

Genetic relationship between glyphosate resistant annual ryegrass (*Lolium rigidum* Gaud.) populations from northern New South Wales

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Summary A number of annual ryegrass (*Lolium rigidum* Gaud.) populations on or near the Liverpool Plains of New South Wales have been identified as being resistant to glyphosate. The evolution of glyphosate resistance is rare and the occurrence of multiple populations in one geographic area has two potential explanations.

Firstly, the farming practices employed in the paddocks where resistance has occurred are similar, and these practices would be considered to be at high risk of developing resistance.

Alternatively, movement of seed through environmental or mechanical means has resulted in multiple populations arising from a few original populations that evolved resistance.

DNA samples were obtained from ryegrass populations from northern New South Wales known to be resistant to glyphosate, as well as several glyphosate susceptible populations. Random amplified polymorphic DNA (RAPD) techniques were used to compare the genetic similarity of the populations.

Results indicate that the samples from within each property generally clustered together. Two of the properties appear to be more closely related to each other than to the third property, despite this not correlating to the geographic relationship of the properties. This suggested that there have been two occurrences of evolution of resistance and that seed movement, rather than previous weed management history, is a possible cause of some of the glyphosate resistant populations.

General farm hygiene and use of integrated weed management practices can reduce the spread of herbicide resistance. When herbicide resistance is known or suspected, it is important to manage the paddock to prevent the resistance moving to other paddocks or properties.

Keywords Glyphosate, resistance, annual ryegrass, RAPD, PCR, genotype.

INTRODUCTION

Occurrences of resistance to glyphosate, the most widely used non-selective herbicide, in annual ryegrass (*Lolium rigidum* Gaud.) populations were first reported in the mid 1990s (Pratley *et al.* 1996,

Powles *et al.* 1998), with further populations having been identified since.

Due to the high level of dependence upon herbicides for weed control, the increasing number of instances of herbicide resistant populations is of particular concern.

A range of techniques is available for undertaking studies of genetic variations between plant populations. Genetic diversity has been shown to be detectable using sophisticated techniques such as simple sequence repeats (SSRs) with canola (Charters *et al.* 1996, Szewc-McFadden *et al.* 1996), wheat (Plashke *et al.* 1995), soybean (Morgante *et al.* 1994, Rongwen *et al.* 1995) and intersimple sequence repeats (ISSRs) with saffron thistle (*Carthamus lanatus* L.) (Ash *et al.* 2003).

Genetic diversity within both crop cultivars and weed species has also been distinguished using simpler techniques such as random amplified polymorphic DNA (RAPD) techniques in *Olea europaea* L. (Belaj *et al.* 2002) and *Capsella bursa-pastoris* (L.) Medik. (Yang *et al.* 1998). This technique has also been used to investigate the introduction pattern of *Crupina vulgaris* (Pers.) Cass. into North America (Garnatje *et al.* 2002).

While SSR markers are not always the most appropriate for phylogenetic studies (Rossetto *et al.* 2002), they have been used to distinguish *L. perenne* L. cultivars (Kubik *et al.* 2001). *L. perenne* cultivars have also been separated using amplified fragment length polymorphism (AFLP) analysis (Guthridge *et al.* 2001) and RAPD markers with as few as four primers (Sweeney and Denneberger 1994, Sweeney and Denneberger 1997).

The purpose of this study was to undertake a preliminary genetic investigation using RAPD markers to determine the genetic relationship between annual ryegrass populations from the Liverpool Plains region of New South Wales (NSW).

MATERIALS AND METHODS

Accessions of ryegrass seed were obtained from two sources. Glyphosate resistant biotypes were obtained from three properties on the Liverpool Plains, NSW (GLR034-GLR037, GLR038-GLR039, and GLR040-GLR041). The Charles Sturt University commercial

herbicide resistance testing service provided a susceptible biotype (20052), also from the Liverpool Plains.

Trays of moist 1:1 sand:peat mixture were established in a glasshouse and 0.2 g of seeds from each accession were germinated and grown to the three leaf stage. Glyphosate resistant populations were treated with 612 g a.i. ha⁻¹ glyphosate to remove susceptible seedlings. Bulk material for DNA analysis was collected from twenty seedlings from each population. All samples were wrapped in aluminium foil and snap frozen using liquid nitrogen prior to being stored at -80°C.

Samples were ground under liquid nitrogen prior to DNA being extracted from 1 g ground material using DNEasy maxikits (Qiagen).

Random amplified polymorphic DNA (RAPD) reactions were performed in 25 µL volumes containing 18.5 µL sterile distilled water, 2.5 µL 10X loading buffer, 2.0 µL 10mM MgCl₂, 0.5 µL deoxy-ribonucleoside tri-phosphate (DNTP), 0.5 µL Primer, 0.5 µL DNA and 0.5 µL Taq polymerase (Promega). Negative controls that contained sterile distilled water in place of DNA samples were included.

Amplification was performed using a Hybaid Sprint polymer chain reaction (PCR) programmed for forty five cycles of denaturation at 94°C for one minute, annealing at 36°C for one minute and extension at 72°C for one minute. A final extension at 72°C for ten minutes was used prior to samples being held at 4°C until removal.

Thirty two primers were screened using two to four arbitrarily chosen DNA samples. Five primers that showed polymorphic banding patterns were chosen for subsequent analysis.

Five microlitres of 6 X loading dye was added to each sample prior to electrophoresis. Three additional samples containing 2µ100 bp DNA ladder (Promega), with 11 bands from 100 bp to 1500 bp, were also run on each gel.

Electrophoresis was conducted on 3% agarose gels in 0.5% Tris-borate-EDTA (TBE) buffer containing 0.0025% ethidium bromide. (Gels were run for approximately five hours at 100 mA until the marker dye had travelled approximately 10 cm.

Gels were imaged under ultraviolet light using GeneSnap (Syngene) software. Images were imported into GeneTools (Syngene) software to assign molecular weights to the 100 bp ladders and to identify bands within each DNA sample.

Statistical analysis Analysis of the images was conducted by two methods. In the first method, data from GeneTools were imported into GeneDirectory (Syngene) and all lane identification information entered.

Molecular weight was analysed using the Dice (Nei and Li 1979) and Jaccard coefficients within GeneDirectory to generate similarity matrices that were exported to NTSYSpc version 2.11S (Exeter software) (Rohlf 1997) for further analysis.

The second method used was to score the bands manually, one for presence and zero for absence at positions, to generate rectangular binary matrices. Similarity matrices were obtained from the binary matrices using the similarity for qualitative (nominal) data (SIMQUAL) routine within NTSYSpc.

Similarity matrices derived by both methods were analysed separately using NTSYSpc. The sequential, agglomerative, hierarchical, nested cluster analysis (SAHN) routine was used to compile unweighted pair group method with arithmetic averaging (UPGMA) dendrograms.

The Mantel test statistic, *Z*, and the product-moment correlation, *r*, were determined by comparing the similarity and cophenetic value matrices using the matrix comparison function in NTSYSpc. These statistics were used to select the clustering with the best fit.

RESULTS

Use of similarity matrices derived from molecular weights produced lower coefficients of similarity than use of similarity matrices derived from manual scoring.

Initial analysis using matrices based on molecular weights showed significant clustering ($r = 0.893$), with the susceptible biotype being markedly dissimilar to the resistant biotypes (Figure 1). The clusters indicate that populations from the second and third properties are more closely related to each other than they are to the first property.

Matrices derived from manually scoring of bands indicated significant differences between the populations ($r = 0.741$), however the clusters did not correlate as well to the three properties (Figure 2). The third property continued to reveal dissimilarity to the other two properties, suggesting that there may have been movement of glyphosate resistant ryegrass between the first and second properties.

DISCUSSION

Bulked samples were used to gain a representative measure of the genetics of each of the populations. Studies using *L. multiflorum* Lam. have shown that genetic diversity of bulk population samples correlates with that obtained from individual samples (Johnson 1998, Guthridge *et al.* 2001). Whilst rare genes are not as easily detected in bulk samples (Sweeney and Denneberger 1994), the selection of resistant plants from within the resistant populations should have ensured an

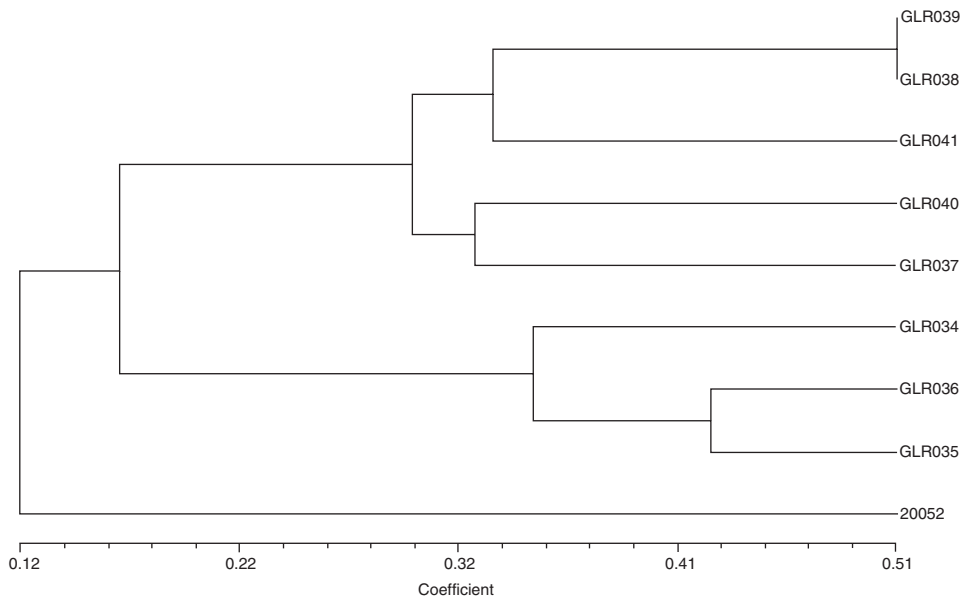


Figure 1. Dendrogram showing genetic relationship of glyphosate resistant *L. rigidum* biotypes from the Liverpool Plains using RAPDs and similarity matrices based on molecular weights.

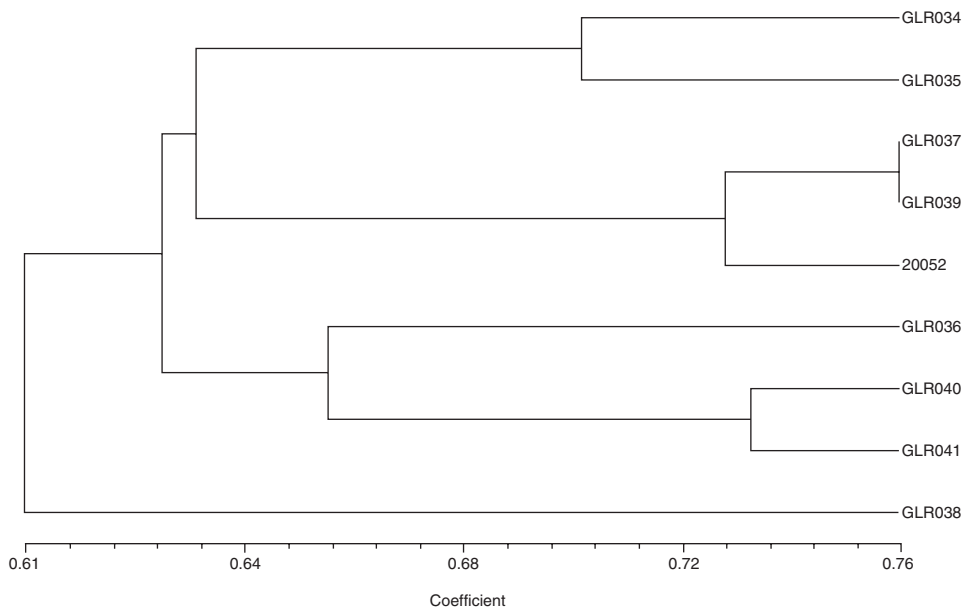


Figure 2. Dendrogram showing genetic relationship of glyphosate resistant *L. rigidum* biotypes from the Liverpool Plains using RAPDs and similarity matrices based on manually scored bands.

adequate level of resistant genetic material to discern differences between populations. However, it would be useful to gain an understanding of the diversity within

the resistant populations to allow clearer conclusions to be drawn regarding the diversity between resistant populations.

A close genetic relationship between glyphosate resistant populations has several management implications. Resistant seed can be spread within and between properties via machinery, contaminated fodder or grain, or through livestock. Management practices need to be followed that minimise the risk of seed transport via these methods. Natural seed movement as a result of wind or water can not be as easily controlled. In these situations, the weed management strategies being used need to be sufficiently diverse to allow any resistant seedling to be controlled to prevent seed set and establishment of a new resistant population.

This preliminary study has indicated that there may be strong genetic similarity between some of the reported glyphosate resistant populations. However, further work needs to be done to fully understand the linkages between resistant populations. The use of more sophisticated techniques, such as SSR or AFLP analysis, may provide a better understanding of the genetic relationships.

ACKNOWLEDGMENTS

The authors would like to thank Monsanto for providing the funds to undertake this work.

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