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Mycobiota associated with the ambrosia beetle *Megaplatypus mutatus*: threat to poplar plantations

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Megaplatypus mutatus (syn. *Platypus sulcatus*) is an ambrosia beetle native to South America, which represents the main forest pest in Argentina of Poplar plantations and is also an emerging pest in Europe, representing a potential risk to forest and fruit plantations globally due to its low specificity. Knowledge of the interactions this insect has with microbes will be important in understanding its impacts and management. In this study, we characterized the fungal diversity associated with *M. mutatus* in *Populus* trees in Argentina. The fungal community of 28 attacked trees was studied by evaluating 1104 gallery fragments and 110 fragments of insects. Fungal isolates were identified using morphology and phylogenetic analysis of the internal transcribed spacer region of nDNA. Nineteen taxa were identified, the most relevant *Fusarium solani* species complex, three species of *Raffaelea* and *Graphium basitruncatum*. Despite the lower frequency occurred by *Raffaelea* spp. and *G. basitruncatum*, we detected a specificity between male/female and location in the gallery. Additionally, the topology of the galleries is described based on analyses with computed tomography and nuclear magnetic resonance. Enhancing these techniques, an image combining both data was produced, suggesting that the water circulation across outer sections might be involved in the modulation of the gallery topology.

Keywords: ambrosia beetle, *Fusarium*, *Raffaelea*, fungal interaction, computed tomography, resonance magnetic nuclear

Introduction

The genus *Populus* is an extended and diverse group with a large number of species and clones (Ceulemans *et al*., 1990). Because of their rapid growth, timber yield and landscaping benefits (Sixto *et al*., 2005), the genus is of great interest for forestry and the production of biomass and biofuel as well as in phytoremediation (Balatinecz and Kretschmann, 2001).

Members of this genus are attacked by *Megaplatypus mutatus* Chapuis an ambrosia beetle considered a major forest pest of *Populus* spp. in Argentina (Alfaro *et al*., 2007). Furthermore, in 2001, this forest pest has achieved the status of invasive species and emergent foreign pest in Europe (Tremblay, 2000; Allegro and Della Beffa, 2001; Funes *et al*., 2011). *Megaplatypus mutatus* (syn. *Platypus mutatus*) is native to South America (Wood, 1993) and has been reported in several countries in the continent (Charles *et al*., 2014). Unlike most bark and ambrosia beetles, *M. mutatus* attacks only living and vigorous trees (Alfaro *et al*., 2007), and it has a wide recorded host range, including broadleaf and coniferous trees, such as *Acacia*, *Acer*, *Citrus*, *Eucalyptus*, *Laurus*, *Pinus* and *Quercus*. Attacks have also been reported on species of *Corylus, Malus, Populus*, *Pyrus* and *Prunus*, causing a great economic impact (Giménez and Etiennot, 2003).

The biology of *M. mutatus* was extensively described by Santoro (1957, 1963, 1965), emphasizing that single attacks are common but multiple attacks are also possible. Galleries built by a unique couple of beetles weaken the trunks causing them to break under the effect of a sixth-grade wind on the Beaufort scale (González-Audino *et al*., 2011; Bobrowsky, 2013), resulting in major yield reductions. Additionally, the dark staining produced by the ambrosial mycelia growing on the galleries walls reduces wood quality (Bascialli *et al*., 2008), making wood unsuitable for certain uses and decreasing its market value (Alfaro *et al*., 2007).

There is an increasing amount of literature on the relationship between fungal consortia with bark and ambrosia beetles, especially on interactions considered of high economic relevance (Belhoucine *et al*., 2011; Endoh *et al*., 2011; Biedermann *et al*., 2013; Freeman *et al*., 2013; Kasson *et al*., 2013; Harrington *et al*., 2014). Earlier studies reported that the mycobiota plays a key role in the adequate establishment and maintenance of beetles (Batra,

1985; Beaver *et al*., 1989; Paine *et al*., 1997; Biedermann *et al*., 2013). Furthermore, mutualistic symbionts can facilitate the establishment of invasive beetles species or enhance their damage (Bennett, 2013; Taerum *et al*., 2013). However, associated fungi of only a small percentage of ambrosia beetles are currently known (Funk, 1965; Batra, 1967; Francke-Grosmann, 1967; Baker and Norris, 1968; Barras and Perry, 1972; Kinuura, 1995; Cassar and Blackwell, 1996; Gebhardt *et al*., 2005), and the nature of the interactions between ambrosia fungi–host–*M. mutatus* along with the role of the fungal community remains mostly unknown.

Concerning *M. mutatus*, Guerrero (1966) identified the first associated fungus, and few, if any, further studies have been published. The main aim of this study was to improve understanding of the processes involved in the establishment of fungal communities and the functional roles of fungal organisms in these interactions, both of which are a prerequisite to develop tools for integrated management of this pest and to estimate the implications on invasion events. The objectives of the research were: (1) to study the fungal community associated with *M. mutatus* in commercial poplar plantations; (2) to characterize the fungal diversity associated with galleries, the beetles and the determination of the pre-existent fungal communities by evaluating the endophytic fungi in the wood of poplar; and (3) to examine and analyse the topology of the beetle galleries.

Methods

Research site and plant material

This study was conducted in Junín, Province of Buenos Aires, Argentina (34°43′56.3″S, 60°51′11.5″W) at 80 m a.s.l, between February and March 2013, in a commercial 12-year-old poplar plantation (*Populus deltoides*, clone Australian I29/60) covering 14 ha, with a 425 trees/ha density (4×4 m spacing), and a mean (±SE) 77.8±0.2 cm diameter at breast height (d.b.h.). The annual average rainfall ranges from 850 to 1050 mm; the average 10-year temperature shows July as the coldest month with 6.9°C and January as the warmest month with 25°C, with extreme temperatures that reach 41°C in summer and −2°C in winter (data from the National Weather Service of Argentina).

Sampling and culturing

In order to isolate and characterize the fungi associated with *M. mutatus*, different sampling methods were used.

Galleries

Active galleries were recognized by the presence of abundant sawdust streaming down the trunk from the entrance/exit holes (Funes *et al*., 2011). A total of 28 trees were studied. Twenty-one trees were sampled using a Pressler borer (Suunto, Vanta, Finland) (Moore and Six, 2015). An area of bark (ca. 3×3 cm) was removed and wood cores, ∼6 cm long, were collected from galleries. Under aseptic conditions, fragments of ca. 0.5 cm long were cut and then placed onto 90-mm Petri dishes containing culture media. Additionally, seven infested trees containing a single beetle gallery were sampled. The procedure for timber maintenance and sampling of gallery fragments was according to Belhoucine *et al*. (2011).

Insects

The relationship between ambrosia beetles and associated fungi was tested at dispersal stage for adults, collected in emergence traps (without bait) (Giménez, 2009); while fourth- and fifth-instar larvae were obtained from inside the galleries. A total of 15 males and 15 females were analysed: in order to study the inoculum transported, an imprint of the ventral side of the body and of the larvae (10) prothoracic segment was made. Additionally, the head and elytra of the insects were dissected without any additional treatments. Fungi cultures were obtained from dissected fragments and imprints of the beetles.

Endophytes

A total of 19 asymptomatic trees were selected at the same location. Selection was made by sampling the nearest asymptomatic tree close to an attacked one. To evaluate the pre-existing microbial community in trees, a 2×2 cm section of bark was removed and wood cores ∼10 cm long were extruded at 1.4 m above soil line by means of a Pressler borer. Wood core fragments were surface-sterilized and analysed for the occurrence of endophytes following Robles *et al*. (2015).

All harvesting instruments used in each type of sampling were sterilized before and after each usage to prevent contamination. The biological material was placed in sterile tubes and kept refrigerated until analysis for no longer than 48 h. All further steps were carried out in a laminar flow hood. The materials obtained (wood and insects) were transferred to 90-mm Petri dishes containing 2 per cent Malt Extract Agar (MEA, Oxoid Malt-Agar). Plates were incubated in the dark at 24°C for 8 weeks and examined periodically. Outgrowing mycelia were isolated, purified, transferred onto slants containing MEA and stored at 4°C.

Fungal identification

For morphological identification purposes, fungal strains were routinely subcultured on MEA and 2 per cent potato dextrose agar (PDA, Difco). Pure cultures were examined periodically for sporulation and morphotypes were selected according to characteristics of the colony and spore morphology. Observations and measurements of fungal strains (n=1004) were carried out on fresh material, mounted in distilled water and phloxine for optical microscopy (Olympus-BX41). Measurements were made with Infinity Analyze v6.4 (Lumenera Corporation). Sterile cultures suspected to belong to the genus *Raffaelea* were also grown on MEA with cycloheximide, following Harrington (1981). Representative strains used in phylogenetic analysis (n=28) are deposited in the Culture Collection of Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina (BAFCcult).

Representative strains of morphotypes were selected for DNA sequence studies (morphotypes representing 95 per cent of the strains obtained). All the strains were grown on MEA and cultures were incubated at 24°C for 7–21 days in the dark. DNA was extracted from growing mycelia (40–80 mg from mycelium), using the UltraCleanTM Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, USA), according to the manufacturer's instructions. The Internal Transcribed Spacer (ITS) region of the nuclear DNA was amplified using the universal primers ITS1 and ITS4 (White *et al*., 1990), using the following PCR conditions: denaturation at 94°C for 3 min, 45 cycles of denaturation at 94°C for 45 s, annealing at 53–55°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 7 min. Reactions were performed in 50.0 μl volumes containing 10 mM PCR buffer supplied by the manufacturer, 50 mM $MgCl_2$, 1 mM of each dNTP, 10 mM of each primer, 1 U Recombinant Taq DNA polymerase (Invitrogen, Brazil) and 4 μl fungal genomic DNA. PCR products were purified by using the UltraCleanTM PCR Clean-Up kit (MO BIO Laboratories Inc.), according to the manufacturer's instructions. Both strands of each fragment were sequenced on an ABI 3730xl DNA Analyzer and then analysed with the software Sequence Scanner (Applied Biosystems). Contiguous sequences were assembled with Vector NTI (Invitrogen). Additional to the sequences generated in this study, 76 sequences from GenBank, representing 52 species, were included in the analyses (Supplementary data, Table S1). All the sequences used in phylogenetic analyses were published by expert taxonomists and deposited in culture collections. Whenever available, sequences of type strains were chosen.

Two dataset were used to aid visual interpretation: (1) *Fusarium* species (including 14 sequences obtained in this work) and (2) multiple genera (including 14 sequences obtained in this work).

The alignments with selected reference sequences were made by Clustal W v.1.4 for multiple alignment of the BioEdit v.7.0.5.3 software (Hall, 1999). Costs assigned were 15 for gap openings and 6 for gap extensions. Sequences were phylogenetically analysed using maximum parsimony (MP) on MEGA version 6 (Tamura *et al.*, 2013) with all the characters equally weighted and gaps scored as fifth state. To determine the support for each clade, a bootstrap analysis with 1000 replications was performed. Additionally, Bayesian inference (BI) was calculated with MrBayes v.3.2.3 with K2P+G model (Ronquist and Huelsenbeck, 2003). Two Markov chains were run from random starting trees for 6.3 and 7 million generations sampled every 5000 generations, for datasets corresponding to multiple genera and the genus *Fusarium*, respectively. The first 25 per cent of the generations were discarded as burn-in. The 50 per cent majority-rule consensus phylogram was computed from the remaining trees. A sequence of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. was chosen as outgroup (CBS-Type-JX010148) in both MP and BI analyses. Sequences were deposited in GenBank (KT828712/739).

Quantitative analysis

The frequency of occurrence for each species was calculated using the following formula:

$$\text{Relative frequency of taxon A} = \left[\frac{\text{number of taxon A}}{\text{total number of all species}}\right] \times 100$$

Species accumulation curves and total richness were inferred with EstimateS v.9.1 (Colwell, 2013). Diversity was estimated by Shannon's diversity index (*H*), whereas similarity was estimated by Sørensen's index.

Computed tomography and nuclear magnetic resonance of attacked timber

Additional to the 28 analysed trees, 9 independent attacked timbers were analysed through multislice computed tomography (CT) (Philips-MX 16-slice). The timber containing the most developed gallery was also analysed by nuclear magnetic resonance (NMR) (Philips-Achieva 3.0 T TX). The X-ray CT technique provides slice images of the sample that correspond to its mass density distribution. On the other hand, due to its physical functioning principle, the NMR provides a strong signal for the most hydrated tissues, thus revealing the water density distribution in the sample. To enhance the information provided by both techniques, an image combining CT and NMR images was generated. Since CT can produce slices with a separation of 0.4 mm and NMR produces slices with a minimum separation of 4.5 mm, a matching-image procedure was necessary. A picture combining both data was produced by using two integrated images generated by adding up 40 slices for CT and 5 for NMR (Dolinko and Kaufmann, 2007). The adding procedure was made in order to account for the information contained in the same region of the timber. To visualize the combined data, we used different basic colours for each integrated image. The integrated CT image was coded in green (light grey for print version), while the integrated NMR image was coded in blue (dark grey for print version). Therefore, a region having high mass density and high content of water simultaneously is visualized in cyan (white for print version).

Results

A total of 1308 fragments corresponding to galleries (n=1104), insects (n=110) and endophytes (n=94) were evaluated and 1004 fungal strains were recovered.

Fungal identifications

A total of 19 species were recognized based on molecular and morphological characters. Phylogenetic studies were carried out to improve the morphological identifications. Within the analyses performed, the genus *Fusarium* was studied by MP analysis and BI of the ITS region (Figure 1). MP and BI analysis showed that *Fusarium* complexes are represented by at least three species: *Fusarium solani* (Mart.) Sacc., *Fusarium asiaticum* O'Donnell, T. Aoki, Kistler and Geiser and *Fusarium oxysporum* Schltdl.

Additionally, reference sequences of *F. oxysporum* appeared in the same clade with one of our sequences assigned to this species. MP and BI analysis clustered the sequences corresponding to *F. solani* in several subclades close to reference sequences, whereas sequences BAFCcult 4495, 4500, 4503 and 4504 clustered as a sister clade of *F. solani* clade, close to reference sequences of *F. ambrosium* (Gadd and Loos) Agnihothr. and Nirenberg, *Fusarium pseudensiforme* Samuels, Nalim and Geiserand., and *Fusarium euwallacea* S. Freeman, Z. Mendel, T. Aoki and O'Donnell, and presumably represent a fourth species of this genus associated with *M. mutatus*. However, further specific taxonomic studies involving multigene analyses and pathogenic test of the strains belonging to *Fusarium* are necessary to clarify if these strains belong to a new or a previously described species.

A multiple genera dataset was analysed by MP and BI of the ITS region (Figure 2). BI analysis showed topologies similar to those of MP. More than 10 groups were found within the analysis. Phylogenetic results supported morphological identifications, especially at genus level, and improved it at species level in some cases.

Raffaelea sequences obtained in this study clustered in one clade separated from other species in the genus and were referred as *Raffaelea* sp. 2 and *Raffaelea* sp. 3. Sequences obtained from strains assigned to *Graphium basitruncatum* (Matsush.) Seifert and G. Okada clustered close to the reference sequences (KF540218/223) temporarily named as *Graphium* sp., currently under taxonomic studies (personal communication, Eskalen, 2015).

Fungal diversity in galleries

From 1104 fragments analysed, 770 strains belonging to 19 different taxa were recovered. Most of the strains (n=594) belonged to *Fusarium solani*. The other representative taxa were *G. basitruncatum* and *Raffaelea arxii* D.B. Scott and J.W. du Toit, represented by 22 strains each. *Trichoderma* Pers. was represented by 20 strains, whereas the family *Dipodascaceae* Engl. and E. Gilg was represented by 47 strains. Among the most recovered strains, 51 were assigned and distributed among *F. solani*, *F. oxysporum*, *F. asiaticum* and *Raffaelea* sp. 2. *Nigrospora sphaerica* (Sacc.) E.W. Mason, *Aspergillus niger* Tiegh complex, *Geotrichum restrictum* de Hoog & M.T. Sm., *Ambrosiozyma platypodis* (J.M. Baker and Kreger-van Rij) Van der Walt, *Raffaelea* sp. 3, *Penicillium* sp., *Bjerkandera adusta* (Willd.) P. Karst, *Coprinellus radians* (Desm.) Vilgalys, Hopple and Jacq. Johnson, and *Mucor circinelloides* Tiegh were represented in a much lower frequency. Table 1 shows the relative frequency of fungal species recovered from the insect galleries.

Direct observations on galleries showed synnematas of *G. basitruncatum* in sections with recent fungal colonization.

Fungal diversity in beetles

A total of 234 strains belonging to 12 different taxa were obtained from 110 fragments and imprints cultured. The most frequent

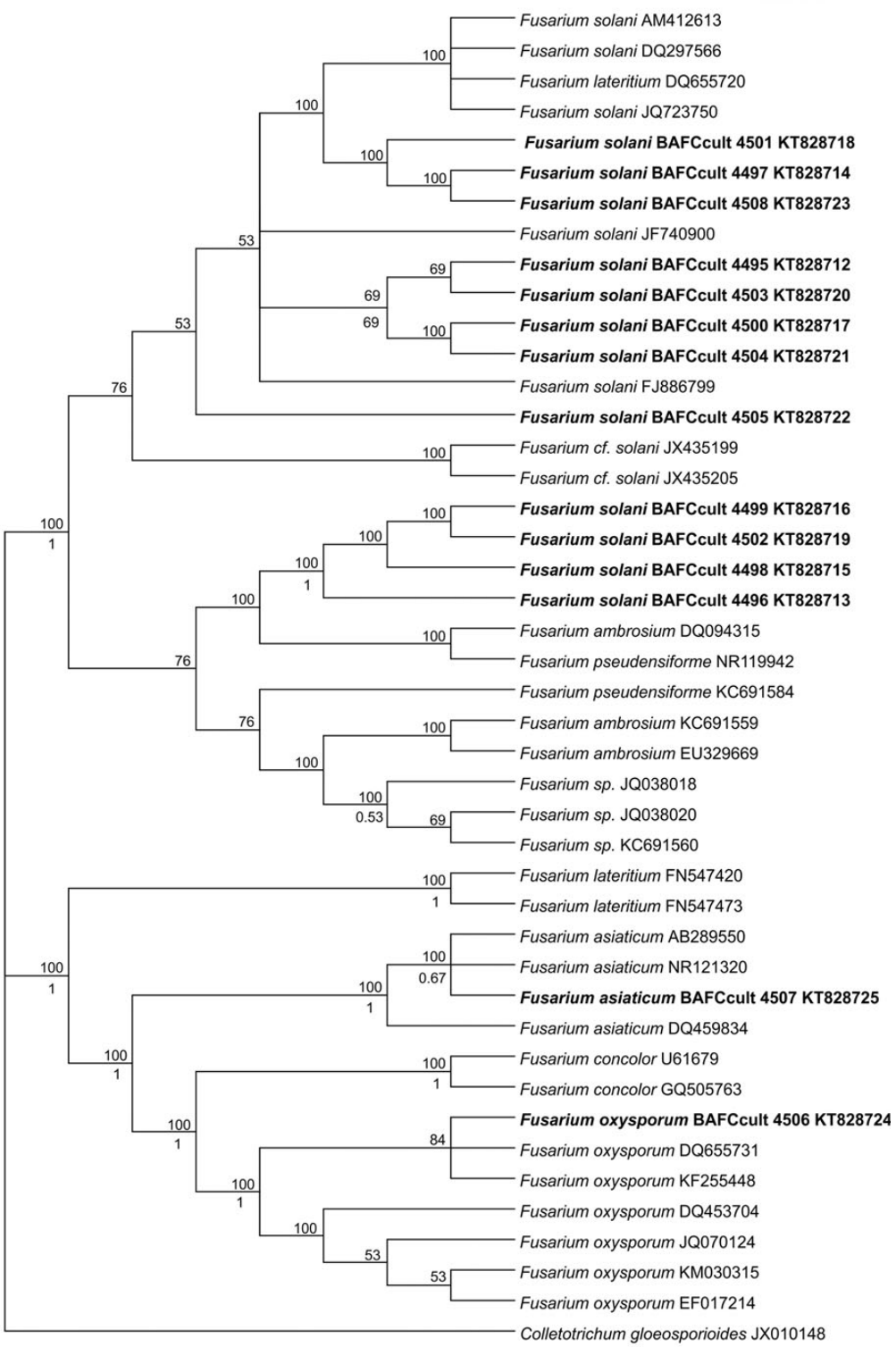

Figure 1 MP and BI analysis of *Fusarium* dataset, based on the ITS1/ITS2 region. Obtained sequences in this study (bold). MP 50% majority-rule consensus tree of *Fusarium* species, dataset of 44 sequences and 504 informative characters. The analyses yielded 13 parsimonious trees (L=672; Ci = 0.71; Ri= 0.87 composite index = 0.67). Bootstrap values higher than 50% are indicated above the line, while BI (K2P+G model) posterior probabilities are indicated under the line.

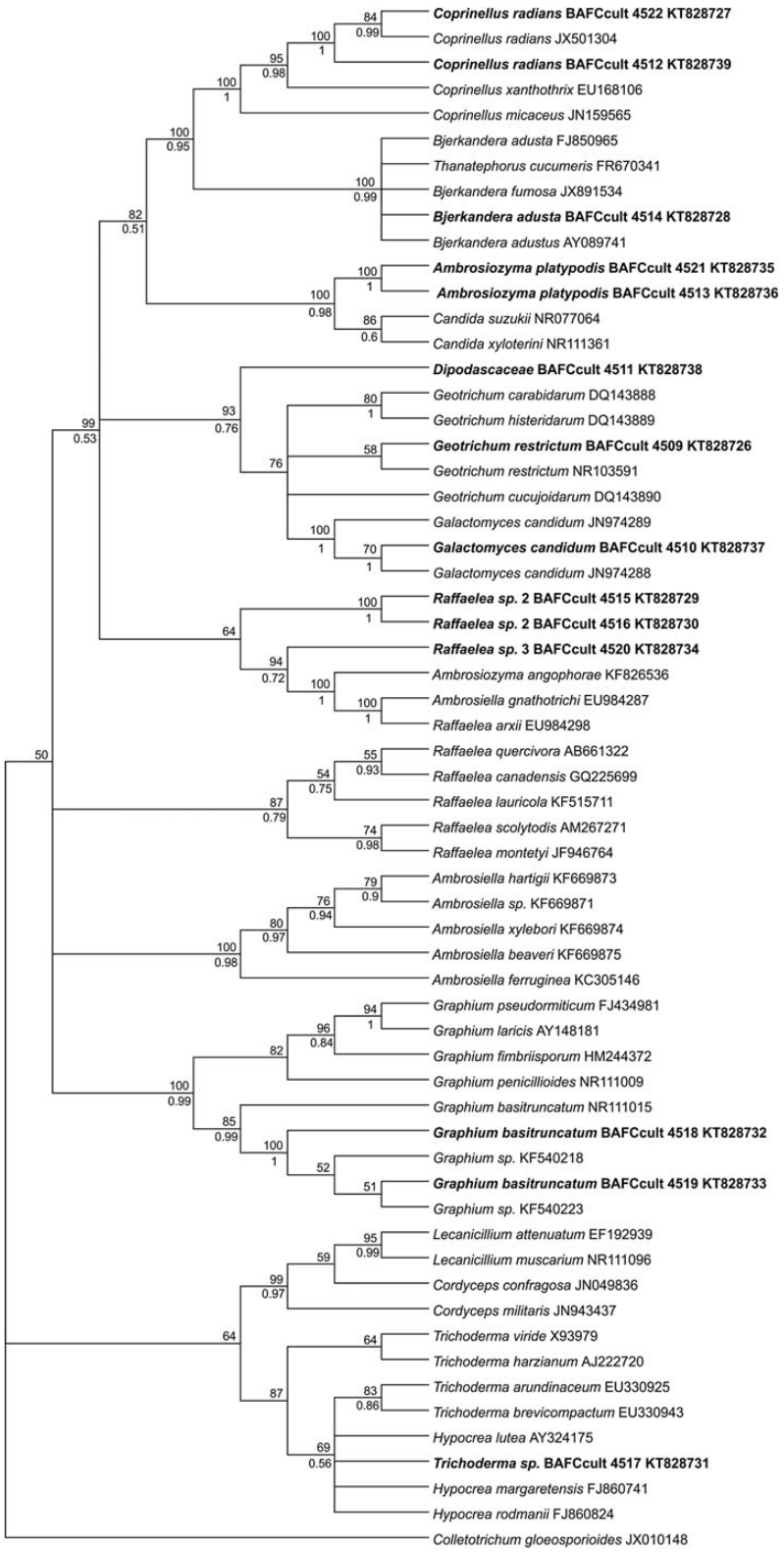

Figure 2 MP and BI analysis of other representative genera, based on the ITS1/ITS2 region. Obtained sequences in this study (bold). MP 50% majority-rule consensus tree of multiple genera analyses, dataset of 61 sequences and 504 informative characters. The analyses yielded 108 parsimonious (L= 2455; Ci = 0.47; Ri = 0.80; composite index = 0.37). Bootstrap values higher than 50% are indicated above the line, while BI (K2P+G model) posterior probabilities are indicated under the line.

Table 1 Fungal species isolated from galleries (28 poplar trees, 1104 gallery fragments) built by *M. mutatus*

Species	Relative frequency
Fusarium solani	77.52
Dipodascaceae	6.10
Graphium basitruncatum	2.86
Raffaelea arxii	2.86
Trichoderma sp.	2.60
Raffaelea sp. 2	2.60
F. oxysporum + *F. asiaticum*	1.82
Nigrospora sphaerica	0.91
Aspergillus niger complex	0.91
Geotrichum restrictum	0.65
Ambrosiozyma platypodis	0.39
Raffaelea sp. 3	0.26
Penicillium sp.	0.13
Bjerkandera adusta	0.13
Coprinellus radians	0.13
Mucor circinelloides	0.13
Total	100

Table 2 Fungi isolated from exoskeleton of *M. mutatus*: males (n=15), females (n=15) and larvae (n=10)

Source	Isolated fungi	Origin			Relative frequency by source
		Head	Elytra	Imprint	
Males	*Fusarium solani*	✓	✓	✓	46.32
	Raffaelea sp. 1	✓	✓	✓	16.84
	Raffaelea arxii	✓	✓	✓	12.63
	Graphium basitruncatum	✓	✓		7.37
	Dipodascaceae	✓			4.21
	Nigrospora sphaerica	✓			4.21
	Raffaelea sp. 3			✓	4.21
	Penicillium sp.	✓			3.16
	Coprinellus radians	✓			1.05
Females	*Fusarium solani*	✓	✓	✓	74.51
	Chaetomium sp.			✓	9.80
	Raffaelea sp. 1		✓	✓	7.84
	Raffaelea arxii	✓			6.86
	Coprinellus radians			✓	0.98
Larvae	*Fusarium solani*	✓	–	✓	32.43
	Chaetomium sp.	✓	–	✓	16.22
	Dipodascaceae	✓	–	✓	16.22
	Mucor circinelloides	✓	–	✓	16.22
	Raffaelea arxii		–	✓	8.11
	Bjerkandera adusta	✓	–	✓	8.11
	Coprinellus radians	✓	–		2.70

species found in males, females and larvae was *F. solani*, represented by 44, 76 and 12 strains, respectively. In males, *Raffaelea* sp. 1, *R. arxii* and *G. basitruncatum* were represented by 16, 12 and 7 strains, respectively. The *Dipodascaceae*, *N. sphaerica* and *Raffaelea* sp. 3 were represented by four strains each, and *Penicillium* sp. and *C. radians* were represented by three strains and one strain, respectively. In females, *Chaetomium* sp. was represented by 10 strains, while *Raffaelea* sp. 1, *R. arxii* and *C.* radians were represented by eight, seven and one strains, respectively. In larvae, *Chaetomium* sp., *Dipodascaceae* and *Mucor circinelloides* were represented by six strains each; *R. arxii* and *B. adusta* were represented by three strains each, and *C. radians* was represented by only one strain. Frequencies and origin of the isolated fungi are shown in Table 2.

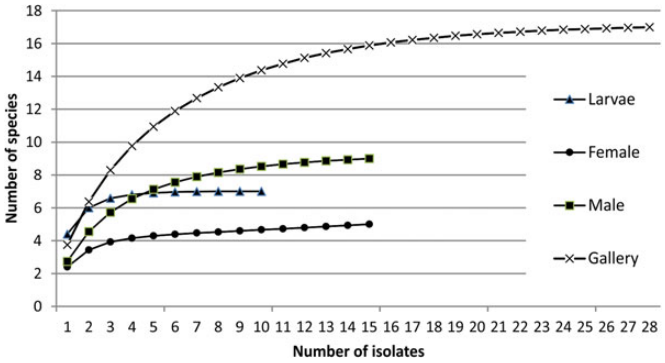

Figure 3 Accumulation curves of fungal species isolated from galleries and *M. mutatus*.

Strains corresponding to *F. solani* were recovered from galleries and insects, whereas *F. oxysporum* and *F. asiaticum* were recovered only from galleries.

It is important to note that the strains referred as *F. solani* and *F. oxysporum* are part of big species complexes and their isolates cannot be distinguished from others in the complexes using ITS region. Tentative names were used to facilitate the interpretation of the results. However, further specific taxonomic studies involving multigene analyses and pathogenic test of the strains belonging to *Fusarium* are necessary to clarify if these strains belong to a new or a previously described species.

The identification to level species of strains belonging to *Raffaelea* was made on a preliminary approach, due to the low resolution of the ITS region and/or the few diagnostic morphological characters.

Endophytic fungi

In order to evaluate the wood endophytic fungi, 19 trees were sampled, but no strains were recovered.

Quantitative analysis

Species accumulation curves of galleries and insects are shown in Figure 3, whereas diversity and richness indexes are shown in Table 3. The similarity (Sørensen's index) between communities of fungal galleries (FG) and males was the highest (0.69), whereas between FG and females was the lowest (0.36).

Computed tomography and nuclear magnetic resonance

In addition to field observations analysis, gallery topology was studied through the use of CT and NMR without performing any mechanical disturbance (Figure 4). The images analysis showed

Table 3 Diversity and richness indexes from galleries and *M. mutatus* (ACE, CHAO1, Shannon, Simpson, Sørensen)

	Gallery	Male	Female	Larvae
ACE	15.29	9.52	4.93	7.00
Chao 1	14.81	8.85	4.68	7.00
Shannon	1.24	1.63	0.86	1.75
Simpson	2.16	3.90	1.74	5.23
Sorensen[a]	–	0.69	0.36	0.50

[a]*vs* Gallery.

that the initial tunnel built by the male is straight for 4–6 cm, and has only one opening to the outside. Afterwards, a loop is formed, clockwise rotation, and the tunnel continues at 4.8 mm higher in relation to the horizontal input. After that, the tunnel has a circular shape, complex multilevel spiral, with multiple tunnels formed at different levels. The diameter is constant, 3.5 mm width, all along the gallery. Tunnels are interconnected within the same plane or with tunnels in different planes. The gallery extension involves both adult and larval activity (stages 4–5). Each larvae is responsible for building its own pupal chamber (personal observation): pupal chambers are perpendicular to the tunnels, closed by a plug of sawdust on the outer tunnel surface and with a generally semi-alternative arrangement. Notably, it is more frequent to find most of the pupal chambers on the right side, opposite to the entrance.

The resulting image shows regions with high content of water in blue (dark grey for print version), regions with high mass density in green (light grey for print version) and regions with high mass density and water content simultaneously in cyan (white for print version).

Discussion

The present research reports the fungal species associated with *M. mutatus* recovered from galleries, insect exoskeleton and fungal endophytes from the tree host, thus providing a comprehensive study of the mycobiota associated with this pest. All the species here reported constitute the first records for the associated *M. mutatus* mycobiota.

Fusarium solani was the most abundant species in every evaluated source. The association between *Fusarium sensu lato* with bark and ambrosia beetles was widely reported (Beaver *et al.*, 1989; Kasson *et al.*, 2013; O'Donnell *et al.*, 2014). This genus plays different roles in different interactions, i.e. entomopathogenic, nutritional, associated fungus and symbiotic partner (Norris, 1979; Teetor-Barsch and Roberts, 1983; Morales-Ramos *et al.*, 2000; Qi *et al.*, 2011; Scully *et al.*, 2012; Castrillo *et al.*, 2013; Freeman *et al.*, 2013).

Among ambrosia fungi, the genus *Raffaelea* is one of the most representative taxa, being a key species in the interactions (Dreaden *et al.*, 2014). Species of this genus maintain a close symbiotic relationship with different wood-boring beetles (Gebhardt *et al.*, 2005; Endoh *et al.*, 2011; Harrington *et al.*, 2014). In our study, *Raffaelea* species achieved a lower frequency than others genera. Nevertheless, the influence of key species is not always correlated with a high diversity by themselves (Hooper *et al.*,

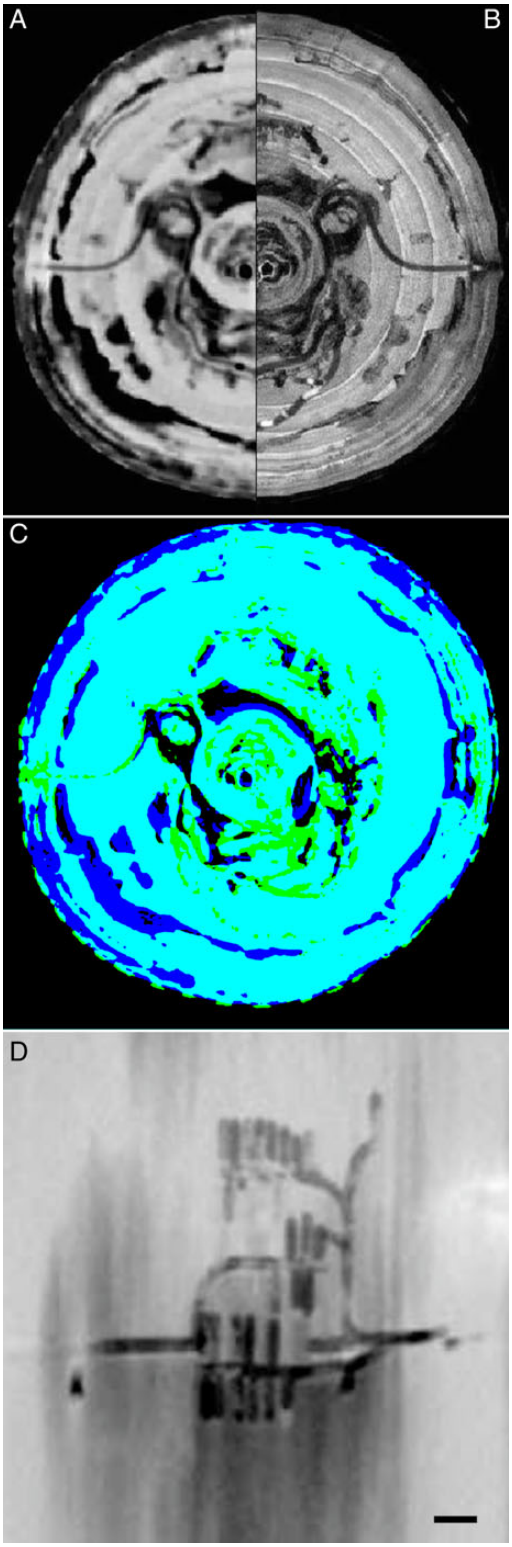

Figure 4 (**A** and **B**) Integrated images generated from slices of CT and NMR, respectively. (**C**) The relationship between density and water content, indicating three different aspects: equal proportion (cyan/white for print version), upper water content (blue/dark grey for print version) or upper density (green/light grey for print version). (**D**) Set of pupal chambers (longitudinal section). Scale bar: 10 mm.

2000). A similar case was found by Henriques *et al*. (2009) in the mycobiota associated with *Platypus cylindrus* galleries. Althought *Raffaelea santoroi* Guerrero was the only associated fungus previously reported for *M. mutatus* (Guerrero, 1966), it was not recovered in the present survey. This could be noted as an evidence that the relationship between *M. mutatus*–*R. santoroi* could be occasional.

Graphium basitruncatum was detected by direct observation in new segments of the gallery (founder male's tunnel and pupal chambers) and on male exoskeleton exclusively. These results suggest that besides *Fusarium* and *Raffaelea*, this species might have a relevant role in the association. Species of *Graphium* were reported as insect vectored (Cruywagen *et al*., 2010), frequently recovered from adult insect and galleries of *Scolytodes unipunctatus* (Hulcr et al., 2007; KOLAŘÍK *et al*., 2015) and from mycangia of *P. cylindrus* (Bellahirech *et al*., 2014). The data support the hypothesis about the relevance of this genus in the interactions between fungi and insect. However, it is not clear if its primary role is related to a nutritional function or it could be relevant in a context of intraspecific communication. This last hypothesis is currently being evaluated by chemical analyses of emitted volatile organic compounds.

Among the communities analysed, only species of *Raffaelea* and *F. solani* were recovered with at least 30 per cent of relative frequency. In male beetles, the main fungal taxa included *Fusarium* spp. and *Raffaelea* spp. (46.32 per cent and 33.68 per cent, respectively). This could indicate that these genera have a key role in the establishment of *M. mutatus*, exploiting different resources in the tree and/or playing distinctive roles in the biology of the beetle (Biedermann *et al*., 2013). The association between these taxa has been previously reported in other interactions (Kasson *et al*., 2013).

Additionally, we found species of *Chaetomium*, *Penicillium* and *Trichoderma* that were previously registered as associated fungi of *P. cylindrus* (Henriques, 2007). Probably, these species represent an opportunistic and saprophytic interaction without a relevant significance in insect–fungi–host interaction.

As expected, the most similar fungal profile was found between galleries and males, in agreement with the hypothesis that males carry the fungal spores that will subsequently constitute the gallery's fungal community. There is no information regarding the structures or mechanisms involved in the transport of the fungi associated with *M. mutatus*. A single, paired or small clusters of specialized mycetangial pores are present on the pronota of other species of *Megaplatypus* (Wood, 1993). However, these pore structures are absent in *M. mutatus* (Alfaro *et al*., 2007). According to Six (2003), the transport of these fungi is enabled by structures as pits, pockets and setae (fungi are carried externally –phoretically – on the insect's exoskeleton).

The absence of fungal endophytes in the analysis carried out indicates that the most important components of *M. mutatus*-associated fungal community would not include wood endophytic fungal communities. This first study was performed using culture-dependent methods and the species accumulation curves indicate that our results reflect the present species richness associated with the insect. Nevertheless, studies using the metagenomic approach are important to detect species that are difficult to obtain because they fail to grow in culture media, or depend on other organisms to develop.

No data about the gallery topology have been reported previously. Data obtained in this work from the gallery topology and the analysis involving density and water content indicate that tunnels never develop through the central cylinder of the trunk and, more importantly, never do so through the active xylem (except for the initial tunnel initiated by the male). This observation suggests that, among other factors, water circulation across the outer sections might be involved in the gallery topology modulation. Santoro (1963) mentioned that gallery tunnels are built following the growing rings of the trees. Here, it is shown that this does not appear to be a relevant factor. Soné *et al*. (1995) studied the galleries built by *Platypus quercivorus* (Murayama) by CT and found that they present principal and secondary tunnels, the loop is not present and constructions are made in a half-circle shape (Sone *et al*., 1998).

Conclusion

Results on the studied communities showed that 19 fungal species are associated to a greater or lesser degree with the forest pest *M. mutatus*. The analyses of CT–NMR combined images enabled us to provide a detailed description of the gallery topology. Data suggest that males of *M. mutatus* are responsible for establishing the fungal inoculum/community and that members of *Fusarium* and *Raffaelea* could play a key role in the interaction. *Graphium basitruncatum* was detected in new segments of the gallery and its exclusive presence on male exoskeleton is being focus of current research. The interaction between *M. mutatus* and *F. solani* species complex is particularly relevant due to its potential role as a phytopathogen. In addition, the ability of *M. mutatus* to establish in new environments and the wide range of susceptible hosts make this interaction a focal point of environmental safety research. The above-mentioned information is useful for future research that aims to clarify the role of the mycobiota associated with *M. mutatus*. Future research will focus on additional geographical regions and hosts to understand whether the fungal species are insect or location-dependent.

Supplementary data

Supplementary data are available at *Forestry* online.

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Conflict of interest statement

None declared.

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