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Research Article

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Assessment of Genetic Diversity Among *Marsilea* Populations of Hadauti Plateau as Revealed by RAPD Markers

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ABSTRACT

Marsilea shows pronounced morphological plasticity within species and as such it is difficult to distinguish species depending on traditional morphology only. In recent years modern techniques viz. molecular markers and PCR analysis have been employed for this study. In the present study genomic analysis through RAPD markers has been employed for the first time to study interpopulational differences between the same plants growing in different habitats. The present work was taken with the specific aim of identifying populations of Marsilea species of hadauti region and to find out whether the population variations are only morphological or are they genetically distinct. The results so obtained indicate sufficient level of genetic diversity.

Keywords*: Marsilea populations, morphological plasticity, genomic analysis, RAPD markers.*

INTRODUCTION

The genus *Marsilea* with approximately 65 species is named after the Italian count Luigi Ferdinando Marsigli by Linnaeus in 1754 [2]. These small plants are of unusual appearance with leaves resembling like four leaf clover held above floating on water or submerged and do not resemble common ferns. *Marsilea* can be broadly classified into two categories-hydrophytic and xerophytic depending upon whether the life history of the species is passed mostly under aquatic or terrestrial environments. The range of morphological plasticity is so much pronounced that different populations of a species growing under diverse habitats appear to be distinct species. The genus *Marsilea* exhibits a remarkable range of morphological and ecological variation and poses problems for the taxonomist and evolutionary biologist, therefore the aim of the present study is to look for molecular indicators that allow the discrimination among the species of the genus *Marsilea* based on genomic analysis.

The water clovers bear few dependable morphological characters on which to base traditional identification. Morphological plasticity and molecular evolution among species of pteridophytes are remarkably high, when compared to the angiosperm families[6]. Das et al,[3] has recently described molecular marker (RAPD) based phylogenetic studies in complementing and supplementing taxonomy of *Selginella* species. DNA sequencing of several plastid regions to attempt to "fingerprint" *Marsilea* specimens from the southeastern U.S. to provide more accurate identifications was recently done by W. Mark Whitten et al.[14].

Recently E. Rolli et al.[10] assessed the genetic stability of *M.quadrifolia* by Random amplified polymorphic DNA by comparing eight randomly selected micropropagated plants derived from repeated subcultures, with donor plant

MATERIALS AND METHODS

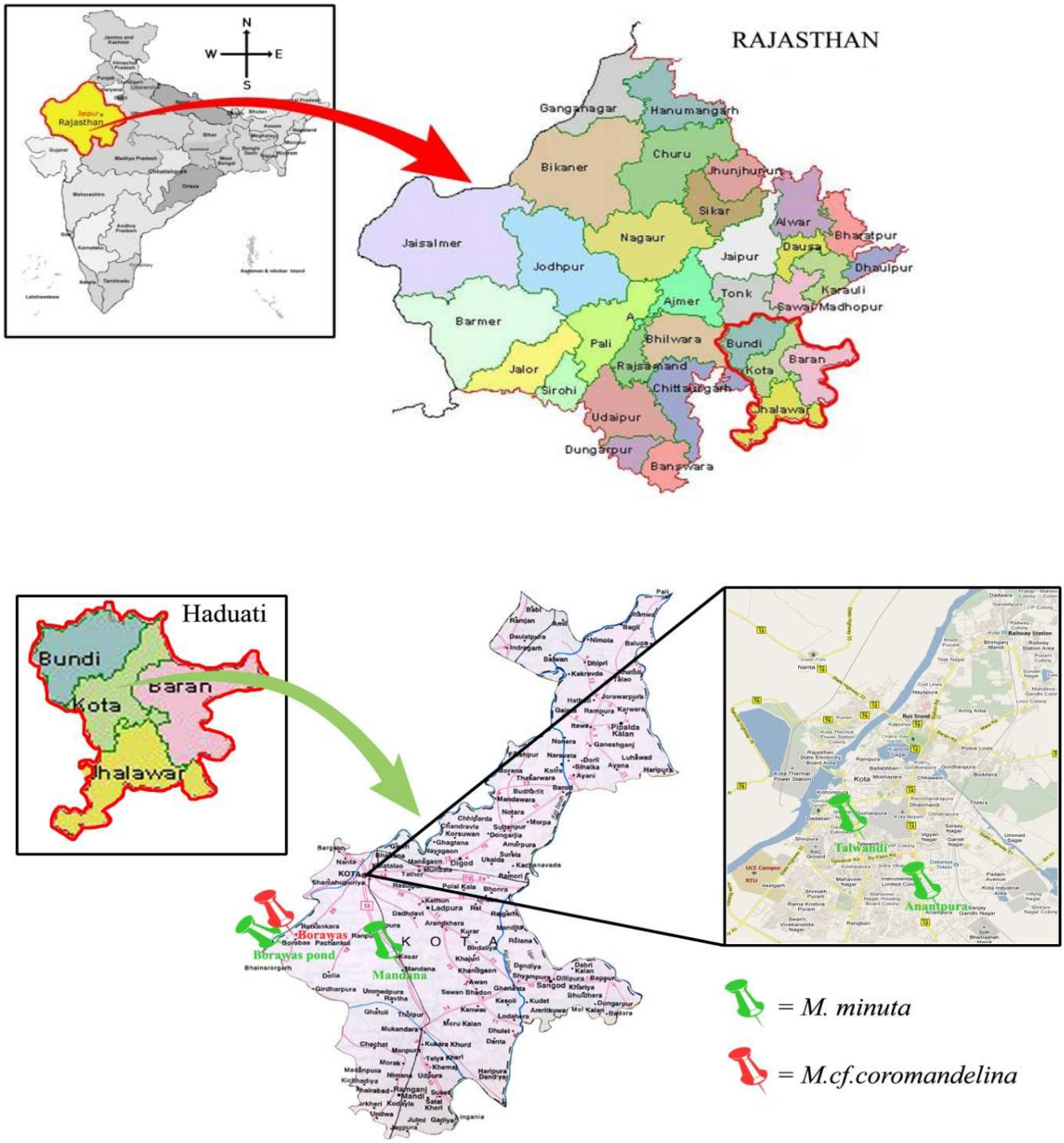
SURVEY AND COLLECTION

Kota situated at the edge of malva plateau at 23°45' to 25°53' N latitudes and 75°09' to 77°26' E longitudes in south eastern corner of Rajasthan state on the banks of Chambal river (Fig-1). *Marsilea* populations were collected from five areas (Table-1) from main city and sub urban areas of Kota region, from January 2013 to February 2014.

Table 1: Surveyed localities of *Marsilea* population of Hadauti Plateau

POPULATION	LOCALITY	SITE
M.minuta [V_1]	Talwandi (Kota city)	A massive patch along the road side situated in the centre of Kota, which remains flooded with water throughout the year.
M.minuta [V_2]	Anantpura (Kota city)	A small patch on the City Mall road representng both hydrophytic and xerophytic condition.
M.minuta (hybrid) [V_3]	Mandana (sub urban)	A small patch near Mandana bus stand
M.cf.coromandelia [V_4]	Borawas (sub urban)	A small patch located ahead of Borawas, 27 Kms away from Kota city.
M.minuta [V_5]	Borawas pond (sub urban)	Dense patch along the edges of the Borawas pond. Flooded with water only during rainy season.

Fig.1: Location map of study area



Genomic DNA isolation (CTAB DNA isolation method)

Fresh leaves obtained from culture and normal were used for extraction of DNA. The non transformed DNA serves as control DNA. Since the leaves of *Marsilea* are rich in polysaccharide and secondary metabolites, the isolation and purification of DNA sample becomes tough. These contaminations can also cause downstream problems in PCR by inhibiting the enzymes. Hence forth, genomic DNA extraction protocol outline was evaluated with minor modifications to isolate intact genomic DNA from transformed leaves. DNA isolation from selected populations of *Marsilea* is shown in Fig-2.

1 gm of leaves were harvested and Grinded in 2 ml of extraction buffer containing (6% Cetyl trimethyl ammonium bromide, 1 M Tris – HCl pH 8.0, 0.5 M EDTA pH 8.0, 5M NaCl, 0.5 % PVP and 100 µl of β-mercaptoethanol) in a pestle and mortar to fine powder and transferred in eppendorf tube. It was than Incubated for 1 hr at 65°C temperatures in dry bath with gentle shaking after each 20 min during incubation. The tube was centrifuged at 5000 rpm for 5 min. The solution was cooled and 800 µl of pre-chilled chloroform: isoamyl alcohol (24 : 1) was added and mixed well for 10-20 times. Again Centrifuged at 6000 rpm for 16 min. The supernatant was pipette out into new eppendorf tube. The procedure was repeated to get clear supernatant. 5µl RNase was added in to supernatant. 100 µl NaCl (2M) added in supernatant. After than equal amount of pre-chilled isopropanol was added to precipitate the DNA. If needed the tube was incubated at 4°C for overnight. The tube was centrifuged at 10,000 rpm for 9 min. The supernatant was pipette out and pellet was taken out. Incubated for 30 min at 50°C in dry bath. The pellet was washed with 100µl of 70% ethanol, dried at 50°C for 20 min in dry bath. Pellet was dissolved in 50 µl TE buffer. Tube incubated at 55°C for 10 min.

PCR AMPLIFICATION

The PCR mixture (25 µl) contained 50 mg of DNA prepared from normal leaves respectively as the template, 1X PCR buffer, 25 pmoles of each primer, 2.5 mM of dNTPs and 1 unit of Taq DNA polymerase (Bangalore Geini). PCR for rol A was carried out by amplifying with initial denaturation at 94ºC for 5 min followed by 35 cycles of 1 min denaturation at 94ºC, 1 min annealing at 55ºC and 1 min extension at 72ºC with a final extension of 72ºC for 10 min using a thermal cycler. Amplification was achieved in a thermal cycler (Bio-Rad, La Jolla, CA) programmed for 35 cycles.

Table: 2 List of RAPD Primers (From operon technologies)

S. No.	Primer code	Sequence
1.	OPA-01	5’-CAGGCCCTTC-3’
2.	OPA-02	5’-TGCCGAGCTG-3’
3.	OPA-03	5’-AGTCAGCCAC-3’
4.	OPA-04	5’-AATCGGGCTG-3’
5.	OPA-05	5’-AGGGGTCTTG-3’
6.	OPA-06	5’-GGTCCCTGAC-3’
7.	OPA-07	5’-GAAACGGGTG-3’
8.	OPA-08	5’-GTGACGTAGG-3’
9.	OPA-09	5’-GGGTAACGCC-3’
10.	OPA-10	5’-GTGATCGCAG-3’

Agarose Gel Electrophoresis

The integrity of DNA isolated was determined by agarose gel electrophoresis. The electrophoresis was carried out in a horizontal gel system on 1% agarose in TAE buffer and 4.5 µl of Ethidium bromide at constant voltage of 100 V for 1 to 2 hrs and visualized under Gel Documentation System (Bio-Rad, La Jolla, CA). The purity of the DNA was checked spectrophotometrically. Electrophoresis pattern of RAPD

profile was studied on 1 percent agarose gel. Only those fragments which consistently amplified were considered for analysis.

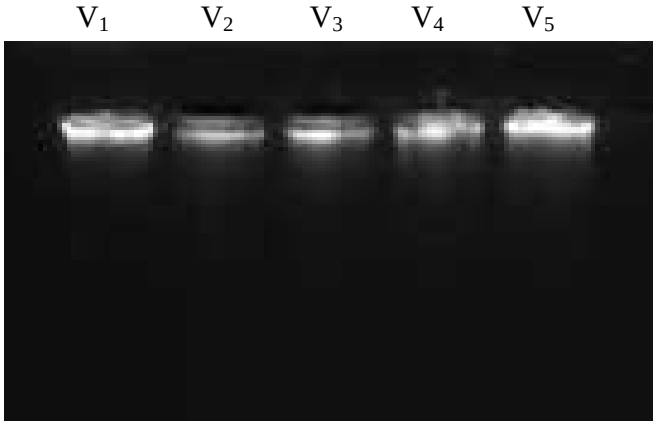
Data Analysis

Each RAPD band was assumed to represent a single locus and data were scored as presence of bands (1) and its absence as (0). The similarity coefficients were utilized to generate dendrogram by using UPGMA (Unweighted Pair Group Method of Arithmetic means).

Genetic Relationship among 5 populations of *Marsilea* and Cluster Analysis Based on Random Amplified polymorphic DNA (RAPD)

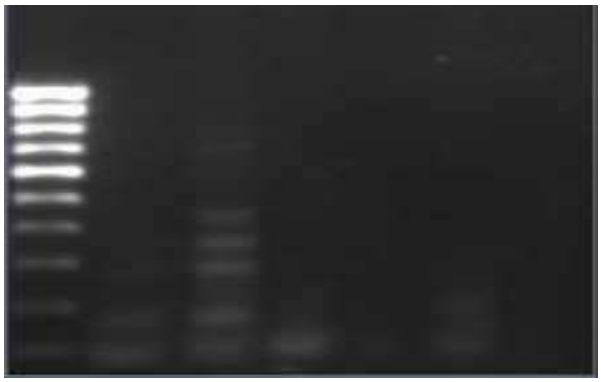
The banding pattern generated and polymorphic pattern was used to calculate the genetic similarity among the 5 selected plants and their genomes were taken for the present study. Genetic similarity estimates based on RAPD banding patterns (Fig-3) were calculated using method of Jaccard's Coefficient Analysis. The similarity coefficient matrix generated for the primers {Table-4} was subjected to algorithm UPGMA (Unweighted Pair Group Method with Arithmetic averages) and dendrogram was generated using NTSYS-pc 2.02 programme (Fig-5).

Fig.2: DNA isolation from different plant sp. of *Marsilea*



Primer OPA-01 (CAGGCCCTTC)

M V1 V2 V3 V4 V5



Primer OPA-02 (TGCCGAGCTG)

M V1 V2 V3 V4 V5

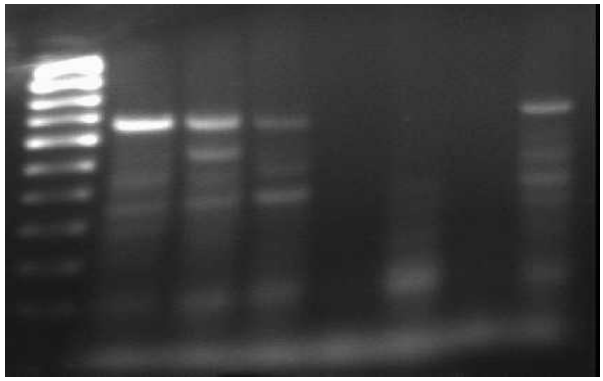
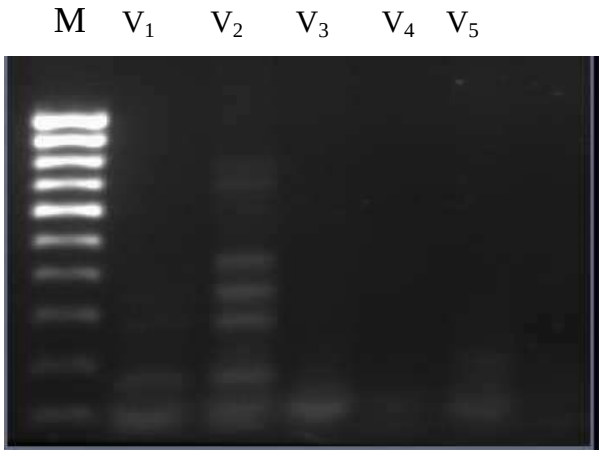
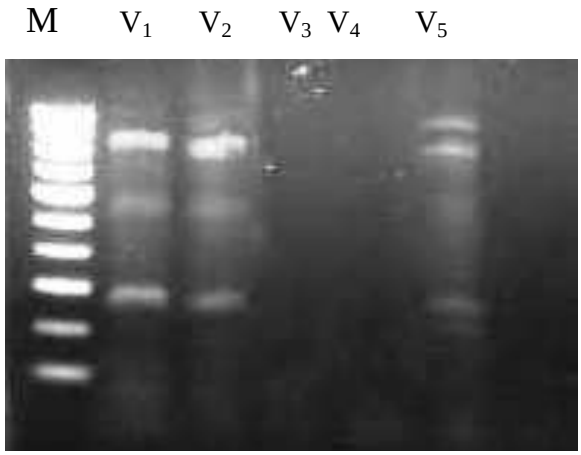


Fig. 3: RAPD band pattern (M=100bp DNA ladder)

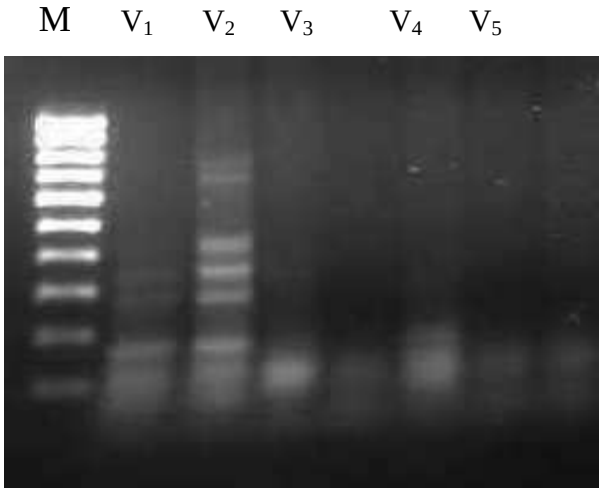
Primer OPA-05 (AGGGGTCTTG)



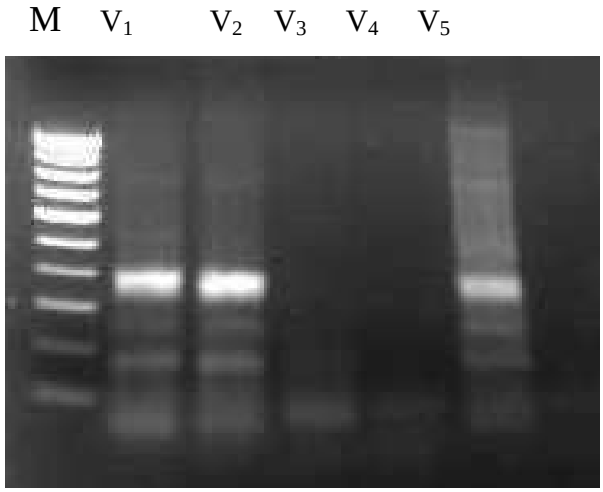
Primer OPA-07(GAAACGGGTG)



Primer OPA- 09 (GGGTAACGCC)



Primer OPA-10 (GTGATCGCAG)



RESULTS

All the selected five population of *Marsilea* growing in Kota and suburban areas of Hadauti Plateau were examined for DNA polymorphism using 10 decamer primers (Banglore Genei) showing high (G+C) content. Ten primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07, OPA-08,OPA-09, OPA-10) were screened for amplification and out of 10 primers only 06 primers (OPA-01, OPA-02, OPA-05, OPA-07,OPA-09, OPA-10) produced amplification and showed variable degree of polymorphism ranging from 0 per cent to 100 per cent. The DNA amplification and polymorphism generated among 5 plants using random primers are presented in Table 3.The representative photographs of electrophoresis gels showing RAPD profiles after amplification are depicted in figure 4,

Six primers (out of ten primers used) on five populations generated a total of 22 bands and all were polymorphic bands. (Table 3). The average number of bands per primer was found to be 3.66 (22/6). The average numbers of polymorphic bands per primer were 3.66 (22/06) (total no of polymorphic band/ no of primers amplified). Overall polymorphism was found to be 100 per cent. Similar results were found by Gupta *et al.*[4] who observed 84.26% polymorphism.

Table 3: Details of the random primers used for amplification of genomic DNA of 5 *Marsilea* populations

Total number of primers	10
Number of primers which show amplification	06
Number of primer which show polymorphism	06
Number of primers which show monomorphism	00
Total number of monomorphic bands	00
Total number of polymorphic bands	22
Total number of bands	85

The similarity coefficient for different genotypes was in the range of 0.00 to 0.90. The maximum similarity coefficient (0.90) was observed between V_5 and V_1. The results obtained were in conformity with the earlier report of Moura et al. (2005) who selected 93 accessions of Jaborandi to study genetic diversity using RAPD marker with a similarity coefficient of 0.86 while the minimum similarity coefficient (0.00) was observed in V_4 from V_1, V_2, V_3 and V_5.

Table 4: Similarity coefficient matrix of five *Marsilea* population obtained from RAPD markers

Marsilea populations	*M.minuta* (Talwandi)	*M.minuta* (Anantpura)	*M.minuta* (Mandana)	*M.cf.coromandelina*(Borawas)	*M.minuta* (Borawas pond)
	V_1	V_2	V_3	V_4	V_5
V_1	1.00				
V_2	0.43	1.00			
V_3	0.30	0.19	1.00		
V_4	0.00	0.00	0.00	1.00	
V_5	0.90	0.41	0.27	0.00	1.00

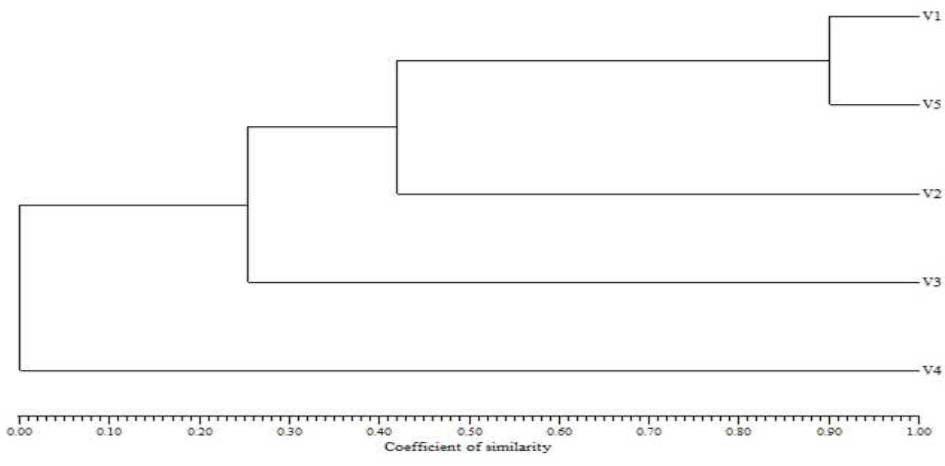
RAPD Dendrogram: (Fig-4)

Dendrogram was constructed using similarity matrix value as determined from RAPD data depicting the relationship among the genotypes of 5 selected plants. The dendrogram generated on the basis of Jaccard's Similarity Coefficient, clearly indicate two clusters. Ndoye-Ndir also studied the genetic diversity of 70 individual samples of *Casuarina equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* growing along the northern coast of Senegal which were characterized by RAPD marker. The result suggested sufficient level of genetic diversity.

Cluster A include four populations namely., V_1, V_2, V_3, V_5 at similarity coefficient of 0.26. This cluster was divided into two sub-clusters A1, A2 at similarity coefficient of 0.43. Sub-cluster A1 includes 3 populations' viz., V_1, V_2, and V_5. In which V_1 and V_5 were showing similarity coefficient of 0.90 while V_2 separated at similarity coefficient of 0.43. Sub-group A2 consists of one plant V_3 which diverged with cluster A1 at similarity coefficient of 0.27. Sub-group A2 joins A1 at similarity coefficient of 0.27.

Cluster B include one plant V_4. This species diverged from Cluster A at similarity coefficient of 0.00. The diversion of this plant V_4 (*Marsilea coromandelina* complex) from other 4 plants at similarity coefficient of 0.00 indicates that this plant is genetically distinct from other selected *Marsilea* populations.

Fig. 4- Dendrogram showing genetic relationship among five *Marsilea* populations as revealed by UPGMA cluster analysis of Jaccard's coefficients based on RAPD markers



DISCUSSION

Marsilea is well known for its range of morphological plasticity. *Marsilea* shows morphological variation within species and as such it is difficult to distinguish species depending on traditional morphology only. Molecular methods were used to enrich this study and testify molecular methods as a tool to find the genetic bases of the differences and similarities among species. The present study is the first molecular study of *Marsilea* population of five surveyed localities in Hadauti plateau. The RAPD-PCR polymorphism and UPGMA study of the genus Marsilea have not been reported till date. Hence RAPD-PCR patterns of both *Marsilea* species (*M.minuta* and *M.cf.coromandelina* complex) are phylogenetically important which would help in relating them at the genetic and evolutionary level.

In this study, minimum numbers of amplified loci were obtained using primer OPA-02 sequence code (5'-TGCCGAGCTG-3'). The genetic similarity matrix of RAPD data for 5 populations were constructed of dendrogram using the UPGMA, as described by Chansiripornchai et al. (2000). The dendrogram showed the genetic relationships among species of *Marsilea* and they seem very closely related, this similarity between them may be due to their common geographical localities. However, *Marsilea coromandelina* complex ($V_{4)}$ species diverged from Cluster A at similarity coefficient of 0.00. The diversion of this plant V_4 (*Marsilea coromandelina complex*) from other 4 plants at similarity coefficient of 0.00 is giving indication that this plant is genetically different (species) from the rest of 4 *Marsilea* populations. It was also observed that *Marsilea coromandelina* complex population is morphologically different from other populations having silver shining streaks (pellucid streaks) between the veins of the leaves and squarish ribbed sporocarp. Accordingly to the genetic distance and relationships illustrated, the ability to resolve genetic variation among different *Marsilea* species may relate to the genomic analysis methodology employed in this study.

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