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Arbuscular mycorrhizal fungi associating with roots of *Alnus* and *Rubus* in Europe and the Middle East

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are ubiquitous microbes in terrestrial habitats with important roles in ecosystem functioning, but knowledge of their large scale biogeography remains particularly limited in some regions and in association with woody host species. The objective of this study was to characterize diversity and community structure of AM fungi associated with the woody plants *Alnus* and *Rubus* in Eastern Europe and Iran-Turkey. AM fungi were identified on the basis of SSU rRNA gene sequences: 39 virtual taxa (VT) were detected across 24 study sites, with high dominance by two Glomeraceae VT (VT113 and VT115 related to *Rhizoglomus intraradices-irregulare* group). Community structure of AM fungi was driven by climatic and spatial variables, while host identity influenced the frequency of AM fungal occurrence. AM fungal communities from species-poor sites were subsets of those in richer sites, indicating nestedness and a progressive loss from the AM fungal species pool.

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1. Introduction

The geographical distribution of living organisms depends on abiotic and biotic factors (Lomolino et al., 2006). Climatic and edaphic variables shape the distribution of organisms through their ecological tolerance, while above- and belowground biotic interactions such as mutualistic or parasitic associations can shape community structures of both partners (Mittelbach, 2012).

Soil microbes have great importance in soil nutrient cycling and mineral nutrition of plants. Arbuscular mycorrhizal (AM) fungi are ubiquitous and obligate plant root symbionts from the phylum Glomeromycota, associating with *c.* 80% of vascular plant species (Smith and Read, 2008). While providing several benefits to host plants, such as enhanced mineral uptake and increased resistance to drought and pathogens (essentially acting as extensions of plant root systems), AM fungi rely on photosynthetically fixed carbon provided by the host (Smith and Read, 2008). It is estimated that the AM symbiosis determines the flow of 5 billion tons of carbon annually (Bago et al., 2000; Soudzilovskaia et al., 2015), while the global biomass of AM fungi in roots has been estimated as 1.4 Pg dry weight (Treseder and Cross, 2006). Thus, Glomeromycota communities have a broad ecological influence as they affect several important ecosystem processes, including soil geochemical cycles, plant productivity, plant diversity and soil structure (van der Heijden et al., 2008; van der Heijden et al., 2015).

Despite their ubiquity, only *c.* 250 morphospecies of AM fungi have been described, while DNA-based estimates of global species numbers double the figure (Öpik et al., 2014; van der Heijden et al., 2015). However, small size and the cryptic lifestyle of AM fungi create difficulties in disentangling biogeography and ecology of Glomeromycota. Moreover, factors driving community structure and diversity of AM fungi can be highly scale and context

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dependent (Davison et al., 2015, 2016). Larger-scale case studies (Moora et al., 2011; Öpik et al., 2013; Davison et al., 2015, 2016) and meta-analyses (Öpik et al., 2006; 2010; Kivlin et al., 2011) suggest important roles for both environmental and spatial factors in shaping AM fungal communities. Dispersal limitation and local abiotic and biotic environmental conditions may determine AM fungal diversity and community composition, but knowledge about their relative importance remains scarce (Chaudhary et al., 2008; Dumbrell et al., 2009; Davison et al., 2015, 2016).

Glomeromycota are frequently regarded as generalists and their communities tend to be more nested than those of fungi forming other mycorrhizal types (van der Heijden et al., 2015). Nevertheless, it is becoming increasingly clear that host identity or host ecological groups can influence AM fungal community composition (Vandenkoornhuyse et al., 2003; Öpik et al., 2009; Davison et al., 2011, 2015, 2016). AM plants can either form only AM symbiosis or be simultaneously involved in several types of root symbioses, e.g. concurrent AM and ectomycorrhiza (Smith and Read, 2008) or AM and association with prokaryotic symbionts, which adds complexity in assessing the importance of AM in particular cases (Mortimer et al., 2008). To address Glomeromycota distribution without a potentially confounding effect of host identity, AM fungal surveys need to be carried out using either locally common or phylogenetically closely related plant taxa across different study sites. Therefore, we selected members of two plant genera, *Alnus* and *Rubus*, for a comparative continental-scale study. Species of these genera are common in mixed temperate and boreal forests throughout Europe. Both genera often co-occur in the same natural habitats and associate with AM fungi. Furthermore, members of these plant genera have different symbiotic profiles: *Rubus* species growing on mineral soil are known to associate only with AM fungi, whereas *Alnus* simultaneously hosts AM and EcM fungi and nitrogen fixing actinobacteria from the genus *Frankia* (Chatarpaul et al., 1989). Such triple symbiosis may mean that AM fungal partners may have smaller importance in the nutrition of the host plant.

This study aimed to assess the relative effects of biotic and abiotic factors on community structure and species richness of AM fungi associating with *Rubus* and *Alnus* roots at the continental scale. We posed two main hypotheses: (i) *Rubus* and *Alnus* have substantially different AM fungal communities; (ii) at the continental scale, host genus is a major determinant of AM fungal community structure compared to environmental variables.

2. Materials and methods

2.1. Study sites and sampling

The study was performed in 24 study sites along a latitudinal gradient in Eastern Europe, Southern Turkey and Northern Iran, covering the hemiboreal, temperate and Mediterranean ecosystems. We selected a subset of sites from Põlme et al. (2013) in which *Alnus* and *Rubus* species co-occurred (Fig. 1; Table S1). Root samples of randomly selected six adult *Alnus* and *Rubus* individuals separated by at least 10 m were collected from each study site, except for two study sites in Iran were only *Alnus* roots were collected. Sampling was performed in September 2009, except for Iran were sampling took place in October 2008 (Table S1). Roots were manually cleaned from adhering soil and wrapped in tissue paper, placed in plastic bags containing silica gel and stored at room temperature until processing. In total, 276 root samples (12 from each study site, except for two Iranian study sites where six root samples were collected) were subjected to molecular analyses.

2.2. Soil analysis

At the rooting depth of each soil core, *c.* 50 g of rhizosphere soil was cleaned from the roots and pooled by sampling areas. Soil samples were dried and subjected to mineral analysis. Total nitrogen was determined by the Kjeldahl method with a Tecator ASN 3313. Phosphorus was extracted using ammonium lactate and measured by flow injection analysis (Ruzicka and Hansen, 1981). Available potassium was determined from the same solution by the flame photometric method (AOAC956.01). Exchangeable calcium and magnesium content in the soil were measured in an ammonium acetate extract (pH = 7.0). The organic matter content was determined based on the loss of gases after ignition for 2 h at 360 °C. Soil pH was measured in a 1 N potassium chloride solution.

2.3. Molecular analyses

DNA was extracted from 30 mg of dried roots from each individual root sample with PowerSoil-htp™ 96 Well Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following Öpik et al. (2013). Glomeromycota were identified in plant roots using PCR, cloning and Sanger sequencing of ca. 560-bp central fragment of the SSU rRNA gene, amplified from root DNA extracts using the Glomeromycota specific primer pair NS31 (5′-ATC AAC TTT CGA TGG TAG GAT AGA-3′) and AML2 (5′-GAA CCC AAA CAC TTT GGT TTC C-3′) (Simon et al., 1992; Lee et al., 2008). PCR and cloning of PCR products were performed as in Öpik et al. (2013). Sixteen positive products of colony PCR per each root sample were sequenced at Macrogen Inc. (Seoul, South Korea) with the primer M13R.

2.4. Bioinformatics

Primer and vector sequences were removed from the sequence data. Short (<517 bp) and low-quality sequences were removed. The remaining sequences were assigned to virtual taxa (resembling operational taxonomic units at species or slightly higher level) following the methodology of Davison et al. (2011). In short, we conducted BLAST searches (soft masking with the DUST filter) against the Maarj*AM* database (Öpik et al., 2010; status as of 26.03.2015) with the following criteria required for a match: (1) sequence similarity ≥97%; (2) the alignment length should not differ from the length of the shorter of the query and subject (reference database sequence) sequences by more than ten nucleotides; (3) a BLAST *e*-value $<1 \times e^{-50}$. All sequences were assigned to known virtual taxa (VT) in Maarj*AM* database.

Sequences of this study were automatically aligned together with type sequences of detected VT, retrieved from the Maarj*AM* database, using MAFFT (Katoh and Toh, 2008). A maximum likelihood (ML) phylogram was constructed in RAxML using the default settings, exhibiting representative sequences of detected virtual taxa from each host and site (Stamatakis et al., 2008). We used *Nectria pseudotrichia* (INSD accession no. JN939687) SSU rRNA gene sequence as an outgroup. All quality filtered sequences were submitted to the European Nucleotide Archive (acc. no. KJ959651-KJ960173).

2.5. Statistical analysis

We used vegan package in R to visualize accumulating AM fungal VT richness in individual root samples as a function of number of clones sequenced (sequencing effort) and across samples (sampling effort) (Oksanen et al., 2007). In order to evaluate whether *Rubus* and *Alnus* exhibit comparable AM frequency in terms of successfully amplified AM fungal sequences from individual root samples, we conducted Fisher's exact test.

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