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RESEARCH

Geographic Location, Management Strategy, and Huanglongbing Disease Affect Arbuscular Mycorrhizal Fungal Communities Across U.S. Citrus Orchards

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ABSTRACT

The benefits of arbuscular mycorrhizal fungi (AMF) to agroecosystems have been well recognized. Citrus is a globally grown fruit tree commonly found in association with AMF. Global citrus production is currently under the threat of the pandemic huanglongbing (HLB) disease. Since its introduction in the United States, the disease has devastated the Florida citrus industry and is now at the doorsteps of commercial orchards in California. Here, we tested how the two distinct climatic zones within the continental United States where citrus is mostly grown (California and Florida) influenced AMF community diversity and composition. We also assessed in what capacity low-input organic farming and HLB disease affected the AMF communities colonizing the citrus roots. Root samples were collected from 88 trees across 10 orchards. Orchards were selected based on conventional or organic practices in California and based on HLB symptom severity in Florida. AMF communities were characterized using high-throughput sequencing of the small-subunit ribosomal RNA gene. Taxa names were assigned based on a phylogenetic analysis that comprised a backbone of AMF reference sequences from Mycobank and virtual taxa from the MaarjAM database. AMF were detected in 78% of citrus root samples, with taxa belonging to six genera (*Dominikia*, *Funneliformis*, *Glomus*, *Rhizophagus*, *Sclerocystis*, and *Septoglomus*) and unknown Glomeraceae genera. Geographical location, management practice, and disease affected AMF community composition. We provide evidence that perennial agroecosystems are composed of generalist and specialist AMF taxa comparable with other ecosystems and identified ubiquitous taxa that could potentially be exploited for agricultural purposes.

Keywords: AMF, arbuscular mycorrhizal fungi, citrus, huanglongbing, microbial diversity, root system

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Arbuscular mycorrhizal fungi (AMF) are biotrophic organisms forming symbiotic associations with more than 70% of land plants across a broad range of terrestrial ecosystems (Brundrett and Tedersoo 2018; Smith and Read 2008). The nature of the symbiosis spans from mutualistic to parasitic (Johnson et al. 1997). The mutualistic symbiosis resides in a trade-off between a more efficient root acquisition of water and nutrients (especially phosphorus) via the mycorrhizal hyphal network, in exchange for photoassimilated carbon. The outcome of this interaction often results in improved plant environmental fitness, with increased tolerance to biotic and abiotic stresses (Chen et al. 2018; Hohmann and Messmer 2017). In addition, AMF improve soil structure by forming stable soil aggregates, thereby limiting erosion and leaching of nutrients (Chen et al. 2018; Wilson et al. 2009). The parasitic interaction between plants and mycorrhizal fungi is only considered when net cost of the symbiosis exceeds net benefits (Johnson et al. 1997).

Plant parts above and below ground are intricately connected and the health status of the root system often determines plant growth and productivity. The rhizosphere microbial diversity is a biomarker of soil fertility and plays a central role in sustainable agricultural

systems (Hartmann et al. 2015; Mäder et al. 2002). Low-input agriculture systems (organic and biodynamic farming) rely on soil biological metabolism and function to support soil fertility and plant root health. In contrast, intensive farming practices characterized by monoculture, input of synthetic agrochemicals, or soil disturbance generally lead to degradation of the soil ecosystem and erosion of AMF biodiversity (Verbruggen et al. 2010). AMF communities are also affected by soil types and land use intensity, and AMF community composition is often characterized by a collection of "specialist" taxa capable of colonizing specific soil habitats and "generalist" taxa associated with a wide range of diverse ecosystems (Oehl et al. 2003, 2010; Verbruggen et al. 2010). Understanding the factors that shape AMF community assemblage under agricultural constraints could lead to deployment of improved sustainable practices.

Citrus is a high-value crop and one of the most popular and widely grown fruit trees globally. It is praised for its nutritional value and benefits to human health as a source of vitamins, fibers, and minerals. Citrus accounts for 16% of the total value of U.S. fruit production (Li et al. 2020), with California and Florida leading the nation's fresh fruit and juice markets, respectively. Symbiotic associations with AMF have been reported in all citriculture production areas and AMF communities are shaped by edaphic characteristics, orchard management practices, and host variety and age (Franca et al. 2007; Nemec et al. 1981; Song et al. 2015; Wang et al. 2012). Adopting low-input farming practices for citrus at a large geographic scale has been challenging because of huanglongbing (HLB), a disease associated with an invasive phloem-limited bacteria in the '*Candidatus* Liberibacter' genus (i.e., '*Candidatus* Liberibacter *asiaticus*', '*Ca.* L. *americanus*', and '*Ca.* L. *africanus*') (Bové 2006). Symptoms of HLB include asymmetrical blotchy mottling of leaves, yellow shoots, thinning of the canopy, and wood dieback, with fruit appearing small, off colored, and lopsided, with a bitter taste (Bové 2006). In the United States, '*Ca.* L. *asiaticus*' is the primary causal agent of citrus HLB and was first detected in Florida in 2005 and California in 2012. In California, the disease has mostly been reported in residential areas in the southern part of the state. In contrast, the disease has now become endemic to Florida, and the citrus industry has already suffered a 74% decline in production, with losses amounting to over $1 billion annually (Court et al. 2018; Li et al. 2020). The pathogen is vectored by an invasive insect (*Diaphorina citri*; the Asian citrus psyllid) and disease management has been mostly achieved by intensive regimens of synthetic insecticide applications to control insect populations (Boina and Bloomquist 2015). In heavily affected orchards, trees are also treated with antibiotics (oxytetracycline and streptomycin) to reduce levels of pathogen inoculum reservoirs (Hu et al. 2018). Those practices have raised environmental concerns due to the risk of unintended consequences for biodiversity and selection for resistance in bacterial and insect populations (McKenna 2019; Wood and Goulson 2017).

In HLB-affected orchards, the tree rhizosphere suffers from microbial dysbiosis and root collapse, thereby weakening the host and its defense response against attack from other pathogens (Fan et al. 2013; Ginnan et al. 2020). Specifically, a study from Florida found that high relative abundance of Glomeromycota species correlated with healthier trees (Ginnan et al. 2020), although the internal transcribed spacer 2 amplicon used in the study limited the resolution of AMF taxonomy and may have omitted key taxa (Lekberg et al. 2021) or biased the identification towards specific taxonomic groups within the phylum Glomeromycota (Davison et al. 2015). AMF can provide protection against citrus root diseases (Tian et al. 2021; Watanarojanaporn et al. 2011) and developing practices that support their biodiversity can offer new ground for management strategies. Previous studies have found at least seven genera of AMF associated with citrus roots (*Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Pacispora*, *Sclerocystis*, and *Scutellospora*), yet little is known about how these communities vary across geographies or management practices. Moreover, many of these studies are morphologically based and may have underrepresented AMF diversity that can be captured with sequencing approaches (Stefani et al. 2020).

Citrus is an emblematic specialty crop to U.S. agriculture. In the wake of the economic and environmental challenges posed by HLB, alternative strategies to farming citrus should be considered. Here, we tested how the two distinct climatic zones within the continental United States where citrus is primarily grown (California and Florida) influenced AMF community diversity and composition. Within each zone, we further evaluated how conventional farming and disease affected AMF diversity and composition. Citrus root-associated AMF were assessed by high-throughput sequencing of the small-subunit (SSU) ribosomal RNA gene (Dumbrell et al. 2011; Lee et al. 2008). Orchards in California, where commercial HLB-positive trees have not been detected, were selected according to the farming practices (organic versus conventional). Orchards in Florida were chosen according to severity of HLB disease symptoms expression (mild, moderate, severe). This study provides insightful information about AMF dynamics within a major perennial agroecosystem and identifies putative generalist taxa that could be exploited for agricultural purposes.

MATERIALS AND METHODS

Root sample collection. Root samples were collected from 88 trees in 10 citrus orchards located in Florida (10 trees/orchard) and California (8 trees/orchard). Roots were collected 0.5 m away from each side of the tree trunk following published protocols (Ginnan et al. 2020). In Florida, 40 root samples were collected in March 2017 from four conventional orchards (Table 1). All trees were rated for HLB symptoms using a disease rating scale ranging from mildly to moderately and severely symptomatic. In California, 48 root samples were collected in October 2017 from four conventional and two organic orchards (Table 1). Gloves were changed and clippers and shovels were sterilized with 30% household bleach between each sampled tree. All samples were immediately placed in bags on ice in a cooler for transit to the laboratory. Root samples were rinsed with autoclaved purified water (Barnstead Mega-Pure System MP-6a; Thermo Fisher Scientific, Waltham, MA, U.S.A.) and approximately 5 g of rinsed root tissue was placed into 50-ml conical tubes, stored at −80°C, then lyophilized (Labconco FreeZone 4.5L, Kansas City, MO, U.S.A.) for 16 to 20 h. Root samples collected in Florida were shipped to University of California–Riverside under United States Department of Agriculture permit number P526P-16-00352 on dry ice.

DNA extraction, library construction, and sequencing. DNA was extracted from roots according to published protocols (Ginnan et al. 2020). Frozen and freeze-dried roots were crushed into small pieces (<0.5 cm) with sterile stainless-steel spatulas on dry ice, and 100 mg of freeze-dried tissue was transferred to 2-ml microcentrifuge tubes (Eppendorf Safe-Lock tubes; Eppendorf, Hamburg, Germany) containing a single 4-mm stainless-steel grinding ball (SPEX SamplePrep, Metuchen, NJ, U.S.A.). Samples were chilled at −80°C for 15 min, then pulverized to a powder using a 2010 Geno/Grinder (SPEX SamplePrep) at 1,680 rpm for 20 to 30 s, twice. Then, 1 ml of 4 M guanidine thiocyanate buffer was added to the pulverized root samples. Samples were incubated at 4°C for 15 min and subsequently centrifuged for 1 h at 17,500 × *g*. DNA was isolated using the MagMAX-96 DNA Multi-Sample Kit

(Thermo Fisher Scientific) with the protocol "MagMAX Express-96 Magnetic Particle Processor". The final DNA was eluted in 100 ml of DNA elution buffer and stored at −20°C prior to Illumina library construction.

DNA was PCR amplified as previously described (Phillips et al. 2019), targeting the 18S region using the universal eukaryote WANDA and the Glomeromycotina-specific AML2 primer sets (Dumbrell et al. 2011; Lee et al. 2008). PCR was conducted in a two-step procedure (Berry et al. 2012), in which first-round amplifications were carried out with primers possessing universal tails synthesized 5′ to the locus-specific sequences (Alvarado et al. 2018) and second-round amplifications ligated Illumina MiSeq flowcell adapters and barcodes (Phillips et al. 2019). PCR1 included 1 μl of template DNA, 12.5 μl of AccuStart II PCR ToughMix (2×) (Quantabio, Beverly, MA, U.S.A.), 0.5 μl of each primer (10 μM), and 10.5 μl of nuclease-free water, resulting in a 25-μl reaction. Thermocycler conditions for PCR1 were as follows: 2 min at 94°C followed by 29 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 68°C. Reaction products were verified on a 1% agarose gel and purified using the AMPure XP magnetic Bead protocol (Beckman Coulter Inc., Brea, CA, U.S.A.). PCR2 was performed in a 25-μl reaction, with 1 μl of the undiluted purified PCR1 product, 6.5 μl of AccuStart II PCR ToughMix (2×) (Quantabio), 2.5 μl of each barcode primer (1 μM), and 12.5 μl of nuclease-free water. Thermocycler conditions for PCR2 were as follows: 2 min at 94°C followed by 9 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. We checked indexed PCR products on an agarose gel and pooled the products by band strength as previously established (Glassman et al. 2018), with 1 μl for strong bands, 2 μl for medium bands, and 3 μl for weak bands prior to AMPure bead purification. The purified library was quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific) and quality checked with an Agilent BioAnalyzer 2100 for size and concentration and sequenced with Illumina MiSeq NanoV2 (2 × 250 bp) at the University of California–Riverside Institute for Integrative Genome Biology. However, there was insufficient overlap between the read pairs to assemble the entire SSU ribosomal RNA amplicon and, thus, we only used the forward sequence for phylogenetic purposes and taxa assignment. Sequences were submitted to the NCBI Sequence Read Archive under accession number PRJNA839101.

Bioinformatics and taxonomy assignment. Initial quality filtering of sequences was done using Trimmomatic (Bolger et al. 2014) truncating reads once the average quality of five consecutive base pairs dropped below a quality score of 20. Sequence reads were further filtered using DADA2's recommended parameters (maxN=, maxEE=, and truncLen=). DADA2 (v 1.14.1) default parameters were also used to dereplicate, learn error rates, and create an amplicon sequence variant (ASV) table (Callahan et al. 2016). Samples with <1,000 reads were removed, as were taxa not identified as fungi via the NCBI database. Rare taxa, defined as taxa which were prevalent in <2% of samples, were also removed. Taxonomy was assigned using BLASTN (Altschul et al. 1990) and the MaarjAM database (https://maarjam.ut.ee/) (Öpik et al. 2010) using a cut-off e-value of 1e-50 and assigning virtual taxa (VT) based on lowest e-value. MaarjAM is an AMF curated database that is standardized, comparable across research projects, and preserved in time (Lekberg et al. 2018). Sequences were aligned in Muscle v.3.7 (Edgar 2004) implemented on the CIPRES Gateway (Miller et al. 2010) using default parameters. The alignment included the VT sequences as well as available consensus sequences from Krüger et al. (2012) and updated by Stefani et al. (2020) and sequences from NCBI GenBank. Genus names were assigned through the curation of a maximum-likelihood bootstrap tree constructed in RAxML v8.2.12 (Stamatakis 2014) implemented on the CIPRES Gateway (Miller et al. 2010) using a GTRGAMMA evolutionary model of nucleotide substitution and with branch support inferred using 1,000 bootstraps. The tree was rooted using *Paraglomus occultum* as the outgroup as per Krüger et al. (2012). The consensus tree was visualized and annotated in Interactive Tree of Life (https://itol.embl.de/) (Letunic and Bork 2019).

Statistical analyses and data visualization. We considered one tree as a single replicate for the three datasets geographical location (Florida = 34 trees and California = 36 trees), cultural practice (organic = 15 trees and conventional = 21 trees), and HLB severity (mild = 12 trees, intermediate = 10 trees, and severe = 11 trees). R Core Team v4.1.1 was used to perform statistical analysis and data visualization with the aid of the phyloseq v1.36.0 (McMurdie and Holmes 2013) and ggplot2 v3.3.5 packages (Wickham 2016). α-Diversity was estimated on untransformed data, as the number of observed taxa of each sample. Statistical

TABLE 1
Orchard location and varieties of citrus sampled indicating the number of root samples where arbuscular mycorrhizal fungi (AMF) was found with the average number of virtual taxa (VT)

Orchard	State[a]	GPS coordinates	*N*[b]	Orchard type	Scion variety	Rootstock	Roots with AMF[c]	VT[d]
1	Florida	27.027723, –80.485323	10	Conventional	Valencia	Swingle	9	12.9
2	Florida	27.446706, –80.325606	10	Conventional	Ruby Red	US897	9	8.6
3	Florida	28.981768, –81.924718	10	Conventional	Parson Brown	Swingle	9	25
4	Florida	28.714208, –81.774555	10	Conventional	Hamlin	Sour orange	6	17.2
5	California	33.322922, –116.988861	8	Conventional	Newhall × Satsuma	Carrizo	7	7.4
6	California	36.353254, –119.059073	8	Conventional	Late Navel Powell	Carrizo	8	12
7	California	35.656439, –119.142610	8	Conventional	Late Navel Powell	Carrizo	2	16
8	California	33.322922, –116.988861	8	Conventional	Late Navel Powell	Carrizo	4	14.5
9	California	35.016722, –118.851070	8	Organic	Newhall x Satsuma	Carrizo	7	24.1
10	California	35.656066, –119.141074	8	Organic	Late Navel Powell	Carrizo	8	17.6

[a] State in the United States.
[b] Number of root samples collected.
[c] Number of root samples with AMF.
[d] Average number of VT.

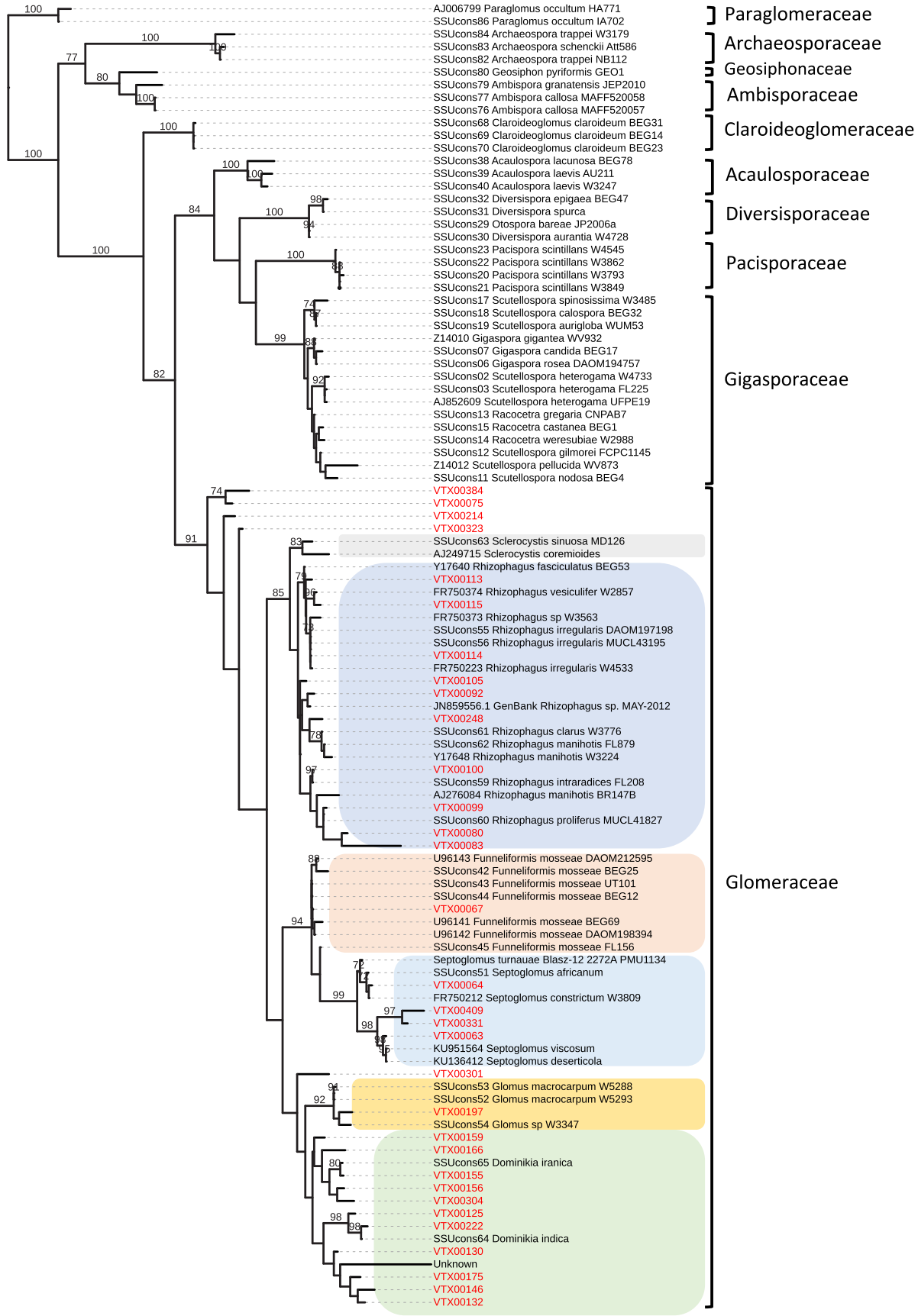

Fig. 1. RAxML phylogenetic tree reconstructed by maximum-likelihood analysis showing the genus taxonomy assignment of the 33 virtual taxa (in red). The tree represents 18 arbuscular mycorrhizal fungi (AMF) genera and virtual taxa (VT) clustered with five colored AMF clades. Bootstrap values greater than 70 are displayed on the nodes.

significance was calculated by a generalized linear model using Poisson regression, and statistical significance on pairwise comparison was performed through Tukey's test using the multcomp packages v1.4.17 (Hothorn et al. 2008). β-Diversity plots were created on proportionally transformed data using the Bray-Curtis dissimilarity matrix and nonmetric multidimensional scaling ordination matrix using the Vegan package v2.5.7 (Oksanen et al. 2020). Permutational analysis of variance (PERMANOVA) as implemented by the vegan package function *adonis* (with 999 permutations) was run to determine statistical differences in community composition between categorical variables. Pairwise PERMANOVA using the RVAideMemoire package v09-81-2 was run for comparison between groups (Hervé 2018). Data was transformed using the variance stabilization method in the DESeq package v1.32.0 (Love et al. 2014) and was used in heat maps to compare abundance between categories. For heatmaps, created using ComplexHeatmap v2.9.4 (Gu et al. 2016), the variance-stabilized transformed data were aggregated to the VT level and dataset split based on category. VT occurring in fewer than three samples of the dataset were removed for ease of visualization. This resulted in 27 taxa in the heatmap comparing California and Florida samples, 15 taxa in the heatmap comparing management strategy, and 17 taxa in the heatmap comparing disease rating. For the Venn diagram, a 10% prevalence filtering was applied to find unique and shared taxa amongst categories. Finally, DESeq2 (V1.32.0) (Love et al. 2014) using the default Wald test and local fit was used to identify taxa with differential abundance analysis among variables of interest. For this analysis, taxa were aggregated to the VT level. VT that had a P value < 0.01 are represented in the heat maps with a asterisk (*) symbol or by colored block.

RESULTS

Quality filtering of the sequences resulted in 1,085,960 reads and 131 ASVs. The MaarjAM database assigned the 131 ASVs to 32 unique VT, and with one sequence left unidentified. The 33 representative VT sequences were aligned to 58 consensus sequences from Krüger et al. (2012) and updated by Stefani et al. (2020) and 12 sequences from GenBank, resulting in a total of 103 sequences and an alignment length of 756 nucleotides. Within this alignment, the portion corresponding to the shorter (220 bp-long) sequences of the 33 VT was 239 bp in length and consisted of 93 conserved, 136 variable, 103 parsimony-informative, and 32 singleton sites.

The maximum-likelihood phylogenetic analysis that included several taxa from Krüger et al. (2012) and the same outgroup (*P. occultum*) yielded a similar tree topology with strong bootstrap support at the family level, and indicated that all the VT belong to the Glomeraceae division (Fig. 1). Taxonomic identification at the genus level was more challenging for some groups given the short nucleotide sequence length (220 bp) of the VT but monophyletic clades with good bootstrap support were obtained for *Sclerocystis*, *Glomus*, and *Septoglomus*. The genus *Rhizophagus* formed a weakly supported clade but was part of a well-supported clade with *Sclerocystis*. Similarly, *Funneliformis*—though itself not a monophyletic group—formed a strongly supported clade with *Septoglomus*. Support for *Dominikia* was low and good bootstrap support

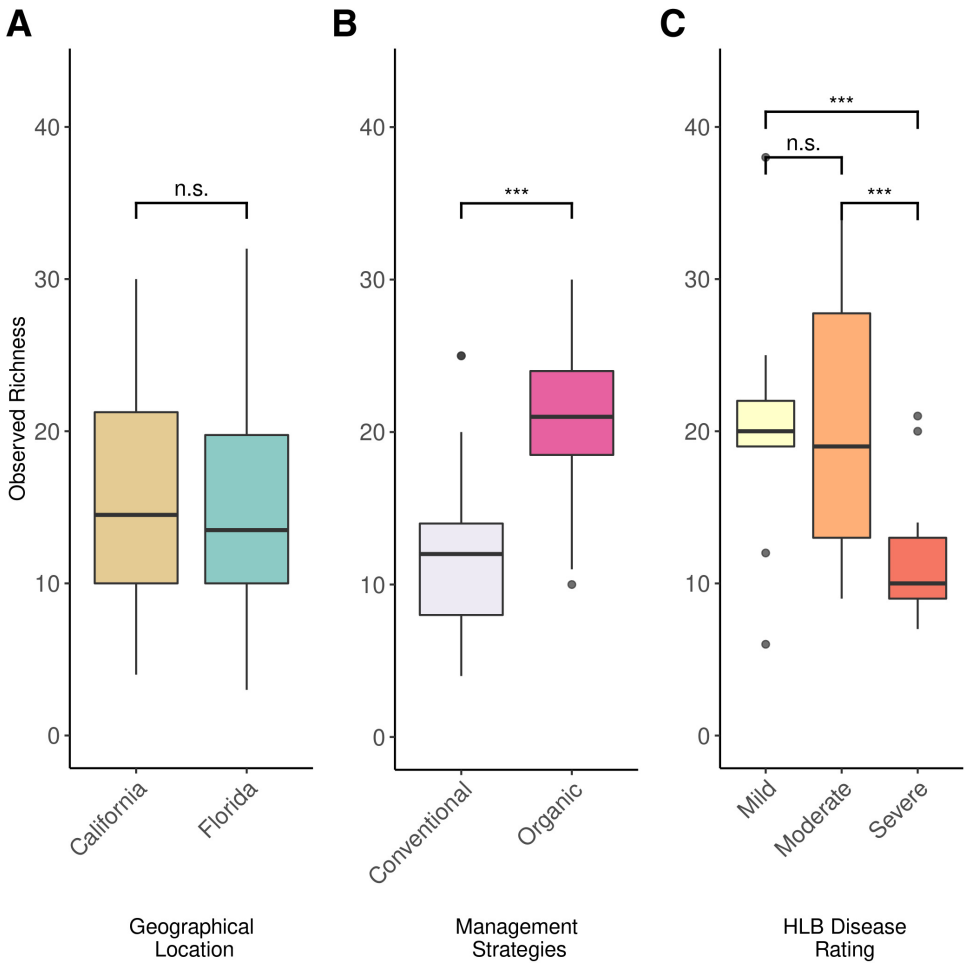

Fig. 2. α-Diversity plots comparing arbuscular mycorrhizal fungi richness across sample types: **A,** geographical location shows no effect on richness, unlike **B,** management strategies and **C,** huanglongbing (HLB) disease. Statistical significance is indicated for $P < 0.001$ (***) based on a Poisson generalized linear model with a pairwise Tukey test. California, $n = 36$ and Florida, $n = 34$; Conventional, $n = 21$ and Organic, $n = 15$; and HLB symptom severity: mild, $n = 12$ trees, intermediate, $n = 10$ trees, and severe, $n = 11$ trees.

was obtained only for a subclade composed of two VT (VTX00222 and VTX00125) with *Dominikia indica*, and for a subclade composed of *D. iranica* and VTX00155. Based on the tree phylogeny, we assigned 12 VT (VTX00125, −130, −132, −146, −155, −156, −159, −166, −175, −222, −304 and -unknown) to *Dominikia*, 10 VT (VTX00080, −083, −092, −099, −100, −105, −113, −114, −115, and −248) to *Rhizophagus*, 4 VT (VTX00063, −064, −331, and −409) to *Septoglomus*, 1 VT (VTX00197) to *Glomus*, and 1 VT (VTX00067) to *Funneliformis*. Placement for five VT (VTX00075, −214, −301, −323, and −384) remained uncertain because they did not cluster in any of those clades, and these were labeled as Glomeraceae species.

We detected AMF in 69 of the total 88 citrus root samples (78%) (Table 1). Geographical location did not affect AMF observed richness in California and Florida citrus orchards, as indicated by similar α-diversity indices (15.8 average ASVs in California versus 15.7 average ASVs in Florida; $P > 0.05$, Poisson generalized linear model with a pairwise Tukey test) (Fig. 2A), although AMF community composition differed significantly between the two states (Adonis $R^2 = 0.176$, $P < 0.001$) (Fig. 3A). Nine VT were commonly associated with citrus in both Florida and California orchards and represent generalists (Fig. 4) They included two *Dominikia* taxa (VTX00156 and VTX00304), three *Rhizophagus* taxa (VTX00092, −100, and −248), one *Septoglomus* taxon (VTX00063), and three unknown taxa within the Glomeraceae family (VTX00214, −301, and −323). The remaining AMF taxa were associated with a single geographical location (VTX00075, −113, −115, −125, −132, −222, and −384 in Florida and VTX00064, −067, −105, −130, −155, −175, −331, −409, and -unknown in California), and under specific management practices or disease phenotype, which could imply specialized functions or unique growth needs (Figs. 4, 5, and 6). Therefore, we refer to these taxa as specialists. Several taxa from both the generalist and specialist groups were differentially abundant across geographical location (Wald's test: $P < 0.01$) (Fig. 4), including three generalist taxa (*Rhizophagus* VTX00092, *Septoglomus* VTX00063, and unknown Glomeraceae VTX00214) and nine specialist taxa, with five from Florida (*Rhizophagus* VTX00113 and −115, *Dominikia* VTX00222 and −125, and unknown Glomer-

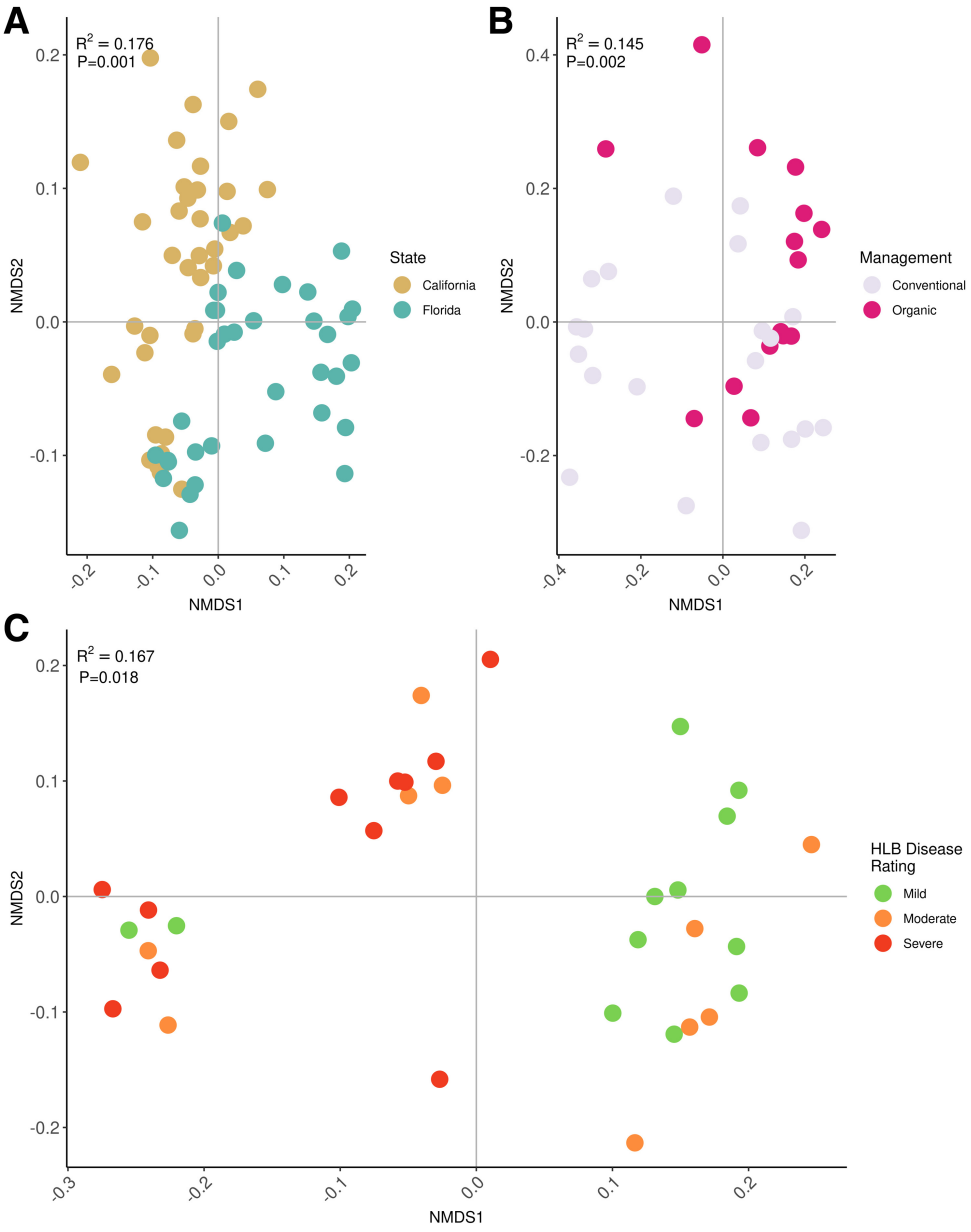

Fig. 3. Nonmetric multidimensional scaling (NMDS) plots indicating that arbuscular mycorrhizal fungi (AMF) β-diversity is significantly affected across sample types based on **A,** geographic location; **B,** management strategies in California; and **C,** huanglongbing (HLB) disease in Florida. Each dot represents the AMF community composition of a single tree. Points are colored by each group. *P* values and R^2 values were measured by permutational multivariate analysis of variance (Adonis) with values shown on the graphs.

aceae VTX00384) and four from California (*Dominikia* VTX00155 and −130 and *Septoglomus* VTX00409 and −064).

Orchard management strategy in California significantly affected both AMF richness and composition, with a significant decrease of observed taxa richness in conventional orchards (12.2 average ASVs in conventional versus 20.7 average ASVs in organic orchards; $P < 0.0001$, Poisson generalized linear model with a pairwise Tukey test) (Fig. 2B) and wider compositional variability (i.e., dispersion) among conventional orchard than organically managed orchards (Adonis $R^2 = 0.145$, $P < 0.01$; betadisper $P < 0.05$) (Fig. 3B). Our data also indicated that organic and conventional California orchards shared 10 AMF taxa, including 8 of the 9 generalist taxa (Fig. 5). Those included three taxa belonging to the genus *Dominikia* (VTX00156, −304, and −155), three to *Rhizophagus* (VTX00092, −248, and −100), two to *Septoglomus* (VTX0063 and VTX00409), and two to unknown genera (VTX00301 and VTX00214). One generalist taxon (unknown Glomeraceae VTX00323) and one specialist (*Funneliformis* VTX00067) were only associated with conventional orchards. In contrast, several AMF taxa were unique to organic orchards, including two *Septoglomus* (VTX00064 and VTX00331), one *Rhizophagus* (VTX00105), and three *Dominikia* (VTX130, −175, and unknown), among which *Dominikia* VTX00130 and *Septoglomus* VTX00064 were significantly enriched (Wald's test: $P < 0.01$) (Fig. 5).

HLB disease status significantly affected the AMF community, with a significant decline in observed richness of severely diseased trees compared with mild and moderately diseased (17.5 average ASVs in mildly diseased versus 20.3 average ASVs in moderately diseased versus 11.5 average ASVs in severely diseased trees; $P < 0.001$, Poisson generalized linear model with a pairwise Tukey test) (Fig. 2C). Shifts in community composition was also seen as disease severity increased from mild to severe (Adonis $R^2 = 0.167$, $P < 0.05$) (Fig. 3C). Nine taxa were not detected in severely affected trees (VTX00075, −099, −113, −115, −125, −132, −146, −222, and −323) (Fig. 6), of which, generalist unknown VTX00323 (Glomeraceae) and VTX00132 (*Dominikia*) were statistically supported (Wald's test: $P < 0.01$) (Fig. 6).

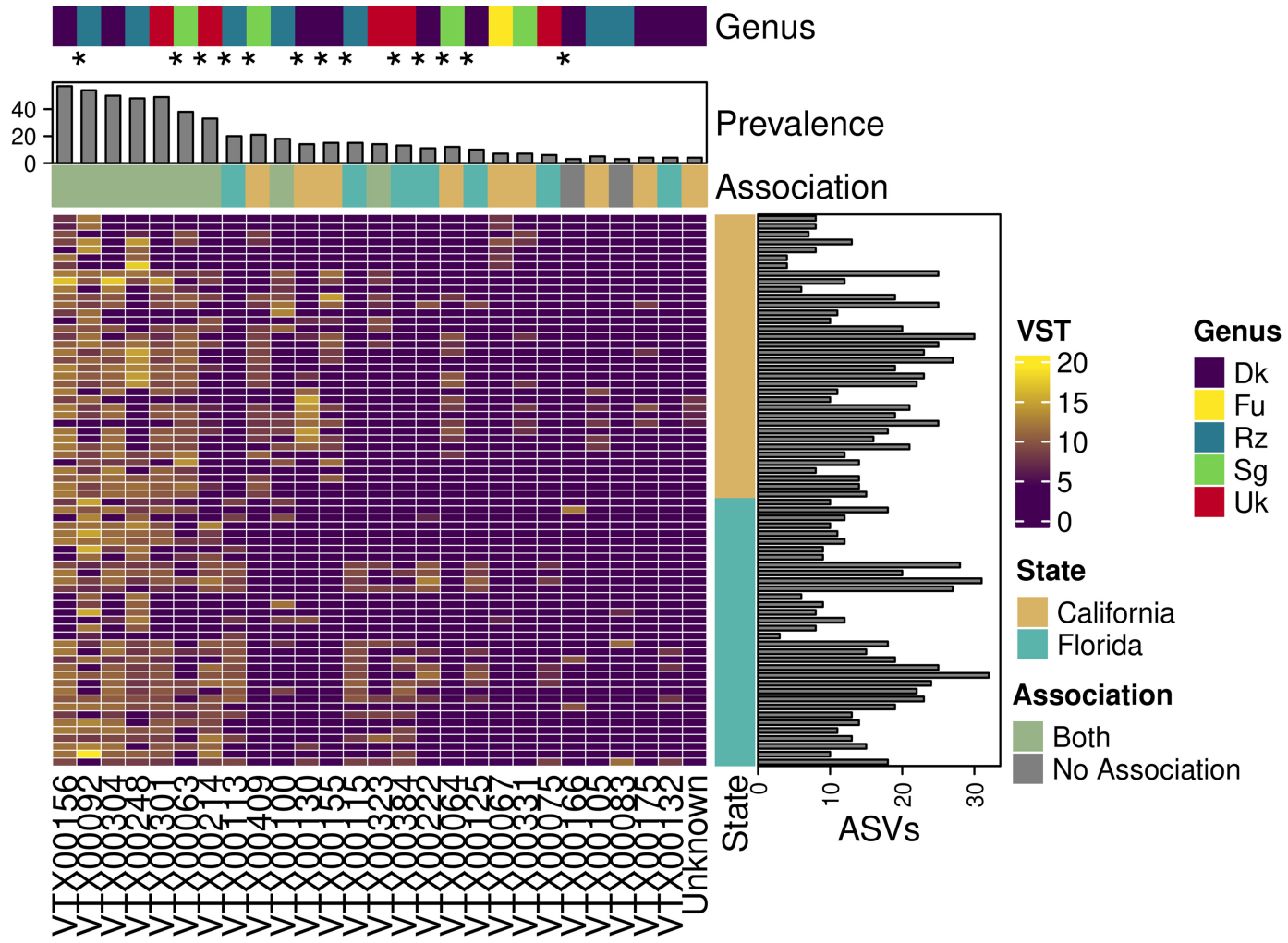

Fig. 4. Heatmap of virtual taxa counts between geographical location following Deseq2 variance stabilization transformation (VST). Each row represents a single root sample and each column represents a unique virtual taxon. For clarity, only virtual taxa that occurred in three or more samples are displayed. An asterisk (*) depicts virtual taxa differentially significantly abundant between geographical locations per DESeq2 Wald's test. The column annotation bar graph depicts the prevalence (number of unique samples) of the virtual taxa. The Association column annotation is a colorimetric Venn diagram with a 10% prevalence cut-off, where yellow squares represent taxa associated with California samples, blue squares represent taxa associated with Florida samples, and green squares represent taxa which were associated with both Florida and California citrus roots. Gray boxes indicate taxa that did not pass the 10% prevalent cut-off to be associated with either category. Row annotations show which samples belong to each geographical location and the bar graph shows the number of unique amplicon sequence variants (ASVs) associated with each sample. Dk = *Dominikia*, Fu = *Funneliformis*, Rz = *Rhizophagus*, Sg = *Septoglomus*, and Uk = unknown.

DISCUSSION

Citrus is globally grown and an iconic crop to U.S. agriculture. Here, we deployed high-throughput amplicon sequencing technology of the citrus root AMF to test whether community diversity and composition was affected by the climatic zones where citrus is grown (California and Florida), the type of farming system farming (organic versus conventional), and the level HLB disease severity.

Our results indicate that commercial citrus trees in the U.S. are commonly found in association with AMF (78%), as reported in other systems (Brundrett and Tedersoo 2018; Smith and Read 2008), but AMF community composition in U.S. orchards was more diverse than previously described. Nemec et al. (1981) used a morphologically based approach to identify AMF taxa inhabiting California and Florida citrus soils, and reported the cosmopolitan genus *Glomus*, as well as the two genera *Gigaspora* and *Sclerocystis*. Morphology and DNA sequencing-based approaches of orchards worldwide also reported that *Glomus* spp. were dominant AMF taxa, including in Brazil (Franca et al. 2007), China (Song et al. 2020; Wang et al. 2012), and Spain (Camprubí and Calvet 1996). In contrast, we found a broader number of AMF genera, including *Dominikia*, *Funneliformis*, *Glomus*, *Rhizophagus*, *Septoglomus*, and likely additional undescribed genera, within the family Glomeraceae. Our results also indicated that *Dominikia* and *Rhizophagus* were the most abundant genera associated with citrus roots, although the *Dominikia* clade was not well supported. Higher taxonomic resolution beyond the genus level could not be achieved because of the short, 220-bp VT amplicon reads. A complete sequence of the WANDA/18S/ALM2 region will need to be obtained from a deeper sequencing run to identify distinct Glomeraceae species with strong branch support.

We achieved resolution of citrus-associated AMF clades by adopting a novel taxonomy assignment approach based on AMF-specific amplicons (Stefani et al. 2020). The disparity in the taxonomic profile between our results and previous reports has several reasons. In part, it can be explained by the recent taxonomic revision of the Glomeromycota phylum, in which the family Glomeraceae was split into several families (http://www.amf-phylogeny.com/) (Krüger et al. 2012) and many *Glomus* spp. were moved to different genera. For example, the *Glomus* VT associated with citrus in China identified by Song et al. (2020), based on the MaarjAM database, clustered in our analysis with *Rhizophagus*, *Glomus*, *Septoglomus*, and *Dominikia*. Furthermore, *Glomus fasciculatus* and *G. constrictus*, identified by Nemec et al. (1981), have now been renamed *Rhizophagus fasciculatus* and *Septoglomus constrictum*, respectively, and they associated with the clades of respective genera in our phylogenetic analysis. Our study also exclusively focused on

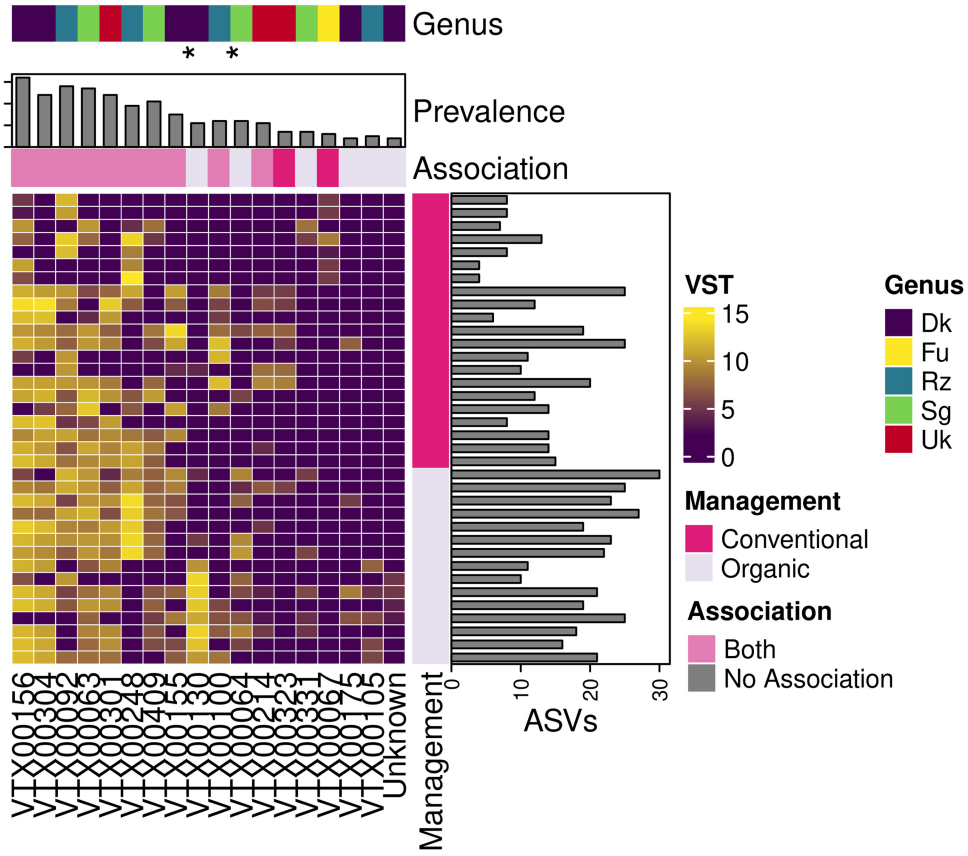

Fig. 5. Heatmap of virtual taxa counts between management strategy from California samples following Deseq2 variance stabilization transformation (VST). Each row represents a single root sample, and each column represents a unique virtual taxon. For clarity, only virtual taxa that occurred in three or more samples are displayed. An asterisk (*) depicts virtual taxa differentially abundant between geographical locations per DESeq2 Wald's test. The column annotation bar graph depicts the prevalence (number of unique samples) of the virtual taxa. The Association column annotation is a colorimetric Venn diagram with a 10% prevalence cut-off, where dark-pink squares represent taxa associated with Conventional samples, lavender squares represent taxa associated with organic samples, and medium-pink squares represent taxa which were associated with both organic and conventional citrus roots. Row annotations show which samples belong to which management strategy and the bar graph shows the number of unique amplicon sequence variants (ASVs) associated with each sample. Dk = *Dominikia*, Fu = *Funneliformis*, Rz = *Rhizophagus*, Sg = *Septoglomus*, and Uk = unknown.

root-associated AMF; however, there have been reports of additional AMF taxa frequently occurring in bulk soil of citrus, as reported by Nemec et al. (1981). These include *Gigaspora* spp. (Gigasporaceae) and *Glomus etunicatus* (now named *Claroideoglomus etunicatum*, Claroideoglomeraceae). Differential AMF composition may also have been driven by sampling size, seasonal and temporal variation (nearly 40 years between sampling events), edaphic properties of orchards, and varieties of citrus (Davison et al. 2021; Gao et al. 2019; Song et al. 2015).

The AMF community in Florida and California was composed of VT that were shared in both states while others were specific to each state. The seven most abundant and common VT within the genera *Dominikia* (VTX00156 and VTX304), *Rhizophagus* (VTX00092 and VTX00248), *Septoglomus* (VTX00063), and unidentified Glomeraceae species (VTX00214 and VTX00301) were also associated with citrus in China (Song et al. 2020), apple in Italy (Turrini et al. 2017), and barrel medic (*Medicago truncatula*) in Tunisia (Mahmoudi et al. 2019), highlighting their cosmopolitan distribution. Both *R. fasciculatus* and *S. constrictum* are known generalist AM fungi capable of colonizing a broad range of soils (Oehl et al. 2010). In fact, *Rhizophagus* spp. have been used broadly in agriculture to improve soil, promote host plant growth, and cope with diseases (Ceballos et al. 2013; Pawlowski and Hartman 2020). Some VT classified as specialist taxa (*Rhizophagus* VTX00113 and VTX00115) from Florida were previously reported in Tunisia (Mahmoudi et al. 2019), reinforcing the need for a larger sampling size in order to profile community structure. Nonetheless, these data support the view of a distribution of ubiquitous taxa across ecosystems (Öpik et al. 2009, 2010). The community segregation within the two distinct citrus climatic zones also indicates that community composition is driven by environmental conditions and ecological requirements of AMF (Öpik et al. 2013).

Our study also tested the impact of farming practices on AMF community richness and composition in citrus orchards. Farming practices and soil characteristics have been recognized to affect soil microbial biodiversity and fertility (Hartmann et al. 2015; Mäder et al. 2002). AMF are major components of soil agroecosystem structure, functionality, and productivity, and low-input agricultural practices (e.g., organic farming) have been shown to be conducive to AMF biodiversity, activity, and root colonization in both annual (Oehl et al. 2003; Verbruggen et al. 2010) and perennial (Franca et al. 2007; Turrini et al. 2017) cropping systems. Our results support those findings because we measured enriched AMF communities, including VTX00105 (*Rhizophagus*) and VTX00064 (*Septoglomus*), with distinct profiles in organic orchards in comparison with nearby conventional orchards. *S. constrictum* and *R. intraradices* have been shown to stimulate plant growth or productivity in several cropping systems and also to increase tolerance to heat and drought stress (Li et al. 2014; Ziane et al. 2017). However, AMF enrichment may also indicate a nutrient deficiency, and that the tree host needs to invest in the symbiotic relationship with AM fungi so that the cost in allocation of photoassimilated carbon

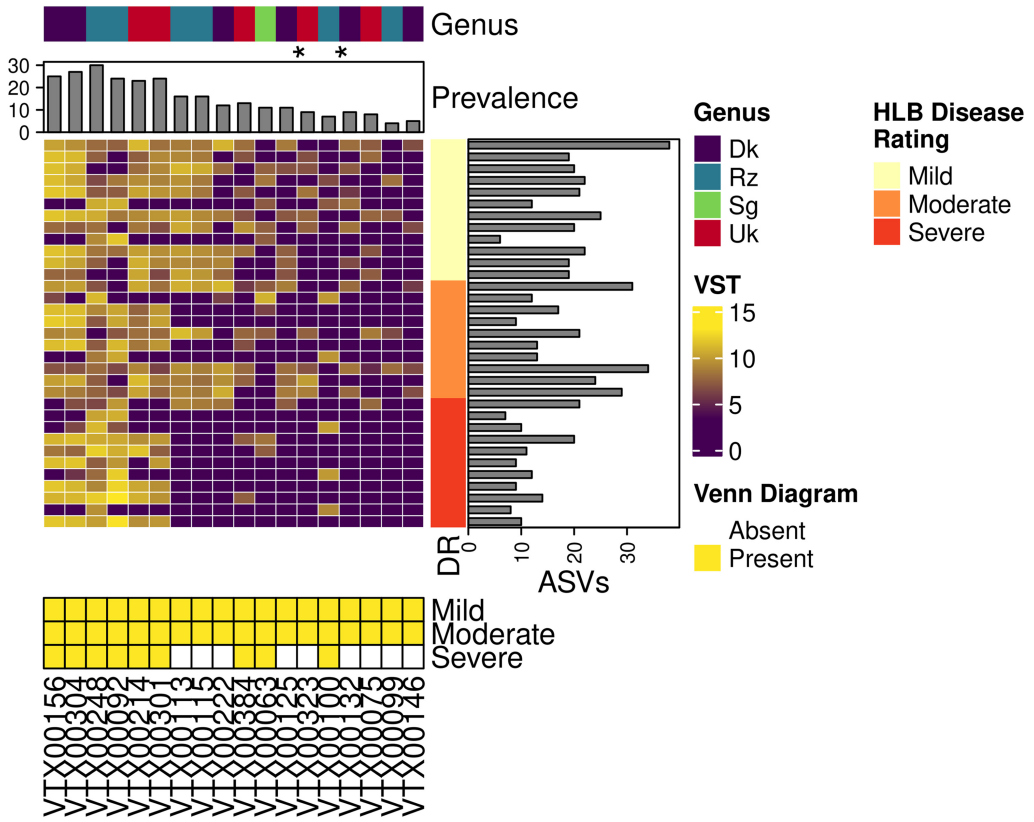

Fig. 6. Heatmap of virtual taxa counts of huanglongbing (HLB) disease severity from Florida samples following Deseq2 variance stabilization transformation (VST). Each row represents a single root sample, and each column represents a unique virtual taxon. For clarity, only virtual taxa that occurred in three or more samples are displayed. An asterisk (*) depicts virtual taxa differentially abundant between geographical locations per DESeq2 Wald's test. The column annotation bar graph depicts the prevalence (number of unique samples) of the virtual taxa. Row annotations show which samples belong to each disease rating severity and the bar graph shows the number of unique amplicon sequence variants (ASVs) associated with each sample. The Venn diagram heatmap with yellow rectangles shows which taxa were found in at least 10% of samples from each disease severity. Dk = *Dominikia*, Rz = *Rhizophagus*, Sg = *Septoglomus*, and Uk = unknown.

is outweighed by the benefits of increased nutrient uptake (Graham et al. 1997; Johnson et al. 1997).

We found conclusive evidence of a significant depletion in AMF richness and shifts in community composition as HLB severity worsened, which corroborates previous findings (Ginnan et al. 2020). Trees affected by HLB expressed a thin canopy, small leaves, and branch dieback symptoms and also showed significant root collapse. Root carbohydrate starvation is a result of carbon sequestration in the aboveground tissues and poor belowground translocation of photoassimilates as a result of phloem sieve tube occlusion from '*Ca.* L. *asiaticus*' infection (Etxeberria et al. 2009). This carbon source–sink unbalance of symptomatic trees is certainly affecting the rhizosphere microbiome and disturbing the symbiosis with AMF. Ginnan et al. (2020) measured enrichment of putative beneficial microbes in the early disease onset which is governed by changes in root signaling for the microbial recruitment events to occur (Bulgarelli et al. 2013). AMF are known to alter root exudate signaling and drive selection of certain microorganisms that would improve fitness under stress conditions (Jung et al. 2012) and may have contributed to the microbial recruitment efforts to protect its host. In the later stage of the disease, Ginnan et al. (2020) measured an enrichment with soilborne parasitic fungi and oomycetes (i.e., *Fusarium* and *Phytophthora* spp.) and it was proposed to contribute to root collapse and hasten the decline of trees affected with HLB. Several studies have highlighted the instrumental role of AMF in delaying disease onset or reducing symptoms against the soilborne pathogen genera *Fusarium* and *Phytophthora* in several pathosystems (Alaux et al. 2018; Wang et al. 2020), including citrus (Tian et al. 2021; Watanarojanaporn et al. 2011). Priming of plant immunity has been described as a major mechanism of AMF-induced disease resistance (Jung et al. 2012). The host defense response can be either localized in the roots or systemic throughout the plant and can be activated either constitutively or primed upon pathogen attack (Hohmann and Messmer 2017). The range of protection conferred by the symbiotic interaction depends on the ability of the AMF to control the plant host defense signaling pathway. Plants associated with AMF are more resistant to necrotrophic pathogens (*Fusarium* and *Phytophthora* spp.) but more susceptible to biotrophs because they can activate jasmonic acid-mediated responses but need to repress salicylic acid-dependent ones (Jung et al. 2012). Given the plant defense benefits that AMF provide, practices that foster AMF diversity may result in extending tree longevity in the context of soilborne diseases and HLB.

In conclusion, this study is a first step to unravel the diversity and composition of AMF communities in citrus. In perennial cropping systems, growers rely on orchard longevity for profits and the root system is a major driver of tree health. Commercial application of mycorrhizae has gained traction as a farming practice but success is limited to the host range of the AMF species used in the product formulation and its biological fitness under agricultural constraints. Identifying beneficial generalist taxa that have potential for commercial application and developing recommendations for best cultural practices that support AMF diversity will help sustainable farming.

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