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Sabiha Acer[1], Zeynel Arslangündoğdu[1] and Asko Lehtijärvi[2]

The occurrence and pathogenicity of fungi associated with *Orthotomicus erosus* on *Pinus brutia* in the Southern Marmara, Türkiye

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Highlights

- Three ophiostomatoid fungi species are recorded, associated with *Orthotomicus erosus* on *Pinus brutia* for the first time in Türkiye.
- *Ceratocystis ips* has the highest frequency. The association between *Leptographium wingfieldii* and *Orthotomicus erosus* occurred with high frequency.
- While all three fungal species are severely pathogenic for pines in some regions, they are weak pathogens for Turkish pine in Türkiye.

Abstract

Fungal pathogens associated with bark beetles constitute one of the most significant problems to forest health. The Turkish pine (*Pinus brutia* Ten.) is a native species in the forests of Türkiye and occurs in the Mediterranean-type climate. The Southern Marmara is a natural occurrence area of Turkish pine in the Marmara Region. In the present study, trap logs were set up in pure *Pinus brutia* forests to investigate fungi associated with *Orthotomicus erosus* (Wollaston) (Mediterranean pine beetle) throughout Southern Marmara. *Orthotomicus erosus* adults, larvae, and their galleries were sampled and individually cultured on a 1% CSMA (cycloheximide–streptomycin malt agar) medium. Three ophiostomatoid fungi were identified using morphological characteristics and molecular genetic analyses: *Ceratocystis* (syn. *Ophiostoma*) *ips* (Rumbold) C. Moreau, *Graphilbum* sp. H.P. Upadhyay & W.B. Kendr., and *Leptographium wingfieldii* M. Morelet. All three species were new in records of the fungal flora of Türkiye. The most dominant of these species, *Ceratocystis ips* was isolated 69%. Unexpectedly, *L. wingfieldii* had a high-frequency association with *O. erosus* (27%). The pathogenicity tests showed that all three species could cause lesions on branches of Turkish pine but were non-pathogenic or weak pathogenic.

Keywords *Ophiostoma*; *Graphilbum*; *Leptographium*; Mediterranean pine beetle; Turkish pine; pathogenicity; Southern Marmara
Addresses [1]Istanbul University-Cerrahpaşa, Department of Forest Entomology and Protection, Faculty of Forestry, 34473, Istanbul, Türkiye; [2]Isparta University of Applied Sciences, Sütçüler Prof. Dr. Hasan Gürbüz Vocational School, 32950, Isparta, Türkiye
E-mail sacer@iuc.edu.tr
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1 Introduction

Pinus brutia Ten. (Turkish or Calabrian pine) is an important representative species of the genus *Pinus* in Türkiye. Although its major distribution area is along the Mediterranean coast of Türkiye, it also grows in small stands in Palestine, Jordan, Syria, Iraq, Lebanon, the Greek Islands, Italy, and Cyprus (Sarıbaş and Ekici 2004; Kavgacı et al. 2017). The natural occurrence area of Turkish pine is in Southern Marmara for Marmara Region (Atalay et al. 1998). Turkish pine is a species with a high growth rate and desirable timber qualities for the wood and paper industries. Today, the proportion of Turkish pine in afforestation ranges from 40% to 50% in Türkiye (Ürgenç 1998).

Turkish pine is a fast-growing coniferous species native to the Mediterranean region that requires high rainfall but tolerates a wide temperature range (Fady et al. 2003). It has a high genetic diversity and adaptability (Korol et al. 2002). *Pinus brutia* requires temperatures of –5 to 12 °C and water-demanding humid/sub-humid climates with annual rainfall ranging from 400 to 1300 mm (Chambel et al. 2013). The elevation limits of this species are between 0–600 m in Marmara Region (Atalay et al. 1998), but in the southern part of its natural range, it can reach 1200–1400 m (Chambel et al. 2013). It shows a distinct preference for south-facing slopes with high insolation and avoids sites with fog or high rainfall. It prefers shallow soils, limestone, schist, and other bedrock types (Atalay et al. 1998; Şentürk et al. 2019).

Climate change alters the distributions and population structures of forest pests and pathogens, the way they interact with trees, and their evolutionary capacity, while also affecting the ability of forest systems to resist and tolerate attacks (Linnakoski et al. 2019). Reis et al. (2018) showed that in a Turkish pine forest characterized by semi-arid extreme summer drought, the annual diameter increments of trees in a local high-humidity area compared to a semi-arid field with the same elevation and aspect characteristics were significantly high. In addition, substantial increases or decreases in precipitation cause significant growth differences in diameter increment in Turkish pine stands where arid climatic conditions prevail. Furthermore, disturbances in water availability, particularly water shortage conditions, can have unfavourable effects on the tree host and benefit the infectivity of the pathogens in the host (Terhonen et al. 2019).

Pure natural stands of Turkish pine are mostly found in fire-prone areas in Türkiye (Turna and Bilgili 2006; Tavşanoğlu and Gürkan 2009; Bilgili et al. 2019). Fire is a major disturbance in *P. brutia* forests, and several adaptations generally contribute to the post-fire regeneration of Turkish pine (Boydak 2004). However, fires cause wounds that disrupt living tissues, including the vascular cambium, resulting in a loss of function in surviving trees (Sutherland and Smith 2000). Post-fire stress causes injured trees to use available carbohydrate storage to replenish dead or injured fine roots, thereby depleting carbohydrate reserves. Then, these fire-weakened trees could succumb to second-order pests, diseases, or climatic stress (Otrosina et al. 1999; Menges and Deyrup 2001; Sword Sayer and Haywood 2006; Morgan Varner et al. 2009; Alexou and Dimitrakopoulos 2014).

Previous studies have shown that *P. brutia* contains a variety of biotic stressors. The pine processionary moth attacks (*Thaumetopoea pityocampa* Dennis & Schiff. and *T. wilkinsoni* Tams.) is a considerable problem faced by pure stands of *P. brutia* (Kanat et al. 2005; Sbay and Zas 2018). They are the primary insect defoliator of Mediterranean pines and can cause significant growth loss and overall decline (Jacquet et al. 2012). Defoliation by pine processionary moths removes photosynthetic material and affects several vital functions. Semiz et al. (2016) reported that the levels of GST (Glutathione S-transferase enzyme) transcripts were significantly high in moth feeding period samples of *P. brutia*. They also suggested that GST could be a valuable marker created by pine processionary moth herbivory stress on *P. brutia*. The combined effects of previous herbivory and drought on tree growth and carbohydrate pools show significant additive effects (Jacquet et al. 2014). *Marchalina hellenica* Genn. is known as the primary source of pine honey production, and

pine honey producers spread the beetle by manually transporting it into different forest areas in Türkiye. It lives on pine trees, especially *P. brutia* and *P. halepensis* Mill. in Türkiye and Greece (Margaritopoulos et al. 2003; Akkuzu et al. 2006). *Marchalina hellenica* feeds by absorbing the sap, which is 20% protein and 80% carbohydrate, from the vascular bundles of trees and directly damages the photosynthetic tissue, weakening and stressing the pine trees (Bacandritsos et al. 2004). Yeşil et al. (2005) and Gallis (2007) showed that *M. hellenica* had a negative effect on the growth of infested *P. brutia* and *P. halepensis* trees in Türkiye and Greece.

Like other coniferous, Turkish pine is stressed and weakened by climate change, forest fires, and invasion of insect defoliators or scale insects and has trouble withstanding attacks by secondary biotic pests (Ciesla 2011; Linnakoski et al. 2019). *Orthotomicus erosus* (Wollaston) is the most abundant bark beetle species that attacks Turkish pine (Kalapanida-Kantartzi et al. 2010; Sarıkaya and Avcı 2011; İbiş and Sarıkaya 2012; Acer et al. 2021). It is named the Mediterranean pine beetle or the Mediterranean pine engraver beetle. Although *O. erosus* can attack *Cedrus*, *Abies*, and *Picea* spp., it primarily attacks pine species and reproduces only in *Pinus* spp. (Mendel and Halperin 1982). The beetle is a secondary pest that infests recently fallen trees and wounded and stressed living trees by forming galleries under the bark and reproducing in them. Stressed trees are more prone to attacks; therefore, attacks are more intense in successive years of drought and in trees ravaged by fire or storms. Weakened, infested trees often die, and where populations are high, massive attacks can lead to the death of healthy trees (Gil Sánchez and Pajares Alonso 1986). In Croatia, it was reported that *O. erosus* occurrence increased owing to cumulative stress on trees caused by drought intensity, frequency and aridification trends. Increased voltinism, high dispersal abilities by flight and easy transportation with the infested material of *O. erosus* altered the population level of this pest and became a crucial forest pest (Pernek et al. 2019). Due to attacks of bark beetles, especially *O. erosus*, from 2005 to 2015 in Balıkesir province alone, control measures were implemented in approximately 8700 ha of coniferous stands (Cebeci and Baydemir 2019).

Beetles carry a great diversity of wood-inhabiting fungi, and environmental factors, vector beetle communities, and, to some extent, fungal source communities are determinants of beetle-associated fungal communities (Seibold et al. 2019). The interactions between beetles, fungi, and the host plant have been well documented (Batra 1903; Beaver 1989; Schowalter and Filip 1993; Krokene and Solheim 1998; Harrington 2005; Six 2012; Hofstetter et al. 2015). Associations among pine species, bark beetles, and ophiostomatoid fungi are among the most significant host-beetle-mycobiota associations (Rane and Tattar 1987; Lieutier et al. 1989; Nevill et al. 1995; Dong Zhou et al. 2001; Jacobs and Wingfield 2001; Solheim et al. 2001; Sabbatini Peverieri et al. 2006; Romón et al. 2007). The ophiostomatoid fungi include nearly 400 species (including asexual stages) in 14 genera classified within the Ophiostomatales and Microascales, of which 134 species belong to the *Ophiostoma* sensu lato (De Beer et al. 2013, 2022). Few studies have investigated the association among Turkish pine-*O. erosus*-ophiostomatoid fungi (Ben Jamaa et al. 2007; Dori-Bachash et al. 2015). Furthermore, it has been reported that *O. erosus* transmits ophiostomatoid fungi to other pine species (Romón et al. 2007).

Only a few previous studies on ophiostomatoid species have been conducted in Türkiye. *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Braiser are responsible for Dutch elm disease in Europe, North America, and Asia. Although the disease agent was not detected, elm deaths were reported in Türkiye by Acatay (1940). Karahan and Maden (1979) isolated *Graphium* (*Ceratocystis*) *ulmi* M.B. Schwarz from elm trees and *Graphium* sp. from poplar trees in Central Anatolia. In 1980, Brasier (1991) reported *O. novo-ulmi* from Southern Türkiye. Sümer (1983) investigated the spread of the disease based on elm deaths in Türkiye. Lehtijärvi et al. (2018) isolated *Ceratocystis platani* (J.M. Walter) Engelbrecht & Harrington, which causes canker stain disease of *Platanus* trees on the European side of Istanbul. Many studies have discussed fungi

associated with bark beetles on pines worldwide (e.g. Jacobs et al. 2004; Sabbatini Peverieri et al. 2006; Jankowiak and Kot 2011; Dori-Bachash et al. 2015). However, no study has been conducted on the fungal species carried by bark beetles associated with any coniferous species in Türkiye.

The objective of this study was to isolate and identify common fungi associated with *O. erosus*, which attacks Turkish pine in both natural and planted Turkish pine forests in the Southern Marmara Region of Türkiye. In addition, the pathogenicity of the isolated fungi was studied by inoculating Turkish pine branches in planted forest.

2 Materials and methods

2.1 Sample collection and morphological observations

Our research area was located in the Balıkesir and Çanakkale Provinces in the southern part of the Marmara Region of Türkiye (39°20′N to 40°45′N, 25°37′E to 28°30′E) (Fig. 1). The area includes coastal regions in the Marmara and Aegean seas of Türkiye and is the location of the Strait of Çanakkale (Doğukan et al. 2008; CSB 2013). The climate in the region is the characteristic transi-

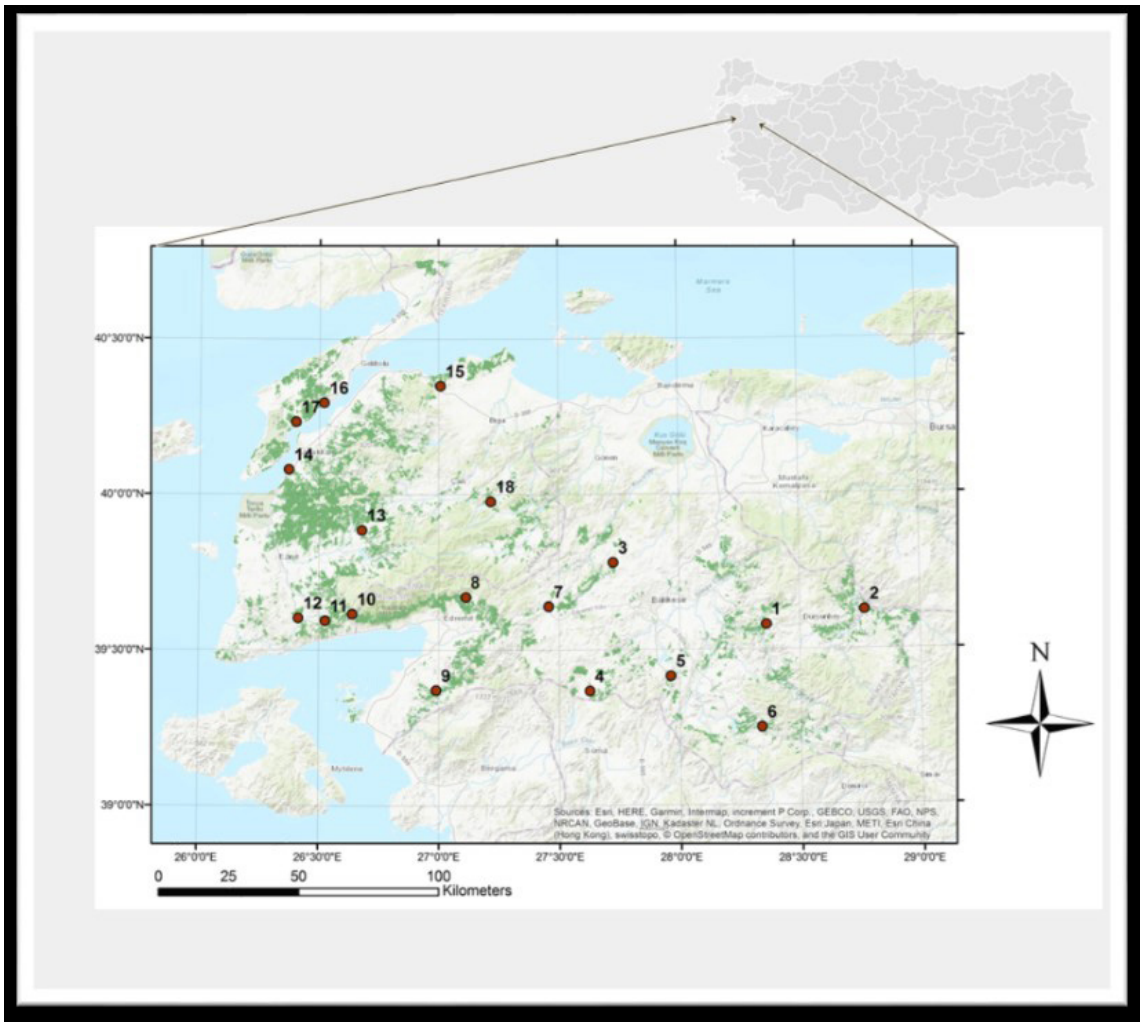

Fig. 1. Map of Southern Marmara, Türkiye, showing study sites denoted by 1–18 (with the distribution area of Turkish pine shown in dark green).

tional climate between the Mediterranean Sea and the Black Sea. The average annual temperature is 14.8 °C, with a maximum summer temperature of 30.6 °C and a minimum winter temperature of 2.7 °C. The average annual rainfall is approximately 615.4 mm (MGM 1998). The prevailing soil types in the FAO system are eutric cambisol and orthic luvisol (FAO 2022). Forty-three percent of the Southern Marmara Region is covered with forests, of which 66% Turkish pine (Karagöz and Demirci 2006). This region is the boundary of the natural distribution of Turkish pine in the north.

Eighteen representative study sites were selected, covering the distribution range of Turkish pine within the study area. The sites contained 20- to 25-year-old pure Turkish pine stands. In February 2014, 15 to 20 trap logs, approximately 1 m long and 0.2 m in diameter (bark thickness ~ 2 cm), were set in each study site, using the technique described by Tribe (1992). To examine the presence of fungi, *O. erosus* individuals and samples of blue-stained wood infested by this bark beetle were collected from trap logs in all 18 Turkish pine stands. The study sites, elevations, and geographical coordinates were also noted (Table 1) and indicated on a map using ArcGIS 10.2 (ESRI 2014) (Fig. 1). Trap logs were checked every 20 days, and three logs at each site were inspected for the presence of beetle entrance holes. All beetles brought to the laboratory were morphologically identified using a LEICA S8APO stereomicroscope (Grüne 1979; Selmi 1998).

2.2 Fungal isolation and identification

Trap logs were debarked and then the bark beetle adults (*O. erosus*), larvae, pupae, and galleries were collected. The larvae, pupae, and adults and pieces of the galleries measuring 1 cm^2 were surface sterilized with 0.05% NaClO, washed three times with distilled water, and blotted on filter paper. Fungal isolations were performed in Petri dishes on autoclaved 1% CSMA (cycloheximide–streptomycin malt agar; 10 g malt extract, 15 g agar, 1000 ml distilled water, 5 ml cycloheximide solution, and 100 mg streptomycin). Each insect and gallery piece was placed separately in a Petri dish. The cultures were incubated at 20 °C in the dark until colonies were formed. The actively growing colonies were subcultured on 2% malt extract agar (MEA) and incubated at 20 °C. The fungal isolates were classified into three morphological groups according to the culture morphology. Representatives of each of these groups were identified based on the morphological characteristics of the ascomata, ascospores, conidiophores, and conidia and DNA sequencing.

Table 1. Geolocation of study sites where wood traps were established in Southern Marmara, Türkiye.

Study site	Geographical position	Elevation (m)	Study site	Geographical position	Elevation (m)
1. Kireç	39°34’46.6”N 28°21’42.1”E	508	10. Küçükkuyu	39°37’02.6”N 26°38’19.3”E	557
2. Gökçedağ	39°37’29.5”N 28°46’12.2”E	467	11. Yeniçam	39°35’43.2”N 26°31’35.2”E	379
3. Balya	39°46’52.1”N 27°43’33.5”E	448	12. Ayvacık	39°36’14.7”N 26°24’52.80”E	269
4. Savaştepe	39°22’07.0”N 27°37’34.9”E	275	13. Bayramiç	39°53’11.2”N 26°40’44.9”E	204
5. Konakpınar	39°24’57.7”N 27°57’44.8”E	442	14. Çınarlı	40°4’53.6”N 26°22’24.9”E	43
6. Sınıdırgı	39°14’58.3”N 28°20’17.4”E	321	15. Dişbudak	40°20’59.8”N 27°00’24.3”E	133
7. İvrindi	39°38’26.2”N 27°27’25.7”E	310	16. Gelibolu	40°17’45.3”N 26°31’11.8”E	26
8. Edremit	39°40’15.3”N 27°06’43.5”E	372	17. Eceabat	40°14’04.0”N 26°24’10.9”E	107
9. Burhaniye	39°22’19.5”N 26°59’19.8”E	311	18. Yenice	39°58’41.6”N 27°13’0.6”E	380

The fruiting structures were mounted in lactophenol on glass slides and characterized using LEICA DM 750 light microscope. Measurements were made of 50 of each morphological structure so that the ranges and average size values could be calculated. The growth ability was determined at different temperatures ranging from 5 °C to 35 °C at intervals of 5 °C; three replicates were used. Agar disks (5 mm in diameter) taken from the edge of a freshly grown colony were placed on 2% MEA medium (20 ml) in 90 mm petri dishes. Colony diameters (two perpendicular measurements) on each plate were determined at 3, 5, and 7 d after incubation, and growth rates were calculated in millimetres per day. Fungal structures were compared with the species descriptions given in the literature (e.g. Jacobs and Wingfield 2001; Wingfield et al. 1993). The cultures used in this study were stored in the culture collection of Istanbul University-Cerrahpaşa, Faculty of Forestry, Department of Forest Entomology and Protection in Istanbul.

Identification based on morphology was confirmed by DNA sequencing of representative isolates (Tab. 1). DNA was extracted from pure fungal cultures after incubation for 8–10 d at 20 ℃ in 2% MEA. For DNA preparation, agar plugs taken from MEA cultures were planted onto sterile cellophane sheets overlaid on 2% MEA plates. After 3–7 d of growth at 20 ℃ in the dark, the mycelium was harvested from the cellophane sheet by scraping the surface with a scalpel (Kim et al. 2003; Roe et al. 2010). DNA extraction was performed using the EURx GeneMATRIX Plant & Fungi DNA Purification Kit, following the manufacturer's instructions. The internal transcribed spacer (ITS) and 5.8S regions of the nuclear rRNA operon were amplified using the primers ITS1 (5'-CCGTAGGTGAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC-3') (White et al. 1990; Kim et al. 2003). Amplification of the D1/D2 region of the nuclear large subunit of the rRNA (LSU) gene was conducted using the primers NL1 (5′-GCATATCAATAAGCGGAG-GAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Guadet et al. 1989; Kolařík et al. 2006). Each PCR amplification was performed in a total volume of 35 µl consisting of 0.3 µM of each primer, 1x PCR buffer, 0.2 µM dNTPs, and 2 U Taq DNA Polymerase (Solis Biodyne FIREPol, Tartu, Estonia). The conditions used for the thermal cycling were as follows: an initial denaturation of the DNA at 95 °C for 5 min, followed by 40 cycles consisting of denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. The PCR products were separated on a 1.5% agarose gel and visualized under UV light. Amplification products were purified with MAGBIO (HighPrep PCR Clean-up System; MagBio Genomics, Inc., Gaithersburg, Maryland, USA) and sequenced at Macrogen Europe B. V. (Amsterdam, The Netherlands). ITS and LSU sequences were compared by a BLAST search against DNA sequences deposited in NCBI GenBank (1988) to identify the most similar available sequences. The ITS sequences of *C. ips* and *L. wingfieldii* and, LSU sequences of *Graphilbum* sp. are deposited in GenBank with accession numbers OM86751–OM867520, OM885001–O M885004, and OM883868 and OM883867, respectively (Table 2).

Information about the fungal strains used in this study is summarized in Tables 2 and 3. ITS and LSU sequence in this study and 47 isolates of ophiostomatoid fungi were aligned with ClustalW using Bioedit v.7.2.5 (Thompson et al. 1994). ML phylogenetic analysis was performed using MEGA v.11.0.13. For the phylogenetic relationship of *C. ips* estimated T92+G evolutionary model determined by Model Test based on Akaike Information Criteria (AIC) was applied. The estimated proportion of the shape parameter for the gamma distribution (G) was set to 0.39. For the phylogenetic relationship of *Graphilbum* sp., the TN93+G evolutionary model and gamma distribution (G) were set to 0.1. The phylogenetic tree of *Graphilbum* sp. was rooted in *Ceratocystiopsis manitobensis* (J. Reid & Georg Hausner) Zipfel, Z.W. de Beer & M.J. Wingf. while *L. wingfieldii* was determined by the T92 evolution model, the tree was rooted in *Ophistoma quercus* (Georgev.) Nannf., in Melin & Nannfeldt. (Tamura et al. 2021). The best-fit model of nucleotide substitution was calculated in MEGA 11 under default parameters using the Bayesian information criterion

Table 2. Representative fungal isolates of Ophiostomatoid fungi associated with *Orthotomicus erosus* on *Pinus brutia* during the current study.

Taxon	Isolate name	Study site	GenBank accession no. LSU	GenBank accession no. ITS	Close match in BLAST	Accessison of match	Identity %
Ceratocystis ips	CZ27.52	18	-	OM867517	*Ophiostoma ips**	OM468593	99.7
	CZ51.1	1	-	OM867518	*O. ips*	OM468597	99.7
	CZ24.21	15	-	OM867519	*O. ips*	OM468593	99.7
	CZ30.21	4	-	OM867520	*O. ips*	OM468597	100
Graphilbum sp.	CZ20.19	11	OM883868	-	*Graphilbum rectanglosporium*	OM514754	99.1
	CZ30.6	4	OM883867	-	*G. rectanglosporium*	OM514754	99.1
	CZ30.24	4	-	-	-	-	-
	CZ44.9	17	-	-	-	-	-
Leptographium wingfieldii	CZ33.40	7	-	OM885001	*Leptographium wingfieldii*	KP691916	99.7
	CZ41.7	14	-	OM885002	*L. wing.fieldii*	KP691916	100
	CZ40.25	13	-	OM885003	*L. wing.fieldii*	KP691916	100
	CZ42.32	15	-	OM885004	*L. wing.fieldii*	KP691916	100

* *Ophiostoma ips* (syn. for *Ceratocystis ips*).

(Schwarz 1978). Support for the nodes was estimated from 1000 replications (Jankowiak 2012). Final adjustments were made in iTOL (https://itol.embl.de) (Letunic and Bork 2021).

2.3 Pathogenicity tests

Pathogenicity tests were conducted using four representative isolates of each fungal species (Table 2) from managed Turkish pine stands in the Istanbul Regional Directorate of Forestry in June 2019. The mean diameter at the breast height of the trees was 20 cm. The inoculum was prepared by growing the fungal isolates on 2% MEA at 20 °C in the dark. Sterile 2% MEA plugs were used as negative controls. Depending on the appropriate trees in the study area, each of the 12 isolates was tested in two trees, and five branches was inoculated in each tree; 24 trees were inoculated in total. Inoculations were made by removing the outer bark from the branches with a 5 mm cork borer, and 5 mm diameter agar plugs cut from the tested isolates were then placed with the mycelium facing downward into the wounds. Controls (a total of 40 branches in 8 trees) were inoculated with sterile 2% MEA. A total of 160 branches in 32 trees, including control, were assigned to pathogenicity treatments. All inoculation points were sealed with removed bark and masking tape to reduce desiccation. Inoculations were harvested after 20 weeks and measured. Re-isolations of the fungi were attempted from the inoculation points. Small pieces of tissue were cut from the edges of necrotic areas with a sterile scalpel and plated onto 1% CSMA. The plates were incubated in the dark at 21 °C for 3 weeks. Re-isolation plates were examined to confirm that the inoculated fungi caused the lesions by assessing colony morphology and microscopic characteristics. Analysis was performed with ANOVA using the GLM procedure in SAS software (SAS 2022). The lesion length data did not meet the normality assumption (Kolmogorov–Smirnov test, $p = 0.13$) and were log-transferred before analysis to correct for heteroscedasticity. The data were compared using Tukey's multiple comparison test. The re-isolation results were tested using the logit model in SPSS in IBM SPSS version 21 for Windows (IBM 2021).

Table 3. List of reference sequences used for the phylogenetic tree in this study and their GenBank accesion.

Species name	Isolate number	Type[1]	Isolated from	Country[2]	Collector	GenBank accession numbers[3]	
						LSU	ITS
Ceratocystiopsis manitobensis	UM237	T	*P. resinosa*/Manitoba beetle gallery	CAN	J. Reid	EU913674	
Graphilbum anningense	CXY1939		*P. yunnanensis/ T. yunnanensis*	CHN	H.M. Wang	MH325162	
G. cf. rectangulosporium	VPRI43763		*P. radiata*	AUS	A.J. Carnegie	MW046118	
G. cf. rectangulosporium	VPRI43843		*P. taeda*	AUS	C. Trollip	MW046119	
G. crescericum	CMW22828	T	*P. radiata/ H. palliatus*	ESP	P. Romón	OM514749	
G. fragrans	CBS 279.54	T		SWE	A. Mathiesen-Kaeaerik	MH868872	
G. fragrans	CBS 279.54		*P. sylvestris*	SWE	T.C. Harrington		AF198248
G. ipis-grandicollis	VPRI43762	T	*Pinus radiata/ I. grandicollis*	AUS	A.J. Carnegie	MW046117	
G. rectangulosporium	CMW26258				M. Procter	OM514754	
Grosmannia aurea	CMW667	A	*Pinus contorta* var. *latifolia*	CAN	R.W. Davidson		OM501387
Leptographium longiclavatum	SL-Kw1436			CAN			AY816686
L. lundbergii	CMW2190	T	*P. sylvestris*	NOR	H. Roll-Hansen		OM501432
L. pyrinum	CMW509	T	*D. adjunctus*	USA	K.R.W. Davidson J.		OM501445
L. terebrantis	CMW29841	T	*D. terebrantis*	USA	S.J. Baras		JF798477
L. wingfieldii	CBS 645.89	E	*T. piniperda*	FRA	M. Morelet		AY935603
L. wingfieldii	MB192		*P. halepensis/ T. destruens* gallery	ISR	M. Dori-Bachash		KP691916
L. wingfieldii	CMW4741		*P. densiflora*	JPN	H. Masuya		OM501461
L. wingfieldii	CBS 648.89	E	*P. brutia*	GRC	Mich.-Ska.		AY935611
L. wingfieldii	MCC 125		*P. densiflora*	JPN	M. Masuya		AY935608
L. wingfieldii	CMW2096		*P. strobus / T. piniperda*	EUR	M. Morelet		AY553398
L. wingfieldii	CMW2096	T		FRA	FIN		AY553398
Ophiostoma bicolor	CBS492.77	T	*Picea glauca*	CAN	S.M. Alamouti		DQ268604
O. floccosum	CMW34182	T	Wood	SWE	A. Mathiesen-Käärik		KU184431
O. fuscum	CMW23196	T	*Picea abies/ P. chalcographus*	FIN	Linnakoski		HM031504
O. ips	AK188		*Ips acuminatus*	UKR	K.V. Davydenko		KU663983
O. ips	CMW7075	T	*I. integer*	USA	CT Rumbold		AY546704
O. ips	MB176		*P. halepensis/ O. erosus*	ISR	Dori-Bachash		KP691908
O. ips	CMW6418		*P. elliottii/O. erosus*	ZAF	XD Zhou		AY546702
O. ips	S36.9		Pine wood	PRT	C.S. Vicente		OM468593
O. ips	S40.1a		*P. pinaster*	PRT	C.S. Vicente		OM468597
O. ips	MCC 023		Tp beetle	JPN	H. Masuya		AY194935
O. japonicum	CMW2202	T	*I. typographus japonicus/Picea jezoensis*	JPN	Y. Yamaoka		OM501492
O. montium	CMW15419		*P. contorta*	USA	B. Bentz		OM501498
O. piceae	C1087	T		DEU	Münch		AF198226
O. pseudobicolor	CFCC52683	T	*I. subelongatus/ Larix gmelinii*	CHN	Q. Lu		MK748188
O. quercus	CMW2467	T	*Quercus* spp.	FRA	M. Morelet		AY466626
O. rectangulosporium	MAFF 238951			JPN	N. Ohtaka	AB235158	

[1]T = ex-type, E = ex-epitype, A = authentic isolate.
[2]AUS: Australia, CAN: Canada, CHN: China, DEU: Germany, ESP: Spain, EUR: Europe, FIN: Finland, FRA: France, GRC: Greece, ISR: Israel, JPN: Japan, NOR: Norway, PRT: Portugal, SWE: Sweden, UKR: Ukraine, USA: United States of America, ZAF: South Africa.
[3]ITS: internal transcribed spacer, LSU: ribosomal large subunit.

3 Results

3.1 Fungal identification

Based on culture morphology, isolates were divided into three distinct groups. The first group produced perithecia, the second formed only mycelia, and the third group contained the *Leptographium* anamorph. The morphological and molecular evidence showed that three ophiostomatoid fungi associated with *O. erosus* were identified from Turkish pine in this study. They were *Ceratocystis ips* (syn. *Ophiostoma ips*), *Graphilbum* sp.*,* and *Leptographium wingfieldii.*

***Ceratocystis ips* (Rumbold) C. Moreau (syn. *Ophiostoma ips* (Rumbold) Nannf.)**, Revue Mycol., Paris 17 (Suppl. Colon. no. 1): 22 (Moreau 1952). The fungus grew optimally at 25 ℃ to 68 mm in diameter on 2% MEA in 10 days. No growth was found either below 5 ℃ or above 35 ℃ on MEA; the colony was pale brown to hyaline (i.e., glossy and translucent); and the hyphae of the fungus were immersed (Fig. 2a). The perithecia were globose and dark brown to black, (203.5–) 305.4 – 473.5 (–635.1) µm in diameter, ornamented with light brown aseptate hyphae and the necks were nearly cylindrical and dark brown, becoming brown at the apex, (137.6–) 1182.2 (–1393.1) µm in length with absent ostiolar hyphae. The ascospores were hyaline and one-celled and had a hyaline gelatinous sheath appearing pillow-shaped, (3.2–) 4.04 – 5.56 (–6.1) × (1.43–) 2.1 – 3.1 (–3.4) µm (Fig. 2b). Regarding the size of the measurements, in this study they were variable, compared to Rumbold (1931), Wingfield and Marasas (1980), Hutchison and Reid (1988), Pérez-Vera et al. (2009), and Kim et al. (2011).

Molecular identification of the CZ24.21, CZ27.52, CZ51.1 and CZ30.21 isolates was made using the amplified sequence of the ITS gene region of the genomic DNA. A BLAST search of the GenBank database using the determined sequence revealed the highest similarity (99.7% to 100%) to that of *Ophiostoma ips.* Three distinct groups are apparent from the phylogenetic analysis. All isolates of *C. ips* were placed in one clade and separated from another containing the sapstain species *O. fuscum* Linnak., Z.W. de Beer & M.J. Wingf., *O. bicolor* R.W. Davidson & D.E. Wells, *O. montium* (Rumbold) Arx. *O. japonicum* Yamaoka & M.J. Wingf., and *Ophiostoma pseudobicolor* Z. Wang & Q. Lu, in Wang, Liu, Wang, Meng, Liu, Decock, Zhang & Lu. The remaing were placed in the third clade (Fig. 2c).

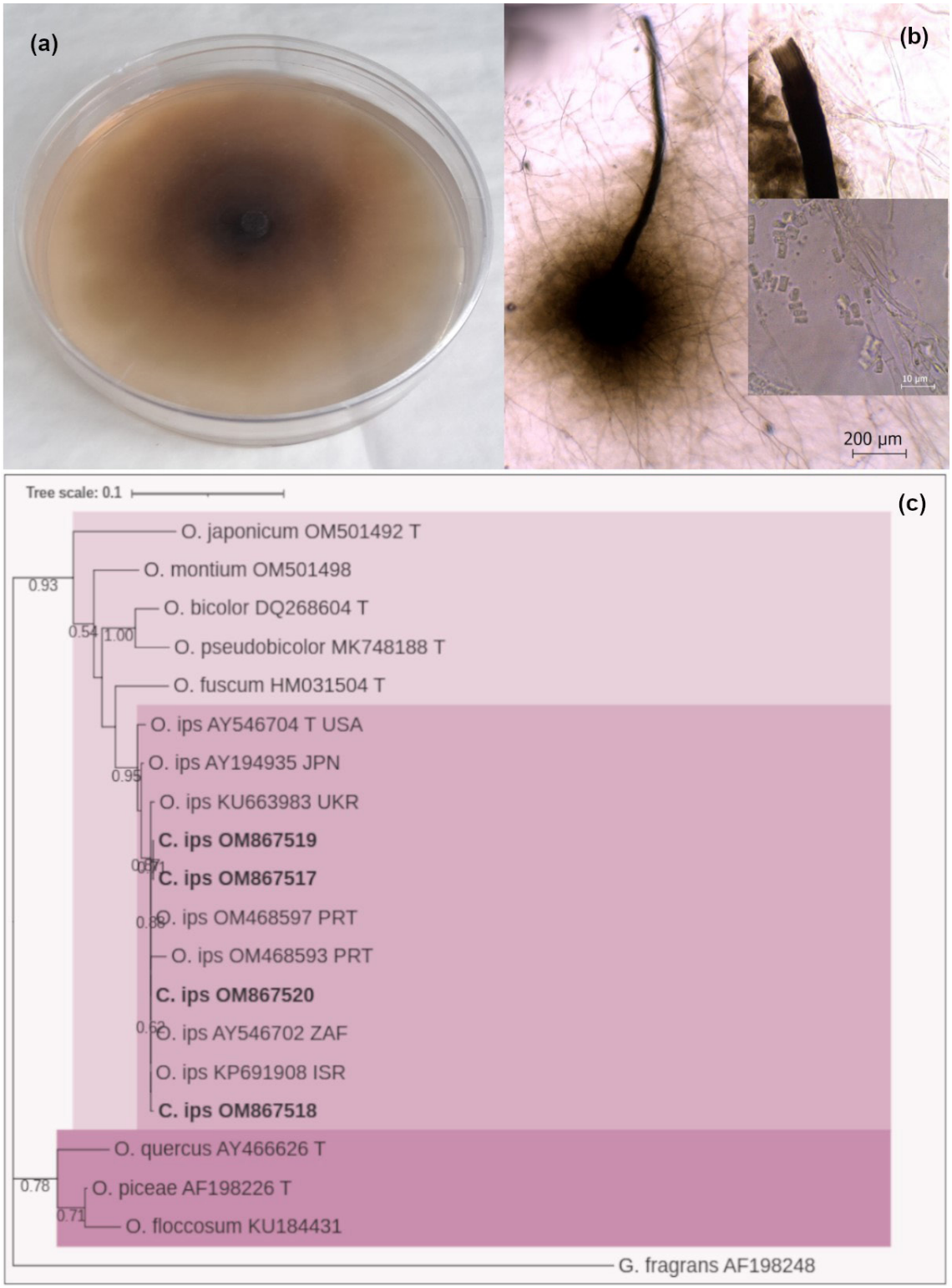

Fig. 2. (a) Colony characteristics of *Ceratocystis ips* on 2% MEA, 25 °C for 10 d: (b) light microscopic micrographs; peritechia, ostium and ascospores of *C. ips*, (c) phylogenetic estimate, based on the ITS and 5.8S regions of the nuclear rRNA operon sequence analysis, showing potential phylogenetic relationships. The tree is rooted in *Graphilbum fragrans*. The tree was constructed with the MEGA program and evaluated using the bootstrap procedure (1000 replicates). Only bootstrap values >50% were provided. The analysis involved 20 nucleotide sequences. There were 1354 positions in the final dataset. The isolates obtained in this study are shown in bold.

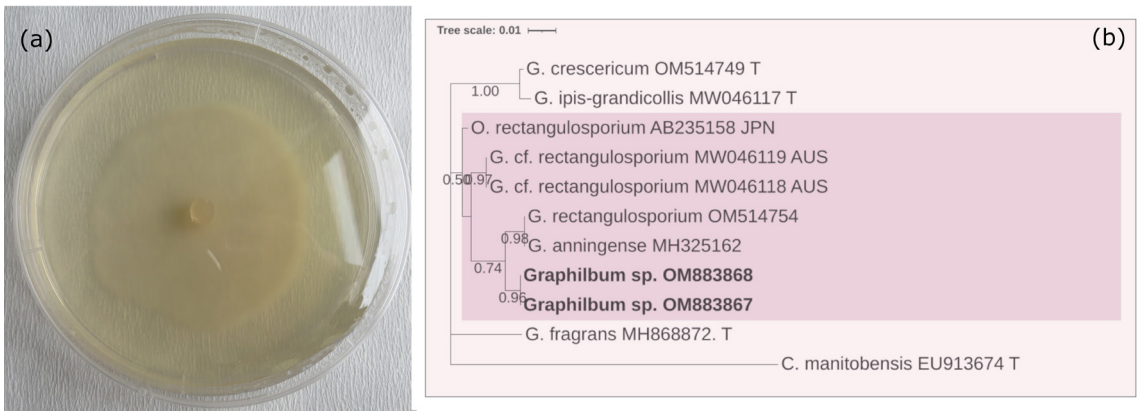

Fig. 3. (a) Colony characteristics of *Graphilbum* sp. grown on 2% MEA, 25 °C for 10 d. (b) Based on the LSU sequence analysis, a phylogenetic estimate showed potential phylogenetic relationships. The tree was constructed with the MEGA program and evaluated using the bootstrap procedure (1000 replicates). Only bootstrap values >50% were provided. This analysis included 11 nucleotide sequences. There were 896 positions in the final dataset. The isolates obtained from this study were printed in bold.

***Graphilbum* sp. H.P. Upadhyay & W.B. Kendr.**, Mycologia 67: 800 (1975). Hyaline colonies on 2% MEA showed optimal growth at 20 °C and were 19–21 mm in diameter after 10 days (Fig. 3a). No growth was observed at either 5 °C or 35 °C. There were only hyaline hyphae observed, with no conidia or perithecium.

A BLAST search of the GenBank database using the determined sequence revealed that the highest similarity (99.1%) was with that of *Graphilbum rectangulosporium* (Ohtaka, Masuya & Yamaoka) Z.W. de Beer & M.J. Wingf. In the phylogenetic tree, our *Graphilbum* sp. isolates were placed in one clade together with *Graphilbum anningense* H.M. Wang, Q. Lu & Zhen Zhang and *G. rectangulosporium* (Fig. 3b).

***Leptographium wingfieldii* M. Morelet**, Ann. Soc. Sci. Nat. Arch. Toulon et du Var 40(1): 43 (Morelet 1988). It grew optimally at 25 °C and covered all 90 mm of the petri dish on 2% MEA medium in 10 d (Fig. 4a). No growth was observed at either –5 °C or 35 °C. The dark brown colonies were immersed and covered with aerial mycelia. Conidiogenous apparatus observed that singly or in groups is light in colour and (63.6–) 66.3 – 93.5 (–117,3) µm long. The conidia were highly variable in length and width and occasionally slightly clavate, transparent, non-segmented, (4.9–) 5.7 – 7.5 (–8.7) × (1.8–) 2.5 – 3.1 (–4.2) µm. The ageing hyphae were dark brown, while the younger hyphae were yellowish. The conidiophores of these isolates also had a slightly yellowish colour, similar to those of the conidiophores of *L. wingfieldii* (Fig. 4b). These findings were like those reported by Jacobs and Wingfield (2001) for this species.

A BLAST search of the GenBank database using the determined sequence revealed that the highest similarity (99.7% to 100%) was with that of *L. wingfieldii.* In the phylogenetic tree, four *L. wingfieldii* isolates obtained from *P. brutia* attacked by *O. erosus* grouped together with other reference isolates of *L. wingfieldii*. Also, *Grosmannia aurea* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. and *Leptographium lundbergii* Lagerb. & Melin, in Lagerberg, Lundberg & Melin, and *Leptographium pyrinum* R.W. Davidson strains were grouped in this section (Fig. 4c).

In total, 694 fungal isolates were obtained from 664 adults, 17 larvae or pupae, and 23 gallery systems of *O. erosus*. Associated with *O. erosus*, 481 *C. ips* isolates were obtained from all but two of the study sites while 24 *Graphilbum* sp. and 187 *L. wingfieldii* isolates were taken from 10 and 11 study sites, respectively. The most dominant species was *C. ips*, with an average isolation frequency of 69%, followed by frequency of 27% *Leptographium wingfieldii* and 4% *Graphilbum* sp. (Table 4).

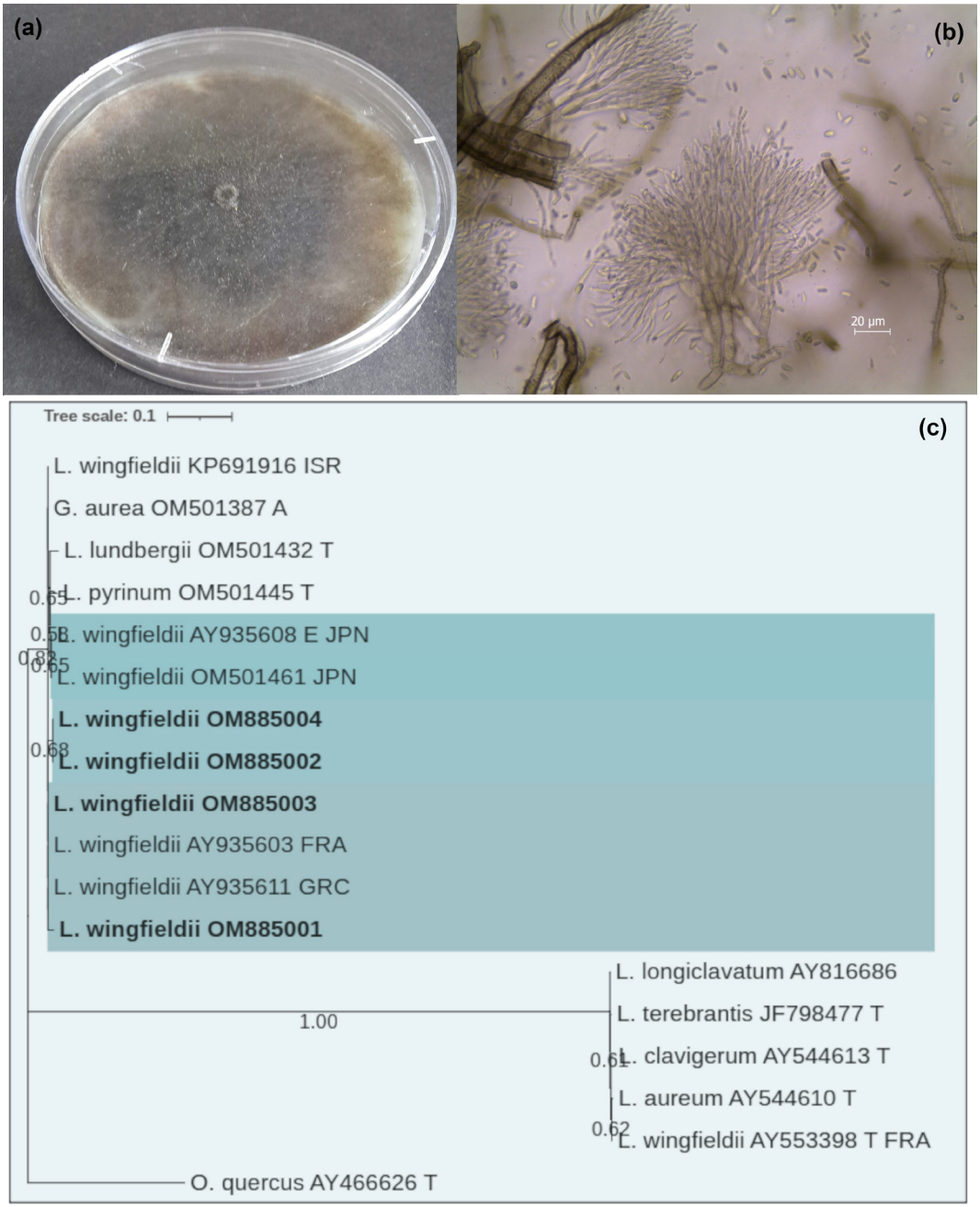

Fig. 4. (a) Colony characteristics of *Leptographium wingfieldii* grown on 2% MEA, 25 °C for 10 d. (b) Light microscopic micrographs: conidiophores and conidia of *L. wingfieldii*, (c) Phylogenetic estimate based on the ITS and 5.8S regions of the nuclear rRNA operon sequence analysis, showing the potential phylogenetic relationships. The tree is rooted in *O. quercus*. The tree was constructed with the MEGA program and evaluated using the bootstrap procedure (1000 replicates). Only bootstrap values >50% were provided. This analysis involved 18 nucleotide sequences. There were 842 positions in the final dataset. The isolates obtained from this study were printed in bold.

Table 4. Fungal species detected from *Orthotomicus erosus* adults, larvae, pupa and gallery (number of fungal isolates frequency) collected at all study sites.

Fungal Species	Study site/s	Gallery	Larvae/ Pupae	Beetle	Total	%
Ceratocystis ips	1,2,3,4,5,6,8,10,11,12,13,14,15,16,17,18	13	15	463	481	69
Graphilbum sp.	1,2,3,4,6,11,12,13,15,17	2	-	24	26	4
Leptographium wingfieldii	1,2,3,4,5,6,7,10,13,14,15	8	2	177	187	27

3.2 Pathogenicity tests

The lesion lengths ($F = 5.52$, $p < 0.0001$) were significantly affected by inoculation treatment in the linear model. In each case, no evidence was found to suggest the inoculation was responsible for wilting, drying, or mortality of branches of Turkish pine trees. All inoculations probably resulted in colonization, achieved by formed phloem necrosis and resin exudation around inoculation points. Phloem necrosis was seen on controls, but only several had resin exudations. The lesion lengths on the phloem caused by *C. ips* (with a lesion length average of 51 mm, CZ51.1) and *L. wingfieldii* (45 mm, CZ41.7) were more significant statistically than the ones caused by the controls ($F = 19.32$, $p < 0.0001$). However, there was a statistically significant difference in the lengths of lesion caused by *C. ips* and *L. wingfieldii*. *Graphilbum* sp. isolates induced significantly shorter lesions compared to *C. ips* and *L. wingfieldii* and did not differ from the control excluding one isolate (CZ20.19) ($F = 4.664$, $p < 0.0001$). There is a significant difference among the inoculated trees, by lesion length ($F = 3.57$, $p < 0.0001$). The third and sixth *P. brutia* individuals inoculated with respectively CZ27.52 and CZ51.1 isolates of *C. ips*, and the twelfth individual inoculated with the CZ41.7 isolate of *L. wingfieldii* caused significantly larger lesions than the others. All tested fungi were re-isolated from tissue surrounding lesions. The colony morphologies and microscopic characteristics of re-isolation colonies were confirmed with original pure cultures. However, infection from inoculated trees was confirmed in 91% *of C. ips*, 64% of *L. wingfieldii* and 52% of *Graphilbum* sp. (Table 5).

Table 5. Length (and standard error) of the lesion in phloem in branches of Turkish pine 4 months after inoculation with Ophiostomatoid spp.

Species	Isolate no.	Tree no.	Mean lesion length (mm)			Re-isolation %
			1th tree	2nd tree	Total	
Ceratocystis ips	CZ24.21	$1^{b}/2^{a}$	52±27.1	15.8±4.9	33.9^{a}±26.5	95.5
	CZ27.52	$3^{c}/4^{b}$	67±11.5	43.4±6.8	55.2^{bc}±15.3	86.4
	CZ51.1	$5^{a}/6^{c}$	55.8±8.6	75.2±30.5	65.5^{c}±30.5	86.4
	CZ30.21	$7^{b}/8^{b}$	50.4±9.9	51.8±10.7	51.1^{bc}±9.7	95.5
	Total			51.4±24.2		91
Leptographium wingfieldii	CZ33.40	$9^{b}/10^{a}$	63.8±69.9	25±11.1	44.4^{b}±51.4	86.4
	CZ41.7	$11^{b}/12^{c}$	48.2±6.7	83.6±22.9	65.9^{c}±24.5	68.2
	CZ40. 25	$13^{a}/14^{a}$	33.6±20.2	35.2±5.8	34.4^{b}±14.0	31.8
	CZ42.32	$15^{a}/16^{b}$	24±15.1	44.6±19.6	34.3^{b}±19.8	68.2
	Total			44.8±32.3		64
Graphilbum sp.	CZ20.19	$17^{b}/18^{a}$	43.6±9.5	28.6±12.2	36.1^{b}±13.0	77.3
	CZ30.6	$19^{a}/20^{a}$	40.6±14.2	21.4±5.3	31.0^{a}±14.3	40.9
	CZ30.24	$21^{a}/22^{a}$	30.6±13.9	28.4±13.8	29.5^{a}±13.1	4.5
	CZ44.9	$23^{a}/24^{a}$	31.8±3.4	26.6±9.3	29.2^{a}±7.2	86.4
	Total			31.5±12.1		52
Control		25^{a}–32^{a}		25.3^{a}±7.7		

Lesion length data were transformed (log10) before analysis though non-transformed values were reported. The same letters within the columns are not significantly different from one another at $p < 0.0001$

4 Discussion

To date, no study of ophiostomatoid species was conducted on conifers in Türkiye. In the present study, based on morphological characteristics, DNA sequence comparisons and phylogenetic tree analysis, three ophiostomatoid fungi species, including *C. ips*, G*raphilbum* sp., and *L. wingfieldii*, were identified associated with *O. ersosus* on *P. brutia* from Türkiye. All were new records for Türkiye's fungal flora but known commonly worldwide.

Ceratocystis ips is well-known both as a sapstainer and also as a tree pathogen (Mathiesen-Käärik 1960; Wingfield and Marasas 1980; Hutchison and Reid 1988; Lieutier et al. 1991; Jacobs and Wingfield 2001; Kirisits 2007; Min et al. 2009; Pérez-Vera et al. 2009; Jankowiak 2012; Davydenko et al. 2017). Zhou et al. (2007) found a higher genetic diversity in the North American than in the European population and suggested that North America could be the possible source region of *C. ips*. The microscopy measurements of *C. ips* were variable (Rumbold 1931; Hutchison and Reid 1988; Pérez-Vera et al. 2009; Kim et al. 2011). One of the three groups formed from phylogenetic analyses includes *C. ips* strains isolated from different geographies accompanying the *C. ips* isolates in the present study. *C. ips* was the most frequent (69%) fungal species isolated from *O. erosus*. Our results were in agreement with previous reports by Zhou et al. (2007) in which *C. ips* constituted 60% of the fungi isolated from *O. erosus* in South Africa. In addition, Dori-Bachash et al. (2015) found that *C. ips* was the most frequently encountered (52.4%) fungal species isolated from *O. erosus* in Israel. *Ceratocystis ips* was first described from *Ips calligraphus* (Germar), which was inhabiting *Pinus echinata* Miller, *Pinus sylvestris* L., and *Pinus rigida* Miller (Rumbold 1941). On the other hand, *C. ips* was isolated from *O. erosus* inhabiting *P. sylvestris*, *Pinus nigra* J.F. Arnold, *Pinus pinaster* Aiton, *P. halepensis*, *P. pinea* L., *Pinus patula* Schiede ex Schltdl. & Cham., *Pinus elliottii* Engelm, and *Pinus radiata* D. Don in preceding studies in other countries (Dong Zhou et al. 2001; Ben Jamaa et al. 2007; Ghaioule et al. 2007; Romón et al. 2007; Dori-Bachash et al. 2015; Musvuugwa et al. 2016).

In the present study, *Graphilbum* sp. produced neither a conidial nor a sexual stage; we identified this species via the results of rDNA sequencing and phylogeny analysis. They had LSU sequences that were similar to the sequence of *G. rectangulosporium*. *Ophiostoma* species without teleomorph or anamorph in culture are unusual (Kim et al. 2011). However, Ohtaka et al. (2006) reported a new species from Japan, *Ophiostoma rectangulosporium*, Ohtaka, Masuya & Yamaoka, with only teleomorph stage in culture via molecular analysis and phylogenetic tree. Besides, *G. rectangulosporium* was identified from *O. erosus*, on Turkish pine in Israel (Dori-Bachash et al. 2015). On the other hand, *Graphilbum* sp. isolates were phylogenetically closely related to *G. anningense* in this study. However, *G. anningense* forms the conidial stage in the 2% MEA medium and differs in colony characteristics and growth rate (Wang et al. 2019).

Microscopic measurements, colony characteristics and growth rate of *L. wingfieldii* isolates in this study were similar to the records in Jacobs and Wingfield (2001). In addition, four *L. wingfieldii* isolates from *P. brutia* attacked by *O. erosus* grouped with referance isolates of *L. wingfieldii* on the ML phylogenetic tree. Additionally, *G. aurea, L. lundbergii*, and *L. pyrinum* strains were placed close to this clade. *L. wingfieldii* has been reported to be the most common fungal species associated with *Tomicus* spp., especially *Tomicus piniperda* (L.) and *Tomicus destruens* (Wollaston) (Solheim and Långström 1991; Långström et al. 1993; Jankowiak and Kurek 2003; Jacobs et al. 2004; Sabbatini Peverieri et al. 2006; Ben Jamaa et al. 2007; Dori-Bachash et al. 2015). It was also reported that *L. wingfieldii* was isolated from *Hylastes opacus* Erichson in England (Wingfield and Gibbs 1991). In North America, the fungus was associated with *T. piniperda* as well as *Ips pini* (Say, T.) and *Dendroctonus valens* LeConte (Jacobs et al. 2004). Romón et al. (2007) obtained only one isolate of *L. wingfieldii* out of 219 isolates from *O. erosus* in Spain. Dori-Bachash et al.

(2015) isolated only one specimen from the gallery created by *O. erosus* in their research. In our study, *L. wingfieldii* was isolated with the second-highest frequency of 27% from 187 *O. erosus* individuals and gallery walls in 11 of 18 study sites. In this aspect, our study was the first to show that there was a significant association between *O. erosus* and *L. wingfieldii*.

We found only a few studies of fungal species associated with bark beetles of Turkish pine. Ben Jamaa et al. (2007) performed pathogenicity tests of fungal species isolated from *O. erosus* and *T. piniperda* on *P. halepensis*, *P. pinaster*, and *P. brutia* in Tunisia. Even though they did not confirm those fungal species (*O. ips* and *Ophiostoma minus* (Hedgc.) Syd. & P. Syd. and *L. wingfieldii*) on Turkish pine, they performed pathogenicity tests with those fungal species from other trees. In addition, they showed that the most susceptible species was Turkish pine. In Israel, three ophiostomatoid species, *C. ips*, *G. rectangulosporium*, and *L. wingfieldii*, were isolated from *O. erosus*, *T. destruens*, and *Pityogenes calcaratus* (Eichhoff) occurring on *P. brutia* and *P. halepensis* in forests (Dori-Bachash et al. 2015). In agreement with these reports, we obtained the same species from *O. erosus* on *P. brutia* but also *L. wingfieldii* in surprisingly high frequency. In the present study, the most abundant isolated fungus associated with *O. erosus* was *C. ips*, consistent with findings reported by Ben Jamaa et al. (2007) and Dori-Bachash et al. (2015).

Graphilbum sp. isolates did not cause significantly different lesions from the control. Hence, those isolates appeared to be non-pathogenic to Turkish pine. In agreement with our results, Dori-Bachash et al. (2015) showed with inoculation experiments that *G. rectangulosporium* isolates did not cause lesions on or deaths of *P. halepensis* or *P. brutia* seedlings. On the other hand, Jankowiak (2012) and Davydenko et al. (2017) showed that the inoculation of *G. rectangulosporium* isolates to Scots pine seedlings created significantly larger necrotic lesions than *C. ips*, as well as a loss of needles, an overall decline in health, and even death.

The lesion lengths on the phloem caused by *C. ips* and *L. wingfieldii* were significantly different from the controls. However, *C. ips* induced significantly larger lesions compared to *L. wingfieldii*. We contradict by Dori-Bachash et al. (2015), who reported that *C. ips* did not cause any lesions or wilting on *P. brutia* and *P. halepensis* in Israel. We agree with Nevill et al. (1995), who showed that single and combination inoculations of *C. ips* caused vertical lesions on 15- to 18-year-old loblolly pine trees, in Alabama.

Ben Jamaa et al. (2007) reported that *C. ips* caused shorter lesions than *L. wingfieldii* on *P. halepensis* in Tunisia. They suggested that *L. wingfieldii* was the most virulent agent and *C. ips* an intermediately virulent agent. In addition, they performed mass inoculations with two isolates of *L. wingfieldii* on *P. brutia* and *P. halepensis* trunks and revealed that *P. brutia* was more susceptible. Lieutier et al. (1989) isolated five ophiostomatoid fungal species, including *C. ips* and *L. wingfieldii*, from bark beetles of *P. sylvestris* in France. They proposed that *L. wingfieldii* was the most aggressive species, while *C. ips* was intermediately aggressive in pathogenicity experiments. Solheim et al. (2001) isolated three ophiostomatoid species, including *L. wingfieldii,* from *Tomicus* spp. on *P. sylvestris*, and then inoculated them to pine trees and showed that *L. wingfieldii* was more virulent. Dori-Bachash et al. (2015) showed that *L. wingfieldii* was responsible for the death of 2-year-old *P. halepensis* and *P. brutia* seedlings. Our results showed that *C. ips* was more aggressive than *L. wingfieldii* and were not in the agreement above studies.

It should be considered that there was a significant difference in lesion length among the inoculated trees in this study. The third and sixth *P. brutia* individuals inoculated with respectively CZ27.52 and CZ51.1 isolates of *C. ips*, and the twelfth individual inoculated with the CZ41.7 isolate of *L. wingfieldii* were more susceptible. These results suggest that susceptibility to each isolate obtained from the current study varies in different Turkish pine individuals.

5 Conclusion

The study included isolations of pure cultures of fungi from Mediterranean pine engraver beetles and their galleries, DNA sequencing, phylogenetic tree, and pathogenicity tests. We identified three species of ophiostomatoids for the first time in Türkiye. The study demonstrated the occurrence of fungi on *O. erosus*, discoloration of infested wood, and pathogenicity to the Turkish pine. Our findings showed that *C. ips* was the most frequent species associated with *O. erosus* on Turkish pine. In addition, our research recorded an important association between *O. erosus* and *L. wingfieldii* for the first time. In Türkiye, the fungi associated with bark beetles, especially those causing damage to a certain tree species, should be studied in detail. In addition, the results of the pathogenicity tests of our pioneering study showed that all three species could cause lesions on Turkish pine branches but that they were weak pathogens.

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Authors' contributions

Sabiha Acer: Served as the primary author of the manuscript and conducted the analysis.
Zeynel Arslangündoğdu: Contributed to field studies and also to manuscript design.
Asko Lehtijärvi: Contributed to the editing and revisions of the manuscript.

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