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Research

Biogeography of ectomycorrhizal fungi associated with alders (*Alnus* spp.) in relation to biotic and abiotic variables at the global scale

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Summary

- Much of the macroecological information about microorganisms is confounded by the lack of standardized methodology, paucity of metadata and sampling effect of a particular substrate or interacting host taxa.
- This study aims to disentangle the relative effects of biological, geographical and edaphic variables on the distribution of *Alnus*-associated ectomycorrhizal (ECM) fungi at the global scale by using comparable sampling and analysis methods.
- Ribosomal DNA sequence analysis revealed 146 taxa of ECM fungi from 22 *Alnus* species across 96 sites worldwide. Use of spatial and phylogenetic eigenvectors along with environmental variables in model selection indicated that phylogenetic relations among host plants and geographical links explained 43 and 10%, respectively,in ECM fungal community composition, whereas soil calcium concentration positively influenced taxonomic richness.
- Intrageneric phylogenetic relations among host plants and regional processes largely account for the global biogeographic distribution of *Alnus*-associated ECM fungi. The biogeography of ECM fungi is consistent with ancient host migration patterns from Eurasia to North America and from southern Europe to northern Europe after the last glacial maximum, indicating codispersal of hosts and their mycobionts.

Introduction

There has been a lot of controversy about whether or not the distribution of microorganisms follows the general biogeographic rules (reviewed in Fierer, 2008). Recent molecular identification studies suggest that typical biogeographic patterns such as latitudinal or altitudinal gradient of diversity and distance decay resulting from dispersal limitation are usually evident in microbes, but these patterns do not always match those observed in plants and animals (Bryant *et al.*, 2008; Fierer *et al.*, 2011; Tedersoo *et al.*, 2012). Furthermore, microbial divisions and kingdoms differ greatly in their ecology and dispersal abilities and could be subject to different selective forces. Micro- and macroorganisms are often involved in symbiotic associations that may constrain the distribution of one or both of the partners.

Among soil-inhabiting microbes, mycorrhizal fungi display mutualistic benefits to most terrestrial plants. Species richness of ectomycorrhizal (ECM) fungi that associate with many ecologically and economically important trees appears to have a unimodal relationship with latitude as based on a metastudy across a wide range of hosts (Tedersoo & Nara, 2010; Tedersoo *et al.*, 2012). ECM fungi also exhibit a declining richness pattern along the altitudinal gradient (Bahram *et al.*, 2012). Both of these diversity gradients were largely ascribed to climatic variables, particularly the mean annual temperature and precipitation. By contrast, Queloz *et al.* (2011) did not find any biogeographic pattern in species composition of *Phialocephala* root endophytes across the northern hemisphere. These patterns found in fungi differ substantially from the peak in biodiversity of plants and animals in ecosystems at low latitudes characterized by high temperature and rainfall (Hillebrand, 2004). Because ECM fungi are obligate

root symbionts, host taxa can have a strong effect on both species richness and community composition of ECM fungi (Ishida *et al.*, 2007; Tedersoo *et al.*, 2008; Bahram *et al.*, 2012). Host identity effect may, however, complicate global ECM fungal richness comparisons because of the poor overlap in host lineages between tropical, temperate and arctic ecosystems. Thus, removal of the potentially confounding host effect should provide a less constrained biogeographic framework for addressing the effects of abiotic factors, such as geographic, climatic and edaphic variables, on ECM fungal biodiversity at the global scale. Therefore, we focused on a single plant genus, *Alnus*, that is widely distributed from tropical to subarctic latitudes and supports a relatively low diversity of ECM fungi.

In *Alnus*, fungal and actinobacterial root symbionts are obligatory and beneficial for obtaining soil mineral nutrients and atmospheric nitrogen, respectively (Yamanaka *et al.*, 2003). Although healthy *Alnus* trees always associate with *Frankia* actinobacteria, the actinorrhizal symbiosis is facultative for the actinobacteria that are ubiquitous free-living soil organisms (Benson & Dawson, 2007). Arbuscular mycorrhizal fungi benefit growth and nutrient uptake of alder seedlings (Chatarpaul *et al.*, 1989), but their colonization is usually low or absent in roots of mature trees (S. Põlme *et al.*, unpublished). By contrast, both seedlings and mature trees of *Alnus* spp. are usually well colonized by ECM fungi, which are thought to play a key role in providing phosphorus and other soil nutrients to their hosts (Chatarpaul *et al.*, 1989; Yamanaka *et al.*, 2003). Approximately 50–60 species of ECM fungi are documented as mycorrhizal symbionts of *Alnus* worldwide (Pritsch *et al.*, 1997; Tedersoo *et al.*, 2009; Kennedy & Hill, 2010; Rochet *et al.*, 2011) and the associated basidiomycetes are strongly specific to their host tree genus (Molina *et al.*, 1992; Tedersoo *et al.*, 2009; Kennedy *et al.*, 2011), but the mechanisms underlying specificity are still under debate (Tedersoo *et al.*, 2009; Kennedy & Hill, 2010; Rochet *et al.*, 2011). While most of the *Alnus*-associated fungal taxa have been recorded only once, a few of the most common species are distributed both in Europe and North and South America (Kennedy & Hill, 2010; Pritsch *et al.*, 2010; Kennedy *et al.*, 2011).

Continental disjunction patterns observed in plant clades support the hypotheses of Beringia serving the primary historical path between Eurasia and North America (Donoghue & Smith, 2004). Because of great pollen production and riparian or pioneer habitat, the palaeoecology and biogeography of *Alnus* are relatively well documented. The genus *Alnus* comprises *c.* 35 species that are widely distributed in the northern hemisphere, but the origin of *Alnus* is considered to be in East Asia, where the highest degree of endemism occurs (Navarro *et al.*, 2003). Fossil evidence suggests that *Alnus* has spread multiple times from Eurasia to North America using both the Beringian and North Atlantic land bridges (Furlow, 1979). *Alnus* was one of the first trees to migrate northward after the receding glacial termini in Europe (Hewitt, 1999). After establishment of the Isthmus of Panama land connection, *Alnus* has been rapidly expanding its distribution into South America using the Andes as a pathway through tropical latitudes (Furlow, 1979). Migration of ECM symbionts may follow migration routes of their host (Murat *et al.*, 2004) or occur via occasional long-distance dispersal events (Moyersoen *et al.*, 2003; Matheny *et al.*, 2009; Geml *et al.*, 2011). Owing to its distribution and restricted range of mycobionts, *Alnus* has been suggested as a good model system for addressing biogeography, coevolution and host specificity of symbiotic microbial taxa (Tedersoo *et al.*, 2009; Rochet *et al.*, 2011). The wide latitudinal range of *Alnus* allows for comparisons of ECM fungal richness across climatic gradients that are not confounded by differences in host genera. However, *Alnus* has some features that are atypical to ECM plants, such as its occurrence in mainly riparian and pioneer habitats and association with actinobacteria, which all may exhibit a strong filtering effect on ECM fungal richness and community composition. Moreover, *Alnus* is absent from tropical lowland forests.

This study aims to disentangle the relative effects of various biotic, geographic, edaphic and climatic factors on taxonomic richness and distribution of *Alnus*-associated ECM fungal communities at the global scale. We hypothesized that: the richness of these root symbionts follows a unimodal relationship with latitudinal gradient and is mainly a function of climatic variables (cf. Tedersoo *et al.*, 2012); host species and their phylogenetic relationships account for the strongest predictor of ECM fungal community composition at the intrageneric level (Rochet *et al.*, 2011); and ECM fungal communities follow the historical migration patterns of their hosts.

Materials and Methods

Sampling design

This study was performed in 96 alder stands (including seven stands from previous study in Estonia; Tedersoo *et al.*, 2009) in Europe, East and West Asia, North America and South America, covering the subalpine, subarctic, boreal, temperate and subtropical ecosystems (Table 1, Supporting Information Table S1). Our sampling covered all continents where *Alnus* is distributed, except North Africa, which shares its single species (*Alnus glutinosa*) with Europe. From the taxonomic perspective, our study comprised 22 alder species out of 29–44 valid species that belong to all three subgenera – *Alnobetula*, *Clethropsis* and *Alnus* (Chen & Li, 2004; Catalogue of Life, www.catalogueoflife.org). We refer to the Catalogue of Life online database for the nomenclature of *Alnus* spp. Following this treatment, we considered European and North American subspecies of *Alnus viridis* together, because they are ecologically and genetically nearly identical (Navarro *et al.*, 2003; Chen & Li, 2004). In our global study, *A. glutinosa* (L.) Gaertn served as a host tree at 18 sites, *A. viridis* (Chaix) DC. at 10 sites, *Alnus hirsuta* (Spach) Rupr. at nine sites, *Alnus subcordata* C.A.Mey. at eight sites, *Alnus incana* (L.) Moench at seven sites, *Alnus nepalensis* (D. Don) at six sites, *Alnus rubra* (Bong.) at five sites, *Alnus japonica* (Thunb.) Steud. and *Alnus mandshurica* (Callier ex C. K. Schneider) at four sites, *Alnus maritima* (Marshall) Muhl. ex Nutt., *Alnus maximowiczii* (Callier) and *Alnus serrulata* (Aiton) Willd. at three sites, *Alnus acuminata* (Kunth), *Alnus fauriei* (H. Lév. & Vaniot), *Alnus firma* (Siebold & Zucc.), *Alnus formosana* (Burkill) Makino, *Alnus orientalis* Deckne and *Alnus sieboldiana* (Matsum.) at two sites, and *Alnus matusmurae*

Table 1 List of sampled sites by countries and hosts

Country	Host species sampled	Number of sites sampled
Northeast US	*Alnus maritima*, *A. serrulata*	6
Northwest US	*A. rhombifolia*, *A. rubra*, *A. viridis*	9
Finland	*A. glutinosa*	5
Lithuania	*A. glutinosa*	1
Poland	*A. glutinosa*	2
Slovakia	*A. incana*	1
Romania	*A. incana*	1
Slovenia	*A. glutinosa*, *A. viridis*	4
Croatia	*A. glutinosa*	3
Italy	*A. incana*	1
Austria	*A. incana*, *A. viridis*	6
Turkey	*A. orientalis*	2
Iran	*A. glutinosa*, *A. orientalis*	9
China	*A. hirsuta*, *A. mandchurica*, *A. nepalensis*	15
Japan	*A. fauriei*, *A. firma*, *A. hirsuta*, *A. japonica*, *A. matsumurae*, *A. maximowiczii*, *A. pendula*, *A. sieboldiana*, *A. trabeculosa*	20
Taiwan	*A. formosana*	2
Ecuador	*A. acuminata*	2
Estonia[1]	*A. glutinosa*, *A. incana*	7
Argentina[2]	*A. acuminata*	2
Mexico[2]	*A. acuminata*, *A. jorrulensis*	4

[1]Data are derived from an earlier study by Tedersoo *et al.* (2009).
[2]Data are derived from previous studies (Becerra *et al.*, 2005; U. Kõljalg, unpublished; Kennedy *et al.*, 2011), which are taken into consideration only in molecular taxonomic unit (MOTU) distribution analyses. See detailed information in Table S1.

(Callier), *Alnus pendula* (Matsum.), *Alnus rhombifolia* (Nutt.) and *Alnus trabeculosa* (Hand.-Mazz.) at a single site.

At each study site, sampling was performed in an area of 2500 m^2. Six soil samples (15 × 15 cm to 10 cm depth) comprising *Alnus* roots were randomly collected at least 10 m apart to secure statistical independence between individual samples (Lilleskov *et al.*, 2004). Soil samples were placed into plastic bags and processed within 48 h after collection. Roots were carefully cleaned under tap water and placed into large Petri dishes filled with water. Tree species were identified under a stereomicroscope based on root morphology (presence and shape of ECM and actinorrhizal root nodules). Only vital alder roots were processed. ECM morphotypes were distinguished based on colour and roughness of mantle, presence of emanating hyphae and rhizomorphs. At least two ECM root tips from each morphotype per soil sample were stored in CTAB buffer (1% cetyltrimethylammonium bromide, 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA) for molecular analyses.

At each site, *c.* 50 g of rhizosphere soil was pooled from the six samples for analysis of soil parameters. In addition to soil pH and concentrations of total soil N, exchangeable P, K, Ca, and Mg were measured (Table S3). The approximate host age was evaluated empirically, using available data of habitats or advice of local experts. Geographical coordinates and altitude were recorded using a Garmin 60CSx GPS (Garmin International Inc., Olathe, KS, USA). Because sampling effort in a previous study in Estonia was greater (18 soil samples per site), data from six randomly selected samples per site were included in the present study.

Molecular analyses

DNA was extracted from ECM root tips using Qiagen's DNeasy 96 Plant Kit according to manufacturer's instructions. In the course of the study, PCR was performed using three alternative products: puReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, UK), 5× HOT FIREPol Blend Master Mix Ready to Load (Solis BioDyne, Tartu, Estonia) or Fermentas PCR mixture (Fermentas, Vilnius, Lithuania). Use of different products did not affect the results, because no cloning was performed and unsuccessfully identified root tip samples were re-extracted and reamplified to maximize recovery. In ECM root tips, the fungal rDNA internal transcribed spacer (ITS) region was amplified with a forward primer ITSOF-T (5′-acttggtcatttagaggaagt-3′) in combination with reverse primers LB-W (5′-cttttcatctttccctcacgg-3′) or TW13 (5′-ggtccgtgtttcaagacg-3′). In case of PCR failure, we combined ITSOF-T with universal primers ITS4 (5′-tcctccgcttattgatatgc-3′) or ITS2 (5′-gctgcgttcttcatcgatgc-3′) to amplify a shorter fragment of fungal DNA. To improve sequence quality, many root tip extracts were reamplified with taxon-specific primers ITS4-Tom (5′-aactcggacgaccagaggca-3′), ITS4-Russ (5′-agcgggtagtctcaccc-3′), ITS4Seb (5′-tcagcgggtartcctactc-3′) (Tedersoo *et al.*, 2011), LR3-Pez (5′-cmtcrggatcggtcgatgg-3′) (Tedersoo *et al.*, 2008), and the newly designed LR3-Aln (5′-cctsagcacgaacgtggta-3′). The latter primer is specific to a small group of Agaricales, including *Alnicola*, *Hebeloma* and *Entoloma* based on several shared mutations in a rapidly evolving D2 region of the rDNA large subunit. PCR reactions were run under the following conditions: 95°C for 15 min; five cycles of 42°C for 30 s, 72°C for 60 s and 92°C for 45 s; 35 cycles of 65°C for 30 s, 72°C for 60 s and 92°C for 45 s, followed by a final cycle of 65°C for 30 s and 72°C for 10 min for all primer combinations. One to three root tips from all rare fungal molecular taxonomic units (MOTUs) that occurred once or twice were subjected to molecular identification of the host to confirm the morphology-based identification. Primers trnH (5′-cgcgcatggtggattcacaatcc-3′) and psbA (5′-gttatgcatgaacgtaatgctc-3′) were used to amplify and sequence the plant plastid trnH-psbA region. PCR products were separated by electrophoresis through a 1.5% agarose gel in 0.5X TBE buffer (45 mM Tris Base, 45 mM boric acid, 1 mM EDTA (pH 8.0)), visualized under UV light and purified using Exo-Sap enzymes (Sigma).

Sequencing of fungal DNA was performed with primers ITS5 (5′-ggaagtaaaagtcgtaacaagg-3′) and ITS4. Sequences were assembled, checked, trimmed and manually corrected in Sequencher 4.10.1 (GeneCodes Corp., Ann Arbor, MI, USA). Sequences were confirmed to belong to ECM fungal lineages (cf. Tedersoo *et al.*, 2010) or *Alnus* host trees by the use of BLASTn searches against the International Sequence Databases (INSD) or UNITE

(Abarenkov *et al.*, 2010). For each ECM fungal lineage, the ITS sequence of a suitable outgroup taxon was downloaded from INSD and aligned automatically using MAFFT 6 (Katoh & Toh, 2008). The alignments were checked and corrected manually in Seaview (Gouy *et al.*, 2010). Maximum likelihood (ML) and fast bootstrap analyses were performed in RAxML (Stamatakis *et al.*, 2008; default settings) as implemented in the Cipres web portal (www.phylo.org/portal2/login!input.action). Consistent with previous studies (Tedersoo *et al.*, 2009; Rochet *et al.*, 2011), these phylograms were used to distinguish MOTUs in *Alnus*-associated ECM fungi based on both bootstrap support and branch length (see Fig. S1 for an example). Many distinct fungal species of *Alnus* exhibit highly similar ITS sequences that prevent their separation by using clustering approaches (Moreau *et al.*, 2006; Rochet *et al.*, 2011). Within-MOTU similarity did not exceed 97.5% in any of the cases, which roughly corresponds to the barcoding gap in Basidiomycota (Schoch *et al.*, 2012) and thus minimizes lumping of biological species.

Host phylogeny

To address the effect of phylogenetic relations among *Alnus* host species on ECM fungal community composition, we created a phylogeny of *Alnus* species based on the ITS region, using *Betula pendula* as an outgroup. ITS sequences of each species were downloaded from the INSD. To construct a phylogram of *Alnus* spp., ML and fast bootstrap analyses with 1000 replications were performed using the GTR + CAT evolutionary model in RAxML. To account for the node ages in the host phylogeny, we used the chronopl function ($\lambda = 0$) in the Ape package of R (Paradis *et al.*, 2004). This function uses a tradeoff between a parametric formulation where each branch has its own rate, and a nonparametric term where changes in rates are minimized between contiguous branches (Sanderson, 2002). We used Mesquite (Maddison & Maddison, 2008) to generate a patristic distance matrix from the derived host phylogeny. Phylogenetic eigenvectors of principal components of neighbour matrices (PCNM) were derived from the patristic distance matrix, forward-selected ($\alpha = 0.05$) in the Packfor package of R (Dray *et al.*, 2007) and used in further statistical analyses. For comparative purpose, we created a phylogram of *Alnus* spp. based on the trnH-psbA region (generated in this study – see 'Molecular analyses' above), and ITS and trnH-psbA jointly, because a combination of these loci discriminates best among *Alnus* species (Ren *et al.*, 2010). Since the phylograms of ITS and trnH-psbA were conflicting (Ren *et al.*, 2010), we decided to use the ITS phylogeny for further analyses, because ITS is a nuclear marker (inherited biparentally) and it supports the traditional classification. The effect of phylogenetic PCNM vectors derived from the combination of trnH-psbA and ITS phylogeny was similar to that of the ITS phylogeny alone (results not shown).

Statistical analyses

The frequency of fungal MOTUs in six root samples per site was used in community-level analyses. All soil nutrient concentrations were log-transformed before analyses. The effect of geographical distance was taken into account by reducing the Euclidean distance matrix into spatial PCNM vectors that account for spatial autocorrelation at different scales (Borcard & Legendre, 2002). Significant PCNM vectors ($\alpha = 0.05$) were forward-selected and used in subsequent analyses.

Estimates of the mean annual temperature and precipitation were retrieved from a high-resolution database of the Earth's surface climate (Hijmans *et al.*, 2005) using the software ArcGIS 9.3 (ESRI, Redlands, CA, USA). This climate database represents a global model of the mean monthly surface climate features over all terrestrial areas with a raster size of 30 s latitude and longitude (*c.* 0.81 km^2 on the equator).

The effects of edaphic and climatic variables on MOTU richness of ECM fungi were tested using generalized least-squares (GLS) analysis as implemented in the Nlme package of R (Pinheiro *et al.*, 2008). The best model describing total MOTU richness per site was chosen based on corrected Akaike information criterion (AICc). Robustness of the best model was further evaluated by averaging models that fell into the 95% AICc confidence set. Beta coefficients (slopes) of individual models were weighted according to their Akaike weight across all models and evaluated as the mean ± 95% confidence intervals. Zero values were conservatively used for nonsignificant variables in individual models. Variables were considered significant when their confidence intervals excluded zero values. EstimateS (Colwell, 2006) was used to create rarefied MOTU accumulation curves comprising all sites to evaluate sufficiency of global sampling effort for the detection of MOTUs of *Alnus*-associated ECM fungi.

To address the relative importance of climatic, edaphic, spatial and biological factors on the community structure of ECM fungi, we used a multivariate ANOVA as implemented in the Adonis routine of the Vegan package of R (Oksanen *et al.*, 2012). Adonis tests the significance of discrete and continuous factors based on permutations. The Bray–Curtis dissimilarity metric was used to calculate the community distance matrix. Using the same options, we constructed a nonmetric multidimensional scaling (NMDS) plot in Ecodist package of R (Goslee & Urban, 2007).

We used the Varpart function in the Vegan package to partition the variation of community dissimilarity by grouping host phylogenetic, edaphic, climatic and spatial variables. Variation partitioning is based on redundancy analysis (RDA), which uses Euclidean distance. Singletons (i.e. MOTUs found from a single site) were excluded from both Adonis and RDA analyses to reduce the effect of rare MOTUs.

Biogeographical analyses

Disjunction patterns of MOTUs were compared among different regions and continents by calculating Sørensen similarity coefficients as implemented in the fossil package of R (Vavrek, 2011). The Sørensen index is a measure of beta diversity, ranging from a value of 0, where there is no MOTU overlap between the communities, to a value of 1 when exactly the same MOTUs are found in both communities. Since the number of sampling sites within regions and continents differed, we used the weighted MOTU frequency (i.e. divided by sample size) to reduce the

effect of sampling effort. To illustrate taxonomic similarity between different regions, host phylogeny and ECM community structure, we employed the Sørensen similarity coefficient in two-way cluster analyses as implemented in PC-ORD 5.0 (McCune & Mefford, 2006). Statistical support of similarity in the area cladograms was tested in the Pvclust package of R (Suzuki & Shimodaira, 2006), which calculates *P*-values based on multiscale bootstrap resampling using 1000 replications.

Results

Taxonomic richness

Out of 1621 ECM root tips subjected to molecular analysis, 1172 (72%) yielded good-quality sequences. Based on ML phylograms, 146 *Alnus*-associated ECM MOTUs were distinguished worldwide (Table S1). These MOTUs included 65 taxa that were found from a single site. Sequencing of plant DNA confirmed that all singletons and doubletons presented here indeed originate from roots of *Alnus* species. Other host genera were identified for an additional 11 fungal MOTUs that were removed from the dataset and all analyses. The rarefied MOTU accumulation curve of *Alnus*-associating ECM fungi did not reach a plateau, indicating that further sampling would reveal additional undiscovered MOTUs (Fig. 1). According to the Chao2 minimum species richness estimator, at least 200 MOTUs associate globally with *Alnus*. MOTU richness per site averaged 6.61 and ranged from 1 to 14.

The likelihood ratio test revealed no significant difference between the best regular and spatial models for explaining ECM fungal MOTU richness. The best model ($F_{1,90} = 5.643$; $R^2 = 0.196$; $P < 0.001$) included soil calcium concentration ($t = 2.882$; $R^2 = 0.121$; $P < 0.005$; Fig. 2a,b), the mean annual precipitation ($t = -1.185$; $R^2 = 0.121$; $P = 0.239$; Fig. 2c,d), host age ($t = 1.533$; $R^2 = 0.040$; $P = 0.128$), mean annual temperature ($t = -2.485$; $R^2 = 0.016$; $P = 0.041$) and soil nitrogen concentration ($t = -1.438$; $R^2 = -0.005$; $P = 0.154$). The averaged model was built on 426 models. Based on the 95% confidence interval of beta coefficients, only soil calcium concentration had a consistently significant positive effect on MOTU richness among the studied variables. Spatial and phylogenetic PCNM vectors had no significant impact on MOTU richness.

Community structure and biogeography

The most MOTU-rich phylogenetic lineages of ECM fungi included /tomentella-thelephora (comprising 32 MOTUs in 92 sites), /cortinarius (24 MOTUs in 51 sites), /hebeloma-alnicola (22 MOTUs in 65 sites), /russula-lactarius (15 MOTUs in 42 sites), /inocybe (13 MOTUs in 20 sites) and /genea-humaria (6 MOTUs in 20 sites; Table S1). While the /tomentella-thelephora lineage was present and common in nearly all sites, some less frequent groups exhibited substantial differences in distribution by hosts and regions. For example, the /genea-humaria lineage was frequent in all sites in northern Iran, but it only occasionally occurred at seven (9.1%) study sites in the rest of the world (Fig. S2). The /inocybe lineage was also relatively common in Iran, inhabiting seven (87.5%) sites compared with 13 (14.8%) sites in other regions.

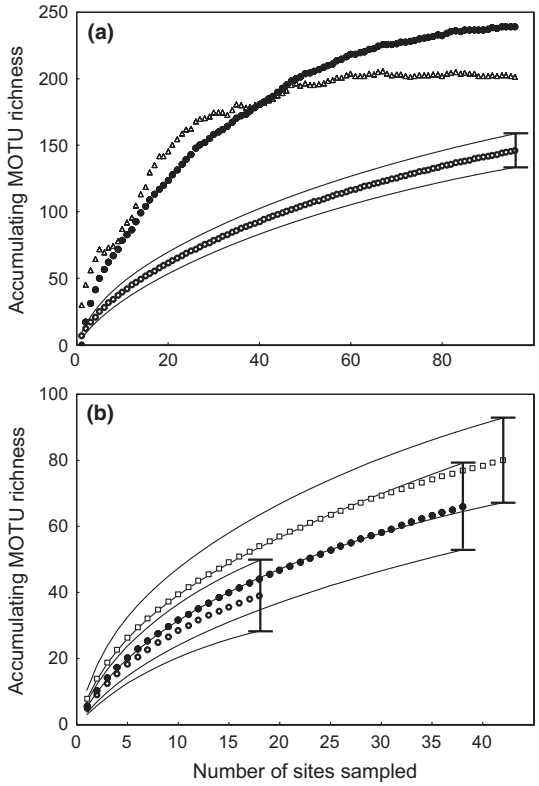
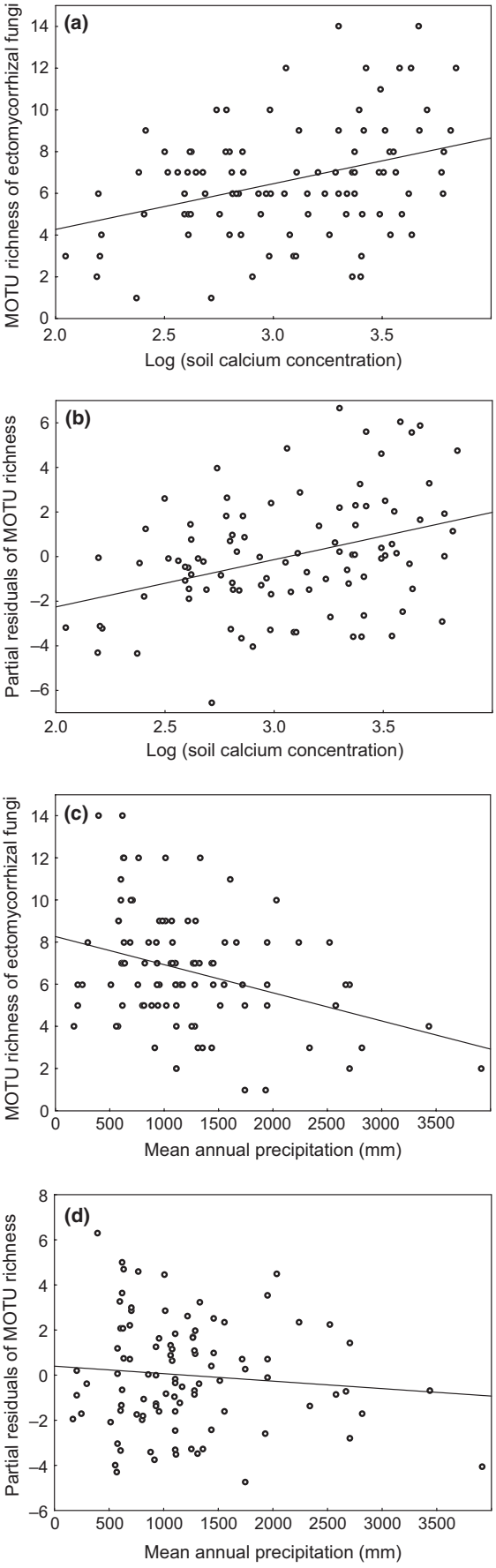


Fig. 1 Minimum richness estimator curves and rarefied molecular taxonomic unit (MOTU) accumulation curve of *Alnus*-associated fungi (circles) and their 95% confidence intervals (lines with terminal bars): (a) at the global scale (squares, Jackknife2 estimator; triangles, Chao2 estimator); and (b) at the continental scale (squares, Europe including Turkey and northern Iran; closed circles, Asia; open squares America).

At the regional scale, northern and southern Europe shared the greatest proportion of species and these areas clustered together ($P = 0.061$), but both differed substantially from Iran–Turkey (Fig. 3; Table S2). The proportion of shared fungal MOTUs was slightly higher between Asia and northwest America than between Asia and Europe (Table S2). However, based on multiscale bootstrap resampling, the clustering of northwest America and Asian regions was marginally nonsignificant ($P = 0.062$; Fig. 3). Biogeographic relationships among other regions were poorly resolved (Fig. 3), indicating that large-scale biogeographic patterns of *Alnus* mycobionts are weak. This is reflected by the panglobal distribution of the most common MOTUs such as *Tomentella aff. sublilacina* #2, *T. aff. ellisii* #3, *T. aff. cf. botryoides* and *T. aff. stuposa* #1 (Fig. S2).

Alnus species belonging to subgenus *Alnobetula* formed three groups with unresolved relationships at the base of the ML phylogram. *Alnus formosana* and *A. maritima* belonging to subgenus *Clethropsis* formed a well-supported clade with species from the subgenus *Alnus* (Fig. 4). Certain species in the subgenus *Alnus* had nearly identical ITS sequences. Nonetheless, host phylogenetic PCNM vectors had the greatest impact (Adonis: $F_{14,70} = 5.09$;



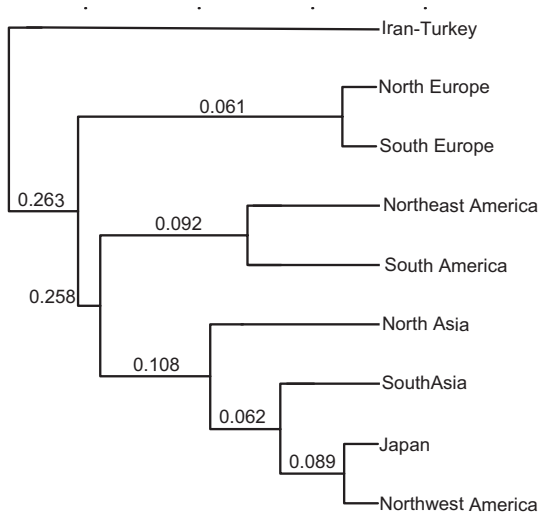


Fig. 3 Cluster dendrogram of *Alnus*-associated ectomycorrhizal (ECM) fungal communities by biogeographic regions. Numbers above branches indicate support by *P*-values, which are computed based on multiscale bootstrap resampling.

$P = 0.001$) on fungal community structure followed by spatial PCNM vectors ($F_{8,70} = 2.005$; $P = 0.001$) that explained 42.9 and 9.7% of variation in the community data, respectively (Table 2; Fig. S3). Among the environmental parameters, soil pH ($F_{1,70} = 5.515$, $R^2 = 0.033$, $P = 0.001$) and mean annual temperature ($F_{1,70} = 3.266$; $R^2 = 0.012$; $P = 0.001$) had a significant but marginal effect on ECM fungal community structure. In total, 42.9% of community variation remained unexplained by the addressed geographical, biological, climatic and edaphic factors (Table 2).

The relatively stronger effects of host phylogeny and spatial distance were confirmed in RDA (Fig. 5), although the overall coefficients of determination were lower than in Adonis. Host phylogeny had a shared effect with climatic, soil and, in particular, spatial variables, but climate had no shared effect with spatial PCNM vectors on the ECM fungal community.

Discussion

Taxonomic richness

This global study confirmed the earlier observations of relatively low within-site MOTU richness of ECM fungi in *Alnus* forests

Fig. 2 The effect of soil calcium concentration and mean annual precipitation on *Alnus*-associated ectomycorrhizal molecular taxonomic unit (MOTU) richness: (a) observed richness vs soil calcium concentration ($F_{1,90} = 14.06$; $R^2 = 0.121$; $P < 0.001$); (b) residual richness vs soil calcium concentration as revealed from the best model accounting for host age, soil nitrogen, the mean annual temperature and precipitation ($F_{1,90} = 4.825$; $R^2 = 0.049$; $P = 0.029$); (c) observed richness vs mean annual precipitation ($F_{1,90} = 14.12$; $R^2 = 0.121$; $P < 0.001$); (d) residual richness vs precipitation as accounting for host age, mean annual temperature, soil nitrogen and calcium concentrations ($F_{1,90} = 0.889$; $R^2 = -0.021$; $P = 0.048$).

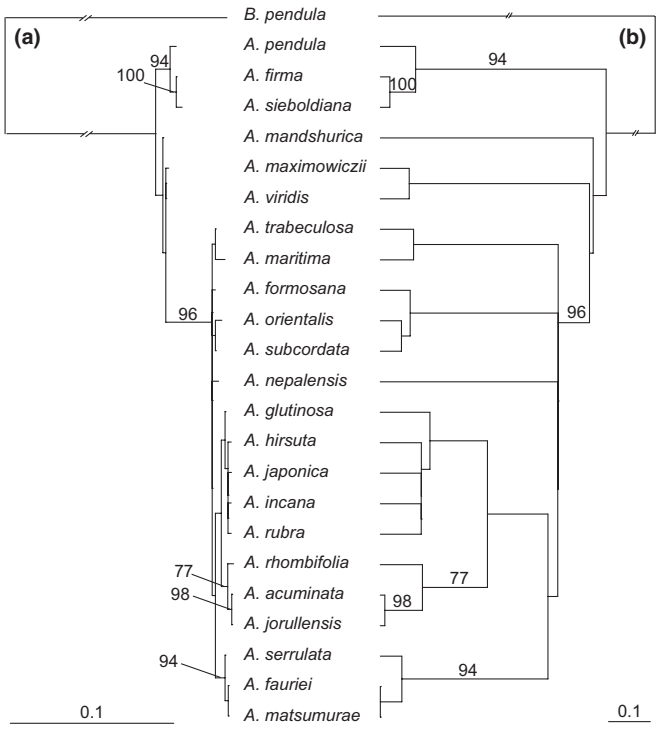


Fig. 4 Maximum likelihood phylograms demonstrating phylogenetic placement of *Alnus* species based on internal transcribed spacer (ITS) gene sequences: (a) unadjusted phylogenetic tree; (b) ultrametric tree allowing shifts in rates of evolution. Bootstrap support > 70 is shown above branches.

Table 2 Relative importance of biological, climatic and edaphic parameters on the community composition of ectomycorrhizal fungi on roots of 22 *Alnus* spp. as revealed from the Adonis function

	Degrees of freedom	*F*-value	R^2	*P*-value
Host phylogeny	14	5.09	0.429	0.001
Spatial vectors	8	2.00	0.097	0.001
Soil pH	1	5.51	0.033	0.001
Mean annual temperature	1	3.26	0.012	0.001
Residuals	70	0.42		
Total	94			

in Europe and America (Pritsch *et al.*, 1997; Becerra *et al.*, 2005; Tedersoo *et al.*, 2009; Kennedy & Hill, 2010). The total richness of 146 MOTUs found worldwide can be ascribed to greater sampling effort in terms of both geographical area and number of host species. While the dominant ECM fungal MOTUs were widely distributed worldwide, the majority of MOTUs were found only once or twice and thus exhibited a restricted geographical range. Both the rarefaction curve and minimum richness estimators suggested that the number of MOTUs colonizing *Alnus* has yet to be saturated and certain isolated locations or rare host species may harbor several potentially endemic taxa (Rochet *et al.*, 2011). Based on these data, however, it remains unknown whether the rare MOTUs are endemic to specific regions or whether they are highly infrequent throughout their global range. Of doubletons, 84.2% were found in a single region and 92.1% in a single continent, indicating that rare MOTUs usually exhibit restricted distribution.

As derived from the GLS model selection, soil calcium concentration was the strongest predictor of MOTU richness, exhibiting a positive effect (Fig. 2a). This unexpected finding contrasts with the conclusions of Tedersoo *et al.* (2012), who demonstrated ECM species richness to be mainly a function of the mean annual temperature and precipitation. That metastudy across various host families, however, suffered from neglect of edaphic variables and use of contrasting sampling and identification protocols. In the present study, both the host genus and methods were held highly similar. Besides methodological differences, certain characteristic ecological traits of the genus *Alnus*, such as the extreme reciprocal specificity with ECM fungi, association with actinobacteria and pioneer or riparian habitat, may contribute to the observed discrepancies between the two studies. In particular, the overall low diversity of *Alnus*-associated ECM fungal species probably results in a limited local species pool, leading to unsaturated richness within study sites, which in turn blurs global richness patterns. In addition, *Alnus* spp. are absent from tropical lowland forests, which shortens the temperature gradient by *c.* 25%, just from the critical point above 20°C, where ECM fungal richness has been suggested to decline (Tedersoo *et al.*, 2012). Nonetheless, our study provided only inconclusive support to the negative relationship between mean annual precipitation and MOTU richness that was ascribed to low oxygen stress in water-saturated soils and/or competition among functional guilds of soil microbes (Tedersoo *et al.*, 2012).

Calcium availability plays a critical role in shaping ecosystem structure, function and response to disturbance (Beier *et al.*, 2012). For example, its low mobility renders calcium a limiting factor in many plant functions (McLaughlin & Wimmer, 1999). Litter layer calcium concentration accounts for the best predictor of soil properties such as carbon and nitrogen concentrations, pH

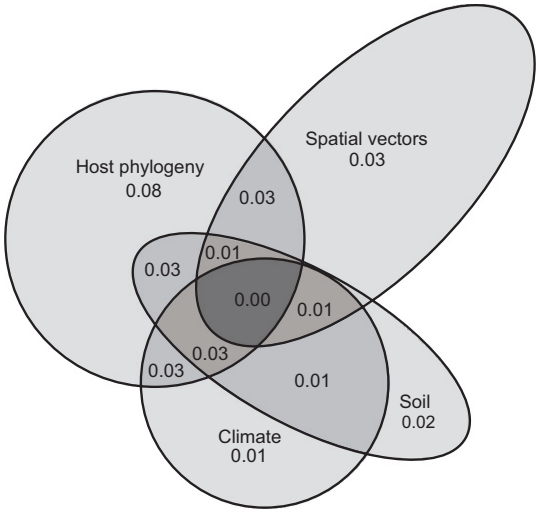


Fig. 5 Pure and shared effects of host phylogeny, spatial structure, edaphic factors and climate on *Alnus*-associated ectomycorrhizal (EcM) fungal community as derived from variation partitioning analysis. Numbers indicate the proportion of explained variation.

and rate of humus horizon turnover (Reich *et al.*, 2005). Consistent with our results, manipulative field studies revealed that calcium displays a substantial role in shaping ECM community structure and affecting the overall fungal richness in forests of *Picea* and *Fagus* (Rineau & Garbaye, 2009), but this may have resulted from stress to liming, marked changes in soil pH and phosphorus availability, as well as altered competitive balance among soil microorganisms and fungal species. In natural ECM fungal communities, greater soil calcium concentration favours generalist species over specialists (Aponte *et al.*, 2010). We suggest three alternative explanations for the observed richness pattern in ECM fungi: elevated concentrations of available soil calcium could enhance the role of ECM symbiosis in mineral nutrition of plants, which in turn broadens the niche for coexistence of more species; calcium uptake and distribution rate are limiting for many key functions of plants (McLaughlin & Wimmer, 1999), and the improved physical condition of host plants may enhance richness of ECM fungi (Swaty *et al.*, 2004); or *Alnus* and its ECM fungi may have radiated in limestone-rich habitats, which are abundant in southern China and Japan, the inferred centers of origin of the host genus (Navarro *et al.*, 2003). Differences in soil pH, mainly driven by soil calcium carbonate concentration (except in ultramafic soils), determine the species pool of vascular plants (Pärtel, 2002). Clearly, these three alternative but partly overlapping hypotheses are not verified and the relative roles of calcium and pH warrant further attention in plant and fungal ecology.

Community structure

Host evolutionary history as measured by multilevel phylogenetic relationships among *Alnus* spp. had the strongest impact on ECM fungal community composition. Treatment of subspecies of *A. viridis* separately in the analyses would have had a marginal influence on our results, because the studied genetic markers were nearly identical in American and European subspecies. Based on extensive fruit-body collections in central Europe, Rochet *et al.* (2011) suggested that host specificity is most evident at the level of *Alnus* subgenus. Because all basidiomycetes associated with alders are regarded as highly host-specific (Molina *et al.*, 1992; Tedersoo *et al.*, 2009), *Alnus* spp. seem to obtain new symbionts both via host shifts from other trees (Tedersoo *et al.*, 2009) and through radiation and coevolution within the host genus (Moreau *et al.*, 2006; Rochet *et al.*, 2011). Speciation via shifts to phylogenetically distant hosts rather than cospeciation seems to predominate in other groups of ECM fungi (Wu *et al.*, 2000; Den Bakker *et al.*, 2004; Suvi *et al.*, 2010).

The global ECM fungal community of *Alnus* spp. resembles the phylogenetic structure reported previously for sites in Europe and America (Pritsch *et al.*, 1997; Becerra *et al.*, 2005; Tedersoo *et al.*, 2009; Kennedy & Hill, 2010; Kennedy *et al.*, 2011), but there were some notable differences in certain regions. In particular, members of the /genea-humaria and /inocybe lineages were among the most common MOTUs in Iran, but were only occasionally found in the rest of the world. Such regional differences have been also reported from Mexico, where species of the /clavulina and /sebacina lineages are among the most species-rich members of the community (Kennedy *et al.*, 2011), albeit never recorded from *Alnus* spp. previously. These discrepant results were suggested to be related to local site conditions, such as volcanic soils, which may favour proliferation of specific fungal groups (Kennedy *et al.*, 2011). While our study confirms that certain members of both the /clavulina and /sebacina lineages are able to colonize roots of *Alnus* spp. in other regions as well, the ECM fungal community composition of *Alnus* assemblages in South America did not include a high number of these groups as would be expected from Central American communities of the same host. It remains unknown whether the characteristic ECM fungal lineages of *Alnus* in Central America and northern Iran have evolved and radiated in these regions or whether they result from natural selection by specific environmental conditions. Community composition of other hosts in these regions provides no evidence to support the hypothesis that these regional environmental conditions favour the abundance of particular ECM fungal lineages (Morris *et al.*, 2009; Bahram *et al.*, 2012).

Biogeographic patterns

Among biogeographic regions, northern and southern Europe had the highest similarity in species composition, probably because of the small geographical distance, shared host species and history. Glacial cycles had a particularly strong impact on European fauna and flora: northern Europe was under ice cover and much of central Europe was affected by permafrost at the last glacial maximum 18 000 yr ago. *Alnus* survived only in southern refugia, and the recolonizing populations became genetically impoverished in northern Europe (King & Ferris, 1998). The lack of similarity between Iran–Turkey and southern Europe can be only partly explained by differences in host species composition, because *A. glutinosa* is shared between these two regions. The Hyrcanian forests served an important refugium of temperate broadleaved trees including *Alnus* during the Quaternary glaciations (Akhani *et al.*, 2010). These discrepant patterns in species composition as observed in Iran–Turkey and Mexico outline the importance of regional historical and potentially environmental processes in shaping the local communities (Ricklefs, 2004).

Intercontinental differences in the ECM fungal MOTU composition were generally as great as intracontinental differences. These biogeographic patterns were driven by the phylogenetic relations among host plants that accounted for a large proportion of variation in fungal community composition. This reflects either specificity for narrow host lineages or mixed patterns of coevolution and comigration in fungi and their host plants. The narrow specificity is unsupported by axenic synthesis experiments and field observations (Molina *et al.*, 1992; Tedersoo *et al.*, 2009), but there is limited evidence for coevolution among *Alnus* and its ECM mycobionts (Rochet *et al.*, 2011).

Besides comigration of plants and fungi resulting from anthropogenic cointroduction to New Zealand (Dickie *et al.*, 2010) and postglacial climate warming in Europe (Murat *et al.*, 2004), more ancient patterns become evident from the clustering of northwest

America with Asian regions rather than with northeast America. Northwest American *Alnus* spp. migrated from Asia via the Beringian land bridge > 5 MA (million years ago), whereas northeastern American species that migrated from Europe probably used the North Atlantic land bridge > 30 MA (Furlow, 1979; Tiffney & Manchester, 2001). The similarity of northwest America and Asian regions in fungal species composition does not resemble the continental-scale disjunction patterns of animals and plants in general. Animal groups exhibit strong links between northeast and northwest America, inferring the importance of recent migration (Sanmartin *et al.*, 2001). Conversely, the most common floristic disjunction patterns between Asia and northeast America suggest a key role of extinctions (particularly in Europe and northwest America) in shaping the present biogeographic patterns of plants in the northern hemisphere (Donoghue & Smith, 2004).

Conclusions

Intrageneric phylogenetic relations among *Alnus* spp. explain a large part of the ECM fungal community structure within this genus at the global scale, indicating that closely related hosts generally exhibit more similar fungal communities largely independent of geographical distance and environmental variables. All *Alnus* spp. associate with a narrow range of ECM fungi, and soil calcium concentration constitutes a key predictor of *Alnus*-associated ECM MOTU richness. Several *Alnus*-associated mycobionts that share 100% ITS similarity occur in Europe, Asia and South America, which is the greatest natural range of ECM fungal species besides the asexual *Cenococcum geophilum* complex. Although ECM fungal communities of *Alnus* were relatively uniform at the global scale, certain regions within continents possessed highly deviating composition of ECM fungal lineages, indicating the importance of regional processes in community development. Both the wide distribution of species and biogeographic similarity between southern and northern Europe, and Asian regions with northwest America are consistent with the hypothesis of host and mycobiont comigration. This needs to be further refined, however, using population genetics and phylogenetics tools based on a group of closely related species or within biological species. Considering the context dependence, spatial and phylogenetic scale of ECM fungal biogeography, this field clearly remains open to further research.

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Supporting Information

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

Fig. S1 Delimitation of MOTUs in the *Tomentella stuposa* complex based on the ITS phylogram.

Fig. S2 Distribution of *Alnus*-associated ECM fungal MOTUs among host plant species and biogeographic regions.

Fig. S3 Nonmetric multidimensional scaling (NMDS) ordination plot with confidence intervals demonstrating the relative effects of (a) geographic regions and (b) host species on the community of *Alnus*-associated ectomycorrhizal fungi.

Table S1. Detailed sampling data

Table S2. Sørensen indices indicating similarity between continents and regions

Table S3. Methods of measuring environmental variables

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