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The spread of resistance to acetolactate synthase inhibiting herbicides in a wind borne, self-pollinated weed species, *Lactuca serriola* L.

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Abstract Resistance to ALS-inhibiting herbicides in *Lactuca serriola* first appeared in the northern Yorke Peninsula in South Australia in 1994, with resistance soon observed at a number of additional sites. The rapid appearance of resistance at many sites could be attributed to a number of independent selection events or to movement of resistant seed from the original field. ISSRs were used to genotype plants collected in 1999 and 2004 from roadsides or fields in an attempt to determine the importance of these two factors in the spread of herbicide resistance in *L. serriola*. In 1999 and 2004, chlorsulfuron-resistant *L. serriola* plants were found in both fields and roadsides with resistant plants being more frequent in fields than roadsides and more frequent in 2004 than in 1999. Genetic relationships generated using UPGMA analysis indicated the presence of more than one genotype within the herbicide resistant populations sampled for both years and suggested independent selection as well as movement of resistant seed had occurred. DNA extracted from samples collected in 1999 was used to sequence a highly conserved region of the ALS gene that coded for a single amino acid modification within the gene. Four different mutations were identified within the resistant samples and these mutations tended to cluster on a geographical basis. Together these data provide evidence for both multiple independent evolutionary events and for the potential movement of individual genotypes as far as 43 km in the region.

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Introduction

Herbicide resistance has evolved in many weed species in a large number of countries (Heap 1997). Herbicide resistance evolves as a consequence of the intensive use of herbicides that control susceptible individuals within a population, but allow resistant individuals to survive and set seed. In this way, herbicide resistance is an example of evolution as a consequence of environmental changes caused by agricultural practices (Maxwell and Mortimer 1994). Wind, water or mechanical dispersal of seed or pollen can influence the pattern of movement of resistance genes.

Wind borne pollen can certainly travel long distances, with distances of 20 km being recorded (Watrud et al. 2004). Such dispersal distances can result in new isolated resistant infestations occurring in sites where resistance had previously not been present despite the efforts of land managers to minimize the risks of herbicide resistance developing. Glyphosate resistant *Conyza Canadensis*, a self-fertilized weed with wind borne seed, now infests more than 44,000 ha of arable land in the USA, with agricultural practices and seed dispersal contributing to the rapid spread of resistant genotypes (Dauer et al. 2007). Long distance dispersal is difficult to quantify and, in a wind borne seed, complicated by wind patterns, including updrafts causing sporadic random long distance movement (Dauer et al. 2007). Thus, despite their best efforts, land managers may face incursions of thousands of resistant seeds in a single

season. Therefore, it is unsurprising that a key question often posed in relation to herbicide resistance is whether resistance is independently selected at each site or whether resistance spreads from one site to another (Andrews et al. 1998; Cavan et al. 1998).

Lactuca serriola is a common weed in fields, waste land and along roadsides (Schoennagel and Waller 1999). It competes with crops, but has its major impact in reducing harvest efficiency because the flower buds are cut together with the grain, are difficult to screen out, with the resulting contamination leading to the value of the grain being discounted (Amor 1986). Likewise, the thick stems of *L. serriola* clog the harvesting machinery and exude a milky sap, which increases the moisture content of the grain and devalues it (Amor 1986). *L. serriola* is self-compatible and self-pollinated with little evidence of interspecific hybridization and reproduces only by seed (Ferakova 1977; Mejias 1994). The seed are light and small (0.40 mg) with a comparatively large pappus (radius 3 mm) and are spread by wind (Weaver and Downs 2003). *L. serriola* has large seed production, a short-lived seedbank (1–3 years) and no primary dormancy (Marks and Prince 1981, 1982; Alcocer-Ruthling et al. 1992).

Herbicide resistance has evolved in *L. serriola*, with sulfonylurea resistance first reported from a no till crop in Idaho in 1987 (Mallory-Smith et al. 1990). Herbicide-resistant *L. serriola* now occurs in several states of the US (Heap 2005). Sulfonylurea-resistant *L. serriola* was first recorded in South Australia in 1994 and has since been reported from a number of sites in this state (Preston et al. 2006). The potential for rapid spread of this weed is great, given the seed is spread by wind. Thus, the potential for spread of *L. serriola* resistant to ALS-inhibiting herbicides is also high. However, in areas where reliance on the use of sulfonylurea herbicides is extensive, there is also the possibility that independent mutation events can, and will, occur. These characteristics make *L. serriola* an ideal species for studying the role of gene flow by seed in the spread of herbicide resistance.

In order to understand the potential spread of herbicide-resistant *L. serriola*, it is important to determine whether individual sites with resistance are related or not. Molecular markers are effective methods to distinguish the genetic relatedness of different populations with resistance. ISSRs (inter simple sequence repeats) have some advantages compared to other molecular markers because they normally generate a higher proportion of polymorphic bands than RAPDs, are more reproducible than RAPDs, the cost is lower than AFLPS and there is no requirement for knowledge of flanking sequences for primer design (Meyer et al. 1993; Gupta et al. 1994; Wu et al. 1994; Zietkiewicz et al. 1994; Souframanien and Gopalakrishna 2004). Furthermore, ISSRs combine many of the benefits of AFLPs, SSRs and RAPDs (Reddy et al. 2002) and are effective in revealing intra-genomic and inter-genomic diversity (Zietkiewicz et al. 1994).

In addition to genetic fingerprinting, it is possible to also use mutations within specific genes to help elucidate patterns of herbicide resistance movement. Eberlein et al. (1997) investigated the mechanism of ALS-inhibiting herbicide resistance in a population of *L. serriola* from Idaho and found resistance was due to a modification of ALS. The mutation conferring resistance to ALS-inhibiting herbicides in *L. serriola* in Idaho was determined to be in Domain A of the ALS gene, with a proline residue changed to a histidine residue (Guttieri et al. 1992). Preston et al. (2006) investigated the mechanism of resistance to ALS-inhibiting herbicides in two South Australian populations and determined the mechanism in these populations was also due to a modification of ALS in these two cases, with the proline residue changed to threonine.

The aim of this research was to determine the genetic relationships among resistant populations of *L. serriola* in order to determine whether gene flow or independent selection were more important for the widespread distribution of resistance across the northern Yorke Peninsula of South Australia. A combination of ISSR markers and partial sequencing of the ALS gene were employed to determine the genetic relationships within and among resistant populations of *L. serriola* to elucidate whether several mutations conferring resistance to ALS-inhibiting herbicides have occurred in *L. serriola* within a relatively small geographical area.

Materials and methods

Plant material and DNA extraction

Individual plants were collected from cereal fields and along adjacent roadsides in 1999 and 2004. In 1999, seed from *L. serriola* plants were collected across a region of approximately 360 km^2 centred on Bute (33°52′S, 138°00′E), South Australia. In 2004, seedlings were collected from roadsides and adjacent fields along 27 km of road within this area. Seed from two susceptible populations (Waite and Paradise) were collected in 1995 from waste ground around Adelaide where no ALS-inhibiting herbicides had ever been used (Preston et al. 2006). Seed from the 1999 collection were germinated in 17-cm diameter pots filled with potting soil. At the 4–6 leaf stage, leaf material was collected from a single seedling and frozen in liquid N_2. The remaining seedlings were treated with 15 g a.i. ha^{-1} chlorsulfuron plus 0.2% non-ionic surfactant. The herbicide was applied in a laboratory-built spray cabinet fitted with a moving boom. The herbicide was delivered through two flat-fan nozzles

positioned 40 cm above the plants. The nozzle output was 103 l ha^{-1} at a speed of 1 m s^{-1} and a pressure of 250 kPa. Seedlings from the 2004 collection were transplanted individually into 17-cm diameter pots and allowed to recover for 14 days. Leaf material was removed from each plant and frozen in liquid N_2, after which the plants were treated with chlorsulfuron at 15 g a.i. ha^{-1} as described above. Seedlings from both collections were assessed 21 days after treatment. Resistant seedlings remained green and continued to grow, whereas susceptible seedlings were killed. DNA was extracted by CTAB method (Doyle and Doyle 1987) from 40 mg frozen leaf material and quantified on agarose gels. DNA was stored at −80°C.

ISSR and data analysis

ISSR PCR reactions were conducted separately on 25 individual plants (one from each of 25 populations) collected in 1999 along with the two control populations and on 39 individuals from six populations collected in 2004. Eight ISSR primers were screened and four primers that amplified a number of bands showing polymorphisms were selected for use (Table 1). PCR amplifications were performed in a total volume of 20 µl, containing 1 mM Tris–HCl (pH 9.0), 5 mM KCl, 0.01% Triton X-100, 1.5mM $MgCl_2$, 0.25 mM dNTP, 1 µM primer, 1 U *Taq* polymerase and 5 ng DNA template, using a program of 30 cycles of 94°C for 15 s; 52°C for 15 s; 72°C for 30 s and finished with 72°C for 2 min. PCR products were separated on polyacrylamide gels (Amersham Biosciences) at 10°C, 200 V, 20 mA, 10 W for 20 min; 380 V, 30 mA, 20 W for 50 min and 450 V, 30 mA, 20 W for 30 min. The gels were stained using the DNA Plus One™ Silver Staining Kit (Amersham Biosciences). At least two PCR amplifications were performed for each sample.

Lanes on the gels were compared and the presence or absence of bands scored. Dendrograms were constructed by UPGMA cluster analysis (Nei 1978) and genetic distances were determined based on Nei's method (1972, 1978) using the software TFPGA (Tools for Population Genetic Analysis). Geographical distances between samples in the 1999 collection were calculated using OziExplorer Version 3.95.4G (D & L Software Pty. Ltd, Australia). Correlation between genetic and geographic distances between the samples was investigated using a Mantel test (Hood 2004), assuming the null hypothesis, that the observed relationship between the two distance matrices could have occurred by any random arrangement in space.

Table 1 Sequences of ISSR primers used for genotyping *L. serriola*

Primer	Sequence
880	$HVH(GT)_7$
888	$BDB(CA)_7$
889	$DBD(AC)_7$
891	$HVH(TG)_7$

Sequencing domain A of the ALS gene

DNA from the samples collected in 1999, and used for ISSR genotyping, was used to amplify a highly conserved region of the ALS gene in *L. serriola*. Two oligonucleotide primers previously described by Boutsalis (1996), 5′-CGTGGATCCTMGTTACTCAACAA-3′ and 5′-GCATG TCTAGAACGTCCTTCCYCGTCACGAACA-3′, were used to amplify a band that was expected to be 193 bp. All PCR amplifications were performed in 50 µl reactions. The PCR mixture consisted of 0.2 µM of each primer; 200 µM of each deoxynucleotide-5′-triphosphate (dNTP), 3.5 mM $MgCl_2$, 5 µl of 10× thermophilic buffer (Promega, USA), 1 µL of DNA sample and 2.5 Units of Taq polymerase (Promega, USA), which was added after the initial denaturation. Amplifications were carried out in a heated lid PCR machine (MJ Research, model PTC-100), with the following cycle: denature at 94°C for 8.5 min, hold at 94°C while adding the Taq polymerase, denature at 94° for 1.5 min, anneal at 50°C for 2 min, elongate at 72°C for 2 min and cycle to second denaturation step 34 more times.

All PCR products were separated on a 2% TAE agarose gel, with a 100 bp standard molecular weight marker to determine the relevant band. This band was excised following ethidium bromide staining and purified using a Wizard DNA kit (Promega, USA). The purified PCR products, believed to contain domain A of the ALS gene, were sequenced at the Biomolecular Research Facility, Newcastle University using an Applied Biosystems (ABI) 377 DNA sequencer. Sequences were aligned using DNAMan (Lynnon Corporation, Canada).

Results

Resistance to chlorsulfuron

In 1999, seed of 85 populations were collected from 67 locations. Of the 85 populations, seed of 58 germinated. Resistance to chlorsulfuron was found in at least one individual in 38 *L. serriola* populations with 20 populations fully susceptible. The proportion of resistant populations was 65.5%. Of the 38 resistant populations, 20 were collected along roadsides and 18 were collected from fields. Resistance was present in 75% of field populations tested and 59% of roadside populations. In 2004, 11 populations were collected from seven sites. At four sites, *L. serriola* was growing in the field and along the adjacent roadside. At the other sites, *L. serriola* was only found growing along

the roadside. Of the 11 populations collected, two were susceptible, the others all contained resistant individuals. The proportion of resistant populations was 82%. All *L. serriola* populations collected from fields in 2004 contained resistant individuals, whereas 74% of the populations from roadsides contained resistant individuals.

ISSR genotypes

The location and resistance status of individuals from the 1999 collection used for fingerprinting are given in Table 2. A total of 179 bands ranging from 200 to 1,500 bp were scored for the 27 samples (25 individuals collected in 1999 and the two control samples) across all four primers. Of these bands, 89 or 49.7% were polymorphic (Table 3). In 2004, between five and eight individuals from each of six populations were genotyped. All individuals genotyped were resistant to chlorsulfuron. The location of samples and number of individuals genotyped for each population are given in Table 4. For the 39 samples collected in 2004, a total of 67 bands were scored across all four primers, of which 15 or 23.9% were polymorphic (Table 5). An example of the banding patterns observed is given in Fig. 1 for primer 891 showing the two control populations, Waite and Paradise, and six populations from the 1999 collection.

Table 2 Individuals in the 1999 collection used in ISSR genotyping with their locations and resistance status

Individual	Source	Resistance status
6-09	Field	Resistant
12-02	Roadside	Susceptible
13-06	Field	Resistant
17-02	Roadside	Susceptible
20-12	Field	Susceptible
22-08	Field	Resistant
24-10	Field	Resistant
25-03	Roadside	Resistant
26-03	Field	Resistant
40-13	Roadside	Susceptible
57-14	Roadside	Resistant
59-01	Field	Resistant
68-15	Roadside	Susceptible
73-30	Roadside	Resistant
76-01	Roadside	Susceptible
81-01	Roadside	Resistant
83-12	Roadside	Resistant
84-04	Roadside	Resistant
86-01	Roadside	Resistant
88-10	Roadside	Resistant
99-01	Field	Resistant
106-04	Field	Resistant
110-01	Roadside	Susceptible
112-08	Field	Resistant
117-08	Roadside	Resistant

Table 3 The number of loci scored and the proportion of polymorphic loci for each of the four primers used on the 25 samples from the 1999 collection and the two control samples, Waite and Paradise

Primer	Loci scored	Number of polymorphic loci	Polymorphic loci (%)
880	46	9	19.6
888	44	21	47.7
889	34	19	55.9
891	55	40	72.7
Total bands analysed	179	89	49.7

Table 4 Location and resistance status of individuals from the 2004 collection used for ISSR genotyping

Population number	Source	Resistance status	Number of individuals genotyped
1	Roadside	Resistant	7
2	Field	Resistant	7
3	Roadside	Resistant	7
4	Field	Resistant	8
10	Roadside	Resistant	5
11	Field	Resistant	5

UPGMA clusters and genetic relationships

The 27 samples in the 1999 collection and the two control samples formed 20 UPGMA clusters (Fig. 2). From these samples, 17 genotypes were represented once and three genotypes more than once. The two outlying control samples from the Adelaide region were genetically the most different among the 27 samples studied. Of the ten samples that grouped, one group of three individuals was susceptible and the other two groups of three and four individuals were resistant. The identical susceptible individuals were

Table 5 The number of loci scored and proportion of polymorphic loci for each of the four primers used on the 39 samples from the 2004 collection

Primer	Loci scored	Number of polymorphic loci	Polymorphic loci (%)
880	19	5	26.3
888	11	4	36.4
889	13	3	23.8
891	24	4	16.7
Total bands analysed	67	16	23.9

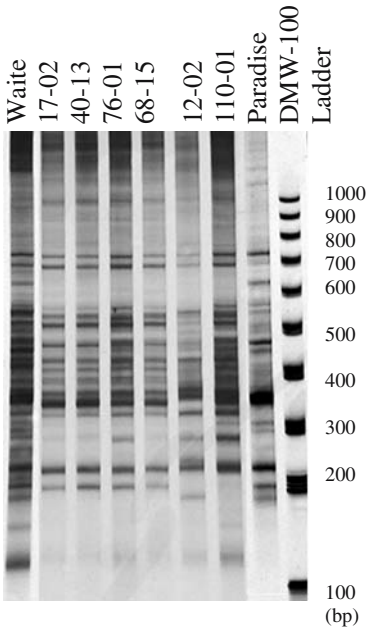

Fig. 1 Example of the ISSR banding patterns for selected *L. serriola* samples collected in Bute, South Australia and in Adelaide (Waite and Paradise) generated by primer 891

not geographically close. Individual 17-02 was 66 km from individual 40-13 and 29 km from individual 20-12. In one of the groups of identical resistant individuals, two individuals, 106-04 and 99-01, were 8 km apart; however, the third individual, 13-06, was located 43 km away. The other group of identical resistant individuals contained four individuals of which three, 84-04, 81-01 and 83-12, occurred within 10 km of each other. The other individual, 22-08, was from 43 km away.

The 39 samples from six populations in the 2004 collection formed nine clusters in the UPGMA dendrogram (Fig. 3). One cluster contained 25 individuals from populations 1, 2, 3 and 4, which were genetically identical. These samples were collected from two sites less than 5 km from each other. The remaining two individuals from population 4 separated into a single cluster, whereas the remaining two individuals in population 1 were in separate clusters. Individuals from populations 10 and 11 separated into five genetic clusters. Individuals 10-7, 10-8 and 10-10 formed two separated clusters, closely related to each other, but more diverse from the other individuals. The genetic distances between all individuals in populations 1, 2, 3 and 4 was small, whereas populations 10 and 11 were both more diverse and more different. The latter were located about 27 km from populations 1 and 2.

Natural migration of genotypes may be influenced by wind or mechanical movement on farm machinery. Spatial analysis of genetic and geographic patterns among the local population can provide evidence that spread of genotypes is random or correlated with distance. The 1999 collection showed no correlation between genotype or geographical distance (Mantel co-efficient $r = 0.055$, $P = 0.10$) indicating an erratic spatial distribution of genotypes in the region surrounding Bute.

Sequencing of domain A of the ALS gene

When domain A of ALS was sequenced, four distinct mutations were identified at the pro_{197} site previously identified

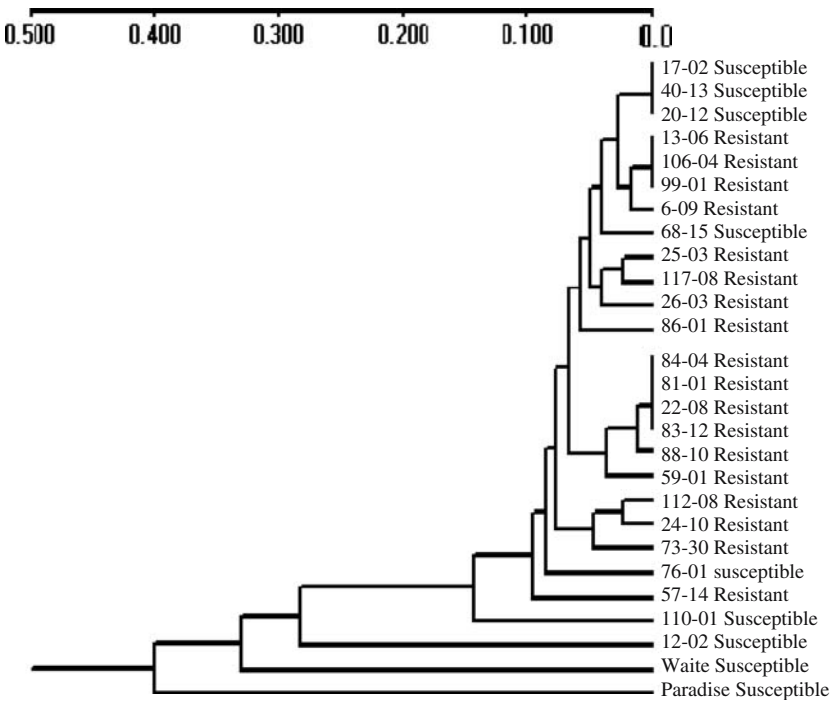

Fig. 2 UPGMA dendrogram of *L. serriola* in the 1999 collection and the two control samples (Waite and Paradise)

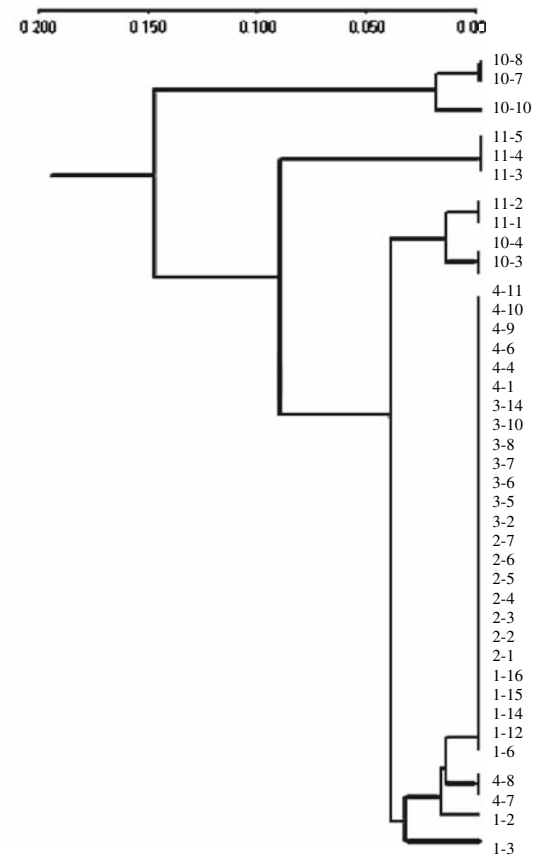

Fig. 3 UPGMA dendrogram of the *L. serriola* in the 2004 collection, individuals from six populations described in Table 2

by Guttieri et al. (1992) (Table 6). These included the pro_{197} to thr and pro_{197} to his mutations already reported in the literature for *L. serriola* (Guttieri et al. 1992; Preston et al. 2006). Two additional mutations were identified. These were pro_{197} to ser and pro_{197} to leu. The only other polymorphism identified in the 193 bp region sequenced was a G to A change, resulting in a cys_{163} to tyr change. This change was observed in two resistant individuals and one susceptible individual. In addition, two samples were tested as resistant to sulfonylurea herbicides, but showed no pro_{197} substitution. It is known that mutations in other conserved regions of the ALS gene also confer resistance to ALS-inhibiting herbicides (Tranel and Wright 2002) and it is likely these two samples contain mutations elsewhere in the ALS gene. There was no apparent relationship between the clusters observed in the ISSR patterns and the polymorphisms observed from the sequence data. This provides additional support for the hypothesis of independent mutations being a major contributor to the evolution of herbicide resistance in the region.

Discussion

The genetic relationship of *L. serriola* samples collected in 1999 and 2004 inferred from the ISSR banding patterns suggested that variation between samples increased with increasing geographic distance when the outliers from Waite and Paradise were considered. The genotypes of the control samples from the Adelaide region were quite different from the samples collected on the Yorke Peninsula in 1999, which is consistent with the geographic distance separating them. The Mantel test on the 1999 collection indicated that the distribution of genotypes in the local area was erratic and not correlated with geographical distance. The first record of resistance in this area was 5 years prior to the 1999 collection. During this time seed may have been moved widely by a variety of means, including those facilitated by human activity, and colonies established and become extinct.

The genetic variance of the samples collected in 1999 is also much greater than the genetic variance of the samples collected in 2004. In 1999 samples were collected over a much larger area and encompassed a much larger number of populations than samples collected in 2004. In the 2004 collection, the genotypes of 25 individuals were identical, reflecting the fact that these individuals had been collected over less than 5 km.

Table 6 The nucleotide sequence at cys_{163} and pro_{197} sites in Domain A of the ALS gene in the haplotypes of individuals from the 1999 collection

Pro substitution		Cys substitution		Individuals with mutation(s)		Resistance status
Mutation	Substitution	Mutation	Substitution	*N*	%	
–	–	–	–	12	48	R,S[a]
–	–	TAT	tyr	1	4	S
ACC	thr	TAT	tyr	2	8	R
ACC	thr	–	–	3	12	R
TCC	ser	–	–	2	8	R
CAC	his	–	–	4	16	R
CTC	leu	–	–	1	4	R

The table gives the number of individuals with each haplotype and their resistance status

[a] *R* resistant, *S* susceptible; two individuals recorded as resistant

There was some evidence from the sequencing data that distinct mutations clustered on a geographical basis, but at least three different mutations conferring herbicide resistance occurred within a 5 km radius (Fig. 4). It is impossible to determine whether the resistant genotypes recorded in the study area are of allochtonous origin or have been transported to the site. However, the genetic relationships inferred by ISSR genotypes combined with the sequence data confirmed that resistance to ALS-inhibiting herbicides in the area surrounding Bute, South Australia has both evolved independently under selection pressure and has probably been distributed by wind borne seed. This is further supported by the stochastic pattern of geographical distribution.

L. serriola is self-pollinated, so gene flow between neighboring plants will be very rare. Therefore, the evolution of resistance in different genotypes suggests that separate selection events had occurred. In the 1999 collection, there were 13 different genotypes present among the resistant individuals, suggesting resistance had evolved at least 13 times in the region. All the individuals genotyped from the 2004 collection were resistant and these separated into nine genotypes. This provided further evidence for the existence of independent selection events for resistance. Lastly, five different mutations within Domain A of ALS were detected by sequencing.

Although independent selection was probably more important in the evolution of resistance in *L. serriola*, seed movement could also have contributed to the spread of herbicide resistance in the area. In 1999 and 2004, resistant individuals with identical genotypes were identified, at least as far as could be determined by ISSRs. The 25 identical individuals from four populations in the 2004 collection were probably from the same progenitor and were likely to have been spread by seed movement in the area within a 5 km in radius. The two groups of identical genetic clusters of resistant plants in the 1999 collection also indicated the importance of seed movement and that spread could be as far as 43 km. Such long distance spread could have occurred over several generations.

Fig. 4 Area surrounding Bute, South Australia, showing spatial distribution of the mutations found in *L. serriola* surveyed in 1999 at the CCC (pro197) site of Domain A of the ALS gene

It was apparent that a considerable percentage of the *L. serriola* plants that occur on roadsides on the Yorke Peninsula are resistant to chlorsulfuron, despite the fact that ALS-inhibiting herbicides are not used on roadside vegetation. Therefore, resistance must have first originated in cropped fields and spread to roadsides. The proportion of resistant populations on the roadsides had increased between 1999 and 2004. This suggests considerable movement of seed from cropped fields to roadsides for this species. Since the seed bank of *L. serriola* is relatively short-lived (Marks and Prince 1981, 1982; Alcocer-Ruthling et al. 1992), the genetic analysis presented here would be consistent with a situation where *L. serriola* populations are transient at any one locality. That is, populations rapidly become locally extinct, but the locality is re-colonised from outside. Therefore, as use of sulfonylurea herbicides continues in fields, it will be expected that the frequency of resistant individuals will increase across the whole area, not just within cropped fields. This indeed appears to be occurring. For example, at one locality in 1999, a resistant population occurred along the roadside, but plants in the adjacent field were susceptible. By 2004, at the same locality plants on both the roadside and in the adjacent field were resistant.

Research on *Avena fatua* has also pointed to the importance of seed movement in the spread of resistance (Andrews et al. 1998). Unlike *L. serriola*, the seed of *A. fatua* does not have structures that aid spread by wind and, therefore, *A. fatua* was most likely spread by harvesting machinery. One consequence of spread by farm machinery is that seed will not move to fields or other sites where the machinery does not go. This makes the spread characteristics different to *L. serriola*, where spread occurs by wind. In contrast, research on localizing the origins of herbicide resistant *Alopecurus myosuroides* suggested no obvious spread between patches (Cavan et al. 1998). The data collected here indicate that independent selection events have been the main driver for the widespread appearance of herbicide resistance in *L. serriola* on the Yorke Peninsula of South Australia. However, there is also evidence of movement of resistant genotypes from one locality to another.

Resistance to ALS-inhibiting herbicides in weed species presents an ongoing challenge to resistance management. This is particularly evident because ALS-inhibiting herbicides are among the most widely used and efficacious herbicides in the world. The pattern of herbicide use by

farmers and growers are significant factors in the evolution of herbicide resistance (Gressel 1982), yet farmers and growers face the issue of reduced options for control if a herbicide becomes ineffective on the spectra of weed species present in their system. Despite the ability of *L. serriola* to move via seed dispersal, the large number of independent mutations in herbicide resistant *L. serriola* detected here indicates rapid selection of resistance through the use of herbicides. Thus, the management of resistance in the area will require farmers to be willing to include alternative control methods for resistant weeds. There is little that can be done to prevent the introduction of resistant seed blown by the wind, but rotation of herbicides and crops is recommended to prevent the evolution and establishment of resistant biotypes, particularly when minimum or no till practices are followed.

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