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Evolution and spread of glyphosate resistance in *Conyza bonariensis* in California and a comparison with closely related *Conyza canadensis*

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Summary

Glyphosate-resistant weeds are an increasing problem in perennial cropping systems in the Central Valley of California, USA. To elucidate the evolutionary origins and spatial spread of resistance, we investigated the geographical distribution of glyphosate resistance and the population genetic diversity and structure of *Conyza bonariensis* and compared the results with previously studied *C. canadensis*. Thirty-five populations from orchards and vineyards across the Central Valley were sampled. Population genetic structure was assessed using microsatellite markers. Population-level resistance was assessed in glasshouse screening of plants grown from field-collected seed. Bayesian clustering and analyses of multilocus genotypes indicated multiple origins of resistance, as observed in *C. canadensis*. Pairwise F_{ST} analysis detected spatial spread of resistance in the south of the Central Valley, also similar to *C. canadensis*. The results strongly indicate that the southern valley was an environment markedly more suitable than the northern valley for resistance spread and that spread in *Conyza* species was driven by increased uniformity of strong selection in the southern valley, due to recent regulation on herbicides other than glyphosate. Accordingly, resistant *C. canadensis* individuals occurred at high frequencies only in the southern valley, but interestingly resistant *C. bonariensis* occurred at high frequencies throughout the valley. Expression of resistance showed varying degrees of plasticity in *C. bonariensis*. The lower selfing rate and substantially greater genotypic diversity in *C. bonariensis*, relative to *C. canadensis*, indicate greater evolutionary potential over shorter time periods. Interspecific hybridisation was detected, but its role in resistance evolution remains unclear.

Keywords: fleabane, herbicide resistance, glyphosate, microsatellite marker, *Erigeron bonariensis*, *Erigeron canadensis*.

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Introduction

In the contemporary evolution of wild populations, human activities are often the drivers of adaptation (Palumbi, 2001). Herbicides are the primary means of controlling weeds in modern agriculture and they exert strong selection on weed populations. Weed populations evolve resistance in response to repeated treatments

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with herbicides (Maxwell *et al*., 1990; Jasieniuk *et al*., 1996; Délye *et al*., 2013). To date, biotypes resistant to herbicides with a broad range of mechanisms of action have been detected in over 200 weed species worldwide (Heap, 2013). Resistance evolution is quickly becoming a major challenge for large-scale conventional agriculture and consequently for global food security.

The herbicide glyphosate has many favourable attributes for managing weeds, including effectiveness on a wide range of species, low toxicity to animals and rapid inactivation in the soil (Baylis, 2000). In recent years, the worldwide use of glyphosate for weed control has increased markedly, due to the cultivation of transgenic glyphosate-resistant (GR) crops and the implementation of reduced tillage systems (Powles, 2008). Reduction in the cost of glyphosate due to the availability of generic equivalents has also led to increases in glyphosate use. Unfortunately, overreliance on glyphosate, with little or no diversity in weed management practices, has led to the evolution of glyphosate-resistant weed populations in many species (Heap, 2013).

In the Central Valley of California, USA, glyphosate has been the primary herbicide used to control weeds in perennial crops and non-crop areas, such as field edges, roadsides and irrigation ditches, for decades. Resistance to glyphosate was first confirmed in 2005 in populations of *Conyza canadensis* (L.) Cronquist (Shrestha *et al*., 2007), a common annual weed in orchards, vineyards and adjacent infrequently disturbed sites. A survey of 42 *C. canadensis* populations across the Central Valley in 2010 revealed a positive correlation between the frequency of resistant plants in a population and the size of groundwater protection areas (GWPA) within counties (Okada *et al*., 2013). Further, it was shown that glyphosate resistance likely originated multiple times and increased in frequency many years before it was detected. Increased use of glyphosate, following the implementation of state regulations restricting pesticides vulnerable to leaching and run-off into groundwater in GWPA, is hypothesised to have increased glyphosate selection for resistance and led to its evolution and detection in *C. canadensis* (Shrestha *et al*., 2007; Okada *et al*., 2013).

Glyphosate-resistant populations of a second *Conyza* species, *C. bonariensis* (L.) Cronquist, were also confirmed in perennial crops of California within 2 years of the identification of resistant *C. canadensis* (Shrestha *et al*., 2008a). The two species are closely related (Noyes, 2000) annuals with similar vegetative phenologies and seed dispersal mechanisms (Shrestha *et al*., 2008a,b), although *C. bonariensis* is allopolyploid ($2n = 6x = 54$; Thébaud & Abbott, 1995), whereas *C. canadensis* is diploid ($2n = 2x = 18$). The geographical distribution, evolutionary origins and patterns of spatial spread of resistance remain to be elucidated in *C. bonariensis* and compared with *C. canadensis*. Also, in our earlier study (Okada *et al*., 2013) using microsatellite markers, we observed a small number of *C. canadensis* individuals exhibiting evidence of interspecific hybridisation with a polyploid *Conyza* species, possibly *C. bonariensis*. Interspecific hybridisation can result in adaptive introgression of herbicide resistance from one weed species to a closely related species (Gaines *et al*., 2012). Although the two *Conyza* species differ in ploidy levels, adaptive introgression has been documented across ploidy barriers (Chapman & Abbott, 2010). It remains unknown whether interspecific hybridisation and introgression has occurred between *C. bonariensis* and *C. canadensis*, resulting in the spread of the resistance trait, but understanding this possibility is important for understanding the evolution of glyphosate resistance and developing resistance management strategies for the two *Conyza* species.

In this study, we characterised the frequency of glyphosate-resistant plants and microsatellite marker variation in *C. bonariensis* in orchards and vineyards across the Central Valley of California and inferred the origin and pattern of spatial spread of glyphosate resistance. We addressed the following questions: (i) how is glyphosate resistance distributed among *C. bonariensis* populations across the area sampled, (ii) is there spatial structuring of molecular marker and glyphosate resistance variation that is indicative of resistance spread, (iii) do the spatial patterns of variation in glyphosate resistance and microsatellite markers in *C. bonariensis* differ from those observed in *C. canadensis* and (iv) is there evidence of interspecific hybridisation between the two *Conyza* species?

Materials and methods

Sampling

Thirty-five populations of *C. bonariensis* were sampled from orchards and vineyards across the Central Valley of California in 2010 (Table 1; Figure S1). Leaf tissue was collected from 30 plants per population. Seeds were also collected from each plant in 31 of the 35 populations. In one additional population, CSU, seed was collected from 30 individuals that were not the plants from which leaf samples were collected. Plants in the remaining three populations were pre-reproductive at the time of leaf sampling and thus were not sampled for seed. In addition to the plants sampled in the field, we collected leaf tissue from 30 plants grown

Table 1 *Conyza bonariensis* populations sampled in California and the proportions of plants surviving glyphosate treatment at two field use rates of glyphosate averaged over two repetitions

ID	Habitat	Latitude (N)	Longitude (W)	Average proportion of plants surviving (SE)	
				1x treatment*	2x treatment†
Northern region				0.88 (0.06)	0.71 (0.08)
A2	Almond	39.692	121.853	0.99 (0.02)	0.90 (0.10)
A1	Almond	39.631	121.871	0.70 (0.30)	**0.24** (0.24)
B2	Walnut	39.353	121.726	0.95 (0.05)	**0.84** (0.14)
B1	Prune/Walnut	39.319	121.688	1.00 (0.00)	0.90 (0.11)
B3	Almond/Walnut	39.069	121.672	1.00 (0.00)	0.95 (0.05)
C3	Almond	39.012	122.070	0.40 (0.17)	0.19 (0.09)
C1	Almond	38.951	122.067	0.99 (0.02)	**0.84** (0.17)
C2	Almond	38.946	122.061	1.00 (0.00)	**0.84** (0.17)
Central region				0.91 (0.06)	0.65 (0.10)
E1	Grape	38.190	121.417	0.45 (0.35)	0.07 (0.04)
E2	Grape	38.190	121.431	0.99 (0.02)	0.95 (0.05)
F2	Olive	37.842	121.100	0.97 (0.04)	**0.82** (0.19)
F1	Almond	37.827	121.135	1.00 (0.00)	**0.80** (0.20)
F3	Almond	37.821	121.108	0.95 (0.05)	**0.85** (0.15)
G1	Almond	37.568	120.675	0.97 (0.04)	**0.57** (0.44)
G2	Almond	37.565	120.664	1.00 (0.00)	**0.54** (0.47)
G3	Grape	37.549	120.646	1.00 (0.00)	**0.59** (0.42)
Southern region				0.92 (0.03)	0.74 (0.06)
H1	Almond	37.040	120.221	0.99 (0.02)	**0.60** (0.41)
H3	Almond	37.014	120.230	1.00 (0.00)	1.00 (0.00)
H2	Pomegranate	36.996	120.241	1.00 (0.00)	0.99 (0.02)
I1	Grape	36.910	120.110	0.95 (0.05)	0.87 (0.14)
I2	Almond	36.885	120.083	1.00 (0.00)	0.95 (0.05)
CSU	Peach	36.818	119.734	1.00 (0.00)	**0.72** (0.29)
KEA	Grape	36.595	119.507	–	–
MCC	Grape/Almond	36.638	119.611	–	–
I3	Grape	36.813	120.205	0.97 (0.04)	**0.62** (0.35)
K2	Prune	36.606	119.670	1.00 (0.00)	**0.85** (0.15)
K1	Grape	36.590	119.827	0.77 (0.10)	0.72 (0.15)
WES	Grape/Almond	36.340	120.106	–	–
L3	Grape	35.906	119.224	0.89 (0.12)	**0.62** (0.39)
L1	Grape	35.906	119.250	0.92 (0.09)	**0.70** (0.30)
L2	Grape	35.892	119.232	0.97 (0.04)	**0.50** (0.47)
M1	Pistachio	35.702	119.857	0.89 (0.12)	**0.67** (0.34)
N1	Grape	35.689	119.379	0.77 (0.24)	**0.74** (0.27)
N2	Pomegranate	35.674	119.360	0.97 (0.04)	**0.85** (0.15)
N3	Almond	35.660	119.388	0.72 (0.29)	**0.52** (0.49)
Reference populations					
R	Roadside	36.488	119.567	0.95 (0.04)	**0.76** (0.23)
S	Grape	36.799	120.217	0.30 (0.27)	0.03 (0.03)

Proportions of plants surviving glyphosate treatment at the 1x (0.84 kg a.e. ha^{-1}) and 2x (1.68 kg a.e. ha^{-1}) rates were assessed 35 days after treatment of the 30 plants per repetition. In repetition 2 of populations B1 and H1, the total number treated was 33 and 31 respectively. Resistant (*R*) and susceptible (*S*) reference populations from Shrestha *et al.* (2008a).

*Homogeneity of odds ratio test among populations showed no difference ($P > 0.05$). The repetitions had a significant effect ($P < 0.001$) based on the common odds ratio over all populations.

†Homogeneity of odds ratio test among populations showed significant differences ($P < 0.001$). Bold text indicates populations with significantly different responses between repetitions, based on significant odds ratios after Bonferroni corrections ($\alpha = 0.05$).

in the glasshouse from seed collected from a glyphosate-resistant population and a glyphosate-susceptible population that were previously characterised (Shrestha *et al.*, 2008a). Sampled leaf tissue was immediately dried in sealed plastic bags filled with silica gel and stored at room temperature until DNA extraction.

Analysis of response to glyphosate

Equal amounts of seed from each plant, by volume, were bulked to produce a seed sample for each of the 32 populations. The bulked population seed samples were germinated on the surface of moist soil in

30-cm-diameter plastic pots. Young seedlings were transplanted into 5 × 5 cm square pots with one seedling per pot. Modified UC soil mix (peat, sand and redwood compost in 1:1:1 ratio) was used. Plants were watered as needed and fertilised biweekly with Hoagland solution. All plants were grown in a single glasshouse under ambient light conditions in Davis, California, USA, and treated with glyphosate at the five- to eight-leaf stage. Because about 50% of the plants from susceptible populations survived treatments in earlier studies (Shrestha *et al*., 2008a), we screened populations for resistance to two rates (1x = 0.84 kg a.e. ha^{-1}, the recommended label rate for *C. bonariensis*, and 2x = 1.68 kg a.e. ha^{-1}) of glyphosate (Roundup WeatherMax, Monsanto Company, St. Louis, MO, USA) with two repetitions per treatment (Table 1). Treatment dates spanned 15 July to 2 August 2011 for repetition one and 17 September to 17 October 2011 for repetition two, due to variation among individuals in time to reach the target size for treatment with glyphosate. The treatments were applied using a track sprayer (Technical Machinery Inc., Sacramento, CA, USA) fitted with an even flat spray tip (TP8001EVS; TeeJet Technologies, Wheaton, IL, USA) at the two rates in a spray volume of 140 L ha^{-1} with deionised water as the carrier at 207 kPa. Thirty plants from each population were screened per repetition, with the exception of populations B1 and H1, in which 33 and 31 plants, respectively, were treated in the second repetition of the 2x treatment. For every repetition and population tested, three glyphosate-resistant and three glyphosate-susceptible plants from previously characterised seed lots (Shrestha *et al*., 2008a) were included for reference. Response to glyphosate was assessed visually for each plant 35 days after glyphosate treatment and characterised at the population level by the proportion of plants surviving glyphosate treatment out of the total number of plants treated per repetition. Because plant response to glyphosate appeared substantially different between repetitions for many populations, comparisons between the repetitions were made using the Mantel–Haenszel Procedure (Sokal & Rohlf, 1995). The analysis was conducted separately for 1x and 2x glyphosate treatments.

Microsatellite marker analysis

DNA was extracted from dried leaf tissue using the CTAB procedure. Individuals were genotyped using 10 microsatellite primer pairs that were developed from *C. canadensis* genomic sequences, but also cross-amplified in *C. bonariensis*. The primer pairs were HW02, HW06, HW29, HW17, HWSSR01, HWSSR03, HWSSR04, HWSSR11, HWSSR09 and HWSSR12 (Okada *et al*., 2013). Polymerase chain reactions (PCR) were performed, and amplification products were separated and sized as described in Okada *et al*. (2013). Three primer pairs amplified two loci and one primer pair amplified three loci (Table S1). In these cases, alleles were attributed to each locus based on non-overlapping size distributions, with the exception of one ambiguous allele, for which scoring was performed to minimise the presence of heterozygotes and null homozygotes.

To estimate genetic diversity at each locus, the total number of alleles detected (T_A), expected heterozygosity (H_E), observed heterozygosity (H_O), inbreeding coefficient (F_{IS}) and fixation index (F_{ST}) were calculated using FSTAT 2.9.3 (Goudet, 2002) for each of the 15 polymorphic disomic loci PCR amplified by the 10 primer pairs in the allopolyploid *C. bonariensis* ($2n = 6x = 54$). Statistical significance of the F_{IS} and F_{ST} values was determined using 1000 randomisations. Random mating was not assumed for estimations of F_{ST}. To estimate genetic diversity within populations over the 15 microsatellite loci, allelic richness (A), H_E, H_O, F_{IS} were calculated for each population using FSTAT 2.9.3. To assess the pattern of mating in populations, the rate of self-fertilisation (s) was estimated as $2F_{IS}/(1 + F_{IS})$.

Genetic differentiation among sampled populations was assessed by calculating pairwise F_{ST} values between all pairs of sampled populations using FSTAT 2.9.3. Statistical significance of the F_{ST} values was assessed with 1000 permutations using the Bonferroni procedure to correct for multiple tests. To elucidate the geographical structuring of genetic variation among populations, we used a distance-based clustering analysis. Nei's genetic distances (Nei, 1978) were computed between all pairs of populations with 1000 bootstrap replications using MICROSATELLITE ANALYSER (Dieringer & Schlötterer, 2003). The program FITCH in PHYLIP version 3.57c (Felsenstein, 2005) was used to construct the dendrogram.

To assess spatial patterns of seed dispersal, the distribution of shared multilocus genotypes (MLGs) among populations was analysed. All MLGs shared among individuals and populations were identified, as in Okada *et al*. (2013). Further, to gain insight into the spread of resistance within populations and the number of potential, independent origins of resistance, MLGs in highly resistant populations were classified as either non-recombinant or recombinant with respect to other MLGs within the populations.

Population structure was further investigated using the model-based Bayesian clustering program INSTRUCT (Gao *et al*., 2007). To determine K, the number of distinct gene pools or genetic clusters within the data,

analyses were conducted for K values ranging from 1 to 37 using MCMC iterations of 100 000 with a burn-in period of 100 000, thinning interval of 100, and a posterior credible interval of 0.95 for five chains at each K. ΔK (Evanno *et al*., 2005) was calculated over the range of K values. Subclustering within clusters at the highest hierarchical level of population structure was assessed by analysing individuals that assigned highly ($q > 0.8$) to a cluster using the program settings in INSTRUCT as described above. The USEPOPINFO option of STRUCTURE (Pritchard *et al*., 2000) was used to assign all individuals to the identified subclusters. The program CLUMPP (Jakobsson & Rosenberg, 2007) was used to assess the consistency among runs and to obtain the probabilities of assignment averaged over five runs.

To visualise the genetic relationships of individuals that appeared to be interspecific hybrids between *C. bonariensis* and *C. canadensis*, based on their microsatellite marker profiles both in this study and in a previous study of *C. canadensis* (Okada *et al*., 2013), the microsatellite marker data sets from both species were combined and analysed using the 10 microsatellite loci that PCR amplified in both species. Pairwise distances between all unique multilocus genotypes and the suspected hybrid individuals were calculated as one minus the proportion of shared alleles (MICROSATELLITE ANALYZER). Principal co-ordinate analysis was performed using the cmdscale function in R 2.14.2 (R Development Core Team, 2011).

Results

Glyphosate resistance

Frequencies of plants that survived treatment with glyphosate at the 1x and 2x rates, averaged over the two repetitions, varied from 0.29 to 1.00 and 0.03 to 1.00, respectively, among the 34 populations tested, which included the two reference populations (Table 1). Predominantly resistant populations were common throughout the Central Valley (Table 1, Fig. 1A and B). Of the 34 populations tested, the average frequency of survivors was greater than 0.50 in 31 populations under the 1x treatment, and in 30 populations under the 2x treatment. Response to the 2x treatment varied substantially among populations observed to be highly resistant under the 1x treatment (Fig. 2A and B), suggesting genetic variation in the resistance trait among resistant populations, possibly due to independent origins (Yuan *et al*., 2010; Okada *et al*., 2013) or the effect of different genetic backgrounds (Chandler *et al*., 2013).

Plant response to glyphosate treatments appeared to vary widely between the two repetitions within many populations (Fig. 2); thus, the effect of the two repetitions was investigated. The magnitude by which the repetitions affected the plant response to glyphosate was not different among populations under the 1x glyphosate treatment (Mantel–Haenszel procedure to test odds ratios for homogeneity among populations,

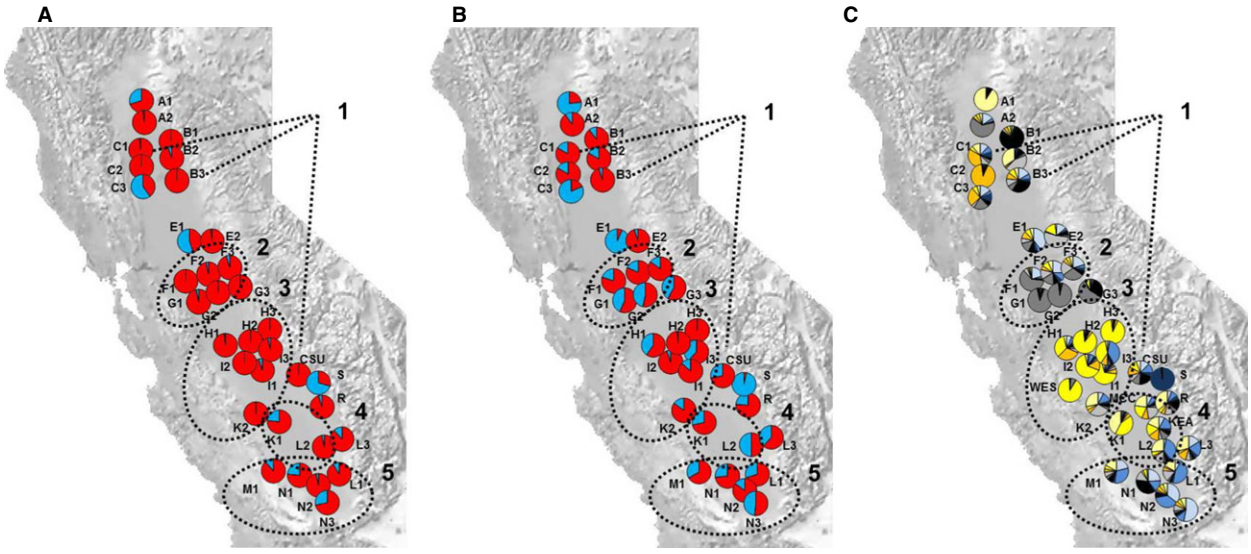

Fig. 1 Map of *Conyza bonariensis* populations, (A) response to 1x glyphosate treatment (0.84 kg a.e. ha^{-1}) in terms of the proportion of resistant (red) and susceptible (light blue) plants based on glasshouse screening of plants grown from field-collected seeds, (B) response to 2x glyphosate treatment and (C) average assignment over individuals per population to the nine genetic clusters inferred by Bayesian clustering analysis in the run with the highest probability of the data. Populations with non-significant pairwise F_{ST} values are indicated as groups 1 through 5.

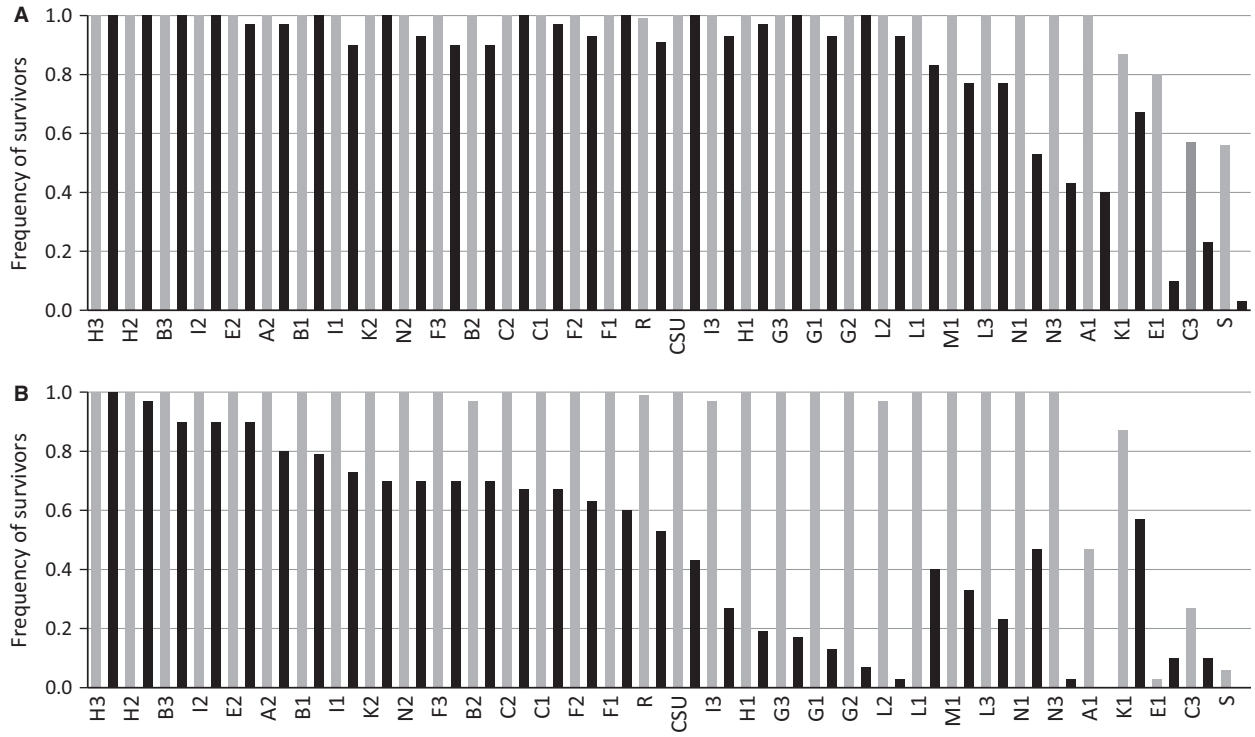

Fig. 2 Frequencies of survivors after glyphosate treatment for repetition 1 (grey) and repetition 2 (black) within 32 populations of *Conyza bonariensis* and resistant (R) and susceptible (S) reference populations from Shrestha *et al.* (2008a), (A) 1x glyphosate treatment at 0.84 kg a.e. ha^{-1} and (B) 2x glyphosate treatment. The populations are sorted by the frequencies of survivors in the 2x treatment.

$\chi^2_{(33)} = 35.85$, $P > 0.05$). Therefore, the effect of repetitions was assessed with a common odds ratio over all populations (Mantel–Haenszel estimator of the odds ratios). The plants were 23.7 times more likely to survive repetition 1 under the 1x treatment, and the effect was significant ($\chi^2_{(1)} = 27\ 224.56$, $P < 0.001$). On the other hand, under the 2x treatment, populations differed in the degree by which the two repetitions affected plant response to glyphosate ($\chi^2_{(33)} = 70.38$, $P < 0.001$). Thus, the effect of the two repetitions was tested in each population separately. The effect of the repetitions was significant in 22 populations of the 34 populations tested after Bonferroni corrections ($\alpha = 0.05$, Table 1). In the 22 populations, the plants more likely survived repetition 1 by 26.95 to 1199.67 times relative to repetition 2. The 22 populations had an average frequency of survivors between 0.24 and 0.85, and the magnitude of the effect of the two repetitions varied widely and continuously (Fig. 2). With the exception of population K1, a non-significant repetition effect was observed in populations with the highest or lowest average frequencies of survivors.

Genetic diversity and structure

The ten PCR primer pairs yielded 15 polymorphic microsatellite marker loci (Table S1). A total of 100 alleles were detected over the 15 loci, and the number of alleles ranged from two to 22 per locus. Observed heterozygosity (H_O) within populations ranged from 0.005 to 0.100, whereas expected heterozygosity (H_S) within populations ranged from 0.009 to 0.513. Expected heterozygosity (H_T) over all samples ranged from 0.009 to 0.828 over loci and F_{ST} ranged from 0.051 to 0.668 with significant differentiation among populations at all loci. With the exception of locus HWSSR09b, inbreeding coefficients (F_{IS}) ranged from 0.359 to 0.869 over loci and were highly significant, consistent with the predominantly selfing mating system of the species. Interestingly, the estimate of F_{IS} for locus HWSSR09b was nearly zero and not significantly different from zero due to 10 individuals that were heterozygous for the only two alleles detected for the locus. There were no homozygous individuals for the less frequent allele at HWSSR09b. The 10 individuals are possibly interspecific F1 hybrids with the co-occurring *C. canadensis* or subsequent hybrid generations, because the 10 individuals were also heterozygous at five to nine other loci of the 15. Possible interspecific hybrids were also detected in *C. canadensis* (Okada *et al.*, 2013). Principal co-ordinate analysis of microsatellite marker variation showed the suspected interspecific hybrids, as well as several other individuals, as intermediate between the two species or within the

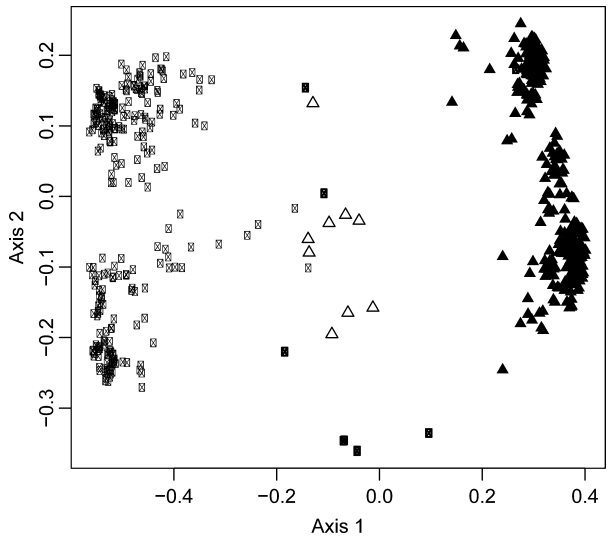

Fig. 3 Plot of the first two axes of the principal co-ordinate analysis of microsatellite marker data. Axis 1 and Axis 2 explained 59.3% and 7.4% of the variation in the data respectively. *Conyza canadensis*, closed circle; *C. bonariensis*, closed triangle; suspected interspecific hybrids collected as *C. canadensis*, open circle; suspected interspecific hybrids collected as *C. bonariensis*, open triangle.

variation of *C. bonariensis* (Fig. 3), indicating interspecific hybridisation and introgression.

Within populations, allelic richness (A) ranged from 1.0 to 2.9 among populations (Table S2). Expected heterozygosity (H_E) varied from 0.00 to 0.35 among populations, whereas observed heterozygosity (H_O) ranged from 0.00 to 0.16. Heterozygous individuals were observed in all sampled populations with the exception of population S. Inbreeding coefficients (F_{IS}) were uniformly positive and significant in all populations, indicating a predominantly selfing mating system. Estimates of selfing rate, s, ranged from 0.709 to 0.990, with an average of 0.882 over 36 polymorphic populations.

Pairwise F_{ST} estimates between populations ranged from 0.01 to 0.97 (Table S3). Most populations were highly differentiated with 639 significant pairwise F_{ST} values between populations of the 666 tested. Population pairs with non-significant F_{ST} values identified five groups of genetically similar populations: B3, C1, CSU; F1, F2, F3, G1, G2; H1, H2, H3, I1, I2, I3, K2, WES; K1, L2, KEA, MCC; and L1, M1, N1, N2, N3, which are shown in Fig. 1. With the exception of Group 1 consisting of populations B3, C1 and CSU, all the populations within the remaining four groups (Groups 2, 3, 4, and 5) were spatially cohesive and predominantly resistant (Fig. 1A and B). The clustering of populations based on pairwise genetic distances was consistent with the pairwise F_{ST} analysis (Figure S2).

A total of 492 unique MLGs were found among the 1097 individuals genotyped at the 15 microsatellite loci. Of the 492 MLGs, 109 were found multiple times and made up 65% of individuals sampled. Of the 109 MLGs, 43 were shared among populations (Figure S3) and accounted for 40% of all plants sampled. Two of the 43 MLGs shared between populations were widespread, occurring 108 and 64 times among the 1097 individuals, and in 14 and five populations. MLGs were analysed and classified into recombinant and non-recombinant MLG within five highly resistant populations (B3, E2, H2, H3, and I2) and two susceptible populations (C3 and E1). The numbers of individuals with recombinant MLGs ranged from five to 17 among the seven populations (Table 2), which suggests outcrossing and possibly spread of resistance by pollen within populations in the populations analysed. However, based on the shared MLGs, resistance spread by seed was predominant within and across all highly resistant populations, with the exception of resistant population B3. Interestingly, most of the recombinant MLGs carried some heterozygosity at microsatellite

Table 2 Numbers of individuals of distinct non-recombinant and recombinant multilocus genotypes (MLGs) in *Conyza bonariensis* populations

	Non-recombinant									Recombinant	
Population	a	b	c	d	e	f	g		Singletons	Homozygous	Heterozygous
B3	2	3							5	1	16
E2			3	14	4				3	1	5
H2			19						4	1	7
H3			22			2			1	0	5
I2			16				2		7	1	4
	h	i	j	k	l	m	n	o			
C3	4	3	3	2	2	2			4	3	7
E1							2	2	4	4	15

Populations, B3, E2, H2, H3 and I2 had average frequencies of glyphosate-resistant individuals (R) of ≥0.95 under 2x treatment (glyphosate 1.68 kg a.e. ha^{-1}) and populations, C3 and E1, with $R < 0.50$ under 1x treatment (glyphosate 0.84 kg a.e. ha^{-1}).

marker loci, indicating that they resulted from outcrossing between non-recombinant MLGs within one or a few generations. Recombinant MLGs with no heterozygosity were substantially lower in frequency within all populations, suggesting that the self-fertilised progeny of outcrossing often do not persist within populations, especially in highly resistant populations.

INSTRUCT analysis (Gao *et al.*, 2007) revealed increasing values of *ln P*(*D*) followed by a slight gradual decrease, but there was no clear maximum of *ln P*(*D*) for *K* values ranging from 1 to 37 (Fig. 4A), indicating a hierarchical pattern of population structure (Evanno *et al.*, 2005). Δ*K* (Evanno *et al.*, 2005) showed the highest peak at *K* = 2 (Fig. 4A), indicating that two genetic clusters best explained the uppermost hierarchical level of genetic structuring. Based on Δ*K*, the two clusters at *K* = 2 exhibited further substructure into six and three clusters, respectively, for a total of nine clusters (Figure S4A and B). Assignment of individuals to the nine clusters (Fig. 4B) showed some localised spatial groupings, in which individuals from populations that were identified as genetically similar by pairwise F_{ST} analysis (Fig. 1C) also assigned to the same cluster(s). Within the highly resistant populations, B3, E2, H2, H3 and I2, there were many highly admixed individuals, but individuals assigning highly (with *q* > 0.9) to six different clusters were also present (Fig. 4B), suggesting multiple independent origins of resistance.

Discussion

Origins and spread of glyphosate resistance in C. bonariensis

Glyphosate-resistant individuals occurred at high frequencies in almost all of the populations sampled throughout the Central Valley of California (Table 1; Figs 1A,B and 2A,B), an outcome that is consistent with decades of use of glyphosate as the most widely used herbicide in orchards and vineyards. In earlier studies of *C. bonariensis*, glyphosate showed good control of the species in areas of the Central Valley (Shrestha *et al.*, 2008a,b), although populations that are completely susceptible were absent among sampled populations in this study. Thus, the plants surviving glyphosate treatments represent evolved resistance.

Population-level mortality in response to glyphosate varied significantly between repetitions. The resistant and susceptible reference populations showed comparable frequencies of plants that survived 1x and 2x glyphosate treatments as shown by Shrestha *et al.* (2007) in the first repetition. However, a considerably lower frequency of survivors than Shrestha *et al.*

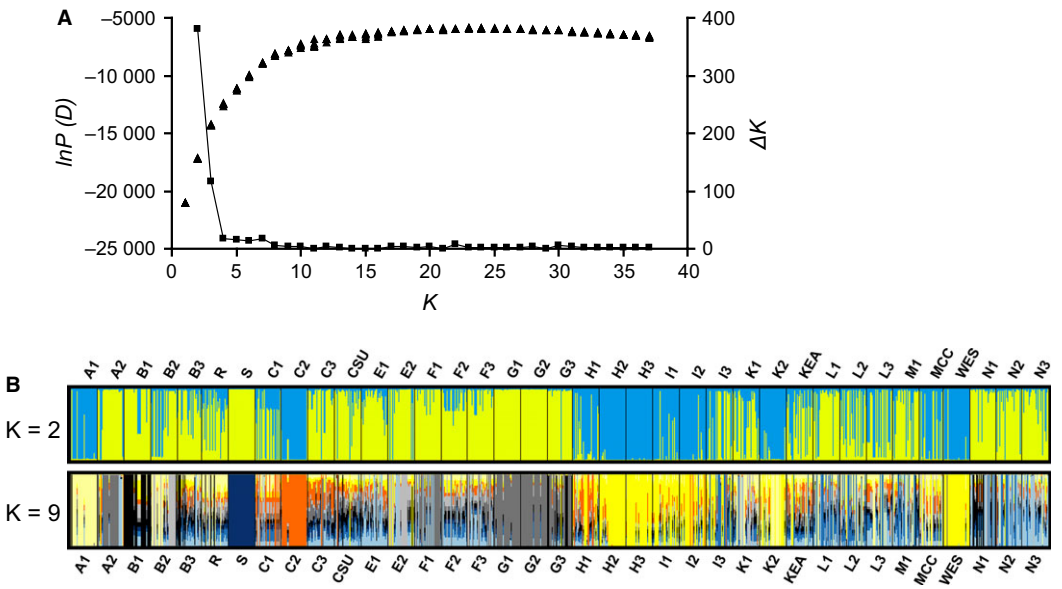

Fig. 4 Bayesian clustering analysis (INSTRUCT, Gao *et al.*, 2007) of *Conyza bonariensis* (A) plot of the log likelihood of the multilocus genotypic data, *ln P*(*D*), (triangles) for five runs at each value of *K*, and the second order rate of change in ln *P*(*D*), Δ*K*, (squares) as a function of the number of clusters or gene pools, *K* and (B) average probabilities of assignment of individuals at *K* = 2 and *K* = 9 over five runs. Each vertical bar represents an individual and the proportion of its genome that assigned to distinct clusters. Subclusters within the two clusters at *K* = 2 were identified by analysing individuals that assigned to each of the clusters at *q* > 0.80 using INSTRUCT. The USEPOPINFO option of STRUCTURE (Pritchard *et al.*, 2000) was used to assign all individuals to the nine subclusters.

(2007) was observed in the second repetition of the 2x treatment in the resistant population, as well as 1x treatment in the susceptible population, indicating lower expression of resistance in late summer conditions or the treatment itself was more effective in the second repetition. However, the magnitude of the variation in response to glyphosate between repetitions also varied among populations under the 2x treatment, suggesting variation among populations in response to environmental conditions. A significant environmental effect on the expression of glyphosate resistance has been previously reported (Urbano *et al*., 2007; Walker *et al*., 2011; Okada & Jasieniuk, 2014) and appears to be a characteristic of the resistance trait in *C. bonariensis* (Moretti *et al*., 2013). Phenotypically, plastic responses to varying environmental conditions may be followed by adaptive evolution (Schwander & Leimar, 2011) and could play a role in the evolution of glyphosate resistance in weeds. Better understanding of the plasticity of the glyphosate resistance trait may aid in improving strategies to control *C. bonariensis* and manage resistance evolution.

The predominantly selfing mating system and the resulting low recombination in *C. bonariensis* allowed tracking of the origin and spread of resistance using neutral molecular markers. Although glyphosate resistance was common throughout the Central Valley, population genetic structuring based on the pairwise F_{ST} analysis, indicative of the pattern of spread of resistance (Fig. 1A–C), strongly suggests that the southern valley was an environment markedly more suitable than the northern valley for resistance spread. Four of the five most highly resistant populations were located in the southern valley and showed spread of resistance within and between populations, predominantly by seed, based on shared MLGs (Table 2). However, the presence of heterozygous recombinant MLGs within populations also indicates substantial outcrossing and the potential for resistance spread by pollen and possible introgression of resistance following gene flow by seed. Interestingly, the greater frequencies of heterozygous recombinant MLGs relative to homozygous recombinant MLGs suggest that spread of resistance by pollen within populations may also be limited.

Population genetic structure combined with the spatial distribution of glyphosate resistance was consistent with multiple independent origins of resistance in *C. bonariensis* (Fig. 1A–C). The presence of multiple non-recombinant MLGs in populations with high frequencies of resistant individuals (Table 2) also indicates multiple independent origins. Multiple origins of resistance in *C. bonariensis* may include the origins of distinct resistance mechanisms in California, based on the multiple patterns of inheritance of resistance detected (Okada & Jasieniuk, 2014). Significant variation in the magnitude by which plants responded to the 2x treatment in the two repetitions may also reflect independent origins of resistance traits with different degrees of plasticity in the expression of resistance (Fig. 2). Continuous variation across populations may reflect different frequencies of individuals with the independently evolved resistance traits. As glyphosate resistance also evolved multiple times in *C. canadensis* within the Central Valley (Okada *et al*., 2013), the resistance trait appears to have originated relatively frequently in both *Conyza* species.

One of the resistance traits in *C. bonariensis* has a simple genetic basis (Okada & Jasieniuk, 2014), similar to *C. canadensis* (Zelaya *et al*., 2004), and is conferred by a single gene. If mutation for resistance occurs frequently in both species, the interspecific hybridisation and introgression detected in this study may not be a major factor contributing to the evolution and spread of resistance in either *Conyza* species. Alternatively, introgression from the other species might be a significant source of alleles conferring resistance and might have had an impact on the evolution of resistance in one or both of the species. The number of hybrids detected in this study was relatively small, but early-generation hybrids could be rare in the field, if hybridisation events are frequent but early-generation hybrids have reduced fitness. Interspecific hybrids between the two *Conyza* species may have reduced fitness due to the difference in ploidy level of the parental species. Because of the strong selection pressure imposed by the herbicide, introgression by backcrossing to either species may have happened relatively quickly. Strong selection pressure for resistance to glyphosate is a favourable condition for interspecific adaptive introgression (Chapman & Abbott, 2010), but because of strong selection, the evidence showing various stages of hybridisation and introgression may be rare and difficult to detect.

Comparison to C. canadensis

In both *Conyza* species, population genetic structure indicated extensive spread of resistance in the southern Central Valley, in agreement with the hypothesis of more intensive and extensive selection in this region (Shrestha *et al*., 2007; Okada *et al*., 2013). Implementation of regulations on the use of herbicides vulnerable to leaching and run-off and the resulting increase in glyphosate use was likely a major factor affecting the spread of glyphosate resistance in both species in the southern Central Valley.

There is a conspicuous and intriguing difference in the frequencies of glyphosate-resistant individuals

within populations of the two species in the northern Central Valley. Whereas populations of *C. bonariensis* are predominantly resistant, populations of *C. canadensis* are largely susceptible in the northern region, based on our sampling. The underlying causes of the difference in geographical distribution of glyphosate resistance between *Conyza bonariensis* and *C. canadensis* in the Central Valley of California are unknown. Only two sampling sites in the northern region contained both species (populations B2 and C3 for *C. bonariensis* corresponding to B1 and C2 for *C. canadensis* respectively). Thus, the selective environments may have differed between the two species to some degree. However, if populations of both species are under similar selective environments, the differences between species could result from biological differences, such as variation in the magnitude of fitness costs (Vila-Aiub *et al.*, 2009), evolutionary constraints associated with distinct molecular bases of resistance (Lande, 1980), or evolution of compensatory traits (Paris *et al.*, 2008). The possible role of multiple mechanisms of resistance in California (Okada & Jasieniuk, 2014) needs investigation.

Environmental conditions affect expression of glyphosate resistance in *C. bonariensis* (Urbano *et al.*, 2007; Walker *et al.*, 2011; Moretti *et al.*, 2013; Okada & Jasieniuk, 2014), as well as in *C. canadensis* (Ge *et al.*, 2011). However, our results suggest that the resistance trait in *C. bonariensis* may be more variable than in *C. canadensis* under different environmental conditions, based on the large differences in resistance observed between repetitions and the degree of phenotypic plasticity appears to vary among populations.

There was substantial genetic variation at microsatellite loci in both *Conyza* species. Overall genetic diversity was greater in *C. canadensis* than *C. bonariensis*, possibly because *C. canadensis* is native to North America, whereas *C. bonariensis* is introduced (Noyes, 2000) and underwent reduction in genetic variation associated with introduction(s). However, *C. bonariensis* had greater observed heterozygosity and a lower selfing rate of 0.88 in comparison with 0.96 in *C. canadensis*. Accordingly, the proportion of unique MLGs among sampled individuals was substantially higher in *C. bonariensis* (0.45) than in *C. canadensis* (0.21). Greater genotypic diversity resulting from a greater number of recombination events suggests greater adaptive potential in *C. bonariensis* over recent time. Consistent with the predominantly selfing mating system in both *Conyza* species, populations were highly differentiated, with F_{ST} of 0.55 in *C. canadensis* and 0.42 in *C. bonariensis*. However, unlike *C. canadensis*, there was little geographical structuring of genetic variation in *C. bonariensis* (Fig. 4), and the genome of most of the individuals was various admixtures of the nine genetic clusters or gene pools identified by Bayesian clustering (Fig. 4). Much admixture in individuals, despite high F_{ST}, suggests that admixture preceded colonisation. The lack of admixture in some highly resistant populations, such as populations H2 and H3, probably reflects strong selection for resistant individuals that happened to be non-admixed.

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References

Baylis AD (2000) Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest Management Science* **56**, 299–308.

Chandler CH, Chari S & Dworkin I (2013) Does your gene need a background check? How genetic background impacts the analysis of mutations, genes, and evolution. *Trends in Genetics* **29**, 358–366.

Chapman MA & Abbott RJ (2010) Introgression of fitness genes across a ploidy barrier. *New Phytologist* **186**, 63–71.

Délye C, Jasieniuk M & LeCorre V (2013) Deciphering the evolution of herbicide resistance in weeds. *Trends in Genetics* **29**, 649–658.

Dieringer D & Schlötterer C (2003) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes*, **3**, 167–169.

Evanno G, Regnaut S & Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611–2620.

Felsenstein J (2005) *PHYLIP (Phylogeny Inference Package) version 3.6*. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.

Gaines TA, Ward SM, Bukun B, Preston C, Leach JE & Westra P (2012) Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in *Amaranthus* species. *Evolutionary Applications* **5**, 29–38.

Gao H, Williamson S & Bustamante CD (2007) A Markov chain Monte Carlo approach for joint inference of population structure and inbreeding rates from multilocus genotype data. *Genetics* **176**, 1635–1651.

Ge X, d'Avignon DA, Ackerman JJH, Duncan B, Spaur MB & Sammons RD (2011) Glyphosate-resistant

horseweed made sensitive to glyphosate: low-temperature suppression of glyphosate vacuolar sequestration revealed by ^{31}P NMR. *Pest Management Science* **67**, 1215–1221.

Goudet J (2002) *FSTAT: A Program to Estimate and Test Gene Diversities and Fixation Indices* (version 2.9.3.2). Available at: http://www2.unil.ch/popgen/softwares/fstat.htm (last accessed 21 September 2012).

Heap I (2013) *The International Survey of Herbicide Resistant Weeds*. Available at: http://www.weedscience.com (last accessed 29 May 2013).

Jakobsson M & Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801–1806.

Jasieniuk M, Brûlé-Babel AL & Morrison IN (1996) The evolution and genetics of herbicide resistance in weeds. *Weed Science* **44**, 176–193.

Lande R (1980) The genetic covariance between characters maintained by pleiotropic mutations. *Genetics* **94**, 203–215.

Maxwell BD, Roush ML & Radosevich SR (1990) Predicting the evolution and dynamics of herbicide resistance in weed populations. *Weed Technology* **4**, 2–13.

Moretti ML, Hanson BD, Hembree KJ & Shrestha A (2013) Glyphosate resistance is more variable than paraquat resistance in a multiple-resistant hairy fleabane (*Conyza bonariensis*) population. *Weed Science* **61**, 396–402.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a number of individuals. *Genetics* **89**, 538–590.

Noyes RD (2000) Biogeographical and evolutionary insights on *Erigeron* and allies (Asteraceae) from ITS sequence data. *Plant Systematics and Evolution* **220**, 93–114.

Okada M & Jasieniuk M (2014) Inheritance of glyphosate resistance in *Conyza bonariensis* from California. *Weed Science* **62**, 258–266.

Okada M, Hanson BD, Hembree KJ *et al.* (2013) Evolution and spread of glyphosate resistance in *Conyza canadensis* in California. *Evolutionary Applications* **6**, 761–777.

Palumbi SR (2001) Humans as the world's greatest evolutionary force. *Science* **293**, 1786–1790.

Paris M, Roux F, Bérard A & Reboud X (2008) The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in *Arabidopsis thaliana*. *Heredity* **101**, 499–506.

Powles SB (2008) Evolved glyphosate-resistant weeds around the world: lessons to be learnt. *Pest Management Science* **64**, 360–365.

Pritchard JK, Stephens M & Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **1555**, 945–959.

R Development Core Team (2011) *R: A Language and Environment for Statistical Computing*. R Development Core Team, Vienna, Austria.

Schwander T & Leimar O (2011) Genes as leaders and followers in evolution. *Trends in Ecology and Evolution* **26**, 143–151.

Shrestha A, Hembree KJ & Va N (2007) Growth stage influences level of resistance in glyphosate-resistant horseweed. *California Agriculture* **61**, 67–70.

Shrestha A, Hembree KJ & Hanson BD (2008a) Glyphosate-resistant hairy fleabane documented in the Central Valley. *California Agriculture* **62**, 116–119.

Shrestha A, Hembree K & Wright S (2008b) *Biology and Management of Horseweed and Hairy Fleabane in California*. University of California Agriculture and Natural Resources publication No. 8314. Available at http://anrcatalog.ucdavis.edu/pdf/8314.pdf.

Sokal RR & Rohlf FJ (1995) *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd Edn, pp. 763–767. W.H. Freeman and Company, New York, USA.

Thébaud C & Abbott RJ (1995) Characterization of invasive *Conyza* species (Asteraceae) in Europe: quantitative trait and isozyme analysis. *American Journal of Botany* **82**, 360–368.

Urbano JM, Borrego A, Torres V *et al.* (2007) Glyphosate-resistant hairy fleabane (*Conyza bonariensis*) in Spain. *Weed Technology* **21**, 396–401.

Vila-Aiub MM, Neve P & Powles SB (2009) Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytologist* **184**, 751–767.

Walker S, Bell K, Robinson G & Widderick M (2011) Flaxleaf fleabane (*Conyza bonariensis*) populations have developed glyphosate resistance in north-east Australian cropping fields. *Crop Protection* **30**, 311–317.

Yuan JS, Abercrombie LLG, Cao Y *et al.* (2010) Functional genomics analysis of horseweed (*Conyza canadensis*) with special reference to the evolution of non-target-site glyphosate resistance. *Weed Science* **58**, 109–117.

Zelaya IA, Owen MDK & Van Gessel MJ (2004) Inheritance of evolved glyphosate resistance in *Conyza canadensis* (L.) Cronq. *Theoretical and Applied Genetics* **110**, 58–70.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Genetic diversity detected at 15 microsatellite loci in 1097 individuals from 37 populations of *Conyza bonariensis*.

Table S2 Genetic diversity and selfing rate estimates within populations of *Conyza bonariensis*.

Table S3 Pairwise F_{ST} values between *Conyza bonariensis* populations.

Figure S1 Geographical distribution of sampled populations of *Conyza bonariensis* and orchard and vineyard cropping systems in the Central Valley of California. Closed triangles indicate populations sampled for leaf tissue and seed used in microsatellite marker genotyping and assessment of response to glyphosate, respectively; open triangles indicate populations sampled only for leaf tissue.

Figure S2 A dendrogram on pairwise distances between *Conyza bonariensis* populations based on the Fitch-Margoliash method. Bootstrap values greater than 50% at nodes are indicated. Five groups of popu-

lations identified in the population pairwise F_{ST} analysis are indicated.

Figure S3 The number of populations and individuals in which multilocus genotypes (MLGs) were found in *C. bonariensis*. The number of individuals of each MLG is indicated on top of the bar. The remaining 66 MLGs were only found in a single population.

Figure S4 Bayesian clustering analysis (INSTRUCT, Gao *et al.*, 2007) of the two subclusters identified when the analysis was performed using all individuals in *Conyza bonariensis*. Plot of the log likelihood of the multilocus genotypic data, *ln* $P(D)$, (diamonds) for five runs at each value of K, and the second order rate of change in ln $P(D)$, ΔK, (circles) as a function of the number of clusters or gene pools, K, within first (a) and second (b) subclusters.