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ORIGINAL ARTICLE

# Multiplex qPCR assays to distinguish individual species of arbuscular mycorrhizal fungi from roots and soil

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## Abstract

Currently, root colonization measurements of arbuscular mycorrhizal fungi (AMF) require staining and microscopy, and species-level identification of the fungi by such observations is not possible. Here, we present novel multiplex real-time PCR assays targeting the glomalin genes of 11 different species of AMF commonly found in temperate agricultural soils, which independently detect and measure the abundance of these fungi using DNA extracts from soil and or root tissue. The availability of these tools will not only increase throughput in determining levels of root colonization but can provide species-specific levels of root colonization from a single sample. This will help to establish which AMF species, or combinations of different species, provide the most benefits to crops, and will aid in the development of AMF for use as biofertilizers.

**Keywords** Soil health · Agricultural practices · Biofertilizer · Mycorrhizae

## Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts which enhance the availability of soil nutrients to plants in exchange for carbon fixed through photosynthesis (Smith and Read 2008). Most crops, aside from those in the Brassicaceae and Amaranthaceae families, form mycorrhizal associations, and as such, AMF are an important consideration for agricultural production and sustainability. Indeed, intentional inoculation with a mixture of AMF species to a variety of crops has demonstrated yield increases of up to 15%, including trials conducted on tomatoes, eggplant, peppers, potatoes, sweet potatoes, and strawberries (Bona et al. 2017; Douds Jr et al. 2007, 2008, 2015, 2017; Douds and Reider 2003).

In general, the goal of AMF inoculation has been to increase the level of colonization of crop roots as early in the season as possible in order to improve nutrient uptake when soil is cool and nutrients, such as P, have limited availability (Douds et al. 2006). It is worthwhile noting that field soils contain indigenous populations of AM fungi, so inoculation experiments must take this into consideration when evaluating root colonization and outcomes on production and nutrient acquisition. In the context of such inoculation trials, molecular analysis for separate quantification of individual fungus species within mixed-species inocula or in the context of native populations previously has not been possible because of the lack of available molecular techniques for these fungi.

The primary obstacles for development of genomic resources for AM fungi have been the inability to culture the organisms axenically and also challenge related to polyploidy and the multinucleate nature of spores and hyphae, which can contain hundreds to thousands of nuclei within a single cytoplasm (Kokkoris et al. 2020). Barcoding based on the ribosomal DNA ITS (internal transcribed spacer) region has been employed for AM fungi, with the caveat that multiple polymorphic sequence variants can be amplified from individual cells, requiring the laborious and time-consuming step of cloning following PCR amplification rather than direct (bulk) sequencing up to dozens of PCR products per accession to get complete coverage (Krüger et al. 2009). Moreover, delimiting taxonomic boundaries within sets of non-identical ITS variants is complex. Despite these complications, various molecular detection assays, including real-time PCR targeting the rDNA ITS region, have been developed over the past two decades, yet these generally fall into one of two classes: those that have narrow specificity for only a few species (Gamper et al. 2008; Jansa et al. 2008; Knegt et al. 2016;

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Thonar et al. 2012), or those that universally consolidate all Glyomeromycota DNA into a single target (Bodenhausen et al. 2021).

Magurno et al. (2019) described a set of primers for universal amplification of the single-copy gene encoding glomalin and presented a dataset showing the utility of this gene as a barcoding locus for taxonomic identification of AM fungi. Glomalin protein, specifically produced by AM fungi, is a key factor affecting soil aggregation with potential implications for soil carbon (C) stability (Wright and Upadhyaya 1996) and makes this locus particularly attractive for functional characterization of different AMF species. In contrast to ITS, glomalin is a single-copy locus that typically produces uniform PCR products that can be sequenced by bulk PCR. We sought to expand upon the foundation laid by Magurno et al. (2019) and we present here PCR primers targeting an expanded genomic region, sequence data from additional taxa, and qPCR assays validated to specifically target 11 different species of AM fungi. The AMF qPCR assays have been combined with a single assay targeting plant DNA (specifically, maize DNA) as an internal control, and the 12 assays have been grouped into three multiplexes to reduce the number of qPCR reactions necessary to measure the abundance of the targets from samples of unknown composition.

## Materials and methods

### Biological material

Whole soil inoculum from pot cultures as a source of spores from accessions of AM fungi was obtained from the collection of the USDA-ARS Eastern Regional Research Center, the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) (Morton et al. 1993), or in the case of *Rhizophagus irregularis* DAOM197198, purchased as a sterile suspension of Mycorise ASP-A (Premier-Tech Horticulture, Riviere-du-Loup, Quebec, Canada). Spores were isolated by wet sieving (US No. 40 and 400 sieves to capture particles between 38 and 425 µm) and flotation on a 40% sucrose step gradient. Accession ID, species identities, and the locations of origin to the extent known of the 43 accessions (one to eight per species) used in this study are indicated in Table 1.

### End-point PCR

Glomalin primers described in Magurno et al. (2019) and new primers designed for this study targeting an expanded portion of the gene were synthesized by Eurofins Genomics (Louisville, KY). The primers are listed in Table 2 and mapped in Fig. 1. Selection of new primers was based on sequence identity among available data for *R. irregularis* isolate DAOM197198 (Tisserant et al. 2013), *Gigaspora rosea* isolates DAOM194757 (Morin et al. 2019), and *Gigaspora margarita* isolate BEG34 (Venice et al. 2020) and spanned the first and third exons of the gene models. Isolated spores, 100 per preparation, were sonicated in 1% SDS for 30 s and rinsed twice with sterile water prior to crushing with micro-pestles. DNA was extracted using the Dynabeads Direct DNA Universal Kit (Thermo-Fisher, Waltham, MA), as in (Schwarzott and Schüßler 2001). Phusion Flash High-Fidelity PCR Master Mix (Thermo-Fisher) was used for all amplifications using the following cycling conditions: initial denaturation of 30 s at 98 °C, followed 40 cycles of 1-s denaturation at 98 °C, 5-s annealing at 56 °C, and 60-s extension at 72 °C, then a final extension of 5-min at 72 °C. Sanger sequencing was completed by Eurofins Genomics. When necessary to improve read quality, PCR products were cloned with the Zero Blunt TOPO PCR Cloning Kit (Thermo-Fisher) prior to sequencing single molecular inserts.

### DNA sequence analysis

Analysis and assembly of raw sequence data were completed in Sequencher version 5.4.6 (GeneCodes Corp., Ann Arbor, MI). Contigs consisting of a minimum of two reads per isolate were assembled. Phylogenetic analysis was completed in MEGA X (Kumar et al. 2018). Alignments were made using the neighbor-joining method (Saitou and Nei 1987), and distances were calculated using the maximum composite likelihood method (Tamura et al. 2004) to generate the phylogenetic tree in Fig. 2.

### Real-time PCR assay design

Standard primers and AffinityPlus qPCR Probes listed in Table 3 were supplied by Integrated DNA Technologies (Coralville, IA) and were designed by inspection of aligned sequence data to identify divergent regions between closely related taxa (i.e., the *Gigaspora* spp., *R. irregularis* and *Rhizophagus intraradices*, and *Claroideoglomus claroideum* and *Claroideoglomus etunicatum*) within introns 1 and 2 of the assembled partial glomalin genes. Locked nucleic acid positions (designated by a + following the position in the probe sequence) improve the specificity for the target sequence over the non-target sequence and alter the annealing conditions. Four fluorophores (FAM, HEX, TEX615, and Cy5) were selected, grouping the assays into three multiplexes (i.e., compatible groups of PCR primers and probes for simultaneous amplification and detection in the same reaction). The compositions of the three multiplexes (A–C) are identified in Table 3. As in an internal control for samples containing corn tissue (or as a spike-in internal control),

**Table 1** Fungal isolates used in this study

| Species | Accession ID | Source | Site of origin | GenBank ID |
|---|---|---|---|---|
| *Claroideoglomus claroideum* | PA104A | INVAM | Kutztown, PA, USA | OK105172 |
| | PA302 | INVAM | Pennsylvania, USA | OK105174 |
| | WV944 | INVAM | West Virginia, USA | OK105175 |
| *Claroideoglomus etunicatum* | PA127B | INVAM | Pennsylvania, USA | OK105176 |
| | PA128 | INVAM | Pennsylvania, USA | OK105178 |
| | PA137 | INVAM | Pennsylvania, USA | OK105179 |
| | NE108A | INVAM | Nebraska, USA | OK105170 |
| | UT316 | INVAM | Utah, USA | OK105173 |
| *Claroideoglomus* spp. | CA101 | INVAM | California, USA | OK105177 |
| | MD210A | INVAM | Maryland, USA | OK105171 |
| | SC186 | INVAM | South Carolina, USA | OK105180 |
| *Funneliformis mosseae* | FL156 | INVAM | Gainesville, FL, USA | OK105162 |
| | KE132 | INVAM | Kenya | OK105166 |
| | OR211 | INVAM | Oregon, USA | OK105161 |
| | OR229 | INVAM | Oregon, USA | OK105165 |
| | PA127A | INVAM | Kutztown, PA, USA | OK105164 |
| | UK115 | INVAM | Ukraine | OK105168 |
| | UT101 | INVAM | Utah, USA | OK105163 |
| | WY111 | INVAM | Wyoming, USA | OK105167 |
| *Gigaspora albida* | FL713 | INVAM | Florida, USA | OK105156 |
| *Gigaspora gigantea* | GGGT002 | ERRC | Pennsylvania, USA | OK105149 |
| | MA401C | INVAM | Bass River Beach, MA, USA | OK105154 |
| | MA453A | INVAM | Massachusetts, USA | OK105153 |
| | MN922A | INVAM | Cedar Creek, MN, USA | OK105152 |
| | NC150 | INVAM | Durham, NC, USA | OK105150 |
| | PA125 | INVAM | Kutztown, PA, USA | OK105155 |
| | PA201 | INVAM | Kutztown, PA, USA | OK105151 |
| *Gigaspora margarita* | NC175 | INVAM | Durham, NC, USA | OK105160 |
| | WV205A | INVAM | Kearneysville, WV, USA | OK105159 |
| *Gigaspora rosea* | FL105 | INVAM | Florida, USA | OK105158 |
| | NY328A | INVAM | New York, USA | OK105157 |
| *Pacispora scintillans* | Sparkle | ERRC | Pennsylvania, USA | OK105190 |
| *Rhizophagus intraradices* | FL208A | INVAM | Florida, USA | OK105181 |
| | FL730 | INVAM | Florida, USA | OK105182 |
| | IA506 | INVAM | Iowa, USA | OK105183 |
| | ID101 | INVAM | Idaho, USA | OK105184 |
| | PL112A | INVAM | Poland | OK105185 |
| | WV795 | INVAM | Jane Lew, WV, USA | OK105186 |
| *Rhizophagus irregularis* | CR316A | INVAM | Costa Rica | OK105188 |
| | DAOM197198 | Premier-Tech | Pont-Rouge, Quebec, Canada | OK105189 |
| | ON205B | INVAM | Ontario, Canada | OK105187 |
| *Scutellospora calospora* | PA103A | INVAM | Pennsylvania, USA | OK105191 |
| *Septoglomus constrictum* | LGEO | ERRC | Pennsylvania, USA | OK105169 |

*INVAM* International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi, West Virginia University, Morgantown West Virginia, USA (Morton et al. 1993), *ERRC* USDA-ARS Eastern Regional Research Center, Wyndmoor, Pennsylvania, USA. Premier-Tech Horticulture, Riviere-du-Loup, Quebec, Canada

the high mobility group (HMG) a qPCR assay (Scholdberg et al. 2009) for amplification of plant DNA was incorporated into multiplex A. qPCR cycling and detection were conducted on a Bio-Rad CFX-384 Instrument (Bio-Rad, Hercules, CA).

**Table 2** Primers used to amplify glomalin genomic DNA from diverse species of AM fungi

| Primer name | Sequence | 5' position | Tm (°C) | Reference |
|---|---|---|---|---|
| Glom_184F | CTAAGGCGGTAGCTGTTACACTTGGTCC | 184 | 68.6 | This publication |
| Glom_221F | GTGTTGATTGAACAACCATATGGTAGC | 221 | 63.8 | This publication |
| Glom_277F | GCTAAAAGTATATCACTTAAAGACAAATTCGAAA | 277 | 63.5 | This publication |
| Glom-FII | GCTGGTTGTAACCCAATGGA | 564 | 58 | (Magurno et al. 2019) |
| Glom-Fout | GGATCTTCGTCGTGGTGTTC | 581 | 56 | (Magurno et al. 2019) |
| Glom-Fin | ATCTTCGTCGTGGTGTTCA | 583 | 53 | (Magurno et al. 2019) |
| Glom_925R | CGCATTCCCTCTGTAATTTCTAATTCATC | 925 | 63.7 | This publication |
| Glom_1000R | TCAATGGTTTTTCAAATTCTACTTTTAGAGATTTAG | 1000 | 64 | This publication |
| Glom-Rin | GCATCAACAAACCGATCTTT | 1514 | 58 | (Magurno et al. 2019) |
| Glom-RinClar | CAATGCATCAACAAATCTATCCTT | 1518 | 59.6 | This publication |
| Glom-Rout | CGCAGCTCGTGTAGCATTTA | 1536 | 53 | (Magurno et al. 2019) |
| Gom-RoutClar | CTGCTGCTCTTGTTGCATTCA | 1537 | 62.9 | This publication |
| Glom-RII | ACGGAACCTTCTTCACCAG | 1709 | 58 | (Magurno et al. 2019) |

## Plant and soil sample DNA extraction and purification

DNA from potting media and roots of bahiagrass (*Paspalum notatum* Flugge) pot cultures was extracted from accessions of each AMF species for qPCR validation. A total of 80 composite root and rhizosphere soil samples derived from 400 sweet corn plants grown under four different management practices within the Vegetable Systems Trial at the Rodale Institute (Kutztown, PA) plants were collected for the proof-of-concept dataset. Twenty samples, each a composite from five individual plants collected from four different plots per treatment, were prepared for each of the following four practices: organic-black plastic mulch (Org-BP), conventional-black plastic mulch (CNV-BP), organic-till (Org-Till, no mulch), and organic-reduced till (Org-RT, roller-crimped cover-crop mulch). Each root sample was a composite of finely chopped roots from 5 individual plants (spanning 1.5-m row-length, 30-cm spacing) grown under one of the four management practices. Soil composition at the selected site is a Comly silt loam; fertility of plots was maintained using a leguminous cover crop and the application of compost and fish emulsion (organic treatments) or application of commercial chemical fertilizer (conventional treatment). Samples were collected at sweet corn ear maturity on August 13–16, 2021. Root systems were carefully extracted with a spade fork and gently shaken to remove loosely attached soil, and then the remaining soil (approx. 250-ml per plant) was collected into a bucket, homogenized among the 5 plants per sample, and subsampled (250-mg) for DNA extraction.

For DNA extraction from root tissue, 50-mg fresh-weight samples were placed in 2-ml screw-cap grinding tubes along with 6, 3-mm zirconium beads (OPS diagnostics, Lebanon, NJ), and homogenized for 60-s at maximum speed (FastPrep-24, MP Biomedicals, Solon, OH), then heated at 56 °C for 30 min and cleared by centrifugation at 13,000×*g* for 60 s. The supernatants were further filtered using 96-well filter plates (NucleoSpin 96 RNA Filter Plate, Macherey–Nagel), then DNA was extracted with the NucleoMag DNA Plant Kit (Macherey–Nagel) on a KingFisher Flex Magnetic Particle Handler (Thermo-Fisher). Similarly, soil samples were homogenized in 2-ml screw-cap tubes filled with 250 mg of crushed garnet and a single 6-mm zirconium bead (OPS Diagnostics) using soil sample lysis buffer SL1 (Macherey Nagel) for 60-s at maximum speed, centrifuged for 60 s at 13,000×*g*, then 400-µl of each supernatant was added to binding buffer and beads of the NucleoMag DNA Food kit (Macherey–Nagel). Aside from the SL1 lysis buffer substitution, all wash buffers and subsequent steps followed the manufacturer's protocol and extraction was automated on the KingFisher Flex.

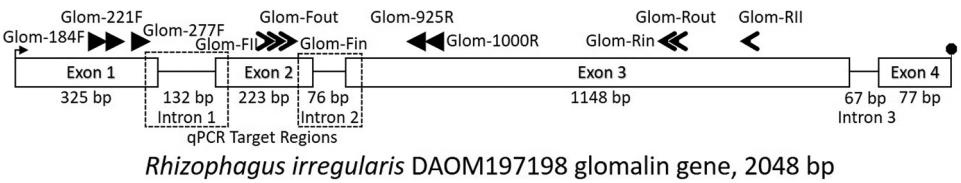


**Fig. 1** Map of *Rhizophagus irregularis* glomalin gene showing exon–intron structure and placement of primers listed in Table 2. Filled arrowheads indicate new primers used in this publication, empty arrowheads are from (Magurno et al. 2019)

**Table 3** Primers and probes for multiplex species-specific qPCR assays of AM fungi

| Multiplex | Species | Forward Primer | Tm (°C) | Reverse Primer | Tm (°C) | Probe[a] | Tm (°C) | Size (bp) | Intron |
|---|---|---|---|---|---|---|---|---|---|
| A | *Gigaspora gigantea* | G_gig_for GTAAAATGT GCTTATACTTATAAC ATAAAATA | 60.6 | G_gig_rev TTAATATAA CGAGAATAATGAGAA TAATGAG | 60.6 | G_gig_pr /56FAM/CGTGTC AA+A+G+AT+C+A+AAA ATTGTG/3IABkFQ/ | 70.5 | 173–196 | 1 |
| A | *Claroideoglomus claroideum* | C_clar_for TTGGTGCAA GGTAAAAAATAATAAT | 60.2 | C_clar_rev CTTGAACAA GCCTAAAATCAAAAA | 60.6 | Clar_spp_pr /5HEX/ CAA+G+T+CTTTCA T+C+A+CATGATT/3IABkFQ/ | 70.4 | 113 | 1 |
| A | *Septoglomus sp.* | Septo_sp_for TGGAATTAA TTTATTGGTTAATAT CATAAACTTGCA | 65.6 | Septo_sp_rev CTTTTCGAA TTCAACCTTTTGAGT TTTAACT | 65.3 | Septo_sp_pr /5TEX615/ TTG+C+T+AC+C+A+TCT CTGC/3IAbRQSp/ | 71.5 | 280 | 2 |
| A | *Zea mays*[b] | ZM-HMG-F TTGGACTAG AAATCTCGTGCTGA | 63.8 | ZM-HMG-R GCTACATAG GGAGCCTTGTCCT | 65.5 | ZM-HMG-Pr /5Cy5/CAATCC ACA/TAO/CAAACGCACGCG TA/3IAbRQSp/ | 68.0 | 79 | |
| B | *Gigaspora margarita* | G_marg_for GTGCAAGGT AAAGAAAATTGTCT | 61.3 | G_marg_rev AATCTGTTA ATATAATGATAATAA ATGATAATAAA | 60.2 | G_marg_pr /56FAM/ TC+A+GTTA+G+C+GTA ATTATT+CTCATTT/3IABkFQ/ | 69.9 | 210 | 1 |
| B | *Claroideoglomus etunicatum* | C_etun_for GGTGCAAGG TAAAAATCTTCG | 60.9 | C_etun_rev TTGAACAAG CCTAAAATCAATACAT | 61.4 | Clar_spp_pr /5HEX/ CAA+G+T+CTTTCA T+C+A+CATGATT/3IABkFQ/ | 70.4 | 112–113 | 1 |
| B | *Pacispora scintillans* | Paci_sp_for ACTTTTAAG CGCGTTATATGTAAC TATGATC | 65.1 | Paci_sp_rev GATCCGTTA TGAAGTATGGGGAGA TG | 65 | Paci_scin_pr /5TEX615/AC+GG A+GGG+TATGC+G+CT/3IA bRQSp/ | 74.3 | 225 | 2 |
| B | *Rhizophagus intraradices* | R_intra_for ACCGATTAT GATGTATTTATCGTA AGATGAATTTCC | 66.8 | R_intra_rev CAAAATTGA AATTTTTTTCTCTGT GAGCAGAATC | 66.5 | R_intra_pr /5Cy5/TA+C+A+ATC TCAG+C+C+AATGGT /3IAbRQSp | 73.9 | 293 | 2 |
| C | *Gigaspora rosea* | G_ros_for AAAATGTGC TTATACTTATAACAT AAATTG | 60.6 | G_ros_rev GTATTTTTA TGATTGATAAGTTGA TGTATTG | 60.9 | G_ros_pr /56FAM/ TTA+A+C+GT+G+T+AAA ATTGTGTGCTC/3IABkFQ/ | 70.8 | 99 | 1 |
| C | *Funneliformis mosseae* | F_mos_for ATTCGTTAT AGCGGTGTATTTATC GTGC | 66.1 | F_mos_rev TCAAATTCA ACCTTTTGAGTTTTG GCA | 66.1 | F_mos_pr /5HEX/ CGG+T+C+AATTAA TAG+C+C+AAC/3IABkFQ/ | 71.7 | 250 | 2 |
| C | *Rhizophagus irregularis* | R_irreg_for TTCACCGAT TATTATAGATTTATC CATAATGAATTTCT | 65.9 | R_irreg_rev CAAAACTGA AATTTTTTTCTCGGA GAGTAGAATT | 66.6 | R_irreg_pr /5TEX615/ TA+C+G+ATCTCG G+C+T+AATGGC/3IAbRQSp/ | 76.1 | 295 | 2 |
| C | *Scutellospora calospora* | S_calo_for GTTAAGGTT AAAAATTCCGCGT | 61.2 | S_calo_rev CAATATAAT GAATATAATGAAATA ATTACGCG | 61.2 | S_calo_pr /5Cy5/AC+C+T+ATC TAT+C+G+GTCA/3IAbRQSp/ | 71.5 | 133 | 1 |

[a]Affinity Plus qPCR Probe notation; + indicated preceding nucleotide is locked, fluorophore and quencher notation and placement determined by IDT, Coralville, IA

[b]For amplification of maize plant DNA, from Scholdberg et al. (2009)

### Real-time PCR cycling and analysis

Real-time PCR reactions, 20-µl, consisted of 10-µl PerfeCTa qPCR ToughMix (Quantabio, Beverly, MA), 2-µl extracted DNA, 0.2-µl of a 100 × stock of multiplexed primers and probes (final concentration 400 nM each primer and 100 nM each probe), and 7.8-µl water. Cycling parameters consisted of a 2-min initial denaturation at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 65 °C. Data were analyzed with CFX-Manager 3.1 (Bio-Rad), and the fluorescence threshold was adjusted to 145 RFU for each fluorophore. All reactions were run in duplicate, and *Ct* values were averaged between replicate reactions.

### Staining and microscopy

To determine the extent to which molecularly characterized levels of root colonization agree with colonization determined by staining and microscopy (20–50 ×), percent root colonization was determined on all samples using the grid-intersect method (Giovannetti and Mosse 1980; McGonigle et al. 1990). In short, root cell contents and root pigment were removed by soaking roots in 10% potassium hydroxide, and roots were acidified for staining in 5% hydrochloric acid and stained with trypan blue (Comas et al. 2014). Roots were randomly dispersed across a 1 cm × 1 cm gridded petri dish and examined at each intersection for the presence of mycorrhizal fungi with a stereoscopic microscope. The percent of the root length colonized was determined as the number of root-grid intersects that were colonized as a portion of total intersects examined (minimum 100 per sample). Normality of the data was confirmed by the Shapiro–Wilk test ($W = 0.99$, $p = 0.810$) and homoscedasticity by the Breusch-Pagan test ($BP = 6.1721$, $p = 0.10$). To determine if mycorrhizal colonization differed significantly between farming practices, we performed one-way analysis of variance (ANOVA). To determine significant differences between treatments, we performed a Tukey–Kramer HSD post hoc analysis.

## Results

### Amplification of an expanded region of the glomalin gene from diverse species of AM fungi

New primers were designed and tested based on conserved regions among available sequences of *R. irregularis* and *Gigaspora* spp. glomalin genes, in an attempt to specifically capture the first intron in addition to the region targeted in (Magurno et al. 2019). Three new forward primers within exon 1 and two reverse primers close to the 5'-end of exon 3 were tested in factorial combinations in single and nested-PCR along with the Magurno et al. primers. A map of all primers is shown in Fig. 1. For *Claroideoglomus* spp. sequences, amplification was improved by modifying the Glom-Rout and Glom-Rin primers to match known sequences from these taxa (new primers Glom-RoutClar and Glom-RinClar, respectively). Outer and nested PCR primer combinations with successful amplifications included Glom_184F/Glom-Rout for *Gigaspora* spp., Glom_184F/Glom-Rout followed by Glom_221/Glom-Rin for *Rhizophagus* spp. and *P. scintillans*, Glom_184F/Glom-Rout followed by Glom_221F/Glom_925R for *S. calospora*, Glom_221F/Glom-RII for *Funneliformis mosseae* and *Septoglomus constrictum*, and Glom_184F/Glom-RoutClar followed by Glom_221F/Glom-RinClar for *Claroideoglomus* spp. Sequencing through intron 2 of products from the *Gigaspora* spp. accessions required cloning because of the presence of 1–2-bp indels within homopolymer runs (only one sequence for each accession was selected for GenBank deposition). Assembled partial glomalin gene sequences, 1.1 to 1.4 Kb, were deposited as GenBank accessions OK105149-OK105191 and are listed in Table 1. A phylogram generated from the alignment of these sequences (Fig. 2) shows the clustering of the isolates as expected from their species designations. Primers and probes for qPCR were strategically placed within introns 1 and 2 to capture the maximum divergence between closely related taxa (i.e., the *Gigaspora* spp., *R. irregularis*, and *R. intraradices*, and *C. claroideum* and *C. etunicatum*), and AffinityPlus qPCR probes were designed to have locked nucleic acid residues selected at the mismatched positions.

### Validation of multiplex glomalin qPCR assays

DNA was extracted from spore isolations and root tissue of actively growing bahiagrass pot cultures; two per each species targeted (except for *S. constrictum* and *Pacispora scintillans* due to only single cultures being available) and used as template for the duplicate reactions in each of the three multiplexes outlined in Table 3. *Ct* values ranged from 24 to 37 cycles for spore DNA (input equivalent to spores isolated from 1 ml of soil) and 25–38 cycles for root tissue collected from the same pots. Cross-reactivity between qPCR assays was not detected (i.e., no off-target amplification was observed among samples). Sequences of qPCR products were verified by Sanger sequencing of bulk PCR products.

### qPCR analysis of AM fungi communities in paired root-rhizosphere field soil samples

By staining, farming practice had a significant effect on mycorrhizal colonization (ANOVA: $F_{3,73} = 7.18$, $p < 0.001$; Fig. 3). Mycorrhizal colonization was higher in the Org-BP treatment (55% ± 4 SE) than in CNV-BP (47% ± 6) and Org-Till (47 ± 8%) but not Org-RT (50 ± 5%), which did not differ between the latter two management practices. By qPCR, major differences between treatment groups were observed in the detection of *C. etunicatum,* with 100% of root samples from the Org-BP and CNV-BP treatments containing this species, and only 65% and 35% of samples from the Org-Till and Org-RT samples testing positive (Table 4). Additionally, the differences in average *Ct* values for the *C. etunicatum* target in samples from the 4 treatment groups (29, 32, 36, and 35 cycles for Org-BP, CNV-BP, Org-Till, and Org-RT, respectively) indicate that the concentration of DNA is approximately eightfold ($=2^{\Delta Ct}$) higher in Org-BP than CNV-BP, and 64–128-fold higher in Org-BP than Org-RT or Org-Till. *Claroideoglomus claroideum* was present in 70–90% of soil samples from each treatment group, but only in 5–15% of corresponding sweet corn root tissue samples. Inversely, in both the Org-BP and CNV-BP sample sets, *F. mosseae* was detected in only a single soil sample (5%) from each set but present in 60% of the root samples from the same sets. Other, less-pronounced changes in relative abundances of AMF taxa within the root and soil samples also were detected.

## Discussion

Building upon the findings of Magurno et al. (2019), we present here an expanded region of the glomalin gene for making phylogenetic inferences about fungi within the Glomeromycota which captures an additional intron relative to the previous dataset. Utilizing the sequence diversity contained within the two introns and flanking regions, we developed species-specific qPCR assays for 11 different species of AM fungi, which we've tested for cross-reactivity and confirmed sequences of genuine amplification products. Future studies may benefit from combining these glomalin qPCR assays with species-specific ITS qPCR assays, such as those of Thonar et al. (2012), or with the universal AMF qPCR assay presented by Bodenhausen et al. (2021).

Analysis of a test sample of field-grown sweet corn roots and paired rhizosphere soil samples has provided evidence of the utility of the glomalin real-time PCR assays (Table 4). We detected differences in abundance of *C. etunicatum* among plots from different management practices. In addition, we found differences among populations present in soil and roots for *C. claroideum and F. mosseae*. This highlights the utility of the assays for characterizing colonization efficiency of different fungi. This evidence suggests our assays will be useful for optimizations of AMF inocula formulations and in characterization of inoculation outcomes. Additionally, these modular assays targeting divergent species of interest will be useful in examining AMF community structure and relative abundance in roots and soils across

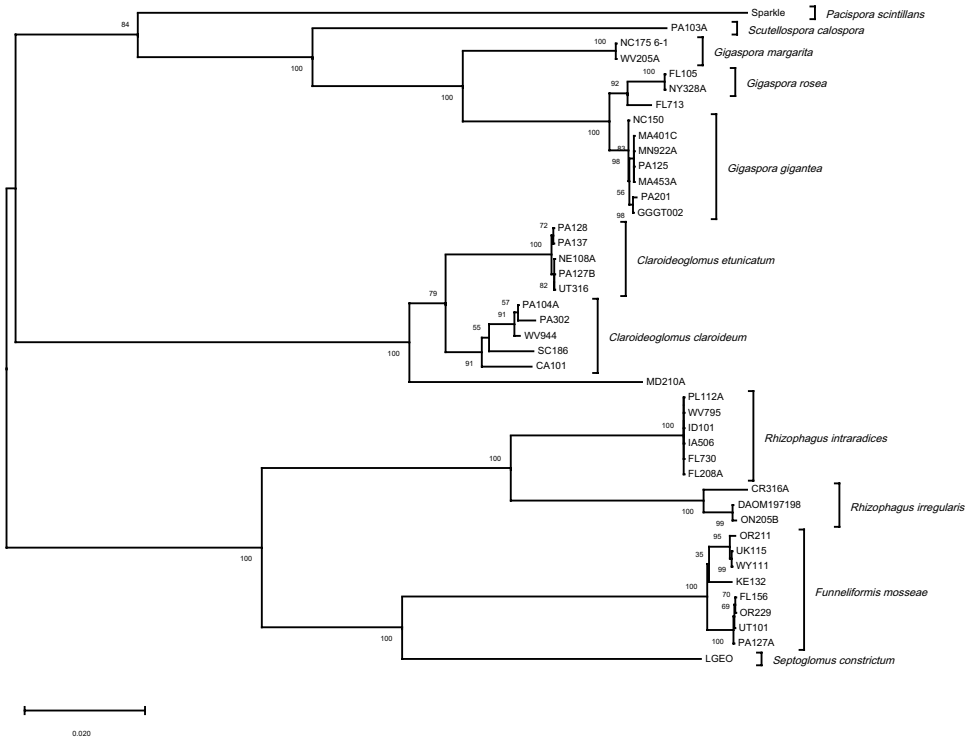


**Fig. 2** Phylogenetic tree based on partial glomalin gene sequence alignments from diverse species of AM fungi, consisting of 1314 positions. Units of the number of base substitutions per site. Bootstrap values are shown. Phylogenetic analyses were conducted in MEGA X (Kumar et al. 2018)

other systems and can give insight into relationships between plants and soil microbial communities.

Mineral fertilizer inputs have been shown to alter soil microbial community composition and ultimately reduce fungal biomass which can lead to reduced root colonization (Jansa et al. 2006). Based on qPCR measurements from the Org-BP and CNV-BP sweet corn samples in our trial, the organic samples had approximately eightfold greater abundance of *C. etunicatum*, indicating mineral fertilizer application may reduce colonization efficiency of this species. In addition, disruption of extraradical hyphae has been associated with reduced infectivity and colonization of plant roots (Evans and Miller 1990; Jasper et al. 1989), and tilling has been shown to reduce AMF spore abundance, disrupt, and reduce fungal hyphal networks, and alter community composition in corn systems (Douds Jr et al. 1995; Galvez et al. 2001; Jansa et al. 2003; Kabir et al. 1998). While mycorrhizal colonization by root-staining showed no significant difference between our Org-RT and Org-Till samples, consistent with those previous findings, some individual species were found to be more abundant in the Org-RT than Org-Till treatments, including *C. claroideum* and *R. intraradices*. These findings demonstrate the utility of the qPCR assays for characterizing species-specific impacts of farm management practices on mycorrhizal community dynamics and plant–microbe interactions which would not be clear from staining alone.

Glomalin has been linked to enhanced soil aggregate stability (Wright and Upadhyaya 1996), which can contribute to physical protection of soil C (Six et al. 2004, 2000). Previous research has shown a loose relationship between levels of root colonization and glomalin protein extracted from root tissue as determined by both Bradford and ELISA assays (Rosier et al. 2008). In addition to determining AMF species-specific levels of root colonization, the glomalin AMF qPCR assays can be used as a proxy for glomalin protein

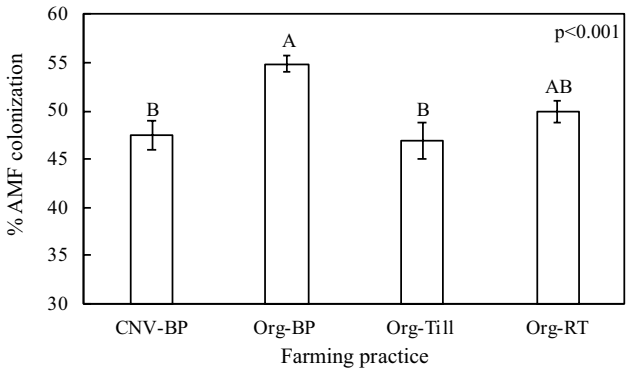


**Fig. 3** Percent root colonization (±SE) in corn farming practices based on the root-intersect method. BP is black plastic. *P* value is from one-way ANOVA. Bars topped by the same letter do not differ at $p \leq 0.05$ according to Tukey–Kramer HSD post hoc analysis

**Table 4** AM fungi detections in root and rhizosphere soil samples from sweet corn grown under different management practices

| | Organic — black plastic | | | | Conventional — black plastic | | | | Organic — till | | | | Organic – reduced till | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Root | | Soil | | Root | | Soil | | Root | | Soil | | Root | | Soil | |
| Target | % Pos | Avg Ct | % Pos | Avg Ct | % Pos | Avg Ct | % Pos | Avg Ct | % Pos | Avg Ct | % Pos | Avg Ct | % Pos | Avg Ct | % Pos | Avg Ct |
| *C. etunicatum* | 100% | 29 | 100% | 31 | 100% | 32 | 100% | 31 | 65% | 36 | 70% | 36 | 35% | 35 | 55% | 36 |
| *C. claroideum* | 15% | 35 | 70% | 34 | 10% | 37 | 80% | 33 | 5% | 33 | 85% | 35 | 15% | 33 | 90% | 34 |
| *F. mosseae* | 60% | 33 | 5% | 32 | 60% | 34 | 5% | 40 | 35% | 33 | 10% | 37 | 15% | 35 | 30% | 35 |
| *R. irregularis* | 35% | 36 | 0% | | 25% | 35 | 0% | | 10% | 34 | 0% | | 30% | 34 | 25% | 37 |
| *R. intraradices* | 35% | 33 | 20% | 37 | 5% | 33 | 5% | 37 | 20% | 38 | 5% | 38 | 10% | 33 | 20% | 36 |
| *G. margarita* | 0% | | 5% | 32 | 5% | 39 | 10% | 37 | 25% | 33 | 20% | 37 | 0% | | 0% | |
| *S. constrictum* | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 5% | 33 | 25% | 37 |
| *G. rosea* | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | |
| *P. scintillans* | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | |
| *G. gigantea* | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | |
| *S. calospora* | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | | 0% | |
| ZM.HMG | 100% | 25 | 100% | 32 | 100% | 25 | 100% | 32 | 100% | 25 | 100% | 33 | 100% | 25 | 100% | 31 |

$n = 20$ samples for each management practice. In the black plastic treatments, soil was tilled into rows and covered with black plastic much prior to planting. In the reduced tillage treatment, the rye cover crop was roller-crimped prior to seedling planting. Organic treatments do not use mineral fertilizer or pesticides. ZM-HMG is the *Zea mays* control primer target

biosynthetic potential, including the production of species-specific glomalin protein variants. While we acknowledge that the current qPCR assays measure abundance of fungal biomass and not glomalin mRNA expression, adaptation of the assays for that purpose is possible. Future research probing the differences in glomalin protein structure, abundance, and stability from different species of AM fungi will be insightful for comparative analysis with soil physiochemical properties including soil aggregation and soil C storage and persistence across a range of soil environments.

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**Data vailability** All sequence data have been deposited in GenBank and biological materials not available elsewhere may be requested from the corresponding author.

**Code availability** Not applicable.

## Declarations

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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