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Isolation and identification of three dominant arbuscular mycorrhizal fungi in the rhizosphere of *Puccinellia tenuiflora* from saline-alkaline grassland of Songnen Plain

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Soil samples in the rhizosphere of *Puccinellia tenuiflora* were collected from saline-alkaline grassland of Songnen Plain, Zhaodong City, Heilongjiang Province, China. Arbuscular mycorrhizal (AM) fungal spores were isolated by wet sieving and sucrose density gradient centrifugation. Fourty species of AM fungi were morphologically identified, which were classified in 17 genera. The three most dominant species *Rhizoglomus intraradices*, *Claroideoglomus etunicatum* and *Funneliformis mosseae* were identified by both morphological and molecular genetical methods. This result can provide a scientific basis not only for the screening of salt-tolerant AM fungi in the rhizosphere of *P. tenuiflora*, but also for further promoting the application of *P. tenuiflora* on the improvement and restoration of the degraded saline-alkaline grassland of Songnen Plain.

Keywords: grassland restoration, salt tolerance AM fungal community composition.

The saline-alkaline grassland of Songnen Plain is located in the northeastern region of China. In the 1950s, soil salinization in the Songnen Plain area was 2.415 million hm^2, with the salinization area expanded to 3.12 million hm^2 in the 1980s. Subsequently, it reached 3.42 million hm^2 in the 1990s, and the trend of soil salinization has increased year-by-year (Li & Wang 2006). Soil salinization has resulted in a decrease in the economic benefits of grassland and farmland, which has seriously affected the production of agriculture and animal husbandry (Zhao et al. 2016). *Puccinellia tenuiflora* is one of the preferred grasses selected by Chinese scientists from 140 kinds of pasture in the past 20 years. It is a perennial grass of the family Poaceae and is not only a good pasture, but also has strong tolerance to salt, drought and cold (Li et al. 2014, Meng et al. 2016, Zhang et al. 2013). Arbuscular Mycorrhizal (AM) fungi play an important role in improving soil structure, promoting plant nutrient uptake, protecting biodiversity and improving the resistance of various plants (Bona et al. 2016, Hashem et al. 2016, Robinson Boyer et al. 2016). AM fungi can form a symbiotic system with approximately 80 % of the terrestrial plants. Although we have found that the roots of *P. tenuiflora* can be infected by AM fungi (Yue 2015), the AM fungi species are still unknown. Whether these AM fungi play a very important role in the salt tolerance of *P. tenuiflora* deserves exploration. This research aims to discover the AM fungi species, especially the dominant ones, in the rhizosphere of *P. tenuiflora*. The results are bound to provide vital basis on the contribution of the AM fungi to the strong salt tolerance of *P. tenuiflora* through further experiment.

Materials and methods

Sampling and separation

Nine soil samples were collected in the rhizosphere of *P. tenuiflora* from saline-alkaline grassland of Songnen Plain in Zhaodong City (E125°30'-125°42', N46°16'-46°17') according to the "multi-point mixing sampling method" and "five-point sampling method". In the process of soil sample collection, the surface sand and large debris were removed, then dug out 10 to 20 cm of soil (about 1 kg) in the rhizosphere of *P. tenuiflora*. All soil samples were placed in a cool ventilated place to

dry out. Subsequently, soil was crushed and sieved to remove impurities and then put into sealed bags. Soil from each sample was weighed (50 g) for spore separation by wet sieving and sucrose density gradient centrifugation (Vilariño & Arines 1990). The spores were stored in the centrifuge tubes.

Morphological identification

Morphological identification is based on a large number of individual spores and staining reaction. Each single spore washed 3–5 times with sterile water was observed under a stereomicroscope (Olympus-DSX500). Close attention was paid to spore staining reaction, size, cytoderm structure, spore hyphae, inclusions and surface decoration. The spores were stained with Melzer's reagent, photographed and preserved. Color and microscope observations refer to the description and pictures of the AM fungi provided by the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal fungi (INVAM; http://invam.wvu.edu/), as well as related literature (e.g. Oehl et al. 2011, Sieverding et al. 2014).

Identification of the dominated AM fungal species

This study mainly identified the dominating AM fungal species according to Frequency (F), Relative Abundance (RA) and Importance Value (IV) according to Yang et al. (2011).

(1) F = The number of times of a certain AM fungal occurrence/Total number of AM fungal species be found in this study × 100 %.

(2) RA=spores of certain AM fungal species/Total AM fungal spores × 100 %.

(3) IV=(F+RA)/2 × 100 %.

Molecular genetics

The three dominant AM fungal spores obtained by morphological identification were stored in centrifuge tubes in a 4°C refrigerator for DNA extraction (Symanczik et al. 2014), for 25S rDNA amplification and sequencing. Micro-pipette tips, centrifuge tubes and other essential instruments were sterilized by autoclaving (120 °C, 2h). A single spore was picked from the centrifuge tube, crushed with a pipette tip and 1 µl sterile ddH_2O was added as diluent. The crushed spore mixture was used as template for the first nested PCR in a reaction volume of 20 µl. The primers were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and NDL22 (5′-TGGTCCGTGTTTCAAGACG-3′). The second PCR reaction takes LR1 (5′-GCATATCAATAAGCGGAGGA-3′) as primer and the first PCR products as template. The nested PCR program was as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and elongation at 72 °C for 1 min; followed by final extension at 72 °C for 10 min. The annealing temperature of the second nested PCR with LR1 primer was 58 °C. After agarose gel electrophoresis, the PCR products were purified by Qiaquick Gel Extraction Kit (TaKaRa), linked into pMD-18T (TaKaRa), and transformed into Trans5α Chemically Competent Cell (CD201-01) competent *Escherichia coli* cells (Trans). Individual bacteria were used for monoclonal verification and selected to culture in LB medium overnight for 12–14 h with a temperature of 37 °C. Then 1 µl of the positive clone liquid was sequenced by Bo Shi Biotechnology Co., Ltd. The sequences of the 25S amplification products and homologous sequences from GenBank were used to arrange the default parameters by MEGA5.1 software, and a maximum likelihood phylogenetic tree was established.

Results

AM fungal species richness

A high diversity of AM fungal spores representing 40 species of 17 genera was isolated from the rhizosphere of *Puccinellia tenuiflora* in the Songnen salt-alkaline grassland (Tab. 1). Among those three species appeared with relatively high IV value, namely *Rhizoglomus intraradices* (61.5%), *Claroideoglomus etunicatum* (38.4%), and *Funneliformis mosseae* (64.6%) (Fig.1).

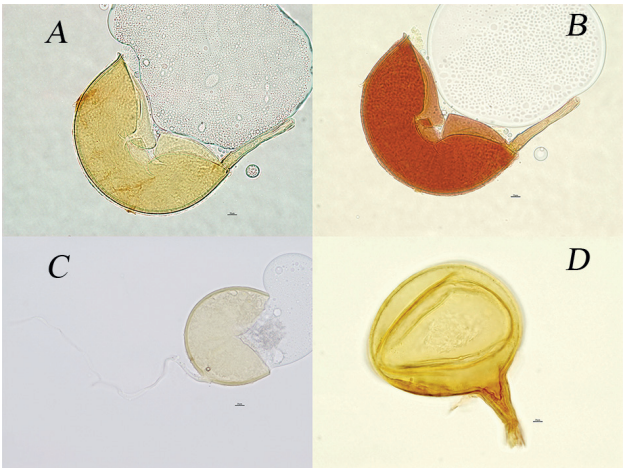

Fig. 1. Spores of three dominanting AM fungal species: **A.** *Rhizoglomus intraradices*. **B.** *Rhizoglomus intraradices* in Melzer's reagent. **C.** *Claroideoglomus etunicatum*. **D.** *Funneliformis mosseae*.

Tab. 1. Fequency, Relative abundance and Importance value of the AM fungi in the roots of *Puccinellia tenuiflora*.

Genus	Taxon	Frequency (%)	Relative abundance (%)	Importance value (%)
Glomus	*G. convolutum*	55.6	2.9	29.2
	G. magnicaule	44.4	2.5	23.5
	G. microcarpum	22.2	2.5	12.3
	G. dolichosporum	44.4	4.1	24.3
	G. sp.1	33.3	3.0	18.1
Acaulospora	*A. delicata*	33.3	3.6	18.5
	A. excavata	55.6	2.5	29.1
	A. gedanensis	22.2	4.1	13.2
	A. laevis	66.7	4.3	35.5
	A. capsicula	44.4	2.6	23.5
	A. cavernata	44.4	2.8	23.6
	A. foveata	33.3	2.9	18.1
	A. lacunosa	11.1	2.9	6.1
	A. scrobiculata	66.7	3.5	35.1
	A. rugosa	22.2	3.5	12.9
Rhizoglomus	*R. fasciculatum*	44.4	3.6	24.0
	R. manihotis	33.3	3.0	18.2
	R. intraradices	100	23.1	61.5
	R. clarum	44.4	4.3	24.4
	R. aggregatum	22.2	2.4	12.3
Dentiscutata	*D. scutata*	22.2	3.9	13.0
Pacispora	*P. chimonobambusae*	55.6	3.7	29.6
Entropnospora	*E. infrequens*	44.4	3.1	23.8
Diversispora	*D. spurca*	44.4	4.3	24.4
	D. pustulatum	44.4	2.2	23.3
Ambispora	*A. leptoticha*	44.4	3.8	24.1
	A. gerdemannii	22.2	4.2	13.2
	A. fecundispora	22.2	2.9	12.6
Funneliformis	*F. mosseae*	100	29.2	64.6
	F. multiforus	55.6	3.4	29.5
Claroideoglomus	*C. etunicatum*	66.7	10.2	38.4
	C. luteum	44.4	3.0	23.7
Racocetra	*Racocetra castanea*	22.2	3.0	12.6
Simiglomus	*S. hoi*	33.3	3.1	18.2
Halonatospora	*H. pansihalos*	33.3	2.7	18.0
Albahypha	*A. walkeri*	22.2	2.6	12.4
Septoglomus	*S. viscosum*	22.2	2.4	12.3
	S. constictum	44.4	3.5	24.0
Sclerocystis	*S. sinuosua*	11.1	3.6	6.2
Kuklospora	*K. colombiana*	11.1	4.1	6.2

Morphological characters of the three dominant species

Rhizoglomus intraradices (Fig. 1 A). – Spores spherical or subspherical, 70–160 µm in diam.; almost transparent inside. Spore wall of mostly three and four layers, pale cream to pale brownish yellow. Subtending hypha single, its wall connected with L1 and L2, and the junction point appearing cylindrical or flamboyant with no septa. With Melzer's reagent (Fig. 1 B) dark brown overall. Hypha wall of a few spores transparent and scattered inside.

Claroideoglomus etunicatum (Fig. 1 C). – Spores spherical or subspherical, 100–260 µm in diam. Single spore orange to reddish brown. Irregular decorations found on the spore surface, L2 always extending to the interior of the subtending hypha. Single subtending hypha colorless, of uneven thickness and long, its wall thickened or forming a crateriform septum at the junction point. With Melzer's reagent spores pale yellowish-brown overall.

Funneliformis mosseae (Fig. 1 D). – Spherical or subspherical, occasionally irregular in shape, 75–200 µm in diam. Single spore with oil droplet analogue of unequal size, surface smooth and glossy. Spore wall three layered. The single subtending hypha mostly short and small, cylindrical or crateri-

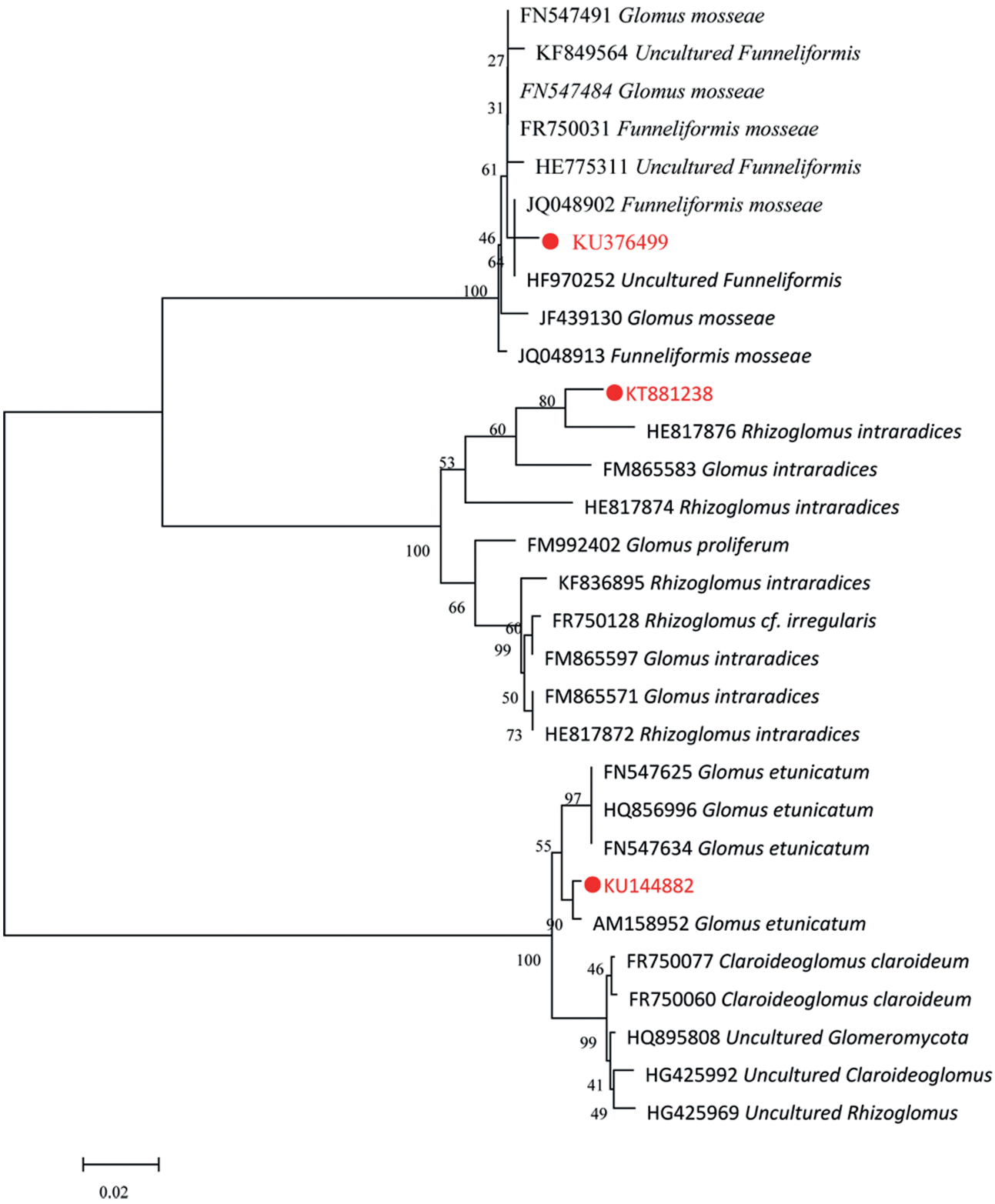

Fig. 2. Phylogenetic ML tree of three dominating AM fungi.

form. The inner layer of the subtending hypha becoming thicker at the junction point where the hyphae is attached to spores, surface not smooth. With Melzer's reagent spore base showing different degrees of yellowish-brown.

Molecular genetic analysis

The nested-PCR amplification products of the three dominant AM fungi single spores were sequenced by a specific DNA fragment to obtain the effective gene sequence with a length of approx. 750 bp. The specific lengths of the 25S rDNA fragments of *R. intraradices*, *C. etunicatum* and *F. mosseae* are 777 bp, 772 bp and 787 bp respectively.

The 25S rDNA sequences were submitted to GenBank with the accession numbers KT881238, KU144882 and KU376499. Newly generated sequences of the three species were blasted in NCBI. The accession number KT881238 has the highest homology and similarity with *R. intraradices* (HE817876) up to 98 %; KU144882 and KU376499 both have 99 % similarity with *C. etunicatum* (AM158952) and *F. mosseae* (JQ048902), respectively. Then ML phylogenetic trees were constructed to determine the phylogenetic relationship between the tested AM fungi and the known AM fungi in GenBank. Phylogenetic tree analyses showed that the sequences with accession numbers KT881238, KU144882, KU376499 clustered with *R. intraradices* HE817876, *C. etunicatum* AM158952, and *F. mosseae* JQ048902, respectively (Fig. 2) thereby identifiying them as the three dominant AM fungal species present in the Songnen Plain.

Discussion

The results showed that there were abundant AM fungal resources in the rhizosphere soil of *Puccinellia tenuiflora* in the Songnen saline-alkaline grassland, namely 40 species of 17 genera. So far, we have carried out a lot of work on the physiological characteristics and resistance of AM fungi. Wang (2017) reports that about 300 AM fungal taxa have been identified by morphology all around the world. Davison (2015) collected all published AM fungi sequences based on NS31-AM1 primer section, and identified 356 AM fungal taxa with 97 % sequences similarity. China with its vast territory and complex ecological environment has the potential for abundant AM fungal species (Qin 2008). In China, 145 taxa belonging to 12 genera of 8 families have been found, among which *Glomus* was the dominant genus (Wang et al. 2018). Studies on AM fungal diversity of saline-alkaline grassland in Songnen Plain are carried out widely. Yang et al. (2015) isolated 40 species of AM fungi of 5 genera from 26 plant species of 11 families, among which *Glomus* and *Acaulospora* were dominantig genera, and *Glomus intraradices* was the dominant species. Subsequently, researchers have identified 37 AM fungi of 12 genera and 37 AM fungi of 8 genera from the rhizosphere of *Iris lactea* (Wang 2018) and *Inula japonica* (Yang et al. 2017).

Based on morphology and molecular genetics, we identified the three dominant AM fungi of *Puccinellia tenuiflora* in the Songnen Plain. According to their Importance Value they were *R. intraradices* (IV=61.5 %), *C. etunicatum* (IV=38.4 %) and *F. mosseae* (IV=64.6 %). Further, from single spore DNA extraction and bands of nested-PCR amplification product on agarose gels up to plasmid DNA sequencing, all the steps of operation were rigorously controlled to ensure the accuracy of experiment results. The results of morphological identification were completely consistent with molecular identification.

Puccinellia tenuiflora can establish symbioses with a lot of AM fungi among which *Rhizoglomus intraradices*, *Claroideoglomus etunicatum* and *Funneliformis mosseae* are dominant, which may be related to the preference of AM fungi infecting the host plants (Jongho et al. 2015). AM fungi have a good performance in improving soil physical and chemical properties. In addition, AM fungi can enhance the absorption of water and nutrients of host plants (Zhang & Guo 2013), enhance the salt-alkali tolerance, improve the balance of plants internal environment (Zhu et al. 2010), enhance the photosynthesis and tissue osmotic adjustment capacity (Carini & Okurowska 2008), promote the absorption of P, K and mineral elements, and enhance the antioxidant system of plants (Hajiboland et al. 2013, Aroca et al. 2008). Hence the excellent salt tolerance of *P. tenuiflora* is probably related to the AM fungi of its rhizosphere. In saline-alkaline soils, AM fungal structure is unique, which may contain AM fungi species with special functions, especially with strong stress resistance (Estrada et al. 2013, Evenlin et al. 2012). According to results of our previous study, *Rhizoglomus intraradices*, *Claroideoglomus etunicatum* and *Funneliformis mosseae* are likely to be the species with strong salt tolerance. Fereshteh et al. (2014) studied *Puccinellia distans* with or without inoculation by *Claroideoglomus etunicatum*, and the results showed that AM fungi increased plants' tolerance with a higher biomass and water-use efficiency, but less proline and malondi-

aldehyde concentration. Nonetheless, how *Rhizoglomus intraradices* and *Funneliformis mosseae* can improve the salt-alkali tolerance of *P. tenuiflora* is still unknown. In addition, the diversity of host plants determines the diversity of AM fungi to some extent, and vice versa. Many studies indicated that indigenous AM fungi tend to show the best ability to inoculate the plant (Klironomos et al. 2003). So we should further explore the diversity of AM fungi in different plant rhizospheres to raise the AM fungal species number in Songnen Plain. And we should go on a step further by selecting the dominant AM fungi species, optimizing the mycorrhizal mutualism systems of saline-alkaline grassland, which would be a certain basis for the improvement and restoration of saline-alkaline land.

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