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Optimizing Palmer amaranth (*Amaranthus palmeri*) genetic testing of seeds using real-time (quantitative) PCR

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Research Article

Cite this article: Bratsch S, Thiede D, Viner D, Sarangi D, Merriman M, Blair S, Cortilet A (2024) Optimizing Palmer amaranth (*Amaranthus palmeri*) genetic testing of seeds using real-time (quantitative) PCR. Weed Sci. **72**: 732–739. doi: 10.1017/wsc.2024.58

Received: 22 May 2024
Revised: 8 August 2024
Accepted: 13 August 2024
First published online: 31 October 2024

Associate Editor:
Patrick J. Tranel, University of Illinois

Keywords:
Amaranthaceae; genetic molecular testing

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Abstract

The *Amaranthus* genus contains numerous agronomic weedy species that are widely distributed across the United States. The seeds of many *Amaranthus* species are morphologically indistinguishable. The Minnesota Department of Agriculture declared Palmer amaranth (*Amaranthus palmeri* S. Watson) a prohibited noxious weed seed in 2016. Any *Amaranthus* spp. seeds that are identified as contaminants during routine seed testing require genetic testing to determine whether *A. palmeri* is present. This research aimed to validate and optimize the molecular detection of *A. palmeri* in seed. We refined the DNA extraction from pools of *Amaranthus* spp. seeds ranging in size from 1 to 100 seeds to improve sample testing throughput. Real-time polymerase chain reaction (qPCR) using primers developed by Murphy and colleagues correctly identified the presence of *A. palmeri* genetics in 84 samples containing 0, 1, 2, 25, or 50 *A. palmeri* seeds in samples containing up to 100 seeds. The method specificity was examined using 17 *Amaranthus* species and 4 hybrids of unknown genetics. The Murphy regular qPCR cycle amplified Watson's amaranth (*Amaranthus watsonii* Standl.), spleen amaranth (*Amaranthus dubius* Mart. ex Thell.), and spiny amaranth (*Amaranthus spinosus* L.), which would result in false-positive calls; however, the fast cycle only identified *A. watsonii* as a potential false positive. Examination of the Murphy primer binding site revealed an identical sequence for *A. palmeri* and *A. watsonii*. Additional markers were evaluated and optimized for use in qPCR to eliminate the risk of a false positive. The additional markers did eliminate the amplification of *A. dubius* and *A. spinosus* but did not eliminate the amplification for *A. watsonii*. Currently, *A. watsonii* is not known to be distributed outside of its limited native range and is not expected to be encountered in samples.

Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson) is regulated as a noxious weed seed in many states, including Minnesota, Iowa, Wisconsin, North Dakota, South Dakota, and Ohio (USDA 2023), making it illegal to sell seeds that are contaminated with this species. Federal and Minnesota risk assessments detail the threat that this species poses to crop production throughout the United States and the need for regulation to prevent widespread establishment in the upper Midwest (MDA NWAC 2014; USDA 2020). Yu et al. (2021) reviewed the regulatory and management actions taken by the Minnesota Department of Agriculture (MDA) to prevent the establishment of this species in Minnesota. That review highlights the pathways for introduction, including seed, screenings, animal feed, and manure in Minnesota. A current map of the infestations in Minnesota, their status, and their sources is available on the MDA's website (https://www.mda.state.mn.us/plants-insects/palmer-amaranth-location-sources).

Based on *A. palmeri*'s listing as a prohibited noxious weed seed in Minnesota, it is not legal to sell seeds, screenings, or animal feed that contain *A. palmeri* in Minnesota. The Association of Official Seed Analysts, Society of Commercial Seed Technologists, and MDA require that if *Amaranthus* seeds are identified in a seed lot during required federal testing, a warning is added to the seed label. Due to the regulations prohibiting *A. palmeri* in Minnesota, MDA requires further genetic testing of sampled seed lots if *Amaranthus* seeds are identified to determine the *Amaranthus* species. The MDA collaborated with the California Department of Food and Agriculture and the company Eurofins BioDiagnostics in 2017 to develop and test a method to identify individual *Amaranthus* seeds to the species level using polymerase chain reaction (PCR)

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and Sanger sequencing of the internal transcribed spacer region (ITS) (Price 2016; Yu et al. 2021). More rapid and less expensive methods employing real-time polymerase chain reaction (qPCR) were published shortly afterward (Murphy et al. 2017).

DNA-based tests to identify *A. palmeri* from among other *Amaranthus* species have been developed for individual plants or seeds. These include a restriction fragment length polymorphism assay (Wetzel et al. 1999) and a PCR-based assay (Wright et al. 2016). Some commercial and government labs initially sequenced the ITS gene region of individual seeds and compared the results to GenBank databases to differentiate *A. palmeri* from other *Amaranthus* spp. (Yu et al. 2021). The development of a fluorescence-based qPCR assay for *A. palmeri* identification facilitated a faster sample testing turnaround time and opened up the possibility of testing groups (also called pools) of seeds up to 100 seeds (Murphy et al. 2017). Competitive allele-specific PCR (KASP) has also been developed but requires different reagents and data analysis than the qPCR assay (Brusa et al. 2021; Oliveira et al. 2018).

This paper describes the MDA's optimization and validation of a published qPCR genetic testing method to detect *A. palmeri* in individual or groups of *Amaranthus* spp. seeds. We evaluated the specificity of three markers for 17 non–*Amaranthus palmeri* species and determined their sensitivity and limit of detection in single seeds and pools of seed. We propose a sequential testing method wherein an initial marker is used to screen samples, and follow-up testing using an additional marker is done to confirm a positive result. This sequential approach lowers the risk of false positives using a qPCR approach.

Materials and Methods

Seed Source and Species Validation

The seeds of *Amaranthus* spp. were obtained from the U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS) Germplasm Resources Information Network (GRIN) (USDA-ARS 2023). These seeds included: tumble pigweed (*Amaranthus albus* L.); sandhill amaranth (*Amaranthus. arenicola* I.M. Johnst.); southern amaranth [*Amaranthus australis* (A. Gray) Sauer]; prostrate pigweed (*Amaranthus blitoides* S. Watson); California amaranth [*Amaranthus californicus* (Moq.) S. Watson]; low amaranth (*Amaranthus deflexus* L.); spleen amaranth (*Amaranthus dubius* Mart. ex Thell.); hybrid amaranth (*Amaranthus* L. hybrid; 4 accessions); smooth pigweed (*Amaranthus hybridus* L.); Prince-of-Wales feather (*Amaranthus hypochondriacus* L.); *A. palmeri*; Powell's amaranth [*Amaranthus powellii* S. Watson ssp. *bouchonii* (Thell.) Costea & Carretero]; redroot pigweed (*Amaranthus retroflexus* L.); spiny amaranth (*Amaranthus spinosus* L.); waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer]; Watson's amaranth (*Amaranthus watsonii* Standl.); and Wright's amaranth (*Amaranthus wrightii* S. Watson) (Supplementary Table S1).

To evaluate marker sensitivity in single-seed and pooled-seed matrices, we used seeds from a single *A. palmeri* plant phenotypically identified by MDA scientists in the field. We confirmed that the seeds from this plant were positive for *A. palmeri* by testing 10 seeds individually at an external lab (Eurofins BioDiagnostics, River Falls, WI, USA). *Amaranthus palmeri*–negative seeds used for seed-pool testing were confirmed to not contain *A. palmeri* through testing of six sets of 100 random seeds by an external lab (University of Illinois Plant Clinic, Urbana, IL, USA).

Nucleic Acid Extraction

Seeds were added to a Lysing Matrix A tube with a second ceramic sphere added (MP Biomedicals, Santa Ana, CA, USA). Tubes were processed for 2 min using a Mini-Beadbeater™-16 (BioSpec Products, Bartlesville, OK, USA), and DNA was subsequently extracted using a MagMAX™ Plant DNA kit (Fisher Scientific, Hanover Park, IL, USA). Lysis buffer A contained 40 mM molecular biology grade dithiothreitol (Fisher Scientific). DNA was purified manually following the manufacturer's directions and eluted with 50 µl of elution buffer (1 to 50 seeds) or 100 µl of elution buffer (50 to 100 seeds).

DNA was quantified using a Nanodrop™ 2000c Spectrophotometer (Fisher Scientific). Samples were diluted to 10 ng μl^{-1} with elution buffer immediately before qPCR reactions were run. Positive control dilution series were made by serial dilution of *A. palmeri* DNA to 10, 2, 0.08, 0.016, and 0.0032 ng μl^{-1} using elution buffer immediately before qPCR reactions were run. DNA was frozen, undiluted, at −20 C if not used the same day.

Oligos

Custom DNA oligos, including primers designed by Murphy et al. (2017) (Murphy forward and reverse) and KASP markers with the FAM or HEX tail removed from Oliveira et al. (2018) (*A. palmeri* Marker 1 [M1] forward and reverse) and Brusa et al. (2021) (*A. palmeri* Markers 2 (M2) and 3 (M3) forward and reverse) were synthesized by IDT (Integrated DNA Technologies, Coralville, Iowa, USA).

qPCR conditions

Twenty-microliter reactions containing 10 µl of 1X Applied Biosystems™ PowerUP™ SYBR™ Green 2X Master Mix (Fisher Scientific) were run for each set of markers. Unless defined otherwise, samples and controls were run in triplicate on an ABI QuantStudio™ 3 Real-Time PCR System (Fisher Scientific). The resulting data were analyzed using the QuantStudio Design and Analysis Software v. 1.2x (Life Technologies, Carlsbad, CA, USA).

Other Marker Cycle Optimization

Markers 1, 2, and 3 were tested for use in an SYBR green qPCR assay. *Amaranthus palmeri*, *A. dubius*, and *A. spinosus* were run in single reactions on an ABI QuantStudio™ 3 Real-Time PCR System (Fisher Scientific) using a standard cycle consisting of 50 C for 2 min, 95 C for 2 min, followed by 35 cycles of 95 C for 1 s, 52/54/56/58/60/62 C for 30 s.

Following the manufacturer's recommendations (Applied Biosystems™ PowerUP™ SYBR™ Green 2X Master Mix), M2 and M3 primers were tested at varied concentrations. Duplicates of no template (water), 10 ng of *A. palmeri* DNA, and 2 ng of *A. palmeri* DNA were tested in a 20-µl reaction with 1 µl of DNA (or water), 10 µl of 1X Applied Biosystems™ PowerUP™ SYBR™ Green 2X Master Mix (Fisher Scientific), primers at 300, 500, or 800 nM, and molecular-grade water to 20 µl.

Optimized qPCR reaction conditions for M2 are 800 nM forward and reverse primers, while M3 uses 800 nM forward primer and 500 nM reverse primer.

qPCR cycles

The Murphy standard cycle consisted of 50 C for 2 min, 95 C for 2 min, followed by 35 cycles of 95 C for 15 s, 59 C for 1 min. The

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Table 1. Composition of seed pools tested by real-time polymerase chain reaction (qPCR) includes individual (single) seeds and pools of 25, 50, or 100 seeds[a]

Pool size	No. of samples	No. of *Amaranthus palmeri* seeds included
Single seeds	10	0 *A. palmeri*
	10	1 *A. palmeri*
25 seeds	10	0 *A. palmeri*
	5	1 *A. palmeri*
	5	2 *A. palmeri*
50 seeds	10	0 *A. palmeri*
	5	1 *A. palmeri*
	5	2 *A. palmeri*
	1	25 *A. palmeri*
	1	50 *A. palmeri*
100 seeds	10	0 *A. palmeri*
	5	1 *A. palmeri*
	5	2 *A. palmeri*
	1	100 *A. palmeri*

[a]The replicates per pool size (number of samples) and number of *A. palmeri* seeds used in each pool are included.

Murphy fast cycle consisted of 50 C for 2 min, 95 C for 2 min, followed by 35 cycles of 95 C for 1 s, 59 C for 30 s.

The M2 standard cycle consisted of 50 C for 2 min, 95 C for 2 min, followed by 40 cycles of 95 C for 15 s, 52 C for 15 s, 72 C for 1 min. The M2 fast cycle consisted of 50 C for 2 min, 95 C for 2 min, followed by 40 cycles of 95 C for 1 s, 52 C for 30 s.

The M3 fast cycle consisted of 50 C for 2 min, 95 C for 2 min, followed by 40 cycles of 95 C for 1 s, 54 C for 30 s. The M3 regular cycle consisted of 50 C for 2 min, 95 C for 2 min, followed by 45 cycles of 95 C for 15 s, 54 C for 15 s, 72 C for 1 min.

Seed-Pool Testing

Eighty-three blind samples were prepared for seed-pool testing, with the analyst running the assay not knowing which samples contained *A. palmeri*. Seed-pool sizes consisted of single seeds (20 samples), 25 seeds (20 samples), 50 seeds (22 samples), and 100 seeds (21 samples). Pools were spiked with 0 to 100 *A. palmeri* seeds (Table 1). The Murphy fast cycle was used to test all 83 seed-pool samples, while the M3 fast qPCR cycle was used to test 4 single-seed pools and 6 pools of 25, 50, and 100 seeds. For each sample, 10 ng of DNA was tested in triplicate.

Limit of Detection

The detection limits of both the fast and regular cycles were tested for Murphy, M2, and M3 primers with the standard and fast cycles using 45 cycles and qPCR reaction conditions, both listed earlier. *Amaranthus palmeri* DNA was serially diluted using MagMAX™ Plant DNA kit elution buffer. Final concentrations tested included 10, 1, 0.05, 0.0125, 0.00625, 0.003125, and 0.0015625 ng μl^{-1}. Concentrations were tested in duplicate wells for the six cycles described.

Primer Species Specificity

DNA was extracted from 5 seeds from each species of *Amaranthus* as described earlier and was run in triplicate wells for each set of primers. The qPCR cycles were completed using Murphy regular, Murphy fast, Brusa M2 regular, and Brusa M3 fast cycles.

ITS Sequence Analysis

The four accessions of hybrid *Amaranthus* and *A. watsonii* were further analyzed by amplifying the ITS1-5.8S rDNA-ITS2 region with ITS1 and ITS4 primers as previously described (White et al. 1990) using Phusion green high-fidelity DNA polymerase (Fisher Scientific). PCR products were purified (PureLink™ PCR Purification Kit, Fisher Scientific) and sequenced bidirectionally (ACGT, Germantown, MD, USA). Geneious Prime® 2023.0.4 bioinformatics software was used to trim and assemble sequences. The four hybrid *Amaranthus* sequences and the *A. watsonii* sequences were submitted to GenBank.

The ITS region of all *Amaranthus* species used in this study were obtained from GenBank or from this study (four hybrid *Amaranthus* and *A. watsonii*). These regions were manually aligned to identify the primer binding sites for the Murphy primers.

Results and Discussion

Nucleic Acid Extraction

DNA extraction from *Amaranthus* spp. seeds was optimized for use with seed samples that contained between 1 and 100 seeds. Due to the hardness and small size of *Amaranthus* spp. seeds (0.9- to 1.7-mm diameter), Murphy et al. (2017) utilized an overnight incubation step to soften the seed coats and repeated the homogenization step to enhance tissue disruption to improve DNA extraction (Stallknecht and Schulz-Schaeffer 1993). We aimed to expedite testing turnaround time to complete extraction in 1 d. The addition of an extra ceramic bead to the homogenization tube to evenly grind all seeds without incubation in lysis buffer overnight was examined and shown to effectively disrupt the seed tissue for extraction of DNA. Nucleic acid concentrations for the different seed-pool sizes (1 to 100) showed consistent DNA concentrations for 1 and 25 seed-pool sizes, while larger seed-pool sizes had a greater concentration range (Figure 1). The differences in nucleic acid concentrations may be due to the range of seed sizes contained in different pools. Larger seeds would have more DNA available for purification.

The variation in seed-pool size (1 to 100) and total DNA extracted from samples (8.7 to 328.2 ng μl^{-1}) necessitated the application of a standard template concentration for use in qPCR. A concentration of 10 ng of template DNA was used for all samples and controls. Samples with higher DNA concentrations were diluted for use in qPCR testing.

The number of *Amaranthus* spp. seeds that are identified in seed noxious weed tests or screening exams can range from one to thousands of seeds (Yu et al 2021). Elimination of an overnight incubation step before DNA extraction improves the testing lab's flexibility and reduces turnaround time. Due to the sensitivity of qPCR, it is possible that seed-pool sizes larger than 100 seeds could also be tested using this method. Additional testing would be required to demonstrate whether this is feasible and what the maximum seed-pool size would be for accurate testing. The maximum seed-pool size is recommended to be 100 seeds until additional work is completed to show larger seed-pool sizes still efficiently detect *A. palmeri*.

Other Marker Cycle Optimization

Although there is a strong interest in detecting different *Amaranthus* species with a molecular method, the assay developed by Murphy et al. (2017) is the only qPCR method that has been

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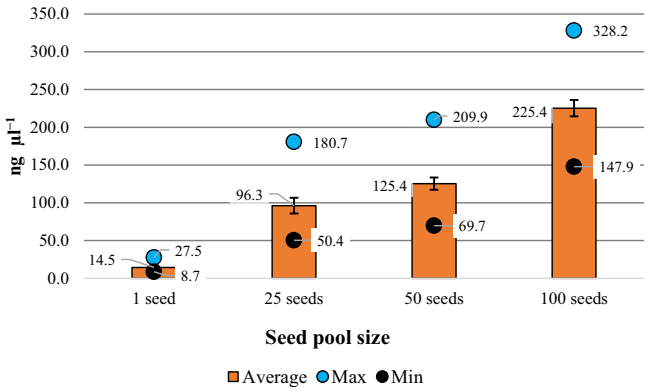


Figure 1. DNA concentration (in ng µl^{-1}) extracted from pools of *Amaranthus* seeds ranging in size from 1 to 100 seeds. The average DNA concentration extracted, maximum DNA concentration extracted, and minimum DNA concentration extracted are shown. Error bars represent the standard deviation of the mean (SE).

published and widely adopted. Other molecular methods like KASP have been published (Brusa et al. 2021). Based on the specificity results from the Murphy marker (see Primer Species Specificity section), we wanted to determine whether additional markers may be needed in regulatory testing. Three additional pairs of primers (M1, M2, M3) were modified for use in qPCR and tested to determine whether any of them would be suitable for use. Initial testing to determine the optimal annealing temperature showed that M1 did not work in an SYBR green qPCR, likely due to the very short target size, and it was not tested further (data not shown). M2 had the most discrete difference in quantification cycle (Cq) values between *A. palmeri* and other *Amaranthus* spp. using an annealing temp of 52 C, while M3 was best at 54 C (Supplementary Figures S1 and S2). The optimal primer concentrations for both M2 and M3 were evaluated, and M2 had the lowest Cq values with 800 nM forward and reverse primer, while M3 had the lowest Cq values with 800 nM forward primer and 500 nM reverse primer (Supplementary Table S2).

Seed-Pool Testing

The Murphy standard qPCR cycle was used to test all 83 seed pools and accurately detected the presence of *A. palmeri* in all seed pools from 1 to 100 seeds (Figure 2A). Seed pools of all sizes that contained 1 or more *A. palmeri* seeds consistently had Cq values less than 25. DNA extracted from pure *A. palmeri* samples resulted in the lowest Cq values. *Amaranthus palmeri*–positive samples ranged in Cq values from 16.2 (50 *A. palmeri* seeds) to 24.2 (1 *A. palmeri* seed in a 50-seed pool). *Amaranthus palmer*–negative samples had Cq values above 34.8, with many samples having a value of undetermined (35 cycles were run).

The pooled-seed testing validation was also completed using M3. The M3 marker was tested using a subset of the 83 samples, including 4 single-seed pools and 6 pools of 25, 50, and 100 seeds containing 0 to 2 *A. palmeri* seeds (Figure 2B). *Amaranthus palmeri*–positive samples had Cq values less than 26.35, while negative samples had Cq values higher than 33.6.

Limit of Detection

The limit of detection was evaluated for Murphy, M2, and M3 primers. Both regular and fast qPCR cycles were tested. The Murphy fast cycle was the most sensitive cycle, yielding the lowest Cq values for all *A. palmeri* DNA concentrations tested (Figure 3). The Murphy regular cycle had lower Cq values than both M2 and M3 cycles. The M3 fast cycle had very similar Cq values to the M2 regular cycle (Figure 3). Although M2 and M3 yielded higher Cq values for *A. palmeri*–positive samples, it would still be feasible to use them for diagnostic testing using a higher Cq level for positive samples than is used for the Murphy qPCR test.

Primer Species Specificity

We tested primer specificity using DNA extracted from seeds of 16 *Amaranthus* spp. (including two *A. palmeri* samples) and four *Amaranthus* hybrids (Figure 4). Both the regular and fast Murphy cycles detected *A. palmeri* with the lowest Cq values across the species tested, with Cq values of 15.61 and 14.84 (regular cycle) or 19.46 and 18.91 (fast cycle). However, both cycles also had amplification of *A. dubius*, *A. spinosus*, and *A. watsonii*. *Amaranthus watsonii* resulted in the lowest Cq values of 17.49 (regular cycle) and 21.24 (fast cycle), while *A. spinosus* had higher Cq values of 23.58 (regular cycle) and 27.85 (fast cycle). Similar to *A. spinosus*, *A. dubius* also had higher Cq values of 23.10 (regular cycle) and 26.86 (fast cycle). The sole use of the Murphy et al. primer may result in a false positive if *A. dubius*, *A. spinosus*, or *A. watsonii* is present in the sample, depending on the testing lab's criteria for identifying positives. None of the four hybrids were amplified in either Murphy cycle. The fast cycle has a higher Cq value for *A. dubius* and could eliminate the potential false positive caused by that species. The strong amplification of *A. watsonii*, with Cq values similar to *A. palmeri*, suggests homologous ribosomal RNA single-nucleotide polymorphisms (SNPs) between *A. watsonii* and *A. palmeri*. Although the Murphy assay cannot differentiate between *A. watsonii* and *A. palmeri*, neither can the M2 or M3 primers.

The M2 regular and M3 fast cycles were tested against the same species. In addition to the Murphy assay, M2 and M3 also showed the highest non-target species amplification of *A. watsonii*. M3 had Cq values of 21.79 and 20.27 for *A. palmeri* and a Cq of 23.56 for *A. watsonii*. M2 had higher Cq values for *A. palmeri* of 25.20 and 23.88 with a similar spread to the Cq value of *A. watsonii* of 27.16. M2 also amplified *A. arenicola*, with a Cq of 27.42, which is a widely distributed native *Amaranthus* spp. in the United States. The amplification of *A. arenicola* limits the effectiveness of M2 for use in an SYBR green qPCR assay as a complementary test to the Murphy assay. The complete genome sequences are not available for either species, but it is likely that these *Amaranthus* species share sequence homology with *A. palmeri* in the DNA region the assays were designed to detect. The M3 marker may be the best additional marker for use in a secondary qPCR assay based on the amplification of only *A. watsonii*, low *A. palmeri* detection limit, and accurate detection of *A. palmeri* in pools of seeds. To implement the most risk averse strategy and limit the risk of false positives in our testing, we decided to take a sequential approach to testing and confirm any positive result identified by the Murphy marker with a follow-up test using the M3 marker. This approach should minimize the risk of a false positive in testing. Confirming a positive result using two independent genetic markers will limit an erroneous result in a regulatory setting.

Future studies may wish to explore the feasibility of utilizing both the Murphy marker and the M3 marker in a probe-based Taqman qPCR assay. Testing both markers simultaneously in one assay would allow for checking for the presence of two independent genetic markers in this group of closely related

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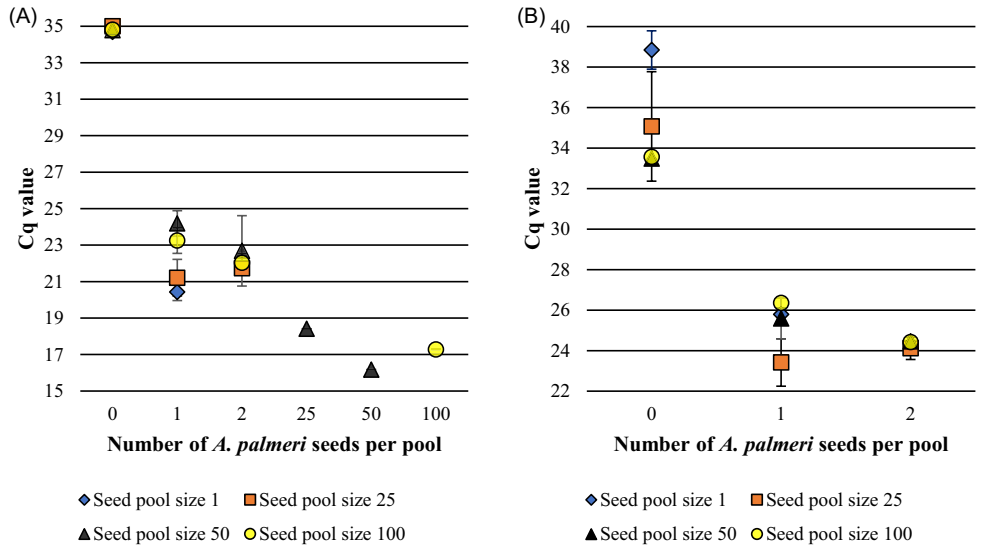


Figure 2. Average quantification cycle (Cq) values for Murphy and M3 real-time polymerase chain reaction (qPCR) with various seed-pool sizes. The average Cq values for Murphy qPCR (A) and M3 qPCR (B) are shown. Seed pools were composed of 1, 25, 50, or 100 *Amaranthus* seeds. The seeds pools contained 0, 1, 2, 25, 50, or 100 *Amaranthus palmeri* seeds (*y* axis). The Murphy cycle included 35 cycles, while M3 included 40 cycles. Error bars represent the standard deviation of the mean (SE).

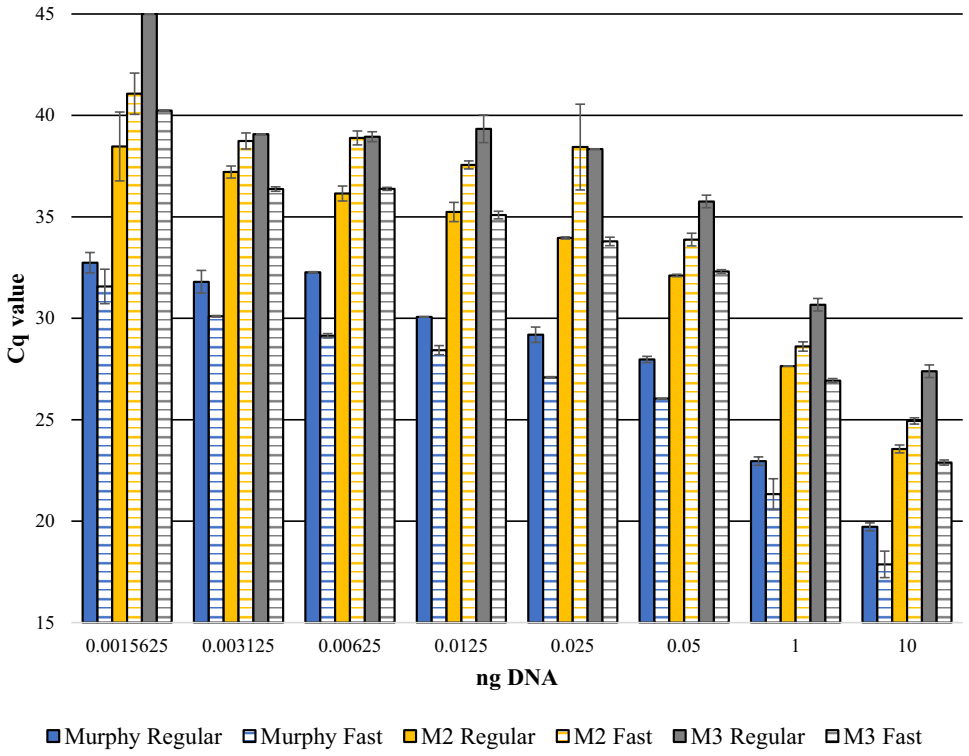


Figure 3. Limit of detection for Murphy, M2, and M3 real-time polymerase chain reaction cycles. *Amaranthus palmeri* DNA ranging in concentration from 0.0015625 to 10 ng μl^{-1} was tested using the regular and fast qPCR cycles for Murphy, M2, and M3. Error bars represent the standard deviation of the mean (SE).

plants. Additional *A. palmeri* or other *Amaranthus* species–specific tests developed should include as many *Amaranthus* species as possible in the assay specificity testing, as GenBank does not contain complete genome sequences for this family. Analysis by computational simulations may not detect regions of high genetic homology between *Amaranthus* species, which could lead to false-positive results when the assay is deployed for real-world use.

ITS Sequence Analysis

The sequences of the ITS1-5.8S rDNA-ITS2 region corresponding to the region amplified by the Murphy qPCR assay were available for 16 out of 17 *Amaranthus* species tested. The sequences of the four hybrids were not available, and the deposited *A. watsonii* sequence had ambiguities in the forward primer binding site. Because the *A. watsonii* sequence for the ITS1-5.8S rDNA-ITS2 region was not available, we sequenced our accession and the four

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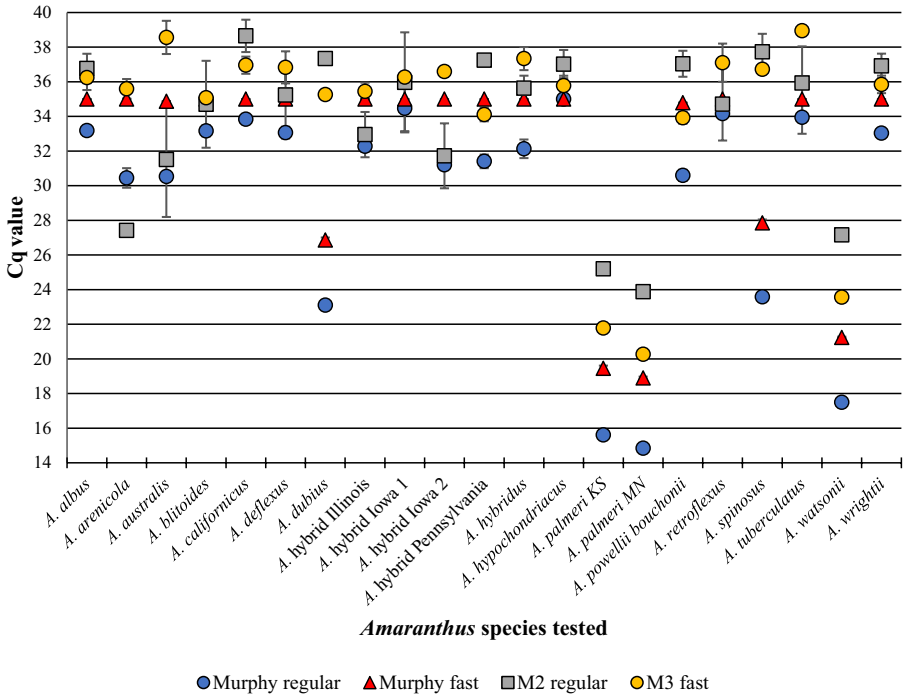


Figure 4. Real-time polymerase chain reaction (qPCR) specificity to detect *Amaranthus palmeri.* The average quantification cycle (Cq) results for the *Amaranthus* species tested using Murphy, M2, and M3 are shown. Two cycle settings are possible using the QuantStudio 3 real-time PCR system and include the regular and fast cycles. The Murphy regular and fast cycles are presented, while only the optimized M2 regular and M3 fast cycles are presented. Error bars represent the standard deviation of the mean (SE). All data points had SE calculated, and points that appear to be missing them had very low error that does not extend beyond the data point's visual.

hybrid *Amaranthus*. The sequences were deposited in GenBank as: OQ996211.1 (hybrid *Amaranthus*), OQ996212.1 (hybrid *Amaranthus*), OQ996213.1 (hybrid *Amaranthus*), OQ996214.1 (hybrid *Amaranthus*), and OQ996215.1 (*A. watsonii*).

Analysis of the forward primer binding site for all *Amaranthus* species tested revealed that *A. watsonii* contained the *A. palmeri* specific SNP the Murphy primer was designed to target (Table 2). *Amaranthus watsonii* and *A. palmeri* have an identical sequence in the forward primer binding site, and alignment of the sequences showed these two species are identical for the entire gene region. The other species that exhibited amplification with the Murphy assay, *A. spinosus*, has one mismatch with the primer of two nucleotides in the middle of the primer binding site and one nucleotide mismatch in the SNP binding region of the primer, substituting a guanosine for a cytosine. This is likely the reason that *A. spinosus* amplifies with the Murphy assay, as both ends of the primer can bind and provide a template for qPCR replication. In addition to *A. spinosus*, *A. dubius* is also able to amplify with the Murphy assay. It is unknown why *A. dubius* is amplified with the Murphy assay, as the forward primer binding site has the same sequence as other species that do not amplify; it may be possible *A. dubius* contains some genetic diversity in that region, and the seed isolate we tested contains a different genetic sequence in this region than the one deposited in GenBank. It is also possible that the *A. dubius* accession in GRIN is not a pure species isolate and may be seed mix or hybridized with other species. Although *A. dubius* and *A. spinosus* amplify with the Murphy regular qPCR cycle, using the fast qPCR cycle decreases the amplification of these two species and would eliminate them as potential false positives. *Amaranthus dubius* and *A. watsonii* were not tested in the initial Murphy paper, so it is not unexpected to identify additional species with some level of cross-reactivity.

In addition to amplifying with the Murphy assay targeting an ITS SNP, *A. watsonii* also amplifies strongly with M2 and M3. These additional markers targeted SNPs from different genomic regions, indicating *A. watsonii* and *A. palmeri* are very closely related. A review of these species shows that *A. palmeri* and *A. watsonii* have been described as sister species based on morphology since 1914 and have recently been a part of several sequencing-based phylogenetic studies of the *Amaranthus* genus (Brenan 1961; Raiyemo et al. 2023; Standley 1914; Waselkov et al. 2018). Analysis of six molecular markers across 58 *Amaranthus* species indicated the close genetic relatedness between *A. palmeri* and *A. watsonii* may be due to a chloroplast capture event and incomplete lineage sorting that are not observed in the greater *Hybridus* clade (Waselkov et al. 2018). Based on their analysis of the male-specific region of the Y chromosome (MSY), Raiyemo et al. (2023) propose two origins of dioecy for the dioecious *Amaranthus* spp. and suggest *A. palmeri* and *A. watsonii* have a different origin than the rest of the clade. The strong genetic homology observed between *A. palmeri* and *A. watsonii* is supported by their overlapping native habitat in arid regions of the southwestern United States and northern Mexico (Sauer 1955). Our sequencing of the *A. watsonii* ITS1-5.8S rDNA-ITS2 gene region revealed an identical sequence to *A. palmeri* and demonstrated the high genetic relatedness of the ribosomal nucleic acid between these species that could not be discerned by the Murphy, M2, or M3 qPCR assays tested.

Although *A. watsonii* would be a potential false positive for the Murphy, M2, and M3 qPCR assay, it currently is not known to be distributed outside its small native range and is not expected to be encountered in samples. Every method has limitations, and this false positive is one such limitation uncovered in this study. Future studies to identify other markers and develop *A. palmeri*–specific

https://doi.org/10.1017/wsc.2024.58 Published online by Cambridge University Press

Table 2. Alignment of the Murphy forward primer with *Amaranthus* species tested

Species	GenBank ID	Sequence[a]
Murphy forward primer	—	GCGAACATGTTTATCATACCTGG
A. albus L.	MG685200.1	GCGAACATGTTTATCATA**AGC**GG
A. arenicola I.M. Johnst.	KC747407.1	GCGAACATGTTTATCATA**AG**TGG
A. australis (A. Gray) Sauer	KC747409.1	GCGAACATGTTTATCATA**AG**TGG
A. blitoides S. Watson	KC747410.1	GCGAACATGTTTATC**G**TA**AG**TGG
A. californicus (Moq.) S. Watson	KC747414.1	GCGAACATGTTTATCATA**AGC**GG
A. deflexus L.	MK905427.1	GCGAACATG**T**TTTATC**G**TA**AGC**GG
A. dubius Mart. ex Thell.	KX079519.1	GCGAACATGTTTATCAT**GAG**TGG
A. hybrid Iowa 1	OQ996211.1	GCGAACATGTTTATCAT**GAG**TGG
A. hybrid Pennsylvania	OQ996212.1	GCGAACATGTTTATCAT**GAG**TGG
A. hybrid Iowa 2	OQ996213.1	GCGAACATGTTTATCATA**AG**TGG
A. hybrid Illinois	OQ996214.1	GCGAACATGTTTATCAT**GAG**TGG
A. hybridus L.	MK261292.1	GCGAACATGTTTATCAT**GAG**TGG
A. hypochondriacus L.	KX079523.1	GCGAACATGTTTATCAT**GAG**TGG
A. palmeri S. Watson	MT622754.1	GCGAACATGTTTATCATAC**G**TGG
A. powellii S. Watson ssp. *bouchonii* (Thell.) Costea & Carretero	KX079530.1	GCGAACATGTTTATCAT**GAG**TGG
A. retroflexus L.	KX079528.1	GCGAACATGTTTATCAT**GAG**TGG
A. spinosus L.	KC747443.1	GCGAACATGTT**AT**ATCATAC**G**TGG
A. tuberculatus (Moq.) Sauer	KC747450.1	GCGAACATGTTTATCATA**AG**TGG
A. watsonii Standl.	OQ996215.1	GCGAACATGTTTATCATAC**G**TGG
A. wrightii S. Watson	KC747453.1	GCGAACATGTTTATCAT**GAG**TGG

[a]Base-pair mismatches are presented in bold, underlined font.

molecular diagnostics should include *A. watsonii* in the analysis to eliminate the possibility of a test reacting with it.

Practical Implications

Regulatory testing of commercially sold seeds requires the identification of contaminating noxious weed seeds to the species level. Molecular testing can assist in seed species identification for seeds obtained during visual purity and noxious weed exams. Method specificity and sensitivity are critical aspects of implementing and validating new methods. Thorough testing of three *A. palmeri* molecular methods using widely distributed *Amaranthus* species identified *A. watsonii*, a geographically isolated sister species to *A. palmeri*, as a potential false positive for all assays. Two assays were shown to be effective at identifying *A. palmeri* from DNA extracted in groups of up to 100 seeds. Regulatory programs need to be knowledgeable about the limitations of tests used in regulatory decisions. This work will support the MDA's efforts to detect and limit the spread of *A. palmeri* in seed, screenings, feed, manure, and other pathways to protect our agricultural and natural lands.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2024.58

Acknowledgments. The authors would like to thank the Minnesota Legislature and the Minnesota Seed Industry for supporting this study. The authors would like to recognize Serena McCoy with support from Tina Seeland who performed preliminary evaluation of DNA extraction methods for *Amaranthus* seeds in the MDA's laboratory. The authors are especially grateful for the shared expertise and research provided by Donald Wyse, Jeff Gunsolus, Roger Becker, Tom Peters, and Anthony Brusa (University of Minnesota) who over the past decade have contributed to the work discussed in this article.

Funding. This research was financially supported by the Minnesota State Legislature through the Minnesota Department of Agriculture (MDA) Noxious and Invasive Plant Program, the MDA Seed and Plant Pathology Laboratory Unit, and the MDA Seed Regulatory Program. The MDA Seed Permit Program also provided support. This research received no specific grant from any funding agency or the commercial or not-for-profit sectors.

Competing interests. The authors declare no conflicts of interest.

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https://doi.org/10.1017/wsc.2024.58 Published online by Cambridge University Press