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Research Article

RESEARCH ARTICLE

Variation in ectomycorrhizal fungal communities associated with Silver linden (*Tilia tomentosa*) within and across urban areas

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One sentence summary: Ectomycorrhizal communities in urban areas are significantly related to soil characteristics, while heavy metal pollution and biogeography had little or no effect.

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ABSTRACT

Trees in urban areas face harsh environmental conditions. Ectomycorrhizal fungi (EcM) form a symbiosis with many tree species and provide a range of benefits to their host through their extraradical hyphal network. Although our understanding of the environmental drivers and large scale geographical variation of EcM communities in natural ecosystems is growing, our knowledge of EcM communities within and across urban areas is still limited. Here, we characterized EcM communities using Illumina miseq sequencing on 175 root samples of the urban tree *Tilia tomentosa* from three European cities, namely Leuven (Belgium), Strasbourg (France) and Porto (Portugal). We found strong differences in EcM richness and community composition between cities. Soil acidity, organic matter and moisture content were significantly associated with EcM community composition. In agreement, the explained variability in EcM communities was mostly attributed to general soil characteristics, whereas very little variation was explained by city and heavy metal

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pollution. Overall, our results suggest that EcM communities in urban areas are significantly associated with soil characteristics, while heavy metal pollution and biogeography had little or no impact. These findings deliver new insights into EcM distribution patterns in urban areas and contribute to specific inoculation strategies to improve urban tree vitality.

Keywords: next generation sequencing; heavy metal pollution; urbanization; EcM; ectomycorrhiza; environmental drivers

INTRODUCTION

Urban areas are the most heavily human-modified ecosystems on earth and are expanding more rapidly than any other type of human land use (Seto, Guneralp and Hutyra 2012). Urban areas, however, face severe environmental problems, including increased temperatures (the urban heat island phenomenon), noise, flooding and air pollution (Fenger 1999; Kalnay and Cai 2003; Kumar *et al.* 2014). Urban trees play a crucial role in mitigating these problems (Escobedo, Kroeger and Wagner 2011) as they can improve air quality, mitigate temperature extremes, reduce storm water run-off and sequester carbon (Mullaney, Lucke and Trueman 2015). Urban trees can also benefit mental health, decreasing mental distress and increasing human well-being (White *et al.* 2013; Kardan *et al.* 2015). Trees in urban areas, however, face harsh environmental conditions. The greatest challenges for urban trees are the restricted volume of root-penetrable soil available to support healthy growth and water stress (Lindsey and Bassuk 1992; Loh, Grabosky and Bassuk 2003). High concentrations of heavy metals in the soil can also hamper root growth, as urban soils can accumulate large amounts of heavy metals originating from incomplete fossil-fuel combustion from diesel powered vehicles or industrial processes (Manta *et al.* 2002; Wei and Yang 2010). Moreover, elevated temperatures in urban areas can increase survival of pathogens and thus the susceptibility of urban trees to diseases (Tubby and Webber 2010). All this commonly results in poor vitality of urban trees (Tubby and Webber 2010; Roman and Scatena 2011) and jeopardizes the provisioning of ecosystem services delivered by urban trees.

For a tree to overcome the environmental challenges of urban areas, it requires a healthy root system. In temperate forests, the roots of many tree species form a symbiosis with Ectomycorrhizal fungi (EcM). In return for plant photosynthates, EcM provide a range of benefits to their host through their extraradical hyphal network. The hyphal network extends the root system, increasing the functional water access area (Lehto and Zwiazek 2011) and plant uptake of mineral nutrients (Chalot and Brun 1998). EcM can also protect their host from heavy metal pollution through both extracellular mechanisms, such as chelation and cell-wall binding, and intracellular mechanisms, such as binding to organic acids and transport into intracellular compartments (Adriaensen *et al.* 2005; Colpaert *et al.* 2011). Moreover, the thick fungal mantle surrounding mycorrhizal roots can physically prevent pathogens from penetrating the roots. Host plants infected by EcM can also develop an enhanced defensive capacity (mycorrhiza-induced resistance) that can protect against a wide range of biotrophic and necrotrophic pathogens, nematodes and herbivorous arthropods (Branzanti, Rocca and Pisi 1999; Itoo and Reshi 2013). Because of these benefits, EcM can improve tree establishment and health, and thus the extent and resilience of ecosystem services delivered by urban trees.

Although our understanding of the environmental drivers and large scale geographical variation of EcM communities in natural ecosystems is growing (Suz *et al.* 2014; Long *et al.* 2016; van der Linde *et al.* 2018), our knowledge of the variation in EcM communities within and across urban areas is still very limited. Some studies based on fungus morphological trait identification and restriction fragment length polymorphism (RFLP) showed that EcM fungal species richness is lower in urban areas compared to rural areas (Bainard, Klironomos and Gordon 2011; Karpati *et al.* 2011). The few studies that used next generation sequencing (NGS) technology have confirmed impoverished EcM communities in urban trees (Jumpponen and Jones 2009; Jumpponen *et al.* 2010; Martinova *et al.* 2016). How EcM communities vary across geographically separated urban areas and which local environmental variables drive EcM community composition and diversity in urban areas remains poorly understood (Newbound, Mccarthy and Lebel 2010). Such knowledge is crucial to devise site specific inoculation strategies for improving urban tree vitality (Bainard, Klironomos and Gordon 2011), as EcM inoculation studies so far have been performed either ex situ, and/or using commercially available inoculants, likely composed of mycorrhizal species that were not adapted to the urban environment, resulting in a poor effectiveness (Wiseman and Wells 2009; Wiseman, Colvin and Wells 2009; Fini *et al.* 2011).

One important driver of EcM community composition in urban areas may be the presence of heavy metals. Whereas much research has focused on the ability of EcM to protect host plants from toxic levels of heavy metals (e.g. Khullar and Reddy 2018), little is known about the impact of heavy metal pollution on EcM diversity and community structure. Using 454 pyrosequencing, Op De Beeck *et al.* (2015) studied the impact of zinc pollution on EcM communities in the soil of a pine plantation in Belgium, polluted by a zinc smelter. They concluded that heavy metal pollution shaped pioneer fungal communities and that the harsh environmental conditions selected metal-tolerant taxa, although still maintaining a relatively high fungal diversity. To our knowledge no studies are available on the impact of heavy metal pollution on the EcM communities in urban areas.

The main objective of this study was to understand how EcM richness and community composition vary across geographically distant urban areas and which environmental drivers are responsible for the variation in EcM communities in urban areas. We characterized EcM communities using Illumina miseq sequencing on 175 root samples of the common urban tree *Tilia tomentosa* from three European cities, namely Leuven (Belgium), Strasbourg (France) and Porto (Portugal), and we specifically aimed to: (i) fully characterize EcM diversity and community composition of *T. tomentosa*; (ii) identify the environmental drivers of EcM richness and community composition and (iii) disentangle variability in EcM communities explained by city, general soil characteristics and heavy metal pollution.

MATERIALS AND METHODS

Study sites and sampling

This study was conducted in three European cities: Leuven (Belgium), Porto (Portugal) and Strasbourg (France) (Figure S1, Supporting Information). Average annual precipitation and temperature are 792 mm and 11.0°C, 635 mm and 15°C and 352 mm and

11°C, respectively. We selected *T. tomentosa* as the study species because of its dependency on EcM and its importance as an urban tree throughout Europe. Across these cities, we sampled 175 *T. tomentosa* trees (Leuven N = 52; Strasbourg N = 56; Porto N = 67), growing under a variety of planting conditions, from trees planted in a small soil pits surrounded by a sealed surface, over trees planted in soil strips surrounded by sealed surface, to trees planted within larger green areas. Trees were selected from a city map indicating all the trees planted in the city. First, sites in the city were selected to obtain a representative sampling across the whole city, accounting for the variety of planting conditions. Within one site, trees were randomly selected for sampling.

We sampled in September 2017. We primarily sampled fine roots, as these are known to contain EcM. In parallel with root sampling, we collected a pooled soil sample near each sampled tree for chemical analysis. We stored root samples at −20°C until further analysis and soil samples at 4°C for maximum 1 week to prevent nitrogen loss. Preservation at 4°C slows down microbial mediated denitrification to the extent that virtually no nitrogen is lost in the sample in the time span of 1 week. In total, we obtained 175 root and 175 soil samples across the three cities.

Soil chemical analyses

For each soil sample, soil pH was quantified using a glass electrode in a 1:10 soil/water mixture. As a measure of plant-available N content of the soil, ammonium and nitrate were quantified by shaking 10 g of soil in 200 mL of 1 M potassium chloride solution for 1 hour and subsequent colorimetric analysis of the extracts using the salicylate method for ammonium and the N-(1-Naphthyl)ethylenediamine method for nitrate (Robertson *et al.* 1999). As a measure of plant-available P content of the soil, Olsen *P* values were quantified by shaking 2 g dry soil for 30 min with 0.5 M sodium bicarbonate at pH 8.5 and colorimetric analysis of the extracts using the molybdenum blue method (Robertson *et al.* 1999). Extracts were analyzed colorimetrically using the Evolution 201 UV-visible Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Moisture content was quantified by the weight loss of 10 g of fresh soil after evaporation of water content at 105°C. Organic matter was quantified by weight loss of 5 g of dry soil after combustion of organic matter at 700°C. Heavy metal concentrations of Cu, Pb, Zn, Cd and Cr in the soil were measured by digesting 50 mg of dried and sieved soil with 7.5 mL concentrated hydrochloric acid and 2.5 mL concentrated nitric acid. The digested solution was diluted to 10 mL and measured with ICP-OES. We chose to measure total heavy metal content in the soil, rather than extractable heavy metal content, as all extraction methods have their advantages and disadvantages, which also depend on the soil type. Total heavy metal content is often highly correlated with extractable heavy metal content and was assumed to be a good estimate, comparable across all our sampling sites spread over three cities.

DNA extraction, PCR amplification and high throughput sequencing

First, the roots with a diameter of 3 mm or less were cut in 1–2 cm pieces and rinsed twice with sterile distilled water. Next, DNA was extracted from 100 mg using the Soil DNA Isolation Kit (Norgen, Ontario, Canada). All 175 root DNA extracts were amplified by PCR in twofold (i.e. two technical replicas) targeting the ITS2 region of the rRNA gene using the sample-specific barcode-labeled versions of the primers ITS86F and ITS4 (Waud *et al.* 2014; dual-index sequencing strategy, Kozich *et al.* 2013). PCR was performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 25 μL, containing 0.25 mM of each dNTP, 0.5 μM of each primer, 1 × HiFi Buffer, 1U HiFi DNA Polymerase (HighQu, Kraichtal, Germany) and 1 μL genomic DNA. DNA samples were denatured at 95°C for 1 min. Next, 35 cycles were ran, consisting of 20 s at 95°C, 30 s at 52°C and 30 s at 72°C. Amplicons were purified using the Agencourt AMPure XP kit (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Next, we quantified purified dsDNA amplicons using the Quant-iT PicoGreen dsDNA Assay Kit and Qubit fluorometer (Invitrogen, Ghent, Belgium), and pooled them in equimolar quantities. The amplicon library was loaded on an agarose gel and the amplicon of the expected size was excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The final amplicon library was diluted to 2 nM and sequenced at the Centre of Medical Genetics Antwerp (University of Antwerp, Antwerp, Belgium) using an Illumina MiSeq sequencer with v2 500 cycle reagent kit (Illumina, San Diego, CA, USA).

Bioinformatics

Sequences from the Illumina run, obtained as de-multiplexed FASTQ files, were clustered into operational taxonomic units (OTUs) using USEARCH (v. 10) following the recommended pipeline (Edgar 2013). First, paired-end reads were merged to form consensus sequences using the *fastq_mergepairs* command. Next, quality filtering of the reads was performed with the *fastq_filter* command, allowing a maximum expected error of 0.5 for the individual sequences. Then, sequences were dereplicated and sorted by abundance. Sequences occurring only once in the entire dataset were removed prior to clustering as this has been shown to improve the accuracy of diversity estimates (Brown *et al.* 2015). Sequences were clustered into OTUs defined at 97% sequence similarity using the UPARSE algorithm implemented in USEARCH, during which chimeric sequences were also removed (Edgar 2013). OTUs were assigned to a taxonomic identity by querying the representative sequence against the annotated UNITE ITS database (reference dataset v. 7.2, 58 049 sequences, release date 2017–12.01, Koljalg *et al.* 2013) using the SINTAX algorithm implemented in USEARCH (Edgar 2016). Taxonomic assignments were considered reliable when genus bootstrap confidence values were equal or exceeded 0.90. Subsequently, OTUs were taxonomically parsed into ecological guilds using FUNGuild (Nguyen *et al.* 2016). Only OTUs identified as ectomycorrhiza were retained in the dataset. Representative sequences for each EcM OTU were deposited in GenBank (accession numbers MH801215 - MH801410).

Data analysis and statistics

To prevent bias due to different sequencing depth (ranging from 11 to 25 950 EcM reads per sample; median of 3047 EcM reads per sample), we randomly resampled reads from each sample to an equal number of reads. To maximize the number of reads and samples retaining in the dataset, we resampled all samples to 866 reads per sample. In this way, 25 samples with fewer than 866 reads per sample were omitted and 150 samples (i.e. 86%) were retained in the dataset.

First, we tested whether soil variables significantly differed between cities using analysis of variance (ANOVA) and *Tukey HSD* post-hoc tests performed in JMP (version 13.1.0, SAS Institute, NC, USA). Next, we modeled EcM richness (i.e. the number of EcM OTUs per sample) against city, general soil characteristics (moisture content, organic matter, pH, Olsen P and N

total) and soil heavy metal concentrations (Cu, Pb, Zn, Cd and Cr) using general linear models (GLM) performed in JMP (version 13.1.0, SAS Institute, NC, USA). We used the Bayesian Information Criterion (BIC) to select the most explanatory model (i.e. with the lowest BIC) out of a range of reduced models compared with the full model. In accordance with Murtaugh (2014), we reported *F* and *P* values of explanatory variables selected in the final model. We calculated Variance Inflation Factors (VIF) to detect multicollinearity between the explanatory variables. VIFs of the explanatory variables were low (<3.1), indicating they can be used together in the models. To account for differences in scale and variation of the environmental predictor variables, they were standardized using the scale function in R, making the mean of every continuous variable 0 and the standard deviation 1.

Next, EcM community composition was related to city, general soil characteristics and soil heavy metal concentrations using canonical redundancy analysis (RDA) using the R-package *vegan*. To determine the explanatory variables that significantly explained variation in the EcM communities, we used forward selection using the *ordistep* function in *vegan* (1000 Monte Carlo permutations, $\alpha < 0.05$). To test the significance of the selected variables in the model, we performed permutation tests on the individual terms (1000 permutations) using the *anova.cca* function (R-package *vegan*). To identify EcM taxa that were significantly indicative for a city, we performed indicator species analysis using the function *multipatt* of the *indicspecies* package in R (De Caceres and Legendre 2009). RDA relating EcM communities to environmental variables were also performed for each city separately to evaluate within city variation of EcM. All RDA analysis was performed on the standardized predictor variables.

Finally, to disentangle the contribution of city, general soil characteristics and heavy metal concentrations to the variation of the EcM communities, we performed variance partitioning using the *varpart* function (Legendre 2008) of the R-package *vegan*. More specifically, we used three explanatory matrices, i.e. (i) city, (ii) general soil variables (moisture content, organic matter, pH, Olsen P and N total) and (iii) heavy metal variables (Cu, Pb, Zn, Cd and Cr). Only the significant explanatory variables, as determined by forward selection (*ordistep* function of the R-package *vegan*), in each of the three explanatory matrices were included in the variance partitioning. The *venneuler* package in R was used to create Venn diagrams to present the variation partitioning results. Furthermore, to test specifically for spatial patterns in EcM communities, we calculated a set of spatial predictors from the geographical coordinates of sampled trees by principle coordinates of neighbor matrices (PCNM), using the *pcnm* function of the vegan package in R (Borcard and Legendre 2002; Borcard *et al.* 2004). We determined the significant spatial predictors by forward selection (ordistep function of the R-package vegan) and tested whether these significant spatial predictors explained additional variation in EcM communities, next to the variable city already included in former analyses, using a partial RDA accounting for the effect of city.

RESULTS

Differences in soil chemical variables and heavy metal concentrations between cities

All soil chemical variables, except moisture content, differed significantly between the three cities (Table 1). Although Porto showed lower moisture content values in the soil compared to Leuven and Strasbourg, no significant differences were found, due to three outliers with high moisture content (19.7, 20.5 and 23.1%; mean without outliers = 6.48±0.43%). Soil acidity was significantly higher in Strasbourg compared to Leuven and Porto. Strasbourg also had the highest Cr concentrations in the soil. Organic matter and Olsen P concentrations were highest in Leuven, while Cu, Pb, Zn and Cd concentrations in the soil were highest in Porto (Table 1).

Ectomycorrhizal richness

After removal of non-ectomycorrhizal sequences, Illumina sequencing yielded a total of 736 247 EcM sequences. To prevent bias due to different sequencing depth, all samples were resampled to 866 EcM sequences per sample, leaving 129 900 sequences and 150 samples for further analysis (Table S1, Supporting Information). After resampling, a total of 197 EcM OTUs across 27 families were detected (Table S2, Supporting Information). The majority of sequences belonged to the Tuberaceae (23.6%, 13 OTUs), Russulaceae (18.7%, 17 OTUs), Sclerodermataceae (10.0%, 11 OTUs), Inocybaceae (9.5%, 38 OTUs), Thelephoraceae (9.5%, 16 OTUs) and Gloniaceae (7.3%, 16 OTUs), whereas the abundance of the other families was less than 3.2% (Table S2, Supporting Information). In Porto, 23 families were observed, while 21 and 20 families occurred in Strasbourg and in Leuven, respectively (Fig. 1). Tuberaceae was the most abundant family in Leuven (34.5%) and Strasbourg (39.3%). In contrast, Russulaceae was the most abundant family in Porto (43.6%), while Tuberaceae was less abundant (1.8%). Three unique families were found in Porto, i.e. Gyroporaceae, Cantharellaceae and Amanitaceae, while Paxillaceae and Gomphaceae were unique for Leuven and Rhizophlyctidaceae and Lyophyllaceae were unique for Strasbourg (Fig. 1).

The model selection procedure relating EcM richness to the environmental variables, selected city ($F = 16.70$, $P < 0.001$) and soil Cu concentration ($F = 8.97$, $P = 0.003$) as the explanatory variables significantly correlated with EcM richness (Fig. 2) (Figure S2, Supporting Information). The final model had a BIC of 937.4, while the full model had a BIC of 971.6. Post-hoc pairwise comparison of EcM richness between cities revealed that Porto had a significant higher EcM richness compared to Leuven and Strasbourg (both comparisons $P < 0.001$) (Fig. 2), whereas Leuven and Strasbourg did not significantly differ ($P = 0.566$). In the final model, the parameter estimate of the relation between EcM richness and Cu concentration is 0.082, indicating a positive relationship between both variables. The final model had an R^2 adjusted of 26.1%.

EcM community composition

RDA with forward selection selected city, pH, organic matter and moisture content as the explanatory variables significantly explaining variation in EcM communities (Fig. 3, Table 2). The final model explained 10.7% of the variation. RDA revealed strong differences in EcM communities between cities ($F = 8.14$) and a strong relation between the EcM communities and soil acidity ($F = 2.71$) (Table 2). The arrow of soil acidity on the RDA ordination plot points away from the Porto samples, indicating that the Leuven and Strasbourg EcM communities coincide with higher pH values, whereas the EcM communities in Porto coincide with lower pH values. The ordination results per city are highly congruent with the RDA performed on the whole dataset (Table S3 and Figure S2, Supporting Information). RDA showed that EcM communities in Leuven were still related to pH, organic matter and moisture content. In agreement with RDA on

Table 1. Meanand standard deviation (S.D.) of the soil chemical properties across the three cities (N = 150). Different letters indicate significant differences at $P < 0.05$.

	Leuven Mean ± S.D.	Strasbourg Mean ± S.D.	Porto Mean ± S.D.
Moisture content (%)	8.73 ± 4.90	7.63 ± 2.72	7.19 ± 3.77
Organic matter (%)	6.13 ± 2.25^{a}	4.50 ± 1.10^{b}	5.69 ± 1.41^{a}
pH	7.65 ± 0.50^{a}	8.24 ± 0.25^{b}	6.58 ± 0.80^{c}
Olsen P (mg kg^{-1})	48.3 ± 13.0^{a}	29.3 ± 23.1^{b}	26.9 ± 23.6^{b}
N total (mg kg^{-1})	11.3 ± 8.1^{a}	21.2 ± 26.2^{ab}	33.1 ± 15.3^{b}
Cu (mg kg^{-1})	13.6 ± 11.1^{a}	26.2 ± 11.5^{b}	28.2 ± 16.7^{b}
Pb (mg kg^{-1})	64.6 ± 72.6^{a}	91.6 ± 52.2^{ab}	126.3 ± 61.1^{b}
Zn (mg kg^{-1})	59.5 ± 30.9^{a}	85.1 ± 45.1^{b}	118.3 ± 43.2^{c}
Cd (mg kg^{-1})	0.17 ± 0.59^{a}	0.04 ± 0.12^{a}	1.16 ± 1.17^{b}
Cr (mg kg^{-1})	17.6 ± 12.1^{a}	42.2 ± 29.1^{b}	20.6 ± 7.3^{a}

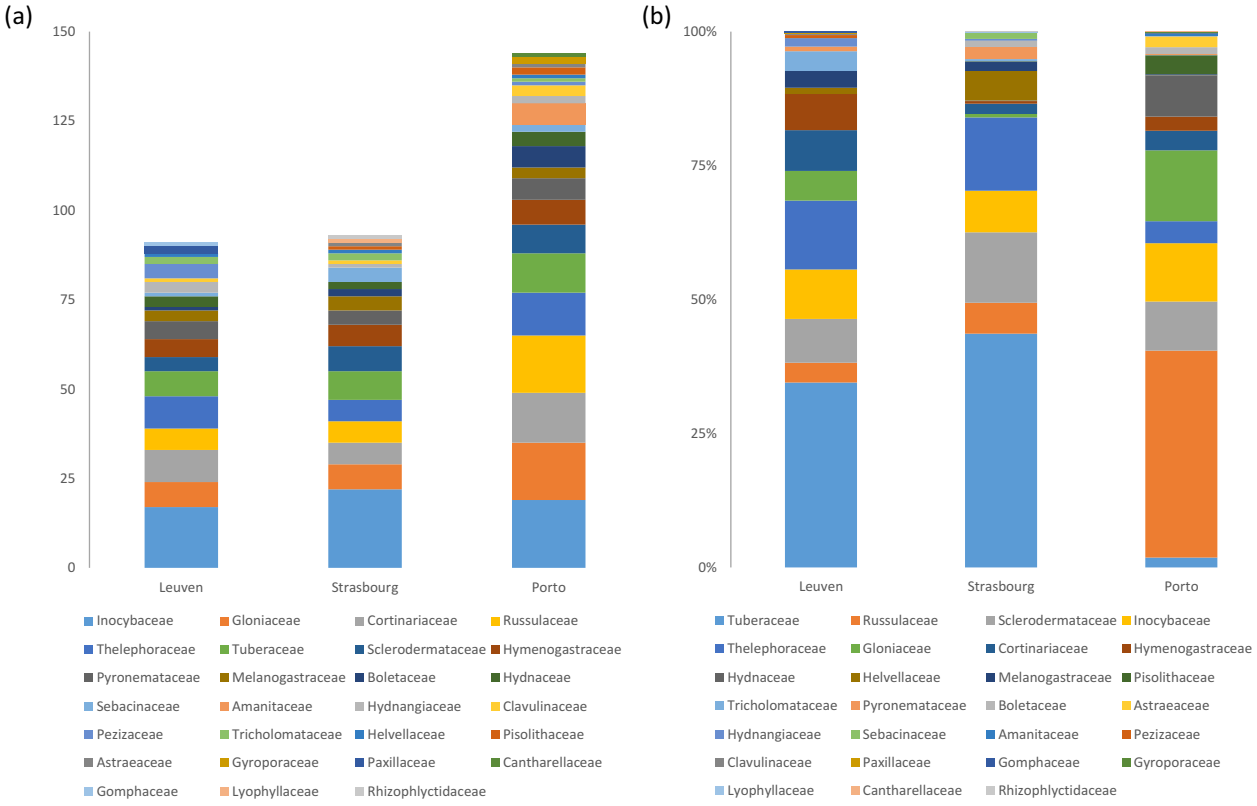

Figure 1. Identified EcM families across Leuven, Strasbourg and Porto per number of EcM OTUs (**a**) and proportion of EcM reads (**b**).

Table 2. Results of the permutation tests of the canonical redundancy analysis (RDA) analysis testing for significant relationships between EcM communities and environmental variables (as selected by forward selection). Results are based on 1000 permutations. The model explained 10.7% of the variation in EcM communities.

	Df	Variance	F	P
City	2	0.084	8.14	<.001
pH	1	0.014	2.72	<.001
Organic matter	1	0.011	2.20	0.002
Moisture content	1	0.009	1.81	0.008

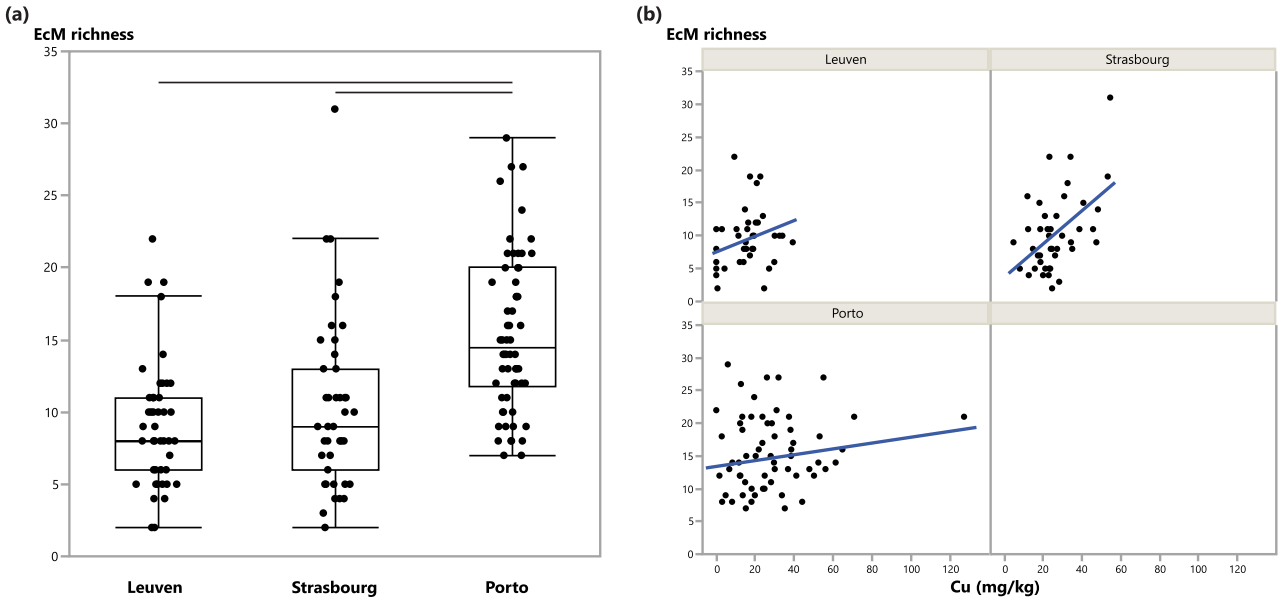

Figure 2. The difference in EcM richness between Leuven, Strasbourg and Porto (**a**), and the relationship between EcM richness and the copper concentration in the soil in the three cities (**b**). Horizontal lines represent significant differences at $P < 0.05$ as determined by Tukey HSD post-hoc tests. Blue lines represent linear trend lines.

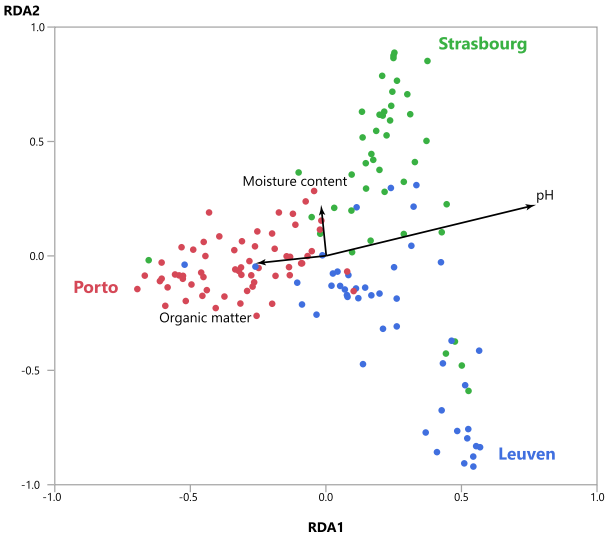

Figure 3. Redundancy analysis (RDA) ordination plot of EcM fungal communities in the roots of *T. tomentosa* (N = 150) across Leuven (blue), Strasbourg (green) and Porto (red). Arrows indicate environmental variables explaining a significant proportion of the EcM communities (as determined with forward selection, Table 2). The arrows represent the direction of the increasing gradient and are proportional to the explained variation in the EcM communities. RDA1 and RDA2 axis accounted for 50.9% and 25.6% of the total explained variation in EcM communities, respectively.

the whole dataset (Fig. 3), EcM communities in Porto were only correlated to soil pH. EcM communities in Strasbourg, however, did not correlate significantly with any environmental variables. This is also reflected in Fig. 3, as the within-city variation of EcM communities in Strasbourg was the smallest of the three cities.

Indicator species

In agreement with the strong difference in EcM communities between cities revealed by the RDA, indicator species analysis detected six OTUs significantly indicative for Leuven, four OTUs for Strasbourg and 14 for Porto (Table 3). In Leuven, the strongest indicator OTU belonged to the genus *Peziza*, while in Strasbourg the strongest indicator OTU belonged to the genus *Basamia*. The other indicator OTUs in Strasbourg were assigned to the genus *Inocybe*. In Porto, 5 out of 14 indicator OTUs were *Cenococcum* taxa, and more specifically *Cenococcum geophilum* as the most indicative.

Variation partitioning

The variable city explained significant variation in the EcM communities ($F = 7.93$, $P < 0.001$). Among the chemical soil variables, the forward selection procedure selected pH ($F = 7.72$, $P < 0.001$), organic matter ($F = 2.77$, $P < 0.001$) and moisture content ($F = 1.62$, $P = 0.029$) as significantly related to the EcM communities. During stepwise selection of the heavy metal variables, Zn ($F = 3.87$, $P < 0.001$), Cr ($F = 3.61$, $P < 0.001$) and Cd ($F = 2.01$, $P = 0.002$) were selected as the most important predictors of EcM communities. All three separate explanatory matrices were significantly related to the EcM communities: city ($F = 7.93$, $P < 0.001$); chemical soil variables ($F = 4.03$, $P < 0.001$) and heavy metal variables ($F = 3.16$, $P < 0.001$). Comparison of the three different explanatory matrices using variance partitioning revealed that the chemical soil variables explained the most unique variation (R^2 adjusted = 0.040), while the heavy metal variables explained very little unique variation (R^2 adjusted = 0.004) in EcM communities (Fig. 4). The variable city explained 2.85 times less unique variation (R^2 adjusted = 0.014) compared to the chemical soil variables. A large part of the variation explained by the soil chemical variables was shared with the heavy metal variables (R^2 adjusted = 0.024) and the variable city (R^2 adjusted = 0.011) (Fig. 4). A large part of the variation explained by the heavy metal concentrations was also shared with city (R^2 adjusted = 0.017). Finally, six spatial predictors (PCNM variables) were retrieved from the geographical coordinates of the sampled trees, and the forward selection procedure selected only PCNM1 as significantly related to the EcM communities ($P < 0.001$). Partial RDA accounting for

Table 3. OTUs indicative for the Leuven, Strasbourg and Porto. Significance levels are obtained by Monte Carlo Permutation tests.

City	OTU_ID	Read abundance rarefied dataset	Family	Genus	Indicator Value	*P*
Leuven	OTU218	134	Pezizaceae	Peziza	0.43	0.001
	OTU54	1079	Tricholomataceae	Tricholoma	0.337	0.003
	OTU221	356	Inocybaceae	Inocybe	0.333	0.005
	OTU427	76	Pezizaceae	Peziza	0.333	0.005
	OTU815	23	Inocybaceae	Inocybe	0.298	0.014
	OTU295	433	Thelephoraceae	Tomentella	0.258	0.044
Strasbourg	OTU94	2507	Helvellaceae	Balsamia	0.463	0.017
	OTU101	1468	Inocybaceae	Inocybe	0.378	0.001
	OTU1127	181	Inocybaceae	Inocybe	0.312	0.011
	OTU855	31	Inocybaceae	Inocybe	0.264	0.023
Porto	OTU19	4774	Russulaceae	Russula	0.643	0.001
	OTU81	5251	Gloniaceae	Cenococcum	0.548	0.007
	OTU18	2931	Inocybaceae	Inocybe	0.5	0.002
	OTU58	1904	Pisolithaceae	Pisolithus	0.421	0.001
	OTU961	82	Russulaceae	Russula	0.417	0.002
	OTU255	139	Gloniaceae	Cenococcum	0.415	0.002
	OTU64	1307	Cortinariaceae	Cortinarius	0.402	0.016
	OTU1003	88	Gloniaceae	Cenococcum	0.402	0.01
	OTU9	4146	Hydnaceae	Hydnum	0.382	0.015
	OTU97	594	Gloniaceae	Cenococcum	0.354	0.004
	OTU176	194	Thelephoraceae	Tomentella	0.311	0.018
	OTU256	82	Gloniaceae	Cenococcum	0.311	0.019
	OTU1237	133	Inocybaceae	Inocybe	0.306	0.02
	OTU492	41	Amanitaceae	Amanita	0.284	0.013

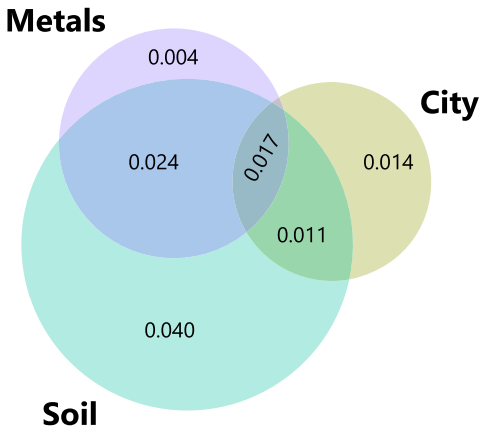

Figure 4. Venn diagrams representing variance partitioning results of EcM communities among three explanatory matrices, i.e. heavy metal variables, soil chemical variables and the variable city. The size of the circles is proportional to the variability in EcM communities as explained by a particular explanatory matrix, while overlap of the circles represents the shared variation among explanatory matrices. Numbers indicate the adjusted R^2 values and thus the variability explained by each partition.

the effect of city found that PCNM1 did not explain any additional variation in EcM communities (R^2 adjusted = 0.006, F = 1.22, $P = 0.170$).

DISCUSSION

To our knowledge, this is the first large-scale study using next generation sequencing technology to investigate the variation in EcM richness and community composition within and across geographically distant urban areas. We found clear differences in EcM richness and community composition between cities. Soil acidity, organic matter and moisture content were significantly related to EcM community composition. In agreement, the explained variability in EcM communities was mostly attributed to general soil characteristics, whereas very little variation was explained by city and heavy metal pollution.

Differences in ectomycorrhizal communities between and within cities

Our results showed that Porto had higher EcM richness compared to Leuven and Strasbourg. A higher EcM richness in Porto may be explained by the lower pH levels (mean = 6.58) compared to Leuven and Strasbourg (mean = 7.65 and 8.24, respectively). The observed increase in EcM richness in acidic soils is in agreement with early research that showed that the EcM symbiosis is favored under these conditions (Read 1991). Aggangan, Dell and Malajczuk (1996), for example, showed that the effectiveness of different EcM taxa in promoting plant growth reduced at more alkaline pH levels, suggesting that alkaline soils negatively affect the growth of the nutrient-absorbing external hyphae. Soudzilovskaia *et al.* (2015) studied global patterns of plant root colonization by EcM and found a strong negative correlation between soil pH and EcM colonization as well. Moreover, most enzymes produced by EcM have a pH optima between 4 and 6 (Pritsch *et al.* 2004; Courty *et al.* 2005), which may explain higher EcM richness in this pH range.

Next to differences in EcM richness, our results also revealed strong differences in EcM community composition between cities. Indeed, we detected large differences in abundance of Tuberaceae and Russulaceae between Leuven and Strasbourg on the one hand, and Porto on the other hand. These differences in

abundance may also be explained by the differences in soil acidity between the cities. Russulaceae generally prefer more acidic conditions, such as the soils in Porto, while Tuberaceae prefer more alkaline conditions, such as the soils in Leuven and Strasbourg (Agerer, Taylor and Treu 1998; Rineau *et al.* 2010). In agreement with the strong differences in EcM communities between cities, we also detected several indicative EcM OTUs per city. Porto experiences high levels of water stress in the summer and, therefore, it was no surprise that *Cenococcum geophilum* was a dominant indicator OTU in this city. *Cenococcum geophilum* tolerates a wide range of stressors and is widely known for its ability to tolerate water stress (Pigott 1982; Jany, Martin and Garbaye 2003). Additionally, climate, metacommunity composition and differences in how seedling stock was sourced, or other factors may also explain the differences in EcM communities between cities.

We also found several EcM OTUs that were common to all three cities. On the one hand, we found that the genera *Inocybe* and *Tomentella* occurred in 79% and 69% of all samples, respectively. These EcM taxa are often considered as *early-stage* fungi as they fruit routinely with seedlings of young trees (Visser 1995; Twieg, Durall and Simard 2007). They generally occur in unstable ecosystems with limited water and nutrient availability (Newton 1992), and therefore it could be expected to detect these EcM taxa in urban areas. On the other hand, we found that the genera *Tuber* and *Russula* occurred in 89% and 67% of the samples, respectively. Although these EcM taxa are often considered as *late-stage* fungi (Visser 1995; Twieg, Durall and Simard 2007), requiring a more stable ecosystem with abundance in water and nutrient supply, they were still abundantly present in urban areas.

We not only found differences between cities, but also observed variation in EcM communities within cities. Our ordination analyses showed that variation in EcM communities in Porto was significantly related to soil acidity, while EcM communities in Leuven were related to moisture content, organic matter content and also soil acidity. Although EcM communities in Strasbourg clearly differed from EcM communities from Leuven and Porto, within-city variation in Strasbourg did not correlate with any measured environmental variables. Strasbourg exhibits less variation in moisture content, organic matter and pH than the other cities so the gradient is shorter, which may explain why environmental variables were not significantly associated with community turnover. Indeed, the variation of pH in Porto, for example, was more than three times larger than the variation of pH in Strasbourg (S.D. 0.80 vs 0.25, respectively).

Impact of metal pollution on ectomycorrhizal diversity in urban areas

Increased concentrations of heavy metals in the soil are known to negatively affect biodiversity. Koptsik *et al.* (2003), for example, showed that plant diversity declined along a heavy metal pollution gradient near a nickel smelter. Many studies showed that heavy metal pollution can also severely decrease microbial diversity in the soil (e.g. Sandaa *et al.* 1999; Gans, Wolinsky and Dunbar 2005). As urban soils can accumulate large amounts of heavy metals originating from incomplete fossil-fuel combustion or industrial processes (Manta *et al.* 2002; Wei and Yang 2010), we expected that high soil heavy metal concentrations would negatively affect EcM diversity. Our results, however, show that the heavy metal concentrations in urban soils were not negatively correlated with EcM richness. These results show that the heavy metal concentrations of Cu, Pb, Zn, Cd and Cr in the soil did not limit EcM diversity. Op De Beeck *et al.* (2015) studied the impact of zinc pollution on EcM communities in the soil of a pine plantation and also did not found a negative effect of metal pollution on fungal diversity. The absence of a negative effect of heavy metals on EcM richness may be explained by the relative low concentrations we observed in urban soils. Compared to the study of Kandeler, Kampichler and Horak (1996), who experimentally contaminated soils with heavy metals to study the impact on soil microbial communities, the mean concentrations of Zn (91.1 mg kg^{-1}), Cu (23.2 mg kg^{-1}) and Cd (0.54 mg kg^{-1}) in our dataset were 4.3, 3.3 and 5.5 times lower than the concentrations these authors considered to be 'light' heavy metal pollution. The mean Pb (103 mg kg^{-1}) and Cr (103 mg kg^{-1}) concentrations were also more than nine times lower than the concentrations in the studies of Konopka *et al.* (1999) and Turpeinen, Kairesalo and Haggblom (2004), who investigated the impact of heavy metal contamination on microbial communities. Although the level of heavy metal pollution was relatively low, EcM have a range of extra- and intra-cellular adaptive mechanisms to accumulate heavy metals (Hartley, Cairney and Meharg 1997; Colpaert *et al.* 2011), possibly making EcM communities more tolerant to heavy metal pollution in comparison to many other groups of microorganism. Our results did not only show the absence of a negative effect of heavy metal pollution on EcM richness, we also observed a positive relation between EcM richness and copper concentration in the soil. This result may be explained by the dependency of many organisms on copper, i.e. copper is a micronutrient playing a unique and critical role in many organisms, promoting structures and chemistries that would otherwise not be available (Festa and Thiele 2011). Therefore, EcM may show a hormetic response to copper in the soil, with a favorable response to low copper concentrations, as observed in our dataset, but a detrimental response to higher concentrations.

Soil acidity, organic matter and moisture content were significantly associated with ectomycorrhizal communities

As discussed above, soil acidity can impact EcM colonization, EcM diversity and the enzymes produced by EcM (Courty *et al.* 2005; Soudzilovskaia *et al.* 2015). Indeed, also our analysis revealed that EcM communities were strongly related with soil acidity. These results were in agreement with Suz *et al.* (2014), who showed that soil acidity explained most of the variation in EcM communities from European temperate oak forests. Moreover, our analysis revealed that EcM communities were related to organic matter, and that the direction of change was opposite to soil pH, indicating a change in EcM communities when organic matter content in the soil increased and pH levels decreased. Increased levels of organic matter are often associated with lower pH levels as decay of organic matter can buffer soil acidity through a constant supply of protons. In agreement, Jumpponen *et al.* (2010) also found that soil organic matter was strongly correlated with EcM communities in oak forests. Finally, our results revealed a small but significant effect of moisture content in the soil on EcM communities. Kennedy and Peay (2007), for example, already showed that low soil moisture levels can decrease EcM colonization of the host and strongly affect the way plants and EcM interact.

Disentangling variability in ectomycorrhizal communities explained by city, soil characteristics and heavy metal pollution

Recent large scale studies observed biogeographical patterns in EcM communities largely ascribed to climatic variables, such as the mean annual temperature and precipitation (Bahram *et al.* 2012; Tedersoo *et al.* 2012). As the three cities sampled in this study largely differ in latitude, we also expected to observe different EcM communities between cities. Although our analysis did indeed reveal strong differences in EcM communities between cities, the variation partitioning showed that city explained 2.85 times less unique variation compared to the general soil characteristics. Specific spatial analysis also did not explain any additional variation in EcM communities. These result suggests that biogeographical patterns had a limited impact on EcM communities in our dataset and that EcM communities were only significantly related to general soil characteristics. A large part of the variation explained by the general soil variables was shared with the variable city, indicating that effects of soil and city could not completely be disentangled. This was expected as cities also strongly differ in soil characteristics. The heavy metal variables explained very little unique variation in EcM communities, indicating that heavy metal pollution had little or no impact on EcM communities. It is, however, possible that the total heavy metal content in the soil is less correlated to biological activity than extractable metal content. Heavy metals may bind to soil mineral and organic fractions and be less available to EcM. Therefore, we suggest future studies investigating the effects of heavy metals on EcM communities to also measure extractable metal concentrations in the soil. Finally, the variability explained by the whole model including all significant environmental variables was relatively small (10.7%). Other studies (e.g. Tedersoo *et al.* 2014; van der Linde *et al.* 2018) are unable to explain larger amounts of variation as well, even when large numbers of predictors variables were included. Yet, this raises the question why the majority of variation (89.3%) in EcM communities is unaccounted for. First, it is possible that more predictor variables are required, such as the (unknown) source of the Tilia seedling stock. Second, stochastic processes such as ecological drift and random dispersion may also contribute to the assembly of EcM communities (Zhou and Ning 2017), and they may explain why environmental variables could only explain a small portion of the variation in EcM communities.

CONCLUSION

Overall, our results showed that EcM communities clearly differed between cities, and that they were significantly associated with soil characteristics, while heavy metal pollution and biogeography had little or no impact. Of all the observed soil characteristics, soil acidity, organic matter and moisture content were significantly related to EcM communities. These findings deliver new insights into EcM distribution patterns in urban areas and contribute to site specific inoculation strategies to improve urban tree vitality and thus the extent and resilience of ecosystem services they deliver.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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