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Environmental heterogeneity structures root-associated fungal communities in *Daphne arbuscula* (Thymelaeaceae), a shrub adapted to extreme rocky habitats

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Abstract

Rocky habitats, globally distributed ecosystems, harbour diverse biota, including numerous endemic and endangered species. Vascular plants thriving in these environments face challenging abiotic conditions, requiring diverse morphological and physiological adaptations. Their engagement with the surrounding microbiomes is, however, equally vital for their adaptation, fitness, and long-term survival. Nevertheless, there remains a lack of understanding surrounding this complex interplay within this fascinating biotic ecosystem. Using microscopic observations and metabarcoding analyses, we examined the fungal abundance and diversity in the root system of the rock-dwelling West Carpathian endemic shrub, *Daphne arbuscula* (Thymelaeaceae). We explored the diversification of root-associated fungal communities in relation to microclimatic variations across the studied sites. We revealed extensive colonization of the *Daphne* roots by diverse taxonomic fungal groups attributed to different ecological guilds, predominantly plant pathogens, dark septate endophytes (DSE), and arbuscular mycorrhizal fungi (AMF). Notably, differences in taxonomic composition and ecological guilds emerged between colder and warmer microenvironments. Apart from omnipresent AMF, warmer sites exhibited a prevalence of plant pathogens, while colder sites were characterized by a dominance of DSE. This mycobiome diversification, most likely triggered by the environment, suggests that *D. arbuscula* populations in warmer areas may be more vulnerable to fungal diseases, particularly in the context of global climate change.

KEYWORDS
amplicon sequencing, arbuscular mycorrhiza, Carpathians, dark septate endophytes, endemism, fungal pathogens

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Zuzana Gajdošová and Miroslav Caboň contributed equally to this work.

1 | INTRODUCTION

Rocky outcrops owe their extraordinary biodiversity to spatial isolation, enduring environmental stability, and pronounced microsite heterogeneity. In addition to exposed open rocky outcrops, formations like shaded inverse gorges, overhangs, and various crevices harbour sites with milder microclimates. This extensive landscape heterogeneity fosters organismal adaptations and the gradual accumulation of species over an extended period (Larson et al., 2000; Matthes & Larson, 2006; Porembski & Barthlott, 2000). Moreover, the light-demanding species persist into interglacial or postglacial periods when these regions remain naturally forest-free, resembling isolated "ecological islands" within the broader landscape (Breman et al., 2020; Coleine et al., 2021; Mráz et al., 2016). On the other hand, rocky outcrops exhibit a stressful abiotic environment, posing significant challenges to the plant communities inhabiting these habitats. Consequently, these habitats are predominantly inhabited by stress-tolerant species that have adapted to the extreme environmental conditions associated with rocky environments (Larson et al., 2000; Matthes & Larson, 2006).

Beyond challenging abiotic conditions, the fitness and survival of rocky species are subject to the influence of biotic factors, notably interactions with the surrounding microbiome. In addition to bacteria, lichens, and algae, fungal communities, in particular, exert a profound influence on the environment and all organisms within these habitats (e.g. Carvalho et al., 2012; Casazza et al., 2017; Krah & March-Salas, 2022; Morón-Ríos et al., 2017). Fungi form diverse ecological interactions with rock-dwelling plants, spanning from beneficial mutualism, asymptomatic endophytism, saprotrophism to detrimental parasitism (Brundrett, 2009; Dighton, 2007; Doehlemann et al., 2017; Kogel et al., 2006; Santos et al., 2021). In these interactions, mycorrhiza is recognized as one of the most fundamental mutually beneficial symbiotic relationships between plants and fungal partners (Brundrett, 2009; Smith & Read, 2008; van der Heijden et al., 2015). The support of fungal partners enhances the ability of host plants to better tolerate challenging abiotic conditions. This positive impact manifests in various ways, such as water and nutrient recovery, promoted plant growth, enhanced stress tolerance, boosted resistance to pathogens, and improved reproductive success (Bothe et al., 2010; Ferrazzano & Williamson, 2013; Hempel et al., 2013; Zabinski & Bunn, 2014). Arbuscular mycorrhiza, where symbiotic fungi form arbuscules in the root of the host, plays a pivotal role not only in the evolution of vascular plants but also in their essential functions (Smith & Read, 2008; Strullu-Derrien et al., 2018; van der Heijden et al., 2015). Indeed, this symbiosis was detected in numerous plants in rocky habitats (AMF; Carvalho et al., 2012; Casazza et al., 2017; Pereira et al., 2019; Sanguin et al., 2016; Xiao et al., 2023; Zubek et al., 2008). Furthermore, beyond saprotrophic fungi, which, together with bacteria, facilitate the decomposition processes and contribute to the generation of available nutrients for plants (Dighton, 2007), endophytic fungi, particularly dark septate endophytes characterized by melanised septate mycelium (DSE; Rodriguez et al., 2009), significantly facilitate the long-term survival and adaptation of plants even in highly challenging environments (e.g. Lugo et al., 2015; Santos et al., 2021). However, not all fungal-plant interactions are necessarily beneficial. An imbalance in the nutrient exchange between fungal colonizers and a host can potentially weaken the fitness of an individual plant or even an entire population (Collinge et al., 2022; Kogel et al., 2006). Prominent among harmful interactions are those involving pathogenic fungi, which function as primary causal agents of plant diseases and mortality (Doehlemann et al., 2017). This is particularly noteworthy because the pathogen can reside within the plant either throughout its life or during specific stages. Diversity and interactions between fungal communities and other organisms are significantly shaped by various environmental factors, predominantly geomorphology and climate. Ecological guilds of soil fungi differ in their ecological niche sizes (Větrovský et al., 2019, 2020) and are highly responsive to climatic changes, which can alter their abundance and composition. These changes have the potential to impact ecosystem processes and plant performance at both micro and macro scales (Baldrian et al., 2022). For instance, elevated temperatures induce drought stress, which, apart from compromising plant resistance to fungal infections, can increase the metabolic rates and aggressiveness of fungal pathogens, resulting in higher infection rates (Dorrance et al., 2003; Francioli et al., 2020; Miller et al., 2022; Ramegowda & Senthil-Kumar, 2015; Thompson et al., 2010). Furthermore, AMF colonization rates are positively correlated with lower elevations (Gai et al., 2012; Geml, 2017; Kotilínek et al., 2017; Zubek et al., 2009) and arid climatic conditions (Kivlin et al., 2013; Porras-Alfaro et al., 2008). In contrast, DSES guilds have been reported to be more abundant in cold and more humid mountain habitats (Bueno de Mesquita et al., 2018; Kivlin et al., 2013; Newsham, 2011; Ranelli et al., 2015). Indeed, recent studies indicate that DSE fungi interact directly with root and soil microbiomes through various mechanisms (Netherway et al., 2024). They can suppress the colonization and growth of plant pathogens (e.g., Narisawa et al., 2004), modulate the development and colonization of arbuscular mycorrhizal fungi (AMF) (Scervino et al., 2009), and even substitute AMF assemblages in harsh mountain environments (Pandey, 2019). In summary, the intricate interplay of plant-fungal-environment interactions, embracing all the aforementioned relationships, highlights the complex nature of ecosystems in natural habitats, including rocky sites. Understanding the dynamics and significance of these interactions is crucial for gaining deeper insights into the adaptation and survival of plants in the challenging environments of rocky habitats (cf. Casazza et al., 2017; Ferrazzano & Williamson, 2013; Sanguin et al., 2016).

To advance our understanding of the intricate dynamics between plants and fungi in rocky habitats, we have opted for *Daphne arbuscula* Čelak (Thymelaeaceae) as a model system for our study. This endemic, dwarf, evergreen shrub thrives in relict limestone crevices and steep slopes in the Muránska planina Mts in the Western Carpathians (Figure 1; Erdelská & Turis, 1995; Erdelská & Turis, 1996). Well-adapted for thriving in demanding rocky environments, it is characterized by a cushion-shaped form, an extensive deep root system, clonal spreading, and leaves with a thick upper

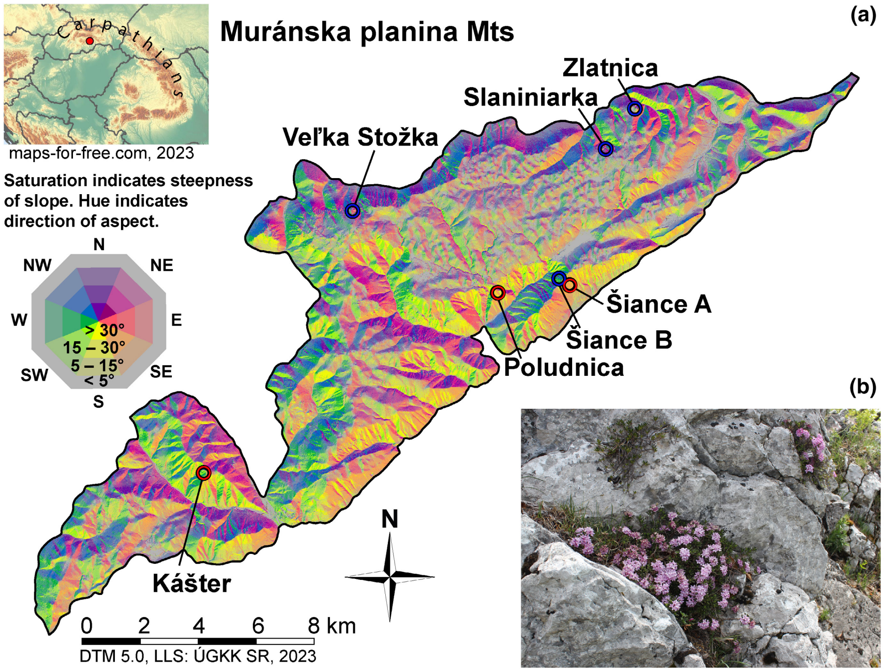

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FIGURE 1 Studied populations of *Daphne arbuscula* in the area Muránska planina Mts in the Western Carpathians. (a) Aspect-slope map showing different slope directions in warmer (red circles) and colder sites (blue circles). (b) Shrub of *Daphne arbuscula* in its natural rocky habitat.

cuticle (Erdelská & Turis, 1995, 1996). The bedrock in these habitats results in poorly developed, skeletal, alkaline soils (pH 7.1–7.9), occasionally featuring a relatively high humus content (32%–54%) in the upper 0.5 cm (Kukla et al., 1996). While AMF have been previously identified in *D. arbuscula* through microscopic examinations (Erdelská & Turis, 1996), and their association with other *Daphne* species has also been confirmed (Harley & Harley, 1987; Wang & Qiu, 2006), the specific species spectrum and the extent of colonization remain unknown. Similarly, despite reports of twig infection by some parasitic-saprotrophic fungal taxa in *D. arbuscula* shrubs (Bacigálová, 1994), their diversity, abundance and role in the survival of this West Carpathian endemic remain elusive. The occurrence of this species has been documented in up to 15 microlocalities and exhibits diverse microclimatic environments (Erdelská & Turis, 1995, 1996; Kochjarová et al., 1999). In general, sites at lower altitudes, facing south, typically have drier and warmer conditions with a prevalence of thermophilous plant communities (association *Pulsatillo slavicae-Caricetum humilis* (Sillinger 1933) Mucina ex Uhlová & Bernátová (2004) (cf. Kochjarová et al., 2015; Valachovič & Jarolímek, 1994)). Conversely, higher-altitude sites on the northern slopes experience colder and wetter conditions, hosting mainly subalpine and dealpine vegetation in garland grassland communities (association *Seslerio-Festucetum tatrae* Sillinger 1933; cf. Valachovič & Jarolímek, 1994; Valachovič & Mucina, 2004) or in relict calcicolous pine forests (alliance *Pulsatillo slavicae-Pinion sylvestris* Fajmonová 1978 (Uhlířová & Bernátová, 2003)). Our recent study indicates that this site microclimatic heterogeneity has also an impact on species' reproductive abilities (Gajdošová et al., 2024). The environmental heterogeneity of *D. arbuscula* sites and the detection of diverse fungal entities within the plant tissues present compelling questions regarding the interactions between the mycobiome, the host plant and the environment. Thus, it remains to be investigated whether this geomorphological configuration influences the overall composition of the root-associated fungal communities and ecological guild in *D. arbuscula* roots and, possibly, how the mycobiome may affect the host plant's survival in these habitats. *Daphne arbuscula* is a long-lived woody plant adapted to rocky habitats with extreme environmental conditions and pronounced microsite heterogeneity (Erdelská & Turis, 1995, 1996). Its long-term survival and fitness likely depend heavily on interactions with the surrounding mycobiome, rendering it an ideal subject for the current study.

Our main objective is to empirically test the hypothesis that the root system of the rock-dwelling endemic shrub accommodates arbuscular mycorrhizal fungi (AMF) and potentially also hosts various other root-associated fungal groups. These endophytes may play a crucial role in enhancing the *Daphne* shrub's survival in rocky habitats. We posit that the composition of fungal endophytes in localities with warmer climates, as opposed to colder environments, may exhibit a discernible bias toward different fungal ecological guilds. To address these hypotheses, our investigation, based on replicates of sites with distinct microclimatic conditions, aims to explore whether the diversity of the root-associated fungal communities correlates with the major climatic characteristics of the localities under examination.

2 | MATERIALS AND METHODS

2.1 | Sampling design of the studied area

Root samples were gathered during the flowering period spanning from May to June 2021. This specific timeframe was selected to minimize potential fluctuations in associated fungal assemblages influenced by seasonal variations in weather conditions, including humidity, temperature and, significantly, the physiological activity of the model species. A total of six populations within the Muránska planina Mts (Western Carpathians, Slovakia) were sampled and categorized into two major groups based on distinct environmental factors (Figure 1a). The first group, termed "warmer localities" (specifically Poludnica and Kášter; Table 1; Figure S1), is situated on south-facing slopes with drier and warmer microclimates, ranging from 780 to 950 meters above sea level. Collection in this group occurred in mid-May 2021. The second group, encompassing "colder localities" (including Zlatnica, Slaniniarka and Veľká Stožka), is predominantly found on northern slopes at higher altitudes (940 to 1275 meters above sea level; Figure S3). These areas experience colder and more humid mountain microclimates, with flowering occurring 3–4 weeks later compared to warmer localities. Sampling in colder localities was conducted in mid-June 2021 to coincide with the flowering season. The specific case represents the population Šiance, situated in an area with notable environmental heterogeneity and spanning both habitat types. The first habitat, "Šiance A," consists of shrubs on small limestone cliffs in full sun exposure, aligning with the microclimate of "warmer localities," and root samples from there were collected in mid-May (designated as "Šiance A"; samples ŠI-A-1, ŠI-A-3, ŠI-A-6, ŠI-A-41; Figure S2a). Conversely, the second habitat ("Šiance B"; samples ŠI-B-47, ŠI-B-6II, ŠI-B-7II; Figure S2b), features plants on a north-oriented slope shaded by forest vegetation with *D. arbuscula* aligning with the microclimate of "colder localities," flowering and being sampled later in mid-June. Unfortunately, no replicates were available for this specific mixed environmental setting due to the absence of a similar population in Muránska planina Mts. Authorization for field research and the collection of *D. arbuscula* samples, designated by permit number 6656/2021-6.3, was granted by the Ministry of Environment of the Slovak Republic.

2.2 | Root collection and preprocessing

Roots were collected from at least six individuals sampled per population, resulting in a total of 42 root samples (Table 1). While collecting root samples, the utmost care was taken to extract them without causing significant disruptions to the root system, ensuring the ongoing survival and well-being of the examined plants. The collected roots were preserved in plastic bags in the refrigerator and transported to the laboratory. Subsequently, the root samples were washed under running tap water to eliminate soil debris. We carefully examined the samples under a stereomicroscope to identify and remove potential root fragments from non-target co-occurring plant species. Subsequently, the fine feeder roots of *D. arbuscula* were collected, dried with a paper towel, and divided into two fractions. The first fraction, consisting of two aliquots (~100 mg fresh weight), was stored at −80°C for subsequent DNA analyses. The remaining roots underwent staining and microscopic assessment to examine fungal structures.

2.3 | Root staining and microscopic observation

The root samples were cleaned with 10% potassium hydroxide and then stained for fungal structures with 0.05% trypan blue in lactoglycerol, according to Koske and Gemma (1989). Given the dark pigmentation of the roots, a post-clearing bleaching step with alkaline hydrogen peroxide was included, as described by Brundrett et al. (1996). Thirty stained root fragments (1–1.5 cm) were carefully mounted on microscope slides in lactoglycerol and examined under a compound microscope at 200-fold magnification (Olympus BX60). Using the magnified intersection method (McGonigle et al., 1990), we scored 100 root intercepts for the presence of AMF (hyphae, arbuscules and vesicles) and DSE structures (hyphae and/or microsclerotia).

2.4 | DNA amplification and amplicon-based sequencing

Both frozen root samples of *D. arbuscula* were subjected to DNA isolation using the DNeasy Plant Mini Kit (Qiagen). Fungal diversity was assessed through amplicon sequencing, targeting the internal transcribed spacer 2 of the ribosomal DNA (hereafter ITS2) with the universal fungal primers fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990). Additionally, the V4/V5 region of the small subunit of

TABLE 1 Sampled localities of *Daphne arbuscula* and their characteristics. Average yearly temperature (t) and precipitation (prec) were extracted from SolarGis.

Locality	Microclimate	Elevation (m.a.s.l.)	Aspect	*t* (°C)	Prec (mm)	Date of collection	No. of samples collected	No. of samples for ITS	No. of samples for SSU
Poludnica	Warmer	780	S–SE	6.8	1053	19.5.2021	6	6	6
Kášter	Warmer	900	S–SW	6.3	984	18.5.2021	6	5	4
Šiance A	Warmer	950	SE	6.4	1033	18.5.2021	4	4	4
Šiance B	Colder	940	NW	5.9	1033	17.6.2021	3	3	3
Zlatnica	Colder	950	NE–E	5.8	1004	15.6.2021	7	6	6
Slaniniarka	Colder	1030	N–NE	4.9	1015	15.6.2021	7	6	6
Veľká Stožka	Colder	1275	N–NE	5.5	1054	16.6.2021	9	6	6
							42	36	35

rDNA (hereafter SSU) was analysed using AMF-specific primers NS31 (Simon et al., 1992) and AML2 (Lee et al., 2008). AMF-specific primers are significantly more efficient in amplifying and capturing a broader diversity of AMF taxa than standard ITS primers (cf. Lee et al., 2008; Řezáčová et al., 2016). All primers were tagged with sample-specific barcodes. The PCR reaction mixes for the amplification of the target SSU and ITS2 regions included 0.5 U Q5 High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA), 1× Q5 reaction buffer, 1× Q5 High GC Enhancer, 0.2 mM of each dNTP, 0.4 μM of each tagged primer, 15 μg of BSA, 2.5 μL of the template in the total volume of 25 μL. Thermal cycling was done in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). The PCR conditions for the primer combination fITS7/ITS4 were as follows: initial denaturation at 94°C for 5 min, 33 cycles of denaturation at 94°C for 30 s, 30 s of annealing at 56°C and 30 s of extension at 72°C and a final 10 min extension at 72°C. The cycling conditions for the primer combination NS31/AML2 were 5 min at 98°C, followed by 35 cycles of 10 s at 98°C, 30 s at 63°C and 20 s at 72°C, with the 10-min final extension at 72°C. Each root subsample underwent two independent PCR reactions to ensure the avoidance of PCR bias. Thus, four PCR products derived from the same plant were pooled and purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Sequencing was carried out using the Illumina MiSeq platform at the company SEQme (Dobříš, Czech Republic). Both molecular markers were sequenced in two independent sequencing runs, with each run covering all samples (Table S1). Raw amplicon sequence data have been deposited in NCBI as BioProject PRJNA1036833 under BioSamples SAMN38152953–SAMN38152987.

2.5 | Sequence data processing

Altogether, 36 samples for the ITS2 region of the internal transcribed spacer and 35 samples for the V4/V5 region of SSU were successfully sequenced (Table 1). Sample Kášter 14 was omitted from the final analyses due to evident contamination with non-*Daphne* roots as identified during data processing. Amplicon sequence data were analysed using a pipeline implemented in SEED 2.1.2. (Větrovský et al., 2018), with comprehensive stepwise sequence counts detailed in Table S1. Prior to delving into further analysis, the ITS2 fastq reads were pair-ended using fastq-join v. 1.1.2 (Aronesty, 2011), with a minimum 40 bp overlap and a maximum 15% difference. SSU fastq reads were demultiplexed based on molecular identifier tags, with sequences bearing tag mismatches excluded from further analyses. Subsequently, sequences with average Phred scores below 30 for ITS2 and 25 for SSU were excluded. Utilizing the barcode motifs, sequences were associated with their respective sample names. Subsequently, barcodes, along with primer sequences, were trimmed, and the sequences were consolidated into a single dataset for each marker. Further refinement was applied to the ITS2 dataset through trimming using ITSx v. 1.0.11 (Bengtsson-Palme et al., 2013). Sequences were clustered into operational taxonomic units (OTUs) using UPARSE in USEARCH v. 11.0.667 (Edgar, 2013), employing a 97% similarity threshold. De novo detected chimeric sequences and pruned low-abundance OTUs (those with fewer than 4 sequences for ITS2 and singletons for SSU) were discarded, yielding 1228 ITS2 OTUs (comprising 763,680 reads) and 315 SSU OTUs (with 138,847 reads; Table S1). To account for potential contamination, OTU read counts were adjusted by subtracting the counts detected in the negative controls (sterile deionized water used instead of a DNA template) from the respective sample counts, although these numbers were negligible. The most abundant sequence from each OTU was retrieved for subsequent taxonomical and fungal ecological guild assignments.

2.6 | Taxonomy and ecology assignment

The most abundant sequence in each OTU in the ITS2 dataset was BLAST searched against the UNITE fungal ITS reference database release 9.0 (Kõljalg et al., 2020; Nilsson et al., 2019). Non-fungal OTUs were excluded resulting in a dataset of 1202 OTUs comprising 519,822 sequences (Table S2). In the SSU dataset, the most abundant sequence of each OTU underwent BLAST search against both the GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and MaarjAM databases (Öpik et al., 2010). Taxonomic classification followed guidelines from the GlobalAMFungi database (Větrovský et al., 2023) and pertinent literature, with non-Glomeromycota OTUs removed, leaving a final set of 270 OTUs encompassing 120,050 reads (Table S3).

Initial fungal ecological guild assignments to ITS2 OTUs were conducted utilizing the FungalTraits database (Põlme et al., 2020). Further refinement occurred manually, corroborated by relevant literature, with a focus on five fungal guilds: plant pathogens, dark septate endophytes (DSE), arbuscular mycorrhizal fungi (AMF), root endophytes and saprotrophs. As the two later guilds eventually exhibit partial overlap and their definitions might be considered ambiguous, for the purposes of this study, we adhere to the definitions provided by Põlme et al. (2020). Specifically, 'other symbiotrophs' include lichenized fungi and ectomycorrhizal, orchid mycorrhizal and ericoid mycorrhizal fungi. Conversely, 'root endophytes' are classified as fungal OTUs known to colonize the interior of plant tissues. Their functions vary, ranging from asymptomatic and commensal to weakly mutualistic (Põlme et al., 2020). The remaining categories (including animal parasites and mycoparasites) were classified as "others." In cases of conflicting literature assignments, OTUs were labelled as "ambiguous." A category "not defined" (ND) included taxa lacking designated ecological functions, unidentified OTUs, or those exhibiting <94% sequence similarity to a reference.

2.7 | Data analyses

Before calculating alpha diversity indices (including OTU richness, Shannon-Wiener diversity index, Shannon entropy, inverse Simpson diversity index, evenness, chao-1 and 80% species richness),

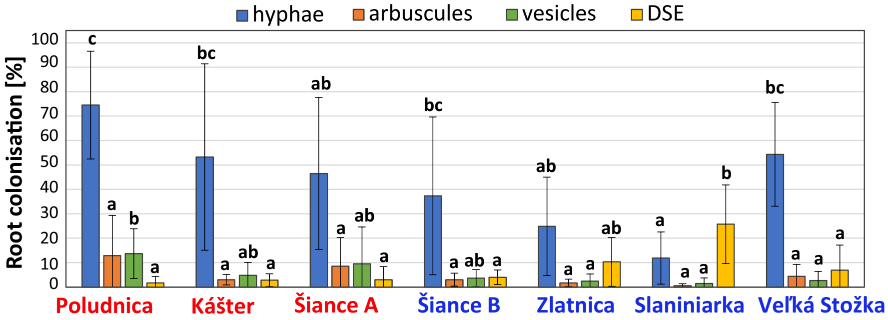

FIGURE 2 The mean rate of root colonization by AMF (hyphae, arbuscules, vesicles) and DSE (hyphae and/or microsclerotia) in the studied populations of *Daphne arbuscula* based on microscopic observations. If there are any statistically significant differences between study sites according to Tukey's test, they are labelled by lowercase letters. Error bars represent the standard deviation of the mean.

sequence reads for each sample were normalized (rarefied) to match the abundance of the least-read sample (1295 reads for ITS2 and 332 reads for SSU). To ensure higher accuracy, sample rarefaction and alpha diversity indices were computed in 10 replications, each with a different set of reads in SEED 2.1.2 (Větrovský et al., 2018), and subsequently averaged. Differences between sites in alpha diversity indices and also fungal colonization were analysed in R 4.1.0 (R Core Team, 2021) using ANOVA with Post hoc comparisons (Tukey's test; 'glht' function of the package 'multcomp'; Hothorn et al., 2008). For all other analyses, the absolute number of sequence reads of both datasets was transformed to relative read abundances. To examine variations in root-fungal communities among different sites, one-way permutational multivariate analysis of variance (PERMANOVA) was conducted based on Bray–Curtis dissimilarity (Bray & Curtis, 1957) of fungal communities between pairs of localities with 9999 permutations. Non-metric multidimensional scaling (NMDS) plots were generated to visualize the similarity of individual samples within our dataset using the Bray–Curtis dissimilarity index. Both PERMANOVA and NMDS analyses were performed in PAST version 4.03 (Hammer et al., 2001) using relative abundances derived from both ITS2 and SSU amplicon data. To mitigate the impact of dominant taxa, we conducted additional PERMANOVA and NMDS analyses on datasets subjected to a binary transformation, following the approach proposed by Caboň et al. (2021). The binary transformation converted the data into a presence/absence matrix, where presence was defined if the OTU's relative abundance of reads in the sample exceeded 0.01%. Indicator OTUs for different sites and their combinations were identified from relative abundances using the 'multipatt' function (func = "IndVal.g") of the 'indicspecies' package in R 4.1.0 (R Core Team, 2021), with 999 permutations and $p < .05$ (De Cáceres & Legendre, 2009). For the identified indicator species, Pearson's phi coefficient of association, with correction for unequal sample sizes (func = "r.g"), was calculated on presence/absence transformed ITS2 and SSU datasets to determine the ecological preferences of fungal OTUs (Chytrý et al., 2002). OTUs with a correlation value higher than 0.5 are considered specific to the locality. The relationship of all indicator species to the specific ecological setup of the localities was further tested using principal components analysis (PCA) in PAST version 4.03 (Hammer et al., 2001). Redundancy analysis (RDA) ordination was performed to examine the relationship between the environmental parameters and fungal communities using the 'rda' function of the 'vegan' package in R 4.1.0 (R Core Team, 2021). The environmental variables used in the analysis included elevation, temperature, precipitation, aspect, longitude, and latitude. Extrapolated climatic data, precisely long-term average yearly temperatures and precipitation for all sites, were obtained from SolarGIS (https://apps.solargis.com/prospect/).

3 | RESULTS

3.1 | Microscopic evidence of fungal colonization of *Daphne arbuscula* roots

Microscopic analysis revealed discernible fungal structures in *D. arbuscula* roots, indicating colonization by AMF, including hyphae, vesicles and arbuscules, as well as structures typical of DSE such as microsclerotia and melanized hyphae (Figure S4). Significant variations in colonization levels were observed among study sites for hyphae, vesicles and DSE, with no significant differences for arbuscules (Figure 2; Table S4). Warmer localities, Poludnica and Šiance A, exhibited a trend toward a little higher AMF colonization levels (particularly vesicles) compared to all colder localities. Conversely, the colder populations of Slaniniarka and Zlatnica showed elevated DSE colonization (25.7% and 10.3%, respectively) compared to warmer sites.

3.2 | Metagenomic evidence of fungal colonization of *Daphne arbuscula* roots

3.2.1 | Mycobiome diversity based on the ITS2 dataset

The majority of fungal ITS2 amplicons, specifically 89% of reads (890 OTUs), were successfully classified up to the family level, while 88% of reads (770 OTUs), can be classified at the genus level (Table S5). Ascomycota dominated the fungal phyla, representing 84% of reads

(882 OTUs), followed by Basidiomycota (15% of reads and 222 OTUs) and Glomeromycota (1% of reads and 76 OTUs). Other phyla were present in negligible amounts, collectively accounting for 0.08% of reads and 21 OTUs (Table S6).

The distribution of fungal classes exhibited variations across study sites with distinct microclimatic conditions (Figures 3a and S5a). In warmer localities, the dominant Ascomycota classes were Sordariomycetes (41%–48.2% of reads; on average 44.4 ± 10.3%) and Dothideomycetes (17.6%–33.1% of reads; on average 25.4 ± 9.4%). Conversely, in colder localities, the most abundant classes included Leotiomycetes (26.8%–44.2% of reads; on average 36.1 ± 17.3%) and Sordariomycetes (14.5%–25% of reads; on average 24.8 ± 19.2%). Other classes were present only in minor proportions.

3.2.2 | Fungal community structure based on the ITS2 dataset

Among the 1202 observed ITS2 OTUs, their richness per sample ranged from 52 to 257 (on average 155 ± 52.5; Table S7). Significant variations in total OTU richness were evident between study sites, with warmer site Poludnica exhibiting the highest richness (174–257 OTUs per sample; on average 209 ± 31.2) and colder site Veľká Stožka recording the lowest (77–156 OTUs; on average 109 ± 27.3; Table S7). Alpha diversity indices revealed comparable diversity across all study sites, and no significant differences were detected in Tukey's test (Table S7). PERMANOVA revealed also significant differences between study sites (Table 2). The warmer localities demonstrated similarity among themselves but were significantly different from all but one (Slaniniarka) of the colder localities. No significant differences were observed among the colder localities, except for Veľká Stožka, which significantly differed from all warmer and colder sites at the $p < .05$ significance level. NMDS analyses (Figure 4a) also confirmed the division into two groups based on colder versus warmer microclimate sites. Similarly, RDA showed that the environmental parameters contributed significantly ($p < .05$) to the fungal community–environment relationship and explained 25.3% of the total variation in the ITS2 fungal dataset (Figure 4c). However, the only significant parameter in the model with relative read abundances was latitude ($p < .01$; Table S9). Additionally, all three analyses (PERMANOVA, NMDS, and RDA) conducted on the transformed presence/absence ITS2 dataset supported this division (Tables S8 and S9; Figure S6a,c). The RDA results also highlighted other significant parameters such as elevation, temperature, and aspect (Figure S6c; Table S9).

3.2.3 | Fungal ecological guilds based on the ITS2 dataset

Fungal OTUs were categorized into six ecological guilds (Table S10): (1) plant pathogens (40.9% of reads; 126 OTUs); (2) DSE (19.8% of reads; 124 OTUs); (3) AMF (0.8% of reads; 54 OTUs); (4) saprotrophs (14% of reads; 216 OTUs); (5) other symbiotrophs (5.8% of reads; 118 OTUs); and (6) root endophytes (2.9% of reads; 11 OTUs). A portion of fungal reads (15% of reads; 524 OTUs) could not be either unambiguously assigned to a specific ecological guild or lacked defined ecological functions. The representation of ecological fungal guilds varied between study sites (Figure 5a) and samples (Figure S7).

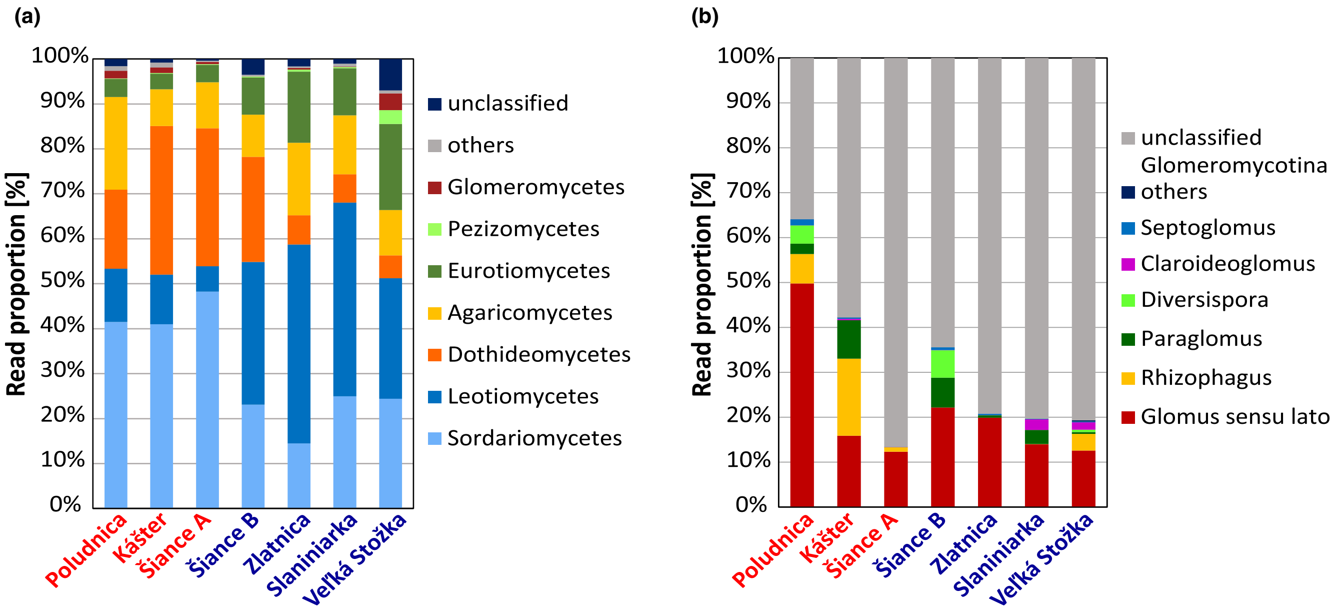

FIGURE 3 Representation of different fungal taxa in the studied sites from warmer (red) and colder (blue) sites based on mean read numbers per studied site generated from (a) the ITS2 dataset (others: Lecanoromycetes, Mortierellomycetes, Orbiliomycetes, Tremellomycetes) and (b) the SSU dataset (others: *Acaulospora*, *Ambispora*, *Archaeospora*; unclassified: other Glomeromycotina unclassified to the genus level).

TABLE 2 One-way PERMANOVA based on relative read abundances and Bray–Curtis dissimilarity of fungal communities between pairs of study sites at 9999 permutations.

							SSU
	Poludnica	.8932	.0180*	.4458	**.0084****	.2321	**.0187***
	.6447	**Kášter**	.0310*	.4053	**.0369***	.2575	.5010
	.4061	.4501	**Šiance A**	.2848	.3766	.1298	.0775
	.0121*	**.0288***	**.0278***	**Šiance B**	.5435	.8860	.3422
	.0063**	**.0254***	**.0099****	.1416	**Zlatnica**	.7756	**.0414***
	.0240*	.0624	**.0389***	.2653	.8589	**Slaniniarka**	**.0449***
ITS2	**.0020****	**.0064****	**.0034****	**.0120***	**.0125***	**.0148***	**Veľká Stožka**

Note: ITS2 dataset: $F = 2.141$; $p = .0001$; SSU dataset: $F = 1.555$; $p = .017$. Significant values are highlighted by bold and gray background and labelled with an asterisk (*$p < .05$, **$p < .01$, ***$p < .001$).

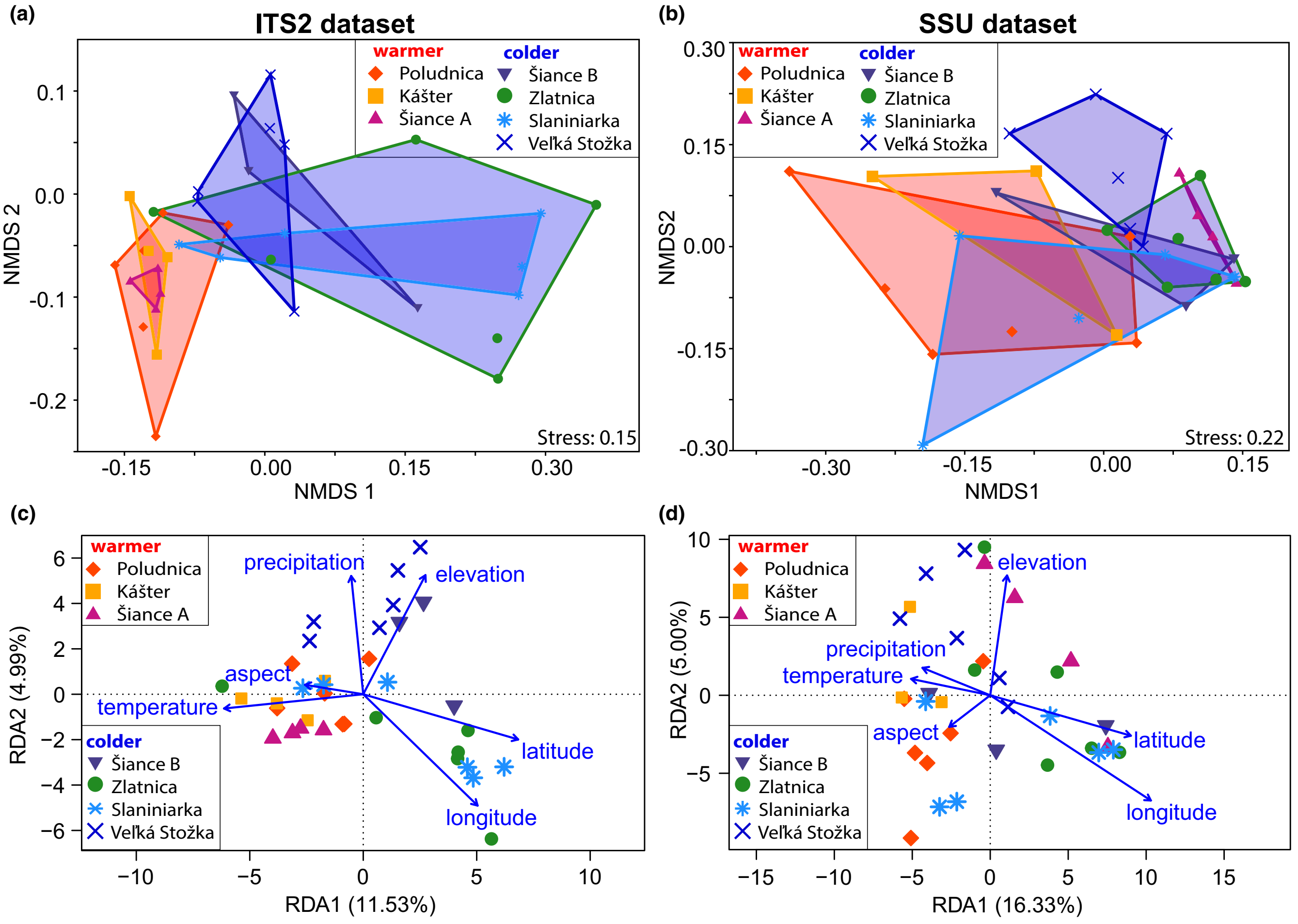

FIGURE 4 Non-metric multidimensional scaling (NMDS) ordination based on a Bray–Curtis dissimilarity matrix of fungal reads associated with roots of *Daphne arbuscula* in warmer (red fill) and colder localities (blue fill) (on the top), and Redundancy analysis (RDA) ordination showing relationship between the environmental variables and fungal communities (on the bottom) generated from (a, c) the ITS2 dataset and (b, d) the SSU dataset. Arrows indicate the direction and magnitude of variables. Different studied sites are represented by different colours and symbol shapes.

Plant pathogens

Plant pathogens were detected across all sites (Figure 5b). Out of them, 71.8% of pathogen reads (46 OTUs) belonged to Hypocreales from Sordariomycetes, and 21.9% of reads (39 OTUs) belonged to Pleosporales from Dothideomycetes (Tables S2 and S10). Pathogens were more prevalent in warmer localities (49.1%–69.1% of total reads; on average 58.9 ± 14.2%), while their percentage was noticeably lower in colder localities (17.6%–30.6% of total reads; on average 26.1 ± 19.2%). Warmer and colder localities also differed in the taxonomic composition of pathogenic fungi (Figure 5b). In warmer sites, species of the genera *Fusarium*, *Phoma*, *Neopyrenochaeta* or *Pyrenochaeta* predominated. In contrast,

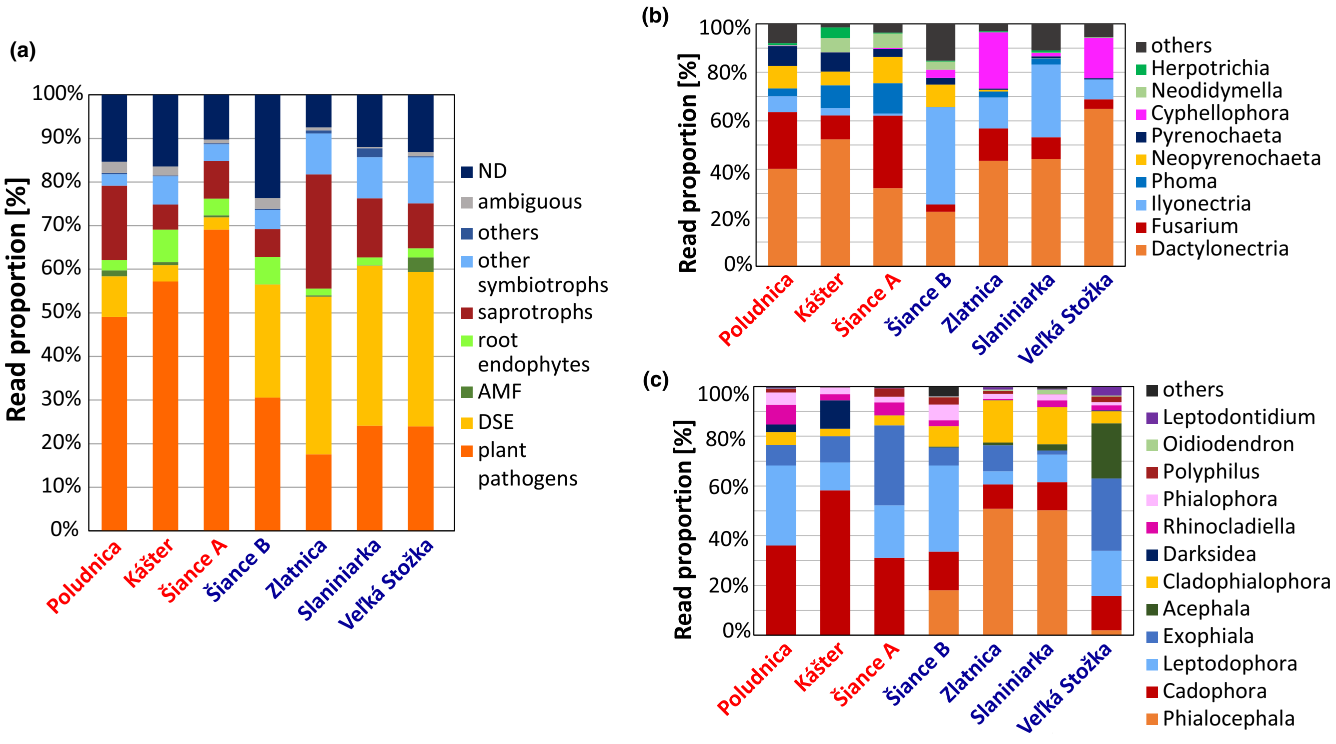

FIGURE 5 Fungal ecological guilds represented in the ITS2 dataset based on mean read numbers per studied site; red—warmer sites; blue—colder sites. (a) Representation of different fungal ecological guilds. (b) Representation of fungal genera in plant pathogens. (c) Representation of fungal genera in DSE.

colder localities predominantly harboured the genera *Ilyonectria* and *Cyphellophora* (Figure 5b). Genus *Dactylonectria* dominated in all localities (22.5%–64.9% of pathogen reads; on average 36.9 ± 26.5%), except for Šiance A. *Dactylonectria macrodidyma* (OTU2) and *Fusarium tricinctum* (OTU8) were both present in 91% of the samples and thus were the most prevalent pathogens across all of the sites (Table S11).

DSE fungi

DSE ranked as the second most abundant group, with the majority of reads assigned to Helotiales from Leotiomycetes (73.1% of DSE reads, 51 OTUs), and a smaller portion to Chaetothyriales from Eurotiomycetes (26.3% of DSE reads, 71 OTUs; Tables S2 and S10). Unlike pathogens, DSE dominated in all colder localities, ranging from 29.9% to 36.7% of total reads (on average 33.8 ± 14.1%). Their representation in warmer sites, however, was significantly lower, ranging from 2.9% to 9.3% of total reads (on average 6 ± 5.4%; Figure 5a).

Members of the genus *Cadophora* were prevalent in warmer sites (31.1%–58.3% of DSE reads; on average 39.7 ± 20.5%) but much rarer in colder ones (9.9%–15.5% of DSE reads; on average 14.8 ± 15.1%). Noteworthy, DSE OTUs detected in the largest number of samples included *Cadophora interclivium* (OTU14; in 97% of samples), *Knufia* sp. and *Exophiala radicis* (OTU23 and OTU96, respectively, in 94% of samples), and *Cadophora ferruginea* (OTU29; each in 91% of samples). Some DSE OTUs dominated at colder sites. For example, *Phialocephala* and *Cladophialophora* species had the highest abundances in Zlatnica and Slaniniarka (50.3% and 50.8% of DSE reads; 17% and 14.9%, respectively). *Acephala applanata* was overrepresented in Veľká Stožka (22.3% of DSE reads; Figure 5c).

Other guilds

The remaining ecological guilds were represented in smaller proportions (Figure 5a; Table S10). AMF were present in most samples (27 out of 35) and all study sites. The remaining ecological guilds were represented in smaller proportions. Over 99% of detected AMF reads belonged to the Glomeraceae family, predominantly the genera *Glomus* (61.2%), *Rhizophagus* (24%), and *Dominikia* (14.2%). Furthermore, saprotrophs emerged as the most abundant group, with *Tetracladium furcatum* (OTU21) being the most commonly identified species, present in 91% of samples. Their relative abundance in localities was consistently low, averaging below 1% (Figure 5a).

3.2.4 | Analyses of indicator species within the ITS2 dataset

The results of PCA analysis, focused on indicator fungal species among localities, consistently confirmed differentiation between colder and warmer sites (Figure 6a). In all three warmer localities, five common indicator OTUs were identified, with four assigned to plant pathogens (*Parophoma chrysanthemicola*, *Pyrenochaeta* sp., *Fusarium oxysporum* and *Fusarium hostae*), and one species, *Clonostachys rosea*,

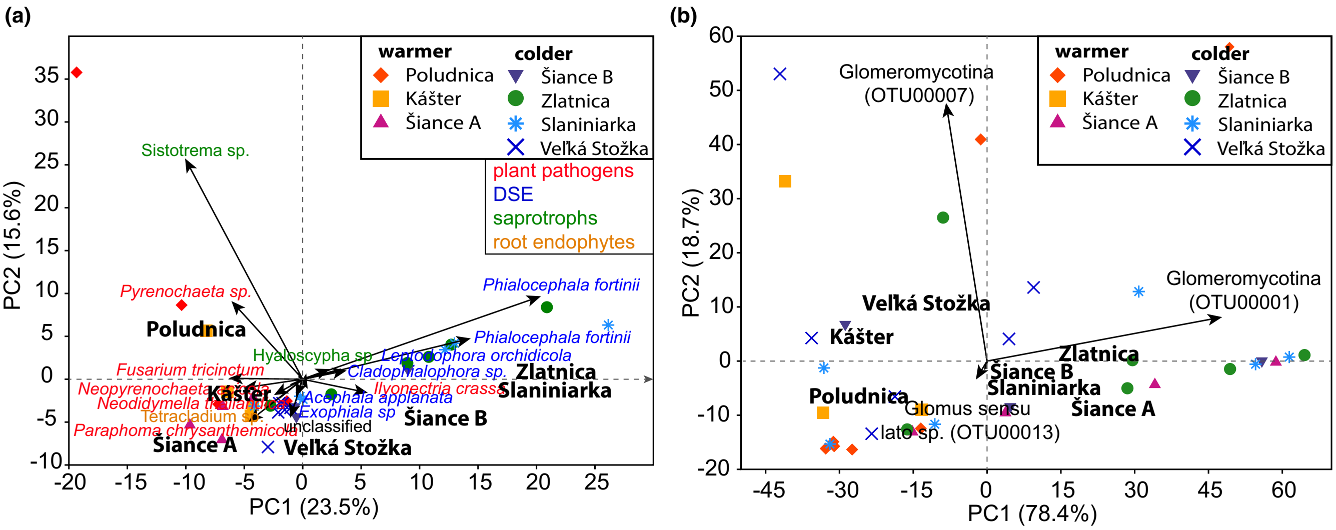

FIGURE 6 Ordination diagram by principal coordinate analysis (PCA) based on all indicator OTUs for different study sites and their combinations generated from (a) the ITS2 and (b) the SSU dataset.

had an ambiguous ecological guild affiliation (Table S12). Among the colder sites, six indicator OTUs were shared, comprising five taxa of DSE (*Cladophialophora* sp.—two taxa, *Cladophialophora nyingchiensis*, *Polyphilus* sp., and *Rhinocladiella* sp.) and one plant pathogen (*Ilyonectria crassa*) (Table S12).

3.2.5 | OTU richness and diversity of the SSU dataset

The SSU dataset included 270 AMF OTUs corresponding to 54 unique virtual taxa (VTX). Most of the AMF sequences belonged to unspecific Glomeromycotina (70.5% of SSU reads; 88 OTUs; Tables S3 and S13). Among OTUs identified to genus level prevailed Glomeraceae in all study sites, especially *Glomus* s. l. (19.9% of SSU reads; 153 OTUs; 27 unique virtual taxa according to MaarjAM database). Less abundant taxa (0%–4.5% of SSU reads; 29 OTUs) detected in all sites were *Septoglomus constrictum*, *Rhizophagus iranicus*, *R. intraradices* and others (for details see, Figures 3b and S5b; Tables S3 and S13). Per sample, we observed from 8 to 72 OTUs (on average 24.2 ± 12.7), which corresponded to 3–20 VTX (on average 11.7 ± 4.5). No significant differences in the diversity and OTU richness were detected between study sites ($p > .05$ according to Tukey's test; Table S7). Neither PERMANOVA (Tables 2 and S8) nor NMDS (Figures 4b and S6b) uncovered significant differences between warmer and colder localities. Only a total of eight indicator OTUs were found in the whole dataset (Table S14). According to RDA, environmental characteristics significantly explained 26.5% of the total variability in the SSU dataset ($p < .05$; Figure 4d; Table S9). The only significant parameters were latitude in the model with relative abundances and elevation in the model with the transformed SSU dataset (Figure S6d; Table S9). Indicators OTU1 and OTU7 (both unspecific Glomeromycotina) explained most of the variability according to PCA (Figure 6b).

4 | DISCUSSION

4.1 | The environmental heterogeneity has triggered a composition divergence of the root-associated fungal communities in rock-dwelling *Daphne arbuscula*

Our study provides empirical support for the hypothesis that the root system of the rock-adapted shrub *Daphne arbuscula* hosts, in addition to the omnipresent arbuscular mycorrhizal fungi (AMF), a diverse array of root-associated fungal communities. We demonstrated that the extensive diversity of root-associated fungal communities is, along with AMF, predominantly composed of plant pathogens, DSE, and saprotrophs (see Figures 2, 5 and S4; Tables S4, S10 and S11). Importantly, the proportion of ecological guilds across studied sites correlates with environmental conditions, indicating a non-random pattern reflecting the response of root-associated fungal communities to microclimatic heterogeneity (cf. Birnbaum et al., 2019; Merges et al., 2018; Tian et al., 2017). Our analyses indicated that environmental parameters might contribute to the composition of fungal communities at the studied localities in both the ITS2 and SSU datasets, as demonstrated by the RDA results (see Figures 4c,d, S6c,d, and Table S9). The fungal communities from the ITS2 dataset appeared to be more closely associated with environmental factors than the AMF communities from the SSU dataset. Latitude emerged as the most significant factor differentiating fungal communities, suggesting a geographic distance-based pattern. However, we believe this primarily reflects the environmentally induced biogeographic setup of *D. arbuscula* populations rather than a geographic distance-based pattern or bias in sampling design. Specifically, sites with colder microclimates are situated in the northern part of the distribution range, lacking localities with south or southwest-oriented slopes and warmer microclimates, and vice versa. This spatial arrangement underscores the role of latitude and associated climatic variables in shaping fungal community structure within the distribution range of *D. arbuscula*. This contrast was also evident in the taxonomic composition, particularly in the prevalence of specific fungal classes between colder and warmer sites. In warmer localities, Sordariomycetes and Dothideomycetes were dominant, whereas colder sites exhibited a dominance of Leotiomycetes and Sordariomycetes (cf. Geml, 2017). The environmental influence on diversification is especially evident in the Šiance population. The sunny and warmer part of the locality (Šiance A) exhibits root-associated fungi diversity resembling that of warmer sites. In contrast, the shaded colder part (Šiance B), just a few meters away on a north-oriented slope, shares a root fungal community composition more akin to colder sites (Figure S2). Furthermore, the most divergent site is Veľká Stožka characterized by the colder microclimate, which exhibits remarkable deviation in composition profile in fungal taxa, AMF composition and in the taxonomic spectrum of DSE (Figure 5c; Table 2). This shift in the root-associated fungal composition is likely influenced by the unique environmental characteristics of the site, particularly its higher elevation (cf. Bonfim et al., 2016). The elevated altitude of this locality, with its more challenging conditions (Table 1), is likely the driving force behind the observed diversification in the root-associated fungal communities. We are, however, aware that Šiance and Veľká Stožka deviated from the rest of the studied sites by their unique environmental configurations and require careful consideration in interpreting our findings. However, the distinctive geomorphologies of both sites are not duplicated in other locations within the Muránska planina Mts, thus unfortunately lacking replicates in our sampling design. We maintain that our recent sampling design has had no remarkable impact on the crucial findings. We believe that this intricate root-associated fungal network of this relictual shrub plays a pivotal role in the adaptation, fitness, and overall survival of this species in the extreme environment of limestone rocky habitats. Last but not least, plant survival and fitness are influenced not only by environmental variables but also by associated microorganisms, particularly root-associated bacterial communities. These microorganisms play a crucial role in plant survival by facilitating nutrient cycling, especially in nutrient-poor substrates (Nicolitch et al., 2017; Zhang et al., 2019). The root-associated mycobiome is also significantly shaped by bacterial assemblages co-inhabiting plant roots, given their established bidirectional biological and ecological interactions (Lastovetsky et al., 2022). Although exploring these complex interactions was beyond the scope of this study, it highlights the potential for future research into the root microbiome of plants in extreme rocky habitats, providing deeper insights into the synergistic relationships that underpin plant resilience and adaptation.

4.2 | The prevalence of plant pathogens in warmer sites suggests their susceptibility to climate fluctuations and global warming

Our study uncovers a significant composition disparity among root-associated fungal communities in colder and warmer environments. Although they share similar fungal taxa and ecological guilds, they exhibit notable proportional differences (Figures 3a, 4a and S5a). Specifically, plant pathogens predominate in warmer sites, while the root systems of shrubs from the colder sites are mostly inhabited by fungi belonging to the DSE guild (Figures 2, 5, and 6; Tables S10–S12). This finding may suggest that the root landscapes of *D. arbuscula* in warmer localities are more susceptible to colonization by plant pathogens, potentially rendering these plants more vulnerable to diseases than those in colder sites. South-exposed rocky formations are more prone to overheating compared to north-oriented sites with a colder microclimate. This environmental configuration may induce alterations in soil water balances, causing variations in the physiological drought stress encountered by shrubs on different aspects (Ritchie, 1998), thereby impacting and modifying the composition of their mycobiome (cf. Ferrazzano & Williamson, 2013; Geml, 2017). Elevated temperatures, besides compromising plant resistance to fungal infections, can increase the metabolic rates and aggressiveness of fungal pathogens, resulting in higher infection rates (Dorrance et al., 2003; Thompson et al., 2010). This might be particularly relevant in the context of ongoing global warming. Responses of soil fungal diversity to climate warming may vary across taxa and guilds (Birnbaum et al., 2019; Looby & Treseder, 2018; Treseder et al., 2016), however, numerous studies consistently propose an elevated presence of plant pathogen taxa within fungal communities in response to environmental changes (Birnbaum et al., 2019; Looby & Treseder, 2018) and especially during drought stress (Francioli et al., 2020; Miller et al., 2022; Ramegowda & Senthil-Kumar, 2015).

Another factor that might influence the abundance of fungal pathogens is the population density of the host species. It has been documented that plants in monoculture-like systems, where the density of a single or very few taxa is high, are more susceptible to increased pathogen loads (Wu et al., 2016). As observed by Erdelská and Turis (1996) and corroborated by subsequent studies, the abundance of *Daphne* shrubs exhibits substantial variability both between and within localities. For instance, the density ranges from 25% to 50% in Poludnica, 1%–5% in Kášter, 25%–50% in Zlatnica, 5%–15% in the colder part of Šiance B, and 5%–50% in Veľká Stožka. These findings indicate that population density is highly variable within each site, regardless of thermal conditions, and is strongly influenced by geomorphological factors. Given this high level of variability, it seems unlikely that population density is a significant factor affecting the level of root colonization by pathogens in this system.

Members of the genus *Daphne* often suffer from various fungal diseases affecting different parts of the plants (Bacigálová, 1994; Halda, 2001; Noshad et al., 2006; White, 2006). The most prevalent taxa in almost all samples were two significant phytopathogenic species (Figure 5b; Tables S2 and S11). The first is *Dactylonectria macrodidyma*, a recognized causal agent causing severe root infections (black foot disease), particularly affecting commercially important woody crops (Cabral et al., 2012; Lombard et al., 2014; Úrbez-Torres et al., 2012; Vitale et al., 2012), while the second is *Fusarium tricinctum*, known as a phytopathogen and mycotoxin producer causing infections across a wide spectrum of plants (Wang et al., 2022, and references therein). In fact, *Fusarium tricinctum* prefers temperatures between 15°C and 25°C for optimal infection and disease development (Yan & Nelson, 2020). While this temperature range can occur in both colder and warmer sites during the growing season, its prevalence and duration of infection are more likely in warmer sites (cf. Erdelská & Turis, 1995, 1996), contributing to a higher rate of infections there. We believe that our findings strongly suggest that the dominance of potential plant pathogens in the root landscape of populations inhabiting warmer climates poses a substantial threat to their overall fitness and long-term survival. However, it is essential to acknowledge that we did not assess the health status of shrubs in the studied populations. At least from a basic visual inspection, we did not observe shrubs with remarkable infestations or fatalities in either colder or warmer sites (authors unpublished data). Moreover, certain strains of *F. oxysporum*, fungal species also detected in our samples (Tables S2 and S11), are typically recognized as vascular plant pathogens but can also exhibit non-pathogenic behaviour toward plants. Instead, they can produce metabolites that inhibit the growth of various phytopathogenic fungi (Michielse & Rep, 2009; Sajeena et al., 2020). This brings us back to recently proposed concepts that emphasize the complex interactions between hosts and fungal infections. Their association with the host plant can range from mutualism and commensalism to amensalism and parasitism (Collinge et al., 2022; Jørgensen et al., 2020). Given our recent data, we are currently limited to speculating about the dynamics of shifts in *D. arbuscula* and root-associated fungal interactions. To gain a more profound understanding, additional comprehensive empirical investigations are imperative.

4.3 | The role of DSE in the survival of *Daphne arbuscula* in rocky habitats

One of the key strategies employed by plants to counteract pathogens or develop resistance against them involves coexisting with AMF or various endophytes, especially DSE (Hempel et al., 2013; Santos et al., 2021; van der Heijden et al., 2015; Zabinski & Bunn, 2014). It was proven that specific members of AMF (Akhtar & Siddiqui, 2008) or DSE (Surono & Narisawa, 2018) can inhibit the growth of potentially pathogenic fungi (Beccari et al., 2022; Constantin et al., 2021; Santos et al., 2021). Our metabarcoding data reveal a distinct pattern, underscoring the prevalence of DSE in colder sites, coupled with a notably lower abundance of pathogens compared to warmer sites (Figures 5 and 6; Table S10).

While direct causality between pathogen rates and DSE in colder versus warmer environments remains unconfirmed by empirical evidence, the observed pattern suggests that the prevalence of DSE, beyond its mutualistic association with *D. arbuscula*, most likely contributes to mitigating pathogens and alleviating environmentally induced abiotic stress (Beccari et al., 2022; Constantin et al., 2021; Santos et al., 2021). Indeed, DSE are better adapted to environmental extremes and tend to be more abundant in mountain habitats at higher elevations with lower temperatures and colder environments (Bueno de Mesquita et al., 2018; Kivlin et al., 2013; Newsham, 2011; Ranelli et al., 2015). The essential role of DSE in benefiting plants arises from their capacity to mineralize organic compounds in the rhizosphere, enhancing the availability of inorganic nitrogen for root uptake. However, once inorganic nitrogen becomes accessible to root systems, the positive impact of DSE diminishes (Alberton et al., 2005; Newsham, 2011). In rocky environments, such as the habitats of *D. arbuscula*, where soil development is limited and the slow decomposition of organic matter is exacerbated by conditions like drought or low temperatures (Erdelská & Turis, 1995, 1996), there is often a reduction in available inorganic nitrogen and phosphorus levels (Santonja et al., 2015; Xiao et al., 2019). Thus, in studied rocky habitats, especially those featuring a colder microclimate, DSE likely plays a pivotal role in mineralizing organic nitrogen and phosphorus (cf. Newsham, 2011). Hence, the higher prevalence of DSE in colder localities harmonizes with the general ecological traits of DSE fungi, likely serving as a mechanism for the enhanced or increased adaptation of *D. arbuscula* in colder environments. Moreover, beyond the variation in DSE abundance between warmer and colder sites, we also observed diversification concerning predominant DSE species. Specifically, in the colder sites of Zlatnica and Slaniniarka, *Phialocephla fortini* dominates, while *Acephla applanata* prevails in Veľká Stožka but is absent in warmer sites (Figure 5c; Tables S2 and S11). Both species are recognized as commonly occurring DSE root-associated fungi with no host specificity (Grünig et al., 2008; Rodriguez et al., 2009; Santos et al., 2021). These DSEs may play a role in mitigating water stress for various host plant species and suppressing fungal pathogens (Santos et al., 2021; Stroheker et al., 2021; Tellenbach et al., 2013). The genus *Cadophora*, which is less abundant in colder sites, emerges as the dominant DSE genus in these environments. *Cadophora* spp. have been shown to suppress the growth of various fungal pathogens in in vitro experiments (Santos et al., 2021; Yakti et al., 2019). However, this antagonistic effect was not confirmed in plants inoculated by both pathogens and *Cadophora* spp., suggesting that plants are likely involved in altering the mutual interaction of these root-associated fungi (Yakti et al., 2019). In conclusion, this nuanced diversity in both abundance and species composition underscores the complex dynamics of DSE communities across different microclimatic conditions. We posit that even subtle temperature fluctuations and variations in moisture regimes may influence the specific types of DSE present in a given environment. Nevertheless, this observation underscores the necessity for more extensive and in-depth investigations, including comparisons to another ecologically similar model system from different localities with a similar ecological setup.

4.4 | Despite its omnipresence, AMF exhibit stable patterns and low diversity across studied sites

Confirming our expectations and aligning with earlier morphology-based investigations (Erdelská & Turis, 1996), we verified the presence of AMF in *D. arbuscula* roots in all study sites. However, their proportion remained consistently stable regardless of the microenvironment of the studied sites (Figure 2). This becomes particularly intriguing in the context of other detected root-associated fungal communities and guilds, which displayed relatively straightforward environmentally induced patterns. AMF colonization rates are often positively correlated with temperature and precipitation (Gai et al., 2012; Huo et al., 2021). It is commonly suggested that colonization increases at lower elevations (Gai et al., 2012; Geml, 2017; Kotilínek et al., 2017; Zubek et al., 2009) and may be linked to arid and semiarid ecosystems, where AMF can potentially enhance drought tolerance in host plants (Kivlin et al., 2013; Porras-Alfaro et al., 2008). Although we did not observe a clear environment-induced pattern in the occurrence of AMF across our study sites, it is essential to remember that our study is designed to capture microscale differences. These differences may be sufficient to diversify other root-associated fungi, yet the composition and spectrum of the AMF may remain without manifesting apparent differentiations (but see e.g. Tipton et al., 2022). Nonetheless, some deviations in mycobiome diversity were noted in Veľká Stožka, which exhibited the highest OTU richness in AMF (Table S7). This suggests a potential correlation between AMF diversity and altitude, given its elevated location compared to the other study sites (cf. Bonfim et al., 2016). However, we hesitate to draw definitive conclusions in this regard due to the relatively modest altitudinal gradient in our study, coupled with the lack of replication for this specific locality (refer to the discussion above). Given their omnipresence across all sites, we posit that AMF constitutes a stable root endophyte community within the root landscape of *D. arbuscula*. These fungi most likely play a substantial role in the adaptation and survival of this species in highly environmentally challenging rocky habitats.

In terms of taxon diversity, the majority of AMF were assigned to unclassified Glomeromycotina (Figures 3b and S5b; Tables S3 and S13) despite the dynamically evolving research in the field of taxonomy and phylogeny of AMF over the last decades (e.g. Morton & Msiska, 2010; Redecker et al., 2013). This observation suggests that rock-dwelling plant communities, including the studied species, present not only excellent models for studying AMF-plant interactions in environmentally extreme habitats but can also serve as a reservoir of recently undiscovered AMF diversity.

5 | CONCLUSIONS

Rocky habitats, defined by their demanding environments, unveil extraordinary geomorphological heterogeneity, prompting investigations into the influence of both microclimatic and macroclimatic conditions on root-associated fungal diversity. Root-associated fungi play diverse ecological roles, ranging from enhancing plant fitness to ensuring survival in challenging environments and fortifying resistance against abiotic stress and pathogens, as indicated by our findings. Apart from the remarkable diversity of root-associated fungi, we uncover a distinct environmentally induced pattern: in rocky environments with warmer conditions, there is a notable increase in plant pathogen abundance. At the same time, colder sites exhibit a higher prevalence of DSE. We posit that the observed pattern may suggest that warmer sites are more susceptible to changing conditions, especially those associated with ongoing climate change. On the other hand, colder sites harbour not only a lower proportion of pathogens but also an increased level of DSE, potentially suppressing or mitigating biotic and abiotic stresses. Interaction among such diverse root-associated fungal classes creates complex, mutualistic and antagonistic networks that are currently underexplored and demand further investigation. Striking a harmonious balance between these positive and negative interactions becomes imperative for the sustained survival of plant communities, especially in rocky habitats subject to highly challenging environments and susceptible to rapid climatic oscillations.

AUTHOR CONTRIBUTIONS

The conception and design of the study were performed by Zuzana Gajdošová, Zuzana Kolaříková and Marek Slovák. Zuzana Gajdošová, Ingrid Turisová, Peter Turis, Jaromír Kučera and Marek Slovák conducted the data collection. Radka Sudová, Jana Rydlová and Zuzana Kolaříková performed the root samples processing in the laboratory, microscopic observations and amplicon data generation. Zuzana Gajdošová and Miroslav Caboň performed data analyses and plotting. The first draft of the manuscript was written by Zuzana Gajdošová, Miroslav Caboň and Marek Slovák, with all authors providing comments on previous versions of the manuscript. Finally, all authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The raw amplicon sequence reads have been deposited in the NCBI (BioProject ID: PRJNA1036833, SRA: SRP470670; BioSamples: SAMN38152953–SAMN38152987).

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