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Evaluation of inter-simple sequence repeat (ISSR) for systematic relationship of some terresetrial species of *Utricularia* L. (Lentibulariaceae)

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Abstract. Inter-simple sequence repeat (ISSR) amplification was evaluated for its applicability as a genetic marker system to establish relationships among ten terrestrial *Utricularia* species. The resultant products were subjected to agarose gel electrophoresis and the banding patterns were compared among *Utricularia alpina*, *U. amethystina*, *U. bifida*, *U. caerulea*, *U. calycifida*, *U. humboldtii*, *U. longifolia*, *U. praelonga*, *U. triflora* and *U. uliginosa*. ISSR amplification generated multiple banding profiles with the six primers from all DNA samples, with an average of 39.3 fragments per primer. A total of 236 bands were detected by six primers. Primer UBC 889 presented the highest number of bands while primer UBC 891 produced the least number. Among the species studied *U. alpina* and *U. longifolia* were found to be most closely related. *Utricularia bifida* and *U. humbodtii* stand the second position in term of similarity whereas *U. cearulea* and *U. triflora* were placed most far from the other species. The genetic relationships of the species estimated by ISSR markers indicate the reliability of ISSR markers for estimation of genotypes. The characteristics feature of ISSR markers (*i.e.*, polymorphism, generation of information and ease of handling) suggest their applicability in molecular systematic studies.

Key words: ISSR, Lentibulariaceae, systematic relationship, terrestrial *Utricularia*

Introduction

Utricularia, a member of the family Lentibulariaceae encompasses about 214 species, is distributed throughout the world except for nival and polar ice zones (Taylor, 1989). Among the carnivorous plants *Utricularia* is unique because of the structural complexity of its traps, thought to be the most intricate in the plant kingdom and the rapid movement of the opening and closing of its trapdoors (Meyers and Strickler, 1979). They have pioneered many habitats including wet grounds, ponds, lakes and other marshy areas, epiphytic conditions and seasonal deserts.

Since *Utricularia* was first reported in "Species Plantarum" where Linnaeus (1753) listed only seven species, it has received considerable attention from many taxonomists. Many important account from different parts of the worlds mainly based on morphology and floristics were produced in the 20th century. For instance, Perrier (1955) paid an attention on the Malagasy species of *Utricularia* while Fernandez-Perez (1964) worked out with Colombian species. The species from Argentina were described

by Dawson (1973). Fromm-Trinta (1972) was successful in producing an account for the Brazilian species of *Utricularia* and Thor (1988) has sorted out the Scandinavian species. In Japan, systematic studies on the Lentibulariaceae was covered by Komiya (1972). Komiya (1973) proposed a new sub-division of the Lentibulariaceae. Komiya and Shibata (1980) showed the distribution of the Japanese Lentibulariaceae. Subramanyum (1979) studied the Indian *Utricularia* intensively. Recently Crow (1992) dealt with *Utricularia* of Costa Rica. However, the most significant systematic work is the monograph of *Utricularia* produced by Taylor (1989). *Utricularia* also received cytological as well as palynological investigation. Casper and Manitz (1975) were most successful in counting chromosome for European species and Kondo (1966, 1973) for non European species. Cytology of Indian species were conducted by Subramanyam and Kamble (1968). The pollens of the *Utricularia* were investigated by Thanikaimoni (1966), Huynh (1968) and Sohma (1975a, b).

Simple sequence repeat (SSRs or microsattelite) consists of tandem arrays of short oligonuleotide sequences 2-6 bases in length. Throughout the eukaryotic genome SSRs are very common (Tautz and Renz, 1984) and highly polymorphic in length (Levinson and Gutman, 1987). Inter-simple sequence repeats (ISSRs) are a new type of DNA marker which are generated from a single primer PCR and the primer is designed from a microsattelite motif. ISSR involves the use of microsattelite sequences directly in the polymerase chain reaction (PCR) for DNA amplifications (Gupta *et al.*, 1994). ISSR primers contain sequences complementary to a SSR motif and also a 1-3 base anchor at either the 3′ or 5′ end. Base positions within the anchor may contain any nucleotide other than the needed to continue the repeat sequence. This technique enables amplification of genomic DNA and provides information about many loci simultaneously. The utility of this approach stems from its simplicity and reproducibility, the higher number of polymorphism revealed and the fact that there is no prior need for DNA sequence information from the organism under study. Zietkiewicz *et al.* (1994) first used this approach to distinguish between closely related plant genotypes. This technique has successfully employed for cultivar identification (Farnandez *et al.*, 2002; Pasqualone *et al.*, 2001; Prevost and Wilkinson, 1999), population study (Crawford *et al.*, 2001; Lai *et al.*, 2001; Qiam and Hong, 2001; Zhou *et al.*, 1999) and interspecific relationships (Huang and Sun, 2000; Joshi *et al.*, 2000; Wolfe and Randle, 2001; Xu and Sun, 2001).

We used ISSR markers first to assess the distinctness of ten terrestrial species of *Utricularia*. The present study is the first report of the applicability of the nuclear DNA marker ISSR-PCR in characterizing genetic affinities at the interspecies level in the genus *Utricularia*. The primary objectives are to establish a baseline of systematic relationships for the *Utricularia* species and to test the utility of ISSR markers in estimating relationships among the species. Our results permit us to establish genetic relationships among ten terrestrial species within the genus *Utricularia*, and show that the ISSR approach is an interesting tool for plant molecular systematics.

Materials and Methods

Plant material *Utricularia alpina* Jacq., *U. amethystina* Salzm. *ex* A. St. Hil., *U. bifida* L, *U. caerulea* L, *U. calycifida* Benj., *U. humboldtii* Schomb., *U. longifolia* Gardner, *U. praelonga* A.St.Hil. *et* Girard, *U. triflora* P. Taylor and *U. uliginosa* Vahl were analyzed in this study. Fresh leaves were collected from plants from *in vitro* culture under long-term conservation conditions at the Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Japan.

DNA extraction Total genomic DNA was extracted from the leaves following Kawahara *et al.* (1995) with slight modification. 1.0-1.8g of leaf was homogenized in a mortar using liquid nitrogen. The powdered tissue was transferred to a 20ml capacity of capped, sterilized centrifuge tube containing 10ml of wash buffer [0.1M Tris-HCl at pH

8.0, 2% 2-Mercaptoethanol (w/v), 1% Polyvinylpyrolidone K-30 (w/v), 0.05M L-Ascorbic acid, dissolved in distilled water]. After shaking gently for 10 min the tube was centrifuged 10,000 rpm at 20℃ for 10 min. The supernatant was discarded from the tube and this process was repeated until the solution becomes transparent. After removing the supernatant, 10ml of CTAB buffer [2% Cetyltrimethylammonium bromide (CTAB; w/v), 1.4M NaCl, 0.1% Tris-HCl at pH 8.0 (w/v), 20mM EDTA-Na2, dissolved in distilled water] and 0.5ml 2-Mercaptoethanol were added to the tube followed by an incubation at 55℃ for 60-90 min with a view to supply the stabilization of DNA. Following that 10ml chloroform:isoamylalcohol (24:1 v/v) 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 centrifuge 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 an another centrifuged tube and 10ml of 2-propanol was added followed by a centrifugation at 10,000 rpm for 15 min at 4℃. After discarding the solution 5ml 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 450μl TE solution (10mM Tris-HCl and 1mM EDTA) with 0.1mg/ml RNase (Sigma). After incubation for 1 hour at 37℃ the solution was transferred in a 1.5ml sterilized ependorf tube. 250μl of neutral equilibrated phenol and 250μl of chloroform:isoamyl alcohol (24:1) were added to the tube. After shaking for 10 min the tube was centrifuged for 10 min. The upper phase was transferred to a new tube. 500μl chloroform:isoamyl alcohol was added and was centrifuged for 10 min. The upper aquous solution was transferred to a new tube. Then 50μl of 3M sodium acetate and 500μl of 99.5% chilled ethanol were placed in the tube and they were kept at –80℃ for 20 min. The DNA pellet was found by spinning in a microcentrifuge at 15,000 rpm for 15 min at 4℃. The supernatant was removed and the pellet was washed with 70% chilled ethanol using centrifugation at 15,000 rpm for 15 min at 4℃. 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℃.

ISSR primers One hundred ISSR primers (primer set # 9) were purchased from the University of British Columbia, Biotechnology laboratory (UBC, Vancouver, Canada). These primers were 15-mer to 22-mer and many of them consisted of di-, tri-, tetra-, and pentanucleotide repeat motifs of which dinucleotide repeats were with anchor. Out of 100 primes procured, a total of 72 primers were screened for PCR amplification. Finally, we selected 6 primers (Table 1), by their number and consistency of amplified fragments, for analyzing ten species of *Utricularia*.

PCR amplification and Electrophoresis DNA amplification were performed in a 10μl reaction volume containing 10ng of template DNA, 1μM of a single primer (UBC, Vancouver, Canada), 1μl of x10 Taq buffer, 0.8μl of dNTP mixture and 0.05μl of Taq polymerase enzyme. The reaction mixture was overlaid with 30μl mineral oil. Amplifications were performed in a PTC-100 thermal cycler programmed for an initial step of 5 min at 94 ℃, followed by 35 cycles of 1m at 94℃, 45s at 50℃ and 2 min at 72℃, and a final 5 min extension at 72℃.

ISSR bands were characterized on 1.5% agarose gels in 1X TAE buffer by loading the entire reaction volumes into prepared wells. Gels were run until a bromophenol blue indicator dye ran morethan 70% from the well. The gels were stained in ethidium bromide and bands were visualized in UV light. The molecular size of the fragments was estimated by reference to a 1kb ladder (Pharmacia).

Data analysis The banding pattern obtained using six ISSR primers were analyzed to estimate genetic relationships among the ten terrestrial *Utricularia* species. The ISSR bands were interpreted as dominant markers and were scored as diallelic characters either as 1 (present) or 0 (absent). The data matrices were analyzed by the SIMQUAL program of the NTSYS-pc version 2.1 (Rohlf, 2000). A pair-wise similarity matrix was calculated for a measure of within species similarities using the simple matching coefficient. This

similarity matrix was employed to construct a dendrogram by the unweighted pair group method with arithmetical averages (UPGMA), using the SAHN-clustering and TREE programs from the NTSYS-pc, version 2.1 package (Rohlf, 2000). A pair-wise dissimilarity matrix was also made using the DIST coefficient.

Results and Discussion

ISSR analysis ISSR amplification using six primers produced a total of 236 fragments from the ten *Utricularia* species. The total number of bands generated six primers in the ten species varied from 18 in *Utricularia amethystina*, *U. humboldtii* and *U. uliginosa* to32 in *U. longifolia*. Figures 1, 2, 3 and 4 represent the amplification patterns generated using primer UBC 842, UBC 888, UBC 889 and UBC 890 across the ten species. The least and highest number of fragments generated per primer in all species studied were 33 (primer UBC 888 and UBC 891) and 48 (primer UBC 889), respectively (Table 1). The number of fragments produced by other primers ranged from 37 (primer UBC 864) to 43 (primer UBC 890). The average number fragments produced by all analysed primers is 39.3.

Genetic relationships among *Utricularia* species

The ISSR marker was found efficient in the study of the genus *Utricularia*, where genetic characterizations of the nuclear genomes are almost lacking. We used the ISSR technique to analyse ten terrestrial *Utricularia* species with a view to determine their genetic relatedness. According to our findings, *Utricularia* species could be characterized using ISSR markers. The estimated similarity coefficient among ten species varied from 5.82 (*U. caerulae*/ *U. longifolia*) to 8.66 (*U. alpina*/ *U. longifolia*).(Table 2). The dendrogram erected by the UPGMA method using 236 ISSR markers scored in the ten species presents 2 defined groups, Group I and Group II. (Fig. 5).

Group I bears the species *U. alpina* and *U. longifolia*. The highest similarity value was found in this group among all taxa studied at a similarity level of 8.6 (Table 2). The dendrogram shows that *U. alpina* and *U. longifolia* seem to be nearest. In contrast, *U. caerulea* show the highest dissimilarity value with *U. longifolia* (Table 3).

Group II forms the cluster between *U. amethystina* and *U. calycifida*; *U. bifida* and *U. humboldtii* ; *U. praelonga* and *U. uliginosa*. The cluster consisting of *U. bifida* and *U. humboldii* also exhibits a high level of similarity (7.63) followed by the cluster containing *U. amethystina* and *U. calycifida* (7.48). In this group the cluster between *Utricularia amethystina* and *U. calycifida* is joined with the cluster *U. bifida* and *U. humblodtii* to which another cluster containing *U. praelonga* and *U. uliginosa* is joined followed by *U. caerulea* while *U. triflora* is the last taxon.

The Lentibulariaceae have not been thoroughly investigated from the molecular point of view although some other carnivorous plant families were investigated using the molecular tools including Droseraceae (Albert *et al.*, 1992) and Saraceniacee (Bayer *et al.*, 1996). Many authors paid an attention on the largest carnivorous genus *Utricularia* based on morphology (Taylor, 1989; Crow, 1992), palynology (Huynh, 1968), and cytology (Casper and Manitz, 1975; Kondo, 1973; Subramanyum and Kamble, 1968). This unique genus, however, did not receive any attention so far from ISSR or other molecular markers. Recently an isozyme study was carried out only for two infraspecific taxa of the aquatic *U. australis* (Araki, 2000).

Our results indicate that the species *Utricularia alpina* and *U. longifolia* show the highest affinity among the species studied. This might be due to sharing a considerable number of markers between these two species which indicates that *U. alpina* and *U. longifolia* form a close genetic assemblage. This result support the morphology of these two species because there share the characteristics by elliptic or obovate leaves, curved filament, ovoid ovary and ovate to ovate-deltoid calyx lobes. However, Taylor

(1989) placed these two species in two different sections. To have the better resolution, some other molecular tools as well as large number of sampling are necessary to be conducted. From the cytological point of view, it is impossible to compare between these species because chromosome number is reported in *U. alpina* (Kondo, 1966) while chromosomal information is missing in *U. longifolia*.

Group II bearing eight species of *Utricularia* forms three subclades including *U. amethystina* with *U. calycifida*; *U. bifida* with *U. humboldtii* and *U. praelonga* with *U. uliginosa*. (Fig. 5). *Utricularia amethystina* form clade with *U. calycifida* showing the close relationship to each other. They are morphologically in the same line by having unequal calyx lobes, globose capsule and obliquely ovoid seeds. In *U. bifida* and *U. humboldtii* some characteristics are found to be similar including subulate spur, distinct anther thecae, basifixed bracts and linear or linear to subulate bracteoles which is supported by our analysis. Although palynlogical information is available (Huynh, 1968), however, our data does not match with that. None of these species was cobered by cytological data. The last clade in Group II comprise the species *U. praelonga* and *U. uliginosa* and these are sharing ovate bracts, short style and subulate or narrowly linear bracteoles.

Utricularia caerulea and *U. triflora* are far away from the other species showing the lower values of similarity with respect to the rest of the species, and this can be explained by the fact is that these taxa present the most distant genetic relationship compared to other species studied.

Out of ten species studied, chromosome numbers have been counted only in *Utricularia alpina* (Kondo 1966 as '*Orchyllium alpinum*'), *U. caerulea* (Kondo, 1973 as '*U. recemosa*') and *U. uliginosa* (Tanaka and Uchiyama, 1988 as '*U. yakusimensis*'). Since the chromosome information is very much lacking and fragmentary therefore our results can not be compared with cytological investigation properly.

In this study we did not compare *Utricularia* with an outgroup species because the aim of this investigation was to establish interspecific relationships rather than phylogenetic relationships. Although we included only ten species in this study, but we are currently evaluating ISSR markers for other species of *Utricularia* as well. Using these efficient markers we will be able to figure out the phylogenetic relationship and evolution of the unique carnivorous genus *Utricularia*.

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Table 1 . List of primer sequences used along with the scorable fragments resulting from each primer

Primer code	Sequencesa	Repeat	No. of fragments scored
UBC 842	GAG AGA GAG AGA GAG AYG	(GA)8YG	42
UBC 864	ATG ATG ATG ATG ATG ATG	(ATG)6	37
UBC 888	BDB CAC ACA CAC ACA CA	(CA)7BDB	33
UBC 889	DBD ACA CAC ACA CAC AC	(AC)7DBD	48
UBC 890	VHV GTG TGT GTG TGT GT	(GT)7VHV	43
UBC 891	HVH TGT GTG TGT GTG TG	(TG)7HVH	33

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

Table 2 : Similarity matrix among ten species of *Utricularia* using the simple matching coefficient based on 236 ISSR fragments scored

Species	*U. alpina*	*U. amethystina*	*U. bifida*	*U. caerulea*	*U. calycifida*	*U. humboldtii*	*U. longifolia*	*U. praelonga*	*U. triflora*	*U. uliginosa*
U. alpina	1									
U. amethystina	7.08	1								
U. bifida	6.61	7.16	1							
U. caerulea	6.22	7.08	7.08	1						
U. calycifida	6.14	7.48	7.16	6.61	1					
U. humboldtii	6.77	7.48	7.63	7.24	7.32	1				
U. longifolia	8.66	6.53	6.37	5.82	6.06	6.85	1			
U. praelonga	6.53	7.08	7.08	6.85	6.61	7.08	6.29	1		
U. triflora	6.53	7.08	6.61	6.85	6.92	6.92	6.14	6.22	1	
U. uliginosa	6.77	7.16	7.01	6.61	7.16	7.16	6.53	7.4	6.29	1

Table 3 : Dissimilarity matrix of ten species of *Utricularia* using DIST coefficient based on 236 ISSR fragments

Species	*U. alpina*	*U. amethystin.*	*U. bifida*	*U. caerulea*	*U. calycifida*	*U. humboldti*	*U. longifolia*	*U. praelonga*	*U. triflora*	*U. uliginosa*
U. alpina	0									
U. amethystina	5.39	0								
U. bifida	5.81	5.32	0							
U. caerulea	6.14	5.39	5.39	0						
U. calycifida	6.21	5.01	5.32	5.81	0					
U. humboldtii	5.68	5.01	4.86	5.24	5.17	0				
U. longifolia	3.65	5.88	6.01	6.46	6.27	5.61	0			
U. praelonga	5.88	5.39	5.39	5.61	5.81	5.39	6.08	0		
U. triflora	5.88	5.39	5.81	5.61	5.54	5.54	6.21	6.14	0	
U. uliginosa	5.68	5.32	5.47	5.81	5.32	5.32	5.88	5.09	6.08	0

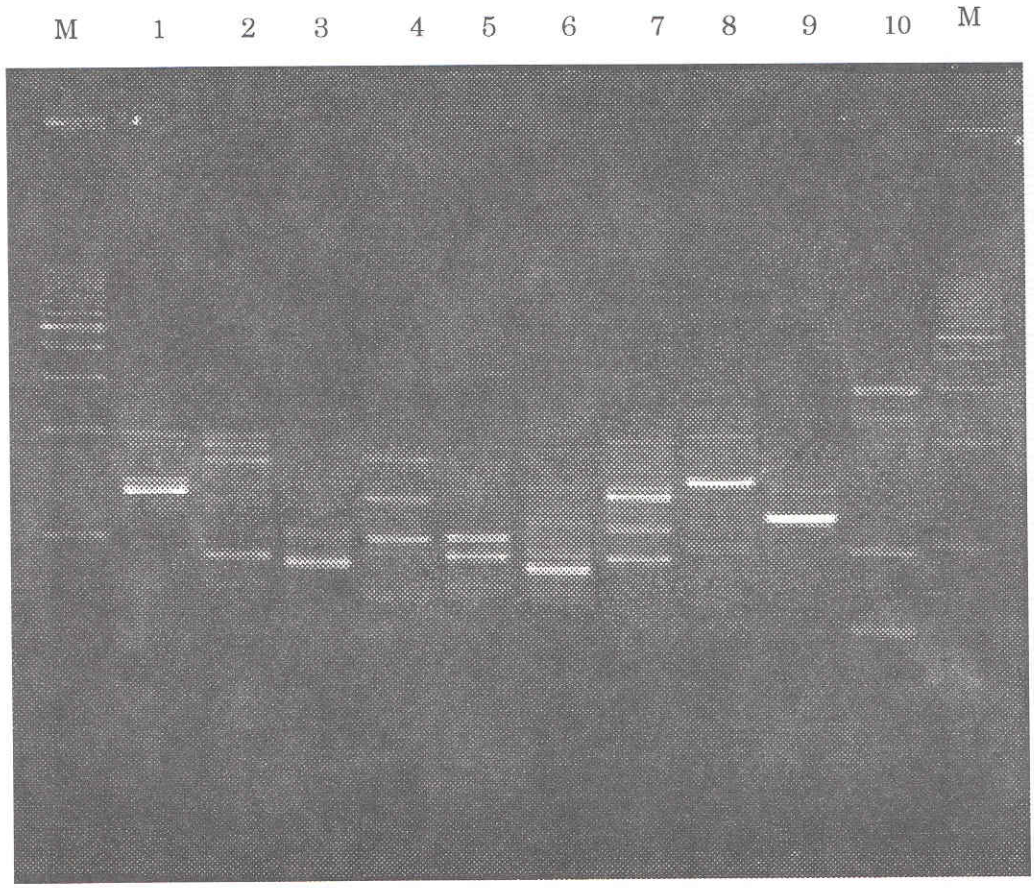

Figure 1 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 842. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

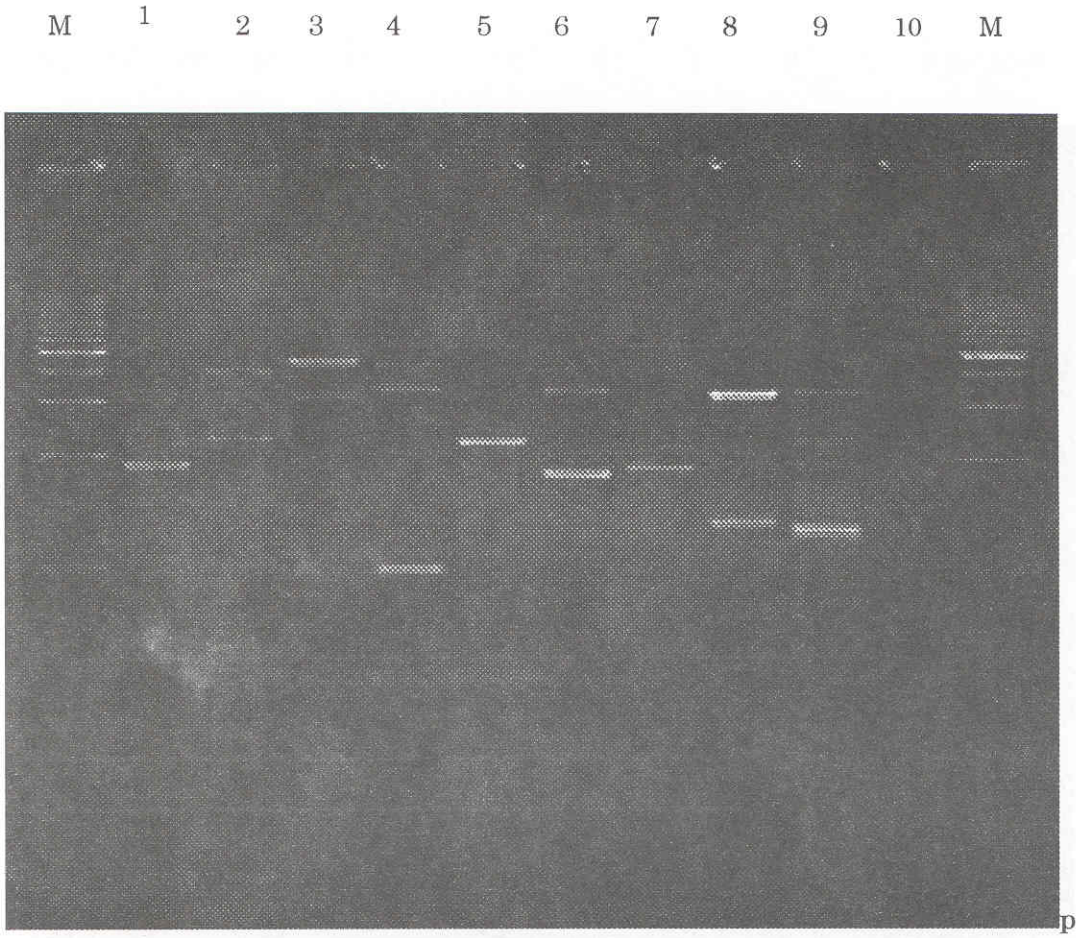

Figure 2 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 888. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

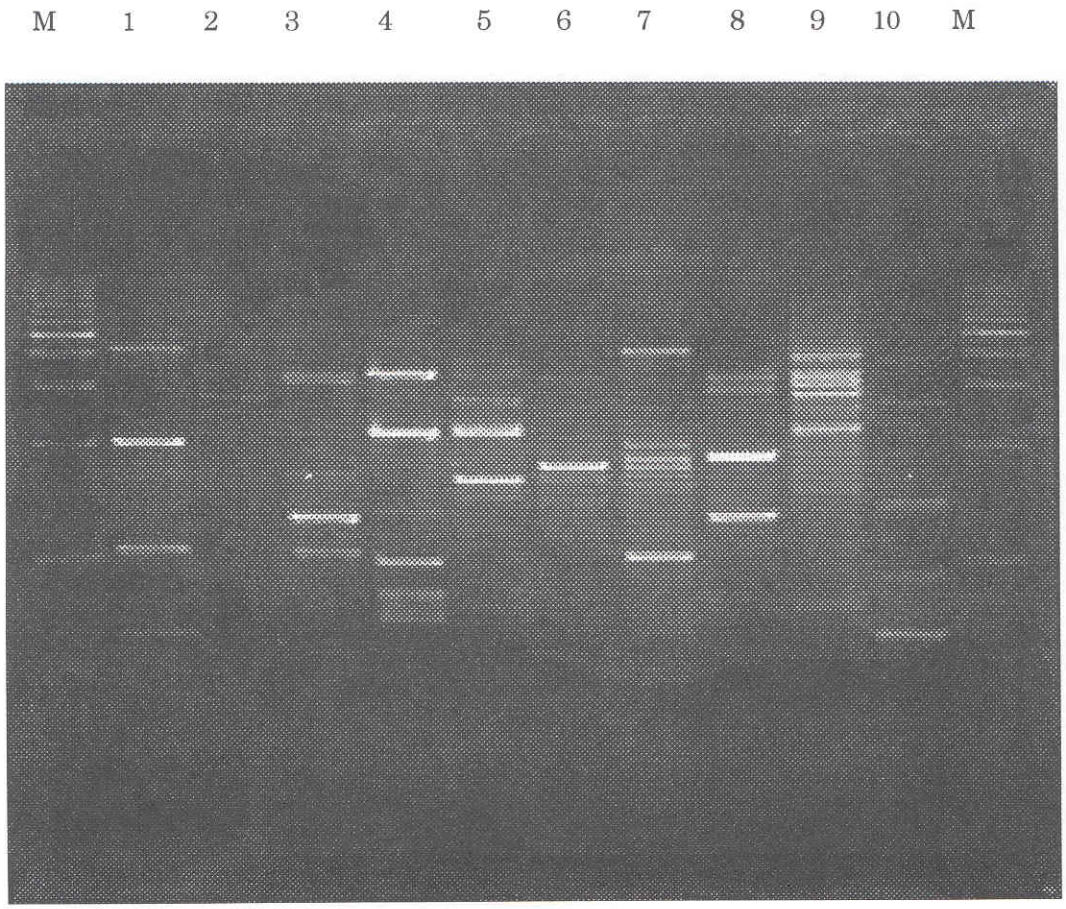

Figure 3 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 889. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

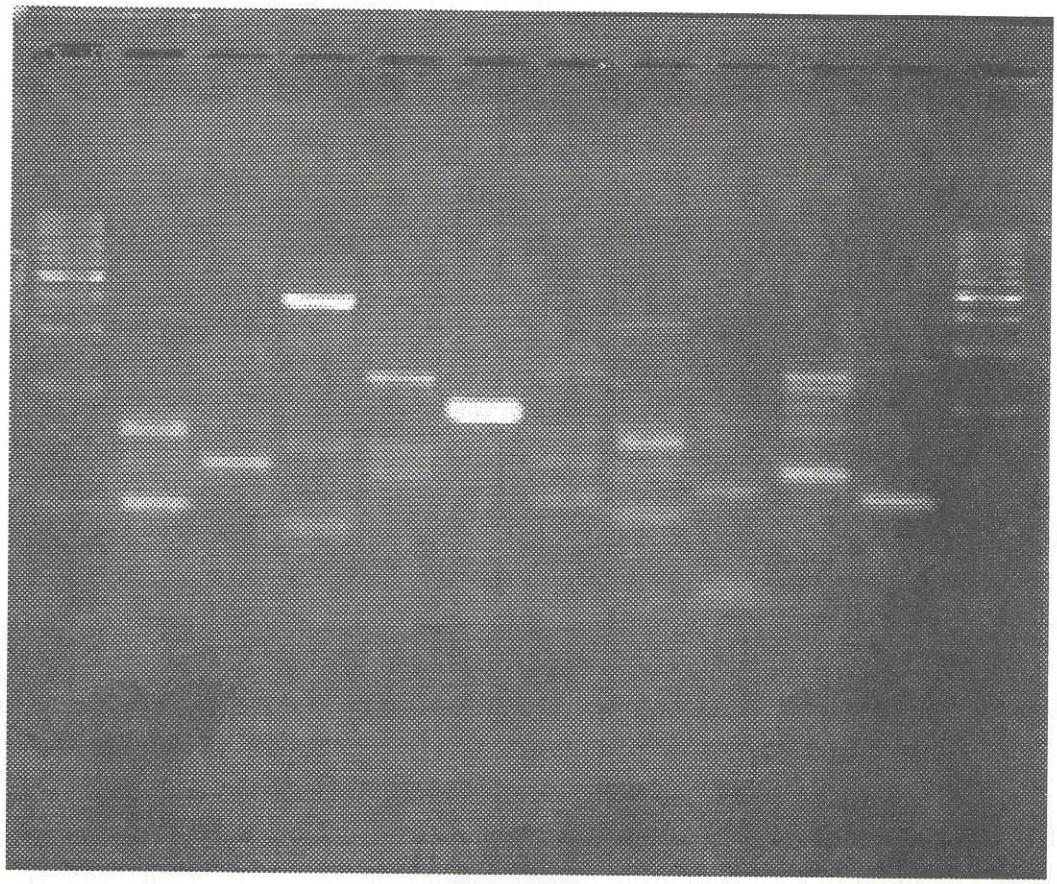

Figure 4 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 890. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

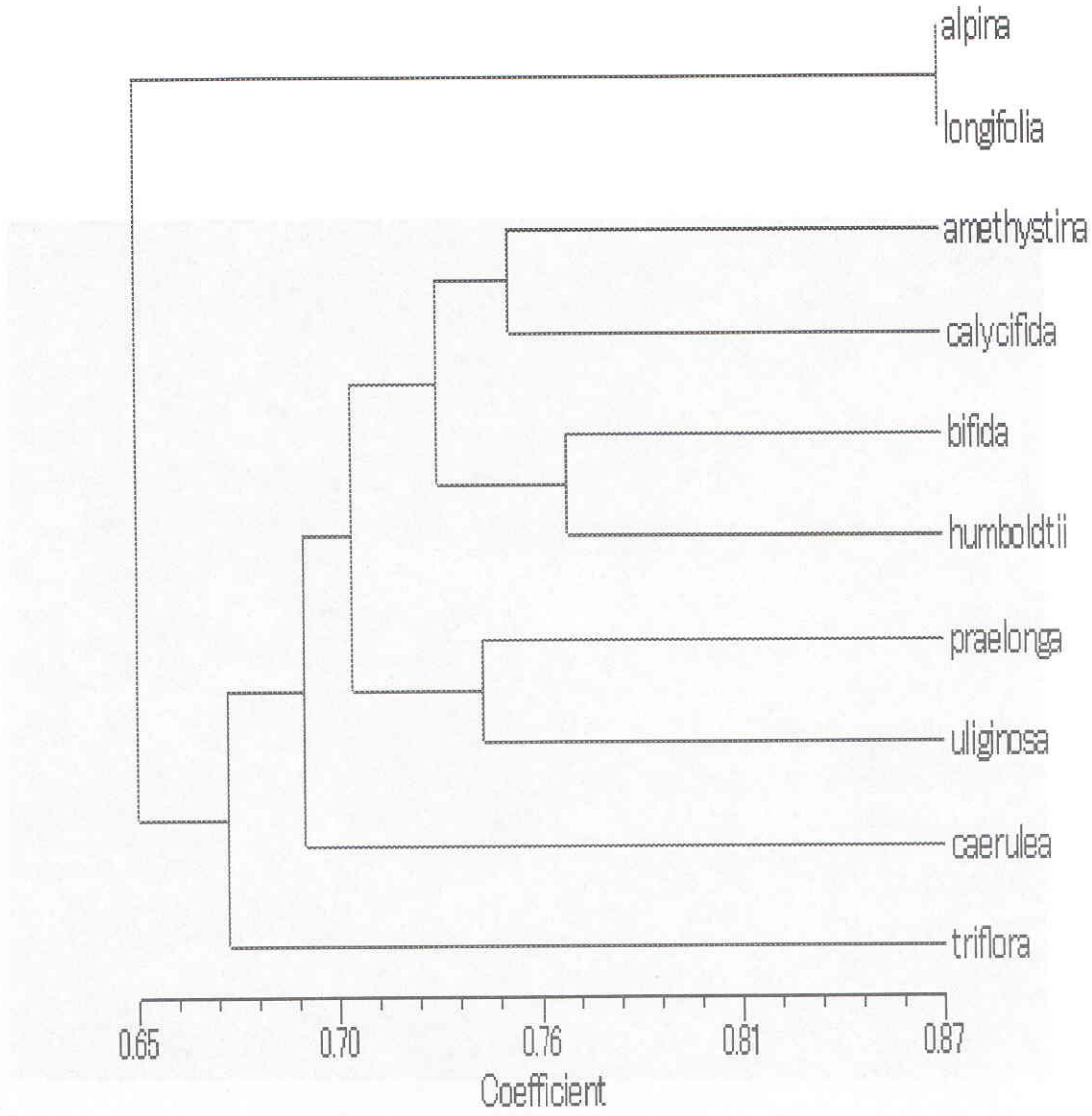

Figure 5 : Dendrogram showing the genetic relationships among ten terrestrial *Utricularia* species constructed by the UPGMA cluster analysis, using simple matching coefficient based on 236 ISSR fragments