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Full Length Research Paper

Mycobiota associated with *Platypus cylindrus* (Coleoptera: Curculionidae, *Platypodidae*) in cork oak stands of North West Algeria, Africa

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Platypus cylindrus* (Coleoptera: Curculionidae, *Platypodidae*) is an important insect pest of the cork oak. These beetles maintain symbiotic relationships with many fungi that serve especially as food for the adults and larvae but also intervene in the mechanisms of establishment of the insect by further weakening the host-tree. 270 samples were taken by 3 sources: Galleries (30), mycangia and intestinal contents of male and female insects and intestinal contents of mature larvae (60 each). The results show the presence of 42 species of ambrosia fungi among which 17 are new to this association. The mycetophagy of these beetles is very rich and consisted essentially of Ophiostomatales. Other groups of fungi playing different roles were also isolated: entomopathogenic, antagonistic, saprophytic but especially pathogenic for the tree host. This group consists of many species and their dissemination by the insect and the inoculation in trees may have fatal consequences by accelerating the cycle of declining affected trees. In the present paper, we discuss the fungal species associated to the beetle, identified on the basis of phenotypic characters and ribosomal DNA sequences analysis, and their relationship with *P. cylindrus.

Key words: Forest of M'sila (Oran- Algeria), *Quercus suber*, *Platypus cylindrus*, Ambrosia fungi.

INTRODUCTION

Some Scolytinae and most Platypodinae, including the genus *Platypus* Herbst, 1793, are known as ambrosia beetles (Chararas, 1979). They transport fungi that are cultivated on the walls of the clean galleries of laying held of dejections and dug deeply in the wood of the plant-hosts (Batra, 1967). The mycelia covering these galleries are very rich in nitrogenized matters, which are essential not only for the adults of *Platypus,* during the period of gallery digging, but also to the larvae unable to attack wood (Balachowsky, 1949; Dajoz, 1980). These xylomycetophagous (most Platypodinae, and some genera of scolytinae: *Xyleborus*, *Xylosandrus*, *Corthylus*, *Gnatotrichus*, *Premnobius*, *Sampsonius*, etc) beetles Gnathotrichus ectosymbiotic (Francke-Grosmann, 1967) relationships of mutualism with the fungi (Beaver, 1989). This association finds its origin in the presence of specialized organs of storage and dissemination of fungi called "mycangia" (Batra, 1963; Krivosheina, 1991; Fraedrich et al., 2008; Moon et al., 2008a, b). These structures contain glands of secretion that maintain fungi spores under favorable conditions during flight and movement of adults (Levieux et al., 1991). The morphology of these structures, as well as their localization on the body, is different depending on

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Abbreviations: PDA, Potato dextrose agar; **MEA,** malt extract agar; **DNA,** deoxyribonucleic acid; **ITS,** internal transcribed spacer; **rDNA,** ribosomal DNA.

Figure 1. Distribution area of *Quercus suber* and location of the forest of M'Sila (Algeria) (taken from http://www.euforgen.org/distribution_maps.html, February 2011).

species and sex. In *Platypus cylindrus* Herbst, 1793, these mycangia are located on the dorsal median surface of the prothorax and are more developed in females than in males (Sousa and Inacio, 2005; Henriques, 2007; Kent, 2008).

Many fungal species are related to this group of Coleoptera. Some of them are pathogenic for the host tree such as *Ceratocystis ulmi* (Buisman, 1932) transmitted by *Scolytus multistriatus* (Marsham, 1802), the agent of the blue stain wood (Dajoz, 1980). A massive attack of these insects with inoculation of pathogenic fungi often ends with a fast decline of the tree (Kirkendall et al., 1997; Š vihra and Kelly, 2004). Several mechanisms were proposed to explain this mortality; fungal production of toxins (Külnholz et al., 2001), penetration of mycelium in the plant tissues, formation of gas vesicles or other substances in the cells blocking the intracellular spaces (Agrios, 2005). Serious physiological disturbances can occur in the infested trees resulting in an extensive economic damage (Batra, 1985; Cassier et al., 1996).

Beetles of the genus *Platypus* attack both healthy and weakened trees (Atkinson, 2004). In Eastern Asia, the record of damaged oaks over the last years has been related with the presence of *P. quercivorus* (Murayama, 1925) in Japan (Inoue et al., 1998; Ito et al., 1998; Kamata, 2002; Kamata et al., 2002; Igeta et al., 2003, 2004) and *P. koryoensis* Wood and Bright, 1992 in Korea (Moon et al., 2008a). In the Western Mediterranean region, the decline of *Quercus suber*, observed since 1980 (Delatour, 1983), matches up with a noteworthy increase of the populations of *P. cylindrus* (Ferreira and Ferreira, 1989). This important insect pest has been responsible for important damages on *Quercus suber* in Portugal (Sousa et al., 1995), Spain (Español, 1964; Soria et al., 1994), France (D.S.F, 2001; Durand et al., 2004) and Morocco (Bakry et al., 1999; Sousa et al., 2005). In Algeria, this beetle has been observed in several cork oak stands since 1893 (Lamey, 1893) and the first major damage on this tree caused by *P. cylindrus*, was observed at the beginning of the last century in the eastern area of the country (G.G.A, 1927).

Since then, little information on *P. cylindrus* has been recorded in Algeria. Some works on this pest have been published in the last years (Bouhraoua et al., 2002; Bouhraoua and Villemant, 2005). The presence of this beetle in some cork oak stands in the western area of the country (Tlemcen, Oran and Mascara) have been largely responsible for damaging trees including cases of severe wasting. The record of this decline from trees varies annually from 1 to 4% (Bouhraoua, 2003). However, the cause of a considerable proportion of this mortality remains unclear particularly in the forest of M'Sila (Oran) where the density of *P. cylindrus* on tree trunks is low. This mortality is probably associated with the plant pathogenic fungi, carried by the adults of *Platypus* and inoculated into the trees during the establishment of the insect.

The mycobiota associated with *P. cylindrus* in the galleries of wood of *Q. suber* was studied in order to determine all the harmful species playing a role in the decline of this tree.

MATERIALS AND METHODS

Research site

This study was conducted in the littoral forest of M'Sila, located 30 km west of Oran in the northwest region of Algeria. This forest covers a total area of 1570 ha of which 460 ha are occupied by the cork oak (Figure 1). The average rainfall is quite low (400 mm per year), but this deficit of precipitation is offset by the sea breeze. The annual mean temperatures is 16.2°C, January is the coldest month with 5.7°C and the warmest August with 30°C on average. Elevation ranges from 365-380 m.

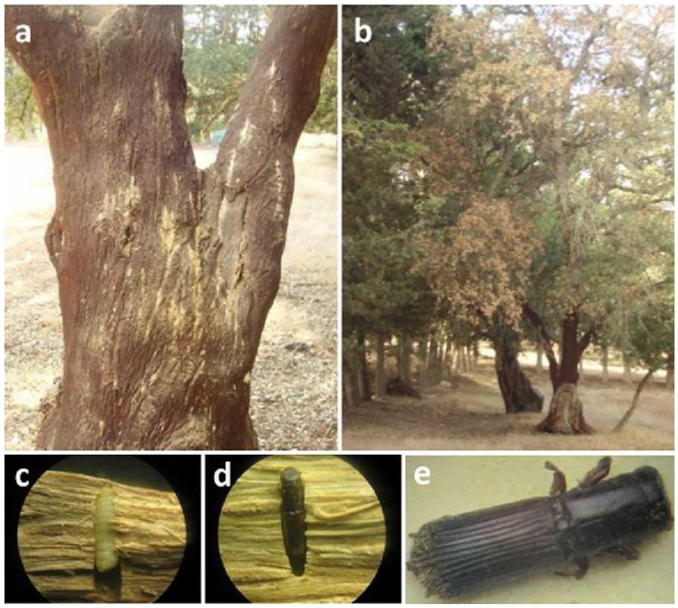

Figure 2. (a) Presence of sawdust indicating tree attack by *Platypus cylindrus*, (b) Symptom of cork oak decline, and biological material used to isolate fungi (c) larvae of 5th stage in a wet gallery, (d) adult insect in a gallery, (e) male insect.

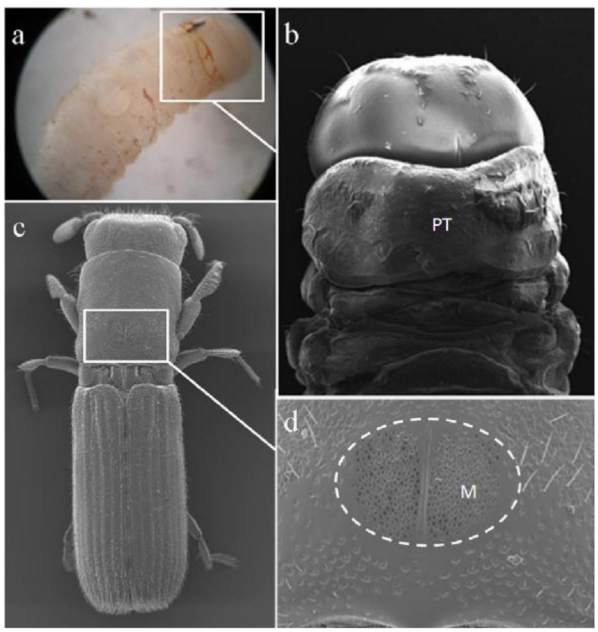

Figure 3. *Platypus cylindrus*: (a) larva of 5th stage, (b) prothoracic segment = PT, (c) female and (d) mycangia = M.

Biological material

In order to isolate and characterize the fungi associated with *P. cylindrus* , the material was collected from several sources: wet galleries produced by adults in the wood, gut contents of larvae and mycangia present in adults (males and females) and their intestinal contents. Three severely infested trees by the insect and newly death were cut between March 2008 and June 2009. Infestation can easily be recognized by the presence of an abundant dust streaming down the trunk through the entrance holes (Figure 2a). The severe decline of the tree is characterized by a total drying of the crown and the completely browning of leaves (Figure 2b). The trunks of the three trees were cut into 1 m logs and deposited on the ground. To maintain adequate humidity inside the timber, which is the limiting factor for insect life and especially the development of associated fungi, the end surfaces of logs were permanently waxed. Washers of 15 cm thick were cut with a chainsaw and then fragmented with chisel in order to recover adult galleries and larvae. For this study, we focused only on adults and larva (Figure 2c-e) of the 4th and 5th instar because the larval size permit to study easily the intestinal products; the fifth instar can be differentiated by the presence of a reddish band at the prothoracic segment (Figure 3a-b) (Strohmeyer, 1907; Korolyov, 1989). The larvae length varies from 5.5 to 7.5 mm; the color is yellow. Adults present mycangia on the dorsal median surface of the prothorax (Figure 3c).

From each cut, 10 larvae, 10 males and 10 females were randomly collected with 5 pieces of moist galleries (0.5 × 0.5 cm). For each sample, six replicates were performed using the method of Sousa (1996). All biological material was collected aseptically and placed individually in Eppendorf tubes for rapid analysis in the laboratory or stored at 4°C until use. All harvesting instruments used were, soaked in alcohol and flamed after each use, in order to

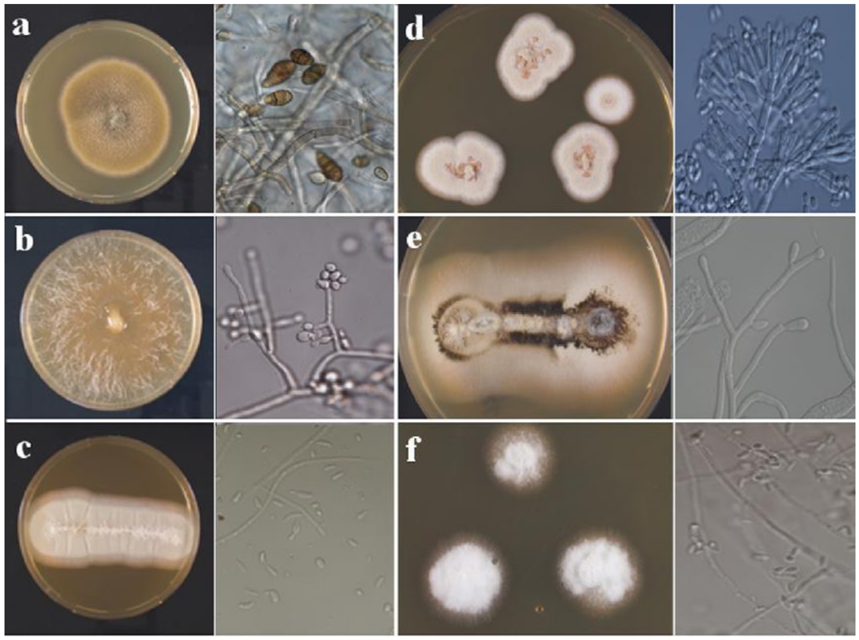

Figure 4. Macroscopic and microscopic (x1000) aspects of some isolated fungi : (a) *Ulocladium botrytis,* (b) *Raffaelea montetyi*, (c) *Ophiostoma nigrocarpum*, (d) *Geosmithia langdonii* , (e) *Ophiostoma quercus*, (f) *Nectria mauritiicola*.

avoid contact between, adults, larvae and fragments of wood.

Preparation of biological material and fungal isolation

The preparation of the material was performed using the technique described by Norris and Baker (1968) . In order to avoid external contaminations, prior to the isolation of fungi, the adults, larvae and wood fragments were separately immersed in a solution of sodium hypochlorite of 1% for 1 min, rinsed with sterilized distilled water, dried with sterilized filter paper and finally placed individually in sterile tubes. Fragments of wood were directly deposited on the agar, previously, poured in the Petri dishes. Larvae and adults were spread individually on a sterile cork plate covered with a sterile filter paper under a dissecting microscope cleansed with alcohol and placed under a laminar flow hood at least 30 min before. An incision of the abdomen using a sterile scalpel was made to clear the intestinal contents, which were directly transferred to an agar culture medium. The mycangia of these same adults were carefully and aseptically removed and transferred directly to culture medium. For fungi isolation, potato dextrose agar (PDA) and malt extract agar (MEA) were used, following Harrington (1981). Streptomycin or Cycloheximide at a dose of 500 mg/L (Upadhay, 1981; Harrington, 1981) was added to the medium. The first antibiotic eliminates bacterial contamination while allowing normal growth of most fungi (Hawksworth et al., 1995). The second, in addition to its bacterial action, acts in a selective manner on fungi and slows to grow only the complex *Ceratocystis/Ophiostoma* and some saprophytes such as Mucorales and *Penicillium* (Harrington, 1981; Hawksworth et al., 1995) . The inoculated plates were incubated at 25 ± 1°C, in conditions of total darkness for 5 to 10 days.

Fungal characterization and identification

The different cultures obtained were classified according to their macroscopic characteristics. A representative of each group was selected and illustrated (Figure 4) for identification based on microscopic and cultural characteristics of their conidia and conidiophores described by Ellis (1971, 1976), Lanier et al. (1978), Botton et al. (1990), Morelet (1998), Mallock (1997) and Barnett and Hunter (1998). Microscopic observations were made using a light microscope (Olympus BH2 and Zeiss Axioskop 2 Plus).

DNA isolation, amplification and analyses

The strains were grown on MEA (Oxoid) for 4 to 7 days at 25°C. Genomic deoxyribonucleic acid (DNA) was isolated using the UltracleanTM Microbial DNA Isolation Kit (MoBio, Solana Beach, U.S.A.) following manufacturer's instructions. Fragments, containing the internal transcribed spacer (ITS) regions were amplified using universal primers V9G and LS266 for the section ITS1-5.8S-ITS2 of ribosomal DNA (rDNA) (Gerrits and Hoog, 1999) and we use for *Ophiostoma* genus only BT2a and BT2b for the partial β -tubulin gene according to Glass and Donaldsson (1994), and subsequently sequenced as described by Houbraken et al. (2007). The use of ITS to identify *Ophiostoma* species, in particular, was recommended by several authors (Harrigton et al., 2001; Masuya et al., 2004; Alves et al., 2005; Kolarik and Hulcr, 2009) in addition to the β-tubulin fragment (Massoumi et al., 2009). The alignments and analyses were performed as described by Samson et al. (2009). The sequences of DNA obtained are compared with those of reference strains deposited in GenBank (ncbi) database. Also an estimated phylogenic tree, based on neighbor- joining test gathering all strain sequences, therefore automatically multi -aligned using Muscle program in MEGA 5 software completed by manual adjustments, was constructed by using MEGA5 software (Tamura et al., 2011).The summary of results is presented in Figure 5. The molecular methods are very important to assure the correct identification of fungal species and especially when there are neither conidia nor conidiophores. The determination of Ophiostomatales species is very difficult; in this moment some groups of researchers are working on this matter; the species determinate here are assured studding large subunit (LSU, 26S) rDNA dataset according to Harrington et al. (2010) and Harrington and Freadrich (2010).

RESULTS

The list of fungi isolated from *P. cylindrus* and its galleries shows a high diversity including 42 species from 11 different orders. Table 1 lists the most important taxa.

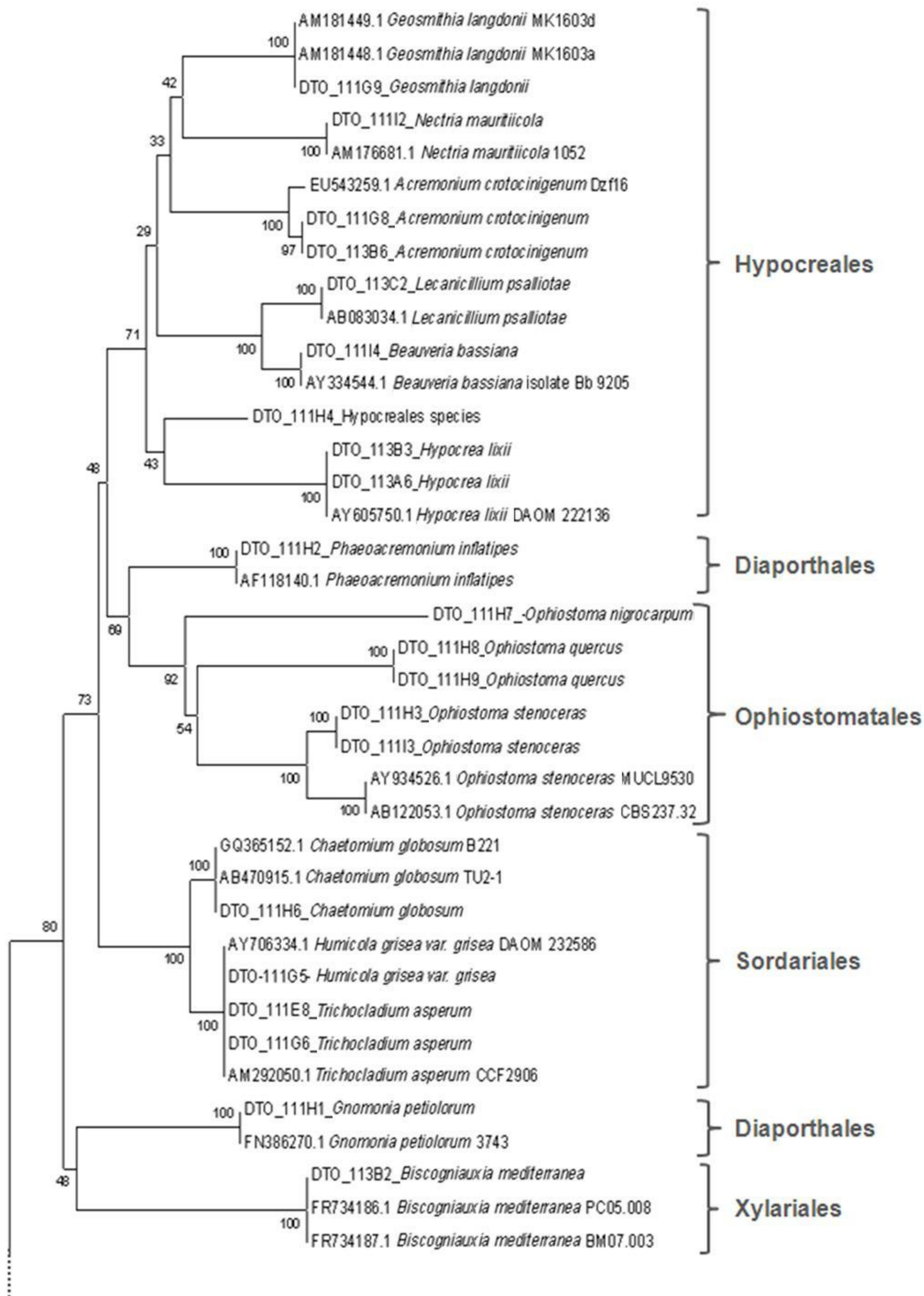

Figure 5. Neighbor -joining phylogenetic analysis of ITS rDNA sequences of fungal strains isolated during this study (marked with DTO) including reference strains from Genbank using p-distance Model. The scale indicates number of changes per site. Bootstrap support values over 50% from 500 replicates.

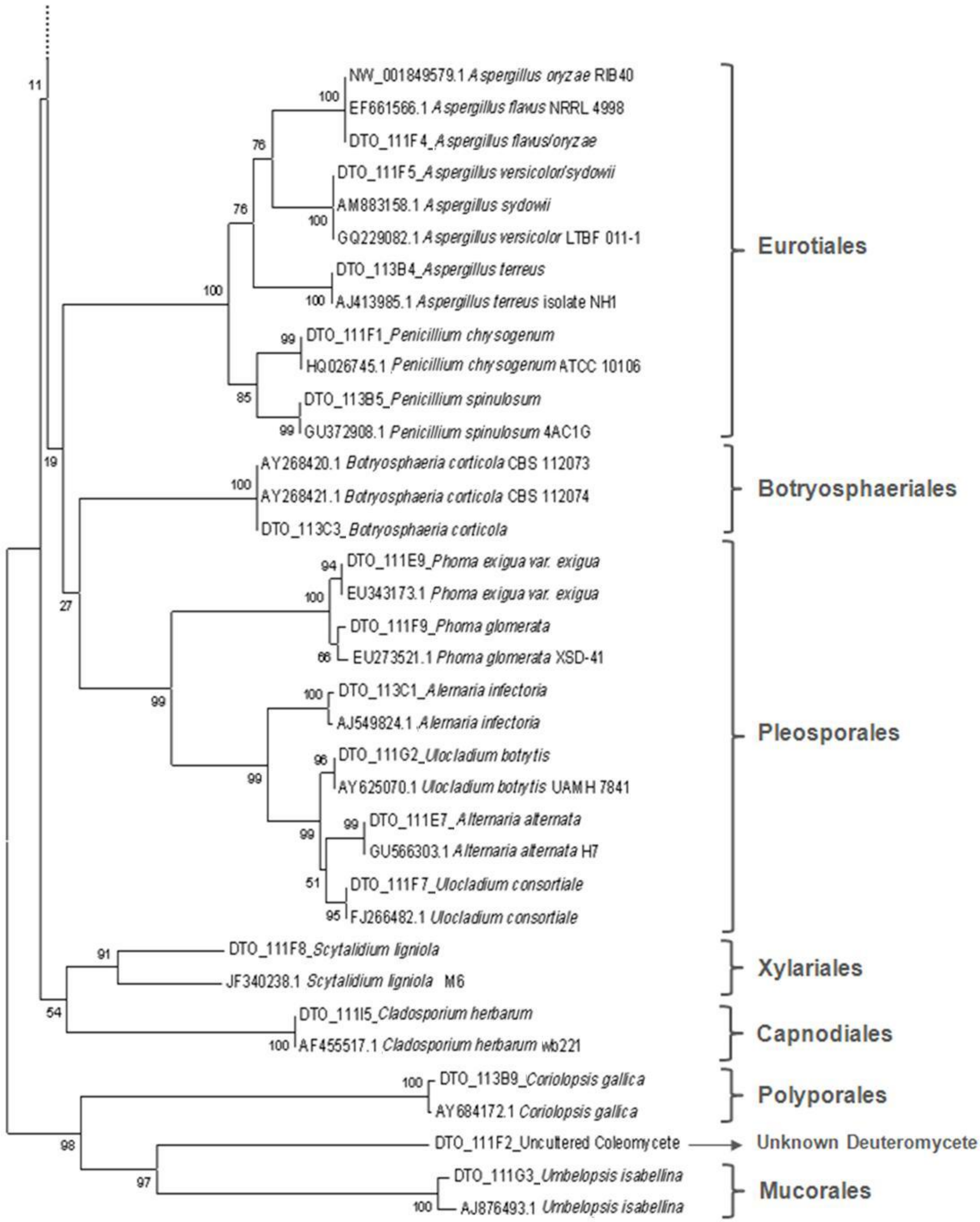

Figure 5. (Cont.)

Table 1. Fungi Isolated from *Platypus cylindrus* and wood of the cork oak according to their origins.

Isolated fungi		**Origin**					
		M. Fe.	**M. Ma.**	**CI. Fe.**	**CI. Ma.**	**CI. Lar.**	**Gal.**
Ophiostomatales	*Ophiostoma stenoceras*	x	x	x	x	X	x
	Ophiostoma nigrocarpum	x	x	x	x	X	x
	Ophiostoma quercus	x	x	x	x	X	x
	Raffaelea montetyi	x	x	x	x	X	x
Hypocreales	*Acremonium crotocinigenum*	x	x	x	x	-	x
	Beauveria bassiana	x	-	-	-	X	-
	Hypocrea lixii	x	-	x	-	X	x
	Geosmithia langdonii	x	x	-	-	X	x
	Nectria mauritiicola	x	x	x	x	-	x
	Lecanicillium psalliotae	x	-	-	-	-	x
	Hypocreales sp.	x	-	-	-	X	x
Xylariales	*Scytalidium lignicola*	x	x	-	-	-	x
	Biscogniauxia mediterranea	x	x	x	-	X	x
Sordariales	*Chaetomium globosum*	-	x	-	-	-	x
	Trichocladium asperum	x	-	x	-	X	x
	Humicola grisea var. *grisea*	-	x	-	-	-	x
Capnodiales	*Cladosporium herbarum*	x	-	x	x	X	x
Eurotiales	*Penicillium spinulosum*	x	x	x	x	X	x
	Penicillium chrysogenum	x	-	-	-	-	x
	Aspergillus flavus/ oryzae	x	x	x	x	X	x
	Aspergillus versicolor/sidowii	x	-	-	-	-	x
	Aspergillus terreus	-	x	-	-	-	x
	Aspergillus niger	x	x	x	x	X	x
Diaporthales	*Gnomonia petiolorum*	x	-	-	-	-	x
	Phaeoacremonium inflatipes	x	-	-	-	-	x
Polyporales	*Coriolopsis gallica*	x	-	-	-	-	x
Botryosphaeriales	*Botryosphaeria corticola*	x	x	-	-	X	x
Pleosporales	*Ulocladium consortiale*	-	x	-	x	X	x
	Ulocladium botrytis	-	x	-	x	-	x
	Phoma glomerata	x	x	-	-	X	x
	Phoma exigua var. *exigua*	x	x	-	-	X	x
	Alternaria alternata	x	x	-	-	X	x
	Alternaria infectoria	x	x	-	-	X	x
Mucorales	*Umbelopsis isabellina*	x	x	-	-	-	x
	Mucorales sp.1	x	x	-	-	-	x
	Mucorales sp.2	x	x	-	-	-	x
	Deuteromycete sp.	x	-	-	x	-	x

M. Fe., Female mycangia; M. Ma., Male mycangia; CI. Fe., Female intestinal content; CI. Ma., Male intestinal content; CI. Lar ., Larvae intestinal content; Gal., Galleries. Species indicated with bold represent new associations with *P. cylindrus*.

Some isolates of *Penicillium* (2) and *Aspergillus* (3) were not identified up to species level. A Deuteromycete fungus and one species with morphological characteristics similar to *Raffaelea montetyi* (Morelet, 1998) were also isolated and they are still subject of further DNA molecular analysis in order to confirm their taxonomic affiliation. *Raffaelea montetyi* has already been related to *P. cylindrus* in France (Morelet, 1998) and Portugal (Henriques, 2007).

All species isolated from the mycangia, except for *Beauveria bassiana*, were found in the galleries dug by the insects in the cork oak wood. Females' mycangia have a significant fungal richness of 37 species (χ^2 = 8,45, 1ddl) against 30 contained in males' mycangia. 25 species are commonly conveyed by both sexes, the most important are Ophiostomatales but also some Hypocreales and Xylariales. The latter two groups are represented respectively by *Acremonium crotocinigenum* and *Biscogniauxia mediterranea* (*Nodulisporium* sp.), which have been also isolated from galleries and mycangia of *P. cylindrus* in Portugal (Sousa and Inacio et al., 2005; Henriques, 2007).

These results confirm the significant role played by females in the transport and inoculation of ectosymbiotic fungal spores (Batra, 1963; Beaver, 1989; Kent, 2008; Moon et al., 2008a). The intestinal contents of the beetle contained 30 fungi of which 12 were collected from both intestines of adults and larvae. They are richly represented by Ophiostomatales and Eurotiales. The intestinal mycobiota of larvae is composed of 25 species of which eight are specific to this stage and belong mainly to Pleosporales (but also to Hypocreales). On the other hand, the intestinal contents of both sexes contain slightly less fungi with a total of 21 species of which only two are common. These are *A. crotocinigenum* and *Nectria mauritiicola*. The intestines of males contain two specific species namely *Ulocladium botrytis* and the Deuteromycete.

The fungi isolated from all sources are richly represented by Ophiostomatales, including three species of *Ophiostoma* (*O. nigrocarpum, O. quercus* and *O. stenoceras*), *R. montetyi* and a majority of Eurotiales.

DISCUSSION

The present study on the mycetophagy of *P. cylindrus* on cork oak shows that adults and larvae feed on a large number of fungi. The four Ophiostomatales species are considered as primary ambrosia fungi that play a primary role in insect diet (Batra, 1967, 1985). Among them *Ophiostoma nigrocarpum* which has also been isolated from mycangia of other insects especially beetles (*Dendroctonus frontalis* and *D. brevicomis*) (Paine and Birch, 1983; Zambino and Harrington, 1990; De Beer et al., 2003b).

Some fungi obtained from the intestines may provide a supplement to the diet of adults during the excavation of galleries (*Acremonium crotocinigenum* and *Nectria mauritiicola*) and to larvae throughout their development (*Geosmithia langdonii*, *Cladosporium herbarum*).

Saprophytic fungi completing the insect feeding diet include indeterminate species of the genera *Aspergillus* and *Penicillium*, and the species *Hypocrea lixii* (*Trichoderma harzianum*). This prevailing fungal group has been largely isolated from many ambrosia beetles as Platypodinae, like *P. cylindrus* on cork oak in Portugal (Sousa and Inacio, 2005; Henriques, 2007) and Morocco (Sousa et al., 2005). Other saprophytic fungal species, for example *Umbelopsis isabellina*, *Trichocladium asperum* and *Humicola grisea* var*. grisea*, have been isolated for the first time from insects. According to Hambleton et al (2005), the ITS sequences of *Trichocladium asperum* and *Humicola grisea* are identical (Figure 5), nevertheless, these two species are morphologically distinct based on conidial characters; we differentiated both species morphologically.

Besides these species which are important as food for the insect, two other categories of fungi have economic and forest importance. The first category includes entomopathogenic fungi belonging to the Hypocreales as *Beauveria bassiana* and *Lecanicillium Psalliota* (*Verticillium lecanii*). These two species are specifically conveyed in females' mycangia and inoculated on the walls of galleries produced in wood. The development of these fungi in galleries may play a significant role in limiting populations of some insect borers living in the cork oak as *P.cylindrus, Cerambyx cerdo* (Figure 6) and *Xyloborus monographus*. These two fungi are used in biocontrol as biopesticides against many pests in many countries (Samson et al., 1988; Goettel et al., 2008). *B. bassiana* has already been associated with *P. cylindrus* on cork oak in Portugal (Inácio et al., 2005; Henriques, 2007), whereas *L. psalliota* seems new to this type of relationship.

The second category includes plant pathogenic fungi, some being the main agents of parasitic diseases. After their introduction in the host trees as spores, these fungi find favorable conditions for germination and growth, strongly affecting trees towards and provoking their decline. Several hypotheses have been proposed to explain the action of these fungi in the host and the mechanisms causing the appearance of decline; degradation of lignin and cellulose causing wood rot (Krivosheina, 1991) and lesion formation within the wood by invading the sapwood (Harrington, 2005). These biochemical and mechanical actions damage sap-conducting vessels, making difficult the flow of water and aggravating water stress of already weakened trees. The onset of symptoms occurs shortly before death of the tree.

The last group of fungi is composed of 17 species of variable economic importance. The most well known are represented by members of *Ophiostoma*.

Figure 6. Insects of two different genera killed by *Beauveria bassiana*: *Cerambyx cerdo* (left) and *Platypus cylindrus* (right).

Many ambrosia beetles are vectors of these fungi causing serious damage to the infested trees such as the Dutch elm disease caused by the fungus *O. ulmi* (Buisman) Nannf. carried by the bark beetle *Scolytus scolytus* F. (Webber and Gibbs, 1989). Also, several other *Ophiostoma* species are known as “blue stain fungi” for their role in the bluish coloring of wood caused by pigmented hyphae (Thwaites et al., 2005). Three species have been isolated here; one of them, *O. nigrocarpum* is not a dangerous species (Harrington, 2005) but the two others, *O. quercus* and *O. stenoceras*, are potentially phytopathogenic and they have already been related to other insects in many countries.

Ophiostoma quercus (*O. querci*) was isolated for the first time in Yugoslavia, from *Quercus pedunculata* (Georgévitch, 1926, 1927). This fungus has been implicated in the decline of various oaks in Central Europe (Cech et al., 1990). In Spain, Luque et al. (2000) have isolated *O. quercus* from cork oak, and it has been considered as the common sapstain or xylem discoloration agent of many other trees (De Beer et al., 2003b; Geldenhuis et al., 2004; Kamgan et al., 2008). *O quercus* is related to many insects but especially to bark beetles (Kirisits, 2004; Zhou et al., 2004, 2006). In Norway, *O. quercus* has been associated with *Scolytus rafzeburgi* in galleries of *Betula* sp. (Linnakoski et al., 2009) and it has been found also in Finland and Russia (Linnakoski et al., 2008).

Ophiostoma stenoceras is conveyed by the beetle *Hylastes ater* and has been isolated from damaged seedlings of *Pinus radiata* (Reay et al., 2002). De Beer et al. (2003a) confirmed that this species is a normal saprophyte of wood in Europe and North America. Piontelli et al. (2006) consider this species as a common contaminant of wood and forest soil becoming potentially pathogenic when conditions are favourable. They isolated it from the Buprestidae *Ectinogonia buquetti* attacking *Eucalyptus* where it causes considerable damage.

The development of phytopathogenic *Ophiostoma* often ends in death of host trees (Lim et al., 2004). To explain the cause of this mortality, Harrington (2005) suggests a combined action of the fungus and the secondary xylophagous insect that disseminates *Ophiostoma*. The fungus may intervene, more in the mechanism of insect population establishment by depleting the tree (Lieutier et al., 1989; Paine et al., 1997).

The species identified morphologically as *Raffaelea montetyi* has already been associated with *P. cylindrus* in France (Morelet, 1998) and Portugal (Henriques et al., 2006; Inacio et al., 2008). However, the effect of the species of *Raffaelea* on the cork oak is still poorly known. In Japan, the pathogenicity of *Raffaelea quercivora* Kubono and Ito (2002) has been proved being a primary ambrosia fungus of *Platypus quercivorus* Murayama; this fungus is the agent of mass mortality of Fagaceae, especially *Q. serra* Thunb, *Q. mongolia* Fich and *Q. cripula* Blume (Kubono and Ito, 2002; Kinuura and Kobayashi, 2006). *Raffaelea lauricola*, a similar fungus isolated from the mycangia of the insect *Xyleborus glabratus* in the USA, was held responsible for Laurel Wilt (*Persea borbonia*) according to Fraedrich et al. (2008) and Harrington et al. (2008).

Studies on oak decline in Europe have shown that the complex of fungi *Ophiostoma*/ *Ceratocystis* are frequent pathogens of the genus *Quercus* (Badler, 1992; Delatour et al., 1992; De Greef, 1992). Santos et al. (1999) recorded the presence of *Ophiostoma* sp. in *Q. suber* in Portugal. However, hitherto no species of *Ophiostoma* has been associated with *P. cylindrus*. Nevertheless, phylogenetic studies using SSU rDNA sequences of

Raffaelea species isolated in Portugal, showed similarities with *Ophiostoma* species (Inacio et al., 2008).

In addition to these fungi, *Biscogniauxia mediterranea* (*Hypoxylon mediterraneum*) has been known as an opportunistic fungus demonstrating its pathogenicity for many weak tree species, causing their decline. This fungus is the agent of charcoal disease of *Quercus* sp. trunks and branches (Mazzaglia et al., 2001a, b) and is widespread throughout the Mediterranean region particularly on cork oak (De Sousa, 2003), where it plays an important role in its decline, both in Europe (Portugal, Spain and Italy) and North Africa (Nugent et al., 2005). The relationship of this fungus with the attack of *P. cylindrus* has already been demonstrated by Sousa and Debouzi (1993) and Santos (2002). *B. mediterranea* is also the agent of canker disease of *Q. cerris* suffering already from a water stress (Vannini and Mugnozza, 1991). This also increases the virulence of the fungus on cork oak (Luque and Girral, 1989; Luque et al., 2000).

Botryosphaeria corticola is part of the plant pathogenic mycoflora in many agricultural and forest trees (*Quercus*, *Pinus*, *Eucalyptus*), and has an important economic significance by causing dieback, cankers and shoot blight (Alves et al., 2004, 2005; De Wet et al., 2008; Barber et al., 2005). Its anamorph is *Diplodia corticola* (Luque et al., 2008). *B. corticola* is considered as one of the agents contributing to the decline of Mediterranean cork oak stands (Becker and Levey, 1982; Luque and Girbal, 1989; Bakry and Abourouh, 1995; Sanchez et al., 2003). The intensity of its pathogenicity increases when the plant is subjected to water stress (Slippers and Wingfield, 2007) resulting in general, in death occurring 2-3 years after onset of symptoms (Luque et al., 2000). The relationship of this fungus with the insects has been reported by Erbilgin et al. (2007) after isolation in California, from *Q. agrifolia*. However, no previous study has reported association of *B. corticola* with *P. cylindrus.*

Many other isolated fungi in this work are considered potentially plant pathogenic but without being able to seriously affect the tree health.

The isolation of *Geosmithia langdonii* from *P. cylindrus* on *Q. suber* is a new association for this beetle. Members of *Geosmithia* genus have not been linked to Platypodinae until now, thought they are usually part of fungal ambrosia beetle galleries (Kirschner, 2001 and Kolarik et al., 2005). The most popular vectors of these fungi are the Scolytinae beetles (Kolarik et al., 2004, 2005, 2007; Kubatova et al., 2004). The effect of *Geosmithia* sp. on trees is still poorly understood but some studies suggest they have significant phytopathogenic potential (Cizkova et al., 2005; Scala et al., 2005).

Representatives of the genus *Acremonium* were often regarded as plant pathogens causing serious damage to plants (Vicente et al., 1999). Some have already appeared in relationships with *P. cylindrus* and many other ambrosia beetles (Francke-Grossmann, 1967; Sousa and Inacio, 2005; Henriques, 2007). The species *A. crotocinigenum* isolated here was described as the causative agent of a leaf disease recognized by its red-brown spots on the leaves of *Syngonium podophyllum* (Uchida and Aragaki, 1982) but the pathogenic action on *Q. suber* needs to be studied.

Nectria species, including the isolated here *N. mauritiicola*, are known as plant pathogens (Vujanovic et al., 2007). They can lead to symptoms such as logs deformations with sapwood staining of *Pinus radiata* (Dick and Crane, 2009) and necrotic disease of *Fagus sylvatica* L (Mihal et al., 2009). Also, this fungus is associated with some insects often attacking *Ulex europaeus* L in New Zealand (Yamoah et al., 2008) but the literature does not provide any information about its presence on *Q. suber* or its relationship with *P. cylindrus*. *Scytalidium lignicola* is a component of the natural mycoflora. Some biotypes are plant pathogenic (Oren et al., 2001). Its relationship with *P. cylindrus* was reported by Sousa (1996) who isolated it from cork oak galleries in Portugal. This is also the case of the genus *Chaetomium* (Henriques, 2007). *C. globosum* is responsible for the deterioration of wood by the production of specific enzymes (Popescu et al., 2010).

Phoma species including *P. glomerata*, are considered serious plant pathogens (De Gruyter et al., 2009; Sreerama Kumar et al., 2005). Thus, *P. glomerata* was used as a biological control agent against the weed *Salvinia molesta* D.S. Mitchell in Bangalore (India) causing its sudden decline in crops (Sreerama-Kumar et al., 2005). Most *Alternaria* species are harmful, causing significant economic damage to many types of crops in the world (Rotem, 1994; Pryor and Gilbertson, 2000). Among the hardwood trees, *A. alternata* and *A. infectoria* have proved their pathogenicity causing twig disease (Erbilgin et al., 2007). The association *Alternaria* sp. / *P. cylindrus* was reported by Cassier et al. (1996).

Among fungi pathogenic to herbaceous plants responsible for many diseases in several countries worldwide, we isolated, *Phaeoacremonium inflatipes* causing Esca and Petri disease on *Vitis vinifera* (Santos et al., 2005; Essakhi et al., 2008), *Ulocladium botrytis* on *Orobanche crenata* (Müller-Stöver and Kroschel, 2005) and *Gnomonia petiotorum* on *Fragaria vesca* (Barr, 1978; Morocko, 2006).

Finally, the fungus known as *Coriolopsis gallica*, agent of white rot in plants, presents a particular industrial interest for its extraordinary powers in the production of many extracellular enzymes able to degrade lignin and cellulose like the laccase. This enzyme is widely used in paper industry for bleaching (Jordaan and Leukes, 2003).

Conclusions

In the last years *P. cylindrus* has been considered as one of the most important biotic agents directly involved in cork oak decline observed in Algeria and elsewhere. Being an ambrosia beetle, it establishes a symbiotic relationship with fungi essential to its life cycle. Knowledge of the associated mycobiota and the roles played by each fungus in the insect-fungus-tree interaction is indispensable for understanding the strategies in the establishment of *P. cylindrus* on cork oak and the direct causes leading to the decline of the tree.

According to our results, *P. cylindrus,* in the cork oak wood maintains a mutualism relationship with a much diversified mycobiota. *Raffaelea montetyi, Biscogniauxia mediterranea, Acremonium crotocinigenum, Scytalidium lignicola, Chaetomium globosum, Beauveria bassiana, Alternaria alternata* and *A. infectoria, Aspergillus, Penicillium* and Mucorales have already been reported to be associated with this insect. However, many other fungi species already related to other ambrosia beetles have been associated in this work with *P. cylindrus* for the first time, such as *Ophiostoma nigrocarpum, O. quercus, O. stenoceras, Nectria mauritiicola, Botryosphaeria corticola, Geosmithia langdonii* and *Cladosporium herbarum.*

In this study we found that *Lecanicillium psalliotae, Humicola grisea* var. *grisea, Gnomonia petiolorum, Phaeoacremonium inflatipes*, *Coriolopsis gallica*, *Ulocladium consortiale, Ulocladium botrytis, Phoma glomerata, Phoma exigua* var. *exigua* and *Umbelopsis isabellina* have not been reported before as associated mycobiota of xylophagous insects including *P. cylindrus*. Thus the symbiotic association between fungi and this insect in the cork oak forests area is here enriched with 17 new species.

The relative importance of the isolated fungi is very variable and debatable. Many species intervene however, in feeding the insect and the presence of Ophiostomatales in all the sources explains their inclusion in this mycetophagy. In addition to these species, larvae feed on a wide variety of other fungi. Besides these fungi, others may be antagonistic or simply saprophytes by getting involved in relations of commensalism with host tree but some are potentially plant pathogens. Among them, we mention especially*, Biscogniauxia mediterranea, Botryosphaeria corticola, Ophiostoma quercus and O. stenoceras*. The transport and inoculation of these fungi by the insect in the tree can have catastrophic consequences for the role that some of them can have in the decline of cork oak forests. This association seems to play a determining role in the establishment strategies of insect populations by weakening the host-tree defense capacity. Results obtained by Bouhraoua et al. (2002) and Belhoucine (2008) confirm that tree mortality increases either as a result of massive attacks of the insect or to the combined action insect /fungi. In this case, the premature death of certain slightly infested trees has rather been attributed to the aggravating action of plant pathogenic fungi spread by the beetle rather than to the action of the beetle itself. Further research is needed to expand our knowledge of the mycobiota associated with *P. cylindrus*. The role of plant pathogenic species on the tree and the action of each fungus in the cycle of cork oak decline must be subject to pathogenicity tests.

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