UNIVERSIDAD DE LEÓN

ESCUELA SUPERIOR Y TÉCNICA DE INGENIERÍA AGRARIA

Departamento de Ingeniería y Ciencias Agrarias

Tesis Doctoral

Chancro en castaño: hongos implicados en la enfermedad y estrategias de control de *Cryphonectria parasitica*

Estefanía Trapiello Vázquez

2017

Este trabajo ha sido realizado en:

- **Servicio Regional de Investigación y Desarrollo Agroalimentario SERIDA. Villaviciosa, Asturias (España).**

- **Swiss Federal Institute for Forest, Snow and Landscape WSL. Birmensdorf (Suiza).**

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SERIDA
Servicio Regional de Investigación y Desarrollo Agroalimentario

INFORME DE LA DIRECTORA DE LA TESIS

La Dra. Ana J. González Fernández, Responsable del Programa de Investigación en Patología Vegetal del Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA).

INFORMA:

Que la Licenciada en Ciencias Ambientales Dña Estefanía Trapiello Vázquez, ha realizado bajo su dirección el trabajo que presenta para optar al Grado de Doctor con el título "Chancro en castaño: hongos implicados en la enfermedad y estrategias de control de *Cryphonectria parasitica*"; y autoriza la presentación de dicho trabajo como Tesis Doctoral ante la Comisión correspondiente, dado que reúne las condiciones necesarias para su defensa.

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REPORT FOR THE INTERNATIONAL DOCTORATE MENTION

Nombre de la examinadora / Name of the examiner
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Categoría académica o profesional / Academic or professional rank
PhD

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Dept. Forest Dynamics Group Swiss Forest Protection	Swiss Federal Institute for Forest, Snow and Landscape Research WSL 8903 Birmensdorf Switzerland

Título de la tesis presentada / Title of the thesis
"Chancro en castaño: hongos implicados en la enfermedad y estrategias de control de *Cryphonectria parasitica* en Asturias"

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INFORME RAZONADO / REASONED REPORT

SOBRE LA CALIDAD CIENTÍFICA DE LA TESIS DOCTORAL / ABOUT THE SCIENTIFIC QUALITY OF PhD

In the PhD thesis entitled 'Chancro en castaño: hongos implicados en la enfermedad y estrategias de control de *Cryphonectria parasitica* en Asturias", the authors studied canker-causing fungi on chestnut trees (*Castanea sativa*) from Asturias (Spain). Moreover they determined biological control strategies against the pathogenic fungus *C. parasitica*.

The work presented is original and valuable to the community of plant pathologists and has excellent scientific and technical merit and accurate planning methodology. Moreover the work peeked in three valuable publications in scientific journals, one of them in a high-ranking journal.

All parts (introduction, materials and methods, results and discussion) are well structured and supported by recent and important bibliography. Interpretations and conclusions are supported by respective results.

General statement: The work meets all the requirements that are considered necessary for a dissertation. The research presented in this dissertation provides results that enable significant progress on the objective of the project. Several novel results are of considerable interest. For example – the reallocation of *Diplodina castanea* to the genus *Sirococcus* and the first report of this fungus in Spain; the finding of hypoviruent isolates in Asturias, especially the hypovirus CHV-1-subtype D (just reported in Germany until this thesis); the finding of an agrochemical product which applied under managed conditions could help to reduce the spread of *C. parasitica*; and the first report of *Gnomoniopsis castaneae* in Spain.

In my opinion, the thesis shows Miss Estefanla Trapiello Vãzquez outstanding qualification as a researcher and her ability to communicate her research findings, therefore she should be awarded the degree of International PhD after carrying out the viva.

Yours faithfully,

Dr. Joana Meyer

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Informo que la calidad científica de esta tesis doctoral es merecedora de obtener la mención Doctor Internacional I inform that the scientific quality of this thesis is worthy of obtaining the International Doctor Mention
Favorable ☒ No favorable / Not favorable ☐

FECHA / DATE: 24/05/2017

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INFORME DE MENCIÓN DOCTOR INTERNACIONAL
REPORT FOR THE INTERNATIONAL DOCTORATE MENTION

Nombre de la examinadora / Name of the examiner
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Categoría académica o profesional / Academic or professional rank
PhD

Departamento / Department	Organismo y país / Institution and country
Dept. Biodiversity and Conservation Group Phytopathology	Swiss Federal Research Institute WSL 8903 Birmensdorf – Switzerland

Título de la tesis presentada / Title of the thesis
"Chancro en castaño: hongos implicados en la enfermedad y estrategias de control de *Cryphonectria parasitica* en Asturias"

Nombre y apellidos del doctorando / Name of the candidate
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INFORME RAZONADO / REASONED REPORT

SOBRE LA CALIDAD CIENTÍFICA DE LA TESIS DOCTORAL / ABOUT THE SCIENTIFIC QUALITY OF PhD

Dear Sir or Madam,

The PhD thesis entitled 'Chancro en castaño: hongos implicados en la enfermedad y estrategias de control de Crypitonectria parasitica en Asturias" deals with chestnut pathogenic fungi involved in cankers formation and shows control strategies for the chestnut blight disease. The candidate presents in this work a well-defined and clearly formulated scientific problem.

First, an introduction with a literature review written in Spanish is presented, which summarizes relevant and actual bibliography, including general aspects about the studied fungi (Cryphonectria parasitica, Diplodina castaneae and Gnomoniopis castaneae) and control methods of the most devastating one (C. parasitica) in northern Spain, Asturias.

The material and methods section is brief and concise, being the methodology used adequate and well explained. Results and discussion are well written using a properly scientific style.
Moreover, results of this thesis have produced four scientific articles, which are included in the work. Two of them have been published and one has been accepted—all in indexed journals. The last one being outstanding of sending to an indexed journal. All these papers clearly support the quality of this thesis.

Finally, the conclusions are well written in Spanish and in English, being relevant and responding correctly to the objectives outlined.

General statement: The research presented in this thesis provides results that enable significant progress on the objective of the project. Precise subjects of research in each chapter are interesting and well-posed. Methodology is updated and experimental work is properly designed. These results are relevant and the articles should be of high impact.

In my opinion, the thesis demonstrates enough merits and Miss EstefanIa Trapiello Vãzquez has shown ability in carrying out relevant experiments with correct methodology and well written, therefore she should be awarded the degree of International PhD after carrying out the viva.

Yours faithfully,

Dr. Carolina Comejo

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Informo que la calidad científica de esta tesis doctoral es merecedora de obtener la mención Doctor Internacional I inform that the scientific quality of this thesis is worthy of obtaining the International Doctor Mention
Favorable ☒ No favorable / Not favorable ☐

FECHA / DATE: 23 / 05 / 2017

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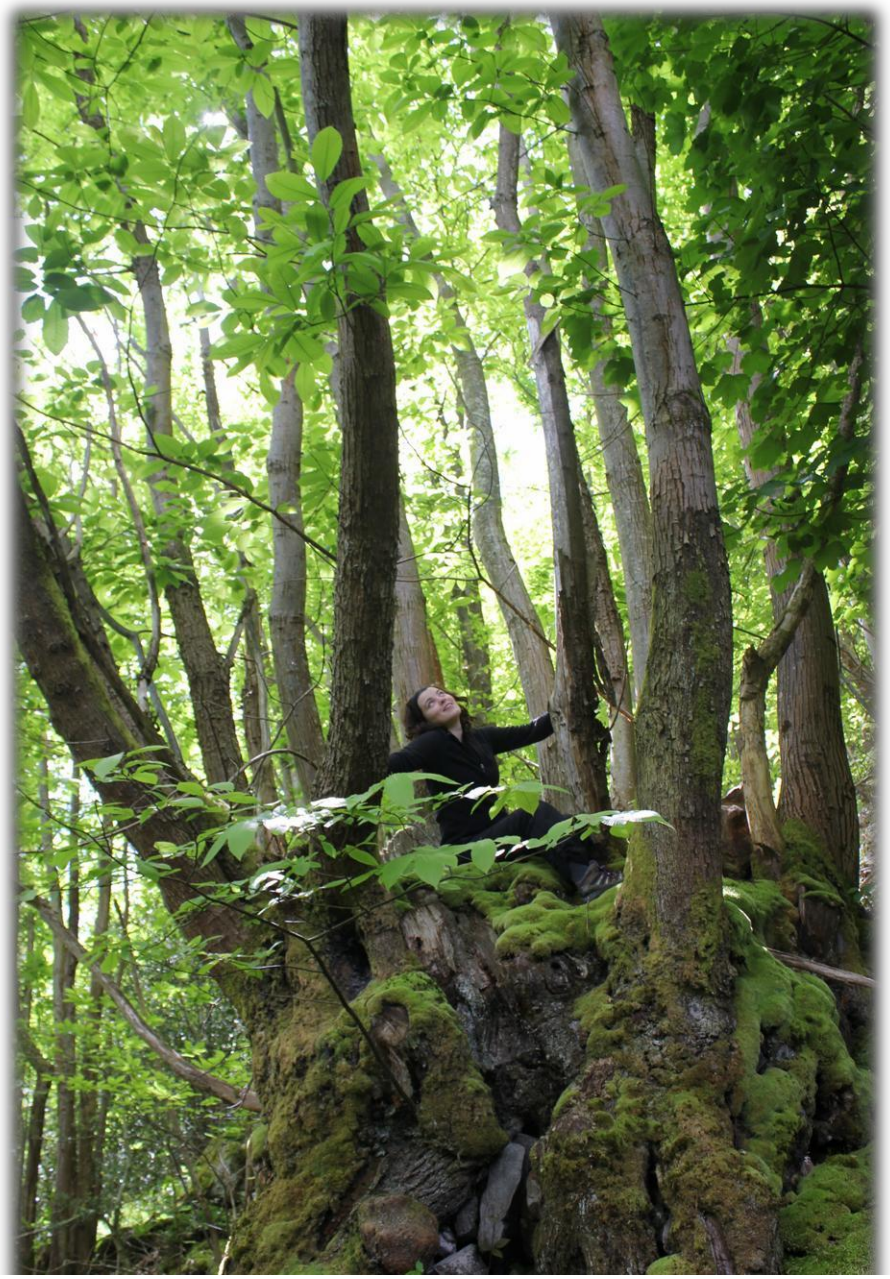

A mis raíces,

a mi tronco,

y a mi fruto.

AGRADECIMIENTOS

Me gustaría manifestar mi agradecimiento a todas las personas que, de alguna manera, me han aportado algo durante el transcurso de este trabajo.

A la directora de esta tesis, Ana J. González, por brindarme la oportunidad de llevarla a cabo, por su confianza, por su ayuda, por sus consejos y por lo que he aprendido.

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A Ramón Antonio Juste, Gerente del SERIDA, y a Carmen Díez, Jefa del Departamento de Investigación, por apoyar la realización del presente trabajo. También a Koldo Osoro, anterior Gerente, por creer en esta tesis y apoyarla desde sus inicios.

A mis queridas compañeras, Luisa y Maite, por su ayuda, consejos y por los buenos momentos compartidos. A otras compañeras que han pasado por el laboratorio de Fitopatología, por los buenos recuerdos. Gracias, porque de todas he aprendido algo.

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A Daniel Rigling, por acogerme y hacerme sentir una más de su equipo, por su trato, por su ayuda, y por hacer que mi estancia en su laboratorio marcara la diferencia en este trabajo. Al resto de compañeros/as del WSL, en especial a Hélène Blauenstein, Nora Borst, Carolina Cornejo, Esther Jung, Joana Meyer y Simone Prospero, por todo lo que me enseñaron, por el buen ambiente de trabajo y por hacer de mi estancia en Suiza un tiempo inolvidable cargado de buenas experiencias.

A Paula Zamora, por sus consejos y por compartir su trabajo conmigo.

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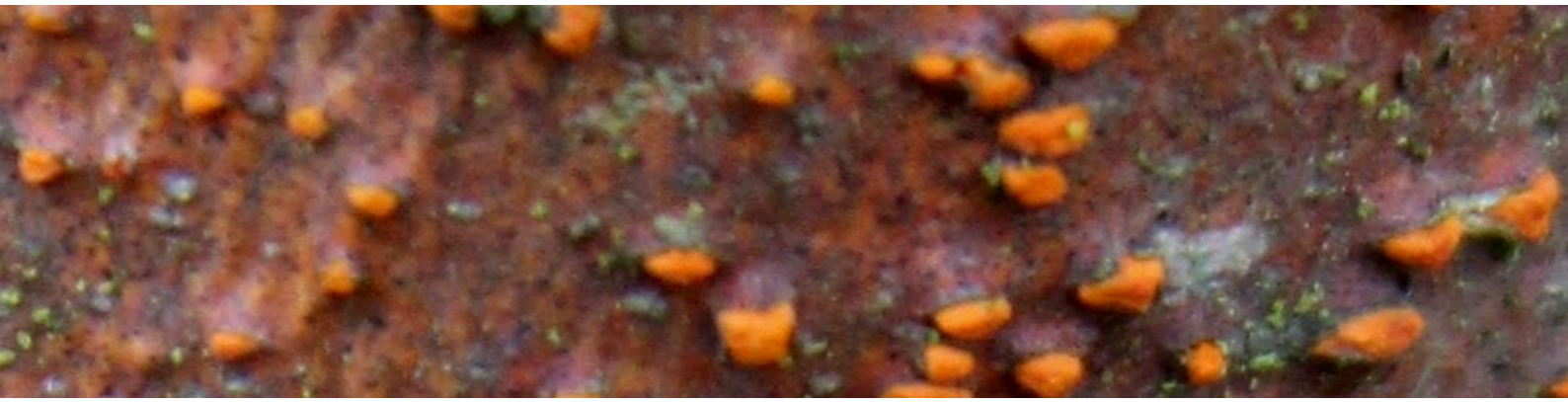
A mi familia, por enseñarme lo que no viene en los libros. En especial: a mi güela, porque con ella aprendí a vivir de la naturaleza, siempre te llevo en mi corazón. A mi güelu, por ayudarme a entender el valor de los castaños. A mis padres, porque sin ellos nada habría sido posible. A mis prim@s, especialmente a Indira y Sergio, porque sí. A Lacho, mi esposo, por su ayuda, paciencia, por amar las plantas y por estar siempre conmigo. A Asur, mi hijo, por llegar en el mejor momento para ser mi motor, por regalarme su tiempo, tan necesario para finalizar este trabajo, y por sus sonrisas, la energía que me ha motivado cada día para llegar hasta aquí.

Y no puedo olvidar a quienes han cuidado de los montes y/o aún lo siguen haciendo, porque gracias a su esfuerzo los castañares asturianos siguen existiendo.

"Si supiera que el mundo se acaba mañana, yo, hoy todavía, plantaría un árbol"

- Martin Luther King -



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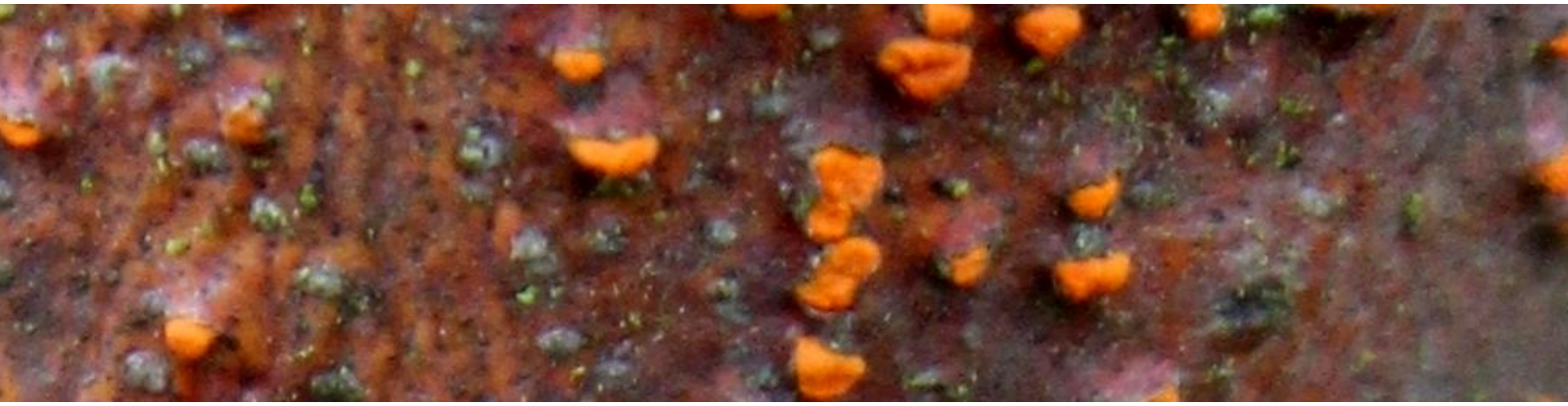

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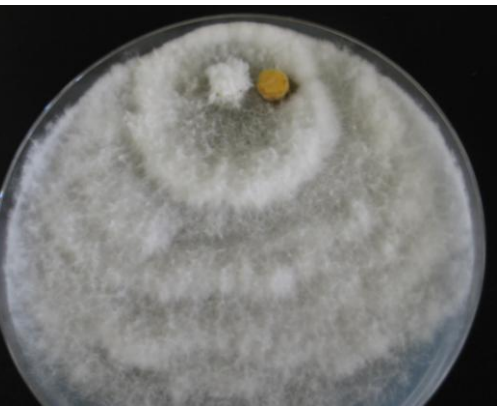


RESUMEN



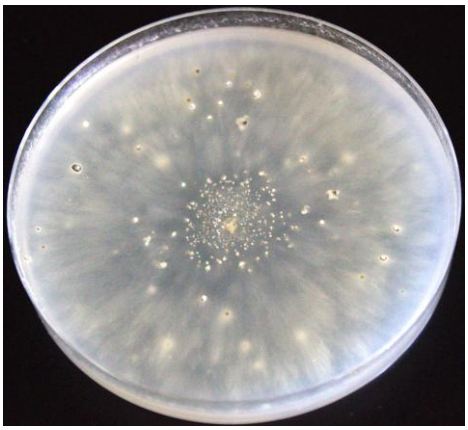
RESUMEN

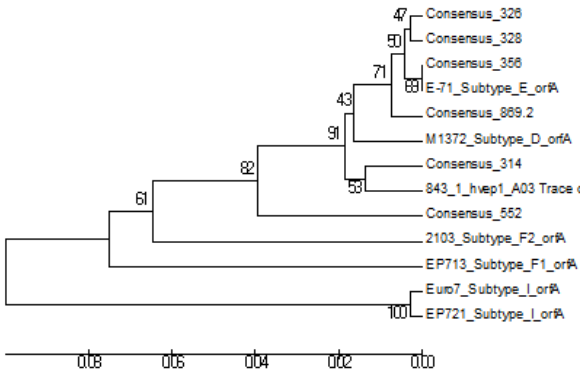
El castaño europeo (*Castanea sativa*), especie de gran importancia ecológica y socio-económica, se encuentra muy afectado por la enfermedad del chancro. Tras la detección en 1947 en España de su principal agente causal, el hongo *Cryphonectria parasitica*, se ha observado en distintas zonas del país. En Asturias se detectó en 1982, y desde entonces se ha dispersado rápidamente diezmando los castañares asturianos.

Experiencias previas de control de la enfermedad en Europa, se han basado en la lucha biológica con cepas hipovirulentas (hvs), las cuales contienen un virus (*Cryphonectria* hypovirus CHV-1) que atenúa la virulencia del hongo. De castaños asturianos con chancros cicatrizados o en cicatrización, se aislaron cepas de *C. parasitica* que, según criterios morfológicos, se clasificaron como potencialmente virulentas e hvs. Tras su caracterización molecular, se confirmó la infección del hipovirus CHV-1 en las potencialmente hvs, del que se identificaron dos subtipos, E y D. La idoneidad como agente de control biológico del subtipo D, descrito sólo en Alemania, es conocida; mientras que la del subtipo E, ya descrito en España, se desconoce. La presencia de cepas hvs compatibles con los tipos de compatibilidad vegetativa (cv) dominantes y la baja diversidad de tipos de cv en la región, favorece la aplicación de control biológico, por lo que se podrían iniciar tratamientos con CHV-1-D, mientras se debería investigar la eficiencia de CHV-1-E.

Por otro lado, el control químico no se ha considerado una alternativa viable debido a su impacto ambiental y a la falta de tratamientos autorizados. Sin embargo, se ha observado que el producto agroquímico epoxiconazol tiene cierta eficacia frente a *C. parasitica* y podría ser útil en determinadas situaciones como la producción de planta en vivero. Por tanto, no se descarta que el control químico bajo condiciones controladas pueda formar parte de la estrategia terapéutica integrada de la enfermedad.

Además de *C. parasitica*, existen otros dos hongos patógenos que causan chancros en castaño, *Gnomoniopsis castaneae* y *Sirococcus castaneae*. En algunas muestras de chancros, junto con *C. parasitica*, se aisló *Diplodina castaneae* que fue caracterizada morfológica y molecularmente, y reclasificada taxonómicamente como *S. castaneae*. En plantas con síntomas similares también se aisló *G. castaneae*. El hallazgo de ambas especies en Asturias, supone su primera detección en España. Se desconoce si son de nueva introducción o si pueden haber pasado desapercibidas y junto con *C. parasitica* ser responsables de la enfermedad del chancro en nuestra región y/o país.

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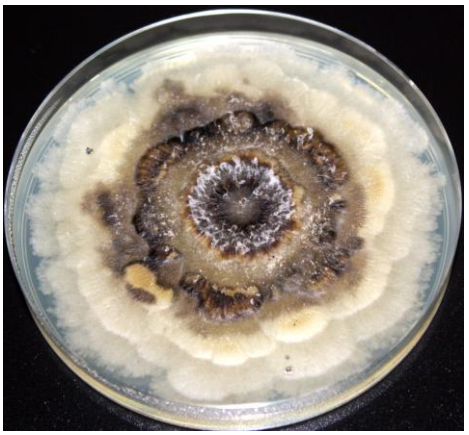
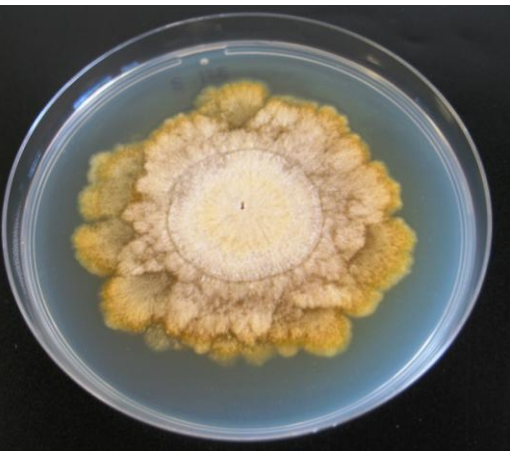
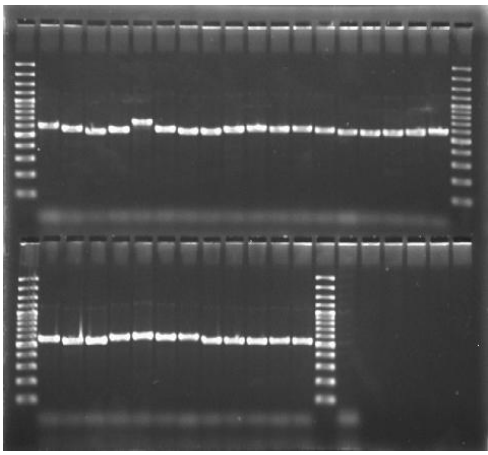


SUMMARY





SUMMARY

The European chestnut (*Castanea sativa*), an ecologically and socioeconomically important species, is being seriously damaged by chestnut blight disease. Since the detection of its main causal agent, the fungus *Cryphonectria parasitica*, in 1947 in Spain it has been observed in different areas of the country. In Asturias it was detected in 1982, and since then it has spread rapidly, declining the Asturian chestnut stands.

Previous experiences of disease control in Europe, were based on biological control using hypovirulent fungal isolates which contain the virus *Cryphonectria* hypovirus CHV-1 that attenuates the fungal virulence. From Asturian chestnuts with healing or healed chestnut cankers sampled, *C. parasitica* isolates were obtained and classified as potentially hypovirus-free or hypovirus-infected according to morphological criteria. After their molecular characterization, the CHV-1 hypovirus infection was verified in all potentially hypovirus-infected isolates, and two subtypes (E and D) were indentified. The ecological fitness of subtype D, only reported in Germany, as biological control agent, is known, whereas the suitability of subtype E, already reported in Spain, is unknown. The presence of hypovirulent isolates compatible with the dominant types of vegetative compatibility (vc) and the low diversity of vc types in this region, favours the application of biological control. Treatments with CHV-1-D could therefore be initiated, whereas the CHV-1-E efficiency should be researched.

On the other hand, the chemical control has not been considered a viable alternative due to its environmental impact and the shortage of authorized treatments. Nevertheless it was observed that the agrochemical epoxiconazol has a certain effectiveness against *C. parasitica* so it could be useful in particular situations such as in the production of plants in nurseries. Thus it is not rejected that the chemical control, under managed conditions, could be part of the integrated therapeutic strategy against the disease.

Besides *C. parasitica*, there are two pathogenic fungi which produce cankers on chestnut trees, *Sirococcus castaneae* and *Gnomoniopsis castaneae*. From some cankers, together with *C. parasitica*, *Diplodina castaneae* was isolated and was morphologically and molecularly characterized, and then taxonomically reclassified as *S. castaneae*. In chestnut plants with similar symptoms, *G. castaneae* was also isolated. The finding of both species in Asturias means their first detection in Spain. It is unknown if they are a new introduction or if they could have previously been unnoticed and together with *C. parasitica* be responsible for the chestnut blight disease in our region and/or country.

LISTADO

DE ARTÍCULOS

LISTADO DE ARTÍCULOS

Los resultados obtenidos a partir de los objetivos planteados en esta Tesis Doctoral han dado lugar o son parte de los siguientes artículos científicos:

I. Trapiello E., Rigling D. y González A.J. (2017). Occurrence of hypovirus-infected *Cryphonectria parasitica* isolates in northern Spain: an encouraging situation for biological control of chestnut blight in Asturian forests. *European Journal of Plant Pathology*, DOI 10.1007/s10658-017-1199-4. **(Objetivos 1, 2 y 3)**

II. Trapiello E., González-Varela G. y González A.J. (2015). Chestnut blight control by agrochemicals in *Castanea sativa* under managed conditions. *Journal of Plant Diseases and Protection*, 122, 120–124. **(Objetivo 4)**

III. Meyer J.[*], Trapiello E.[*], Senn-Irlet B., Sieber T.N., Cornejo C., Aghayeva D., González A.J. y Prospero S. (2017). Phylogenetic and phenotypic characterization of *Sirococcus castaneae* comb. nov. (synonym *Diplodina castaneae*), a fungal endophyte of European chestnut. *Fungal Biology*, DOI: 10.1016/j.funbio.2017.04.001.
[*] Ambas autoras contribuyeron por igual al artículo. **(Objetivo 5)**

IV. Trapiello E., Feito I. y González A.J. (2017). First report of the fungus *Gnomoniopis castaneae*, a chestnut canker agent, in Spain. (Elaborado para enviar a *Plant Disease*). **(Objetivo 5)**

En el texto se hará referencia a cada artículo con su número correspondiente (I- IV).

INTRODUCCIÓN

El castaño

El castaño europeo (*Castanea sativa* Mill.), con una extensión de más de 80.000 ha en el Principado de Asturias (Anónimo 2013), es considerado uno de los árboles más representativos de la flora asturiana. Esta frondosa constituye un elemento natural, social y paisajístico, clave para la biodiversidad de la región. Cultivado desde la antigüedad para aprovechar su madera y su fruto, la mayor parte de los ejemplares presentes en territorio asturiano corresponden a plantaciones (Ballesteros y Benito 2006). Sin embargo, hoy en día, su cultivo y aprovechamiento se encuentran en declive (Figura 1) por diversos factores, siendo una de las principales causas el estado sanitario en el que se encuentran.

Figura 1. Masa de castaños o "castañeu", tradicionalmente manejado y actualmente abandonado en el concejo de Aller, Asturias.

El chancro del castaño y su agente causal

La principal enfermedad de los castañares asturianos es la conocida como chancro del castaño, provocada por el hongo ***Cryphonectria parasitica*** (Murr.) Barr. (Syn. *Endothia parasitica* (Murr.) And. & And.) (Figura 2). Este ascomiceto es considerado uno de los patógenos más agresivos que afecta a castaño (*Castanea spp.*), ya que desde su aparición en la Península Ibérica, la producción de esta especie forestal, tanto de madera como de fruto, se ha visto mermada drásticamente en calidad y cantidad (Ana-Magán *et al.* 1993 en Homs 2001).

Figura 2. A la izquierda, *Cryphonectria parasitica* en placa de APD. A la derecha, síntomas de chancro en castaño.

El micelio del hongo atraviesa la corteza e invade su parte interior (floema, cambium y xilema) destruyendo los tejidos de transporte y crecimiento del árbol, y provocando así la marchitez de las partes vegetales situadas por encima (Figura 3). En la corteza, como consecuencia de la reacción de la planta frente a la desecación de los tejidos infectados por el hongo, se originan áreas algo deprimidas con coloraciones rojizo-anaranjadas que se van agrietando, formando el típico síntoma de chancro que da nombre a la enfermedad (Figura 2).

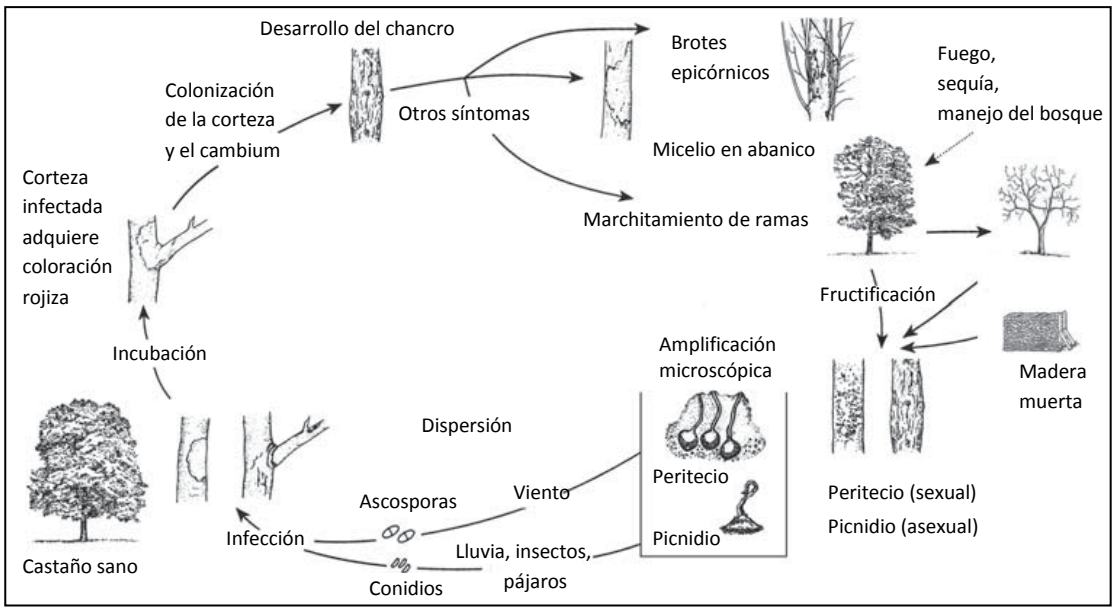


Figura 3. Ciclo de vida de *Cryphonectria parasitica*, el hongo responsable del chancro del castaño (Adaptado de Prospero y Rigling 2013).

Con frecuencia, se pueden observar en estas zonas de la corteza unas pústulas de color anaranjado donde se encuentran embebidas las estructuras de reproducción asexual y sexual del hongo, picnidios y peritecios, respectivamente. Bajo la corteza, se puede apreciar el crecimiento del micelio del hongo en forma de abanico (Heiniger y Rigling 1994) (Figura 4).

Figura 4. A la izquierda, corteza de castaño con cuerpos de fructificación. En el centro, detalle de los mismos. A la derecha, micelio característico del hongo *C. parasitica* en el interior de la corteza.

Los chancros pueden crecer rápidamente, rodeando y estrangulando el tallo o rama, y provocando así la muerte de las partes distales de la planta. Los síntomas que se observan son la presencia de ramas con hojas secas que no se caen (Figura 5). Cuando el follaje presenta estos síntomas, en las ramas pueden aparecer chancros elipsoidales hundidos con la madera expuesta en el centro (García-Benavides y Monte 2005).

Figura 5. Ramas secas de castaño con hojas y erizos que se mantienen en el árbol.

Además, es común observar bajo los chancros la proliferación de numerosos brotes epicórnicos (Anagnostakis 1987), como posible respuesta de supervivencia del árbol

frente a la infección. Una vez infectado por el hongo, el árbol se debilitará notablemente, e incluso, llegará a morir (Figura 6).

Figura 6. Castaños infectados por *Cryphonectria parasitica*. En primer plano, ejemplar con ramas y hojas secas. En segundo plano, árbol totalmente seco. (Foto tomada en el concejo de Llanera, Asturias).

Los síntomas en el castaño europeo se manifiestan de manera distinta dependiendo de la edad del individuo afectado y de la virulencia de la cepa responsable de la infección (Prospero y Rigling 2013).

El ascomiceto *C. parasitica*, introducido desde el este de Asia, fue observado por primera vez en Norteamérica en 1904 (Merkel 1905) dónde ha provocado la devastación del castaño americano (*Castanea dentata* [Marsh.] Borkh) (Anagnostakis 1988). En Europa, el hongo fue detectado por primera vez en Italia en 1938 (Biraghi 1946), y desde entonces, se ha extendido rápidamente por la mayor parte del área europea productora de castaño (Robin y Heiniger 2001). En España se observó por primera vez en 1947, concretamente en Vizcaya (Elorrieta 1949), y años más tarde, ya se había extendido infectando masas de castaño por todo el norte peninsular (Ana-Magán 1984). La primera cita en Asturias es de 1982, en 2000 ya estaba presente en 60 concejos (Valdezate *et al.* 2001) y en 2007 se encontraba en 69 de los 78 concejos de la región (González-Varela *et al.* 2011).

Hipovirulencia

Mientras que el castaño americano ha estado cerca de extinguirse por la epidemia del chancro, el castaño europeo se ha recuperado espontáneamente en muchas zonas debido a la aparición de un fenómeno natural: la hipovirulencia. Este fenómeno se debe a la infección de *C. parasitica* por hipovirus, que causan la reducción de la virulencia del hongo y su capacidad reproductiva, principalmente de la reproducción sexual (Gobbin *et al.* 2003).

La hipovirulencia fue descrita por primera vez por Grente (1965), y desde entonces, ha sido y es, objeto de diversos estudios (Griffin 1986, Heiniger y Rigling 1994, MacDonald y Fullbright 1991, Milgroom y Cortesi 2004, Nuss 1992, Van Alfen *et al.* 1975, Van Alfen 1982, Bryner *et al.* 2012, Brusini y Robin 2013, Rigling y Prospero 2017).

Los virus de ARN de doble cadena pertenecientes al género Hypovirus se localizan en el citoplasma del hongo (Choi y Nuss 1992). Se han descrito cuatro especies: *Cryphonectria* hypovirus CHV-1, CHV-2, CHV-3 y CHV-4 (Milgroom y Cortesi 2004). El más conocido hasta el momento es CHV-1, parásito obligado dependiente del metabolismo de su huésped (Nuss 2005). Este hipovirus presenta un genoma de 12,7 kb con dos regiones de marco abierto de lectura (ORFs) que codifican poliproteínas multifuncionales. La región ORF A codifica la poliproteína p69, que es procesada en dos polipéptidos, p29 y p40 (Choi *et al.* 1991). La región ORF B codifica la poliproteína p48 (Shapira y Nuss 1991), pero poco más se conoce sobre esta región (Hillman y Suzuki 2004). El esquema de organización de ambas regiones se muestra a continuación en la Figura 7.

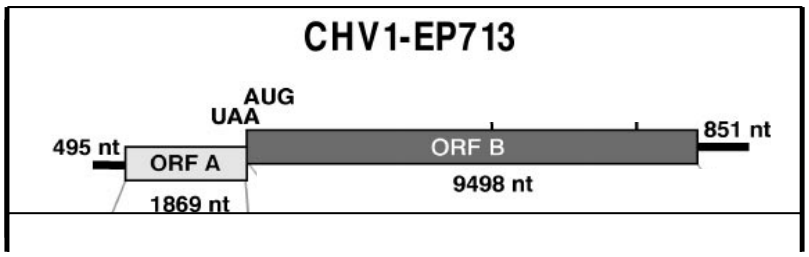


Figura 7. Esquema de la organización genómica del hipovirus CHV-1/EP713. El pentanucleótido 5'-UAAUG-3' sirve de unión entre ORF A y ORF B, siendo UAA el codón de parada de la región ORF A, y AUG el codón de inicio de la región ORF B. (Adaptado de Dawe y Nuss 2001).

La proteína p29 está asociada con síntomas del huésped y acumulación de ARN (Suzuki *et al*. 2003), además de influenciar la respuesta antivírica (Dawe y Nuss 2013). También hay indicios de que podría jugar un papel importante en fomentar la transmisión del virus a través de los conidios (Suzuki *et al*. 2003), y en disminuir la pigmentación del hongo y la acumulación del enzima lacasa (Dawe y Nuss 2001). Además, Dawe y Nuss proponen que esta proteína proporciona información relativa al origen del hipovirus.

La proteína p40 interviene en la acumulación de ARN viral, relacionada con la severidad de los síntomas del huésped (Suzuki y Nuss 2002).

La proteína p48 está relacionada con los fenotipos asociados a la hipovirulencia, como reducción de la esporulación y pigmentación (Deng y Nuss 2008). También puede proporcionar información relativa al origen del hipovirus (Dawe y Nuss 2001). Los determinantes responsables de las diferencias en la morfología de las colonias fúngicas y de los chancros residen principalmente en la región ORF B (Figura 8).

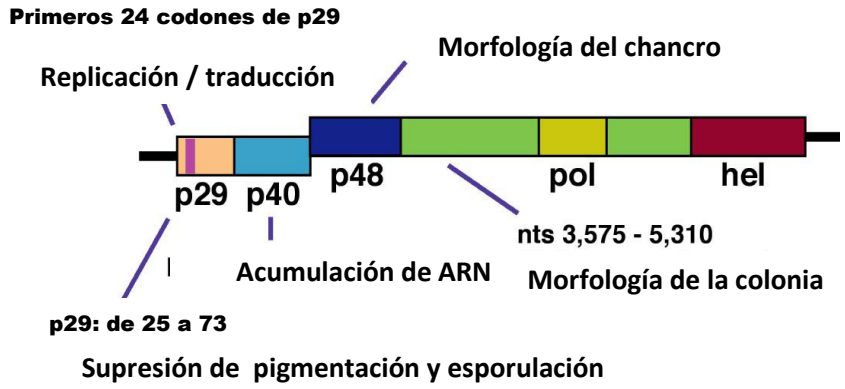


Figura 8. Mapa de CHV1-EP713 que muestra las poliproteínas codificadas por las regiones ORF's y sus implicaciones funcionales. **(Adaptado de Dawe y Nuss 2001).**

Aunque las proteínas virales y sus productos son aún poco conocidos, está claro que ciertos elementos en el genoma del hipovirus juegan un papel específico en la replicación y determinación del fenotipo del hongo (Dawe y Nuss 2013).

Según las variaciones encontradas en las secuencias de la región ORF A, se han descrito cinco subtipos del micovirus CHV-1 (I, F1, F2, D y E). El subtipo I es el más extendido, y ha sido citado en Francia, Grecia, Hungría, Bosnia-Herzegovina (Allemann *et al.* 1999), España (Homs *et al.* 2001), Italia, Suiza, Croacia (Gobbin *et al.* 2003),

Macedonia (Sotirovski *et al.* 2006), Eslovenia (Krstin *et al.* 2011) y Turquía (Akıllı *et al.* 2013). Los subtipos F1 y F2 han sido observados en Francia (Robin *et al.* 2010), y además, F1 se ha encontrado en España (Zamora *et al.* 2012) y F2 en el Este de Turquía (Akıllı *et al.* 2013). El subtipo E se ha encontrado en España (Gobbin *et al.* 2003) y recientemente en Francia (Feau *et al.* 2014). El subtipo D presenta una distribución mucho más limitada, ya que hasta el momento sólo ha sido observado en Alemania (Gobbin *et al.* 2003). La distribución geográfica de los subtipos del hipovirus CHV-1 se presenta a continuación en la Figura 9.

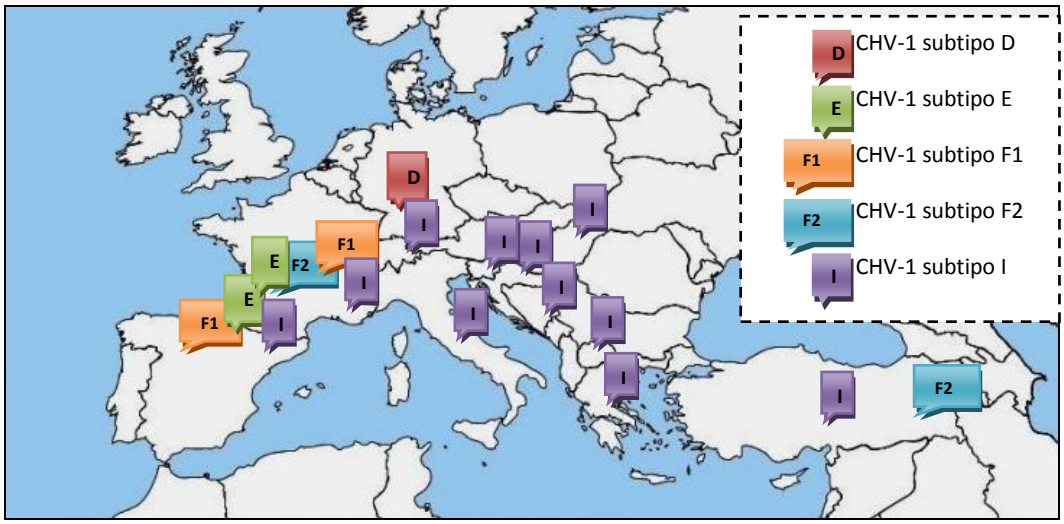


Figura 9. Mapa de la distribución de los subtipos del hipovirus CHV-1.

Recientemente, Feau *et al.* (2014) han realizado una nueva clasificación de los subtipos mediante el análisis de las secuencias parciales de ORF A y ORF B, que revela un patrón de distribución de CHV-1 en Europa más complejo (Figura 10). En el nuevo análisis, estos autores han definido varios linajes dónde se incluyen las cepas de referencia (EP713, 2103, Euro7, M1147 y M1372) que han dado lugar a los subtipos previamente descritos (subtipo F1 en linaje A1-B1, F2 en A2-B1, I en A3-B3, E en A4-B1 y D en A4-B2).

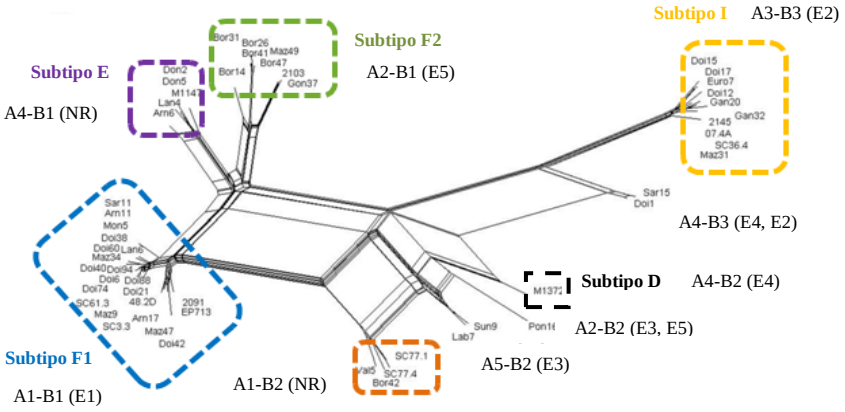


Figura 10. Esquema filogenético del análisis de secuencias concatenadas de las regiones ORF A y ORF B de *Cryphonectria* hypovirus 1, CHV-1 (Adaptado de Feau *et al.* 2014).

La transmisión de los hipovirus puede tener lugar de dos formas. Por una parte, pueden ser transmitidos verticalmente en los conidios o esporas asexuales del hongo (Russin y Shain 1984, Peever *et al.* 2000), que se dispersan a cortas distancias con la lluvia y animales (también largas distancias), pero no en las ascosporas o esporas sexuales (Anagnostakis 1988, Carbone 2004, Prospero *et al*. 2006), dispersadas a largas distancias por el viento. Y por otra parte, puede ocurrir una transmisión horizontal mediante la anastomosis hifal entre aislamientos infectados por virus y aislamientos no infectados (Anagnostakis y Day 1979). El proceso de anastomosis hifal, por el cual hay un intercambio de material citoplasmático de un aislamiento a otro, depende de la compatibilidad vegetativa (cv) de los aislamientos, factor controlado por varios genes de incompatibilidad vegetativa (vic) que presenta el hongo. Según Cortesi *et al*. (2001), la transmisión de hipovirus entre aislamientos fúngicos de diferentes tipos de cv, es decir, que presentan distintos alelos en uno o varios vic, está restringida.

Otro factor que afecta a la hipovirulencia, es el modo de reproducción. *C. parasitica* tiene un sistema de apareamiento o *mating system* con dos alelos MAT-1 y MAT-2 en un único locus (Zhang y Van Alfen 1994), limitándose así la reproducción sexual a los cruces entre cepas con alelos opuestos (Kronstad y Staben 1997). La reproducción sexual en el hongo es un obstáculo para la diseminación del hipovirus, ya que sólo contribuye a la difusión de la forma virulenta del hongo. Además, la reproducción sexual, a través de la recombinación de genes vic, puede aumentar la diversidad de tipos

de cv (Milgroom y Cortesi 1999). Por todo ello, tanto la diversidad de tipos de compatibilidad vegetativa de los aislamientos fúngicos, como la reproducción sexual entre ellos, son factores clave a tener en cuenta para el éxito de la hipovirulencia en una zona determinada.

Sin embargo, cabe destacar que hay estudios más recientes llevados a cabo por Bryner y Rigling (2012b), dónde se ha demostrado que la diversidad de tipos de cv es menos importante de lo que anteriormente se creía. En poblaciones de alta diversidad de tipos de cv se han encontrado virus de alta virulencia, lo que indica que estos virus podrían haber interferido en el sistema de reconocimiento propio del hongo y, por tanto, facilitado la transmisión de virus entre hongos vegetativamente incompatibles (Bryner y Rigling 2012a).

Control biológico de la enfermedad

En zonas europeas, donde la hipovirulencia natural estaba establecida, se observó que la enfermedad del chancro aún persistía causando mortalidad, por lo que se recurrió a la introducción artificial del hipovirus (Heiniger y Rigling 1994). Este método consiste en inocular los chancros con cepas hipovirulentas del mismo tipo de cv que el de la cepa o cepas virulentas presentes en esos chancros, o en su defecto, presentes en otros castaños del área objeto de tratamiento. En la práctica, y con carácter previo al tratamiento, habría que realizar un muestreo de la zona a tratar para conocer los tipos de cv, seguido de la obtención en laboratorio de la cepa seleccionada, preferiblemente de esa zona, infectada por el hipovirus (Figura 11).

Figura 11. A la izquierda, cepa virulenta (v, naranja) e hipovirulenta (hv, blanca) de *C. parasitica*. A la derecha, transmisión del hipovirus de cepa hv a v, la cual adquiere color blanco en el proceso.

Los chancros causados por cepas hipovirulentas se conocen como inactivos, son normalmente superficiales y se encuentran cicatrizados o en estado de cicatrización (Figura 12). En la etapa inicial de la infección del árbol, los chancros pueden mostrar características similares a las producidas por las cepas virulentas, pero su expansión es más lenta y se pueden formar nuevas capas de corteza bajo las áreas afectadas, causando hinchamiento y agrietamiento de la corteza exterior (Prospero y Rigling 2013). En este caso, debido a que el hongo infectado no coloniza y destruye el cambium del árbol, las partes distales del mismo sobreviven a la infección y no se forman brotes epicórnicos bajo el chancro.

Además del efecto terapeútico del hipovirus en los chancros tratados, se espera que su espontánea diseminación también controle la cicatrización de los chancros no tratados. De hecho, el éxito de un tratamiento se valora por la relación de los chancros cicatrizados entre los chancros tratados y no tratados (Robin *et al.* 2010).

El empleo de hipovirus de *C. parasitica* es un método de control biológico que se ha venido estudiando intensamente en las últimas décadas, y cuya eficacia ya ha sido demostrada en algunos países europeos como Francia, Italia y Suiza (Heiniger y Rigling 1994), y recientemente en España (Zamora *et al.* 2014).

Figura 12. Chancros inactivos, cicatrizados o en estado de cicatrización, originados de forma natural por *Cryphonectria parasitica*.

Control químico

Además de la estrategia biológica, otra posible forma de control de las enfermedades es el control químico. El uso de fungicidas puede conseguir una disminución significativa de la incidencia de una enfermedad (Denman *et al.* 2004).

Respecto al control del chancro del castaño, a lo largo de los años se han realizado ensayos con distintos productos fitosanitarios. Sin embargo, no se ha encontrado ninguna estrategia química cuya aplicación sea sostenible en campo a largo plazo (Prospero y Rigling 2013).

Resultados de trabajos previos realizados por González-Varela y González en 2007, muestran productos con buena capacidad para inhibir *in vitro* el crecimiento del hongo *C. parasitica*. A pesar de ello, no se considera una solución para el control de la enfermedad, por los condicionantes ambientales que tiene este tipo de tratamientos en zonas especialmente sensibles como son los bosques, pero sí como una posible alternativa bajo condiciones controladas.

Otros hongos que causan chancros en el castaño

A pesar de que el hongo *C. parasitica* es conocido como principal responsable del chancro del castaño, enfermedad actualmente considerada una de las más graves que afectan a esta especie, también existen otros hongos patógenos capaces de originar chancros en los castaños, los cuales se describen a continuación.

El ascomiceto ***Diplodina castaneae*** Prill. et Del. es agente causal de la conocida como enfermedad de Javart. Al igual que *C. parasitica* es patógeno del castaño europeo (*C. sativa*) y produce, como principal síntoma, la aparición de chancros (Figura 13). El nombre de la enfermedad deriva del término veterinario francés "Javart", referido a las úlceras de los cascos de los caballos, ya que los chancros que este hongo origina se asemejan a dichas úlceras (Day 1930). El hongo suele atacar más a las ramas jóvenes, que terminan secando.

La enfermedad se encuentra principalmente en Francia, dónde fue detectada por primera vez en torno al año 1860. Desde entonces, se ha extendido rápidamente afectando a la mayoría de las plantaciones de castaño del país (Prillieux y Delacroix 1893). También ha sido observada en Bélgica, el norte de Italia y Alemania (Saccardo 1895). En 1930, comenzó una severa epidemia causada por *D. castaneae* en Inglaterra (Day 1930). Desde entonces, parece que la enfermedad ha sido menos prevalente y, aunque el hongo ha continuado dispersándose, los brotes nuevos han sido más localizados. Así, más recientemente, se ha detectado en Bulgaria y Suiza (Vanev *et al*. 1997, Bissegger y Sieber 1994) y en Eslovaquia (Juhásová 1999). También ha sido detectado en Japón en el castaño japonés *(C. crenata)* (Farr y Rossman 2013).

Figura 13. Chancro en castaño originado por *D. castaneae*. (Foto cedida por D. Robert Perroulaz).

Recientemente ha sido descrito otro hongo, ***Gnomoniopsis castaneae*** Tamietti (syn. *G. smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew y D.I. Guest) (Shuttleworth 2012, Visentin *et al.* 2012, Shuttleworth *et al*. 2015), como responsable de la formación de chancros en castaño (Pasche *et al.* 2016). Este hongo, taxonómicamente similar a *D. castaneae*, ya se conocía como patógeno de castaño y había sido descrito como agente causal de la podredumbre de la castaña (Shuttleworth 2012, Visentin *et al.* 2012, Maresi *et al.* 2013, Dennert *et al.* 2015).

Los síntomas en el fruto son lesiones de color marrón con variaciones de tono desde pálido a oscuro, que ocurren en el endospermo y el embrión de la castaña, donde

aparecen manchas (Shuttleworth 2012). Los síntomas en la madera son chancros y fructificaciones (Figura 14). Aunque son muy similares a los síntomas causados por el hongo *C. parasitica*, se pueden diferenciar visualmente de éstos por el color de los picnidios. Mientras que los picnidios de *C. parasitica* presentan un color más naranja, los de *G. castaneae* son de color más rojizo que con el tiempo se oscurece cambiando a negro.

Figura 14. Chancro en planta de castaño originado por la inoculación de *Gnomoniopsis castaneae*. (Foto cedida por Dª. Joana Meyer).

G. castaneae, considerado un patógeno emergente en Europa, ha sido descrito en Italia, Francia, Suiza (Visentin *et al.* 2012), Australia (Shuttleworth *et al.* 2013) y Nueva Zelanda (Pasche *et al.* 2016).

PLANTEAMIENTO

Y

OBJETIVOS

PLANTEAMIENTO Y OBJETIVOS

El castaño es una frondosa que por su valor ecológico, socio-económico y cultural, tiene gran relevancia en el Principado de Asturias. Actualmente, se encuentra en decadencia debido a la aparición de enfermedades, que han mermado su producción y provocado la muerte de muchos ejemplares, siendo la más importante el chancro del castaño. Por ello, se planteó la realización de esta tesis cuyos objetivos principales fueron **estudiar posibles estrategias de control del hongo *Cryphonectria parasitica*, principal responsable de la enfermedad del chancro del castaño, y estudiar la presencia de otros hongos formadores de chancros en castaño.** Estos objetivos generales se concretaron en los siguientes objetivos parciales:

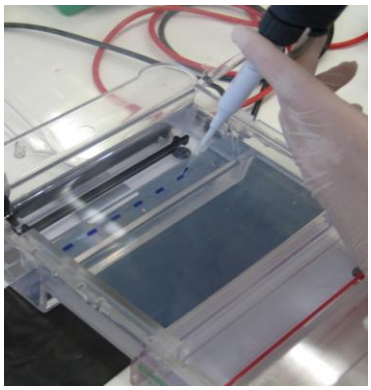
1. Muestrear castaños con chancros cicatrizados, o en estado de cicatrización, en distintos concejos asturianos para estudiar la presencia natural de cepas hipovirulentas (hvs) de *C. parasitica* (I).

2. Determinar el tipo de compatibilidad vegetativa (cv) de las cepas obtenidas en el muestreo y caracterizarlas morfológicamente para realizar una selección de las posibles hvs (I).

3. Caracterizar molecularmente las cepas potencialmente hvs para confirmar la presencia del hipovirus CHV-1, estudiar la prevalencia y distribución de la infección vírica y determinar el subtipo de hipovirus mediante análisis filogenéticos (I).

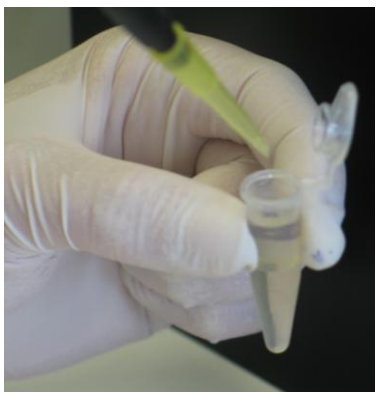
4. Completar la evaluación de la eficacia del control químico, como posible estrategia complementaria al control biológico, finalizando un estudio previo realizado con tres productos fitosanitarios (II).

5. Describir y caracterizar otros posibles agentes causales de chancro en castaño (III, IV).


MATERIAL
Y MÉTODOS






Cepas (I, II, III, IV)

- 937 cepas del hongo *Cryphonectria parasitica* (I, II)
  - 50, procedentes de seis concejos del Principado de Asturias y obtenidas en este trabajo (I).
  - 813, de la colección de hongos del Laboratorio de Fitopatología del Principado de Asturias (LPPAF), obtenidas anteriormente (Valdezate *et al*. 2001, González-Varela 2009, Trapiello 2010) (I, II).
  - 74 cepas control de los tipos europeos de compatibilidad vegetativa (cv) (Cortesi y Milgroom 1998) (Robin *et al*. 2000) (I).
- Diez cepas del hongo *Sirococcus castaneae* (III)
  - Ocho, obtenidas en este trabajo en cuatro concejos asturianos.
  - Dos procedentes de dos regiones de Suiza.
- Tres cepas del hongo *Gnomoniopsis castaneae* (III, IV)
  - Una procedente de la colección de hongos del Laboratorio de Fitopatología del Swiss Federal Institute for Forest, Snow and Landscape (WSL) de Suiza, obtenida en trabajos previos por Meyer *et al*. (2015) (III).
  - Dos procedentes de Asturias y obtenidas en este trabajo (IV).

Medios de cultivo (I, II, III, IV)

- Agar agua. Se añadieron 15 g de agar por litro de agua destilada (I, III).
- Agar patata dextrosa, APD (Gams *et al*. 1998). Se cocieron durante 20 minutos 200g de patatas peladas. Al caldo de cocción, filtrado a través de una muselina, se le añadieron 20 g de glucosa, 16 g de agar y agua destilada hasta completar un litro (I, II, III).
- Agar patata dextrosa, APD. BD Difco PDA (Chemie Brunschwig AG, Basel, Switzerland). Se añadieron 39 g por litro de agua destilada (III).
- Agar extracto de malta, AEM. Se añadieron 12 g de extracto de malta (Chemie Brunschwig AG) y 15 g de agar a un litro de agua destilada (III).

Todos los medios fueron esterilizados en autoclave (20 min. a 120ºC) y conservados a una temperatura de 4º C hasta su uso.

Iniciadores (I, III, IV)

Todos los iniciadores utilizados en este trabajo se recogen a continuación en la Tabla I.

Tabla 1. Iniciadores utilizados en este estudio para las cepas de *Cryphonectria parasitica* (azul claro), ***Sirococcus castaneae*** **y** ***Gnomoniopsis castaneae*** (azul oscuro).

Producto génico	Región	Iniciador	Secuencia del iniciador (5´→3´)	LP (pb)	Referencia
Marco abierto de lectura (ORF)	ORF A	hvep 1(d)	TGACACGGAAGCTGAGTGTC	693	Gobbin *et al.* 2003
		hvep 2(r)+	AGCGCGAATTTCTTGTCG		
		EP 721-4(r)	GGAAGTCGGACATGCCCTG	353	Bryner *et al.* 2012
	ORF B	ORF B-12F(d)	ATCGGGTCTCCCTTCAAGTT	780	Feau *et al.* 2014
		ORF B-12R(r)	CACGACGAGTTCGTTGAGRA		
Factor de elongación 1-alfa (EF-1α)	EF-1α-1	EF1-728F(d)	CATCGAGAAGTTCGAGAAGG	577	Carbone y Kohn 1999
		EF1-1199R(r)	GGGAAGTACCMGTGATCATGT		Walker *et al.* 2010
	EF-1α-2	EF1-728F(d)*	CATCGAGAAGTTCGAGAAGG	521	Carbone y Kohn 1999
		EF1-1567R(r)	ACHGTRCCRATACCACCRATCTT		Rehner y Buckley 2005
		EF1-983F(d)**	GCYCCYGGHCAYCGTGAYTTYAT		
ARNr 18S, ITS1, ARNr 5.8S, ITS2, y ARNr 28S	ITS	ITS1(d)	TCCGTAGGTGAACCTGCGG	593	White *et al. 1990*
		ITS4(r)	TCCTCCGCTTATTGATATGC		
ARNr 28S	LSU	LR0R(d)	ACCCGCTGAACTTAAGC	1339	Rehner y Samuels 1994
		LR7(r)	TACTACCACCAAGATCT		Vilgalys y Hester 1990
ARN polimerasa II	RPB2-1	fRPB2-5F(d)	GAYGAYMGWGATCAYTTYGG	1163	Liu *et al.* 1999
		fRPB2-7cR(r)	CCCATRGCTTGYTTRCCCAT		
Beta tubulina	β-tub-1	T1(d)	AACATGCGTGAGATTGTAAGT	1597	O'Donnell *et al.* 1997
		T22(r)	TCTGGATGTTGTTGGGAATCC		

pb: pares de bases, d: directo, r: reverso, +En los casos en los que el iniciador EP 721-4 no funcionó.*Utilizado sólo para la amplificación, **Utilizado sólo para la secuenciación.

Plantas de castaño (II)

Para la evaluación del control químico se utilizaron 126 plantas de castaño, mantenidas en invernadero, que fueron inoculadas con la cepa *C. parasitica* LPPAF-187 y tratadas con fitosanitarios en trabajos previos (González-Varela 2009).

Figura 15. Plantas de castaño inoculadas con *Cryphonectria parasitica* y tratadas con agroquímicos.

Muestreo (I, III)

Entre 2013 y 2014, se muestrearon 60 castaños con síntomas típicos de hipovirulencia como chancros superficiales, en estado de cicatrización o cicatrizados. El muestreo se realizó en tres concejos asturianos de la zona central (Aller, Lena y Llanera) y tres de la oriental (Colunga, Peñamellera Baja y Villaviciosa). El procedimiento consistió en recoger tres discos de corteza con un sacabocados estéril (5mm de diámetro) de las partes superior, central e inferior del chancro, y sólo se muestreó un chancro por árbol. Las muestras se procesaron en el laboratorio 24-48 horas tras su recogida.

Aislamiento de hongos (I, III, IV)

Las muestras, desinfectadas con alcohol, se colocaron con la corteza hacia arriba (Anagnostakis 1988) en placas con agar- agua y se incubaron a temperatura ambiente. Tras cinco/siete días, el micelio crecido en la placa (uno por chancro o más, en caso de diferencias morfológicas) fue repicado a otra placa con APD y en algunos casos, se obtuvieron los cultivos monoconidiales mediante el método de dilución seriada.

Figura 16. A la izquierda, placa con micelio del hongo *Cryphonectria parasitica* procedente de los trozos de corteza. A la derecha, cultivos monoconidiales del hongo.

Determinación de los tipos de compatibilidad vegetativa (cv) de *C. parasitica* (I)

El tipo de cv se determinó mediante el método barrera/fusión (Anagnostakis *et al*. 1986). Las cepas se sembraron en APD tal como describen Cortesi *et al*. (1998) y se incubaron a 25º C, siete días en oscuridad y siete con fotoperiodo 16h luz/8h oscuridad. Los enfrentamientos se realizaron primero entre las cepas de Asturias, y las resultantes se enfrentaron con las de la colección europea. El ensayo se repitió tres veces.

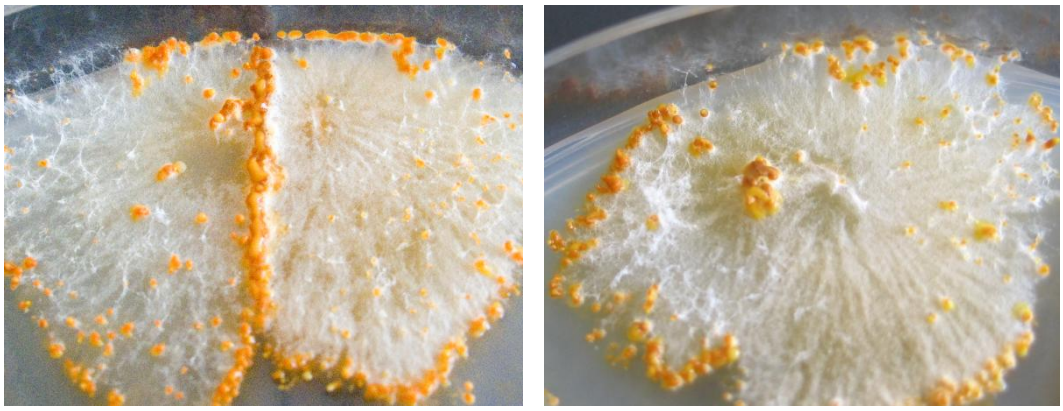


Figura 17. Método de barrera/fusión. En la izquierda, barrera entre dos cepas de *Cryphonectria parasitica* incompatibles. En la derecha, fusión de dos cepas compatibles.

Caracterización morfológica (I, III, IV)

- **De las cepas de *C. parasitica* (I)**

  Las cepas se sembraron en APD y se mantuvieron siete días en oscuridad y siete en condiciones ambientales. A continuación, se valoró la presencia o ausencia de características típicas de cepas hvs (Bissegger *et al*. 1997).

- **De las cepas de *S. castaneae* y *G. castaneae* (III, IV)**

  Se sembraron las cepas en placas con dos medios distintos (APD y AEM). La incubación, durante 21 días, se desarrolló bajo dos condiciones diferentes, oscuridad completa y fotoperíodo de 16 h de luz/ 8 h de oscuridad.

Identificación molecular (I, III, IV)

- **De las cepas hvs de *C. parasitica* (I)**

  Se realizó la extracción de ARN, síntesis de ADN complementario ADNc, reacción en cadena de la polimerasa PCR y secuenciación de las regiones ORF A y/o ORF B del hipovirus CHV-1 (Allemann *et al*. 1999). Las secuencias se compararon con las de la base de datos del NCBI (National Center for Biotechnology Information) mediante BLAST (Altschul *et al*. 1990) y se utilizaron para construir árboles filogenéticos que sirvieron para clasificar los hipovirus en subtipos o grupos (Gobbin *et al*. 2003, Feau *et al*. 2014). Todos los datos fueron analizados con el software CLC Genomics Workbench version 8.0.1 (http://www.clcbio.com).

- **De las cepas de *S. castaneae* y *G. castaneae* (III, IV)**

  Se realizó la extracción de ADN, amplificación y secuenciación de las cinco regiones genómicas que se especifican en Tabla 1 y las secuencias se compararon con las del NCBI como se describe anteriormente. Las secuencias se alinearon mediante ClustalW (Thompson *et al*. 1994) y se construyeron árboles filogenéticos utilizando la herramienta informática MEGA (Molecular Evolutionary Genetics Analysis) software 6.06 (Tamura *et al.* 2013).

Prevalencia de la infección vírica en *C. parasitica* (I)

Se calculó la presencia de virus en cepas procedentes de castaños con chancros activos e inactivos, es decir, cicatrizados o en estado de cicatrización. Para el análisis se utilizaron los métodos estadísticos que se explican en el apartado "Análisis estadístico".

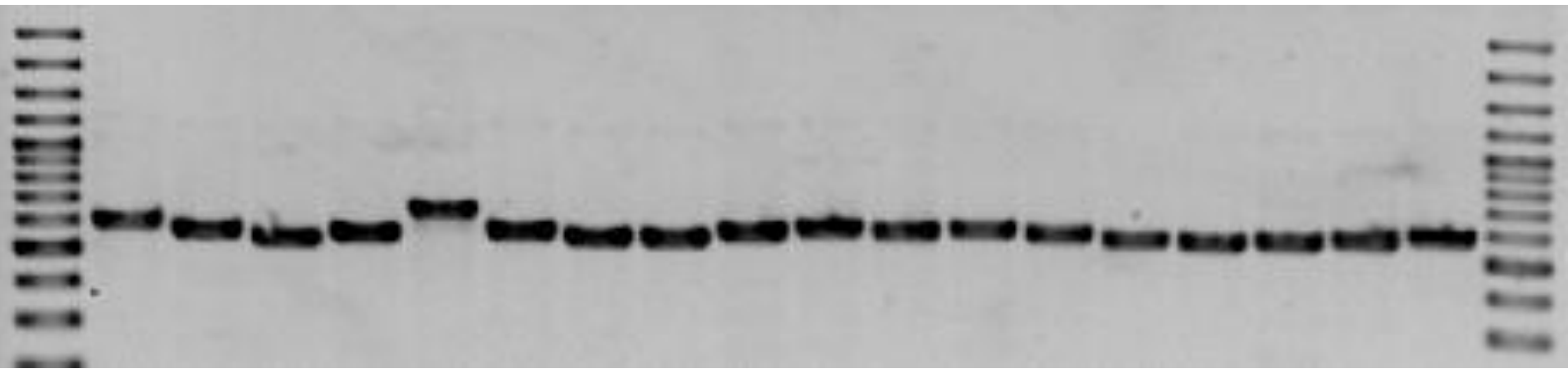
Evaluación de un ensayo de control químico de *C. parasitica* (II)

Se evaluó la eficacia de tres productos (epoxiconazol EP, carbendazima CA, y mezcla de carbendazima más flutriafol CA-F) aplicados mediante tres métodos (preventivo-pulverización P, curativo-pulverización CP y curativo-rodillo CR) en un ensayo previo realizado por González-Varela (2009). Los parámetros evaluados cinco meses tras el último tratamiento, fueron longitud de la lesión (cm), esporulación (0-5), presencia de brotes sanos (0/1) y grietas (0-5), y tres años después, el parámetro mortalidad (ratio).

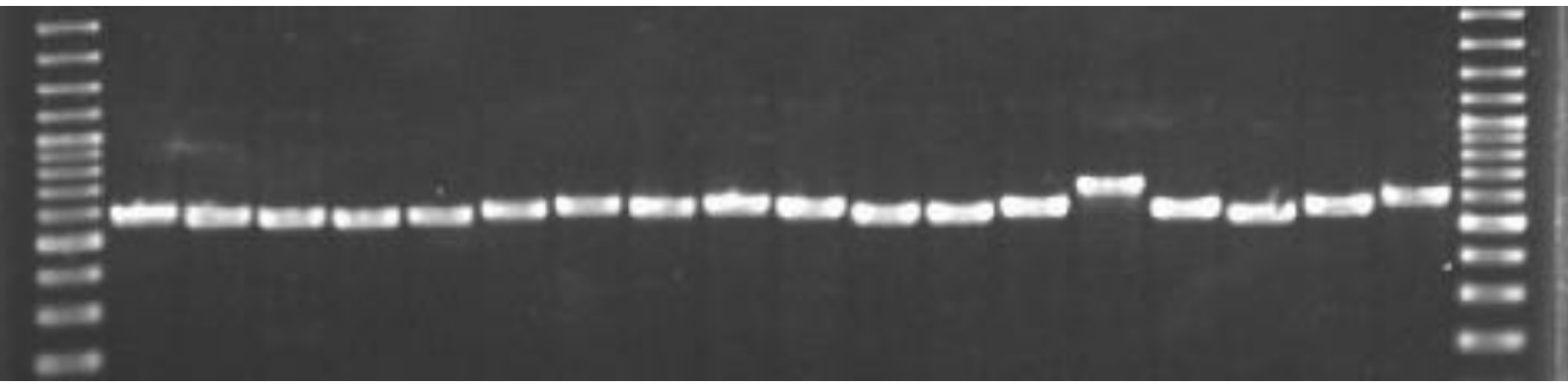
Análisis estadístico (I, II)

- Cálculo de la media aritmética, la desviación estándar y el error estándar (II).
- Realización de la prueba Levene (I) (parámetro: longitud de la lesión, II).
- Análisis de la varianza (one-way ANOVA) (presencia de virus- chancro cicatrizado, I) (método de aplicación y producto- longitud de la lesión, II).
- Pruebas de rango post hoc (Tukey) y comparaciones múltiples (Scheffé) (longitud de la lesión, II).
- Contraste no paramétrico Kruskal-Wallis (esporulación y mortalidad, II).

Los análisis se realizaron con el programa SPSS Statistics 19 (c Copyright 1989,2010 SPSS,Inc).



RESULTADOS



RESULTADOS

1. Muestreo de chancros inactivos (I)

En los seis concejos del Principado de Asturias muestreados en este trabajo (Aller, Colunga, Lena, Llanera, Peñamellera Baja y Villaviciosa), se hallaron 60 castaños con síntomas típicos de infección por cepas hvs, como chancros cicatrizados y/o en estado de cicatrización, De los 60 chancros muestreados, se obtuvieron un total de 50 cepas del hongo *C. parasitica*. Por tanto, hubo 10 en los que no se aisló el hongo.

2. Diversidad de tipos de compatibilidad vegetativa (cv) de *C. parasitica* (I)

Las 50 cepas del hongo, previamente obtenidas, se pudieron asignar a tres tipos de compatibilidad vegetativa (cv): EU-1, EU-3 y EU-13. El tipo mayoritario fue EU-1 con una frecuencia del 92%, seguido de EU-13 con un 6% y EU-3 con un 2%. Teniendo en cuenta su distribución geográfica en la región, se observó la presencia del tipo de cv EU-1 en todos los concejos, mientras que EU-13 y EU-3 sólo se encontraron en la zona oriental, en el concejo de Villaviciosa (ambos tipos de cv) y en el concejo de Peñamellera Baja (EU-13).

3. Caracterización morfológica de las cepas e identificación y distribución de las potencialmente hvs en la región (I)

Tres de las 50 cepas obtenidas en el muestreo de chancros inactivos de este trabajo, es decir, el 6%, mostraron baja pigmentación y esporulación, características típicas de cepas hvs. Estas tres cepas procedían de tres concejos diferentes, el concejo de Aller, localizado en la zona central de la región, y los concejos de Peñamellera Baja y Villaviciosa, en la zona oriental.

Respecto a las cepas de la colección de hongos LPPAF, obtenidas de chancros activos, 27 de las 813 (3,32%) presentaron características de cepas hvs. No se

observaron diferencias significativas entre ambos análisis ($\chi 2 = 28.958$, df = 1, P = 0.333).

En total, 30 de las 863 cepas de *C. parasitica* analizadas (3,48%), fueron caracterizadas como potencialmente hvs al presentar características morfológicas típicas de hipovirulencia.

4. Caracterización molecular de las cepas potencialmente hvs, prevalencia y distribución de la infección vírica, y determinación del subtipo de hipovirus (CHV-1) (I)

Las 30 cepas potencialmente hvs (100%) presentaron un resultado positivo en la detección del hipovirus CHV-1. Además, otras 30 cepas (procedentes de este trabajo y de la colección LPPAF) con características típicas de cepas virulentas, como coloración naranja y esporulación abundante, que fueron objeto del mismo análisis molecular, presentaron un resultado negativo.

En cuanto a la prevalencia de la infección vírica, los datos coinciden con los presentados en el apartado anterior, al ser confirmada la presencia de hipovirus en todas las cepas potencialmente hvs.

Respecto a la distribución de las cepas hvs en la región, la mitad (15) procedían de la zona oriental de Asturias (cinco de Peñamellera Baja, tres de Caravia, dos de Villaviciosa, dos de Parres, una de Peñamellera Alta, una de Onís y una de Nava). De la zona central de Asturias, se obtuvieron 12, es decir, el 40%, distribuidas en los concejos de Lena (cinco), Aller (dos), Grado, San Martín del Rey Aurelio, Morcín, Carreño y Oviedo (una cepa por concejo). Las tres cepas restantes (10%) procedían de la zona occidental, dos de Cangas del Narcea y una de Villayón.

Según la diversidad de tipos de cv, se encontraron dos tipos entre las 30 cepas hvs. El tipo mayoritario EU-1 se observó en 28 cepas hvs (93,34%) y el tipo EU-13 en las dos restantes (6,67%), procediendo estas dos últimas del mismo concejo (Peñamellera Baja), situado en la zona oriental.

La caracterización de los hipovirus se basó en las regiones específicas ORF A y ORF B. Sólo se obtuvieron secuencias de la región ORF A de 20 cepas hvs, mientras que de las 10 restantes se obtuvieron secuencias de ambas regiones. Considerando el análisis filogenético de la región ORF A, las cepas hvs asturianas se agruparon con las secuencias de referencia de los subtipos E y D, dentro del grupo A4. Considerando el análisis de ORF B, siete cepas (70%) se agruparon con el subtipo E (grupo B1) y tres (30%) con el subtipo D (grupo B2). El análisis conjunto de ambas regiones, presentó los mismos resultados que el análisis independiente de la región ORF B.

Figura 18. Mapa de los concejos asturianos con la presencia del hongo *Cryphonectria parasitica* marcada en tono oscuro y la presencia de cepas hipovirulentas, en tono claro.

5. Evaluación de la eficacia de tres productos fitosanitarios frente a *C. parasitica* (II)

En la evaluación de los parámetros longitud de la lesión y esporulación, se observaron diferencias significativas en cuanto a los distintos métodos de aplicación (longitud de la lesión: P = 0.018, ANOVA; esporulación: $\chi 2$ = 13.542, df = 3, P = 0.004, Kruskall-Wallis KW) y en cuanto a los distintos productos empleados (longitud de la lesión: P = 0.007, ANOVA; esporulación $\chi 2$ = 20.923, df = 3, P = 0.000, KW).

Respecto a la longitud de la lesión, el método curativo aplicado con rodillo (CR) fue el de mayor eficacia (longitud de la lesión de las plantas L = 5,97 cm), y el

método preventivo aplicado por pulverización (P) el de menor eficacia (L = 6,93 cm) (P = 0.047, Scheffé; P = 0.025, Tukey). Respecto a los productos, las plantas tratadas con epoxiconazol (EP) presentaron la lesión más corta (5,8 cm) mientras que las tratadas con la mezcla de carbendazima más flutriafol (CAF) presentaron la más larga (6,8 cm) (P =0.037, Scheffé; P =0.019, Tukey).

En cuanto a la esporulación, CR fue el método de aplicación más eficaz (1,83) frente a P y CP (ambos 2,7) ($\chi 2$ = 13.542, df = 3, *P* = 0.004, KW). Las plantas tratadas con EP presentaron la cantidad más baja de pústulas (1,73), y las tratadas con CA-F la más alta (3) ($\chi 2$ = 20.923, df = 3, *P* = 0.000, KW).

En la evaluación de la mortalidad no se observaron diferencias significativas entre los métodos de aplicación, pero sí entre los productos. La tasa más alta de mortalidad tuvo lugar en plantas tratadas con CA-F (0,4) y la tasa más baja en plantas tratadas con EP (0,2).

Los parámetros presencia de grietas y brotes sanos no presentaron diferencias estadísticamente significativas, por lo que esos datos han sido omitidos.

6. Detección, descripción y caracterización de *Sirococcus castaneae* (III)

De los 60 chancros muestreados, se obtuvieron ocho cepas (13,3%) de otro hongo, que estuvo presente en las dos áreas muestreadas, siendo más abundante en la zona central, de donde se aislaron siete cepas: tres en Aller, dos en Lena y dos en Llanera. La octava cepa se aisló en Peñamellera Baja, en la zona oriental.

La identificación molecular indicó que, todos los genes analizados de las cepas del hongo estudiadas, compartían una similitud del 99-100% con la cepa de colección perteneciente a la especie *Diplodina castaneae* (CBS212.90). Esta especie está descrita como patógena de castaño, siendo uno de sus síntomas principales la formación de chancros.

Morfológicamente, se observaron diferentes características según las condiciones de incubación y el medio de cultivo utilizado. Los aislamientos incubados en AEM, cubiertos de micelio blanco, presentaron aspecto glabro y

márgenes difusos. En APD, presentaron márgenes ondulados y color marrón bajo condiciones de luz, y blanco-parduzco con presencia de micelio blanco algodonoso bajo condiciones de oscuridad. En ambas condiciones, presentaron unos patrones concéntricos de tonalidad marrón mate. En todos los casos, las colonias fueron planas y se observaron menos conidios en oscuridad que en luz, presentando éstos una variación de color entre gris-pardo y rosado. Paralelamente, se mantuvo en incubación bajo las mismas condiciones una cepa de referencia de *Gnomoniopsis castaneae*, que se utilizó como cepa control por ser una especie morfológicamente similar.

Las colonias de *G. castaneae* en medio AEM mostraron forma espiral y coloración parduzca bajo condiciones de luz y, en medio APD, abundante micelio de color blanco parduzco en condiciones de luz y blanco en oscuridad. Los conidios, viscosos y anaranjados, se distribuyeron uniformemente por el medio y presentaron forma espiral en condiciones de luz.

Basándose en las características filogenéticas y morfológicas de las cepas estudiadas en el presente trabajo, se ha asignado una nueva posición taxonómica a la especie *D. castaneae*, incluyéndola en el género *Sirococcus*, y nombrándola *Sirococcus castaneae*. En todos los chancros en los que se aisló *S. castaneae*, también se aisló *C. parasitica*. Esta es la primera cita de este hongo en España.

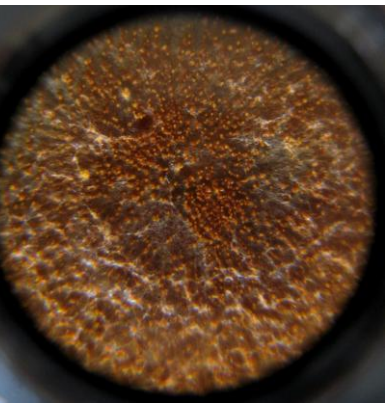
7. Detección y descripción del hongo *Gnomoniopsis castaneae* en Asturias (IV)

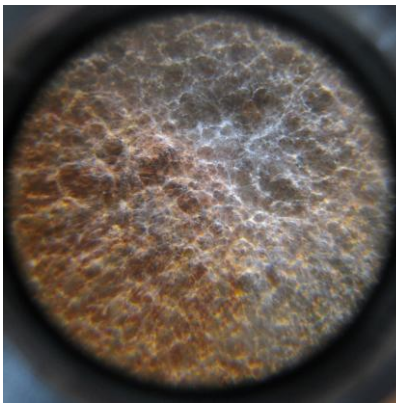
A partir de dos muestras de plantas de castaño procedentes del concejo de Grado, recogidas en la propia finca del SERIDA, con lesiones de coloración rojiza, se obtuvieron dos aislamientos de un hongo morfológicamente similar a *S. castaneae* y *G. castaneae*.

La caracterización morfológica de ambos aislamientos mostró que eran idénticos y resultó coincidente con la morfología previamente descrita (apartado 6) para *G. castaneae*.

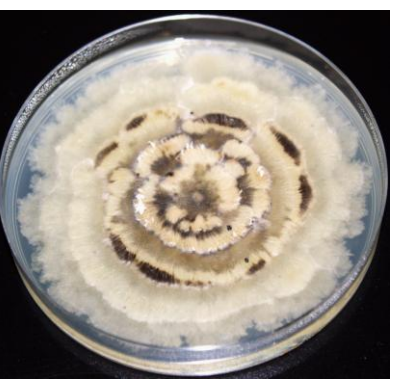
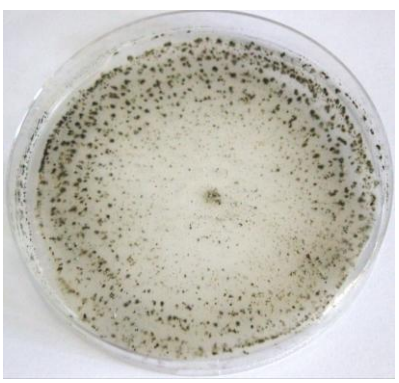
La identificación molecular, mediante la secuenciación de la región ITS, verificó el resultado de la caracterización morfológica. Las secuencias de los dos aislamientos obtenidos fueron idénticas y presentaron un 100% de similitud con la cepa de referencia ICMP14079, identificada como *G. smithogilvyi* (nº de accesión KC145860), sinónimo de *G. castaneae*. Se depositó en EMBL (*European Molecular Biology Laboratory*) la secuencia de una de las cepas (LPPAF-935) con el nº de accesión LT837820.

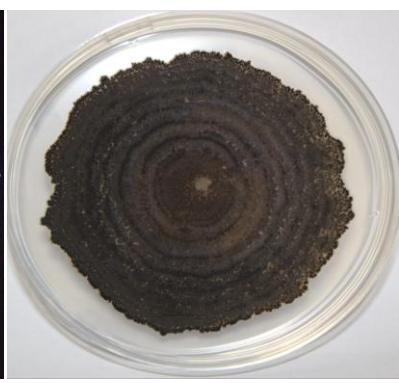
La identificación de *G. castaneae* sería la primera cita de este patógeno en España.



DISCUSIÓN



DISCUSIÓN

La gran superficie forestal ocupada por el castaño en Asturias y la incidencia del chancro en ella, hace que el control de la enfermedad sea un tema de sumo interés para nuestra Comunidad Autónoma. Actualmente, el método de lucha utilizado en Europa es el control biológico con cepas hipovirulentas del hongo.

En Asturias, en un trabajo previo (González-Varela 2009) se había descrito la situación de la enfermedad y se habían seleccionado una serie de cepas de *C. parasitica* que presentaban características típicas de las hipovirulentas, aunque no se había llegado a comprobar la presencia del hipovirus.

Por otra parte, la presencia de castaños con chancros inactivos, es decir, que presentaban chancros cicatrizados y/o en estado de cicatrización, cuya morfología se asocia a la presencia de hipovirulencia, y denominados así porque el daño provocado en el árbol es pequeño o nulo (Heiniger y Rigling 1994, Milgroom y Cortesi 2004), motivó la realización de un nuevo **muestreo** dirigido a este tipo de chancros en las zonas central y oriental de la región, con el fin de seleccionar el mayor número de cepas hvs posible.

De los 60 **chancros inactivos** muestreados sólo se pudo aislar *C. parasitica* en 50 de ellos, y para explicar este resultado se pueden plantear varias hipótesis. Por una parte, el hongo estaría presente pero el aislamiento podría haber sido fallido, y por otra parte, el hongo podría haber estado presente en algún momento pero no haber sobrevivido, bien porque el hipovirus fuera tan agresivo que hubiera llegado a matar al hongo, o bien por la presencia de otro enemigo natural del patógeno. Por último, ya hemos mencionado que otros hongos son capaces de producir chancro en castaño, por lo que podemos considerar también la posibilidad de que el chancro no hubiera sido producido por *C. parasitica*.

Este nuevo muestreo nos ha permitido comprobar que la **diversidad de tipos de cv** en la región se mantiene baja, ya que en este trabajo se han encontrado los mismos tipos que en trabajos previos (González-Varela 2009, González-Varela *et al.* 2011), que cubrieron todos los concejos que conforman el Principado de Asturias. Estos tipos son

EU-1, EU-3 y EU-13. Incluso las frecuencias obtenidas en el presente trabajo se mantienen en rangos muy similares a las descritas por González-Varela (2009).

El grupo EU-1 sigue siendo mayoritario con un 92% de los aislamientos, frente al 95% que cita González-Varela (2009), seguido de EU-13 con un 6%, frente al 5% del muestreo anterior, y por último, EU-3 con un 2% frente al <1% citado con anterioridad.

En cuanto a la distribución geográfica de los tipos de cv, EU-1 se encontró en todos los concejos muestreados, situados en la zona central y oriental, tal como se había descrito previamente. González-Varela (2009) encontró EU-13 en 11 concejos distribuidos por toda la región, mientras que en el presente trabajo se muestrearon dos de ellos, Aller y Peñamellera Baja, siendo este último concejo el único en el que se describe la presencia de EU-13 en ambos estudios.

En este trabajo se confirmó la presencia de EU-13 en Villaviciosa tal como se había encontrado en un trabajo anterior (Trapiello 2010) y contrariamente a lo descrito por González-Varela (2009), lo que podría indicar que este tipo pudo haber ampliado su distribución en ese tiempo, aunque no se puede descartar también que hubiera pasado desapercibido en el muestreo anterior.

EU-3 ha sido detectado en el presente estudio en la zona oriental, en Villaviciosa, lo que apunta a una reciente dispersión de este tipo, convirtiendo a este concejo en uno de los más diversos de la región al presentar los tres tipos de cv, que hasta este momento sólo se habían detectado en la zona central, en el concejo de Sobrescobio.

Comparando estos resultados con otras regiones del norte peninsular, podemos observar que en Galicia hay cuatro tipos de cv descritos (Aguin *et al.* 2008, Montenegro *et al.* 2008), en Cataluña cinco (Castaño *et al.* 2015) y en Castilla y León cinco conocidos y seis no conocidos previamente (Zamora *et al.* 2012), siendo EU-1 el único tipo que estas zonas comparten con Asturias. Los tipos EU-3 y EU-13 no han sido descritos en otras zonas de España.

En Europa, el tipo EU-1 también está ampliamente extendido (Robin y Heiniger 2001); sin embargo, la distribución de los tipos EU-3 y EU-13 está limitada a países de Europa central como Bosnia-Herzegovina (Trestic *et al.* 2001), Hungría (Radócz 2001) y Eslovaquia (Adamčíková *et al*. 2006, Juhásová *et al.* 2012). El tipo EU-13 ha sido

encontrado en República checa (Jankovský *et al*. 2010) y en Eslovenia (Krstin *et al.* 2011).

Cabe mencionar que la diversidad de tipos podría llegar a aumentar ya que los idiomorfos MAT-1 y MAT-2 (3:1) están presentes en la región, lo que implica la posibilidad de que se produzca reproducción sexual, aunque con baja probabilidad, ya que su proporción difiere significativamente de la esperada (1:1) en una población que se está reproduciendo sexualmente (González-Varela 2009).

En referencia a la **caracterización morfológica de las cepas** obtenidas en el muestreo de chancros inactivos, podemos decir que se obtuvieron pocas cepas con débil esporulación y pigmentación, lo que implica una baja prevalencia (6%) de cepas potencialmente hvs. También se obtuvo un bajo porcentaje (3,32%) de cepas potencialmente hvs en el análisis de cepas recogidas de la colección LPPAF que procedían de chancros activos muestreados con anterioridad (Valdezate *et al*. 2001, González-Varela 2009, Trapiello 2010). Reseñar que no se observaron diferencias significativas entre el número de cepas potencialmente hvs aisladas de chancros activos e inactivos, resultado que apoya el estudio de Bryner *et al.* (2013), donde no se detectaron chancros con características morfológicas que se puedan asociar a la presencia o ausencia de infección vírica. Estos autores, contrariamente a lo que se había descrito hasta entonces, sugieren que el aspecto del chancro no está claramente relacionado con la presencia de cepas hvs.

Todas las cepas caracterizadas morfológicamente como potencialmente hvs, resultaron ser efectivamente hipovirulentas, al comprobarse molecularmente la **presencia del hipovirus CHV-1**. Resultados similares se observaron en otros estudios donde todas las cepas de *C. parasitica* con fenotipo blanco presentaron el hipovirus CHV-1 (Montenegro *et al.* 2008, Krstin *et al.* 2011, Peters *et al.* 2012, Zamora *et al.* 2012, Zamora *et al.* 2015). Nuestros datos, por tanto, coinciden con lo descrito anteriormente (Van Alfen *et al.* 1978, Elliston 1985, Choi y Nuss 1992), resultando ser la caracterización morfológica un criterio fiable como primer paso en la selección de cepas hvs. Sin embargo, cabe destacar que tanto las condiciones de incubación como el tipo de medio APD utilizado para el estudio morfológico, son criterios muy importantes que pueden alterar el resultado.

La proporción similar de cepas hvs procedentes de chancros activos e inactivos, nos lleva a plantear hipótesis similares a las citadas para explicar la ausencia del hongo en el 16% de los chancros inactivos muestreados.

Por un lado, este resultado podría indicar que, a pesar de que los castaños muestreados presentaban signos típicamente asociados a la infección de *C. parasitica* por el hipovirus CHV-1, como chancros superficiales de menor crecimiento y esporulación que los chancros activos causados por el hongo (Heiniger y Rigling 1994), no la había. Esto podría ser debido a que el muestreo se realizó siguiendo este criterio morfológico, el cual puede no resultar siempre fiable según Bryner *et al.* (2013), tal y como se explica con anterioridad.

Por otro lado, podría existir infección vírica pero el hipovirus no haber sido aislado con éxito, ya que un movimiento incompleto del virus en el micelio del hongo dificultaría su detección (Bryner *et al.* 2013).

Además, como todos los virus, CHV-1 es un parásito obligado y totalmente dependiente del metabolismo de su huésped (Nuss 2005). La infección vírica hace que el hongo crezca más lentamente, lo que puede acarrear la desaparición del propio virus.

En los 50 chancros cicatrizados de los que se ha obtenido *C parasitica*, un hallazgo a destacar es que se aislaron tanto cepas virulentas (vs) como hipovirulentas (hvs), y además, en algunos chancros se han encontrado los dos tipos de cepas, lo que parece indicar que, o bien son dos cepas diferentes que conviven en el mismo árbol, o bien se trata de la misma cepa que presenta parte del micelio infectado y parte no infectado, tal y como se ha observado en estudios previos, donde el micelio del hongo en un chancro puede estar sólo parcialmente infectado por el virus (Bryner *et al.* 2013).

En trabajos realizados tanto en España (Castaño *et al.* 2015) como en Europa (Prospero *et al.* 2006) ya se han citado dificultades en el aislamiento de cepas hvs de chancros definidos como inactivos, según criterios morfológicos.

Por otra parte, a pesar de que no existe una alta **prevalencia de cepas hvs** en nuestra región, cabe destacar que cuentan con una amplia distribución geográfica, estando presentes en siete concejos de la zona oriental asturiana, siete de la zona central y dos de la occidental.

La baja prevalencia de cepas hvs coincide con la observada en otras regiones europeas como Portugal, donde sólo se encontró una cepa infectada entre 617 analizadas (Bragança *et al.* 2007), o Alemania, que presentó un porcentaje de prevalencia vírica del 10% (Peters *et al.* 2014). Por el contrario, en otras zonas se han encontrado prevalencias mucho más altas; por ejemplo, en el sudeste de Francia se han observado plantaciones de castaño que presentaban del 31 al 90% de los chancros infectados (Robin *et al.* 2010), en el sur de Suiza del 43 al 52% y en los Balcanes del 44 al 85% (Bryner y Rigling 2012b).

Además, Robin y Heiniger (2001) han observado una leve correlación entre el año de detección de la enfermedad y la aparición de chancros cicatrizados o en cicatrización en diferentes países europeos. En España, el chancro del castaño fue detectado en 1940 (Molina 1984) mientras que los chancros inactivos y las cepas hvs no se detectaron hasta 48 años más tarde (Allemann *et al.* 1999). En Asturias, el hongo *C. parasitica* fue observado por primera vez en 1982 (Valdezate *et al.* 2001), mientras que el primer muestreo donde se recogen cepas con características morfológicas de cepas hvs ocurre unos 25 años más tarde (González-Varela 2009) y no se corrobora su infección hasta el presente trabajo. La presencia relativamente reciente del hipovirus podría explicar la baja prevalencia de la hipovirulencia en esta región, pudiendo estar el hipovirus en una etapa inicial de dispersión.

Resultados similares han sido obtenidos en otras regiones del norte peninsular. En Galicia, se detectaron cuatro cepas hvs entre 610 (Aguín *et al.* 2008). En Cataluña, también se ha observado una baja prevalencia del hipovirus CHV-1 (35 cepas hvs de 312) (Castaño *et al.* 2015). En un estudio llevado a cabo en Galicia y en León, se observaron 15 cepas infectadas de un total de 539, siendo esas 15 originarias de León (4,6%) (Montenegro *et al.* 2008). Zamora *et al.* (2012) han descrito también una baja prevalencia (3,1%) en esta provincia, atribuida a la detección relativamente reciente, en 1978, del hongo. En general, se ha observado con frecuencia un bajo nivel de hipovirulencia en las áreas donde el hongo *C. parasitica* ha sido recientemente establecido (Hoegger *et al.* 2000).

Respecto a los **subtipos del hipovirus CHV-1** presentes en España, han sido descritos tres: subtipo E presente en Navarra (Allemann *et al.* 1999), Galicia (Aguín *et al.* 2008) y Cataluña (Castaño *et al.* 2015); subtipo F1 presente en León (Montenegro *et al.* 2008,

Zamora et *al.* 2012) y subtipo I en Cataluña (Homs *et al.* 2001). En este trabajo, se han encontrado dos subtipos en Asturias: el subtipo E (CHV-1- E) y el subtipo D (CHV-1-D). Ambos se encontraron distribuidos en las zonas central (concejo de Lena) y oriental (concejo de Peñamellera Baja) de la región, áreas donde el chancro está más presente (González-Varela 2009).

El subtipo D no había sido detectado en España hasta el presente trabajo, por lo que este hallazgo supone el aumento del número de subtipos del hipovirus CHV-1 descritos en España, convirtiéndose éste en uno de los países con mayor diversidad de subtipos de CHV-1 de Europa. CHV-1-D sólo se había citado en Alemania, dónde no se detectaron más subtipos (Peters *et al.* 2014).

Aunque sea necesario llevar a cabo más investigaciones para determinar el potencial de ambos subtipos como agentes de biocontrol y su nivel de adaptabilidad ecológica, podemos decir que el hipovirus alemán (D) ha resultado ser apropiado como agente de control biológico según estudios previos (Bryner y Rigling 2011 y 2012a, Peters *et al.* 2012). Sin embargo, y, a pesar de que fue descrito mucho antes, no hay datos sobre la eficacia del subtipo español (E), que es el más frecuente en la región. Las cepas hvs correspondieron a los dos tipos de cv mayoritarios de la región (EU-1 y EU-13), y el subtipo E se encuentra asociado a ambos. Este dato podría sugerir la ocurrencia de una transmisión horizontal del subtipo E entre cepas de diferentes tipos de cv, lo que propiciaría su uso como agente de control biológico.

Este resultado coincide con estudios previos en los que se ha demostrado que el hipovirus CHV-1 se transmite con frecuencia en la naturaleza, incluso entre aislamientos de tipos de cv diferentes (Carbone *et al.* 2004), y está bien establecido en poblaciones con una alta diversidad de tipos de cv (Krstin *et al.* 2008, Robin *et al.* 2010, Krstin *et al.* 2011). El hallazgo del mismo subtipo de virus en cepas de diferentes tipos de cv, sugiere que el control mediante el uso de cepas hvs de *C. parasitica* podría ser más efectivo y sostenible de lo que, en principio, se había pensado.

Respecto a la **caracterización molecular**, la mayoría de las cepas hvs asturianas fueron asignadas al grupo genético A4-B1, que incluye al subtipo E de referencia. Este linaje es considerado no recombinante y podría incluir cepas hvs que fueron originalmente introducidas desde Asia hasta Francia o norte de España, cuyos hipovirus sirvieron como parentales en algún evento de recombinación (Feau *et al.* 2014). El resto de las

cepas hvs fueron asignadas al grupo genético A4-B2, que incluye el subtipo D de referencia. Este grupo podría haber derivado de un evento de recombinación entre cepas ancestrales de los linajes A4-B1 (subtipo E) y A3-B3 (subtipo I) (Feau *et al.* 2014), ambos presentes en España. La baja prevalencia del subtipo D y su ausencia en otras zonas de España, podría indicar que el evento de recombinación es reciente y que aún no ha tenido suficiente tiempo de dispersarse. La presencia del subtipo D en Asturias, indica que este hipovirus podría haber sido introducido desde España a Alemania, tras el evento recombinante asumido. Sin embargo, la clasificación actual de los hipovirus detectados en España está basada únicamente en la región ORFA y, tal y como se muestra en Feau *et al.* 2014, una clasificación basada en ambas regiones (ORF A y ORF B) proporcionaría mucha más información. Por tanto, sería conveniente realizar una clasificación completa de todos los hipovirus presentes en España, para así obtener una visión más exhaustiva sobre la diversidad y evolución de la hipovirulencia en nuestro país.

Por otra parte, desde la aparición de la enfermedad del chancro se han probado diferentes estrategias de **control químico** (Jaynes y Van Alfen 1974, Elkins *et al.* 1978, Aksoy y Serdar 2004), pero ninguna ha demostrado ser eficaz (Prospero y Rigling 2013). Sin embargo, ensayos *in vitro* realizados por González-Varela y González (2007) mostraron productos con una alta eficacia frente al hongo *C. parasitica*. Tres de esos productos se probaron en un **ensayo *in vivo*** mediante distintos métodos de aplicación (González- Varela 2009) y la **evaluación** de su eficacia a medio plazo se ha realizado en el presente trabajo.

Respecto a los métodos de aplicación ensayados, el mejor a corto plazo fue el curativo aplicado con rodillo, mientras que el preventivo presentó los peores resultados. En cuanto a los productos, el epoxiconazol (EP) resultó ser el más eficaz, y la mezcla de carbendazima y flutriafol (CA-F) el menos eficaz, lo cual nos plantea una cuestión difícil de explicar y es cómo puede ser más eficaz la carbendazima sola que con el flutriafol, más si tenemos en cuenta que *C. parasitica* puede adquirir resistencia tras aplicaciones continuas de carbendazima (Delen 1980), y que el flutriafol (F) tiene un mecanismo de acción similar al del epoxiconazol.

EP se considera un fungicida de alta eficacia, pero que no inhibe el crecimiento del hongo hasta después de la infección, por lo que no influye en la activación de las defensas de la planta ni en la senescencia (Bertelsen *et al.* 2001). Por ello, este producto no es apropiado en un tratamiento preventivo y su modo de acción podría haber influenciado los resultados de la aplicación preventiva.

EP y F, ambos inhibidores de ergosterol, no mostraron grandes diferencias en el ensayo *in vitro* pero sí en el ensayo *in vivo*. Esto podría ser debido a la influencia de factores externos ambientales que intervinieran en la degradación del producto. Es importante tener en cuenta que la eficacia de un producto depende de varios factores como degradación, persistencia, modo de acción o interacción con otros componentes (González-Varela y González 2007). A pesar de que los resultados *in vitro* fueron muy alentadores, las diferencias entre productos y métodos *in vivo* fueron reducidas en el ensayo planteado, aunque hay que tener en cuenta que no se realizó un calendario de tratamientos sistemático y la última evaluación se realizó tres años después de la última aplicación del producto realizada. Se observaron siempre diferencias entre plantas tratadas y control, excepto en el caso de la mortalidad dependiendo del método, lo que podría indicar que el método de aplicación no influye a largo plazo. También se observaron siempre diferencias significativas entre las plantas tratadas con epoxiconazol, y las tratadas con otros productos, siendo el epoxiconazol el más eficaz. Por tanto, el epoxiconazol podría ser una alternativa para el control de la enfermedad en plantas en condiciones similares a las del presente estudio.

Sin embargo, estudios adicionales sobre el modo de acción, la concentración, el intervalo de aplicación, la fitotoxicidad y el desarrollo de resistencia deberían ser desarrollados antes de que este producto sea propuesto para castaño. Además, autores como Chen *et al.* (2012) sugieren que la combinación de epoxiconazol con otros fungicidas podría implicar una mayor eficacia, por lo que también sería interesante estudiar este tipo de interacciones entre productos.

En este trabajo, se propone que el control químico podría formar parte de una estrategia integrada de control de la enfermedad del chancro del castaño bajo determinadas condiciones, como pueden ser plantas de vivero o algún árbol centenario con un especial interés de conservación, pero no como una solución para la enfermedad ya que no se considera una alternativa ambientalmente aceptable.

A pesar de que la enfermedad del chancro está ampliamente extendida por toda la región, el conocimiento de su distribución no es un indicador de la severidad de la enfermedad. Los datos de incidencia no son suficientes para estimar correctamente el nivel de la enfermedad del chancro en los castañares (Robin y Heiniger 2001). Por ello, con carácter previo a la implementación de estrategias de control del hongo *C. parasitica,* y con el objetivo de priorizar las zonas y/o plantas objeto de tratamiento, sería conveniente estimar la severidad de la enfermedad. Se entiende como severidad, la proporción de la planta que es afectada por la enfermedad, y varía en función de distintos factores epidemiológicos como el poder patógeno del hongo, los caracteres genéticos de los castaños, y los factores ambientales y de manejo de la enfermedad, entre otros (Tizado *et al.* 2012).

Otro aspecto relevante a comentar es que de los chancros inactivos muestreados, además de *C. parasitica*, se obtuvo con cierta frecuencia (13,3%) un hongo identificado en un principio como ***Diplodina castaneae***. Este hongo es responsable de la enfermedad de "Javart" (Prillieux y Delacroix 1893), que se caracteriza por la formación de chancros en los tallos de brotes jóvenes, que pueden provocar su muerte (Day 1930). No hay muchos datos de esta enfermedad desde la epidemia que ocurrió en Inglaterra en 1930, lo que nos lleva a pensar que o bien su presencia ha ido reduciéndose, o bien ha pasado desapercibido como consecuencia de la introducción de *C. parasitica* en Europa a finales de los años 30. En este tiempo, las consecuencias devastadoras del nuevo patógeno y su dispersión por la mayoría de las regiones de castaño europeas, centraron el interés de la comunidad científica y de los productores de castaño. Por ello, es posible que, en algunos casos, los daños e incluso la muerte de ejemplares originados por *D. castaneae* fueran erróneamente atribuidos a *C. parasitica*.

D. castaneae se aisló en todos los chancros conjuntamente con *C. parasitica*, lo que coincide con lo observado por Adamčíková *et al.* (2013) que citan la presencia de ambos hongos coexistiendo en el mismo árbol.

Además de las cepas aisladas de Asturias, en el presente estudio se incluyeron cepas procedentes de Suiza y Azerbaiyán, y se observó una baja diversidad genética entre ellas, no encontrándose ningún patrón geográfico. Estos hallazgos son consistentes con estudios previos de otro patógeno de castaño genéticamente parecido, *Gnomoniopsis*

castaneae, llevados a cabo en Europa, Nueva Zelanda y Australia (Dennert *et al.* 2015, Shuttleworth *et al.* 2015). Sin embargo, un estudio reciente de la misma especie usando microsatélites ha revelado la existencia de diferentes subpoblaciones putativas en Europa (Sillo *et al.* 2017). Por tanto, para una mejor comprensión de la genética de poblaciones en *D. castaneae*, serían necesarios estudios complementarios en este sentido.

En este trabajo **se ha reclasificado taxonómicamente** la especie *D. castaneae* **en el género *Sirococcus*, como *S. castaneae*.** Esta nueva posición taxonómica está apoyada en los análisis filogenéticos y morfológicos realizados. En cuanto a los análisis filogenéticos, las secuencias de *D. castaneae* se incluyeron en un grupo que contenía las especies de *Sirococcus*.

Diplodina es un grupo heterogéneo que incluye sinónimos de especies en otros géneros (*Ascochyta, Discella, Microdiplodia* y *Phloeospora*), por ejemplo, la cepa tipo *D. salicis* es considerada sinónimo de *D. microsperma* (Sutton 1980) y anamorfo de *Plagiostoma apiculatum*, por lo que *D. microsperma* se ha transferido al género *Plagiostoma* (Sogonov *et al.* 2008, Mejía *et al.* 2011, Rossman *et al.* 2015). Como la nomenclatura de la especie *D. castaneae* presenta gran ambigüedad taxonómica, se ha propuesto incluirla en el género *Sirococcus* que es el más cercano. Este género incluye especies de patógenos importantes de coníferas (Rossman *et al.* 2008).

Esta reclasificación está apoyada también por descripciones morfológicas realizadas por Sutton (1980), que indica que ambos géneros no se diferencian entre sí; y por nuestras propias observaciones, ya que la morfología de nuestras cepas encaja bien con la descripción de *Sirococcus*. Si bien es cierto que la elección de un nuevo género para acomodar la especie *D. castaneae* estaría justificada, ya que la distancia filogenética entre ésta y otras especies de *Sirococcus* presenta igual rango que la de otros géneros bien conocidos (por ejemplo, *Cryphonectria-Chrysoporthe* o *Apioplagiostoma-Pleuroceras*), ésta opción ha sido rechazada ya que no se ha encontrado ninguna característica morfológica que distinga *D. castaneae* del género *Sirococcus*.

Por otra parte, en el presente trabajo también se ha encontrado el hongo ***Gnomoniopsis castaneae***, responsable de la podredumbre de la castaña y de la formación de chancros. Se ha aislado de la corteza de plantas jóvenes de castaño, cuyos tallos presentaban

lesiones similares a las producidas por *C. parasitica*, de las que sólo se diferenciaban por presentar una coloración más rojiza. Este hongo no ha sido aislado de los chancros muestreados en Asturias, o porque realmente no estaba presente, o porque ha pasado desapercibido en la fase de aislamiento con *D. castaneae*. Sin embargo, ha sido frecuentemente recogido de chancros de *C. parasitica*, cicatrizados o en estado de cicatrización en Suiza (D. Rigling, datos sin publicar). También ha sido aislado de agallas formadas por la avispilla del castaño (*Dryocosmus kuriphilus*) en Suiza, que a su vez estaban colonizadas por *C. parasitica* (Meyer *et al.* 2015). Estos autores, observaron que *G. castaneae* podría estar compitiendo eficazmente con el patógeno *C. parasitica*, reduciendo así su cantidad de inóculo en las agallas.

Las fructificaciones de este hongo en la corteza de castaño, similares a las de *C. parasitica*, podrían haber llevado a confusiones entre ambos. Pasche *et al.* (2016) consideran que la alta mortalidad en viveros y plantaciones jóvenes, que ha sido atribuida exclusivamente a *C. parasitica*, podría deberse también a *G. castaneae*. Además, este hongo podría contribuir a la presencia de los síntomas de chancro que surgieron en los últimos años, desde que se ha observado una disminución de la incidencia del chancro por la presencia natural de cepas hipovirulentas de *C. parasitica* en Europa (Milgroom y Cortesi 2004). No se conoce el comportamiento de *G. castaneae* en plantas viejas, por lo que aún podría ser confundido con *C. parasitica*.

CONCLUSIONES

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1. En todos los concejos muestreados en este trabajo se han hallado castaños que presentaban chancros inactivos, es decir cicatrizados y/o en estado de cicatrización, típicos de la presencia de cepas hipovirulentas (hv).

2. La diversidad de tipos de compatibilidad vegetativa (cv) en la región se mantiene baja, con los tres tipos previamente descritos y en la misma proporción, siendo EU-1 el mayoritario, seguido de EU-13 y EU-3. Esta baja diversidad de tipos de cv convierte a los castañares asturianos en un escenario idóneo para la aplicación de control biológico.

3. Un bajo porcentaje (3,48%) de cepas presentaron características morfológicas típicas de cepas hipovirulentas, baja esporulación y pigmentación, sin observarse diferencias significativas entre la proporción presente en chancros activos e inactivos.

4. En todas las cepas caracterizadas morfológicamente como potencialmente hipovirulentas (hvs) se detectó el hipovirus CHV-1, confirmándose así la presencia natural de cepas hvs en Asturias.

5. La presencia de dos subtipos de hipovirus en Asturias: subtipo E (linaje A4-B1) y subtipo D (linaje A4-B2), citado por primera vez en España, sitúa al país como una de las áreas con mayor diversidad de subtipos, y amplía las probabilidades de éxito en el control de la enfermedad. Aún no hay estudios sobre la eficacia de CHV-1-E como agente de control pero CHV-1-D es una buena alternativa para iniciar la implementación de programas de control biológico en la región.

6. El ensayo *in vivo* de tres productos fitosanitarios ha mostrado que el epoxiconazol tiene una eficacia relativa en el control de *C. parasitica*. El tratamiento químico con epoxiconazol bajo condiciones controladas, podría ser una alternativa en determinadas situaciones como producción de plantas en viveros o mantenimiento de ejemplares singulares, siendo una parte de la

estrategia integrada de control. Sin embargo, es necesario llevar a cabo estudios sobre modo de acción, concentración, intervalo de aplicación, fitotoxicidad, y desarrollo de resistencia, entre otros, antes de proponer la utilización de este producto en castaño.

7. *Diplodina castaneae*, responsable de la formación de chancros en castaño, se ha aislado junto con *C. parasitica* de los chancros cicatrizados o en estado de cicatrización muestreados en este trabajo. En esta tesis se cita por primera vez su presencia en España, momento hasta el que ha podido pasar desapercibido por la acción de *C. parasitica*. Además, la caracterización filogenética y morfológica realizada, ha llevado a reclasificarlo taxonómicamente dentro del género *Sirococcus*, como *S. castaneae*.

8. *Gnomoniopsis castaneae* se cita por primera vez en España en el presente trabajo y es responsable de la podredumbre de la castaña, además de la formación de chancros en castaño, al igual que *C. parasitica* y *S. castaneae*. Su presencia ha sido puntual por lo que se desconoce si es de nueva introducción o también ha podido estar enmascarado por la acción devastadora de *C. parasitica*.

CONCLUSIONS

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1. In all the councils sampled in this research, chestnut trees have shown inactive cankers, meaning healed and/or healing cankers, which are typical from trees with hypovirulence (hv).

2. Diversity of types of vegetative compatibility (vc) in the region remains low, with the three types previously reported. The proportions also remains the same, EU-1 being the main vc type, followed by EU-13 and EU-3. This low diversity of vc types places the Asturian chetnuts stands into an ideal situation to apply biological control.

3. A low prevalence of isolates (3.48%) showed morphological features typical of hvs, such as low sporulation and pigmentation, without having observed significant differences between the number of isolates originating from active cankers and from healed or healing cankers.

4. In all the isolates characterized as potentially hypovirulent (hvs), the hypovirus CHV-1 was detected, thus the natural presence of hvs isolates in Asturias has been verified.

5. The presence of two hypoviral subtypes in Asturias, the subtype E (lineage A4-B1) and the subtype D (lineage A4-B2), which was first reported in Spain, makes the country one of the highest subtype diversity spots and increases the probability of success in the disease control. There are no studies about the effectiveness of the CHV-1-E virus as control agent yet, but the CHV1-D virus is a good alternative to initiate the implementation of biological control programs in the region.

6. The *in vivo* assay has shown that epoxiconazole has a relative effectiveness to control *Cryphonectria parasitica*. Chemical control with epoxiconazole under managed conditions could be an alternative in particular situations such as the production of plants in nurseries or the maintenance of singular specimens, as

being part of the integrated control strategy. Nevertheless, it is necessary to carry out studies about the mode of action, product concentration, application intervals, phytotoxicity, and the development of resistance, among others, before proposing the use of this product in chestnut trees.

7. *Diplodina castaneae*, responsible for the formation of cankers in chestnut trees, has been isolated together with *C. parasitica* from healed or healing cankers sampled in this study. Its presence in Spain is first reported in this thesis, until that moment it had been unnoticed due to the action of *C. parasitica*. Furthermore, with the phylogenetic and morphologic characterization done, it could be taxonomically reclassified into the genus *Sirococcus*, as *S. castaneae*.

8. *Gnomoniopsis castaneae* is first reported in Spain in the present thesis and it is responsible for the chestnut rot as well as the formation of cankers in the chestnut bark, the same as *C. parasitica* and *S. castaneae*. Its presence has been a one-time find so far, therefore it is unknown if it is a new introduction or if it was also disguised by the devastating action of *C. parasitica*.

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ARTÍCULOS

ARTÍCULO I

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Título

Presencia de aislamientos de *Cryphonectria parasitica* infectados por hipovirus en el norte de España: una situación alentadora para el control biológico del chancro del castaño en los bosques asturianos

Resumen

La presencia de aislamientos hipovirulentos (infectados por hipovirus) del hongo causante del chancro del castaño, *Cryphonectria parasitica*, en Asturias, norte de España, se describe por primera vez mediamte evaluaciones morfológicas y moleculares. La hipovirulencia fue detectada en 16 de los 69 concejos donde el hongo fue previamente observado. En total, 30 de los 863 aislamientos de *C. parasitica* analizados resultaron estar infectados por hipovirus. Los aislamientos pertenecían a los dos tipos de compatibilidad vegetativa (cv) principales (EU-1 y EU-13) de la región. Los hipovirus asturianos fueron caracterizados genéticamente por secuenciación de las regiones específicas ORF A y ORF B del ARN hipoviral. Los resultados muestran que el 70% de los aislamientos hipovirulentos investigados contenían el subtipo español (subtipo E) mientras que el 30% de los aislamientos contenían el subtipo alemán (D) del hipovirus 1 de *Cryphonectria* (CHV-1). La presencia de aislamientos hipovirulentos compatible con los tipos de cv dominantes y la baja diversidad de tipos de cv en esta región, favorece la aplicación de control biológico. Se podría realizar un esfuerzo inicial en el tratamiento de la enfermedad aplicando el hipovirus del subtipo D, mientras se deberían llevar a cabo más investigaciones relacionadas con las propiedades de biocontrol del hipovirus del subtipo E.

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Occurrence of hypovirus-infected *Cryphonectria parasitica* isolates in northern Spain: an encouraging situation for biological control of chestnut blight in Asturian forests

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Abstract The presence of hypovirulent (hypovirus-infected) isolates of the chestnut blight fungus *Cryphonectria parasitica* in Asturias, northern Spain, is first reported by using both morphological and molecular assessments. Hypovirulence was detected in 16 of the 69 councils where the fungus was previously observed. In total 30 of the 863 *C. parasitica* isolates analyzed were found to be hypovirus-infected. The isolates belonged to the two main vegetative compatibility (vc) types (EU-1 and EU-13) of the region. Asturian hypoviruses were genetically characterized by sequencing of ORFA and ORFB specific regions of the hypoviral RNA. The results show that 70% of the investigated hypovirulent isolates contained the Spanish subtype (subtype E) while 30% isolates the German subtype (D) of *Cryphonectria* hypovirus 1 (CHV-1). The presence of hypovirulent isolates compatible with the dominant vc types and the low vc diversity in this region favours the application of biological control. An initial disease management effort could be applied by using the hypovirus of subtype D, whereas further research related to the biocontrol properties of the subtype E hypovirus should be conducted.

Keywords Forest pathogen · *Castanea sativa* · RT-PCR detection · *Cryphonectria hypovirus* · Vegetative compatibility · Healing cankers

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Introduction

The ascomycete fungus *Cryphonectria parasitica* (Murrill) Barr, is the causal agent of chestnut blight, one of the most severe diseases of chestnut (*Castanea spp.*). The pathogen, introduced from East Asia, was first observed in the early twentieth century in North America where it was responsible for the devastation of American chestnut (*Castanea dentata* [Marsh.] Borkh) stands (Anagnostakis 1988). In Europe, chestnut blight was first observed in 1938 in Italy (Biraghi 1946), and since then, it has been recorded in almost all chestnut growing areas (Robin and Heiniger 2001; Bragança et al. 2007; Krstin et al. 2008, 2011; Akıllı et al. 2009; Erincik et al. 2011; Peters et al. 2012; Prospero et al. 2013; Risteski et al. 2013) causing the decline of European chestnut (*Castanea sativa* Mill.). However, whereas the American chestnut was nearly eliminated by chestnut blight (Anagnostakis 1988), the European chestnut has recovered from the disease in many areas due to the spontaneous appearance of hypovirulence (Heiniger and Rigling 1994; Robin et al. 2000; Homs

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et al. 2001; Milgroom and Cortesi 2004; Perlerou and Diamandis 2006; Sotirovski et al. 2006; Montenegro et al. 2008; Zamora et al. 2008; Bryner et al. 2012; Peters et al. 2014).

Hypovirulence refers to an infection of *C. parasitica* by hypoviruses, which cause a reduction in fungal virulence and in its reproductive capacities, notably sexual reproduction. The phenomenon of hypovirulence was first described by Grente (1965), and later intensively studied by different authors (for reviews see Griffin 1986; Heiniger and Rigling 1994; MacDonald and Fullbright 1991; Milgroom and Cortesi 2004; Nuss 1992; Van Alfen et al. 1975; Van Alfen 1982). *Cryphonectria* hypoviruses (CHV) are RNA viruses in the genus Hypovirus located in the cytoplasm of the fungus. They are vertically transmitted into asexual spores (conidia) but not into sexual spores of the fungus (Prospero et al. 2006). Horizontal virus transmission can occur via hyphal anastomosis between virus-infected and virus-free isolates (Anagnostakis and Day 1979; Choi and Nuss 1992). The formation of hyphal anastomosis depends on vegetative compatibility (vc), which is controlled by several vegetative incompatibility (*vic*) genes. Hypovirus transmission between fungal isolates of different vc types, meaning individuals that have different alleles at one or several *vic* locus, is restricted (Cortesi et al. 2001). Therefore, the level of vc type diversity is considered a critical factor for the success of biological control using hypovirulence. Another factor, which affects hypovirulence is the mode of reproduction. Sexual reproduction of *C. parasitica* is an obstacle to the dissemination of the hypovirus since it only contributes to the spread of the virulent form of the fungus (Prospero et al. 2006). Furthermore, the occurrence of sexual reproduction could lead to an increase in vc type diversity through recombination of polymorphic *vic* genes (Milgroom and Cortesi 1999).

The most researched hypovirus type is *Cryphonectria* hypovirus 1 (CHV-1), which has a 12.7 kb genome with two open reading frames (ORFs) encoding multifunctional polyproteins. CHV-1 is the only member of the genus Hypovirus that have been reported in Europe to date. Five different CHV-1 subtypes have been identified based on sequence variations found within ORF A (Gobbin et al. 2003). Subtype I was the most widespread, being reported in France (Allemann et al. 1999), Spain (Homs et al. 2001), Italy and Switzerland (Gobbin et al. 2003), Croatia (Krstin et al. 2008), Macedonia (Sotirovski et al. 2006), Slovenia (Krstin et al. 2011) and Turkey (Akıllı et al. 2013). The other subtypes have a more restricted distribution, both subtypes F1 and F2 being observed in France (Robin et al. 2010), F1 in Spain (Zamora et al. 2012) and F2 in Eastern Turkey (Akıllı et al. 2013); whereas subtypes E and D were detected in Spain and Germany, respectively (Gobbin et al. 2003; Peters et al. 2014). By analysing partial sequences of both ORF A and ORF B, Feau et al. 2014 revealed a more complex subtype pattern of CHV-1 in Europe. They defined several lineages of CHV-1 with A1-B1 comprising subtype F1, A2-B1 subtype F2, A3-B3 subtype I, A4-B1 subtype E, and A4-B2 subtype D.

In the Principado de Asturias, an autonomous community in northern Spain, chestnut blight was first reported in 1982, and by 2000, it had spread throughout much of the chestnut growing area (Valdezate et al. 2001). With more than 77,000 ha of *Castanea sativa* stands (Ortega et al. 2011), chestnut is one of the most important tree species in the Asturian forests. According to previous works by González-Varela et al. (2011), a low diversity of vc types was observed in Asturias where only three vc types were found. Populations with low vc types diversity present an ideal situation for deploying hypovirulence (Heiniger and Rigling 1994; Milgroom and Cortesi 2004; González-Varela et al. 2011). Considering the seriousness of the chestnut blight disease and the socio-economic significance of the chestnut tree in this region, we initiated a first study on hypovirulence in Asturias in order to evaluate the natural potential for biological control in this region. For this purpose, the main objectives of the present study were: (i) to sample *C. parasitica* from chestnut trees with symptoms of natural hypovirulence; (ii) to assess morphological features of *C. parasitica* isolates; iii) to determine the vc type of the isolates; (iv) to screen the isolates for the presence of CHV-1; and (v) to characterize the hypoviruses by sequence analysis.

Material and methods

Sample collection and fungal isolates

For the years 2013 and 2014, 60 chestnut trees presenting symptoms of hypovirulence (i.e. superficial or callusing chestnut blight cankers) were sampled. They were located in six councils of Asturias, three of them (Aller, Lena and Llanera) belonging to the central part

and three (Colunga, Peñamellera Baja and Villaviciosa) to the eastern part of Asturias. For isolation of *C. parasitica*, a bark sample was removed with a sterile cork-borer (5 mm diameter) from the middle, upper and lower margin of each canker. Only one canker per tree was sampled. Bark samples were plated on 1.5% water agar and Petri plates were maintained at room temperature for 5 days. Mycelium from one bark sample per canker (or from more samples in case isolates showed different culture morphology) was transferred to a new plate containing potato dextrose agar (PDA) and incubated at room temperature. In addition to the isolates collected in this study, 813 isolates obtained from 69 councils of Asturias in previous samplings (Valdezate et al. 2001; Trapiello 2010; González-Varela et al. 2011) and recovered from the fungal culture collection of the Phytopathology Laboratory of Principado de Asturias (LPPAF) were used.

Vegetative compatibility (vc) typing

The vc type of the isolates was determined by assessing the barrage/merging response of pairs of isolates grown together on agar medium (Anagnostakis et al. 1986). Six pairings were inoculated into each PDA plate using the conidial inoculation method as described by Cortesi et al. 1998. All isolates from the same site were first paired in all combinations to identify compatible isolates of the same vc type. Next, one isolate was randomly chosen to represent each vc type in that site. All site-specific testers were then paired with each other in all combinations to get a common collection of vc type testers in Asturias. After that, representative isolates from each vc type were paired with the European vc type testers EU-1 to EU-64 (Cortesi and Milgroom 1998). Each pairing was repeated at least three times. Incubation was conducted at 25 °C for 7 days in the dark, followed by 7 days under day light conditions on the laboratory bench. After this time, pairs were considered incompatible when a barrage was observed between the two mycelia, and compatible when both mycelia completely merged. Finally, all isolates were assigned to known European vc types.

Morphological characterization

863 *C. parasitica* isolates (813 from the LPPAF fungal culture collection and 50 isolated in this work) were tested for typical morphological features of hypovirulent isolates such as reduced pigmentation and sporulation (Bissegger et al. 1997). For this analysis, isolates were cultured on PDA and incubated for 7 days at room temperature in the dark, followed by 7 days at room temperature under day light conditions. Finally, the potentially hypovirus-infected isolates, according to the morphological assessment, were analyzed for the presence of CHV1 by total RNA extractions and hypovirus-specific RT-PCR. 30 isolates with normal looking culture morphology on PDA (i.e. orange pigmentation and abundant sporulation), three from a different council randomly selected, were included in this analysis as controls.

Total RNA extraction, first strand cDNA synthesis and polymerase chain reaction (PCR)

A sample of 60 isolates was tested for the presence of CHV-1. This sample included all isolates which showed hypovirulent morphology and the same number of normal looking isolates which were randomly selected. Total RNA extraction was carried out by using the Plant/Fungi RNA Purification Kit (Norgen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from the RNA with random hexamer primers (Fermentas) using the Maxima Reverse Transcriptase (Promega, Madison, WI, USA) as described by Allemann et al. (1999). PCR amplification was conducted with the specific primers hvep 1 (Gobbin et al. 2003) and EP 721–4 (Bryner et al. 2012) to amplify a 693 bp region of the open reading frame A (ORF A) corresponding to positions 1473–2165 in the nucleotide sequence of CHV1/Euro7 (Chen and Nuss 1999). In cases where amplification did not work, primers hvep 1 and hvep 2 (Gobbin et al. 2003) were used to amplify a 353 bp region of ORF A. The primers ORF B-12F and ORF B-12R were used to amplify a 780 bp region of open reading frame B (ORF B) corresponding to positions 6252–6991 in the nucleotide sequence of CHV1/EP713 (Feau et al. 2014).

Hypovirus sequence analysis and subtype determination

In order to determine the CHV1 subtype of the isolates, the ORF A and ORF B specific regions of the hypoviral RNA were sequenced by using the primers hvep 1/EP721–4 and ORF B-12F/ORF-B-12R, respectively. Forward and reverse sequences of each isolate were

assembled and edited to obtain a consensus sequence. The sequences were then aligned and used to construct a phylogenetic tree using the unweighted pair group method with arithmetic mean (UPGMA) together with the Kimura 80 (K80) model. Separate analysis of the ORF A and ORF B regions was first performed, followed by an analysis of both regions concatenated. All data were analyzed with the software CLC Genomics Workbench version 8.0.1 (http://www.clcbio.com). One reference sequence for each previously reported CHV1 subtype or cluster according to Gobbin et al. 2003 and Feau et al. 2014, respectively (M1372, subtype D / cluster A4B2; M1147, subtype E / cluster A4B1; EP713, subtype F1 / cluster A1B1; 2103, subtype F2 / cluster A2B1; Euro7, subtype I / cluster A3B3) was included in the phylogenetic analysis. Additional reference sequences for each genetic cluster were obtained from Feau et al. 2014. Sequences for each hypovirus subtype previously reported in Spain were also included in the phylogenetic tree derived from ORF A. All sequences obtained in this work were deposited in GenBank (see Table 1 for accession numbers).

Statistical analysis

The presence or absence of virus infection in chestnut trees with and without healing cankers was calculated. Virus infection data were first analyzed by Levene's test to check if variance homogeneity was fulfilled. As it was fulfilled, data were analyzed using one-way ANOVA with virus presence as independent variable and healing canker as dependent variable. The analysis was performed using the software package IBM SPSS Statistics 19 (c Copyright 1989, 2010 SPSS, Inc.).

Results

Fungal isolates

Chestnut blight was observed in the two sampled areas, central and eastern part of the Principado de Asturias. Healing cankers were found in both areas, a total of 50 *C. parasitica* isolates being recovered from them. These isolates were subjected to further analyses, together with the 813 *C. parasitica* isolates recovered from the LPPAF culture collection.

Vegetative compatibility (vc) types

Three vc types were found among the 50 isolates of *C. parasitica*, all of which were compatible with known vc types: EU-1, EU-13 and EU-3 (Cortesi and Milgroom 1998) and previously reported in Asturias (González-Varela et al. 2011). 46 of 60 isolates were compatible with EU-1 (92%), three isolates were compatible with EU-13 (6%) and one isolate was compatible with EU-3 (2%). EU-1 was the dominant type being found in all the six councils sampled in this work.

Culture morphology

Among the 813 isolates from the LPPAF culture collection, a total of 27 isolates (proportion: 3.32%) originating from 16 councils of Asturias (Table 1) showed typical morphological features of hypovirulent isolates such as reduced pigmentation and sporulation on PDA. Among the 50 isolates collected in this work from chestnut blight cankers with symptoms of hypovirulence, three isolates (proportion: 6%) originating from three different councils (Aller, Peñamellera Baja and Villaviciosa) showed typical morphological characteristics of hypovirulent isolates. In total, 30 isolates (3.48%) out of 863 analyzed presented morphological characteristics of hypovirulence. All these isolates identified as potentially hypovirus-infected were subsequently subjected to molecular analysis.

Molecular characterization and phylogenetic analysis

All the 30 normal looking isolates were tested negative for CHV1 by virus-specific RT-PCR. In contrast, all the 30 isolates, which showed typical morphological features of hypovirus-infected isolates, were tested positive. From these, 20 viral isolates could only be sequenced in the ORFA region, whereas for the remaining 10 viral isolates sequences of the ORF A and ORF B regions were obtained. An alignment of the 10 sequences of both CHV1 genomic regions, including reference sequences retrieved from the NCBI database was constructed.

Considering the ORF A sequences, the phylogenetic tree grouped all viral isolates from Asturias together with the reference isolates of subtype E (M1147) and D (M1372) with 99% bootstrap support (Fig. 1). This group corresponds to the CHV1 genetic cluster A4 defined by Feau et al. (2014), which also contains viral

Table 1 Characterization of hypovirulent isolates of *Cryphonectria parasitica* obtained from Asturias, northern Spain

Isolate number	Origin of isolate	Vc type	Accession numbers of ORF A sequenced region	RT-PCR ORFB	Accession numbers of ORF B sequenced region	CHV1 cluster (subtype) according to ORF A region	CHV1 cluster (subtype) according to ORF B region	CHV1 cluster (subtype) according to both regions
91	Aller	EU-1	KY002090	−		A4[1] (D)[2]		
201	Caravia	EU-1	KY002091	−		A4[1] (D)[2]		
202	Caravia	EU-1	KY002092	−		A4[1] (D)[2]		
268.1	Peñamellera Baja	EU-1	KY002093	−		A4[1] (D)[2]		
268.2	Peñamellera Baja	EU-1	KY002094	+	KY002080	A4[1] (E)[2]	B2[1] (D)[2]	A4-B2[1] (D)[2]
292	Peñamellera Alta	EU-1	KY002095	−		A4[1] (D)[2]		
305	Peñamellera Baja	EU-1	KY002096	−		A4[1] (D)[2]		
313	Parres	EU-1	KY002097	−		A4[1] (D)[2]		
314	Parres	EU-1	KY002098	+	KY002081	A4[1] (D)[2]	B1[1] (E)[2]	A4-B1[1] (E)[2]
319	Nava	EU-1	KY002099	−		A4[1] (D)[2]		
326	Lena	EU-1	KY002100	+	KY002082	A4[1] (E)[2]	B2[1] (D)[2]	A4-B2[1] (D)[2]
327	Lena	EU-1	KY002101	−		A4[1] (E)[2]		
328	Lena	EU-1	KY002102	+	KY002083	A4[1] (E)[2]	B2[1] (D)[2]	A4-B2[1] (D)[2]
356	Caravia	EU-1	KY002103	+	KY002084	A4[1] (E)[2]	B1[1] (E)[2]	A4-B1[1] (E)[2]
358	Peñamellera Baja	EU-13	KY002104	+	KY002085	A4[1] (E)[2]	B1[1] (E)[2]	A4-B1[1] (E)[2]
368	Peñamellera Alta	EU-1	KY002105	−		A4[1] (D)[2]		
390	Cangas de Narcea	EU-1	KY002106	−		A4[1] (D)[2]		
391	Cangas de Narcea	EU-1	KY002107	−		A4[1] (E)[2]		
398.1	Grao	EU-1	KY002108	−		A4[1] (D)[2]		
421	San Martín del Rey Aurelio	EU-1	KY002109	−		A4[1] (D)[2]		
485	Villaviciosa	EU-1	KY002110	−		A4[1] (D)[2]		
552	Onís	EU-1	KY002111	+	KY002086	A4[1] (D)[2]	B1[1] (E)[2]	A4-B1[1] (E)[2]
572	Morcín	EU-1	KY002112	−		A4[1] (D)[2]		
629	Carreño	EU-1	KY002113	−		A4[1] (D)[2]		
745	Lena	EU-1	KY002114	−		A4[1] (D)[2]		
746	Lena	EU-1	KY002115	+	KY002087	A4[1] (E)[2]	B1[1] (E)[2]	A4-B1[1] (E)[2]
767	Villayón	EU-1	KY002116	−		A4[1] (D)[2]		
843.1	Villaviciosa	EU-1	KY002117	+	KY002088	A4[1] (E)[2]	B1[1] (E)[2]	A4-B1[1] (E)[2]
869.2	Peñamellera Baja	EU-13	KY002118	+	KY002089	A4[1] (E)[2]	B1[1] (E)[2]	A4-B1[1] (E)[2]
884.1	Aller	EU-1	KY002119	−		A4[1] (E)[2]		

[1] CHV-1 cluster according to Feau et al. 2014

[2] CHV-1 subtype according to Gobbin et al. 2003

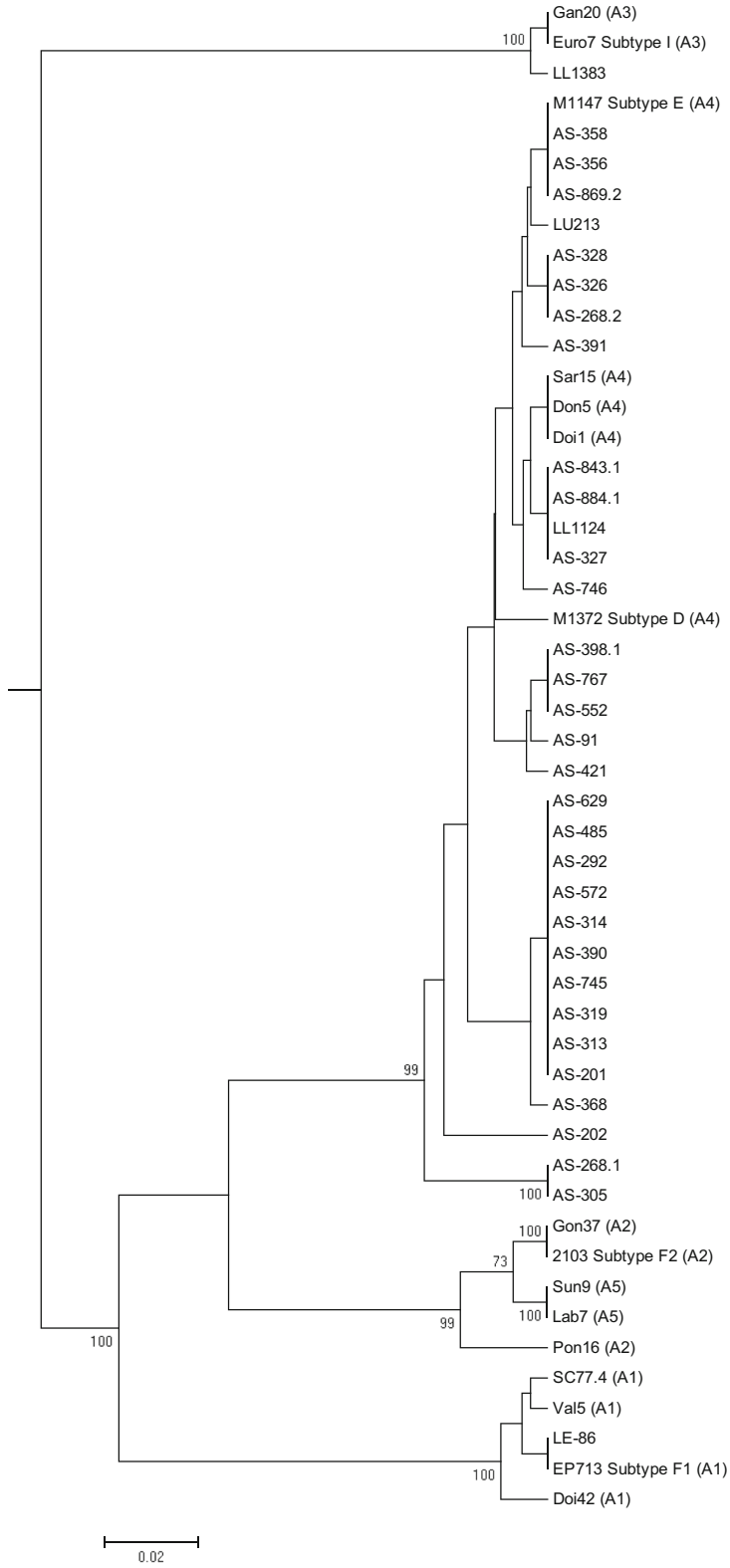

Fig. 1 UPGMA tree derived from partial ORF A sequences of *Cryphonectria* hypovirus (CHV1) isolates from Asturias (codified with "AS"). Sequences of references isolates for each known CHV1 subtype (M1372, M1147, EP713, 2103, Euro7) (Gobbin et al. 2003) and for each known CHV1 genetic cluster (Don5, Gon37, Doi42, Val5, SC77.4, Sun9, Lab7, Pon16, Gan20, Sar15, Doi1) (Feau et al. 2014) were included. Sequences of isolates from northern Spain: LU213 (Aguín et al. 2008), LE-86 (Zamora et al. 2012), LL1124 and LL1383 (Castaño et al. 2015) were also included. Bootstrap values >70% resulting from 1000 replicates are shown on the internodes. The CHV-1 cluster designation of the reference strains according to Feau et al. 2014 is given in brackets

isolates from Navarra, Spain (Don5) and France (Sar15 and Doi1) (Feau et al. 2014). The viral isolates LU213 (Aguín et al. 2008) and LL1124 (Castaño et al. 2015) from northern Spain, Galicia and Cataluña respectively, also grouped in this cluster. Other isolates from northern Spain, LE86 from León (Zamora et al. 2012), which was previously defined as CHV1 subtype F1 and LL1383 from Cataluña (Castaño et al. 2015), which was previously defined as CHV1 subtype I, were assigned to the A1 and A3 cluster, respectively.

In respect to ORF B, three important clusters were resolved and supported with a high bootstrap value (1.00) (Fig. 2). One cluster consisted of seven hypoviruses from *C. parasitica* isolates originating from Asturias, of the 10 collected during this study, which grouped together with the reference isolate M1147 (subtype E / cluster B1). The remaining three hypoviruses (AS-328, AS-326 and AS-268.2) grouped into the B2 cluster with M1371 as the reference isolate of subtype D, and other isolates from northeastern Spain (Sun9, Lab7 and Val5) and France (Pon16 and SC77.1) (Feau et al. 2014). Reference isolates EP713 and 2103, defined as subtype F1 and F2 respectively, were included in cluster B1 with viral isolates from Spain (Don5) and France (Doi42, Gon37) (Feau et al. 2014). For the joined sequences of the ORF A and ORF B regions (Fig. 3), results were the same as obtained for the ORF B region. Seven viral isolates (AS-358, AS-869.2, AS-356, AS-552, AS-746, AS-843.1, and AS-314) grouped together with the reference strain M1147 thus belonging to the CHV-1 cluster A4B1, which corresponds to subtype E (Table 1). Included in this cluster is the Spanish viral strain Don5 (Feau et al. 2014), which originated, as M1147 from Navarra. The remaining three viral isolates from Asturias (AS-268.2, AS-328, AS-326) belonged to the CHV-1 cluster A4B2 (Table 1), represented by the subtype D reference strain M1372.

Prevalence of virus infection

Regarding the isolates from the LPPAF culture collection, the hypovirus prevalence was 3.3%, whereas among the isolates sampled in the present study the prevalence was 6.0%. This difference observed between isolates from the culture collection recovered from active cankers, and isolates from this work collected from healing cankers was statistically not significant ($\chi 2 = 28.958$, df = 1, $P = 0.333$).

Discussion

Morphological features in culture and molecular characterization (RT-PCR and sequencing) consistently indicated that 30 out of 863 *C. parasitica* isolates from Asturias analyzed in this study were infected by CHV-1.

In concordance with previous studies (Van Alfen et al. 1978; Elliston 1985; Choi and Nuss 1992) we observed that morphology assessment is a reliable criterion to determine which isolates are infected by the hypovirus CHV-1. All isolates with a white cultural phenotype were tested positive for CHV-1 whereas all orange isolates were found to be hypovirus-free. This result is in line with other studies where all *C. parasitica* isolates presenting the white phenotype contained CHV-1 (Montenegro et al. 2008; Krstin et al. 2011; Peters et al. 2012; Zamora et al. 2012).

The 30 hypovirus-infected isolates originated from 16 different Asturian councils (Table 1). Twelve of these isolates were collected in seven councils located in the central part of Asturias, 15 isolates were from seven councils in the eastern part, and three isolates from two councils in the western part. This result indicates that hypovirulence is present across Asturias, however, with an overall low prevalence (3.48%). The prevalence of hypovirulence appears to be much lower in the western part compared to the central and eastern parts. This could be due to the fact that in this area the incidence of chestnut blight is also much lower too. In western Asturias, chestnut forests are less abundant and the movement of chestnut wood is less intense than in other parts of the region (Valdezate et al. 2001). There are still councils located in this area where chestnut blight so far has not been detected (González-Varela et al. 2011).

Asturias is a region with a very low diversity of vc types, only three being previously detected by González-Varela et al. (2011), and no more vc types being detected to date. In that study, EU-1 was found to be the most frequent vc type of the region (95%) followed by EU-13 (5%) and EU-3 (<1%). Similar frequencies of these three vc types (92%, 6% and 2%, respectively) were obtained in the present work. These results are similar to other studies from northern Spain where also low vc type diversity was observed. For example, in Galicia, the neighbouring region of Asturias, four vc types were reported; EU-1 and EU-66 by Montenegro et al. (2008), and EU-9 and EU-72 by Aguín et al. (2008). In Cataluña, northeastern Spain, five vc types (EU-2, EU-1, EU-5, EU-67 and EU-12)

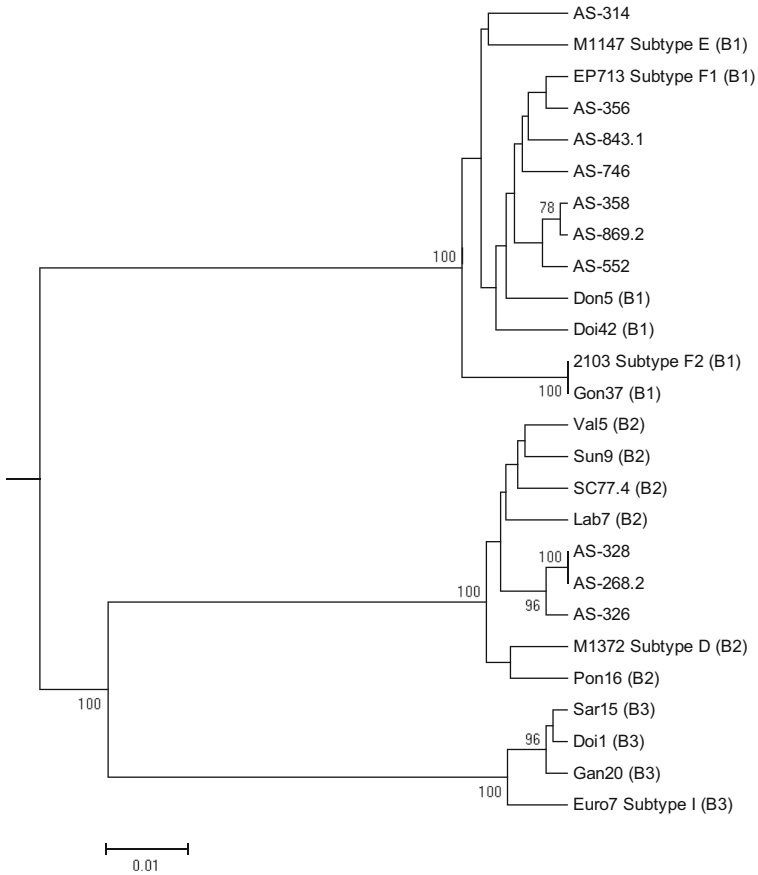

Fig. 2 UPGMA tree derived from partial ORF B sequences of *Cryphonectria* hypovirus (CHV1) isolates from Asturias (codified with "AS"). Sequences of references isolates for each known CHV1 subtype (M1372, M1147, EP713, 2103, Euro7) (Gobbin et al. 2003) and for each known CHV1 genetic cluster (Don5, Gon37, Doi42, Val5, SC77.4, Sun9, Lab7, Pon16, Gan20, Sar15, Doi1) (Feau et al. 2014) were included. Bootstrap values >70% resulting from 1000 replicates are shown on the internodes. The CHV-1 cluster designation of the reference strains according to Feau et al. 2014 is given in brackets

were observed by Castaño et al. 2015. Nevertheless, in Castilla y León, a region which borders Asturias on the south, the diversity was higher, five known vc types (EU1, EU11, EU12, EU28 and EU66) and six not previously known vc types being found (Zamora et al. 2012).

Among the 30 hypovirus-infected isolates, only two vc types were found with 28 isolates belonging to EU-1 (93.3%) and two isolates to EU-13 (6.6%). Noteworthy, the two EU-13 isolates originated from the same council (Peñamellera Baja).

Hypovirus infection of *C. parasitica* typically leads to superficial cankers colloquially called healing or healed cankers because they do little or no harm to the tree (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). Regarding prevalence of the hypovirus in healing and active cankers, no significant difference was detected. Thus, by sampling healing cankers the recovery of hypovirus-infected isolates could not be significantly increased. Similar results were also recently reported from Cataluña in Spain, where a low prevalence of CHV1 among healing cankers was observed (Castaño et al. 2015). These findings are consistent with the study of Bryner et al. (2013), which revealed that presence or absence of hypovirus infection in *C. parasitica* is not clearly correlated with canker morphology. Incomplete movement of the hypovirus within the fungal mycelium or a possible disappearance of CHV1 in healed cankers (Bryner et al. 2012) might explain the low recovery of hypovirus-infected isolates from healing cankers.

The low prevalence of hypovirus-infected isolates observed in Asturias is in line with other European regions such as Portugal, where only one hypovirus-infected isolate was found among 617 isolates (Bragança et al. 2007) or Germany with a hypovirus prevalence of only 10% (Peters et al. 2014). In contrast, a much higher prevalence of hypovirulence has been reported in many other regions in Europe. For example in local chestnut stands in south-eastern France, 31 to 90% of the cankers were found to be hypovirus-infected (Robin et al. 2010), in southern Switzerland, 43 to 52%; and in the Balkans, 44 to 85% (Bryner and Rigling

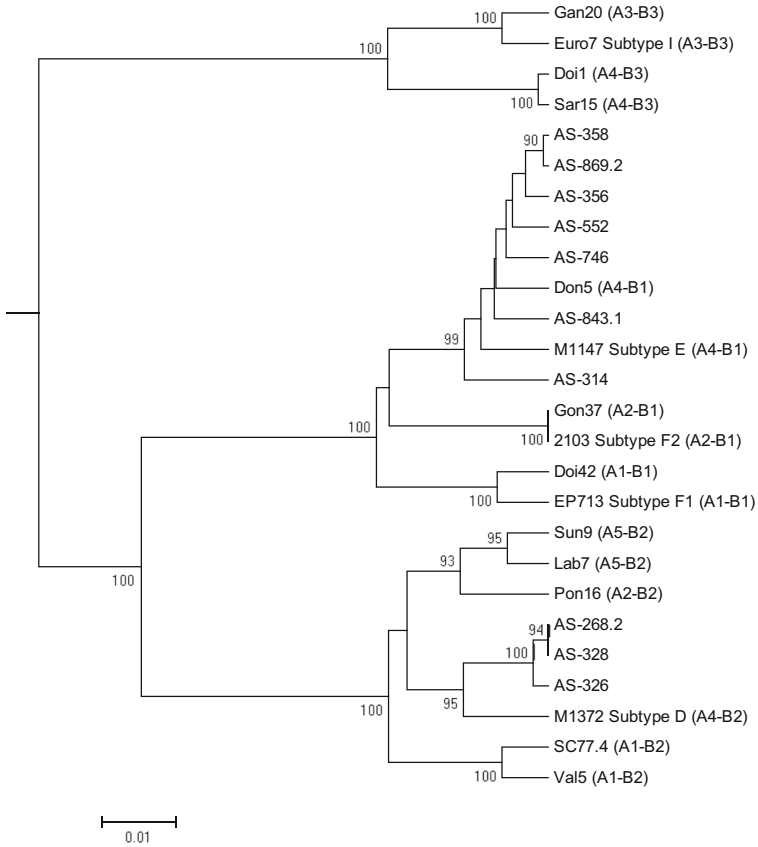

Fig. 3 UPGMA tree derived from concatenated sequences of ORF A and ORF B of *Cryphonectria* hypovirus (CHV1) isolates from Asturias (codified with "AS"). Sequences of references isolates for each known CHV1 subtype (M1372, M1147, EP713, 2103, Euro7) (Gobbin et al. 2003) and for each known CHV1 genetic cluster (Don5, Gon37, Doi42, Val5, SC77.4, Sun9, Lab7, Pon16, Gan20, Sar15, Doi1) (Feau et al. 2014) were included. Bootstrap values >70% resulting from 1000 replicates are shown on the internodes. The CHV-1 cluster designation of the reference strains according to Feau et al. 2014 is given in brackets

2012b). A weak correlation between the year of first observation of chestnut blight and the appearance of healing cankers and white isolates was reported by Robin and Heiniger (2001). Their study suggested that there is a time lag of 20 to 30 years between the two observations. Chestnut blight in Spain was first detected in 1940 (Molina 1984) whereas healing cankers and white isolates were not detected until 48 years later, in 1988 (Allemann et al. 1999). In Asturias, *C. parasitica* was first reported in 1982 (Valdezate et al. 2001), hypovirus-infected isolates not being detected until the present work. The recent detection of the hypovirus could explain the low prevalence of hypovirulence currently found in this region, i.e. the hypovirus might be in an early stage of spreading. Similar results were obtained from other regions in northern Spain. In Galicia, northwestern Spain, four hypovirus- infected isolates among 610 *C. parasitica* isolates were found (Aguín et al. 2008). In a study in Galicia and León, a province of Castilla y León, 15 out of 539 isolates were found to be hypovirus-infected (Montenegro et al. 2008). All these 15 isolates were sampled in León resulting in a prevalence of 4.6% in this province. A low prevalence of hypovirulence (3.1%) in León has also been reported by Zamora et al. (2012) and attributed to the relative recent introduction (1978) of chestnut blight in this region. A low level of hypovirulence has often been observed in areas in Europe where *C. parasitica* has recently established (Hoegger et al. 2000). In northeastern Spain, a low prevalence of CHV1 was also observed: a recent study conducted in Cataluña revealing 35 hypovirulent isolates among 312 analyzed (Castaño et al. 2015).

With respect to the diversity of CHV1, three subtypes have been observed so far in Spain: Subtype E in Navarra (Allemann et al. 1999) corresponding to lineage A4B1 (Feau et al. 2014), subtype F1 in León (Montenegro et al. 2008; Zamora et al. 2012) corresponding to lineage A1B1 (Feau et al. 2014), and subtype I in Cataluña (Homs et al. 2001) corresponding to lineage A3B3 (Feau et al. 2014). In addition, two more lineages (A1B2 and A5B2) were found in Navarra by Feau et al. 2014. The presence of several CHV1 subtypes or lineages in Spain supports the hypothesis of

multiple introductions of hypovirulence (Montenegro et al. 2008; Castaño et al. 2015). The same situation was observed in France where three subtypes (F1, F2 and I) were reported (Allemann et al. 1999; Robin et al. 2010). In contrast, in other countries such as Bosnia-Herzegovina, Croatia, Greece, Hungary, Italy, Macedonia, Switzerland, and Slovenia only one subtype (subtype I) was detected (Allemann et al. 1999; Sotirovski et al. 2006; Krstin et al. 2008, 2011; Prospero and Rigling 2013).

In Asturias we found two CHV1 subtypes, E and D. The subtype E (CHV1 genetic cluster A4-B1) has been previously detected in northern Spain, being first reported in 1998 in Navarra (Allemann et al. 1999), in 2007 in Galicia (Aguín et al. 2008) and recently also in Cataluña (Castaño et al. 2015). Interestingly, the subtype E has not been found in Castilla y León, the neighbouring region of Asturias. Likewise, subtype F1, which occurs in León was not detected in Asturias. Possibly the mountain range Cordillera Cantábrica between both regions acts as a barrier for hypovirus spread in both directions. The subtype D (CHV1 genetic cluster A4-B2) has not been previously detected in Spain, being first reported in the present work. Therefore, this finding increases the number of CHV1 subtypes present in Spain and indicates that this country harbours one of the highest diversity of CHV1 subtypes in Europe. Concerning the distribution of CHV1 subtypes across Asturias, both E and D subtypes were present in the central (Lena) and eastern part (Peñamellera Baja) of the region. In terms of ecological fitness, more studies should be conducted for both subtypes, despite that the German hypovirus subtype (D) was previously studied and supposed to be suitable as biological control agent (Bryner and Rigling 2011 and Bryner and Rigling 2012a; Peters et al. 2012).

Results of our study support the clear split of the CHV1 subtypes E and D into two clusters based on additional sequencing in ORFB. Most of our viral isolates (70%) were assigned to the genetic cluster A4-B1, which also includes the reference strain of subtype E (Feau et al. 2014). In contrast to several other CHV1 clusters, which apparently derived from recombination events, the A4-B1 cluster was considered non-recombinant (Feau et al. 2014). This cluster possibly comprises CHV-1 isolates, which were originally introduced from Asia into France or northern Spain, and which subsequently served as ancestral isolates in some of the recombination events (Feau et al. 2014). The remaining Asturian viral isolates (30%) were assigned to the genetic cluster A4-B2, which also includes the reference strain of subtype D. This cluster is supposed to have been derived from a recombination event involving ancestral isolates in the A4-B1 (subtype E) and A3-B3 (subtype I) lineages (Feau et al. 2014). Both parental lineages are present in Spain suggesting that the recombination event might have occurred here. The low prevalence of subtype D in Asturias compared to subtype E, and the absence of this subtype in other parts of Spain to date, could indicate that the recombination event was quite recent and the subtype D hypoviruses would not have had enough time to spread efficiently.

Interestingly, the CHV1 subtype D has so far only been detected in Germany where no other subtypes are present (Peters et al. 2014). The finding of subtype D in Asturias could indicate that this CHV1 subtype was introduced (after the assumed recombination event) from Spain into Germany. This hypothesis is supported by the fact that the putative ancestral isolates of subtype D are also present in Spain and that chestnut blight has a longer disease history in Spain than in Germany.

The most frequent CHV1 subtype in Asturias, subtype E, is associated with two different vc types (EU-1 and EU-13). This finding could suggest frequent horizontal transmission of subtype E between different *C. parasitica* vc types and promotes its use as biological control agent.

Regarding other Spanish hypovirus isolates there is a lack of ORFB sequence data, i.e. the current subtype classification is only based on ORFA. As shown by Feau et al. (2014) and the present study, a classification based on both regions is needed to compare hypoviruses from Spain and to obtain a broader overview of the diversity and evolution of *Cryphonectria* hypoviruses.

In conclusion, this is the first report about the occurrence of hypovirus-infected *C. parasitica* isolates in Asturias. The natural appearance of hypovirulence together with the low diversity of fungal vc types, are ideal conditions for a sustainable biological control of chestnut blight in this region. Nevertheless, further research is needed to increase the knowledge of the dynamics of the *Cryphonectria*-hypovirus interaction in areas where hypovirulence is naturally established. Two genetically distinct CHV1 subtypes (E and D) were detected in Asturias with subtype D being first reported outside of Germany. Subtype E has been previously reported in other areas in Spain and France, however, compared to subtype D little is known about its interaction with the

fungal host. Further studies about the effects of the subtype E on the fungal host as well as its dissemination and conversion capacity should be conducted before its application as biological control agent. Nevertheless, the presence of CHV1 subtype D in Asturias, previously characterized as suitable for biological control, provides a good alternative to implement a successful biocontrol programme against chestnut blight in Asturias.

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ARTÍCULO II

Trapiello E., González-Varela G. y González A.J. (2015). Chestnut blight control by agrochemicals in *Castanea sativa* under managed conditions. *Journal of Plant Diseases and Protection*, 122, 120–124.

Título

Control del chancro del castaño mediante agroquímicos en *Castanea sativa* bajo condiciones controladas

Resumen

Esta investigación fue llevada a cabo con el objetivo de evaluar la eficacia *in vivo* de tres productos agroquímicos para controlar la enfermedad del chancro del castaño en plantas de *Castanea sativa* infectadas por el hongo *Cryphonectria parasitica* bajo condiciones controladas. Se ensayaron tres productos (epoxiconazol, carbendazima y una mezcla de carbendazima más flutriafol) usando tres métodos diferentes de aplicación: i) método preventivo aplicado por pulverización, ii) método curativo aplicado por pulverización y iii) método curativo aplicado directamente sobre la lesión con un rodillo. Los parámetros longitud de la lesión, esporulación y mortalidad fueron evaluados presentando diferencias estadísticamente significativas. Respecto a los métodos, los resultados mostraron que el método curativo aplicado directamente sobre la lesión es eficaz cuando el patógeno no se ha extendido aún, siendo el método de aplicación más efectivo a corto plazo. Respecto a los productos, se observaron siempre diferencias significativas entre controles y plantas tratadas, siendo el epoxiconazol el fungicida más eficaz para el control del chancro del castaño.

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Chestnut blight control by agrochemicals in *Castanea sativa* under managed conditions

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Abstract

This research was conducted with the aim of evaluating the *in vivo* effectiveness of three agrochemical products to control chestnut blight disease in *Castanea sativa* infected by the fungus *Cryphonectria parasitica* under managed conditions. Three products (epoxiconazole, carbendazim and a mixture of carbendazim plus flutriafol) were tested using three different methods of application: i) preventive method applied by spraying, ii) curative method applied by spraying and iii) curative method applied directly over the lesion with a paint roller. Lesion length, sporulation and mortality parameters were evaluated presenting statistically significant differences. According to the methods, results showed that the curative method applied directly over the lesion is effective when the pathogen has not already spread, being the most effective application method in the short term. Regarding products, significant differences between control and treated chestnut plants were always observed, epoxiconazole being the most effective fungicide for chestnut blight control.

Key words: chemical control, chestnut tree, *Cryphonectria parasitica*, epoxiconazole

Non-standard abbreviations CA – carbendazim, CA-F – carbendazim plus flutriafol, CR – curative paint roller-treatment, CS – curative spray-treatment, EP – epoxiconazole, P – preventive spray-treatment

Introduction

The European chestnut (*Castanea sativa*) is a socioeconomically important tree whose wood and fruit production is seriously affected by *Cryphonectria parasitica*, the causal agent of chestnut blight. This fungus, introduced from East Asia, was first observed in United States in 1904 (Merkel 1905) and in Europe in 1938 (Biraghi 1946). It spread quickly throughout Italy and into southern France and Switzerland (Heiniger & Rigling 1994), and later on into the Balkans, Spain and Portugal (Robin & Heiniger 2001). *C. parasitica* is a wound parasite which infects branches and stems of *C. sativa*, causing the smooth bark of young branches to become reddish and sunken (Heiniger & Rigling 1994). Other disease symptoms are bark cankers, foliage wilting, formation of epicornic sprouts below the canker and presence of withered leaves and branches. Orange fungal stromata push through the epidermis where pycnidia and perithecia are found (Anagnostakis 1987). In Europe, *C. parasitica* is listed as an A2 quarantine organism by the European and Mediterranean Plant Protection Organization (Eppo 2005). Quarantine regulations against the fungus focus on the trading of chestnut wood, bark and plants, in order to prevent its further spread (Seemann 2001).

Chestnut occupies an area upper than 77 000 ha in Asturias, Northern Spain (Ortega et al. 2011) where it is one of the most common trees in natural forests. In this region, chestnut blight was first reported in 1982, and since then it has rapidly spread across the region (González-Varela et al. 2011) so control measures are necessaries at least to prevent the disease where it could be a source of fungus spread such as nurseries. In nursery cultivation, breeding is mostly conducted via cuttings, a potential way of pathogen infection. In addition, chestnut grafts and pruning cuts are particularly vulnerable to the disease (Prospero & Rigling 2013). In order to prevent the spread of chestnut blight, a research of suitable fungicides for disease control was carried out. Results of a previous assay about agrochemicals effectiveness against *C. parasitica* showed products with great ability to inhibit fungal growth *in vivo* (González-Varela & González 2007). Epoxiconazole (12.5%) was the most effective since it inhibited the growth from the lowest concentration (1 $\mu g\ ml^{-1}$) showing fungicidal activity. According to this result, we chose epoxiconazole and the following most effective product: a combination of carbendazim plus flutriafol. We also chose carbendazim because it is one of the mixture compounds and it had been used in similar works (Delen 1980, Aksoy & Serdar 2004).

The aim of the present work was to evaluate the *in vivo* effectiveness of *C. parasitica* chemical control for which the three fungicides chosen according to *in vitro* results and expected to control the disease were tried out by three application methods on infected chestnut plants under managed conditions.

Material and methods

Chestnut plants, culture conditions and inoculation

A total of 126 two-year-old plants from seeds collected in the Parque Natural of Somiedo (Asturias) were used. These plants were transplanted into seven-litre-containers of peat (50%) + coconut fibre (25%) with a slow release fertilizer (Osmocote Exact).

Plants were placed in a greenhouse measuring 40 metres long, 4 m wide and 3 m high. An anti-aphid mesh (density:

134 g m^{-2}, spacing: 375 μm) and an anti-weed mesh (density: 150 g cm^{-2}) were used for cover and ground, respectively. A direct drip irrigation system was installed.

For inoculation one strain of the Phytopathology Laboratory Collection of Asturias (LPPAF-187) was chosen. A quarter of a 5 mm diameter PDA (Potato dextrose agar) (Gams et al. 1980) disc containing the fungus was used as inoculum. Inoculations were carried out making a lesion with a scalpel in the bark through which the inoculum was introduced. Lesions were covered with laboratory film.

Phytosanitary products and application methods

Epoxiconazole 12.5% (Lovit, BASF Española) (hereafter, EP); carbendazim 50% (Bavistin FL, BASF Española) (hereafter, CA); and carbendazim (20%) plus flutriafol (9.4%) (Impact-R, Agrodan) (hereafter, CA-F) were utilized. These products are registered in Spain for cereal crops. Plants were treated with the maximum dose specified by the manufacturer.

For each product, three application methods were performed. A preventive (hereafter, P) applied by spraying nine days before inoculation; a curative by spraying (hereafter, CS); and a curative applied over the lesion with a paint roller (hereafter, CR). Both methods were applied 21 days after inoculation on three occasions each separated 21 days. Pulverization and direct application methods were conducted according to previous works (Aksoy & Serdar 2004) with modifications, such as spraying on the whole plant surface instead of only on the leaves, and applying products over the lesion by paint roller instead of on the carved areas by brush.

Assay design

Plants were divided into nine assay units corresponding to combinations of products and methods assayed. Each individual unit consisted in ten treated inoculated plants, two untreated inoculated controls and two untreated controls without inoculation.

Evaluation of results

The fungicides and the method effectiveness were evaluated. Parameters analyzed were: i) lesion length (cm); ii) sporulation, evaluated using a scale from 0 (without fruiting structures) to 5 (fruiting structures covering the whole lesion); iii) dead chestnut plants (ratio). Two evaluations were carried out. In the first, five months after the last application, data concerning lesion, cracks presence, healthy shoots presence and sporulation were collected; whereas in the second, three years after the last application, data from mortality were gathered.

Statistical analysis

Arithmetic mean, standard deviation and standard error were calculated. Lesion length data were first analyzed by Levene's test to check if variance homogeneity was fulfilled. Data which fulfilled it were analyzed using two-way ANOVA with method and product as independent variables and lesion length as repeated measure. It was followed by two post-hoc tests (Tukey's HSD and Scheffe's) when significant variation was observed. Sporulation and mortality data were analyzed by Kruskall-Wallis non parametric test. All analyzes were performed using the software package IBM SPSS Statistics 19 (© Copyright 1989, 2010 SPSS, Inc.).

Results

Results of all evaluated parameters were statistically analyzed. All data are presented in Table 1 and Fig. 1. Cracks and healthy shoots presence did not show any statistically significant difference so these data were omitted.

Lesion length

The variance homogeneity was fulfilled (F = 1.892; $P = 0.062$, Levene's test). Two-way ANOVA results showed no significant application method × product interaction ($P = 0.330$), so both variables could be interpreted. Regarding application methods, significant differences were observed ($P = 0.018$). Differences between treated and control plants were significant ($P < 0.005$, Scheffe's and Tukey's HSD post hoc tests). Concerning each method, there were significant differences between P and CR ($P = 0.047$, Scheffe's; $P = 0.025$, Tukey's HSD), being CR the most effective method since CR-treated plants presented the shortest lesions (5.97 cm) and P the least effective (6.93 cm).

Table 1: Analysis of variance (two-way ANOVA) of the lesion length parameter data

Source	DF	SS	MS	F value	*P* value
Application method	2	14.156	7.078	4.173	0.018 *
Product	2	17.689	8.844	5.215	0.007 **
Application method × product	4	7.911	1.978	1.166	0.330
Error	98	166.200	1.696		

Data in bold show significant differences (levels of statistical significance: * P < 0.05, ** P < 0.01).

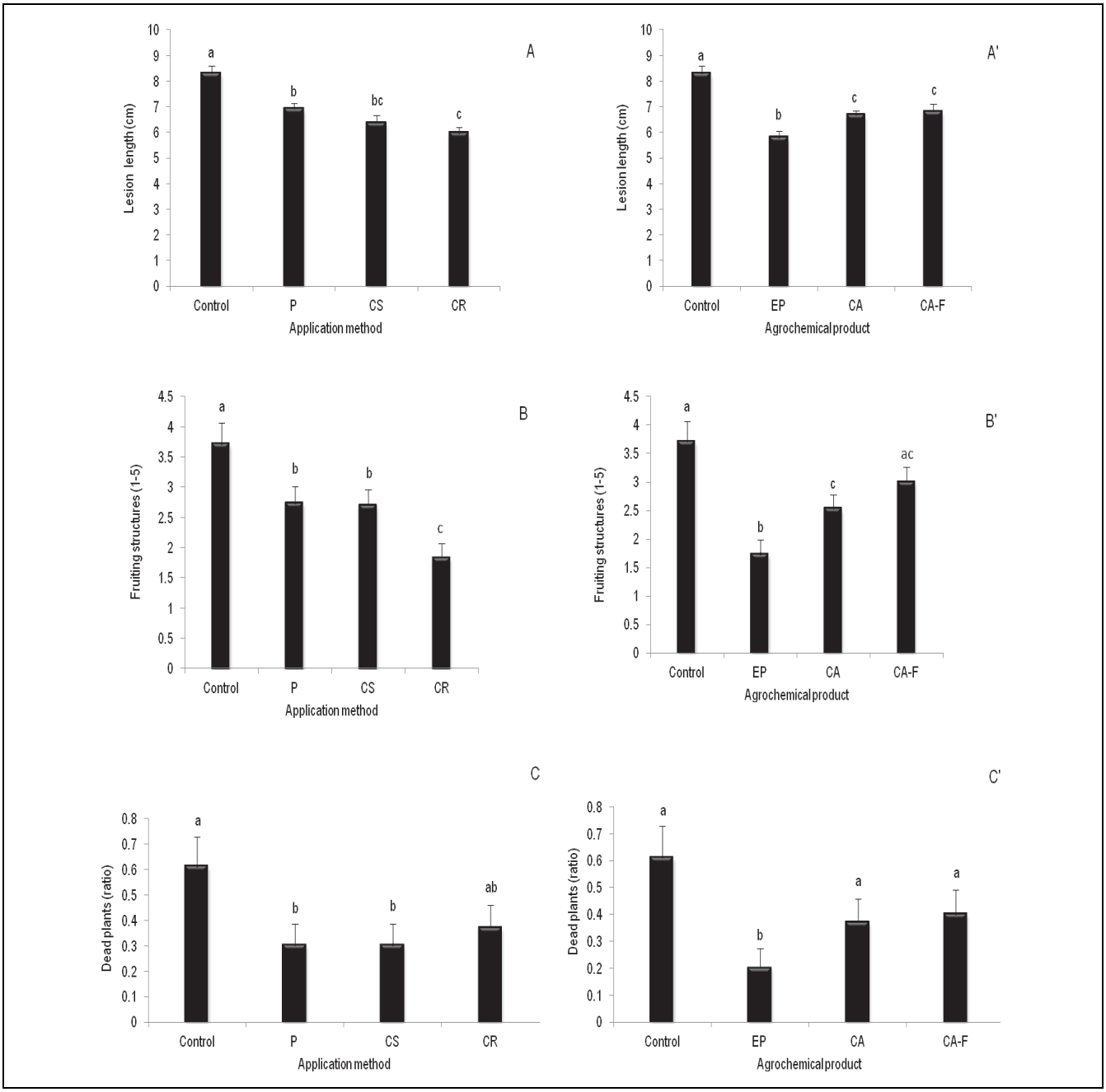

Fig. 1: The arithmetic mean (x) and the standard error (SE) of the lesion length (A, A'), sporulation (B, B') and mortality (C, C') parameters of chestnut plants according to the application method (A, B, C) and the agrochemical product (A', B', C'). Bars topped by the same letter do not differ significantly from each other ($P > 0.05$). P: preventive spray-treatment; CS: curative spray-treatment; CR: curative paint roller-treatment; EP: epoxiconazole; CA: carbendazim; CA-F: carbendazim plus flutriafol.

Regarding products, significant differences were observed ($P = 0.007$). Differences between treated and controls were significant ($P < 0.005$, Scheffe's and Tukey's HSD post hoc tests). With respect to each product, significant differences between EP and CA-F were observed by Scheffe's ($P = 0.037$) and Tukey HSD post hoc test ($P = 0.019$). EP-treated plants showed the shortest lesion (5.8 cm) whereas CA-F-treated plants presented the longest (6.8 cm), therefore EP was the most effective product according to lesion length.

Sporulation

According to the method used as well as the product applied, Kruskall-Wallis test showed significant differences.

Concerning application methods differences observed were significant ($\chi^2 = 13.542$, df = 3, $P = 0.004$, Kruskall-Wallis test). Between methods, CR was the most effective due to CR-treated plants presented the lowest fruiting structures formation (1.83) whereas the other two methods presented the same (2.7).

Regarding products, differences observed were significant (χ^2 = 20.923, df = 3, P = 0.000, Kruskall-Wallis test), showing EP-treated plants the lowest fruiting structures formation (1.73) and CA-F-treated plants the highest.

Just as in the lesion length study, CR method showed the most effectiveness, whereas P showed the least. Concerning products, results are also consistent, EP being the most effective and CA-F the least.

Plant mortality

CR-treated plants presented the highest mortality (0.37) compared to P + C and CS-treated plants (0.3, both). Kruskall-Wallis test showed no statistically significant difference for mortality between application methods (χ^2 = 5.696, df = 3, P = 0.127).

However, differences observed in relation to products were statistically significant (χ^2 = 8.245, df = 3, P = 0.041). CA-F-treated plants presented the highest mortality rate (0.4) whereas EP-treated plants presented the lowest (0.2), so epoxiconazole was the most effective. This result is in line with lesion and sporulation data depending on the product applied.

Discussion

Chestnut blight disease appeared long time ago and since then different chemical control strategies have been tested. Jaynes & Van Alfen (1974) tested the fungicide methyl-2-benzimidazole carbamate by injection into trunks, a possible appropriate method for treating individual trees. Soils injections with benomyl were tested by Elkins et al. (1978), results showing that this product has not been so effective. Aksoy & Serdar (2004) tested the efficacy of various chemicals (copper oxychloride, benomyl and carbendazim), the combinations copper oxychloride/carbendazim and copper oxychloride/benomyl presenting the best results.

Nevertheless, none of the chemical alternative approaches has found a large-scale and systematic application in the field (Prospero & Rigling 2013).

The agrochemicals expected to have a great potential to control the fungus by González-Varela & González (2007) were tested *in vivo* in the present work. These products are not registered for the *C. parasitica* management in Spain, therefore we propose to study if they are able to control the disease in order that their application under managed conditions could be approved.

We suggest chemical therapeutic treatment only in enclosed areas such as nurseries or in particular conditions as the case of a singular specimen with an outstanding interest of conservation, but not in forests where it could not be a solution because it is contaminant, difficult, expensive and fairly long-term effective. At present, the most effective strategy for limiting the impact of chestnut blight in Europe is the biological control with hypovirulence (Prospero & Rigling 2013), that it is a viral disease in the pathogen population with a high potential to control natural infections.

According to results from this work, the preventive application has not been able to prevent the disease establishment due to P-treated plants presenting the longest lesion and the highest sporulation. CR method presents the best results concerning both parameters, however it showed the worst result depending on mortality. This could indicate that this method is short-term effective on young localized lesions, but in the long term it has not been able to stop the pathogen spread.

About products, in both evaluations and according to all parameters the same result was observed: epoxiconazole is the most effective product whereas the mixture of carbendazim plus flutriafol is the worst. Epoxiconazole is considered a highly efficient triazole fungicide which does not generally inhibit fungal growth until after initial infection, thus possibly not influencing defence activation and senescence promotion (Bertelsen et al. 2001). Therefore, the mode of action of this fungicide could have influenced results of the preventive application, epoxiconazole not being appropriate as a preventive treatment. Aksoy & Serdar (2004) reported that combinations of carbendazim with copper oxychloride or benomyl could be useful in the chestnut blight control, but they did not use epoxiconazole or flutriafol. Interestingly, both are inhibitors of ergosterol, nevertheless differences in their effectiveness were observed. Differences between them from the *in vitro* trial were not large and could be explained by the proportion of active matter (epoxiconazole 12.5%, flutriafol 9.4%) (González-Varela & González 2007). However, in the *in vivo* assay EP showed the best result whereas CA-F the worst; a fact that could have been affected by other factors such as environmental factors resulting in product degradation. It is important to remember that the efficacy of a product will depend on a variety of factors such as: degradability, persistence, mode of action and interaction with other compounds (González-Varela & González 2007). Furthermore, in this case the carbendazim could have had a long-term effect in the combination due to Delen (1980) reported that *C. parasitica* could acquire resistance after continuous applications of carbendazim, therefore this product could have influenced the development of resistance.

Despite *in vitro* results were so encouraging, *in vivo* differences between products and methods were reduced. Significant differences were always observed between treated and control plants except in the case of mortality depending on the method. It could prove that results of treated plants are going to be similar over time regardless of the method applied. Significant differences were also observed between plants treated with epoxiconazole and the other two products-treated plants, epoxiconazole being the most effective. This result shows that epoxiconazole could be an alternative for disease control in plants with similar conditions to those belonging to this study. Nevertheless, additional studies about mode of action, product concentration, application interval, phytotoxicity and development of resistance need to be conducted before this product can be proposed for chestnuts. According to Chen et al. (2012), epoxiconazole (a sterol demethylation inhibitor DMIs) could not be extensively used for disease control because it is at-risk fungicide, and

resistance to DMIs has already been reported in previous studies (Nikou et al. 2009). In addition, it would be interesting to study the interaction of epoxiconazole with other effective fungicides with different modes of action. Chen et al. (2012) suggested that the combination of fungicides could achieve greater efficacy and they proposed an integrated use of epoxiconazole and pyraclostrobin as a good alternative to carbendazim for the control of Fusarium head blight.

Some authors consider that regular prophylactic use of fungicides can result in an appreciable decrease in disease incidence (Denman et al. 2004). Chestnut blight is a big problem for Asturian forestry, therefore we suggest that a judicious use of epoxiconazole could be an alternative to further study in order to reduce the spread of *C. parasitica,* chemical control under managed conditions being a part of the therapeutic strategy to prevent the disease occurrence in chestnut forests.

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ARTÍCULO III

Meyer J.[*], Trapiello E.[*], Senn-Irlet B., Sieber T.N., Cornejo C., Aghayeva D., González A.J. y Prospero S. (2017). Phylogenetic and phenotypic characterization of *Sirococcus castaneae* comb. nov. (synonym *Diplodina castaneae*), a fungal endophyte of European chestnut. *Fungal Biology*, DOI: 10.1016/j.funbio.2017.04.001.

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Título

Caracterización filogenética y fenotípica de *Sirococcus castaneae* comb. nov. (sinónimo *Diplodina castaneae*), un hongo endófito del castaño europeo.

Resumen

En esta publicación resolvemos el estatus taxonómico del hongo *Diplodina castaneae* (Ascomycetes, Diaporthales, Gnomoniaceae) presente en el castaño europeo (*Castanea sativa*) como endófito y como agente causal de la enfermedad Javart. Especímenes de Suiza, España y Azerbaiyán fueron secuenciados en cinco loci nucleares (β-tubulin, EF-α, ITS, LSU y RPB2). Las filogenias inferidas situaron *D. castaneae* en la familia Gnomoniaceae. Además, se evaluaron las tasas de crecimiento y las características morfológicas en diferentes medios y se compararon con las de *Gnomoniopsis castaneae*, que puede ser fácilmente confundido con *D. castaneae*. Basándonos en las características morfológicas y filogenéticas, proponemos recolocar *D. castaneae* en el género *Sirococcus*, como *S. castaneae* comb. nov.

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Phylogenetic and phenotypic characterisation of *Sirococcus castaneae* comb. nov. (synonym *Diplodina castaneae*), a fungal endophyte of European chestnut

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ABSTRACT

In this paper we resolve the taxonomic status of the fungus *Diplodina castaneae* (Ascomycetes, Diaporthales, Gnomoniaceae) which occurs on the European chestnut (*Castanea sativa*) as endophyte and as the causal agent of Javart disease. Specimens from Switzerland, Spain, and Azerbaijan were sequenced at five nuclear loci (β-tubulin, EF-1α, ITS, LSU, and RPB2). Phylogenies were inferred to place *D. castaneae* in the Gnomoniaceae family. Moreover, growth rates and morphological characteristics on different agar media were assessed and compared to those of *Gnomoniopsis castaneae*, which can easily be confused with *D. castaneae*. Based on morphological and phylogenetic characteristics, we propose to reallocate *D. castaneae* to the genus *Sirococcus*, as *S. castaneae* comb. nov.

Introduction

The European chestnut (*Castanea sativa* Mill.) is a multipurpose tree species in Europe that has been cultivated for centuries for its edible nuts and valuable wood (e.g. high durability) (Conedera *et al.* 2004). Chestnut stands are thus considered as significant agro-forest ecosystems of great ecological, recreational, and cultural value. Unfortunately, several

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pathogens and pests affect *C. sativa* in Europe, among them the introduced chestnut blight fungus *Cryphonectria parasitica* (Murr.) Barr (Ascomycetes, Diaporthales, Cryphonectriaceae) and the chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera, Cynipidae). *Cryphonectria parasitica* is a wound pathogen that infects the bark and cambium of susceptible chestnut (*Castanea*) species, causing perennial cankers on the stem and branches (Rigling & Prospero 2017). The pathogen was first introduced into Europe at the end of the 1930s and is now widespread in all of the major European regions where *C. sativa* occurs. *Dryocosmus kuriphilus*, a gall wasp native to China, is considered as the most significant pest for *Castanea* species worldwide (EPPO 2005). In Europe, this insect was detected for the first time in 2002 in Italy, and in the following years it rapidly spread to various countries (Brussino *et al.* 2002; Avtzis & Matošević 2013). The presence of galls is usually non-lethal for the affected tree, but leads to reduced growth and fruit production (e.g. Kato & Hijii 1997; Sartor *et al.* 2015).

When attempting to isolate *C. parasitica* from abandoned *D. kuriphilus* galls (i.e. necrotic, empty galls, after the emergence of the adults; Meyer *et al.* 2015) and chestnut blight cankers in Switzerland, Spain, and Azerbaijan, a fungus was recurrently recovered. On potato dextrose agar (PDA), this fungus showed a culture morphology similar to that of *Gnomoniopsis castaneae* Tamietti (syn. *G. smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest), a chestnut endophyte which also causes brown rot of chestnut fruits (Sieber *et al.* 2007; Shuttleworth 2012; Visentin *et al.* 2012; Maresi *et al.* 2013; Shuttleworth *et al.* 2015; Dennert *et al.* 2015; Lione *et al.* 2016) and is frequently reported on necrotic chestnut galls (Magro *et al.* 2010; Vannini *et al.* 2017). Recently, Pasche *et al.* (2016) showed that *G. castaneae* can also induce bark cankers on European chestnut. Comparing the internal transcribed spacer (ITS) sequence of this unknown fungal species against reference sequences in GenBank (www.ncbi.nlm.nih.gov) revealed a complete match with the specimen *Gnomoniopsis* sp. ICMP14082 (KC145849). Hence, we initially defined this fungus as *Gnomoniopsis* sp. (Meyer *et al.* 2015). However, additional microscopic analyses that we performed using the key proposed by Bissegger & Sieber (1994) revealed that, based on morphology and size of the asexual spores (conidia), the unknown fungus was actually *Diplodina castaneae* Prill. et Del. (Ascomycetes, Diaporthales, Gnomoniaceae) rather than a *Gnomoniopsis* species.

Diplodina castaneae has been previously found as an endophyte or pathogen on *C. sativa* in several European countries, including Belgium, Bulgaria, England, France, Germany, Italy, Slovakia, and Switzerland (Saccardo 1895; Day 1930; Bissegger & Sieber 1994; Vanev *et al.* 1997; Adamčíková *et al.* 2013). In Japan, *D. castaneae* occurs on the Japanese chestnut *Castanea crenata* (Farr & Rossman 2013). In Europe, this species is the causal agent of the 'maladie de Javart', which has been documented in France since the end of the 19th century (Prillieux & Delacroix 1893). The disease is recognisable by deep, small, elongated cankers appearing as bright brown patches on the bark, frequently beginning at the stem base (Prillieux & Delacroix 1893; Day 1930). The infected bark seems to have been severely bruised and becomes depressed. Subsequently, it dries up and falls off in patches, exposing the wood. *Diplodina castaneae* is considered to be an endophyte of the European chestnut (Bissegger & Sieber 1994). However, in coppice stands it can eventually kill young shoots that have been weakened by other biotic or abiotic stresses (Prillieux & Delacroix 1893; Saccardo 1895; Day 1930; Berthon *et al.* 1953).

Grove (1935) and Ellis & Ellis (1997) considered *D. castaneae* to be the anamorphic state of *Cryptodiaporthe castaneae* (Tul. & C. Tul.) Whem., whereas Bissegger & Sieber (1994) found *D. castaneae* and *Discella castaneae* (Sacc.) Arx (the anamorphic state of *C. castaneae* according to Smith *et al.* (1988)) to be distinctive species based on culture morphology and conidial dimensions. *Diplodina* species do not form a taxonomically homogeneous group because they are synonyms of species in many other genera (e.g. *Ascochyta*, *Discella*, *Microdiplodia*, and *Phloeospora*). The type species of *Diplodina* Sutter is *Diplodina salicis* Westend. 1857 (Rosmann *et al.* 2015) which includes *Diplodina microsperma* B. Sutton 1977, known to be the anamorph form of *Plagiostoma apiculatum* (Wallr.) L. C. Mejía (Mejía *et al.* 2011). For this reason, Sogonov *et al.* (2008) used the name *Plagiostoma* instead of *Diplodina* in their analysis, even if the type species *Plagiostoma euphorbiae* (Fuckel) Fuckel was first described only in 1870 (Rossman *et al.* 2015).

The morphology of *D. castaneae* has been described by several authors (Prillieux & Delacroix 1893; Day 1930; Grove 1935; Bissegger & Sieber 1994; Ellis & Ellis 1997; Adamčíková *et al.* 2013). The taxonomic status of *D. castaneae*, however, is still unresolved. In this study, we aimed to shed light on the taxonomic position of *D. castaneae*, in particular regarding its relationship with the genera *Plagiostoma* and *Gnomoniopsis*. To achieve this goal, specimens from Switzerland, Spain, and Azerbaijan were sequenced at five nuclear loci (β-tubulin, EF-1α, ITS, LSU, and RPB2) and phylogenies were inferred using two previously published datasets (Sogonov *et al.* 2008; Walker *et al.* 2010). Moreover, for a subset of isolates, growth rates and morphological characteristics on different agar media were assessed and compared to *G. castaneae*, the other chestnut endophyte and potential pathogen, which can easily be confused with *D. castaneae*.

Materials and methods

Origin of the Diplodina castaneae *isolates*

Diplodina castaneae isolates were recovered from two different tissues of European chestnut trees, specifically abandoned galls of *Dryocosmus kuriphilus* and bark cankers caused by *Cryphonectria parasitica*. The galls all originated from eight chestnut stands in Switzerland and were sampled for a previous study in which we determined the incidence of *C. parasitica* on abandoned galls (Meyer *et al.* 2015). Of the stands used in this previous study, only those in the region Ticino were considered for canker samples (80 specimens) because the presence of *D. castaneae* was not recorded in the cankers from the other region (Chablais). Additionally, 14 cankers were sampled in 2014 in Gilly, Switzerland (Canton Vaud). In Azerbaijan, 257 bark cankers were sampled in 2015 in chestnut stands located in seven regions (Qabala, Sheki, Ismayilli, Oghuz, Qakh, Zagatala, Balakan). In Spain, 60 bark cankers were sampled in 2014 in four chestnut stands each located in a different county (Aller, Lena, Llanera, and Peñamellera

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Baja) of the Principado de Asturias (Northern Spain). In Switzerland and Spain, three bark samples were taken from each canker (upper, middle, and lower part) with a fine bone marrow biopsy needle (Jamshidi gauge, 2 mm diameter, Baxter, Deerfield, IL, USA) or a cork borer (5 mm diameter). In Azerbaijan, only one bark sample (2 × 2 cm) per canker was taken with a knife sterilized with 95 % ethanol. Additionally, one *D. castaneae* isolate was obtained from a canker in Bex-Montet (Canton Vaud), although systematic sampling was not conducted in this chestnut population. For the coordinates of the chestnut stands in which the *D. castaneae* isolates analysed in this study were recovered, see Table S1 in the Supplementary Materials.

Isolation and cultivation of Diplodina castaneae

The sampled galls and pieces of bark cankers were dipped in 70 % ethanol, briefly flamed, and placed on 1.5 % water agar for one week in dark conditions at room temperature (Meyer *et al.* 2015). Outgrowing fungal hyphae were then transferred to potato dextrose agar (BD Difco PDA; 39 g l^{-1}; Chemie Brunschwig AG, Basel, Switzerland) and incubated at room temperature in the dark for one week and then under light on the laboratory bench for two weeks. After this period, 81 fungal colonies showed the morphological characteristics of *D. castaneae* (i.e. brown/black mycelium, curled margins and dull brownish-grey to pink conidia). A total of 32 colonies (the Swiss isolates referred to as Dca, the Spanish as LPPAF- and the Azerbaijani as M9240; Table 1) were used for DNA extraction. These colonies also included two *D. castaneae* isolates present in the WSL collection, namely isolate Dca87 (Table 1) recovered from the healthy bark of a chestnut twig from southern Switzerland (Canton Ticino; Contone) and isolate Dca85 (Table 1) obtained from a bark canker in Bex-Montet.

Statistical analysis of the incidence of Diplodina castaneae

The incidence of *D. castaneae* in a chestnut stand was calculated as the number of isolates from the galls divided by the total number of galls analysed in that stand. To test if the incidence of gall-colonizing *D. castaneae* differed significantly between regions and chestnut stands, a linear mixed-effects regression model ('glmmPQL') with a logit link binomial distribution was fitted using the R software package *MASS* (Venables & Ripley 2002). The mixed model considers the nested structure of the experimental design, with four chestnut stands within each region (Ticino and Chablais, fixed effects) and five trees within each stand (nested random effects). To detect statistical differences in the fungal incidence between chestnut stands, a Kruskal–Wallis test was used within each region. As the majority of *D. castaneae* specimens were recovered from galls in Switzerland, all statistical analyses were only performed on this dataset and not on cultures from Spain and Azerbaijan.

Cardinal growth temperatures

Mycelial plugs (0.5 cm diameter) were excised with a sterile cork borer from the edge of actively growing 17-d-old cultures of the *Diplodina castaneae* strain Dca90 and of the *Gnomoniopsis castaneae* strain Gca1 (Meyer *et al.* 2015; sequence with GenBank accession number KT823778, Tables 1 and S2). The plugs were placed in the centre of a PDA plate (8.4 cm inner diameter) and incubated at 10, 15, 20, 25, 30, and 35 °C in the dark. Five replicated plates were prepared for each fungal strain and temperature. The experiment was conducted twice and the radial growth (mm d^{-1}) of the colonies was assessed after 4 and 5 d. As most colonies showed an elliptic shape, the minimum and maximum diameter were measured with a millimetre ruler and the geometric mean diameter was then calculated.

Fungal growth on different media

For comparing culture morphology, four isolates of *Diplodina castaneae* (LPPAF-866.4 and LPPAF-885.4 from Spain, Dca90 and Dca98 from Switzerland) and one isolate of *Gnomoniopsis castaneae* Gca1 (Tables 1 and S2) were cultured on two different media, i.e. PDA and malt extract agar (MEA; 12 g l^{-1} of malt extract (Chemie Brunschwig AG) and 15 g l^{-1} of agar). In parallel with the growth experiment, a mycelial plug (0.5 cm diameter) was placed in the centre of a Petri plate (8.4 cm inner diameter) and incubated at 25 °C under two different conditions: i) complete dark and ii) 16/8 h photoperiod of light and dark. Five replicates per strain and light condition were produced, and the trial was conducted twice. After 21 d, morphological characteristics were assessed and documented photographically.

Microscopy

For microscopic examination, free-hand sections were used from the strains *Gnomoniopsis castaneae* Gca1 (Tables 1 and S2), Gca5, Gca10, and Gca21 (Meyer *et al.* 2015; all isolates correspond to GenBank accession number KT823778) and *Diplodina castaneae* Dca90, Dca98, Dca101, Dca113 (Tables 1 and S2) grown on PDA mounted in water and cotton blue. Photographs and measurements of microscopic features were taken using a Leica DM1000 LED microscope with bright-field illumination with a Leica DFC 290 HD digital camera including the LAS software also provided by Leica (Wetzlar, Germany). At least 20 spores per isolate were measured to calculate the 95 % confidence interval of the spore sizes.

DNA extraction and PCR reaction

Genomic DNA was extracted from 10 to 20 mg of lyophilized fungal mycelium using the DNeasy Plant Mini Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's instructions. The following five genomic regions were amplified by PCR: (1) part of the nuclear ribosomal DNA, including the 5.8S rDNA and the adjacent internal transcribed spacers ITS1 and ITS2; (2) two adjacent, partly overlapping fragments of the elongation factor 1-alpha (EF-1α), referred to as EF-1α-1 and EF-1α-2; (3) two different sections of the beta-tubulin (β-tub), referred to as β-tub-1 and β-tub-2 (internal to β-tub-1); (4) two different sections of the RNA polymerase II large subunit (RPB2), referred to as RPB2-1 and RPB2-2 (internal to RPB2-1); and (5) the large subunit nuclear ribosomal DNA (LSU). Forward and reverse primers used for PCR and sequencing for each region are listed in Table S3. For the EF-1α-2, the primer pair

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Table 1 – Fungal cultures used in this study.

Fungal species	Isolate	Country/Region/Location			Host species	Host tissue	Month of collection	Collector	Herbarium number	Collection number
Sordariomycetes/Sordariomycetidae/Diaporthales/Gnomoniaceae:										
Diplodina castaneae	Dca40	CH	Vd	Bex-Montet	*Castanea sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca60	CH	Vd	Bex-Creux	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca77	CH	Vs	Malevoz	*C. sativa*	Gall	Sep. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca79	CH	Ti	Biasca	*C. sativa*	Gall	Jul. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca80	CH	Ti	Stabio	*C. sativa*	Gall	Jul. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca83	CH	Vd	Gilly	*C. sativa*	Canker	Sep. 2015	J.B. *Meyer*	–	–
Diplodina castaneae	Dca85	CH	Vd	Bex-Montet	*C. sativa*	Canker	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca87	CH	Ti	Contone	*C. sativa*	Twig	Jul. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca90	CH	Vd	Bex-Creux	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	CBS142041
Diplodina castaneae	Dca91	CH	Vd	Bex-Montet	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca92	CH	Vd	Bex-Montet	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca93	CH	Vd	Bex-Creux	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca94	CH	Vd	Bex-Montet	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca95	CH	Vd	Bex-Creux	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca97	CH	Ti	Mugena	*C. sativa*	Gall	Jul. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca98	CH	Vs	Malevoz	*C. sativa*	Gall	Sep. 2014	J.B. *Meyer*	–	CBS142042
Diplodina castaneae	Dca99	CH	Vs	Malevoz	*C. sativa*	Gall	Sep. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca100	CH	Vs	Malevoz	*C. sativa*	Gall	Sep. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca101	CH	Ti	Mugena	*C. sativa*	Gall	Jul. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca102	CH	Vs	Choëx	*C. sativa*	Gall	Sep. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca103	CH	Vs	Choëx	*C. sativa*	Gall	Sep. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca104	CH	Vd	Bex-Montet	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	Dca105	CH	Vd	Bex-Montet	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Diplodina castaneae	LPPAF-851.2	ES	As	Llanera	*C. sativa*	Canker	Apr. 2014	E. *Trapiello*	–	–
Diplodina castaneae	LPPAF-855.1	ES	As	Lena	*C. sativa*	Canker	Apr. 2014	E. *Trapiello*	–	–
Diplodina castaneae	LPPAF-860.1	ES	As	Lena	*C. sativa*	Canker	May 2014	E. *Trapiello*	–	–
Diplodina castaneae	LPPAF-862.1	ES	As	Lena	*C. sativa*	Canker	May 2014	E. *Trapiello*	–	–
Diplodina castaneae	LPPAF-866.4	ES	As	Peñamellera Baja	*C. sativa*	Canker	May 2014	E. *Trapiello*	–	–
Diplodina castaneae	LPPAF-883.2	ES	As	Aller	*C. sativa*	Canker	May 2014	E. *Trapiello*	–	–
Diplodina castaneae	LPPAF-885.4	ES	As	Aller	*C. sativa*	Canker	May 2014	E. *Trapiello*	–	–
Diplodina castaneae	LPPAF-886.2	ES	As	Aller	*C. sativa*	Canker	May 2014	E. *Trapiello*	–	–
Diplodina castaneae	M9240	AZ	Ismayilli	Istisu	*C. sativa*	Canker	May 2015	D. *Aghayeva*	–	CBS142043
Gnomoniopsis castaneae	Gca1	CH	Vd	Bex-Montet	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	CBS142044
Gnomoniopsis castaneae	Gca5	CH	Vd	Bex-Creux	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Gnomoniopsis castaneae	Gca10	CH	Vd	Bex-Montet	*C. sativa*	Gall	Jun. 2014	J.B. *Meyer*	–	–
Gnomoniopsis castaneae	Gca21	CH	Vs	Monthey	*C. sativa*	Canker	Jul. 2014	S. *Prospero*	–	–
Cryptosporella jaklitschii	LCM112.04 LCM112.01	US	New York	Marcy Dam	*Alnus serrulata*	Twig	Jun. 2007	L.C. *Mejia*	*BPI879231*	CBS125665
Diplodina acerina	cult_08_A08	DE	Saxony	Burgaue	*Fraxinus excelsior*	Living leaf	Oct. 2008	A. *Reiher*	–	–
Diplodina castaneae	–	FR	Normandy	Forêt de Rouvray	*C. castanea*		1986	M. *Morelet*	–	CBS212.90
Gnomoniopsis sp.	–	NZ	Mid Canterbury	Lincon	*C. sativa*×*C. crenata*	Fruit	May 1999	K.D.R. *Wadia*	–	ICMP14082
Melanconis alni	AR4016	AT	Steiermark	Kleinsoelk	*Alnus alnobetula*	–	Aug. 2003	W. *Jaklitsch*	*BPI843621*	–

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Ophiognomonia ischnostyla	–	CH	Vd	Aigle	*Carpinus betulus*	–	May 1979	M. *Monod*	–			CBS838.79
Plagiostoma apiculatum	AR3455	AT	Vienna	Marchfeldkanalweg	*Salix* sp.	–	May 2000	*W. Jaklitsch*	BPI747938			CBS109775
Plagiostoma conradii	AR3488	US	New Jersey	Island Beach State Park	*Hudsonia tomentosa*	Leaves, Stems	Mar. 1995	G. Bills	SBML746482	BPI746482		CBS109761
Sirococcus piceicola	AR3962	CH	–	–	*Picea abies*	Cones	–	G. *Stanosz*	BPI871166			CBS119621
Sirococcus tsugae	AR3953	US	Alaska	Mendenhall Valley	*Tsuga mertensiana*	–	–	G. *Stanosz*	BPI871167			CBS119626

728F and 1567R was used for PCR and the pair 983F and 1567R was used for sequencing. For the sequencing reaction of the LSU, RPB2-1, and β-tub-1, the PCR primers, as well as one (LSU) or two (RPB2-1 and β-tub-1) internal primers were used (Table S3). In all cases, the PCR mixture (20 μl) consisted of 1 μl DNA diluted 20 times (10–50 ng), 7.2 μl JumpStart REDTaq Ready Reaction Mix 2x (Sigma–Aldrich, Buchs, Switzerland), 1 μl of each primer (20 pmol μl^{-1}), and 9.8 μl sterile water (Lichrosolv water for chromatography, Merck AG, Zug, Switzerland). Thermal cycling parameters for ITS and the two EF-1α fragments were: initial denaturation at 94 °C for 2 min, followed by 35 amplification cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C, and a final extension at 72 °C for 10 min. For β-tub-2, RPB2-1, RPB2-2, and LSU sequences, the 35 amplification cycles consisted of a denaturation step for 30 s at 94 °C, followed by annealing for 30 s at 54–55 °C or for 55 s at 50–52 °C for the β-tub-1 sequence. Successful PCR amplification was verified on 1.4 % agarose gel. For PCR product purification, 2 μl APEX-Enzymatic PCR seq (Fisher Scientific, Reinach, Switzerland) were added to 5 μl PCR product. This mixture was incubated at 37 °C for 15 min, then at 80 °C for 15 min.

Sequencing of the ITS, EF-1α, β-tub, RPB2, and LSU gene fragments

The purified PCR products were diluted to a concentration of about 5 $\mu g\ ml^{-1}$, and 3 μl of this diluted solution were combined with 4.5 μl of a master mix containing 0.75 μl Big Dye Sequencing Buffer (Big Dye Terminator v3.1 Kit, Life Technologies, Carlsbad, CA, USA), 1.5 μl forward or reverse primer, 1.5 μl Big Dye Ready Reaction Premix (2.5x), and 0.75 μl sterile water (Lichrosolv water for chromatography, Merck AG). The cycle sequencing program consisted of 1 min at 96 °C for initial denaturation, 25 cycles of 10 s at 96 °C for denaturation, 5 s at 50 °C for annealing, and 1 min at 60 °C for extension. All sequencing reaction products were purified with the BigDye XTerminator Purification Kit (Life Technologies) and the sequences were run on a 3130xl DNA Analyzer (Life Technologies). The resulting forward and reverse DNA strands were assembled to contigs in DNA Dynamo Sequence Analysis Software (Blue Tractor Software Ltd). For species identification, sequences were compared with available sequences in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

Phylogenetic analysis

Two phylogenies were inferred based on the multilocus datasets (β-tub-1, EF-1α-1, ITS) of Walker *et al.* (2010) and those (EF-1α-2, LSU, RPB2-1) of Sogonov *et al.* (2008), each obtained from the authors. Sequences of two Spanish (LPPAF-855.1 and LPPAF-862.1), two Swiss (Dca90 and Dca98), and one Azerbaijani (M9240) Dca isolate were included, as well as sequences of the collection cultures *Diplodina castaneae* CBS212.90 and *Gnomoniopsis castaneae* Gca1, previously isolated from chestnut galls (Meyer *et al.* 2015), *Plagiostoma apiculatum* L.C. Mejia CBS109775, and *Plagiostoma conradii* (Ellis) M.E. Barr CBS109761. After a BLAST search, the β-tub-1, EF-1α-2, ITS, and RPB2-1 sequences of this latter strain were found to share the highest sequence identity with the genes of our *D.*

castaneae isolates (see Results). The dataset of concatenated β-tub-1, EF-1α-1, and ITS (Walker *et al.* 2010), also included sequences of *Cryptosporella jaklitschii* (Fr.) Sacc. CBS125665, *Gnomoniopsis* sp. ICMP14082, *Melanconis alni* Tul. & C. Tul. BPI843621, and *Ophiognomonia ischnostyla* (Desm.) Sogonov CBS838.79, and the dataset of EF-1α-2, LSU, RPB2-1 (Sogonov *et al.* 2008) included sequences of *Sirococcus piceicola* Castl., D.F. Farr & Stanosz CBS119621 and *Sirococcus tsugae* Castl., D.F. Farr & Stanosz CBS119626. GenBank accession numbers of all sequences used in this study are listed in Table S2. As the congruence among the single-locus trees were already verified statistically in Walker *et al.* (2010) and in Sogonov *et al.* (2008), analyses were not repeated here. Nevertheless, we visually evaluated all single-locus trees for possible incongruences. Single-locus phylogenies for each of the four genomic regions (EF-1α-1, β-tub-2, ITS, RPB2-2) were also inferred from *D. castaneae* isolates separately, including isolates of 20 Swiss, six Spanish, one Azerbaijani *D. castaneae*, and *D. castaneae* CBS212.90, with *P. conradii* CBS109761 as the outgroup. The LSU phylogeny was not inferred, as all LSU sequences of *D. castaneae* isolates were monomorphic (see Results). The sequences of LPPAF-851.2, LPPAF-885.4, Dca77, Dca87, and Dca100 (Table S2) were not included in the single-locus phylogenies because the β-tub-1 region was missing.

For each genomic region, sequences were aligned and manually edited using Molecular Evolutionary Genetics Analysis (MEGA) software 6.06 (Tamura *et al.* 2013). Ambiguous sites in the aligned sequences were excised using GBlocks 0.91b (Castresana 2000; Talavera & Castresana 2007) on the Phylogeny.fr platform (Dereeper *et al.* 2008, 2010). This program identifies regions in an alignment with high homology among sites, thus containing reliable phylogenetic information. The best-fit model of nucleotide substitution was estimated from the aligned sequences using jModelTest 0.1.1 (Guindon & Gascuel 2003; Posada *et al.* 2006; Posada 2008). The analyses were performed for 88 models, considering 11 substitution types, equal or unequal base frequencies (+F), invariable sites (+I), and the gamma shape parameter (+G). Base trees were calculated with the maximum likelihood (ML) option. The Akaike Information Criterion (AIC) (Akaike 1973) was used to select the best-fit model for multilocus and single-locus alignments.

Maximum likelihood (ML) analyses were performed for the concatenated as well as the single-locus datasets, and Bayesian MCMC relaxed clock (BRC) analyses were performed for the concatenated datasets only. The topologies were inferred with PhyML 3.0 (Guindon & Gascuel 2003; Guindon *et al.* 2010) and BEAST 1.7.5 (Drummond & Rambaut 2007), as explained in Cornejo & Scheidegger (2015). ML and BRC phylograms were produced with TreeGraph2 (Stöver & Müller 2010) and FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Incidence of Diplodina castaneae

In Switzerland, *D. castaneae* cultures were recovered from 67 out of 1973 (3.4 %) analysed abandoned *Dryocosmus kuriphilus* galls. From the totality of the galls infected by *D. castaneae*, 64 (95.5 %) came from the chestnut stands in the Chablais (Bex-Montet: 34 galls; Bex-Creux: 17; Malevoz: 12; Choex: 1) and only three originated from the Ticino (4.5 %) (Fig 1). The difference between the two regions was significant ($p = 0.041$) (Table S4). In the Ticino, no significant difference was found between chestnut stands ($p = 0.27$), whereas in the Chablais *D. castaneae* colonized significantly more galls in Bex-Montet than in the three other chestnut stands ($p = 0.003$, Table S4).

The overall incidence of *D. castaneae* in the cankers was comparable to that in the abandoned galls (14 out of 411 cankers colonized, 3.4 %). In northern Spain, *D. castaneae* was found in all four sampled stands in a total of eight out of 60

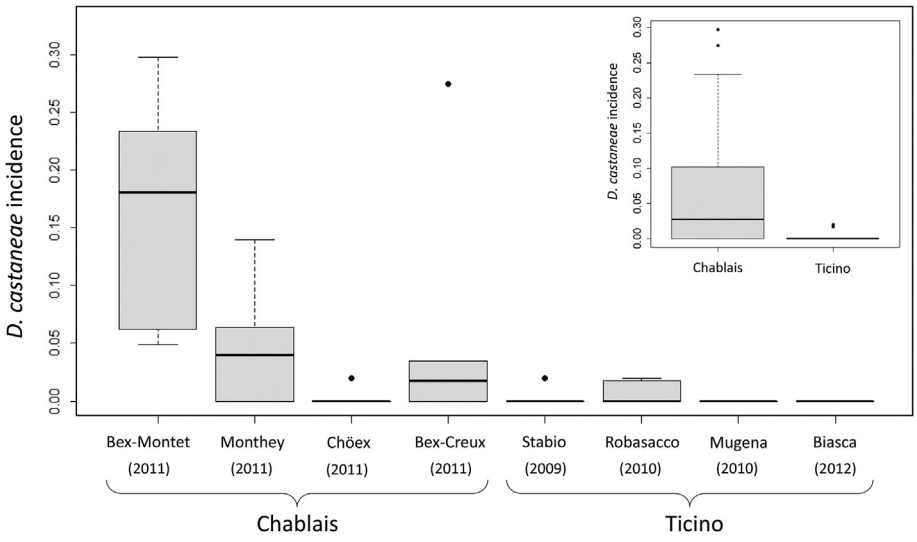

Fig 1 – Incidence of *Diplodina castaneae* on abandoned galls of *Dryocosmus kuriphilus* in eight chestnut stands in Switzerland. The distribution of the incidence of *D. castaneae* is shown in boxplots, where the bold line within the boxes indicates the median incidence, box edges indicate quartiles, and whiskers indicate the 0.01 and 0.99 quantiles. The upper-right panel shows the incidence of *D. castaneae* in the two regions Chablais and Ticino.

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bark cankers (13.3 %). In Azerbaijan, *D. castaneae* was isolated from four out of 257 cankers (1.6 %) in three different regions (Ismayilli: 1, Qabala: 1, and Sheki: 2). In Switzerland, the species was recovered in one out of 80 cankers (1.2 %) in Ticino and one out of 14 (7.1 %) in Gilly.

Cardinal growth temperatures

Growth of Dca90 and *Gnomoniopsis castaneae* Gca1 at different temperatures was recorded in two experimental replicates. The growth rates were similar in the two replicates, and thus these data were combined. Dca90 and Gca1 growth curves followed a similar pattern, but the daily growth rate of the *Diplodina castaneae* isolate was about 0.2 mm d^{-1} slower than that of *G. castaneae* over almost the whole range of temperatures (Fig 2). The optimum temperature was 30 °C for both strains, whereas the daily growth rate differed between the strains, with 1.04 mm d^{-1} for Dca90 and 1.22 mm d^{-1} for Gca1. Both fungal strains nearly stopped growing at 35 °C.

Morphological culture characterization and microscopy

The colony of *Gnomoniopsis castaneae* grown on MEA was characterized by a smooth white mycelium with diffuse margins, which acquired a brownish colour with a spiral shape under light conditions (Fig S1). The colony grown on PDA showed abundant mycelia that were brownish white under light conditions and white under dark conditions. Orange viscous conidia were observed under both dark and light conditions. These elements were uniformly spread throughout the media and had a spiral shape in light conditions. Conidia were 5.7–10.3 × 1.9–3.0 µm in size (length/width ratio, Qmean = 3.00–3.50), ellipsoid, apically obtuse, with truncate base, non-septate, hyaline, and with few small guttules (Table 2). The culture characteristics of *Diplodina castaneae* are presented in the Taxonomy Section at the end of the article. Morphological confusion between cultures of both species could arise because of the brownish (in the web version) colouration they acquire on MEA and PDA under light conditions,

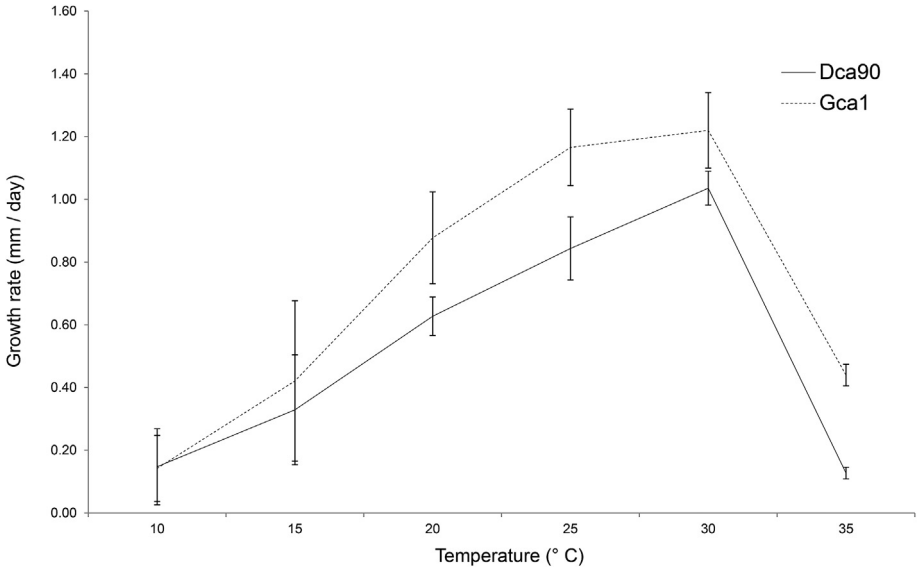

Fig 2 – Growth rates (mm d^{-1}) of the two Swiss isolates *Diplodina castaneae* Dca90 and *Gnomoniopsis castaneae* Gca1 at different temperatures. Error bars represent standard deviations.

Table 2 – Size (µm) of conidia of *Diplodina castaneae* and *Gnomoniopsis castaneae* isolates.

Species	Isolate[a]	Length[b] (µm)		Width[b] (µm)		L/W ratio[b]		Mean values			
		Lmin	Lmax	Wmin	Wmax	Qmin	Qmax	N	Lmean	Wmean	Qmean
D. castaneae	Dca90	9.1	12.2	1.7	3.2	2.30	5.90	58	9.90	2.40	4.20
D. castaneae	Dca98	7.6	11.5	2.7	3.9	1.90	3.97	29	9.60	3.30	3.00
D. castaneae	Dca101	8.0	13.6	2.1	3.2	2.42	5.76	41	10.8	2.60	4.10
D. castaneae	CBS212.90	6.5	10.9	2.4	3.3	1.90	3.88	30	8.20	2.90	2.90
G. castaneae	Gca1	5.7	8.9	2.1	2.8	2.05	4.05	36	7.30	2.40	3.00
G. castaneae	Gca5	6.5	9.3	1.9	3.0	2.32	4.21	30	7.91	2.45	3.27
G. castaneae	Gca10	6.9	10.3	2.1	2.9	2.69	4.30	31	8.60	2.50	3.50
G. castaneae	Gca21	6.4	8.8	2.0	3.0	2.16	2.93	22	7.61	2.53	3.05

a Name of the specific isolate (see Table 1).
b For length (L), width (W), and L/W ratio (Q) of the conidia, minimal, maximal and mean values are given.

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and by the spiral collocation of the conidia on PDA under light conditions (Figs 3 and S1).

Molecular identity and phylogeny of Diplodina castaneae

The EF-1α-1, EF-1α-2, β-tub-1, β-tub-2, ITS, LSU, RPB2-1, and RPB2-2 genomic regions were sequenced for 33 *D. castaneae* isolates and deposited in GenBank (Table S2). All analysed gene fragments of the collection culture *D. castaneae* CBS212.90 (Table S2) shared 99–100 % identity with the *D. castaneae* isolates studied here. The best BLAST match in the NCBI database was obtained by the rDNA ITS gene sequence (553 bp) of the isolate Dca90, which shared 100 % identity with sequences of the *Gnomoniopsis* sp. ICMP14082 (accession no. KC145849) and *Gnomoniopsis* sp. Gall_18a–b–c (KT823779–80–81). The best matches (maximum score) in the NCBI for the other gene regions of *D. castaneae* Dca90 were: LSU (1305 bp), which shared 99 % identity with *Cryptosporella hypodermia* AFTOL-ID2124 (DQ862028); RPB2-1 (1039 bp), which shared 98 % identity with *Plagiostoma conradii* CBS109761 (EU219294); EF-1α (1006 bp), which shared 97 % identity with *Gnomoniopsis clavulata* CBS121255 (GU320807); and β-tub-1 (1553 bp), which shared 86 % identity with *Cryptosporella jaklitschii* CBS125665 (GU826006). The second intron in the EF-1α-1 sequence (536 bp in Dca90) was considerably shorter in *D. castaneae* isolates (intron length 86 bp in Dca90) compared to the *Gnomoniopsis* specimen analysed here (intron lengths 144–158 bp, e.g. *G. castaneae* Gca1: 153 bp) and all other

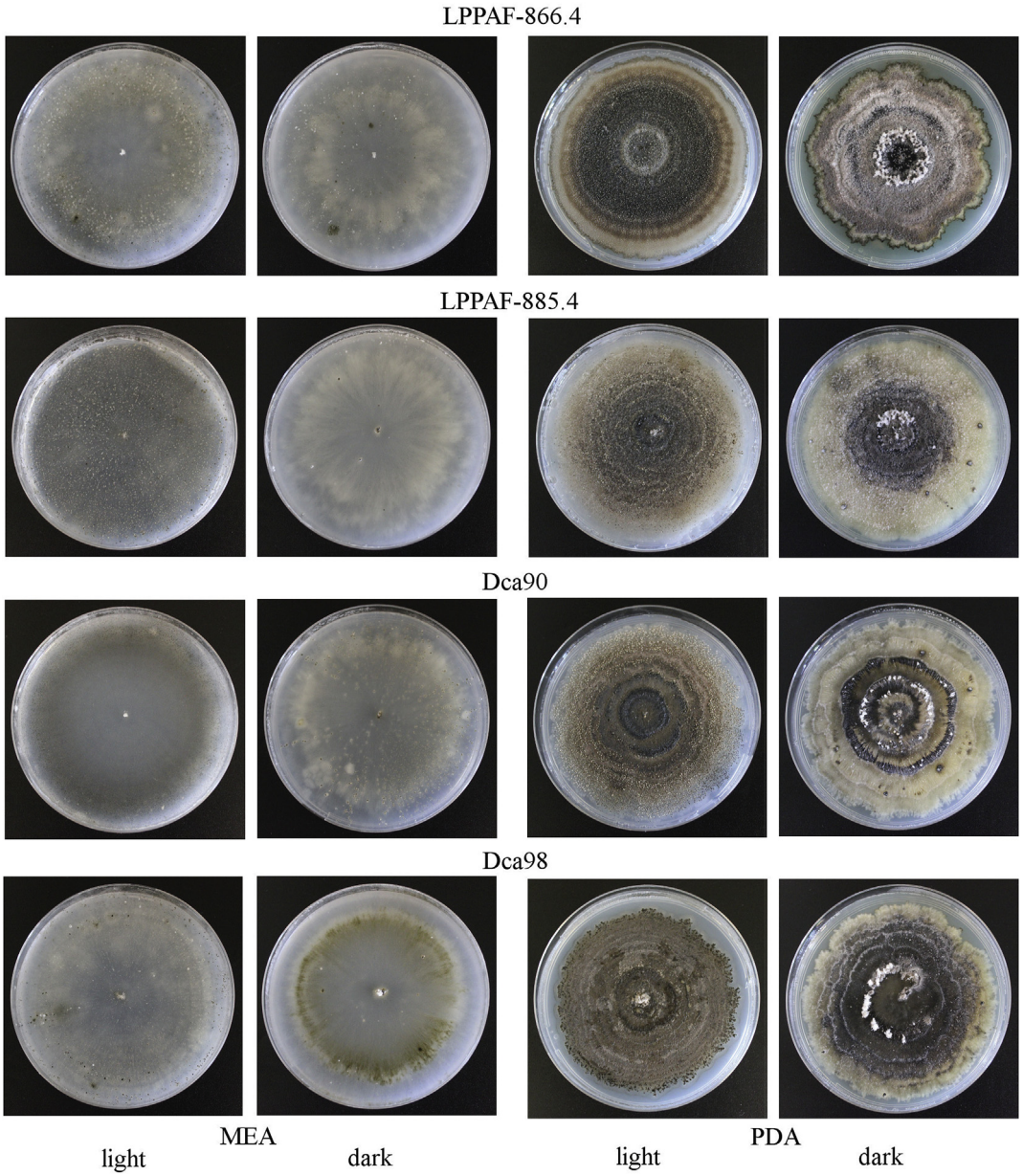

Fig 3 – Morphology on potato dextrose agar (PDA) and malt extract agar (MEA) of the two Swiss isolates of *Diplodina castaneae* Dca90 and Dca98 and the Spanish isolates LPPAF-885.4 and LPPAF-866.4. Colonies were grown at 25 °C for 21 d under i) complete darkness and ii) 16 h/8 h light/dark regime.

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Gnomoniaceae included in this study, such as *P. conradii* CBS109761 (163 bp) (Table S2). For this reason, this intron was excluded from the alignments for further phylogenetic analysis.

As ML and BRC analyses resulted in similar phylograms, only the ML trees are included in this paper. The ML tree produced with EF-1α-2, LSU, and RPB2-1 comprised 38 sequences containing 2735 characters (Fig 4A), and that produced with β-tub-1, EF-1α-1, and ITS (Fig 4B) consisted of 29 sequences with 1335 characters. In both phylogenetic trees, all *D. castaneae* isolates (i.e. two Spanish, two Swiss, one Azerbaijani, the culture collection CBS212.90) and *Gnomoniopsis* sp. ICMP14082 (in the β-tub-1, EF-1α-1, ITS dataset) segregated together within a monophyletic clade with a maximum likelihood bootstrap value (100 %). The closest relative to this clade was *P. conradii* CBS109761 (Fig 4A: 100 % and Fig 4B: 100 %), which was separated from all other *Plagiostoma* isolates (*P. aesculi* CBS109765, *Papiculatum* CBS109775, and *Peuphorbiae* CBS340.78), which clustered together in a highly supported monophyletic clade (Fig 4A: 97 % and Fig 4B: 100 %). Both phylogenies placed the *D. castaneae* group as a sister clade of *Sirococcus* species. This finding was moderately supported by the ML analysis (Fig 4A: 95 %; Fig 4B: 57 %), but strongly supported by the Bayesian model (Bayesian posterior probabilities, Fig 4A: 0.99, and Fig 4B: 0.96).

Single-locus phylogenies were inferred on the loci β-tub-2 (506 characters), EF-1α-1 (461 characters), ITS (554 characters), and RPB2-2 (521 characters) for 29 isolates (or 28 in the case of β-tub-2 because the CBS212.90 sequence was missing; Fig S2) originating from all types of chestnut tissues and geographic origins (Azerbaijan, France, Spain, and Switzerland). A LSU phylogeny was not inferred because all *D. castaneae* isolates had an identical LSU sequence. As the single-locus trees were discordant, no multilocus analysis was performed. Single-locus trees indicated a low genetic diversity within the 27 isolates. The trees divided the *D. castaneae* isolates into three groups that shared 97–99 % (for both RPB2-2 and EF-1α-1) and 99 % (ITS) sequence identity and two groups that shared 98 % sequence identity (β-tub-2).

Discussion

The main goal of the present study was to morphologically and phylogenetically characterise a fungal species that we isolated from different tissues of *Castanea sativa* growing in three different countries. Although this fungus can be confused with *Gnomoniopsis castaneae* based on its morphology, phylogenetic analyses of five genomic regions placed all analysed samples in a monophyletic group. This group includes the strain CBS212.90, i.e. *Diplodina castaneae*, and is clearly separated from the genus *Gnomoniopsis*.

Diplodina castaneae is the causal agent of the disease referred to as 'Javart', a veterinary term for ulcers on horses hooves, because cankers on chestnut shoots resemble these ulcers (Day 1930). The disease has mainly been found in France, where it was first reported around 1860. Afterwards, *D. castaneae* spread rapidly and affected the majority of French

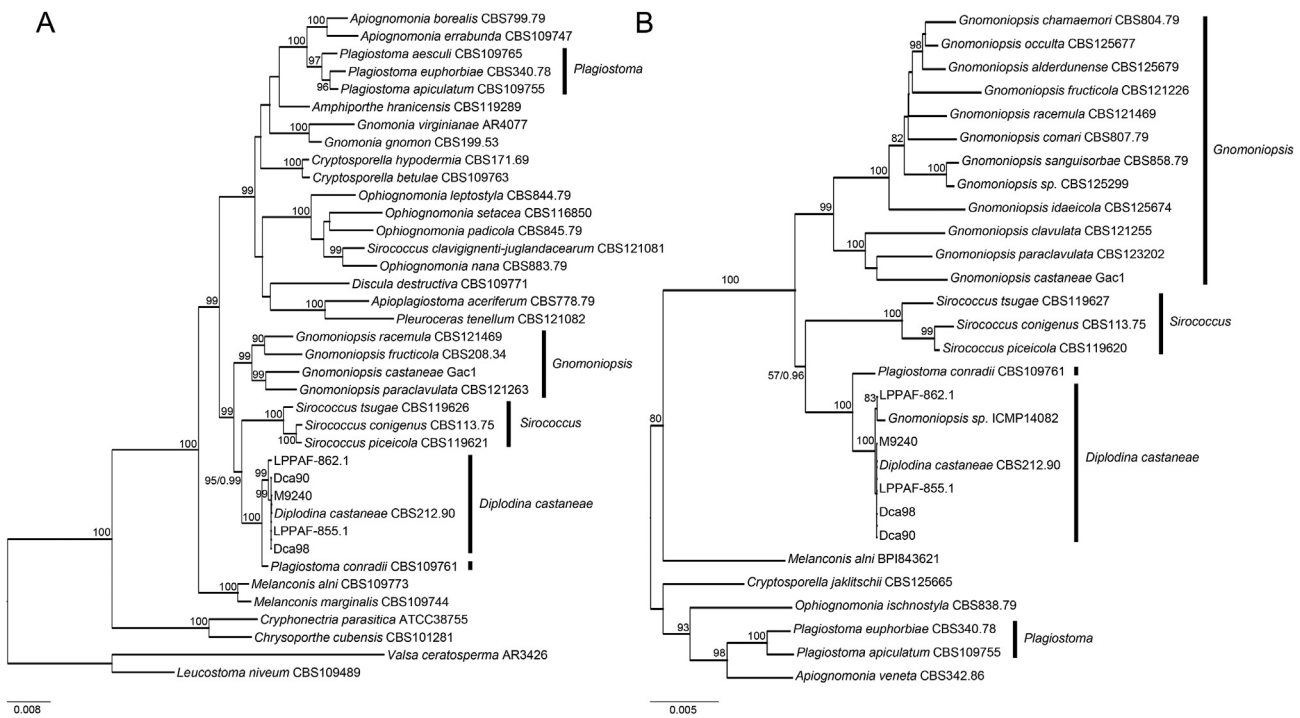

Fig 4 – Phylogenetic relationships of *Diplodina castaneae* isolates among genera of the Gnomoniaceae (A) and *Gnomoniopsis* spp. (B). The Maximum Likelihood (ML) trees were inferred from concatenated sequences of EF-1α-2, LSU, and RPB2-1 (2735 characters; A) and of β-tub-1, EF-1α-1, and ITS (1335 characters; B). Only bootstrap values higher than 70 % are shown. Scale bar = substitutions per site. Origins of the *D. castaneae* isolates, Dca: Switzerland, LPPAF-: Spain, M9240: Azerbaijan, and CBS212.90: culture collection from France.

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coppice stands (Prillieux & Delacroix 1893). In the 1930s, a serious dieback of European chestnut caused by *D. castaneae* was also observed in England (Day 1930). Since then, the disease has become less prevalent and new outbreaks have been reported rarely and only locally. This is most likely a consequence of the introduction of *Cryphonectria parasitica*, the causal agent of chestnut blight, in Europe in the late 1930s. At that time, the potentially devastating ecological consequences of this new invasive pathogen captured the interest of the scientific community and of interested parties (e.g. wood producers, chestnut growers). It is also possible that, in some cases, chestnut dieback caused by *D. castaneae* was confused with damages due to *C. parasitica*, which since 1938 rapidly spread to and became predominant in most European chestnut regions.

The incidence of *D. castaneae* in bark cankers caused by *C. parasitica* was low (<5 %) in Azerbaijan and Switzerland. Comparably low *D. castaneae* incidences were found by Bissegger & Sieber (1994) in the healthy bark of coppice shoots in Switzerland. In contrast, in northern Spain *D. castaneae* was isolated from 13.3 % of the cankers. This higher incidence rate may be explained by the fact that only inactive cankers (callused or callusing; Rigling & Prospero 2017) were sampled in Spain. In such cankers, *C. parasitica* may coexist with other fungi, including *D. castaneae*. In the abandoned galls of *Dryocosmus kuriphilus*, *D. castaneae* showed an overall incidence similar to that in healthy chestnut bark. However, since we only sampled necrotic galls, it is not possible to determine if *D. castaneae* was already present as endophyte in the green galls or if it colonised the galls after the tissue died.

To better characterize the intraspecific genetic diversity of *D. castaneae*, we analysed 20 different isolates from Switzerland, six from Spain and one from Azerbaijan. All isolates clustered together, regardless of their geographic origin and the chestnut tissue (i.e. galls, cankers, and healthy twigs) from which they originated. Gene trees indicated a low genetic diversity, with one to three haplotypes per genetic region. Furthermore, no geographical pattern could be found, even between the European isolates and the isolate from Azerbaijan. This finding is consistent with previous studies on *G. castanea*, another common chestnut endophyte, which is genetically homogenous (considering ITS and EF-1α loci) over Europe, New Zealand, and Australia (Dennert *et al.* 2015; Shuttleworth *et al.* 2015). However, a recent study conducted on the same species using polymorphic microsatellite loci revealed the presence of at least two putative subpopulations in Italy, France, and Switzerland (Sillo *et al.* 2017). Therefore, for comprehensive population genetic studies on *D. castaneae* additional, variable loci would be needed.

In our study, both phylogenetic analyses placed the *D. castaneae* isolates in a statistically well-supported group containing *Sirococcus* species and the strain CBS109761 known as *Plagiostoma conradii* in GenBank. Although Sogonov *et al.* (2008) is given as a reference for several sequences of the strain CBS109761 in GenBank, none of these sequences is mentioned in that paper. Mejía *et al.* (2011) suggest, based on a LSU analysis, that the strain CBS109761 represents a species from a genus within the *Gnomoniaceae*, other than *Plagiostoma*. However, as we could not find any conidiomata on CBS109761 cultures, we cannot make any statement on the morphological similarity between the CBS109761 and *D. castaneae*/*Sirococcus* species. Noteworthy, the holotype of *P. conradii* was isolated from the plant *Corema conradii*, a member of the Ericaceae, whereas CBS109761 strain was recovered from a plant species from the Cistaceae, an only distantly related plant family, suggesting that CBS109761 was not identified correctly.

Our analyses additionally demonstrate that application of the genus name *Diplodina* is problematic. To our knowledge, no other *Diplodina* species is phylogenetically close to *D. castaneae*. For example, *Diplodina acerina*, an endophyte of maples (*Acer* spp.) (Sieber & Dorworth 1994), groups with the *Apiognomonia* genus based on its ITS sequence (data not shown). Furthermore, *Diplodina* is a taxonomically heterogeneous group that includes synonyms of species in many other genera (e.g. *Ascochyta*, *Discella*, *Microdiplodia*, and *Phloeospora*). *Diplodina microsperma* (Sutton 1980) is the anamorph of *Plagiostoma apiculatum* and is considered identical with *Diplodina salicis*, the type strain of *Diplodina* and consequently transferred into the genus *Plagiostoma* (Sogonov *et al.* 2008; Mejía *et al.* 2011; Rossman *et al.* 2015). Unfortunately, there appears to be no other name available for the species known as *D. castaneae*, and taxonomic ambiguity regarding this species will remain until a systematic proposal for a more appropriate name is formulated. To our knowledge, *Sirococcus* is the most closely related genus to the fungal strain studied here; therefore, we propose that *D. castaneae* should be included in the genus *Sirococcus*. This genus comprises asexually reproducing species including important pathogens of conifers (Rossman *et al.* 2008). This re-arrangement is supported by the morphological descriptions provided in Sutton (1980), which indicated that the two genera do not differ essentially from each other, and by our own observations that the morphology of our samples fits well with the description of *Sirococcus*. Since the phylogenetic distance between *D. castaneae* and other *Sirococcus* species is in the same range of that between other phylogenetically well-supported genera (e.g. *Cryphonectria*—*Chrysoporthe*, *Apioplagiostoma*—*Pleuroceras*), erection of a new genus to accommodate *Diplodina castaneae* could be justified. However, as none of the morphological characteristics of *D. castaneae* deviates distinctly from the genus concept of *Sirococcus*, we rejected this option.

Taxonomy

Sirococcus castaneae (Prill. & Delacr.) J.B. Meyer & B. Senn-Irlet & T.N. Sieber, **comb. nov.**

MycoBank No.: MB821535; Figs 3 and 5.

Basionym: *Diplodina castaneae* Prill. & Delacr. — Bull. Soc. mycol. Fr. 9: 277 (1893).

≡ *Diplodinula castaneae* (Prill. & Delacr.) Tassi — Bulletin Labor. Orto Bot. de R. Univ. Siena **5**: 44 (1902).

Asexual morphology

Conidiomata stromatic separate, occasionally confluent, multilocular, dark brown to black, surface brown felty up to 1 mm diam; conidiomatal wall 30—50 μm diam, textura globulosa-angularis of dark brown thick-walled cells of

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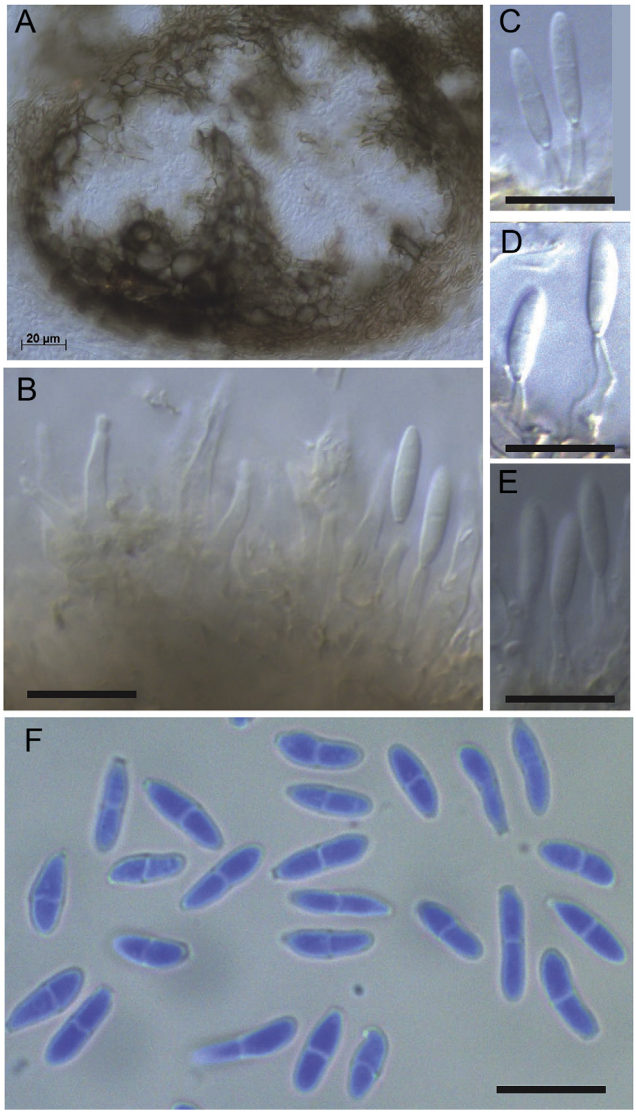

Fig 5 – Micromorphology of *Diplodina castaneae* Dca90 grown on PDA for 28 d at 20 °C. (A) Multilocular stromatic conidioma. (B–E) Conidiophores and conidiogenous cells with conidia in different developmental stages. (F) Conidia of Dca98 isolate stained in cotton blue. Scale bars in (B–F) represent 10 µm.

3.5–15 µm (Fig 5A). *Conidiophores* hyaline, smooth, sometimes branched at the base, but mostly consisting of the conidiogenous cell only, formed from the tissue lining the loci. *Conidiogenous cells* enteroblastic, phialidic, hyaline, smooth, collarette and channel minute, up to 10 µm long and 2 µm wide (Fig 5B–E). *Conidia* 6.5–13.6 × 1.7–3.9 µm, hyaline, smooth, l/w ratio (Qmean) = 2.90–4.20, 0–1 septate, septum located at 45–55 % of conidiospore length, ellipsoid, polymorphic, i.e. cylindrical, fusoid, not constricted at septum and with few very small guttules (Fig 5F, Table 2), or ellipsoid, with obtuse end, slightly constricted at septum and with several small guttules, straight or inequilateral, with acyanophilous cell walls.

Culture characteristics – The colony grown on MEA, under both light and dark conditions, was glabrous with diffuse margins and overlaid by white loose mycelium. The colony grown on PDA presented curled margins and was brown in light conditions and brownish-white in dark conditions. Short white cottony-like mycelium was observed in isolates incubated in the dark, and dull brown patterns in the centre were shown under both conditions. In all cases, fungal colonies were flat, and abundant dull brownish grey to pink conidia were observed under light conditions whereas fewer conidia were observed under dark conditions.

Specimens examined – All material examined is listed in Table 1.

Notes – The description of the micromorphology of *D. castaneae* is based on fruiting bodies formed in culture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2017.04.001.

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Table S1. Coordinates of the chestnut stands in which the *Diplodina castaneae* isolates analysed in this study were sampled.

Chestnut stand	Country	Region/County	Coordinates (WGS84)	Reference
Biasca	Switzerland	Ticino	46.353812, 8.976810	Meyer et al. (2015)
Robasacco	Switzerland	Ticino	46.142016, 8.944519	Meyer et al. (2015)
Mugena	Switzerland	Ticino	46.050261, 8.885494	Meyer et al. (2015)
Stabio	Switzerland	Ticino	46.846565, 8.920165	Meyer et al. (2015)
Bex-Creux	Switzerland	Chablais	46.239145, 7.012009	Meyer et al. (2015)
Bex-Montet	Switzerland	Chablais	46.257859, 7.014974	Meyer et al. (2015)
Choëx	Switzerland	Chablais	46.241162, 6.971451	Meyer et al. (2015)
Monthey	Switzerland	Chablais	46.258282, 6.940308	Meyer et al. (2015)
Gilly	Switzerland	La Côte	46.460450, 6.292540	-
Aller	Spain	Asturias	43.118729, 5.583930	-
Lena	Spain	Asturias	43.130833, 5.733333	-
Llanera	Spain	Asturias	43.442627, 5.857354	-
Peñamellera Baja	Spain	Asturias	43.295275, 4.610715	-
Istisu	Azerbaijan	Ismayilli	48.075833, 40.938388	-
Vandam	Azerbaijan	Qabala	47.941083, 40.944694	-
Bash Kungut	Azerbaijan	Shaki	47.319337, 41.138457	-

Table S2 Fungal cultures and NCBI sequences used in this study.

Isolate name	Strain	Collection number	ITS[a]	EF-1α[a]	LSU[a]	RPB2[a]	β-tub[a]
Sordariomycetes/Sordariomycetidae/Diaporthales/Gnomoniaceae:							
Diplodina castaneae	Dca40	-	KX929736	KX929702	KX929771	KX929806	KX958437
Diplodina castaneae	Dca60	-	KX929737	KX929703	KX929772	KX929807	KX958438
Diplodina castaneae	Dca77	-	KX929738	KX929704	KX929773	KX929808	-
Diplodina castaneae	Dca79	-	KX929739	KX929705	KX929774	KX929809	KX958439
Diplodina castaneae	Dca80	-	KX929740	KX929706	KX929775	KX929810	KX958440
Diplodina castaneae	Dca83	-	KX929741	KX929707	KX929776	KX929811	KX958441
Diplodina castaneae	Dca85	-	KX929742	KX929708	KX929778	KX929812	KX958442
Diplodina castaneae	Dca87	-	KX929743	KX929709	KX929777	KX929813	-
Diplodina castaneae	Dca90	CBS142041	KX929744	KX929710	KX929779	KX929814	KX958443
Diplodina castaneae	Dca91	-	KX929745	KX929711	KX929780	KX929815	KX958444
Diplodina castaneae	Dca92	-	KX929746	KX929712	KX929781	KX929816	KX958445
Diplodina castaneae	Dca93	-	KX929747	KX929713	KX929782	KX929817	KX958446
Diplodina castaneae	Dca94	-	KX929714	KX929748	KX929783	KX929818	KX958447
Diplodina castaneae	Dca95	-	KX929749	KX929715	KX929784	KX929819	KX958448
Diplodina castaneae	Dca97	-	KX929750	KX929716	KX929785	KX929820	KX958449
Diplodina castaneae	Dca98	CBS142042	KX929751	KX929717	KX929786	KX929821	KX958450
Diplodina castaneae	Dca99	-	KX929752	KX929718	KX929787	KX929822	KX958451
Diplodina castaneae	Dca100	-	KX929753	KX929719	KX929788	KX929823	-
Diplodina castaneae	Dca101	-	KX929754	KX929720	KX929789	KX929824	KX958452
Diplodina castaneae	Dca102	-	KX929755	KX929721	KX929790	KX929825	KX958453
Diplodina castaneae	Dca103	-	KX929756	KX929722	KX929791	KX929826	KX958454
Diplodina castaneae	Dca104	-	KX929757	KX929723	KX929792	KX929827	KX958455
Diplodina castaneae	Dca105	-	KX929758	KX929724	KX929793	KX929828	KX958456
Diplodina castaneae	LPPAF-851.2	-	KX929759	-	KX929794	KX929829	-
Diplodina castaneae	LPPAF-855.1	-	KX929760	KX929730	KX929801	KX929830	KX958457
Diplodina castaneae	LPPAF-860.1	-	KX929761	KX929725	KX929795	KX929831	KX958458
Diplodina castaneae	LPPAF-862.1	-	KX929762	KX929726	KX929800	KX929832	KX958459
Diplodina castaneae	LPPAF-866.4	-	KX929763	KX929727	KX929796	KX929833	KX958460
Diplodina castaneae	LPPAF-883.2	-	KX929764	KX929728	KX929799	KX929834	KX958461

Diplodina castaneae	LPPAF-885.4	-	KX929765	KX929731	KX929797	KX929835	-
Diplodina castaneae	LPPAF-886.2	-	KX929766	KX929729	KX929798	KX929836	KX958462
Gnomoniopsis castaneae	Gca1	CBS142044	KT823778	KX929733	KX929802	KX929837	KX958464
Cryptosporella jaklitschii	LCM112.04 LCM112.01	BPI879231 CBS125665	GU826089	GU826048	-	-	GU826007
Diplodina acerina	cult_08_A08	-	HQ414605	-	-	-	-
Diplodina castaneae	-	CBS212.90	KX929734	KX929700	KX929769	KX929805	-
Gnomoniopsis sp.	-	ICMP14082	KC145849	KC145964	-	-	-
Melanconis alni	AR4016	BPI843621	EU254863	EU221894	-	-	EU219122
Ophiognomonia ischnostyla		CBS838.79	EU254891	JQ414142	-	-	EU219162
Plagiostoma apiculatum	AR3455	BPI747938 CBS109775	DQ323529	EU221916	AF408345	EU199141	GU367008
Plagiostoma conradii	AR3488	BPI746482 CBS109761	EU254761	EU221888	KX929768	EU219294	EU219094
Sirococcus piceicola	AR4038	BPI871166 CBS119621	-	EU221923	EU199135	EU199158	-
Sirococcus tsugae	AR3953	BPI 871167 CBS119626	-	EU221925	EU199136	EU199159	-

[a]ITS: internal transcribed spacer (ITS) 1, 5.8S ribosomal RNA gene, ITS2; EF-1α: translation elongation factor-1 alpha gene; LSU: 28S rRNA gene; RPB2: RNA polymerase II gen; β-tub: beta-tubulin gene.

Table S3 Primers used in this study.

Gene product	Region label	Primer name	Primer sequence (5'→3')	Primers use[a]	Product lenght (bp) With / without primer sequences	Reference
Translation elongation factor-1 alpha (EF-1α) gene	EF-1α-1	EF1-728F	CATCGAGAAGTTCGAGAAGG	PCR and seq EF-1α-1	577 / 536	Carbone & Kohn, 1999
		EF1-1199R	GGGAAGTACCMGTGATCATGT	PCR and seq EF-1α-1		Walker et al., 2010
	EF-1α-2	EF1-728F	CATCGAGAAGTTCGAGAAGG	PCR and seq for EF-1α-2	475 / 521	Rehner & Buckley 2005
		EF1-1567R	ACHGTRCCRATACCACCRATCTT	PCR EF-1α-2		
		EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	seq EF-1α-2		
18S rRNA[c] gene, ITS[d]1, 5.8S rRNA gene, ITS2, and 28S rRNA gene	ITS	ITS1	TCCGTAGGTGAACCTGCGG	PCR and seq ITS	593 / 553	White et al., 1990
		ITS4	TCCTCCGCTTATTGATATGC	PCR and seq ITS		
28S rRNA gene	LSU	LR0R	ACCCGCTGAACTTAAGC	PCR and seq LSU	1339 / 1305	Rehner & Samuels 1994;
		LR7	TACTACCACCAAGATCT	PCR and seq LSU		Vilgalys & Hester 1990
		LSU-R	CGCCGAAGCTCTCACCTAAT	seq LSU		This study
RNA polymerase II gene	RPB2-1	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	PCR and seq RPB2-1	1163 / 1123	Liu et al., 1999
		fRPB2-7cR	CCCATRGCTTGYTTRCCCAT	PCR and seq RPB2-1		This study
	RPB2-2	pol2-for	TCAAYGGTACCTGGGTTGGYG	PCR RPB2-2 / seq RPB2-1 and 2	561 / 521	
		pol2-rev	CGCAGATCCCCARAAGCAT	PCR RPB2-2 / seq RPB2-1 and 2		
Beta-tubulin gene	β-tub-1	T1	AACATGCGTGAGATTGTAAGT	PCR and seq β-tub-1	1597 / 1555	O'Donnell et al., 1997
		T22	TCTGGATGTTGTTGGGAATCC	PCR and seq β-tub-1		This study
		Gnom_tub2_F	CGTYCTCGTCGATCTCGAGC	seq β-tub-1		
	β-tub-2	Gnom_tub1_F	TCACCTCCAGACCGGYCAAT	PCR β-tub-2 / seq β-tub-1 and 2	544 / 504	
		Gnom_tub1_R	GCTCGAGATCGACGAGRACG	PCR β-tub-2/ seq β-tub-1 and 2		

[a] PCR: primers used for the PCR amplification; seq: primers used for the sequencing reaction. E.g. RPB2-1 (see column "Region label") was amplified with fRPB2-5F and fRPB2-7cR and either sequenced only with fRPB2-5F and fRPB2-7cR or with fRPB2-5F, fRPB2-7cR, pol2-for and pol2-rev;

[b]The indicated product lengths were obtained with isolate *Diplodina castaneae* LPPAF-883.2;

[c]rRNA: ribosomal RNA;

[d] ITS: internal transcribed spacer.

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Table S4 Script and output for the statistical tests described in Materials and Methods.

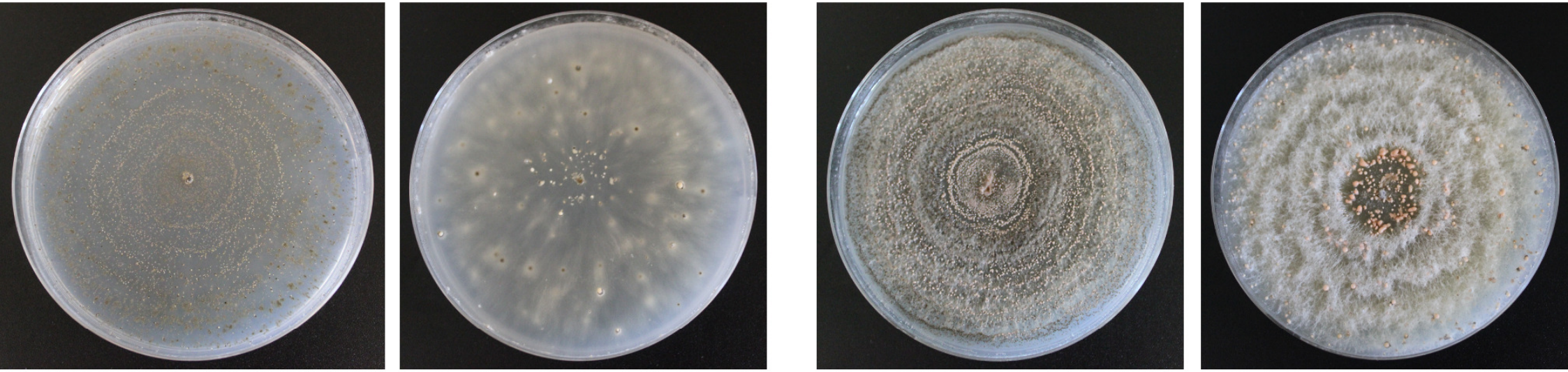
(1) Testing differences between the two regions Chablais and Ticino:

```
> glmm_proportion_model_MASS <- glmmPQL(Abundance_Dca ~ Region, random = ~1 | Chestnut_stand, fa
mily = binomial(link = "logit"), data = Diplodina_castaneae)
> summary(glmm_proportion_model_MASS)
Linear mixed-effects model fit by maximum likelihood
 Data: tt2
  AIC BIC logLik
   NA  NA     NA

Random effects:
 Formula: ~1 | Place
        (Intercept)  Residual
StdDev:    0.802928 0.2313887

Variance function:
 Structure: fixed weights
 Formula: ~invwt
Fixed effects: Ab_Dca ~ Region
                Value Std.Error DF   t-value p-value
(Intercept)  -2.802502 0.4785004 32 -5.856843  0.0000
RegionTicino -3.063677 1.1789421  6 -2.598666  0.0407
 Correlation:
             (Intr)
RegionTicino -0.406

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-1.1997434 -0.6348502 -0.2137867 -0.1684716  3.7385977

Number of Observations: 40
Number of Groups: 8
```

(2) Testing differences between the chestnut stands within the regions:

```
> kruskal.test(Abundance_Dca ~ Chestnut_stand, data = subset(Diplodina_castaneae, Region == "Chablais"))

        Kruskal-Wallis rank sum test

data:  Ab_Dca by Place
Kruskal-Wallis chi-squared = 9.0944, df = 3, p-value = 0.02806

> kruskal.test(Abundance_Dca ~ Chestnut_stand, data = subset(Diplodina_castaneae, Region == "Ticino"))

        Kruskal-Wallis rank sum test

data:  Ab_Dca by Place
Kruskal-Wallis chi-squared = 3.9333, df = 3, p-value = 0.2688

> kruskal.test(Abundance_Dca ~ Chestnut_stand, data = subset(Diplodina_castaneae, Region == "Chablais" &
Place != "Bex-Montet"))

        Kruskal-Wallis rank sum test

data:  Ab_Dca by Place
Kruskal-Wallis chi-squared = 2.6353, df = 2, p-value = 0.2678
```

Gnomoniopsis castaneae strain Gca1



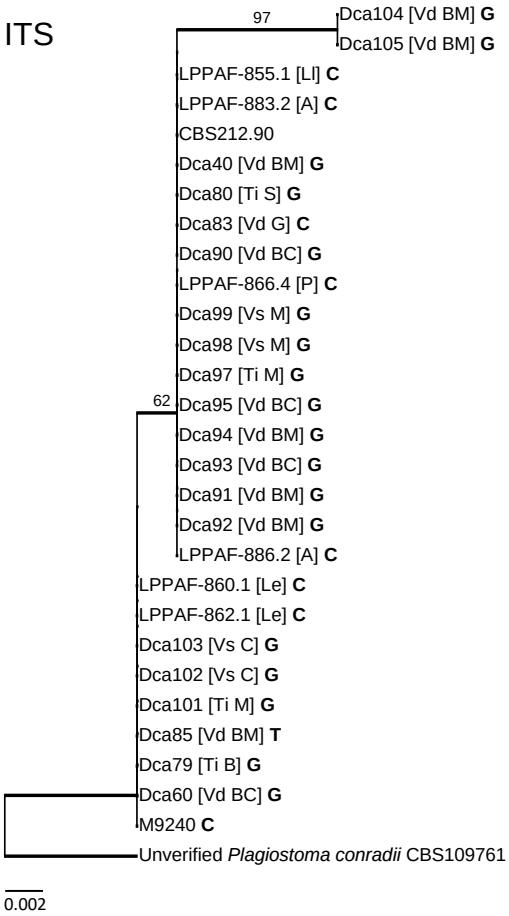
MEA

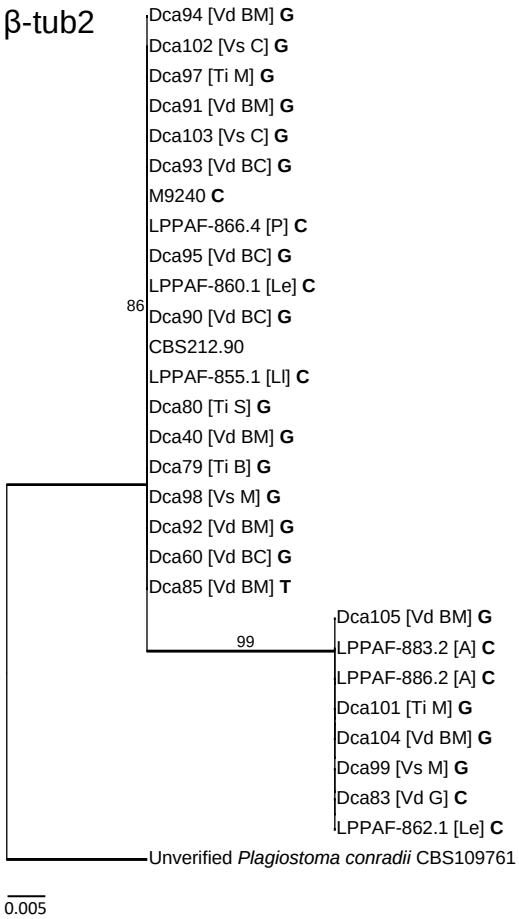
light dark

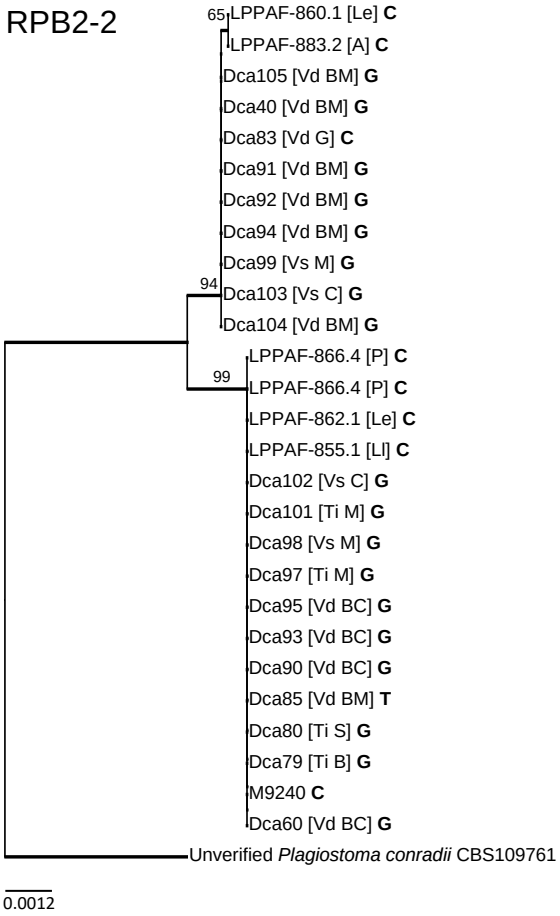
PDA

light dark


EF-1α-1
LPPAF-860.1 [Le] C
Dca105 [Vd BM] G
Dca83 [Vd G] C
LPPAF-866.4 [P] C
Dca93 [Vd BC] G
LPPAF-886.2 [A] C
Dca95 [Vd BC] G
LPPAF-862.1 [Le] C
69
LPPAF-883.2 [A] C
Dca104 [Vd BM] G
87
Dca99 [Vs M] G
Dca101 [Ti M] G
64
Dca103 [Vs C] G
72
LPPAF-855.1 [Ll] C
Dca102 [Vs C] G
Dca98 [Vs M] G
Dca97 [Ti M] G
Dca94 [Vd BM] G
Dca92 [Vd BM] G
Dca91 [Vd BM] G
Dca90 [Vd BC] G
Dca85 [Vd BM] T
Dca80 [Ti S] G
Dca79 [Ti B] G
Dca60 [Vd BC] G
Dca40 [Vd BM] G
CBS212.90
M9240 C
Unverified Plagiostoma conradii CBS109761
0.04


ITS
97
Dca104 [Vd BM] G
Dca105 [Vd BM] G
LPPAF-855.1 [Ll] C
LPPAF-883.2 [A] C
CBS212.90
Dca40 [Vd BM] G
Dca80 [Ti S] G
Dca83 [Vd G] C
Dca90 [Vd BC] G
LPPAF-866.4 [P] C
Dca99 [Vs M] G
Dca98 [Vs M] G
Dca97 [Ti M] G
62
Dca95 [Vd BC] G
Dca94 [Vd BM] G
Dca93 [Vd BC] G
Dca91 [Vd BM] G
Dca92 [Vd BM] G
LPPAF-886.2 [A] C
LPPAF-860.1 [Le] C
LPPAF-862.1 [Le] C
Dca103 [Vs C] G
Dca102 [Vs C] G
Dca101 [Ti M] G
Dca85 [Vd BM] T
Dca79 [Ti B] G
Dca60 [Vd BC] G
M9240 C
Unverified Plagiostoma conradii CBS109761
0.002


β-tub2
Dca94 [Vd BM] G
Dca102 [Vs C] G
Dca97 [Ti M] G
Dca91 [Vd BM] G
Dca103 [Vs C] G
Dca93 [Vd BC] G
M9240 C
LPPAF-866.4 [P] C
Dca95 [Vd BC] G
LPPAF-860.1 [Le] C
86
Dca90 [Vd BC] G
CBS212.90
LPPAF-855.1 [Ll] C
Dca80 [Ti S] G
Dca40 [Vd BM] G
Dca79 [Ti B] G
Dca98 [Vs M] G
Dca92 [Vd BM] G
Dca60 [Vd BC] G
Dca85 [Vd BM] T
Dca105 [Vd BM] G
99
LPPAF-883.2 [A] C
LPPAF-886.2 [A] C
Dca101 [Ti M] G
Dca104 [Vd BM] G
Dca99 [Vs M] G
Dca83 [Vd G] C
LPPAF-862.1 [Le] C
Unverified Plagiostoma conradii CBS109761
0.005


RPB2-2
65
LPPAF-860.1 [Le] C
LPPAF-883.2 [A] C
Dca105 [Vd BM] G
Dca40 [Vd BM] G
Dca83 [Vd G] C
Dca91 [Vd BM] G
Dca92 [Vd BM] G
Dca94 [Vd BM] G
Dca99 [Vs M] G
94
Dca103 [Vs C] G
Dca104 [Vd BM] G
LPPAF-866.4 [P] C
99
LPPAF-866.4 [P] C
LPPAF-862.1 [Le] C
LPPAF-855.1 [Ll] C
Dca102 [Vs C] G
Dca101 [Ti M] G
Dca98 [Vs M] G
Dca97 [Ti M] G
Dca95 [Vd BC] G
Dca93 [Vd BC] G
Dca90 [Vd BC] G
Dca85 [Vd BM] T
Dca80 [Ti S] G
Dca79 [Ti B] G
M9240 C
Dca60 [Vd BC] G
Unverified Plagiostoma conradii CBS109761
0.0012

ARTÍCULO IV

Trapiello E., Feito I. y González A.J. (2017). First report of the fungus *Gnomoniopis castaneae*, a chestnut canker agent, in Spain. (Pendiente de enviar a *Plant Disease*).

Título

Primera cita del hongo *Gnomoniopis castaneae*, un agente de chancro en castaño

Resumen

De dos muestras de castaño, recogidas en Asturias en 2016, que presentaban lesiones rojizas, se obtuvieron dos aislamientos fúngicos morfológicamente idénticos. Las colonias crecidas en APD mostraron abundante micelio de color blanco parduzco bajo condiciones de luz y blanco en oscuridad. En ambas condiciones se observaron conidios viscosos de color naranja, distribuidos en espiral bajo condiciones de luz. Tras la extracción de ADN, amplificación y secuenciación del ITS de los dos aislamientos, se comprobó que la secuencia era idéntica y similar en 100% con secuencias correspondientes a *Gnomoniopsis castaneae*. Esta especie es un conocido agente causal de la enfermedad de la podredumbre de la castaña, y patógeno responsable de la formación de chancros en castaño. Por lo que sabemos, esta es la primera cita del patógeno en España.

First report of the fungus *Gnomoniopis castaneae*, a chestnut canker agent, in Spain

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Gnomoniopsis castaneae Tamietti (syn. *G. smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew y D.I. Guest) is the causal agent of chestnut rot disease (Visentin et al. 2012). It is recently known as responsible of wood cankers on chestnut, these cankers being similar to ones due to *Cryphonectria parasitica* infection on twigs and scions of chestnut trees (Pasche et al. 2016). From two samples presenting similar symptoms to those of chestnut blight disease (figure 1) and collected in 2016 in Asturias, northern Spain, two fungal isolates morphologically identical, and different from *C. parasitica*, were isolated. Colonies grown on PDA showed abundant brownish-white mycelia under light conditions and white under dark conditions (figure 2). Orange viscous conidia were always observed, they being uniformly spread throughout the media with a spiral shape in light (figure 2). The morphology of these isolates was similar to that described for *G. castaneae* by Meyer et al. (2017). To confirm its identity, DNA from one of them was extracted by using Terra ^{TM} PCR Direct Polymerase Mix Clontech TAKARA, and their nuclear ribosomal internal transcribed spacer (ITS) was amplified with two universal primers, ITS1 and ITS4 (White et al. 1990). The rDNA ITS gene sequence of the isolate (LPPAF-935, 552 bp) shared the highest sequence identity (100%) with the *G. smithogilvyi* strain ICMP14079 (accession number KC145860) according to the best BLAST match in the NCBI database. The phylogenetic relationship is depicted in figure

3. Sequences were aligned by ClustalW (Thompson et al. 1994). Phylogenetic tree was constructed using Mega 6.06 (Tamura et al. 2013) The sequence was deposited in GenBank and the accesion number is LT837820. To our knowledge, this is the first report of *Gnomoniopsis castaneae* in Spain.

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Figures

Figure 1. Chestnut samples showing infection with discoloration on twigs.

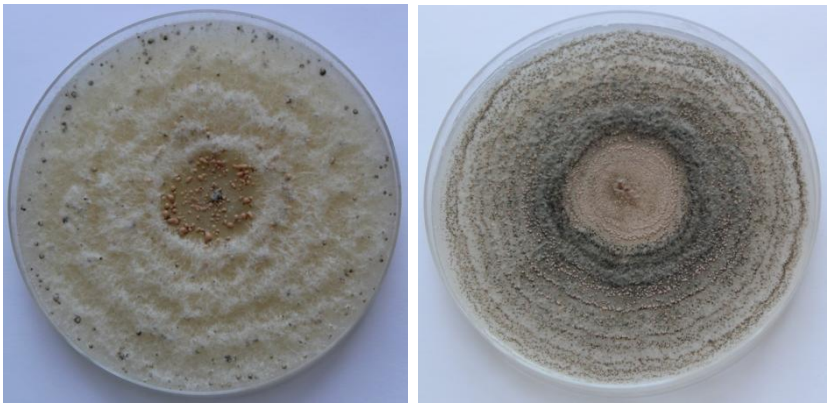

Figure 2. Colonies of *Gnomoniopsis castaneae* grown on PDA, under dark conditions (left) and under light conditions (right).

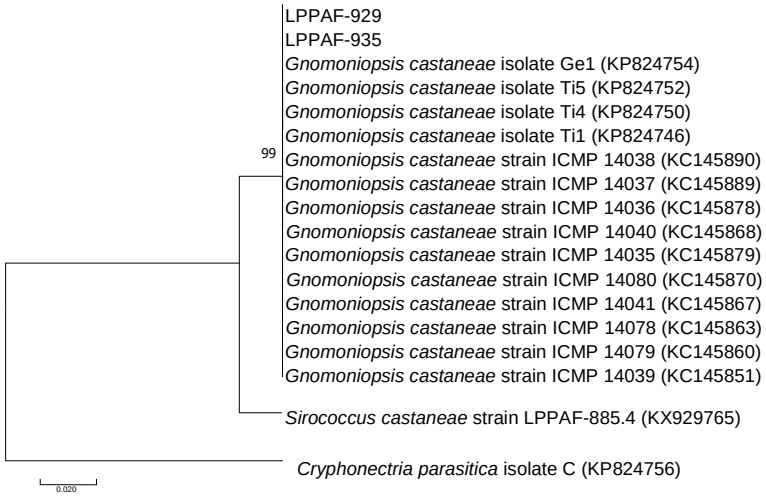

Figure 3. Phylogenetic tree depicting the relationship among our isolates (LPPAF-929 and LPPAF-935) and the species *Gnonomiopsis castaneae*, *Sirococcus castaneae* and *Cryphonectria parasitica*.