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

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13

14 **Summary**

- 15 • Changes in species richness and distributions of ectomycorrhizal (ECM) fungal  
16 communities along altitudinal gradients have been attributed to changes in both host  
17 distributions and abiotic variables. However, few studies have considered altitudinal  
18 relationships of ECM fungi associated with a single host to identify the role of abiotic  
19 drivers. To address this, ECM fungal communities associated with one host were  
20 assessed along five altitudinal transects in Scotland.
- 21 • Roots of Scots pine (*Pinus sylvestris*) were collected from sites between 300 and 550-  
22 600 m altitude and ECM fungal communities were identified by 454 pyrosequencing  
23 of the fungal ITS region. Soil moisture, temperature, pH, C : N ratio and organic  
24 matter content were measured as potential predictors of fungal species richness and  
25 community composition.
- 26 • Altitude did not affect species richness of ECM fungal communities but strongly  
27 influenced fungal community composition. Shifts in community composition along  
28 the altitudinal gradient were most clearly related to changes in soil moisture and  
29 temperature.
- 30 • Our results show that a 300 metre altitudinal gradient produced distinct shifts in ECM  
31 fungal communities associated with a single host, and that this pattern was strongly  
32 related to climatic variables. This finding suggests significant climatic niche  
33 partitioning among ECM fungal species.

34

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

## 37 **Introduction**

38 Altitudinal gradients are characterised by decreasing temperature with increasing altitude  
39 (Körner, 2007). Precipitation, solar radiation and atmospheric pollution deposition can also  
40 vary with elevation thus creating complex environmental changes along elevation gradients  
41 (Körner, 2007). How these changes impact on ecosystems has been studied for many years  
42 (Rahbek, 1995) and patterns of species richness, composition and productivity have been  
43 identified for a broad range of organisms in relation to changing altitude (Kitayama & Aiba,  
44 2002; McCain, 2004; Romdal & Grytnes, 2007; Hoiss *et al.*, 2012). The availability of high-  
45 throughput molecular techniques in recent years has enabled the investigation of altitudinal  
46 patterns in diversity to be extended to micro-organisms (Bryant *et al.*, 2008; Geml *et al.*,  
47 2014).

48 Ectomycorrhizal (ECM) fungi are symbionts of many woody plants, including many  
49 temperate and boreal tree species (Smith & Read, 2008). Previous investigations have  
50 indicated that, in common with many other organisms, the species richness of ECM fungi can  
51 be affected by altitude. Fungal richness has been observed to either decrease at high altitude  
52 (Kernaghan & Harper, 2001; Bahram *et al.*, 2012), or peak at mid-altitude (Miyamoto *et al.*,  
53 2014), patterns which are commonly observed in both macro- and micro-organisms (Rahbek,  
54 1995; Bryant *et al.*, 2008). Declines in species richness of ECM fungi with altitude have been  
55 attributed to harsher climatic conditions, decreased energy availability and/or less favourable  
56 soil conditions at high altitude while the mid-domain effect has been hypothesised to cause a  
57 mid-altitude peak in richness (Kernaghan & Harper, 2001; Bahram *et al.*, 2012; Miyamoto *et*  
58 *al.*, 2014). In addition to changes in species richness, several studies have shown changes in  
59 the composition of ECM fungal communities with altitude (Kernaghan & Harper, 2001;  
60 Bahram *et al.*, 2012; Scattolin *et al.*, 2014).

61 The richness and composition of ECM fungal communities are known to be influenced by a  
62 range of factors including host identity and environmental conditions, both of which can  
63 change with altitude. The identity of the host species has been consistently found to be a  
64 strong determinant of ECM fungal communities (Ishida *et al.*, 2007; Tedersoo *et al.*, 2008,  
65 2013). Changes in host vegetation with altitude are therefore likely to be important in  
66 producing patterns in ECM fungal diversity with altitude. In a study of altitudinal gradients in  
67 Iran, host identity was shown to have a greater effect on community change than abiotic  
68 variation (Bahram *et al.*, 2012). As yet, little research has focused on the role of abiotic

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

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

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

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

## 119 **Materials and Methods**

### 120 *Study site*

121 An area of Scots pine (*Pinus sylvestris* L.) forest, approximately 9 km<sup>2</sup>, in the Cairngorms,  
122 Scotland was identified which includes an altitudinal range between 300 and 600 metres  
123 above sea level (latitude 57.122, longitude -3.818 at centre of study area). The forest is on the  
124 northern to western slopes of the Cairngorm mountain range and the majority of the area has  
125 been continuously forested since at least the 1700s (Steven & Carlisle, 1959). Ground  
126 vegetation mainly consisted of ericoid species, including *Calluna vulgaris* (L.) Hull and  
127 *Vaccinium* spp. (Bunce, 1977). The only other ECM hosts present are occasional birch  
128 (*Betula* spp.) trees. Soil types range from humus-iron and peaty podzols on the north-facing  
129 slopes to peaty subalpine soils on the western slopes (Soil Indicators for Scottish Soils,  
130 <http://sifss.macaulay.ac.uk>). Five altitudinal transects were demarcated, each ranging from  
131 300 m to 550 or 600 m altitude (Figure 1, Table 1). Transects were at least 300 m apart and  
132 sampling sites were positioned at 50 m altitudinal intervals (Figure 1). A barometric altimeter  
133 (Suunto, Finland) was used to determine altitude in the field. Samples were collected between  
134 late July and early September 2011.

### 135 *Sample collection*

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

### 149 *Needle and soil chemistry*

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

#### 155 *Preparation of amplicon libraries*

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

166 Amplicon libraries for each transect point were prepared by amplifying the fungal ITS2  
167 region using primers fITS7 and ITS4 (Ihrmark *et al.*, 2012). The ITS4 primer was tagged  
168 with an 8 base region ('tag') which allows identification of samples after multiplexed  
169 sequencing. Each sampling point was assigned a separate tag with at least two bases  
170 difference between tags. PCR reactions consisted of 500 nM primer fITS7 (5'-3';  
171 GTGARTCATCGAATCTTTG), 300 nM primer ITS4 (5'-3';  
172 XXXXXXXXTCCTCCGCTTATTGATATGC, where X indicates a base belonging to the  
173 multiplex identifier tag), 2.75 mM MgCl<sub>2</sub>, 0.19 mM of each dNTP, 0.5 units of DreamTaq  
174 Taq polymerase (Thermo Fisher Scientific, Waltham, MA), 1x DreamGreen buffer (Thermo  
175 Fisher Scientific, Waltham, MA), 0.2 µg/µl bovine serum albumin (BSA), 10 µl DNA  
176 template and 3.88 µl of deionised water to a final reaction volume of 20 µl. Thermal cycling  
177 conditions were as follows; initial denaturing at 94°C for 5 minutes followed by 31 - 35  
178 cycles of 94°C for 30 seconds, 56.8°C for 30 seconds and 72°C for 30 seconds and a final  
179 elongation step at 72°C for 7 minutes. The number of PCR cycles was adjusted per sample  
180 based on the DNA concentration of extracts to avoid entering the lag phase of PCR. Negative



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

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

#### 194 *Bioinformatic analyses*

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

208 Denoised sequences were reorientated where necessary to 5' to 3' direction and clustered to  
209 determine operational taxonomic units (OTUs). Clustering was performed at 97% similarity  
210 to avoid producing artefactual OTUs that can be produced at higher clustering thresholds  
211 (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](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 reads and 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

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

249 Community composition was analysed based on the presence or absence of OTUs as there are  
250 known issues with quantitative use of read numbers from 454 sequencing (Amend *et al.*,  
251 2010). OTUs that occurred in only one sample were removed before analysis as rare OTUs  
252 can have a large impact on ordination results (Poos & Jackson, 2012). Differences in OTU  
253 community composition between samples were assessed using non-metric multidimensional  
254 scaling with Sørensen distances using the vegan package. To minimise the stress of the  
255 ordination a three dimensional solution was used. Vector fitting of altitude, transect and  
256 measured environmental variables was carried out using the 'envfit' function to assess  
257 significant variables affecting community structure. To check for an influence of variation in  
258 sequencing depth between samples on community composition the number of reads was also  
259 fitted as a vector. Permutations were restricted to within transects using the 'strata' argument.  
260 Because there were more replicates of the environmental measures (96 samples, one sample  
261 per tree) than the community data (32 samples), environmental conditions at each transect  
262 point were averaged. Spatial autocorrelation in community composition was tested for using  
263 partial Mantel tests after accounting for variation due to abiotic variables.

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

## 273 **Results**

### 274 *Change in environmental variables with altitude*

275 High altitude sampling points had lower soil temperature and higher soil moisture than low  
276 altitude sites (Figure 2). Soil temperature decreased by  $0.48 \pm 0.17$  °C with every 50 m  
277 increase in altitude ( $\beta = -0.01$ ,  $P < 0.001$ ) (Figure 2a). Soil C : N ratio also decreased with  
278 altitude ( $\beta = -0.026$ ,  $P = 0.004$ ) while soil moisture increased ( $\beta = 0.07$ ,  $P < 0.001$ ) (Figure  
279 2b,c). There was no change in needle C : N ratio or soil pH (Figure 2d,e). Soil organic matter  
280 content showed a quadratic relationship with the highest values at mid-altitude (linear  $\beta =$   
281  $0.002$ ,  $P = 0.002$ , quadratic  $\beta = 3 \times 10^{-6}$ ,  $P < 0.001$ ) (Figure 2f). Variation in intercepts  
282 between transects were observed in all variables but altitudinal trends were similar along  
283 transects, except for soil temperature which declined most rapidly along transect five (Figure  
284 2 a).

### 285 *Fungal community description*

286 A total of 232,290 sequence reads were obtained from pyrosequencing. After filtering and  
287 assignment to tags 165,347 quality checked ectomycorrhizal reads were recovered with a  
288 mean of 5,166 reads per sample (Supporting Information Table S1). The number of root tips  
289 estimated during the root cleaning process was 26,262 across all 32 samples, suggesting a  
290 high level of sequencing redundancy. A total of 64 ectomycorrhizal OTUs were delimited at  
291 97% clustering, from 18 different genera (Table 2). The number of OTUs per sample was not  
292 related to the number of root tips sampled, nor to the number of reads obtained (Supporting  
293 Information Figure S1). Read numbers were massively dominated by *Suillus variegatus*  
294 which comprised 75% of all reads in this study (Table 2). Relatively low diversity of  
295 ascomycete taxa was recorded with only 15 reads from two OTUs being assigned to  
296 ascomycete taxa.

297 Altitude had a significant quadratic relationship with the number of tips per sample (linear  $z$   
298  $= 6.21$ ,  $P < 0.001$ ; quadratic  $z = -6.59$ ,  $P < 0.001$ ) and number of reads per sample (linear  $z =$   
299  $15.74$ ,  $P < 0.001$ ; quadratic  $z = -14.59$ ,  $P < 0.001$ ) with the highest numbers of tips and reads  
300 at intermediate altitudes. However, there was no relationship between altitude and the  
301 rarefied OTU richness per sample, nor was there any trend in the total number of species per  
302 altitudinal zone (Supporting Information Table S2). One sample outlier was observed with a

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

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

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

#### 318 *Relationships between fungal taxa and altitude*

319 Relationships between the occurrence of individual taxa and altitude were assessed by binary  
320 logistic models. Relationships were assessed at both OTU and genus levels (Figure 5a-f).  
321 Five OTUs and one genus were observed to show significant patterns of occurrence in  
322 relation to altitude. *Piloderma sphaerosporum* (Figure 5b) was most frequent in low altitude  
323 sites while *Russula sardonia* (Figure 5c) was most frequent at higher altitudes. Quadratic  
324 relationships were seen in three taxa with *Cortinarius semisanguineus* (Figure 5a) and an  
325 unknown Cantharellales (Figure 5e) peaking in occurrence at mid altitudes while an unknown  
326 Atheliaceae OTU was most frequent at low and high altitudes (Figure 5d). At a genus level  
327 only *Russula* displayed a significant pattern, increasing in occurrence with altitude (Figure  
328 5f). Further inspection of the OTU data showed that three other *Russula* species were also  
329 found more often at high altitudes but were too infrequent for a significant pattern to be  
330 observed at the OTU level (data not shown).

331 **Discussion**

332 Altitude was a major driver of variation in fungal community composition in the absence of  
333 host vegetation change. A 300 metre difference in altitude was associated with large changes  
334 in measured climate and soil variables, and in the community composition of ECM fungi.  
335 Ordination suggested that variation in soil moisture and temperature were important drivers  
336 of community variation along the transects studied, supporting previous findings of an  
337 influence of climatic variables on ECM fungal communities (Bahram *et al.*, 2012; Jarvis *et*  
338 *al.*, 2013; Counce *et al.*, 2014). Soil temperature and moisture were highly correlated and it  
339 was not possible to separate their effects on fungal communities. Both variables have been  
340 shown to influence fungal communities in experimental conditions (Allison & Treseder,  
341 2008; Deslippe *et al.*, 2011; Richard *et al.*, 2011) and it is likely that fungi respond to a  
342 combination of both variables through a variety of mechanisms. Soil organic matter and pH  
343 were also found to affect community structure supporting previous results (Rosling *et al.*,  
344 2003; Kjølner & Clemmensen, 2009; Geml *et al.*, 2014; Counce *et al.*, 2014). Here, variation  
345 in soil organic matter and pH was not as strongly correlated with altitude as the climatic  
346 variables and appeared to explain variation in community composition not related to  
347 altitudinal differences.

348 Soil temperature and moisture were only measured on a single occasion and could not capture  
349 seasonal variation and extreme events that may influence the fungal community. Despite this  
350 problem, both measurements were strongly correlated with altitude, suggesting the single  
351 measurements did reflect real gradients. Soil temperature decreased by approximately 1°C  
352 per 100m, and as soil temperature is likely to be less variable than air temperature, a similar  
353 or larger change in air temperature would be expected. Although single measurements will  
354 never fully describe the climatic conditions, soil temperature and moisture may be more  
355 appropriate explanatory variables than low resolution interpolated climate data for altitudinal  
356 studies focusing on fungal communities. It is also worth considering that altitude may  
357 influence communities through mechanisms which were not assessed in this study. In  
358 particular exposure to high winds has strong influences on the vegetation at high altitudes and  
359 high exposure has also been linked to changes in fungal communities (Kernaghan & Harper,  
360 2001; Scattolin *et al.*, 2008).

361 Analysing the community response to altitude further we found that several OTUs showed  
362 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.

394 In contrast to the clear response of community composition to altitude there was no change in  
395 the species richness of ECM fungi along the altitudinal gradients surveyed. Some previous  
396 studies have suggested that species richness of ECM fungi declines at high altitude  
397 (Kernaghan & Harper, 2001; Bahram *et al.*, 2012) or peaks at mid-altitude (Gómez-  
398 Hernández *et al.*, 2011; Miyamoto *et al.*, 2014) but neither pattern was supported here.  
399 Interestingly, Counce *et al.* (2014) also found no relationship between diversity and altitude in  
400 their study of fungal communities of a single host species. They suggest that the changes in  
401 species richness observed in previous studies may have been related to changes in host plant  
402 diversity which were not controlled for (Counce *et al.*, 2014), although Kernaghan and Harper  
403 (2001) found no relationship between host and ECM richness along an altitudinal gradient.  
404 Alternatively, the failure to fully saturate the sampling curve may explain the absence of a  
405 richness relationship in our study as it was not possible to identify all species present. In  
406 addition, although the gradients sampled here represented the entire altitudinal extent of  
407 *Pinus sylvestris* at the study site, ectomycorrhizal hosts also occur above the treeline in the  
408 form of dwarf shrubs such as *Arctostaphylos uva-ursi* (L.) Spreng. If the mechanism behind  
409 altitudinal changes in ECM diversity is not host-specific, for example decreased energy  
410 availability at high altitude, it may be that a pattern may emerge by including a larger  
411 altitudinal range.

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

420

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422

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549

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).

584 **Supporting Information**

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

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

588 **Fig. S2** Sequence based rarefaction curves for each sample

589

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

591

592 **Table S2** Total number of taxa at each altitude sampled

593

594 **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

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

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

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

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

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

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

605

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

607