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Genetic diversity of glyphosate resistant *Echinochloa colona* in Australia

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ABSTRACT

Echinochloa colona (L) Link. is a problematic annual grass weed in summer fallows in northern Australian cropping regions where repeated use of glyphosate has resulted in the evolution of glyphosate resistance. Pot trials conducted on *E. colona* populations collected from northern Australia identified 34 glyphosate resistant populations out of 65 populations tested, with resistance levels varying between 2 and 11-fold. The technique of AFLP (amplified fragment length polymorphism) was used to investigate genetic diversity within and between two resistant and one susceptible population. Within these three populations, a total of 354 fragments were identified with 99.2% being polymorphic. The frequency of polymorphic fragments within the 30 individuals from each of the two resistant populations (81.0 and 83.9%) was similar to the susceptible population (80.8%), suggesting no apparent selection bottleneck associated with resistance evolution. The large genetic diversity present within populations suggests a significant level of outcrossing between individuals. A high level of genetic diversity among the individuals was identified across a single individual examined from each of 62 populations. These individuals clustered into four main groups with three isolated accessions. Individuals did not cluster geographically; additionally, individuals did not cluster by resistance or susceptibility to glyphosate either. The results of this study suggest glyphosate resistance evolved independently across a wide geographical region in northern Australia and the large genetic diversity within populations likely contributed to rapid resistance evolution.

Introduction

Echinochloa colona (L.) Link (junglerice) is an annual weed commonly occurring in paddy fields throughout rice-growing regions around the world (Mooney and Hobbs, 2000). It is considered an important grass weed in agriculture worldwide, due to its ability to compete with major agricultural crops (Holm et al. 1977). *E. colona* is distributed throughout agricultural areas in the tropics, especially in Asia, Australia, Pacific Islands, South America and the Caribbean, and is native

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to India (Holm et al. 1977). *E. colona* is widely distributed across Australia, with the exception of the arid west and Tasmania (Friend, 1983). However, it is most common in the grain-growing areas and summer fallows of northern Australia (Rew et al. 2005; Osten et al. 2007). *E. colona* is the most troublesome grass weed for many crops in central and southern Queensland (QLD), as well as in central and northern New South Wales (NSW) (Osten et al. 2007).

Glyphosate has been the main means of managing *E. colona* in fallows in northern Australia. This herbicide was introduced to world agriculture in 1974 (Duke et al. 2003) and is now the world's most widely used herbicide. However, the intensive use of herbicides, including glyphosate, has resulted in the evolution of herbicide resistance in weed species (Norsworthy et al. 1998). At present, 43 weed species worldwide have been reported as glyphosate resistant. In addition to resistance to glyphosate, *E. colona* has evolved resistance to another six herbicide modes of action (Heap, 2019). Herbicide resistance can spread by dispersal of pollen, seed or other propagules (Thill and Mallory-Smith, 1997; Christoffers, 1999; Delye et al. 2010). Factors contributing to dispersal include wind, water, animals or machinery (Benvenuti, 2007). Herbicide resistance can increase the costs and difficulty of managing weeds, as well as reducing crop productivity (Orson, 1999; Danquah et al. 2002; Beltran et al. 2012). Knowledge of how herbicide resistance is spread can allow better decisions to be made with respect to managing resistance (Thill and Mallory-Smith, 1997; Llewellyn and Allen, 2006). For example, glyphosate resistant genotypes of common waterhemp (*Amaranthus rudis*) in Missouri have spread rapidly over an area of 503 ha, possibly through pollen movement (Legleiter and Bradley, 2008). Genetic variability in weed populations occurs through variation in individual genotypes within a weed population. The frequency of individual genotypes within a population changes when weed populations are subjected to a changing selection pressure, such as the repeated use of herbicides. The proportion of susceptible to resistant genotypes declines under herbicide selection (Jasieniuk et al. 1996). Genetic studies can help better understand of the evolution and adaptation of weed populations. Ruiz-Santaella et al. (2006) used RAPD markers to analyse genetic variability of six *Echinochloa* species and observed that genotypes of the same species were similar. It was also found that *E. colona* is more closely related to *Echinochloa crus-galli* and *Echinochloa utilis* than other *Echinochloa* species (Ruiz-Santaella et al. 2006).

The objectives of the current study were to investigate suspected glyphosate resistant *E. colona* populations from NSW and QLD in Australia, and to evaluate genetic variability of populations collected from different locations in these two states to understand the potential spread of glyphosate resistance in this weed species.

Materials and Methods

Plant material

Seeds of suspected glyphosate resistant *E. colona* were obtained from surviving plants in summer fallow fields in dryland cropping regions of QLD and NSW (Figure 1) during 2008 to 2011. All 65 populations were confirmed as *E. colona* through floral and morphological characteristics described in taxonomic keys by Michael (1983). The seeds were treated with 95% H_2SO_4 for 30 minutes, rinsed under running water for 60 minutes and germinated on 0.6% (w/v) agar in an environmentally controlled cabinet with 12h light/dark periods at 22°C with 30 µmol $m^{-2}s^{-1}$ during the light period. Seedlings at the one leaf stage were transplanted into 8.5 by 9.5 by 9.5 cm pots (Masrac Plastics Pty Ltd., South Australia) containing standard potting mix, with nine seedlings per pot, and transferred to a growth room set at 25/23°C day/night temperature and a 12-h photoperiod at 553 µmol $m^{-2}s^{-1}$.

Figure 1. Geographical sites of towns where are nearest to origins of 65 *E. colona* populations used in this study (in Table 1). The numbers adjacent to the location names (in parentheses) are the quantity of resistant (R) and susceptible (S) populations.

Response to glyphosate

Twenty- seven seedlings from each of all populations were tested for resistance to glyphosate (Roundup PowerMax®, Nufarm Australia Limited) at a single discriminating dose of 270 g a.e. ha^{-1}

to determine whether they were resistant to glyphosate. Subsequently, a dose response experiment was conducted on ten resistant and one susceptible population chosen from those initially tested. These populations: 12, 15, 23, 31, 33, 38, 41, 45, 51, 52, 53 (Table 1), exhibited marked differences in response to glyphosate among populations in the initial test. Glyphosate was applied at rates of 0, 270, 540, 1080 and 2160 g a.e. ha^{-1}, with three replicates for each rate (nine seedlings per replicate). Non-ionic surfactant (alcohol alkoxylate, BS 1000, Crop Care) at 0.2% (v/v) was added to the glyphosate solution. The glyphosate application was carried out using a moving-boom laboratory twin nozzle sprayer (Hardi ISO F-110-01 standard flat fan, Hardi, Adelaide) placed 40 cm above the seedlings with a water volume of 109.6 L ha^{-1}, a pressure of 250 kPa and a boom speed of one m s^{-1}. There were three replicates of nine plants for each herbicide rate. Survival was assessed 21 days after glyphosate application, with plants having new green leaf tissue considered survivors.

Mortality data were analysed using PriProbit ver. 1.63 (Sakuma, 1998) to determine the relationship of glyphosate dose to number of survivors. LD_{50} (dose required to control 50% of individuals in a population) estimates generated from the Probit analysis were used to calculate the resistance index (resistance/susceptibility - R/S) to compare the resistance level of populations.

Table 1. Location and resistance phenotype of *E. colona* populations used in this study

Population number	Location	Phenotype	Population number	Location	Phenotype
1	Bellata, NSW	Resistant	34	North Star, NSW	Resistant
2	Bellata, NSW	Resistant	35	North Star, NSW	Resistant
3	Bellata, NSW	Resistant	36	North Star, NSW	Susceptible
4	Bellata, NSW	Resistant	37	North Star, NSW	Susceptible
5	Bellata, NSW	Resistant	38	North Star, NSW	Resistant
6	Bellata, NSW	Resistant	39	North Star, NSW	Susceptible
7	Boggabilla, NSW	Susceptible	40	North Star, NSW	Susceptible
8	Coonamble, NSW	Resistant	41	Tamworth, NSW	Susceptible
9	Croppa Creek, NSW	Susceptible	42	Yallaroi, NSW	Susceptible
10	Croppa Creek, NSW	Susceptible	43	Dalby, QLD	Susceptible
11	Croppa Creek, NSW	Susceptible	44	Glenmorgan, QLD	Resistant
12	Croppa Creek, NSW	Resistant	45	Glenmorgan, QLD	Resistant
13	Croppa Creek, NSW	Susceptible	46	Goondiwindi, QLD	Susceptible
14	Dubbo, NSW	Susceptible	47	Goondiwindi, QLD	Resistant
15	Garah, NSW	Resistant	48	Goondiwindi, QLD	Susceptible

Population number	Location	Phenotype	Population number	Location	Phenotype
16	Gilgandra, NSW	Susceptible	49	Goondiwindi, QLD	Susceptible
17	Gurley, NSW	Susceptible	50	Goondiwindi, QLD	Resistant
18	Moree, NSW	Susceptible	51	Goondiwindi, QLD	Resistant
19	Moree, NSW	Resistant	52	Goondiwindi, QLD	Resistant
20	Moree, NSW	Susceptible	53	Goondiwindi, QLD	Resistant
21	Moree, NSW	Susceptible	54	Goondiwindi, QLD	Susceptible
22	Moree, NSW	Susceptible	55	Goondiwindi, QLD	Resistant
23	Moree, NSW	Resistant	56	Meandarra, QLD	Susceptible
24	Moree, NSW	Resistant	57	Meandarra, QLD	Susceptible
25	Moree, NSW	Susceptible	58	Meandarra, QLD	Resistant
26	Moree, NSW	Resistant	59	Millmerran, QLD	Resistant
27	Moree, NSW	Susceptible	60	Moonie, QLD	Resistant
28	Moree, NSW	Susceptible	61	Pittsworth, QLD	Resistant
29	Moree, NSW	Susceptible	62	Pittsworth, QLD	Resistant
30	Moree, NSW	Resistant	63	Coonamble, NSW	Resistant
31	North Star, NSW	Resistant	64	Croppa Creek, NSW	Resistant
32	North Star, NSW	Susceptible	65	Moree, NSW	Susceptible
33	North Star, NSW	Resistant			

NSW: New South Wales, QLD: Queensland.

AFLP (amplified fragment length polymorphism) analysis

To examine genetic diversity within and between populations, DNA was extracted from young green leaf tissue of 30 individuals representing 30 plants sampled in each from 3 populations of *E. colona*, two resistant populations (63 and 64) and one susceptible population (65), using the DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions. In addition, the overall variation across populations of *E. colona* was examined taking DNA from one individual from each of 62 populations (populations 1 to 62 in Table 1) using the same method. In this case, leaf tissue was sampled prior to applying glyphosate at 270 g a.e. ha^{-1} and the survival of sampled individuals was determined after herbicide treatment.

The AFLP technique described by Vos et al. (1995) with minor modifications was used to investigate the genetic diversity within and between populations of *E. colona*. Adaptors were first prepared by adding 50 µM of each *Mse*I adaptor and 5 µM each *Pst*I adaptor and heating for 3 minutes at 90°C followed by cooling at room temperature for 30 minutes. Genomic DNA (120 ng) was then digested and adaptors ligated in a single reaction as follows: *Pst*I (10 units) and *Mse*I (2.5

units) restriction enzymes, adaptors (0.08 µM *Pst*I and 0.83 µM *Mse*I), 1× RL Buffer (50 mM Tris-HCl at pH 7.5, 50 mM Mg-acetate, 250 mM K-acetate and 25 mM DTT), 0.2 mM ATP Cofactor and 1 unit of T4 DNA ligase enzyme were combined in a final volume of 60 µl. This reaction was incubated at 37^{o}C for three hours. Initial PCR amplification was conducted in a volume of 25 µl including 5.5 µl digested DNA, 0.4 µM each of *Pst*I + A and *Mse*I + C primers (A and C were selective nucleotides joined to the 3'-end of adaptors), 1× ImmoBuffer [160 mM $(NH_4)_2SO_4$, 1M Tris-HCl pH 8.3, 0.1% Tween-20], 2 mM $MgCl_2$, 1.6 mM dNTPs and 1 unit of *Taq* Immolase™. The amplification was performed by an automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany) with cycle parameters as follows: an initial denaturing step of 95^{o}C for 10 minutes, followed by 21 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 60^{o}C for 1 minute and extension at 72^{o}C for 1 minute.

The initial PCR reaction was subsequently diluted with nanopure water at a ratio of 1:6.4. Selective PCR amplification was then carried out using 5.5 µl of the diluted initial PCR reaction as template. Duplicate reactions were performed, both using *Pst*I + AGC primer (0.4 µM) but differing in the *Mse*I selective primers (0.4 µM): either *Mse*I + CAA (Fluro-VIC) or *Mse*I + CAT (Fluro-FAM). The *Mse*I primers at this step were fluorescently labelled. The other components of the reaction were the same as that in the initial PCR. The following thermal cycles were applied: an initial denaturation for 10 minutes at 95^{o}C, followed by cycling of denaturation at 94^{o}C for 30 s, annealing at 65^{o}C for 30 s and extension at 72^{o}C for 90 s, with a decrease in the annealing temperature by 1^{o}C each cycle until 56^{o}C was reached. This was then followed by 24 cycles with denaturation at 94^{o}C for 30 s, annealing at 56^{o}C for 30 s and extension at 72^{o}C for 90 s. PCR products were analysed basing on capillary electrophoresis using an Applied Biosystems 3730, fluorescence-based DNA analyser by the Australian Genome Research Facility (AGRF), Australia using GeneScan™ 500 ROX™ dye Size Standard (ThermoFisher Scientific, Scoresby, Vic).

AFLP data were viewed and edited using GeneMapper® software ver. 4.0 (Applied Biosystems, USA) and informative peaks were recorded as a binary data set with 0 for absence and 1 for presence at each locus. The binary data were used to analyse the genetic relationships, and similarity and distance matrices were computed with Jaccard's coefficient using DendroUPGMA, a dendrogram construction utility (Garcia-Vallve et al. 1999). The phenograms were displayed using TreeView software ver. 1.6.6 (Page, 1996).

Results and Discussion

Response to glyphosate

Of the 65 *E. colona* populations collected from northern Australia that were treated with glyphosate at 270 g a.e. ha^{-1} to determine resistance status, 34 populations had greater than 20% survival and were classified as resistant to glyphosate (Table 1). These comprised 13 populations collected from QLD and 21 from NSW (Figure 1). The remaining 31 populations were considered susceptible to glyphosate.

The results of the dose-response experiment showed a range of responses to glyphosate. The one susceptible population (41) was easily controlled with glyphosate and had an LD_{50} (concentration of glyphosate required to kill 50% of the population) of 110 g a.e. ha^{-1}, well below the normal use rate of this herbicide (Table 2). The LD_{50} of the other populations tested ranged from 234 to 1289 g a.e. ha^{-1} making them 2 to 11-fold resistant to glyphosate compared with the susceptible population. The most resistant population (23) was from Moree in NSW, whereas the next most resistant population (45) was from Glenmorgan in QLD (Figure 1). The level of resistance to glyphosate in *E. colona* is variable among the populations tested.

Table 2. Glyphosate resistance levels in ten *E. colona* populations. LD_{50} values (dose required to kill 50% of individuals in a population) are presented along with a resistance index (R/S) calculated by dividing the LD_{50} of each resistant population with the LD_{50} of susceptible population 41.

Population	LD_{50} (g a.e. ha^{-1})	R/S
23 (R)	1289	11.6
45 (R)	1069	9.7
33 (R)	867	7.8
31 (R)	635	5.7
51 (R)	595	5.4
53 (R)	508	4.6
52 (R)	392	3.5
15 (R)	279	2.5
12 (R)	263	2.4
38 (R)	234	2.1
41 (S)	110	-

Widespread resistance to glyphosate in *E. colona* was identified in the grain-growing regions of NSW and QLD in Australia. In this region, summer fallows are typically treated with herbicides

several times a season to control weeds and conserve summer moisture. Glyphosate is the most commonly used herbicide for this purpose (Osten et al. 2007). Resistance to glyphosate was first reported in *E. colona* in NSW in 2008 (Storrie et al. 2008). The present study has confirmed that resistance is now present in at least 34 populations in NSW and QLD. Recently, glyphosate resistance was also reported in a population of *E. colona* from Western Australia (Gaines et al. 2012), more than 2000 km from the locations in NSW and QLD.

The ten glyphosate resistant populations chosen for full dose response experiments showed varying levels of resistance to glyphosate (Table 2). There are a number of mechanisms known to confer resistance to glyphosate (Shaner et al. 2012), and the different mechanisms result in different levels of resistance (Preston et al. 2009). While the resistance mechanisms present within the populations of *E. colona* are not known, the different responses to glyphosate suggest populations have different resistance alleles.

Genetic diversity across E. colona in the surveyed region

To assess genetic diversity across the *E. colona* samples collected and to test whether resistant populations may have originated from a small number of sources, AFLPs arising from the two primer combinations were used to analyse polymorphisms in a single individual from 62 populations. A total of 70 fragments ranging in length from 45 to 300 bp were reliably detected by the two primer combinations in this experiment (Table 3). The primer combination of *Pst*I with *Mse*I + CAT produced 49 fragments and all fragments were polymorphic across the populations. The primer pair *Mse*I + CAA produced 21 fragments and again all fragments were polymorphic.

Table 3. Across-population genetic structure: fragment lengths, total number of fragments, number and percentage of polymorphic fragments produced by each primer set used to analyse the polymorphisms of one individual from each of 62 *E. colona* populations.

Primer	Fragment lengths	Total number of fragments	Number of polymorphic fragments	Polymorphic percentage
*Mse*I + CAT	45-300	49	49	100
*Mse*I + CAA	45-300	21	21	100
Total		70	70	
Average				100

The phenogram produced using UPGMA grouped the 62 individuals into four distinct clusters, with three individuals ungrouped (25, 33 and 45) (Figure 2). The largest cluster contained 44 individuals with the other three clusters containing between two and nine individuals. Only one cluster (cluster II) contained individuals from a single location (Goondiwindi); all other clusters contained individuals from many different locations. The phenogram also showed that individuals did not cluster according to herbicide resistance. For example, individuals from six resistant populations (1 to 6) sampled from near Bellata in NSW were split between two clusters (I and IV).

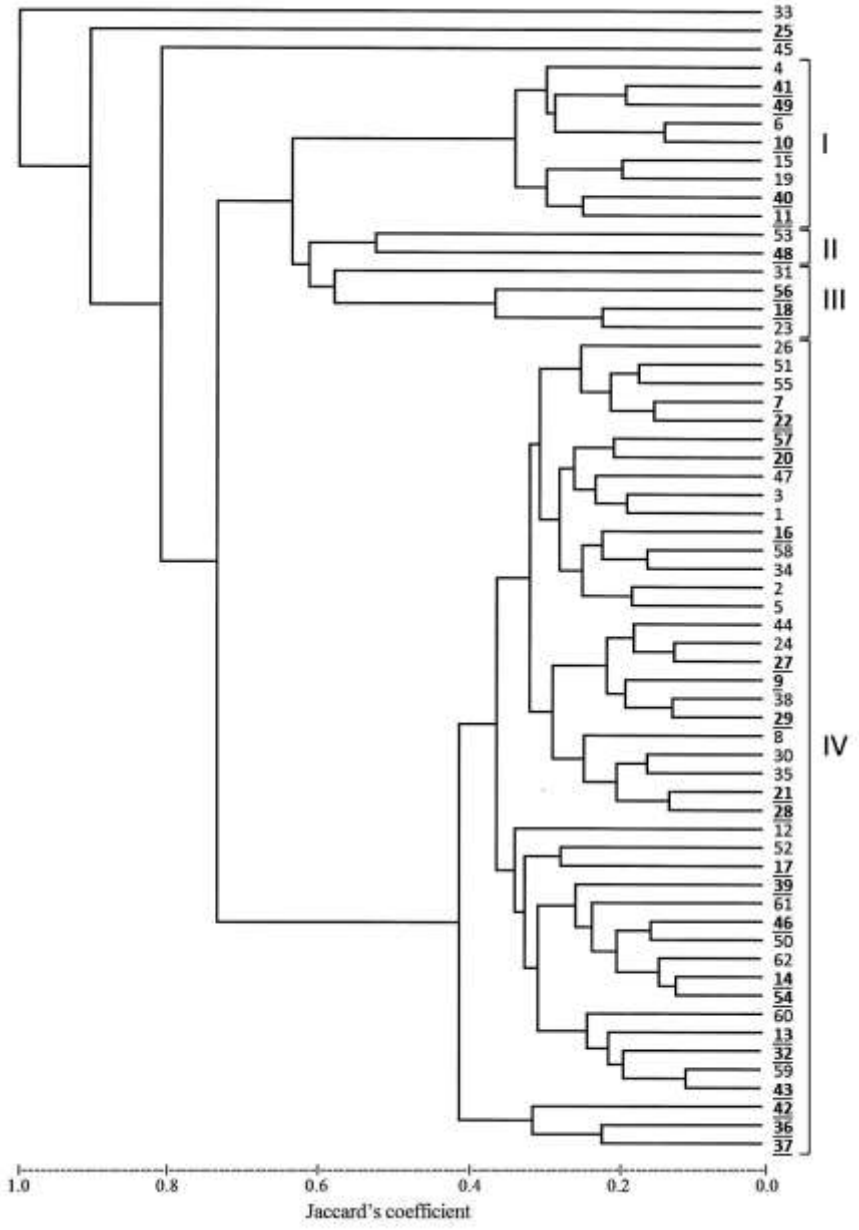

Figure 2. UPGMA phenogram of the genetic relationship between *E. colona* populations collected across NSW and QLD, Australia. The numbers on the phenogram correspond to the number given to each population in Table 1. The susceptible populations are bold and underlined.

Further confirmation of extensive genetic variation across *E. colona* in the surveyed region of Australia was also obtained when the genetic variation among a single individual from each of 62 populations was examined (Figure 2). These individuals failed to cluster by geography or by resistance status. While a single large cluster contained 44 of the samples, even within this cluster there was considerable genetic diversity (Figure 2). High genetic diversity within species will increase the likelihood of herbicide resistance evolving (Powles and Yu, 2010).

This study also demonstrated no genetic grouping of glyphosate resistant individuals from across NSW and QLD. This suggests glyphosate resistant populations have evolved from local susceptible populations, rather than resistance arising a few times and then spreading across the region. The fact that six resistant individuals from different sites near Bellata in NSW were spread across two clusters (I and IV) supports the conclusion that resistance to glyphosate has occurred independently even within a small area. Other studies comparing the genetic relatedness of resistant and susceptible weed populations have come to similar conclusions for *Lactuca serriola* L. in South Australia (Lu et al. 2007), *C. album* in Europe (Aper et al. 2010), *Echinochloa oryzoides* (Ard.) Fritsch in California (Osuna et al. 2011) and *A. palmeri* in the USA (Chandi et al. 2013). However, in several studies there was evidence of spread of resistance between sites as well as independent selection (Baker et al. 2007; Lu et al. 2007; Osuna et al. 2011; Okada et al. 2013).

Genetic diversity within populations

Genetic diversity was examined using 30 *E. colona* individuals from each of three populations and two primer pairs: *Pst*I with *Mse*I + CAT and *Mse*I + CAA. The primers produced a total of 354 AFLP fragments ranging in length from 15 to 615 bp. Of the 354 fragments, 351 were polymorphic, giving an average polymorphism percentage of 99.2% (Table 4). The frequency of polymorphic fragments was similar between populations ranging from 80.8% to 83.9% across the three populations. For all three populations, the primer combination of *Pst*I + AGC and *Mse*I + CAA produced fewer fragments. The diversity in genotypes of the resistant populations used was as high as that of the susceptible population. Hence there is no evidence in this data of a founder effect resulting from selection for glyphosate resistance.

Table 4. Within-population genetic structure: fragment lengths, total number of fragments, number and percentage of polymorphic fragments produced by each primer set used to analyse the polymorphisms of one individual from each of two glyphosate resistant *E. colona* populations (63 and 64) and one susceptible population (65) (30 individuals for each population)

Population	Primer	Fragment lengths	Total number of fragments	Number of polymorphic fragments	Polymorphic percentage
63 (R)	*Mse*I + CAT	15-615	203	174	85.7
	*Mse*I + CAA	15-615	151	124	82.1
	Total		354	298	
	Average				83.9
64 (R)	*Mse*I + CAT	15-615	203	162	79.8
	*Mse*I + CAA	15-615	151	124	82.1
	Total		354	286	
	Average				81.0
65 (S)	*Mse*I + CAT	15-615	203	160	78.8
	*Mse*I + CAA	15-615	151	125	82.8
	Total		354	285	
	Average				80.8
Primers and populations combined			354	351	99.2

The genetic relationship among individuals within the three populations was generated by UPGMA (Figure 3). In the phenogram, all but five individuals grouped into five major clusters. All these five ungrouped samples were derived from the two resistant populations 63 (63-19) and 64 (64-17, 64-18, 64-21 and 64-30). All clusters, except cluster IV, were composed of individuals from more than one population. This suggests there is considerable genetic diversity within populations of *E. colona* in northern Australia.

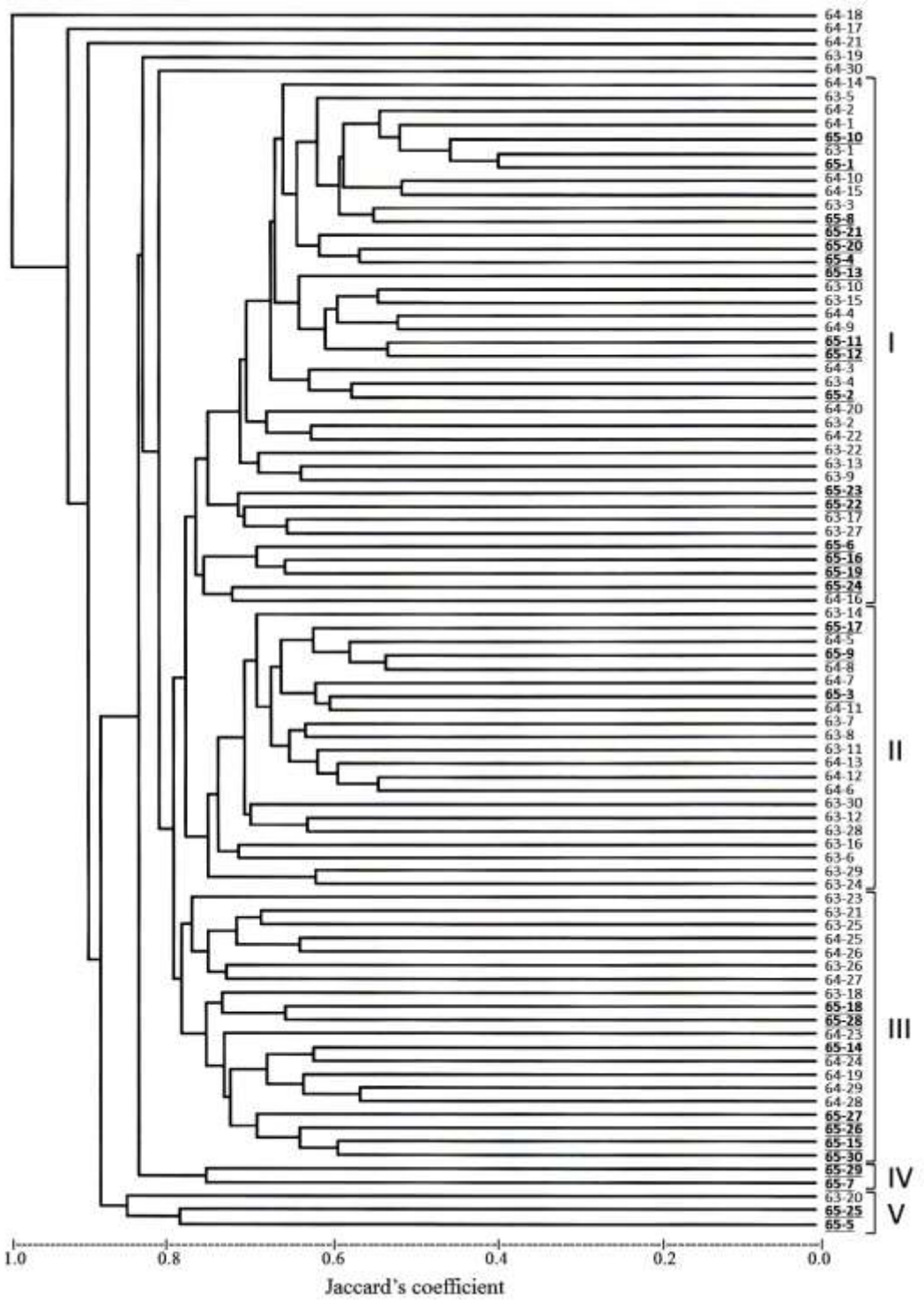

Figure 3. UPGMA phenogram showing the genetic relationship within two resistant populations (63 and 64 in Table 1) and the susceptible population (65) of *E. colona* collected from three separate fields in NSW. The number before the dash is the number assigned to these populations in Table 1 and the number after the dash is an individual plant from that population. The susceptible individuals are bold and underline.

AFLP analysis showed that genetic variation within three populations of *E. colona* collected from fields within NSW State was high. *Echinochloa* spp. are generally considered to be largely self-pollinating (Maun and Barrett, 1986; Honek and Martinkova, 1996; Osuna et al. 2011). Therefore, the high genetic diversity obtained within *E. colona* populations was unexpected. A high level of genetic variation suggests either frequent movement of seed material between sites or significant out-crossing occurs in *E. colona*. In addition, in the data collected no evidence of a genetic bottleneck was evident in the two resistant populations, as these populations had similar genetic variation to the susceptible population (Table 4). Founder effects are expected to occur with selection for herbicide resistance where only a small number of individuals in the original population carry the resistance allele (Jasieniuk et al. 1996). Founder effects have previously been identified in herbicide resistant weed populations of *Poa annua* L. (Mengistu et al. 2000) and *Chenopodium album* L. (Aper et al. 2010). Several factors may mediate against the identification of founder effects in populations. For example, if there is cross-pollination and possession of a dominant herbicide resistant trait, the trait will be readily shared among individuals of the population.

The UPGMA phenogram of the 90 individuals across the three populations showed no clustering by population (Figure 3). The lack of clustering suggests high genetic variation within populations or considerable gene flow between populations across the region. Glyphosate resistant *Amaranthus palmeri* S. Wats. populations from Georgia and North Carolina demonstrated some clustering with four populations clustering together and separately from four other populations (Chandi et al. 2013). However, this clustering was neither geographic, nor related to glyphosate resistance status. Okada et al. (2013) showed that glyphosate resistant *Conyza canadensis* (L.) Cronq. populations in California showed clustering based on geographical areas. They further concluded that glyphosate resistance had occurred and spread well before it was detected in California. *E. colona* seed has no specific modifications for long-distance seed movement; therefore, any movement has most likely occurred as a contaminant on farm machinery, seed for sowing or by flood waters.

Conclusion

This study has shown glyphosate resistance is widespread in populations of *E. colona* from northern NSW and QLD. The levels of glyphosate resistance compared to a known susceptible population ranged from 2- to almost 12-fold. There was high genetic diversity within glyphosate resistant *E. colona* populations and across the region, indicating glyphosate resistance has evolved numerous times in this species. Therefore, over-reliance on glyphosate for weed control has been the most important factor in the current extent of resistance in this weed species.

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Conflicts of Interest

Authors declare no conflict of interest.

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