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Fungal communities associated with bark and ambrosia beetles trapped at international harbours

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Abstract

Bark and ambrosia beetles (Coleoptera; Scolytinae) establish trophic relationships with fungi, which could be also agents of plant diseases. *Orthotomicus erosus* (Wollaston) and *Xyleborinus saxesenii* (Ratzeburg) are two species of Palaearctic origin that have been introduced in several countries around the world. Here, we investigated their associated fungal communities using individuals trapped at harbours in their native range, without strictly focusing on nutritional symbionts. Targeting the ITS2 region of the fungal rDNA through pyrosequencing, we retrieved taxa known to be agents of plant diseases, taxa never previously reported associated with these beetle species, and sequence clusters not linked to any known fungus. These findings underline that surveillance at harbours should be extended to the fungi associated with trapped bark and ambrosia beetles, taking into account their role as potential vectors of plant pathogens.

Keywords: *Orthotomicus erosus*; *Xyleborinus saxesenii*; Ophiostomataceae; ITS2; Scolytinae; pyrosequencing; metabarcoding

Introduction

The existence of specific associations between insects and fungi has been widely documented for several different taxa, ranging from mutualistic to antagonistic interactions (Klepzig et al., 2001). In many cases, however, the nature of the interaction is still unknown (Six, 2012; Malacrinò et al., 2015). The type of interaction is especially relevant if it involves plant pathogens, as sometimes occurs in bark and ambrosia beetles (Coleoptera; Curculionidae; Scolytinae) (Kirisits, 2007; Six, 2012; Ploetz et al., 2013; Carrillo et al., 2014). In bark beetles, in particular, this association was considered functional for tree colonization (Paine et al., 1997; Lieutier et al., 2009; Villari et al., 2012), although the ecological role of these fungi has been questioned for many years (Six and Wingfield, 2011). As globalization is leading to a sharp increase in the number of bark and ambrosia beetles moved outside their native range, one could raise the question whether fungi introduced together with the beetles can cause severe damage to invaded ecosystems (Hulcr and Dunn, 2011; Ploetz et al., 2013). Examples come from the elm bark beetles *Scolytus* spp. and fungi belonging to the genus *Ophiostoma*, which have been destructive to elms in both North America and Europe, despite being vectored by native beetles (Brasier and Kirk, 2010).

The association with fungi is usually different between bark and ambrosia beetles. Bark beetles build galleries in the phloem, and feed on fungi to supplement their diet (Ayres et al., 2000; Six, 2003; Bleiker and Six, 2007; Kirisits, 2007). Ambrosia beetles, instead, make their galleries in the xylem and feed on fungi cultivated on the galleries' walls (Kirisits, 2007). These differences are not always clear, as some ambrosia beetles infest the phloem-sapwood interface (Roeper et al., 2015). In both groups, fungi can be transported in specialized structures called mycangia (Kostovcik et al., 2015), in the gut (Biedermann et al., 2013), or phoretically on the beetle cuticle (Six, 2003; Harrington, 2005). Fungal symbionts are usually vertically transmitted from one generation to the next, but horizontal transmission from one species to another has been demonstrated to occur in

both bark and ambrosia beetles (Harrington and Fraedrich, 2010; Six and Wingfield, 2011; Carrillo et al., 2014). Such transmission can occur, for example, when a species interacts with another species' brood gallery and its associated fungi, or via fungus-feeding phoretic mites (Klepzig et al., 2001; Moser et al., 2010; Six, 2012) and can involve plant pathogenic symbionts (Carrillo et al., 2014).

Bark and ambrosia beetles can be easily moved around the world within wood-packaging materials, wood chips, and logs (Haack, 2001; Rassati et al., 2015a). Harbours, receiving large amounts of imported commodities, represent the most likely points of entry for non-native species (Stanaway et al., 2001; Campolo et al., 2014; Palmeri et al., 2015). Since the risk of new introductions has strongly increased in recent decades due to increasing globalized trade (Hulme, 2009; Prigigallo et al., 2015a; Prigigallo et al., 2015b), specific measures have been taken to prevent the arrival and establishment of non-native species, including international standards (ISPM, 2013) and early-detection programs carried out at harbours using baited traps (Brockerhoff et al., 2006; Rassati et al., 2015a; Rassati et al., 2015b). These traps can capture not only a number of individuals of non-native species, but also several individuals of native species. These native species could have originated either from the natural areas surrounding harbours, potentially representing invaders for countries receiving shipped goods, or from the imported wood packaging materials, potentially representing re-introduced individuals belonging to populations established in foreign countries (Rassati et al., 2015a; Rassati et al., 2015b). Thus the analysis of their associated fungi can give information on native fungi that can be potentially spread, or even introduced in non-native areas, as well as on non-native fungi vectored by beetles re-introduced from non-native to native countries (Wingfield et al., 2010; Taerum et al., 2013; Wingfield et al., 2016).

Here we characterized the fungal community associated with a bark and an ambrosia beetle frequently trapped at Italian international harbours (Rassati et al., 2015a; Rassati et al., 2015b),

84 revealing potential plant threatening agents, and providing insights into their ecology and diffusion
85 pathways. We did not focus on the primary symbionts nutritionally associated with bark or ambrosia
86 beetles, since they are widely described in the literature and mostly harmless to plants (Romon et
87 al., 2007; Hulcr et al., 2011). Analyses were conducted with the bark beetle *Orthotomicus erosus*
88 (Wollaston) and the ambrosia beetle *Xyleborinus saxesenii* (Ratzeburg). These species were selected
89 because: (i) they represented the most commonly trapped species at Italian harbours for two
90 consecutive years (2012-2013) (Rassati et al., 2015a; Rassati et al., 2015b); (ii) they are of Palaearctic
91 origin but they have been introduced in several other Countries (Wood and Bright, 1992; Haack,
92 2001; Gómez and Martínez, 2013; Gómez et al., 2013). A culture-independent high-throughput
93 metabarcoding approach, based on fungal ITS2 region amplification and 454 pyrosequencing, was
94 performed to analyse both *O. erosus* and *X. saxesenii* associates. Fungal communities were analysed
95 according to the beetle species, and the harbours in which beetles were trapped.
96

97 ## Materials and methods

98 ### *Beetles trapping*

99 The individuals of *O. erosus* and *X. saxesenii* analysed in the present study were collected in 2013
100 during a nationwide trapping program carried out at 15 Italian harbours and aimed at improving the
101 early-detection of non-native wood-boring beetles (Rassati et al., 2015a). At each site, three 12-unit
102 black multi-funnel traps (Econex, Murcia, Spain) were placed within the harbour area, hanging them
103 about 2 m above the ground. Traps were baited with a multi-lure blend composed of (−)α-pinene,
104 ipsenol, ipsdienol, 2-methyl-3-buten-2-ol, and ethanol (Contech Enterprises Inc., Victoria, BC,
105 Canada). Traps were checked every 2 weeks, trapped beetles were sorted to species, and then
106 preserved at −80°C in Eppendorf 1.5 ml tubes, filled with 95% ethanol. An insecticide (FERAG IDTM

– SEDQ, Spain) was placed within the collector cup of each trap to quickly kill the insects, and no liquid was added. In this way, we tried to keep cross-contamination among samples as low as possible. In this regard, our results did not show evidence of cross-contamination between individuals of the two beetle species: their fungal communities were, in fact, clearly different, and their shared core taxa are known as ubiquitous fungi (see Results). Nonetheless, we are conscious that fungal spores could have moved among individuals of the same or different species when simultaneously present within the collector cup, and we cannot exclude that contamination occurred (Viiri, 1997). We did not surface sterilized the individuals used for the analysis in order to describe the whole fungal community associated to the beetle, including those fungi carried outside mycangia that may be transmitted to the colonized trees. A sufficient number of individuals of both species to allow for analyses were collected only in three out of the fifteen monitored harbours (Fig. S1 – Marghera - 45° 43’ N, 12° 31’ E; Ravenna - 44° 49’ N, 12° 28’ E; Salerno - 40° 67’ N, 14° 64’ E). All three harbours are known to import commodities from foreign countries (Assoporti, 2014).

DNA extraction and library preparation

DNA was extracted from samples made up of 10 randomly selected individuals, with a total of 3 replicates from each harbour for each beetle species for a total of 18 samples (3 harbours by 2 beetle species by 3 replicates). Before DNA extraction, ethanol used to store the samples was evaporated using a vacuum evaporator (Eppendorf® Concentrator Plus, Hamburg, Germany). Each sample (10 individuals) was crushed in an extraction buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with the aid of a bead mill homogenizer, and the mixture was then treated with Proteinase K (5Prime GmbH, Germany) following the producer’s protocol. Total DNA was extracted as described by Schena and Cooke (2006), analysed by electrophoresis in TBE buffer and 1.5% agarose gel stained

with GelRed™ nucleic acid stain (Biotium, USA), and then visualized with UV light using a Gel Doc™ system (Bio Rad, USA). DNA concentration and quality was assessed by means of a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA).

Libraries for 454 GS FLX+ sequencing were built using fusion primers (http://www.454.com/) targeting the fungal ITS2 region of the ribosomal DNA (rDNA). PCR reactions were conducted in a total volume of 25 µl and contained 1 µl of extracted DNA (about 50 µg), 1X Taq buffer, 1.5 mM MgCl2, 40 µM dNTPs, 1 unit of Taq polymerase (AccuPrime™, Thermo Fisher), and 0.5 µM of primers ITS3 and ITS4 (White et al., 1990). Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 94°C for 3 min, 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, repeated 35 times, and ended with 10 min of extension at 72°C. A non-template control, in which target DNA was replaced by nuclease-free water, was included in all PCR reactions. PCR products were analysed by electrophoresis as described above, and purified using Agencourt AMPure XP kit (Beckman Coulter Inc., CA, USA). Samples were amplified in triplicate, to decrease the stochastic variability among reactions (Schmidt et al., 2013). The concentration of PCR products in each sample was measured with Qbit Instrument (Thermo Fisher Scientific, USA), and normalized diluting amplicons in molecular biology grade water. Ten µl of each purified sample were pooled together and sequenced by Macrogen Inc. (Seoul, Korea) on one 1/8th regions of a sequencing plate on a 454 GS FLX+ System (454 Life Sciences, Branford, CT, USA).

Data processing

Raw sequencing data were processed using QIIME 1.8.0 (Caporaso et al., 2010), setting the minimum quality score to 25 and without allowing mismatches in the primer sequence. Sequences <150 bp, >1000bp, containing homopolymers >10 bp and with >6 ambiguous bases were discarded. Reads were denoised using denoise wrapper (Reeder and Knight, 2010), and chimeric sequences

were removed using USEARCH 6.1 algorithm (Edgar et al., 2011) combining a reference-based with a *de novo* detection method. Fungal ITS2 sequences were extracted using ITSx (Bengtsson-Palme et al., 2013), as it is known that conserved flanking regions can lead to errors in clustering, taxonomic and similarity results (Bruns and Shefferson, 2004; Kostovcik et al., 2015). Reads were then clustered into Operational Taxonomic Units (OTUs) using BLAST method, with 0.99 similarity threshold to the UNITE dynamic reference database (Kõljalg et al., 2013) accessed on March 2015 (http://unite.ut.ee/). When reads failed to hit to the reference database, sequences were clustered as *de novo*, and singletons were discarded from analyses. The UNITE database was employed, using BLAST algorithm, for the taxonomic identifications of representative sequences of each detected OTU.

The most commonly associated OTUs in each analysed sample were identified using the method of core/satellite taxa as described by Magurran and Henderson (2003). This approach involves an iterative process to decompose the Species Abundance Distribution (SAD) into two parts: core OTUs and satellite OTUs. The threshold between the two categories was set at the number of samples at which the SAD fitted a log-normal distribution. The core dataset was employed for taxonomic and ecological inferences, while the whole community composition was used in alpha and beta diversity analyses.

The alpha diversity was estimated through the Species Accumulation Curves (SAC) and a set of diversity indices (Dominance, Shannon and Chao1), calculated for both insect species. Comparison between species and among the sampling sites was performed through a PERMANOVA non-parametric approach (999 permutations), and visualized through a Principal Coordinates Analysis (PCoA) with 95% confidence ellipses, calculated on a Bray-Curtis matrix supported by jackknifed UPGMA tree inference. All analyses were performed with QIIME (Caporaso et al., 2010) and Microsoft® Excel® 2013.

178

179 ***Taxonomic identification of core taxa***

180 Since the UNITE databases enabled a reliable identification of fungi only at the genus level (Knief,
181 2014), the identification of representative sequences of all detected OTUs were further confirmed
182 by means of a MegaBLAST search. To keep the taxonomic assignment as reliable as possible, we
183 used as thresholds an E-value <0.001, a coverage >80%, and an identity >95%. Sequences meeting
184 these criteria were classified to genus level. Furthermore, OTUs classified as "core" OTUs and
185 putative plant pathogens were analysed along with validated reference sequences of closely related
186 species, to determine their phylogenetic placement at the highest possible level of accuracy
187 (Abdelfattah et al., 2015; Malacrinò et al., 2016). OTUs associated to unknown or uncultured fungi,
188 or with unreliable taxonomic information, were not phylogenetically analysed and classified as
189 "unknown fungi". Specifically, using the phylogenetic approach we analysed all sequences belonging
190 to the genera *Alternaria*, *Aureobasidium*, *Cladosporium*, *Geosmithia*, *Ophiostoma*, and *Ramularia*.
191 For each fungal genus, sequences were aligned using MUSCLE (Edgar, 2004) and phylogenetically
192 analysed with RAxML 8.0.0 using a GTR + Γ model (Stamatakis, 2014).

193

194 ## Results

195 In total, 59,247 reads were retrieved after quality filtering, denoising and chimera discarding.
196 Sequences were demultiplexed, obtaining an average read count of 3,291 and a mean length of
197 250bp. Using a 0.99 cut-off, and deleting singletons from the analyses, a total of 294 OTUs were
198 retrieved. The flattening of Species Accumulation Curve (SAC), as the number of analysed sequences
199 increased, indicated a sufficient sequencing depth to reconstruct the fungal community of both
200 beetle species (Fig. S2). The fungal community of *X. saxesenii* showed a higher average number of

OTUs identified than that of *O. erosus*, however, the two beetle species had similar values of Shannon diversity indices (Table 1 - $F_{1, 16} = 0.21$; $P = 0.64$).

Fungal communities associated to *O. erosus* and *X. saxesenii*

One hundred sixty-nine fungal OTUs were identified in *O. erosus*. The analysis of SAD divided the dataset into core and satellite taxa (log-normal, goodness of fit $\chi^2=6.308$; $P = 0.70$), classifying as "core" those OTUs with a persistence of ≥6 samples (Fig. 1 A-B). This approach suggested 20 OTUs as core taxa associated to this bark beetle, accounting for 79.7±5.41% of sequences. Inside the core taxa, 50.5±9.48% of the sequences were identified at least at genus level, 49.1±11.11% were associated to different yeast genera and 0.32±0.23% (1 OTU) were classified as unknown fungi. Filamentous fungi identified to genus or species level included: *Aspergillus* spp., *Devriesia* sp., *Geosmithia* sp. (Fig. 2A), *Stemphylium* sp., *Fusarium* sp. (*incarnatum-equiseti* species complex), *Ophiostoma* spp. (Fig. 2B), *Alternaria* sp. (Fig. 2 C), *Botrytis* sp. and *Boeremia* sp. (Table 2). Among yeasts, we retrieved sequences that matched with the following genera: *Candida* sp., *Ogataea* sp., *Sporobolomyces* sp., *Pichia* sp., *Myxozyma* sp., *Rhodosporium* sp., *Rhodotorula* sp. The phylogenetic analysis enabled the identification of OTUs *OPH1*, *OPH2*, *OPH3* and *OPH4* as *Ceratocystiopsis* sp., *Ophiostoma* sp., *O. saponiodorum* and *Graphilbum* sp., respectively (Fig. 2B). Furthermore, the OTU *ALT1* was associated to *Alternaria* sect. Alternata (Fig. 2C).

For *X. saxesenii*, the core/satellite taxa approach divided the SAD into two parts, identifying as core OTUs those with a persistence ≥4 samples (Fig. 1 C-D). Of the total 96 OTUs, 15 were classified as the core part of SAD (goodness of fit $\chi^2=12.71$; $P = 0.47$), including 59.62±9.48% of sequences associated to filamentous fungi, 6.51±3.35% to different yeast species and 33.87±8.19% to unknown fungi (5 OTUs). The phylogenetic approach allowed us to identify the *CLA1* core OTU cluster as part of *Cladosporium herbarum* s.c. (Fig. 2D), while the cluster *AUR1* was identified as

Aureobasidium pullulans (Fig. 2F). Identified fungi belonged to *Alternaria* sp., *Aspergillus* spp., *Aureobasidium pullulans*, *Botrytis* sp., *Cladosporium herbarum* s.c. and *Ramularia hydrangea-macrophylla* (Fig. 2E), while among core OTUs we found the yeasts *Candida* sp. and *Cryptococcus* sp (Table 2).

Analysis of the occurrence of fungal species

A clear clustering of fungal communities appeared when samples were labelled according to the beetle species (Fig. 3A) but not when they were labelled according to harbours. These results were supported by a PERMANOVA analysis that highlighted differences between beetle species (PseudoF = 8.29; $P = 0.01$) but not among harbours (PseudoF = 1.48; $P = 0.15$).

Comparing the whole fungal community of the two beetle species, 52 OTUs were shared between *O. erosus* and *X. saxesenii* (Fig. 3B) and, among them, the shared core OTUs were identified as *Aspergillus* spp., *Aureobasidium pullulans*, *Botrytis* spp., *Devriesia* sp., *Cladosporium* sp., *Stemphylium* sp., *Alternaria* sp., and *Fusarium* sp. Comparing the three harbours, 27 OTUs were shared among them whereas 7 to 34 OTUs were shared in pairwise comparison of harbours (Fig. 3B).

Focusing on core OTUs, one associated to the genus *Aspergillus* was shared between Marghera and Salerno, whereas 4 shared core OTUs associated to the genera *Fusarium* (2 OTUs), *Stemphylium* and *Botrytis* were shared between Ravenna and Salerno. We did not find any shared core OTU between Ravenna and Marghera. Interestingly, we found OTUs associated to *Geosmithia*, *Graphium* and *Graphilbum* shared between the samples collected in Salerno and Ravenna. On the other hand, 3 OTUs associated to the genera *Acremonium* and *Ophiostoma* were found only in Ravenna, and 2 OTUs (one *Geosmithia* and one *Ophiostoma*) were found only in Salerno (Fig. 3B).

Discussion

We investigated the fungal communities associated with the bark beetle *O. erosus* and the ambrosia beetle *X. saxesenii* through a HTS (High Throughput Sequencing) metabarcoding approach. This approach has been previously used to analyse symbionts of both bark (Miller et al., 2016) and ambrosia beetles (Hulcr et al., 2012; Kostovcik et al., 2015; Li et al., 2015) but, instead of focusing on nutritional symbionts, we investigated the potential role of the two beetle species as carriers of fungal plant pathogens. Our results provide novel information on the fungal communities of two widely distributed species of Scolytinae, suggesting new associations with fungi potentially representing agents of plant diseases, including species of the genera *Ophiostoma*, *Ceratocystiopsis*, *Graphilbum*, *Acremonium*, *Ramularia* and *Fusarium*. For *O. erosus* we retrieved sequences that can be associated to generalist fungi, which might allow this bark beetle species to exploit new food sources. For *X. saxesenii*, on the other hand, we retrieved a high number of sequences for which the identification was not possible because of uninformative sequences or their absence in public databases. These sequences can be associated to unknown fungi or fungi that have not been cultured, and since *X. saxesenii* has a fungus farming behaviour, these unidentified OTUs could be undisclosed symbionts, pathogens or both. Future endeavours should focus on their *in vitro* isolation, obtaining pure cultures and allowing their identification to species level.

Overall, our data are consistent with those available in literature and here we report novel information worthy of being further explored. Among Ophiostomatales, we obtained a total of 4 OTUs, identified to belong to the *Ceratocystiopsis*, *Ophiostoma* and *Graphilbum* genera. The taxonomy of OTU *OPH1* was not clearly identified to species level through the phylogenetic approach, although both this and BLAST search suggested that this cluster could be associated to the genus *Ceratocystiopsis*. Within the genus *Ophiostoma* we retrieved 2 OTUs, of which one (*OPH3*) was clearly identified as *O. saponiodorum* through the phylogenetic approach. More doubtful was,

instead, the phylogenetic identification of the OTU *OPH2*, which clustered together with both *O. pulvinisporum* and *O. ips*. However, the BLAST analysis matched much more sequences of *O. ips*. Instead, the OTU *OPH4*, was identified to belong to the genus *Graphilbum*. Among these taxa, *O. ips* has been previously reported as one of the main associate for *O. erosus* (Romon et al., 2007; Zhou et al., 2007), while *O. saponiodorum* has never been found to be associated with *O. erosus*. Regarding the genus *Graphilbum*, we were not able to discriminate the species, however *G. rectangulosporium* has been already retrieved for this beetle species in the past (Romon et al., 2007). Some ophiostomatoid fungi are known to be either important pathogens of conifers or agents of bluestain on logs and freshly-cut wood (Zhou et al., 2006), discoloration that can lead to the reduction of wood price up to 50% (Romón et al., 2014a). Microascales are also considered symbionts of bark and ambrosia beetles; *Graphium* species are mostly reported from bark beetles, but have been rarely found in ambrosia beetles (Linnakoski *et al.* 2012). In our study, we retrieved one satellite OTU assigned to *Graphium*, which was associated with *O. erosus,* confirming the existence of *O. erosus* and *Graphium* spp. association (Jacobs et al., 2003).

For Hypocreales, we obtained a total of 12 OTUs belonging to the genera *Geosmithia*, *Acremonium*, and *Fusarium*. We retrieved 3 OTUs that were associated to *Geosmithia* spp., of which one (*GEO1*) was included among the core OTUs of *O. erosus*, and the other two were classified as satellite sequences of *X. saxesenii*. The phylogenetic analysis did not allow us to push the identification to species level, but it is known that *Geosmithia* spp. are regularly associated to many Scolytinae (about 30 species worldwide), including *O. erosus* (Kolařík et al., 2007; Kolařík et al., 2008; Dori-Bachash et al., 2015), but their relationship is still poorly understood (Kolařík et al., 2008). These fungi lack entomochory-related adaptations, such as sticky conidia or ascospores and their phytopathogenic activity is still unclear and confirmed only in one case (*Geosmithia morbida* on

296 walnuts). It is possible, instead, that they play an important role as nutrient suppliers (Kolařík et al.,
297 2011; Dori-Bachash et al., 2015).

298 *Acremonium* spp. have been reported as fungal associates of several bark and ambrosia
299 beetles (Belhoucine et al., 2011; Giordano et al., 2013; Repe et al., 2013) but, to our knowledge,
300 never with *O. erosus* or *X. saxesenii*. We identified two satellite OTUs belonging to the genus
301 *Acremonium*, one associated only to *O. erosus*, and the other one shared between the two beetle
302 species. As reported by Belhoucine et al. (2011), these fungi may be nutritionally beneficial as they
303 could be isolated from the gut of adult *Platypus cylindrus* F.

304 We reported 7 *Fusarium* OTUs of which 2 (*FUS1* and *FUS2*) were identified as core OTUs of
305 *O. erosus*, belonging to the *F. incarnatum-equiseti* species complex (s.c.). The OTU *FUS2* was also
306 detected in *X. saxesenii* as satellite OTU. The satellite taxa clustered into *F. incarnatum-equiseti* s.c.,
307 *F. solani* s.c., *F. lateritium* (shared between beetle species), *F. oxysporum* s.c. and *F. brachygibbosum*
308 (associated only to *O. erosus*). Fungi belonging to the genus *Fusarium* have been reported to be
309 associated both with bark beetles, including *O. erosus* (Romón et al., 2008), and ambrosia beetles
310 (Kasson et al., 2013), but never with *X. saxesenii*. It is worth noting that ambrosial *Fusarium* species
311 belong to a specific monophyletic group within the *Fusarium solani* s.c., and could potentially allow
312 carrying beetles to exploit new food sources, even in non-native ecosystems (Kasson et al., 2013).
313 One OTU associated to *X. saxesenii* was identified as *Ramularia hydrangea-macrophylla*. This fungal
314 genus is known to cause plant diseases, and in particular leaf spots with different degree of severity
315 (Videira et al., 2016). This is the first time that *Ramularia* is reported as associated with *X. saxesenii*.

316 A total of 16 OTUs were associated with the genus *Aspergillus*, among which one (*ASP1*) was
317 part of the core OTUs of *O. erosus*, and *ASP2*, *ASP3,* and *ASP4* were associated with *X. saxesenii*.
318 Considering that these fungi are ubiquitous and widely distributed generalist pathogens of plants
319 and foodstuffs, as well as saprotrophs, they cannot be considered beneficial for the beetle. In fact,

320 although other studies reported *Aspergillus* spp. on bark and ambrosia beetles, a strict association
321 has never been demonstrated (Iqbal and Saeed, 2012; Repe et al., 2013). The analyses highlighted
322 one OTU belonging to the *Alternaria* sect. Alternata (*ALT1*) shared between *O. erosus* and *X.*
323 *saxesenii*. As for *Aspergillus*, however, *Alternaria* fungi are widely distributed and reported both in
324 bark and ambrosia beetles (Belhoucine et al., 2011; Repe et al., 2013) and it is unlikely that they can
325 play any beneficial role for the beetle. Unfortunately, for both *Aspergillus* and *Fusarium*, the ITS
326 region of rDNA is not variable enough to distinguish among the different species, therefore the
327 identification is possible and reliable only at section/complex level (Balajee et al., 2009).
328 We identified other core OTUs in our study belonging to the genera *Devriesia*, *Stemphylium*,
329 *Boeremia*, and *Botrytis*. These fungal genera comprise widely distributed species with a known
330 saprophytic habit. Moreover, we retrieved a high number of ITS sequences of yeasts taxa: 49.17%
331 for *O. erosus* and 6.51% for *X. saxesenii*. Yeasts are known to be commonly associated with bark and
332 ambrosia beetles, contributing to their development, reproduction, nutrition, and defence, and
333 participating in other ecological relationships with plants and other microorganisms (Six, 2003).
334 About the 33% of the core sequences of *X. saxesenii* was classified as “unknown fungus”. This can
335 be due to a series of factors, including the high presence of sequences with unsettled nomenclature
336 in public databases. Furthermore, many fungal species associated to bark and ambrosia beetles are
337 still unknown or their ITS barcode not publicly available.
338 To amplify the ITS2 region of the rDNA, a widely accepted gene as official barcode for fungi
339 (Bellemain et al., 2010; Schoch et al., 2012), we used the ITS3 and ITS4 primers. This approach has,
340 however, both advantages and disadvantages when it comes to HTS metabarcoding. On one hand,
341 it allows both a reduction in sequencing bias due to the avoidance of the highly conserved 5.8S
342 region that could lead to the formation of chimeric ITS1-ITS2 amplicons from different species
343 (Ihrmark et al., 2012). On the other hand, this approach leads to problems when applied to the study

344 of fungal communities, especially when dealing with fungi belonging to the order Ophiostomatales
345 such as *Ambrosiella* spp. and *Raffaelea* spp. First of all, amplification of the ITS2 region of those fungi
346 is very difficult due to the formation of a secondary structure in the GC-rich region where 5' primers
347 anneal (Fraedrich et al., 2008). In addition, their representation in public databases is very low
348 (Fraedrich et al., 2008; Kostovcik et al., 2015), their nomenclature is not well defined, and the ITS2
349 region is not sufficiently variable to discriminate among closely related species (O'Donnell, 2000;
350 Kostovcik et al., 2015). Given our goal of finding plant pathogenic fungi potentially carried by *O.*
351 *erosus* and *X. saxesenii* and not well-known symbionts, we decided to amplify the ITS2 region with
352 ITS3 and ITS4 primers. We tried to keep the cross-contamination among trapped individuals as low
353 as possible avoiding the use of preservative liquid within the collector cup. Indeed, the fungal
354 communities of the two beetle species were clearly different, and their shared core taxa are known
355 as ubiquitous fungi, so it is not surprising that they were present on both species. Nonetheless, the
356 sampling procedure did not allow us to completely exclude the occurrence of cross-contamination
357 among trapped individuals (Viiri, 1997). To deal with this issue, we used the core-satellite approach
358 to focus the analysis on those fungal species that are more constantly associated with the beetles,
359 and therefore unlikely representing an occasional environmental contamination. Furthermore, we
360 were aware that pooling together more beetles lowered the resolution of the analysis but, on the
361 other hand, this approach allowed to focus on those taxa that are more closely associated with both
362 beetles, "diluting" the occasional external contaminants.

363 Overall, these findings underline the importance of considering bark and ambrosia beetles
364 as potential carriers of unknown fungi that can even be plant pathogens. For this reason, beetles
365 trapped or intercepted at harbours should be not only identified, but also analysed in order to
366 characterize their fungal associates. Further studies should focus on the genetic comparison of
367 individuals from populations sampled in both their native and non-native ranges, allowing to better

368 elucidate the mechanisms involved in the spread of novel beetle/fungus associations in both native
369 and invaded environments.
370

Data accessibility

Raw data from 454 sequencing is available on NCBI SRA database under the Project Accession ID: PRJNA298522. Sequences used for phylogenetic analysis are available as Supplementary Material.

Author contribution

All authors designed the research. AB, LS and VP supervised and coordinated the work. DR, MR and AB provided samples. AM performed lab work, data analysis and data interpretation. AM and DR wrote the first draft of the manuscript. All authors contributed to, corrected and approved the final manuscript.

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Figures

Fig. 1 Separation of core and satellite OTUs of the fungal community for both *O. erosus* (A, B) and *X. saxesenii* (C, D). Species abundance distribution (A and C) based on OTU abundance (number of retrieved sequences) and persistence (number of samples containing each OTU), with dashed lines on the threshold between core and satellite OTUs set through a process iterated until the abundance class distribution (B and D) fit a log-normal distribution.

Figure 2. Phylogenetic identification of detected sequence types (STs). Trees were built using unique sequences representative of STs of the most relevant fungal genera detected in the present study and ITS2 sequences retrieved from *O. erosus* and *X. saxesenii* (●) along with validated reference sequences from *Geosmithia* spp. (A) (Kolařík et al., 2008), *Ophiostoma* spp. (B) (Harrington et al., 2001; Zhou et al., 2006; Lee et al., 2008; Lu et al., 2009; Jankowiak and Bilański, 2013; Romón et al., 2014a; Romón et al., 2014b), *Alternaria* spp. (C) (Woudenberg et al., 2013), *Cladosporium* spp. (D) (Bensch et al., 2012), *Ramularia* spp. (E) (Videira et al., 2016), and *Aureobasidium* spp. (F) (Zalar et al., 2008). Numbers on nodes represent the posterior probabilities for the Randomized Axelerated Maximum Likelihood (RAxML) method. (*) The OTU named ALT1 is common to both species.

Figure 3. (A) Principal Coordinates Analysis (PCoA) of fungal community diversity for *O. erosus* (black— $n = 9$) and *X. saxesenii* (grey — $n = 9$). Each point represents a samples (same species, same location), and those marked with (*) have a similar composition of fungal community, resulting in overlapping points. (B) Venn diagrams showing the shared OTUs between the two beetle species sampled at three harbour sites in Italy.

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646

Table 1
Diversity indices of the fungal community associated with *O. erosus* and *X. saxesenii*.

Species	Observed OTUs	Dominance (D)	Shannon (H)	Chao1
O. erosus	18.56 ± 3.19	0.23 ± 0.07	2.05 ± 0.26	18.79 ± 3.24
X. saxesenii	45.56 ± 7.85	0.37 ± 0.12	1.84 ± 0.38	51.10 ± 9.09

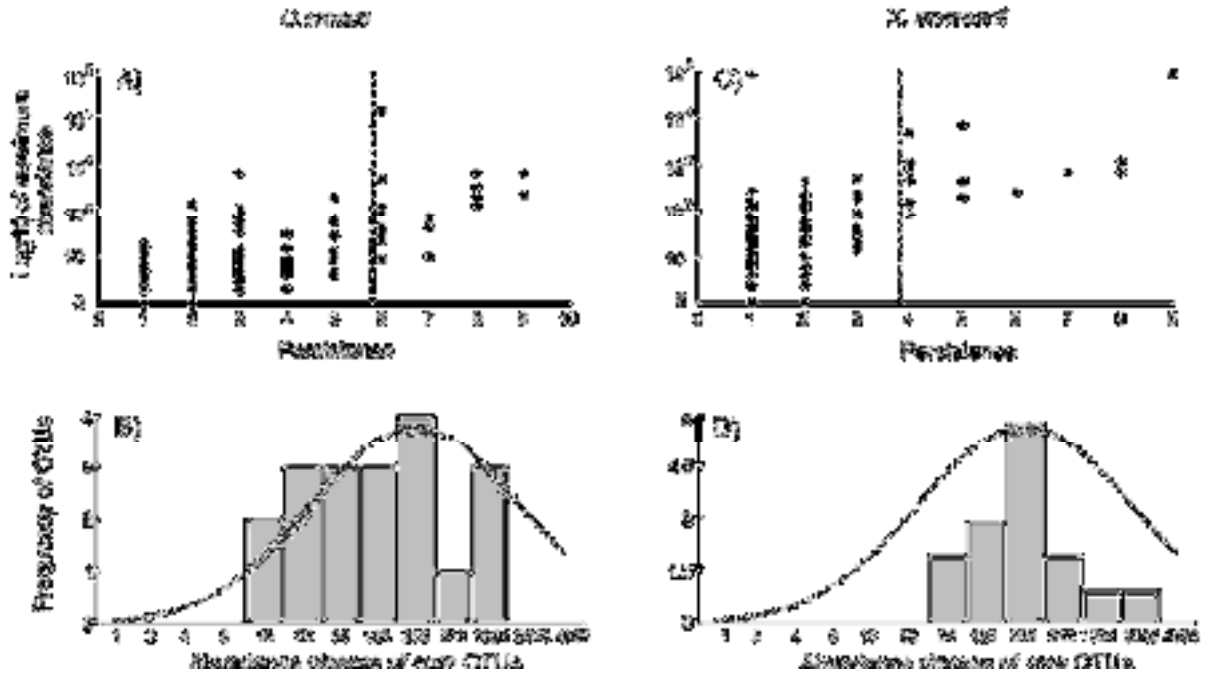


Fig. 1. Separation of core and satellite OTUs of the fungal community for both O. erosus (A, B) and *X. saxesenii* (C, D). Species abundance distribution (A and C) based on OTU abundance (number of retrieved sequences) and persistence (number of samples containing each OTU) are reported, with dashed lines on the threshold between core and satellite OTUs set through a process iterated until the abundance class distribution (B and D) !t a log-normal distribution.

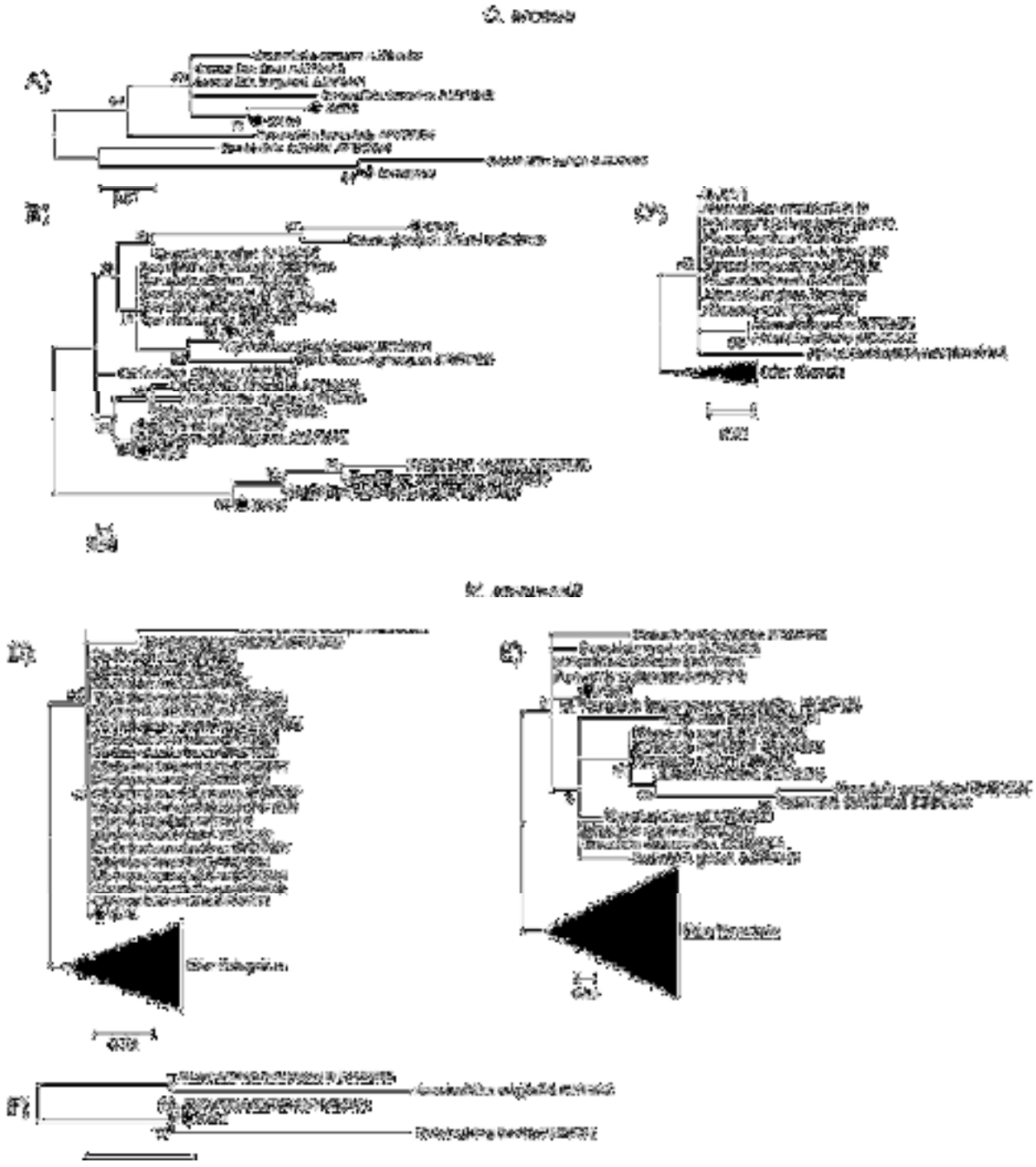
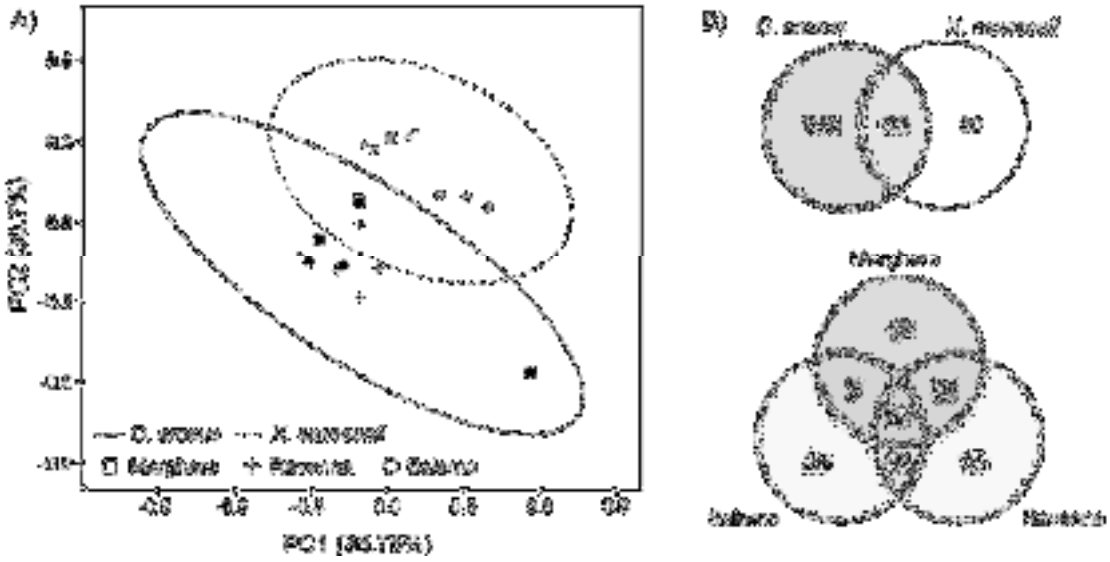


Fig. 2. Phylogenetic identi!cation of detected sequence types (STs). Trees were built using STs of the most relevant fungal genera detected in the present study and ITS2 sequences retrieved from *O. erosus* and *X. saxesenii* (C) along with validated reference sequences from *Geosmithia spp.* (A) (Kola$rík et al., 2008), *Ophiostoma spp.* (B) (Harrington et al., 2001;

aZhou et al., 2006; Lee et al., 2008; Lu et al., 2009; Jankowiak and Bila#nski, 2013; Rom#on et al., 2014a; Rom#on et al., 2014b), Alternaria spp. (C) (Woudenberg et al., 2013), Cladosporium spp. (D) (Bensch et al., 2012), Ramularia spp. (E) (Videira et al., 2016), and Aureobasidium spp. (F) (Zalar et al., 2008). Numbers on nodes represent the posterior probabilities for the Randomized Axelerated Maximum Likelihood (RAxML) method. (*) The OTU named ALT1 is common to both species.

Table 2
Filamentous fungi classified as core OTUs with the SAD analysis (Fig. 1), that were classified at least to genus level.

Beetle species	OTU	Fungal taxa	Percentage (x ± SE)
O. erosis	ASP1	*Aspergillus* sp.	31.13 ± 15.32
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
P. saccardi	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]



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668 Fig. 3. (A) Principal coordinates analysis (PCoA) of fungal community diversity for *O. erosus* (blackd
669 n9) and *X. saxesenii* (greyn9). Each point represents a sample (same species, same location), and
670 those marked with (*) have a similar composition of fungal community, resulting in overlapping
671 points. (B) Venn diagrams showing the shared OTUs between the two beetle species sampled at
672 three harbour sites in Italy.