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AMF diversity in citrus rhizosphere

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ABSTRACT

Microbial diversity in any soil is considered as bio-indicator towards the maintenance of soil ecosystem service. Citrus is one of the most widely grown commercial fruit crops and is heavily dependent on arbuscular mycorrhizal fungi (AMF). In the proposed work, small subunit ribosomal RNA (SSU rRNA) was used to identify the AMF diversity in roots and rhizospheric soils of 29-yr-old Satsuma mandarin *(Citrus unshiu* Marc) grafted on trifoliate orange (*Poncirus trifoliata* L.). As many 193 and 190 operational taxonomic units (OTUs) were observed in the SSU rRNA clone library of plant roots and soils. Soil native mycorrhizal fungi mostly colonized citrus roots, because 178 OTUs co-existed in both plant roots and soils. While, *Glomus* was predominant in roots, and *Claroideoglomus* and *Glomus* were examined in rhizosphere. In the species levels, the clone, Glomus Glo20, had the highest relative and read abundance in all the root and soil samples than other clones. As many, four soil samples were grouped with higher taxonomic similarity with the database sequences compared to root samples. These results provide the new perspectives at the molecular level to highlight the community of AMF in citrus.

Key words : Citrus, *Claroideoglomus*, *Glomus*, Mycorrhiza, SSU rRNA

Globally citrus is one of the leading fruit trees grown in more than 140 countries, with China as the biggest citrus producing country (Srivastava and Singh 2008). In Satsuma mandarin (*Citrus unshiu* Marc) growing belts, citrus is predominantly grown on hilly and mountainous regions, where soils of citrus orchards are coarse textured with high infiltration rate and poor structure, with heavy sub. soils representing taxonomically the two most predominant soil orders, viz. Alfisols and Ultisols (Srivastava and Singh 2002). Such unfavorable soil conditions strongly inhibit the growth performance and fruit production of citrus trees (Srivastava and Malhotra 2014).

Citrus is heavily dependent on arbuscular mycorrhizal fungi (AMF), due to shallow root systems and less developed root hairs (Srivastava *et al*. 2002, Wu *et al*. 2013). Therefore, the presence of arbuscular mycorrhizas in citrus rhizosphere can help the host plant to absorb nutrients and water from soils, enhance the tolerance against abiotic stresses, improve soil structure, and induce superior root development (Srivastava *et al*. 2008, Ortas 2012, Liu *et al*. 2016, Tuo *et al*. 2016). Earlier studies showed forty-five species of AMF within citrus rhizosphere, belonging to as many seven genera (Wu and Srivastava 2012, Wu *et al*. 2013). However, the identification of AMF species in past studies was based on morphological features of spores, which is so arduous because of limited information available on morphotypes (Krüger *et al*. 2012). On the other hand, the relative abundance of spores in soils is not considered as a good indicator of AMF community composition and dynamics (Vandenkoornhuyse *et al*. 2002). An improved technique of molecular analysis could identify AMF actively growing within the roots as endophytes and within the rhizosphere, though independent of morphotypes, but in corroboration with spore morphological features (Lee *et al*. 2008). Earlier studies in the past showed that ribosomal RNA (rRNA) could provide suitable insights into the AMF identification (Lee *et al*. 2008, Spruyt *et al*. 2014). Of late, other techniques such as internal transcribed spacer (ITS) sequences, the nested AML primer pair targeting a portion of the SSU rRNA region (Spruyt *et al*. 2014) and AMV4.5NF-AMDGR (Geel *et al*. 2014) aided effectively in identifying AMF species at various level of taxonomy.

In this background, the objective of the proposed study was to analyze the community diversity of AMF in roots of *Citrus unshiu* Marc trees grafted on trifoliate orange and within rhizosphere and soils citrus using SSU rRNA gene sequences.

MATERIALS AND METHODS

The experimental site selected was 29-yr-old *C. unshiu* trees grafted on trifoliata orange (*Poncirus trifoliata* L.)

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(30°36′N and 112°14′E) located in the Yangtze University Campus, Jingzhou, China. The citrus orchard represented the north subtropical humid monsoon climate, with four distinct seasons, 4367–4576 MJ/m annual total radiation, 1823–1987 h annual sunshine hour, 16.2–16.6ºC annual average temperature, and 1100–1300 mm annual precipitation. The orchard was maintained with no-tillage soil management with natural grass cover. The physico-chemical properties of the yellow-brown soil from the citrus orchard consisted of pH 6.2, organic matter 9.4 g/kg, and Olsen-P 16.2 mg/kg.

Rhizosphere soils and fine root samples were collected at random locations from four citrus trees having more or less uniform growth vigor. The samples were collected within the perimeter of tree canopy at 5–15 cm depth in July, 2016 representing typical monsoon time so that rhizosphere is microbially very active. Soil and root samples from four trees per block were well mixed as a composite sample and stored in -80ºC. Each sample replicated four times.

The total genomic DNA was extracted from rhizosphere soils and roots with the DNA purification ELISA kits and checked by the 0.8% agarose gel for the quality level. Partial SSU rRNA gene fragments were amplified with the TOYOBO KOD-Plus-Neo DNA Polymerase by the PCR reaction using the specific AM fungal primers (Geel *et al*. 2014): AML1 (F): 5'-ATCAACTTTCGATGGTAGGATAGA-3'; AML2 (R): 5'-GAACCCAAACACTTTGGTTTCC-3'; AMV4.5NF (F): 5'-AAGCTCGTAGTTGAATTTCG-3'; AMDGR (R): 5'-CCCAACTATCCCTATTAATCAT-3'. The Applied Biosystems® Gene Amp® PCR System 9700 was used here. The PCR reaction was ended in the linear amplification stage. The PCR product was visualised on 1.5% agarose gels and recycled with the QIAquick gel Extraction Kit (Qiagen). TE buffer solution eluted the target DNA fragments.

After detecting the quality of the PCR products with GE NanoVue System, the product was diluted, and the library was constructed using the TruSeq DNA PCR-Free Sample Prep Kit (FC-121-3001/3002). The sequencing was done with the HiSeq Reagent Kit v2.

The sequences were filtrated, labeled, weighed and sorted. In 97% similarity level, operational taxonomic units (OTUs) were clustered by the UPARSE-OUT algorithm (Edgar 2013). A BLAST was used on the sequence data, in combination with the online database, MAARJAM (www.maarjam.botany.ut.ee), to divide the species taxonomy, as well to remove the OUT from non-Glomeromycota (Öpik *et al*. 2010). The relative abundance in genus and species levels was described with the R (2011) software (www.R-project.org). The clustering analysis was done with the unweighted/Pair group method using arithmetic mean.

RESULTS AND DISCUSSION

AMF diversity

OTUs, clustered based on the rRNA gene, have been

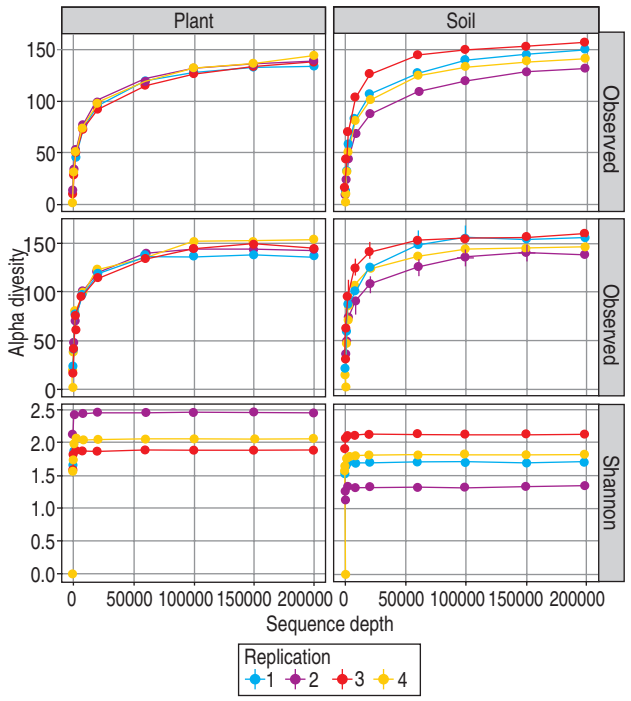

Fig 1 Rarefaction curve of OTUs based on alpha diversity of sequence depth in the plant and soil of *Citrus unshiu* grafted on *Poncirus trifoliata*.

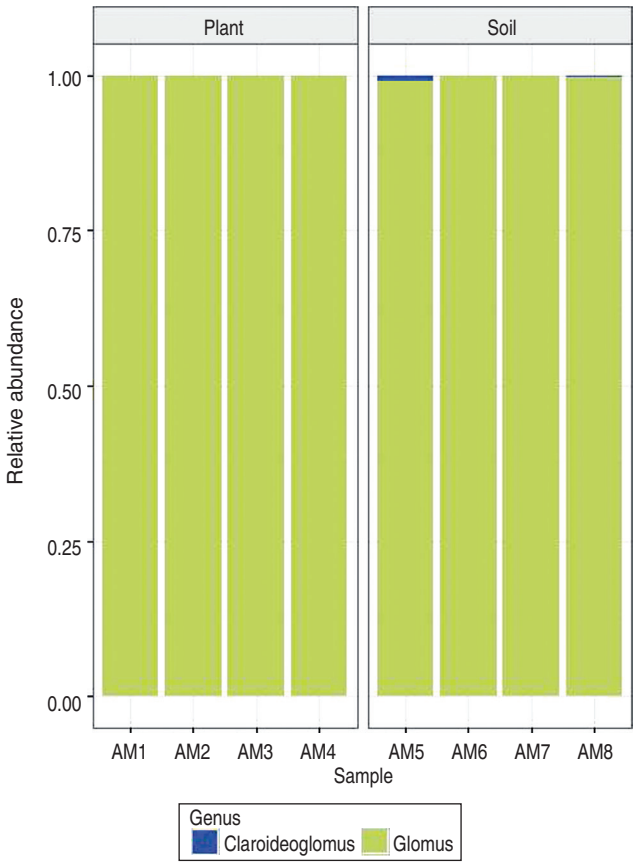

Fig 2 Relative abundance of AMF in the genus level in the plant and soil of *Citrus unshiu* grafted on *Poncirus trifoliata*. Here, AM1 to AM 4 are the four samples from plant roots, and AM5 to AM8 from soils.

widely used to evaluate fungal and bacterial species (Koeppel and Wu 2013). In this citrus orchard, 193 and 190 OTUs were observed in the SSU rRNA clone library of plant roots and rhizosphere soils, respectively, suggesting that a relative similar OTU quantity was found in two samples. As many, 178 overlapping OTUs were observed in common considering plant roots and rhizosphere soils indicating that soil native mycorrhizal fungi mostly colonized citrus roots.

The rarefaction curves showed that the curves became flatter when the number of the clones rose to 120 (Fig 1). And, plant samples represented a highly consistent, and soil samples had a relatively disperse trait, meaning that the diversity of AMF species in soils is more variable than in the plant roots.

AMF community

The microbial community at the genus level indicated that *Glomus* was observed in citrus roots, and both *Claroideoglomus* and *Glomus* were observed within the citrus rhizosphere (Fig 2). This result indicated that *Glomus* was the most predominant genus amongst AMF community, which is in agreement with earlier studies by Wu *et al*. (2013).

The AMF community on the species level revealed that top five AMF clones were *Glomus*; Glo20, sp, Wirsel_OTU16, Glo7 and acnaGlo2 in plants and *Glomus*. Glo20, viscosum, Glo7, sp, and Wirsel_OTU16 in soils (Fig 3). The clone, *Glomus*; Glo20 was observed in all the root and rhizosphere soil samples as well, with the highest relative abundance and read abundance (Fig 3). The relative abundance of AMF clone was relatively higher within rhizosphere soils than in roots, indicating that the AMF strain is a stronger inhabitant in rhizosphere soils than roots, as AMF is the soil inhabited fungi. However,

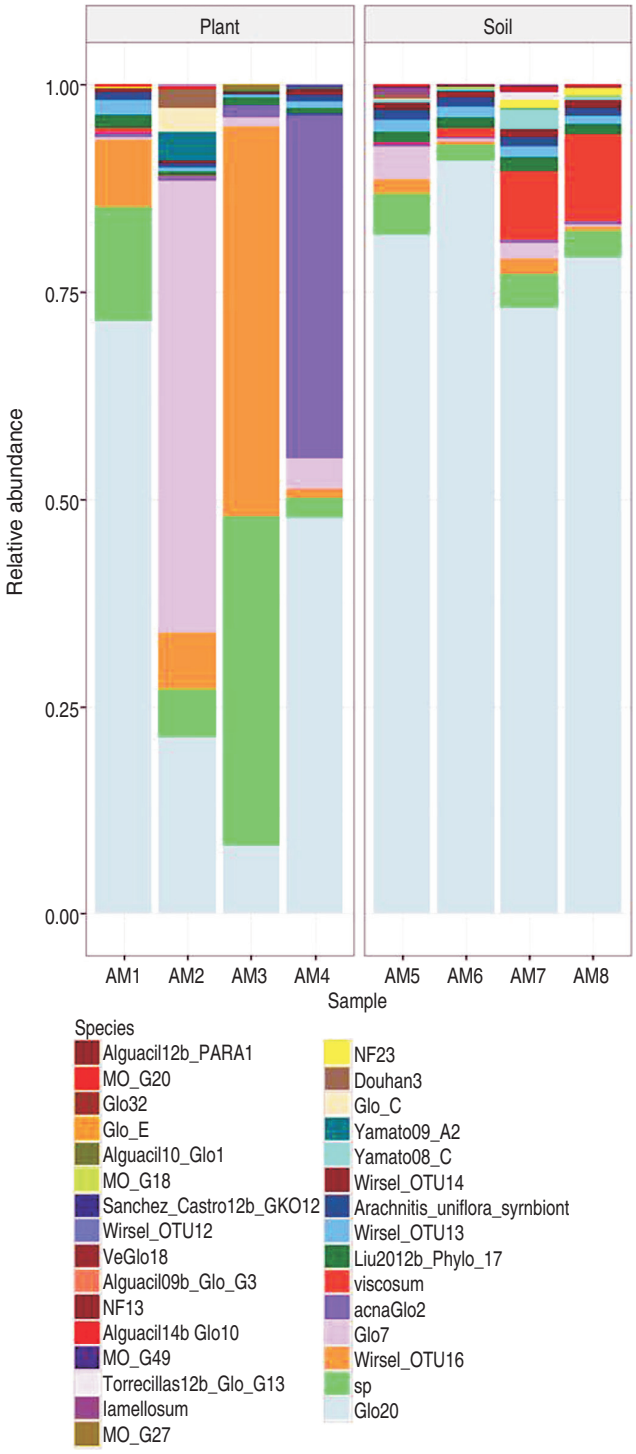

Fig 3 Relative abundance of AMF in the species level in the plant and soil of *Citrus unshiu* grafted on *Poncirus trifoliata*. Here, AM1 to AM 4 are the four samples from plant roots, and AM5 to AM8 from soils.

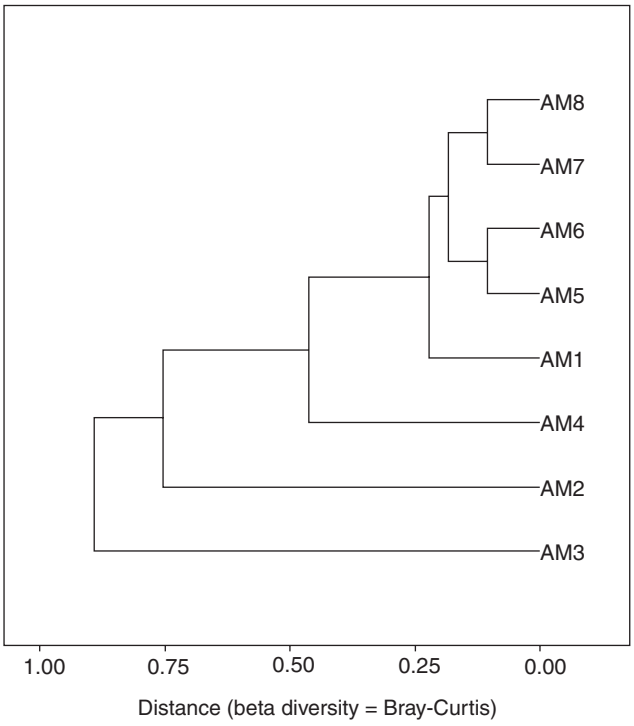

Fig 4 Neighbor-joining tree in terms of SSU rRNA sequences the plant and soil of *Citrus unshiu* grafted on *Poncirus trifoliata*. Here, AM1 to AM 4 are the four samples from plant roots, and AM5 to AM8 from soils.

the clone, *Glomus*; sp had higher read abundance in plants than in rhizosphere soils, indicating that the AM fungal species preferably inhabited within the roots compared to soils.

According to the clustering analysis, all the four soil samples were grouped with the high taxonomic similarity with the database sequences (Fig 4). Four root samples were clustered with the low similarity with the database sequences. These results suggested that within the diversity of the AMF community, citrus rhizosphere soils had greater similarity than plant roots. Hence, in citrus orchard, 193 and 190 OTUs were observed in the SSU rRNA clone library of Unshiu roots and rhizosphere, respectively. Soil native mycorrhizal fungi mostly colonized citrus roots, since 178 OTUs co-existed together both in roots and rhizosphere. *Glomus* was observed in predominantly roots, while *Claroideoglomus* and *Glomus* were observed within the rhizosphere. These results could be seen offering strong perspectives at molecular level to exploit the AMF diversity of citrus mycorrhizosphere in developing an AMF consortium.

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