Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses

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Ammophila arenaria and *Elymus farctus* are two grasses which grow in sympatry in sand dunes of the Atlantic coasts of Europe. Culturable fungal endophytes were isolated from leaf and rhizome tissues of eigthy four plants of each species, sampled in 12 different locations in beaches of the northern coast of Galicia (Spain). Morphological and molecular techniques were used for the identification of fungi. One hundred and three different endophytic species were identified in both grasses, 75 in *Ammophila* and 54 in *Elymus*. The mean number of species identified did not significantly differ between leaves or rhizomes for any of the grasses. The endophytic assemblages of both grasses were dominated by species capable of infecting both hosts. Endophytes found in both grasses comprised 25% of all species recorded, but produced 61% of all isolates obtained. A statistically significant inverse relationship existed between the similarity of endophytic assemblages and their distance. This spatial effect and species accumulation curves suggested that increasing the number of plants or locations examined would reveal new endophytic species, mostly singletons represented by single isolates, on both grasses.

Key words: Biodiversity, endophytes, molecular taxonomy, rDNA.

Article Information

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Introduction

Endophytic fungi may be isolated from healthy plant tissues, and all plants may house endophytes (Saikkonen *et al*., 1998; Stone *et al*., 2004; Schulz and Boyle, 2005; Arnold, 2007). Fungal endophytes are abundant and taxonomically diverse. The use of molecular techniques has enabled identification of difficult species and several reports indicate more than 100 species of fungi may be associated to a single host species (Arnold *et al*., 2000; Guo et al., 2000; Stone *et al*., 2004). In addition, the composition of fungal communities may differ among plant species, tissues, geographically distant regions, and environment (Collado *et al*., 1999, Higgins *et al*., 2007).

The identification of endophytes associated to plant species in environments where biotic or abiotic stress factors are present has led to the discovery of several species of mutualistic endophytes which may improve plant adaptation (Redman *et al*., 2002; Arnold *et al*., 2003; Schardl *et al*., 2004; Waller *et al*., 2005). A practical application of this knowledge is that mutualistic endophytes, like some *Neotyphodium* and *Epichloë* species, are currently being used for the improvement of forage and turfgrass cultivars (Bouton and Easton, 2005; Schardl *et al*., 2004). These two genera include the most studied endophytes, but some surveys suggest that they only represent a small fraction of the endophytic species which may be associated to grasses (e.g. Morakotkarn *et al*., 2006; Neubert *et al*., 2006; Sánchez *et al*., 2007)

In this work, we have studied the endophytic mycobiota of *Ammophila arenaria* and *Elymus farctus* (=*Agropyron junceiforme*), two perennial grasses which grow in sand dunes on beaches, where they are often buried by sand or have their roots flooded by seawater at high tide. The objectives of the work were to describe the endophytic assemblages of these grasses. This would include the identification

and quantification of the species associated to each grass, including multihost species capable of infecting both species, as well as studying the differences in endophytic species composition observed at different locations.

Materials and methods

Plants and collection sites

Ammophila arenaria and *Elymus farctus* are native to the Atlantic coasts of Europe (Hubbard, 1984). On the northern coast of the Iberian Peninsula, they grow in sympatry in beach foredunes. To propagate and to overcome sand burial both species produce vertical and horizontal rhizomes. *Ammophila* plants are larger and grow in more compact tufts than *Elymus*. Plants of both species were obtained in twelve locations on seven sandy beaches of the northern coast of Galicia, in the Atlantic coast of Spain (Figure 1). This coast consists of tall rock cliffs with some interspersed beaches, and it has a humid Atlantic climate. In four beaches (Doniños, Esteiro, Lago, and Villarrube) plants were obtained from two or three locations, while on the three remaining beaches, plants were obtained at only one location (Table 1). Different sampling locations within the same beach were at least 500 m apart. At each location seven plants of each species were sampled, leaving a distance of at least 10 m between pairs of plants. In total, 84 plants of each species were obtained. The plants were processed for endophyte isolation in less than 48 hours after sampling.

Isolation of fungi

Endophytes were isolated from samples of 4-5 leaves of each of the seven plants of each species obtained at each location, and from 4-5 segments obtained from a single rhizome from four of the seven plants. Samples of asymptomatic leaves and rhizomes from each plant were cut transversally into 4 mm long fragments which were surface-disinfected and plated in potato dextrose agar (Sánchez *et al*., 2007). The effectiveness of the surface disinfection methods was tested with imprints of leaf and rhizome fragments made in PDA plates (Schulz *et al*., 1998). All isolates obtained from each leaf and rhizome sample were classified according to their morphological appeareance into morphotypes, for each sample only one isolate of each morphotype was kept for further identification.

Morphological and molecular identification

To induce sporulation in non-sporulating isolates not producing spores in PDA, the strains were plated in water agar, and water agar containing sterilized pieces of leaves of their host, *Ammophila* or *Elymus*. Whenever possible, the identification of endophytes was based on morphological and molecular characters. The molecular marker used for identification was the nucleotide sequence of the ITS1-5.8S rRNA-ITS2 region. Amplicons of this region were obtained using the method described by Sánchez *et al*. (2007), and sequenced using primers ITS4 and ITS5 (White *et al*., 1990). Isolates whose sequences had a similarity greater than 95% were considered to belong to the same species.. This 5% difference used to define species boundaries appears to correlate well with differences among known endophytic species (Arnold and Lutzoni, 2007).

Sequence-based identifications were made by searching with FASTA algorithms the EMBL/Genbank database of fungal nucleotide sequences (Pearson, 1990). Genus and species of the database match were accepted whenever identity between our sequence and that of the database was greater than 97.0%; only the genus was accepted when identity to a database match was from 96.9 to 95.0%, and when the similarity was less than 95%, the isolates were considered unidentified. Morphological examination was used to clarify ambiguities and to confirm results of sequence similarity searches.

Fungal diversity estimations

All identified endophytes were classified into species isolated exclusively from *Ammophila* or from *Elymus*, and species isolated from both hosts. Multi-host endophytes belonging to the last group were considered generalists.

Shannon's diversity index (H') was calculated for each set of endophytic species observed at each location on each host, and for the set of all species observed on each host

(Zac *et al*., 2004). The average species diversity (H'), and the mean number of species isolated at each location were compared between hosts using a Student's t test with α = 0.05.

Species accumulation curves were used to plot the relationship between the number of plants analyzed and fungal species encountered (Colwell, 2005). To estimate the total number of endophytic species which could be associated with *Ammophila* and *Elymus*, several non-parametric estimators of species richness (Chao 1, Chao 2, ACE, ICE, Michaelis-Menten, Bootstrap) were evaluated (Magurran, 2004).

Tissue and location effects

Differences in the average number of species present in leaves and rhizomes were tested with a Student´s t test with α = 0.05. The data used was the number of species observed in four leaf samples and four rhizome samples at each location.

The similarity of the species composition of each pair of locations was estimated using Jaccard´s index of similarity (Magurran, 2004). That index was calculated for each grass from presence/absence data for all endophytic species occurring at more than one location. The relationship between the index of similarity and the distance among locations was tested by linear regression (Arnold *et al*., 2003; Gange *et al*., 2004). Regression was applied after a Kolmogorov-Smirnov test confirmed that the Jaccard similarity data for each plant species followed a normal distribution.

Spatial effects on the presence and specificity of generalist species were estimated by comparing the similarity indexes of the assemblages of both grasses at the same location with those of each grass at different locations, or of both grasses at different locations. Comparisons of the similarity indexes obtained between hosts at each location (n=12) with those of all possible pairwise combinations the same host in different locations (n=66), and those of interhost combinations at different locations (n=144) were made using a Student´s t test with α = 0.05. The normal distribution of the above similarity data was checked with a Kolmogorov-Smirnov test.

Results

Fungal isolation and species identification

Endophytes were isolated from all plants processed. The imprint tests indicated that the surface disinfection procedures efficiently eliminated epiphytic mycobiota.

From the leaf and rhizome samples obtained from 84 plants of each species, 950 isolates were obtained. After grouping isolates belonging to the same morphotype, 211 representative isolates, 128 from *Ammophila* and 83 from *Elymus*, were selected for sequencing. These 211 sequences were analyzed and those differing by less than 5% homology were considered to belong to the same species; as a result 94 different taxa were identified by means of ITS sequences. Thirty eight of these species were sterile mycelium, and their identification was based exclusively on molecular characters. The remaining 56 species were identified using both morphological and molecular characters. In addition, 9 species were identified solely with morphological characters.

In total, 103 different species were identified, 24 of these species could not be identified to genus rank, but could be classified as ascomycetes, basidiomycetes, or assigned to an order or family (e.g., Helotiales, *Xylariaceae*) (Tables 2-4). Except for 5 basidiomycete taxa (*Cryptococcus*, *Kondoa*, *Meira*, *Phlebia*, and unknown basidiomycete 1), all species belonged to the *Ascomycota*.

Excluding unidentified species, the endophytic assemblage of both grasses belonged to 62 genera. The 10 most abundant genera were *Alternaria, Acremonium, Podospora, Penicillium, Microdochium, Arthrinium, Leptosphaeria, Epicoccum, Cladosporium*, and *Beauveria*. Sixty two percent of all isolates obtained belonged to these genera. Unknown ascomycete 1 was also one of the most abundant endophytes. In contrast to the above genera, which were isolated from both grasses, this endophyte only occurred in *Ammophila*.

Host effects and diversity

The endophytic fungi isolated from *Ammophila arenaria* could be assigned to 75 different species, and those from *Elymus farctus* to 54. Forty nine species were found exclusively in plants of *Ammophila* (Table 2), 28 only in *Elymus* (Table 3), and 26 species were generalists common to both grasses (Table 4).

The mean number of species found at each location (Table 1) was significantly greater for *Ammophila* than for *Elymus* ($t = 2.5721$, $P<0.05$). Across locations, Shannon's diversity index, which is a function of the number of species and isolates, was also significantly greater for *Ammophila* than for *Elymus* ($t = -2.4518$, $P<0.05$).

Forty eight of the 103 species identified in both plants were plurals represented by more than one isolate, the remaining species were singletons, represented by a single isolate. In *Ammophila* 48% of the endophytic species were plurals, and in *Elymus* 52%.

Species accumulation curves for all species identified in each grass were non-asymptotic, but the curves showing the accumulation of plural species approached asymptotic growth for both grasses (Figure 2). All estimators of the total number of fungal species that were evaluated produced non-asymptotic species accumulation curves for both *Ammophila* and *Elymus*. The Chao 1 and Chao 2 estimators produced greater estimates of total number of species for *Elymus* than for *Ammophila*. This unexpected difference occurred because these estimators are based on the ratio of singleton to doubleton species found in the sample (Magurran, 2004), and this ratio was greater for *Elymus* (33 singletons and 4 doubletons) than for *Ammophila* (43 singletons and 13 doubletons). The ACE, ICE, Bootstrap and Michaelis-Menten estimators did not cause this overestimate. The highest estimates of the total number of species were obtained with the incidence-based coverage estimate (ICE), with 154.58 species for *Ammophila* and 116.27 for *Elymus*; the lowest estimates were provided by the Bootstrap estimator, with 92.65 species for *Ammophila* and 66.89 for *Elymus*. Since the accumulation curves produced by all estimators were non-asymptotic, their numbers should be considered lower bound estimates of the total number of species (Gotelli and Colwell, 2001).

The endophytic mycobiota of each grass species was dominated by generalist species (Table 4). In *Ammophila*, seven of the 10 most abundant species were generalists, these 10 species were represented by 158 isolates, comprising 59% of all isolates identified in this grass. In *Elymus* 9 of the 10 most abundant species were generalists, and represented 64% of all *Elymus* isolates.

Tissue and location effects

In *Ammophila* 51 species were isolated from leaves and 38 from rhizomes; in *Elymus* 36 species came from leaves and 34 from rhizomes (Table 1). The average number of species isolated from rhizomes did not significantly differ between both plant hosts ($t= 0.1236$, $P>0.05$), but significantly more species were found in *Ammophila* than in *Elymus* leaves ($t= 3.6446$, $P<0.01$).

When species data from 4 leaf and 4 rhizome samples at each location was analyzed to find out if there were differences between these tissues, no significant differences were observed in *Ammophila* (6.58 species in leaves and 5.08 in rhizomes; $t = -1.6912$, $P>0.05$) or in *Elymus* (4.92 species in leaves and 4.92 in rhizomes, $t = 0.0$; $P>0.05$).

Fourteen species from *Ammophila* (19%) and 7 from *Elymus* (30%) were isolated from leaves and rhizomes. Because isolate numbers were small, it is impossible to assert if some species are exclusive to leaves or rhizomes; however, species like *Gliomastix murorum* (Table 2) or *Epicoccum nigrum* (Table 4) were found exclusively in rhizomes or leaves.

As expected from the high number of singleton species observed, many species occurred only at one location. The most cosmopolitan species was *Alternaria sp.*, which was found at all 12 locations in both grasses. Other taxa found at five or more locations were *Podospora*, *Acremonium*, *Epicoccum*, *Penicillium*, and Unknown ascomycete 1.

A statistically significant inverse relationship between the similarity in endophytic species composition and distance among locations occurred for *Ammophila* ($R= -0.3049$; $P<0.05$) and for *Elymus* assemblages

(R: -0.3573, P<0.01) (Figure 3). The Jaccard similarity data obtained for all pairs of locations was found to adjust to a normal distribution for both *Ammophila* (Kolmogorov – Smirnov d= 0.1126, P> 0.05) and *Elymus* (d= 0.0950, P> 0.05).

The mean similarity of the assemblages of generalist species was greater between both grasses at the same location (0.317), than among assemblages of the same grass at different locations (*Ammophila*= 0.245; *Elymus*= 0.239), or between both grasses at different locations (0.273). The difference in the mean similarity between different host species in the same location, and that within the same species in different locations was statistically significant for *Elymus* (t= -2.0979, P<0.05), but not for *Ammophila* (t= -1.790, P > 0.05), or for both grasses compared at different locations (t= -1.1215, P> 0.05).

Discussion

Both *Elymus* and *Ammophila* sustain a highly diverse culturable endophytic mycobiota. One hundred and three different fungal species were isolated from both hosts, 75 from *Ammophila* and 54 from *Elymus* (Tables 2-4). The non-asymptotic species accumulation curves, and the variation observed among locations, suggest that more endophytic species would have been found if more plants or locations were analyzed (Figures 2, 3). The slope of the last 20 data points of the species accumulation curve for *Ammophila* was 0.55, and for *Elymus* was 0.41. These numbers suggest that for every two or three additional plants analyzed, one more endophytic species would be found. The high proportion of singleton species, 53% of all species but only 11% of all isolates, had an important influence in the shape of the species accumulation curves. When curves were based only on data from plural species, those having more than one isolate, the resulting curves were asymptotic (Figure 2). These results suggest that there is a limited number of plural species infecting each grass, and our survey identified most them. On the other hand, an extremely diverse group of singleton species only occasionally infect the plants. Therefore, increased plant sampling would most likely extend the identification of new singleton species.

Based on the data obtained, the ICE and Bootstrap estimators of total number of species point out that at least 155 to 93 species could be found in *Ammophila*, and 116 to 67 in *Elymus*. It is very likely that these numbers underestimate the endophytic mycobiota of the plants analyzed. For instance, different isolation media or sample processing techniques, like particle filtration, could have produced different or even more culturable species (Collado *et al*., 2007). Furthermore, the isolation method we used does not allow for the detection of non culturable endophytic species (Kowalchuk *et al*., 1997; Neubert *et al*., 2006).

The endophytic assemblage of each grass was dominated by a relatively small number of plurivorous species. In each grass, 10% of its endophytic species accounted for more than 50% of the isolates obtained, and multihost species were the majority in this group (Table 4). This situation of dominance by multihost species was also observed in other studies of endophytic assemblages of sympatric hosts (Seena and Sridar, 2004; Gange *et al*., 2007; White and Backhouse, 2007). Several of these dominant multihost species (e.g. *Acremonium*, *Alternaria*, *Cladosporium*, *Epicoccum*) are ubiquitous endophytes present in other grasses and plant families (Stone *et al*., 2004; Schulz *et al*., 2005; Sánchez *et al*., 2007).

Host-specific endophytes were difficult to identify because many taxa were only represented by one or few isolates. However, “unknown ascomycete 1” (Table 2) appears to be a host-specific endophyte, twenty two isolates were obtained from *Ammophila* plants at five different locations. “Unknown ascomycete 12” from *Elymus* (Table 3) could also represent a host-specific taxon. These “unknown ascomycetes”, as well as some of the most frequent generalist taxa could be good candidates to test if they maintain a mutualistic relationship with their host grasses.

Some mutualistic fungal endophytes have been found in very high frequencies (0.75-1.00) in host populations (Redman *et al*., 2002; Schardl *et al.*, 2004). In this study we

have not identified any endophytic species occurring in most individuals. The highest frequencies of infection occurred with *Alternaria*, found in 63% and 55% of the *Ammophila* and *Elymus* plants, respectively (Table 4).

The mean number of endophytic species per location, as well as the diversity (H'), were significantly greater for *Ammophila* than from *Elymus* (Table 1). A similar observation was made in a survey of fungi from senescent leaves and stems in the same grasses (Apinis and Chesters, 1964). In our study, the mean number of species isolated from rhizomes did not significantly differ between both grasses, but in the case of leaves, that number was significantly greater for *Ammophila*. The aerial parts of *Ammophila* plants are larger, and their ramets are much more densely clumped than those of *Elymus*. These anatomical differences could make *Ammophila* leaves more prone to trap aerial inoculum, and could explain why its leaves harbour more endophytes than those of *Elymus*.

Rhizomes supported a mycobiota as rich as that of leaves. When an equal number of leaf and rhizome samples were analyzed at each location, we found that the mean number of fungal species isolated from leaves or rhizomes were not significantly different in *Ammophila* or *Elymus*.

The variation in the geographical distribution of the endophytic species was remarkable. About two thirds of the species identified on each grass were found only at one location, 65.8% in *Ammophila* and 66.7% in *Elymus*. When species occurring at more than one location were considered, it was found that the distance among locations was inversely related to the similarity of endophytic assemblages (Figure 3). Other situations where distance is inversely related to the similarity of endophytic assemblages have been described (Arnold *et al*., 2003; Gange *et al*, 2007). This effect of distance between plants on species composition is also supported by the fact that the similarity index of multihost species assemblages was greater between both grasses in the same location (0.32), than between both grasses (0.27), or each grass at different locations (*Ammophila*= 0.25, *Elymus*= 0.24). The mean similarity between both grasses at the same location was significantly greater than that among *Elymus* plants at different locations. These results suggest that in general, multihost species assemblages are more strongly influenced by the location than by fungal preference for one of the host plants. This effect of distance on species composition may explain why surveys of fungi fructifying on senescent stems and leaf litter of *Ammophila* and *Elymus* in particular locations of England or Portugal show very little overlap with the endophytic species we found (Apinis and Chesters, 1964; Dennis, 1983)

The 103 different species identified in both grasses belonged to 62 genera, 56 of which were in the *Ascomycota*. A predominance of ascomycetes has been observed in other surveys of endophytes and saprophytes of grasses (Wirsel *et al*., 2001; Wong and Hyde, 2001; Barata, 2002; Morakotkarn *et al*., 2006; Sánchez *et al*., 2007). Twenty four endophytic taxa cold not be identified to genus rank because they were sterile and their sequences were not similar to any taxon registered in the EMBL/Genbank database. It is a possibility that some of these isolates represent unknown species. This result also suggests that emphasis on the endophytic world may may strenghten our still weak knowledge of fungal taxonomic diversity (Hawksworth and Rossman, 1997).

Only a few of the genera identified as endophytes in this survey contain pathogens previously described in *Elymus* (*Gaeumannomyces*, *Leptosphaeria*, *Phaeosphaeria*, *Cladosporium*, *Drechslera*, *Curvularia*, *Fusarium*) or in *Ammophila* (*Lophodermium, Ustilago, Alternaria*) (Farr *et al*., 1989). Although latent pathogens can behave for a time period as endophytes (Mostert *et al*., 2000; Photita *et al*., 2004), this survey and a previous one (Sánchez *et al*., 2007), indicate that pathogens do not appear to constitute a considerable fraction of the endophyte assemblage of grasses.

Although by definition endophytes are not expected to sporulate in their hosts, the spores of *Alternaria, Cladosporium, Penicillium, Aspergillus niger, Aureobasidium pullulans, Chaetomium globosum, Acremonium strictum,* and *Epicoccum nigrum*, can be abundant in indoor and outdoor air and dust

samples (Fang *et al*., 2005; Vesper *et al*., 2008). This suggests that these endophytes may complete their life cycles in alternate substrates or hosts, or may be cryptic saprophytes, whose reproductive cycle starts with the death of the plant hosts (Promputtha *et al*., 2007). Some of the endophytic species identified have known ecological roles such as insect pathogens (*Beauveria bassiana*, *Lecanicillium lecanii*), pathogens in other plant (*Plectosphaerella cucumerina*, *Anthostomella eucalyptorum*) or animal species (*Phialemonium dimorphosporum*), or wood rotting fungi (*Phlebia radiata*).

In conclusion, this study shows that two sympatric grasses can support a very rich endophytic assemblage. In both grasses, rhizomes supported a mycobiota as rich as that observed in leaves. The assemblage of each grass was dominated by several multihost species; species accumulation curves indicated that most of these dominant species were detected in the present survey. However, numerous singleton species that were detected are likely to increase in number if new plant samples or locations would have been studied. Variation in the composition of the mycobiota was very strong among locations, but when plural species were considered, an inverse relationship between distance among locations and similarity of endophyte assemblages was detected.

References

Apinis A.E., Chesters C.G.C. (1964). Ascomycetes of some salt marshes and sand dunes. Transactions of the British Mycological Society 47: 419-435.

Arnold A.E., Maynard Z., Gilbert G.S., Coley P.D., Kursar T.A. (2000). Are tropical fungal endophytes hyperdiverse?. Ecology Letters 3: 267-274.

Arnold A.E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biology Reviews 21:51-66.

Arnold A.E., Lutzoni F. (2007). Diversity an host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots?. Ecology 88: 541-549.

Arnold A.E., Mejia L.C., Kyllo D., Rojas E.I., Maynard Z., Robbins N., Herre E.A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences USA 26: 15649-15654.

Barata M. (2002). Fungi on the halophyte *Spartina maritima* in salt marshes. In: *Fungi in marine environments* (ed. K.D. Hyde). Vol.7. Fungal Diversity Research Series: 179-193.

Bouton J., Easton S. (2005). Endophytes in forage cultivars. In: *Neotyphodium in cold season grasses* (eds. C.A. Roberts, C.P. West, D.E. Spiers). Blackwell publishing, Iowa: 327-340.

Collado J., Platas G., González I., Peláez F. (1999). Geographical and seasonal influences on the distribution of fungal endophytes in *Quercus ilex*. New Phytologist 144: 525-532.

Collado J., Platas G., Paulus B., Bills, G.F. (2007). High-throughput culturing of fungi from plant litter by a dilution-to-extinction technique. FEMS Microbiology Ecology 60: 521–533.

Colwell R.K. (2005). EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. Persistent URL <purl.oclc.org/estimates>.

Dennis R.W.G. (1983). Fungi of *Ammophila arenaria* in Europe. Revista de Biología 12:15-48.

Fang Z.G., Ouyang Z.Y., Hu L.F., Wang X.K., Zheng H., Lin X.Q. (2005) Culturable airborne fungi in outdoor environments in Beijing, China. Science of the Total Environment 350:47-58.

Farr D.F., Bills G.F., Chamuris G.P., Rossman A.Y. (1989). Fungi on plants and plant products in the United States. APS Press, USA.

Gange A.C., Dey S., Currie A.F., Sutton B.C. (2007). Site-and species specific differences in endophyte occurrence in two herbaceous plants. Journal of Ecology 95: 614-622.

Gotelli N.J., Colwell R.K. (2001). Quatifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecology Letters 4: 379-391.

Guo L.D., Hyde K.D., Liew E.C.Y. (2000). Identification of endophytic fungi from *Livistonia chinensis* based on morphology and rDNA sequences. New Phytologist 147: 617-630.

Hawksworth D.L., Rossman A.Y. (1997). Where are all the undescribed fungi?. Phytopathology 87: 888-891.

Higgins K.L., Arnold A.E., Miadlikowska J., Sarvate S.D., Lutzoni F. (2007). Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. Molecular Phylogenetics and Evolution 42: 543-555.

Hubbard C.E. (1984). Grasses. A guide to their structure, identification, uses and distribution in the British Isles. Penguin Books, London, UK.

Kowalchuk G.A., Gerards S., Woldendorp J.A. (1997). Detection and characterization of fungal infections of *Ammophila arenaria* (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. Applied and Environmental Microbiology 63: 3858-3864.

Magurran A.E. (2004). Measuring Biological Diversity. Blackwell Publishing, Oxford.

Morakotkarn D., Kawasaki H., Seki T. (2006). Molecular diversity of bamboo-associated fungi isolated from Japan. FEMS Microbiology Letters 266:.10-19.

Mostert L., Crous P.W., Petrini O. (2000). Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. Sydowia 54: 46-58.

Neubert K., Mendgen K., Brinkmann H., Wirsel S.G.R. (2006). Only a few fungal species dominate highly diverse mycofloras associated with the common reed. Applied and Environmental Microbiology 72: 1118-1128.

Pearson W.R. (1990). Rapid and Sensitive Sequence Comparison with PASTP and FASTA. Methods in Enzymology 183: 63-98.

Photita W., Lumyong S., Lumyong P., McKenzie E.H.C., Hyde K.D. (2004). Are some endophytes of *Musa acuminata* latent pathogens?. Fungal Diversity 16:.131-140.

Promputtha I., Lumyong S., Dhanasekaran V., McKenzie E.H.C., Hyde K.D., Jeewon R. (2007). A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microbial Ecology 53: 579-590.

Redman R.S., Sheehan K.B., Scout R.G., Rodriguez R.J., Henson J.M. (2002). Thermotolerance generated by plant/fungal symbiosis. Science 298: 1581.

Rodriguez R.J., Redman R.S., Henson J.M. (2004). The role of fungal symbioses in the adaptation of plants to high stress environments. Mitigation and Adaptation Strategies for global Change 9: 261-272.

Saikkonen K., Faeth S.H., Helander M., Sullivan T.J. (1998). Fungal endophytes: A continuum of interactions with host plants. Annual Review of Ecology and Systematics 29: 319-343.

Sánchez S., Bills G.F., Zabalgogeazcoa I. (2007). The endophytic mycobiota of the grass *Dactylis glomerata*. Fungal Diversity 27: 171-195.

Schardl C.L., Leuchtmann A., Spiering M.J. (2004). Symbioses of grasses with seedborne fungal endophytes. Annual Review of Plant Biology 55: 315-340.

Schulz B., Boyle C. (2005). The endophytic continuum. Mycological Research 109: 661-686.

Schulz B., Guske S., Dammann U., Boyle C. (1998). Endophyte-host interactions II. Defining symbiosis of the endophyte-host interaction. Symbiosis 25: 213-227.

Seena S., Sridar K.R. (2004). Endophytic fungal diversity of two sand dune wild legumes from the southwest coast of India. Canadian Journal of Microbiology 50: 1015-1021.

Stone J.K., Polishook J.D., White Jr. J.F. (2004). Endophytic fungi. In: *Biodiversity of fungi* (eds. Mueller G.M., Bills G.F., Foster M.S.). Inventory and monitoring methods, Elsevier Academic Press, USA: 241-270.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876-4882.

Vesper S., McKinstry C., Haugland R., Neas L., Hudgens E., Heidenfelder B., Jane Gallagher J. (2008). Higher Environmental Relative Moldiness Index (ERMIsm) values measured in Detroit homes of severely asthmatic children. Science of the Total Environment 394:192-196.

Waller F., Achatz B., Baltruschat H., Fodor J., Becker K., Fischer M., Heier T., Hückelhoven R., Neumann C., von Wettstein D., Franken P., Kogel K.H. (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proceedings of the National Academy of Sciences 102: 13386-13391.

White I.R., Backhouse D. (2007). Comparison of fungal endophyte communities in the invasive panicoid grass *Hyparrhenia hirta* and the native grass *Botriochloa macra*. Australian Journal of Botany 55: 178-185.

White T.J. Bruns T., Lee S., Taylor J. (1990). Amplification and direct sequencing of fungal

ribosomal RNA genes for phylogenetics. In: *PCR protocols. A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfland, J.J. Sninsky, T.J. White). Academic Press, USA: 315-322.

Wirsel S.G.R., Leibinger W., Ernst M., Mendgen K. (2001). Genetic diversity of fungi closely associated with common reed. New Phytologist 149: 589-598.

Wong M.K.M., Hyde K.D. (2001). Diversity of fungi on six species of *Graminae* and one species of *Cyperaceae* in Hong Kong. Mycological Research 105: 1485-1491.

Zak J.C., Willig M.R. (2004). Fungal biodiversity patterns. In: *Biodiversity of fungi. Inventory and monitoring methods*. (eds. G.M Mueller., G.F. Bills, M.S. Foster) Elsevier Academic Press, USA: 59-75.

Table 1. Number of fungal species identified in leaves and rhizomes of *Ammophila arenaria* (Aa) and *Elymus farctus* (Ef) at twelve locations on beaches in the northern coast of Galicia, Spain. At each location endophytes were isolated from leaf samples from seven plants and rhizome samples from four plants of each species. Shannon's diversity (H') index was estimated from the total number of endophytic species observed at each location, and also for all species found in each host grass (All plants). Differences between grass hosts in the average number of endophytic species found in leaves, rhizomes, and both organs were tested with a Student's t test. Bold type numbers in the average line indicate significant differences with $p<0.05$

Location	**Number of endophytic species observed**						**Species diversity (H')**	
	Leaves		**Rhizomes**		**Total**			
	Aa	**Ef**	**Aa**	**Ef**	**Aa**	**Ef**	**Aa**	**Ef**
Doniños, A	9	5	7	0	13	5	2.24	1.61
Doniños, B	8	5	6	1	12	5	2.31	1.55
Espasante	4	5	3	1	6	5	1.54	1.54
Esteiro, A	5	6	3	8	8	12	2.08	2.23
Esteiro, B	10	4	3	7	12	9	2.27	1.92
Lago, A	5	5	5	5	9	9	2.09	2.16
Lago, B	12	11	4	7	15	16	2.52	2.53
Morouzos	12	5	7	8	17	10	2.71	2.09
Pantín	5	2	7	4	11	5	2.30	1.47
Villarrube, A	10	7	4	9	14	14	2.55	2.50
Villarrube, B	10	6	10	2	18	6	2.70	1.60
Villarrube, C	14	11	2	5	15	13	2.71	2.37
Average	**8.67**	**6.00**	5.08	4.75	**12.5**	**9.08**	**2.33**	**1.96**
All plants	51	36	38	34	75	54	3.67	3.27

Accession	*Morphological identification*	Sequence-based identification	% FASTA similarity	Proposed identification	Number of isolates	
					Leaves	Rhizomes
AM921701	Sterile mycelium	*Coniosporium* sp.	91.53	Unknown Ascomycete 1	19	3
882F	*Arthrinium* sp .	n.s.[1]	--	*Arthrinium* sp.	5	2
AM921702	Sterile mycelium	*Gliomastix murorum*	98.29	*Gliomastix murorum*	0	5
AM921703	Helotiales	*Heyderia abietis*	88.82	Unidentified Helotiales A	2	2
AM921735	Sterile mycelium	*Scutellinia* sp.	74.04	Unknown Ascomycete 2	0	4
AM921704	Sterile mycelium	*Cordyceps sinensis*	96.41	Unknown Hypocreales	4	0
AM921705	Sterile mycelium	*Lophodermium actinothyrium*	95.46	*Lophodermium* sp.	3	0
AM921738	Unidentified yeast	*Cryptococcus victoriae*	100.00	*Cryptococcus victoriae*	0	2
AM921706	*Dactylaria* sp.	*Dactylaria* sp.	97.72	*Dactylaria* sp.	0	2
AM921707	*Nigrospora* sp.	*Nigrospora oryzae*	97.84	*Nigrospora* oryzae	2	0
AM921708	*Periconiella* sp.	*Periconiella* sp.	94.59	*Periconiella* sp.	2	0
AM921709	*Stagonospora* sp.	*Stagonospora* sp.	98.31	*Stagonospora* sp.	2	0
AM921710	*Trichoderma* sp.	*Trichoderma viride*	99.81	*Trichoderma viride*	2	0
AM921711	Sterile mycelium	Limestone ascomycete	89.26	Unknown Ascomycete 3	2	0
AM921739	Sterile mycelium	*Scolecobasidium variabile*	70.97	Unknown Ascomycete 4	0	2
AM921712	Sterile mycelium	Fungal sp.	90.37	Unknown Ascomycete 5	2	0
AM930536	*Acremonium* sp.	*Sepedonium chlorinum*	71.91	*Acremonium* sp. A	0	1
AM921713	*Aspergillus niger*	*Aspergillus niger*	100.00	*Aspergillus niger*	0	1
AM921714	*Aspergillus* sp.	*Aspergillus versicolor*	99.78	*Aspergillus versicolor*	1	0
AM921736	*Chaetomium* sp.	*Chaetomium globosum*	98.66	*Chaetomium globosum*	1	0
AM921740	Sterile mycelium	*Coprinellus radians*	97.76	*Coprinellus radians*	1	0
AM921715	Unidentified yeast	*Debaryomyces hansenii*	97.37	*Debaryomyces hansenii*	0	1
AM921716	Sterile mycelium	*Engyodontium album*	99.81	*Engyodontium album*	1	0
AM921717	*Fimetariella rabenhorstii*	Fungal endophyte	96.41	*Fimetariella rabenhorstii*	0	1

Table 2. Endophytic species isolated only from plants of *Ammophila arenaria*.

[1] n.s. = not sequenced.

Table 2 continued. Endophytic species isolated only from plants of *Ammophila arenaria*.

Accession	*Morphological identification*	Sequence-based identification	% FASTA similarity	Proposed identification	Number of isolates	
					Leaves	Rhizomes
AM921741	*Helgardia* sp.	*Helgardia anguioides*	99.78	*Helgardia anguioides*	1	0
AM921718	*Kabatiella* sp.	*Fungal sp.*	85.49	*Kabatiella* sp.	1	0
AM921719	*Leptosphaeria* sp.	*Leptosphaeria* sp.	98.17	*Leptosphaeria* sp. A	0	1
AM921720	*Lophiostoma* sp.	*Cercophora coprophila*	90.66	*Lophiostoma* sp.	1	0
AM921721	Sterile mycelium	*Macrophomina phaseolina*	100.00	*Macrophomina phaseolina*	0	1
AM921742	*Meira* sp.	*Meira* sp.	98.99	*Meira* sp.	1	0
AM921743	*Penicillium* sp.	*Penicillium brevicompactum*	98.67	*Penicillium brevicompactum*	1	0
2908IR	*Phaeosphaeria* sp.	n.s.	--	*Phaeosphaeria* sp.	1	0
AM921722	*Phialemonium* sp.	*Phialemonium* dimorphosporum	99.79	*Phialemonium dimorphosporum*	1	0
AM921744	Sterile mycelium	*Phlebia radiata*	98.63	*Phlebia radiata*	1	0
AM921723	*Phomopsis* sp.	*Phomopsis* sp.	96.21	*Phomopsis* sp. A	0	1
AM921724	Sterile mycelium	*Phomopsis* sp.	96.58	*Phomopsis* sp. B	1	0
AM921725	Sterile mycelium	*Phyllosticta pyrolae*	98.62	*Phyllosticta pyrolae*	0	1
AM921726	Sterile mycelium	*Pyrenochaeta lycopersici*	94.66	*Pyrenochaeta* sp.	0	1
AM921727	Sterile mycelium	*Sarea* sp.	99.80	*Sarea* sp.	1	0
AM921728	Sterile mycelium	Dothioraceae sp.	99.81	*Sydowia polyspora*	1	0
AM921729	Pleosporales	Ascomycete sp.	95.63	Unidentified Pleosporales A	0	1
AM921730	Pleosporales	Fungal sp.	91.03	Unidentified Pleosporales B	0	1
AM921731	*Xylariaceae*	*Muscodor albus*	84.89	Unidentified Xylariaceae	1	0
AM921737	Sterile mycelium	*Dactylaria appendiculata*	93.08	Unknown Ascomycete 6	0	1
AM921732	Sterile mycelium	*Zopfiella karachiensis*	93.32	Unknown Ascomycete 7	0	1
AM921745	Sterile mycelium	*Trimmatostroma salinum*	91.08	Unknown Ascomycete 8	1	0
AM921746	Sterile mycelium	*Preussia isomera*	76.99	Unknown Ascomycete 9	0	1
AM921733	Sterile mycelium	*Eutypa lata*	82.31	Unknown Ascomycete 10	1	0
AM921734	Sterile mycelium	Fungal endophyte	80.95	Unknown Ascomycete 11	1	0

Table 3. Endophytic species isolated exclusively from plants of *Elymus farctus*.

Accession	Morphological identification	Sequence-based identification	% FASTA similarity	Proponed identification	Number of isolates	
					Leaves	Rhizomes
AM922199	Sterile mycelium	Fungal sp.	90.64	Unknown Ascomycete 12	1	5
AM922200	*Phaeosphaeria* sp.	*Phaeosphaeria pontiformis*	97.46	*Phaeosphaeria* sp.	3	0
AM922201	*Xylaria* sp.	*Xylaria hypoxylon*	92.11	*Xylaria* sp. B	3	0
AM922202	*Chaetomium* sp.	*Chaetomium* sp.	99.44	*Chaetomium* sp. B	0	2
3093IR	*Drechslera* sp.	*n.s.*	--	*Drechslera* sp.	0	2
AM922203	Sterile mycelium	Foliar endophyte	75.38	Unknown Ascomycete 13	2	0
AM922204	*Acremonium* sp.	*Acremonium alternatum*	96.51	*Acremonium* sp. C	1	0
AM922205	*Anthostomella* sp.	*Anthostomella eucalyptorum*	98.01	*Anthostomella eucalyptorum*	1	0
AM922206	*Arthrinium* sp.	*Arthrinium* sp.	100.00	*Arthrinium* sp. B	1	0
AM922225	Sterile mycelium	*Chaetosphaeria* sp.	95.25	*Chaetosphaeria* sp.	0	1
AM922221	*Coelomycete*	*Epacris microphylla* root	89.65	*Coelomycete*	1	0
AM922207	*Coniothyrium* sp.	*Coniothyrium cereale*	100.00	*Coniothyrium cereale*	0	1
AM922208	*Emericellopsis* sp.	*Emericellopsis* sp.	98.34	*Emericellopsis* sp.	1	0
AM922209	*Fusarium* sp.	*Fusarium* sp.	99.44	*Gibberella avenacea*	0	1
AM922210	Sterile mycelium	*Hypoxylon perforatum*	97.66	*Hypoxylon* sp.	1	0
AM922224	Unidentifed yeast	*Kondoa aeria*	99.62	*Kondoa aeria*	1	0
AM922211	Sterile mycelium	*Neofabraea alba*	100.00	*Neofabraea alba*	1	0
AM922212	Sterile mycelium	*Phialocephala* sp.	99.83	*Phialocephala* sp.	0	1
AM922213	Sterile mycelium	*Cadophora luteo-olivacea*	99.35	*Phomopsis* sp. C	0	1
AM922214	*Schizothecium* sp.	*Podospora tetraspora*	99.77	*Schizothecium* sp.	1	0
AM922215	Cytospora sp.	*Valsa fabianae*	100.00	*Valsa fabianae*	1	0
AM922222	*Verticillium* sp.	*Verticillium nigrescens*	100.00	*Verticillium nigrescens*	0	1
AM922216	*Verticillium* sp.	*Verticillium balanoides*	96.07	*Verticillium* sp.	0	1
AM922217	*Xylaria* sp.	*Xylaria* sp.	97.69	*Xylaria* sp.	0	1
AM922218	Pleosporales	*Leptosphaeria contecta*	92.43	Unidentified Pleosporales C	0	1
AM922219	Xylariales	*Hypoxylon multiforme*	93.70	Unidentified Xylariales	1	0
AM922220	Sterile mycelium	*Nodulisporium* sp.	90.45	Unknown Ascomycete 14	0	1
AM922223	Sterile mycelium	*Plicaturopsis crispa*	77.96	Unknown Basidiomycete	1	0

Table 4. Endophytic species isolated from leaves (L) and rhizomes (R) of *Ammophila arenaria* (Aa) and *Elymus farctus* (Ef).

Accession	Morphological identification	Sequence-based identification	% FASTA similarity	Proposed identification	Number of isolates			
					Aa		Ef	
					L	R	L	R
1883IR	*Alternaria* sp.	n.s.	--	*Alternaria* sp.	53	9	46	20
1892IR	*Podospora* sp.	n.s.	--	*Podospora* sp.	13	4	13	4
1869IR	*Acremonium* sp.	n.s.	--	*Acremonium* sp.	11	5	6	5
AM924149	*Acremonium* sp.	*Nectria mauritiicola*	94.27	*Acremonium* sp. B	10	0	1	3
AM924150	*Microdochium* sp.	*Microdochium* sp.	100.00	*Microdochium* sp.	0	8	1	4
884F	*Penicillium* sp.	n.s.	--	*Penicillium* sp.	3	2	5	3
1901IR	*Epicoccum* nigrum	n.s.	--	*Epicoccum* nigrum	6	0	5	0
AM924151	*Leptosphaeria* sp.	*Leptosphaeria* sp.	98.17	*Leptosphaeria* sp. B	0	3	0	8
AM924152	*Acremonium* sp.	*Acremonium strictum*	99.82	*Acremonium strictum*	2	2	2	4
1913IR	*Cladosporium* sp.	n.s.	--	*Cladosporium* sp.	3	2	6	0
AM924153	*Beauveria bassiana*	*Cordyceps bassiana*	100.00	*Beauveria bassiana*	4	0	3	2
AM924154	*Gaeumannomyces* sp.	*Gaeumannomyces cylindrosporum*	99.28	*Gaeumannomyces cylindrosporum*	0	5	0	1
AM924155	Sterile mycelium	*Pestalotiopsis* sp.	98.80	*Pestalotiopsis* sp. B	3	0	2	1
AM924156	*Thielavia* sp.	*Thielavia coactilis*	95.71	*Thielavia* sp.	1	0	3	2
AM924157	*Curvularia* sp.	*Curvularia inaequalis*	100.00	*Curvularia inaequalis*	4	0	1	1
AM924158	Helotiales	Ericoid mycorrhizal sp.	92.23	Unidentified Helotiales B	0	1	1	3
AM924159	*Arthrinium* sp.	*Arthrinium* sp.	100.00	*Arthrinium* sp. A	1	1	2	0
AM924160	*Acremonium* sp.	*Acremonium alternatum*	99.10	*Acremonium alternatum*	1	0	0	2
AM924161	*Aureobasidium pullulans*	*Aureobasidium pullulans*	100.00	*Aureobasidium pullulans*	1	1	1	1
AM924162	Sterile mycelium	*Stemphylium solani*	99.09	*Stemphylium solani*	2	0	1	1
AM924163	*Lecanicillium lecanii*	*Torrubiella confragosa*	99.65	*Lecanicillium lecanii*	1	1	2	0
878F	*Chaetomium* sp.	n.s.	--	*Chaetomium* sp.	1	1	1	1
AM924164	*Pestalotiopsis* sp.	*Pestalotiopsis* sp.	100.00	*Pestalotiopsis* sp. A	1	0	0	1
AM924165	*Plectosphaerella* sp.	*Plectosphaerella cucumerina*	99.06	*Plectosphaerella cucumerina*	0	1	0	1
AM924166	Sterile mycelium	*Preussia australis*	96.36	*Preussia australis*	1	1	1	1
AM924167	Sterile mycelium	*Emarcea castanopsidicola*	87.11	Unknown Ascomycete 15	1	0	0	1

Figure 1. Location of beaches in the northern coast of Galicia (Spain) where plants were sampled. The square in the map of the Iberian peninsula shows the position of the larger map. The locations indicated by numbers are Esteiro (1), Espasante (2), Morouzos (3), Villarrube (4), Pantín (5), Lago (6), and Doniños (7). In beaches 1, 4, 6 and 7, plants were sampled at more than one location.

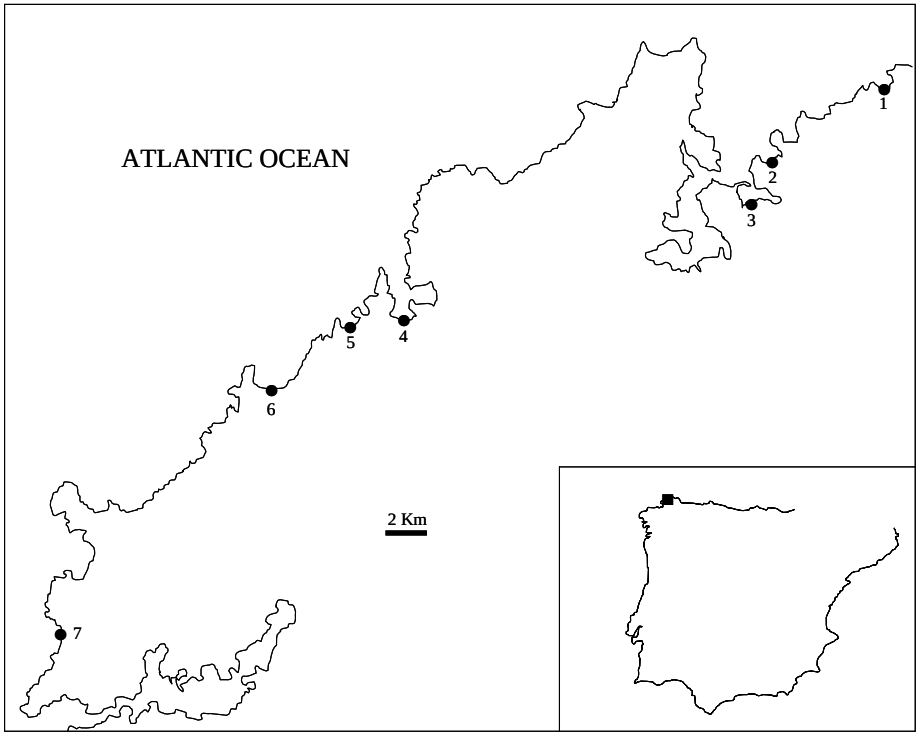

Figure 2. Species accumulation curves showing total number of species (continuous lines), and plural species consisting of at least two isolates (dashed lines) identified in plants of *Ammophila arenaria* (Aa) and *Elymus farctus* (Ef)

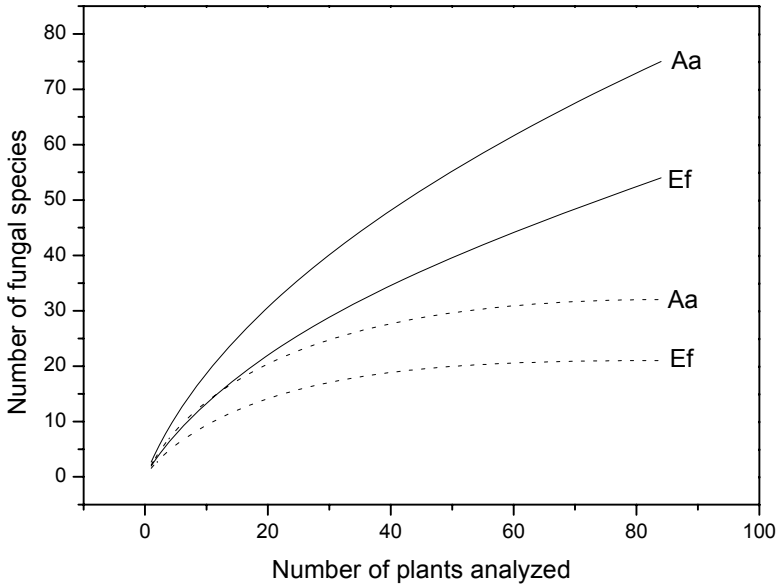

Figure 3. Relationship between the similarity in species composition among pairs of locations and their distance. Assemblages of leaf endophytes of *Ammophila arenaria* (A) and *Elymus farctus* (B) were compared at 12 locations. Only species present at more than one location were considered for the comparisons, there were 26 such species in *Ammophila* and 18 in *Elymus*. Jaccard coefficients were used to estimate the similarity in the endophyte assemblages for all pairs of locations, and the relationship between similarity and distance was tested by linear regression.