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Strong altitudinal partitioning in the distributions of ectomycorrhizal fungi along a short (300 m) elevation gradient

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Summary

- Changes in species richness and distributions of ectomycorrhizal (ECM) fungal communities along altitudinal gradients have been attributed to changes in both host distributions and abiotic variables. However, few studies have considered altitudinal relationships of ECM fungi associated with a single host to identify the role of abiotic drivers. To address this, ECM fungal communities associated with one host were assessed along five altitudinal transects in Scotland.
- Roots of Scots pine (*Pinus sylvestris*) were collected from sites between 300 and 550-600 m altitude and ECM fungal communities were identified by 454 pyrosequencing of the fungal ITS region. Soil moisture, temperature, pH, C : N ratio and organic matter content were measured as potential predictors of fungal species richness and community composition.
- Altitude did not affect species richness of ECM fungal communities but strongly influenced fungal community composition. Shifts in community composition along the altitudinal gradient were most clearly related to changes in soil moisture and temperature.
- Our results show that a 300 metre altitudinal gradient produced distinct shifts in ECM fungal communities associated with a single host, and that this pattern was strongly related to climatic variables. This finding suggests significant climatic niche partitioning among ECM fungal species.

Keywords: Climate, Ectomycorrhizal, Elevation, Forest, Ordination, *Pinus sylvestris*, Soil temperature

Introduction

Altitudinal gradients are characterised by decreasing temperature with increasing altitude (Körner, 2007). Precipitation, solar radiation and atmospheric pollution deposition can also vary with elevation thus creating complex environmental changes along elevation gradients (Körner, 2007). How these changes impact on ecosystems has been studied for many years (Rahbek, 1995) and patterns of species richness, composition and productivity have been identified for a broad range of organisms in relation to changing altitude (Kitayama & Aiba, 2002; McCain, 2004; Romdal & Grytnes, 2007; Hoiss *et al.*, 2012). The availability of high-throughput molecular techniques in recent years has enabled the investigation of altitudinal patterns in diversity to be extended to micro-organisms (Bryant *et al.*, 2008; Geml *et al.*, 2014).

Ectomycorrhizal (ECM) fungi are symbionts of many woody plants, including many temperate and boreal tree species (Smith & Read, 2008). Previous investigations have indicated that, in common with many other organisms, the species richness of ECM fungi can be affected by altitude. Fungal richness has been observed to either decrease at high altitude (Kernaghan & Harper, 2001; Bahram *et al.*, 2012), or peak at mid-altitude (Miyamoto *et al.*, 2014), patterns which are commonly observed in both macro- and micro-organisms (Rahbek, 1995; Bryant *et al.*, 2008). Declines in species richness of ECM fungi with altitude have been attributed to harsher climatic conditions, decreased energy availability and/or less favourable soil conditions at high altitude while the mid-domain effect has been hypothesised to cause a mid-altitude peak in richness (Kernaghan & Harper, 2001; Bahram *et al.*, 2012; Miyamoto *et al.*, 2014). In addition to changes in species richness, several studies have shown changes in the composition of ECM fungal communities with altitude (Kernaghan & Harper, 2001; Bahram *et al.*, 2012; Scattolin *et al.*, 2014).

The richness and composition of ECM fungal communities are known to be influenced by a range of factors including host identity and environmental conditions, both of which can change with altitude. The identity of the host species has been consistently found to be a strong determinant of ECM fungal communities (Ishida *et al.*, 2007; Tedersoo *et al.*, 2008, 2013). Changes in host vegetation with altitude are therefore likely to be important in producing patterns in ECM fungal diversity with altitude. In a study of altitudinal gradients in Iran, host identity was shown to have a greater effect on community change than abiotic variation (Bahram *et al.*, 2012). As yet, little research has focused on the role of abiotic

drivers in producing altitudinal patterns in ectomycorrhizal communities. Previous studies have identified climatic variation as an important driver of ectomycorrhizal species richness and community composition at both local and global scales (Tedersoo *et al.*, 2012; Jarvis *et al.*, 2013) suggesting that changes in climate along altitudinal gradients could affect ECM communities. ECM fungi might also be affected by changes in pH and other soil characteristics occurring along altitudinal gradients (Coince *et al.*, 2014). By focusing on a single host species it is possible investigate abiotic drivers of community composition independently of host vegetation change. Two recent studies have used this approach to observe changes in ECM communities associated with holm oak (*Quercus ilex* L.) and beech (*Fagus sylvatica* L.) along altitudinal gradients (Scattolin *et al.*, 2014; Coince *et al.*, 2014). Scattolin *et al*. found altitude was a driver of ECM community change in *Quercus ilex* forests in the absence of host vegetation change, but no information was gathered on potential abiotic drivers and they did not investigate changes in diversity. Coince *et al.* found a change in community composition with altitude in beech forests but no change in species richness along a 1400 m elevation gradient. Both temperature and soil pH were found to be related to differences in community composition (Coince *et al.*, 2014). Identifying the abiotic drivers of altitudinal differences in ECM communities is important in understanding whether fungal communities are likely to respond to environmental change in a similar way to their host plants, for example shifting their ranges upwards in response to increased temperatures (Lenoir *et al.*, 2008).

In order to link changes in fungal communities along altitudinal gradients to abiotic variation it is necessary to collect suitable data on potential environmental drivers. In particular, data need to be collected at a high enough resolution to capture any variation associated with altitudinal changes. This can be difficult, particularly for climate related variables where high resolution data are lacking, and previous studies have relied on interpolated large scale climate data to produce explanatory variables for community analysis (Bahram *et al.*, 2012; Coince *et al.*, 2014). These data are likely to be poor representations of altitudinal variation in climate because the resolution of the data (typically 1-5 km) is too low to capture variation in topography. Long term measurements of air temperature and rainfall are prohibitively costly to obtain at a local scale and single time point measurements are unlikely to be informative due to large seasonal and diurnal variations. Soil temperature and moisture generally fluctuate less than air temperature and rainfall (Paul *et al.*, 2004) and single point

measurements may provide a useful alternative measure to capture altitudinal climatic variation, particularly for soil dwelling organisms such as ectomycorrhizal fungi.

In this study we investigated the ectomycorrhizal communities occurring along an altitudinal gradient using a single host (Scots pine; *Pinus sylvestris* L.) and focused on the roles of abiotic drivers of community composition, including soil temperature and moisture. Previous work has identified the native pinewoods of Scotland as suitable forest type in which to study climatic impacts on ECM fungi as the pinewoods occur over a wide climatic range and in a predominantly monospecific stand structure (Jarvis *et al.*, 2013). The pinewoods occur at elevations between just above sea level on the west coast of Scotland up to the current semi-natural treeline of Scots pine at 700-750 metres in central Scotland (Steven & Carlisle, 1959). The natural treeline in Scotland occurs at low altitudes due to the oceanic climate; air temperature decreases with altitude at a rate of up to 1°C per 100m (Grace, 1997) producing significant temperature differences over short altitudinal gradients. By utilising field measurements of climatic variables it was possible to investigate the effects of altitudinal variation on fungal communities at a smaller spatial scale and at a higher sampling resolution (every 50 metres elevation) than previously possible. The current study investigated the effect of altitude on ECM communities in one native Scots pine forest in the Cairngorm mountain range in the UK.

Materials and Methods

Study site

An area of Scots pine (*Pinus sylvestris* L.) forest, approximately 9 km^2, in the Cairngorms, Scotland was identified which includes an altitudinal range between 300 and 600 metres above sea level (latitude 57.122, longitude -3.818 at centre of study area). The forest is on the northern to western slopes of the Cairngorm mountain range and the majority of the area has been continuously forested since at least the 1700s (Steven & Carlisle, 1959). Ground vegetation mainly consisted of ericoid species, including *Calluna vulgaris* (L.) Hull and *Vaccinium* spp. (Bunce, 1977). The only other ECM hosts present are occasional birch (*Betula* spp.) trees. Soil types range from humus-iron and peaty podzols on the north-facing slopes to peaty subalpine soils on the western slopes (Soil Indicators for Scottish Soils, http://sifss.macaulay.ac.uk). Five altitudinal transects were demarcated, each ranging from 300 m to 550 or 600 m altitude (Figure 1, Table 1). Transects were at least 300 m apart and sampling sites were positioned at 50 m altitudinal intervals (Figure 1). A barometric altimeter (Suunto, Finland) was used to determine altitude in the field. Samples were collected between late July and early September 2011.

Sample collection

At each 50 metre sampling interval on each transect, ECM roots were collected from three trees at three points around each tree. Trees were selected to be as close to the sampling altitude as possible and were approximately 5 metres apart. Sampling points were 1.5 m away from the base of the tree and roughly equidistant from each other. At each point a 15 cm x 15 cm x 10 cm deep soil sample was taken from which roots were extracted in the field. A total of 288 samples were collected (32 transect points with three trees each and three samples per tree). Soil samples from the same sampling points were also collected, pooled per tree and placed in separate polythene bags. All root and soil samples were stored chilled and then returned to the laboratory within four days. At each sampling point, soil temperature and soil moisture at 6 cm depth were measured with a temperature probe attached to a Diligence EV N2011 datalogger (Comark, Norwich, UK) and a theta probe attached to a HH2 datalogger (Delta T Devices, Cambridge, UK), respectively. Five, one year old, needles from one branch on each tree were collected for analysis of needle carbon and nitrogen.

Needle and soil chemistry

Soil samples were air dried at 30°C and sieved before organic matter content was calculated by loss on ignition at 450°C in a muffle furnace for 12 hours. Soil pH was measured from 2.5 g of air dried soil in 45 ml of deionised water using a Jenway 3310 pH meter. Total carbon and nitrogen in the soil and needles was measured using a Fisons NA 15000 NCS elemental analyser.

Preparation of amplicon libraries

Root samples were stored at -20°C until processed. Each sample was defrosted and 20 g of root material cleaned under a dissecting microscope to remove any adhering organic matter and roots of other plant species. The number of ectomycorrhizal root tips on cleaned root systems was counted and the cleaned fine roots (< 2 mm diameter) were placed into 2 ml Retsch® tubes then lyophilised. Lyophilised roots were milled to a fine powder in the microcentrifuge tubes with stainless steel beads using an adaptor rack in a Retsch® mill. Root samples were pooled for each tree (three samples per tree, 96 trees in total). DNA was extracted from 50 mg of milled root material using a Qiagen DNeasy Plant Mini kit following the manufacturer's instructions. DNA extracts from each tree were then pooled within sampling points to produce 32 samples in total.

Amplicon libraries for each transect point were prepared by amplifying the fungal ITS2 region using primers fITS7 and ITS4 (Ihrmark *et al.*, 2012). The ITS4 primer was tagged with an 8 base region ('tag') which allows identification of samples after multiplexed sequencing. Each sampling point was assigned a separate tag with at least two bases difference between tags. PCR reactions consisted of 500 nM primer fITS7 (5'-3'; GTGARTCATCGAATCTTTG), 300 nM primer ITS4 (5'-3'; XXXXXXXXTCCTCCGCTTATTGATATGC, where X indicates a base belonging to the multiplex identifier tag), 2.75 mM $MgCl_2$, 0.19 mM of each dNTP, 0.5 units of DreamTaq Taq polymerase (Thermo Fisher Scientific, Waltham, MA), 1x DreamGreen buffer (Thermo Fisher Scientific, Waltham, MA), 0.2 µg/µl bovine serum albumin (BSA), 10 µl DNA template and 3.88 µl of deionised water to a final reaction volume of 20 µl. Thermal cycling conditions were as follows; initial denaturing at 94°C for 5 minutes followed by 31 - 35 cycles of 94°C for 30 seconds, 56.8°C for 30 seconds and 72°C for 30 seconds and a final elongation step at 72°C for 7 minutes. The number of PCR cycles was adjusted per sample based on the DNA concentration of extracts to avoid entering the lag phase of PCR. Negative

controls, where sterile deionised water was added in place of DNA extract, were added to all PCR runs and no products were observed to originate from control samples.

Five PCR replicates were conducted per sample to account for possible variation in the communities recovered from individual PCR runs (Tedersoo *et al.*, 2010b). PCR replicates were pooled and purified using an AMPure PCR purification kit (Beckman Coulter, High Wycombe, UK). DNA concentrations of PCR products were measured using a QuBIT 2.0 Fluorometer (Life Technologies Ltd, Paisley, UK). PCR products were pooled in equimolar concentrations to create the final amplicon library and purified a second time with a GeneJET PCR purification kit (Thermo Fisher Scientific, Waltham, MA). The amplicon library was checked for final DNA concentration with the QuBIT fluorometer and for purity with a BioAnalyzer (Agilent Technologies Ltd, Wokingham, UK). Adaptor sequences were ligated to amplicons and sequencing was performed on a quarter of a pyrosequencing plate using Titanium chemistry on a GLX 454 sequencer (Roche Diagnostics Ltd, Burgess Hill, UK).

Bioinformatic analyses

Reads and quality scores obtained from the pyrosequencing platform were filtered in QIIME v. 1.6.0 (Caporaso *et al.*, 2010) to remove low quality reads where the average Phred score was less than 25, sequence length was less than 200 base pairs or where there were any mismatches in primer or tag sequences. In addition, a sliding window quality check of 50 base pairs was applied to identify low quality regions (average Phred score less than 25) and any reads with low quality regions were removed. Due to the adaptor ligation process, reads were present in both 5'-3' and 3'-5' orientations, therefore quality filtering and demultiplexing were applied to both the raw reads and the reverse complement of reads and quality scores to retain the maximum number of sequences. Filtered reads were used as input for denoising with Denoiser (Reeder & Knight, 2010) implemented in QIIME using the flowgram files provided by the sequencing centre. Due to the variation in orientation between reads, denoising was applied to reads in each orientation separately. Flowgram files are archived at the NCBI Sequence Read Archive under BioProject PRJNA253816.

Denoised sequences were reorientated where necessary to 5' to 3' direction and clustered to determine operational taxonomic units (OTUs). Clustering was performed at 97% similarity to avoid producing artefactual OTUs that can be produced at higher clustering thresholds (Tedersoo *et al.*, 2010b). Open reference based clustering with the UClust method was

212 conducted in QIIME using a combined UNITE and INSD fungal database as the reference
213 database (version 12_11, available from http://qiime.org/home_static/dataFiles.html). OTUs
214 represented by only one read were removed as these are likely to represent sequencing errors
215 (Tedersoo *et al.*, 2010b).

216 Resulting OTU assignments were individually checked by BLAST against the live UNITE
217 database (http://unite.ut.ee) or the INSD database if no match was found in the UNITE
218 database. Individual checking of OTU assignments allowed the identification of potential
219 chimeric sequences by detection of partial matches. Reads identified as originating from non-
220 ECM taxa (following Tedersoo *et al.*, 2010a) were removed from the dataset to restrict the
221 analysis to taxa with evidence of ectomycorrhizal status. To avoid assignment errors due to
222 tag switching (Carlsen *et al.*, 2012) if an OTU occurred at an abundance of at least 100 reads
223 in one sample, and then occurred as a single read in another sample, it was excluded from the
224 second sample.

225 *Statistical analyses*

226 All analyses were conducted in R v.3.0.0 (R Core Team, 2013). To investigate whether soil
227 and needle variables changed over the altitudinal gradient, linear mixed models were
228 constructed with altitude as a fixed effect and transect as a random effect. Models were built
229 using the nlme package (Pinheiro *et al.*, 2012). To allow for potential differences in the
230 altitude effect between transects, a random slope effect of altitude conditioned on transect
231 was included and retained in the model if the variance in slopes was greater than zero. The
232 significance of the fixed effect was tested using a *t* statistic. Where model diagnostic plots
233 showed heterogeneity of variance, weights terms were added to the models to allow variance
234 to differ between transects. Organic matter content was arcsine transformed to approximate a
235 normal distribution before analysis.

236 Relationships between the number of 454 readsand number of root tips collected per sample
237 with altitude were assessed using generalised linear mixed models with Poisson distributions.
238 There was no evidence that the number of pyrosequencing reads was related to the number of
239 OTUs per sample (Supporting Information Figure S1), suggesting a high level of sequencing
240 redundancy. However, inspection of OTU accumulation curves for each sample showed that
241 not all samples had been sequenced to extinction (Supporting Information Figure 2).
242 Therefore, rarefaction was conducted using the sample with the lowest number of reads as the

sampling size and the relationship between rarefied OTU richness and altitude was assessed with a linear mixed model.Altitude was included in all models as both a linear and a quadratic term to allow for a mid-altitude peak in richness. A sample based OTU accumulation curve was fitted using the 'specaccum' function in the vegan package (Oksanen *et al.*, 2011) and the predicted local species richness was estimated with the Chao2 estimator (Chao, 1987).

Community composition was analysed based on the presence or absence of OTUs as there are known issues with quantitative use of read numbers from 454 sequencing (Amend *et al.*, 2010). OTUs that occurred in only one sample were removed before analysis as rare OTUs can have a large impact on ordination results (Poos & Jackson, 2012). Differences in OTU community composition between samples were assessed using non-metric multidimensional scaling with Sørensen distances using the vegan package. To minimise the stress of the ordination a three dimensional solution was used. Vector fitting of altitude, transect and measured environmental variables was carried out using the 'envfit' function to assess significant variables affecting community structure. To check for an influence of variation in sequencing depth between samples on community composition the number of reads was also fitted as a vector. Permutations were restricted to within transects using the 'strata' argument. Because there were more replicates of the environmental measures (96 samples, one sample per tree) than the community data (32 samples), environmental conditions at each transect point were averaged. Spatial autocorrelation in community composition was tested for using partial Mantel tests after accounting for variation due to abiotic variables.

Relationships between the occurrence of individual OTUs and altitude were assessed by binary logistic regression, using the presence or absence of a particular OTU as the response variable. Relationships were also assessed after grouping OTUs into genera to assess whether similar responses were seen at a higher taxonomic level. Transect was included as a random intercept term in all models. As only two transects had sampling plots at 600m, this elevation was removed from the regression analysis. At the OTU level, only OTUs with three or more occurrences were included in the models (38 OTUs). Models were fitted with both linear and quadratic altitudinal terms and the best model chosen using the Akaike information criterion (AIC).

Results

Change in environmental variables with altitude

High altitude sampling points had lower soil temperature and higher soil moisture than low altitude sites (Figure 2). Soil temperature decreased by 0.48 ± 0.17 °C with every 50 m increase in altitude (β = -0.01, $P < 0.001$) (Figure 2a). Soil C : N ratio also decreased with altitude (β = -0.026, $P = 0.004$) while soil moisture increased (β = 0.07, $P < 0.001$) (Figure 2b,c). There was no change in needle C : N ratio or soil pH (Figure 2d,e). Soil organic matter content showed a quadratic relationship with the highest values at mid-altitude (linear β = 0.002, $P = 0.002$, quadratic $\beta = 3x10^{-6}$, $P < 0.001$) (Figure 2f). Variation in intercepts between transects were observed in all variables but altitudinal trends were similar along transects, except for soil temperature which declined most rapidly along transect five (Figure 2 a).

Fungal community description

A total of 232,290 sequence reads were obtained from pyrosequencing. After filtering and assignment to tags 165,347 quality checked ectomycorrhizal reads were recovered with a mean of 5,166 reads per sample (Supporting Information Table S1). The number of root tips estimated during the root cleaning process was 26,262 across all 32 samples, suggesting a high level of sequencing redundancy. A total of 64 ectomycorrhizal OTUs were delimited at 97% clustering, from 18 different genera (Table 2). The number of OTUs per sample was not related to the number of root tips sampled, nor to the number of reads obtained (Supporting Information Figure S1). Read numbers were massively dominated by *Suillus variegatus* which comprised 75% of all reads in this study (Table 2). Relatively low diversity of ascomycete taxa was recorded with only 15 reads from two OTUs being assigned to ascomycete taxa.

Altitude had a significant quadratic relationship with the number of tips per sample (linear z = 6.21, $P < 0.001$; quadratic z = -6.59, $P < 0.001$) and number of reads per sample (linear z = 15.74, $P < 0.001$; quadratic z = -14.59, $P < 0.001$) with the highest numbers of tips and reads at intermediate altitudes. However, there was no relationship between altitude and the rarefied OTU richness per sample, nor was there any trend in the total number of species per altitudinal zone (Supporting Information Table S2). One sample outlier was observed with a

much higher number of reads, attributed to either error in preparing the final amplicon mix or tag bias during later steps.

Computation of an OTU accumulation curve (Figure 3) indicated that taking more samples would have increased the number of OTUs recovered as sampling did not appear to be saturated,. The Chao2 species richness estimator predicted a local diversity of 87.1 ± 14.8 species.

Ordination and vector fitting showed altitude, soil moisture, soil temperature, pH and organic matter content to be significantly correlated with community structure (Figure 4, Table 3). Two main axes of variation were recovered, one related to altitude and climatic effects and the other to soil pH and organic matter content. The partial Mantel test did not identify any spatial autocorrelation in community composition was identified once environmental variation was accounted for. The number of reads was not a significant vector in the ordination indicating the variation in read numbers between samples did not influence the ordination structure. R code to produce an interactive three dimensional visualisation of the ordination is available in Supporting Information Note S1.

Relationships between fungal taxa and altitude

Relationships between the occurrence of individual taxa and altitude were assessed by binary logistic models. Relationships were assessed at both OTU and genus levels (Figure 5a-f). Five OTUs and one genus were observed to show significant patterns of occurrence in relation to altitude. *Piloderma sphaerosporum* (Figure 5b) was most frequent in low altitude sites while *Russula sardonia* (Figure 5c) was most frequent at higher altitudes. Quadratic relationships were seen in three taxa with *Cortinarius semisanguineus* (Figure 5a) and an unknown Cantharellales (Figure 5e) peaking in occurrence at mid altitudes while an unknown Atheliaceae OTU was most frequent at low and high altitudes (Figure 5d). At a genus level only *Russula* displayed a significant pattern, increasing in occurrence with altitude (Figure 5f). Further inspection of the OTU data showed that three other *Russula* species were also found more often at high altitudes but were too infrequent for a significant pattern to be observed at the OTU level (data not shown).

Discussion

Altitude was a major driver of variation in fungal community composition in the absence of host vegetation change. A 300 metre difference in altitude was associated with large changes in measured climate and soil variables, and in the community composition of ECM fungi. Ordination suggested that variation in soil moisture and temperature were important drivers of community variation along the transects studied, supporting previous findings of an influence of climatic variables on ECM fungal communities (Bahram *et al.*, 2012; Jarvis *et al.*, 2013; Coince *et al.*, 2014). Soil temperature and moisture were highly correlated and it was not possible to separate their effects on fungal communities. Both variables have been shown to influence fungal communities in experimental conditions (Allison & Treseder, 2008; Deslippe *et al.*, 2011; Richard *et al.*, 2011) and it is likely that fungi respond to a combination of both variables through a variety of mechanisms. Soil organic matter and pH were also found to affect community structure supporting previous results (Rosling *et al.*, 2003; Kjøller & Clemmensen, 2009; Geml *et al.*, 2014; Coince *et al.*, 2014). Here, variation in soil organic matter and pH was not as strongly correlated with altitude as the climatic variables and appeared to explain variation in community composition not related to altitudinal differences.

Soil temperature and moisture were only measured on a single occasion and could not capture seasonal variation and extreme events that may influence the fungal community. Despite this problem, both measurements were strongly correlated with altitude, suggesting the single measurements did reflect real gradients. Soil temperature decreased by approximately 1°C per 100m, and as soil temperature is likely to be less variable than air temperature, a similar or larger change in air temperature would be expected. Although single measurements will never fully describe the climatic conditions, soil temperature and moisture may be more appropriate explanatory variables than low resolution interpolated climate data for altitudinal studies focusing on fungal communities. It is also worth considering that altitude may influence communities through mechanisms which were not assessed in this study. In particular exposure to high winds has strong influences on the vegetation at high altitudes and high exposure has also been linked to changes in fungal communities (Kernaghan & Harper, 2001; Scattolin *et al.*, 2008).

Analysing the community response to altitude further we found that several OTUs showed significant relationships with altitude, with some taxa occurring more frequently at low

363 altitude and others at high altitude. Unfortunately, it was not possible to investigate
364 relationships between individual taxa and the measured environmental variables due to the
365 high covariance between variables and low number of replicates. However, it is worth noting
366 that *Piloderma sphaerosporum*, shown here to have a preference for low altitude sites, was
367 found to be strongly associated with low rainfall and soil moisture in a larger scale study of
368 the same ecosystem (Jarvis *et al.*, 2013). *Piloderma* taxa have been observed to occur more
369 often at low altitudes by Bahram *et al.* (2012) and Kernaghan and Harper (2001), although it
370 was not possible to attribute this observation to abiotic variables in either study. Soil moisture
371 has previously been hypothesised as an important variable in niche segregation of ECM fungi
372 as fungi have been observed to have relatively narrow range of optimal soil moisture contents
373 (Erland & Taylor, 2002). The large variation in soil moisture observed could have
374 contributed to the community shift observed along the short altitudinal gradient in this study.
375 There was no evidence that the turnover in fungal community was related to changes in plant
376 nutrient status as there was no change in host needle carbon to nitrogen ratio along the
377 altitudinal gradient.

378 Many of the OTUs recovered in this study occurred too infrequently for statistical
379 relationships with altitude to be assessed, a common feature of fungal studies where many
380 taxa are rare. To avoid this problem relationships can be analysed after grouping species at
381 higher taxonomic levels e.g. genus or lineage (Tedersoo *et al.*, 2010a; Bahram *et al.*, 2012).
382 In this analysis, the occurrence of the genus *Russula* increased with altitude but only one
383 *Russula* species had sufficient occurrences to produce a significant species level response.
384 Investigation of the less frequent *Russula* species suggested that all followed the same
385 pattern, with increasing occurrence at higher altitudes, demonstrating the utility of grouping
386 taxa into genera. However, grouping makes the assumption that all taxa within a genus or
387 lineage have a shared response. Only one species each from the *Cortinarius* and *Piloderma*
388 genera showed a significant relationship with altitude and neither genus showed an overall
389 relationship. For *Piloderma* this effect is explained by low frequency of the other species but
390 several *Cortinarius* species were equally frequent yet showed no relationship with altitude.
391 Species within a genus, therefore, may not always share environmental preferences;
392 intrageneric niche differentiation in ECM fungi has been observed in several contexts
393 (Rosling *et al.*, 2003; Beiler *et al.*, 2012) and could also occur along altitudinal gradients.

In contrast to the clear response of community composition to altitude there was no change in the species richness of ECM fungi along the altitudinal gradients surveyed. Some previous studies have suggested that species richness of ECM fungi declines at high altitude (Kernaghan & Harper, 2001; Bahram *et al.*, 2012) or peaks at mid-altitude (Gómez-Hernández *et al.*, 2011; Miyamoto *et al.*, 2014) but neither pattern was supported here. Interestingly, Coince *et al.* (2014) also found no relationship between diversity and altitude in their study of fungal communities of a single host species. They suggest that the changes in species richness observed in previous studies may have been related to changes in host plant diversity which were not controlled for (Coince *et al.*, 2014), although Kernaghan and Harper (2001) found no relationship between host and ECM richness along an altitudinal gradient. Alternatively, the failure to fully saturate the sampling curve may explain the absence of a richness relationship in our study as it was not possible to identify all species present. In addition, although the gradients sampled here represented the entire altitudinal extent of *Pinus sylvestris* at the study site, ectomycorrhizal hosts also occur above the treeline in the form of dwarf shrubs such as *Arctostaphylos uva-ursi* (L.) Spreng. If the mechanism behind altitudinal changes in ECM diversity is not host-specific, for example decreased energy availability at high altitude, it may be that a pattern may emerge by including a larger altitudinal range.

This study has demonstrated that an altitudinal gradient of only 300 metres can produce shifts in fungal community composition without host vegetation change. Both soil temperature and soil moisture were correlated with altitudinal changes in community composition while soil pH and organic matter content were associated with community variation unrelated to altitude. The study highlights the importance of local scale climatic variation in maintaining local diversity of fungal communities and the findings indicate that ECM fungi might be expected to respond to climate change through upwards expansion of their range with potential consequences for high elevation ecosystems.

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550 **Figure legends**

551

552 **Figure 1.** Map of altitudinal transects used to investigate changes in ectomycorrhizal fungal
553 communities of *Pinus sylvestris*. The extent of the native pinewood is indicated by the shaded
554 area (Caledonian Pinewood Inventory, Forestry Commission), some sampling points appear
555 to be outside this area due to slight inaccuracies in the resolution of spatial mapping and
556 because some small clumps of trees are not included in the outline. The altitudinal gradient is
557 shown by the topographical contours at 50 metre intervals (Ordnance Survey Land-Form
558 PANORAMA, downloaded from http://edina.ac.uk/digimap). Insert shows the location of the
559 study area in Scotland. Both maps are orientated so that the top of the map faces north.

560 **Figure 2.** Relationships between soil temperature (a), soil moisture (b), soil C : N ratio (c),
561 needle C : N ratio (d), soil pH (e) and soil organic matter content (f) and altitude along five
562 altitudinal transects in Scotland. Transects are distinguished by different symbols as shown
563 by the legend in Figure 2 a. Random intercepts for each transect were included in all models
564 and for soil temperature the slope of the relationship was also allowed to vary between
565 transects. Lines indicate the predicted relationships from linear mixed models. †Variable is
566 arcsine transformed.

567 **Figure 3.** Rarefaction curve of number of ectomycorrhizal operational taxonomic units
568 against the number of samples taken. Dashed grey lines indicate the standard deviation of the
569 rarefaction curve.

570 **Figure 4.** Non-metric multidimensional scaling (NMDS) plot of ectomycorrhizal
571 communities of *Pinus sylvestris* along an altitudinal gradient (stress of the three dimensional
572 solution = 0.18). Each sample on the NMDS is represented by the altitude and a symbol
573 which identifies the transect as shown in Figure 2. Abbreviations are as follows: OM, organic
574 matter.

575 **Figure 5.** Significant relationships between ECM fungal OTUs and genera and altitude along
576 an altitudinal gradient. Plots represent significant relationships between *Cortinarius*
577 *semisanguineus* (a; linear $\beta = -5.125$, $P = 0.039$; quadratic $\beta = -6.254$, $P = 0.056$), *Piloderma*
578 *sphaerosporum* (b; $\beta = -2.506$, $P = 0.013$) *Russula sardonia* (c; $\beta = 1.017$, $P < 0.001$),
579 Uncultured Atheliaceae OTU 2 (d; linear $\beta = -0.511$, $P = 0.252$; quadratic $\beta = 1.124$, $P =$
580 0.043), Uncultured Cantharellales OTU 2 (e; linear $\beta = 0.8134$, $P = 0.230$; quadratic $\beta =$ -

581 2.067, $P = 0.026$), the genus Russula (f; $\beta = 1.501$, $P = 0.027$) and altitude. The size of the
582 points reflects the number of transects where the species was present or absent (the larger the
583 point, the more transects it represents).

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Number of operational taxonomic units recovered plotted against the number of ectomycorrhizal root tips and number of pyrosequencing reads

Fig. S2 Sequence based rarefaction curves for each sample

Table S1 Number of root tips, pyrosequencing reads and OTUs per sample

Table S2 Total number of taxa at each altitude sampled

Note S1 R code to produce interactive 3D visualisation of ordination plot

595 **Table 1.** Environmental characteristics of the five sampled transects used to assess the effect
596 of altitude on ectomycorrhizal fungal communities associated with *Pinus sylvestris* in
597 Scotland

Transect	Location of transect start (latitude, longitude)	Altitudinal range (m)	Soil temperature range (°C)	Soil moisture range (% VWC)
1	57.1276, -3.8409	300 - 600	9.7 – 12.4	24.1 – 48.1
2	57.1328, -3.8304	300 - 600	11.5 – 13.1	29.8 – 45.1
3	57.1425, -3.8099	300 - 550	11.8 – 14.4	22.6 – 45.8
4	57.1472, -3.7916	300 - 550	10.2 – 11.4	34.1 – 43.5
5	57.1484, -3.7813	300 - 550	6.9 – 11.7	17.0 – 50.4

598 VWC, Percentage volumetric water content defined as the percentage of soil volume
599 occupied by water.

Table 2. List of ectomycorrhizal operational taxonomic units (OTUs) discovered in this study with accession numbers and taxonomies of top BLAST matches, most frequently occurring taxa are shown first.

Taxonomy	Accession number of top match	Taxonomy of top match	E value	% coverage	% match	Number of reads	Number of samples
Suillus variegatus	UDB015800	*Suillus variegatus*	e-178	100	99	124780	32
Tomentellopsis cf. *submollis*	UDB016634	*Tomentellopsis submollis*	e-145	99	95	1828	27
Uncultured Atheliaceae OTU 1	UDB008299	Atheliaceae	e-154	100	99	987	27
Pseudotomentella tristis	UDB000032	*Pseudotomentella tristis*	0	100	100	796	22
Uncultured *Chroogomphus*	EF619654	Uncultured Chroogomphus clone	e-167	100	97	1023	19
Suillus bovinus	UDB015816	*Suillus bovinus*	0	100	100	9526	16
Piloderma sphaerosporum	UDB001750	*Piloderma sphaerosporum*	e-162	100	99	595	15
Uncultured Atheliaceae OTU 2	UDB008299	Atheliaceae	e-134	100	96	33	15
Russula paludosa	UDB011277	*Russula paludosa*	0	100	99	6607	14
Uncultured Cantharellales OTU 1	AM087245	Uncultured ectomycorrhiza (Clavulinaceae)	0	100	99	548	14
Uncultured Cantharellales OTU 2	AY641465	Uncultured ectomycorrhiza (Basidiomycota)	0	100	98	719	14
Rhizopogon luteolus	UDB001618	*Rhizopogon luteolus*	0	95	99	3175	13
Russula sardonia	UDB011197	*Russula sardonia*	0	100	99	473	13
Cortinarius albovariegatus subgroup B	UDB001547	*Cortinarius acutus*	e-145	96	98	439	10
Russula decolorans	UDB011326	*Russula decolorans*	0	100	99	3079	10
Sistotrema sp.	FR838002	cf. *Sistotrema* sp. P37	e-165	100	99	341	10
Suillus flavidus	UDB011444	*Suillus flavidus*	0	100	99	1238	10
Cortinarius cf. *acutus* OTU 1	UDB001543	*Cortinarius* sp.	e-126	100	94	84	9
Cortinarius cf. *mucifluus*	UDB015965	*Cortinarius mucifluus*	e-176	100	99	99	9

Uncultured Sebacinales	DQ309224	Uncultured fungus	e-156	100	98	18	9
Cortinarius semisanguineus	UDB001178	*Cortinarius semisanguineus*	e-163	96	98	190	8
Tomentella stuposa	UDB000248	*Tomentella stuposa*	e-159	100	97	534	8
Uncultured Cantharellales OTU 3	EF077524	Uncultured ectomycorrhiza (Basidiomycota)	0	100	98	3353	8
Cortinarius cf. *laetus*	UDB001046	*Cortinarius laetus*	e-144	100	96	62	7
Tomentellopsis submollis	UDB016634	*Tomentellopsis submollis*	e-178	100	99	17	7
Lactarius quieticolor	UDB015750	*Lactarius quieticolor*	e-182	100	97	1825	6
Pseudotomentella griseopergamacea	UDB001617	*Pseudotomentella griseopergamacea*	e-173	98	99	314	6
Russula vinosa	UDB011328	*Russula vinosa*	0	100	99	70	5
Cortinarius cf. *obtusus* OTU 1	UDB000127	*Cortinarius obtusus*	e-163	100	99	52	4
Cortinarius cf. *obtusus* OTU 2	UDB013156	*Cortinarius* sp.	e-126	100	94	13	4
Cortinarius glandicolor	UDB015919	*Cortinarius glandicolor*	e-167	100	99	30	4
Russula emetica	UDB015973	*Russula emetica*	0	100	100	60	4
Acephala macrosclerotium	HM189720	*Acephala macrosclerotium*	e-130	100	99	5	3
Cenococcum geophilum	HM189727	*Cenococcum geophilum*	e-148	100	100	10	3
Cortinarius cf. *acutus* OTU 2	UDB001002	*Cortinarius acutus*	e-157	100	98	80	3
Cortinarius quarciticus	UDB000748	*Cortinarius quarciticus*	e-160	100	100	21	3
Rhizopogon cf. *roseolus*	UDB015451	*Rhizopogon roseolus*	e-157	100	95	4	3
Suillus luteus	UDB016610	*Suillus luteus*	0	100	99	465	3
Tomentella sublilacina	UDB000970	*Tomentella sublilacina*	e-178	100	99	304	3
Tomentellopsis cf. *echinospora*	UDB008250	*Tomentellopsis* sp.	e-172	100	98	4	3
Uncultured Gomphidiaceae	GU187544	*Suillus bresadolae*	e-113	100	90	7	3
Amanita porphyria	UDB011151	*Amanita porphyria*	0	100	100	3	2
Boletus edulis	UDB015697	*Boletus edulis*	0	100	99	251	2

Cortinarius anomalus	UDB015930	*Cortinarius anomalus*	e-172	100	99	4	2
Hydnum repandum	UDB015778	*Hydnum repandum*	e-176	100	100	2	2
Lactarius musteus	UDB015417	*Lactarius musteus*	0	100	100	22	2
Piloderma byssinum	UDB016381	*Piloderma byssinum*	e-162	100	99	449	2
Russula nuoljae	UDB002540	*Russula nuoljae*	0	100	98	2	2
Russula versicolor	UDB011297	*Russula versicolor*	e-176	100	98	195	2
Sistotrema alboluteum	UDB002253	*Sistotrema alboluteum*	e-172	100	100	52	2
Tomentella badia	UDB000961	*Tomentella badia*	e-177	99	99	4	2
Cantharellus lutescens	UDB011212	*Cantharellus aurora*	0	95	99	3	1
Cortinarius cf. *livido-ochraceus*	UDB001049	*Cortinarius livido-ochraceus*	e-174	100	99	3	1
Hebeloma pusillum	UDB011806	*Hebeloma pusillum*	e-173	100	99	11	1
Lactarius tabidus	UDB015806	*Lactarius tabidus*	0	100	100	125	1
Lactarius vietus	UDB015785	*Lactarius vietus*	0	100	100	2	1
Piloderma olivaceum	UDB001746	*Piloderma olivaceum*	e-158	100	99	2	1
Russula amethystina	UDB000303	*Russula amethystina*	0	100	98	8	1
Russula aquosa	UDB015988	*Russula aquosa*	0	100	99	4	1
Russula integra	UDB011319	*Russula integra*	0	100	100	3	1
Sebacina cf. *vermifera*	GQ907128	Uncultured *Sebacina* voucher JD271.2	e-167	100	99	2	1
Tomentellopsis zygodesmoides	UDB000187	*Tomentellopsis zygodesmoides*	e-167	96	99	3	1
Uncultured Thelephoraceae	EF619795	Uncultured Thelephoraceae	e-171	100	99	244	1
Uncultured *Tomentella*	FR852180	Uncultured *Tomentella*	e-163	100	98	135	1

602 The number of samples in which the OTU occurred is out of a maximum of 32

603 **Table 3.** Environmental vectors fit against the non-metric multidimensional scaling
604 ordination of ectomycorrhizal fungal communities displayed in Figure 4.

	R^2	*P*
Altitude	0.432	**0.002**
Easting	0.376	0.158
Northing	0.169	0.205
Soil pH	0.252	**0.029**
Soil moisture	0.400	**0.003**
Soil temperature	0.329	**0.001**
Soil OM content	0.306	**0.003**
Soil C : N ratio	0.133	0.180
Needle C : N ratio	0.148	0.111

605

606 OM, organic matter. Statistically significant values are indicated in bold text.

607