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Comparison of Culturing and Metabarcoding Methods to Describe the Fungal Endophytic Assemblage of *Brachypodium rupestre* Growing in a Range of Anthropized Disturbance Regimes

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Simple Summary: The richness (number of species) of the fungi kingdom is estimated at 1.5 million species, but the vast majority remains unknown. Many of them inhabit plants—the so-called fungal endophytes—and may establish different types of interactions with their host plant. Fungal endophytes have been traditionally studied by letting them grow in appropriate culturing media in petri dishes, but novel massive DNA sequencing techniques which do not require a cultivation step (metabarcoding) are gaining ground. Both techniques were applied and compared to characterize the mycobiome of plants of a tall grass (*Brachypodium rupestre*) growing in high-mountain grasslands with different plant diversity (low and high). The two methods showed similar trends comparing endophyte richness between plant tissue types (root > rhizome > shoot) and between grasslands (low-diversity > high-diversity). However, the metabarcoding identified almost six times more endophyte species than the culturing although the most isolated fungal species via culturing, *Omnidemptus graminis*, was not recognized via metabarcoding. We conclude that the complementation of both techniques is still the best option to obtain a complete characterization of the fungal endophytic assemblage of the plant species.

Abstract: Fungal endophytes develop inside plants without visible external signs, and they may confer adaptive advantages to their hosts. Culturing methods have been traditionally used to recognize the fungal endophytic assemblage, but novel metabarcoding techniques are being increasingly applied. This study aims to characterize the fungal endophytic assemblage in shoots, rhizomes and roots of the tall grass *Brachypodium rupestre* growing in a large area of natural grasslands with a continuum of anthropized disturbance regimes. Seven out of 88 taxa identified via metabarcoding accounted for 81.2% of the reads (Helotiaceae, *Lachnum* sp. A, *Albotricha* sp. A, Helotiales A, Agaricales A, *Mycena* sp. and Mollisiaceae C), revealing a small group of abundant endophytes and a large group of rare species. Although both methods detected the same trends in richness and fungal diversity among the tissues (root > rhizome > shoot) and grasslands (low-diversity > high-diversity grasslands), the metabarcoding tool identified 5.8 times more taxa than the traditional culturing method (15 taxa) but, surprisingly, failed to sequence the most isolated endophyte on plates, *Omnidemptus graminis*. Since both methods are still subject to important constraints, both are required to obtain a complete characterization of the fungal endophytic assemblage of the plant species.

Keywords: *Brachypodium rupestre*; mycobiome; fire; grazing; metabarcoding; culturing

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1. Introduction

The study of microorganisms in their natural environment is a recent branch of research compared to microbial investigations undertaken in disciplines such as medicine

and agronomy, with high impact on human health and development [1,2]. Nowadays, microbial ecology, i.e., their diversity in nature, their response to prevailing and future environmental conditions, the associations they establish with plants and the complex network of interactions and functions they are involved in, are gaining ground in ecological research [3–5].

One example involves examining the associations that endophytic fungi establish with plants. These associations were first studied in agronomic grasses [6–8] and the research has extended to natural plant communities in recent decades [9–11]. Scientific literature has shown that these hidden associations are ubiquitous in nature and that all plants harbor an endophyte assemblage that delivers different functions and constitutes a collective and complex holobiont [12].

Nowadays, two techniques, culturing and metabarcoding, are used for the determination of fungal endophyte assemblages [13]. The protocols of culturable techniques have a longer record and have been implemented in many laboratories [14]. In this method, important constraints include the possibility that some fungal species are unculturable on artificial medium and the accumulation of inaccuracies and errors due to different sterilization times, diverse species growth rates and the presence of surface contaminants [15]. Metabarcoding techniques (culture-independent) [16], despite appearing very promising, still remain costly and lack a complete repository of sequences with taxonomic identification, a task which is under way [17,18]. In the latter, the potential for providing quantitative data based on the proportion of read sequences makes it a very powerful ecological tool [19,20].

The genus *Brachypodium* encompasses several perennial tall grasses, native to European calcareous grasslands, which have been expanding aggressively in the last decades due to the global change conditions (*B. pinnatum*, *B. genuense* and *B. rupestre)* [21–25]. This tall grass expansion causes a decline of the biodiversity of the natural grasslands and also has an impact on the ecosystem service of provisioning [26]. The competitive strategies of this group of species that explain the expansive process is a matter of interest [27–33], as it is the study of the mycobiome that may help to understand these advantages. To date, the research in the *Brachypodium* genus has focused on the systemic fungi of the Clavicipitaceae family hosted by *B. sylvaticum* [34,35], *B. phoenicoides* [36,37] and *B. pinnatum* [38]. Only a previous study of our research team has characterized the systemic and non-systemic mycobiome of *Brachypodium rupestre* under a gradient of grazing and fire disturbances using culturable techniques [39].

The aim of this research is to provide a characterization of the endophytic mycobiome of the tall grass species *Brachypodium rupestre* and to compare culture and metabarcoding techniques applied to conditions with restricted sampling effort due to the high cost of the novel technique. The comparison includes the aboveground (shoot) and the underground (rhizome and root) component of a set of *B. rupestre* individuals growing in the same region but subjected to different levels of anthropic disturbance (grasslands with different regimes of grazing and prescribed burning and, consequently, encompassing a different plant community composition). Through this range of regional variation, and considering different tissues and different environmental drivers, we are interested in determining the capacity of the two methods to identify and characterize the fungal endophyte assemblage of *B. rupestre*.

2. Materials and Methods

2.1. The Study Area

The Aezkoa valley (Navarra county, Spain) is the westernmost valley of the southern Pyrenees (42.53–43.3′ N, 1.8–1.17′ W) (Figure 1d). The climate is snowy and cold in winter, and mild and foggy in summer. The annual temperature averages 9.3 °C and the accumulated precipitation reaches 1856 mm per year (Irabia climatic station, http://meteo.navarra.es accessed on 17 October 2021). The landscape is a mosaic of forests (e.g., *Fagus sylvatica*, *Abies alba*), shrubland communities (e.g., *Erica* spp., *Ulex gallii*) and

grasslands. The area of study is part of the Special Area of Conservation (SAC) Roncesvalles-Selva de Irati (code ES0000126; Figure 1f) and is located in the north of the valley. High-altitude grasslands (800–1400 m asl) comprise diverse communities of perennial grasses (*Festuca* gr. *rubra*, *Agrostis capillaris*, *Brachypodium rupestre*, *Danthonia decumbens*), forbs (*Achillea millefolium*, *Potentilla erecta*, *Gallium saxatile*) and legumes (*Trifolium repens*, *Lotus corniculatus*). Sandstones and calcareous clays dominate the substrate, upon which develop acidic, deep and organic soils, with clay-loamy and loamy textures.

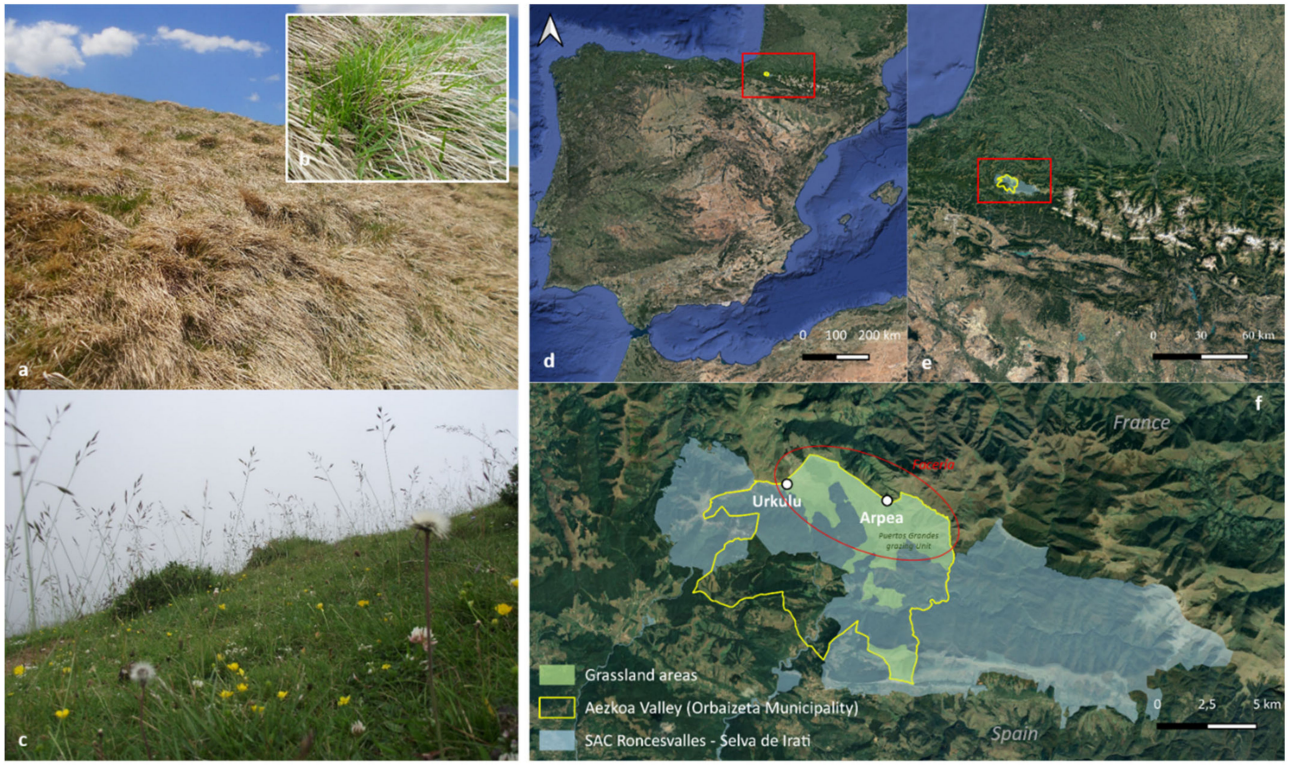

Figure 1. The appearance of low (**a**,**b**) and high (**c**) diversity grasslands. Location of the Aezkoa Valley in Spain (**d**) and within the western Pyrenees (**e**). The two locations (Arpea and Urkulu) where the samples were collected in the Roncesvalles-Selva de Irati SAC (**f**).

Depending on the grazing pressure of the livestock during the summer months, farmers schedule different types of burnings to control the build-up of litter and resprouting of woody species. As a result, traditional (bush-to-bush) burnings applied every 6–7 years coexist with more intense fire regimes, applied across the whole surface every 1–2 years in the less grazed areas. The regional plant community composition reflects the dominant grazing/burning regime, which leads to a mosaic of high-diversity grasslands (more grazed, less burned) and low-diversity grasslands highly dominated by *B. rupestre* (less grazed, more burned). Based on previous floristic surveys undertaken in the area [26], we selected two representative locations according to the percentage of *B. rupestre* cover. A low-diversity grassland (LD) in Arpea, with a dominant cover of *B. rupestre* up to 80%, and a high-diversity grassland (HD) located in Urkulu, with a *B. rupestre* cover lower than 25% (Table 1).

Table 1. General description of the study sites.

	Study Site	ARPEA	URKULU
	Type of Grassland	**LD = Low Diversity**	**HD = High Diversity**
General description	Location	−1°10′ 57″ W 43°2′ 12″ N	–1°14′ 38″ W 43°2′ 49″ N
	Soil classification (WRB)	Cambic Umbrisol	Dystric Cambisol
	Altitude (m.a.s.l.)	893	1256
	Slope (%)	40	45
Management	Burning recurrence	High 1–2 years	Low 6–7 years
	Type of burning	Large grassland areas	Bush-to-bush
	Grazing level	Low to nonexistent	Moderate to high
	B. rupestre cover (%)	>80%	<25%

2.2. Plant Sampling

In summer 2018, a total of 10 turfs of *B. rupestre* were collected (turfs included shoots, rhizomes and roots surrounded by soil) from the two locations (Figure 1f). The distance between turf samples was ca 150 m to avoid collecting clonal individuals. Turfs were transported to the UPNA laboratory and processed in the following days.

One *B. rupestre* plant with high biomass was selected from each turf. Tissues were separated (shoots, rhizomes and roots) and cut into fragments of ca 2 cm, surface-disinfected via immersion in a solution of 20% commercial bleach (1% active chlorine) containing 0.02% Tween 80 (v:v) for 10 min and finally rinsed with sterile water. The rhizome and root fragments were also treated with an aqueous solution of 70% ethanol for 30 s. Thirty fragments (10 shoots, 10 rhizomes and 10 roots) assigned to the metabarcoding method were ground using a pestle with liquid nitrogen and preserved at −20 °C until shipment.

2.3. Isolation and Identification of Fungi Using the Culturing Method

We plated 300 tissue fragments of *B. rupestre* onto 30 culture media plates (10 fragments/tissue/plate, 90 mm diameter), containing PDA medium (potato dextrose agar) with chloramphenicol (200 mg/L). Dishes with tissue fragments were kept at room temperature and ambient light and checked daily for 4 weeks. Any emerging mycelium was transferred and individually isolated in a new mini petri dish (60 mm diameter). Isolates with the same morphological characteristics (colony color, exudates, growth type and general appearance) were grouped into morphotypes, and at least one of them was genotyped for taxonomic analysis.

A small amount of mycelium was collected and its DNA extracted using a Phire Plant Direct PCR Kit (Thermo Fisher Scientific). The complete ITS region (ITS1-5.8S-ITS2) was amplified using ITS4 and ITS5 primers [40]. The amplification cycles followed were: 98 °C for 5 min, 95 °C for 5 s (35 repeated cycles), 54 °C for 5 s, 72 °C for 20 s and a final phase of 72 °C for 1 min. PCR amplicons were purified (Favor PrepTM Plant Genomic DNA Extraction Mini Kit, Favorgen) and sequenced using the Sanger method, copying single-stranded DNA, at STABVIDA enterprise. The returned DNA sequences were grouped using the CD_HIT program at 97% identity threshold [41,42], considering the clustered sequences to represent the same taxon. A representative sequence of each cluster was selected and contrasted to the closest match of the ITS region from fungal types at the National Centre for Biotechnology Information (NCBI) using the BLAST algorithm [43]. The database UNITE was also interrogated for sequences.

2.4. Metabarcoding Analysis and Taxonomic Assignment

A total of 30 samples was sent to AllGenetics services for metabarcoding analysis. The DNA of samples was isolated using a Dneasy PowerSoil DNA isolation kit (Qiagen, Hilden, Germany), and the complete ITS2 region was amplified using the primers ITS86F and ITS4 [40,44], to which the Illumina sequencing primer sequences were attached to their 5′ ends. The PCR cycle consisted of an initial denaturation at 95 °C for 5 min, followed by

35 cycles of 95 °C for 30 s, 49 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 10 min. The index sequences required for multiplexing libraries were attached in a second PCR with the same conditions but only 5 cycles and 60 °C as the annealing temperature. Libraries were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek, Norcross, GA, USA), pooled in equimolar amounts and sequenced in a MiSeq PE300 run (Illumina, San Diego, CA, USA).

The Illumina raw files R1 (forward) and R2 (reverse) reads were trimmed and checked using the software FastQC (www.bioinformatics.babraham.ac.uk accessed on 17 October 2021). FLASH2 was used to merge reads and CUTADAPT software 1.3 to remove sequences that did not contain the PCR primers and those shorter than 100 nucleotides [45,46]. The sequences were filtered by quality using Qiime v1.9.1. and the FASTA file was processed using VSEARCH [47]. Sequences were dereplicated, sorted and clustered at a similarity threshold of 100%. Artefacts were detected and filtered using the UCHIME algorithm implemented in VSEARCH [48]. Sequences were then assigned to OTUs, and those occurring at a frequency below 0.005% in the whole dataset were removed. In the same way as the sequences obtained from the culture method, sequences were grouped using the CD_HIT program at 97% identity threshold [41,42]; we considered that the clustered OTUs were the same taxon. A representative OTU of each cluster was selected and compared with the NCBI and UNITE data using the BLAST algorithm [43].

2.5. Data Analysis

For the metabarcoding data, we estimated accumulation curves with and without singletons (OTUs and taxa that were only present in one sample) to evaluate the sampling effort and to compare the importance of rare taxa/OTUs between grasslands and tissue types. We calculated the OTU richness and Shannon and Simpson diversity indexes and we analyzed the effects of tissue and grassland type on fungal endophyte richness and diversity using two-way ANOVAs [49]. We calculated the relative abundance at the taxonomic level of phyla, orders, families and OTUs grouped into taxa using read sequences, within each tissue (shoot, rhizome and root) and each grassland type (LD and HD). We evaluated the effects of tissue and grassland type on fungal endophyte assemblages of *B. rupestre* using nonmetric multidimensional scaling (NMDS) with a Bray-Curtis dissimilarity index matrix, and we identified the distinctive fungal endophytes of a specific tissue and grassland type using indicator species tests [50], measuring the fidelity of the taxa to a particular situation [51].

3. Results

3.1. Comparison of B. rupestre Mycobiome Obtained by Culturing and Metabarcoding Methods

For the culture method, we obtained 28 isolates which were classified into a total of 19 morphotypes. Their corresponding sequences were matched in databases, a total of 15 taxa were obtained and classified to species (2), genus (9), family (3) and order (1) rank (Table 2). Ten taxa were isolated in plants collected in the LD grassland (66.6%), while eight were from the HD grassland (53.3%). We identified 2, 5 and 11 taxa from shoots, rhizomes and roots, respectively (Table 3).

The thirty samples of *B. rupestre* analyzed using the metabarcoding method produced 1,622,980 reads from 1822 OTUs before filtering and 513,671 reads from 352 OTUs after the filtering process. We obtained 316 OTUs from the LD grassland (61.1%) and 246 OTUs from the HD grassland (38.9%). There were 19,197 and 340 OTUs from shoots, rhizomes and roots, respectively. The OTU clustering process returned a total of 88 taxa: 38 assigned to genus, 16 to family, 19 to order, 9 to class and the remaining 6 to phylum or still unidentified (Appendix A). According to grassland type, 75 taxa were identified in the LD grassland (85.2%) and 52 in the HD grassland (59.1%). According to tissue type, 15, 37 and 82 taxa were identified in shoots, rhizomes and roots, respectively (Table 3).

Table 2. Fungal endophytes isolated from *B. rupestre* via the culturing method, their greatest percentage identity in both databases (NCBI and UNITE), the proposed taxon and the available accession number in GenBank.

	Match Taxon (NCBI)			Match Taxon (UNITE)				
		Accessio Number	Greatest Percentage Identity (%)		Accession Number	Greatest Percentage Identity (%)	Taxon Proposed	GenBank Accession Number
1	*Lachnellula hyalina*	NR_165202	90.11	*Albotricha* sp.	HM136666	98.22	*Albotricha* sp.	MW789554
2	*Codinaea paniculata*	NR_166297	99.74	*Codinaea* sp.	MT118230	99.74	*Codinaea* sp.	MW789567
3	*Paracamarosporium* sp.	NR_154318	94.28	*Paracamarosporium* sp.	MT882131	97.6	Didymosphaeriaceae	MW789559
4	*Drechslera* sp.	NR_153992	94.43	*Drechslera* sp.	UDB0174425	100	*Drechslera* sp	MW789560
5	*Falciphora oryzae*	NR_153972	96.69	*Falciphora* sp.	UDB0162916	99.76	*Falciphora* sp.	MW789558
6	*Glarea lozoyensis*	NR_137138	96.18	*Glarea* sp.	KF617491	99.58	Helotiaceae	MW789565
7	*Ilyonectria leucospermi*	NR_152889	99.36	*Ilyonectria crassa*	MT294410	100	*Ilyonectria* sp.	MW789566
8	*Lachnellula hualina*	NR_165202	88.89	*Lachnum virgineum*	MT133783	98.15	*Lachnum* sp.	MW789564
9	*Microdochium phragmitis*	NR_132916	100	*Microdochium phragmitis*	MH861162	100	*Microdochium phragmitis*	MW789562
10	*Mollisia asteliae*	NR_173037	96.44	*Mollisia* sp.	KJ188683	98.69	*Mollisia* sp.	MW789555
11	*Phialocephala spaheroides*	NR_121302	95.71	*Loramyces* sp.	KF618060	99.36	Mollisiaceae	MW789556
12	*Neoascochyta dactylidis*	NR_170041	100	*Neoascochyta* sp.	MT185527	100	*Neoascochyta* sp.	MW789561
13	*Omnidemptus graminis*	NR_164058	100	*Omnidemptus graminis*	MK487758	100	*Omnidemptus graminis*	MW789553
14	*Phialocephala sphaeroides*	NR_121302	89	*Phialocephala* sp.	JN995646	98.87	*Phialocephala* sp.	MW789563
15	*Paraphaeosphaeria michotii*	NR_155640	91.41	Pleosporales	MN450621	100	Pleosporales	MW789557

Table 3. Total number of reads, OTUs and taxa associated with *B. rupestre* tissues and the type of grassland where plants were collected (LD: low-diversity grassland, HD: high-diversity grassland).

		Type of Grassland		Tissue		
				Shoot	Rhizome	Root
Metabarcoding method	Reads	LD	313,621	4680	47,268	261,673
		HD	200,050	3204	29,692	167,154
		Total	513,671	7884	76,960	428,827
	OTUs	LD	316	12	165	305
		HD	246	11	58	236
		Total	352	19	197	340
	Taxa	LD	75	10	27	69
		HD	52	10	23	45
		Total	88	15	37	82
Culture method	Taxa	LD	10	2	3	6
		HD	8	1	3	5
		Total	15	2	5	11

The culturing method isolated 13 taxa out of 88 sequenced via metabarcoding. Since we used a conservative approach in the process of identification, it is likely that we arrived at different taxonomic levels of identification depending on the methodology, for example, *Codinaea* sp. (culturing) vs. Chaetosphaeriaceae (metabarcoding), Didymosphaeriaceae (culturing) vs. *Paracamarosporium* sp. (metabarcoding) and *Mollisia* sp. and *Phialocephala* sp. (culturing) vs. Mollisiaceae (metabarcoding). The rest of the isolated taxa did match at the taxonomic level assigned (*Albotricha* sp., *Drechslera* sp., *Falciphora* sp., Helotiaceae, *Lachnum* sp., *Microdochium phragmitis* and *Neoascochyta* sp.). Table 4 shows the complete information obtained from both methods for each sample, as well as the samples where the same taxon was isolated via the culturing method and also sequenced via the metabarcoding analysis. The two taxa isolated via culturing but not sequenced via metabarcoding were *Ilyonectria* sp. and *Omnidemptus graminis*. The latter was the most isolated fungal endophyte from shoots of *B. rupestre* using the culturing method.

Table 4. Culturing and metabarcoding comparison. Total number of reads, OTUs and taxa and match identification for each sample.

Sample		Culture Method	Match Methods	Metabarcoding		
		Isolated Taxa		Taxa	OTUs	Reads
Shoot LD	1	*Neoascochyta* sp.	✓	5	5	233
	2	*Omnidemptus graminis*	×	5	6	1639
	3	*Omnidemptus graminis*	×	7	9	1619
	4	*Omnidemptus graminis*	×	3	3	207
	5		×	4	4	982
Shoot HD	6		×	2	2	229
	7	*Omnidemptus graminis*	×	2	2	37
	8	*Omnidemptus graminis*	×	5	5	644
	9	*Omnidemptus graminis*	×	1	1	13
	10		×	3	3	2281
Rhizome LD	1		×	8	102	12,546
	2		×	11	21	24,377
	3	Didymosphaeriaceae Helotiaceae	× ✓	4	6	1312
	4		×	5	7	831
	5	Mollisiaceae	✓	12	41	8202
Rhizome HD	6	Helotiaceae	✓	6	22	5621
	7	Helotiaceae	✓	3	4	267
	8	*Phialocephala* sp.	×	11	17	15,380
	9		×	4	5	2035
	10	*Microdochium phragmitis*	×	11	18	6389
Root LD	1	Didymosphaeriaceae	×	15	180	49,606
	2	*Falciphora* sp. *Codinaea* sp.	✓ ×	29	53	40,482
	3	Didymosphaeriaceae	×	27	184	70,132
	4	*Mollisia* sp.	×	34	73	52,335
	5	Pleosporales Didymosphaeriaceae *Lachnum* sp.	× × ✓	31	95	49,118
Root HD	6	Helotiaceae	✓	18	141	62,044
	7	Mollisiaceae	✓	20	116	23,703
	8	*Albotricha* sp.	✓	16	32	12,379
	9	*Albotricha* sp.	✓	20	55	47,814
	10	*Drechslera* sp. *Ilyonectria* sp.	✓ ×	17	59	21,214

Despite the remarkable differences in the number of sequences obtained using the two methods (28 isolates vs. 513,671 reads), the pattern of fungal endophyte richness and diversity among grassland and tissue types followed a similar trend, with the highest values in the root tissue and plants collected from the LD grassland.

3.2. The Mycobiome of B. rupestre According to the Metabarcoding Method

3.2.1. Fungal Endophytic Richness and Diversity

The quantitative data from metabarcoding, based on read sequences, allowed an exhaustive characterization of the endophytic diversity of *B. rupestre*. Both the OTUs (352) and the clustering of OTUs into taxa (88) produced non-asymptotic species accumulation curves (Figure 2). However, 23 out of 88 taxa and 71 out of 352 OTUs were sequenced in only one sample (designated as singletons). Additional curves were constructed without singletons, suggesting that an increase in sampling effort would increase the number of rare taxa/OTUs but not the more common ones (Figure 2a,d). Accumulation curves comparing tissues and grassland types did not approach horizontal asymptotes (Figure 2b,c,e,f), therefore, greater sampling effort is required for reliable richness estimates.

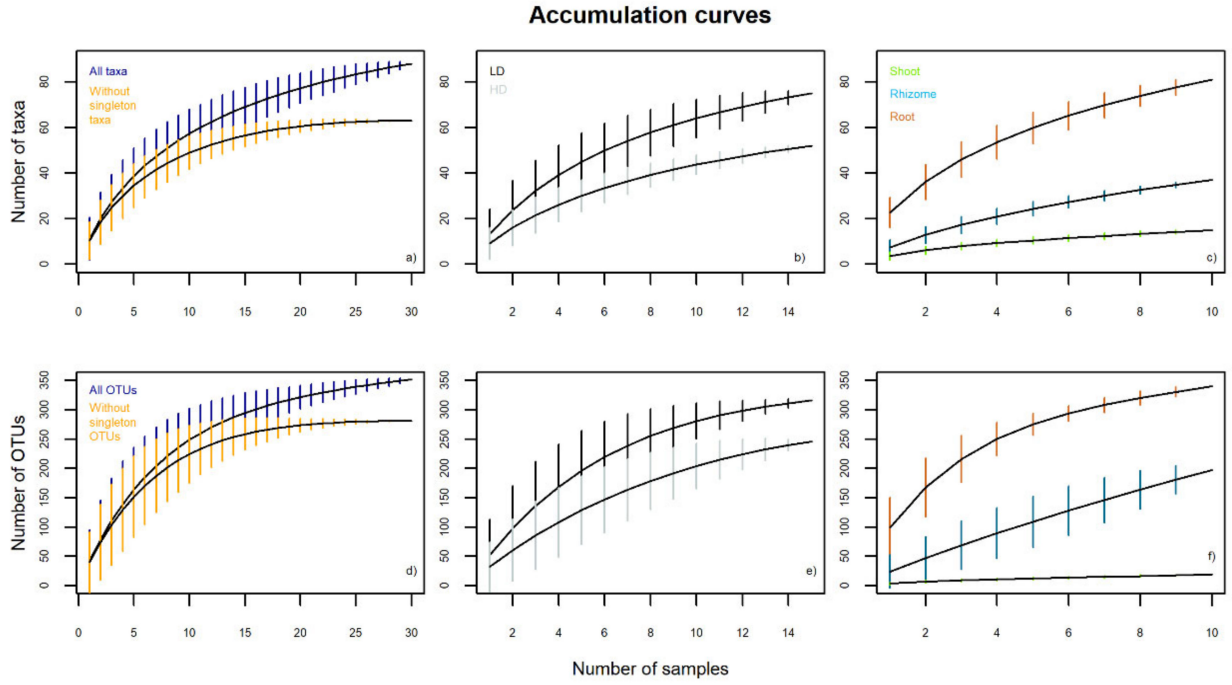

Figure 2. Taxon and OTU accumulation curves for the endophytic community of *B. rupestre* from metabarcoding (LD: low-diversity grassland, HD: high-diversity grassland). Black line shows the total number of taxa/OTUs, and vertical colored lines indicate the standard deviation.

The two factor ANOVA showed a significant effect of plant tissue (F = 19.9, $p < 0.001$) but not of grassland type (F = 2.5, $p = 0.126$) on OTU richness, whereas Shannon and Simpson indexes showed significant differences between grassland types (F = 5.1, $p = 0.033$ and F = 4.4, $p = 0.046$, respectively) and tissues (F = 32.7, $p < 0.001$ and F = 9.5, $p < 0.001$, respectively) (Figure 3).

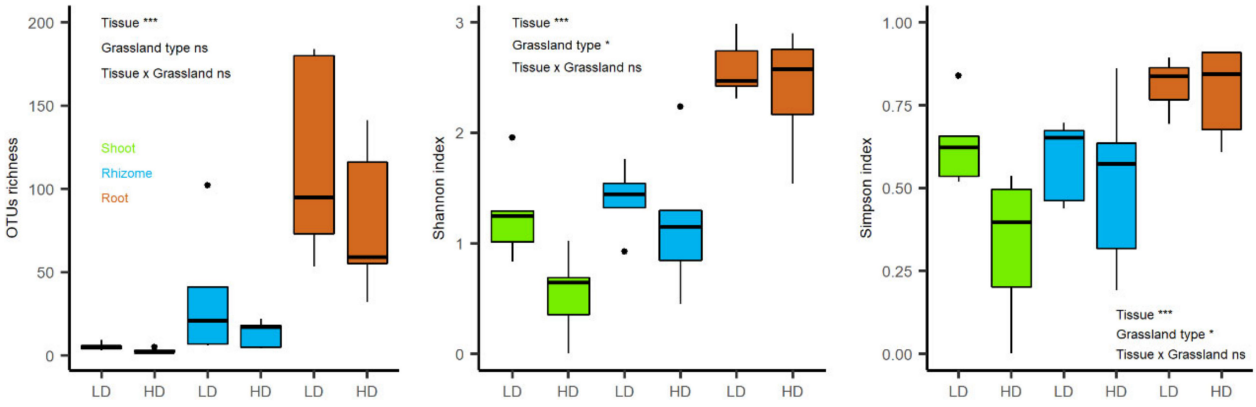

Figure 3. OTU richness and diversity indexes (Shannon and Simpson) for the endophytic community of *B. rupestre* from different tissues and grasslands (LD: low-diversity grassland, HD: high-diversity grassland). *** p-value < 0.001; * p-value < 0.05 and ns = no significance. Black points represent outliers.

3.2.2. Taxonomic Assemblages for Grassland Types and Tissues

The relative abundance of phyla, orders and families was estimated from the read sequences. Most taxa were included in the phyla Ascomycota (71.21%) and Basidiomycota (21.21%). Figure 4 shows the relative abundance of orders and families according to tissue and grassland type.

Figure 4. Taxonomic structure (orders, left and families, right) of fungal endophytes in *B. rupestre* tissues (shoot, rhizome and root) in the different grassland types (LD: low-diversity grassland, HD: high-diversity grassland).

Pleosporales dominated in shoots of plants from both grassland types (52.59% LD and 65.39% HD), followed by Phyllachorales (20.49%) and Pucciniales (16.05%) in the LD grassland and Hypocreales (17.45%) and Capnodiales (12.73%) in the HD grassland. The other orders did not exceed 4%, except Xylariales in the LD grassland (5.58%). Helotiales dominated in the belowground tissues of both grassland types ranging from 85.81% (Rhizome-HD) to 77.47% (Rhizome-LD), followed by Agaricales ranging from 20.93% (Rhizome-LD) to 7.17% (Root-LD). The other orders did not exceed 2.5% except Pleosporales in roots from the HD grassland (6.74%) (Figure 4, left).

Phaeosphaeriaceae dominated in shoots from both grassland types (63.48% HD and 34.08% LD), followed by unidentified family A (22.28%), Didymellaceae (16.94%) and Pucciniaceae (16.05%) in the LD grassland and unidentified family A (17.45%) and Mycosphaerellaceae (12.73%) in the HD grassland. The rest of the families did not exceed 5.5%. Hyaloscyphaceae dominated in belowground tissues ranging from 54.47% (Rhizome-HD) to 32.72% (Root-HD), followed by Helotiaceae ranging from 35.05% (Root-HD) to 13.16% (Rhizome-HD). Other families with relatively high abundance were Tricholomataceae and unidentified family A, ranging from 20.93% (Rhizome-LD) to 5.18% (Root-LD) and from 24.21% (Root-LD) to 8.18% (Root-HD), respectively. The other families did not exceed 4% (Figure 4, right).

The relative abundance of endophytic taxa after the OTU clustering process according to their high genetic similarity (97% threshold) was estimated from the read sequences. The most abundant read sequences were located in the root tissue and were reached by Helotiaceae (22.60%), *Lachnum* sp. A (21.94%), Helotiales A (8.29%) and *Albotricha* sp. A (7.00%). All of these were more abundant in plants collected in the LD grassland, with the exception of *Albotricha* sp. A.

In the roots, taxa with abundances higher than 5% were *Lachnum* sp. A (35.08%), Helotiaceae (24.51%) and Helotiales A (12.43%) in LD grassland plants and Helotiaceae (31.09%), *Albotricha* sp A (18.83%), *Lachnum* sp. A (12.50%), Agaricales A (9.65%) and Helotiales A (6.03%) in HD grassland plants (Table 5).

Table 5. List of the most abundant taxa in *B. rupestre* underground tissues. The relative abundance is based on number of reads, number of OTUs and infected plants (out of five). Shaded taxa were sequenced in both underground tissues. The complete table is available in Appendix B.

Endophyte Taxon	ROOT								RHIZOME							
	Relative Abundance (%)		Reads		OTUs		Infected Plants		Relative Abundance (%)		Reads		OTUs		Infected Plants	
	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Helotiaceae	24.51	31.09	64,132	51,968	114	116	2	4	25.95	16.49	12,267	4897	94	18	2	4
***Lachnum* sp. A**	35.08	12.5	91,790	20,889	36	20	5	5	29.13	9.01	13,771	2676	5	7	4	4
***Albotricha* sp. A**	1.71	18.83	4465	31,473	7	6	3	3	5.23	50.58	2473	15,018	4	6	3	3
Helotiales A	12.43	6.03	32,534	10,072	40	28	5	5	10.98	2.77	5188	823	25	1	2	3
Agaricales A	3.55	9.65	9281	16,124	3	3	2	4	0.05	0	24	0	2	0	1	0
***Mycena* sp. A**	2.03	0.17	5323	289	10	2	4	1	20.88	0	9870	0	1	0	1	0
Mollisiaceae C	4.17	0.45	10,913	745	2	1	4	1	0.42	0.04	198	13	2	1	1	1
Pleosporales A	0.56	4.04	1476	6751	2	4	3	5	0	0.04	0	12	0	1	0	1
***Glarea* sp.**	0.41	3.94	1060	6589	2	1	2	1	0	0.29	0	86	0	1	0	1
Mollisiaceae B	0.43	1.89	1118	3161	1	3	2	3	2.35	1.44	1111	429	1	3	2	2
Mollisiaceae D	0.89	1.07	2330	1782	1	2	2	1	0.78	3.56	369	1056	1	2	2	2
Chaetosphaeriaceae	1.76	0	4608	0	4	0	1	0	0.07	0	33	0	1	0	1	0
***Mycena* sp. B**	0	2.08	0	3479	0	3	0	1	0	3.34	0	993	0	1	0	1
Tricholomataceae B	0	1.48	0	2474	0	1	0	2	0	4.21	0	1251	0	1	0	1
***Lachnum* sp. B**	0.38	1.27	1007	2119	11	7	4	4	0.87	0.14	411	43	1	1	1	1
Cantharellales	1.3	0	3397	0	2	0	1	0								
***Parasola* sp.**	0	0.9	0	1503	0	1	0	1	0	5.81	0	1725	0	3	0	1
Unidentified A	1.21	0.01	3174	19	2	1	1	1								
***Ophiosphaerella* sp.**	0.96	0.32	2513	535	2	1	2	1	0.11	0.06	50	17	1	1	1	1
Mollisiaceae A	0.88	0.4	2309	666	4	3	4	5	0.03	0.28	13	83	1	1	1	1
***Drechslera* sp.**	0.03	1.43	87	2388	2	2	2	5								

The dominant taxon in the shoots was Phaeosphaeriaceae (34.08% LD and 58.80% HD). In LD grasslands, it was accompanied by Phyllachorales (20.49%), *Puccinia* sp. (16.05%), *Neoascochyta* sp. A (14.94%) and *Microdochium* sp. (5.58%) and in HD grasslands by Sordariomycete A (17.45%), Mycosphaerellaceae (12.73%) and *Ophiosphaerella* sp. (4.68%). The remaining taxa did not exceed 4% (Table 6).

Table 6. List of taxa in the *B. rupestre* shoots and their relative abundance based on number of reads, number of OTUs and infected plants (out of five).

Endophyte Taxon	SHOOT							
	Relative Abundance (%)		Reads		OTUs		Infected Plants	
	LD	HD	LD	HD	LD	HD	LD	HD
Phaeosphaeriaceae	34.08	58.80	1595	1884	2	1	4	2
Phyllachorales	20.49	0	959	0	1	0	4	0
Puccinia sp.	16.05	0	751	0	3	0	1	0
Neoascochyta sp. A	14.94	0.53	699	17	1	1	4	1
Sordariomycetes A	0	17.45	0	559	0	1	0	1
Mycosphaerellaceae	0	12.73	0	408	1	0	0	1
Microdochium sp.	5.58	1.59	261	51	1	1	4	1
Dothideales	3.65	0	171	0	1	0	2	0
Ophiosphaerella sp.	0	4.68	0	150	0	1	0	1
Epicoccum sp.	2.01	0.75	94	24	1	1	1	1
Helotiaceae	1.41	1.56	66	50	1	2	2	3
Periconia sp.	1.56	0	73	0	1	0	1	0
Lachnum sp. A	0	1.28	0	41	0	1	0	1
Phragmocephala sp. B	0	0.63	0	20	1	0	0	1
Unidentified C	0.23	0	11	0	1	0	1	0
	100	100	4680	3204				

The dominant taxa in the rhizomes of the LD grassland were *Lachnum* sp A. (29.13%), Helotiaceae (25.95%), *Mycena* sp. A (20.88%), Helotiales A (10.98%) and *Albotricha* sp. A (5.23%) and in the rhizomes of the HD grassland *Albotricha* sp. A (50.58%), Helotiaceae (16.49%), *Lachnum* sp. A (9.01%), *Parasola* sp. (5.81%) and Tricholomataceae (4.21%) (Table 5).

3.2.3. Indicator Species of the Fungal Assemblages

NMDS analysis showed that the fungal endophyte assemblage from above and belowground tissues of *B. rupestre* was clearly different (Figure 5a). In addition, fungal assemblages from root tissues (Figure 5d), unlike shoots (Figure 5b) and rhizomes (Figure 5c), displayed significant differences between grassland types.

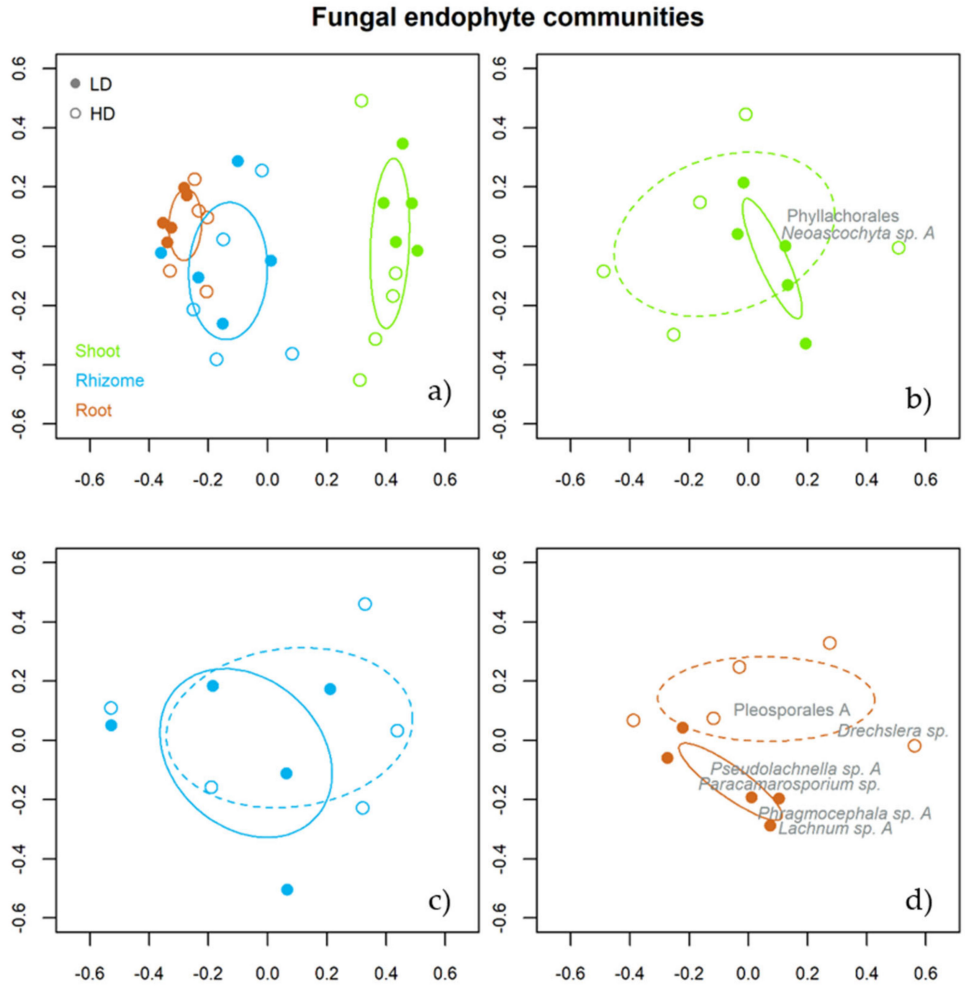

Figure 5. Non-metric multidimensional scaling analysis (NMDS) for the endophytic community of *B. rupestre* according to the effect of tissue (**a**) and plant diversity (shoot 1b, rhizome 1c & roots 1d). The ellipse formed by the solid line encompasses the fungal composition of *B. rupestre* tissues (**a**). The ellipses formed by broken and solid lines encompass the fungal composition of the low and high-diversity grassland, respectively (**b**–**d**). The taxon names in the graphs for shoots (**b**) and roots (**d**) are the indicator species for the effect of plant diversity.

The indicator species for shoot tissues were Phaeosphaeriaceae (p = 0.002), *Neoascohyta* sp. A (p = 0.005) and Phyllachorales (p = 0.029) and for root tissues were Helotiales A (p = 0.001), *Lachnum* sp. A (p = 0.001), Mollisiaceae A (p = 0.001), Pleosporales A (p = 0.001), *Drechslera* sp. (p = 0.001), *Lachnum* sp. B (p = 0.002), Agaricales A (p = 0.001), *Cladophialophora* sp. (p = 0.003), *Leohumicola* sp. (p = 0.003), *Pseudolachnella* sp. A (p = 0.007), Mollisiaceae C (p = 0.011), *Pseudolachnella* sp. B (p = 0.023), Unidentified B (p = 0.041),

Phragmocephala sp. A (p = 0.019) and *Paracamarosporium* sp. (p = 0.036). No species were indicative of rhizome tissues.

The indicator species for grassland type were Phyllachorales (p = 0.044) and *Neoascochyta* sp. A (p = 0.035) in shoots collected in the LD grassland (Figure 5b). *Drechslera* sp. (p = 0.012) and Pleosporales A (p = 0.036) were indicators in roots from the HD grassland, while *Lachnum* sp. A (p = 0.007), *Phragmocephala* sp. A (p = 0. 042), *Paracamarosporium* sp. (p = 0.037) and *Pseudolachnella* sp. A (p = 0.047) were indicators in roots from the LD grassland (Figure 5d). No species in the rhizome tissues were indicative of plant community (Figure 5d).

4. Discussion

4.1. The Mycobiome of B. rupestre According to the Metabarcoding Data

The results of the metabarcoding showed that 88 taxa constituted the mycobiome of *B. rupestre* and that only seven taxa sequenced from the belowground tissues accounted for 81.2% of the total reads (Helotiaceae, *Lachnum* sp. A, *Albotricha* sp. A, Helotiales A, Agaricales A, *Mycena* sp. A and Mollisiaceae C), while the other 81 taxa were responsible for the remaining 18.8%, and 25 of them were only sequenced in a single sample. Therefore, a restricted sampling effort using the metabarcoding method was able to identify a small group of abundant fungal endophytes and a large group of rare species. The accumulation curves also supported the idea that extension of the sampling effort would enrich the group of rare species but not the most common species. This pattern of fungal endophyte distribution seems common to grasses [52] and indicates that a limited sampling effort is enough to provide good characterization of the dominant fungal species in plants, which is important considering the high cost of metabarcoding. However, when addressing studies on fungal richness and diversity, more extended sampling appears necessary to avoid an underestimation of the values.

The results of the study also highlight the importance of sampling the different tissues of plants to obtain a reliable characterization of its mycobiome [53,54]. Aboveground fungal assemblages were much poorer in species, less diverse and taxonomically different from those of rhizomes and roots, and this pattern was consistent between the grassland types, as observed by other authors in different plant species and different habitats [55–57]. The soil rhizosphere is the main route of fungal transmission to plants [58,59], and the high biomass of rhizome and roots developed by *B. rupestre* offers a large surface in contact with the soil microbiome. The majority of taxa identified were specific to a tissue, or exhibited a strong preference for it, and only five taxa appeared in all tissues (Helotiaceae, *Lachnum* sp. A, *Ophiosphaerella* sp., *Microdochium* sp. and *Epicoccum* sp.). As expected, the relative abundances of taxonomic orders and families also varied between tissues, with Pleosporales and Phaeosphaeriaceae more abundant in shoots and Helotiales and Hyaloscyphaceae more abundant in rhizomes and roots.

When comparing these results with previous characterizations of fungal endophyte assemblages in perennial temperate grasses based on culture techniques and extensive surveys, we realize the power of the metabarcoding tool, which is capable of identifying a large set of taxa with much less sampling effort. In *Dactylis glomerata*, 22 and 48 taxa were identified using culturing methods from the leaves and the roots of 120 samples [60], and in *Holcus lanatus*, 77 and 79 were identified in the same tissues of 77 samples [61]. The results of our survey of the leaves and roots of *B. rupestre* (2 and 11 taxa identified using the culturing method and 12 and 82 taxa identified using metabarcoding) obtained from a small number of samples in a regional sampling suggest that the real diversity and richness of the endophytic fungal assemblages of the previously studied grass species have probably been underestimated and would increase greatly if the novel metabarcoding techniques were used.

4.2. Culturing vs. Metabarcoding Methods

Modern massive sequencing techniques are gaining ground over traditional culturing methods due to the quantitative power of data that they are able to generate. With equal sampling effort, metabarcoding identified 13, 32 and 71 more taxa than culturing methods in shoots, rhizomes and roots, respectively, which means around ×5.8 times more species identified by the novel technique consistently in the three tissues. In similar studies comparing both methods, the metabarcoding identified ×5.2 and ×4.3 times more OTUs in roots of *Elymus repens* and *Deschampsia flexuosa* respectively than the culturing technique [62,63]. A parallel study using 240 plants of *B. rupestre* recognized 45 fungal endophytic taxa using the culturing method [39], in contrast to the 88 taxa sequenced using metabarcoding from 10 plants in the current survey. In this parallel study, the singletons isolated accounted for 48.9% of the taxa identified via culturing methods and 28.4% of the taxa identified via metabarcoding (with OTUs clustered with a 97% of similarity threshold).

Regarding belowground tissues, four fungal species with high incidence in root tissues were identified via both methodologies: *Albotricha* sp., Helotiaceae, *Lachnum* sp. and Mollisiaceae. In shoots, surprisingly, the most frequent shoot endophyte identified via the culturing method, *Omnidemptus graminis*, was not identified using the metabarcoding technique. *O. graminis* is a recently described taxon, included in a family associated with ongoing taxonomic changes due to molecular advances [64,65]. Its fast mycelial growth observed on culture plates may suggest the encrypting of other endophytes, but how *O. graminis* escaped the sequencing process of the metabarcoding is a matter that needs further study.

At this point, some issues need to be discussed when comparing the technical procedures of sequencing in both techniques. The ITS region is a universal and commonly used DNA barcode marker for fungi [66], and in the metabarcoding study undertaken by an external company, only the ITS2 region was amplified to identify the fungal sequences [67,68]. In the culturing method undertaken in the UPNA's lab, the fungal mycelium was collected and the complete ITS region was amplified (ITS1-5.8S-ITS2), generating longer DNA sequences. We suggest that, since the ITS2 region is more restrictive, taxonomic inconsistencies may occur when short sequences are compared in the databases, thus affecting taxon identification [18]. The percentages of taxa identified for the metabarcoding were in the range 78.1–100%, and 97.6–100% for the culture sequencing, evidencing this restriction and indicating the value of sequencing the complete ITS region to achieve better fungal taxa identification. As a particular example, the taxon proposed as *Codinaea* sp. reached a match of 99.74% with the complete ITS region sequenced, while this percentage decreased to 97.52% when considering only the ITS2 region. As a consequence, the species was identified as Chaetosphaeriaceae in the metabarcoding, following a more conservative approach, although it was probably the same taxon. Similar situations may occur in other closely related taxa, when there is no reference specimen in the database [43,69]. Taxa identified as Mollisiaceae in our study probably belong to the genera *Mollisia* and/or *Phialocephala* [70,71] and the family Helotiaceae to the genera *Glarea* and/or *Hymenoscyphus* [72]. Both families were abundant in our samples. Other highly inclusive taxa, such as Pleosporales, raised similar doubts in the identification due to the still high uncertainty in the genetic characterization of the type specimens.

Despite the remarkable differences between the quantitative data generated using the two methods, the characteristics of the fungal assemblages in the different plant communities and tissues types are consistent between methods. Root tissues display the most diverse and rich fungal assemblages, and the endophytic community in plants collected in more disrupted, LD grasslands had the highest diversity and richness. Similar patterns have been reported in previous research in the area, conducted with a much greater sampling effort and using the culturing method [39], that analyzed the fungal assemblages in terms of the ecological mechanisms favored by the different disturbance regimes.

5. Conclusions

The endophytic mycobiome of *B. rupestre* is composed of a few abundant and many rare species, the identification of which depends on the sampling effort. Despite the restricted sampling effort, the two methodologies produced consistent results and detected the same trends in endophytic richness and diversity among tissues (roots > rhizomes > shoot) and grassland types (low-diversity > high-diversity). Comparatively, the metabarcoding method allowed the identification of a much larger number of taxa than the culturing method and revealed differences in richness and diversity that were not apparent with the culturing method (even when a larger number of samples was collected [39]).

Despite the promising results of the metabarcoding technique, the data indicate that a combination of the two methodologies is the best current option to obtain an adequate characterization of the plant fungal assemblage. In this study, metabarcoding did not identify *Omnidemptus graminis*, the most abundant fungal endophyte isolated in shoots via culturing; this recently described species is included in a family where there have been repeated taxonomic restructurings as a result of molecular advances [65].

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequencing data have been deposited in GenBank at NCBI with their accession number.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Table with the 88 identified taxa from the metabarcoding method.

	Match Taxon (NCBI)			Match Taxon (UNITE)				
		Accession Number	Greatest Percentage Identity (%)		Accession Number	Greatest Percentage Identity (%)	Taxon Proposed	Accession Number
1	*Gerronema* sp.	NR_166278	82.37	*Delicatula integrella*	UDB034203	99.77	Agaricales A	OK430888
2	*Gerronema* sp.	NR_166278	78.9	*Mycena* sp.	KT224934	89	Agaricales B	OK430889
3	*Ramariopsis flavescens*	NR_119913	85.06	Agaricales	JX456916	95.2	Agaricales C	OK430890
4	*Gerronema indigoticum*	NR_166278	78.06	Mycenaceae	KT224934	94.29	Agaricales D	OK430891
5	*Laccaria aurantia*	NR_154113	78.57	*Mycena floridula*	MH856660	99.35	Agaricales E	OK430892
6	*Radulotubus resupinatus*	NR_153458	84.66	Agaricomycetes	LR864837	99.32	Agaricomycetes	OK430893
7	*Lachnellula hyalina*	NR_165202	93.02	*Albotricha* sp.	JN995639	100	*Albotricha* sp. A	OK430894
8	*Lachnellula hyalina*	NR_165202	91.59	*Albotricha* sp.	JN995639	98.71	*Albotricha* sp. B	OK430895
9	*Lachnellula hyalina*	NR_165202	91.59	*Albotricha* sp.	HM136666	100	*Albotricha* sp. C	OK430896
10	*Funiliomyces biseptatus*	NR_159862	96.39	*Acremonium* sp.	MT911439	100	Ascomycota A	OK430897
11	*Tricladium terrestre*	NR_160144	93.67	Ascomycota sp.	KR266584	93.67	Ascomycota B	OK430898
12	*Auricularia scissa*	NR_125807	80.48	*Oliveonia* sp.	MT235652	97.16	Auriculariales	OK430899
13	*Hydnum albidum*	NR_164025	78.7	*Sistotrema* sp.	KC965692	93.87	Cantharellales	OK430900
14	*Codinaeae* sp.	NR_168799	97.52	*Codinaea* sp.	MT626587	98.35	Chaetosphaeriaceae	OK430901
15	*Chalara hyalocuspica*	NR_137568	91.25	*Chalara* sp.	MK965778	98.33	*Chalara* sp.	OK430902
16	*Cladophialophora tengchongensis*	NR_172399	90.07	*Cladophialophora* sp.	KP889848	100	*Cladophialophora* sp.	OK430903
17	*Coccomyces pinicola*	NR_158295	83.54	*Coccomyces dentatus*	KU986782	93.82	*Coccomyces* sp.	OK430904
18	*Conlarium duplumascospora*	NR_138382	94.9	*Conlarium* sp.	MK164654	96.85	*Conlarium* sp.	OK430905
19	*Laburnicola centaurear*	NR_154131	93.6	*Laburnicola* sp.	MK018553	97.95	Didymosphaeriaceae	OK430906
20	*Pseudoseptoria collariana*	NR_156560	97.63	*Pseudoseptoria donacis*	MH859141	99.6	Dothideales	OK430907
21	*Roussoella thailandica*	NR_155717	80.56	Dothideomycetes	KJ827952	95	Dothideomycetes A	OK430908
22	*Pirozynskiella laurisilvica*	NR_153488	91	Capnodiales	KX403688	91	Dothideomycetes B	OK430909
23	*Drechslera* sp.	NR_164466	92.89	*Drechslera* sp.	MT816433	99.6	*Drechslera* sp.	OK430910
24	*Entoloma luteofuscum*	NR_152900	95.24	*Entoloma conferendum*	MT741744	100	*Entoloma* sp.	OK430911
25	*Epicoccum phragmospora*	NR_165920	99.19	*Epicoccum* sp.	MW054426	100	*Epicoccum* sp.	OK430912
26	*Falciphora oryzae*	NR_153972	98.86	*Falciphora oryzae*	MH201898	99.23	*Falciphora* sp.	OK430913

Table A1. *Cont.*

	Match Taxon (NCBI)			Match Taxon (UNITE)				
		Accession Number	Greatest Percentage Identity (%)		Accession Number	Greatest Percentage Identity (%)	Taxon Proposed	Accession Number
27	*Glarea lozoyensis*	NR_137138	98.48	*Glarea* sp.	KT268823	100	*Glarea* sp.	OK430914
28	*Glarea lozoyensis*	NR_137138	95.96	*Glarea* sp.	KF617491	100	Helotiaceae	OK430915
29	*Loramyces macrosporus*	NR_138379	89.8	*Loramyces* sp.	KF618060	99.58	Helotiales A	OK430916
30	*Loramyces macrosporus*	NR_138379	89.07	*Mollisia sp.*	UDB0778890	99.59	Helotiales B	OK430917
31	*Triposporium cycadicola*	NR_156587	89.71	*Hymenoscyphus* sp.	HQ625461	99.58	Helotiales C	OK430918
32	*Bisporella shangrilana*	NR_153628	97.02	Helotiales	LR863043	99.58	Helotiales D	OK430919
33	*Hyaloscypha finlandica*	NR_121279	92.27	*Hyaloscypha vraolstadiae*	KC876248	96.23	Hyaloscyphaceae	OK430920
34	*Lachnellula hyalina*	NR_165202	91.12	*Lachnum* sp.	MT913626	96.61	*Lachnum* sp. A	OK430921
35	*Lachnum fusiforme*	NR_154122	89.91	*Lachnum* sp.	MK808968	97.45	*Lachnum* sp. B	OK430922
36	*Proliferodiscus* sp.	NR_164304	86.67	*Lachnum* sp.	MH628228	99.57	*Lachnum* sp. C	OK430923
37	*Leohumicola minima*	NR_121307	100	*Leohumicola* sp.	FM999596	100	*Leohumicola* sp.	OK430924
38	*Variabilispora flava*	NR_165906	86.83	Helotiales	AY969994	95.65	Leotiomycetes	OK430925
39	*Menispora ciliata*	NR_171740	99.5	*Menispora ciliata*	MH860017	99.12	*Menispora* sp.	OK430926
40	*Microdochium phragmitis*	NR_132916	100	*Microdochium phragmitis*	MN077456	100	*Microdochium* sp.	OK430927
41	*Phialocephala* sp.	NR_119482	90.38	*Phialocephala* sp.	MG066460	97.88	Mollisiaceae A	OK430928
42	*Mollisia scopiformis*	NR_119460	93.22	*Phialocephala* sp.	MK808244	98.72	Mollisiaceae B	OK430929
43	*Mollisia monilioides*	NR_171261	96.22	*Phialocephala* sp.	MT911435	100	Mollisiaceae C	OK430930
44	*Mollisia prismatica*	NR_171258	91.9	*Phialocephala* sp.	MK965789	99.57	Mollisiaceae D	OK430931
45	*Mollisia asteliae*	NR_173037	95.15	*Mollisia* sp.	MH633925	100	Mollisiaceae E	OK430932
46	*Mollisia diesbachiana*	NR_171259	96.77	*Mollisia* sp.	MT179560	100	Mollisiaceae F	OK430933
47	*Mortierella gemmifera*	NR_111559	94.81	Mortierellaceae	LR863033	99.43	*Mortierella* sp.	OK430934
48	*Podila horticola*	NR_111572	99.09	*Mortierella* sp.	DQ388818	99.7	Mortierellaceae	OK430935
49	*Mycena fulgoris*	NR_163300	93.29	*Mycena* sp.	JF519186	98.4	*Mycena* sp. A	OK430936
50	*Mycena fulgoris*	NR_163300	93.29	*Mycena* sp.	MK961197	99.67	*Mycena* sp. B	OK430937
51	*Mycena fulgoris*	NR_163300	93.31	*Mycena arcangeliana*	JF908402	99.35	*Mycena* sp. C	OK430938
52	*Mycena fulgoris*	NR_163300	87.99	*Mycena* sp.	UDB020406	100	*Mycena* sp. D	OK430939
53	*Mycena fulgoris*	NR_163300	89.64	*Mycena* sp.	HQ625481	99.32	*Mycena* sp. E	OK430940
54	*Cercospora coniogrammes*	NR_147260	97.89	*Cercospora* sp.	MN970528	97.89	Mycosphaerellaceae	OK430941
55	*Myrmecridium spartii*	NR_155376	96.25	*Myrmecridium* sp.	MW133876	98.32	*Myrmecridium* sp.	OK430942
56	*Pseudomassariella vexata*	NR_164217	87.78	*Fusidium* sp.	HG936132	100	Nectriaceae	OK430943
57	*Neoascochyta europaea*	NR_136131	97.03	*Neoascochyta europaea*	MK190674	97.17	*Neoascochyta* sp. A	OK430944
58	*Neoascochyta soli*	NR_158269	100	*Neoascochyta paspali*	MT373264	100	*Neoascochyta* sp. B	OK430945
59	*Ophiosphaerella aquatica*	NR_154352	89.96	*Ophiosphaerella* sp.	MH063799	98.38	*Ophiosphaerella* sp.	OK430946
60	*Paracamarosporium fagi*	NR_154318	99.18	*Paracamarosporium fagi*	MN244221	99.18	*Paracamarosporium* sp.	OK430947
61	*Parasola parvula*	NR_160509	94.43	*Parasola schroeteri*	UDB024639	99.67	*Parasola* sp.	OK430948
62	*Periconia epilithographicola*	NR_157477	94.55	*Periconia* sp.	MG543950	100	*Periconia* sp.	OK430949
63	*Pezicula rhizophila*	NR_155659	100	*Pezicula* sp.	MN385513	100	*Pezicula* sp.	OK430950
64	*Parastagonospora poagena*	NR_168147	97.94	*Parastagonospora nodorum*	MN313349	99.17	Phaeosphaeriaceae	OK430951
65	*Phragmocephala garethjonessi*	NR_147636	92.21	*Phragmocephala garethjonessi*	MN660752	92.21	*Phragmocephala* sp. A	OK430952
66	*Phragmocephala garethjonessi*	NR_147636	90.2	*Phragmocephala atra*	MN660752	90.61	*Phragmocephala* sp. B	OK430953
67	*Phyllachora* sp.	NR_156611	85	*Phyllachora graminis*	AF257111	96.68	Phyllachorales	OK430954
68	*Pleotrichocladium opacum*	NR_155696	94.21	Pleosporales	KY228531	99.58	Pleosporales A	OK430955
69	*Camposporium multiseptatum*	NR_171863	100	*Camposporium* sp.	MN758889	100	Pleosporales B	OK430956
70	*Anteaglonium rubescens*	NR_164489	89.92	*Lophiostoma* sp.	EU977287	93.17	Pleosporales C	OK430957
71	*Pseudolachnella fusiformis*	NR_154280	94.24	*Pseudolachnella fusiformis*	AB934080	94.24	*Pseudolachnella* sp. A	OK430958
72	*Pseudolachnella fusiformis*	NR_154280	93.78	*Pseudolachnella fusiformis*	AB934080	93.77	*Pseudolachnella* sp. B	OK430959
73	*Puccinia aizazii*	NR_158929	99.2	*Puccinia brachypodii*	GQ457303	100	*Puccinia* sp.	OK430960
74	*Plectosphaerella niemeijerarum*	NR_156677	88.24	Plectosphaerellaceae	MK762215	88.23	Sordariomycetes A	OK430961
75	*Phaeoacrenonium cinereum*	NR_132066	80.62	Sordaryomycetes	KP050604	80.62	Sordariomycetes B	OK430962
76	*Cordana pauciseptata*	NR_154771	88.98	Sordariales	UDB067041	96.69	Sordariomycetes C	OK430963
77	*Neomyrmecridium guizhouense*	NR_170024	82.45	Sordariomycetes	LR865231	100	Sordariomycetes D	OK430964
78	*Atractospora verruculosa*	NR_153542	89.53	Sordariales	EU754966	100	Sordariomycetes E	OK430965
79	*Subulicistidium oberwinkleri*	NR_159060	86.42	Trechisporales	JF519283	100	Trechisporales A	OK430966
80	*Subulicystidium oberwinkleri*	NR_159060	80.53	Trechisporales	UDB020436	83.77	Trechisporales B	OK430967
81	*Trichoderma hispanicum*	NR_138451	99.25	*Trichoderma koningii*	MT781958	99.24	*Trichoderma* sp.	OK430968
82	*Corinarius hadrocroceus*	NR_131854	79.62	Tricholomataceae	KX115676	100	Tricholomataceae A	OK430969
83	*Mycena seminau*	NR_154170	88.82	Tricholomataceae	MH016642	99.67	Tricholomataceae B	OK430970
84	*Phialocephala humicola*	NR_103570	87.7	Chaetosphaeriales	HM136627	100	Unidentified A	OK430971
85	*Rhodosporidiobolus fluvialis*	NR_077089	93.65	Agaricomycetes	UDB0327559	100	Unidentified B	OK430972
86	*Mycosymbioces mycenaphila*	NR_137807	85.06	Helotiales	UDB0779249	100	Unidentified C	OK430973
87	*Mollisia monilioides*	NR_171261	90.34	Helotiales	KT203037	96.61	Unidentified D	OK430974
88	*Linteromyces quintiniae*	NR_171989	86.25	Xylariales	MN218782	99.62	Xylariales	OK430975

Appendix B

Table A2. Complete table with all identified taxa in underground tissues of the *B. rupestre* via metabarcoding method. The relative abundance is based on number of reads, number of OTUs and infected plants (out of five). Shaded taxa were sequenced in both underground tissues.

Endophyte Taxon	ROOT								RHIZOME							
	Relative Abundance (%)		Reads		OTUs		Infected Plants		Relative Abundance (%)		Reads		OTUs		Infected Plants	
	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Helotiaceae	24.51	31.09	64,132	51,968	114	116	2	4	25.95	16.49	12,267	4897	94	18	2	4
***Lachnum* sp. A**	35.08	12.5	91,790	20,889	36	20	5	5	29.13	9.01	13,771	2676	5	7	4	4
***Albotricha* sp. A**	1.71	18.83	4465	31,473	7	6	3	3	5.23	50.58	2473	15,018	4	6	3	3
Helotiales A	12.43	6.03	32,534	10,072	40	28	5	5	10.98	2.77	5188	823	25	1	2	3
Agaricales A	3.55	9.65	9281	16,124	3	3	2	4	0.05	0	24	0	2	0	1	0
***Mycena* sp. A**	2.03	0.17	5323	289	10	2	4	1	20.88	0	9870	0	1	0	1	0
Mollisiaceae C	4.17	0.45	10,913	745	2	1	4	1	0.42	0.04	198	13	2	1	1	1
Pleosporales A	0.56	4.04	1476	6751	2	4	3	5	0	0.04	0	12	0	1	0	1
***Glarea* sp.**	0.41	3.94	1060	6589	2	1	2	1	0	0.29	0	86	0	1	0	1
Mollisiaceae B	0.43	1.89	1118	3161	1	3	2	3	2.35	1.44	1111	429	1	3	2	2
Mollisiaceae D	0.89	1.07	2330	1782	1	2	2	1	0.78	3.56	369	1056	1	2	2	2
Chaetosphaeriaceae	1.76	0	4608	0	4	0	1	0	0.07	0	33	0	1	0	1	0
***Mycena* sp. B**	0	2.08	0	3479	0	3	0	1	0	3.34	0	993	0	1	0	1
Tricholomataceae B	0	1.48	0	2474	0	1	0	2	0	4.21	0	1251	0	1	0	1
***Lachnum* sp. B**	0.38	1.27	1007	2119	11	7	4	4	0.87	0.14	411	43	1	1	1	1
Cantharellales	1.3	0	3397	0	2	0	1	0								
***Parasola* sp.**	0	0.9	0	1503	0	1	0	1	0	5.81	0	1725	0	3	0	1
Unidentified A	1.21	0.01	3174	19	2	1	1	1								
***Ophiosphaerella* sp.**	0.96	0.32	2513	535	2	1	2	1	0.11	0.06	50	17	1	1	1	1
Mollisiaceae A	0.88	0.4	2309	666	4	3	4	5	0.03	0.28	13	83	1	1	1	1
***Drechslera* sp.**	0.03	1.43	87	2388	2	2	2	5								
***Paracamarosporium* sp.**	0.92	0	2419	0	1	0	4	0	0.06	0	28	0	1	0	2	0
Agaricales C	0.58	0.07	1514	114	1	1	2	1								
Auriculariales	0.5	0	1308	0	2	0	1	0								
Tricholomataceae A	0.48	0	1266	0	1	0	1	0								
Unidentified B	0.13	0.43	340	718	1	1	3	2	0.19	0.29	90	86	1	1	1	1
***Pseudolachnella* sp. B**	0.42	0.04	1097	72	1	1	3	2	0.11	0	51	0	1	0	1	0
***Trichoderma* sp.**	0.41	0.01	1076	15	1	1	2	1								
Didymosphaeriaceae	0	0.58	0	963	0	2	0	1								
***Conlarium* sp.**	0.35	0	925	0	1	0	3	0	0.07	0	35	0	1	0	1	0
Helotiales C	0.34	0	900	0	1	0	1	0								
***Phragmocephala* sp. A**	0.28	0	734	0	2	0	4	0								
Agaricales B	0.26	0.02	675	32	2	1	1	1								
***Menispora* sp.**	0.27	0	702	0	1	0	2	0								
***Cladophialophora* sp.**	0.23	0.04	604	74	1	1	3	3	0.04	0	18	0	1	0	1	0
Pleosporales B	0.11	0.2	299	341	1	2	1	2								
***Pseudolachnella* sp. A**	0.23	0.01	606	11	2	1	4	1								
Mortierellaceae	0.11	0	283	0	2	0	1	0	0.43	0	201	0	3	0	1	0
Mollisiaceae F									1.01	0	476	0	1	0	1	0
***Chalara* sp.**	0.06	0.03	168	57	2	2	3	2	0.4	0.09	187	26	1	1	1	1
Sordariomycetes D	0.17	0	435	0	1	0	1	0								
Helotiales B	0.03	0	87	0	1	0	1	0	0.21	0.73	99	218	1	1	1	1
Agaricomycetes	0.12	0	325	0	1	0	1	0								
Ascomycota B	0.1	0	266	0	1	0	1	0	0.09	0	41	0	1	0	1	0
Mollisiaceae E	0	0.13	0	216	0	1	0	1	0	0.31	0	91	0	1	0	1
***Microdochium* sp.**	0.07	0	187	0	1	0	3	0	0.25	0	119	0	1	0	1	0
***Mortierella* sp.**	0.12	0	305	0	2	0	1	0								
Sordariomycetes B	0.11	0	297	0	1	0	1	0								
***Pezicula* sp.**	0	0.16	0	272	0	1	0	2								
***Coccomyces* sp.**	0.09	0.02	227	31	1	1	2	1								
***Albotricha* sp. B**	0	0.13	0	217	0	2	0	1	0	0.06	0	19	0	1	0	1
***Leohumicola* sp.**	0.06	0.03	166	57	1	1	4	2	0.03	0	13	0	1	0	1	0
***Lachnum* sp. C**	0.09	0	228	0	1	0	1	0								
Pleosporales C	0	0.13	0	213	0	1	0	3								
Nectriaceae	0.08	0	212	0	1	0	1	0								
***Phragmocephala* sp. B**	0.06	0.03	147	45	1	1	1	2								
Sordariomycetes C	0	0.11	0	188	0	1	0	2								

Table A2. *Cont.*

Endophyte Taxon	ROOT								RHIZOME							
	Relative Abundance (%)		Reads		OTUs		Infected Plants		Relative Abundance (%)		Reads		OTUs		Infected Plants	
	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Ascomycota A	0.06	0	168	0	1	0	3	0								
Sordariomycetes E	0.06	0	167	0	1	0	1	0								
Trechisporales B	0	0.09	12	151	1	1	1	1								
Trechisporales A	0.03	0	76	0	1	0	2	0	0	0.28	0	82	0	1	0	1
Agaricales D	0.06	0	153	0	1	0	1	0								
***Myrmecridium* sp.**	0.06	0	149	0	1	0	2	0								
Dothideomycetes A	0.04	0	117	0	1	0	1	0	0.06	0	30	0	1	0	1	0
***Entoloma* sp.**	0.06	0	144	0	1	0	1	0								
***Falciphora* sp.**	0.05	0	141	0	1	0	1	0								
Leotiomycetes	0.05	0	139	0	1	0	1	0								
***Mycena* sp. D**	0.05	0.01	120	14	1	1	1	1								
***Mycena* sp. E**	0	0.08	0	133	0	1	0	1								
Unidentified D	0.05	0	132	0	1	0	1	0								
Agaricales E	0.05	0	128	0	1	0	1	0								
Dothideomycetes B	0	0.08	0	127	0	1	0	1								
Unidentified C	0.05	0	124	0	1	0	1	0								
***Albotricha* sp. C**	0.05	0	123	0	1	0	1	0								
Helotiales D	0.05	0	121	0	1	0	1	0								
***Neoascochyta* sp. B**	0.01	0	17	0	1	0	1	0	0.22	0	102	0	1	0	3	0
***Mycena* sp. C**	0.03	0	86	0	1	0	1	0	0	0.07	0	22	0	1	0	1
Hyaloscyphaceae	0.03	0.02	73	27	1	1	2	1								
***Periconia* sp.**	0.02	0	51	0	1	0	2	0								
***Epicoccum* sp.**	0	0	12	0	1	0	1	0	0	0.09	0	26	0	1	0	1
Phaeosphaeriaceae	0	0.02	0	28	0	1	0	1								
Xylariales sp.	0.04	0.01	105	12	1	1	2	1								
	100	100	261673	167154					100	100	47268	29692				

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