environmental microbiology

society for applied microbiology sfam

Environmental Microbiology (2016) **18**(8), 2418–2434 doi:10.1111/1462-2920.13112

The local environment determines the assembly of root endophytic fungi at a continental scale

Kyriaki Glynou,[1,2] Tahir Ali,[3] Ann-Katrin Buch,[2,3] Sevda Haghi Kia,[1,2] Sebastian Ploch,[3†] Xiaojuan Xia,[3] Ali Çelik,[4] Marco Thines[1,2,3] and Jose G. Maciá-Vicente[1,2*]

[1]*Institute of Ecology, Evolution and Diversity, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany.*

[2]*Integrative Fungal Research Cluster (IPF), Georg-Voigt-Str. 14-16, 60325 Frankfurt am Main, Germany.*

[3]*Biodiversity and Climate Research Centre (BiK-F), Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, 60325 Frankfurt am Main, Germany.*

[4]*Department of Biology, Faculty of Science and Arts, Pamukkale University, 20070 Denizli, Turkey.*

Summary

Root endophytic fungi are found in a great variety of plants and ecosystems, but the ecological drivers of their biogeographic distribution are poorly understood. Here, we investigate the occurrence of root endophytes in the non-mycorrhizal plant genus *Microthlaspi*, and the effect of environmental factors and geographic distance in structuring their communities at a continental scale. We sampled 52 plant populations across the northern Mediterranean and central Europe and used a cultivation approach to study their endophytic communities. Cultivation of roots yielded 2601 isolates, which were grouped into 296 operational taxonomic units (OTUs) by internal transcribed spacer sequencing of 1998 representative colonies. Climatic and spatial factors were the best descriptors of the structure of endophytic communities, outweighing soil characteristics, host genotype and geographical distance. OTU richness was negatively affected by precipitation, and the composition of communities followed latitudinal gradients of precipitation and temperature. Only six widespread OTUs belonging to the orders Pleosporales, Hypocreales and Helotiales represented about 50% of all isolates. Assessments of their individual distribution revealed particular ecological preferences or a cosmopolitan occurrence. Our findings support a strong influence of the local environment in determining root endophytic communities, and show a different niche occupancy by individual endophytes.

Received 15 August, 2015; revised 26 October, 2015; accepted 27 October, 2015. *For correspondence. E-mail maciavicente@em.uni-frankfurt.de; Tel. +49 069 798-42090. †Present address: Institute of Population Genetics, University of Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany.

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Introduction

Plant roots establish symbioses with a large diversity of microorganisms, some of which are able to penetrate the outer root boundaries and constitute endophytic assemblages different from those in the surrounding rhizosphere and rhizoplane (Lundberg *et al*., 2012). Although some are transient colonizers that enter the roots due to stochastic events, others present adaptations that allow them to persist for long periods confined in particular compartments, or to more effectively invade the tissues and establish an active metabolic interaction with the host (Hardoim *et al*., 2008). A single plant might contain a complex assembly of root endophytic fungi (Vandenkoornhuyse *et al*., 2002), and plants in all terrestrial ecosystems have these associations. They can reach considerable microbial loads (Maciá-Vicente *et al*., 2012), thereby constituting an important cost to the host as photosynthetic carbon is diverted to the symbionts. In exchange, some endophytic mycorrhizae provide their host plants with benefits, most prominently assisting in the uptake of nutrients and water, or protecting against stress (Kiers and van der Heijden, 2006; Van Der Heijden *et al*., 2008; Kiers *et al*., 2011). Other endophytes constitute a unidirectional sink for plant resources and develop parasitic or pathogenic relationships of varying magnitudes (Tellenbach *et al*., 2011; Keim *et al*., 2014; Mandyam and Jumpponen, 2014). Through these processes, endophytic fungi contribute to the functioning of land ecosystems by modulating plant productivity and diversity, alongside their implication in the cycling of soil carbon.

The largest fraction of the endophytic mycobiome remains poorly characterized. Although endophytes are hypothesized to impact plant fitness, experimental work has been unable to assign decisive functions to most of them (Mandyam and Jumpponen, 2005; 2014; Newsham, 2011). Because the function of organisms is necessarily

linked to their habitat, their potential ecological roles can be inferred from their natural occurrence, from the identification of the ecological factors affecting their communities, and from understanding how they affect them. For instance, dominant plant species characteristic of major biomes associate with different types of mycorrhizae, which develop distinct symbiotic functions in relation to the specific soil properties (Read, 1991; Read *et al.*, 2004). There is substantial evidence that non-mycorrhizal root endophytes also have preferences towards ranges of hosts and environments (for an extensive review see Sieber and Grünig, 2013). Their local or regional occurrence can be linked to environmental variables like soil type and biotic factors like host phylogeny (Maciá-Vicente *et al.*, 2008a; 2012). However, knowledge of the large-scale biogeographic patterns of non-mycorrhizal root endophytes is very limited, at best.

The biogeography of organisms is driven by environmental, geographic and historical factors, together with features intrinsic to them such as their lifestyle, their dispersal capabilities, or their biotic interactions (Prosser *et al.*, 2007). As with other microorganisms, fungi were assumed to occur ubiquitously owing to large population sizes and a nearly unlimited ability to disperse (Fitter, 2005). This implies that their diversity is high locally, but comparably low at larger scales because the same species occur across landscapes, as summarized by the tenet 'everything is everywhere, but, the environment selects' (Baas-Becking, 1934). Evidence challenging this view has accumulated and depicts a more complex scenario for the distribution of different fungal guilds (Taylor *et al.*, 2006; Amend *et al.*, 2010; Tedersoo *et al.*, 2014; Van der Gast, 2015). Non-mycorrhizal root endophytes have been suggested not to follow a biogeographic pattern (Queloz *et al.*, 2011), as opposed to other above-ground and root-plant symbionts (Arnold and Lutzoni, 2007; Kivlin *et al.*, 2011; Tedersoo *et al.*, 2012; U'Ren *et al.*, 2012). This could indicate that different processes govern the diversity of different fungal functional groups (Tedersoo *et al.*, 2012). Alternatively, this could be a consequence of the lesser efforts devoted to study the broad-scale patterns of root endophytes.

Here, we investigate the biogeographic distribution of non-mycorrhizal root fungal endophytes at a continental scale, and evaluate the effects of geographic distance, local environment and the biogeography of their hosts in their community composition, and in the occurrence of dominant phylotypes. As for the host plant, we focus on closely related members of the annual genus *Microthlaspi* F.K. Meyer (Brassicaceae), which were until recently included in the species *Microthlaspi perfoliatum* (L.) F.K. Meyer (Ali *et al.*, 2015). These comprise both diploid and polyploid cytotypes that are morphologically similar, but phylogenetically dissimilar. Current data have shown that they represent two distinct species that form predominantly selfing populations (Ali *et al.*, 2015). Assessments of the distribution of endophytes have often focused on several unrelated host plants that were not represented in all sampling sites (Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008; Maciá-Vicente *et al.*, 2008a; Herrera *et al.*, 2010; 2013; U'Ren *et al.*, 2012). Because host phylogeny is one of the main factors determining the composition of plant-associated communities (U'Ren *et al.*, 2012; Wehner *et al.*, 2014), focusing on one host with a widespread occurrence may allow for more accurate biogeographical inferences. *Microthlaspi* has a broad distribution over nearly all of Europe (Meyer, 2003), allowing for samplings across a wide range of environmental gradients. As most Brassicaceae, *Microthlaspi* also lacks classical mycorrhizal associations and alternative adaptations for the efficient capture of soil nutrients because it dwells in habitats where these are not limiting (Fitter, 2005). This could leave additional niches open to other root colonizers with different effects on the host.

The aim of this study is to unravel the broad-scale biodiversity patterns of root endophytes and identify their key ecological drivers. We use *Microthlaspi* as a model host system and rely on a cultivation approach to characterize its endophytic mycobiome. The collection of an extensive inventory of fungal cultures will warrant further phylogenetic and ecological studies on these endophytes and on their interaction with plants.

Results

Diversity of root endophytes

A total of 424 plants were processed for isolation of root endophytic fungi, originating from 52 populations distributed along an area spanning four parallels and five meridians (Table 1). Out of the total plants sampled, 414 (97.4%) yielded endophytic fungal growth in at least 1 of the 10 root pieces plated. We recorded 2601 fungal colonies developing from 2359 out of the total 4240 root pieces, accounting for an overall colonization percentage of 55.6% (i.e., the proportion of root pieces yielding at least one isolate), and an averaged colonization per population of 56.7 ± 18% (mean ± standard deviation).

The fungal isolates were grouped into 296 OTUs by sequencing of the internal transcribed spacer (ITS) rDNA region of a subset of representative pure cultures (Fig. 1A). On average we obtained 16.5 ± 6.3 OTUs per population of *Microthlaspi* (Fig. 1B). The overall number of operational taxonomic units (OTUs) obtained was below the maximum expected richness of 344.3 OTUs as assessed by Bootstrap analysis, and the 564.3 OTUs as assessed by the Chao estimator. This translates into an average value of 4 ± 1.8 or 11.7 ± 13.7 OTUs that went undetected in each plant population respectively

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Table 1. Description of *Microthlaspi* populations studied in this work, and results of fungal colonization and diversity.

								Observed richness		Estimated richness		Diversity indices	
Country	Site	Coordinates	Elevation (m.a.s.l.)	Host's ploidy	*n*[a]	Isolates	Colonization (%)[b]	*S*[c]	Av. *S*[d]	Boot[e]	Chao[f]	*H'*[g]	*J'*[h]
Bulgaria	BG-007	42.50 N / 22.82 E	614	Polyploid	6	60	86.7 ± 13.7	17	5.2 ± 1.9	20.6 (2.3)	62 (30.1)	2.3	0.8
	BG-010	42.70 N / 22.83 E	770	Diploid	9	78	81.1 ± 19	18	4.6 ± 1.3	21.8 (1.7)	27.3 (8.8)	2.4	0.8
	BG-011	42.67 N / 22.84 E	740	Mixed	5	43	76 ± 8.9	14	4.2 ± 1.8	17.2 (2.3)	15.7 (2.2)	2.3	0.9
	BG-012	42.66 N / 22.81 E	773	Polyploid	9	66	67.8 ± 13	25	3.8 ± 1.5	31.8 (3)	38.2 (10.2)	2.9	0.9
	BG-013	42.63 N / 22.73 E	837	Polyploid	9	65	64.4 ± 19.4	23	4.6 ± 2.2	28.5 (3)	62 (30.3)	2.7	0.8
	BG-014	42.59 N / 22.72 E	711	Diploid	4	37	85 ± 10	14	4.8 ± 1	17.6 (2.1)	29 (12.8)	2.0	0.8
	BG-015	42.57 N / 22.69 E	685	Diploid	9	84	77.8 ± 15.6	28	5.6 ± 1.5	34.5 (3)	35.3 (5.7)	2.9	0.9
	BG-023	42.91 N / 22.83 E	621	Polyploid	6	52	80 ± 22.8	14	4.3 ± 1.9	16.3 (2)	14.5 (1)	2.3	0.9
Germany	D-100	49.54 N / 09.34 E	415	Polyploid	10	82	69 ± 12.9	31	5.4 ± 2.2	38.8 (3.6)	48 (10.7)	2.8	0.8
	D-101	49.68 N / 10.00 E	278	Diploid	10	48	44 ± 29.5	20	3.4 ± 2.1	24.8 (2.8)	47.5 (22.7)	2.7	0.9
	D-102	49.45 N / 09.82 E	281	Diploid	10	32	29 ± 24.2	22	3.1 ± 3.2	28.3 (3.8)	46 (16.4)	2.9	1.0
	D-103	49.27 N / 09.84 E	299	Diploid	10	40	39 ± 26	21	3 ± 1.5	27.4 (2.7)	89 (48.6)	2.6	0.8
	D-104	48.61 N / 09.53 E	515	Diploid	10	51	41 ± 23.3	18	3.4 ± 1.6	22.5 (2.4)	40 (17.4)	2.3	0.8
	D-105	48.55 N / 10.12 E	481	Diploid	10	42	40 ± 13.3	23	3.6 ± 0.8	28.5 (2.3)	32.4 (7.2)	3.0	1.0
	D-11a	50.37 N / 07.22 E	504	Diploid	10	73	61 ± 24.7	20	4.1 ± 1.9	24.6 (2.8)	24 (3.9)	2.5	0.8
	D-11b	50.37 N / 07.22 E	504	Diploid	10	61	55 ± 21.2	17	3.7 ± 1.6	20.1 (1.7)	20.8 (4.2)	2.5	0.9
Spain	ES-001	38.04 N / 02.48 W	1630	Polyploid	10	54	50 ± 20	20	3.7 ± 1.2	25.4 (2.2)	46 (20)	2.5	0.8
	ES-002	38.05 N / 02.54 W	1612	Polyploid	10	58	50 ± 20.5	15	3.7 ± 1.6	17.7 (1.4)	25.5 (10.5)	2.3	0.8
	ES-003	38.09 N / 02.56 W	1253	n.d.	10	65	60 ± 18.3	21	3.5 ± 1.6	27.1 (2.6)	34.2 (10.2)	2.3	0.8
	ES-004	37.97 N / 02.45 W	1204	Polyploid	10	85	80 ± 15.6	17	3.6 ± 1.2	20.9 (2)	21.7 (4.5)	1.9	0.7
	ES-005	37.14 N / 03.48 W	1351	Polyploid	10	47	46 ± 23.7	11	2.2 ± 0.9	13.3 (1.6)	11.3 (0.7)	2.1	0.9
	ES-006	37.13 N / 03.43 W	1669	Polyploid	10	59	57 ± 14.9	11	2.9 ± 0.7	13 (1.3)	14.3 (4.1)	1.9	0.8
	ES-010	42.81 N / 04.25 W	1055	Polyploid	10	64	59 ± 18.5	24	3.3 ± 0.8	31.1 (2.5)	69.3 (31.8)	2.5	0.8
	ES-012	42.87 N / 04.15 W	1305	Polyploid	10	62	59 ± 27.7	17	3.2 ± 1.6	20.9 (2.3)	22.3 (5.4)	2.4	0.8
France	F-001	47.41 N / 06.56 E	285	Diploid	10	36	33 ± 25	13	2.2 ± 1.1	16.1 (1.8)	18 (5.5)	2.2	0.9
	F-002	47.14 N / 06.20 E	553	Diploid	10	45	43 ± 15.7	17	3 ± 0.7	20.8 (1.8)	18.3 (1.7)	2.6	0.9
	F-004	47.03 N / 06.33 E	699	Polyploid	10	27	27 ± 13.4	12	2.2 ± 1.2	15 (1.7)	26 (13.1)	2.0	0.8
	F-007	47.11 N / 06.07 E	543	Diploid	9	64	67.8 ± 18.6	16	3.8 ± 1.3	19.1 (1.6)	16.8 (1.3)	2.2	0.8
	F-008	47.08 N / 06.07 E	533	Diploid	9	32	34.4 ± 25.1	11	1.9 ± 1.1	14.2 (1.6)	12.5 (2.2)	2.0	0.8
	F-009	47.18 N / 05.46 E	216	Polyploid	9	77	75.6 ± 14.2	18	4.6 ± 1.6	22.3 (2.1)	23.6 (5.3)	2.3	0.8
	F-010	47.20 N / 05.43 E	198	Diploid	9	56	60 ± 17.3	18	3.8 ± 1.1	22.3 (2.1)	30 (10.7)	2.4	0.8
	F-011	47.32 N / 04.60 E	446	Polyploid	6	29	48.3 ± 23.2	8	2.2 ± 1	10 (1.5)	18 (10.1)	1.3	0.6
	F-013	47.30 N / 03.59 E	215	n.d.	6	35	55 ± 18.7	16	4 ± 1.5	19.7 (2.1)	38.5 (19.2)	2.5	0.9
	F-014	47.19 N / 01.20 E	121	Polyploid	7	62	75.7 ± 5.3	16	4.1 ± 1.1	19.6 (1.8)	18 (2.6)	2.3	0.8
	F-015	46.41 N / 00.22 E	112	Polyploid	9	42	43.3 ± 24.5	26	3.7 ± 2.2	33.5 (4.1)	48.7 (14.9)	3.1	0.9
	F-021	44.58 N / 05.38 E	1260	Polyploid	7	65	74.3 ± 21.5	17	5 ± 2.3	20.1 (2.1)	20.8 (4.2)	2.3	0.8
	F-023	44.49 N / 05.44 E	1095	Polyploid	9	59	57.8 ± 12	30	5 ± 1.7	37.5 (3.8)	47.1 (11.5)	3.2	0.9
	F-024	44.50 N / 05.42 E	734	Diploid	7	42	55.7 ± 23.7	20	4 ± 1.9	25 (2.9)	31.3 (9.5)	2.8	0.9
Greece	GR-001	39.81 N / 20.77 E	1065	Polyploid	10	87	82 ± 13.2	18	4.1 ± 1.9	22.2 (2.6)	33 (12.8)	2.1	0.7
	GR-002	38.94 N / 21.76 E	1410	Polyploid	10	71	61 ± 16	24	4.1 ± 1.4	29.8 (2.6)	37.2 (10.2)	2.7	0.9
	GR-003	38.91 N / 21.74 E	1283	Polyploid	10	57	55 ± 25.5	15	2.8 ± 1.5	18.3 (1.7)	17 (2.6)	2.1	0.8
	GR-004	38.91 N / 21.83 E	905	Polyploid	8	54	62.5 ± 17.5	20	3.9 ± 1.7	24.9 (2.7)	27.5 (6.3)	2.6	0.9
Croatia	HR-021	44.16 N / 15.58 E	795	Diploid	7	12	15.7 ± 17.2	8	1.4 ± 1.4	10.2 (1.4)	11.3 (4.1)	2.0	1.0
	HR-022	44.19 N / 15.52 E	574	Diploid	8	57	61.3 ± 24.2	17	4.3 ± 2	20.5 (2.1)	17.4 (0.9)	2.6	0.9
	HR-023	44.24 N / 15.54 E	760	Polyploid	4	12	27.5 ± 9.6	7	2.3 ± 1	8.9 (1.1)	12 (5.9)	1.7	0.9
	HR-025	44.46 N / 15.40 E	755	Diploid	10	47	46 ± 32	11	2.1 ± 1.2	14.1 (2.1)	18.5 (8.1)	1.6	0.6
	HR-028	44.59 N / 15.44 E	525	Diploid	8	14	17.5 ± 28.7	5	0.9 ± 1.1	6.4 (1.1)	8 (4.4)	1.3	0.8
Turkey	T-024	38.33 N / 30.64 E	1101	Polyploid	3	25	66.7 ± 15.3	9	5.3 ± 2.3	10.3 (1.6)	9.3 (0.9)	2.0	0.9
	T-025	38.39 N / 30.67 E	1180	Polyploid	3	17	53.3 ± 30.6	4	2 ± 1	4.9 (0.9)	7 (4.3)	0.7	0.5
	T-026	38.57 N / 30.58 E	1166	Polyploid	3	34	93.3 ± 11.5	5	3.7 ± 1.5	5.4 (0.7)	5 (0.4)	1.3	0.8
	T-027	38.79 N / 30.28 E	1105	Polyploid	3	20	50 ± 26.5	9	4.3 ± 2.3	10.6 (1.6)	11 (2.9)	2.0	0.9
	T-028	38.86 N / 30.00 E	1210	Polyploid	3	15	46.7 ± 41.6	8	3 ± 3	10.1 (2.3)	11.3 (4.1)	1.8	0.9

a. Number of plants sampled.
b. Percentage of root pieces yielding at least one fungal colony (±SD).
c. Overall observed OTU richness.
d. Mean observed OTU richness (±SD) across plant individuals.
e. Bootstrap incidence-based richness estimator (SE).
f. Unbiased Chao abundance-based richness estimator (SE).
g. Shannon's diversity index.
h. Pielou's evenness index.
n.d., not determined. SD, standard deviation; SE, standard error.

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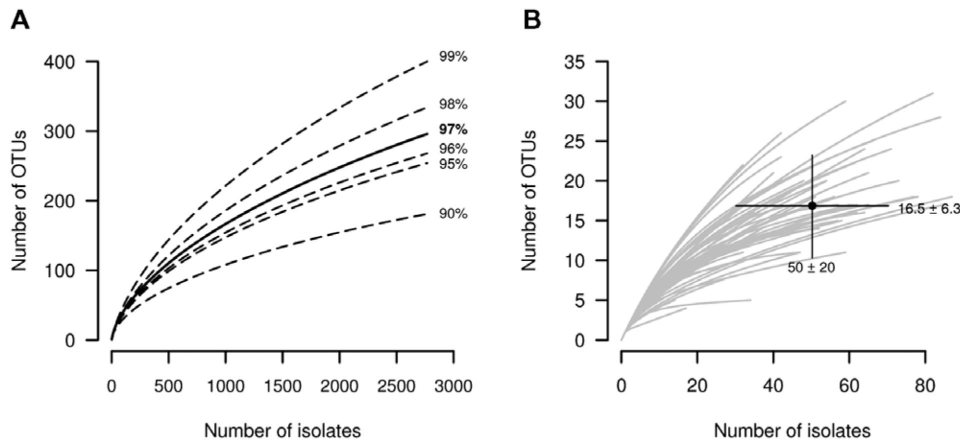

Fig. 1. Rarefaction curves of OTU accumulation with sampling effort, consisting of the total number of isolates developing from root pieces plated in a cultivation medium.
A. Accumulation curves for the entire study showing the effect of different sequence similarity thresholds for OTU definition.
B. Accumulation curves for individual *Microthlaspi* populations. The point with error bars indicate average and standard deviation for the number of isolates obtained per population (*x* axis), and number of OTUs per population (*y* axis), based on individual plant values.

(Table 1). The lack of saturation of the fungal diversity was confirmed by rarefaction curves calculated for the overall survey and for every individual plant population, which in most cases failed to reach an asymptote even when using similarity thresholds to define OTUs as low as 90% (Fig. 1).

Taxonomic classification of isolates

OTUs were classified at varying taxonomic precision by comparing ITS sequences with reference databases. They were assigned to 16 fungal orders, most of them within the Ascomycota (95%; Table S1). The Pleosporales and Hypocreales were the most represented orders, both in terms of the number of OTUs (43.2% and 19.6% respectively) and of the frequency of counts (Fig. 2A). The order Helotiales followed with 14.9% of the OTUs and a frequency of 11.6% of the colony counts, whereas the remaining orders were marginally represented (Fig. 2A; Table S1). Only six OTUs accounted for 50% of the isolates recorded (Fig. 2B). Three of these could be assigned to the order Hypocreales, two within the genus *Fusarium* – with affinities to the species *Fusarium tricintum* and *Fusarium avenaceum* (OTU001) and *Fusarium oxysporum* (OTU003) – and one within the genus *Ilyonectria* (OTU005). Another two of these OTUs belonged to the Pleosporales, the most abundant (OTU002) within the genus *Alternaria* – with close affinity to *Alternaria tellustris* – and another (OTU004) within *Pyrenochaeta* – with closest BLAST hits on *Pyrenochaeta lycopersici*. The sixth OTU in abundance (OTU006) was classified as *Cadophora* sp. Apart of their overall frequency, these OTUs had a widespread distribution and occurred in most plant populations, often representing an important proportion of communities (Fig. 2C). They were followed in abundance mostly by members of the Hypocreales and Pleosporales (Table S1). The remaining OTUs were in general infrequent, with 161 of the total 296 (54.4%) represented by a single isolate, and 47 (15.9%) and 21 (7.1%) by 2 and 3 isolates respectively.

Effect of environmental factors on endophytic diversity

Fungal assemblages differed significantly across populations in OTU richness ($H_{51} = 135.2$, $P < 0.001$), Shannon's diversity ($H_{51} = 121.2$, $P < 0.001$) and Pielou's evenness ($H_{51} = 93.1$, $P < 0.001$; Table 1). We compared endophytes' richness and diversity across environmental factors by using plant averages to correct for the different sampling sizes at each site (Table 1). None of these variables was significantly correlated with latitude (Fig. 3A), but linear regression showed a strong negative relationship of richness and diversity ($P < 0.002$) with various factors related to precipitation (Fig. 3B). These included annual precipitation at each site (Fig. 3B), precipitation of the wettest month, and precipitation of the wettest and coldest quarters of the year. Soil physico-chemical variables had no significant relationships with either richness or diversity of endophytic communities.

Effect of environmental factors on community structure

The unconstrained non-metric multidimensional scaling (NMDS) ordination of Horn–Morisita distances (stress: 20%) revealed a clear structure of endophytic communities along a latitudinal gradient (Fig. 4A). Differences among sites were significant when country of origin or climatic region were used as grouping variables in permu-

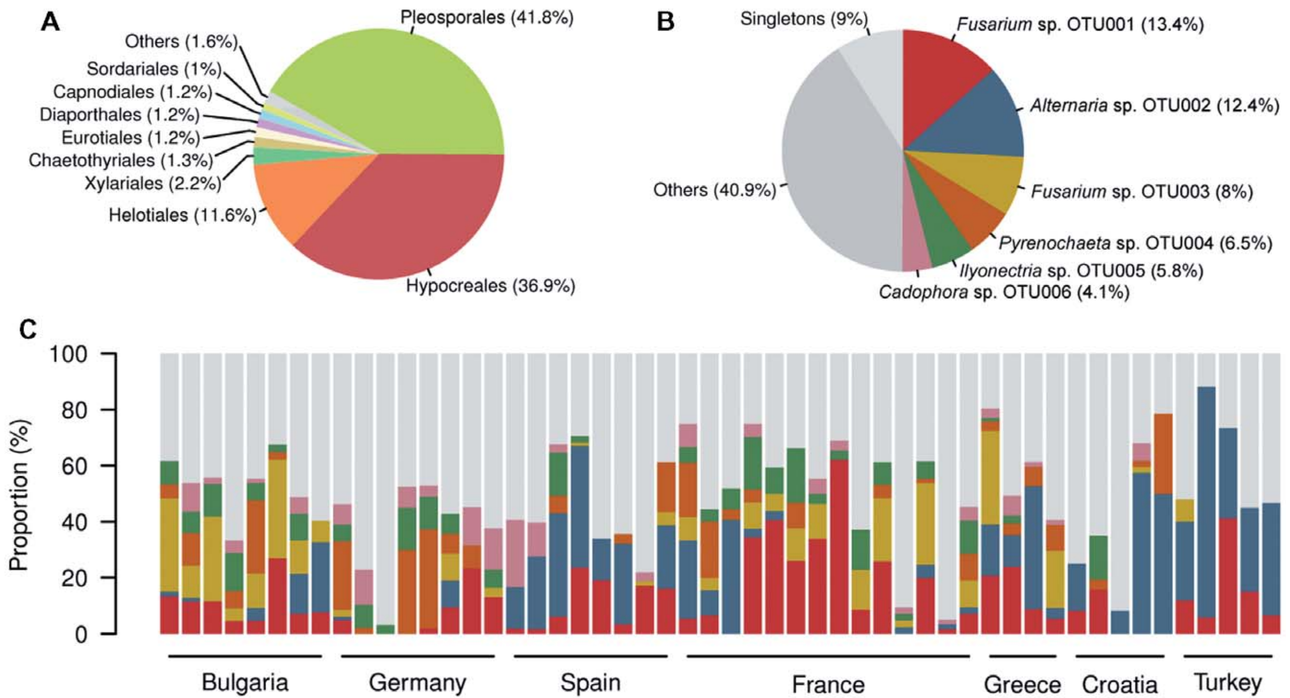

Fig. 2. Relative proportion of fungal taxa among the total number of endophytic isolates.
A. Proportion of isolates belonging to the most frequent fungal orders.
B. Proportion of isolates belonging to the most frequent OTUs.
C. Relative proportion of dominant OTUs across plant populations. Colours as in (B).

tation analysis of variance (PERMANOVA) ($P < 0.001$). All environmental variables retained in the forward selection as potential descriptors of communities had significant correlations with the ordination of sites and were strongly collinear with the latitudinal axis (Fig. 4A). Among these, the only soil variable with a certain degree of correlation with communities was Mg content (pseudo-$F_{40} = 1.8$, $P = 0.046$). We explored other variables not included in the forward selection in an attempt to explain variation in the axis perpendicular to latitude and found that the overall degree of endophytic colonization was the best fitting (pseudo-$F_{51} = 5.7$, $P = 0.001$; Fig. 4A).

An assessment of the distribution of the most common orders showed distinctive patterns of occurrence of Hypocreales in contrast to both Pleosporales and Helotiales (pseudo-$F_{57} = 1.8$, $P = 0.002$). Hypocreales tended to accumulate in communities leftwards in the ordination plot, perpendicular to the main axis of influence of environmental factors and positively correlated with overall colonization (Fig. 4B). Pleosporales and Helotiales on the other hand did not show a clear preference towards any factor.

Variation partitioning was used to assess the individual effect of climatic, spatial and host-related variables on the

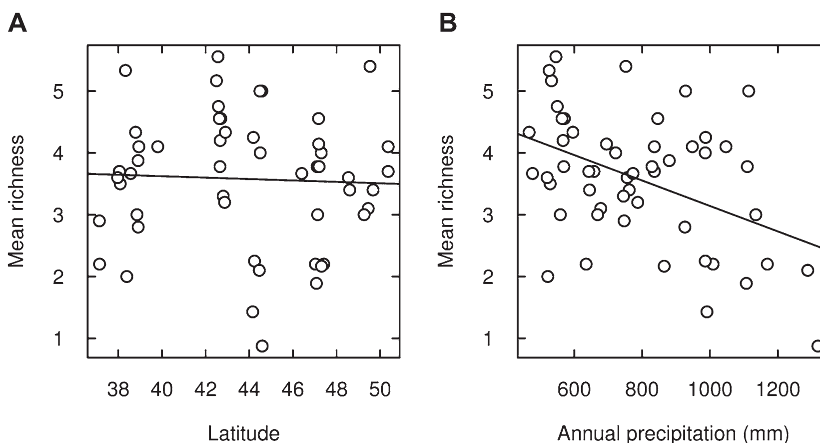

Fig. 3. Relationship between OTU richness in each *Microthlaspi* population, calculated as the average richness observed in each plant of the populations, and the respective latitude (A) and mean annual precipitation (B). Lines denote the linear regression model of interaction between both variables.

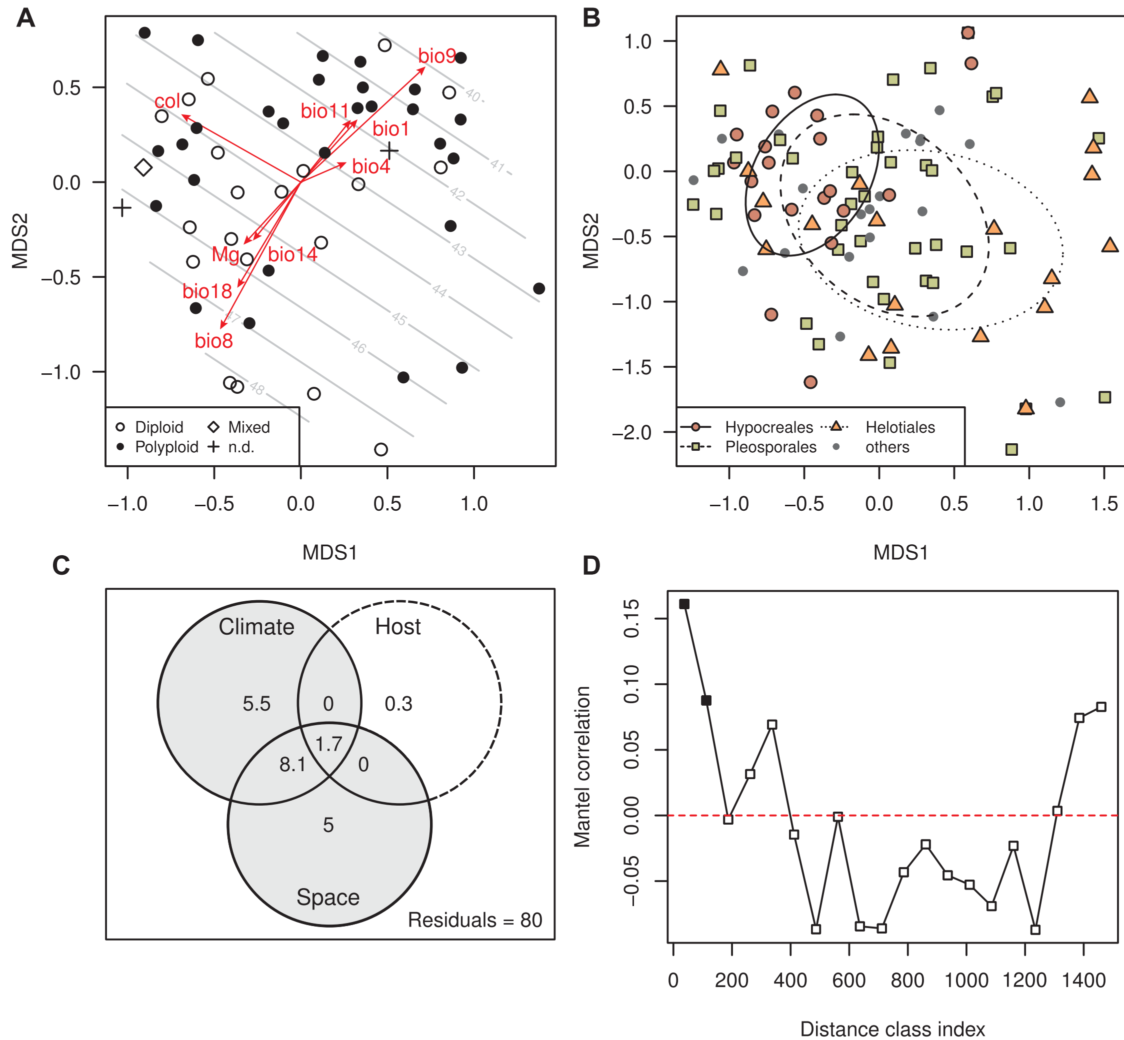

Fig. 4. Effect of ecological factors in whole-community structure of root endophytes.
A. Unconstrained non-metric multidimensional scaling (NMDS) analysis of communities displaying distances among populations, and depicting the relative influence of selected variables (arrows). The latitudinal gradient is represented as surface lines.
B. Species scores of the NMDS ordination in (A), highlighting the three dominant fungal orders. Ellipses delimit 95% confidence intervals around the mean values for each order.
C. Partition of the community variance into a climatic, a spatial and a host component. The numbers inside the sections indicate the percentage of the variation explained. Grey sectors with solid line indicate that the values comprised are significant ($P < 0.05$), whereas the value in an empty sector with dashed lines is not significant.
D. Mantel correlogram showing Mantel correlations among communities across distance classes. Solid symbols denote significant ($P < 0.05$) correlations for each class. Comparisons beyond 1500 km were not calculated due to the low number of samples included beyond this distance.
Abbreviations: bio1, annual mean temperature; bio4, temperature seasonality (standard deviation); bio8, mean temperature of wettest quarter; bio9, mean temperature of driest quarter; bio11, mean temperature of coldest quarter; bio14, precipitation of driest month; bio18, precipitation of warmest quarter; col, mean colonization percentage per population; Mg, magnesium soil content.

structure of endophytic communities (Fig. 4C). These three components explained 20% of the variance, and each accounted for a significant proportion individually according to pseudo-*F* tests ($P < 0.05$). However, the host predicted only a 1.7% of the overall variation ($P = 0.02$), indistinguishable from the contribution by climate or space. Each of the climatic and the spatial components explained individually around 15% of the total community variance, of which about 10% was jointly attributed to both categories (Fig. 4C). Sampling size accounted for a 3.3% of the overall variation, of which 2.4%, 1.8% and 1.1% were undistinguishable from the effect of climatic, spatial and host variables respectively. Sampling size alone explained a 0.7% ($P = 0.15$) of community variance.

Geographic distance had no overall effect on the similarities among communities ($R = 0.02$, $P = 0.32$). To investigate a potential effect across distance classes, we built a multivariate Mantel correlogram (Fig. 4D), which showed

a patchy distribution of communities separated by up to 115 km ($P < 0.002$).

Effect of ecological factors on individual endophyte populations

Maps of OTU occurrence and variance partition of individual fungal populations showed distinctive patterns in their distribution (Fig. 5). Occurrences of *Fusarium* sp. OTU001 (Fig. 5A) and *Pyrenochaeta* sp. OTU004 (Fig. 5D) were unaffected by the ecological components considered, and the models for their distribution were not significant. *Alternaria* sp. OTU002 (Fig. 5B) and *Ilyonectria* sp. OTU005 (Fig. 5E) showed clear but opposed latitudinal gradients of occurrence, mostly driven by confounding climatic and spatial factors. *Cadophora* sp. OTU006 was the only dominant endophyte the occurrence of which was consistent with a local distribution determined by soil factors, especially pH and Mg content (Fig. 5F). In this case, a fraction (5%) of the soil component overlapped with a significant effect ($P = 0.002$) of the sampling size. The contribution of the individual ecological variables to the occurrence of each OTU is shown in Table 2.

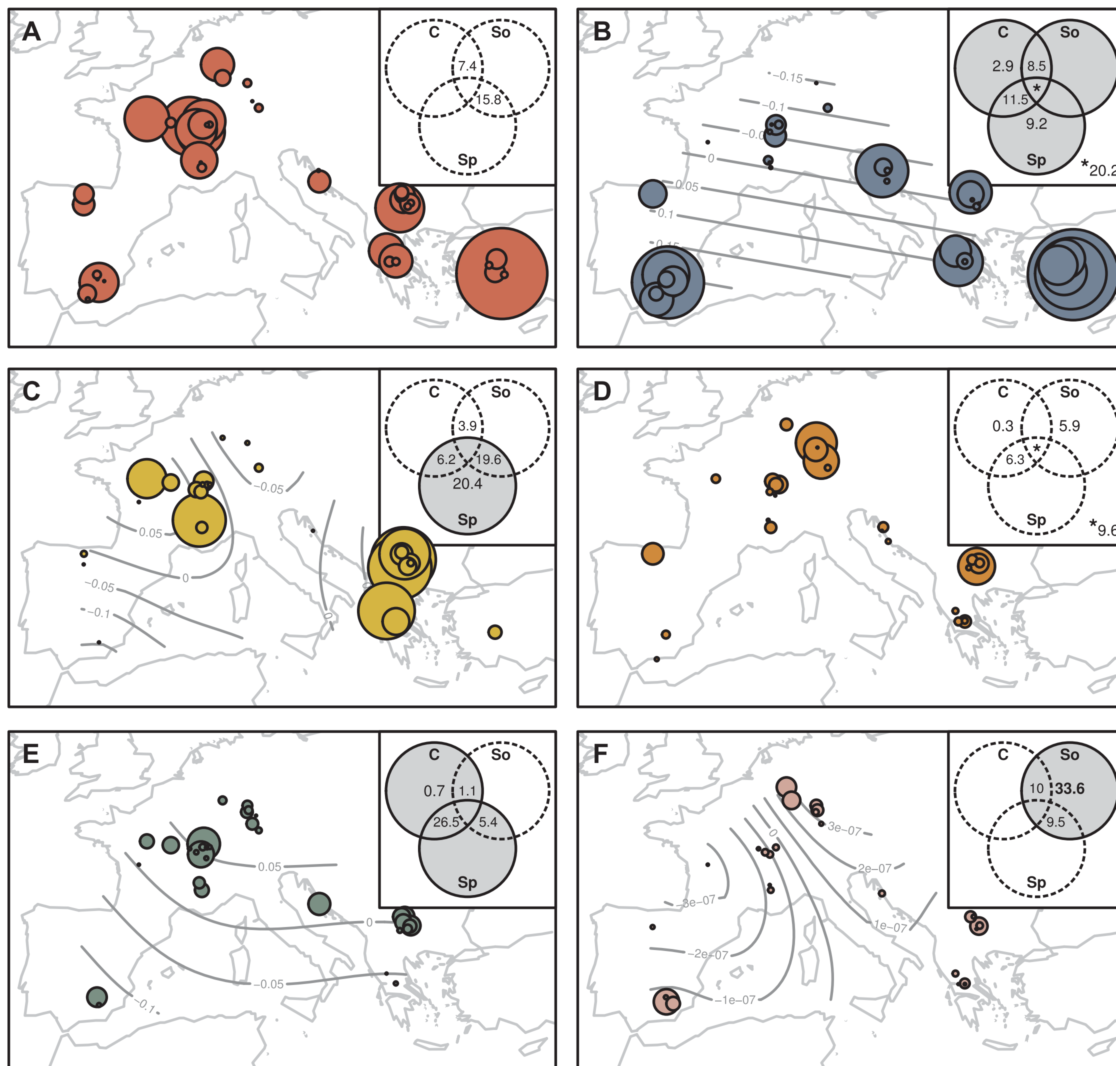

Fig. 5. Distribution and frequency of the six dominant OTUs across the sampling area: *Fusarium* sp. OTU001 (A), *Alternaria* sp. OTU002 (B), *Fusarium* sp. OTU003 (C), *Pyrenochaeta* sp. OTU004 (D), *Ilyonectria* sp. OTU005 (E) and *Cadophora* sp. OTU006 (F). Bubble sizes indicate relative frequency for each OTU at every location, and surface lines represent the fitted scores of redundancy analysis, depicting the variation explained by significant ecological components (samples from Croatia and Turkey not included in models, due to missing data on soil properties). Insets represent the variation partitioning results in a climatic (C), a spatial (Sp) and a soil (So) component. Grey sectors with solid line indicate significant values ($P < 0.05$), and empty sectors with dashed lines are not significant. Sectors without numbers indicate no variance explained at all.

Table 2. Variance partitioning for the occurrence of individual dominant endophytes into ecological categories of factors, including climatic, soil and spatial components.

Component	Factor[a]	OTU001		OTU002		OTU003		OTU004		OTU005		OTU006	
		Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*
Climate	Annual temperature range (Bio 7)	–	–	0.2	0.002	–	–	–	–	–	–	–	–
	Mean temperature of wettest quarter (Bio 8)	–	–	0.19	0.004	–	–	–	–	–	–	–	–
	Mean temperature of driest quarter (Bio 9)	–	–	0.25	0.002	–	–	–	–	0.26	0.002	–	–
	Precipitation seasonality (Bio 15)	–	–	0.24	0.001	–	–	–	–	0.22	0.002	–	–
	Precipitation of warmest quarter (Bio 18)	–	–	0.13	0.015	–	–	–	–	0.23	0.002	–	–
Soil	Magnesium soil content (Mg)	–	–	0.14	0.01	–	–	–	–	–	–	0.24	0.003
	pH	–	–	–	–	–	–	–	–	–	–	0.26	0.001
Spatial[b]	Latitude	–	–	0.36	0.001	–	–	–	–	0.22	0.003	–	–
	MEM1	0.11	0.027	–	–	–	–	–	–	0.11	0.024	–	–
	MEM2	–	–	0.21	0.003	0.1	0.03	0.08	0.04	0.21	0.003	–	–
	MEM3	–	–	–	–	0.21	0.004	–	–	–	–	–	–
	MEM5	–	–	–	–	0.08	0.038	–	–	–	–	–	–

a. Ecological factors explaining a significant fraction of the occurrence of at least one fungal OTU, selected via a forward selection of all factors in this study.
b. MEMs (Moran's Eigenvector Maps) represent spatial structures in the data not explained by measured variables.

Co-occurrence and in-plate interactions of dominant endophytes

We found positive co-occurring patterns involving OTU001, OTU003 and OTU005, among each other and between OTU004 and OTU005 (Fig. 6). Relations among OTU001, OTU002 and OTU003 were strongly affected by a positive spatial autocorrelation, but the significance and magnitude of the relations persisted after correcting the spatial effect in linear regressions (slope = 0.30, $P = 0.014$ for the OTU001-OTU003 interaction). There was a strong negative correlation between OTU002 and OTU005 ($P < 0.001$), but this could not be linked with antagonistic interactions in culture (Fig. 6). Only *Pyrenochaeta* sp. OTU004 presented a consistent presence of inhibitory halos with other colonies. Both *Fusarium* strains tended to overgrow other fungi via direct contact between colonies because of their fast radial growth.

Discussion

The diversity and structure of fungal assemblages within roots of *Microthlaspi* were largely determined by the local environment to which plants were subjected. Of the ecological variables directly measured, climatic rather than soil conditions were the best descriptors of the broad-scale structure of endophytic communities. They were also strongly influenced by other factors that were lumped in the so-called spatial effect. This explains non-random spatial structures of the data not accounted for by the variables measured, and might include processes of environmental, historical or biological nature (Peres-Neto and Legendre, 2010; Dray *et al*., 2012). In contrast to the climatic and spatial effect, geographic distance among locations had a negligible influence in defining the composition of communities. The pattern arising when examining the turnover of OTUs across distance classes supported such view because communities that were close or very far away had greater commonalities than communities within intermediate distances. An effect purely due to distance and consequently to a limited dispersal, on the contrary, would imply a steady decay in community similarity with distance (the distance–decay relationship; Green *et al*., 2004; Peay *et al*., 2007).

In line with previous studies (Green *et al*., 2004; Amend *et al*., 2010; Queloz *et al*., 2011; U'Ren *et al*., 2012), our results do not reject the Baas–Becking hypothesis of a ubiquitous dispersal for fungi. Our findings are somewhat surprising because many root endophytes commonly lack specialized structures for dispersal in culture or field conditions (Jumpponen and Trappe, 1998; Sieber, 2002; Addy *et al*., 2005; Maciá-Vicente *et al*., 2008a), and even those able to produce spores have important constraints hindering their long-range dissemination (e.g., due to

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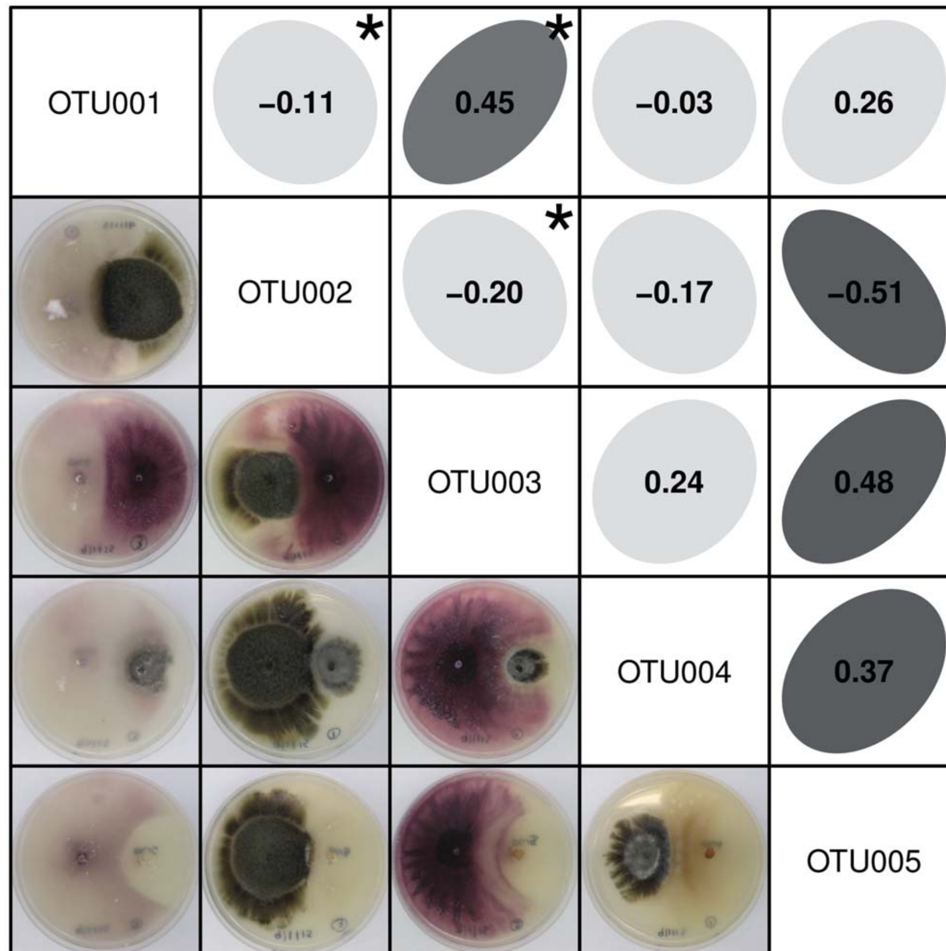

Fig. 6. Co-occurrence patterns and *in vitro* interactions among the five most frequent endophyte OTUs. Boxes with ellipses show the magnitude of the correlation between the co-occurrence of OTU pairs in roots of *Microthlaspi* populations. Values and ellipticity represent Spearman's ρ, and darker ellipses denote significance at $P < 0.05$. Asterisks indicate relationships with a significant autocorrelation, which were further assessed by spatial autoregressive models. Images in the lower diagonal represent interaction between colony pairs in dual culture assays. OTUs in each row are shown to the right in each interaction.

structural characteristics of the spores or their release points; Peay *et al.*, 2010). Alternative mechanisms for their efficient dispersal must therefore exist. The hitchhiking with host dispersal could be relevant for endophytes that colonize the plant systemically and reach the seeds or fruits, but this mechanism cannot account for the majority of root endophytes that are restricted to below-ground plant organs (Rodriguez *et al.*, 2009; Herrera *et al.*, 2010; Maciá-Vicente *et al.*, 2012). Other mechanisms of dissemination could imply animal transportation of plant material via herbivory and deposition, especially for some fungal groups that develop resistance structures within the plant tissues like microsclerotia (Currah *et al.*, 1993; Porras-Alfaro *et al.*, 2008), or processes in common with soil-borne fungi, like wind transportation by carrier soil particles, adhesion to invertebrates or spore washings (Dix and Webster, 1995).

Two considerations have to be taken into account with respect to the ubiquitous occurrence of root endophytes found in this study. First, our sampling could not achieve a complete description of the fungal richness constituting communities, and therefore the main results are driven by dominant endophytes. Thus it cannot be ascertained whether rare OTUs detected locally have a truly restricted distribution, or if such a finding would be due to under-sampling. Such under-representation is common in assessments of microbial diversity and has only been tackled by reaching a considerable sampling depth in species-poor habitats (Taylor *et al.*, 2014). Second, the definition of fungal OTUs based on ITS similarity might not be sufficient to resolve closely related species (even when using stringent clustering parameters), and thus might mask hidden biogeographic patterns. Queloz and colleagues (2011) could not detect a biogeographic

pattern in an assembly of root endophytes distributed globally, after applying several molecular markers. However, based on observations in other eukaryotic microorganisms (including fungi; Taylor *et al*., 2006; Gazis *et al*., 2011; Ryšánek *et al*., 2015), and given the broad taxonomic diversity uncovered in our study, we do not discard the possibility of cryptic patterns in the distribution of some fungal groups that showed a cosmopolitan occurrence.

Environmental descriptors of community structure

Endophytic communities were clearly structured along a latitudinal gradient. Latitude gathers a set of co-varying historical, abiotic and biotic gradients that have a strong influence on the distribution of all sorts of organisms (Hillebrand, 2004; Mittelbach *et al*., 2007), including soil and plant-associated fungi (Hoffman and Arnold, 2008; Herrera *et al*., 2010; Tedersoo *et al*., 2012; 2014; U'Ren *et al*., 2012). In our study, latitude determined community composition but not richness and diversity, possibly because the latitudinal range covered was shorter than in other works (Amend *et al*., 2010; Tedersoo *et al*., 2012; 2014). Instead, OTU richness and diversity were negatively correlated with various variables reflecting local precipitation. This situation is similar to what has been found in mycorrhizal fungi (Tchabi *et al*., 2008; Tedersoo *et al*., 2012), but not for above-ground endophytes (U'Ren *et al*., 2012), including those in plants within the Brassicaceae, like *Microthlaspi* (García *et al*., 2013). This pattern contradicts well-known positive effects of rainfall on soil fungal richness (Tedersoo *et al*., 2014, but see Hawkes *et al*., 2011), and hence suggests processes of environmental filtering specific for root symbionts. Water deficiency could increase fungal richness within roots by favouring an active hyphal growth towards roots with a higher water content than the surrounding soil, by compromising host defences against fungal colonization through water stress or by a direct functional modulation of rhizosphere microbial consortia (Van Der Heijden *et al*., 2008; Hawkes *et al*., 2011).

Climatic variables collinear with the latitudinal gradient were the strongest determinants of community composition. These factors were related to annual temperature and precipitation ranges that clearly differentiated endophytic communities from southern areas, with hot and dry summers and wet winters, from those in northern temperate regions, characterized by wetter and colder seasons. Both temperature and rainfall are well-known broad-scale descriptors of fungal occurrence (Arnold and Lutzoni, 2007; Amend *et al*., 2010; Herrera *et al*., 2010; Hawkes *et al*., 2011; Tedersoo *et al*., 2012; 2014; Timling *et al*., 2014). They impose physiological constraints to fungal growth with a differential effect across taxa, affecting growth, spore formation and germination (Torres *et al*., 2003). In addition, the effect of bioclimatic variables on fungal communities might be indirect because they are likely to modulate the structure and productivity of plant communities, and this in turn could affect the microbial diversity associated with particular plants (e.g., Mohamed and Martiny, 2011; Blaalid *et al*., 2012).

Soil characteristics had a negligible influence on endophytic communities at the scale of this study. Soil physicochemistry is a well-known determinant of below-ground fungal assemblages, with pH being the factor best explaining large-scale differences (Taylor *et al*., 2014; Tedersoo *et al*., 2014; Timling *et al*., 2014). However, soil features are most likely decisive at local and regional scales, where closely adjacent soil patches can have heterogeneous edaphic conditions. This was shown by Maciá-Vicente and colleagues (2012), who described a profound shift in the structure of root endophytic communities of a single plant species along a gradient of soil salinity of only a few meters. Besides, *Microthlaspi* has specific edaphic preferences that determine only a slight variation in soil characteristics across samples (Koch and Bernhardt, 2004), which are unlikely to exceed the ranges of tolerance for the majority of fungi and thus to represent an important selective factor.

Effect of host phylogeography on endophyte assemblages

Host phylogeny is one of the best descriptors of the plant-associated fungal communities, when widely divergent plant species are considered (Wehner *et al*., 2014). However, the biogeographic structure identified in our study appears to be host independent, which may reflect the relatedness of the plants sampled. While the host genotype had a weak effect in determining whole community structure, it was collinear with other latitude-associated climatic factors that better explained fungal occurrence. Diploid and polyploid *Microthlaspi* species have divergent biogeographic distributions owing to different climate preferences (Ali *et al*., 2015). These explain a somewhat latitudinal distribution of cytotypes (Koch and Bernhardt, 2004), with polyploid *M. perfoliatum* having a wider distribution but preferentially occurring in southern regions, and diploid *M. erraticum* occurring in cooler regions.

Niche occupancy by dominant root endophytes

Our data reveal a clear pattern of distinctive preferences for specific niches or ecological conditions by individual endophytes. Niche occupancy is not only delimited by the distribution of relevant environmental properties, but is also driven by the interaction with competitor species (Silvertown, 2004). In the current study, however, positive

or negative co-occurrences among dominant endophytes seemed to reflect their shared or opposing ecological needs, rather than direct interactions. For example, *Alternaria* sp. OTU002 and *Ilyonectria* sp. OTU005 had latitudinally opposed distributions, which were largely determined by the climate, while their colonies showed a neutral interaction *in vitro*. An alternative explanation for their exclusive presence could be the competition of both groups for the same resources. *Fusarium* sp. OTU001 and *Pyrenochatea* sp. OTU004 had a cosmopolitan occurrence, which was unaffected by environmental or spatial variables. The distribution of *Fusarium* sp. OTU003 was likewise independent from the environment and purely driven by spatial autocorrelation, which defined two apparent foci of occurrence. Interestingly, *Cadophora* sp. OTU006 was the only endophyte the distribution of which was largely determined by soil conditions, showing a negative interaction with pH. Dark septate endophytes within the Helotiales – to which *Cadophora* belongs – are often associated with acidic soils (Sieber and Grünig, 2013), and a recent work found several accessions phylogenetically similar to OTU006 to be strongly correlated with soil properties, including pH (Taylor *et al*., 2014).

Taxonomic identity of Microthlaspi *endophytes*

The largest proportion of root endophytes in *Microthlaspi* belonged to the phylum Ascomycota, consistent with findings for most plants (except for ecto-mycorrhizal trees) based on both cultivation-based and molecular approaches (Sieber, 2002; Porras-Alfaro *et al*., 2008; Herrera *et al*., 2010; Maciá-Vicente *et al*., 2012; Pecoraro *et al*., 2012; Obase and Matsuda, 2014; Wehner *et al*., 2014). The dominance in diversity and frequency of Pleosporales and Hypocreales, the latter with a high proportion of Fusaria, also reflects common patterns of fungal occurrence in roots (Maciá-Vicente *et al*., 2008a; 2012; Márquez *et al*., 2010). Both constitute species-rich orders containing functionally versatile species adapted to a variety of habitats, and their relative presence appears to be modulated by the environment (Maciá-Vicente *et al*., 2008a; Porras-Alfaro *et al*., 2008). Because the occurrence of hypocrealean endophytes was correlated with overall root colonization, it is possible that cultivation methods positively bias towards them, since they often have fast growth rates and easily overgrow other fungi in the isolation plates. Alternatively, this could indicate a systemic colonization of roots by these fungi, which would possibly explain their growth from most of the root pieces plated. The order Helotiales, being the third-most frequent order, contains instances of dark septate root endophytes that predominate in woody hosts in temperate and boreal regions (Sieber, 2002; Sieber and Grünig, 2013).

Several of the frequent OTUs found here overlap with those in a previous description of the cultivable root mycobiota of *Microthlaspi*, from specimens collected in Germany 1 year prior to our sampling (Keim *et al*., 2014). This included species of *Fusarium*, *Ilyonectria*, *Alternaria*, *Pyrenochaeta* and multiple others related to strains identified in our sampling, suggesting a temporal stability of the fungal communities associated with this plant. Remarkably, a large proportion of the endophytic diversity that can be found in healthy wild plants is from genera containing known plant pathogens, many of which are of economic importance in crops. Our work complements previous studies that disclose a cryptic biology of fungi traditionally considered as *bona fide* pathogens because they were first described from diseased plants or are prevalent in agricultural systems (Malcolm *et al*., 2013). This could hint at a switch to pathogenicity because of the highly artificial environment created by intensive forms of agriculture. Besides, it also highlights the problem of over-simplifying the functional roles of root-associated fungi, which is frequent in ecological studies (Aguilar-Trigueros *et al*., 2014) and might lead to the erroneous interpretations of the participation of the fungal biodiversity in the functioning of ecosystems.

Conclusions

Understanding the distribution patterns of fungal root endophytes will help infer the potential functions they play in natural ecosystems, which are as yet largely cryptic. This information will be essential for the long-term monitoring of the global fungal biodiversity, especially in the context of current environmental threats. Here, we show that the distribution of fungal endophytes in roots of an annual plant is determined by the local environment at a continental scale. Geographic distance was a poor descriptor of community structure, suggesting efficient mechanisms for dispersion in this group of fungi. The large-scale changes are principally driven by climatic factors that define a latitudinal gradient of community structure, while soil conditions and host factors appear to have little or a locally restricted effect. Our results also demonstrate particular ecological preferences by individual groups of endophytes, suggesting that they play different functional roles in the ecosystems. To date, there is a limited number of studies on the biogeography of non-mycorrhizal root endophytes. Additional studies based on cultivation-free molecular approaches are ongoing and will provide a more comprehensive view of the spatial scaling of the endophytic fungal diversity. Lastly, the availability of an extensive collection of endophyte strains will warrant the performance of laboratory ecological studies that will help draw a link between their distribution and their potential functional roles.

Experimental procedures

Sample collection

Microthlaspi plants were collected from 52 sites distributed across six European countries (Spain, France, Germany, Croatia, Greece and Bulgaria) and Turkey (Table 1). The samplings were performed in 2013, from mid-April up to early June, roughly corresponding to the flowering period of the plant, and consisted of several field campaigns. Sites were selected according to the presence of an individual *Microthlaspi* population, defined as a cluster of several plant individuals. Populations were separated from one another by a minimum of 2 km. The only exception were populations D-11a and D-11b (Table 1), which grew adjacently but formed clearly different clusters, each with a particular accompanying vegetation. We collected 3–10 healthy-looking and medium-sized plants per population (Table 1), which we carefully uprooted to minimize disruption of roots and stored in cool conditions in food-grade plastic bags until their processing in the laboratory.

Acquisition of environmental and host data

In 42 out of the 52 sites we collected soil samples to characterize the chemical properties of the substrate in which the plants grew. For each site we took multiple soil subsamples from points covering the area of distribution of the plant population, and then pooled them in a single sample. Soils were analysed for pH, conductivity, organic/inorganic carbon and content of macronutrients (N, P, K, S, Na, Mg and Ca; Table S2) by the Soil Science Laboratory Unit of the Goethe University (Frankfurt am Main, Germany). Besides, for all sites we gathered data on elevation and geographic coordinates, which were used to retrieve several bioclimatic variables from the WorldClim (http://www.worldclim.org/; Hijmans *et al*., 2005) and the Consortium for Spatial Information (CGIAR-CSI; Trabucco and Zomer, 2009) data sets. The data set includes 19 variables derived from temperature and precipitation measurements (O'Donnell and Ignizio, 2012), and the degree of aridity (Table S2).

The genotype of the host plants was considered as an additional factor likely to influence the distribution of endophytes. The ITS regions of the ribosomal DNA were sequenced for up to three representative plants of most populations. DNA ploidy levels of these representative plants were determined by flow cytometry calibrated by chromosome counts for reference *Microthlaspi* specimens and ITS sequence comparisons (Ali *et al*., 2015). The estimated ploidy levels were used as a categorical variable in later analyses. Additionally, one representative ITS sequence per population was used to generate a matrix of pairwise genetic distances among populations to include host phylogeny as a numeric variable in statistical analyses. Selection of only one sequence per population was done after assessing a high sequence similarity within populations. One mixed population containing both cytotypes of *Microthlaspi* was excluded from analyses aimed at testing the effect of host on endophytic communities.

Isolation of endophytic fungi from roots

The processing of samples in the laboratory took place in most cases within 72 h after their collection. Roots from every plant were detached and treated individually in every step of the process to isolate endophytic fungi. We chose a mild surface-sterilization protocol for the elimination of microbial epiphytes to avoid over-disinfecting the roots, given their reduced thickness. The protocol consisted of a first wash under running tap water to remove adhered soil particles, and then a surface-sterilization with a 0.5% (v/v) sodium hypochlorite solution for 1 min, followed by three rinses with sterilized deionized water. Roots were then dry-blotted onto sterilized filter paper and cut into *c*. 3-mm long pieces. Ten randomly picked root pieces per plant were plated on a Petri plate containing 0.5% (w/v) Malt Extract Agar (AppliChem, Darmstadt, Germany) supplemented with 0.5 g l^{-1} chloramphenicol to minimize development of bacteria, and with 0.1% (v/v) Triton X-100 (Amresco, Solon, OH, USA) to restrict the spread of fast-growing fungi. We tested the effectiveness of the surface-sterilization protocol by imprinting one third of all root pieces (representing all samples) in the same medium before plating them into the final cultivation plates (Hallmann *et al*., 2006). This yielded fungal growth in 1.7% of the imprints, which we considered acceptable given the large number of root pieces handled and the overall variability in root morphology among individual plants and populations.

The plates with root pieces were incubated at room temperature for a period of 2 months. During this time we recorded the occurrence of fungal colonies as they emerged, and we classified them into morphotypes. We isolated in pure culture one representative colony from each morphotype per plate, yielding a total of 2006 cultures representing 2601 colony counts. All isolates have been deposited in the living fungal cultures collection of IPF hosted at Goethe University, and are available upon request from the authors.

Molecular characterization of strains

We processed 1998 isolates representing all morphotypes for sequencing of their ITS rDNA region. Genomic DNA was extracted from all cultures using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) on a KingFisher Flex 96 robotic workstation (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The ITS region was amplified with the fungal-specific primer pair ITS1F and ITS4 (White *et al*., 1990; Gardes and Bruns, 1993) in 20 μl of polymerase chain reactions containing 1 μl of DNA template, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer, and 0.5 U Taq polymerase (VWR International, Darmstadt, Germany). Temperature cycles were carried out in a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) and consisted of an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension step of 72°C for 5 min. The size of amplicons ranged between 500 and 600 bp in most cases, although some reactions yielded products of up to 1000 bp. The amplified products were sequenced using the same primers by the sequencing laboratory of the Biodiversity and Climate Research Centre (Frankfurt am Main, Germany).

Isolates were assigned to OTUs according to pairwise similarities of ITS sequences, as calculated with the BLASTCLUST tool (ftp://ftp.ncbi.nih.gov/blast/documents/blastclust.html) from the NCBI-BLAST package (Altschul *et al.*, 1990), using cut-off values that ranged from 90% to 99%. For subsequent analyses, we selected the data set based on the clustering at 97% similarity because it has been shown to provide a good approximation to biological species in studies spanning wide fungal diversities (Taylor *et al.*, 2014). Besides, in our case this clustering matched well individual identification of strains, and downstream analyses with data sets based on 98% and 99% cut-offs yielded similar results. The classification of strains was based on comparisons of all sequences with NCBI GenBank entries using BLAST, and with the curated UNITE database (Kõljalg *et al.*, 2013) using the Naïve Bayesian classifier running under MOTHUR v1.34.4 (Wang *et al.*, 2007; Schloss *et al.*, 2009), with a bootstrap support of 80%. Additionally, we repeated the latter approach with an in-house database with sequence and taxonomic data for all fungal ITS sequences in GenBank identified to species level, formatted for its use in MOTHUR. A consensus taxonomy was built for every OTU by either method considering a within-group sharing of at least 51% of the hits at each taxonomic level. Finally, we combined all taxonomic data for a definitive assignment of OTUs up to genus level. Conflicting assignments between the UNITE and the in-house databases were checked manually against GenBank using BLAST. When this was not conclusive, the lowest taxonomic level at which both databases agreed was selected. The taxonomic assignment for each OTU and the GenBank accessions for all sequences are shown in Table S1.

Dual plate assays

To understand potential in-culture interactions among different fungal groups that could have biased the root isolation results, we performed a dual-plate assay as described in Maciá-Vicente and colleagues (2008b). Briefly, representative cultures of the five most frequent fungal OTUs found were confronted in all pairwise combinations in the same medium used for their isolation. Assays were performed in triplicate, and plates were incubated for 1 month after which we recorded presence/absence of inhibitory interactions between colonies (e.g., formation of inhibition halos).

Data analysis

Fungal diversity. All analyses were carried out in R v3.0.2 (R Core Team, 2013) using relevant packages. To analyse fungal diversity and community data, we mostly relied on the package VEGAN v2.2-1 (Oksanen *et al.*, 2015). OTU count records for individual root pieces were first assembled into a data matrix containing group-wise colonization percentages per plant, calculated as in Fröhlich and colleagues (2000). This was used to compute overall and averaged values of OTU frequency, richness and diversity indices and richness estimators (Magurran and McGill, 2011) for each plant population. Statistical support for these comparisons was determined with the Kruskal–Wallis Rank sum test (Hollander *et al.*, 2013) at a significance level of 0.05. Potential links among richness and diversity data, frequencies of individual OTUs and ecological factors were explored by the calculation of pairwise correlations with the Spearman's rank statistic and linear regression. Because the sampling design included clusters of sites closely spaced and unevenly separated, our data were sensitive to spatial autocorrelation that could inflate type I error in significance tests and invalidate them (Peres-Neto *et al.*, 2006). Therefore, we estimated autocorrelation in all bivariate tests using Moran's *I* (Li *et al.*, 2007) and corrected it when present using spatial autoregressive models (Dormann *et al.*, 2007).

Community analyses. To compare overall fungal communities across plant populations, we calculated dissimilarities in OTU composition among assemblages using the Horn–Morisita index (Horn, 1966). Prior to this we removed singletons (defined as OTUs occurring in only one plant specimen over the survey), and then square-root-transformed the data to reduce the weight of dominant OTUs in the dissimilarities. The utilization of other distance indices or transformation methods yielded similar downstream results, and hence we considered these parameters appropriate. All environmental variables recorded were fitted to the dissimilarities among samples to investigate potential relationships, and significance of these correlations was tested with PERMANOVA (Anderson, 2001). Distances among samples and their correlation with significant factors were visualized by means of an NMDS. The effect of geographic distance on communities was investigated with a Mantel test and a Mantel correlogram by comparing ecological and geographical distances among sites at different ranges.

Variation partitioning. To determine the contribution of ecological factors as predictors of the endophytic community, we used the variance partitioning method following procedures described in Borcard and colleagues (2011) and Legendre and Legendre (2012). This was used to decompose the variation of OTU assemblages into four independent components gathering climatic, soil, spatial and host factors. The spatial component – accounting for unmeasured processes, either intrinsic to the organisms (e.g. dispersal) or environmental (Peres-Neto and Legendre, 2010) – was obtained by the calculation of Moran's eigenvector maps (MEMs; Dray *et al.*, 2006), which represent the multivariate structure of the data at all scales covered by the sampling. MEMs were the resulting ordination axes of a principal coordinate analysis (PCoA) of geographic distances among sites, following a weighted Delaunay triangulation connectivity matrix. The geographic coordinates of sites were also included in the spatial component, because MEMs do not cover linear trends associated with latitude and longitude. On the other hand, we excluded elevation from this component because of its strong collinearity with latitude (Spearman's $\rho = -0.86$, $P < 0.001$). The host component included the categorical variable ploidy, and vectors representing the phylogenetic relationship among populations, obtained as the resulting axes of a PCoA ordination of genetic distances in a manner similar to MEMs (Desdevises *et al.*, 2003).

Variation partitioning of the community data relied on constrained redundancy analyses (RDAs). We first transformed the singleton-free community matrix using a Hellinger conversion (Legendre and Gallagher, 2001), and then included it

as response variable. The explanatory matrices included factors that individually explained a significant proportion of the variation of the community data, as determined by a forward selection using the R package PACKFOR v0.0-8. We decided to exclude the soil component from these analyses because it only predicted a marginal proportion of the variation, and it reduced considerably the number of observations due to missing data. To assess potential effects of the number of plants collected at each site on the observed structure of communities, we included it as an additional explanatory variable and reported its contribution to the variance explained by ecological components. After variance partitioning, the significance of the variance fractions explained by each component was assessed using constrained RDA with pseudo-*F* tests.

Distribution of dominant OTUs. A modification of the above procedure was used to determine the contribution of the ecological components to the individual variation of the six most frequent OTUs (Peres-Neto and Legendre, 2010). In this case, RDA is equivalent to multiple linear regression because only one response variable is included. We forward-selected factors individually for each OTU and retained all those selected at least once, which were then used as explanatory variables. We excluded the host component from these analyses because of its poor contribution to the variation, and instead we included soil factors at the expense of reducing the number of observations in the models because we deemed them important in explaining the occurrence of particular fungi. Repetition of these analyses with climate and spatial effects alone yielded similar results for these components (data not shown). Fitted scores for linear models representing significant fractions of the variance were represented as surfaces in distribution maps for each OTU.

Acknowledgements

We thank Viola Ziller for technical assistance. This study was supported by LOEWE (Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz) of the state of Hesse and was conducted within the framework of the Cluster for Integrative Fungal Research (IPF). The authors declare no conflict of interest.

Author contributions

MT and JGMV conceived the study. KG, TA, AKB, SP, XX, MT and JGMV collected plant and soil samples. AÇ helped to coordinate samplings in Turkey. KG, SHK and JGMV isolated and processed fungal endophytes. KG and TA characterized plant genotypes. MT and JGMV contributed material/reagents. KG and JGMV analysed the data. KG and JGMV wrote the manuscript with contributions from the other authors.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sequence accessions, taxonomic information, and isolation results for the OTUs described in this study.
Table S2. Ecological factors used in this study.