J. Aquat. Plant Manage. 36: 107-111

Random Amplified Polymorphic DNA Analysis of Water Spinach (*Ipomoea aquatica*) in Florida

THAI K. VAN AND P. T. MADEIRA[1]

ABSTRACT

The genetic relationship between three biotypes of water spinach (*Ipomoea aquatica* Forsk.), including one 'upland' agricultural cultivar and two floating wild biotypes collected from Hillsborough County, Florida, were evaluated using Random Amplified Polymorphic DNA (RAPD) markers. Forty-eight decamer primers were screened; eighteen of these were informative and yielded 188 resolvable bands, of which 58 (31%) were polymorphic. Five primers produced unique DNA fingerprints useful for identification of the biotypes and fingerprints of the biotypes from two primers are presented. Phenetic analysis of the banding patterns grouped the three biotypes within unique clusters which bootstrap analysis further confirmed. Furthermore, while there is bootstrap evidence that the 'Cultivated' type is distinct, there is no evidence that the cultivated variety has diverged from the wild types to any greater extent than the wild types are different from each other. Marker variation occurs mainly between the biotypes (78%) which may reflect the limited number of farmers, limited wild type introductions, and frequent clonal reproduction, but also reflects the severely limited sample size.

Key words: RAPD, molecular marker, DNA fingerprint, bootstrap, AMOVA.

INTRODUCTION

Water spinach (*Ipomoea aquatica* Forsk.), a member of the morning-glory family, Convolvulaceae, and a native to southern Asia, is a fast-growing herbaceous vine commonly found creeping on muddy stream banks or floating in freshwater marshes and ponds. Water spinach has been widely cultivated as a vegetable in much of the tropics of the Old World. Due to its rapid and prolific growth, however, water spinach has often become weedy even in its native range, in rice paddies, fish ponds, irrigation and drainage canals, and other aquatic systems (Patnaik 1976).

Because it has been cultivated for centuries (the earliest known record is from China in the third century A.D.), there are a significant number of cultivars reported worldwide (Snyder et al. 1981). Nevertheless, two main wild biotypes are reported throughout southeast Asia (Cornelis et al. 1985, Westphal 1993). The first is called 'Red' and is characterized by green/purple stems, dark green leaves with sometimes purple petioles and veins, and light purple to white flowers. The second is called 'White' and is characterized as plants with green/white stems, green leaves with green/white petioles, and white flowers.

In Florida, two floating wild biotypes and at least one 'upland' cultivated form have been identified (Kipker, Florida Department of Environmental Protection, personal communication). All three were sent to Dan Austin, Professor of Botany at Florida Atlantic University, an expert on the morning-glory family, who confirmed their identification as *Ipomoea aquatica*. The wild forms include a 'Red' type with red flowers, green/purple stems, and dark green leaves with sometimes purple petioles and veins, and a 'White' type

[1]US Department of Agriculture, Agricultural Research Service, 3205 College Avenue, Fort Lauderdale, FL 33314. Received for publication November 5, 1997 and in revised form July 7, 1998.

with white flowers and light green stems. The two wild forms are usually found floating in freshwater marshes and ponds. In contrast, the 'upland' cultivar, also having white flowers and light green stems, roots in non-inundated soils and is grown commercially in raised beds under terrestrial conditions.

Water spinach is currently listed in the U.S. Federal Noxious Weed Act and is also listed under Florida law as a Class I Prohibited Aquatic Plant, where possession is a crime that can carry a $500 fine for the first offense. The plant is cultivated, however, in Florida, Texas, and California, because of availability of profitable domestic and international markets for the vegetable crop. Some farmers have claimed that cultivation has bred the "weedy, invasive" character out of their plants. This question is pertinent to those responsible for regulation of the species and for determination of whether the 'upland' agricultural cultivar would become invasive in aquatic habitats.

This lab was initially requested to conduct a study of regrowth from irradiated tissue, as the Florida Department of Environmental Protection was considering various methods to prevent the escape of water spinach due to illicit planting of the product along waterways by individuals purchasing the commercial food product. As part of this study the control plants provided some information on the growth habits of the three types.

We found few differences in growth characteristics between the cultivated form and the wild forms in our greenhouse cultures. Without frequent clippings, as normally practiced in commercial plantings, all three forms exhibited a vining habit, typical of many members of the morning-glory family. Similar observations were noted also in State of Florida greenhouse cultures (Burks, Florida Department of Environmental Protection, personal communication). Additionally, total growth in the controls, measured as dry weight, for the three biotypes in two media (either floating in pond water or rooting in moist soil), was not significantly different. To provide further insight we decided to look at the biotypes, as provided by the growers, using Random Amplified Polymorphic DNA (RAPD) markers, to: 1) ascertain whether there were markers that could distinguish the types (generate DNA fingerprints for biotype identification), and 2) look at the phenetic relationship between the biotypes, using Dice similarity measures, UPGMA clustering, and bootstrap analysis to see if they indicate distinct 'types', and whether there is a possibility that the 'cultivated' biotype has diverged from the 'wild' types.

Random amplified polymorphic DNA markers are anonymous genetic loci generated by polymerase chain reaction (PCR) amplification of genomic DNA using short random primers and low annealing temperatures (Welsh and McClelland 1990, Williams et al. 1990). In contrast to other molecular techniques, the RAPD technique is easy to perform, inexpensive, and requires no knowledge of the genomes being investigated. For these reasons, the RAPD technique and related methods have attracted widespread interest as a way to generate markers for various genetic variation studies, including genomic fingerprinting and strain identification (Hosaka et al. 1994, Ko et al. 1994, Keil and Griffin 1994, Lanham et al. 1995, Sobral and Honeycutt 1994), and the generation of genetic distances and phenograms (Connolly et al. 1994, Figueira et al. 1994, Ko et al. 1994, Lanham et al. 1995, Madeira et al. 1997, Marmey et al. 1994, M'Ribu and Hilu 1994, Ramser et al. 1996).

MATERIALS AND METHODS

Plant material. Two floating wild forms of water spinach, 'Red' and 'White', were collected originally from Hillsborough County, Florida, and maintained in greenhouse cultures by vegetative propagation. The cultivated form was obtained from two commercial growers, 'A' and 'B', from the same geographical area. Dried herbarium specimens of plants from these accessions have been examined by Dr. Dan Austin (Florida Atlantic University, personal communication), an authority on the morning-glory family, who indicated that all three accessions are *Ipomoea aquatica*.

DNA extraction. Three plants from each accession were sampled. For each individual plant, 50 mg of young leaf tissues were excised and frozen at -80 C until DNA extraction. Total DNA was extracted using the CTAB method of Doyle and Doyle (1990) with minor modifications. Plant samples were removed from the freezer, the extraction buffer (0.5 ml) added, and the tissue was immediately ground. The extraction buffer contained 3% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% PVP-40, and 1% mercaptoethanol. Because of the small amount of tissue and the chilled mortar and pestle, no liquid nitrogen was necessary and the tissue was rapidly disrupted. After grinding, the tissue was transferred to a 1.5 ml microcentrifuge tube and incubated for 30 min at 60 C, followed by chloroform:isoamyl alcohol (24:1) extraction. Purity of DNA is greatly enhanced by repeated mixing of the phases until thoroughly emulsified. The phases were separated by centrifugation at 12,000 g for 30 min, the upper phase was pipetted to a 1.5 ml microcentrifuge tube, and 0.1 volume of 10% CTAB, 0.7 M NaCl added followed by a 5 min incubation at 65 C. The tubes were cooled and the chloroform:isoamyl extraction repeated. The nucleic acids were then precipitated with 0.6 volume isopropanol and pelleted by centrifuging at 3000 g for 15 min, then the supernatant was decanted and the tube inverted over a paper towel to drain residual alcohol. Resuspension buffer [50 µl; 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1M NaCl], 20% SDS (1.2 µl), and proteinase K (to 100 µg/ml) were added and the mixture was incubated at 37 C for 1 hr. The mixture was then extracted with an equal volume of Tris- saturated phenol: chloroform: isoamyl alcohol (25:24:1), thoroughly mixed, and centrifuged at 12,000 g for 15 min. The upper aqueous phase was transferred to another 1.5 ml microcentrifuge tube and precipitated with 2 volumes of ethanol, then centrifuged at 12,000 g for 15 min and drained by inverting. The pellet was washed with 70% ethanol, decanted, and air dried. The pellet was then resuspended in 50 µl of TE solution pH 8, and 2 µl of RNAse (2 mg/ml stock) added, followed by incubation for 1 hr at 37 C. The DNA solution was stored at 4 C.

RAPDs Amplification. RAPDs reactions contained 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.1 mM each dATP, dCTP, dGTP, and dTTP, 0.2 mM primer (Operon

TABLE 1. SUMMARY OF RAPD LOCI AND BIOTYPES BY BRIGHTNESS AND DEGREE OF POLYMORPHISM.

	Number of bands			Resolved bands	
By band brightness	Unresolved	Monomorphic	Polymorphic	Total	% Polymorphic
bright	0	23	4	27	15
moderate	3	48	21	69	30
dim	101	59	33	92	36
all	104	130	58	188	31
By *Ipomoea* biotype					
'Red' Wild		168	3	171	2
'White' Wild		155	19	174	11
Cultivated		151	15	166	9

Technologies[2], Alameda, CA), 0.02 U/µl AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), and 0.4 ng/µl of genomic DNA. Reactions were amplified as a total volume of 18 µl overlain with mineral oil. DNA amplification reactions were performed in a thermal cycler (PTC-100; MJ Research, Watertown, MA) programmed for 1 cycle (pseudo-hot start) of 8 min at 94 C followed by 45 cycles of 94 C for 1 min, 37 C for 1 min, and 72 C for 2 min, for denaturing, annealing, and primer extension, respectively. After the final cycle, samples were incubated for a further 5 min at 72 C to ensure complete extension. Amplification products were resolved by electrophoresis on a 1.4% agarose gel in 0.5% TBE buffer, stained with ethidium bromide and visualized under UV illumination.

Screening and Analysis. Forty-eight decamer primers (Operon Technologies; kits A, B, & G) were screened with 46 producing at least some amplified product. Eighteen primers were chosen with a bias towards those producing more and brighter products; however, no selection was performed for those with more polymorphism. Loci were scored as presence (1) or absence (0) of bands, and characterized by the primer used, followed by the size in base pairs as determined by reference to a 100 bp DNA ladder (Gibco BRL, Bethesda, MD). Amplifications were replicated at least three times, and loci classified as bright, moderate, or dim, according to intensity. Dice similarity matrices and the corresponding UPGMA (unweighted pair-group method, arithmetic average) phenograms were generated in the NTSYS (v 1.8) program (Exeter Software, Setauket, N.Y.). A cophenetic matrix was generated for the UPGMA phenogram and compared to the actual Dice similarity matrix to generate a matrix correlation. Bootstrap Analysis (Felsenstein 1985) of the UPGMA phenogram was generated using the RAPDBOOT program[3]. The Analysis of Molecular Variance (AMOVA) procedure (Excoffier et al. 1992) was used along with the Euclidean metric as defined by Huff et al. (1993) for RAPDs. The analysis was carried out using the WINAMOVA program (ver 1.55) provided by Laurent Excoffier (Dept. of Anthropology and Ecology, U. of Geneva, Switzerland). The Euclidean metric amounts to, for binary data, the sum of the band differences between individuals

[2]Mention of a trademark name, proprietary product, or specific equipment does not constitute a warranty by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that also may be suitable.

[3]Black IV, W. C., 1995. FORTRAN programs for the analysis of RAPD-PCR markers in populations. Colorado State University, Ft Collins, CO 80523.

RESULTS AND DISCUSSION

A summary of the loci generated in this study is given in Table 1. A total of 188 resolvable and 58 (31%) polymorphic loci were identified. The total number of loci and the number of unresolved loci increased as the brightness decreased. Additionally, the brightest loci were the least polymorphic. Loci of moderate brightness, which were for the most part still easy to resolve, were only slightly less polymorphic than the dim ones. One purpose of this study was to generate DNA fingerprints for biotype identification. Five primers (Table 2) were individually capable of distinguish-

TABLE 2. PRIMERS CAPABLE OF DISTINGUISHING THREE BIOTYPES IN *IPOMOEA AQUATICA* INDIVIDUALS EXAMINED.

			Presence (1) or absence (0) of loci		
Primer	Loci size (bp)	Loci brightness	'Red' Wild	'White' Wild	Cultivated
A18	500	moderate	1	0	0
	870	moderate	1	1	0
	1220	moderate	1	0	0
	1400	dim	1	0	0
	1720	dim	0	1	1
B10	320	dim	0	1	1
	640	bright	1	0	1
	710	dim	1	0	0
	1850	moderate	1	1	0
B12	380	dim	1	0	0
	450	dim	1	0	1
	580	moderate	0	1	1
G5	450	dim	1	1	0
	1020	moderate	0	1	1
	1160	dim	0	1	1
	1220	dim	1	0	0
	1400	moderate	1	0	0
G10	450	dim	1	0	1
	1230	moderate	0	1	1

ing the three water spinach biotypes based on the limited samples compared. Even if the limited sample size failed to pick up exceptions within certain diagnostic bands, use of multiple primers should allow for the types to be distinguished. Primers A18 (5'-AGGTGACCGT-3') and B10 (5'-CTGCT GGGAC-3') were the most powerful diagnostic fingerprints in that moderate and bright loci provided the definition. Figure 1, which shows the amplification patterns obtained with these two primers, illustrates the criteria we used to consider the different levels of brightness for the loci as indicated in Table 1.

The second objective was to look at the phenetic relationship between the biotypes, using Dice similarity measures and UPGMA clustering, to see if they indicate a possibility that the cultivated variety has diverged from the wild types. The matrix correlation (normalized Mantel Z statistic) between the actual similarities and those generated by the UPGMA procedure was 0.97, indicating a high correlation. The results of the UPGMA analysis of the Dice similarities is presented in Figure 2. Note that each biotype of water spinach (Red, White, and Cultivated) clustered uniquely. Bootstrap values add further evidence that these clusters by 'type' are significant. High bootstrap scores (>90%) suggest strong support for a particular cluster. The major clusters all exhibited high bootstrap values (Red-100%; White-99%; Cultivated-100%), indicating strong support. Cultivated plants did not cluster uniquely by grower, however. The cultivated biotype clustered somewhat closer to the 'White' than to the 'Red' wild type, as might be expected from phenotypic characteristics. Note however, that the bootstrap support is only 67%, indicating additional data is needed to confirm whether the 'White/Cultivated' cluster is supported. Note also, in Table 2, the loci at B10-640, B12-450, and G10-450 were absent only in the 'White' type, indicating that some traits of the cultivated genotype may derive from the 'Red' wild type. There is therefore evidence that the 'Cultivated' type is distinct, but little evidence that phenetic divergence from, for example, the 'White' type, is any greater than the phenetic divergence between the wild types.

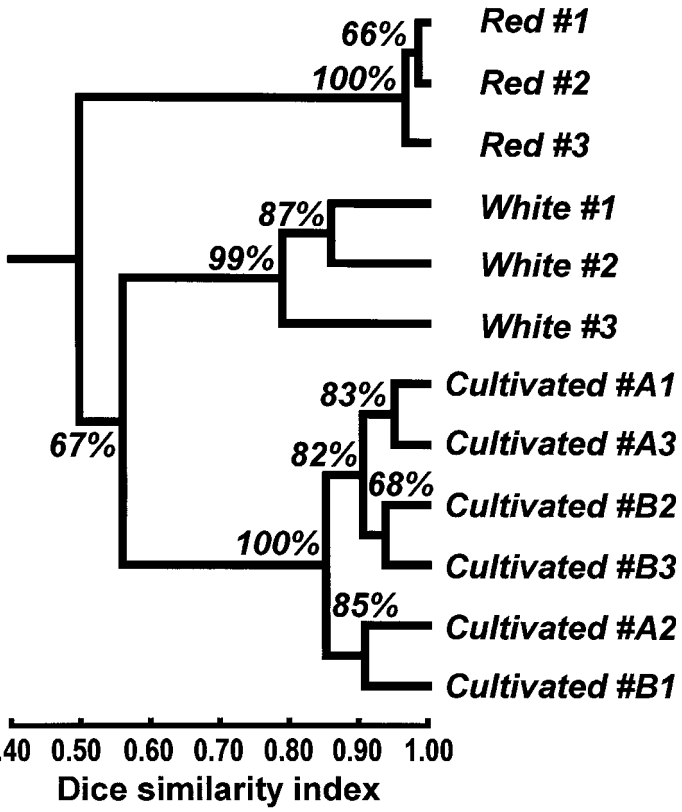

Figure 2. UPGMA phenogram generated from Dice similarity index using polymorphic bands. The numbers at the forks indicate the % of times, out of 4500 bootstrap trees, the individuals to the right of that fork all appeared within the cluster. Bootstrap values of greater than 90% are generally considered to provide strong support.

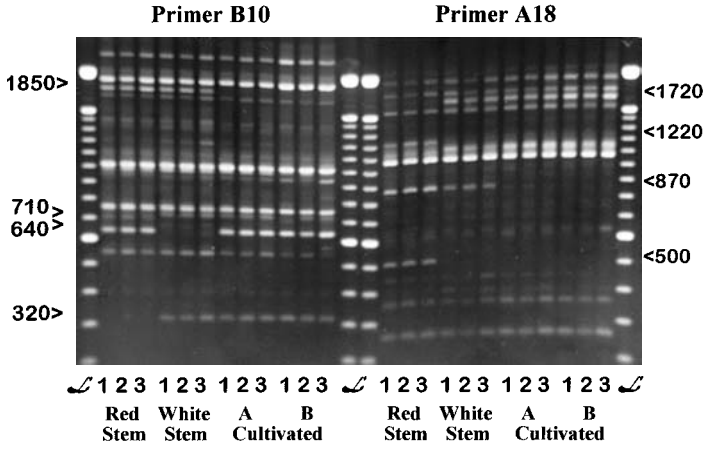

Figure 1. Gel electrophoresis of two primers, B10 and A18, capable of distinguishing the three genotypes in *Ipomoea aquatica* individuals collected in Florida. Gibco BRL DNA ladder (L), which displays a band every 100 nucleotides from 100 bp to 1500 bp, was used to size and identify the loci. Loci of interest are indicated by their size and location.

The AMOVA analysis allows partition of the variation in a hierarchical fashion in much the same way as an ANOVA does, except that the significance levels for the variance component estimates are generated by non-parametric permutational procedures. Two components were analyzed, variation 'between' the biotypes, which accounted for 78% of the variation, and variation 'within' the biotypes, which accounted for 22% of the variation. The probability of obtaining these values by random permutation alone is <0.001. Once again, the 'within' biotypes value is probably distorted downward due to limited 'individuals/biotype' sample size and range. It probably also reflects the reality of a limited number of farmers, limited wild type introductions, and frequent clonal reproduction.

In summary, RAPD analysis of *I. aquatica* in Florida revealed five primers which produced unique DNA fingerprints capable of distinguishing the three biotypes. Phenetic analysis clearly clusters the three biotypes uniquely, and bootstrap analysis provides confidence. However, there is no evidence that the cultivated variety has diverged from the wild types to any greater extend than the wild types are different from each other. Cautions are indicated due to limited sample size.

ACKNOWLEDGMENTS

The authors thank Be Le of Be Le Farm in Hillsborough County, Florida, for supplying the water spinach biotypes. The help of Suzanne Cady, Hillsborough County Extension Service is also greatly appreciated. Kathy Burks, Robert Kipker, Robert Pemberton, Fred Ryan, and David Sutton reviewed the manuscript.

LITERATURE CITED

Connolly, A. G., I. D. Godwin, M. Cooper, and I. H. DeLacy. 1994. Interpretation of randomly amplified polymorphic DNA marker data for fingerprinting sweet potato (*Ipomoea batatas* L) genotypes. Theor. Appl. Genet. 88: 332-336.

Cornelius, J., J. A. Nugteren, and T. Westphal. 1985. Kangkong (*Ipomoea aquatica* Forsk.): an important leaf vegetable in Southeast Asia. Abstracts on Tropical Agriculture. 10: 9-21.

Doyle, J. J. and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction sites. Genetics 131: 479-491.

Felenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39: 783-791.

Figueira, A., J. Janick, M. Levy, and P. Goldsbrough. 1994. Reexamining the classification of *Theobroma cacao* L. using molecular markers. J. Amer. Soc. Hort. Sci. 119(5): 1073- 1082.

Hosaka, K., M. Mori, and K. Ogawa. 1994. Genetic relationships of Japanese Potato Cultivars assessed by RAPD analysis. Am. Potato J. 71: 535-545.

Huff, D. R., R. Peakall, and P. E. Smouse. 1993. RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.]. Theor. Appl. Genet. 86: 927-934.

Keil, M. and A. R. Griffin. 1994. Use of random amplified polymorphic DNA (RAPD) markers in the discrimination and verification of genotypes in *Eucalyptus*. Theor. Appl. Genet. 89: 442-450.

Ko, H. L., D. C. Cowen, R. J. Henry, G. C. Graham, A. B. Blakeney, and L. G. Lewin. 1994. Random amplified polymorphic DNA analysis of Australian rice (*Oryza sativa* L.) varieties. Euphytica 80: 179-189.

Lanham, P. G., R. M. Brennan, C. Hackett, and R. J. McNicol. 1995. RAPD fingerprinting of blackcurrant (*Ribes nigrum* L.) cultivars. Theor. Appl. Genet. 90: 166-172.

Madeira, P. T., T. K. Van, K. K. Steward, and R. J. Schnell. 1997. Random amplified polymorphic DNA analysis of the phenetic relationships among world-wide accessions of *Hydrilla verticillata*. Aquat. Botany. 59: 217-236.

Marmey, P., J. R. Beeching, S. Hamon, and A. Charrier, 1994. Evaluation of cassava (*Manihot esculenta* Crantz) germplasm collections using RAPD markers. Euphytica. 74: 203-209.

M'Ribu, H. K. and K. W. Hilu. 1994. Detection of interspecific and intraspecific variation in *Panicum* millets through random amplified polymorphic DNA. Theor. Appl. Genet. 88: 412-416.

Patnaik, S. 1976. Autoecology of *Ipomoea aquatica* Forsk. J. Inland Fish. Soc. India 8: 77-82.

Ramser, J., C. Lopez-Peralta, R. Wetzel, K. Weising, and G. Kahl. 1996. Genomic variation and relationships in aerial yam (*Dioscorea bulbifera* L.) detected by random amplified polymorphic DNA. Genome 39: 17-25.

Snyder, G. H., J. F. Morton, and W. G. Genung. 1981. Trials of *Ipomoea aquatica*, nutritious vegetable with high protein- and nitrate-extraction potential. Proc. Fla. State Hort. Sci. 94: 230-235.

Sobral, B. W. S. and R. J. Honeycutt. 1994. Genetics, plants, and the polymerase chain reaction. *In*: The Polymerase Chain Reaction, K. B. Mullis, F. Ferre, R. A. Gibbs (eds.), Birkauser Boston, pp. 304-319.

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18: 7213-7218.

Westphal, E. 1993. *Ipomoea aquatica* Forskal. In: Plant Resources of South-East Asia. Vol 8, Vegetables, Siemonsma, J. S. and K. Piluek (eds.), Pudoc Scientific Publishers, Wageningon, The Netherlands, pp. 181-184.

Williams, J. G. K., A. E. Kubelik, K. J. Livac, J. A. Rafalski, and S. C. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.