Received: 4 October 2024 | Revised: 11 December 2024 | Accepted: 13 December 2024

DOI: 10.1002/ppp3.10634

Plants People Planet PPP Open Access

RESEARCH ARTICLE

Arbuscular mycorrhiza in the urban jungle: *Glomeromycotina* communities of the dominant city tree across Amsterdam

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Funding information
European Research Council, Grant/Award Number: 101045057

Societal Impact Statement

Trees in cities provide a great number of benefits to people and nature, but they are challenged by harsh conditions. Trees rely on helpful fungi in their roots to get essential nutrients from the soil, but we do not know which of these fungi are resistant to city landscapes. By studying these fungi, we can learn how their communities are affected by cities and which of them survive best there. Understanding this can help us develop effective ways to make use of these microbes or to improve city conditions for the benefit of urban trees and their fungi and, therefore, us.

Summary

- Urban trees are important green features in cities. Arbuscular mycorrhizal fungi (AMF) in roots may alleviate urban environmental pressures affecting urban trees. These pressures also appear detrimental for AMF, reducing root colonization rates, spore production and spore diversity. However, a limited number of molecular studies on urban AMF contrast these findings, but the matter remains unresolved. Therefore, we investigated the hitherto understudied AMF communities in the roots of urban trees, their diversity, spatial and neighbouring-tree effects and interaction networks.
- We did this by metabarcoding fungi from tree roots across an urbanization gradient in Amsterdam (the Netherlands), focussing on Dutch elm (*Ulmus x hollandica*). Samples were collected from three urbanization classes, differentiated by the degree of soil sealing and management: an urban forest, park and street. In the urban forest and park, root samples were collected in a grid, allowing the construction of interaction networks and assessment of neighbouring-tree root effects, whereas the street trees stood in solitary pits.
- Using the ITS2 barcode, we detect distinct, diverse and heterogenous AMF communities. The (phylogenetic) diversity of AMF increased with urbanization. Contrary to our expectations, we found no evidence that AMF communities in street trees were more spatially homogenous. Moreover, neighbouring root diversity did not appear to affect AMF diversity.
- Our findings suggest a strong response of AMF communities to urbanization. An urban-induced change in mycorrhizal partners, rather than a loss of partners and

interaction complexity, demonstrates the high adaptability of the arbuscular mycorrhizal symbiosis to urban stressors, which has important management implications.

KEYWORDS
arbuscular mycorrhiza, community structure, diversity, metabarcoding, network analysis, spatial, urban ecology, urban trees

1 | INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) in the subphylum *Glomeromycotina* (Spatafora et al., 2016) are the most prevalent type of mycorrhiza, associating with over 70% of vascular plant species (Brundrett & Tedersoo, 2018). Mycorrhizal fungi provide access to otherwise inaccessible soil nutrients and improve drought, pollution and disease resistance (Jayne & Quigley, 2014; Johnson & Gehring, 2007; van der Heijden et al., 2015). In exchange, plants invest parts of their photosynthetic yield to the mycorrhizal fungi (Andrino et al., 2021; Jakobsen & Rosendahl, 1990; Kiers et al., 2011). However, the carbon-for-nutrients exchange rates may not always constitute a 'fair trade' (Durant et al., 2023), and both plants and fungi can occupy any part of the mutualist–antagonist spectrum (Hoeksema et al., 2010; Merckx, 2013; Van Der Heijden & Horton, 2009). When colonizing the roots of two or more plants simultaneously, mycorrhizal fungi can establish common mycorrhizal networks (sensu Selosse et al., 2006), which, in some cases, can facilitate the exchange of carbon between plant hosts (Merckx et al., 2024).

Besides, their crucial role in natural ecosystems, mycorrhizal fungi are important for the health of urban trees (Stewart et al., 2024). The importance of urban trees, their underground symbionts and urban green spaces are increasingly recognized for their role in climate change adaptation, human well-being and biodiversity conservation (Dearborn & Kark, 2010; Geneletti & Zardo, 2016; Stevenson et al., 2020; Wolf et al., 2020). However, in urban areas, trees are often adversely affected by urban environmental conditions such as soil restriction, soil sealing, water stress, heat stress, pollution and pests (Esperon-Rodriguez et al., 2022; Flückiger & Braun, 1999; Impens, 1999). Mycorrhizal associations may alleviate the vulnerability of trees to these urban stressors (Aalipour et al., 2019; Calvo Polanco et al., 2008; Fini et al., 2011; Rewald et al., 2015).

Consequently, the response of mycorrhizal fungi to urbanization has received considerable attention in previous research. Most of these studies have assessed either ectomycorrhiza (e.g., Alzetta et al., 2012; Bainard et al., 2011; Hui, Liu, et al., 2017; Jumpponen et al., 2010; Karpati et al., 2011; Martinová et al., 2016; Tonn & Ibáñez, 2017), the AMF root colonization rate (e.g., Bainard et al., 2011; Karliński et al., 2014; Rusterholz et al., 2020; Stabler et al., 2001; Tonn & Ibáñez, 2017; Tyburska et al., 2013; Whitehead, Hempel, & Rillig, 2022; Wiseman & Wells, 2005) or used morphological identification of spores to reconstruct AMF communities (e.g., Bills & Stutz, 2009; Buil et al., 2021; Carrenho & Gomes-da-Costa, 2011; Cousins et al., 2003; Egerton-Warburton & Allen, 2000; Gupta et al., 2018). Of the relatively few studies using molecular analyses to identify urban AMF, one directly sequenced fungal DNA in the roots of herbaceous plants (Lin et al., 2021), while all others identified AMF from soil samples (e.g., Chen et al., 2021; Donald et al., 2021; Lin et al., 2020; Reese et al., 2016; Whitehead, Roy, et al., 2022).

None of these molecular studies have directly assessed the AMF symbionts in the roots of urban trees, despite arbuscular mycorrhizal (AM) trees being major green features in cities. Indeed, in Amsterdam (the Netherlands), at least 45% of the ca. 300.000 trees managed by the municipality (which excludes urban forests) are AM trees, following the classification of Brundrett and Tedersoo (2020), with Elm as the most abundant AM tree and most abundant tree overall (Gemeente Amsterdam, n.d.-a). Moreover, data from the Global Urban Tree Inventory (Ossola et al., 2020) shows that, of the top 20 tree species that occur in the highest number of cities globally, 80% are AM. Nevertheless, many aspects of arbuscular mycorrhizal tree symbioses are understudied in urban environments. At the same time, urban expansion is expected to continue to increase worldwide throughout the first half of the 21st century, at the expense of natural habitat (Angel et al., 2011; van Vliet, 2019). Moreover, the majority of tree species in cities are projected to be adversely affected by climate change (Esperon-Rodriguez et al., 2022). Therefore, in this study, we used metabarcoding to characterize the community composition, diversity, network topology (i.e., the patterns of association between fungal and plant species) and spatial and phylogenetic aggregation of urban tree AMF across an urbanization gradient. By directly sequencing roots, AMF communities of urban trees can be more accurately investigated compared to spore identification (Kowalchuk et al., 2002), with the added benefit of enabling the construction of plant-AMF interaction networks.

Previous molecular studies show a positive (Lin et al., 2021; Whitehead, Roy, et al., 2022) or neutral (Lin et al., 2020; Reese et al., 2016) effect of urbanization on AMF diversity, while in two studies, this was not directly assessed (Chen et al., 2021; Donald et al., 2021). Additionally, in an experiment designed to assess the effect soil sealing—a key feature of urbanization (Scalenghe & Marsan, 2009)—no significant differences in AMF diversity were found between tree roots from uncovered soils, sealed soils and soils with a permeable cover (Grassi et al., 2023). Nevertheless, the AMF assemblages underneath a permeable soil cover were found to be congruent to those from uncovered soils, whereas sealed soil communities diverged from the other treatments (Grassi et al., 2023). In

contrast, measures of AMF colonization rate and AMF spore diversity and density predominantly show a negative response to urbanization (as well as ectomycorrhizal [EM] diversity). Furthermore, Glomeraceae and *Glomus* were previously identified as the most abundant taxonomic groups in urban environments, both in terms of richness and relative abundance (Lin et al., 2020, 2021), as well as *Rhizophagus* and *Funneliformis* (both Glomeraceae; Buil et al., 2021; Chen et al., 2021). *Glomus* was also found to be particularly prominent in paved soils (Grassi et al., 2023). In another study, urbanity was associated with increased Archaeosporales diversity, and *Scutellospora calospora* (Diversisporales) was identified as an indicator species for highly urbanized sites (Whitehead, Roy, et al., 2022). Lastly, Lin et al. (2021) showed reduced nestedness in the interaction networks between AMF and herbs. In mutualistic networks, nestedness measures the degree to which specialists associate with a subset of the partners of generalists (Bascompte et al., 2003) and is associated with species coexistence and reduced extinctions (Bastolla et al., 2009; Thébault & Fontaine, 2010).

Therefore, following the results previous molecular studies, we expect AMF diversity to increase or to the stay the same with increasing urbanization, while featuring distinct community compositions across our sampled gradient. Congruent with the urban homogenization hypothesis (Delgado-Baquerizo et al., 2021; McKinney, 2006) and the findings of previous research, we expect to find a strong association of Glomeraceae taxa, particularly *Glomus*, with the most urbanized locations in our study, in addition to *Rhizophagus* and *Funneliformis*, and *Scutellospora calospora*. Moreover, due to the adverse environmental conditions in cities, we expect trees in increasingly urbanized environments to feature a proportionally higher degree of closely related lineages, consistent with environmental filtering (Maherali & Klironomos, 2007). In turn, this is expected to lead to less nested interaction networks. Lastly, due to street level homogeneity of the environment and plant hosts (i.e., single species tree lanes), we expect AMF communities to be more spatially homogenous with increasing urbanization. By jointly studying AMF community composition, diversity, spatial and phylogenetic aggregation and, for the first time, the network structure of urban trees and their AMF, this study contributes to understanding the effects of urbanization on mycorrhizal communities and interactions. In doing so, urban planning strategies may be informed to promote the beneficial effects of subterranean microbiomes on the health of urban trees and the urban environment (Averill et al., 2022; Stevenson et al., 2020; Stewart et al., 2024).

2 | MATERIALS AND METHODS

2.1 | Sample collection

Root tips (ca. 2 cm) were collected between December 2021 and February 2022 in three increasingly urbanized locations in Amsterdam (the Netherlands): an urban 'forest' (Vliegenbos; 52.389°N, 4.933°E), park (Vondelpark; 52.3580°N, 4.8680°E) and street (Keizersgracht, 52.3745°N, 4.8855°E; Figure 1). Hereafter, the urban 'forest' location is referred to simply as forest, although we recognize it does not constitute a natural forest.

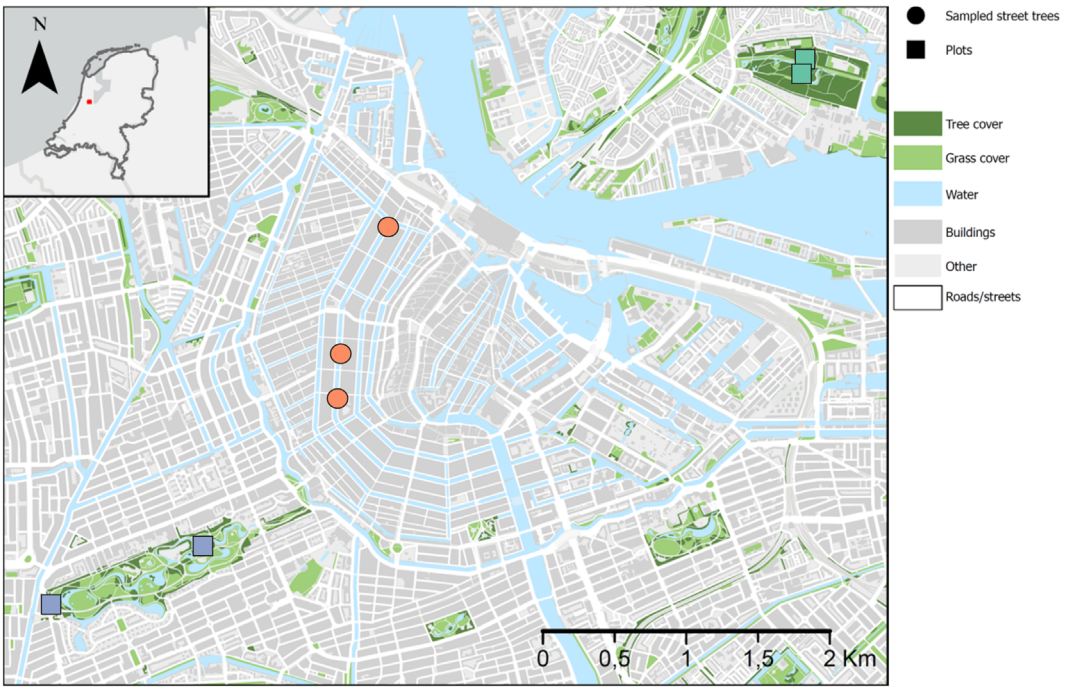

FIGURE 1 Map of the sampling locations. The sampling locations were, in order from north to south: forest plot 1 (F1), forest plot 2 (F2), street site 1 (S1), street site 2 (S2), street site 3 (S3), park plot 1 (P1) and park plot 2 (P2). The forest, street and park locations are coloured green, orange and blue, respectively.

Urbanization was measured as the percentage of built surface area at each location (Rafiee et al., 2016). Built surface areas were estimated from the Dutch land registry map (Basisregistratie Topografie TOP10NL; Kadaster, 2017), using the calculate geometry attributes function in ArcGIS pro (esri, CA, USA). The forest, park and street featured 18%, 28% and >99% built surface, respectively. Furthermore, the forest is extensively managed with spontaneous regeneration of trees and limited accessibility for the public, whereas the park is more intensively managed, with the primary policy goal of facilitating high visitor numbers, recreation and accessibility, in addition to more lenient restrictions on paved surfaces and facilities (see policy notes [in Dutch]; Gemeente Amsterdam, n.d.-b).

To allow for comparisons between locations, we chose sampling sites where Dutch elm (*Ulmus x hollandica*), the most abundant tree in Amsterdam (Gemeente Amsterdam, n.d.-a), was present. For the forest and park locations, samples were collected from two plots each. Each plot was 10.5 by 10.5 m and was divided into 49 grid cells of 1.5 by 1.5 m (7 rows by 7 columns). Two root tips were randomly collected from the topsoil in each grid cell, resulting in 98 samples per plot. Root tips that obviously belonged to herbaceous understory plants (i.e., *Ficaria verna)* were disregarded. An inventory of tree and shrub species was recorded with a buffer of 3 m surrounding each plot (the plant identity of the collected roots are listed in Table S1).

In the street, three sets of three neighbouring Dutch elm trees were selected, distributed across the street. The trees within a set were regularly spaced in tree pits at approximately 10 m apart. Two root tips were randomly collected at three points around each tree, either by lifting a pavement brick or directly from the tree pit, approximately 0.5–2 m from the base of the tree, depending on obstacles such as parked cars and bicycles. Fifty-four samples were collected in the street, resulting in a total of 446 samples overall. During collection, the root samples were rinsed with water to remove dirt and debris. The samples were preserved in CTAB buffer and stored at −20°C. Additionally, composite soils samples were collected to indicate soil conditions and nutrient status at each location. From the plots, composite soil samples were taken from the topsoil in each quadrant, consisting of five randomly distributed samples. In the street location, three composite samples were collected for each site. Soil samples were stored at −20°C.

2.2 | Soil properties

Soil organic matter (SOM) was determined using the loss on ignition method (Heiri et al., 2001). First, thoroughly mixed and sieved subsamples were dried at 105°C for 24 h and weighted to determine the soil moisture content. Subsequently, the SOM was ignited by heating at 550°C for 4 h in a muffle furnace and weighted again to determine the SOM content.

The remaining soil fractions were used to determine pH, nitrate (NO_3^-), ammonium (NH_4^+) and orthophosphate (PO_4^{-3}) content. A soil: water-ratio of 1:2 was used to determine the pH. Potassium chloride (KCl; 1 M) and calcium chloride (CaCl; 0.01 M) extraction methods were used to measure nitrate and ammonium, and orthophosphate content, respectively (Houba et al., 2000; Kachurina et al., 2000). The soil properties are detailed in Table S2.

2.3 | DNA extraction, amplification and library preparation

After rinsing with 70% ethanol, the root samples were cut into approximately 0.5 cm pieces. Larger root branches containing no first-order roots were discarded, as mycorrhizal colonization occurs primarily in the first and second orders of branching (Guo et al., 2008). The roots were subsequently disrupted for 4 × 60 s at 30 Hz using the TissueLyser II (Qiagen, Hilden, Germany) and 3 mm tungsten carbide beads. The sample clamps were rotated after each cycle. DNA was extracted using the DNeasy Plant kit Mini/Pro (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with an additional centrifugation step (20,000*g* for 2 min) after using the final wash buffer. Subsequently, the DNA concentration and purity were measured using a NanoPhotometer N60 (IMPLEN, München, Germany).

The ITS2 region was amplified by polymerase chain reaction (PCR) using the ITS3NGS1-5 (forward) and ITS4NGS (reverse) primer mix, which includes a *Glomeromycotina*-specific primer (Tedersoo et al., 2014). ITS barcodes have been used previously in urban microbiome studies to make inferences about AMF (Chen et al., 2021; Donald et al., 2021; Reese et al., 2016). As this primer mix co-amplifies plant DNA, we could, in combination with field surveys, identify the plant species of the root sample (as in Perez-Lamarque et al., 2022), alongside the AMF in the same reaction. The PCR was performed in a 20 µL reaction volume containing 10.4 µL ultrapure water (MilliQ), 4 µL Phire green PCR buffer (5×), 0.8 µL BSA (10 mg/mL), 1 µL forward primer mix (10 pMol/µL), 1 µL reverse primer (10 pMol/µL), 0.4 µL dNTP (2.5 mM) and 0.4 µL Phire Hot Start II DNA polymerase (Thermo scientific, Waltham, MA, USA) and 2 µL template DNA (2.5 ng/µL). The PCR was conducted starting with an initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 5 s, annealing at 54°C for 5 s and extension at 72°C for 15 s. Afterwards, a final extension step was performed at 72°C for 5 min. Successful amplification was confirmed by gel electrophoresis. Samples showing no band on the gel were diluted 10 times, after which the protocol was repeated. The PCR products were purified with magnetic beads (0.9× sample volume) using the automated C.WASH platform (CYTENA, Freiburg, Germany). Subsequently, a second round of PCR (PCR2) was performed to attach Illumina MiSeq index sequences to the primer ends of the DNA. PCR2 consisted of the same reaction volume and five cycles of the protocol described above.

The concentration of the desired fragment lengths was measured with a Fragment Analyser (Agilent, Santa Clara, CA, USA). All but one sample showed successful amplification, which was discarded. Next, the PCR2 products were pooled equimolarly in two end pools using the QiAgility (QIAGEN, Hilden, Germany), after which an additional

magnetic bead cleaning step was performed. The PCR products were sequenced by BaseClear (Leiden, the Netherlands) using the Illumina MiSeq (PE300) next-generation sequencing platform.

2.4 | Sequence processing, filtering and normalization

All data processing and analyses were performed in R, version 4.1.2 (R core Team, 2021), unless stated otherwise. Raw amplicon data were processed using the DADA2 pipeline (Callahan et al., 2016). First, the forward and reverse primers were removed using Cutadapt (Martin, 2011). Next, the library was filtered by discarding reads containing ambiguous nucleotides, and truncating reads shorter than 210 or larger than 250 nucleotides. Sequences with more than two expected errors were removed. A parametric model of the error rates was used to denoise the filtered and dereplicated reads (Callahan et al., 2016). Paired forward and reverse reads were merged, discarding non-overlapping sequences. Chimeric sequences were removed with the *removeBimeraDenovo*-function using the consensus method in the 'dada2' R-package (Callahan et al., 2016). Two samples with unexpectedly low read counts were discarded (Figure S1).

The resulting amplicon sequence variants were clustered into operational taxonomic units (OTUs) at 97% similarity. Taxonomy was assigned to the OTUs using a naïve Bayesian classifier (Wang et al., 2007), with the *AssignTaxonomy*-function in the 'dada2' R-package, and the UNITE reference database (version 8.3, Abarenkov et al., 2021). Afterwards, all non-AMF OTUs were discarded, including the plant OTUs that were used to determine the identity of the root samples. If the remaining OTUs had less than five reads in a sample, they were discarded from that sample to eliminate spurious OTUs. As a result, global singletons were also removed.

Read counts were normalized using scaling with ranked subsampling (SRS; Beule & Karlovsky, 2020). The normalization depth (C_{min}) was chosen by iteratively increasing C_{min} from 0 to 1000 to balance the amount and incidence of AMF OTUs, and the retained number of samples. Trading off these factors, a balanced C_{min} was found at 285, where both the number of AM OTUs and AM OTU incidence plateaued, while retaining 90% of the collected samples (Figure S2). This approach is enabled by the negligible variability in the outcome of SRS (Beule & Karlovsky, 2020); thus, it does not require large amounts of iterations, as would be the case with rarefaction (Cameron et al., 2021).

2.5 | Community composition, diversity and structure

From the normalized AMF community matrix, the beta-diversity of the three sampling locations were visualized using non-metric multidimensional scaling (NMDS) in four dimensions with extended dissimilarities, using the Jaccard index (Jaccard, 1901). We used read counts as presence/absence data, here and in further analyses, since different AMF differentially invest biomass in roots or in soil (Barceló et al., 2020; Hart & Reader, 2002; Maherali & Klironomos, 2007); thus, read counts from either compartment may not accurately reflect the true abundance of a taxon. The analyses were conducted for Dutch elm samples and the overall sampled plant communities separately (the latter is mainly shown in the Supporting information).

After verifying the homogeneity of variance (using the *betadisper*-function in the 'Vegan' R-package; Oksanen et al., 2022), community differences between locations were tested with permutational multivariate analysis of variance (PERMANOVA) and the Jaccard distance matrix, in the 'vegan' R-package (Oksanen et al., 2022; version 2.6–4). If the PERMANOVA showed a significant difference (at $\alpha = 0.05$), a post hoc pairwise PERMANOVA was performed with a Benjamini-Hochberg adjustment for multiple comparisons (Benjamini & Hochberg, 1995). Similarly, pairwise differences in variance were tested using Tukey's honest significant difference (HSD) test (Tukey, 1949). An Euler diagram was drawn to show the overlap of OTUs between locations, using the 'eulerr' R-package (Larsson, 2022). The communities were represented by ellipses, enabling the areas of each ellipse and their intersections to be accurately drawn proportionally to the number of OTUs (Micallef & Rodgers, 2014).

Hill number based rarefaction and extrapolation was used to assess the diversity and phylodiversity of the AMF communities, using the 'iNEXT' (version 3.0.0) and 'iNextPD' (version 0.3.1) R-packages, respectively (Hsieh et al., 2016; Hsieh & Chao, 2017). Hill numbers provide diversity measures that are intuitive to interpret and compare across different community structures and are well suited for metabarcoding studies (Alberdi & Gilbert, 2019). For the OTU diversity, Hill number rarefaction and extrapolation curves were generated with 1000 bootstrap replications for orders (q) 0, 1 and 2, corresponding to effective measures of OTU richness, Shannon-diversity and Simpson-diversity (Chao et al., 2014), respectively. For the phylodiversity, $q = 0$, 1 and 2 correspond to effective branch lengths of Faith's PD, Allen's H_p and Roa's Q, respectively (Chao et al., 2010). For $q = 0$, rare and abundant OTUs are weighted equally; thus, overestimating the influence of rare taxa, for $q = 1$, OTUs are weighted by their frequency, reflecting the diversity of common OTUs, and lastly, $q = 2$ yields the effective diversity or branch lengths of dominant OTUs (Chao et al., 2014). Rarefaction and extrapolation curves were drawn with 84% confidence intervals, where non-overlapping confidence intervals correspond to a significance level of $\alpha = 0.05$ (MacGregor-Fors & Payton, 2013; Payton et al., 2003).

For the phylogenetic diversity, a phylogenetic tree of the AMF OTUs was constructed by first generating a reference tree of *Glomeromycotina* from long read barcodes spanning the SSU-ITS-LSU region (around 3000 bp), provided by Krüger et al. (2012). The highest likelihood reference tree was generated using RAxML (version 8.2.11; Stamatakis, 2014) under the GTR + I + G model of substitution on an alignments generated with MAFFT (v7.490; Katoh & Standley, 2013) in Geneious Prime (version 2021.2.2; Dotmatics, Boston, MA, USA). Next, the ITS2 sequences of the OTUs obtained in this study were aligned with the reference sequences from Krüger et al. (2012), using MAFFT. *Paraglomus occultum* was selected as outgroup (Krüger

et al., 2012). The reference sequences were then trimmed to the ITS2 region, and a tree was constructed with RAxML, where the relative positions of the reference sequences were constrained by their positions in the reference tree. An ultrametric tree was subsequently generated using treePL (Smith & O'Meara, 2012) with the root node dated at 505 Ma (Davison et al., 2015).

Pairwise cophenetic distances from the resulting phylogenetic tree were used to estimate the phylogenetic community structure, using the 'picante' R-package (version 1.8.2; Kembel et al., 2010). Phylogenetic dispersion was calculated as the standardized effect sizes of the mean nearest taxon distances (MNTD) and the mean pairwise distance (MPD), and compared to null models, testing whether the observed phylogenetic structure differs from random associations. A thousand randomizations of the community matrices were performed, using the 'independent swap' method, preserving marginal sums (Gotelli, 2000). The standardized effect sizes of MNTD and MPD were multiplied by −1 to yield the nearest taxon index (NTI) and the nearest relative index (NRI), respectively (Webb et al., 2002). Values of NTI and NRI smaller than 0 indicate that that the community is phylogenetically overdispersed, while values larger than 0 indicate phylogenetic clustering. NTI is driven by phylogenetic clustering near the tips of the tree, while NRI measures clustering across the phylogeny (Webb et al., 2002). It is expected that overdispersed communities are structured by interspecific competition, which selects for divergence, while phylogenetically clustered communities are expected to be structured by environmental filtering, which selects for a conserved set of traits (Webb et al., 2002). Differences in phylogenetic structures between sites were tested using Kruskal–Wallis rank sum tests on the non-standardized MNTD and MPD values, followed by a post hoc Dunn's test.

Lastly, to find association between AMF and the study sites, the group equalized indicator value index (IndVal.g; Dufrêne & Legendre, 1997) was calculated using the *multipatt*-function in the 'indicspecies' R-package (Cáceres & Legendre, 2009). Confidence intervals were calculated with the *strassoc*-function from the same package. The IndVal.g is calculated for each OTU and is the square root of the product of two indicator components: A, the site specificity (i.e., the probability that a sample belongs to a particular urbanization class, given the occurrence of the target OTU); and B, the sensitivity (i.e., the probability that a sample from an urbanization class contains the target OTU). The significance and confidence intervals were calculated from 10,000 permutations and bootstrap replications, respectively, and *p*-values were corrected with a Benjamini-Hochberg adjustment for multiple comparisons.

2.6 | Spatial aggregation

The Jaccard community distance and the spatial Euclidian distance were used to determine the spatial heterogeneity of AMF assemblages. To do so, Mantel tests (Mantel, 1967) using Pearson's correlation were performed on the two distance matrices for each site, using the 'vegan' R-package (Oksanen et al., 2022). Significance was assessed from 10,000 permutation and the results were visualized in distance-decay plots. When the whole plant community was considered, permutations were restricted within plant species, thus assessing whether AMF communities within the same host species change as a function of distance for the overall plant community.

The influence of neighbouring-tree roots on AMF richness was tested with a negative binomial generalized linear model (GLM), using pooled data from all plots. A root was considered a 'neighbour' if it occurred within one grid cell distance in our sampling grid. The AM and EM plant richness and the total AMF richness of neighbouring roots were used as explanatory variables for AMF richness. The root samples from grid cells on the edges of the plots were excluded from the response variable, as these roots had neighbours outside the plot. These samples were retained however for the explanatory variables as they constitute neighbours of the inner grid cells. Since not all samples passed the filtering and normalization, the number of neighbouring samples differed between grid cells and was thus included as a covariate. The plant identity of each root and the plot were considered as random effects in a mixed-effects model; however, this resulted in a singular fit with the variance of the random effects close to zero; thus, these effects were not included in the final model.

2.7 | Network analyses

Lastly, interaction networks were constructed from species/OTU level plant-AMF incidence matrices using Gephi (version 0.10.1; Bastian et al., 2009) and the 'ForceAtlas2' layout algorithm (Jacomy et al., 2014). Network nestedness was analysed using the 'bipartite' (Dormann et al., 2009) and 'maxnodf' (Hoeppke & Simmons, 2021) R-packages. The weighted nestedness based on overlap and decreasing fill ($NODF_w$) was used to assess whether the observed nestedness significantly differed from randomized null models, using z-scores (Almeida-Neto & Ulrich, 2011). The incidence frequencies of OTUs in plant species were used as weights for the interaction. Networks were randomized 1000 times using 'quasiswap' randomization in the 'vegan' R-package (Oksanen et al., 2022), where interactions are randomized, but the overall number of links (connectance) and the marginal sums are preserved.

Furthermore, the combined nestedness ($NODF_c$) was used to assess differences in nestedness between observed networks. Typically, nestedness is strongly influenced by network size and connectance, complicating comparisons between networks (Song et al., 2017). Therefore, Song et al. (2017) proposed the $NODF_c$, which normalizes NODF based on the maximum possible NODF-value of the observed network, while correcting for differences in network size and connectance, allowing comparisons between networks (Song et al., 2017). Network size (S) gives the total number of taxa in the network (number of plant species + number of AMF OTUs), whereas connectance gives the proportion of realized connections out of the total number of possible connections. Species level network analysis could not be conducted for the street, as there was only one sampled tree species.

3 | RESULTS

3.1 | Community structure and diversity

In total, 198 AMF OTUs were recovered represented by a total of 69,913 reads in 443 samples (see Figure S1 for total read counts). Read count normalization reduced the number of AM OTUs to 177. No AMF were found in 134 samples, 46 of which were from ectomycorrhizal trees (see also Table S1). Of the 184 collected Dutch elm roots tips, 38 contained no AMF. The proportions of Dutch elm roots containing no AMF were 0.20, 0.26 and 0.16 for the forest, park and street locations, respectively. Out of 177 total AMF OTUs, 147 were found in Dutch elm roots.

AMF communities significantly differed between the three sites (Figure 2a; PERMANOVA; $p = 0.001$; Figure 2b; PERMANOVA; $p = 0.001$). Pairwise post hoc tests subsequently showed significant differences between all sites for the Dutch elm AMF (post hoc pairwise PERMANOVA with Benjamini-Hochberg adjustment; forest-park $p = 0.001$, forest-street $p = 0.001$, park-street $p = 0.001$). However, dispersion significantly differed between forest and park locations (*betadisper* followed by Tukey's HSD; forest-park $p = 0.005$, forest-street $p = 0.127$, park-street $p = 0.497$); thus, the pairwise PERMANOVA between forest and park should be interpreted with caution (Anderson & Walsh, 2013). Similarly, when the whole plant community was considered, AMF communities significantly differed between all locations (post hoc pairwise PERMANOVA with Benjamini-Hochberg adjustment; forest-park $p = 0.001$, forest-street $p = 0.001$, park-street $p = 0.001$).

Glomeraceae accounted for >80% of OTUs in Dutch elm across locations (Figure 3a). In the forest location, *Glomus* was the most frequently identified genus in Dutch elm roots, while *Dominikia* was most frequently identified in the park and street. *Septoglomus*, *Diversispora*

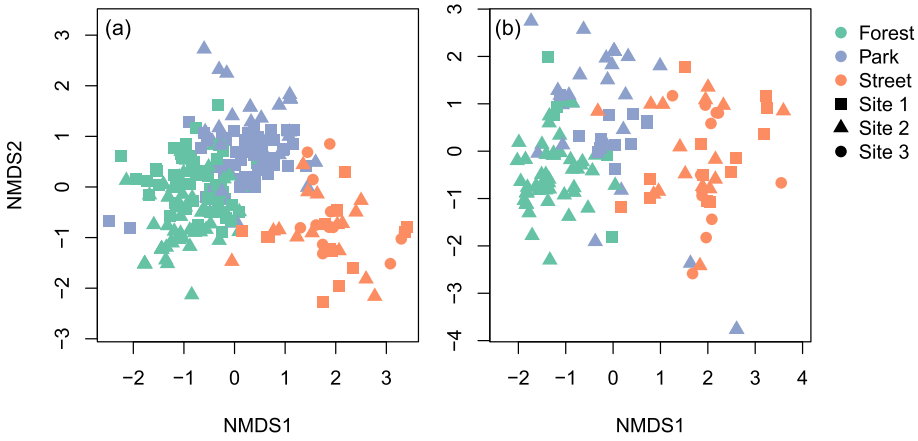

FIGURE 2 Non-metric multidimensional scaling (NMDS) of arbuscular mycorrhizal fungi Jaccard community distances from (a) all collected roots (stress = 0.141) and (b) Dutch elm roots (stress = 0.096). Locations are indicated by colour, while shapes indicate sites within each location.

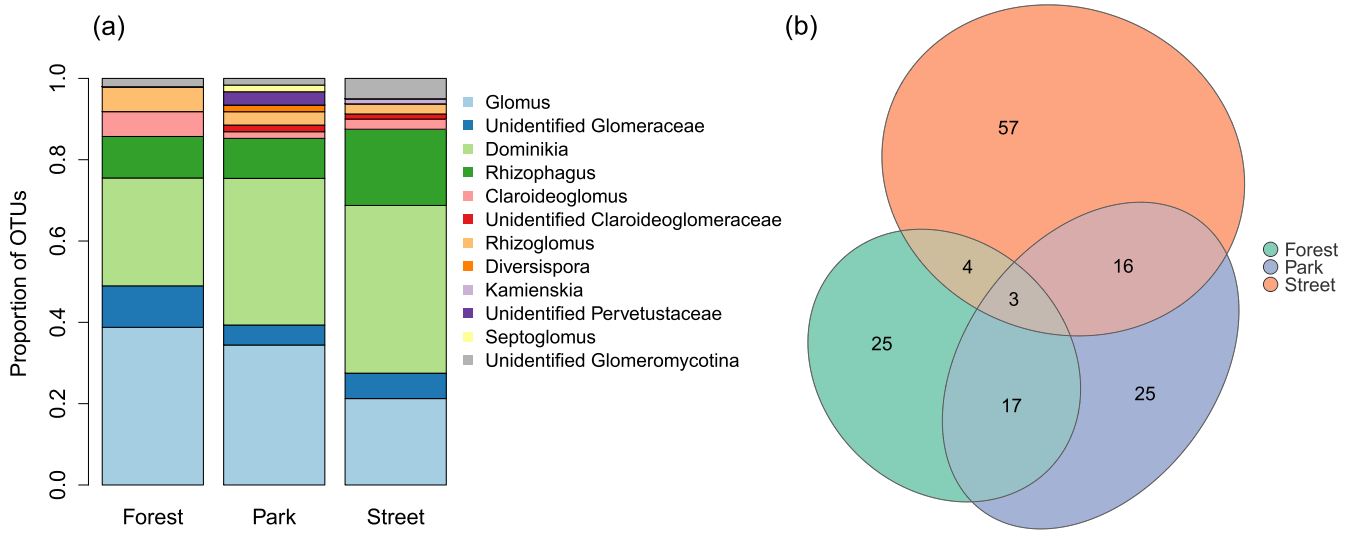

FIGURE 3 Proportion of recovered arbuscular mycorrhizal fungi (AMF) operational taxonomic units (OTUs) from Dutch elm roots at genus level per location (a). OTUs that could not be identified to genus level are grouped in the lowest taxonomic group that could be identified. Euler diagram showing the number of unique and shared AMF OTUs between Dutch elm roots from all locations (b). The area of each section is proportional to the number of OTUs.

and two unidentified Pervetustaceae OTUs were only identified in Dutch elm roots from the park, and *Kamienskia* was only found in the street. Three OTUs were found in all locations (Figure 3b): Two *Glomus* OTUs and one *Dominikia*, though they were not identified to the species level. Furthermore, more OTUs were shared between the forest and the park, and the park and the street, than between the forest and the street.

The indicator species analysis identified different *Rhizoglomus* OTUs as indicators for the forest, park and street locations. Furthermore, *Dominikia achra* was an indicator for the park location, alongside two other *Dominikia* OTUs. Four unidentified *Glomus* OTUs were indicators for the street, as well as *Dominikia aurea*, *Dominikia duoreactiva* and *Rhizophagus irregularis*, amongst others (see Table S3).

The effective AMF OTU richness (Figure 4a; q = 0) in Dutch elm was highest in the street, followed by the park and forest, although the observed richness was not saturated. Extrapolation of AMF richness indicates that the street and park locations might not differ at larger sample sizes, although both still richer than the forest. The effective Faith PD (Figure 4b; q = 0) did not differ between the park and the street for both the observed and the extrapolated diversities, although both were higher than the forest. The higher order Hill number diversities (Figure 4a,b; q = 1 and 2) consistently showed significantly higher diversity of AMF in the street, followed by the park, and after that, the forest, for both the observed and extrapolated diversities. When considering the whole plant community, the street consistently showed the highest diversity for orders q = 1 and 2, although no difference was found for OTU richness and faith's PD (q = 0; Figure S3).

The phylogenetic community structures of AMF tended towards clustering for both NRI and NTI, although both did not significantly differ from random expectation as a whole for each location (Figure 5a,b). MNTD did not differ between sites (Kruskal–Wallis test; $p = 0.4346$), although the MPD of the street communities was significantly lower than that in the forest (Kruskal–Wallis test; $p = 0.003$, post hoc Dunn's test adjusted-p: forest-park = 0.078, forest-street = 0.003, park-street = 0.304). Similarly, for the whole

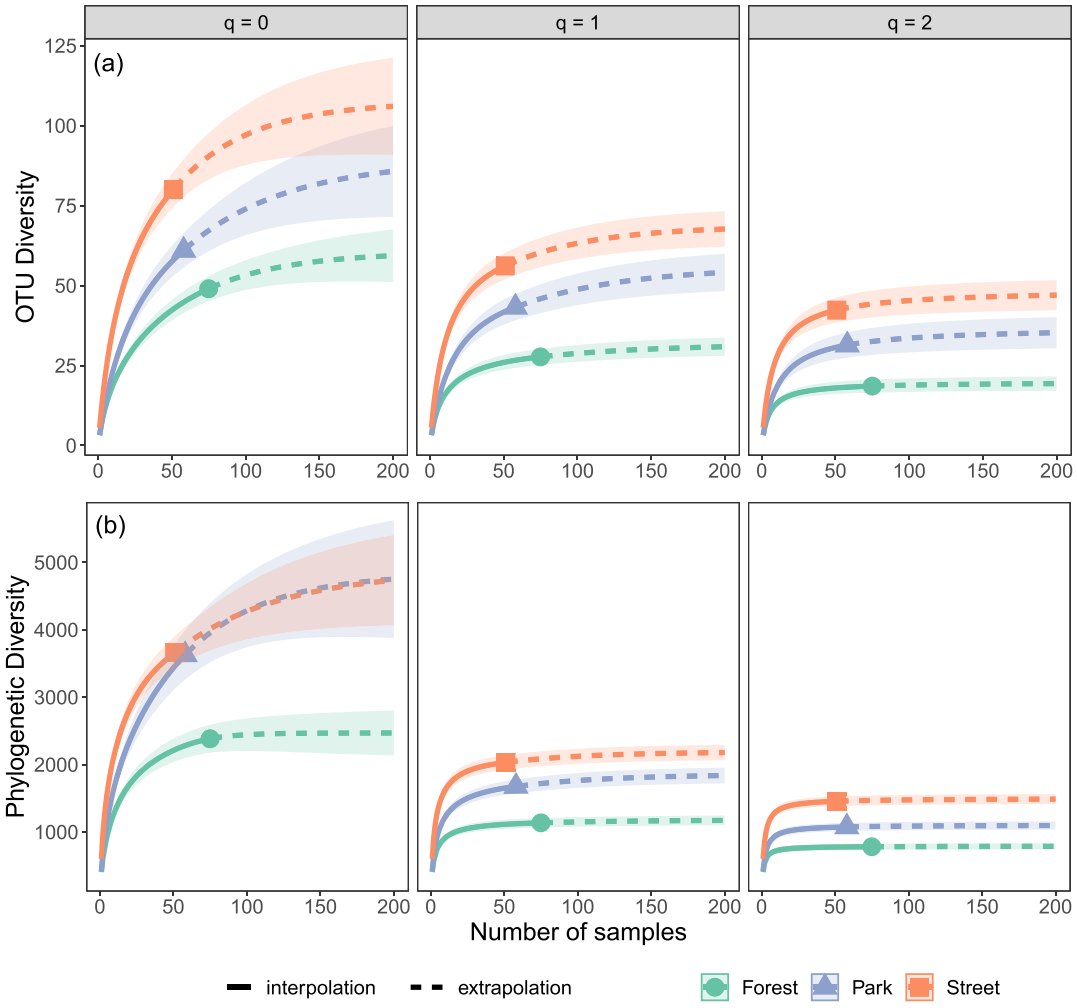

FIGURE 4 Operational taxonomic unit (OTU) (a) and phylogenetic (b) diversity rarefaction and extrapolation curves for Hill numbers (q) 0, 1 and 2 of forest, park and street Dutch elm arbuscular mycorrhizal fungi communities. Shaded area shows the 84% confidence interval, indicating significant differences at $\alpha = 0.05$.

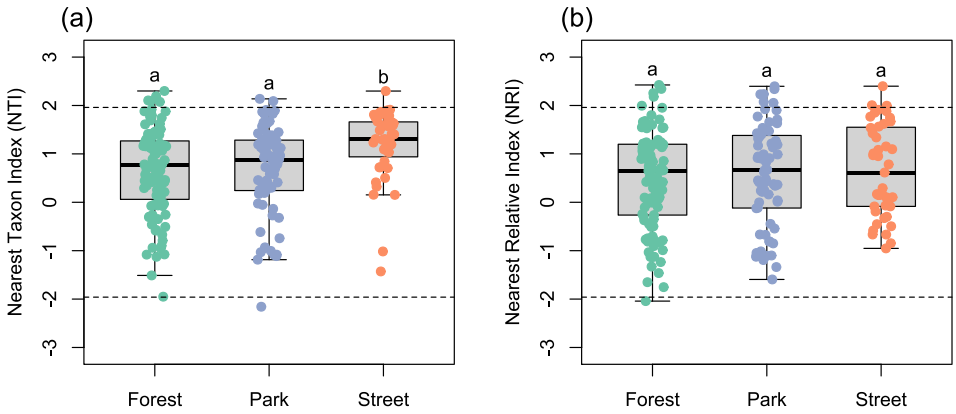

FIGURE 5 Phylogenetic community structure of Dutch elm arbuscular mycorrhizal fungi. Coloured circles show the nearest taxon index (NTI; a) and the nearest relative index (NRI; b) values of individual samples, which are summarized in boxplots for each location. The horizontal dashed lines at 1.96 and −1.96 indicate the significant difference threshold compared to null models (z-scores), indicating clustered or overdispersed communities, respectively. Letters indicate significant differences (Kruskal–Wallis test followed by a post hoc Dunn's test) in non-standardized mean nearest taxon distances (MNTD; a) and the mean pairwise distances (MPD; b).

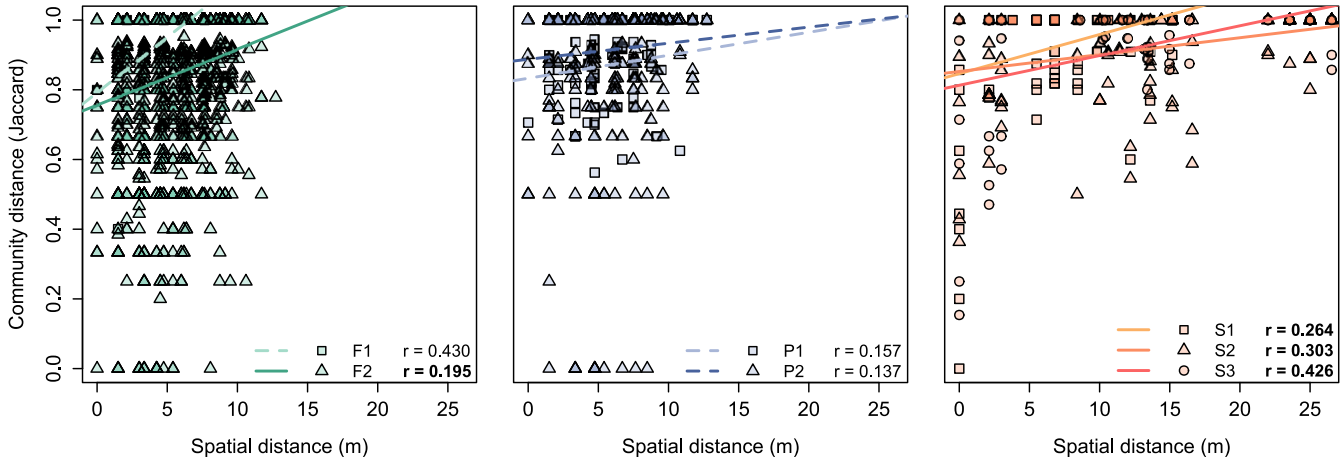

FIGURE 6 Distance-decay plots of Dutch elm arbuscular mycorrhizal fungi community distance (Jaccard) over spatial distance (m) for each sampling site: forest plot 1 (F1), forest plot 2 (F2), park plot 1 (P1) and park plot 2 (P2) street site 1 (S1), street site 2 (S2) and street site 3 (S3). Pairwise community and spatial distances of samples are shown as points (shapes indicate sites within each location). Each data point is transparently coloured; thus, darker shades indicate overlapping points. Mantel's r statistics and regression lines are shown in bold and as solid lines, respectively, to indicate significant Mantel's r values.

plant communities, phylogenetic community structure did not differ from random expectations (Figure S4a,b), although street AMF communities were more clustered than forest and park communities (Figure S4a).

3.2 | Spatial aggregation

AMF communities tended to be more dissimilar the further apart the roots were collected (Figure 6). The strongest significant AMF community change over distance (slope) was observed in the forest plot 2 (Figure 6; $\Delta_{F2\ Jaccard} = 0.016$ [±0.002] m^{-1}, Mantel's $r_{F2} = 0.195$, $p_{F2} < 0.001$), while no significant correlation between community turnover and distance was found for forest plot 1 (Mantel's $r_{F1} = 0.430$, $p_{F1} = 0.058$). For the street location, a significant correlation between AMF community distance and spatial distance was observed for all sites. Here, the highest turn-over by distance was observed in street site 1 ($\Delta_{S1\ Jaccard} = 0.011$ [±0.004] m^{-1}, Mantel's $r_{S1} = 0.264$, $p_{S1} = 0.007$), followed by street site 3 ($\Delta_{S3\ Jaccard} = 0.008$ [±0.002] m^{-1}, Mantel's $r_{S3} = 0.426$, $p_{S3} = 0.001$) and site 2 ($\Delta_{S2\ Jaccard} = 0.004$ [±0.001] m^{-1}, Mantel's $r_{S2} = 0.303$, $p_{S2} = 0.005$). In both park plots, no significant change over distance was observed (Mantel's $r_{P1} = 0.157$, $p_{P1} = 0.119$; $r_{P2} = 0.137$, $p_{P2} = 0.063$). When the whole plant community was considered, Mantel's r-values of the park plots were again not significant, while both forest plots showed significant positive correlations (Figure S5). Lastly, the negative binomial GLM indicate that AMF richness was not significantly affected by neighbouring root AMF richness or plant richness (Table 1).

3.3 | Network analysis

The observed $NODF_w$ values for forest plots 1 and 2, and park plots 1 and 2, were, respectively 28.36, 32.42, 38.86 and 8.54. The park 1 network was nested, while all other networks were non-nested (Figure 7). $NODF_c$ values indicate that the AMF networks in the forest were less nested than the networks in the park (Figure 7), although too few replicate plots were collected for statistical testing.

4 | DISCUSSION

In this study, we have characterized the understudied AMF communities of urban Dutch elm trees and tree assemblages, their diversity, spatial and phylogenetic structures and network properties across an urbanization gradient. Using the ITS2 barcode, we found distinct AMF communities in each urbanization class (Figure 2), with few AMF overlapping between all three sampled locations in Dutch elm (Figure 3). The park shared features of both the street and forest locations, while sharing of AMF was limited between the forest and the street. Several *Glomus* OTUs were significantly associated with Dutch elm in the street location in (Table S3), corroborating studies identifying *Glomus* as a predominant feature of urban and paved soils (Grassi et al., 2023; Lin et al., 2020, 2021).

Additionally, for Dutch elm trees, we showed that *Rhizophagus irregularis* and other *Rhizophagus* OTUs had a significant association with the street location. *R. irregularis* (formerly *Glomus intraradices*) is commonly considered a generalist with a global distribution across a range of environments, including disturbed anthropogenic soils (Oehl et al., 2010; Öpik et al., 2006). Multiple *Dominikia* OTUs were associated with both the street and park locations. Broadly, *Dominikia* spp. are shown to be tolerant to disturbance, owing to their rapid growth and high biomass allocation to roots (Cahyaningtyas & Ezawa, 2024). This genus has not been previously described as a typical feature of urban AMF communities, potentially due to underestimation of taxa with high root abundance when assessing soil AMF communities. Differences with previous studies may also be the result of primer biases or differences in the underlying factors associated with urbanization. Lastly, different *Rhizoglomus* OTUs were identified as significant indicator taxa for all three locations. This may be the result of oversplitting of taxa when clustering the sequences in to OTUs or unresolved taxonomic differences and habitat preferences within the genus. Alternatively, it could be speculated that *Rhizoglomus* spp. constitute part of the AMF 'core microbiome' (sensu Neu et al., 2021) of Dutch elm in Amsterdam, although more research is needed to verify if this extends to other cities.

We show that AMF were most diverse in the street, followed by the park and forest (Figure 4; Figure S3). This pattern was robust when taking into account the phylogenetic relationships between OTUs, and rarefaction and extrapolation curves show that the

TABLE 1 Negative binomial generalized linear model (GLM) parameter estimates (±standard error) and *p*-values (significant values are highlighted in bold) of variables testing the effect neighbouring root diversity on arbuscular mycorrhizal fungi richness.

	Estimate ± SE	*p*-Value
Intercept	1.995 ± 0.924	**0.031**
AMF richness in neighbouring roots	0.014 ± 0.015	0.356
AM plant richness of neighbouring roots	−0.044 ± 0.149	0.770
EM plant richness of neighbouring roots	−0.165 ± 0.164	0.316
Number of sequenced neighbours	−0.054 ± 0.053	0.303

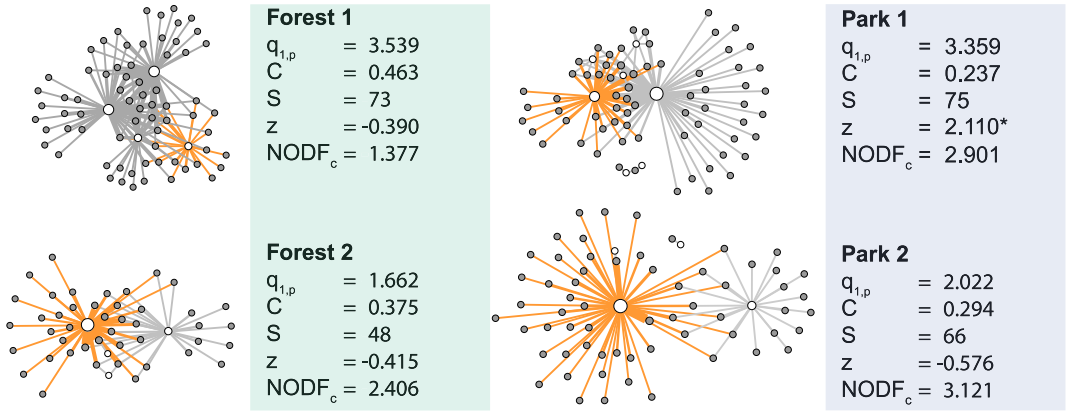

FIGURE 7 Species level networks and network properties of each plot. Lines (links) between circles (nodes) indicate that an arbuscular mycorrhizal fungi operational taxonomic unit (OTU; grey circles) was found in a root of the corresponding plant species (white circles). The breadth of the link indicates the incidence of that OTU in the linked plant species and the size of each node indicates the total number of links. Dutch elm links are highlighted in orange. The plant diversity of the sampled roots ($q_{1,p}$) is noted as the Hill number for q = 1, along with the connectance (C), the network size (S), the z-scores of the weighted nestedness metric based on overlap and decreasing fill ($NODF_w$; z, asterisk indicates significant difference form null models) and the combined nestedness metric ($NODF_c$).

observed pattern was consistent for samples sizes larger than ca. 10 samples. Differences between locations were less clear when considering the effective OTU richness or Faith's PD ($q = 0$). However, richness metrics can be strongly biased by rare and/or spurious OTUs (Bálint et al., 2016). Therefore, the OTU richness should be interpreted with caution, even though we minimized these biases through filtering and normalization. Overall, these results are in agreement with sequencing studies that indicated higher AMF biodiversity in urban soils (Whitehead, Roy, et al., 2022) and herbaceous plant roots (Lin et al., 2021), but contrast earlier studies on spore diversity (Buil et al., 2021; Carrenho & Gomes-da-Costa, 2011; Egerton-Warburton & Allen, 2000; Gupta et al., 2018). More broadly, the diversity of the urban soil microbiome (i.e., bacteria, archaea, non-AM fungi, protists and other eukaryotes) was previously shown to match or exceed the diversity of natural biomes (Delgado-Baquerizo et al., 2021; Hui, Jumpponen, et al., 2017; Ramirez et al., 2014), but, to the best of our knowledge, this is the first time this pattern has been shown for AMF in urban trees and urban tree assemblages.

It is currently unknown why urbanity seems to be associated with increased AMF diversity. In their study on the urban soil microbiome (which did not include AMF), Hui, Jumpponen, et al. (2017) link their findings to the intermediate disturbance hypothesis (Connell, 1978), which suggests that diverse communities are facilitated by disturbances of intermediate frequency and severity, maintaining a non-equilibrium and high biodiversity state. Whitehead, Roy, et al. (2022) speculate the same mechanism may play a role in urban environments, alongside the occurrence of novel niches and the filtering of usually dominant taxa, leaving unoccupied niches for other fungi. Alternatively, Lin et al. (2021) suggests that plants undergoing physiological stress from environmental pressures are increasingly reliant on root symbionts, thus opening up a wider niche space in their roots. Interestingly, in an experiment, Johnson et al. (2004) showed that roots of *Plantago lanceolata* bioassays that were planted in mesocosms with either high plant diversity (12 species), two different monocultures or bare soil, had the highest AMF diversity combined with the lowest colonization rates when planted in bare soil, and one of the monocultures matched the AMF diversity of the high plant diversity mesocosm. To explain this result, they offer the hypothesis that, when colonization occurs from isolated propagules (i.e., spores or viable hyphal fragments in the absence of an extensive mycelium sustained by other plants), no preselection of compatible/dominant fungi by the plant community takes place, thus avoiding a loss of AMF diversity, whereby a large fraction of the available propagules can establish in the host, but with a low colonization rate overall (Johnson et al., 2004). When colonization occurs from a well-established mycelium (i.e., sustained by other plants), the subset of AMF with extensive hyphae and/or rapid growth will be favoured when colonizing new roots, thus resulting in a relatively lower AMF diversity in those roots. This hypothesis can be used to explain both the high AMF diversity of isolated street trees in the present study and previous findings showing low colonization rates in urban trees. However, these hypotheses are not mutually exclusive and further experimental investigation is needed.

The phylogenetic community structure showed that the Dutch elm AMF tended to be significantly clustered for some samples from each location (Figure 5), indicating that those AMF communities are structured by habitat filtering rather than competition (Webb et al., 2002). However, the overall mean of the phylogenetic community structure from each location was not different from random expectations. However, the street AMF community was significantly less clustered than the forest community. This might indicate that the street community is structured proportionally more by competition. This was contrary to our expectations and contrasts earlier finding by Lin et al. (2021) who showed a reduction in competition by evaluating the aggregation patterns of AMF in interactions networks. More research, ideally combining the phylogenetic and network approaches, is needed to assess the community structure and aggregation patterns of urban AMF.

Our data did not support the hypothesis that AMF communities are more spatially homogenous in increasingly urbanized locations (Figure 6), although more replicate sites are needed to validate this. In contrast, at a global scale, Delgado-Baquerizo et al. (2021) show that, more broadly, urban soil microbial communities are more homogenous compared to natural environments, although they did not include AMF. In future studies, AMF should be included in international and global urban microbiome biodiversity research, given their importance to urban green infrastructure. Collaborations with municipalities and managers of urban trees, who often regularly survey tree health in cities, may provide particularly fruitful avenues for AMF research.

In our neighbour analysis, we found no evidence that the AMF richness in a root is influenced by the surrounding root richness or the AMF richness therein (Table 1). These results are consistent with findings that the plant community is only a weak predictor of AMF communities at similar spatial scales (Horn et al., 2017) and that, at a global scale, AMF richness seems unrelated to plant richness, owing to the non-specificity of AMF to plant hosts (Tedersoo et al., 2014; but see also Zhang et al., 2021). Instead, AMF communities at small spatial scales seem to be driven by spatial distance and phylogenetic clustering (Horn et al., 2014, 2017). Adding to these previous findings, in this study, we measured the plant diversity by randomly sampling roots in a spatially divined grid. This method yields an estimate of plant diversity that more directly applies to the perspective of AMF compared to above ground surveys. That a higher plant root richness does not necessarily lead to higher AMF diversity in urban environments is further supported by our results showing a higher AMF diversity in monoculture street trees even when the whole tree communities from the forest and park plots were included (Figure S3). Nevertheless, plant community composition strongly influences the composition of AMF communities and both are likely codependent (Kokkoris et al., 2020; Mony et al., 2024).

Lastly, network analysis revealed frequent sharing of AMF between Dutch elm and surrounding trees (Figure 6). We found that the topology of these networks tended to be less nested in the forest location than in the park, although more replicate plots are needed to test this statistically. This was contrary to our expectations based on previous work by Lin et al. (2021), who showed decreased nestedness in more urbanized locations. However, nested interactions are

thought to promote community resilience, and, based on this, it is expected that frequent environmental disturbances select for network topologies that are more nested (Song et al., 2017). The trend in our data supports this hypothesis. Future studies and experiments should therefore include measures of (soil) disturbance in their analyses to better understand the observed network patterns.

Although the use of the ITS2 barcode likely limited the taxonomic coverage of *Glomeromycotina* in our study (Delavaux et al., 2022), studies comparing the performance of *Glomeromycotina*-specific SSU primers to general fungi ITS primers show that community composition and diversity changes in response to environmental gradients were congruent, despite preferential amplification of Glomeraceae taxa by the ITS primers (Berruti et al., 2017; Lekberg et al., 2018). It should be considered that the Glomeraceae amplification bias could have resulted in an underestimation of AMF diversity in our forest location, as this location may be expected to feature relatively more disturbance intolerant AMF taxa (e.g., Diversisporales), even though our study locations overall were skewed towards disturbed rather than natural sites. Nevertheless, similar diversity patterns to what we observed have been noted in previous studies investigating the response of AMF to urbanization using LSU and SSU primers (Lin et al., 2021; Whitehead, Roy, et al., 2022).

Further bias may have been introduced by sampling in winter. In grasslands, AMF communities have been shown to have marginally lower diversity in winter (Montero Sommerfeld et al., 2013), but winter increases are also shown, possibly related to relaxed competitive interactions amongst AMF as a result of restricted carbon availability (Dumbrell et al., 2011). Furthermore, network properties change over the growing season (June, July, October), and this response can vary between successional stages in temperate forests (Bennett et al., 2013). For these reasons, future research should consider temporal variation in the study design.

Nevertheless, using ITS2 barcodes, we have shown that AMF communities in urban Dutch elm trees and tree assemblages in Amsterdam are distinct, diverse and heterogenous. An urbanity-associated increase in mycorrhizal partners and change in composition, rather than a loss of partners and interaction complexity, demonstrates the high adaptability of the arbuscular mycorrhizal symbiosis to urban stressors. These findings have important implications for urban tree management. Mycorrhizal inoculation of urban trees (i.e., the intentional introduction of AMF propagules, such as spores) has been suggested as means to improve the establishment, stress tolerance and longevity of urban trees, although the outcomes are not always conclusive (Stevenson et al., 2020). Our research suggests that a lack of mycorrhizal inoculum may not be not a limiting factor for mycorrhizal colonization of urban trees, as was previously indicated by Wiseman and Wells (2005). Therefore, the mycorrhizal inoculum that is already present at a site and in planted trees should be considered before applying an inoculum (Appleton et al., 2003; Stewart et al., 2024), and the potential inoculum should be tailored to the application site (Fini et al., 2011). First, however, more research on urban AMF is needed to understand their functional differences in different types of urban green spaces and natural areas. Importantly, the disparity between the effects of urbanization on AMF colonization rates and diversity should be an urgent topic of investigation in order to elucidate the function of AMF in urban green infrastructure, as both diversity and colonization rate influence the plant-benefit derived from the interaction (Gange & Ayres, 1999; Maherali & Klironomos, 2007).

AUTHOR CONTRIBUTIONS

Casper Verbeek: Formal analysis; investigation; writing—original draft; visualization. **Sofia Gomes:** Conceptualization; methodology; writing—review and editing. **Vincent Merckx:** Conceptualization; methodology; investigation; writing—review and editing; supervision; funding acquisition.

ACKNOWLEDGEMENTS

We would like to thank, in no particular order, Melvin Stigter for his help in selecting sites in het Vliegenbos, Edwin Peterse for his help sampling underneath the pavement on the Keizersgracht, and Geert Timmermans and Hans Kaljee for their help in conceptualizing this study. Moreover, we would like to thank Betsie Voetdijk, Nafiessa Ibrahim and Megan Verhagen for their help with the DNA extraction and library preparation, and Annemiek van Dijke for her work on the soil property analyses. This work was funded by an European Research Council (ERC) consolidator grant (MIXOTROPH, project ref.: 101045057) to C.T.V. and V.S.F.T.M.

CONFLICT OF INTEREST STATEMENT

We declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence reads files are openly available in the Sequence Read Archive (of the National Center for Biotechnology Information, NCBI) at https://www.ncbi.nlm.nih.gov/sra, reference number PRJNA1195707.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Verbeek, C. T., Gomes, S. I. F., & Merckx, V. S. T. F. (2025). Arbuscular mycorrhiza in the urban jungle: *Glomeromycotina* communities of the dominant city tree across Amsterdam. *Plants, People, Planet*, 1–17. https://doi.org/10.1002/ppp3.10634