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Variation in lanceleaved waterplantain (*Alisma lanceolatum*) in southeastern Australia

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Lanceleaved waterplantain is an exotic weed of rice that was introduced into Australia in the 1930s. Since its introduction, it has spread throughout much of the rice-growing region in southeastern Australia. The variability of lanceleaved waterplantain in these regions was studied using polymerase chain reaction-based, inter simple sequence repeat (ISSR) analysis. Deoxyribonucleic acid fingerprints from samples of the weed from southeastern Australia were compared between locations and with two samples of common waterplantain, a closely related species. The analysis indicated that there were two distinct groups of lanceleaved waterplantain that correlated with location. From the results of multidimensional scaling analysis, it is hypothesized that the Griffith group did not arise from hybridization between lanceleaved waterplantain and common waterplantain, and that it is more likely that the group arose from a separate introduction into the area. It is also suggested that there is seed movement between areas in the Murray Valley and Colleambally Irrigation areas. The implications of this variation for biological control of the weed are discussed.

Nomenclature: Common waterplantain, *Alisma plantago-aquatica* L. ALSPA; lanceleaved waterplantain, *Alisma lanceolatum* With. ALSLA; rice, *Oryza sativa* L.

Key words: Aquatic weed, invasive weed, inter simple sequence repeat, ISSR.

Members of the family *Alismataceae* are monocotyledenous aquatic herbs. The family is worldwide in distribution with 11 genera and 100 species. Within Australia, there are nine species in four genera. Some members of the family are native to Australia, such as *Damasonium minus* (R. Br.) Buchenau and common waterplantain, whereas species such as California arrowhead (*Sagittaria montevidensis* Cham. & Schltdl.) and lanceleaved waterplantain are exotic. Lanceleaved waterplantain is considered native to Europe, North Africa, and West Africa (Aston 1973). In Europe, it is rare in the northern regions. As a weed, it has been reported in southern Europe (Catizone 1983; Ferrero et al. 2002) and in rice crops in California (Barrett and Seaman 1980).

The weed was introduced into Victoria at Creswick in the 1930s with willow (*Salix* sp.) cuttings (Aston 1973) and has been reported in Echuca, Victoria, and the Murray Valley and Coleambally Irrigation areas of New South Wales (McIntyre and Newnham 1988). It is favored by shallow, slow-moving, or still water but can survive drought periods. In rice, the weed is favored by aerial sowing, although herbicides or cultivation can be used to control it. In 1983, 12 rice farms in the Coleambally region were found to be infested with lanceleaved waterplantain. This infestation was considered disjunct from the infestations at Echuca. The weed has continued to spread mainly through agricultural machinery and water movement. In 1994, a new outbreak was reported from the Griffith area. A similar infestation at Derriboota was reported at the same time. The spread of lanceleaved waterplantain is viewed with concern by the rice industry. It is difficult to control with herbicides such as (4-chloro-2-methylphenoxy)acetic acid (MCPA) and bensulfuron-methyl, and farms known to be infested are reportedly more difficult to sell (M. Hedditch, personal communication).

Suspected development of herbicide resistance in lanceleaved waterplantain and confirmation of resistance in *D. minus* has encouraged the development of biological control based on the fungus *Rhynchosporium alismatis* (Cother and Gilbert 1994; Cother et al. 1994). Because subpopulations within a species may harbor variations in resistance to pathogens, knowledge of variation within the target weed species is important in the selection of biological control agents. The variation within lanceleaved waterplantain in Australia is not known, and hence, the selection of a representative sample for glasshouse trials associated with the study of the biology of *R. alismatis* is more problematic. Major variation within the species has been reported from outside Australia, which can be attributed to the existence of two morphologically similar cytotypes of lanceleaved waterplantain ($n = 26$ and $n = 28$) and its ability to form hybrids with common waterplantain. The occurrence of this hybridization in nature is extremely rare because of self-fertility of the species (Pogan 1971). Any hybrids formed are generally sterile or have reduced seed set (Björkqvist 1967; Pogan 1971).

Molecular methods have afforded the opportunity to explore not only genetic diversity but also the spread of weeds and have allowed us to theorize about the origin of weed introductions and the production of hybrids. There is a range of molecular methods available to study genetic diversity. The choice of marker system to be used depends on the aims, technical considerations, availability of laboratory facilities, costs, and on the genetic diversity of the population under study (O'Hanlon et al. 1999). Inter simple sequence repeats (ISSR) are polymerase chain reaction (PCR)-based markers that require no sequence information and are composed of single primers based on a simple repeat with the addition of a degenerate nucleotide or a selective anchor. These primers amplify the region between microsatellites, the ISSR region. They rely on the presence of palindromic microsatellites within amplifiable distances. ISSR have been

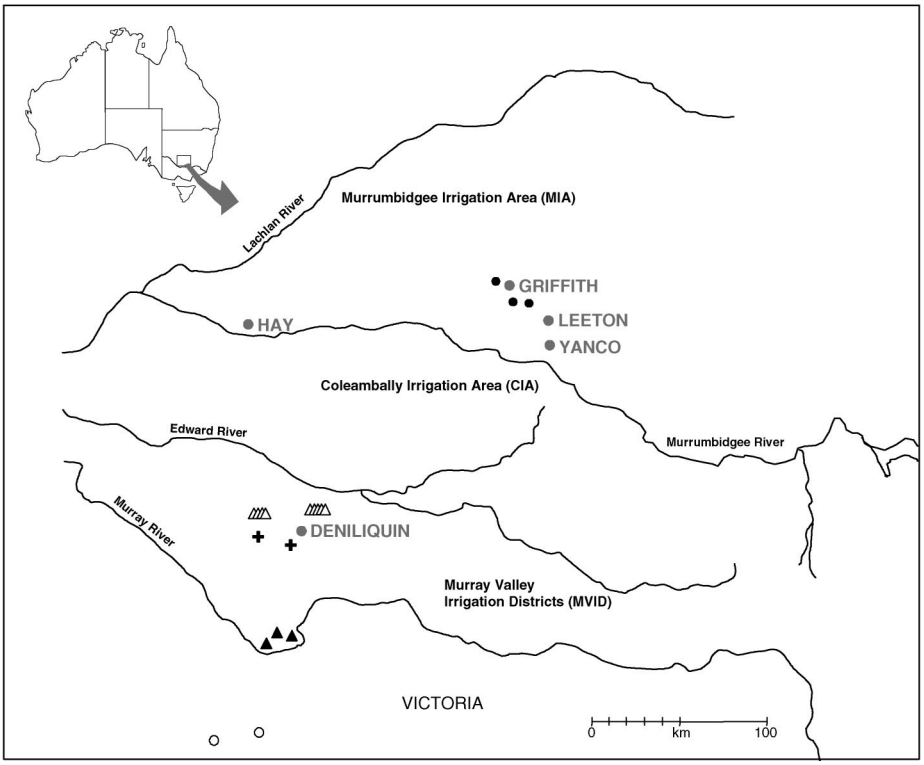

FIGURE 1. Major population centers, irrigation areas, and locations of common waterplantain (cross symbols) and lanceleaved waterplantain samples (all other symbols) collected for this study. Each symbol represents a single plant from which seed was harvested. Up to four daughter plants were grown from each plant sampled.

shown to generate similar or higher levels of polymorphism than restriction length polymorphisms or random amplified polymorphic deoxyribonucleic acid (RAPD-DNA) (Godwin et al. 1997; Hollingsworth et al. 1998; Nagaoka and Ogihara 1997) but are generally less variable than simple sequence repeats or amplified fragment length polymorphism (O'Hanlon et al. 1999). Recently, ISSR PCR has been used for fingerprinting weeds such as distaff thistle (*Carthamus lanatus* L. [Ash et al. 2003b]), *D. minus* (Jahromi et al. 2002), and *Orbanche crenata* (Román et al. 2002).

In this article, we use PCR ISSR to describe the variation in lanceleaved waterplantain and provide evidence for multiple introductions of the weed into southeastern Australia.

Materials and Methods

Plant Material

Seed of lanceleaved waterplantain was collected from all known infestations in southern New South Wales and from representatives of common waterplantain from southern New South Wales and northern Victoria in December 1994 (Figure 1). Seed was collected from individual plants and kept as separate lots. Because infestations were not widespread, plants were selected at random from within the area of infestation. Seed from each lot was returned to the glasshouse and germinated in flooded pots containing field soil. Floating leaves were harvested from up to four plants from each seed lot, washed in 10 g L^{-1} sodium hypochlorite (to remove algae), and used in DNA extraction.

DNA Extraction

One gram of leaf material from glasshouse-grown plants was ground in liquid nitrogen in a mortar and pestle. The ground material was added to 10 ml of preheated (60 C) extraction buffer (2% cetyltrimethylammonium bromide [wt/v], 50 mg polyvinyl-pyrrolidone, 0.04% β-mercaptoethanol, pH 8.0) and allowed to return to room temperature over a period of 25 min. Fifteen milliliters of chloroform–iso-amyl alcohol (24:1) was added and gently mixed. This was centrifuged for 13 min at 12,000 rpm at 4 C to separate the phases. The aqueous phase was removed to a separate tube, and a half volume of 5 M NaCl and two volumes of ethanol (− 20 C) were added to precipitate the DNA for 1 h at − 20 C. It was then centrifuged at 12,000 rpm for 20 min, the aqueous phase removed, and the pellet air-dried. The pellet was then resuspended in 200 μl of TE buffer (10 mM Tris–HCl, 1.0 mM ethylenediaminetetraacetic acid, pH 8.0). The DNA was diluted to approximately 30 ng μl^{-1} and stored at − 20 C until use.

Oligonucleotide Primers

One hundred primers[1] were evaluated for their ability to produce polymorphic banding patterns in lanceleaved waterplantain.

ISSR Analysis

PCR was performed in 500 mM KCl, 100 mM Tris–HCl pH 9.0, and 1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.2 μM of prim-

TABLE 1. Sequence of inter simple sequence repeat primers that revealed polymorphism in lanceleaved waterplantain.

Primer identifier	Sequence[a]	Polymorphic bands	Melting temperature (C)
UBC 811	GAG AGA GAG AGA GAG AC	4	54
UBC 834	AGA GAG AGA GAG AGA GYT	2	51
UBC 835	AGA GAG AGA GAG AGA GYC	3	55
UBC 836	AGA GAG AGA GAG AGA GYA	3	51
UBC 840	AGA AGA GAG AGA GAG AYT	2	53
UBC 841	GAG AGA GAG AGA GAG AYC	3	53
UBC 845	CTC TCT CTC TCT CTC TRG	4	52
UBC 848	CAC ACA CAC ACA CAC ARG	4	52
UBC 854	TCT CTC TCT CTC TCT CRG	4	52
UBC 855	ACA CAC ACA CAC ACA CYT	5	51
UBC 856	ACA CAC ACA CAC ACA CYA	6	51
UBC 858	TGT GTG TGT GTG TGT GRT	6	50
UBC 860	TGT GTG TGT GTG TGT GRA	6	51
UBC 872	GAT AGA TAG ATA GAT A	2	40
UBC 881	GGG TGG GGT GGG GTG	7	54

[a] Y = C,T; R = A,G.

er, 1 unit of *Taq* polymerase,[2] and 30 ng of genomic DNA per 25 μl of reaction volume. The amplifications were carried out in an OMN-E thermocycler programmed for initial denaturation temperature of 95 C for 1 min and then 40 cycles of denaturation at 94 C for 1, 2 min at annealing temperature specific for each primer and extension at 72 C for 3 min, with a 5 min final extension at 72 C. Annealing temperature of a given primer was calculated according to the melting temperature as described by Gupta et al. (1994). PCR products were separated on 1.4% agarose gels electrophoresed in 1× Tris-acetate-EDTA buffer (Sambrook et al. 1989) and stained with ethidium bromide. Banding patterns were observed using ultraviolet radiation and photographed using Gel Pro image analysis software and an ISIS cooled CCD camera.

Gel Scoring and Cluster Analysis

All the amplified bands were treated as dominant genetic markers. Only clearly discernable, bright bands were scored. Bands were scored for presence (1) or absence (0) and scores assembled in a rectangular data matrix. Data were analyzed using the Numerical Taxonomy System.[3] A similarity matrix was obtained using similarity for qualitative (nominal) data. Sequential, agglomerative, hierarchical, nested cluster analysis was performed on the data matrix using the unweighted pair group method with the arithmetic averaging algorithm and 25 iterations. The validity of the clustering was determined by comparing the similarity and cophenetic (ultrametric) value matrices using the matrix comparison module of NTSYS-pc. The program takes two symmetric similarity or dissimilarity matrices and plots one matrix against the other element by element. The degree of relationship between the two matrices is determined using the product–moment correlation, *r*, and the Mantel test statistic, *Z*. These statistics were then used to select the most appropriate clustering. The stability of this clustering was further tested using multidimensional scaling (MDS), an ordination technique similar to principal component analysis (PCA), using NTSYS-pc. MDS is preferable to PCA because it preserves the small interplot distances more faithfully. The MDS was initially performed in a two-dimensional space. The data were prepared using the DCENTER (double center) routine, which transforms a symmetric similarity matrix to scalar product form so that its eigenvalues and eigenvectors can be computed using the EIGEN routine. The eigenvalues and random initial values were used in the initial computation. The output from this was then used as the initial values for a three-dimensional MDS. The stress factor was minimized and compared with the values of Kruskal (1964) to determine goodness of fit of the data. The groups identified from this analysis were described using the Shannon's Diversity Index using POPGENE version 1.31 (Yeh et al. 1997).

Results and Discussion

Of the 100 primers evaluated for polymorphism, 15 were found to produce clearly discernible, variable bands (Table 1). When these primers were tested across all the samples, they produced 61 bands that could be readily scored. Forty-nine unique genotypes were identified from all of the samples of lanceleaved waterplantain analyzed.

The product–moment correlation, r (0.982), and the Mantel test statistic, Z product ($t = 10.77$) were highly significant ($P < 0.01$) for the clustering shown in Figure 2. This figure shows that the collections of lanceleaved waterplantain fall into two clearly defined groups that are separated from the common waterplantain samples at a similarity coefficient of 34%. A number of primers were identified, which could differentiate the two groups identified, with primer UBC858 ($(TG)_8RT$) displaying four polymorphic bands between the two groups.

MDS of the genetic distance matrix produced a plot with a minimum stress value of 0.036 between a final MDS value and the original matrix. This represents an excellent fit of the data (Kruskal 1964). The three-dimensional scatter plot of the MDS (Figure 3) shows the clear separation of the two groups of lanceleaved waterplantain identified in the initial cluster analysis from the common waterplantain representatives (Figure 2). The data in Figure 3 also demonstrates that although there appear to be different groups originating from the Murray Valley Irrigation Area and the Colleambly Irrigation Area, these groups are not disjunct. This may indicate seed movement between locations. The separation of the lanceleaved waterplantain and the common

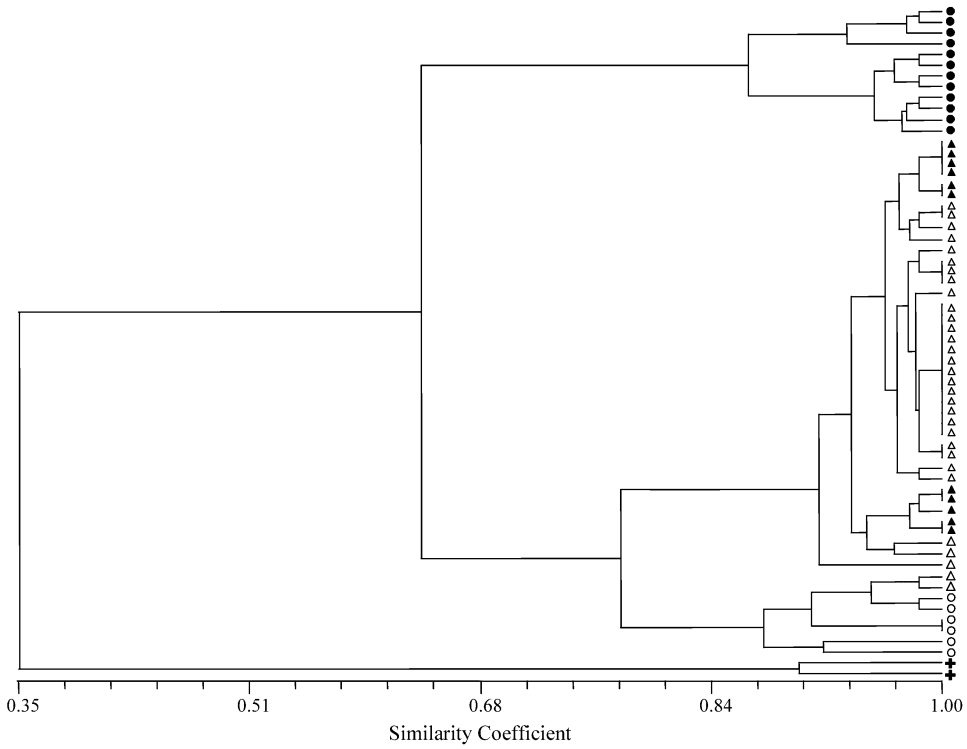

FIGURE 2. Dendogram constructed by unweighted pair group method with the arithmetic averaging showing the relationship between samples of lanceleaved waterplantain and common waterplantain collected from southeastern Australia. The locations from which the samples were taken are shown in Figure 1.

waterplantain into different planes within the three-dimensional space also demonstrates that the different groups were unlikely to have arisen from hybridization of the two species. The Shannon Indexes from the two groups were low (Griffith, − 0.1219 and elsewhere − 0.1992). However, these values may be artifacts of low sample sizes.

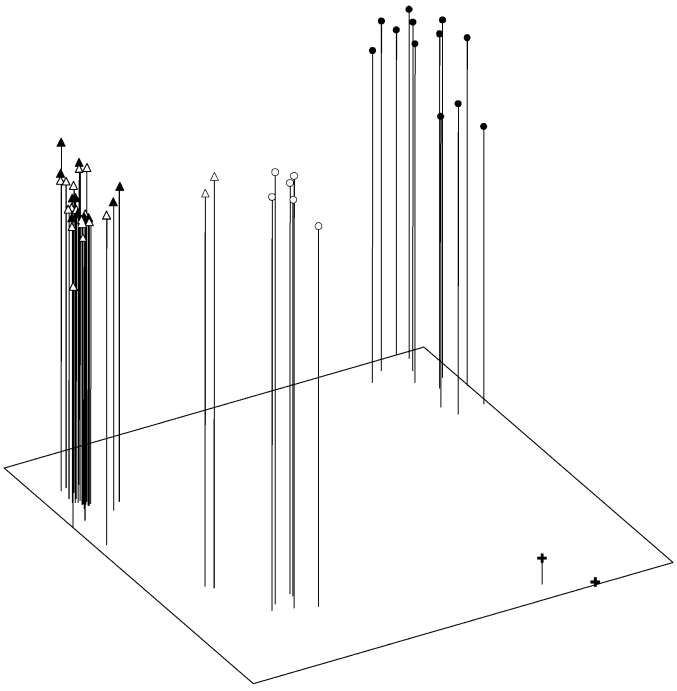

FIGURE 3. Three-dimensional multidimensional scaling plot of distances between samples of lanceleaved waterplantain and common waterplantain. The key for the symbols is from Figure 1.

In this study, we demonstrated the variability in lanceleaved waterplantain in southeastern Australia using PCR of ISSR regions within the genome. The ISSR method proved to be robust and reproducible while providing a simple method to examine the variability within the plant population. The ISSR method combines the simplicity of techniques such as RAPD-PCR with greater reproducibility afforded by the longer primers, and hence more stringent PCR conditions. As ISSR are dominant markers, the analysis of the data is correlated with differences in genes but cannot distinguish allelic differences.

Within the region studied, two clearly defined groups were shown to be present. The smaller group was centered on Griffith in southern New South Wales with the other group being widespread in all other areas sampled. These two groups could have arisen from separate introductions of the weed, the existence of separate cytotypes of the weed, or through hybridization of lanceleaved waterplantain and common waterplantain. The latter two are contributors to variation in this species outside of Australia (Björkqvist 1967). Analysis of the three-dimensional plot arising from the MDS indicates that the two lanceleaved waterplantain groups are on separate axes to the common waterplantain specimens. Therefore, the groups shown must have arisen from different introductions into New South Wales. From this initial study, the genetic diversity within and between the groups in the geographic area could be the subject of further study using higher intensity hierarchical sampling.

The distinct groups found in the lanceleaved waterplantain in, or adjacent to, the major rice growing areas in Australia may have ramifications for attempts at biological control of the weed. In the search for exotic biological control agents, attempts would need to be made to identify the

origin of diverse groups so that insects or pathogens from the native range could be identified. Furthermore, it has been suggested that there is a negative correlation between the likely success of biological control and the genetic diversity of the target species (Burdon and Brown 1986; Burdon and Marshall 1981). Therefore, the underlying genetic diversity of the weed should be taken into account when determining the priorities for biological control (Nissen et al. 1995). The genetic diversity of the weed could also be important when developing inundative biological control for weeds. In the case of lanceleaved waterplantain, an endemic fungus is being developed as a mycoherbistat (Crump et al. 1999) for the control of this and other closely related weed species (Ash et al. 2003a; Cother 1999; Cother and Gilbert 1994; Jahromi et al. 2001). In contrast to the structure of lanceleaved waterplantain, Jahromi et al. (2002) have shown that low levels of variation occur in *D. minus*, the main target of a mycoherbistat based on *R. alismatis*. Although the volume of inoculum and the addition of low rates of herbicides may negate any differences between groups, samples from different groups of lanceleaved waterplantain should be included in any screening of candidate isolates of this biological control agent.

Sources of Materials

[1] UBC primer set # 9, Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4.

[2] Taq DNA polymerase, Promega, 2800 Woods Hollow Road, Madison, WI 53711.

[3] Numerical Taxonomy System Software, NTSYS-pc, Version 2.02, Applied Biostatistic Inc., Exeter Software, 47 Route 25A, Suite 2, Setauket, NY 11733-2870.

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Literature Cited

Ash, G. J., E. J. Cother, F. G. Jahromi, W. M. Pitt, V. M. Lanoiselet, and S. Cliquet. 2003a. Status and future for biological control of rice in Australia. Page 51 *in* Proceedings of 8th International Congress of Plant Pathology, Christchurch, New Zealand.

Ash, G. J., R. Raman, and N. S. Crump. 2003b. An investigation of genetic variation in *Carthamus lanatus* in New South Wales, Australia, using inter simple sequence repeats (ISSR) analysis. Weed Res. 43:1–7.

Aston, H. I. 1973. Aquatic Plants of Australia. Melbourne, Australia: Melbourne University Press.

Barrett, S.C.H. and D. E. Seaman. 1980. The weed flora of Californian ricefields. Aquat. Bot. 9:351–376.

Björkqvist, I. 1967. Studies in *Alisma* L. I. Distribution, variation and germination. Opera Bot. 17:12–28.

Burdon, J. J. and A.H.D. Brown. 1986. Population genetics of *Echium plantagineum*, a target weed for biological control. Aust. J. Biol. Sci. 39:369–378.

Burdon, J. J. and D. R. Marshall. 1981. Biological control and the reproductive mode of weeds. J. Appl. Ecol. 18:649–658.

Catizone, P. 1983. Farmer's weed control technology in rice in southern Europe. Pages 183–191 *in* Weed Control in Rice (International Rice Research Institute). Los Banos, Philippines: IRRI.

Cother, E. J. 1999. Host range studies of the mycoherbistat fungus *Rhynchosporium alismatis*. Australas. Plant Pathol. 28:149–155.

Cother, E. J. and R. L. Gilbert. 1994. Efficacy of a potential mycoherbicide for control of *Alisma lanceolatum* and *Damasonium minus* in Australian rice crops. Aust. J. Exp. Agric. 34:1043–1050.

Cother, E. J., R. L. Gilbert, and D. C. Pollock. 1994. First record of *Rhynchosporium alismatis* on *Alisma lanceolatum* and *Damasonium minus*. Australas Plant. Pathol. 23:46–49.

Crump, N. S., E. J. Cother, and G. J. Ash. 1999. Clarifying the nomenclature in microbial weed control. Biocontrol Sci. Technol. 9:89–97.

Ferrero, A., M. Tabacchi, and F. Vidotto. 2002. Italian rice field weeds and their control. Pages 535–544 *in* J. E. Hill and B. Hardy, eds. Second Temperate Rice Conference. Proceedings of the Second Temperate Rice Conference, June 13–17, 1999; Sacremento, California, USA. Los Baños, Philippines: International Rice Research Institute.

Godwin, I. D., E.A.B. Aitken, and L. W. Smith. 1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis 18:1524–1528.

Gupta, M., Y.-S. Chyi, J. Romero-Severson, and J. L. Owen. 1994. Amplification of DNA markers from evolutionary diverse genomes using single primers of simple-sequence repeats. Theor. Appl. Genet. 89: 998–1006.

Hollingsworth, P. M., M. Tebbitt, K. J. Watson, and R. J. Gornall. 1998. Conservation genetics of an arctic species, *Saxifgra rivularis* L., in Britain. Bot. J. Linn. Soc. 128:1–14.

Jahromi, F. G., G. J. Ash, and E. J. Cother. 2002. Genetic variability identified in populations of the rice weed starfruit (*Damasonium minus*) by simple sequence repeats. Plant Prot. Q. 17:151–154.

Jahromi, F. J., E. J. Cother, and G. J. Ash. 2001. The use of a fungal pathogen to reduce weed competition in Australian rice. Page 345 *in* Proceedings of 13th Biennial Conference of the Australasian Plant Pathology Society, Cairns, Australia.

Kruskal, J. B. 1964. Multidimensional scaling by optimising goodness of fit to a nonmetric hypothesis. Psychometrika 29:1–27.

McIntyre, S. and M. R. Newnham. 1988. Distribution and spread of the Alismataceae in the rice-growing region of New South Wales. Cunninghamia 2:25–38.

Nagaoka, T. and Y. Ogihara. 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Appl. Genet. 94:597–602.

Nissen, S. J., R. A. Masters, D. J. Lee, and M. L. Rowe. 1995. DNA-based marker systems to determine genetic diversity of weedy species and their application to biocontrol. Weed Sci. 42:504–513.

O'Hanlon, P. C., R. Peakall, and D. T. Briese. 1999. Amplified fragment length polymorphism (AFLP) reveals introgression in weedy *Onopordum* thistles: hybridization and invasion. Mol. Ecol. 8:1239–1246.

Pogan, E. 1971. Karyological studies in a natural hybrid of *Alisma lanceolatum* With. × *Alisma plantago-aquatica* L. and its progeny. Genet. Polon. 12:219–222.

Román, B., Z. Staovic, D. Rubiales, A. M. Torres, J. I. Cubero, N. Katzir, and D. M. Joel. 2002. Variation among and within populations of the parasitic weed *Orbanche crenata* from Spain and Israel revealed by inter simple sequence repeat markers. Phytopathology 92:1262–1266.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Yeh, F. C., R.-C. Yang, T.B.J. Boyle, Z.-H. Ye, and J. X. Mao. 1997. POPGENE, the User-Friendly Shareware for Population Genetic Analysis. Edmonton, Canada: Molecular Biology and Biotechnology Centre, University of Alberta, Canada.

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