iForest
Biogeosciences and Forestry

Research Article
doi: 10.3832/ifor4559-017
vol. 17, pp. 378-385

The complexity of mycobiota associated with chestnut galls induced by *Dryocosmus kuriphilus* in Galicia (Northwestern Spain)

María Fraga Meizoso [1],
Olga Aguín [2],
María López-Burgos [1],
Carmen Salinero [2],
J Pedro Mansilla [2]

The European chestnut tree (*Castanea sativa*) is a highly valued deciduous species in Galicia (Northwestern Spain), mainly due to economic, landscape, and social or cultural reasons. However, the Asian wasp *Dryocosmus kuriphilus*, one of the biggest threats to *C. sativa*, is severely affecting tree vigor and reducing chestnut yields. Some studies indicated that this wasp and the galls that it produces may play an important role in spreading fungal disease. The present work aimed to characterize the complex of fungi associated with galls induced by *D. kuriphilus* in Galician chestnut trees, focusing on the study of plant-pathogen diversity. For this purpose, branches with necrotic galls were collected from seventy-eight chestnut stands located in the four Galician provinces (A Coruña, Lugo, Ourense, and Pontevedra). In total, 1170 necrotic galls of the year of study were collected at the different sampling sites. In the laboratory, four galls were randomly selected from each point, thus analyzing 312 galls, which were surface disinfected and dissected, and tissue fragments placed on culture media. From the selected galls, 308 fungal colonies were isolated and then identified by the morphology of their fruiting bodies and the molecular analysis of the ITS, beta-tubulin, elongation factor, and histone regions. They were classified into 27 genera and 53 species, of which 5 genera and 29 species of fungi were reported for the first time associated with *D. kuriphilus* galls, including phytopathogenic fungi. Results show that further research is needed to study in detail the role of galls as entry points and reservoirs of pathogenic fungi.

Keywords: Asian Chestnut Gall Wasp, *Castanea sativa*, Endophytes, Phytopathogens

Introduction

The European chestnut tree (*Castanea sativa* Mill.) registered under the Protected Geographical Indication (PGI) regulation "*Castaña de Galicia*" is a highly valued deciduous species in Galicia (Northwestern Spain), mainly due to economic, landscape, and social or cultural reasons. Chestnut trees are of great economic importance in this area due to their nutritional value, high fruit yield, and excellent wood quality. Spain is the second largest producer in the world, where approximately 60% of the European nut production originates, and 66% of the Spanish stands are located in Galicia, representing 92% of the country's total nut production (FAO 2023).

The Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera, Cynipidae) is one of the most important pests of *C. sativa*. It prevents fruit production by altering the growth and physiology of the host plant, interrupting photosynthetic organ development, and altering flower growth (EFSA 2010). Consequently, it affects tree growth and reproduction, resulting in decay, fewer fruits and reduced crop yields, and, in severe cases, tree death (EPPO 2021). According to various studies, chestnut crop yields can be reduced by 50%-80% depending on the variety and the biological control measures used (EFSA 2010, EPPO 2021).

This insect is listed as a harmful organism in Annex III (List of protected zones and the respective protected zone quarantine pests and their respective codes) of the Commission Implementing Regulation (EU) 2019/2072 and has been included in the A2 list of the European and Mediterranean Plant Protection Organization (EPPO) since 2003 (EPPO 2021).

The wasp is native to China, although it is currently present in Southeast Asia, most of Europe and the east coast of the United States (EPPO 2021). In Europe, this wasp was first recorded in Italy in 2002, spreading and invading the rest of the continent. In Spain, it was detected for the first time in 2012 in Catalonia and, two years later, in Galicia and northern Portugal (Pérez-Otero & Mansilla 2014). According to data collected in annual samplings carried out by the Regional Government of Galicia (*Xunta de Galicia*), this pest is now widespread in Galicia and its incidence has greatly increased. This wasp can spread locally as an adult since it is capable of flying. Additionally, it can be carried by the wind, transported by humans, or travel long distances due to the movement of infested plant material. The wasps may be inconspicuous within the plant tissues, making them difficult to detect visually (EFSA 2010).

D. kuriphilus is a univoltine cynipid (produces one generation per year) and reproduces by thelytokous parthenogenesis; that is, only females develop from unfertilized eggs, thus no males of the species are known (EPPO 2021). During their life cycle, *D. kuriphilus* larvae feed for 20-30 days before pupating, which induces green to red-

---

✉ (1) Fauna Útil SL, Ctra. A Guarda-Tui km 194, Tomiño, Pontevedra (Spain); (2) Estación Fitopatolóxica Areeiro, Deputación de Pontevedra, Subida a la Robleda s/n, Pontevedra (Spain)

@ María Fraga Meizoso (mariafragameizoso@gmail.com)

Received: Jan 11, 2024 - Accepted: Aug 27, 2024

Citation: Fraga Meizoso M, Aguín O, López-Burgos M, Salinero C, Mansilla JP (2024). The complexity of mycobiota associated with chestnut galls induced by *Dryocosmus kuriphilus* in Galicia (Northwestern Spain). iForest 17: 378-385. - doi: 10.3832/ifor4559-017 [online 2024-12-14]

Communicated by: Matteo Marchioro

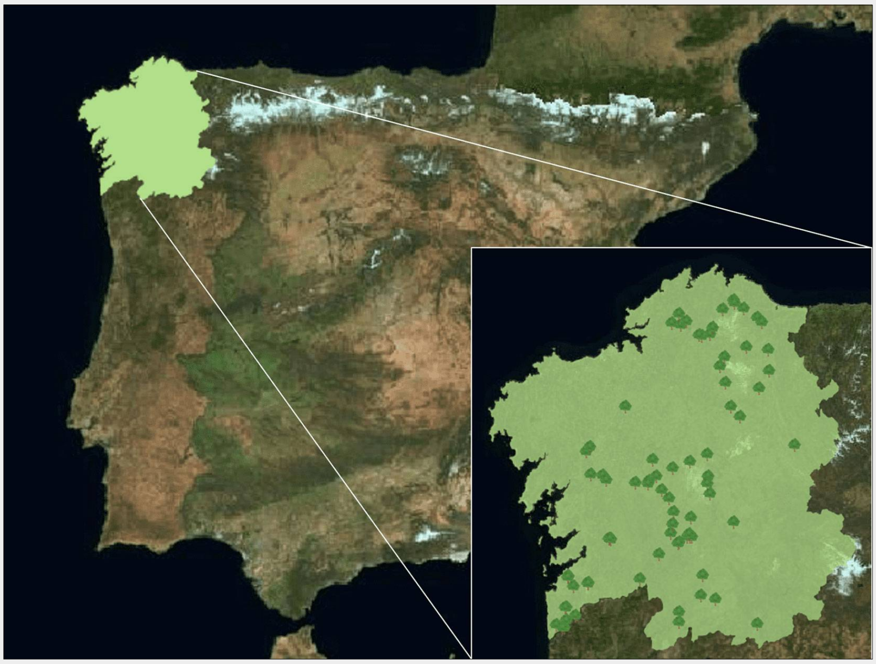

Fig. 1 - The location of Galicia in Northwestern Spain and the geographical distribution of chestnut sampling points in the region.

dish gall formation on buds, leaves, and male catkins (Pérez-Otero & Mansilla 2014). Depending on the climatic conditions of the area and the chestnut cultivar, pupation takes place between mid-May and July. Adult females emerge from the galls between late May and July and can infect new shoots immediately (Pérez-Otero & Mansilla 2014). After emergence, galls dry, become wood-like, and remain on the tree for several years (EPPO 2021).

According to some studies, the galls produced by this wasp can act as entry points and sources of inoculum, thereby causing an increase in the incidence of chestnut diseases, such as chestnut blight, caused by *Cryphonectria parasitica* (Murrill) M.E. Barr (Meyer et al. 2015), or brown rot, produced by *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest (syn. *G. castaneae* Tamietti – Lema et al. 2023). On the other hand, several studies suggest that some endophytic species present in galls could be used as biological control agents against *D. kuriphilus* and other pests (Addario & Turchetti 2011, Tosi et al. 2015).

This work aimed to characterize, for the first time in Galicia, the complex of fungi associated with galls induced by *D. kuriphilus* in chestnut trees, focusing on the study of plant-pathogen diversity.

Material and methods

Study area and sampling sites

Seventy-eight chestnut stands randomly selected in the four Galician provinces (A Coruña, Lugo, Ourense, and Pontevedra) were examined during dormancy. Of these stands, 70% belong to the area covered by the PGI *Castaña de Galicia* (Fig. 1).

In each site, three specimens of *C. sativa* showing symptoms caused by *D. kuriphilus* were selected. Then, several branches with necrotic galls were collected (Fig. 2), totaling five galls per tree, thus obtaining 15 galls per site. In total, 1170 necrotic galls of the year of study were collected at the different sampling sites (120 from A Coruña, 375 from Lugo, 255 from Ourense, and 420 from Pontevedra). Samples were then coded and stored cold until analysis.

Isolation and morphological characterization

In the laboratory, four galls randomly selected from each site were surface sterilized with 1% sodium hypochlorite for one minute and rinsed twice with sterile water for at least five minutes. Subsequently, they were dried with blotting paper, labeled, and cut into small fragments (5 × 5 mm). Around 5-10 fragments were placed in 90 mm Petri dishes containing the culture media Malt Extract Agar with streptomycin 0.5 g L^{-1} (AMs) and kept in a laminar flow chamber under sterile conditions. Plates were sealed with Parafilm® and incubated in the dark at 24 °C. Fragments were checked daily under the microscope, and after 4-5 days, a portion of each mycelium grown from the seeded fragments was transferred to a new plate with Potato Dextrose Agar (PDA) to obtain pure cultures.

Fungal isolates were first identified according to their morphology at the genus level and then by molecular techniques at the species level.

For the morphological study of each isolate, macroscopic features of the mycelium, such as colony shape, color, and texture, and microscopic features (size and shape of the mycelium and size, shape, and color of the conidia) were recorded for each isolate using a Nikon Eclipse® E600 microscope and compared with different fungal taxonomic keys.

Molecular identification

Molecular analysis was performed for all morphologically identified isolates by amplification, sequencing, and phylogeny of different fungal regions. Genomic DNA was extracted from 7-day-old pure colonies

Fig. 2 - Necrotic galls produced by *D. kuriphilus* on chestnut branches.

grown in PDA culture medium using the commercial E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA) following the short protocol. According to the genus determined by morphological analysis, different molecular markers were amplified and sequenced for each isolate: ITS, beta-tubulin, elongation factor 1-alpha, and histone (Tab. 1).

Selected regions were amplified in a SureCycler® 8800 thermal cycler (Agilent Technologies, Santa Clara, CA, USA). PCR products were separated on a 2% (w/v) electrophoresis gel in 0.5× TBE, stained with Midori Green® (Nippon Genetics, Europe), and examined under ultraviolet light. PCR products were purified with the Ilusta ExoProStar® 1-Step kit (GE Healthcare Life Sciences, Chicago, IL, USA). Amplicons obtained were sequenced in both directions (forward and reverse) using the two primers with the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) on the ABIPrism 3500 Genetic Analyzer.

Tab. 1 - Primers from the ITS, beta-tubulin, elongation factor and histone regions used in this study.

Region	Primer name	Reference
ITS	ITS1F	Gardes & Bruns (1993)
	ITS4	White et al. (1990)
	Bot2R	García-Figueres et al. (2004)
Beta-tubulin	T1	O'Donnell & Cigelnik (1997)
	BT2B	Glass & Donaldson (1995)
Elongation factor 1-alpha	EF1	Geiser et al. (2004)
	EF2	Geiser et al. (2004)
	EF1-728F	Carbone & Kohn (1999)
Histone	H3-1b	Glass & Donaldson (1995)
	CYLH3F	Crous et al. (2004)

Bioinformatic analysis

The consensus sequence was obtained with the Mega v. 7 software (https://www.megasoftware.net/) and compared with the homologous sequences using the BLASTn search application of the NCBI GenBank nucleotide database (https://blast.ncbi.nlm.nih.gov/). Subsequently, a mono- or multi-phylogenetic analysis was performed for each isolate of the more complex genera *Fusarium* and *Trichoderma* analyzed, according to the loci used in the molecular identification process.

The sequences of one representative isolate per species were deposited in GenBank, and the accession numbers are specified in Tab. S1 (Supplementary material).

Results

From the analysis of 312 *D. kuriphilus* galls we identified 308 fungal colonies. Of the isolates, 91.6% belong to the Ascomycota phylum, being the Hypocreaceae (31.8%) and Nectriaceae (24.7%) the most abundant families (Fig. 3). At the morphological level, the isolates were classified into 27 different genera (Tab. S1 in Supplementary material), of which five were reported for the first time: *Abortiporus* Murrill, *Neurospora* Shear & B.O. Dodge, *Phlebia* Fr., *Rosellinia* De Not., & D. Hawksw. and *Schizophyllum* Fr. The most abundant genera were *Fusarium* Link with 76 isolates (24.7%) and *Trichoderma* Persoon ex Gray, having 98 isolates (31.8%) (Tab. S1 in Supplementary material).

The morphological results combined with the molecular analysis of the ITS, beta-tubulin, elongation factor, and histone regions allowed the identification of 53 different species (Tab. S1 in Supplementary material), thus showing the great diversity of fungi in the analyzed galls. In fact, no references of presence in galls have been found for 29 species, accounting for 55% of the species detected (Tab. 2). A conclusive morphological and molecular identification at the species level was not obtained for 28 isolates, which is approximately 10% of all isolates. These unidentified isolates belong to 11 different genera, which can be consulted in Tab. S1 (Supplementary material). The presence of the genus *Gnomoniopsis* is

Tab. 2 - Taxonomic classification, number of isolates and relative abundance of the species reported for the first time associated with *D. kuriphilus* galls.

Species	Phylum	Family	No. isolates	Relative abundance (%)
Abortiporus biennis	Basidiomycota	Podoscyphaceae	1	0.32
Colletotrichum fioriniae	Ascomycota	Glomerellaceae	17	5.52
Diaporthe eres	Ascomycota	Diaporthaceae	4	1.30
Diaporthe phaseolorum	Ascomycota	Diaporthaceae	1	0.32
Diaporthe phillipsii	Ascomycota	Diaporthaceae	1	0.32
Diplodia corticola	Ascomycota	Botryosphaeriaceae	2	0.65
Diplodia mutila	Ascomycota	Botryosphaeriaceae	3	0.97
Diplodia sapinea	Ascomycota	Botryosphaeriaceae	5	1.62
Dothiorella iberica	Ascomycota	Botryosphaeriaceae	1	0.32
Fusarium acuminatum	Ascomycota	Nectriaceae	2	0.65
Fusarium culmorum	Ascomycota	Nectriaceae	2	0.65
Fusarium foetens	Ascomycota	Nectriaceae	3	0.97
Fusarium graminearum	Ascomycota	Nectriaceae	7	2.27
Fusarium solani	Ascomycota	Nectriaceae	3	0.97
Fusarium sporotrichioides	Ascomycota	Nectriaceae	18	5.84
Fusarium temperatum	Ascomycota	Nectriaceae	1	0.32
Fusarium torulosum	Ascomycota	Nectriaceae	1	0.32
Neofusicoccum eucalyptorum	Ascomycota	Botryosphaeriaceae	1	0.32
Neurospora crassa	Ascomycota	Sordariaceae	1	0.32
Pestalotiopsis brachiata	Ascomycota	Sporocadaceae	1	0.32
Pestalotiopsis paeoniicola	Ascomycota	Sporocadaceae	1	0.32
Phlebia acerina	Basidiomycota	Meruliaceae	16	5.19
Phlebia radiata	Basidiomycota	Meruliaceae	1	0.32
Rhizopus microsporus	Mucoromycota	Rhizopodaceae	2	0.65
Rosellinia corticium	Ascomycota	Xylariaceae	2	0.65
Schizophyllum commune	Basidiomycota	Schizophyllaceae	2	0.65
Talaromyces cecidicola	Ascomycota	Trichocomaceae	2	0.65
Trichoderma gamsii	Ascomycota	Hypocreaceae	10	3.25
Trichoderma koningiopsis	Ascomycota	Hypocreaceae	2	0.65

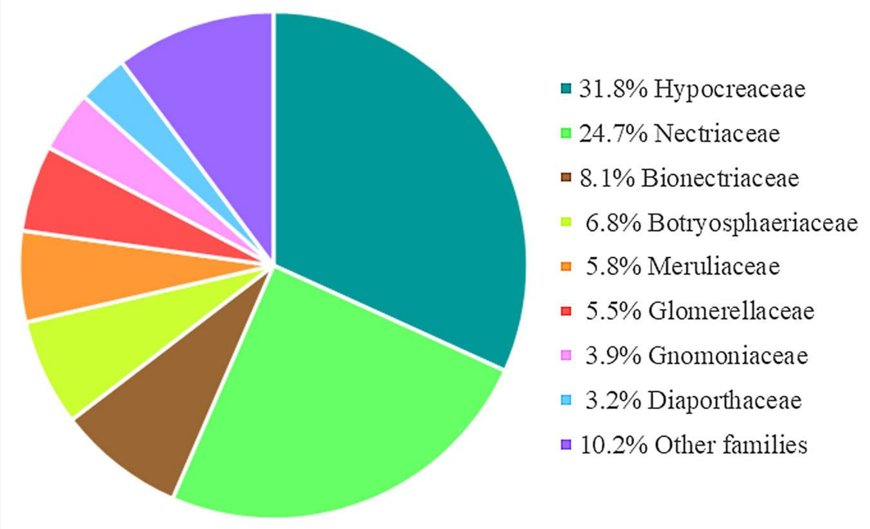

Fig. 3 - Relative abundance of the isolated fungi at the family level.

highlighted since, in recent years, new species such as *G. daii, G. castanopsidis, G. fagacearum, G. guangdongensis, G. hainanensis, G. rossmaniae* and *G. silvicola* (Jiang et al. 2021) have been described. The 5 isolates that could not be identified may belong to a new *Gnomoniopsis* species not yet described, as this genus is currently under study.

Regarding life strategies, two large trophic groups stand out: plant pathogens and saprotrophs. Of the 53 species identified, according to the literature review, 37 species proved to be pathogenic. Of them, 21 were reported to cause damage to chestnut trees (Tab. 3), such as *Gnomoniopsis smithogilvyi* (Lema et al. 2023), with 2 isolates in A Coruña, 2 in Lugo, 2 in Pontevedra, and 1 in Ourense) and *Cryphonectria parasitica* (Meyer et al. 2015), although in this case, it was isolated from a single gall (see Tab. S1 in Supplementary material).

As mentioned above, the genera *Fusarium* and *Trichoderma* were the most abundant. The multilocus phylogenetic analysis allowed the identification of isolates at the species level. The galls' condition at the time they were collected could explain the presence of a great diversity of species belonging to these genera, since they were necrotic and thus a great diversity of saprophytic fungi was expected to be found. In the case of the genus *Fusarium*, isolates belonging to 13 different species were detected, being *F. avenaceum* (Fr.) Sacc., *F. graminearum* Schwabe, *F. oxysporum* Schltdl. and *F. sporotrichioides* Sherbakoff those with the highest incidence (Tab. S1 in Supplementary material). For the first time, 8 species were reported in association with *D. kuriphilus* galls: *F. acuminatum* Ellis & Everhart, *F. culmorum* (Wm. G. Sm.) Sacc., *F. foetens* Schroers, O'Donnell, Baayen & Hooftman, *F. graminearum, F. solani* (Mart.) Sacc., *F. sporotrichioides, F. temperatum* Scaufl. & Munaut and *F. torulosum* (Berk. & M.A. Curtis) Nirenberg (Tab. 2). The isolates obtained from the genus *Trichoderma* corresponded to 7 different species, mainly *T. atroviride* Bissett and *T. harzianum* Rifai (Tab. S1 in Supplementary material), being *T. gamsii* Samuels & Druzhinina and *T. koningiopsis* Samuels, Suarez & Evans first reported associated with *D. kuriphilus* galls (Tab. 2).

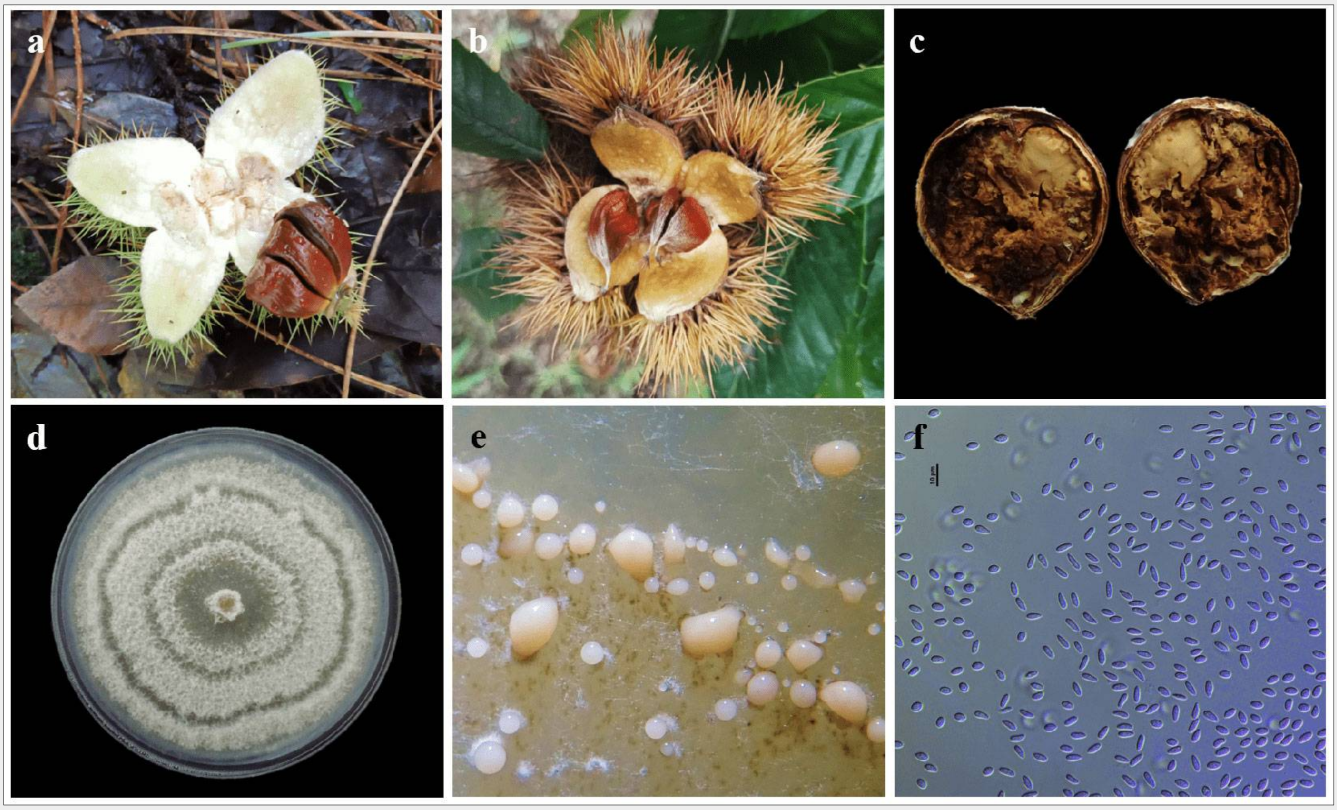

Fig. 4 - *Gnomoniopsis smithogilvyi* symptoms and morphology: (a) premature fall of burrs with mummified nuts; (b) mummified nuts and premature opening of burrs affected by the pathogen; (c) characteristic brown rot of nuts; (d) colony morphology on PDA; (e) conidiomata formed on PDA; (f) conidia (scale bar: 10 μm).

Tab. 3 - List of phytopathogenic fungal species isolated in this work and their presence in symptomatic chestnut samples.

Pathogen	Distribution	Host plants	Reported on *Castanea*	Plant organs	Reference
Alternaria alternata	Cosmopolitan	Broad host range	Yes	Leaves, fruits	Ren et al. (2021), Cakar & Akilli (2023)
Botryosphaeria dothidea	Cosmopolitan	Broad host range	Yes	Bark, fruits	Akilli et al. (2009), Hamasaki et al. (2016)
Botrytis cinerea	Cosmopolitan	Broad host range	Yes	Fruits	Donis-González et al. (2016), Rodrigues et al. (2022)
Colletotrichum fioriniae	Cosmopolitan	Broad host range	Yes	Fruits	Cakar (2024)
Cryphonectria parasitica	Cosmopolitan	*Castanea* spp., *Quercus* spp., *Acer* spp., *Carpinus betulus*	Yes	Bark of stems and branches, fruits	Rigling & Prospero (2018), Cakar (2024)
Diaporthe amygdali	Cosmopolitan	Broad host range	Yes	Bark	Aghayeva et al. (2017)
Diaporthe eres	Cosmopolitan	Broad host range	Yes	Fruits, leaves	Ivić & Novak (2018), Jiang et al. (2011)
Diaporthe phaseolorum	Cosmopolitan	Broad host range	No	-	-
Diaporthe phillipsii	Europe	*Vaccinium corymbosum*	No	-	-
Diplodia corticola	Africa, America, Europe	*Quercus* spp., *Vitis vinifera*	No	-	-
Diplodia mutila	America, Europe	Broad host range	No	-	-
Diplodia sapinea	America, Asia, Europe	Broad host range	No	-	-
Diplodia seriata	Cosmopolitan	Broad host range	Yes	Branches and stems	Dar & Rai (2017)
Dothiorella iberica	Cosmopolitan	Broad host range	Yes	Branches	Batista et al. (2020)
Epicoccum nigrum	Cosmopolitan	Broad host range	Yes	Bark	Aghayeva et al. (2017)
Fusarium acuminatum	Cosmopolitan	Broad host range	Yes	Fruits	Rodrigues et al. (2022)
Fusarium avenaceum	Cosmopolitan	Broad host range	No	-	-
Fusarium culmorum	Cosmopolitan	Broad host range	Yes	Fruits	Donis-González et al. (2016)
Fusarium foetens	Cosmopolitan	Mainly *Begonia* × *Hiemalis*	No	-	-
Fusarium graminearum	Cosmopolitan	Broad host range	Yes	Fruits	Donis-González et al. (2016)
Fusarium lateritium	Cosmopolitan	Broad host range	No	-	-
Fusarium oxysporum	Cosmopolitan	Broad host range	Yes	Fruits	Rodrigues et al. (2022)
Fusarium proliferatum	Cosmopolitan	Broad host range	Yes	Fruits	Ivić & Novak (2018)
Fusarium sambucinum	Cosmopolitan	Broad host range	No	-	-
Fusarium solani	Cosmopolitan	Broad host range	Yes	Fruits	He et al. (2001)
Fusarium sporotrichioides	Cosmopolitan	Broad host range	No	-	-
Fusarium temperatum	Cosmopolitan	*Zea mays*	No	-	-
Fusarium torulosum	Cosmopolitan	Broad host range	No	-	-
Gnomoniopsis smithogilvyi	Cosmopolitan	*Castanea* spp., *Buxus sempervirens*, *Corylus avellana*, *Fraxinus ornus*, *Pinus pinaster*, *Quercus cerris*, *Quercus ilex*	Yes	Leaves, flowers, fruits, shoots, stems, branches	Lema et al. (2023)
Mucor hiemalis	Cosmopolitan	Broad host range	Yes	Fruits	Jermini et al. (2006)
Neofusicoccum eucalyptorum	Cosmopolitan	*Eucalyptus* spp. and other Myrtaceae	No	-	-
Neofusicoccum parvum	Cosmopolitan	Broad host range	Yes	Bark, stems, branches, fruits	Ciordia et al. (2022), Seddaiu et al. (2021)
Penicillium glabrum	Cosmopolitan	Broad host range	Yes	Fruits	Overy et al. (2003)
Pestalotiopsis paeoniicola	America, Asia	*Paeonia suffruticosa*, *Pouteria sapota*	No	-	-
Rhizopus microsporus	Cosmopolitan	*Oryza sativa*, *Zea mays*, *Helianthus annuus*, *Brassica juncea*	No	-	-
Rhizopus arhizus	Cosmpolitan	Broad host range	No	-	-
Schizophyllum commune	Cosmopolitan	Broad host range	Yes	Bark, stems, branches	Takemoto et al. (2010)

Discussion

The presence of *D. kuriphilus* is one of the greatest threats to chestnut trees, as it limits tree vigor and nut yields. Moreover, this wasp and its galls may play an important role in spreading fungal diseases. Both in our work and in previous studies, it has been observed that most isolated fungal species are phytopathogenic, thus providing evidence that galls can serve as a reservoir for plant pathogens as well as other tissues, mainly leaves, stems, shoots, or bark (Nicoletti et al. 2021).

As previously reported, according to the literature review, of the 53 species identified, 37 were found to be pathogenic (Tab. 3) and can be transmitted horizontally to other plant species by means of free-living or gall-inducing insects. Of these pathogenic fungi, 21 species were found damaging chestnut trees, mostly fruits (Tab. 3).

Among the pathogenic fungi causing significant damage to chestnut trees, *C. parasitica* and *G. smithogilvyi* are the most important. *C. parasitica* causes chestnut blight, a serious disease that forms perennial necrotic lesions on the bark of stems and branches, leading to their death (Rigling & Prospero 2018). This pathogen requires natural openings or wounds to penetrate the host tissue, so the galls produced by *D. kuriphilus* represent an entry point that could be related to the increased incidence of the disease in areas where both species are present (Meyer et al. 2015). However, in the present work, it has only been detected in one gall, probably due to the time of the year and the randomness of the sampling. This opens the possibility for conducting a detailed study of gall-associated fungi in trees with canker and proving whether the presence of *C. parasitica* in the galls could influence the incidence of the disease, as reported in previous studies. *G. smithogilvyi*, first described in 2012, is an emerging pathogen recently found in chestnuts trees in Galicia that significantly affects crop yield (Aguín et al. 2022). The most characteristic symptoms of this disease are the formation of cankers, leaf, flower, and gall necrosis, fruit mummification, and rot, both pre- and post-harvest, damaging nuts still present on the tree (Lema et al. 2023 – Fig. 4). In several studies, this fungus was found as an endophyte, being isolated from galls, shoots, leaves, stems, flowers and asymptomatic immature fruits, and producing symptoms in them once they mature (Aguín et al. 2022, Lema et al. 2023).

Among other phytopathogenic fungi, the genus *Fusarium* stands out due to the diversity of species present and the large number of isolates obtained. These fungi produce different types of degradative enzymes and entomopathogenic mycotoxins such as fumonisins (Nelson et al. 1993) and, in several studies, strains belonging to the *Fusarium incarnatum-equiseti* complex and the species *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg were isolated in necrotic galls produced by *D. kuriphilus*, which showed a high wasp mortality rate in laboratory tests (Addario & Turchetti 2011, Tosi et al. 2015). However, according to Cooper & Rieske (2010), the mortality of *D. kuriphilus* inside the galls could be related to the dryness and hardness of their necrotic tissues, which would make it difficult for adults to emerge and rule out a direct entomopathogenic effect. The genus *Fusarium* is not only associated with galls but it has also been detected as an endophyte in other chestnut tissues such as leaves, buds, stems, and fruits (Nicoletti et al. 2021). In terms of pathogenicity, according to previous studies, there is evidence of chestnut fruit damage on *F. acuminatum, F. culmorum, F. graminearum, F. oxysporum, F. proliferatum,* and *F. solani,* species identified in this study (Tab. 3).

Furthermore, eight species of the Botryosphaeriaceae family were identified, known to be important pathogens in Europe and worldwide, namely *Botryosphaeria dothidea, Diplodia corticola, D. mutila, D. sapinea, D. seriata, Dothiorella iberica, Neofusicoccum eucalyptorum* and *N. parvum* (Tab. 3). This family includes some of the most important emerging and invasive plant pathogens worldwide (Aiello et al. 2023, Batista et al. 2021). Many of these species were reported for the first time on chestnut and on the *D. kuriphilus* galls in the present work, but only 4 of them were found causing symptoms in chestnuts: *B. dothidea* in bark (Akilli et al. 2009) and fruits (Hamasaki et al. 2016), *D. seriata* in branches and stems (Dar & Rai 2017), *D. iberica* in branches (Batista et al. 2020), and *N. parvum* in bark, stems, branches (Ciordia et al. 2022) and fruits (Seddaiu et al. 2021).

On the other hand, fungal spoilage of commercially purchased chestnuts is a cause for concern as the fungal colonization may have occurred at any stage of flowering, harvesting, storage, sorting, or transport. It can cause great economic losses and poses a serious health risk to consumers due to their mycotoxins (Overy et al. 2003, Rodrigues et al. 2022). Among these fungi, those belonging to the genera *Aspergillus* and *Penicillium* stand out as mycotoxin producers, secondary metabolites showing toxic, mutagenic, and teratogenic effects, including potential immunosuppressive activity and carcinogenic effects, having a long-term chronic or cumulative effect on human health (Prencipe et al. 2018a, 2018b).

Beside the pathogenic species, it is worth highlighting the presence of fungi that can be used as biological control agents, such as *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (Jensen et al. 2022), and *Trichoderma* spp. (Hermosa et al. 2012). The latter is an important genus due to its diversity and abundance, including *T. atroviride* and *T. harzianum*, which are the main species found in this study. *Trichoderma* is a genus of fungi frequently associated with soils and the rhizosphere, though they also appear to colonize different parts of plants, such as leaves; that is, they are opportunistic symbionts (Harman et al. 2004). These fungi exhibit an antagonistic behavior against various pathogens, especially fungi, by inhibiting their growth through direct interactions, namely by antibiosis, competition, or mycoparasitism, or indirect ones, by increasing plant vigor and tolerance to stress (Hermosa et al. 2012, Harman et al. 2004). Various studies evaluated different species against important pathogens of *C. sativa*, such as *T. harzianum, T. parceramosum* Bissett and *T. viride* Persoon, which showed antagonistic and protective effects against *C. parasitica* (Akilli et al. 2011, Arisan-Atac et al. 1995) and *T. atroviride* against *G. smithogilvyi* (Pasche et al. 2016). Therefore, further studies need to be conducted with *Trichoderma* strains isolated from Galician chestnut trees and evaluate their antagonism against new threats, such as *G. smithogilvyi,* which is one of the most important diseases found in chestnut stands in Galicia.

Conclusions

This work is the first study of fungal communities in *D. kuriphilus* galls in Galicia. The presence of a wide diversity of fungi, both phytopathogenic and saprophytic, has been identified, and some genera and species were reported for the first time. Therefore, this study provides interesting results for managing *D. kuriphilus* at the local scale and comparing it with fungal communities in galls found in other regions. However, further research is needed to determine the role of *D. kuriphilus* in dispersing these fungi.

Acknowledgments

The authors would like to thank Carmela Menduíña, *Estación Fitolpatolóxica Areeiro*, Pontevedra, Spain, for her contribution to identifying the isolates, and Marisol Guerreiro Lemos, Fauna Útil S.L., Pontevedra, Spain, for taking part in the samplings.

The authors declare that part of the data included in this paper was communicated as a poster presented at the 8th Spanish Forestry Congress held in Lleida (Catalonia, Spain) from 27 June to 1 July 2022 and included in the proceedings (in Spanish) under the title "*Hongos asociados a agallas producidas por Dryocosmus kuriphilus en castaños de Galicia*" (https://8cfe.congresoforestal.es/sites/default/files/actas/8CFE-1077.pdf).

References

Addario E, Turchetti T (2011). Parasitic fungi on *Dryocosmus kuriphilus* in *Castanea sativa* necrotic galls. Bulletin of Insectology 64 (2): 269-273. [online] URL: http://www.bulletinofinsectology.org/pdfarticles/vol64-2011-269-273addario.pdf

Aghayeva DN, Rigling D, Meyer JB, Mustafabeyli E (2017). Diversity of fungi occurring in the bark of *Castanea sativa* in Azerbaijan. In: Proceedings of the “VI International Chestnut Symposium”. Samsun (Turkey) 9-13 Oct 2017. ISHS Acta Horticulturae 1220: 79-86. - doi: 10.17660/ActaHortic.2018.1220.12

Aguín O, Rial C, Piñón P, Sainz MJ, Mansilla JP, Salinero C (2022). First report of *Gnomoniopsis smithogilvyi* causing chestnut brown rot on nuts and burrs of sweet chestnut in Spain. Plant Disease 107(1): 218. - doi: 10.1094/PDIS-02-22-0322-PDN

Aiello D, Bregant C, Carlucci A, Guarnaccia V, Gusella G, Linaldeddu BT, Mugnai L, Raimondo ML, Polizzi G (2023). Current status of Botryosphaeriaceae species in Italy: impacts on agricultural crops and forest ecosystems. Phytopathologia Mediterranea 62 (3): 381-412. - doi: 10.36253/phyto-14711

Akilli S, Katircioglu YZ, Maden S (2009). Chestnut cankers in Black Sea region of Turkey. In: Proceedings of the “International Workshop on Chestnut Management in Mediterranean Countries - Problems and Prospects”. Bursa (Turkey) 23-25 Oct 2007. ISHS Acta Horticulturae 815: 247-252. - doi: 10.17660/ActaHortic.2009.815.32

Akilli S, Katircioglu YZ, Maden S (2011). Biological control of chestnut canker, caused by *Cryphonectria parasitica*, by antagonistic organisms and hypovirulent isolates. Turkish Journal of Agriculture and Forestry 35 (5): 515-523. - doi: 10.3906/tar-0912-579

Arisan-Atac I, Heidenreich E, Kubicek CP (1995). Randomly amplified polymorphic DNA fingerprinting identifies subgroups of Trichoderma viride and other *Trichoderma* sp. capable of chestnut blight biocontrol. FEMS Microbiology Letters 126 (3): 249-255. - doi: 10.1111/j.1574-6968.1995.tb07426.x

Batista E, Lopes A, Alves A (2020). Botryosphaeriaceae species on forest trees in Portugal: diversity, distribution and pathogenicity. European Journal of Plant Pathology 158 (3): 693-720. - doi: 10.1007/s10658-020-02112-8

Batista E, Lopes A, Alves A (2021). What do we know about Botryosphaeriaceae? An overview of a worldwide cured dataset. Forests 12 (3): 313. - doi: 10.3390/f12030313

Cakar D, Akilli S (2023). Role of the fungal flora on kernel rot of chestnuts. International Journal of Agriculture and Wildlife Science 9 (2): 143-152. - doi: 10.24180/ijaws.1252736

Cakar D (2024). Significance of *Gnomoniopsis smithogilvyi* as kernel rot of sweet chestnut in Turkey. Journal of Phytopathology 172 (2): e13293. - doi: 10.1111/jph.13293

Carbone I, Kohn LM (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91 (3): 553-556. - doi: 10.2307/3761358

Ciordia M, Loureiro MD, González AJ (2022). First report of *Neofusicoccum parvum* causing canker on *Castanea sativa* in Spain. Plant Disease 106(4): 1299. - doi: 10.1094/PDIS-06-21-1231-PDN

Cooper WR, Rieske LK (2010). Gall structure affects ecological associations of *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae). Environmental Entomology 39 (3): 787-797. - doi: 10.1603/EN09382

Crous PW, Groenewald JZ, Risède JM, Simoneau P, Hywel-Jones NL (2004). *Calonectria* species and their *Cylindrocladium* anamorphs: species with sphaeropedunculate vesicles. Studies in Mycology 50: 415-430. - doi: 10.3114/sim.55.1.213

Dar M, Rai M (2017). First report of *Diplodia seriata* causing canker on *Castanea sativa* in India. New Disease Reports 35: 19. - doi: 10.5197/j.2044-0588.2017.035.019

Donis-González IR, Guyer DE, Fulbright DW (2016). Quantification and identification of microorganisms found on shell and kernel of fresh edible chestnuts in Michigan. Journal of the Science of Food and Agriculture 96 (13): 4514-4522. - doi: 10.1002/jsfa.7667

EFSA (2010). Risk assessment of the oriental chestnut gall wasp, *Dryocosmus kuriphilus* for the EU territory and identification and evaluation of risk management options. EFSA Journal 8(6): 1619. - doi: 10.2903/j.efsa.2010.1619

EPPO (2021). EPPO datasheets on pests recommended for regulation: *Dryocosmus kuriphilus*. [online] URL: http://gd.eppo.int/taxon/drycku/datasheet

FAO (2023). FAOSTAT online database: crops and livestock products. FAO-UN, Rome, Italy, website. [online] URL: http://www.fao.org/faostat/es/

García-Figueres F, Torres E, Luque J, Martos S (2004). Evaluación de la especificidad de los cebadores para la detección de hongos fitopatógenos de madera de vid [Evaluation of the specificity of primers for the detection of phytopathogenic fungi of grapevine wood]. In: Proceedings of the “XII Congreso de La Sociedad Española de Fitopatología”. Lloret de Mar (Cataluña, Spain) 26 Sept-1 Oct 2004, pp. 134. [in Spanish]

Gardes M, Bruns TD (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology 2 (2): 113-118. - doi: 10.1111/j.1365-294X.1993.tb00005.x

Geiser DM, Del Mar Jiménez-Gasco M, Kang S, Makalowska I, Veeraraghavan N, Ward TJ, Zhang N, Kuldau GA, Donnell K (2004). FUSARIUM-ID v. 1.0: a DNA sequence database for identifying Fusarium. European Journal of Plant Pathology 110 (5): 473-479. - doi: 10.1023/B:EJPP.0000032386.75915.a0

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61 (4): 1323-1330. - doi: 10.1128/aem.61.4.1323-1330.1995

Hamasaki K, Kawaradani M, Shibao M (2016). Control of the chestnut weevil, *Curculio sikkimensis* (Heller), and the black rot fungus, *Botryosphaeria dothidea* (Mougeot) Cesati and De Notaris, using hot water treatment. Annual Report of the Kansai Plant Protection Society 58: 51-55. - doi: 10.4165/kapps.58.51

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004). *Trichoderma* species - opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2 (1): 43-56. - doi: 10.1038/nrmicro797

He W, Shen R, Wang X (2001). Pathogenicity of pathogens contributing to dry rot of Chinese chestnut and their infection process. Journal of Beijing Forestry University 23 (2): 36-39. - doi: 10.5555/20013164745

Hermosa R, Viterbo A, Chet I, Monte E (2012). Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology 158 (1): 17-25. - doi: 10.1099/mic.0.052274-0

Ivić D, Novak A (2018). Fungi associated with nut rot in sweet chestnut, with the first record of *Gnomoniopsis smithogilvyi* in Croatia. Pomologia Croatica: Glasilo Hrvatskog Agronomskog Društva 22 (1-2): 13-22. - doi: 10.33128/pc.22.1-2.2

Jensen DF, Dubey M, Jensen B, Karlsson M (2022). *Clonostachys rosea* to control plant diseases. In: “Microbial bioprotectants for plant disease management” (Köhl J, Ravensberg W eds). Burleigh Dodds Science Publishing, Cambridge, UK, pp. 44. [online] URL: http://library.oapen.org/handle/20.500.12657/61518

Jermini M, Conedera M, Sieber TN, Sassella A, Schärer H, Jelmini G, Höhn E (2006). Influence of fruit treatments on perishability during cold storage of sweet chestnuts. Journal of the Science of Food and Agriculture 86 (6): 877-885. - doi: 10.1002/jsfa.2428

Jiang S, Liu C, Wang Q, Jia N, Li C, Ma H (2011). A new chestnut disease-brown margin leaf blight and the pathogen identification. Scientia Silvae Sinicae 47 (5): 177-180. - doi: 10.11707/j.1001-7488.20110530

Jiang N, Voglmayr H, Bian DR, Piao CG, Wang SK, Li Y (2021). Morphology and phylogeny of *Gnomoniopsis* (Gnomoniaceae, Diaporthales) from fagaceae leaves in China. Journal of Fungi 7(10): 792. - doi: 10.3390/jof7100792

Lema F, Baptista P, Oliveira C, Ramalhosa E (2023). Brown rot caused by *Gnomoniopsis smithogilvyi* (syn. *Gnomoniopsis castaneae*) at the level of the chestnut tree (*Castanea sativa* Mill.). Applied Sciences 13(6): 3969. - doi: 10.3390/app13063969

Meyer JB, Gallien L, Prospero S (2015). Interaction between two invasive organisms on the European chestnut: does the chestnut blight fungus benefit from the presence of the gall wasp? FEMS Microbiology Ecology 91(11): fiv122. - doi: 10.1093/femsec/fiv122

Nelson PE, Desjardins AE, Plattner RD (1993). Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. Annual Review of Phytopathology 31 (1): 233-252. - doi: 10.1146/annurev.py.31.090193.001313

Nicoletti R, Beccaro GL, Sekara A, Cirillo C, Di Vaio C (2021). Endophytic fungi and ecological fitness of chestnuts. Plants 10 (3): 542. - doi: 10.3390/plants10030542

O’Donnell K, Cigelnik E (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7 (1): 103-116. - doi: 10.1006/mpev.1996.0376

Overy DP, Seifert KA, Savard ME, Frisvad JC (2003). Spoilage fungi and their mycotoxins in commercially marketed chestnuts. International Journal of Food Microbiology 88 (1): 69-77. - doi: 10.1016/S0168-1605(03)00086-2

Pasche S, Crovadore J, Pelleteret P, Jermini M, Mauch-Mani B, Oszako T, Lefort F (2016). Biological control of the latent pathogen *Gnomoniopsis smithogilvyi* in European chestnut grafting scions using *Bacillus amyloliquefaciens* and *Trichoderma atroviride*. Dendrobiology 75: 113-122. - doi: 10.12657/denbio.075.011

Pérez-Otero R, Mansilla JP (2014). El cinípido del castaño *Dryocosmus kuriphilus* Yasumatsu, 1951 llega a Galicia (NO de la Península Ibérica) [The chestnut cinipid *Dryocosmus kuriphilus* Yasumatsu, 1951 arrives in Galicia (NW Iberian Peninsula)]. Arquivos Entomolóxicos 12: 33-36. [in Spanish] [online] URL: http://dialnet.unirioja.es/servlet/articulo?codigo=6391081

Prencipe S, Siciliano I, Contessa C, Botta R, Garibaldi A, Gullino ML, Spadaro D (2018a). Characterization of *Aspergillus* section Flavi isolated from fresh chestnuts and along the chestnut flour process. Food Microbiology 69: 159-169. - doi: 10.1016/j.fm.2017.08.004

Prencipe S, Siciliano I, Gatti C, Garibaldi A, Gullino ML, Botta R, Spadaro D (2018b). Several species of *Penicillium* isolated from chestnut flour processing are pathogenic on fresh chestnuts and produce mycotoxins. Food Microbiology 76: 396-404. - doi: 10.1016/j.fm.2018.07.003

Ren F, Dong W, Shi SQ, Wang HH, Dou GM, Yan DH (2021). Primary study on causes and associated pathogens for chestnut leaf scorch. Forest Research 34 (2): 185-192. - doi: 10.13275/j.cnki.lykxyj.2021.02.021

Rigling D, Prospero S (2018). Cryphonectria parasitica, the causal agent of chestnut blight: invasion history, population biology and disease control. Molecular Plant Pathology 19 (1): 7-20. - doi: 10.1111/mpp.12542

Rodrigues P, Driss JO, Gomes-Laranjo J, Sampaio A (2022). Impact of cultivar, processing and storage on the mycobiota of European chestnut fruits. Agriculture 12 (11): 1930. - doi: 10.3390/agriculture12111930

Seddaiu S, Mello A, Sechi C, Cerboneschi A, Linaldeddu BT (2021). First report of *Neofusicoccum parvum* associated with chestnut nut rot in Italy. Plant Disease 105 (11): 3743. - doi: 10.1094/PDIS-01-21-0072-PDN

Takemoto S, Nakamura H, Imamura Y, Shimane T (2010). *Schizophyllum commune* as a ubiquitous plant parasite. Japan Agricultural Research Quarterly 44 (4): 357-364. - doi: 10.6090/jarq.44.357

Tosi L, Beccari G, Rondoni G, Covarelli L, Ricci C (2015). Natural occurrence of *Fusarium proliferatum* on chestnut in Italy and its potential entomopathogenicity against the Asian chestnut gall wasp *Dryocosmus kuriphilus*. Journal of Pest Science 88: 369-381. - doi: 10.1007/s10340-014-0624-0

White TJ, Bruns T, Lee SJWT, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: "PCR protocols: a guide to methods and applications" (Innis MA, Gelfand DH, Sninsky JJ, White TJ eds). Academic Press, London, UK, pp. 315-322. - doi: 10.1016/B978-0-12-372180-8.50042-1

Supplementary Material

Tab. S1 - Taxonomic classification, relative abundance, GenBank Accession Number, and previous references of its association with *D. kuriphilus* galls of the fungal species identified.

Link: Fraga_4559@suppl001.pdf