Mycorrhiza (2002) 12:199–211
DOI 10.1007/s00572-002-0172-y

ORIGINAL PAPER

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The arbuscular mycorrhizal fungus *Glomus geosporum* in European saline, sodic and gypsum soils

Received: 7 September 2001 / Accepted: 25 March 2002 / Published online: 6 June 2002
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Abstract Plants of saline and sodic soils of the Hungarian steppe and of gypsum rock in the German Harz mountains, thus soils of high ionic strength and electric conductivity, were examined for their colonization by arbuscular mycorrhizal fungi (AMF). Roots of several plants of the saline and sodic soils such as *Artemisia maritima*, *Aster tripolium* or *Plantago maritima* are strongly colonized and show typical AMF structures (arbuscules, vesicles) whereas others like the members of the Chenopodiaceae, *Salicornia europaea*, *Suaeda maritima* or *Camphorosma annua*, are not. The vegetation of the gypsum rock is totally different, but several plants are also strongly colonized there. The number of spores in samples from the saline and sodic soils examined is rather variable, but high on average, although with an apparent low species diversity. Spore numbers in the soil adjacent to the roots of plants often, but not always, correlate with the degree of AMF colonization of the plants. As in German salt marshes [Hildebrandt et al. (2001)], the dominant AMF in the Hungarian saline and sodic soils is *Glomus geosporum*. All these isolates provided nearly identical restriction fragment length polymorphism (RFLP) patterns of the internal transcribed spacer (ITS) region of spore DNA amplified by polymerase chain reaction (PCR). Cloning and sequencing of several PCR products of the ITS regions indicated that ecotypes of the *G. geosporum*/*Glomus caledonium* clade might exist at the different habitats. A phylogenetic dendrogram constructed from the ITS or 5.8S rDNA sequences was nearly identical to the one published for 18S rDNA data (Schwarzott et al. 2001). It is tempting to speculate that specific ecotypes may be particularly adapted to the peculiar saline or sodic conditions in such soils. They could have an enormous potential in conferring salt resistance to plants.

Keywords Arbuscular mycorrhizal fungi · Halophytes · Restriction fragment length polymorphism analysis · Salt resistance · *Glomus geosporum*

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Introduction

The older reviews (Juniper and Abbot 1993; Peat and Fitter 1993) state that high salinity in soils has adverse effects on the colonization of plants by AMF. However, there are reports from all over the world scattered amongst the literature that plants of salt marshes can be colonized by AMF (Mason 1928; Boullard 1959; Kahn 1974; Hoefnagels et al. 1993; Brown and Bledsoe 1996). Even families which are generally considered as non-mycorrhizal like the Chenopodiaceae *Salicornia europaea* and *Suaeda maritima* have been reported to show significant colonization by AMF under high salt stress (Kim and Weber 1985; Rozema et al. 1986; Van Duin et al. 1989; Sengupta and Chaudhari 1990). In a detailed study, this laboratory (Hildebrandt et al. 2001) screened plants of several salt marshes both of the North and Baltic Sea and of German inland salt habitats for their colonization by AMF. Members of the Asteraceae, *Aster tripolium* and *Artemisia maritima*, the plantains *Plantago maritima* and *P. coronopus* and *Oenanthe lachenalii* of the Apiaceae showed a high degree of mycorrhizal colonization, and low, though distinct, signs of AMF were scored in samples of the grasses *Puccinellia maritima* and *P. distans* and even of *Salicornia europaea* of the Chenopodiaceae, at inland salt marshes, whereas other species like the grass *Spartina anglica*, the Juncaceae *Juncus gerardii*, and the Juncaginaceae, *Triglochin maritimum*, were non-mycorrhizal. The soils of all salt marshes investigated contained spores of AMF in high numbers. Their distribution was patchy and highly vari-

Table 1 Characteristics of the sites investigated. *ND* Not determined

Site no.	1	2	3	4	5	6	7	8	9
Name	Apaj	Apaj	Szabadszállás	Szabadszállás	Sarród	Dinnyés	Nyírölapos Hortobágy	Zám, Hortobágy	Sachsenstein
Description of the site	Meadow, occasionally pastured by sheep	Meadow, extensively pastured by cattle	Border of lake Zabszék	Not farmed, ~500 m from lake Zabszék	Meadow, close to lake Neusiedel	Not farmed, close to lake Velence	Meadow, ~20 km west of Debrecen	Meadow, ~40 km west of Debrecen	Gypsum rock at Bad Sachsa/Harz mountains
Geographic coordinates (N, E)	47°5,2'N 19°5,8'E	47°7,2'N 19°6,9'E	46°50,6'N 19°10,6'E	46°51,2'N 19°12,1'E	47°40,3'N 16°50,4'E	47°10,3'N 18°33,1'E	47°33,6'N 21°18,3'E	47°31,7'N 21°2,4'E	52°04'N 10°55'E
Soil type	Na_2CO_3-Na_2SO_4, Solonetz[a]	Na_2CO_3-Na_2SO_4, Solonetz	Na_2CO_3, Solonchak[a]	Na_2CO_3, Solonchak	Na_2SO_4-NaCl, Solonchak	Na_2SO_4-NaCl, Solonetz	Na_2SO_4-Na_2CO_3, Solonetz	NaCl, Solonetz	$CaSO_4$, pure gypsum
pH value (1:5)	7.9–9.8	9.8–10.4	9.6–10.2	8.4–9.8	8.3–9.2	8.2–10.9	6.8–11.1	5.1–5.8	5.0–8.2
Electrical conductivity of 1:5 diluted suspension (mS/cm)	0.1–8.8	0.6–4.5	0.4–1.0	0.2–0.9	0.9–4.7	0.2–6.7	0.1–2.5	1.1–3.2	0.6–1.9
Sampling dates	August 2000	August 2000	August 2000	August 2000	August 2000	June 2000	April 2000	April 2000	October 2000
Typical plants[b]	*Artemisia maritima*, *Aster tripolium*, *Camphorosma annua*, *Plantago maritima*, *Puccinellia limosa*	*Aster tripolium*, *Plantago maritima*, *Puccinellia limosa*	*Suaeda maritima*, *Puccinellia limosa*, *Plantago maritima*	*Artemisia maritima*, *Aster tripolium*, *Crypsis aculeata*	*Aster tripolium*, *Plantago maritima*, *Salicornia europaea*, *Bupleurum tenuissimum*	*Artemisia maritima*, *Aster tripolium*, *Camphorosma annua*, *Plantago maritima*, *Salicornia europaea*, *Puccinellia limosa*, *Scorzonera parviflora*	*Camphorosma annua*, *Puccinellia limosa*, *Artemisia maritima*	*Suaeda maritima*, *Salicornia europaea*, *Artemisia maritima*	*Gypsophila repens*, *Sesleria varia*, *Cardaminopsis petraea*, *Festuca glauca*, *Hippocrepis comosa*, *Calluna vulgaris*[c]
Equilibrium ion percentages									
Ca	1	ND	0.1	ND	6	0.2	0.1	14	91
Mg	0.3		0.2		20	1	0.1	16	7
Na	99		99		74	99	99	70	0.7
K	0.3		0.2		0.3	0.4	0.2	0.3	0.7
SO_4^-	39		25		67	87	91	4	85
CO_3^-	43		50		4	4	8	0	1
HCO_3^-	10		13		13	0.1	0.2	0.1	8
Cl^-	9		12		16	8	0.4	96	6

[a] For the terms Solonchak and Solonetz see Wendelberger (1950) and Horvat et al. (1974); [b] Plant names are as in Adler et al. (1994); [c] Two *Calluna vulgaris* probes were sampled in June 2001

able from soil sample to sample, but, in sum, statistically higher than in the non-saline vicinity. Molecular biological techniques revealed that 80%, on average, of these spores belonged to one single species, *Glomus geosporum*, which did not – or at best in low amounts – occur in the non-saline habitats examined.

The Hungarian steppe offers the possibility to study adaptations of plants to different alkaline salinities. NaCl, Na_2CO_3 and Na_2SO_4 soils occur, and have long attracted the attention of botanists (Woenig 1899; Stocker 1928; Wendelberger 1950). For comparison, a gypsum ($CaSO_4$) soil in the southern part of the Harz mountains of Germany was included in the present examination. Typical plants of these different salt-affected soils were screened for colonization by AMF, and the AMF spore content was determined in soil samples. This study should also show whether the AMF spores mainly belong to *G. geosporum*, irrespective of the type of salt found in such soils. The usefulness of molecular techniques [restriction fragment length polymorphism (RFLP) analysis] for assessing the occurrence of specific AMF in such soils is also documented in the present communication. Polymerase chain reaction (PCR) products of the internal transcribed spacer (ITS) regions of spores from several locations were sequenced. The new sequences allowed us to construct phylogenetic dendrograms based on the ITS and 5.8S rDNA regions.

Materials and methods

Details of the sites investigated are given in Table 1. Plants, named as in the standard Austrian flora (Adler et al. 1994) and the Hungarian plant illustration (Javorka and Csapody 1979) belonged to the following families: Asteraceae: *Artemisia maritima* L. (sea wormwood), *Aster tripolium* L. (sea aster), *Scorzonera parviflora* Jacq. (small flowering viper's "grass"), Caryophyllaceae: *Spergularia marina* (L.) Griseb.=*S. salina* J. et C. Presl. (lesser sand-spurrey), *Gypsophila repens* L. (creeping gypsophila), Chenopodiaceae: *Salicornia europaea* L. (glasswort, marsh samphire), *Suaeda maritima* (L.) Dum. (annual seablite), *Camphorosma annua* Pall.=*Camphorosma ovata* W. et K. (camphor weed, Hungarian steppe species), Plantaginaceae: *Plantago maritima* L. (sea plantain), Poaceae: *Puccinellia limosa* (Schur) Holmberg (salt marsh grass, Hungarian steppe species), *Festuca pseudovina* Hack. (false sheep's fescue), *Crypsis aculeata* (L.) Ait. (thorngrass, Hungarian steppe species), *Sesleria varia* (Jacq.) Wettst.=*Sesleria caerulea* (L.) Ard. (blue sesleria), *Festuca glauca* Lam.=*Festuca pallens* Host (blue fescue), Fabaceae: *Hippocrepis comosa* L. (horseshoe vetch), Ericaceae: *Calluna vulgaris* (L) Hull (common heather), Brassicaceae: *Cardaminopsis petraea* (L.) Hiitonen (rock cress). The English names are as in Blamey and Grey-Wilson (1989). The fungi quoted were: *G. geosporum* (Nicolson and Gerdemann) Walker, *G. intraradices* Schenck and Smith, *G. mosseae* (Nicol. and Gerd.) Gerdemann and Trappe.

For comparison, this publication contains sequence data of a *G. geosporum* isolate, collected from the soil adhering to the roots of *Aster tripolium* at Secovljske Soline, Bay of Piran, Slovenia, 45°29'318''N, 13°35'807''E (at the immediate border of the Hrvatska Republic) on 27 July 2001.

Analyses

The degree of mycorrhizal colonization of the roots was counted by a slightly modified version of the gridline intersect method (Giovanetti and Mosse 1980) after staining with lactophenol blue exactly as described in the preceding publication (Hildebrandt et al. 2001). For assessing the amount of extraradical hyphae, those fungal structures outside the roots were counted which were distinctly stained after taking the plants out of the soils and rinsing them with tap water. The isolation of AMF spores by differential sieving has also been described (Esch et al. 1994; Hildebrandt et al. 1999). Soil samples for analysing the chemical composition were collected from the surface layer. Electric conductivity in the soil was determined by a WTW LF 537 microprocessor conductivity meter. For this, 2 g soil was suspended in 10 ml double-distilled water and gently stirred to obtain a homogeneous suspension. After the sample stood for 1 h it was stirred again, and the conductivity was determined at room temperature.

For the characterization of the DNA by PCR, the spores were mechanically crushed on a microscope slide and transferred to 0.5-ml microtubes. The method of White et al. (1990) was used to amplify the DNA region between the end of the 18S rRNA, ITSI, 5.8S rRNA, ITSII and the beginning of the 28S rRNA using the primers ITS1 and ITS4 or ITS5 and ITS4. For nested PCR, the primers R1 of the 18S rDNA with the sequence GGG ATT CTC AACC CTC CAGT GAT and R2 of the 28S rRNA with GAA ACT TCA TCG TGC TGG GGA were first employed; the PCR products obtained were diluted up to 1,000-fold and then used for the second PCR with ITS5 and ITS4 or with ITS1 and ITS4. The PCR products were separated by electrophoresis on an 1% (v/v) agarose gel (GibcoBRL ultrapure) followed by staining the DNA with ethidium bromide and photographing. Another aliquot of the PCR products was restricted by *Alu*I, *Hin*fI, *Hpa*II, *Hae*III, *Bsu*RI and *Taq*I (all from MBI Fermentas). Digests were separated on 2% agarose gels, and the DNA was stained with ethidium bromide for photographing.

For sequencing, the PCR products were cloned into the vector pGEM-T Easy following the standard protocol of the manufacturer (Promega, Madison, Wis.) and transformed into competent *Escherichia coli* XL1-Blue by the heat-shock method. Sequencing was performed on an ABI sequencer using the ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer, Foster City, USA). Sequence data were compared with the NCBI databank using the BLAST program (Altschul et al. 1997). Alignment of DNA sequences was done with the ClustalX program (Thompson 1997), and phylograms were edited with TreeView (Page 1996). Sequences of the PCR products were always analysed without the primers. Phylograms were constructed by the neighbour joining method with 1,000 replicate trees (Saitou and Nei 1987).

The sequences were deposited in the EMBL-Genbank database with the accession nos. AF413088–AF413093 in the order: Hungary 1a (related to the sequence from *G. geosporum*), Hungary 2 (*G. mosseae*), Bad Sachsa 1 (*Glomus* sp.), Bad Sachsa 2 (*G.* sp.), Hungary 3 (ascomycete), and Hungary 1b (*Cladosporum* sp.). Slovenia 1 (*G. geosporum*) was deposited as AF479651.

Results

At all eight Hungarian locations, typical plant species were distinctly AMF colonized (Table 2). The Asteraceae, *Artemisia maritima* and *Aster tripolium*, and the Plantaginaceae, *Plantago maritima*, showed a high degree of mycorrhizal colonization in all samples counted. Characteristic arbuscules in addition to intraradical hyphae and vesicles were detected. Among the grasses, *Festuca pseudovina* was highly colonized at Apaj (location 1). However, since the taxonomic affiliation of the *Festuca ovina/pseudovina* agg. is not finally resolved according to standard floras, this grass was not examined at the other locations. The other grass, *Puccinellia limosa*, showed a highly variable degree of colonization,

Table 2 Degree of mycorrhizal colonization in Hungarian salt marshes. Average values and SDs are given for the counts. High scores are given in *italics*

Plant	Samples counted	Total colonization (%)	Arbuscules (%)	Vesicles %	Intraradical mycelium (%)	Extraradical mycelium (%)
Location 1, Apaj, meadow, Na_2CO_3						
Artemisia maritima	6	*51±26*	16±10	28±18	46	15
Aster tripolium	2	*62±16*	40±20	45±19	59	14
Camphorosma annua	3	0±0	0±0	0±0	0	0
Festuca pseudovina	2	*82±0*	20±0	47±9	81	21
Plantago maritima	4	*54±29*	10±7	27±18	50	21
Puccinellia limosa	3	40±6	14±9	9±3	31	16
Location 2, Apaj, meadow, Na_2CO_3						
Aster tripolium	2	47±6	11±7	30±4	45	5
Plantago maritima	2	*54±7*	6±6	34±18	45	25
Puccinellia limosa	3	0±0	0±0	0±0	0	0
Location 3, Szabadszállás, border of the lake, Na_2CO_3						
Plantago maritima	1	13	0	4	9	5
Puccinellia limosa	1	3	0	0	1	3
Suaeda maritima	1	1	0	0	0	1
Location 4, Szabadszállás, around 500 m distant from the lake, Na_2CO_3						
Artemisia maritima	2	*64±2*	19±5	1±0	63	18
Aster tripolium	2	*60±30*	17±8	1±1	60	34
Crypsis aculeata	2	8±6	0±0	6±5	5	1
Location 5, Sarród, Na_2CO_3						
Aster tripolium	3	*53±9*	15±6	28±16	50	16
Plantago maritima	3	*50±26*	29±34	19±9	47	4
Salicornia europaea	3	2±2	0±0	0±0	1	1
Location 6, Dinnyés, NaCl						
Artemisia maritima	1	18	3	2	18	8
Aster tripolium	4	*59±18*	9±7	40±22	57	23
Camphorosma annua	1	4	0	0	4	1
Plantago maritima	1	2	0	0	2	0
Puccinellia limosa	1	2	0	0	2	0
Salicornia europaea	3	2±2	0±0	0±0	2	0
Spergularia salina	1	0	0	0	0	0
Location 7, Hortobágy, Na_2SO_4						
Puccinellia limosa	2	18±6	1±1	8±1	16	7
Location 8, Hortobágy, NaCl						
Salicornia europaea	1	0	0	0	0	0
Suaeda maritima	1	0	0	0	0	0
Location 9, Sachsenstein/Bad Sachsa, Harz mountains, $CaSO_4$ soil						
Calluna vulgaris	1	0	0	0	0	0
Gypsophila repens	4	*41±25*	2±3	22±23	41	13
Hippocrepis comosa	4	16±8	0±0	12±7	15	4
Sesleria varia	4	36±19	1±1	14±14	36	13
Festuca glauca	7	10±3	0±0	5±3	9	4

depending on the plant and site examined, as observed also with the related species *Puccinellia distans* and *P. maritima* in the German coastal and inland salt marshes (Hildebrandt et al. 2001). The Chenopodiaceae, *Salicornia europaea*, *Suaeda maritima* and *Camphorosma annua*, as well as the Caryophyllaceae, *Spergularia salina*, were not colonized, as counts $<3\%$ were not accompanied by positive data for arbuscules and vesicles and are therefore not significant.

The gypsum rock at Bad Sachsa (Table 2, location 9) carries a totally different vegetation. The Fabaceae, *Hippocrepis comosa*, the grasses *Sesleria varia* and *Festuca glauca* and, unexpectedly, the Caryophyllaceae, *Gypsophila repens*, were distinctly colonized by AMF, whereas

Table 3 Numbers of the spores isolated from the soil adhering to the roots of halophytes at the different locations. Data are given as spore numbers/g dry weight of soil, isolated by differential sieving and sucrose gradient centrifugation. Average values and SDs are given. High scores are given in *italics*

Plant	Number of determinations	Average number of spores/g soil	Average degree of mycorrhizal colonization (%)
Location 1, Apaj, Na_2CO_3			
Artemisia maritima	*7*	*58±51*	*51*
Aster tripolium	5	28±6	62
Camphorosma annua	7	1±1	0
Festuca pseudovina	3	33±14	82
Plantago maritima	15	39±32	54
Puccinellia limosa	8	34±28	40
Root free soil	54	28±38	–
Location 2, Apaj, Na_2CO_3			
Aster tripolium	4	54±40	47
Plantago maritima	2	4±0	54
Puccinellia limosa	6	16±3	0
Root free soil	12	24±31	–
Location 3, Szabadszállás, border of the lake, Na_2CO_3			
Plantago maritima	4	15±9	17
Puccinellia limosa	2	5±0	3
Suaeda maritima	2	9±0	1
Root free soil	8	9±10	–
Location 4, Szabadszállás, Na_2CO_3			
Artemisia maritima	4	*199±65*	*64*
Aster tripolium	4	*191±76*	*60*
Crypsis aculeata	4	25±3	8
Root free soil	12	138±99	–
Location 5, Sarród, $NaCl/Na_2CO_3$			
Aster tripolium	*6*	*102±45*	53
Plantago maritima	6	*86±15*	*50*
Salicornia europaea	6	48±24	2
Root free soil	18	79±39	–
Location 6, Dinnyés, $NaCl/Na_2SO_4$			
Artemisia maritima	1	13	18
Aster tripolium	*4*	*21±8*	*59*
Camphorosma annua	1	27	4
Plantago maritima	1	4	2
Puccinellia limosa	1	1	2
Salicornia europaea	3	11±11	2
Spergularia salina	1	0	0
Root free soil	12	11±12	–
Location 7, Hortobágy, Na_2CO_3/Na_2SO_4			
Camphorosma annua	2	4±0	2
Puccinellia limosa	*4*	*53±43*	*18*
Root free soil	6	28±41	–
Location 8, Hortobágy, NaCl			
Salicornia europaea	2	9±0	0
Suaeda maritima	2	7±0	0
Root free soil	4	8±2	–
Location 9, Bad Sachsa, $CaSO_4$			
Calluna vulgaris	2	4±1	0
Festuca glauca	*9*	*45±30*	*10*
Gypsophila repens	*5*	*43±14*	*41*
Sesleria varia	6	*50±14*	*36*
Root-free soil	30	39±24	–

the three samples of *Calluna vulgaris*, collected at different times, did not show signs of colonization by either an ericoid or any other mycorrhizal fungus.

Mycorrhizal plants from all locations of the Hungarian steppe showed a dense, highly branched intraradical mycelium which was not the case with the samples from the gypsum soil of Bad Sachsa. Such visual impressions can hardly be quantified but this has never been observed with any other of the samples from the many locations examined in the laboratory in the past. In some cases, e.g. in roots of *Festuca pseudovina*, *Artemisia maritima* or *Plantago maritima*, hyphal coils have been seen which resemble those of the *Paris* type (Smith and Read 1997).

Soils from all locations contained AMF spores, but in highly variable numbers (Table 3, data for root-free soil). Highest scores were obtained for the Na_2CO_3 meadows at Szabadszállás (location 4) and at Sarród (location 5), whereas samples taken from the coast-line of the small lake Szabadszállás which is flooded periodically had lower numbers of AMF spores. It must be stressed that SDs were high despite the fairly high number of counts, indicating that the distribution of spores in such soils was patchy. However, on average, the spore content in these soils was definitively higher than in non-saline soils (Hildebrandt et al. 2001). The gypsum soil of Bad Sachsa was also rich in spores (Table 3).

Soil samples were taken directly from the roots of the plants for spore counting (Table 3). In several cases, a high degree of mycorrhizal colonization of the plants correlated with a high spore content in the soils adjacent to the roots (e.g. with *Artemisia maritima* at locations 1 and 4, *Aster tripolium* at 1, 2, 4 and 5 or *Plantago maritima* at 1 and 5). Conversely, a low degree of mycorrhizal colonization sometimes matched a low spore number in the soils in the vicinity of the roots (for *Camphorosma annua* at locations 1 and 7 or *Puccinellia limosa* at 3 and 6). Such correlation was, however, not general (e.g. *Plantago maritima* at location 2 with a high mycorrhizal colonization but a low spore content in the soil or, conversely, *Salicornia europaea* at 5).

Attempts were made to correlate the data for mycorrhizal colonization of the plant roots and spore numbers in soil samples taken from the roots with the values for pH and electrical conductivity. At all sites investigated, both pH and electrical conductivity was highly variable within short distances (Table 1). The electrical conductivity was rather high close to the roots of *Camphorosma annua* (4.2±2.0 mS/cm), *Puccinellia limosa* (2.0±0.8 mS/cm), *Salicornia europaea* (2.4±1.0 mS/cm) at all Hungarian locations, irrespective of the pH value measured. Soil samples from the roots of these non-mycorrhizal or poorly colonized plants contained low spore numbers (Table 3). In samples taken from the gypsum rock, the electric conductivity was high (1.8±0.2 mS/cm) and plants were distinctly AM-colonized there (the exception was *Calluna vulgaris*). At the Hungarian sites, no correlation was apparent between the mean values for electric conductivity and pH on the one hand and spore content and AMF colonization on the other (data not shown). Logistic problems hindered further more detailed analyses.

Soils of the Hungarian salt steppe apparently contained only a few AMF spore morphotypes. The main type amounted to roughly 70% of all spores. It was bright light yellow, had a diameter of about 100 µm and morphologically resembled *G. geosporum* which had been isolated previously from the German inland salt marshes (Hildebrandt et al. 2001). At the different locations other types were occasionally seen but in low numbers only. It was not possible to classify these spores by simple morphological criteria, particularly since the high ionic strength in such soils may alter the appearance of the spores. The gypsum soil from Bad Sachsa mainly

Table 4 Abundance of the RFLP pattern of the PCR products obtained from spores of the different locations in the Hungarian steppe and in the gypsum soil of Bad Sachsa. For locations, see Tables 2 and 3

Restriction pattern	Number of RFLP patterns								
	Location							Sum	Abundance (%)
	1	2	3	4	5	6	7		
Hungary 1	5	5	6	3	7	9	4	39	67
Hungary 2	1				5			6	9
Hungary 3	3	1						4	7
Hungary 4					3			5	
Hungary 5				1				1	2
Hungary 6					2				
Hungary 7	1							1	2
Hungary 8	1							1	2
Hungary 9	1							1	2
Total								58	100
Bad Sachsa 1								8	36
Bad Sachsa 2								5	23
Bad Sachsa 3								4	18
Other pattern in Bad Sachsa (only once each)								5	23
Total								22	100

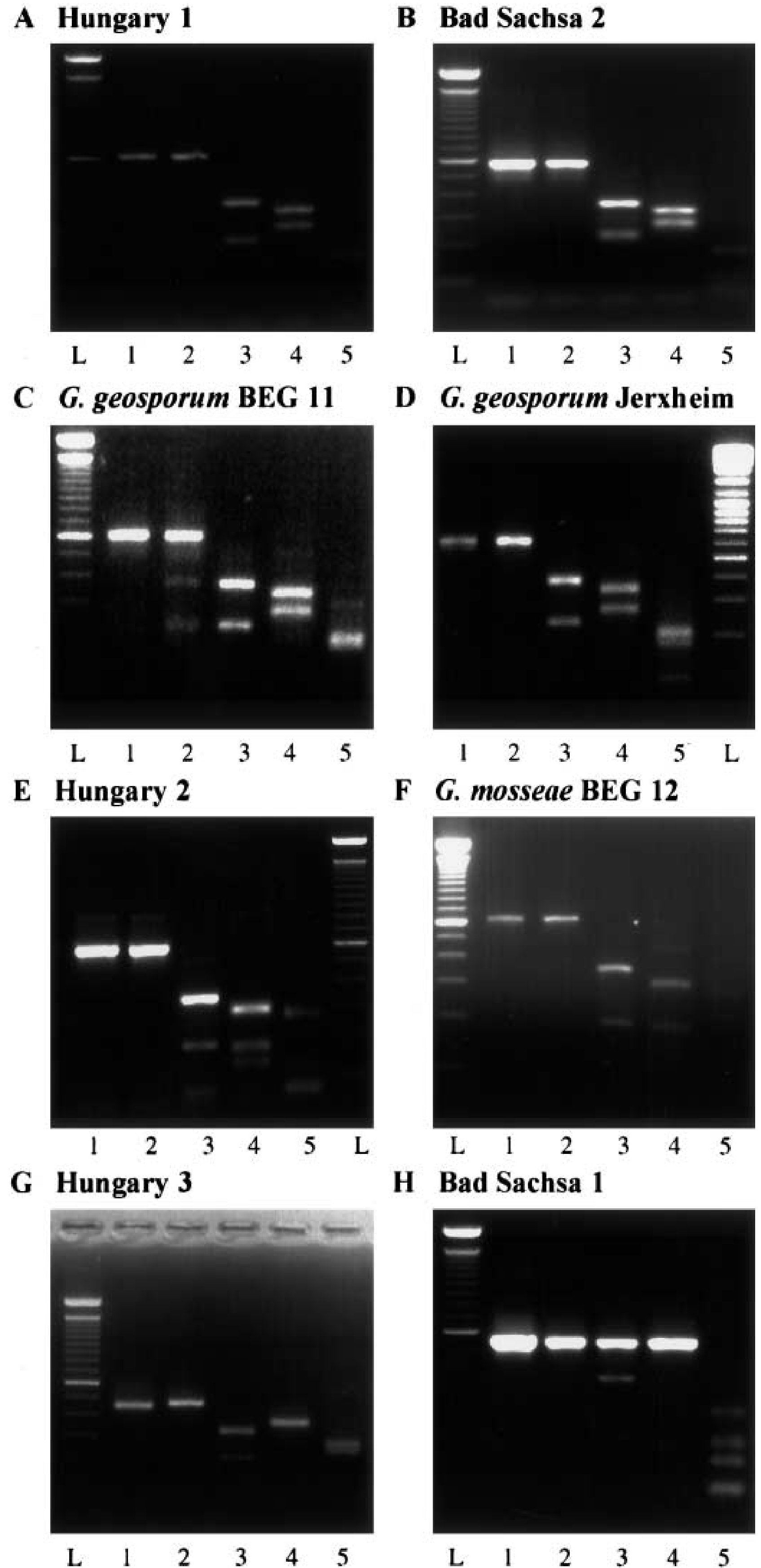


Fig. 1A–H RFLP analysis of PCR products obtained from single spores of the different locations. For the PCR reactions, the primers used were: R1/R2 and then ITS1/ITS4 (nested PCR) for Hungary 1 and Bad Sachsa 1, 2 and ITS4/ITS5 for the rest. The PCR products were restricted and separated on 2% agarose gels. *lane L* Restriction with 100-bp DNA standard (Gibco), *lane 1* restriction with *Alu*I, *lane 2* restriction with *Bsu*RI, *lane 3* restriction with *Hin*fI, *lane 4* restriction with *Hpa*II, *lane 5* restriction with *Taq*I

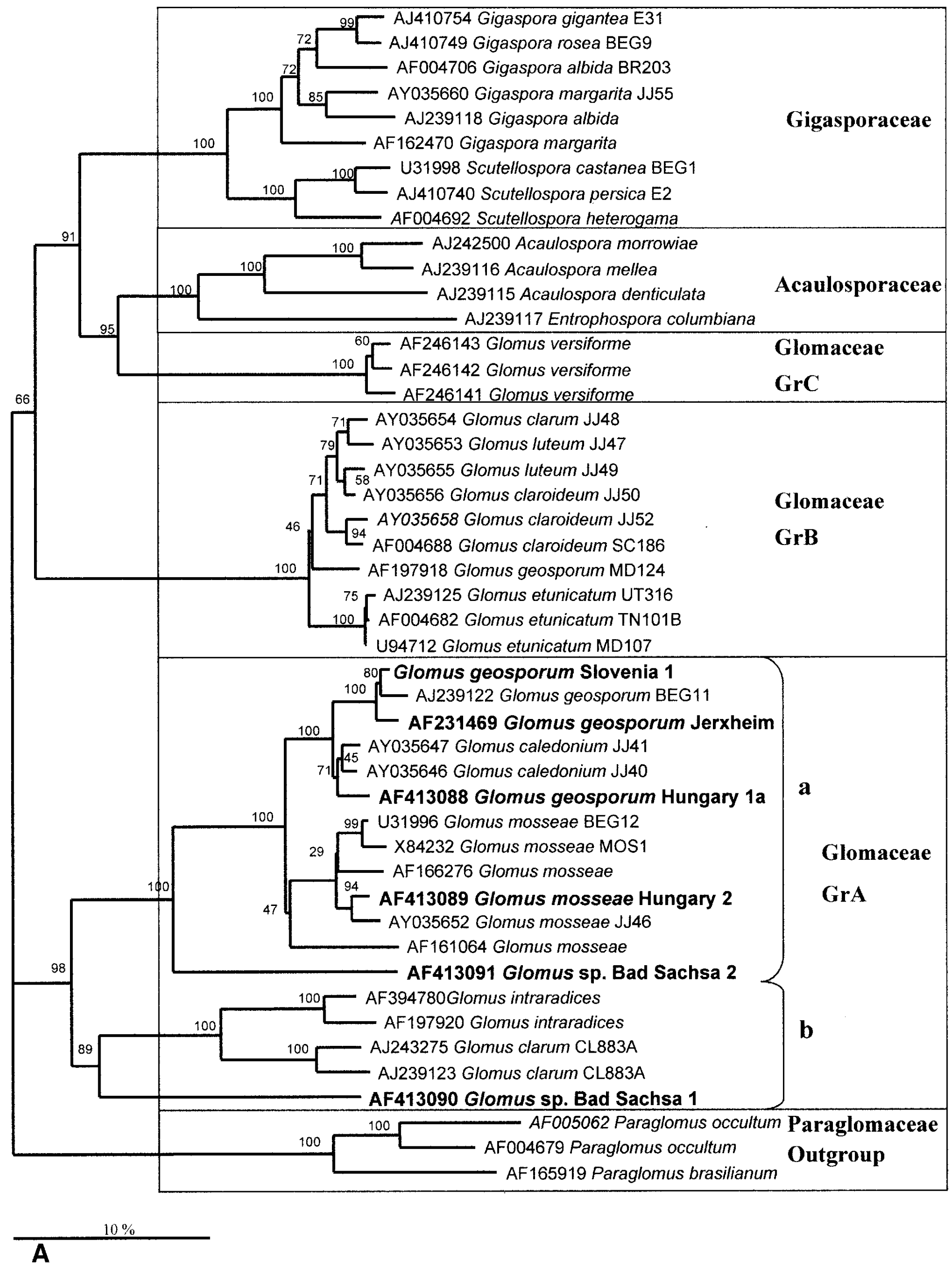


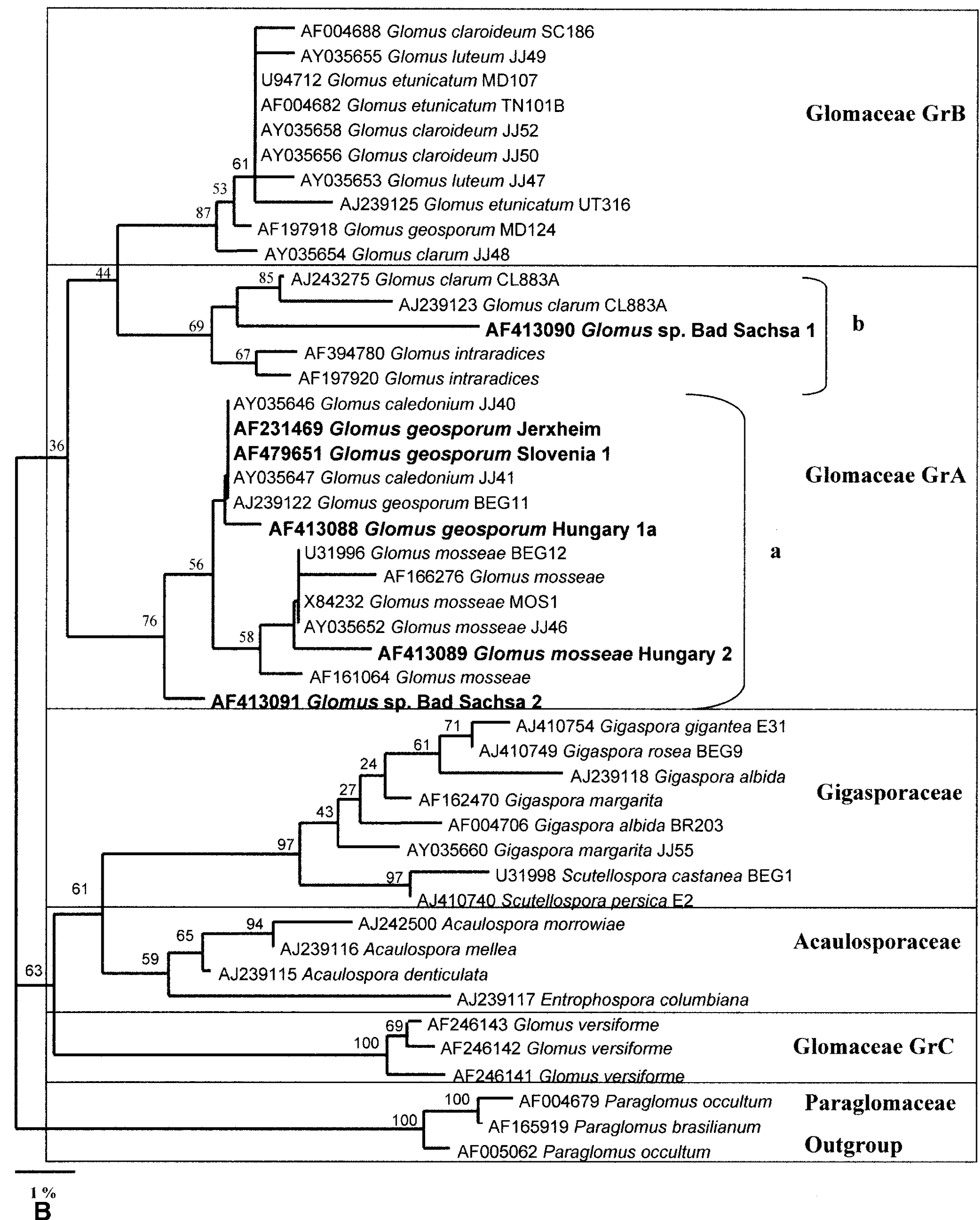
Fig. 2 **A** Phylogenetic dendrogram based on the ITS plus 5.8S rDNA sequences showing the position of the newly obtained sequences within the Glomaceae of group A. The tree was obtained by distance analysis with the neighbour-joining method. The sequences of the Paraglomaceae served as outgroup. The *scale bar* indicates ten nucleotide substitutions within 100 bases. The own sequences are indicated in bold. Numbers at the nodes indicate the proportional occurrence of the respective nodes in a bootstrap analysis of 1,000 resamplings. **B** Phylogenetic dendrogram based on 5.8S rDNA sequences only. The neighbour-joining method and the automatic alignment was done as in the dendrogram of **A**. The *scale bar* indicates one nucleotide substitution within 100 bases

contained three different AMF spore morphotypes: (1) yellow orange (abundance 30–40%), (2) red-brownish (30–35%), and (3) yellow to dark-yellow spores (25–30%), all middle-sized in diameter (80–100 μm). Other spore types accounted for <5% of the total.

To obtain a molecular characterization of the AMF population in these soils, DNA of spores was amplified by PCR and subjected to RFLP analysis. With DNA from single spores, the primer combination ITS1 and ITS4 (White et al. 1990) provided PCR products in 3–38%, whereas the yield increased to about 40% with the primers ITS4 and ITS5. Approximately 5% of the trials provided more than one single PCR band with DNA from single spores. When nested PCR was employed (first using the primers R1 and R2 and subsequently ITS4 and ITS5), the yield was 40–70%, but more than one band, often a smear, was obtained in 30% of the PCR amplifications. Distinct restriction patterns were subsequently obtained only when the PCR provided a single band.

Altogether 58 PCR products of DNA from Hungarian spores were subjected to RFLP analysis. The restriction pattern Hungary 1 (Fig. 1) was obtained with DNA isolated from spores of all the seven locations examined (Table 4). It was the major pattern in all cases and represented 67% of the 58 PCR products. Remarkably, it was identical to the major RFLP pattern seen with spores of the German inland salt marshes, which was very closely

Fig. 2 B



related to the pattern detected with DNA from *G. geosporum* BEG 11 spores [Fig. 1; see also Hildebrandt et al. (2001)]. Restriction pattern Hungary 2 was frequently found with spores of the Sarród salt marsh (location 5) and matched the *G. mosseae* BEG 12 pattern (Fig. 1). Other restriction patterns occurred only occasionally (Table 4). Spores of Bad Sachsa provided three major restriction patterns (termed Bad Sachsa 1, 2 and 3) at almost equal frequency. It should be noted that DNA from spores of Hungary 1 and Bad Sachsa 2 gave an almost, but not completely, identical restriction patterns (Fig. 1).

The most frequently occurring PCR products (Hungary 1, 2, 3, Bad Sachsa 1 and 2) were cloned. Prior to sequencing, plasmid DNA of the clones was reamplified by the use of the primers ITS1 and ITS4 and subjected to RFLP analysis to affirm that the original PCR products had been cloned. Indeed, the RFLP patterns of Hungary 2, 3 and Bad Sachsa 2, 3 did not show any significant differences to the original patterns. In the case of Hungary 1, two different patterns were obtained, one of which (1a) was closely related to the original Hungary 1, whereas the other one (1b) gave a different restriction pattern, particularly when *Hpa*II was used (Fig. 1). Sequencing of these clones and the comparisons with the data in the Genbank [BLASTN program; Altschul et al. (1997)] revealed that the ITS regions of Hungary 1a, Hungary 2 as well as Bad Sachsa 1 and 2 scored with those of *Glomus* species (Table 5). It was noteworthy

Table 5 Sequence identity of the selected cloned PCR products. The BLASTN search program was used. For other details see Materials and methods

ITS sequence	Highest sequence identity in the sequenced rRNA-ITS region to	Identical bases/total bases	Sequence identity (%)
Hungary 1a	*G. geosporum* BEG 11	510/565	90
Hungary 1b	*Cladosporum* sp. (Ascomycete) isolate 4/97–17	469/504	93
Hungary 2	*G. mosseae* BEG 57	549/566	97
Hungary 3	*Leptosphaeria* sp. ITSc1 (Ascomycete)	481/565	95
Bad Sachsa 1	*G. intraradices* Ey 118	373/485	77
Bad Sachsa 2	*G. geosporum* BEG 11	415/570	73
Slovenia 1	*G. geosporum* BEG 11	520/560	92

that in the BLAST search Hungary 1 and Bad Sachsa 2 were next to *G. geosporum*, whereas Hungary 2 showed distinct sequence identities to *G. mosseae* and Bad Sachsa 1 to *G. intraradices*. Somewhat surprisingly, the ITS regions of both Hungary 1b and Hungary 3 were similar to those of ascomycetes (Table 5). The identity between *G. geosporum* Hungary 1 and Hungary 3 (the sequence of an Euascomycete, termed *Leptosphaeria* sp., Hosny et al. 1999) was 56%.

The ITS sequences of the present study and of the databanks were used to construct a phylogenetic dendrogram (Fig. 2a). To improve the resolution of the phylogram, the sequence of an isolate recently obtained from a Slovenian salt marsh at the Adriatic Sea was included. All new sequences clustered in the *Glomus* group A (Schwarzott et al. 2001). The sequences of *G. geosporum* from Jerxheim, Slovenia 1, Hungary 1 and BEG 11 were closely related to each other within the *G. geosporum/caledonium* clade (Fig. 2a). However, the sequence divergence among each of them exceeded 2%, which is estimated to be the maximal error due to PCR amplification and sequencing. Both sequences obtained from Bad Sachsa samples, although scoring next to *G. geosporum* and *G. intraradices*, respectively, in the databanks using BLASTN (Table 5), might represent other species (Fig. 2a, b). *Glomus* sp. Bad Sachsa 1 could be placed into subgroup b and *Glomus* sp. Bad Sachsa 2 into a, although they may also be members of own subclades. This separation is very tentative at present.

The dendrogram based on the ITS region including the 5.8S rDNA was obtained by automatic alignment. Since deletions or insertions abundantly occur in the ITS regions, depending on the isolate, it could be argued that such an automatic alignment produces artefacts. Therefore, a further dendrogram was constructed solely based on the approximately 160 bp of the 5.8S rDNA (Fig. 2b). The similarities between the dendrograms based on the 5.8S rDNA, ITS regions and 18S rDNA sequences (Schwarzott et al. 2001) were striking. However, in the case of the 5.8S rDNA dendrogram, members of the group Ab more closely clustered with the isolates of group B, but only with low support by the bootstrap values (Fig. 2b). Any further analysis was beyond the scope of the present study. The pivotal result was, however, obvious in the dendrograms based on ITS and 5.8S rDNA sequences: all isolates from the saline and sodic soils and also the isolate Bad Sachsa 2 clustered within group Aa in close proximity to the *Glomus geosporum*/*G. caledonium* clade.

Discussion

It was shown in the preceding publication (Hildebrandt et al. 2001) that AMF spores abundantly occur in neutral salt (NaCl) marshes, both at the North and Baltic Sea and at inland saline habitats. The present study extends these findings to extreme alkaline soils (pH values up to 11), independently of the Solonetz or Solonchak (Horvat et al. 1974) soil type and irrespective of NaCl, Na_2CO_3, Na_2SO_4 or $CaSO_4$ being the major salt present. The degree of mycorrhizal colonization of the roots, like the numbers of arbuscules and vesicles, however, varied from one individual to the next; which has also been described for plants of other locations (Smith and Read 1997). As the degree of mycorrhizal colonization and in particular the content of arbuscules may vary even in a plant within the vegetative period, any statistical evaluation of the data has to meet large difficulties. Some plants like the frequently dominant salt marsh grass *Puccinellia* sp. (this study and Hildebrandt et al. 2001) show an extremely variable but, in general, low degree of colonization, in accordance with data recently published for this grass in a Portuguese salt marsh (Carvalho et al. 2001).

Some current data are not in accord with those of the literature. Heather (*Calluna vulgaris*) has been given as an example of an ericoid mycorrhizal plant (Brundrett 1991; Smith and Read 1997). *Calluna* roots from the gypsum soil of Bad Sachsa were not colonized when examined in late spring or autumn. The Chenopodiaceae, *Salicornia europaea*, showed typical AMF structures including arbuscules in roots from the German inland salt marshes (Hildebrandt et al. 2001), however, not from the Hungarian steppe. One can only speculate about these differences. In the case of *Salicornia*, the inland salt marshes at the leeside of the Harz mountains suffer from periodic drought and have a high soil electric conductivity. Plants occurring there have to cope with the extremely negative water potential and therefore endure a "physiological drought" (Schimper 1898). Under such extremely adverse conditions, the monoculture stands of

Salicornia may utilize the fungi for water-exploitation of the soils; alternatively the fungi may get access to the residual water stored in the succulent stems of *Salicornia* under prolonged periods of drought.

Due to their size and ease with which they can be enriched, AMF spores can hardly be confused with spores of other organisms (Arora et al. 1991). The present study demonstrated that the average spore content in the saline and sodic soils is high, but also shows a large variation from sample to sample examined. To our knowledge, a correlation between the number of spores in the soils of the adjacent rhizosphere and the degree of mycorrhizal colonization has been reported only once in the literature (Kim and Weber 1985) but not by others (Kahn 1974; Brundrett 1991). Spore formation in soils might depend on complex physiological and ecological parameters (Redecker et al. 2000) and on the genotypes of the plants and the fungi (Clapp et al. 1995). AM fungi which sporulate poorly (Sparkling and Tinker 1978) may be effective in colonizing plants by also using propagules other than spores (Requena et al. 1996). Thus the high spore content in soil samples and the intense mycorrhizal colonization of the roots does indicate that AMF activity plays a role under such harsh conditions in saline and sodic soils, in contrast to statements in the literature (Juniper and Abbot 1993; Peat and Fitter 1993). The large variations in the counts from one sample to the next unfortunately do not permit to draw further statistically sound conclusions.

The attempt to correlate the degree of mycorrhizal colonization of plants and spore content of soils with parameters like pH or conductivity failed in the present study. This was not unexpected. With regard to the plant life in saline and sodic habitats in Hungary, Horvat et al. (1974) stated that: "the vegetation is mosaic like and changes within small distances. Small changes, often in deeper layers which are not visible at the surface, may cause alterations in the vegetation. The real determining factor is the salt concentration, partly also the balance between the different salt ions. All other factors may enhance or lower the detrimental effects of the salts." Such statements might also apply to the mycorrhizal life in such soils. The elucidation of other easily measurable factors like soil redox potential or O_2 content (partial anaerobiosis) will unlikely contribute much to understanding the complex life in such soils. As stated by Horvat et al. (1974), tedious measurements made over years may eventually, if at all, resolve the factors which govern the effectiveness of plant life under high salinity.

Morphology-based identification is of limited use in ecological studies, because spore production is highly dependent on physiological parameters (Redecker et al. 2000). Extensive examination of the spores is generally required for their taxonomic classification (Giovannetti and Gianinazzi-Pearson 1994). The present communication confirms the usefulness of the RFLP analysis of the ITS regions for the classification of AMF spores, despite the fact that DNA of one spore can provide more than one PCR product (Sanders et al. 1995; Antoniolli et al. 2000). Multiple PCR products of different length were obtained in up to 30% of the trials with nested PCR. However, when DNA was amplified by only one PCR, about 95% of the spores gave only one PCR product with one RFLP pattern, which was apparently indicative of specific *Glomus* isolates. It showed only slight alterations when a specific restriction enzyme was used [e.g. with *Bsu*RI in the case of *G. geosporum* isolated from Jerxheim and *G. geosporum* BEG 11 (Fig. 1), and see Fig. 1 in Hildebrandt et al. (2001); or with *Taq*I in the case of *G. geosporum* Hungary 1 and Bad Sachsa 2 (Fig. 1)]. The ITS regions of *G. geosporum* and *G. caledonium* have identical restriction sites for the enzymes used in the present study. Therefore the RFLP analysis cannot differentiate between these two closely related species. It should also be mentioned that only 10–30% of the spores analysed immediately after their removal from the soils gave PCR products. Unpublished experience in the laboratory indicates that the yield of PCR products even decreases when spores are stored in the deep-freeze or in the refrigerator prior to PCR experiments. The failure to obtain PCR products with 70–90% of the spores may have manifold explanations (non-vital spores, degraded DNA, salt load interfering with the DNA amplification, etc.). For those AMF providing the correct PCR product of one size only, this and the previous study (Hildebrandt et al. 2001) showed that the *G. geosporum*/*G. caledonium* group provides the most dominant AM fungi occurring in all saline and sodic soils. In the vicinity of these areas, *G. geosporum* was detected in much lower spore numbers in non-saline soils (Hildebrandt et al. 2001).

It should be stressed that the statements of the present study are based on determinations with spores. A high spore content of one AMF species in a soil may not reflect its dominance in the roots of the plants living there (Clapp et al. 1995). However, the occurrence of spores of almost only the *G. geosporum*/*G. caledonium* clade in the saline and sodic soils investigated suggests, but does not prove, that these fungi are the dominant root colonizers in such soils.

The present study also showed that characterization of the spore population simply by RFLP analysis is not sufficient, but that the PCR products obtained have to be cloned and sequenced beforehand at least once. As known, because of its sensitivity, PCR can amplify false DNA even when present in small amounts. The primers used (White et al. 1990) are not specific for AMF. Thus it is not surprising that two PCR products of ascomycete DNA have been obtained. It is, however, surprising that *Leptosphaeria* sp. has also been found in a single spore of *Scutellospora castanea* (Hosny et al. 1999) possibly indicating that this *Leptosphaeria* sp. is a widespread contaminant in AMF.

For molecular classifications, sequences of the 18S rDNA and of the ITS regions have been used in the past. Sequencing of 18S rDNA segments resolves deeply branching lineages (Redecker et al. 2000; Schwarzott et al. 2001). In contrast, ITS regions are not sufficiently

useful to produce robust results in the higher taxonomic ranks (Redecker et al. 1999) but can be used as diagnostic tools to differentiate isolates within species or genera. The aim of the present study was to show whether closely related ecotypes occur in the different salt marshes. The phylogenetic tree based on the ITS regions is not as comprehensive as the 18S rDNA dendrogram for which more sequences are available in the databanks. The comparison of the sequences of the databanks indicated that AF197919 deposited as *G. geosporum* MD124 belongs to the *Glomus* group B (Fig. 2) and, therefore, probably has to be reclassified. This is not the case for the sequence of *G. geosporum* BEG 11 (deposited as AJ239122).

To some surprise, the dendrogram based on ITS and 5.8S rDNA sequences (this study, Fig. 2a) matched even in detail the one constructed from the 18S rDNA data [Schwarzott et al. (2001), their Fig. 6]. All isolates from the salt marshes belonged to group Aa, and by far the most dominant spore type in salt marshes of Germany (Hildebrandt et al. 2001) and Hungary (this study) clustered with *G. geosporum*, although the current sets of information in the databanks do not permit one to differentiate between *G. geosporum* and *G. caledonium*. The present study indicated that apparently few AMF species, however, in high spore numbers occur in saline and sodic habitats. A similar situation was also observed in heavy metal soils (Hildebrandt et al. 1999; Ouziad 1999; Tonin et al. 2001). In the salt marshes, *G. geosporum* ecotypes, possibly being particularly adapted to their specific habitat, may exist, as the sequences of the spores from Jerxheim (Lower Saxony), Hungary 1, Slovenia and from BEG 11 were not completely identical. The presence of *G. geosporum* or other members of the subgroup Aa (*G. mosseae*) in highly saline soils world-wide has been described (Sengupta and Chaudhari 1990; Brown and Bledsoe 1996; Aliasgharzadeh et al. 2001; Carvalho et al. 2001).

In the case of the gypsum soil, the BLASTN search program indicated *G. geosporum* had the top score with the sequence Bad Sachsa 2. However, the ITS plus 5.8S rDNA sequences of both Bad Sachsa 1 and 2, though both belonging to *Glomus* group A, showed a deep branching of the lineages; therefore these sequences might come from other species. Similar to the vegetation, the AMF population might be dissimilar on the gypsum soil and in the salt marshes. In many aspects, the gypsum rock might be the most interesting location, since the other microoganisms living there, in particular the cyanobacteria, exhibit unusual features (A. Mergel et al., this laboratory, unpublished data).

The occurrence of *G. geosporum/G. caledonium* spores in high numbers in salt marshes suggests that these fungi confer salt tolerance to plants. Fungal ecotypes characterized by RFLP analysis and sequencing may differ in their efficacy with respect to conferring salt tolerance. If this were the case, the potential use of this fungus for all kind of applications would be enormous, as about 7% of the global soil surface is saline. AMF were described to protect plants against salinity (Ruiz-Lozano and Azcón 2000). Studies to reveal the underlying mechanisms of salt tolerance are in the centre of the current interest.

Acknowledgements This work was kindly supported by a grant to both groups from the European Community, project MYCOREM, contract no. QLR 3–99–00097. The stimulating discussions about the content of this manuscript in the COST Action meetings on Mycorrhiza of the European Community are also gratefully acknowledged. This manuscript is dedicated to Professor Gustav Wendelberger, Vienna, on behalf of his pioneering work in 1950 on the continental halophyte vegetation.

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