The endophytic mycobiota of the grass *Dactylis glomerata*

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Fungal endophytes were isolated from asymptomatic and symptomatic plants of *Dactylis glomerata* sampled in different ecosystems in Spain. Fungi were identified using morphological, as well as molecular methods based on internal transcribed spacer (ITS) and ribosomal DNA sequencing. Molecular data provided a framework for identification and assessing the phylogenetic position of isolates. One hundred and nine different fungal species were identified. Eighteen of these species were potentially unknown. The endophytic assemblage consists of grass-specific, as well as generalist species, and is quite different from those described for perennial woody species. Species richness curves showed that the survey discovered most species commonly infecting this grass, but the number of sporadic infections of singleton species continued to increase with more sampling effort. A large endophytic assemblage consisting of fungi with diverse ecological roles, and potentially unknown species, was found in a small herbaceous plant.

Key words: biodiversity, endophytes, rDNA, ITS

Introduction

Endophytic fungi are those that live in the interior of apparently healthy and asymptomatic hosts. Fungi fitting this description appear to be ubiquitous; indeed, no study has yet shown the existence of a plant species without endophytes (Promputtha *et al*., 2007). High species diversity is another characteristic of endophytic mycobiota. It is quite common for endophyte surveys to find assemblages consisting of more that 30 fungal species per host plant species (Stone *et al*., 2004; Ganley *et al*., 2006; Kauhanen *et al*., 2006).

Culture-dependent assessments of endophytic fungi are based on isolations from surface-sterilized plant tissue samples, which are subsequently plated on culture media (Bills, 1996; Stone *et al*., 2004; Devarajan and Suryanarayanan, 2006). Fungi that emerge from these samples can be identified

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by means of phenotypic (morphological) or genotypic (molecular) characters. Since the sequencing of ribosomal DNA and internal transcribed spacers (ITS) was applied to fungal taxonomy, improved taxonomic information has been accumulated from sterile isolates obtained in endophyte surveys (Guo *et al*., 2000; Wirsel *et al*., 2001; Promputtha *et al*., 2005; Crozier *et al*., 2006; Higgins *et al*., 2007).

Certain vertically-transmitted endophytes can have a beneficial influence on their plant hosts. Some of the best known organisms in this category are the *Epichloë/Neotyphodium* systemic endophytes, whose grass hosts contain fungal alkaloids toxic to herbivores, and have shown advantages in certain situations of biotic and abiotic stress (Clay and Schardl, 2002; Wang *et al*., 2005). Other plant-endophyte associations result in improved plant adaptation to salt and thermal stress, increased biomass, or resistance to pathogen damage (Redman *et al*., 2002; Arnold *et al*., 2003; Waller *et al*., 2005). As a result, fungal endophytes could be very useful for plant improvement. In fact, some cultivars of forage and turf grasses artificially infected by select endophytes are commercially available (Bouton and Easton, 2005). In addition, the production of antimicrobial and toxic secondary metabolites is relatively common in this group of fungi, and their potential as a source of drugs may also be important (Strobel, 2002; Wang *et al*., 2007). At the other extreme of the endophyte spectrum exist species that behave as latent and weak pathogens (Photita *et al*., 2004; Gonthier *et al*., 2006).

This work describes a wide range of endophytic species associated with *Dactylis glomerata*, a perennial grass native to the temperate zones of Europe, Asia, and North Africa. Commercial cultivars of this grass are used for forage production, usually in mixtures with other plants like ryegrass, alfalfa, or clovers. *Dactylis* is a monospecific genus, but several subspecies, some of them differing in ploidy level have been described (Lumaret, 1988). In Spain, wild plants of *D. glomerata* are common in many ecosystems, in dry areas of the central part of the country, as well as in the humid north.

The objectives of this study were to identify the endophytic mycobiota of *Dactylis glomerata* from different habitats, and to determine if potential pathogens of the plant host as well as of cereal crops behave as endophytes. Also, we wanted to compare the assemblage of endophytes of this grass with those found in other plant groups, like woody perennials.

Materials and methods

Sample collection

The collected plants of *Dactylis glomerata* lacked obvious disease symptoms such as chlorosis, leaf spots, or other types of pathogen-induced

lesions. Plants were sampled at ten locations in the province of Salamanca, one location in the province of Ávila, one location in the province of Cáceres, and two locations in the province of La Coruña (Table 1). Salamanca, Ávila, and Cáceres are located in central-western Spain, and their climate is of Mediterranean type with a continental trend (cold winters and dry warm summers). La Coruña, located in northern Spain, has a milder humid Atlantic climate. In Salamanca, plants were obtained from different habitats, such as river banks, semiarid grasslands, or sulphurous water springs (Table 1). All of these locations represent a set of ecologically different habitats. The number of plants sampled varied among locations, and at each location a distance of more than 10 meters was left between sampled plants.

In addition to the asymptomatic plants, in Montemayor del Río (Salamanca), 11 plants showing disease symptoms, e.g., leafspots or other types of leaf lesions, were collected in order to isolate pathogens from the diseased tissue. Dry culms were also collected at two locations in Salamanca: Montemayor del Río (14 plants), and Muñovela (5 plants). Fungal isolates were obtained from fructifications in these culms.

Plants were sampled during the summer and fall of 2003 and throughout the year in 2004 and 2005. Whole plants were dug up in the field and transported to the laboratory, where they were processed for the isolation of fungi.

Isolation of fungi

To isolate endophytes from the plants, small leaf pieces, measuring about 5 mm in length were washed in tubes containing a solution of 20% commercial bleach (1% active chlorine) for 10 minutes. The treatment was followed by a rinse in sterile water, and plating on potato dextrose agar (PDA) containing chloramphenicol (200 mg/l). Root fragments were surface-disinfected by means of a 5 minute rinse with ethanol, followed by treatment with a 1% active chlorine solution for 15 minutes, 2 minutes in ethanol, and a final rinse in sterile water (Bills, 1996). For each one of the 120 sampled plants, two plates, each containing about 15 leaf pieces, were prepared and kept in the dark at room temperature (22-26ºC). Stem fragments were also prepared as above described, but only from 7 plants. Two similar plates of root fragments were prepared from 82 plants. As mycelium emerged from plant tissues into the agar, mycelial fragments were transferred to new PDA plates. These isolates were maintained under natural light at room temperature.

In plants with disease symptoms, small pieces of tissue were cut from the margins of leaf lesions, and plated on PDA after surface disinfection. Fungal

Table 1. Locations and habitat types where asymptomatic plants were sampled, showing the number of isolates obtained, and of species identified at at each location.

Location[a]	Type of habitat	Number of plants	Number of isolates	Isolates per plant	Number of species
Beco, Cedeira. Co	Coastal meadow	15	50	3.33	35
Calvarrasa de Arriba, Sa	River bank	8	47	5.88	34
Casas del Conde, Sa	River bank	1	1	1	1
Cristo de Cabrera, Sa	Road ditch	9	18	2	14
El Cabaco, Sa	*Quercus pyrenaica* woodland	13	29	2.23	26
Faro, Cedeira. Co	Coastal meadow	15	35	2.33	21
Fuente Roldán, Sa	Sulphurous spring	2	11	5.50	10
Los Montalvos, Sa	Road ditch	7	9	1.29	8
Montemayor del Río, Sa	Sheep track	3	6	2	4
Muñovela, Sa	*Quercus ilex* grassland	6	34	5.67	19
Puente Mocho, Sa	River bank	12	36	3	21
Sagos, Sa	*Quercus ilex* grassland	2	5	2.50	4
Valvellidos, Ca	Meadow	18	25	1.39	16
Villafranca de la Sierra, Av	River bank	9	10	1.11	7

Note: [a]Provinces of Co: La Coruña, Sa: Salamanca, Ca: Cáceres, Av: Ávila.

samples from fructifications in dry culms were obtained using needles, or excising fructifications, cleaning them on water agar, and plating.

In order to induce sporulation in isolates not producing spores in the PDA medium, fungi were cultured in three other media: malt extract agar, water agar, and water agar containing sterilized pieces of leaves of *Dactylis glomerata*. These growth media also contained 200 mg/l of chloramphenicol.

To test whether the disinfection methods were effective in eliminating surface fungi, imprints of leaf fragments were made by pressing them against the surface of some PDA plates, then these plates were incubated without plant parts. The plates were periodically observed to determine if fungi emerged from the prints (Schulz *et al*., 1998).

DNA amplification and sequencing

Because many isolates failed to sporulate on any growth medium, identifications were approximated by means of the nucleotide sequence of the ITS1-5.8S rRNA-ITS2 region. DNA was extracted from small mycelial fragments scraped from the surface of culture plates using a commercial kit

(RedExtract-N-Amp Plant PCR, Sigma Aldrich). One volume of phenol saturated with 10 mM Tris-HCl pH 8 was added to the DNA extract obtained with the kit, and the aqueous phase was recovered after centrifugation at 13,000 × g for 10 minutes. This phase was reextracted with one volume of chloroform, centrifuged at 13,000 × g for 5 minutes, and the aqueous extract containing DNA was used for PCR amplification. The ITS1-5.8S rRNA-ITS2 region was amplified in a PCR which included 2 μl of DNA extract and primers ITS4 and ITS5 (White *et al*., 1990). Amplification conditions were: 95ºC for 2 min, followed by 35 cycles of 94ºC for 1 min, 54ºC for 1 min, and 72ºC for 1 min; after these cycles the reaction was kept at 72ºC for 10 minutes. PCR amplicons were purified by filtration (Montage PCR, Millipore), and sequenced in a 3100 Genetic Analyzer (Applied Biosciences). Only one strand of the PCR amplicon was sequenced. The sequencing reaction was started at the 5' end of the ITS1-5.8S rRNA-ITS2 region, using primer ITS4. The quality of the sequences obtained was analyzed by means of the sequencing reaction chromatograms, visualized with Chromas 1.45 software (Technelysium, Australia). Only sequences whose chromatograms showed discrete peaks, and no ambiguous sections were used.

For a subset of 12 isolates, both strands of the ITS amplicons were sequenced using primers ITS4 and ITS5 (Table 2). These complete ITS1-5.8S rRNA-ITS2 sequences were used to analyze the reliability of the taxonomic information obtained with the corresponding partial one-sided sequences.

Nucleotide sequences were trimmed at the 5' end of the ITS1 region. In most sequences the beginning of this region was identified by means of the conserved sequence GATCAT, which is found at the end of the 18S rRNA gene. The 3' end of each sequence was trimmed at places where the sequence chromatogram showed that the sequence quality was good, and not ambiguous.

Molecular taxonomy

To find ITS sequences similar to the ones obtained from the *Dactylis* isolates, the FASTA algorithms (Pearson, 1990) were used to interogate the EMBL/Genbank database of fungal nucleotide sequences.

To visualize the diverse fungal taxa identified by means of molecular characters, a sequence similarity dendrogram was made with the ITS1-5.8S rRNA-ITS2 nucleotide sequences of the isolates. Isolate sequences were aligned using the program ClustalX (Thompson *et al*. 1997) with the default settings, and the dendrogram was made with MEGA 3.1 using the neighb our-

Table 2. Value of the partial sequences lacking part of the 3' end of ITS2 region for isolate identification.

Isolate accession number	Partial sequence size[a] (nt)	Complete sequence Size[b] (nt)	FASTA ID obtained with partial sequence	E[c] value	FASTA ID obtained with complete sequence	E value
AM262408	353	472	*Beauveria bassiana*	1.9e-71	*Beauveria bassiana*	3.2e-70
AM262444	503	594	*Mortierella alpina*	3.8e-84	*Mortierella alpina*	2.3e-90
AM262418	468	482	*Embellisia eureka*	5.8e-66	*Embellisia eureka*	4.8e-72
AM262430	520	535	*Helgardia anguioides*	2.5e-66	*Helgardia anguioides*	2.3e-77
AM262441	452	499	*Rhodotorula minuta*	1.3e-58	*Rhodotorula minuta*	6.4e-69
AM262371	479	517	*Valsa ceratosperma*	6.3e-53	*Valsa ceratosperma*	3.1e-67
AM262439	483	604	*Mycena murina*	5.5e-76	*Mycena murina*	9.8e-93
AM262979	535	547	*Ustilago williamsii*	1.9e-57	*Ustilago williamsii*	1.7e-57
AM262403	452	500	*Epacrid* root endophyte	2.4e-45	*Epacrid* root endophyte	3.5e-52
AM262343	415	507	*Talaromyces ohiensis*	5.9e-36	*Talaromyces ohiensis*	2.9e-41
AM262424	457	466	*Eurotium amstelodami*	1.7e-59	*Eurotium amstelodami*	6.8e-61
AM262431	479	515	*Rhizosphaera kalkhoffii*	6e-53	*Rhizosphaera kalkhoffii*	1.2e-54

Note: [a]Partial sequences were obtained with sequencing reactions primed with primer ITS4 (White *et al*., 1990), which produces sequences with characteristics like those shown in Table 5.[b]Complete sequences were obtained by sequencing with primers ITS4 and ITS5 both strands of the replicon containing the ITS1-5.8S rRNA-ITS2 region. [c]Number of database matches as good as the observed one which could occur by chance.

joining method with Kimura 2-parameter distances (Kumar *et al*., 2004). Groups of sequences at close proximity within the same branch of the dendrogram were individually aligned with ClustalX to determine their percentage of similarity. Because for most fungal species the range of intraspecific variation in ITS sequences is unknown (Taylor *et al*., 2000), sequences with a similarity greater than 97% were considered to belong to the same species. This distance is an arbitrary number which has been used in other studies (O'Brien *et al*., 2005; Neubert *et al*., 2006; Higgins *et al*., 2007).

Quantification of fungal diversity

Species accumulation curves, showing the relationship between the number of plants sampled and the number of fungal species identified, were made by random sampling without replacement of the fungal species data obtained from each plant sample (Colwell and Coddington, 1994). These calculations were made with EstimateS 7.5 software (Colwell, 2005). Species accumulation curves were also plotted with a data set which only contained plural species, represented by more than one isolate, and with a dataset of singleton species, each represented by a single isolate.

To estimate the possible total number of endophytic species which could be associated to *Dactylis glomerata*, the incidence-based coverage estimator (ICE), and the Chao 2 estimator of total species richness were calculated (Chazdon *et al*., 1998).

Shannon's index of diversity (H') was estimated from the relative abundance of each taxon identified (Zak and Willig, 2004).

Results

Isolation and morphological characteristics of fungi

Fungi did not grow out of plates where leaf imprints were made (Schulz *et al*., 1998), indicating that the surface sterilization methods efficiently eliminated epiphytes, and the fungal isolates obtained correspond to fungi with an endophytic growth habit. This is an excellent method for testing protocols for isolating endophytes and should be used in all endophyte studies.

From a total of 120 field-sampled plants, approximately 1400 isolates were obtained. An initial visual screening was carried out to avoid selecting several identical isolates from the same plant. As a result, a total of 316 fungal isolates were selected and identified (Tables 1 and 3). On the average, 2.63 species were identified on each plant, and only 13 plants did not yield any

Table 3. Endophytic isolates identified by means of morphological and/or molecular characters, and isolates which could not be identified due to sterility and low homology to known nucleotide sequences, or high homology to sequences of unknown fungi in the EMBL fungi database.

Isolate accession number	**Morphological identification**	***Sequence-based identification*[a]**	**% FASTA identity**[a]	**Proposed identification**	**Presence in leaves**	**Presence in roots**
176	*Cladosporium* sp.	*n.s.*[c]	-	*Cladosporium* sp.	17	3
AM262430	*Helgardia* sp.	*Helgardia* sp.	96.95	*Helgardia* sp.	11	7
AM262390	*Acremonium* sp.	*Acremonium strictum*	99.80	*Acremonium strictum*	17	0
1471	*Penicillium* sp.	n.s.[c]	-	*Penicillium* sp.	6	10
1463	*Epicoccum* sp.	n.s.[c]	-	*Epicoccum* sp.	10	4
1365	*Podospora* sp.	n.s.[c]	-	*Podospora* sp.	9[f]	3
1794	*Phaeosphaeria* sp.	n.s.[c]	-	*Phaeosphaeria* sp.	8[f]	2
AM262420	*Epichloë typhina*	*Epichloë typhina*	100	*Epichloë typhina*	8	0
AM262425	*Fusarium* sp.	*Fusarium* sp.	100	*Fusarium* sp. A	5	3
AM262393	*Alternaria* sp.	Alternaria *sp*.	100	Alternaria *sp*.	7	0
AM262400	*Chaetomium* sp.	*Chaetomium* sp.	99.60	*Chaetomium* sp. A	6[f]	1
AM262435	Sterile mycelium	*Lewia infectoria*	99.82	*Lewia infectoria*	5	2
AM262340	*Microdochium phragmitis*	*Microdochium phragmitis*	100	*Microdochium phragmitis*	6	0
AM262407	Sterile mycelium	Coniothyrium cereale	100	Coniothyrium cereale	5	0
AM262414	Sterile mycelium	*Drechslera* sp.	99.83	*Drechslera* sp.	2	3
AM262433	Sterile mycelium	*Leptodontidium orchidicola*	98.38	*Leptodontidium orchidicola*	3	2
AM262426	*Fusarium* sp.	*Fusarium culmorum*	100	*Fusarium culmorum*	3	1
AM262344	*Penicillium sp*.	*Penicillium* sp.	99.04	*Penicillium* sp. A	1	3
AM262345	*Penicillium sp*.	*Penicillium* sp.	98.85	*Penicillium* sp. B	2	2
AM262347	*Penicillium sp*.	*Penicillium* sp.	99.61	*Penicillium* sp. D	2	2
AM262348	*Penicillium sp*.	Penicillium *sp*.	100	Penicillium *sp. E*	2	2
AM262351	Sterile mycelium	*Phaeosphaeria* sp.	99.60	*Phaeosphaeria* sp. A	2	2
AM262405	*Colletotrichum sp*.	*Glomerella* sp.	97.33	*Glomerella* sp.	3	0

Table 3. continued. Endophytic isolates identified by means of morphological and/or molecular characters, and isolates which could not be identified due to sterility and low homology to known nucleotide sequences, or high homology to sequences of unknown fungi in the EMBL fungi database.

Isolate accession number	**Morphological identification**	***Sequence-based identification*[a]**	**% FASTA identity**[a]	**Proposed identification**	**Presence in leaves**	**Presence in roots**
AM262408	*Beauveria bassiana*	*Cordyceps bassiana*	100	*Cordyceps bassiana*	3	0
AM262416	*Drechslera biseptata*	*Drechslera biseptata*	99.82	*Drechslera biseptata*	3[f]	0
AM262417	Sterile mycelium	*Drechslera dactylidis*	99.82	*Drechslera dactylidis*	2	1
AM262428	*Fusarium* sp.	*Fusarium oxysporum*	99.36	*Fusarium oxysporum*	1	2
AM262434	Sterile mycelium	*Leptosphaeria* sp.	99.58	*Leptosphaeria* sp.	3	0
AM262360	Sterile mycelium	*Podospora decipiens*	100	*Podospora decipiens*	3[f]	0
AM262370	*Trichoderma* sp.	*Trichoderma viride*	100	*Trichoderma viride*	2	1
AM262392	*Acremonium* sp. B[b]	*Nectria mauritiicola*	91.37	*Acremonium* sp. B	2	0
AM262394	*Arthrinium* sp.	*Arthrinium* sp.	92.62	*Arthrinium* sp. A	2	0
AM262395	*Arthrinium* sp.	*Arthrinium* sp.	100	*Arthrinium* sp. B	2	0
AM262410	Sterile mycelium	*Cyathicula* sp.	97.70	*Cyathicula* sp.	1[f]	1
AM262412	Sterile mycelium	*Davidiella tassiana*	100	*Davidiella tassiana*	2	0
AM262422	*Penicillium* sp.	*Eupenicillium* sp.	98.43	*Eupenicillium* sp.	0	2
AM262443	*Laetisaria arvalis*[b, d]	*Amauroderma subresinosum*	77.15	*Laetisaria arvalis*	2	0
AM262343	*Paecilomyces* sp.[b]	*Talaromyces ohiensis*	94.63	*Paecilomyces* sp.	2	0
AM262346	*Penicillium sp.*	*Penicillium* sp.	99.81	*Penicillium* sp. C	0	2
AM262353	Sterile mycelium	*Phaeosphaeria avenaria*	98.54	*Phaeosphaeria avenaria*	2	0
AM262364	*Sordaria* sp.	*Sordaria macrospora*	99.81	*Sordaria macrospora*	0	2
AM262368	Sterile mycelium	*Stemphylium solani*	99.23	*Stemphylium solani*	2	0
AM262371	Sterile mycelium	*Valsa* sp.	95.65	*Valsa* sp.	2	0
AM262391	*Acremonium* sp. A[b]	Nectria mauritiicola	89.72	*Acremonium* sp. A	1	0
1521	*Arthrinium* sp.	*n.s.*[c]	-	*Arthrinium* sp.	1	0
AM262396	Sterile mycelium	*Ascochyta* sp.	96.15	*Ascochyta* sp.	1	0

Table 3. continued. Endophytic isolates identified by means of morphological and/or molecular characters, and isolates which could not be identified due to sterility and low homology to known nucleotide sequences, or high homology to sequences of unknown fungi in the EMBL fungi database.

Isolate accession number	**Morphological identification**	***Sequence-based identification*[a]**	**% FASTA identity**[a]	**Proposed identification**	**Presence in leaves**	**Presence in roots**
AM262397	*Aspergillus* sp.	*Aspergillus terreus*	99.18	*Aspergillus terreus*	0	1
AM262398	*Auxarthron compactum*?	*Auxarthron conjugatum*	99.78	*Auxarthron conjugatum*	1	0
AM262399	*Phialophora-like anamorph*	*Calycina herbarum*	98.64	*Calycina herbarum*	1	0
AM262401	*Chaetomium* sp.	*Chaetomium* sp.	95.10	*Chaetomium* sp. B	0	1
AM262402	*Chaetomium* sp.	*Chaetomium funicola*	98.65	*Chaetomium funicola*	1	0
AM262403	*Chloridium* sp.[b]	*Epacrid* root endophyte	91.45	*Chloridium* sp.	1	0
AM262404	Sterile mycelium	*Cladosporium oxysporum*	100	*Cladosporium oxysporum*	1	0
AM262406	*Coniochaeta* sp.[b]	Ascomycete sp.	92.55	*Coniochaeta* sp.	0	1
AM262409	*Libertella* anamorph of *Creosphaeria sassafras*	*Creosphaeria sassafras*	99.78	*Creosphaeria sassafras*	1	0
AM262437	Pink yeast	*Cryptococcus* sp.[d]	99.09	*Cryptococcus* sp.	1	0
AM262436	Pink yeast	*Cryptococcus paraflavus*[d]	99.02	*Cryptococcus paraflavus*	1	0
AM262445	*Cunninghamella elegans*	*Cunninghamella elegans*[e]	99.50	*Cunninghamella elegans*	0	1
AM262411	*Cylindrotrichum* sp.[b]	*Glomerella cingulata*	85.06	*Glomerella cingulata*		
AM262438	Orange yeast	*Cystofilobasidium macerans*	100	*Cystofilobasidium macerans*	1	0
AM262413	Coelomycete	*Discula quercina*	100	*Discula quercina*	1[f]	0
AM262415	Sterile mycelium	*Drechslera andersenii*	100	*Drechslera andersenii*	1	0
AM262418	Sterile mycelium	*Embellisia* sp.	98.44	*Embellisia* sp.	1	0
AM262419	*Engydontium album*	*Engydontium album*	99.43	*Engydontium album*	1	0
AM262421	*Epicoccum* sp.	*Epicoccum nigrum*	99.80	*Epicoccum nigrum*	1	0
AM262423	*Penicillium* sp.	*Eupenicillium tropicum*	99.73	*Eupenicillium tropicum*	0	1

Table 3. continued. Endophytic isolates identified by means of morphological and/or molecular characters, and isolates which could not be identified due to sterility and low homology to known nucleotide sequences, or high homology to sequences of unknown fungi in the EMBL fungi database.

Isolate accession number	Morphological identification	*Sequence-based identification*[a]	% FASTA identity[a]	Proposed identification	Presence in leaves	Presence in roots
AM262424	*Eurotium amstelodami*	*Eurotium amstelodami*	99.41	*Eurotium amstelodami*	0	1
AM262427	*Fusarium* sp.	*Fusarium equiseti*	100	*Fusarium equiseti*	0	1
AM262429	*Fusarium* sp.	*Fusarium poae*	98.67	*Fusarium poae*	0	1
AM262431	*Hormonema* sp.[b]	*Rhizosphaera kalkhoffii*	91.15	*Hormonema* sp.	1[f]	0
AM262432	Sterile mycelium	*Lachnum pygmaeum*	97.61	*Lachnum pygmaeum*	0	1
AM262444	*Mortierella alpina*	*Mortierella alpina*[e]	99.35	*Mortierella alpina*	1	0
AM262439	Basidiomycete	*Mycena* sp.[d]	95.10	*Mycena* sp.	0	1
AM490816	*Aspergillus fumigatus*	*Neosartorya* sp.	98.43	*Aspergillus fumigatus*	0	1
AM262341	*Nigrospora* sp.	Fungal endophyte	96.77	*Nigrospora* sp.	1	0
AM262342	*Oidiodendron* sp.	*Oidiodendron* sp.	99.54	*Oidiodendron* sp.	0	1
AM262349	Sterile mycelium	*Periconia macrospinosa*	100	*Periconia macrospinosa*	0	1
AM262350	*Phaeoacremonium* sp.	*Phaeoacremonium rubrigenum*	99.78	*Phaeoacremonium rubrigenum*	1	0
AM262352	Sterile mycelium	*Phaeosphaeria* sp.	95.05	*Phaeosphaeria* sp. B	1	0
AM262354	Sterile mycelium	*Phoma* sp.	98.93	*Phoma* sp.	1	0
AM262355	*Phoma* sp.	*Phoma exigua*	99.78	*Phoma exigua*	1	0
AM262356	*Phomopsis* sp.	*Phomopsis* sp.	99.38	*Phomopsis* sp. A	0	1
AM262357	*Phomopsis sp*.	*Phomopsis* sp.	96.23	*Phomopsis* sp. B	0	1
AM262358	Sterile mycelium	*Podospora* sp.	95.26	*Podospora* sp.	0	1
AM262359	*Podospora* sp.	*Podospora coprophila*	99.80	*Podospora coprophila*	0	1
AM262361	Sterile mycelium	*Podospora tetraspora*	99.59	*Podospora tetraspora*	1	0
AM262362	*Pseuderotium sp*.	*Pseuderotium bakeri*	100	*Pseuderotium bakeri*	0	1
AM262440	Unidentified yeast	*Rhodotorula bacarum*[d]	99.39	*Rhodotorula bacarum*	1	0
AM262441	Unidentified yeast	*Rhodotorula minuta*[d]	99.79	*Rhodotorula minuta*	1	0

Table 3. continued. Endophytic isolates identified by means of morphological and/or molecular characters, and isolates which could not be identified due to sterility and low homology to known nucleotide sequences, or high homology to sequences of unknown fungi in the EMBL fungi database.

Isolate accession number	**Morphological identification**	***Sequence-based identification*[a]**	**% FASTA identity**[a]	**Proposed identification**	**Presence in leaves**	**Presence in roots**
AM262363	*Sagenomella* sp.[b]	*Talaromyces purpureus*	85.83	*Sagenomella* sp.	0	1
AM262367	Sterile mycelium	*Stagonospora arenaria*	99.50	*Stagonospora arenaria*	1	0
AM262365	Sterile mycelium	*Stagonospora sp*.	98.92	*Stagonospora sp*. A	1	0
AM262366	Sterile mycelium	*Stagonospora* sp.	95.20	*Stagonospora* sp. B	1	0
AM262369	*Lecanicillim lecanii*	*Torrubiella confragosa*	99.24	*Torrubiella confragosa*	1	0
AM262442	Basidiomycete	*Trametes versicolor*[d]	99.27	*Trametes versicolor*	1	0
148	*Ulocladium* sp.	n.s.[c]	-	*Ulocladium* sp.	1	0
AM262979	Unidentified yeast	*Ustilago* sp.[d]	95.04	*Ustilago* sp.	1	0
AM262387	Yeast-like anamorph	Calycina herbarum	92.01	Unknown Ascomycete 1	1	0
AM262372	Sterile mycelium	*Stenella araguata*	81.92	Unknown Ascomycete 2	1	0
AM262373	Sterile mycelium	Dactylaria ampulliformis	79.38	Unknown Ascomycete 3	1	0
AM262374	Sterile mycelium	Magnaporthe grisea	91.22	Unknown Ascomycete 4	1	0
AM262377	Sterile mycelium	Ascomycete sp.	97.20	Unknown Ascomycete 5	1	0
AM262375	Sterile mycelium	Fungal endophyte	90.68	Unknown Ascomycete 6	1	0
AM262376	*Xylariaceae*	*Xylaria cornu-damae*	89.58	Unknown Ascomycete 7	1	0
AM262389	Sterile mycelium	Verticillium *sp*.	100	Unknown Ascomycete 8	1	0
AM262378	*Acremonium* sp.	Acremonium strictum	74.94	Unknown Ascomycete 9	1	0
AM262379	Sterile mycelium	*Cistella grevillei*	92.34	Unknown Ascomycete 10	1	0
AM262380	Sterile mycelium	*Stachybotrys cylindrospora*	71.68	Unknown Ascomycete 11	1	0
AM262388	*Acremonium* sp.	Leaf litter Ascomycete	79.57	Unknown Ascomycete 12	1	0
AM262381	Sterile mycelium	Leaf litter Ascomycete	92.75	Unknown Ascomycete 13	1	0
AM262385	Sterile mycelium	Ascomycete sp.	90.82	Unknown Ascomycete 14	1	0
AM262382	Sterile mycelium	Fungal endophyte	90.65	Unknown Ascomycete 15	0	1

Table 3. continued. Endophytic isolates identified by means of morphological and/or molecular characters, and isolates which could not be identified due to sterility and low homology to known nucleotide sequences, or high homology to sequences of unknown fungi in the EMBL fungi database.

Isolate accession number	Morphological identification	*Sequence-based identification*[a]	% FASTA identity[a]	Proposed identification	Presence in leaves	Presence in roots
AM262383	Sterile mycelium	Podospora cochleariformis	94.78	Unknown Ascomycete 16	1	0
AM262384	Sterile mycelium	*Epacris microphylla* root associated fungus	99.54	Unknown Ascomycete 17	1	0
AM262386	Sterile mycelium	Bamboo Basidiomycete	95.04	Unknown Basidiomycete	1	0

Note: [a]Similarity to nucleotide sequences stored in the EMBL/Genbank database of fungal sequences was the criteria used to adscribe most isolates to a taxonomic group. Nucleotide sequences were searched with FASTA program. [b]Morphological identification was considered the correct option in cases where the database match is a different taxon and similarity is less than 95%. [c](n.s.: not sequenced). [d,e]All species in the list are ascomycetes, except for nine basidiomycetes [d], and two zygomycetes[e]. [f] Isolates were obtained from stem samples of 7 plants. For the five taxa showing more than one isolate, isolates were also obtained from leaf samples.

endophytes. On the PDA plates, fungi grew out of the plant fragments relatively fast; most isolates emerged in less than 10 days after the placement of plant samples on the plates.

Only 18% of the isolates obtained produced spores on PDA medium during the period of 6 to 8 weeks after isolation. The remaining isolates produced sterile mycelia. When sterile isolates were plated again on additional media, particularly on water agar with pieces of *D. glomerata* leaves, more isolates sporulated and could be morphologically identified. In total, 53% of all endophytic species could be identified by morphological characters. If the isolates identified as "unknown fungi" are excluded from the count (Table 3, bottom), then, 66% of the species could be identified with the use of phenotypic characters.

Molecular identification of isolates

The partial sequences obtained contained the complete nucleotide sequence of ITS1 and 5.8S rRNA, but most of them were incomplete at the 3' end of the ITS2 region. On the average, the sequences contained about 92% of the total ITS2 sequence (Table 4). In order to test if partial sequences of these characteristics were reliable for isolate identification, complete sequences were obtained for a subset of 12 isolates randomly chosen. In these 12 cases, the entry retrieved with FASTA from the EMBL/Genbank database was the same using a partial or a complete sequence (Table 2). This result suggests that partial sequences missing information at the 3' end may be as reliable as the complete versions for approximating an identification.

The following criteria were used to interpret matches provided by FASTA search of the EMBL fungal database: when sequence identity was greater than 97%, genus and species of the database result were accepted, when identity was 97 to 95%, only genus was accepted; when identity was less than 95% isolates were labelled as "unknown fungus". Nevertheless, there were situations where similarity values were almost equally high for several species, in those cases species rank was doubtful and not accepted.

There were several cases in which nucleotide sequence homology was low (<95%), and the taxa indicated by the sequence did not correspond to the morphological identification. In such cases, the morphological identification was accepted.

A sequence similarity dendrogram consisting of all sequences was used to identify groups of very similar sequences. Each branch clustering very similar sequences was analyzed, and sequences differing by less than 3% were considered to belong to the same species. Establishing species differences

Table 4. A sample of 16 randomly chosen sequences showing the percentage of ITS1, 5.8S rRNA, ITS2, obtained with a one-sided sequencing reaction and percentage of the total nucleotide sequence obtained.

Isolate accession number	% sequence obtained*			% of total sequence	EMBL reference sequence
	ITS1	5.8rRNA	ITS2		
AM262369	100	100	100	100	AB079127
AM262367	100	100	95.2	98.4	SAU77360
AM262417	100	100	88.3	96.1	AY004781
AM262413	100	100	100	100	AY853199
AM262394	100	100	89.5	96.5	ASP279479
AM262445	100	100	91.1	97	AF346409
AM262405	100	100	100	100	AB233343
AM262407	100	100	100	100	CCE293812
AM262347	100	100	100	100	AY373928
AM262402	100	100	100	100	CFU279450
AM262409	100	100	72.1	917	AJ390425
AM262349	100	100	96.1	98.7	PMA246159
AM262430	100	100	86.8	95.6	AY266144
AM262419	100	100	82.4	94.1	AF346409
AM262397	100	100	83.1	94.4	AJ413985
AM262357	100	100	92.1	97.4	AJ246145
Average	**100**	**100**	**92.3**	**97.5**	

*To estimate the percentage of the total sequence obtained, each partial sequence was compared to the complete (ITS1-5.8S rRNA-ITS2) reference sequence of the most similar EMBL database entry.

among sequences differing by more than 3% implied that in genera such as *Arthrinium*, *Chaetomium*, *Penicillium*, *Phaeosphaeria*, *Stagonospora*, and *Phomopsis*, multiple species were encountered, and isolates were grouped in different species denominated A, B, C, etc. (Table 3).

Using morphological and molecular characteristics for identification, 91 different species of fungi belonging to 63 genera could be identified (Tables 3 and 6). An additional set of sterile fungi belonging to 18 different taxa could not be identified because they had sequences different to any entry from the EMBL fungal database, or were similar to entries not assigned to any taxonomic group (Table 3, bottom). In total, 316 isolates representing 109 different species were obtained from the 107 plants infected by endophytes. Nucleotide sequences of each species were submitted to the EMBL/Genbank nucleotide database.

With respect to the plant tissue infected by the endophytes, 48 of the 91 identified species were found on leaves, 22 only on roots, and 21 species were found in both above and belowground parts (Table 3). Eight species were isolated from a set of stem samples of 7 plants. Three of these species were obtained only from the stem samples, isolates of the other five were also obtained from leaf samples (Table 3). Only 82 of the 120 plants had their roots plated for endophyte isolation. Therefore, the number of species obtained from aerial parts was probably proportionally greater than the number of species obtained from roots. However, the surface sterilization method used for roots was more aggressive than that used for leaves, and perhaps killed endophytes living close to the root surface.

The fungi isolated from diseased tissue obtained from plants showing symptoms, and from fructifications in dry culms are listed in Table 5.

Species diversity of the endophytic mycobiota

Most species identified were ascomycetes, only 9 species of basidiomycetes and 2 of zygomycetes were identified (Table 3). The identified Ascomycetes belonged to 54 different genera, and most could be grouped within 22 families (Table 6).

Seventy species were singletons, represented by only one isolate, and 39 species were plural, sampled more than once. The cumulative species curve calculated from all isolates (Fig. 1, curve a) suggests that increasing the number of plants analyzed would yield additional species. However, when a cumulative species curve was plotted with data from plural species, the curve approached asymptotic growth (Fig. 1, curve c). On the other hand, the shape of the species accumulation curve plotted for singleton species (Fig. 1, curve b) resembled the non-asymptotic curve obtained for all species.

The genera most abundant in terms of the number of isolates collected were: *Penicillium* (34 isolates), *Cladosporium* (21 isolates), *Acremonium* (20), *Helgardia* (18), *Podospora* (18), *Fusarium* (17), *Phaeosphaeria* (17), *Epicoccum* (15), *Epichloë* (8), *Alternaria* (7), *Chaetomium* (9), and *Lewia* (7). These 12 genera accounted for 57% of all isolates obtained, but represented only 25% of all species recorded.

Estimates of total species richness ranged from 261.52 (ICE) to 326 (Chao 2 estimator) When the values of ICE and Chao 2 estimators for each number of plant samples were plotted, none of them became asymptotic.

Shannon's index of diversity equalled 4.27 when all 109 fungal species were considered, and 3.45 when calculated only for the subgroup of plural species represented by more than one isolate. These values appear to be as high

as other endophytic communities, and suggested that this grass represents an ecosystem rich in endophytic mycobiota (Zak and Willig, 2004; Higgins *et al*., 2007).

Table 5. Fungi isolated from lesion margins in diseased plants and from fructifications in dry culms.

On lesions	On dry culms
Alternaria sp.	*Alternaria sp*.
Ampellomyces humuli	*Ampellomyces humuli*
Cercospora sp.	*Cladosporium sp*.
Cladosporium sp.	*Colletotrichum acutatum*
Colletotrichum acutatum	*Dreschlera dactylidis*
Colletotrichum falcatum	*Epicoccum sp*.
Drechslera sp.	*Epicoccum nigrum*
Drechslera biseptata	*Glomerella acutata*
Dreschlera dactylidis	*Fusarium poae*
Dothideales sp.	*Fusarium lateritium*
Embellisia eureka	*Hypocrea sp*.
Epichloë typhina	*Lewia infectoria*
Epicoccum nigrum	*Phaeosphaeria pontiformis*
Epicoccum sp.	*Phaeosphaeria sp*.
Fusarium lateritium	*Phomopsis sp*.
Fusarium poae	*Pyrenophora tritici-repenti*
Glomerella acutata	
Glomerella graminicola	
Helgardia sp.	
Hypocrea sp.	
Lewia infectoria	
Phaeosphaeria sp.	
Phaeosphaeria avenaria	
Phaeosphaeria pontiformis	
Phoma sp.	
Phoma glomerata	
Phoma exigua	
Phomopsis sp.	
Rhexoscercosporidium sp.	
Septoria passerinii	
Stagonospora arenaria	
Stemphylium solani	
Torrubiella confragosa	

Discussion

In studies where ITS sequences are used to approximate identifications, the sequences are usually obtained by sequencing both complementary strands of a PCR replicon containing the ITS1-5.8S rRNA-ITS2 region (Guo *et al*. 2000, Wirsel *et al*., 2001). In the present study, nucleotide sequences were obtained from only one strand in a reaction driven by primer ITS4, upstream from the 5´end of ITS1. As shown in Table 4, the nucleotide sequences obtained by this method were missing approximately 10% of the 3' end of ITS2. However, a comparison of database results obtained with partial and complete sequences suggested that partial sequences were equally effective as the whole sequences for identification purposes (Table 2). Further evidence of the value of these partial sequences comes from the fact that there was agreement in the molecular and morphological identification, at least to genus rank, for all isolates whose identity to database entries was greater than 95% (Table 3). Therefore, although limited in value for rigorous phylogenetic analysis, partial sequences derived from single sequencing reactions can be useful for database interogation when large numbers of isolates are processed.

In this survey, 91 different species of endophytic fungi belonging to 63 genera were identified (Tables 3 and 6). Eighteen additional species, representing 16.5% of the total number of species, could not be identified because they were sterile hyphae, and their ITS sequences did not resemble any species identified in the EMBL/Genbank database (Table 3, bottom). It is possible that some of these unidentified species are known species whose ITS sequences are not included in the database. Other species from the list of unidentified isolates may be truly unknown. Such results argue in favor of the potential of endophytic ecosystems for harboring some of the numerous undocumented fungal species (Hawksworth and Rossman, 1997; Pinnoi *et al*., 2006).

In terms of isolate abundance, the *Dactylis* mycobiota ranged from a group of 70 singleton species, to a group of 39 plural species represented by 2 or more isolates. The shape of the species accumulation curves produced by the plural and singleton species data suggests that increasing sampling effort would yield new endophytic species (Fig. 1, curve a). However, the trend to an asymptotic curve seen in the case of plural species (Fig. 1, curve c) suggested that the sampling in this study detected most plural species associated with *Dactylis glomerata*. In contrast, the species accumulation curve of singleton species (Fig. 1, curve b) resembles the total species curve, showing a direct relationship between newly encountered fungal species and plants analyzed. This analysis of species accumulation curves suggested that sampling more

Table 6. Summary of endophytic taxa isolated from *Dactylis glomerata*.

PHYLUM/Order/*Family*	Number of genera	Number of species
ASCOMYCOTA		
Chaetosphaeriales		
Chaetosphaeriaceae	2	2
Coniochaetales		
Coniochaetaceae	1	1
Diaporthales		
Valsaceae	3	4
Dothideales		
Botryosphaeriaceae	1	1
Eurotiales		
Trichocomaceae	6	11
Helotiales		
Hyaloscyphaceae	2	2
Uncertain	1	1
Hypocreales		
Clavicipitaceae	4	4
Hypocreaceae	1	1
Nectriaceae	1	5
Mycosphaerellales		
Mycosphaerellaceae	2	2
Onygenales		
Onygenaceae	1	1
Phyllachorales		
Glomerellaceae	1	1
Pleosporales		
Leptosphaeriaceae	2	2
Phaeosphaeriaceae	2	6
Pleosporaceae	6	9
Sordariales		
Lasiophaeriaceae	3	8
Xylariales		
Hyponectriaceae	1	1
Xylariaceae	1	1
Uncertain		
Apiosporaceae	2	2
Dermataceae	1	1
Myxotrichaceae	1	1
Pseudeurotiaceae	1	1
Uncertain	8	12
BASIDIOMYCOTA		
Corticiaceae	1	1
Cystofilobasidiaceae	1	1
Polyporaceae	1	1
Tricholomataceae	1	1
Ustilaginaceae	1	1
Uncertain	2	4
ZYGOMYCOTA		
Mortierellaceae	1	1
Cunninghamellaceae	1	1
TOTAL	63	91

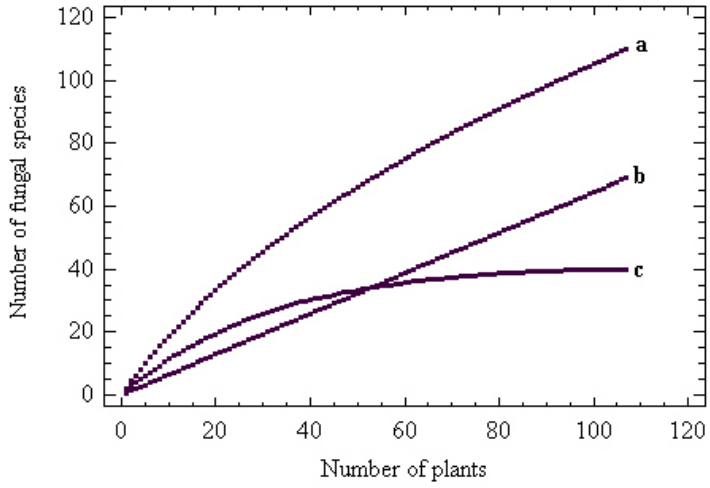

Fig. 1 Species accumulation curves showing the relationship between the number of plants analyzed and the total number of fungal species found (curve a). The curves for singleton (b) and plural (c) species were made with data subsets of species which were represented by one isolate (singletons), or of species represented by two or more isolates (plurals).

plants of *Dactylis glomerata* would yield new species, and the species found would most probably be singletons. Abundance of singleton species (Kauhanen *et al*., 2006; Neubert *et al*., 2006; Pinnoi *et al*., 2006), as well as non asymptotic collection effort curves have been found in other endophyte surveys (Higgins *et al*., 2007).

Estimators of total species richness can be used to predict where species accumulation curves may plateau. The estimates of the total species richness based on the data from Fig.1 (curve a) ranged from 326 (Chao 2 estimator) to 262 (ICE estimator). Because of the relatively high and constant proportion of singleton species, the curves produced by all estimators were non-asymptotic. Therefore, the values obtained should be interpreted as lower bound estimates of species richness (Gotelli and Coldwell, 2001).

The endophytic assemblage of *Dactylis* may be greater than what it is suggested by the estimates obtained. Technical constraints limited the number of endophytes identified; for example, some species may not have grown isolated with the media used, and obligate biotrophs could not be detected with the methods used. Nevertheless, methods useful to detect and identify

unculturable endophytes have been developed (Duong *et al*., 2006; Neubert *et al*., 2006).

Considering the number of endophytes identified, and the values for total species richness estimated for this grass, if the species richness of endophytic mycobiota were positively correlated to host plant size, many surveys of endophyte species in trees and shrubs probably underestimate the number of endophytic species (Stone *et al*., 2004). The shortfall may be due to the fact that exclusively morphological identification was used in many earlier studies, and sterile isolates were not identified.

Webster (1956, 1957) studied the fungi appearing on decaying *Dactylis* culms, and recorded the sequence in which those species appeared after seed development in inflorescences. The first species recorded on mature culms were *Cladosporium herbarum*, *Epicoccum purpurascens*, *Alternaria tenuis*, *Leptosphaeria microscopica* (= *Phaeosphaeria microscopica*), and *Pleospora vagans* (= *Phaeosphaeria vagans*). All these fungi belong to genera that we isolated frequently. Perhaps those early colonizers of decaying stems were already present in the living plants as endophytes; becoming saprophytes after stem senescence. We also isolated from dry culms fungi belonging to some of the genera of primary saprophytes described by Webster (Table 5).

The most extensive list of fungi identified on *D. glomerata* is a compilation of literature records made by Farr *et al*. (1989). Sixty-eight fungal species belonging to 41 genera were listed. Only 10 genera are common between that list and the one compiled in the present study: *Epichloë*, *Phaeosphaeria*, *Drechslera*, *Fusarium*, *Periconia*, *Ascochyta*, *Colletotrichum*, *Phoma*, *Stagonospora*, and *Ustilago*. In the list of Farr *et al*. (1989), species of the above genera are associated with disease symptoms in plants. Therefore, it is very likely that some of the endophytes of the above genera were latent or weak *Dactylis* pathogens. This fact is supported by the fact that, except for *Periconia*, species of all of the above genera were also isolated from lesions of diseased plants (Table 5).

Most of the species isolated from diseased tissues (Table 5) are pathogens of grasses (Mathre, 1982; Wiese, 1987, Farr *et al*., 1989). However, most of these species were also isolated from healthy plants (Table 3). Therefore, those fungi isolated from diseased and healthy plants may represent a group of latent pathogens.

Several genera of potential pathogens of cereal crops, e.g., wheat or barley, were present in asymptomatic plants of *Dactylis*. In those cereals, *Alternaria*, *Acremonium*, *Ascochyta*, *Aureobasidium*, *Cladosporium*, *Colletotrichum*, *Cryptococcus*, *Drechslera*, *Epicoccum*, *Fusarium*, *Laetisaria*, *Leptosphaeria*, *Microdochium*, *Phoma*, *Stagonospora*, *Trichoderma*,

Ulocladium, and *Ustilago* are associated to several diseases (Mathre, 1982; Wiese, 1987, Farr *et al*., 1989). Genera such as *Alternaria*, *Acremonium*, *Cladosporium*, *Epicoccum*, *and Fusarium*, were frequently isolated as *Dactylis* endophytes. In addition, *Helgardia* sp., a pathogen associated to eyespot disease of cereals (Crous *et al*., 2003) was one of the most abundant endophytes of *Dactylis*. Eighteen isolates of *Helgardia* sp., were obtained from plants at one location in La Coruña, five in Salamanca, and one in Ávila. *Helgardia* could be a pathogen of *Dactylis*, because it was also isolated from leaf lesions from diseased plants (Table 5). The above results implied that *D. glomerata*, a common grass species in Spain, could act as an alternative host and reservoir of potential pathogens of cereal crops.

The ascomycete predominance of the *Dactylis* mycobiota, and the presence of genera such as *Acremonium*, *Alternaria*, *Cladosporium*, *Coniothyrium*, *Epicoccum*, *Fusarium*, *Stagonospora*, *Penicillium*, *Phoma*, and *Phomopsis* are characteristics common to endophytic assemblages from many plant species (Bills, 1996; Stone *et al*., 2004; Schulz and Boyle, 2005). On the other hand, out of a group of 21 endophytic taxa that we could identify to species level, at least 6 appear to be specific of grasses: *Drechslera dactylidis*, *Epichloë typhina*, *Laetisaria arvalis*, *Periconia macrospinosa*, *Phaeosphaeria avenaria*, and *Stagonospora arenaria*; these species have not been described in hosts of other families (Farr *et al*., 1989).

Although ascomycetes seem to dominate endophytic assemblages (Stone *et al*., 2004; Duong *et al*., 2006; Ganley and Newcombe, 2006; Morakotkarn *et al*., 2006; Higgins *et al*., 2007), exceptions were basidiomycetes prevail have been described (Crozier *et al*., 2006). The incidence of Zygomycete endophytes appears to be very low (Gonthier *et al*., 2006).

Many endophytic genera described in other grasses such as *Phragmites australis* (Wirsel *et al*., 2001), *Achnatherum sibiricum* (Wei *et al*., 2007), or bamboo (Morakotkarn *et al*., 2006), were also present in *Dactylis*. In contrast, the endophytic assemblage of *Dactylis* is quite different from that of woody perennials: out of 68 genera described as endophytes of leaves of woody perennials, only eight were found in *Dactylis*; and of 97 genera from bark and shoots of trees, only 10 genera were present in this grass (Stone *et al*., 2004).

This study demonstrates that a small herbaceous plant can be considered to be an ecosystem which sustains a rich endophytic ensemblage. This mycobiota is composed of a relatively small number of species commonly associated with the host, including several potential pathogens, and a predominant background of singleton species. Most endophytes identified appear to be host-generalists, because they have been described in other plant families.

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