Identification of Ectomycorrhizae in Dipterocarp Roots using DNA Metabarcoding in Tropical Urban Parks

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Research Article

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Abstract

Ectomycorrhizae (ECM) are important symbionts for multiple host plants. This study used morphology and DNA metabarcoding to identify ECM in Dipterocarpaceae, the dominant tree family of Southeast Asian forests. ECM fruiting bodies were first visually documented and identified across five urban parks in Singapore. Under host Dipterocarp *Hopea odorata* trees, 50 soil and root samples were collected. This was done together with another 50 root samples taken from ten species of host Dipterocarp seedlings at the Singapore Botanic Gardens' Plant Resource Centre nursery. Eight genera of ECM were found in parks, one was identified to species level and three genera were only found from fungal Amplicon Sequence Variants (ASVs) using DNA metabarcoding. Although the nursery had more Dipterocarp species, only four genera of ECM were present. ECM communities differed slightly across host species, but not host genera. *Tomentella* spp. were the most common ECM found in parks and Dipterocarp seedlings.

Introduction

Soil underpins the health of natural ecosystems and people's livelihoods. It is also the foundation that supports biodiversity and its ecosystem services (Wagg et al., 2014; Wall et al., 2015). Unfortunately, threats such as climate change, habitat loss, urban development, poor agricultural practices, and anthropogenic pollutants have impacted soil health severely (Tibbett et al., 2020). The deterioration of soil health would adversely affect the numerous taxa that lives in the soil, such as invertebrates, bacteria, archaea, protists, and fungi (Decaëns, 2010). Many with important ecosystem functions would disappear without ever being identified (Cameron et al., 2018). Fortunately, soil is receiving increasing attention because of its role in carbon sequestration, agricultural productivity, and ecosystem services (Bhattacharyya et al., 2022; Brevik et al., 2018). Many of these services are provided by the beneficial symbiotic fungi of plants, the ectomycorrhizae (ECM).

ECM contribute to the survival of its hosts and stabilise soil structures. They grow large networks of hyphae known as mycelia that transport nutrients to the host plant directly in exchange for photosynthates (Figueiredo et al., 2021). ECM protect plant hosts from toxic chemicals, pathogens, and predators (Lalaymia et al., 2014; Schausberger et al., 2012). ECM can make up more than a quarter of living biomass in soils, they lock carbon belowground and protect soil from erosion (Averill & Hawkes, 2016; Frey, 2019).

Despite the ubiquity of ECM, they face increasing threats that will undermine their abilities to provide ecosystem services. Habitat destruction is the ultimate challenge for the conservation of ECM(Nic Lughadha et al., 2020) and this includes habitat fragmentation which exposes sensitive fungal species to harsher environments (Boeraeve *et al.*, 2016). When natural ecosystems are replaced by human developments, the original soil and belowground biodiversity are significantly altered (Rusterholz et al., 2020). Poor agricultural practices such as excessive use of fertilizers and pesticides change these networks (Vincent & Declerck, 2021). Chemical pollution from farms severely affects ECM (Arnolds, 1991). These threats will cascade through the food web, depriving arthropods and other fauna that

depend on fungi as a food source (Fitter & Garbaye, 1994; Suz et al., 2021). With ECM playing critical roles in soil integrity, and the many-pronged anthropogenic threats known thus far, many studies exploring ECM emerged in the last few decades.

ECM research was mainly conducted in temperate regions. Research has shown complex relationships of ECM with multiple taxa, including its host. Not only do ECM channel mineral nutrition to their plant hosts, but other herbaceous parasitic plants such as temperate myco-heterotrophs also tap into the mycelial network (Cullings et al., 1996; Feild & Brodribb, 2005). The fungal network helps temperate trees to withstand droughts (Claire, 2019). Responses to environmental changes have been documented using isotopes to track the movement of carbon, nitrogen and phosphorous (Pellitier et al., 2021). New technologies are being used to visually capture the network, create global models, and measure the percentage colonisation of fungi on plant roots (Freschet et al., 2021; Wu et al., 2022). Unlike the temperate areas and its new technologies, there are much fewer studies in the tropics (Corrales et al., 2018).

Tropical ECM are associated with the Dipterocarpaceae, Fabaceae and many others (Brearley, 2011; Founoune *et al.*, 2002). Like their temperate fungal counterparts, these associations are found in polyphyletic plant families. Although identifications of ectomycorrhiza, cultivation of ECM fungi with their tree hosts and their benefits have been reported with on-going research, physiological and ecological approaches are still lacking (Brearley, 2011; Brearley et al., 2016; Kaewgrajang et al., 2013; S. S. Lee et al., 2008). As there is greater diversity of plant taxa in the tropics, ECM may display different levels of host specificity (Peay et al., 2015a). One of the most studied ECM are those associated with Dipterocarps in Southeast Asia (Corrales et al, 2022).

Dipterocarps are the dominant tree family in Southeast Asian Forest, characterised by their winged fruits (Appanah *et al.*, 1998; Ashton et al., 2021). Those nearest to the equator undergo masting, a synchronised flowering and fruiting period for many Dipterocarp species (Kurten et al., 2018). The trees are mainly pollinated by insects such as thrips and seeds can be dispersed by wind and water (Momose et al., 1998). Masting also provides food for many animals (Nakagawa et al., 2003). Local communities also used them as butter, as the seeds are rich in oil and fats (Seirbet, 1996). Seeds are recalcitrant and germinate quickly when environmental conditions are right (Umarani et al. 2015). Dipterocarps are one of the most threatened plant family due to deforestation, legal as well as illegal harvest of its valuable timber. In situ conservation is a major challenge, while ex situ conservation efforts are dampened by irregular mast fruiting and recalcitrant seeds- both leading to shortage of seedlings. Also, lack of information on reforestation methodologies, especially on ECM relationships, among others complicate matters further.

Dipterocarp ECM belong to the same families as those in the temperate forests of Amanitaceae, Boletaceae, Russulaceae, Thelophraceae and studies into their identities are still ongoing (Essene et al., 2017; Segnitz et al., 2020). The research in tropical Asia has shown mixed results in understanding ECM host specificity and benefits. Research shows that composition of soil affects what species of fungi grow on the trees (Essene et al., 2017). The mycorrhizal strategies used in dipterocarp forests also determine

the community structure of other soil microbes (Cowan et al., 2023). In tree nurseries, Dipterocarps inoculated with ECM were tested under different light conditions with varying results for different species such as the *Shorea* spp. (Brearley et al., 2016; Ramadhani et al., 2018; Rimbun & Chaidir, 2016; Saner et al., 2011). Such studies should be conducted more frequently in tropical urban environments like Singapore.

Singapore is an island nation located near the equator in Southeast Asia. It is highly urbanised but has substantial areas of greenery in remaining forest fragments and parks. In Singapore, identities of fruiting bodies of ECM were compiled as part of the major fungal surveys (Corner & Bas, 1962; Lee, 2019; Tan et al., 2011; Tham & Watling, 2017). The fungal biodiversity list is incomplete as only fungi with visible fruiting bodies have been captured and DNA work was only done sporadically. For the DNA identification of ectomycorrhiza, the nuclear internal transcribed spacer (ITS) is generally sequenced. This region contains the linear order of 18S small subunit, 5.8S, and 28S large subunit with ITS1 and ITS2 sub-regions in between (Gardes & Bruns, 1993; Janowski et al., 2019; Joos et al., 2020; Manter & Vivanco, 2007).

There is much needed research into ECM in tropical urban city such as Singapore to better harness nature-based climate solutions and mitigate climate change. The first step to understanding any ecosystem is to identify the organisms. This is also true for urban parks. Urban parks are known to provide essential ecosystem services in cities and such services vary depending on the biodiversity richness (Mexia et al., 2018). Nurseries which provide the trees to urban parks need to be investigated as well. According to Trappe (2003), nurseries are sources of ECM for trees that grow in parks. The introduction of ECM into parks through its plant hosts can shape its belowground biodiversity such as rhizosphere bacteria communities (Cowan et al, 2023). After identification of these ECM, the ecological functions can be interpreted in future studies. It is also important to understand the factors that affect the ECM communities such as soil properties. With this understanding, we can inoculate specific ECM in urban parks to maximise its ecosystem services. Given the importance of Dipterocarps to the eco-region, this study aimed to answer two main questions: 1) What are the ECM found in Dipterocarps planted in parks, *Hopea odorata* in particular? 2) What are the ECM found in Dipterocarp seedlings grown in a nursery?

Materials and Methods

Selection of Parks and Trees

In Singapore, *Hopea odorata* trees are commonly planted along selected streetscape or in some local parks, thus making it ideal as the target species for sampling. It is a Dipterocarp tree from dry seasonal forests of Peninsular Malaysia and Indo China (Appanah *et al.*, 1998). The five main sites chosen were Bishan Ang Mo Kio Park (BA), Bukit Batok Nature Park (BB), Singapore Botanic Gardens (BG), Yishun Park (YP) and Zheng Hua Nature Park (ZH). These parks were chosen as they had numerous *Hopea*

odorata trees planted. Two separate locations from every park were selected. Each location had at least five conspecific trees.

Sampling fruiting bodies, roots and soil

When ECM fruiting bodies appeared near the tree in these parks, depending on the number of fruiting bodies found, one to five fruiting bodies of each species would be collected and brought back to the laboratory in a Ziplock bag inside a cooler box. As for root and soil samples, areas with a maximum radius of 2 m were demarcated around each of the five chosen trees in the location. Rubber gloves, hand spade, secateurs and the PVC pipes were sterilised with ethanol (70%) and a clean C-fold towel before every sample. Five samples were then taken from random points found within the areas. Cylinder soil cores (10 cm deep and 4 cm in diameter) were dug using a modified Polyvinyl Chloride (PVC) pipe. An extra soil core was also taken near the original core to measure soil properties. Feeder roots of *Hopea odorata* were identified by tracing it to the larger roots of the main tree and 15 cm of the roots were cut with secateurs. The root and soil samples were stored similarly as the fruiting bodies. A map with locations of fruiting bodies was plotted with ArcGIS (Esri Inc., 2021).

Sampling Seedlings at Singapore Botanic Gardens Nursery

As for existing seedlings at the Singapore Botanic Gardens nursery, ten species of potted Dipterocarps from various origins and ages were sampled (Table 1). Five pots of each species were randomly selected. They were grown under shade and watered daily in the nursery. Root sampling was done in the sheltered nursery work shed over a clean plastic sheet. Before each sample was taken, the plastic sheet, rubber gloves and secateurs were sprayed with ethanol (70%). A bucket was also sterilised and soaked with 10% bleach solution. Each plant was taken out of its pot and washed under clean water over the bucket to remove soil from its roots. Three sections of the seedlings' terminal roots were cut at 10 cm from the tip. The roots were then placed in the Ziplock bag to be brought back to the laboratory later.

Table 1
List of the ten Dipterocarp seedling species sampled at the Singapore Botanic Gardens nursery with their dates sown.

Dipterocarp species	Date sown
Dipterocarpus intricatus (Di)	6-May-19
Dipterocarpus sublamellatus (Ds)	9-Mar-20
Hopea cf. *apiculata* (Ha)	8-Aug-19
Hopea nutans (Hn)	1-Nov-19
Shorea foxworthyi (Sf)	3-Oct-19
Shorea gibbosa (Sg)	3-Oct-19
Shorea singkawang (Ssi)	2-Dec-19
Shorea cf. *sumatrana* (Ssu)	2-Dec-19
Vatica bella (Vb)	30-Dec-19
Vatica cf. *perakensis* (Vp)	27-Oct-14

Soil study

pH

Each soil core was air dried and sieved for particles under 2000 microns. For the pH test, 10 g of soil were mixed with 10 ml of Milli-Q water in Duran borosilicate bottles. The Ohaus pH meter was calibrated at the start of each use. Once the electrode (made of a glass rod) had been calibrated and ready, it was fully submerged into the soil suspension. The reading was recorded automatically under room temperature of 22^{o}C. This was repeated for all the samples and the electrode was cleaned with Milli-Q water and Kimwipes each time.

Soil Particle Size

To test for soil particle size, another 50 g of sieved soil were mixed with 100 ml of 5% sodium hexametaphosphate solution in the borosilicate bottles (Karkanis *et al*., 1991). The bottles were placed on an automatic shaker overnight for 12 to 16 hours. After shaking, the soil suspension was poured into a 1 litre measuring cylinder and topped up with water until the 1000 ml mark. The top of the measuring cylinder was sealed with parafilm before being tilted upside down ten times. After 40s, the first hydrometer reading was taken. The second reading was recorded seven hours later. A blank hydrometer

reading was taken for 100 ml 5% sodium hexametaphosphate solution mixed with 900 ml water. Below was the summary of calculations with the readings:

$$\text{Percentage of Clay and Silt} = \frac{\text{First Reading-Blank}}{0.05\ \text{kg}}$$

$$\text{Percentage of Sand} = 1\text{- Percentage of Clay and Silt}$$

$$\text{Percentage of Clay} = \frac{\text{Second Reading-Blank}}{0.05\ \text{kg}}$$

$$\text{Percentage of Silt} = \text{Percentage of Clay and Silt-Percentage of Clay}$$

Identification of ECM Fruiting Bodies

In the laboratory, the fruiting bodies of ECM were dissected and photographed on a black velvet cloth. Whenever possible, spore prints were taken and their microscopic features such as spores and hyphae were documented under Differential Interference Contrast (DIC) microscope (Olympus BX53). Their lengths and widths were measured. After analysis, the fruiting bodies were dehydrated in an air dryer over night at 60°C. The dried fruiting bodies were stored in an airtight container for preservation.

Root Microscopy

For fresh roots, six root tips of each sample were rinsed under water and removed for DNA analysis first. The remaining nearby root tips with similar morphology were then cleaned thoroughly over running water before being placed in petri dishes. The Leica stereo microscope (M205 FA) captured photos of the root on white paper. After the photos were taken, razor blades were used to make cross sections of the root. Only the thinnest section was observed under the DIC microscope. The type of mantle and hartig net were documented and later classified following the definition on DEEMY, an information catalogue for the characterization and determination of ECM (Rambold, & Agerer, 1997).

DNA Metabarcoding

Root samples were placed in 2 ml tubes with two metal beads added and stored in a -80°C freezer for cryopreservation. When samples were ready for DNA extraction, they were inserted inside the Qiagen Tissue Lyser adapters for another half an hour in the freezer. While waiting, a master extraction solution was prepared, 300 µl CTAB (Cetrimonium bromide) buffer, 1 µl RNAase enzyme and 3 µl of BME (Beta-mercaptoethanol) were mixed for each sample (Janowski et al., 2019). The adapters were then placed inside the Qiagen Tissue Lyser for one minute at maximum speed. For samples that were not lysed properly, they underwent another round of mechanical lysis or further grinding using a sterile rod. The master extraction solution was added immediately into the sample after mechanical lysis. Tubes were

vortexed and spun down before being incubated in a 65℃ heat block for 15min. After samples cooled down, 300 µl Chloroform was added inside each sample, vortexed and centrifuged at maximum speed for 5 min. Inside a fume hood, the supernatant layer of the sample was pipetted and transferred to another 1.5 ml tube. 210 µl of isopropanol was added into transferred tube and mixed well by inverting it slowly and continuously. The 1.5 ml tube had to be stored for at least 20 minutes in the − 20℃ freezer before the clean-up step. When the sample was removed from the freezer, it was centrifuged at maximum speed again. The solution in the tube was removed, leaving the solid DNA pellet at the bottom. The pellet in the tube was washed with 500 µl of ethanol (70%). The clean-up step was repeated except the pellet was dissolved in 100 µl TE buffer at the end of extraction. The solution was stored in -20℃ freezer for PCR.

For the first PCR step, each tube was mixed with 1 µl 10× Taq Buffer, 1 µl $MgSO_4$, 1 µl of 2mM dNTP, 4.96 µl of water, 1 µl sample DNA, 0.04 µl of Taq polymerase, 0.5 µl Forward ITS1F primer and 0.5 µl Reverse LR21 primer (Gardes & Bruns, 1993). All primers used in PCRs were listed in Table 2. The tubes underwent 14 cycles of 30s at 95℃, followed by 30s at annealing temperature of 55℃ and 45s at 72℃. For the second PCR, the samples from the first round were diluted a hundred times in water. The same reagents were added into the second-round tubes except with a different forward ITS1Fa primer and the reverse ITS2 primer attached with the Illumina overhangs. The tubes underwent 32 cycles of 30 s at 95℃, followed by 30 s at annealing temperature of 55℃ and 10s at 72℃. To check for the presence of desired DNA bands via gel electrophoresis, the gel was prepared by adding 50 ml of TAE buffer to 0.5 g of Agarose A powder and microwaved for a minute. 2 µl of SYBR safe dye was pipetted into the gel solution before being poured into the caste. After half an hour, the gel was settled in the electrophoresis machine. 1.5 µl of 1 kb Gene ruler ladder was used. 1 µl of loading dye and 1.5 µl of DNA sample was pipetted into individual wells. The gel ran for 20 minutes under 120 V. After confirmation of the bands, the third PCR was conducted where three times the volume of reagents and DNA template were used. Primers were replaced with the Illumina adapters of 501–508 and 701–712. The tubes underwent 35 cycles of 30s at 95℃, followed by 30s at annealing temperature of 60℃ and 11s at 72℃. Gel electrophoresis was repeated for the third PCR step. The relative intensity value of the gel bands for each DNA sample was recorded and used for normalisation. Before normalisation, high sensitivity Qubit measured the exact concentrations of the samples with the highest and lowest gel band intensities for each round of DNA extraction. The concentrations of both samples were averaged and applied to obtain the relative concentrations of the remaining samples. The volume of each sample was calculated to standardise to the same mass of DNA. Samples were then pooled and cleaned with 0.9 volume of Polyethylene Glycol solution and ethanol (70 %). The purified DNA as dissolved in 200 µl of water and stored at -20℃ freezer before being sequenced by Illumina iSeq 100 System. The forward and reverse DNA sequences of 150 bp were merged to become Amplicon Sequence Variants (ASVs) when grouped into putative species based on significant abundance with R package ‘dada2 (version 1.22.0)’ (Callahan et al., 2016) and UNITE database (Kõljalg et al., 2005; UNITE Community, 2017). According to Forster et al. (2019), ASVs may overestimate putative species richness as ASVs may represent variants of the same species. Thus, for this DNA metabarcoding study, the ECM were grouped into genera to avoid this overestimation. They were then analysed with R package ‘phyloseq’ (McMurdie & Holmes, 2013).

Table 2
Primers used in PCRs

Primers	Sequences
ITS1F	CTTGGTCATTTAGAGGAAGTAAAAGT
LR21	ACTTCAAGCGTTTCCCTTT
ITS1Fa	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTAACAAGGTTTCCGTAGG
ITS2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGAGCCAAGAGATCCGTTG

Results

Visual Sightings of Fruiting Bodies at Parks

Based on visual observations between mid-June 2021 to mid-January 2022, five genera of ECM were recorded at the five parks surveyed. The locations of these fruiting bodies were marked in Fig. 1. Many of them are close to one another. Only one species of ECM could be identified from the fruiting body while others remained at the genus level. The ECM identified were: *Clavulina* sp., *Lactarius* sp., *Russula* sp., *Scleroderma* sp. and *Spongispora temasekensis*.

The macro and microscopic features were recorded (Table 3). The mycelium under the fruiting bodies were observed only in *Russula* sp. and *Spongispora temasekensis*. Most identifications were also confirmed by DNA analysis with their closest sequence IDs, except *Clavulina* sp.

The forward and reverse reads of *Spongispora temasekensis* were uploaded separately on GenBank as they could not be assembled. All ECM of this study had distinct colours, shapes and textures which made them easy to differentiate for both macro and microscopic features. *Spongispora temasekensis* had pores instead of gills and produced the largest spores among the ECM in this study. The spores of *Clavulina* sp. had no ornamentation unlike the other ECM. *Lactarius* sp. and *Russula* sp. had similar cystidia present.

In Fig. 2, Yishun Park had the least number of ECM genera identified from fruiting body. All ECM were found at Bukit Batok Nature Park. *Spongispora temasekensis* was the rarest ECM fruiting body, present only at Singapore Botanic Gardens and Bukit Batok Nature Park. *Lactarius* sp., *Russula* sp. and *Scleroderma* sp. were the most common and observed in at least four out of five parks surveyed.

Root Morphotypes at Parks

There were 50 root samples taken from *Hopea odorata* trees across the five parks in Singapore. The prominent root morphotypes were classified using DEEMY in the table below based on their dominant colour, including their location and ECM present. Pictures of these distinct morphotypes were taken with

the stererozoom and their cross sections captured with the DIC to aid identification. The black morphotype was found in most roots. From the sequencing data, the ECM found in the black morphotype belonged to *Tomentella* spp., based on its relative abundance. The presence of clamp connections and pseudoparenchyma mantle were the usual characters in the black morphotype and confirmed its identification (Jakucs & Erős-Honti, 2008). The brown morphotype harboured the *Russula* spp., with cystidia present, like in its fruiting body. It also had *Tomentella* spp. present. Only *Tomentella* spp. was found in the orange morphotype, however, there were no clamp connections present. The orange morphotype likely belonged to another ECM species that DNA metabarcoding could not match. Other morphotypes had three or more ECM of varying relative abundances, hence it was difficult to discern which species they belonged to.

ECM identified by DNA Metabarcoding at Parks

The maximum number of (Amplicon Sequence Variants) ASVs detected in a single sample was 14 at Singapore Botanic Gardens location 1 and Yishun Park location 2. Six samples had only one ASV. Kruskal-Wallis non-parametric statistical test indicated there was no significant difference in the number of ASVs found between locations (Kruskal-Wallis chi-squared = 14.372, df = 9, p-value = 0.1097). For the fungal communities sequenced and detected in each sample (Fig. 3), there were 123 ASVs in total. These ASVs comprised mainly of ECM fungi. Other types included endophytes, pathogens, and saprotrophs, all classified as non-ECM. Non-ECM also included other fungi that could not be identified to the genus level. ECM with more than one ASV belonging to that genus were written as spp.. The most common fungal genera found in the samples were: *Russula* spp., *Lactarius* spp., *Tomentella* spp. and *Scleroderma* spp.. The location that was dominated by *Russula* spp. was Singapore Botanic Gardens location 1. *Lactarius* spp. occupied Bishan Ang Mo Kio Park location 1. *Tomentella* spp. was found in most parks, with high abundances in Zheng Hua Nature Park location 1. Yishun Park had high proportions of *Scleroderma* spp.. Other ECM ASVs included *Clavulina* sp., *Lactifluus* sp. and *Inocybe* sp. which were much rarer.

Analysis of Similarities (ANOSIM) was performed on the fungal communities After 999 permutations, the R value is 0.202 and the p value is 0.001, hence there was a significant difference in the fungal community structure between park locations, but it was small.

Using the same bipartite network analysis (Fig. 4), three more genera of ECM were found with DNA metabarcoding, bringing the total count to eight genera associated with *Hopea odorata* found in parks surveyed. Compared to visual sightings, Yishun Park had the greatest number of ECM found, including one *Inocybe* sp.. *Lactifluus* sp. was found only at the Singapore Botanic Gardens. *Clavulina* sp. was observed visually at Zheng Hua Nature Park, Bishan Ang Mo Kio Park and Bukit Batok Nature Park but could only be detected at Yishun Park with DNA metabarcoding. Like the fruiting bodies, *Spongispora temasekensis* unassembled reads were only found through the sequence files for Bukit Batok Nature Park. However, it was not observed in the sequence files of Singapore Botanic Gardens. Zheng Hua

Nature Park and Bishan Ang Mo Kio Park had the least number of ECM, three genera were only identified there.

Soil Tests

Across the parks, the soil samples were mostly acidic, between pH 4 to 8. Few samples were slightly basic, above pH 7. When soil texture boxplots were plotted for different locations, sand had the highest proportion in all samples. The highest value recorded for sand was 90% while the lowest value was 76%. For silt and clay, their proportions ranged from 0–16%. This showed most locations were filled with sand. As for percentages of sand, silt and clay, the results also showed that soil textures did differ significantly at various locations. Table 5 is a summary of the soil results.

Table 5
Summary of Kruskal-Wallis test results for soil properties at different locations.

Soil Properties	Kruskal-Wallis chi-squared value	df	p-value
pH	33.639	9	< 0.001
sand	26.791	9	< 0.050
silt	32.041	9	< 0.001
clay	23.127	9	< 0.050

Factors affecting Fungal Communities at Parks

Canonical correspondence analysis was performed on the fungal communities with geographical coordinates of samples, locations, and soil properties. After the analysis, model selection was then applied to test the significance of adding or removing variables in the model for fungal communities. This determined which variables were key in shaping the fungal community. Forward and backward stepwise simplification of the model showed that adding location to the model produced the most significant result (D.f = 9, AIC = 154.88, F statistic = 1.2632, p-value = 0.005). Therefore, location was a factor in shaping the ECM communities in Singapore parks from the results.

Root Morphotypes at Singapore Botanic Gardens Nursery

Like the park study, 50 root samples were taken across the different Dipterocarp seedlings at the Singapore Botanic Gardens. There were only two prominent root colour morphotypes: black and black/white (Table 6).

Table 6
Nursery ECM colour root morphotypes and its samples. irregular: ir, monopodial: mo, smooth: sm, stringy: st, woolly: wo, pseudoparenchyma: ps, prosenchyma: pr, paraepidermal: pa, periepidermal: pe, present: +, absent: -, *Lactarius* sp.: La, *Russula* sp.: Ru, *Scleroderma* spp.: Sc, *Tomentella* spp.: To.

ECM colour morphotype	black	black/white
Shape	ir/mo/-	ir
Texture	sm/st/wo	st/wo
Rhizomorph	+/-	-
Mantle Surface	ps/pr/-	pr/-
Mantle	ps/pr/-	pr/-
Hartig Net	pa/pe/-	pa/-
Clamp Connection	+/-	+/-
Cystida	-	-
Samples	Most samples	Ssi_1
		Ssi_2
		Ssi_4
		Ssi_5
		Ssu_2
ECM ASVs	La	La
	Ru	Ru
	Sc	Sc
	To	To

Closer images of the roots and their cross section were also taken below (Fig. 5, 6, 7). Some were stained with lactophenol cotton blue for clearer inspection under microscope. Clamp connections were very abundant in the black morphotype, indicating prevalence of *Tomentella* spp.. No cystidia was found in the roots despite *Russula* spp. and *Lactarius* spp. being present.

ECM identified by DNA Metabarcoding at the Singapore Botanic Gardens Nursery

Shorea sumatrana and *Shorea singkawang* seedlings had the highest number of ASVs of 20 and much more ASVs in their samples unlike the other Dipterocarp species (Fig. 8). Twelve seedlings had only one ASV detected. Kruskal-Wallis non-parametric statistical test showed there was a significant difference in the number of ASVs found between species (Kruskal-Wallis chi-squared = 21.194, df = 9, p-value = 0.01181). When the Dipterocarp hosts were classified to the genus level instead, there was no significant difference between genera (Kruskal-Wallis chi-squared = 0.43989, df = 3, p-value = 0.9319).

For the different genera found in each seedling at the Singapore Botanic Gardens, there were 120 ASVs found. Most seedlings, regardless of host species, were occupied by the *Tomentella* spp.. *Dipterocarpus sublamellatus* had the smallest proportion of ECM DNA present among host species. *Shorea sumatrana* and *Shorea singkawang* had higher relative abundances of other ECM such as *Lactarius* sp., *Russula* sp. and *Scleroderma* spp. compared to other host species.

Bray Curtis Non-metric Multidimensional Scaling (NMDS) plots were created for the fungal communities classified into host species and genera. Both plots did not show a clear distinction in fungal communities among the different taxa. There were many overlaps between the genera and species. Only for the NMDS plot under host species (Fig. 10), points of *Vatica bella* clustered closely to one another and did not overlap with *Dipterocarpus sublamellatus*. Other host species had wider distributions of points.

ANOSIM was done for the seedlings for both host genera and species levels. The R value at the host species level is 0.1657 and the p-value was 0.003, thus communities were significantly different from each other, but the difference was small like those found in park locations. When done at the genus level, the R statistic value was 0.01973 and had a p value of 0.237. This meant that among Dipterocarp genera, the fungal communities were not different.

Figure 11 shows a summary of ECM present in the different Dipterocarp host species. *Dipterocarpus intricatus*, *Shorea gibbosa* and *Vatica bella* had only the *Tomentella* spp. present. *Scleroderma* spp. was the second most common while *Russula* sp. and *Lactarius* sp. were only present in two host species. *Shorea sumatrana* had all four ECM present in its roots, making it the most promiscuous host Dipterocarp species.

Discussion

Visual Sightings of ECM at Parks

This study was one of the few investigations into urban park ECM associated with *Hopea odorata* in Singapore using both visual sightings and DNA metabarcoding. From visual sightings, the bipartite network showed that *Hopea odorata* can have more than one species of ectomycorrhiza. The map also showed that ECM co-existed with one another under the same host. Based on the fruiting body morphological characters, genus level identifications were possible as the fungi were distinct. Characters of the fruiting bodies need to be analysed by fungal taxonomists to confirm species identification. When using the ‘dada2’ package, the *Clavulina* sp. fruiting body did not have congener ASVs matched to it. This

could explain the absence of *Clavulina* sp. in parks where its fruiting bodies were found when DNA metabarcoding was used. The DNA metabarcoding primers in this study might not have been suitable to find *Clavulina* sp. Apart from *Spongispora temasekensis*, DNA only gave genus identifications as there were insufficient species sequences to match online. *Lactarius* sp. and *Russula* sp. matched at more than 90% to ECM in Thailand (Kaewgrajang et al., 2013; Phosri et al., 2012) where it is part of the natural distribution of *Hopea odorata*. None of the fruiting bodies recorded belonged to Ascomycota phylum. Hence, visual sightings of fruiting bodies could only serve as a preliminary study into the ECM of Dipterocarps. From visual sightings, there were differences in the number of genera spotted at various parks. These differences could be attributed to limited sampling and other factors. Some ECM that might not have emerged yet were not recorded. There should have been greater numbers of ECM fruiting bodies found in Singapore as there were many unexplored parks with *Hopea odorata* trees planted, like Pasir Ris Park.

Root Morphotypes at Parks

The root morphotypes were much harder to differentiate based on taxa compared to the fruiting bodies found throughout the parks. Most roots had the black morphotype due to the prevalence of *Tomentella* spp.. *Tomentella* spp. do not have visible, erect fruiting bodies as they are corticioid fungi (smooth, effused and flat against substrate). Despite this, *Tomentella* spp. could be the most abundant ECM for Singapore's *Hopea odorata* trees based on its morphotype abundance and DNA metabarcoding. The genus has a global distribution and occupies many tree hosts in forest habitats (Jakucs & Erős-Honti, 2008). The reproductive ecology of *Tomentella* spp. could have made it common in parks as many animals harbour and disperse its viable spores for one species of *Tomentella*, (Lilleskov & Bruns, 2017), allowing it to spread to multiple locations compared to other ECM that rely on wind dispersal. A population genetic study and fauna sampling could confirm its distribution. Based on root morphotype alone, it was hard to discern the other ECM. Only the brown morphotype consistently had the *Russula* spp. and *Tomentella* spp. In the root's cross sections, *Tomentella* spp. was distinguished by its compact pseudoparenchyma mantle surface. For mantles that had prosenchyma, those probably belonged to other genera of ECM. The hartig net character was not consistent among samples and hard to tell the morphotypes apart. Presence of cystidia, like those found in the fruiting bodies of ectomycorrhiza, was the common character for the *Russula* spp. DNA also might not have picked out the actual ECM that occupied the orange morphotype. Thus, relying on DNA metabarcoding alone to determine root morphotypes may not be feasible for all ECM. From basic root morphological identification, root colour morphotypes could only suggest the presence of common ECM such as the *Tomentella* spp. and *Russula* spp.

ECM ASVs found at Parks

When ASV richness was compared between locations in parks, there was no significant difference, each location thus has similar ASV richness but in different proportions and fungal genera. Majority of

samples were occupied by ECM. Locations were dominated by different ECM genera, this showed that *Hopea odorata* could rely on a few genera of ECM instead of a single ECM genus or species. Host Dipterocarps were not symbiont specific as mentioned in a previous study (Peay et al., 2015b). Dominance of certain ECM could be due to the different origins of host trees before they were planted or the historical legacy of ECM in parks (Lofgren et al., 2018). Before the trees were planted in parks, they could have also hosted the ECM from the nursery which continue to dominate in the parks (Stenström & Ek, 1990). As many rare ASVs were found in samples, Non-metric Multidimensional Scaling (NMDS) could not be applied to the samples. Even though DNA metabarcoding could find three more genera of ECM that occupied *Hopea odorata* roots, it could not detect visible fungi like the *Clavulina* sp. in other locations where its fruiting bodies were present at. The R package and UNITE fungal database need to be updated to include other ECM like *Spongispora temasekensis*. Given the weaknesses of both visual inspection and DNA metabarcoding methods, future studies should continue to use both methods together for a more comprehensive investigation of the ECM community.

The variable ITS region could have resulted in some unidentified ECM. The primers used in this study were inferred from literature and confirmed with other ECM species. However, the primers did not have degenerate bases. Thus, some ECM might have been filtered out due to primer and region bias within the ITS region. The ITS region was one of the most sequenced for fungal DNA metabarcoding, but arbuscular mycorrhiza and other types of fungi were often left out as they possibly required other specific primers (Lee et al., 2008). Arbuscular mycorrhizas are harder to find using the microscope as they are within roots. There is a possibility that the ECM could have outcompeted the arbuscular mycorrhizas in most sections of the roots. Arbuscular mycorrhizas could have offered a different perspective about host symbiont specific relationships. Moreover, DNA metabarcoding might still be in its infancy to provide fungal species identification without sufficient species barcodes available online.

Factors affecting Park ECM Communities

The results from ANOSIM and model selection showed that the fungal communities between locations are slightly different from one another. Factors such as pH and soil texture did not have the same effect despite being significantly different in various locations. CCA also did not indicate any strong correlation between certain ASVs to the soil variables. Therefore, there was a small difference in fungal communities between locations, but not for soil pH and soil texture. Location differences might constitute a variety of factors beyond the edaphic properties. This included climatic, historical, and anthropogenic differences that could not be accounted for in this study as these were outside the scope. Differences in tree management among locations could lead to fungal community differences in terms of leaf litter or fertiliser application. There could also be leaching from urban structures into nearby soils. Other plants that do not host ECM may affect the ECM community through root exudates (Wang et al., 2022). This challenge can be dealt with by identifying the plant roots in samples using DNA and confirming the results with plants nearby. The method will also guarantee the ECM host roots were properly sampled by DNA. Soil texture might not affect the fungal communities as previously thought as most location

samples were sandy despite being different from one another. Hence, it would have been difficult to find major differences in the ECM community structure based on soil properties alone. Like the visual observations, limited soil and root sampling might have also only picked up the few dominant ECM that shaped the community structure. Increased sampling efforts in places like streetscapes and forests or other factors might provide a better insight as to why the ECM communities showed differences in locations.

ECM of Nursery Dipterocarp Seedling Species

At the Singapore Botanic Gardens, there were more host Dipterocarp species sampled but it only detected four genera of ECM compared to the single tree species investigated at various parks in Singapore. All four genera were the same ones found in parks as well. This study showed that host specificity might not have a strong effect in the ECM communities found in Dipterocarp seedlings at a young age. Although *Vatica* cf. *perakensis* was much older than the other seedlings, it did not show any difference with other host species. As the seedlings were all sampled at the Singapore Botanic Gardens nursery, the same soil used and proximity of seedlings despite being in separate pots could have encouraged the dominance of the *Tomentella* spp.. In such environments, animals, especially small invertebrates, could spread the fungal spores. From the root morphotypes found in seedlings, it was also less diverse as expected although they were different species. The other black/white morphotypes seen in the seedlings came from the *Shorea sumatrana* and *Shorea singkawang* which had the *Lactarius* sp., *Russula* sp. and *Scleroderma* spp.. Cystidia were not present in the black/white morphotypes, but the mantle did show prosenchyma tissue like those found in the park ECM. Moreover, ASV richness were higher for these two host species. This meant for certain hosts, they were able to take advantage of more ECM species other than *Tomentella* spp. Host genera did not have any significant effect on the fungal communities in terms of ASV richness.

As for the NMDS, the plot did not indicate any distinct groups of fungal communities in the seedling species or genera. However, ANOSIM found that there were slight differences between host species, most probably because the ellipses of *Vatica bella* and *Dipterocarpus sublamellatus* did not overlap. Therefore, there might be slight differences between the fungal communities for host species only. ECM have been thought to be host specific, but this experiment showed, at least for seedlings, most hosted *Tomentella* sp.. *Shorea sumatrana* and *Shorea singkawang* were promiscuous with multiple symbiotic partners, according to the root morphotypes and DNA metabarcoding. Unlike some host species with restricted distributions, the most promiscuous host species *Shorea sumatrana* can be found in Peninsular Thailand, Peninsular Malaysia, Sumatra (Ashton, 1982). A wide species distribution could have allowed individuals to naturally accept more ECM species. More studies need to be done in this area.

Conclusion

To conclude, this project was one of the few urban park ECM community studies using DNA metabarcoding and served as baseline study on ECM currently present in *Hopea odorata* trees in Singapore (Ngiam J.J., pers. comm., 2021). Eight ECM genera in Singapore parks were found with visual observations and DNA metabarcoding. Both methods were needed to understand ECM distribution. Although the study suggested location specificity of ECM instead of soil properties, more sampling is needed and other attributes of location specificity such as local climate and humus measured. The nursery Dipterocarp seedlings were not as host specific as expected. The ten species shared four ECM genera. *Tomentella* spp. were common in both parks and nursery seedlings. More investigations into its biology will explain its widespread distribution. Results from further investigations will guide reforestation and fungal species recovery efforts in Singapore and the region. By assessing the species of ECM in the nursery, the nursery seedlings planted in urban environments can be monitored to ensure they are resilient, increasing the success of ecosystem restoration and rehabilitation. Therefore, this project established preliminary information on the urban ECM in Singapore which will benefit future research into these important fungi and its benefits to restoration efforts.

Declarations

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Authors declare that they have no known competing financial interests or personal relationships that could have affected the research and outcome in this paper.

Authors' contributions:

Png Jun Qiang, Karl led the research and wrote the manuscript. Amy Choong Mei Fun supervised and suggested the research idea. Hu Donghui supported the DNA extraction and sequencing work. Elango Velautham provided access to Dipterocarp seedlings for sampling and advised on the research idea. Chae Eunyoung provided access to the laboratory and supervised the DNA metabarcoding effort.

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Tables

Table 3 and 4 are available in the Supplementary Files section.

Figures

Figure 1

Visual sightings of ECM fruiting bodies' locations at different parks.

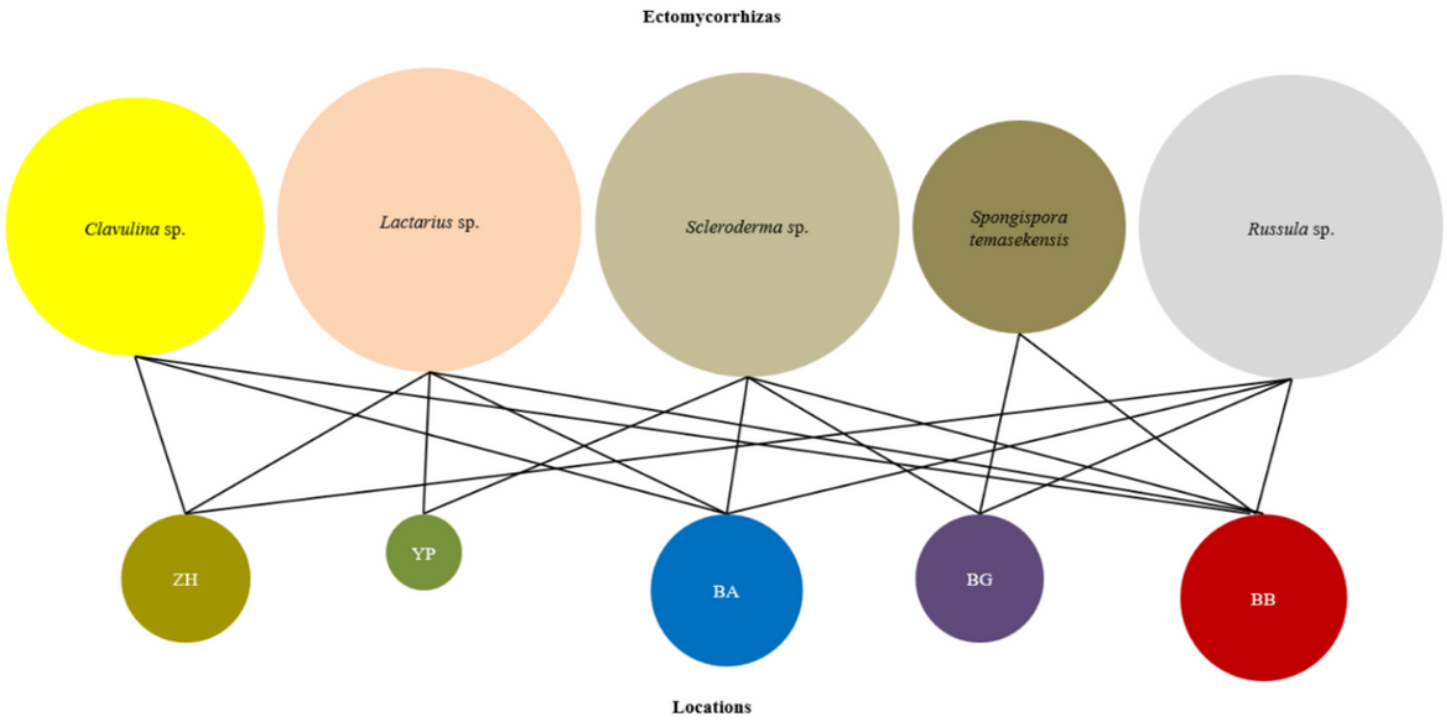

Figure 2

Bipartite network analysis of ECM visual sightings in parks.

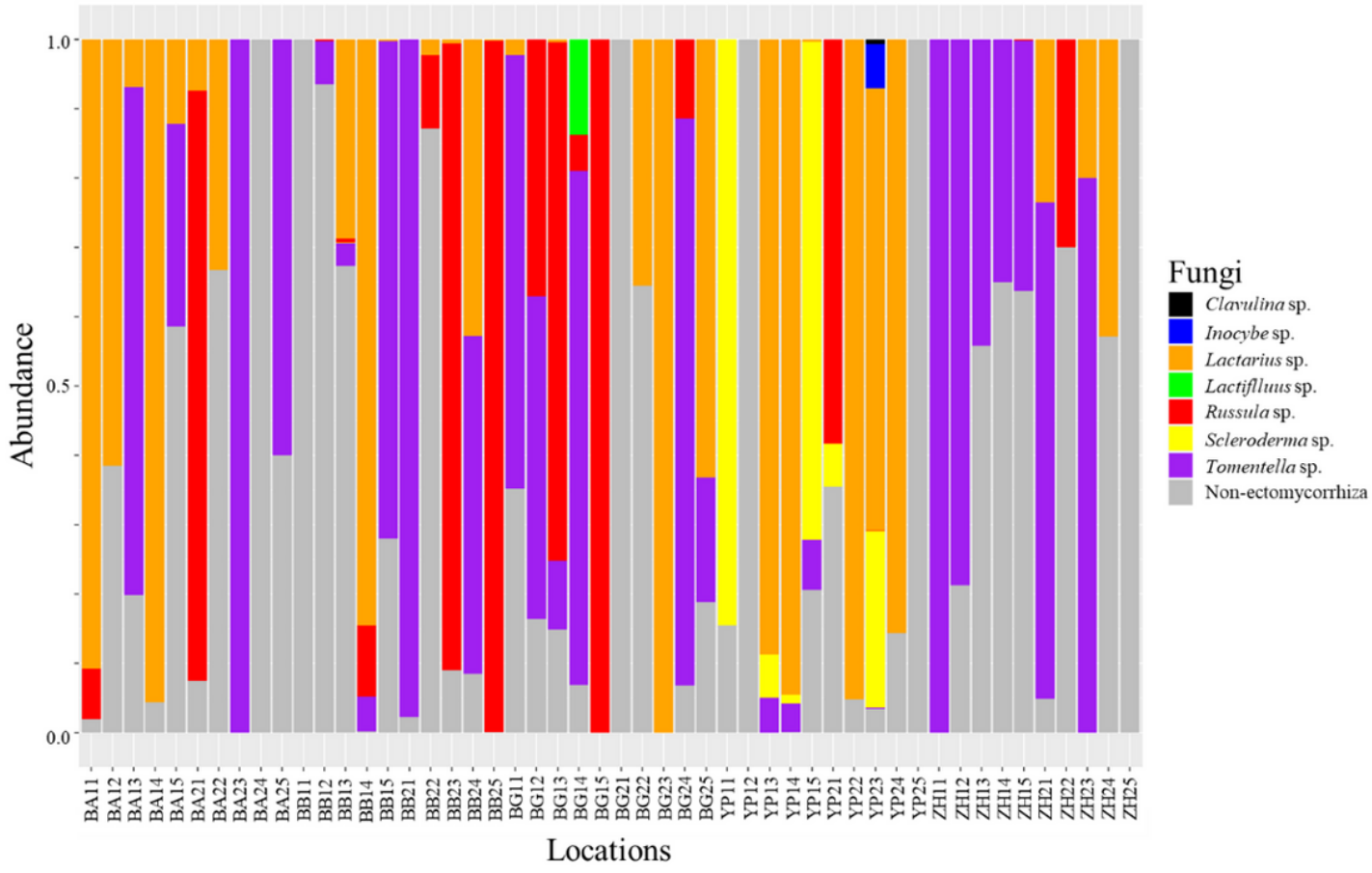

Figure 3

Relative abundances of ECM ASVs in location samples.

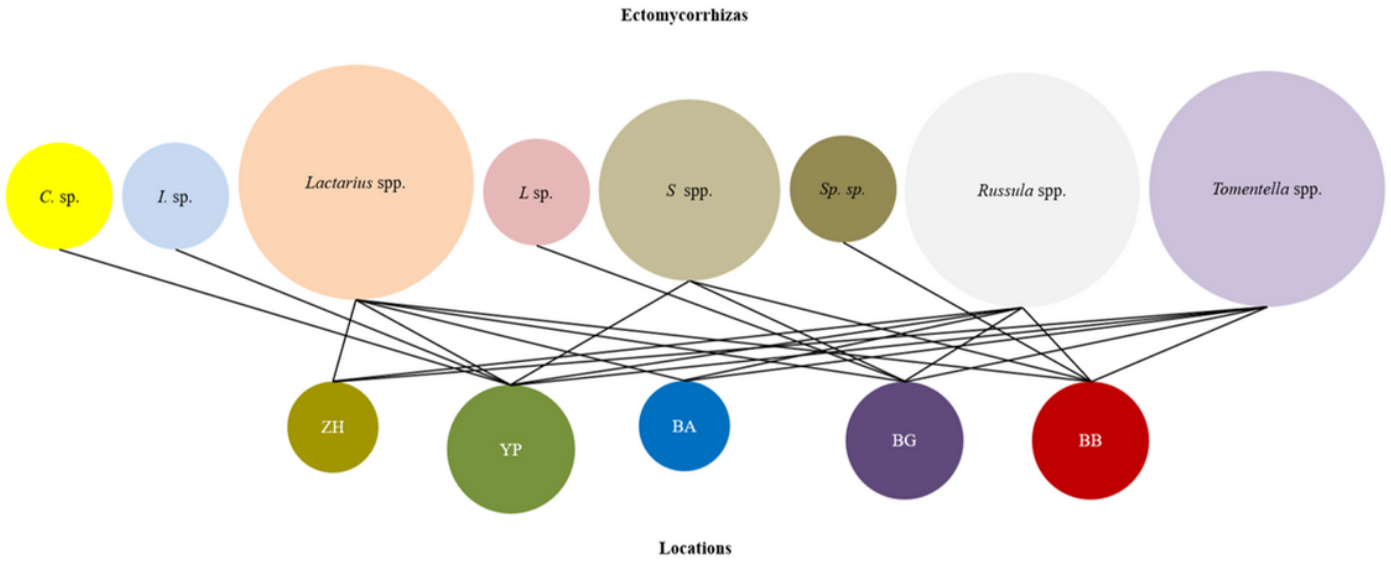

Figure 4

Bipartite network analysis of ECM in parks detected by DNA metabarcoding. *Clavulina* sp.: *C.* sp., *Inocybe* sp.: *I.* sp., *Lactifluus* sp.: *L.* sp., *Scleroderma* spp.: *S.* spp., *Spongispora temasekensis*: *Sp.* sp.

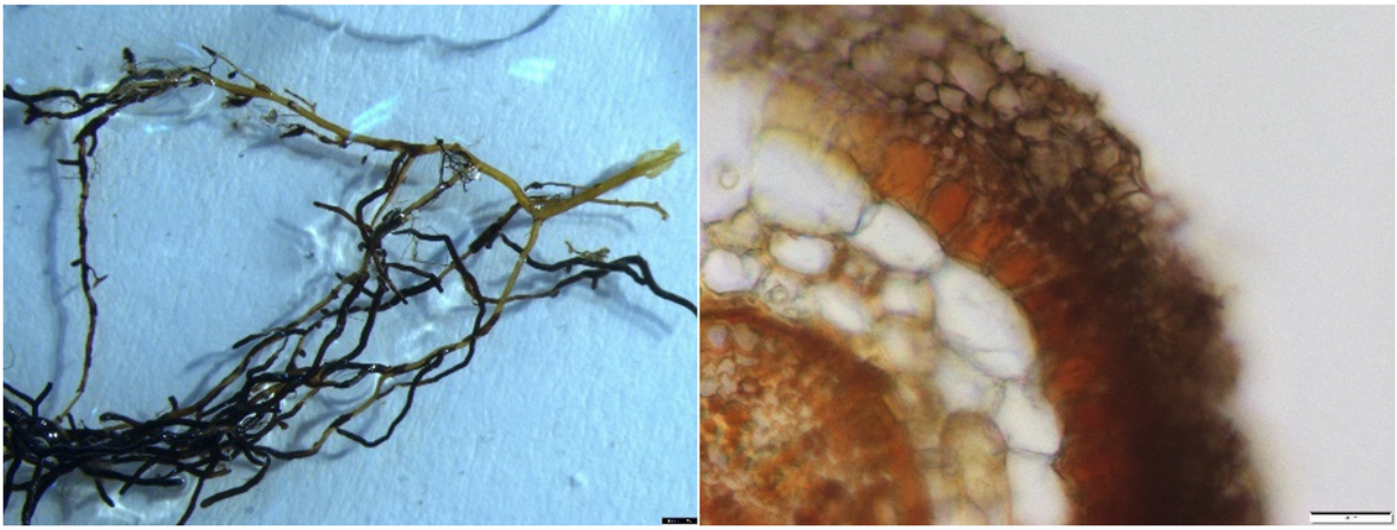

Figure 5

Black morphotype: roots and its cross section found in Ssu_5.

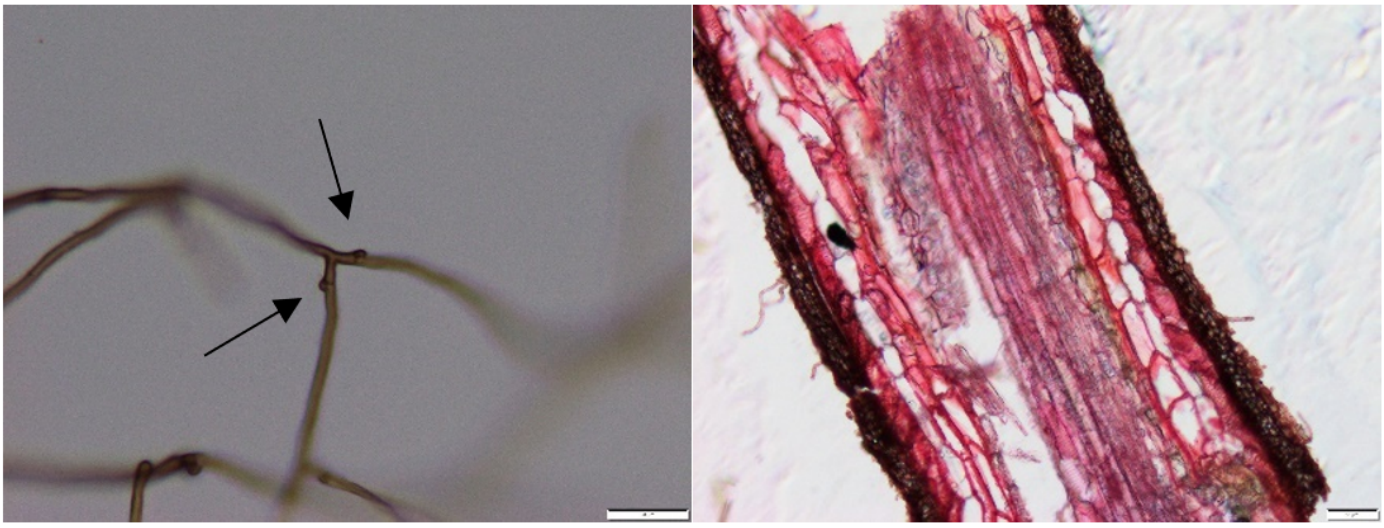

Figure 6

Black morphotype: black arrows pointing at clamp connections and its transverse section with pseudoparenchyma mantle found in Ha_1

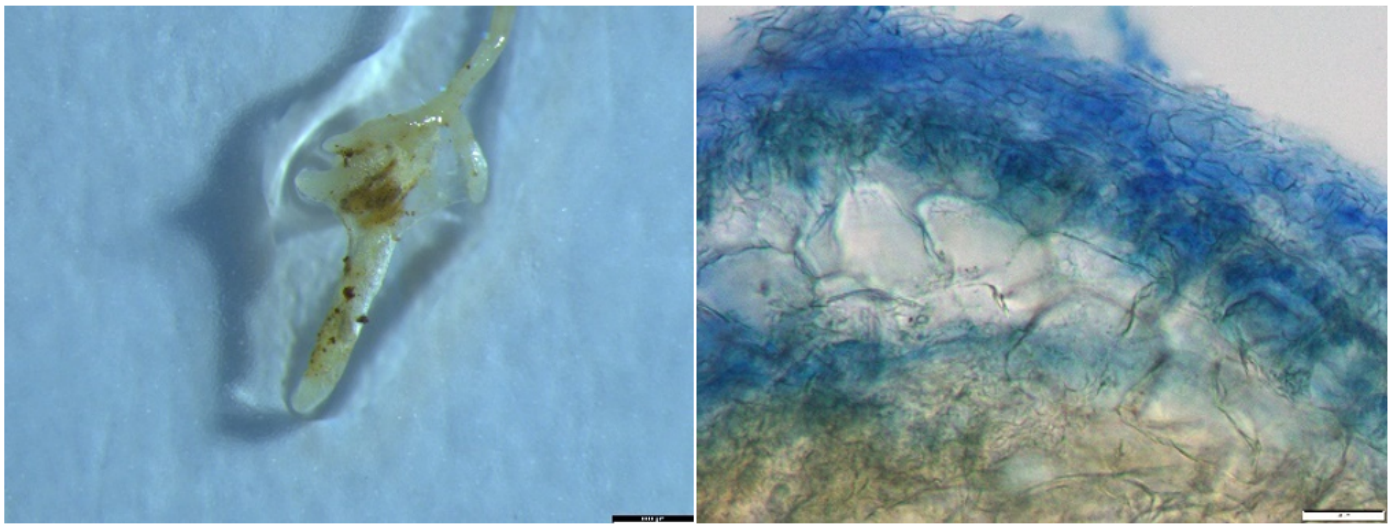

Figure 7

Black/white morphotype: roots and its cross section with prosenchyma mantle found in Ssu_2.

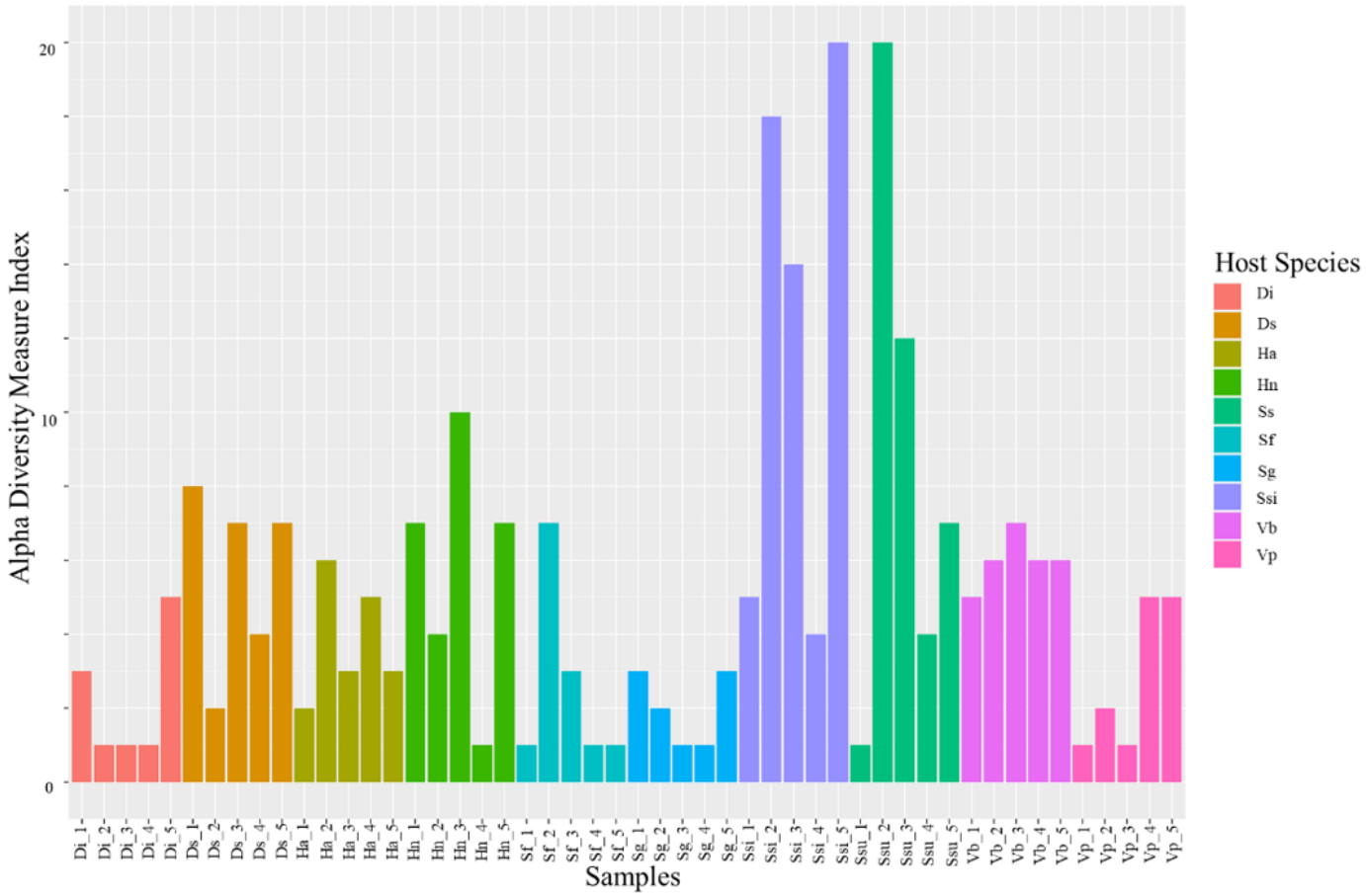

Figure 8

Alpha diversity measure of different host species, coded by colours.

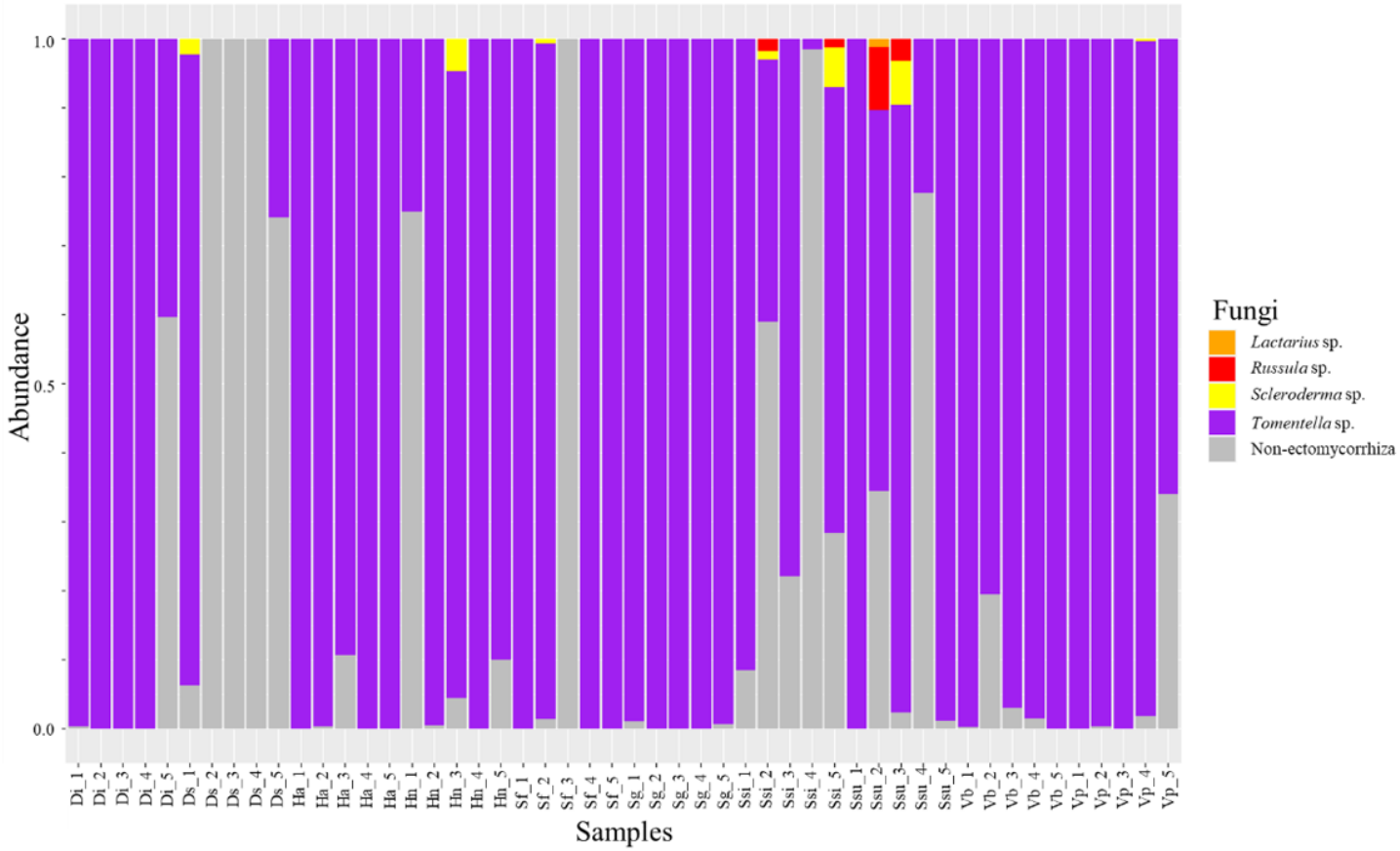

Figure 9

Relative abundance of ECM ASVs in each Dipterocarp species sample.

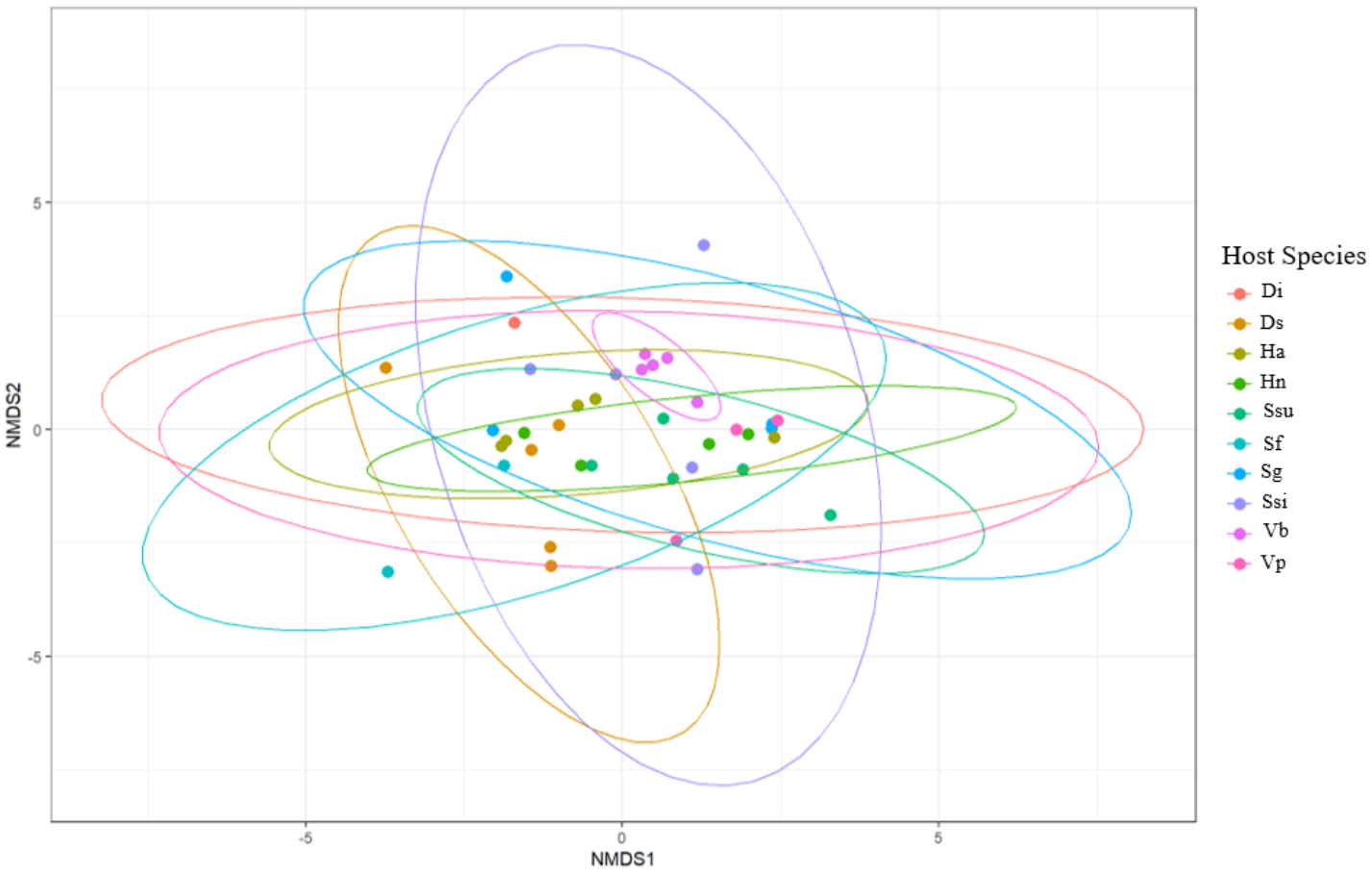

Figure 10

Bray-Curtis NMDS of fungal communities found in different Dipterocarp host species with ellipses.

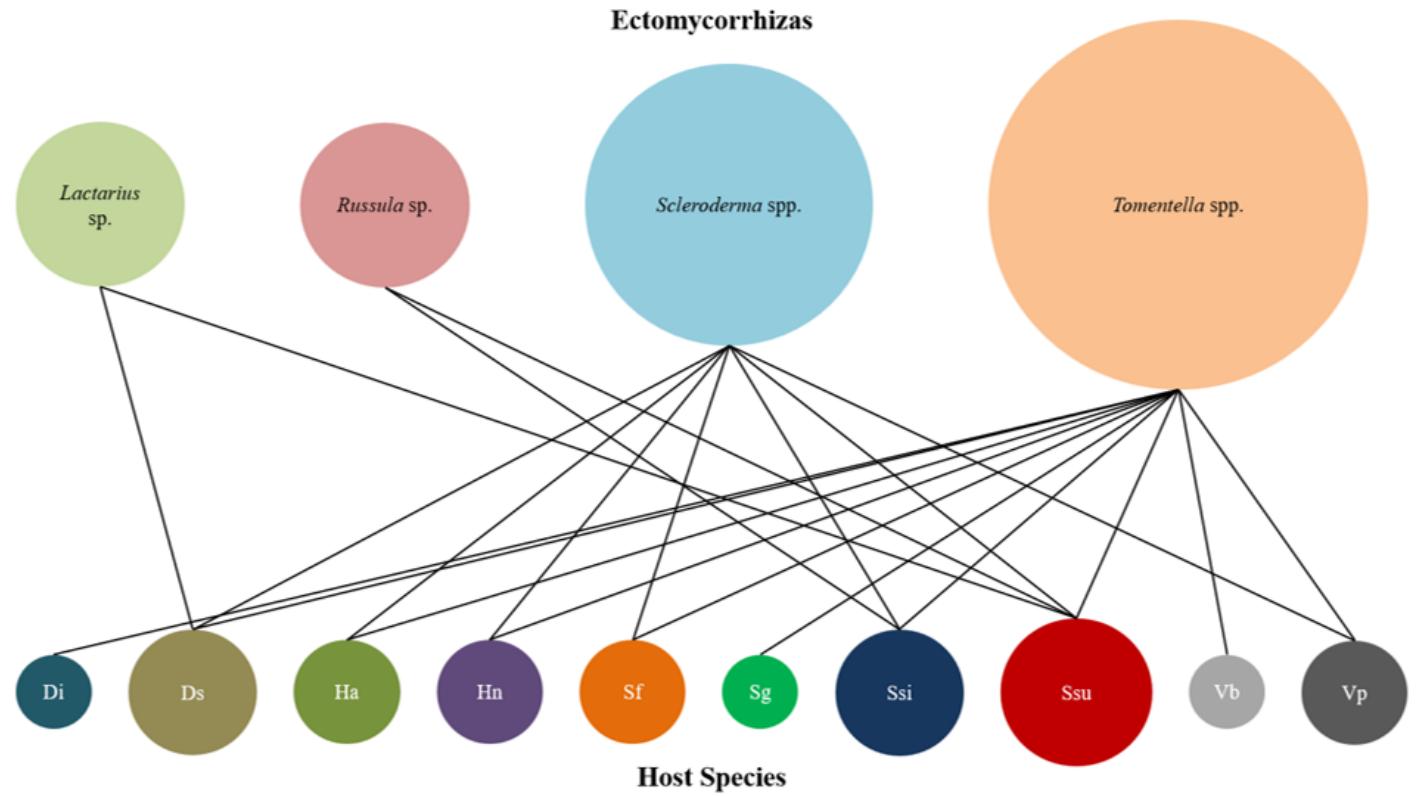

Figure 11

Bipartite network analysis of ECM in Dipterocarp host species detected by DNA metabarcoding.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table3and4.docx