Fungal Endophytes Diversity and Influencing Factors in *Liquidambar orientalis* Mill. in Türkiye

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Fungal endophytes were isolated from the leaves and petioles of *Liquidambar orientalis* Mill., an endangered species in Türkiye. Plant material was collected from 10 sites in September 2023, yielding 499 fungal isolates, classified into 38 morphological groups. DNA extraction and polymerase chain reaction (PCR) amplification of ITS and Beta-tubulin regions were conducted on representative isolates. All fungi belonged to the Ascomycota phylum, comprising 11 genera and 26 species across 15 families, with one group unidentified. The most prevalent families were *Diaporthaceae* (34.9%), *Pleosporaceae* (23.4%), and *Botryosphaeriaceae* (22.2%), with *Diaporthe eres* (15.0%) and *Phomopsis* sp. (12.4%) being dominant species. Fungal diversity was assessed using Shannon, Simpson, and Chao1 indices, revealing tissue type as the strongest factor influencing species diversity, followed by media and spatial factors. The presence of pathogenic families, such as *Botryosphaeriaceae*, highlights potential threats to the species. This is the first study to report fungal endophytes in *L. orientalis*, as well as the first records in Türkiye for several species, including *Alternaria destruens*, *Alternaria alstroemeriae*, *Stemphylium majusculum*, *Diaporthe cynaroidis*, *Pseudopithomyces rosae*, *Nothophoma variabilis*, *Cladosporium endophyticum*, *Cladosporium colombiae*, *Muyocopron* sp., *Sphaerulina rhododendricola*, *Constantinomyces macerans*, and *Aequabiliella effusa.*

DOI: 10.15376/biores.20.1.1251-1272

Keywords: Anatolian sweetgum; Endophytic fungi; Fungal pathogen; Mycobiota; Türkiye

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INTRODUCTION

Endophyte research dates back to 1866, when German botanist Anton de Bary defined microorganisms residing within plant tissues as “endophytes” (de Bary 1866). The discovery of taxol present in an endophytic fungus associated with *Taxus* species (Stierle *et al.* 1993) drew significant attention to the species diversity and bioactive potential of these microorganisms (Reis *et al.* 2022). Endophytes, which include both fungi and bacteria, colonize the internal tissues of plants without causing visible harm, although certain species may become pathogenic under specific stress conditions (Wilson 1995; Tejesvi *et al.* 2007; Rodriguez and Redman 2008). Some fungi can transition from being asymptomatic endophytes to latent or opportunistic pathogens, particularly when triggered by environmental stressors, such as drought, hail, extreme temperatures, or mechanical injury (Swart and Wingfield 1991; Diekmann *et al.* 2002; Blumenstein *et al.* 2021). These stressors can lead to sudden outbreaks of diseases, and cryptic or latent pathogens are now recognized as major contributors to emerging fungal diseases in forests (Ghelardini *et al.* 2016).

Endophytes have garnered substantial scientific interest due to their multifaceted roles in plant health, development, and defense mechanisms (Arnold *et al.* 2003; Hartley and Gange 2009). They can colonize various plant organs, such as leaves, stems, and roots, forming complex associations with their hosts. Beyond their ecological roles, endophytes are prolific sources of bioactive compounds with applications in medicine, agriculture, and biotechnology industry (Trejo-Estrada *et al.* 1998; Guo *et al.* 2008; Priti *et al.* 2009). Exploring the diversity and biochemical capabilities of endophytes continues to unlock new opportunities for benefiting both plant and human health (Kusari and Spiteller 2012).

Extensive research on fungal endophytes has shown that they inhabit a wide range of taxonomic groups, vegetation types, and ecological settings (Arnold *et al.* 2000; Porras-Alfaro and Bayman 2011). The production of bioactive secondary metabolites can vary based on factors such as environmental conditions, geography, host species, and tissue type (Bacon and White 2000). Understanding endophyte communities in different plant tissues and species is essential to fully harness the potential of these valuable resources (Singh *et al.* 2017b). Many fungal genera are host-specific, with colonization influenced by the phytochemistry and nutrient content of plant tissues (Arnold *et al.* 2003). Furthermore, the richness and diversity of endophytes within the same plant species can be highly dynamic, affected by various biotic and abiotic factors (Özdemir *et al*., 2017; Reis 2022; Özdemir 2024).

Liquidambar orientalis, known as the “sweetgum tree” in Türkiye, is a paleoendemic species that first appeared around 60 million years ago (Davis 1982). It is part of the genus *Liquidambar*, the subfamily Buclanoidae, and the family Hamamelidaceae. Historically widespread, this relict species is now found only in limited regions, specifically in southwestern Türkiye and on the island of Rhodes (Kurt and Ketenoğlu 2008; Güner 2012). The name "*Liquidambar*" derives from the Latin “Liquidus,” meaning liquid, and the Arabic “Amber,” which refers to a resinous substance, describing the balsam found in the tree’s trunk. *L. orientalis* is classified as “Endangered” by the IUCN, with its last assessment in 2017 listing it under criterion A2c due to habitat loss and other environmental threats (Kavak and Wilson 2018). Significant threats include agricultural activities, fires, pollution, contaminated water, tourism, and overgrazing. Changes in water availability due to rainfall variability and climate change-induced droughts are also critical threats to the species’ habitat (IUCN 2024). Its conservation status is crucial for regional ecosystems and biodiversity preservation.

The decline of *L. orientalis* habitats in Türkiye is well documented and reflects a broader trend of habitat loss. In 1949, sweetgum forests covered 6,312 hectares, but by 2016, this had decreased to 1,416 hectares (GDF 2021). Efforts to conserve this protected species continue, given its ecological, cultural, and economic significance. *L. orientalis* is not only important for biodiversity but is also used in various contexts. Its pleasant aroma has made it valuable in products ranging from cosmetics and perfumes to parasiticides and medicines. Additionally, it is used for its calming effects in therapy forests and as incense in religious and cultural ceremonies (Lee *et al.* 2009; Selim and Sönmez 2015).

The restricted distribution and historical context of *L. orientalis* highlight its importance in understanding environmental changes and conservation needs. Due to ongoing and emerging threats, continuous monitoring and protection of this species are essential for maintaining biodiversity and ecosystem health. Moreover, many studies globally have emphasized the role of such plant species in providing habitats for endophytic fungi. These plants often thrive in unique ecosystems that offer specific conditions, making them suitable for hosting microorganisms such as endophytes.

Research on these plant species serves as a crucial resource for discovering new fungal species, thereby enriching the scientific understanding of global fungal diversity. Such efforts not only aid in developing effective conservation approaches but also improve predictions related to global fungal biodiversity.

The diversity of endophyte communities in trees is influenced by numerous factors, making it challenging to identify and understand the specific contributions of individual elements to these communities. However, studies with adequate sample sizes that account for various environmental and methodological variables can help elucidate these interactions and their individual contributions. Understanding these factors is essential for advancing knowledge on the ecology and dynamics of endophytic communities.

Despite the importance of *L. orientalis* and its potential as a host for diverse endophytes, no previous research has specifically investigated its endophyte associations. This study is the first comprehensive report on the diversity of fungal endophytes within *L. orientalis*, exploring both the Turkish and global contexts. In Muğla province, various natural populations of *L. orientalis* were identified, and 10 sampling sites were selected based on distinct geographical features. The selection aimed to include representative sites with diverse habitat characteristics to capture ecological variability. Samples from these sites were collected and analyzed to identify endophytic fungi using both morphological and molecular approaches. The research investigated variations in endophyte communities concerning spatial, individual, directional, tissue type, tissue region, and nutrient media factors.

EXPERIMENTAL

Determination of Sample Sites

In Muğla province, various natural populations of *Liquidambar orientalis* were identified, resulting in the selection of 10 different sampling sites based on their distinct geographical features. The identification of these sites utilized management plans, which allowed for the mapping of areas where *L. orientalis* occurs in a digital format. Topographic variables, such as elevation and aspect, as well as climate variables, including annual average temperature and total annual precipitation, were defined for these areas.

Elevation data were downloaded from the EarthData database with a resolution of 30 m. The data were used in ArcMap (Environmental Systems Research Institute, Inc., version 10.2, Redlands, CA, USA) to create an aspect map (EarthData 2024). Climate data were obtained from the WorldClim database, using maps with a resolution of 30 arc seconds (~1 km). These four variables were transferred to ArcMap, where the distribution areas of *L. orientalis* were overlaid on the maps.

Finally, the most representative sampling sites with diverse habitat characteristics were selected for field sampling. Geographic Information Systems (GIS) were employed throughout the process of site selection and sample collection. Using ArcGIS, the study area in Muğla was divided into a grid system, creating sub-sampling areas. Random points were generated within these grids, and trees located at these points were selected for sampling (Oruç *et al.* 2017).

Sampling

The sampling was conducted in September 2023 at 10 different sites located in the Muğla district in southwestern Türkiye (Fig. 1). Beram and Akyol collected 40 leaf and

petiole samples from 20 trees. In each sub-sampling area, two sample trees were selected, spaced 50 meters apart to represent different microhabitats.

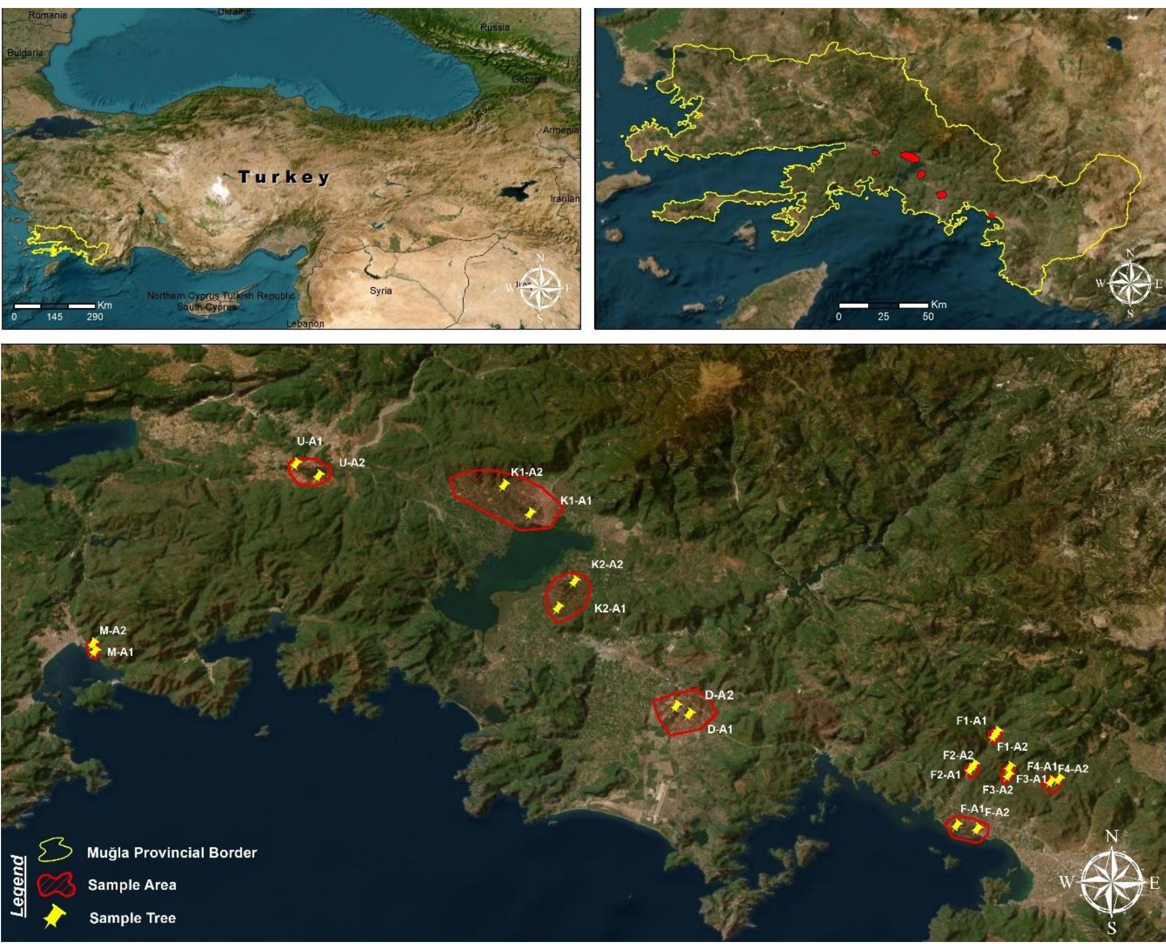

Fig. 1. Location of the sampling sites in Muğla province, south-western Türkiye (Sampling Sites: K1, Köyceğiz; K2, Köyceğiz; KZ, Kızılkaya; D1, Dalaman; M, Marmaris; F, Fethiye; F1, Fethiye; F2, Fethiye; F3, Fethiye; F4, Fethiye; A1, Tree1; A2 Tree2)

The host characteristics of the stands, including age (Haglöf Sweden increment borer), diameter (Haglöf Mantax black 1020 mm diameter gauge), and height (measured with a Blume Barl Leiss height meter), along with current stand type (Table 1), were recorded in the field notebook.

Samples of healthy leaves and petioles from branches 4 to 8 m above the ground were collected using pruning shears (Meşem, Türkiye) from one-year-old growth on the selected trees (Oono *et al.* 2015) (Fig. 2). This height range was chosen to standardize sampling from similar canopy levels across all trees. Leaves were taken from the leaf just below the terminal shoot leaf, following a consistent sampling method for each tree (Gamboa and Bayman 2001). A total of four leaves (two from the north and two from the south) showing no signs of disease were collected (Dos Reis *et al.* 2022).

The collected leaves were transported in labeled sealed bags with the corresponding sampling site number, sample number, and sampling date, using ice packs to maintain suitable conditions in the laboratory. The samples were stored at 4 °C ± 1 °C until analyzed.

Table 1. Characteristics of Sampling Sites and Sampling Trees in Muğla District, South-Western Türkiye

Sampling Site Codes	District Name	Age of Tree (tree1-tree2)	Diameter of Tree (tree1-tree2) (cm)	Height of Tree (tree1-tree2) (m)	Coordinates (tree1-tree2)	Aspect	Elevation (m)
F	Fethiye	35 to 32	27 to 25	16 to 15	36º57’42’’N-28º40’20’’E/ 36º59’28’’N-28º38’59’’E	South	5
F1	Fethiye1	25 to 15	37 to 20	19 to 14	36º52’34’’N-28º41’59’’E/ 36º54’14’’N-28º42’43’’E	Southwest	100
F2	Fethiye2	20 to 22	25 to 35	15 to 18	36º50’34’’N-28º17’19’’E/ 36º50’57’’N-28º17’08’’E	South	70
F3	Fethiye3	33 to 28	27 to 30	15 to 18	37º00’28’’N-28º27’58’’E/ 36º59’58’’N-28º29’16’’E	Northeast	110
F4	Fethiye4	15 to 18	32 to 33	16 to 14	36º47’09’’N-28º48’46’’E/ 36º47’35’’N-28º48’07’’E	South	120
D	Dalaman	25 to 27	22 to 23	22 to 25	36º46’21’’N-29º05’14’’E/ 36º46’00’’N-29º05’09’’E	South	20
K1	Köyceğiz1	15 to 13	33 to 20	14 to 16	36º44’35’’N-29º04’01’’E/ 36º44’13’’N-29º03’52’’E	South	10
K2	Köyceğiz2	23 to 25	20 to 21	37 to 39	36º44’28’’N-29º05’56’’E/ 36º43’59’’N-29º05’50’’E	Southeast	10
M	Marmaris	28 to 22	28 to 24	21 to 19	36º43’49’’N-29º08’28’’E/ 36º43’55’’N-29º08’07’’E	Southwest	12
KZ	Kızılyaka	36 to 40	27 to 34	30 to 33	36º41’16’’N-29º02’59’’E/ 36º40’57’’N-29º04’02’’E	Southwest	100

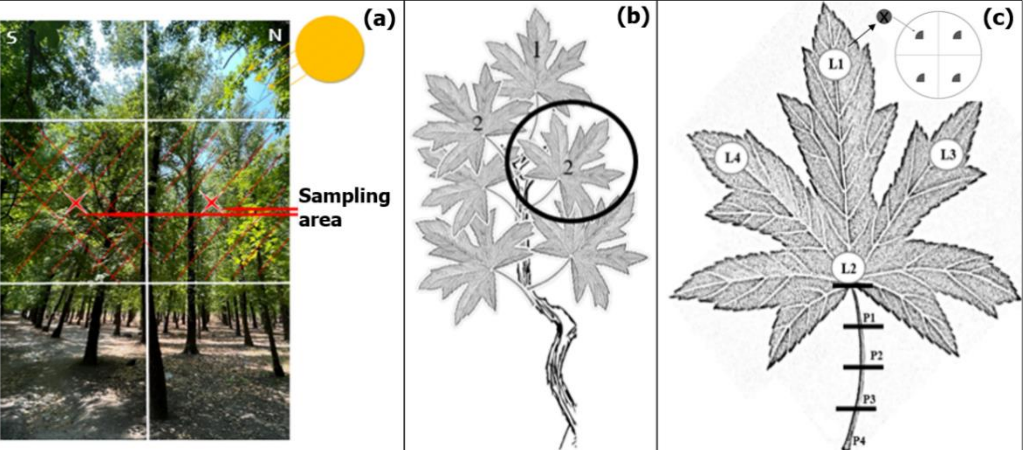

Fig. 2. Sampling and shredding method: (a) Selection of the area for leaf sampling on the tree, (b) Leaf selection for sampling, and (c) Shredding method for leaves and petioles

Fungal Culture Isolation

Isolation is a critical step in the identification of fungi. The isolates obtained during this process, representing pure fungal colonies, were employed in both morphological and molecular diagnostic studies. During the isolation process, media-dependent variations were analyzed to enhance the understanding of how methodological factors influence fungal diversity.

Surface Sterilization of Plant Material

The sterilization procedure was performed to eliminate external contamination and epiphytic fungi, ensuring the accurate isolation of endophytic fungi for analysis. The leaves and petioles underwent surface sterilization procedures. For surface sterilization, each sample was initially thoroughly rinsed in distilled water. Subsequently, the samples were subjected to a surface sterilization process, wherein each sample was immersed in 75% ethanol for 1 min, followed by a 5-min immersion in 5% sodium hypochlorite (NaOCl), and then dipped in 75% ethanol for 30 s. Finally, the samples underwent a sequential rinse of 1 min in sterile distilled water, and this was repeated five times (Gamboa *et al.* 2003; Ibrahim *et al.* 2021). Post-sterilization, the leaves and stems were left to air-dry within a under sterile conditions in a biological safety cabinet.

Cultivation of Fungi from Leaf and Petiole Samples

Each leaf was dissected into four approximately 10 x 5 mm^2 fragments using a sterile scalpel: one near the tip along the midrib, one near the base along the midrib, one from the area closest to the right edge, and another from the area closest to the left edge. Subsequently, each of these fragments was further subdivided into four equal sub-samples. Each sub-sample was individually placed onto Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA), Water Agar (WA), and Malt Extract Agar supplemented with plant material (PL-MEA), allowing the cultivation of leaf samples from all four fragments on each growth medium (Fig. 2) (Cannon and Simmons 2002).

The same procedure was applied to petioles. Each stem was initially divided into four equal vertically along the length segments, and then each of these segments was further divided into four equal transverse sections. Petri dishes were incubated in the dark at 25 ± 2 °C for 21 days. Regular microscopic examinations were conducted daily using a

stereomicroscope. Fungal hyphae actively developing in plant tissues were transferred to the same type of culture media for purification, and the obtained cultures were incubated again at 25 ± 2 °C (Khalil *et al.* 2020).

Morphological and Molecular Identification of Fungal Isolates

Fungal isolates grown on plates were initially grouped based on their morphological characteristics, including colony shape, size, color, texture, growth pattern, and reproductive structures, to tentatively identify them at the genus level. Distinct morphotypes were selected for molecular identification. Genomic DNA was extracted from fresh mycelium using the High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche, Basel, Switzerland), following the manufacturer's protocol. Molecular identification involved PCR amplification and sequencing of the ITS1-5.8S-ITS2 region (ITS1-2) using the universal primer pairs ITS1 and ITS4 (White *et al.* 1990), along with the beta-tubulin (tub2) gene using primers Bt2a and Bt2b (Glass and Donaldson 1995). The PCR was performed using Xpert Fast Hotstart Mastermix (Grisp, Portugal) in a 25 µL reaction mixture.

The PCR products were sequenced at BMLabosis (Ankara, Türkiye), and the DNA sequences were analyzed and edited using MEGA 11 software (MEGA Development Team, MEGA 11 version, Philadelphia, PA, USA). The sequences were compared against the NCBI GenBank database using BLAST (Altschul *et al.* 1990), with species-level identification requiring a minimum of 98% coverage and 98.5% identity. Genus-level identification was set between 94% and 97% similarity (Singh *et al.* 2017a). For ambiguous species assignments, published studies were used for confirmation (Hernández *et al.* 2023).

Evolutionary relationships were analyzed using the Neighbor-Joining method (Tamura *et al.* 2004), with bootstrap values calculated from 500 replicates (Felsenstein 1985). Evolutionary distances were measured using the Maximum Composite Likelihood method, and ambiguous positions were removed *via* pairwise deletion. Only isolates identified with both markers were considered accurately identified at the species level.

Finally, selected isolates underwent further morphological characterization using an Olympus compound microscope (Olympus Corporation, Tokyo, Japan) and the Olympus DP-Soft program. Representative isolates were preserved and stored in triplicate at the Fungal Biotechnology Laboratory, Department of Biology, Pamukkale University, Denizli, Türkiye.

Statistical Analysis of Fungal Endophyte Diversity

To evaluate the diversity of the fungal endophyte community across sampling sites and tissue types, statistical analyses were conducted. The statistical analysis was performed using R (R Core Team, R 4.3.1 Version, Auckland, New Zealand). Shannon diversity indices and the Bray-Curtis similarity index (Shannon 1948; Bray and Curtis 1957) were used to characterize the diversity and composition of fungal communities, while the Simpson index was employed to estimate dominance (Simpson 1949). Beta diversity has been employed to identify the dissimilarities among the sampling sites (Bray and Curtis 1957; Jost 2007; Legendre and De Cáceres 2013; Ricotta *et al.* 2021). In this study, a universal beta diversity calculation based on Shannon entropy was preferred. Additionally, the Chao1 diversity index was calculated for parameters with one or two OTUs (Operational Taxonomic Unit).

RESULTS

This study includes findings related to the prevalence of fungi obtained from the leaves and petioles of *L. orientalis* collected from 10 different sampling sites within Muğla province. A total of 40 leaf and petiole samples were collected from 20 trees, and these samples were inoculated onto 1280 Petri dishes.

From the collected samples, 499 isolates were obtained. All isolated fungi were identified as members of the Ascomycota. Morphological and molecular analyses resulted in the identification of a total of 37 fungi from 15 different families, including 26 species at the species level and 11 genera at the genus level. Additionally, one unidentified species was obtained (Table 2).

Upon reviewing the data, the most common families identified were as follows: *Diaporthaceae* with 174 isolates (34.9%), *Pleosporaceae* with 117 isolates (23.4%), and *Botryosphaeriaceae* with 111 isolates (22.24%). These three families accounted for 80.5% of all isolates, indicating their dominance within the fungal community. Other families contributed less significantly, such as Didymosphaeriaceae (7.4%), Aspergillaceae (6.8%), and Hypocreaceae (3.4%). At the species level, *D. eres* (75 isolates - 15.0%) was the most commonly isolated and dominant species across all sites, followed by *Phomopsis* sp. (62 isolates - 12.4%) and *Alternaria* sp. (56 isolates - 11.2%).

Fungal diversity was assessed using Shannon and Simpson alpha diversity indices, as well as the Chao1 index. The analyses considered various factors, including spatial, individual, orientational, tissue type, tissue region, and media-dependent variations. These findings indicate that specific environmental and methodological factors influence fungal diversity. Among the isolates obtained, 71.0% were recovered from all tissue types. While these endophytes were widespread, some were specific to particular environmental and methodological conditions.

Notably, tissue type emerged as the strongest factor affecting species diversity within the endophytic community, followed closely by media-dependent and spatial factors, with only minor differences between them. Collectively, these factors shaped the structure of the endophytic mycobiota community in *L. orientalis*. Additionally, this research provides the first records of the following species in Türkiye: *Alternaria destruens*, *Alternaria alstroemeriae*, *Stemphylium majusculum*, *Diaporthe cynaroidis*, *Pseudopithomyces rosae*, *Nothophoma variabilis*, *Cladosporium endophyticum*, *Cladosporium colombiae*, *Muyocopron* sp., *Sphaerulina rhododendricola*, *Constantinomyces macerans*, and *Aequabiliella effusa*.

Spatial Alpha Diversity Analysis

Upon examining the data collected from the sampling sites, the most dominant species were FBL7, FBL23, and FBL56, each with a frequency value of 10. These were followed by FBL6, FBL11, and FBL32, with a frequency value of 9. Shannon and Simpson alpha diversity indices have been calculated. The sites with the highest diversity for both Shannon and Simpson indices were F and F2, while the sites with the lowest diversity were F3 and K1 (Table 3).

Table 2. Classification of Isolated Fungi, Morphological and Molecular (Based on ITS Region) Identification of Different OTUs, Their Closest Match from NCBI Database with Their Accession Number, Query Coverage (%QC) and Similarity (%ID)

Family	OTU Acronym	Closest NCBI Match	Reference Accession No. ITS	QC/ID % ITS	Accession No./ ITS
Pleosporaceae	FBL7	*Alternaria* sp.	OR245528.1	100/98	PQ373062
	FBL56	*Alternaria destruens*	NR_137143.1	99/98	PQ373063
	FBL3	*Alternaria alstroemeriae*	NR_163686.1	100/97	PQ373064
	FBL53	*Bipolaris subramanianii*	NR_147496.1	99/100	PQ373065
	FBL50	*Stemphylium* sp.	OR562057.1	100/97	PQ373066
	FBL13	*Stemphylium botryosum*	NR_163547.1	99/99	PQ373067
	FBL39	*Stemphylium majusculum*	NR_160116.1	99/98	PQ373068
Diaporthaceae	FBL11	*Diaporthe bohemiae*	NR_164425.1	98/99	PQ373069
	FBL29	*Diaporthe cynaroidis*	MH863230.1	100/97	PQ373070
	FBL23	*Diaporthe eres*	NR_144923.1	99/100	PQ373071
	FBL6	*Phomopsis* sp.	OR122531.1	100/100	PQ373072
Didymosphaeriaceae	FBL12	*Paraconiothyrium brasiliense*	NR_163552.1	99/98	PQ373073
	FBL26	*Pseudopithomyces rosae*	NR_157539.1	100/97	PQ373074
Botryosphaeriaceae	FBL15	*Neofusicoccum* sp.	OR916288.1	100/99	PQ373075
	FBL21	*Neofusicoccum* sp.	OR803190.1	100/99	PQ373076
	FBL33	*Neofusicoccum* sp.	PP701999.1	100/98	PQ373077
	FBL36	*Neofusicoccum* sp.	KX226449.1	97/98	PQ373078
	FBL27	*Neofusicoccum* sp.	LC698678.1	100/100	PQ373079
Didymellaceae	FBL43	*Nothophoma variabilis*	NR_158280.1	100/97	PQ373080
	FBL51	*Didymella prosopidis*	NR_137836.1	100/98	PQ373081
Aspergillaceae	FBL8	*Aspergillus niger*	NR_111348.1	98/99	PQ373082
	FBL28	*Penicillium* sp.	KP994293.1	100/97	PQ373083
	FBL55	*Penicillium rubens*	NR_111815.1	99/99	PQ373084
	FBL48	*Penicillium italicum*	NR_153215.1	100/98	PQ373085
Hypocreaceae	FBL35	*Trichoderma harzianum*	NR_174890.1	100/97	PQ373086
	FBL40	*Trichoderma lixii*	NR_131264.1	99/100	PQ373087
Cladosporiaceae	FBL52	*Cladosporium endophyticum*	NR_158360.1	99/98	PQ373088
	FBL32	*Cladosporium colombia*	NR_119729.1	99/100	PQ373089
Discinellaceae	FBL14	*Pseudopezicula* sp.1	PP447733.1	100/100	PQ373090
	FBL45	*Pseudopezicula tetraspora*	NR_164096.1	98/97	PQ373091

	FBL49	*Pseudopezicula tracheiphila*	NR_170833.1	100/99	PQ373092
Muyocopronaceae	FBL42	*Muyocopron* sp.	PP313039.1	99/100	PQ373093
Cucurbitariaceae	FBL22	*Neocucurbitaria cava*	NR_160112.1	100/98	PQ373094
Mycosphaerellaceae	FBL44	*Sphaerulina rhododendricola*	NR_137839.1	98/99	PQ373095
Meruliaceae	FBL47	*Constantinomyces macerans*	NR_164011.1	100/97	PQ373096
Cyphellaceae	FBL58	*Aequabiliella effusa*	NR_132005.1	99/98	PQ373097
Glomerellaceae	FBL19	*Gnomoniopsis idaeicola*	NR_166025.1	99/99	PQ373098
Unknown type	FBL9				

Table 3. Spatial Diversity Analysis Data

Plot	Simpson	Shannon	Chao-1	Species Richness (S)	Number of Individuals (N)
F	0.9407	2.845	38.62	18	55
F1	0.9207	2.666	33.56	16	41
F2	0.9387	2.827	20.66	17	44
F3	0.8892	2.392	14.96	13	48
F4	0.8596	2.198	13.92	11	39
M	0.9023	2.38	12.16	12	67
D	0.8913	2.401	46.5	12	24
U	0.9106	2.528	14.08	14	59
K1	0.8499	2.245	14.96	13	52
K2	0.9164	2.64	24.39	17	70

The community with the highest number of individuals was K2. However, the diversity in areas F, F1, and F2 was higher. For instance, the species richness in F2 and K2 was equal, but K2 had a higher number of individuals. Despite this, the Shannon and Simpson diversity indices for F2 were higher (Fig. 3). In F2, Shannon diversity was 2.827, with a Simpson index of 0.9387, whereas K2 had values of 2.64 and 0.9164, respectively. These differences highlight how the number of individuals does not always correlate with overall diversity. Additionally, Chao1 analysis revealed that F2 had a lower estimate for rare species compared to K2, suggesting distinct community structures. The community with the lowest species richness and number of individuals was D, resulting in lower Shannon and Simpson diversity indices. However, contrary to this, the Chao-1 diversity index changes the result when calculated (Fig. 3).

Individual Beta Diversity Analysis

Beta diversity has been used to compare the values obtained from measurements of different trees (T1 and T2) in the sampling areas. Beta diversity refers to the variation in species composition between different ecosystems or habitats (Whittaker 1960). In general, beta diversity refers to the difference between two communities. It is attributed to the unique characteristics of living communities. In other words, the variation that remains outside the similarity between at least two communities corresponds to beta diversity.

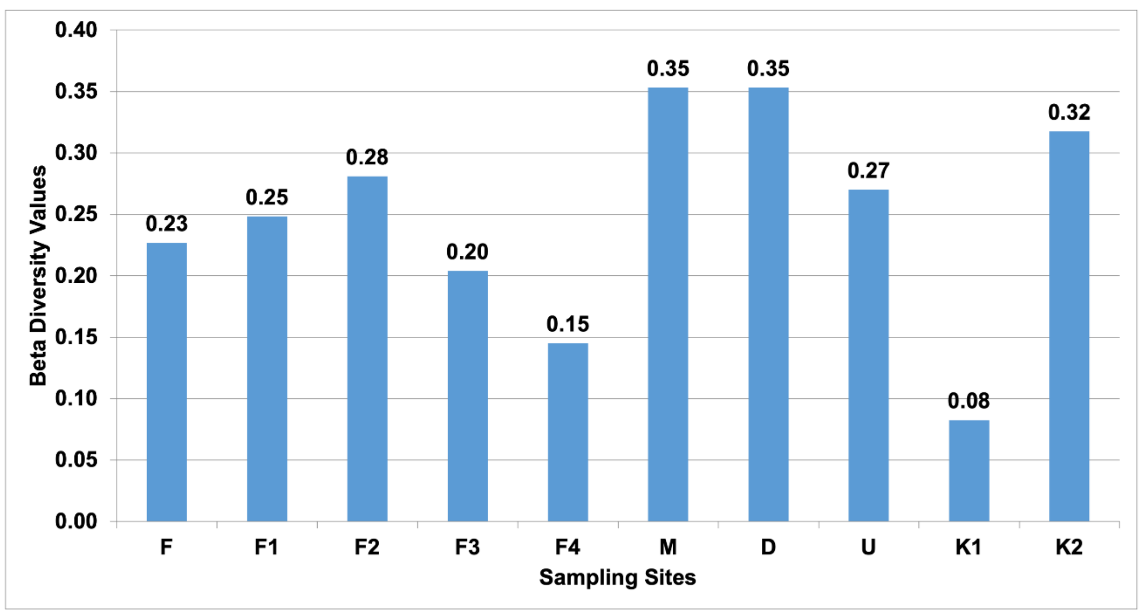

Fig. 3. Results of the Shannon entropy based universal beta diversity

As demonstrated in Fig. 3, community D exhibited the highest beta diversity value. This indicates a higher level of disssimilarity among the measurements taken from different trees within the D community. In other words, the figure illustrates the differences in isolate species identified on different trees within the same sampling area. From this perspective, the D community stands out compared to the others (Fig. 4).

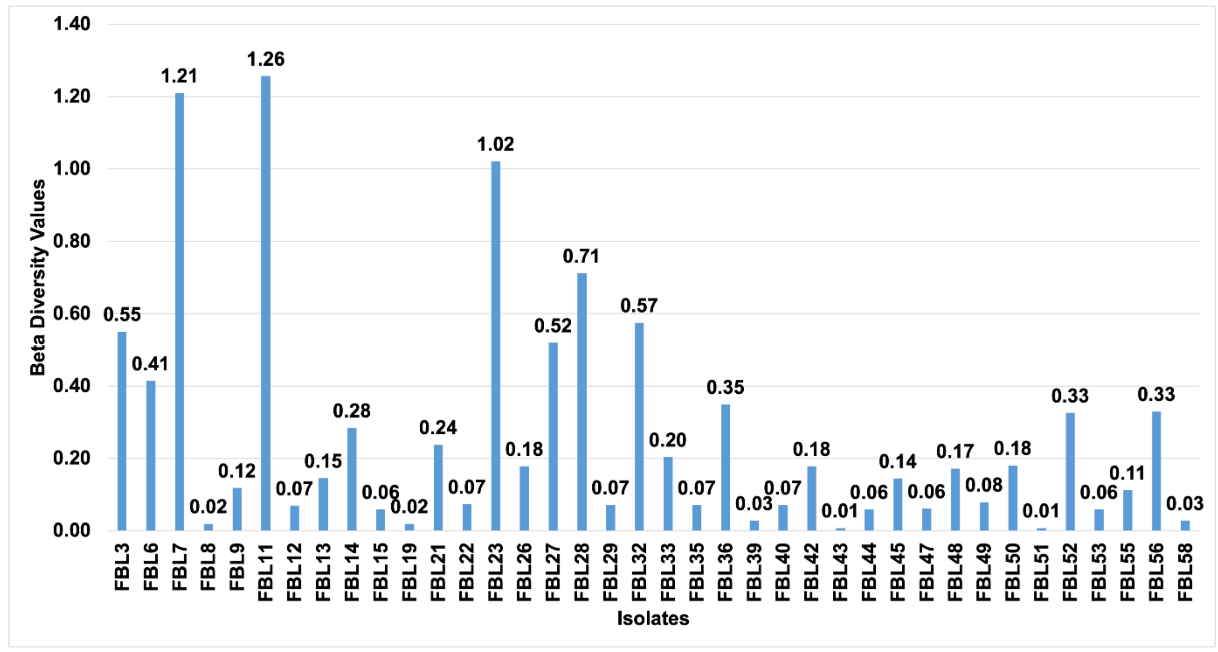

Fig. 4. Species contributings most to Shannon entropy based universal beta diversity across all communities

Directional Alpha Diversity Analysis

A total of 260 isolates were identified in the north and 239 in the south. The southern region showed higher Shannon and Simpson diversity values, while the Chao-1

index was higher in the north. Isolates FBL15, FBL19, FBL44, FBL47, FBL51, FBL53, and FBL58 were unique to the north, while FBL8, FBL12, FBL26, FBL29, FBL35, FBL39, FBL40, and FBL43 were found only in the south. The remaining 23 isolates were found in both regions. For leaves and stems, 34 species were identified in leaves and 31 in stems, with species counts of 264 for leaves and 235 for stems. Shannon, Simpson, and Chao1 diversity indices were higher for leaves. Twenty-seven species were common to both leaves and stems, while FBL8, FBL15, FBL19, FBL39, FBL43, FBL49, and FBL55 were found only in leaves, and FBL47, FBL51, FBL53, and FBL58 only in stems. In leaf parts, L1 had the highest species richness (23 species), while L2 and L4 had the lowest (18 species each). Shannon diversity ranked L1 highest, followed by L4, L3, and L2, while for Simpson, the order was L4, L1, L3, and L2. Chao1 results placed L1 highest, followed by L3, L2, and L4. In stems, P4 had the highest species richness (20 species), and P2 the lowest (15 species). Shannon diversity ranked P4 highest, followed by P3, P2, and P1, with Simpson showing a similar pattern. Chao1 ranked P4 highest, followed by P1, P3, and P2. Specific isolates were associated with certain stem parts, including FBL9, FBL12, FBL13, FBL22, FBL27, and FBL58 for P4, and FBL51 and FBL53 for P3. For media types, diversity was highest in PL-MEA, followed by PDA, PCA, and WA, with the Chao1 index ranking WA highest. Isolates FBL8, FBL43, and FBL55 were exclusive to PCA; FBL15, FBL35, FBL39, FBL40, FBL53, and FBL58 to PL-MEA; FBL49 and FBL51 to PDA; and FBL19 to WA (Table 4).

Table 4. Alpha Diversity Results

		Shannon	**Simpson**	**Chao-1**	**Species Richness (S)**	**Number of Individuals (N)**
Tree Aspect	**K**	2.80656	0.91751	37.2	30	260
	G	2.82401	0.91879	33.1	31	239
Tissue Type	**L**	2.86113	0.92284	49.6	34	264
	P	2.79804	0.91278	42	31	235
Tissue Regions: Leaf (Lamina)	**L1**	2.75284	0.91506	36.2	23	66
	L2	2.58479	0.9128	25.5	18	95
	L3	2.68642	0.91373	34.75	21	61
	L4	2.70886	0.9229	21.5	18	42
Tissue Regions: Stem (Petiole)	**P1**	2.38119	0.88279	24	17	81
	P2	2.49045	0.90358	20	15	47
	P3	2.51204	0.90023	21.25	16	42
	P4	2.59876	0.90083	42.5	20	65
Media Types	**PDA**	2.74855	0.91189	32	25	123
	PCA	2.63235	0.89914	30	23	146
	WA	1.88984	0.74888	40.5	18	112
	PL-MEA	2.82965	0.91396	29.625	27	118

DISCUSSION

Species identification of fungal endophytes often requires molecular analysis because morphological characteristics alone can be misleading. DNA barcoding, particularly the ITS1-5.8S-ITS2 rDNA region, is a common and effective method for accurate species identification (Schoch *et al.* 2012; Wu *et al.* 2019). However, in some fungal groups, the ITS region may lack sufficient variation to distinguish species, necessitating the use of additional gene regions such as TEF1-α, RPB2, or beta-tubulin for better precision (Kõljalg *et al.* 2013; Stielow *et al.* 2015). Increasing the number of ITS sequences in databases and improving their quality would enhance identification accuracy (Vu *et al.* 2016; Hawksworth and Lücking 2017).

This study found that all isolated fungi from *Liquidambar orientalis* belonged to the Ascomycota phylum, consistent with previous findings (Bullington and Larkin 2015; Singh *et al.* 2017b). Dominant families included *Diaporthaceae* (34.86%), *Pleosporaceae* (23.44%), and *Botryosphaeriaceae* (22.24%), with prevalent species being *Diaporthe eres*, *Phomopsis* sp., and *Alternaria* sp. (Gomes *et al.* 2013). Their widespread presence and easy isolation under laboratory conditions further support their dominance (Santos *et al.* 2011; Udayanga *et al.* 2014). Studies have consistently found species like *Diaporthe* in various tree species, suggesting these endophytes have adapted to coexist as consistent residents within plant tissues (Saikkonen *et al.* 2004).

The prevalence of Ascomycota and specifically families such as *Pleosporaceae* is typical in broad-leaved plants. Research on tropical and temperate trees has shown frequent occurrences of common endophytic genera including *Colletotrichum*, *Xylaria*, *Fusarium*, and *Phomopsis* across different environments (Arnold *et al.* 2000; Suryanarayanan *et al.* 2011). The rare endophytes found in this study, such as *Muyocopron* sp., could be due to diverse sampling and focusing on an endemic host, *L. orientalis*, which may harbor unique fungal communities.

Geographic location, environment, and host-specific factors play crucial roles in shaping endophytic communities (Huang 2020; Thangavel *et al.* 2022). Different studies have demonstrated unique fungal communities tied to specific regions, emphasizing how climate, season, and local environmental factors influence endophyte composition (Arnold and Lutzoni 2007; U'Ren *et al.* 2010). For example, factors such as plant density, host plant characteristics, and changes in environmental conditions can lead to either a stable or fluctuating diversity of endophytes (Herrera *et al.* 2010). Climate change impacts, such as water stress, are also significant, as they can alter fungal communities and increase the pathogenic potential of otherwise benign endophytes (Kivlin *et al.* 2013; Lu *et al.* 2021).

Although endophytic fungi are usually harmless or beneficial to their hosts, certain species, including *D. eres*, *Phomopsis* sp., and *Neofusicoccum* sp., can become pathogenic when the host is stressed or weakened, such as under conditions of climate change (Koike *et al.* 2003; Sieber 2007). The presence of fungi from families including *Pleosporaceae*, *Diaporthaceae*, and *Botryosphaeriaceae* in this study underscores the importance of monitoring these species, especially as stressors like global warming may exacerbate their pathogenicity (Rodriguez *et al.* 2009; Delaye *et al.* 2013). Previous studies have highlighted the prevalence of these genera across diverse regions and conditions, indicating their adaptive strategies and interactions with different environmental factors (Zimmerman and Vitousek 2012).

Recording new fungal species within a specific region contributes valuable information to the understanding of local ecosystems. This study provides the first records

in Türkiye for species such as *Alternaria destruens*, *Alternaria alstroemeriae*, and *Diaporthe cynaroidis*. The discovery of new or rare species species is significant because fungi play critical roles in ecosystem functions, including nutrient cycling, plant health, and soil stability. Identifying new or rare species helps reveal the complexity of biological diversity and provides insights into the ecological balance and resilience of ecosystems. Moreover, such findings enhance predictions about global fungal diversity, emphasizing their importance for biodiversity conservation and ecosystem sustainability (Petrini 1991; Taylor et al. 2006). Furthermore, an undescribed species was found, which future studies could characterize and document as a new species.

Geographical factors, as demonstrated in various studies, significantly affect fungal diversity. For example, research in Brazil's Atlantic Rainforest showed that location-based differences can impact the composition of fungal communities (Correia *et al.* 2018). Similarly, studies on *Laurus nobilis* in western Türkiye highlighted the influence of local environments on endophytes (Göre and Bucak 2007). Additionally, environmental factors like altitude have been linked to variations in endophytic diversity (Yin *et al.* 2021), indicating the importance of geographic context in understanding these microbial communities.

Sampling methods also play a crucial role in accurately assessing fungal diversity. In contrast to permanent organs, leaves undergo seasonal changes that can influence fungal colonization. In this study, consistent sampling of leaves at the same age in September was chosen to minimize seasonal variation, acknowledging that higher rainfall in autumn may increase species richness (Vaz *et al.* 2014). However, sampling during other seasons might have uncovered additional fungi, highlighting the need for more comprehensive, year-round sampling to capture the full diversity (Oliveira *et al.* 2020; Debbarma *et al.* 2024).

The orientation of sampling within the plant significantly influences endophytic diversity. For example, variations in microclimate conditions, such as sunlight exposure, between different sides of a tree (e.g., northern versus southern exposure) can affect fungal communities (Bacon and White, 2000). In this study, higher overall diversity was observed in the south-facing samples, potentially due to increased sunlight exposure, which can enhance microbial activity by creating favorable growth conditions (Atsatt and Whiteside 2014). Conversely, the Chao1 index, which reflects the abundance of rare or less common species, was higher in the north-facing samples. This suggests that the cooler and more humid microclimate on the northern side may provide conditions favorable for the growth and persistence of these less common fungal species. Additionally, factors such as increased organic matter accumulation, distinct soil structure, and other abiotic conditions on the northern side likely contribute to these differences in diversity. These findings underscore the importance of considering microhabitat variability when analyzing fungal community composition.

Tree proximity also impacts endophytic diversity. Trees located close to each other are likely to share similar environmental conditions, leading to similar microbial communities (Rodriguez *et al.* 2009). However, increasing distances can introduce variations in microhabitats, which may result in more diverse or distinct fungal communities (Arnold and Herre 2003). In this study, intermediate distance sampling (50 to 100 m apart) showed that even within the same area, different trees could host distinct fungal populations, demonstrating how local environmental factors shape community structure.

Endophytic diversity is also influenced by plant tissue types. Each tissue, whether leaf, stem, or root, provides distinct microhabitats that can support varied fungal

communities (Rodriguez *et al.* 2009). For instance, leaves tend to host more diverse fungi due to their higher concentration of photosynthesis products, while root-associated fungi are more influenced by soil conditions (Carroll 1988; Arnold and Herre 2003). This study found more diversity in leaf tissues, with significant differences between tissues, supporting previous research (Rim *et al.* 2021).

The selection of culture media is another critical factor. Different media can support varying types of fungi, influencing the diversity observed during isolation (Bills and Polishook 1992). While PDA is a common medium, it may not capture slower-growing or less competitive species. In this study, PL-MEA mimicked natural conditions and showed the highest diversity, while WA was effective for isolating rare species, suggesting the benefits of using a variety of media (Arnold and Lutzoni 2007). However, culture-dependent techniques alone may miss certain species, which highlights the importance of integrating culture-independent methods, such as next-generation sequencing (NGS), for a more comprehensive view (Sun *et al.* 2012; Zhang and Yao 2015).

CONCLUSIONS

1. This study successfully identified a diverse community of fungal endophytes within the leaves and petioles of *Liquidambar orientalis*, highlighting the presence of 26 species across 15 families, all belonging to the Ascomycota phylum. The results demonstrate considerable diversity, especially within the families *Diaporthaceae*, *Pleosporaceae*, and *Botryosphaeriaceae*, indicating the adaptability and ecological significance of these endophytes.
2. The use of DNA barcoding, including ITS and Beta-tubulin regions, enabled precise species identification, overcoming limitations of morphological classification. This method allowed the detection of several species recorded for the first time in Türkiye, demonstrating the value of molecular approaches in fungal biodiversity studies.
3. Fungal diversity analyses using Shannon, Simpson, and Chao1 indices revealed that tissue type was the primary factor influencing endophyte distribution, followed by culture media and spatial variation. This suggests that specific plant tissues provide unique microhabitats that shape endophytic communities.
4. The presence of pathogenic families, such as *Botryosphaeriaceae*, underscores potential threats to *L. orientalis*, particularly under conditions of environmental stress. Monitoring these species is essential for understanding their impact on plant health and conservation efforts.
5. This research provided the first records of several fungal species in Türkiye, including A*lternaria destruens*, *Alternaria alstroemeriae*, *Stemphylium majusculum*, and others. These findings contribute to the broader understanding of fungal diversity in the region and highlight the ecological importance of *L. orientalis* as a host species.
6. As the first comprehensive study of fungal endophytes in *L. orientalis*, this research sets a foundation for future studies on the ecological roles, potential benefits, and risks associated with endophytic fungi in endangered plant species. Further investigations, especially with culture-independent techniques, could enhance the understanding of fungal diversity and inform conservation strategies.

ACKNOWLEDGMENTS

The authors are thankful to Pamukkale University BAP department (Project no: 2023FEBE037) for their financial support.

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Article submitted: October 20, 2024; Peer review completed: November 15, 2024; Revised version received: November 19, 2024; Accepted: November 26, 2024; Published: December 9, 2024.
DOI: 10.15376/biores.20.1.1251-1272