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Soil Studies for Fungal Diversity to Enable the Conservation Translocation of Green-Winged Orchid

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Abstract: Conservation Translocation (CT), which includes reintroduction, reinforcement and introduction, is gaining momentum, responding to serious population decline in many orchids. Orchid conservation underpinned by a greater emphasis on understanding biotic and abiotic factors of habitats is critical for successful recovery and restoration programmes. *Anacamptis morio*, commonly known as green-winged orchid (GWO), is a terrestrial orchid found throughout Europe, but populations in England rapidly declined in its native range in the last several decades. The current study explored the relationship between soil abiotic characteristics and the community composition of key fungal groups. Wild sites in Essex and Cumbria in England, where successful colonies are currently present, were compared to potential sites for CT in Cumbria. The Cumbrian sites managed by cattle grazing include the wild site and three potential CT sites, with two of them hosting no GWO plants. The Essex site, fOxley Meadow, where no cattle is used for grassland management, hosts the largest population of GWO in England. The aim of this study was to understand whether the community composition of fungi and soil characteristics of the potential CT sites in Cumbria are nearly compatible with that of Oxley Meadow. Oxley Meadow, with around 65,000 plants, stands out as a unique habitat compared to all Cumbria sites, as it showed low organic content. Nitrate and phosphate content were smaller for Oxley Meadow compared to other Cumbrian sites. The proportion of Basidiomycota fungi was greater in Oxley Meadow compared to all Cumbrian sites where Ascomycota dominated. The abundant fungal group found in Oxley Meadow was Agaricales. From Agariclaes, Hygrophoraceae or waxcaps fungi are considered an indicator group of fungi and were the most abundant group in Oxley Meadow. They have a negative correlation with targeted key fungal groups and abiotic parameters. More in-depth assessments using additional primers are essential to better understand the fungal diversity and how this diversity translates to the resilience of orchid habitats. This preliminary study points to future studies to assess whether sites are near-compatible to the wild site where large colonies are present using additional primers collected over different time scales.

Keywords: community composition; orchid mycorrhizal fungi; Conservation Translocation; edaphic effect; orchids; soil DNA; barcoding; *Anacamptis morio*

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1. Introduction

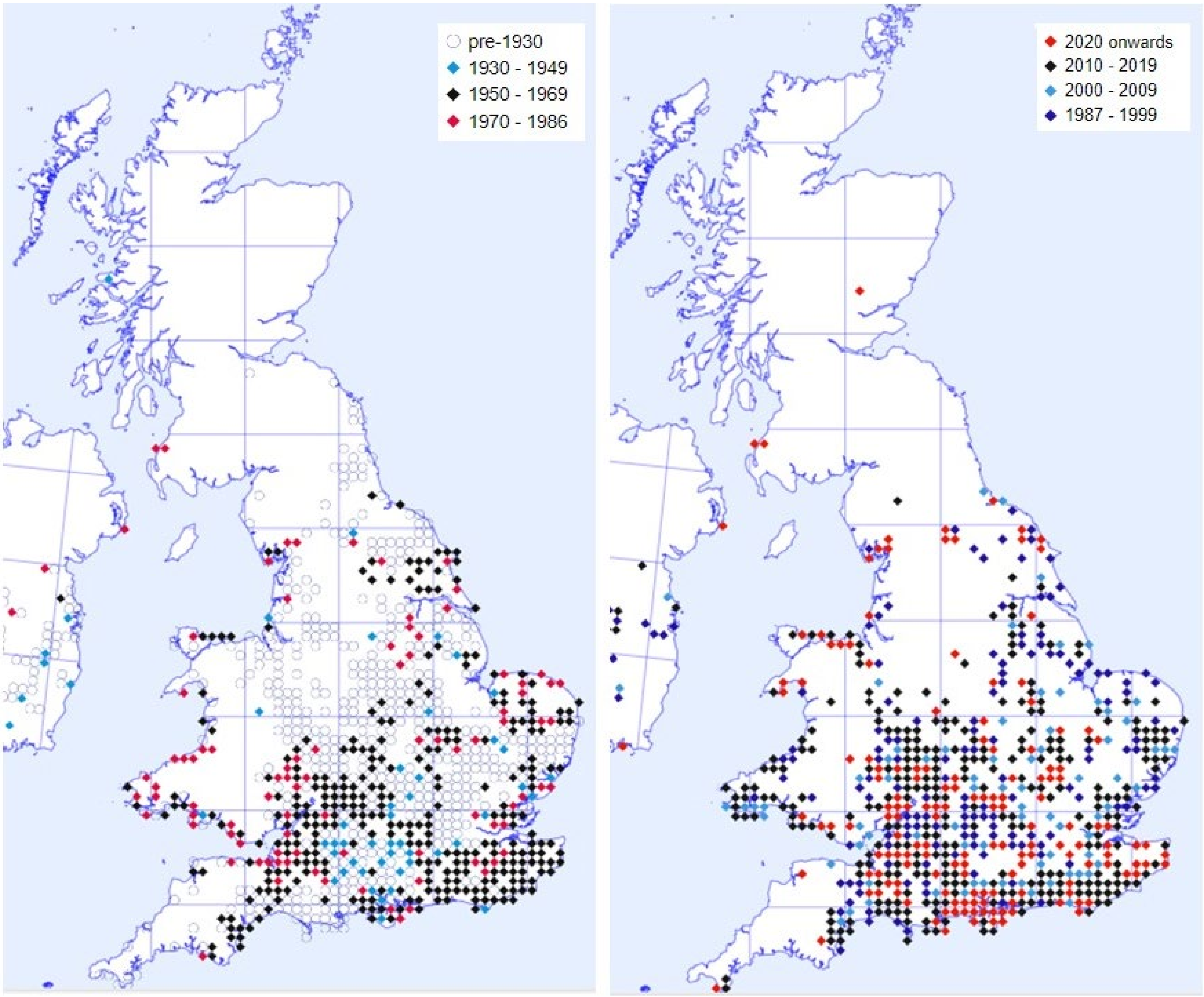
With about 28,000 described taxa and new species being discovered each year, Orchidaceae is one of the largest families of flowering plants in the world [1]. While orchids have not been found to provide any large-scale ecosystem services, orchids are valuable members of their ecosystems, and their ecological importance is often overlooked. Their sensitivity to biotic and abiotic disturbances makes them good bioindicators, and orchids are often used as warning systems for the health of ecological communities, with the conservation strategies developed for orchids benefitting other taxa in the communities in which they occur.

Anacamptis morio (L.) R.M. Bateman, Pridgeon & M.W. Chase, commonly known as green-winged orchid, GWO (Figure 1), is an orchid colonised mainly in unimproved grasslands [2]. In England, its population declined mainly due to habitat loss through the agricultural improvement of old hay meadows [3]. Between 1930 and 2000, *A. morio* declined by about 49% in England [4] (Figure 2) and continued to decline.

(**a**) (**b**)

Figure 1. Green-winged orchid (*Anacamptis morio*) with green lip (**a**) in a grassland habitat in England (**b**).

Wild populations of rare plants like orchids are undergoing rapid decline, habitat numbers, and populations within habitats due to land use change and climate change, which support the need for interventions to conserve habitats and Conservation Translocation of taxa. These declines are due to historical land use changes and possibly climate change [5–9]. Studies in Central Europe found that most of the long-term population has been on the increase since the late 1970s. They also found that climatic change could be beneficial for established populations of *A. morio* [10]. The reliance of orchids on compatible orchid mycorrhizal fungi (OMF) is critical for orchids as the microscopic seeds are devoid of nutritional reserves, which means the development of the heterotrophic protocorm requires colonization by mycorrhiza [11,12]. Due to the important role of OMF in orchid seedling recruitment and establishment, it has been proposed that the distribution of OMF in the landscape could be critical, which may constrain the establishment of orchids in the wild [13]. According to McCormick et al. [14], many OMFs are probably widespread at a greater landscape level, which means orchid distribution is essentially limited by seed dispersal. But at the local fine-scale levels, spatial distribution patterns may be driven by other factors, which leads to small population sizes and a scattered occurrence.



(**a**) (**b**)

Figure 2. Map of the UK showing recorded populations of *Anacamptis morio* categorised by year (pre-1930 till 1986) (**a**), and distribution from 1987 till last recorded (**b**) from Distribution data from BSBI 2024.

In addition to the support from OMFs, an understanding of abiotic factors is also important to orchid recovery and establishment in the wild [15–17]. In some cases, selected groups of fungi are in decline in some habitats, as reported before [18]. Several studies undertaken at a fine-scale habitat and at a large landscape level for monitoring of species have found that relevant environmental data for vegetation niche modelling and community composition of fungi [13,15,19–23] are examples that can help develop strategies for Conservation Translocation of rare orchids. Species like GWO prefer unimproved grasslands that are either managed by grazing or mowing, and orchids prefer non-dominant vegetation. However, in the case of orchids, each species has niche requirements that make these processes expensive.

The aim of this investigation is to (a) understand soil characteristics such as organic matter, nitrate, and phosphate and (b) the fungal community composition of key fungal groups in soils of wild and potential Conservation Translocation for GWO. By comparing these characteristics in the wild for potential Conservation Translocation, their suitability for the successful establishment of GWO can be evaluated, and evidence-based conservation actions can be implemented.

2. Materials and Methods

2.1. Soil Collecting

Soil samples were collected in 2021–2022 to compare wild population sites in Cumbria and Essex (Lots Field and Oxley Meadow) to two potential introduction sites where the orchids had previously failed to establish (Jack Scout and Humphrey Head) and one new potential site for future introduction (Gait Barrows) to determine their similarity and, therefore, suitability. At each sample spot, a trowel was used to dig away the top vegetation and put ~250 g of topsoil into Ziploc bags. Three samples were collected at each site. At The Lots, the orchids were found in the entire area, so three locations were arbitrarily chosen about 10 m apart to sample within the site, and all samples were pooled as a single sample. At Humphrey Head, all three samples were taken in an area where there is a small existing orchid population, and the samples were pooled. At Jack Scout, all three samples were taken close to the plants present on the site. At Gait Barrows, all three samples were taken within a meter of the only orchid present. Soil samples were collected from four sites in Cumbria (Figure 3), and lab analyses were performed to identify the soil characteristics from each location. Oxley Meadow site has orchids, and soil samples were taken very close to the orchid.

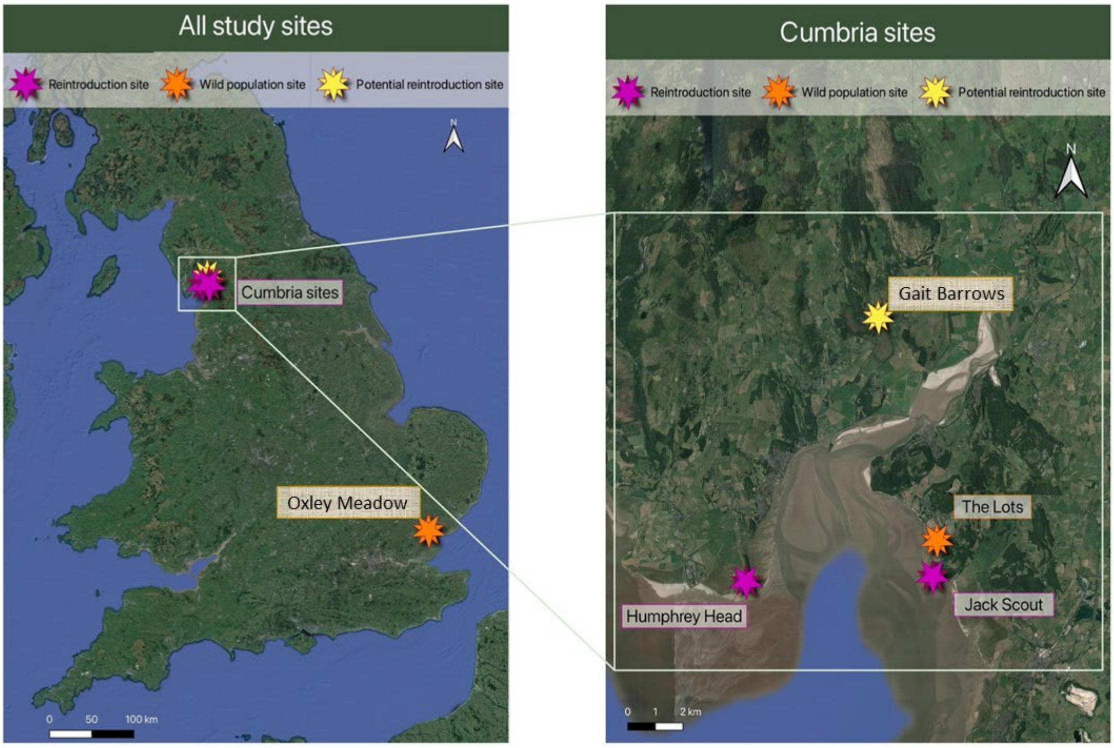


Figure 3. Map of all study sites in England, UK.

2.2. Soil Processing and Chemical Analyses

Fresh soil samples from individual plots were divided into two subsamples, and the first subsample was used for water content. The remaining subsamples were air-dried and passed through a 2 mm sieve before the following chemical analyses. Soil pH and electrical conductivity (EC) were measured in triplicate using calibrated HANNA HI8424 pH and EXTECH EC400 EC meters (Camlab, Cambridge, UK).

Soil nitrate was measured using calorimetry (cadmium reduction method) in triplicates. This process involved two stages of adding reagents. First, the NitraVer 6 was added to the diluted soil extract. After the reaction, the NitriVer 3 was added, and the colour intensity of the resultant solution was measured using a calibrated Hach DR900 (Camlab, Cambridge, UK) with a measurement wavelength of 520 nm; calibration involved the use of a blank sample of deionised water.

Similarly, phosphate was also determined in triplicates using colorimetry (USEPA Ascorbic Acid Method). This method is a one-stage process, adding PhosVer3 to the soil extract. The intensity of the colour of the resultant solution was measured using a calibrated Hach DR900 with a measurement wavelength of 610 nm.

2.3. Soil DNA Extraction

DNA extraction and purification were conducted as described by Dove et al. [14] on soil samples stored in BashingBead™ Lysis Tubes (Zymo Research, Cambridge Bioscience, UK) using Quick-DNATM Fecal/Soil Microbe Miniprep Kits (Zymo Research, USA (Zymo Research, Cambridge Bioscience, UK), following the manufacturer's instructions. The DNA concentration and quality of all 20 eluted samples were assessed using a Nanodrop 2000/2000c Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.4. Metabarcoding

DNA metabarcoding was conducted by Nature Metrics (Guildford, UK), as described by Dove et al. [15]. Detailed methodology is as follows.

DNA samples were purified and amplified by PCR in the internal transcribed spacer 2 (ITS2) region, targeting fungi as part of the eDNA survey fungi pipeline. The analysis included 3 replicate PCRs per sample, with the primers used in the metabarcoding step originating as described by White [24]. Sequences were quantified with a Qubit broad range kit (Thermofisher, Swindon, UK) following the manufacturer's protocol. The final library was sequenced using an Illumina MiSeq V3 kit (San Diego, CA, USA). Resulting sequence data went through processing using a specialised bioinformatics pipeline, clustering similar sequences into Operational Taxonomic Units (OTUs), and aligning a representative sequence from each cluster with a reference database.

Sequences were demultiplexed based on the combination of the i5 and i7 index tags with bcl2fastq (v2.20.0.422; https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversionsoftware.html (accessed on 28 September 2021). Paired-end FASTQ reads for each sample were merged with USEARCH v11 [25], requiring a minimum overlap of 80% of the total read length. Following this, dereplicated sequences were processed with ITSx [26] (v1.1b1;) to extract only fungal ITS2 sequences, removing the primers and any remaining ribosomal sequence. Unique ITS2 sequences from all samples were denoised following a single analysis with UNOISE [27], requiring retained zero-radius OTUs (ZOTUs). Taxonomic assignments were made using sequence similarity [28] searches of the ZOTU sequences against two reference databases—the NCBI nucleotide (NCBI nt; Available online: https://www.ncbi.nlm.nih.gov/nuccore/ (downloaded and accessed on 28 September 2021)) database and UNITE (v8.2). Hits were needed to ensure a minimum e-score of $1 \times 10-20$ and cover at least 90% of the query sequence.

Consensus taxonomic assignments were made for each OTU using sequence similarity searches against the UNITE (v8.2) and NCBI nt (GenBank) reference database. Where there was consistency in the matches, assignments were made to the lowest possible taxonomic level. Minimum similarity thresholds of 98%, 95%, and 92% were used for species-, genus-, and higher-level taxonomic groups, respectively. In cases where there were matches to multiple species, public records from GBIF were used to assess likely to be present in the United Kingdom.

When resolution was not satisfactory, higher-level taxonomic identifications or multiple potential identifications were provided. Afterward, the OTU table underwent filtering to exclude low-abundant OTUs from each sample, using a threshold of < 10 reads.

Minimum similarity thresholds of 98%, 95%, and 92% were applied for taxonomic groups such as species-, genus-, and higher-level assignments, respectively. Using the USEARCH tool to cluster OTUs, a 97% similarity threshold was used. In USEARCH, an OTU-by-sample table was generated by mapping dereplicated reads for each sample to the representative sequences of the OTUs at an identity threshold of 97%. Values in the resulting OTU table were calculated and expressed as copy numbers. Each sample is represented with the values indicating the number of reads obtained for each OTU sequence in the respective sample.

2.5. Statistical Analysis

Due to an unequal number of observations and variance, the effect of geographical location (Table 1) on soil properties was analysed using Welch ANOVA followed by Dunnet's post hoc test for pair-wise comparison of these locations. Pearson correlations (two-tailed with a confidence interval set at 95%) were computed between soil parameters (pH, EC, nitrate, phosphate), diversity indices (Shannon and Simpson), and fungal orders (abundance) using GraphPad Prism (V9 for Mac). The orders considered for the correlations were Cantharellales, Sebacinales, Agaricales Helotiales, and Hypocreales. Genus- and family-level analyses for correlation with soil characteristics were impossible due to insufficient data for all the key fungal groups. Heatmaps with Pearson's 'r' ranging from +1 (blue) to −1 (red).

Table 1. Locations and purposes of each study site.

Site Name	County (England)	Site Type
Lots Field (LF)	Cumbria	Wild site
Jack Scout (JS)	Cumbria	Potential receiver site
Humphrey Head (HH)	Cumbria	Potential receiver site
Gait Barrows (GB)	Cumbria	Potential receiver site
Oxley Meadow (OM)	Essex	Wild site

3. Results

3.1. Soil Analysis

The ANOVA results showed that the effect of locations was statistically significant for organic matter ($p < 0.0001$), EC ($p < 0.0001$), and phosphate ($p = 0.0264$); however, there was no significant effect of locations on pH ($p = 0.0562$) and nitrate ($p = 0.1767$). Pair-wise comparison of sites showed there was a significant difference between HH and LF as well as LF and OM for pH, as shown in Figure 4. Similarly, broadly, there were significant differences between sites for EC, whereas OM was significantly different from all other sites for OM.

There are significant differences between sample sites (Figure 4), especially for pH, electrical conductivity, and organic matter. Based on organic matter values, Oxley Meadow stands out as unique compared to all Cumbria sites. Humphrey Head and Oxley Meadow have similar electrical conductivity average values. Nitrate and phosphate content are also smaller for Oxley Meadow. Overall, Jack Scout is an outlier for nitrate and phosphate.

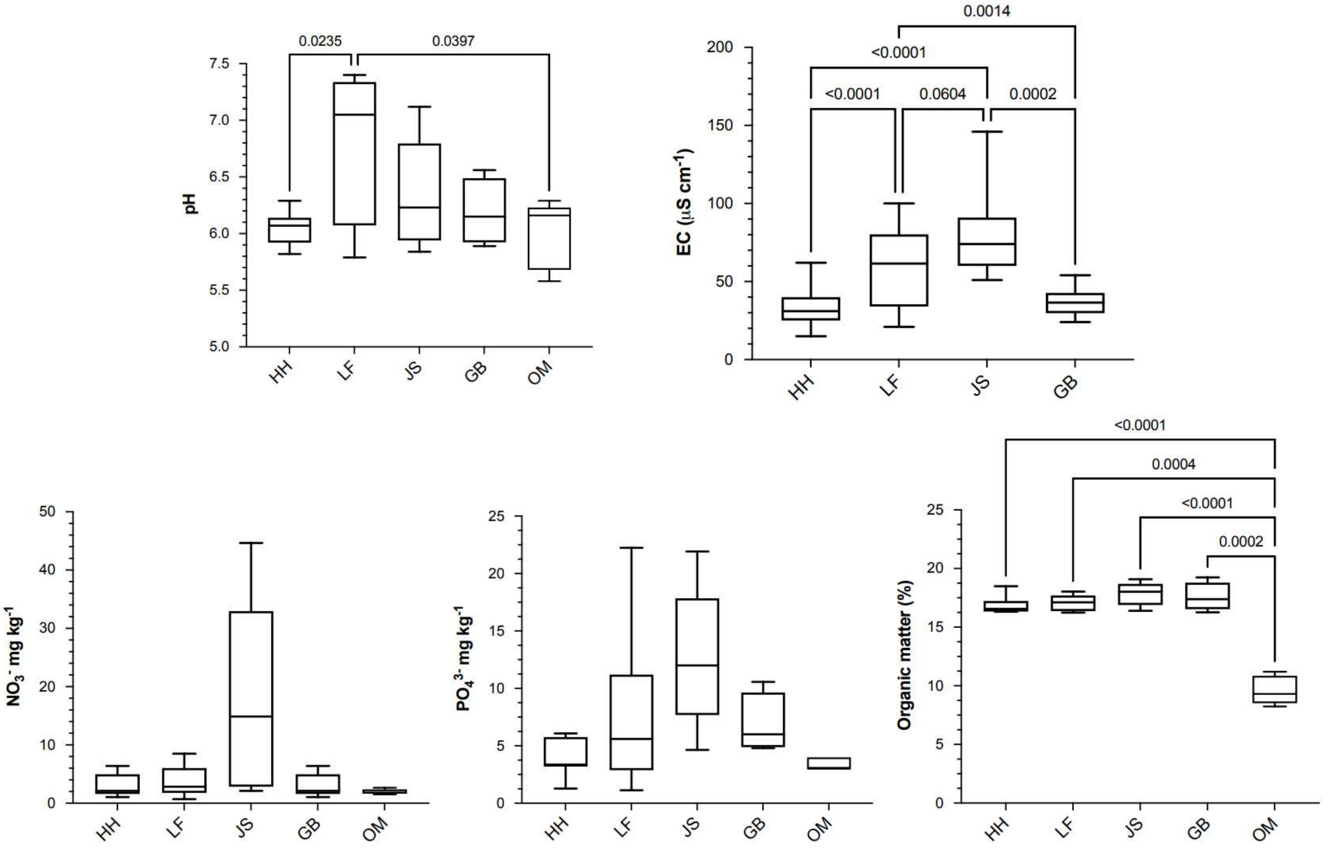
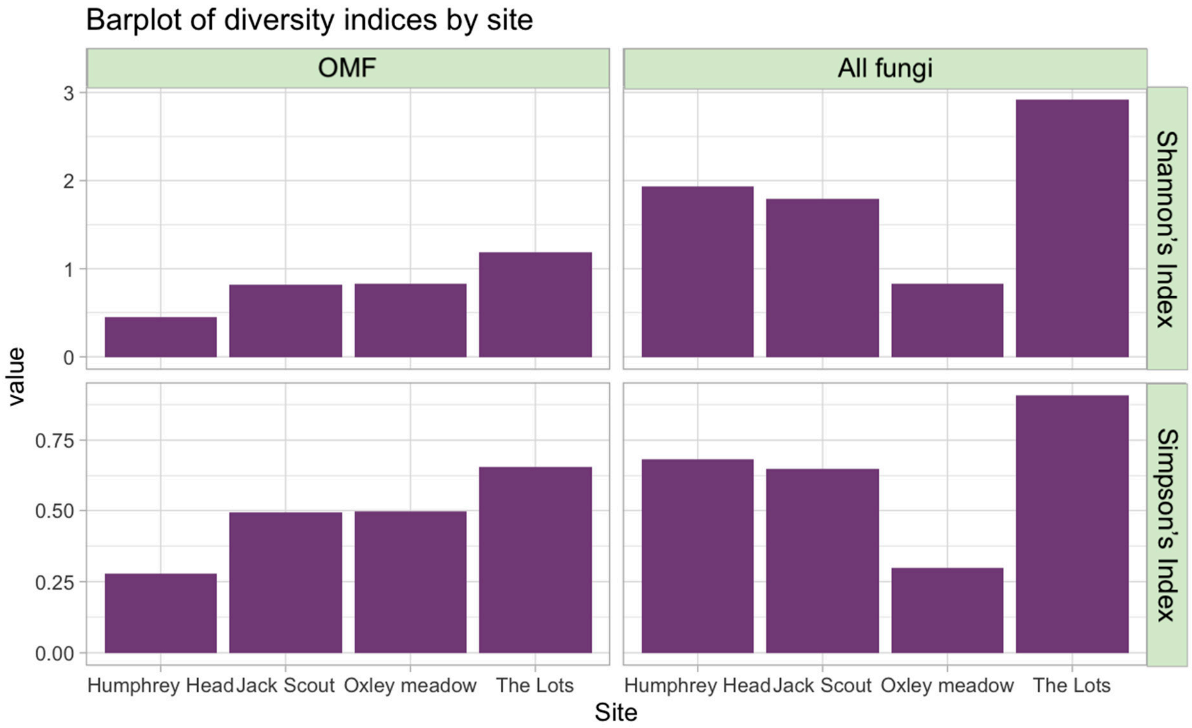


Figure 4. Boxplots (range with median line in the box) for each soil analysis (pH, electrical conductivity, nitrate, phosphate, and organic matter) showing the distribution of samples from each site in Cumbria (HH—Humphrey Head, LF—Lots Field, JS—Jack Scout, GB—Gait Barrows), and Oxley Meadow (OM).

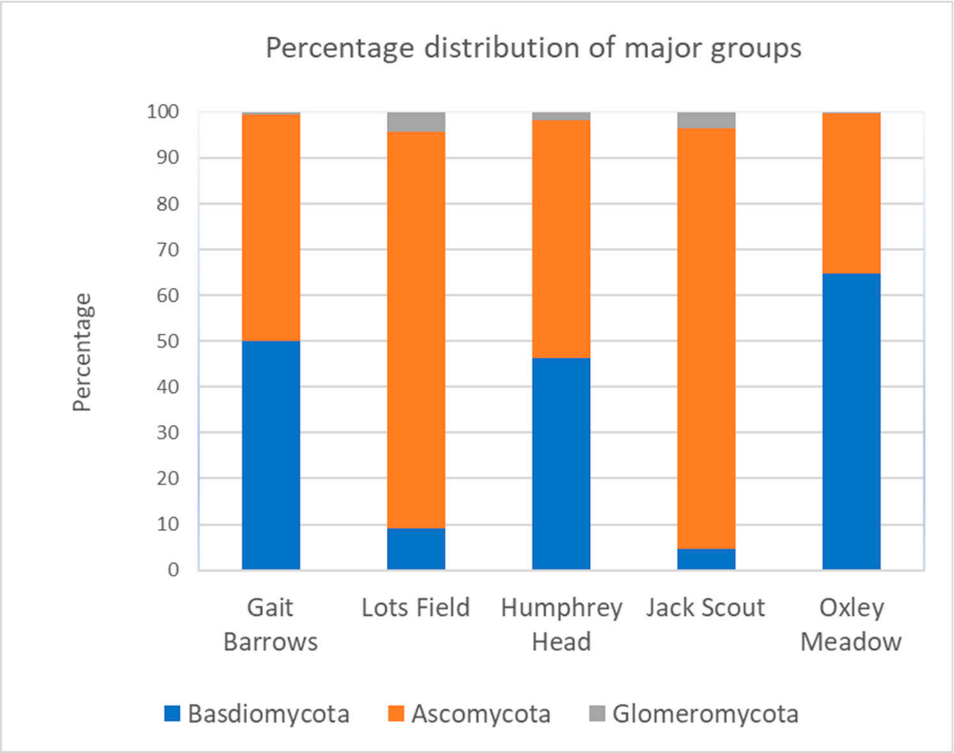
3.2. Fungal Distribution and Abundance

Both Simpson's diversity and Shannon's indices in the diversity plot (Figure 5a) indicate that Orchid mycorrhizal fungi (OMF) diversity is highest in The Lots site. Jack Scout and Oxley Meadow and Jack Scout were similar for mycorrhizal fungi, but the lowest level was recorded at Humphrey Head. However, a different pattern is found when looking at all fungi, where The Lots has the most diverse fungi composition of the four sites, but Oxley Meadow has the lowest. Jack Scout has a slightly lower diversity than Humphrey Head, but they are within the same range. When compared to the Essex site, Oxley Meadow (which has more than 65,000 green-winged plants), with Cumbrian wild site Lots Field and other potential Conservation Translocation sites, community composition and abundance values showed substantial differences. The proportion of Basidiomycota fungi (rare fungi) was greater in Oxley Meadow compared to all Cumbrian sites (Figure 5b). Except for the first location, other locations had a higher proportion of Basidiomycota fungi. Gait Barrows and Humphrey Head looked similar, although abundance values were quite high in Gait Barrows.

Gait Barrows had most OTUs (424) with almost equal distribution for Ascomycota and Basidiomycota while Oxley Meadow had 65% of the OTUs were from Basidiomycota from just 154 OTUs. Humphrey Head had 169 OTUs with more than 50% Basidiomycota while Lots Field had 226 OTUs with less than 10% of Basidiomycota. Jack Scout with 150 OTUs had more than 95%of the OTUs from Ascomycota, showing the highest proportion among all sites studied (Figure 6a–c).



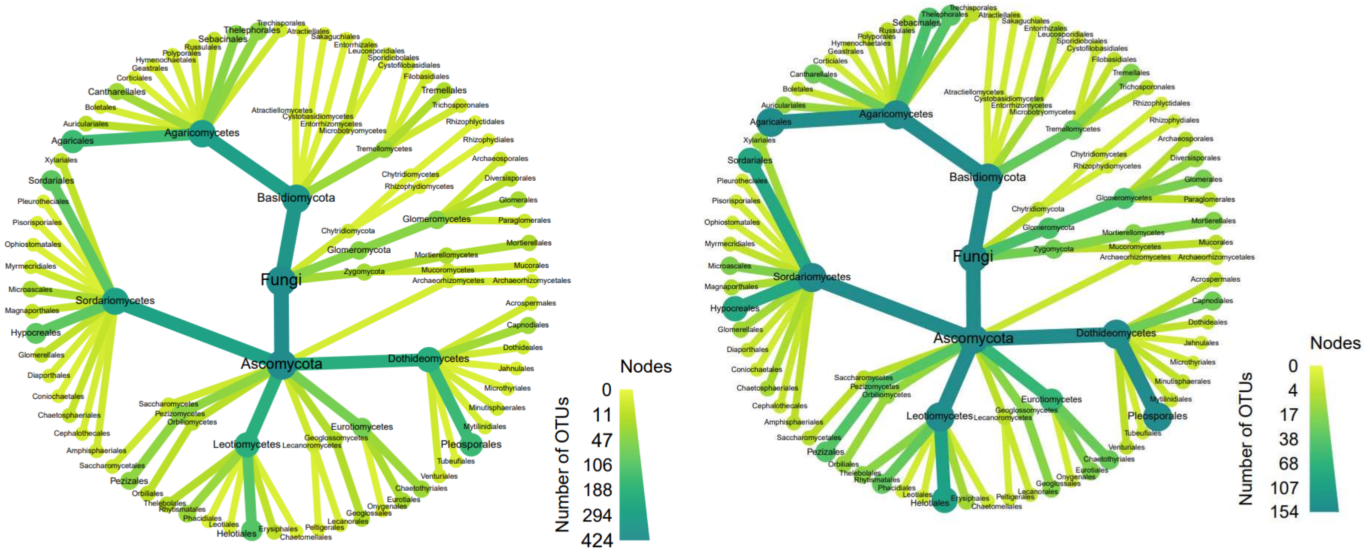
(**a**)



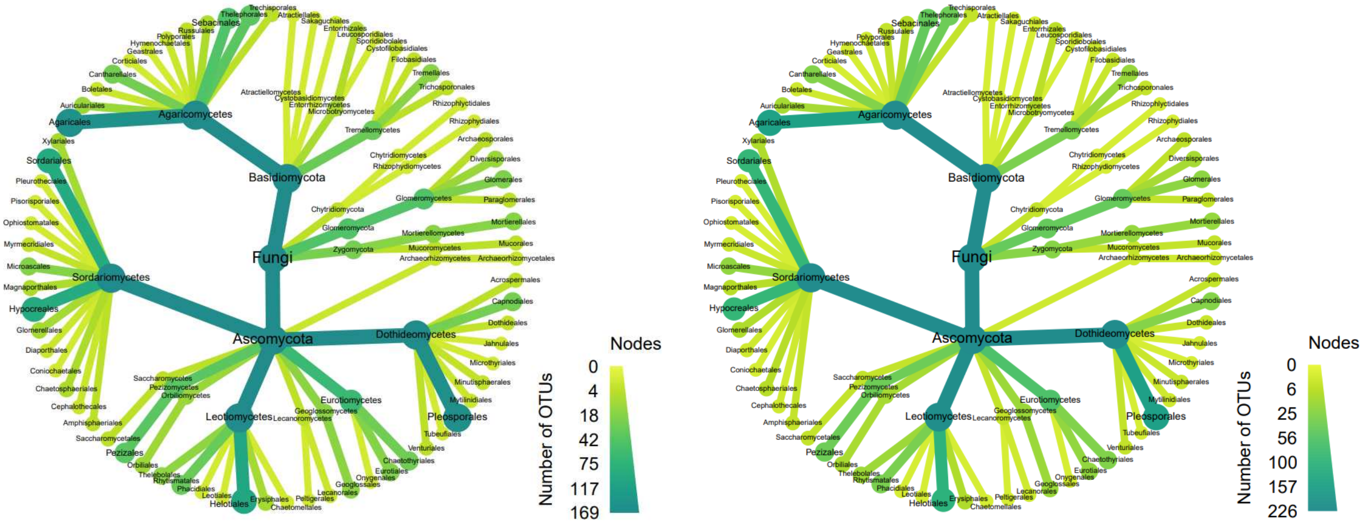
(**b**)

Figure 5. Diversity indices of families of orchid mycorrhizal fungi (OMF) and All fungi (**a**) for the four sites (Gait Barrow is not included in the analysis); and Community composition and abundant percentage of major fungal groups (**b**) in Cumbria (The Lots Field with wild population and potential Conservation Translocation sites such as Gait Barrows, Humphrey Head and Jack Scout), and Colchester, Essex (Oxley Meadow).

Helotiales and Hypocreales were the dominant groups of fungi in most orchid habitats. When compared with mycorrhizal fungal orders (Figure 7), Oxley Meadow showed an extremely low abundance of Hypocrealeas, with the potential Conservation Translocation site Jack Scout in Cumbria showing the highest.

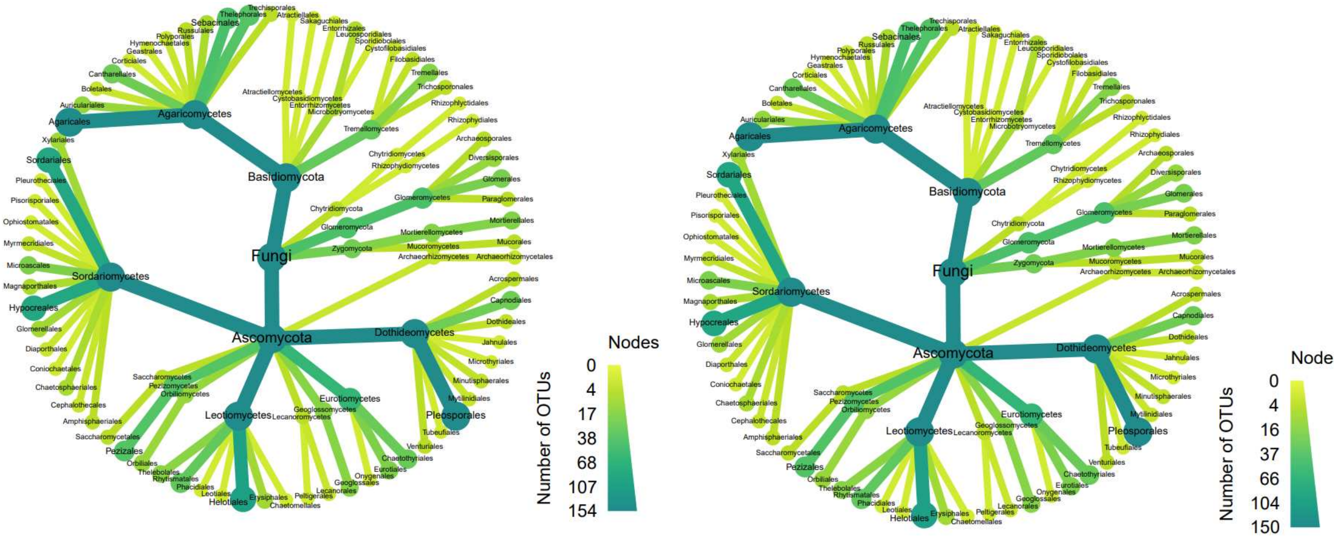


Gait Barrows **(a)** Oxley Meadow



Humphrey Head **(b)** Lots Field

Figure 6. *Cont.*



(c)

Oxley Meadow

Jack Scout

Figure 6. Heat trees comparing for number of OTUs of Ascomycota and Basidiomycota at Gait Barrows with Oxley Meadow (**a**); Humphrey Head with Lots Field (**b**); and Oxley Meadow with Jack Scout (**c**).

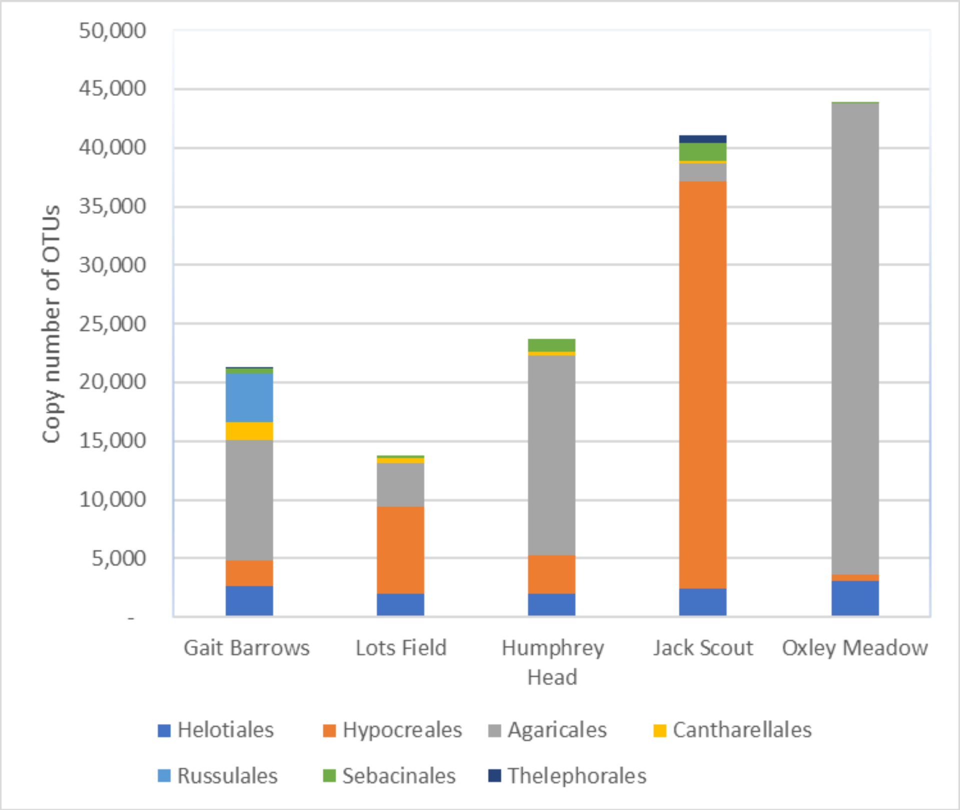


Figure 7. Community composition and abundance of selected orders of fungi in Cumbria (The Lots Field with wild population and potential Conservation Translocation sites such as Gait Barrows, Humphrey Head, and Jack Scout) and Colchester, Essex (Oxley Meadow).

Ceratobasidiaceae OTUs were recorded from all sites except Lots Field (with wild orchids present), with Gait Barrows showing the highest number of OTUs. Sebacinaceae

OTUs were present at all sites, with Gait Barrows with the highest number of OTUs and abundance, followed by Oxley Meadow for the number of OTUs (Figure 8b). When key mycorrhizal families were assessed, except for Jack Scout, soils from both Cumbrian sites and Oxley Meadow hosted OTUs of Hygrophoraceae (Figure 8b). There are similarities between the Cumbrian potential Conservation Translocation site, Humphrey Head, and the Oxley Meadow in Essex, especially in relation to the abundance of Hygrophoraceae, wax cap fungi, and some mycorrhizal families (Figure 8). However, Humphrey Head is an exception for its diversity of 'taxa', which has less Hygrophoraceae than Oxley Meadow with only narrow diversity (Figure 9). Humphrey Head hosts nine OTUs (combined total reads of just under 12,300) of wax cap fungi compared to two OTUs (*Gliophorus psittacinus* with 40,498 reads and another OTU *Hygrocybe* 1 with 70,043 reads) at Oxley Meadow. The abundance of these two OTUs at OM is nearly 10 times that of HH.

Agaricales abundance is negatively correlated with Hypocreales and Helotiales, the two most abundant Ascomycetous fungi found in most grassland landscapes. As Hygrophoraceae is the most abundant Agariclaes family, this is significant. The abundance of Agaricales is negatively correlated with soil characteristics such as pH, phosphate, and nitrate (Figure 10).

Correlation analysis showed a negative correlation for Agaricales with pH, phosphate, and nitrate in Oxley Meadow. Similarly, Cantharellales and Sebacinales abundance was negatively correlated with organic matter (Figure 10). However, the Ascomycota orders Helotiales and Hypocreales were positively correlated with pH, nitrate, and phosphate in Oxley Meadow. Nevertheless, these were not statistically significant.

Gait Barrows was selected for comparison with Oxley Meadow as this site has the greatest number of OTUs with almost equal distribution of Ascomycota and Basidiomycota fungi. When Gait Barrows soil abiotic components were tested for correlation with an abundance of key fungal groups, the main finding was the positive and significant ($p = 0.045$) correlation recorded for Hypocreales and Cantharellales with phosphate (Figure 11).

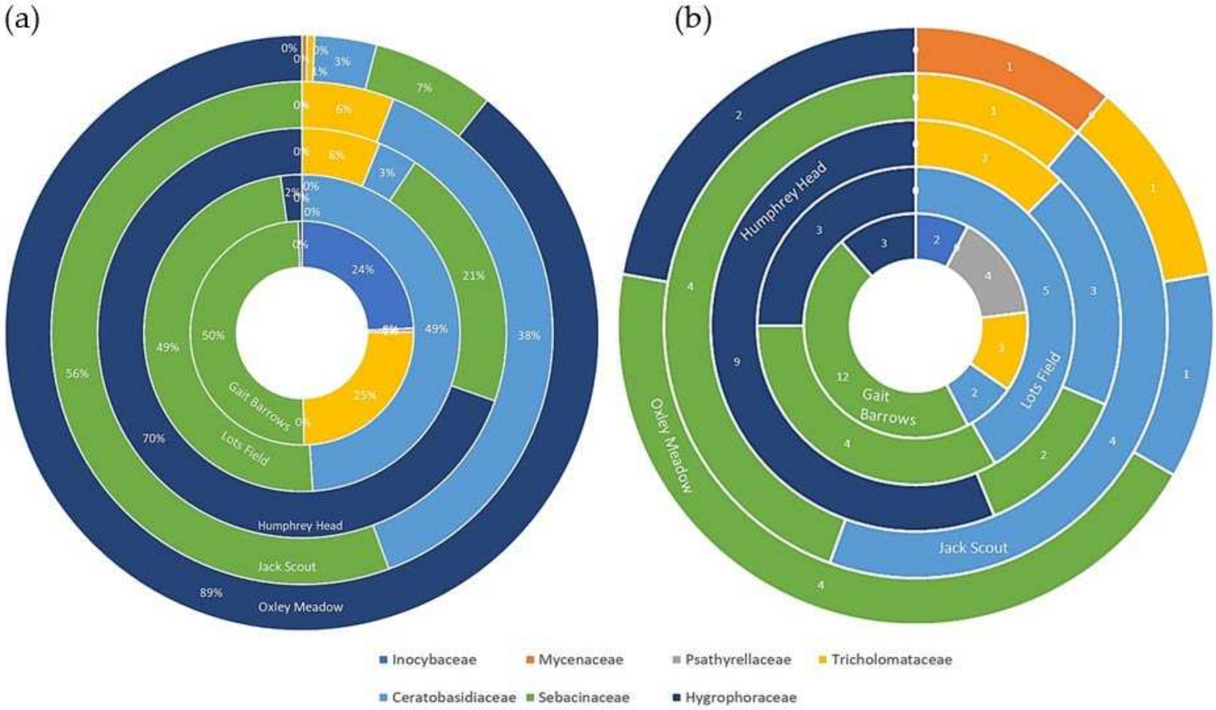


Figure 8. Abundance based on read numbers (relative abundance of the selected families), (**a**) and diversity (number of OTUs), (**b**) for the key mycorrhizal families at various sites in Cumbria (Gait Barrows, Jack Scout, Humphrey Head, and Lots Field) and Essex (Oxley Meadow).

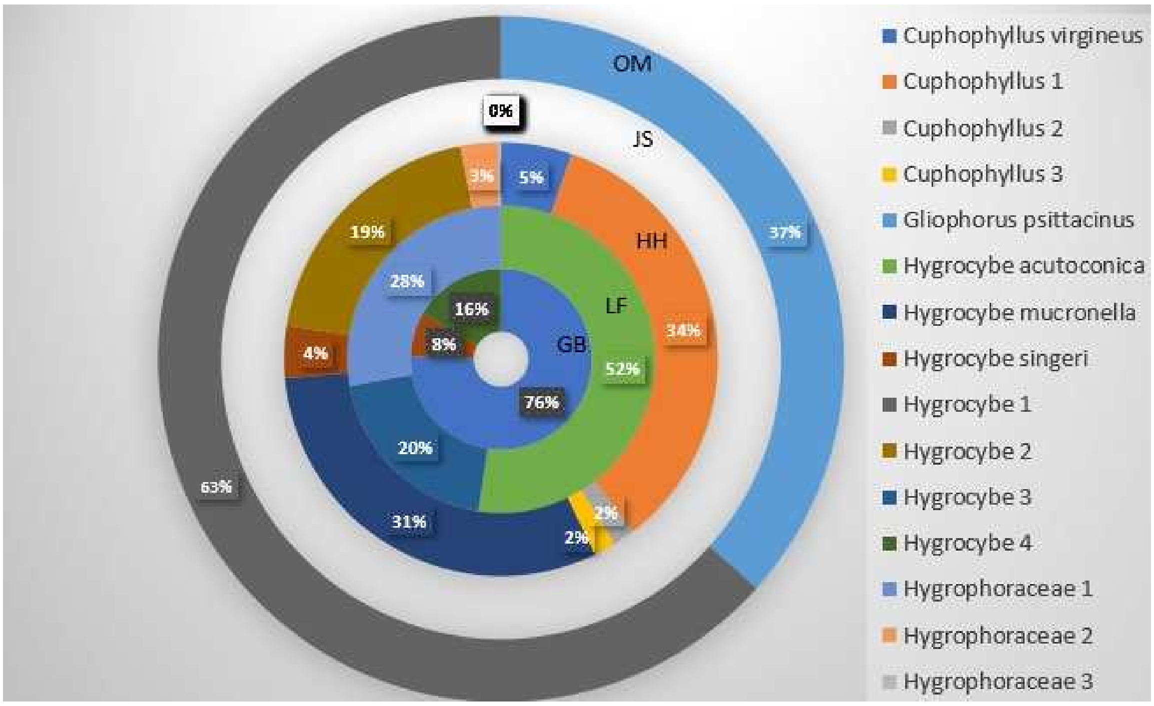
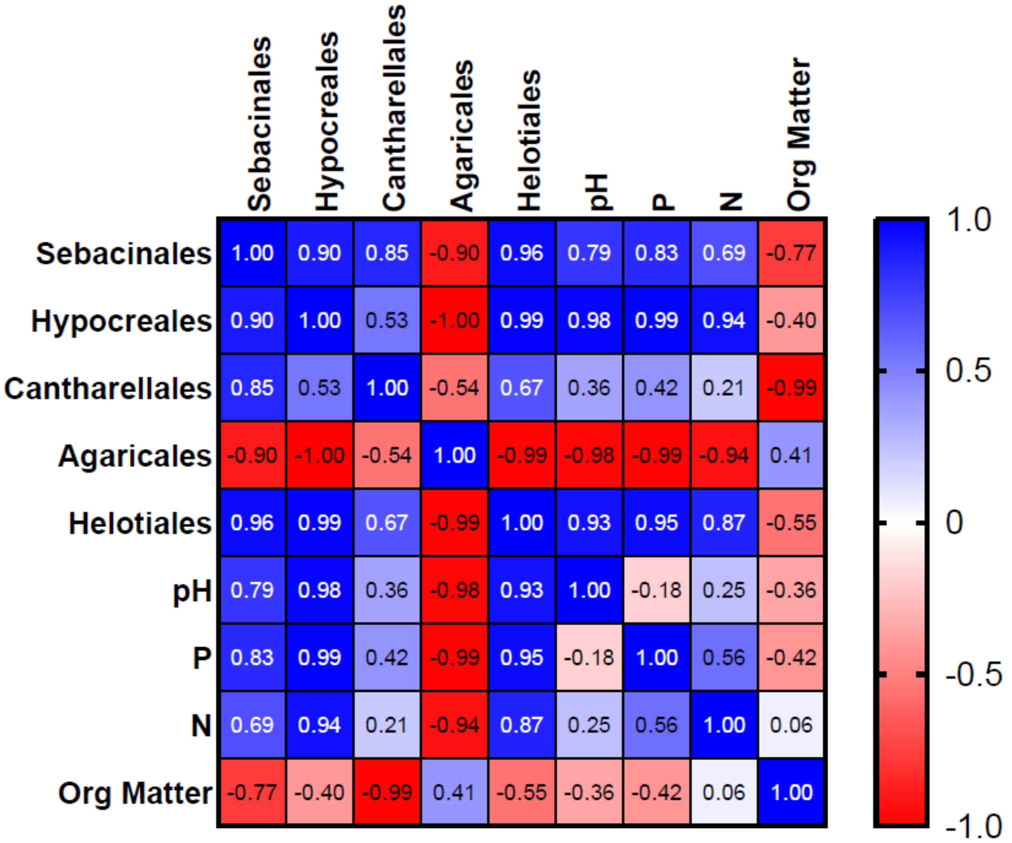
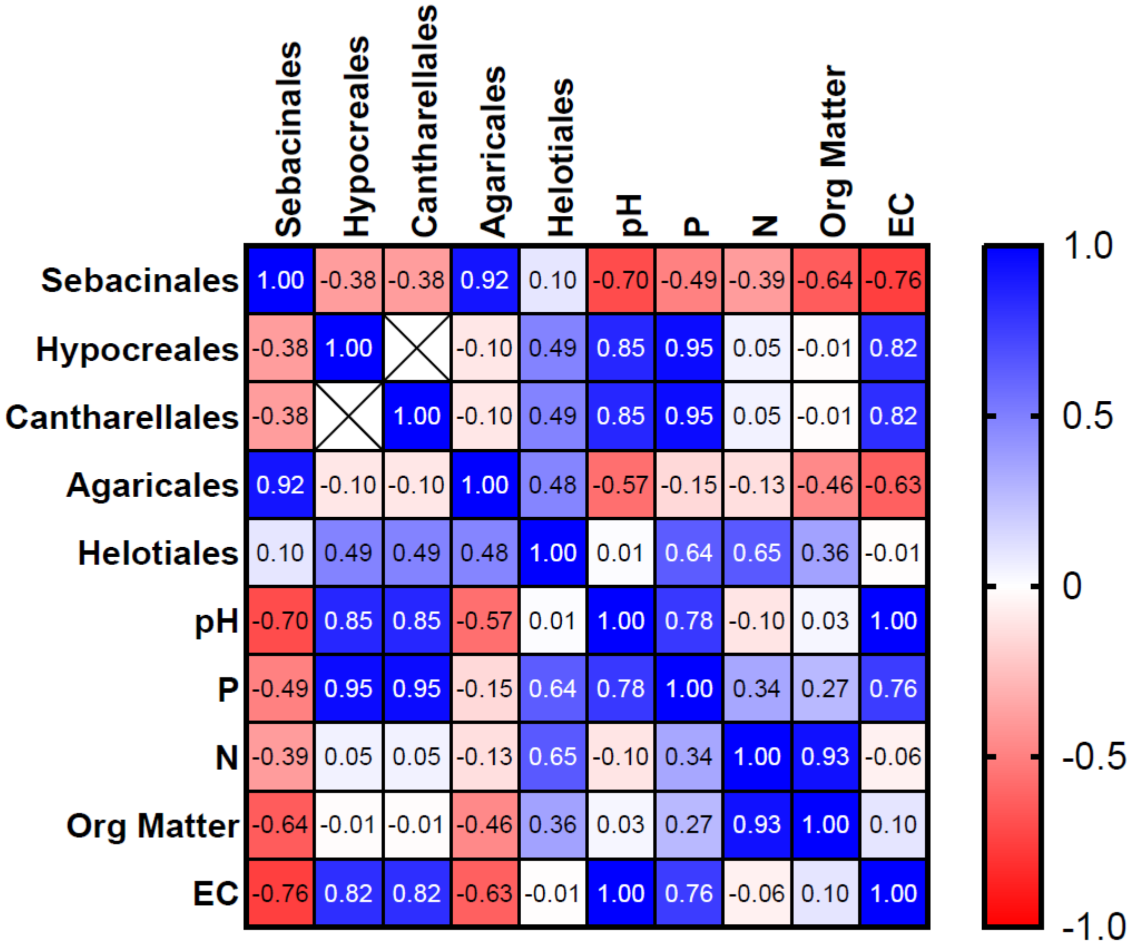


Figure 9. Taxa and relative abundance values based on copy number of OTUs for Hygrophoraceae in Cumbria (Lots Field (LF) with wild population and potential Conservation Translocation sites such as Gait Barrows (GB), Humphrey Head (HH) and Jack Scout, (JS)) and Colchester, Essex (Oxley Meadow, OM).



	Sebacinales	Hypocreales	Canthareliales	Agaricales	Helotiales	pH	P	N	Org Matter
Sebacinales	1.00	0.90	0.85	-0.90	0.96	0.79	0.83	0.69	-0.77
Hypocreales	0.90	1.00	0.53	-1.00	0.99	0.98	0.99	0.94	-0.40
Canthareliales	0.85	0.53	1.00	-0.54	0.67	0.36	0.42	0.21	-0.99
Agaricales	-0.90	-1.00	-0.54	1.00	-0.99	-0.98	-0.99	-0.94	0.41
Helotiales	0.96	0.99	0.67	-0.99	1.00	0.93	0.95	0.87	-0.55
pH	0.79	0.98	0.36	-0.98	0.93	1.00	-0.18	0.25	-0.36
P	0.83	0.99	0.42	-0.99	0.95	-0.18	1.00	0.56	-0.42
N	0.69	0.94	0.21	-0.94	0.87	0.25	0.56	1.00	0.06
Org Matter	-0.77	-0.40	-0.99	0.41	-0.55	-0.36	-0.42	0.06	1.00

Figure 10. Correlation analysis of key groups of fungi in relation to pH, nitrate, phosphate, and organic matter at Oxley Meadow in Essex.



	Sebacinales	Hypocreales	Cantharellales	Agaricales	Helotiales	pH	P	N	Org Matter	EC
Sebacinales	1.00	-0.38	-0.38	0.92	0.10	-0.70	-0.49	-0.39	-0.64	-0.76
Hypocreales	-0.38	1.00	☒	-0.10	0.49	0.85	0.95	0.05	-0.01	0.82
Cantharellales	-0.38	☒	1.00	-0.10	0.49	0.85	0.95	0.05	-0.01	0.82
Agaricales	0.92	-0.10	-0.10	1.00	0.48	-0.57	-0.15	-0.13	-0.46	-0.63
Helotiales	0.10	0.49	0.49	0.48	1.00	0.01	0.64	0.65	0.36	-0.01
pH	-0.70	0.85	0.85	-0.57	0.01	1.00	0.78	-0.10	0.03	1.00
P	-0.49	0.95	0.95	-0.15	0.64	0.78	1.00	0.34	0.27	0.76
N	-0.39	0.05	0.05	-0.13	0.65	-0.10	0.34	1.00	0.93	-0.06
Org Matter	-0.64	-0.01	-0.01	-0.46	0.36	0.03	0.27	0.93	1.00	0.10
EC	-0.76	0.82	0.82	-0.63	-0.01	1.00	0.76	-0.06	0.10	1.00

Figure 11. Correlation analysis of key groups of fungi in relation to pH, nitrate, phosphate, and organic matter at Gait Barrow in Cumbria.

4. Discussion

The current study found organic matter content in Cumbrian sites was higher than in Oxley Meadow in Essex (with nearly 65,000 green-winged orchid plants). It is not explicitly clear why Oxley Meadow has lower organic matter except the fact that the Cumbrian sites are managed farmlands grazed by cattle, which must have contributed to the increased organic content. Oxley Meadow in Essex is also a grassland site that is mowed, but no cattle are used for the grassland management. Terrestrial orchid colonisation is underpinned by poor nutrient content, which means soil health is a primary factor [16], with low nitrate and phosphate levels being important for *Cynorkis* spp. Orchids are considered canaries in the coal mine, especially terrestrial ones, as successful colonisation is better in unimproved habitats [16]. Discovered low nitrate and phosphate levels were important for *Cynorkis* spp. Soil organic matter influences the distribution and abundance of orchids [29,30]. A major study on soils and orchid distribution, including *A. morio* in Serbia, found that ultramafic soils, natural metalliferous soils derived from the weathering of ultramafic rocks, are low in nitrate and phosphate [31]. Changes in the community composition and abundance of mycorrhizal fungi can have an influence on orchid distribution and fitness [32].

Heat trees of fungal community composition as a number of OTUs showed interesting readings. The potential Conservation Translocation sites Gait Barrow (GB) and Jack Scout (JS) in Cumbria may not be suitable based on their proportions of common and rare fungi. Compared to Oxley Meadow, common and rare fungi were distributed equally in GB, while in JS, the abundant fungi were 95%. Rare fungi, Basidiomycota, in Oxley Meadow were 65% with Humphrey Head hosting 50% Basidiomycota OTUs compared to Lots Field with 10% of Basidiomycota. This is interesting as Lots Field has already obtained nearly 10,000 plants of GWO. This means further studies are required both temporally and spatially to obtain better quality data that can be used for detailed statistical analyses, which are lacking in this study.

Ascomycetous fungal groups such as Helotiales and Hypocreales are known to be distributed abundantly in orchid habitats [15]. On the other hand, Basidiomycetous fungi with most of the orchid mycorrhizal fungal groups can be considered 'rare' fungi [33]. Oxley Meadow (OM), with nearly 65,000 green-winged orchid plants, when compared to the wild site in Cumbria and other potential Conservation Translocation sites, was found to have a proportion of Basidiomycota fungi greater than OM compared to all Cumbrian sites. The Cumbrian potential Conservation Translocation site Humphrey Head is, in some ways, similar to OM in the diversity of fungi and abundance values for key mycorrhizal fungal groups.

It was difficult to conduct correlation analysis for fungal families with soil abiotic characteristics as the sample size was small. When correlation analysis was conducted with a few selected abundant orders of fungi, it was noticed that a negative correlation was recorded for Agaricales with pH, phosphate, and nitrate in Oxley Meadow. Hygrocybe, a genus from Hygrophoraceae (waxcap fungi), is known to inhabit unimproved grasslands [34] like orchid mycorrhizal fungi as orchids prefer nutrient-poor soils [35]. Interestingly, the abundant saprophytic fungal orders such as Hypocreales and Helotiales were less abundant in Oxley Meadow as there is a string negative correlation existed with Agaricales. All sites except Jack Scout recorded less than 10 mg/kg of total available phosphate in the soil. This was also the case for total available nitrate. Therefore, Jack Scout is an outlier for these two key minerals, which probably demonstrates that fungal distribution and abundance are different in Jack Scout compared to Oxley meadow, which has the highest number of green-winged orchids in the UK.

Jack Scout showed the highest Ascomycota to Basidiomycota ratio compared to any other site) and was the only site without Hygrophoraceae. In Europe, pastures were characterized by an increased abundance of Hygrophoraceae, mainly represented by *Hygrocybe* spp., especially in nutrient-poor pastures [36]. The presence and high abundance of waxcap fungi, Hygrophoraceae taxa, in these habitats may not be critical for GWO colonisation, but from all the data collected, it demonstrates it may have a positive effect on soil hosting the GWO, especially for Oxley Meadow in Essex. According to Lodge et al. [37], it may be construed as *Hygrocybe* spp. being not only saprotrophic but also functional as mutualists. The abundance value for waxcap fungi recorded from Oxley Meadow may demonstrate that soil conditions are optimum for both green-winged orchids and Hygrophoraceae waxcap fungi. Whether a strong correlation exists must be studied in detail. It may be feasible that Oxley Meadow is compatible with the orchid due to the right soil conditions abiotically, and that is supplemented by the high abundance of waxcap fungi. In a detailed study, if this can be proved, potential Conservation Translocation sites can be selected based on orchid mycorrhizal fungi and fungal groups such as waxcap fungi, which may have a mutualistic role.

Cantharellales abundance was negatively correlated with organic matter. However, the Ascomycota orders Helotiales and Hypocreales were positively correlated with pH, nitrate, and phosphate in Oxley Meadow. The abundance of Agaricales was negatively correlated with Sebacinales in Oxley Meadow; however, a positive correlation was recorded between them in Gait Barrows, a potential Conservation Translocation site. In Gait Barrows, the abundance of Cantharellales was positively correlated with pH and phosphate. This indicates, through a direct comparison with Oxley Meadow, that Gait Barrow may not be a compatible site. It is interesting when potential translocation sites such as Gait Barrow and Jack Scout with no existing orchid population showed the presence of many lineages of OMFs. Compared to other vascular plants that host ecto- and arbuscular mycorrhizal fungi, orchids have a relatively reduced root system [38]. Typically, OMFs could use their host plants for survival and persistence in the environment [39,40]. They could eventually be stimulated to grow and proliferate into orchid roots as a mechanism to prevail and colonise.

Therefore, OMFs distribute closer to orchids, which may contribute to their restricted distribution in soil. OMF abundance has been found to be positively correlated with orchid abundance [19], and while OMF diversity and its relationship with orchid success have not been studied in detail, there is reason to believe they could be linked [41]. Further studies at a temporal and greater landscape level will help understand this better.

When looking at the overall fungal community, diversity is highest in Lots Field, the wild site for GWO in Cumbria, which also has the highest number of fungus families. Overall diversity for mycorrhizal families is greater in Gait Barrows, and the lowest numbers are shared by Oxley Meadow and Jack Scout. Hygrophoraceae, waxcap fungi, are rare in many grazed and mown nutrient-poor grasslands, which are abundant in both Oxley Meadow and Humphrey Head. Some of the taxa from Hygrophoraceae could be potentially mycorrhizal as has been reported before [42]. They concluded that Hygrophoraceae are unlikely to be saprotrophs and are probably in symbiosis with bryophytes or other understory plants. While certain species of Hygrophoraceae are ectomycorrhizal and form associations with trees, they have not been shown to associate with orchids [37]. However, the presence and high abundance of waxcap fungi could be considered indicators of the overall health of the grassland. As reported before, they are sensitive to anthropogenic disturbance, which makes them excellent indicator organisms for the status of natural and semi-natural grassland [43].

The waxcap fungal hotspots are usually found in biocide-free; unimproved; or semi-improved, grazed, or mown grasslands with minimum soil disturbance and long periods of ecological continuity [44,45]. This fits in with Oxley Meadow and Humphrey Head, of which Oxley Meadow is a mown grassland with an extremely high colonisation of GWO. Considering the high level of abundance of two different types of waxcap fungi and GWO plants, this site is of exceptional significance for protection as these fungi can be considered bioindicators [44].

The study shows that in addition to the community composition and their abundance values for orchid mycorrhizal fungi, key fungal groups like Hygrophoraceae shed light on the quality of the grassland at Oxley Meadow. Any site which has near-optimal similarity would be a compatible site to host GWO. Further studies on orchid colonisation and waxcap fungi in unimproved grasslands in Britain may help understand a hitherto unknown link between soil health and unique fungal groups.

5. Conclusions

This study was limited in space, time, and sample size. Future studies must explore both soil and root samples at different times of the year, spreading larger areas where green-winged orchids colonise successfully. Within these large landscapes, microsites must also be studied for diverse groups of minerals in addition to nitrate and phosphate, as this may help understand habitat preferences for all groups of orchid mycorrhizal fungi. As waxcap fungi, Hygrophoraceae, are a threatened group of fungi, their distribution and abundance in unimproved grasslands will help understand soil health in the context of orchid distribution and diversity. Most terrestrial orchids are present in unimproved or nutrient-poor habitats. Studying other key fungal groups in addition to orchid mycorrhizal fungi will help understand factors that influence orchid rarity.

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