1000 bp) was added to identify the molecular weight of corresponding bands. The phylogenetic tree made by UPGMA helps to correlate the ancestor relationship between both the species. This phylogenetic tree has helped to show the most nearly similar polymorphic characteristics in between them (fig. 4). Hence in the study, with great extent of similarities we can inter-relate and compare these two plants.

CONCLUSION

The *Marsilea minuta* Linn. and *Marsilea quadrifolia* Linn. are one of the Ayurvedic plant species with great vital medicinal values. The RAPD PCR polymorphism and UPGMA study of these plants have not been reported yet. Hence RAPD PCR patterns of both these important species would help in relating them at the genetic and evolutionary level. These plants can further be evaluated on the basis of Proteomics, Metabolomics and Phytochemistry to a greater extent.

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