Mycorrhiza (2014) 24:79–94
DOI 10.1007/s00572-013-0516-9

ORIGINAL PAPER

Differential effects of abiotic factors and host plant traits on diversity and community composition of root-colonizing arbuscular mycorrhizal fungi in a salt-stressed ecosystem

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Received: 8 February 2013 / Accepted: 8 July 2013 / Published online: 31 July 2013

Abstract Arbuscular mycorrhizal fungi (AMF) were investigated in roots of 18 host plant species in a salinized south coastal plain of Laizhou Bay, China. From 18 clone libraries of 18S rRNA genes, all of the 22 AMF phylotypes were identified into *Glomus*, of which 18 and 4 were classified in group A and B in the phylogenetic tree, respectively. The phylotypes related to morphologically defined *Glomus* species occurred generally in soil with higher salinity. AMF phylotype richness, Shannon index, and evenness were not significantly different between root samples from halophytes vs. non-halophytes, invades vs. natives, or annuals vs. perennials. However, AMF diversity estimates frequently differed along the saline gradient or among locations, but not among pH gradients. Moreover, UniFrac tests showed that both plant traits (salt tolerance, life style or origin) and abiotic factors (salinity, pH, or location) significantly affected the community composition of AMF colonizers. Redundancy and variation partitioning analyses revealed that soil salinity and pH, which respectively explained 6.9 and 4.2 % of the variation, were the most influential abiotic variables in shaping the AMF community structure. The presented data indicate that salt tolerance, life style, and origin traits of host species may not significantly affect the AMF diversity in roots, but do influence the community composition in this salinized ecosystem. The findings also highlight the importance of soil salinity and pH in driving the distribution of AMF in plant and soil systems.

Keywords AM fungi · Community structure · Environmental variables · Plant traits · Salt tolerance · SSU rDNA

Introduction

Much effort has been made to study the diversity and distribution of widespread arbuscular mycorrhizal fungi (AMF) in saline soils and roots of salt-tolerant plants. Based on fungal spore morphology, *Glomus intraradices*, *Glomus versiforme*, and *Glomus etunicatum* were identified as the most predominant AMF species in saline soils in Iran (Aliasgharzadeh et al. 2001), while a single AMF species, *G. geosporum*, was reported to be frequent in European salt marshes and saline soils (Carvalho et al. 2001; Landwehr et al. 2002; Grzybowska 2004; Wilde et al. 2009). Wang et al. (2004) examined the diversity of AMF spores in the rhizosphere of five wild plants in saline-alkaline soils of the Yellow River Delta, China, and found three genera, with *G. caledonium* being the dominant species. The distribution of AMF spores in saline soils does not necessarily follow the salinity gradient (Aliasgharzadeh et al. 2001; Wilde et al. 2009), whereas the rate of AMF colonization of a wetland legume tree *Pterocarpus officinalis* was found to decrease with the increasing salt levels (Saint-Etienne et al. 2006) and was limited by edaphic factors including salinity in the halophyte *Puccinellia nuttalliana* (Johnson-Green et al. 2001).

Spore analyses alone may poorly represent AMF population structure in an ecosystem, and molecular identification of AMF in roots remains the most informative approach for ecological studies (Kowalchuk et al. 2002; Santos et al. 2006). Molecular diversity of AMF associated with salt-tolerant plants has been primarily investigated in plants in coastal environments. These studies have usually examined roots from a single or a couple of plant species using 28S

Electronic supplementary material The online version of this article (doi:10.1007/s00572-013-0516-9) contains supplementary material, which is available to authorized users.

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(Rosendahl and Stukenbrock 2004; Stukenbrock and Rosendahl 2005), 18S (Sonjak et al. 2009b; Yamato et al. 2008, 2012), or ITS (Wilde et al. 2009) rDNA sequences. Recently, Yamato et al. (2008, 2012) showed that AMF populations could adapt to a salt-stressed environment, and that the AMF community associated with a coastal plant *Ixeris repens* showed a significant relationship between the distribution of phylotypes and the environmental variables that were examined (Yamato et al. 2012). Results from investigations by Wang et al. (2011) in three dominant mangrove species indicated that the communities of AMF in wetland ecosystems are not necessarily low in diversity and that both host species and tide level affected community structure of AMF. Sonjak et al. (2009a) examined the molecular diversity of AMF colonizing 12 halophytes using temporal temperature gradient gel electrophoresis and found only six species. Nevertheless, knowledge about AMF diversity of plant species in salt-affected ecosystems remains insufficient, and the environmental constraints and host effects in saline ecosystems have yet to be investigated. The AMF communities of plants from different origins (Greipsson and DiTommaso 2006; Moora et al. 2011; Jordan et al. 2012; Lekberg et al. 2013) and life style (Alguacil et al. 2012; Lugo et al. 2012; Torrecillas et al. 2012) have been investigated, but again, little is known about the effect of the origin and life style of plant species on AMF diversity and community in salt-stressed ecosystems.

In this study, we investigated the root-associated AMF communities in 11 typical halophytes and 7 amphiphytes and zerophytes, which are all dominant species in salt marshes and in freshwater meadows in a long-term salinized coastal ecosystem. Research aims were: (1) to characterize the molecular diversity and community composition of AMF in roots of dominant plant species in the salt-stressed ecosystem, (2) to test the hypothesis that diversity and community composition of AMF are different in terms of host plant species' salt tolerance (halophytes vs. non-halophytes), life style (annuals vs. perennials), and origin (invaders vs. natives), and (3) to explore the possible relationship between AMF and soil properties (especially salinity) in the salt-stressed system.

Materials and methods

Study sites and sampling

The South Coastal Plain of Laizhou Bay (Bohai Sea, Shandong Peninsula) covering approximately 2,870 km^2 in the Yellow River Delta has been the most serious and largest saltwater intrusion area in China since the 1980s (Fig. 1). The climate is temperate, with an average annual rainfall of 600 mm and a mean annual temperature of 12 °C. The saltwater intrusion is largely due to the overexploitation of freshwater in aquifers, and to paleogeographic and sedimentary environmental change. This has induced soil salinization with soil salinity generally increasing seawards (Chen et al. 1997; Zhang et al. 2008a). The modern land surface geomorphology of this area consists mainly of alluvial deposit, alluvial-marine deposit, and marine deposit from south to north. The strata are gravel and coarse sandstone in the upper proluvial fan and turn to fine sand, silt, sandy clay, and silty clay towards the coast (Chen et al. 1997; Zhang et al. 2008a; Han et al. 2011). Consequently, three obvious land types can be identified in this area: sand marsh (SM), coastal meadow (CM), and farm land (FL).

Many plants of this area are halophytes that can survive in saline soils with low nutrient loads and low water holding capacity, and dominant plant species are distinct across land types. According to previous surveys in the south coastal plain of the Laizhou Bay, the dominant plants of vegetation in middle and high tide foreshore wetlands are halophytes such as *Suaeda heterobtera* and *Tamarix chinensix*, whereas halophytes (e.g., *Phragmites communis*) and amphiphytes (e.g., *Aeluropus littoralis*) dominate in freshwater marsh and meadow. Much of the salt marsh and freshwater marsh have evolved into meadow, and much of the vegetation characteristic of salt marshes has disappeared due to saltwater intrusion, climate change and the construction of salterns and ponds for mariculture in the last 30 years (Zhang et al. 2006).

Five sites (A–E) in the south coastal plain were surveyed on 22 April 2011, with two sites (A, B) located along the Jiaolai River and three sites (C–E) along the Di River. The Jiaolai River is about 130 km long, receiving extensive agricultural discharges, and the Di River is relatively shorter (23 km), flowing through a town with both domestic and industrial sewage inputs. The spatial distribution of AMF in plant roots was analyzed by sampling on the same day, thus minimizing the effects of seasonal fluctuations, since temperature has been shown to be a driving force in regulating the dynamics of AMF communities (Heinemeyer and Fitter 2004; Dumbrell et al. 2010). This enabled a focus on the possible effects of plant hosts and soil physiochemical factors (especially the salinity gradient) on AMF diversity. The five sites were selected to represent the three typical habitats (salt marsh, A and C; coastal meadow, B and D and farm land E) and the soil salinity gradient (Fig. 1). The area covered 5.6 km from site A to B, and about 5 km between two neighboring sites of C, D, and E, with a distance between the two rivers of about 22 km. In each study site, five replication blocks (10×10 m) were established, and the most abundant species available in the blocks were sampled. Roots of ten different plant individuals of the same species in each site were taken and pooled to form one sample, which was thereafter referred to as A1-6, B1-2, etc. (see Table 1). The sampled plant species were typically in their yearly growing period.

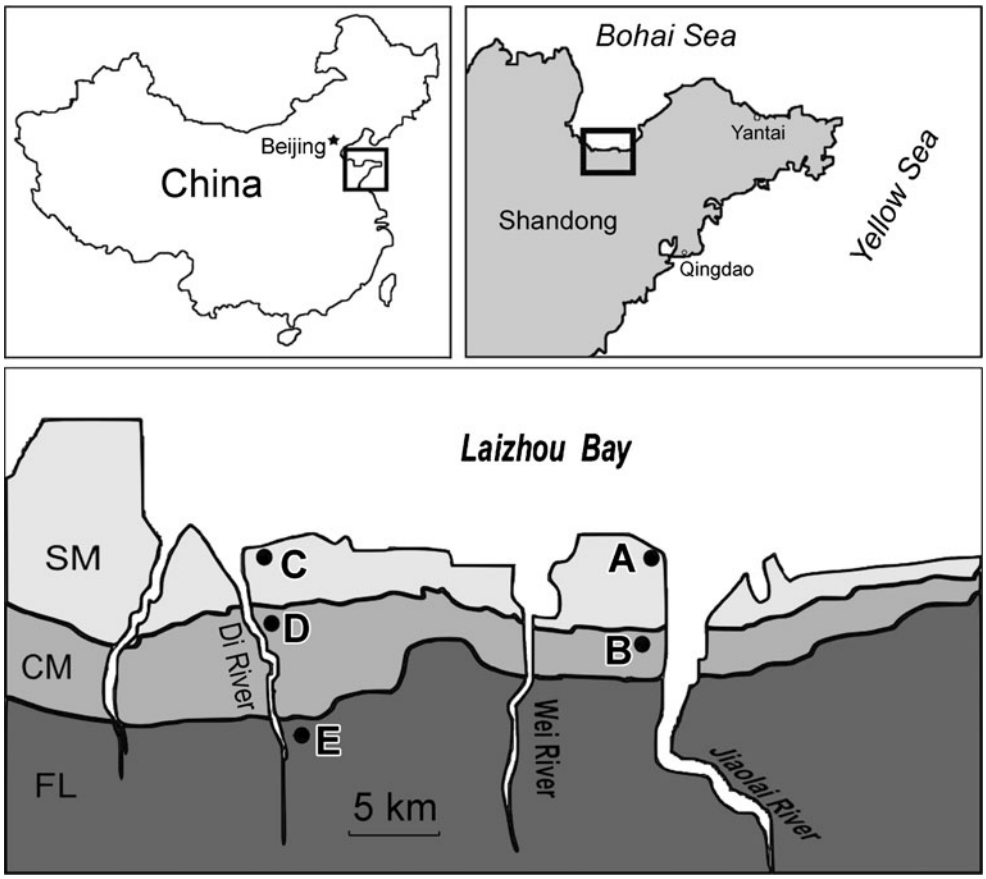

Fig. 1 Location of study sites and sampling zones in the south plain of Laizhou Bay

For each individual plant from a given plot, surface soil was removed, plant roots together with their rhizosphere soil were carefully excavated, placed in plastic bags, and returned to the laboratory at 4 °C. Roots were then rinsed thoroughly with sterilized distilled water, cut into approximately 1-cm-long pieces, and stored at −80 °C until DNA extraction. Finally, 18 samples corresponding to 18 different plant species were obtained. Plants were morphologically identified and classified according to their salt tolerance (11 halophytic and 7 non-halophytic), life style (8 annual and 10 perennial), and origin (14 native and 4 invasive) according to the literature (Zhang et al. 2006, 2008b, 2009), the Subject Database of Chinese Plant (http://www.plant.csdb.cn/), and Plant Collection Database (http://www.plantpic.csdb.cn/).

Soil chemical analysis

Soil total nitrogen and organic carbon contents were analyzed using the Vario Micro Cube Elemental Analyser (Elementar Analysensysteme, Hanau, Germany) with the burning method at 450 and 1,250 °C, respectively. Soil available phosphorus was extracted using the $NaHCO_3$ method and measured colorimetrically. Soil pH was measured in 1 M KCl (10 g soil per 50 mL solution). Soil salinity (Sal) was determined by measuring the electrical conductivity/salt content in water extracts at soil/water ratios of 1:5. Water content was measured gravimetrically following the drying method (Gardner 1986). Classification of soil types was according to Richards (1954): non-saline (Sal <1.2‰), slightly saline (1.2–2.4‰), moderately saline (2.4–4.8‰), strongly saline (4.8–9.6‰), and severely saline (Sal >9.6‰). Soil pH was divided into acidic (<6.80), neutral (6.80–7.10), and alkaline (>7.10).

DNA extraction and PCR amplification

For each sample (or plant species), about 1 g fresh root fragments from ten different plant individuals was homogenized in liquid nitrogen using a mortar and pestle, and 0.1 g of the resulting homogenate was used for genomic DNA extraction using the Plant DNA Extraction Kit (Tiangen Biotech, China) following the manufacturer's instructions. The concentrations of extracted and purified DNA were quantified using a ND-2000 spectrophotometer (NanoDrop Technologies, Delaware, USA). Partial fragments (~790 bp) of fungal 18S rDNA were PCR amplified with primer pairs AML1 and AML2 (Lee et al. 2008) in a Tprofessional thermocycler (Biometra, Göttingen, Germany). Each PCR tube had a final volume of 25 μl containing 2 μl extracted

Table 1 Characteristics of host plants and rhizosphere soils

Sample ID	Scientific name	Life span	Origin	Salt tolerance	pH	Soil salt content (‰)	Soil water content (%)	Total N (g kg^{-1})	Total C (g kg^{-1})	C/N ratio	Available P (mg kg^{-1})
A1	*Atriplex centralasiatica*	An	Na	H	6.73±0.02	12.5±0.39	2.12±0.09	1.14±0.01	15.30±0.02	13.5±1.54	11.1±0.06
A2	*Juncus effusus*	Pe	Na	NH	6.12±0.06	9.50±0.31	0.45±0.27	0.45±0.01	6.46±0.30	14.4±1.98	28.2±2.11
A3	*Artemisia annua*	An	I	H	6.64±0.31	6.50±0.30	0.45±0.17	0.47±0.01	6.77±0.09	14.3±1.99	20.5±3.45
A4	*Bidens* sp.	An	I	H	6.84±0.19	16.5±0.45	6.68±0.30	0.48±0.02	6.80±0.28	15.2±1.15	31.2±4.92
A5	*Artemisia littoricola*	Pe	Na	H	7.02±0.33	8.00±0.26	13.8±0.21	0.54±0.01	6.96±0.21	13.3±0.85	18.8±1.23
A6	*Calamagrostis adans*	An	Na	NH	6.84±0.07	4.50±0.09	13.9±0.43	0.90±0.01	10.2±0.08	11.4±0.86	17.9±1.37
B1	*Plantago asiatica*	Pe	Na	NH	6.89±0.45	3.50±0.01	9.59±0.09	0.67±0.01	7.68±0.03	11.5±0.21	26.3±1.62
B2	*Phragmites communis*	Pe	Na	H	7.31±0.32	2.00±0.02	21.6±0.91	0.99±0.01	9.68±0.14	9.81±0.45	12.1±0.61
C1	*Limonium sinense*	Pe	Na	H	7.12±0.33	8.00±0.05	12.6±0.16	0.43±0.01	5.90±0.01	13.9±0.85	16.1±3.67
C2	*Suaeda salsa*	An	Na	H	7.04±0.09	18.5±0.36	16.2±0.34	0.64±0.01	10.4±0.08	16.1±0.74	10.3±1.49
C3	*Hieracium pilosella*	Pe	I	NH	6.89±0.15	17.0±0.34	5.51±0.12	0.55±0.01	7.25±0.02	13.5±3.61	16.0±1.79
C4	*Tamarix chinensis*	Pe	Na	H	6.80±0.06	9.50±0.22	8.95±0.36	0.73±0.01	9.84±0.01	13.6±1.10	20.5±0.65
D1	*Setaria viridis*	An	Na	NH	7.26±0.14	4.50±0.06	2.59±0.36	0.44±0.01	6.36±0.08	14.3±0.46	31.2±3.01
D2	*Aeluropus littoralis*	Pe	Na	H	7.40±0.03	4.50±0.06	3.65±0.07	0.99±0.01	9.21±0.05	9.36±0.66	37.0±3.61
D3	*Polygonum aviculare*	An	Na	NH	7.35±0.11	1.50±0.01	0.91±0.07	0.99±0.01	8.60±0.02	8.67±0.28	35.6±0.85
E1	*Hypochaeris radicata*	Pe	I	H	6.80±0.23	1.00±0.01	0.88±0.03	0.80±0.01	8.30±0.02	10.3±0.87	60.6±1.23
E2	*Xanthium sibiricum*	An	Na	NH	6.61±0.04	3.50±0.03	15.9±0.05	0.69±0.01	13.90±0.12	20.3±1.90	42.1±1.20
E3	*Artemisia argyi*	Pe	Na	H	7.41±0.26	2.50±0.05	24.1±2.49	0.58±0.01	9.47±0.05	16.6±2.30	7.53±1.94

Values are provided as mean and standard errors

An annual, *Pe* perennial, *I* Invasive, *Na* native, *H* halophyte, *NH* non-halophyte

DNA, 0.5 μM each primer, 0.1 mM each dNTP, 0.5 mM $MgCl_2$, 2.5 μl manufacturer's PCR buffer, and 0.5 units *Taq* DNA polymerase (Tiangen Biotech, China). Amplification was run under the following conditions: 94 °C for 10 min, followed by 35 cycles of 94 °C for 0.5 min, 59 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Five PCR replicates were carried out independently for each sample. Products were then pooled in order to reduce PCR biases and minimize the effects of root spatial heterogeneity (Schwarzenbach et al. 2007). The resulting PCR products were verified on a 1 % agarose gel and purified using a Purification Kit (Tiangen).

Cloning, RFLP typing, and sequencing

Initially, a nested PCR-DGGE approach was used to characterize the AMF communities associated with the 18 plant species (Anderson and Cairney 2004). The first round PCR used primers AML1/AML2 (Lee et al. 2008) and the second NS31-GC (which corresponds to NS31 described by Simon et al. 1992 plus a 5′GC clamp sequence described by Kowalchuk et al. 1997) and Glo1 (Cornejo et al. 2004). Although 35 different bands (OTUs) across all samples were detected from the DGGE gel, sequencing of the sliced bands demonstrated that many of them were from non-AMF microorganisms (e.g., algae), and only 11 AMF sequences could be obtained. This recalled the drawbacks of recovering sequence data from excised bands (Liang et al. 2008). To obtain relatively longer sequences with higher phylogenetic resolution and more phylotypes of root-associated AMF, clone libraries were constructed using the primer pair AML1/AML2 which proved to be highly AMF-specific and allowed the amplification of a small subunit (SSU) rDNA fragment of ~790 bp (Lee et al. 2008).

PCR products of each sample were pooled, inserted into pGM-T vector (Tiangen Biotech, China), and transferred into *Escherichia coli* DH5α (Tiangen Biotech, China) according to the manufacturer's instructions. A total of 18 libraries were constructed (one library per plant species). For each library, 100 white clones were randomly selected for inserted PCR products with the primers AML1/AML2 under the same conditions as mentioned above. The PCR products with correct size were digested with *Hinf* I (NEB, Lithuania) and examined on a 2.5 % (*w*/*v*) agarose gel. Three clones of

each restriction fragment length polymorphism (RFLP) types were randomly selected for sequencing (Sangon Biotech, China).

Sequence analysis

Chimeric sequences were identified using Bellerophon (Huber et al. 2004) and KeyDNAtools (http://keydnatools.com/). Representative sequences for each phylotype were subject to BLAST searches with default settings against the Maarj*AM* (http://maarjam.botany.ut.ee/; Öpik et al. 2010) and GenBank databases. The Maarj*AM* database contains representative sequences from published environmental Glomeromycota sequence groups and known taxa. As of May 2013, it contained a total of 6,064 records that could be associated with SSU sequence-based taxa, or so-called virtual taxa. Representative sequences retrieved from the GenBank and Maarj*AM* database then were aligned with our newly obtained sequences using ClustalW (Thompson et al. 1994). A neighbor-joining (NJ) tree was constructed using MEGA 4.0 (Tamura et al. 2007) with K2-parameter model (Kimura 1980), and a maximum likelihood (ML) tree was built with RAxML (Katoh et al. 2009) using GTR+G+I model that was selected by ModelTest (Posada and Crandall 1998). Bootstrap analysis of 1,000 replications was carried out in both phylogenetic analyses (Felsenstein 1985).

Shannon diversity (H) of AMF communities was calculated as $H=-\sum_{i}^{n}{}_{=1}Pi*\ln(Pi)$, where Pi is the relative abundance of each phylotype. Richness (S) was the number of phylotypes, and evenness (E) as $E=H/\ln(S)$. Rarefaction analysis was performed by plotting the number of phylotypes detected against the number of sequences with the aRarefactWin software package (S. Holland, University of Georgia).

Statistical analyses

A parametric one-way analysis of variance (ANOVA), followed by a least significance difference test at the 0.05 confidence level, was used to determine differences in soil properties among samples. Mean values of diversity estimates were compared with pairwise t tests, or one-way ANOVA following multiple comparisons. Pearson correlation coefficients were calculated to explore the relationship between AMF diversity and soil properties (e.g., water and salt content, pH, and C/N ratio). All these analyses were performed using program SPSS 13.0 (SPSS Inc., Chicago, IL, USA). To examine the composition and clustering of AMF communities, principal coordinate analysis (PCoA) was performed using weighted UniFrac (Lozupone et al. 2006). Differences in AMF composition clustered by life style (annual vs. perennial), origin (invasive vs. native), and salt tolerance (halophyte vs. non-halophyte) of host plants, and by saline and pH grades and location, were pairwise or globally tested based on unweighted UniFrac metric. Following detrended correspondence analysis determining the length of the environmental gradient, redundancy analysis (RDA) was performed to reveal the relationships between soil environmental variables and AMF communities using CANOCO 4.5 (ter Braak and Smilauer 2002). Major abiotic factors were retested by variation partitioning analysis within the *vegan* package in R (R Development Core Team 2008). A non-halophytic, annual, and native plant species (D3: *Polygonum aviculare*) with no detectable AMF sequences was excluded from these analyses.

Sequence deposition

The non-redundant sequences of AMF 18S rDNA genes obtained in this study have been deposited in the GenBank with accession numbers JX144109-JX144126, JX144128-JX144130, JX144132-JX144139, and KC579403-KC579433.

Results

Soil and plant properties

All soil parameters differed significantly (ANOVA, $P<0.05$) among samples except for pH. The sampled rhizosphere soils were generally neutral, with a few samples slightly acidic (A1) or slightly alkaline (D2 and E3; Table 1). Salt content (1–18.5‰) and water content (0.45–24.1 %) varied greatly among samples. Basically, the mean salinity of each site increased seawards. Soil carbon to nitrogen ratios ranged from 8.67 (D3) to 20.3 (E2). The highest records of both total N (1.14 g kg^{-1}) and total organic C (15.3 g kg^{-1}) were detected from the sample A1, whereas the lowest records were from the sample C1. Soil available phosphorous ranged from 7.5 to 60.6 mg kg^{-1}.

Clone libraries

For all the 18 libraries constructed for AMF colonizing the roots of salt-tolerant plant species, a total of 1,800 clones were screened, and 78 types were identified by RFLP analysis. The three clones sequenced for each RFLP type did not always give rise to the same phylotype, and some RFLP types were found in only one or two clones. A total of 228 sequences were obtained, of which 10 sequences (4.3 %) were likely chimeras and hence excluded. BLAST against GenBank showed that only 136 sequences (62.4 %) had high sequence similarity with members of Glomeromycota, and non-AMF sequences accounted for a substantial proportion (37.6 %; see Fig. S1). No AMF sequences were detected from the sample D3, which was an annual flat weed (*P. aviculare*), collected from a non-saline soil during this

Table 2 Distribution of arbuscular mycorrhizal fungal sequences (clones) observed in each AMF phylotype within the root of 18 plant species

Taxon	Halophyte									
	Suaeda salsa	*Biden* sp.	*Atriplex centralasiatica*	*Aeluropus littoralis*	*Artemisia argyi*	*Phragmites communis*	*Tamarix chinensis*	*Artemisia littoricola*	*Limonium sinense*	*Artemisia annua*
LGlo1		38	33	15			36	50		8
LGlo2			28							
LGlo3										
LGlo4					3					
LGlo5		2								
LGlo6		1						25		
LGlo7						6				
LGlo8					38					
LGlo9				49	7	6				
LGlo10									2	
LGlo11						11				
LGlo12					16					
LGlo13				2	3					
LGlo14				16		1				
LGlo15				3						
LGlo16					1					
LGlo17				2						
LGlo18										
LGlo19					1					
LGlo20					3					
LGlo21					2					
LGlo22	3									
Total	3	41	61	87	74	24	36	75	2	8
S	1	3	2	6	9	4	1	2	1	1
H	0	0.31	0.69	1.23	1.50	1.18	0	0.64	0	0
E	0	0.28	1.00	0.69	0.68	0.85	0	0.92	0	0

Taxon	Halophyte	Non-halophyte						Total
	Hypochaeris radicata	*Hieracium pilosella*	*Juncus effusus*	*Calamagrostis adans*	*Setaria viridis*	*Plantago asiatica*	*Xanthium sibiricum*	*Polygonum aviculare*	
LGlo1		12		47					239
LGlo2	52	35	23				17		155
LGlo3	2								2
LGlo4			1						4
LGlo5		1		15					18
LGlo6									26
LGlo7		4							10

Table 2 (continued)

Taxon	Halophyte	Non-halophyte							Total
	Hypochaeris radicata	*Hieracium pilosella*	*Juncus effusus*	*Calamagrostis adans*	*Setaria viridis*	*Plantago asiatica*	*Xanthium sibiricum*	*Polygonum aviculare*	
LGlo8			12			3	16		69
LGlo9	15				23	8	8		116
LGlo10	15	1			15				33
LGlo11						3			14
LGlo12	4		2			5			27
LGlo13									5
LGlo14	4						6		27
LGlo15									3
LGlo16							6		7
LGlo17									2
LGlo18					3				3
LGlo19			1						2
LGlo20			4			1			8
LGlo21									2
LGlo22									3
Total	92	53	43	62	41	20	53	0	775
S	6	5	6	2	3	5	5	0	62
H	1.27	0.96	1.23	0.55	0.88	1.43	1.50	0	
E	0.71	0.59	0.69	0.80	0.80	0.89	0.94	0	

Novel phylotypes are underlined

S richness, *H* Shannon index, *E* evenness

survey (Fig. S1B), whereas all sequences obtained from a perennial wetland grass *Phragmites communis* (sample B2) belonged to AMF (see Table 2, Fig. S1B). No significant correlation was found between the relative proportion of AMF sequences in each library and soil salinity (r=−0.25, P=0.33). The rarefaction curves showed that the clones screened in each sample were sufficient to detect the majority of phylotypes present in roots, as the curves approached saturation (data not shown).

Phylogenetic analyses

Both NJ and ML trees showed a congruent topology (Fig. 2), in which the known families (e.g., Glomeraceae, Paraglomeraceace, Gigasporaceae) were well recognized (Liu et al. 2011). Based on the phylogenetic tree, a total of 22 AMF phylotypes were recognized, and all were members of the genus *Glomus*. Specifically, *Glomus* group A had three clusters (clusters 1–3), hosting 18 phylotypes and 760 clones; the remaining four phylotypes (LGlo19-22) belonged to the *Glomus* group B. Two phylotypes were closely related to the morphologically described species: one was LGlo1, which clustered with *G. intraradices*, *Glomus fasciculatum*, and *Glomus vesiculiferum* in *Glomus* group A, and accounted for 30.8 % (239 out of 775) AMF clones, representing the most abundant phylotype in our study; the other was LGlo19, which clustered with *Glomus lamellosum* in the *Glomus* group B, and accounted for only 0.26 % (2 out of 775 clones) of the AMF community. Among the other 20 phylotypes, 9 showed high sequence similarity to uncultured AMF; the other 11 phylotypes represented previously undescribed sequences since they exhibited low (<97 %) sequence similarities with those publicly available. Seven phylotypes (LGlo15-18, LGlo20-22) were highly divergent from the genus *Glomus*, but were firmly placed in the family Glomeraceae, suggesting these could represent new genera within the family.

According to the Maarj*AM* database, the 22 phylotypes detected in our study were closely related to 7 molecular virtual taxa (VT00105, VT00113, VT00114, VT00154, VT00156, VT00166, and VT00193), with 6 virtual taxa of *Glomus* group A and one of group B. The novel 11 phylotypes closely matched to VT00113 (LGlo5), VT00156 (LGlo11-13, LGlo15, and LGlo18), VT00154 (LGlo16), VT00166 (LGlo17), and VT00193 (LGlo20-22), with sequence similarities ranging from 95 to 97 %. There were 297 clones (four phylotypes) belonged to VT00113, accounting for 38.3 % in our dataset. The second (29.4 %) and the third most dominant phylotypes (20.3 %) were VT00156 and VT00114, respectively.

Interestingly, four out of six phylotypes assigned in Cluster 1 (i.e., LGlo1, 5, 6, and 7) were detected from plant species that occurred in soil with relatively higher salt content (12.5–18.5‰), while the other four phylotypes of cluster 1, and those of clusters 2 and 3 (only except sequence LG0428 and LG0221 of LGlo10) were all observed from samples with lower salt content (1–4.5‰, Fig. 2).

The numbers of phylotypes that were unique or shared between plant types are summarized in Fig. 3. Seven, 11, and 12 phylotypes were observed in the halophytes, perennials, and natives, respectively. A majority of the new phylotypes was only detected in native (81.8 %) or perennial (63.6 %) plant species (Fig. 3). Five new phylotypes were shared by halophytes and non-halophytes, and a few phylotypes were shared between invaders and natives (1), or between perennials and annuals (2). Nevertheless, six phylotypes (LGlo1, 2, 5, 9, 10, and 14), which all were affiliated with clusters 1 and 2 in the phylogenetic tree, were detected in all the plant types.

Richness, Shannon diversity, and evenness

The number of AMF phylotypes (richness) colonizing roots of a given plant species examined in this study ranged from 0 to 9 (Table 2). As mentioned above, no AMF was detected in plant D3 (*P. aviculare*), while *Artemisia argyi* (E3) harbored the highest number of phylotypes. Most plant species were colonized by two or more AMF phylotypes, except for *Artemisia annua* (A3), *Limonium sinense* (C1), *Suaeda salsa* (C2), and *Tamarix chinensis* (C4), which were colonized by only one phylotype. Three phylotypes were most frequently detected: LGlo1 in eight host plants (A1, 3, 4, 5, B1, C3, 4, and D2), LGlo2 in five plants (C1, 2, D1-3), and LGlo9 in seven plants (B1, 2, D1, 2 and E1, 2, 3). These three phylotypes accounted for 65.8 % of the total sequence abundance in all samples. Apparently, no phylotype was common to all plants sampled in this study. Shannon index varied from 0 (only one phylotype observed) to 1.50 (in *Xanthium sibiricum* and *A. argyi*), and evenness from 0 to 1 (Table 2).

ANOVA and t tests were performed to compare the diversity estimates (richness, Shannon index, and evenness) of AMF from different plant types (i.e., salt tolerance, life style, and origin), soil saline levels, pH, and locations (Table 3). Overall, saline levels and locations seemed to be more influential than other factors on the diversity estimates of AMF in the host plant. Although the average values of all the three estimates of AMF diversity seemed to be lower in the halophytes (vs. the non-halophytes) or in the annuals (vs. the perennials), the differences were not statistically supported at a confidence level of 95 % (Table 3). Differences in AMF diversity estimates were not detected between invasive and native plant samples (t test, P>0.20), nor between soil pH ranges (ANOVA, P>0.50). However, in general, the diversity estimates were significantly different among soil saline levels or locations (ANOVA, P<0.05), except that no significant differences were found for the phylotype evenness (Table 3). The highest AMF diversity was usually found in

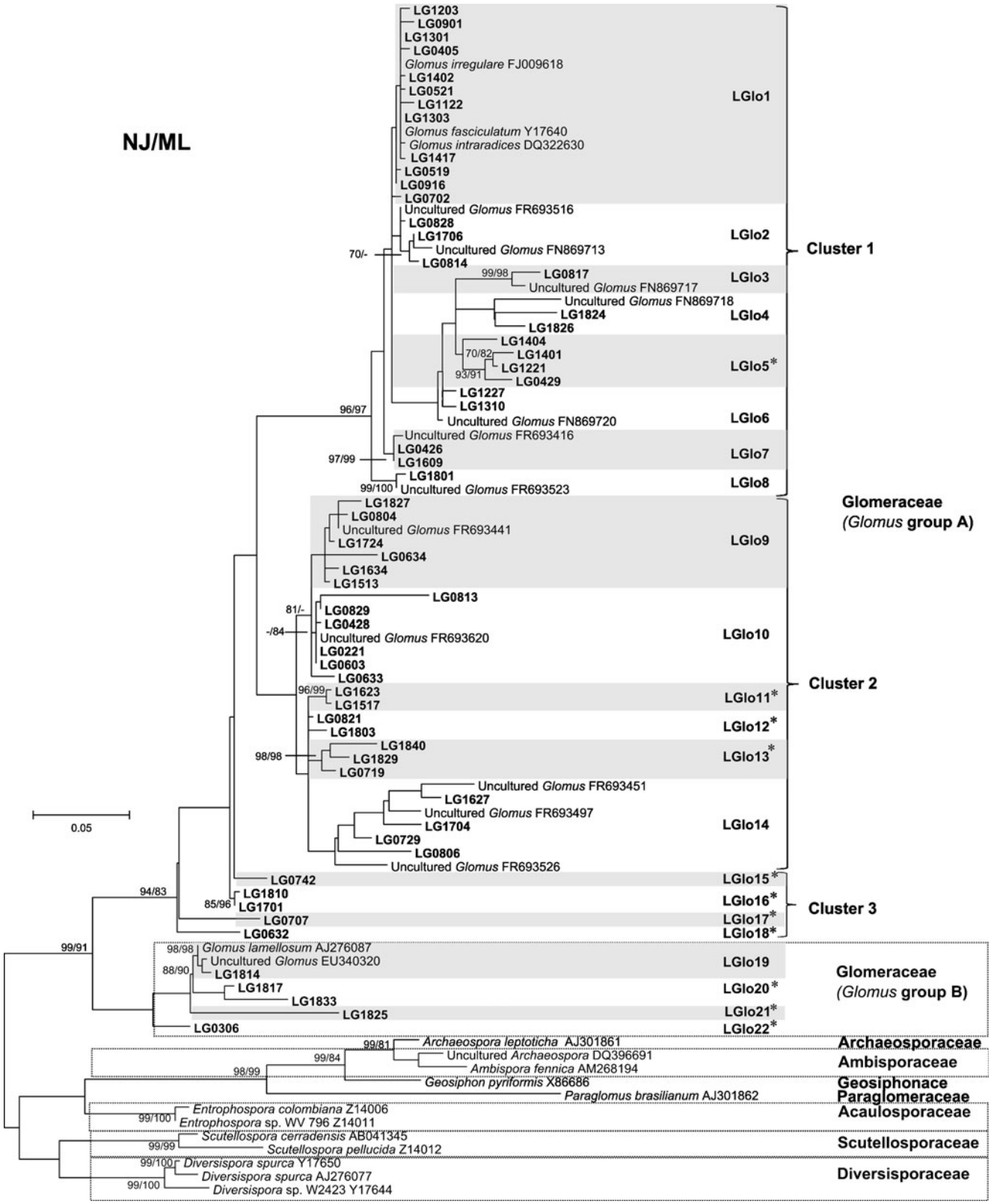

Fig. 2 A maximum likelihood tree showing the phylogenetic positions of the AMF phylotypes obtained from roots of 17 plant species. Nodal bootstrap values >70 % are shown. Sequences obtained in the present study are shown in *black*. The 11 novel AMF phylotypes are labeled with *asterisks*

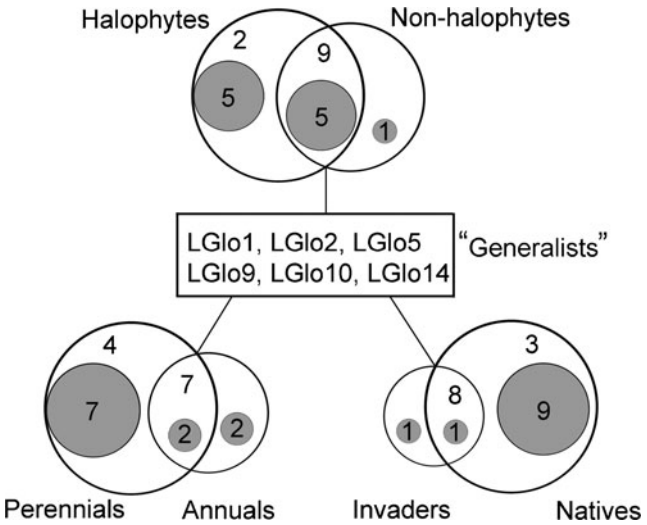

Fig. 3 Numbers of AMF phylotypes that were shared or exclusively found in plant types. The numbers of new phylotypes are shown in *solid circles*. The six "generalist" phylotypes are those detected in all plant types

the moderately saline samples, at site E, or in the farm land zone (Table 3).

Effects of plants and soils on community composition

UniFrac significance tests were performed to explore whether two groups of AMF communities were significantly different (Table 4). The results showed that effects of plant traits on AMF community composition were generally evident. The invaders and natives had significantly different AMF communities ($P<0.001$), as did the annuals and perennials ($P<0.02$). The composition of AMF colonizers in halophytic and non-halophytic samples was marginally different ($P=0.06$; Table 4).

AMF communities were also significantly different among saline levels, pH ranges, or locations ($P<0.05$, Table 4). Pairwise comparison showed that samples of the four saline grades were generally different from each other, except for the

Table 3 Comparison of diversity estimates of AMF from different plant hosts or soils using two-tailed *t* test or one-way ANOVA

	Richness	Shannon index	Evenness
Salt tolerance			
Halophyte	3.27±0.77	0.62±0.17	0.47±0.12
Non-halophyte	4.33±0.25	1.09±0.06	0.79±0.02
P value	0.39	0.10	0.09
Life span			
Annual	2.43±0.49	0.57±0.19	0.56±0.16
Perennial	4.50±0.73	0.94±0.17	0.59±0.09
P value	0.07	0.20	0.89
Origin			
Invasive	3.75±0.66	0.64±0.15	0.40±0.10
Native	3.62±0.96	0.83±0.25	0.64±0.14
P value	0.92	0.55	0.27
Saline grade			
Slight	5.00±0.71	1.23±0.03ab	0.78±0.05ab
Moderate	5.00±0.91	1.18±0.14a	0.80±0.04a
Strong	2.20±0.87	0.37±0.22b	0.32±0.17b
Severe	2.75±0.74	0.49±0.18b	0.47±0.19ab
P value	**0.05**	**0.01**	0.07
Location			
Sand marsh	2.40±0.53b	0.44±0.13b	0.43±0.12
Coastal meadow	4.50±0.56ab	1.18±0.10a	0.81±0.04
Farm land	6.67±0.98a	1.42±0.06a	0.78±0.07
P value	**0.01**	**0.00**	0.12
pH			
Acidic	3.50±0.91	0.78±0.25	0.56±0.17
Neutral	3.00±0.62	0.65±0.19	0.58±0.14
Alkaline	4.60±1.22	0.96±0.23	0.60±0.14
P value	0.55	0.69	0.98

Values are provided as mean and standard errors. Lowercase letters and the values highlighted in bold indicate significant difference ($P<0.05$)

Table 4 Comparisons of AMF community composition between host plant or soil groups using unweighted UniFrac significance test

Group pairs	*P* value
Halophyte vs. non-halophyte	0.06
Annual vs. perennial	**0.02**
Invasive vs. native	**<0.001**
Saline grade	**<0.001**
Slight vs. moderate	**0.05**
Slight vs. strong	0.96
Slight vs. severe	**0.05**
Moderate vs. strong	**0.05**
Moderate vs. severe	**0.05**
Strong vs. severe	1.00
Sampling zones	**0.04**
Sand marsh vs. coastal meadow	0.12
Sand marsh vs. farm land	0.24
Coastal meadow vs. farm land	0.21
pH	**0.03**
Acidic vs. neutral	0.33
Acidic vs. alkaline	0.27
Neutral vs. alkaline	**0.05**

The values highlighted in bold are statistically significant ($P<0.05$)

slightly and strongly, or the strongly and severe saline samples ($P>0.05$). AMF community composition showed a distinct pattern according to location ($P=0.04$), which agreed with the plotting of PCoA, in which the two principal coordinate axes explained 84.2 % of the cumulative variance, with the first axe explained 58.5 % of AMF communities (Fig. 4).

Linking diversity and community structure with environmental variables

Correlation analysis of soil properties and diversity estimates showed that a significant relationship only existed between AMF Shannon index and soil salinity ($r=-0.56$, $P<0.05$). RDA was further performed to correlate the community structure data with environmental parameters (Fig. 5). In the RDA biplot, the eigenvalues of axes 1 and 2 were 0.32 and 0.1, respectively. The first two axes explained 59.9 and 18.1 % of the AMF–environment relationship variance, respectively, and all four axes explained 93.7 %. The species–environment correlation of the first axis was 0.88 and was defined by soil salinity, in contrast to 0.85 of the second axis and defined by pH. Soil salt content significantly contributed to AMF distributions ($P<0.05$, Monte Carlo permutation tests). Variation partitioning analyses further supported that soil salt content and pH were more influential than any other variables, explaining 6.89 and 4.24 % of the AMF community variation observed, respectively.

Discussion

To our knowledge, this is the first molecular diversity study of AMF colonizing roots of a range of salt-tolerant plant species in a salinized coastal plain in China. Our study revealed a total of 22 AMF phylotypes in 17 plant species, with a frequency of about 3.6 (62/17) AMF phylotypes per host species (Table 2), which is comparable to that recorded for another coastal zone (2–4, Yamato et al. 2008) or a wetland (3–6, Wang et al. 2011). However, considerably higher numbers of AMF phylotypes per host plant species have been reported for other ecosystems, such as polluted sites (5.2), temperate forests (5.6), tropical forests (18.2), grasslands (8.3), high altitude sites of the Tibet Plateau (10.5), and extreme geothermal soils (16) (Öpik et al. 2006; Liu et al. 2011, Appoloni et al. 2008). Although plants in coastal areas appear to harbour fewer AMF phylotypes than in other ecosystems, care should be taken when interpreting these data because the number of samples, the size of a root, sampling season, or molecular methodologies (rDNA markers, screened/sequenced of clone number, OUT/phylotype definition) can significantly affect the number of root-colonizing AMF detected (Kowalchuk et al. 2002; Heinemeyer and Fitter 2004; Öpik et al. 2008; Dumbrell et al. 2010).

Half of the 22 phylotypes detected in the present study are not well affiliated to known AMF species sequences, and another 9 phylotypes are related to environmental sequences that are not identified to species level (Fig. 2), suggesting that there is a substantially high and novel diversity of AMF taxa in the salinized ecosystem of Laizhou Bay. In agreement with previous studies (van der Heijden et al. 2008; Wang et al. 2011), the present results thus also support the hypothesis that there may be many AMF taxa awaiting discovery, especially in less-studied geographic regions and biomes (Liu et al. 2011).

For the AMF phylotypes obtained in this study, those of cluster 1 dominated in the highly salt-stressed environments. This is not surprising because most phylotypes in cluster 1 are ubiquitous (VT00113, a globally widespread taxon as shown in the Maarj*AM* database, Öpik et al. 2006, 2010). The results of BLAST against GenBank indicate that taxa of the clusters 2 and 3 were mostly found from harsh environments (heavy metal polluted soils, semi-arid, degraded land, and endangered African Pencil Cedar), which suggests that they are less salinity-tolerant, though may be competitive in polluted or moderately stressed environments.

Data from the present study show that *Glomus* are dominant AMF colonizing plants in the south coastal plain of the Laizhou Bay, which is in accordance with previous studies on salt-stressed environments (Wilde et al. 2009; Stukenbrock et al. 2005; Yamato et al. 2008, 2012). The most dominant phylotype LGlo1 shares the highest sequence similarity with morphologically identified species like *G. intraradices*, *G.*

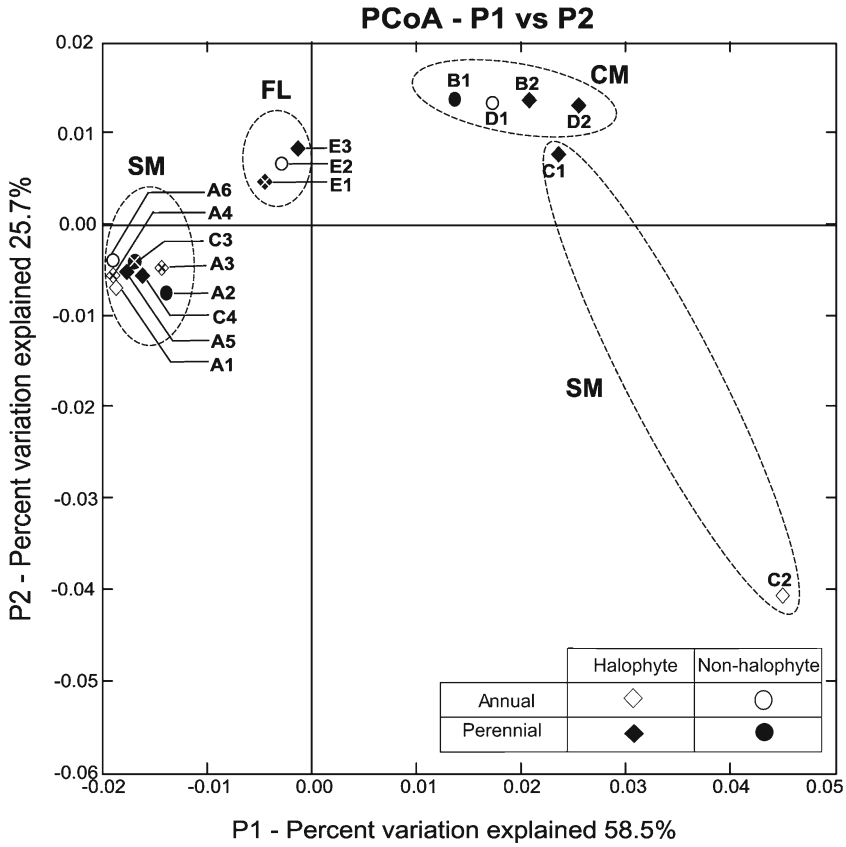

Fig. 4 Principal coordinate analysis of AMF assemblages of 17 samples from halophytic, non-halophytic, perennial, annual, invasive, and native host plant species based on weighted UniFrac distances. The clustering of community structure by locations (SM, CM, and FL) is distinct. The samples from invasive plant species are indicated by a *cross in the symbols*

fasciculatum and *G. irregulare*. This is not surprising because other identified species of this genus were often reported to be dominant in saline soils (Aliasgharzadeh et al. 2001; Carvalho et al. 2001; Landwehr et al. 2002; Grzybowska 2004; Wilde et al. 2009).

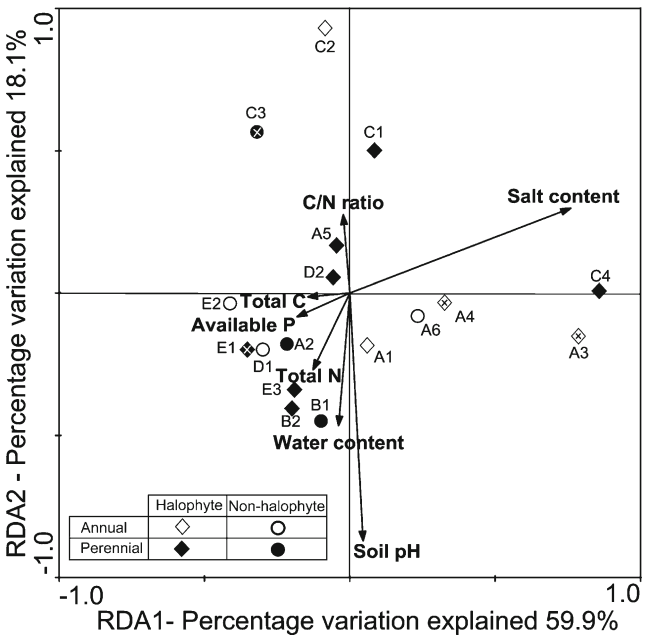

Fig. 5 A biplot of redundancy analysis of AMF 18S rDNA data set. Environmental variables are represented by *arrows*. The samples from invasive plant species are indicated by a *cross in the symbols*. Soil salinity and pH are shown as the most influential abiotic factors

All the AMF phylotypes found in this study belonged to the two groups of the genus *Glomus*. This is in accordance with many previous studies reporting that Glomeraceae is the predominant taxon in the roots of various plants (Li et al. 2010; Hijri et al. 2006; Yamato et al. 2008), including those from wetlands (Wirsel 2004; Wilde et al. 2009). This seems reasonable since *Glomus* spp. have been frequently found to be dominant in salinity soils (Wang et al. 2004) as well as in various other habitats (e.g., Renker et al. 2005; Vallino et al. 2006; Zhao and Zhao 2007; Appoloni et al. 2008; Li et al. 2010; Hassan et al. 2011). The frequent detection of *Glomus* species in roots may be related to the use of primer pairs (e.g., AM1-NS31) that amplify mainly *Glomus*-related sequences (Dumbrell et al. 2010), although it has been suggested that their propagation mechanisms (mycelial fragments and mycorrhizal root fragments) make them more resilient and widespread than other AMF that require spore germination (Helgason and Fitter 2009). No sequences were detected for phylotypes belonging to taxa of other families (e.g., Scutellosporaceae, Acaulosporaceae, and Gigasporaceae). This contrasts with other studies reporting that these taxa have frequently been detected in other saline soils (Camprubí et al. 2010; Wang et al. 2004; Wang et al. 2011; Yamato et al. 2012),

highlighting the need to reveal the less abundant AMF taxa that may escape general clone library-based analysis. For any future study of AMF diversity in saline soils, next-generation sequencing technologies should be employed to reveal the rare phylotypes in these stressed environments, as has been done in several recently conducted studies (e.g., Lin et al. 2012).

Effects of plant traits on AMF diversity and community structure

We compared, for the first time, the root-colonizing AMF communities in halophytes (n=11) and non-halophytes (n=6) in a salinized ecosystem. We found that the plant species of these two ecological categories hosted marginally different Shannon diversity and composition of AMF (P<0.10; Tables 3 and 4), suggesting a rather weak effect of salt tolerance of host plants on root-colonizing AMF diversity. For AMF phylotype richness and Shannon index, their statistical insignificance (α=0.05) may be attributed to the large variations of the phylotype numbers (ranging from 1 to 9) and clone numbers (from 2 to 92) across the 11 halophytic species (Table 2). In fact, variable degrees of AMF colonization in halophytes have long been observed: some species of halophytes can be poorly and occasionally colonized by AMF, whereas others can be strongly AMF positive (Hildebrandt et al. 2001; Sonjak et al. 2009b; Bothe 2012).

In this study, the perennials and annuals, two groups of host species as a whole, hosted similar diversity but significantly different composition of AMF phylotypes (Tables 3 and 4). This finding is in line with two recent studies which both showed the annuals and perennials had distinct AMF community composition in roots (Torrecillas et al. 2012; Alguacil et al. 2012). However, they found a higher (Torrecillas et al. 2012) or lower (Alguacil et al. 2012) diversity of AMF in the annuals than the perennials. These contrasting results could be related to the different numbers of sampled plant species and sampling scale. In these two studies, two or three plant species from close (<50 m) plots were investigated, which could oversimplify the putative heterogeneity of host plant species of the same life style and minimize the environmental gradients, which may play a role in AMF distribution. A recent investigation surveyed 35 plant species from a large altitudinal range (3,320–4,314 m), and found little effect of life style of the host plants on AMF colonization (Lugo et al. 2012), which supports our augment. All these suggest that the perennials and annuals have different AMF colonizers, and effect of plant life style on the AMF diversity could be pronounced locally, but minor when viewed at a large scale or along a great environmental gradient.

The relationship between AMF community and plant invasion has received increasing attention (Pringle et al. 2009). Recent studies have revealed that roots of native species had higher (Jordan et al. 2012) or lower (Lekberg et al. 2013) AMF richness than these of the invaders. By contrast, the composition of AMF colonizers was significantly different between invasive and native hosts, but no effect of plant origin was detected for the AMF diversity in our study, indicating the invaders, as the native plant species, might have also benefited from a substantial number of AMF taxa for thriving in the salt-stressed ecosystem. Among 9 of the 11 novel phylotypes were exclusively detected in native plants (Fig. 3), suggesting the difference of AMF community in both plant types should primarily attribute to natives hosting these specialized colonizers. In addition, eight out of the ten phylotypes in invaders found in this study have already been detected in many previous studies, implying the dominance of widespread generalists in samples of invasive plant species, which agrees with Moora et al. (2011) in that the generalist AMF phylotypes (e.g., LGlo1 and LGlo2) dominated.

Salinity and pH are primary abiotic factors shaping AMF community

Our study indicates that salinity is the primary abiotic factor influencing the distribution pattern of AMF colonizers, which is in coincident with the role of salinity in global pattern of bacterial distribution (Lozupone and Knight 2007). The strong effects of saline grade on the richness and Shannon diversity are congruent to the significant correlation between soil salinity and Shannon index (r=−0.56, P<0.05), supporting that the diversity of AMF colonizing roots decreases with increasing soil salinity, a pattern was also demonstrated by Yamato et al. (2012). This pattern can be well explained since AMF spore infection also decreases with increasing soil salinity (Saint-Etienne et al. 2006). Nevertheless, the salinity effect was not observed when the distribution of AMF spores in soils was investigated (Aliasgharzadeh et al. 2001; Wilde et al. 2009; Sonjak et al. 2009b). This discrepancy can be attributed to the remarkably different composition of AMF taxa in roots and in soil (Hempel et al. 2007). If roots only recruit a fraction of the AMF taxa pool present as spores in soils (Johnson et al. 2003), and the different pattern of AMF in roots and in soils responding to soil salinity are supported, then it can be deduced the host plants and/or the soil salinity play an important role in affecting the colonization of AMF from soils to roots in the salt-stressed ecosystem. In fact, it has been reported that salt stress hinders root-colonization levels and stimulates sporulation (Tressner and Hayes 1971). Furthermore, colonization of AMF on halophytes such as *P. nuttalliana* was limited by edaphic factors including salinity (Johnson-Green et al. 2001).

Although no effects of pH on AMF richness, Shannon diversity, or evenness were detected, pH was found as the

second important factor in shaping the AMF composition in these plant species. This is corroborated by the UniFrac significance tests of samples with different levels of pH in this study. Again, our finding shows similar importance of pH in structuring AMF colonizers as previously demonstrated for the global distribution of soil bacteria (Fierer and Jackson 2006).

In conclusion, this study of AMF in 18 dominant plant species dwelling in the south coastal plain of Laizhou Bay reveals a previously unrecognized diversity of these colonizers in the salt-stressed ecosystem. By grouping the plant species according to their salt tolerance, life style, and origin, we could statistically analyze the effects of these plant traits on the diversity and community composition of AMF in roots. Compared with previous studies, the insignificant effects of plant traits on diversity estimates found in this study could be due to a relatively larger number of host plant species sampled and the great environmental gradients surveyed. Although the relative importance of host plants and abiotic factors on the AMF distribution is not determined in this study, we provide compelling evidence that both host plant traits and abiotic factors determine the composition and distribution of root-colonizing AMF taxa in the salinized ecosystem. By and large, our study implies that the pattern of AMF distribution on a large scale may not so different from what have been known for bacteria in soils, in which salinity and pH are usually the most influential.

Acknowledgments This work was supported by the Main Direction Program of Knowledge Innovation of CAS (grant no. KSCX2-EW-G-12B), the One Hundred Talent Program of CAS, the Natural Science Foundation for Distinguished Young Scholars of Shandong (no. JQ201210), and the Yantai Double Hundred Talent Plan awarded to JG. Thanks are due to Dr. Bin Ma and Dr. Xiaoli Zhang for helps in statistical analysis. The constructive comments of anonymous reviewers are greatly appreciated.

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