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BRIEF COMMUNICATION

Morphological and molecular variations in the epiphytic CAM fern *Pyrrosia piloselloides*

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Abstract

Variation in shape and size of mature sterile fronds of the epiphytic fern, *Pyrrosia piloselloides* (L.) Price, was observed. These morphological differences were also linked to genotypic variations of the different fern populations studied. Genetic polymorphism in different populations of *P. piloselloides* was investigated using random amplified polymorphic DNA markers.

Additional key words: frond morphology, genetic variation, RAPD DNA markers.

Pyrrosia piloselloides (L.) Price is a common epiphytic fern exhibiting dimorphism in frond morphology; sterile fronds differ from fertile fronds in shape and size. However, the shape and size of mature sterile fronds are also commonly observed to vary under natural conditions. Shapes of these fronds range from almost circular to lanceolate with rounded apices. Fertile fronds are usually elongated and do not exhibit the same degree of variation as mature sterile fronds.

In this study, naturally growing populations of *P. piloselloides* with sterile fronds of different shapes were characterized to determine the extent of genetic variability within and between the different populations of the fern.

Fresh specimens of *P. piloselloides* were collected from several sites within the campus of the National University of Singapore. Length, width and length/width ratio of the mature sterile fronds were determined. For RAPD assays, three populations of *P. longifolia* were also collected for comparison.

All fronds collected were rinsed with distilled water and surface-sterilized with 10 % (v/v) *Clorox*™ for 10 min. They were then ground to a fine powder in liquid nitrogen. Genomic DNA was isolated with DNA extraction buffer [1.875 % CTAB,

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37.5 mM Tris-HCl at pH 8.0, 7.5 mM EDTA and 1.3 M NaCl, 29 mM mercapto-ethanol, 20 mg cm^{-3} PVP and 2 % (m/v) SDS in sterile distilled water] (Lim *et al.* 1997). The mixture was incubated at 65 °C, followed by a chloroform extraction. A second extraction with 0.2 volumes of 10X CTAB buffer (10 % CTAB, 0.7 M NaCl) was carried out before another chloroform extraction step. The DNA was, then, precipitated with ethanol and treated with DNase-free RNase.

Polymerase chain reactions were conducted in a total volume of 0.05 cm^3 of thermophilic buffer (50 mM KCl, 10 mM Tris-HCl at pH 9 with 1% Triton X-100), 100 μM of each deoxyribonucleotide, 2 mM $MgCl_2$, 1 μM RAPD primer (*Primer Kit U, Operon Technologies Inc.*, Alameda, USA), 3 μg cm^{-3} DNA sample and 0.04 unit mm^{-3} *Taq* DNA polymerase (*Promega,* Madison, USA), 45 cycles of denaturation (95 °C, 1 min); annealing (32 °C, 3 min) and extension (72 °C, 2 min) followed (see Lim *et al.* 1997). The RAPD fragments were, then, separated by electrophoresis through a 2 % agarose gel. DNA molecular mass markers (*Lambda Hind III* and *ϕx174-Hae III*) were used as molecular size standards. The DNA bands were visualized under UV light and photographs were taken.

The RAPD bands were scored either for presence or absence. To compare between populations, RAPD data for each population were consolidated from three replicates. Pair-wise comparisons were then conducted with these data by calculating the coefficient of genetic similarity with the equation, $F = 2n_{xy}/(n_x + n_y)$ in which n_x and n_y are the numbers of fragments in populations X and Y, respectively, while n_{xy} is the number of fragments shared by the two populations (Nei and Li 1979). The similarity coefficient was expressed as percentage genetic similarity, GS [%]. The higher GS the greater the similarity was between two genotypes.

Fronds collected from different populations of *P. piloselloides* and *P. longifolia* were characterized morphologically according to the length and width of the fronds (Table 1); the length/width ratio of fronds of each fern population was also calculated to indicate the shape of the mature sterile fronds.

Table 1. Frond length, width and length/width ratio in several populations of *Pyrrosia piloselloides* (named A, B, C, D, E, F, G). Means ± standard error (n = 50); means in the same row with the same letter are not significantly different at the 95 % confidence interval.

Parameter	A	B	C	D	E	F	G
Length [cm]	2.44±0.001^b	1.11±0.000^a	3.95±0.001^c	1.16±0.001^a	2.44±0.001^b	6.05±0.230^d	7.73±0.170^e
Width [cm]	1.52±0.001^c	1.07±0.001^a	2.00±0.001^f	1.19±0.001^b	1.55±0.001^e	1.37±0.001^d	1.32±0.001^c
Ratio	1.64^b	1.09^a	3.00^c	1.09^a	1.64^b	4.46^d	6.00^e

Analyses of RAPD profiles of *P. piloselloides* and *P. longifolia* indicated genetic polymorphism in all fern populations investigated. The number of DNA bands generated with each of the nine primers used ranged from two to 14 (Table 2). It was found that replicates within a particular population of fern species showed high genetic similarities (average GS = 94 %) (data not shown). The data for these replicates were then consolidated to allow pair-wise comparisons between different

fern populations. The similarity matrix constructed showed that populations of *P. piloselloides* with different length/width ratios were genetically different (Table 3). Their GS ranged from 67 to 84 %. The results also showed that GS between populations with fronds of almost similar length/width ratios was high as indicated by the higher number of RAPD bands common to these populations (Table 3). Genetic similarity between fronds with different length/width ratios was lower (Table 3).

Table 2. Sequences of primers (5′ - 3′) used for RAPD amplifications of *Pyrrosia piloselloides* and the number of monomorphic (present in all populations), polymorphic (present in more than one population) and unique (present in only one population) bands obtained from each primer.

Primer	Primer sequence	Monomorphic	Polymorphic	Unique	Total
OPU-01	ACGGACGTCA	1	7	6	14
OPU-03	CTATGCCGAC	1	9	3	13
OPU-04	ACCTTCGGAC	2	9	2	13
OPU-05	TTGGCGGCCT	3	1	0	4
OPU-06	ACCTTTGCGG	5	6	1	12
OPU-07	CCTGCTCATC	1	0	1	2
OPU-08	GGCGAAGGTT	1	10	1	12
OPU-16	CTGCGCTGGA	0	7	1	8
OPU-17	ACCTGGGGAG	1	4	3	8

The low GS between populations and higher GS within populations indicated that *P. piloselloides* could be an inbreeding species (Layton and Ganders 1984, Van Dijk *et al.* 1988). Inbreeding within a population would restrict gene flow, isolating it from gene pools of other populations and, thus, accentuating genetic variability. On the other hand, pair-wise comparisons between populations of *P. piloselloides* and *P. longifolia* showed an average GS of 56 %. This value was lower than the average value of GS (76 %) scored for pair-wise comparisons between *P. piloselloides* populations. The results of this study agreed with those of Chalmers *et al.* (1992).

Table 3. A similarity matrix showing pair-wise comparisons between populations A to G of *Pyrrosia piloselloides* and *P. longifolia* (PL). Genetic similarities were calculated from RAPD data. Numbers within parentheses show genetic similarities [GS (%)] while numbers without parentheses show the total number of common RAPD bands shared by the two populations. The RAPD analysis were replicated three times for all fern populations.

	A	B	C	D	E	F	G	PL
A	-	42	44	40	38	38	34	24
B	(80)	-	42	39	34	38	39	24
C	(82)	(77)	-	39	37	39	38	28
D	(83)	(80)	(77)	-	36	35	32	21
E	(82)	(72)	(76)	(84)	-	36	31	22
F	(75)	(75)	(74)	(74)	(80)	-	34	23
G	(67)	(76)	(72)	(68)	(69)	(69)	-	23
PL	(54)	(53)	(60)	(51)	(56)	(63)	(54)	-

They studied the partitioning of genetic variability within and between tree populations using RAPD markers in *Gliricidia sepium* and *G. maculata*, and found that extensive genetic variability was detected between species and genetic variability was higher between populations than within populations. In fact, RAPD has been used to determine genetic variability within plant species and to show phylogenetic relationships between populations. For instance, RAPD was used to determine the levels of genetic diversity and phylogenetic relations in several species of *Stylosanthes*, *Carica papaya*, *Azolla*, and *Lycopersicon esculentum* (Kazan *et al.* 1993, Stiles *et al.* 1993, Van Coppenolle *et al.* 1993, Williams and St. Clair 1993).

This study has effectively demonstrated the use of RAPD assay to detect genetic variation between populations of *P. piloselloides*. Although RAPD markers could not categorically define the existence of distinct populations within *P. piloselloides*, it had shown that genetic similarity was high within populations but lower between populations. Apart from genomic sequence diversity that could be detected with RAPD assay, other genomic changes such as the occurrence of autoploidy, aneuploidy or allopolyploidy could account for variations in morphology (Soltis and Soltis 1993). In addition, methylation of certain DNA sequences could possibly account for variations in shape. In this study, it was not possible to determine whether such methylation effects occurred. RAPD assays indicating DNA polymorphisms in *P. piloselloides* did not provide an insight into the cause of genetic variation nor the site of variation within the genome.

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