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Diversity of AMF associated with *Ammophila arenaria* ssp. *arundinacea* in Portuguese sand dunes

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Abstract Dune vegetation is essential for the formation and preservation of sand dunes and the protection of the coast line. Coastal sand dunes are harsh environments where arbuscular mycorrhizal fungi (AMF) play an important role in promoting plant establishment and growth. We present a study of the diversity of AMF associated with *A. arenaria* ssp. *arundinacea* in two locations of the Portuguese coast under a Mediterranean climate. These two locations were selected to compare a well-preserved dune system from a protected area with a degraded dune system from a public beach. AMF diversity was assessed mainly by cloning and sequencing of a fragment of the ribosomal SSU using the primer NS31 and AM1. Most of the 89 AMF clones obtained from the rhizosphere and roots of *A. arenaria* belonged to the genus *Glomus*, the largest clade within the *Glomeromycota*. Higher AMF diversity was found in the least disturbed site, in which spores of *Scutellospora persica*, *Glomus constrictum* and *Glomus globiferum* were found in the rhizosphere of *A. arenaria*.

Keywords Coast · Marram grass · Mediterranean climate · Molecular diversity · Polymerase chain reaction

Introduction

Vegetation plays a key role in the stabilization and preservation of coastal sand dunes, which are among the most threatened habitats in areas under Mediterranean climate. Nonetheless, the establishment and survival of pioneer plants in sand dunes is hampered by harsh abiotic conditions (low soil fertility, strong wind, sand accretion). Arbuscular mycorrhizal fungi (AMF) are instrumental in promoting plant establishment and growth in these environments by enhancing plant nutrient and water uptake, protecting plants from root herbivores and pathogens and improving soil structure (Allen 1996; Azcón-Aguilar and Barea 1996; de la Peña et al. 2006; Koske 1975; Koske and Polson 1984).

Ammophila arenaria (L.) Link (marram grass) is the most important sand-fixing species in the European coast. This is a perennial, rhizomatous grass that occurs in coastal foredunes of Europe and North Africa (Tutin et al. 1980), and needs regular sand-burial to grow vigorously. Thus, *A. arenaria* is the dominant species at the seaward slope and top of foredunes where sand accretion is intense (Greig-Smith 1961; Huiskes 1979). Two subspecies of *A. arenaria* grow naturally in Europe: *A. arenaria* ssp. *arenaria* in the northern and western coast down to the north coast of the Iberian Peninsula and *A. arenaria* ssp. *arundinacea* in areas under Mediterranean climate (Tutin et al. 1980). Previous studies have shown typical structures of AMF (coils, arbuscules, vesicles and hyphae) inside the roots of *A. arenaria* as well as spores and mycelia in its rhizosphere (Giovannetti and Nicolson 1983; Kowalchuk et al. 2002; Maremmani et al. 2003). The growing evidence of the multifunctionality and importance of AMF diversity for ecosystem functioning have led to a great effort to identify the species that colonize plants in natural systems. Several *Glomus*, *Scutellospora* and *Acaulospora* species have been found associated with *A. arenaria* ssp. *arenaria* in European coastal dunes (Blaszkowski 1994; Blaszkowski and Tadych 1997; Blaszkowski et al. 2001; Kowalchuk et al. 2002). Spore morphology has been used as the main tool

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to identify AMF species but in recent years molecular techniques have also been successfully applied in species description (Kramadibrata et al. 2000; Redecker et al. 2000) and in the study of AMF diversity in natural systems (Douhan et al. 2005; Ferrol et al. 2004; Husband et al. 2002; Kowalchuk et al. 2002; Öpik et al. 2003; Rosendahl and Stukenbrock 2004; van Tuinen et al. 1998; Vandenkoornhuyse et al. 2003). An analysis based solely on spore counting and identification is problematic because sporulation depends on the biology of particular AMF species and their interaction with environmental conditions. Also, an accurate identification of spores extracted from field samples may be hampered because spores may be unrecognisable due to degradation. One of the main advantages of molecular techniques is the possibility of direct analysis of AMF *in planta*. Molecular techniques have also shown an unexpected high genetic diversity of AMF, although the true extent of variability within species or individuals remains obscure (Sanders 2004).

About 75% of the Mediterranean coastal sand dunes have been destroyed or severely degraded in the last 100 years as a consequence of increased human pressure (Carter 1988). When restoration programs have been implemented, they have invariably used *A. arenaria* to create and stabilize the primary dunes. Although AMF are assumed to be important in the success of restoration projects in coastal dunes, little is known about the diversity of these organisms associated with *A. arenaria* in the Mediterranean coast (Giovannetti 1985; Giovannetti and Nicolson 1983). We present a study of the diversity of AMF associated with *A. arenaria* ssp. *arundinacea* in two Portuguese coastal locations under Mediterranean climatic conditions. These two locations were selected to compare a well-preserved dune system from a protected area with a degraded dune system from a public beach.

The diversity of AMF *in planta* was studied with nested-PCR and sequencing targeting the 18S-rRNA. Additional sequences were obtained from superficially healthy spores extracted both from field samples and pot cultures. Spores extracted from field samples and pot cultures were identified to species where possible.

Materials and methods

Sampling

Monospecific stands of *Ammophila arenaria* (L.) Link ssp. *arundinacea* were selected in the foredunes from two Portuguese coastal locations, the Natural Reserve of São Jacinto (N40°41′ W08°44′) and Comporta (N38°23′, W08° 48′). The dune system in the Natural Reserve of São Jacinto is well-preserved with a healthy vigorous population of *A. arenaria* dominant in the foredunes. Comporta is a public beach where the vegetation of the foredunes is heavily disturbed. Plants of *A. arenaria* in this site are fewer and smaller than in São Jacinto.

Roots and soil from the rhizosphere were collected from four different foredunes separated by 50 m following a line parallel to the coastline in both locations. Samples were collected in July 2003, November 2003 and April 2004 from individual plants. In each sampling, the dune was excavated to expose the roots of *A. arenaria* and 500 g of soil and roots were collected from that point.

Extraction and identification of spores

The rhizosphere soil collected in July and November 2003 was used to set up trap cultures using *Zea mays* (L.) as host plant. After 3 months, pots were checked for spore production and spores with different morphology were separated to attempt establishment of single-species cultures with one to five spores of the same morphology. Pure cultures were harvested and checked for mycorrhizal colonisation and spore production 3 months after the establishment. Only three cultures were successful, one started with spores originally from São Jacinto and the other two from Comporta. Healthy spores from each culture were selected under a dissecting microscope to be used for DNA extraction. Permanent slides were also mounted with PVLG (polyvinyl alcohol, lactic acid, glycerine) and PVLG + Melzer's reagent (1:1). Dr. Chris Walker examined the spores for species determination using both fresh spores from the trap cultures and the collection of permanent slides.

DNA extraction from spores and roots

Individual spores that were apparently healthy when examined through a dissecting microscope were selected from field samples and trap cultures, sonicated and rinsed with autoclaved water three times, washed with 2% Chloramin-T for 15 min and rinsed again with autoclaved water. A single spore was crushed in 40 µl of TE buffer 10 mM (pH 8.0) in a 1.5-ml tube using a micropestle. Afterwards, 10 µl of Chelex 20% was added to the solution, tubes were incubated for 10 min at 95°C and then left to cool down on ice. Finally, the samples were centrifuged at 10,000 ×*g* for 1 min and the supernatant, containing the DNA, was transferred to a new tube (Kowalchuk et al. 2002).

Roots collected from *A. arenaria* were stained with ink (Blue Quink, Parker) (Walker 2005). Roots were cleared in 2.5% (w/v) KOH for 1 h at 90°C, rinsed with tap water and immersed in 1% (v/v) HCl overnight. The following day, roots were stained with 1% (v/v) ink in 1% HCl for 30 min

at 60°C. AMF colonization was examined under a compound microscope. Root colonization was around 40% in all samples. DNA was extracted from 1-cm root fragments using the Qiagen DNAeasy Plant Minikit (Qiagen). All DNA samples were stored at −20°C.

DNA amplification

A nested-PCR was used to selectively amplify fungal DNA from the extracts. The first PCR used the forward primer NS1 in combination with the reverse primer ITS4, covering the region from the beginning of the 18S rRNA gene through the 5′ end of the 25S rRNA gene (De Souza et al. 2004; White et al. 1990). PCRs were performed in a final volume of 20 µl using 200 µM of each dNTP (Amersham), 1.5 M $MgCl_2$, 0.4 µM of each primer, and 1 U of the Taq DNApolymerase (Amersham Pharmacia). 5 µl were used from the DNA extracts of spores, and 1 µl of a 1:10 dilution of the DNA extracts from the roots. The PCR program was: 94°C for 4 min; 30 cycles of (94°C, 30 s; 55°C, 40 s; 68°C 2 min+5 s per cycle); and 68°C for 7 min. All reactions were carried out in a GeneAmp PCR 9700 (Perkin Elmer). The products of this first PCR were diluted (250 µl of sterile water were added when the PCR product was visible in an agarose gel and 150 µl to those reactions without product) and used in the second PCR with the primers NS31 (Simon et al. 1992) and AM1 (Helgason et al. 1998) targeted at the region V3–V4 of the 18S rRNA gene and designed to specifically amplified AMF sequences. The reactions were performed in a final volume of 25 µl using 1 µl of DNA template and the same conditions as detailed above with the following program: 94°C for 2 min; 35 cycles of (92°C, 30 s; 61°C, 60 s; 68°C 50 s+1 s per cycle); and 68°C for 5 min. All reactions were carried out in a GeneAmp PCR 9700 (Perkin Elmer). The products from the second PCR were examined by standard 1% (w/v) agarose gel electrophoresis with ethidium bromide staining, to confirm product integrity and estimate yield.

DNA purification, cloning and sequencing

The products from the second PCR were purified using the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) with a final elution volume of 30 µl. Cloning of the purified products was done using the pGEM-T Easy Vector System from Promega (Madison, WI, USA) according to the manufacturer's protocol. Three colonies from each cloning reaction were grown overnight at 37°C with shaking at 200 r.p.m. in 3 ml Luria–Bertani medium supplemented with 100 mg/ml ampicillin. Plasmids were purified using the Rapid Plasmid DNA daily mini-prep kit (Bioron GmbH, Ludwigshafen, Germany) following the manufacturer's protocol. Positive clones were sequenced, only in one direction, using ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit according to manufacturer's instructions (Perkin Elmer, CA, USA).

All sequences were compared to sequences in Internet databases using BLAST to check for similarities with previously described species (Altschul et al. 1997) and were checked for putative chimeric sequences using the online chimera checker program at the Ribosomal Database Project (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). The sequences obtained in this study have been deposited in the GenBank (for accession numbers see Table 1). The closest BLAST hits from described species, the sequences used by Schwarzott et al. (2001), and two outgroup taxa (*Endogone pisiformis* Link (X58724), *Mortierella polycephala* Coem. (X89436)) were acquired from GenBank/EMBL databases and used in the phylogenetic analysis. Sequences were aligned using BioEdit (Hall 1999). Consensus trees were constructed from a 1,000-fold bootstrapped neighbour-joining (NJ) analyses based on Kimura's two-parameter distances (Kimura 1980) using PHYLIP 3.5 (Felsenstein 1993). Trees were visualized with TreeView 1.6.6 (Page 2001).

Results

Spores from *Scutellospora* and *Glomus* species were found in the rhizosphere and inside fine roots collected under plants of *A. arenaria* in São Jacinto and Comporta. The determination of species was done for the three most abundant morphological types found in São Jacinto. These species, extracted from both field samples and trap cultures, were identified as *Scutellospora persica*, *Glomus constrictum* and *Glomus globiferum*. Pure cultures starting from one to five spores from trap cultures were attempted for those species but successful cultures were only obtained for *S. persica*. No species were identified from spores extracted from the field samples from Comporta, but two successful pure cultures were obtained which are currently being examined by Dr. Chris Walker to achieve species identification. One of those cultures contained dark glomoid spores of about 100–150 µm diameter and thick external wall component. The second pure culture from Comporta contained yellow glomoid spores grouped in clusters and sporulating inside moribund roots.

Obtained from the *A. arenaria* roots and spores collected in Comporta and São Jacinto were 89 different fungal sequences (Table 2). A first analysis divided the sequences into two main groups (Fig. 1). BLAST analysis showed that the biggest cluster contained 69 sequences with high homology to members of the *Glomeromycota*. The second group contained 20 sequences that were more similar to organisms in either the *Ascomycota* or the *Basidiomycota*.

Table 1 Correspondence of GenBank accession numbers with the clone names used in the manuscript

Accession # GenBank	Clone	Sample	Site	Fungi
AY882582	SJsp11	Spore	São Jacinto	*Glomeromycota*
AY882583	SJsp21	Spore	São Jacinto	*Glomeromycota*
AY882584	SJsp77	Spore	São Jacinto	*Glomeromycota*
DQ357072	Csp29	Spore	Comporta	*Glomeromycota*
DQ357073	Csp30	Spore	Comporta	*Glomeromycota*
DQ357074	Csp81	Spore	Comporta	*Glomeromycota*
DQ357075	Csp82	Spore	Comporta	*Glomeromycota*
DQ357076	Csp84	Spore	Comporta	*Glomeromycota*
DQ357077	Csp85	Spore	Comporta	*Glomeromycota*
DQ357079	SJsp79	Spore	São Jacinto	*Glomeromycota*
DQ357080	CCons2	Root	Comporta	*Glomeromycota*
DQ357081	C207	Root	Comporta	*Glomeromycota*
DQ357082	C210	Root	Comporta	*Glomeromycota*
DQ357083	C22	Root	Comporta	*Glomeromycota*
DQ357087	C311	Root	Comporta	*Glomeromycota*
DQ357088	C312	Root	Comporta	*Glomeromycota*
DQ357089	C313	Root	Comporta	*Glomeromycota*
DQ357090	C415	Root	Comporta	*Glomeromycota*
DQ357091	C51	Root	Comporta	*Glomeromycota*
DQ357092	C518	Root	Comporta	*Glomeromycota*
DQ357093	C520	Root	Comporta	*Glomeromycota*
DQ357094	C521	Root	Comporta	*Glomeromycota*
DQ357095	C54	Root	Comporta	*Glomeromycota*
DQ357101	Ca21	Root	Comporta	*Glomeromycota*
DQ357102	Ca211	Root	Comporta	*Glomeromycota*
DQ357103	Ca22	Root	Comporta	*Glomeromycota*
DQ357104	Ca25	Root	Comporta	*Glomeromycota*
DQ357105	Ca26	Root	Comporta	*Glomeromycota*
DQ357107	Ca41	Root	Comporta	*Glomeromycota*
DQ357108	Ca410	Root	Comporta	*Glomeromycota*
DQ357109	Ca411	Root	Comporta	*Glomeromycota*
DQ357110	Ca412	Root	Comporta	*Glomeromycota*
DQ357111	Ca413	Root	Comporta	*Glomeromycota*
DQ357112	Ca414	Root	Comporta	*Glomeromycota*
DQ357113	Ca416	Root	Comporta	*Glomeromycota*
DQ357114	Ca417	Root	Comporta	*Glomeromycota*
DQ357115	Ca42	Root	Comporta	*Glomeromycota*
DQ357116	Ca43	Root	Comporta	*Glomeromycota*
DQ357117	Ca45	Root	Comporta	*Glomeromycota*
DQ357118	Ca47	Root	Comporta	*Glomeromycota*
DQ357119	Ca48	Root	Comporta	*Glomeromycota*
DQ357120	Ca49	Root	Comporta	*Glomeromycota*
DQ357121	ConsCa1	Root	Comporta	*Glomeromycota*
DQ357122	C25	Root	Comporta	*Ascomycota*
DQ357123	C27	Root	Comporta	*Ascomycota*
DQ357124	C28	Root	Comporta	*Ascomycota*
DQ357125	C61	Root	Comporta	*Basidiomycota*
DQ357126	C62	Root	Comporta	*Basidiomycota*
DQ357127	C65	Root	Comporta	*Basidiomycota*
DQ357128	C81	Root	Comporta	*Ascomycota*
DQ357129	C83	Root	Comporta	*Ascomycota*
DQ357130	Ca29	Root	Comporta	*Ascomycota*
DQ357131	SJ1	Root	São Jacinto	*Ascomycota*
DQ357132	SJ13	Root	São Jacinto	*Ascomycota*
DQ357133	SJ14	Root	São Jacinto	*Ascomycota*
DQ357134	SJ15	Root	São Jacinto	*Ascomycota*

Table 1 (continued)

Accession # GenBank	Clone	Sample	Site	Fungi
DQ357135	SJ16	Root	São Jacinto	*Ascomycota*
DQ357136	SJ2	Root	São Jacinto	*Ascomycota*
DQ357137	SJ3	Root	São Jacinto	*Ascomycota*
DQ357138	SJ32	Root	São Jacinto	*Ascomycota*
DQ357139	SJ33	Root	São Jacinto	*Ascomycota*
DQ357140	SJ4	Root	São Jacinto	*Ascomycota*
DQ357141	SJcons	Root	São Jacinto	*Ascomycota*
DQ357152	SJ41	Root	São Jacinto	*Glomeromycota*
DQ357153	SJ42	Root	São Jacinto	*Glomeromycota*
DQ357154	SJ43	Root	São Jacinto	*Glomeromycota*
DQ357155	SJ44	Root	São Jacinto	*Glomeromycota*
DQ357156	SJ441	Root	São Jacinto	*Glomeromycota*
DQ357157	SJ442	Root	São Jacinto	*Glomeromycota*
DQ357158	SJ45	Root	São Jacinto	*Glomeromycota*
DQ357159	SJ649	Root	São Jacinto	*Glomeromycota*
DQ357160	SJa11	Root	São Jacinto	*Glomeromycota*
DQ357161	SJa110	Root	São Jacinto	*Glomeromycota*
DQ357162	SJa111	Root	São Jacinto	*Glomeromycota*
DQ357163	SJa112	Root	São Jacinto	*Glomeromycota*
DQ357164	SJa113	Root	São Jacinto	*Glomeromycota*
DQ357165	SJa12	Root	São Jacinto	*Glomeromycota*
DQ357166	SJa15	Root	São Jacinto	*Glomeromycota*
DQ357167	SJa17	Root	São Jacinto	*Glomeromycota*
DQ357168	Sja19	Root	São Jacinto	*Glomeromycota*
DQ357169	SJa33	Root	São Jacinto	*Glomeromycota*
DQ357170	SJa34	Root	São Jacinto	*Glomeromycota*
DQ357171	SJa37	Root	São Jacinto	*Glomeromycota*
DQ357172	SJa39	Root	São Jacinto	*Glomeromycota*
DQ357173	SJacon	Root	São Jacinto	*Glomeromycota*
DQ357174	SJaCons1	Root	São Jacinto	*Glomeromycota*
DQ357175	SJaCons2	Root	São Jacinto	*Glomeromycota*
DQ380231	Sjsp711	Spore	São Jacinto	*Glomeromycota*
DQ380232	SJsp71	Spore	São Jacinto	*Glomeromycota*

Origin of each sequence and fungal group to which they belong (based on BLAST analysis)

Most of the sequences with similarity to members of the phylum *Glomeromycota* fell into the order *Glomerales* (Fig. 2). Only four sequences obtained from field samples from São Jacinto belonged to the order *Diversisporales*. Three of those sequences (SJsp711, SJsp71, SJsp79) had a high similarity with sequences from *D. spurcum* and a species morphologically similar to *G. etunicatum* obtained from GenBank and clustered in the family *Diversisporaceae*. The spores from which these sequences originated were found occupying *A. arenaria* roots (SJsp71) and inside a dead *S. persica* spore (SJsp79, SJsp711). The fourth sequence was obtained from one spore determined to be of *S. persica* (SJsp77) and clustered with other *Scutellospora* sequences obtained from GenBank and within the family *Gigasporaceae*. Sequencing was also done from spores extracted from the single-species cultures of *S. persica* and the two *Glomus* types from Comporta. Two similar sequences were obtained from spores of

Table 2 Number of sequences obtained from *A. arenaria* roots and AMF spores from each sampling site

Site	DNA from	*Glomeromycota* sequences	*Ascomycota* sequences	*Basiodiomycota* sequences
São Jacinto	Root	24	11	0
	Spore	6	0	0
Comporta	Root	33	6	3
	Spore	6	0	0
Total		69	17	3

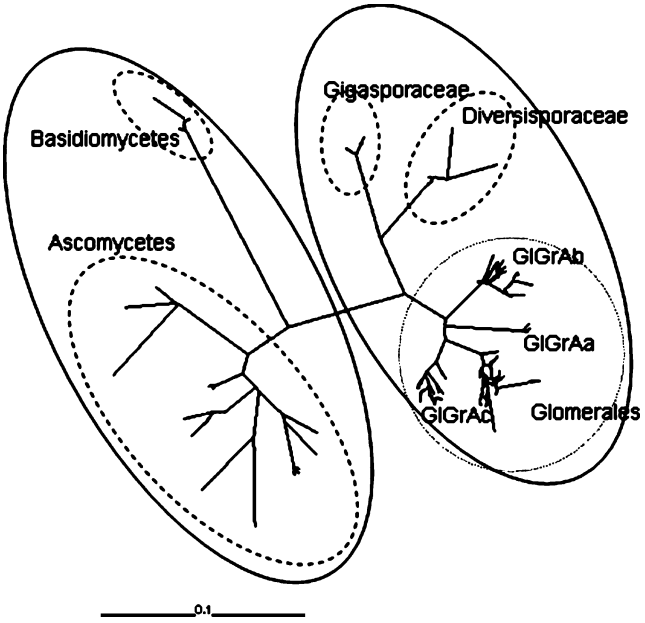


Fig. 1 Unrooted tree based on Kimura's two parameter distances inferred from partial SSU rDNA fungal sequences from the clones obtained from *A. arenaria* roots and AMF spores. Taxonomic classification follows Schwarzott et al. (2001). GlGrAa, Ab and Ac are the subclades defined within the clade GlGrA (*Glomus* Group A). All sequences obtained in this study belonging to the genus *Glomus* clustered into the clade GlGrA

S. persica (SJsp11, SJsp21) and clustered within the *Gigasporaceae*. The three sequences (SJsp77, SJsp11, SJsp21) obtained from spores of *S. persica* were grouped together with high bootstrap values. The sequences obtained from the spores from Comporta (Csp81, Csp82, Csp84, Csp85 for the first morphotype, and Csp29, Csp30 for the second one) clustered in two different groups within the *Glomeraceae* (*Glomus* group A).

The clones of *Glomeromycota* fungi obtained from the root samples were grouped in four clusters within the *Glomeraceae* family. A BLAST search revealed high levels of homology between some of the sequences obtained in this study and *Glomus* sequences obtained in studies from grasslands (Wirsel 2004), sand dunes (Kowalchuk et al. 2002), and with species identified as *G. intraradices*, *G. fasciculatum*, *G. hoi* and *G. constrictum*. Five sequences obtained from root samples clustered with *G. constrictum* (AJ506090), confirming the presence inside *A. arenaria* roots of this species identified from spores. Several sequences obtained from the roots from Comporta clustered with the sequences Csp81–84 from spores, also indicating the presence inside the roots of those AMF species detected in the rhizosphere.

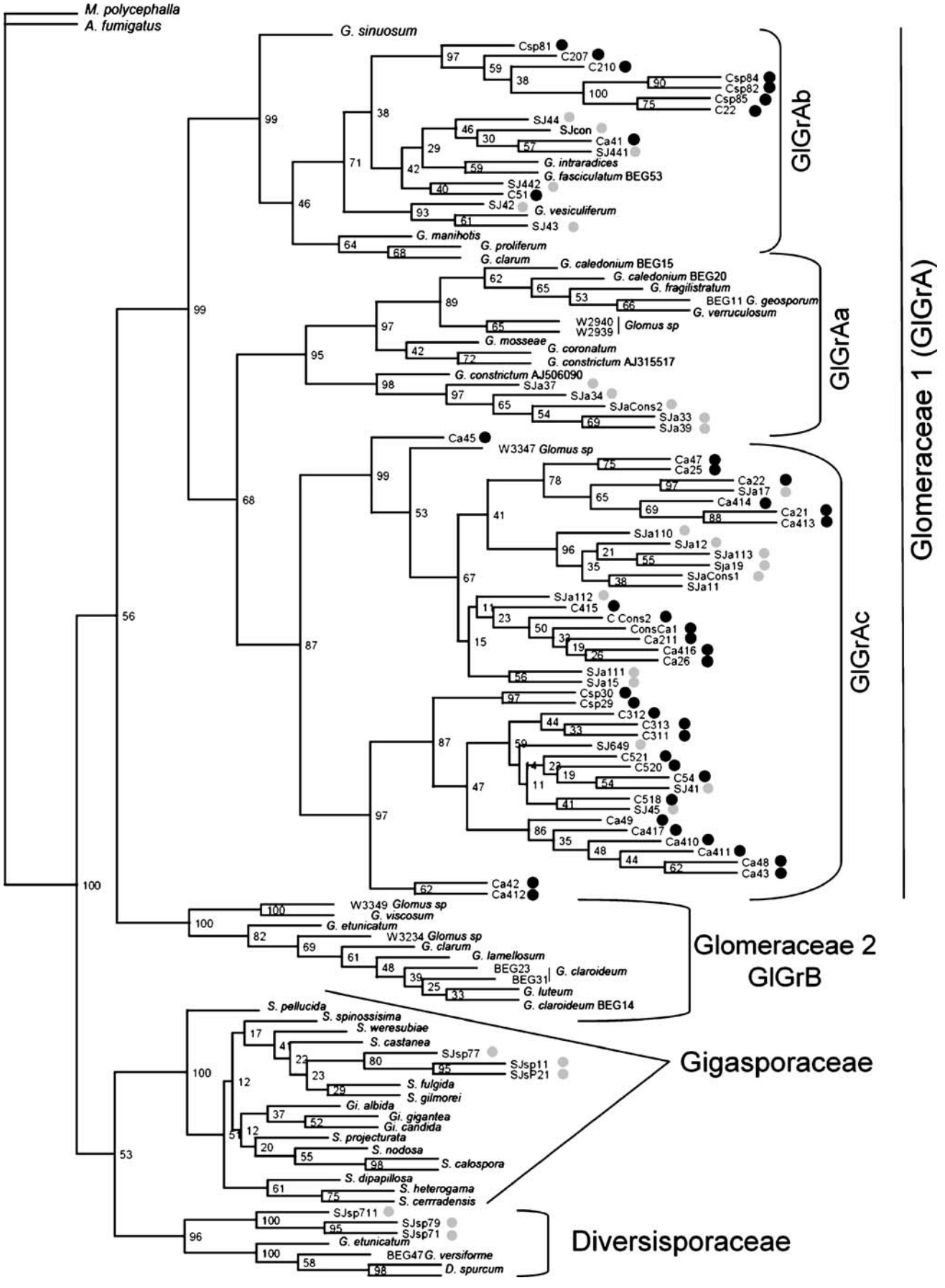
Three non-glomeromycotan sequences from samples from Comporta showed a high similarity with the basidiomycotan yeasts *Dioszegia hungarica* and *Bullera armeniaca* (Fig. 3). The remaining 17 non-glomeromycotan sequences from Comporta and São Jacinto corresponded to ascomycotous fungi. Four clusters were obtained in the phylogenetic analysis using our sequences and those of the closest BLAST hits (Fig. 3). Out of 11 sequences obtained from São Jacinto, 9 displayed a high similarity and clustered together with plant pathogens such as *Fusarium cerealis*, *F. culmorum* and *Verticillum longisporum* and with the nematophagous fungi *Plectosphaerella cucumerina*. The other two sequences from São Jacinto clustered with one sequence from Comporta and the opportunistic human pathogens *Cladophialophora carrionii*, *Phialophora verrucosa* and *Cyphelophora laciniata*. Two sequences from Comporta clustered with *Geomyces pannorum*, a common soil saprophytic fungus that is also an opportunistic human pathogen, and the grass pathogen *Blumera graminis*. The fourth group contained three sequences from Comporta, the cereal pathogens *Microdochium nivale* and *Monographella nivalis*, and, with lower bootstrap values, the saprophytic fungi *Hyponectria buxi* and *Pestalotiopsis* sp.

Discussion

The AM1–NS31 primer pair has been extensively used to selectively amplify the SSU rDNA from AMF (Helgason et al. 1998; Kowalchuk et al. 2002). However, our results agree with recent studies that have found low stringency for the pair AM1–NS31 and amplification of DNA from *Glomeromycota*, *Ascomycota* and *Basidiomycota* fungi (Douhan et al. 2005; Ma et al. 2005). Although AM1 was first defined as specific for AMF, the sequence of the AM1 region is as variable within the *Glomeromycota* members as between those members of the *Ascomycota* and *Basidiomycota* (Douhan et al. 2005). The amplification of DNA from ascomycetes and basidiomycetes shows that care should be taken when using this primer combination for the analysis of AMF communities of field samples, especially if sequencing is not involved in the study.

All the AMF sequences obtained from the roots of *A. arenaria* fit into the genus *Glomus*, the largest within the phylum *Glomeromycota* (Schüßler et al. 2001), and within the group GlGrA (Schwarzott et al. 2001). Only sequences from São Jacinto grouped within the cluster GlGrAa (Schwarzott et al. 2001), together with *G. mosseae*, *G. coronatum*, *G. geosporum* and *G. constrictum*. Five clones from São Jacinto and nine clones from Comporta

Fig. 2 Neighbour joining analysis based on Kimura's two parameter distances inferred from partial SSU rDNA sequences from AMF. The sequences obtained in this study are marked with black (Comporta) and grey (São Jacinto) dots. Taxonomic classification follows Schwarzott et al. (2001). Within the Glomeraceae, only clades GlGrA and GlGrB are represented here. GlGrAa, Ab and Ac are the subclades defined within the clade GlGrA (*Glomus* Group A). Bootstrap values are based on 1,000 replicates, percentage values are shown


M. polycephalla
A. fumigatus
G. sinuosum
Csp81
C207
C210
Csp84
Csp82
Csp85
C22
SJ44
SJcon
Ca41
SJ441
G. intraradices
G. fasciculatum BEG53
SJ442
C51
SJ42
G. vesiculiferum
SJ43
G. manihotis
G. proliferum
G. clarum
G. caledonium BEG15
G. caledonium BEG20
G. fragilistratum
BEG11 G. geosporum
G. verruculosum
W2940
W2939
Glomus sp
G. mosseae
G. coronatum
G. constrictum AJ315517
G. constrictum AJ506090
SJa37
SJa34
SJaCons2
SJa33
SJa39
Ca45
W3347 Glomus sp
Ca47
Ca25
Ca22
SJa17
Ca414
Ca21
Ca413
SJa110
SJa12
SJa113
Sja19
SJaCons1
SJa11
SJa112
C415
C Cons2
ConsCa1
Ca211
Ca416
Ca26
SJa111
SJa15
Csp30
Csp29
C312
C313
C311
SJ649
C521
C520
C54
SJ41
C518
SJ45
Ca49
Ca417
Ca410
Ca411
Ca48
Ca43
Ca42
Ca412
W3349 Glomus sp
G. viscosum
G. etunicatum
W3234 Glomus sp
G. clarum
G. lamellosum
BEG23
BEG31
G. claroideum
G. luteum
G. claroideum BEG14
S. pellucida
S. spinossisima
S. weresubiae
S. castanea
SJsp77
SJsp11
SJsP21
S. fulgida
S. gilmorei
Gi. albida
Gi. gigantea
Gi. candida
S. projecturata
S. nodosa
S. calospora
S. dipapillosa
S. heterogama
S. cerradensis
SJsp711
SJsp79
SJsp71
G. etunicatum
BEG47G. versiforme
D. spurcum
GIGrAb
GIGrAa
GIGrAc
Glomeraceae 1 (GIGrA)
Glomeraceae 2
GIGrB
Gigasporaceae
Diversisporaceae

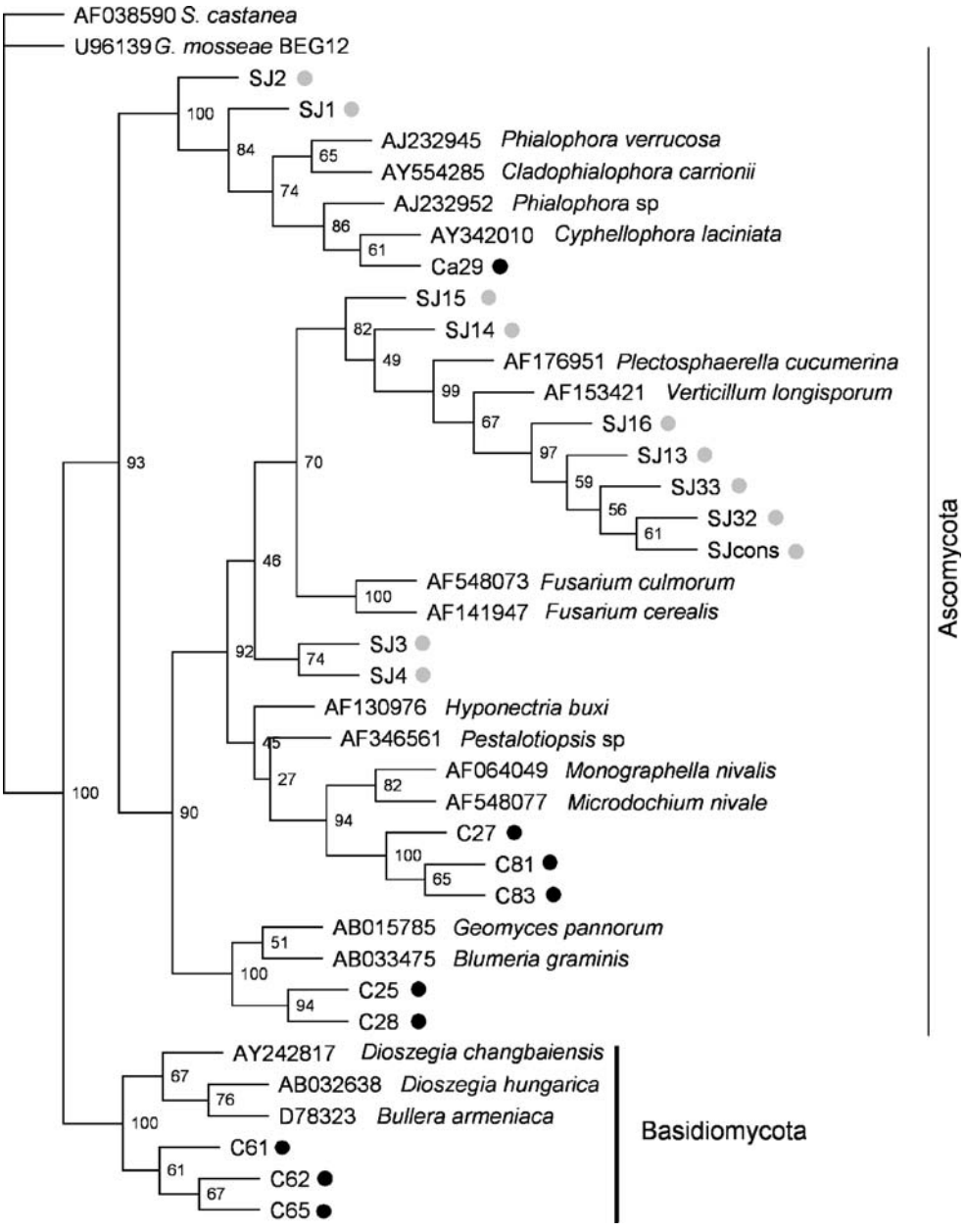


Fig. 3 Neighbour joining analysis based on Kimura's two parameter distances inferred from partial SSU rDNA sequences from the non-AMF sequences found in this study and sequences from *Ascomycota* and *Basidiomycota* members obtained from the GenBank. The sequences obtained in this study are marked with black (Comporta) and grey (São Jacinto) dots. Taxonomic classification follows Schwarzott et al. (2001). Bootstrap values are based on 1,000 replicates, percentage values are shown

(four of them from spores) grouped within the cluster GlGrAb (Schwarzott et al. 2001). Half of the remaining clones clustered with the isolate G W3347, which was an outlier in the phylogenetic analysis of the genus *Glomus* by Schwarzott et al. (2001), and could be part of the proposed group GlGrAc.

As in other molecular studies of AMF *in planta*, most of the sequences obtained in this study did not match previously described AMF species suggesting a higher natural AMF diversity than acknowledged from culture collections. However, the true meaning of this genetic variation is still unknown (Munkvold et al. 2004; Sanders 2004). Nevertheless, the highest diversity of AMF sequences was found in the samples from São Jacinto. Since the vegetation in this site has a much better conservation status, these data would agree with other studies that suggest a positive correlation between AMF diversity and ecosystem functioning (Johnson et al. 2004; Kowalchuk et al. 2002; Newsham et al. 1995).

Cosmopolitan *Glomus* spp were found in *A. arenaria* as revealed by the similarity of some clones and AMF sequences obtained from other plant species and ecosystems (Öpik et al. 2003; Vandenkoornhuyse et al. 2003; Wirsel 2004). Only one clone showed homology with sequences from a study of AMF in *A. arenaria* in The Netherlands (Kowalchuk et al. 2002). Two subspecies of *A. arenaria* grow in Europe, the subspecies *arenaria* grows in the northern coast and ssp. *arundinacea* appears in areas

under a Mediterranean climate. Our data question whether these two subspecies preferentially associate to different AMF species, or whether the differences found in the AMF communities are a consequence of the distance between the sites studied by Kowalchuk et al. (2002) and those studied in this work. Biogeographical variation is likely to happen, as it was also found between the root samples from Comporta and São Jacinto, but genetic specificity in the symbiosis cannot be excluded.

DNA sequencing proved to be useful to find the *Glomus* species identified from spores inside *A. arenaria* roots, a result that cannot be achieved by morphological studies. However, none of the clones obtain from *A. arenaria* roots showed similarity with the sequences from *S. persica* spores, showing that molecular methods can also be biased. This species is commonly associated with *A. arenaria* as it has been previously reported from coastal dunes in Poland (Blaszkowski and Tadych 1997). This AMF species must be colonising *A. arenaria* roots because this plant grows in monocultures and *S. persica* spores were very abundant in the soil samples. However, if the levels of root colonization by *S. persica* are much lower than those of *Glomus* spp. our molecular design might have failed detecting the presence of *S. persica* inside the roots because of the preferential amplification of more abundant sequences during the PCR.

The diversity of non-AMF sequences was higher in the samples from Comporta than in those from São Jacinto. Although the plants in Comporta do not look as healthy as those in São Jacinto, the higher fungal diversity did not result in more plant pathogenic fungi associated with the roots of plants from Comporta. Three of the clones from Comporta represent *Basidiomycota* fungi with a high similarity with the yeasts *Dioszegia hungarica* and *Bullera armenica*. These species are commonly associated with mycorrhizal roots and AMF spores (Renker et al. 2004), but the putative interactions between AMF and yeasts is yet unknown. The remaining non-AMF clones were from ascomycetes closely related to plant and human pathogens or saprophytic fungi. From those *Ascomycota* fungi, only *Fusarium culmorum* and *Microdochium* sp were previously reported from the roots of *A. arenaria* (de Rooij-van der Goes 1995; Kowalchuk et al. 1997).

Basic knowledge of the fungal diversity associated with *A. arenaria* may be useful for the conservation and restoration of coastal sand dunes. This study has detected, using cloning and partial sequencing of the SSU rDNA, fungal species newly associated with the sand dune plant *A. arenaria*. Although the subspecies *arundinacea* occurs in the highly threatened Mediterranean coastal dunes, the information on the AMF diversity associated with this plant is still scarce. Furthermore, to our knowledge, this is the first molecular study of AMF associated with *A. arenaria* spp. *arundinacea*. In short, most of the clones belonged to the genus *Glomus* sensu lato, the larger clade within the *Glomeromycota* fungi. Members of the families *Diversisporaceae* and *Gigasporaceae* were detected in the ecosystem that was better preserved, showing a higher systematic diversity of mutualistic fungi in the healthy dune system than in the disturbed dunes.

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