The endophytic community of *Dactylis glomerata*

S. SÁNCHEZ MÁRQUEZ[1], G.F. BILLS[2] and I. ZABALGOGEAZCOA[1]
[1]*Instituto de Recursos Naturales y Agrobiologia, CSIC, Salamanca, Spain*
[2]*Centro de Investigación Básica de España, Merck, Sharp & Dohme, Madrid, Spain*
izabalgo@usal.es

Abstract

Morphological and molecular methods were used to identify the endophytic mycobiota of the grass *Dactylis glomerata*. Fungal endophytes belonging to 109 different species were isolated from asymptomatic plants sampled in different ecosystems in Spain. Species accumulation curves showed that most species commonly infecting this grass have been identified, but the number of singleton species occasionally infecting the plants is likely 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 number of plants.

Keywords: endophytes, *Dactylis glomerata*, diversity, abundance

Introduction

Evidence accumulated in surveys suggests that all plant species are associated with fungal endophytes. High species diversity per plant species is a characteristic of the endophytic mycobiota (Stone *et al.* 2004). In grasses, systemic endophytes belonging to the *Epichloë* and *Neotyphodium* genera are well known. However, not much is known about other endophytic species associated with grasses. This paper describes a wide range of endophytic species associated with *Dactylis glomerata,* a grass common in many different ecosystems in Spain.

Methods

Plant collection and endophyte isolation

Asymptomatic plants of *Dactylis glomerata* were sampled at 10 locations in the province of Salamanca, one in Ávila, one in Cáceres, and two in La Coruña. These locations represent different habitats (e.g. semiarid grasslands, coastal meadows, roadsides, etc.).

To isolate endophytes, leaf and stem pieces were surface-disinfected with a solution of 1% active chlorine for 10 minutes. Root fragments were disinfected with a 5 minute rinse in ethanol, followed by treatment with a 1% active chlorine solution for 15 minutes, and 2 minutes in ethanol. The treated fragments were rinsed in sterile water and plated on potato dextrose agar (PDA) containing 200 mg/L of chloramphenicol.

To induce sporulation in sterile isolates in PDA medium, fungi were cultured in three other media: malt extract agar, water agar, and water agar containing autoclaved pieces of leaves of *Dactylis glomerata*.

DNA amplification and molecular taxonomy

DNA was extracted from mycelium with a commercial kit (RedExtract-N-Amp Plant PCR, Sigma Aldrich). The ITS1-5.8S rRNA-ITS2 region was amplified with a polymerase chain reaction (PCR) using primers ITS4 and ITS5 (White *et al.* 1990). Only one strand of the PCR amplicon was sequenced in a reaction started with primer ITS4. In subset of 12 isolates, both strands of the amplicons were sequenced; these complete sequences were used to analyse the reliability of the taxonomic information obtained with the corresponding partial one-sided sequences.

The FASTA algorithms (Pearson 1990) were used to interrogate the EMBL/Genbank database of fungal nucleotide sequences. 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 arbitrary distance has been used in other studies (O'Brien *et al*. 2005; Neubert *et al*. 2006).

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 using EstimateS 7.5 software (Colwell 2005). To estimate the possible total number of endophytic species which could be associated to *Dactylis glomerata*, the incidence-based coverage estimator (ICE), and the Chao2 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 & Willig 2004).

Results and Discussion

Isolation and identification of endophytes

From 120 plants, a total of 316 fungal isolates were identified. On the average, 2.63 species were identified on each plant; only 13 plants did not yield any endophytes.

Using morphological and molecular characteristics for identification, 91 different species of fungi belonging to 63 genera could be identified (Table 1). An additional set of sterile fungi belonging to 18 different species could not be identified because their sequences were different to any entry from the EMBL fungal database, or were similar to database entries corresponding to unidentified fungi.

In total, 53% of all endophytic species could be identified by morphological characters. If only the isolates that could be identified at the genus or species level are considered (Table 1), 66% of them could be identified with the use of phenotypic characters.

The partial sequences obtained contained the complete nucleotide sequence of ITS1 and 5.8S rRNA, but most were incomplete at the 3'end of the ITS2 region. On the average, these sequences contained about 92% of the total ITS2 sequence. When 12 partial sequences of these characteristics were compared to their corresponding complete sequences for identification purposes, in all cases, the entry retrieved with FASTA from the Genbank database was the same using a partial or a complete sequence. This result suggests that partial sequences missing information at the 3' end may be as reliable as the complete versions for approximating identification. 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 identities were similar to database entries and were greater than 95% (Table 1). Although limited in value for rigorous phylogenetic analysis, partial sequences derived from single sequencing reactions can be useful for database interrogation where large numbers of isolates are processed.

Species diversity of the endophytic mycobiota

Most species were ascomycetes, only nine species of basidiomycetes and two of zygomycetes were identified (Table 1). The identified ascomycetes belonged to 54 different genera, and most could be grouped within 22 families.

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 shows that this survey of the endophytic mycobiota is incomplete (Fig. 1). The non-asymptotic shape of the curve suggests that increasing the number of plants analysed would yield additional endophytic species. However, a cumulative species curve plotted with data from plural species approached asymptotic growth (Fig. 1). This curve suggested that most plural species, which could be considered as common endophytes of *Dactylis glomerata,* were identified. On the other hand, the shape of the species accumulation curve plotted with singleton species indicates that increased sampling effort will yield mainly singleton species (Fig. 1).

Estimates of total species richness ranged from 261.52 (ICE) to 326 (Chao 2 estimator). Because of the high number 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 & Coldwell 2001). The endophytic assemblage of *Dactylis* may be even greater than what the estimates suggest. Some species may not have grown isolated with the media used, and obligate biotrophs could not be detected with the methods used.

Shannon's index of diversity equalled 4.27 when all 109 fungal species were considered, and 3.45 when calculated for the subgroup of plural species. These values suggest that this grass represents an ecosystem rich in endophytic mycobiota.

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.

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*., 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.

Out of a group of 21 endophytic taxa that we could identify to species level, only 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).

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.

REFERENCES

Chazdon, R.L.; Colwell, R.K.; Denslow, J.S.; Guariguata, M.R. 1998. Statistical methods for estimating species richness of woody regeneration in primary and secondary rain forests of NE Costa Rica. pp. 285-309. *In*: Forest biodiversity research, monitoring and modelling: Conceptual background and Old World case studies. Eds. Dallmeier, F.; Comiskey, J.A. Parthenon Publishing, Paris.

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

Colwell, R.K.; Coddington, J.A. 1994. Estimating terrestrial biodiversity through extrapolation. *Philosophical Transactions of the Royal Society. London, Series B 345*: 101-118.

Farr, D.F.; Bills, G.F.; Chamuris, G.P.; Rossman, A.Y. 1989. Fungi on plants and plant products in the United States. American Phytopathological Society Press, St.Paul, Minnesota.

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

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.

O'Brien, H.E.; Parrent, J.L.; Jackson, J.A.; Moncalvo, J.M.; Vilgalys, R. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Applied and Environmental Microbiology 71*: 5544-5550.

Pearson, W.R. 1990. Rapid and sensitive sequence comparison with PASTP and FASTA. *Methods in Enzymology 183*: 63-98.

Stone, J.K.; Polishook, J.D.; White Jr., J.F. 2004. Endophytic fungi. pp. 241-270 *In*: Biodiversity of Fungi. Inventory and Monitoring Methods. Eds. Mueller. G.M.; Bills, G.F.; Foster, M.S. Elsevier Academic Press, San Diego.

Taylor, J.W.; Jacobson, D.J.; Kroken, S.; Kasuga, T.; Geiser, D.M.; Hibbett, D.S.; Fisher, M.C. 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology 31*: 21-32.

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.

White, T.J.; Bruns, T.; Lee, S.; Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315-322. *In*: PCR Protocols. A Guide to Methods and Applications. Eds. Innis, M.A.; Gelfland, D.H.; Sninsky, J.J.; White, T.J. Academic Press, San Diego.

Table 1 Endophytic species isolated from asymptomatic plants of *Dactylis glomerata*, and identified by means of morphological and/or molecular characters.

Isolate accession number	Morphological identification	Identification based on sequence similarity[a]	% FASTA identity	Presence in stems or leaves	Presence in roots	Isolates
AM262390	*Acremonium* sp.	*Acremonium strictum*	99.80	+	-	17
AM262391	*Acremonium* sp. A[b]	*Nectria mauritiicola*	89.72	+	-	1
AM262392	*Acremonium* sp. B[b]	*Nectria mauritiicola*	91.37	+	-	2
AM262393	*Alternaria* sp.	*Alternaria* sp.	100.00	+	-	7
1521	*Arthrinium* sp.	n.s.[c]	-	+	-	1
AM262394	*Arthrinium* sp.	*Arthrinium* sp. A	92.62	+	-	2
AM262395	*Arthrinium* sp.	*Arthrinium* sp. B	100.00	+	-	2
AM262396	Sterile mycelium	*Ascochyta* sp.	96.15	+	-	1
AM262397	*Aspergillus* sp.	*Aspergillus terreus*	99.18	-	+	1
AM262398	*Auxarthron compactum*?	*Auxarthron conjugatum*	99.78	+	-	1
AM262399	*Phialophora*-like anamorph	*Calycina herbarum*	98.64	+	-	1
AM262400	*Chaetomium* sp.	*Chaetomium* sp. A	99.60	+	+	7
AM262401	*Chaetomium* sp.	*Chaetomium* sp. B	95.10	-	+	1
AM262402	*Chaetomium* sp.	*Chaetomium* funicola	98.65	+	-	1
AM262403	*Chloridium* sp.[b]	*Epacrid* root endophyte	91.45	+	-	1
176	*Cladosporium* sp.	n.s.	-	+	+	20
AM262404	Sterile mycelium	*Cladosporium* oxysporum	100.00	+	-	1
AM262405	*Colletotrichum* sp.	*Glomerella* sp.	97.33	+	-	3
AM262406	*Coniochaeta* sp.[b]	Ascomycete sp.	92.55	-	+	1
AM262407	Sterile mycelium	*Coniothyrium cereale*	100.00	+	-	5
AM262408	*Beauveria bassiana*	*Cordyceps bassiana*	100.00	+	-	3
AM262409	*Libertella* anamorph of *Creosphaeria sassafras*	*Creosphaeria sassafras*	99.78	+	-	1
AM262437	Pink yeast	*Cryptococcus* sp.	99.09	+	-	1
AM262436	Pink yeast	*Cryptococcus paraflavus*	99.02	+	-	1
AM262445	*Cunninghamella elegans*	*Cunninghamella elegans*	99.50	-	+	1
AM262410	Sterile mycelium	*Cyathicula* sp.	97.70	+	+	2
AM262411	*Cylindrotrichum* sp.[b]	*Glomerella cingulata*	85.06			1
AM262438	Orange yeast	*Cystofilobasidium macerans*	100.00	+	-	1
AM262412	Sterile mycelium	*Davidiella tassiana*	100.00	+	-	2
AM262413	*Coelomycete*	*Discula quercina*	100.00	+	-	1
AM262414	Sterile mycelium	*Drechslera* sp.	99.83	+	+	5
AM262415	Sterile mycelium	*Drechslera andersenii*	100.00	+	-	1
AM262416	*Drechslera biseptata*	*Drechslera biseptata*	99.82	+	-	3
AM262417	Sterile mycelium	*Drechslera dactylidis*	99.82	+	+	3
AM262418	Sterile mycelium	*Embellisia* sp.	98.44	+	-	1
AM262419	*Engydontium album*	*Engydontium album*	99.43	+	-	1
AM262420	*Epichloë typhina*	*Epichloë typhina*	100.00	+	-	8
1463	*Epicoccum* sp.	*n.s*	-	+	+	14
AM262421	*Epicoccum* sp.	*Epicoccum nigrum*	99.80	+	-	1
AM262422	*Penicillium* sp.	*Eupenicillium* sp.	98.43	-	+	2
AM262423	*Penicillium* sp.	*Eupenicillium tropicum*	99.73	-	+	1

Isolate accession number	Morphological identification	Identification based on sequence similaritya	% FASTA identity	Presence in stems or leaves	Presence in roots	Isolates
AM262424	*Eurotium amstelodami*	*Eurotium amstelodami*	99.41	-	+	1
AM262425	*Fusarium* sp.	*Fusarium* sp. A	100.00	+	+	8
AM262426	*Fusarium* sp.	*Fusarium culmorum*	100.00	+	+	4
AM262427	*Fusarium* sp.	*Fusarium equiseti*	100.00	-	+	1
AM262428	*Fusarium* sp.	*Fusarium oxysporum*	99.36	+	+	3
AM262429	*Fusarium* sp.	*Fusarium poae*	98.67	-	+	1
AM262430	*Helgardia* sp.	*Helgardia* sp.	96.95	+	+	18
AM262431	*Hormonema* sp.[b]	*Rhizosphaera kalkhoffii*	91.15	+	-	1
AM262432	Sterile mycelium	*Lachnum pygmaeum*	97.61	-	+	1
AM262443	*Laetisaria arvalis*[b]	*Amauroderma subresinosum*	77.15	+	-	2
AM262433	Sterile mycelium	*Leptodontidium orchidicola*	98.38	+	+	5
AM262434	Sterile mycelium	*Leptosphaeria* sp.	99.58	+	-	3
AM262435	Sterile mycelium	*Lewia infectoria*	99.82	+	+	7
AM262340	*Microdochium phragmitis*	*Microdochium phragmitis*	100.00	+	-	6
AM262444	*Mortierella alpina*	*Mortierella alpina*	99.35	+	-	1
AM262439	*Basidiomycete*	*Mycena* sp.	95.10	-	+	1
1663	*Aspergillus fumigatus*	*Neosartorya* sp.	98.43	-	+	1
AM262341	*Nigrospora* sp.	Fungal endophyte	96.77	+	-	1
AM262342	*Oidiodendron* sp	*Oidiodendron* sp.	99.54	-	+	1
AM262343	*Paecilomyces* sp.[b]	Talaromyces ohiensis	94.63	+	-	2
1471	*Penicillium* sp.	n.s.	-	+	+	16
AM262344	*Penicillium* sp.	*Penicillium* sp. A	99.04	+	+	4
AM262345	*Penicillium* sp.	*Penicillium* sp. B	98.85	+	+	4
AM262346	*Penicillium* sp.	*Penicillium* sp. C	99.81	-	+	2
AM262347	*Penicillium* sp.	*Penicillium* sp. D	99.61	+	+	4
AM262348	*Penicillium* sp.	*Penicillium* sp. E	100.00	+	+	4
AM262349	Sterile mycelium	*Periconia macrospinosa*	100.00	-	+	1
AM262350	*PhaeoAcremonium* sp.	*PhaeoAcremonium rubrigenum*	99.78	+	-	1
1794	*Phaeosphaeria* sp.	*n.s.*	-	+	+	10
AM262351	Sterile mycelium	*Phaeosphaeria* sp. A	99.60	+	+	4
AM262352	Sterile mycelium	*Phaeosphaeria* sp. B	95.05	+	-	1
AM262353	Sterile mycelium	*Phaeosphaeria avenaria*	98.54	+	-	2
AM262354	Sterile mycelium	*Phoma* sp.	98.93	+	-	1
AM262355	*Phoma* sp.	*Phoma exigua*	99.78	+	-	1
AM262356	*Phomopsis* sp.	*Phomopsis* sp. A	99.38	-	+	1
AM262357	*Phomopsis* sp.	*Phomopsis* sp. B	96.23	-	+	1
1365	*Podospora* sp.	*n.s.*	-	+	+	12
AM262358	Sterile mycelium	*Podospora* sp.	95.26	-	+	1
AM262360	Sterile mycelium	*Podospora decipiens*	100.00	+	-	3
AM262359	*Podospora* sp.	*Podospora coprophila*	99.80	-	+	1
AM262361	Sterile mycelium	*Podospora tetraspora*	99.59	+	-	1
AM262362	*Pseuderotium* sp.	*Pseuderotium bakeri*	100.00	-	+	1
AM262440	Unidentified yeast	*Rhodotorula bacarum*	99.39	+	-	1
AM262441	Unidentified yeast	*Rhodotorula minuta*	99.79	+	-	1
AM262363	*Sagenomella* sp.[b]	*Talaromyces purpureus*	85.83	-	+	1
AM262364	*Sordaria* sp.	*Sordaria macrospora*	99.81	-	+	2
AM262367	Sterile mycelium	*Stagonospora arenaria*	99.50	+	-	1
AM262365	Sterile mycelium	*Stagonospora* sp. A	98.92	+	-	1

Isolate accession number	Morphological identification	Identification based on sequence similarity[a]	% FASTA identity	Presence in stems or leaves	Presence in roots	Isolates
AM262366	Sterile mycelium	*Stagonospora* sp. B	95.20	+	-	1
AM262368	Sterile mycelium	*Stemphylium solani*	99.23	+	-	2
AM262369	*Lecanicillium lecanii*	*Torrubiella confragosa*	99.24	+	-	1
AM262442	*Basidiomycete*	*Trametes versicolor*	99.27	+	-	1
AM262370	*Trichoderma* sp.	*Trichoderma viride*	100.00	+	+	3
148	*Ulocladium* sp.	*n.s.*	-	+	-	1
AM262979	Unidentified yeast	*Ustilago* sp.	95.04	+	-	1
AM262371	Sterile mycelium	*Valsa* sp.	95.65	+	-	2

[a] Similarity to nucleotide sequences stored in the EMBL/Genbank database of fungal sequences was the criteria used to ascribe most isolates to a taxonomic group. Nucleotide sequences were searched with FASTA program.
[b] Morphological identification is 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).

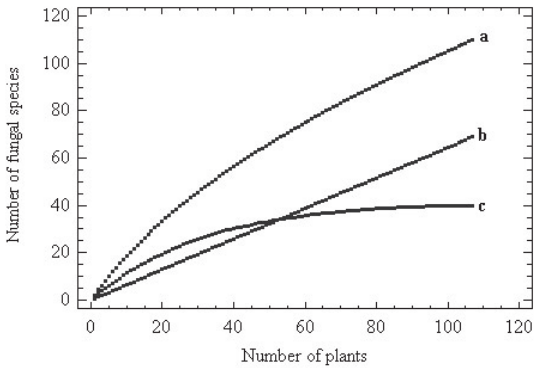

Figure 1 Species accumulation curves showing the relationship between the number of plants analysed and the total (a) number of fungal species found. The curves for singleton (b) and plural (c) species were made with data subsets of species which were represented by one isolate (singletons), or of species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