Use of environmental sequencing in evaluating fungal response to peatland degradation

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SUMMARY

Central European peatlands are critically endangered by climate change, eutrophication, disturbed water regime and inappropriate management. This leads to their degradation, which affects all components of these habitats including fungi, and significantly fungi participating in the carbon cycle such as saprotrophs, mycorrhizal symbionts and parasites. Nature conservation efforts focus mainly on macrofungi, especially 'fungi important for nature conservation' (FINC) which are rare or associated with endangered habitats. To find out whether standard environmental DNA (eDNA) sequencing methods can be used to monitor the effect of peatland degradation on FINC and fungal communities, we established pairs of plots in degraded and well-preserved parts of ten peatlands in the Třeboň Protected Landscape Area (Czech Republic). Peatland degradation was locally caused by a combination of different factors (water level and chemistry) followed by vegetation succession, whose effect on fungi was evaluated using multivariate analyses. A total of 28 FINC species were detected using macrofungal surveying, whereas 25 were found using eDNA of *Sphagnum* and peat (Illumina sequencing of ITS2). These two methods agreed on 13 predominantly abundant FINC species, most of which grew in both well-preserved and degraded plots. However, their sensitivity to the studied degradation could not be assessed due to the low abundance of most species. Fungal communities found on the basis of both methods (macrofungal survey, eDNA) were affected by water chemistry, whereas a significant effect of vegetation and locally expansive plants was demonstrated only in fungal communities found using macrofungal survey. Optimisation (e.g. specific primers) for local conditions leading to a better FINC capture must be carried out if eDNA is to be used in the practice of evaluating management effects on peatlands.

KEY WORDS: eDNA, macrofungi, nature conservation, peatland management, vegetation succession

INTRODUCTION

Fungi are an important group of organisms which fulfil several key functions in ecosystems. Many of them decompose organic matter and thus return nutrients into nutrient cycles. Mycorrhizal species support their symbiotic plants, and parasitic species influence populations of their hosts. At the same time, fungi are an important food source for many other organisms. In nature conservation it is mainly macrofungi (= macromycetes, fungi producing macroscopic fruitbodies at least 2 mm in size) that are taken into account. Thanks to field mycologists, the ecology and distribution of macrofungi are much better known compared to microfungi (fungi whose morphological structures can only be observed microscopically) (Senn-Irlet *et al*. 2007). Macrofungal surveys are often conducted alongside botanical and zoological surveys to characterise protected nature areas and to evaluate the appropriateness of their management (Heilmann-Clausen *et al*. 2015, Bazzicalupo *et al*. 2022). However, due to the large number of macrofungi (an estimate for temperate Europe is 4000–7000 species; Læssøe & Petersen 2019), these surveys often include hundreds of species so are financially demanding as well as time-consuming. Nevertheless, such macrofungal surveys can be very useful, especially in habitats with a low diversity of plants, because fungi respond very sensitively and faster than vegetation to habitat changes (Griffith *et al.* 2013, Caboň *et al.* 2021). Due to the limited number of field mycologists, efforts have been made to reduce these detailed surveys and to focus on, e.g., the so-called 'fungi important for nature conservation' (FINC) species only, and thus increase capacity to build a better understanding of their ecological requirements. FINC are macrofungal

species whose occurrence is rare and/or the habitat they are associated with is threatened. Given the fact that fruitbodies of most macrofungi occur for only a short period under favourable weather conditions, the question arises whether other methods such as environmental DNA sequencing (eDNA) could be used to detect their mycelia (Frøslev *et al*. 2019).

The advantage of environmental sequencing is that fungi which have not formed fruitbodies can be detected. This option could be advantageous in the case of time-limited studies during which we are not able to record fruitbodies of all species due to seasonality or sub-optimal weather conditions for fungal growth. Additionally, it could serve as a solution for the lack of field mycologists in the case of large-scale studies or repeated monitoring. In inaccessible or inhospitable areas of the planet which have never been explored mycologically, environmental sequencing can estimate the expected biodiversity and composition of fungi (Tedersoo *et al*. 2015). On the other hand, environmental sequencing has many disadvantages and pitfalls which must be taken into account at the beginning of any study. The first problem is the sampling methodology itself. It is important to note that sample collection cannot cover an entire study area and, as a result, not all species actually occurring there will be detected (Frøslev *et al*. 2019, Vašutová *et al*. 2023). In addition, Vašutová *et al*. (2021) demonstrated that a high substrate heterogeneity limits the use of environmental sequencing and results in missing fungal species associated with unsampled substrates. Another pitfall is that environmental sequencing cannot assess whether the sequences come from hyphae growing at the site or spores which arrived accidentally from elsewhere, and thus cannot confirm that the organism is viable or has grown there (Wright *et al*. 2019). In the next step of environmental sample processing, several critical points are important including a correct choice of primers for the target fungal groups (Balami *et al*. 2020) and optimisation of the PCR reaction (primer annealing temperature, optimal number of cycles). Based on the study by Tedersoo *et al*. (2015), the choice of primers influences the underestimation or overestimation of some fungal groups or causes absence of some sequences. The depth of sequencing (i.e. number of sequences per sample) also affects the resulting data (Shirazi *et al*. 2021). Sequencing depth could be solved by random selection of the same number of sequences or converting sequence abundances to percentages, etc., which is just another way of affecting the resulting data (Taberlet *et al*. 2018). Also, the error rate of DNA polymerase and incorrect pairing of reads (resulting in formation of chimeras) can cause the resulting OTUs to be not biologically relevant (Tedersoo *et al*. 2015).

Peatlands are habitats which are permanently waterlogged and where organic matter decomposes very slowly. They provide several ecological functions ranging from water retention, cooling the local environment and carbon storage to biodiversity maintenance (Joosten *et al*. 2017, Rowland *et al.* 2021). They are critically threatened by climate change, and by human activities at global scale (e.g. high nitrogen input) as well as at local scale (e.g. drainage, local eutrophication, lack of management). The Třeboňsko Protected Landscape Area (Czech Republic), where the study was conducted, is a wetland landscape known for fish farming and has numerous peatlands. Most of the peatlands were initiated at the beginning of the Holocene (Jankovská 1978) when springs and margins of glacial lakes warmed up, starting slow peat formation processes at their edges and resulting in terrestrialisation of the lakes. In the Middle Ages, huge peatlands were replaced by ponds for fish farming and today's peatlands are only remnant edges or on sites of former springs (Navrátilová & Navrátil 2005). With the change in pond water trophic levels (i.e. increased nutrient content), the chemistry of the peatlands is also fundamentally changing, leading to replacement of the original peatland vegetation by more competitive other species (Navrátilová *et al*. 2006). Warming and associated droughts are undoubtedly contributing to the degradation of peatlands too. Nature conservation is trying to counter this negative development by improving the water regime and vegetation management (regular mowing, tree removal) and by simultaneous monitoring of the effects of these interventions on different groups of organisms (Assandri & Bazzi 2022, Hájková *et al*. 2022).

The aims of this study were:

(1) to compare results of standard environmental DNA sequencing with data from macrofungal surveys to evaluate its practical use for nature conservation;

(2) to determine how macrofungi respond to peatland degradation; and

(3) to find out which variables influence their occurrence.

We expected that environmental sequencing would detect fungal species which are abundant; that FINC would occur more frequently in well-preserved than in degraded parts of peatlands; and that FINC occurrence would be mainly influenced by vegetation.

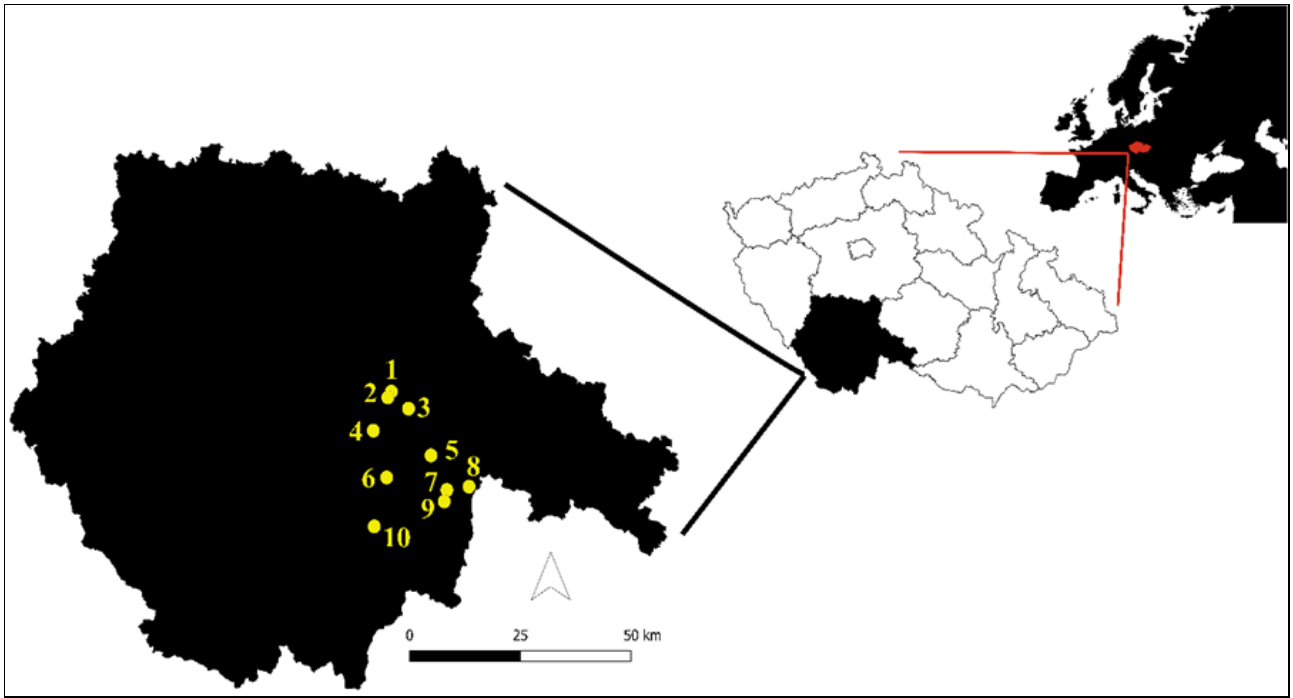
METHODS

Study area and plots

Ten poor fens threatened by degradation were selected in the Třeboňsko Protected Landscape Area and its surroundings in the South Bohemian Region, Czech Republic (Figure 1). Four of them are regularly managed by the Třeboň Protected Landscape Area Administration and the regional office of the national government's Department of the Environment, Agriculture and Forestry. Both preserved and degraded parts of the peatlands are managed to conserve or restore these habitats.

The altitudes of the study sites range from 410 to 550 m above sea level. The mean annual temperature is 7.8 °C, the coldest month is January (–2.8 °C) and the warmest is July (18 °C). The mean annual precipitation is 570 mm (Tolasz *et al*. 2007). Local causes of peatland degradation are a combination of different factors followed by vegetation succession. The dominant elements of this succession (locally expansive plants, based on the author's field experience) are summarised in Table 1 and used as a proxy for degradation. At each site, two study plots (9 × 9 m) were established, one in a well-preserved part and the other in a degraded part (see Appendix and Table S1 in Supplementary Material).

PVC tubes (diameter 5 cm, length 150 cm with transverse notches for the first 50 cm from one end) were installed at each plot with the notched (perforated) parts embedded in peat. Five times per season, the water level in each tube was measured manually then water samples were collected for analyses of pH and electrical conductivity (EC). Water samples for estimation of C, N, P, NH_4^+, NO_3, Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+} and Fe^{2+} content were collected from the same tubes in September 2021 (Table S1).



Studied site		Coordinates		Area (ha)	Reg man?	Locally expansive plants
		N	E			
1	Ruda	49.1506503	14.6896992	70.2	Yes	*Salix cinerea, Molinia caerulea*
2	Hliníř	49.1367100	14.6792369	4.7	No	*Phragmites australis*
3	Rod	49.1205125	14.7498275	6.6	No	*Molinia caerulea, Polytrichum commune, Pinus sylvestris*
4	Dvořiště	49.0704353	14.6498428	25.9	Yes	*Molinia caerulea, Pinus sylvestris*
5	Starý Vdovec	49.0396894	14.8361128	0.1	No	*Molinia caerulea*
6	V Rájích	48.9862014	14.7089131	0.5	Yes	*Molinia caerulea*
7	Staré Jezero	48.9788300	14.8973303	3.7	Yes	*Phragmites australis*
8	Staňkovský rybník	49.0168744	14.9965536	7.9	No	*Molinia caerulea, Polytrichum commune, Pinus sylvestris*
9	Kukla	48.9566408	14.8949094	0.7	No	*Pinus sylvestris, Betula pendula, Frangula alnus, Calamagrostis canescens*
10	Žemlička	48.8913119	14.6903172	0.2	No	*Molinia caerulea*

Figure 1. Map of the study area with locations and details of the studied sites. The column headed "Reg man?" indicates whether or not the site is regularly managed for nature conservation.

For the determination of dissolved organic C (DOC) and dissolved N (DN), a fresh water sample (10 g) was placed in 50 ml of deionised water (dH_2O) and shaken at 80 oscillations per minute for 45 min. Then the suspension was centrifuged for 30 min and passed through a 0.45-µm membrane filter. Filtrates were frozen and stored at -20 °C until analysis. DOC and DN contents were measured using a TOC analyser (model TOC-LCPH/CPN, Shimadzu). The NH_4^+ content was determined with a modified Berthelot reaction (Schinner *et al*. 1996). The determination was based on the reaction of sodium salicylate with NH_3 in the presence of sodium dichloroisocyanurate, which forms a green-coloured complex. Sodium nitroprusside was used as a catalyst. NO_3^- ions were assessed by UV absorption at 210 nm (Schinner *et al*. 1996). Controls were performed by reducing nitrate with copper-sheathed granulated zinc. Elemental determination was performed using an ICP-QQQ mass spectrometer (Agilent, Japan). Samples were dispensed automatically using an SPS 4 autosampler (Agilent, Japan). Na, Al and Mg were measured in single quadrupole mode, and helium (He) collision mode was used for K, Ca and Fe measurements. Analytica and CPAchem standards were used for verification.

The vegetation was sampled in July 2021 by making phytosociological relevés in the 9 × 9 m plots using the Braun-Blanquet scale (Kent & Coker 1992) (Table S2). Vascular plant nomenclature follows Kaplan *et al.* (2021) and that of bryophytes Kučera *et al*. (2012).

In the Czech Republic, FINC are considered to be species listed in:

(i) Regulation No. 395/1992 of the Czech Ministry of Environment;

(ii) the Methodology for Fungal Species Conservation (Dvořák & Hrouda 2020);

(iii) the Red list of Fungi (Macromycetes) of the Czech Republic (Holec & Beran 2006); and

(iv) the List of Indicator Species of Animals and Fungi for Particular Habitat Types according to the Habitat Catalogue of the Czech Republic (Hofmeister & Hošek 2016).

We also included species which are known to be very rare but are not mentioned in the lists above (Table 1). Assessment of fungal guilds was based on Põlme *et al*. (2020). Due to the uncertainty of the saprotrophic/ parasitic status of several important FINCs (*Arrhenia, Galerina*), only ectomycorrhizal and CHEGD (*Clavariaceae, Hygrocybe, Entoloma, Geoglossaceae, Dermoloma* (also *Porpoloma* and *Camarophyllopsis* spp.); Griffith *et al*. 2013) fungi were assessed.

Macrofungal survey

The macrofungal surveys were conducted once per month from August to November 2020 and from April to November 2021 (Table S3). Fruitbodies not identified directly in the field were dried at 40 °C and identified under the microscope using the following keys and monographs: Breitenbach & Kränzlin (1984), Breitenbach & Kränzlin (1986), Breitenbach & Kränzlin (1991), Noordeloos (1992), Breitenbach & Kränzlin (1995), Breitenbach & Kränzlin (2000), Kränzlin (2005), Jamoni (2008), Bernicchia & Gorjón (2010), Boertmann (2010), Knudsen & Vesterholt (2012) and Aronsen & Læssøe (2016). If necessary, fruitbodies were sequenced and identified by comparing them with reference sequences from the GenBank database. DNA was extracted with the DNeasy Plant Kit (Qiagen). The ITS area was amplified with primers ITS1F (White *et al*. 1990) and ITS4 (Gardes & Bruns 1993) following Vašutová *et al*. (2023). PCR products were cleaned with Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (0.5:1) (ThermoFisher) and sequenced by GATC Biotech (Konstanz, Germany). Sequences are deposited in Genbank under DOS637-DOS653.

Sample collection, DNA extraction and sequencing

In September 2020, a mixed peat sample and a mixed decayed *Sphagnum* sample (both approximately 300 g) were collected from each plot using a sterile trowel and disposable gloves. Each mixed sample was composed of five subsamples from the corners and the middle of the plot. Peat samples were passed separately through a sterile 3 mm mesh sieve, *Sphagnum* samples were cut into smaller parts with sterile scissors, and ~60 g from each sample was stored in a freezer at -20 °C.

DNA was extracted from ~200 mg peat samples using the NucleoSpin Soil Kit (Machery-Nagel Gmbh & Co., Germany). Approximately 200 mg of decayed *Sphagnum* was ground with sterile sand, and DNA was extracted with the DNeasy Plant Mini Kit (Qiagen). The ITS2 region was amplified in triplicate with barcoded primers gITS7 and ITS4 (White *et al*. 1990, Ihrmark *et al*. 2012). The 10 µl PCR reaction mixture contained 5.2 µl of water, 2 µl of HF Buffer, 0.2 µl of dNTP, 1 µl of each primer, 0.1 µl of Phusion polymerase (New England Biolabs), and 0.5 µl of template DNA. The amplification program setup was: an annealing temperature of 98 °C for one minute, followed by 40 cycles of 98 °C for ten seconds, 66 °C for 30 seconds, 72 °C for 45 seconds, and a final extension of 72 °C for 10 minutes. Three PCR replicates were pooled and cleaned with the MinElute PCR Purification Kit (Qiagen). DNA concentration was measured with the Qubit 2.0

Fluorometer. Samples were sequenced with Illumina MiSeq (SeqMe, Czech Republic), using 250 bp paired-end chemistry. Sequence data are available in SRA under no. SRR24605436-469.

Molecular data analysis

The sequenced data were analysed with SEED version 2.1.2 (Větrovský *et al*. 2018). Pair-ended sequences with 15 % maximum difference and minimum overlap of 40 bp were further analysed applying recommended settings. Sequences with quality less than 30 were removed. Chimeric sequences were removed with the UCHIME programme (Edgar *et al*. 2011). For the ITS extraction, ITSx implemented in SEED (Bengtsson-Palme *et al*. 2013) was used, and only full-length ITS2 sequences were kept. In total, 827,980 sequences were clustered into 6,038 operational

Table 1. Fungi important for nature conservation (FINCs) detected in macrofungal surveys and by eDNA sequencing. Blue boxes indicate finds from environmental sequencing only, yellow boxes that we recorded fruitbodies only, and the darker green boxes that the species was found as fruitbodies and in environmental sequencing data. Z = well-preserved plot, Deg = degraded plot. Red list categories (Holec & Beran 2006): CR = Critically Endangered, EN = Endangered, NT = Near Threatened, VU = Vulnerable, DD = Data Deficient. Fungal lifestyle (Pölme *et al*. 2020): S = Saprotroph, ECM = Ectomycorrhizal, P = Parasite, CHEGD = species characteristic of less-disturbed unfertilised grasslands, possibly symbiotrophs (Griffith *et al*. 2013).

FINC	Lifestyle	Red list	Peatland indicator species	Kukla Z	Kukla Deg	Staré Jezero Z	Staré Jezero Deg	V Rájich Z	V Rájich Deg	Rod Z	Rod Deg	Ruda Z	Ruda Deg	Žemlička Z	Žemlička Deg	Starý Vdovec Z	Starý Vdovec Deg	Staňkovský rybník Z	Staňkovský rybník Deg	Hliniť Z	Hliniť Deg	Dvořiště Z	Dvořiště Deg
Arrhenia bigelowii	S																						
Arrhenia gerardiana	S	EN	×																				
Cortinarius alnetorum	ECM	EN																					
Cortinarius huronensis	ECM	DD																					
Cortinarius chrysolitus	ECM	NT																					
Cortinarius fulvescens agg.	ECM																						
Cortinarius tubarius	ECM	NT																					
Cudoniella clavus	S	NT																					
Entoloma anatinum	CHEGD																						
Entoloma caesiocinctum	CHEGD																						
Entoloma cuspidiferum	CHEGD																						
Entoloma poliopus	CHEGD																						
Exobasidium vaccinii	P		×																				
Exobasidium vaccinii-uliginosi	P		×																				
Galerina hybrida	S		×																				
Galerina paludosa	S		×																				
Geoglossum glabrum	CHEGD	CR																					
Geoglossum umratile	CHEGD																						
Geoglossum simile	CHEGD																						
Hygrocybe cantharellus	CHEGD	DD																					
Hygrocybe coccineocrenata	CHEGD	EN	×																				
Hygrocybe conica var. *conicopalustris*	CHEGD																						
Hypholoma elongatum	S		×																				
Hypholoma udum	S																						
Inocybe acutella	ECM	DD																					
Lyophyllum palustre	P		×																				
Mitrula paludosa	S		×																				
Mycena megaspora	S	CR	×																				
Myriosclerotinia dennisii	P		×																				
Phaeonematoloma myosotis	S	VU	×																				
Psathyrella typhae	S	DD																					
Russula laccata	ECM																						
Russula subrubens	ECM	DD																					
Russula sphagnicola	ECM	VU																					
Sarcoleotia turficola	S	CR																					
Scutellinia heterosculpturata	S																						
Simocybe cetunculus var. *laevigata*	S																						
Suillus flavidus	ECM	EN																					
Thuemenidium atropurpureum	CHEGD																						
Trichoglossum hirsutum	CHEGD	EN	×																				

taxonomic units (OTUs) including 2,362 singletons at a 97 % level of similarity. The most abundant sequence of each OTU was compared with the GenBank database using the BLASTn algorithm. Species level was assigned only if the sequence identity was higher than 97 % and coverage higher than 70 %. Genus level was assigned in cases where the sequence similarity was higher than 90 % (Vašutová *et al*. 2021). The 150 most abundant OTUs were utilised in further analysis.

Statistical analysis

The vegetation data (Table S2), data from macrofungal surveys (Table S3) and the eDNA sequencing (Table S4) were analysed in Canoco version 5.0 (ter Braak & Šmilauer 2012), calculating presence/absence data to compare various communities equally (Frøslev *et al*. 2019). Only OTUs with an abundance of more than 0.01 % per sample were included. The data from peat and *Sphagnum* at each plot were merged before the analysis. We evaluated the effect of environmental variables (Table S1), namely pH, EC, water level, C, N, P, NH_4, NO_3, Na, Mg, Al, K, Ca, Fe, cover of the moss (E0), herb (E1), shrub (E2) and tree (E3) layers, presence of peatland degradation and presence of management, by forward selection with the Bonferroni correction. The effect of each environmental factor was evaluated separately with Canonical Correspondence Analysis (CCA). The general effect of vegetation on fungal communities was evaluated by Co-Correspondence Analysis (CoCA). The effect of locally expansive plants (*Polytrichum commune*, *Calamagrostis canescens*, *Molinia caerulea*, *Phragmites australis*, *Alnus glutinosa*, *Betula pendula*, *Frangula alnus*, *Picea abies* and *Pinus sylvestris*) was evaluated with Canonical Correspondence Analysis (CCA).

Macrofungal surveys were focused on fungi forming fruitbodies, which was influenced by the weather, whereas the eDNA dataset contained a notable amount of microfungi. Due to the large differences between the datasets from macrofungal survey and eDNA, we tested them separately. In addition, we filtered out Agaricomycetes and Geoglossomycetes (according to Frøslev *et al*. 2019) from the datasets containing OTUs with an abundance of more than 0.01 % per sample.

RESULTS

Comparison of macrofungal survey with eDNA data

During the field survey, a total of 58 species of macrofungi were found in the studied plots (Table S3), of which 28 are considered to be FINCs. The most abundant orders included: Agaricales (67.2 %), Boletales (6.9 %), Helotiales (6.9 %), Exobasidiales (5.2 %) and Russulales (5.2 %). A total of 27 % of the recorded species were ectomycorrhizal and 15.5 % were CHEGD fungi. Analysis of eDNA detected a total of 875 OTUs with > 0.01 % per sample, of which 18 were identified as FINCs. Additionally, seven FINC species were found amongst less abundant OTUs. The most abundant orders were Helotiales (30.9 %), Agaricales (9.1 %), Pleosporales (6.2 %), Hypocreales (4.4 %) and Eurotiales (3.9 %). In total, 4.2 % of the 875 OTUs were ectomycorrhizal and 1.6 % were CHEGD fungi. Altogether, 13 of the 40 identified FINCs were common to both datasets. Nine FINCs were detected using both methods in the same plot (37 cases; Table 1), most of them abundant species (*Galerina hybrida*, *Galerina paludosa*, *Hygrocybe coccineocrenata*, *Hypholoma elongatum* and *Lyophyllum palustre*) (Tables S3 and S4). Filtering of Agaricomycetes and Geoglossomycetes from the eDNA data (all OTUs with more than 0.01 % abundance per sample) gave a total of 115 OTUs.

Degradation and FINC

It is evident from the obtained data (Table 1) that most FINC species grew on both well-preserved and degraded parts of the peatland. A total of 33 FINC species were detected in well-preserved plots and 28 in degraded plots. Unfortunately, most FINCs were found in one or two plots only (Figure 2). Therefore, it was not possible to assess their sensitivity to the studied degradation.

Degradation and fungal community

Based on the macrofungal survey, both well-preserved and degraded plots show dominance of Agaricales (65.9 % and 70 %), followed in the case of well-preserved plots by Boletales (9.8 %), Helotiales (9.8 %), Exobasidiales (7.3 %) and Cantharellales (2.4 %); and in the case of degraded plots by Helotiales (7.5 %), Boletales (5 %), Exobasidiales (5 %) and Russulales (5 %). Based on the eDNA dataset, the differences between the most dominant orders are even smaller. The most abundant Helotiales (33.7 % in well-preserved plots, 32.5 % in degraded ones) are followed by Agaricales (8.3 %, 7.9 %), Pleosporales (5.3 %, 6.5 %), Hypocreales (4.8 %, 4.6 %) and Eurotiales (4 %, 4 %).

Based on a joint analysis of water level and chemical properties (CCA) (Figure 3), it is evident that fungal communities in the studied plots were significantly influenced by the content of magnesium

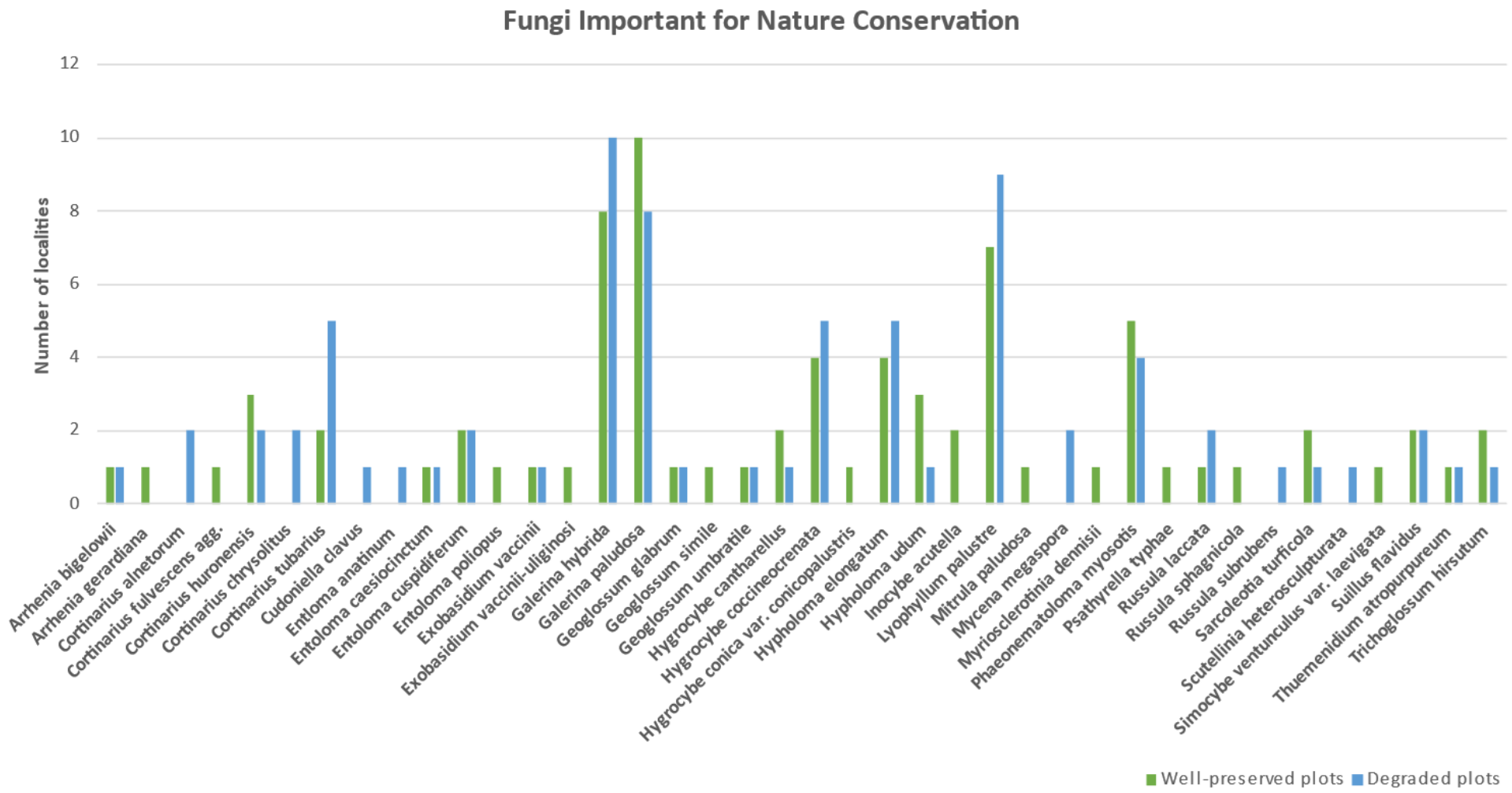


Figure 2. Number of localities where individual FINC species were detected on well preserved and/or degraded plots.

(Mg) only (macrofungal survey F = 2.0, p = 0.032; eDNA F = 1.6, p = 0.032). Marginal testing of each environmental factor separately without covariates using CCA revealed significant effects of carbon (DOC), magnesium (Mg), calcium (Ca) and management on fungal communities detected by macrofungal surveys, and of phosphorus (DP), magnesium (Mg), calcium (Ca) and management on fungal communities detected by eDNA (Table S5). The filtered Agariomycetes + Geoglossomycetes dataset was affected by the same factors as the whole eDNA dataset. In addition, a significant effect of carbon (DOC) was confirmed.

The fungal communities detected by the macrofungal surveys were influenced significantly by vegetation. Co-correspondence analysis (CoCA) was significant for all axes (p = 0.008), and the correlation coefficient for the first axis was 0.994.

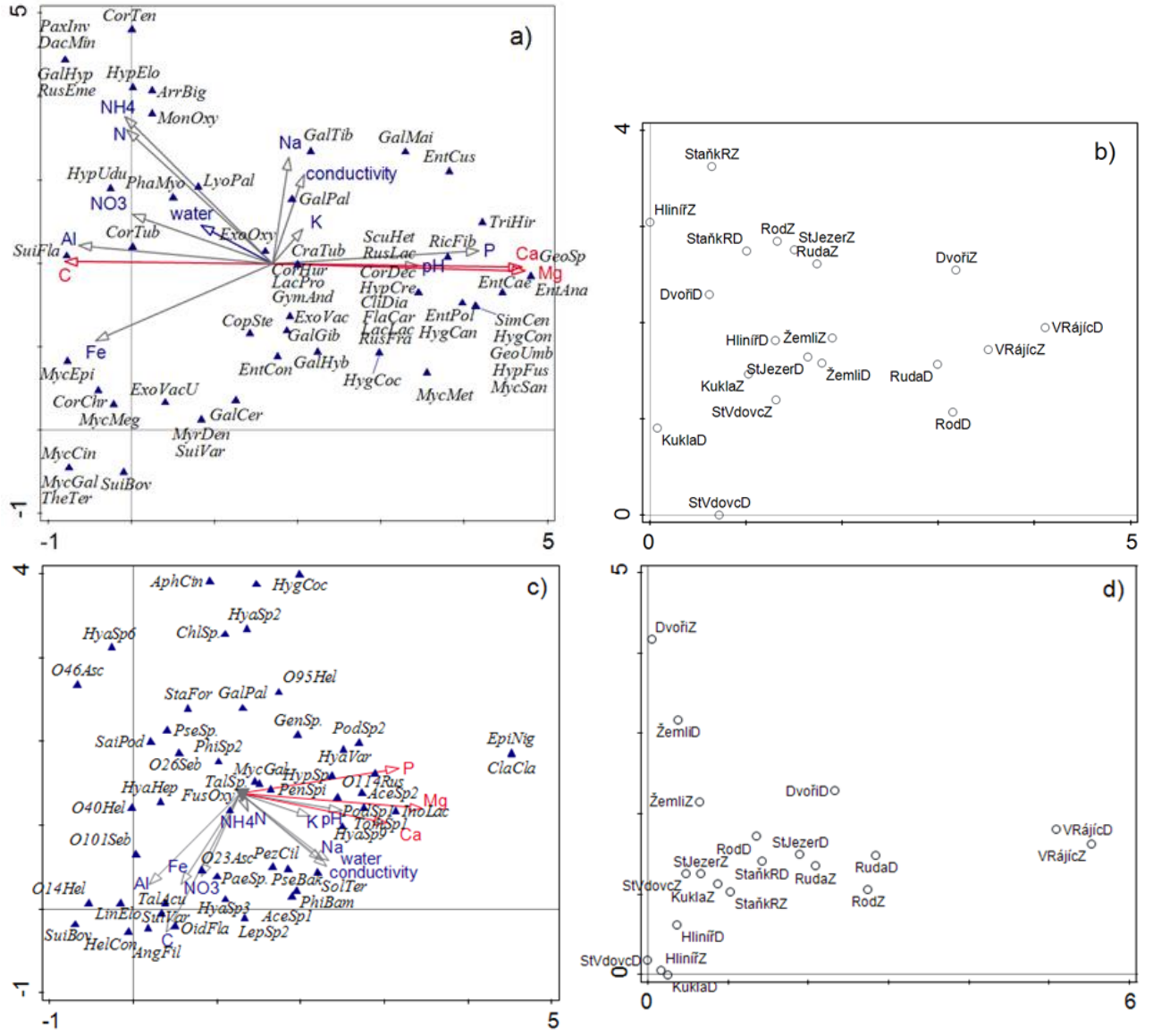


Figure 3. Results of analyses. a) DCA ordination diagrams summarising the variation of macrofungal species from field sampling alongside selected environmental variables. Those which were statistically significant (CCA) are indicated in red. b) DCA ordination summarising the variation in sampled localities. Eigenvalues corresponding to the first and second axis, respectively: λ1 = 0.6916, λ2 = 0.4042. The first axis explains 12.4 % of total variability and the second axis 7.3 %. c) DCA ordination diagram summarising the variation of eDNA species alongside selected environmental variables. Those which were statistically significant (CCA) are indicated in red. Only the 50 OTUs with the highest weight are displayed. d) DCA ordination diagram summarising the variation of sampled localities based on eDNA results. Eigenvalues corresponding to the first and second axis, respectively: λ1 = 0.6628, λ2 = 0.5262. The first axis explains 10.0 % of total variability and the second axis 8.0 %. For abbreviations see Tables S2, S3 and S4 in the Supplementary Material.

For the dataset based on eDNA, the effect of vegetation was non-significant (p = 0.052), just as for the filtered dataset of Agaricomycetes and Geoglossomycetes (p = 0.27).

The effects of locally expansive plants on fungal communities (macrofungal survey) were significant in the cases of the following species: *Polytrichum commune* (F = 1.8; p = 0.002), *Frangula alnus* (F = 1.3; p = 0.032), *Picea abies* (F = 1.4; p = 0.036), *Salix cinerea* (F = 1.6; p = 0.010) and *Alnus glutinosa* (F = 2.0; p = 0.004) (Figure 4). The effect of locally expansive plants on fungal communities detected by eDNA was not significant (F = 1.1; p = 0.060) (Figure 4). Similarly, no significant effect was proved in the case of the Agaricomycetes + Geoglossomycetes dataset (p = 0.184).

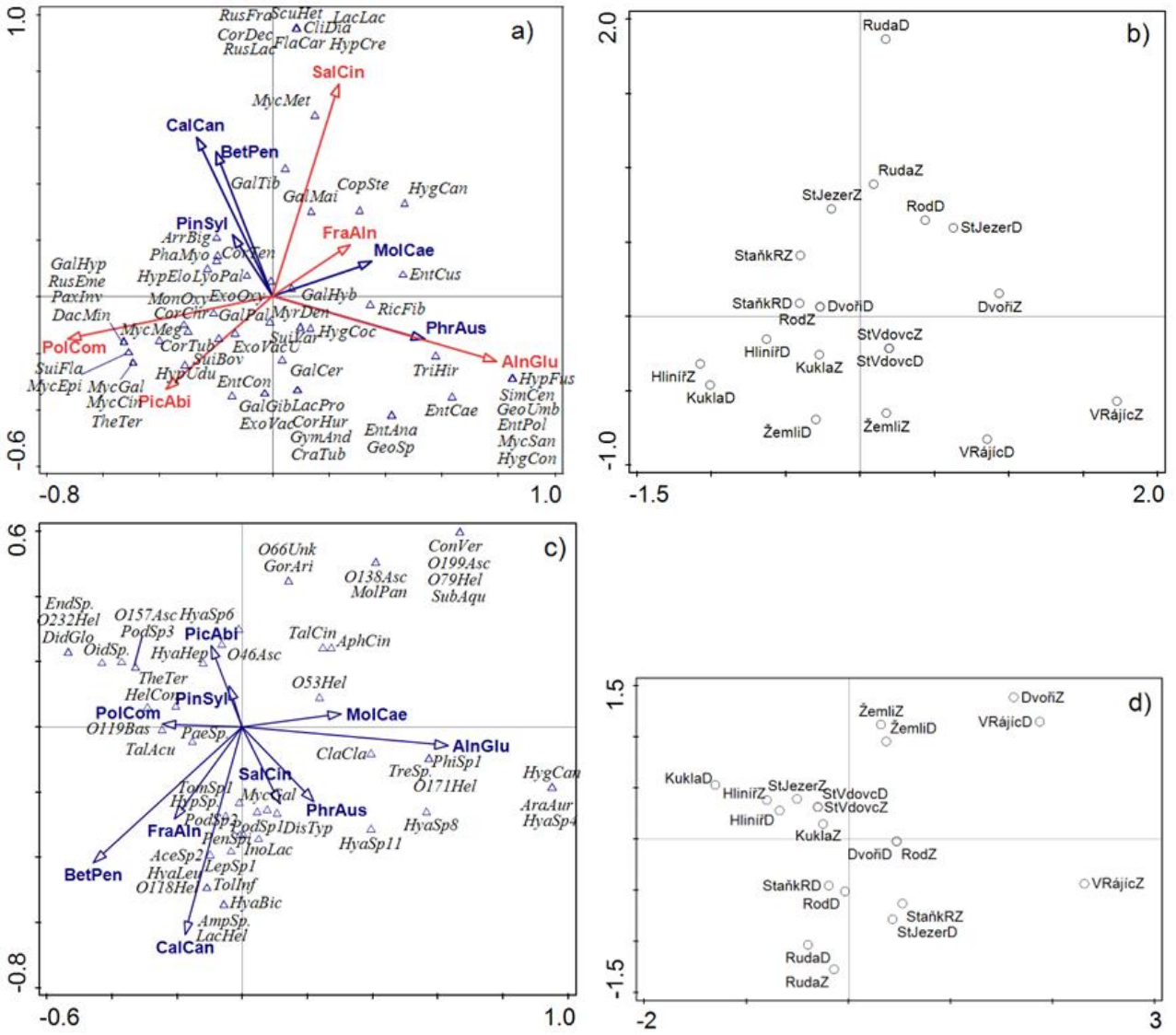


Figure 4. Results of constrained analyses. a) CCA ordination diagram summarising the variation of macrofungal species from field surveys alongside locally expansive species of plants. Those which were statistically significant (CCA) are indicated in red. b) CCA ordination diagram summarising the variation of sampled localities based on results from field surveys. Eigenvalues corresponding to the first and second axis, respectively: λ1 = 0.591, λ2 = 0.552. The first axis explains 11.44 % of total variability and the second axis 10.68 %. c) DCA ordination diagram summarising the variation of eDNA species alongside locally expansive species of plants. Those which were statistically significant (CCA) are indicated in red. Only the 50 OTUs with the highest weight are displayed. d) DCA ordination diagram summarising the variation of sampled localities based on eDNA results. Eigenvalues corresponding to the first and second axis, respectively: λ1 = 0.526, λ2 = 0.480. The first axis explains 7.95 % of total variability and the second axis 7.26 %. For abbreviations see Tables S2, S3 and S4 in the Supplementary Material.

DISCUSSION

Macrofungal surveys versus standard eDNA

Our study confirmed that environmental sequencing can detect more abundant FINC species, but for less abundant species the detection is more random. Several species not recorded as fruitbodies were identified by means of eDNA (e.g. *Cortinarius alnetorum, Cudoniella clavus, Inocybe acutella, Sarcoleotia turficola*, *Thuemenidium* sp., etc.). The possible reason for absence of their fruitbodies may be unsuitable weather conditions or reduced fungus vitality due to removal of a mycorrhizal partner or substrate, decreased humidity, etc. For example, *Sarcoleotia turficola* is a species which is often found in environmental sequencing datasets from peatlands (Vašutová *et al*. 2021) but forms fruitbodies very rarely, mainly in places with high water level (Stasińska 2011, Vašutová *et al*. 2013). *Inocybe acutella* could not grow in a studied plot because its mycorrhizal host (willow) was absent. However, the presence of its sequences may have resulted from the introduction of spores from another part of the peatland where the presence of its fruitbodies under willows was documented (Vašutová 2019). In the worst case, DNA from dead mycelia may have been sequenced (Gordon & Van Norman 2014, 2021).

On the other hand, many species forming fruitbodies in the studied plots were not detected by eDNA. Some of them (*Exobasidium*, *Simocybe*) were strongly associated with aboveground parts of plants whilst others grew mainly on litter (*Scutellinia*) or in the interface of living and decaying *Sphagnum* (*Arrhenia, Trichoglossum, Geoglossum umbratile*), which was not or not sufficiently sampled. This constraint has already been discussed by Vašutová *et al*. (2021, 2023). Another case is presented by species whose fruitbodies were recorded extremely rarely (*Hygrocybe conica* var. *conicopalustris*, *Cortinarius chrysolitus*). If the number of their fruitbodies was correlated with the abundance of their mycelia, the chance of detecting them was very low. Similarly, if the mycelia of rather common species tend to grow in patches, their distribution is not regular, and so they can be omitted because of the sampling design. More detailed sampling and analysis of multiple mixed samples from a single plot could solve this problem. However, this would result in more damage to the plot and significantly increased costs.

Another constraint of environmental sequencing is the lack of reference sequences for some rare fungal species in public databases (e.g. *Ramariopsis subartica*) which are thus impossible to identify. The accuracy of identification using ITS2 is also limiting. Mainly *Cortinarius* species cannot be distinguished based on the commonly used 97 % threshold in OTUs clustering (Liimatainen *et al*. 2020).

Thus, for nature conservation purposes, a macrofungal survey cannot be fully replaced by eDNA methods but these provide a unique supplement. Also, eDNA can be used effectively in some special cases of large-scale sampling to detect prospective sites for rare species (Gordon & Norman 2021), with later verification by field mycologists.

Response of FINC to degradation of peatlands

FINC species were found approximately equally in well-preserved and degraded parts of the peatlands. Most of the FINC records suggest that there is no observable sensitivity of these species to the studied peatland degradation. We assume that the detected species respond to peatland degradation, but there was either not enough time for their sensitivity to manifest, or they have a higher degree of tolerance to peatland degradation than we expected.

By focusing in more detail on species forming fruitbodies or detected by environmental sequencing in degraded parts of the peatlands only (*Cortinarius alnetorum, Cortinarius chrysolitus, Cudoniella clavus, Entoloma anatinum, Mycena megaspora, Russula subrubens* and *Scutellinia heterosculpturata*), the following conclusions can be drawn. Half of these species are ectomycorrhizal symbionts, needing ectomycorrhizal partners (trees) which are found mainly in degraded parts of peatlands. However, based on field experience and analysis of the requirements of selected species (Vašutová *et al*. 2023), it is known that these ectomycorrhizal FINC species are found only under solitary trees or shrubs with a high water table. If degradation continued, they would be replaced by common forest ectomycorrhizal species or by saprotrophs in the cases of increased E1 (herb) or litter cover, and/or subsequent local eutrophication. Other explanations for the occurrence of some FINC fruitbodies in degraded parts of peatlands only may be that the formation of fruitbodies of some species is induced by stressful conditions (Moore *et al*. 2020). Also, the improved microclimate of a degraded site (moisture, shading) could be the reason for formation of fruitbodies. At the same time, species such as *Cortinarius alnetorum*, *Cudoniella clavus* and *Russula subrubens* have been recorded in degraded plots by environmental sequencing only, making their real situation uncertain for the reasons given in the previous section of this Discussion. Species recorded only in plots on preserved parts of the peatlands (*Arrhenia gerardiana*, *Entoloma*

poliopus, *Exobasidium vaccinii-uliginosi*, *Hygrocybe conica* var. *conicopalustris*, *Inocybe acutella*, *Myriosclerotinia dennisii*, *Mitrula paludosa*, *Psathyrella typhae* and *Simocybe centunculus* var. *laevigata*) were mostly found in a single plot, so no specific conclusions can be drawn.

Response of fungal communities to degradation of peatlands

Both datasets showed an effect of calcium and magnesium, which is, however, not related to degradation but to natural differences between sites. Analysis of the relationship between vegetation, locally expansive plants and fungal communities detected by eDNA showed that the species (= OTUs) captured in this way were not very sensitive to site degradation. The reason may be that the most common species in the eDNA dataset were soil saprotrophic microfungi (e.g. *Podospora*, *Talaromyces*, *Pseudeurotium*) and yeasts (e.g. *Saitozyma podzolica*, *Solicoccozyma terricola*). Frøslev *et al.* (2019) faced a similar problem of having significant numbers of soil microfungi obtained from eDNA. These dominant micromycetes are somewhat generalist and do not have such strong specific associations with particular plant species as macrofungi. Despite the filtering out of Agaricomycetes and Geoglossomycetes, the results are not different. Therefore, according to our study, the presented standard eDNA method may not be suitable for regular monitoring of the success of management interventions in peatlands. If primer combinations targeting most FINCs (primers for *Agaricomycetes* + *Geoglossomycetes*) were used to partially eliminate moulds and yeasts, the results could be more applicable. However, these would not detect FINC species of other groups such as the genera *Myriosclerotinia*, *Cudoniella* and *Mitrula*.

On the other hand, macrofungal surveys indicated that the influence of vegetation on fungal communities was significant. This agrees with the specific association of some peatland macrofungi with the vascular plant and moss species that are now present - as their ectomycorrhizal symbionts, saprotrophs or parasites. When focusing on locally expansive plants, the influence of *Polytrichum commune*, *Frangula alnus*, *Picea abies* and *Salix cinerea* was significant. A number of ectomycorrhizal macrofungi are symbionts of willow and spruce, whose dead wood or fallen leaves serve as a source of nutrients for some saprotrophic macrofungi. The influence of *P. commune* on fungal communities cannot be explained by simply trophic relationships. Only two species of the genus *Galerina* and, rarely, *Phaeonematoloma myosotis* were found in *P. commune* tufts. Since *P. commune* was recorded in about half of the plots, this is probably a cumulative effect of a number of species without a causal relationship. Studies regarding *F. alnus* indicate that its litter contains significant amounts of nitrogen (3.2 % of dry weight; Stokdyk & Herrman 2016), which may affect fungal growth in the surrounding area (Branco *et al*. 2022).

Concluding remarks

Our study has shown that environmental sequencing can detect abundant species of fungi important for nature conservation as well as some other rare FINC species. However, it is not possible to say with certainty whether the rare species are viable (i.e. forming fruitbodies) at the site.

A comparison of well-preserved and degraded parts of the peatlands in terms of FINC occurrence demonstrated that most FINC species grow in both preserved and degraded parts and, so far, do not show sensitivity to the studied degradation. Given that ectomycorrhizal FINC species occur mainly in degraded areas, their conservation is dependent on at least some solitary trees being retained at otherwise open (treeless) sites during peatland management.

The analysis of the influence of environmental factors on fungal communities identified by both methods showed a significant influence of magnesium and calcium content. According to the data from macrofungal surveys, the fungal communities were influenced by vegetation and some locally expansive plants. Surprisingly, the importance of these factors was not apparent in data obtained from environmental sequencing. This is probably due to the high abundance of soil saprotrophic microfungi that are weakly associated with specific plants, which also affected the presence of OTUs in the filtered Agaricomycetes + Geoglossomycetes dataset. Thus, without costly and time-consuming optimisation of the standard eDNA method for local conditions leading to better FINC capture, eDNA may not be useful in practice for evaluating management effects on peatlands.

ACKNOWLEDGEMENTS

We thank Jakub Čech for assistance with fieldwork, Alžběta Manukjanová for laboratory work and identification of mosses, Karel Prach for the selection of sites and plots and help with identification of vascular plants. Aleš Jirsa assisted in the field and identified resupinate fruitbodies, Sujan Balami

helped with peat and *Sphagnum* sampling, Veronica Jílková analysed water chemistry, Jan W. Jongepier performed language revision. We are also grateful to the Třeboň Protected Landscape Area Administration and the Regional Office of the South Bohemian Region for permission to establish plots and collect samples. Finally, we thank Miroslav Caboň and Mateusz Wilk for their valuable review comments which have improved the article. This study was supported by the Czech Science Foundation (Grant No. 31-19-15031S).

AUTHOR CONTRIBUTIONS

HS: investigation, molecular analysis, data analysis, writing (preparation of original draft); AM: data analysis, writing (review and editing); MV: methodology, writing (review and editing).

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Submitted 21 Nov 2023, revision 12 May 2024
Editors: Gerald Jurasinski and Olivia Bragg

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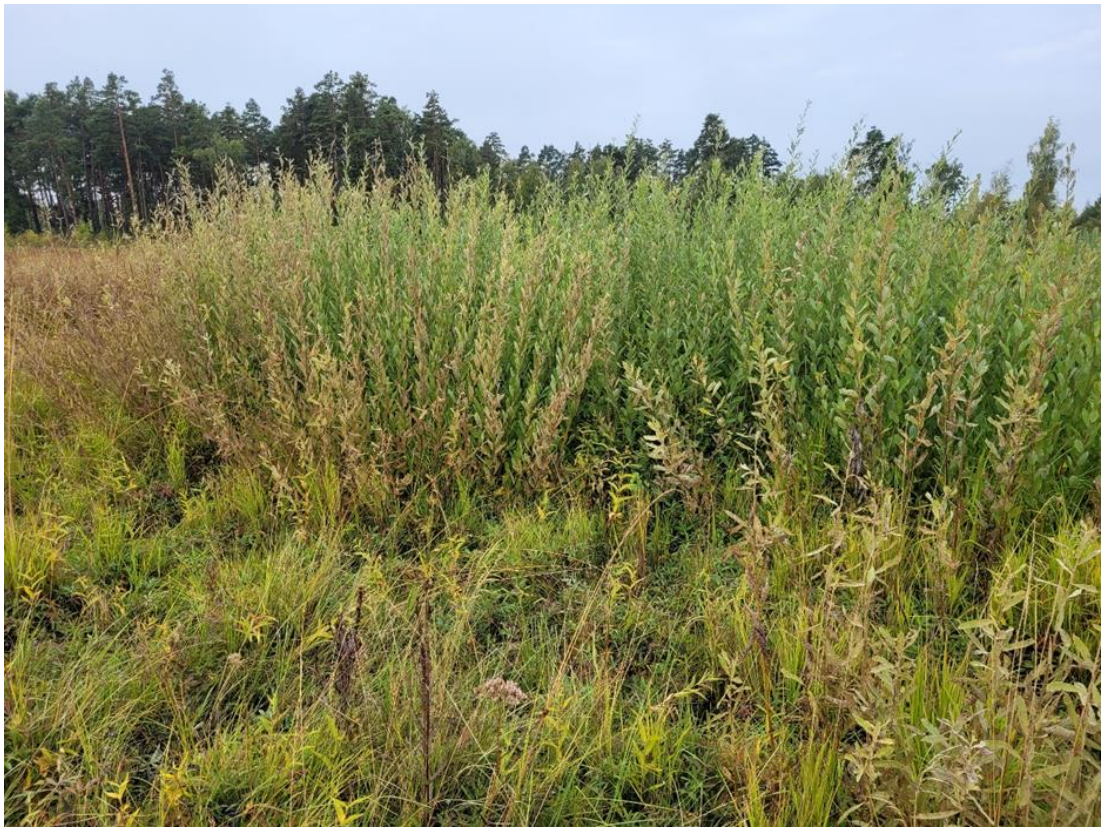
Appendix: Photographs of the well preserved (WP) and degraded (Deg.) study plot at each site.

1. Ruda

WP

12 Sep 2021



Deg.

12 Sep 2021

2. Hliníř

WP

31 Oct 2020

Deg.

31 Oct 2020

3. Rod

WP

31 Oct 2020

Deg.

27 Sep 2020

4. Dvořiště

WP

12 Sep 2021

Deg.

12 Sep 2021

5. Starý Vdovec

WP

25 Jul 2022

Deg.

30 Oct 2020

6. V Rájích

WP

12 Sep 2021

Deg.

12 Sep 2021

7. Staré jezero

WP

13 Sep 2021

Deg.

21 Aug 2021

8. Staňkovský rybník

WP

13 Sep 2021

Deg.

13 Sep 2021

9. Kukla

WP

13 Sep 2021

Deg.

21 Aug 2021

10. Žemlička

WP

12 Sep 2021

Deg.

12 Sep 2021