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EVALUATION OF RAPD MARKERS FOR TAXONOMIC RELATIONSHIPS IN SOME AQUATIC SPECIES OF *UTRICULARIA* L. (LENTIBULARIACEAE)

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Abstract

Random amplified polymorphic DNA (RAPD) markers were used to assess relationship across nine aquatic species of *Utricularia*. The highest numbers of RAPD bands were detected in *Utricularia bremii* and *U. intermedia*. The highest genetic similarity was observed between *U. australis* and *U. dimorphantha*; between *U. australis* and *U. vulgaris*; and between *U. dimorphantha* and *U. macrorhiza* indicating that these species are closely related. Unweighted pair group method with arithmetic mean (UPGMA) analysis based on the RAPD profile in aquatic *Utricularia* resolved the taxa into three clusters: the first cluster included *U. aurea, U. australis, U. dimorphantha, U. macrorhiza* and *U. vulgaris*; the second cluster constituted from *U. bremii* and *U. gibba* while *U. intermedia* formed the third cluster with *U. minor*. The Neighbour Joining (NJ) tree showed that aquatic species could be placed in two main groups and the results obtained from the NJ analysis were coherent with that of the UPGMA clustering method. The molecular findings revealed from this study support the systematic relationships in *Utricularia* inferred from morphological investigations.

Introduction

The development of random amplified polymorphic DNA (RAPD) markers generated by polymerase chain reaction (PCR) using arbitrary primers has resulted in alternative molecular markers for the detection of nuclear DNA polymorphism (Williams *et al.* 1990). RAPD markers detect nucleotide sequence polymorphisms, providing fingerprints for individuals and populations. These sequence polymorphisms may arise due to changes in the random priming sites on the template or because of insertions or deletions in the DNA downstream of the site, changing the size of the amplified fragment. RAPD method employs a lower annealing temperature during amplification ($35\text{-}39^0$C compared to the usual $50\text{-}55^0$C) because of using random primers (Bowditch *et al*. 1994). The resulting PCR products from RAPD analysis are electrophorized on 1.5-2.0% agarose gel and stained with ethidium bromide. Sometimes polyacrylamide gel and silver staining are also used. Huff and Bara (1993) found silver-stained RAPD markers more reliable than RAPD analysis using agarose gel and ethidium bromide staining. However, the agarose gel with ethidium bromide staining were used successfully by Sweeney and Danneberger (1995). Electrophoresis of a set of products translated from different DNA regions chosen by the primer produces a series of bands in this technique (Schierwater 1995).

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The RAPD technique has many advantages such as detection of polymorphism, relatively inexpensive, fast, and reliable. In addition, it utilizes primers of arbitrary sequences which may be used for different species and does not require previous knowledge of DNA sequence. RAPD markers have found application in many fields including DNA fingerprinting (Caetano-Anollés *et al.* 1991, Micheli *et al*. 1994), assessment of genetic diversity (Bolaric *et al.* 2005, Bodo Slotta and Porter 2006), cultivar identification (Koller *et al.* 1993, Al-Khalifah and Askari 2003), estimation of population genetic parameters (Oiki *et al.* 2001, Sales *et al.* 2001), hybridization (Caraway *et al.* 2001, Triest 2001), systematics (Díaz Lifante and Aguinagalde 1996, Vilatersana *et al*. 2005), phylogeny reconstruction (Rath *et al.* 1998, Ahmed *et al*. 2005) and genome mapping (Stockinger *et al*. 1996, Krutovskii *et al*. 1998). RAPD markers have been used in different group of plants. These markers have also been applied successfully in many aquatic plants. San Martín *et al*. (2003) applied these markers for detecting genetic variation in *Aldrovanda vesiculosa*. Madeira *et al*. (1997) determined phenetic relationships among accessions of *Hydrilla verticillata*. Waycott (1995) assessed genetic variation in seagrass *Posidonia australis* employing RAPD and allozyme analyses.

Utricularia, a member of the family Lentibulariaceae, encompasses 214 species and is distributed throughout the world with the largest number of species in tropical and central America (Taylor 1989). Many important contributions were made on *Utricularia* based on morphology (Taylor 1989, Crow 1992), cytology (Casper and Manitz 1975, Pogan *et al.* 1990) and palynology (Huynh 1968, Sohma 1975). However, molecular techniques have not been widely used in this genus to evaluate genetic variation and to infer systematic relationships except a few investigations (Müller *et al.* 2002, Rahman and Kondo 2003). RAPD markers have never been tested in this important carnivorous genus. The present study offers a methodological approach using RAPDs to investigate partitioning of variation and taxonomic relationships among nine aquatic species of *Utricularia*. This investigation examines the suitability of RAPDs as a tool for identifying *Utricularia* species and detecting genetic variability among the species.

Materials and Methods

Plant materials: The species of *Utricularia* employed in this study are listed in Table 1. The species were grown in both *in vitro* and *in vivo* cultures at the Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Japan. In order to grow the species *in vitro* culture B5 medium was used (Gamborg *et al*. 1968).

Isolation of DNA: Total DNA was isolated from the leaf tissue ranging from 1.0g to 1.8g using CTAB (Cetyl trimethyl ammonium bromide) method. See Rahman and Kondo (2003) for detailed protocol for DNA extraction. The isolated DNA was dissolved in TE buffer and stored at -20^0C.

RAPD amplification : RAPD primers were purchased from Operon Technology. The following oligonucleotide primers were examined for RAPD analysis which provided good amplified products: OPA1, OPA2, OPA3, OPA4, OPA7, OPA9, OPA13, OPB1, OPB4, OPB5, OPB6, OPB8, OPB11, OPB15, OPB17 and OPB18. Each PCR included 20ng of DNA, 10 pmol primer, 1µl 10X Ex Taq buffer, 1µl of dNTP mixture, 0.05µl Taq polymerase enzyme, and sterile, deionised water up to final volume of 12µl. The reaction mixture was overlaid with 30µl mineral oil. PCR reaction was performed in a PTC-100 thermal cycler as per following temperature profile: 1 min at 94ºC, 1 min at 35ºC and 2 min at 72ºC followed by 45 cycles. A final 5 min extension at 72ºC ensured full extension of all amplified fragments.

Table 1. List of the aquatic species of *Utricularia* employed in the present study.

Name of the species	Cultivation procedure	Amount of leaf (g)	Distribution*
U. aurea Lour.	*In vitro*	1.2	NAS, TAS, MAL, ANZ
U. australis R. Br.	*In vivo*	1.5	EUA, NAS, AFR, TAS, MAL, ANZ
U. bremii Heer ex. Kölliker	*In vitro*	1.8	EUA
U. dimorphantha Makino	*In vivo*	1.5	NAS
U. gibba L.	*In vitro*	1.4	NAM, CAM, SAM, EUA, NAS, AFR, TAS, MAL, ANZ
U. intermedia Hayne	*In vivo*	1.0	NAM, EUA, NAS
U. macrorhiza LeConte	*In vivo*	1.3	NAM, CAM, NAS
U. minor L.	*In vivo*	1.2	NAM, EUA, NAS, TAS, MAL
U. vulgaris L.	*In vivo*	1.1	EUA, NAS

* After Taylor (1989)

CAM: Central America from Mexico to Panama, including all the islands in the Caribbean from the Bahamas southwards to Trinidad; **SAM**: South America from Colombia to Argentina and Chile, including the Galapagos Islands; **NAM**: North America, including the whole of the United States, Canada and Greenland. **NAS**: North Asia, including USSR east to the Urals, China, Mongolia, Korea and Japan; **TAS**: Tropical Asia from Pakistan to Indo-China; **MAL**: Malesia from the Malay peninsula to New Guinea, including the islands of Guam and Palau; **ANZ**: Australia, New Zealand and New Caledonia; **EUA**: Europe, North Africa, and the Middle eastern countries to Afghanistan; **AFR**: Africa, south of Sahara with Madagascar and the Mascarene Islands.

Gel electrophoresis: Amplified products were mixed with bromophenol dye and were analyzed on 1.5% agarose gel following ethidium bromide staining for 30 minutes. The bands were visualized under ultra-violet radiation and photographed.

Data analysis: RAPD bands were recorded in a binary data matrix scored as presence (1) or absence (0). Simple Matching coefficient was used for measuring genetic similarity among the species analyzed. UPGMA (unweighted pair group method with arithmetic mean) tree was generated by clustering the similarity data and SAHN (sequential, agglomerative hierarchical and nested clustering) method. Dist coefficient was employed

to know the dissimilarity level between the species examined. A Neighbour Joining (NJ) tree was constructed based on Jukes and Cantor (1969) distance coefficient. NTSYS-pc package, version 2.1 was used for all analyses (Rohlf 2000).

Results and Discussion

RAPD fingerprints and genetic variation: RAPD markers generated reproducible fingerprints across the aquatic species tested. Some RAPD primers were ignored because of presenting artifacts. The electrophoretic pattern of PCR amplified RAPD fingerprints generated by the primer OPB15 is shown in Figure 1. The highest number of RAPD bands was observed in *Utricularia intermedia* followed by *U. bremii*. In contrast, *U. vulgaris* showed the least number of bands.

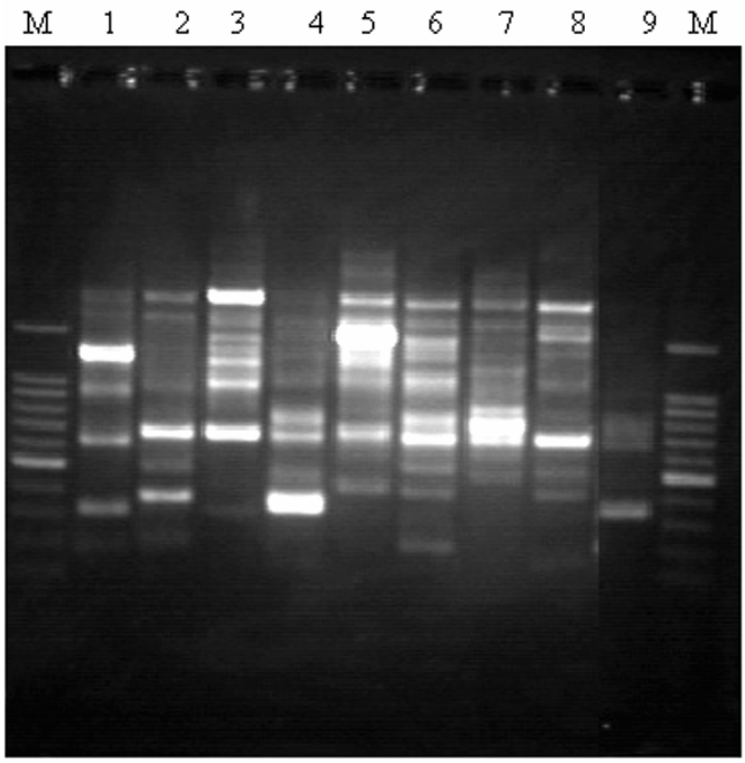

Fig. 1. Electrophoretic pattern of PCR amplified RAPD banding profile on 1.5% agarose gel for aquatic *Utricularia* species with the primer OPB15. M. Molecular size marker (100 bp), 1. *U. aurea*, 2. *U. australis*, 3. *U. bremii*, 4. *U. dimorphantha*, 5. *U. gibba*, 6. *U. intermedia*, 7. *U. macrorhiza*, 8. *U. minor* and 9. *U. vulgaris.*

Across the aquatic species of *Utricularia*, the highest genetic similarity (9.37) has been found between *U. australis* and *U. dimorphantha*; between *U. australis* and *U.*

vulgaris; and between *U. dimorphantha* and *U. macrorhiza* indicating that these species are very closely related (Table 2). *Utricularia bremii* was found close to *U. gibba* showing a high genetic affinity (8.75). On the other hand, the highest dissimilarity (7.07) was detected between *U. aurea* and *U. bremii*; *U. aurea* and *U. intermedia*; *U. bremii* and *U. dimorphantha;* and *U. dimorphantha* and *U. intermedia*.

Table 2. Similarity matrix among the aquatic species of *Utricularia* studied by RAPD markers using Simple Matching coefficient.

Species	*U. aurea*	*U. australis*	*U. bremii*	*U. dimorphantha*	*U. gibba*	*U. intermedia*	*U. macrorhiza*	*U. minor*	*U. vulgaris*
U. aurea	1								
U. australis	6.87	1							
U. bremii	5.00	5.62	1						
U. dimorphantha	6.25	9.37	5.00	1					
U. gibba	6.25	6.87	8.75	6.25	1				
U. intermedia	5.00	5.62	5.00	5.00	6.25	1			
U. macrorhiza	6.87	8.75	5.62	9.37	6.87	5.62	1		
U. minor	6.25	6.87	5.00	6.25	6.25	7.50	6.87	1	
U. vulgaris	7.50	9.37	6.25	8.75	7.50	6.25	8.12	7.50	1

Taxonomic relationships: For aquatic species of *Utricularia*, dendrograms were constructed both from UPGMA and NJ analyses. UPGMA dendrogram in aquatic species based on Dist coefficient resulted in producing three clusters (Fig. 2). In the first cluster *U. australis* grouped with *U. dimorphantha* and *U. macrorhiza* was joined with this group in which *U. vulgaris* was joined, and morphologically, these species are closely related. *Utricularia aurea* was found sister to this group. The lowest dissimilarity (25%) was obtained in this group between *U. australis* and *U. dimorphantha* indicating that they are very close to each other. The second cluster made from *U. bremii* and *U. gibba* was found as a sister group to the cluster having *U. aurea*, *U. australis*, *U. dimorphantha*, *U. macrorhiza* and *U. vulgaris* (Fig. 2). The third cluster consisted of *Utricularia minor* and *U. intermedia* and these two species were found far away from other aquatic species employed.

The NJ tree constructed on the basis of Jukes and Cantor coefficient revealed that aquatic species could be placed into two main groups: the first group possessed five species including *U. aurea*, *U. australis*, *U. dimorphantha*, *U. macrorhiza* and *U. vulgaris* and the second one consisted of four species, namely, *U. bremii*, *U. gibba*, *U. intermedia and U. minor* (Fig. 3). The second group contained two clusters where *U. bremii* and *U. gibba* formed one cluster and the second one was made by *U. intermedia*

and *U. minor*. The NJ analysis showed the similar results with that of the UPGMA clustering method.

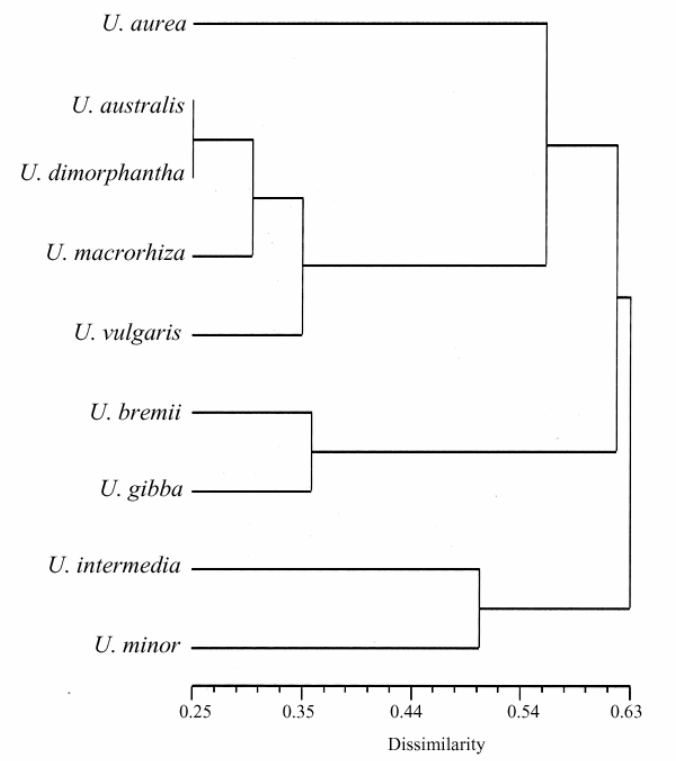

Fig. 2. Dendrogram showing relationships among aquatic *Utricularia* species based on UPGMA analysis generated by RAPD markers.

In recent years, several DNA markers were developed for genome analysis and have been found suitable in molecular systematic studies. The DNA markers commonly used in molecular studies include RFLP (Restriction fragment length polymorphism; Botstein *et al*. 1980), SSR (Simple sequence repeat; Tautz and Renz 1984), RAPD (Williams *et al.* 1990), ISSR (Inter simple sequence repeat; Zietkiewicz *et al*. 1994) and AFLP (Amplified fragment length polymorphism; Vos *et al.* 1995). Among these marker systems available RAPD is the most popular approach that has wide range of applications. Application of these markers in aquatic plants is quite evident (Piquot *et al.* 1996, Padgett *et al*. 1998, Madeira *et al.* 2000).

In the genus *Utricularia*, application of DNA markers is very limited. Recently, Rahman and Kondo (2003) applied ISSR markers in terrestrial *Utricularia* and found them useful for species delimitation. However, RAPD markers were not tested in this genus earlier. The present investigation revealed that RAPD markers are useful to

characterize aquatic *Utricularia* species. The RAPD analyses in aquatic *Utricularia* showed that *U. australis, U. dimorphantha, U. macrorhiza* and *U. vulgaris* could be grouped together. In another study, these aquatic species of *Utricularia* were investigated by some other molecular markers, like ISSR, and *U. australis* was found very close to *U. dimorphantha*, while *U. gibba* was found near to *U. bremii* (M. Oliur Rahman, unpublished). The interspecific relationships in aquatic *Utricularia* based on RAPD

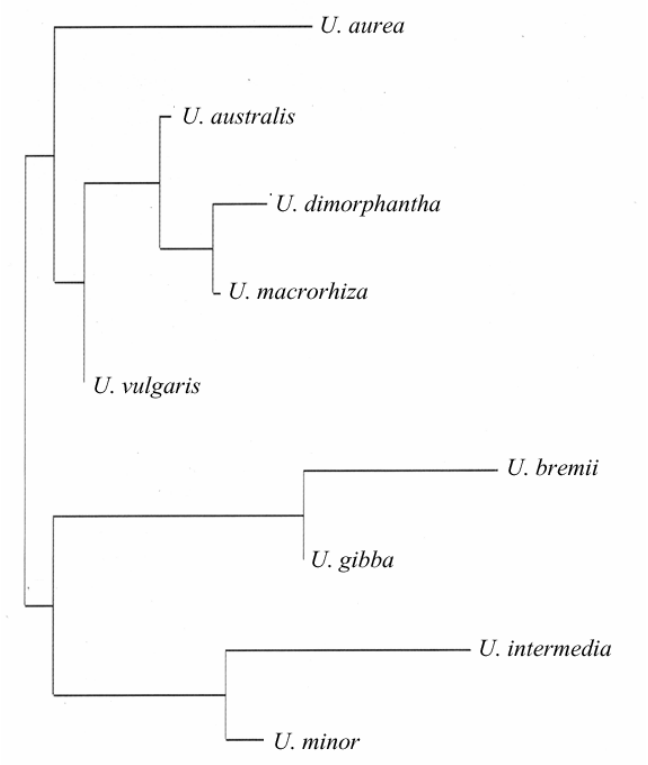

Fig. 3. Neighbour Joining tree for aquatic *Utricularia* species using Jukes and Cantor coefficient based on RAPD data.

analysis is in agreement with the results obtained from the previous study inferred from ISSR analysis. Morphologically, *U. dimorphantha* is allied to *U. australis* by having basifixed bracts, curved filaments, globose ovary, globose capsule and prismatic seeds. A close relationship was evident among *U. australis, U. macrorhiza* and *U. vulgaris* in the present study, which was concordant with their morphology. For instance, leaves of these species divided into two primary segments each of which pinnately divided and the secondary segments divided into further segments. These species showed dimorphic traps and the basal traps were ovoid (Taylor 1989). Cytologically, *Utricularia australis* and *U. macrorhiza* presented the same gametic number of chromosome (Casper and Manitz

1975). *Utricularia macrorhiza* is also close to *U. vulgaris* in terms of somatic chromosome numbers (Löve 1954, 1982). The RAPD data revealed that *U. minor* was genetically closely related to *U. intermedia*. Some morphological characters such as ovate or ovate-deltoid bracts, subequal calyx lobes, curved filament and globose ovary placed these two species in the same line (Taylor 1989).

In conclusion, RAPD markers were found suitable to detect genetic variation and species relationships in aquatic *Utricularia*. In addition, the results obtained from RAPD analysis in *Utricularia* were in agreement with previous studies based on morphological, cytological and molecular approaches.

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