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DNA Fingerprinting in *Utricularia* L. Section *Utricularia* as Revealed by PCR Based Assay

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Abstract: DNA fingerprinting from Polymerase Chain Reaction (PCR) based assay ISSR (inter simple sequence repeat) has been investigated in order to detect interspecific variation and molecular relationships among nine aquatic species of the section *Utricularia*. PCR was performed with ISSR primers following agarose gel electrophoresis with ethidium bromide staining. The primer UBC 889 based on $(AC)_n$ repeat presented the highest number of fingerprints while the primer UBC 891 based on $(TG)_n$ repeat produced the least. *Utricularia bremii* showed the highest number of DNA fingerprints followed by *U. gibba* while *U. aurea* showed the least number of bands. The highest genetic affinity was detected between *U. gibba* and *U. bremii* and the highest genetic distance was found between *U. bremii* and *U. dimorphantha* among the species analyzed. Phenetic analysis based on the ISSR fingerprints using the Neighbour Joining analysis resolved the aquatic species into two groups: i) *Utricularia aurea*, *U. australis*, *U. dimorphantha*, *U. intermedia*, *U. macrorhiza*, *U. minor* and *U. vulgaris* ii) *U. bremii* and *U. gibba*. Principal coordinates analysis in aquatic *Utricularia* resulted in three clusters and the genetic relationships obtained from this analysis were found consistent with that of Neighbour Joining analysis. Molecular relationships obtained from PCR-based DNA fingerprinting is an agreement of taxonomy of *Utricularia* based on non-morphological approaches.

Key words: Aquatic *Utricularia*, DNA fingerprints, genetic relationships, ISSR-PCR, neighbor joining, principal coordinates analysis

INTRODUCTION

DNA fingerprinting is an important tool for molecular characterization of various groups of plants. It offers a faster and more precise way of determining relationships among closely related species than that of morphological investigation because morphological characteristics are subject to environmental influence and thorough and extensive studies of mature plants are often necessary for taxonomic classification. During planning DNA fingerprinting, one of the most important decision is the marker systems and techniques to be employed. Several DNA marker systems and associated techniques are available today for fingerprinting and many of them are based on Polymerase Chain Reaction (PCR). The commonly used PCR-based techniques include random amplified polymorphic DNA (RAPD; Williams *et al*., 1990), amplified fragment length polymorphism (AFLP; Vos *et al*., 1995) and inter simple sequence repeat (ISSR; Zietkiewicz *et al*., 1994). Among these techniques ISSR appears as an easy, reliable, speedy and highly reproducible. ISSR markers have the ability to detect variation without prior sequence information. They are more advantageous than other DNA markers and overcome many of the limitations of these markers (Pujar *et al*., 2002). In addition, they are found most economical when a cost comparison was made between RAPD, RFLP (Restriction fragment length polymorphism) and ISSR methods (Yang *et al*., 1996).

The ISSR assay has been successfully used for DNA fingerprinting (Vijayan, 2004; Pharmawati *et al*., 2005), genetic diversity (Huang and Sun, 2000; Ge *et al*., 2005), phylogenetic and species relationships (Joshi *et al*., 2000; Wolfe and Randle, 2001), germplasm characterization (Moreno *et al*., 1998; Lanham and Brennan, 1999), cultivar identification (Fang and Roose, 1997; Chowdhury *et al*., 2002), population studies (Camacho and Liston, 2001; Xie *et al*., 2005), gene tagging (Akagi *et al*., 1996; Ratnaparkhe *et al*., 1998), genome mapping (Sankar and Moore, 2001; Carlier *et al*., 2004) and biogeography (Smissen *et al*., 2003).

Utricularia belongs to the family Lentibulariaceae is a carnivorous group of plants and consists of 35 sections (Taylor, 1989). It is found world-wide except for the Antarctic, but the greatest species richness is in tropical regions with the largest number of species originating from Central and South America. They are unique because of the structural complexity of their traps and the rapid movement of the opening and closing of the trapdoors. Many important accounts were made in the genus

Utricularia based on morphology, cytology and palynology (Taylor, 1989; Casper and Manitz, 1975; Sohma, 1975). Out of 35 sections reported the section *Utricularia* includes all aquatic species and the members of this section are found in different ponds, lakes, ditches, marshy areas and other aquatic bodies. There is limited knowledge about the taxa of this section (Meyers and Strickler, 1979; Sasago and Sibaoka, 1985). In comparison with morphological studies molecular works in *Utricularia* are very scanty (Müller *et al*., 2002; Rahman and Kondo, 2003). DNA fingerprinting pattern has not been detected in aquatic species to determine genetic variability and to characterize them. Therefore, the present study was undertaken to evaluate the potential of PCR-based ISSR fingerprinting for detecting genetic variation and inferring molecular relationships among aquatic species of the section *Utricularia* for the first time. The results elucidate that ISSR markers are valuable for determining interspecific variation and molecular characterization in *Utricularia*.

MATERIALS AND METHODS

Plant materials: The plant materials employed in this study have been listed in Table 1. Plants were grown both *in vivo* and *in vitro* culture (B5 medium-Gamborg *et al*., 1968) at the Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Japan.

Genomic DNA extraction: Genomic DNA was extracted from the leaf tissue. 1.0-1.8 g of leaf was homogenized in a mortar using liquid nitrogen. The powdered tissue was transferred to a 20 mL capacity of capped, sterilized centrifuged tube containing 10 mL of wash buffer [0.1 M Tris-HCl at pH 8.0, 2% 2-Mercaptoethanol, 1% Polyvinylpyrolidone K-30, 0.05M L-Ascorbic acid, dissolved in distilled water]. After shaking gently for 10 min the tube was centrifuged at 10,000 rpm at 20°C for 10 min. The supernatant was discarded from the tube and this process was repeated until the solution becomes transparent. After removing the supernatant, 10 mL of CTAB buffer [2% Cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.1% Tris-HCl at pH 8.0, 20 mM EDTA-Na_2, dissolved in distilled water] and 0.5 mL 2-Mercaptoethanol were added to the tube followed by an incubation at 55°C for 60-90 min in order to supply the stabilization of DNA. Following that 10 mL chloroform:isoamylalcohol (24:1) was added to the tube and was shaken gently for 10 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new sterilized centrifuged tube and it was continued until there was no precipitation on the border of the supernatant layer and chloroform: isoamylalcohol layer. The final supernatant was transferred to another centrifuged tube and 10 mL of 2-propanol was added followed by a centrifugation at 10,000 rpm for 15 min at 4°C. After discarding the solution, 5 mL 70% chilled ethanol was added to wash the pellet and was centrifuged at 10,000 rpm for 5 min. DNA was dried after decanting the ethanol and the dried DNA pellet was dissolved in 450 μL TE solution (10 mM Tris-HCl and 1mM EDTA) with 0.1 mg mL^{-1} RNAse (Sigma). After incubation for 1 h at 37°C the solution was transferred in a 1.5 mL sterilized ependorf tube. Two hundred and fifty microlitter of neutral equilibrated phenol and 250 μL of chloroform: isoamylalcohol (24:1) were added to the tube. The tube was centrifuged for 10 min after shaking for 10 min. The upper phase was transferred to a new tube. Five hundred microlitter chloroform:isoamyl alcohol was added and was centrifuged for 10 min. The upper aquous solution was transferred to a new tube. Then 50 μL of 3M sodium acetate and 500 μL of 99.5% chilled ethanol were added to the tube and they were kept at -80°C for 20 min. The DNA pellet was found by spinning in a microcentrifuge at 15,000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed with 70% chilled ethanol using centrifugation at 15,000 rpm for 15 min at 4°C. The ethanol was discarded and the DNA was dried into a Halogen Vacuum Concentrator for 3-5 min. The isolated DNA was dissolved in TE buffer and stored at -20°C.

PCR amplification: Seventy-two ISSR primers were screened for PCR amplification of *Utricularia* DNA, out of which the primers UBC 864[$(ATG)_6$], UBC 888[$(CA)_7$BDB], UBC 889[$(AC)_7$DBD], UBC 890[$(GT)_7$VHV] and UBC 891[$(TG)_7$HVH] were finally used because of their ability to produce clear and reproducible patterns of bands (Table 2). The PCR amplification was performed in a 10 μL reaction volume containing 10 ng of template DNA, 1 μM of a single primer (UBC, Vancouver, Canada), 1 μL of X10 Taq buffer, 0.8 μL of dNTP mixture and 0.05 μL of Taq polymerase enzyme and the remaining was filled with deionized distilled water. The mixture was overlaid with 30 μL mineral oil and subjected to PCR. Amplifications were carried out using a PTC-100 thermocycler with an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 45 sec at annealing temperature 50°C and 2 min extension at 72°C. A final extension step for 5 min at 72°C was included. PCR products were visualized using 1.5% agarose gel electrophoresis stained with ethidium bromide. The electrophoretic gels were photographed under ultraviolet radiation.

Table 1: List of the aquatic *Utricularia* species used in the present study

Species	Cultivation	Distribution*
U. aurea Lour.	*In vitro*	North Asia, Tropical Asia, Malaysia, Australia, New Zealand and New Caledonia
U. australis R. Br.	*In vivo*	Europe, North Asia, Africa, Tropical Asia, Malaysia, Australia, New Zealand and New Caledonia
U. bremii Heer ex. Kölliker	*In vitro*	Europe
U. dimorphantha Makino	*In vivo*	North Asia
U. gibba L.	*In vitro*	North America, Central America, South America, Europe, North Asia, Africa, Tropical Asia, Malaysia, Australia, New Zealand and New Caledonia
U. intermedia Hayne	*In vivo*	North America, Europe and North Asia
U. macrorhiza LeConte	*In vivo*	North America, Central America and North Asia
U. minor L.	*In vivo*	North America, Europe, North Asia, Tropical Asia and Malaysia
U. vulgaris L.	*In vivo*	Europe and North Asia

* After Taylor (1989)

Table 2: ISSR primers used for analyses of aquatic *Utricularia* species

Primer name	Nucleotide sequences[a]	Repeat	Percentage of fragments
UBC 864	ATG ATG ATG ATG ATG ATG	$(ATG)_6$	20
UBC 888	BDB CAC ACA CAC ACA CA	$(CA)_7$BDB	20
UBC 889	DBD ACA CAC ACA CAC AC	$(AC)_7$DBD	23
UBC 890	VHV GTG TGT GTG TGT GT	$(GT)_7$VHV	16
UBC 891	HVH TGT GTG TGT GTG TG	$(TG)_7$HVH	21

a: Y stands for pyrimidine; B for non-A, D for non-C; V for non-T and H for non-G residues

Data scoring and analysis: Each ISSR band was considered as a character and the presence or absence of the band was scored in a binary code (present = 1, absent = 0). A data matrix was generated using the binary code. Data analyses were performed using the NTSYS-pc (Numerical Taxonomy System, Rohlf, 2000) version 2.1 computer program package. Genetic similarity was measured with the SIMQUAL (Similarity for qualitative data) program that computes similarity coefficients for qualitative data using Simple Matching coefficient. Genetic distance was calculated employing Jukes and Cantor (1969) coefficient and a Neighbour Joining tree was constructed. Among the ordination approaches, the Principal Coordinates Analysis (PCO) was conducted to view the clustering pattern of the taxa more precisely. In PCO, the distance matrix based on Dist coefficient was transformed using the double-centering option, eigenvectors calculated and the multidimensional hyper ellipsoid viewed as a three-dimensional model.

RESULTS AND DISCUSSION

The potential of ISSR fingerprints has been observed for genetic analysis in the section *Utricularia*. Five ISSR primers employed generated a total of 250 ISSR fragments across nine aquatic species of the section *Utricularia*. *Utricularia bremii* presented the highest number of fingerprints followed by *U. gibba*. *Utricularia aurea* showed the least number of fragments and *U. intermedia* and *U. vulgaris* gave same number of fragments. Figure 1 represents the amplification patterns generated using the primer UBC 864 across nine aquatic species of *Utricularia*. The primer UBC 889 presented the highest number of bands (Fig. 2) followed by the primer UBC 891 (Fig. 3) in all species. The average number of fragments generated by all analyzed primers was 50.

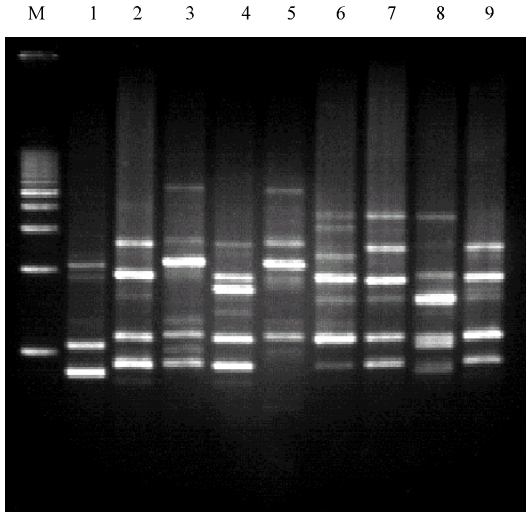

Fig. 1: DNA fingerprints in aquatic *Utricularia* species revealed by ISSR primer UBC 864. M. Molecular size marker (1 kb). 1. *U. aurea*. 2. *U. australis*. 3. *U. bremii*. 4. *U. dimorphantha*. 5. *U. gibba*. 6. *U. intermedia*. 7. *U. macrorhiza*. 8. *U. minor* and 9. *U. vulgaris*

The similarity index has been calculated in aquatic *Utricularia* species using Simple Matching coefficient based on ISSR fingerprints. The estimated genetic similarity index indicates that *U. bremii* was most closely allied to *U. gibba* among the aquatic species employed. *Utricularia australis* showed a high affinity with *U. dimorphantha* and *U. vulgaris*. *U. macrorhiza* is closely allied to *U. vulgaris* while *U. intermedia* is genetically close to *U. minor* (Table 3).

The genetic distance in the species studied has been calculated employing Jukes and Cantor coefficient. The

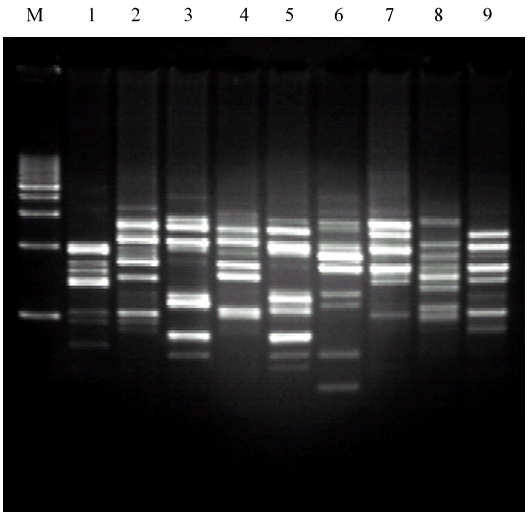

Fig. 2: DNA fingerprints in aquatic *Utricularia* species revealed by ISSR primer UBC 889. M. Molecular size marker (1 kb). 1. *U. aurea*. 2. *U. australis*. 3. *U. bremii*. 4. *U. dimorphantha*. 5. *U. gibba*. 6. *U. intermedia*. 7. *U. macrorhiza*. 8. *U. minor* and 9. *U. vulgaris*

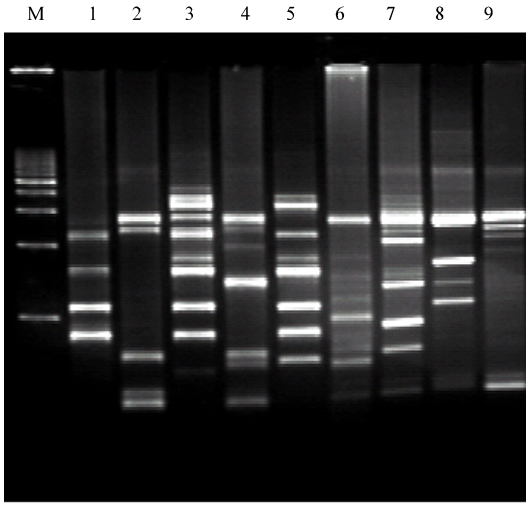

Fig. 3: DNA fingerprints in aquatic *Utricularia* species revealed by ISSR primer UBC 891. M. Molecular size marker (1 kb). 1. *U. aurea*. 2. *U. australis*. 3. *U. bremii*. 4. *U. dimorphantha*. 5. *U. gibba*. 6. *U. intermedia*. 7. *U. macrorhiza*. 8. *U. minor* and 9. *U. vulgaris*

highest genetic distance across the species was observed between *U. bremii* and *U. dimorphantha* showing that these two species were most far from each other among the species analyzed. *Utricularia bremii* also exhibited a high genetic distance with *U. macrorhiza*. The same genetic distance was found between *U. bremii* and *U. vulgaris* and between *U. dimorphantha* and *U. gibba*. Among the species analyzed the lowest genetic distance was identified between *U. bremii* and *U. gibba* (Table 4).

Cluster analysis of the genetic distance values was carried out to generate a dendrogram indicating relationships between aquatic species studied. A Neighbour-joining (NJ) tree was constructed based on Jukes and Cantor coefficient. The NJ tree resolved that the aquatic species could be placed in two groups: One consisted of *Utricularia aurea*, *U. australis*, *U. dimorphantha*, *U. intermedia*, *U. minor*, *U. macrorhiza* and *U. vulgaris* and the other group constituted from *U. bremii* and *U. gibba* (Fig. 4). The first group is subdivided into two subgroups: one possessed *U. aurea*, *U. australis*, *U. dimorphantha*, *U. macrorhiza* and *U. vulgaris*. *Utricularia dimorphantha* has been found closest to *U. australis* in this cluster and the other subgroup contained *U. intermedia* and *U. minor*.

Principal Coordinates Analysis (PCO) was performed to more effectively view the clustering pattern of the taxa. The PCO analysis of ISSR data in aquatic species resulted in three clusters where *Utricularia bremii* and *U. gibba* grouped together, *U. minor* and *U. intermedia* formed another cluster and the third cluster consisted of *U. aurea*, *U. australis*, *U. dimorphantha*, *U. macrorhiza* and *U. vulgaris* (Fig. 5). The results obtained from the PCO analysis in the aquatic species studied were found coherent to the Neighbour Joining analysis.

DNA fingerprinting employing different marker systems is quite evident these days (Vanderpoorten *et al*., 2003; Roy *et al*., 2006). Recent studies revealed that ISSR markers are potential in molecular characterization of different groups of angiospermic taxa viz., *Amaranthus* (Xu and Sun, 2001), *Gossypium* (Liu and Wendel, 2001), *Tolpis* (Mort *et al*., 2003), *Cicer* (Sudupak, 2004), *Phaseolus* (Sicard *et al*., 2005), *Juniperus* (Meloni *et al*., 2006) and *Morus* (Vijayan *et al*., 2006). ISSR markers have also been found application in other groups of plant including phytoplankton (Bornet *et al*., 2004), fern (Thomson *et al*., 2005) and gymnosperm (Xiao and Gong, 2006).

Many authors paid acute attention on *Utricularia* and those studies were mainly based on morphological investigations. Taylor (1989) produced an excellent world monograph of *Utricularia* while Casper and Manitz (1975) did the pioneer chromosomal work of the genus. The pollen morphology was studied extensively by Huynh (1968). However, very little information is available on molecular studies of this genus. Although a previous

Table 3: Similarity matrix among the aquatic *Utricularia* species studied by ISSR markers

Species	*U. aurea*	*U. australis*	*U. bremii*	*U. dimorphantha*	*U. gibba*	*U. intermedia*	*U. macrorhiza*	*U. minor*	*U. vulgaris*
U. aurea	1								
U. australis	0.66	1							
U. bremii	0.58	0.55	1						
U. dimorphantha	0.62	0.82	0.48	1					
U. gibba	0.60	0.56	0.83	0.54	1				
U. intermedia	0.61	0.66	0.63	0.64	0.62	1			
U. macrorhiza	0.68	0.69	0.51	0.71	0.55	0.68	1		
U. minor	0.63	0.60	0.59	0.62	0.66	0.72	0.68	1	
U. vulgaris	0.70	0.76	0.54	0.73	0.60	0.65	0.74	0.61	1

Table 4: Pair-wise genetic distance among the aquatic *Utricularia* species studied by ISSR markers

Species	*U. aurea*	*U. australis*	*U. bremii*	*U. dimorphantha*	*U. gibba*	*U. intermedia*	*U. macrorhiza*	*U. minor*	*U. vulgaris*
U. aurea	0								
U. australis	0.46	0							
U. bremii	0.62	0.68	0						
U. dimorphantha	0.52	0.19	0.88	0					
U. gibba	0.56	0.66	0.19	0.70	0				
U. intermedia	0.54	0.46	0.51	0.49	0.52	0			
U. macrorhiza	0.41	0.39	0.80	0.37	0.68	0.41	0		
U. minor	0.51	0.56	0.58	0.52	0.46	0.35	0.41	0	
U. vulgaris	0.38	0.28	0.70	0.34	0.56	0.47	0.32	0.54	0

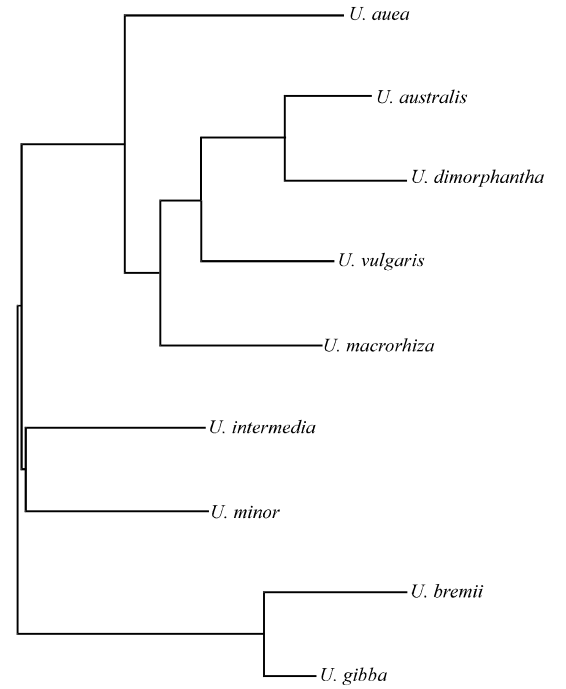

Fig. 4: Neighbor-Joining tree for aquatic *Utricularia* species obtained from ISSR fragments based on Jukes and Cantor coefficient

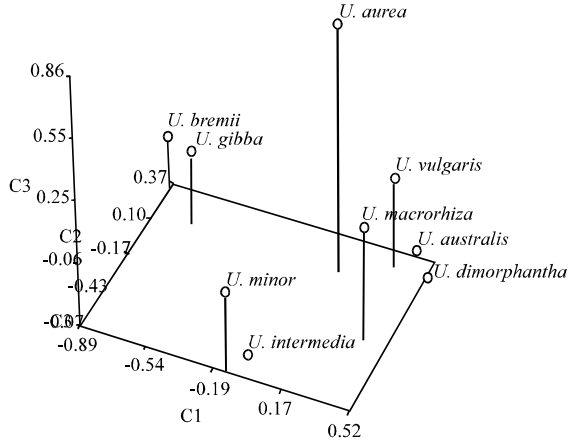

Fig. 5: Principal Coordinates Analysis for aquatic *Utricularia* species based on ISSR fingerprints

study demonstrated the genetic variation among some terrestrial species of *Utricularia*, that study provided no information among the aquatic ones (Rahman and Kondo, 2003).

The present study shows that ISSR markers are very useful for genetic variability and species relationship in *Utricularia*. DNA fingerprinting from PCR-based ISSR analysis in aquatic *Utricularia* revealed that *U. australis, U. dimorphantha, U. macrorhiza* and *U. vulgaris* grouped together (Fig. 4 and 5). The close relationship among these species obtained from this work support the previous intrageneric classification of the genus based on morphological characters (Taylor, 1989). Cytologically, *Utricularia dimorphantha* was found close to *U. vulgaris* showing the same chromosome numbers. Recently Rahman *et al.* (2001) counted the somatic chromosome number 2n = 44 in *U. dimorphantha* which was same as in *U. vulgaris* (Pogan *et al.*, 1987). The close relationship between these two species from cytological studies has been found coherent with the present investigation. This work further demonstrates that *Utricularia bremii* is genetically closest to *U. gibba* among the species studied and morphologically, these species are closely allied. The highest genetic distance among the aquatic species has

been detected between *U. bremii* and *U. dimorphantha* (Table 4), which is in accordance with the morphology and cytology of these species (Taylor, 1989; Rahman *et al*., 2001). Hence, the present study has been found corroborated with morphological and cytological investigations indicating the potential of ISSR fingerprints for molecular analysis of the aquatic species of the section *Utricularia*.

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