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

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19

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21 **Abstract**

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

32

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

35

36 **Introduction**

37 The existence of specific associations between insects and fungi has been widely documented for
38 several different taxa, ranging from mutualistic to antagonistic interactions (Klepzig et al., 2001). In
39 many cases, however, the nature of the interaction is still unknown (Six, 2012; Malacrinò et al.,
40 2015). The type of interaction is especially relevant if it involves plant pathogens, as sometimes
41 occurs in bark and ambrosia beetles (Coleoptera; Curculionidae; Scolytinae) (Kirisits, 2007; Six, 2012;
42 Ploetz et al., 2013; Carrillo et al., 2014). In bark beetles, in particular, this association was considered
43 functional for tree colonization (Paine et al., 1997; Lieutier et al., 2009; Villari et al., 2012), although
44 the ecological role of these fungi has been questioned for many years (Six and Wingfield, 2011). As
45 globalization is leading to a sharp increase in the number of bark and ambrosia beetles moved
46 outside their native range, one could raise the question whether fungi introduced together with the
47 beetles can cause severe damage to invaded ecosystems (Hulcr and Dunn, 2011; Ploetz et al., 2013).
48 Examples come from the elm bark beetles *Scolytus* spp. and fungi belonging to the genus
49 *Ophiostoma*, which have been destructive to elms in both North America and Europe, despite being
50 vectored by native beetles (Brasier and Kirk, 2010).

51 The association with fungi is usually different between bark and ambrosia beetles. Bark
52 beetles build galleries in the phloem, and feed on fungi to supplement their diet (Ayres et al., 2000;
53 Six, 2003; Bleiker and Six, 2007; Kirisits, 2007). Ambrosia beetles, instead, make their galleries in the
54 xylem and feed on fungi cultivated on the galleries' walls (Kirisits, 2007). These differences are not
55 always clear, as some ambrosia beetles infest the phloem-sapwood interface (Roeper et al., 2015).
56 In both groups, fungi can be transported in specialized structures called mycangia (Kostovcik et al.,
57 2015), in the gut (Biedermann et al., 2013), or phoretically on the beetle cuticle (Six, 2003;
58 Harrington, 2005). Fungal symbionts are usually vertically transmitted from one generation to the
59 next, but horizontal transmission from one species to another has been demonstrated to occur in

60 both bark and ambrosia beetles (Harrington and Fraedrich, 2010; Six and Wingfield, 2011; Carrillo
61 et al., 2014). Such transmission can occur, for example, when a species interacts with another
62 species' brood gallery and its associated fungi, or via fungus-feeding phoretic mites (Klepzig et al.,
63 2001; Moser et al., 2010; Six, 2012) and can involve plant pathogenic symbionts (Carrillo et al.,
64 2014).

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

82 Here we characterized the fungal community associated with a bark and an ambrosia beetle
83 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

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

120

121 ***DNA extraction and library preparation***

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

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

133 Libraries for 454 GS FLX+ sequencing were built using fusion primers (<http://www.454.com/>)
134 targeting the fungal ITS2 region of the ribosomal DNA (rDNA). PCR reactions were conducted in a
135 total volume of 25 µl and contained 1 µl of extracted DNA (about 50 µg), 1X Taq buffer, 1.5 mM
136 MgCl₂, 40 µM dNTPs, 1 unit of Taq polymerase (AccuPrime™, Thermo Fisher), and 0.5 µM of primers
137 ITS3 and ITS4 (White et al., 1990). Amplifications were performed in a Mastercycler Ep Gradient S
138 (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
139 35 times, and ended with 10 min of extension at 72°C. A non-template control, in which target DNA
140 was replaced by nuclease-free water, was included in all PCR reactions. PCR products were analysed
141 by electrophoresis as described above, and purified using Agencourt AMPure XP kit (Beckman
142 Coulter Inc., CA, USA). Samples were amplified in triplicate, to decrease the stochastic variability
143 among reactions (Schmidt et al., 2013). The concentration of PCR products in each sample was
144 measured with Qbit Instrument (Thermo Fisher Scientific, USA), and normalized diluting amplicons
145 in molecular biology grade water. Ten µl of each purified sample were pooled together and
146 sequenced by Macrogen Inc. (Seoul, Korea) on one 1/8th regions of a sequencing plate on a 454 GS
147 FLX+ System (454 Life Sciences, Branford, CT, USA).

148

149 ***Data processing***

150 Raw sequencing data were processed using QIIME 1.8.0 (Caporaso et al., 2010), setting the
151 minimum quality score to 25 and without allowing mismatches in the primer sequence. Sequences
152 <150 bp, >1000bp, containing homopolymers >10 bp and with >6 ambiguous bases were discarded.
153 Reads were denoised using denoise wrapper (Reeder and Knight, 2010), and chimeric sequences

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

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

171 The alpha diversity was estimated through the Species Accumulation Curves (SAC) and a set
172 of diversity indices (Dominance, Shannon and Chao1), calculated for both insect species.
173 Comparison between species and among the sampling sites was performed through a PERMANOVA
174 non-parametric approach (999 permutations), and visualized through a Principal Coordinates
175 Analysis (PCoA) with 95% confidence ellipses, calculated on a Bray-Curtis matrix supported by
176 jackknifed UPGMA tree inference. All analyses were performed with QIIME (Caporaso et al., 2010)
177 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

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

203

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

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

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

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

229

230 **Analysis of the occurrence of fungal species**

231 A clear clustering of fungal communities appeared when samples were labelled according to the
232 beetle species (Fig. 3A) but not when they were labelled according to harbours. These results were
233 supported by a PERMANOVA analysis that highlighted differences between beetle species (PseudoF
234 = 8.29; $P = 0.01$) but not among harbours (PseudoF = 1.48; $P = 0.15$).

235 Comparing the whole fungal community of the two beetle species, 52 OTUs were shared
236 between *O. erosus* and *X. saxesenii* (Fig. 3B) and, among them, the shared core OTUs were identified
237 as *Aspergillus* spp., *Aureobasidium pullulans*, *Botrytis* spp., *Devriesia* sp., *Cladosporium* sp.,
238 *Stemphylium* sp., *Alternaria* sp., and *Fusarium* sp. Comparing the three harbours, 27 OTUs were
239 shared among them whereas 7 to 34 OTUs were shared in pairwise comparison of harbours (Fig.
240 3B).

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

248

249 **Discussion**

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

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

273 instead, the phylogenetic identification of the OTU *OPH2*, which clustered together with both *O.*
274 *pulvinisporum* and *O. ips*. However, the BLAST analysis matched much more sequences of *O. ips*.
275 Instead, the OTU *OPH4*, was identified to belong to the genus *Graphilbum*. Among these taxa, *O. ips*
276 has been previously reported as one of the main associate for *O. erosus* (Romon et al., 2007; Zhou
277 et al., 2007), while *O. saponiodorum* has never been found to be associated with *O. erosus*.
278 Regarding the genus *Graphilbum*, we were not able to discriminate the species, however *G.*
279 *rectangulosporium* has been already retrieved for this beetle species in the past (Romon et al.,
280 2007). Some ophiostomatoid fungi are known to be either important pathogens of conifers or
281 agents of bluestain on logs and freshly-cut wood (Zhou et al., 2006), discoloration that can lead to
282 the reduction of wood price up to 50% (Romón et al., 2014a). Microascales are also considered
283 symbionts of bark and ambrosia beetles; *Graphium* species are mostly reported from bark beetles,
284 but have been rarely found in ambrosia beetles (Linnakoski *et al.* 2012). In our study, we retrieved
285 one satellite OTU assigned to *Graphium*, which was associated with *O. erosus*, confirming the
286 existence of *O. erosus* and *Graphium* spp. association (Jacobs et al., 2003).

287 For Hypocreales, we obtained a total of 12 OTUs belonging to the genera *Geosmithia*,
288 *Acremonium*, and *Fusarium*. We retrieved 3 OTUs that were associated to *Geosmithia* spp., of which
289 one (*GEO1*) was included among the core OTUs of *O. erosus*, and the other two were classified as
290 satellite sequences of *X. saxesenii*. The phylogenetic analysis did not allow us to push the
291 identification to species level, but it is known that *Geosmithia* spp. are regularly associated to many
292 Scolytinae (about 30 species worldwide), including *O. erosus* (Kolařík et al., 2007; Kolařík et al., 2008;
293 Dori-Bachash et al., 2015), but their relationship is still poorly understood (Kolařík et al., 2008).
294 These fungi lack entomochory-related adaptations, such as sticky conidia or ascospores and their
295 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

371 **Data accessibility**

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

374 **Author contribution**

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

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390

391 **Figures**

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

397 **Figure 2.** Phylogenetic identification of detected sequence types (STs). Trees were built using unique
398 sequences representative of STs of the most relevant fungal genera detected in the present study
399 and ITS2 sequences retrieved from *O. erosus* and *X. saxesenii* (●) along with validated reference
400 sequences from *Geosmithia* spp. (A) (Kolařík et al., 2008), *Ophiostoma* spp. (B) (Harrington et al.,
401 2001; Zhou et al., 2006; Lee et al., 2008; Lu et al., 2009; Jankowiak and Bilański, 2013; Romón et al.,
402 2014a; Romón et al., 2014b), *Alternaria* spp. (C) (Woudenberg et al., 2013), *Cladosporium* spp. (D)
403 (Bensch et al., 2012), *Ramularia* spp. (E) (Videira et al., 2016), and *Aureobasidium* spp. (F) (Zalar et
404 al., 2008). Numbers on nodes represent the posterior probabilities for the Randomized Accelerated
405 Maximum Likelihood (RAxML) method. (*) The OTU named ALT1 is common to both species.

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

411

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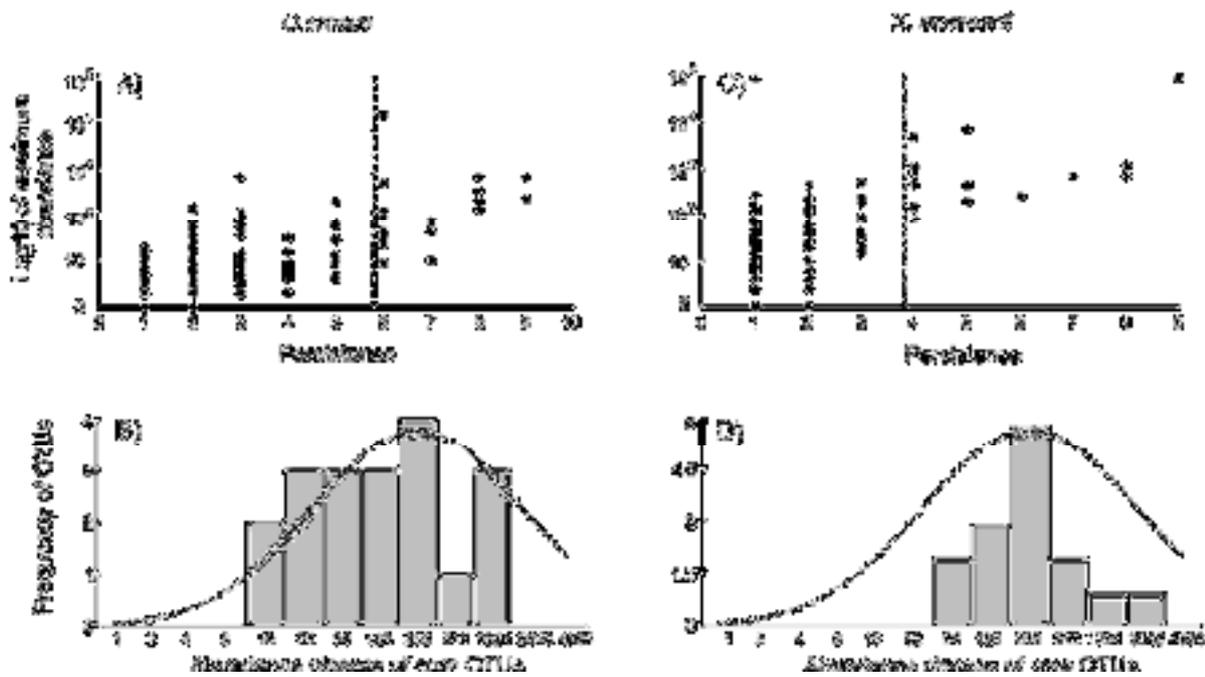
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646

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

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

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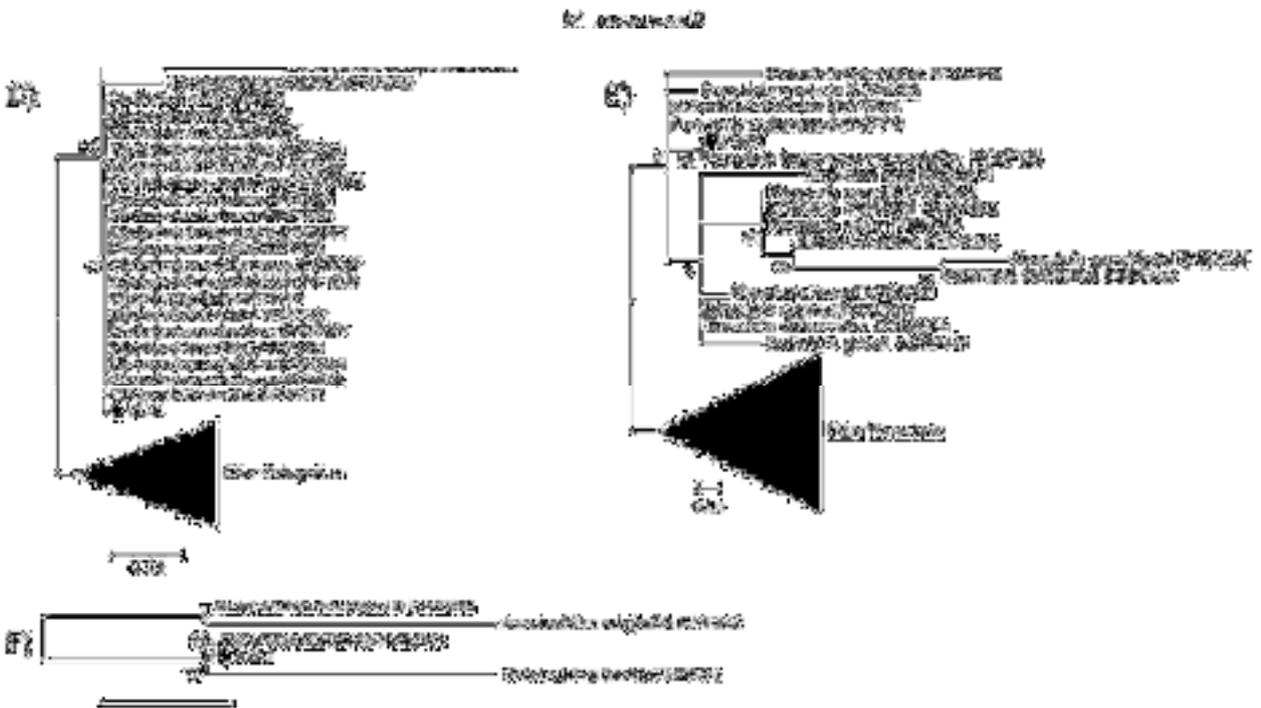


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649 Fig. 1. Separation of core and satellite OTUs of the fungal community for both *O. erosus* (A, B) and
 650 *X. saxesenii* (C, D). Species abundance distribution (A and C) based on OTU
 651 abundance (number of retrieved sequences) and persistence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



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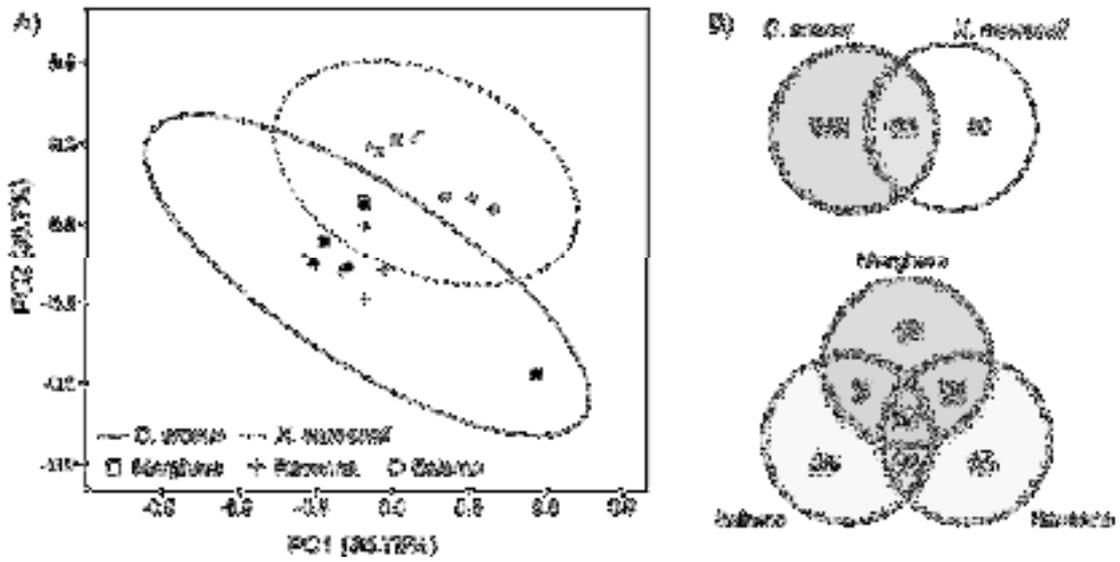
657 Fig. 2. Phylogenetic identification of detected sequence types (STs). Trees were built using STs of the
 658 most relevant fungal genera detected in the present study and ITS2 sequences
 659 retrieved from *O. erosus* and *X. saxesenii* (C) along with validated reference sequences from
 660 *Geosmithia*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

661 aZhou et al., 2006; Lee et al., 2008; Lu et al., 2009; Jankowiak and Bilański, 2013; Romon et al.,
 662 2014a; Romon et al., 2014b), *Alternaria* spp. (C) (Woudenberg et al., 2013), *Cladosporium* spp. (D)
 663 (Bensch et al., 2012), *Ramularia* spp. (E) (Videira et al., 2016), and *Aureobasidium* spp. (F) (Zalar et
 664 al., 2008). Numbers on nodes represent the posterior probabilities for the Randomized Accelerated
 665 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 ($\bar{x} \pm SE$)
<i>O. erosus</i>	ASP1	<i>Aspergillus</i> sp.	31.13 \pm 15.52
	WSP1	<i>Zizytrichia</i> sp.	7.45 \pm 2.43
	ORC1	<i>Geosmithia</i> sp.	2.82 \pm 2.09
	STK1	<i>Stemphylium</i> sp.	2.78 \pm 1.37
	WSP1	<i>Fusiclone incrustans</i> -synonym n.s.	2.31 \pm 1.01
	WSP2	<i>Opitostoma</i> sp.	1.88 \pm 1.62
	ASP1	<i>Aspergillus</i> sp.	1.15 \pm 0.88
	ALT1	<i>Alternaria</i> sp.	0.81 \pm 0.22
	WSP1	<i>Sterigmatia</i> sp.	0.42 \pm 0.14
	WSP2	<i>Fusiclone incrustans</i> -synonym n.s.	0.34 \pm 0.14
	WSP1	<i>Saccaria</i> sp.	0.25 \pm 0.13
<i>N. succensoides</i>	WSP2	<i>Sterigmatia</i> sp.	17.82 \pm 4.78
	CLA1	<i>Cladomyces leucosporus</i> n.s.	8.58 \pm 3.41
	ASP2	<i>Aspergillus</i> sp.	6.52 \pm 2.48
	WSP1	<i>Saccaria</i> sp.	2.38 \pm 2.31
	ALT1	<i>Arthroascus guttulatus</i>	6.50 \pm 3.29
	ASP3	<i>Aspergillus</i> sp.	4.18 \pm 2.95
	ASP4	<i>Aspergillus</i> sp.	3.31 \pm 1.48
	ALT1	<i>Alternaria</i> sp.	2.88 \pm 1.70

666



667

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.