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Fungal community associated with adults of the chestnut gall wasp *Dryocosmus kuriphilus* after emergence from galls: Taxonomy and functional ecology

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ABSTRACT

The diversity of the fungal community associated with adults of *Dryocosmus kuriphilus* following emergence was examined using HTS analysis. Ascomycota dominated the fungal core-biome community. The functional guilds of the 90 taxa forming the core-biome were assessed, demonstrating three main groups: saprotrophs, plant pathogens and entomopathogens. Twenty-nine OTUs out of 90 were resolved to species level identifying 26 different fungal species. Among these species, many were cosmopolitan or previously recorded in Europe. Ten taxa were previously recorded on chestnut, including some recognized plant pathogens associated with foliage and green tissues such as *Epicoccum nigrum*, *Gnomoniopsis castanea*, *Colletotrichum acutatum*, *Stromatoseptoria castaneicola*, *Ramularia endophylla*. *Beauveria bassiana*; within the core microbiome, *Fusarium larvarum* represented the most abundant entomopathogenic species. Some of these species are known to impact directly or indirectly the vitality of the insects in the galls. The chestnut blight pathogen, *Cryphonectria parasitica*, was never found associated with *D. kuriphilus*. Based on the present study, an active role for *D. kuriphilus* as a vector of chestnut fungal endophyte/pathogens cannot be demonstrated but neither ruled out.

1. Introduction

Invasion by alien plant pests (used here to mean both insects and pathogens) represents an important component of global biological invasions, having a highly significant direct impact on host species in the invaded environments (Eschen et al., 2019). Moreover, the introduction and establishment of an alien species in a new environment results in additional ecosystems changes resulting from indirect effects on ecosystem functioning. Indirect interactions occur when one species influences a second via its interactions with a third species (Waser et al., 2015). In the context of alien invasive pests, these interactions can affect densities of ecological communities, for example by providing, or depleting, a source of nutrients, altering the availability of physical factors such as light, moisture, and temperature, or modifying their behavioral traits, for example by interacting with patterns of dispersal or colonization (Gandhi and Herms, 2010).

Changes in the microbial community have been suggested in sweet chestnut (*Castanea sativa* Mill.) ecosystems following the introduction, establishing and successive spreading in Europe of the chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (Bernardo et al., 2013). The direct impact of *D. kuriphilus* on *C. sativa* includes a reduction in fruit production; reduction in leaf area, functionality and photosynthetic capacity (Sartor et al., 2015); tree decline and death (Dixon et al., 1986). Moreover, *D. kuriphilus* invasion indirectly produced changes in chestnut ecosystems, for instance in communities of natural parasitoids, by altering their densities and host niches (Panzavolta et al., 2013; Speranza et al., 2008); recent studies also suggest changes in densities and host niches of microbial communities, native and exotic, commonly associated with chestnut trees (Fernández et al., 2018; Maresi et al., 2013; Meyer et al., 2015; Vannini et al., 2018). The outbreak of chestnut kernel brown rot caused by *Gnomoniopsis castanea* Tamietti is believed to be associated with the invasion of *D. kuriphilus* (Vannini et al., 2017)

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which provided the fungus with an optimal substrate, the galls, for growth and production of secondary inoculum (Magro et al., 2010; Maresi et al., 2013; Vannini et al., 2017). A suggested additional indirect effect of *D. kuriphilus* invasion is the occurrence of current year shoot-flagging recently reported by Vannini et al. (2018) in Central Italy and associated with *Cryphonectria parasitica* (Murr.) Barr infection of cryptic dried buds. Vannini et al. (2018) suggested a role for *D. kuriphilus* in facilitating the inoculation and penetration of *C. parasitica* into dormant buds during oviposition. The most conservative hypothesis limited the role of *D. kuriphilus* during oviposition activity by causing fresh wounds on buds providing entry points for *C. parasitica* external inoculum (e.g. spores). The second hypothesis also considers the role of *D. kuriphilus* as a vector in the horizontal transmission of *C. parasitica* from natural sources of inoculum, i.e. bark cankers and abandoned galls, into the buds. The latter is based on the widely reported association between gall-forming insects and fungi (Raman et al., 2012), ranging from 'vague' association to true symbiotic interactions. Although data on the presence and role of fungal taxa in green and abandoned galls were provided by some authors (Fernández et al., 2018; Maresi et al., 2013; Meyer et al., 2015; Vannini et al., 2018), studies of the fungal community associated with adults of *D. kuriphilus* during the flying period are limited to the molecular detection of *G. castanea* by Lione et al. (2016). In the present study, the fungal community associated with adults of *D. kuriphilus* was assessed by means of metabarcoding with High Throughput Sequencing (HTS). The resulting taxa were subjected to metacommunity and functionality analysis to understand the diversity among samples, to study the core microbiome, intended as the microbial community found in at least 80 % of the insects, and to contribute to a better understanding of the epidemiology of this recently described pathosystem.

2. Materials and methods

2.1. Sampling area

The sampling area was located in Monti Cimini (Viterbo Province), one of the largest chestnut districts in Central Italy. Highly productive, managed orchards and coppices characterize this chestnut growing area. *D. kuriphilus* has been present since 2006 causing severe infestations and damage (Speranza et al., 2008; Vannini et al., 2017).

Orchard trees were chosen along a linear transect, approximately 1.5 km in length. The transect was located north of the volcanic cone of Vico Lake (42°22′10″N 12°13′15″E) in the municipality of Canepina. Five adult *C. sativa* trees were tagged along the transect.

2.2. Sampling of D. kuriphilus adults

For each of the 5 trees, on June 10, 2016, two terminal branches with fresh *D. kuriphilus* galls (at least 15) were tagged, one without visible *C. parasitica* canker on the previous year's growth (NC) one with canker visible on the previous year's growth (C). Each branch was enclosed in an insect mesh bag (17-mesh net, 1.22 × 1.61 mm) to capture the adults of *D. kuriphilus* after emergence from mature galls. After 30 d the branches were excised and taken to the laboratory. Adults of *D. kuriphilus* were pooled from each bag and carefully collected in sterile 2 mL Eppendorf® test tubes and stored at 5 °C. Adults of *D. kuriphilus* captured from galls on branches with and without *C. parasitica* cankers were coded CD and NCD respectively.

2.3. DNA extraction and amplification

Total DNA from an average of a bulk 6 adults per sample (branch) was extracted using the DNeasy PowerSoil Kit (Qiagen, Germany), following the manufacturer's instructions. The ITS1 region was amplified with a dual indexing primer using the tagged primer pair ITS1F (5′-xxxxCTYGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5-xxxxGCHRCGTTCTTCATCGDTGC-3′), where xxx represents the barcoding key. The PCR reaction mixture comprised 12.5 μl of Maxima Hot Start PCR Master Mix (2X) (Thermo Fisher Scientific, USA) and 1 μM of each primer in a total volume of 25 μl containing 24 μl of reaction mixture and 1 μl template. The thermal cycle was an initial denaturation at 94 °C for 10 min followed by 30 cycles of 95 °C for 40 s, 60 °C for 40 s and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. Eight PCRs were carried out and pooled per sample. Amplicons were purified using the MagJET NGS Cleanup (Thermo Scientific, USA), quantified with the Qubit Quantitation kit (Invitrogen, USA) and pooled at equal concentrations for sequencing. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq sequencer by Eurofins Genomics GmbH (Germany).

2.4. Bioinformatics analysis

Data sets were analyzed out following the pipeline described by Gómez et al. (2019). To reduce the phenomenon of cross-contamination and false assignments, only reads containing the combination of 5′barcode and forward primer as well as the expected 3′barcode and reverse primer were paired and used in the analyses; moreover, for the identification of barcode and primer sequences no mismatches were allowed. Raw read pairs were quality filtered (limit = 0.05) and trimmed using CLC Genomic Workbench Version 8.5.1 (QIAGEN bioinformatics, Aarhus, Denmark) filtering out all sequences containing "N"s and sequences with a minimum length of 100 nucleotides or a maximum length of 400 nucleotides. After this, the paired-end reads were assembled. If there were mismatches between the overlapping fragments of the forward and reverse reads, these were corrected according to the base call with the higher sequencer-assigned quality score.

After quality filtering, paired-end assembly and demultiplexing, the sequences were processed, and similarity clustering performed based on the UPARSE pipeline of USEARCH v8 (Edgar, 2010) using a 97 % clustering threshold (Lindahl et al., 2013). Sequences failing alignment or identified as chimeric were removed before downstream analysis.

Consensus OTUs were identified using the BLAST tool in the Genbank database with the algorithm parameters: word size = 11, match/mismatch scores = 2,−3, gap cost existence = 5 and gap cost extension = 2. The xml file from the BLAST and the blasted fasta file were imported into MEGAN (Huson et al., 2007) to compute and explore the taxonomical content of the data set, employing the NCBI taxonomy to summarize and order the results. Lowest common ancestor parameters were: Min score = 170; Max. expected = 0.01; Top percent = 2.0, Min support percent = 0.3; Min support = 1 and LCA percent = 40) and with the following minimum requirements of similarity to accept the proposed taxonomy: Species 99 %, Genus 97 %, Family 95 %, Order 90 %, Class 85 %, and Phylum 80 %.

Finally, an OTU abundance table was generated with USEARCH v8 (Bálint et al., 2014; Edgar, 2010). Any OTU representing less than 0.001 % of the total filtered sequences was removed to avoid the inclusion of erroneous reads, which could lead to inflated estimates of diversity (Parks et al., 2013). The core-microbiome was obtained by selecting the OTUs present in at least 4 of the 5 samples in each case (branch with canker and without). The Venn diagram was calculated using the online application available on http://bioinformatics.psb.ugent.be/webtools/Venn.

The reads generated in this work are available in the NCBI Sequence Read Archive (SRA) https://www.ncbi.nlm.nih.gov/bioproject/PRJNA543312 under the project name “Dryocosmus and associated fungi”, accession number PRJNA543312.

Each OTU was classified into an ecological guild using FUNGuild (Nguyen et al., 2016). FUNGuild is a two—component system that includes an online community annotated database and a python script that assigns functional guilds to OTUs obtained from next-generation sequencing. The FUNGuild database is based on a genus level of classification. A downstream check was carried out on the output table by extending the identification to species level where applicable and choosing the appropriate guild, when more than one option was provided, with the support of the USDA Fungal Database (Farr and Rossman, 2019). OTUs ranked as ‘possible’ and not passing the downstream check were considered ‘unclassified’ in the final output table.

The OTU abundance and taxonomy tables, and representative-sequences Fasta files were transformed in qza format for use in QIIME2 2018.2, https://qiime2.org (Caporaso et al., 2010) for diversity (Evenness and Faith-PD indexes indices) and taxonomy analyses. Non-metric multidimensional scaling (NMDS) was carried out with the software PAST 3.24 (Hammer et al., 2001).

3. Results

From the 10 insect bulk samples (5 trees x 2 samples, with and without cankers) analyzed, 3,580,326 reads were clustering to a 97 % similarity level in 748 OTUs with a minimum frequency of 215,038 reads per sample and a median frequency of 357,365 reads. Based on representation (presence in a minimum of 4 of the 5 samples per thesis, C and NC, and never below 50 % of the total samples), 90 OTUs formed the core microbiome clustering 96,72 % (3,463,105 reads of which 1,683,053 from NCD and 1,780,052 from CD samples) of the reads with a median frequency of 350,381 reads per sample. Alpha rarefaction curves constructed for each of the 10 insects bulk samples showed saturated curves of the fungal community, suggesting that most fungal biodiversity in the samples was detected (Fig. 1).

Eighty-seven out of the 90 OTUs representing the core microbiome were shared between the NCD and CD groups. No significant differences in Evenness and Faith-PD indices were found between the NCD and CD groups (Kruskal—Wallis, $P > 0.05$). NMDS analysis using the Jaccard Similarity index is shown in Fig. 2. The convex hulls showed that NCD and CD samples formed distinct clusters, although the 95 % ellipses suggested there was no-significant distance between groups. Stress value was 0.13. Similar results were obtained with Bray-Curtis and Euclidian distance indices (data not shown).

Ascomycota dominated the fungal core community with 3,446,873 sequences (99.5 %). The remaining 0.5 % community was Basidiomycota. The most abundant families were Cladosporiaceae (47.7 %), Didymellaceae (16.4 %), Pleosporaceae (15.8 %), Glomerellaceae (6.7 %), Aspergillaceae (4.7 %) and Mycosphaerellaceae (3.5 %) (Fig. 3).

Assignment of the functional guilds to the 90 OTUs following downstream checks is illustrated in Fig. 4. Plant pathogens and saprotrophs were the largest groups present, while over 40 % of the taxa were not assigned to a functional guild.

Twenty-nine OTUs out of 90 were resolved to the species level, identifying 26 different fungal species (Table 1).

Among these species, many were cosmopolitan or previously recorded in Europe. Ten taxa were previously recorded on sweet chestnut, including several recognized plant pathogens associated with foliage and green tissues, such as *Epicoccum nigrum*, *G. castanea*, *Colletotrichum acutatum*, *Stromatoseptoria castaenicola*, *Ramularia endophylla* (anamorph of *Mycosphaerella punctiformis*; Syn. *Mycosphaerella maculiformis*). The number of reads of the 26 fungal species in the 10 samples is shown in the heatmap in Fig. 5. The most abundant nine species were recorded from all samples: *E. nigrum*, *Penicillium brevicompactatum*, *C. acutatum*, *Stemphylium vesicarium*, *Cladosporium aggregatumcicatricatum*, *Stromatoseptoria castaneicola*, *Geosmithia pallida*, *G. castanea,* and *Taphrina carpini*. The entomopathogens *Beauveria bassiana* and, in the core microbiome, *Fusarium larvarum* (OTU 55) were unevenly present in the 10 samples (7 out of 10 samples for a total of 45,019 reads, and 4 out of 10 samples for a total of 863 reads, respectively). Specifically, 99.6 % of reads referring to *B. bassiana* were recorded in one sample (5NC). Out of the core microbiome, OTU’s 33, 53 and 125 were for two distinct *Fusarium* sp. and to *Fusarium equiseti*, respectively. Twenty-five out of twenty-six species were cosmopolitan and reported in Europe on a wide range of hosts. OTU170. *Devriesia fraseriae*, is the sole taxon not previously recorded in Europe.

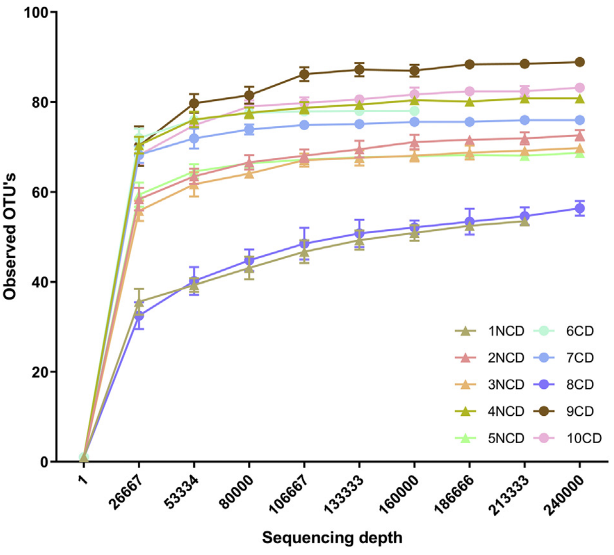

Fig. 1. Alpha rarefaction curves constructed for each of the 10 insect bulk samples. CD, adults of *D. kuriphilus* captured from galls on branches with *C. parasitica* cankers; NCD, adults of *D. kuriphilus* captured from galls on branches without *C. parasitica* cankers.

4. Discussion

In the present study, the fungal community associated with adults of *D. kuriphilus*, after their emergence from the galls, was examined by HTS analysis. The choice to analyze emerged adults was taken to investigate the possible role of *D. kuriphilus* in the epidemiology of the newly reported flagging symptoms associated with *C. parasitica* infection, described by Vannini et al. (2018). The assumptions were that, as reported by Raman et al. (2012), (i) almost every insect-induced gall is associated with one or more species of fungus during the developmental stages, and that (ii) *C. parasitica* occurs as an endophyte in from green galls, even if at very low frequency (Vannini et al., 2018). However, the absence of *C. parasitica* among the fungal OTUs associated with *D. kuriphilus* adults in both CD and NCD samples did not support direct involvement of the insect as an occasional vector of the pathogen to dormant buds, whereas an indirect effect of *D. kuriphilus* in providing penetration sites to *C. parasitica*, via oviposition wounds, remains a possibility.

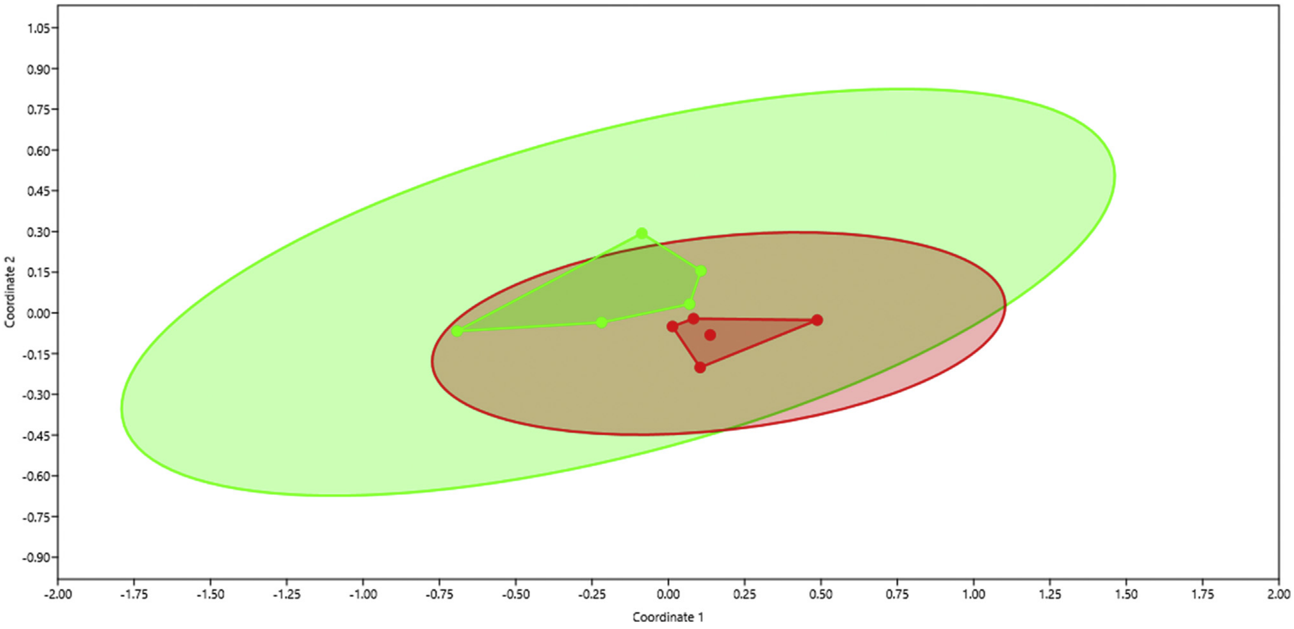

Fig. 2. NMDS representing dissimilarities in the composition of fungal communities between the NCD (red) and CD (green) groups of samples. CD, adults of *D. kuriphilus* captured from galls on branches with *C. parasitica* cankers; NCD, adults of *D. kuriphilus* captured from galls on branches without *C. parasitica* cankers. The stress value associated with this representation was 0.13, indicating a fair fit. Ellipses represent 95 % confidence intervals around the centroids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

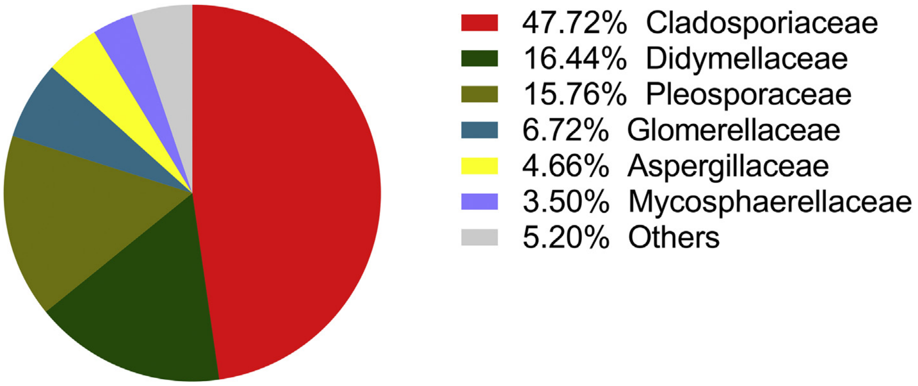

Fig. 3. Distribution among families of the 90 fungal taxa representing the core microbiome as defined in Materials and Methods.

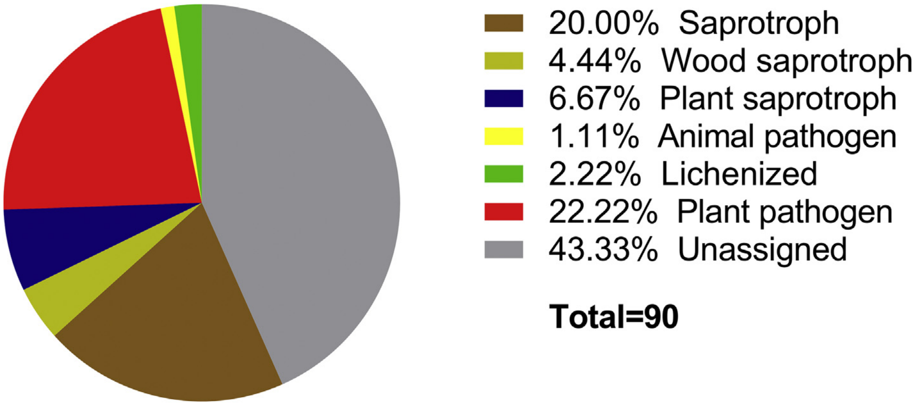

Fig. 4. Assignment of the functional guilds (%) to the 90 fungal taxa representing the core microbiome, following downstream checks.

Table 1
Taxonomic position, life strategy, occurrence and recorded hosts/substrate for each of the twenty-six fungal species identified by HTS analysis.

OTU	Fungus		Life strategy	Occurrence	Reported host range	Reported substrate	Reported on *Castanea*	Ref.
	Species	Family						
2, 17	*Epicoccum nigrum*	Didymellaceae	Plant pathogen	cosmopolitan	broad-host-range	leaves, galls	yes	Fernández et al. (2018)
5	*Penicillium brevicompactum*	Aspergillaceae	Plant pathogen	cosmopolitan	broad-host range	generic	no	Farr & Rossman (2019)
7	*Colletotrichum acutatum*	Glomerellaceae	Plant pathogen	cosmopolitan	broad-host-range	leaves, shoots, buds, fruits, galls	yes	Vannini et al. (2018)
8	*Stemphylium vesicarium*	Pleosporaceae	Plant pathogen	cosmopolitan	broad-host-range	leaves, roots, seeds	no	Farr & Rossman (2019)
9	*Cladosporium aggregatocicatricatum*	Cladosporiaceae	Saprotroph	USA, Europe	*Vitis vinifera*, *Asteriscus sericeus*	generic	no	Farr & Rossman (2019)
11, 18, 137	*Stromatoseptoria castaneicola*	Mycosphaerellaceae	Plant pathogen	cosmopolitan	*Castanea* spp., *Aesculus* spp., *Chrysanthemum* sp.	leaves	yes	Farr & Rossman (2019)
12	*Beauveria bassiana*	Cordycipitaceae	Entomopathogen	cosmopolitan	broad-host range	insects	no	Farr & Rossman (2019)
15	*Geosmithia pallida*	Bionectraciae	Plant pathogen	cosmopolitan	*Quercus* spp., *Ulmus* spp.	bark	no	Farr & Rossman (2019)
19	*Gnomoniopsis castanea*	Gnomoniaceae	Plant pathogen	cosmopolitan	*Castanea* spp.	leaves, shoots, fruits, buds, galls	yes	Vannini et al. (2017)
21	*Taphrina carpini*	Taphrinaceae	Plant pathogen	Europe, Asia	*Carpinus* spp., *Quercus pyrenaica*	leaves	no	Farr & Rossman (2019)
25	*Ramularia endophylla*	Mycosphaerellaceae	Plant pathogen	cosmopolitan	broad-host range	leaves	yes	Videira et al. (2015)
28	*Botrytis cinerea*	Sclerotinaceae	Plant pathogen	cosmopolitan	broad-host-range	Stems, twigs, leaves, fruit, pods.	yes	Farr & Rossman (2019)
38	*Mycocalicium victoriae*	Mycocaliciaceae	Symbiont/lichen	Europe, USA, Australia	broad-host-range	trunk, soil, limestone	no	Nádvornik (1942)
59	*Periconia byssoides*	Periconiaceae	Endophyte	cosmopolitan	broad-host-range	leaf and stem spots, blight, twig dieback, fruit mold	no	Farr & Rossman (2019)
65	*Pyrigemmula aurantiaca*	Chaetosphaeriaceae	Saprotroph	Europe	woody hosts	bark	yes	Magyar et al. (2011)
74	*Monochaetia monochaeta*	Xylariaceae	Plant pathogen	cosmopolitan	woody hosts	leaves, stems	yes	Farr & Rossman (2019)
121	*Naevala minutissima*	Dermataceae	Saprotroph	Europe	*Quercus* spp.	leaves, litter	no	Voriskova and Baldrian (2013)
130	*Penicillium adametzioides*	Aspergillaceae	Saprotroph	Europe, Japan	woody hosts	fruits, various organs	no	Farr & Rossman (2019)
152	*Petrophila incerta*	Extremaceae	Saprotroph	Europe	na	na	no	Isola et al. (2016)
170	*Devriesia fraseriae*	Teratosphaeriaceae	Saprotroph	Australia	*Maleleuca* sp.	Leaves	no	Crous et al. (2010)
206	*Cladosporium sphaerospermum*	Cladosporiaceae	Saprotroph	cosmopolitan	broad-host range	generic	yes	Farr & Rossman (2019)
294	*Scoliciosporum umbrinum*	Lecanoraceae	Symbiont/lichen	cosmopolitan	different woody hosts	tree bark/rock	yes	Loppi e Putortì (2001)
468	*Cladosporium langeronii*	Cladosporiaceae	Saprotroph	cosmopolitan	broad-host range	generic	no	Zalar et al. (2007)
23	*Filobasidium wieringae*	Filobasidiaceae	Saprotroph/yeast	cosmopolitan	broad-host-range	generic	no	Glushakova and Kachalkin (2017)
45	*Vishniacozyma victoriae*	Bulleribasidiaceae	Yeast	Europe, South America	broad-host-range	generic	no	Gramisci et al. (2018)
238	*Rhodotorula mucilaginosa*	Sporidiobolaceae	Saprotroph/yeast	cosmopolitan	broad-host-range	generic	no	Wirth and Goldani (2012)

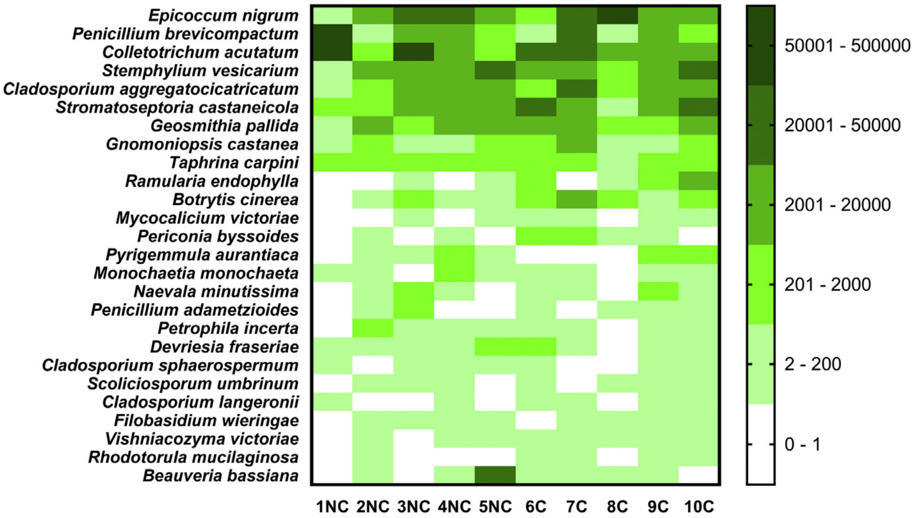

Fig. 5. Heatmap of the number of reads attributed to each of the 26 fungal taxa identified to species level among the 10 samples.

The nature of the interactions between *D. kuriphilus* and the associated fungal community appeared to be complex and diverse. Possible associations between *D. kuriphilus* infestation and the outbreak of kernel brown rot by *G. castanea* (Lione et al., 2016; Vannini et al., 2017), and between *D. kuriphilus* and new flagging symptoms by *C. parasitica* (Vannini et al., 2018) were suggested previously. However, as suggested by Lione et al. (2016), and based on the results of the present study, it appears unlikely that *D. kuriphilus* has a role as a vector in either disease.

The origin of the insects from galls on sampled trees along a 1,5 km transect did not significantly affect the composition of associated fungal communities, suggesting that the taxa within the core microbiome belong to peculiar endophytic and/or epiphytic communities associated with galls on chestnut, rather than to random aerial inoculum, the composition of which is more likely to be determined by micro-site conditions (tree level). Furthermore, most fungal species identified in the present study have life strategy associated with terminal branches of woody hosts including foliage, buds, flowers, fruits, and bark (see Table 1 for literature).

Saprotrophs represented an important component of the fungal communities identified, with some genera, such as *Penicillium* and *Cladosporium*, highly represented in terms of abundance; the two most abundant species, *Penicillium brevicompactum* and *Cladosporium aggregatocicatricatum* are typical saprotrophs on generic substrates, and were previously recorded in different geographic areas and hosts, although never recorded on chestnut (Farr and Rossman, 2019). Two additional *Cladosporium* spp. were recorded, *Cladosporium langeronii* and *Cladosporium sphaerospermum*, the latter previously reported on *Castanea mollissima* in China (Zhang et al., 2003). It is notable that insects from 8 samples out of 10, were contaminated with *Vishniacozyma victoriae*, a Basidiomycota yeast known as a biocontrol agent of *P. expansum* and *Botrytis cinerea* (Gramisci et al., 2018), the latter also found associated with *D. kuriphilus* in the present study. Contamination of the insects with epiphytic fungal taxa, including also the above-mentioned genera *Cladosporium* and *Penicillium*, may occur after adult emergence but, possibly, during adult flight toward the buds for oviposition. A similar process might also occur with *T. carpini* that was detected in all samples. The mycelial dikaryotic phase of *T. carpini* is associated with *Carpinus* spp., while the haploid yeast phase, referred as *Lararia carpini*, was described from Portugal on the phylloplane of *Quercus pyrenaica*, like sweet chestnut also a member of the Fagaceae, where it seems to behave as a saprotroph (Inácio et al., 2004). Recently, *T. carpini* was detected using molecular methods from leaves of oak, chestnut and hornbeam (*Carpinus betulus*) forest patches (Fort et al., 2016), although the authors did not specify if the yeast was detected solely from hornbeam leaves. In general, this is an interesting finding of a pathogen with a biotrophic lifestyle in its dikaryotic phase, which, ultimately, can colonize epiphytically, and saprophytically, green tissues of non-host tree species in the same forest type. However, it appears unlikely that a monophagous species such as *D. kuriphilus* could play a significant role in vectoring *T. carpini* between the tree species.

Ten out of 26 fungal species, including *T. carpini*, have pathogenic lifestyles on several woody hosts. Some species have a cosmopolitan distribution and a broad host range including the genus *Castanea*, such as *E. nigrum* (Fernández et al., 2018), *C. acutatum* (Vannini et al., 2018), *R. endophylla* and *B. cinerea* (Farr and Rossman, 2019). Others, such as *S. vesicarium* and *G. pallida*, were not previously recorded on chestnut (Farr and Rossman, 2019), while two species, *G. castanea*, and *S. castaneicola*, have main hosts within the genus *Castanea*. These species show an endophytic behavior in healthy host tissues: *E. nigrum*, *C. acutatum*, *G. castanea* have been reported as endophytes in healthy chestnut tissues (Aghayeva et al., 2017; Vannini et al., 2018); *R. endophylla* is known as a leaf endophyte in oak (Verkley et al., 2004). *S. castaneicola* was recently reported as a component of the foliar fungal community of oak, chestnut and hornbeam in forest patches in France (Fort et al., 2016). *S. vesicarium* is reported as an endophyte, specifically in foliage and petioles of Proteaceae species (Swart et al., 2000), although, considering the known broad-host-range (more than 700 hosts recorded) (Farr and Rossman, 2019), it is likely to occur as an endophyte in other hosts. Finally, *G. morbida*, as fungal endophyte, is limited to a single record in grapevine in Iran (Hergoli et al., 2015), although the genus *Geosmithia* is considered to contain several true endophytic species (Kolařík and Kirkendall, 2010). Among entomopathogens, *B. bassiana* is also considered to have an endophytic lifestyle in plant tissues (Quesada-Moraga et al., 2014) and *F. larvarum* was reported as an endophyte in *Pinus halepensis* (Botella and Diez (2011). *F. larvarum* is considered a natural biocontrol agent against scale insects and aphids, producing insecticidal compounds affecting the survival of insects at different development stages (Ganassi et al., 2001).

Associations between cynipid gall wasps (Hymenoptera Cynipidae) and fungi is well known (Raman et al., 2012). Endophytic fungal communities colonizing gall tissues are commonly characterized by Type II endophyte taxa. Type II endophytes are Ascomycota and are typically horizontally transmitted through different strategies, including via biotic vectors such as insects (Carroll, 1988). In general, this category of fungi is reported directly or indirectly impact the vitality of the insects into the galls. Regarding the fungal lifestyle, they can be generic or specific parasites of the insect or secondary plant pathogens whose activities in necrotizing gall and surrounding plant tissues limit the survival of the insect especially in the larval stages (Butin, 1992; Raman et al., 2012). Several publications (Graziosi and Rieske, 2015; Magro et al., 2010; Vannini et al., 2017), reported the pathogenicity of *Colletotrichum* sp. and *G. castanea* on *D. kuriphilus* galls and surrounding leaf blades, and indirect effects on insect survival in galls. A similar interaction was postulated by Wilson (1995) on *Quercus garriana* between the Cynipidae *Besbicus mirabilis* and the endophytic fungus *Discula quercina*. Similarly to *G. castanea*, it was demonstrated that the fungal endophyte grows from the leaf into the gall and infects all gall tissue but does not directly kill the gall making larva. Comparable behavior may be exerted by *S. castaneicola* and *R. endophylla*, known to cause necrotic lesions on chestnut leaves (Farr and Rossman, 2019), and by *E. nigrum*, one of the most abundant taxa isolated from necrotic galls on chestnut in Spain (Fernández et al., 2018).

The detection of the entomopathogen *B. bassiana* associated with *D. kuriphilus* is new to science; however, this fungus was previously reported associated with other species of Cynipidae (Quesada-Moraga et al., 2014). A positive effect of the endophytic entomopathogen *B. bassiana* in protecting opium poppy from the gall wasp *Iraella luteipes* (Hymenoptera: Cynipidae) was suggested by Quesada-Moraga et al. (2014), who found larvae of the insect naturally infected by the fungus in the gall. In the present study, the high number of reads related to *B. bassiana* limited to one sample would suggest the mass colonization of one or a few adults. However, the mechanisms through which the endophytic *B. bassiana* exerts biocontrol potential are still unclear (McKinnon et al., 2017).

The role of Cynipids in the dispersal of Type II endophytes has not been investigated and no data are available in the literature. Other Hymenoptera, with different behaviors, were shown to disperse fungi, such as ants (Formicidae) involved in the spread of airborne fungi in hospital environments (Aquino et al., 2013). Leach (1940) established four rules to confirm that an insect was the vector of a given pathogen: (1) a close association of the insect with diseased plants; (2) regular visits to healthy plants by the insect; (3) an association of the pathogen with the insect; and (4) the development of the disease in healthy plants after interaction with pathogen-infested insects. Rules 1 and 2 are easily demonstrated for *D. kuriphilus*; rule 3 has been demonstrated but based only on the results of DNA metabarcoding that does not provide any information on nature and vitality of the insect-associated inoculum; rule 4 is difficult to demonstrate primarily because some of the associated fungal pathogens do not necessarily express immediate or recognizable symptoms on chestnut (e.g. *C. acutatum*, *E. nigrum*); others, such as *S. cataneicola* and *R. endophylla*, were causing seasonal symptoms (leaf spots) even before the introduction of the *D. kuriphilus* in Europe. Incidence of brown rot of chestnut fruits by *G. castanea* was effectively related to *D. kuriphilus* infestation, although inoculation of female flowers is more likely to occur via airborne inoculum vectored by wind or water (Shuttleworth et al., 2013). In conclusion, an active role of *D. kuriphilus* in the dispersal of chestnut fungal endophytes/pathogens cannot be demonstrated but neither ruled out. The interaction between endophytic fungi and gall-forming insects and, more generally, between fungi and insects, represents an extremely complex field of investigation because of the large number of variables determining the final nature of interaction, ranging from occasional or unexpected to strictly symbiotic and stable. The introduction of new techniques for description of fungal communities and assessment from environmental samples, and the production of large metabarcoding data sets, represent new challenges for the advancement of knowledge in the biology, ecology and functionality of fungal-insect interactions.

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