Use of eDNA analysis of soil samples to evaluate the fungal conservation value of grassland areas in the South West Peak: lessons for woodland creation proposals

Pink waxcap (*Porpolomopsis calyptriformis*) © Alex Hyde

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FINAL REPORT

Use of eDNA analysis of soil samples to evaluate the fungal conservation value of grassland areas in the South West Peak; lessons for woodland creation proposals

Gareth W Griffith, Andrew P Detheridge and Ruby Bye
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Abstract (186wds)
Analysis of environmental DNA (eDNA) extracted from soil cores collected from 116 quadrats (900m2 area) at 25 sites across the South West Peak area was undertaken in autumn 2021. eDNA metabarcoding via high-throughput sequencing was used to examine fungal populations present in each quadrat, with particular focus on macrofungi of conservation concern, namely waxcaps and allied groups, often collectively referred to as CHEGD fungi. Many members of this group of fungi face a risk of global extinction. A total of 137 CHEGD species were detected, with 74 or more species found at the best five sites (seven sites had 20 or more waxcap species). This range of species included one assessed as globally Endangered and 10 assessed as globally Vulnerable on the IUCN Global Red List. Numbers of CHEGD species present correlated positively with soil pH but negatively with other soil parameters including soil moisture, organic matter, nitrogen, phosphorus, potassium content. Attempted correlations with land management parameters showed weaker correlations. Four of these sites were being considered for woodland creation but all were home to 49+ CHEGD species (including 12-21 waxcaps and 7-12 Global Red List species.

Executive Summary

Soil samples were collected from 116 grassland quadrats (each ca. 900 m^2) at 25 sites across the South West Peak area (Staffordshire, Cheshire, Derbyshire) of the Peak District National Park, during autumn 2021. After freeze-drying, grinding, DNA extraction and PCR (polymerase chain reaction) amplification of part of ribosomal RNA fungal DNA barcode region, high throughput DNA sequencing was conducted using an Illumina MiSeq High Throughput DNA sequencer. This yielded a total of 4,616,636 DNA sequences for the ITS2 locus (primary DNA barcode locus for Fungi (mean 37,493 per quadrat; range 6,207-167,593). These were clustered into 5020 distinct OTUs (operational taxonomic units) (mean 1146/quadrat). The UNITE database was used to classify these OTUs, of which 2322 corresponded to named species (the remainder were listed as numbered OTUs).

Ascomycete and basidiomycete fungi dominated all samples (mean 48% and 34% respectively of all sequences), with the majority of basidiomycete sequences present belonging to the CHEGD fungi (23%). Waxcaps (Hygrophoraceae) and coral fungi (Clavariaceae) were the most abundant of these (overall mean 12% and 6% respectively; mean 16%). For Hygrophoraceae, *Microglossum* (green earthtongues) and *Dermoloma* spp. (cracked caps), 99% of sequences could be identified to species but for Clavariaceae, Entolomataceae (pink gills) and Geoglossaceae (black earthtongues), ca. 35% of sequences could not be linked to named species; this illustrates the need for fundamental taxonomic studies of these taxa and potentially the presence of hitherto undiscovered species, for instance one unidentified *Gliophorus* sp. (AR2018a). The relative abundance of CHEGD fungi ranged from 0.9% to 47% across all the 116 quadrats. The combined CHEGD score for all quadrats (based only on named species, not unnamed OTUs) was (C34H41E37G21D4=137) but for individual quadrats the CHEGD scores ranged from 3 (C0H0E3G0D0) to 46 (C17H11E12G6D0), averaging 28.2. Of the 137 named CHEGD fungi detected, 19 have hitherto been found in Europe but not the UK and 6 only outside Europe. Four potential species new to science were detected. Amongst the named CHEGD fungi one is categorised as Endangered on the IUCN Global Red List (*Gloioxanthomyces vitellinus*) and 26 as either Vulnerable (10), assessed as Vulnerable but not yet published (16) or Vulnerable and subsequently split into a number of different species (5). For the purposes of this report we assume all of these species to be categorised as Vulnerable. Several of these species are also listed in Section 41 of the NERC Act (2006).

Management regimes and a range of edaphic (soil) parameters were also obtained for the 116 quadrats in order to determine whether these showed correlation with the diversity and/or abundance of CHEGD fungi. Correlation of eDNA sequence data for fungi with a range of soil parameters revealed a positive correlation of CHEGD species richness with pH but a negative correlation with soil moisture, soil organic carbon content, total nitrogen content, total/available phosphorus content and available potassium content. Thus in more acidic peaty soils (which also have higher N content), there was reduced diversity/abundance of CHEGD fungi. Use of Taqman qPCR to estimate fungal and bacterial biomass in the soils did not reveal any significant ($P<0.05$) correlations with CHEGD diversity/abundance, and methodologies for quantification of fungal/microbial biomass need further development.

The correlation of several management practises with CHEGD diversity/abundance was assessed. Grazed pastures showed greater Hygrophoraceae and total CHEGD species richness than hay meadows (opposite was found for arbuscular mycorrhizal fungi) and cattle grazing was associated with reduced Hygrophoraceae species richness and CHEGD relative sequence abundance. Care must be taken in assessing cause and effect when interpreting such data, since hay meadows or cattle grazing would less likely be located on sloping or high altitude fields.

Based on comparison of the eDNA and data from past fruitbody surveys, most of the 25 sites have high conservation value for grassland fungi with the best meriting consideration for SSSI designation. Specifically the four sites at risk of afforestation all merit protection of their grassland fungi, with even the least species-rich of these (Gag Aye) having 12 CHEGD species classified as VU (vulnerable) on the IUCN global Red List.

List of Figures, Tables (also in Excel file) and Appendices (Excel file only)

Introduction

Most of our knowledge of the distribution of fungi is based on the occurrence of their reproductive structures (basidiocarps [mushrooms], ascocarps etc.) which occur only ephemerally and in a highly season and weather-dependent manner. Thus, establishing which fungi are present at a given site requires detailed and time-consuming field surveys. We have adapted new developments in DNA sequencing technology (often called NextGen sequencing) to devise a method whereby extraction of DNA from soil samples can be used to assess which fungi are present.

Specifically we are developing the use of this technology to elucidate the distributions of grassland macrofungi, many of which (notably the waxcaps but also including other 'CHEGD' fungi [coral fungi-Clavariaceae, earth tongues-Geoglossaceae, pink gills-Entolomataceae, cracked cap-*Dermoloma*/*Porpoloma*]) are of conservation concern. The acronym 'CHEG' was first suggested by Rotheroe et al. (1996), with *Dermoloma* / *Porpoloma* [now *Pseudotricholoma*] / *Camarophyllopsis* merged into the 'H' (Hygrophoraceae) score. These three genera were separated out as a separate group ('D') by Griffith et al. (2013), to follow the convention originally established by Nitare (1988) and also used by McHugh et al. (2001). However, shortly thereafter phylogenetic analysis confirmed that the agaricoid genus *Camarophyllopsis* was in fact clearly placed within the mainly coralloid family Clavariaceae (Birkebak et al., 2016). Moreover, it was shown that *Camarophyllopsis*, as originally circumscribed, was polyphyletic, so several species were reassigned to *Hodophilus* (e.g. *H. atropunctus*, *H. foetens*). Due to these taxonomic changes *Camarophyllopsis* / *Hodophilus* are included in 'C' for our CHEGD counts, though we note that Bosanquet et al. (2018) did not account for recent taxonomic changes and retained *Camarophyllopsis* / *Hodophilus* in 'D'. In Table 1, CHEGD scores from Neil Barden's fruitbody surveys are reported and it should be noted that he reported any *Camarophyllopsis* / *Hodophilus* in category 'D'.

It should also be noted that *Microglossum* spp. (incl. *Thueminidium*), belonging to class Leotiomycetes of phylum Ascomycota, are classified as 'G', despite being only distantly related to the other species belonging to the *Geoglossum* spp. and relatives (belonging to class Geoglossomycetes of phylum Ascomycota) which comprise the majority of species placed in group 'G'.

It is important to note that there has been a taxonomic reappraisal by Lodge et al. (2014) of the Hygrophoraceae family (which contains the waxcaps but also some other lichenised fungi and ectomycorrhizal species). This has resulted in the creation of some name changes (e.g. *H. calyptriformis* [pink waxcap] is now *Porpolomopsis calyptriformis*; and some of what were formerly known as *Hygrocybe* spp. are now placed in the genera *Chromosera*, *Cuphophyllus*, *Gliophorus*, *Gloioxanthomyces*, *Humidicutis*, *Neohygrocybe*). However, the specific names are preserved. It is important to note the contribution of David Boertmann's (2010) book 'The Genus *Hygrocybe'* which provided clear and pragmatic concepts for (nearly) all the species found in Europe, and more recently for phylogenetic analyses (Ainsworth et al., 2013; Lodge et al., 2014). It is hoped that an updated edition of this monograph, accounting for the new species recently discovered and the modified concepts of existing species will be published in the near future.

These new methods are dependent upon the existence of genetic information (DNA barcodes) relating to each of the species of interest. The genes used as DNA barcodes for fungi differ from those used for animals and (to some extent) plants. For fungi it is the ribosomal RNA genes that are used, notably the internal transcribed spacer (ITS) and large subunit (LSU) regions; it should be noted that the ITS contains the 5.8S rRNA gene flanked by two intronic regions, ITS1 and ITS2, the latter more commonly used for eDNA metabarcoding. We regularly analysed both regions but in this project have opted for ITS2 since it allows more accurate identification, despite being less reliable for relative quantification. The ITS locus provides by far

the best reference sequence coverage across all fungi coverage but with a few exceptions. For example, more LSU reference sequences are available for Clavariaceae (fairy clubs) than ITS. However, in the case of Clavariaceae and also Entolomataceae, the underlying taxonomy is still unclear. Not only are there uncertainties in the taxonomy of numerous species, many species remain to be DNA barcoded and thus in urgent need of fundamental revision. The reason that the LSU locus provides improved quantification is that it is less variable, so PCR products from different fungal groups are more conserved in length, resulting in less bias in PCR amplification. In the current project, the priority is to obtain the most accurate species identification possible, hence our use of ITS2. It should be noted that whilst comparison of LSU and ITS2 datasets would show higher relative abundance of CHEGD species in the former, comparison of the quadrats all assessed via ITS2 would provide accurate relative abundance ranking.

DNA barcodes are available for most of the CHEGD fungi found in semi-natural grasslands, though some of the current barcodes relate to specimens from non-UK locations (but which are likely to differ slightly in DNA sequence). Other groups of fungi are less well-studied and thus fewer barcodes are available. As a result, it is sometimes only possible to identify DNA sequences to genus or family. These 'mystery' barcodes may represent undiscovered species or alternatively known species for which no DNA barcodes have been established (note the unclassified Entolomataceae etc. in Table3).

The issue of how quantitative DNA metabarcoding is (i.e. how much reliance can be placed on read abundance) has been much discussed (Lamb et al., 2019). Primer mismatches and taxon-related differences in PCR product (amplicon) length may cause bias. However, for the primers we use (Tedersoo et al., 2014), the primer binding sequences are identical for all the CHEGD fungi. However, the amplicon length can vary by ca. 40% across all fungi, with the lower fungi tending to have shorter ITS2 regions and being somewhat preferentially amplified at the expense of higher fungi, especially basidiomycetes which have longer ITS2 sequences. This contrasts with the less widely used LSU barcode locus which we pioneered (Detheridge et al., 2016) where there is little length polymorphism (<10 bp across all fungi) and no primer mismatch. Use of LSU primers provides better relative quantification of different fungi but at the expense of accurate species identification.

Whereas for the ITS locus, much higher sequence variability provides better taxonomic discrimination but the PCR products are more variable in length and subject to PCR amplification bias, for example with basidiomycetes (which have a relatively long ITS region) being slightly under-represented.

To ensure PCR amplification of the ITS locus for all fungal groups (including the oomycetes), we use a mix of primers suggested by Tedersoo et al. (2014). Bias may also result from differential efficiency of extraction of DNA from different fungal tissues. For example, it is likely that extraction of DNA from the (thick-walled) fungal spores is less efficient than from actively-growing mycelia. Additionally, the rRNA operon is a multicopy operon (as tandem repeats, visible as the nucleolus in microscopy) and it is estimated that on average 110 copies of this operon are present in each fungal nucleus. Differences in rRNA copy number (commonly ranging from 50-200), as recently identified by Lofgren et al. (2019) could also cause bias but to establish copy number for different species is not a simple matter.

Analysis of the huge numbers of sequences from NextGen sequencing (typically ca. 20,000 per sample) can provide not only identification but also relative abundance information. However, as noted above, the alignment of 'genetic' and morphological species concepts for many CHEGD fungi is still not complete and the taxonomy of some fungal families examined here is currently in flux. We also do not yet know the extent to which fungal biomass fluctuates on an annual basis but it is known that the grassland fungi of conservation interest are long-lived

organisms fruiting in the same locations each year and thus very likely to be present at similar relative abundance throughout the year.

The last factor (often not sufficiently accounted for in many peer-reviewed publications) is the sampling strategy. We have adopted a 900 m^2 quadrat. This is a moderately large area, which will fit into most grassland field plots; these are conveniently compatible with permanent quadrats which we established across Wales in 2003-4 for fruitbody surveying (Griffith et al., 2006) and also our main reference field site (Brignant long-term experiment; https://www.ecologicalcontinuitytrust.org/brignant/; (Detheridge et al., 2018)). Within these quadrats, the 36 cores taken on a grid pattern weigh ca. 500-700 g, suitable for convenient freezing. To obtain a representative sample for DNA extraction this soil must be well mixed. Freeze drying the sample allows us to grind and mix the sample in such a way that the subsample taken for DNA extraction is likely to contain all the species present in the whole.

Soil sampling for mycelial eDNA can be conducted at any time of year and for some fungi (and plants) an obvious seasonal pattern is observed, consistent with above ground abundance in the case of many higher plants. However, for grassland fungi which are long-lived perennial organisms, we have observed very little seasonal variation (Griffith and Detheridge, unpublished data). Therefore, we do not think that significant changes in relative abundance or numbers of CHEGD species detected would result from sampling at different times of year.

In addition to assessment of fungal biodiversity in the soils from each quadrat, a small amount of the sequencing 'effort' was directed to providing information about the plants present. This was achieved by including a PCR primer (Chen et al 2010) which specifically amplifies the ITS2 region of plants (Kingdom Archaeplastida; includes mosses, liverworts, green algae, as well as higher plants). Botanical information derived from DNA in roots, from plant litter or potentially seed provides useful contextual data for interpretation. This element was not part of the original project specification.

Whilst eDNA metabarcoding is a highly informative method for biodiversity assessment, it is relatively costly (ca. £300 per sample with some economy of scale). Sequencing costs have reduced significantly over the past decade.

Aims of this study: The aim of this study was to investigate CHEGD soil fungal populations and thereby assess the conservation importance of the various grassland sites across the South West Peak area of the Peak District using NextGen sequencing. Additionally, the identification of areas with diverse grassland fungal populations (and thus of high conservation value, despite reduced botanical conservation value due to high grazing pressure) would allow the Peak District National Park Authority (NPA) to better protect such habitats, especially areas of marginal land currently at risk of afforestation for attempted carbon sequestration. Assessment of a range of soil chemical parameters and total fungal/bacterial biomass alongside details of site management/history was also undertaken in order to identify potential proxies for high grassland fungal diversity more rapidly than costly eDNA analyses or time-consuming fruitbody surveys but which could also permit assessment of site quality outside the autumn fruiting period.

Sites were selected in five categories: highest ranked sites (international importance for Hygrophoraceae, national importance for other groups, these formed 'control' sites comparing field survey results with lab analysis results); hay meadows (typically circum-neutral, dry to damp grasslands managed for hay making); mediocre or degraded sites (changes in field survey results had been noted from earlier surveys); unknown sites (sites not previously included in field mycology survey); 'at risk' sites (sites under consideration for tree planting, but where there could be potential fungal interest). Landowner consent was then sought and

secured, thus 25 sites were selected. Each site was allocated a unique numerical code allowing for anonymity for those landowners who had requested it.

Within these sites quadrat location was chosen to reflect the fungal hotspots within the highest ranked sites; all hay meadows on the hay meadow sites; fields with former fungal interest, or where habitat indicated suitability but field survey had unexpectedly yielded poor results; fields where vegetation data indicated unimproved or good semi-improved acid/neutral grassland, or relevant NVC classifications on unsurveyed sites; and fields proposed for tree planting on 'at risk' sites.

Fruitbody surveys were previously conducted by Neil Barden at 11 of the sites and these in part guided the location of quadrats. At other sites quadrat were placed in areas with high densities of 'indicative' CHEGD-associated plant species such *Plantago*, *Agrostis* and *Rhytidiadelphus squarrosus*.

Results and Discussion

A) Soil sampling and transfer to Aberystwyth

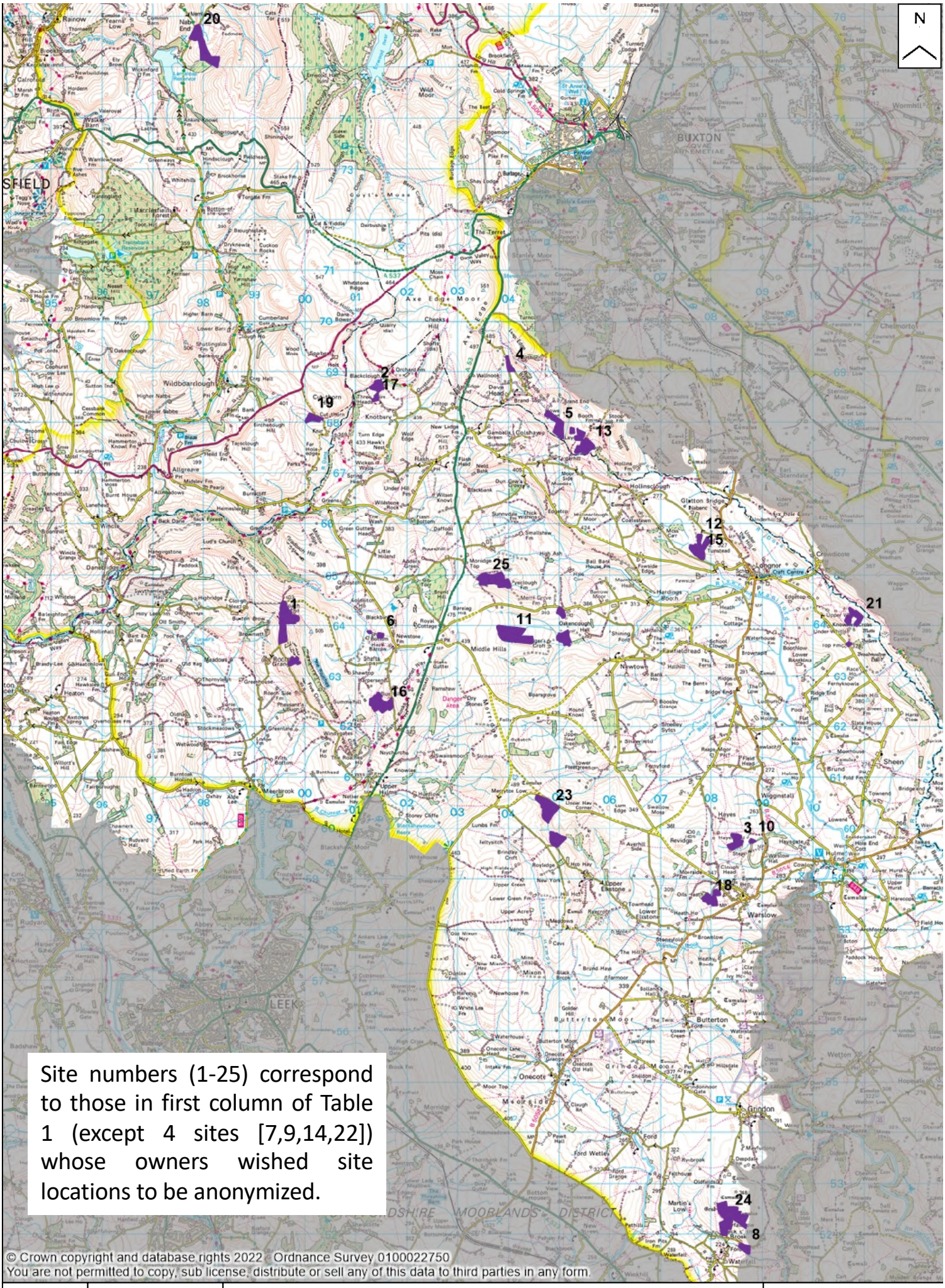
A total of 116 quadrats (ca. 30x30m) were surveyed (Fig. 1; Table 1, App1). Sampling was conducted by Mr. Neil Barden between 5th August and 2nd November 2021. Soil cores were taken (with an 18 mm steel auger to a depth of ca. 10cm. The top layer of vegetation was removed and used to cover the original hole. Cores were taken following an approximate grid pattern, with a spacing of ca. 5 m in each of the 25 grassland areas across a 30 m x 30 m quadrat giving 36 cores per quadrat. Quadrat locations were recorded by GPS. Where topography or areas of suitable habitat did not lend themselves to a grid format, a less defined pattern was followed. Cores were pooled in a plastic bag (fresh weight of 883-2182 g/sample; mean 1488 g; App5) and stored in a coolbox until they could be placed in a fridge at 4°C (ca. 4-8 hrs after start of sampling). Samples were sent with bubblewrap insulation by delivery courier in several batches. On arrival at Aberystwyth samples were immediately stored in a -80°C freezer. Transit time between soil sampling and freezing at Aberystwyth ranged from 2-12 days (App5).

Fig. 1: Map of South West Peak and sites sampled
Table 1: Summary of sites, CHEGD ranking and species detected
App1: Full details of sites and management (from Karen Shelley-Jones)
App5: Full list of soil data for all quadrats

B) High throughput DNA sequencing

Following the sequencing run, the quality of sequences was assessed and short reads not covering the whole barcode region or sequences of poor quality were removed, leaving a total of 4,616,636 DNA sequences. Sequences were clustered to group identical sequences, and clusters containing a single sequence (120,726 'singletons') were discarded. Inclusion of a small amount of the plant forward PCR primer (Chen et al., 2010) allowed some amplification of plant DNA barcodes (146,745 sequences) with the remainder being fungi (4,349,165). Of the remainder, 146,745 were identified as plants and the rest (120,726) unknown.

Sequences were then split using the unique 10 bp identifier tags at each end of the PCR amplicon to separate the 116 samples. The total number of fungal sequences per sample ranged from 6,207 to 167,593 (mean 37,493). Amongst these sequences 5020 distinct OTUs (operational taxonomic units) were identified, of which 2322 were recognised by the UNITE database. The remainder were listed as numbered OTUs) but in most cases these are classified at least partially (i.e. to family or order level).



Drawn by:	Shelley-Jones Karen
Date:	04 May 2022
Drawing No:	1
Scale:	1:70000 at A3

Fig. 1. Distribution of the 25 sites across the Peaks NPA area

PEAK DISTRICT NATIONAL PARK

Table 1. CHEGD counts based on eDNA and FB surveys

						SPECIES COUNTS											
SITE	CHEGD RANK ORDER	Farm Name	CODE	No. Quads	CHEGD TOT	Clav spp. (/34)	Hyg spp. (/41)	Ent spp. (/37)	Geo spp. (/17)	Mglo spp. (/4)	Derm spp. (/4)	IUCN spp.	CHEGD profile (NB)	NVC	Agri Env Sch	SSSI	Owner type	Rationale for inclusion
1	1	Brownsetts	1131	12	83	21	26	24	9	1	2	17	C5 H18 E8 G2 D0	U4	Yes	Part	F	Mediocre site (survey data quite old)
24	2	Waterfall Low	797	6	82	26	19	18	12	3	4	15	C1 H5 E1 G0 D0		Yes	No	F	Degraded site (change in management?)
13	3	Leycote	2137	10	81	20	27	22	9	1	2	16	C8 H31 E18 G4 D0	MG5	Yes	No	F	High quality site (not surveyed for some time, high quality site, inc. hay meadows)
23	4	Lower Fleetgreen o'l	712	10	75	21	23	22	8	0	1	12	C0 H5 E3 G0 D0	CG	Yes	Part	F	Potentially threatened (Tree planting proposals)
16	5	Franklins Farm Bluehills	2536	5	74	19	18	24	12	0	1	10	C7 H23 E15 G2 D0		Yes	No	F	High quality site (Has management changed? 2013 survey was poorer)
20	6	Summer Close Farm	403	6	69	19	21	19	7	1	2	11	C2 H10 E9 G0 D1		Yes	No	F	Unsurveyed site (Part of and adjacent to Lamaload clough LWS)
14	7	Anon3 (SH)	23	6	66	20	21	13	10	0	2	10	no data		No	No	F	Unsurveyed site
25	8	Pyeclough Farm/Head	83	3	65	19	18	19	8	0	1	8	C0 H2 E1 G0 D0		Part	No	F	Potentially threatened (Tree planting proposals, under-surveyed valley, in AES)
7	9	Anon1 (BL)	1526	4	65	18	20	15	9	1	2	14	C9 H17 E18 G3 D0		Yes	No	F	High quality site
5	10	Howe Green	1404	3	57	18	17	14	8	0	0	6	C2 H11 E4 G2 D0	MG5	Yes	No	F	Hay meadows (Unsurveyed site, interesting management history?)
4	11	Greenland Farm	1324	3	57	16	14	18	8	0	1	12	C5 H20 E18 G0 D0	MG5	No	No	F	Hay meadows (High quality site)
22	12	Anon4 (F)	5	3	54	19	13	9	10	0	3	7	C7 H21 E10 G3 D0		No	No	F	High quality site
2	13	Knotbury Common	1153	2	53	15	15	14	8	0	1	8	no data		No	No	WT	Unsurveyed site
19	14	Cut Thorn	3152	3	52	13	18	13	7	0	1	7	C3 H10 E4 G0 D0		No	No	F	Potentially threatened site (new owners interested in tree planting)
8	15	Waterfall Farm	3095	4	51	20	10	7	9	1	4	7	C0 H1 E0 G0 D0		Yes	Part	F	Degraded site
18	16	Oils Heath	507	6	49	11	17	10	9	0	2	9	C3 H17 E3 G0 D0	MG5	Yes	No	NPA	Hay meadows (High quality site. PDNPA owned land, management info known)
12	17	Gag Aye	2127	3	49	12	20	10	6	0	1	7	C3 H13 E5 G1 D0		Yes	No	F	Potentially threatened site (new owners interested in tree planting)
17	18	Knotbury1	2885	2	48	13	10	18	7	0	0	6	no data	U4	Yes	Yes	NPA	Unsurveyed site (Unsurveyed moorland fringe site. PDNPA owned land.)
15	19	Tunstead	2391	7	46	15	13	10	7	0	1	8	C7 H15 E6 G0 D0		No	No	CF	Unsurveyed site (also hay meadows)
11	20	Badger's Croft	1914	4	43	15	10	9	8	0	1	3	C3 H3 E1 G0 D0		No	Part	F	Degraded site (prev. survey on small part of holding, could be wider interest)
21	21	Under Whitle	46	4	42	14	9	6	10	0	3	4	C5 H17 E1 G0 D0	MG5	Yes	No	CF	Hay meadows
3	22	Upper Brownhills	335	3	42	14	8	9	8	0	3	5	C0 H2 E0 G0 D0	MG5	Yes	No	NPA	Unsurveyed site (hay meadows)
9	23	Anon2 (RK)	1655	4	41	13	9	7	10	0	2	6	C0 H2 E2 G0 D0	MG5	No	No	F	Unsurveyed site
6	24	Shawbottom	1470	2	33	11	8	6	8	0	0	5	C3 H10 E6 G1 D0	U4	Yes	Yes	WT	Unsurveyed site
10	25	Steps Farm	1702	1	17	5	6	0	5	0	1	1	C0 H1 E0 G0 D0	MG6	Yes	No	NPA	Unsurveyed site (hay meadows)

Top5-Green
Bottom5-Red
Middle-Orange

Red=National
Green=Regional
Lilac=Local
Rald (1985) scoring system

NOTE: In Neil Barden's CHEGD scores any Camarophyllopsis/Hodophilus spp. found are counted in 'D', whereas for all eDNA data they are counted in 'C' (since they have recently been shown to belong to family Clavariaceae)

Analysis of the sequence data (App2) across all quadrats showed that fungal populations were dominated by Ascomycota (cup fungi; 48.1%), Basidiomycota (mushrooms and allies; 33.6%) and Mortierellomycota (pin moulds; 9.3%). The most abundant species across all quadrats were a dark septate endophyte (a *Leohumicola* sp.), two soil yeasts (*Solicoccozyma* sp and *Saitozyma* sp) and two pin moulds (*Mortierella elongata* and *Mortierella* sp.).

App2: Full list of all fungi detected in each quadrat (5,020 OTUs)

C) Plant diversity:
The number of plant sequences obtained ranged from 23 to 19411 (mean 1146/quadrat; 3.0% of all sequences [range 0.14%-21.2%]). Although not part of the original contract specification, inclusion of the Chen S2F primer provided some useful contextual data.

A total of 91 higher plant species (plus 9 bryophytes) were detected across all the quadrats (mean 13.1 spp./quadrat [range 4-30 spp.). Chlorophyta (green algae; e.g. *Enallax costatus*, *Elliptochloris_marina*, *Prasiola* cf. *delicata*) were also detected, accounting for 6.8% of all the plant sequence (range 0-58.7%) but these data are not presented (Table 2; App3). Many plants (especially wind pollinated species) have a propensity to form hybrids and cannot be separated by the ITS2 gene marker (e.g. *Agrostis stolonifera* and *A. canina*, so referred to here are "Agrostis_stol_can"). Some unexpected species (for grassland soils) were detected, for example tomato (likely via human faeces or compost) and also some tree species (e.g. beech/ash, possibly from windblown leaves). It should be noted that the DNA detected here would mostly be derived from root tissues and recent plant litter (possibly seeds). We have found that patterns of plant abundance does vary seasonally, especially for annual plants such as *Rhinanthus* minor.

Table 2: List of higher plant species detected
App3: Full list of plant species (excl. algae) detected in each quadrat (100 spp.)

Knowledge of the plant communities in these quadrats provides useful ground-truthing information (e.g. *Helianthemum nummularium* at 797F5Q1 [24D] suggests a calcareous site and *Nardus stricta* at site 83 [25ABC] suggests an acidic nutrient poor soil) but the relatively low numbers of sequences in some quadrats (<100 sequences in 6 quadrats; App3) means that there was not good coverage of plant diversity, so it would not be appropriate to attempt to correlate plant diversity with diversity of CHEGD fungi. Several other lines of evidence suggest that attempts to find links between higher plant and CHEGD diversity would not necessarily be correlated.

First, plant diversity is heavily correlated with the nature and intensity of grazing pressure at a site (Liu et al., 2015; Olff and Ritchie, 1998; Scimone et al., 2007), whereas soil fungi are much less sensitive to this. Second, many waxcap species are globally distributed and outside Europe are mainly found in forest ecosystems dominated by non-ectomycorrhizal hosts (Halbwachs et al., 2018). Waxcaps and other CHEGD fungi are clearly obligate biotrophs and very likely mycorrhizal but there is no evidence that they exhibit any distinct host specificity and attempts to correlate CHEGD fungal diversity with any particular plant species have been unsuccessful (Griffith et al., 2014).

The rarity of waxcaps and other CHEGD fungi in the woodlands of Europe dominated by tree species which host ectomycorrhizal (ECM) fungi suggests that the latter outcompete the CHEGD fungi. It would be very interesting to explore CHEGD diversity in grassland habitats containing a high density of shrubby ECM hosting plants such as *Helianthemum* or *Dryas* (Harrington and Mitchell, 2005) but several sites in the Peak District where *Helianthemum* is abundant also host diverse CHEGD populations (Neil Barden, per. comm). The high abundance of moss at many sites with diverse CHEGD populations has led to speculation about a possible

Table 2. List of higher plant species found across the 116 quadrats

class	order	family	species	Count	mulative To	Mean	Max	Min
Liliopsida	Asparagales	Orchidaceae	Dactylorhiza_maculata	1	0.11%	0.11%	0.11%	0.11%
Liliopsida	Poales	Cyperaceae	Carex_caryophyllea	7	3.59%	0.51%	1.20%	0.12%
Liliopsida	Poales	Cyperaceae	Carex_nigra	8	4.79%	0.60%	2.54%	0.08%
Liliopsida	Poales	Cyperaceae	Carex_panicea	2	0.89%	0.45%	0.56%	0.33%
Liliopsida	Poales	Poaceae	Agrostis_cap_gig	106	367.26%	3.46%	34.21%	0.05%
Liliopsida	Poales	Poaceae	Agrostis_stol_can	3	1.60%	0.53%	0.63%	0.46%
Liliopsida	Poales	Poaceae	Alopecurus_pratensis	25	24.69%	0.99%	6.03%	0.03%
Liliopsida	Poales	Poaceae	Anthoxanthum_odoratum	68	105.95%	1.56%	13.04%	0.08%
Liliopsida	Poales	Poaceae	Avenella_flexuosa	2	2.73%	1.37%	2.53%	0.20%
Liliopsida	Poales	Poaceae	Briza_media	2	0.54%	0.27%	0.34%	0.20%
Liliopsida	Poales	Poaceae	Cynosurus_cristatus	44	29.09%	0.66%	2.59%	0.05%
Liliopsida	Poales	Poaceae	Dactylis_glomerata	17	5.79%	0.34%	1.09%	0.03%
Liliopsida	Poales	Poaceae	Deschampsia_cespitosa	4	3.37%	0.84%	1.45%	0.36%
Liliopsida	Poales	Poaceae	Festuca_ovina	19	33.53%	1.76%	10.87%	0.07%
Liliopsida	Poales	Poaceae	Festuca_rubra	73	79.78%	1.09%	5.31%	0.03%
Liliopsida	Poales	Poaceae	Holcus_lanatus	66	92.78%	1.41%	7.90%	0.03%
Liliopsida	Poales	Poaceae	Koeleria_macrantha	3	1.48%	0.49%	0.65%	0.34%
Liliopsida	Poales	Poaceae	Lolium_perr_mult	41	105.10%	2.56%	16.76%	0.03%
Liliopsida	Poales	Poaceae	Nardus_stricta	7	9.66%	1.38%	4.32%	0.13%
Liliopsida	Poales	Poaceae	Poa_annua	2	6.48%	3.24%	4.42%	2.06%
Liliopsida	Poales	Poaceae	Poa_prat_calc_parv	11	27.00%	2.45%	15.79%	0.11%
Liliopsida	Poales	Poaceae	Poa_trivialis	29	54.39%	1.88%	16.75%	0.06%
Liliopsida	Poales	Poaceae	Trisetum_flavescens	13	7.43%	0.57%	1.85%	0.08%
Streptophyta	Apiales	Apiaceae	Conopodium_majus	29	280.93%	9.69%	90.99%	0.15%
Streptophyta	Apiales	Apiaceae	Heracleum_sphondylium	8	16.81%	2.10%	9.52%	0.18%
Streptophyta	Asterales	Asteraceae	Achillea_millefol_ptarmica	22	320.64%	14.57%	64.09%	0.11%
Streptophyta	Asterales	Asteraceae	Bellis_perennis	11	18.44%	1.68%	5.26%	0.27%
Streptophyta	Asterales	Asteraceae	Centaurea_nigra	9	45.57%	5.06%	10.51%	0.25%
Streptophyta	Asterales	Asteraceae	Cirsium_arvense	3	42.66%	14.22%	31.14%	0.82%
Streptophyta	Asterales	Asteraceae	Cirsium_palustre	17	270.15%	15.89%	74.76%	0.24%
Streptophyta	Asterales	Asteraceae	Hieracium_sp.	15	75.11%	5.01%	14.52%	0.17%
Streptophyta	Asterales	Asteraceae	Hypochaeris_radicata	29	163.56%	5.64%	28.49%	0.11%
Streptophyta	Asterales	Asteraceae	Jacobaea_vulgaris	5	7.02%	1.40%	3.31%	0.28%
Streptophyta	Asterales	Asteraceae	Leontodon_hispidus	8	164.47%	20.56%	51.28%	0.25%
Streptophyta	Asterales	Asteraceae	Leucanthemum_vulgare	9	65.23%	7.25%	42.56%	0.07%
Streptophyta	Asterales	Asteraceae	Pilosella_officinarum	19	190.07%	10.00%	36.61%	0.28%
Streptophyta	Asterales	Asteraceae	Scorzoneroides_autumnalis	12	49.05%	4.09%	27.97%	0.33%
Streptophyta	Asterales	Asteraceae	Taraxacum_officinale_agg.	48	471.44%	9.82%	54.12%	0.04%
Streptophyta	Asterales	Campanulaceae	Campanula_rotundifolia	16	151.77%	9.49%	44.36%	0.29%
Streptophyta	Brassicales	Brassicaceae	Cardamine_prat_flex	24	129.36%	5.39%	32.87%	0.10%
Streptophyta	Caryophyllales	Caryophyllaceae	Cerastium_glomeratum	27	46.82%	1.73%	12.56%	0.05%
Streptophyta	Caryophyllales	Caryophyllaceae	Sagina_procumbens	1	2.05%	2.05%	2.05%	2.05%
Streptophyta	Caryophyllales	Montiaceae	Montia_fontana	2	3.78%	1.89%	3.33%	0.46%
Streptophyta	Caryophyllales	Polygonaceae	Rumex_acetosa	63	339.99%	5.40%	23.63%	0.11%
Streptophyta	Caryophyllales	Polygonaceae	Rumex_obtusifolius	2	0.72%	0.36%	0.40%	0.32%
Streptophyta	Dipsacales	Caprifoliaceae	Scabiosa_columbaria	1	2.46%	2.46%	2.46%	2.46%
Streptophyta	Dipsacales	Caprifoliaceae	Valeriana_officinalis	1	1.68%	1.68%	1.68%	1.68%
Streptophyta	Ericales	Ericaceae	Vaccinium_myrtillus	9	24.83%	2.76%	11.11%	0.17%
Streptophyta	Ericales	Primulaceae	Primula_vulgaris	1	1.19%	1.19%	1.19%	1.19%
Streptophyta	Fabales	Fabaceae	Lathyrus_pratensis	13	30.13%	2.32%	21.41%	0.10%
Streptophyta	Fabales	Fabaceae	Lotus_corniculatus	29	244.78%	8.44%	43.26%	0.13%
Streptophyta	Fabales	Fabaceae	Lotus_uliginosus	1	1.31%	1.31%	1.31%	1.31%
Streptophyta	Fabales	Fabaceae	Trifolium_dubium	11	24.21%	2.20%	15.41%	0.05%
Streptophyta	Fabales	Fabaceae	Trifolium_micranthum	4	1.85%	0.46%	1.39%	0.14%
Streptophyta	Fabales	Fabaceae	Trifolium_pratense	42	485.04%	11.55%	86.28%	0.16%
Streptophyta	Fabales	Fabaceae	Trifolium_rep_occi_nigr	105	2624.70%	25.00%	93.41%	0.09%
Streptophyta	Fabales	Fabaceae	Vicia_cracca	7	45.99%	6.57%	36.54%	0.60%
Streptophyta	Fabales	Fabaceae	Vicia_sepium	9	91.54%	10.17%	51.99%	0.02%
Streptophyta	Fabales	Polygalaceae	Polygala_serpyllifolia	7	13.63%	1.95%	5.56%	0.06%
Streptophyta	Fagales	Fagaceae	Fagus_sylvatica	2	16.12%	8.06%	15.87%	0.26%
Streptophyta	Gentianales	Rubiaceae	Galium_obtusum	3	10.08%	3.36%	4.95%	0.41%
Streptophyta	Gentianales	Rubiaceae	Galium_saxatile	37	378.12%	10.22%	63.89%	0.11%
Streptophyta	Gentianales	Rubiaceae	Galium_verum	2	4.77%	2.38%	4.43%	0.34%
Streptophyta	Lamiales	Lamiaceae	Ajuga_reptans	1	18.07%	18.07%	18.07%	18.07%
Streptophyta	Lamiales	Lamiaceae	Prunella_vulgaris	21	83.92%	4.00%	27.10%	0.15%
Streptophyta	Lamiales	Lamiaceae	Thymus_sp.	1	5.89%	5.89%	5.89%	5.89%
Streptophyta	Lamiales	Oleaceae	Fraxinus_excelsior	4	99.16%	24.79%	57.06%	0.24%
Streptophyta	Lamiales	Oleaceae	Fraxinus_ornus	1	10.18%	10.18%	10.18%	10.18%
Streptophyta	Lamiales	Orobanchaceae	Euphrasia_spp.	6	17.74%	2.96%	8.43%	0.20%
Streptophyta	Lamiales	Orobanchaceae	Pedicularis_sylvatica	1	11.75%	11.75%	11.75%	11.75%
Streptophyta	Lamiales	Orobanchaceae	Rhinanthus_minor	4	3.60%	0.90%	2.64%	0.22%
Streptophyta	Lamiales	Plantaginaceae	Plantago_lanceolata	66	798.68%	12.10%	54.07%	0.18%
Streptophyta	Lamiales	Plantaginaceae	Veronica_chamaedrys	19	150.79%	7.94%	35.61%	0.13%
Streptophyta	Lamiales	Plantaginaceae	Veronica_officinalis	4	13.11%	3.28%	8.90%	1.16%
Streptophyta	Lamiales	Plantaginaceae	Veronica_serpyllifolia	1	18.27%	18.27%	18.27%	18.27%
Streptophyta	Malpighiales	Linaceae	Linum_catharticum	2	23.51%	11.75%	18.87%	4.63%
Streptophyta	Malpighiales	Violaceae	Viola_lutea	1	7.62%	7.62%	7.62%	7.62%
Streptophyta	Malpighiales	Violaceae	Viola_riviniana	11	55.09%	5.01%	27.40%	0.46%
Streptophyta	Malvales	Cistaceae	Helianthemum_nummulariu	1	0.42%	0.42%	0.42%	0.42%
Streptophyta	Ranunculales	Ranunculaceae	Ficaria_verna	12	176.24%	14.69%	41.42%	0.41%
Streptophyta	Ranunculales	Ranunculaceae	Ranunculus_acris_occid	55	602.69%	10.96%	52.76%	0.15%
Streptophyta	Ranunculales	Ranunculaceae	Ranunculus_bulb_repe	55	438.78%	7.98%	54.21%	0.04%
Streptophyta	Rosales	Rosaceae	Alchemilla_mollis	2	9.53%	4.76%	5.35%	4.18%
Streptophyta	Rosales	Rosaceae	Crataegus_monogyna	1	0.06%	0.06%	0.06%	0.06%
Streptophyta	Rosales	Rosaceae	Potentilla_erecta	20	250.21%	12.51%	58.33%	0.15%
Streptophyta	Rosales	Rosaceae	Prunus_spinosa_domestica	1	1.91%	1.91%	1.91%	1.91%
Streptophyta	Rosales	Rosaceae	Sanguisorba_minor	3	16.17%	5.39%	11.97%	1.97%
Streptophyta	Rosales	Rosaceae	Sanguisorba_officinalis	3	0.74%	0.25%	0.39%	0.15%
Streptophyta	Rosales	Urticaceae	Urtica_dioica	4	18.91%	4.73%	15.53%	0.09%
Streptophyta	Saxifragales	Saxifragaceae	Saxifraga_sp.	1	1.38%	1.38%	1.38%	1.38%
Streptophyta	Solanales	Solanaceae	Solanum_lycopersicum	1	1.37%	1.37%	1.37%	1.37%
Bryopsida	Bryales	Bryaceae	Bryum_argenteum	1	1.26%	0.01%	1.26%	1.26%
Bryopsida	Bryales	Bryaceae	Bryum_pseudotriquetrum	2	5.50%	0.05%	3.27%	2.23%
Bryopsida	Hypnales	Brachytheciacea	Brachythecium_rivulare	8	1.26%	0.01%	0.26%	0.03%
Bryopsida	Hypnales	Brachytheciacea	Kindbergia_praelonga	5	0.95%	0.01%	0.43%	0.05%
Bryopsida	Hypnales	Hylocomiaceae	Rhytidiadelphus_squarrosus	12	10.19%	0.09%	2.08%	0.09%
Bryopsida	Hypnales	Hypnaceae	Hypnum_cupressiforme	2	2.25%	0.02%	1.59%	0.66%
Bryopsida	Hypnales	Pylaisiaceae	Calliergonella_lindbergii	5	1.85%	0.02%	0.65%	0.17%
Bryopsida	Pottiales	Pottiaceae	Ephemerum_minutissimum	1	0.39%	0.00%	0.39%	0.39%
Polytrichopsic	Polytrichales	Polytrichaceae	Polytrichum commune	1	1.85%	0.02%	1.85%	1.85%
						Mean	Max	Min
					GRASS TOTAL	16.21%	91.45%	0.00%
					HERB TOTAL	75.70%	98.82%	4.43%
					MOSS TOTAL	0.22%	3.70%	0.00%
					ALGAE TOTAL	6.84%	58.65%	0.00%
					Viridiplantae Total	1194	19259	23
					Unidentified PLANTS (Likely FUNGI)	1.02%	14.56%	0.00%
					NUMBER OF HIGER PLANT SPP (Not moss)	13.09	30	4

mutualistic interaction (Griffith et al., 2002; Seitzman et al., 2011). However, mosses have rhizoids rather than roots, as confirmed by the low abundance of moss eDNA in the soil samples studied here (mean abundance 0-3.7%), so it is not likely that they would form an association with soil fungi. Indeed, killing of moss with a specific biocide had no effect on CHEGD fruiting (Griffith et al., 2014), whilst several grasslands in drier lowland areas have diverse CHEGD population but very limited moss cover. It is more likely that CHEGD fungi and mosses co-occur in many grasslands because they are both sensitive to elevated levels of inorganic nitrogen (Halbwachs et al., 2018).

There are well-established indicators of ancient woodland (Allen, 2018) (https://www.gov.uk/guidance/ancient-woodland-and-veteran-trees-protection-surveys-licences) and there have been some attempts to provide similar definitions for ancient grasslands (Karlík and Poschlod, 2019). Since plant diversity is directly affected by heavy grazing, especially by sheep, proxy (non-plant) indicators (i.e. of potential plant diversity if sympathetic grazing management were imposed), for instance anthills, have been suggested (Fagan et al., 2010), although these are themselves susceptible to destruction by harrowing. There have also been occasional suggestions that the diversity of grassland fungi, a direct indicator of the low nutrient and undisturbed status of the soil is one of the most effective indicators, and the data obtained during the present study adds weight to this suggestion.

D) Possible sample degradation in transit:
In an earlier study (Clasen et al., 2020), we explored optimal conditions for sample transit (from field sampling to -80°C storage at Aberystwyth). We found that soil samples stored at 4°C would exhibit few changes in the fungi later detected for up to 14d. In this study we also observed that certain fungi were potentially useful markers for sample degradation, notably *Metarhizium carneum* and *Mortierella* spp. These microfungi are likely important in degradation of fungal chitin in soils so would be expected to proliferate where other mycelia were dying.

Soil samples were stored in a fridge (4°C) within a few hours of collection but difficulties were experienced with unreliable couriers, so the transit times ranged from 4 to 12d. We did find some positive correlation between the relative abundance of *Mortierella* spp. but not *Metarhizium carneum* with increased transit time (Fig. 2), suggesting that some spoilage may have occurred. The possible effect of this could have been to reduce the relative abundance of the target CHEGD taxa but there was no correlation between *Mortierella* abundance with either the relative abundance or number of CHEGD species.

Fig. 2. Correlation of abundance of chitinolytic spoilage fungi with duration of transit from sampling to arrival at Aberystwyth.

E) CHEGD species detected
A total of 137 CHEGD species were identified from the 116 quadrats (C34:H41:E37:G21:D4) (Table 3, App4). Of the 50 most abundant fungal species present across all quadrats, 15 were CHEGD fungi, mostly waxcaps (family Hygrophoraceae) (App2). Overall CHEGD fungi comprised 24.5% of the sequences obtained from the different quadrats (range 0.9% to 46.6%), with waxcaps comprising 12.2%, Clavariaceae 6.5%, Entolomataceae 0.6%, Geoglossaceae 3.3%, *Microglossum* spp 1.1% and *Dermoloma* spp. 3.5% (App2, App4). These proportions are consistent with our observations at other undisturbed grassland sites such as Hardcastle Crags (Griffith et al., 2019) and The Leasowes (Griffith et al., 2018). However, these data must be viewed in the context of the taxonomic uncertainties relating to several of the groups of CHEGD fungi (but less so waxcaps), as discussed below.

App4: List of all CHEGD fungi (and relative abundance) detected in each quadrat.

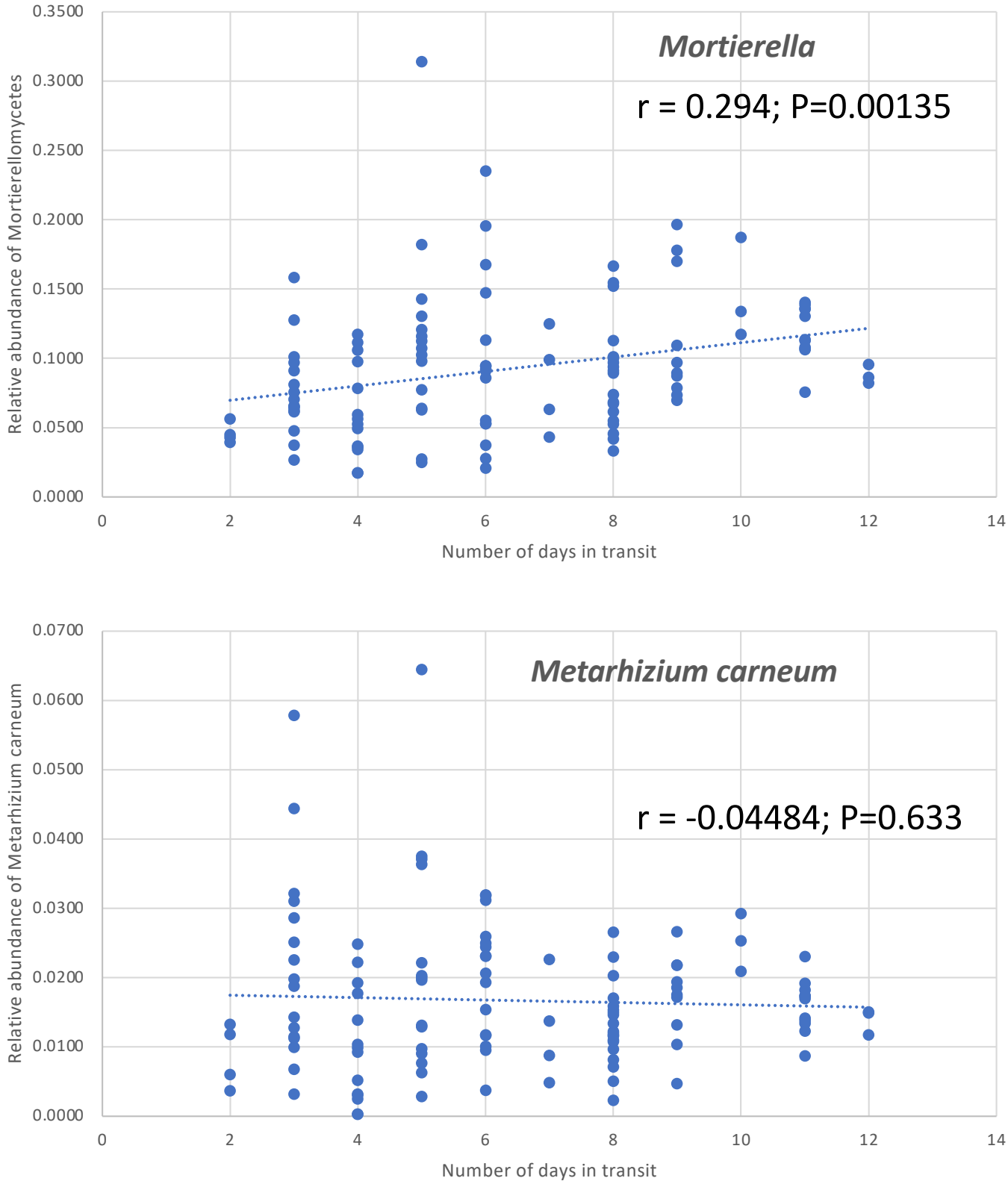


Fig. 2. Correlation of abundance of chitinolytic spoilage fungi with duration of transit from sampling to arrival at Aberystwyth. *Mortierella* spp. were more abundant in samples which took longer in transit

P = probability of linear (Pearson's) correlation: <0.05 is significant
r = correlation coefficient (0=no correlation +1= 100% positive correlation and -1= 100% negative correlation

The term CHEG was coined by Rotheroe (1999, 2001), and as a result of continuing taxonomic revisions, it evolves gradually, now CHEGD (Griffith et al., 2013). For instance, the agaricoid members of family Clavariaceae (*Camarophyllopsis*, *Hodophilus*) were previously placed under category D until the taxonomic revisions of Adamčík et al. (2017a; 2017b). *Microglossum* spp. are still classified under "G" though it is known that they belong to an unrelated order of ascomycetes (Helotiales) following recent taxonomic revisions (Hustad et al., 2014; Hustad and Miller, 2015; Kučera et al., 2017), but to try to minimise confusion the acronym CHEGMD has not yet been adopted.

There are several examples where morphologically similar CHEGD taxa are recorded from North America and Europe; it is currently not clear whether these represent sister species or variants of the same species, and there are examples where the concepts for these species differ between the US and Europe.

F) CHEGD species of conservation concern

Of the 137 CHEGD species (Table 3), four were potentially new species to science (see below). For Hygrophoraceae, *Microglossum* (green earthtongues) and *Dermoloma* spp. (cracked caps), 99% of sequences could be identified to species, but for Clavariaceae, Entolomataceae (pink gills) and Geoglossaceae (black earthtongues), ca. 35% of sequences (App4) could not be linked to named species. It is likely that at least some of these partially identified sequences represent species for which no established barcode exists or which are also new to science. This illustrates the need for fundamental taxonomic studies of these taxa.

Table 3. Summary of CHEGD species across the 116 quadrats

A further 17 spp. were previously recorded from continental Europe but not from the UK and four only from outside Europe (Tables 4, 5). For example, *Cuphophyllus flavipesoides* was only very recently named from several locations in Norway (it is closely related to *C. flavipes*) but our data suggest that it also occurs in the UK (Crous et al., 2021). Armed with the detailed quadrat location and description of the basidiocarp morphology, it should be possible to make a first UK record though resurveying of the three quadrats this autumn. Bearing in mind that estimates for the total number of fungal species suggests that >90% of species remain to be discovered (Hawksworth and Lücking, 2017), this situation in not surprising. However, for macrofungi (i.e. with more obvious fruitbodies) inhabiting the better explored parts of the world, some explanation is required. Morphological species concepts and those based on phylogenetic reconstructions generally show good consistency (congruence) but for some groups where there has been limited detailed study and hitherto a dearth of molecular phylogenetic study, there are often examples of cryptic speciation (where current morphological concepts encompass two unrelated species) or morphological traits that can mislead the classification of species into true natural groupings. Furthermore, insufficient survey effort to date means that new species are regularly discovered or their range is extended (i.e. 1st UK record etc).

Table 4. Summary of the rarer species found and those of conservation concern

					Global Red List		
	Total Spp.	Non-UK	Non-Europe	New Sp.	EN	VU	NT
CLAV	34	6	3	0	0	2	0
HYG	41	2	1	2	1	16	1
ENT	37	6	1	1	0	7	0
GEOG	17	5	2	0	0	1	0
Microglossum	4	0	0	1	0	4?	0
DERM	4	0	0	0	0	1	0

Table 3. Summary of CHEGD species across the 25 sites

						SITE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Clavariaceae	25	157.9%	6.32%	10.94%	2.69%		6.47%	5.80%	7.23%	8.17%	6.95%	3.61%	7.23%	10.94%	4.29%	4.87%	2.69%	5.67%	7.37%	6.09%	5.95%	6.41%	3.06%	7.50%	5.98%	7.59%	7.13%	10.34%	5.36%	7.37%	3.82%
Hygrophoraceae	25	293.4%	11.73%	25.18%	2.47%		20.13%	13.19%	3.21%	12.68%	9.20%	2.47%	14.49%	9.71%	20.19%	12.60%	10.49%	25.18%	9.30%	11.08%	11.87%	16.25%	9.30%	13.40%	11.52%	14.91%	10.54%	10.65%	7.72%	5.64%	7.63%
Entolomataceae	25	15.1%	0.60%	1.56%	0.17%		0.87%	0.44%	0.61%	1.34%	0.65%	0.47%	0.66%	0.17%	0.53%	0.65%	0.41%	0.59%	0.55%	0.61%	0.26%	1.56%	0.70%	0.38%	0.21%	0.92%	0.22%	0.40%	0.78%	0.47%	0.60%
Geoglossaceae	25	99.7%	3.99%	20.81%	1.12%		1.80%	3.02%	4.67%	1.77%	3.46%	1.55%	2.32%	3.15%	3.84%	20.81%	3.95%	4.21%	2.67%	3.06%	5.00%	2.64%	5.03%	4.65%	1.61%	1.12%	6.35%	6.11%	2.46%	2.66%	1.78%
Microglossum	6	6.4%	1.07%	2.48%	0.02%		2.48%						0.02%	1.57%					0.03%							0.47%				1.87%	
Tricholomataceae	22	58.0%	2.64%	14.24%	0.00%		1.38%	0.01%	2.89%	0.00%			4.52%	4.85%	0.20%	1.49%	0.35%	0.03%	3.66%	3.73%	1.93%	1.96%		6.90%	0.09%	2.39%	14.24%	2.71%	0.93%	2.94%	0.76%
CHEGMD TOT	25	614.6%	24.59%	40.43%	8.10%		29.93%	22.45%	18.61%	23.96%	20.26%	8.10%	29.22%	29.21%	29.00%	40.43%	17.71%	35.66%	22.08%	23.33%	22.22%	27.26%	18.08%	32.84%	19.36%	25.82%	38.48%	30.21%	16.68%	19.40%	14.33%

\Species	IUCN gRDL	Site Count	Cum Tot	Mean	Max	Min	1131(12	1153(2)	335(3)	1324(3)	1404(3)	1470(2)	1526(4)	3095(4)	1655(4)	1702(1)	1914(4)	2127(3)	2137(10	23(6)	2391(7)	2536(5)	2885(2)	507(6)	3152(3)	403(6)	46(4)	5(3)	712(10)	797(6)	83(3)
Geoglossum barlae	ne	20	4.78%	0.24%	0.84%	0.01%	0.80%	0.07%		0.47%	0.84%	0.14%	0.10%	0.10%	0.03%		0.52%	0.39%	0.25%	0.14%		0.23%	0.06%		0.04%	0.01%		0.07%	0.12%	0.21%	0.20%
Geoglossum chamaecyparinum	ne	1	0.93%	0.93%	0.93%	0.93%																								0.93%	
Geoglossum fallax	ne	23	9.74%	0.42%	3.10%	0.01%	0.46%	0.05%	0.46%	0.10%	0.15%	0.33%	0.16%	0.06%	0.10%		0.63%	3.10%	0.46%	0.49%	0.41%	0.17%		0.16%	0.34%	0.17%	0.22%	0.28%	1.35%	0.07%	0.01%
Geoglossum glabrum	ne	5	0.65%	0.13%	0.56%	0.01%						0.02%			0.05%							0.56%								0.01%	0.02%
Geoglossum simile	ne	24	12.33%	0.51%	1.71%	0.00%	0.54%	0.65%	0.61%	0.09%	0.93%	0.18%	0.17%	0.50%	0.63%	1.32%	1.71%		0.44%	0.04%	0.27%	0.31%	1.40%	0.31%	0.00%	0.14%	0.30%	0.31%	0.04%	1.00%	0.43%
Geoglossum umbratile	ne	11	1.57%	0.14%	0.63%	0.00%	0.03%			0.25%				0.03%				0.63%	0.02%	0.20%		0.06%					0.13%	0.01%		0.21%	0.00%
Geoglossum variabilisporum	ne	1	0.01%	0.01%	0.01%	0.01%																								0.01%	
Glutinoglossum glutinosum	ne	25	8.89%	0.36%	0.88%	0.04%	0.21%	0.61%	0.08%	0.07%	0.04%	0.21%	0.12%	0.12%	0.25%	0.37%	0.77%	0.88%	0.17%	0.57%	0.36%	0.54%	0.11%	0.21%	0.33%	0.33%	0.55%	0.47%	0.79%	0.23%	0.48%
Glutinoglossum heptaseptatum	ne	7	3.68%	0.53%	1.82%	0.01%			0.10%				0.01%			1.82%	0.36%				0.71%			0.27%			0.41%				
Glutinoglossum orientale	ne	10	2.75%	0.28%	1.21%	0.00%		0.02%				0.50%					1.21%	0.02%	0.01%	0.55%		0.02%		0.16%	0.00%				0.26%		
Hemileucoglossum alveolatum	ne	23	4.11%	0.18%	0.65%	0.02%	0.09%	0.19%	0.02%	0.42%	0.56%	0.09%	0.28%	0.05%	0.04%	0.13%		0.14%	0.18%	0.36%	0.65%	0.02%	0.03%	0.08%	0.10%	0.05%	0.22%	0.13%		0.10%	0.20%
Leucoglossum leucosporum	ne	4	1.18%	0.30%	1.03%	0.02%			0.02%						0.02%												1.03%	0.12%			
Nothomitra cinnamomea	ne	1	0.04%	0.04%	0.04%	0.04%																	0.04%								
Trichoglossum hirsutum	ne	6	3.67%	0.61%	1.56%	0.07%	0.07%							0.18%						0.60%		1.56%						0.82%		0.44%	
Trichoglossum octopartitum	ne	11	3.47%	0.32%	1.63%	0.01%				0.11%	0.14%		0.07%		0.02%	1.63%	0.37%					0.49%	0.01%	0.02%			0.16%		0.45%		
Trichoglossum variabile	ne	18	11.60%	0.64%	2.90%	0.02%	0.06%	0.02%	0.08%		0.64%		0.55%	2.26%	0.40%				0.19%	1.05%	0.20%	0.39%		0.72%		0.03%	0.99%	2.90%	0.89%	0.05%	0.16%
Trichoglossum walteri	VU	22	15.32%	0.70%	2.27%	0.01%	0.76%	0.01%	0.41%	0.84%	0.69%	0.22%	0.45%	0.35%	1.34%		0.05%		1.22%	0.66%	2.18%	0.21%	0.68%	2.27%	0.75%	0.24%	0.85%	0.08%	0.18%	0.87%	
UNID. GEOGLOSSACEAE	x	25	45.98%	1.84%	15.54%	0.08%	0.37%	1.42%	3.42%	0.08%	1.53%	0.51%	1.20%	1.30%	1.68%	15.54%	2.08%	0.41%	0.65%	0.39%	2.75%	0.77%	2.71%	1.59%	0.25%	0.35%	3.58%	1.56%	0.48%	0.54%	0.84%
Microglossum nudipes	VU?	1	1.57%	1.57%	1.57%	1.57%								1.57%																	
Microglossum rufescensAFF1	VU?	4	2.06%	0.52%	1.26%	0.02%	1.26%						0.02%													0.47%				0.32%	
Microglossum truncatum	VU?	2	0.04%	0.02%	0.03%	0.02%													0.03%											0.02%	
Microglossum rufescens	VU?	1	1.54%	1.54%	1.54%	1.54%																								1.54%	
Camarophyllopsis atrovelutina	ne	25	16.82%	0.67%	2.92%	0.01%	0.74%	1.84%	0.46%	2.92%	0.42%	0.47%	0.47%	0.13%	0.16%	2.09%	0.16%	0.18%	0.65%	0.16%	0.35%	0.93%	0.25%	1.38%	0.08%	1.52%	0.92%	0.01%	0.16%	0.32%	0.09%
Camarophyllopsis hymenocephal	ne	1	0.02%	0.02%	0.02%	0.02%																								0.02%	
Camarophyllopsis schulzeri	[VU]	18	9.00%	0.50%	2.24%	0.02%	0.70%	0.05%		0.76%	0.56%	0.43%	0.02%	0.04%				1.72%	1.01%		0.72%	0.04%	0.02%	2.24%	0.03%	0.26%			0.03%	0.18%	0.19%
Clavaria amoenoides	ne	1	0.01%	0.01%	0.01%	0.01%														0.01%											
Clavaria acuta	ne	23	2.76%	0.12%	0.56%	0.00%	0.18%	0.02%	0.00%	0.27%	0.46%	0.07%	0.18%	0.05%	0.03%			0.16%	0.15%	0.08%	0.02%	0.56%	0.11%	0.03%	0.00%	0.02%	0.16%	0.01%	0.06%	0.08%	0.03%
Clavaria flavipes	ne	25	6.37%	0.25%	0.65%	0.01%	0.17%	0.14%	0.13%	0.51%	0.39%	0.21%	0.33%	0.11%	0.12%	0.12%	0.19%	0.05%	0.43%	0.17%	0.65%	0.26%	0.29%	0.34%	0.01%	0.22%	0.58%	0.11%	0.20%	0.42%	0.23%
Clavaria fragilis	ne	9	2.91%	0.32%	1.66%	0.00%								0.05%	0.04%		1.66%		0.43%	0.10%		0.00%			0.00%				0.01%	0.61%	
Clavaria fumosa	ne	8	2.60%	0.33%	0.93%	0.01%	0.17%			0.93%			0.10%					0.06%	0.84%	0.22%						0.28%			0.01%		
Clavaria griseobrunnea	ne	9	0.29%	0.03%	0.10%	0.01%							0.01%					0.02%	0.01%	0.02%	0.04%					0.01%			0.07%	0.01%	0.10%
Clavaria incarnata	ne	20	1.58%	0.08%	0.35%	0.01%	0.07%		0.04%	0.10%	0.01%		0.19%	0.04%	0.14%	0.01%	0.01%	0.05%	0.08%	0.07%	0.35%	0.02%	0.01%		0.12%	0.09%	0.11%		0.05%	0.03%	
Clavaria macounii	nd	1	0.01%	0.01%	0.01%	0.01%	0.01%																								
Clavaria neonigrita	nd	2	0.09%	0.05%	0.08%	0.01%																						0.01%		0.08%	
Clavaria pullei	nd	3	0.14%	0.05%	0.10%	0.01%		0.01%																				0.03%		0.10%	
Clavaria redoleoalii	nd	1	0.03%	0.03%	0.03%	0.03%																								0.03%	
Clavaria rosea	ne	8	0.82%	0.10%	0.59%	0.01%		0.08%	0.02%		0.03%															0.01%	0.59%	0.03%		0.01%	0.04%
Clavaria subacuta	ne	1	0.03%	0.03%	0.03%	0.03%	0.03%																								
Clavaria tyrrhenica	ne	12	0.58%	0.05%	0.18%	0.01%		0.11%	0.02%		0.06%			0.01%	0.05%		0.06%				0.18%	0.01%					0.01%	0.02%	0.01%		0.03%
Clavaria zollingeri	VU	4	2.98%	0.74%	2.66%	0.01%				2.66%										0.01%									0.03%		0.28%
Clavulinopsis corniculata	ne	22	15.51%	0.70%	2.07%	0.03%	0.57%		1.18%	0.40%	2.07%	0.75%	0.12%	1.64%	1.03%	0.12%	0.93%		0.36%	1.14%	1.37%	0.58%	0.03%	0.06%		0.27%	0.32%	0.97%	0.25%	0.73%	0.62%
Clavulinopsis gracillima	ne	21	8.20%	0.39%	1.22%	0.02%	0.42%	0.02%	0.21%	0.09%	0.95%		0.23%	0.85%	0.33%		0.27%		0.29%	0.49%	0.35%	0.67%	0.05%	0.23%		0.07%	1.22%	0.72%	0.18%	0.47%	0.10%
Clavulinopsis helvola	ne	24	20.57%	0.86%	4.10%	0.10%	1.40%	0.90%	0.20%	0.42%	0.44%	0.11%	0.89%	0.46%	0.38%		0.53%	1.31%	0.75%	1.22%	0.57%	0.32%	0.10%	1.70%	4.10%	1.35%	0.20%	0.89%	1.56%	0.30%	0.46%
Clavulinopsis hisingeri	ne	5	0.24%	0.05%	0.12%	0.00%	0.03%							0.00%								0.12%						0.02%		0.06%	
Clavulinopsis laeticolor	ne	24	13.57%	0.57%	1.91%	0.01%	0.29%	0.64%	0.69%	0.09%	0.11%	0.18%	0.32%	1.61%	0.45%	0.23%	0.14%		0.33%	0.87%	1.80%	0.58%	0.07%	0.56%	0.16%	0.32%	0.28%	0.88%	1.91%	1.07%	0.01%
Clavulinopsis luteoalba	ne	22	7.83%	0.36%	1.10%	0.03%	0.71%	0.21%	0.09%	0.11%	0.03%	0.41%	0.14%	0.19%				0.74%	0.20%	0.61%	0.36%	0.36%	0.05%	0.30%	1.10%	1.04%	0.03%	0.14%	0.72%	0.12%	0.18%
Clavulinopsis luteonana	ne	13	0.44%	0.03%	0.09%	0.00%	0.04%	0.01%			0.04%		0.00%	0.04%			0.01%		0.09%	0.04%		0.07%			0.00%	0.01%				0.07%	0.00%
Clavulinopsis umbrinella	ne	17	14.75%	0.87%	4.06%	0.01%	1.15%	0.07%			2.02%	0.11%	4.06%	2.84%			0.02%	0.20%	0.49%	0.28%	0.01%	0.84%	0.20%			1.43%			0.84%	0.12%	0.06%
Hodophilus atropunctus	ne	5	0.74%	0.15%	0.35%	0.06%								0.06%			0.08%							0.09%		0.15%	0.35%				
Hodophilus foetens	ne	16	6.26%	0.39%	1.97%	0.01%	0.78%		0.08%	0.01%	0.04%		1.22%	0.05%			0.02%		0.06%	0.36%		0.03%				0.01%	0.06%	1.97%	0.09%	1.03%	0.44%
Hodophilus micaceus	ne	18	3.36%	0.19%	1.36%	0.01%	0.10%	1.36%	0.01%	0.05%	0.03%		0.06%	0.05%	0.08%			0.37%	0.14%	0.15%	0.26%	0.03%				0.24%		0.13%	0.11%	0.07%	0.12%
Lamelloclavaria petersenii	ne	15	8.46%	0.56%	2.67%	0.00%	0.38%			0.02%	0.13%	0.12%	1.05%		0.06%		0.02%		0.55%			0.70%	0.01%		0.00%			2.67%	1.74%	1.01%	0.01%
Ramariopsis asperulospora	ne	2	0.62%	0.31%	0.61%	0.01%													0.01%									0.61%			
Ramariopsis kunzei	ne	6	0.58%	0.10%	0.44%	0.01%	0.01%													0.44%					0.01%			0.01%	0.03%	0.07%	
Ramariopsis pulchella	ne	2	0.06%	0.03%	0.05%	0.01%								0.01%																0.05%	
Ramariopsis subtilis	ne	24	2.31%	0.10%	0.28%	0.01%	0.05%	0.05%	0.05%	0.05%	0.13%	0.15%	0.05%	0.07%	0.07%		0.02%	0.07%	0.08%	0.07%	0.05%	0.26%	0.01%	0.12%	0.14%	0.04%	0.04%	0.13%	0.25%	0.28%	0.11%
UNID. CLAVARIACEAE	x	25	53.67%	2.15%	5.06%	0.43%	1.27%	1.15%	4.23%	1.05%	1.84%	1.43%	2.29%	5.06%	1.83%	2.31%	1.15%	1.27%	2.95%	1.88%	2.36%	2.02%	2.01%	2.79%	0.43%	1.41%	4.05%	2.64%	1.69%	2.81%	1.77%
Alboleptonia sericella	nd	2	0.04%	0.02%	0.02%	0.02%		0.02%																						0.02%	
Clitopilus hobsonii	nd	3	0.04%	0.01%	0.03%	0.01%								0.01%					0.01%											0.03%	
Clitopilus passeckerianus	nd	4	0.06%	0.02%	0.03%	0.01%								0.02%			0.03%				0.01%						0.01%				
Clitopilus scyphoides	nd	1	0.03%	0.03%	0.03%	0.03%																0.03%									
Entocybe nitida	nd	6	0.04%	0.01%	0.01%	0.00%							0.01%						0.01%	0.01%					0.00%	0.01%			0.01%		
Entoloma asprellum	nd	16	1.38%	0.09%	0.42%	0.01%	0.11%	0.01%		0.16%	0.03%	0.08%	0.42%					0.01%	0.04%	0.01%		0.04%	0.05%			0.20%		0.03%	0.04%	0.07%	0.09%
Entoloma bloxamii	VU	4	0.26%	0.06%	0.18%	0.02%	0.04%			0.18%								0.03%				0.02%									
Entoloma byssisedum	nd	21	0.28%	0.01%	0.02%	0.00%	0.01%		0.01%	0.01%	0.02%	0.02%	0.01%	0.01%	0.01%		0.02%		0.01%	0.01%	0.01%	0.02%	0.00%		0.00%	0.01%	0.01%	0.02%	0.02%	0.01%	0.02%
Entoloma caesiocinctum	nd	1	0.02%	0.02%	0.02%	0.02%																							0.02%		
Entoloma calongeAFF	nd	4	0.45%	0.11%	0.30%	0.00%			0.14%												0.30%						0.01%		0.00%		
Entoloma catalaunicum	nd	2	0.05%	0.03%	0.05%	0.00%	0.05%																							0.00%	
Entoloma cetratum	nd	12	0.20%	0.02%	0.06%	0.00%	0.02%						0.01%					0.06%	0.01%	0.02%		0.01%	0.00%	0.01%	0.00%	0.02%			0.01%		0.02%

Table 3. (cont.)

Entoloma chalybeum	nd	10	0.23%	0.02%	0.09%	0.00%	0.01%				0.05%								0.02%		0.01%				0.03%	0.01%			0.01%				0.09%	0.00%	0.02%			
Entoloma clandestinum	nd	19	2.36%	0.12%	1.08%	0.00%	0.01%	0.03%	0.00%	0.11%	0.02%		0.24%	0.07%	0.62%		0.01%		0.03%		0.01%	1.08%	0.01%	0.00%		0.01%		0.01%	0.02%	0.02%	0.04%						
Entoloma conferendum	nd	24	1.35%	0.06%	0.10%	0.01%	0.04%	0.10%	0.02%	0.05%	0.08%	0.10%	0.05%	0.01%	0.06%		0.02%	0.06%	0.05%	0.04%	0.03%	0.08%	0.08%	0.07%	0.10%	0.10%	0.01%	0.03%	0.09%	0.03%	0.04%						
Entoloma cuspidiferum	nd	1	0.17%	0.17%	0.17%	0.17%													0.17%																		
Entoloma dysthales	nd	20	0.40%	0.02%	0.07%	0.00%	0.01%	0.01%	0.00%		0.05%		0.02%	0.02%	0.04%			0.01%	0.01%	0.01%	0.07%	0.02%	0.01%	0.00%	0.01%	0.04%	0.01%	0.01%	0.02%		0.01%						
Entoloma exile	nd	10	0.72%	0.07%	0.36%	0.00%	0.02%	0.01%		0.02%	0.01%											0.03%	0.01%		0.01%				0.36%	0.23%	0.00%						
Entoloma griseocyaneum	VU	10	0.31%	0.03%	0.12%	0.00%	0.06%			0.01%			0.01%						0.01%	0.12%	0.01%	0.01%			0.00%			0.01%		0.06%						
Entoloma henricii	[VU]	10	0.76%	0.08%	0.17%	0.00%		0.04%			0.01%	0.12%	0.17%		0.01%				0.10%			0.13%				0.10%				0.09%	0.00%					
Entoloma hirtipes	nd	4	0.03%	0.01%	0.01%	0.00%													0.01%			0.01%	0.00%					0.01%								
Entoloma lividocyanulum	nd	12	0.47%	0.04%	0.10%	0.00%	0.09%	0.01%	0.01%		0.10%						0.05%		0.08%			0.03%	0.02%	0.00%		0.06%			0.01%		0.01%					
Entoloma madidum	VU?	1	0.04%	0.04%	0.04%	0.04%	0.04%																														
Entoloma neglectum	[VU]	1	0.00%	0.00%	0.00%	0.00%																	0.00%													
Entoloma ochreoprunuloides	nd	6	4.24%	0.71%	1.62%	0.09%	0.60%			0.09%			0.68%					1.09%		1.62%										0.16%							
Entoloma piceinum	nd	9	0.06%	0.01%	0.01%	0.00%	0.01%						0.00%						0.01%			0.01%		0.00%	0.01%	0.01%			0.01%		0.00%					
Entoloma pleopodium	nd	13	0.78%	0.06%	0.45%	0.00%	0.01%	0.01%		0.06%	0.08%		0.02%				0.45%	0.05%		0.01%		0.02%	0.02%			0.01%			0.04%		0.00%					
Entoloma poliopus	nd	12	0.36%	0.03%	0.08%	0.00%	0.01%	0.03%		0.02%								0.01%	0.03%	0.02%		0.03%			0.00%	0.07%			0.08%	0.06%	0.01%					
Entoloma porphyrophaeum	VU	2	0.04%	0.02%	0.04%	0.00%				0.04%														0.00%												
Entoloma proterum	nd	12	0.12%	0.01%	0.02%	0.00%	0.01%	0.01%		0.01%	0.01%							0.01%			0.02%	0.01%	0.01%	0.01%	0.00%	0.01%			0.01%								
Entoloma prunuloides	VU	9	1.32%	0.15%	0.45%	0.01%	0.24%	0.04%		0.45%		0.05%	0.06%									0.13%	0.01%						0.31%		0.02%						
Entoloma pseudocoelestinum	nd	16	0.38%	0.02%	0.10%	0.01%	0.02%	0.01%	0.03%	0.01%	0.03%								0.03%	0.02%	0.01%	0.01%	0.10%		0.02%	0.02%	0.02%		0.01%	0.01%	0.05%					
Entoloma rhombisporum	nd	13	0.27%	0.02%	0.06%	0.00%				0.01%					0.02%		0.05%		0.01%		0.02%	0.03%	0.04%	0.01%	0.00%	0.01%			0.06%	0.02%	0.01%					
Entoloma saccharioleus	nd	16	0.62%	0.04%	0.27%	0.00%	0.05%	0.01%	0.02%	0.01%	0.27%	0.04%	0.02%		0.03%				0.04%			0.06%	0.02%	0.00%		0.01%		0.02%		0.02%	0.02%					
Entoloma serrulatum	nd	6	0.27%	0.04%	0.09%	0.01%	0.09%												0.03%	0.09%		0.02%						0.01%		0.04%						
Entoloma setastipes	nd	13	0.66%	0.05%	0.22%	0.00%	0.05%		0.05%	0.22%	0.01%			0.06%			0.01%		0.01%				0.00%			0.20%		0.01%	0.02%	0.02%	0.00%					
Entoloma turbida	nd	11	1.20%	0.11%	0.55%	0.02%	0.15%				0.03%		0.02%					0.04%	0.08%	0.10%		0.07%			0.02%	0.55%			0.11%		0.02%					
UNID. ENTOLOMATACEAE	x	25	4.98%	0.20%	0.65%	0.04%	0.16%	0.16%	0.08%	0.15%	0.20%	0.17%	0.13%	0.07%	0.26%	0.65%	0.23%	0.06%	0.22%	0.20%	0.06%	0.29%	0.35%	0.31%	0.04%	0.07%	0.13%	0.19%	0.36%	0.20%	0.23%						
Cuphophyllus angustifolius	nd	3	0.77%	0.26%	0.56%	0.00%			0.56%																				0.20%		0.00%						
Cuphophyllus aurantius	nd	4	1.76%	0.44%	1.07%	0.02%	1.07%												0.20%							0.02%			0.48%							
Cuphophyllus colemannianus	nd	2	0.02%	0.01%	0.01%	0.01%																						0.01%		0.01%						
Cuphophyllus flavipesoides	nd	3	3.73%	1.24%	2.52%	0.11%	1.10%																	2.52%	0.11%											
Cuphophyllus fornicatus	nd	5	8.63%	1.73%	3.64%	0.12%													0.12%			3.64%					2.73%	0.63%		1.51%						
Cuphophyllus lacmus	VU	3	10.51%	3.50%	6.09%	1.75%																		6.09%		2.67%			1.75%							
Cuphophyllus pratensis	nd	25	50.42%	2.02%	6.21%	0.01%	6.21%	3.06%	0.03%	2.76%	0.76%	0.99%	4.98%	0.86%	0.01%	0.22%	0.43%	4.55%	3.05%	2.30%	3.45%	4.15%	0.51%	0.45%	2.34%	3.48%	0.99%	0.92%	2.42%	1.49%	0.01%						
Cuphophyllus virgineus	nd	24	37.66%	1.57%	5.04%	0.00%	1.67%	0.82%	0.60%		3.47%	0.14%	0.85%	1.82%	1.26%	3.20%	5.04%	0.04%	0.78%	4.23%	3.55%	1.04%	0.03%	1.79%	0.00%	0.16%	2.50%	2.17%	0.50%	1.57%	0.43%						
Gliophorus europerplexus	VU	1	0.64%	0.64%	0.64%	0.64%													0.64%																		
Gliophorus irrigatus	nd	18	8.50%	0.47%	2.57%	0.01%	0.61%	0.01%		0.21%	0.08%		0.05%				0.35%	2.57%	0.25%	0.55%	0.35%	0.06%	0.04%	0.40%	0.80%	0.48%	0.26%		0.90%		0.53%						
Gliophorus laetus	nd	14	18.38%	1.31%	4.99%	0.02%	0.93%				0.02%	1.99%	0.69%				0.28%	2.70%	1.69%	0.40%		4.99%		0.88%	0.22%	2.10%			1.21%		0.30%						
Gliophorus psittacinus	nd	22	45.65%	2.07%	6.33%	0.01%	2.71%	1.35%			0.91%	0.03%	3.92%	4.72%	1.09%	0.01%	6.33%	4.59%	0.92%	0.97%	1.72%	2.99%	2.26%	4.02%	1.23%	1.97%	1.32%		2.16%	0.39%	0.03%						
Gliophorus reginae	VU	9	2.49%	0.28%	0.87%	0.00%			0.00%				0.69%						0.68%	0.03%				0.87%		0.06%	0.01%		0.01%		0.15%						
Gliophorus sp:AR2018a	nd	2	0.03%	0.02%	0.03%	0.01%							0.03%						0.01%																		
Gloioxanthomyces vitellinus	EN	2	0.09%	0.05%	0.05%	0.04%	0.04%																						0.05%							
Hygrocybe acutoconica	nd	3	2.65%	0.88%	2.60%	0.01%		0.01%												0.04%		2.60%														
Hygrocybe aurantiosplendens	[VU]	4	4.31%	1.08%	2.13%	0.02%								1.08%						1.09%								2.13%		0.02%						
Hygrocybe cantharellus	nd	16	7.07%	0.44%	1.79%	0.01%	0.30%	0.01%		0.67%	0.01%	0.66%	0.14%					1.79%	0.22%	0.20%		0.33%	0.14%		0.04%	0.12%			0.73%	1.64%	0.07%						
Hygrocybe ceracea	nd	7	5.62%	0.80%	1.65%	0.24%	0.48%	1.65%										0.24%						0.81%	0.47%	0.77%			1.21%							
Hygrocybe chlorophana	nd	16	28.57%	1.79%	4.29%	0.01%	3.25%	0.15%		0.01%	2.42%		1.46%		4.29%			2.27%	1.94%	1.42%	3.11%		2.73%	0.05%	2.01%	0.93%				1.08%	1.44%						
Hygrocybe citrinovirens	VU	11	7.50%	0.68%	1.86%	0.03%	0.19%		1.86%				1.05%		0.49%				1.30%	0.03%	0.13%			0.07%		0.11%				1.61%	0.66%						
Hygrocybe coccinea	nd	18	30.22%	1.68%	5.07%	0.02%	5.07%	0.20%		4.97%	0.09%		0.61%	0.95%		0.02%		3.04%	2.09%	0.03%		0.45%		1.04%	1.95%	3.23%		1.43%	2.13%	0.43%	2.49%						
Hygrocybe conica	nd	23	67.13%	2.92%	16.94%	0.01%	0.70%	0.64%	0.04%	0.45%	4.10%		3.21%	2.58%	16.94%	9.06%	1.69%	0.94%	0.25%	2.22%	1.55%	6.90%	4.58%	0.63%		0.01%	4.32%	1.59%	0.90%	0.21%	3.64%						
Hygrocybe constrictospora AFF	nd	5	3.71%	0.74%	1.66%	0.03%		0.03%										0.48%		0.50%	1.66%							1.04%								
Hygrocybe glutinipes	nd	24	24.01%	1.00%	3.25%	0.06%	0.73%	1.09%	0.27%	0.22%	0.32%	0.85%	2.51%	0.99%	0.75%	0.10%	1.45%	2.28%	1.56%	0.63%	1.52%	0.48%	0.36%	3.25%	1.02%	0.63%	0.06%	0.22%	0.09%		2.63%						
Hygrocybe helobia	[NT]	8	7.42%	0.93%	2.80%	0.05%	0.42%			0.70%								2.05%		0.15%		1.04%			2.80%				0.21%		0.05%						
Hygrocybe insipida	nd	17	4.66%	0.27%	1.18%	0.01%	0.54%			0.06%	0.01%		0.01%	0.02%			0.11%	0.08%	0.24%	0.54%		0.38%		1.18%	0.32%	0.46%		0.04%	0.25%	0.40%	0.02%						
Hygrocybe intermedia	[VU]	4	5.98%	1.50%	3.24%	0.41%												3.24%	0.80%									1.53%		0.41%						
Hygrocybe miniata	nd	10	3.01%	0.30%	1.22%	0.00%	0.42%				0.01%							0.45%	0.02%	1.22%		0.47%			0.00%	0.01%			0.41%		0.00%						
Hygrocybe mucronella	[VU]	9	1.61%	0.18%	0.49%	0.01%	0.29%			0.41%									0.01%	0.25%		0.01%			0.49%			0.04%	0.03%	0.09%						
Hygrocybe phaeococcinea	[VU]	6	0.15%	0.03%	0.08%	0.01%	0.01%	0.01%		0.08%			0.03%						0.02%				0.02%														
Hygrocybe punicea	VU	6	9.73%	1.62%	2.85%	0.27%	2.31%			1.40%	0.27%			0.74%							2.85%				2.17%												
Hygrocybe quieta	[VU]	18	33.28%	1.85%	5.39%	0.01%	2.75%	5.39%	0.11%	2.90%	0.22%		0.13%	1.69%	0.01%		0.72%	0.09%	1.54%	3.60%	0.33%					4.20%	5.18%	3.57%	0.80%	0.04%							
Hygrocybe reidii	[DD]	14	12.30%	0.88%	2.60%	0.02%	2.60%				0.02%	0.08%	0.97%				0.48%	1.58%	0.15%		0.96%	0.78%			0.24%	0.55%			2.02%	1.85%	0.02%						
Hygrocybe russocoriacea	nd	4	3.71%	0.93%	2.77%	0.27%				0.38%	2.77%								0.30%											0.27%							
Hygrocybe spadicea	VU	1	1.34%	1.34%	1.34%	1.34%																								1.34%						
Hygrocybe splendidissima	VU	1	0.54%	0.54%	0.54%	0.54%													0.54%																		
Hygrocybe subpapillata	[VU]	6	2.76%	0.46%	1.76%	0.01%	0.23%						0.29%									0.01%	0.01%	1.76%					0.46%								
Neohygrocybe ingrata	VU	4	5.20%	1.30%	2.33%	0.39%												2.33%	1.58%							0.89%				0.39%						
Neohygrocybe nitrata	VU	3	1.34%	0.45%	1.19%	0.03%	1.19%						0.12%												0.03%												
Porpolomopsis calyptriformis	VU	15	5.77%	0.38%	2.19%	0.01%	0.06%	0.11%			0.26%	0.04%	0.34%		0.01%			0.08%	0.56%	0.10%	0.31%	0.03%		0.53%		2.19%			1.12%		0.02%						
UNID. HYGROPHORACEAE	x	22	7.42%	0.34%	3.05%	0.01%	0.40%	0.16%	0.28%	0.16%	0.05%	0.15%	0.02%	0.01%	0.10%			0.18%	0.37%	0.44%	3.05%	0.30%	0.03%	0.06%		0.03%	0.01%	1.44%	0.05%	0.09%	0.03%						
Dermoloma cuneifolium	nd	11	17.64%	1.60%	6.09%	0.01%	2.10%		0.74%				0.03%	2.08%	0.01%					3.61%				0.23%		6.09%	1.60%	0.20%		0.95%						
Dermoloma josserandii	nd	2	0.24%	0.12%	0.23%	0.01%								0.23%																0.01%						
Dermoloma magicum	[VU]	22	57.50%	2.61%	18.44%	0.00%	0.65%	0.01%	2.15%	0.00%			6.01%	3.33%	0.30%	1.49%	0.35%	0.03%	4.31%	2.06%	1.93%	1.98%		6.82%	0.09%	0.53%	18.44%	2.64%	0.93%	2.68%	0.76%						
Dermoloma pseudocuneifolium	nd	6	1.55%	0.26%	1.03%	0.00%			0.00%					0.04%					0.44%								0.03%	0.00%		1.03%						
UNID. DERMOLOMA	x	2	0.55%	0.28%	0.38%	0.17%								0.17%																0.38%						

SPECIES COUNTS																																				
Clavariaceae (/34)	21	15	14	16	18	11	18	20	13	5	15	12	20	20	15	19	13	11	13	19	14	19	21	26	19											
Hygrophoraceae (/41)	26	15	8	14	17	8	20	10	9	6	10	20	27	21	13	18	10	17	18	21	9	13	23	19	18											
Entolomataceae (/37)	24	14	9	18	14	6	15	7	7	0	9	10	22	13	10	24	18	10	13	19	6	9	22	18	19											
Geoglossaceae (/17)	9	8	8	8	8	8	9	9	10	5	8	6	9	10	7	12	7	9	7	7	10	10	8	12	8											
Microglossum (/4)	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	3	0											
Dermoloma	2	1	3	1	0	0	2	4	2	1	1	1	2	2	1	1	0	2	1	2	3	3	1	4	1											
CHEGD TOT	83	53	42	57	57	33	65	51	41	17	43	49	81	66	46	74	48	49	52	69	42	54	75	82	65											
CHEGD RANK ORDER	1st	13th	22nd	11th	10th	24th	9th	15th	23rd	25th	20th	17th	3rd	7th	19th	5th	18th	16th	14th	6th	21st	12th	4th	2nd	8th											

In the case of *Microglossum* spp., (green earthtongues) a number of new species have been named in the past decade, notably by Kučera et al. (2017). In a project funded by Natural England we have been undertaking DNA barcoding and phylogenetic and ecological studies using samples collected across the UK. Previously these species were grouped amorphously as *M. olivaceum* agg. (aggregate) but it is now clear that 12 species are found in the UK, of which seven require some taxonomic clarification (work in progress). In the case of the four species discovered here, it may be necessary to split *M. rufescens* into two species (including the species "*Microglossum rufescens* AFF1" detected in this study; we have ca. 10 vouchers of this species from across the UK), whilst the *M. nudipes* is in need of epitypification (i.e. there is no valid type specimen). From a legal perspective, it is not clear whether all of these species merit the same level of protection as *M. olivaceum* (which also needs epitypification) and *Thuemenidium atropurpureum* (needs renaming to *Microglossum* and comprises two cryptic species). Both these 'species' are named in the Natural Environment and Rural Communities (NERC) Act 2006 (Section 41: Species of Principal Importance in England), are listed as VU (vulnerable) on the IUCN Global Fungal Red List and as Priority Fungi Species (including lichens) (2007) in UK BAP. A similar position pertains to *Entoloma bloxamii* (also a Section 41 and IUCN VU species) which was also taxonomically complex, with several new species recently named from this species complex (Ainsworth et al., 2018), including *E. madidum* found in this study.

Table 5. Known distribution of CHEGD species and conservation status

Cuphophyllus aurantius and *Cuphophyllus angustifolius* are two poorly studied species, known only from the USA, and both related to *C. pratensis* (Lodge et al., 2014). Both species were each found at four different sites (no overlap), and in moderate abundance (>1% of all fungi at two sites). It is possible that these species were overlooked due to their superficial similarity to *C. pratensis* and *C. virgineus* respectively. A novel and unnamed species "*Gliophorus* sp:AR2018a" is close to *G. irrigatus* and known from one barcoded fruitbody (white in colour) and one eDNA sequence. A species closely related to this (possibly the same species) was recently discovered in Pembrokeshire, and we are preparing a manuscript to name this new taxon at present.

One species found in this study (*Gloioxanthomyces vitellinus*), found in three quadrats is listed as EN (endangered) on the IUCN Global Fungal Red List (same level of extinction threat as the blue whale). A further 10 species are listed as VU (vulnerable) (same level of extinction threat as the giant panda and snow leopard), with a further 16 assessed as VU but not yet published (a total of 16 waxcaps). Furthermore, taxonomic revision of the *Entoloma bloxamii* species complex has resulted in the naming of several new species, one of which (*E. madidum*) was found here. We are currently revising the *Microglossum olivaceum* species complex; whilst *M. olivaceum* was not found, four other species were (two are not yet formally named). In both cases since the species complex is listed as VU, the individual compnents are equally/more rare. Thus the true total of VU species would be 31.

Additionally, a subset of these species (*Entoloma bloxamii* species complex, *Hygrocybe spadicea*, *Microglossum* spp.) are also listed in Section 41 of the NERC Act 2016. Note that for several species (bracketed in Table 5; e.g. [VU]), IUCN assessment is complete but not yet formally published. Of the 116 quadrats studied here, 114 have at least one species listed as VU on the IUCN Global Fungal Red List and 19 quadrats have six or more such species (and one quadrat had 9) (App4). The Global fungal Red List is a relatively recent project (Mueller et al., 2014) but is already having a huge positive effect on global fungal conservation, not least because it provides a more objective and more effective/meaningful alternative to National Red Lists which are highly inconsistent and often very out of date. It also provides a more accurate

Table 5. Known distribution of CHEGD species and conservation status

(IUCN data from iucn.ekoo.se/ and GBIF data from www.gbif.org/)

Species	Count (No .Q)	Cum. Total	Mean (per Q)	Max (per Q)	Min (per Q)	Confirm	IUCN Global Redlist	GBIF distribution	Notes
Geoglossum barlae	59	18.37%	0.31%	3.94%	0.002%	OK	ne		
Geoglossum chamaecyparinum	1	0.93%	0.93%	0.93%	0.926%	CHECK	ne	Non-UK (present in S/E Europe)	New sp. from 2014
Geoglossum fallax	71	36.25%	0.51%	3.96%	0.002%	OK	ne		
Geoglossum glabrum	6	0.67%	0.11%	0.56%	0.002%	OK	ne		
Geoglossum simile	62	29.22%	0.47%	2.87%	0.005%	OK	ne		
Geoglossum umbratile	21	2.77%	0.13%	0.63%	0.002%	OK	ne		
Geoglossum variabilisporum	1	0.01%	0.01%	0.01%	0.007%	CHECK	ne	Non-UK (present in S/E Europe)	New sp. from 2014
Glutinoglossum glutinosum	92	33.34%	0.36%	2.09%	0.007%	OK	ne		
Glutinoglossum heptaseptatum	11	5.31%	0.48%	1.82%	0.006%	CHECK	ne	Non-UK (present in NW Europe)	
Glutinoglossum orientale	16	4.16%	0.26%	1.21%	0.002%	CHECK	ne	Non-UK (Not Europe)	
Hemileucoglossum alveolatum	59	10.84%	0.18%	2.41%	0.003%	CHECK	ne	Non-UK (Not Europe)	
Leucoglossum leucosporum	5	1.30%	0.26%	1.03%	0.016%	CHECK	ne	Non-UK (present in E Europe)	
Nothomitra cinnamomea	1	0.04%	0.04%	0.04%	0.040%	CHECK	ne	Non-UK (present in E Europe)	
Trichoglossum hirsutum	14	6.47%	0.46%	1.56%	0.042%	OK	ne		
Trichoglossum octopartitum	13	4.00%	0.31%	1.63%	0.012%	OK	ne		
Trichoglossum variabile	36	26.01%	0.72%	4.70%	0.005%	OK	ne		
Trichoglossum walteri	65	59.32%	0.91%	4.53%	0.009%	OK	VU		
UNID GEOGLOSSACEAE	**109**	**140.34%**	**1.29%**	**15.54%**	**0.001%**	**NonID**	x		
Microglossum nudipes	1	1.57%	1.57%	1.57%	1.567%	OK	VU?	NERC/S41? (UK BAP?)	
Microglossum rufescensAFF1 (New sp.?)	5	3.32%	0.66%	2.48%	0.015%	OK	VU?	NERC/S41? (UK BAP?)	To be named
Microglossum truncatum	2	0.04%	0.02%	0.03%	0.016%	OK	VU?	NERC/S41? (UK BAP?)	
Microglossum rufescens	1	1.54%	1.54%	1.54%	1.535%	OK	VU?	NERC/S41? (UK BAP?)	
Camarophyllopsis atrovelutina	88	61.17%	0.70%	4.54%	0.004%	OK	ne		
Camarophyllopsis hymenocephala	1	0.02%	0.02%	0.02%	0.024%	OK	ne		
Camarophyllopsis schulzeri	46	29.33%	0.64%	4.48%	0.002%	OK	[VU]		
Clavaria amoenoides	1	0.01%	0.01%	0.01%	0.008%	OK	ne		
Clavaria acuta	70	10.41%	0.15%	1.80%	0.002%	OK	ne		
Clavaria flavipes	104	28.66%	0.28%	1.30%	0.006%	OK	ne		
Clavaria fragilis	14	3.77%	0.27%	1.66%	0.002%	OK	ne		
Clavaria fumosa	21	7.33%	0.35%	2.13%	0.006%	OK	ne		
Clavaria griseobrunnea	9	0.29%	0.03%	0.10%	0.006%	OK	ne		
Clavaria incarnata	51	4.56%	0.09%	0.58%	0.002%	OK	ne		
Clavaria macounii	1	0.01%	0.01%	0.01%	0.014%	CHECK	nd	Non-UK (present in NW Europe)?	
Clavaria neonigrita	4	0.18%	0.05%	0.14%	0.008%	CHECK	nd	Non-UK (Not Europe)	
Clavaria pullei	3	0.14%	0.05%	0.10%	0.011%	CHECK	nd	Non-UK? (present in NW Europe)	
Clavaria redoleoalii	1	0.03%	0.03%	0.03%	0.028%	CHECK	nd	Non-UK (Not Europe)	
Clavaria rosea	9	0.85%	0.09%	0.59%	0.010%	OK	ne		
Clavaria subacuta	1	0.03%	0.03%	0.03%	0.027%	CHECK	ne	Non-UK (Not Europe)	
Clavaria tyrrhenica	18	0.69%	0.04%	0.18%	0.002%	CHECK	ne	Non-UK? (present in S/E Europe	New sp. from 2017
Clavaria zollingeri	4	2.98%	0.74%	2.66%	0.012%	OK	VU		
Clavulinopsis corniculata	67	50.99%	0.76%	4.10%	0.005%	OK	ne		
Clavulinopsis gracillima	63	25.77%	0.41%	3.61%	0.010%	OK	ne		
Clavulinopsis helvola	95	95.83%	1.01%	6.43%	0.010%	OK	ne		
Clavulinopsis hisingeri	9	0.51%	0.06%	0.23%	0.002%	CHECK	ne	Non-UK? (present in NW Europe)	
Clavulinopsis laeticolor	82	51.70%	0.63%	4.03%	0.002%	OK	ne		
Clavulinopsis luteoalba	86	37.94%	0.44%	3.03%	0.005%	OK	ne		
Clavulinopsis luteonana	21	0.90%	0.04%	0.16%	0.004%	CHECK	ne	Non-UK? (present in NW Europe)	
Clavulinopsis umbrinella	45	42.61%	0.95%	5.43%	0.007%	OK	ne		
Hodophilus atropunctus	6	0.89%	0.15%	0.35%	0.014%	OK	ne		
Hodophilus foetens	22	10.58%	0.48%	3.35%	0.007%	OK	ne		
Hodophilus micaceus	42	6.42%	0.15%	1.36%	0.004%	OK	ne		
Lamelloclavaria petersenii	27	16.80%	0.62%	4.76%	0.002%	CHECK	ne	Non-UK? (present in NW Europe)	
Ramariopsis asperulospora	4	1.84%	0.46%	1.27%	0.009%	OK	ne		
Ramariopsis kunzei	7	0.59%	0.08%	0.44%	0.008%	OK	ne		
Ramariopsis pulchella	2	0.06%	0.03%	0.05%	0.008%	OK	ne		
Ramariopsis subtilis	88	9.23%	0.10%	0.55%	0.006%	OK	ne		
UNID CLAVARIACEAE	**116**	**250.18%**	**2.16%**	**7.50%**	**0.043%**	**NonID**	x		
Alboleptonia sericella	3	0.06%	0.02%	0.04%	0.009%	CHECK	nd	Non-UK? (present in NW Europe)	
Clitopilus hobsonii	3	0.04%	0.01%	0.03%	0.005%	OK	nd		
Clitopilus passeckerianus	4	0.06%	0.02%	0.03%	0.006%	OK	nd		
Clitopilus scyphoides	2	0.06%	0.03%	0.06%	0.004%	OK	nd		
Entocybe nitida	10	0.06%	0.01%	0.01%	0.001%	OK	nd		
Entoloma asprellum	51	4.40%	0.09%	0.82%	0.003%	OK	nd		
Entoloma bloxamii	5	0.43%	0.09%	0.35%	0.008%	OK	VU	NERC/S41 (UK BAP)	
Entoloma byssisedum	42	0.60%	0.01%	0.04%	0.001%	OK	nd		
Entoloma caesiocinctum	2	0.04%	0.02%	0.03%	0.009%	OK	nd		
Entoloma calongeAFF (New sp.?)	7	1.20%	0.17%	0.46%	0.004%	CHECK	nd	Non-UK? (present in S/E Europe)	
Entoloma catalaunicum	2	0.05%	0.03%	0.05%	0.004%	OK	nd		

Table 5. (cont.)

Entoloma cetratum	28	0.54%	0.02%	0.13%	0.001%	OK	nd	
Entoloma chalybeum	18	0.39%	0.02%	0.09%	0.004%	OK	nd	
Entoloma clandestinum	40	6.18%	0.15%	4.15%	0.003%	OK	nd	
Entoloma conferendum	105	6.03%	0.06%	0.18%	0.005%	OK	nd	
Entoloma cuspidiferum	1	0.17%	0.17%	0.17%	0.167%	OK	nd	
Entoloma dysthales	54	1.11%	0.02%	0.14%	0.002%	OK	nd	
Entoloma exile	19	0.85%	0.04%	0.36%	0.003%	OK	nd	
Entoloma griseocyaneum	19	0.76%	0.04%	0.21%	0.003%	OK	VU	
Entoloma henricii	17	1.50%	0.09%	0.32%	0.005%	OK	[VU]	
Entoloma hirtipes	4	0.03%	0.01%	0.01%	0.003%	OK	nd	
Entoloma lividocyanulum	21	0.91%	0.04%	0.20%	0.002%	OK	nd	
Entoloma madidum	1	0.04%	0.04%	0.04%	0.038%	OK	VU?	NERC/S41? (UK BAP?)
Entoloma neglectum	1	0.00%	0.00%	0.00%	0.003%	CHECK	[VU]	Non-UK? (present in NW Europe)
Entoloma ochreoprunuloides	10	6.21%	0.62%	1.62%	0.004%	CHECK	nd	Non-UK? (present in NW Europe)
Entoloma piceinum	14	0.10%	0.01%	0.01%	0.002%	CHECK	nd	Non-UK? (present in NW Europe)
Entoloma pleopodium	23	1.12%	0.05%	0.45%	0.002%	OK	nd	
Entoloma poliopus	21	0.68%	0.03%	0.15%	0.002%	OK	nd	
Entoloma porphyrophaeum	2	0.04%	0.02%	0.04%	0.002%	OK	VU	
Entoloma proterum	26	0.24%	0.01%	0.02%	0.003%	CHECK	nd	Non-UK? (present in NW Europe)
Entoloma prunuloides	20	3.81%	0.19%	1.12%	0.008%	OK	VU	
Entoloma pseudocoelestinum	36	0.90%	0.02%	0.19%	0.003%	OK	nd	
Entoloma rhombisporum	25	0.70%	0.03%	0.14%	0.002%	OK	nd	
Entoloma sacchariolens	36	1.25%	0.03%	0.27%	0.002%	OK	nd	
Entoloma serrulatum	14	0.75%	0.05%	0.38%	0.008%	OK	nd	
Entoloma setastipes	17	1.18%	0.07%	0.44%	0.002%	CHECK	nd	Non UK-US only?
Entoloma turbida	38	5.24%	0.14%	2.12%	0.004%	OK	nd	
UNID ENTOLOMATACEAE	**115**	**22.42%**	**0.19%**	**1.06%**	**0.012%**	**NonID**	**x**	
Cuphophyllus angustifolius	4	1.33%	0.33%	1.04%	0.002%	OK	nd	Non-UK (present in NW Europe)
Cuphophyllus aurantius	4	1.76%	0.44%	1.07%	0.017%	OK	nd	
Cuphophyllus colemannianus	2	0.02%	0.01%	0.01%	0.007%	OK	nd	
Cuphophyllus flavipesoides	3	3.73%	1.24%	2.52%	0.111%	OK	nd	Non-UK. Recently named (2021) from Norway
Cuphophyllus fornicatus	6	9.26%	1.54%	3.64%	0.082%	OK	nd	
Cuphophyllus lacmus	3	10.51%	3.50%	6.09%	1.752%	OK	VU	
Cuphophyllus pratensis	88	237.57%	2.70%	14.94%	0.003%	OK	nd	
Cuphophyllus virgineus	80	130.53%	1.63%	9.43%	0.003%	OK	nd	
Gliophorus europerplexus	2	1.28%	0.64%	0.80%	0.478%	OK	VU	
Gliophorus laetus	35	45.39%	1.30%	7.16%	0.002%	OK	nd	
Gliophorus psittacinus	72	175.60%	2.44%	18.78%	0.004%	OK	nd	
Gliophorus reginae	15	4.85%	0.32%	2.00%	0.002%	OK	VU	
Gliophorus sp:AR2018a (New sp.?)	2	0.03%	0.02%	0.03%	0.007%	NEW	nd	Non-UK (Not Europe); To be named
Gloioxanthomyces vitellinus	3	0.14%	0.05%	0.09%	0.008%	OK	EN	
Hygrocybe acutoconica	3	2.65%	0.88%	2.60%	0.009%	OK	nd	
Hygrocybe aurantiosplendens	4	4.31%	1.08%	2.13%	0.015%	OK	[VU]	
Hygrocybe cantharellus	43	18.87%	0.44%	2.72%	0.005%	OK	nd	
Hygrocybe ceracea	16	13.58%	0.85%	2.31%	0.007%	OK	nd	
Hygrocybe chlorophana	36	70.71%	1.96%	7.42%	0.004%	OK	nd	
Hygrocybe citrinovirens	28	23.61%	0.84%	3.22%	0.003%	OK	VU	
Hygrocybe coccinea	39	90.17%	2.31%	14.19%	0.009%	OK	nd	
Hygrocybe conica	83	223.14%	2.69%	27.29%	0.002%	OK	nd	
Hygrocybe constrictospora AFF	5	3.71%	0.74%	1.66%	0.033%	NEW?	nd	Possibly to be named as new sp.
Hygrocybe glutinipes	59	68.64%	1.16%	10.69%	0.006%	OK	nd	
Hygrocybe helobia	16	14.97%	0.94%	4.18%	0.002%	OK	[NT]	
Neohygrocybe ingrata	5	6.78%	1.36%	2.33%	0.391%	OK	VU	
Hygrocybe insipida	35	12.28%	0.35%	2.04%	0.008%	OK	nd	
Hygrocybe intermedia	6	7.59%	1.26%	3.24%	0.308%	OK	[VU]	
Gliophorus irrigata	44	26.09%	0.59%	5.83%	0.006%	OK	nd	
Hygrocybe miniata	11	3.43%	0.31%	1.22%	0.002%	OK	nd	
Hygrocybe mucronella	13	2.42%	0.19%	0.68%	0.005%	OK	[VU]	
Neohygrocybe nitrata	3	1.34%	0.45%	1.19%	0.031%	OK	VU	
Hygrocybe phaeococcinea	6	0.15%	0.03%	0.08%	0.008%	OK	[VU]	
Hygrocybe punicea	11	21.82%	1.98%	9.44%	0.038%	OK	VU	
Hygrocybe quieta	40	92.07%	2.30%	14.90%	0.006%	OK	[VU]	
Hygrocybe reidii	22	26.68%	1.21%	9.78%	0.006%	OK	[DD]	
Hygrocybe russocoriacea	6	4.36%	0.73%	2.77%	0.139%	OK	nd	
Hygrocybe spadicea	1	1.34%	1.34%	1.34%	1.344%	OK	VU	NERC/S41 (UK BAP)
Hygrocybe splendidissima	1	0.54%	0.54%	0.54%	0.543%	OK	VU	
Hygrocybe subpapillata	11	8.52%	0.77%	5.45%	0.007%	OK	[VU]	
Porpolomopsis calyptriformis	25	13.16%	0.53%	3.06%	0.006%	OK	VU	
UNID HYGROPHORACEAE	**50**	**13.93%**	**0.28%**	**3.05%**	**0.002%**	**NonID**	**x**	
Dermoloma cuneifolium	24	38.69%	1.61%	6.09%	0.006%	OK	nd	
Dermoloma josserandii	4	0.71%	0.01%	0.67%	0.008%	OK	nd	
Dermoloma magicum	56	193.34%	1.67%	25.25%	0.005%	OK	[VU]	
Dermoloma pseudocuneifolium	6	1.55%	0.01%	1.03%	0.002%	OK	nd	
UNID DERMOLOMA	5	1.48%	0.30%	0.71%	0.083%	**NonID**	**x**	

global context to nature conservation, highlighting for example how 27% of the global population of *H. citrinovirens* is found in the UK (based on GBIF data. In contrast, there are no UK mammal species and very few birds (e.g. Puffin [*Fratercula arctica*]; www.iucnredlist.org) listed as VU on the IUCN Global Red List (Stanbury et al., 2021).

In the context of biodiversity conservation, the use of eDNA is novel. Using traditional fungal survey methods, it is usual to count the numbers of mature individuals (i.e. a fungal colony forming basidiocarps or ascocarps). It is more difficult to extrapolate from relative abundance of a particular species to a count of mature individuals. We recently attempted to do this (Detheridge and Griffith, 2021) but even if a particular species is present at high abundance, this DNA could potentially come from numerous small immature individuals rather than fewer mature individuals. For this reason, it is not likely that species counts based on eDNA surveys will be admissible for SSSI notification in the near future. Nonetheless, use of eDNA is likely to be a game-changer in fungal conservation since it permits "eDNA-guided surveying". If a particular rare species is known to be present in a quadrat, then detailed fruitbody surveys in subsequent autumn fruiting periods are very likely to discover the associated fruitbodies. This approach was recently followed at The Leasowes Country Park in Halesowen, leading to the discovery of four new species and thus directly contributing to the notification of this site as a SSSI (ttps://www.aber.ac.uk/en/news/archive/2019/02/title-221042-en.html.).

As an attempted proxy for the presence of mature individuals and based on the calculations presented by Detheridge and Griffith (2021), we undertook species counts based only on those species present in a quadrat at an abundance of 0.05% or higher (corresponding to a mean of 19 sequences or higher) (App4). The resulting mean CHEGD score fell from 29.1 (range 3-46) per quadrat to 18.8 (range 1-33) per quadrat (for waxcaps alone the mean fell from 3.9 to 3.0) but the rank order of quadrats in terms of CHEGD score did not change appreciably (of the top 20 quadrats,15 were ranked in top 20 by both methods). Although the choice of 0.05% abundance as a threshold is rather arbitrary (and would need some form of validation in future studies), this more cautious approach may offer a means for closer comparison of eDNA counts with fruitbody survey data (Detheridge and Griffith, 2021).

G) Soil chemical and biotic parameters

In addition to eDNA metabarcoding of soil fungal populations, a range of edaphic (soil) parameters were measured, including soil nutrients, organic matter, moisture and pH. Additionally, total bacterial and fungal DNA content, as a proxy for biomass, was estimated using quantitative PCR (Taqman probe). These data are presented in App5 and the linear correlations between these factors and with CHEGD fungal parameters are shown in Fig. 3.

Fig. 3. Linear correlations between all soil and CHEGD parameters
App5: Full list of soil data for all quadrats

Soil moisture, pH and organic matter content are relatively simple measurements to make and potentially useful indicators of CHEGD fungal diversity/abundance. Soil carbon content can be estimated indirectly via loss on ignition (=organic matter content) or direct measurement of soil carbon (Elementar; App5) and these parameters correlate closely (Fig. 4) with the ratio of LOI:%C averaging in these soils 1.80±0.19, lower in soils with higher N content. The mean C content of the soils is 9.6% (median 8.7), with the majority (52%) lying in the range 7.4-10.5%. There is a significant negative correlation between species richness and abundance of CHEGD fungi and soil carbon content. It should be noted that soil %C measurement potentially includes inorganic C (e.g. limestone). Of the five quadrats with pH values above 6, two (Howe Green [1404-F1Q1] and Under Whitle [46-F1Q1] have low LOI:C% ratios (ca. 1.65, relative to overall mean of 1.80), possibly due to presence of carbonates (Howe Green is close to the limestone White Peak). The Summerclose quadrat 403-F3Q1 also had an unexpectedly low LOI:C% ratio (0.99, next lowest was 1.42) but the reason for this is unclear (pH was 5.02)(App5).

Fig. 3. Linear correlations between all soil and CHEGD parameters

P-value

	DNA (ng/mg soil)	FungD NA ng/µl tot	NA ng/mg soil	Fung % Total	Bact DNA ng/µl	F:B	Lab % Moist	Field % Moist	Lab pH	Field pH	n Similar Manage	C%	LOI %	N%	Total P mg/kg	Avail P mg/kg	Total K mg/kg	Avail K mg/kg	Av Ca	LOI:%C	C:N	C:totP	C:avP	N:totP	N:avP	CLAV%	HYG%	ENT%	GEO%	DER%	CHEG D%	AMF	CLAV sp	HYG sp	ENT sp	GEO sp	DER sp	CHEG sp
Fungal DNA ng/µl	0.5504																																				
Fungal DNA ng/mg soil	0.7407	0.0000						How to read these matrices:																														
% Fungal of Total	0.0003	0.0000	0.0000					In this matrix the significant correlations are shaded.																														
Bacterial DNA ng/µl	0.1451	0.0000	0.0000	0.0002				If shaded, there is a correlation between the two parameters, so consult the matrix below to see whether the correlation is positive (green) or negative (red)																														
F:B	0.4196	0.0718	0.0728	0.1172	0.2652			Cells within the black square (bottom left of matrix) show the correlations between soi and CHEGD parameters																														
Lab% Moisture	0.0344	0.3840	0.1366	0.7997	0.0202	0.3550																																
Field % Moisture	0.0344	0.4735	0.4613	0.0660	0.0068	0.8496	0.0000																															
Lab pH	0.0036	0.2302	0.1714	0.0035	0.1179	0.9352	0.0377	0.0187																														
Field pH	0.0079	0.3764	0.2930	0.9947	0.0254	0.1119	0.0039	0.0076	0.0000				Yellow = P<0.05 (Significant Correlation																									
ength similar management	0.8448	0.1045	0.0718	0.3307	0.1004	0.0355	0.0013	0.0583	0.0895	0.0027			Green= P<0.0001 (Highly Significant Correlation)																									
C%	0.9789	0.4179	0.0503	0.0897	0.7717	0.8259	0.0000	0.1701	0.0358	0.2089	0.0667																											
LOI %	0.7439	0.7912	0.1355	0.2456	0.2187	0.9721	0.0001	0.0272	0.0453	0.3568	0.3340	0.0000																										
N%	0.3744	0.0299	0.0015	0.0190	0.0483	0.2754	0.0000	0.6309	0.3008	0.0173	0.0001	0.0000	0.0000																									
Total P mg/kg	0.8093	0.0029	0.0011	0.0117	0.0000	0.4561	0.0000	0.0702	0.0071	0.0042	0.0013	0.2403	0.4493	0.0000																								
Avail P mg/kg	0.1545	0.6590	0.5399	0.2706	0.0089	0.7345	0.9196	0.3081	0.0664	0.1271	0.4066	0.3180	0.2687	0.7999	0.0000																								
Total K mg/kg	0.1171	0.0071	0.0160	0.2521	0.0022	0.4084	0.3846	0.2359	0.0211	0.0343	0.0012	0.9721	0.5706	0.0017	0.0000	0.9169																						
Avail K mg/kg	0.0453	0.1975	0.0877	0.5101	0.0331	0.2247	0.0969	0.3329	0.8529	0.0032	0.0022	0.0000	0.0001	0.0000	0.0000	0.6657	0.0000																					
Av Ca	0.0044	0.0401	0.0513	0.0023	0.0122	0.3092	0.3076	0.1194	0.0003	0.0070	0.5152	0.0491	0.0028	0.9971	0.1218	0.4540	0.4225	0.1315																				
LOI:%C	0.2509	0.1741	0.1583	0.1256	0.0096	0.4077	0.1687	0.0110	0.6241	0.0861	0.0108	0.0716	0.0746	0.0015	0.0770	0.0988	0.2847	0.2545	0.0063																			
C:N	0.0805	0.0212	0.0161	0.1374	0.0000	0.0462	0.0011	0.0610	0.1262	0.0031	0.0000	0.5694	0.1966	0.0000	0.0000	0.1567	0.0000	0.0000	0.0003	0.0156																		
C:totP	0.3613	0.0143	0.0283	0.0900	0.0019	0.8532	0.3101	0.0488	0.0469	0.4610	0.3954	0.0003	0.0001	0.4347	0.0000	0.0176	0.0000	0.2424	0.0157	0.1351	0.0001																	
C:avP	0.2969	0.2506	0.2203	0.4508	0.6782	0.8357	0.2343	0.8955	0.0059	0.6329	0.1076	0.0000	0.0082	0.0021	0.0283	0.0000	0.7131	0.7424	0.6772	0.0076	0.2762	0.0083																
N:totP	0.1142	0.0611	0.1048	0.1507	0.0326	0.7723	0.8263	0.1044	0.0451	0.8081	0.9421	0.0003	0.0004	0.0245	0.0000	0.0166	0.0037	0.6569	0.1842	0.5149	0.2686	0.0000	0.0080															
N:avP	0.0944	0.0897	0.0744	0.3750	0.4998	0.5671	0.0437	0.4555	0.0163	0.4782	0.0162	0.0001	0.0315	0.0000	0.2629	0.0000	0.1618	0.1797	0.2057	0.0007	0.2909	0.0924	0.0000	0.0182														
CLAV%	**0.5978**	**0.0612**	**0.1436**	**0.1822**	**0.1404**	**0.2991**	**0.0045**	**0.1278**	**0.0066**	**0.5915**	**0.4576**	**0.0001**	**0.0001**	**0.0169**	**0.6159**	**0.1723**	**0.0014**	**0.4125**	**0.8257**	**0.9873**	**0.1427**	**0.0407**	**0.2122**	**0.1070**	**0.4363**														
HYG%	**0.8117**	**0.6013**	**0.5160**	**0.3227**	**0.1454**	**0.4401**	**0.0506**	**0.7655**	**0.5346**	**0.0899**	**0.0003**	**0.9403**	**0.8925**	**0.2876**	**0.0369**	**0.0877**	**0.1452**	**0.0069**	**0.3143**	**0.7728**	**0.1443**	**0.9086**	**0.3786**	**0.7886**	**0.5430**	0.2702												
ENT%	**0.8856**	**0.3016**	**0.4453**	**0.5156**	**0.3143**	**0.1247**	**0.4564**	**0.6179**	**0.7101**	**0.4684**	**0.1683**	**0.3130**	**0.3774**	**0.3083**	**0.0104**	**0.2492**	**0.1629**	**0.0810**	**0.1446**	**0.9731**	**0.0006**	**0.0080**	**0.2243**	**0.0929**	**0.6328**	0.4133	0.2577											
GEO%	**0.2603**	**0.4658**	**0.4333**	**0.3732**	**0.9605**	**0.5917**	**0.0036**	**0.0833**	**0.0002**	**0.0701**	**0.0747**	**0.1628**	**0.2939**	**0.6763**	**0.0022**	**0.4560**	**0.6982**	**0.5753**	**0.4968**	**0.5380**	**0.0008**	**0.0073**	**0.0669**	**0.0377**	**0.1990**	0.2734	0.7893	0.2846										
DER%	**0.6974**	**0.0357**	**0.0356**	**0.0534**	**0.1017**	**0.5838**	**0.0810**	**0.7946**	**0.4573**	**0.2725**	**0.5564**	**0.2437**	**0.3260**	**0.2237**	**0.0054**	**0.7231**	**0.0887**	**0.2319**	**0.4219**	**0.7876**	**0.0001**	**0.0156**	**0.0277**	**0.1545**	**0.1613**	0.7222	0.1497	0.0319	0.2268								
CHEGD%	**0.8255**	**0.0776**	**0.0896**	**0.0710**	**0.9645**	**0.2336**	**0.3403**	**0.6891**	**0.0927**	**0.5500**	**0.0588**	**0.0379**	**0.0493**	**0.3048**	**0.9521**	**0.0554**	**0.5220**	**0.0717**	**0.4561**	**0.9329**	**0.1043**	**0.0294**	**0.2794**	**0.0683**	**0.4774**	0.0000	0.0000	0.5328	0.0006	0.0004							
AMF	**0.0166**	**0.4742**	**0.4841**	**0.4914**	**0.0005**	**0.6799**	**0.0000**	**0.0017**	**0.0000**	**0.0161**	**0.0002**	**0.9872**	**0.6030**	**0.3188**	**0.0010**	**0.0003**	**0.5412**	**0.7851**	**0.9963**	**0.0530**	**0.0283**	**0.2829**	**0.6397**	**0.4073**	**0.9296**	0.7322	0.0667	0.4608	0.0665	0.2971	0.2410						
CLAV sp	**0.6432**	**0.7137**	**0.3647**	**0.1260**	**0.6828**	**0.0331**	**0.0002**	**0.5002**	**0.1028**	**0.2482**	**0.4662**	**0.0017**	**0.0034**	**0.0005**	**0.0002**	**0.0003**	**0.0554**	**0.0835**	**0.1226**	**0.3226**	**0.1018**	**0.5512**	**0.6508**	**0.7309**	**0.7124**	0.0000	0.0839	0.1066	0.3610	0.2743	0.0070	0.2381					
HYG sp	**0.1102**	**0.7693**	**0.9628**	**0.5767**	**0.2273**	**0.2558**	**0.0000**	**0.2239**	**0.0056**	**0.0000**	**0.0575**	**0.0313**	**0.0738**	**0.0024**	**0.0000**	**0.0217**	**0.0901**	**0.0207**	**0.1983**	**0.3099**	**0.0475**	**0.2480**	**0.2938**	**0.4117**	**0.2404**	0.0048	0.0000	0.3641	0.1274	0.0797	0.0005	0.0003	0.0000				
ENT sp	**0.9926**	**0.4531**	**0.2606**	**0.1953**	**0.1797**	**0.0811**	**0.0464**	**0.8521**	**0.5444**	**0.0281**	**0.3661**	**0.6396**	**0.8840**	**0.0029**	**0.0000**	**0.0233**	**0.0316**	**0.0001**	**0.2017**	**0.2068**	**0.0000**	**0.0004**	**0.1103**	**0.0225**	**0.4200**	0.0930	0.1006	0.0000	0.0025	0.0020	0.9696	0.9572	0.0000	0.0000			
GEO sp	**0.3259**	**0.1190**	**0.0704**	**0.0200**	**0.2105**	**0.8460**	**0.6047**	**0.8517**	**0.0000**	**0.1970**	**0.4207**	**0.0449**	**0.1281**	**0.1882**	**0.3028**	**0.0089**	**0.1210**	**0.9765**	**0.1466**	**0.5066**	**0.1941**	**0.6915**	**0.4590**	**0.8383**	**0.5128**	**0.0001**	0.6336	0.7086	0.0035	0.0975	0.0014	0.5393	0.0000	0.0680	0.1355		
DER sp	**0.2051**	**0.1342**	**0.2015**	**0.6704**	**0.0419**	**0.3057**	**0.7066**	**0.2574**	**0.0003**	**0.1471**	**0.0319**	**0.1277**	**0.2281**	**0.1338**	**0.0001**	**0.5573**	**0.0000**	**0.0006**	**0.8491**	**0.3671**	**0.0000**	**0.0004**	**0.0261**	**0.0231**	**0.2674**	0.0002	0.2826	0.0631	0.0971	0.0005	0.0344	0.1209	0.1358	0.3590	0.0391	0.0033	
CHEG sp	**0.9971**	**0.5982**	**0.2940**	**0.1621**	**0.2539**	**0.0510**	**0.0001**	**0.3571**	**0.4843**	**0.0137**	**0.3593**	**0.0063**	**0.0244**	**0.0002**	**0.0000**	**0.0003**	**0.7712**	**0.0089**	**0.3026**	**0.1414**	**0.0211**	**0.1239**	**0.9229**	**0.3069**	**0.8759**	**0.0000**	0.0035	0.0060	0.2002	0.1229	0.0017	0.2020	0.0000	0.0000	0.0000	0.0000	0.2871
OTU Count	**0.4243**	**0.8956**	**0.7289**	**0.4279**	**0.3471**	**0.3933**	0.0261	**0.4560**	**0.0642**	**0.9291**	**0.0002**	**0.5374**	**0.6364**	**0.4481**	**0.1850**	**0.5681**	**0.0716**	**0.2472**	**0.9916**	**0.7423**	**0.0084**	**0.7611**	**0.0727**	**0.7593**	**0.2740**	0.0495	0.0056	0.8069	0.5942	0.7557	0.2313	0.0000	0.1172	0.8855	0.0225	0.0130	0.0000	0.0118

R-coefficient

	DNA (ng/mg soil)	FungD NA ng/µl tot	NA ng/mg soil	Fung % Total	Bact DNA ng/µl	F:B	Lab % Moist	Field % Moist	Lab pH	Field pH	n Similar Manage	C%	LOI %	N%	Total P mg/kg	Avail P mg/kg	Total K mg/kg	Avail K mg/kg	Av Ca	LOI:%C	C:N	C:totP	C:avP	N:totP	N:avP	CLAV%	HYG%	ENT%	GEO%	DER%	CHEG D%	AMF	CLAV sp	HYG sp	ENT sp	GEO sp	DER sp	CHEG sp
Fungal DNA ng/µl	0.061																																				
Fungal DNA ng/mg soil	0.034	0.982						R-coefficient relates to Pearson's linear correlation, which goes from -1 (perfect negative correlation) to +1 (perfect positive correlation)																														
% Fungal of Total	-0.358	0.848	0.874					Correlations of not are in cells with black bounary and bold text																														
Bacterial DNA ng/µl	0.148	0.536	0.529	0.368				Beware of autocorrelations (e.g. HYG% contributes to CHEGD% so unsurprising that they exhibit strong positive correlation)																														
F:B	-0.082	-0.182	-0.181	-0.158	-0.113																																
Lab% Moisture	0.213	0.088	0.151	0.026	0.233	-0.094																																
Field % Moisture	0.213	-0.073	-0.075	-0.186	0.270	-0.019	**0.447**																															
Lab pH	0.290	-0.122	-0.139	-0.290	0.158	-0.008	0.209	0.236																														
Field pH	0.266	0.090	0.107	-0.001	0.225	-0.161	0.288	0.267	**0.407**																														
ength similar management	-0.020	-0.164	-0.182	-0.099	-0.166	0.212	-0.318	-0.191	-0.172	-0.298																												
C%	-0.003	0.082	0.197	0.171	-0.030	-0.022	0.412	-0.139	-0.211	0.127	-0.185																											
LOI %	0.033	0.027	0.151	0.118	-0.125	0.004	0.373	-0.222	-0.202	0.094	-0.098	0.924																										
N%	0.090	0.218	0.316	0.235	0.199	-0.111	0.526	-0.049	-0.105	0.239	-0.381	0.846	0.723																									
Total P mg/kg	0.025	0.296	0.322	0.253	0.458	-0.076	0.432	0.183	0.269	0.286	-0.319	0.119	0.077	0.428																								
Avail P mg/kg	-0.144	0.045	0.062	0.112	0.262	-0.035	-0.010	0.103	0.185	0.154	-0.084	-0.101	-0.112	-0.026	0.497																							
Total K mg/kg	0.159	0.269	0.242	0.116	0.305	-0.084	-0.088	-0.120	0.232	0.213	-0.321	0.004	-0.058	0.311	0.467	-0.011																						
Avail K mg/kg	0.202	0.131	0.173	0.067	0.214	-0.123	0.168	-0.098	-0.019	0.294	-0.305	0.416	0.372	0.672	0.420	0.044	0.633																					
Av Ca	0.284	-0.207	-0.196	-0.303	-0.251	0.103	-0.104	-0.158	0.354	0.269	0.066	0.198	0.297	0.000	-0.157	-0.076	0.082	0.153																				
LOI:%C	0.116	-0.138	-0.143	-0.155	-0.259	0.084	-0.139	-0.255	-0.050	-0.173	0.255	-0.182	0.180	-0.314	-0.179	-0.167	-0.109	-0.116	0.273																			
C:N	-0.177	-0.231	-0.241	-0.150	-0.402	0.201	-0.324	-0.189	-0.155	-0.295	0.436	0.058	0.131	-0.458	-0.554	-0.143	-0.516	-0.556	0.356	0.242																		
C:totP	0.093	-0.245	-0.221	-0.171	-0.309	0.019	-0.103	-0.199	-0.200	-0.075	0.086	0.355	0.382	0.079	-0.639	-0.238	-0.415	-0.119	0.242	0.151	0.391																	
C:avP	0.106	0.117	0.124	0.077	-0.042	-0.021	0.121	0.013	-0.275	0.049	-0.163	0.416	0.264	0.305	-0.220	-0.484	0.037	0.033	-0.042	-0.267	0.111	0.264																
N:totP	0.160	-0.189	-0.164	-0.146	-0.215	-0.029	-0.022	-0.164	-0.202	-0.025	-0.007	0.355	0.350	0.226	-0.548	-0.240	-0.289	0.045	0.135	0.066	0.112	0.949	0.265															
N:avP	0.169	0.171	0.180	0.090	0.069	-0.058	0.203	0.076	-0.241	0.072	-0.241	0.390	0.216	0.404	-0.114	-0.477	0.142	0.136	-0.128	-0.335	-0.107	0.170	0.964	0.237														
CLAV%	-0.054	0.189	0.148	0.135	0.149	0.105	-0.283	-0.154	0.271	-0.055	-0.076	-0.373	-0.379	-0.240	-0.051	-0.138	0.318	-0.083	0.022	0.002	-0.148	-0.206	-0.126	-0.163	-0.079													
HYG%	-0.024	0.053	0.066	0.100	-0.147	0.078	-0.197	-0.030	-0.063	-0.171	0.354	-0.008	-0.014	-0.108	-0.210	-0.173	-0.147	-0.270	-0.102	-0.029	0.148	0.012	0.089	-0.027	0.062	0.112												
ENT%	-0.015	0.105	0.078	0.066	-0.102	0.155	-0.076	-0.051	-0.038	-0.074	0.140	0.102	0.090	-0.103	-0.256	-0.117	-0.141	-0.176	0.148	-0.003	0.338	0.265	0.123	0.170	0.049	0.083	0.115											
GEO%	0.114	-0.074	-0.080	-0.090	-0.005	0.055	0.290	0.175	0.363	0.183	-0.180	-0.141	-0.107	0.042	0.304	-0.076	0.039	0.057	0.069	0.063	-0.332	-0.268	-0.185	-0.209	-0.130	0.111	-0.027	-0.109									
DER%	-0.040	0.211	0.212	0.195	0.165	-0.056	0.176	-0.026	0.076	0.111	-0.060	-0.118	-0.100	0.123	0.278	0.036	0.172	0.121	-0.082	0.027	-0.384	-0.243	-0.221	-0.144	-0.142	0.036	-0.146	-0.216	0.123								
CHEGD%	-0.022	0.178	0.172	0.182	-0.005	0.121	-0.097	-0.041	0.170	-0.061	0.191	-0.209	-0.198	-0.104	0.006	-0.193	0.065	-0.182	-0.076	0.009	-0.164	-0.219	-0.110	-0.184	-0.072	0.463	0.762	0.063	0.339	0.347							
AMF%	0.240	0.073	0.071	-0.070	0.345	-0.042	0.414	0.311	0.467	0.241	-0.370	-0.002	-0.053	0.101	**0.325**	**0.355**	0.062	-0.028	0.000	-0.195	**-0.221**	-0.109	-0.048	-0.084	0.009	0.035	-0.185	0.075	0.185	-0.106	-0.119						
CLAV sp	0.047	-0.037	-0.092	-0.155	-0.042	0.214	**-0.371**	-0.069	0.165	-0.117	0.074	-0.311	-0.292	-0.345	-0.363	-0.354	0.193	-0.175	0.156	0.100	0.165	0.061	0.046	0.035	0.038	0.588	0.175	0.163	-0.093	-0.111	0.270	-0.120					
HYG sp	-0.162	0.030	0.005	0.057	-0.122	0.115	**-0.434**	-0.123	-0.276	-0.459	0.192	-0.217	-0.180	-0.302	-0.410	-0.231	-0.171	-0.232	-0.130	0.103	0.200	0.117	-0.107	0.083	-0.119	0.282	0.464	0.092	-0.154	-0.177	0.343	-0.353	0.423				
ENT sp	0.001	-0.076	-0.114	-0.131	-0.136	0.176	**-0.201**	-0.019	-0.062	-0.221	0.092	-0.048	-0.015	-0.297	-0.456	-0.228	-0.216	-0.377	0.129	0.128	0.448	0.346	0.162	0.229	0.082	0.170	0.166	0.475	-0.300	-0.306	0.004	0.005	0.485	0.462			
GEO sp	0.100	-0.158	-0.183	-0.234	-0.127	0.020	-0.053	-0.019	0.405	0.131	-0.082	-0.202	-0.154	-0.133	-0.105	-0.262	0.157	-0.003	0.147	0.068	-0.132	-0.040	-0.075	-0.021	-0.067	0.382	0.048	0.038	0.290	0.167	0.317	0.062	0.484	0.184	0.151		
DER sp	0.128	0.152	0.129	0.043	0.205	-0.104	0.038	-0.115	0.358	0.147	-0.216	-0.154	-0.122	0.152	0.386	0.060	0.598	0.340	0.019	0.092	-0.517	-0.348	-0.224	-0.228	-0.113	0.361	-0.109	-0.187	0.168	0.342	0.213	0.157	0.151	-0.093	-0.208	0.293	
CHEG sp	0.000	-0.054	-0.107	-0.142	-0.116	0.197	-0.380	-0.094	0.071	-0.247	0.093	-0.273	-0.226	-0.368	-0.444	-0.356	0.030	-0.262	0.105	0.149	0.232	0.156	0.010	0.104	-0.016	0.510	0.290	0.274	-0.130	-0.156	0.312	-0.129	0.847	0.711	0.754	0.554	0.108
OTU Count	0.081	-0.013	-0.035	-0.081	0.096	-0.087	0.224	0.076	0.187	0.009	-0.361	-0.063	-0.048	0.077	0.134	0.058	0.182	0.117	-0.001	0.033	-0.263	-0.031	-0.181	0.031	-0.111	0.198	-0.276	0.025	0.054	0.032	-0.121	**0.447**	0.158	0.015	0.229	0.249	0.398	0.252

Fig. 4. Correlation of measurements of Organic matter, pH, moisture

Soils with higher %C are more acidic, so there is also a positive correlation between pH and CHEGD species richness /abundance (but not Hygrophoraceae species richness where there is a negative correlation, possibly because of the various waxcaps (e.g. *G. laetus*) that show a preference for more acidic soils; ca. 4.5 or below). It would be expected that soil moisture would provide a useful indicator for soil organic matter and clear positive correlation is found between soil moisture and LOI%/%C (0.373 and 0.412 respectively. However, this relationship is somewhat confounded by the fact that during the 4 month period when soils were collected (when rainfall was gradually increasing), there was also a positive correlation between collection date and moisture level (0.430; $P<0.001$).

Measurements of pH and moisture were made using field-based meters at the time of sample collection and also later in the lab. The latter are clearly the more reliable but comparisons allow assessment of field-based measurements. If reliable, these would be useful proxies, as noted above. The field-based measurement of moisture showed a stronger correlation ($r=0.447$) with lab readings than did pH ($r=0.407$) but for the former, some samples with highly aberrant readings were obtained (Fig. 4C).

Total and available P and K (also available Ca) as well as total N were also quantified. N, P and K levels showed predominantly negative correlation with species richness of CHEGD fungi (Fig. 3), consistent with the negative effects of synthetic (ie. NPK) fertiliser addition. P is a highly immobile and insoluble nutrient, so soil P levels provide a good indicator of past fertiliser application and this may explain the observed negative correlation.

Our study of fungal diversity focused on CHEGD fungi, however, arbuscular mycorrhizal fungi (AMF) belonging to phylum Glomeromycota, are known to be important for plant nutrition in grassland/arable habitats and are by far the best studied of the grassland fungi (Smith and Read, 2010). Although AMF account for only a small proportion of the total fungal biomass (mean 0.25%; range 0.075-0.57%), we have found that these fungi are highly active compared to CHEGD fungi (Detheridge et al., unpublished data). Correlation of the relative abundance of AMF showed some interesting contrasts to the CHEGD fungi, for instance their positive correlation with soil moisture and also total/available P levels. The latter suggests that they are associated with more fertile soils than are CHEGD fungi (consistent with the observation that increased abundance of AMF correlated negatively with species richness of waxcaps).

The data presented here from eDNA metabarcoding indicate relative abundance of different fungal species. It would be more useful to be able to provide actual biomass data of each species but this would require a means of estimating total fungal biomass. This can be done by use of the fungal-specific biomarker ergosterol which comprises ca. 0.5% dry wt. of fungal mycelia (Detheridge et al., 2018; Detheridge and Griffith, 2021). In the current study we tested the utility of a Taqman-based quantitative PCR assay (FUNquant) to estimate total fungal biomass (Liu et al., 2012b), the same technology used for the COVID PCR test. This showed a positive correlation with levels of total N/P/K, which is plausible since the latter are the key limiting nutrients for microbial growth. However, such Taqman-based assays have not been widely used to estimate fungal biomass in soils and are potentially susceptible to differences in DNA extraction efficiency, and this may vary according to differences in the abundance of soil metabolites that bind DNA or otherwise alter the DNA extraction process. Ground-truthing against independent measurements of soil biomass (e.g. ergosterol) would be required to determine the accuracy of this assay.

There is strong evidence from the scientific literature that low soil fertility is associated with an increase in fungal biomass relative to bacterial biomass (Karimi et al., 2019; Morriën et al.,

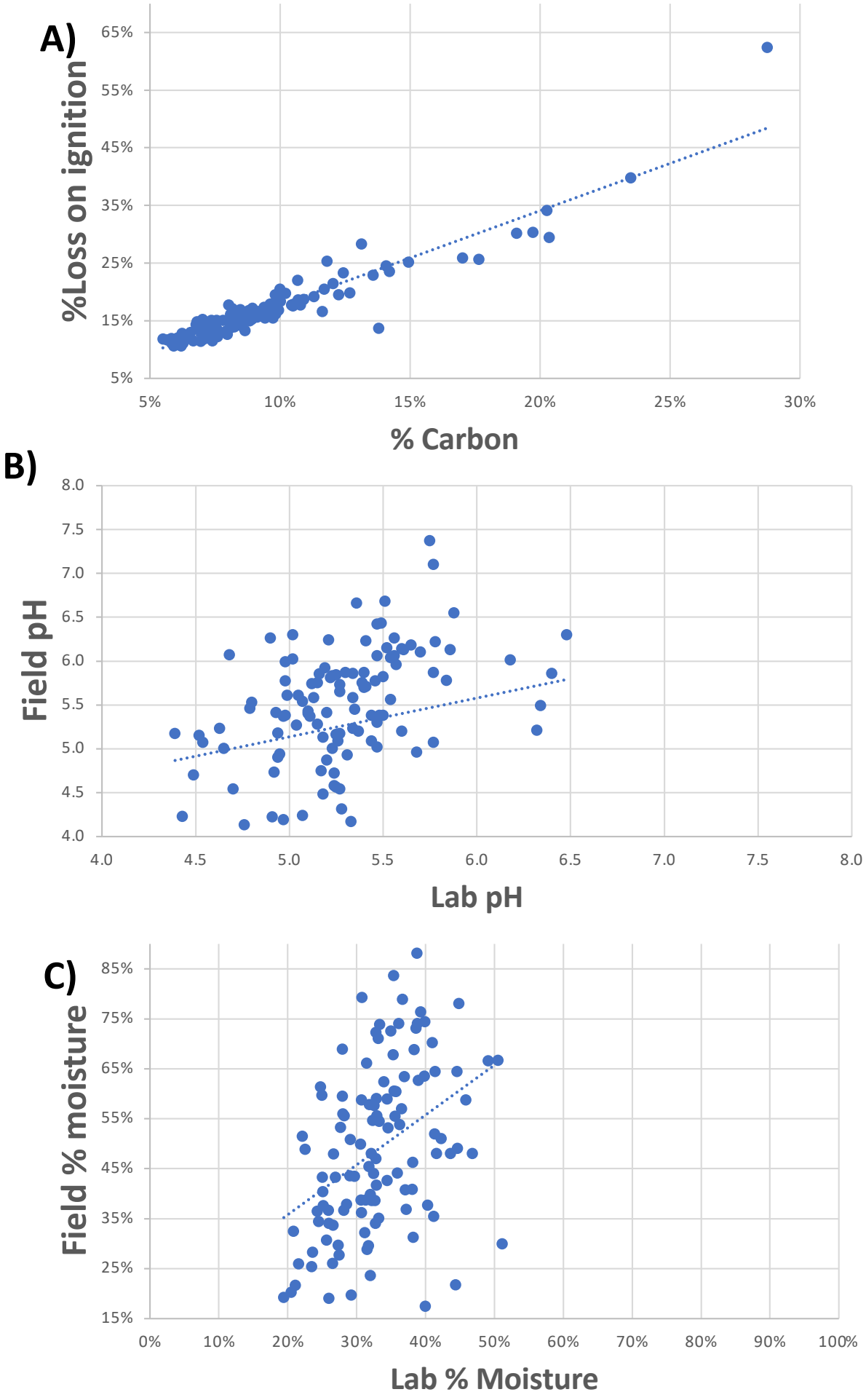


Fig 4. Correlations of different measurements of A) Soil organic matter (%Carbon vs Loss on Ignition),; B) pH (Lab vs field measurement); C) Soil moisture (Lab vs field measurement)

2017), often termed the F:B ratio. This term was coined by Bardgett et al. (1996; Bardgett and McAlister, 1999), who quantified relative levels of PLFAs (phospholipid fatty acids, a key component of cell membranes) using a method devised by Bååth et al. (1992). This method has been widely used but remains very laborious and costly (ca. £100/sample), requiring extraction of total lipids from soil, the isolation of polar lipids and subsequent quantification by GC-MS (Gas chromatography-mass spectrometry). Furthermore, there is no fungal-specific PLFA (those present in fungi are also present in plants and other eukaryotes), so the F:B ratios quoted in the literature use oleic/linoleic acids as proxies for fungal biomass (since most eukaryote biomass in soil is fungal).

Here we deployed a Taqman-base assay (BACTquant; (Liu et al., 2012a)) to quantify total bacterial biomass. This showed a strong positive correlation with the amount of fungal DNA (assessed by the FUNquant assay), which would be expected since total soil biomass would be expected to correlate with soil nutrient levels. F:B levels derived from the results of these Taqman assays did show a positive correlation with C:N ratio (with high C:N ratio indicating low fertility). Thus the assay does show some promise as a proxy (it is straightforward to undertake once DNA is extracted) for the PLFA-derived F:B ratio, but as noted above, DNA extraction efficiency from different soils would need to be tested and compared with PLFA/ergosterol estimates of biomass before placing great faith in these data.

H) Effect of land management on grassland fungal populations

Detailed management data was obtained for all the quadrats, alongside data relative to the dominant vegetation (App1). The dominant vegetation was noted for all quadrats, but it was not possible to undertake quantitative comparisons with CHEGD data. Vegetation data are highly dependent on recent grazing patterns and, as noted above, we have previously found no correlation between CHEGD diversity/abundance and the abundance of particular components of grassland vegetation. Grasslands heavily grazed by sheep over long periods may be botanically depauperate but the presence of diverse populations of grassland fungi would suggest that, given suitable management, more diverse populations of higher plants (and potentially associated animals) could develop following imposition of more sympathetic sward management.

For the most part the management data were categorical (ie. yes/no) rather than quantitative. However, for duration of management (range 1-70yrs; mean 28.8yrs), which indicates the stability of management and for the higher values the absence of any ploughing or synthetic fertiliser addition, there was a positive correlation with the number of waxcap species and a negative correlation with AMF abundance, both consistent with reduced levels of soil fertility in these quadrats. When these sites were split into those with more (>30yrs) or less (<30yrs) stable management, the relative abundance of waxcaps and overall CHEGD fungi were significantly greater in more stably managed quadrats (Table 6).

Table 6. Results of statistical analyses (ANOVA) of site management and CHEGD fungi.
(red=not significant; orange=close to significance threshold; green=significant; dark green=highly significant)

ANOVA (P)	CLAV sp	HYG sp	CHEG sp	CLAV%	HYG%	CHEGD%	AMF%
Management type (P/H)	0.0403	0.0021	0.0105	0.8460	0.1827	0.2366	0.0064
Livestock type (C/S/CS/SH)	0.0156	0.0002	0.0092	0.0069	0.0301	0.0736	0.2523
Length of similar management (<30/>30)	0.0718	0.1921	0.4214	0.4468	0.0151	0.0105	0.0165
In AgriEnv Scheme (Y/N)	0.1208	0.0674	0.0477	0.4468	0.8883	0.4413	0.6202
SSSI (Y/N)	0.0918	0.9454	0.3644	0.0208	0.9291	0.0538	0.3643

For several of the management parameters no significant effects on CHEGD fungi species richness or abundance were found, for instance liming, nutrient use (i.e. manure or synthetic fertiliser etc.) and herbicide application. Lack of significant correlation for such observational ('survey') data must be interpreted carefully (e.g. there were no data on when and how much lime was added), so it is not possible to conclude from these data whether these treatments are beneficial or harmful. However, for other management parameters, significant effects on CHEGD populations were found, as summarised in Table 6 and illustrated in Figs. 5/6.

Fig. 5. Box and jitter plots showing which CHEGD measurements were significantly different (ANOVA P<0.05) between: **A/B/C/D:** Pasture (P) vs Hay meadow (H) or **E/F/G:** Duration of continuous management

Fig. 6. Box and jitter plots showing which CHEGD measurements where significantly different (ANOVA P<0.05) between: **A/B/C/D/E:** Different types of grazing, F: membership of Agri-environment scheme and G: SSSI status.

In particular, hay meadow management was associated with less diverse/abundant CHEGD fungal populations than pastures (Fig. 5A,B,C), as were quadrats with a shorter history of extensive management (Fig. 5D,E). With regard to grazing, cattle-grazed quadrats consistently showed lower species richness and relative sequence abundance than quadrats grazed by sheep alone or with horses (Fig. 6A-E). CHEGD species richness was higher in quadrats managed under an Agri-Environment Scheme agreement but the P-value was marginal (P=0.0477), whilst quadrats within an SSSI showed lower Clavariaceae relative sequences abundance (again marginal; P=0.0208).

Whilst interesting to note these differences, care must be taken not to infer causality. It is likely that sheep-grazing and pasture management will predominate on steeper slopes at higher altitudes where fields are less accessible to mechanised agriculture (relative to flatter areas which are more likely to be managed as hay meadows and/or be cattle-grazed). Other factors may also come into play, for instance field size, with three of the best four hay meadows being at Greenland Farm and all being small (<0.5 ha), making it more difficult/inefficient to undertake mechanised agriculture.

I) Muiltivariate analyses of fungal communities

The analyses presented above are univariate (comparing variables in pairwise combinations). We also undertook multivariate analyses of the complete fungal communities in each quadrat using Principal Coordinates Ordination implemented in PAST3 [http://folk.uio.no/ohammer/past/]). This ordination method, similar to detrended correspondence analysis, is widely used in molecular ecology with dots closer together being more closely related (Fig. 7). It can be seen that quadrats taken from the same site (same symbol) tend to cluster. Additionally, the first principal component (x-axis) shows some separation of the quadrats with the higher CHEGD score ordinating to the right of the graph and the poorer sites/quadrats ordinating to the left.

Fig. 7. Principal Coordinates Ordination (PCO) of fungal communities across all 116 quadrats.

A related multivariate analytic method (distance-based redundancy analysis; dbRDA; Fig. 8) permits correlations to be made with environmental data (soils and management parameter). In this figure, the direction and length of the lines representing these environmental variables indicates how they correlate with the fungal communities in the different quadrats. For example, the quadrats for two best sites out of the 25 surveyed (797: Waterfall Low and 1131: Brownsetts) ordinate toward the bottom of the graph (i.e. they are separated from the poorer sites primarily on the y-axis, with pH, N:avP ratio and av. Ca pointing in this direction). Only the main environmental factors contributing to the ordination are shown (as ranked in the sequential

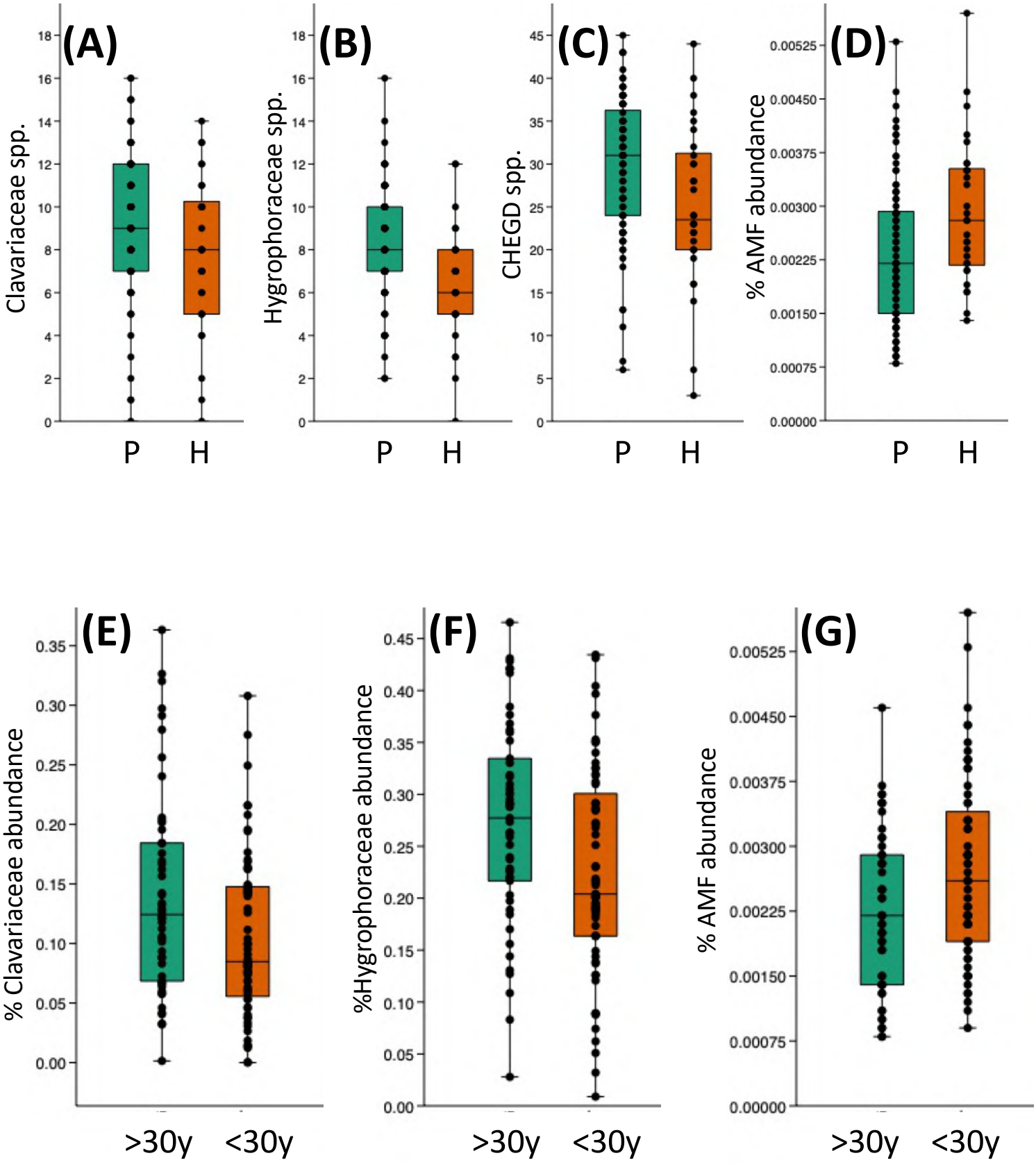


Fig. 5. Box and jitter plots showing which CHEGD measurements where significantly different (ANOVA P<0.05) between: A/B/C/D: Pasture (P [n=90]) vs Haymeadow (H [n=30]) management and E/F/G: Duration of continuous management (unchanged for more [n=51] or less [n=65] than 30 years). Relevant P-values are presented in Table 6. On the plot, the box represents the interquartile range middle 50% of the datapoints, the horizontal line within the box shows the median, with the outlier values indicated outside the box.

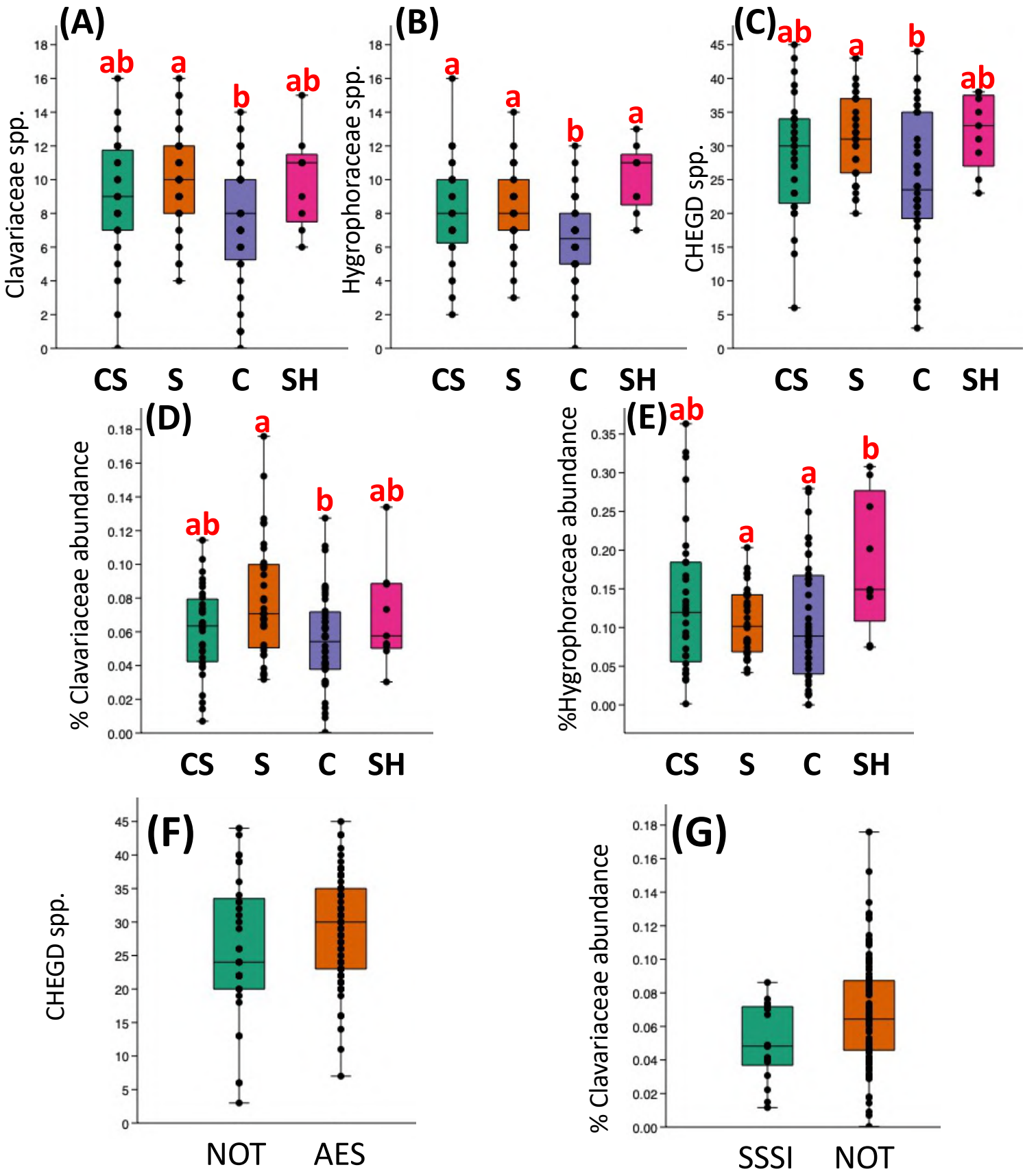


Fig. 6. Box and jitter plots showing which CHEGD measurements where significantly different (ANOVA P<0.05) between: **A/B/C/D/E**: Different types of grazing (CS: cattle-sheep [n=32]; S: sheep [n=35]; C: cattle [n=40]; SH: sheep-horse [n=9]); **F**: membership of Agri-environment scheme (AES)[n=83] or not; **G**: Site notified as SSSI [n=18] or not. Relevant P-values are presented in Table 6. On the plot, the box represents the interquartile range middle 50% of the datapoints, the horizontal line within the box shows the median, with the outlier values indicated outside the box. On plots **A-E**, bars which do not share a common letter (in red above the bars) are significantly different.

tests; beneath Fig. 8) though some others, for example duration of current management (P=0.01; contribution 2.3%), were significant but smaller.

The correlation of the total N:available P ratio with fungal communities with high numbers of CHEGD species is unexpected and intriguing. We found N:avP ratio to have a mean of ca. 800:1 for the soil (range 0.05:1 to 4350:1). The correlation was not apparent in the linear correlation analyses (Fig. 3) suggesting that the connection relates to the whole soil fungal community rather than just the CHEGD fungi. It is reasonable to speculate that the cellular composition of cells would reflect that of the soil they inhabit (i.e. that microbes inhabiting a soil with a high N:avP ratio would themselves have a high cellular N:avP ratio). This situation is reminiscent of the Redfield Ratio that is applied to marine plankton (Redfield, 1934; Zimmerman et al., 2014).

Overall marine plankton have an average N:P ratio of 16:1 ("the Redfield ratio") but within different groups of phytoplankton, some have an N:P ratio higher than this due to greater investment in proteins (which contain N but no P) required for nutrient acquisition, relative to investment in ribosomal RNA (rRNA; where most of the cell's P resides) which are needed for growth. Thus, organisms with a high N:P ratio are efficient in nutrient acquisition in conditions where nutrients are present at very low concentration; thus, they are capable of growth in nutrient-poor condition but propagate slowly (they are 'survivalists'; a concept akin to K-selection). In contrast, organisms with a low N:P ratio invest more in rRNA and are thus capable of rapid growth when nutrients become available; these are often the phytoplankton species that cause 'algal blooms' (akin to r-selection). When nutrients are in short supply, these 'bloomer' organisms are dormant.

The basic concept of the Redfield Ratio has occasionally been applied to terrestrial ecosystems, for instance higher plants (Koerselman and Meuleman, 1996) but not to our knowledge to soil organisms. In the context of the soil fungal populations studied here, the CHEGD fungi could be considered as the 'survivalists', with other groups, for example the AMF, as the 'bloomers', which are capable of efficient exploitation of sudden nutrient inputs. However, these ideas need to be developed further, not least because the N:avP ratio of the substrates (i.e. soil) would be expected to be much higher than that of the organisms present in the soil.

Fig. 8. Distance-based redundancy analysis (dbRDA) plot of fungal communities in relation to key environmental variables.

Conclusions (Take home messages)

1) Afforestation: All of the four sites at risk of afforestation host diverse grassland fungal populations and should be protected from damage by intensive tree-planting. It is not clear how quickly the grassland fungi will decline if trees are planted, likely slowly (e.g. over 10 year timescale). From our current knowledge of the ecology of these fungi, the likely driver of loss will be the proliferation of ectomycorrhizal fungi associated with the trees (Sepp et al., 2021). Current grants for afforestation stipulate high density planting of sapling, similar to the requirements of plantation forestry. However, low density planting (i.e. a more agroforestry) or natural regeneration resulting from possible reduced future grazing levels would pose a much lesser threat to grassland fungi. There would be some loss of waxcap grassland habitat as part of such a return to a wood pasture-type landscape.

2) Additional conservation actions: Several of the sites host exceptionally diverse grassland fungal populations and merit notification as SSSIs. Of the top 10 sites (Table 1), all with ≥ 17 waxcap spp., only two have partial legal protection within SSSIs (both within the Leek Moors SSSI). Grassland fungi are not a notified interest feature of Leek Moors SSSI but operations such as applying fertilisers and lime, and changes to the grazing regime, are amongst those

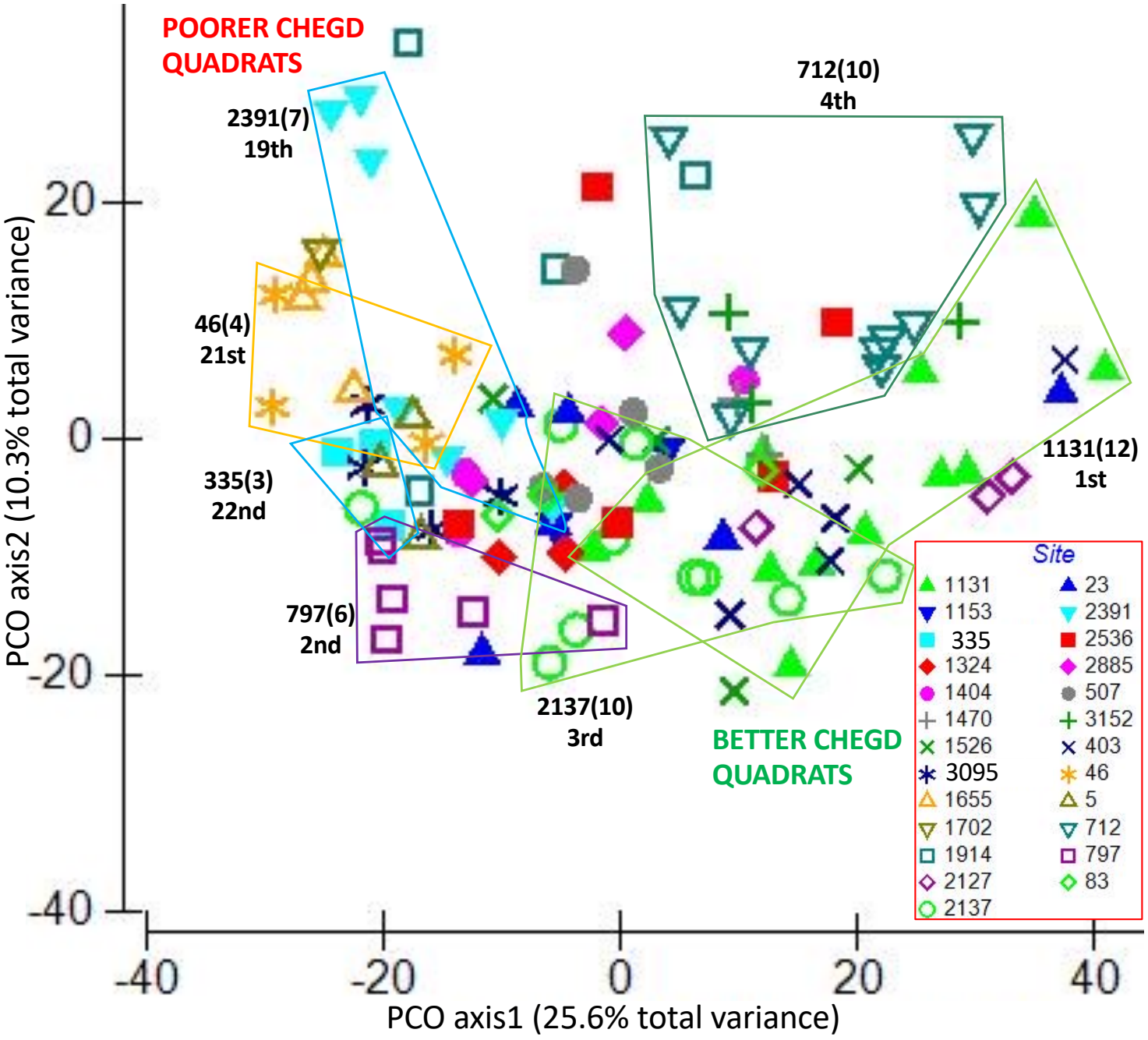


Fig. 7. Principal Coordinates Ordination (PCO) of fungal communities across all 116 quadrats. Points closer together on this plot have more similar fungal communities. A total of 35.9% of the variance in the whole dataset is explained by these two axes. Data were subjected to square root Bray-Curtis transformation prior to ordination.
Quadrats from several of the best and worst sites (based on total CHEGD score) are grouped with polygons, with the site number and ranking indicated. Number in brackets indicates number of quadrats at that site.

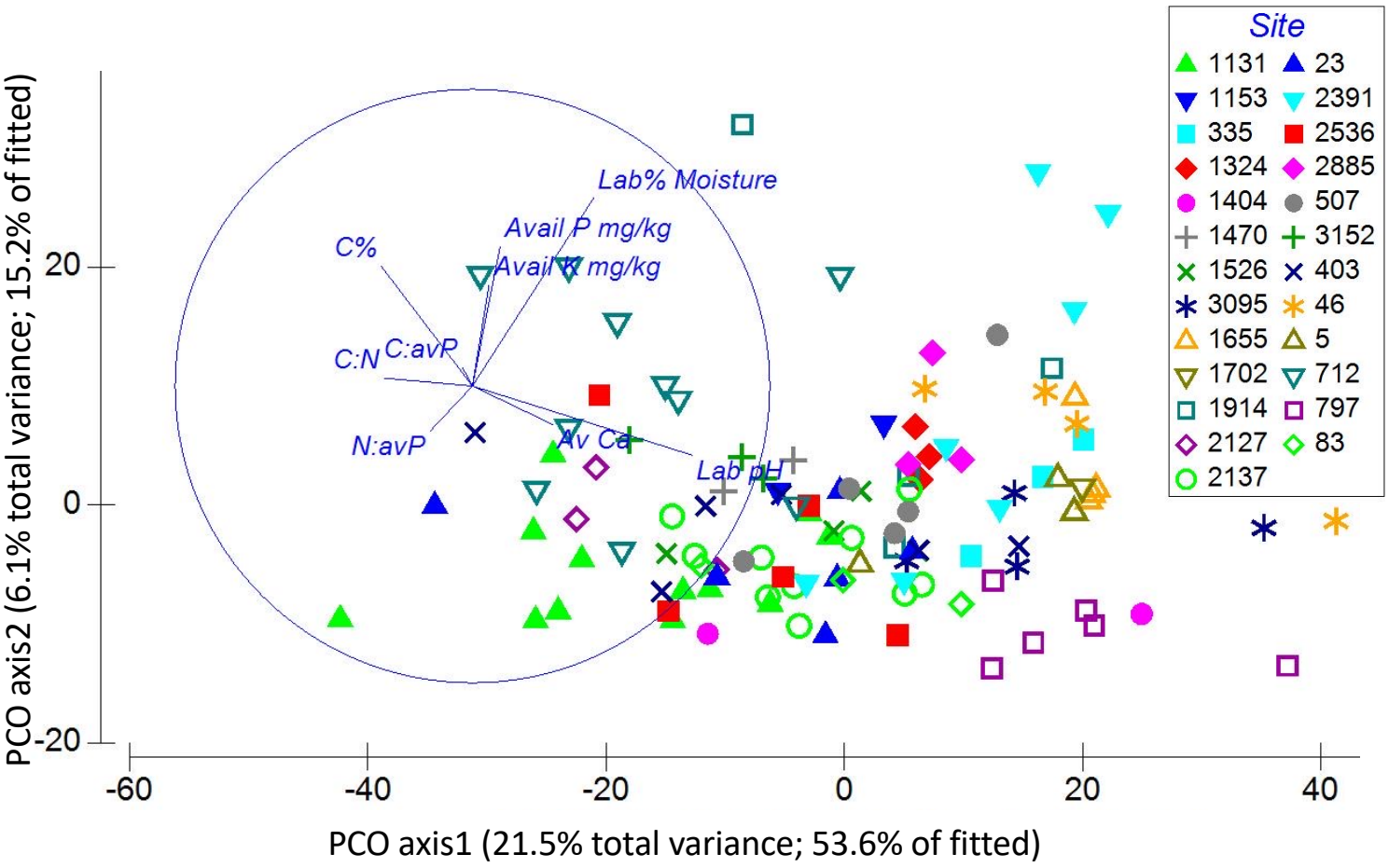


Fig. 8. Distance-based redundancy analysis (dbRDA) plot of fungal communities in relation to key environmental variables. The ordination explains 27.6% of the total variance within the data set and 68.8% of the fitted variance. Data were subjected to square root Bray-Curtis transformation prior to ordination.

Only environmental variables with statistically significant correlation are shown (hence absence 'Fungal DNA' and 'Fungal:bacterial DNA ratio').

Environmental variables (blue lines within the circle) pointing in the same direction are highly correlated (eg. % Loss on ignition [LOI%], total N, % carbon), whereas those pointing in opposite direction are inversely correlated (e.g. high pH inversely correlated with high %carbon).

DistLM2 Sequential Tests

(showing relative contribution of the environmental variables. i.e. linked to lengths of blue lines in circle)

Variable	% Contribution	Cumul.%	AICc	SS(trace)	Pseudo-F	P	res.df
Lab pH	17.78	17.8%	803.4	24648	24.65	0.001	114
Lab% Moisture	6.10	23.9%	796.5	8449	9.05	0.001	113
C%	3.67	27.5%	792.9	5092	5.68	0.001	112
Avail K (mg/kg)	3.49	31.0%	789.4	4844	5.63	0.001	111
Avail P (mg/kg)	2.72	33.8%	787.0	3770	4.52	0.001	110
Av Ca (mg/kg)	1.82	35.6%	786.0	2528	3.09	0.001	109
C:N	1.98	37.6%	784.7	2746	3.43	0.001	108
N:avP	1.27	38.8%	784.6	1759	2.22	0.001	107
C:avP	1.33	40.2%	784.5	1845	2.36	0.001	106

requiring Natural England's consent and there is also protection from agricultural improvement under Environmental Impact Regulations.

3) Proxies for identification of sites/quadrats with diverse grassland fungal populations: Whilst areas with diverse higher plant populations will likely also host diverse populations of CHEGD fungi due to lack of past disturbance via ploughing or fertiliser application, heavy grazing can result in loss of plant diversity yet leave the CHEGD fungal populations undamaged. Simple measurements such as pH and soil moisture appear to provide useful information (i.e. sites with pH 4.8-5.8 and soil moisture [late summer/autumn] of 25-35% but the interpretation of soil nutrient (N,P,K, Ca) levels is less straightforward as described above (Results G).

4) Soil organic matter levels: A key driver for tree planting is to enhance carbon sequestration but there is low awareness beyond the scientific community that most carbon is sequestered below ground, rather than in visible vegetation. All the soils in this study had moderate C levels in the top 10 cm sampled (mean 9.6%C = ca. 96 T/ha) and likely at least twice as much in deeper soil horizons (i.e. 200-300 T/ha in total), consistent with the estimates of Milne and Brown (1997). For comparison, the trees in a 50 year old sitka spruce plantation (used as an example of a fast-growing tree species) could contain up to ca. 100 T/ha C in above-ground vegetation, whereas native broadleaved species may achieve similar levels but over a longer period.

Aside from questions as to how well-sequestered the harvest wood is (depends on intended use), on organo-mineral soils, there is invariably more C in the soil than visible in vegetation. To our knowledge, there are no studies showing net gain of soil C following tree planting on undisturbed grasslands but there are many that demonstrate loss of soil C following tree planting, for example, Upson et al. (2016) and Poeplau et al. (2011). Bradfer-Lawrence et al. (2021) specifically note that tree planting on C-rich organo-mineral soils should be avoided.

Regularly ploughed land has much lower levels of soil C (below 50 T/ha) and in these areas tree planting would lead to gradual C accumulation. Reseeded grass leys are regularly ploughed and thus greatly depleted in soil C. It is important to emphasise the distinction between such grasslands and undisturbed pasture both in terms of biodiversity and the fact that they are already stable reservoirs for large amounts of C, and thus any disturbance will imperil this.

5) Recommendations for management of waxcap grasslands: The longstanding recommendations for 'waxcap grasslands' (avoidance of synthetic fertiliser) should be followed (Griffith et al., 2004; Halbwachs et al., 2018). The effects of periodic lime addition are less clear; very high levels can have a detrimental effect on fruiting (Halbwachs et al., 2018) but the longer term effect of a normal application rate (up to 5 T/ha at 5-10 yr intervals) is potentially beneficial for some soils, since it prevents excessively low (<4.5) soil pH levels which are detrimental to CHEGD abundance/species richness (Fig. 3; Fig. 7), though a few species (e.g. *G. laetus*) exhibit a preference for acidic soils and could be adversely affected by liming.

Sward height is known to affect fruiting of CHEGD fungi (Griffith et al., 2012) but there is no evidence that it influences levels of mycelium in the soil. Thus the eDNA approach offers a useful approach to assess the potential of under-grazed/scrubby grasslands to assess CHEGD fungal diversity since fruitbody surveys would likely yield an underestimate. CHEGD fungi are long-lived so do not need to reproduce annually and even absence of any grazing for several years would not necessarily lead to decline. Ultimately failure to reproduce would lead to decline on decade/century timescales. Similarly, long term absence of grazing would lead to successional changes in plant communities, though as noted above there is no evidence of any specific correlation between any CHEGD fungi and particular plant species (Griffith et al., 2014; Halbwachs et al., 2018).

Some positive correlations between particular sward management regimes and CHEGD diversity/abundance were observed here. However, our analyses suggesting that pasture management and sheep grazing were associated with more diverse CHEGD populations does not necessarily indicate a causative correlation. For example, some upland pastures are less likely to be disturbed due to inaccessibility and for the same reasons less likely to be managed as hay meadows or grazed by cattle. However, since CHEGD-rich hay meadows are much rarer than CHEGD-rich sheep-grazed pastures, their conservation value is greater, for example, Greenland Farm (1324) and Howe Green Farm (1404). The issue of soil compaction from agricultural vehicles, not investigated here, is worthy of further study, since this is linked to field management practices.

6) Taxonomic considerations: The approach used here has only recently been developed and is thus undergoing continued improvement, most importantly in the accuracy with which species are identified. For the Hygrophoraceae, there are reference barcodes for nearly all the UK species. A cautious approach is needed when considering the introduction of new names; unless the new taxa are not easily distinguished from each other morphologically, then there is scope for taxonomic confusion, with surveyors unable to attribute names reliably without recourse to genetic analyses. The recent revision of the *Entoloma bloxamii* species complex (Ainsworth et al., 2018) provides a good example of how the problem of cryptic speciation may be resolved. In the case of the *H. conica* species complex, we are undertaking an extensive investigation of samples from across Europe and combining genetic analyses, with investigation of $^{15}N/^{13}C$ isotopic profiles and basidium/spore morphology. This continues the research already published by Halbwachs et al. (2018).

7) Continuing need for fruitbody surveys: eDNA provides accurate identification and evidence that a particular species is present. However, whether the mycelia detected represent mature individuals is more difficult to assess via eDNA. Therefore, confirmation of the occurrence of (especially) rarer species should be obtained by targeted fruitbody surveying. The apparent rarity of some fungal species may be attributable to their infrequent fruiting. For example, *Dermoloma magicum*, though considered to be rare in the UK and globally with only 5 UK records listed in GBIF (https://www.gbif.org/species/2531880) was found at 56/116 quadrats, often in abundance. This species is clearly more widespread and common than fruitbody surveys would lead us to believe (we have observed the same pattern at many other sites), likely because it fruits only very rarely.

8) The importance of retaining vouchers from field surveys: Even with access to high quality field guides and expertise in microscopy, some species identifications are still difficult. The fact that there are often discrepancies between eDNA data and field survey data is consistent with this, as is the recent discovery that a moderate number of samples deposited at Royal Botanic Gardens Kew were originally misidentified. For sites of higher value in terms of fungal biodiversity and which may be candidates for subsequent designation, we suggest that dried specimens are routinely retained from field surveys. Such collections are not bulky and if kept in a plastic box with desiccant (after freezing for 24 h to kill eggs of any insects present), it will be possible to extract DNA for future DNA barcoding (and potentially other scientific uses) for many years.

Future work

A) Awareness raising: The broader priority across the UK is to raise awareness that tree-planting is not necessarily a beneficial activity even if generous subsidies are available. It needs to be communicated that this is for two reasons, first that grassland habitats containing valuable biodiversity at a global level may be destroyed, and second that 'marginal' grasslands (less productive and thus where the business case for afforestation is stronger) are already high in soil carbon (mean 9.6% carbon here), so the potential for increased soil C sequestration is low.

Since tree roots tend to dry out soils, leading to loss of soil C (amongst other factors (Anderson, 2021)), there is a clear short-term (and potentially long-term) danger of net C loss resulting from afforestation. A wide range of nature conservation organisations, including Woodland Trust, are aware of the dangers of inappropriate afforestation. These represent allies that can provide support and pressure on regulators to avoid loss of valuable habitats.

There needs to be some flexibility in determining the balance of woodland/grassland, possibly aiming towards a tree pasture type habitat, for example by allowing a degree of natural reinvasion of scrub/trees. Whilst some grassland is lost to small patches of trees/scrub, overall biodiversity is enhanced in more heterogeneous/natural landscapes. It is very likely (and there is no evidence to the contrary) that CHEGD fungi mycelia will survive periods of several years under rank sward, though they would likely not form basidiocarps. Thus temporary withdrawal of grazing could be considered (possibly for several years), to allow successional processes to continue, with later management involving reimposition of grazing.

In any event, it is a significant problem that afforestation of grassland is being proposed and often permitted because there is a lack of data about any fungi present. It is therefore important to undertake more field surveys and to ensure that resulting data reach Natural England and Local Environmental Record Centres so it can be used to inform future targeting. Waxcap grassland is not represented in the Priority Habitat Inventory which is used for targeting of AES and woodland creation. This omission needs to be addressed with urgency.

B) Ensuring that regulations are enforced: In Wales it is clear that the 'light touch' assessment of afforestation programmes results in inadequate biodiversity assessment (i.e. no consideration of grassland fungal populations). Often no on-site biodiversity (EIA) assessment is done, and for smaller sites (<2 ha), no EIA assessment is required. In England there is a threshold of 2 hectares (ha), either in one block or as a total on one holding, below which an EIA screening decision is not required; although Natural England will consider proposals that affect land of a smaller area that is semi-natural, has heritage features, and/or has special landscape features, e.g. historic parkland. Planting or natural regeneration of less than 0.5 hectares is not considered afforestation under the forestry EIA regulations unless it is adjacent to another afforestation project completed in the preceding five years. There is a presumption against there being a likely significant effect from planting between 0.5 and 2ha within a National Park unless within a defined 'sensitive area', however the Forestry Commission must provide an opinion. Forestry Commission England EIA scoping guidance advises describing communities of plants and animals but not fungal populations.

C) Methodological improvements: Whilst the cost of high-throughput sequencing has fallen substantially, the limiting factor cost-wise is person-time for the collection of soil samples and preparation of eDNA for sequencing. At sufficient scale, the cost of the latter could be reduced. Since eDNA analysis, as conducted here, is a direct measurement of fungal diversity, it is not likely that there will be radical technological advances in the short-term (<5yrs). From an ecological perspective, economical/reliable quantification of microbial biomass in soil is a priority. We have found ergosterol analysis to be reliable for assessment of fungal biomass (this allows the relative sequence abundance data to be converted to actual biomass data). We would likely be able to develop this protocol further but its routine deployment requires additional method development funding.

D) Rapid assessment of waxcap grasslands: With incomplete habitat data, inadequate regulation and poor enforcement of existing regulations, there is a high likelihood that many biodiverse grasslands will be lost to afforestation, or other changes in management. Rapid assessment of grassland habitats, for example with citizen scientists using novice waxcap keys, originally suggested by Griffith et al. (2004) and recently promoted by a Plantlife campaign, app and leaflet (Harries and Lamacraft, 2014) App6) would cover many sites at low cost.

Coordinated promotion of such a citizen scientist driven approach would not only raise awareness of the importance of grassland habitats and their fungi but also identify sites which could be prioritised initially for eDNA survey and if good fruitbody survey. The rational for this approach is that funds will always be limited and the bulk of any costs will be person time. Fruitbody surveys are placed third because the lack of suitably skilled surveyors will be a limiting factor but if any formal protection is being considered then mature individuals (i.e fruitbodies) need to be detected. Guided by species lists and locations generated from initial eDNA surveys, field surveys will thus become more efficient.

The issue of permission to access private land presents a problem. Promising sites could be later assessed via eDNA; for some sites in Wales such eDNA assessment has been required by NRW as part of the assessment process (transferring cost to the landowner).

The use of field-based pH/moisture meters is potentially helpful but probably more useful would be to directly measure moisture from a small soil sample (i.e. wet/dry weight measurement) which can be undertaken without a laboratory (especially if standardised: assessment in autumn and use of nearby reference sites to account for annual rainfall variation). Similarly, measurement of pH off-site from soil slurry would provide more reliable measurement than field-deployed meters. Direct assessment of soil carbon requires laboratory analysis but some methods have been developed for colour-based assessment of soil organic matter which can be conducted in the field (Aitkenhead et al., 2015). Such soil samples could be collected as part of initial citizen scientist surveys suggested above.

Methodology

Sample preparation: On receipt of each sample the soil was weighed and immediately frozen at -80°C. After 24 hours the soils were freeze dried to remove the water without it entering the liquid phase hence suppressing biological activity during drying, which could affect the fungal community profile. Dried soil samples were finely ground by passing through a 2 mm wire sieve. After thorough mixing, a 50 g subsample was further ground through a 0.5 mm sieve. The moisture content of the samples was in the range 19-51% (mean 33%; App5). Compared to the majority of the samples we have analysed from the more westerly/northerly parts of the UK, the samples provided in this survey were larger than we usually receive (generally 600-900 g), due to the use of an 18 vs 15 mm auger but it is unlikely that this had a significant effect on the fungi detected. Following grinding, 150 mg of soil was taken for DNA extraction using the Qiagen *Powersoil* Soil DNA extraction kit.

Genetic analysis: PCR amplification of a 3-400 bp portion of the ITS2 region of the ribosomal RNA locus was amplified with the primer mix devised by Tedersoo et al. (2014). These primers are specific but also amplify Oomycetes. In order to allow several samples to be sequenced in a single sequencing run, a second round PCR amplification was undertaken to add unique 10bp identifier tags to sequences from each quadrat. Following PCR amplification, PCR products were quantified using a Qubit fluorometer (Invitrogen) and pooled in equimolar concentrations. The pooled library was purified using AMPure XP beads (Beckman Coulter) and the library checked and quantified with a Bioanalyzer High Sensitivity DNA analysis (Agilent). The pooled sample DNA was sequenced using an Illumina MiSeq High Throughput DNA sequencer employing the MiSeq Reagent Kit v3 (600-cycle) to give 2x300 bp paired end reads (Brennan et al., 2019).

The unpaired sequence reads were paired using the PEAR program. Sequences were then dereplicated and singleton sequences removed before clustering at 97% using the UPARSE algorithm (Edgar, 2013). Taxonomy was assigned using a naïve Bayesian classifier (Wang et

al., 2007). against the UNITE database (Abarenkov et al., 2010) and data formatted in as an Excel matrix. More detail on bioinformatic analyses can be found in Detheridge et al. (2016; 2018).

Chemical analysis of soils

Soil pH was measured using 5g suspended in 25 ml of double distilled water in a 50ml tube and shaken for an hour (120 oscillations min^{-1}) on a reciprocating shaker to form a slurry. The tubes were then allowed to settle for 30 minutes before pH was measured using a Mettler Toledo LE438 pH probe. The measurement was taken after the reading had settled, usually between 30 seconds and a minute.

Soil moisture:. After completion of the drying the soil samples were re-weighed and the percentage moisture content calculated as water content (wet soil weight – dry soil weight) / wet soil weight * 100.

Organic matter was measured using loss on ignition. Approximately 5 g of dried soil was added to a porcelain crucible of known weight and the weight of the soil and crucible recorded. The crucibles were placed in a muffle furnace and ignited at 400 °C for 16 hours. After ignition the crucibles were reweighed and organic matter % was calculated as soil weight / original soil weight * 100.

Total carbon and nitrogen were measured on a CN analyser (Elementar Vario Max Cube) using 350 mg dry weight of soil.

Total nutrients (P, K, Mg, Ca): 5g of freeze-dried soil was weighed into conical flasks and 20 ml of concentrated nitric acid added and the flask capped with upturned crucible lid. The flasks were left overnight and the following day heated to bring the acid to boil and simmered with the lid on for 2 hours. The lid was removed and the nitric acid volume reduced to leave a slurry. The flasks were then removed from the heat and allowed to cool before 20ml of 0.1 M nitric acid was added and the slurry resuspended. The slurry was then filtered using Whatman No. 1 paper into a 50 ml volumetric and a further 20 ml 0.1M nitric acid added and the filtration repeated. Each volumetric flask was then brought up to 50 ml with 0.1 M nitric acid. The elements were then measured using a Vista MPX simultaneous ICP-OES with 5-point standards for each element.

Available nutrients (P, K, Mg, Ca): 3g of freeze-dried soil was added to a 5ml tube before 30ml of Mehlich III extraction buffer (20g ammonium nitrate, 0.55g ammonium fluoride, 0.29g EDTA, 11.5ml glacial acetic acid, 0.825 ml nitric acid made up to 1 L with distilled water) was added and shaken for 5 min (120 oscillations min^{-1}) on a reciprocating shaker. The extract was then filtered (Whatman No 1) into 15ml tubes and the elements measured as for total nutrients.

Taqman qPCR quantification of total fungal and bacterial biomass:

For the fungal and bacterial Taqman qPCR assays we used established primers and probes: FungiQuant (Fwd GGRAAACTCACCAGGTCCAG, Rev GSWCTATCCCCAKCACGA, Probe (6FAM) TGGTGCATGGCCGTT-30 (MGBNFQ) (Liu et al., 2012b); BactQuant (Fwd CCTACGGGDGGCWGCA, Rev GGACTACHVGGGTMTCTAATC, Probe (6VIC) 5′-CAGCAGCCGCGGTA-3′ (MGBNFQ)) (Liu et al., 2012a). Internal standards were run for each plate created from DNA extracts of pure cultures of *Penicillium roqueforti* as the fungal standard and *Escherichia coli* as the bacterial standard in 10^1–10^5 10-fold serial dilutions. Also included on each plate were no DNA template controls. Each standard and sample were run in triplicate and the response averaged to reduce the effect of pipetting differences. The reaction mix volume was 20 µl using qPCRBIO Probe Mix (PCR Biosystems Ltd) with 2 µl DNA. The forward and reverse primers were added to a final concentration of 500 nM and the probe to 225 nM

and the primers and probes were duplexed in a single reaction. Each qPCR was run for 40 cycles at an annealing/extension temperature of 60 °C on a Roche LightCycler 480 measuring the FAM and VIC fluorophores.

Crossing points of the amplification curves for each sample were converted to a fungal or bacteria absolute DNA concentration using the standards and this converted to a DNA quantity per gram of soil.

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Box 3 A novice's guide to fungal diversity at grassland sites

Small (<2cm cap diameter) brown/grey/white basidiomes (basidiomycete fruit bodies)	☐ (1 pt)
Basidiomes on dung	☐ (1 pt)
Field mushroom (*Agaricus*) basidiomes	☐ (1 pt)
Puffballs or other larger basidiomes (Cap >4cm diameter)	☐ (1 pt per species)
Pink gills (*Entoloma*) – narrow pink/off-white gills; caps white to brown	☐ (2 pts per species)
Earth tongues (black/dark green; tough) or fairy clubs (yellow/white; fragile)	☐ **(5 pts per species)**
Purple Fairy Club *Clavaria zollingerii* (distinctive forked structures)	☐ **(10 pts)**

Waxcaps *Hygrocybe* (caps often slimy when wetted; gills thick, well spaced and white or similar colour to cap)

Cap colour	white (*H. virginea*)	☐ (2 pts)
	orange/yellow, turning black (*H. conica*)	☐ **(2 pts)**
	yellow (*H. chlorophana, glutinipes*)	☐ **(2 pts)**
	green (*H. psittacina*)	☐ **(2 pts)**
	light brown (*H. pratensis*)	☐ (3 pts)
	orange (*H. reidii*; honey odour *H. laeta*)	☐ (3 pts)
	red (*H. coccinea, punicea, splendidissima*, etc.)	☐ **(7 pts)**
	pink (*H. calyptriformis*)	☐ **(10 pts)**

Based on a single visit:
Scores of <10 would be expected for more intensively managed grasslands
Scores of 10-30 indicate some mycological potential
Scores of >30 would indicate a good 'waxcap grassland'
For species read distinct morphological type. The groups shown in bold are those which are fairly unmistakable. Note that sites should not be 'written off' because of a low score; scores will be higher from mid-October onwards.

Griffith et al. (2004)

How do I know if I have a good waxcap grassland?

Look for the different coloured mushroom-like fungi and for each colour-group add together the relevant points.

Red (eg *H. coccinea, punicea, splendidissima*)	5 points
Pink (eg *H calyptriformis*)	5 points
Orange (eg *H. reidii, quieta, laeta*)	2 points
Buff/brown (eg *H. pratensis*)	2 points
Yellow (eg *H. chlorophana, glutinipes*)	2 points
Orange/yellow turning black (eg *H. conica*)	1 point
Green (eg *H. psittacina*)	1 point
White (eg *H. virginia*)	1 point

Are there other grassland fungi? Add the points for the following groups.

Violet coral (*Clavaria zollingeri*)	5 points
Yellow/white coral (not illustrated)	1 point
Beige/brown coral (not illustrated)	2 points
Earthtongue (any)	2 point

Harries & Lamacraft (2014)

What is your final score?

Scores of 0-4 indicate more intensively managed grasslands, probably with low grassland fungi interest. But, be aware that this is not always the case; fungi do not always fruit e.g. if the vegetation is too dense, or the weather too dry. **You should not dismiss a site with a low score straight away**, as sites are best visited over several years to assess their fungi interest.
Scores of 5-11 would indicate sites that may be of grassland fungi interest and may be worth further investigation (see below).
Scores of 12-29 would indicate sites that are good for grassland fungi and worth further survey, especially by an expert (see below).

What to do if you find an interesting waxcap grassland?

If you find an interesting site for grassland fungi eg scoring 5+ please inform the landowner and one of the two fungi organisations mentioned in the "Information and advice" section. The site may be worth further investigation and these organisations will be able to find a local expert to undertake a more comprehensive survey.

Full leaflet as pdf from:
https://www.plantlife.org.uk/application/files/6915/0460/9899/Waxcap_ID_guide_low_res_website.pdf

Appendix 6: Novice keys for waxcap grassland assessment