Bulletin of the National Research Centre

RESEARCH Open Access

Genetic diversity and spread of glyphosate-resistant flaxleaf fleabane

Mohammed Hussein Minati*, Christopher Preston and Jenna Malone

Abstract

Background: Continual application of herbicides for flaxleaf fleabane control readily results in the evolution of herbicide resistance. Flaxleaf fleabane has evolved resistance to different modes of action herbicides in many countries. Due to the comprehensive geographical distribution of flaxleaf fleabane in Australia, it was classified as a cosmopolitan weed and it therefore has no specific climatic requirement.

Results: The high percentage of polymorphisms in the samples of the susceptible population (79.8%, 75%) suggests that susceptible populations of flaxleaf fleabane, even within one site, originated from a number of parents. However, the percentage of polymorphism in the resistant populations (51.5%, 66.8%) suggests that resistant populations of flaxleaf fleabane within one site could have originated from fewer parents. In addition, any site containing resistant and susceptible populations of flaxleaf fleabane may have been populated by a large number of parents, evidenced by the relatively high percentage of polymorphisms detected by amplified fragment length polymorphism (AFLP) analysis (86.5%). Despite the large geographic distances between collection locations, populations from across states clustered in several groups showing a close genetic relationship among these populations over these large distances. These high levels of genetic diversity within and between populations confirmed in the sequencing of enolpyruvylshikimate-3-phosphate above support the results of AFLP studies and gave the author more confidence to report the genetic diversity seen within and between population studies.

Conclusion: To prevent further resistance spread, flaxleaf fleabane management in infested areas should focus on decreasing seed movement from resistant sites as well as reducing the selection pressure for resistance to glyphosate by adopting alternative management strategies.

Keywords: AFLP, Flaxleaf fleabane, Genetic variation, Herbicide resistance

Introduction

Flaxleaf fleabane (*Conyza bonariensis*) is a weed species belonging to the family *Asteraceae* (Wu 2009) that predominantly occurs in cropping systems of northern, southern, and Western Australia. As a result of intensive use of the herbicide glyphosate in summer fallow operations, glyphosate-resistant populations of *C. bonariensis* have begun to appear, with the first resistant population (Randall 2017). This species is a self-fertilized annual weed, which is often found in pastures and summer and winter field crops including cotton, maize, chickpea, soybean, and wheat (Wu 2009; Heap 2008; Shrestha et al. 2008a). Importantly, Wu et al. (2006) reported that flaxleaf fleabane provides significant competition with crops for water and nutrients, particularly for stored soil moisture in crops of dryland sorghum and wheat, causing significant yield reduction (up to 31%).

The specific effect of glyphosate is to inhibit the biosynthesis of the aromatic amino acids (phenylalanine, tryptophan, and tyrosine) in plants. This inhibition causes a number of metabolic instabilities, such as protein synthesis inhibition and blocking of the shikimate pathway, resulting in metabolic disturbance (Nandula et al. 2005). Glyphosate binds to 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway which catalyzes the condensation of shikimate-3-phosphate and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate. Once plants come in contact with glyphosate, the shikimate pathway is blocked, hindering carbon flow to aromatic amino acids (Harring et al. 1998). Glyphosate is

* Correspondence: abo_azher70@yahoo.com
School of Agriculture, Food & Wine, Faculty of Sciences, The University of Adelaide, Waite Campus, Urrbrae, Australia

translocated rapidly to metabolic sites and kills the target weeds slowly after application (Franz et al. 1997; Duke et al. 2003).

Efforts are required to advance knowledge regarding the genetic diversity of resistant populations, which is needed to understand seed dispersal of flaxleaf fleabane to determine the best management strategies. A common technique used to study genetic diversity is amplified fragment length polymorphism (AFLP). It provides the opportunity to illustrate diversity at the genetic level, allows observation of polymorphism at a very large number of loci, and produces extremely reproducible markers with no need for prior sequence knowledge (Vos et al. 1995; Acquaah 2009). In this study, genetic diversity was investigated within single populations as an initial study to examine the possible level of outcrossing. In addition, the genetic diversity between populations was investigated to examine the evolution and spread of resistance. The main objectives of this study were to assess genetic variation and to estimate genetic relationships within and between populations of flaxleaf fleabane, using AFLP markers and sequencing of EPSP synthase.

Material and methods

Plant materials

Eighty-two populations of *C. bonariensis* had been previously collected in a 2011 roadside survey across Australia (Table 1, (Malone et al. 2012)). These populations had been tested for glyphosate resistance using 1500 g ha^{-1} (Touchdown 500 g/L, Monsanto, Melbourne, Victoria, Australia), and results had shown that approximately 50% were susceptible and 50% were resistant (Malone et al. 2012). To assess genetic relationships within and between populations of flaxleaf fleabane, a single individual from each of these populations was used in a "between populations" analysis, while 20 individuals of the susceptible population FBTAR and the resistant population SEQLD07 were used for a "within populations" study.

For comparison with the target populations of flaxleaf fleabane, two populations of tall fleabane (*Conyza sumatrensis*) were collected from two different locations in South Australia: Dernancourt (FTALLH) and Paradise (FTALLP) as controls.

Seed germination and plant growth

In a glasshouse, distinct trays (30 × 20 × 10 cm) comprising coca peat soil, roughly 0.1–0.2 g of seeds from each population, were sown. Seeds were sown on the surface of soil and watered regularly as needed until a suitable number of seedlings had developed for each population. For the "within populations" experiment, seedlings were transferred into normal pots at a rate of 5–10 seedlings per pot. The plants were well-looked-after under natural growing conditions (outdoor) during the growing period and sprayed with a mist spray daily as required until sampling for DNA extraction. Also, the two populations of tall fleabane were germinated in separate trays (30 × 20 × 10 cm). At 6 weeks after germination, plants were transplanted into standard pots and transferred outdoors to be maintained until sampling for DNA extraction.

The "within population" experiment was carried out at the Waite Campus of the University of Adelaide, South Australia, in March 2012.

Sampling and plant DNA extraction

Leaf tissue was collected from a single individual from all 82 fleabane populations for the "between populations" study, as well as 20 susceptible individuals from population FBTARS and the same number of resistant individuals from population SEQLD7R for the "within population" study.

For the "between populations" study, leaf tissue was collected with no herbicide application, while for the "within populations" study, leaf tissues were collected first, and then, all individuals were treated to confirm susceptibility or resistance to glyphosate. Approximately 0.25 g of young leaf tissue was cut with sterilized scissors from plants and transferred to a sterile 1.5 mL microcentrifuge tube. Tubes were placed in a small ice box, transported to the laboratory, and stored in a freezer at − 80 °C until DNA extraction. Three-millimeter ball bearings were inserted into each micro-centrifuge tube containing the leaf samples and were homogenized under liquid nitrogen using a vortex or Retsch mill (Retsch GmbH, Haan, Germany). DNA was extracted using Isolate Plant DNA Mini Kit (Bioline, Australia) as per the manufacturer's instructions. The concentration of extracted DNA was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) at 280 nm. DNA samples were stored in the freezer at − 20 °C until DNA amplification.

Amplified fragment length polymorphisms

The AFLP protocol designed by Vos et al. (1995) provided the initial basis for the AFLP technique used here, with the exception that the methylation-sensitive restriction enzyme *Pst*I was substituted for *Eco*RI as it is identified to yield low-copy DNA constructively (Burr et al. 1988). Oligonucleotide sequences of the adaptors are shown in Table 2. Standard solutions were set at 200 μM for both adaptors [(*Mse*I adapters at 50 μL each + 100 μL nanopure water) and (*Pst*I adapters at 5 μL each + 190 μL nanopure water)]. Stocks were heated at 90 °C for 3 min and kept at room temperature for 30 min. All digestion/ligation reactions were performed in 60 μL reaction mixtures containing 20 μL of isolated genomic

Table 1 Names, locations, and response to glyphosate of the 82 populations of flaxleaf fleabane used in this AFLP analysis. Data in boldface shows populations selected for the dose response experiment

Location	Study label	Response to glyphosate
SNSW	**FLE01**	**Resistant**
SNSW	**FLE02**	**Resistant**
SNSW	FLE03	Resistant
SNSW	FLE04	Resistant
SNSW	FLE05	Resistant
SNSW	FLE06	Resistant
SNSW	FLE07	Resistant
SNSW	**FLE08**	**Resistant**
SNSW	FLE09	Resistant
SNSW	FLE10	Resistant
SNSW	FLE11	Resistant
SNSW	FLE12	Resistant
SNSW	FLE13	Resistant
SNSW	FLE14	Susceptible
SNSW	FLE15	Resistant
SNSW	FLE16	Susceptible
SNSW	FLE17	Resistant
SNSW	FLE18	Susceptible
SNSW	FLE19	Resistant
SNSW	FLE20	Resistant
SNSW	FLE21	Susceptible
SNSW	FLE22	Resistant
SNSW	**FLE23**	**Resistant**
SNSW	FLE24	Resistant
SNSW	FLE25	Resistant
SNSW	FLE26	Resistant
SNSW	FLE27	Resistant
SA	**FB01C**	**Susceptible**
SA	**FB02C**	**Resistant**
SA	**FB03C**	**Resistant**
SA	FB04C	Resistant
SA	**FB05C**	**Resistant**
SA	FB06C	Resistant
SA	**FB07C**	**Resistant**
SA	FB08C	Susceptible
SA	FBWIR	Susceptible
SA	FBTAR	Susceptible
NNSW	NNSW01	Susceptible
NNSW	NNSW02	Susceptible
NNSW	NNSW03	Susceptible
NNSW	NNSW04	Resistant
NNSW	NNSW05	Susceptible
NNSW	**NNSW06**	**Resistant**
NNSW	NNSW08	Resistant
NNSW	NNSW09	Resistant
NNSW	NNSW10	Susceptible
NNSW	NNSW11	Resistant
NNSW	NNSW12	Susceptible
NNSW	NNSW13	Resistant
NNSW	**NNSW14**	**Resistant**
NNSW	NNSW15	Susceptible
SEQSLD	SEQLD01	Resistant
SEQSLD	SEQLD02	Susceptible
SEQSLD	SEQLD3A	Resistant
SEQSLD	**SEQLD3B**	**Resistant**
SEQSLD	SEQLD04	Susceptible
SEQSLD	**SEQLD05**	**Resistant**
SEQSLD	SEQLD6	Resistant
SEQSLD	SEQLD07	Resistant
SEQSLD	SEQLD08	Resistant
VIC	FB01	Susceptible
VIC	FB02	Susceptible
VIC	FB03	Susceptible
VIC	FB04	Susceptible
VIC	FB05	Susceptible
VIC	FB06	Susceptible
VIC	FB07	Susceptible
VIC	FB08	Susceptible
VIC	FB09	Susceptible
VIC	FB10	Susceptible
VIC	FB11	Susceptible
VIC	FB12	Susceptible
VIC	FB13	Susceptible
VIC	FB14	Susceptible
WA	WA04	Susceptible
WA	WA17	Susceptible
WA	WA20	Susceptible
WA	WA24	Susceptible
WA	WA25	Susceptible
WA	WA26	Susceptible
WA	WA29	Susceptible
WA	WA30	Susceptible

SNSW Southern New South Wales, *SA* South Australia, *NNSW* Northern New South Wales, *SEQSLD* South-eastern Queensland, *VIC* Victoria, *WA* Western Australia

Table 2 Sequences (5′–3′) of adapters and primers used in adjusted AFLP for the within and between population studies and sequences (5′–3′) of primers used for the sequencing of the conserved region of EPSP. Data in boldface shows the most informative primers in terms of number of bands and polymorphisms

Adapters		
*Mse*I	*Mse*I adapter 1 *Mse*I adapter 2	GACGATGAGTCCTGAG TACTCAGGACTCAT
*Pst*I	*Pst*I adapter 1 *Pst*I adapter 2	CTCGTAGACTGCGTACATGCA TGTACGCAGTCTAC
Pre-amplification primers		
*Pst*I pre-selective primer A	*Pst*I + A primer	GACTGCGTACATGCAG-A
*Mse*I pre-selective primer C	*Mse*I + C primer	GATGAGTCCTGAGTAA-C
Selective amplification primers		
***Pst*I selective primer ATC**	***Pst*I + ATC primer**	**GACTGCGTACATGCAG-ATC**
*Mse*I selective primer CAG + (TET)	*Mse*I + CAG Fluro (TET) primer	GATGAGTCCTGAGTAA-CAG
*Mse*I selective primer CAC + (NED)	*Mse*I + CAC Fluro (NED) primer	═ -CAC
***Mse*I selective primer CAT + (FAM)**	***Mse*I + CAT Fluro (FAM) primer**	**═ -CAT**
***Mse*I selective primer CAA + (VIC)**	***Mse*I + CAA Fluro (VIC) primer**	**═ -CAA**
*Mse*I selective primer CC + *Pst*I + primer AC	*Mse*I + CC primer + *Pst*I + AC primer	(GATGAGTCCTGAGTAA-CC+ GACTGCGTACATGCAG-AC)
*Mse*I selective primer CC + *Pst*I + primer AG	*Mse*I + CC primer + *Pst*I + AG primer	(GATGAGTCCTGAGTAA-CC + GACTGCGTACATGCAG-AG)
EPSPS primers		
Forward primer Reverse primer	Lr-EPSP_F Lr-EPSP_R	TGCAAAAAGAGCTGTAGTCGTTGGCT AGTTGTTTCAAACCGACAACTAAGTC

DNA and 40 μL of Master Mix (6 μL of RL buffer 10× (50 nM Tris-HCL at PH 7.5, 50 mM Mg-acetate, 250 mM K-acetate, and 25 mM DTT), 1 μL of each of the two restriction enzymes *Mse*I adaptors (5 units) and *Pst*I adapters (10 units), 1.2 μL of 10 mM ATP cofactor, 0.5 μL of *Mse*I Tru 9 l enzyme (5 units), 1 μL of *Pst*I CUTTER enzyme (10 units), 1 μL of T4 DNA ligase (1 unit/μL) enzyme, and 28.3 μL of nanopure water). The ligation/digestion was performed at 37 °C for 3 h.

Pre-amplification reactions were performed in 20 μL reaction mixtures containing 5.5 μL of the digested DNA and 14.5 μL of PCR Master Mix (2 μL of 10× ImmoBuffer, 0.8 μL of $Mgcl_2$ solution, 2 μL of dNTP Mix, 1 μL of *Mse*I + C primer (Table 2), 1 μL of *Pst*I + A primer (Table 2), 0.2 μL of Taq Immolase™, and 7.5 μL of nanopure water). PCR (Gradient Eppendorf Mastercycler®, Germany) was performed for 11 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 60 s, and an extension at 72 °C for 90 s and then dropping the annealing temperature by 0.7 °C per cycle, followed by 20 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s. At the end of the 20 cycles, a final extension was applied at 72 °C for 7 min and held at 4 °C until the next step (selective PCR). The pre-amplification reactions were diluted in the ratio of 1:8 with nanopure water (20 μL of DNA and 160 μL of nanopure water).

Selective amplification reactions were performed in duplicate on the selected samples with different primers to determine which primer yielded high numbers of visibly scorable polymorphic bands (Table 2). A first batch was performed as same as for the pre-amplification PCR above, except that 1 μL of *Pst*I + ATC primer and 1 μL of *Mse*I + CAT (FAM) primer were added. The second batch was prepared in the same way but in place of 1 μL of *Mse*I + CAA (VIC). Primer PCR was run using the following protocol: 94 °C for 30 s for denaturing, 65 °C for 30 s for annealing, and a 90-s extension at 72 °C, followed by 9 cycles for further denaturation, annealing, and extension in which each annealing cycle was reduced by 1 °C, followed by 25 PCR cycles of 94 °C for 30 s, 56 °C for 30 s, and 72°C for 90 s.

Capillary electrophoresis was performed using an Applied Biosystems 3730 analyzer by the Australian Genome Research Facility (AGRF), Australia.

Sequencing a conserved region of EPSP synthase

DNA extracted from leaf tissue of 13 populations of flaxleaf fleabane shown in boldface in Table 1 was used to amplify a conserved region of EPSPS following the methods of Wakelin and Preston (2006) using primers LrEPSP_F and LrEPSP_R (Table 2). The DNA fragments produced by the PCR reaction were sequenced by AGRF to obtain both forward and reverse sequence data using the same primers (LrEPSP_F and LrEPSP_R).

Data analysis

Recognizable AFLP bands were visually scored with Genemapper® (Applied Biosystems, Australia) software

to conclude or score genetic relationships. PopGene® (Canada, Department of Renewable Resources, http://www.ualberta.ca/~fyeh/popgene.pdf) software was used to calculate genetic distance. PopGene® is a Windows software-based application for analysis of genetic variation among and within populations through dominant markers including AFLPs. TreeView software version 1.6.6 (UK, Taxonomy Zoology, http://taxonomy.zoology.gla.ac.uk/rod/treeview/help/contents.html) was used to produce dendrograms for each population analysis, based on Nei's (1973) regular and unbiased inherited distance processes.

Sequence data of EPSP were assembled, compared, and analyzed using BioEdit Sequence Alignment Editor, version 7.1.3.0 Hall (Hall 1999).

Results

Within population study

Genetic diversity within a population was investigated in this experiment to examine possible outcrossing within populations. As found in several studies of other plant species, AFLPs have played an important role in genetic polymorphism investigation, and proved highly informative in assessing diversity in plants (Vos et al. 1995; Acquaah 2009; Teulat et al. 2000; Green et al. 2001).

*Mse*I selective primers, CAG + (TET) and CAC + (NED); *Mse*I selective primers, CC + *Pst*I + AC primer; and *Pst*I + AG primer all produced insufficient visibly scorable polymorphic bands and therefore were not used. Two of the 6 primer combinations used (shown in boldface in Table 2) were the most informative in terms of number of bands and polymorphisms. The number of peaks obtained from these primers was different depending on the primer combination used. Peaks lower than 900 and very close to each other were not considered. Of the loci, 194 were scored for the selective primer pair *Pst*I + ATC and *Mse*I + CAT primer (FAM), and 205 loci were scored for the selective primer pair *Pst*I + ATC and *Mse*I + CAA primer (VIC), resulting in a total of 399 loci. The 2 primer combinations together generated a total of 399 bands, ranging in length from 35 to 500 bp. Three hundred forty-five bands were polymorphic, resulting in an average polymorphism percentage of 86.5% (Table 3). Polymorphic fragments were dispersed through the whole size range, with the greatest ratio noticed between 45 and 320 bp.

The AFLP profiles among the resistant and susceptible samples differed by a number of bands, showing a different percentage of polymorphism (Table 3).

The dendrogram formed by UPGMA of the Jaccared similarity matrix from the collective data of 2 primer combinations from the 40 samples of the resistant and the susceptible populations of flaxleaf fleabane (FSEQLD7R, FBTARBS) is shown in Fig. 1a. Four main clusters containing a number of sub-clusters were identified from the dendrogram. The susceptible population (FBTARBS) is represented by 9 accessions clustered in 1 main cluster 5S and 2 sub-clusters 2Sb and 2Sa (standalone). The resistant population (FSEQLD7R) is represented by 8 accessions clustered in 2 main clusters 5R and 1R (standalone) and 1 sub-cluster 2R.

The percentage of similarity among both resistant and susceptible samples was very high, giving only weak support for this sub-clustering. However, the susceptible samples were not as tightly clustered as the resistant samples. The remaining accessions of both susceptible and resistant populations, however, were intermixed, represented by 22 accessions grouped together in 3 sub-clusters 7S+1R, 2S+5R, and 6R+1S. The sub-clusters of resistant and susceptible populations did not cluster together, but were mixed. This suggests there is no clear genetic distinction between the resistant and susceptible populations.

The high percentage of polymorphisms in the samples of the susceptible population (79.8%, 75%, Table 3) suggests that susceptible populations of flaxleaf fleabane, even within one site, originated from a number of parents. However, the percentage of polymorphism in the resistant populations (51.5%, 66.8%, Table 3) suggests that resistant populations of flaxleaf fleabane within one site could have originated from fewer parents. In addition, any site containing resistant and susceptible populations of flaxleaf fleabane may have been populated by a large number of parents, evidenced by the relatively

Table 3 Summary of the polymorphisms detected by AFLP system in 20 samples of the selected resistant population (FSEQLD7R) and 20 samples of the susceptible population (FBTARPBS) of flaxleaf fleabane

Primers	Total no. of bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)
R*FAM**	194	94	100	51.5
S***FAM	194	40	154	79.8
R VIC****	205	69	136	66.8
S VIC	205	52	153	75.0
RS, VIC, and FAM	399	54	345	86.5

*R** 20 samples of resistant population (FSEQLD7R), *FAM*** PstI + primer ATC + Fluro (VIC), *S**** 20 samples of susceptible population (FBTARPBS), *VIC***** PstI +primer ATC + Fluro (FAM)

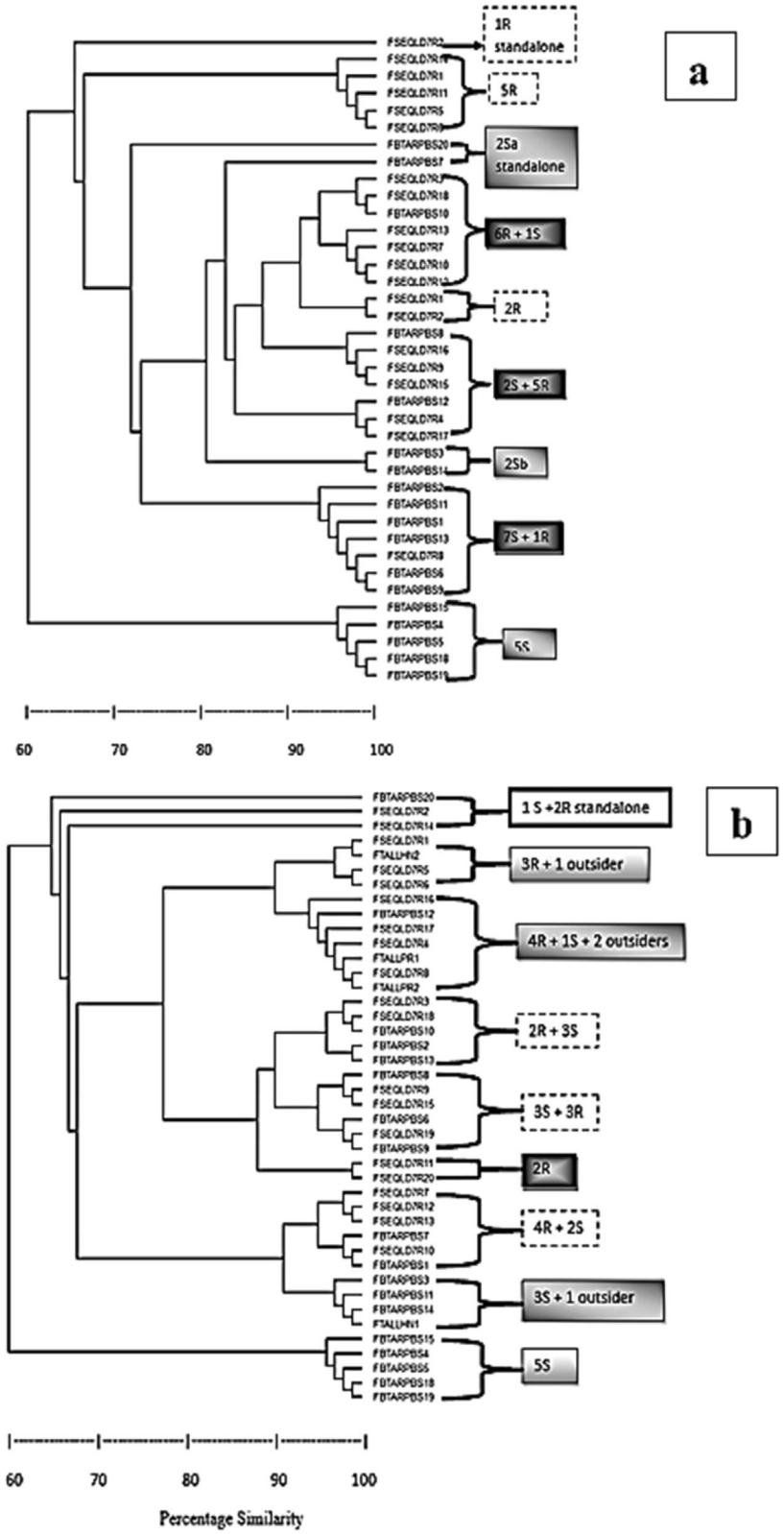

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 UPMGA dendrogram shows the relationship between the resistant (FSEQLD7R) and the susceptible (FBTARPBS) populations of flaxleaf fleabane for the within population study. **a** White clusters (represent resistant accession), gray clusters (represent susceptible accessions), and black clusters (represent resistant and susceptible accessions clustered together). **b** With the outsider populations of tall fleabane, bordered white clusters (resistant and susceptible populations' standalone), white clusters (a group of resistant and susceptible populations clustered together), black clusters (resistant populations grouped together), light gray clusters (susceptible populations grouped together), and gray clusters (resistant and susceptible populations clustered with the outsider populations)

high percentage of polymorphisms detected by AFLP analysis (86.5%).

Information from other weed species related to flaxleaf fleabane can be used to increase knowledge of the genetic relationships. Therefore, two outsider populations of tall fleabane were chosen for comparison with the target populations of flaxleaf fleabane as controls. These two outsider populations were informatively matched with both the resistant and susceptible populations of flaxleaf fleabane with the *Mse*I + CAT Fluro (FAM) and *Pst*I + ATC primers (Fig. 1b). The tall fleabane samples were mixed with the flaxleaf fleabane samples, suggesting that while morphologically different, they may not be genetically different.

Between population study

Eighty-two susceptible and resistant populations (Table 1) collected from different locations across Australia were selected for AFLP analysis to find the relationships between these populations and to determine the evolution and spread of resistance.

AFLP analysis produced the dendrogram formed by UPGMA of the Jaccared similarity matrix from the collective data of 2 primer combinations (Fig. 2), which shows that the 82 populations fall into 16 separate clusters. Only 4 populations were standalone as independent clusters (FB7c and FB2c from South Australia, SEQLD05 from Southern-eastern Queensland, and Fle16 from

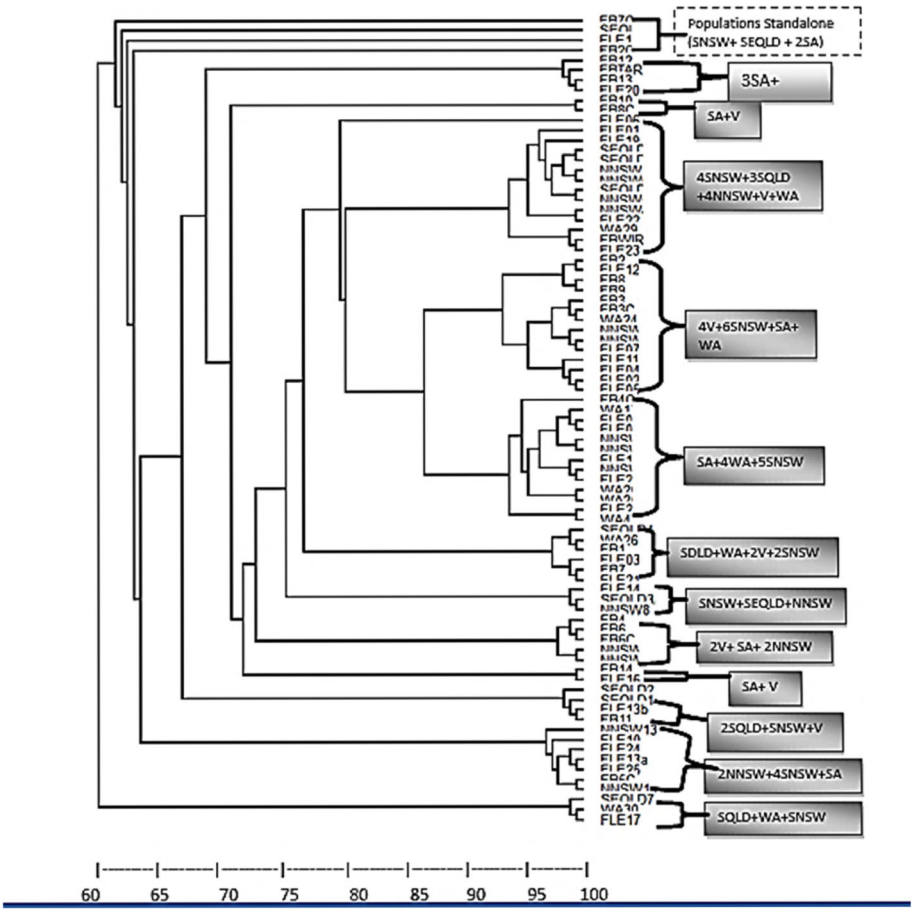

Fig. 2 UPMGA dendrogram shows the relationship among 82 populations of flaxleaf fleabane collected from different locations across Australia for the between population study. White clusters represent populations sourced from one location (standalone), and gray clusters represent populations sourced from different locations.

Southern New South Wales). The remaining 78 populations, both susceptible and resistant, were intermixed. Although there were some indications of environmental clustering, the dendrogram formed from these populations using 564 scored loci, which shows a large genetic diversity existing in Australia. The data also indicate clustering of both resistant and susceptible populations from different sources, such as populations from Western Australia with those from Southern and Northern New South Wales, Southern-eastern Queensland, and Victoria (4SNSW+3SQLD+4NNSW+V+WA). Despite the large geographic distances between collection locations, populations from across states that clustered in several groups showed a close genetic relationship among these populations over these large distances. Further, the genetic distances between most clusters were very small.

Four populations (Fle13, FBWIR, SEQLD01, and WA30) were duplicated from the same DNA for AFLP analysis to confirm the outcomes of this experiment. Figure 3 shows some similarities and differences between two samples of one of the duplicated populations (Fle13). Duplicated populations were expected to be the same as they were sourced from the same DNA samples. As a result of this unexpected outcome, some optimizations were carried out on the PCR to improve results following recommendations of Brandariz-Fontes et al. (2015), such as lowering dNTP concentrations to increase the specificity and the fidelity of PCR products, reducing the amount of Mg^{2+} to increase the enzyme fidelity and the level of specific amplification, and using high denaturation temperature to decrease the probability of occurrence of any mutation in the PCR products and also to achieve specific amplification. Despite these optimizations, identical outcomes were not obtained. Therefore, due to time constraints, conclusive results were not determined in this experiment, and further investigations are thus required.

Despite the high level of genetic diversity seen among populations based on AFLP study, the author was not fully confident to report these results due to unidentified outcomes of the duplicated populations (Fig. 3). However, the results of sequencing of EPSP synthase were determined at high levels of genetic diversity within and between populations.

Sequencing of EPSP synthase

Thirteen populations with confirmed resistance or susceptibility to glyphosate with different levels of resistance were included in this study. Approximately 300 bp region of the *EPSPS* gene was sequenced from extracted DNA of the 13 populations of flaxleaf fleabane as shown in Fig. 4a. After alignment of the 13 sequences, polymorphisms were present at 20 positions (from 374 to 435 bp) among the sequences. The substitutions were not in the predicted amino acids but in the consensus nucleotides, namely in the second and third positions of codons as silent changes such as C (cytosine) for T (thymine) or G (guanine) (positions 374, 422, 427, and 432), G for A (adenine) or A for G (positions 377, 389, 415, and 434), G for C (positions 393 and 415), and T for A or G (positions 418 and 423).

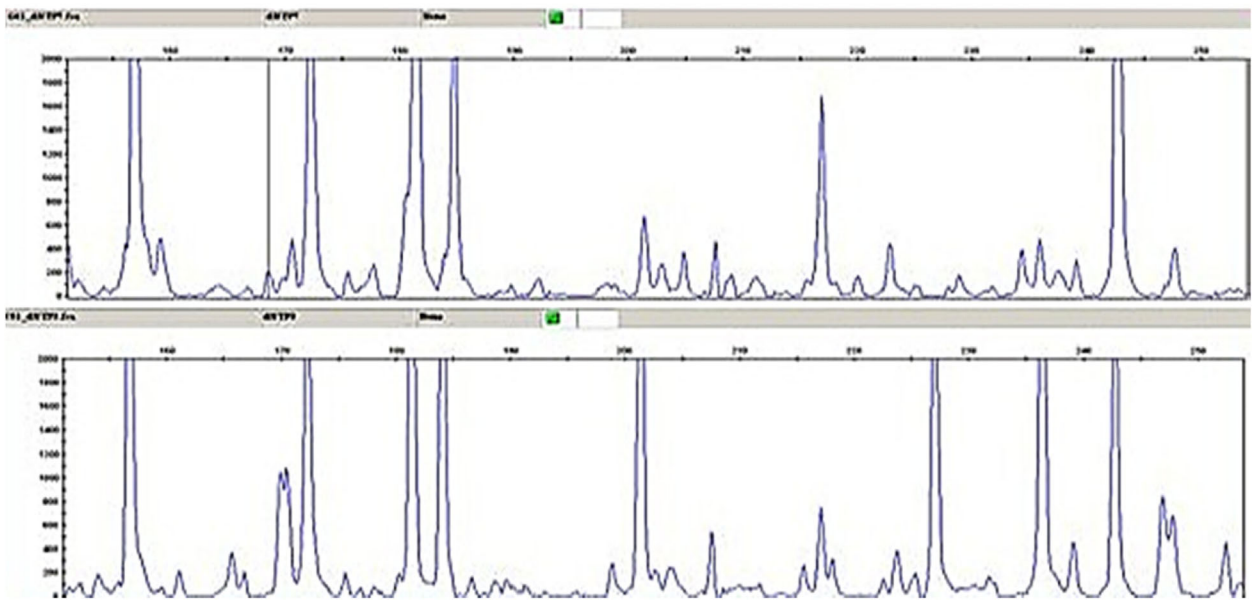

Fig. 3 AFLP electropherograms obtained with the primers *Pst*I + ATC and *Mse*I + Fluro (FAM). The duplicated population (Fle13) was coming from the same DNA sample and showing different alleles (peaks), which are unidentified outcomes.

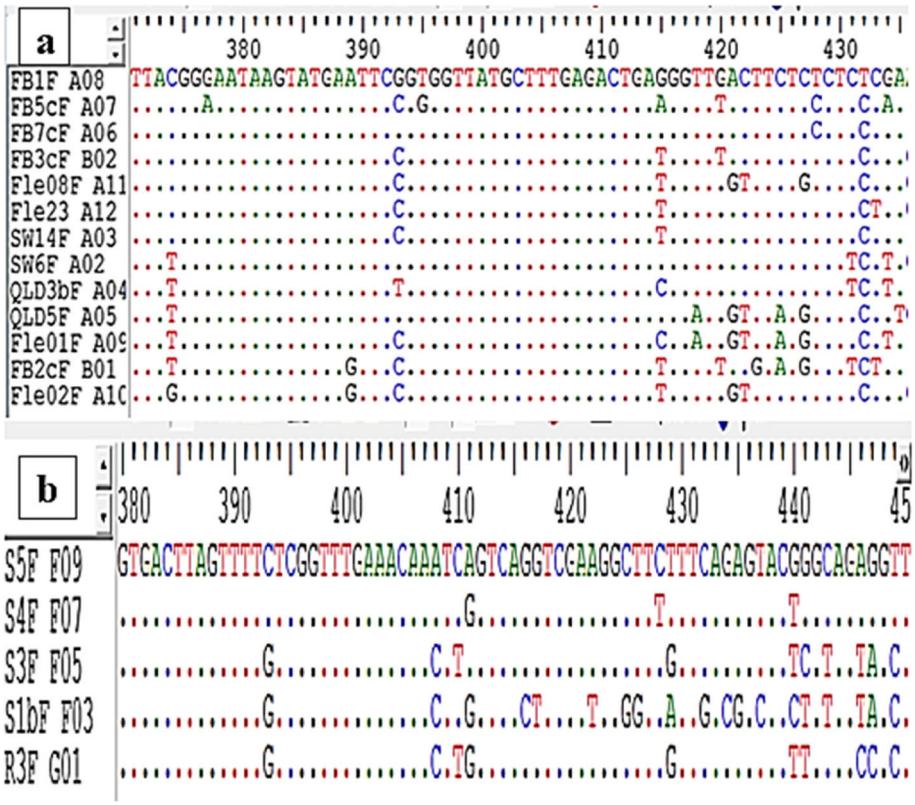

Fig. 4 Polymorphic sites detected by using primers LrEPSP_F and LrEPSP_R in the sequencing of EPSP study. **a** For the 13 flaxleaf fleabane populations collected from different locations across Australia. **b** For the 4 individuals of the susceptible population (FBTAR) and 1 individual of the resistant population (FSEQLD7) of flaxleaf fleabane. All polymorphic sites are displayed comparative to *Lolium rigidum* sequence. Polymorphic sites are shown, while dots point to nucleotides identical to *Lolium rigidum*. The numbers above the partial gene sequences represent the base pair numbers between 371 to 435 bp for **a** and between 380 to 450 bp for **b**

To determine the occurrence of polymorphisms within populations, the 300-bp region of the *EPSPS* gene was sequenced from the extracted DNA of 4 individuals of the susceptible population (FBTAR) and 1 individual of the resistant population (FSEQLD7) as shown in Fig. 4b. Polymorphisms in the resistant sequence (R3) occurred at 10 positions, while polymorphisms from the 4 individual susceptible populations (S5, S4, S3, and S1b) occurred at 20, 3, 10, and 19 positions, respectively.

Discussion

Padgette et al. (1991) have reported that mutations in a specific region of EPSP synthase result in an enzyme with increased tolerance to glyphosate. The *EPSP* gene was sequenced herein to test whether these mutations were the molecular basis for resistance in flaxleaf fleabane. The outcome showed that the resistant populations of this species did not contain amino acid modifications within the active site. However, the differences among the 13 sequences indicated a high level of genetic diversity between these populations (Fig. 4a). As well as, genetic diversity within the susceptible population (FBTAR) was very high (Fig. 4b).

These high levels of genetic diversity within and between populations confirmed in the sequencing of EPSP above support the results of AFLP studies and gave the author more confidence to report the genetic diversity seen in the within and between population studies.

The level of diversity observed within the populations using AFLPs was high. Although the resistant and susceptible populations of flaxleaf fleabane were collected from different states in Australia (Table 1), genetic variations exceeding 95% were found between resistant and susceptible samples. These data support the hypothesis, as in the dendrogram clusters based on genetic relationships, that flaxleaf fleabane occurred as genetically variable populations in the different locations.

Self-pollination is expected to reduce the levels of genetic variation within populations and among populations (Bartkowska and Johnston 2009). It has been generally assumed that flaxleaf fleabane is a self-pollinated species with wind dispersed seed (Wu 2009; Heap 2008; Shrestha et al. 2008a). However, the UPGMA dendrogram produced in

the within population study (Fig. 1) suggests much greater diversity than would be expected from a self-pollinated species. The high genetic diversity seen here suggests an infrequent level of outcrossing could occur.

The movement of seeds could possibly be another factor for the occurrence of genetic variation. The numerous light furry seeds of flaxleaf fleabane are freely spread by wind over large distances (Shrestha et al. 2008b). The flaxleaf fleabane populations used in this study were sampled as multiple plants from a specific location and seeds mixed together for each population. This suggests that the original source of seeds for the collected populations of this study could have originated from different areas due to seed movement by wind.

To the best of the author's knowledge, this is the first paper employing the use of AFLPs for reviewing genetic diversity in flaxleaf fleabane. The results of this research are not consistent with the other studies on herbicide-resistant species. For example, Danquah et al. (2002) reported that AFLP analysis revealed similarities up to 75% among *Echinochloa* spp. populations collected from four countries (Bangladesh, India, Philippines, and the Cote d'Ivoire) whether these populations were resistant or susceptible. Rutledge et al. (2000) postulated that there was no evidence for relationship among populations of *Echinochloa crus-galli* on the basis of their resistance or susceptibility to propanil when herbicide resistance occurs.

Conclusion

Genetic diversity based on the sequencing of EPSP experiments was clearly observed within and between populations of flaxleaf fleabane regardless of their resistance or susceptibility to glyphosate. These results suggest that flaxleaf fleabane populations are highly diverse and resistance has likely evolved multiple times in Australia. To prevent further resistance spread, flaxleaf fleabane management in infested areas should focus on decreasing seed movement from resistant sites as well as reducing the selection pressure for resistance to glyphosate by adopting alternative management strategies.

Abbreviations

AFLP: Amplified fragment length polymorphism; AGRF: Australian Genome Research Facility; Bp: Base pair; DNA: Deoxyribonucleic acid; dNTP: Deoxynucleoside triphosphate; EPSP: Enolpyruvylshikimate-3-phosphate; NNSW: Northern New South Wales; PCR: Polymerase chain reaction; R: Resistant; S: Susceptible; SA: South Australia; SEQSLD: South-eastern Queensland; SNSW: Southern New South Wales; UK: United Kingdom; UPGMA: Unweighted pair group method with arithmetic mean; VIC: Victoria; WA: Western Australia

Acknowledgements

Appreciation is extended to the AusAID organization for the financial support during my study, the University of Adelaide for giving this opportunity to complete my master's degree, and also to the Iraqi Agriculture Ministry for allowing me 4 years study leave.

Authors' contributions

All work was done by MH under the supervision and advice of CP and JM. MH was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Funding

This research was fully funded by AusAID organization.

Availability of data and materials

Not applicable in this section

Ethics approval and consent to participate

Not applicable in this section

Consent for publication

Not applicable in this section

Competing interests

The authors declare that they have no competing interests.

Received: 21 September 2019 Accepted: 2 February 2020
Published online: 12 February 2020

References

Acquaah G (2009) Principles of plant genetics and breeding. Wiley
Bartkowska MP, Johnston MO (2009) Quantitative genetic variation in populations of Amsinckia spectabilis that differ in rate of self-fertilization. Evolution 63(5):1103–1117
Brandariz-Fontes C, Camacho-Sanchez M, Vila C, Vega-Pla JL, Rico C, Leonard JA (2015) Effect of the enzyme and PCR conditions on the quality of high-throughput DNA sequencing results. Sci Rep 5:8056
Burr B, Burr FA, Thompson KH, Albertson MC, Stuber CW (1988) Gene mapping with recombinant inbreds in maize. Genetics. 118(3):519–526
Danquah EY, Johnson DE, Riches C, Arnold GM, Karp A (2002) Genetic diversity in Echinochloa spp. collected from different geographic origins and within rice fields in Cote d'Ivoire. Weed Res 42(5):394–405
Duke SO, Baerson SR, Rimando AM (2003) Glyphosate. In: Plimmer JR, Gammon DW, Ragsdale NN (eds) Encyclopedia of Agrochemicals. Wiley, New York
Franz JE, Mao MK, Sikorski JA (1997) Glyphosate: a unique global herbicide. American Chemical Society, Washington, p 653
Green JM, Barker JHA, Marshall EJP, Froud-Williams RJ, Peters NCB, Arnold GM et al (2001) Microsatellite analysis of the inbreeding grass weed Barren Brome (Anisantha sterilis) reveals genetic diversity at the within-and between-farm scales. Mol Ecol 10(4):1035–1045
Hall TA (ed) (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series. Information Retrieval Ltd, London, pp c1979–c2000
Harring T, Streibig JC, Husted S (1998) Accumulation of shikimic acid: a technique for screening glyphosate efficacy. J Agric Food Chem 46(10):4406–4412
Heap I (ed) (2008) International survey of herbicide-resistant weeds-survey results and criteria to add cases. Proceedings of the 16th Australian Weeds Conference, Cairns Convention Centre, North Queensland, Australia, 18-22 May, 2008. Queensland Weed Society
Malone J, Cook A, Wu H, Hashem A, Preston C (2012) Management of glyphosate resistant weeds in non-agricultural areas. NSW 41:31
Nandula VK, Reddy KN, Duke SO, Poston DH (2005) Glyphosate-resistant weeds: current status and future outlook. Outlooks Pest Manag 16(4):183
Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Nat Acad Sci 70(12):3321–3323
Padgette SR, Re DB, Gasser CS, Eichholtz DA, Frazier RB, Hironaka CM et al (1991) Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. J Biol Chem 266(33): 22364–22369
Randall RP (2017) A global compendium of weeds: RP Randall
Rutledge J, Talbert RE, Sneller CH (2000) RAPD analysis of genetic variation among propanil-resistant and-susceptible Echinochloa crus-galli populations in Arkansas. Weed Sci 48(6):669–674
Shrestha A, Hembree K, Wright S (2008a) Biology and management of horseweed and hairy fleabane in California. University of California, Agriculture and Natural Resources

Shrestha A, Hembree K, Wright S (2008b) Biology and management of horseweed and hairy fleabane in California. University of California, Division of Agriculture and Natural Resources, California, p 9
Teulat B, Aldam C, Trehin R, Lebrun P, Barker JHA, Arnold GM et al (2000) An analysis of genetic diversity in coconut (Cocos nucifera) populations from across the geographic range using sequence-tagged microsatellites (SSRs) and AFLPs. Theor Appl Genet 100(5):764–771
Vos P, Hogers R, Bleeker M, Reijans M, Lee T, Hornes M et al (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23(21):4407–4414
Wakelin AM, Preston C (2006) Inheritance of glyphosate resistance in several populations of rigid ryegrass (Lolium rigidum) from Australia. Weed Sci 54(2): 212–219
Wu H (2009) Conyza bonariensis (L.) Cronquist. The biology of Australian weeds. 3:85–101
Wu H, Walker S, Taylor I, Robinson G (eds) (2006) Biology and management of flaxleaf fleabane (*Conyza bonariensis* (L.) Cronquist). 15th Australian Weeds Conference, Adelaide

Publisher's Note