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# Fungal colonization of the roots of selected halophytes from Sečovlje salterns

## Glivna kolonizacija korenin izbranih halofitov iz Sečoveljskih solin

Silva SONJAK[1], Tamara GLAVINA[2], Metka UDOVIČ[3], Marjana REGVAR[1]*

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia; Tel: +386-1-4233388, Fax: +386-1-2573390; E-mail (*Corresponding author): marjana.regvar@bf.uni-lj.si
[2]Monteko d.o.o. Šmarje, Šmarje 10, SI-6274 Šmarje, Slovenia.
[3]Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia.

**Abstract.** To reveal their mycotrophic status, we have here analysed the fungal colonisation of the roots, and the identity of these fungi, of the most abundant halophytic plant species from Sečovlje salterns (Slovenia): *Aster tripolium*, *Limonium angustifolium* and *Salicornia europaea*. The highest frequency of fungal colonisation was seen for the roots of *A. tripolium*, followed by those of *L. angustifolium* and *S. europaea.* Hyphae and occasional microsclerotia, the assumed structures of dark septate endophytes, were seen in the roots of all three species, whereas arbuscules, as typical structures of arbuscular mycorrhiza, were seen in the roots of *A. tripolium* and in one specimen of *L. angustifolium*. Fungal partial small subunit ribosomal DNA (SSU-rDNA) fragments were amplified from total root DNA extracts for further restriction fragment length polymorphism (RFLP) analyses of the structures of the fungal root communities. Fifteen different RFLP profiles were obtained that grouped into two major clusters. Two RFLP profiles strongly dominated in samples of all three of the plant species. Sequencing revealed that one of these profiles corresponds to the species *Cylindrobasidium laeve* (Basidiomycota), and the second to *Capnobotryella* sp. / *Phaeotheca fissurella*, putative dark septate endophytes from the class Dothideomycetes (Ascomycota). Intraspecific RFLP polymorphisms were demonstrated for both species. To our knowledge, this is the first report on dark septate endophyte fungi occurrence in roots of *A. tripolium*, *L. angustifolium* and *S. europaea*.

**Keywords:** *Aster tripolium*, *Limonium angustifolium*, *Salicornia europaea*, halopytes, mycorrhizal fungi, dark septate endophytes, restriction fragment length polymorphism

**Izvleček.** Analizirali in določili smo glivno kolonizacijo korenin treh najpogostejših halofitskih rastlinskih vrst iz Sečoveljskih solin (Slovenija): *Aster tripolium*, *Limonium angustifolium* in *Salicornia europaea*, da bi ugotovili njihov mikotrofni status. Najvišjo frekvenco glivne kolonizacije smo opazili v koreninah vrste *A. tripolium*, sledili sta vrsti *L. angustifolium* in *S. europaea*. Hife in mikrosklerocije, verjetne strukture temnih septiranih endofitov, smo opazili v koreninah vseh treh rastlinskih vrst, arbuskule, tipične strukture arbuskularne mikorize, pa le v koreninah vrste *A. tripolium* in ene rastline vrste *L. angustifolium*. Iz celokupne koreninske DNA smo pomnožili glivne DNA fragmente dela majhne ribosomske podenote (SSU-rDNA) in v nadaljevanju analizirali polimorfizem dolžin restrikcijskih fragmentov (RFLP) struktur koreninskih glivnih združb. Dobili smo petnajst različnih RFLP profilov, ki so se združevali v dve glavni gruči. V vzorcih vseh treh rastlinskih vrst sta prevladovala dva RFLP profila, sekveniranje pa je pokazalo, da eden ustreza vrsti *Cylindrobasidium laeve* (Basidiomycota), drugi pa *Capnobotriyella/ Pheotheca fissurella*, domnevnemu temnemu septiranemu endofitu iz razreda Dothideomycetes

(Ascomycota). V nadaljevanju smo ugotovili, da pri obeh vrstah obstaja intraspecifični RFLP polimorfizem. Kot nam je znano, je to prvo poročilo o pojavljanju temnih septiranih glivnih endofitov v koreninah vrst *A. tripolium*, *L. angustifolium* in *S. europaea*.

**Ključne besede:** *Aster tripolium*, *Limonium angustifolium*, *Salicornia europaea*, halofiti, mikorizne glive, temni septirani endofiti, polimorfizem dolžin restrikcijskih fragmentov

## Introduction

Salterns represent one of the most extreme of environments, where drought and salinity are the most important abiotic factors that limit plant growth (Ruiz-Lozano 2003). Plants that can establish themselves, grow to maturity and reproduce under these conditions have developed many efficient mechanisms to overcome the osmotic and ionic stresses (Larcher 1995). Halophytes are thus recognized as a diverse group of vascular plants with adaptation at the morphological, anatomical and cellular levels that allow them to avoid these stresses or to increase their tolerance to them (Bray 1997). In addition to such morpho-physiological adaptations, plants can have associated soil microorganisms that alleviate the stress symptoms. Symbiosis with mycorrhizal fungi has been repeatedly demonstrated to enhance the water and mineral nutrient supply for plants and to protect them against diverse biotic and abiotic stresses (Smith & Read 1997). Many beneficial effects of this symbiosis have been described for host plants and for ecosystems, including the enhancement of plant tolerance to drought and salt stress (Smith & Read 1997, Ruiz-Lozano & Azcon 2000). Among these symbiotic microorganisms, the cosmopolitan arbuscular mycorrhizal fungi (AMF) have been extensively studied (Smith & Read 1997, Gupta & al. 2002), with their occurrence also having been confirmed in European saline environments (Hildebrandt & al. 2001, Landwehr & al. 2002). Dark septate endophytes (DSEs), on the other hand, are widespread organisms that can form mutualistic, mycorrhiza-like, associations with their host plants (Jumpponen 2001). They are especially common in stressful environments (Jumpponen & Trappe 1998, Barrow & Aaltonen 2001, Barrow 2003), including those that are extremely arid (Ruotsalainen & al. 2007). Despite this, little is known about their ecology and identity or about the effects that they have on the plants that they inhabit (Jumpponen & Trappe 1998).

Plants growing in high saline soils belong to families that are frequently reported as non-mycotrophic; nevertheless, mycorrhizal colonization has been recorded in plant species from northern (above 51° N) European salt marshes and that belong to the families: *Chenopodiaceae*, *Caryophyllaceae* and *Juncaceae* (Hildebrandt & al. 2001, Landweher & al. 2002). *Aster tripolium* (Asteraceae), *Limonium vulgare* (Plumbaginaceae) and *Salicornia europaea* (Chenopodiaceae) are among the species documented for their maintenance of arbuscular mycorrhizae (Wang & Qiu 2006); however, there have been no reports regarding the mycorrhizal status of *L. angustifolium*, and *Salicornia* spp. are frequently reported to be non-mycorrhizal (Harley & Harley 1987). The aim of the present study was therefore to examine the root fungal colonization of these three halophytic plant species growing in Sečovlje salterns (Slovenia), and to identify their fungal endophytes using the molecular techniques of cloning, RFLP and sequencing.

## Materials and methods

### Site description and sampling

The Sečovlje salterns are situated in south-eastern Piran Bay in the Gulf of Trieste (northern Adriatic Sea) on the sediment of the Dragonja River. The bottom of the pans consists of flysch silt and clay, and on average the water salinity in the Gulf of Trieste reaches 3.7% (Kaligarič 1988). The climate is Mediterranean, with most of the annual precipitation falling in autumn (934 mm) (SORS 2005). The plants were collected in October 2002 and 2003 from the abandoned southern sections of the salterns, at

Fontanigge. The water content of the soil in the sampling area was on average 27%, the organic matter 3.88%, with a pH of 7.9 and soil extract conductivity of 1880 µS/cm (Udovič 2004). We selected two plant species that have been frequently reported as non-mycorrhizal: *L. angustifolium* (Tausch) Degen [*Statice serotina* Rchb., *L. vulgare* Mill. subsp. *serotinum* (Rchb.) Gams] (Plumbaginaceae) and *S. europaea* L., which is considered as an aggregate of closely related species in the literature (Chenopodiaceae), and additionally a moderately mycorrhizal plant *A. tripolium* L. [*Tripolium vulgare* Nees] (Asteraceae) for comparison (Martinčič & al. 2007). Six specimens (three from each year) of each plant species were collected for the analysis of their mycorrhizal colonization, which was estimated according to Trouvelot & al. (1986). Fifteen root fragments per plant were cleaned with tap water, cleared in 10% KOH, and stained with trypan blue (Philips & Heymann 1970). The frequency (F%) and intensity (M%) of their fungal colonisation, along with their arbuscular (A%), vesicular (V%) and microsclerotial (MS%) densities, were determined under light microscopy (Zeiss).

**DNA extraction**

Roots of three specimens of each plant species from the two consecutive years were used for further DNA analysis. The roots were washed thoroughly with tap water and then sterile water, randomly sampled (~150 mg of roots from each plant) and stored either at room temperature (in 2002) or frozen at –20 °C (in 2003) until use. The total DNA was extracted from the roots after they had been ground in liquid nitrogen, using the DNeasy Plant Mini Kit (Qiagen), following the manufacturer recommendations. After isolation, 10 µl of 20% (w/v) Chelex 100 was added to 50 µl of DNA eluents (to scavenge multivalent metal ions that could inhibit the PCR amplification), and after an incubation for 1 min, the samples were centrifuged and the supernatants used for the PCR reactions.

**Nested PCR amplification**

Amplifications of the partial fungal small subunit ribosomal DNAs (SSU-rDNAs) were carried out in a final volume of 50 µl, using 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U *Taq* DNA polymerase (all from Fermentas), 0.4 µM primers and 2 µl DNA extract or PCR product. In the first PCR reaction, a 1330 bp SSU-rDNA fragment was amplified using a crude DNA extract and the MH2 and MH4 primers (Vandenkoornhuyse & Leyval 1998), with the following cycle conditions: 95 °C for 2 min, followed by 33 cycles at 94 °C for 1 min, 48 °C (–0.1 °C per cycle) for 1.5 min, 72 °C for 2 min, and a final extension at 72 °C for 8 min. The second, nested PCR was then performed on 1 µl of the MH2/MH4 PCR product. The universal eukaryotic primer NS31 (Simon & al. 1992) and an AMF-specific primer AM1 (Helgason & al. 1998) were used, with the latter designed to amplify a 550 bp fragment of SSU-rDNA from Glomerales from colonized roots. The PCR cycle conditions were as follows: 95 °C for 2 min, followed by 30 cycles at 95 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 8 min. All of the PCR amplifications were performed with a Thermal Cycler (Biozym). To check the size and quality of the PCR products, they were visualized under UV light after electrophoresis with 1% (w/v) agarose gels and staining with 0.5 mg/l ethidium bromide (Biorad).

**Cloning, RFLP, sequencing and sequence analysis**

Single PCR bands of ~550 bp were excised from the agarose gels, purified with the Wizard® SV Gel and PCR Clean-up System (Promega), and cloned using the pGEM-T Easy Vector Systems II with JM109 competent cells (Promega), following the manufacturer protocols. The transformants were selected using blue/ white screening on LB agar containing X-Gal, isopropyl-1-thio-β-D-galactopyranoside (IPTG) and ampicilin (Sigma). For the RFLP analysis, up to 20 positive clones of each of the SSU-rRNA gene libraries were randomly selected and re-amplified (333 in all; by colony

PCR) with the NS31 and AM1 primers, as described above. Re-amplified fragments were digested with the *Hph*I, *Hinf*I and *Mbo*I restriction enzymes (Fermentas), according to the manufacturer recommendations, and analysed by 3% agarose gel electrophoresis. The restriction enzymes were selected through an analysis of several SSU sequences from GenBank, using the Webcutter 2.0 (Heiman 1997) and NebCutter 2.0 (Vincze & al. 2003) programmes, and according to the literature (Helgason & al. 2002, Vandenkoornhuyse & al. 2002). The DNA restriction fragment patterns were visualized using a UVItec gel imaging and documentation system (UVItec Limited), and they were analysed using ImageQuant TL (Molecular Dynamics). Each restriction fragment was treated as a unit character and scored for its presence or absence. The dendrogram was constructed using the Jaccard distance equation and simple average clustering. Calculations were carried out with the Biodiversity pro application programme (The Natural History Museum, London). Representatives of most frequent RFLP patterns were selected for sequencing. Therefore, the plasmids were isolated using the Wizard® Plus Minipreps DNA Purification System (Promega). The sequencing reactions were performed using the SP6 or T7 primers and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer instructions, and the sequencing was carried out using an ABI prism 377 (Perkin-Elmer Corporation), as provided by the Omega company (Omega d.o.o, Slovenia). The sequences obtained were compared to the available sequences of the National Center for Biotechnology Information (NCBI) using the BLAST-n programme (Altschul & al. 1990, 1997). The sequences were aligned using CLUSTAL W (Thompson & al. 1994), and for the phylogenetic analysis, the neighbor-joining method (Saitou & Nei 1987) was used. Data were first analyzed using a Kimura 2-parameter model (Kimura 1980), which was than used to construct the neighbor-joining tree with MEGA3 software (Kumar & al. 2004). To determine the support for each clade, bootstrap analysis was performed with 10,000 replications. The sequences generated through this study have been deposited with GenBank, and their Accession numbers are given in Table 1.

## Results

### Fungal colonization of the roots

Fungi were seen to be present in the roots of all three of the selected halophytic plant species (Fig. 1). The highest frequency of colonisation was seen for the root fragments of *A. tripolium*, following by *L. angustifolium* and *S. europaea* (Fig. 1; F%). The roots of *A. tripolium* were clearly colonized by AMF, since both vesicles and arbuscules were seen. For the roots of *L. angustifolium*, there were vesicles, whereas arbuscules were only seen in one specimen (Fig. 1; A%). Hyphae and extremely rare vesicles were present in the roots of *S. europaea*, but no arbuscules were seen. There were also melanized hyphae and microsclerotia of DSE fungi in the roots of all three of these halophytic plant species; however, microsclerotia were only seen in one specimen of each species (Fig. 1; MS%).

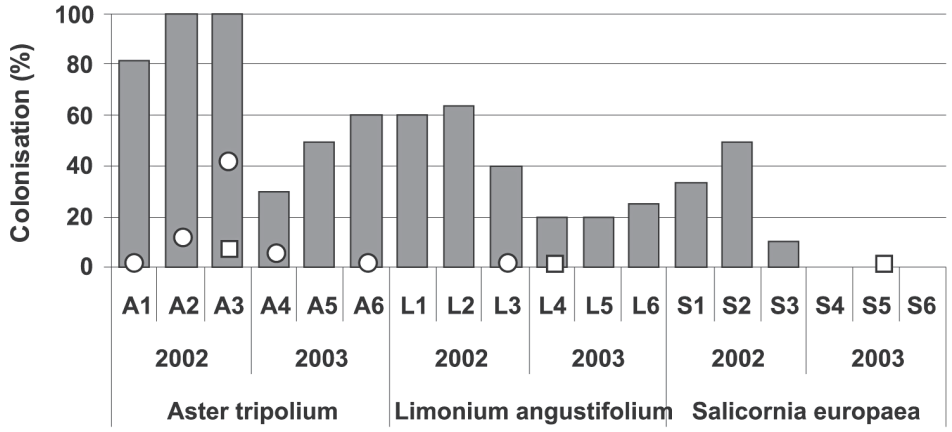


Figure 1: Fungal colonisation in the roots of the selected halophytic plant species *Aster tripolium*, *Limonium angustifolium* and *Salicornia europaea*, sampled in two consecutive years (2002, 2003). ■, frequency of colonisation (F%); ○, density of arbuscules (A%); □, density of microsclerocia (MS%).

Slika 1: Glivna kolonizacija korenin izbranih halofitskih rastlinskih vrst *Aster tripolium*, *Limonium angustifolium* in *Salicornia europaea*, vzorčenih v dveh zaporednih letih (2002, 2003). ■, frekvenca kolonizacije (F%); ○, gostota arbuskulov (A%); □, gostota mikrosklerocijev (MS%).

**RFLP and sequence identification**

Only after the second (nested) PCR were adequate and equivalent amounts of the amplified partial SSU-rDNAs obtained for all 18 of the samples. These samples were cloned, and up to 20 clones from each were subjected to RFLP analysis. Fifteen different RFLP profiles were obtained with the *Hph*I (6) and *Hinf*I (5) restriction enzymes. Since no additional profiles were obtained with *Mbo*I, these results were not included in the subsequent analyses. Profiles 1 and 12 dominated strongly and were obtained for each of the 18 samples, with the exception of profile 1 in sample A4 (Fig. 2a). For each plant species, ~80% of all of the clones analysed resulted in these two profiles (Fig. 2b), regardless of the year of sampling (Fig. 2c), whereas all of the other profiles taken together represented the remaining 20% (Fig. 2b).

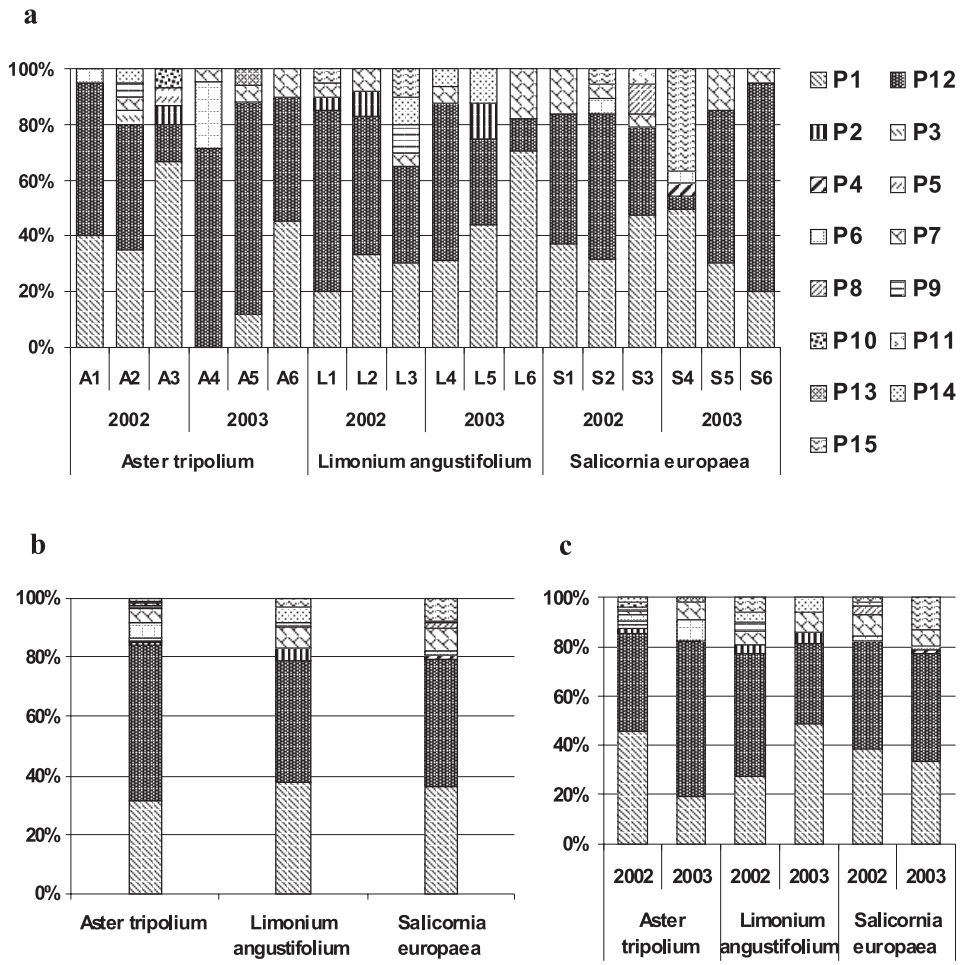


Figure 2: Percentages for the different RFLP profiles obtained: a) for each plant specimen; b) as means for each species: *Aster tripolium*, *Limonium angustifolium* and *Salicornia europaea*; and c) as means for each species within each single year (2002, 2003).

Slika 2: Procenti posameznih RFLP profilov pridobljenih: a) za vsako rastlino; b) kot povprečje za vsako rastlinsko vrsto: *Aster tripolium, Limonium angustifolium* in *Salicornia europaea*; in c) kot povprečje za vsako vrsto v enem letu (2002, 2003).

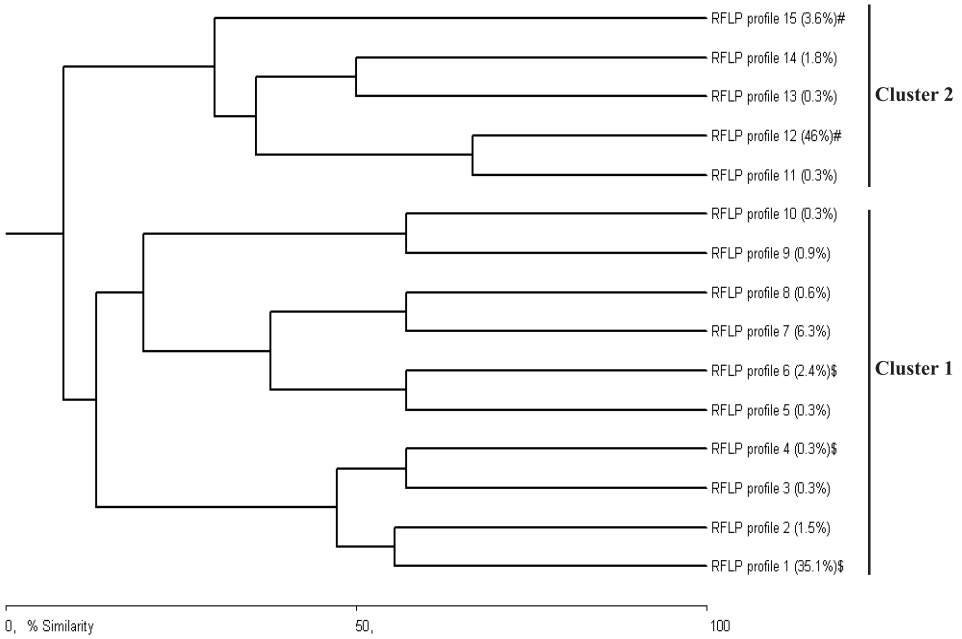


Figure 3: Dendrogram generated from the RFLP data. Altogether, 333 clones with fungal SSU-rDNA fragments were obtained from 18 root samples of the halophytic plant species analysed: *Aster tripolium*, *Limonium angustifolium* and *Salicornia europaea*. The percentages of each of the RFLP profiles obtained are given in parenthesis.
$, sequences of the representative clones that showed 99% similarity with *Cylindrobasidium laeve*; #, sequences of the representative clones that showed up to 99% similarity with species from the class Dothideomycetes.

Slika 3: Dendrogram narejen na osnovi RFLP podatkov. V celoti je bilo pridobljenih 333 klonov z glivnimi SSU-rDNA fragmenti iz 18 koreninskih vzorcev analiziranih halofitskih rastlinskih vrst: *Aster tripolium*, *Limonium angustifolium* in *Salicornia europaea*. V oklepajih je podan delež, ki ga predstavlja vsak posamezen RFLP profil.
$, sekvence reprezentativnih klonov so kazale 99 % podobnosti z vrsto *Cylindrobasidium laeve*; #, sekvence reprezentativnih klonov so kazale do 99 % podobnosti z vrsto iz razreda Dothideomycetes.

In the dendrogram constructed from the RFLP data, profiles 1–10 grouped into the first main clusters, and profiles 11–15 into the second (Fig. 3). Representative clones of the dominant profiles 1 (one clone of *A. tripolium*: A2-16 and three clones of *S. europaea*: S1-1, S4-24, S4-30) and 12 (S4-26) were selected for sequencing. Additionally, clones were selected for sequencing from some of the less frequent profiles: 4 (S4-12), 6 (A1-20, S4-31) and 15 (S4-29). Furthermore, a PCR product was obtained by single amplification with the NS31-AM1 primer pair of sample S4, and this was cloned, with three of these clones selected for sequencing. The sequences of the partial SSU-rDNAs that corresponded to profiles 1, 4 and 6 showed 99% similarity with the sequence of the fungal species *Cylindrobasidium laeve* (Basidiomycota), whereas those corresponding to profiles 12 and 15 showed 98%-99% similarity with the sequences belonging to members of the class Dothideomycetes (Ascomycota) *Capnobotryella* sp. / *Phaeotheca fissurella* (Table 1). The sequences obtained from sample S4 after one NS31-AM1 PCR amplification were shown to be chimeric. Approximately 100 bp of these sequences showed 96% similarity with sequences corresponding to representatives of the phylum Glomeromycota (Schübler & al. 2001) (data not shown).

Table 1: Nearest GenBank matches and percentages of similarity with the sequences obtained. The GenBank accession numbers of the deposited fungal sequences and the number of the corresponding RFLP profile and clone are given.

Tabela 1: Najbližji zadetki iz podatkovne baze GenBank in procenti podobnosti s pridobljenimi sekvencami. Podane so številke dostopanja do glivnih sekvenc deponiranih v bazo GenBank in oznake pripadajočega RFLP profila in klona.

| **Fungal phylum / class** | **RFLP profile/ clone** | **Accession number*** | **Nearest GenBank match** | | **% similarity** |
|---|---|---|---|---|---|
| **Basidiomycota** | 1 / A2-16 | EU189957 | AF518576 | *Cylindrobasidium laeve* | 99 |
| **Agaricomycetes** | 1 / S1-1 | EU189952 | AF518576 | *Cylindrobasidium laeve* | 99 |
| | 1 / S4-24 | EU189953 | AF518576 | *Cylindrobasidium laeve* | 99 |
| | 1 / S4-30 | EU189954 | AF518576 | *Cylindrobasidium laeve* | 99 |
| | 4 / S4-12 | EU189955 | AF518576 | *Cylindrobasidium laeve* | 99 |
| | 6 / A1-20 | EU189951 | AF518576 | *Cylindrobasidium laeve* | 99 |
| | 6 / S4-31 | EU189956 | AF518576 | *Cylindrobasidium laeve* | 99 |
| **Ascomycota** | 12 / S4-26 | EU189958 | AJ972854 | *Capnobotryella* sp. | 98 |
| **Dothideomycetes** | | | Y18697 | *Phaeotheca fissurella* | 98 |
| | 15 / S4-29 | EU189959 | AJ972854 | *Capnobotryella* sp. | 99 |
| | | | Y18697 | *Phaeotheca fissurella* | 99 |

## Discussion

Species from hypersaline environments have frequently been reported as non-mycorrhizal, including *S. europaea* (Chenopodiaceae) (Harley & Harley 1987, Landwehr & al. 2002, Wang & Qiu 2006). The association of plants with mycorrhizal fungi has traditionally relied on morphological characterisation after trypan blue staining, which appears not to be a sensitive enough method in cases of low colonisation levels (Regvar & al. 2003). We saw arbuscules in *A. tripolium* and in one specimen of *L. angustifolium*, but not in *S. europaea*, and this therefore confirms the presence of AMF using this traditional approach only for the first two species here. In addition, although frequently neglected in AMF studies, melanized hyphae and microsclerotia of presumed DSE fungi were also seen in all three species.

Nested PCR had to be performed to obtain enough products for our molecular analyses. The PCR-RFLP technique has been previously applied in molecular identification studies of mycorrhizal fungi to reduce the need for further sequencing (Helgason & al. 2002, Vandenkoornhuyse & al. 2002). The 15 different RFLP profiles that we obtained in the present study grouped into two main clusters (Fig. 3) and were numbered consecutively from 1 to10 in cluster 1 and from 11 to 15 in cluster 2. Profile 1 strongly dominated in cluster 1 and profile 12 dominated in cluster 2. The sequences of the selected clones showed that the RFLP profiles 1, 4 and 6 from cluster 1 represent the species *Cylindrobasidium laeve* (Basidiomycota). RFLP profiles 12 and 15 from cluster 2 correspond to species from the class Dothideomycetes (Ascomycota). As different RFLP profiles were obtained for the same species, this shows intraspecific RFLP polymorphisms of the fungi belonging to both of these clusters, which has previously been seen for some ectomycorrhizal fungi (Horton 2002). Using the blast searching, two closest matches were obtained for the clones of profiles 12 and 15 with the same percentage of similarity, namely *Capnobotryella* sp. and *Phaeotheca fissurella* (Table 1). We were therefore not able to determine the precise taxonomic positions of the sequences obtained even after constructing the phylogenetic tree (Fig. 4).

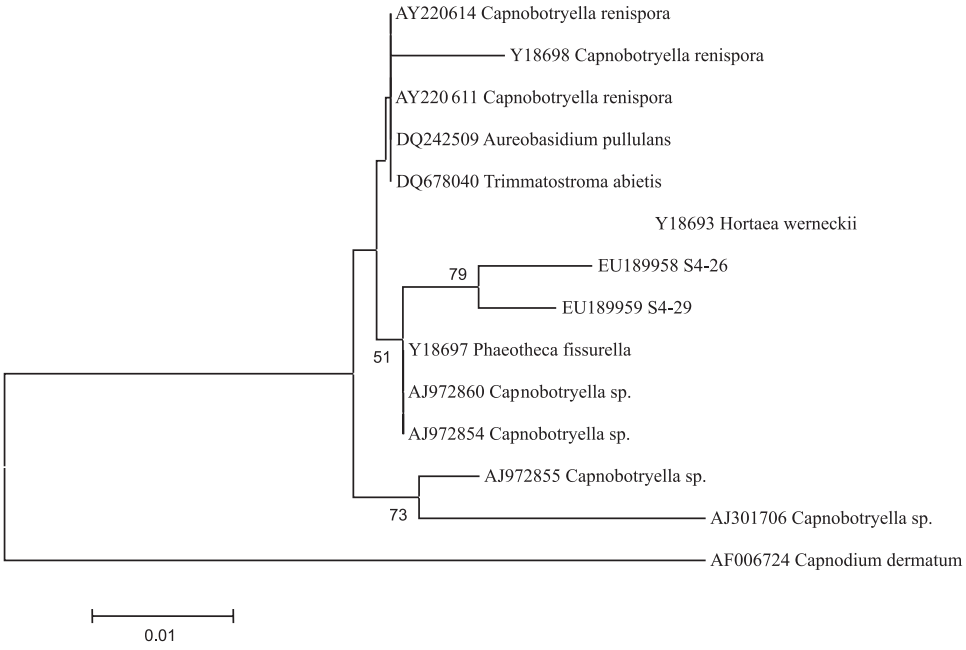


Figure 4: Phylogenetic tree inferred from neighbor-joining analysis of the partial SSU-rRNA gene sequences. The numbers at the nodes represent the bootstrap values of >50% (out of the 10,000 replications). The number of nucleotide changes between the taxa is given by the branch length.

Slika 4: Filogenetsko drevo narejeno na osnovi analize združevanja najbližjega soseda delnih SSU-rRNA genskih sekvenc. Številke ob razvejitvah predstavljajo vrednosti >50 % (od 10000 ponovitev) statistične metode vezenja. Število nukleotidnih sprememb med taksoni je ponazorjeno z dolžino veje.

Except for the chimeric sequences, no other sequences showed similarities with sequences of species from the phylum Glomeromycota. The abundance of arbuscular mycorrrhizal fungal spores found in saline environments has been shown to be rather variable, although with an apparent low species diversity, whereas the degree of root colonisation varies with the individual plant, plant species and vegetation period (Hildebrandt & al. 2001, Landwehr & al. 2002). Although the AM1 primer (Helgason 1998) was designed to amplify species from some of the AMF groups, its broader lack of specificity was only recently reported (Douhan & al. 2005). There are only a few base mismatches in the annealing sequences of the AM1 primer that were also seen in some of the species from the phyla Ascomycota and Basidiomycota (Douhan & al. 2005). The specificity of primers usually depends on the content and quality of the target DNA (Anderson & Cairney 2004), and thus a small amount of AM sequences in the total DNA extract can lead to loss of specificity of the NS31-AM1 primer pair, which therefore amplifies extended non-target DNA (Douhan & al. 2005). Nevertheless, the NS31-AM1 primer pair is still widely used for detection and identification of AMF *in planta* (Santos & al. 2006, Santos-González & al. in press). The specificity of the amplification can also be reduced using the nested PCR reaction (Landwehr & al. 2002). In addition, freezing of the AMF spores drastically reduces the DNA concentration in the PCR products (Landwehr & al. 2002), which may also apply for the samples in the present study, in which the root samples were dried (2002) or frozen (2003) prior to the DNA extraction. Thus, the higher sensitivity of the DNA of the AMF to all of the above-mentioned processes may have contributed to the results.

Clones with RFLP profiles related to species *C. laeve* and *Capnobotryella* sp./ *P. fissurella* were obtained from samples of all three of the selected halophytic plants. *C. laeve* is known as a wooden saprophyte (Phillips 1994); however, it can obviously penetrate into the roots of green plants. The frequent detection of the corresponding RFLP profiles in our analysed root extracts from all three of the species indicates the extensive presence and association with plant roots of this fungus in the section of the Sečovlje salterns that was sampled, where the presence of decayed wooden and plant material supports its growth. *Capnobotryella* sp. and *P. fissurella* are mitosporic fungi that have previously been isolated from plants (Zalar & al. 1999, Hambelton & al. 2003). According to some of their morphological features, production of microsclerotia and thick-walled conidiogenous cells impregnated with numerous melanin-like granules (Zalar & al. 1999, Hambelton & al. 2003) and their relation to the identified endophytic putative DSE fungi from the class Dothideomycetes (Jumpponen & Trappe 1998), they could be described as DSE fungi. Typical structures of DSEs, such as septate melanised hyphae and distinct microsclerotia, were seen in the roots of all three of our sampled halophytes. The desiccation-tolerant, melanin-rich cell wall of DSE fungi is one of their main characteristics that allows them to tolerate unfavourable environments (Jumpponen & Trappe 1998). Furthermore, the intracellular DSE microsclerotia may also provide a mechanism for the fungi to withstand unfavourable environments and/or may serve as dispersal propagules in heavily eroded areas (Ruotsalainen & al. 2007). Extensive root colonisation by DSE fungi of these three selected halophytes that thrive in Sečovlje salterns therefore indicates their functional importance for those species in the environment where the propagules of AMF are clearly reduced and the demands for symbiotic associations are considerable.

Thus, fungi from the phyla Ascomycota and Basidiomycota extensively colonise plants in extremely saline environments; however, the nature of these associations and the significance for their hosts are not easily determined. Finding compatible DSE fungal symbionts of such halophytic plant species may be of importance for the applications of comparable symbiosis in saline agricultural environments.

## Conclusions

- AMF colonisation was demonstrated for *L. angustifolium* and *A. tripolium* on the basis of microscopic examinations, whereas it was not confirmed by molecular identification, apparently due to the combination of reduced colonisation levels, the sensitivity of the DNA of the AMF to drying/freezing, and the lack of specificity of the NS31-AM1 primer pair in the case of insufficiency of target DNA from Glomeromycota.
- Melanized hyphae and microsclerotia of DSE fungi were seen in all three of the species. Sequencing revealed the presence of the putative dark septate fungi *Capnobotryella* sp. / *Phaeotheca fissurella* from the class Dothideomycetes.
- Additionally, sequences related to the saprophyte *Cylindrobasidium laeve* were also obtained from all three of the plant species.
- RFLP analysis showed two dominating profiles out of the 15 profiles identified. However, sequencing revealed that more than one of the RFLP profiles corresponded to the same fungal species, indicating high intraspecific RFLP polymorphism.
- This is to our knowledge the first report on the occurrence of DSE fungi in the roots of *L. angustifolium*, *S. europaea* and *A. tripolium*.

## Povzetek

Soline predstavljajo ekstremno okolje, kjer sta suša in slanost glavna dejavnika, ki omejujeta rast rastlin. V takem okolju lahko uspešno živijo in se razmnožujejo le rastline, ki so odporne na osmotski in ionski stres. Pri tem jim pomagajo tudi mikorizne glive, med katerimi so najbolj proučevane arbuskularno mikorizne (AM) glive. Temne septirane endofitske (DSE) glive so zelo pogoste v stresnih okoljih, vendar je o njih zelo malo znanega. Rastline, ki uspevajo v ekstremno slanih okoljih, pogosto sodijo v družine, ki veljajo za nemikorizne. Halofitske vrste *Aster tripolium* (Asteraceae), *Limonium vulgare* (Plumbaginaceae) in *Salicornia europaea* (Chenopodiaceae) sicer lahko vzdržujejo mikorizo, vendar pa ne poznamo mikoriznega statusa vrste *L. angustifolium*, medtem ko rod *Salicornia* pogosto opisujejo kot nemikorizen.

V predstavljenem delu smo analizirali glivno kolonizacijo korenin omenjenih treh vrst iz Sečoveljskih solin. S klasičnim morfološkim pregledom pobarvanih korenin smo najvišjo frekvenco glivne kolonizacije opazili v koreninah vrste *A. tripolium*, sledili sta vrsti *L. angustifolium* in *S. europaea*. Temne septirane hife in mikrosklerocije, verjetne strukture DSE gliv, smo opazili v koreninah vseh treh rastlinskih vrst, arbuskule, tipične strukture arbuskularne mikorize, pa le v koreninah *A. tripolium* in ene rastline *L. angustifolium*. Z vgnezdeno PCR reakcijo smo z uporabo začetnega nukleotidnega para MH2-MH4 v prvi in NS31-AM1 v drugi PCR reakciji iz celokupne koreninske DNA pomnožili glivne fragmente SSU rDNA, pripravili gensko knjižnico in izvedli RFLP analizo z restrikcijskimi encimi *Hph*I, *Hinf*I in *Mbo*I. Dobili smo petnajst različnih RFLP profilov, ki so se v dendrogramu združevali v dve glavni gruči. V primeru vseh treh rastlinskih vrst sta prevladovala dva profila, sekveniranje pa je pokazalo, da eden ustreza saprofitski vrsti *Cylindrobasidium laeve* (Basidiomycota), drugi pa domnevni DSE vrsti iz razreda Dothideomycetes (Ascomycota), za katero je filogenetska analiza pokazala, da je sorodna vrstama *Capnobotryella* sp. in *Phaeotheca fissurella.* V nadaljevanju smo ugotovili, da pri obeh vrstah obstaja intraspecifični RFLP polimorfizem.

AMF kolonizacije opažene pri vrstah *A. tripolium* in *L. angustifolium* nismo uspeli potrditi z molekularno identifikacijo. Začetni oligonukleotid AM1 so sicer razvili z namenom pomnoževanja AM gliv, vendar pa se je izkazalo, da je predvsem v primeru nezadostne količine tarčne DNA zelo nespecifičen. Poleg tega je DNA AM gliv zelo občutljiva na procese sušenja oziroma zmrzovanja, kar zmanjšuje uspešnost pomnoževanja. Glive iz debel Basidiomycota in Ascomycota očitno predstavljajo zelo razširjene kolonizatorje rastlin v ekstremno slanih okoljih. Velik pomen za rastline v ekstremnih okoljih naj bi imeli temni septirani glivni endofiti. Kot nam je znano, je to prvo poročilo o prisotnosti DSE gliv v koreninah vrst *A. tripolium*, *L. angustifolium* in *S. europaea*.

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