

MOLECULAR MARKER TOOLS FOR THE IDENTIFICATION OF WEEDY SPOROBOLUS SPECIES IN AUSTRALIA

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ABSTRACT

Nine species of Sporobolus having overlapping morphological characteristics have been included in 'Sporobolus indicus complex'. Five of these nine species are noxious weeds in various states of Australia. These species are major constraints to pasture production causing significant losses in the dairy and beef industries. In this study, a DNA-based molecular investigation was undertaken to help develop improved integrated management strategies for these noxious weeds. This study employed 40 Sporobolus seed collections coming from 14 species. Polymerase Chain Reaction (PCR) based Random Amplified Polymorphic DNA (RAPD) analysis generated a series of species-specific molecular markers that can be employed for the identification purpose. However, as the technique was highly sensitive to PCR reaction and cycling conditions, an alternative molecular approach, involving DNA sequencing of Internal Transcribed Spacers (ITS) of nuclear Ribosomal DNA (rDNA) repeat units was employed to develop a diagnostic tool for these species. The rDNA ITS sequencing approach could also be used for a phylogenetic study which revealed clear cut boundary between the weedy and the non-weedy species within the 'S. indicus complex'. In addition to the molecular phylogenetic study, PCR-Restriction Fragment Length Polymorphism (RFLP) approach was used to develop a diagnostic tool for the weedy species. Using this approach, single restriction enzyme (MvnI) was identified which could discriminate all five noxious weedy species from all others. The molecular genetic and phylogenetic information thus generated and the molecular diagnostic tool thus developed from this study can be effectively utilized for the integrated management of these noxious weeds in Australia.

Keywords: Giant rats tail grasses, Internal Transcribed Spacers (ITS), molecular diagnostics Random Amplified Polymorphic DNA (RAPD), Polymerase Chain Reaction (PCR),

INTRODUCTION

Weedy *Sporobolus* grasses (WSG) are the members of the '*Sporobolus indicus* complex', a cluster of nine closely related species

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that are to be found in Australia (Simon 1999) and with a pantropical distribution around the globe. Five of these species [*viz.* giant rats tail grasses (GRTGs) [*S. pyramidalis* P. Beauv and *S. natalensis* (Steud.) Dur. & Schinz.] Giant Parramatta grass (GPG) [*S. fertilis* (Steud.) Clayton], Parramatta grass (PG) [*S. africanus* (Poir.) Robyns & Tourney] and American Rat's tail grass (ARTG) [*S. jacquemontii* Kunth] are considered serious weeds of pastures in many of the locations (Ensby and Betts, 1998; Simon and Jacobs, 1999; DPIF, 2007). These five species are collectively known as the WSG (DPIF, 2007).

WSGs are now reported to infest an estimated 450,000 ha of grazing land in eastern Queensland and New South Wales. They grow in lower rainfall areas (600 mm annual rainfall areas), but are most prevalent in areas with an average annual rainfall of > 700 mm (DPIF, 2007). WSG are adapted to a wide range of Australian soil types and climatic conditions and have a potential to establish in areas receiving as little as 500 mm of annual rainfall. This makes over 60 % of Queensland (108 million ha), or 30 % of Australia (223 million ha) at risk to infestation.

Seeds of the WSG can be set throughout the year but most are produced during the warmer summer season. Seed production is high with up to 85 000 seeds being produced per m² in case of *S. pyramidalis* (Vogler and Bahnisch, 2002). Most seeds that enter the soil can remain viable for up to 10 years. A soil seed bank quickly develops and sizes of up to 20,000 seeds per m² can be present. GPG is also capable of producing up to 85,000 seeds per m² each year with an initial seed viability of 90%. It has been reported that a significant proportion of its seed can remain viable for up to 10 years in the soil (Queensland Government, 2011).

WSGs have been major threats to the dairy and beef industries as their infestations have dramatically reduced the economic viability of pastures leading to a lowering of land values and it has been estimated that current infestations are collectively costing the pastoral industry c. \$60 million per year in terms of lost production and control costs (DPIF, 2007). They become dominant grasses in many sown and native pastures and pose a serious threat to the viability of many rural industries. GPG is reported to cause losses in terms of carrying capacity and decreased production by as much as 80% (Queensland Government, 2011).

WSGs being invasive weeds and have the potential to spread to larger areas of Australia via different means, and therefore an integrated weed management strategy should be developed for them. So far, several research studies, focusing on biology and mode of transmission, have been carried out on various weedy *Sporobolus*

species (Andrews, 1995; Vogler and Bahnisch, 2002; Vogler and Bahnisch, 2006) and a number of weed management strategies have been devised for their management and include the use of chemical control (Andrews, 2009), mechanical and biological control (Yobo *et al.*, 2009; Queensland Government, 2011).

The present study aims to understand the genetic and phylogenetic relationships of the various members of the '*S. indicus* complex' and develop a robust molecular marker technique for the reliable identification of five major weedy *Sporobolus* species of Australia. Three different molecular marker techniques *viz.* 1) Polymerase Chain Reaction (PCR)-based Random Amplified Polymorphic DNA (RAPD) 2) PCR and DNA sequencing-based on Nuclear Ribosomal DNA (rDNA) Internal Transcribed Spacers (ITS) and 3) PCR-Restriction Fragment Length Polymorphism (RFLP) were used to achieve the overall objective.

MATERIALS AND METHODS

Plant Materials

Altogether 56 seed samples coming from 14 *Sporobolus* species were used for the entire study (Shrestha *et al.*, 2003; Shrestha *et al.*, 2005).

DNA Extraction

DNA extraction was carried out from seed tissue (10-50 mg of the caryopsis) using the technique described by Edwards *et al.* (1991). DNA estimation of the various samples was carried out using a spectrophotometric method (GeneQuant II, RNA/ DNA calculator, Amersham Pharmacia, Biotech, Australia).

Random Amplified Polymorphic DNA (Rapid) Based Study

A Polymerase Chain Reaction (PCR)-based RAPD technique (Williams *et al.*, 1990) was used for the generation of RAPD markers specific to the five major weedy species and for the study of inter and intra specific genetic diversity among the various *Sporobolus* species under study. The optimized RAPD-PCR reaction and cycling conditions used for the *Sporobolus* RAPD profiling and RAPD data analysis are those described in Shrestha *et al.*, (2005).

Phylogenetic Study Based On Nuclear rDNA ITS Sequences

Of the total 56 *Sporobolus* samples (from 14 species), 40 samples were used for ITS region sequencing. Two other species of Poaceae [*Heteropogon contortus* (L.) P. Beauv. Ex Roem & Schult and *Pennisetum alopecuroides* (L.) Spreng] were also sequenced as out group species for phylogenetic analysis.

For this phylogenetic study, same DNA samples extracted for the RAPD investigation were used. The PCR amplification of the entire ITS region, DNA sequencing, sequence analysis and phylogenetic analysis were carried out as mentioned in Shrestha *et al.* (2003).

Diagnostic Development Using PCR-RFLP-Based Marker Technique

The consensus sequences generated for the phylogenetic analysis were subjected to web cutter program (Web cutter program, 2000; <http://www.firstmarket.com/cutter/cut2.html>). Of the five restriction enzymes identified (*AccII*, *BstUI*, *Bsh1236I*, *MvnI* and *ThaI*.) from the restriction maps that could be used for the diagnostic purpose, *MvnI* was finally selected and used in restriction analysis involving all 14 species of *Sporobolus* under study. Validation of the PCR-RFLP assay was carried out on 23 randomly and blindly selected *Sporobolus* seed samples (Shrestha et al., 2010)

RESULTS

Identification of Weedy Species-Specific RAPD Markers

The important identification RAPD markers discovered were those of UBC 51₄₉₀ for *S. pyramidalis* and *S. natalensis*, UBC 43₃₁₀, UBC43₂₁₀₀ and UBC43₂₀₀₀ for *S. fertilis* and *S. africanus* and UBC 43₄₇₀ and OPA20₈₅₀ for *S. jacquemontii*. More importantly, the DNA fingerprint profile generated by a single primer UBC43 could also be used to positively identify all five major weedy *Sporobolus* species found in Australia (Shrestha et al., 2005).

Genetic Diversity and Relationship Based On RAPD Data

The RAPD similarity matrix generated using (the qualitative data) NTSYS pc program revealed the extent of the inter and intra-specific genetic diversity to be found among the various *Sporobolus* species under study (Shrestha et al., 2005).

In the phenograms generated from the Dice and Jaccard's coefficients, all the species of the '*S. indicus* complex' were found to group into one major cluster and the three Australian native species studied formed a second and distinct cluster (Shrestha et al., 2005).

ITS sequence-based Molecular phylogeny of '*S. indicus* complex'

A strict consensus tree of 64 Maximally Parsimonious Trees (MPTs) separated the various species under study into two major clades, one clade comprising of the species of '*S. indicus* complex' (11 species) and another one comprising the three native species. Separation of species into these two major clades was supported by an absolute level of confidence (100 % bootstrap values). Weedy and the non-weedy species of the complex formed different clades in the cladogram (Figure1.) (Shrestha et al. 2003).

Molecular Diagnostic Development Based on ITS-RFLP

The ITS sequences generated for the phylogenetic study were successfully utilized for the development of a molecular tool diagnostic based on an ITS PCR-RFLP approach (Shrestha et al., 2010). Single

restriction enzyme *MvnI* could discriminate between the weedy and the non-weedy species of the complex (Plate 1). When tested on 23 randomly selected seed samples, identification was accomplished with 100 % accuracy.

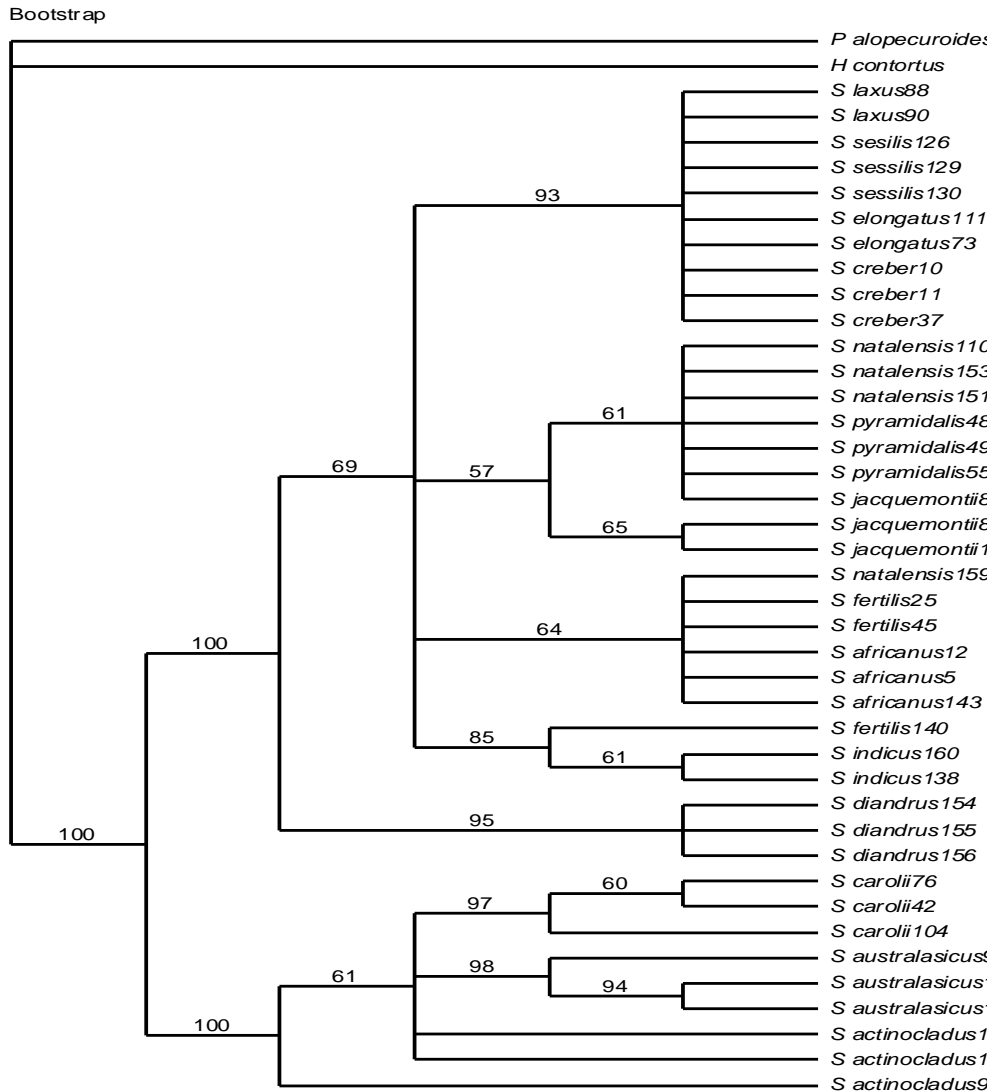


Figure 1. Bootstrap consensus tree generated from maximum parsimony analysis using PAUP. Bootstrapping performed with 1000 replicates. Values above nodes indicate bootstrap values.

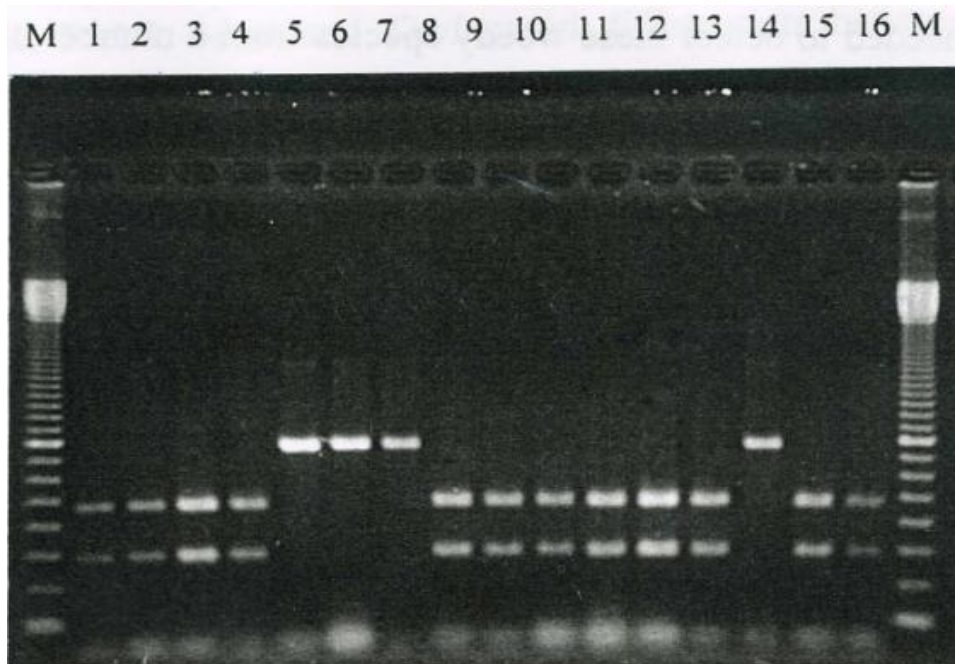


Plate 1. The restriction digestion pattern of the ITS fragments of 16 blindly selected samples with *MvnI*. The digested samples were those that were members of the major weedy species. Lanes marked M, are the 100 bp ladder molecular weight markers. Note that the ITS fragment of the non-weedy species are not restricted.

DISCUSSION

When naturally occurring protein-based and DNA-based molecular markers are interpreted as genealogical markers, they offer extraordinary power to illuminate such topics as human forensics, disease diagnostics, species diagnostics, wild life forensics, reproductive modes, population structure, intra-specific phylogeography, phylogeny, taxonomy, systematic, conservation biology etc. (Jagouix *et al.*, 1994; Hocquellet *et al.*, 1999; Ortiz-Diaz and Culham, 2000; Li *et al.*, 2002; Shrestha *et al.*, 2003; Avise, 2004; Shrestha *et al.*, 2005; Yashuda *et al.*, 2007; Shrestha *et al.*, 2010).

In the present investigation, three molecular marker approaches have been employed in order to understand the genetic diversity and phylogenetic relationship pertinent to weedy *Sporobolus* species of Australia as well as to identify the five noxious weedy species from all natives. Our RAPD-based study successfully generated five weedy species-specific markers as well as DNA fingerprint profiles

that can be used for a similar diagnostic purpose (Shrestha, 2002; Shrestha *et al.*, 2005). Fingerprint profile generated by a single primer UBC 43 could discriminate all five noxious weedy *Sporobolus* species.

Genetic diversity studies at the molecular level have great implications for formulating weed management strategies (Lopez-Martinez *et al.*, 1999). The present investigation has put forth the inter and intra specific genetic diversity estimates and relationship among nine species of the complex based on RAPD qualitative data. Although carefully optimized RAPD technique have been used widely in various biological disciplines for genetic diversity studies, this technique is very sensitive to the PCR reaction and cycling conditions, and therefore its reproducibility has to be checked across laboratories using different PCR machines and reagents.

The ITS phylogenetic study revealed the '*S. indicus* complex' to be a monophyletic group, which is consistent with the study of Ortiz-Diaz and Culham (2000). The way in which species of the complex cluster, producing a monophyletic group and then sub-divide into various sub-clades is similar to that seen in the RAPD phenograms using RAPD data in which several species can be better resolved (Shrestha *et al.*, 2003).

Our ITS sequence based phylogenetic study has not only generated the inherent phylogenetic relationship within the '*S. indicus* complex' but has also resulted into a robust molecular diagnostic tool that can be employed for the positive identification of the five major WSGs. Whenever it is felt that these weeds are escaping identification (whether based on gross morphology of plants or based on seed morphology) and this has resulted in the rapid spread of these weeds in Australia, the molecular diagnostic tool developed can now be used to help prevent this.

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REFERENCES CITED

Andrews, T.S. 1995. Dispersal of seeds of giant *Sporobolus* spp. After ingestion by grazing cattle. *Aus. J. Exp. Agric.* 35: 353-356.

- Andrews, T.S. 2009. Preventing giant Parramatta grass [*Sporobolus fertilis* (Steud.) Clayton] reinfestation with reduced rates of fluoroanate herbicide. *Plant Protec. Quart.* 24(3):118-119.
- Avise, J.C. 2004. *Molecular markers natural history and evolution*. Second Edition. Sinauer Associates, Inc. Publishers Sunderland, Massachusetts.
- DPIF. 2007. *Weedy Sporobolus grasses*. Best Practice Manual. The State of Queensland, Department of Primary Industries and Fisheries.
- Edwards, K., C. Johnstone and C. Thompson. 1991. A simple and rapid method for preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* 19: 1349.
- Ensby, R. and J. Betts. 1998. Giant Rat's Tail Grass identification and control. *Agnote*. NSW Agriculture publication, NSW.
- Hocquellet, A., P. Toorawa, J.M. Bove and M. Garnier. 1999. Detection and identification of the two *Candidatus Liberibacter* species associated with citrus Huanglongbing by PCR amplification of ribosomal protein genes of the β operon. *Molec. Cell. Prob.* 13: 373-379.
- Jagoueix, S., J.M. Bove and M. Garnier. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the Proteobacteria. *Int. J. Syst. Bact.* 44 (3): 379-386.
- Li, X., X. Ding, B. Chu, G. Ding, S. Gu, L. Qian, Y. Wang, and Q. Zhou. 2007. Molecular authentication of *Alisma orientale* by PCR-RFLP and ARMS. *Planta Medica* 73(1): 67-70.
- Lopez-Martinez, N., A.P. Salva, R.P. Finch, and R. de Prado. 1999. Molecular markers indicate intraspecific variation in the control of *Echonocloa* spp. with quinclorac. *Weed Sci.* 47:310-315.
- Ortiz-Diaz, J. And A. Culham. 2000. Phylogenetic relationship of the genus *Sporobolus* (Poaceae: Eragrostideae) based upon nrDNA ITS sequences. *In: Jacobs, S. W. L. and Everett, J. (Eds.). Grass Systematic and Evolution. Proceedings of Third International Symposium on Grass Systematics and Evolution, 1998.* CSIRO Publishing, Sydney, NSW. pp. 184-188.
- Queensland Government 2011. American rat's tail grass. QLD government primary industries and Fisheries. <http://www.dpi.qld.gov.au>.
- Queensland Government 2011. Giant Parramatta grass. QLD government primary industries and Fisheries. <http://www.dpi.qld.gov.au>
- Shrestha, S. 2002. *Molecular systematics of weedy Sporobolus species of Australia*. Thesis submitted for the degree of Doctor of

- Philosophy (Ph. D.) in the School of Land and Food Sciences, University of Queensland, Australia.
- Shrestha, S., S.W. Adkins, G.C. Graham and D.S. Loch. 2003. Phylogeny of the '*Sporobolus indicus* complex' based on Internal Transcribed Spacers (ITS) sequences. *Aus. Syst. Bot.* 16: 165-176.
- Shrestha, S., Adkins, S.W., Graham, G.C. and Loch, D.S. (2005). An identification tool for the Australian weedy *Sporobolus* species based on random amplified polymorphic DNA (RAPD) profiles. *Aus. J. Agric. Res.* 56(2): 157-167.
- Shrestha, S., G.C. Graham, D.S. Loch and S.W. Adkins. 2010. Molecular Identification of weedy *Sporobolus* species by PCR-RFLP. *Weed Res.* 50:383-387
- Simon B.K. 1999. Taxonomy of the '*Sporobolus indicus* complex'. In: Proceedings of the Workshop 'Weedy *Sporobolus* Grasses, Research Update'. Gympie, 19th November, 1999. pp. 41-47. Published by Queensland Department of Primary Industries (QDPI).
- Simon, B.K. and S.W.L. Jacobs. 1999. Revision of the genus *Sporobolus* (Poaceae, Chloridoideae) in Australia. *Aus. Syst. Bot.* 12: 375-448.
- Vogler, W.D and L.M. Bahnisch. 2002. The seed ecology of giant rats tail grass: implication for management. In 'Proceedings of the 7th Queensland weed symposium'. (The Weed Society of Queensland: Brisbane).
- Vogler, W. D. and L. M. Bahnisch. 2006. Effect of growing site, moisture stress, and seed size on viability and dormancy of *Sporobolus pyramidalis* (giant rats tail grass) seed. *Aus. J. Exp. Agric.* 46: 1473-1479.
- Webcutter program (2000). <http://www.firstmarket.com/cutter/cut2.html>
- Williams, J.G.K, A.R. Kubelic, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA Polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.
- Yasuda, K., A. Yano, Y. Nakayama and H. Yamaguchi. 2002. Molecular identification of *Echinochloa oryzicola* Vasing. and *E. crus-galli* (L.) Beauv. using a polymerase chain reaction-restriction fragment length polymorphism technique. *Weed Bio. and Manag.* 2: 11-17.
- Yobo, K.S., M.D. Liang, W.A. Palmer and R.G. Shivas. 2009. Evaluation of *Ustilago sporoboli-indici* as a classical bio-control agent for invasive *Sporobolus* grasses in *Australia*. *Bio. control* 50:7-12.