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Research Article

RESEARCH ARTICLE

Plant species identity and soil characteristics determine rhizosphere soil bacteria community composition in European temperate forests

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One sentence summary: Rhizosphere soil bacterial community composition shows high habitat dependency. This study reported the influence of plant identity, soil chemistry, climate, nitrogen deposition and land-use history on its dynamics across Europe.

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ABSTRACT

Soil bacteria and understorey plants interact and drive forest ecosystem functioning. Yet, knowledge about biotic and abiotic factors that affect the composition of the bacterial community in the rhizosphere of understorey plants is largely lacking. Here, we assessed the effects of plant species identity (*Milium effusum* vs. *Stachys sylvatica*), rhizospheric soil characteristics, large-scale environmental conditions (temperature, precipitation and nitrogen (N) deposition), and land-use history (ancient vs. recent forests) on bacterial community composition in rhizosphere soil in temperate forests along a 1700 km latitudinal gradient in Europe. The dominant bacterial phyla in the rhizosphere soil of both plant species were *Acidobacteria*, *Actinobacteria* and *Proteobacteria*. Bacterial community composition differed significantly between the two plant species. Within plant species, soil chemistry was the most important factor determining soil bacterial community

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composition. More precisely, soil acidity correlated with the presence of multiple phyla, e.g. *Acidobacteria* (negatively), *Chlamydiae* (negatively) and *Nitrospirae* (positively), in both plant species. Large-scale environmental conditions were only important in *S. sylvatica* and land-use history was not important in either of the plant species. The observed role of understorey plant species identity and rhizosphere soil characteristics in determining soil bacterial community composition extends our understanding of plant-soil bacteria interactions in forest ecosystem functioning.

Keywords: forest age; herbaceous layer; macroclimate; N deposition; soil acidity; soil bacterial diversity

INTRODUCTION

Understorey plants in temperate forest ecosystems play a critical role for the maintenance of biodiversity, nutrient and carbon (C) cycling, evapotranspiration and tree regeneration (Gilliam 2007). Soil bacteria are one of the most abundant and diverse organisms on earth (Bardgett and van der Putten 2014; Delgado-Baquerizo *et al.* 2018). Despite their importance for ecological processes, rhizospheric bacterial diversity under different understorey plant species in temperate forests are less studied. The ecological importance of soil bacteria (e.g. in biogeochemical cycling) in different ecosystems has only being elucidated recently because of advanced analytical methods (reviewed in Llado, Lopez-Mondejar and Baldrian 2017). In forest ecosystems, bacteria are among the most abundant microorganisms in soils (Lauber *et al.* 2009), and their composition varies substantially across the globe owing to distinct biotic and abiotic conditions in different habitats (Delgado-Baquerizo *et al.* 2018). Understanding the driving factors for soil bacterial diversity and composition is essential for maintaining ecosystem functioning because the dynamics of the soil bacterial community determines multiple ecological processes, for instance, litter decomposition and nitrogen (N) fixation (Bardgett and van der Putten 2014; Wu *et al.* 2018). Yet, knowledge about the extent to which soil bacterial community can be affected by understorey plant species and associated abiotic environmental drivers, such as soil chemistry, climatic conditions, N deposition and land-use history, in temperate forests is still limited.

Plant species can affect the soil bacterial community around the root systems via rhizosphere resources (e.g. root exudates, allelochemicals and soil nutrients) (Haichar *et al.* 2008; Eilers *et al.* 2010). Root exudates produced by different plant species may result in species-specific interactions with different soil microbial groups and finally shift bacterial community composition. For instance, nitrification inhibitory compounds can constrain the activity and change the composition of nitrifying bacteria (Gopalakrishnan *et al.* 2009). Additionally, plants can modify soil bacterial community composition through effects on soil C resources and nutrients because plants translocate up to 16% of plant N and 40% of photosynthetically fixed C into the soil (Berendsen, Pieterse and Bakker 2012; Bulgarelli *et al.* 2013). However, there are inconsistent results of plant species effects on soil bacterial community composition. Dawson *et al.* (2017) compared soil bacterial community composition under 19 herbaceous grassland species and found that plant species identity was not a significant factor in explaining the composition of soil bacterial community. In forest understoreys, studies have mainly focused on the driving effects of understorey plant species diversity, richness and abundance on soil microbial community assembly (Wardle *et al.* 2006; McIntosh Macdonald and Quideau 2013). Studies on a specific understorey plant species affecting its rhizosphere bacterial community composition will help to understand the underlying mechanisms of plant–bacteria interaction in temperate ecosystems.

Abiotic conditions are highly correlated with the dynamics and activity of soil microbes (Bissett *et al.* 2013). Among those, soil chemistry is one of the overarching reasons that drive soil microbial community assembly. For instance, soil pH and nutrients are key drivers for microbial catabolic activities (the process of breaking down molecules into small units) and nutrient utilisation (Lauber *et al.* 2009; Klimek *et al.* 2015). Lauber *et al.* (2009) specifically focused on soil bacteria and characterized soil bacterial community composition across North and South America and found that the relative abundance of three phyla (i.e. *Acidobacteria*, *Actinobacteria* and *Bacteroidetes*) was strongly affected by soil pH. Klimek *et al.* (2015) compared soil bacteria along an altitudinal gradient and indicated that the utilization of amines differs significantly in bacterial communities that inhabited different sites. These biogeographical patterns between soil chemistry and bacteria can be used to predict soil bacterial dynamics at large geographical scales. Recently, a global-scale study on soil bacterial abundance and diversity again advocated that the high divergence of habitat preferences in terms of chemical and climatic characteristics is responsible for soil bacterial community assembly (Delgado-Baquerizo *et al.* 2018).

At larger spatial scales, also climate and N deposition loads affect the plant as well as the soil bacterial community, both directly and indirectly. The direct effects of soil temperature and moisture capacity on the activity of soil bacteria and the structure of the soil bacterial community composition are evidenced as explanatory mechanisms for soil microbial diversity change and biochemical kinetics (Avrahami and Bohannan 2007; Santana and Gonzalez 2015; Borowik and Wyszkowska 2016). Similarly, increased N deposition may favour copiotrophic bacteria taxa (species that have a high requirement of N) but suppress the diversity of oligotrophic bacteria taxa (Fierer *et al.* 2012). Indirectly, soil bacterial community assembly can be affected through changes in aboveground plant community composition (Zak *et al.* 2011). As such, soil microbes inhibited under understorey plants are influenced by resource inputs and allelochemicals.

Finally, past land use is also a key driver of forest communities (Perring *et al.* 2018). The past land use influences both biotic and abiotic conditions in forests and past use imprints can persist for decades to centuries, reflecting on dissimilarities in vegetation, and chemical and physical soil properties (Jangid *et al.* 2011; Aggemyr and Cousins 2012; Bachelot *et al.* 2016). These dissimilarities, together with current climate change and N deposition, can modulate the activity and composition of soil microorganisms (Dupouey *et al.* 2002; Ma *et al.* 2018). Thus, the soil bacterial community may differ between ancient forests (i.e. those forests already present on the oldest available land use maps, typically > 200 years old (Hermy and Verheyen 2007), and more recently established forests on former agricultural land. Apart from knowing the difference of soil bacterial assemblages between ancient and recent forests, the most important rational is to understand and predict soil microbial succession in the face of land fragmentation and land-use change relative to the formation of understory vegetation.

Here, we used a 16S rRNA gene marker approach to assess soil bacterial community composition under two widespread temperate forest understorey plant species (*Milium effusum* and *Stachys sylvatica*). Rhizosphere soils under each plant species were sampled in ancient and recent forests along a 1700 km latitudinal gradient in Europe. The importance of biotic (plant species identity) and abiotic (soil chemistry, large-scale environmental conditions and land-use history) factors on differences in soil bacterial community composition was assessed at the European scale. Our aims were to address (i) whether the soil bacterial community is affected by the plant species identity; and (ii) within each plant species, which abiotic factors affect the soil bacterial community composition.

MATERIALS AND METHODS

Study species

Milium effusum L. (Poaceae), and *Stachys sylvatica* L. (Lamiaceae), covering distinct plant functional types, are both perennial and rhizomatous understorey plant species. *Milium effusum* is a hemicryptophyte, early-summer flowing grass; *S. sylvatica* is a protohemicryptophyte, summer forb (Taylor and Rowland 2010; De Frenne *et al.* 2017). Both plant species are characteristic for the understorey layer across European temperate forests and may be used, in some regions, as indicators for ancient forests (Wulf 1997). However, both species are good colonizers of non-isolated recent forest patches across ancient-recent forest ecotones (De Frenne *et al.* 2011a; Brunet *et al.* 2012). Their interaction with soil microbes may differ owing to different habitat preferences in physicochemical conditions. At the global scale, *M. effusum* commonly grows on moderately acid, mesotrophic soils (De Frenne *et al.* 2017), while *S. sylvatica* prefers weakly acid or basic, eutrophic soils (Taylor and Rowland 2010). The seeds of both species are wind- and gravity-dispersed, but epizoochory also occurs (Hermy *et al.* 1999; Graae 2002; Grime, Hodgson and Hunt 2007). Reproduction from seed is the main regeneration mode, but *M. effusum* also produces stolons for vegetative spread and stolons of *S. sylvatica* have been found to be effective for local clonal reproduction (Taylor and Rowland 2010, De Frenne *et al.* 2011b).

Soil sampling

We collected soil samples in eight regions, i.e. Northern France (NF), Belgium (Be), Poland (Po), Western Germany (WG), Eastern Germany (EG), Southern Sweden (SS), Central Sweden (CS) and Estonia (Es) in June and July 2015 (Fig. 1). Within each region, soil samples for each species were taken from two pairs of forest patches differing in their time of origin, i.e. each pair consisted of one ancient (having existed continuously as forest based on the oldest maps (*ca.* 1750) in Europe, (Hermy and Verheyen 2007)) and one recent (established on former agricultural lands, (De Frenne *et al.* 2011a)). Within each forest, we surveyed the entire patch until we found the two plant species, and the sampling sites for the four plant species within each patch were at least 50 m away from each other. When the four species did not occur in the same forest patch in a certain region, we sampled from other forests but with consistent land-use history within the region. At each sampling site, we selected one healthy individual (with no signs of damage from herbivores or pathogens) growing at least 10 m away from the nearest forest edge. Tree species composition within a 5 × 5 m^2 range around each sampling site was recorded to assess the forest uniformity and litter quality across the sites. This record was used as background information but not a determinant of site selection because it was not possible to select focal individuals with consistent tree species composition across all regions (Table S1 and S2, Supporting Information).

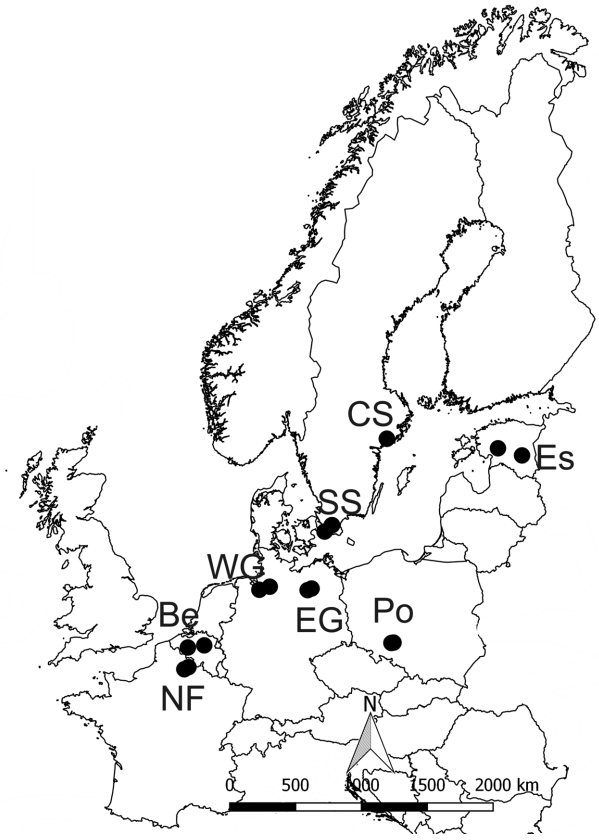

Figure 1 Sampling regions along the latitudinal gradient in Europe used for soil collection: Northern France (NF), Belgium (Be), Poland (Po), Western Germany (WG), Eastern Germany (EG), Southern Sweden (SS), Central Sweden (CS) and Estonia (Es).

For each focal individual, we cut the stem at about 1 cm above the ground and took one soil sample using an auger with a diameter of 3 cm till a depth of 10 cm after removal of the litter layer around the base of the plant stem. All sampling was performed between June and August 2015. Sampling time across regions and species was standardized by only sampling at the moment of complete seed maturation of the focal plant species. Stem diameters of focal individuals fluctuated around 5 mm. Debris and stones were removed from the soil samples immediately after sampling. Soil was brushed off from the plant root systems and used for further analyses. We defined the used soils here as rhizosphere soil because of their distances to plant roots (*ca.* 10 mm) and the high likelihood that this soil is both abiotically and biotically affected by plant roots (McNear Jr 2013). In total, we collected 62 soil samples (8 regions × 4 sites × 2 plant species, but *M. effusum* was collected from three ancient forests but not from recent forests in Belgium, and *S. sylvatica* was absent in one recent forest in Estonia). Soil samples were stored at 4°C and transported in plastic bags until treatments in the central lab in Belgium. All samples were immediately sieved through a 1-mm mesh upon arrival and stored at −18 °C until the start of soil DNA extraction (the mesh was cleaned and sterilized with 75% ethanol in between samples). A subsample of each soil sample was taken and dried at 40 °C for 48 h for subsequent chemical analyses.

Soil bacterial community

Total DNA extraction from soil samples was carried out with the PowerSoil®DNA Isolation kit and purified by means of the Wizard®DNA Clean-Up System, following the manufacturer's instructions. The 16S rRNA gene v3-v4 region was amplified by PCR using the barcoded versions of the primers described by Klindworth *et al.* (2013). The PCR mix included 1 μL of DNA extract, 15 pmol of both the forward primer 341F 5'- NNNNNNNN NTCCTACGGGNGGCWGCAG-3' and the reverse primer 785R 5'-NNNNNNNNNNTGACTACHVGGGTATCTAAKCC-3' in 20 μL volume of MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 μL of BioStabII PCR Enhancer (Sigma). For each extracted sample, the forward and reverse primers had the same unique 10-nt barcode sequence. PCRs were carried out with an initial denaturation of 2 min at 96°C, followed by 20 cycles of 15 s at 96°C, 30 s at 50°C, 90 s at 70°C and a final extension of 5 min at 72°C.

The DNA concentration of the amplicons of interest was determined by gel electrophoresis. Next, 20 ng amplicon DNA of each sample was pooled. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MinElute columns (Qiagen). Finally, about 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries by means of adaptor ligation using the Ovation Rapid DR Multiplex System 1–96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq platform.

Contigs were created by merging paired-end reads based on the Phred quality score (of both reads) heuristic (Schloss, Gevers and Westcott 2011; Kozich *et al.* 2013), in MOTHUR (v.1.38) (Schloss *et al.* 2009). Contigs were aligned to the Silva database (v123), and filtered from those with (i) very divergent lengths (outside of the 2.5%–97.5%) and ambiguous bases, (ii) sequences falling outside of the alignment space and with more homopolymers (maximum of the alignment database), (iii) those not corresponding to the v3-v4 region. The aligned sequences were filtered and dereplicated, while sequencing errors were removed using the *pre.cluster* command. Chimera removal was performed with the *uchime* command. The sequences were compared to RDP 16S rRNA reference version 10 and clustered into operational taxonomic units (OTUs) at 97% similarity with the *cluster* command (opticlust algorithm). Rarefaction curves were plotted to show how many bacterial species were discovered with increased sampling efforts (Fig. S1, Supporting Information). Soil bacterial biomass was determined by assessing phospholipid fatty acids (PLFAs). In total, 15 PLFA biomarkers were assigned to bacteria, including *Actinobacteria*: 10MeC16:0, 10MeC18:0; Gram-positive bacteria iC15:0, aC15:0, iC16:0, aC16:0, iC17:0; Gram-negative bacteria: 16:1ω7c, cy17:0, C18:1ω7t and non-specific bacteria C14:0, C15:0, C16:0, C17:0, C18:0. The concentration of each PLFA (μg/g) was added together to indicate the total bacterial biomass. A full detailed description of the methods can be found in Ma *et al.* (2018).

Soil chemistry

Soils were combusted at 1200°C, and the gases were measured using a thermal conductivity detector in a CNS elemental analyser (vario Macro Cube, Elementar, Germany) for total carbon (C) and nitrogen (N). Total phosphorus (P) was measured after complete destruction of the soil samples with $HClO_4$ (65%), HNO_3 (70%) and H_2SO_4 (98%) in Teflon bombs for 4 h at 150°C. The concentrations of total P were measured colorimetrically according to the malachite green procedure (Lajtha *et al.* 1999). Bioavailable phosphorus (Olsen P), which is available for plants within one growing season (Gilbert, Gowing and Wallace 2009), was measured by using extraction in $NaHCO_3$ (according to ISO 11 263:1994 (E)) and colorimetric measurement according to the malachite green procedure (Lajtha *et al.* 1999). Potassium (K), calcium (Ca), magnesium (Mg) and aluminum (Al) were measured by extracting soil samples with NH_4 Ac-EDTA and by analysing with atomic absorption spectrophotometry. Soil pH-H_2O was measured after mixing 10 g of soil and 50 mL of water and shaking for 5 min at 300 rpm using a pH meter Orion 920A (with pH electrode model Ross sure-flow 8172 BNWP, Thermo Scientific Orion, USA). See Supporting Information (Table S3) for soil chemistry description for each sampling region.

Large-scale environmental conditions

We calculated mean annual temperature and precipitation for each sampling site at the scale of 30 arc-seconds (approximately 1 km^2 at the equator) using WorldClim version 2 (http://worldclim.org/version2) (Fick and Hijmans 2017). Atmospheric N deposition at each sampling site was calculated for the year 2015 as the sum of wet and dry depositions of oxidised (NO_y) and reduced (NH_x) N based on modelled EMEP deposition data; and the model results of the 2016 version (data edition 2015v2016, 50 km resolution; http://www.emep.int/mscw/mscw_ydata.html#NCdata; Table S3).

Data analysis

Data analyses of the bacterial community were conducted after proportional normalization. We normalized OTUs data by first taking the proportion of each OTU reads in the total number of reads, then multiplying the result with the minimum sample size (3338 reads) and rounding to the nearest integer to account for sample size differences (McMurdie and Holmes 2014). This resulted in a scaled taxon-abundance matrix comprised of 10 729 OTUs which were classified into 38 phyla. OTU reads in each soil sample were multiplied with bacterial biomass (abundance) in the corresponding soil sample to calculate the absolute abundance of read count. Comparing with traditional relative abundance based on OTU reads, the action of multiplication considers one more parameter, i.e. bacterial abundance (Props *et al.* 2017). The absolute abundances were used for all composition and diversity analyses. Predictors for soil bacterial community diversity and community composition were plant species identity (biotic) and three abiotic factor groups (soil chemistry, large-scale environmental conditions and land-use history). We did not include latitude and longitude in our data analyses because spatial autocorrelation tests were not significant. Additionally, trees can significantly affect soil chemistry (de Schrijver *et al.* 2012). Therefore, we also considered the effect of tree species composition on bacterial diversity and community composition due to tree compositional divergence among sampling sites. Litter quality (LQ) scores were used as an indicator of litter decomposition rate. The score for individual tree species ranges from 1 to 5 (1: very low decomposition rate, 5: very high decomposition rate) (Table S2, Supporting Information). At each sampling site, tree canopy cover weighted average of LQ score was calculated and used for further data analyses. More details about sources for extracting LQ scores can be found in Table S1 and S2 (Supporting information).

To visualise the composition of soil bacterial community, we calculated the absolute abundance of shared OTUs and unique

OTUs (Schmidt et al. 2016) in M. effusum and S. sylvatica, following a chi-square test to assess the differences of absolute abundances in the unique OTUs between the two plant species. Phylum composition in the shared and unique group was plotted separately using the ggplot2 package (Wickham 2009). Non-metric multidimensional scaling (NMDS) was used to visualize the difference of soil bacterial community composition (both OTU and taxonomic phylum level) between the two plant species. We did not test at family level because 69% to 82% of bacterial families were unclassified making the results uninformative. The significance of the difference was tested using PERMANOVA (site nested within region) with Bray–Curtis distance and performed within the function adonis implemented in the vegan package (Oksanen et al. 2016).

Three alpha diversity indices, i.e. species richness, Shannon diversity and the inverse Simpson index were calculated at the OTU level. We assessed the effects of 16 predictor variables: plant species, land-use history, soil chemistry (10 variables), large-scale environmental conditions (three variables) and tree species composition on each alpha diversity index, using functions glmer (species richness) and lmer (Shannon diversity and inverse Simpson) (site nested within region as random effect) in the lme4 package (Bates et al. 2015). To test whether the predictor variable affected alpha diversity (response variables) significantly, we constructed an effect model (including the predictor) and a null model (only including an intercept) for each response (alpha diversity index) and predictor variable using chi-square tests following Zuur et al. (2009). Each model comparison was implemented separately on a one-by-one basis. The same model comparison was applied to test the differences of the absolute abundance of each bacterial phylum between plant species, and for the chemical soil characteristics depending on the land-use history and plant species. Values of Shannon diversity and inverse Simpson were log- or sqrt- transformed to meet the assumptions of the statistical tests. Significances were determined with likelihood ratio tests.

Variation partitioning was used to evaluate the strength of plant species identity, soil chemistry, large-scale environmental conditions and land-use history, as well as the three abiotic factor groups and one biotic factor (tree species composition) within each plant species, in explaining the variation in soil bacterial community composition (phylum and OTU level). The base of this statistic was redundancy analysis (RDA). Phylum and OTU data were Hellinger-transformed before variation partitioning. Adjusted R^2 values were used due to the unbalanced number of variables in each variable category. The significance of each factor was tested using function anova.cca in the vegan package (Oksanen et al. 2016).

In order to find the most significant abiotic drivers determining soil bacterial community composition in each plant species, all variables in soil chemistry, large-scale environmental conditions and land-use history were combined and then were selected using the function forward.sel in the package adespatial with 999 permutations. Site scores were extracted from the RDA results and selected significant variables were plotted using the package ggplot2 (Wickham 2009).

RESULTS

Soil bacterial community composition

The main bacterial phyla in our samples were Acidobacteria, Actinobacteria and Proteobacteria. These three phyla accounted for 76% of the absolute abundance of all OTUs in Milium effusum and 72% in Stachys sylvatica. The absolute abundance of the unique OTUs was significantly ($P < 0.001$) higher in M. effusum (60 712) than S. sylvatica (45 048) (Fig. 2A and B). The absolute abundance of each phylum in the unique OTUs showed evident differences between the two plant species (Fig. 2B). The absolute abundance of shared OTUs accounted for 93% (M. effusum) and 95% (S. sylvatica) of the overall abundance (Fig. 2A–C).

Soil bacterial community differed between the plant species

Soil bacterial community represented by OTUs (Fig. 3A) and phylum (Fig. 3B) both differed significantly between M. effusum and S. sylvatica (PERMANOVA, $P < 0.001$). Twelve phyla showed significant differences between the two plant species (Table S4, Supporting Information). For instance, one of the main phyla Acidobacteria was significantly more abundant under M. effusum, while nitrite-oxidizer bacteria Nitrospirae was nearly four times more abundant in S. sylvatica.

The effect of biotic and abiotic factors on alpha diversity

Of the three alpha diversity indices, richness was the most affected index, with significant effects of understorey plant species identity, multiple chemical characteristics, mean annual precipitation and litter quality (Table 1). Land-use history had no influence on any of the measured indices. The ratio of soil C to N and mean annual precipitation had contrasting effects and were the only two factors that had impacts on all alpha diversity indices, i.e. indices' values increased with the ratio of soil C to N, whereas decreased with mean annual precipitation.

Soil bacterial community composition is mainly driven by plant species identity and soil chemistry

Taking all biotic and abiotic factors together, plant species identity, soil chemistry and large-scale environmental conditions were significant drivers in the variation of soil bacterial community composition at phylum level (Fig. 4). Plant species identity with soil chemistry jointly explained 8%, soil chemistry purely explained 27% and large-scale environmental conditions purely explained 5% of the variation in bacterial community composition. Within each plant species, soil chemistry explained nearly half (47%) of the variation in M. effusum, and 27% in S. sylvatica. Large-scale environmental conditions explained only the variation in bacterial community composition in S. sylvatica significantly (11%). Land-use history did not result in differences in terms of soil chemistry (Table S5, Supporting Information) and was not significant in explaining the variation neither in M. effusum nor in S. sylvatica (Fig. 3). This absence of evidence was also found for litter quality (Fig. S2, Supporting Information). The observed significant effects at the phylum level also occurred at the OTU level (except insignificant effect of large-scale environmental conditions in S. sylvatica) (Fig. S3, Supporting Information).

The correlation between soil bacteria and significant abiotic drivers

Soil pH, Ca and total N concentration were the most significant drivers for bacterial community composition in the rhizospher soil under M. effusum, and pH and Al concentration were important drivers in S. sylvatica (Fig. 5). All significant chemical drivers were associated with soil acidity. Nitrospirae was positively correlated with soil pH in both plant species, while Actinobacteria

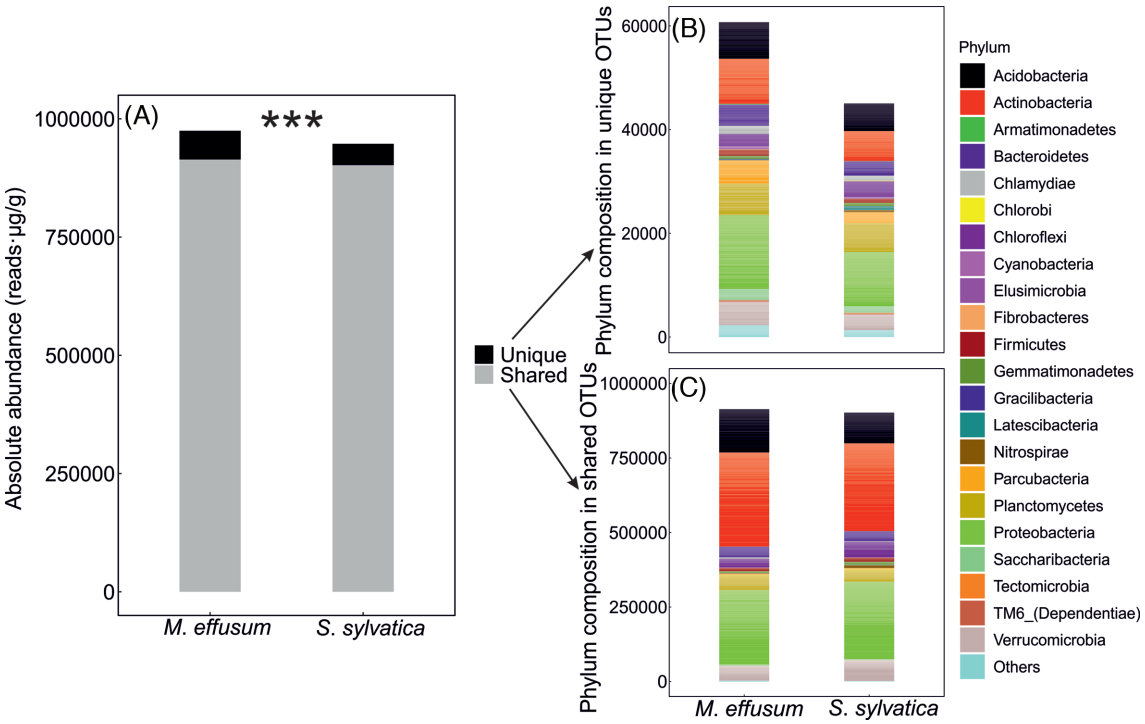

Figure 2 The absolute abundance of the number of unique and shared operational taxonomic units (OTUs) in rhizosphere soil samples of *Milium effusum* and *Stachys sylvatica* **(A)**. The community composition of bacterial phyla in unique **(B)** and shared **(C)** OTUs in *M. effusum* and *S. sylvatica*. *** indicates a significant difference in unique OTUs between the two plant species based on a chi-square test ($P < 0.001$). The phylum group 'Others' includes 15 bacterial Phyla whose absolute abundances were lower than 100.

showed a positive correlation with pH in *M. effusum*, but a negative correlation with pH in *S. sylvatica*. *Acidobacteria*, *Chlamydiae*, *Firmicutes* and *Proteobacteria* all had negative correlation with soil pH under both plant species.

DISCUSSION

In this study, we assessed the soil bacterial community composition in the rhizosphere of two common understorey plant species with distinct functional types at the continental scale. The results demonstrated that local factors, i.e. plant species identity and associated rhizosphere soil chemistry, were the main determinants of soil bacterial community diversity and composition. Each plant species formed a specific composition of bacterial phyla, and this correlated with plant species identity through rhizospheric chemical soil conditions, particularly soil acidity. Large-scale factors, i.e. climate and N deposition loads were only important in determining soil bacterial community composition in *S. sylvatica*.

Plant species identity and soil bacteria community composition

The dominant phyla were the same in both *M. effusum* and *S. sylvatica*, i.e. *Acidobacteria*, *Actinobacteria* and *Proteobacteria*. This

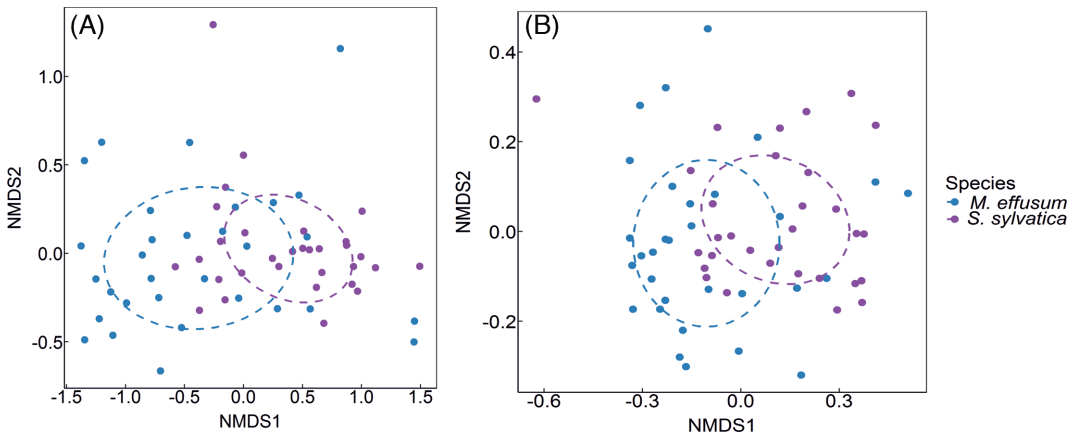

Figure 3 The compositional differences in the rhizosphere soil bacterial community based on non-metric multidimensional scaling (NMDS) in *Milium effusum* and *Stachys sylvatica* (distance metric = Bray). Data used in **(A)** were the absolute abundance of all OTUs, and in **(B)** only per phylum, see Fig. 2 for the phylum composition. The stress value is 0.10 (A) and 0.15 (B). Significance tests (PERMANOVA) for (A) and (B): $P < 0.001$***. Ellipses represent standard deviation of the samples within the species.

Table 1. Effects of biotic and abiotic predictor variables on alpha diversity of soil bacterial community represented by three indexes (species richness, Shannon index and inverse Simpson) based on generalized linear and linear mixed-effect models. For each alpha diversity index and each predictor variable, an effect model and a null model were constructed. Values (χ2-value) were obtained from each model comparison (the effect model vs. null model) by using maximum likelihood estimation.

Variable	Richness (Df = 1)	Shannon index (Df = 1)	Inverse Simpson (Df = 1)
Plant species	↑26.4***	2.8 ns §	0.6 ns §
Land-use history	<0.1 ns	<0.1 ns §	0.2 ns §
C %	↓53.9***	2.2 ns §	1.6 ns §
N %	↓56.2***	1.6 ns §	1.4 ns §
C/N	↓38.5***	↓12.2*** §	↓8.2** §
P (mg/kg)	↓28.1***	0.3 ns §	0.6 ns §
Olsen P (mg/kg)	↓10.6**	0.1 ns §	<0.1 ns §
K (mg/kg)	↓10.8**	0.8 ns §	2.3 ns §
Ca (mg/kg)	↓25.1***	1.5 ns §	1.1 ns §
Mg (mg/kg)	↓34.6***	0.1 ns §	1.5 ns §
Al (mg/kg)	↓9.5**	3.6 ns	0.1 ns §
pH (H_2O)	3.0 ns	2.3 ns §	0.3 ns §
MAT	2.4 ns	<0.1 ns §	0.8 ns §
MAP (mm yr^{-1})	↑17.0***	↑6.0* §	↑3.0(*) §
Ndep (kg/ha)	2.7 ns	0.1 ns §	<0.1 ns §
LQ	↑11.0***	0.5 ns	0.8 ns §

The direction of the effect is displayed as an arrow: ↑ indicates a positive effect and ↓ indicates a negative effect, ↑ in the row of plant species indicates higher species richness in *S. sylvatica*. ¶ A Poisson error distribution was applied. (*) $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns: non-significant ($P > 0.1$). § Log10-transformed. Sqrt-transformed. C: total carbon, N: total nitrogen, C/N: the ratio of soil carbon to nitrogen, P: total phosphorus, Olsen P: bioavailable phosphorus, K: potassium, Ca: calcium, Mg: magnesium, Al: aluminium. MAT: mean annual temperature; MAP: mean annual precipitation; Ndep: nitrogen deposition. LQ: litter quality. Df: the degree of freedom. Two outliers in richness were removed.

observation is consistent with the main trend of dominant bacteria in forest ecosystems (Lauber *et al.* 2009; Ma *et al.* 2018). However, the two plant species did show differences with respect to bacterial phylum abundance and uniqueness, and plant species identity significantly explained variation in soil bacterial community composition. Notably, our unique OTUs in *M. effusum* and *S. sylvatica* did not exclude rare OTUs (singleton OTUs in one soil sample) because of the risk of underestimating the diversity of soil bacteria (Brown *et al.* 2015). These rare OTUs accounted for 90% of the total OTUs in each plant species. The percentage of the rare OTUs in the shared group (singleton OTUs occurred in one soil sample of each species) decreased to *ca.* 25%. To our knowledge, this is the first study demonstrating rhizospheric bacterial community composition under individual understorey plant species in temperate forests. Our results are consistent with other studies, which have shown that tree (Chodak *et al.* 2015), moss (Bach, Frostegard and Ohlson 2009) and fern (Liu *et al.* 2012) identity have significant influences on soil microbial community composition in various ecosystems (i.e. temperate forests, grasslands, tundra). In grasslands, Dassen *et al.* (2017) conducted comparisons of soil bacterial community composition among legumes, grasses, small and large herbs and found that distinct soil bacterial communities occur especially between small herbs and grasses. Our observed significant effects of understorey plant species identity on bacterial community composition but not litter quality of tree layers (although a significant effect on species richness was found) imply strong correlations between soil bacteria and the root surface. One of the mechanisms is that plant traits, such as rhizodeposition, can impact rhizosphere bacterial community composition. According to Taylor and Rowland (2010), *S. sylvatica* showed a lower Ca but higher K-concentration in plant tissue than most other forest herb species. This characteristic may indirectly evidence the different chemical compounds released into rhizosphere soil via root systems between the two studied species. M-tyrosine, a chemical component of root exudates of the grass *Festuca rubra*, has been found important in regulating soil microbial activities and community composition (Kaur *et al.* 2009). Alternatively, litter quality produced by host plant species is highly selective in soil decomposers at a fine scale, leading to distinct soil microbial assemblage between plant species (De Deyn *et al.* 2004; Sayer *et al.* 2017). The characteristics of grasses (*M. effusum* in our study), such as tougher leaves, a longer life span and lower decomposition rates (Scharfy *et al.* 2011), may stimulate the accumulation of divergent bacterial species compared to forbs (*S. sylvatica* in this study). Although we observed little influence of tree species composition (litter quality) on soil bacterial community composition, the selective role of tree litter quantity was not assessed in this study and can be considered in the future because trees usually dominate forest ecosystems and have strong influences on both biotic and abiotic conditions for all biomes at large scales (Lu, Turkington and Zhou 2016). Whether litter quality of host plants or chemical composition of root exudates is the main mechanism is still open for the discussion. However, both are directly correlated with host plant species identity and soil chemistry of the rhizosphere (see Table S6, Supporting Information for the assessed chemical differences between the two plant species). Therefore, the effects of understorey plant species identity on soil bacterial diversity and composition demonstrated here facilitate our understanding of plant-soil interactions and soil physicochemical cycling in forest ecosystem. Yet, our study only focused on soil bacterial community. In addition, there is a large number of fungi, protozoa, archaea and soil fauna that occur simultaneously with bacteria. Changes in one community can lead to shifts in other communities, resulting in complex biotic interactions. Therefore, further studies are needed to disentangle plant-soil feedbacks in the face of global change.

Soil chemistry and bacterial community composition

Apart from the biotic factor of plant species identity, we assessed three groups of abiotic factors and found that soil chemistry

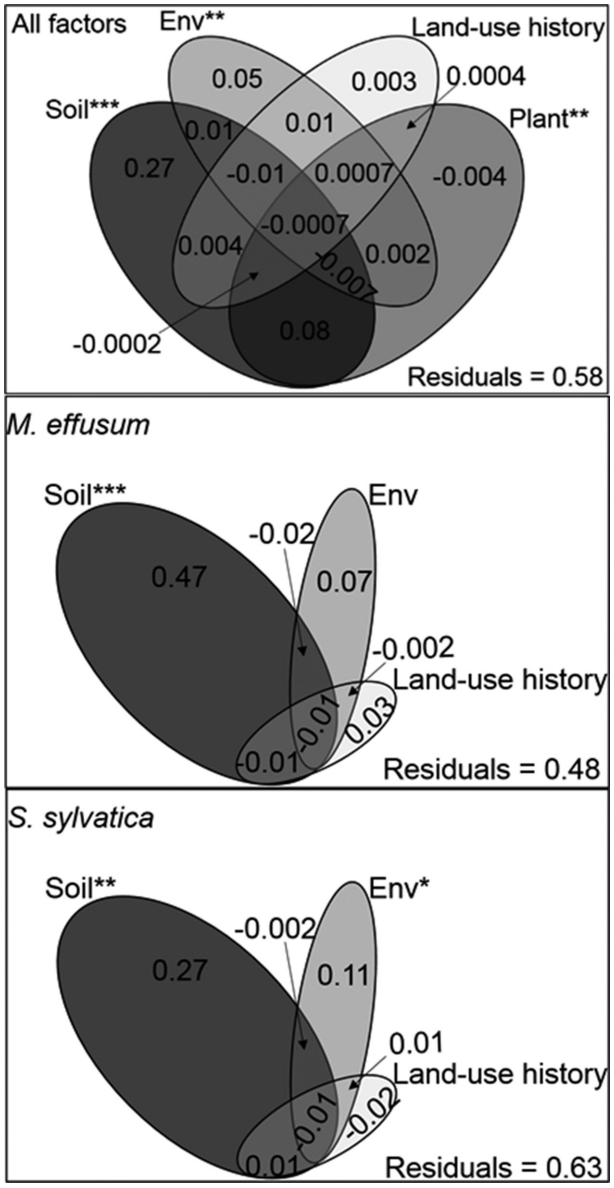

Figure 4. Variation partitioning of the rhizosphere soil bacterial community composition explained by all investigated factors, i.e. plant species identity (Plant), soil chemistry (Soil), large-scale environmental conditions (Env) and land-use history, and by three abiotic factors in each plant species. Adjusted R^2 values in each fraction indicate the explained proportion of the variation for that variable category. Residuals indicate the unexplained variation. Adjusted R^2 values may cause small negative values. Asterisks show the significance of permutation tests for each explanatory factor. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

was the most important determinant in soil bacterial community composition in each plant species. The critical role of soil chemistry is not surprising as soil bacteria and chemical properties are intimately correlated. Specifically, soil pH was one of the most influential chemical soil properties in the correlation with soil bacteria. This is consistent with previous studies conducted at different large geographical scales (Lauber *et al.* 2009; Iovieno, Alfani and Baath 2010; Shen *et al.* 2013). In our study, the two plant species spontaneously displayed a positive relationship between the bacterial phylum *Nitrospirae* and soil pH. *Nitrospirae* is one of the main nitrite-oxidizer bacterial phyla and occurs widely in terrestrial and aquatic ecosystems (Lucker *et al.* 2010). Thus, the process of ammonia and nitrite conversion is partly regulated by the abundance of nitrite-oxidizers (Lucker *et al.* 2010). Soil pH has been widely used as the best predictor for *Acidobacteria* (Jones *et al.* 2009), with negative relationship between the abundance of *Acidobacteria* and soil pH (the same pattern as we observed). *Chlamydiae*, a bacterial phylum including many pathogens, was also negatively correlated with soil pH. In addition, soil Ca and N in the rhizosphere of *M. effusum* and Al in *S. sylvatica* were also significantly correlated with certain bacteria, for instance *Actinobacteria*. However, the correlation was positive in *M. effusum*, while negative in *S. sylvatica*, suggesting the dissimilarity of both chemical conditions and actinobacterial communities under different plant species. This again supports the importance of taking understorey plant species identity into consideration when exploring the effects of abiotic factors on soil microbial dynamics. Within the selected study sites, soil Al concentration was negatively correlated with pH, while soil Ca concentration showed a positive correlation with pH. All these soil chemical properties are good indicators of soil acidity (Fig. S4, Supporting Information). However, despite the large differences of the mean values of each chemical characteristic, there were no statistical significances (except the ratio of soil C to N) among regions, probably due to a large variation of soil chemistry within regions. Additionally, there is no clear boundary between rhizosphere and bulk soil (McNear Jr 2013). The definition of rhizosphere soil applied in this study (3-cm diameter around plant stems) cannot exclude unaccidental mixture with bulk soil if a more close area to plant roots (e.g. soil particles attached on roots after shaking) is defined as the rhizosphere. Further studies on the comparison of soil microbial community composition in different soil fractions depending on their distance to the root surface will be helpful in this respect.

Large-scale environmental conditions and soil bacterial community composition

The effects of large-scale environmental conditions (temperature, precipitation and N deposition loads) on soil bacterial community composition depended on plant species, with significant effects under *S. sylvatica*. This observation partly supports the mechanism of divergent soil bacterial community assembly in rhizosphere soil due to the ability of each plant species in selecting specific soil microbes and accumulating different pathogens (Dassen *et al.* 2017; Dawson *et al.* 2017). Across all studied sites, temperature and N deposition loads were positively correlated with precipitation (Fig. S4, Supporting Information). Different precipitation regimes can have selective effects on specific bacteria. For instance, less precipitation facilitates the assembly of Gram-positive bacteria, while higher precipitation increases the abundance of Gram-negative, anaerobic and sulphate-reducing bacteria (Drenovsky *et al.* 2010). In our study, precipitation did increase bacteria richness and alpha diversity across the two studied plant species (Table 1). The underlying mechanisms between precipitation regimes on specific bacteria taxa are still unknown. Sampling regions along the latitudinal gradient differ significantly with respect to temperature and N deposition (Table S3, Supporting Information). The two drivers can affect bacterial taxa which are involved in the processes of nitrification (sequential oxidation of ammonia to nitrate) and subsequently nutrient transportation (Lucker *et al.* 2010; Osborne, Baron and Wallenstein 2016). Unexpectedly, when combined with chemical soil variables, the three drivers in large-scale environmental conditions were cancelled out because of less contribution relative to soil acidic drivers in explaining the variation of soil bacte-

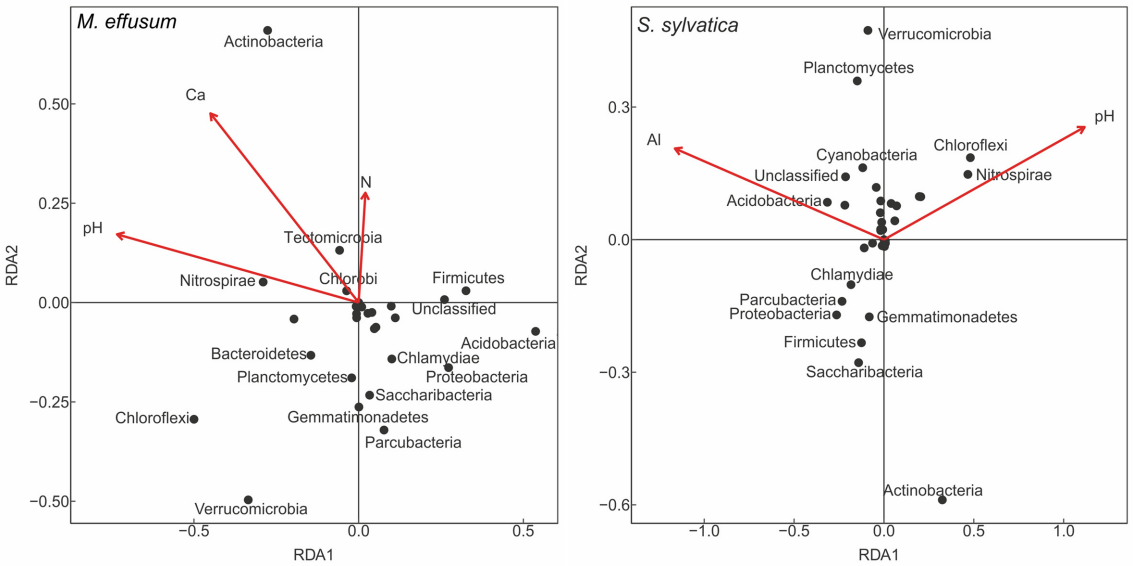

Figure 5 Redundancy analysis (RDA) of soil bacterial taxa in the rhizosphere soils of *Milium effusum* and *Stachys sylvatica* with significant explanatory variables produced by forward selection. The predictor variables in these analyses included fourteen variables, i.e. land-use history, soil C, total N, the ratio of soil C to N, total P, Olsen P, K, Ca, Mg, Al, pH, MAT (mean annual temperature), MAP (mean annual precipitation), and N deposition. Each dot represents a bacterial taxon. To avoid text overlapping, dots located around the centre were not aligned with taxon names.

rial community composition. Yet, we cannot exclude that there might be indirect effects of temperature, precipitation and N deposition among studied regions on rhizosphere soil bacterial community composition through changes in aboveground plant community composition and local edaphic properties (reviewed in Classen *et al.* 2015).

Land-use history and soil bacterial community composition

As for land-use history, we observed a weak influence on soil bacterial community composition, which was unexpected. Previous studies have shown that the legacy effect of land-use change on biodiversity (e.g. plants, soil microbes) is profound and enduring (Goodale and Aber 2001; Aggemyr and Cousins 2012). However, forest age matters in the process of plants and soil microbes' recovery (Krause *et al.* 2016). In the study of Jangid *et al.* (2011), post-agricultural lands which were established in 1951 showed high similarity in terms of soil microbial community composition with that in ancient forest. Moreover, the attribute of short life turnover of soil bacteria can also contribute to fast recovery after land-use change or disturbance (Baath 1998). In our study, recent forests were mostly afforested in the early 20th century (except the sites in Poland, which are younger and their age ranges from 15 to 40 years) and thus stand more than one century. Long-term succession in recent forests may be one of the main reasons of lack of differences in soil bacteria between ancient and recent forests. Additionally, soil chemistry of ancient and recent forests partly supported this insight, as we observed no significant differences between the two types of forests (Table S5, Supporting Information).

CONCLUSIONS

We assessed the rhizospheric soil bacterial community composition in temperate forests across Europe, and found the community was dominated by *Acidobacteria*, *Actinobacteria* and *Proteobacteria*. Soil bacterial community composition in the rhizosphere soil was strongly affected by plant species identity and soil acidity (as overarching characteristic of the soil chemistry). For different plant species, variation in soil chemical conditions helps to predict specific bacterial abundance. A long time after land-use change (more than one century), the rhizospheric soil bacterial community showed no difference between ancient and recent forests. Our results further our understanding of interaction mechanisms between the microbial community and soil chemistry, and elucidate the ecological importance of understorey plants in determining microbial diversity. The observed correlations between nitrifying bacteria and chemical soil characteristics can be a focus for further bacterial functioning research in forests.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

Data accessibility

The sequencing data are available from NCBI BioProject under accession ID: PRJNA494394 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA494394).

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Author contributions

S.M., N.B., K.V. and P.D.F. planned and designed the research. S.M., P.D.F., J.B., S.A.O.C., G.D., A.K., I.L., J.L., T.N., A.O., J.P. and M.W.

conducted fieldwork; S.M., P.D.F. analysed the data; all authors contributed to the writing of the manuscript.

Conflicts of interest. None declared.

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