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Original Research Article

Fungi associated with *Platypus cylindrus* Fab. (Coleoptera: Curculionidae) from *Quercus suber* L. in North-Eastern Algeria

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The oak pinhole borer, *Platypus cylindrus,* is an ambrosia beetle capable of establishing symbiotic relationships with many fungi serve, among other things, as a feeding source and/or facilitate tree decline, which is the process of wood tissues decomposition. The aims of this study are to identify the diversity of fungi associated with the oak pinhole borer in cork oak forest of El Kala isolated from galleries, mycangia and intestinal contents of insects. Likewise, this investigation allows to acquire more information on the role of this interaction. A diversity of eleven fungi (*Botryosphaeria* sp., *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp., *Mucor* sp., three *Ophiostoma* sp., *Raffaelea montetyi, Raffaelea canadensis-like* and *Biscogniauxia mediterranea*) belonging to the six systematic orders and originating mainly from Ascomycota were isolated. Molecular characterization was performed based on amplification and sequencing of the D1/D2 domain of large subunit (LSU) ribosomal DNA (rDNA) to confirm the morphological identification of *Ophiostoma* sp. The sequences obtained revealed 86% similarity with *Ophiostoma piliferum* and 84% similarity with *Ophiostoma* sp. In this study, the presence of the species *Raffaelea montetyi* and *Ophiostoma piliferum* associated with *P. cylindrus* confirms its pathogenicity with respect to the health status of the cork oak.

Keywords: Ambrosia beetle, fungi, ophiostomatales, cork oak, decline.

INTRODUCTION

The decline of cork oak forests has been reported in its range areas (Bakry and Abourouh, 1995; Kubono and Ito, 2002). The oak pinhole borer, *Platypus cylindrus,* was initially considered a secondary pest because its attacks generally result weakening of trees (Espanõl, 1964). Severe infestations have been observed on apparently healthy cork oaks in the Iberian Peninsula (Sousa, 1996; Sousa and Debouzie, 2002) resulting in widespread tree death from three months to a year and a half after attack. This period depends on the vigor and resistance of the host.

In Algeria, *P. cylindrus* outbreaks observed in cork oak forests in the north-western region (Tlemcen, Oran and Mascara) have been considered the main factor of plantation forests degradation (Belhoucine and Bouhraoua, 2012). The spatial and temporal distribution study of this xylomycetophagous insect has been analyzed at stand scale, and a high attack rate has been noted (33%) in the north-eastern region (Amoura et al., 2014). The cause of the consequent mortality of *Quercus suber* trees remains to be elucidated.

Tree trunks are often colonized by a limited density of pests (Belhoucine and Bouhraoua, 2012). *Platypus cylindrus*

is referred to as an ambrosia beetle because its larvae and adults feed mainly on fungi (ambrosia fungi) in the gallery (Batra, 1963) and is one of the few ambrosia beetles found in the Mediterranean basin. This insect-fungi relation is expressed by an ectosymbiosis in which the fungi live outside the insect body but are temporarily stored in special prothoracic organs for dissemination (Francke-Grosmann, 1967), known as mycangia. The insects carry a viable fungal inoculum in these sac-like structures, located in the prothorax; the inoculum is protected throughout the beetle's life and is disseminated to new breeding sites when the tunnel constructed (Batra, 1963, Sousa and Inàcio, 2005).

Batra (1985) characterized ambrosia fungi as primary and auxiliary. Primary ambrosia fungi are carried by specific insect and their distribution corresponds to those of species symbionts. They are present and dominant in tunnels serving as feeding for larvae and isolated regularly from the mycangia of the beetles during the flight stage or when excavating tunnels. These obligate mutualistic fungi are extremely susceptible to drought; generally, they are localized in the mycangia and in the beetle's gallery (Batra, 1985). Auxiliary ambrosia fungi are transitory and non-specific with respect to symbiont insect, and can appear after insect development. They may not be present in larval cradles or in adult beetles, and their habitat and distribution range are unrestricted and unrelated to that of ambrosia beetles (Batra, 1985). Most of the auxiliary fungi are easy to cultivate and some have been confused with the primary ambrosia fungi (Lévieux and Cassier, 1994).

The ambrosia fungi taxonomy is complex, and belongs to the four anamorph genera, *Ambrosiella*, *Raffaelea*, *Monacrosporium* and *Phialophoropsis*. Other genera can be noted, including *Fusarium*, *Acremonium*, *Candida* and *Graphium* (Batra, 1963; Franck-Grosmann, 1967; Baker and Norris, 1968). In addition to fungi directly involved in insect feeding, others have been found to be associated with them, such as host pathogenic fungi that play a role in insect selection and host colonization (*Botryodiplodia*, *Ceratocystics*, *Graphium*, *Leptographium* and *Ophiostoma)* (Subramanian, 1983). In Portugal, Sousa and Inàcio (2005) and Inàcio et al. (2010) insolated different fungi from *P. cylindrus* and their galleries in *Q. suber*. *Raffaellea* species are considered to be the principal ambrosia fungi. These asexual symbionts occur in a monophyletic clade within the genus *Ophiostoma sensu lato*, which is an important group of fungal pathogens widely distributed on hard-woods (Harrington, 1981; De Beer and Wingfield, 2013). Most of these fungi have been shown to be pathogenic for *Q. suber* plants (Inàcio et al., 2012).

The genus *Streptomyces*, representing nonpathogenic gram-positive filamentous bacteria, was reported as being related to *P. cylindrus* in Portugal by Henriques et al. (2006) and Inàcio et al. (2010). This group was introduced by Selman-Waksman (1943), it was considered as fungi for a long time. These bacteria are mainly located on the surface of soils and are used for antibiotics production. This work focuses on the mycobiota associated with *P. cylindrus* in *Q. suber* forests. The study also aims to highlight their importance and implications in the cork oak decline.

MATERIALS AND METHODS

The samples were collected in 2013 from cork oak logs showing decline symptoms in the El Kala area forest. In this investigation, the cork oak tree infested by *P. cylindrus* was repered and we assigned it a slaughter process. The trunk having the most infested parts was cut in logs of 50 cm height. The sections were protected with paraffin and stored in the laboratory. *P. cylindrus* adults were extracted from the cut timber of the infested cork oak trees and maintained in 70% alcohol. Adults sexing was identified by using a stereoscopic microscope (Leica, Germany). The excised fungi were prepared on clear lactophenol slides according to method proposed by Inaçio et al. (2012).The pics were realized using stereomicroscope (Olympus BX41TF)

Fungal isolation

Biological material is collected from parental galleries, mycangia and adult intestinal contents of both sexes. A total, 10 males and 10 females adults of *P. cylindrus* were randomly collected from the lot. Likewise, five 0.5 cm cubic-shaped wood fragments were considered. Six replicates are performed for each collection according to the established method by Inàcio et al. (2008). The harvested biological material was immersed in a sodium hypochlorite solution (1%) for 1 min and rinsed with sterilized distilled water. Then, the samples were placed on malt extract agar (MEA, Difco) or Potato Dextrose Agar (PDA, Difco), added with cycloheximide (500 mg/L), a specific antibiotic for the isolation of *Ophiostoma* fungi (Harrington, 1981).

Morphological characterization

The cultures were performed on PDA and incubated at 25 ± 1° C, in total darkness for two weeks. The cultures obtained were classified according to their macroscopic characteristics. A representative of each group was selected for identification on the basis of the cultural and morphological characteristics of their conidia and conidiophores established by Ellis (1971, 1976), Lanier et al. (1978) and Kiffer and Morelet (1997). The macroscopic characteristics of the colonies were described after 21 days. The names of the colors are taken from Saccarado (1891). The observations were conducted using a photon microscope (Olympus, BX41TF), taking images at different magnifications (200x, 400x, 600x) from the pathology laboratory of INIAV (Oeiras, Portugal).

Molecular characterization of Ophiostomatales

All isolates belonging to the genus *Ophiostoma* were used for molecular analysis which was performed based on the

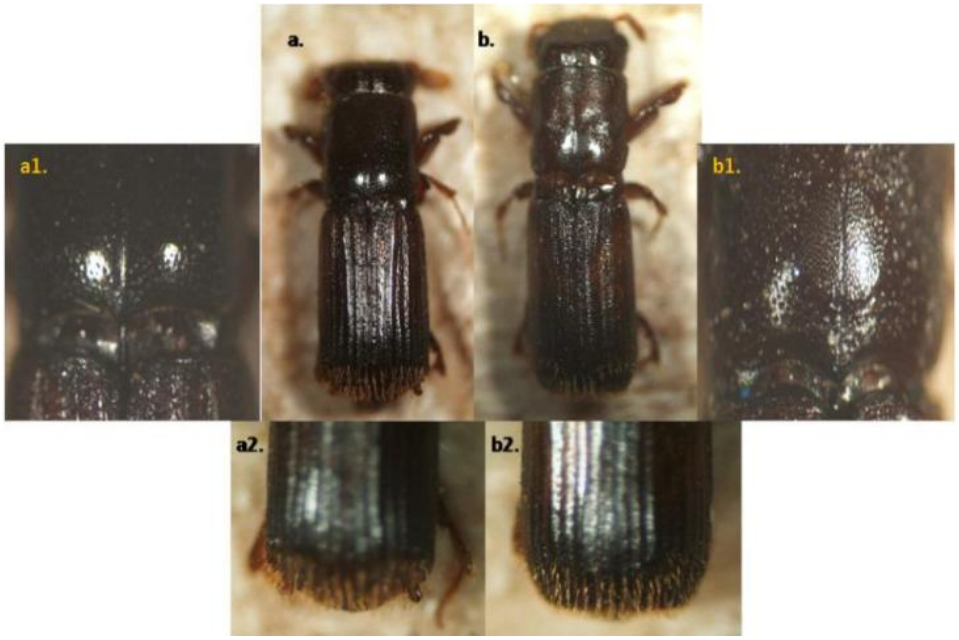

Figure 1: Sexual dimorphism of *Platypus cylindrus:* a. Male, a1. Male mycangia. a2. Male distinctive elytra, b. Female, b1. Female mycangia, b2. Female elytra

amplification and sequencing of the D1/D2 domain of the large subunit (LSU) ribosomal DNA (rDNA). DNA was extracted from approximately 100 mg of fresh mycelium using the DNeasy Plant Mini kit (Qiagen, Germany). PCR amplification was performed by LROR (5'ACCCGCTGAACTTAAGC3') and LR5 (5'TCCTGAGGGAAACTTCG3') (Vilgalys and Hester 1990). PCR reactions were carried out using the Dream Taq PCR Master Mix (2X) (Qiagen, Germany) in a Biometra Gradient thermocycler (Biometra, GmbH).Each reaction mixture was performed in a final volume of 25 µL containing 1 µL of template DNA, 0.4 M of each primer and 1x Master Mix PCR buffer, which included 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. Thermal cycling conditions were as follows: initial denaturation at 94 °C for 2 min followed by 35 cycles of 1 min at 94 °C, annealing at 50°C for 1min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10min.

The amplified products were visualized on a 2% agarose gel containing 0.5x Tris-borate-EDTA buffer (TBE) and 0.5 mg/ml Ethidium bromide using the VersaDoc Gel imaging system (Bio-Rad, USA). The PCR fragments were purified using the QIAquick PCR purification kit (Fermentas) according to the manufacture's protocol (Qiagen, Germany). The DNA amplicons were sequenced in both directions using the same primers at the STAB Vida Sequencing Laboratory (Lisbon, Portugal) on an ABI PRISM 3730xl DNA analyzer (Applied Biosystems). Nucleotide sequences were edited and analyzed using BioEdit software version 7.2.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) (Hall, 2007) and compared to reference sequences available at NCBI Gen Bank database using the BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS AND DISCUSSION

Mycobiota associated with *P. cylindrus*

The adults sexing of *P. cylindrus* was easily observed by the presence of two unequal teeth developed on the posterior extremity of the male elytra as already mentioned by Barbey (1925) and Balachowsky et al. (1963). The observation of *P. cylindrus* mycangia showed spheroidal cavities symmetrically laid out as ovoid shape on the insect "prothorax" in both sexes (Figure 1) and are more consequent in the female. *Platypus cylindrus* is a carrier of various fungi that influence the cork oak in its natural area (Sousa et al., 1997; Henriques, 2006; Inàcio et al., 2008;; Bellahirech et al., 2014). In total, eight genera belonging to six orders were isolated (Table 1). The majority of taxa belong to the Ascomycota branch except Mucorales (Zygomycota). Many saprobes are frequently present in insect galleries, such as *Aspergillus* spp., *Penicillium* spp., *Mucor* spp., *Trichoderma* spp. and these have a commensal relationship (Cassier et al., 1996; Inàcio et al., 2008). The presence of these fungi associated with the insect indicates their importance in symbiosis, such as cellulose decomposers and/or antagonists that inhibits the growth of other fungi (Henriques et al., 2009). *Penicillium* and *Aspergillus* sp. were also found in the adults intestinal contents.

The fungi were isolated from the adult mycangia and their intestinal contents as well as from the walls of the

Table 1. Fungi isolated from *Platypus cylindrus* and cork oak galleries

Taxonomic characteristics of identified Species					
			Origin		
Orders	**Genera**	**Species**	**Mycangia**	**Intestinal contents**	**Gallery**
Botryosphaeriales	*Botryosphaeria*	*Botryosphaeria sp.*			*
Eurotiales	*Aspergillus*	*Aspergillus sp.*		*	*
	Penicillium	*Penicillium sp.*		*	*
Hypocreales	*Trichoderma*	*Trichoderma sp.*			*
Mucorales	*Mucor*	*Mucor sp.*			*
Ophiostomatales	*Ophiostoma*	*Ophiostoma sp. 001*	*	*	*
		Ophiostoma sp. 002	*	*	*
		Ophiostoma sp. 003	*	*	*
	Raffaelea	*Raffaelea montetyi*	*	*	*
		Raffaelea canadensis-like			
Xylariales	*Biscogniauxia*	*Biscogniauxia mediterranea*			*

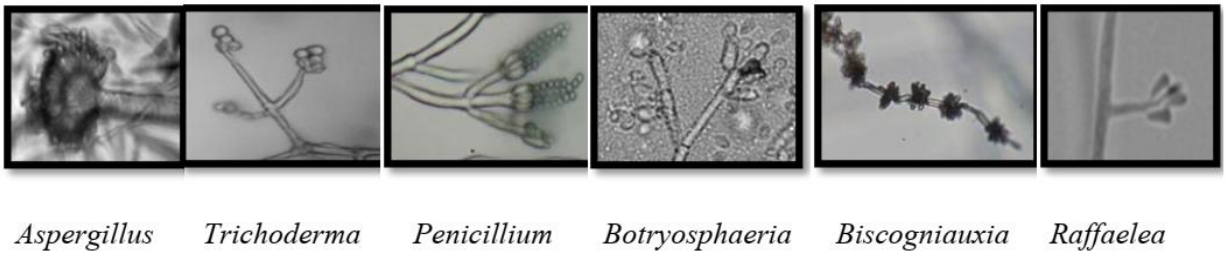

Figure 2: Fungi genera isolated from *P. Cylindrus*

galleries dug by the insect in the cork oak wood (Figure 2). These results showed the presence of Ophiostomatales representatives in all sources of isolation, i.e. *Ophiostoma* sp. with three unidentified isolates and *Raffaelea* sp. with two isolates identified on the basis of the morphological appearance (*Raffaelea montetyi* and *Raffaelea Canadensis-like*). These species have been considered as fungi of nutritional interest (ambrosia fungi) and studied by different authors (Henriques et al*.,* 2009). The study conducted by Inàcio et al*.* (2010) confirms the pathogenicity of the species *Raffaelea montetyi* on cork oak in Portugal.

The main pathogenic fungi from cork oak vectored by *P. cylindrus* belong to Ophiostomatales and Xylariales are presented by *Biscogniauxia mediterranea*. They have been isolated from the galleries and mycangia of *P. cylindrus* in Portugal by several authors (Santos, 2003; Inàcio et al., 2010; Henriques et al., 2015).

In this study, this species was obtained exclusively from galleries, which allows to consider that this fungus is not transported by *P. cylindrus*; it remains a secondary fungus. *Biscogniauxia mediterranea* is considered as an endophyte in cork oak tissues (Henriques et al., 2015) and the fungus responsible for the charcoal stroma of oak trunks and branches while having a particular effect on cork oak (Mazzaglia et al., 2001). It is also considered to be the main cause of the decline of *Quercus suber* in Sardinia (Evidente et al., 2005). It was first reported in M'Sila (north-western Algeria) in 1980 on twigs and trunks of dead trees (Lanier et al., 1978). *Botryosphaeria* species of the order of Botryosphaeriales were isolated from the galleries. Transport and direct inoculation of this pathogen by *P. cylindrus* could contribute to enhance disease dispersal and the decline of the Portuguese cork oak stand, as case in oak forest at Algeria north-western (Belhoucine and Bouhraoua, 2012).

The highest diversity of fungal species was detected in Portuguese cork oak stands by Inàcio et al. in 2008. The most frequent fungi isolated from *P. cylindrus* and its galleries belong to *Raffaelea* genus. It has been obtained in particular from intestinal contents and mycangia, about 30% and 40% of all fungi isolated, in both organs, respectively of the females and males, respectively (Henriques, 2009).

The cultures of *Raffaelea* species on PDA are effuse, yeast-like to farinaceous, with long, sparse, vigorous aerial mycelium, light density, pale brown color similar to the description of Inàcio et al*.* (2008). This species, unlike most *Raffaelea* spp., grows and establishing itself rapidly in the host facilitating colonization by insects. *Raffaelea montetyi* was previously isolated from *P. cylindrus* by Morelet (1998).

The *Ophiostoma* fungi are the second most frequent species. This group isolates have also been obtained from declining cork oaks with visible aborted attacks of *P. cylindrus* (Inàcio et al., 2012). Considering that these

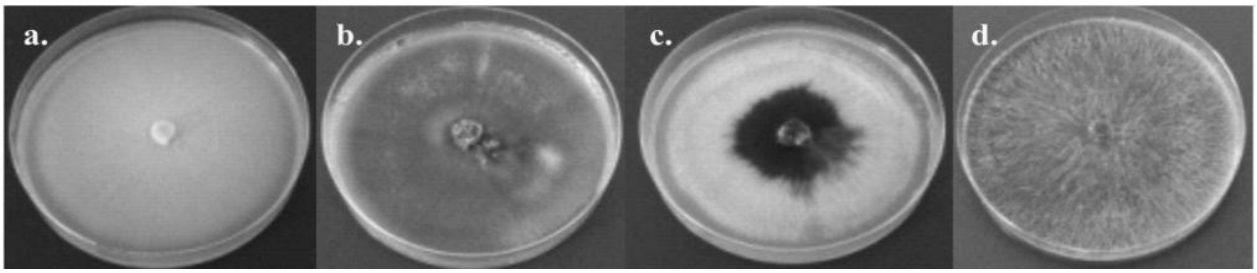

Figure 3: Photograph of 21-day-old cultures of Ophiostomatales isolated from *Platypus cylindrus* adult (mycangia and intestinal contents) and their galleries from cork oak, growing of MEA 90mm Petri plates with cycloheximide. Isolates: a, b, c: *Ophiostoma* sp.; d: *Raffaelea montetyi*.

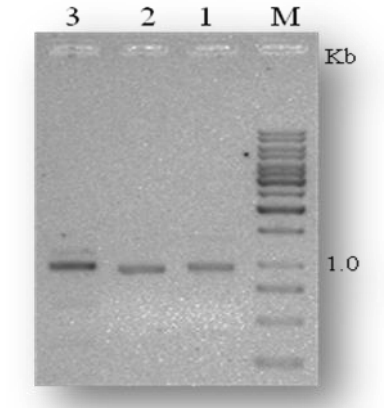

Figure 4: Agarose gel development of PCR amplification products specific to the D1/D2 region LSU. M: DNA marker. (Generuler 1kb DNA Ladder, Fermentas)

Ophiostomatoid fungi have been isolated from cork oak galleries, from the mycangia and intestinal contents of *P. cylindrus,* it is most likely that they were transferred to oak trees by this insect. Thus, even if the insects did not succeed to colonize the tree, they inoculate pathogens into a susceptible host. Without *P. cylindrus* as a vector, it is unlikely that these fungal species can infect new hosts. Moreover, Ophiostomatales fungi require pre-existing wounds to infect their hosts (Nkuekam et al., 2012). Without *P. cylindrus*, it would not be possible for the fungal species to continue their life cycle, as without fungi, the beetles would have enormous difficulties in colonizing new trees. As such, it is an obligatory symbiosis. Inside the host, fungus can rapidly spread from several points (Inàcio et al. 2012).

These fungi have a variety of different anamorphs; most of them produce their conidia in the form of viscous droplets. The droplets of the sticky spores can fix on the insects organs that facilitate their dispersion. The morphological similarity of the Ophiostomatales fungi is the result of a convergent evolution, as an adaptation to the insect's dispersion (Spatafora and Blackwell, 1994). In this regard, Inàcio et al. (2012) noted that the Ophiostomatales: *Raffaelea montetyi*, *Raffaelea canadensis* and *Ophiostoma* sp. are related to the decline of cork oak in Portugal. In this study, diverse Cycloheximide tolerant fungi belonging to the *Ophiostoma*/*Raffaelea* genus were obtained (Figure 3). Cycloheximide is an antibiotic that inhibits the protein synthesis in the majority of the eukaryotic organisms. However, *Ophiostoma* species have a peculiar cell wall (composed of cellulose and rhamnose) whose structure prevents the antibiotic from entering the cell, making them tolerant to Cycloheximide (Inàcio et al., 2012).

Molecular analysis of Ophiostomatales

Amplification of the D1/D2 domain of the large subunit (LSU) ribosomal DNA (rDNA), using the conserved primers LROR and LR5, generated a fragment of approximately 1.0 Kb (Figure 4). All sequences obtained were compared with the GenBank database (NCBI). Only the sequences corresponding to the PCR amplification of the D1/D2 region

Table 2. Similarity of D1/D2 region LSU sequences with sequences from retrieved GenBank

Sample	LSU	
	Accession n.	Similarity
002 -b-	AF221010 – *Ophiostoma piliferum*	233/270 (86%)
003 -c-	JF946765.1|- *Ophiostoma sp.*	321/384 (84%)

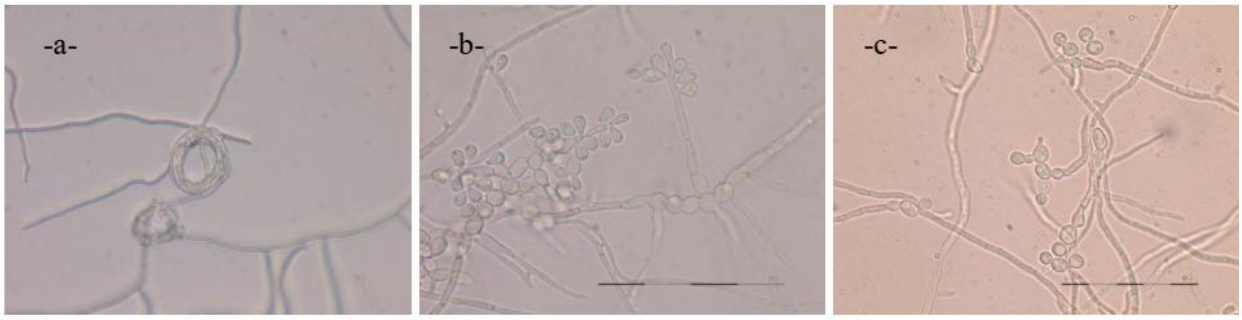

Figure 5: Conidia and conidiophores of *Ophiostoma* a, b, c. isolated from *Platypus cylindrus* observed under light microscope: sample 001 -a- (x200); sample 002 -b- (x600); sample 003 -c- (x600).

(LSU) of the samples 002 (Fig. 3-b) and sample 003 (Figure 3-c) showed a similarity presented in Table 2.

The sequences obtained showed a similarity: sample –b presented a 86% similarity with *Ophiostoma piliferum* and sample -c presented a 84% similarity with *Ophiostoma* sp. Despite the low similarity obtained for the two samples, these incipient results coupled with the morphological analysis suggest the presence of fungi belonging to the genus *Ophiostoma*.

The *Ophiostoma* isolates were identified using LROR and LR5 primers of the D1/D2 region (LSU). Sample 2, of species *Ophiostoma piliferum*, showed a 86% similarity. The second species confirming the belonging to the genus Ophiostoma.

Microscopic observations of conidia and conidiophores of *Ophiostoma* genus fungi isolated from mycangia and intestinal contents of *P. cylindrus* were conducted out to confirm the molecular results (Figure 5).

The morphological structures coupled with the molecular analysis confirm the presence of fungi belonging to the genus *Ophiostoma* transmitted by *P. cylindrus*, as well as the identification of the *Ophiostoma piliferum* species. The presence of *O. piliferum* in association with *P. cylindrus* is the first report. This fungus causes wood staining; it does not alter wood structure, but it affects its aesthetic. For the sawmill industry, the losses associated with this staining are significant (Morin et al., 2006).

The genus *Ophiostoma* characterizes the order Ophiostomatales (De Beer et al., 2013). The species *O. piliferum* was described as *Sphaeria pilifera* from stained conifer wood in Sweden by Fries in 1823.

The term "Ophiostomatoid" gathers more than one hundred Ascomycetes and Deuteromycetes fungi who are characterized by the production of sexual or asexual reproductive structures heavily melanized (Wingfield et al., 1993; Jacobs and Wingfield, 2001). Some Ophiostomatoid fungi are saprotrophs that and they colonize the xylem of the trees; they cause a color called blue stain. Although they do not corrupt the mechanical properties of the wood, the blue stain causes important economic losses since the attained wood is sold at a lower price. Moreover, some Ophiostomatoid are phytopathogenic agents that are very aggressive and are responsible of several diseases such vascular wilt and cankers development (Morin et al., 2006). The species *O. piliferum* and *Ophiostoma* sp. to be identified could be considered as the primary ambrosia fungi of *P. cylindrus* in cork oak stands. In this study, they were found in the mycangia and intestinal contents of insects of both sexes, providing a fundamental role in the feeding of both adults and larvae.

CONCLUSION

This study reports the presence of Ophiostomatales, considered as the primary ambrosia fungi of *P. cylindrus* in cork oak area. Cultures of ambrosia fungi were identified as *Raffaelea montetyi*, *Raffaelea canadensis*-like, *Ophiostoma piliferum* and *Ophiostoma sp.* They were found in the mycangia and intestinal contents of insects of both sexes, thus having a key role in the feeding of adults as well as larvae. The confirmation of its implication in cork oaks decline remains to be clarified.

The data collected highlighted evidence of *Ophiostoma* species associated with *Platypus cylindrus* in cork oak stands. The first report of *Ophiostoma piliferum* to association with *P. cylindrus* is noted. The identification of *Ophiostoma* genus fungi is important to indicate the major factor that has influenced the defense capacity of the host infected by *P. cylindrus*. Phytopathogenic tests should be

carried out to confirm the effect of the fungi in cork oak forests, where *P. cylindrus* is considered a secondary pest.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in design, execution, interpretation, or writing of the study.

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