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Distinguishing between weedy *Amaranthus* species based on intron 1 sequences from the 5-enolpyruvylshikimate-3-phosphate synthase gene†

Alice A Wright, William T Molin* and Vijay K Nandula

Abstract

BACKGROUND: Hybridization between *Amaranthus* species and the potential for herbicide resistance to be transferred by hybridization are of growing concern in the weed science community. Early detection of evolved herbicide resistance and hybrids expressing resistance to single or multiple herbicides is important to develop an effective control strategy.

RESULTS: A PCR test was developed for quick identification of weedy amaranths and any hybrids. The sequences of intron 1 for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) gene were determined for *Amaranthus palmeri*, *A. spinosus*, *A. retroflexus*, *A. blitoides*, *A. viridis*, *A. tuberculatus* and *A. hybridus*. These sequences were aligned and primers were developed in areas where the sequence differed between species. Species-specific primers and cycle conditions were successfully developed. These primers produce a single robust band only for the species for which they were designed.

CONCLUSION: The PCR techniques described here allow identification of a weedy amaranth or suspect hybrid in a few hours. Using a similar target, it may be possible to design simple PCR tests to identify even more difficult to distinguish weed species or weeds prone to interspecific hybridization.
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Keywords: PCR; hybrids

1 INTRODUCTION

Herbicide resistance is a growing problem in agriculture, as has been exemplified by weedy amaranths. Resistance to acetolactate synthase (ALS) inhibitors, photosystem II (PSII) inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) inhibitor, protoporphyrinogen oxidase (PPO) inhibitors, microtubule inhibitors and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors has been reported in populations of weedy amaranths.[1] Some of these populations exhibit resistance to multiple herbicides, making them difficult to manage. To make matters worse, there is the potential for hybridization between weedy amaranths with transfer of herbicide resistance alleles; this has been known for over a decade now.[2–5] Franssen *et al.*[2] showed a low (0.1%) rate of transfer of ALS inhibitor resistance from *A. palmeri* to *A. rudis* in a growth chamber. Tranel *et al.*[3] found that ALS inhibitor resistance could be transferred from monoecious ALS-inhibitor-resistant *A. hybridus* to dioecious *A. rudis*. In subsequent backcrosses (BC1 and BC2) with *A. rudis* as the recurrent parent, although BC1 progeny had reduced fertility, a low number of offspring were obtained. Further backcrossing produced plants with greater fertility, and 35% had the resistance allele, demonstrating that the resistance allele was inherited.[3] Trucco *et al.*[4] found a hybridization frequency of 33% between ALS-inhibitor-sensitive female *A. tuberculatus* and ALS-inhibitor-resistant *A. hybridus* under field conditions. These authors also determined that hybrid frequencies were influenced by distance between the parental species, and pollen availability may have been a limiting factor to hybridization. Gaines *et al.*[5] performed field and greenhouse crosses between glyphosate-resistant male *A. palmeri* and five other *Amaranthus* species. Successful hybridizations occurred between *A. palmeri* and *A. hybridus*, *A. spinosus* and *A. tuberculatus* at distances of 1–3 m from the pollen source.[5]

Recently, a pigweed population resistant to glyphosate and having characteristics of both *A. palmeri* and *A. spinosus* was found in a cotton field near Water Valley, Mississippi.[6] These plants were tall like *A. palmeri* and had spines like *A. spinosus*. The roadside borders of the field had both species growing in close proximity to one another. This field and other neighboring row crop fields were located close to pastures that had populations of *A. spinosus*. The putative resistant hybrids collected from this field were heterozygous for EPSPS exons and introns from *A. palmeri* and *A. spinosus*.[6] Although the EPSPS exons differed by only 29

* Correspondence to: WT Molin, Crop Production Systems Research Unit, USDA-ARS, Stoneville, MS 38776, USA. E-mail: william.molin@ars.usda.gov

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Crop Production Systems Research Unit, USDA-ARS, Stoneville, MS, USA

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nucleotides between *A. palmeri* and *A. spinosus*, intron 1 sequences from *A. palmeri* and *A. spinosus* differed markedly, and alignments of intron 1 sequences from the hybrids aligned with intron 1 from *A. palmeri*. These plants also exhibited ALS inhibitor resistance. They contained a point mutation in ALS known to confer resistance to ALS inhibitors and had sequence upstream to the ALS gene that aligned to *A. palmeri* and not *A. spinosus*.[7] These results demonstrated that hybridization does occur naturally under field conditions, with transmission and retention of functional resistance alleles and mechanisms such as amplification.

Phylogenetic relationships among *Amaranthus* species have been examined on the basis of the presence or absence of restriction site polymorphisms in two chloroplast genes (ribulose-1,5-biphosphate carboxylase gene and open reading frame, and intergenic spacer between *trn*T and *trn*L) and nuclear genes (internal transcribed spacer).[8] Overall, there was a low level of variation, which generated poorly resolved trees among 28 species. Wetzel *et al.*[9] created a two-step test in which ribosomal DNA was amplified and then digested with five different restriction enzymes. Different species had different patterns, allowing for identification. Other studies, many of which were trying to develop a phylogeny for *Amaranthus*, involve more complicated molecular techniques. Wassom and Tranel[10] used amplified fragment length polymorphism (AFLP) to develop a phylogeny for several weedy amaranths. Random amplified polymorphic DNA (RAPD) has also been developed for weedy and crop amaranths to determine genetic relationships.[11]

The marked differences in EPSPS intron sequences between *A. palmeri* and *A. spinosus* prompted the question: to what extent would intron sequences vary among other amaranths and, if variable, could these sequences be used to identify seedling stage amaranths? The goal of the present study was to determine sequence variation in intron 1 of the EPSPS gene from seven common *Amaranthus* species to create a tool by which individual species at the seedling stage could be identified. In this paper, we present a simple PCR-based method for quick identification of weedy *Amaranthus* species. Species-specific PCR primers have been developed using sequence differences present in the first intron of EPSPS. These primers generate a single band only with the species for which they were developed, providing users with a simple and definitive yes or no answer that can be achieved in a few hours.

2 EXPERIMENTAL METHODS

2.1 Seed and plant collection, germination and growth conditions

A. spinosus seed was obtained from the National Plant Germplasm System (PI 632248, North Central Regional Plant Introduction Station, Iowa State University, Ames, IA). Seeds from *A. palmeri*, *Abutilon theophrasti* and *Sida spinosa* were collected from single plants from the USDA-ARS Crop Protection Systems Research Farm in Stoneville, Mississippii. Seeds from *A. tuberculatus*, *A. retroflexus*, *A. blitoides* and *A. viridis* were from USDA-ARS in-house collections. *A. hybridus* seed was provided by Dr Pat Tranel, University of Illinois. In the summers of 2013, 2014 and 2015, *A. spinosus* × *A. palmeri* hybrids were collected from a field in Water Valley, Mississippii, where the original hybrids were documented.[6] Hybrid plants were collected, along with roots, and transferred to 10 L pots containing field soil, and were allowed to grow in a greenhouse set to 25/20 °C day/night, 12 h photoperiod, under natural sunlight conditions supplemented with high-pressure sodium lights providing 400 mmol m^{-2} s^{-1} of light intensity. All greenhouse studies were conducted under these growing conditions.

Seeds were planted at 0.5 cm depth in 50 × 20 × 6 cm plastic trays with holes containing a commercial potting mix (Metro-Mix 360). Two weeks after emergence, seedlings were transplanted into 6 × 6 × 6 cm pots containing the potting mix. Trays and pots were maintained in a greenhouse set to the conditions described previously. Plants were fertilized once with a nutrient solution (Miracle-Gro; The Scotts Company LLC, Marysville, OH) containing 200 mg L^{-1} each of N, P_2O_5 and K_2O, 1 week after transplanting, and subirrigated as needed thereafter. All studies were conducted from 2014 to 2015 at the Jamie Whitten Delta States Research Center of USDA-ARS in Stoneville, Mississippi.

To establish the specificity of the test, seeds from the various species were blindly assigned numbers and planted. When seedlings had grown to the four-leaf stage, 1–2 leaves were collected for DNA extraction. Plants were subsequently grown to flowering to authenticate species identity.

2.2 Intron 1 sequencing

For each species or hybrid, 1–2 leaves, depending on size, were removed for DNA extraction. Leaf tissue was homogenized in an extraction buffer[12] using a mortar and pestle. The homogenate was transferred to a microcentrifuge tube and incubated at 65 °C for 1 h. Cellular debris was collected by centrifugation at 13 000 rpm for 4 min, and 266 μL of the supernatant was transferred to a fresh tube. The DNEasy Plant Mini kit (Qiagen, Valencia, CA) protocol was followed from this point on. RNA was also extracted, using a RNEasy Plant Mini kit (Qiagen), from *A. retroflexus*, *A. blitoides* and *A. viridis*, for sequencing of the EPSPS coding sequence to develop primers to amplify intron 1. Quality and quantity of DNA and RNA were assessed by an $A_{260/280}$ reading and by visualization of the DNA or RNA on a 1% agarose 1× TAE (0.04 M Tris-acetate and 0.001 M EDTA) gel stained with ethidium bromide.

The EPSPS cDNA was amplified for *A. blitoides* (GenBank accession number KT833342) and *A. viridis* (KT833340) with primers AW128 and AW264, and for *A. retroflexus* (KT833341) with primers AW263 and AW264 (sequences for all primers are given in Table 1). A one-step RT-PCR Master Mix w/Thermo Prime Taq (Fisher Scientific, Waltham, MA) was used to amplify the cDNA. Reactions consisted of ∼50 ng of RNA, 5 μM of primers, 1× Master Mix, 2.5 μL of RT enhancer, 2 μL of enzyme mix and H_2O to 50 μL. Cycle conditions were as follows: 50 °C for 15 min; 92 °C for 2 min; 35 cycles of 95 °C for 20 s, 52 °C for 30 s and 72 °C for 3 min; 72 °C for 5 min; and a 4 °C hold. The PCR product was purified by gel extraction using the GenElute™ Gel Extraction kit (Sigma-Aldrich, St Louis, MO). Purified PCR product was ligated into the pCR™ 2.1 vector using the TA Cloning® kit (Life Technologies, Grand Island, NY). Reactions consisted of a 3:1 ratio of insert to vector (25 ng), 1× buffer, 5 units of ligase and H_2O to 10 μL, and were incubated at 16 °C overnight. Chemically competent cells were prepared and transformed with 2 μL of ligation reaction, as described by Sambrook *et al.*[13] The resulting colonies were streaked for isolation and incubated at 37 °C overnight. These clones were screened for the presence of the insert using the primers described above. For positive clones, 800 μL of an overnight culture was added to 200 μL of 80% glycerol and stored at −80 °C. Positive clones were submitted to the Genomics and Bioinformatics Research Unit, USDA-ARS, Stoneville, Mississippii, for plasmid isolation and sequencing.

Intron 1 of EPSPS was amplified from genomic DNA for *A. tuberculatus* (KT833345) with primers AW127 and AW205,

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Table 1. Primers used in PCR amplification of EPSPS and EPSPS intron 1 in weedy amaranths

Name	Sequence	Name	Sequence
AW90	GTTGTGAGTTCGATACACTGC	AW448	CATTGAAGGATAAGTTTGCCTACT
AW100	GAGTTCTGAGAGCGTCCA	AW449	CTAGCGGTTCATAAGTGAACA
AW127	CTACGACTGCCCTTTTGGCTG	AW450	GTTATCGTGTGTTGCAGTTTGAGA
AW128	CTCCAAAGTTCATGTCTTTAACCA	AW453	GAGCCAGGGTCGAGC
AW155	CAGTAGGTAAACCGTGTTG	AW454	CAGTCAGCAAACTGTGTGAACA
AW205	GCCTGGGTCAAAGTCTTTATC	AW455	GAGTCGGCGTTAAGTGC
AW263	GCTCAAGCTACTACCATCAACAAT	AW456	GCTGTATAGCATCGGACACG
AW264	CAATGCTTGGCGAACTTTTCTA	AW457	CAGGCTGTTGCCGATG
AW386	GAGAAGATTGATTTGGTCGTG	AW458	GACCTTGCTTCCGCTGTC
AW388	GAGTCAAAGAGATACTGTATGACG	AW464	CTTAGTCATGTCTAGTATCATCCT
AW415	CATCCCTCGCACAGAGGATAC	AW465	GATCCCAATCTCACACGCT
AW416	GTTGAAGGCTAAACCTTATCACTG	AW466	GTCCTTCGACTATGGATTTACT
AW418	GAATATCATCACTATACAGCAAGT	AW467	CGACTTAGCGTTAAGGCA
AW419	CTGCCCTTTTGGCTGTA	AW468	GCTTAAATAAACTGTATTCCAGGC
AW436	GCAACCATCAGGGATTGTG	AW469	CATATTGCTGAGGAATACTTGGGT
AW437	GCGTTCGTGATTCTGTATTGGA	AW471	GCGAGACACATTGTTATCCCT
AW438	CTAGCGGTTCATAAGTGAACA	AW473	CACAAGAGACACATTATTATCCCT
AW439	GATCAATCACGAAGAACTTGAGAT	AW474	CGTTTTTGTGACTCAAAGATCGA
AW443	GCTGTTGCCGATGGGATAT	AW477	GTGGCTAATCATGTTTACAGGAC
AW444	CACATTGATAGTGAACCACGTTA	AW482	CTTAGAGCTCAAGTATTATCTAGT
AW445	CAACATGCAGCACATTTGACCA	AW483	CTTGGAGCCCAAGTATAATC
AW446	GCCATAGTTTAGAGCACCAAATGT	AW490	CTGTTGCCGCATGTTTTC
AW447	GATCCTTTGGTTGCTGGTTTCT	AW493	GTCTAAGTGATGTTGGATAACT

for *A. retroflexus* (KT833348) and *A. blitoides* (KT833343) with primers AW128 and AW418, for *A. viridis* (KT833346) with primers AW128 and AW419 and for *A. hybridus* (KT833344) with primers AW128 and AW100. PCR was performed using the Takara LA PCR kit v.2.1 (Clontech, Mountain View, CA) as follows: ∼50 ng of DNA, 200 nM of primers, 2.5 mM of Mg^{2+}, 1× buffer, 400 μM of dNTPs, 5 units of polymerase and H_2O to 50 μL. Cycle conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min; 72 °C for 5 min; and a 4 °C hold. PCR products were purified, cloned and sequenced as described above. Sequencing was performed using M13F and M13R primers. Additional sequencing primers were AW415 and AW416 for *A. tuberculatus*, AW436, AW437, AW443 and AW444 for *A. retroflexus*, AW438, AW439, AW445, AW446, AW455, AW456, AW464 and AW465 for *A. viridis*, AW447, AW448, AW457 and AW458 for *A. hybridus* and AW449, AW450, AW453, AW454, AW466 and AW467 for *A. blitoides*. The sequences were analyzed and assembled using Geneious Bioinformatics Software v.7.1.9.[14] The intron 1 sequences were submitted to GenBank with the following accession numbers: KT833343 (*A. blitoides*), KT833344 (*A. hybridus*), KT833345 (*A. tuberculatus*), KT833346 (*A. viridis*) and KT833348 (*A. retroflexus*).

2.3 Development of species-specific primers

Intron 1 sequences for each species were aligned using Geneious Bioinformatics Software to identify sequence differences between species for primer design. Primers and cycle conditions for each species are given in Table 2. PCR reactions were performed using Thermo Scientific Maxima Hot Start Green PCR Master Mix (Fisher Scientific) and consisted of ∼50 ng of DNA, 100 nM of primers, 1× Master Mix and H_2O to 10 μL. Each primer pair was tested against a representative of each species to check for specificity. Blind tests were performed to check the specificity of the primers. Seeds from *A. spinosus*, *A. tuberculatus*, *A. retroflexus*, *A. hybridus*, *A. blitoides*, *A. viridis*, *A. albus*, *Abutilon theophrasti* and *Sida spinosa* were planted and assigned numbers. DNA was harvested as described above and by using the QuickExtract™ plant DNA extraction solution (Epicentre, Madison, WI). Briefly, a leaf disc was collected by snapping a 0.2 mL PCR tube shut over a leaf. A quantity of

Table 2. Primers and PCR cycle conditions for identifying weedy *Amaranthus* species

Species	Primers	Cycle conditions	Size of PCR product (bp)
A. palmeri	AW90 × AW155	94 °C 4 min; 30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 2 min; 72 °C 5 min; 4 °C hold	697
A. spinosus	AW386 × AW388	94 °C 4 min; 30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 2 min; 72 °C 5 min; 4 °C hold	1335
A. tuberculatus	AW468 × AW469	94 °C 4 min; 30 cycles of 94 °C 30 s, 56 °C 30 s, 72 °C 2 min; 72 °C 5 min; 4 °C hold	992
A. retroflexus	AW471 × AW482	94 °C 4 min; 30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 1 min 30 s; 72 °C 5 min; 4 °C hold	1616
A. hybridus	AW473 × AW483	94 °C 4 min; 30 cycles of 94 °C 30 s, 58 °C 30 s, 72 °C 2 min; 72 °C 5 min; 4 °C hold	1623
A. blitoides	AW474 × AW490	94 °C 4 min; 30 cycles of 94 °C 30 s, 60 °C 30 s, 72 °C 2 min; 72 °C 5 min; 4 °C hold	1486
A. viridis	AW477 × AW493	94 °C 4 min; 30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 1 min 30 s; 72 °C 5 min; 4 °C hold	1215

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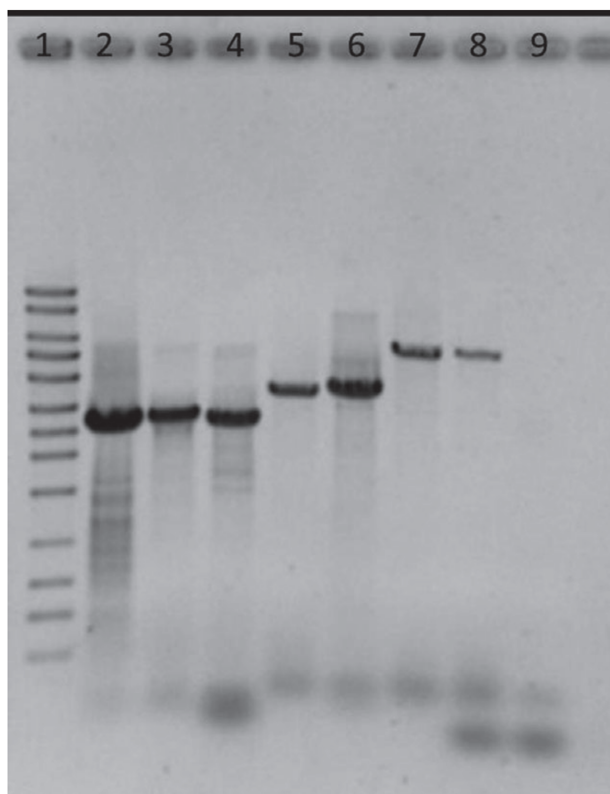

Figure 1. Amplification of intron 1 for seven *Amaranthus* spp. Lane 1: 1 kb molecular marker with bands of 10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.7, 0.5 and 0.3 kb. Lanes 2 to 8: *A. palmeri*, *A. spinosus*, *A. tuberculatus*, *A. retroflexus*, *A. hybridus*, *A. blitoides* and *A. viridis*. Lane 9 is a negative (no DNA) control.

100 µL of solution was added to the leaf disk, and the solution was incubated at 65 °C for 6 min, and then at 98 °C for 2 min. The species-specific primers were screened against each species for each extraction following the conditions outlined in Table 2. PCR products were analyzed by gel electrophoresis.

3 RESULTS

3.1 Intron 1 sequencing

PCR amplification and sequencing of the EPSPS intron 1 for each of the seven *Amaranthus* species revealed diversity in both size and sequence. For size, the species fell into three distinct groups (Figs 1 and 2). *A. palmeri*, *A. spinosus* and *A. tuberculatus* had intron 1 sizes of 2416, 2585 and 2307 bp respectively. *A. retroflexus* and *A. hybridus* had intron 1 sizes of 2915 and 2920 bp. *A. blitoides* and *A. viridis* had the largest intron 1 sizes, which were 4432 and 4467 bp. These size differences between groups were large enough to be distinguished by gel electrophoresis. Five of these intron 1 PCR fragments were selected for cloning; *A. palmeri* and *A. spinosus* intron 1 sequences were available from previous studies.[6,15]

Sequencing data revealed many differences in intron 1 sequences between the seven species. Although there were differences, some of the species were similar to each other. To determine how similar, a series of pairwise alignments were performed, comparing each intron 1 sequence to every other intron 1 sequence. The percentage identity was recorded for each alignment (Table 3). The species fell into the same groups as they did when organized by intron 1 size. *A. blitoides* and *A. viridis* shared 95.9% sequence identity and only 55–60% sequence identity when compared with other *Amaranthus* species. *A. hybridus* shared 97.5% sequence identity with *A. retroflexus* and only 55–76% with the other species. *A. palmeri*, *A. spinosus* and *A. tuberculatus* grouped together, sharing 82–83% sequence identity. This similarity can be observed in the alignment of all seven intron 1 sequences (Fig. 2).

3.2 Primer test development

Using the intron 1 alignment, primers were developed at unique sequences for each species. Primers were then tested against representatives of each species, and cycle conditions were optimized such that one band was produced for the appropriate species and all other lanes were clear. The primer pairs and cycle conditions developed for each species are given in Table 3. The resulting gel images for each of these primer sets and cycle conditions are shown in Fig. 3. For each species, the identifying primers generated a band in the corresponding species and in that species alone. These PCR tests should serve as a way of correctly identifying young *Amaranthus* plants.

A blind test was developed in which seed from the species for which the primers were designed and seed for other species having seedling morphologies that were difficult to distinguish from amaranths were planted and assigned numbers. DNA was extracted from seedlings using a quick extraction method (QuickExtract™ from Epicentre) and a longer, column-based format (DNEasy Plant Mini kit from Qiagen). Each sample was screened with each set of primers, and from those screens the species was predicted (Table 4). All were correct except sample 7. The seeds were labeled as being from *A. retroflexus*, but the seedlings tested positive as *A. hybridus*. These two species are very difficult to distinguish prior to flowering, and even then identification may be a challenge. The plants were grown to flowering, and it was confirmed that both species were present in the seed lot, meaning that the lot was contaminated. Wetzel *et al.*,[9] using restriction enzyme analysis of ribosomal gene cluster ITS regions to identify pigweed species, found a purported spiny amaranth to be misidentified, attesting to the specificity of these methods.

The extraction methods were also compared. Two gels for sample 3 are shown in Fig. 4. In both, the *A. tuberculatus* primers are the only ones to generate a signal, indicating that the plants belonged to that species. However, the signal is much stronger from the samples prepared with the Qiagen kit compared with the QuickExtract™ solution. The former method is more time intensive and requires a centrifuge, but it is more effective when used with these primers.

3.3 Application

Hybridization is very important among weedy amaranths, as this is a means by which resistance can spread. Plants were collected from a field near Water Valley, Mississippii, where *A. palmeri* × *A. spinosus* hybrids had been identified in previous years.[6] These were screened with *A. palmeri*- and *A. spinosus*-specific primers (Fig. 5). Five of the plants, 90, 92, 95, 98 and 99, were *A. spinosus*; one, 94, was *A. palmeri*; two, 93 and 97, were hybrids of the two species. This test clearly demonstrates which plants were hybrids and proves that hybrids are still present in that area. This test can be applied in other areas where hybrids are suspected.

4 DISCUSSION

Proper identification of weeds is critical to developing an effective weed management program. This is particularly important

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Figure 2. Alignment of EPSPS intron 1 sequences for seven *Amaranthus* species. In the consensus sequence identity, green represents a perfect match across all sequences, brown a moderate match and red a poor consensus. The height of the consensus sequence identity line is an indication of degree of consensus (green where all are the same is tallest, followed by brown and red). The thick gray lines are the sequence for each *Amaranthus* species, and colors (green, yellow, blue and red) indicate the base pair difference from the consensus sequence when one is present. Gaps are indicated by thin gray lines. This is a visual representation of the data given in Table 3.

Table 3. Percentage identity for each alignment of the intron 1 sequence

	A. blitoides	*A. hybridus*	*A. palmeri*	*A. retroflexus*	*A. spinosus*	*A. tuberculatus*	*A. viridis*
A. blitoides	100	55	56.2	54.7	58.8	59.7	95.9
A. hybridus	55	100	76.4	97.5	68.8	69.1	54.7
A. palmeri	56.2	76.4	100	76.6	82.4	82.5	55.9
A. retroflexus	54.7	97.5	76.6	100	68.9	69.1	55.1
A. spinosus	58.8	68.8	82.4	68.9	100	83.3	59.1
A. tuberculatus	59.7	69.1	82.5	69.1	83.3	100	60.3
A. viridis	95.9	54.7	55.9	55.1	59.1	60.3	100

because herbicide resistance is a persistent and growing problem in the *Amaranthus* genus.[1] It is important to know the potential for hybridization in a field because one of the parental species may not be considered a problem; controlling the less problematic weed species may lessen the opportunity for hybridization, and therefore prevent a potentially larger problem from occurring. Furthermore, as in the case where *A. spinosus* × *A. palmeri* hybrids were found, the hybrids were at the interface where a pasture and agronomic field converged, and hybrids radiated outward. Management in these areas differs, but without management the hybrids spread.

Here, we have created a simple PCR-based method for identifying seven weedy *Amaranthus* species and hybrids between those species. The first intron of EPSPS proved to be an ideal choice for development of an identification assay because, unlike internal transcribed spacer (ITS) sequences, intron 1 had greater sequence diversity, allowing species-specific primers to be designed. The diversity present in the intron sequence is probably due to the fact that it is not under the constraints facing coding sequences: a change in coding sequence could alter, reduce or abolish the function of the gene product. Fewer penalties for changes to the intron sequence means that changes can accumulate at a greater rate and provide more diversity between closely related species than exon sequences. This diversity in intron sequences has been used by many researchers to create phylogenetic trees for groups of species.[16–18] EPSPS intron 1 was also chosen, in part, because EPSPS was one of the few genes for which there was sequence data available within weedy amaranths and the other sequences available, ALS and ITS, did not provide the sequence diversity needed to develop primers. Of the seven introns in EPSPS, intron 1 was selected because prior research had shown that this sequence was very diverse between *A. palmeri* and A. spinosus,[6] and it was suspected that other species within the genus would show enough diversity to allow for primer development.

The intron 1 sequence also provided some insight into relatedness among weedy amaranths. The seven species could be

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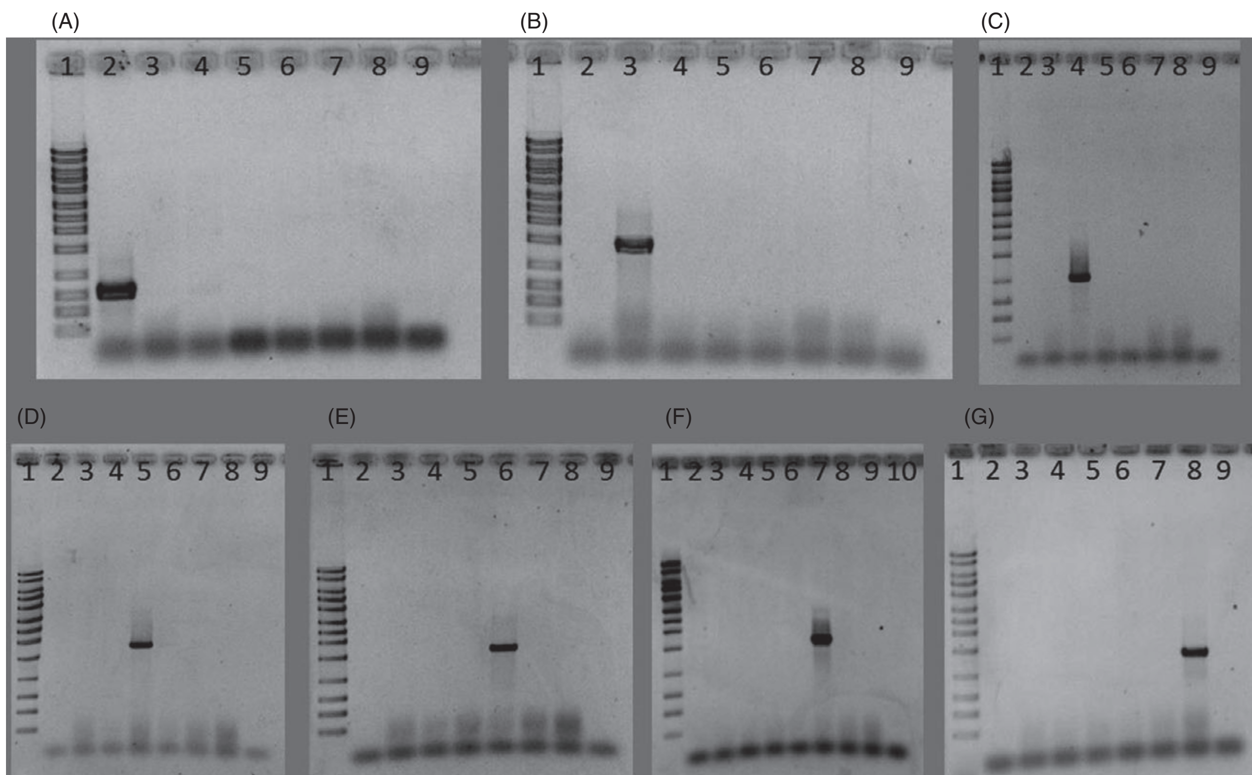

Figure 3. Gel images of PCR with species-specific primers. Primer sets are for (A) *A. palmeri*, (B) *A. spinosus*, (C) *A. tuberculatus*, (D) *A. retroflexus*, (E) *A. hybridus*, (F) *A. blitoides* and (G) *A. viridis*. Lane 1 is a 1 kb ladder with bands of 10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.7, 0.5 and 0.3 kb. In each gel picture, lanes 2 to 8 are *A. palmeri*, *A. spinosus*, *A. tuberculatus*, *A. retroflexus*, *A. hybridus*, *A. blitoides* and *A. viridis*. Lane 9 is a negative control. In F, lane 9 is *A. albus* and lane 10 is the negative control.

Table 4. Results of blind test confirming the ability of the PCR test correctly to identify weedy amaranths. Plants were labeled by sample number only

Sample number	Species	Primer test
1	*A. hybridus*	*A. hybridus*
2	*A. spinosus*	*A. spinosus*
3	*A. tuberculatus*	*A. tuberculatus*
4	*Sida spinosa*	None
5	*A. albus*	None
6	*A. viridis*	*A. viridis*
7	*A. retroflexus*	*A. hybridus*
8	*A. blitoides*	*A. blitoides*
9	*Abutilon theophrasti*	None

divided into three groups based on size of intron 1 and sequence similarity (Figs 1 and 2). *A. hybridus* and *A. retroflexus* were most similar to each other at 97.5% identity (Table 3), and *A. hybridus* at 2920 bp had only five additional base pairs. *A. blitoides* and *A. viridis* were next with 95.9% identity and were both over 4400 bp in size. *A. spinosus*, *A. palmeri* and *A. tuberculatus* were most variable, with 82–83% identity and 2307–2585 bp in size. These results are similar to those obtained by Wassom and Tranel.[10] In their study, *A. spinosus* and *A. palmeri* clustered together, as did *A. hybridus* and *A. retroflexus*. *A. tuberculatus* was separate from *A. spinosus* and *A. palmeri*.[10] Analysis of additional intron sequences and sequencing of many populations may provide a robust phylogeny for this genus.

Specific primers and cycle conditions (Table 2) allowed different species to be distinguished from one another by PCR (Fig. 3). Each primer pair amplified a segment of intron 1 only in species for which the primer had been designed. This is a clear and simple way to ascertain the species. The advantage of this method, over others that have been developed, is that there are three main steps: extract DNA, perform PCR and confirm PCR products by gel electrophoresis. Someone with a basic background in molecular biology should be able to perform these steps and identify the plant of interest within a few hours. This differs from other methods in which an additional reaction, such as a restriction digest, is required or multiple bands are produced, such as in AFLP. This PCR generates a single, robust band on a gel. The blind test confirmed the ability of these primers to distinguish seedlings. The test also showed that not all extraction methods are equal and that a column format is likely to produce a more intense PCR product (Fig. 4). It is also recommended that labs that choose to use this method for confirming the identity of weedy amaranths have a positive control. This can confirm that the PCR is working properly in the lab and that a negative result truly means that the plant in question does not belong to the species for which it was being tested.

This PCR-based identification method can also be used to detect hybridization events. Hybridization is of concern among weed scientists as it is a way by which herbicide resistance alleles can spread. In the field and greenhouse, transfer of resistance alleles has been documented among closely related species. Zelaya *et al.*[19] confirmed that glyphosate resistance could be transferred from *Conyza canadensis* to *C. ramasissima*. More recently, the transfer of MCPA resistance from *Raphanus raphanistrum* to the crop

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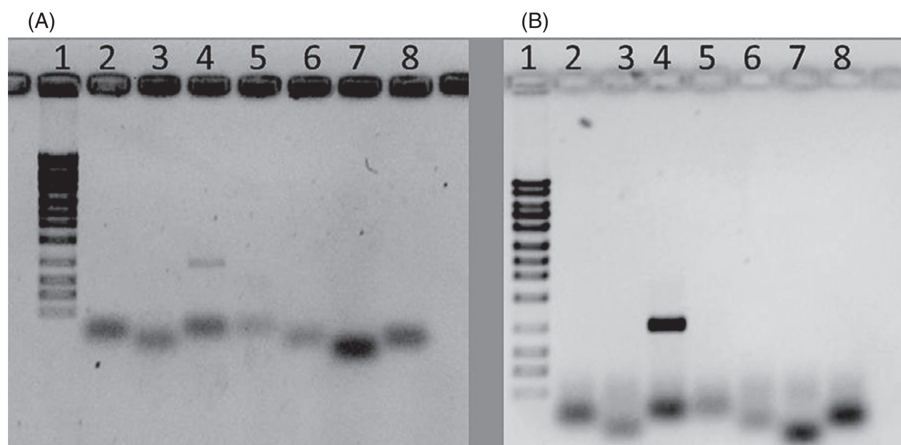

Figure 4. The type of extraction affects the robustness of the results. The lanes are ordered as in Fig. 3. DNA was extraction using (A) the QuickExtract™ plant DNA extraction solution (Epicentre) and (B) the DNeasy Plant Mini kit (Qiagen).

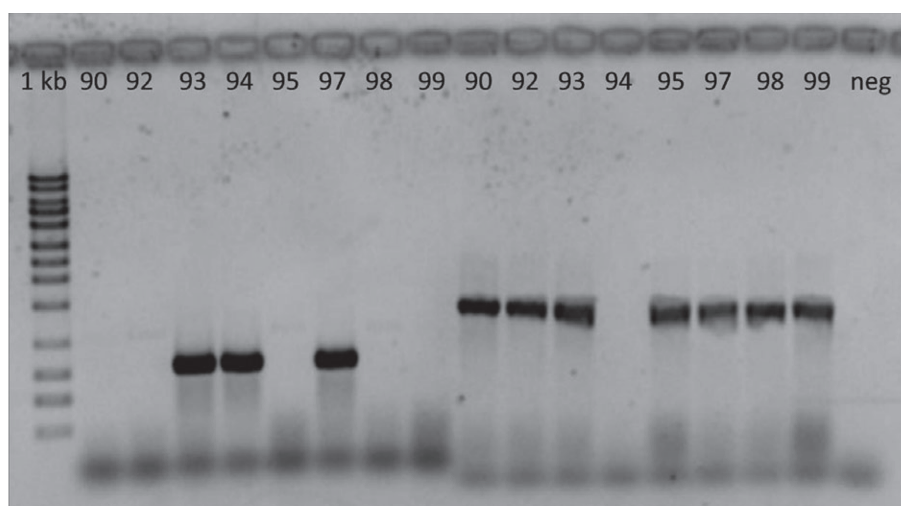

Figure 5. Screen for hybrid plants using *A. palmeri*- and *A. spinosus*-specific primers. Sample designations are listed at the top of each lane. The first set of reactions are for *A. palmeri*, and the second set for *A. spinosus*.

R. sativus was demonstrated.[20] In 1998, there was evidence for hybridization between *Avena fatua* and *Avena sterilis* in the field, increasing the likelihood of the spread of fenoxaprop resistance alleles.[21] Hybridization has been particularly well studied among weedy *Amaranthus* species. *A. tuberculatus* can hybridize with *A. hybridus* and *A. palmeri* in the field and greenhouse.[2–4] In a later study, *A. palmeri* was crossed with *A. spinosus*, *A. hybridus* and *A. tuberculatus* in both the field and greenhouse.[5] Even though hybridization rates were low, given the number of *Amaranthus* populations and the amount of pollen and seed produced, it was only a matter of time before hybridization occurred independently in the field. In 2014 a study was published showing a field population to be the result of hybridization between *A. palmeri* and *A. spinosus*.[6] Worse, glyphosate resistance and ALS inhibitor resistance from the *A. palmeri* parent were transferred to the hybrid offspring.[7] Although only one case has been documented so far, it is inevitable that additional hybrid populations will be found. Having a rapid way of testing for hybridization, such as the assay described in this paper, will facilitate the confirmation of suspect hybrid populations. This was illustrated by screening suspect hybrids collected from the field in which the original *A. spinosus* × *A. palmeri* hybrids were found. The screen clearly identified two hybrids among the eight plants tested (Fig. 5). The importance of identifying hybrids for management is that resistance is now known to be transferred from one species to another via hybridization. This can alert growers to the need to control both parental species, even if one is not typically regarded as a problem weed, and to scout for and remove hybrids.

Amaranthus species are more easily distinguished when they reach the flowering stage than when they are seedlings. However, some species are difficult to distinguish throughout their entire life cycle, such as members of the *Lolium*, *Echinochloa* or *Setaria* genera. It is hoped that this diagnostic test will serve as a springboard or template for the development of similar tests in these challenging species.

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