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New insights in *Ilyonectria* black foot disease of grapevine

Tese apresentada para a obtenção do grau de doutor em Engenharia Agronómica

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Lisboa
2011

Novas perspectivas sobre o pé negro da videira causado por *Ilyonectria*

RESUMO

Considerando a crescente importância do pé negro da videira, este estudo visa aprofundar o conhecimento sobre aspectos taxonómicos, genéticos, biológicos e de patogenicidade dos agentes causais, anteriormente atribuídos maioritariamente a *Ilyonectria liriodendri* e *I. macrodidyma.* A análise multigénica e morfológica de uma colecção de isolados de *Ilyonectria* permitiu descrever 12 espécies no complexo *I. radicicola* e quatro no complexo *I. macrodidyma*. Estudos de patogenicidade mostraram que *I. lusitanica*, *I. estremocensis* e *I. europaea* são mais virulentas para a videira que *I. liriodendri* e *I. macrodidyma.* Foram obtidos os loci de "mating-type" de *I. liriodendri* e de espécies do complexo *I. macrodidyma*. A estrutura do idiomorfo de *I. macrodidyma* mostrou-se semelhante à de outros fungos heterotálicos Hypocreales, mas a organização dos loci MAT em *I. liriodendri* é singular, sugerindo um potencial pseudo-heterotalismo. O inóculo existente no solo contribui de forma significativa para a doença. A amplificação de DNA de amostras de solo por qPCR demonstrou que a rotação permite reduzir a quantidade de *Ilyonectria* nos solos dos viveiros, e que o nível de infestação nos solos vitícolas é inferior ao verificado nos solos de viveiro e de plantas-mãe. Foi optimizado um protocolo de transformação de protoplastos de *I. liriodendri* para a integração estável do gene GFP, o que permitirá estudos futuros de genética funcional.

Palavras chave

Pé-negro da videira; *Cylindrocarpon*; *Ilyonectria*; Filogenia; Sistemática; genes de "mating-type"; patogenicidade; transformação de protoplastos; inóculo no solo; PCR quantitativo.

ABSTRACT

Considering the growing importance of black foot disease of grapevine, this study was aimed to deeply understand details on taxonomy, genetics, biology and pathological behaviour of its main causal agents, previously attributed mostly to *Ilyonectria liriodendri* and *I. macrodidyma*. A multi-gene analysis of a collection of *Ilyonectria* isolates, along with morphological characterisation, enabled the description of 12 species from *I. radicicola* and four from *I. macrodidyma* complexes. Among these, pathogenicity experiments revealed *I. lusitanica*, *I. estremocensis* and *I. europaea* as more virulent to grapevine than *I. liriodendri* and *I. macrodidyma*. The entire mating-type loci of *I. liriodendri* and of species from the *I. macrodidyma* complex were obtained. While the idiomorph structure of species from the latter matches that of other heterothallic Hypocreales, the organization of the mating-type loci in *I. liriodendri* seems unique, suggesting a potential pseudo-heterothallism. Soilborne inoculum is accepted to contribute significantly to initiate black foot disease in grapevine plants. qPCR amplification from DNA soil samples demonstrate that rotation can reduce the levels of *Ilyonectria* in nurseries, and that levels of infestation in vineyard soils are lower than in nursery or mother-plant soils. Additionally, a protoplast transformation protocol is presented for the stable integration of the GFP gene in the genome of *I. liriondendri*, enabling future downstream functional genetic studies.

Keywords

Black foot disease of Grapevine; Cylindrocarpon; *Ilyonectria*; Phylogeny; Systematics; Mating-type genes; pathogenicity; protoplast transformation; soilborne inoculum; quantitative PCR;

ACKNOWLEDGMENTS

The accomplishment of this thesis would not have been possible without the advice and collaboration of numerous persons which helped and encouraged its execution neither without the support of different institutions. This work was carried out in the context of the Research Group II - Production Systems and Technologies, of Biosystems Engineering (CEER) at Instituto Superior de Agronomia and of the Evolutionary Phytopathology group at CBS-KNAW Fungal Biodiversity Centre. This work was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/24790/2005; project PTDC/AGR-AAM/099324/2008).

First of all, I would like to thank Professor Helena Oliveira for the supervision that she gave me during my PhD, all the motivation and encouragement, friendship, valuable and constructive suggestions and a great guidance during all these years.

I would like to thank Professor Pedro Crous the co-supervision of this thesis. It was a privilege to work with you. Your enthusiasm is contagious, the innumerous suggestions, your expertise and knowhow that lead to produce the data present here and for the great opportunity to work in the CBS-KNAW Fungal Biodiversity Centre.

I am grateful to Eng. Cecília Rego co-supervisor of this thesis for her patience, motivation, enthusiasm, and immense knowledge on black foot disease. Her friendship and encouragement were also extremely important. She is also acknowledged for keeping the *Cylindrocarpon* culture collection (stored at the Laboratório de Patologia Vegetal 'Veríssimo de Almeida') in good conditions and available for this study.

At ISA/UTL I would like to acknowledge all the people of Departamento de Ciências e Engenharia de Biossistemas, Agrupamento Funcional de Protecção de Plantas for help and technical assistant, friendship and encouragement. To Eng. Teresa Nascimento for the help on molecular biology, scientific discussion and friendship. To Professors Arlindo Lima and Paula Ramos, and Eng. Filomena Caetano for their encouragement, valuable support, kindness and friendship. Amélia Marques, Olga Nogueira and Joaquim Antunes are thanked for the technical assistance especially during the greenhouse trials, re-isolations and media preparations. To Paula Pinto for office support and technical assistance. To my lab colleagues Ana Teresa Vaz, Inês Diniz, Margarida Cardoso, Marta Rocha, Patrícia Pinto, Pedro Reis and Sara Belchior for their continuous support, help and friendship. To Doctor Elsa Silva, Manuel Cariano and Vera Zina with whom I shared the office I am sincerely grateful for the encouragements, pleasant and

productive atmosphere and for providing unforgettable moments. Hopefully, there are still more to come!

At CBS-KNAW I would like to thank to the all the people that receive me always with a smile and were helpful and supportive. A special thank you to the people of the Evolutionary Phytopathology group that received me as a "guest", and made me feel like "home". In particular special thanks to Doctor Ewald Groenewald that has always been there for me, since the first instance, for his kindness and valuable knowledge that he transmitted to me. To Doctor Marizeth Groenewald, who first introduced me to the mating type world. To Doctor Madhi Arzanlou who was a great support, for all the scientific discussions, the technical help and all the trips we had in the Netherlands. To Doctor Lute-Harm Zwiers for the help in the last stage of the mating type work, his expertise was a valuable help for me.

To Doctor Maria do Céu Silva (Coffee Rust Research Centre/Tropical Research Institute, Portugal) for making equipment available, and for assisting with the use of the cryostat for perithecial sectioning. Doctors Josep Armengol (Univ. Politécnica de Valencia, Spain), Keith A. Seifert (Agriculture and Agri-Food, Canada), Maria de Lourdes Inácio (Instituto Nacional de Recursos Biológicos/INIA, Portugal) Pierre Lecomte (Institut National de la Recherche Agronomique, Bordeaux-Aquitaine, France) and Walter D. Gubler (Univ. California, Davis, USA) are thanked for making several strains available, without which this study would not have been possible. The curator of Cornell Plant Pathology Herbarium (CUP) is acknowledged for making the neotype *"C." destructans* available for examination, as the nursery and grapevine growers for their collaboration.

To my friends that are always there for me, even if we are not together as much as we want, for their encouragements, social life and friendship. Thank you for sharing these years with me!

My deepest gratitude goes to my family. Thanks for all support and encouragement, constant love, assistance and patience.

Finally I would like to thank my husband Pedro there are no words enough to express my gratitude for his understanding and patience throughout these years, and to Matilde for the smiles and kisses and all. Both of you were the greatest incentive to carry out this thesis!

Thank you all!

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CHAPTER 1

GENERAL INTRODUCTION

Black foot is an important trunk disease that affects grapevines in most of the vine-producing countries of the world with increasing severity since the 1990s. The disease affects both nurseries and young vineyards, provoking typical darkening of the basal end of the plant. Infected vineyards exhibit declining plants with slow growth, reduced vigour, retarded sprouting, shortened internodes, sparse and chlorotic foliage, resulting frequently in plant death, and forcing growers to uproot and replant considerable areas (Rego *et al.*, 2000; Halleen *et al.*, 2006a). For decades, black foot was associated with fungal species belonging to the genus *Cylindrocarpon*, but the taxonomy of these pathogens has endured several revisions particularly in recent times.

The causes for the recent increase of black foot incidence and severity are still poorly understood, which has led to a renewed interest aiming to understand the biology and pathology of the causal agents. In this context, the present work focuses on key aspects of the pathogens causing black foot disease of grapevines, namely taxonomy, regulation of sexual behaviour (mating type genes), pathogenicity and cross-infection potential, dynamics in nursery and vineyard soils, and the development of genetic transformation tools.

The black foot disease of grapevines

Economic importance and symptoms

Black foot disease is economically important because it can appear either in nursery plants as well as in young vineyards. Symptoms may develop soon after transplantation or 2-10 years. In young plantations the diseased plants have to be replaced, resulting in negative consequences, such as the direct economic cost of the replantation, the heterogeneity of the vineyard in terms of the age of plants at the beginning of full grape production, and strong limitations on the employment of herbicides. In extremely high severity situations, the entire viability of the vineyard can be compromised (Dumot *et al.*, 1999; Oliveira *et al.*, 2003). When the disease affects young vines, death occurs quickly, but as the vines age, infections result in a more gradual decline, and death can take more than one year to occur. However, death seems to be inevitable when vines less than 10 years are infected (Gubler *et al.*, 2004). In nurseries black foot makes the selection of healthy plant material more difficult, and also reduces the productivity and income (Dumot *et al.,* 1999). Disease incidence is highly variable even in adjacent fields, ranging from only a few plants affected to over 50% incidence in the most serious situations (Dumot *et al.*, 1999).

In Portugal black foot disease of grapevines was first reported in 1992 in young vineyards at the Oeste and, in 1994, in different locations at Alto Alentejo. By then this was acknowledged as a new and serious disease causing decline and death of young grapevines and rooted rootstocks (Rego, 1994). Later the disease was reported in several grapevine growing regions, sharing common rootstocks originating from the same region, or even from the same nursery (Oliveira *et al*., 1998; Rego *et al.*, 1998, 2000). Due to benefits from improvements in plant breeding, training methods and irrigation, the 1990's witnessed a strong increase in the establishment of new vineyards, with the subsequent intensification of nursery activity, with a parallel shift towards bench-grafts and an increase in domestic and international exchange of plant material (Rego, 2004), enhancing the spread of soil-borne diseases.

Plants affected by black foot disease reveal poor growth, reduced vigour as well as sparse and chlorotic foliage (Scheck *et al.*, 1998a; Rego *et al.*, 2000). Nursery plants present a reduction in root biomass and root hairs with sunken necrotic lesions, with black wood discolouration and dark-brown to black streaking in the vascular tissue (Grasso & Magnano di San Lio, 1975; Scheck *et al.*, 1998a). Transverse sections through symptomatic wood tissues reveal the xylem vessels plugged with tyloses and/or brown gum inclusions (Sweetingham, 1983), and internal necrosis developing from the bark to the pith (Larignon, 1999; Fourie & Hallleen, 2001). Longitudinal

sections also reveal black wood discolouration and dark-brown to black streaking in the vascular tissue, particularly at the rootstock base (Rego *et al*., 2000). When the disease affects older vines, symptoms are noticed early in the growing season, and typically include poor new growth, failure to form shoots and death of plants throughout the growing season or in the subsequent dormant winter period (Sweetingham, 1983). Internal symptoms are similar to those reported for nursery plants (Fig. 1).

Although remaining healthy in a first stage, roots frequently do not grow deep into the soil. Later on, roots become necrotic, appearing grey to black in colour. Frequently, diseased plants produce new roots in the upper rootstock, enabling a temporary survival of the plant (Dumot *et al.*, 1999; Larignon, 1999). The expression of symptoms is exacerbated during periods of high water demand, as blocked xylem vessels accentuate the water stress and lead to insufficient water and nutrient supply to the vegetative plant parts (Larignon, 1999).

Field symptoms of black foot disease are frequently indistinguishable from those of Petri disease caused by *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams, as well as numerous species of the genus *Phaeoacremonium* (Crous *et al.*, 1996; Mugnai *et al.*, 1999; Groenewald *et al.*, 2001) suggesting that diagnosis should never be made solely on the basis of visual symptoms (Scheck *et al.*, 1998b; Rego *et al.*, 2000; Fourie & Halleen, 2001). Environmental factors and host stresses such as malnutrition, poor drainage, soil compaction, heavy crop loads on young plants and planting vines in poorly prepared soil also play an important role in the development of black foot and Petri diseases (Larignon, 1999; Fourie & Halleen, 2001). High temperatures during summer are also important in symptom expression, since the debilitated root and vascular system of diseased plants may not be able to cope with the water requirements during summer (Larignon, 1999).

Black foot diseased plants appear randomly dispersed in vineyards, rather than aggregated, suggesting that the infection occurred before planting. The contribution of nursery infected materials on pathogen dissemination is well documented (e.g., Rego *et al.*, 2000; Rego *et al*., 2001a; Gubler *et al.*, 2004; Rego, 2004; Gramaje, 2011). In Portugal, black foot incidence is variable, being reported both from wine cultivars and from table grape cultivars, grafted on diverse rootstocks (Rego *et al*., 2000).

Fig. 1 Grapevine black foot symptoms: A-C, reduced vigour and chlorotic vegetation; D, dead plant; E, F, precocious yellowing/reddening of leaves in young plants; G, H, longitudinal sections in the rootstock base, showing black wood discolouration and dark-brown to black streaking in the vascular tissue; I, transversal section of an infected root; J, transversal section in the base of a rootstock, showing punctuations and necroses in the xylem. (Photos: C. Rego and H. Oliveira).

Surveys of naturally infected materials demonstrated that all major rootstock/scion combinations are susceptible to fungal trunk diseases. Nevertheless, Gubler *et al.* (2004) found some resistance of the rootstocks *Vitis riparia* "O39-16" and "Freedom" towards black foot of grapevine. Jaspers *et al.* (2007) evaluated the susceptibility of the more commonly planted grapevine rootstocks in New Zealand under greenhouse conditions, showing that all rootstocks included in that study were susceptible to black foot pathogens to some degree. Similarly, Alaniz *et al.* (2010) evaluated the susceptibility of the grapevine rootstocks most commonly used in Spain to black foot pathogens and found that all rootstocks inoculated were affected by the disease.

Taxonomy and sexuality

For decades, black foot disease was associated with two species of the genus *Cylindrocarpon* Wollew., *C. destructans* (Zinnsm.) Scholten and *C. obtusisporum* (Cooke & Harkn.) Wollew. The first report of *C. destructans* on grapevine was in France in 1961 (Maluta & Larignon, 1991). Since then, it has been reported in several countries, such as Australia (Sweetingham, 1983), Italy (Grasso, 1984), Portugal (Rego, 1994), Argentina (Gatica *et al*., 2001), Germany (Fischer & Kassemeyer, 2003), New Zealand and South Africa (Halleen *et al*., 2004), Brazil (Garrido *et al*., 2004) and USA (Petit & Gubler, 2005). *C. obtusisporum* was first associated to the disease in Italy (Grasso & Magnano di San Lio, 1975), and afterwards in USA (Scheck *et al*., 1998b) and Portugal (Rego, 2004).

The taxonomy of the causal agents went through several revisions in recent years. Halleen *et al*. (2004) studied a collection of *Cylindrocarpon* and *Cylindrocarpon*-like isolates obtained from grapevine and nurseries in France, South Africa, New Zealand and Australia and, based on morphological characters and phylogenetic inference, described three new taxa, *C. macrodidymum* Schroers, Halleen & Crous, *Campylocarpon fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous. Subsequently, *C. macrodidymum* was found in Portugal (Rego *et al*., 2005), USA (Petit & Gubler, 2005), Chile (Auger *et al*., 2007), Spain (Alaniz *et al*., 2007) and Uruguay (Abreo *et al*., 2010). Halleen *et al*. (2004) speculated that isolates identified as *C. obtusisporum* could in fact be *C. macrodidymum*. In this study it was found that a collection of *C. destructans* isolates obtained from diseased grapevines form a phylogenetically distinct member in the *Neonectria radicicola* complex sensu Seifert *et al*. (2003) and in a subsequent publication (Halleen *et al*., 2006b) these isolates were grouped with the type-strain of *C. liriodendri* J.D. MacDon. & E.E. Butler, a species previously associated with root

rot of tulip tree (*Liriodendron tulipifera*) in the USA (MacDonald & Butler, 1981). *C. liriodendri* was identified in France, Portugal and South Africa (Halleen *et al*., 2006b), Australia (Whitelaw-Weckert *et al*., 2007), USA (Dubrovsky & Fabritius, 2007; Petit & Gubler, 2007), Spain (Alaniz *et al*., 2007) and Iran (Mohammadi *et al*., 2009). Schroers *et al*. (2008) reported another species from grapevines with black foot disease symptoms, *C. pauciseptatum*, from Slovenia and New Zealand. Since this first report, it has also been detected in Uruguay (Abreo *et al*., 2010) and Spain (Martín *et al*., 2011). However, its potential role and relative importance as plant pathogen has yet to be determined, although it was able to produce necrotic root lesions in 110R rootstock cuttings (Alaniz *et al.*, 2009a).

In his taxonomic revision of *Cylindrocarpon*, Booth (1966) divided this genus into four groups based on the presence or absence of microconidia or chlamydospores. The type species of the genus *Cylindrocarpon*, *C. cylindroides*, belonged to group 1 (microconidia present, mycelial chlamydospores lacking), while the type of the genus *Neonectria*, *N. ramulariae*, which is the teleomorph of *C. obtusiusculum* (= *C. magnusianum*; Braun, 1993), belongs to group 4 (microconidia lacking, mycelial chlamydospores present). Group 2 (lacking both microconidia and mycelial chlamydospores) contained *Cylindrocarpon* species predominantly connected with teleomorphs of "*Neonectria*" *mammoidea*. Group 3 (microconidia and mycelial chlamydospores present) contained *C. destructans*, which was considered to be a species complex comprising various taxa, including *C. macroconidialis*, *C. coprosmae* and *C. liriodendri* (Seifert *et al*., 2003; Halleen *et al.*, 2006b). Most of the teleomorphs of *Cylindrocarpon* species have been classified in *Neonectria* (Rossman *et al*., 1999; Mantiri *et al*., 2001; Brayford *et al*., 2004; Halleen *et al*., 2004, 2006b). However, several phylogenetic studies have revealed that *Neonectria/Cylindrocarpon* is paraphyletic (Mantiri *et al*., 2001; Halleen *et al.,* 2004; Hirooka *et al.,* 2005; Castlebury *et al.,* 2006; Halleen *et al.*, 2006b). A further phylogenetic study (Chaverri *et al*., 2011) divided the *Neonectria* complex into four genera based on a combination of characters linked to perithecial anatomy and conidial septation: *Neonectria/Cylindrocarpon sensu stricto* (Booth's groups 1 and 4), *Rugonectria*, *Thelonectria* (group 2) and *Ilyonectria* (group 3). According to this treatment, only *Neonectria* has *Cylindrocarpon* anamorphs, while the remaining genera have *Cylindrocarpon*-like anamorphs. In this thesis, a single generic name was proposed for each clade in an attempt to move towards a single nomenclature for pleomorphic fungi, meaning that the *Cylindrocarpon*-like anamorphs of *Ilyonectria, Rugonectria* and *Thelonectria* were placed in teleomorph genera, as recently done with other groups of pleomorphic fungi (Crous *et al*., 2006, 2007, 2009; Lombard *et al*., 2010; Gräfenhan *et al*., 2011; Schroers *et al*., 2011).

Sexuality plays a key role in the evolution and species differentiation in these fungi. Mating-type (*MAT*) genes are involved in the regulation of sexual behaviour and are also very useful for phylogenetic analysis because of their high evolution rates (Turgeon, 1998; Pöggeler, 2001). Although the existence of mating types in fungi has been recognised for over a century (Blakeslee, 1904), mating type genes were first identified in ascomycetes in the late 1980's (Glass *et al*., 1988). The mating type locus contains genes that regulate functions involved in mating processes, and thus significant insights in the sexual reproduction of fungi were provided by the study of the structure, function and evolution of these genes (Metzenberg & Glass, 1990; Nelson, 1996; Coppin *et al.*, 1997; Kronstad & Staben, 1997; Casselton, 2008). In heterothallic (self-sterile) ascomycetes, compatible mating types are defined by the two alternate and dissimilar forms of the *MAT* locus termed idiomorphs (Metzenberg & Glass, 1990). These *MAT* genes encode proteins containing conserved DNA binding domains involved in transcription regulation (Nelson, 1996; Coppin *et al*., 1997; Kronstad & Staben, 1997). They were shown to complement each other by regulating the attraction among compatible mating types in order to initiate the mating process and to be involved in later steps of sexual development by controlling the regulation of internuclear recognition (Casselton, 2002; Stanton & Hull, 2007; Turgeon & Debuchy, 2007). In contrast, in homothallic (self-fertile) ascomycetes, *MAT* genes from both idiomorphs are usually present within a single individual. The expression of the genes in such an individual can bypass the cellular recognition step between genes that are otherwise located in different individuals, resulting in selfing (Casselton, 2002; Lin & Heitman, 2007; Stanton & Hull, 2007; Turgeon & Debuchy, 2007).

Mating experiments performed for *C. liriodendri* and *C. macrodidymum* suggest a bipolar mating system, as no teleomorph has been obtained when each isolate was self-crossed, but it was obtained when crossing different isolates (Rego, 2004; Halleen *et al*., 2004, 2006b). However, the genetic regulation of the mating system of these fungi has not yet been sufficiently investigated.

Epidemiology and pathogenicity

Cylindrocarpon spp. are recognised as soil-borne saprobes occurring on dead plant material, or acting as weak pathogens infecting wounds on roots and stems of various hosts (Halleen *et al*., 2006b; Schroers *et al*., 2008). Also, the production of chlamydospores would guarantee long-term survival of *Cylindrocarpon* spp. in the soil (Booth, 1966). For instance, *C. destructans* behaves as an equilibrium or opportunistic parasite, causing disease only under certain

circumstances, namely related to plant susceptibility and favourable environmental conditions. No single factor can explain the occurrence of the disease, neither are there simple strategies to avoid it (Dumot *et al.*, 1999). Factors that build up plant stress and its increased receptivity are replantation, lesions caused in the preparation of cuttings, bud removal, grafting and pruning, and the colonisation of plants by other wood fungi.

Soil pH seems to strongly affect the relative population balance between *Fusarium* and *Cylindrocarpon* spp. (Peterson, 1958). In the rhizosphere of red clover and wheat, *Fusarium* was vastly dominant (and *Cylindrocarpon* nearly absent) in acid sandy soils, but *Cylindrocarpon* was dominant (and *Fusarium* rare) in alkaline limestone soils. Identical proportions of both populations were found in neutral clay soils. A similar contrast was observed by Matturi & Stenton (1964) in forest soils. Following this trend, *C. destructans* was isolated more frequently from healthy roots of *Picea abies* growing in neutral or alkaline soils than from acid soils (Holdenrieder & Sieber, 1992).

Weeds can also act as inoculum reservoir, as Agustí-Brisach *et al*. (2011) demonstrated that *C. macrodidymum* could be successfully isolated from the roots of a total of 26 weed species in a total of 56 species surveyed in South-Eastern Spain.

Grapevine propagation techniques use rootstock and scion mother vines, from which dormant cuttings are taken for bench grafting or rooting. Nursery practices involve cold storage of cuttings until late winter or early spring, water-soaking and bench-grafting. The grafting union is protected by wax, and graftlings are held under growth-stimulating, warm and humid callusing conditions until callus forms at the base of the cutting and around the graft union. Following successful callusing, vines are planted in the nursery field for nine months, and then they are uprooted, selected, pruned, buds protected with paraffin and sold to farmers. Thus, there are many opportunities for infection by *Cylindrocarpon* spp. and other trunk disease pathogens during the propagation process, as wounds are made at every stage of production. Improperly healed graft unions are also vulnerable to infection in the nursery and, if the vines survive, after planting in the vineyard. Poor cold storage and transport conditions can also result in stress and cross contamination (Gramaje, 2011).

Several reports suggest that the pathogens are isolated at much higher frequencies from rooted cuttings and graftlings in nurseries than from rootstock canes, suggesting that infections occurred mainly from soil-borne inoculum (Rego *et al*., 2000; Fourie & Halleen, 2001; Halleen & Crous, 2001; Rego *et al.*, 2001b). This inoculum was largely built up after the nursery

replantation, because highest disease incidence and severity was observed during the second-year plantation, compared to the first year.

Since 2006, rules governing grapevine nurseries in Portugal made rotation compulsory, where at least three years without grapevine cultivation are required (typically rotation is carried out with vegetable crops) ("Decreto-Lei 194/2006"). However, although several reports indicate the presence of the black foot causal agents (and other grapevine trunk pathogens) in nursery plants at the end of the rooting process, other authors still question the role of these fungi in the decline of vineyards settled with these vines (Gramaje, 2011). For instance, Rumbos & Rumbou (2001) found very low incidence of *Cylindrocarpon* spp. in nursery plants with failed graft unions, and concluded that these pathogens could not be the cause of young grapevine decline. Furthermore, they suggested that abiotic causes, such as lesions from improperly healed rootstock disbudding sites and graft unions made in the nursery, as well as improper storage and transportation conditions of the propagated material must also have played a role and made the decline more acute.

To understand the contribution of black foot pathogens and/or abiotic factors in the decline of young vines, the dynamics of populations of black foot pathogens in nursery and vineyard soils should be investigated. Also, the relative importance of infection occurring on nursery versus vineyard soils, or the factors affecting infection, have not been fully resolved.

While some studies reported no virulence differences among isolates from the various species responsible for black foot (Halleen *et al*., 2004; Petit & Gubler, 2005; Alaniz *et al*., 2007), other pathogenicity trials detected variation in virulence among groups of *C. macrodidymum*, previously distinguished based on Inter-Simple Sequence Repeat markers, and further showed that *C. macrodidymum* appears to be more virulent than *C. liriodendri* (Alaniz *et al.*, 2009b). Also, variability in virulence was detected and characterised in a collection of 54 *C. destructans* (later renamed *C. liriodendri*) isolates, but no correlation could be established to the source (geographic origin or scion/root stock varieties) of these isolates (Rego, 2004).

Although the disease cycle of *Cylindrocarpon* spp. on grapevine is poorly known, their behaviour on other hosts has been studied (Kernaghan *et al.*, 2007; Tewoldemedhin *et al*., 2011). These fungi produce slimy spores that are dispersed in free water and chlamydospores that allow the organism to survive in soil. After a spore comes in contact with the root surface, the hypha enters the roots and decomposes the cortex cells, eventually restricting the uptake and subsequent transport of soil-derived nutrients to the shoots, and restricts the movement of photosynthates to the roots. Over time, plants become more and more stunted, as their capacity

continually declines. *Neonectria radicicola* perithecia were observed in *C. destructans*-inoculated rootstocks (Larignon, 1999), suggesting the possible formation of such structures in the field may contribute for the survival of the fungus in the bark and for genetic recombination events leading to increased diversity.

Despite the economic importance and pathologic complexity of black foot disease, no transformation tools have been developed for these fungi, impairing subsequent studies on the molecular mechanisms of pathogenicity. For instance, transformation would enable tagged mutation experiments, which could help identifying key genes involved in pathogenicity, or could deliver silencing vectors that would confirm the role in pathogenicity of genes previously identified.

Objectives

In an attempt to deepen our knowledge on key aspects of the biology and pathology of fungi causing black foot disease of grapevine, the main objectives of this work were:

-to elucidate the morphological variation present within the *I. radicicola* complex, and to link fresh collections to older names introduced for species in this complex. This was addressed by combining morphological and culture characteristics with DNA sequence data derived from the Internal Transcribed Spacers of the nrRNA gene operon (ITS), and partial β-tubulin (TUB), histone H3 (HIS), and translation elongation factor 1-α (TEF) genes (**Chapter 2**);

-to study a collection of *Cylindrocarpon*-like isolates originating from grapevines that appear to be closely related to *I. macrodidyma* using morphological/culture characterisation and a phylogenetic analysis derived from a multi-locus (TUB, HIS, TEF and ITS) data set (**Chapter 3**);

-to characterize the mating type genes of *I. macrodidyma* (and closely related species), and *I. liriodendri* and their proportions in *Ilyonectria* spp. populations from grapevine (and other hosts) from Portugal and other countries (**Chapter 4**);

-to assess the importance of alternative hosts as inoculum sources capable of causing grapevine black-foot (both on nursery and vineyard), namely analysing the virulence of *Ilyonectria* spp. isolates obtained from grapevine, as well as analysing the pathogenicity and virulence of *Ilyonectria* spp. isolates obtained from different hosts to grapevine (**Chapter 5**);

-to optimize a stable genetic transformation protocol for *I. liriodendri*, by testing both a protoplast transformation protocol and an *Agrobacterium tumefaciens*-mediated transformation protocol (**Chapter 6**);

-to perform a quantitative comparison of *Ilyonectria* spp. in different nursery and vineyard soils using a quantitative PCR approach (**Chapter 7**).

Thesis structure

Each chapter in this thesis is formatted as a journal article, according to the style of the journal where they were published/submitted/planned to submit:

-chapter 2, “Cylindrocarpon root rot: multi-gene analysis reveals novel species within the *Ilyonectria radicicola* species complex”, *Mycological Progress*;

-chapter 3, “Multi-gene analysis and morphology reveal novel *Ilyonectria* species associated with black foot disease of grapevines”, *Fungal Biology*;

-chapter 4, “Characterization of mating type genes in *Ilyonectria* species causing black foot of grapevine”, *Fungal Genetics and Biology*;

-chapter 5, “Virulence and cross-infection potential of *Ilyonectria* spp. to grapevine”, *Phytopathologia Mediterranea*;

-chapter 6, “A protocol for stable genetic transformation of *Ilyonectria liriondendri*, a fungal pathogen causing root rot of woody plant species”, *Journal of Plant Diseases and Plant Protection*;

-chapter 7, “Relative quantification of *Ilyonectria* spp. from grapevine nursery and vineyard soils”, *Australian Journal of Grape and Wine Research*.

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CHAPTER 2

CYLINDROCARPON ROOT ROT: MULTI-GENE ANALYSIS REVEALS NOVEL SPECIES WITHIN THE *ILYONECTRIA RADICICOLA* SPECIES COMPLEX

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Mycological Progress (submitted)

Keywords Cylindrocarpon root rot, Nectria-like fungi, phylogeny, systematics;

Abstract

Ilyonectria radicicola and its *Cylindrocarpon*-like anamorph represent a species complex that is commonly associated with root rot disease symptoms on a range of hosts. During the course of this study, several species could be distinguished from *I. radicicola sensu stricto* based on morphological and culture characteristics. DNA sequence analysis of the partial β-tubulin, histone H3, translation elongation factor 1-α and nuclear ribosomal RNA-Internal Transcribed Spacer (nrRNA-ITS) genes were employed to provide further support for the morphological species resolved among 68 isolates associated with root rot disease symptoms. Of the various loci screened, nrRNA-ITS sequences were the least informative, while histone H3 sequences were the most informative, resolving the same number of species as the combined dataset across the four genes. Within the *Ilyonectria radicicola* species complex, 12 new taxa are delineated occurring on a diverse range of hosts, the most common being *Cyclamen*, *Lilium*, *Panax*, *Pseudotsuga*, *Quercus* and *Vitis*.

Introduction

The genus *Cylindrocarpon* was introduced in 1913 by Wollenweber, with *C. cylindroides* as type. *Cylindrocarpon* and *Cylindrocarpon*-like species have since been commonly associated with root and decay of woody and herbaceous plants (Domsch et al. 2007). Cylindrocarpon root rot causes losses up to 30% on ginseng (*Panax quinquefolium*) (Seifert et al. 2003), and plays an important role in black foot rot of grapevines (Halleen et al. 2004, 2006a), apple replant disease (Tewoldemedhin et al. 2010), and beech cankers (Castlebury et al. 2006), to name but a few hosts of economic importance.

In his taxonomic revision of *Cylindrocarpon*, Booth (1966) divided this genus into four groups based on the presence or absence of microconidia or chlamydospores. Booth's group 4 represents *Neonectria s. str*., as it accommodates the type species *N. ramulariae* (anamorph: *C. obtusiusculum*). Most of the teleomorphs of *Cylindrocarpon* species have since this date been classified in *Neonectria* (Brayford et al. 2004; Halleen et al. 2004, 2006; Mantiri et al. 2001; Rossman et al. 1999). Several phylogenetic studies have, however, revealed that *Neonectria/Cylindrocarpon* is paraphyletic (Castlebury et al. 2006; Halleen et al. 2004, 2006b, Hirooka et al. 2005; Mantiri et al. 2001). The first step in resolving this issue was taken by Halleen et al. (2004), who proposed *Campylocarpon* for species resembling *Cylindrocarpon* with 3–5-septate, curved macroconidia, and lacking microconidia. A further phylogenetic study (Chaverri et al. 2011) divided the *Neonectria* complex into four genera based on a combination of characters linked to perithecial anatomy and conidial septation: *Ilyonectria, Neonectria/Cylindrocarpon s. str.*, *Rugonectria* and *Thelonectria*. In this study, a single generic name was proposed for each clade in an attempt to move towards a single nomenclature for pleomorphic fungi, meaning that the *Cylindrocarpon*-like anamorphs of *Ilyonectria, Rugonectria* and *Thelonectria* were placed in teleomorph genera, as recently done with other groups of pleomorphic fungi (Crous et al. 2006, 2007, 2009a; Gräfenhan et al. 2011; Lombard et al. 2010; Schroers et al. 2011).

Cylindrocarpon root rot is commonly associated with "*Cylindrocarpon*" *destructans* in literature (Halleen et al. 2004; Samuels and Brayford 1990). This fungus was originally described as *Ramularia destructans* from roots of ginseng (*Panax quinquefolium*) collected in the USA (Zinssmeister 1918). Furthermore, it has been linked to the teleomorph *Ilyonectria radicicola* (Booth 1966; Chaverri et al. 2011; Samuels and Brayford 1990), which Gerlach and Nilsson (1963) described from rotting bulbs of *Cyclamen persicum* collected in Sweden. Samuels and Brayford (1990) commented on the morphological variation in collections of *I. radicicola* and its

anamorph *"C." destructans*. Seifert et al. (2003) showed that there was more than one *"C." destructans*-like species occurring on *Panax,* and that none of the resolved clades correlated to the ex-type strain of *I. radicicola*, leading Halleen et al. (2006b) to question the purported anamorph/teleomorph link between *I. radicicola* (from *Cyclamen*, Sweden) and *"C." destructans* (from *Panax*, USA). Based on a phylogenetic analysis of ITS nrRNA gene sequences, Schroers et al. (2008) concluded that the *I. radicicola* complex includes *"C." destructans*, *"C." destructans* var. *crassum*, *I. coprosmae*, *I. liriodendri, N. austroradicicola* and *N. macroconidialis*.

The aim of the present study was to elucidate the morphological variation present within the *I. radicicola* complex, and to link fresh collections to older names introduced for species in this complex. This was addressed by combining morphological and culture characteristics with DNA sequence data derived from the Internal Transcribed Spacers (ITS) of the nrRNA gene operon, and partial β-tubulin (TUB), histone H3 (HIS), and translation elongation factor 1-α (TEF) genes.

Material and methods

Isolates

This study (Table 1) included 42 "*C.*" *destructans s. lat.* isolates [including the ex-type strains of *I. radicicola* (CBS 264.65) and "*C.*" *destructans* f.sp. *panacis* (CBS 124662), "*C.*" *destructans* var. *destructans* and "*C.*" *destructans* var. *crassum*], six "*C.*" *didymum* isolates, six *I. liriodendri* isolates, one *N. macroconidialis* isolate and one *I. coprosmae* isolate, all deposited at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS). Included are also two isolates that were previously identified as *Ramularia mors-panacis* (CBS 306.35) and *R. panacicola* (CBS 307.35) by Hildebrand (1935).

Besides those, 10 "*Cylindrocarpon*" spp. isolates were obtained in Portugal from grapevine plants showing decline symptoms, either 1–6-year-old plants in vineyards (Cy22, Cy155, Cy158, Cy190, CBS 129078, CBS 129080, CBS 129081, CBS 129082) or from rootstock nurseries (Cy23), and from a 25-year-old grapevine plant with esca symptoms (CBS 129084). Furthermore, isolates were obtained from a young *Malus domestica* (Cy164) and from the stem of a young *Quercus suber* (Cy232) plant, both showing decline symptoms, and from *Thymus* sp. (Cy231) and *Ficus* sp. (Cy228). One isolate (Cy131) was made available by P. Lecomte (Institut National de la Recherche Agronomique, Bordeaux-Aquitaine, France) and was obtained from an internal lesion of a stem of *Actinidia chinensis* 'Hayward'. Another isolate (Cy122) was made available by W.D.

Gubler (Univ. California, Davis, USA) and was obtained from *Vitis* sp. All of these isolates are stored in a culture collection at the Laboratório de Patologia Vegetal "Veríssimo de Almeida" (LPVVA-ISA, Lisbon, Portugal).

An additional 25 "*C.*" *destructans* isolates used during this study were made available by K.A. Seifert (Agriculture and Agri-Food, Canada), and were isolated from commercial *Panax quinquefolium* gardens (CBS 120359–120369, CBS 129079, CBS 129083, CD1666, CPC 13535, CPC 13537, NSAC-SH2, NSAC-SH2.5), *Picea glauca* (94-1628, CPC 13539), *Poa pratensis* (CPC 13534), *Pseudotsuga menziesii* (CBS 120370-120372, CPC 13536) and *Prunus cerasus* (CPC 13532) (Seifert et al. 2003).

Another 109 isolates were also included in the analysis to add phylogenetic support to this study and represent strains of the following taxa: *C. cylindroides*, *C. obtusisporum*, *C. pauciseptatum*, species 1 to 6 (Mostert et al., in prep.; Cabral et al., in prep.), *I. macrodidyma*, *N. ditissima*, *N. major*, *N.neomacrospora* and *N. ramulariae*.

DNA isolation, sequencing and phylogenetic analysis

For each isolate, genomic DNA was isolated from mycelium following the protocol of Möller et al. (1992), adapted by Crous et al. (2009b). Sequencing of the ITS and part of the β-tubulin (TUB), histone H3 (HIS) and translation elongation factor 1-α (TEF) genes was performed after PCR amplification using 1× PCR buffer (Bioline, London, UK), 1.5 mM $MgCl_2$, 32 µM of each dNTPs, 0.24 µM of each primer, 0.5 units *Taq* DNA Polymerase (Bioline), and 1 µl of diluted gDNA in a final volume of 12.5 µl. The cycle conditions in a iCycler thermocycler (BioRad, Hercules, USA) were 94 ºC for 5 min, followed by 40 cycles at 94 ºC for 30 s, 52 ºC for 30 s and 72 ºC for 80 s, and a final elongation at 72 ºC for 10 min. Primers were V9G (de Hoog and Gerrits van den Ende 1998) and ITS4 (White et al. 1990) for ITS, T1 (O'Donnell and Cigelnik 1997) and Bt-2b (Glass and Donaldson 1995) for TUB, CYLH3F and CYLH3R (Crous et al. 2004b) for HIS, and EF1 and EF2 (O'Donnell et al. 1998) or CylEF-1 (5'- ATG GGT AAG GAV GAV AAG AC-3'; J.Z. Groenewald, unpubl.) and CylEF-R2 (Crous et al. 2004b) for TEF. For TEF the following modifications were made to the amplification protocol: 2.0 mM of $MgCl_2$, 40 µM of each dNTPs and addition of 5% of Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Netherlands).

After confirmation by agarose gel electrophoresis, amplicons were sequenced in both directions with the corresponding PCR primers and a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Netherlands) according to manufacturer's recommendations. The products were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, USA).

Sequences were assembled and edited to resolve ambiguities, using the EditSeq and SeqMan modules of the Lasergene software package (DNAStar, Madison, USA). Consensus sequences for all isolates were compiled into a single file (Fasta format) and aligned using CLUSTAL X v. 2.0.11 (Larkin et al. 2007). Following manual adjustment of the alignment by eye where necessary, the alignment was subjected to phylogenetic analyses as described by Crous et al. (2004b). Optimal models were analysed for each locus using MrModeltest v. 2.2 (Nylander 2004). Ambiguous alignment areas were excluded from the analyses only in the ITS alignment, namely alignment positions 247–255, 267–276 and 566–572 (see TreeBASE for alignment). Novel sequences were lodged in GenBank (Table 1), taxonomic novelties in MycoBank (Crous et al. 2004a) and the alignments and phylogenetic trees in TreeBASE (http://www.treebase.org).

Morphology

Isolates were grown for up to 5 wk at 20 ºC on synthetic nutrient poor agar (SNA; Nirenberg 1976) with and without two 1 cm^2 filter paper pieces, carnation leaf agar (CLA; Crous et al. 2009b), PDA, and oatmeal agar (OA; Crous et al. 2009b) under continuous n-UV light (NUV, 400–315 nm; Blacklight-Blue, Sylvania, Netherlands).

Measurements were done on a 1 cm^2 agar plug removed from the colony margin, placed on a microscope slide, to which a drop of water and coverslip were added. For each isolate, 30 measurements were obtained for each structure. Measurements were done at 1000× magnification using a Nikon Eclipse 80i microscope, or a Leica DM2500. Images were captured using a Nikon DS-Fi1 digital camera with NIS-Elements Software, or a Leica DFC295 digital camera with the Leica Application Suite. Measurements for length and width of conidia and ascospores are given as (Minimum) Lower Limit of a 95% Confidence Interval – Upper Limit of a 95% Confidence Interval (Maximum). For other measurements, only the extreme values are given.

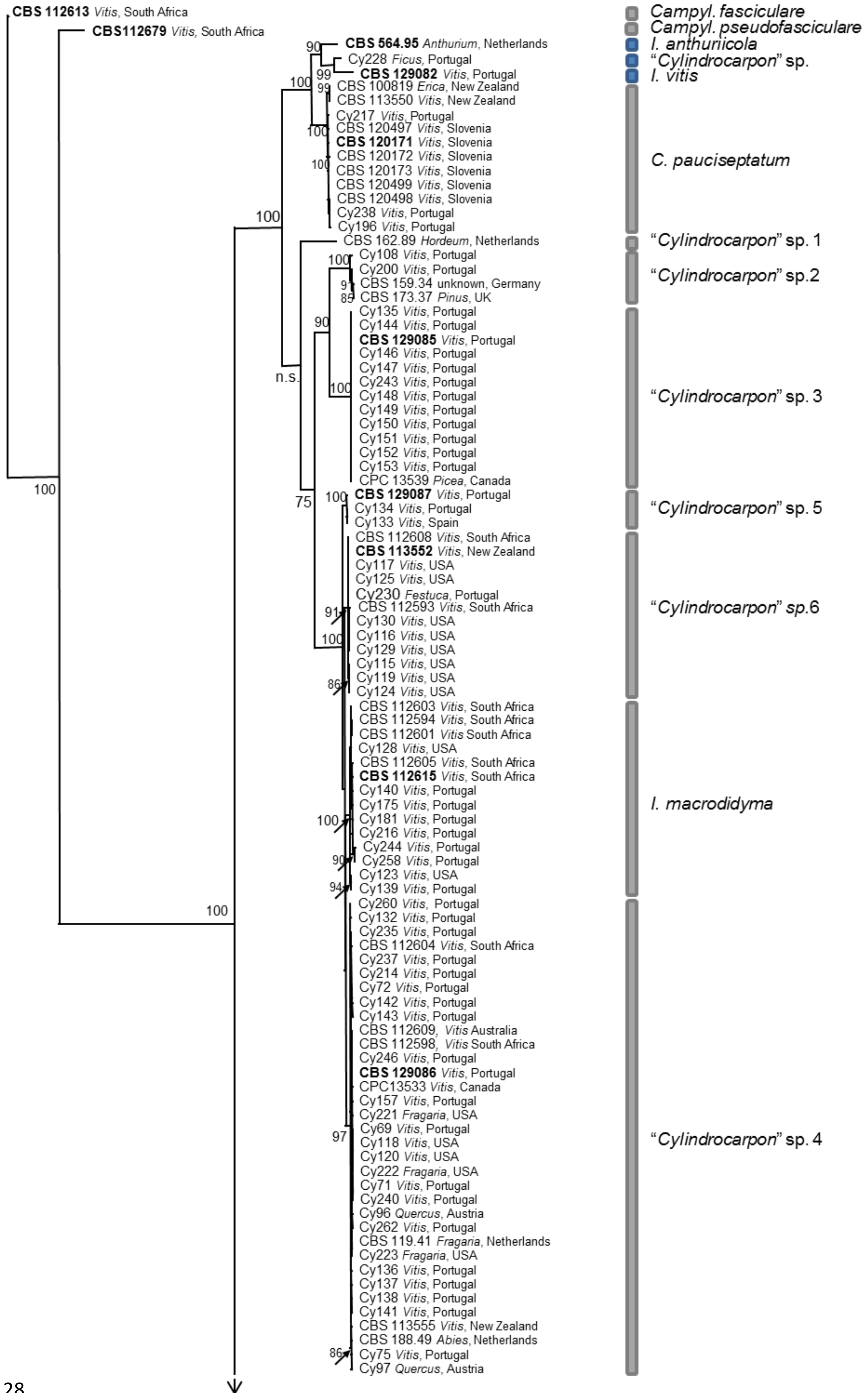
Culture characteristics (texture, density, colour, growth front, transparency and zonation) were described on PDA after incubation at 20 ºC in the dark for 14 d. Colour (surface and reverse) was described using the colour chart of Rayner (1970). Cardinal temperatures for growth were assessed by inoculating 90 mm diam PDA dishes with a 3 mm diam plug cut from the edge of an actively growing colony. Growth was determined after 7 d in two orthogonal directions. Trials were conducted at various temperatures (4, 10, 15, 18, 20, 22, 25, 30 and 35 ºC) with three replicate plates per strain at each temperature.

To induce the formation of perithecia, isolates were crossed in 60 mm diam Petri dishes containing a minimal salts medium supplemented with two sterile birch toothpicks (Guerber and Correll 2001). The plates were incubated at 20 ºC under n-UV light for 8–20 wk. Two strains were considered sexually compatible if perithecia were formed that exuded masses of viable ascospores. The colour reaction of the perithecia was checked in 3% KOH and in lactic acid. For sectioning, perithecia were mounted in Jung Tissue Freezing Medium (Leica) or in Arabian Gum, and cut in 10–15 µm thick sections using a Leica cryostat CM3050 S or CM1850 at -20°C.

Results

Phylogeny

Amplification products of approximately 700 bases (ITS), 650 bases (TUB), 500 bases (HIS) and 600–800 bases (TEF) were obtained for the isolates listed in Table 1. The manually adjusted combined alignment contains 189 sequences (including the two outgroup sequences) and the statistical parameters for the combined and individual analyses are presented in Table 2. For the combined analysis, only a maximum of 1000 equally most parsimonious trees were saved, the first of which is presented as Fig. 1. Phylogenetic trees derived from the individual loci are available in TreeBASE. The combined analysis of the four genes enabled the identification of 37 species. However, the analysis of HIS data alone was enough to resolve these taxa. Sequences of TEF could not distinguish species 6, *I. robusta*, *I. europaea*, *I. lusitanica*, *I. rufa* and *N. ditissima*; whereas sequences of TUB could not separate *I. robusta*, species 4, and 6, while *"C." macrodidymum*, species 5, *I. liliigena* and *I. pseudodesctructans* were supported by low bootstrap values, and CBS 120370 clustered apart from the remaining isolates of *I. crassa*. Of all loci screened, ITS proved to be the least informative, being unable to resolve 22 of the species in this study. Neighbour-Joining (NJ) analyses using the three substitution models, as well as the parsimony analysis, yielded trees with similar topology and bootstrap support values for the individual and combined gene analyses. The trees obtained supported the same clades, sometimes with rearrangements in the order of these clades between the different analyses (data not shown). The results of the phylogenetic analyses are highlighted below under the taxonomic notes or in the Discussion, where applicable.


CBS 112613 Vitis, South Africa
CBS112679 Vitis, South Africa
CBS 564.95 Anthurium, Netherlands
Cy228 Ficus, Portugal
CBS 129082 Vitis, Portugal
CBS 100819 Erica, New Zealand
CBS 113550 Vitis, New Zealand
Cy217 Vitis, Portugal
CBS 120497 Vitis, Slovenia
CBS 120171 Vitis, Slovenia
CBS 120172 Vitis, Slovenia
CBS 120173 Vitis, Slovenia
CBS 120499 Vitis, Slovenia
CBS 120498 Vitis, Slovenia
Cy238 Vitis, Portugal
Cy196 Vitis, Portugal
CBS 162.89 Hordeum, Netherlands
Cy108 Vitis, Portugal
Cy200 Vitis, Portugal
CBS 159.34 unknown, Germany
CBS 173.37 Pinus, UK
Cy135 Vitis, Portugal
Cy144 Vitis, Portugal
CBS 129085 Vitis, Portugal
Cy146 Vitis, Portugal
Cy147 Vitis, Portugal
Cy243 Vitis, Portugal
Cy148 Vitis, Portugal
Cy149 Vitis, Portugal
Cy150 Vitis, Portugal
Cy151 Vitis, Portugal
Cy152 Vitis, Portugal
Cy153 Vitis, Portugal
CPC 13539 Picea, Canada
CBS 129087 Vitis, Portugal
Cy134 Vitis, Portugal
Cy133 Vitis, Spain
CBS 112608 Vitis, South Africa
CBS 113552 Vitis, New Zealand
Cy117 Vitis, USA
Cy125 Vitis, USA
Cy230 Festuca, Portugal
CBS 112593 Vitis, South Africa
Cy130 Vitis, USA
Cy116 Vitis, USA
Cy129 Vitis, USA
Cy115 Vitis, USA
Cy119 Vitis, USA
Cy124 Vitis, USA
CBS 112603 Vitis, South Africa
CBS 112594 Vitis, South Africa
CBS 112601 Vitis South Africa
Cy128 Vitis, USA
CBS 112605 Vitis, South Africa
CBS 112615 Vitis, South Africa
Cy140 Vitis, Portugal
Cy175 Vitis, Portugal
Cy181 Vitis, Portugal
Cy216 Vitis, Portugal
Cy244 Vitis, Portugal
Cy258 Vitis, Portugal
Cy123 Vitis, USA
Cy139 Vitis, Portugal
Cy260 Vitis, Portugal
Cy132 Vitis, Portugal
Cy235 Vitis, Portugal
CBS 112604 Vitis, South Africa
Cy237 Vitis, Portugal
Cy214 Vitis, Portugal
Cy72 Vitis, Portugal
Cy142 Vitis, Portugal
Cy143 Vitis, Portugal
CBS 112609, Vitis Australia
CBS 112598, Vitis South Africa
Cy246 Vitis, Portugal
CBS 129086 Vitis, Portugal
CPC13533 Vitis, Canada
Cy157 Vitis, Portugal
Cy221 Fragaria, USA
Cy69 Vitis, Portugal
Cy118 Vitis, USA
Cy120 Vitis, USA
Cy222 Fragaria, USA
Cy71 Vitis, Portugal
Cy240 Vitis, Portugal
Cy96 Quercus, Austria
Cy262 Vitis, Portugal
CBS 119.41 Fragaria, Netherlands
Cy223 Fragaria, USA
Cy136 Vitis, Portugal
Cy137 Vitis, Portugal
Cy138 Vitis, Portugal
Cy141 Vitis, Portugal
CBS 113555 Vitis, New Zealand
CBS 188.49 Abies, Netherlands
Cy75 Vitis, Portugal
Cy97 Quercus, Austria
90
99
100
99
100
100
100
100
100
91
85
90
100
n.s.
100
75
100
91
100
86
100
90
94
100
97
86
Campyl. fasciculare
Campyl. pseudofasciculare
I. anthuriicola
"Cylindrocarpon" sp.
I. vitis
C. pauciseptatum
"Cylindrocarpon" sp. 1
"Cylindrocarpon" sp.2
"Cylindrocarpon" sp. 3
"Cylindrocarpon" sp. 5
"Cylindrocarpon" sp.6
I. macrodidyma
"Cylindrocarpon" sp. 4

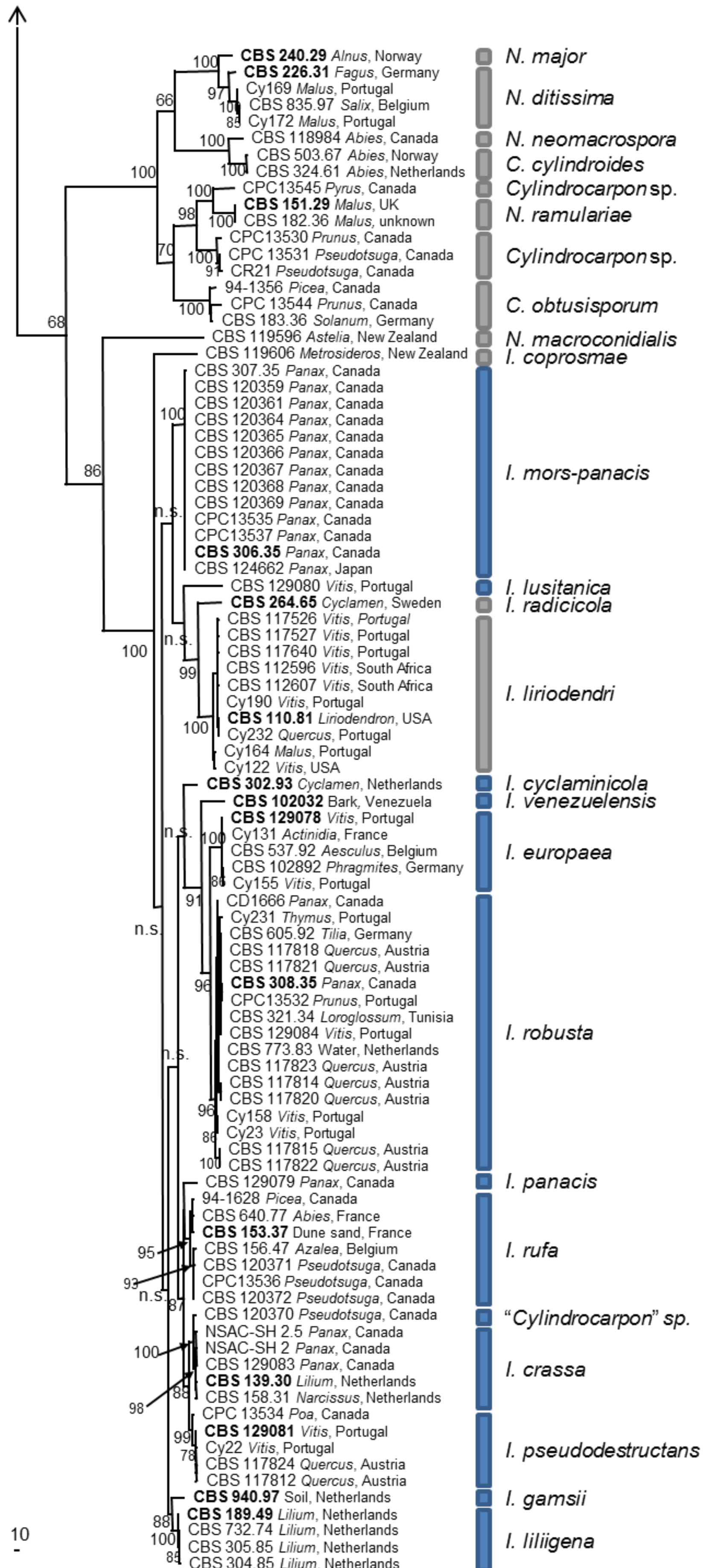

CBS 240.29 Alnus, Norway
CBS 226.31 Fagus, Germany
Cy169 Malus, Portugal
CBS 835.97 Salix, Belgium
Cy172 Malus, Portugal
CBS 118984 Abies, Canada
CBS 503.67 Abies, Norway
CBS 324.61 Abies, Netherlands
CPC13545 Pyrus, Canada
CBS 151.29 Malus, UK
CBS 182.36 Malus, unknown
CPC13530 Prunus, Canada
CPC 13531 Pseudotsuga, Canada
CR21 Pseudotsuga, Canada
94-1356 Picea, Canada
CPC 13544 Prunus, Canada
CBS 183.36 Solanum, Germany
CBS 119596 Astelia, New Zealand
CBS 119606 Metrosideros, New Zealand
CBS 307.35 Panax, Canada
CBS 120359 Panax, Canada
CBS 120361 Panax, Canada
CBS 120364 Panax, Canada
CBS 120365 Panax, Canada
CBS 120366 Panax, Canada
CBS 120367 Panax, Canada
CBS 120368 Panax, Canada
CBS 120369 Panax, Canada
CPC13535 Panax, Canada
CPC13537 Panax, Canada
CBS 306.35 Panax, Canada
CBS 124662 Panax, Japan
CBS 129080 Vitis, Portugal
CBS 264.65 Cyclamen, Sweden
CBS 117526 Vitis, Portugal
CBS 117527 Vitis, Portugal
CBS 117640 Vitis, Portugal
CBS 112596 Vitis, South Africa
CBS 112607 Vitis, South Africa
Cy190 Vitis, Portugal
CBS 110.81 Liriodendron, USA
Cy232 Quercus, Portugal
Cy164 Malus, Portugal
Cy122 Vitis, USA
CBS 302.93 Cyclamen, Netherlands
CBS 102032 Bark, Venezuela
CBS 129078 Vitis, Portugal
Cy131 Actinidia, France
CBS 537.92 Aesculus, Belgium
CBS 102892 Phragmites, Germany
Cy155 Vitis, Portugal
CD1666 Panax, Canada
Cy231 Thymus, Portugal
CBS 605.92 Tilia, Germany
CBS 117818 Quercus, Austria
CBS 117821 Quercus, Austria
CBS 308.35 Panax, Canada
CPC13532 Prunus, Portugal
CBS 321.34 Loroglossum, Tunisia
CBS 129084 Vitis, Portugal
CBS 773.83 Water, Netherlands
CBS 117823 Quercus, Austria
CBS 117814 Quercus, Austria
CBS 117820 Quercus, Austria
Cy158 Vitis, Portugal
Cy23 Vitis, Portugal
CBS 117815 Quercus, Austria
CBS 117822 Quercus, Austria
CBS 129079 Panax, Canada
94-1628 Picea, Canada
CBS 640.77 Abies, France
CBS 153.37 Dune sand, France
CBS 156.47 Azalea, Belgium
CBS 120371 Pseudotsuga, Canada
CPC13536 Pseudotsuga, Canada
CBS 120372 Pseudotsuga, Canada
CBS 120370 Pseudotsuga, Canada
NSAC-SH 2.5 Panax, Canada
NSAC-SH 2 Panax, Canada
CBS 129083 Panax, Canada
CBS 139.30 Lilium, Netherlands
CBS 158.31 Narcissus, Netherlands
CPC 13534 Poa, Canada
CBS 129081 Vitis, Portugal
Cy22 Vitis, Portugal
CBS 117824 Quercus, Austria
CBS 117812 Quercus, Austria
CBS 940.97 Soil, Netherlands
CBS 189.49 Lilium, Netherlands
CBS 732.74 Lilium, Netherlands
CBS 305.85 Lilium, Netherlands
CBS 304.85 Lilium, Netherlands
N. major
N. ditissima
N. neomacrospora
C. cylindroides
Cylindrocarpon sp.
N. ramulariae
Cylindrocarpon sp.
C. obtusisporum
N. macroconidialis
I. coprosmae
I. mors-panacis
I. lusitanica
I. radicicola
I. liriodendri
I. cyclaminicola
I. venezuelensis
I. europaea
I. robusta
I. panacis
I. rufa
"Cylindrocarpon" sp.
I. crassa
I. pseudodestructans
I. gamsii
I. liliigena
10

Fig. 1 The first of 1000 equally most parsimonious trees obtained from the combined ITS, TUB, HIS and TEF sequence alignment of Cylindrocarpon isolates and relatives with a heuristic search using PAUP v. 4.0b10. The tree was rooted using Campylocarpon isolates as outgroup sequences and bootstrap support values are indicated near the nodes, where"ns" designates not supported. Type strains are indicated by bold type isolate name.

Table 2. Statistical information on the individual datasets and number of equally most parsimonious trees for each locus [Internal Transcribed Spacers (ITS) of the nuclear ribosomal RNA, β-tubulin (TUB), histone H3 (HIS) and translation elongation factor 1-α (TEF) genes]

	ITS	TUB	HIS	TEF	Combined
Aligned characters (including gaps)	475	502	440	696	2113
Parsimony-informative characters	122	212	215	364	913
Variable and parsimony-uninformative characters	31	38	11	43	123
Constant characters	322	252	214	289	1077
Equally most parsimonious trees obtained	136	384	1	60	1000
Tree length	294	603	1095	1149	3259
Consistency index (Cl)	0.718	0.660	0.468	0.611	0.559
Retention index (RI)	0.978	0.972	0.946	0.966	0.959
Rescaled Consistency index (RC)	0.702	0.642	0.442	0.590	0.537

Taxonomy

The present study treats isolates that have been freshly collected, or previously identified and maintained in culture collections as "*Cylindrocarpon destructans*", meaning cylindrical, rarely curved, 3-septate macroconidia with obtuse apices, abundant microconidia and chlamydospores (Samuels and Brayford 1990). The latter species has in the past been acknowledged as anamorph of *I. radicicola* (Booth 1966; Chaverri et al. 2011; Samuels and Brayford 1990). However, an examination of the neotype of *"C." destructans* in this study [CUP-011985, conidia (18.0)23.0–30.0(35.0) × (6.0)6.5(7.0) µm], found conidia to be considerably smaller than those of *I. radicicola* (24.0)33.1(47.0) × (4.9)6.4(7.8) µm (Gerlach and Nilsson 1963) (also confirmed in the present study by examination of CBS 264.65, ex-type), revealing them to represent two distinct species. Furthermore, based on the phylogenetic and morphological data obtained in the present study, several novel species could be distinguished that are phylogenetically distinct from *I. radicicola*, and morphologically distinct based on a range of characters linked to culture characteristics, conidiophores, macro- and microconidium morphology. Some of these could be linked to older names, or taxa long regarded as potential syonyms of "*destructans*", which could now be resurrected. These taxa are treated below:

Ilyonectria anthuriicola A. Cabral & Crous, *sp. nov.* Fig. 2.

MycoBank 560108.

Etymology: Named after its host, *Anthurium*.

Cylindrocarpi destructantis morphologice simile, sed longitudine media conidiorum longiore, 29.5–32.2 µm, distinguitur.

Conidiophores simple or complex to sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched bearing up to three phialides, 1–3-septate, 40–95 µm long; phialides monophialidic, more or less cylindrical but slightly tapering towards the tip, 10.5–20.5 µm long, 2.5–3.5 µm wide at the base, 3.0–4.5 µm at widest point, 1.5–2.5 µm near the aperture. Conidiophores giving rise to microconidia, formed on mycelium at agar surface, penicillately mono- or bi-verticillate; phialides monophialidic, narrowly flask-shaped, typically with widest point near the middle, 8–15 µm long, 2.0–3.0 µm wide at the base, 2.5–4.5 µm at widest point, 1.0–2.0 µm near the apex. *Sporodochial conidiophores* irregularly branched; phialides cylindrical, mostly widest near the middle. *Macroconidia* formed in flat domes of slimy masses, (1–)3-septate, straight or minutely curved, cylindrical with both ends more or less obtusely rounded, mostly without a visible hilum; 1-septate, (20.0)23.5–26.7(29.0) × (5.5)5.9–6.8(7.0) µm (average = 25.1 × 6.4 µm), with a length:width ratio of 3.6–4.8; 2-septate, (25.0)26.6–29.3(32.0) × (6.5)6.8–7.8(8.5) µm (av. = 27.9 × 7.3 µm), with a length:width ratio of 3.2–4.8; 3-septate, (25.0)29.5–32.2(38.0) × (6.0)7.5–8.1(9.0) µm (av. = 30.8 × 7.8 µm) with a length:width ratio of 3.1–5.2. *Microconidia* 0(–1)-septate, subglobose to ovoid, rarely ellipsoid, mostly with a visible centrally located or slightly laterally displaced hilum; aseptate microconidia, (4.9)5.0–8.1(12.0) × (4.0)4.3–5.5(6.5) µm (av. = 6.5 × 4.9 µm), with a length:width ratio of 1.0–1.8; 1-septate, (11.0)11.6–16.7(18.0) × (5.0)5.4–6.1(6.0) µm (av. = 14.1 × 5.8 µm), with a length:width ratio 1.8–3.0. *Chlamydospores* globose to subglobose to ellipsoid, 8–14 × 7–12 µm, smooth, but often appearing rough due to deposits, thick-walled, formed intercalary in chains or in clumps and also in the cells of macroconidia, hyaline, becoming golden-brown.

Holotype: Netherlands, Bleiswijk, root rot of *Anthurium* sp., 1995, coll./isol. R. Pieters, holotype CBS H-20555, culture ex-type CBS 564.95.

Culture characteristics: Mycelium felty with average density. Surface on OA chestnut, with aerial mycelium sparse, saffron; margin pure yellow to orange. Surface on PDA, chestnut with saffron

aerial mycelium, growth at margin luteous; zonation absent, transparency homogeneous, margin even; reverse similar to surface, but chestnut to cinnamon on OA, and chestnut on PDA. Colonies on PDA do not grow at 4 ºC after 7 d. Optimum temperature 20 ºC when colonies reach 25–27 mm, after 7 d. Colony diam was 20–22 mm at 25 ºC, after 7 d. Hardly grows at 30 ºC (2 mm colony diam after 7d).

Isolate studied: CBS 564.95 (Table 1).

Host and distribution: Roots of *Anthurium* sp. (Netherlands).

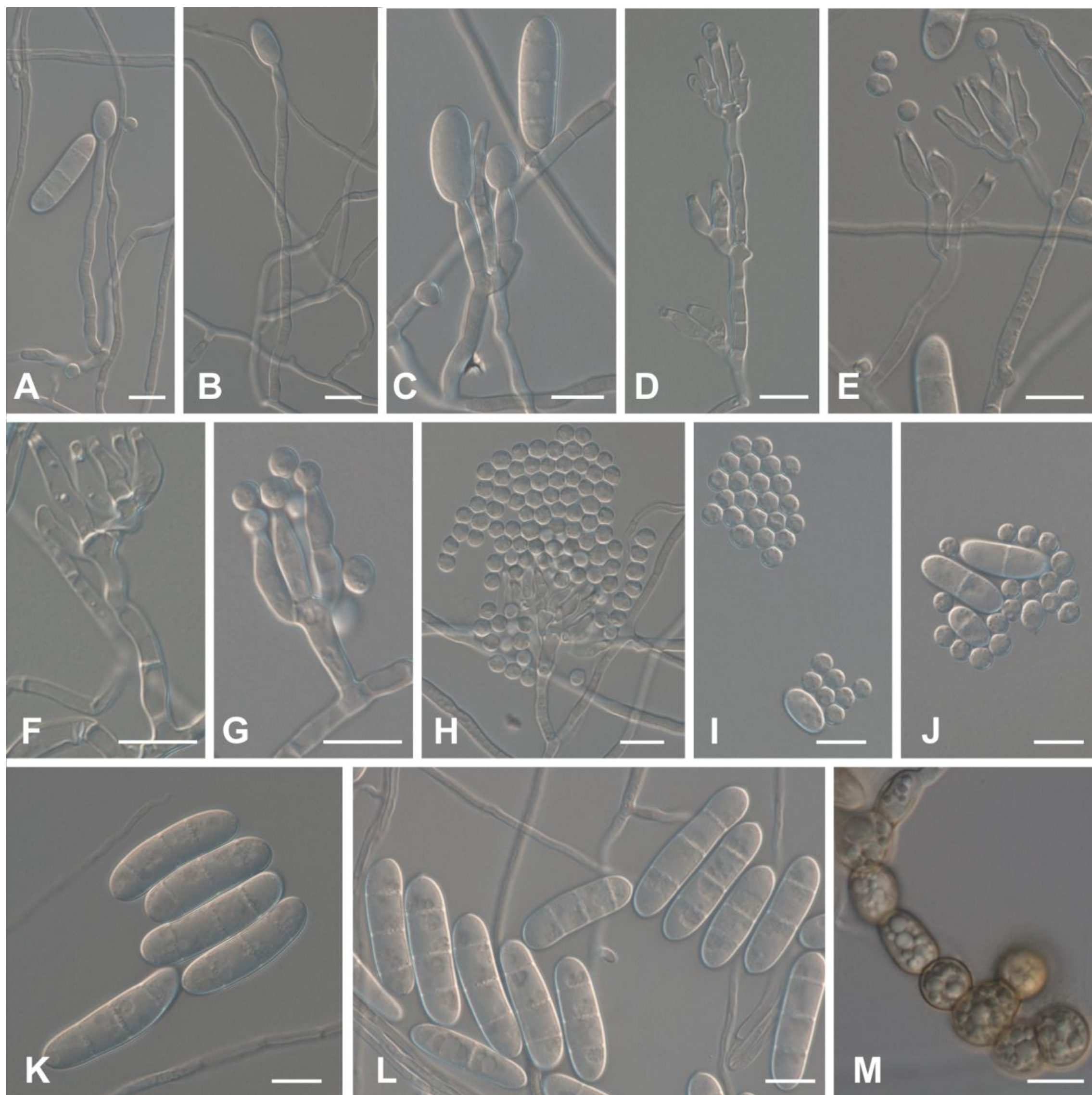


Fig. 2 *Ilyonectria anthuriicola* (CBS 564.95). (a–c) Simple conidiophores on aerial mycelium. (d–g) Conidiophores giving rise to microconidia, formed on mycelium at agar surface, penicillately mono- or bi-verticillate. (h–l) Micro- and macroconidia. (m) Chlamydospores in mycelium. Bars: 10 µm

Ilyonectria crassa (Wollenw.) A. Cabral & Crous, *comb. et stat. nov.* Fig. 3.

MycoBank 560109.

Basionym: *Cylindrocarpon radicicola* var. *crassum* Wollenw., Z. Parasitenkunde 3: 495. 1931.

≡ *Cylindrocarpon destructans* var. *crassum* (Wollenw.) C. Booth, Mycol. Pap. 104: 37. 1966.

Conidiophores simple or complex, to sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched bearing up to two phialides, rarely consisting only of phialides, 1–4-septate, 40–180 µm long; phialides monophialidic, cylindrical to subulate, 20–55 µm long, 2.5–4.0 µm wide at the base, 1.5–2.0 µm near the apex. *Complex conidiophores* aggregated in small sporodochia (on carnation leaf), repeatedly and irregularly branched; phialides more or less cylindrical, but tapering slightly in the upper part towards the apex, or narrowly flask-shaped, mostly with widest point near the middle, 17–24 µm long, 2.0–3.0 µm wide at the base, 2.5–3.5 µm at the widest point, and 1.5–2.5 µm wide near the apex. *Macroconidia* predominating, formed on both type of conidiophores, on SNA formed in flat domes of slimy masses, 1–3-septate, straight, cylindrical, but may narrow towards the tip, more or less broadly rounded, and the base appearing somewhat acute due to the presence of the hilum, mostly centrally located; 1-septate, (21.0)25.7–27.3(34.0) × (4.5)5.0–5.3(6.5) µm (av. = 26.5 × 5.1 µm), with a length:width ratio of 3.8–6.7; 2-septate, (23.0)28.5–30.3(37.0) × (4.5)5.3–5.6(6.5) µm (av. = 29.4 × 5.4 µm)with a length:width ratio of 4.2–6.7; 3-septate, (29.0)34.1–36.0(49.0) × (5.0)5.6–5.8(7.0) µm (av. = 35.1 × 5.7 µm), with a length:width ratio of 4.8–8.9. *Microconidia* 0–1-septate, ellipsoid to subcylindrical, more or less straight, with a visible, truncate hilum; aseptate microconidia, (7.0)9.7–10.9(15.0) × (3.0)3.3–3.6(4.5) µm (av. = 10.3 × 3.5 µm), with a length:width ratio of 1.8–4.3; 1-septate, (12.0)14.2–15.2(19.0) × (3.0)3.8–4.2(5.0) µm (av. = 14.7 × 4.0 µm), with a length:width ratio 2.7–5.0. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. *Chlamydospores* globose to subglobose to cylindrical, 7–15 × 6–10 µm, smooth, but often appearing rough due to deposits, thick-walled, terminal on short lateral branches, rarely intercalary, single, in chains or in clumps, and also in the cells of the macroconidia, hyaline, becoming pale brown.

Lectotype: The Netherlands, on *Lilium* bulbs, Dec. 1930, coll./isol. W.F. van Hell, lectotype designated here CBS H-20556, culture ex-lectotype CBS 139.30.

Culture characteristics: Mycelium cottony to felty with average to strong density. Surface on OA cinnamon, with aerial mycelium sparse, buff. Surface on PDA saffron with aerial mycelium sparse buff to saffron to pale luteous. No zonation was observed, transparency was homogeneous and growth at margin even. Reverse similar to surface, except in colour, saffron to cinnamon on OA, and chestnut to sienna on PDA. Colonies on PDA grow 5–8 mm diam at 4 ºC after 7 d. Optimum temperature at 20 ºC, when colonies reach 31–46 mm diam, after 7 d. Colony diam was 19–34 mm at 25 ºC, after 7 d. No growth was observed at 30 ºC.

Isolates studied: CBS 139.30; CBS 158.31; CBS 129083; NSAC-SH-2; NSAC-SH-2.5 (Table 1).

Hosts and distribution: *Lilium* sp. (bulbs), *Narcissus* sp. (roots) (Netherlands), *Panax quinquefolium* (roots) (Canada).

Notes: In the original description, Wollenweber (1931) cites *Cylindrocarpon radicicola* var. *crassum* as occurring on roots of *Ulmus*, *Taxus* and *Lilium* in Europe (Germany and the Netherlands). He did not designate any type specimen. However, he specifically refers to a culture sent to him by prof. J. Westerdijk on *Lilium* from the CBS in the Netherlands in 1930, which was regarded as authentic for the species. This culture is represented by CBS 139.30 (accessioned in 1930, from *Lilium*, the Netherlands), and thus we designate a dried, sporulating culture as lectotype for the species.

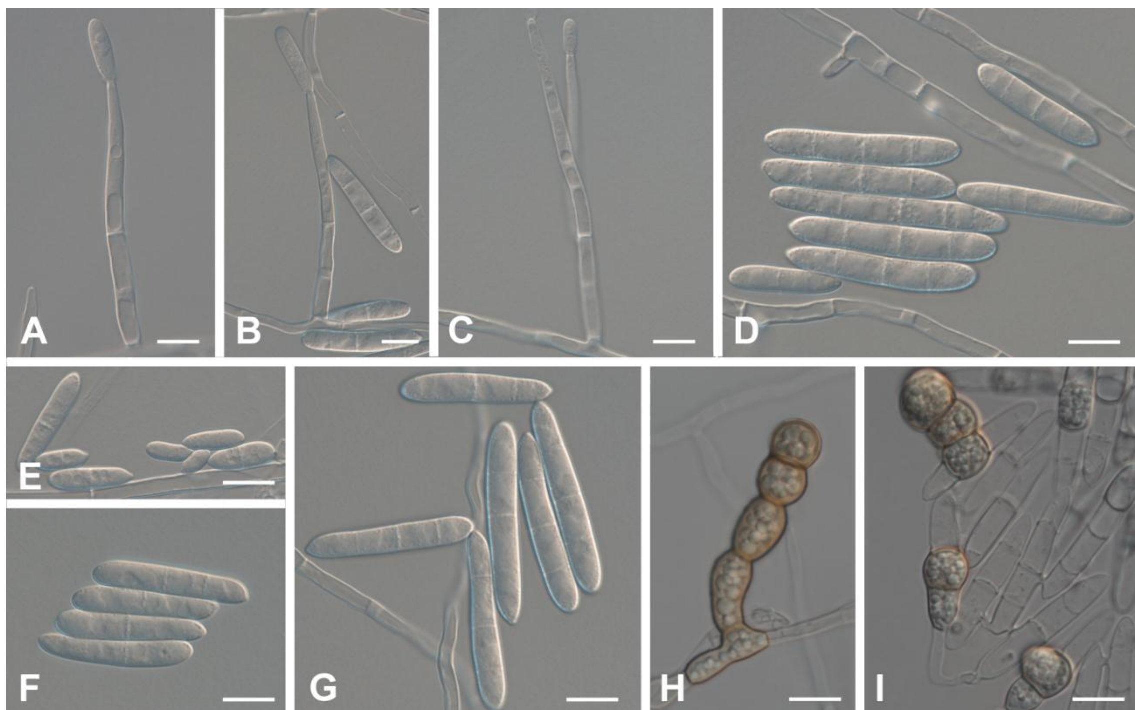


Fig. 3 *Ilyonectria crassa* (CBS 129083). (a–c) Simple conidiophores on aerial mycelium. (d–g) Micro- and macroconidia. (h–i) Chlamydospores and macroconidia. Bars: 10 µm

Ilyonectria cyclaminicola A. Cabral & Crous, sp. nov. Fig. 4

MycoBank 560110.

Etymology: Named after the host from which it was isolated, *Cyclamen* sp.

Cylindrocarpi destructantis morphologice simile, sed longitudine media conidiorum longiore, 26.9–31.9 µm, distinguitur.

Conidiophores simple or complex to sporodochial. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to two phialides, 1–3-septate, 60–120 µm long; phialides monophialidic, more or less cylindrical but slightly tapering towards the tip, 20–60 µm long, 2.0–4.0 µm wide at the base, 3.0–4.5 µm at widest point, 1.5–2.5 µm near the aperture. Conidiophores giving rise to microconidia formed by mycelium at agar surface, penicillate to mono-verticillate; phialides monophialidic, more or less cylindrical, but with slight taper towards the tip, 19–34 µm long, 1.5–2.5 µm wide at the base, 2.0–3.0 µm at widest point, 1.0–2.0 µm near the apex. Sporodochial conidiophores irregularly branched; phialides more or less cylindrical, but slightly tapering towards the tip, or narrowly flask-shaped, with widest point near the base, 14–26 µm long, 2.5–3.5 µm wide at the base 3.0–4.0 µm at widest point, 1.0–2.0 µm near the apex. Macroconidia formed in flat domes of slimy masses, 1(–3)-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, sometimes with a constriction at the septa, mostly without a visible hilum; 1-septate, (19.2)21.3–23.6(29.8) × (4.4)5.4–6.0(7.3) µm (av. = 22.5 × 5.7 µm), with a length:width ratio of 3.4–5.5; 2-septate, (23.8)24.0–28.4(29.8) × (5.0)5.5–7.3(8.0) µm (av. = 26.2 × 6.4 µm), with a length:width ratio of 3.1–5.1; 3-septate, (25.3)26.9–31.9(33.6) × (5.8)5.9–6.5(6.9) µm (av. = 29.4 × 6.2 µm), with a length:width ratio of 3.7–5.6. Microconidia formed in heads or on the agar surface, 0–1-septate, subglobose to ovoid to subcylindrical, mostly with a visible, centrally located or slightly laterally displaced hilum; aseptate microconidia, (3.9)7.6–8.9(12.9) × (2.2)3.6–3.9(5.4) µm (av. = 8.2 × 3.7 µm), with a length:width ratio of 1.2– 3.4; 1-septate, (11.5)13.8–15.2(17.5) × (3.7)4.6–4.9(5.5) µm (av. = 14.5 × 4.7 µm), with a length:width ratio of 2.3–3.9. Chlamydospores globose to subglobose, 10–18 × 10–16 µm, smooth, but often appearing rough due to deposits, thick-walled, formed in lateral branches, rarely intercalary, mostly isolated, hyaline, becoming medium brown.

Holotype: Netherlands, Roelofarendsveen, NAKS laboratory, Cyclamen bulb, May 1993, coll./isol. M. Hooftman, iden. E.J. Hermanides-Nijhof, holotype CBS H-20557, culture ex-type CBS 302.93.

Culture characteristics: Mycelium felty with average density. Surface on OA sepia to chestnut. Surface on PDA sepia to chestnut, with sparse, rust, aerial mycelium; no zonation was observed, and transparency was homogeneous; margins predominantly even. Reverse similar to surface, except in colour, sepia to dark brick on OA and chestnut on PDA. Colonies on PDA do not grow at 4 ºC after 7 d. Optimum temperature at 22 ºC, when colonies reach 68–70 mm diam, after 7 d. Colony diam was 63–64 mm at 25 ºC, after 7 d. No growth was observed at 30 ºC.

Isolate studied: CBS 302.93 (Table 1).

Host and distribution: Bulb of *Cyclamen* sp. (Netherlands).

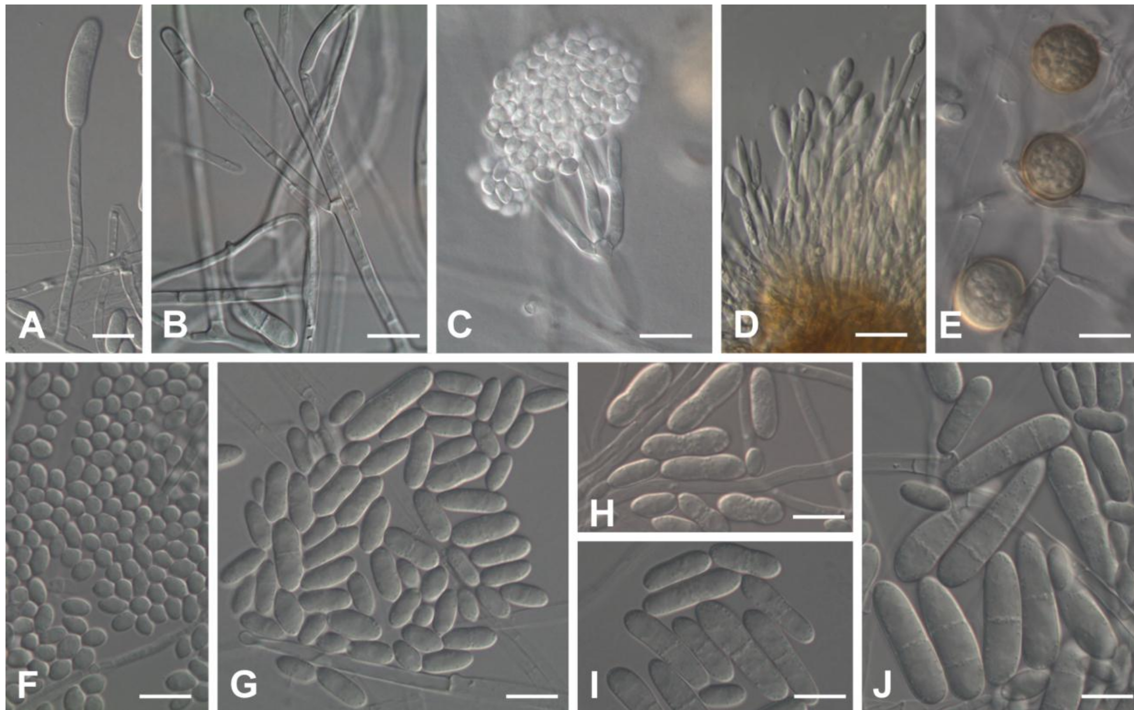


Fig. 4 *Ilyonectria cyclaminicola* (CBS 302.93). (a, b) Simple conidiophores on aerial mycelium. (c) Pennicilate conidiophores with aseptate microconidia. (d) Sporodochial conidiophore on carnation leaf agar. (e) Isolated chlamydospores formed in lateral branches. (f-j). Micro- and macroconidia. Bars: 10 µm

Ilyonectria europaea A. Cabral, Rego & Crous, *sp. nov*. Fig. 5

MycoBank 560103.

Etymology: Named after the European continent, where this fungus appears to be widely distributed.

Ilyonectriae robustae morphologice similis, sed longitudine media macroconidiorum breviore, 29.7–31.5 µm, distinguitur.

Conidiophores simple or complex to sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to three phialides, 1–3-septate, 50–120 µm long; phialides monophialidic, cylindrical to subulate, 26–60 µm long, 2.5–3.5 µm wide at the base, 1.5–2.5 µm near the apex. *Complex conidiophores* aggregated in small sporodochia (on carnation leaf), repeatedly and irregularly branched. *Macroconidia* predominating, formed on both type of conidiophores, on SNA formed in flat domes of slimy masses, 1(–3)-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, but may narrow towards the tip, mostly without a visible hilum; 1-septate, (16.4)21.9–23.4(34.0) × (4.0)5.2–5.6(7.8) µm (av. = 22.7 × 5.4 µm), with a length:width ratio of 3.2–5.4; 2-septate, (22.0)26.4–28.1(34.0) × (4.4)5.9–6.4(8.0) µm (av. = 27.2 × 6.1 µm), with a length:width ratio of 3.4–6.4; 3-septate, (22.0)29.7–31.5(40.0) × (5.0)6.5–6.9(8.6) µm (av. = 30.6 × 6.7 µm), with a length:width ratio of 3.5–6.0. *Microconidia* 0–1-septate, ellipsoid to ovoid, more or less straight, without a visible hilum; aseptate microconidia sometimes curved towards one end, (3.0)8.5–9.8(17.0) × (1.7)3.3–3.5(5.0) µm (av. = 9.1 × 3.4 µm), with a length:width ratio of 1.5–3.4; 1-septate, (9.2)13.4–14.6(18.9) × (3.0)4.0–4.4(5.9) µm (av. = 14.0 × 4.2 µm), with a length:width ratio 2.6–4.0. *Conidia* formed in heads or on simple conidiophores as white (OA) or unpigmented (SNA) masses. *Chlamydospores* globose to subglobose, 9–14 × 7–14 µm, smooth, but often appearing rough due to deposits, thick-walled, terminal on short or long lateral branches or intercalary, single, in chains or in clumps, golden-brown.

Holotype: Portugal, Vidigueira, at basal end of a 2-year-old *Vitis vinifera* plant; scion Petit Verdot, rootstock 110R, 2008, coll./isol. C. Rego, holotype CBS H-20558, culture ex-type CBS 129078 = Cy241 = CPC 19165.

Culture characteristics: Mycelium felty with average density. Surface on OA chestnut, with saffron aerial mycelium. Sienna to saffron on PDA, with luteous aerial mycelium. Concentric zonation, with homogeneous transparency, margins predominantly even. Reverse similar to surface, except in the colour; sepia on OA, and chestnut to umber on PDA. Colonies on PDA grow poorly, 1–5 mm diam at 4 ºC after 7 d. Optimum temperature for growth is 22 ºC, when colonies reach 43–57 mm diam, after 7 d. Colony diam was 37–47 mm at 25 ºC, after 7 d. No growth was observed at 30 ºC.

Isolates studied: Cy131; Cy155; CBS 537.92; CBS 102892; CBS 129078 (Table 1).

Hosts and distribution: *Actinidia chinensis* 'Hayward' (internal lesion of stem) (France), *Aesculus hippocastanum* (wood) (Belgium), *Phragmites australis* (stem) (Germany), *Vitis vinifera* (Portugal).

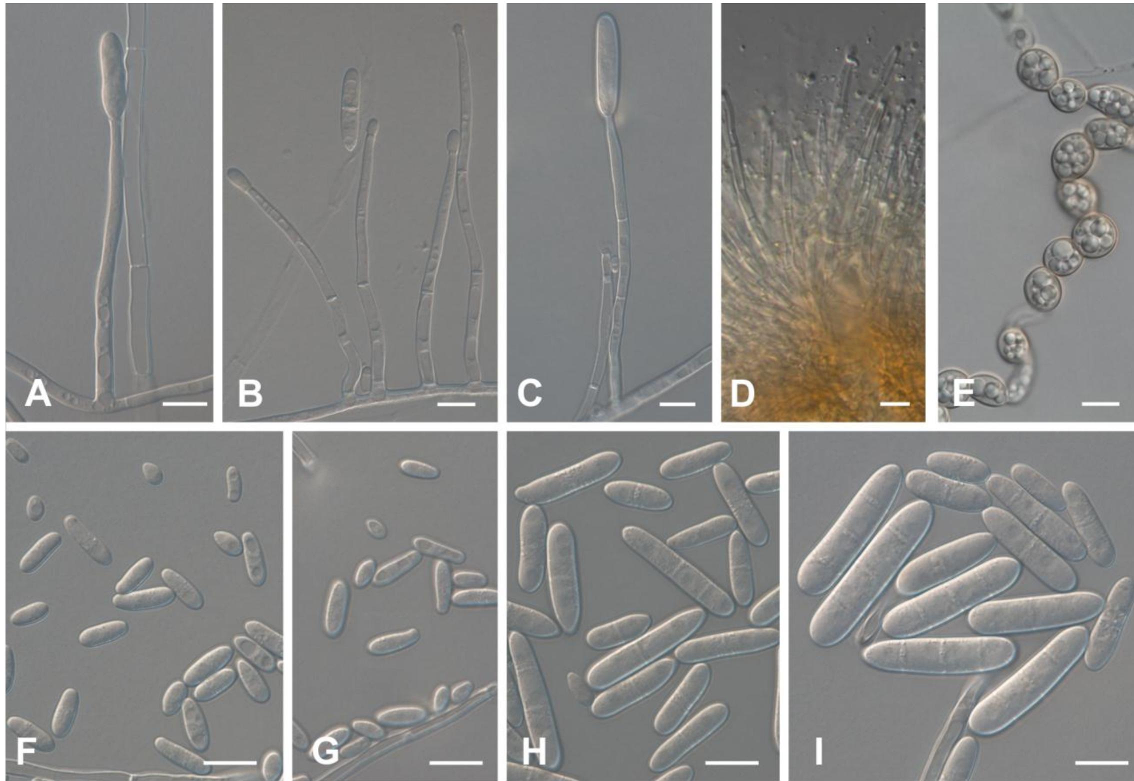


Fig. 5 *Ilyonectria europaea* (CBS 129078). (a-c) Simple conidiophores on aerial mycelium. (d) Sporodochial conidiophore on carnation leaf agar. (e) Chlamydospores in aerial mycelium. (f–i) Micro- and macroconidia. Bars: 10 µm

Ilyonectria gamsii A. Cabral & Crous, *sp. nov*. Fig. 6

MycoBank 560112.

Etymology: Named after Prof. dr Walter Gams, who has made a major contribution to our knowledge of Hypocrealean soil fungi.

Ilyonectriae panacis morphologice similis, sed longitudine media macroconidiorum breviore, 34.3–38.5 µm, distinguitur.

Conidiophores simple or complex to sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to two phialides, 1–3-septate, 50–150 µm long; phialides monophialidic, cylindrical to subulate, 30–60 µm long, 2.5–3.5 µm wide at the base, 1.5–2.0 µm near the

aperture. *Sporodochial conidiophores* irregularly branched; phialides cylindrical, mostly widest near the base. *Macroconidia* predominating, formed on simple conidiophores, on SNA formed in flat domes of slimy masses, 1–3-septate, straight, cylindrical with both ends broadly rounded, with mostly visible, centrally located hilum; 1-septate, (22.0)25.7–27.9(33.0) × (4.0)5.1–5.5(6.0) µm (av. = 26.8 × 5.3 µm), with a length:width ratio of 4.3–6.2; 2-septate, (25.0)28.2–31.7(39.0) × (5.0)5.5–5.9(6.5) µm (av. = 29.9 × 5.7 µm), with a length:width ratio of 4.2–7.1; 3-septate, (24.0)34.3–38.5(44.0) × (5.0)5.9–6.3(7.0) µm (av. = 36.4 × 6.1 µm), with a length:width ratio of 4.3–7.3. *Microconidia* 0–1-septate, ellipsoid to subcylindrical, more or less straight, mostly with a visible hilum; aseptate microconidia (4.0)6.9–8.0(10.0) × (3.0)4.0–4.5(5.0) µm (av. = 7.4 × 4.3 µm), with a length:width ratio of 1.3–2.9; 1-septate, (8.0)12.9–15.7(18.0) × (4.0)4.2–4.7(5.5) µm (av. = 14.3 × 4.4 µm), with a length:width ratio 1.8–4.0. *Chlamydospores* globose to subglobose to ellipsoidal, 8–14 × 7–12 µm, smooth, but often appearing rough due to deposits, thick-walled, mostly intercalary, rarely terminal on short lateral branches, single, in chains or in clumps, hyaline, becoming medium brown.

Holotype: Netherlands, Lelystad, soil, June 1997, coll./isol. J.T. Poll, iden. W. Gams, holotype CBS H-20559, culture ex-type CBS 940.97.

Culture characteristics: Mycelium cottony, dense. Surface on OA cinnamon, with sparse, buff aerial mycelium, on PDA umber to chestnut, with buff to saffron aerial mycelium; zonation absent, transparency homogeneous, margin even; reverse similar to surface, but chestnut on PDA. Colonies on PDA grow 6–7 mm diam at 4 ºC after 7 d. Optimum temperature at 22 ºC when colonies reach 44–45 mm diam, after 7 d. Colony diam is 22–24 mm at 25 ºC, after 7 d. No growth observed at 30 ºC.

Isolate studied: CBS 940.97 (Table 1).

Habitat and distribution: Soil (Netherlands).

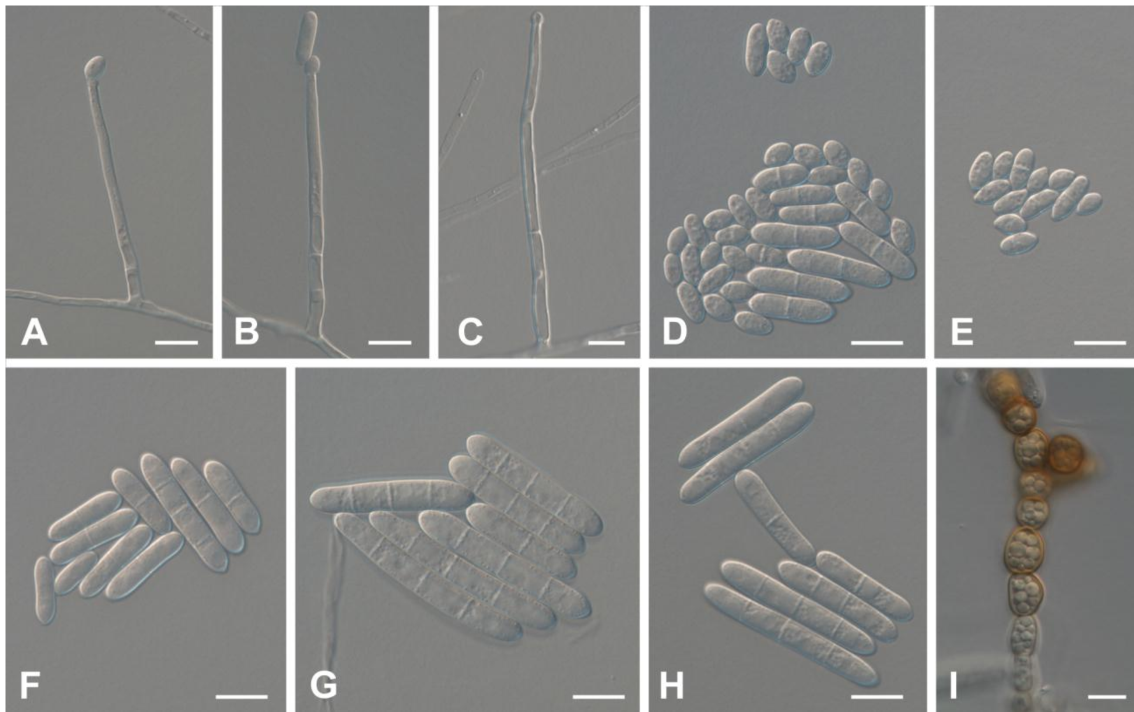


Fig. 6 *Ilyonectria gamsii* (CBS 940.97). (a-c) Simple conidiophores on aerial mycelium. (d-h) Micro- and macroconidia. (i) Chlamydospores on mycelium. Bars: 10 µm

Ilyonectria liliigena A. Cabral & Crous, sp. nov. Fig. 7

MycoBank 560114.

Etymology: Named after its host, *Lilium regale*.

Ilyonectriae panacis morphologice similis, sed longitudine media macroconidiorum 3-septatorum breviore, 27.9–29.8 µm, distinguitur.

Conidiophores simple or complex or sporodochial. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to two phialides, 1–4-septate, 50–170 µm long; phialides monophialidic, cylindrical to subulate, 30–65 µm long, 2.0–3.5 µm wide at the base, 1.5–2.0 µm near the apex. Sporodochial conidiophores irregularly branched; phialides cylindrical, mostly widest near the base. Macroconidia predominating, formed on simple conidiophores, on SNA formed in flat domes of slimy masses, 1(–3)-septate, straight or frequently minutely curved, cylindrical or sometimes typically minutely widening towards the tip, therefore appearing somewhat clavate, mostly without a visible hilum; 1-septate, (19.0)22.9–24.6(30.0) × (3.3)4.2–4.5(5.2) µm (av. = 23.8 × 4.3 µm), with a length:width ratio of 4.0–7.0; 2-septate, (21.0)26.1–27.7(32.1) × (4.0)4.7–

5(5.7) µm (av. = 26.9 × 4.9 µm)with a length:width ratio of 3.8–7.0; 3-septate, (23.9)27.9–29.8(35.0) × (3.9)4.7–5.1(6.0) µm (av. = 28.9 × 4.9 µm), with a length:width ratio of 4.0–8.3. Microconidia 0–1-septate, ellipsoidal to subcylindrical, more or less straight, mostly with a visible hilum; aseptate, microconidia (5.9)8.9–10.3(17.0) × (2.5)3.0–3.2(4.4) µm (av. = 9.6 × 3.1 µm), with a length:width ratio of 2.0–4.6; 1-septate, (10.0)12.9–14.3(18.0) × (2.5)3.3–3.6(4.5) µm (av. = 13.6 × 3.4 µm), with a length:width ratio 2.8–5.6. Conidia formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. Chlamydospores globose to subglobose, 6–14 × 5–12 µm, smooth but often appearing rough due to deposits, thick-walled, mostly in terminal on short lateral branches or rarely intercalary, single, in chains or in clumps, hyaline, becoming slightly brown at margins.

Holotype: Netherlands, Hoorn, bulb rot of *Lilium regale*, 1949, coll./isol. M.A.A. Schipper, holotype CBS H-20560, culture ex-type CBS 189.49.

Culture characteristics: Mycelium felty, with an average to strong density. Surface on OA sienna, with sparse, saffron, aerial mycelium. Surface on PDA sepia to cinnamon, with saffron to buff aerial mycelium. Zonation absent or concentric, with homogeneous transparency. Margins were even, or sometimes slightly uneven. Reverse similar to surface, except in colour; on OA pale vinaceous to cinnamon; on PDA buff to saffron to chestnut. Colonies on PDA grew poorly (1–4 mm diam) at 4 ºC after 7 d. Optimum temperature at 22 ºC, when colonies reach 34–45 mm diam, after 7d. Colony diam was 16–29 mm at 25 ºC, after 7 d. No growth was observed at 30 ºC.

Isolates studied: CBS 189.49; CBS 732.74; CBS 304.85; CBS 305.85 (Table 1).

Host and distribution: *Lilium regale* bulbs (Netherlands).

Fig. 7 *Ilyonectria liliigena* (CBS 189.49). (a–d) Simple conidiophores on aerial mycelium. (e) Chlamydospores on mycelium. (f–i) Micro- and macroconidia. Bars: 10 µm

Ilyonectria lusitanica A. Cabral, Rego & Crous, *sp. nov.* Fig. 8

MycoBank 560105.

Etymology: Named after the latin name for the country from where it was collected, Portugal.

Ilyonectriae europaeae morphologice similis, sed longitudine media macroconidiorum breviore, 25–28.4 µm, distinguitur.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to two phialides, 1–4-septate, 60–220 µm long; phialides monophialidic, cylindrical to subulate, 20–70 µm long, 2.5–3.5 µm wide at the base, 1.5–2.5 µm near the aperture. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched. *Macroconidia* predominating, formed by both type of conidiophores, on SNA formed in flat domes of slimy masses, 1(–3)-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, but may narrow towards the tip, without a visible hilum, and may have a constriction on the septa in older cultures; 1-septate, (14.0)17.3–18.8(21.0) ×

(4.0)4.6–5(5.5) µm (av. = 18.1 × 4.8 µm), with a length:width ratio of 2.8–4.8; 2-septate, (18.0)20.5–22.1(27.0) × (4.0)4.9—5.2(6.0) µm (av. = 21.3 × 5.1 µm), with a length:width ratio of 3.5–5.4; 3-septate, (18.0)25.0–28.4(38.0) × (4.5)5.2–5.5(6.0) µm (av. = 26.7 × 5.4µm), with a length:width ratio of 3.6–6.8. *Microconidia* 0–1-septate, ellipsoid to ovoid, more or less straight, without a visible hilum, and may have a constriction at the septum; aseptate, (5.0)6.9–8.2(10.0) × (2.5)3.0–3.3(4.0) µm (av. = 7.6 × 3.2 µm), with a length:width ratio of 1.7–3.3; 1-septate, (8.0)10.0–11.0(14.0) × (3.0)3.4–3.7(4.0) µm (av. = 10.5 × 3.6 µm), with a length:width ratio 2.0–3.7. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. *Chlamydospores* rarely observed, globose to subglobose to cylindrical, 9–13 × 7–11 µm, smooth, but often appearing rough due to deposits, thick-walled, intercalary, hyaline, becoming slightly brown at the margin.

Holotype: Portugal, Melgaço, Alvaredo, on *Vitis vinifera,* below grafting zone, 6–year-old plant; scion Alvarinho on rootstock 196-17, 2005, coll./isol. N. Cruz, holotype CBS H-20563, culture ex-type CBS 129080 = Cy197 = CPC 19166.

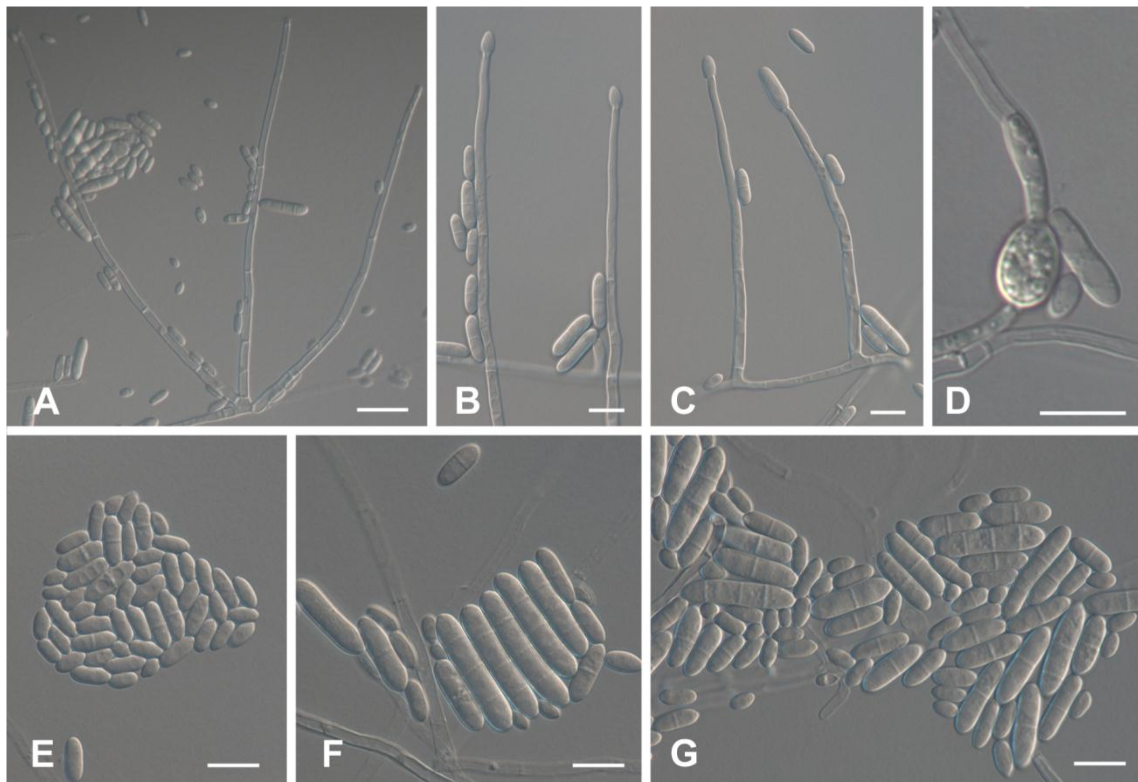


Fig. 8 *Ilyonectria lusitanica* (CBS 129080). (a–c) Simple conidiophores of the aerial mycelium. (d) Chlamydospores on mycelium. (e–g) Micro- and macroconidia. Bars: a = 20 µm, others = 10 µm

Culture characteristics: Mycelium felty with average density. Surface on OA cinnamon, with aerial mycelium sparse, buff. Surface on PDA, cinnamon, with sparse, ochreous to buff aerial

mycelium. Zonation absent, transparency homogeneous, margin even. Reverse similar to surface but buff to cinnamon on OA, and chestnut to cinnamon on PDA. Colonies on PDA grow 5–6 mm at 4 ºC after 7 d. Optimum temperature between 20–22 ºC, with colonies reaching 42–46 mm and 43–46 mm, respectively, after 7 d. Colony diam was 31–32 mm at 25 ºC, after 7 d. No growth observed at 30 ºC.

Isolate studied: CBS 129080 (Table 1).

Host and distribution: *Vitis vinifera* (Portugal).

Ilyonectria mors-panacis (A.A. Hildebr.) A. Cabral & Crous, *comb. nov.* Fig. 9

MycoBank 560115.

Basionym: *Ramularia mors-panacis* A.A. Hildebr., Canadian Journal of Research, Section C 12: 101. 1935.

= *Cylindrocarpon panacis* Matuo & Miyaz., Trans. Mycol. Soc. Japan 9: 111. 1969.

≡ *Cylindrocarpon destructans* f.sp. *panacis* Matuo & Miyaz., Ann. Phytopath. Soc. Japan 50: 390. 1984.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, rarely consisting only of phialides, 1–3-septate, 45–170 µm long; phialides monophialidic, cylindrical to subulate, 23–55 µm long, 2.0–3.0 µm wide at the base, 1.5–3.0 µm near the apex. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched. *Macroconidia* predominating, formed on simple conidiophores, on SNA formed in flat domes of slimy masses, 1(–3)-septate, straight, cylindrical with both ends more or less broadly rounded, mostly without a hilum; 1-septate, (21.0)28.2–31.6(40.0) × (5.0)5.8–6.3(7.5) µm (av. = 29.9 × 6.1 µm), with a length:width ratio of 3.3–7.0; 2-septate, (28.0)30.5–38.4(42.0) × (5.0)5.9–6.4–7.0(7.1) µm (av. = 34.4 × 6.4 µm), with a length:width ratio of 4.0–6.0; 3-septate, (37.8)39.0–44.2(45.0) × (6.9)7.0–7.5(7.5) µm (av. = 41.0 × 7.2 µm), with a length:width ratio of 5.3–6.0. *Microconidia* 0–1-septate, ellipsoid to subcylindrical, more or less straight, without a visible hilum; aseptate, (5.0)8.9–10.4(17.0) × (2.5)3.6–3.9(5.0) µm (av. = 9.6 × 3.8 µm), with a length:width ratio of 1.3–3.4; 1-septate, (9.0)12.5–14.1(19.0) × (3.5)4.4–4.8(5.5) µm (av. = 13.3 × 4.6 µm), with a length:width ratio 2.0–4.0. *Conidia* formed in heads on simple

conidiophores or as white, creamy (OA) or hyaline (SNA) masses. *Chlamydospores* globose to subglobose, 8–16 × 7–15 µm, smooth, but often appearing rough due to deposits, thick-walled, terminal on short lateral branches or intercalary, single, in chains or in clumps, hyaline, becoming medium brown.

Lectotype: Canada, Ontario, on living roots of *Panax quinquefolium,* June 1935, A.A. Hildebrand, lectotype designated here CBS H-20561, culture ex-lectotype CBS 306.35.

Culture characteristics: Mycelium felty with an average density. Surface on OA and PDA chestnut, with sparse, buff to rosy-buff to cinnamon or saffron aerial mycelium. Concentric zonation, with homogeneous transparency, and even margins. Reverse similar to surface, ochreous to fulvous, or sepia to dark vinaceous on OA, and chestnut to sienna on PDA. Colonies on PDA grow 3–9 mm diam at 4 ºC after 7 d. Optimum temperature for growth is 18 ºC, when colonies reach 22–40 mm diam, after 7 d. Colony diam was 31–40 mm at 25 ºC after 7 d. No growth was observed at 30 ºC.

Isolates studied: CBS 306.35; CBS 307.35; CBS 120359; CBS 120360; CBS 120361; CBS 120362; CBS 120363; CBS 120364; CBS 120365; CBS 120366; CBS 120367; CBS 120368; CBS 120369; CBS 124662; CPC 13535; CPC 13537 (Table 1).

Hosts and distribution: *Panax ginseng* (Japan), *P. quinquefolium* (Canada).

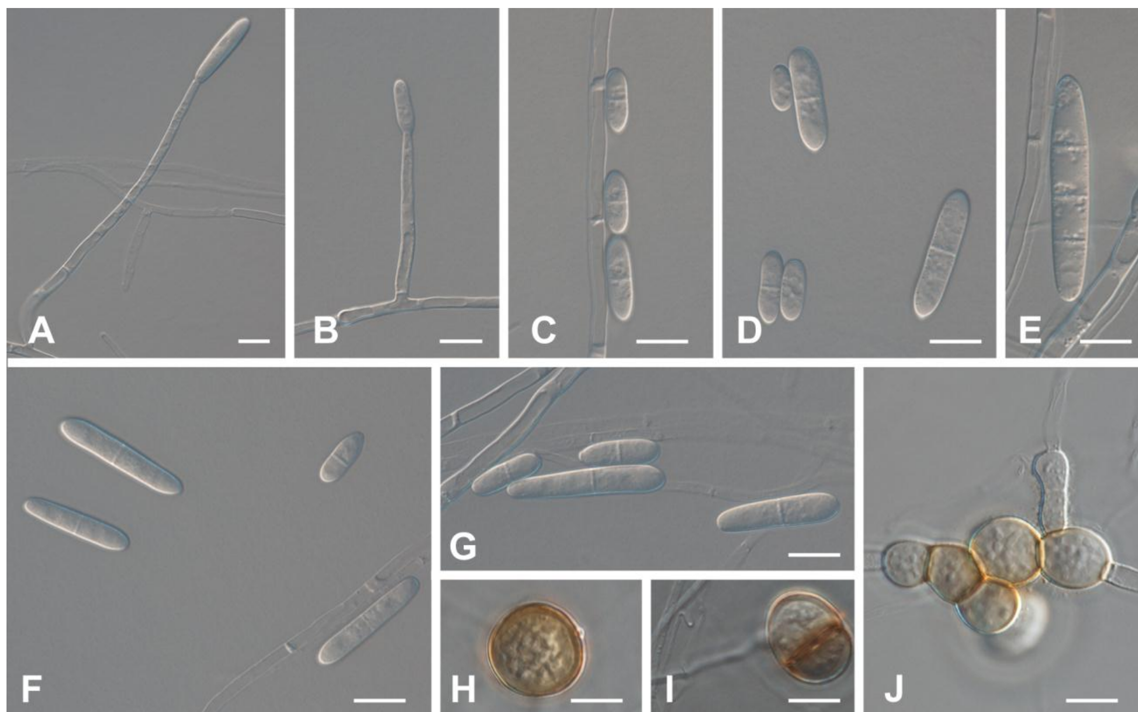


Fig. 9 *Ilyonectria mors-panacis* (CBS 120363). (a–b) Simple conidiophores on aerial mycelium. (c–g) Micro- and macroconidia. (h–j) Chlamydospores on mycelium. Bars: 10 µm

Notes: *Ilyonectria mors-panacis* is distinct from "*C.*" *destructans* (anamorph: "*C*". *destructans*, neotype CUP-011985, conidia (18.0)23.0–30.0(35.0) × (6.0)6.5(7.0) µm) in having larger conidia, and indistinct hila (being prominent, flat, 2 µm diam in *I. radicicola,* also see Samuels and Brayford 1990, fig. 1). "*Ramularia" panacicola* is distinct by also having shorter conidia than *I. mors-panacis*, 5.5–34.2 × 2.5–7.2 µm (Zinssmeister 1918), and appears to be another potential synonym of "*C.*" *destructans*. However, no authentic material could be located of "*R.*" *panacicola*, and the only isolate deposited under this name, was a Canadian strain collected by Hildebrand (1935), which in fact represented *I. mors-panacis* (Fig. 1). The oldest name for the species on *Panax* treated here, therefore, is *R. mors-panacis* (CBS 306.35), with the Japanese collections ("*C.*" *panacis* ≡ "*C.*" *destructans* f.sp. *panacis*, CBS 124662 = NBRC 31881) being later synonyms (see Fig. 1).

Ilyonectria panacis A. Cabral & Crous, *sp. nov.* Fig. 10

MycoBank 560104.

Etymology: Named after its host, *Panax quinquefolium*.

Ilyonectriae liliigenae morphologice similis, sed longitudine media macroconidiorum longiore, 31–35 µm, distinguitur.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched bearing up to three phialides, 1–5-septate, 60–220 µm long; phialides monophialidic, cylindrical to subulate, 20–65 µm long, 2.5–3.0 µm wide at the base, 1.5–2.0 µm near the aperture. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched. *Macroconidia* predominating, formed on both type of conidiophores, on SNA formed in flat domes of slimy masses, 1(–3)-septate, straight, cylindrical with both ends more or less broadly rounded, mostly with a visible centrally located hilum; 1-septate, (20.0)23.7–25.9(32.0) × (4.0)4.7–5.0(5.5) µm (av. = 24.8 × 4.8 µm), with a length:width ratio of 4.0–6.0; 2-septate, (23.0)27.0–30.3(37.0) × (4.8)5.0–5.4(6.0) µm (av. = 28.7 × 5.2 µm), with a length:width ratio of 4.6–6.7; 3-septate, (27.0)31.2–35.0(49.0) × (5.0)5.4–5.8(6.0) µm (av. = 33.1 × 5.6 µm), with a length:width ratio of 4.9–8.2. *Microconidia* 0–1-septate, ellipsoid to ovoid to subcylindrical, more or less straight, mostly with a visible hilum; aseptate, (6.0)8.0–9.8(13.0) × (3.5)3.7–3.9(4.0) µm (av. = 8.9 × 3.8 µm), with a length:width ratio of 1.7–3.3; 1-septate, (8.0)11.3–13.7(16.0) ×

(3.5)3.8–4.2(4.5) µm (av. = 12.5 × 4.0 µm), with a length:width ratio 1.8–4.3. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. *Chlamydospores* globose to subglobose to ellipsoidal, 8–14 × 6–10 µm, smooth, but often appearing rough due to deposits, thick-walled, terminal on short lateral branches or intercalary, single, in chains or in clumps, hyaline, becoming medium brown.

Holotype: Canada, Alberta, *Panax quinquefolium*, 1998, coll./isol. K. F. Chang, holotype CBS H-20562, culture ex-type CBS 129079 = CDC-N-9A = CPC 19167.

Culture characteristics: Mycelium felty with strong density. Surface on OA chestnut to sienna, with aerial mycelium sparse, vinaceous-buff. Surface on PDA chestnut to cinnamon, with aerial mycelium sparse, buff to saffron. No zonation was observed, and transparency was homogeneous; margins predominantly even. Reverse similar to surface, except in the colour, fawn to cinnamon on OA, and chestnut on PDA. Colonies on PDA grow 5 mm diam at 4 ºC after 7 d. Optimum temperature at 20 ºC, with colonies reaching 40–42 mm diam, after 7 d. Colony diam was 15 mm at 25 ºC after 7 d. No growth observed at 30 ºC.

Isolate studied: CBS 129079 (Table 1).

Host and distribution: *Panax quinquefolium* (Canada).

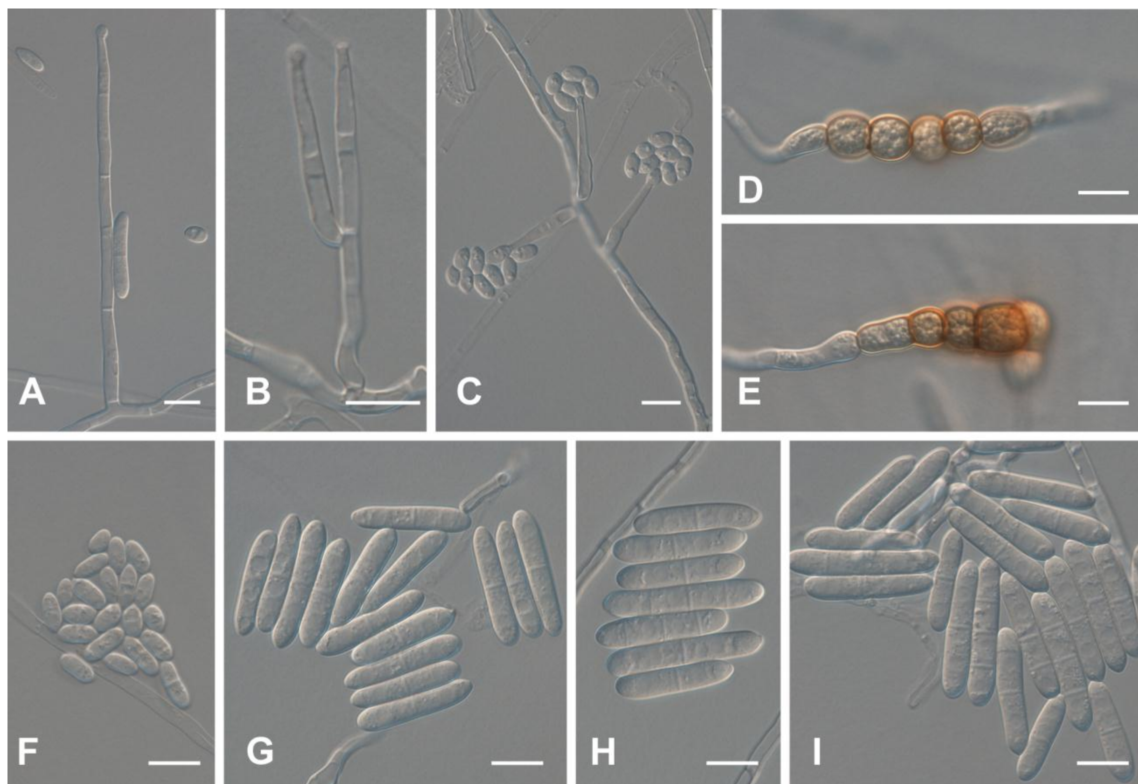


Fig. 10 *Ilyonectria panacis* (CBS 129079). (a–c) Simple, unbranched or sparsely branched conidiophores on aerial mycelium. (d, e) Chlamydospores on mycelium. (f–i) Micro- and macroconidia. Bars: 10 µm

Notes: Several species have in the past been described on *Panax* in the genera *Ramularia* and *Cylindrocarpon*. The only unresolved species is *"C." destructans* (and its potential synonym, "*Ramularia" panacicola,* see above). *"Cylindrocarpon" destructans* is clearly different from *I. panacis*, which has larger conidia, (27.0)31.2–33.1–35.0(49.0) × (5.0)5.4–5.6–5.8(6.0) µm.

Ilyonectria pseudodestructans A. Cabral, Rego & Crous, *sp. nov.* Fig. 11

MycoBank 560106.

Etymology: Named after its morphological similarity to the "*Cylindrocarpon" destructans*.

Ilyonectriae crassae morphologice similis, sed macroconidiis clavatis distinguitur.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to two phialides, 1–3-septate, 50–180 µm long; phialides monophialidic, cylindrical to subulate, 30–58 µm long, 2.5–3.5 µm wide at the base, 1.5–2.0 µm near the aperture. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched. *Macroconidia* predominating, formed by simple conidiophores, on SNA formed in flat domes of slimy masses, 1–3(–4)-septate, straight, typically clavate, mostly centrally located hilum; 1-septate, (19.0)25.8–27.5(35.0) × (4.0)5.0–5.3(6.5) µm (av. = 26.7 × 5.2 µm), with a length:width ratio of 3.8–6.6; 2-septate, (23.0)30.0–31.7(38.0) × (5.0)5.3–5.5(6.0) µm (av. = 30.9 × 5.4 µm), with a length:width ratio of 4.3–7.4; 3-septate, (28.0)34.2–36.2(48.0) × (5.0)5.9–6.2(7.0) µm (av. = 35.2 × 6.0 µm), with a length:width ratio of 4.6–7.4. *Microconidia* 0–1-septate, ellipsoid to ovoid to subcylindrical, more or less straight, with a visible, centrally located hilum; aseptate (6.0)10.5–11.8(15.0) × (3.0)3.6–3.8(4.5) µm (av. = 11.2 × 3.7 µm), with a length:width ratio of 1.5–4.3; 1-septate, (10.0)14.6–15.6(18.0) × (3.0)4.1–4.4(5.0) µm (av. = 15.1 × 4.2 µm), with a length:width ratio of 2.4–5.0. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. *Chlamydospores* globose to subglobose to ellipsoid, 9–18 × 8–14 µm, smooth but often appearing rough due to deposits, thick-walled, terminal on short lateral branches or intercalary, in chains or in clumps, and also in the cells of macroconidia, hyaline, becoming medium brown.

Holotype: Portugal, São Paio, Gouveia, *Vitis vinifera,* 4-year-old, showing decline symptoms, scion Malvasia fina; rootstock 1103P, 1996, coll./isol. C. Rego, holotype CBS H-20564, culture ex-type CBS 129081 = Cy20 = CPC 19164.

Culture characteristics: Mycelium felty, with average to strong density. Surface on OA cinnamon, with sparse, buff to saffron or chestnut to sienna aerial mycelium. Surface on PDA cinnamon to vinaceous, with sparse, saffron to buff or chestnut to sienna aerial mycelium. Zonation absent, with homogeneous transparency; margins even. Reverse similar to surface, except in colour, sepia to cinnamon on OA and chestnut to cinnamon on PDA. Colonies on PDA grow poorly (4–6 mm diam), at 4 ºC after 7 d. Optimum temperature between 20–22 ºC, when colonies reach 32–44 mm and 37–41 mm diam, respectively, after 7 d. Colony diam was 22–29 mm at 25 ºC after 7 d. No growth was observed at 30 ºC.

Isolates studied: CPC 13534; CBS 117812; CBS 117824; CBS 129081; Cy22 (Table 1).

Hosts and distribution: *Poa pratensis* (Canada), *Quercus* sp. (Austria), *Vitis vinifera* (Portugal).

Notes: *Ilyonectria pseudodestructans* is reminiscent of "*Cylindrocarpon" destructans,* in having a similar conidial morphology (3-septate, with central, truncate hilum). However, conidia of *I. pseudodestructans* are somewhat longer than those of *I. radicicola*.

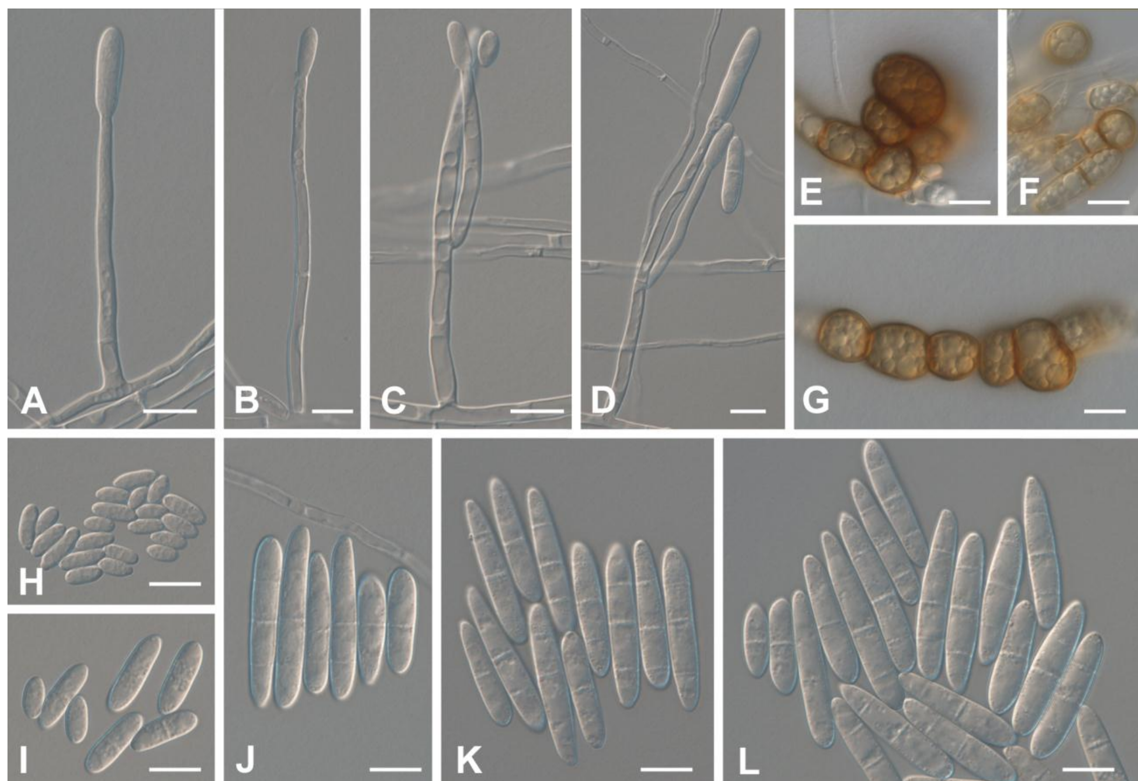


Fig. 11 *Ilyonectria pseudodestructans* (all from CBS 129081, except g and e from CBS 117824). (a–d) Simple, unbranched or sparsely branched conidiophores on aerial mycelium. (e–g) Chlamydospores on mycelium and macroconidia. (h–l) Micro- and macroconidia. Bars: 10 µm

Ilyonectria robusta (A.A. Hildebr.) A. Cabral & Crous, *comb. nov.* Fig. 12, 13

MycoBank 560113.

Basionym: *Ramularia robusta* A.A. Hildebr. Can. J. Res. 12: 102. 1935.

Perithecia formed heterothallically *in vitro*, disposed solitarily or in groups, developing directly on the agar surface or on sterile pieces of birch wood, ovoid to obpyriform, with a flattened apex, up to 70 µm wide, orange to red, becoming purple-red in 3 % KOH (positive colour reaction), smooth to warted, up to 250 µm diam and high; *perithecial wall* consisting of two regions; outer region 11–36 µm thick, composed of 1–3 layers of angular to subglobose cells, 10–30 × 6–24 µm; cell walls up to 1 µm thick; inner region 8–14 µm thick, composed of cells that are flat in transverse optical section and angular to oval in subsurface optical face view, 5–11 × 2.5–5 µm; *Asci* narrowly clavate to cylindrical, 40–50 × 4.5–6 µm, 8-spored; apex subtruncate, with a minutely visible ring. *Ascospores* medianly 1-septate, ellipsoid to oblong-ellipsoid, somewhat tapering towards both ends, smooth to finely warted, frequently guttulate, hyaline, (8.2)9.4–9.7–10.0(11.5) × (2.5)2.9–3.0–3.1(3.7) µm. *Conidiophores* simple or complex or sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to three phialides, 1–4-septate, 55–160 µm long; phialides monophialidic, cylindrical to subulate, 20–60 µm long, 2.0–3.0 µm wide at the base, 1.5–2.0 µm near the apex. *Complex conidiophores* aggregated in small sporodochia (on carnation leaf agar; Crous et al. 2009b), repeatedly and irregularly branched; phialides more or less cylindrical, but tapering slightly in the upper part towards the apex, or narrowly flask-shaped, mostly with widest point near the middle, 15–20 µm long, 2.5–3.5 µm wide at the base, 3.0–4.0 µm at the widest point, and 1.0–2.0 µm wide near the apex. *Macroconidia* predominating, formed on simple conidiophores, on SNA formed in flat domes of slimy masses, 1–3-septate, straight, minutely curved or sometimes distorted, cylindrical with both ends more or less broadly rounded, but may narrow towards the tip, mostly without a visible hilum; 1-septate, (15.0)22.8–24.6(35.0) × (4.5)6.3–6.7(8.0) µm (av. = 23.7 × 6.5 µm), with a length:width ratio of 2.7–5.2; 2-septate, (20.0)26.2–28.1(38.0) × (5.0)6.9–7.2(8.0) µm (av. = 27.2 × 7.0 µm), with a length:width ratio of 2.9–5.2; 3-septate, (24)32.3–34.7(58) × (6.0)7.2–7.5(9.0) µm (av. = 33.5 × 7.4 µm), with a length:width ratio of 3.1–7.3. *Microconidia* 0–1-septate, ellipsoid to ovoid to subcylindrical, more or less straight, without a visible hilum; aseptate, (4.0)8.0–9.3(14.0) × (2.5)3.6–4.0(5.5) µm (av. = 8.7 × 3.8 µm), with a length:width ratio of 1.3–4.0; 1-septate, (9.0)13.5–14.7(18.0) × (3.5)4.7–5.1(6.0) µm (av. = 14.1 × 4.9 µm), with a

length:width ratio 1.5–4.5. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. *Chlamydospores* globose to subglobose, 7–14 × 6–13 µm, smooth, but often appearing rough due to deposits, thick-walled, mostly occurring intercalary in chains, hyaline, becoming golden-brown.

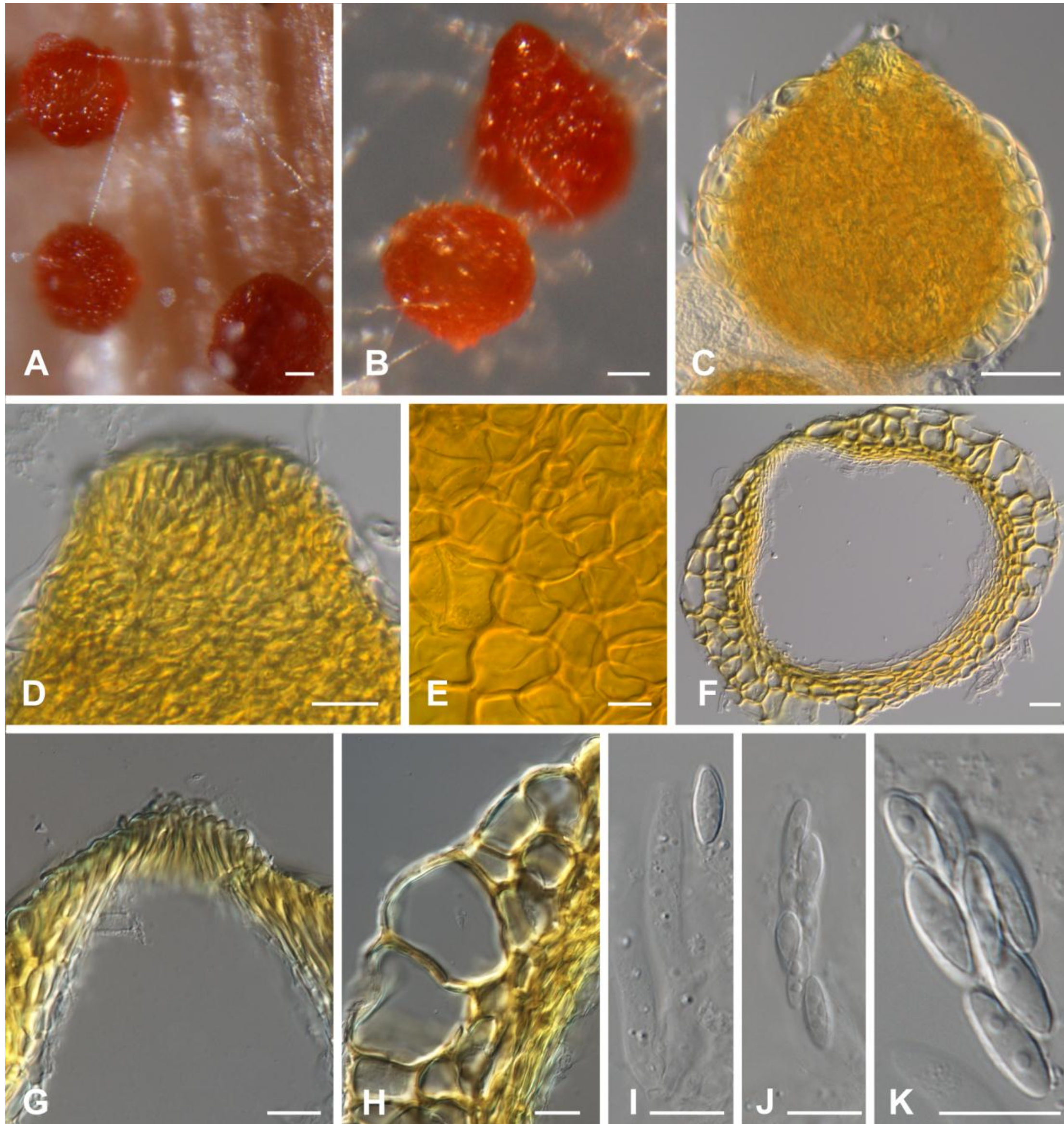


Fig. 12 *Ilyonectria robusta* (a, b from CPC 13532 × CBS 308.35; c–k from CPC 13532 × CBS 117813). (a, b) Development of perithecia on the surface of a birch toothpick or agar. (c–e) Perithecium mounted in lactic acid. (d) Ostiolar area. (e) Surface view of perithecium wall region. (f–h) Longitudinal sections of perithecia showing details of ostiole and wall. (i–k) Asci and ascospores. Bars: a–c = 50 µm; d, f = 20 µm; e, g–k = 10 µm

Lecto- and teleotype: Canada, Ontario, on living roots of *Panax quinquefolium,* 1935, A.A. Hildebrand, lectotype designated here CBS H-20565, as dried culture of CBS 308.35; teleotype

designated here CBS H-20566, including fertile perithecia of the teleomorph (CPC 13532 × CBS 308.35), culture ex-lectotype CBS 308.35.

Fertile matings: Perithecia observed after 4 wk in crossings of strains: CPC 13532 × CBS 308.35, CPC 13532 × CBS 773.83, CPC 13532 × CBS 605.92, CPC 13532 × CBS 117813, CBS 129084 × CBS 308.35, CBS 129084 × CBS 605.92, CBS 129084 × CBS 117813.

Culture characteristics: Mycelium felty with an average density. Surface on OA sienna to sepia with aerial mycelium sparse, buff. Surface on PDA cinnamon, with aerial mycelium buff to cinnamon, or rosy buff on PDA. Zonation absent to concentric, with homogeneous transparency; margins predominantly even, but sometimes uneven. Reverse similar to surface, except in the colour, sienna on OA and chestnut at the centre, and sienna to orange towards the margin on PDA. Colonies on PDA grow 4–7 mm at 4 ºC after 7 d. Optimum temperature at 22 ºC when colonies reach 40–52 mm diam, after 7 d. Colony diam was 35–48 mm at 25 ºC after 7 d. No growth to slight growth (0–2 mm) was observed at 30 ºC.

Isolates studied: CBS 321.34; CBS 308.35; CBS 773.83; CBS 605.92; CBS 117813; CBS 117814; CBS 117815; CBS 117817; CBS 117818; CBS 117819; CBS 117820; CBS 117821; CBS 117822; CBS 117823; CBS 129084; CD1666; CPC 13532; Cy23; Cy158; Cy231 (Table 1).

Hosts and distribution: *Loroglossum hircinum* (root) (Tunisia), *Panax quinquefolium* (root) (Canada), *Prunus cerasus, Thymus* sp., *Vitis vinifera* (basal end of rootstock) (Portugal), *Quercus robur* (root), *Quercus* sp. (root) (Austria), *Tilia petiolaris* (rootstock) (Germany), water (in aquarium with *Anodonta* sp.) (Netherlands).

Notes: When Hildebrand (1935) described *Ramularia robusta* from living roots of *Panax quinquefolium* in Ontario, Canada, he did not indicate a type specimen. However, he deposited an original culture in the CBS. A sporulating, dried-down culture is thus herewith designated as lectotype, and a new name proposed in *Ilyonectria*, with a teleotype represented by a fertile mating between CPC 13532 × CBS 308.35.

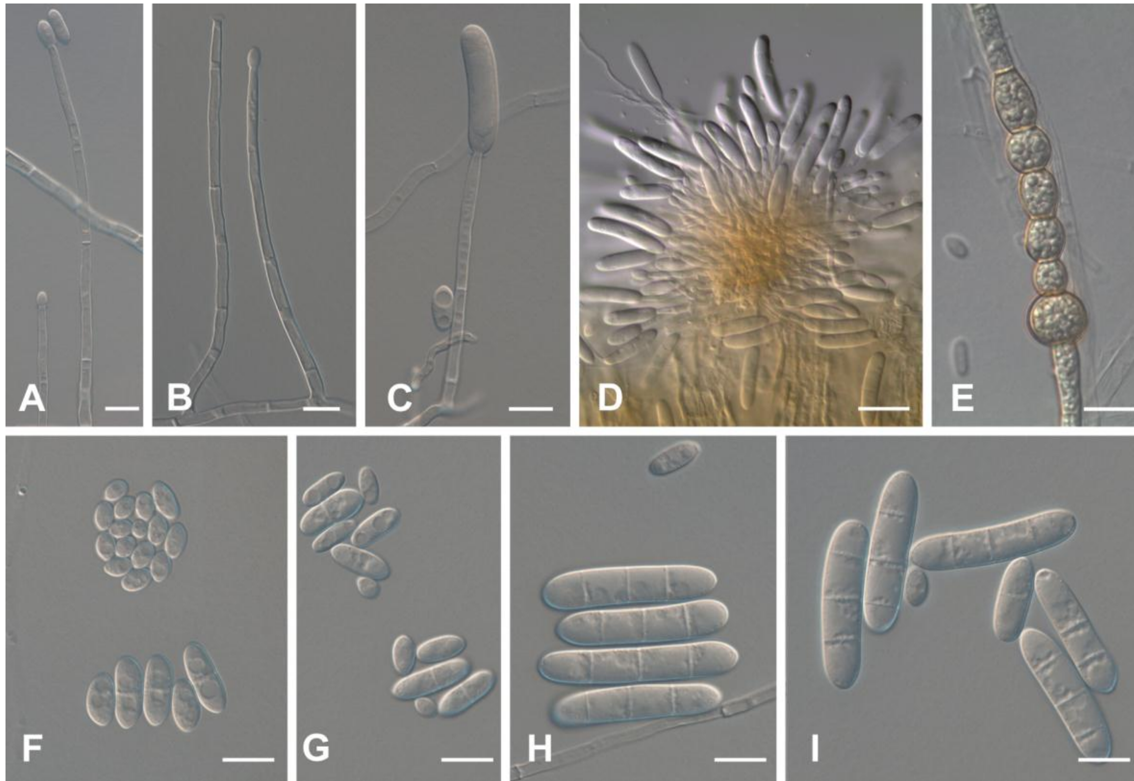


Fig. 13 *Ilyonectria robusta* (All from CBS 129084, except f from CBS 605.92). (a–c) Simple conidiophores on aerial mycelium. (d) Sporodochial conidiophore on carnation leaf agar. (e) Chlamydospores on mycelium (f–i) Micro- and macroconidia. Bars: 10 µm

Ilyonectria rufa A. Cabral & Crous, *sp. nov.* Fig. 14

MycoBank 560116.

Etymology: The epithet “*rufa*” referring to “*Coleomyces rufus*”, a provisional name proposed for this species by Moreau and Moreau (1937).

Ilyonectriae crassae morphologice similis, sed macroconidiis brevioribus, 28–31.2 µm longis, distinguitur.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to two phialides, 1–5-septate, 55–210 µm long; phialides monophialidic, cylindrical to subulate, 20–57 µm long, 2.5–3.5 µm wide at the base, 1.5–2.0 µm near the aperture. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched. *Macroconidia* predominating, formed on both types of conidiophores, on SNA formed in flat domes of slimy masses, 1(–3)-septate, straight, cylindrical with both ends broadly round,

mostly centrally located hilum; 1-septate, (17.0)22.3–23.8(29.0) × (4.0)5.1–5.4(6.0) µm (av. = 23.1 × 5.3 µm), with a length:width ratio of 3.1–5.6; 2-septate, (19.0)24.5–26.6(32.0) × (4.0)5.2–5.5(6.5) µm (av. = 25.5 × 5.4 µm), with a length:width ratio of 3.4–6.0; 3-septate, (23.0)28.6–31.2(37.0) × (5.0)5.5—5.9(7.0) µm (av. = 29.9 × 5.7 µm), with a length:width ratio of 3.4–7.2. *Microconidia* 0–1-septate, ellipsoid to subcylindrical, more or less straight, with a visible, centrally located hilum; aseptate, (4.0)8.4–9.8(15.0) × (3.0)3.5–3.8(5.0) µm (av. = 9.1 × 3.6 µm), with a length:width ratio of 1.3–4.0; 1-septate, (9.0)12.1–13.3(17.0) × (3.0)4.2–4.6(5.5) µm (av. = 12.7 × 4.4 µm), with a length:width ratio 2.2–3.8. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. *Chlamydospores* globose to subglobose to cylindrical, 7–12 × 6–9 µm, smooth, but often appearing rough due to deposits, thick-walled, terminal on short, lateral branches, or intercalary, single, in chains or in clumps, and also in the cells of the macroconidia, hyaline, becoming slightly brown in the outer wall.

Holotype: France, dune sand, Feb. 1937, coll./isol. F. Moreau, holotype CBS H-20567, culture ex-type CBS 153.37.

Culture characteristics: For CBS 153.37, CBS 156.47, CPC 13536 and 94-1628: Mycelium felty with average to strong density. Surface on OA buff to saffron, aerial mycelium sparse, buff. On PDA rosy-buff to cinnamon, with aerial mycelium sparse, buff to rosy-buff or pale luteus in the centre. For CBS 640.77, CBS 120371 and CBS 120372: Mycelium felty, with low to average density. Surface on OA cinnamon to sienna, aerial mycelium sparse, saffron to cinnamon. On PDA saffron to cinnamon, with aerial mycelium cinnamon to rust. Zonation absent or concentric, with homogeneous transparency; margins even or sometimes uneven. Reverse similar, except in colour, saffron on OA, and cinnamon to rosy-buff on PDA, or sienna with pigments, pale vinaceous in OA and umber to chestnut on PDA. Colonies on PDA grow poorly, (2–7 mm diam) at 4 ºC, after 7 d. Optimum temperature between 20–22 ºC, when colonies reach 28–42 mm, 31–46 mm diam, respectively, after 7 d. Colony diam was 19–24 mm at 25 ºC after 7 d. No growth observed at 30 ºC.

Isolates studied: CBS 153.37; CBS 156.47; CBS 640.77; CBS 120371; CBS 120372; CPC 13536; 94-1628 (Table 1).

Hosts and distribution: *Azalea indica* (Belgium), dune sand (France), *Picea glauca*, *Pseudotsuga menziesii* (Canada).

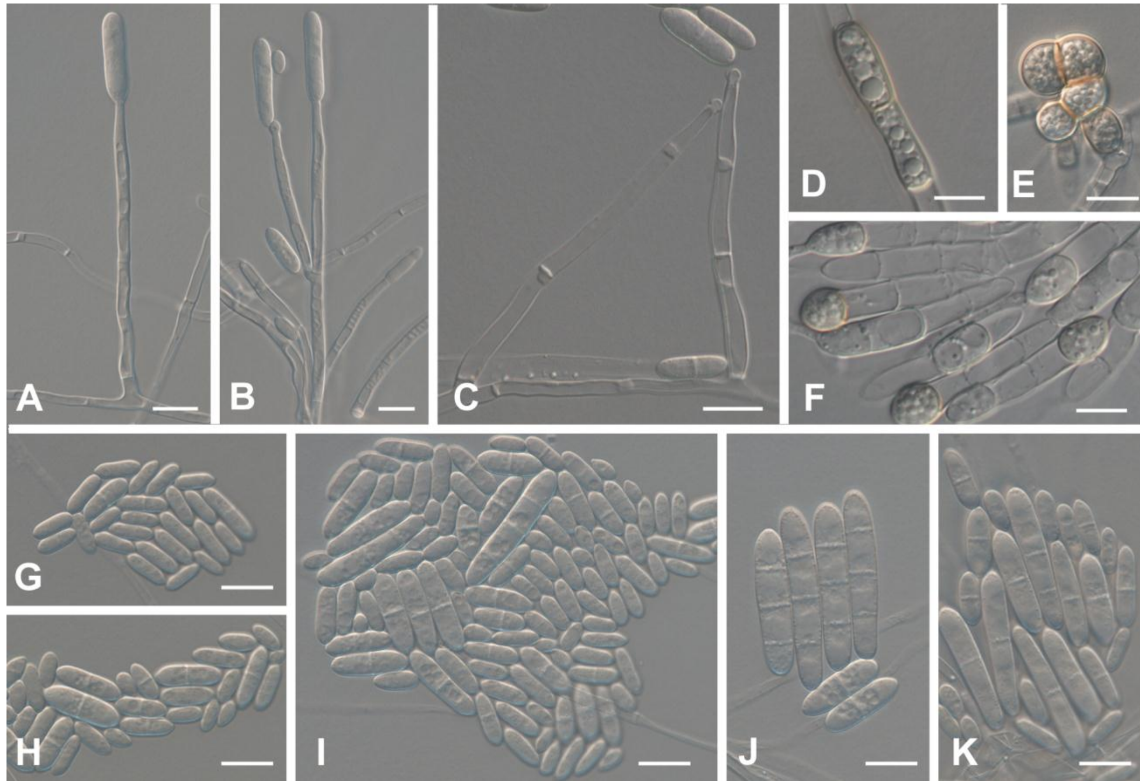


Fig. 14 *Ilyonectria rufa* (All from CBS 156.47, except c from CBS 120372). (a–c) Simple, sparsely branched conidiophores on aerial mycelium. (d–f) Chlamydospores in mycelium and in macroconidia. (g–k) Micro- and macroconidia. Bars: 10 µm

Notes: The genus *Coleomyces* represents a later synonym of *Cylindrocarpon* (Booth 1966). However, *Coleomyces*, which is based on *C. rufus* (Moreau and Moreau 1937), was published as "*ad interim*", suggesting that Moreau and Moreau were planning to validate the name later, which was not the case. Based on the International Code of Botanical Nomenclature (Art. 34.1, Ex. 6), Chaverri et al. (2011) correctly chose to ignore the name. However, an original strain of *C. rufus* was deposited in the CBS (CBS 153.37), and the species epithet is herewith validated for the species.

Ilyonectria venezuelensis A. Cabral & Crous, *sp. nov.* Fig. 15

MycoBank 560117.

Etymology: Named after the country from where it was collected, Venezuela.

Ilyonectriae robustae morphologice similis, sed conidiophoris cum verticillo terminali phialidum distinguitur.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium or from agar surface, solitary to loosely aggregated, unbranched, or bearing terminal, penicillate phialides, 1–4-septate, 35–200 µm long; phialides monophialidic, cylindrical to subulate, 40–60 µm long, 2.5–3.5 µm wide at the base, 1.5–2.5 µm near the apex, or narrowly flask-shaped, 16–40 µm long, 2.0–3.0 µm wide at the base, 3.0–4.0 µm at the widest point, 1.5–2.5 µm near the apex. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched. *Macroconidia* predominating, formed by both types of conidiophores, on SNA formed in flat domes of slimy masses, 1–3-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, but may narrow towards the tip, mostly without a visible hilum; 1-septate, (22.0)24.6–27.3(35.0) × (5.0)5.3–5.7(6.5) µm (av. = 26.0 × 5.5 µm), with a length:width ratio of 3.8–7.0; 2-septate, (25.0)26.3–37.4(44.0) × (5.9)6.0–6.6(7.0) µm (av. = 31.9 × 6.3 µm), with a length:width ratio of 4.2–6.8; 3-septate, (28.0)36.5–41.7(50.0) × (6.0)6.9–7.5(8.0) µm (av. = 39.1 × 7.2 µm), with a length:width ratio of 4.0–6.7. *Microconidia* 0–1-septate, ellipsoid to ovoid, more or less straight, without a visible hilum; aseptate, (5.0)8.4–10.5(13.0) × (3.0)3.3–3.7(4.0) µm (av. = 9.5 × 3.5 µm), with a length:width ratio of 1.7–3.4; 1-septate, (11.0)14.5–16.3(19.0) × (3.5)3.9–4.3(5.0) µm (av. = 15.4 × 4.1 µm), with a length:width ratio 2.8–4.8. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses. *Chlamydospores* ovoid to ellipsoidal, 6–13 × 5–7 µm, smooth, but often appearing rough due to deposits, thick-walled, terminal on short lateral branches or intercalary, single, in chains or in clumps, hyaline, becoming slightly brown at the margin.

Holotype: Venezuela, Amazonas, Cerro de la Neblina, tree bark, 1985, coll./isol. A. Rossman, holotype CBS H-20568, culture ex-type CBS 102032.

Fig. 15 *Ilyonectria venezuelensis* (CBS 102032). (a, b) Simple conidiophores on aerial mycelium. (c–e) Conidiophores bearing terminal, penicillate phialides. (f–j) Micro- and macroconidia. Bars: 10 µm

Culture characteristics: Mycelium cottony with average to strong density. Surface on OA saffron, with aerial mycelium sparse, buff, on PDA buff to saffron, with aerial mycelium saffron to pale luteous; zonation absent, transparency homogeneous, margin even; reverse similar to surface, but saffron to cinnamon on PDA. Colonies on PDA grow poorly (2–3 mm) at 4 ºC, after 7 d. Optimum temperature at 20 ºC, with colonies reaching 49 mm diam, after 7 d. Colony diam was 35–36 mm at 25 ºC after 7 d. No growth was observed at 30 ºC.

Isolate studied: CBS 102032 (Table 1).

Host and distribution: Tree bark (Venezuela).

Ilyonectria vitis A. Cabral, Rego & Crous, *sp. nov.* Fig. 16

MycoBank 560107.

Etymology: Named after the host from which it was collected, *Vitis vinifera*.

Ilyonectriae anthuriicolae morphologice similis, sed longitudine media macroconidiorum longiore, 41.6–43.5 µm, distinguitur.

Conidiophores simple or complex or sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to three phialides, 1–3 septate, 30–70 µm long; monophialides more or less cylindrical, but tapering slightly towards the tip, 11–21 µm long, 2.0–3.0 µm wide at the base, 3.0–4.5 µm at widest point, 1.5–2.5 µm near the apex. Conidiophores forming microconidia arising from mycelium at agar surface, reduced to monophialides, or a stipe with a terminal arrangement of phialides, ranging from 2 to a dense cluster; sparsely branched or penicillate; monophialides narrowly flask-shaped, typically with widest point near the middle, 10–17 µm long, 1.5–3.0 µm wide at the base, 2.5–4.0 µm at widest point, 1.0–2.0 µm near the apex. *Sporodochial conidiophores* irregularly branched; phialides more or less cylindrical but slightly tapering towards the tip, or narrowly flask-shaped, with widest point near the middle, 14–20 µm long, 2.5–3.5 µm wide at the base, 3.0–4.5 µm at widest point, 1.5–2.5 µm near the apex. *Macroconidia* formed in flat domes of slimy masses, predominantly 3-septate, rarely 1–2- or 4-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, mostly without a visible hilum; 3-septate conidia (34.9)41.6–43.5(51.6) × (6.2)7.9–8.2(9.5) µm (av. =42.5 × 8.0 µm), with a length:width ratio of 3.9–6.7. *Microconidia* on SNA formed in heads, aseptate, subglobose to ovoid, rarely ellipsoid, mostly with a visible, centrally located or slightly laterally displaced hilum, (3.7)4.9–5.4(6.7) × (3.2)3.7–4.0(4.6) µm (av. = 5.1 × 3.9 µm), with a length:width ratio of 1.1–1.7. *Chlamydospores* globose to subglobose to ellipsoid, 9–18 × 6–13 µm, smooth, but often appearing rough due to deposits, thick-walled, formed intercalary in chains or in clumps, and also in the cells of macroconidia, hyaline, becoming golden-brown.

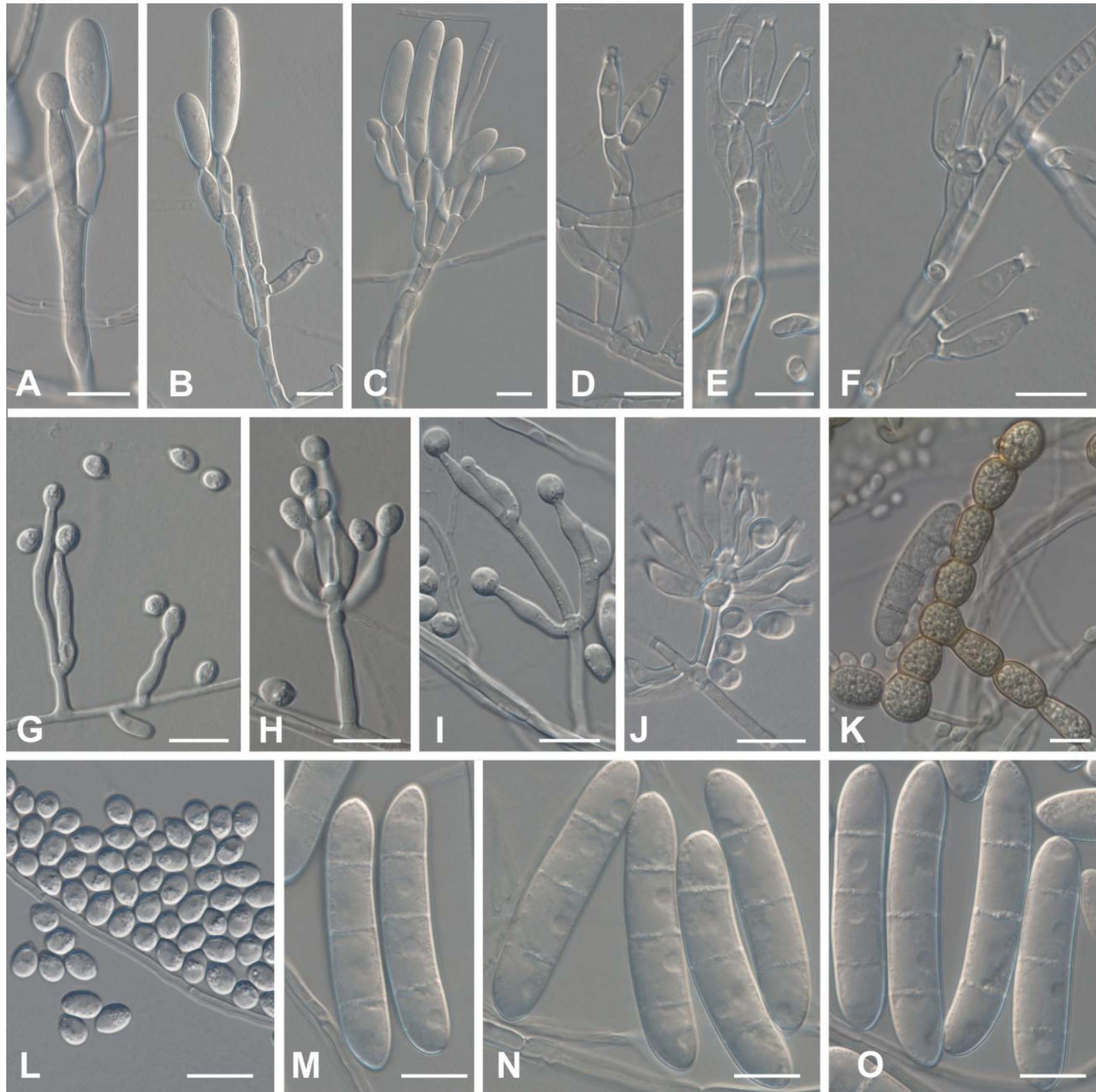


Fig. 16 *Ilyonectria vitis* (CBS 129082). (a–c) Simple conidiophores on aerial mycelium. (d–g) Conidiophores forming microconidia arising from mycelium at agar surface, reduced to a stipe with a terminal arrangement of phialides, ranging from 2 to a dense cluster; sparsely branched or penicillate. i–l) Micro- and macroconidia. (m) Chlamydospores on mycelium. Bars: 10 µm

Holotype: Portugal, Vidigueira, *Vitis vinifera,* basal end of a 2-year-old plant; scion Touriga Nacional; rootstock 110R, 2008, coll./isol. C. Rego, holotype CBS H-20569, culture ex-type CBS 129082 = Cy233 = CPC 19168.

Culture characteristics: Mycelium felty with density low to average. Surface on OA sienna, with sparse, saffron aerial mycelium, and luteous growth at margin. Surface on PDA chestnut, with sienna aerial mycelium, with luteous margin. Zonation was absent (OA) or concentric (PDA), transparency was homogeneous (PDA) or not (OA). Growth at margin even to uneven. Reverse similar to surface, except in colour, sienna to saffron on OA, and chestnut to umber on PDA.

Colonies on PDA do not grow at 4 ºC after 7 d. Optimum temperature at 20 ºC, when colonies reach 29–30 mm diam, after 7 d. Colony diam was 39–40 mm at 25 ºC and 8–9 mm at 30 ºC after 7d. No growth was observed at 35 ºC.

Isolate studied: CBS 129082 (Table 1).

Host and distribution: *Vitis vinifera* (Portugal).

Key to species treated

1 Growth at margin on OA after 14 d at 20 ºC, lacking yellow pigmentation

2 Colony diameter on PDA after 7 d at 25 ºC < 30 mm

3 Macroconidia forming chlamydospores

4 Macroconidia 1–3-septate, 3-septate macroconidia mean range 34.1–36.2 µm long

5 Macroconidia cylindrical, with the base appearing somewhat acute *I. crassa*

5* Macroconidia clavate *I. pseudodestructans*

4* Macroconidia predominantly 1-septate; 3-septate macroconidia smaller, mean range 28–31.2 µm long *I. rufa*

3* Macroconidia lacking chlamydospores

6 Macroconidia predominantly curved *I. liliigena*

6* Macroconidia straight

7 Macroconidia lacking visible hilum *I. mors-panacis*

7* Macroconidia with a visible, centrally located hilum

8 Three-septate macroconidia mean range 31.0–35.0 µm long *I. panacis*

8* Three-septate macroconidia mean range 34.3–38.5 µm long *I. gamsii*

2* Colony diameter after 7 d at 25 ºC > 30 mm

9 Colony diameter after 7 d at 25 ºC, >50 mm *I. cyclaminicola*

9* Colony diameter after 7 d at 25 ºC, 30–50 mm

10 Conidiophores bearing a terminal whorl of phialides *I. venezuelensis*

10* Conidiophores unbranched, or different from above

11 Teleomorph known, and can be induced in culture

12 Three-septate macroconidia mean range 32.3–34.7 µm long; ascospores mean range 9.4–10.0 µm long *I. robusta*

12* Three-septate macroconidia mean range 30.0–36.0 µm long; ascospores mean range longer, 10–13 µm long *I. radicicola*[1]

11* Teleomorph unknown

13 Mean range of 3-septate macroconidia, 29.7–31.5 × 6.5–6.9 µm *I. europaea*

13* Mean range of 3-septate macroconidia smaller, 25.0–28.4 × 5.2–5.5 µm *I. lusitanica*

1* Yellow pigmentation present at margin

14 Macroconidia 3-septate, mean range 29.5–32.2 µm long *I. anthuriicola*

14* Macroconidia 3-septate, mean range 41.6–43.5 µm long *I. vitis*

[1]No authentic cultures of *"C." destructans*, conidia (18.0–)23.0–30.0(–35.0) × (6.0–)6.5(–7.0) µm are presently available.

Discussion

"Cylindrocarpon" destructans is a cosmopolitan soil-borne pathogen causing disease on a wide number of herbaceous and woody plant species (Samuels and Brayford 1990). The wide morphological and pathogenic amplitude of *"C." destructans* makes it a commonly identified species, with many diseases from the *Cylindrocarpon*-complex being attributed to it, and ranking at the top of all "*Cylindrocarpon"* spp. deposited in the NCBI nucleotide database.

In this study, *"C." destructans* isolates from the CBS culture collection (deposited under the wider concept of the species) were analysed using a multigene approach in order to clarify taxonomic aspects of this species complex. Molecular analyses show that these isolates cluster in various clades supported by high bootstrap support values. A previous study (Seifert et al. 2003) included a subset of the strains used here, and already highlighted the existence of unexpected divergence in "*C.*" *destructans*, as opposed to a large homogeneity in e.g. *Neonectria ditissima*. Several species have in recent years been separated from the "*C.*"

destructans complex, including "*C.*" *macroconidialis*, "*C.*" *coprosmae* and "*C.*" *austroradicicola* based on morphological (Samuels and Brayford 1990) and molecular characters (Schroers et al. 2008; Seifert et al. 2003). Furthermore, several isolates causing black foot disease of grapevine, previously considered as "*C.*" *destructans*, were recently identified as *I. liriodendri* (Chaverri et al. 2011; Halleen et al. 2006b), along with the ex-type strain from *Liriodendron tulipifera* (CBS 110.81) and a strain from *Cyclamen* (CBS 301.93). In this study, two further strains isolated from young *Malus domestica* and *Quercus suber* trees showing decline symptoms, were also identified as *I. liriodendri*.

Altogether we analysed 68 strains putatively belonging to "*C.*" *destructans*, but none of them clustered together with the ex-type culture of *I. radicicola* (CBS 264.65), suggesting that this species may not be as common as previously accepted. Halleen et al. (2006b) identified a single strain (IMI 313237, isolated from arecoid palm) clustering with CBS 264.65. This also raises questions relating to the correlation between the anamorph, "*C.*" *destructans,* and its purported teleomorph, *I. radicicola*.

"Nectria" radicicola was described by Gerlach and Nilson (1963) from decayed leaves, flowers stalks and corms of *Cyclamen persicum* collected in Sweden, with a "*Cylindrocarpon"* anamorph they identified as *"C." radicicola*.

In 1924, Wollenweber introduced "*C.*" *radicicola* (McAlpine) Wollenw. as a new combination, based on *Septocylindrium radicicola* McAlpine (1899), described from *Citrus* trees in Australia. Later, Wollenweber (1928) noted that *Septocylindrium radicicola*, with catenulate conidia, was different from "*C.*" *radicicola*, and the name was therefore based on Wollenweber's (1928) description. Because of this confusion in names, Booth (1966) suggested that "*C.*" *radicicola* should be dropped, and that the name to be used as anamorph for *I. radicicola* should be "*C.*" *destructans* [originally described by Zinssmeister (1918) on *Panax quinquefolia* from Wisconsin, USA]. Furthermore, Booth (1966) designated a neotype for "*C.*" *destructans*, obtained from *P. quinquefolia* in USA, KY, Washington Co., Springfield; collected by W.B. Edwards in 1922, available as herbarium material in Cornell Plant Pathology Herbarium, CUP-011985. This specimen was re-examined in the present study [conidia (18.0)23.0–30.0(35.0) × (6.0)6.5(7.0) µm] [original description by Zinssmeister (1918), 9.0–32.4 × 3.2–8.1 µm], thus revealing conidia to be smaller than those formed by *I. radicicola,* which are (24.0)33.1(47.0) × (4.9)6.4(7.8) µm. From these observations we conclude that "*C.*" *destructans*, which occurs on *P. quinquefolia* in the USA, represents yet another species distinct from *I. radicicola*, which is not yet represented in our phylogenetic tree (Fig. 1).

A strain deposited in CBS culture collection by Hildebrand in 1935, as "*Ramularia" mors-panacis,* was found to represent original material (ex-type CBS 306.35), collected from living roots of *Panax quinquefolium* in Ontario, Canada. The epithet "*mors-panacis"* is therefore resurrected for this clade, while the Japanese collection identified as "*Cylindrocarpon destructans"* f. sp. *panacis* (ex-type CBS 124662 = NBRC 31881), isolated from *Panax ginseng* in Japan, is treated as synonym.

The ex-type strain of "*Ramularia" robusta* (CBS 308.35), isolated from living roots of *Panax quinquefolium* in Ontario, Canada, can be resurrected for a large clade representing isolates from a range of hosts and continents. Similarly, an authentic strain of "*C.*" *destructans* var. *crassum* (Booth 1966; Wollenweber 1931) is available for a species occurring on *Panax* and *Pseudotsuga* in Canada, *Lilium* and *Narcissus* in the Netherlands, and can thus be resurrected as *I. crassa*. Although strain CBS 120370 clustered together with other strains of *I. crassa* for most genes (no nucleotide differences in ITS and TEF, two nucleotide differences in HIS), this strain was not included in that species because of an 8-bp difference in TUB, a slower growth rate (e.g., 21 mm colony diam at 20 ºC for 7 d, as opposed to 31–46 mm for other isolates), a lower conidial length:width ratio [e.g. for 3-septate conidia 4.0–6.5, as opposed to 4.8–8.9 and smaller conidia (e.g. 3-septate conidia ranging from (26.0)31.2–34.0(40.0) × (6.0)6.6–7.1(8.0) µm (av. 32.6 × 6.9 µm) , as opposed to (29.0)34.1–36(49.0) × (5.0)5.6–5.8(7.0) µm (av. 35.1 × 5.7 µm)]. Further studies should thus be conducted in order to clarify the taxonomy of this strain.

Ilyonectria anthuriicola and *I. vitis* are very similar in morphology to "*C.*" *pauciseptatum*. These species all have predominantly 3-septate macroconidia after 10 d in culture. *Ilyonectria anthuriicola* is easier to distinguish than "*C.*" *pauciseptatum* as the 3-septate conidia are smaller and narrower, ranging from (25.0)29.5–32.2(38.0) × (6.0)7.5–8.1(9.0) µm, while in "*C.*" *pauciseptatum* they range from (37.0)42.0–47.0(54.0) × (7.0)8.5–9.5(10.0) (Schroers et al. 2008). For *I. vitis* 3-septate macroconidia are of similar size to those of "*C.*" *pauciseptatum*, (34.9)41.6–43.5(51.6) × (6.2)7.9–8.2(9.5), making it difficult to distinguish them based on this character. Growth rate at 20 ºC is slower in *I. vitis* than in "*C.*" *pauciseptatum*, and they also differ regarding colony characteristics and colours. Morphologically, however, they remain difficult to distinguish.

In this study, the genetic structure of the *I. radicicola* complex was analysed using a multi-locus approach along with morphological and culture characters. Three major groups were identified based on this approach, each group containing several species. Although we have been able to clarify several aspects related to the host range and distribution of taxa in the *I. radicicola*

species complex, further collections, especially from *Panax* in the USA, will be essential to elucidate the status of *"C." destructans*.

Acknowledgements The first author would like to thank Dr M.C. Silva (Coffee Rust Research Centre / Tropical Research Institute, Portugal) for making equipment available, and for assisting with the use of the cryostat for perithecial sectioning. Drs F. Caetano (Instituto Superior de Agronomia, Portugal), J. Armengol (Univ. Politécnica de Valencia, Spain), K.A. Seifert (Agriculture and Agri-Food, Canada), M.L Inácio (Instituto Nacional de Recursos Biológicos/INIA, Portugal) P. Lecomte (Institut National de la Recherche Agronomique, Bordeaux-Aquitaine, France) and W.D. Gubler (Univ. California, Davis, USA) are thanked for making several strains available, without which this study would not have been possible. The curator of CUP is acknowledged for making the neotype *"C." destructans* available for examination. This article is part of a Ph.D. dissertation (Instituto Superior de Agronomia, Technical University of Lisbon, Portugal). This work was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/24790/2005; project PTDC/AGR-AAM/099324/2008).

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Table 1. Details pertaining to isolates investigated during this study

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
Campylocarpon fasciculare, Holotype	CBS 112613; STE-U 3970; C 76	F. Halleen, 2000	*Vitis vinifera*, trunk of young grapevine showing decline symptoms; scion Cabernet Sauvignon; rootstock Richter 99	South Africa, Western Cape, Riebeeck Kasteel	AY677301	AY677221	JF735502	JF735691
Campylocarpon pseudofasciculare, Holotype	CBS 112679; STE-U 5472; HJS-1227	F. Halleen, 2000	*Vitis vinifera*, roots, asymptomatic nursery grapevine plant; scion Sultana; rootstock Ramsey	South Africa, Western Cape, Wellington	AY677306	AY677214	JF735503	JF735692
Neonectria macroconidialis	CBS 119596; ICMP 9349; IMI 332705; GJS 85-59	G.J. Samuels, 1985	*Astelia* sp.	New Zealand, Gisborne, Urewera National Park	JF735259	JF735372	JF735504	JF735693
Ilyonectria coprosmae	CBS 119606; GJS 85-39	G.J. Samuels, 1985	*Metrosideros* sp.	Canada, Ontario	JF735260	JF735373	JF735505	JF735694
Ilyonectria radicicola, type strain	CBS 264.65	L. Nilsson, 1961	*Cyclamen persicum*	Sweden, Skåne, Bjärred	AY677273	AY677256	JF735506	JF735695
Ilyonectria liriodendri, type strain of *"C." liriodendri*	CBS 110.81; IMI 303645	J.D. MacDonald & E.E. Butler, 1978	*Liriodendron tulipifera*, root	USA, California, Yolo Co., Davis	DQ178163	DQ178170	JF735507	JF735696
Ilyonectria liriodendri	CBS 117526; Cy68	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 99 R, clone 179F	Portugal, Ribatejo e Oeste	DQ178164	DQ178171	JF735508	JF735697
Ilyonectria liriodendri	CBS 117527; Cy76	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 110 R, clone 164E	Portugal, Ribatejo e Oeste	DQ178165	DQ178172	JF735509	JF735698
Ilyonectria liriodendri	CBS 117640; IMI 357400; Cy1	C. Rego, 1992	*Vitis vinifera*, 4-year-old plant showing decline symptoms; scion Seara Nova; rootstock 99R	Portugal, Torres Vedras, Dois Portos	DQ178166	DQ178173	JF735510	JF735699
Ilyonectria liriodendri	CBS 112596; STE-U 3994; C 14	F. Halleen, 1999	*Vitis vinifera*, roots	South Africa, Western Cape, De Wet	AY677264	AY677239	JF735511	JF735700
Ilyonectria liriodendri	CBS 112607; STE-U 3986; C 81	F. Halleen, 2000	*Vitis vinifera*, basal end of trunk	South Africa, Western Cape, Robertson	AY677269	AY677241	JF735512	JF735701

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
Ilyonectria liriodendri	Cy164	C. Rego, 1997	*Malus domestica*; cultivar Lysgolden; rootstock MM106	Portugal, Porto de Mós, Valbom	AM419079	AM419112	JF735513	JF735702
Ilyonectria liriodendri	Cy122	W.D. Gubler	*Vitis vinifera*	USA, California	JF735261	JF735374	JF735514	JF735703
Ilyonectria liriodendri	Cy190	N. Cruz, 2005	*Vitis vinifera*, basal end of 6-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Monção, Cortes	JF735262	JF735375	JF735515	JF735704
Ilyonectria liriodendri	Cy232	L. Inácio & J. Henriques, 2007	*Quercus suber*, stem	Portugal, Macedo de Cavaleiros	JF735263	JF735376	JF735516	JF735705
Ilyonectria robusta	CBS 321.34	-	*Loroglossum hircinum*, root	Tunisia, Tunis	AY677275	AY677253	JF735517	JF735706
Ilyonectria robusta, type strain of *Ramularia robusta*	CBS 308.35	A.A. Hildebrand	Panax quinquefolium	Canada, Ontario	JF735264	JF735377	JF735518	JF735707
Ilyonectria robusta	CBS 773.83	J. Hemelraad	water, in aquarium with *Anodonta*	Netherlands, Utrecht	AY677276	AY677254	JF735519	JF735708
Ilyonectria robusta	CBS 605.92	R. Schröer, 1992	*Tilia petiolaris*, root	Germany, Hamburg	EF607078	EF607065	JF735520	JF735709
Ilyonectria robusta	CBS 117813; IFFF 84	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden	-	JF735378	-	-
Ilyonectria robusta	CBS 117814; IFFF 85	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf	JF735265	JF735379	JF735521	JF735710
Ilyonectria robusta	CBS 117815; IFFF 86	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf	JF735266	JF735380	JF735522	JF735711
Ilyonectria robusta	CBS 117817; IFFF 88	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf	-	JF735381	-	-
Ilyonectria robusta	CBS 117818; IFFF 89	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf	JF735267	JF735382	JF735523	JF735712
Ilyonectria robusta	CBS 117819; IFFF 90	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden	-	JF735383	-	-
Ilyonectria robusta	CBS 117820; IFFF 91	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden	JF735268	JF735384	JF735524	JF735713

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
Ilyonectria robusta	CBS 117821; IFFF 93	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden	JF735269	JF735385	JF735525	JF735714
Ilyonectria robusta	CBS 117822; IFFF 94	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden	JF735270	JF735386	JF735526	JF735715
Ilyonectria robusta	CBS 117823; IFFF 95	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden	JF735271	JF735387	JF735527	JF735716
Ilyonectria robusta	CD1666	R. D. Reeleder, 1998	*Panax quinquefolium*	Canada, Nova Scotia	AY295331	JF735388	JF735528	JF735717
Ilyonectria robusta	CPC 13532; DAOM 139398; K 18-3A	-	*Prunus cerasus* cultivar Montmorency	Canada, Ontario	AY295330	JF735389	JF735529	JF735718
Ilyonectria robusta	Cy23	C. Rego, 1997	*Vitis* sp. rootstock 99R clone 179F in nursery	Portugal, Ribatejo e Oeste	AJ875333	AM419093	JF735530	JF735719
Ilyonectria robusta	Cy158	C. Rego & T. Nascimento, 2004	*Vitis vinifera*, 1-year-old, died before sprouting; scion Alicante Bouschet; rootstock 1103P	Portugal, Lamego, Cambres	JF735272	JF735390	JF735531	JF735720
Ilyonectria robusta	CBS 129084; Cy192	N. Cruz, 2005	*Vitis vinifera*, basal end of 25-year-old plant; scion Alicante; rootstock 196-17	Portugal, Monção	JF735273	JF735391	JF735532	JF735721
Ilyonectria robusta	Cy231	F. Caetano, 2005	*Thymus* sp.	Portugal, Lisbon	JF735274	JF735392	JF735533	JF735722
Ilyonectria crassa	CBS 139.30	W.F. van Hell, 1930	*Lilium* sp., bulb	Netherlands	JF735275	JF735393	JF735534	JF735723
Ilyonectria crassa	CBS 158.31; IMI 061536; NRRL 6149	1930	*Narcissus* sp., root	Netherlands	JF735276	JF735394	JF735535	JF735724
Ilyonectria crassa	CBS 129083; NSAC-SH-1	S. Hong, 1998	*Panax quinquefolium*	Canada, Nova Scotia	AY295311	JF735395	JF735536	JF735725
Ilyonectria crassa	NSAC-SH-2	S. Hong, 1998	*Panax quinquefolium*	Canada, Nova Scotia	AY295313	JF735396	JF735537	JF735726
Ilyonectria crassa	NSAC-SH-2.5	S. Hong, 1998	*Panax quinquefolium*	Canada, Nova Scotia	AY295314	JF735397	JF735538	JF735727
“Cylindrocarpon” sp.	CBS 120370; CR 20	P. Axelrood, 1998	*Pseudotsuga menziesii*	Canada, British Columbia	AY295317	JF735398	JF735539	JF735728
Ilyonectria rufa Authentic strain of *Coleomyces rufus*	CBS 153.37	F. Moreau, 1937	dune sand	France	AY677271	AY677251	JF735540	JF735729

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					ITS	TUB	H3	EF1
Ilyonectria rufa	CBS 156.47; IAM 14673; JCM 23100	-	*Azalea indica*	Belgium, Amandsberg	AY677272	AY677252	JF735541	JF735730
Ilyonectria rufa	CBS 640.77	F. Gourbière, 1977	*Abies alba*	France, Villeurbanne	JF735277	JF735399	JF735542	JF735731
Ilyonectria rufa	CBS 120371; CR 26	P. Axelrood, 1998	*Pseudotsuga menziesii*	Canada, British Columbia	AY295318	JF735400	JF735543	JF735732
Ilyonectria rufa	CBS 120372; CR 29	P. Axelrood, 1998	*Pseudotsuga menziesii*	Canada, British Columbia	JF735278	JF735401	JF735544	JF735733
Ilyonectria rufa	CPC 13536; DAOM 226721; CR36	P. Axelrood, 1998	*Pseudotsuga menziesii*	Canada, British Columbia	JF735279	JF735402	JF735545	JF735734
Ilyonectria rufa	94-1628	R.C. Hamelin, 1994	*Picea glauca*	Canada, Quebec	AY295315	JF735403	JF735546	JF735735
Ilyonectria mors-panacis	CBS 120359; CD1561	R. D. Reeleder, 1996	*Panax quinquefolium*	Canada, Ontario	AY295309	JF735404	JF735547	JF735736
Ilyonectria mors-panacis	CBS 120360; CD1567	R. D. Reeleder, 1996	*Panax quinquefolium*	Canada, Ontario	-	AY297200	-	-
Ilyonectria mors-panacis	CBS 120361; CD1596	R. D. Reeleder, 1996	*Panax quinquefolium*	Canada, Ontario	JF735280	JF735405	JF735548	JF735737
Ilyonectria mors-panacis	CBS 120362; CD1598	R. D. Reeleder, 1996	*Panax quinquefolium*	Canada, Ontario	-	AY297202	-	-
Ilyonectria mors-panacis	CBS 120363; CD1635	R. D. Reeleder, 1997	*Panax quinquefolium*	Canada, Ontario	-	AY297204	-	-
Ilyonectria mors-panacis	CBS 120364; CD1636	R. D. Reeleder, 1997	*Panax quinquefolium*	Canada, Ontario	JF735281	JF735406	JF735549	JF735738
Ilyonectria mors-panacis	CBS 120365; CD1637	R. D. Reeleder, 1997	*Panax quinquefolium*	Canada, Ontario	JF735282	JF735407	JF735550	JF735739
Ilyonectria mors-panacis	CBS 120366; CD 1639	R. D. Reeleder, 1997	*Panax quinquefolium*	Canada, Ontario	JF735283	JF735408	JF735551	JF735740
Ilyonectria mors-panacis	CBS 120367; CD1640	R. D. Reeleder, 1997	*Panax quinquefolium*	Canada, Ontario	AY295321	JF735409	JF735552	JF735741
Ilyonectria mors-panacis	CBS 120368; CD1641	R. D. Reeleder, 1997	*Panax quinquefolium*	Canada, Ontario	JF735284	JF735410	JF735553	JF735742
Ilyonectria mors-panacis	CBS 120369; CD1642	R. D. Reeleder, 1997	*Panax quinquefolium*	Canada, Ontario	JF735285	JF735411	JF735554	JF735743
Ilyonectria mors-panacis	CPC 13535; DAOM 221059; CD 0265	R. D. Reeleder, 1989	*Panax quinquefolium*	Canada, Ontario	JF735286	JF735412	JF735555	JF735744
Ilyonectria mors-panacis	CPC 13537; DAOM 226727; CD 1570	R. D. Reeleder, 1996	*Panax quinquefolium*	Canada, Ontario	JF735287	JF735413	JF735556	JF735745

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Ilyonectria mors-panacis, type of *Ramularia mors-panacis*	CBS 306.35	A.A. Hildebrand	*Panax quinquefolium*	Canada, Ontario	JF735288	JF735414	JF735557	JF735746
Ilyonectria mors-panacis	CBS 307.35	A.A. Hildebrand	*Panax quinquefolium*	Canada, Ontario	JF735289	JF735415	JF735558	JF735747
Ilyonectria mors-panacis, type of *"C." destructans* f.sp. *panacis*	CBS 124662; NBRC 31881; SUF 811	Y. Miyazawa	*Panax ginseng*	Japan, Nagano, Kitasaku-gun	JF735290	JF735416	JF735559	JF735748
Ilyonectria pseudodestructans	CPC 13534; DAOM 150670; Berkenkamp 1	B. Berkenkamp, 1974	*Poa pratensis*	Canada, Alberta, Lacombe	AY295319	JF735417	JF735560	JF735749
Ilyonectria pseudodestructans	CBS 117812; IFFF 83	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf	JF735291	JF735418	JF735561	JF735750
Ilyonectria pseudodestructans	CBS 117824; IFFF 98	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf	JF735292	JF735419	JF735562	JF735751
Ilyonectria pseudodestructans	CBS 129081; Cy20	C. Rego, 1996	*Vitis vinifera*, 4-year-old, showing decline symtoms, scion Malvasia Fina; rootstock 1103P	Portugal, Gouveia, São Paio	AJ875330	AM419091	JF735563	JF735752
Ilyonectria pseudodestructans	Cy22	C. Rego, 1996	*Vitis vinifera*, 5-year-old, showing decline symtoms, scion Aragonez; rootstock 99R	Portugal, Viseu, Silgueiros	AJ875331	AM419092	JF735564	JF735753
Ilyonectria europaea	Cy131	P. Lecomte & S. Chamont, 2000	*Actinidia chinensis* 'Hayward', internal lesion of stem	France, St. Chicq-du-Gaue	AM419067	AM419103	JF735565	JF735754
Ilyonectria europaea	Cy155	C. Rego & H. Oliveira, 2004	*Vitis vinifera*, 2-year-old, showing decline symtoms, scion Alfrocheiro; rootstock SO4	Portugal, Alter do Chão	JF735293	JF735420	JF735566	JF735755
Ilyonectria europaea	CBS 129078; Cy241	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira	JF735294	JF735421	JF735567	JF735756
Ilyonectria europaea	CBS 537.92	V. Demoulin, 1992	*Aesculus hippocastanum*, wood	Belgium, Liège	EF607079	EF607064	JF735568	JF735757
Ilyonectria europaea	CBS 102892; No.5/97-12	W. Leibinger, 1997	*Phragmites australis*, stem	Germany, Lake Constance	JF735295	JF735422	JF735569	JF735758

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					ITS	TUB	H3	EF1
Ilyonectria lusitanica	CBS 129080; Cy197	N. Cruz, 2005	*Vitis vinifera*, below grafting zone, 6-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço, Alvaredo	JF735296	JF735423	JF735570	JF735759
Ilyonectria venezuelensis	CBS 102032; ATCC 208837; AR2553	A. Rossman, 1985	bark	Venezuela, Amazonas, Cerro de la Neblina	AM419059	AY677255	JF735571	JF735760
Ilyonectria panacis	CBS 129079; CDC-N-9a	K. F. Chang, 1998	*Panax quinquefolium*	Canada, Alberta	AY295316	JF735424	JF735572	JF735761
Ilyonectria liliigena	CBS 189.49; IMI 113882	M.A.A. Schipper	*Lilium regale*, bulb	Netherlands, Hoorn	JF735297	JF735425	JF735573	JF735762
Ilyonectria liliigena	CBS 732.74	G.J. Bollen, 1973	*Lilium* sp.	Netherlands, Heemskerk	JF735298	JF735426	JF735574	JF735763
Ilyonectria liliigena	CBS 304.85	G.J. Bollen, 1985	*Lilium* sp., bulb	Netherlands	JF735299	JF735427	JF735575	JF735764
Ilyonectria liliigena	CBS 305.85	G.J. Bollen, 1985	*Lilium* sp., bulb	Netherlands	JF735300	JF735428	JF735576	JF735765
Ilyonectria gamsii	CBS 940.97	J.T. Poll, 1997	soil	Netherlands, Lelystad	AM419065	AM419089	JF735577	JF735766
"*Cylindrocarpon*" sp.	Cy228	F. Caetano, 2003	*Ficus* sp.	Portugal, Lisbon	JF735301	JF735429	JF735578	JF735767
Ilyonectria anthuriicola	CBS 564.95; PD 95/1577	R. Pieters, 1995	*Anthurium* sp., root	Netherlands, Bleiswijk	JF735302	JF735430	JF735579	JF735768
Ilyonectria vitis	CBS 129082; Cy233	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Touriga Nacional; rootstock 110R	Portugal, Vidigueira	JF735303	JF735431	JF735580	JF735769
Ilyonectria cyclaminicola	CBS 302.93	M. Hooftman, 1993	*Cyclamen* sp., bulb	Netherlands, Roelofarendsveen	JF735304	JF735432	JF735581	JF735770
Cylindrocarpon pauciseptatum	CBS 100819; LYN 16202/2	H.M. Dance, 1998	*Erica melanthera*, root	New Zealand, Tauranga	EF607090	EF607067	JF735582	JF735771
Cylindrocarpon pauciseptatum	CBS 113550	2003	*Vitis* sp., blackening areas in wood and base of trunk	New Zealand, Keesbury Estate	EF607080	EF607069	JF735583	JF735772
Cylindrocarpon pauciseptatum	CBS 120497; KIS 10763	H.-J. Schroers, 2006	*Vitis* sp.brownish spots of healthy looking root of ca. 12-year-old, possibly dead, in vineyard	Slovenia, Mrzlak	EF607085	EF607071	JF735584	JF735773

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					ITS	TUB	H3	EF1
Cylindrocarpon pauciseptatum	CBS 120498; KIS 10775	M. Žerjav, 2006	*Vitis* sp., decayed secondary roots with black areas of 3-year-old, dead	Slovenia, Ljutomer	EF607087	EF607072	JF735585	JF735774
Cylindrocarpon pauciseptatum	CBS 120499; KIS 10780	M. Žerjav, 2006	*Vitis* sp., decayed secondary roots with black areas of 3-year-old, dead	Slovenia, Ljutomer	EF607084	EF607074	JF735586	JF735775
Cylindrocarpon pauciseptatum, type	CBS 120171; KIS 10467	M. Žerjav, 2005	*Vitis* sp., partly decayed roots of 4-year-old plant, still living but badly shooting; in vineyard	Slovenia, Krŝko	EF607089	EF607066	JF735587	JF735776
Cylindrocarpon pauciseptatum	CBS 120172; KIS 10729	M. Žerjav, 2006	*Vitis* sp., strongly decayed, blackish brown root of ca. 9-year-old plant, possibly dead; in vineyard	Slovenia, Žužemberk	EF607086	EF607070	JF735588	JF735777
Cylindrocarpon pauciseptatum	CBS 120173; KIS 10468	M. Žerjav, 2005	*Vitis* sp., partly decayed roots of 4-year-old plant, still living but badly shooting; in vineyard	Slovenia, Krŝko	EF607088	EF607068	JF735589	JF735778
Cylindrocarpon pauciseptatum	Cy196	N. Cruz, 2005	*Vitis vinifera*, basal end of 4-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço/Monção	JF735305	JF735433	JF735590	JF735779
Cylindrocarpon pauciseptatum	Cy217	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Gouveio	Portugal, Torres Vedras	JF735306	JF735434	JF735591	JF735780
Cylindrocarpon pauciseptatum	Cy238	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira	JF735307	JF735435	JF735592	JF735781
"*Cylindrocarpon*" sp. 1	CBS 162.89	M. Barth, 1988	*Hordeum vulgare*, root	Netherlands, Noordoostpolder, Marknesse, Lovinkhoeve	AM419060	AM419084	JF735610	JF735799
"*Cylindrocarpon*" sp. 2	Cy108	C. Rego, 1999	*Vitis vinifera*, basal end of a 4-year-old plant showing decline symptoms; scion Aragonez; rootstock SO4	Portugal, Nelas	JF735316	AM419100	JF735611	JF735800
"*Cylindrocarpon*" sp. 2	Cy200	N. Cruz, 2005	*Vitis vinifera*, basal end of a 16-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço	JF735317	JF735445	JF735612	JF735801

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					ITS	TUB	H3	EF1
"*Cylindrocarpon*" sp. 2	CBS 159.34; IMI 113891; MUCL 4084; VKM F-2656	H.W. Wollenweber, 1934	-	Germany	JF735318	JF735446	JF735613	JF735802
"*Cylindrocarpon*" sp. 2	CBS 173.37; IMI 090176	T.R. Peace, 1937	*Pinus laricio*, associated with dieback	UK, England, Devon, Haldon	JF735319	JF735447	JF735614	JF735803
"*Cylindrocarpon*" sp. 3	Cy135	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419069	AM419105	JF735615	JF735804
"*Cylindrocarpon*" sp. 3	Cy144	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419074	AM419107	JF735616	JF735805
"*Cylindrocarpon*" sp. 3	CBS 129085; Cy145	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735320	JF735448	JF735617	JF735806
"*Cylindrocarpon*" sp. 3	Cy146	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735321	JF735449	JF735618	JF735807
"*Cylindrocarpon*" sp. 3	Cy147	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735322	JF735450	JF735619	JF735808
"*Cylindrocarpon*" sp. 3	Cy148	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735323	JF735451	JF735620	JF735809
"*Cylindrocarpon*" sp. 3	Cy149	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735324	JF735452	JF735621	JF735810
"*Cylindrocarpon*" sp. 3	Cy150	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735325	JF735453	JF735622	JF735811

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					ITS	TUB	H3	EF1
"*Cylindrocarpon*" sp. 3	Cy151	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5-year-old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735326	JF735454	JF735623	JF735812
"*Cylindrocarpon*" sp. 3	Cy152	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5-year-old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735327	JF735455	JF735624	JF735813
"*Cylindrocarpon*" sp. 3	Cy153	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5-year-old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735328	JF735456	JF735625	JF735814
"*Cylindrocarpon*" sp. 3	Cy243	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old-plant; scion Touriga Nacioal; rootstock 110R	Portugal, Vidigueira	JF735329	JF735457	JF735626	JF735815
"*Cylindrocarpon*" sp. 3	CPC 13539; 94-1685; CCFC226730	R. C. Hamelin, 1994	*Picea glauca*	Canada, Quebec	JF735330	JF735458	JF735627	JF735816
"*Cylindrocarpon*" sp. 5	Cy133; IAFM Cy9-1	J. Armengol	*Vitis vinifera*	Spain, Valencia, L'Alcudia	JF735331	JF735459	JF735628	JF735817
"*Cylindrocarpon*" sp. 5	Cy134; IAFM Cy20-1	J. Armengol	*Vitis vinifera*	Spain, Ciudad Real, Villarubia de los Ojos	JF735332	AM419104	JF735629	JF735818
"*Cylindrocarpon*" sp. 5	CBS 129087; Cy159	A. Cabral & H. Oliveira, 2004	*Vitis vinifera*, basal end of a 3-year-old plant with root discolouration and decline symptoms; scion Sangiovese; rootstock 1103P	Portugal, Alcácer do Sal, Torrão	JF735333	AM419111	JF735630	JF735819
"*Cylindrocarpon*" sp. 6	CBS 112593; STE-U 3990; C 107	F. Halleen, 2000	*Vitis vinifera*, roots of an asymptomatic nursery plant; scion Pinotage; rootstock 101-14 Mgt	South Africa, Western Cape, Wellington, Voorgroenberg	AY677281	AY677236	JF735631	JF735820
"*Cylindrocarpon*" sp. 6	CBS 112608; STE-U 3987; C 62	F. Halleen, 2000	*Vitis vinifera*, roots, scion Chardonnay; rootstock 101-14 Mgt	South Africa, Western Cape, Citrusdal	AY677288	AY677235	JF735632	JF735821
"*Cylindrocarpon*" sp. 6	CBS 113552; STE-U 5713; HJS-1306; NZ C 41	R. Bonfiglioli, 2003	*Vitis* sp. decline of nursery plants dead rootstocks	New Zealand, Candy P New Ground	JF735334	AY677237	JF735633	JF735822
"*Cylindrocarpon*" sp. 6	Cy115	W.D. Gubler	*Vitis vinifera*	USA, California	JF735335	JF735460	JF735634	JF735823
"*Cylindrocarpon*" sp. 6	Cy116	W.D. Gubler	*Vitis vinifera*	USA, California	AJ875322	JF735461	JF735635	JF735824

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"*Cylindrocarpon*" sp. 6	Cy117	W.D. Gubler	*Vitis vinifera*	USA, California	AJ875321	JF735462	JF735636	JF735825
"*Cylindrocarpon*" sp. 6	Cy119	W.D. Gubler	*Vitis vinifera*	USA, California	JF735336	JF735463	JF735637	JF735826
"*Cylindrocarpon*" sp. 6	Cy124	W.D. Gubler	*Vitis vinifera*	USA, California	JF735337	JF735464	JF735638	JF735827
"*Cylindrocarpon*" sp. 6	Cy125	W.D. Gubler	*Vitis vinifera*	USA, California	AM419066	JF735465	JF735639	JF735828
"*Cylindrocarpon*" sp. 6	Cy129	W.D. Gubler	*Vitis vinifera*	USA, California	JF735338	JF735466	JF735640	JF735829
"*Cylindrocarpon*" sp. 6	Cy130	W.D. Gubler	*Vitis vinifera*	USA, California	JF735339	JF735467	JF735641	JF735830
"*Cylindrocarpon*" sp. 6	Cy230	F. Caetano, 2005	*Festuca duriuscula*	Portugal, Lisbon	JF735340	JF735468	JF735642	JF735831
Ilyonectria macrodidyma	CBS 112594; STE-U 3991; C 111	F. Halleen, 2000	*Vitis vinifera*, roots of an asymptomatic nursery plant; scion Pinotage; rootstock Richter 99	South Africa, Western Cape, Malmesbury, Jakkalsfontein	AY677282	AY677231	JF735643	JF735832
Ilyonectria macrodidyma	CBS 112601; STE-U 3983; C 82	F. Halleen, 1999	*Vitis vinifera*, roots with black foot symtoms; scion Pinotage; rootstock US 8-7	South Africa, Western Cape, Tulbagh	AY677284	AY677229	JF735644	JF735833
Ilyonectria macrodidyma	CBS 112603; STE-U 4007; C 8	F. Halleen, 1999	*Vitis vinifera*, trunk of a plant showing decline symptoms, scion Sauvignon blanc; rootstock Richter 110	South Africa, Western Cape, Darling	AY677285	JF735469	JF735645	JF735834
Ilyonectria macrodidyma	CBS 112605; STE-U 3984; C 106	F. Halleen, 2000	*Vitis vinifera*, basal end of an asymptomatic nursery plant; scion Sultana; rootstock 143-B Mgt	South Africa, Western Cape, Malmesbury, Jakkalsfontein	AY677287	AY677230	JF735646	JF735835
Ilyonectria macrodidyma, holotype of *C. macrodidymum*	CBS 112615; STE-U 3976; C 98	F. Halleen, 2000	*Vitis vinifera*, roots, asymptomatic nursery grapevine plant scion Sultana; rootstock 143-B Mgt	South Africa, Western Cape, Malmesbury, Jakkalsfontein	AY677290	AY677233	JF735647	JF735836
Ilyonectria macrodidyma	Cy123	W.D. Gubler	*Vitis vinifera*	USA, California	JF735341	JF735470	JF735648	JF735837
Ilyonectria macrodidyma	Cy128	W.D. Gubler	*Vitis vinifera*	USA, California	JF735342	JF735471	JF735649	JF735838
Ilyonectria macrodidyma	Cy139	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419071	AM419106	JF735650	JF735839

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					ITS	TUB	H3	EF1
Ilyonectria macrodidyma	Cy140	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735343	JF735472	JF735651	JF735840
Ilyonectria macrodidyma	Cy175	C. Rego, 2004	*Vitis vinifera*, basal discolouration in rootstocks; scion Touriga Nacional; rootstock 1103P	Portugal, Torre de Moncorvo	JF735344	JF735473	JF735652	JF735841
Ilyonectria macrodidyma	Cy181	C. Rego, 2005	*Vitis vinifera*, scion 140-Ru; rootstock Aragonês	Portugal, Alcácer do Sal	JF735345	JF735474	JF735653	JF735842
Ilyonectria macrodidyma	Cy216	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Marssanne	Portugal, Torres Vedras	JF735346	JF735475	JF735654	JF735843
Ilyonectria macrodidyma	Cy244	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira	JF735347	JF735476	JF735655	JF735844
Ilyonectria macrodidyma	Cy258	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735348	JF735477	JF735656	JF735845
"*Cylindrocarpon*" sp.4	CBS 119.41	H.C. Koning	*Fragaria* sp., root	Netherlands, Baarn	JF735349	JF735478	JF735657	JF735846
"*Cylindrocarpon*" sp.4	CBS 188.49	J.A. von Arx	*Abies nordmanniana*, root	Netherlands, Egmond	AM419063	AM419087	JF735658	JF735847
"*Cylindrocarpon*" sp.4	CBS 112604; STE-U 4004; C 10	F. Halleen, 1999	*Vitis vinifera*, roots; scion Cabernet Sauvignon ; rootstock 101-14 Mgt	South Africa, Western Cape, Paarl	AY677286	AY677227	JF735659	JF735848
"*Cylindrocarpon*" sp.4	CBS 112609; STE-U 3969; HJS-1217	M. Sweetingham, 1979	*Vitis* sp., dark brown discolouration in trunk; scion Cabernet Sauvignon	Australia, Tasmania, Bream Creek	AY677289	AY677226	JF735660	JF735849
"*Cylindrocarpon*" sp.4	CBS 113555; STE-U 5715; HJS-1309; NZ C 60	R. Bonfiglioli, 2003	*Vitis* sp., blackening areas in wood and roots; scion Pinot Noir; rootstock 101-14	New Zealand, Fiddlers Green	JF735350	AY677234	JF735661	JF735850
"*Cylindrocarpon*" sp.4	CBS 112598; STE-U 3997; C 115	F. Halleen, 2000	*Vitis vinifera*, roots of an asymptomatic plant; scion Sultana; rootstock Ramsey	South Africa, Western Cape, Wellington, Lelienfontein	JF735351	JF735479	JF735662	JF735851
"*Cylindrocarpon*" sp.4	CPC 13533; CCFC 144524; Dias 2B	H.F. Dias, 1972	*Vitis vinifera*, Concord Bradt grapes, roots and stems	Canada, Ontario	AY295332	JF735480	JF735663	JF735852

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
"*Cylindrocarpon*" sp.4	Cy69	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock SO4, clone 102F	Portugal, Ribatejo e Oeste	AJ875332	AM419095	JF735664	JF735853
"*Cylindrocarpon*" sp.4	Cy71	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 99R, clone 96F	Portugal, Ribatejo e Oeste	AJ875335	AM419096	JF735665	JF735854
"*Cylindrocarpon*" sp.4	Cy72	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock clone 113F	Portugal, Ribatejo e Oeste	AJ875336	AM419097	JF735666	JF735855
"*Cylindrocarpon*" sp.4	Cy75	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 99R	Portugal, Ribatejo e Oeste	AJ875334	AM419098	JF735667	JF735856
"*Cylindrocarpon*" sp.4	Cy96	E. Halmschlager	*Quercus* sp., root	Austria, Patzmannsdorf	JF735352	JF735481	JF735668	JF735857
"*Cylindrocarpon*" sp.4	Cy97	E. Halmschlager	*Quercus* sp., root	Austria, Patzmannsdorf	JF735353	JF735482	JF735669	JF735858
"*Cylindrocarpon*" sp.4	Cy118	W.D. Gubler	*Vitis vinifera*	USA, California	JF735354	JF735483	JF735670	JF735859
"*Cylindrocarpon*" sp.4	Cy120	W.D. Gubler	*Vitis vinifera*	USA, California	AJ875320	AM419101	JF735671	JF735860
"*Cylindrocarpon*" sp.4	Cy132; IAFM Cy1-1	J. Armengol	*Vitis vinifera*	Spain, Alicante	JF735355	JF735484	JF735672	JF735861
"*Cylindrocarpon*" sp.4	Cy136	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735356	JF735485	JF735673	JF735862
"*Cylindrocarpon*" sp.4	Cy137	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419070	JF735486	JF735674	JF735863
"*Cylindrocarpon*" sp.4	Cy138	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735357	JF735487	JF735675	JF735864
"*Cylindrocarpon*" sp.4	Cy141	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735358	JF735488	JF735676	JF735865

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
"*Cylindrocarpon*" sp.4	Cy142	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735359	JF735489	JF735677	JF735866
"*Cylindrocarpon*" sp.4	Cy143	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735360	JF735490	JF735678	JF735867
"*Cylindrocarpon*" sp.4	Cy157	H. Oliveira, 2004	*Vitis vinifera*, scion Touriga Nacional; rootstock 99R	Portugal, Alenquer	AM419077	AM419110	JF735679	JF735868
"*Cylindrocarpon*" sp.4	Cy214	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Grenache	Portugal, Torres Vedras	JF735361	JF735491	JF735680	JF735869
"*Cylindrocarpon*" sp.4	CBS 129086; Cy218	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Chenin	Portugal, Torres Vedras	JF735362	JF735492	JF735681	JF735870
"*Cylindrocarpon*" sp.4	Cy221	L. Leandro	*Fragaria x ananassa*	USA, North Carolina, Asheville	JF735363	JF735493	JF735682	JF735871
"*Cylindrocarpon*" sp.4	Cy222	L. Leandro	*Fragaria x ananassa*	USA, North Carolina, Asheville	JF735364	JF735494	JF735683	JF735872
"*Cylindrocarpon*" sp.4	Cy223	L. Leandro	*Fragaria x ananassa*	USA, North Carolina, Asheville	JF735365	JF735495	JF735684	JF735873
"*Cylindrocarpon*" sp.4	Cy235	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735366	JF735496	JF735685	JF735874
"*Cylindrocarpon*" sp.4	Cy237	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Chardonnay; rootstock 110R	Portugal, Vidigueira	JF735367	JF735497	JF735686	JF735875
"*Cylindrocarpon*" sp.4	Cy240	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Touriga Nacional; rootstock 140RU	Portugal, Vidigueira	JF735368	JF735498	JF735687	JF735876
"*Cylindrocarpon*" sp.4	Cy246	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Antão Vaz; rootstock 110R	Portugal, Vidigueira	JF735369	JF735499	JF735688	JF735877

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
"*Cylindrocarpon*" sp.4	Cy260	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735370	JF735500	JF735689	JF735878
"*Cylindrocarpon*" sp.4	Cy262	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735371	JF735501	JF735690	JF735879
Neonectria major, type strain	CBS 240.29; IMI 113909	H.W. Wollenweber	*Alnus incana*, canker	Norway	JF735308	DQ789872	JF735593	JF735782
Neonectria ditissima, authentic strain of *C. willkommii*	CBS 226.31; IMI 113922	H.W. Wollenweber	*Fagus sylvatica*	Germany, Tharandt	JF735309	DQ789869	JF735594	JF735783
Neonectria ditissima, representative strain of *N. galligena*	CBS 835.97	W. Gams, 1997	*Salix cinerea*, dead branch of still living tree	Belgium, Marais de Sampant	JF735310	DQ789880	JF735595	JF735784
Neonectria ditissima	Cy169	H. Oliveira, 1997	*Malus domestica*	Portugal, Alcobaça	AM419080	AM419113	JF735596	JF735785
Neonectria ditissima	Cy172	T. Nascimento, 2004	*Malus domestica*; scion Oregon; rootstock MM107	Portugal, Caldas da Rainha	AM419081	AM419114	JF735597	JF735786
Neonectria neomacrospora, representative strain	CBS 118984; GJS 03-28	L. Reitman, 2005	*Arceuthobium tsugense*, parasiting *Abies balsams*	Canada, British Columbia, Vancouver Island, Spider Lake	JF735311	DQ789882	JF735598	JF735787
Cylindrocarpon cylindroides, representative strain	CBS 324.61; DSM 62489; IMB 9628	J.A. von Arx	*Abies concolor*	Netherlands, Zwolle	JF735312	DQ789875	JF735599	JF735788
Cylindrocarpon cylindroides	CBS 503.67	F. Roll-Hansen	*Abies alba*, wood	Norway, Hordaland, Fana	AY677261	JF735436	JF735600	JF735789
Cylindrocarpon sp.	CPC 13545; DAOM 185212; # 5	J.A. Traquair & B. Harrison, 1982	*Pyrus* sp.	Canada, Ontario, Harrow	AY295303	JF735437	JF735601	JF735790

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
Neonectria ramulariae, authentic strain of *C. obtusiusculum* (=*C. magnusianum*)	CBS 151.29; IMI 113894; MUCL 28083; MUCL 28094	H.W. Wollenweber	*Malus sylvestris*, fruit	UK, England, Cambridge	JF735313	JF735438	JF735602	JF735791
Neonectria ramulariae	CBS 182.36; IMI 113893; UPSC 1903	H.W. Wollenweber	*Malus sylvestris*, fruit	-	JF735314	JF735439	JF735603	JF735792
Cylindrocarpon sp.	CR21	P. Axelrood	*Pseudotsuga menziesii*	Canada, British Columbia	JF735315	JF735440	JF735604	JF735793
Cylindrocarpon sp.	CPC 13530; DAOM 185722; JAT 1591	J.A. Traquair, 1983	*Pyrus* sp., lesions on seedlings	Canada, Ontario, Harrow	AY295302	JF735441	JF735605	JF735794
Cylindrocarpon sp.	CPC 13531; CCFC 226722; DAOM 226722; CR6	P. Axelrood	*Pseudotsuga menziesii*, root	Canada, British Columbia	AY295301	JF735442	JF735606	JF735795
Cylindrocarpon obtusisporum	CBS 183.36; IMI 113895	H.W. Wollenweber, 1936	*Solanum tuberosum*, tuber	Germany	AM419061	AM419085	JF735607	JF735796
Cylindrocarpon obtusisporum	CPC 13544; DAOM 182772; JAT 1366	J.A. Traquair, 1982	*Prunus armenica*, twigs	Canada, Ontario, Ruthven	AY295306	JF735443	JF735608	JF735797
Cylindrocarpon obtusisporum	94-1356	R. C. Hamelin, 1994	*Picea mariana*	Canada, Quebec	AY295304	JF735444	JF735609	JF735798

[a]**ATCC**: American Type Culture Collection, USA; **CBS**: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **CCFC**: Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, Canada; **CPC**: Culture collection of Pedro Crous, housed at CBS; **Cy**: *Cylindrocarpon* collection housed at Laboratório de Patologia Vegetal 'Veríssimo de Almeida' - ISA, Lisbon, Portugal; **DAOM**: Agriculture and Agri-Food Canada National Mycological Herbarium, Canada; **DSM**: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; **GJS**: Gary J. Samuels collection; **HJS**: Hans-Josef Schroers collection; **IAFM**: Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain; **IAM**: Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan; **ICMP**: International Collection of Microorganisms from Plants, Auckland, New Zealand; **IFFF**: Institute of Forest Entomology, Forest Pathology and Forest Protection, Austria; **IMI**: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; **JAT**: J. A. Traquair collection; **JCM**: Japan Collection of Microorganisms, Japan; **KIS**: Agricultural Institute of Slovenia, Ljubljana, Slovenia; **LYN**: Lynchburg College, Biology Department, USA; **MUCL**: Mycothèque de l'Université Catholique de Louvain, Belgium; **NBRC**: NITE Biological Resource Center, Japan; **NRRL**: Agricultural Research Service Culture Collection, USA; **STE-U**: Stellenbosch University, South Africa; **UPSC**: Fungal Culture Collection at the Botanical Museum, Uppsala University, Uppsala, Sweden; **VKM**: All-Russian Collection of Microorganisms, Russia.

CHAPTER 3

MULTIGENE ANALYSIS AND MORPHOLOGY REVEAL NOVEL *ILYONECTRIA* SPECIES ASSOCIATED WITH BLACK FOOT DISEASE OF GRAPEVINES

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Fungal Biology (in preparation)

Keywords Black foot disease of grapevines; *Cylindrocarpon macrodidymum*; *Nectria*-like fungi; phylogeny; systematics.

Abstract

Black foot is an important disease of grapevines, which has in recent years been recorded with increased incidence and severity throughout the world, affecting grapevines both in nurseries and young vineyards. In the past the disease has been associated with infections by *Ilyonectria macrodidyma*, *I. liriodendri*, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare*. Based on published data, a high level of genetic diversity was detected among isolates of *I. macrodidyma*. To resolve this issue, we employed a multi-gene analysis strategy (based on the β-tubulin, histone H3, translation elongation factor 1-α and the internal transcribed spacers on both sides of the 5.8S nuclear ribosomal RNA gene) along with morphological characterisation to study a collection of 81 *I. macrodidyma*-like isolates from grapevine and other hosts. Morphological characters (particularly conidial size) and molecular data (highest resolution achieved with histone H3 nucleotide sequence) enabled the distinction of six monophyletic species within the *I. macrodidyma* complex, four of which (*I. alcacerensis*, *I. estremocensis*, *I. novozelandica* and *I. torresensis*) are described here. This work forms part of an effort by the International Council on Grapevine Trunk Diseases to resolve the species associated with black foot disease, which we believe will clarify their taxonomy, and therefore help researchers to devise control strategies to reduce the devastating impact of this disease.

Introduction

Black foot is an important disease of grapevines in most of the wine-producing countries of the world. The disease has increased in incidence and severity over the past few years, affecting both nurseries and young vineyards, provoking typical darkening of the basal end of plant rootstocks (Halleen et al. 2004; Oliveira et al. 2004). Infected vineyards show a high percentage of declining plants with slow growth, reduced vigour, retarded sprouting, shortened internodes, sparse and chlorotic foliage (Rego et al. 2000; Halleen et al. 2006a), resulting frequently in plant death, and forcing growers to uproot and replant considerable areas.

Based on current data, there are at least four causal agents of black foot disease of grapevine, namely *Ilyonectria liriodendri* and *I. macrodidyma* (Halleen et al. 2004, 2006b), and two *Campylocarpon* species, *Campyl. fasciculare*, and *Campyl. pseudofasciculare* (Halleen et al. 2004). Similar black foot symptoms are caused by these pathogens, and while some studies found no virulence differences among isolates from these four species (Alaniz et al. 2007; Halleen et al. 2004; Petit and Gubler 2005), other pathogenicity trials detected variation in virulence among groups of *I. macrodidyma*, previously distinguished based on Inter-Simple Sequence Repeat markers, and further showed that *I. macrodidyma* appears to be more virulent than *I. liriodendri* (Alaniz et al. 2009a). Although the relative importance, frequency and geographic distribution of these pathogens are still poorly understood, *I. liriodendri* and *I. macrodidyma* are the two species most commonly isolated from affected grapevines (Petit & Gubler 2005; Halleen et al. 2006b; Alaniz et al. 2007), whereas the *Campylocarpon* spp. have thus far only been reported from South Africa (Halleen et al. 2004) and Uruguay (Abreo et al. 2010). Schroers et al. (2008) reported a fifth species, '*Cylindrocarpon*' *pauciseptatum* (a *Cylindrocarpon*-like species pending revision of taxonomic placement; Cabral et al. 2011), which was associated with diseased roots of *Vitis* sp. in South-Eastern Europe (Slovenia) as well as New Zealand. Since this first report, it has also been detected in Uruguay (Abreo et al. 2010), Spain (Martín et al. 2011) and Portugal (Cabral et al. 2011), showing that it is present on at least three continents. Its potential role as plant pathogen, however, has yet to be determined, although it has been able to produce necrotic root lesions in 110R rootstock cuttings (Alaniz et al. 2009b).

The genus *Ilyonectria* represents one of several newly established genera for fungi with Cylindrocarpon-like anamorphs (Chaverri et al. 2011). This followed on previous work by Booth (1966), who segregated the genus *Cylindrocarpon* in four groups based on the presence or absence of microconidia and chlamydospores. The type species of the genus *Cylindrocarpon*, *C. cylindroides*, belongs to group 1 (microconidia present, mycelial chlamydospores lacking), while

the type of the genus *Neonectria*, *N. ramulariae*, which is the teleomorph of *C. obtusiusculum* (= *C. magnusianum*; Braun 1993), belongs to group 4 (microconidia lacking, mycelial chlamydospores present). Group 2 (lacking both microconidia and mycelial chlamydospores) contains *Cylindrocarpon* species predominantly connected with teleomorphs of '*Neonectria*' *mammoidea*. Group 3 (microconidia and mycelial chlamydospores present) contains *C. destructans*, which is considered to be a species complex comprising various taxa, including *C. macroconidialis*, *C. coprosmae* and *C. liriodendri* (Seifert et al. 2003; Halleen et al. 2006b). Further studies recently led to the introduction of several novel *Ilyonectria* spp., including four species (*I. europaea*, *I. lusitanica*, *I. pseudodestructans* and *I. robusta*) associated with grapevine black foot disease symptoms (Cabral et al. 2011). Most of the teleomorphs of *Cylindrocarpon* (groups 1, 2 and 4; Booth 1966) have been classified in *Neonectria* (Rossman et al. 1999; Mantiri et al. 2001; Brayford et al. 2004; Halleen et al. 2004). Species of *Neonectria* were divided into three to five groups based on the anatomical characters of the perithecial wall, and partly on ascospore characters (Booth 1959; Rossman et al. 1999; Mantiri et al. 2001; Brayford et al. 2004). Based on results of a recent phylogenetic study, *Neonectria* was divided into four genera based on a combination of characters linked to perithecial anatomy and conidial septation: *Neonectria/Cylindrocarpon sensu stricto* (Booth's groups 1 and 4), *Rugonectria*, *Thelonectria* (group 2) and *Ilyonectria* (group 3) (Chaverri et al. 2011). According to this treatment, only *Neonectria* has *Cylindrocarpon* anamorphs, while the remaining genera have *Cylindrocarpon*-like anamorphs, and are referred to as 'Cylindrocarpon' in this text.

The aim of the present study was to characterise a collection of *Cylindrocarpon*-like isolates originating from grapevines that appeared to be closely related to *I. macrodidyma*. To this end nucleotide sequences were derived from the β-tubulin (TUB), histone H3 (HIS), translation elongation factor 1-α (TEF), and the internal transcribed spacers (ITS) on both sides of the 5.8S nuclear ribosomal RNA gene, and a multi-locus phylogeny was constructed. These data were further supplemented with culture characteristics and morphological features to elucidate possible cryptic taxa.

Materials and methods

Isolates

This study addressed 68 *I. macrodidyma*-like isolates from grapevine and 13 from other hosts (Table 1). Forty isolates were obtained in Portugal mainly from 1.5–4 yr old vineyards showing decline symptoms, or from rootstock nurseries. Small pieces of blackened tissue were cut from

either the base of the rootstock, or from the grafting zone. Tissue pieces were disinfected for 1 min in a NaClO solution (0.35 % w/w as active chlorine), rinsed with sterile distilled water and placed in Petri dishes containing potato-dextrose agar (PDA, Difco, USA) amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppliChem, Germany). Dishes were incubated at 20 ºC for up to 2 wk, in order to allow for the identification of *Cylindrocarpon* colonies. Single-conidial cultures were obtained and stored in the collection at the Laboratório de Patologia Vegetal 'Veríssimo de Almeida' (LPVVA-ISA, Lisbon, Portugal), and representative strains deposited at the CBS-KNAW Fungal Biodiversity Centre (Utrecht, Netherlands). Additional isolates used during this study were obtained from: CBS; the working collection of Pedro Crous (CPC) housed at CBS; F. Caetano (LPVVA-ISA); J. Armengol (Univ. Politécnica de Valencia, Spain); K.A. Seifert (Agriculture and Agri-Food, Canada); L. Leandro (Iowa State University, Department of Plant Pathology, USA) and W.D. Gubler (Univ. California, Davis, USA).

DNA isolation, sequencing and phylogenetic analysis

For each isolate, genomic DNA was obtained from mycelium following the protocol by Möller et al. (1992) adapted by Crous et al. (2009). Sequencing of the ITS and of part of TUB, HIS and TEF genes was performed after PCR amplification using 1× PCR buffer (Bioline, UK), 1.5 mM $MgCl_2$, 32 µM of each dNTPs, 0.24 µM of each primer, 0.5 units *Taq* DNA Polymerase (Bioline) and 1 µL of diluted gDNA in a final volume of 12.5 µL. The cycle conditions in a iCycler thermocycler (BioRad, USA) were 94 ºC for 5 min, followed by 40 cycles at 94 ºC for 30 s, 52 ºC for 30 s and 72 ºC for 80 s, and a final elongation at 72 ºC for 10 min. Primers were V9G (de Hoog and Gerrits van den Ende 1998) and ITS4 (White et al. 1990) for ITS, T1 (O'Donnell and Cigelnik 1997) and Bt-2b (Glass and Donaldson 1995) for TUB, CYLH3F and CYLH3R (Crous et al. 2004b) for HIS and EF1 and EF2 (O'Donnell et al. 1998) or CylEF-1 (5'- ATG GGT AAG GAV GAV AAG AC-3'; J.Z. Groenewald, unpublished) and CylEF-R2 (Crous et al. 2004b) for TEF. For TEF the following modifications were made to the amplification protocol: 2.0 mM of $MgCl_2$, 40 µM of each dNTPs and addition of 5 % of Dimethyl sulfoxide (DMSO; Sigma-Aldrich, the Netherlands).

After confirmation by agarose gel electrophoresis, amplicons were sequenced in both directions with the corresponding PCR primers and a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, the Netherlands) according to manufacturer's recommendations. The products were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, USA).

Sequences were assembled and edited to resolve ambiguities, using the EditSeq and SeqMan modules of the Lasergene software package (DNAStar, USA). Consensus sequences for all

isolates were compiled into a single file (Fasta format) and aligned using CLUSTAL X v.2.0.11 (Larkin et al. 2007). Following manual adjustment of the alignment by eye where necessary, the alignment was subjected to phylogenetic analyses as described by Crous et al. (2004b). Optimal models were analysed for each locus using MrModeltest v. 2.2 (Nylander 2004). Ambiguous alignment areas were excluded from the analyses only in the ITS alignment, namely alignment positions 247–255, 267–276 and 566–572 (see TreeBASE for alignment). Novel sequences were lodged in GenBank (Table 1), alignments and phylogenetic trees in TreeBASE (http://www.treebase.org), and taxonomic novelties in MycoBank (Crous et al. 2004a).

Morphology

Isolates were grown for up to 5 wk at 20 ºC on synthetic nutrient agar (SNA; Nirenberg 1976) with and without two 1 cm^2 filter paper pieces, PDA and oatmeal agar (OA; Crous et al. 2009) under continuous n-UV light (NUV; 400–315 nm; Sylvania Blacklight-Blue, Netherlands).

Measurements were done by removing a 1 cm^2 agar square, and placing this on a microscope slide, to which a drop of water was added and a cover slip laid. For each isolate, 30 measurements were obtained for each structure. Measurements were done at 1000× magnification using a Nikon Eclipse 80i microscope, or a Leica DM2500. Images were captured using a Nikon DS-Fi1 digital camera with NIS-Elements Software, or a Leica DFC295 digital camera with the Leica Application Suite. Measurements are given as (minimum–) lower limit of a 95 % confidence interval – average – upper limit of a 95 % confidence interval (–maximum).

Culture characteristics (texture, density, colour, growth front, transparency and zonation) were described on PDA after incubation at 20 ºC in the dark for 14 d. Colour (surface and reverse) was described using the colour chart of Rayner (1970). Cardinal temperatures for growth were assessed by inoculating 90 mm diam PDA dishes with a 3 mm diam plug cut from the edge of an actively growing colony. Growth was determined after 7 d in two orthogonal directions. Trials were conducted at 5–35 ºC in 5 ºC intervals, with three replicate plates per strain at each temperature.

To induce perithecial formation within each prospective species, all isolates were crossed to each other in 60 mm diam Petri dishes containing a minimal salts medium supplemented with two birch toothpicks (Guerber and Correll 2001). The plates were incubated at 20 ºC under n-UV light during 8–20 wk. Two strains were considered sexually compatible if perithecia exuding masses of viable ascospores were produced. The colour reaction of the perithecia was checked in 3 % KOH and in lactic acid. For sectioning, perithecia were mounted in Jung Tissue Freezing

Medium (Leica) or in Arabian Gum, and cut in 10–15 µm thick sections using a Leica cryostat CM3050 S or CM1850 at –20 °C.

Results

Phylogeny

Amplification products of approximately 700 bases for ITS, 630 bases (TUB), 500 bases (HIS) and 700–800 bases (TEF) were obtained for the isolates listed in Table 1. The manually adjusted combined alignment contains 83 sequences (including the two outgroup sequences) and the combined analysis was performed on 2201 characters. Of these, 591 were parsimony informative, 1474 were constant and 136 variable characters were parsimony-uninformative. The partition homogeneity test indicated congruence between the different loci included (P value = 0.212) and the combined analysis yielded 455 equally most parsimonious trees, the first of which is presented as Fig 1 (Tree length = 1017, CI = 0.875, RI = 0.963 and RC = 0.843). The results of the phylogenetic analyses are highlighted below, under Taxonomy or Discussion, as appropriate.

Phylogenetic trees derived from the individual loci are available in TreeBASE and discussed in more detail in the next paragraph. An analysis by MrModeltest proposed the following optimal models for each locus: ITS, equal proportion of bases, substitution model Jukes Cantor, an equal among-site rate variation, and no proportion of invariant sites; TUB: base frequencies set to (0.2167 0.3335 0.2256), substitution models Kimura two-parameter and HKY85, the transition/transversion ratio set to 3.1655, an equal among-site rate variation and proportion of invariant sites set to 0.5477; HIS: base frequencies set to (0.2135 0.3693 0.2182), substitution model General Time Reversible, the matrix of relative substitution rates set to (2.2982 3.4747 1.1882 0.0268 9.6735), a gamma-distributed among-site rate variation (Shape=0.7645) and proportion of invariant sites set to 0.5383; and TEF: base frequencies set to (0.2081 0.3147 0.2146), substitution model General Time Reversible, the matrix of relative substitution rates set to (5.0420 11.9045 4.7344 2.8027 17.5985), an equal among-site rate variation and no proportion of invariant sites. Looking at individual gene trees obtained using the models proposed by MrModeltest, the HIS tree enables the separation of all species with high bootstrap values. The same occurs for TUB, but the bootstrap values are low in the macrodidyma cluster and *I. torresensis* is basal to *I. alcacerensis* and *I. macrodidyma*. For TEF, all species could be resolved, except I. novozelandica, which is divided into two separate groups. The ITS tree does not resolve any species. Neighbour-Joining (NJ) analyses using the three substitution models

(uncorrected ('p'), Kimura two-parameter or HKY85), as well as the parsimony analysis (Fig. 1), yielded trees with similar topology and bootstrap support values for the combined analysis. In addition, a comparison between the tree derived from the combined alignment using optimised evolutionary models per locus vs. applying the same model (General Time Reversible) across all loci COMBcustom=COMBstandard did not reveal any incongruences in the obtained clades between the analyses.

The four gene combined data set enabled the distinction of four species within the isolates previously identified as *I. macrodidyma*. The ITS or TUB genes were ineffective in resolving any of these species, as nucleotide sequences were indistinguishable for ITS, and only revealed four positions with nucleotide differences in TUB. This contrasts with up to 20 differences in HIS and 14 in TEF. *Ilyonectria alcacerensis* is the most distinct species, with 12–14 bp differences to the remaining species in HIS, and 10–12 bp differences to *I. macrodidyma* and *I. torresensis* and four *to I. novozelandica* in TEF. Among these differences, seven polymorphisms are unique to *I. alcacerensis* in HIS, four in TEF and one in TUB. The differences between *I. torresensis*, *I. novozelandica* and *I. macrodidyma* are quite similar, with 9–11 bp in HIS and 6–8 bp in TEF (Table 2). Four polymorphisms each are unique for *I. torresensis* in HIS and TEF, while only three polymorphisms are unique to *I. novozelandica* in HIS besides one in TUB.

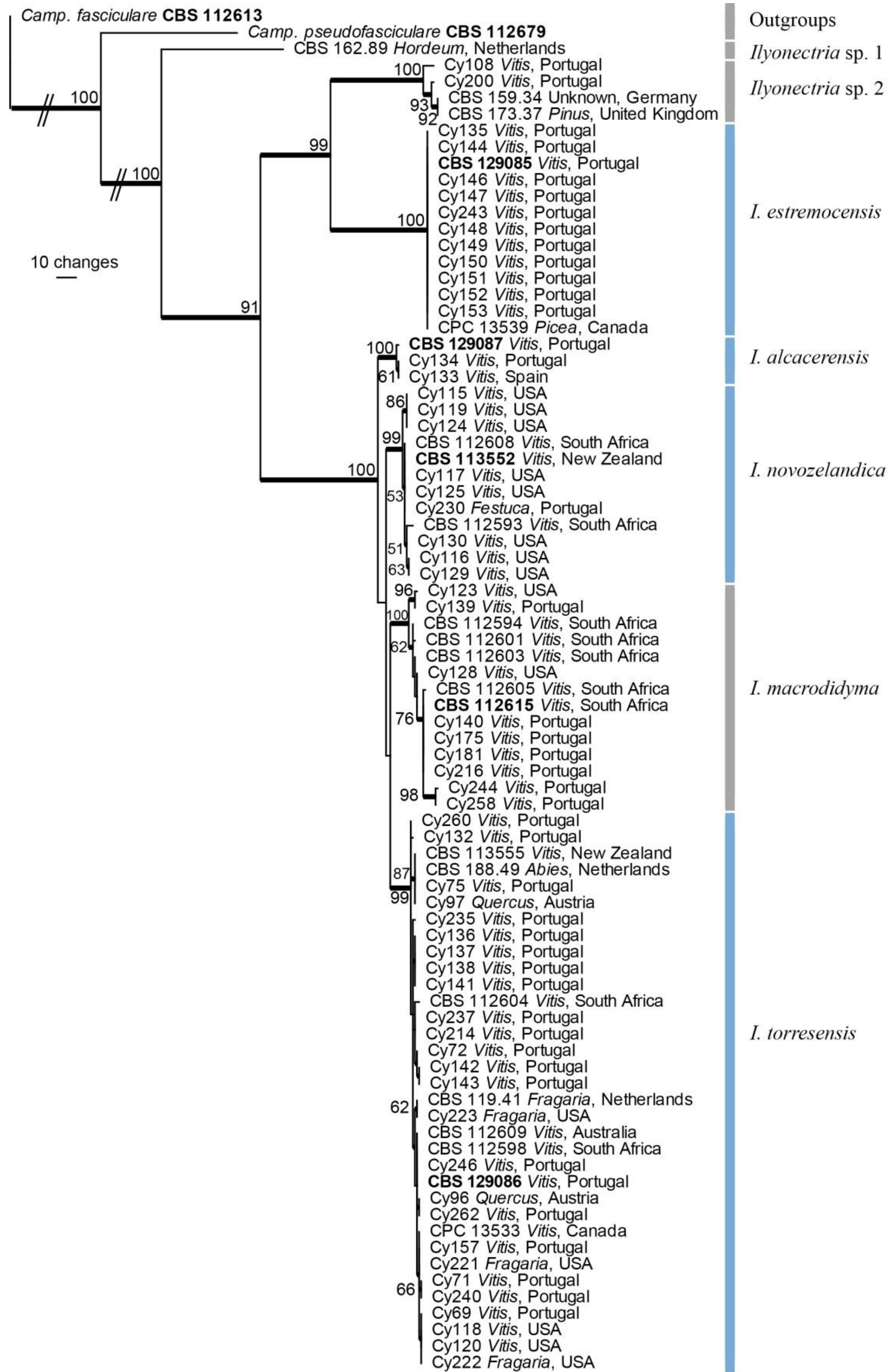

Fig. 1. The first of 455 equally most parsimonious trees obtained from the combined ITS, TUB, HIS, and TEF sequence alignment of *Ilyonectria* isolates and relatives with a heuristic search using PAUP v.4.0b10. The tree was rooted using *Campylocarpon* isolates as outgroup sequences and bootstrap support values are indicated near the nodes. Ex-type strains are indicated in bold and those branches present in the strict consensus tree are thickened. Newly described species are indicated by blue boxes. Scale bar shows ten changes.

Table 2 Nucleotide differences for partial gene sequences of β-tubulin (TUB), histone H3 (HIS), translation elongation factor 1-α (TEF) for isolates belonging to *I. alcacerensis*, *I. macrodidyma*, *I. novozelandica* and *I. torresensis*. Position (bp) refers to the nucleotide position on each sequence of TUB, HIS and TEF of isolate CBS 112615, the holotype of *I. macrodidyma*

TUB	**Position (bp)**			
Species	**156**	**331**	**353**	**421**
I. macrodidyma	C	A	T	T (C in Cy123, Cy139)
I. torresensis	C	G	T	C
I. alcacerensis	A	G	T	C
I. novozelandica	C	G	A	C

HIS	**Position (bp)**									
Species	**36**	**79**	**102**	**111**	**119**	**122**	**124**	**216**	**280**	**292**
I. macrodidyma	T	T	A	G	A	C	T	C	T	C
I. torresensis	T	T	C	T	A	C/T	C	T	C	C
I. alcacerensis	C	C	C	A	G	C	C	C	T	T
I. novozelandica	T	T	C	A	A	T	C	C	T	C
	293	**295**	**297**	**303**	**323**	**324**	**347**	**365**	**395**	**428**
I. macrodidyma	C	T	A	T	G	T	C	T	C	C
I. torresensis	C	T	T	C	T	T	T	T	T	C
I. alcacerensis	T	C	A	C	T	T	T	C	T	C
I. novozelandica	C	T	A	C	C	C	C	T	T	T

TEF	**Position (bp)**													
Species	**30**	**36**	**37**	**68**	**102**	**128**	**137**	**249**	**441**	**450**	**521**	**529**	**535**	**552**
I. macrodidyma	G	T	C	G	T	A	A	C	C	-	T	A	G	A
I. torresensis	G	T	C	G	T	C	G	A	T	T	C	A	G	A
I. alcacerensis	A	-	-	A	A	C	A	A	C	-	T	C	C	T
I. novozelandica	G	-	-	G	A	C	A	A	C	-	T	C	G	A

Taxonomy

Based on the phylogenetic data derived in the present study, six new species could be distinguished in the *I. macrodidyma* species complex. Four of these taxa are named in this study, while the two other species will be treated separately.

Ilyonectria alcacerensis A. Cabral, Oliveira & Crous, **sp. nov.** MycoBank 560152. Fig 2.

Etymology: Named after the Portuguese city of Alcácer do Sal, where the holotype was collected.

Ilyonectriae macrodidymae similis, sed macroconidiis (1–)3(–6)-septatis, majoribus, (33.0–)43.9–46–48.1(–68.0) × (6.0–)7.2–7.4–7.7(–9.0) µm.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary, unbranched, bearing up to two phialides, 1–6-septate, 29–190 µm long; phialides monophialidic, more or less cylindrical, but tapering slightly in the upper part towards the apex, 16–42 µm long, 2.0–3.5 µm wide at the base, 2.5–4 µm at the widest point, and 1.5–2.5 µm near the apex. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched; phialides more or less cylindrical, but tapering slightly in the upper part towards the apex, or narrowly flask-shaped, mostly with the widest point near the middle, 15–27 µm long, 2.5–3.5 µm wide at the base, 3.0–3.5 µm at the widest point, and 2.0–2.5 µm wide at the apex. *Macroconidia* predominating, formed by both types of conidiophores; on SNA formed in flat domes of slimy masses, (1–)3(–6)-septate, straight or minutely curved, cylindrical, or minutely widening towards the tip, appearing somewhat clavate, particularly when still attached to the phialide, with apex or apical cell typically slightly bent to one side and minutely beaked; base mostly with a visible, centrally located or laterally displaced hilum; 1-septate conidia (21.0–)26.0–27.1–28.1(–39.0) × (4.5–)5.3–5.4–5.6(–7.0) µm, with a length : width ratio of (3.8–)4.8–5.0–5.1(–6.8); 2-septate conidia (26.0–)33.2–34.5–35.9(–45.0) × (5.0–)6.0–6.2–6.5(–7.5) µm, with a length : width ratio of (4.4–)5.4–5.6–5.7(–7.0); and 3-septate conidia (33.0–)43.9–46–48.1(–68.0) × (6.0–)7.2–7.4–7.7(–9.0) µm, with a length : width ratio of (4.5–)5.9–6.2–6.5(–9.8) µm. *Microconidia* 0–1-septate, more or less straight, with a minutely or clearly laterally displaced hilum; constricted at the septum; aseptate microconidia globose to subglobose, (8.0–)11.3–11.8–12.4(–18.0) × (3.0–)4.0–4.1–4.3(–5.0) µm, with a length : width ratio of (1.8–)2.7–2.9–3.0(–4.0) µm; 1-septate microconidia ellipsoidal to ovoid, (11.0–)15.0–

15.6–16.2(–20.0) × (3.5–)4.4–4.5–4.6(–5.0) µm, with a length : width ratio of (2.4–)3.3–3.5–3.6(–4.5) µm. *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses on complex conidiophores. *Chlamydospores* rarely occur, globose to subglobose, 6–10 × 5–8 µm, smooth but often appearing rough due to deposits, thick-walled, mostly occurring in chains.

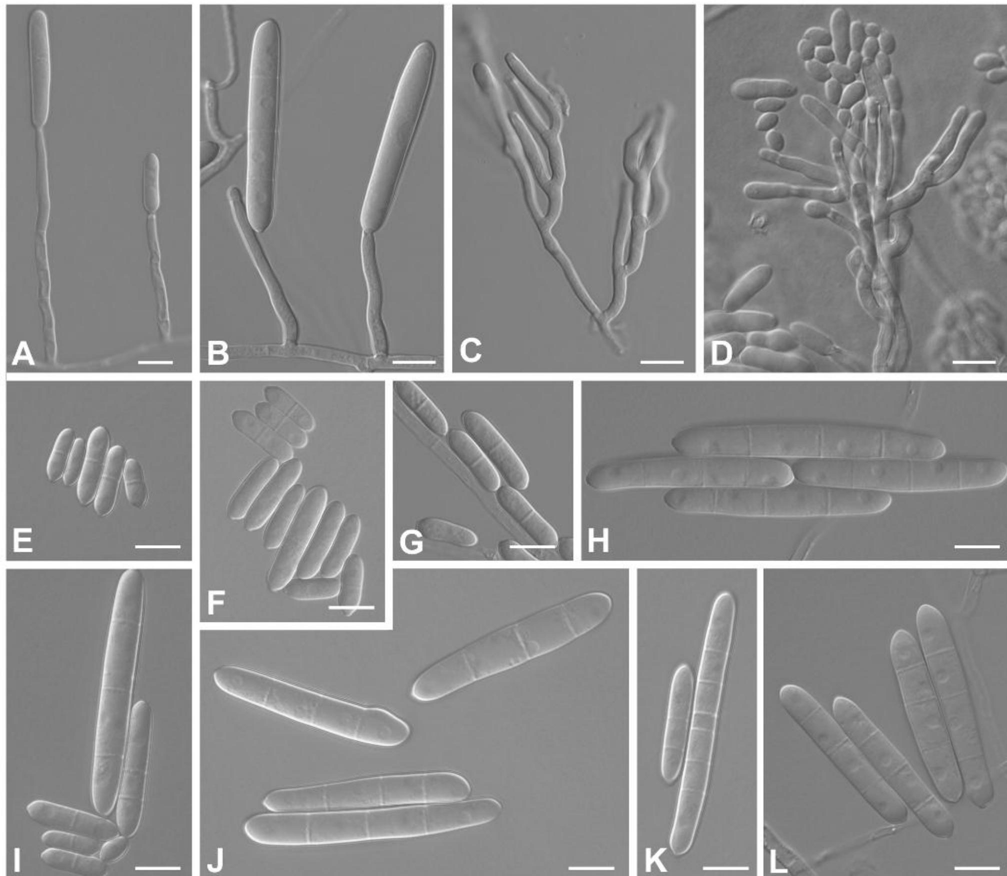

Fig. 2. *Ilyonectria alcacerensis* (A-C) Simple, sparsely branched conidiophores of the aerial mycelium. (D) Complex conidiophores. (F-L) Micro and macroconidia. Bars 10 µm. All from isolate CBS 129087.

Holotype: **Portugal:** Alcácer do Sal, Torrão, *Vitis vinifera*, base of a 3 yr old plant with root discolouration and decline symptoms; scion Sangiovese; rootstock 1103P, 2007, coll./isol. A. Cabral and H. Oliveira, CBS H-20573, culture ex-type CBS 129087 = Cy159.

Cardinal temperatures for growth: Colonies on PDA grow poorly (0.5–2 mm) at 5 ºC after 7 d. Optimum temperature between 20–25 ºC, with colonies reaching 21–28 mm and 31–33 mm diam respectively. Maximum temperature around 30 ºC, with colonies reaching 2–6 mm; no growth observed at 35 ºC.

Culture characteristics: Mycelium felty to slightly cottony with average density. Surface on OA buff to sienna; margin amber to pure yellow. On PDA buff to saffron; margin luteous; zonation absent, transparency homogeneous, margin even to somewhat uneven; reverse similar, but chestnut to saffron on PDA.

Isolates studied: CBS 129087; Cy133; Cy134 (Table 1).

Host and distribution: *Vitis vinifera* (Portugal, Spain).

Ilyonectria estremocensis A. Cabral, Nascimento & Crous, **sp. nov.** MycoBank 560153. Fig 3.

Etymology: Named after the Portuguese city of Estremoz, where the holotype was collected.

Ilyonectriae macrodidymae similis, sed microconidiis cylindricaceis et macroconidiis fere 1-septatis.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to three phialides, 1–3-septate, 40–150 µm long; phialides monophialidic, cylindrical to subcylindrical, tapering slightly in the upper part towards the apex, 15–42 µm long, 2–3 µm wide at the base, 2.5–3.5 µm at the widest point, and 1.5–2.0 µm at the apex. *Sporodochial conidiophores* irregularly branched; phialides cylindrical, mostly widest near the base. *Micro*- and *macroconidia* present on both types of conidiophores. *Macroconidia* predominating, formed on simple conidiophores; on SNA formed in flat domes of slimy masses, 1(–3)-septate, straight or slightly curved, cylindrical, but typically with a minutely widening towards the apex, appearing somewhat clavate; apex obtuse; base mostly with a visible, centrally located or laterally displaced hilum; 1-septate conidia (22.0–)29.0–30.2–31.4(–45.0) × (3.4–)5.1–5.2–5.4(–7.0) µm, with a length : width ratio of (4.4–)5.5–5.7–5.9(–7.5); 2-septate conidia (28.0–)38.8–40.0–41.1(–48.0) × (5.0–)5.9–6.1–6.2(–7.0) µm, with a length : width ratio of (4.9–)6.4–6.6–6.8(–9.2) µm; 3-septate conidia (38.0–)44.1–45.2–46.3(–54.0) × (5.0–)6.3–6.4–6.6(–7.5) µm with a length : width ratio of (5.3–)6.8–7.1–7.3(–9.8) µm. *Microconidia* 0–1-septate, cylindrical, more or less straight, with a minutely or clearly laterally displaced hilum, (6.0–)13.3–13.9–14.5(–21.0) × (3.0–)3.8–3.9–4(–5.0) µm with a length : width ratio of (1.5–)3.3–3.5–3.7(–5.4), 1-septate (12.0–)16.6–17.1–17.6(–20.0) × (4.0–)4.4–4.6–4.7(–5.0) µm with a length : width ratio of (2.8–)3.6–3.8–3.9(–5.0). *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) sporodochial masses. *Chlamydospores* globose to

subglobose to ellipsoidal, 8–20 × 7–14 µm, smooth but often appearing rough due to deposits, thick-walled, mostly occurring in chains or irregular clusters, becoming medium brown, and formed abundantly in mature colonies.

Holotype: **Portugal**: Estremoz, *Vitis vinifera*, base of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C, 2003, coll./isol. C. Rego and T. Nascimento, CBS H-20574, culture ex-type CBS 129085 = Cy145.

Cardinal temperatures for growth: Minimal temperature not determined, at 5 ºC after 7 d colonies on PDA grew 5–8 mm. Optimum temperature between 20 and 25 ºC, when colonies reached 33–41 mm, and 37–43 mm, respectively. For some isolates no growth was observed at 30 ºC, while others grew 1–4 mm; no growth was observed at 35 ºC.

Culture characteristics: Mycelium cottony to felty, with an average to strong density. Surface on OA buff to saffron to cinnamon; margin amber to pure yellow. On PDA buff to sienna; margin luteous. No zonation was observed, and transparency was homogeneous. Margins were even, or sometimes slightly uneven. In reverse colonies were similar in colour, except on PDA, where they varied from buff to saffron to chestnut.

Isolates studied: CBS 129085; Cy135; Cy144; Cy146; Cy147; Cy148; Cy149; Cy150; Cy151; Cy152; Cy153; Cy243, CPC 13539 (Table 1).

Hosts and distribution: *Picea glauca* in Canada and *Vitis vinifera* (base and grafting zone) in Portugal.

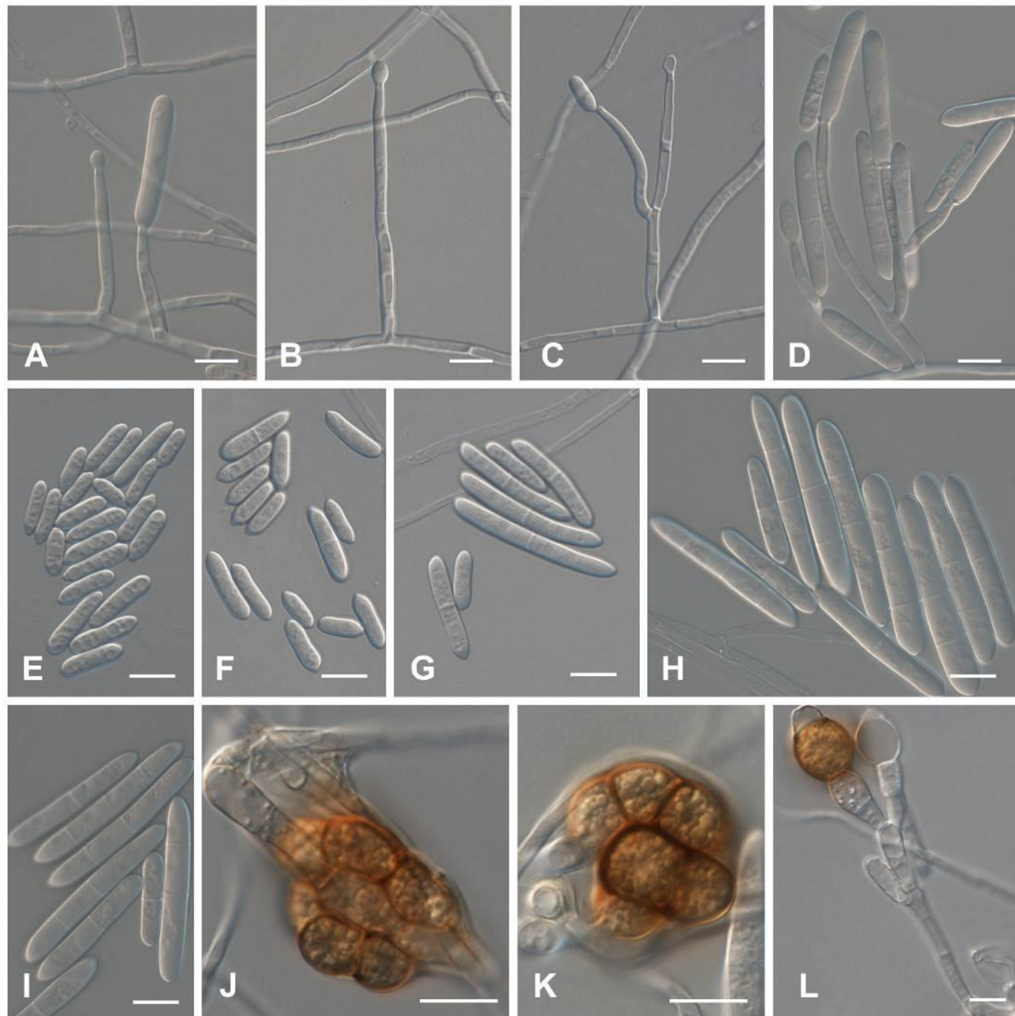

Fig. 3. *Ilyonectria estremocensis*. (A-D) Simple, sparsely branched conidiophores of the aerial mycelium. (E-I) Micro and macroconidia. (J-L) Chlamydospores. Bars 10 µm. All from isolate CBS 129085.

Ilyonectria novozelandica A. Cabral & Crous, **sp. nov.** MycoBank 560154. Figs 4, 5.

Etymology: Named after the country from where the holotype was collected, New Zealand.

Ilyonectriae macrodidymae similis, sed macroconidiis majoribus, (23.0–)36.8–38.4–40.3(–55.0) × (5.0–)6.3–6.5–6.8(–8.5) µm.

Perithecia formed heterothallically *in vitro*, disposed solitarily or in groups, developing directly on the agar surface or on sterile pieces of birch wood, ovoid to obpyriform, dark-red, becoming purple-red in 3 % KOH (positive colour reaction), smooth to finely warted, 220–270 × 300–350 µm high when rehydrated; without recognisable stroma; perithecial wall consisting of two poorly distinguishable regions; outer region 18–35 µm thick, composed of 1–3 layers of angular to subglobose cells, 9–30 × 5–17 µm; cell walls up to 2 µm thick; inner region up to 15 µm thick, composed of cells that are flat in transverse optical section and angular to oval in subsurface

optical face view; walls in the outer and inner region sometimes locally thinning to form pseudopores in conjunction with matching structures in adjacent cells; Asci clavate to narrowly clavate, ca. 55–65 × 8–10 µm, 8-spored; apex rounded, with a minutely visible ring. Ascospores divided into two cells of equal size, ellipsoidal to oblong-ellipsoidal, somewhat tapering towards the ends, smooth to finely warted, (10.9–)13.5(–15.2) × (3.3–)4.2(–6.3) µm.

Fertile matings: Perithecia observed after 4 wk in crossings of strains: CBS 113552 × CBS 112593; CBS 113552 × Cy130; CBS 113552 × CBS 112608.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to three phialides, 1–4-septate, 40–150 µm long; phialides monophialidic, more or less cylindrical, but tapering slightly in the upper part towards the apex, 20–45 µm long, 2.0–3.5 µm wide at the base, 2.5–3.5 µm at the widest point, and 1.5–2.5 µm wide at the apex. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched; phialides more or less cylindrical, but tapering slightly in the upper part towards the apex, or narrowly flask-shaped, mostly with the widest point near the middle, 15–23 µm long, 2.5–3.5 µm wide at the base, 2.5–4.0 µm at the widest point, and 1.5–2.5 µm wide at the apex. *Macroconidia* predominant, formed on both types of conidiophores; on SNA formed in flat domes of slimy masses, (1–)3(–4)-septate, straight or minutely curved, cylindrical or minutely widening towards the tip, appearing somewhat clavate, particularly when still attached to the phialide; apex or apical cell typically slightly bent to one side and minutely beaked; base mostly with a visible, centrally located or laterally displaced hilum; 1-septate conidia (20.0–)26.1–27.4–28.7(–42.0) × (4.0–)5.2–5.4–5.6(–7.0) µm with a length : width ratio of (3.8–)4.9–5.1–5.2(–7.0); 2-septate conidia (22.0–)27.9–29.1–30.3(–40.0) × (5.0–)5.6–5.8–6.0(–7.0) µm, with a length : width ratio of (3.7–)4.9–5.1–5.2(–6.2)µm, and 3-septate conidia (23.0–)36.8–38.4–40.3(–55.0) × (5.0–)6.3–6.5–6.8(–8.5) µm, with a length : width ratio of (4.6–)5.7–5.9–6.2(–8.7) µm. *Microconidia* 0–1-septate, ellipsoid to ovoid, more or less straight, with a minutely or clearly laterally displaced hilum, constricted at the septum; 0-septate microconidia (6.0–)9.8–10.5–11.3(–17.0) × (3.5–)4.0–4.1–4.2(–5.0) µm, with a length : width ratio of (1.5–)2.4–2.6–2.8(–4.3); 1-septate conidia (10.0–)14.1–14.7–15.3(–19.0) × (3.5–)4.3–4.4–4.5(–5.0) µm, with a length:width ratio of (2.4–)3.2–3.3–3.5(–4.8) µm. Conidia formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses as well as on complex conidiophores. *Chlamydospores* rarely occur, globose to subglobose, 7–11 × 6–8 µm, smooth but often appearing rough due to deposits, thick-walled, mostly occurring in chains.

Holotype: **New Zealand:** Candy P New Ground, *Vitis vinifera*, 2003, coll./isol. R. Bonfiglioli, CBS H-20575, culture ex-type CBS 113552. The teleomorph is represented by a fertile mating between CBS 113552 × CBS 112593.

Cardinal temperatures for growth: Colonies on PDA grow poorly (1–5 mm diam) at 5 ºC after 7 d. Optimum temperature between 20–25 ºC, when colonies reach 28–37 mm and 31–41 mm respectively. Maximum temperature around 30 ºC, when colonies reach 3–8 mm; no growth was observed at 35 ºC.

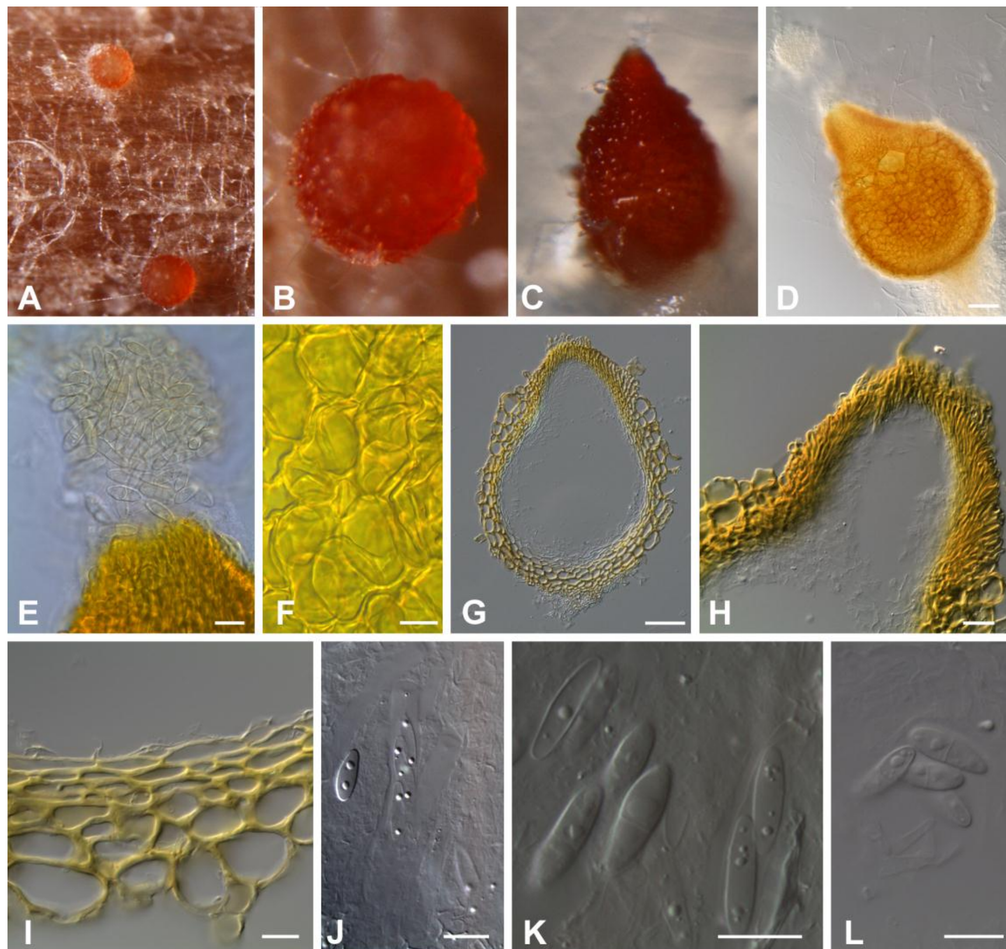

Fig. 4. *Ilyonectria novozelandica* (A-C) Development of perithecia on the surface of birch toothpick or agar.(D-F) Perithecium mounted in lactic acid, (E) ostiolar area, (F) surface view of perithecium wall region(G-I) Longitudinal sections of perithecia showing details of ostiolo and wall. (J) Ascos and ascospores. (K-L) Ascospores. Bars A-C, D, G - 50 µm; H - 20 µm; E,F,I-L - 10 µm. All from crossing of CBS 113552 × CBS 112593.

Culture characteristics: Mycelium cottony to felty with average to strong density. Surface on OA buff to amber; margin buff to luteous. Surface on PDA buff to saffron to chestnut; margin buff to

luteous; no zonation was observed, and transparency was homogeneous; margins predominantly even. Reverse similar to surface, except chestnut to buff to saffron on PDA.

Isolates studied: CBS 112593; CBS 112608; CBS 113552; Cy115–119; Cy124; Cy125; Cy129; Cy130; Cy230 (Table 1).

Hosts and distribution: *Festuca duriuscula* (Portugal), *Vitis vinifera* (New Zealand, South Africa, USA).

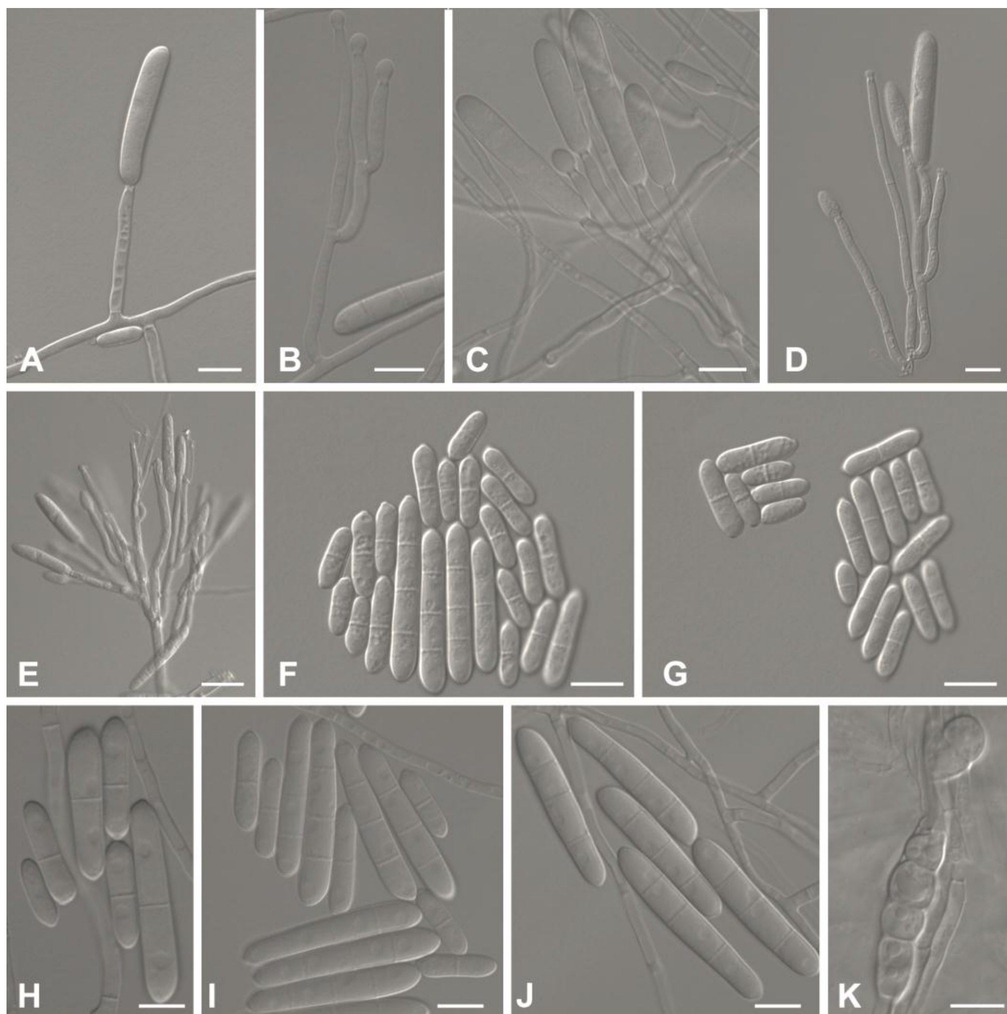

Fig. 5. *Ilyonectria novozelandica* (A-B) Simple, sparsely branched conidiophores of the aerial mycelium. (C-E) Complex conidiophores. (F-J) Micro and macroconidia. (K) Chlamydospores on mycelium. Bars 10 µm. A, D-G from Cy130; B-C, H-K from CBS 113552.

Ilyonectria torresensis A. Cabral, Rego & Crous, **sp. nov.** MycoBank 560155. Figs 6, 7.

Etymology: Named after the Portuguese city of Torres Vedras, where the holotype was collected.

Ilyonectriae macrodidymae similis, sed macroconidiis majoribus, (30.0–)38.3–39.4–40.6(–56.0) × (5.0–)6.7–6.8–7.0(–9.0) µm.

Perithecia formed heterothallically *in vitro*, disposed solitarily or in groups, developing directly on the agar surface or on sterile pieces of birch wood, ovoid to obpyriform, dark-red, becoming purple-red in 3 % KOH (positive colour reaction), smooth to finely warted, 210–270 × 260–320 µm high when rehydrated; without recognisable stroma; perithecial wall consisting of two poorly distinguishable regions; outer region 17–30 µm thick, composed of 1–3 layers of angular to subglobose cells, 13–22 × 7–13 µm; cell walls up to 2 µm thick; inner up to 10 µm thick, composed of cells that are flat in transverse optical section and angular to oval in subsurface optical face view; walls in the outer and inner region sometimes locally thinning to form pseudopores in conjunction with matching structures in adjacent cells. Asci clavate to narrowly clavate, ca. 55–65 × 8–10 µm, 8-spored; apex rounded, with a minutely visible ring. Ascospores divided into two cells of equal size, ellipsoid to oblong-ellipsoid, somewhat tapering towards the ends, smooth to finely warted, (10.1–)13.9(–15.8) × (4.1–)5.3(–6.4) µm.

Fertile matings: Perithecia observed after 4 wk in crossings of strains: Cy71 × Cy222; Cy118× Cy222; Cy120× Cy222; Cy137 × Cy222; Cy223 × Cy222; Cy240 × Cy222; CBS 129086 × Cy222, CBS 129086 × Cy214.

Conidiophores simple or complex, sporodochial. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched, bearing up to three phialides, 1–6-septate, 28–180 µm long; phialides monophialidic, more or less cylindrical, with slight taper towards the apex, 18–40 µm long, 2.0–3.5 µm wide at the base, 2.5–3.5 µm at the widest point, and 1.5–2.5 µm wide at the apex. *Complex conidiophores* aggregated in small sporodochia, repeatedly and irregularly branched; phialides more or less cylindrical, but tapering slightly in the upper part towards the apex, or narrowly flask-shaped, mostly with widest point near the middle, 17–22 µm long, 2.5–3.0 µm wide at the base, 3.5–4.0 µm at the widest point, and 1.5–2.0 µm wide near the apex. *Macroconidia* predominating, formed on both types of conidiophores; on SNA formed in flat domes of slimy masses, (1–)3(–4)-septate, straight or minutely curved, cylindrical, or with minute widening towards the tip, appearing somewhat clavate, particularly when still attached to the phialide, with apex or apical cell typically slightly bent to one side and minutely beaked; base mostly with a visible, centrally located or laterally displaced hilum; 1-septate conidia (20.0–)26.5–27.7–28.9(–43.0) × (4.5–)5.5–5.6–5.8(–7.0) µm, with a length : width ratio of (3.3–)4.7–4.9–5.1(–7.2)

µm, 2-septate conidia (24.0–)31.4–32.5–33.6(–44.0) × (5.0–)6.0–6.2–6.4(–8.0) µm, with a length : width ratio of (3.7–)5.1–5.2–5.4(–6.7) µm, and 3-septate conidia (30.0–)38.3–39.4–40.6(–56.0) × (5.0–)6.7–6.8–7.0(–9.0) µm, with a length : width ratio of (4.3–)5.7–5.8–6.0(–7.9) µm. *Microconidia* 0–1-septate, ellipsoidal to ovoid, more or less straight, with a minutely or clearly laterally displaced hilum, with a constriction on the septum; 0-septate microconidia (9.0–)11.8–12.3–12.7(–16.0) × (3.5–)4.2–4.3–4.4(–5) µm with a length : width ratio of (2.0–)2.8–2.9–3(–4.0) µm, 1-septate conidia (11.0–)15.0–15.5–16.0(–20.0) × (3.5–) 4.3–4.4–4.5(–5.5) µm with a length : width ratio of (2.4–)3.4–3.6–3.7(–4.8) µm. Conidia formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses, as well as on complex conidiophores. *Chlamydospores* rarely occur, globose to subglobose, 6–15 × 5–13 µm, smooth but often appearing rough due to deposits, thick-walled, mostly occurring in chains.

Holotype: **Portugal**: Torres Vedras, *Vitis vinifera*, asymptomatic; scion Chenin, 2007, coll./isol. A. Cabral, CBS H-20576, culture ex-type CBS 129086 = Cy218. The teleomorph is represented by a fertile mating between CBS 129086 × Cy222.

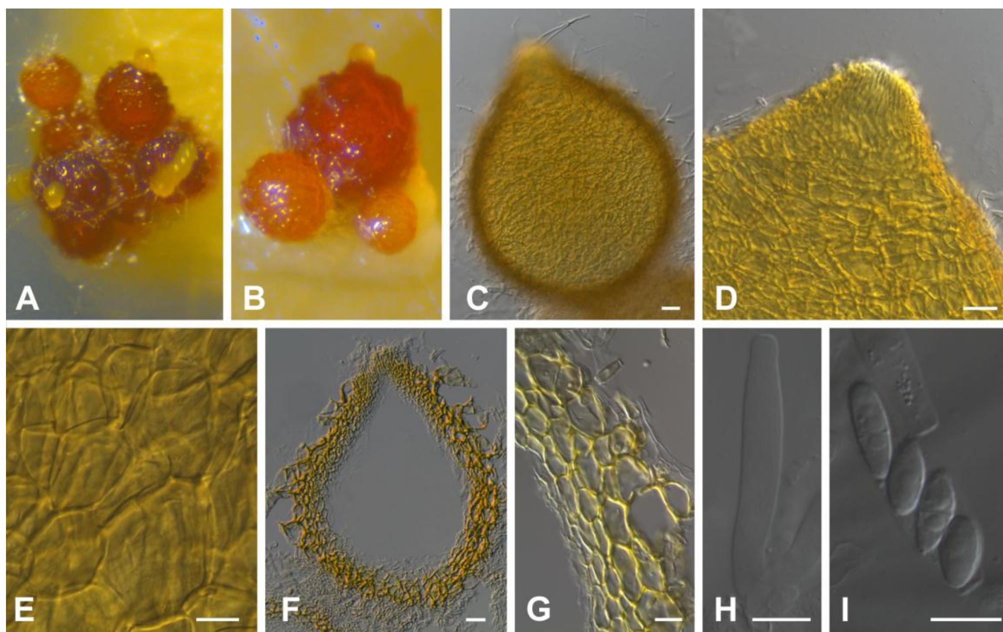

Fig. 6. *Ilyonectria torresensis* (A-B) Development of perithecia on the surface of birch toothpick .(C-E) Perithecium mounted in lactic acid, (D) ostiolar area, (E) surface view of perithecium wall region(F-G) Longitudinal sections of perithecia showing detail of wall. (H) Asci. (I) Ascospores. Bars A-B - 50 µm; C-D,F - 20 µm; E,G-I- 10 µm. A-B from crossing of CBS 129086 × Cy222; C-E, H-I from crossing of Cy118 × Cy222 and F-G from crossing of Cy120 × Cy222.

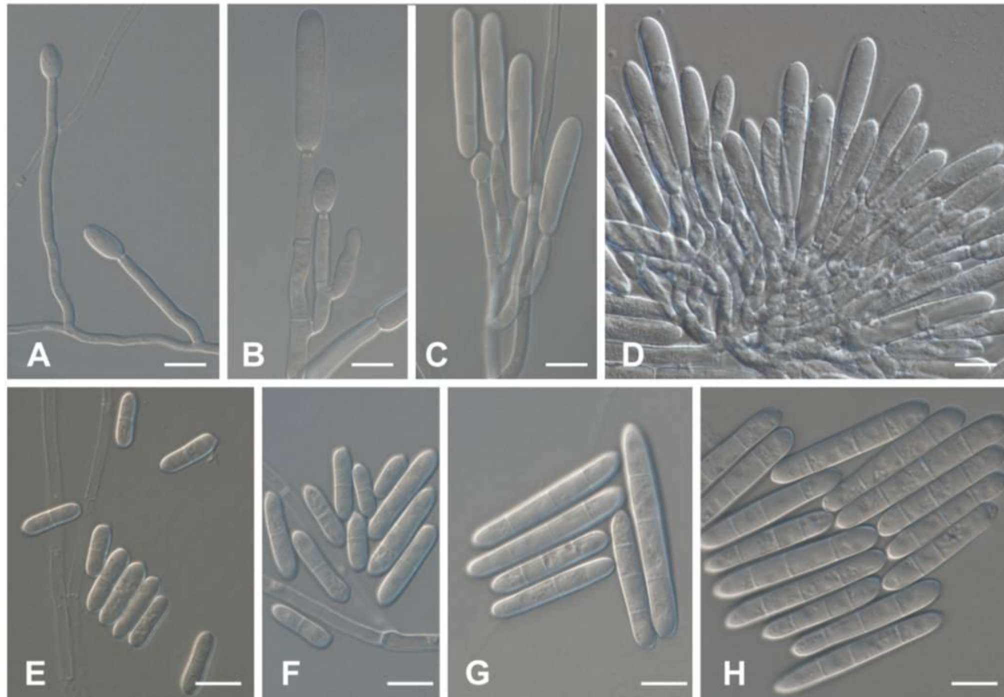

Fig. 7. *Ilyonectria torresensis* (A-C) Simple, sparsely branched conidiophores of the aerial mycelium. (D) Complex conidiophores. (E-H) Micro and macroconidia. Bars 10 µm. All from isolate CBS 129086.

Cardinal temperatures for growth: Colonies on PDA grow poorly (1–6 mm diam) at 5 ºC after 7 d. Optimum temperature for growth is between 20–25 ºC, when colonies reach 21–38 mm and 31–44 mm, respectively. For some isolates no growth was observed at 30 ºC, whereas others grew 1–6 mm; no growth was observed at 35 ºC.

Culture characteristics: Mycelium cottony to felty with an average to strong density. Surface on OA buff to saffron to chestnut, with a saffron to luteous margin. On PDA pale buff to chestnut; aerial mycelium buff to luteous, and margin pale buff to amber. Zonation absent to concentric, with homogeneous transparency; margins predominantly even. Colonies similar in reverse, except on PDA, buff to umber to chestnut.

Isolates studied: CBS 119.41; CBS 188.49; CBS 112604; CBS 112609; CBS 113555; CBS 112598; CBS 129086; CPC 13533; Cy69; Cy71; Cy72; Cy75; Cy96; Cy97; Cy118; Cy120; Cy132; Cy136–138; Cy141–143; Cy157; Cy214; Cy221–223; Cy235; Cy237; Cy240; Cy246; Cy260; Cy262 (Table 1).

Hosts and distribution: *Abies nordmanniana* (root) (Netherlands), *Fragaria* sp. (root) (Netherlands), *Fragaria* × *ananassa* (USA), *Quercus* sp. (root) (Austria), *Vitis vinifera* (roots, basal end and grafting zone) (Australia, Canada, New Zealand, Portugal, South Africa, Spain, USA).

The comparative analysis of morphological results shows that *I. torresensis*, *I. alcacerensis*, *I. novozelandica* and *I. macrodidyma* are similar in broad terms, but some characteristics can be used to distinguish these species. *Ilyonectria alcacerensis* is the most contrasting species, presenting conidia with up to six septa (the remaining species having only up to four septa), longer and wider conidia (particularly for 3-septate conidia). *Ilyonectria novozelandica* has slightly more elongated and shorter 3-septate conidia, and less septate and shorter conidiophores than *I. torresensis*. The 3-septate conidia of *I. macrodidyma* are on average the smallest.

Ilyonectria estremocensis can clearly be distinguished on both morphology and DNA sequence from the group represented by *I. torresensis*, *I. alcacerensis*, *I. novozelandica* and *I. macrodidyma*, since in *I. estremocensis* microconidia are cylindrical, and not ellipsoid to ovoid; 1-septate macroconidia are predominant, instead of 3-septate conidia; the macroconidial apex is round, and not slightly bent to one side nor minutely beaked; on average, conidia of the other species tend to be longer, and have a larger length:width ratio than *I. estremocensis*.

Discussion

Black foot disease of grapevine has in the past been mainly attributed to three species, namely *I. liriodendri*, *I. macrodidyma* and *'Cylindrocarpon' pauciseptatum*. Since the first description of *I. macrodidyma* as a new species (Halleen et al. 2004), several additional reports have implicated this pathogen as the causal agent of the disease (Abreo et al. 2010; Alaniz et al. 2007; Auger et al. 2007; Petit and Gubler 2005; Rego et al. 2005).

In the present study we compared the Biological Species Concept (sexual compatibility within lineages) (Mayr 1963) to the Morphological Species Concept (morphological divergence), and the Phylogenetic Species Criterion (divergence based on DNA sequence data) (Taylor et al. 2000). As phylogenetic species could still retain interspecies compatibility (O'Donnell et al. 2004), and species in the *I. macrodidyma* complex are morphologically rather similar, we followed Genealogical Concordance Phylogenetic Species Recognition to recognise species within this complex (Taylor et al. 2000, Dettman et al 2003, Schoch et al. 2009, Lombard et al. 2010). By employing this concept on a collection of 81 *I. macrodidyma*-like isolates, mainly collected from young vineyards or rootstock nurseries showing black foot symptoms, and 13 from other hosts (Table 1), six new species of *Ilyonectria* could be recognised. Four of the latter were named in this study, namely *I. estremocensis*, *I. torresensis*, *I. alcacerensis* and *I. novozelandica*, while a further two *Ilyonectria* spp. will be treated elsewhere.

Ilyonectria estremocensis, isolated from grapevine in Portugal and white spruce (*Picea glauca*) in Canada, is characterised by straight to slightly curved, predominantly 1-septate macroconidia with round apices and abundant chlamydospores. Crosses between isolates of *I. estremocensis* failed to produce perithecia with viable ascospores. *Ilyonectria estremocensis* can clearly be distinguished on both morphology and DNA sequence level from the group formed by *I. torresensis*, *I. alcacerensis*, *I. novozelandica* and *I. macrodidyma*. *Ilyonectria torresensis* was the species with the widest occurrence, being present on four continents, and associated with *Vitis vinifera, Abies nordmanniana* (root), *Fragaria* sp., and *Quercus* sp. (root). *Ilyonectria alcacerensis* on the other hand, was so far only isolated from *Vitis vinifera* on the Iberian Peninsula. *Ilyonectria novozelandica* is mainly associated with *Vitis vinifera* in New Zealand, South Africa and USA but was also identified in *Festuca duriuscula* in Portugal.

Within the species identified close to *I. macrodidyma*, *I. alcacerensis* is the most contrasting species, having conidia with up to six septa (the remaining species having up to four septa) and longer and wider conidia (the later only for 3-septate conidia). *Ilyonectria torresensis* rarely produced conidia with four septa, and 3-septate conidia are wider than *I. novozelandica*. A comparison to data from Halleen et al. (2004) shows that the 3-septate conidia of *I. macrodidyma* are the shortest. The employment of ITS, TUB, HIS, and TEF sequence diversity analysis to a collection of isolates previously identified as *I. macrodidyma* made it possible to identify a level of polymorphism that enabled the description of four novel species. The resolving capacity of the different genes under study ranged from a minimum for ITS, with which none of the species could be distinguished, to a maximum for HIS, with 1.6 (between *I. novozelandica* and *I. macrodidyma*; *I. novozelandica* and *I. torresensis*) to 2.6 (between *I. macrodidyma* and *I. alcacerensis*) percent diversity.

In a previous study, genetic diversity among *I. macrodidyma* isolates had been characterised both phenotypically and by nucleotide sequence analysis of ribosomal genes (LSU, SSU and ITS) and part of the TUB gene, but low levels of diversity had been found in nucleotide sequences. Halleen et al. (2004) found four variable sites in the partial TUB gene among *I. macrodidyma* isolates, but this variation did not appear to correlate with host diversity or geographical patterns. Furthermore, Petit and Gubler (2005) also found very little DNA variation in *I. macrodidyma* (ITS rDNA, partial TUB gene, and mtSSU rDNA sequencing), contrasting with the wide range of geographical origin of isolates, which included South Africa, Chile, and four counties in California.

Alaniz et al. (2007) reported low variation in the partial TUB gene data generated from a collection of Spanish isolates of *I. macrodidyma,* but found high levels of diversity among the

same isolates using ISSR markers and pathogenicity tests. However, no direct comparison can be made to the present study, because just two isolates are common to both studies (Alaniz et al. 2009a). Menkis and Burokienė (2011) studied a collection of 123 isolates of *I. macrodidyma* from forest nurseries, and reported two distinct IGS types, each respectively comprising 11 and 14 genotypes as revealed by an arbitrary primed PCR fragment analysis.

Although morphological characteristics play a major role in the description of fungal species (Brasier 1997; Taylor et al. 2000), the use of such characters alone to delimit these new species has proved insufficient, thus highlighting the usefulness of DNA sequence characters for such purpose. These results reinforce the applicability of these genes for species delimitation in *Nectriaceae*, as has been shown recently in *Calonectria* (Lombard et al. 2010).

Acknowledgements: We would like to thank Dr M.C. Silva (Coffee Rust Research Centre / Tropical Research Institute, Portugal) for making equipment available, and assisting with the use of the cryostat for perithecial sectioning. Drs F. Caetano (Instituto Superior de Agronomia, Portugal), J. Armengol (Univ. Politécnica de Valencia, Spain), K.A. Seifert (Agriculture and Agri-Food, Canada), L. Leandro (Iowa State University, Department of Plant Pathology, USA) and W.D. Gubler (Univ. California, Davis, USA) and are thanked for making several strains available, without which this study would not have been possible. This article is part of a Ph.D. dissertation (Instituto Superior de Agronomia, Technical University of Lisbon, Portugal). This work was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/24790/2005; project PTDC/AGR-AAM/099324/2008).

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Table 1. Details pertaining to isolates investigated during this study. Ex-type strains are marked in bold type

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
Campylocarpon fasciculare	**CBS 112613; STE-U 3970; C 76**	F. Halleen, 2000	*Vitis vinifera*, trunk of young grapevine showing decline symptoms; scion Cabernet Sauvignon; rootstock Richter 99	South Africa, Western Cape, Riebeeck Kasteel	AY677301	AY677221	JF735502	JF735691
Campylocarpon pseudofasciculare	**CBS 112679; STE-U 5472; HJS-1227**	F. Halleen, 2000	*Vitis vinifera*, roots, asymptomatic nursery grapevine plant; scion Sultana; rootstock Ramsey	South Africa, Western Cape, Wellington	AY677306	AY677214	JF735503	JF735692
***Ilyonectria* sp. 1**	CBS 162.89	M. Barth, 1988	*Hordeum vulgare*, root	Netherlands, Noordoostpolder, Marknesse, Lovinkhoeve	AM419060	AM419084	JF735610	JF735799
***Ilyonectria* sp. 2**	Cy108	C. Rego, 1999	*Vitis vinifera*, basal end of a 4 yr old plant showing decline symptoms; scion Aragonez; rootstock SO4	Portugal, Nelas	JF735316	AM419100	JF735611	JF735800
	Cy200	N. Cruz, 2005	*Vitis vinifera*, basal end of a 16 yr old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço	JF735317	JF735445	JF735612	JF735801
	CBS 159.34; IMI 113891; MUCL 4084; VKM F-2656	H.W. Wollenweber, 1934		Germany	JF735318	JF735446	JF735613	JF735802
	CBS 173.37; IMI 090176	T.R. Peace, 1937	*Pinus laricio*, associated with dieback	UK, England, Devon, Haldon	JF735319	JF735447	JF735614	JF735803
I. estremocensis	Cy135	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419069	AM419105	JF735615	JF735804
	Cy144	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419074	AM419107	JF735616	JF735805

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
	CBS 129085; Cy145	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735320	JF735448	JF735617	JF735806
	Cy146	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735321	JF735449	JF735618	JF735807
	Cy147	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735322	JF735450	JF735619	JF735808
	Cy148	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735323	JF735451	JF735620	JF735809
	Cy149	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735324	JF735452	JF735621	JF735810
	Cy150	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735325	JF735453	JF735622	JF735811
	Cy151	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5 yr old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735326	JF735454	JF735623	JF735812
	Cy152	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5 yr old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735327	JF735455	JF735624	JF735813
	Cy153	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5 yr old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735328	JF735456	JF735625	JF735814
	Cy243	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Touriga Nacioal; rootstock 110R	Portugal, Vidigueira	JF735329	JF735457	JF735626	JF735815
	CPC 13539; CCFC226730; 94-1685	R. C. Hamelin, 1994	*Picea glauca*	Canada, Quebec	JF735330	JF735458	JF735627	JF735816

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
I. alcacerensis	Cy133; IAFM Cy9-1	J. Armengol	*Vitis vinifera*	Spain, Valencia, L'Alcudia	JF735331	JF735459	JF735628	JF735817
	Cy134; IAFM Cy20-1	J. Armengol	*Vitis vinifera*	Spain, Ciudad Real, Villarubia de los Ojos	JF735332	AM419104	JF735629	JF735818
	CBS 129087; Cy159	A. Cabral and H. Oliveira, 2004	*Vitis vinifera*, basal end of a 3 yr old plant with root discolouration and decline symptoms; scion Sangiovese; rootstock 1103P	Portugal, Alcácer do Sal, Torrão	JF735333	AM419111	JF735630	JF735819
I. novozelandica	CBS 112593; STE-U 3990; C 107	F. Halleen, 2000	*Vitis vinifera*, Pinotage/101-14 Mgt	South Africa, Western Cape, Wellington, Voorgroenberg	AY677281	AY677236	JF735631	JF735820
	CBS 112608; STE-U 3987; C 62	F. Halleen, 2000	*Vitis vinifera*, Chardonnay/101-14 Mgt	South Africa, Western Cape, Citrusdal	AY677288	AY677235	JF735632	JF735821
	CBS 113552; STE-U 5713; HJS-1306; NZ C 41	R. Bonfiglioli, 2003	*Vitis* sp. decline of nursery plants dead rootstocks	New Zealand, Candy P New Ground	JF735334	AY677237	JF735633	JF735822
	Cy115	W.D. Gubler	*Vitis vinifera*	USA, California	JF735335	JF735460	JF735634	JF735823
	Cy116	W.D. Gubler	*Vitis vinifera*	USA, California	AJ875322	JF735461	JF735635	JF735824
	Cy117	W.D. Gubler	*Vitis vinifera*	USA, California	AJ875321	JF735462	JF735636	JF735825
	Cy119	W.D. Gubler	*Vitis vinifera*	USA, California	JF735336	JF735463	JF735637	JF735826
	Cy124	W.D. Gubler	*Vitis vinifera*	USA, California	JF735337	JF735464	JF735638	JF735827
	Cy125	W.D. Gubler	*Vitis vinifera*	USA, California	AM419066	JF735465	JF735639	JF735828
	Cy129	W.D. Gubler	*Vitis vinifera*	USA, California	JF735338	JF735466	JF735640	JF735829
	Cy130	W.D. Gubler	*Vitis vinifera*	USA, California	JF735339	JF735467	JF735641	JF735830
	Cy230	F. Caetano, 2005	*Festuca duriuscula*	Portugal, Lisbon	JF735340	JF735468	JF735642	JF735831

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
I. macrodidyma	CBS 112594; STE-U 3991; C 111	F. Halleen, 2000	*Vitis vinifera*, Pinotage/Richter 99	South Africa, Western Cape, Malmesbury, Jakkalsfontein	AY677282	AY677231	JF735643	JF735832
	CBS 112601; STE-U 3983; C 82	F. Halleen, 1999	*Vitis vinifera*, Pinotage/US 8-7	South Africa, Western Cape, Tulbagh	AY677284	AY677229	JF735644	JF735833
	CBS 112603; STE-U 4007; C 8	F. Halleen, 1999	*Vitis vinifera*, Sauvignon blanc/Richter 110	South Africa, Western Cape, Darling	AY677285	JF735469	JF735645	JF735834
	CBS 112605; STE-U 3984; C 106	F. Halleen, 2000	*Vitis vinifera*, Sultana/143-B Mgt	South Africa, Western Cape, Malmesbury, Jakkalsfontein	AY677287	AY677230	JF735646	JF735835
	CBS 112615; STE-U 3976; C 98	F. Halleen, 2000	*Vitis vinifera*, roots, asymptomatic nursery grapevine plant scion Sultana; rootstock 143-B Mgt	South Africa, Western Cape, Malmesbury, Jakkalsfontein	AY677290	AY677233	JF735647	JF735836
	Cy123; C08	W.D. Gubler	*Vitis vinifera*	USA, California	JF735341	JF735470	JF735648	JF735837
	Cy128; C20	W.D. Gubler	*Vitis vinifera*	USA, California	JF735342	JF735471	JF735649	JF735838
	Cy139	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419071	AM419106	JF735650	JF735839
	Cy140	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735343	JF735472	JF735651	JF735840
	Cy175	C. Rego, 2004	*Vitis vinifera*, basal discolouration in rootstocks; scion Touriga Nacional; rootstock 1103P	Portugal, Torre de Moncorvo	JF735344	JF735473	JF735652	JF735841
	Cy181	C. Rego, 2005	*Vitis vinifera*, scion 140-Ru; rootstock Aragonês	Portugal, Alcácer do Sal	JF735345	JF735474	JF735653	JF735842
	Cy216	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Marssanne	Portugal, Torres Vedras	JF735346	JF735475	JF735654	JF735843
	Cy244	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira	JF735347	JF735476	JF735655	JF735844

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
	Cy258	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735348	JF735477	JF735656	JF735845
I. torresensis	CBS 119.41	H.C. Koning	*Fragaria* sp., root	Netherlands, Baarn	JF735349	JF735478	JF735657	JF735846
	CBS 188.49	J.A. von Arx	*Abies nordmanniana*, root	Netherlands, Egmond	AM419063	AM419087	JF735658	JF735847
	CBS 112604; STE-U 4004; C 10	F. Halleen, 1999	*Vitis vinifera*, Cabernet Sauvignon/101-14 Mgt	South Africa, Western Cape, Paarl	AY677286	AY677227	JF735659	JF735848
	CBS 112609; STE-U 3969; HJS-1217	M. Sweetingham, 1979	*Vitis* sp., dark brown discolouration in trunk; scion Cabernet Sauvignon	Australia, Tasmania, Bream Creek	AY677289	AY677226	JF735660	JF735849
	CBS 113555; STE-U 5715; HJS-1309; NZ C 60	R. Bonfiglioli, 2003	*Vitis* sp., blackening areas in wood and roots; scion Pinot Noir; rootstock 101-14	New Zealand, Fiddlers Green	JF735350	AY677234	JF735661	JF735850
	CBS 112598; STE-U 3997; C 115	F. Halleen, 2000	*Vitis vinifera*, Sultana/Ramsey	South Africa, Western Cape, Wellington, Leliefontein	JF735351	JF735479	JF735662	JF735851
	CPC 13533; CCFC 144524; Dias 2B	H.F. Dias, 1972	*Vitis vinifera*, Concord Bradt grapes, roots and stems	Canada, Ontario	AY295332	JF735480	JF735663	JF735852
	Cy69	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock SO4, clone 102F	Portugal, Ribatejo e Oeste	AJ875332	AM419095	JF735664	JF735853
	Cy71	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 99R, clone 96F	Portugal, Ribatejo e Oeste	AJ875335	AM419096	JF735665	JF735854
	Cy72	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock clone 113F	Portugal, Ribatejo e Oeste	AJ875336	AM419097	JF735666	JF735855
	Cy75	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 99R	Portugal, Ribatejo e Oeste	AJ875334	AM419098	JF735667	JF735856
	Cy96	E. Halmschlager	*Quercus* sp., root	Austria, Patzmannsdorf	JF735352	JF735481	JF735668	JF735857

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
	Cy97	E. Halmschlager	*Quercus* sp., root	Austria, Patzmannsdorf	JF735353	JF735482	JF735669	JF735858
	Cy118; C07	W.D. Gubler	*Vitis vinífera*	USA, California	JF735354	JF735483	JF735670	JF735859
	Cy120; C12	W.D. Gubler	*Vitis vinífera*	USA, California	AJ875320	AM419101	JF735671	JF735860
	Cy132; IAFM Cy1-1	J. Armengol	*Vitis vinifera*	Spain, Alicante	JF735355	JF735484	JF735672	JF735861
	Cy136	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735356	JF735485	JF735673	JF735862
	Cy137	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419070	JF735486	JF735674	JF735863
	Cy138	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735357	JF735487	JF735675	JF735864
	Cy141	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735358	JF735488	JF735676	JF735865
	Cy142	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, grafting zone of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735359	JF735489	JF735677	JF735866
	Cy143	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 yr old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735360	JF735490	JF735678	JF735867
	Cy157	H. Oliveira, 2004	*Vitis vinifera*, scion Touriga Nacional; rootstock 99R	Portugal, Alenquer	AM419077	AM419110	JF735679	JF735868
	Cy214	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Grenache	Portugal, Torres Vedras	JF735361	JF735491	JF735680	JF735869
	CBS 129086; **Cy218**	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Chenin	Portugal, Torres Vedras	JF735362	JF735492	JF735681	JF735870

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
	Cy221 MTF6BH2	L. Leandro	*Fragaria x ananassa*	USA, North Carolina, Asheville	JF735363	JF735493	JF735682	JF735871
	Cy222 MT1 17BD1	L. Leandro	*Fragaria x ananassa*	USA, North Carolina, Asheville	JF735364	JF735494	JF735683	JF735872
	Cy223 MT2 20AD2	L. Leandro	*Fragaria x ananassa*	USA, North Carolina, Asheville	JF735365	JF735495	JF735684	JF735873
	Cy235	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735366	JF735496	JF735685	JF735874
	Cy237	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Chardonnay; rootstock 110R	Portugal, Vidigueira	JF735367	JF735497	JF735686	JF735875
	Cy240	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Touriga Nacional; rootstock 140RU	Portugal, Vidigueira	JF735368	JF735498	JF735687	JF735876
	Cy246	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Antão Vaz; rootstock 110R	Portugal, Vidigueira	JF735369	JF735499	JF735688	JF735877
	Cy260	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735370	JF735500	JF735689	JF735878
	Cy262	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 yr old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735371	JF735501	JF735690	JF735879

[a]CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CCFC: Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, Canada; CPC: Culture collection of Pedro Crous, housed at CBS; Cy: Cylindrocarpon collection housed at Laboratório de Patologia Vegetal 'Veríssimo de Almeida' - ISA, Lisbon, Portugal; HJS: Culture collection of Hans-Josef Schroers; IAFM: Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain; IMI: International Mycological Institute, CABI-Bioscience, Egham, U.K.; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain, Belgium; STE-U: Stellenbosch University, Stellenbosch, South Africa; VKM: All-Russian Collection of Microorganisms, Moscow, Russia.

CHAPTER 4

CHARACTERIZATION OF MATING TYPE GENES IN *ILYONECTRIA* SPECIES CAUSING BLACK FOOT OF GRAPEVINE

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Fungal Genetics and Biology (in preparation)

Keywords: *Ilyonectria*, Mating-type, population structure, *Vitis vinifera*

Abstract

The genus *Ilyonectria* comprises most of the causal agents of black foot disease of grapevines. The recent increase in incidence and severity of this disease, along with the increase in genetic diversity of its causal agents, led to the need to better understand the occurrence of recombination in such species, which in turn requires a thorough knowledge of the mating types in these fungi. In the present study, a chromosome walking strategy was followed to obtain the entire mating-type loci of *I. liriodendri* and of *Ilyonectria* spp. from the *I. macrodidyma* species complex. From these results, the idiomorph structure of species belonging to the *I. macrodidyma* species complex was observed to match that of other heterothallic hypocrealean fungi. In contrast, however, the organization of the mating-type locus in *I. liriodendri* was completely different. Two types, A and B, could be distinguished. Both types contained *MAT1-2-1* and *MAT1-1-1*, but the type B also contained *MAT1-1-2* and *MAT1-1-3,* making it genetically homothallic. However, all 82 *I. liriodendri* isolates (both type A and B) tested were self-sterile and functionally heterothallic as the presence of the other type was required to complete the sexual cycle. This "pseudo-heterothallic" behaviour and the organization of the MAT locus, questions the feasibility of using *MAT1-1-1* and *MAT1-2-1* to define the mating type. Specific PCR primers were designed to screen 238 isolates from 23 *Ilyonectria* species from the *macrodidyma*, *radicicola* and *pauciseptatum* clusters for the presence of *MAT1-1-1* and/or *MAT1-2-1*. All isolates tested appeared to be heterothallic. The relative proportions of *MAT1-1* and *MAT1-2* found within *Ilyonectria* spp. collected from grapevine in Portugal during 1992-2008 suggest the occurrence of active recombination in these species. The correlation of the increased virulence of isolates with the occurrence of mating types that were not present earlier, together with the increased frequency of isolation of species that were absent during the 1990's, suggest that the appearance of new genotypes of black foot pathogens is largely responsible for the increased incidence and severity of the disease.

Introduction

In ascomycetes, sexual development is controlled by the mating type locus (*MAT*). In heterothallic (self-sterile) species, sexual reproduction occurs only between individuals of opposite mating-type. These alternative mating-types (*MAT1-1* and *MAT1-2*), are characterized by the presence of dissimilar sequences (idiomorph) at the mating-type locus (Metzenberg and Glass, 1990). Due to the lack of significant sequence similarity this specific portion of the genome does not undergo homologous recombination during meiosis (Coppin et al., 1997; Kronstad and Staben, 1997). Although the organization of the idiomorph varies greatly between fungal classes, the *MAT1-1* idiomorph always contains *MAT1-1-1*, encoding a protein with an alpha box motif, and the *MAT1-2* idiomorph always contains *MAT1-2-1*, encoding a protein with a high mobility group (HMG) DNA-binding motif.

Homothallic (self-fertile) species do not require the presence of complementary isolates for sexual reproduction to occur. These fungi contain all mating type genes in a single genome (physically linked or unlinked), organised in structures that are unique to each homothallic species (Coppin et al., 1997). MAT proteins were shown to complement each other by regulating the attraction between compatible mating types as a preliminary step to the mating process, besides being involved in later stages of sexual development by controlling the regulation of internuclear recognition (Casselton, 2002; Stanton and Hull, 2007; Turgeon and Debuchy, 2007; Zaffarano et al., 2010). In homothallic strains, the expression of the genes can circumvent the cellular recognition step between genes that are otherwise located in different individuals, thus resulting in selfing (Casselton, 2002; Lin and Heitman, 2007; Stanton and Hull, 2007; Turgeon and Debuchy, 2007).

Pseudo-homothallic species (*e.g.*, *Gelasinospora tetrasperma*, *Podospora anserina* and *Neurospora tetrasperma*; Sordariaceae) behave apparently as homothallic (self-fertile) organisms. This is because, due to physical changes to normal meiosis, each ascospore is heterokaryotic with one nucleus from each of the mating types, which in turn will germinate to produce homothallic (self-fertile) mycelia (Merino et al., 1996). Multiple independent transitions between heterothallism and homothallism are likely to have occurred in many genera, although in certain genera it is clear that homothallic species have arisen from heterothallic ancestors (Yun et al., 1999; Lee et al., 2010; O'Donnell et al., 2004; Martin et al., 2011).

Mating-type genes are very useful for phylogenetic analysis because of their high evolution rates and because of their direct involvement in mating and reproduction (Pöggeler, 2001; Turgeon, 1998). Because of the latter, population studies addressing the distribution and frequency of the

different idiomorphs in heterothallic species can be of utmost importance to understand recent-time population dynamics either on local, regional or global scales. This is of great relevance for phytopathogenic fungi, as it enables us to understand recent events of sexual recombination vs. clonality, eventually leading to the forecast of potential epidemics.

In most self-incompatible Sordariomycetes, (*e.g.*, the heterothallic Hypocreales *Gibberella fujikuroi*, *Claviceps purpurea*, *Cordyceps subsessilis*, *Ephelis japonica*, *Epichloë typhina* and *Metarhizium anisopliae*) the organization of the mating-type is similar to the organization in the model species *Neurospora crassa* (Arie et al., 1999; Glass et al., 1990; Staben and Yanofsky, 1990; Yokoyama et al., 2006; Yun et al., 2000). The *MAT1-1* idiomorph typically contains three genes (*MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*) whereas a single gene (*MAT1-2-1*) is found in the *MAT1-2* idiomorph.

In homothallic Sordariomycetes, such as *Fusarium graminearum*, all four *MAT* genes are adjacent within the MAT locus (Yun et al., 2000). Recently a new MAT gene was identified in a heterothallic *Fusarium* MAT1-2 idiomorph, termed *MAT1-2-3*, showing homology to FG08894 located adjacent to *MAT1-2-1* in the genome of homothallic *F. graminearum*. Putative homologs of MAT1-2-3 were also identified in two additional hypocrealean genomes (Martin et al., 2011).

The genus *Ilyonectria* was recently established for species with *Cylindrocarpon* anamorphs (Chaverri et al., 2011). *Ilyonectria* spp. (and other "*Cylindrocarpon*"-like fungi) are soil-borne ascomycetes, responsible for diseases in various host plants, *e.g.* black foot disease of grapevines (*Vitis vinifera*). In recent years, this disease has been recorded with increased incidence and severity throughout the world, affecting grapevines both in nurseries and young vineyards (Halleen et al., 2004; Oliveira et al., 2004). Furthermore, genetic diversity and even variability in virulence have been reported within these pathogens (Alaniz et al., 2009; Cabral et al., 2011a,b; Rego et al., 2001; Rego, 2004), leading to the identification of numerous species within the macrodidyma, radicicola and pauciseptatum clusters (as exemplified in Table 1). Within the macrodidyma species complex, the morphologically similar species *I. alcacerensis*, *I. macrodidyma, I. novozelandica* and *I. torresensis* were identified using Genealogical Concordance Phylogenetic Species Recognition (Taylor et al., 2000). In the radicicola species complex, *I. liriodendri* stands out as the most notorious pathogen associated with black foot of grapevines (Alaniz et al., 2007; Halleen et al., 2006). The recent increase in incidence and severity of black foot disease of grapevines as well as the levels of genetic diversity of the associated pathogens raises the need to understand population dynamics both at local and global scales. By determining patterns of sexual behaviour, the pathogen population structure

can be elucidated, and the disease spread forecast. This will potentially enable the deployment of more efficient plant protection strategies.

However, no information was available about the structure of mating type loci in *Ilyonectria* spp. Therefore, the objectives of this study were to characterise the structure of the *MAT* locus in *Ilyonectria* spp., and to identify the mating-type distribution within of several *Ilyonectria* spp., involved in black foot disease of grapevine. To achieve these objectives, the following strategy was employed: (1) the full-length mating type loci of *I. alcacerensis*, *I. macrodidyma, I. novozelandica*, *I. torresensis* and *I. liriodendri* were sequenced and characterized using PCR-based techniques; (2) a PCR method for rapid identification of the *MAT1-1-1* and *MAT1-2-1* genes was developed to determine the frequencies of the mating types in different Ilyonectria populations.

Material and Methods

Fungal material

All *Ilyonectria* isolates were obtained from culture collections as indicated in Table 1. Genomic DNA was extracted from pure cultures following the protocol by Möller et al. (1992) adapted by Crous et al. (2009). The full sequence of the *MAT* locus was obtained for eight *I. liriodendri* isolates [Cy27, Cy33, Cy36, Cy68 (CBS 117526), Cy76 (CBS 117527), Cy107, Cy 109 and Cy111] and for 20 isolates in the macrodidyma species complex (Cy123, Cy175, Cy216, CBS 112601, CBS 112603 and CBS 112615 from *I. macrodidyma*; Cy118, Cy137, Cy214, Cy222, Cy237, Cy246, CBS 112604 and CBS 113555 from *I. torresensis*; Cy133, Cy134 and Cy159 from *I. alcacerensis*; Cy130, CBS 112593 and CBS 113552 from *I. novozelandica*). Within each species, sexually compatible isolates were chosen based on results from previous studies (Halleen et al., 2004, 2006; Rego, 2004).

Population diversity studies based on mating type-specific PCR primers were performed for all 238 isolates listed in Table 1, including 82 *I. liriodendri* isolates (74 from grapevine in Portugal representing diverse regions and years), 46 *I. torresensis* isolates (40 from grapevine, mostly from Portugal), 15 *I. macrodidyma* isolates (from grapevine in Portugal, South Africa and USA), 15 *I. robusta* isolates (8 from *Quercus* spp. and the remaining from various hosts), 14 *I. mors-panacis* isolates (from *Panax* spp. in Canada), 12 *I. novozelandica* isolates (11 from grapevine in New Zealand, South Africa and USA), 10 *I. estremocensis* isolates (9 from grapevine in Portugal), nine *Cylindrocarpon pauciseptatum* isolates (eight from grapevine in Portugal and Slovenia), five

I. rufa isolates (three from Douglas-fir), five *I. crassa* isolates (three from American ginseng), four *I. pseudodestructans* isolates (two from grapevine), four *I. europaea* isolates (two from grapevine), three *Ilyonectria* sp2 isolates (from various hosts), three *I. alcacerensis* isolates (from grapevine in Portugal and Spain), and one isolate each from *I. anthuriicola*, *I. liliigena*, *I. lusitanica*, *I. panacis*, *I. venezuelensis*, *I. vitis* and *Ilyonectria* sp1.

To induce the formation of perithecia in *I. liriodendri* isolates, six isolates (Cy27, Cy68, Cy76, Cy107, Cy111 and CBS 110.81) were chosen to be crossed with all 82 isolates of *I. liriodendri* studied. Furthermore, all 82 isolates were self-crossed. Crosses were performed in 60 mm diam Petri dishes containing a minimal salts medium supplemented with two sterile birch toothpicks (Guerber and Correll, 2001). The plates were incubated at 20 ºC under n-UV light for 8–20 wk. Two isolates were considered sexually compatible if perithecia were formed that exuded masses of viable ascospores (i.e., capable of germinating).

Strategy for sequencing mating type loci

In an attempt to amplify the conserved region encoding the α box domain present in *MAT1-1-1*, the primer pair Fα1 and Fα2, designed by Kerényi et al. (2004) for *Fusarium* spp. was tested, but with no success. Based on *MAT1-1* sequences available for Hypocreales, namely *Nectriaceae* (*Fusarium/Gibberella)* and *Clavicipitaceae* a new primer set was designed: CylMat1-Fa (CGNCCNCTNAAYGSNTTYATNGC), CylMat1-Ra (RTANACYTTRGCAATYARNGYCCA) and CylMat1-Rb (RTANACYTTRGCRAYYARNRYCCA), where CylMat1-Ra and CylMat1-Rb are two alternative reverse primers differing in the degree of degeneracy. These primers targeted an amplicon of ca. 200 bp.

Similarly, for *MAT1-2*, the degenerated primers previously described by Arie et al. (1997), NcHMG1 and NcHMG2, were unsuccessful in the studied *Ilyonectria* isolates. Therefore, a new set of primers was designed based on sequences from species closely allied to *Ilyonectria*, such as *Gibberella*/*Fusarium*, CyHMG-F (CCIAAYGCITAYATYYTITACCG) and CyHMG-R (GGICKRTAYTGRTAITCIGGRT), targeting an amplicon of ca. 300 bp.

All primers were designed with PRIMER3 (Rozen & Skaletsky, 2000) and synthesised by Biolegio (the Netherlands) or by STABvida (Portugal).

Each PCR reaction contained 1 × PCR buffer (Bioline, UK), 3.5 mM $MgCl_2$, 40 µM of each dNTP, 0.64 µM of each degenerated primer, 1 U *Taq* DNA Polimerase (Bioline), 2 µl of diluted gDNA in a final volume of 25 µl. The amplification was carried out in a Bio-Rad thermocycler with an

initial denaturation step at 94ºC for 5 min, followed by 15 cycles of 94ºC for 30 sec, 55ºC (decreasing 0.5ºC in each cycle) for 30 sec and 72ºC for 50 sec, followed by 35 cycles of 94ºC for 30 sec, 48ºC for 30 sec and 72ºC for 50 sec and a final elongation at 72ºC for 7 min.

PCR products were cloned using pGEM®-T Easy Vector System TA cloning kit (Promega, USA), sequenced using DYEnamic ET terminator Cycle Sequencing kit (Amersham Biosciences, the Netherlands) on an ABI Prism 3700 DNA Sequencer (Applied Biosystems, USA) using the vector primers M13F and M13R and checked for homology to mating type proteins using BLAST (Basic Local Search Tool) module “BLASTX 2.2.26” at NCBI (http://www.ncbi.nlm.nih.gov) (Altschul et al. 1997).

To obtain the entire sequence of the *MAT1-1* and *MAT1-2* locus, a bidirectional chromosome walking strategy was followed, according to the DNA Walking SpeedUp™ Premix Kit protocol (Seegene, USA).

The sequences obtained with the chromosome walking strategy were assembled using the SeqMan module from the Lasergene package (DNAstar, USA). Gene predictions were made using the FGENESH gene prediction software (http://www.softberry.com) (Salamov and Solovyev 2000) with the Fusarium *graminearum* dataset as reference and the annotations were assessed by BLASTX and BLASTP analysis (Altschul et al. 1997; Altschul et al. 2005). A comparative sequence analysis was conducted with the graphical alignment programme GATA (Nix and Eisen, 2005), based on the Bl2Seq algorithm (Tatusova and Madden, 1999), enabling the identification of putative duplication and inversion events.

In order to compare the potential phylogenetic informativeness of the *MAT* loci to other commonly used markers, the nucleotide diversity (π) was determined for *MAT1-1* and *MAT1-2* idiomorphs obtained from species belonging to the macrodidyma species complex. The sequences of 12 *MAT1-1* idiomorphs (originating from *I. alcacerensis*, Cy133, Cy134 and Cy159, *I. macrodidyma*, Cy216, CBS 112601 and CBS 112603, *I. novozelandica*, Cy130 and CBS 112593, and *I. torresensis*, Cy118, Cy137, CBS 112604 and CBS 113555) and from five *MAT1-2* idiomorphs (*I. macrodidyma*, Cy175 and CBS 112615, *I. novozelandica*, CBS 113552, and *I. torresensis*, Cy222 and Cy237) were aligned using MEGA5.03 (Tamura et al., 2011). Subsequently, DNASP v5 (Rozas et al., 2003), including a sliding window analysis, with a window length of 50 bp and a step size of 25 bp was used to estimate π. These π values were compared to those calculated for sequences previously obtained for the same isolates using the rDNA-ITS, β-tubulin, histone H3, and translation elongation factor 1-α markers (Cabral et al., 2011a).

Table 1. Isolates of *Ilyonectria* and *Cylindrocarpon* spp. studied, with collection details and mating type according to results of this study

Species	Isolate	Collector, date	Location	Host	Mating type [a]
radicicola cluster					
I. liriodendri	Cy1	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Seara Nova/99R	Type A
I. liriodendri	Cy2	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Seara Nova/99R	Type A
I. liriodendri	Cy3	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Corvo Nero/99R	Type A
I. liriodendri	Cy4	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Corvo/99R	Type A
I. liriodendri	Cy5	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Boal Branco/99R	Type A
I. liriodendri	Cy6	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Boal Branco/99R	Type A
I. liriodendri	Cy7	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Perrum/99R	Type A
I. liriodendri	Cy8	C. Rego, 1992	Portugal, Torres Vedras, Dois Portos	*Vitis vinifera*, Perrum/99R	Type A
I. liriodendri	Cy9	C. Rego, 1994	Portugal, Borba	*Vitis vinifera*, -/99R	Type A
I. liriodendri	Cy10	C. Rego, 1994	Portugal, Borba	*Vitis vinifera*, -/99R	Type A
I. liriodendri	Cy11	C. Rego, 1994	Portugal, Borba	*Vitis vinifera*, -/99R	Type A
I. liriodendri	Cy12	C. Rego, 1995	Portugal, Caldas da Rainha, Salir do Porto	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy13	C. Rego, 1995	Portugal, Caldas da Rainha, Salir do Porto	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy14	C. Rego, 1995	Portugal, Caldas da Rainha, Salir do Porto	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy15	C. Rego, 1995	Portugal, Caldas da Rainha, Salir do Porto	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy16	C. Rego, 1995	Portugal, Caldas da Rainha, Salir do Porto	*Vitis vinifera*, 118F/110R	Type A
I. liriodendri	Cy17	C. Rego, 1995	Portugal, Caldas da Rainha, Salir do Porto	*Vitis vinifera*, 118F/110R	Type A
I. liriodendri	Cy18	C. Rego, 1996	Portugal, Almeirim	*Vitis vinifera*, Fernão Pires/SO4	Type A
I. liriodendri	Cy19	C. Rego, 1996	Portugal, Gouveia, São Paio	*Vitis vinifera*, Malvasia Fina/1103P	Type A
I. liriodendri	Cy21	C. Rego, 1996	Portugal, Gouveia, São Paio	*Vitis vinifera*, Malvasia Fina/1103P	Type B
I. liriodendri	Cy24	C. Rego, 1997	Portugal, Ribatejo e Oeste	*Vitis vinifera*, -/110R	Type A
I. liriodendri	Cy25	C. Rego, 1997	Portugal, Ribatejo e Oeste	*Vitis vinifera*, -/SO4	Type A
I. liriodendri	Cy26	C. Rego, 1997	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy27	C. Rego, 1997	Portugal, Peso da Régua	*Vitis vinifera*, Tinta Barroca/110R	Type A
I. liriodendri	Cy28	C. Rego, 1997	Portugal, Peso da Régua	*Vitis vinifera*, Touriga Nacional/99R	Type A
I. liriodendri	Cy29	C. Rego, 1997	Portugal, Peso da Régua	*Vitis vinifera*, Tinta Franca/110R	Type A
I. liriodendri	Cy30	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy31	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 196-17/99F	Type B
I. liriodendri	Cy32	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy33	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 101-14/3F	Type A
I. liriodendri	Cy34	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy35	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 113F/1103P	Type A
I. liriodendri	Cy36	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 139F/110R	Type B

Species	Isolate	Collector, date	Location	Host	Mating type [a]
I. liriodendri	Cy37	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 31Opp/SO4	Type A
I. liriodendri	Cy38	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy39	C. Rego, 1998	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 96F/99R	Type A
I. liriodendri	Cy66	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 196-17/199F	Type A
I. liriodendri	CBS 117526; Cy68	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 179F/99R	Type B
I. liriodendri	Cy70	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 139F/110R	Type A
I. liriodendri	Cy73	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 179F/99R	Type A
I. liriodendri	Cy74	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, -/1103P	Type B
I. liriodendri	Cy76	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 164E/110R	Type A
I. liriodendri	Cy77	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 196-17/99F	Type A
I. liriodendri	Cy103	C. Rego, 1999	Portugal, Nelas	*Vitis vinifera*, Trincadeira/99R	Type A
I. liriodendri	Cy104	C. Rego, 1999	Portugal, Almeirim	*Vitis vinifera*, Trincadeira/1103P	Type A
I. liriodendri	Cy105	C. Rego, 1999	Portugal, Mealhada	*Vitis vinifera*, Castelão/110R	Type A
I. liriodendri	Cy106	C. Rego, 1999	Portugal, Mealhada	*Vitis vinifera*, Baga/99R	Type A
I. liriodendri	Cy107	C. Rego, 1999	Portugal, Nelas	*Vitis vinifera*, Alvarinho/196-17	Type B
I. liriodendri	Cy109	C. Rego, 1999	Portugal, Nelas	*Vitis vinifera*, Jaen/SO4	Type B
I. liriodendri	Cy110	C. Rego, 1999	Portugal, Abrantes, Tramagal	*Vitis vinifera*, Trincadeira/99R	Type A
I. liriodendri	Cy111	C. Rego, 1999	Portugal, S. João da Pesqueira	*Vitis vinifera*, Touriga Nacional/99R	Type A
I. liriodendri	Cy112	C. Rego, 1999	Portugal, Sabrosa, Pinhão	*Vitis vinifera*, Tinta Franca/99R	Type A
I. liriodendri	Cy113	C. Rego, 1999	Portugal, Setúbal, Azeitão	*Vitis vinifera*, Castelão/99R	Type A
I. liriodendri	Cy121; C13	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	Type B
I. liriodendri	Cy122; C01	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	Type B
I. liriodendri	Cy127; C15	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	Type B
I. liriodendri	Cy164	C. Rego, 1997	Portugal, Porto de Mós, Valbom	*Malus domestica*, Lysgolden/MM106	Type A
I. liriodendri	Cy176	C. Rego, 2004	Portugal, Torre de Moncorvo	*Vitis vinifera*, -/1103P	Type B
I. liriodendri	Cy180	L. Mugnai (donor)	Italy, Sicíly	*Vitis* sp.	Type B
I. liriodendri	Cy185	N. Cruz, 2005	Portugal, Monção, Moreira	*Vitis vinifera*, Alvarinho/196-17	Type B
I. liriodendri	Cy186	N. Cruz, 2005	Portugal, Monção, Moreira	*Vitis vinifera*, Alvarinho/196-17	Type A
I. liriodendri	Cy187	N. Cruz, 2005	Portugal, Monção, Moreira	*Vitis vinifera*, Alvarinho/196-17	Type A
I. liriodendri	Cy188	N. Cruz, 2005	Portugal, Monção, Pias	*Vitis vinifera*, Alvarinho/196-17	Type A
I. liriodendri	Cy191	N. Cruz, 2005	Portugal, Monção, Vila	*Vitis vinifera*, Alvarinho/196-17	Type B
I. liriodendri	Cy194	N. Cruz, 2005	Portugal, Monção, Mazedo	*Vitis vinifera*, Alvarinho/196-17	Type A
I. liriodendri	Cy195	N. Cruz, 2005	Portugal, Monção, Pias	*Vitis vinifera*, Alvarinho/196-17	Type A
I. liriodendri	Cy198	N. Cruz, 2005	Portugal, Melgaço, Alvaredo	*Vitis vinifera*, Alvarinho/196-17	Type A
I. liriodendri	Cy201	N. Cruz, 2005	Portugal, Melgaço, Paços	*Vitis vinifera*, Alvarinho/196-17	Type A

Species	Isolate	Collector, date	Location	Host	Mating type [a]
I. liriodendri	Cy202	N. Cruz, 2005	Portugal, Melgaço, Paços	*Vitis vinifera*, Alvarinho/196-17	Type B
I. liriodendri	Cy203	N. Cruz, 2005	Portugal, Monção, Pias	*Vitis vinifera*, Alvarinho/196-17	Type B
I. liriodendri	Cy204	N. Cruz, 2005	Portugal, Melgaço/Monção	*Vitis vinifera*, Alvarinho/196-17	Type A
I. liriodendri	Cy209	C. Rego & H. Oliveira, 2006	Portugal, Nelas	*Vitis vinifera*, Trincadeira/110R	Type B
I. liriodendri	Cy212	C. Rego & H. Oliveira, 2006	Portugal, Nelas	*Vitis vinifera*, Trincadeira/110R	Type B
I. liriodendri	Cy215	A. Cabral, 2007	Portugal, Torres Vedras	*Vitis vinifera*, Marssanne	Type A
I. liriodendri	Cy232	L. Inácio & J. Henriques, 2007	Portugal, Macedo de Cavaleiros	*Quercus suber*, stem	Type A
I. liriodendri	Cy253	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	Type A
I. liriodendri	Cy254	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	Type B
I. liriodendri	Cy255	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	Type B
I. liriodendri	Cy256	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	Type A
I. liriodendri	Cy257	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	Type A
I. liriodendri	CBS 110.81	J. MacDonald & E. Butler, 1978	USA, California, Yolo Co., Davis	*Liriodendron tulipifera*, root	Type B
I. liriodendri	CBS 112596	F. Halleen, 1999	South Africa, De Wet	*Vitis vinifera*, C. Sauvignon/Richter 99	Type A
I. radicicola	CBS 264.65	L. Nilsson, 1961	Sweden, Skåne, Bjärred	*Cyclamen persicum*	*MAT1-1*
I. robusta [c]	CBS 308.35	A.A. Hildebrand	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. robusta	CBS 773.83	J. Hemelraad	Netherlands, Utrecht	water, in aquarium with Anodonta	*MAT1-2*
I. robusta	CBS 605.92	R. Schröer, 1992	Germany, Hamburg	*Tilia petiolaris*, root	*MAT1-2*
I. robusta	CBS 117813; IFFF 84	E. Halmschlager, 1993	Austria, Niederweiden	*Quercus robur*, root	*MAT1-2*
I. robusta	CBS 117814; IFFF 85	E. Halmschlager, 1993	Austria, Patzmannsdorf	*Quercus* sp., root	*MAT1-1*
I. robusta	CBS 117815; IFFF 86	E. Halmschlager, 1993	Austria, Patzmannsdorf	*Quercus* sp., root	*MAT1-1*
I. robusta	CBS 117818; IFFF 89	E. Halmschlager, 1993	Austria, Patzmannsdorf	*Quercus* sp., root	*MAT1-2*
I. robusta	CBS 117820; IFFF 91	E. Halmschlager, 1993	Austria, Niederweiden	*Quercus robur*, root	*MAT1-1*
I. robusta	CBS 117821; IFFF 93	E. Halmschlager, 1993	Austria, Niederweiden	*Quercus robur*, root	*MAT1-2*
I. robusta	CBS 117822; IFFF 94	E. Halmschlager, 1993	Austria, Niederweiden	*Quercus robur*, root	*MAT1-1*
I. robusta	CBS 117823; IFFF 95	E. Halmschlager, 1993	Austria, Niederweiden	*Quercus robur*, root	*MAT1-2*
I. robusta	CD1666	R. D. Reeleder, 1998	Canada, Nova Scotia	*Panax quinquefolium*	*MAT1-1*
I. robusta	CPC 13532; DAOM 139398; K 18-3A	-	Canada, Ontario	*Prunus cerasus*, Montmorency	*MAT1-1*
I. robusta	CBS 129084; Cy192	N. Cruz, 2005	Portugal, Monção	*Vitis vinifera*, Alicante Bouchet/196-17	*MAT1-2*
I. robusta	Cy231	F. Caetano, 2005	Portugal, Lisbon	*Thymus* sp.	*MAT1-1*
I. crassa	CBS 139.30	W.F. van Hell, 1930	Netherlands	*Lilium* sp., bulb	*MAT1-1*
I. crassa	CBS 158.31; IMI 061536; NRRL 6149	1930	Netherlands	*Narcissus* sp., root	*MAT1-1*

Species	Isolate	Collector, date	Location	Host	Mating type [a]
I. crassa	CBS 129083; NSAC-SH-1	S. Hong, 1998	Canada, Nova Scotia	*Panax quinquefolium*	*MAT1-2*
I. crassa	NSAC-SH-2	S. Hong, 1998	Canada, Nova Scotia	*Panax quinquefolium*	*MAT1-2*
I. crassa	NSAC-SH-2.5	S. Hong, 1998	Canada, Nova Scotia	*Panax quinquefolium*	*MAT1-2*
"*Cylindrocarpon*" sp.	CBS 120370; CR 20	P. Axelrood, 1998	Canada, British Columbia	*Pseudotsuga menziesii*	*MAT1-1*
I. rufa [d]	CBS 153.37	F. Moreau, 1937	France	dune sand	*MAT1-2*
I. rufa	CBS 156.47; IAM 14673; JCM 23100	-	Belgium, Amandsberg	*Azalea indica*	*MAT1-2*
I. rufa	CBS 120371; CR 26	P. Axelrood, 1998	Canada, British Columbia	*Pseudotsuga menziesii*	*MAT1-2*
I. rufa	CBS 120372; CR 29	P. Axelrood, 1998	Canada, British Columbia	*Pseudotsuga menziesii*	*MAT1-1*
I. rufa	CPC 13536; DAOM 226721; CR36	P. Axelrood, 1998	Canada, British Columbia	*Pseudotsuga menziesii*	*MAT1-1*
I. rufa	94-1628	R.C. Hamelin, 1994	Canada, Quebec	*Picea glauca*	*MAT1-2*
I. mors-panacis	CBS 120359; CD1561	R. D. Reeleder, 1996	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120360; CD1567	R. D. Reeleder, 1996	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120361; CD1596	R. D. Reeleder, 1996	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120362; CD1598	R. D. Reeleder, 1996	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120363; CD1635	R. D. Reeleder, 1997	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120364; CD1636	R. D. Reeleder, 1997	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120365; CD1637	R. D. Reeleder, 1997	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120366; CD1639	R. D. Reeleder, 1997	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120367; CD1640	R. D. Reeleder, 1997	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120368; CD1641	R. D. Reeleder, 1997	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 120369; CD1642	R. D. Reeleder, 1997	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CPC 13537; DAOM 226727; CD 1570	R. D. Reeleder, 1996	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis [e]	CBS 306.35	A.A. Hildebrand	Canada, Ontario	*Panax quinquefolium*	*MAT1-2*
I. mors-panacis	CBS 307.35	A.A. Hildebrand	Canada, Ontario	*Panax quinquefolium*	*MAT1-1*
I. pseudodestructans	CPC 13534; DAOM 150670; Berkenkamp 1	B. Berkenkamp, 1974	Canada, Alberta, Lacombe	*Poa pratensis*	*MAT1-1*
I. pseudodestructans	CBS 117824; IFFF 98	E. Halmschlager, 1993	Austria, Patzmannsdorf	*Quercus* sp., root	*MAT1-2*
I. pseudodestructans	CBS 129081; Cy20	C. Rego, 1996	Portugal, Gouveia, São Paio	*Vitis vinifera*, Malvasia Fina/1103P	*MAT1-1*
I. pseudodestructans	Cy22	C. Rego, 1996	Portugal, Viseu, Silgueiros	*Vitis vinifera*, Aragonez/99R	*MAT1-2*
I. europaea	Cy131	P. Lecomte & S. Chamont, 2000	France, St. Chicq-du-Gaue	*Actinidia chinensis*, Hayward	*MAT1-1*
I. europaea	Cy155	C. Rego & H. Oliveira, 2004	Portugal, Alter do Chão	*Vitis vinifera*, Alfrocheiro/SO4	*MAT1-2*
I. europaea	CBS 129078; Cy241	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	*MAT1-1*

Species	Isolate	Collector, date	Location	Host	Mating type [a]
I. europaea	CBS 537.92	V. Demoulin, 1992	Belgium, Liège	*Aesculus hippocastanum*, wood	*MAT1-1*
I. lusitanica	CBS 129080; Cy197	N. Cruz, 2005	Portugal, Melgaço, Alvaredo	*Vitis vinifera*, Alvarinho/196-17	*MAT1-1*
I. venezuelensis	CBS 102032; ATCC 208837; AR2553	A. Rossman, 1985	Venezuela, Amazonas, Cerro de la Neblina	bark	*MAT1-2*
I. panacis	CBS 129079; CDC-N-9a	K. F. Chang, 1998	Canada, Alberta	*Panax quinquefolium*	*MAT1-2*
I. liliigena	CBS 189.49; IMI 113882	M.A.A. Schipper	Netherlands, Hoorn	*Lilium regale*, bulb	*MAT1-2*
macrodidyma cluster					
Ilyonectria sp 1	CBS 162.89	M. Barth, 1988	Netherlands, Noordoostpolder, Marknesse, Lovinkhoeve	*Hordeum vulgare*, root	*MAT1-1*
Ilyonectria sp2	Cy108	C. Rego, 1999	Portugal, Nelas	*Vitis vinifera*, Aragonez/SO4	*MAT1-2*
Ilyonectria sp2	CBS 159.34; IMI 113891; MUCL 4084; VKM F-2656	H.W. Wollenweber, 1934	Germany		*MAT1-1*
Ilyonectria sp2	CBS 173.37; IMI 090176	T.R. Peace, 1937	UK, England, Devon, Haldon	*Pinus laricio*, associated with dieback	*MAT1-1*
I. estremocensis	Cy135	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	Cy144	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	CBS 129085; Cy145	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	Cy146	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	Cy149	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	Cy150	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	Cy151	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	Cy152	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. estremocensis	Cy243	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-1*
I. estremocensis	CPC 13539; 94-1685; CCFC226730	R. C. Hamelin, 1994	Canada, Quebec	*Picea glauca*	*MAT1-2*
I. alcacerensis	Cy133; IAFM Cy9-1	J. Armengol	Spain, Valencia, L'Alcudia	*Vitis vinifera*	*MAT1-1*
I. alcacerensis	Cy134; IAFM Cy20-1	J. Armengol	Spain, Ciudad Real, Villarubia de los Ojos	*Vitis vinifera*	*MAT1-1*
I. alcacerensis	CBS 129087; Cy159	A. Cabral & H. Oliveira, 2004	Portugal, Alcácer do Sal, Torrão	*Vitis vinifera*, Sangiovese/1103P	*MAT1-1*
I. novozelandica	CBS 112593; STE-U 3990; C 107	F. Halleen, 2000	South Africa, Western Cape, Wellington, Voorgroenberg	*Vitis vinifera*, Pinotage/101-14 Mgt	*MAT1-1*
I. novozelandica	CBS 112608; STE-U 3987; C 62	F. Halleen, 2000	South Africa, Western Cape, Citrusdal	*Vitis vinifera*, Chardonnay/101-14 Mgt	*MAT1-1*
I. novozelandica	CBS 113552; STE-U 5713; HJS-1306; NZ C 41	R. Bonfiglioli, 2003	New Zealand, Candy P New Ground	*Vitis* sp.	*MAT1-2*
I. novozelandica	Cy115	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. novozelandica	Cy116	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*

Species	Isolate	Collector, date	Location	Host	Mating type [a]
I. novozelandica	Cy117	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. novozelandica	Cy119	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. novozelandica	Cy124	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. novozelandica	Cy125	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. novozelandica	Cy129	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. novozelandica	Cy130	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. novozelandica	Cy230	F. Caetano, 2005	Portugal, Lisbon	*Festuca duriuscula*	*MAT1-2*
I. macrodidyma	CBS 112594; STE-U 3991; C 111	F. Halleen, 2000	South Africa, Western Cape, Malmesbury, Jakkalsfontein	*Vitis vinifera*, Pinotage/Richter 99	*MAT1-2*
I. macrodidyma	CBS 112601; STE-U 3983; C 82	F. Halleen, 1999	South Africa, Western Cape, Tulbagh	*Vitis vinifera*, Pinotage/US 8-7	*MAT1-1*
I. macrodidyma	CBS 112603; STE-U 4007; C 8	F. Halleen, 1999	South Africa, Western Cape, Darling	*Vitis vinifera*, Sauvignon Blanc/Richter 110	*MAT1-1*
I. macrodidyma	CBS 112605; STE-U 3984; C 106	F. Halleen, 2000	South Africa, Western Cape, Malmesbury, Jakkalsfontein	*Vitis vinifera*, Sultana/143-B Mgt	*MAT1-2*
I. macrodidyma [b]	CBS 112615; STE-U 3976; C 98	F. Halleen, 2000	South Africa, Western Cape, Malmesbury, Jakkalsfontein	*Vitis* sp., 143-B Mgt	*MAT1-2*
I. macrodidyma	Cy123; C08	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-2*
I. macrodidyma	Cy128; C20	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. macrodidyma	Cy139	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. macrodidyma	Cy140	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. macrodidyma	Cy175	C. Rego, 2004	Portugal, Torre de Moncorvo	*Vitis vinifera*, Touriga Nacional/1103P	*MAT1-2*
I. macrodidyma	Cy181	C. Rego, 2005	Portugal, Alcácer do Sal	*Vitis vinifera*, Aragonês	*MAT1-2*
I. macrodidyma	Cy216	A. Cabral, 2007	Portugal, Torres Vedras	*Vitis vinifera*, Marssanne	*MAT1-1*
I. macrodidyma	Cy244	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	*MAT1-2*
I. macrodidyma	Cy250	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Chardonnay/110R	*MAT1-2*
I. macrodidyma	Cy258	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-2*
I. torresensis	CBS 119.41	H.C. Koning	Netherlands, Baarn	*Fragaria* sp., root	*MAT1-1*
I. torresensis	CBS 188.49	J.A. von Arx	Netherlands, Egmond	*Abies nordmanniana*, root	*MAT1-2*
I. torresensis	Cy221; MTF6BH2	L. Leandro	USA, North Carolina, Asheville	*Fragaria x ananassa*	*MAT1-2*
I. torresensis	Cy223; MT2 20AD2	L. Leandro	USA, North Carolina, Asheville	*Fragaria x ananassa*	*MAT1-1*
I. torresensis	Cy118; C07	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. torresensis	Cy120; C12	W.D. Gubler, received 1999	USA, California	*Vitis vinifera*	*MAT1-1*
I. torresensis	Cy96	E. Halmschlager	Austria, Patzmannsdorf	*Quercus* sp., root	*MAT1-1*
I. torresensis	Cy97	E. Halmschlager	Austria, Patzmannsdorf	*Quercus* sp., root	*MAT1-1*

Species	Isolate	Collector, date	Location	Host	Mating type [a]
I. torresensis	CBS 112609; STE-U 3969; HJS-1217	M. Sweetingham, 1979	Australia, Tasmania, Bream Creek	*Vitis vinifera*, Cabernet Sauvignon	*MAT1-2*
I. torresensis	CPC 13533; CCFC 144524; Dias 2B	H.F. Dias, 1972	Canada, Ontario	*Vitis vinifera*, Concord Bradt	*MAT1-2*
I. torresensis	Cy69	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 102F/SO4	*MAT1-1*
I. torresensis	Cy71	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 96F/99R	*MAT1-1*
I. torresensis	Cy72	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis vinifera*, 113F	*MAT1-2*
I. torresensis	Cy75	C. Rego, 1999	Portugal, Ribatejo e Oeste	*Vitis* sp.; 99R	*MAT1-2*
I. torresensis	Cy136	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-1*
I. torresensis	Cy137	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-1*
I. torresensis	Cy138	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-1*
I. torresensis	Cy141	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-1*
I. torresensis	Cy142	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. torresensis	Cy143	C. Rego & T. Nascimento, 2003	Portugal, Estremoz	*Vitis vinifera*, Aragonez/3309C	*MAT1-2*
I. torresensis	Cy157	H. Oliveira, 2004	Portugal, Alenquer	*Vitis* sp., 99R	*MAT1-2*
I. torresensis	Cy214	A. Cabral, 2007	Portugal, Torres Vedras	*Vitis vinifera*, Grenache	*MAT1-2*
I. torresensis	CBS 129086; Cy218	A. Cabral, 2007	Portugal, Torres Vedras	*Vitis vinifera*, Chenin	*MAT1-1*
I. torresensis	Cy234	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-2*
I. torresensis	Cy235	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-1*
I. torresensis	Cy236	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-1*
I. torresensis	Cy237	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Chardonnay/110R	*MAT1-2*
I. torresensis	Cy240	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/140RU	*MAT1-1*
I. torresensis	Cy242	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-1*
I. torresensis	Cy245	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	*MAT1-2*
I. torresensis	Cy246	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Antão Vaz/110R	*MAT1-2*
I. torresensis	Cy247	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-2*
I. torresensis	Cy248	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-1*
I. torresensis	Cy249	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-2*
I. torresensis	Cy251	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Chardonnay/110R	*MAT1-1*
I. torresensis	Cy259	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-2*
I. torresensis	Cy260	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-1*
I. torresensis	Cy261	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-1*
I. torresensis	Cy262	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-1*
I. torresensis	Cy263	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-1*
I. torresensis	Cy264	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	*MAT1-2*

Species	Isolate	Collector, date	Location	Host	Mating type [a]
I. torresensis	Cy265	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-2*
I. torresensis	Cy132; IAFM Cy1-1	J. Armengol	Spain, Alicante	*Vitis vinifera*	*MAT1-2*
I. torresensis	CBS 112598; STE-U 3997; C 115	F. Halleen, 2000	South Africa, Western Cape, Wellington, Lelienfontein	*Vitis vinifera*, Sultana/Ramsey	*MAT1-2*
I. torresensis	CBS 112604; STE-U 4004; C 10	F. Halleen, 1999	South Africa, Western Cape, Paarl	*Vitis vinifera*, C. Sauvignon/101-14 Mgt	*MAT1-1*
I. torresensis	CBS 113555; STE-U 5715; HJS-1309; NZ C 60	R. Bonfiglioli, 2003	New Zealand, Fiddlers Green	*Vitis vinifera*, Pinot Noir/101-14	*MAT1-1*
pauciseptatum cluster					
I. vitis	CBS 129082; Cy233	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Touriga Nacional/110R	*MAT1-1*
"*Cylindrocarpon*" sp.	Cy228	F. Caetano, 2003	Portugal, Lisbon	*Ficus* sp.	*MAT1-1*
I. anthuriicola	CBS 564.95; PD 95/1577	R. Pieters, 1995	Netherlands, Bleiswijk	*Anthurium* sp., root	*MAT1-1*
C. pauciseptatum	Cy217	A. Cabral, 2007	Portugal, Torres Vedras	*Vitis vinifera*, Gouveio	*MAT1-2*
C. pauciseptatum	Cy238	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, Petit Verdot/110R	*MAT1-2*
C. pauciseptatum	Cy239	C. Rego, 2008	Portugal, Vidigueira	*Vitis vinifera*, C. Sauvignon/110R	*MAT1-2*
C. pauciseptatum	CBS 120497; KIS 10763	H.-J. Schroers, 2006	Slovenia, Mrzlak	*Vitis* sp.	*MAT1-2*
C. pauciseptatum	CBS 120499; KIS 10780	M. Žerjav, 2006	Slovenia, Ljutomer	*Vitis* sp.	*MAT1-2*
C. pauciseptatum	CBS 120171; KIS 10467	M. Žerjav, 2005	Slovenia, Krŝko	*Vitis* sp.	*MAT1-1*
C. pauciseptatum	CBS 120172; KIS 10729	M. Žerjav, 2006	Slovenia, Žužemberk	*Vitis* sp.	*MAT1-2*
C. pauciseptatum	CBS 120173; KIS 10468	M. Žerjav, 2005	Slovenia, Krŝko	*Vitis* sp.	*MAT1-2*
C. pauciseptatum	CBS 100819; LYN 16202/2	H.M. Dance, 1998	New Zealand, Tauranga	*Erica melanthera*, root	*MAT1-2*

[a] Type A – *MAT 1-1-1, MAT 1-2-1*; Type B – *MAT 1-1-1, MAT 1-1-2, MAT 1-1-3, MAT 1-2-1*;
[b] ex-holotype of *C. macrodidymum*; [c] ex-type strain of *Ramularia robusta*; [d] authentic strain of *Coleomyces rufus*; [e] ex-type of *Ramularia mors-panacis*.

Diagnostic PCR for mating type determination in *Ilyonectria* species

Based on the sequences of the *I. macrodidyma* and *I. liriodendri MAT* locus, we designed PCR primers directed against the conserved α box domain encoding region in *MAT1-1-1* (primers CylMat1-Fd and CylMat1-Re; Table 2) and the HMG box domain encoding region in *MAT1-2-1* (primers Cy-HMG-F2 and Cy-HMG-R2; Table 2). These primers were used to amplify DNA from a collection of *Ilyonectria* species (Table 1). The PCR mix contained 1 × PCR buffer with 2 mM of $MgCl_2$ (MBI Fermentas, Lithuania), 32 µM of each dNTP, 320 nM of each primer, 0.5 U Dream *Taq* DNA Polymerase (MBI Fermentas), 1.25 µl of diluted gDNA in a final volume of 12.5 µl. The amplification was carried out in a S1000 (Bio-Rad, USA) thermocycler with a initial denaturation step at 94ºC for 5 min, followed by 40 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 90 sec, followed by a final elongation at 72ºC for 10 min.

Additionally, four primers (NmMAT111-F1, NmMAT111-R1, NmMAT121-F and NmMAT121-R; Table 2) were designed with the purpose of performing a multiplex PCR to screen the presence/absence of *MAT1-1-1* and *MAT1-2-1* in the *I. macrodidyma* species complex. A similar strategy was employed to distinguish between *MAT1-1* and *MAT1-2* isolates of *I. liriodendri* (NliMAT111-F, NliMAT111-R, NliMAT121-F and NliMAT121-R; Table 2).

Finally, four primers (NliType1-F, NliType1-R, NliType2-F, NliType2-R; Table2) were designed for the discrimination of the *I. liriodendri* A and B type isolates. The primers NliType1-F, NliType1-R were designed in the region between *MAT1-2-1* and *MAT1-1-1* in type 1 isolates targeting a fragment with approx. 450 bp, and the primers NliType2-F and NliType2-R, located in the second exon of *MAT1-1-3* and *MAT1-1-2* in type 2 isolates, respectively targeting a fragment with approx. 1070 bp. The PCR reaction contained 1 × PCR buffer with 2 mM of $MgCl_2$ (MBI Fermentas), 64 µM of each dNTPs, 0.4 µM of each primer, 0.5 U Dream *Taq* DNA Polymerase (MBI Fermentas), 1.25 µl of diluted gDNA in a final volume of 12.5 µl. The amplification was carried out in a Bio-Rad S1000 thermocycler with an initial denaturation step at 94ºC for 5 min, followed by 40 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 90 sec, followed by a final elongation at 72ºC for 10 min.

The conserved region of DNA lyase that flanks the mating type idiomorphs was also amplified with the primers NmDNALyaseF3 andNmDNALyaseR1 (Table 2), following the PCR conditions described above.

Population frequencies were statistically analysed using the χ^2-test to calculate the probability of the population from a given species to conform to a 1:1 *MAT1-1*/*MAT1-2* ratio.

Table 2. Primers used to amplify *MAT1-1-1* and *MAT1-2-1* in *Ilyonectria liriodendri*, and species from the macrodidyma species complex

Primer name	Primer sequence (5' -> 3')	Region amplified	Amplicon size (bp)
NmMAT111-F1	TCAGAGATCCTACGTCATGCAG	*MAT1-1-1* of macrodidyma complex	
NmMAT111-R1	AGAGAGTTGTAGTCCGCATGG	*MAT1-1-1* of macrodidyma complex	~1200
NmMAT121-F	GGAACAAGTCACCGAAAGAGTC	*MAT1-2-1* of macrodidyma complex	
NmMAT121-R	TAGTAGCCTCATCATCGGAAGC	*MAT1-2-1* of macrodidyma complex	~650
NliMAT111-F	ACGAGACGATGCTCCAAGTC	*MAT1-1-1* of *I. liriodendri*	
NliMAT111-R	TCGTAGGGGCTGTCAATGTC	*MAT1-1-1* of *I. liriodendri*	~1100
NliMAT121-F	TTTCCCCTTTCGTCTATGTCC	*MAT1-2-1* of *I. liriodendri*	
NliMAT121-R	CTTGTAGTCGGGGTGTTTCTTG	*MAT1-2-1* of *I. liriodendri*	~800
NliType1- F	GCTTGGTTTTGTTCATCATCCT	Type A of *I. liriodendri*	
NliType1-R	GCTGGGGATAGAATGGGAGT	Type A of *I. liriodendri*	~450
NliType2-F	GACGCTGTTCCCATCAAGAC	Type B of *I. liriodendri*	
NliType2-R	AAAACAATGGCACCTCGAAC	Type B of *I. liriodendri*	~1070
CylMat1-Fd	CCYCTSAATGCATTCATGGC	Alpha box of *MAT1-1-1*	
CylMat1-Re	GATSATGTTCATGGYGGGACAG	Alpha box of *MAT1-1-1*	~270-290
CyHMG F2	CTCCMAAYGCGTAYATCTTGTACCG	HMG box of *MAT1-2-1*	
CyHMG R2	GRCGSGGGCGGTACTTG	HMG box of *MAT1-2-1*	~260-400
NmLyaseF3	CAACAACGGTAGTCGGATCG	DNA Lyase	
NmLyaseR1	CATTCCGATTCCTTCTCCTTCT	DNA Lyase	~950

Results

Amplification and chromosome walking of mating type locus

The 200 bp sequence obtained for *I. torresensis* isolate Cy137 with the primer pairs CylMAT1Fa and CylMAT1Ra or CylMAT1Rb revealed homology at protein level to other *MAT1-1-1* of Hypocreales deposited in GenBank. Chromosome walking steps were performed until the DNA Lyase was reached in the upstream direction, and the full *MAT1-1-1* was obtained downstream. A total of 7905 bp was sequenced (Supplementary material S1).

After the first chromosome walking on genomic DNA of *I. torresensis* isolate Cy137, a new primer set was designed against the region encoding the alpha box domain in order to amplify the *MAT1-1-1* gene in *I. liriodendri* isolates. The primer pair CylMat1-Fb (AARCGACCRYTRAATGCWTTYATBGC) and CylMat1-Rc (CGARTANACYTTBGCRAYYADDGCCCA) was tested in *I. liriodendri* isolates using the same PCR mix and program as that used with *I. torresensis* isolate Cy137. After cloning, sequencing and identity confirmation, a chromosome walking strategy was again applied. This resulted in a total of 6384 bp for *I. liriodendri* isolate Cy33 (Supplementary material S3) and of 6433 bp for *I. liriodendri* isolate Cy109 (Supplementary material S4).

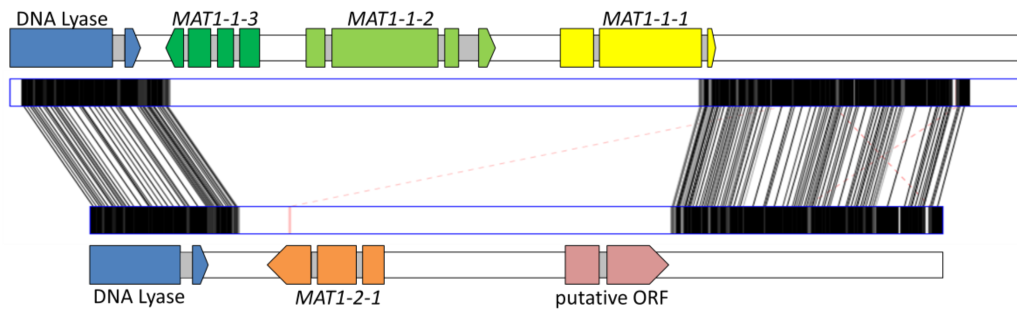
Amplification of the HMG box of *MAT1-2-1* was achieved using the primer pair CyHMG-F / CyHMG-R for *I. torresensis* isolate Cy237. From this first fragment a chromosome walking step was performed in upstream and downstream direction, reaching a 6640 bp sequence (Supplementary material S2). Analysis of the sequences obtained for the *I. torresensis MAT1-1* isolate Cy137 (Fig. 1, Table 3 and Supplementary material S1) revealed the presence of a partial ORF encoding a protein with highest similarity (BLASTX) to *APN2* (DNA lyase) of *Epichloë festucae* (AEI72618, e-value 10^{-103}), a gene with high similarity (e-value; 9 x 10^{-54}) to *MAT1-1-3* of *Metarhizium anisopliae* (BAE93596), a gene highly similar (e-value of 3 x 10^{-85}) to *MAT1-1-2* of *Hypocrea jecorina* (ACR78245), and finally, a putative *MAT1-1-1* sequence (e-value; 4 x 10^{-90}) similar to *Epichloë festucae* (ACN59937).

The annotation of the sequence for the *I. torresensis MAT1-2* isolate Cy237 (Fig. 1, Table 3 and Supplementary material S2) revealed the presence of an incomplete DNA Lyase, and of a putative *MAT1-2-1* with highest homology to *MAT1-2-1* of *Fusarium oxysporum* (EGU79454; 1 x 10^{-85}) and *Epichloë festucae* (AEI72619; 5 x 10^{-66}). Interestingly, a putative ORF was predicted using FGENESH. The deduced protein showed no similarities to the NCBI EST, nucleotide or protein databases (using tblastn or blastp, respectively) at e-value $<10^{-1}$.

BLAST2seq analysis of the *I. torresensis MAT1-1* and *MAT1-2* mating type loci of (represented respectively by the sequences for Cy137 and Cy237) identified a *MAT1*-1 idiomorph of 4.1 kb and a *MAT1*-2 idiomorph of 3.4 kb (Fig. 1).

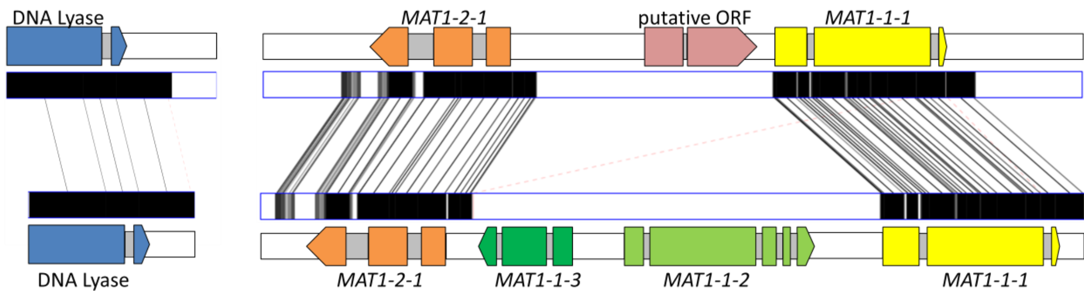
In *I. liriodendri* all the isolates screened both *MAT1-1-1* and *MAT1-2-1* were found, indicative of a homothallic species. Interestingly, two different types of organization of the mating-type loci could be distinguished (designated type A and type B). The type A isolates (e.g. Cy33) contain *MAT1-1-1*, *MAT1-2-1*, and a putative ORF. Type B isolates contain all mating-type genes (*MAT1-1-1, MAT1-2-1*, *MAT1-1-2* and *MAT1-1-3)* and thus can be considered to be genetically homothallic (Fig. 1, Table 3 and Supplementary materials S3 and S4). For both type A and B isolates (Cy33 and Cy109) a fragment was amplified harbouring an incomplete DNA Lyase with highest homologies to *Fusarium oxysporum* (EGU79454; 1 x 10^{-96}). Attempts to join by long-range PCR this DNA Lyase contig with the one containing the mating type genes were unsuccessful (Fig. 1 and Supplementary Material S5). BLAST2 analysis of the *I. liriodendri* type A and type B mating type loci showed the presence of dissimilar regions between type A and type B isolates of 1.8 kb and 3.2 kb respectively.

I. torresensis MAT1-1



I. torresensis MAT1-2

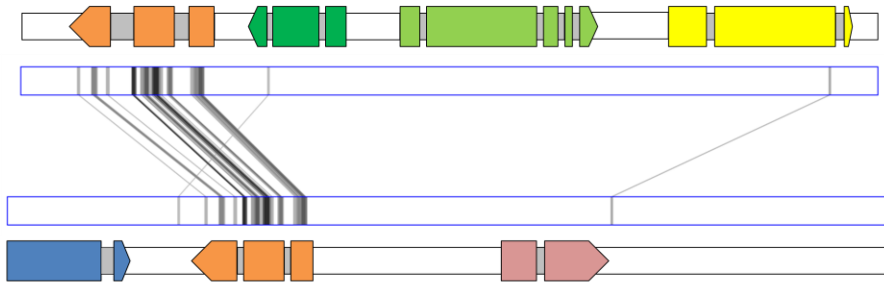
I. liriodendri “type A”



I. liriodendri “type B”

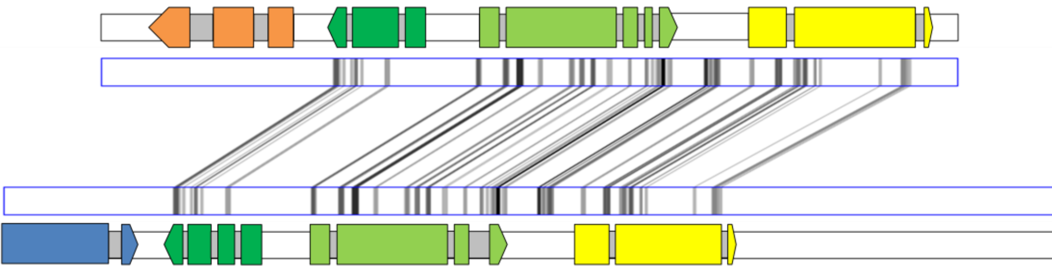
500 bp

I. liriodendri “type B”



I. torresensis MAT1-2

I. liriodendri “type B”



I. torresensis MAT1-1

500 bp

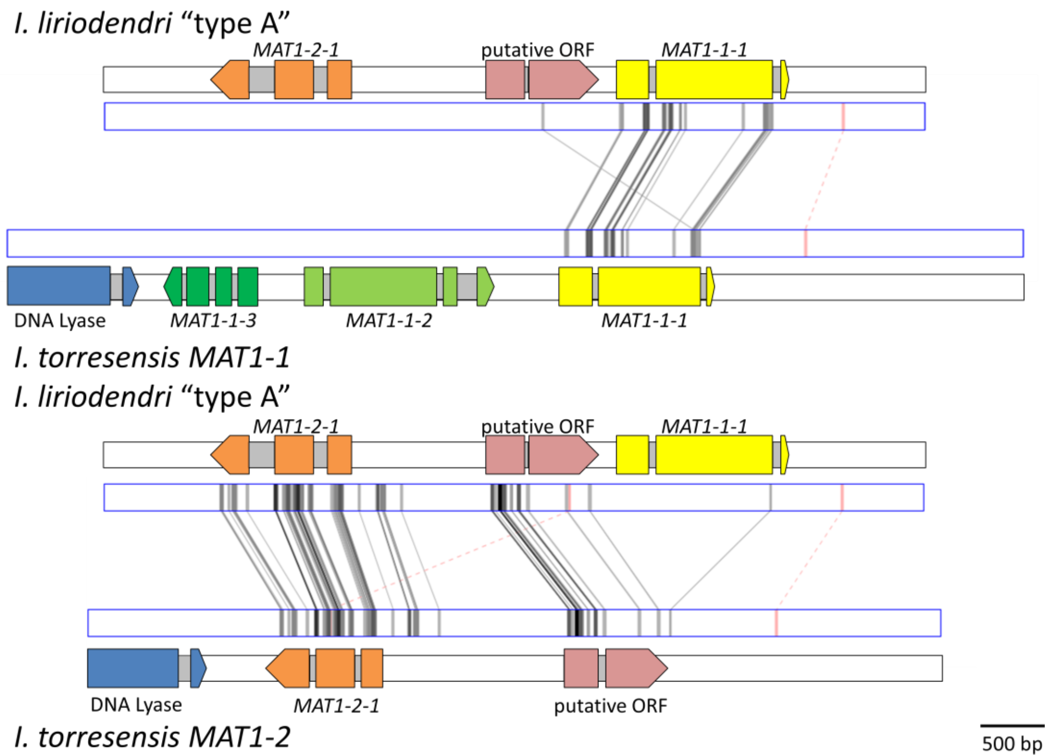


Fig. 1. Schematic representation of the structure of *MAT1-1* and *MAT1-2* loci of *Ilyonectria torresensis* (representing the macrodidyma species complex) and of type A and B mating type loci of *I. liriodendri*. Regions of strong homology are shaded and connected by lines. The intensity of shading indicates the strength of homology. Red shading with dashed lines indicates inverted homology. Genes are represented by box arrows. Intra-cluster diversity in the macrodidyma species complex (*I. alcacerensis*, *I. macrodidyma*, *I. novozelandica* and *I. torresensis*) is illustrated by the nucleotide diversity (π) analysis in Fig. 2.

Table 3. Predicted gene and intron size of the *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3* and *MAT1-2-1* genes in *Ilyonectria torresensis* isolates Cy137 and Cy237 (identical results were obtained for *I. alcacerensis*, *I. macrodidyma* and *I. novozelandica*) and *I. liriodendri* isolates Cy33 and Cy109 and number of amino acids present in the encoded proteins. Commas separate the intron sizes where more than one intron is present

Species	Gene	Gene size (bp)	Intron size (bp)	Nr. amino acids
I. torresensis	*MAT1-1-3*	738	44, 52, 48	197
	MAT1-1-2	1476	54, 52, 156	404
	MAT1-1-1	1211	47, 48	371
I. torresensis	*MAT1-2-1*	907	48, 49	269
	Putative ORF	807	60	262
I. liriodendri type A	*MAT1-2-1*	1096	199, 108	262
	Putative ORF	872	29	280
	MAT1-1-1	1277	56, 63	385
I. liriodendri type B	*MAT1-2-1*	1079	173, 108	265
	MAT1-1-3	734	44, 51	212
	MAT1-1-2	1481	51, 52, 49, 54	424
	MAT1-1-1	1379	64, 64	416

Crossing experiments among 82 *I. liriodendri* isolates revealed that crosses were only successful, as judged by the occurrence of perithecia with viable ascospores, when both type A and type B

isolates were present together (Table 4), suggesting that despite the “homothallic” molecular organization of the *I. liriodendri* mating-type locus this species is functionally heterothallic.

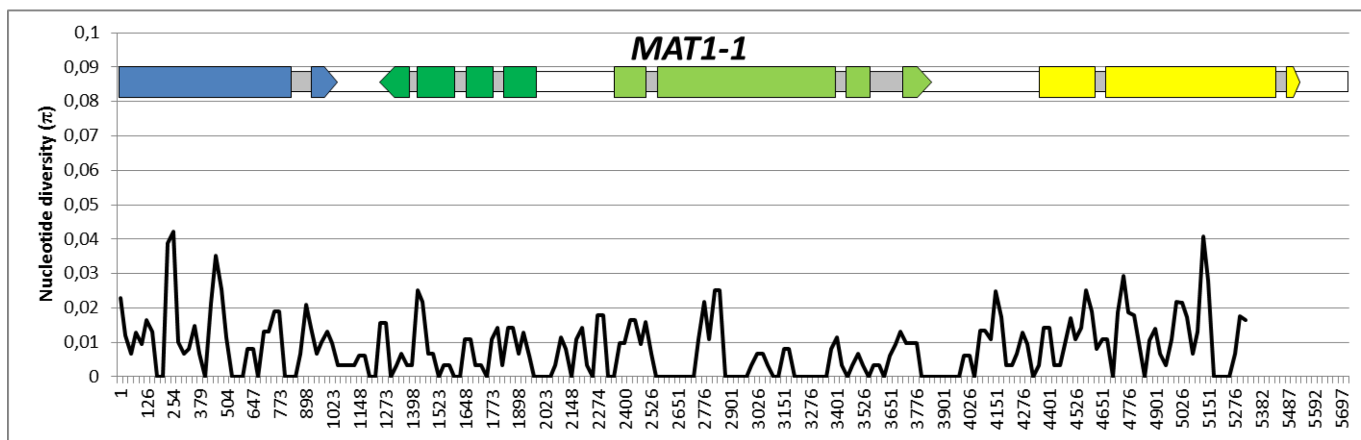
Table 4. Results of the crossing experiments among *Ilyonectria liriodendri* tester isolates from type A and B, showing the production of fertile perithecia (+) or the incapacity of producing them (-)

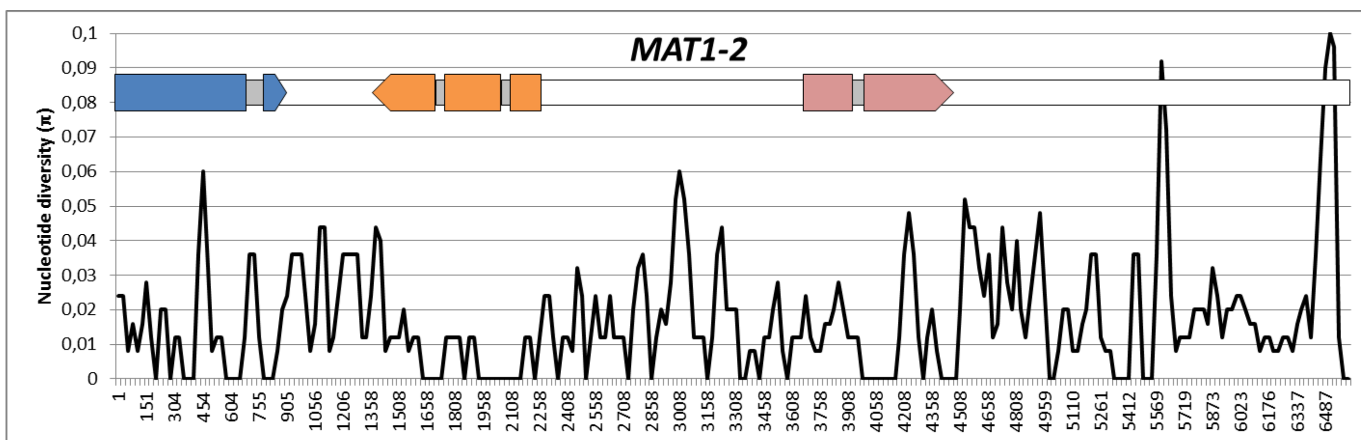
Isolate	Mating type	Type A			Type B		
		Cy27	Cy76	Cy111	Cy68	Cy107	CBS110.81
Cy27	A	-	-	-	+	-	-
Cy76	A	-	-	-	+	+	+
Cy111	A	-	-	-	+	-	-
Cy68	B	+	+	+	-	-	-
Cy107	B	-	+	-	-	-	-
CBS110.81	B	-	+	-	-	-	-

Alignments between the predicted proteins of *I. torresensis* and *I. liriodendri* were made using the BLAST2SEQ/BLASTP. The *MAT1-1-1* proteins have 52% similarity (e-value 10^{-108}) between the predict proteins of *I. torresensis MAT1-1* (Cy 137) and *I. liriodendri* type A (Cy33), and 53% (2 x 10^{-120}) between Cy137 and *I. liriodendri* type B (Cy109). Also, the similarity of *MAT1-1-1* protein between both types of isolates of *I. liriodendri* is 98% (0.0). The predicted *MAT1-2-1* proteins of *I. torresensis MAT1-2* (Cy237) and Cy33 had a similarity of 80% (2 x 10^{-108}), Cy237 and Cy109 had 75% similarity (6 x 10^{-112}) and Cy33 and Cy109 had 99% similarity (2 x 10^{-135}). For Cy137 and Cy109, similarity in *MAT1-1-3* was 52% (4 x 10^{-65}) and in *MAT1-1-2* was 64% (10^{-164}). The predicted ORF in Cy237 has 47% similarity (4 x 10^{-63}) to the predicted ORF of Cy33.

Nucleotide diversity was tested across the *MAT1-1* locus by comparing 12 sequences representing four species belonging to the macrodidyma species complex, *I. alcacerensis* (Cy133, Cy134 and Cy159), *I. macrodidyma* (Cy216, CBS 112601 and CBS 112603), *I. novozelandica* (Cy130 and CBS 112593) and *I. torresensis* (Cy118, Cy137, CBS 112604 and CBS 113555). Overall nucleotide diversity (π) was 0.00823, with a maximum value (0.04212) in an exon of DNA lyase at position 350-399 (Fig. 2). No intron or intergenic region reached a higher value.

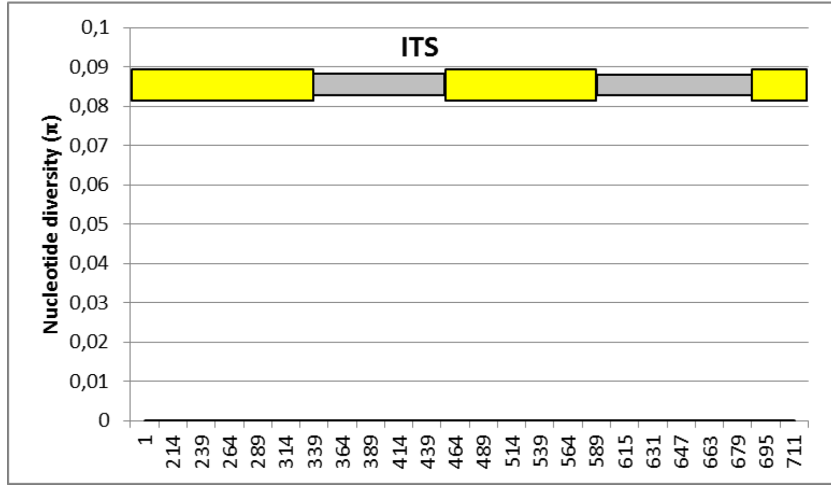
Similarly, nucleotide diversity was tested across the *MAT1-2* locus by comparing five sequences representing three species, *I. macrodidyma* (Cy175 and CBS 112615), *I. novozelandica* (CBS 113552) and *I. torresensis* (Cy222 and Cy237). Overall nucleotide diversity (π) was 0.01764, with a maximum value (0.1) in an intergenic region at position 6554-6612 (Fig. 2). These values were compared (Fig. 2) to those of the rDNA-ITS, β-tubulin, histone H3, and translation elongation factor 1-α markers for sequences from the same isolates previously obtained (Cabral et al., 2011a), showing null nucleotide diversity for ITS (*MAT1-1* and *MAT1-2*), 0.00370 and 0.00313 for β-tubulin (*MAT1-1* and *MAT1-2*, respectively), 0.00812 and 0.00790 for the translation elongation factor 1-α, and 0.02421 and 0.02285 for histone H3.

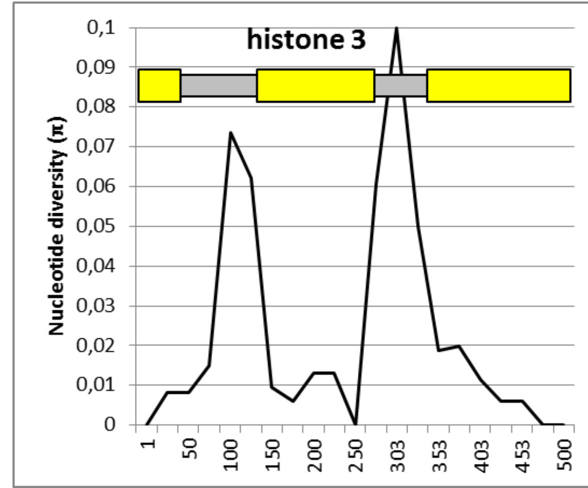


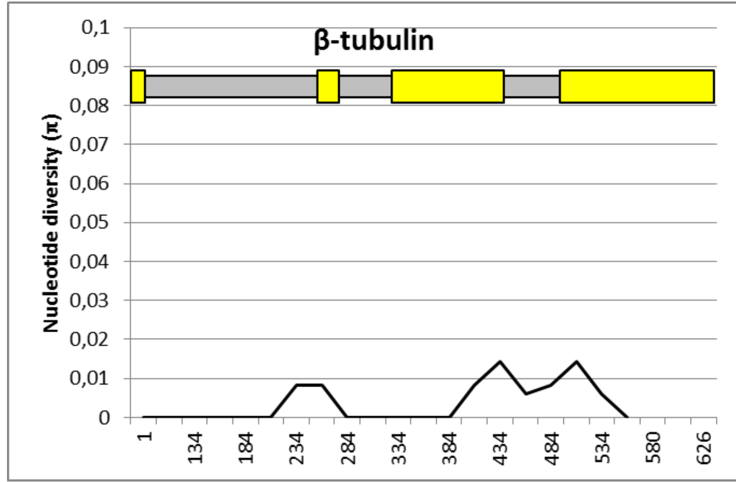


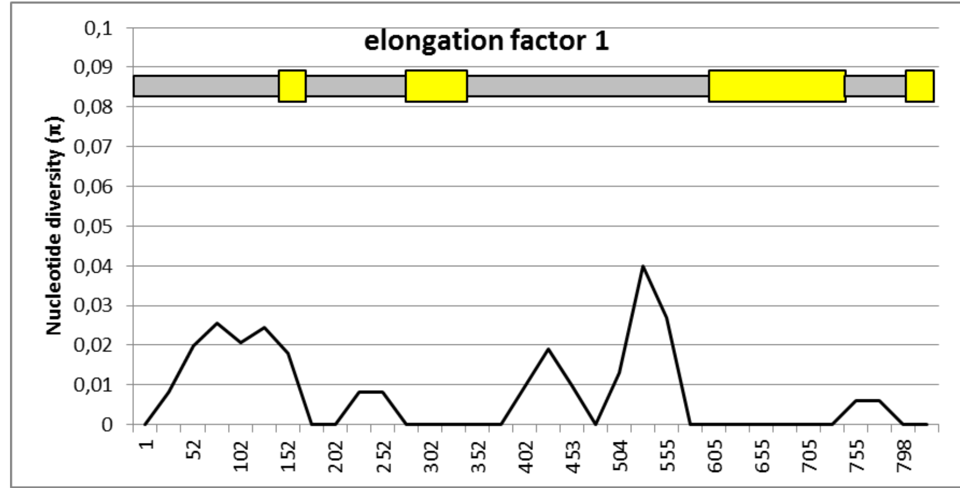
A

B









C

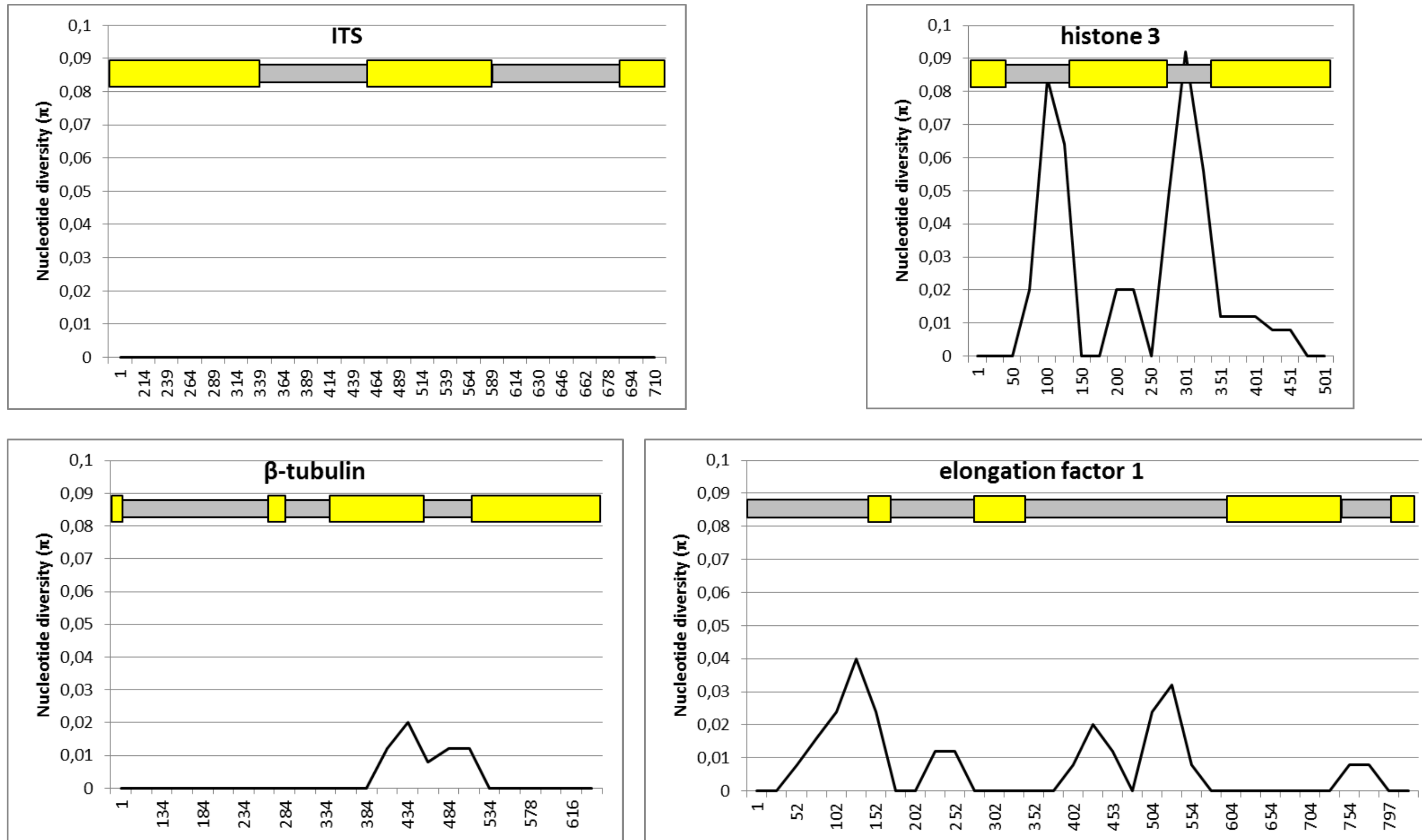


Fig. 2. Sliding window analysis of the nucleotide diversity (π) estimated from *MAT1-1* and *MAT1-2* locus (panel A) compared to nucleotide diversity of sequences from the ITS, β-tubulin, histone 3 and elongation factor 1 markers representing *Ilyonectria* spp. isolates from the macrodidyma species complex clustering in *MAT1-1* (panel B) and *MAT1-2* (panel C) locus. The window length is 50 bp and the step size is 25 bp. Each plot includes a schematic representation of gene exons (coloured boxes; colour codes for panel A are presented in Fig. 1) and introns (grey boxes) and intergenic regions (white boxes).

The use of the primer pairs CyHMG F2 - CyHMG R2, that target the HMG domain encoded by the *MAT1-2-1* gene, and CylMat1-Fd - CylMat1-Re, that target the alpha box domain of *MAT1-1-1* gene, enabled the amplification of part of these genes in several species of *Ilyonectria* listed in Table 1, and also showed that all isolates from those species only contain either *MAT1-1-1* or *MAT1-2-1,* in contrast to *I. liriodendri*. This was observed for species belonging to the macrodidyma species complex as well as for species more closely related to *I. liriodendri* such as *I. radicicola* (Fig. 3).

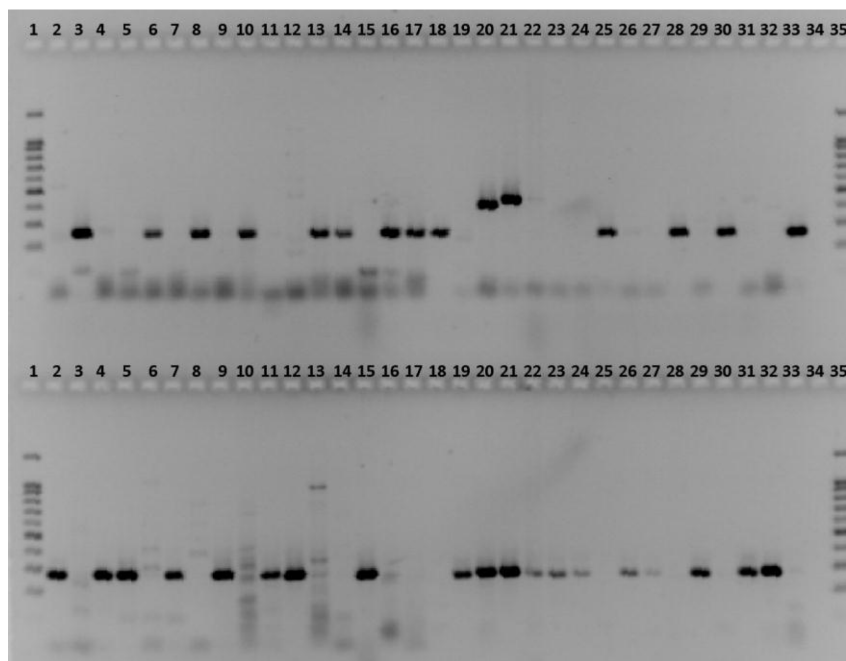


Fig. 3. Agarose gel electrophoresis showing the PCR amplification of *Ilyonectria* spp. DNA using primers CyHMG F2 and CyHMG R2 (upper panel), that target the HMG encoded part of *MAT1-2-1*, and primers CylMat1-Fd and CylMat1-Re (lower panel), that target the alpha box domain encoded part of *MAT1-1-1*. 100bp Blue extended DNA Ladder (BIORON, Germany) (1, 35); *I. radicicola*: CBS 264.65 (2); *I. robusta*: CBS 117813, CPC 13532 (3, 4); *I. crassa*: CBS 139.30, CBS 129083 (5, 6); "*Cylindrocarpon*" sp.: CBS 120370 (7); *I. rufa*: CBS 153.37, CBS 156.47 (8, 9); *I. pseudodestructans*: CBS 117824, Cy20 (10,11); *I. lusitanica*: Cy197 (12); *I. venezuelensis*: CBS 102032 (13); *I panacis*: CBS 129079 (14); *I. europaea*: Cy131, Cy155 (15, 16); *I. liliigena*: CBS 189.49 (17); *I. mors-panacis*: CBS 120359, CBS 307.35 (18, 19); *I. liriodendri*: Cy33, Cy68 (20, 21); "*Cylindrocarpon*" sp.: Cy228 (22); *I. anthuriicola*: CBS 564.95 (23); *I. vitis*: CBS 129082 (24); *C. pauciseptatum*: CBS 100819, CBS 120171 (25, 26); *Ilyonectria* sp. 1: CBS 162.89 (27); *Ilyonectria* sp.2: Cy108, CBS 159.24 (28, 29); *I. estremocensis*: CBS 129085, Cy243 (30, 31); *I. novozelandica*: CBS 112593, CBS 113552 (32, 33); Negative control (34).

Mating type distribution

The employment of diagnostic PCR tests to 82 *I. liriodendri* isolates enabled the identification of 61 isolates as type A (containing *MAT1-1-1* and *MAT1-2-1*) and 21 isolates as type B (containing *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3* and *MAT1-2-1*). From the 74 isolates isolated from grapevine in Portugal, 78% cluster in type A and 22% in type B. However, this proportion varies along the

years, with type B rising from 0% in the first half of the 1990's, up to 40% in 2008 (Fig. 4). Among other grapevine isolates, three isolates received in 1999 from California (USA) clustered in type B, along with isolate Cy180 from Sicily (Italy), while isolate CBS 112596, from South Africa, clustered in type A.

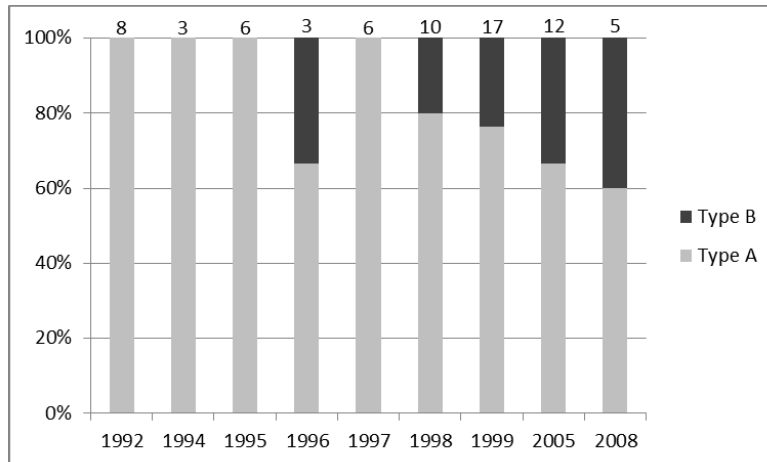


Fig. 4. Variation in the relative proportion of types A and B of *Ilyonectria liriodendri* from 1992 until 2008 in grapevines in Portugal. Values above each bar represent the total number of isolates studied.

Among the remaining species, the employment of diagnostic PCR to 184 isolates enabled the identification of 70 isolates as *MAT1-1* and 86 isolates as *MAT1-2*. Within the overall macrodidyma cluster, 46 isolates are *MAT1-1* and 44 *MAT1-2*. However, the relative proportions of the two mating types are different for the various examined species (Fig. 5). In detail, while the two mating types are nearly equiproportional distributed in *I. torresensis* (54% *MAT1-1* and 46% *MAT1-2*), *MAT1-1* is more frequent in *I. novozelandica* and *I. alcacerensis* (83% and 100% respectively) and *MAT1-2* is more frequent in *I. macrodidyma* and *I. estremocensis* (73% and 90% respectively). A Chi-square test indicated that only the examined *I. novozelandica* (p=0.0209) and *I. estremocensis* (p=0.0114) did not conform to the expected 1:1 *MAT1-1/MAT1-2* ratio. The single *Ilyonectria* sp.1 isolate tested also clustered in *MAT1-2*, while the three *Ilyonectria* sp.2 isolates clustered in *MAT1-1* (two) and *MAT1-2* (one).

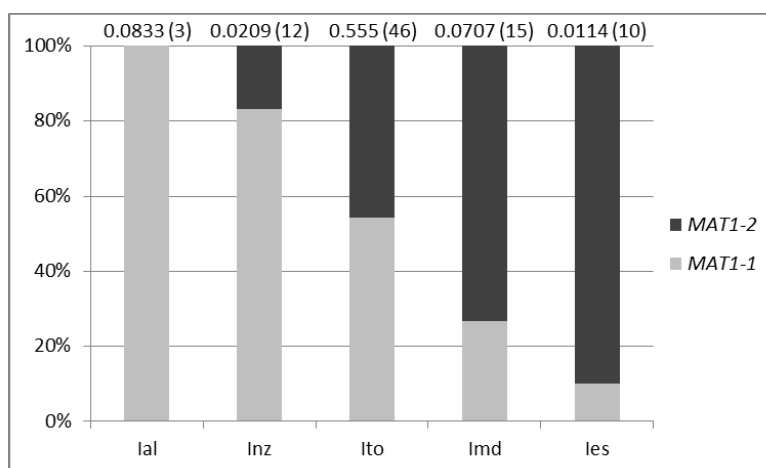


Fig. 5. Relative frequency of *MAT1-1* and *MAT1-2* within *Ilyonectria* species from the macrodidyma cluster (Ial – *I. alcacerensis*, Ies – *I. estremocensis*, Imd – *I. macrodidyma*, Inz – *I. novozelandica*, Ito – *I. torresensis*). Values above each bar represent the probability (p) of values conforming to a 1:1 *MAT1-1*/*MAT1-2* ratio (χ^2 test). Values in brackets represent sample size.

Within *I. novozelandica*, all grapevine isolates from the USA (8) and South Africa (2) clustered in MAT1, while one isolate each from grapevine in New Zealand and from *Festuca* in Portugal clustered in *MAT1-2*. Similarly, all *I. estremocensis* isolates from grapevine collected in 2003 at Estremoz (Portugal) clustered in *MAT1-2*, while a single grapevine isolate collected in 2008 at Vidigueira (Portugal) clustered in *MAT1-1*. An isolate obtained from *Picea glauca* in Canada clustered in *MAT1-2*. Considering the *I. macrodidyma* isolates from grapevines in South Africa, three isolates collected in 2000 at Jakkalsfontein (Malmesbury, Western Cape) clustered in *MAT1-2*, while two isolates collected in 1999 at two different locations in the Western Cape province clustered in *MAT1-1*. Similarly, eight *I. macrodidyma* isolates from grapevines in Portugal collected in different years (2003-2008) and locations clustered in *MAT1-2*, while a single isolate collected in 2007 at Torres Vedras (Portugal) clustered in *MAT1-1*. Besides these, two *I. macrodidyma* grapevine isolates collected in California (USA) clustered in *MAT1-1* and *MAT1-2* respectively.

A different scenario is revealed when dissecting the distribution of mating types within *I. torresensis* isolates, with both mating types found in the same location/year in several occasions. For instance, grapevine isolates obtained in Portugal at Ribatejo e Oeste in 1999 clustered 50% in *MAT1-1* and 50% in *MAT1-2*; at Estremoz in 2003 clustered 67% in *MAT1-1* and 33% in *MAT1-2*; at Torres Vedras in 2007 clustered one isolate each in both MAT1 and MAT2 types; at Vidigueira in 2008 clustered 53% in *MAT1-1* and 47% in *MAT1-2* (from a total of 19 isolates). Similarly, both mating types were recorded among strawberry isolates collected in Asheville (USA, North Carolina). Also, both *MAT1-1* and *MAT1-2* occur in the set of isolates representing

single hosts or locations (*MAT1-1* – strawberry from the Netherlands, *Quercus* sp. in Austria; *MAT1-2* – *Abies nordmanniana* from the Netherlands, grapevine from Australia, Canada, New Zealand, South Africa and Spain).

In the *I. radicicola* species complex (Fig. 6), 21 isolates cluster in *MAT1-1* and 34 cluster in *MAT1-2*. While similar proportions of both mating types are recorded for *I. crassa* (40% *MAT1-1* and 60% *MAT1-2*), *I. robusta* (47% *MAT1-1* and 53% *MAT1-2*) and *I. pseudodestructans* (50% each), *MAT1-1* predominates in *I. europaea* (75% *MAT1-1* and 25% *MAT1-2*) and *MAT1-2* is prevalent in *I. rufa* (33% *MAT1-1* and 67% *MAT1-2*) and *I. mors-panacis* (7% *MAT1-1* and 93% *MAT1-2*). *MAT1-1* was also identified in the single isolates representing *I. lusitanica*, *I. radicicola* and a *Cylindrocarpon* sp. (CBS 120370), while *MAT1-2* was identified in the single isolates representing *I. liliigena*, *I. panacis* and *I. venezuelensis*.

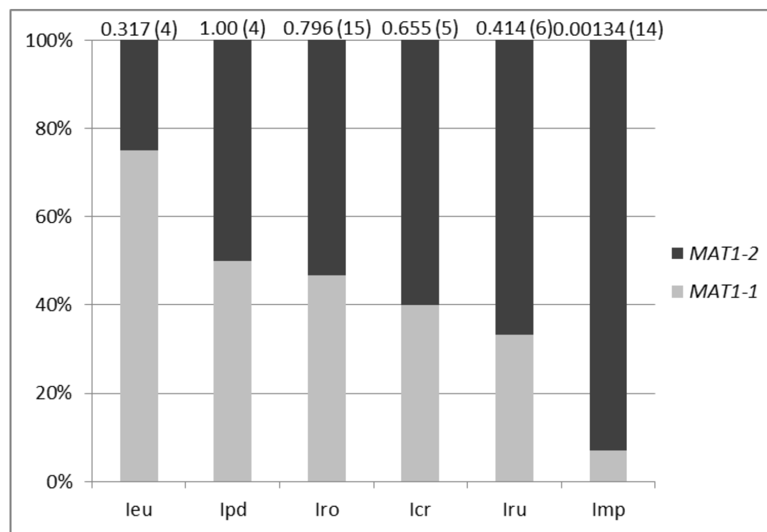


Fig. 6. Relative frequency of *MAT1-1* and *MAT1-2* within *Ilyonectria* species from the destructans cluster (Icr – *I. crassa*, Ieu – *I. europaea*, Imp – *I. mors-panacis*, Inz – *I. pseudodestructans*, Iro – *I. robusta*, Iru – *I. rufa*). Values above each bar represent the probability (p) of values conforming to a 1:1 *MAT1-1*/*MAT1-2* ratio (χ^2 test). Values in brackets represent sample size.

Although very limited in the number of samples, it still worth noting the fact that the two *I. europaea* isolates from grapevines collected in Portugal in 2004 and 2008 at two different locations in the Alentejo (Alter do Chão and Vidigueira) cluster respectively in *MAT1-2* and *MAT1-1*. The remaining two isolates from kiwi in France and *Aesculus hippocastanum* in Belgium cluster both in *MAT1-1*. Similarly, two *I. pseudodestructans* isolates from grapevine in Portugal, collected in 1996 at two different locations at Beira Alta (Gouveia and Viseu), cluster respectively in *MAT1-1* and *MAT1-2*. Another two isolates, from *Poa pratensis* in Canada and *Quercus* sp. in Austria, cluster respectively in *MAT1-1* and *MAT1-2*.

Among the eight *I. robusta* isolates collected from *Quercus* spp. in Austria in 1993, both mating types occur at similar frequencies (at Patzmannsdorf, two isolates cluster in *MAT1-1* and one in *MAT1-2*, while at Niederweiden, two isolates cluster in *MAT1-1* and three in *MAT1-2*). Two *Panax quinquefolium* collected in Canada at separate decades in the 20th century cluster each in *MAT1-1* and *MAT1-2*. Two other *I. robusta* isolates from *Prunus cerasus* and *Thymus* sp. cluster in *MAT1-1*, while three isolates from grapevine, *Tilia petiolaris* and water from an aquarium cluster in *MAT1-2*.

Furthermore, both mating types were recorded among three isolates of *I. rufa* collected in Canada (British Columbia) from *Pseudotsuga menziesii* (two *MAT1-1* and one *MAT1-2*), while a Canadian (from Quebec) isolate from *Picea glauca* cluster in *MAT1-2*. In *I. rufa*, a Belgian isolate from *Azalea indica* also clustered in *MAT1-2*.

Among the five *I. crassa* isolates analysed, three isolates from *Panax quinquefolium* from Canada cluster in *MAT1-2*, while two isolates collected in 1930 in the Netherlands from *Lilium* sp. and *Narcissus* sp. cluster in *MAT1-1*.

Contrasting results can be pointed out from the analysis of the mating types identified for the 14 *I. mors-panacis* isolates studied, all from *Panax quinquefolium* in Canada (Ontario): while two isolates collected in the 1930’s cluster in each of the mating types, 12 isolates collected in 1996 and 1997 cluster only in *MAT1-2*.

In the pauciseptatum cluster, single isolates representing *I. anthuriicola*, *I. vitis* and a *Cylindrocarpon* sp. (Cy228) cluster in *MAT1-1*, while for *C. pauciseptatum*, one isolate clusters in *MAT1-1* and eight in *MAT1-2*. Among these, three grapevine isolates from Portugal cluster in *MAT1-2*, while among five Slovenian grapevine isolates collected in 2005 and 2006 one clusters in *MAT1-1* and four in *MAT1-2*.

Results concerning mating type frequencies for each species need be considered in relation to the frequency of detection of each species throughout the years (Fig. 7). For grapevine isolates in Portugal, the frequency of isolation of *I. liriodendri* has decreased from 1992 to 2008, with an average frequency of isolation of 91% in 1992-1999 and 39% in 2000-2008, paralleled by an increase on the relative frequency of type B compared to type A. On the contrary, the frequency of detection of *I. torresensis* has increased from 2.6% in 1992-1999 (in fact, it was first detected in grapevine in Portugal in 1999) to 26% in 2000-2008, with both mating types detected at similar proportions throughout the years. Although at lower levels, the frequency of detection of *I. macrodidyma* has been increasing (0% in 1992-1999 and 11% in 2000-2008). Other species

were detected sporadically, although in some occasions their frequencies are relevant (e.g., *I. estremocensis* in 2003).

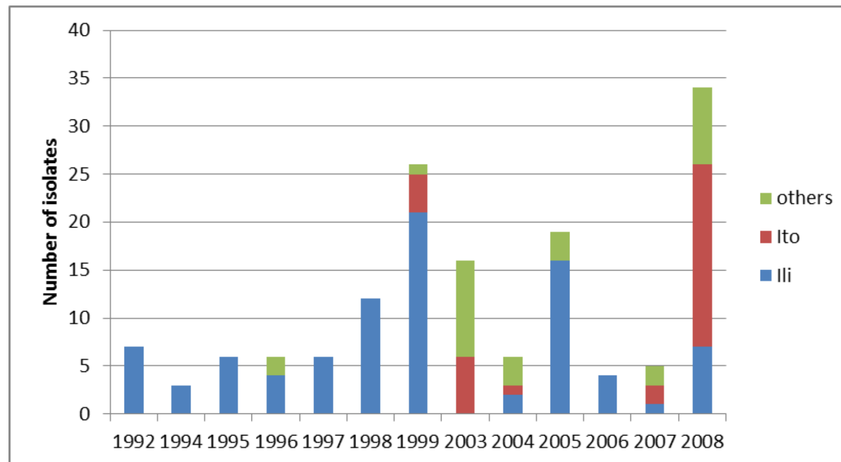


Fig. 7. Variation of the number of isolates identified as *Ilyonectria liriodendri* (Ili), *I. torresensis* (Ito) or others in grapevines in Portugal during 1992-2008.

Discussion

In the present study the DNA sequence for the *MAT1-1* and *MAT1-2* locus of *Ilyonectria* spp. from the macrodidyma species complex was obtained, including *I. alcacerensis* (Cy133, Cy134 and Cy159), *I. macrodidyma* (Cy175, Cy216, CBS 112601, CBS 112603 and CBS 112615), *I. novozelandica* (Cy130, CBS 112593 and CBS 113552) and *I. torresensis* (Cy118, Cy137, Cy222, Cy237, CBS 112604 and CBS 113555). The structure of these loci corresponds to the standard structure of Sordariomycetes, with species from the genera *Fusarium*, *Epichloë*, *Hypocrea* and *Metarhizium* giving the best hit in homology searches. Similarly, the two types identified for *I. liriodendri* mat gene sequences (Cy27, Cy33, Cy36, Cy68, Cy76, Cy107, Cy109 and Cy111) were sequenced, with *Fusarium*, *Epichloë*, *Metarhizium* and *Nectria* as the genera of the best hit species.

Distinct mating types were identified for nearly all species addressed in this study for which more than one isolate was available, indicative of probable heterothallism. These species are *"C." pauciseptatum*, *I. crassa*, *I. estremocensis*, *I. europaea*, *I. macrodidyma*, *I. mors-panacis*, *I. novozelandica*, *I. pseudodestructans*, *I. robusta*, *I. rufa* and *I. torresensis*. Only *I. alcacerensis* had all three isolates clustering in a single mating type. However, a very particular situation was identified in *I. liriodendri*, with two different types of locus organisation identified, both of them containing components of a *MAT1-1* and *MAT1-2* isolate. These could be considered genetically homothallic, but in practice they are self-sterile. This raises the question why type B isolates are

not capable of selfing despite the fact that they contain all mating-type genes. Further functional or transcriptomic studies would be required to ascertain whether *MAT1-2-1* or *MAT1-1-1* are not expressed or not fully complete. As opposed to pseudo-homothallism (Merino et al., 1996), where genetically heterothallic strains behave as homothallic, the current situation of genetically homothallic but functionally heterothallic strains in *I. liriodendri* could be considered a case of pseudo-heterothallism, eventually representing an evolutionary transition from heterothallism to homothallism as suggested in other occasions (Martin et al., 2011). An idiomorph is characterized by the fact that it contains dissimilar sequences at the same chromosomal location, and normally it contains either *MAT1-1-1* or *MAT1-2-1*. In *I. liriodendri* both types contain *MAT1-1-1* and *MAT1-2-1* and the dissimilar sequences are located between these two genes, containing either a putative ORF (type A isolates) or *MAT1-1-3* and *MAT1-1-2* (type B). This putative ORF is also present in *MAT1-2* mating type loci of the heterothallic species of the macrodidyma complex, while *MAT1-1-3* and *MAT1-1-2* are present in *MAT1-1* mating type loci of the same species. Conceptually, this is a very interesting case with an idiomorph lacking sequences that are normally present within the idiomorph but now are shared by the two mating types. This questions whether *MAT1-1-1* and *MAT1-2-1* can be properly used to define mating types. Further studies should be done in order to verify if the putative ORF is expressed in heterothallic species of the macrodidyma complex and in type A isolates of *I. liriodendri*.

The potential phylogenetic informativeness of the mat sequences was assessed by analysing the nucleotide diversity across *MAT1-1* and *MAT1-2* idiomorphs of the macrodidyma species complex and comparing it to the nucleotide diversity of sequences from rDNA-ITS, β-tubulin, histone H3, and translation elongation factor 1-α markers from the same isolates, previously obtained and used for taxonomic purposes (Cabral et al., 2011a). Results show that both average and maximum nucleotide diversity parameters of the mat locus are higher than that of rDNA-ITS, β-tubulin, and translation elongation factor 1-α, but lower than that of histone H3. Although species in the macrodidyma complex are morphologically similar and were resolved mostly based on a phylogenetic analysis, the levels of nucleotide diversity recorded among them are higher than those observed (both for the mating type genes and histone H3) among nine species from the *F. graminearum* complex (O'Donnell et al., 2004), further validating the delimitation of species in the macrodidyma complex (Cabral et al., 2011a).

The frequencies of each mating type across the various species analysed varied strongly. Among grapevine isolates, *MAT1-1* was exclusively observed in *I. alcacerensis* (along with single isolates from *I. lusitanica* and *I. vitis*) and predominant in *I. novozelandica*, while similar proportions were recorded in *I. europaea*, *I. pseudodestructans* and *I. torresensis*, and *MAT1-2* prevailed in *C.*

pauciseptatum, *I. estremocensis*, *I. macrodidyma*, and was exclusively detected in *I. robusta* and *Ilyonectria* sp.2. Similarly, *I. liriodendri* type A (*MAT1-1-1*; *MAT1-2-1*) prevails over type B (*MAT1-1-1*; *MAT1-1-2*; *MAT1-1-3*; *MAT1-2-1*). Although type B was undetected in Portugal in the early 1990's, its frequency increased since 1996, when it was first detected, reaching up to 40% in 2008. Moreover, grapevine isolate Cy68 (type B) was shown to be more virulent than grapevine isolates Cy5 and Cy253 (type A), or even isolates Cy 164, Cy 232 (type A) and CBS 110.81 (type B) from other hosts (Cabral *et al*. 2011b). Both mating types were recorded simultaneously in the same region, year or even in the same vineyard in Portugal. Although more limited, samples from the USA also suggest a similar trend. The appearance of type B in Portugal and the increase in frequency seems to be associated with a higher virulence of some *I. liriodendri* isolates (Cabral et al., Chapter 5) which were shown in the present study to cluster in type B of *I. liriodendri*.

Among *I. estremocensis* isolates obtained from grapevine in Portugal, only *MAT1-2* was identified among eight isolates collected at Estremoz in 2003, while *MAT1-1* was identified only in 2008, although isolate Cy243 (*MAT1-2*) proved (Cabral et al. 2011b) more virulent than Cy135, CBS 129085 and Cy152 (all *MAT1-1*). As for *I. liriodendri*, the appearance of a new mating type seems to be linked to an increase in virulence.

Furthermore, the single *MAT1-2* grapevine isolate (Cy155) identified in *I. europaea* this far is more virulent than a representative of *MAT1-1* isolates (Cabral et al. 2011b).

While both mating types were recorded among *I. macrodidyma* grapevine isolates from Portugal, South Africa and the USA, only in the latter case they were both reported from the same vineyard. Interestingly, one of such isolates (Cy128 – *MAT1-1*) was recorded as the most virulent *I. macrodidyma* isolate (Cabral et al. 2011b).

In contrast to these species, only *MAT1-1* was recorded among *I. novozelandica* grapevine isolates. Interestingly, four of such isolates included in pathogenicity tests to grapevines were among the least virulent in a range of *Ilyonectria* spp. black foot pathogens (Cabral et al. 2011b), not differing significantly from *I. novozelandica* isolate Cy230 (*MAT1-2*) from *Festuca duriuscula*.

Similarly to *I. liriodendri*, both *MAT1-1* and *MAT1-2* were recorded in *I. torresensis* grapevine isolates in Portugal in the same year, region or even vineyard, often at similar proportions, with no evident differences in virulence between isolates from each of the mating types (Cabral et al. 2011b).

The increased virulence of isolates clustering with mating types that were not present previously (e.g., type B of *I. liriodendri*, *MAT1-1* in *I. macrodidyma*, *MAT1-2* in *I. estremocensis* and *I. europaea*), together with the increased frequency of isolation of species that were absent during

the 1990's (e.g., type B of *I. liriodendri* and *I. torresensis*), suggests that the appearance of new genotypes of black foot pathogens is largely responsible for the increased incidence and severity of the disease.

Acknowledgments

Marizeth Groenewald and Madhi Arzanlou (both at CBS-KNAW) and Inês Diniz (at ISA/UTL) are thanked for technical help and scientific discussions. This article is part of a Ph.D. dissertation (Instituto Superior de Agronomia, Technical University of Lisbon, Portugal). This work was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/24790/2005; project PTDC/AGR-AAM/099324/2008).

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Supplementary material S1

DNA and protein sequences of the *MAT1-1* locus of *Ilyonectria torresensis* (isolate Cy137) including the genes *DNA lyase* (Blue), *MAT1-1-3* (light green), *MAT1-1-2* (dark green) and *MAT1-1-1* (yellow)

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1    TCTGCCAACAACGGTAGTCGGATCGACTACATCCTGTGTAGCGACGGTATCAAGGATTGGTTCACTTCTGCCAACATACAAGAAGGCTTGATGGGATCTG
101  ACCACTGCCCAGTGTTCGCGACCATGGCAGACAAGCTCGCAGTCAAAGGAAAGGAGCATGCCCTCCTGGAGCTCTTAAACGCCCAGGGCACATTTCAAGA
201  CGACCGACGTCTTCGCGAATGGGGACCAAAAGACTTGCTTCCTTTGTCAGCGAGGCTGATACCAGAATTCGATCGTCGACAGAGCATTCGCGATATGTTC
301  ACAAAGAAACCCAATTCGACCTTGGAGCAGAGCTCGAAGCACAAGGTGGTGACCGAAAATCTCCACCCACAACCGACCACGAAGCGGCCGATTGCTACAG
401  GTGAAAGTATGTCTGAGCGGGCTTTGAAGAAGACCAAAGCGCCATCAAATTCCGTCAACGCCAAGAACAAAGCCGTTACGGGACAACGAACGCTTCAAGG
501  TTTCTTCAAGCCAAAAGCCTCAACGCCTCAGCCCGAGGCTCCAAGCGTGGGCGGCACGGGAAGAGCAACGCCATCGCCGACAACAAAGACCTCGCTTGGC
601  TCACCCAACGGTCCGCCTAGCCAGCGGCTTCCGCCTCAGCCGCCCGAGACTTTGTCGAACGGGCATTCTGAAAGAGTCTTCGACCCAATCGAAGCGAAGG
701  AATCCTGGTCGAAGCTCCTCCGTAAGCGAGTTGCCCCGGAATGTGAGCATCACGAGCCTTGCATCAGCCTCACTACCAAGAAGCCGGGCGTCAACTGCGG
801  TAAGTTCGCGATCCATATTTAGTTTGCGGGCCTGCACGTATTGGGACATGGAACGTTGTCTCGGTTATGATAGGGAAGCTAATACGAGGCACAGGGCGGT
901  CCTTCTACATTTGTCCGCGACCTCTTGGCCCCTCTGGGGAGAAGGAGAAGGAATCGGAATGGCGGTGCGGGACGTTCATTTGGAGCAGCGACTGGAACTC
1001 TACGTCTTCGGGATGATGACATGAGACGGGCGCAGCATAAGACAGCACGTAGAGAATATAATGTTCGAACTTCTTTTGATTCCAACTACATGACCTGTTC
1101 GTGGGTATCGGCCGCCAACGCGTTTAATAGGCAGCTTTAGTCCATAATGTTTCCACCAAGACAAAGGCACTGGCTTCGTTCATGGGAATTATGGTTAAGG
1201 ACCACGGCCATAATCATGTCGTCTTCTTCTTCGTGGTGAATTTATAGCCCGGGTACTGGGCCTTGTGTCGCCGATCCTCGTCCTCGGCAAGAGCTTGCCA
1301 ATAAGCTCTGACCTCTGGCGTCTCGTCCTTCCAGAGTTGGGAAATGATAGTGGCTATATTCGTTAGACATGCTTCCATGCTTGATGAAGAGACCTACAGG
1401 TTTCAGCGGCAGTGATTCCAGGGTTTCGCTTAGCGACCTCCTGGGACTTGGCCTGGCGATACAGAATCCATTTGTTTCTGGGACGAGGAATATGAGGCTC
1501 CGATTCGGATGCAGTAGGGGCTGTCGTCTCCTCTTTGTCCCTCCCTTCAGCGTCCACCTCAGTGGATGCATCTATGTTTGGCGTTAGCTCTGCGTAGTTC
1601 GCGACTCCGAGGAATGTCCATACCACAACGAAAGCAGAATTGACCGTATATCGGGAGTTCCACCATCACATGGTATGAGAGCGGACAGAGGCGGTATTTC
1701 TGACGGTGAATGTCATGGAAGACTGTGACTGACTGGTTGATGCGTCGGCTGTTACAGTTAGTAAGGCTTGAGCCTCATGTAGCAATCTGTTCGTACCTGA
1801 AGATCATTGCGATTTTGCGCACGATCTTGATGTTAAACGTCTCGGGCAAAAACACATTGACATCACCCTGCATAGCAGAGGTCACGAAGATGAAGGGCTG
1901 CTTCAAGCATAACTGGGGAATGGCGTTTTGTATTTCGGCAAAGGAGGACATGGCGACTTTGCTGTGGCCGGTGTTGTCGTGACGTTGGGAGAGGTGAACT
2001 CTTCGAGCGAAGTTTTGTGGTGATGAGTGAGGAAGAAAGCTGCCTCACAATTTTGGTGAGCCCGAGAATTTTATAGTGCGCCTTGCACTAAACAGAGAAC
2101 AATGGCTGCCTATCTACCTACTTACGTACCTAAGTAGGTACCTAGGTTGTTTGGTGCCCAATGGTTGTTCAGTGAATGGCTGTTTGTTTGGCGCACTTTG
2201 TCTTCGCAGCAGACGCGCAGCTGCAACTCCAAACTCCCAACATTTACCTCCCACCTCCACCTCCCACCTTTCACTTCTCACAATCTTTCCCTTTCAACAC
2301 CACAACAATCATCATGGACAATCTGTTGTTCTATAGACCACTTTGGGAGGAAGAGGCCTTGATTTTTGATTCCTCGAAGCATATTGAGAAGCTCCGCCTA
2401 AAGAGCATCGGGATCCTCTTGCACAAGCTTCCTGTGGACGTCAATCCTCCATCTGTGGAAGGTAAGAGCTTTGATCTGATACCTTTCGTACGCGTGTCAA
2501 CTTACCCAATACTAGCGGTGATCACTTCCAGCATCCACATCATCAAGACATTGCTGGAGGATGTTTCTGATGACAATGCGATTTTGCAAAAAATACGAAA
2601 TCATGCGACCGAGGTTGGAGTCGACCCAAATCTTGTCGTTCGAGGTGCCATCGTTTTGTGGTATTCGAGTGTTCTCCCCATTCTGTCAAGGGATCCAGAT
2701 CAAGGCTTGCCCCATCACGCGATCGTTGCGGGAGGCCTTGCGTTGCCGTGGGTACAAAAGTTGGCCGAGAATCAATACTTCATTGGGAACTTGGGATACT
2801 TGTCGATGTTATTGACTGCTGAAGACTGGGAAAAACCAACTCATCCGAAACTACAGGTCGCGACTTTGATCACAAATGCGATGGTTTCAATTATCTACGC
2901 AGCTTTCGTCATCACGCCGCATCTGAACGCAAACTTGAAGTGGCGTGAGTTGCTCATGAAGTCGGGCAGCAGAGGCGGCTACGAATTGAGGGTCTTTCTC
3001 AAAACATACTGGCACGCTTCTAGGGAGGGATGGGAAAAGCTCAACTACCCTCCCGGCTCTGAGTTTTCTGCTTTGGAGTCTGAAGCTAGGATGTCCCCTG
3101 ATGGGAAAAAACTGTTGACTCGTGTTGGCCAAGGAAACTGGCACGAGGCGCCATTCTGGCACCCCCTCAGAACCGTTCCGGGTTCTCCATGGAACAAATT
3201 CATCAAGAACAAGAAGCAGAAGGTCTTCCCGGTCCTCCCACGGAGAGGCAACAGTGTCCGATACATGGTTCCAGGGTCGGCGACTGGGTTGATCGGTTCC
3301 TGTGAGACTTACTATGGCATCTTGCGTTCGCAGTTTGATGACGTGAGCGATTCATTCTCCATTGTTCGGGCTTTGAGCTAACGATTTATTCCAGACAGAA
3401 ACACCACGAGTCGAGGTTTCGGATGCGGAGCGAATTGAACAATTCAAGCGTTTAGCTGAGGGAACAGGTCTCGAATACCCTAAGACGAGACCAGTCGTAG
3501 AGGTAGGTTCTCGTCTCTTCCAGAAACACAATGGGAGGCTCACCAGACTAGAACTCCATGATCCGTGATTACTCAGGAGCAGAACACATGTACAATGTAC
3601 CTACGGTATACTCGCCCTTTCCCAGAGTTTCATTCAGAATACGCTAATTTCCATTTAGATTAAAAAGAACGCTTCGGACTTGAGCGGCAACTTGACTCTG
3701 CCGTTTTTGACCACCACCGCTCGAGCAATGAGGTTCACCGAGGAGCCTGACTTCGTCGACGAGGATTTCCACGCAATGGTTTTCTTCCAGTAGGCTCATG
3801 GTTCGCGCATGAATTGCCTGGATATCAGCTGCAAAGCAGCACGAAGAGGCGGTGGATTGCTCATTCTTCATTGGAAACAGAGAAGCAAGGCAGTGTTTTT
3901 CAATTGTGTGTAGACCACAGTAGTACCTGAAACCATCTCTTCTCAAGGAATCATGTCTCGCCTGTGCGTAGTGTGCCTTGTATGGTAGATTGGGGTCTTT
4001 GGTTGAGTCTGGGGCAAGTGGTCACAGCTGCCAACAATGGCAGCAACTGACAACGGCTGGTATGTATCCCCACTAGCTTCGCGCATTCACAACGCCTCGC
4101 GGCAAGCCACCAAACACAGAAATCCCATTGTCACTTCTGTCAACATTGTTTTTACTGGAACCACACCTGCAGAGGGGCTAGAAcCCCCCCTCCCTCTCGC
4201 CCTCTTAAATCACCTCCCCCTCCTTCTTTTCTCTCTCTTTCTTGACCCAATACCCAAATTCTCTGATATCTTCACAACCTCACATCATTCCAAAATGGTC
4301 ACTCAGAGATCCTACGTCATGCAGCAGTTGGCGTCTCTGTCCACAGCAGAGATTCTCAGTCTGCTCACCGATGACACCATTCTCGAAGTTGCTGCCAGGT
4401 ACTTCGAGATCAATGCCAACACCATGGATGCCAACGAACCCTTGAGCCCTTCGGGTACCGACATCGTTACGACACCCCCTGCTCAAGATGTGGCTGGTAA
4501 CGACTCTGCTCCTCGTGCCAAGCGTCCCCTCAATGCATTCATGGCGTTTCGCAGTAAGTCCACCTGTCTCTTCTGAGTTCTTGCTGCTTACCACCTCCAG
4601 CTTTCTACATGAAGTTGTTTCCGGATATCCAGCAAAAAACCGCATCTGGTTTCCTAACCACTCTCTGGAACAAAGATCCCTTCAGAAACAAATGGGCATT
4701 GATTGCCAAAGTCTACTCATTTGTGAGAGATGAGGTTGGCAAAGGGAATATTTCACTGTCACGCTTCCTCGATGTGTGCTGTCCCACCATGAACATCATC
4801 GAGCCAAGAGCCTATATTGCCGCCCCTCGGCTGGACTGTCAAACATGATGACACAGGCTCCCAGGTCCTTCTCGAAGGCACACCAACCGACCTTTCTCGTT
4901 TCCAGGATGAGTCGGTTCCGAACACGGAAATGGAACTTCTTCGTGGCCTTCTCAACACAGGATATCTCTCCGATCGCGGCCTGGCTTTGATGGAGCGACT
5001 GGGCGAAAGCTCCAACGGTATCATGACGACCTATGGAGCCTCGATGGaAGCACCAGTGATTTTGACTACGGAGAAACTTGACTTCGTGGATCTCGTCCGG
5101 GAGAACCCGCAGGAAGCGGTGAGAGCATTGGTCGATGACACGGCACGCGAATTCGTTTTCCAGCAGGAGATTCCCGTGTACGATGTCTATGATCTCAGCA
5201 ACGCTCCTTTCCACTCTATCCGAGGCCCTCAGCCGAACCCGTCACAGAACTACACTTTCTCTAATTCGAGCTTGGGACTCTACCCTGTTCGAGCCCTGAC
5301 ACTCAGCAGTGtTGGTGAATTCGATGCAATCGACCTTGACAGCCCCTTTGACATCGACGTCATAATGGGACACACTCGATCGGAAGGCGAGAGAAGTAAG
5401 TGAATTCACAATAACTTTTCAAATGCCTACTGACATCTGATAGCTCGTGACCAGCCGGAAGGCGAGCCTTTCRACCCCCATGCGGACTACAACTCTCTCC
5501 TTTGAGCTTGGACTCTCTCGTGACGGAAATCGAGGCACAGAATGATGGCGGTACGCAAACGAAGTGGGATGGGGGACGACACTAGGAGAAGAACTTGTCT
5601 TTTTTAGTCACTTTAGCTCACAACTCGAACACGAAACAGTCTTGATGTTTTTGCGCAATGACTTGAGGTATCGTCGCTCACTTGTGCACACGAAGTGAAC
5701 GGTCCGGTGCCAAGACTACAAGACGTGATGTATGAGTYGTGGCTTTTTCGCCCAAGTCCTGATCCACCTTTAGCACTGAAGGGTTGTCCTTTTCTACAGA
5801 GTGGATATGGACGTGGGTGGCTTCAACGTGGGAACAGCTGCTGAAGAGGTGGGCGTAGTCGGCAATGGAGAATTTACGGGCATAGCGATAATTGGGGGGC
5901 GTCGCTTGGCATTTGGGATGTTTCAGTTTAGTGAGAGAGGTGGAGCCAATGACCATGGGAGAGTGAGGTGGATTGATGCTGACCTAGGAGGCTAAGATA
6001 AGGTGAGGTGAGGAAAGCGGTTGGGGTTTAGTGCTTGCGAACGGAGGAAGCTGAAGCCGGTTGGAGTGGGCACTCAATCAACCACCCTCCCAAGGCTAGG
6101 GACTAAACTTACGCAGAATAAGGCAGCTAGCTTAGACATGCTGAACCAGTCTCGAAGTGATGTTCGATCAAGCAGCTCCTTAGGCTTCCGGGGATCGGGC
6201 TTTGGCGGGCCGGAGGAGGGTGGAGGGTCTGAGGCAAGGAGCACTTGTGGAGTCTGGACGGGAACTGCCAGCTAGCAGATGATTACAAAACAATAGGAA
6301 ATAGGATTGCCACTAATTGACGGAGGTGCCAGACACGCCAGCAGTGTGAAAAAACCAAGGACGTAGTCTAATAAATGCTACGAAACGCAAGGGACGATGA
6401 GGCGTCGGGGAGTCGGCCATCCATCCATCCATCCATCCATCCTGGATGCTGATACGCAAGTTTACTTTAGAGAGTGCCGTACCGAATTGGCGCTATCTTCAAG
6501 CCCTACAAGTGGGGGGCTGCGGCGCTAAGGAGCACTAACTCTGAGGCTGTCCAACTGCCATGTCTGGACGACCACAGACACACACGCACAGCCCAGCTTG
6601 AATCATTGACACACCTGGACAAATCGTTAACAAGTCCATGCAATATGGAATGCTTTGACTCGAGCCCTGATGAAGAATTCCACCTCCGACGCCTACTCTA
6701 AGCCGGAAACACTAGCCATTCGAGCCAACGCTCAATTAATCTGTTTACGTTTCATACCTTATTCATGTTTTTATCATCAATTTGTCACGTCCATCTGTTA
6801 ACGAAGGCTGTCACCTCCGTTTTGGCAGATGGGGTCTGTGGACAAGAAGTGGAGTGAACTCTCCGACGTCGTATGTTAATTGGATTGCACCCTTCCTAGA
6901 CGTGCGGCTGCCGCATGCCTGGCCGTTGAGAATTTAGTGGAGCTTCCCTcCCTCTCTTCTCCCCCCTCCAAAAAAAACAGGGGACTGCACGAACCACTGC
7001 CGGCAGTGAGGTCACAGGCGCCAGGCGCCAGGTGCCAGACAGACGCAAACATGGCGATCCAAACAGGGTCATACAGTGGGGCTGTTCTTGTGGCTACCTC
7101 TAGTGTGGGGGCTTTTGTGTTGTTTGGTGGCTGTTTGGTGGCCGTTTGGTGATCGTTTGGTGGTCGTTTGGCACATCTGAGGACAATTGTGGCCCGAGAC
7201 CTCAGTTAAACATAGTTAAAACATGGCATCAGTGCTTCATTGCTTTGGCTTGCTTGCCCTGTTCGATTTCTAGCCTGGTAGCACCGCCGTCTGGGGGGAT
7301 TTCGTCTGTCCGTCTGTCTGTCTGTCTGCCTGCCTGTCTGCCTGTCTGGATGAGATGAGCCGAGATGAGATGGATGGATGAGATGGATGAATGAGATGGA
7401 TGGATGAGATGGATCGCCCTCAATCTAAGGTGGGTACCATTCGGCGATCCCCAGTTACGCTAGAACATGAGTCGCCGGCCCCTGGCTCGAGCATTCCTC
7501 CACCACACGGACTGCCATGAGGTAAATGCTGAATGCCGACCACCCTCAGGTCGTTCAGTCGCCAGTCGCCAATCGCCAGTCGCCAGTCGCCAGTCGTCTG
7601 TCCATCTGGGCGCTTAGTGCGTGCGAACAGTCGCAGCCGCACCTTGGCGCAGCCACGCTAAGAAAAGCCCCCCCAGATCTGATGAGCGGGGGGGCATTTT
7701 TTAGCTTCGTGAGCTGCAAGGCTCAAGCGGCCGACTGCGAGTGGCTGAGTTACGCACCCACCCACGCACAGTGTAATAAGACGGTATCCGATGRACCGCA
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7801 TGCCTCTCGATTGTCTTTAACTCTGAGCGGGCGGCGAGACGAACTTCAGCCCGAGATTGATTTGGCCCAGACCAAAATTGAACCAGCTCGAGAGTCTGAT
7901 CTCA
```

DNA lyase

```
SANNGSRIDYILCSDGIKDWFTSANIQEGLMGSDHCPVFATMADKLAVKGKEHALLELLNAQGTFQDDRRLREWGPKDLLPLSARLIPEF
DRRQSIRDMFTKKPNSTLEQSSKHKVVTENLHPQPTTKRPIATGESMSERALKKTKAPSNSVNAKNKAVTGQRTLQGFFKPKASTPQPEA
PSVGGTGRATPSPTTKTSLGSPNGPPSQRLPPQPPETLSNGHSERVFDPIEAKESWSKLLRKRVAPECEHHEPCISLTTKKPGVNCGRSF
YICPRPLGPSGEKEKESEWRCGTFIWSSDWNSTSSG
```

Best three blastx hits

```
>gb|EGU79454.1| Hypothetical protein FOXB_10039 [Fusarium oxysporum Fo5176]
Length=593

 Score =  336 bits (862),  Expect = 2e-109
 Identities = 187/344 (54%), Positives = 215/344 (63%), Gaps = 44/344 (13%)
 Frame = +1

Query  4    ANNGSRIDYILCSDGIKDWFTSANIQEGLMGSDHCPVFATMADKLAVKGKEHALLELLNA  183
            ANNGSRIDY+LCSDGIK WF  +NIQEGLMGSDHCPVFA ++DK+ V  KE ALLE++N
Sbjct  252  ANNGSRIDYVLCSDGIKSWFNYSNIQEGLMGSDHCPVFANLSDKVTVGDKECALLEMMNP  311

Query  184  QGTFQDDRRLREWGPKDLLPLSARLIPEFDRRQSIRDMFTKKP-----------------  312
             G F  D RLR+W PKD LPLS++LIPEFDRRQSIRDMFTKK
Sbjct  312  PGMFNGDERLRDWSPKDHLPLSSKLIPEFDRRQSIRDMFTKKAAPPREPTRTDTPAEPLN  371

Query  313  --NSTLEQSSKHKVVTENL----------HPQPTTKRPIATGESMSERALKKTKAPSNSV  456
              NS+   S  +   T  L            QP++KRP  T    + R  KKTK+ + +
Sbjct  372  NGNSSASGSPTNASSTPRLGETTNSTKLSASQPSSKRP-GTAADTTSRPFKKTKSFTGAN  430

Query  457  NAKNKAVTGQRTLQGFFKPKASTPQP---------EAPSV----GGTGRATPSPTTKTSL  597
            + K+K   GQRTLQGFFKPKA   Q            PS      G+G+A P+   + S
Sbjct  431  DTKSKVAQGQRTLQGFFKPKAPAAQDGKAELVAANSTPSTTKKPAGSGKA-PASAQRLSN  489

Query  598  GspngppsqrlppqppETLSNGHSERVFDPIEAKESWSKLLRKRVAPECEHHEPCISLTT  777
                 P  +  P  P       S+RVFDPIEAKESWSKLL KRVAP CEH EPCIS TT
Sbjct  490  TPQATPTEKSSPTVPLRGKDPDPSDRVFDPIEAKESWSKLLGKRVAPRCEHDEPCISFTT  549

Query  778  KKPGVNCGRSFYICPRPLGPSGEKEKESEWRCGTFIWSSDWNST  909
            KKPGVNCGR FYICPRPLGPSGEKE+ SEWRC TFIWSSDW  +
Sbjct  550  KKPGVNCGRMFYICPRPLGPSGEKERNSEWRCSTFIWSSDWTGS  593


>gb|AEI72618.1|  DNA lyase [Epichloë festucae]
Length=642

 Score =  323 bits (827),  Expect = 1e-103
 Identities = 183/361 (51%), Positives = 219/361 (61%), Gaps = 57/361 (16%)
 Frame = +1

Query  4    ANNGSRIDYILCSDGIKDWFTSANIQEGLMGSDHCPVFATMADKLAVKGKEHALLELLNA  183
            ANNGSRIDYILCSDGIKDWFT ANIQEGLMGSDHCPVFAT+AD+++ KG + ALL+++N
Sbjct  281  ANNGSRIDYILCSDGIKDWFTFANIQEGLMGSDHCPVFATIADRVSFKGNDTALLDVMNP  340

Query  184  QGTFQDDRRLREWGPKDLLPLSARLIPEFDRRQSIRDMFTKKPN----------------  315
             G F   +R+RE   +DLLPLSA+LIPEFDRRQSIRDMF KK +
Sbjct  341  PGVFAQGKRVRELAQRDLLPLSAKLIPEFDRRQSIRDMFFKKSSTIGVAADGAPSPGQPA  400

Query  316  ---STLEQSSKHKVVTENLHPQPT---TKRPIATGESMSERA------------------  423
               S++ ++    V   N +       T   +  G S + RA
Sbjct  401  KRASSITETGLRPVSNNNSNNNTDRNGTSEDLLGGASANSRAANAQQAESQKRPAEPIDP  460

Query  424  ----LKKTKAPSNSVNAKNKAVTGQRTLQGFFKPKASTPQPEAPS------VGGTGRATP  573
                LK++KA +++ ++K K   GQ+TL+GFFKP  S P    PS          T   T
Sbjct  461  APRQLKRSKAGADTNSSKLKTAPGQKTLKGFFKPVDSAPVHPTPSSLQESRTNNTSPRTA  520

Query  574  SPTTKTSLGspngppsqrlppqppETLSNGHSE-------RVFDPIEAKESWSKLLRKRV  732
            + +T  +      P    L    P+   + HSE       RVFDPIEAKESWSKLL KRV
Sbjct  521  TDSTSPTKSVSGEPVGSELTSPSPKYSQSHHSEPIPRLPDRVFDPIEAKESWSKLLGKRV  580

Query  733  APECEHHEPCISLTTKKPGVNCGRSFYICPRPLGPSGEKEKESEWRCGTFIWSSDWNSTS  912
             P CEH EPCISL TKKPGVN GRSFYICPRPLGPSGEKEK SEWRCGTFIWSSDWN T+
Sbjct  581  VPRCEHEEPCISLVTKKPGVNRGRSFYICPRPLGPSGEKEKGSEWRCGTFIWSSDWNGTA  640

Query  913  S  915
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            +
Sbjct  641  A  641


>gb|EFY88584.1|  DNA lyase [Metarhizium acridum CQMa 102]
Length=634

 Score =  317 bits (812),  Expect = 2e-101
 Identities = 182/357 (51%), Positives = 217/357 (61%), Gaps = 58/357 (16%)
 Frame = +1

Query  4    ANNGSRIDYILCSDGIKDWFTSANIQEGLMGSDHCPVFATMADKLAVKGKEHALLELLNA  183
            ANNGSRIDYILCS+GIKDWFTSANIQEGLMGSDHCPVFAT+A  +A  G++ ++ +++N
Sbjct  280  ANNGSRIDYILCSNGIKDWFTSANIQEGLMGSDHCPVFATIAGTVASNGRQVSINDVMNP  339

Query  184  QGTFQDDRRLREWGPKDLLPLSARLIPEFDRRQSIRDMFTKKPNSTLEQSSKHKVVTENL  363
             G F++  R+R+   KD+LPLSA+LIPEFDRRQSIRDMF KK  S   +++ H   + +
Sbjct  340  AGMFENGTRVRDLAQKDILPLSAKLIPEFDRRQSIRDMFFKKVTSAERRTTPHNSESSSF  399

Query  364  HPQPT------------------------TKRPIATGESMSE-----------RALKKTK  438
              + T                        T +P A   S              R LK++K
Sbjct  400  KAEDTSQASDNDASSQIDNALQTVNTNTPTNKPAALPYSQPRKRSPDPIDPVPRQLKRSK  459

Query  439  APSNSVNAKNKAVTGQRTLQGFFKPKAST-------PQPEAPSVGGTGRATPSPTTKTS-  594
            + ++ + +K K   GQ TL+GFFKP +S          PEA S       AT  P TKTS
Sbjct  460  SAADPIGSKQKISAGQTTLKGFFKPVSSVLPSPAVLADPEAQS-----NATDLPATKTSP  514

Query  595  --LGspngppsqrlppqppET--------LSNGHSERVFDPIEAKESWSKLLRKRVAPEC  744
               GS     S    P   E         S    +RVFDPI+AKESWSKLL KRV P C
Sbjct  515  STKGSSENLTSNEFMPPFSEQPAHDSLVDTSPKSPDRVFDPIQAKESWSKLLGKRVVPRC  574

Query  745  EHHEPCISLTTKKPGVNCGRSFYICPRPLGPSGEKEKESEWRCGTFIWSSDWNSTSS  915
            EH EPCISL TKKPGVN GRSFYICPRPLGPSGEKE+ SEWRCGTFIWSSDWN TSS
Sbjct  575  EHDEPCISLVTKKPGVNRGRSFYICPRPLGPSGEKERGSEWRCGTFIWSSDWNGTSS  631
```

MAT1-1-3

MSSFAEIQNAIPQLCLKQPFIFVTSAMQGDVNVFLPETFNIKIVRKIAMIFSRRINQSVTVFHDIHRQKYRLCPLSYHVMVELPIYGQFC
FRCDASTEVDAEGRDKEETTAPTASESEPHIPRPRNKWILYRQAKSQEVAKRNPGITAAETSTIISQLWKDETPEVRAYWQALAEDEDRR
HKAQYPGYKFTTKKKTT

Best three blastx hits

```
>dbj|BAE93596.1|  MAT1-1-3 [Metarhizium anisopliae]
Length=193

 Score =  176 bits (447),  Expect = 9e-54
 Identities = 88/173 (51%), Positives = 120/173 (69%), Gaps = 4/173 (2%)
 Frame = -1

Query  549  LKQPFIFVTSAMQGDVNVFLPETFNIKIVRKIAMIFSRRINQSVTVFHDIHRQKYRLCPL  370
            L  P  FVTS +QG+V++F+PETF+  +V  IA  FSRR+ QSV+VFHD +RQK+R+CPL
Sbjct  13   LDTPVTFVTSEIQGNVHIFIPETFHRNLVEVIASNFSRRVQQSVSVFHDAYRQKFRICPL  72

Query  369  SYHVMVELPIYGQFCFRCDASTEVDAEGRDKeettaptasesePHIPRPRNKWILYRQAK  190
            +    V    YG   F CD+S   + + + K      ++S    HIPRPRN WILYRQ K
Sbjct  73   TPGSAVNPTNYGILLFTCDSSGPKELKAQAKTAEPEDSSS----HIPRPRNSWILYRQFK  128

Query  189  SQEVAKRNPGITAAETSTIISQLWKDETPEVRAYWQALAEDEDRRHKAQYPGY  31
            S+E+ K +PGITA+E ST+IS LWK+E+   +A+WQ +A++EDR HK +YPGY
Sbjct  129  SRELRKDHPGITASELSTLISNLWKNESDGEKAFWQKMAQEEDRMHKEKYPGY  181


>gb|AEI72616.1|  mating type A-1-3 HMG1/2 [Epichloë festucae]
Length=193

 Score =  174 bits (441),  Expect = 7e-53
 Identities = 89/170 (52%), Positives = 116/170 (68%), Gaps = 4/170 (2%)
 Frame = -1

Query  540  PFIFVTSAMQGDVNVFLPETFNIKIVRKIAMIFSRRINQSVTVFHDIHRQKYRLCPLSYH  361
            P  FVTS +QG+V+VF+PETF+  +V  +A  FSRR+ Q V+VFHD +RQK+R+CPL
Sbjct  16   PVTFVTSEVQGNVHVFVPETFHGNLVEVLAKNFSRRVQQPVSVFHDTYRQKFRICPLPLG  75

Query  360  VMVELPIYGQFCFRCDASTEVDAEGRDKeettaptasesePHIPRPRNKWILYRQAKSQE  181
```

```
             MV+   YG   F CD S   D         +  T+S    HIPRPRN WILYRQ KS+E
Sbjct  76   AMVDTTKYGTPFFSCDLSDPKDLPADTSVVESEDTSS----HIPRPRNSWILYRQFKSRE  131

Query  180  VAKRNPGITAAETSTIISQLWKDETPEVRAYWQALAEDEDRRHKAQYPGY  31
            + K + GITA+E ST+IS LWK+ET E +A+WQ +A++EDR HK +YPGY
Sbjct  132  LRKDHSGITASELSTMISSLWKNETDEEKAFWQKMAQEEDRLHKEKYPGY  181
```

```
>gb|EFZ01123.1|  MAT1-1-3 like protein [Metarhizium anisopliae ARSEF 23]
Length=259

 Score =  176 bits (445),  Expect = 1e-52
 Identities = 88/173 (51%), Positives = 119/173 (69%), Gaps = 4/173 (2%)
 Frame = -1

Query  549  LKQPFIFVTSAMQGDVNVFLPETFNIKIVRKIAMIFSRRINQSVTVFHDIHRQKYRLCPL  370
            L  P  FVTS +QG+V++F+PETF+  +V  IA  FSRR+ QSV+VFHD +RQK+R+CPL
Sbjct  2    LDTPVTFVTSEIQGNVHIFIPETFHRNLVEVIASNFSRRVQQSVSVFHDAYRQKFRICPL  61

Query  369  SYHVMVELPIYGQFCFRCDASTEVDAEGRDKeettaptasesePHIPRPRNKWILYRQAK  190
                 V    YG   F CD+S   + + K      ++S    HIPRPRN WILYRQ K
Sbjct  62   PPGSAVNPTNYGILLFTCDSSGPKELKAQAKTAEPEDSSS----HIPRPRNSWILYRQFK  117

Query  189  SQEVAKRNPGITAAETSTIISQLWKDETPEVRAYWQALAEDEDRRHKAQYPGY  31
            S+E+ K +PGITA+E ST+IS LWK+E+   +A+WQ +A++EDR HK +YPGY
Sbjct  118  SRELRKDHPGITASELSTLISNLWKNESDGEKAFWQKMAQEEDRMHKEKYPGY  170
```

MAT1-1-2

```
MDNLLFYRPLWEEEALIFDSSKHIEKLRLKSIGILLHKLPVDVNPPSVEAVITSSIHIIKTLLEDVSDDNAILQKIRNHATEVGVDPNLV
VRGAIVLWYSSVLPILSRDPDQGLPHHAIVAGGLALPWVQKLAENQYFIGNLGYLSMLLTAEDWEKPTHPKLQVATLITNAMVSIIYAAF
VITPHLNANLKWRELLMKSGSRGGYELRVFLKTYWHASREGWEKLNYPPGSEFSALESEARMSPDGKKLLTRVGQGNWHEAPFWHPLRTV
PGSPWNKFIKNKKQKVFPVLPRRGNSVRYMVPGSATGLIGSCETYYGILRSQFDDTETPRVEVSDAERIEQFKRLAEGTGLEYPKTRPVV
EIKKNASDLSGNLTLPFLTTTARAMRFTEEPDFVDEDFHAMVFFQ
```

Best three blastx hits

```
>gb|ACR78245.1|  MAT1-1-2 [Hypocrea jecorina]
Length=434

 Score =  273 bits (697),  Expect = 3e-85
 Identities = 158/433 (36%), Positives = 244/433 (56%), Gaps = 36/433 (8%)
 Frame = +1

Query  1     MDNLLFYRPLW---EEEALIFDSSKHIEKLRLKSIGILLHKL-PVDVN-PPSVEAVITSS  165
             M+N+  + PLW   E +A   D    I  +RL+S+ I L +   +D+  PPS++ ++  S
Sbjct  1     MENICSFEPLWKNIESDAPQTD----ISNIRLESMKIFLQENGSLDIQQPPSMDYIVAES  56

Query  166   IHIIKTLLEDVSDDNAILQKIRNHATEVGVDPNLVVRGAIVLWYSSVLPILSRDPDQGLP  345
             + ++K LL      N I+ ++R  A +   DP + V GAI LWY+S + IL RD  QG+
Sbjct  57    LKVMKHLLSFWEGFNPIIARLRTVAVQTEADPIIAVEGAIALWYTSSVSILERDIHQGVS  116

Query  346   HHAIVA----GGLALPWVQKLAENQYFIGNLGYLSMLLTAEDWEKPTHPKLQVATLITNA  513
                 +A    G +   W +  A  ++++GNLG ++MLLT E W  P  PKLQVATL++
Sbjct  117   SEEEIATFEDGTVVATWNRDFAAEKHYVGNLGLMAMLLTTEVWLPPKDPKLQVATLVSCG  176

Query  514   MVSIIYAAFVITPHLNANLKWRELLMKSGSRGGYELRVFLKTYWHASREGWEKLNYPPGS  693
               ++++AA++I+  + +   WR  L  S   G   +++FL++ W  +++  ++   PPG
Sbjct  177   ATTMLFAAYMISSRMPSYNAWRPAL--SACPGTDGIKLFLQSAWRLAQKPVDEAGTPPGI  234

Query  694   EFSALESEARMSPDGKKLLTRVGQGNWHEAPFWHPLRTVPGSPWNKFIKNKKQKVFPVLP  873
             +F A   E R+  +GK LL++VG+ +W +APFWHP R VPGS WNK+I N  + +FP  P
Sbjct  235   DFGAHVDEIRLCRNGKGLLSKVGRADWGKAPFWHPCRKVPGSHWNKWINNTCRPIFPKSP  294

Query  874   RRGN---SVRYMVPGSATGLIGSCETYYGILRSQFDDTETPRVEVSDAERIEQFKRLAEG  1044
               G+    + + +P SA  L  +   YY  LR++FD T  PR  ++  E  +QF+ LA
Sbjct  295   GDGSRDIRIIFRLPSSAAILTSAFGDYYSTLRTRFDQTNRPRSRLALEECEKQFRDLAAQ  354

Query  1045  TGLEYPKTRPVVE------------------IKKNASDLSGNLTLPFLTTTARAMRFTEE  1170
             TG EYP   P +E                  +KK  SD +GNLTLPFLTT  RA+RF E+
Sbjct  355   TGCEYPALIPALETAASVTHLDQEYLYNLPMVKKPISDPTGNLTLPFLTTAIRALRFGED  414

Query  1171  PDFVDEDFHAMVF  1209
             PD ++++   M F
Sbjct  415   PDEIEQNLFMMTF  427
```

```
>gb|AEI72617.1|  mating type A-1-2 protein [Epichloë festucae]
Length=331

 Score =  232 bits (591),  Expect = 1e-70
 Identities = 123/338 (36%), Positives = 195/338 (58%), Gaps = 11/338 (3%)
 Frame = +1

Query  1     MDNLLFYRPLWEEEALIFDSSKHIEKLRLKSIGILLHKLPVDVNPP-SVEAVITSSIHII  177
             M+N+  + PLWE   LIF   + IE+LR+KS+   LH    +   P ++  V+     +I
Sbjct  1     MENIHHFSPLWERLELIFKPEQAIEELRIKSLQAFLHNHRSEAAKPLNLNQVVLECTCVI  60

Query  178   KTLLEDVSDDNAILQKIRNHATEVGVDPNLVVRGAIVLWYSSVLPILSRDPDQGLPHHAI  357
             + LL++ +D+N ILQ++RN       DP  +V+ A+VLWY+   PI SRDP QGLP  +
Sbjct  61    QQLLDNNNDENEILQRLRNSGQG---DPLAIVKKALVLWYAGSCPIFSRDPHQGLPPDSE  117

Query  358   V---AGGLALPWVQKLAENQYFIGNLGYLSMLLTAEDWEKPTHPKLQVATLITNAMVSII  528
             +   +G   L W ++    Q+ IGNLG ++ML+T+E W  P H KL+ A+LI+ A  +I+
Sbjct  118   LEDDSGAPILTWSRRFFHEQHAIGNLGLMAMLMTSETWLSPKHEKLKAASLISTASATIL  177

Query  529   YAAFVITPHLNANLKWRELLMKSGSRGGYELRVFLKTYWHASREGWEKLNYPPGSEFSAL  708
             +A+++I   +  +  W   +  S ++    + +F+++ W  +RE  E  + PPG EF A
Sbjct  178   FASYLICTEV-MHRPWAHNV--SSAQSSEAMALFIRSSWQVARENSEIFDSPPGREFGAT  234

Query  709   ESEARMSPDGKKLLTRVGQGNWHEAPFWHPLRTVPGSPWNKFIKNKKQKVFPVLPRRGNS  888
               E ++S DGK+ LT++G  +WHEAP+WHP R VPGS WNK+++N    +FP      N
Sbjct  235   LKEVKLSDDGKRFLTKIGHESWHEAPYWHPCRRVPGSSWNKYLRNFACPLFPT-HLTTND  293

Query  889   VRYMVPGSATGLIGSCETYYGILRSQFDDTETPRVEVS  1002
             +   +P +   L+   E YY  LR +FD     + + S
Sbjct  294   IHISLPTTMFSLVQPWEAYYAELRIRFDQVRHSKEKFS  331


>dbj|BAD72611.1|  MAT1-1-2 [Epichloë typhina]
Length=337

 Score =  226 bits (575),  Expect = 3e-68
 Identities = 122/331 (37%), Positives = 191/331 (58%), Gaps = 14/331 (4%)
 Frame = +1

Query  1    MDNLLFYRPLWEEEALIFDSSKHIEKLRLKSIGILLHKLPVDVNPP----SVEAVITSSI  168
            M+N+  + PLW+   L+F   + IE+LR+KS+   LH    +   P        V+
Sbjct  1    MENIHHFSPLWQRLELVFKPEQAIEELRIKSLQAFLHNHRSEAAKPLNLNRTYLVVLECT  60

Query  169  HIIKTLLEDVSDDNAILQKIRNHATEVGVDPNLVVRGAIVLWYSSVLPILSRDPDQGLPH  348
             II+ LLE+ +D+N ILQ++  H++  G DP  +V+ A+VLWY+    I SRDP QGLP
Sbjct  61   CIIQQLLENNNDENEILQRL--HSSGQG-DPLAIVKKALVLWYAGSSSIFSRDPHQGLPP  117

Query  349  HAIV---AGGLALPWVQKLAENQYFIGNLGYLSMLLTAEDWEKPTHPKLQVATLITNAMV  519
             + +   +G   L W ++    QY IGNLG ++ML+T+E W  P H KL+ A+LI+ A
Sbjct  118  DSELEDDSGAPILTWSRRFFHEQYAIGNLGLMAMLMTSETWLSPKHEKLKAASLISTASA  177

Query  520  SIIYAAFVITPHLNANLKWRELLMKSGSRGGYELRVFLKTYWHASREGWEKLNYPPGSEF  699
            +I++A+++I   +  +  W   +  S ++    + +F+++ W  +RE  E  + PPG EF
Sbjct  178  TILFASYLICTEV-MHRPWAHDV--SSAQSSEAMALFIRSSWQVARENSEIFDSPPGREF  234

Query  700  SALESEARMSPDGKKLLTRVGQGNWHEAPFWHPLRTVPGSPWNKFIKNKKQKVFPVLPRR  879
             A   E ++S DGK+ LT++G  +WHEAP+WHP R VPGS WNK+++N    +FP
Sbjct  235  GATLKEIKLSDDGKRFLTKIGHESWHEAPYWHPCRRVPGSSWNKYLRNFASPLFPT-HHT  293

Query  880  GNSVRYMVPGSATGLIGSCETYYGILRSQFD  972
             N +   +P +   L+   E YY  LR +FD
Sbjct  294  TNKIHISLPTTMFSLVQPWEAYYAELRIRFD  324
```

MAT1-1-1

```
MVTQRSYVMQQLASLSTAEILSLLTDDTILEVAARYFEINANTMDANEPLSPSGTDIVTTPPAQDVAGNDSAPRAKRPLNAFMAFRTFYM
KLFPDIQQKTASGFLTTLWNKDPFRNKWALIAKVYSFVRDEVGKGNISLSRFLDVCCPTMNIIEPRAYIAALGWTVKHDDTGSQVLLEGT
PTDLSRFQDESVPNTEMELLRGLLNTGYLSDRGLALMERLGESSNGIMTTYGASMEAPVILTTEKLDFVDLVRENPQEAVRALVDDTARE
FVFQQEIPVYDVYDLSNAPFHSIRGPQPNPSQNYTFSNSSLGLYPVRALTLSSVGEFDAIDLDSPFDIDVIMGHTRSEGERTRDQPEGEP
FXPHADYNSLL

YLRMAGW)
Y-[LMIF] x(3)-G [WL]
```

Best three blastx hits

```
>gb|ACN59937.1|  mating-type A-1 protein [Epichloë festucae]
Length=369

 Score =  282 bits (721),  Expect = 4e-90
 Identities = 151/369 (41%), Positives = 231/369 (63%), Gaps = 12/369 (3%)
 Frame = +1

Query  13    RSYVMQQLASLSTAEILSLLTDDTILEVAARYFEINANTMDANEPLSPSGTDIVTTPPAQ  192
             R+ +MQ+L+ L T E+L  L D+T+L++A+RYF+           ++P+     TT  +
Sbjct  4     RAELMQRLSMLPTQELLQYLKDETMLDIASRYFDTTFQPT-----MTPTFNQ--TTMNSH  56

Query  193   DVAGNDSAPRAKRPLNAFMAFRTFYMKLFPDIQQKTASGFLTTLWNKDPFRNKWALIAKV  372
               +      +AKRPLNAFMAFR++Y+KL+PD QQKTASGFLTTLWN+DPFRNKWALIAKV
Sbjct  57    LKSKVQICEKAKRPLNAFMAFRSYYLKLWPDQQQKTASGFLTTLWNRDPFRNKWALIAKV  116

Query  373   YSFVRDEVGKGNISLSRFLDVCCPTMNIIEPRAYIAALGWTVKHDDTGSQVLLEGTPTDL  552
             YSFVRDEVGK  +SL+ FL   CP M I+EP+AY++ LGW+V+  +   +++ +     L
Sbjct  117   YSFVRDEVGKDKVSLAYFLGFACPVMGIVEPQAYLSILGWSVEGVEPLQRLVQDEAVAAL  176

Query  553   --SRFQDESVPNTEMELLRGLLNTGYLSDRGLALMERLGESSNGIMTTYGASMEAPVILT  726
               S F     P+TE+ELL GL+N GYL ++G+ L+++LG +SN IM T    +  PV  T
Sbjct  177   GQSHFSGCEYPSTELELLSGLVNVGYLPEQGIDLIDKLGTNSNTIMATSNPRLTLPVSYT  236

Query  727   TEKLDFVDLVRENPQEAVRALVDDTAREFVFQQ-EIPVYDVYDLSNAPFHSIRGPQPNPS  903
              EKL F++ +R +P +A + L  D   ++  Q   +  +DV +L +     ++   P+P
Sbjct  237   PEKLQFMNTIRSDPVQATKELFGDAYDDYTIQMLGVKSHDVENLDSINHLPMQMDLPDPR  296

Query  904   --QNYTFSNSSLGLYPVRALTLSSVGEFDAIDLDSPFDIDVIMGHTRSEGERTRDQPEGE  1077
                NY+ S+S L +     L   ++ + +  D+D+P+D+D ++G ++SEG+RT   P
Sbjct  297   YYYNYSTSHSQLAVNGAPILRFENIPDHETFDIDNPWDVDAMLGQSQSEGQRTISHPRSP  356

Query  1078  PFBPHADYN  1104
               + H D++
Sbjct  357   RHDSHEDFH  365


>gb|EFZ01122.1|  mating-type A-1 protein [Metarhizium anisopliae ARSEF 23]
Length=376

 Score =  280 bits (716),  Expect = 3e-89
 Identities = 155/370 (42%), Positives = 230/370 (62%), Gaps = 30/370 (8%)
 Frame = +1

Query  13    RSYVMQQLASLSTAEILSLLTDDTILEVAARYFEINANTMDANEPLSPSGTDIVTTPPAQ  192
             R+ +MQ+L+ L T E+L +L D+TI ++AA+YF+ +   M +N  + PS T ++
Sbjct  4     RAELMQRLSMLPTQELLHILKDETIFDIAAQYFDAS---MQSN--ILPSTTPVIHNTHKT  58

Query  193   DVAGNDSAPRAKRPLNAFMAFRT----------------FYMKLFPDIQQKTASGFLTTL  324
                 N    +AKRPLNAFMAFR+                 Y+KLFPD+QQK+ASGFLTTL
Sbjct  59    K---NTVCDKAKRPLNAFMAFRSKFITKPISDPFLPNSGHYLKLFPDVQQKSASGFLTTL  115

Query  325   WNKDPFRNKWALIAKVYSFVRDEVGKGNISLSRFLDVCCPTMNIIEPRAYIAALGWTVKH  504
             WNKDPFRNKWALIAKVYSFVRDE+GK  I+L+ FL   CP M IIEP++Y+  LGW+V
Sbjct  116   WNKDPFRNKWALIAKVYSFVRDEIGKDKITLASFLSFSCPVMGIIEPQSYLTILGWSVGG  175

Query  505   DDTGSQVLLEGTPTDL--SRFQDESVPNTEMELLRGLLNTGYLSDRGLALMERLGESSNG  678
             D    +++ + T   L  SR Q + VP+TE+ELL  L+N GYL ++G+ LM +LG +++
Sbjct  176   DSNSQRLIQDETLAALGQSRLQSDGVPSTELELLTALVNVGYLPEQGIDLMGKLGANNSS  235

Query  679   IM-TTYGASMEAPVILTTEKLDFVDLVRENPQEAVRALVDDTAREFVFQQ-EIPVYDVYD  852
             IM T     +  PV  T EKL F+  +R +P +A + L+ D   ++  Q   +  ++V D
Sbjct  236   IMATATQPRLSLPVSYTPEKLQFMGTIRSDPVQATKELLGDVYDDYTIQMLGVKAHNVED  295

Query  853   LSNAPFHSIRGPQPNPS--QNYTFSNSSLGLYPVRALTLSSVGEFDAIDLDSPFDIDVIM  1026
             L +     ++   P+P    NY+ S++ L L     ++  ++ E ++ D+DSP+D+D I+
Sbjct  296   LDSINHLPMQVEMPDPRYYYNYSTSHAQLSLAGAPTMSFENIPEHESFDIDSPWDVDTIL  355

Query  1027  GHTRSEGERT  1056
             G T+SEG+R+
Sbjct  356   GQTQSEGDRS  365


>gb|ACR78244.1|  MAT1-1-1 [Hypocrea jecorina]
Length=379
```

```
 Score =  258 bits (660),  Expect = 8e-81
 Identities = 152/371 (41%), Positives = 223/371 (60%), Gaps = 11/371 (3%)
 Frame = +1

Query  13    RSYVMQQLASLSTAEILSLLTDDTILEVAARYFEINANTMDANEPLSPSGTDIVTTPPAQ  192
             R  + + L+ L T EIL  L DDT+LE+A  YF         N+ +  + +   ++
Sbjct  4     REELEKHLSVLRTDEILHFLRDDTLLELANTYFTSLGIGAQQNQAMKQNTSGDASSSALI  63

Query  193   DVAGND-SAPRAKRPLNAFMAFRTFYMKLFPDIQQKTASGFLTTLWNKDPFRNKWALIAK  369
                G + S  +AKRPLNAFMAFR++Y++LFP++QQKTASGFLTTLWNKDP+RNKWALIAK
Sbjct  64    PTQGAETSTDKAKRPLNAFMAFRSYYLRLFPEVQQKTASGFLTTLWNKDPYRNKWALIAK  123

Query  370   VYSFVRDEVGKGNISLSRFLDVCCPTMNIIEPRAYIAALGWTVKHDDTGSQVLLEGTPTD  549
             VYSFVRD++G+  +SLS FL+V CP M I EP  Y+++ GW+V+ DD GS  L +
Sbjct  124   VYSFVRDQLGRDKVSLSYFLNVSCPIMKITEPSIYLSSFGWSVE-DDAGSPRLFQADGAS  182

Query  550   LSRFQD-ESVPNTEMELLRGLLNTGYLSDRGLALMERLGESSNGIMTTYGASMEAPVILT  726
             +++  D +  PNTE +LL  ++  GYL D  + LM+R+  +SNGIMTT  A+   PVI T
Sbjct  183   IAQPMDSDDHPNTENDLLSAIIQVGYLPDDSMNLMDRMNANSNGIMTT--ATSSVPVIST  240

Query  727   TEKLDFVDLVRENPQEAVRALVDDTARE-FVFQQEIPVYDVYDLSNAPFHSIRGPQPNPS  903
              EK +F+ +V  +P +A + L+     E  V    +  + V DL+      ++   P+P
Sbjct  241   KEKSEFMKVVEADPFQAAKELLGGHYEEKRVAALGVKSHLVEDLNAVSHLPLQFAYPDPR  300

Query  904   QNYTFSNSSLGL-YPVRALTL---SSVGEFDAIDLDSPFDIDVIMGHTRSEGERTRDQPE  1071
             Q Y +++S+    Y      L   +++ E D  DLD+P+D D ++G+  +EG+R
Sbjct  301   QIYNYASSTAAQPYQPEMPQLDYFATMTESDTFDLDNPWDFDKMLGYKENEGDRVVIPAA  360

Query  1072  GEPFBPHADYN  1104
             G P  P+  YN
Sbjct  361   GLP-QPNNQYN  370
```

Supplementary material S2

DNA and protein sequences of the *MAT1-2* locus of *Ilyonectria torresensis* (isolate Cy237) including the genes *DNA lyase* (blue), *MAT1-2-1* (orange) and a putative ORF (purple)

```
1    GGATCTGACCACTGCCCAGTGTTCGCGACCATGGCAGACAAGCTCACAGTCAAAGGAAAGGAGCATGCCCTCCTGGAGCTCTTAAACGCGCAGGGCACAT
101  TTCAAGGCGACCGACGTCTTCGCGAATGGGGACCAAAAGACTTGCTTCCTTTGTCAGCGAGGCTGATACCGGAATTCGATCGTCGACAGAGCATCCGCGA
201  TATGTTCACAAAGAAACCCAATTCGACCTTGGAGCAGAGCTCGAAGCACAAGGTTGTGACCGAAAACCCCACCCACAACCGACCACGAAGCGGCCGATT
301  GCTACAGGTGAAAGTATGTCTGAGCGGGCTTTGAAAAAGACCAAAGCGCCATCAAATTCCGTCAACGCCAAGAACAAAGCCGTTACGGGACAACGAACGC
401  TTCAAGGTTTCTTCAAGCCAAAAGCCTCAACGCCTCAGCCCGAGGCTCCAACCGTGGGCGGCACGGGAAGAGCAACGCCATTGCCGACAACAAAGACCTC
501  GCTTGGCTCACCCAACGGTCCGCTCAGCCAGCGGCTTCGCCTCAGCCGCCCGAGACTTTGTCGAATGGGCATTCTGAAAGAGTCTTCGACCCAATCGAA
601  GCGAAGGAATCCTGGTCGAAGCTCCTCCGTAAGCGAGTTGCCCCAGAATGTGAGCATCACGAGCCTTGCATCAGCCTCACTACCAAGAAGCCGGGCGTCA
701  ACTGCGGTAAGTTCGCGATCCATATTTAGTTTGCGGGCCTGCACGTACTAGGACATGGAACGTTGTCTCGGATATGATAGGGAAGCTAATACGAGGCACA
801  GGGCGGTCCTTCTACATTTGTCCGCGACCTCTTGGCCCCTCTGGGGAGAAGGAGAAGGAATCGGAATGGCGGTGCGGGACGTTCATTTGGAGCAGCGACT
901  GGAACTCTGCGTCTTCGGGATCATGACATGAGACGGGCACAGCATAAGACAGCACGTAAAGAACATAATGTTCGAACTTCCTTTGATTCCAACTACATGA
1001 CCTGTTCGTAGCTATCGGCCGCCAACGCGTTTAATAGGCAGCTTTAGTCCATAATGTTTTCACCAAGACAAAGGCACTGGCTTCGTTCATGGGAATTGTG
1101 GTTAAGGACCCCCTGCATAATCCTGTCGTCTTCTTCTTCGTGGTCAACTTGTAGCCCGGGTACCGAACGTTGCGTCTCCAATCTTCATCCACAGCCAAAA
1201 TCCGTCTTCGCCTCGTGCGTCTCATCCTTTTGCATCTTAGGGCCAATATGCTAAACTCGTTAGTGACATTTCATAAATTGATCTGGGGACTTACAAGTTC
1301 TGGGGCAGGGATTACAGCAATCTGCTTCGCGACTTTCAAGGTTTGCTCGACGGTGCAGACGGAATTTGCCCAAGGCCACAGTTTAGGACGCCTCAACAGT
1401 GGGAACAGGGGTTTCAGCGGTGGGAGCAGTAGCCTCATCATCGGAAGCAGGGGCTTCATCAGTGGGTTCAGGATCGGCTTCATCAGCGGGGGCAGCGACA
1501 GGAACTGCCTGAGAAGCATCGACAACGATGTCCGCAAGAGCATCTTCAGCTTCCTCGGTCCGCTCGGCGATGCGGCGGCGACGTCTCTCAGAGGGACGAC
1601 GGGGGCGGTACTTGTACTCCGGATGTCTCTTGATCAACTCAGCCTTAATGGCGTCGGCTCTCTGCTTATACTTGGCGCGGATGGCCTCAGACTCAAGGTT
1701 CCAGGCGCGACCAAGGATCTGGGCTACTGAAGTTAGTTATTGTTTCCACTCGTTTCAAGGTGCTCACTCACAAATCTCATTGTTGTGGATTCCAGGCTGG
1801 CTCGCCTTGACGAAGCGATGACGATCCGAGCGATACAAGATATACGCATTTGGAGGTCGGGGAATCTTGGCGCACTTCTTGGGGAGAGCTGGAGGAACGA
1901 CCAGGATATGCTGGATTCCGTCATCGGGACGCTTGACCCAAATCGCTTCCTTGGTGTGGAGAGCATGAACGATCATTCCAGAGCCCGCGAGAAAGAAGCG
2001 TGGAGAACCGAGGAAGTAACGATCGCCATCAGTTCCGTCACGGCAGTACATGACTCTTTCGGTGACTTGTTCCCTGAAAAGAAGGTTAGTGGAGTAGAAG
2101 ATTGAAGAATTGTGATACTCACATGAACTGCTTGGCAATGAACTGCTTGGCTCCATCGTCGAGCACACGGTAGAGGGGTCCTTCGATGCAAAGAACATAC
2201 ACAAAAGGAGAAACTTGAGCCTTCAGCTGGTCCCAGATGGCATCGAGTTGATCCTGAGTGTAGTTAGAGGCAACATTCGCACGAGGCATGATGACTTGAG
2301 ATGGCTTGGGTAATTGATGTTGATGAAAGAAGGGGAAGAAAGGCTGGACAAGAGGTTCTTGTTCTGTGAACAAGAATGTTCGAAGGTGTGGAGGAGAGAG
2401 AATCCTTTGTTTATTTTCGAAGTGGGAGTGGTTTTTGTAGGCGACTGAAACATTGGTCGTCGGCTGAGAGGCAGCTGGTAGCGGCAACTCACCGAAGTAC
2501 GTACCAACCTGAGGTTATCCTGAGGTGGTATTGGTTCCTTTGTTCAATAGAACGCTCATGCAAGCCACCTCACTTAATGCGGGGACAATTTAGCAACAAC
2601 AAACGTGAGCACTGTATCGCCACCGTATGCAGACGGAGTACGCTCATTAGCGTTCTTCGCCCAGTCCTGACGCCAAGCACAGGCGAAAGAAGTTAGAGTG
2701 AATGAAGCCAGCGCGGGCTGTCCAGAAATTGAGTTTAGATTGCATTCTTACCTGATGACAGGCCAACTAGAAACATGGTTTGGTCATCGGCCTGGGTAGC
2801 GTGGCATCCCTGAGTCTTATCCAGAGCGTGCACATTGTGAATATTTTGATCATCATTTTGGTGGTGATTATGACTTCACAGGTCTCGGTTCTCACCGTTA
2901 TTATCCTCGCGGGTGAACGCCAGAAGGATAATTTGGCAAGCTGAAAGTTACCAGAAACTTGTGAAAGCATCTTTTGACACCATTTCAAGCAACTCATCCA
3001 CTTCCCAGTGTCTAAGTTACACTGGCCATGGATGATGTAGATGCTGCAACCTATCTAACTCACTCACTACGTATTGTGATACTCGAATTATTTGGTTACT
3101 AGCAAGTCATGGCAATCAAATCTTTCTAGAGAACGAAACATCGCAACATGGCTAGGGGGTGGGAGATGTTCAGCGGGTGTTTTTGCTCCTAATCTCAAAG
3201 TACATCCTCAAGCGAATTGCATTCTCCCAAGTCATGTCTAACAAGAACCTATGGGAGCGCCCAGACCCGGTGGCTTCTCCTAAGCTTCAAGAAAGTTAAT
3301 GATAAACGACAAATTCTGGCGTTCTTTGACAACAACCAGCCACAAACGGAATACTGCCAAACGAACAAGGGTGGTGGGTAGACCCAGGTACCTCCCTACG
3401 TTGGAGCATTACCTATCTAGGTACCTGGGTACTGGATGATCAAACGGACAGCGGGAGGCGGCACGCGCTTAGTGGCCTCCACAAGAATTTACAGGTGTTG
3501 CCAACTGGAACCTGAAATGCTGCAGGTATTTGAGGCACATTACATTTTGTCTCGCCCAACTCCCGGCTCCATTCTCCTTCTCTCCACGAGTCAGAAACCT
3601 TTCACTCCCGCATCCCTCAAATGACCAACACTTTCCTCTCCAACCGATTCTTTCTCCGATCAGCTCTTTCTCAAATCACCTTTTCGCCTACTGCACCGGA
3701 ATGGCGAACCTTCCTCTACCTCCAGACCACCCTGGTGCGGCCATGTACCAGTTCACCCATGGACCTGGGCTTCATGACATTCAGCCGCAGCGAGAAGGCT
3801 TTTGGACCTTAGCAGGGCATGATGCTGAGCTGTTTCGGCTTGGCTTTGTTCATACTCCTCCTGGCCAGGCCACAGCACAGCTTCTGTACCAGGTCTTCCG
3901 CCCCATCAAAAGCCTAGACGTCCTTGAGACACACGCCTCTGATCTCATCTATGAATATGAAGGCGGTAAGCACGACTTCCATTCAAACCTGCAACTCGAT
4001 GAAACCAAGCTGAGACATGAAACAGATTTCGCCTCAATTCCCCAGCACGACGAAGGTTTTGCCGCTCAACCCACAGTTCGCGATGATCTAGTCCACATCA
4101 GCCATTCCGACCTCGCTGAGGAGGGCGTGATGAACCAAGGCCAAAATATTGTTCAGGATGATCTTCAGGTTGAGCAAGGTTCTGTCATTCAAAGCGACAA
4201 CTCCCTCATCTCGCAGATGGTGTCTGAGCCCGATGCTCAAATCCTTTCACCTCTTCTGGATGACAATCTTCTGGCCGCTTCCCAGAGCATGGATGACGGC
4301 AACTGGGCCTTTCCCCCTGACGTTTTTCAGTCTCAGGATTACAATTTGCTTAATGGTACTCCTGAAATGAATGTTGAGGATCTTGGAATGAACCAAGAGC
4401 AAATGGTCGCCTCTGGGATGCCACATACACAGGAGTGGGCTGAGTTGCCAGATGCCGACAACGATCAACTCCAGGGAGATGATCAAATGATTCTCCCAGA
4501 CTTCTAAGCTGTTAGCATAGAAGATTTGATCGGAGGGCGAGAGAAGTAACAGAATCCACGATCACTGTTCAAATGCCTACTGACATCTGATAGCTCGCGG
4601 CTAGCCGGAAGGCGCGCCATTCGACCTCCATGCGGACTACAACTCTCTCCTTTGGGCTTGGACTCTCTCGTGACGGAAATGGAGACACAGAATGATGGCG
4701 GTACGCAAACGAAGTGGGATGGGGGACGACACTAGGAGAAGAACTTGTCTTTTTTAGTTACTTTAGCCCACAACTCGAACACGAAACAGTCTTGATGTTT
4801 TTGCGCAATTACTTGAGATATCCTCGCTCAATTGAGCACACGAAGTGAACGGTCCGGTGCCAAGACTACAAGACGTGATGTATGAGTCGTGGCTTTTTCG
4901 CCCAAGTCCCGATCCACCTTTAGCACTGAAGGGTTGTCCTTTTCTACAGAGGGGATATGGACGTGGGGTGACTTCAACGTGGGAGCAGCTGCTGAAGAGG
5001 TGGGCGTAGTCGGCAATGGAGAATTTACGGGCATAGCGATAATTGGGGGCGTCGCTTGGCATTTGGGATGTTTCAGTTTAGTGAGACGTGGAGCCAATGA
5101 CCATGGGAGAGTGAGGTGGAATTGATGCTGACCTAGGAGGCTAAGATAAGGTGAGGTGAGGAAAGCGGTTGGGGTTTAGTGCTTGCGAACGGAGGAAGCT
5201 GAAGCCGGTTGGAGTGGGCACTCAATCAACCACCCTCCCAAGGCTAGGCACTAAACTTGCGCAGAATAAGGCAGCTAGCTTAGACATGCTGAACCAGTCT
5301 CGAAGTGATGTTCGATCAAGCAGCTCCTTAGGCTTCCGGGGATCGGGCTTTGGCGGGCCGGAGGAGGGTGGGAGGGTCTGAGGCAAGGAGCACCTGTGGA
5401 GTCTGGACGGGAACTGCCAGCTAGCAGATGATTACAAAACAATAGGAAATAGGATTGCCACTAATTGGCGGAGGTGCCAGACACGCCAGCAGTGTGAAAA
5501 AACCAAGAACGTAGTCTAATAAATGCTACGAAACGCAAGGGGCGATGAGGCGTCGGGGAGTCGGCCATCCATCCATCCCATCCATGCTGGATGCTTGATA
5601 CGCAGGTTTACTTTAGAGGAGTGCCGTACCGAATTGGCGCTACCTTCAAGCCCTACAAGTGGGGGGCTGCGGCGCTAAGGAGCACTAACTCTGAGGCTGT
5701 CCAACTACCATGTCTGGACGACCACAGACACACACAGCCCAGCTTGAATCATTGACACACCTGGGCAAATCGTTAACAAGTCCATGCAATATGGAATGCT
5801 TTGACTCGAGCCCTGATGAAGAATTCCACCTCCGACGCCTACTCTAAGCCGGAAACACTAGCCATTCGAGCCAACGCTCAATTAATTTGTTTACGTTTCA
5901 TAACTTATTCATGTTTTTATCATCAATTTGTCACGTCCATCTGTTAACGAAGGTTGTCACCTCCGTTTTGGCAGATGGGGCCTGTGGACAAGAAGTGGAG
6001 TGAACTCTCCGACGTCGTATCTTAATTGGATTGCACCCTTCGTAGACGTGCGGCTGCCGCATGCCTGGCCGTTGAGAATTTAGTGGAGCTTCCCCCCTCT
6101 CTTCTCCCCCTCCAAAAAAAAAACCAGGGGACTGCACGAACCACTGCCGGCAGTGAGGTCACAGGCGCCAGGCGCCAGGTGCCAGACAGACGCAAACATG
6201 GCGATCCAAACAGGGTCATACAGTGGGGCTGTTCTTGTGGCTACCTCTAGTGTGGGGGCTTTTGTGTTGTTTGGTGGCTGTTTGGTGGCCGTTTGGTGAT
6301 CGTTTGGTGGTCGTTTGGTGGTCGTTTGGCACATCTGAGGACAATTGTGGCCCGAGACCTCAGTTAAACATAGTTAAAACACGGCATCAGTGCTTCATTG
6401 CTTTGGTTTGCTTGCCCTGTTCGATTTCTAGCCTGGTAGCACCGCCGTCTGGGGGTATTTCGTCTGTCCGTCTGTCTGTCTGCCTGCCTGCCTGTCTGCC
6501 TGTCTGCCTGTCTGGATGAGATGAGCCGAGATGAGATGGATGGATGAGATGGATGGAGGAGATGGATCGCCCTCAATCTAAGGGTGGGTACCATTCGGCG
6601 ATCCCCAGTTACGCTAGAACATGAGTCGCCGGCCCCTGGC
```

DNA lyase

```
GSDHCPVFATMADKLTVKGKEHALLELLNAQGTFQGDRRLREWGPKDLLPLSARLIPEFDRRQSIRDMFTKKPNSTLEQSSKHKVVTENP
HPQPTTKRPIATGESMSERALKKTKAPSNSVNAKNKAVTGQRTLQGFFKPKASTPQPEAPTVGGTGRATPLPTTKTSLGSPNGPPSQRLP
PQPPETLSNGHSERVFDPIEAKESWSKLLRKRVAPECEHHEPCISLTTKKPGVNCGRSFYICPRPLGPSGEKEKESEWRCGTFIWSSDWN
SASSGS
```

Best three blastx hits

>gb|EGU79454.1| hypothetical protein FOXB_10039 [Fusarium oxysporum Fo5176]
Length=593

```
 Score =  274 bits (700),  Expect = 1e-85
 Identities = 157/307 (51%), Positives = 181/307 (59%), Gaps = 44/307 (14%)
 Frame = +1

Query  1    GSDHCPVFATMADKLTVKGKEHALLELLNAQGTFQGDRRLREWGPKDLLPLSARLIPEFD  180
            GSDHCPVFA ++DK+TV  KE ALLE++N  G F GD RLR+W PKD LPLS++LIPEFD
Sbjct  282  GSDHCPVFANLSDKVTVGDKECALLEMMNPPGMFNGDERLRDWSPKDHLPLSSKLIPEFD  341

Query  181  RRQSIRDMFTKKP-----------------NSTLEQSSKHKVVTENPH------------  273
            RRQSIRDMFTKK                  N     S      +  P
Sbjct  342  RRQSIRDMFTKKAAPPREPTRTDTPAEPLNNGNSSASGSPTNASSTPRLGETTNSTKLSA  401

Query  274  PQPTTKRPIATGESMSERALKKTKAPSNSVNAKNKAVTGQRTLQGFFKPKASTPQP----  441
             QP++KRP  T    + R  KKTK+ + + + K+K   GQRTLQGFFKPKA   Q
Sbjct  402  SQPSSKRP-GTAADTTSRPFKKTKSFTGANDTKSKVAQGQRTLQGFFKPKAPAAQDGKAE  460

Query  442  -----EAPTV----GGTGRATPLPTTKTSLGspngppsqrlppqppETLSNGHSERVFDP  594
                   P+      G+G+A P    + S      P  +  P  P        S+RVFDP
Sbjct  461  LVAANSTPSTTKKPAGSGKA-PASAQRLSNTPQATPTEKSSPTVPLRGKDPDPSDRVFDP  519

Query  595  IEAKESWSKLLRKRVAPECEHHEPCISLTTKKPGVNCGRSFYICPRPLGPSGEKEKESEW  774
            IEAKESWSKLL KRVAP CEH EPCIS TTKKPGVNCGR FYICPRPLGPSGEKE+ SEW
Sbjct  520  IEAKESWSKLLGKRVAPRCEHDEPCISFTTKKPGVNCGRMFYICPRPLGPSGEKERNSEW  579

Query  775  RCGTFIW  795
            RC TFIW
Sbjct  580  RCSTFIW  586
```

>gb|AEI72618.1| DNA lyase [*Epichloë* festucae]
Length=642

```
 Score =  246 bits (628),  Expect = 1e-74
 Identities = 147/322 (46%), Positives = 181/322 (56%), Gaps = 57/322 (18%)
 Frame = +1

Query  1    GSDHCPVFATMADKLTVKGKEHALLELLNAQGTFQGDRRLREWGPKDLLPLSARLIPEFD  180
            GSDHCPVFAT+AD+++ KG + ALL+++N  G F   +R+RE   +DLLPLSA+LIPEFD
Sbjct  311  GSDHCPVFATIADRVSFKGNDTALLDVMNPPGVFAQGKRVRELAQRDLLPLSAKLIPEFD  370

Query  181  RRQSIRDMFTKKPN-------------------STLEQSSKHKVVTENPHPQPT---TKR  294
            RRQSIRDMF KK +                   S++ ++    V   N +       T
Sbjct  371  RRQSIRDMFFKKSSTIGVAADGAPSPGQPAKRASSITETGLRPVSNNNSNNNTDRNGTSE  430

Query  295  PIATGESMSERA----------------------LKKTKAPSNSVNAKNKAVTGQRTLQG  408
             +  G S + RA                      LK++KA +++ ++K K   GQ+TL+G
Sbjct  431  DLLGGASANSRAANAQQAESQKRPAEPIDPAPRQLKRSKAGADTNSSKLKTAPGQKTLKG  490

Query  409  FFKPKASTPQPEAPT------VGGTGRATPLPTTKTSLGspngppsqrlppqppETLSNG  570
            FFKP  S P    P+         T   T   +T  +      P    L    P+   +
Sbjct  491  FFKPVDSAPVHPTPSSLQESRTNNTSPRTATDSTSPTKSVSGEPVGSELTSPSPKYSQSH  550

Query  571  HSE-------RVFDPIEAKESWSKLLRKRVAPECEHHEPCISLTTKKPGVNCGRSFYICP  729
            HSE       RVFDPIEAKESWSKLL KRV P CEH EPCISL TKKPGVN GRSFYICP
Sbjct  551  HSEPIPRLPDRVFDPIEAKESWSKLLGKRVVPRCEHEEPCISLVTKKPGVNRGRSFYICP  610

Query  730  RPLGPSGEKEKESEWRCGTFIW  795
            RPLGPSGEKEK SEWRCGTFIW
Sbjct  611  RPLGPSGEKEKGSEWRCGTFIW  632
```

>dbj|BAC66502.1| DNA lyase [Isaria tenuipes]
Length=611

```
 Score =  243 bits (621),  Expect = 8e-74
 Identities = 136/302 (45%), Positives = 171/302 (57%), Gaps = 42/302 (14%)
 Frame = +1

Query  1    GSDHCPVFATMADKLTVKGKEHALLELLNAQGTFQGDRRLREWGPKDLLPLSARLIPEFD  180
            GSDHCPV+ATM D +   GKE AL E++N    F+  +R+R W  +D L LSA+LIPEFD
Sbjct  302  GSDHCPVYATMRDTVVKGGKEIALAEVVNPTNMFRDGKRIRNWEQRDALALSAKLIPEFD  361

Query  181  RRQSIRDMFTKK-PNSTLEQSSKHKVVTENPHP-----QPTTKRPIATGESMSER-----  327
            RR++IRDMFT K  +++ ++ +K + V  +        + T  R  + G    ER
Sbjct  362  RRRNIRDMFTNKAAHASFDRGNKTRSVARDAEEMKQLGKTTMPRLQSEGSVGVERERERE  421

Query  328  ----------------ALKKTKAPSNSVNAKNKAVTGQRTLQGFFKPKASTPQP------  441
                             +   K    S   K K   GQ TLQGFFKPK++T
Sbjct  422  REWVSQLKRHAGPVQVGIPANKKSKGSSETKTKVAGGQTTLQGFFKPKSTTTADVQGGPL  481

Query  442  ----EAPTVGGTGRATPLPTTKTSLGspngppsqrlppqppETLSNGHSERVFDPIEAKE  609
                + P V G  +  PLP +  SL                + +    S +VFDPIEAKE
Sbjct  482  AGPCQVPGVPGRAQTPPLPASAASL-----QTPASWQTGTVKPVEEKSSGKVFDPIEAKE  536

Query  610  SWSKLLRKRVAPECEHHEPCISLTTKKPGVNCGRSFYICPRPLGPSGEKEKESEWRCGTF  789
            SWSKLL KRV P+CEH EPCISL TKKPGVNCGRSFYICPRPLGPSG+KE+++EWRCGTF
Sbjct  537  SWSKLLGKRVVPKCEHDEPCISLVTKKPGVNCGRSFYICPRPLGPSGDKERDTEWRCGTF  596

Query  790  IW  795
            IW
Sbjct  597  IW  598
```

MAT1-2-1

```
MPRANVASNYTQDQLDAIWDQLKAQVSPFVYVLCIEGPLYRVLDDGAKQFIAKQFMEQVTERVMYCRDGTDGDRYFLGSPRFFLAGSGMI
VHALHTKEAIWVKRPDDGIQHILVVPPALPKKCAKIPRPPNAYILYRSDRHRFVKASQPGIHNNEISQILGRAWNLESEAIRAKYKQRAD
AIKAELIKRHPEYKYRPRRPSERRRRRIAERTEEAEDALADIVVDASQAVPVAAPADEADPEPTDEAPASDDEATAPTAETPVPTVEAS
```

Best three blastx hits

>gb|AEI72619.1| mating type 2 HMG1/2 protein [*Epichloë* festucae]
Length=246

```
 Score =  212 bits (540),  Expect = 5e-66
 Identities = 103/188 (55%), Positives = 131/188 (70%), Gaps = 1/188 (1%)
 Frame = -1

Query  789  SNYTQDQLDAIWDQLKAQVSPFVYVLCIEGPLYRVLDDGAKQFIAKQFMEQVTERVMYCR  610
            S +  DQL AIW QL+ QV+PFV VLC++G LYR+LD GAK FIA+ F+  V E V+YC
Sbjct  10   SQWNADQLRAIWSQLQLQVNPFVQVLCLDGNLYRMLDTGAKTFIAQNFINHVKESVLYCI  69

Query  609  DGTDGDRYFLGSPRFFLAGSGMIVHALHTKEAIWVKRPDDGIQHILVVPPALPKKCAKIP  430
            DGT  DR FLG+PR F+AG GM++      E  WV R +    +   V  P++  K  KIP
Sbjct  70   DGTGHDRVFLGAPRHFVAGGGMLLQP-GGSEPFWVVRSEAKFRTPAVCSPSVSAKTTKIP  128

Query  429  RPPNAYILYRSDRHRFVKASQPGIHNNEISQILGRAWNLESEAIRAKYKQRADAIKAELI  250
            RPPNAYILYR +RH  VK + PGI NNEISQILGRAWNLE+  +R +YK  AD +K  L+
Sbjct  129  RPPNAYILYRKERHNTVKDANPGITNNEISQILGRAWNLETRDVRQRYKDMADRVKQALL  188

Query  249  KRHPEYKY  226
            ++HP+Y+Y
Sbjct  189  EKHPDYQY  196
```

>gb|EFY88585.1| MAT1-2-1 like protein [Metarhizium acridum CQMa 102]
Length=246

```
 Score =  212 bits (539),  Expect = 7e-66
 Identities = 99/188 (53%), Positives = 130/188 (69%), Gaps = 1/188 (1%)
 Frame = -1

Query  789  SNYTQDQLDAIWDQLKAQVSPFVYVLCIEGPLYRVLDDGAKQFIAKQFMEQVTERVMYCR  610
            S +  DQL  IW QL+ QV+PF  VLC++G LYR+LDDGAK FI + F+  V E V+YC
Sbjct  11   SQWNLDQLRGIWSQLQTQVNPFARVLCLDGNLYRMLDDGAKNFIVQNFIHHVKEPVLYCI  70

Query  609  DGTDGDRYFLGSPRFFLAGSGMIVHALHTKEAIWVKRPDDGIQHILVVPPALPKKCAKIP  430
            DGT  DR +LG+PR F+ G G+++     + +  WV R +  ++   + PP +  K  KIP
Sbjct  71   DGTGPDRVYLGAPRHFVTGGGILIQPSGS-DPFWVIRNETKLKTATICPPPVSTKVTKIP  129
```

```
Query  429  RPPNAYILYRSDRHRFVKASQPGIHNNEISQILGRAWNLESEAIRAKYKQRADAIKAELI  250
            RPPNAYILYR +RH  VK + PGI NNEISQILGRAWNLES  +R KYK  AD +K  L+
Sbjct  130  RPPNAYILYRKERHNTVKEANPGITNNEISQILGRAWNLESREVRQKYKDMADRVKQALL  189

Query  249  KRHPEYKY  226
            ++HP+Y+Y
Sbjct  190  EKHPDYQY  197


>gb|EGR49870.1|  mating type protein MATa1 [Trichoderma reesei QM6a]
Length=241

 Score =  203 bits (516),  Expect = 2e-62
 Identities = 97/182 (53%), Positives = 130/182 (71%), Gaps = 4/182 (2%)
 Frame = -1

Query  768  LDAIWDQLKAQVSPFVYVLCIEGPLYRVLDDGAKQFIAKQFMEQVTERVMYCRDGTDGDR  589
            L  IWD LK Q++PF+ +LCI G +YR+LDD  +Q++A++FME V E VM+CRDG   DR
Sbjct  18   LAGIWDGLKEQINPFLQILCINGNIYRMLDDYGRQYVARKFMEHVKEAVMFCRDGNGEDR  77

Query  588  YFLGSPRFFLAGSGMIVHALHTKEAIWVKRPDDG-IQHILVVPPALPKKCAKIPRPPNAY  412
            ++LG+PR+F+A  G +VH+    E  W    DDG I+       ++P K  KIPRPPNAY
Sbjct  78   HYLGAPRYFIANGG-VVHSPDGLEPFWTM--DDGSIRLETTAVCSIPAKPTKIPRPPNAY  134

Query  411  ILYRSDRHRFVKASQPGIHNNEISQILGRAWNLESEAIRAKYKQRADAIKAELIKRHPEY  232
            ILYR DRH  VKA+ PGI NNEISQILGRAWN ES  +R +YK+ ++ IK  L+++HP+Y
Sbjct  135  ILYRKDRHNLVKAANPGITNNEISQILGRAWNQESREVRQRYKEMSEEIKLALLEKHPDY  194

Query  231  KY  226
            +Y
Sbjct  195  QY  196
```

Putative ORF

MANLPLPPDHPGAAMYQFTHGPGLHDIQPQREGFWTLAGHDAELFRLGFVHTPPGQATAQLLYQVFRPIKSLDVLETHASDLIYEYEGDF
ASIPQHDEGFAAQPTVRDDLVHISHSDLAEEGVMNQGQNIVQDDLQVEQGSVIQSDNSLISQMVSEPDAQILSPLLDDNLLAASQSMDDG
NWAFPPDVFQSQDYNLLNGTPEMNVEDLGMNQEQMVASGMPHTQEWAELPDADNDQLQGDDQMILPDF

Deduced protein with no similarities to the NCBI EST, nucleotide or protein databases (using tblastn or blastp, respectively) at e-value <e-1.

Supplementary material S3

DNA and protein sequences of the type A *MAT* locus of *Ilyonectria liriodendri* (isolate Cy33) including the genes *MAT1-2-1* (orange), a putative ORF (purple) and *MAT1-1-1* (yellow)

```
1    TCAGCTAGCAATGGGTGATGATCAAATGAGCGATGATGGATGGGCAATGATGGATGGGCGATGATGGATGACAATGGACATGGACGGGATTCAGGTTGGA
101  GGCATCCTCGGGTTCAGCATGGACATGACGGGAAGTGTGTTCACATCACTGGAGGGGGAAGTGTATTCACGTCACTTTCAAATCACCAGCAATCACCAGA
201  ATCACCAGCAATCACTAAATCACCAGAATCACCAGAATCACCAGCAATCACCAGCAATCACCAGAATCACCAGCAATCACTAAATCACCAG
301  AATCACCAGAATCACTAAATCACCAGAATCACCAGAATCACCAGCAATCACTAAATCACCAGCAATCACCAGAATCACCAGAATCACCAGAATCACCAGC
401  AATCACCAGAATCACCAGCAATCACCAAATCACCAGCAATCACCAGAATCACCAGAATCACCAGCAGGGAAATCAAATCGGGTTCAACTGTTATTCATCC
501  TTCCTGTATTCATCCTTCCTGATCCTCCTRGCTACGTACGGGAGCGCCCAAACGTTTCGAGAATCATGTTGTTTGCACCTTTCCATGGTGAATGTGCCAT
601  GAACCAGARGCGAAAAGTGGCAATGAAAGGTGAAAGTGCCAATGAAAGGTGAAAAGTGGCAATGAAAGCAAGGTGAAAAAGTCGCACACTTTCCATGGTG
701  CACACTTTCCATGGTGAATGCCCACTTTCCACGGTGAATGTCAACTGTCCATGGTGAATGCTTCAAGAACCACATTCAAGARCCGCATTCAAGAACCACA
801  TTCAAGAACCACATTCAAGAACCACATGCTCAAGAAGCCTCATCGGCGGAAGGCGGAGGGACACCGGCGGAAGGCGGAGGGACACCAGCGGAAGGCGGAG
901  GGACAGGCACGGCCTGGGAAGCATCGACGGCGACGGCCGCCAGCGCGTTCTCCGACTCCTGGGTGCGCTCCGCGACGAGGCGGCGGCGGCGCTCCGAGGG
1001 CCGGCGCGGGCGGTACTTGTAGTCGGGGTGTTTCTTGATCAGGGCCTCCTTGATGTCGTTGGCCATCTTTTGGTACTTGGATCTGATGGCGGTCGACTCG
1101 ACGTTCCAGGCCCGGCCGAGGATTTGGGCTGTTTTTTCAAAGTCAGTGTTTTTTCAAAGTCGGTTTTTTTTCAAAGTCAGTGTTGTTTTGGGAGTGATGG
1201 GTGAGAGGTGTTTTGAGACGATGCGAGGGGTAGGTGTTGTTTTGAGGGTGGTGGTTTGAGACGATGCGAGGGGTAGGTGTTGTTTTGAAGGGTGATAAGG
1301 GTGAAGGGTGATGAGGGCGGTACTCACAAATCTCATTGTTGTGAATCCCAGGCTTGCTGAGCTTGATGTAGCGGTGACGGTCCCGGCGGTACAAGATGTA
1401 CGCGTTGGGAGGACGTGGGATCTTGGCGCACTTGTTTGGGAGAGCTGGGGGGACAACGAGGATGTGCTGGAGCCCGTCGTCGGGGCGCTTGACCCAGACC
1501 GCTTCCTTGGTGTGGAGAGCGTGGACAATCATTCCAGACCCTGCGAGGAAGAAGCGAGGCGCGCCGAGAAAGTAGCGGTCGCCTTCGGTACCGTCGCGAC
1601 AGTACATGACCTTTTCGGTGACGTGATCCCTGGGGAATGAGTTAGAAGAAGAAAAAGCTAGGCAGAGGGAGAGGGAGACAGAGGGAGAGGGAGAGAGGGG
1701 AATGGTGTCTGGTGGCACTTAGTCCCGTGGAACTTACATGAACTGGCGGGCAATGAACTCTTTGGCCCCGGCGTCGAGCACGCGGAAGAGGCTTCCTTCG
1801 ATGCAGAGGACATAGACGAAAGGGGAAACTTGAGTCTTGAGCTGGTCCCAGACGGCATTGAGCTGGTCTTGGGAGTACTGCGAGACGACGGGGTGAGAGA
1901 CGGGGTGAGAGGTCAGAGACGCCATTGTGACGGGGGTGTTGATGAAAAGAGAAGGAGAAAAGAGAAGGAGAATGGGTGGACAAATATGTTGGAAGGTGTG
2001 AAGAGGAAGAAGAATTCCTGAGCTCGAGAGGTGCAGAGGACGTTTTTGTAGTCGGGCTGAGCGGAGGGGGCGGACTGGCAACCGGCTGGCAGCGCAGCAC
2101 CAACGGCGATATCTCACCATGGTACGTACCATCCTGAGGTTGTCCATATTCCTTTGTTCAATAGAGCATTCATGCAAGCCACCTGATTTATTGTGGGGCA
2201 AGGGCAAACGCCAGCACTGTGCACTTACTGTGCACTTAGCATCTCCACCCTGACGCCAAACGCAGGGCGTAGCCGCTAGAACGGATGGGGCCAAGGCTTC
2301 TGAATATTTGAGTTTGGACTGACATTCTCACTGCCAACAACCCAACTCCAGCTGGAAACGCTATTTGGCGTAGCTGAAACCTCTGAAAACCCCGCACCTTG
2401 GAAGGCCGACTCTCTCAGCTGGGTTTTTTATTTGTCTCACCGTAATTTGACCGCTTCGCTGGTTCATTGGGCACATGTTTCTGACTCCAATGAATATCAC
2501 CTCGATTCATGTTTGGGCTGCTACCAGTCCGGAGCTTCTGGAGGCCGTTCTCTTCAGATTGGGAAATCAGCAAACAACCTTTCGCTAAACCTCAGGAATG
2601 TGCTCCAGAACTTTGCTCCAGAACTTTCCTCAACATCTTAACTTGTTTTGTTCAAGCTTCTTGACTACCACCCCTCCCCACATAGCCACCCTGGCGAGGT
2701 GAGCACGTGCTACGCRCACCCCACCGGAGTCGCAGGTGCTGCCAGCTGCCGGCCTGAAAACGCTTGTCCACTTGGGGCACGTTGGACCAACTCAATGTTC
2801 GTTGTCTCCACCTCCATYTCGTTTTCCCCGAGAAGTCAAAAACCCAGTCTCATTTTCCAGTGCCCTTCTCTTTACTTCCTTCCAACAACTTTCTCTGATC
2901 TCTTCTTTCTTTTAATCTCATTTCCACCCCTTTCGCAGACTTTCAGCAACGACGCCTTCGAGGACTTCACAATGACGACGAACATTCCTCCACCTCCCAC
3001 CGATCCCAATGCCGCCATGTACCATTTCACCCACGGCCCAGGGCTCCACGACCTTCAGCCCCAGTCGCAAGGCTTTTGGACCTTAGCAGGGCATGATGAG
3101 GAACTTTTTCGGCTTGGTTTTGTTCATCATCCTCCCGGCGCGGCTGCGGCACAGGTTCACTTCCAAGTTTTCCGTCCCATCAAAACTTTGAACGATCTTG
3201 AGACACACGCGGAAGACCTCGTCTATGAATATGACGGCGGTGAGAATTATTCCAATCCCAAACCCGAGCTTCGTGAGACTGGGTACTGACAAATGAAACA
3301 GAGTTCGGCTCGGTTCCTCAGAAAGCAGACGGCTTTGCTCCTCCTGATGCTCCCAAGGACAATGCCGTCCATCTCACCCAACCCCGCTCCGTTGAGGAAG
3401 ATTTTGTCAGCCCCTTTGAGATCCCAGGAAGTGCCATGGTTCAGAACCAGAATGTTGTTCAGCCAGCATTCAATCCTGAACCGAGCGCTCTCGTCCAACT
3501 TTCTCCATCTGCTGTGTCGCAGATGATGCCTCATTACAATACTCCCATTCTATCCCCAGCGGTAGATCAGCACCAGCCTCAGGCTGCTCAGGCCATGGAT
3601 GATGGCAGCTGGAACTTCGCTCCTCCAGCTCTCCCGGCTCAAGATGATGGCATGGTTCTCCCGGCTCAAGATGATGGCATGGTCGCTGGAGTTCCCATGA
3701 TGAATGTTGGGGTTCAGGACATGCCTCAGCAGCAGGTGGTGGTGGTCCCCCAGATGTGGCAGACTCAAGCGTGGGCCGACTTGCCAGACGCCAACAACAA
3801 CCAGGACAGTGGGGAGGACCAGGTTTTCCCCCCAAACTTCTAAGTGTAGGGTCACTCTATAGCAAATAGGTAGACACTGCACCTCTCTCCGGCACAAGTA
3901 GGGAGTGAACAGAACACAAATTCAAATGCTGAAATTCATTCTTCCTACTGATTCTTTTGATCGCTTTCACAGCCTCGTCTGAAGTCATGCGTGCGTTGTC
4001 CGCTCTGTCAACCGCCGAGATCCTCAGTCTCCTCACGGACGAGACGATGCTCCAAGTCGCGGCCAAGTACTTTGCGACGCACGCCGCGACCATGGACGTC
4101 AACGCGCCTCTGAGCCCTACCGAGGCGGACGCCATTCTTTCTGTCCAGCAGATGACTACTCCTTTTTCTGTCCAGTCGGGTGGTGCGCCTGCTCCTCGTG
4201 CCAAGCGGCCTCTGAATGCATTCATGGCATTCCGCAGTACGTTTTTCTTTGTCCTTGTCCTTTTCTGTCCAACCCCCACTGACAACTGGCAGCCTTCTAC
4301 ATGAGAATCTTCCCAGACATCCAGCAAAAGAGCGCCTCCGGCTTCCTGACCACCCTCTGGAACAAGGACCCGTTCCGCGCCAAATGGGCCCTCATCGCCA
4401 AAGTCTACTCGTTTGTGCGCGACGAGGTCGGCAAGGGCCACATCGCCCTCGCGCGCTTCCTGGACGTCGCCTGTCCCGCCATGAACATGATCCGCCCGAG
4501 CGGCTACCTCGCCGCGCTGGGCTGGTCTGTCAGTTATGACGACGACGACTCTCTCGTCCTGGACCAGACCCTCCCGACAAACCTGGGCCGCTTCCAGCAC
4601 GAGGCCGTCCCCCGCACCGAGATGGAGCTGCTCCACGCGCTCCTGCAGACGGGGTACCTCTCCGAGCGGGGGCTGGCCCTCCTGGAGCGACTCAGCGCAA
4701 ACAACAACAGCGGGTTCGTCAACGCCAGCGGATTCGTCGGCGCCGCCAACACCAGCGGATTCGTCACCACCACCAACGGGTCGTCCTCCTCTGCTGCTCC
4801 TCCTCAAGCTCCTCCTGAGACTCCTCCCCCCCCTCCCCTTCAAACCCCAGAGAAACTCAGCCTCGTCRACACCATCCGCGCCGACCCCATCCAGGCCGCC
4901 AAGGAGCTGTTCCGCGCCCGCGAGGACGAGTACCTCGACCCCAAGCACGTCGCCACCTACGAGGTCTACGATCTGAGCTACGCGCCCTACGCCGCCATGC
5001 TGGCGCCCCAGCCGGAACCCCTCCAGTACTACGACTTTGCCGGGGCCAGCCTGGGACTCCGCGGAAGCGGAAGCGGAGAAGAAGCACCCCCCACCTTCAA
5101 CCCCCTCGGCAGCGTCGACGAGTTTGACGCCATCGACATTGACAGCCCCTACGACGTCGACGCCATGATGGGAAACACTGAATCCGAGGGCGAGAGAAGT
5201 GAGTTTTTTTTTCCTTTTTTTTGAAAATCGTTGAAAGGGAAACACGGCTAACACATCATAGCCGCTGATCAGGAGGACACCAACCCGTATGATCCTCAGT
5301 CCGAATATCACCCCCTCGCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAATCGCATTCATTTTTTGGCACTGGATGATGGCACTTGTGTCAAGTGTGGAAT
5401 TGGGCACTTGGTCGGACGCTCAGAACGAAATTGGCTCTGTTTTAGTTGACTCACTTCAATACGAAATTGACACTTTTTGATTCTTTGCACAACCCATGGC
5501 CTGACGAGGGGCGAAACATGGCGTGCTTTGTGATTATTCCCACATGTTTCAATGAGTCTTCGAACCTGTGGCCGCCAGTTGCAGCACACCCAGGTTCAAA
5601 GAGGCCTCACTGCCAGGTCTCCCTCTCGATCCCTCCATCAGGGCGGCACATGCATCCATCTGCAAACTCCACTGTAGCGTCCACTGTGACTCCACTGATC
5701 ACCTCCACTGATCCACTGATCACCTCCACTGGTTCCCCTCCCCTCCCCCCCCGGCCCGCCCGAGCCGCAGTCCCCGGAGGATCCCAGTCCCACAGTTCCA
5801 CAGTTCCGCAGTCCCACAGTGTCACACGTCACGCTAAGCCCAGTTGGGTCCACAGTCCCACAGTGTCACACGTCACGCTAAGCCAAGTTGGTAGGGTGGT
5901 GGGGCTCACTAAAACGTTGCATTTGCCCTACTGCGCGCCATCGCCTCAAACGCTAAATCCGACGTTCGAATTTCTCACCTTGGCGCTTCAGCCCTTCCTC
6001 ACTCTCCCACCTCACCTTTCACTCCCACCTCACCTTCACTCCCACCTCAACTTCTCCTCACTTCTCCTCACCTTCCATTGAAGCCGCGCGTCATGCCAAG
6101 AAACGCCGCCGCCCACCACCATCGGGATCTCAGGGGGCCTCACCTCACCTCACCTCACCTCACTCCCACCACCTCACTCACTCCCACCACCTCACTTCTC
6201 CTCACCTTTGAAGCGCGCGTCATGCCAAGAAACGCCGCCGCCCACCACCAGTYGGATCTCAGGCCGGCCCCTCCCTCCGCTTTGTGTGAATCAAAGGCAG
6301 AGTTGGGCTGAGTGAGGAGAGTGTGAGGAGGCAGGGGTCGGCTGCTTCTCGTTGCCGCTCCCCACAACCTCGCGCTTGGCTCGA
```

MAT1-2-1

```
MASLTSHPVSHPVVSQYSQDQLNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHVTEKVMYCRDGTEGDRYFLGAPRFF
LAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPPALPNKCAKIPRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRS
KYQKMANDIKEALIKKHPDYKYRPRRPSERRRRLVAERTQESENALAAVAVDASQAVPVPPPSAGVPPPSAGVPPPSADEAS
```

Best three blastx hits

```
>gb|AEI72619.1|  mating type 2 HMG1/2 protein [Epichloë festucae]
Length=246

 Score =  224 bits (571),  Expect = 9e-71
 Identities = 106/192 (55%), Positives = 142/192 (74%), Gaps = 1/192 (1%)
```

```
 Frame = +1

Query  31   HPVVSQYSQDQLNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHVTEKV  210
            H  VSQ++ DQL A+W QL+ QV+PFV VLC++G+L+R+LD GAK FIA+ F++HV E V
Sbjct  6    HWAVSQWNADQLRAIWSQLQLQVNPFVQVLCLDGNLYRMLDTGAKTFIAQNFINHVKESV  65

Query  211  MYCRDGTEGDRYFLGAPRFFLAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPPALPNKC  390
            +YC DGT  DR FLGAPR F+AG GM++      E  WV R +   +   V  P++  K
Sbjct  66   LYCIDGTGHDRVFLGAPRHFVAGGGMLLQP-GGSEPFWVVRSEAKFRTPAVCSPSVSAKT  124

Query  391  AKIPRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRSKYQKMANDIK  570
             KIPRPPNAYILYR++RH  +K + PGI NNEISQILGRAWN+E+  +R +Y+ MA+ +K
Sbjct  125  TKIPRPPNAYILYRKERHNTVKDANPGITNNEISQILGRAWNLETRDVRQRYKDMADRVK  184

Query  571  EALIKKHPDYKY  606
            +AL++KHPDY+Y
Sbjct  185  QALLEKHPDYQY  196


>gb|EFY88585.1|  MAT1-2-1 like protein [Metarhizium acridum CQMa 102]
Length=246

 Score =  221 bits (564),  Expect = 1e-69
 Identities = 102/189 (54%), Positives = 139/189 (74%), Gaps = 1/189 (1%)
 Frame = +1

Query  40   VSQYSQDQLNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHVTEKVMYC  219
            VSQ++ DQL  +W QL+TQV+PF  VLC++G+L+R+LD GAK FI + F+ HV E V+YC
Sbjct  10   VSQWNLDQLRGIWSQLQTQVNPFARVLCLDGNLYRMLDDGAKNFIVQNFIHHVKEPVLYC  69

Query  220  RDGTEGDRYFLGAPRFFLAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPPALPNKCAKI  399
             DGT  DR +LGAPR F+ G G+++    + +  WV R +  L+   + PP +  K  KI
Sbjct  70   IDGTGPDRVYLGAPRHFVTGGGILIQPSGS-DPFWVIRNETKLKTATICPPPVSTKVTKI  128

Query  400  PRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRSKYQKMANDIKEAL  579
            PRPPNAYILYR++RH  +K + PGI NNEISQILGRAWN+ES  +R KY+ MA+ +K+AL
Sbjct  129  PRPPNAYILYRKERHNTVKEANPGITNNEISQILGRAWNLESREVRQKYKDMADRVKQAL  188

Query  580  IKKHPDYKY  606
            ++KHPDY+Y
Sbjct  189  LEKHPDYQY  197


>gb|EGR49870.1|  mating type protein MATa1 [Trichoderma reesei QM6a]
Length=241

 Score =  203 bits (517),  Expect = 1e-62
 Identities = 99/197 (50%), Positives = 138/197 (70%), Gaps = 6/197 (3%)
 Frame = +1

Query  25   VSHPVVSQYSQDQ--LNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHV  198
            ++ P  SQ  Q    L  +WD LK Q++PF+ +LCI G+++R+LD   ++++AR+FM+HV
Sbjct  3    LAQPTSSQDVQTAVYLAGIWDGLKEQINPFLQILCINGNIYRMLDDYGRQYVARKFMEHV  62

Query  199  TEKVMYCRDGTEGDRYFLGAPRFFLAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPP-A  375
             E VM+CRDG   DR++LGAPR+F+A  G +VH+    E  W    DDG   +     +
Sbjct  63   KEAVMFCRDGNGEDRHYLGAPRYFIANGG-VVHSPDGLEPFWTM--DDGSIRLETTAVCS  119

Query  376  LPNKCAKIPRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRSKYQKM  555
            +P K  KIPRPPNAYILYR+DRH  +K + PGI NNEISQILGRAWN ES  +R +Y++M
Sbjct  120  IPAKPTKIPRPPNAYILYRKDRHNLVKAANPGITNNEISQILGRAWNQESREVRQRYKEM  179

Query  556  ANDIKEALIKKHPDYKY  606
            + +IK AL++KHPDY+Y
Sbjct  180  SEEIKLALLEKHPDYQY  196
```

Putative ORF

```
MTTNIPPPPTDPNAAMYHFTHGPGLHDLQPQSQGFWTLAGHDEELFRLGFVHHPPGAAAAQVHFQVFRPIKTLNDLETHAEDLVYEYDGG
ENYSNPKPELQFGSVPQKADGFAPPDAPKDNAVHLTQPRSVEEDFVSPFEIPGSAMVQNQNVVQPAFNPEPSALVQLSPSAVSQMMPHYN
TPILSPAVDQHQPQAAQAMDDGSWNFAPPALPAQDDGMVLPAQDDGMVAGVPMMNVGVQDMPQQQVVVVPQMWQTQAWADLPDANNNQDS
GEDQVFPPNF
```

Deduced protein with no similarities to the NCBI EST, nucleotide or protein databases (using tblastn or blastp, respectively) at e-value <e-1.

MAT1-1-1

```
MRALSALSTAEILSLLTDETMLQVAAKYFATHAATMDVNAPLSPTEADAILSVQQMTTPFSVQSGGAPAPRAKRPLNAFMAFRTFYMRIF
PDIQQKSASGFLTTLWNKDPFRAKWALIAKVYSFVRDEVGKGHIALARFLDVACPAMNMIRPSGYLAALGWSVSYDDDDSLVLDQTLPTN
LGRFQHEAVPRTEMELLHALLQTGYLSERGLALLERLSANNNSGFVNASGFVGAANTSGFVTTTNGSSSSAAPPQAPPETPPPPPLQTPE
KLSLVXTIRADPIQAAKELFRAREDEYLDPKHVATYEVYDLSYAPYAAMLAPQPEPLQYYDFAGASLGLRGSGSGEEAPPTFNPLGSVDE
FDAIDIDSPYDVDAMMGNTESEGERTADQEDTNPYDPQSEYHPLA
```

Best three blastx hits

```
>gb|ACN59937.1| mating-type A-1 protein [Epichloë festucae]
Length=369

 Score =  236 bits (601),  Expect = 1e-71
 Identities = 155/405 (38%), Positives = 225/405 (56%), Gaps = 50/405 (12%)
 Frame = +1

Query  1      MRALSALSTAEILSLLTDETMLQVAAKYFATHAATMDVNAPLSPTEADAILSVQQMTTPF  180
             M+ LS L T E+L  L DETML +A++YF T          ++PT         Q T
Sbjct  8      MQRLSMLPTQELLQYLKDETMLDIASRYFDT-----TFQPTMTPT-------FNQTTMNS  55

Query  181    SVQSGGAPAPRAKRPLNAFMAFRTFYMRIFPDIQQKSASGFLTTLWNKDPFRAKWALIAK  360
              ++S      +AKRPLNAFMAFR++Y++++PD QQK+ASGFLTTLWN+DPFR KWALIAK
Sbjct  56     HLKSKVQICEKAKRPLNAFMAFRSYYLKLWPDQQQKTASGFLTTLWNRDPFRNKWALIAK  115

Query  361    VYSFVRDEVGKGHIALARFLDVACPAMNMIRPSGYLAALGWsvsyddddslvldQTLPTN  540
             VYSFVRDEVGK  ++LA FL  ACP M ++ P  YL+ LGWSV   +    ++
Sbjct  116    VYSFVRDEVGKDKVSLAYFLGFACPVMGIVEPQAYLSILGWSVEGVEPLQRLVQDEAVAA  175

Query  541    LGR--FQHEAVPRTEMELLHALLQTGYLSERGLALLERLSANNNSGFVNASGFVGAANTS  714
             LG+  F     P TE+ELL  L+  GYL E+G+ L+++L  N+N+
Sbjct  176    LGQSHFSGCEYPSTELELLSGLVNVGYLPEQGIDLIDKLGTNSNT---------------  220

Query  715    GFVTTTNGssssaappqappetppppplqtpeklsLVBTIRADPIQAAKELFRAREDEY-  891
               + T+N                  P   TPEKL  ++TIR+DP+QA KELF    D+Y
Sbjct  221    -IMATSN-------------PRLTLPVSYTPEKLQFMNTIRSDPVQATKELFGDAYDDYT  266

Query  892    LDPKHVATYEVYDLSYAPYAAMLAPQPEPLQYYDFagaslglrgsgseeaPPTFNPLGS  1071
             +    V +++V +L    +  M    P+P  YY+++ +   L  +G+            +
Sbjct  267    IQMLGVKSHDVENLDSINHLPMQMDLPDPRYYYNYSTSHSQLAVNGAPILR------FEN  320

Query  1072   VDEFDAIDIDSPYDVDAMMGNTESEGERTADQEDTNPYDPQSEYH  1206
             + + +  DID+P+DVDAM+G ++SEG+RT     +  +D   ++H
Sbjct  321    IPDHETFDIDNPWDVDAMLGQSQSEGQRTISHPRSPRHDSHEDFH  365


>gb|EFZ01122.1|  mating-type A-1 protein [Metarhizium anisopliae ARSEF 23]
Length=376

 Score =  229 bits (584),  Expect = 5e-69
 Identities = 160/405 (40%), Positives = 216/405 (53%), Gaps = 66/405 (16%)
 Frame = +1

Query  1      MRALSALSTAEILSLLTDETMLQVAAKYFATHAATMDVNAPLSPTEADAILSVQQMTTPF  180
             M+ LS L T E+L +L DET+  +AA+YF    A+M  N  + P+    I +  +
Sbjct  8      MQRLSMLPTQELLHILKDETIFDIAAQYFD---ASMQSN--ILPSTTPVIHNTHK-----  57

Query  181    SVQSGGAPAPRAKRPLNAFMAFRT----------------FYMRIFPDIQQKSASGFLTT  312
                +      +AKRPLNAFMAFR+                 Y+++FPD+QQKSASGFLTT
Sbjct  58     ---TKNTVCDKAKRPLNAFMAFRSKFITKPISDPFLPNSGHYLKLFPDVQQKSASGFLTT  114

Query  313    LWNKDPFRAKWALIAKVYSFVRDEVGKGHIALARFLDVACPAMNMIRPSGYLAALGWsvs  492
             LWNKDPFR KWALIAKVYSFVRDE+GK   I LA FL  +CP M +I P  YL  LGWSV
Sbjct  115    LWNKDPFRNKWALIAKVYSFVRDEIGKDKITLASFLSFSCPVMGIIEPQSYLTILGWSVG  174

Query  493    yddddslvldQTLPTNLG--RFQHEAVPRTEMELLHALLQTGYLSERGLALLERLSANNN  666
              D +   ++       LG  R Q + VP TE+ELL AL+  GYL E+G+ L+ +L ANN+
Sbjct  175    GDSNSQRLIQDETLAALGQSRLQSDGVPSTELELLTALVNVGYLPEQGIDLMGKLGANNS  234

Query  667    SGFVNASGFVGAANTSGFVTTTNGssssaappqappetppppplqtpeklsLVBTIRADP  846
             S    A+        S  V+ T                        PEKL  + TIR+DP
Sbjct  235    SIMATAT----QPRLSLPVSYT------------------------PEKLQFMGTIRSDP  266
```

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Query  847   IQAAKELFRAREDEY-LDPKHVATYEVYDLSYAPYAAMLAPQPEPLQYYDFagaslglrg  1023
             +QA KEL     D+Y +    V  + V DL    +  M    P+P  YY++
Sbjct  267   VQATKELLGDVYDDYTIQMLGVKAHNVEDLDSINHLPMQVEMPDPRYYYNY-----STSH  321

Query  1024  sgsgeeaPPTFNPLGSVDEFDAIDIDSPYDVDAMMGNTESEGERT  1158
             +       PT +   ++ E ++ DIDSP+DVD ++G T+SEG+R+
Sbjct  322   AQLSLAGAPTMS-FENIPEHESFDIDSPWDVDTILGQTQSEGDRS  365


>gb|ACR78244.1|  MAT1-1-1 [Hypocrea jecorina]
Length=379

 Score =  218 bits (556),  Expect = 8e-65
 Identities = 142/405 (35%), Positives = 211/405 (52%), Gaps = 47/405 (12%)
 Frame = +1

Query  10    LSALSTAEILSLLTDETMLQVAAKYFATHAATMDVNAPLSP-TEADAILSVQQMTTPFSV  186
             LS L T EIL  L D+T+L++A  YF +       N  +   T  DA  S    T
Sbjct  11    LSVLRTDEILHFLRDDTLLELANTYFTSLGIGAQQNQAMKQNTSGDASSSALIPT-----  65

Query  187   QSGGAPAPRAKRPLNAFMAFRTFYMRIFPDIQQKSASGFLTTLWNKDPFRAKWALIAKVY  366
             Q       +AKRPLNAFMAFR++Y+R+FP++QQK+ASGFLTTLWNKDP+R KWALIAKVY
Sbjct  66    QGAETSTDKAKRPLNAFMAFRSYYLRLFPEVQQKTASGFLTTLWNKDPYRNKWALIAKVY  125

Query  367   SFVRDEVGKGHIALARFLDVACPAMNMIRPSGYLAALGWsvsyddddslvldQTLPTNLG  546
             SFVRD++G+  ++L+ FL+V+CP M +  PS YL++ GWSV  D     +       +
Sbjct  126   SFVRDQLGRDKVSLSYFLNVSCPIMKITEPSIYLSSFGWSVEDDAGSPRLFQADGASIAQ  185

Query  547   RFQHEAVPRTEMELLHALLQTGYLSERGLALLERLSANNNSGFVNASGFVGAANTSGFVT  726
                 +  P TE +LL A++Q GYL +  + L++R++AN+N     A+  V   +T
Sbjct  186   PMDSDDHPNTENDLLSAIIQVGYLPDDSMNLMDRMNANSNGIMTTATSSVPVISTK----  241

Query  727   TTNGssssaappqappetppppplqtpeklsLVBTIRADPIQAAKELFRAR-EDEYLDPK  903
                                        EK   +  + ADP QAAKEL     E++ +
Sbjct  242   ---------------------------EKSEFMKVVEADPFQAAKELLGGHYEEKRVAAL  274

Query  904   HVATYEVYDLSYAPYAAMLAPQPEPLQYYDFagaslglrgsgsgeeaPPTFNPLGSVDEF  1083
              V ++ V DL+   +  +    P+P Q Y++A ++               P  +   ++ E
Sbjct  275   GVKSHLVEDLNAVSHLPLQFAYPDPRQIYNYASSTAAQPYQPEM----PQLDYFATMTES  330

Query  1084  DAIDIDSPYDVDAMMGNTESEGERTAD-----QEDTNPYDPQSEY  1203
             D  D+D+P+D D M+G  E+EG+R          +  N Y+P +E+
Sbjct  331   DTFDLDNPWDFDKMLGYKENEGDRVVIPAAGLPQPNNQYNPHNEF  375
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Supplementary material S4

DNA and protein sequences of the type B *MAT* locus of *Ilyonectria liriodendri* (isolate Cy109) including the genes *MAT1-2-1* (orange), *MAT1-1-3* (dark green), *MAT1-1-2* (light green) and *MAT1-1-1* (yellow)

```
1    TCGAGAATCATGTTGTTTGCACACTTTCCATGGTGAATGTGCCATGAACCAGAGGCGAAAAGTGGCAATGAAAGCAAGGTGAAAAGTGGCAATGAAAGGT
101  GAAAGTGCCAATGAAAGGTGAAAAGTGCCAATGAAAGGTGAAAGTGCCAGTGAAAGGTGAAAATGCCAATGAACGCAAGGTGAAAAAGTTGCACAACCTT
201  CCATGGTGAATGCACACTTTCCACGGTGAATGTCAACTTCCATGGTGAATGTCAACTTCCATGGTGAATGTCAACTTCCATGGTGAATGTCAACCTTCCA
301  CGGTGAATGTGTCAAGAACCACATGCTTCAAACCACATGCTTCAAACCACATGCTTCAAGCCTCGTCAGTGGAAGGGACATCAGTGGAAGGGACATCAGC
401  GGAAGGGACATCAGAAGGGACATCAGAAGGGGCAGGGACAGGCATGGCCTGGGAAACATCAACGGCAACACCCGCCAGCGCATTCTCCGAGGCCTGAGTC
501  CGCTCCGCGACGAGCCGGCGGCGGCGCTCCGAGGGCCGGCGCGGGCGGTACTTGTAGTCGGGGTGTTTCTTGATCAGGGCCTCCTTGATGTCGTTGGCCA
601  TCTTTTGGTACTTGGATCTGATGGCGGTCGACTCGACGTTCCAGGCCCGGCCGAGGATTTGGGCTGTTTTTTCAAAGTCAGTGTTTTTTCAAAGTCAGTG
701  TTTTTTCAAAGTCGGTTTTTTTTTCAAAGTCAGTGTTGTTTTGAGAGTGATGGGTGAGAGGTGTTTTGAGACGATGCGAGGGGTAGGTGTTGTTTTGAAGG
801  GTGATAAGGGTGAAGGGTGATGAGGGCGGTACTCACAAATCTCATTGTTGTGAATCCCAGGCTTGCTGAGCTTGATGTAGCGGTGACGGTCCCGGCGGTA
901  CAAGATGTACGCGTTGGGAGGACGTGGGATCTTGGCGCACTTGTTTGGGAGAGCTGGGGGGACAACGAGGATGTGCTGGAGCCCGTCGTCGGGGCGCTTG
1001 ACCCAAACCGCTTCCTTGGTGTGGAGAGCGTGGACAATCATTCCAGACCCTGCAAGGAAGAAGCGAGGCGCGCCGAGAAAGTAGCGGTCGCCTTCGGTAC
1101 CGTCGCGACAGTACATGACCTTTTCGGTGACGTGATCCCTGGGGAATGAGTTAGAAGAAGAAAAAGCTAGGCAGAGGGAGAGGGAGACAGAGGGAGAGGG
1201 AGAGAGGGGAATGGTGTCTGGTGGCACTTAATCCCGTGGAACTTACATGAACTGGCGGGCAATGAACTCTTTGGCCCCGGCGTCGAGCACGCGGAAGAGG
1301 CTTCCTTCGATGCAGAGGACATAGACGAAAGGGGAAACTTGAGTCTTGAGCTGGTCCCAGACGGCATTGAGCTGGTCTTGGGAGTACTGCGAGACGACGG
1401 GGTGAGAGACGGGGTGAGAGGTCAGAGACGCCATTGTGACGGGGGTGTTGTTGAAAAGAGAAGGAGAAAAGAGAAGGAGAAAAGAGAAGGAGAATGGGTG
1501 GACAAATATGTTGGAAGGTGTGAAGAGGAAGAAGAATTCCTGAGCTCGAGAGGTGCAGAGGTCGTTTTTGTAGTCGGGCTGAGCGGAGGGGGCGGACTGG
1601 CAACCGGCTGGCAGCGCAGCACCAACGGCGATATCTCACCATGGTACATCCCAGGCTGGCCACGGCGTCGTGAACAGACTTTAAGTAGTCTTTTTCGAGG
1701 GGGTGAATTTGTAGTTCGGTTGCTGTTCGTTGTGTTTTCGAGCCTCATCCTCGGCCAAAGCTTGCCAATAAGCCTTGACCTCGGGTGTCTCATCTTTCCA
1801 CATCTTGGCAATGATATTGGCTAAAATTGTTAGAGGTGATTTGGTACTTGATCTGGGGAGTTACAGGCTGCAGTGTCAATGATTCCAGGGTTCTGTCGGG
1901 CGACCTCCTGGGACTTTGCTTGACGGTATAGCATCCACCGATTCTTGGGACGGGAAACGTGAGGCTCCGGTTCAGCGACAGTCGTTGTTGGCGCTTGGCC
2001 TGCTTCAAGGCGTTTGTGATCAGATTCATCTTCAAATTTCGGGTTAGTAGTGCGCAAATCGTAGTTCACTAGGGAGTTCGTACCGACTGCAAAGCAAAAA
2101 TGCCCAAACGACGCTGTTCCCATCAAGACATGGTAAGAGAAAGGACAGAGACGATACTTCTGACGATGACGATCGTGAAACACCGTGACTTGCTGATTGA
2201 CGCGACGGCTAACAAGGTTAGCCAGACTACAACTCATGTGCATTGGAGTATCGCCCTACCTCAGGGTCAACGAGACTCGGTACACGACTCTGACGTCAAA
2301 GGATTCAGGCAAGAAGAGGTGGACATCACCTTGTGTCGCGGAGAACACAAAAGTGACTGGGACGTCGCTCGGCAGGTGCAAAGCAGCTCTGGGGATGTCA
2401 GCCCGAGTTGTCATGGTGATGGTCGTGGTTGGAGAGTAGCGGGATGTAAGGTAATGCGAGGAAACTATCGTGGTGAAGAATGAAAGAGAGAGAGCGAGAT
2501 GGCGTGCATCTTAAGGCCTAAGAAAATGGGTGCCTCAGTTGGTGCCTTCAGAACAATAGCCGGCAGAACAATAGCCGGCAGAACAATAGCCGGCAGAACA
2601 ATAGCCGGCAGAACAATAGCCGGCAAAACAATAGCCTTCGGAACAATAGCCTTCAAAACAACAGCTTCTCKTTTGGTGCCTAAGGTACCTGTAGCTGTTT
2701 TGTTTGGGTGAATGGCCGTCATTTGTTAACAGGCGGCCTAGCACGCGACTTTATTTACCAGTGCACAACACCTGTGCTTCCCACATTTCTTCACCTTTCA
2801 CCATCACAAAAGCCAAGATGGACAATTTGTTGTTCTACAGACCCCTTTTGGAGGAGAAAGCCTTGGCGACGTCTTCTTCAAAGCATCTTGAAAGTCTTCG
2901 CATGGATATCATGAAGAAACTCTTGCACGCGCTGCCAGTGGATGTGAAACCACCAACGGTGGCAGGTAGGAACTCATCGCCAACACTTTGTCCTCATGAA
3001 GCTTACCAATCTGCAGCTGTGGTCACTTCCAGCATCGAAATCATCAAGACATTGTTCCAGGAGGCTTCTGGCAGCAACGCTATCCTACAGAAAATCTTGG
3101 ATCACTCCAAGGAATCCGGTGTCGACCCAAATCTCGTCGTTCGAGGTGCCATTGTTTTGTGGTATACCAGCGCTTCTCCCATCCTTTCGCGCGACCCTAA
3201 CTTAGGTTTGCCTCCCAAGGCAATCTCGGGGGGAGGTCTGGCACTTCCATGGGTCCAGAGATTGGCCGAGTCGCAGTTCTTCATCGGAAACCTGGGCTAC
3301 TTGTCCATGTTGATGACAGCCGAAACCTGGGAGAAGCCATCTCACCCAAAACTGCAGGTAGCAACCCTGATGGCAAATGCGATCATCTCCTTTCTTTTCA
3401 CTACCTTCGTCATTACACCTCACCTGAAAACCCATACCCATTGGCGCCAACATCTTTCAAGCTCGAACAGCAGAGGTTACAAAGAGATGACGGTCTTCCT
3501 CAAGACCACATGGCACGTCTCCAGGGAGGGGTGGGAAAAGTTCGACCACCCACCCGGTGCCCAATTTTCTGCTCTGAACACTGAAGTCAAGATGTCCCGC
3601 GATGGGAGAAAACTCTTGACTCGCGTTGGCTGTCCGGACTGGCATGTTGCACCATATTGGCATCCTTTCCGGGCCGTGCCTGGTTCCCCATGGAACAAGT
3701 TCATCAGAAACAGACGGCAGAAAATCTTTCAGATCTTTCCAACTTTGAATAATCGCGTCCGGTACATGGTCCCGGGATCCGCCATTGCACTTACCGGTTC
3801 CTGCAAAGCTTACTATGGCGTCCTCCGCTCGCAGTTTGACTCCGTGAGTAGTTTCTTATCATTGATCCTGGATTTGGCTCACCTTTTGCTTTCAGACGAA
3901 CACAGAACGAGCCAGGCCTTCATTCCTGGAGCGTAAGCGGCAGTTCTGGCGGTTGGCCGAGGGGACTGGTCTCCAATACCCCACTGTGAGGCCAACAGAG
4001 CAGGTAAGTTCAAATATTCTTCAGAAGCGGCGAGAGGCTCACCAACCACCAGGACGCAACCAGCATCCAGGACTACGGACAAGCTGAGCACATGTACAAC
4101 GTACCATTGGTATGCACACCTCTTTTACAGAGTTTTATTAAGCCTACGCTAACTCCCACCTAGATCAAAAAGGCCGCTGCGGACTTAAGCGGGAACTTAA
4201 CCCCACCATTTTTGACCACCACCGCTCGAGCACTACGATTTGGGGTGGAACCTGAGTCCGTCGATGAGGACTTCTACGCTATGGTTTTTGAGCACTAGGT
4301 TGCATTCCCAGGCATGGAGACGTGAAGTTGTCATCAACTGTAAACCGATGAGAAGGCGTGGTAGCTGCTCTTTTTTTGTGTGACCGTCTACACTACAAAT
4401 AGTGCCAACAGAGTAGACAAAGGATATGACAGTGCTTCTAAACTTGTTCGCGTTCCTAAATTGGATGTTTCACGCGATGCTCCCCTCTTCCAAGCATCCA
4501 CAGTGTCTCATTCCTGTGTCTCATTCCTGTGCAGGTGGTCACAGCTGCCAACAATGTTTCCACTGACAACAGCTGGCATGCATCCCCACCCGTTTAGTGC
4601 ATTCAAAACGCCTCGTGGCCAGCCACCAAACACAAGAAACCCATTGTCACTTTTCTGCGAACAAGATTTTACTGGCACCACAGCCAACATTCTGCACACC
4701 CTACATTCTGCACACTCTCTCCCCCCCCCATTTAAATCCCCATCTCTCTCTCCCTCTTCCTCCTTTTCCACCAAACACTCAACTTCTTTCATCACTTTAC
4801 ATCAAAACTTTCTCCTCCTTTCAACATGGTTCAGTCTCGATCTGAAGTCATGCGTGCGTTGTCCGATCTGTCAACCGCCGAGATCCTCAGTCTCCTCACG
4901 GACGAGACGATGCTCCAAGTCGCGGCCAAGTACTTTGCGACGCACGCCGCGACCATGGACGTCAACGCGCCTCTGAGCCCTACCGAGGCGGACGCCATTC
5001 TTTCTGTCCAGCAGATGACTTGTTCTGTTCAGACTCCTTTTGTTCAGTCGGGTGGTGCGCCTGCTCCTCGTGCCAAGCGGCCTCTGAATGCATTCATGGC
5101 ATTCCGCAGTACGTTTTTCTTTGTCTTTGTCCCCCTCATTCCCTGTGACAAACCCCCACTCACAACTGGCAGCCTTCTACATGAGAATCTTCCCAGACAT
5201 CCAGCAAAAGAGCGCCTCTGGCTTCCTGACCACCCTCTGGAACAAGGACCCGTTCCGCGCCAAATGGGCCCTCATCGCCAAAGTCTACTCGTTTGTGCGC
5301 GACGAGGTCGGCAAGGGCCACATCGCCCTCGCGCGCTTCCTGGACGTCGCCTGTCCCGCCATGAACATGATCCGCCCGAGCGGCTACCTCGCCGCGCTGG
5401 GCTGGTCTGTCAGCTACGACGACGACGGCTCCCTGATCCTGGACCAGACCCTCCCGACAAACCTGGGCCGCTTCCAGCACGAGGCCGTCCCCCGCACCGA
5501 GATGGAGCTCCTCCACGCGCTCCTGCAGACGGGGTACCTCTCCGAGCGGGGGCTGGCCCTCCTGGAGCGACTCAGCGCAAACAACAACAGCGGGTTCGTC
5601 AACGCCAGCGGGTTCGTCGCCGCCGCCAACACCAGCGGATTCGTCACCACCACCAACGGGTCGTCCTCCTCTGCTGCTCCTCCTCAAGCTCCTCCTGAGA
5701 CTCCTCCCCCCCCTCCCCTTCAAACCCCAGAGAAACTCAGCCTCGTCGACACCATCCGCGCCGACCCCATCCAGGCCGCCAAGGAGCTGTTCCGCGCCCG
5801 CGAGGACGAGTACCTCGACCCCAAGCACGTCGCCACCTACGAGGTCTACGATCTGAGCTACGCGCCCTACGCCGCCATGCTGGCGCCCCAGCCGGAACCC
5901 CTCCAGTACTACGACTTTGCCGGGGCCAGCCTGGGACTCCGCGGAAGCGGAAGCGGAGAAGAAGCACCACCAACCTTCAACCCCCTCGGCAGCGTCGACG
6001 AGTTTGACGCCATCGACATTGACAGCCCCTACGACGTCGACGCCATGATGGGAAACACTGAATCCGAGGGCGAGAGAAGTGAGTTTTTTTTTTCCTTTTT
6101 TTTGAAAATCGTTGAAAGGGAAACACGGCTAACACATCATAGCCGCTGATCAGGAGGACACCAACCCGTATGATCCTCAGTCCGAATATCACCCCCTCGC
6201 TTAAAAAAAAAAAAAAAAAAAAAAAATCGCATTCATTTTTGGCACTGGATGATGGCACTTGTGTCAAGTGTGGAATTGGGCACTTGGTCGGACGCTCAG
6301 AACGAAATTGGCTCTGTTTTAGTTGACTCACTTCAATACGAAATTGACACTTTTTGATTCTTTGCACAACCCATGGCCTGACGAGGGGCGAAACATGGCG
6401 TGCTTTGTGATTATTCCCACATGTTCAATGATC
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MAT1-2-1

MASLTSHPVSHPVVSQYSQDQLNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHVTEKVMYCRDGTEGDRYFLGAPRFF
LAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPPALPNKCAKIPRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRS
KYQKMANDIKEALIKKHPDYKYRPRRPSERRRRLVAERTQASENALAGVAVDVSQAMPVPAPSDVPSDVPSADVPSTDVPSTDEA

Best three blastx hits

>gb|AEI72619.1| mating type 2 HMG1/2 protein [*Epichloë* festucae]
Length=246

Score = 224 bits (571), Expect = 9e-71

```
 Identities = 106/192 (55%), Positives = 142/192 (74%), Gaps = 1/192 (1%)
 Frame = +1

Query  31   HPVVSQYSQDQLNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHVTEKV  210
            H  VSQ++ DQL A+W QL+ QV+PFV VLC++G+L+R+LD GAK FIA+ F++HV E V
Sbjct  6    HWAVSQWNADQLRAIWSQLQLQVNPFVQVLCLDGNLYRMLDTGAKTFIAQNFINHVKESV  65

Query  211  MYCRDGTEGDRYFLGAPRFFLAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPPALPNKC  390
            +YC DGT  DR FLGAPR F+AG GM++      E  WV R +   +   V  P++  K
Sbjct  66   LYCIDGTGHDRVFLGAPRHFVAGGGMLLQP-GGSEPFWVVRSEAKFRTPAVCSPSVSAKT  124

Query  391  AKIPRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRSKYQKMANDIK  570
             KIPRPPNAYILYR++RH  +K + PGI NNEISQILGRAWN+E+  +R +Y+ MA+ +K
Sbjct  125  TKIPRPPNAYILYRKERHNTVKDANPGITNNEISQILGRAWNLETRDVRQRYKDMADRVK  184

Query  571  EALIKKHPDYKY  606
            +AL++KHPDY+Y
Sbjct  185  QALLEKHPDYQY  196


>gb|EFY88585.1|  MAT1-2-1 like protein [Metarhizium acridum CQMa 102]
Length=246

 Score =  221 bits (564),  Expect = 1e-69
 Identities = 102/189 (54%), Positives = 139/189 (74%), Gaps = 1/189 (1%)
 Frame = +1

Query  40   VSQYSQDQLNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHVTEKVMYC  219
            VSQ++ DQL  +W QL+TQV+PF  VLC++G+L+R+LD GAK FI + F+ HV E V+YC
Sbjct  10   VSQWNLDQLRGIWSQLQTQVNPFARVLCLDGNLYRMLDDGAKNFIVQNFIHHVKEPVLYC  69

Query  220  RDGTEGDRYFLGAPRFFLAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPPALPNKCAKI  399
             DGT  DR +LGAPR F+ G G+++    + +  WV R +  L+   + PP +  K  KI
Sbjct  70   IDGTGPDRVYLGAPRHFVTGGGILIQPSGS-DPFWVIRNETKLKTATICPPPVSTKVTKI  128

Query  400  PRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRSKYQKMANDIKEAL  579
            PRPPNAYILYR++RH  +K + PGI NNEISQILGRAWN+ES  +R KY+ MA+ +K+AL
Sbjct  129  PRPPNAYILYRKERHNTVKEANPGITNNEISQILGRAWNLESREVRQKYKDMADRVKQAL  188

Query  580  IKKHPDYKY  606
            ++KHPDY+Y
Sbjct  189  LEKHPDYQY  197
>gb|EGR49870.1|  mating type protein MATa1 [Trichoderma reesei QM6a]
Length=241

 Score =  203 bits (517),  Expect = 1e-62
 Identities = 99/197 (50%), Positives = 138/197 (70%), Gaps = 6/197 (3%)
 Frame = +1

Query  25   VSHPVVSQYSQDQ--LNAVWDQLKTQVSPFVYVLCIEGSLFRVLDAGAKEFIARQFMDHV  198
            ++ P  SQ  Q    L  +WD LK Q++PF+ +LCI G+++R+LD   ++++AR+FM+HV
Sbjct  3    LAQPTSSQDVQTAVYLAGIWDGLKEQINPFLQILCINGNIYRMLDDYGRQYVARKFMEHV  62

Query  199  TEKVMYCRDGTEGDRYFLGAPRFFLAGSGMIVHALHTKEAVWVKRPDDGLQHILVVPP-A  375
             E VM+CRDG   DR++LGAPR+F+A  G +VH+    E  W    DDG   +      +
Sbjct  63   KEAVMFCRDGNGEDRHYLGAPRYFIANGG-VVHSPDGLEPFWTM--DDGSIRLETTAVCS  119

Query  376  LPNKCAKIPRPPNAYILYRRDRHRYIKLSKPGIHNNEISQILGRAWNVESTAIRSKYQKM  555
            +P K  KIPRPPNAYILYR+DRH  +K + PGI NNEISQILGRAWN ES  +R +Y++M
Sbjct  120  IPAKPTKIPRPPNAYILYRKDRHNLVKAANPGITNNEISQILGRAWNQESREVRQRYKEM  179

Query  556  ANDIKEALIKKHPDYKY  606
            + +IK AL++KHPDY+Y
Sbjct  180  SEEIKLALLEKHPDYQY  196
```

MAT1-1-3

MTTRADIPRAALHLPSDVPVTFVFSATQGDVHLFLPESFDVRVVYRVSLTLSRRVNQQVTVFHDRHRQKYRLCPFSYHVLMGTASFGHFC
FAVGTNSLVNYDLRTTNPKFEDESDHKRLEAGQAPTTTVAEPEPHVSRPKNRWMLYRQAKSQEVARQNPGIIDTAASNIIAKMWKDETPE
VKAYWQALAEDEARKHNEQQPNYKFTPSKKTT

Best three blastx hits

>ref|XP_389066.1| hypothetical protein FG08890.1 [Gibberella zeae PH-1]

```
Length=181

 GENE ID: 2790116 FG08890.1 | similar to mating type protein MAT1-1-3
[Gibberella zeae PH-1] (10 or fewer PubMed links)

 Score =  158 bits (400),  Expect = 1e-46
 Identities = 78/191 (41%), Positives = 111/191 (58%), Gaps = 21/191 (11%)
 Frame = +1

Query  46   SDVPVTFVFSATQGDVHLFLPESFDVRVVYRVSLTLSRRVNQQVTVFHDRHRQKYRLCPF  225
            ++VP+T ++S +Q D+H+F+PE+  + +V RV+  LSRRV Q V VFHD  R+KYRLCP
Sbjct  5    AEVPITIIYSRSQADIHVFIPETASMTMVNRVADNLSRRVQQPVKVFHDEARKKYRLCPI  64

Query  226  SYHVLMGTASFGHFCFAVGTNSLVNYDLRTTNPKFEDESDHKRLEAGQAPTTTVAEPEPH  405
               +   T++FG +CFA                   D+S    + A      T+ E
Sbjct  65   PKDIFANTSTFGRYCFA------------------RDQSTPATVSASDP---TIGEGGKR  103

Query  406  VSRPKNRWMLYRQAKSQEVARQNPGIIDTAASNIIAKMWKDETPEVKAYWQALAEDEARK  585
            + RP+N WMLYRQAKSQ++  Q+ G+     S II+ MW  ETPE +AYW+ LAEDE  +
Sbjct  104  IPRPRNSWMLYRQAKSQQIIPQHEGLTAGELSTIISNMWSSETPETQAYWRKLAEDEDAE  163

Query  586  HNEQQPNYKFT  618
            H    P YK++
Sbjct  164  HKRLYPGYKYS  174


>gb|AAG42812.1|  mating type protein MAT1-1-3 [Gibberella zeae]
Length=181

 Score =  157 bits (396),  Expect = 6e-46
 Identities = 78/191 (41%), Positives = 110/191 (58%), Gaps = 21/191 (11%)
 Frame = +1

Query  46   SDVPVTFVFSATQGDVHLFLPESFDVRVVYRVSLTLSRRVNQQVTVFHDRHRQKYRLCPF  225
            ++VP+T + S +Q D+H+F+PE+  + +V RV+  LSRRV Q V VFHD  R+KYRLCP
Sbjct  5    AEVPITIIHSRSQADIHVFIPETASMTMVNRVADNLSRRVQQPVKVFHDEARKKYRLCPI  64

Query  226  SYHVLMGTASFGHFCFAVGTNSLVNYDLRTTNPKFEDESDHKRLEAGQAPTTTVAEPEPH  405
               +   T++FG +CFA                   D+S    + A      T+ E
Sbjct  65   PKDIFANTSTFGRYCFA------------------RDQSTPATVSASDP---TIGEGGKR  103

Query  406  VSRPKNRWMLYRQAKSQEVARQNPGIIDTAASNIIAKMWKDETPEVKAYWQALAEDEARK  585
            + RP+N WMLYRQAKSQ++  Q+ G+     S II+ MW  ETPE +AYW+ LAEDE  +
Sbjct  104  IPRPRNSWMLYRQAKSQQIIPQHEGLTAGELSTIISNMWSSETPETQAYWRKLAEDEDAE  163

Query  586  HNEQQPNYKFT  618
            H    P YK++
Sbjct  164  HKRLYPGYKYS  174


>gb|EGU79453.1|  hypothetical protein FOXB_10038 [Fusarium oxysporum Fo5176]
Length=204

 Score =  157 bits (396),  Expect = 1e-45
 Identities = 78/195 (40%), Positives = 114/195 (58%), Gaps = 21/195 (11%)
 Frame = +1

Query  46   SDVPVTFVFSATQGDVHLFLPESFDVRVVYRVSLTLSRRVNQQVTVFHDRHRQKYRLCPF  225
            ++VP+T V+S +Q D+H+FLPE+  + ++  V+   SRRV Q V VFHD+ R KYRLCP
Sbjct  22   AEVPITMVYSQSQADIHIFLPENASLMLINHVADKFSRRVQQPVRVFHDKARSKYRLCPI  81

Query  226  SYHVLMGTASFGHFCFAVGTNSLVNYDLRTTNPKFEDESDHKRLEAGQAPTTTVAEPEPH  405
               V   T+++G FCF            ++T  K  DE               TV E
Sbjct  82   PEDVSPDTSTYGRFCFTRD---------QSTPVKVSDEDP------------TVGEGGCR  120

Query  406  VSRPKNRWMLYRQAKSQEVARQNPGIIDTAASNIIAKMWKDETPEVKAYWQALAEDEARK  585
            + RP+N W+LYRQ+KSQE+ ++   GI  +  S +I +MW +ETPE++AYW  +AE E
Sbjct  121  IPRPRNCWLLYRQSKSQEITKKVEGITASELSRVIGRMWGEETPEIQAYWYDMAEKEEFN  180

Query  586  HNEQQPNYKFTPSKK  630
            H +  P YK+TP+K+
Sbjct  181  HKQHYPGYKYTPAKE  195
```

MAT1-1-2

```
MDNLLFYRPLLEEKALATSSSKHLESLRMDIMKKLLHALPVDVKPPTVAAVVTSSIEIIKTLFQEASGSNAILQKILDHSKESGVDPNLV
VRGAIVLWYTSASPILSRDPNLGLPPKAISGGGLALPWVQRLAESQFFIGNLGYLSMLMTAETWEKPSHPKLQVATLMANAIISFLFTTF
VITPHLKTHTHWRQHLSSSNSRGYKEMTVFLKTTWHVSREGWEKFDHPPGAQFSALNTEVKMSRDGRKLLTRVGCPDWHVAPYWHPFRAV
PGSPWNKFIRNRRQKIFQIFPTLNNRVRYMVPGSAIALTGSCKAYYGVLRSQFDSTNTERARPSFLERKRQFWRLAEGTGLQYPTVRPTE
QDATSIQDYGQAEHMYNVPLIKKAAADLSGNLTPPFLTTTARALRFGVEPESVDEDFYAMVFEH
```

Best three blastx hits

```
>gb|ACR78245.1|  MAT1-1-2 [Hypocrea jecorina]
Length=434

 Score =  279 bits (714),  Expect = 2e-87
 Identities = 156/434 (36%), Positives = 253/434 (58%), Gaps = 19/434 (4%)
 Frame = +1

Query  1     MDNLLFYRPL---LEEKALATSSSKHLESLRMDIMKKLLHAL-PVDVK-PPTVAAVVTSS  165
             M+N+  + PL   +E  A  T  S    ++R++ MK  L     +D++ PP++  +V  S
Sbjct  1     MENICSFEPLWKNIESDAPQTDIS----NIRLESMKIFLQENGSLDIQQPPSMDYIVAES  56

Query  166   IEIIKTLFQEASGSNAILQKILDHSKESGVDPNLVVRGAIVLWYTSASPILSRDPNLGLP  345
             ++++K L     G N I+ ++    + ++  DP + V GAI LWYTS+  IL RD + G+
Sbjct  57    LKVMKHLLSFWEGFNPIIARLRTVAVQTEADPIIAVEGAIALWYTSSVSILERDIHQGVS  116

Query  346   PK----AISGGGLALPWVQRLAESQFFIGNLGYLSMLMTAETWEKPSHPKLQVATLMANA  513
              +        G +   W +  A  + ++GNLG ++ML+T E W  P  PKLQVATL++
Sbjct  117   SEEEIATFEDGTVVATWNRDFAAEKHYVGNLGLMAMLLTTEVWLPPKDPKLQVATLVSCG  176

Query  514   IISFLFTTFVITPHLKTHTHWRQHLSSSNSRGYKEMTVFLKTTWHVSREGWEKFDHPPGA  693
               + LF  ++I+  + ++  WR  LS+    G   + +FL++ W ++++  ++   PPG
Sbjct  177   ATTMLFAAYMISSRMPSYNAWRPALSACP--GTDGIKLFLQSAWRLAQKPVDEAGTPPGI  234

Query  694   QFSALNTEVKMSRDGRKLLTRVGCPDWHVAPYWHPFRAVPGSPWNKFIRNRRQKIFQIFP  873
              F A   E+++ R+G+ LL++VG  DW  AP+WHP R VPGS WNK+I N  + IF   P
Sbjct  235   DFGAHVDEIRLCRNGKGLLSKVGRADWGKAPFWHPCRKVPGSHWNKWINNTCRPIFPKSP  294

Query  874   ---TLNNRVRYMVPGSAIALTGSCKAYYGVLRSQFDSTNTERARPSFLERKRQFWRLAEG  1044
                + + R+ + +P SA  LT +   YY  LR++FD TN  R+R +  E ++QF  LA
Sbjct  295   GDGSRDIRIIFRLPSSAAILTSAFGDYYSTLRTRFDQTNRPRSRLALEECEKQFRDLAAQ  354

Query  1045  TGLQYPTVRPTEQDATSIQDYGQAEHMYNVPLIKKAAADLSGNLTPPFLTTTARALRFGV  1224
             TG +YP + P  + A S+    Q E++YN+P++KK  +D +GNLT PFLTT  RALRFG
Sbjct  355   TGCEYPALIPALETAASVTHLDQ-EYLYNLPMVKKPISDPTGNLTLPFLTTAIRALRFGE  413

Query  1225  EPESVDEDFYAMVF  1266
             +P+ ++++ + M F
Sbjct  414   DPDEIEQNLFMMTF  427
```

```
>dbj|BAD72611.1|  MAT1-1-2 [Epichloë typhina]
Length=337

 Score =  226 bits (577),  Expect = 3e-68
 Identities = 125/331 (38%), Positives = 186/331 (56%), Gaps = 14/331 (4%)
 Frame = +1

Query  1    MDNLLFYRPLLEEKALATSSSKHLESLRMDIMKKLLHALPVDVKPPT----VAAVVTSSI  168
            M+N+  + PL +   L     + +E LR+  ++  LH    +   P        VV
Sbjct  1    MENIHHFSPLWQRLELVFKPEQAIEELRIKSLQAFLHNHRSEAAKPLNLNRTYLVVLECT  60

Query  169  EIIKTLFQEASGSNAILQKILDHSKESGVDPNLVVRGAIVLWYTSASPILSRDPNLGLPP  348
             II+ L +  +  N ILQ++  HS   G DP  +V+ A+VLWY  +S I SRDP+ GLPP
Sbjct  61   CIIQQLLENNNDENEILQRL--HSSGQG-DPLAIVKKALVLWYAGSSSIFSRDPHQGLPP  117

Query  349  KAI---SGGGLALPWVQRLAESQFFIGNLGYLSMLMTAETWEKPSHPKLQVATLMANAII  519
             +      G   L W +R    Q+ IGNLG ++MLMT+ETW  P H KL+ A+L++ A
Sbjct  118  DSELEDDSGAPILTWSRRFFHEQYAIGNLGLMAMLMTSETWLSPKHEKLKAASLISTASA  177

Query  520  SFLFTTFVITPHLKTHTHWRQHLSSSNSRGYKEMTVFLKTTWHVSREGWEKFDHPPGAQF  699
            + LF +++I   +  H  W   +SS+ S   + M +F++++W V+RE  E FD PPG +F
Sbjct  178  TILFASYLICTEVM-HRPWAHDVSSAQSS--EAMALFIRSSWQVARENSEIFDSPPGREF  234

Query  700  SALNTEVKMSRDGRKLLTRVGCPDWHVAPYWHPFRAVPGSPWNKFIRNRRQKIFQIFPTL  879
             A   E+K+S DG++ LT++G   WH APYWHP R VPGS WNK++RN    +F   T
```

```
Sbjct  235  GATLKEIKLSDDGKRFLTKIGHESWHEAPYWHPCRRVPGSSWNKYLRNFASPLFPTHHT-  293

Query  880  NNRVRYMVPGSAIALTGSCKAYYGVLRSQFD  972
             N++   +P +  +L    +AYY  LR +FD
Sbjct  294  TNKIHISLPTTMFSLVQPWEAYYAELRIRFD  324
```

```
>gb|AEI72617.1|  mating type A-1-2 protein [Epichloë festucae]
Length=331

 Score =  225 bits (574),  Expect = 7e-68
 Identities = 124/336 (37%), Positives = 188/336 (56%), Gaps = 11/336 (3%)
 Frame = +1

Query  1    MDNLLFYRPLLEEKALATSSSKHLESLRMDIMKKLLHALPVDV-KPPTVAAVVTSSIEII  177
            M+N+  + PL E    L     + +E LR+  ++  LH     +  KP  +  VV     +I
Sbjct  1    MENIHHFSPLWERLELIFKPEQAIEELRIKSLQAFLHNHRSEAAKPLNLNQVVLECTCVI  60

Query  178  KTLFQEASGSNAILQKILDHSKESGVDPNLVVRGAIVLWYTSASPILSRDPNLGLPPKAI  357
            + L    +  N ILQ++ +  +    DP  +V+ A+VLWY  + PI SRDP+ GLPP +
Sbjct  61   QQLLDNNNDENEILQRLRNSGQG---DPLAIVKKALVLWYAGSCPIFSRDPHQGLPPDSE  117

Query  358  ---SGGGLALPWVQRLAESQFFIGNLGYLSMLMTAETWEKPSHPKLQVATLMANAIISFL  528
                 G   L W +R    Q  IGNLG ++MLMT+ETW  P H KL+ A+L++ A  + L
Sbjct  118  LEDDSGAPILTWSRRFFHEQHAIGNLGLMAMLMTSETWLSPKHEKLKAASLISTASATIL  177

Query  529  FTTFVITPHLKTHTHWRQHLSSSNSRGYKEMTVFLKTTWHVSREGWEKFDHPPGAQFSAL  708
            F +++I    +  H  W  ++SS+ S    + M +F++++W V+RE  E FD PPG +F A
Sbjct  178  FASYLICTEVM-HRPWAHNVSSAQSS--EAMALFIRSSWQVARENSEIFDSPPGREFGAT  234

Query  709  NTEVKMSRDGRKLLTRVGCPDWHVAPYWHPFRAVPGSPWNKFIRNRRQKIFQIFPTLNNR  888
              EVK+S DG++ LT++G   WH APYWHP R VPGS WNK++RN    +F    T N+
Sbjct  235  LKEVKLSDDGKRFLTKIGHESWHEAPYWHPCRRVPGSSWNKYLRNFACPLFPTHLTTND-  293

Query  889  VRYMVPGSAIALTGSCKAYYGVLRSQFDSTNTERAR  996
            +   +P +  +L    +AYY  LR +FD     + +
Sbjct  294  IHISLPTTMFSLVQPWEAYYAELRIRFDQVRHSKEK  329
```

MAT1-1-1

```
MVQSRSEVMRALSDLSTAEILSLLTDETMLQVAAKYFATHAATMDVNAPLSPTEADAILSVQQMTCSVQTPFVQSGGAPAPRAKRPLNAF
MAFRTFYMRIFPDIQQKSASGFLTTLWNKDPFRAKWALIAKVYSFVRDEVGKGHIALARFLDVACPAMNMIRPSGYLAALGWSVSYDDDG
SLILDQTLPTNLGRFQHEAVPRTEMELLHALLQTGYLSERGLALLERLSANNNSGFVNASGFVAAANTSGFVTTTNGSSSSAAPPQAPPE
TPPPPPLQTPEKLSLVDTIRADPIQAAKELFRAREDEYLDPKHVATYEVYDLSYAPYAAMLAPQPEPLQYYDFAGASLGLRGSGSGEEAP
PTFNPLGSVDEFDAIDIDSPYDVDAMMGNTESEGERTADQEDTNPYDPQSEYHPLA
```

Best three blastx hits

```
>gb|EFZ01122.1|  mating-type A-1 protein [Metarhizium anisopliae ARSEF 23]
Length=376

 Score =  245 bits (626),  Expect = 4e-75
 Identities = 165/413 (40%), Positives = 223/413 (54%), Gaps = 69/413 (17%)
 Frame = +1

Query  10    SRSEVMRALSDLSTAEILSLLTDETMLQVAAKYFATHAATMDVNAPLSPTEADAILSVQQ  189
             +R+E+M+ LS L T E+L +L DET+   +AA+YF    A+M  N  + P+    I +  +
Sbjct  3     TRAELMQRLSMLPTQELLHILKDETIFDIAAQYFD---ASMQSN--ILPSTTPVIHNTHK  57

Query  190   MTCSVQTPFVQSGGAPAPRAKRPLNAFMAFRT----------------FYMRIFPDIQQK  321
                +V            +AKRPLNAFMAFR+                  Y+++FPD+QQK
Sbjct  58    TKNTV-----------CDKAKRPLNAFMAFRSKFITKPISDPFLPNSGHYLKLFPDVQQK  106

Query  322   SASGFLTTLWNKDPFRAKWALIAKVYSFVRDEVGKGHIALARFLDVACPAMNMIRPSGYL  501
             SASGFLTTLWNKDPFR KWALIAKVYSFVRDE+GK  I LA FL  +CP M +I P  YL
Sbjct  107   SASGFLTTLWNKDPFRNKWALIAKVYSFVRDEIGKDKITLASFLSFSCPVMGIIEPQSYL  166

Query  502   AALGWSVSYDDDGS-LILDQTLPT-NLGRFQHEAVPRTEMELLHALLQTGYLSERGLALL  675
               LGWSV  D +   LI D+TL      R Q + VP TE+ELL AL+  GYL E+G+ L+
Sbjct  167   TILGWSVGGDSNSQRLIQDETLAALGQSRLQSDGVPSTELELLTALVNVGYLPEQGIDLM  226

Query  676   ERLSANNNSGFVNASGFVAAANTSGFVTTTNGssssaappqappetppppplqtpeklsL  855
              +L ANN+S    A+    +   S                             TPEKL
Sbjct  227   GKLGANNSSIMATATQPRLSLPVS----------------------------YTPEKLQF  258
```

```
Query  856    VDTIRADPIQAAKELFRAREDEY-LDPKHVATYEVYDLSYAPYAAMLAPQPEPLQYYDFa  1032
              + TIR+DP+QA KEL     D+Y +    V  + V DL    +  M    P+P  YY++
Sbjct  259    MGTIRSDPVQATKELLGDVYDDYTIQMLGVKAHNVEDLDSINHLPMQVEMPDPRYYYNY-  317

Query  1033   gaslglrgsgsgeeaPPTFNPLGSVDEFDAIDIDSPYDVDAMMGNTESEGERT  1191
                      +       PT +   ++ E ++ DIDSP+DVD ++G T+SEG+R+
Sbjct  318    ----STSHAQLSLAGAPTMS-FENIPEHESFDIDSPWDVDTILGQTQSEGDRS  365


>gb|ACN59937.1|  mating-type A-1 protein [Epichloë festucae]
Length=369

 Score =  243 bits (620),  Expect = 2e-74
 Identities = 157/414 (38%), Positives = 230/414 (56%), Gaps = 55/414 (13%)
 Frame = +1

Query  10     SRSEVMRALSDLSTAEILSLLTDETMLQVAAKYF-ATHAATMDVNAPLSPTEADAILSVQ  186
              +R+E+M+ LS L T E+L  L DETML +A++YF  T   TM      + T  ++ L  +
Sbjct  3      TRAELMQRLSMLPTQELLQYLKDETMLDIASRYFDTTFQPTM--TPTFNQTTMNSHLKSK  60

Query  187    QMTCSVQTPFVQSGGAPAPRAKRPLNAFMAFRTFYMRIFPDIQQKSASGFLTTLWNKDPF  366
                 C               +AKRPLNAFMAFR++Y++++PD QQK+ASGFLTTLWN+DPF
Sbjct  61     VQICE--------------KAKRPLNAFMAFRSYYLKLWPDQQQKTASGFLTTLWNRDPF  106

Query  367    RAKWALIAKVYSFVRDEVGKGHIALARFLDVACPAMNMIRPSGYLAALGWSVSYDDDGSL  546
              R KWALIAKVYSFVRDEVGK  ++LA FL  ACP M ++ P  YL+ LGWSV   +
Sbjct  107    RNKWALIAKVYSFVRDEVGKDKVSLAYFLGFACPVMGIVEPQAYLSILGWSVEGVEPLQR  166

Query  547    ILDQTLPTNLGR--FQHEAVPRTEMELLHALLQTGYLSERGLALLERLSANNNSGFVNAS  720
              ++       LG+  F     P TE+ELL  L+  GYL E+G+ L+++L  N+N+
Sbjct  167    LVQDEAVAALGQSHFSGCEYPSTELELLSGLVNVGYLPEQGIDLIDKLGTNSNT------  220

Query  721    GFVAAANTSGFVTTTNGssssaappqappetppppplqtpeklsLVDTIRADPIQAAKEL  900
                         + T+N                  P   TPEKL  ++TIR+DP+QA KEL
Sbjct  221    ----------IMATSN-------------PRLTLPVSYTPEKLQFMNTIRSDPVQATKEL  257

Query  901    FRAREDEY-LDPKHVATYEVYDLSYAPYAAMLAPQPEPLQYYDFagaslglrgsgsgeea  1077
              F    D+Y +    V +++V +L    +  M    P+P  YY+++ +   L  +G+
Sbjct  258    FGDAYDDYTIQMLGVKSHDVENLDSINHLPMQMDLPDPRYYYNYSTSHSQLAVNGAPILR  317

Query  1078   PPTFNPLGSVDEFDAIDIDSPYDVDAMMGNTESEGERTADQEDTNPYDPQSEYH  1239
                      ++ + +  DID+P+DVDAM+G ++SEG+RT     +  +D   ++H
Sbjct  318    ------FENIPDHETFDIDNPWDVDAMLGQSQSEGQRTISHPRSPRHDSHEDFH  365
```

Supplementary material S5

DNA and protein sequences of the DNA lyase fragment for types A and B of *Ilyonectria liriodendri* (isolates Cy33 and Cy109, respectively) including *DNA lyase* (yellow) and a putative ORF (pink)

Type A - Cy33

```
1    GGATTGGTTCACTTCTGCCAATATTCAAGAAGGCTTGATGGGATCTGATCACTGCCCAGTATTCGCAACGCTAGGAGACACAAAGGACAGGGAGCATACT
101  CTCTTGGAGCTCTTGAACCCGCCAGGCGTGTTTCAAGGCGATCAGCGCCTCCGTGATTGGAGCCCCAAAGACTTGCTTCCGCTGTCTGCAAGGTTGATAC
201  CAGAATTTGATCGTCGACAGAGCATTCGCGACATGTTCACAAAGCAAGTAACCCCGCATGCGAGCACGGATACGGCCGAAAAGCTGTTAGGCCCTCGACC
301  GATTGGGAAACGGCCTGCAGACACGGCTGAGAAGGCGCCGAAAGGGGCTTTGAAGAGGAATAAGCCGTCCTCAAATTCCGTCGATGCCAAGAACAAAGCA
401  GCTGCTGGACAAAGAACTCTGCAAGGCTTCTTCAAGCCGAAAGCCTCAGCGCCACAGCCCGAGAACCCGCACGTGGAGGCCACGGGAAGAACAACGCCTC
501  CGCCACCAAGGAAAACCTCGATCGGCTCTTCCAACGGTCTGCTTAGGCAGCAACAAACTCCGCCTTTGACATCAGAGACTCTTTCAGCAGAAGCTTTYGC
601  CAACACATCTGAAAGAGTGTTCGACCCAATCGAAGCTAAGGAATCTTGGTCAAAGCTGCTGAGCAAGCGAGTTGCACCTCGGTGCGAGCATCAGGAACCA
701  TGCATCAGCCTGACTACTAAGAAGCCTGGCGTCAATTGCGGTAAGTCTGCGGCTCATACCGCCACGATGCTAGATTTTACGCGTATGATGCAGGAGCTAA
801  CTTCGAGCGCAGGGCGGTCGTTCTACATCTGCCCGCGGCCTCTTGGGCCTTCTGGCGAGAAGGAGAAGGGGACGGAATGGCGGTGTGGGACTTTCATATG
901  GAGTAGTGACTGGAGTAGTTCATCATTAGGCTCGTAAATGTTAGGTGCTCGTACCCCGTCTCCCCCGTCTCTCGATCCCCCCCACCGCTCCTCTAACACC
1001 GCCTCGCTTTGCCGGCCGACGACCAAGGCCATCTCAGCGCGATAGAGCTTGTGGTCTTCAATGCGACCGACGAAGAGCCCTCGGAGCACGTGGATTCTAA
1101 CAATCCTGCGAGGAAGTGGGCTTTCGAAGCCCAGCGGCAACATCACAAGTTATCTCCCCCTTCTCCCTGCTCCATCATGGCTTCTCCTGGCAGTGCAATG
1201 GCGGGGTCTCCGGATGACATATAAATGTAAAGCTGAATTTTTCTTTTTAAAAAAAGCCTGCTTATTTTAAAAACACCCTCTCAATCCATTGTCGATAATC
1301 CGTTGCAATCCAATCCCCAAGTTTCCGCACTGCAGCAACAAGCGTATACAGCCCCAGCAAGGTCCTAGTATCCCCCAGTGCAAGCTCCYCCGCGATCTCA
1401 ATCCTATCCCGCTGCCAGYATGCAAATGACAGCCTCCAGTCATGTCCGACGATATACAAGAGTGGCAGCGTTACGATACCATGCTGGGCAACCCCGTCAG
1501 GCACTAGCTTGATTATACGTTGATACCATGCGGAGGTCCAGACGGAAAGTTGTAACTGCCCTGCTTGCTGCATTGTCTATACCTTGGTCTCAATATTGCA
1601 CACCGTTTCAGTTAACCACACCGACTTTAGA
```

DNA lyase

```
DWFTSANIQEGLMGSDHCPVFATLGDTKDREHTLLELLNPPGVFQGDQRLRDWSPKDLLPLSARLIPEFDRRQSIRDMFTKQVTPHASTD
TAEKLLGPRPIGKRPADTAEKAPKGALKRNKPSSNSVDAKNKAAAGQRTLQGFFKPKASAPQPENPHVEATGRTTPPPPRKTSIGSSNGL
LRQQQTPPLTSETLSAEAFANTSERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISLTTKKPGVNCGRSFYICPRPLGPSGEKEKGTEWR
CGTFIWSSDWSSSSLGS
```

Best three blastx hits

```
>gb|EGU79454.1|  hypothetical protein FOXB_10039 [Fusarium oxysporum Fo5176]
Length=593

 Score =  302 bits (774),  Expect = 1e-96
 Identities = 165/318 (52%), Positives = 190/318 (60%), Gaps = 44/318 (14%)
 Frame = +1

Query  4     WFTSANIQEGLMGSDHCPVFATLGD---TKDREHTLLELLNPPGVFQGDQRLRDWSPKDL  174
             WF  +NIQEGLMGSDHCPVFA L D     D+E  LLE++NPPG+F GD+RLRDWSPKD
Sbjct  270   WFNYSNIQEGLMGSDHCPVFANLSDKVTVGDKECALLEMMNPPGMFNGDERLRDWSPKDH  329

Query  175   LPLSARLIPEFDRRQSIRDMFTKQVTPH--------------------------------  258
             LPLS++LIPEFDRRQSIRDMFTK+   P
Sbjct  330   LPLSSKLIPEFDRRQSIRDMFTKKAAPPREPTRTDTPAEPLNNGNSSASGSPTNASSTPR  389

Query  259   -ASTDTAEKLLGPRPIGKRPADTAEKAPKGALKRNKPSSNSVDAKNKAAAGQRTLQGFFK  435
                T  + KL    +P  KRP    A+    +    K+ K   + + D K+K A GQRTLQGFFK
Sbjct  390   LGETTNSTKLSASQPSSKRPGTAADTTSR-PFKKTKSFTGANDTKSKVAQGQRTLQGFFK  448

Query  436   PKASAPQPENPHVEAtgrttppppprktSIGSS-NGLLRQQQTPPLTSETLSAEAF-----  597
             PKA A Q      + A    T      +      G +      R    TP   T     S+
Sbjct  449   PKAPAAQDGKAELVAANSTPSTTKKPAGSGKAPASAQRLSNTPQATPTEKSSPTVPLRGK  508

Query  598   -ANTSERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISLTTKKPGVNCGRSFYICPRPLG  774
               + S+RVFDPIEAKESWSKLL KRVAPRCEH EPCIS TTKKPGVNCGR FYICPRPLG
Sbjct  509   DPDPSDRVFDPIEAKESWSKLLGKRVAPRCEHDEPCISFTTKKPGVNCGRMFYICPRPLG  568

Query  775   PSGEKEKGTEWRCGTFIW  828
             PSGEKE+ +EWRC TFIW
Sbjct  569   PSGEKERNSEWRCSTFIW  586


>gb|EFY88584.1|  DNA lyase [Metarhizium acridum CQMa 102]
Length=634

 Score =  280 bits (715),  Expect = 2e-87
 Identities = 163/328 (50%), Positives = 201/328 (61%), Gaps = 54/328 (16%)
 Frame = +1
```

```
Query  1    DWFTSANIQEGLMGSDHCPVFATLGDT---KDREHTLLELLNPPGVFQGDQRLRDWSPKD  171
            DWFTSANIQEGLMGSDHCPVFAT+  T     R+ ++ +++NP G+F+   R+RD + KD
Sbjct  297  DWFTSANIQEGLMGSDHCPVFATIAGTVASNGRQVSINDVMNPAGMFENGTRVRDLAQKD  356

Query  172  LLPLSARLIPEFDRRQSIRDMFTKQVT-------PHAS----------------------  264
            +LPLSA+LIPEFDRRQSIRDMF K+VT       PH S
Sbjct  357  ILPLSAKLIPEFDRRQSIRDMFFKKVTSAERRTTPHNSESSSFKAEDTSQASDNDASSQI  416

Query  265  --------TDTAEKLLGPRPIG---KRPADTAEKAPKGALKRNKPSSNSVDAKNKAAAGQ  411
                    T+T        P     KR  D  +  P+  LKR+K +++ + +K K +AGQ
Sbjct  417  DNALQTVNTNTPTNKPAALPYSQPRKRSPDPIDPVPR-QLKRSKSAADPIGSKQKISAGQ  475

Query  412  RTLQGFFKPKASA-PQPE---NPHVEAtgrttpppprktSI-GSSNGLLRQQQTPPLTSE  576
             TL+GFFKP +S  P P    +P ++     P      S GSS  L   +  PP  SE
Sbjct  476  TTLKGFFKPVSSVLPSPAVLADPEAQSNATDLPATKTSPSTKGSSENLTSNEFMPPF-SE  534

Query  577  TLSAEAFANTS----ERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISLTTKKPGVNCGR  744
              + ++  +TS    +RVFDPI+AKESWSKLL KRV PRCEH EPCISL TKKPGVN GR
Sbjct  535  QPAHDSLVDTSPKSPDRVFDPIQAKESWSKLLGKRVVPRCEHDEPCISLVTKKPGVNRGR  594

Query  745  SFYICPRPLGPSGEKEKGTEWRCGTFIW  828
            SFYICPRPLGPSGEKE+G+EWRCGTFIW
Sbjct  595  SFYICPRPLGPSGEKERGSEWRCGTFIW  622
```

```
>gb|AEI72618.1|  DNA lyase [Epichloë festucae]
Length=642

 Score =  277 bits (709),  Expect = 2e-86
 Identities = 164/336 (49%), Positives = 200/336 (60%), Gaps = 61/336 (18%)
 Frame = +1

Query  1    DWFTSANIQEGLMGSDHCPVFATLGDT---KDREHTLLELLNPPGVFQGDQRLRDWSPKD  171
            DWFT ANIQEGLMGSDHCPVFAT+ D    K  +  LL+++NPPGVF   +R+R+ + +D
Sbjct  298  DWFTFANIQEGLMGSDHCPVFATIADRVSFKGNDTALLDVMNPPGVFAQGKRVRELAQRD  357

Query  172  LLPLSARLIPEFDRRQSIRDMFTKQVTP--------------------------------  255
            LLPLSA+LIPEFDRRQSIRDMF K+ +
Sbjct  358  LLPLSAKLIPEFDRRQSIRDMFFKKSSTIGVAADGAPSPGQPAKRASSITETGLRPVSNN  417

Query  256  --HASTD---TAEKLLGPRPIG------------KRPADTAEKAPKGALKRNKPSSNSVD  384
              + +TD   T+E LLG                 KRPA+  + AP+  LKR+K  +++
Sbjct  418  NSNNNTDRNGTSEDLLGGASANSRAANAQQAESQKRPAEPIDPAPR-QLKRSKAGADTNS  476

Query  385  AKNKAAAGQRTLQGFFKPKASAPQPENPHV--EAtgrttpppprktSIGSSNGLLRQQQT  558
            +K K A GQ+TL+GFFKP  SAP    P    E+    T P     S   +  +  +
Sbjct  477  SKLKTAPGQKTLKGFFKPVDSAPVHPTPSSLQESRTNNTSPRTATDSTSPTKSVSGEPVG  536

Query  559  PPLTS------ETLSAEAFANTSERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISLTTK  720
              LTS      ++  +E      +RVFDPIEAKESWSKLL KRV PRCEH+EPCISL TK
Sbjct  537  SELTSPSPKYSQSHHSEPIPRLPDRVFDPIEAKESWSKLLGKRVVPRCEHEEPCISLVTK  596

Query  721  KPGVNCGRSFYICPRPLGPSGEKEKGTEWRCGTFIW  828
            KPGVN GRSFYICPRPLGPSGEKEKG+EWRCGTFIW
Sbjct  597  KPGVNRGRSFYICPRPLGPSGEKEKGSEWRCGTFIW  632
```

Putative ORF

CNIETKV.TMQQAGQLQLSVWTSAWYQRIIKLVPDGVAQHGIVTLPLLYIVGHDWRLSFAXWQRDRIEIAXELALGDTRT
LLGLYTLVAAVRKLGDWIATDYRQWIERVFLK

Best two blastx hits

```
>gb|EGU71805.1|  hypothetical protein FOXB_17686 [Fusarium oxysporum Fo5176]
Length=500

 Score =  146 bits (369),  Expect = 2e-35
 Identities = 76/112 (68%), Positives = 94/112 (84%), Gaps = 0/112 (0%)
 Frame = -2

Query  1600  CNIETKV*TMQQAGQLQLSVWTSAWYQRIIKLVPDGVAQHGIVTLPLLYIVGHDWRLSFA  1421
             CNIETK  ++Q  G+LQLSVWT+AWYQR+  LVP+ +AQHGI+TLPLLYI+GHDW+LSFA
Sbjct  388   CNIETKTSSVQHDGELQLSVWTAAWYQRMNMLVPERIAQHGIITLPLLYIIGHDWKLSFA  447
```

```
Query  1420   XWQRDRIEIAXELALGDTRTLLGLYTLVAAVRKLGDWIATDYRQWIERVFLK  1265
                W+ +RIEI   LALGDT TLLG YT+VA +RK+GDWI T YR WIE++FL+
Sbjct  448    CWRGNRIEIMGPLALGDTTTLLGTYTIVAVLRKIGDWITTVYRDWIEKMFLQ  499
```

```
>ref|XP_003041238.1|  hypothetical protein NECHADRAFT_55546 [Nectria haematococca mpVI
77-13-4]
 gb|EEU35525.1|  hypothetical protein NECHADRAFT_55546 [Nectria haematococca mpVI
77-13-4]
Length=143

 GENE ID: 9675963 NECHADRAFT_55546 | hypothetical protein
[Nectria haematococca mpVI 77-13-4]

 Score =  107 bits (268),  Expect = 7e-25
 Identities = 59/112 (53%), Positives = 79/112 (71%), Gaps = 3/112 (3%)
 Frame = -2

Query  1600   CNIETKV*TMQQAGQLQLSVWTSAWYQRIIKLVPDGVAQHGIVTLPLLYIVGHDWRLSFA  1421
              CN+ETK+ T    G+LQL VWT+AW+ R+     P G      VT+PLLY+V H WR+SFA
Sbjct  33    CNVETKLGTAITDGRLQLGVWTAAWFCRMETFRPKGCPW---VTIPLLYVVDHSWRISFA  89

Query  1420   XWQRDRIEIAXELALGDTRTLLGLYTLVAAVRKLGDWIATDYRQWIERVFLK  1265
                + D IEI  E+ +GDTR+L+G+Y L A +R+L  WI+T YR+WIERVFL+
Sbjct  90    CHRSDCIEILEEMDIGDTRSLVGIYQLTAVLRELATWISTTYREWIERVFLE  141
```

Type B - Cy109

```
1    GGCATCAAGGATTGGTTCACTTCTGCCAATATTCAAGAAGGCTTGATGGGATCTGATCACTGCCCAGTATTCGCAACGCTAGGAGACACAAAGGACAGGG
101  AGCATACTCTCTTGGAGCTCTTGAACCCGCCAGGCGTGTTTCAAGGCGATCAGCGCCTCCGTGATTGGAGCCCCAAAGACTTGCTTCCGCTGTCTGCAAG
201  GTTGATACCAGAATTTGATCGTCGACAGAGCATTCGCGACATGTTCACAAAGCAAGTAACCCCGCATGCGAGCACGGATACGGCCGAAAAGCTGTTAGGC
301  CCTCGACCGATTGGGAAACGGCCTGCAGACACGGCTGAGAAGGCGCCGAAAGGGGCTTTGAAGAGGAATAAGCCGTCCTCAAATTCCGTCGATGCCAAGA
401  ACAAAGCAGCTGCTGGACAAAGAACTCTGCAAGGCTTCTTCAAGCCGAAAGCCTCAGCGCCACAGCCCGAGAACCCGCACGTGGAGGCCACGGGAAGAAC
501  AACGCCTCCGCCACCAAGGAAAACCTCGATCGGCTCTTCCAACGGTCTGCTTAGGCAGCAACAAACTCCGCCTTTGACATCAGAGACTCTTTCAGCAGAA
601  GCTTTCGCCAACACATCTGAAAGAGTGTTCGACCCAATCGAAGCTAAGGAATCTTGGTCAAAGCTGCTGAGCAAGCGAGTTGCACCTCGGTGCGAGCATC
701  AGGAACCATGCATCAGCCTGACTACTAAGAAGCCTGGCGTCAATTGCGGTAAGTCTGCGGCTCATACCGCCACGATGCTAGATTTTACGCGTATGATGCA
801  GGAGCTAACTTCGAGCGCAGGGCGGTCGTTCTACATCTGCCCGCGGCCTCTTGGGCCTTCCGGCGAGAAGGAGAAGGGGACGGAATGGCgGTGTGGGACT
901  TTCATATGGAGTAGTGACTGGAGTAGTTCATCATTAGGCTCGTAAATGTTAGGTGCTCGTACCCCGTCTCCCCCGTCTCTCGATCCCCCCCACCGCTCCT
1001 CTAACACCGCCTCGCTTTGCCGGCCGACGACCAAGGCCATCTCAGCGCGATAGAGCTTGTGGTCTTCAATGCGACCGACGAAGAGCCCTCGGAGCACGTG
1101 GATTCTAACAATCCTGCGAGGAAGTGGGCTTTCGAAGCCCAGCGGCAACATCACAAGTTATCTCCCCCTTCTCCCTGCTCCATCATGGCTTCTCCTGGCA
1201 GTGCAATGGCGGGGTCTCCGGATGACATATAAATGTAAAGCTGAATTTTTCTTTTTAAAAAAAGCCTGCTTATTTtAAAAACACCCTCTCAATC
```

DNA lyase

```
GIKDWFTSANIQEGLMGSDHCPVFATLGDTKDREHTLLELLNPPGVFQGDQRLRDWSPKDLLPLSARLIPEFDRRQSIRDMFTKQVTPHA
STDTAEKLLGPRPIGKRPADTAEKAPKGALKRNKPSSNSVDAKNKAAAGQRTLQGFFKPKASAPQPENPHVEATGRTTPPPPRKTSIGSS
NGLLRQQQTPPLTSETLSAEAFANTSERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISLTTKKPGVNCGRSFYICPRPLGPSGEKEKGT
EWRCGTFIWSSDWSSSSLGS
```

Best three blastx hits

```
>gb|EGU79454.1|  hypothetical protein FOXB_10039 [Fusarium oxysporum Fo5176]
Length=593

 Score =  308 bits (789),  Expect = 8e-99
 Identities = 168/322 (52%), Positives = 193/322 (60%), Gaps = 44/322 (14%)
 Frame = +1

Query  1    GIKDWFTSANIQEGLMGSDHCPVFATLGD---TKDREHTLLELLNPPGVFQGDQRLRDWS  171
            GIK WF  +NIQEGLMGSDHCPVFA L D     D+E  LLE++NPPG+F GD+RLRDWS
Sbjct  266  GIKSWFNYSNIQEGLMGSDHCPVFANLSDKVTVGDKECALLEMMNPPGMFNGDERLRDWS  325

Query  172  PKDLLPLSARLIPEFDRRQSIRDMFTKQVTPH----------------------------  267
            PKD LPLS++LIPEFDRRQSIRDMFTK+   P
Sbjct  326  PKDHLPLSSKLIPEFDRRQSIRDMFTKKAAPPREPTRTDTPAEPLNNGNSSASGSPTNAS  385

Query  268  -----ASTDTAEKLLGPRPIGKRPADTAEKAPKGALKRNKPSSNSVDAKNKAAAGQRTLQ  432
                   T  + KL    +P  KRP    A+     +    K+ K   + + D K+K A GQRTLQ
Sbjct  386  STPRLGETTNSTKLSASQPSSKRPGTAADTTSR-PFKKTKSFTGANDTKSKVAQGQRTLQ  444

Query  433  GFFKPKASAPQPENPHVEAtgrttppppprktSIGSS-NGLLRQQQTPPLTSETLSAEAF-  606
            GFFKPKA A Q      + A    T       +      G +      R    TP    T      S+
Sbjct  445  GFFKPKAPAAQDGKAELVAANSTPSTTKKPAGSGKAPASAQRLSNTPQATPTEKSSPTVP  504

Query  607  -----ANTSERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISLTTKKPGVNCGRSFYICP  771
                 + S+RVFDPIEAKESWSKLL KRVAPRCEH EPCIS TTKKPGVNCGR FYICP
Sbjct  505  LRGKDPDPSDRVFDPIEAKESWSKLLGKRVAPRCEHDEPCISFTTKKPGVNCGRMFYICP  564
```

```
Query  772  RPLGPSGEKEKGTEWRCGTFIW  837
            RPLGPSGEKE+ +EWRC TFIW
Sbjct  565  RPLGPSGEKERNSEWRCSTFIW  586


>gb|EFY88584.1|  DNA lyase [Metarhizium acridum CQMa 102]
Length=634

 Score =  285 bits (730),  Expect = 1e-89
 Identities = 166/331 (50%), Positives = 204/331 (62%), Gaps = 54/331 (16%)
 Frame = +1

Query  1    GIKDWFTSANIQEGLMGSDHCPVFATLGDT---KDREHTLLELLNPPGVFQGDQRLRDWS  171
            GIKDWFTSANIQEGLMGSDHCPVFAT+  T     R+ ++ +++NP G+F+   R+RD +
Sbjct  294  GIKDWFTSANIQEGLMGSDHCPVFATIAGTVASNGRQVSINDVMNPAGMFENGTRVRDLA  353

Query  172  PKDLLPLSARLIPEFDRRQSIRDMFTKQVT-------PHAS-------------------  273
             KD+LPLSA+LIPEFDRRQSIRDMF K+VT       PH S
Sbjct  354  QKDILPLSAKLIPEFDRRQSIRDMFFKKVTSAERRTTPHNSESSSFKAEDTSQASDNDAS  413

Query  274  -----------TDTAEKLLGPRPIG---KRPADTAEKAPKGALKRNKPSSNSVDAKNKAA  411
                       T+T        P     KR  D  +  P+  LKR+K +++ + +K K +
Sbjct  414  SQIDNALQTVNTNTPTNKPAALPYSQPRKRSPDPIDPVPR-QLKRSKSAADPIGSKQKIS  472

Query  412  AGQRTLQGFFKPKASA-PQPE---NPHVEAtgrttppprktSI-GSSNGLLRQQQTPPL  576
            AGQ TL+GFFKP +S  P P    +P  ++     P      S  GSS  L   +  PP
Sbjct  473  AGQTTLKGFFKPVSSVLPSPAVLADPEAQSNATDLPATKTSPSTKGSSENLTSNEFMPPF  532

Query  577  TSETLSAEAFANTS----ERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISLTTKKPGVN  744
             SE  + ++  +TS    +RVFDPI+AKESWSKLL KRV PRCEH EPCISL TKKPGVN
Sbjct  533  -SEQPAHDSLVDTSPKSPDRVFDPIQAKESWSKLLGKRVVPRCEHDEPCISLVTKKPGVN  591

Query  745  CGRSFYICPRPLGPSGEKEKGTEWRCGTFIW  837
             GRSFYICPRPLGPSGEKE+G+EWRCGTFIW
Sbjct  592  RGRSFYICPRPLGPSGEKERGSEWRCGTFIW  622


>gb|AEI72618.1|  DNA lyase [Epichloë festucae]
Length=642

 Score =  283 bits (724),  Expect = 1e-88
 Identities = 167/339 (49%), Positives = 203/339 (60%), Gaps = 61/339 (18%)
 Frame = +1

Query  1    GIKDWFTSANIQEGLMGSDHCPVFATLGDT---KDREHTLLELLNPPGVFQGDQRLRDWS  171
            GIKDWFT ANIQEGLMGSDHCPVFAT+ D    K  +  LL+++NPPGVF   +R+R+ +
Sbjct  295  GIKDWFTFANIQEGLMGSDHCPVFATIADRVSFKGNDTALLDVMNPPGVFAQGKRVRELA  354

Query  172  PKDLLPLSARLIPEFDRRQSIRDMFTKQVTP-----------------------------  264
             +DLLPLSA+LIPEFDRRQSIRDMF K+ +
Sbjct  355  QRDLLPLSAKLIPEFDRRQSIRDMFFKKSSTIGVAADGAPSPGQPAKRASSITETGLRPV  414

Query  265  -----HASTD---TAEKLLGPRPIG------------KRPADTAEKAPKGALKRNKPSSN  384
                 + +TD   T+E LLG                 KRPA+  + AP+  LKR+K  ++
Sbjct  415  SNNNSNNNTDRNGTSEDLLGGASANSRAANAQQAESQKRPAEPIDPAPR-QLKRSKAGAD  473

Query  385  SVDAKNKAAAGQRTLQGFFKPKASAPQPENPHV--EAtgrttppprktSIGSSNGLLRQ  558
            +  +K K A GQ+TL+GFFKP  SAP    P    E+    T P     S   +  +  +
Sbjct  474  TNSSKLKTAPGQKTLKGFFKPVDSAPVHPTPSSLQESRTNNTSPRTATDSTSPTKSVSGE  533

Query  559  QQTPPLTS------ETLSAEAFANTSERVFDPIEAKESWSKLLSKRVAPRCEHQEPCISL  720
                 LTS      ++  +E      +RVFDPIEAKESWSKLL KRV PRCEH+EPCISL
Sbjct  534  PVGSELTSPSPKYSQSHHSEPIPRLPDRVFDPIEAKESWSKLLGKRVVPRCEHEEPCISL  593

Query  721  TTKKPGVNCGRSFYICPRPLGPSGEKEKGTEWRCGTFIW  837
             TKKPGVN GRSFYICPRPLGPSGEKEKG+EWRCGTFIW
Sbjct  594  VTKKPGVNRGRSFYICPRPLGPSGEKEKGSEWRCGTFIW  632
```

CHAPTER 5

VIRULENCE AND CROSS-INFECTION POTENTIAL OF *ILYONECTRIA* SPP. TO GRAPEVINE

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Phytopathologia Mediterranea (in preparation)

Keywords: black foot disease; Cylindrocarpon root rot; pathogenicity; *Vitis vinifera*.

Summary

Black foot is an important disease of grapevines, affecting vines in nurseries as well as in young plantations. In recent years the disease has increased in incidence and severity throughout the world. Black foot is associated with at least two *Campylocarpon* and 12 *Ilyonectria* species, most of which have only recently been described. The recognition of previously unknown species, together with published reports of variability in virulence between and within isolates identified as *I. macrodidyma* and *I. liriodendri*, underlined the need to compare the virulence of isolates from these complexes. A further objective of this work was to determine the cross-infection potential of isolates of these species from other hosts to grapevine. Results from this study revealed recently described species such as *I. lusitanica*, *I. estremocensis* and *I. europaea* to be more virulent to grapevine than the species previously accepted as the main causal agents of black foot, such as *I. liriodendri* and *I. macrodidyma*. Furthermore, these results also provided support for isolates obtained from non-grapevine hosts to be as virulent to grapevines as isolates obtained from grapevine, underlying the cross-infection potential of these pathogens.

Introduction

Black foot is an important disease of grapevines in most countries throughout the world. In recent years the disease has been reported with an increased incidence and severity, affecting both nurseries and young plantations, causing typical darkening of the basal end of rootstock plants (Halleen *et al*., 2004; Oliveira *et al*., 2004). Declining plants are frequently found in infected vineyards, showing slow growth, reduced vigour, retarded sprouting, shortened internodes, sparse and chlorotic foliage (Rego *et al*., 2000; Halleen *et al*., 2006a), resulting frequently in plant death and forcing growers to replant considerable areas.

Black foot is caused by several *Cylindrocarpon*-like species residing in the genera *Ilyonectria* and *Campylocarpon*. Two species of *Campylocarpon* have been reported, namely *Campyl. fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous (Halleen *et al*., 2004), although these have thus far only been reported from South Africa (Halleen *et al*., 2004) and Uruguay (Abreo *et al*., 2010). The genus *Ilyonectria* was recently established within what was formerly treated as *Neonectria s. lat.*, accommodating well-known pathogens such as *Ilyonectria liriodendri* (Halleen, Rego & Crous) P. Chaverri & C. Salgado and *I. macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & C. Salgado (Chaverri *et al*., 2011). In fact, *I. liriodendri* and *I. macrodidyma* are the species most commonly reported from affected grapevines (Petit and Gubler, 2005; Halleen *et al*., 2006b; Alaniz *et al*., 2007). Recent studies have shown, however, that many of these records actually represent some newly described species (Cabral *et al*., 2011a, 2011b). These include *I. alcacerensis* A. Cabral, Oliveira & Crous, *I. estremocensis* A. Cabral, Nascimento & Crous, *I. novozelandica* A. Cabral & Crous and *I. torresensis* A. Cabral, Rego & Crous which were described from within the *I. macrodidyma* species complex (Cabral *et al*., 2011b), and *I. europeaea* A. Cabral, Rego & Crous, *I. lusitanica* A. Cabral, Rego & Crous, *I. pseudodestructans* A. Cabral, Rego & Crous, *I. robusta* (A.A. Hildebr.) A. Cabral & Crous and *I. vitis* A. Cabral, Rego & Crous, which emerged from the *I. radicicola* (Gerlach & L. Nilsson) P. Chaverri & C. Salgado species complex (Cabral *et al*., 2011a). *Ilyonectria torresensis* was found to be associated with *Vitis vinifera, Abies nordmanniana*, *Fragaria* sp. and *Quercus* sp. in countries throughout the world. In contrast, *I. alcacerensis* has thus far only been reported from *V. vinifera* in the Iberian Peninsula. *Ilyonectria novozelandica* was associated with *V. vinifera* in New Zealand, South Africa and the USA, but also reported on *Festuca duriuscula* in Portugal. *Ilyonectria estremocensis* was isolated from *V. vinifera* in Portugal and *Picea glauca* in Canada (Cabral *et al*., 2011b). *Ilyonectria europaea*, *I. pseudodestructans* and *I. robusta* were found on *V. vinifera* in Portugal and on other host plants in different parts of the world, while *I. lusitanica* and *I. vitis* were thus far exclusively reported from grapevines (Cabral *et al*., 2011a). Besides

these, "*Cylindrocarpon*" *pauciseptatum* Schroers & Crous was associated with diseased roots of *Vitis* spp. in New Zealand and Slovenia (Schroers *et al*., 2008), in Uruguay (Abreo *et al*., 2010), in Portugal (Cabral *et al*., 2011a) and in Spain (Martín *et al*., 2011).

Ilyonectria macrodidyma has been reported as more virulent to grapevines than *I. liriodendri*, although variation in virulence among groups of *I. macrodidyma* was also found (Alaniz *et al*., 2009). However, no other comparative virulence studies have been reported among the pathogens causing black foot disease of grapevine. This is becoming particularly relevant, as at least 12 species are currently recognised to be associated with this disease. Moreover, most of these species are not exclusive to grapevine, and infect several other hosts, underlining the cross-infection potential of isolates from other hosts to grapevines. Therefore, the objective of this work was to compare the virulence of isolates from different species associated with black foot disease of grapevines, as well as to test the pathogenicity of isolates from other hosts to grapevine.

Materials and Methods

A total of 60 isolates were analysed, 36 of which are from grapevines (Table 1). The other hosts include *Olea europaea* (five isolates) and *Quercus* spp. (five isolates), among others. Species covered in this study include "*C.*" *pauciseptatum* (three isolates from grapevine and one from *Olea europaea*), *I. alcacerensis* (two isolates from grapevine), *I. estremocensis* (four isolates from grapevine), *I. europaea* (two isolates from grapevine, one from *Actinidia chinensis* and one from *Aesculus hippocastanum*), *I. liriodendri* (four isolates from grapevine, one from *Liriodendron tulipifera*, one from *Malus domestica,* and one from *Quercus suber*), *I. lusitanica* (one isolate from grapevine), *I. macrodidyma* (three isolates from grapevine and two from *Olea europaea*), *I. novozelandica* (five isolates from grapevine and one from *Festuca duriuscula*), *I. pseudodestructans* (two isolates from grapevine and two from *Quercus* sp.), *I. robusta* (three isolates from grapevine, two from *Quercus* spp., two from *Panax quinquefolium*, one from *Prunus cerasus*, one from *Tilia petiolaris*, one from *Thymus* sp. and one from an aquarium with *Anodonta*), *I. vitis* (one isolate from grapevine), *I. torresensis* (four isolates from grapevine, one from *Fragaria* x *ananassa* and one from *Olea europaea*), an *I. estremocensis*-like undescribed species, here referred as *Ilyonectria* sp2 (L. Mostert, pers. comm.; two isolates from grapevine, one from *Pinus laricio* and one from an unknown host) and an *I. venezuelensis*-like undescribed species, here referred as *Ilyonectria* sp1 (Cabral *et al*., unpubl. data).

Cuttings of the susceptible rootstock 1103P (Alaniz *et al*., 2010) were rooted for 1.5 to 2 months at 20°C in a rooting bench containing perlite and sand, and irrigated by overhead nebulisation for 5 s every 10 min.

After the rooting period, plants were removed from the bench and the roots were slightly pruned. The wounded cuttings were dip-inoculated by immersing the roots and the basal end of the cuttings in a 10^6 mL^{-1} conidial suspension (for each isolate listed in Table 1), for 60 min. Conidia were harvested by flooding 14 d old potato-dextrose agar (PDA, Difco, USA) cultures with sterile distilled water, and dislodged with a sterile glass rod. The spores and mycelium were then filtered through a double layer of cheesecloth, and the conidial concentration estimated using a haemocytometer, which was then adjusted with sterile distilled water. After inoculation, the rooted cuttings were planted in 1 L bags containing a mixture of soil, peat and sand (2:1:1, v/v/v), and maintained in a greenhouse at 24±5°C (day) and 18°C (night) with approximately a 12 h photoperiod. For negative control plants, sterile distilled water was used instead of conidial suspension.

The plants were grown on the greenhouse for 4.5 months and, following this period, results were evaluated for each isolate (10-12 plants per isolate, including the control), and compared to the control. The parameters analysed were focused on the loss of root (number and root dry weight, and the length of the longest root) and shoot (number of shoot nodes and the length and shoot dry weight; usually a single shoot was formed) biomass and on the intensity of wood colonisation by the pathogens (percentage of reisolation). For the latter, 10 pieces of wood from the basal end of each rootstock plant (at least 2 cm above the bottom) were excised, disinfected (for 1 min in a NaClO solution with 0.35 % w/w as active chlorine), rinsed with distilled water and placed in Petri dishes containing PDA amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppiChem, Germany). The dishes were incubated at 20°C for up to 2 weeks and observed for the presence of *Ilyonectria* colonies, which was confirmed through morphological appearance of colonies and conidial characteristics. The percentage of reisolation was calculated as the number of wood pieces from which *Ilyonectria* colonies were recovered, divided by the total number of pieces of wood for each plant.

All data were subjected to a one-way ANOVA and means compared using the Tukey's test at a 5% significance level (STATISTICA 8.0). Before analysis, percentage data were arcsine-square root transformed.

To confirm results from this experiment, data from a subsequent, smaller experiment were used for comparison under the same conditions as stated above. Isolates tested were from the

following species: *I. estremocensis* (isolates Cy135, Cy144, Cy145, Cy152 and Cy153 from grapevine), *I. europaea* (isolate Cy131 from *Actinidia chinensis*), *I. liriodendri* (isolates Cy5, Cy68 and Cy76 from grapevine, Cy164 from *Malus domestica* and Cy232 from *Quercus suber*), *I. novozelandica* (isolate Cy230 from *Festuca* sp.), *I. pseudodestructans* (isolates Cy20 and Cy22 from grapevine, and CBS 117812 from *Quercus* sp.), *I. robusta* (CBS 117818 from *Quercus* sp. and Cy231 from *Thymus* sp.), *I. torresensis* (isolates OL1 from *Olea europaea*, Cy96 from *Quercus* sp. and Cy221 and Cy222 from *Fragaria* x *ananassa*) and *Ilyonectria* sp2 (isolate OL2 from *Olea europaea*).

Results

At the end of the first experiment, root rot symptoms were visible in inoculated plants, while no symptoms were seen in the uninoculated controls. Symptoms included root lesions, vascular discolouration, and necrosis in the basal plant tissues, although the quantification of these lesions and discolouration was not possible. Symptoms related to reduced vigour were more readily quantifiable. In general, inoculated plants had shorter shoots with less nodes, as well as fewer and shorter roots, although significant differences were found among isolates (Table 2).

The percentage of reisolation ranged from a minimum of 18.6% for isolate OL2 (*Ilyonectria* sp1, from *Olea europaea*) to a maximum of 96.5% for isolate CBS 537.92 (*I. europaea*, from *Aesculus hippocastanum*). *Ilyonectria* could not be re-isolated from the control plants, differing significantly from all tested isolates except OL2 and Cy230. This trait had the fewest homogeneous groups among all the traits studied.

The average number of roots in the control plants was 36.3, which did not differ significantly from the maximum value recorded from inoculated plants (35.8 for isolate CBS 112615; *I. macrodidyma* from grapevine). The smallest value for NR was 19.2 for isolate CBS 117526 (*I. liriodendri*, from grapevine), which represents a 47% reduction in the number of roots.

Root dry weight ranged from a maximum of 4.50 g for isolate OL2 (which did not differ significantly from the control plants; 4.08 g) to a minimum of 0.49 for Cy243 (*I. estremocensis*, from grapevine; 88% reduction from control).

The length of the longest root for the control plants was 49.6 cm, with all inoculated plants showing a significant reduction from that value, ranging from a minimum reduction of 23% for isolate OL2 to a maximum of 66% (16.8 cm) for isolate Cy243.

The average number of shoot nodes in the control plants was 15.9, ranging for the inoculated plants from 13.2 nodes for isolate Cy233 (*I. vitis*, from grapevine), which did not differ significantly from the control, to 8.1 nodes for isolate CBS 129086 (*I. torresensis*, from grapevine), which represents a 49% reduction.

The average shoot length was 52.2 cm in the control plants, ranging from 43.0 cm for isolate Cy200 (*Ilyonectria* sp2, from grapevine; 43.0 cm, 18% reduction) to 18.1 cm for isolate Cy243 (18.1 cm, 65% reduction).

The shoot dry weight ranged from a maximum of 1.08 g for isolate CBS 113552 (*I. novozelandica*, from grapevine), which did not differ significantly from the control (0.95 g), to a minimum of 0.22 g (Cy243), which represents an 80% reduction.

Considering the isolates obtained from grapevine separate from the isolates from other hosts, significant differences were observed among species and to the control (Figure 1). The percentage of reisolation ranged between 39.4% for *I. pseudodestructans* and 85.0% for *I. vitis* for grapevine isolates, all of them differing significantly from the control. Results for isolates from other hosts ranged between 88.1% for *I. torresensis* and 18.6% for *Ilyonectria* sp1 (which did not differ from the control, along with *I. novozelandica*; the latter was the single species with significant differences among isolates from grapevine and other hosts).

For grapevine isolates, the number of roots ranged between a maximum of 33.3 for *Ilyonectria* sp2 (the single species that did not differ significantly from the control; 36.3) and a minimum of 20.7 for *I. europaea*, representing a 43% reduction in the number of roots. Among the isolates from other hosts, "*C.*" *pauciseptatum*, *I. macrodidyma* and *I. novozelandica* did not differ statistically from the control (non-grapevine isolates from "*C.*" *pauciseptatum* and *I. novozelandica* differed significantly from grapevine isolates), while inoculations with *I. robusta* resulted in the lowest number of roots (a 32% reduction).

In all treatments the longest root was significantly shorter than the longest root of the control plants, ranging between a maximum of 33.8 cm for *I. pseudodestructans* (a 32% reduction from the control) and a minimum of 22.9 cm for *I. estremocensis* (54% reduction) for grapevine isolates, and between 38.2 cm for *Ilyonectria* sp1 and 24.9 cm for *Ilyonectria* sp2 for isolates from other hosts. Significant differences were recorded, however, for "*C.*" *pauciseptatum* inoculations between grapevine (25.4 cm) and non-grapevine isolates (33.7 cm).

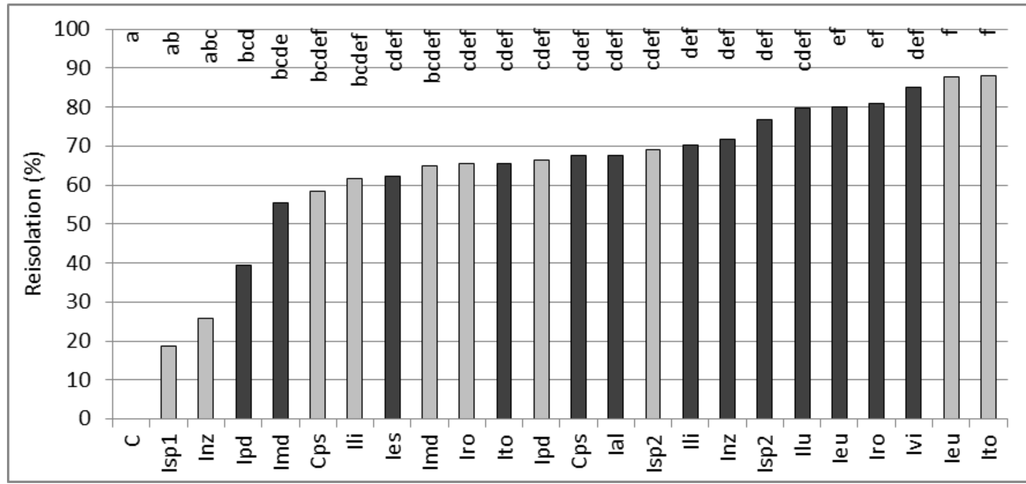
For grapevine isolates, the root dry weight of inoculated plants ranged between a maximum of 2.98 g for *I. vitis* (the single species that does not differ significantly from the control; 4.08 g) and a minimum of 1.28 g for *I. lusitanica* (a 69% reduction from the control). Among non-grapevine

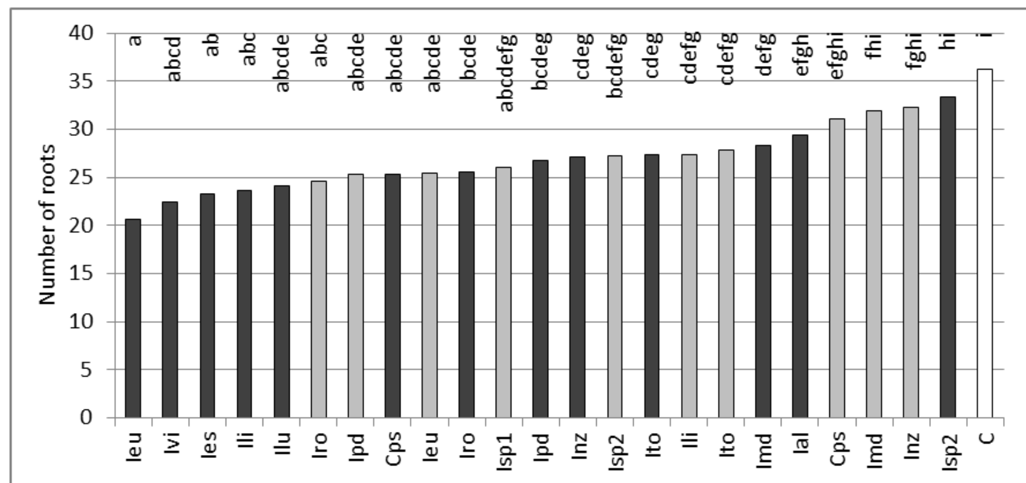
isolates, "*C.*" *pauciseptatum* and *Ilyonectria* sp1 did not differ statistically from the control (and "*C.*" *pauciseptatum* non-grapevine isolates differed significantly from grapevine isolates), while inoculations with *Ilyonectria* sp2 resulted in a root dry weight of 1.95 g (a 52% reduction).

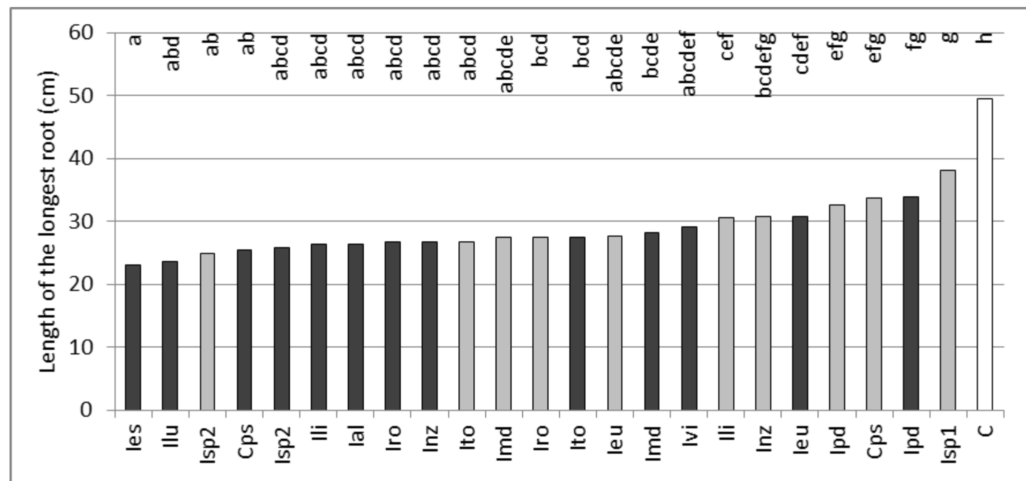
Similarly, the number of shoot nodes ranged between a maximum of 13.2 for plants inoculated with *I. vitis* (the single species that did not differ significantly from the control; 15.9) and a minimum of 8.9 for plants inoculated with *I. lusitanica* (a 44% reduction from the control) for grapevine isolates, and between 12.5 for "*C.*" *pauciseptatum* and 8.8 for *Ilyonectria* sp1 among isolates from other hosts. For each species, no significant differences were found between grapevine and non-grapevine isolates.

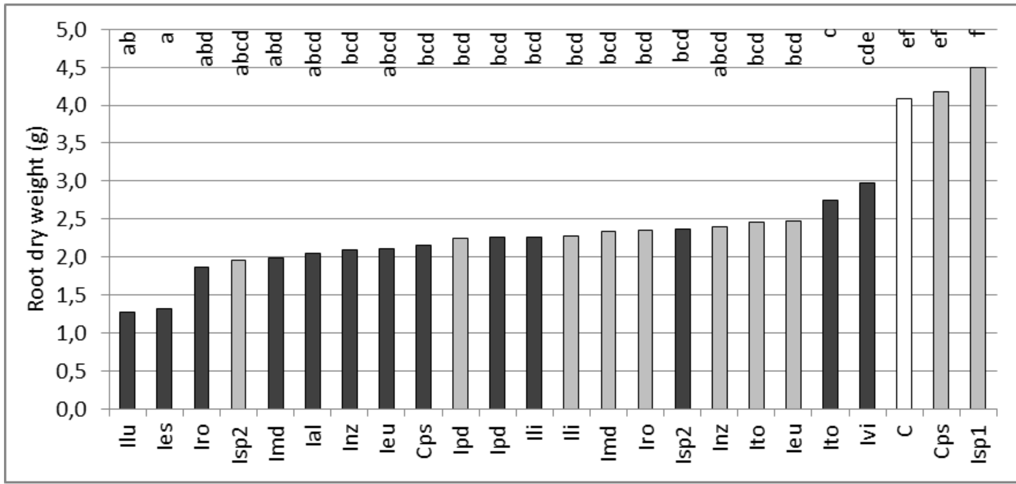
Shoot length was significantly shorter than that of the control for all samples, ranging between a maximum of 41.4 cm for *Ilyonectria* sp2 (a 21% reduction from the control) and a minimum of 24.3 for *I. lusitanica* (53% reduction) for grapevine isolates, and between 36.7 cm for *I. torresensis* and 21.8 cm for *Ilyonectria* sp1 for isolates from other hosts. Non-grapevine isolates had significantly higher values than grapevine isolates for several species, such as "*C.*" *pauciseptatum*, *I. liriodendri*, *I. macrodidyma* and *I. torresensis*, while the opposite was recorded for *Ilyonectria* sp2.

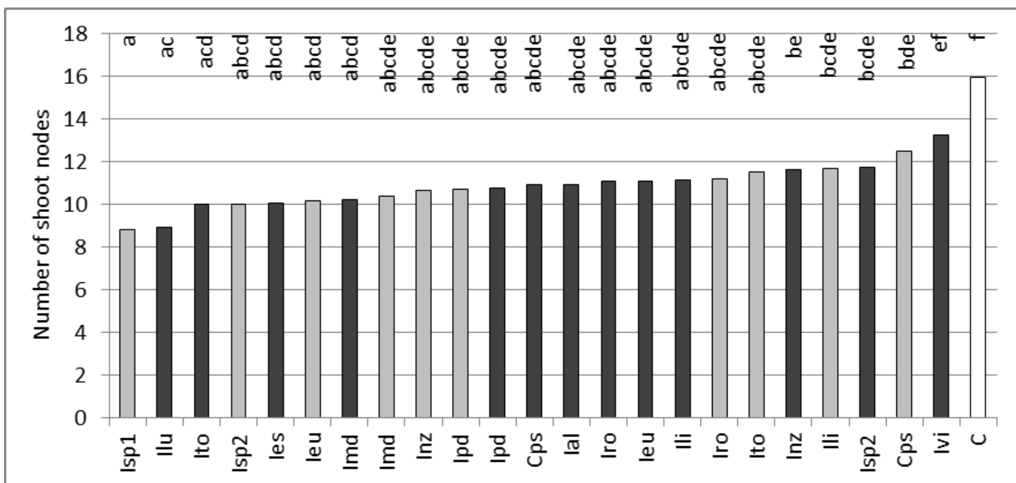
The shoot dry weight ranged between a maximum of 0.96 g for *Ilyonectria* sp2 and a minimum of 0.41 g for *I. lusitanica* (a 57% reduction from the control) for grapevine isolates ("*C.*" *pauciseptatum*, *I. estremocensis*, *I. liriodendri*, *I. lusitanica* and *I. macrodidyma* were significantly lower than the control) and of 0.45 g for *Ilyonectria* sp1 for non-grapevine isolates. Differences between grapevine and non-grapevine isolates were only recorded for *Ilyonectria* sp2 (0.96 g and 0.55 g, respectively).

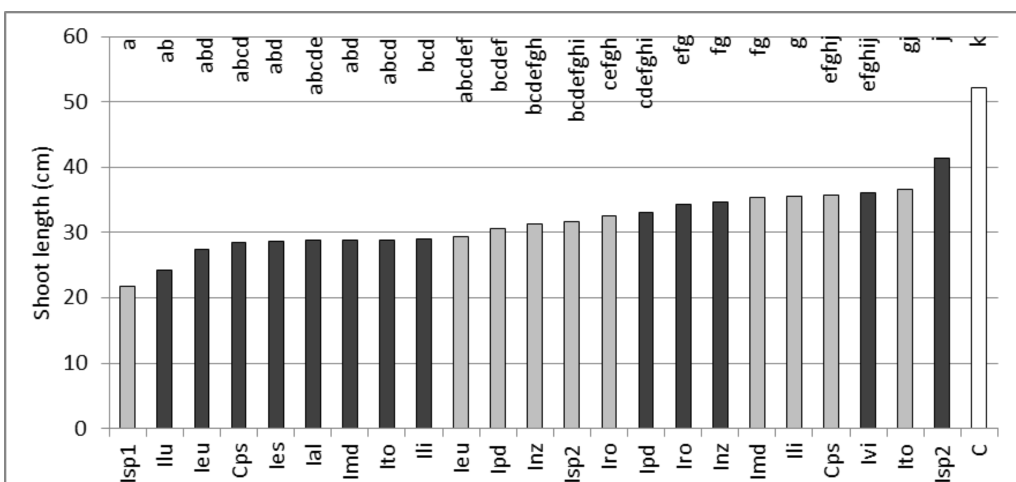

Reisolation (%)
a ab abc bcd bcde bcdef bcdef cdef bcdef cdef cdef cdef cdef cdef cdef def def def cdef ef ef def f f
C Isp1 Inz Ipd Imd Cps Ili Ies Imd Iro Ito Ipd Cps Ial Isp2 Ili Inz Isp2 Ilu Ieu Iro Ivi Ieu Ito


Number of roots
a abcd ab abc abcde abc abcde abcde abcde bcde abcdefg bcdeg cdeg bcdefg cdeg cdefg cdefg defg efgh efghi fhi fghi hi i
Ieu Ivi Ies Ili Ilu Iro Ipd Cps Ieu Iro Isp1 Ipd Inz Isp2 Ito Ili Ito Imd Ial Cps Imd Inz Isp2 C


Length of the longest root (cm)
a abd ab ab abcd abcd abcd abcd abcd abcd abcde bcd bcd abcde bcde abcdef cef bcdefg cdef efg efg fg g h
Ies Ilu Isp2 Cps Isp2 Ili Ial Iro Inz Ito Imd Iro Ito Ieu Imd Ivi Ili Inz Ieu Ipd Cps Ipd Isp1 C


Root dry weight (g)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
5,0
ab a abd abcd abd abcd bcd abcd bcd bcd bcd bcd bcd bcd bcd bcd abcd bcd bcd c cde ef ef f
Ilu Ies Iro Isp2 Imd Ial Inz Ieu Cps Ipd Ipd Ili Ili Imd Iro Isp2 Inz Ito Ieu Ito Ivi C Cps Isp1


Number of shoot nodes
0
2
4
6
8
10
12
14
16
18
a ac acd abcd abcd abcd abcd abcde abcde abcde abcde abcde abcde abcde abcde abcde abcde abcde be bcde bcde bde ef f
Isp1 Ilu Ito Isp2 Ies Ieu Imd Imd Inz Ipd Ipd Cps Ial Iro Ieu Ili Iro Ito Inz Ili Isp2 Cps Ivi C


Shoot length (cm)
0
10
20
30
40
50
60
a ab abd abcd abd abcde abd abcd bcd abcdef bcdef bcdefgh bcdefghi cefgh cdefghi efg fg fg g efghj efghij gj j k
Isp1 Ilu Ieu Cps Ies Ial Imd Ito Ili Ieu Ipd Inz Isp2 Iro Ipd Iro Inz Imd Ili Cps Ivi Ito Isp2 C

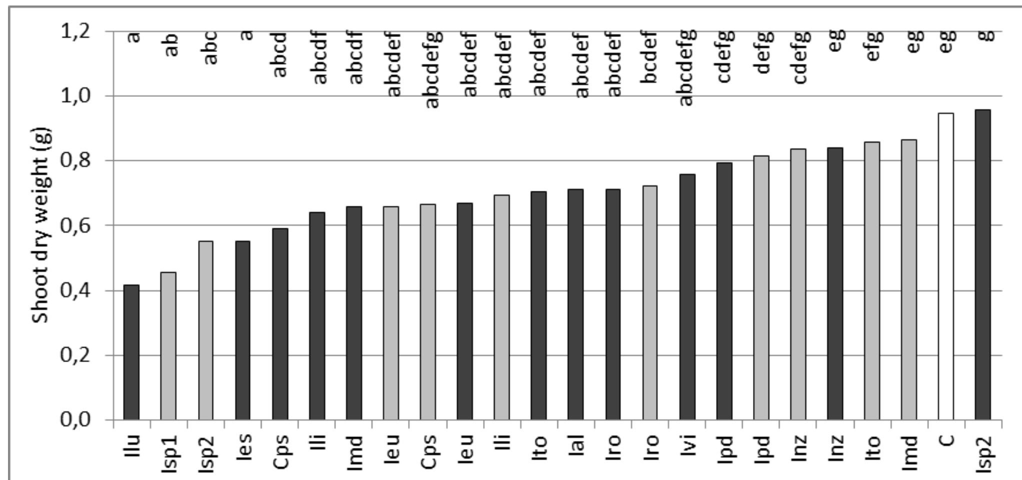


Fig. 1. Comparison among *Ilyonectria* spp. isolates from grapevines (black bars) and from other hosts (grey bars) for their effect on grapevine rootstock 1103P in: frequency of reisolation; number of roots; length of the longest root; root dry weight; number of shoot nodes; shoot length; shoot dry weight. Bars followed by the same letter do not differ statistically according to Tukey's test (p=0.05). C – Control (white bars); Cps – "*C.*" *pauciseptatum* (average of three isolates from grapevine and one isolate from another host); Ial – *I. alcacerensis* (two isolates from grapevines); Ies – *I. estremocensis* (four isolates from grapevines); Ieu – *I. europaea* (two isolates from grapevines and two from other hosts); Ili – *I. liriodendri* (four isolates from grapevines and three from other hosts); Ilu – *I. lusitanica* (one isolate from grapevines); Imd – *I. macrodidyma* (four isolates from grapevines and two from other hosts); Inz – *I. novozelandica* (four isolates from grapevines and one from another host); Iro – *I. robusta* (three isolates from grapevines and eight from other hosts); Isp1 – *Ilyonectria* sp1 (one isolate from olive); Ipd – *I. pseudodestructans* (two isolates from grapevines and two from other hosts); Isp2 – *Ilyonectria* sp2 (two isolates from grapevines and two from other hosts); Ito – *I. torresensis* (four isolates from grapevines and two from other hosts); Ivi – *I. vitis* (one isolate).

Inoculated plants in the second experiment also revealed typical black foot symptoms, with significant reductions in root and shoot biomass as compared to the control plants (Figure 2). Considering the species for which grapevine isolates were analysed, *I. estremocensis* was slightly more virulent than *I. liriodendri* and *I. pseudodestructans*, particularly in parameters concerning the aerial plant part, although the frequency of reisolation was significantly lower than that of *I. liriodendri*. Furthermore, results confirmed most non-grapevine isolates to be as virulent as grapevine isolates.

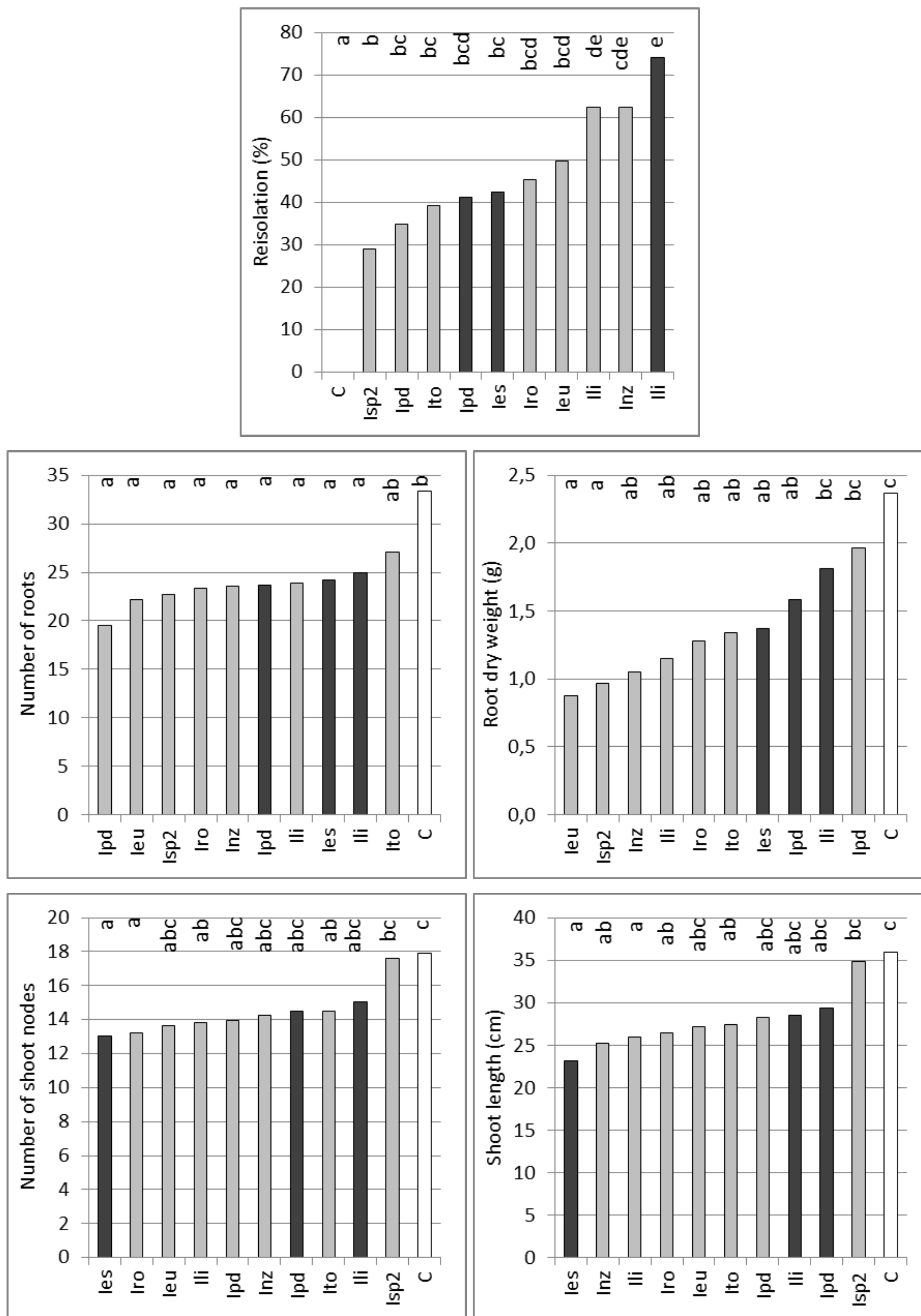


Fig. 2. Comparison among *Ilyonectria* spp. isolates from grapevines (black bars) and from other hosts (grey bars) for their effect on grapevine rootstock 1103P in: frequency of reisolation; number of roots; root dry weight; number of shoot nodes; shoot length. Bars affected by the same letter do not differ statistically according to Tukey's test (p=0.05). C – Control (white bars); Ies – *I. estremocensis* (five isolates from grapevines); Ieu – *I. europaea* (one isolate from another host); Ili – *I. liriodendri* (three isolates from grapevines and two from other hosts); Inz – *I. novozelandica* (one isolate from another host); Ipd – I. pseudodestructans (two isolates from grapevine); Iro – *I. robusta* (three isolates from other hosts); Isp1 – *Ilyonectria* sp1 (one isolate from olive); Ipd – *I. pseudodestructans* (two isolates from grapevines and two from other hosts); Isp2 – *Ilyonectria* sp2 (one isolate from another host); Ito – *I. torresensis* (four isolates from other hosts).

Discussion

Black foot disease symptoms recorded at the end of the experiment were associated with a reduction in plant growth and vigour, fewer shoot internodes and roots, and shorter and thinner shoots. These are illustrated by a reduction in the number of roots (up to 47%), shoot nodes (up to 49%), shoot length (up to 65%), length of the longest root (up to 66%), shoot dry weight (up to 80%), and root dry weight (up to 88%).

Frequency of reisolation was the least informative character, only separating the control plants and the isolates OL2 (*Ilyonectria* sp1) from *Olea europaea* and Cy230 (*I. novozelandica*) from *Festuca duriuscula*, from the remaining isolates. Traits related to the roots were slightly more informative than those related to the aerial plant parts, thus corroborating results from Alaniz *et al*. (2010).

In general, grapevine isolates from the species *I. lusitanica*, *I. estremocensis*, *I. europaea* and *"C." pauciseptatum* were the most virulent, while those from species *I. novozelandica*, *I. pseudodestructans*, *I. vitis* and *Ilyonectria* sp2 were the least virulent, with intermediate results for *I. robusta*, *I. liriodendri*, *I. macrodidyma*, *I. torresensis* and *I. alcacerensis*. For some species however, differences were recorded between characters related to the roots and to the aerial part of the plants. Symptoms related to inoculations by *I. lusitanica*, *I. estremocensis* and *"C." pauciseptatum* isolates were equally prominent based on root and aerial part parameters. In contrast, symptoms caused by *I. europaea*, *I. novozelandica* and *I. robusta* isolates were more prominent on roots than on aerial parts, while symptoms of *I. torresensis* and *I. macrodidyma* were more noticeable on aerial plant parts. However, the effect of these pathogens in the aerial parts should be interpreted while taking into consideration that only ungrafted rootstocks were studied here. Experiments using grafted plants would be necessary to reach conclusions on the effect of these pathogens on the aerial parts of grapevine plants. In spite of this, the results obtained here reveal that different *Ilyonectria* species and "*C.*" *pauciseptatum* originate diverse levels of severity on the aerial plant parts. This observation may be relevant in infected fields of rootstock mother-plants, because, most likely, the canes will be shorter, thinner and of poorer quality, thus compromising the later success of cuttings made from such vines.

A comparison among all isolates, revealed isolates Cy243 (*I. estremocensis*), Cy197 (*I. lusitanica*), Cy23 (*I. robusta*), Cy238 ("*C.*" *pauciseptatum*) and Cy128 (*I. macrodidyma*), all from grapevines, to be the most virulent, while the least virulent were isolates OL-CM3 ("*C.*" *pauciseptatum*) from *Olea europaea*, Cy200 (*Ilyonectria sp2*) from grapevine, CBS 129081 (*I. pseudodestructans*) from grapevine, CBS 112593 (*I. novozelandica*) and Cy164 (*I. liriodendri*) from *Malus domestica*.

Virulence to the roots varied among isolates, which in turn exhibit different effects on the aerial parts. Isolates Cy23 (*I. robusta*), Cy128 (*I. macrodidyma*), Cy152 (*I. estremocensis*), Cy196 (“*C.*” *pauciseptatum*), CBS 110.81 (*I. liriodendri*, from *Liriodendron tulipifera*) or CBS 117824 (*I. pseudodestructans*, from *Quercus* sp.) showed high virulence in roots, but limited effects on the aerial parts. On the contrary, isolates CBS 129086 (*I. torresensis*), Cy250 (*I. macrodidyma*), CBS 537.92 (*I. europaea*, from *Aesculus hippocastanum*), CBS 159.34, and particularly isolate OL2 (*Ilyonectria* sp1, from *Olea europaea*) had low reisolation frequency and caused little effects on roots, but very strong effects on the above ground parts of inoculated plants. When isolate OL2 was inoculated on olive plants, it was found to be highly virulent (Cabral *et al*., unpublished data), inducing not only aerial symptoms but also root and crown necroses. This indicates that *Ilyonectria* sp1 may be more host-specialized than the other species studied here, suggesting that although there are taxa with wide host ranges, host specialisation also occurs in some species of *Ilyonectria*. However, the unexpected pattern of symptoms produced by OL2 or other isolates, suggests that further work is required to fully elucidate the grapevine-*Ilyonectria* pathosystem. To date, little information exists on the mechanisms of host infection and root colonization, as well as the concomitant mechanisms of host-defense response. In apple trees for example, it was hypothesized that the most virulent “Cylindrocarpon” isolates do not proliferate extensively within the host tissue, but rather cause damage to the host by the secretion of cell wall degrading enzymes or toxins (Tewoldemedhin *et al*., 2011).

For each fungal species, comparisons between grapevine and non-grapevine isolates could not suggest specific trends, with the notable exception of isolates from *Olea europaea* (and to some extent, of *Festuca duriuscula*), which were always less virulent than grapevine isolates of the same species (“*C.*” *pauciseptatum*, *I. macrodidyma* and *I. torresensis*). However, frequency of reisolation did not differ significantly to other isolates, suggesting that these isolates are fully capable of infecting and colonizing the inoculated plants. The capacity of isolates from hosts such as *Actinidia chinensis*, *Fragaria* x *ananassa*, *Malus domestica* and *Quercus* spp. to be as virulent as the grapevine isolates, including isolates from some of the most virulent species, such as *I. europaea*, raises the importance of the cross-infection potential of isolates from other hosts to grapevine, particularly for plants that are likely to precede grapevine in cultivation, either in a vineyard or nursery. In fact, a recent study addressing apple replant disease (Tewoldemedhin *et al*., 2011) revealed the involvement of species also pathogenic to grapevine in the present study, such as “*C.*” *pauciseptatum*, *I. macrodidyma* and *I. liriodendri*, supporting their polyphagous nature.

Furthermore, many isolates of the *I. macrodidyma* species complex were obtained from roots of several monocotyledons and dicotyledons weed families sampled in Spanish vineyards. When inoculated on grapevines, these isolates were able to induce typical black foot disease symptoms (Agustí-Brisach *et al.,* 2011). In addition to the hosts referred to above, therefore, weeds may represent an important inoculum source of *I. macrodidyma* s. lat. in vineyards.

Besides the importance of cross-infection potential as well as indications of host specificity, the present study also showed that grapevine isolates from newly described species such as *I. lusitanica*, *I. estremocensis* and *I. europaea* are more virulent to grapevine than the species previously accepted to represent the main causal agents of black foot, such as *I. liriodendri* and *I. macrodidyma*.

Acknowledgments

We thank M.Sc. Teresa Nascimento, M.Sc. Ana Teresa Vaz, Mrs. Amélia Marques and Mrs. Olga Natividade for technical assistance. This article is part of a Ph.D. dissertation (Instituto Superior de Agronomia, Technical University of Lisbon, Portugal). This work was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/24790/2005; project PTDC/AGR-AAM/099324/2008).

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Table 1. List of isolates studied with collection details

Strain number [a]	Collected/isolated by, Year	Isolated from	Location
"Cylindrocarpon" pauciseptatum			
OL-CM3	C. Rego, 2008	*Olea europaea*	Portugal, Campo Maior
Cy196	N. Cruz, 2005	*Vitis vinifera*, basal end of 4-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço/Monção
Cy217	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Gouveio	Portugal, Torres Vedras
Cy238	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira
Ilyonectria vitis			
CBS 129082; Cy233	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Touriga Nacional; rootstock 110R	Portugal, Vidigueira
Ilyonectria alcacerensis			
Cy134; IAFM Cy20-1	J. Armengol	*Vitis vinifera*	Spain, Ciudad Real, Villarubia de los Ojos
CBS 129087; Cy159	A. Cabral & H. Oliveira, 2004	*Vitis vinifera*, basal end of a 3-year-old plant with root discolouration and decline symptoms; scion Sangiovese; rootstock 1103P	Portugal, Alcácer do Sal, Torrão
Ilyonectria sp2			
CBS 173.37; IMI 090176	T.R. Peace, 1937	*Pinus laricio*, associated with dieback	UK, England, Devon, Haldon
Cy108	C. Rego, 1999	*Vitis vinifera*, basal end of a 4-year-old plant showing decline symptoms; scion Aragonez; rootstock SO4	Portugal, Nelas
Cy200	N. Cruz, 2005	*Vitis vinifera*, basal end of a 16-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço
CBS 159.34; IMI 113891	H.W. Wollenweber, 1934	-	Germany
Ilyonectria estremocensis			
Cy135	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz
CBS 129085; Cy145	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz
Cy152	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5-year-old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz

Strain number [a]	Collected/isolated by, Year	Isolated from	Location
Cy243	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old-plant; scion Touriga Nacioal; rootstock 110R	Portugal, Vidigueira
Ilyonectria macrodidyma			
OL_CM4	C. Rego, 2008	*Olea europaea*	Portugal, Campo Maior
OL_CM6	C. Rego, 2008	*Olea europaea*	Portugal, Campo Maior
Cy128	W.D. Gubler	*Vitis vinifera*	USA, California
Cy175	C. Rego, 2004	*Vitis vinifera*, basal discolouration; scion Touriga Nacional; rootstock 1103P	Portugal, Torre de Moncorvo
Cy250	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Chardonnay rootstock 110R	Portugal, Vidigueira
CBS 112615	F. Halleen, 2000	*Vitis vinifera*, roots, asymptomatic nursery plant; scion Sultana; rootstock 143-B Mgt	South Africa, Western Cape, Malmesbury, Jakkalsfontein
Ilyonectria novazelandica			
Cy230	F. Caetano, 2005	*Festuca duriuscula*	Portugal, Lisbon
CBS 113552	R. Bonfiglioli, 2003	*Vitis* sp. decline of nursery plants dead rootstocks	New Zealand, Candy P New Ground
Cy129	W.D. Gubler	*Vitis vinifera*	USA, California
Cy130	W.D. Gubler	*Vitis vinifera*	USA, California
CBS 112593	F. Halleen, 2000	*Vitis vinifera*, roots of asymptomatic nursery plant; scion Pinotage; rootstock 101-14 Mgt	South Africa, Western Cape, Wellington, Voorgroenberg
Ilyonectria torresensis			
Cy222	L. Leandro	*Fragaria x ananassa*	USA, North Carolina, Asheville
HC7	C. Rego, 2007	*Olea europaea*	Portugal, Avis
Cy118	W.D. Gubler	*Vitis vinifera*	USA, California
Cy214	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Grenache	Portugal, Torres Vedras
CBS 129086; Cy218	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Chenin	Portugal, Torres Vedras
Cy260	C. Rego, 2008	*Vitis vinifera*, basal end of 2-year-old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira
Ilyonectria europaea			

Strain number [a]	Collected/isolated by, Year	Isolated from	Location
Cy131	P. Lecomte & S. Chamont, 2000	*Actinidia chinensis* 'Hayward', internal lesion of stem	France, St. Chicq-du-Gaue
CBS 537.92	V. Demoulin, 1992	*Aesculus hippocastanum*, wood	Belgium, Liège
CBS 129078; Cy241	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira
Cy155	C. Rego & H. Oliveira, 2004	*Vitis vinifera*, 2-year-old, with decline symptoms, scion Alfrocheiro; rootstock SO4	Portugal, Alter do Chão
Ilyonectria liriodendri			
CBS 110.81; IMI 303645	J.D. MacDonald & E. Butler, 1978	*Liriodendron tulipifera*, root	USA, California, Yolo Co., Davis
Cy164	C. Rego, 1997	*Malus domestica*; cultivar Lysgolden; rootstock MM106	Portugal, Porto de Mós, Valbom
Cy232	L. Inácio & J. Henriques, 2007	*Quercus suber*, stem	Portugal, Macedo de Cavaleiros
Cy5	C. Rego, 1992	Vitis vinifera, 4-year-old, with decline symptoms; scion Boal Branco; rootstock 99R	Portugal, Torres Vedras, Dois Portos
CBS 117526; Cy68	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 99 R, clone 179F	Portugal, Ribatejo e Oeste
Cy190	N. Cruz, 2005	*Vitis vinifera*, basal end of a 6-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Monção, Cortes
Cy253	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira
Ilyonectria lusitanica			
CBS 129080; Cy197	N. Cruz, 2005	*Vitis vinifera*, below grafting zone, 6-year-old; scion Alvarinho; rootstock 196-17	Portugal, Melgaço, Alvaredo
Ilyonectria sp1			
OL2	C. Rego & H. Oliveira, 2007	*Olea europaea*	Portugal, Évora
Ilyonectria pseudodestructans			
CBS 117824	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf
CBS 117812	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf
CBS 129081; Cy20	C. Rego, 1996	*Vitis vinifera*, 4-year-old, with decline symptoms, scion Malvasia Fina; rootstock 1103P	Portugal, Gouveia, São Paio
Cy22	C. Rego, 1996	*Vitis vinifera*, 5-year-old, with decline symptoms, scion Aragonez; rootstock 99R	Portugal, Viseu, Silgueiros

Strain number [a]	Collected/isolated by, Year	Isolated from	Location
Ilyonectria robusta			
CD1666	R. D. Reeleder, 1998	*Panax quinquefolium*	Canada, Nova Scotia
CBS 308.35	A.A. Hildebrand	*Panax quinquefolium*	Canada, Ontario
CPC 13532; DAOM 139398	-	*Prunus cerasus* cultivar Montmorency	Canada, Ontario
CBS 117813	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden
CBS 117815	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf
Cy231	F. Caetano, 2005	*Thymus* sp.	Portugal, Lisbon
CBS 605.92	R. Schröer, 1992	*Tilia petiolaris*, root	Germany, Hamburg
Cy23	C. Rego, 1997	*Vitis* sp. rootstock 99R clone 179F in nursery	Portugal, Ribatejo e Oeste
Cy158	C. Rego & T. Nascimento, 2004	*Vitis vinifera*, 1-year-old, died before sprouting; scion Alicante Bouschet; rootstock 1103P	Portugal, Lamego, Cambres
CBS 129084; Cy192	N. Cruz, 2005	*Vitis vinifera*, basal end of 25-year-old plant; scion Alicante; rootstock 196-17	Portugal, Monção
CBS 773.83	J. Hemelraad	water, in aquarium with *Anodonta*	Netherlands, Utrecht

[a] CBS: CBS-KNAW Fungal Biodiversity Centre (Centraalbureau voor Schimmelcultures), Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; Cy,OL and HC: Cylindrocarpon collection housed at Laboratório de Patologia Vegetal "Veríssimo de Almeida" - ISA, Lisbon, Portugal; DAOM: Agriculture and Agri-Food Canada National Mycological Herbarium, Canada; IAFM: Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.

Table 2. Comparison (average and homogeneous groups) among *Ilyonectria* spp. isolates for their effect on the frequency of reisolation (%RI), on the number of roots (NR), length of the longest root (LR, cm) root dry weight (RDW, g), number of shoot nodes (NSN), shoot length (SL, cm) and shoot dry weight (SDW, g) of grapevine rootstock 1103P

Species	Strain Number	Host	%RI [a]	NR	LR	RDW	NSN	SL	SDW
Control			0 a	36.3 t	49.6 s	4.08 tuv	15.9 g	52.2 o	0.95 g-k
"Cylindrocarpon" pauciseptatum	OL-CM3	*Olea europaea*	56 b-g	31.3 m-t	33.8 o-r	4.25 uv	12.5 c-f	35.8 j-n	0.67 b-j
	Cy196	*Vitis vinifera*	90 efg	23.7 a-k	22.4 abc	1.63 b-h	10.5 a-f	33.0 f,h-m	0.53 a-e
	Cy217	*Vitis vinifera*	52 b-g	29.6 i-s	30.1 d-p	3.82 r-v	11.4 a-f	30.1 b-l	0.72 b-j
	Cy238	*Vitis vinifera*	56 b-g	22.8 a-i	23.7 a-f	0.99 abc	10.8 a-f	22.4 abc	0.51 a-d
Ilyonectria vitis	Cy233	*Vitis vinifera*	85 d-g	22.4 a-h	29.1 c-p	2.98 k-s	13.2 efg	36.1 j-n	0.76 b-k
Ilyonectria alcacerensis	Cy134	*Vitis vinifera*	51 b-g	33.2 q-t	27.7 b-o	2.10 d-n	10.3 a-f	30.3 c-l	0.74 b-j
	CBS 129087	*Vitis vinifera*	83 d-g	25.2 a-m	25.0 b-i	2.00 c-l	11.6 a-f	27.2 b-i	0.68 b-j
Ilyonectria sp2	CBS 173.37	*Pinus laricio*	68 b-g	25.0 a-n	24.9 b-j	1.92 b-l	10.0 a-f	31.8 d-m	0.53 a-e
	Cy108	*Vitis vinifera*	76 c-g	33.8 rst	26.0 b-j,l	2.06 d-l,n	10.7 a-f	40.0 mn	0.93 g-k
	Cy200	*Vitis vinifera*	78 c-g	32.9 p-t	25.6 b-j	2.71 i-q	12.8 efg	43.0 n	0.99 i,k
	CBS 159.34	Unknown	70 b-g	29.0 g-r	25.0 b-i	1.98 c-l	10.0 a-f	31.5 d-l	0.57 a-f
Ilyonectria estremocensis	Cy135	*Vitis vinifera*	63 b-g	25.7 a-n	23.2 a-d	1.57 b-g	11.2 a-f	34.8 h-n	0.67 b-j
	CBS 129085	*Vitis vinifera*	71 b-g	27.1 d-q	26.0 b-l,n	1.68 b-j	10.3 a-f	31.7 e-l	0.71 b-j
	Cy152	*Vitis vinifera*	63 b-g	20.3 abc,e	25.4 b-j	1.49 a-f	9.8 a-f	30.0 b-l	0.61 b-g
	Cy243	*Vitis vinifera*	53 b-g	20.3 abc,e	16.8 a	0.49 a	8.9 abc	18.1 a	0.22 a
Ilyonectria macrodidyma	OL_CM4	*Olea europaea*	57 b-g	30.3 k-t	24.8 b-h	1.40 a-e	10.5 a-f	35.3 i-n	0.82 d-k
	OL_CM6	*Olea europaea*	74 b-g	33.8 rst	30.3 e-p	3.36 q-u	10.2 a-f	35.5 i-n	0.92 f-k
	Cy128	*Vitis vinifera*	61 b-g	20.9 abc,ef	22.0 abc	1.63 b-h	9.8 a-f	28.8 b-k	0.56 a-e
	Cy175	*Vitis vinifera*	70 b-g	26.4 c-o	28.2 b-o	2.20 d-n	11.0 a-f	34.2 h-m	0.79 c-k

Species	Strain Number	Host	%RI [a]	NR	LR	RDW	NSN	SL	SDW
	Cy250	*Vitis vinifera*	33 bcd	30.0 j-s	29.8 d-p	2.14 d-n	10.2 a-f	23.3 a-d	0.50 a-d
	CBS 112615	*Vitis vinifera*	58 b-g	35.8 st	32.7 k,m-r	1.98 c-l	10.0 a-f	29.0 b-k	0.77 c-k
Ilyonectria novazelandica	Cy230	*Festuca duriuscula*	26 abc	32.3 o-t	30.7 e-p	2.39 e-q	10.6 a-f	31.4 d-l	0.84 d-k
	CBS 113552	*Vitis* sp.	71 b-g	24.4 a-l	27.7 b-o	2.11 d-n	10.4 a-f	35.2 i-n	1.08 k
	Cy129	*Vitis vinifera*	75 c-g	25.2 a-m	26.2 b-l	1.97 c-k	12.1 b-f	35.6 i-n	0.70 b-j
	Cy130	*Vitis vinifera*	72 b-g	27.0 d,g-p	24.3 b-e	1.75 b-h	11.7 b-f	33.1 h-m	0.65 b-h
	CBS 112593	*Vitis vinifera*	69 b-g	32.1 n-t	29.9 d-p	2.63 h-q	12.4 c-f	35.5 i-n	0.97 h-k
Ilyonectria torresensis	Cy222	*Fragaria* x *ananassa*	92 efg	27.5 d,g-r	28.6 b-o	2.28 d-p	11.6 a-f	37.7 lmn	0.95 g-k
	HC7	*Olea europaea*	84 d-g	28.3 g-r	25.2 b-j	2.63 h-q	11.5 a-f	35.7 j-n	0.77 b-k
	Cy118	*Vitis vinifera*	70 b-g	25.0 a-l	25.4 b-j	1.71 b-i	10.9 a-f	36.3 j-n	0.63 b-h,j
	Cy214	*Vitis vinifera*	76 b-g	27.4 d,f-r	27.7 b-o	3.08 m-t	9.8 a-f	27.9 b-k	0.78 b-k
	CBS 129086	*Vitis vinifera*	73 b-g	25.3 a-m	28.8 c-o	3.25 o-u	8.1 a	23.4 a-e	0.70 b-j
	Cy260	*Vitis vinifera*	37 b-e	30.7 l-t	28.0 b-o	3.03 k-t	11.0 a-f	28.5 b-k	0.71 b-j
Ilyonectria europaea	Cy131	*Actinidia chinensis*	73 b-g	22.6 a-h	31.0 e-r	2.19 d-o	10.8 a-f	32.3 f-m	0.71 b-j
	CBS 537.92	*Aesculus hippocastanum*	96 g	28.1 g-r	24.8 b-h	2.73 i-q	9.6 a-f	26.6 a-h	0.61 b-g
	CBS 129078	*Vitis vinifera*	76 c-g	21.3 a-f	30.6 f-p	2.03 d-l	11.1 a-f	28.0 b-j	0.71 b-j
	Cy155	*Vitis vinifera*	85 d-g	19.8 abc	31.0 e-r	2.21 d-o	11.1 a-f	26.6 a-h	0.62 b-h
Ilyonectria liriodendri	CBS 110.81	*Liriodendron tulipifera*	75 b-g	27.5 d-r	23.8 a-h	0.77 ab	10.0 a-f	36.7 j-n	0.73 b-k
	Cy164	*Malus domestica*	51 b-g	29.0 h-r	33.0 m,o-r	2.90 k-r	13.0 fg	37.1 lmn	0.63 b-g
	Cy232	*Quercus suber*	64 b-g	25.2 a-n	33.3 m-r	2.77 j-r	11.3 a-f	32.7 f,h-m	0.74 b-k
	Cy5	*Vitis vinifera*	51 b-g	23.6 a-j	33.0 k-r	3.10 m,o-t	11.6 a-f	33.0 f,h-m	0.77 b-k
	CBS 117526	*Vitis vinifera*	88 efg	19.2 a	38.0 qr	1.99 c-l	11.3 a-f	31.8 e-m	0.66 b-j

Species	Strain Number	Host	%RI [a]	NR	LR	RDW	NSN	SL	SDW
	Cy190	*Vitis vinifera*	75 b-g	25.4 a-m	24.9 b-h	1.65 b-h	10.7 a-f	32.2 f,h-m	0.76 b-k
	Cy253	*Vitis vinifera*	67 b-g	26.0 a-o	32.4 j-r	3.97 s-v	11.0 a-f	26.1 a-h	0.61 b-h
Ilyonectria lusitanica	Cy197	*Vitis vinifera*	80 c-g	24.1 a-l	23.7 a-f	1.28 a-d	8.9 a-d	24.3 a-g	0.41 ab
Ilyonectria sp1	OL2	*Olea europaea*	19 ab	26.1 b-o	38.2 r	4.50 v	8.8 ab	21.8 ab	0.45 abc
Ilyonectria pseudodestructans	CBS 117824	*Quercus* sp.	83 d-g	20.0 ab	31.2 h-r	1.34 a-d	9.8 a-f	30.8 c-l	0.88 e-k
	CBS 117812	*Quercus* sp.	48 b-f	30.6 l-t	34.1 o-r	3.06 m,o-s	11.5 a-f	30.5 c-l	0.75 b-k
	CBS 129081	*Vitis vinifera*	53 b-g	28.2 g-r	36.2 pqr	3.35 p-u	11.3 a-f	33.8 h-m	0.97 g-k
	Cy22	*Vitis vinifera*	32 bcd	26.3 b-o	31.8 i-r	1.49 a-f	10.3 a-f	32.5 f,h-m	0.67 b-j
Ilyonectria robusta	CD1666	*Panax quinquefolium*	42 b-e	21.3 a-f	26.7 b-n	1.53 b-f	9.7 a-e	23.8 a-e,g	0.54 a-d
	CBS 308.35	*Panax quinquefolium*	74 b-g	25.7 a-o	24.0 a-g	1.45 a-f	11.1 a-f	34.5 h-m	0.62 b-g
	CPC 13532	*Prunus cerasus*	77 b-g	22.2 a-g	32.8 k-r	3.44 q-v	12.5 c-g	34.9 h-n	0.99 ijk
	CBS 117813	*Quercus robur*	72 b-g	23.1 a-i	30.8 e-q	2.60 g-q	11.4 a-f	34.9 h-n	0.74 b-k
	CBS 117815	*Quercus* sp.	75 b-g	27.5 d,g-r	21.4 ab	2.47 f-q	12.6 d-g	36.5 k-n	0.64 b-j
	Cy231	*Thymus* sp.	96 fg	26.6 b-q	27.0 b-o	3.40 p-v	11.6 a-f	35.7 h-n	0.60 a-j
	CBS 605.92	*Tilia petiolaris*	50 b-g	24.3 a-l	28.0 b-o	1.49 a-f	10.2 a-f	31.5 e-l	0.91 f-k
	Cy23	*Vitis vinifera*	92 efg	20.0 a-f	20.7 ab	1.52 a-h	10.1 a-f	32.1 e-m	0.65 b-j
	Cy158	*Vitis vinifera*	80 c-g	25.9 a-o	26.2 b-n	2.01 c-l,n	11.3 a-f	34.8 h-n	0.71 b-j
	CBS 129084	*Vitis vinifera*	71 b-g	28.8 g-r	31.0 g-q	1.96 c-l	11.5 a-f	34.7 h-m	0.78 b-k
	CBS 773.83	water	40 b-e	28.0 g-r	28.6 b-o	3.00 l-s	10.9 a-f	31.8 e-m	0.73 b-j

[a] In each column, values followed by the same letter do not differ statistically according to Tukey's test (p=0.05)

CHAPTER 6

A PROTOCOL FOR STABLE GENETIC TRANSFORMATION OF *ILYONECTRIA LIRIONDENDRI*, A FUNGAL PATHOGEN CAUSING ROOT ROT OF WOODY PLANT SPECIES

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Journal of Plant Diseases and Plant Protection (in preparation)

Keywords: *Agrobacterium tumefaciens*-mediated transformation; black foot disease; *Cylindrocarpon liriondendri*; green fluorescent protein; protoplast transformation; soil borne pathogen; *Vitis vinifera*.

Abstract

Ilyonectria liriondendri is a fungal pathogen causing black foot disease of grapevine and root rot in other woody hosts. Despite the economic importance and pathologic complexity of these diseases, no transformation tools have been developed for this fungus, impairing subsequent studies on the molecular mechanisms of pathogenicity. In this work, a protoplast transformation protocol is presented for the stable transformation of *I. liriondendri*, with subsequent confirmation of transformation by PCR amplification of the transgene and microscopic observation of green fluorescent protein-tagged mycelium. Several variations to an *Agrobacterium tumefaciens*-mediated transformation protocol are presented, nevertheless resulting unsuccessful for the transformation of *I. liriondendri*.

Introduction

Root rots are important diseases of woody plants, frequently associated with decline of young or nursery plants, where they can cause severe losses. The economic importance of these diseases, along with the complex host infection and colonization mechanisms, suggest the need of in-depth studies to understand key steps of plant-pathogen interactions, and histopathology and epidemiology of their pathogens. The development of efficient transformation methods conducing to a stable integration of reporter genes, such as the green fluorescence protein gene (*gfp*), that can be visualised by fluorescence microscopy and also studies of gene function by disruption or silencing (Knight et al., 2009) are fundamental for subsequent studies on the molecular mechanisms regulating the pathogenicity life styles and host preference.

Ilyonectria liriondendri (anamorph "*Cylindrocarpon*" *liriondendri*) is a fungal pathogen causing root rot of different woody plant species, such as apple, oak and tulip trees, including black foot disease of grapevine. Although several studies have been conducted on the pathogenicity and virulence of this pathogen (e.g., Cabral et al., 2011; Tewoldemedhin et al., 2011), no genetic transformation tools have thus far been reported for *I. liriodendri*.

In this work we report the optimisation of a protoplast transformation protocol for *I. liriodendri*, upon several unsuccessful attempts to adapt an *Agrobacterium tumefaciens*-Mediated Transformation (ATMT) protocol, previously developed for *Fusarium oxysporum* (Mullins et al., 2001). Stability of protoplast transformation was then tested by PCR amplification of the transgenes both in mycelia of the transformed fungus and in grapevine plants inoculated with the transformed fungus.

Materials and methods

Fungal strains and transformation vector

Ilyonectria liriondendri isolate Cy5 was selected for this study among virulent isolates from grapevine (Rego et al., 2001; Rego, 2004). The transformation vector used was the plasmid pBHt2-GFP (Talhinhas et al., 2008), a 11.2 kb binary vector containing the hygromycin B resistance (*hph*) gene under the *Aspergillus nidulans trp*C promoter and the *gfp* gene with *A. nidulans gpd*A promoter and *trp*C terminator.

Protoplast transformation

Protoplast transformation of fungal cells was adapted from Rogers et al. (2004). Conidia of *I. liriondendri* were inoculated into Potato Dextrose Broth (PDB, Difco) and cultivated for 3 d (enough just for slight mycelial growth) at 22ºC under gentle agitation. The mycelium was recovered by centrifugation for 10 min at 2300 g, the liquid was discarded, and the mycelial pellet washed twice with sterile water. Approximately 2 g of fresh mycelia was ressupended in 10 ml of filter-sterilized (0.2 µm) GCC lytic mix (10 mg ml^{-1} β-D-glucanase (InterSpex Products), 1 mg ml^{-1} chitinase from *Streptomyces griseus* (Sigma-Aldrich) and 20 mg ml^{-1} cellulase Onozuka R10 from *Trichoderma viride* (Duchefa) prepared in filter-sterilized OM buffer (1.2 M $MgSO_4$, 10 mM sodium phosphate buffer pH 5.8) and incubated for 3 h at 29°C at 150 rpm. After the incubation period, 30 ml of filter-sterilized OM buffer was added. The suspension was carefully overlaid with 4 ml of ST buffer [0.8 M sorbitol (Sigma-Aldrich), 100 mM Tris pH 7.5] by pipetting it slowly down the inside of the tube. The tubes were centrifuged 20 min at 1500 g, and the protoplasts were recovered from the ST/OM interface with a wide-bore pipette. Five volumes of ST buffer were added and the tubes were centrifuged 5 min at 1000 g. The pellet was washed twice with 20 ml ST buffer and once with 20 ml STC buffer (0.8 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris pH 7.0). The protoplasts were resuspended in STC buffer at aprox. 10^8 protoplasts ml^{-1}. Plasmid pBHt2-GFP (2 µg) were added to 10^7 protoplasts (in 100 µl) and incubated on ice for 30 min. After the incubation period, 900 µl PTCS buffer [40% Polyethyleneglycol 4000 (BDH), 0.8 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris pH 7.5] were added with gentle mixing by inverting the tube several times and incubated at room temperature for 30 min. Another 500 µl PTCS were added to the tubes and immediately plated onto osmotically stable selective media [Potato Dextrose Agar (PDA, Difco) + 0.8 M sucrose + 100 µg ml^{-1} hygromycin (Sigma-Aldrich)]. The plates were incubated at 24 ºC until colonies emerged.

The emerging colonies were transferred to PDA supplemented with 100 µg ml^{-1} hygromycin and after 15 d the mycelium was scraped and visualized by fluorescence microscopy to check for GFP expression. To check the stability of the transformation, the cultures were replicated three times to PDA without hygromycin and after that were plated again in PDA with hygromycin.

PCR detection of the transgene

The presence of the transgene in the genome of the fungus growing in selection medium was checked by PCR upon DNA extraction using a rapid freeze-boil protocol (Talhinhas et al., 2008). DNA was amplified using specific primers for the hygromycin resistance gene (HPHF – 5’

CGATCTTAGCCAGACGAGCG 3' and HPHR – 5' TTGCCCTCGGACGAGTGCTG 3') and the GFP gene (eGFP361 – 5' GGCCACAAGTTCAGCGTGTC 3' and eGFP998 – 5' AGCTCGTCCATGCCGAGAGT 3'). In a 12.5 µl volume, PCR reaction included 2.5 mM $MgCl_2$, 0.5 U Taq DNA Polymerase (MBI Fermentas), including the appropriate buffer, 50 mM dNTPs, 0.5 µM of each primer and 2.5 µl DNA. The PCR amplification was performed in a T-Gradient thermocycler (Biometra) programmed for 94ºC for 5 min, followed by 30 cycles of 94ºC for 45 s, 55ºC for 30 s and 72ºC for 1 min and a final extension of 72ºC for 5 min (Talhinhas et al., 2008).

Viability of transformants

In order to test the pathogenic viability of the transformed fungus, a transformed strain was inoculated in plants of the grapevine rootstock 1103P as previously described (Cabral et al., 2011). Wild-type fungus was used as control. Briefly, cuttings were rooted for 1.5 to 2 months, roots were dipped in a 10^6 ml^{-1} conidial suspension, and plants grown in 1 l bags containing a mixture of soil, peat and sand (2:1:1, v/v/v) under greenhouse conditions (at 24±5°C day and 18°C night) for one year. Sections obtained at 0-2.5 cm, 2.5-5 cm and 5-10 cm from the base of the cutting, as well as from roots, were used for DNA extraction employing the protocol by Cenis (1992), with the following adaptations: sections of plants were ground to a fine powder in a mortar aided by liquid nitrogen; the extraction buffer included 2.5% SDS and 1% PVP 40; plant material was incubated in the extraction buffer for 10 min at 65ºC; incubation at -20ºC in the presence of sodium acetate was extended to 20 min.; a ½ vol. chloroform extraction was performed prior to isopropanol precipitation. Extracted DNA was subjected to PCR amplification with *hph* and *gfp* specific primers (as described above), as well as with *Ilyonectria* spp./*Cylindrocarpon* spp. specific primers for the rDNA-ITS gene (Nascimento et al., 2001).

Agrobacterium tumefaciens–mediated transformation

Agrobacterium tumefaciens–mediated transformation was tested using the protocol by Mullins et al. (2001) with modifications. Competent *A. tumefaciens* strain AGL-1 (penn.) cells were subcultured from a -80ºC stock and grown at 26ºC for 2 d in plates containing Luria-Bertani Medium (LB) supplemented with kanamycin (30 µg ml^{-1}) and rifampicin (80 µg ml^{-1}). After this, a colony was picked and inoculated on 5 ml of LB medium supplemented with kanamycin (30 µg ml^{-1}) and incubated overnight at 26ºC in the dark on a rotary shaker (150 rpm). The bacterial suspension (2 ml) was transferred to a flask containing 25 ml of minimal medium [MM – 11.8 mM K_2HPO_4 +10.7 mM KH_2PO_4, pH 7.0; 2.0 mM $MgSO_4.7H_2O$; 2.6 mM NaCl; 456.4 µM

$CaCl_2.2H_2O$; 10.1 mM glucose; 9 µM $FeSO_4.7H_2O$; 3.8 mM $(NH_4)_2SO_4$ (Hooykaas et al., 1979)] supplemented with kanamycin (30 µg ml^{-1}) and incubated as described above. The bacterial suspension was diluted to (optical density) OD_{660} = 0.15 in induction medium [IM – as MM, with the following modifications: 40 mM 2-(N-Morpholino)-ethanesulfonic acid and 0.5% glycerol, 5 mM glucose (Bundock et al., 1995)] in the presence of 200 µM acetosyringone (AS) and kanamycin (30 µg ml^{-1}). The cells were grown at 26ºC under agitation (150 rpm) for an additional period of 6 h before mixing them with the fungal culture.

Conidial suspensions with 10^6 conidia ml^{-1} were prepared from 5-d old PDB cultures. For each isolate four different combinations of conidial suspension and *A. tumefaciens* were mix together (100 µl/100 µl, 100 µl/200 µl, 200 µl/100 µl, 200 µl/200 µl) on IM agar plates containing 200 µM AS and cellophane discs (P32J film, Cannings packaging Ltd).

Different co-cultivation periods were tested (at 22 and 24ºC), from 1 to 5 d, including a 2 d pre-cultivation of *I. liriodendri*. Co-cultivation was also conducted with 100 µl bacteria/100 µl of 10^6 conidia ml^{-1}, at 22ºC for 2 d. Co-cultivation was also tested in liquid medium (IM without agar + 200 µM AS) under gentle agitation at 22ºC for 2 or 3 d in 1.5-ml tubes by mixing 100 µl bacterial suspension (in IM containing 200 µM AS) and 100 µl of 10^6 conidia ml^{-1} suspension. After the co-cultivation, the cellophane discs were cut into eighths and transferred to two PDA selection plates containing 100 µg ml^{-1} hygromycin and 200 µM cefotaxime. The product of co-cultivation in liquid medium was spread directly into selection plates. In both cases, selection plates were incubated at 22ºC for up to two months.

Colletotrichum acutatum was used as positive control (Talhinhas et al., 2008). Three co-cultivation plates were used for each modality (quantity of conidia and number of days of pre-germination).

Results and discussion

Co-cultivation of *I. liriodendri* conidia with *A. tumefaciens* strain AGL1 containing vector pBHt2-GFP consistently yielded no transformants in more than five independent experiments, regardless of variations in the co-cultivation time or in the fungi/bacteria proportions. These results, in opposition to the successful transformation of the closely related species *F. oxysporum* (Mullins et al., 2001) or of the positive control *C. acutatum*, with similar efficiencies to those reported by Talhinhas et al. (2008), suggest that *I. liriodendri* may not be amenable for

ATMT. Although ATMT of fungi is usually successful, there are also cases of fungal strains recalcitrant to ATMT (Michielse et al., 2005).

Although more time consuming and much less efficient in general terms, transformation of protoplasts proved the best strategy for transforming *I. liriodendri*. In this work, an average of 2 transformants was obtained per co-cultivation plate, which corresponds to an efficiency of transformation of 2 transformants per 10^7 protoplasts. Stability of transformation was tested by successful cultivation of transformants in medium containing hygromycin after cultivation for three times in medium without hygromycin. Hyphae from the transformants expressed the green fluorescent protein, according to epifluorescence microscopy visualisation (Figure 1). No fluorescence was observed for the wild-type fungi.

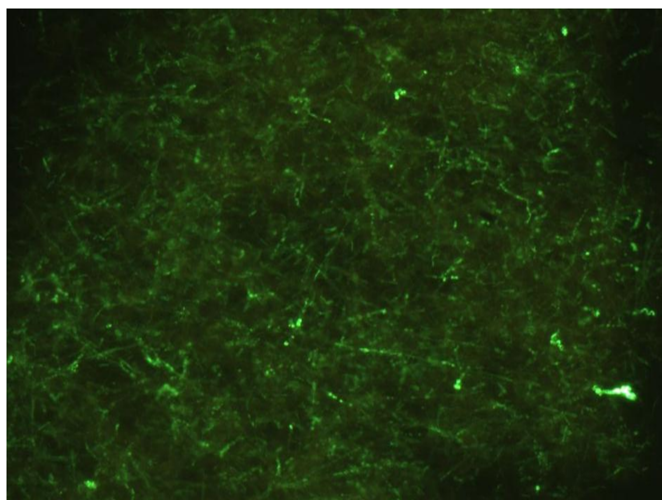

Fig. 1 Hyphae of transformed *Ilyonectria liriodendri* visualized in fluorescence microscopy showing the expression of the Green Fluorescent Protein.

PCR amplification of *hph* and *gfp* genes from DNA extracted from mycelia obtained in artificial culture yielded fragments of the expected sizes for a selection of transformants and no fragments for the wild type fungi, showing the presence of the transgene in the genome of *I. liriodendri* (Figure 2).

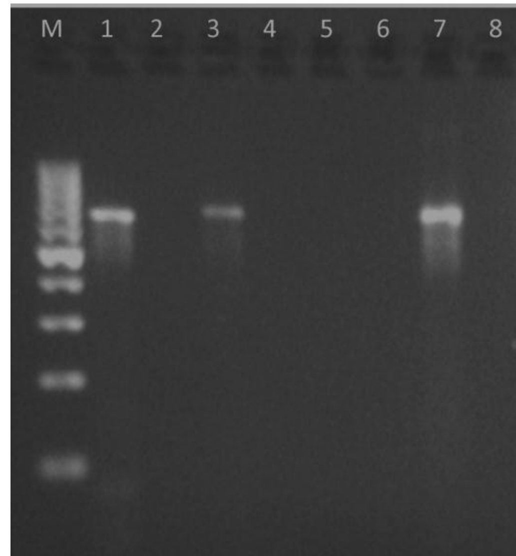

Fig. 2 PCR amplification using the *hph*-specific primers (similar results were obtained with *gfp*-specific primers; data not shown) of DNA isolated from mycelium of a transformed *Ilyonectria liriodendri* strain (lane 1) and of a wild-type strain (lane 2) and from stems (lanes 3 and 5) and roots (lanes 4 and 6) of grapevine plants inoculated with transformed- (lanes 3 and 4) and wild-type strains (lanes 5 and 6); positive control is DNA of vector pBHt2-GFP (lane 7); lane 8 represents the no-template control M – Low Range DNA marker (MBI Fermentas; bright band is 500 bp).

Similarly, PCR amplification of *hph* and *gfp* genes from DNA extracted from infected plants yielded fragments of the expected sizes and no fragments for the wild type fungi, for which only amplification with *Ilyonectria* spp./*Cylindrocarpon* spp. specific primers was achieved (data not shown). Positive results were achieved for all stem samples, but not for root samples, suggesting that the pathogen migrated to the stem over the one-year infection (Figure 2). Further, these results demonstrate that the transformants retained the insertion of the transgene, showing the stability of the transformation.

This is the first report of successful transformation of *I. liriodendri*. The protocol described here enables the stable insertion of genes of interest in *I. liriodendri* genome, opening routes for tagged mutagenesis, gene knock-out or heterologous expression studies. Further, strains expressing the GFP may be used for histopathology studies, enabling detailed insights into the complex infection and colonization mechanisms of *I. liriodendri*.

Acknowledgements

This article is part of a Ph.D. dissertation (Instituto Superior de Agronomia, Technical University of Lisbon, Portugal). This work was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/24790/2005; project PTDC/AGR-AAM/099324/2008).

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CHAPTER 7

RELATIVE QUANTIFICATION OF *ILYONECTRIA* SPP. FROM GRAPEVINE NURSERY AND VINEYARD SOILS

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Australian Journal of Grape and Wine Research (in preparation)

Keywords: Black foot disease of grapevines; nursery; *Cylindrocarpon*; quantitative PCR; soil DNA extraction.

Abstract

Background and Aims: Black foot is an important disease of grapevines, with global distribution, and recent increased incidence and severity, affecting nurseries and young plantations. The disease is caused by soil-borne fungi, predominantly representing *Ilyonectria* spp. The objective is to compare the level of *Ilyonectria* spp. infestation in nursery soils at different stages of the four-year rotation cycle, and in the soils of mother-plant fields and vineyards.

Methods and Results: Employing quantitative PCR using specific primers, no reliable detection occurred bellow 100 fg genomic DNA, with a window of linearity of 1 ng-1 pg. Results suggest the presence of inhibitors in the soil DNA samples affecting qPCR results, leading to the employment of a 1000-fold dilution in the analysis.

Conclusions: Results of qPCR amplification in soil samples demonstrate the effect of rotation in the reduction of levels of the black foot pathogens in nursery soils, and corroborate other reports indicating that levels of *Ilyonectria* infestation in vineyard soils are lower than in nursery or mother-plant soils. A field that was abandoned due to rooting problems yielded the highest *Ilyonectria* spp. DNA content, estimated in 87 $ng.g^{-1}$ of soil.

Significance of the study: Black foot pathogens are more frequent in nursery and rootstock mother-vine soils than in vineyard soils.

Introduction

Black foot is an important disease of grapevines in most countries throughout the world, with recent increased incidence and severity. The disease affects both nurseries and young plantations, causing typical darkening of the basal end of the rootstock plants (Halleen et al. 2004, Oliveira et al. 2004). Declining plants are frequently found in infected vineyards, showing slow growth, reduced vigour, retarded sprouting, shortened internodes, sparse and chlorotic foliage (Rego et al. 2000, Halleen et al. 2006), resulting frequently in plant death, forcing growers to replant considerable areas (Scheck et al. 1998). The disease is caused by several *Cylindrocarpon*-like species, 11 of which cluster in the genus *Ilyonectria* (Cabral et al. 2011a, 2011b) and two in the genus *Campylocarpon* (Halleen et al. 2004). *Campylocarpon fasciculare* and *Campyl. pseudofasciculare* seem to be infrequent and geographically restricted, and are not addressed in this study. Within *Ilyonectria*, *I. liriodendri*, *I. macrodidyma* and "*Cylindrocarpon" pauciseptatum* (a *Cylindrocarpon*-like species pending black foot, although recently several species were described: *I. alcacerensis*, *I. estremocensis*, *I. novozelandica* and *I. torresensis* from the *I. macrodidyma* species complex (Cabral et al. 2011b); *I. europeaea*, *I. lusitanica*, *I. pseudodestructans* and *I. robusta* from the *I. radicicola* species complex, and *I. vitis* which is close to "*C.*" *pauciseptatum* (Cabral et al. 2011a).

Ilyonectria spp. are common soilborne fungi, but factors determining their occurrence are poorly understood, namely which aspects influence the population dynamics of *Ilyonectria* spp. in the soil. Also, the relative importance of infection occurring on nursery *versus* vineyard soil is not fully understood, nor the factors affecting it, although several reports suggest that the pathogens are isolated at much higher frequencies from nursery plants than from vineyard plants (Rego et al. 2000, Fourie and Halleen 2001, Halleen and Crous 2001, Rego et al. 2001).

In Portugal, rules governing grapevine nurseries made rotation compulsory since 2006, where at least three years without grapevine cultivation are required (typically rotation is carried out with horticultural crops). However, no studies have been undertaken to monitor *Ilyonectria* spp. in the soil during the rotation cycle.

Methods for detection and quantification of soil-borne fungal pathogens in soils by quantitative PCR (qPCR) have been optimised with the purpose of rapidly and reliably assess the disease risk of a particular soil, for example in *Pythium intermedium* in forest soils (Li et al. 2010) and in *Nectria haematococca* causing pea footrot disease (Etebu and Osborn 2010), since fungal inoculum densities assessed by culture-based assays have been shown to have a high and significant correlation with molecular assays (e.g., Mahuku et al. 1999, Etebu and Osborn 2010).

Furthermore, some reports suggest that qPCR could be a more reliable measure of fungal populations than bioassay or plating data (Kernaghan et al. 2007). In *Cylindrocarpon*/*Ilyonectria*, qPCR quantification of pathogens have been reported for *C. destructans* f. sp. *panacis* (recently renamed *I. mors-panacis*; Cabral et al. 2011a) causing root rot and replant diseases in ginseng (Kernaghan et al. 2007) and for four *Cylindrocarpon*-like spp. associated with apple tree root rot (Tewoldemedhin et al. 2011). Detection of pathogens causing black foot disease of grapevine has thus far only been described by means of conventional PCR (Nascimento et al., 2001, Damm and Fourie 2005, Dubrovsky and Fabritius 2007), and the PCR primers employed are inadequate for qPCR and for the breadth of *Ilyonectria* species diversity associated with this disease.

The objective of this study was to perform a quantitative comparison of *Ilyonectria* spp. in different nursery and vineyard soils. Samples from nursery soils include the final stage of the rooting process (representing potentially the maximum of *Ilyonectria* spp. infestation) and the end of the three-year cultivation with horticultural crops, just prior to the planting of graftlings (representing potentially the minimum of *Ilyonectria* spp. infestation). Samples from vineyard soils represent different levels of disease incidence and severity in vineyards and mother-plant fields.

Material and Methods

Experimental design

Sampling was conducted in grapevine nurseries, in fields with rootstock and scion mother-vines and vineyards with different histories of black foot disease (Table 1). Also different soil samples taken from fields during the compulsory crop rotation cycle, or intended for use in nurseries were sampled.

Nurseries, located in the main grapevine nursery area of Portugal (Bombarral, Óbidos and Caldas da Rainha), were surveyed in late Autumn, just at the end of the rooting process. Nurseries 1-4 were surveyed in 2005 and Nursery 5 was surveyed in 2006, and the following samples were collected: a field cultivated with eucalypts, which would be a nursery in the next year (sample N1); a field cultivated with grapevine nursery materials (mainly graftlings of Tinta Roriz/1103P; sample N2); a field cultivated with rootstock nursery material, following a rotation with horticultural crops, that would also be a nursery in the next year (sample N3); a field that was abandoned as nursery, because of recurring problems in grapevine materials related with failure to root (sample N4); a field cultivated with grapevine nursery materials (mainly graftlings of

Tinta Roriz/1103P) sampled in 2006 (sample N5). Nurseries 6-13 were surveyed in 2010: samples N6 to N10 were obtained from fields at the end of the graftling cultivation cycle following three-year rotations (previous grapevine nursery material cultivated in 2006; samples N6 to N8) or a longer rotation cycle (no grapevine nursery since 2006; samples N9 and N10); three samples were obtained from fields at the end of the three-year horticultural crop rotation (grapevine nursery in 2006 and 2007; samples N11 and N12) or after long term perennial ryegrass cultivation (no grapevine nursery since 2006; sample N13). Nurseries N14 to N18 were also surveyed in 2010, all sharing the fact that these fields were at the end of a rotation period, prior to use as grapevine nurseries, except for sample N15, which has not been used as a nursery for 5 years.

Adult mother plants were sampled in 2010, either from rootstock-mother plants (R110 – sample MP1, and 1103P – samples MP2 and MP3) or from scion-mother plants (Touriga Nacional – MP4, and Tinta Roriz – MP5). Vineyards were sampled in 2007 (samples V1 to V3) and 2010 (samples V4 and V5), at different locations (Arruda dos Vinhos – samples V1 to V3; Estremoz – sample V4; Gouvinhas, Sabrosa – sample V5), and representing different black foot severity levels (no symptoms – samples V1, V4 and V5, and presence of dead plants – samples V2 and V3).

Table 1. List of soil samples used in this study

Sample	Description	Year	Previous crop	Cq value	ng DNA per gram of soil
MP1	A field with rootstock mother-plants (R110)	2010	not applicable	33.4	6.53
MP2	A field with rootstock mother-plants (1103P)	2010	not applicable	34.2	3.75
MP3	A field with rootstock mother-plants (1103P)	2010	not applicable	35.6	1.58
MP4	A field with scion mother-plants (Touriga Nacional)	2010	not applicable	33.8	4.92
MP5	A field with scion mother-plants (Tinta Roriz)	2010	not applicable	34.7	2.79
N1	A forest field (*Eucalyptus*), which would be a nursery next year	2005	not applicable	36.0	1.21
N2	Grapevine nursery at the end of the rooting process	2005	vegetables	no Cq	0.00
N3	Grapevine nursery at the end of the rooting process	2005	vegetables	37.0	0.61
N4	A field that was abandoned as nursery, because of recurrent problems in grapevine materials related with failure to root	2005	grapevine	29.4	87.2
N5	Grapevine nursery at the end of the rooting process	2006	vegetables	35.0	2.25
N6	Grapevine nursery at the end of the rooting process; sandy soil, pH 6.8	2010	3-yr rotation with vegetables	35.0	2.30
N7	Grapevine nursery at the end of the rooting process; sandy soil, pH 7.8	2010	3-yr rotation with vegetables	34.7	2.78
N8c	Grapevine nursery at the end of the rooting process; sandy soil, pH 6.8	2010	3-yr rotation with vegetables	35.1	2.16
N9	Grapevine nursery at the end of the rooting process; sandy soil, pH 6.3	2010	long rotation	36.6	0.837
N10	Grapevine nursery at the end of the rooting process; sandy soil, pH 6.3	2010	long rotation	36.5	0.863
N11	A field at the end of the three-year horticultural crops cultivation; sandy soil, pH 6.9	2010	3-yr rotation with vegetables	no Cq	0.00
N12	A field at the end of the three-year horticultural crops cultivation; sandy soil, pH 6.1	2010	3-yr rotation with vegetables	42.9	0.0136
N13	A field after long term perennial ryegrass cultivation; sandy soil, pH 6.1	2010	perennial ryegrass	40.0	0.0888
N14	A field in rotation, which will be a nursery next year; sandy soil, pH 7.2	2010	3-yr rotation with vegetables	33.6	5.75
N15	A field in rotation, which will be a nursery next year; sandy soil, pH 7.0	2010	3-yr rotation with vegetables	32.7	9.89
N16	A field in rotation, which will be a nursery next year; sandy soil, pH 7.3	2010	3-yr rotation with vegetables	34.9	2.43
N17	A field in rotation, which will be a nursery next year; sandy soil, pH 7.2	2010	3-yr rotation with vegetables	34.9	2.43
N18	A field in rotation, which will be a nursery next year; sandy soil, pH 6.7	2010	3-yr rotation with vegetables	33.1	7.77
V1	An healthy young vineyard	2007	not applicable	44.1	0.00642
V2	An young vineyard with dead plants	2007	not applicable	35.0	2.37
V3	An young vineyard with dead plants	2007	not applicable	34.7	2.75
V4	An healthy vineyard; medium texture soil, pH 7.5	2010	not applicable	no Cq	0.00
V5	An healthy young vineyard; fine texture soil, pH 5.0	2010	not applicable	no Cq	0.00

Soil sampling and DNA extraction

In each field, 15 samples were collected randomly, at 5-20 cm deep, close to plants but avoiding roots, and close to diseased plants in the case of grapevines with black foot symptoms. Composite samples, representing each field, were carefully homogenised, sieved (2 mm) and stored at 4ºC until use. All material used was disinfected before handling new samples.

DNA was extracted from 0.5 g of soil following a protocol adapted from Crous *et al.* (2009). In a 2-ml screw-cap tube, 0.5 g each of 0.1 mm and 2 mm diameter glass beads were added to the soil sample along with 500 µl TES buffer (100 mM Tris pH 8.0, 10 mM EDTA pH 8.0 and 2% SDS). The tubes were shaken horizontally on a vortex shaker for 10 min at maximum speed, boiled for 3 min and then placed on ice water for 10 min. After this period, 10 µl of Proteinase K (20 mg/ml) were added to each tube and the tubes were incubated for 30 min at 65ºC. After that, the salt concentration was increased by adding 140 µl of 5 M NaCl. 65 µl of 10% (w/v) Cetyltrimethylammonium bromide were added to each tube which was incubated again for 30 min at 65ºC. After the incubation period, 1 ml of chloroform:isoamylacohol (24:1) was added to each tube and mixed carefully by hand and then incubated during 30 min at 0ºC (on ice water). The tubes were centrifuged for 10 min at 4ºC at 12 000 rpm, and the top supernatant was transferred to a clean tube. 225 µl 5 M ammonium acetate was added and tubes were mixed carefully. The tubes were placed for at least 30 min on ice water, centrifuged 10 min at 4ºC at 12 000 rpm. The supernatant was transferred to a clean sterile tube. 0.55 volumes of ice-cold isopropanol were added (filling until 1.5 ml) to the supernatant, mixed carefully and incubated for 30 min on ice water. Samples were centrifuged directly for 10 min at 12 000 rpm at 4 ºC, the supernatant discard and the pellet washed with ice-cold ethanol 70% (centrifuging for 10 min and discarding the supernatant). The pellet was air-dried and resuspended in ca. 50 µl TE buffer. Subsequently, the DNA extracted was purified using polyvinylpolypyrrolidone columns, as described by Damm and Fourie (2005).

The concentration and quality of DNA was evaluated by spectrophotometry (BioTek Synergy HT), and 0.8% agarose gel electrophoresis.

All soil samples were extracted twice and treated as repetitions.

PCR inhibitors

With the purpose of avoiding the potential inhibitory effect of soil components (e.g., humic acids) in PCR, dilution series (1/10, 1/50, 1/100, 1/500, 1/1000, 1/5000 and 1/10000) of two

contrasting soil samples were established and subjected to PCR and qPCR in order to determine the dilution level below which no inhibition effect was recorded.

Design of PCR primers

Primers designed for the purpose of amplifying *Ilyonectria* pathogens responsible for black foot disease of grapevines, should not amplify fungi responsible for other grapevine diseases (including *Campylocarpon* spp.). Therefore, the nucleotide sequences of the rDNA-ITS gene block from 27 *Ilyonectria* spp. were aligned along with some *Neonetria* spp., and *Campyl. fasciculare* and *Campyl. pseudofasciculare* (Cabral et al. 2011a). Primers (ITSrtF - CATCGAATCTTTGAACGCAC and ITSrtR - GTGTGCTACTACGCAGAGGAAG) are located in the 5.8 S and in the ITS2 regions respectively, amplifying a 177-180-bp fragment, except for *Neo. macroconidialis* that amplified a fragment with 218 bp. Primer 3 software (Rozen & Skaletsky 2000) was used to analyse primers melting temperature, self-homology, cross-homology and internal stability. The specificity of the primers was tested experimentally using DNA extracted as previously described (Nascimento et al. 2001) from pure cultures of isolates clustering in 27 species of *Ilyonectria* and *Cylindrocarpon*-like (Cabral et al. 2011a and b), namely from species isolated from grapevines (such as "*Cylindrocarpon*" sp.2, "*C.*" *pauciseptatum*, *I. alcacerensis*, *I. estremocensis*, *I. europaea*, *I. liriodendri*, *I. lusitanica*, *I. novozelandica*, *I. pseudodestructans*, *I. robusta*, *I. torresensis* and *I. vitis*), and annealing temperature optimised by gradient PCR (58-64°C) following the protocol described in next section for ITSrtF – ITSrtR with 40 amplification cycles. Negative control for this test was DNA obtained from fungi commonly found in grapevine tissues: "*Botryosphaeria obtusa*", "*B.*" *viticola*, *Botrytis cinerea*, *Campylocarpon fasciculare*, *Campyl. pseudofasciculare*, *Colletotrichum acutatum*, *Fusarium oxysporum*, *Pestalotiopsis menezesiana*, *Phaeomoniella chlamydospora*, *Phomopsis* sp. and *Truncatella angustata*.

PCR and qPCR

For the conventional PCR, a nested-PCR strategy was used in order to amplify the fungi from soil. Initially, the DNA from the soil was amplified combining the fungal-specific primer ITS1F (Gardes and Bruns, 1993) along with the universal primer ITS4 (White *et al.*, 1990). Subsequently the resulting PCR product was used in a second amplification stage with primers ITSrtF and ITSrtR. All amplifications were carried out in a 12.5 µl volume, including 0.5 U Dream Taq DNA Polimerase (MBI Fermentas), 1× PCR Buffer with 2.0 mM $MgCl_2$, 32 µM dNTPs, 0.24 µM of each primer and 2.5 µl of 100× diluted DNA extracted from soil for the first PCR or 1 µl of DNA amplified in the first stage for the second PCR. PCRs were performed in a S1000 (Bio-Rad)

thermocycler programmed for 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and a final extension of 72°C for 10 min. The second PCR began at 95°C for 5 min, followed by 25 cycles of 95°C for 15 s and 60°C for 30 s and a final extension of 72°C for 5 min. Amplified products from PCR reactions were separated by electrophoresis in 2% agarose gel in TBE buffer (45 mM Tris-HCl pH8.0, 45 mM H_3BO_3 and 1 mM EDTA) for 2 h at 120 V, stained with ethidium bromide (0.5 µg L^{-1}) and photographed under ultraviolet light.

All qPCR amplifications were performed using a MJ Mini thermocycler (Bio-Rad) equipped with a MiniOpticon Real Time PCR 610 System (Bio-Rad), using SsoFastEvaGreen Supermix (Bio-Rad). Each 15 µl reaction comprised 3 µl diluted DNA sample, 1x SsoFastEvaGreen Supermix and 300 nM of each primer. The reactions were subjected to an initial denaturation step at 98°C during 2 min, followed by 45 cycles at 98°C for 10 s, 60°C for 20 s. A melting curve analysis was performed at the end of the PCR run over the range 60-95°C, increasing the temperature in a stepwise fashion by 0.5°C every 10 s. Each PCR reaction was performed in duplicate and the specificity of the amplicons was checked by melting curve analysis and by 3% agarose gel electrophoresis.

Reference samples

Reference DNA samples for qPCR were obtained from pure cultures of *Ilyonectria* (*Ilyonectria* sp. 1, isolate CBS 162.89; *I. liriodendri*, isolates CBS 117526; *I. novozelandica*, isolate Cy130; *I. pseudodestructans*, isolates CBS 129081 and Cy22; *I. torresensis*, isolate Cy72). These fungi were grown and DNA extracted as previously described (Nascimento et al. 2001). DNA concentration was established by spectrophotometry and confirmed by electrophoresis in 0.8% agarose gels. Eight 10x serial dilutions of the DNA samples were used for each isolate, in the 10 ng -1 fg range (values refer to quantity of DNA per reaction, in 15 µl reactions). Regression equations were calculated for each sample between the logarithm of DNA concentration and Cq values. An overall equation was established and used to estimate the quantity of DNA in soil samples, taking in consideration the dilution factor employed according to the analysis regarding qPCR inhibitors.

Analysis of qPCR data

The qPCR data generated were analysed using LinRegPCR software (Ramakers et al. 2003), from which Cq values were derived for each sample. Replicates were averaged and Cq values were converted into quantity (ng) of *Ilyonectria* spp. DNA in each gram of soil.

Results and Discussion

PCR specificity, sensitivity and inhibitors

The PCR primers developed in this study were shown to be specific for *Ilyonectria* spp., as amplification of PCR products (ca. 180 bp) was only achieved for the *Ilyonectria* spp. samples, and not for *Campylocarpon* spp., *Colletotrichum* sp., *Fusarium* spp. or *Phaeomoniella* sp. (data not shown).

Regression analysis between the logarithm of the amount of genomic DNA and qPCR Cq values for six isolates from different species revealed that, in most cases, no reliable detection could be achieved bellow 100 fg of genomic DNA, and that the window of linearity ranged from 1 ng to 1 pg, with correlation coefficients (r^2) of 0.999 for all isolates and slopes of regression curves ranging from 3.26 to 3.77, leading to efficiencies of 84 to 102% (Figure 1).

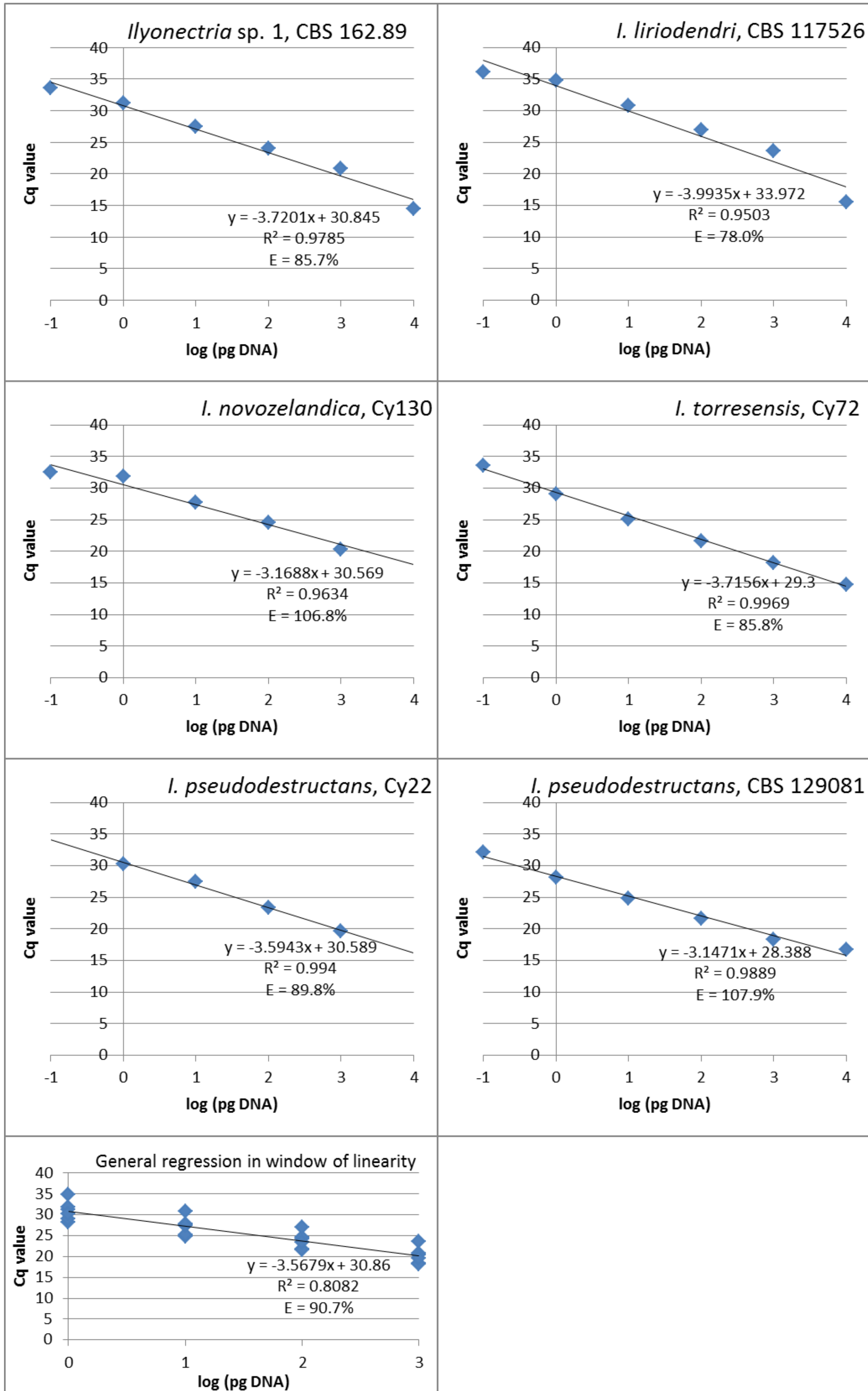
Ilyonectria sp. 1, CBS 162.89
y = -3.7201x + 30.845
R² = 0.9785
E = 85.7%
I. liriodendri, CBS 117526
y = -3.9935x + 33.972
R² = 0.9503
E = 78.0%
I. novozelandica, Cy130
y = -3.1688x + 30.569
R² = 0.9634
E = 106.8%
I. torresensis, Cy72
y = -3.7156x + 29.3
R² = 0.9969
E = 85.8%
I. pseudodestructans, Cy22
y = -3.5943x + 30.589
R² = 0.994
E = 89.8%
I. pseudodestructans, CBS 129081
y = -3.1471x + 28.388
R² = 0.9889
E = 107.9%
General regression in window of linearity
y = -3.5679x + 30.86
R² = 0.8082
E = 90.7%
Cq value
log (pg DNA)

Fig. 1. Correlation between the logarithm of the amount of genomic DNA and Cq values for six *Ilyonectria* spp. isolates. The efficiency of amplification for each isolate was calculated using the formula: E=-1+10(-1/slope).

The qPCR analyses of serial dilutions of soil samples showed that no amplification was obtained for the 1/10 dilutions (Figure 2) and that the 1/50 dilutions originated higher Cq values than those obtained in the 1/100 dilutions. From this dilution onwards Cq values followed a linear relation to the dilution factor. These results suggest the presence of inhibitors in the soil DNA samples affecting qPCR results at least in the 1/10 and 1/50 dilutions. As a precaution, the 1/1000 dilution was chosen for all soil samples screened in this study.

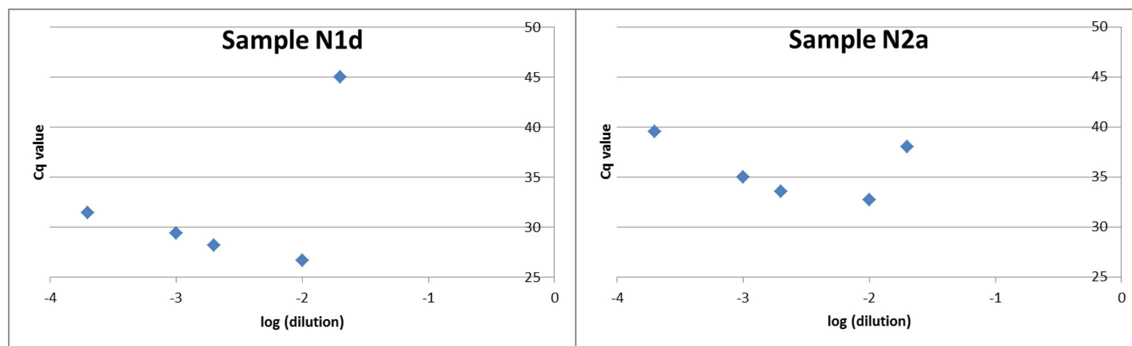

Fig. 2. Analyses for qPCR inhibitors in serial dilutions of two samples (dilutions plotted are 1/50, 1/100, 1/500, 1/1000 and 1/5000).

Relative quantification of *Ilyonectria* spp. in soil samples

Conventional PCR amplification from the 28 soil samples yielded a positive result for all samples after the second stage amplification (ITSrtF - ITSrtR), although PCR amplification after the first round (ITS1F - ITS4) yielded no bands or occasionally faint bands (data not shown), suggesting relatively low levels of *Ilyonectria* spp. DNA in soils and corroborating the low levels of DNA in soil obtained after DNA extraction (data not shown).

The qPCR results from the 1/1000 diluted DNA soil samples (Table 1) ranged from a minimum Cq value of 29.37 for sample N4 (corresponding to 87 ng of *Ilyonectria* spp. DNA per g of soil) to no amplification in samples N2, V4 and V5 (for these, amplification was only achieved for the 1/100 dilution). Average DNA concentration was 7.1 ng.g^{-1} of soil for nursery samples, 3.9 ng.g^{-1} for grapevine mother-plants and 1.0 ng.g^{-1} for vineyards.

Among grapevine mother-plants, DNA of *Ilyonectria* spp. in soil samples varied from 1.6 to 6.5 $ng.g^{-1}$ of soil, but no relation could be established to the type of mother-plant (rootstock or scion).

Contrasting with this range of values, vineyards varied greatly in the concentration of *Ilyonectria* spp. DNA in soil, ranging from no (V4 and V5) or very low (V1) detection in apparently healthy vineyards to an average of 2.6 $ng.g^{-1}$ of soil in two vineyards with dead plants (V2 and V3).

In nursery samples N1-N5, fields under rotation either with horticultural crops or forest trees showed similar levels of *Ilyonectria* spp. infestation (samples N1 and N3, with an average of 0.9 $ng.g^{-1}$ of soil). The most striking result from nurseries N1-N5, however, was the high level of detection of *Ilyonectria* DNA in sample N4 (87 $ng.g^{-1}$ of soil), a field which in fact had been abandoned as nursery because of recurrent problems in grapevine materials related to failure to root. This could represent 69 $CFU.g^{-1}$ of soil considering the formula estimated for *P. intermedium* by Li et al. (2010). In nurseries N6-N13, a field after long term perennial ryegrass cultivation (sample N13) attained higher levels of infestation (0.089 ng $DNA.g^{-1}$ of soil) than a couple of fields at the end of the three-year horticultural crops cultivation (samples N11 and N12), with no or low (0.014 $ng.g^{-1}$) detection of *Ilyonectria* spp. DNA. Still, these values are much lower than those obtained at the end of the rooting process (average 1.8 $ng.g^{-1}$ of soil for samples N6a-N10), clearly indicating that rotation can reduce *Ilyonectria* spp. infestation by over 10-fold. Among these five samples, over two-fold differences could be recorded between those following a three-year rotation (samples N6-N8, average 2.4 $ng.g^{-1}$ of soil) and those following a long rotation cycle (samples N9 and N10, average 0.8 $ng.g^{-1}$ of soil), suggesting the beneficial effect of long rotations. Samples from fields at the end of rotation cycles from five different nurseries originated high levels of *Ilyonectria* DNA detection (from 2.4 to 9.9 $ng.g^{-1}$ of soil; samples N14-N18). Although no comparative analyses can be made with these results, they still highlight the importance and spread of these pathogens. Moreover, the comparison between samples collected in 2005 and 2010, suggests a tendency for an increase in the levels of *Ilyonectria* spp. infestation.

The role of rootstock mother vines as a primary source of grapevine trunk pathogens is well established, but the role of scion mother vines is subject of debate (Rego et al. 2000, Fourie and Halleen 2001, Halleen and Crous 2001, Rego et al. 2001, Gramaje 2011).

Overall, the present results demonstrate the effect of rotation in the reduction of levels of the black foot pathogens in nursery soils and indicate that the levels of *Ilyonectria* spp. infestation in grapevine soils are lower than in nursery or mother-plant soils.

Acknowledgments We would like to thank to all the nursery and grapevine growers for their collaboration. This article is part of a Ph.D. dissertation (Instituto Superior de Agronomia, Technical University of Lisbon, Portugal). This work was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/24790/2005; project PTDC/AGR-AAM/099324/2008).

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CHAPTER 8

CONCLUSIONS AND PERSPECTIVES

In recent years, black foot disease of grapevines has proved to be an increasingly important disease, causing economic losses in grapevine nurseries and young vineyards. Co-operative research at international level on this disease and on its causal agents led to important advances and the research reported in this thesis aims to contribute to such advances in diverse areas, namely taxonomy, distribution of mating type genes in pathogen populations (sexual behaviour governing recombination potential), pathogenicity and cross-infection potential, dynamics on the nursery and vineyard soils, and the development of genetic transformation tools.

Prior to the present study, black foot disease of grapevines was attributed to *Ilyonectria liriodendri* and *I. macrodidyma* (along with other less frequently detected species – "*Cylindrocarpon*" *pauciseptatum*, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare*). From this work, the combined analysis of morphological and cultural characteristics along with DNA sequence data derived from the Internal Transcribed Spacers of the nrRNA gene operon, and partial β-tubulin, histone H3, and translation elongation factor 1-α genes enabled the identification and description of the following new species: *I. alcacerensis* A. Cabral, Oliveira & Crous, *I. estremocensis* A. Cabral, Nascimento & Crous, *I. novozelandica* A. Cabral & Crous and *I. torresensis* A. Cabral, Rego & Crous within the macrodidyma complex and *I. europeaea* A. Cabral, Rego & Crous, *I. lusitanica* A. Cabral, Rego & Crous, *I. pseudodestructans* A. Cabral, Rego & Crous, *I. robusta* (A.A. Hildebr.) A. Cabral & Crous and *I. vitis* A. Cabral, Rego & Crous, sisters to *I. liriodendri*, in the *I. radicicola* complex. This analysis further led to the identification of eight species within the *I. radicicola* complex, containing isolates not related to black foot disease of grapevine, such as *I. anthuriicola* A. Cabral & Crous, *I. crassa* (Wollenw.) A. Cabral & Crous, *I. cyclaminicola* A. Cabral & Crous, *I. gamsii* A. Cabral & Crous, *I. liliigena* A. Cabral & Crous, *I. mors-panacis* (A.A. Hildebr.) A. Cabral & Crous, *I. panacis* A. Cabral & Crous, *I. rufa* A. Cabral & Crous and *I. venezuelensis* A. Cabral & Crous, contributing to the clarification of the taxonomy of the causal agents of root rots in several host plants (e.g., from the genera *Cyclamen*, *Lilium*, *Panax*, *Pseudotsuga* and *Quercus*). Within the pathogens causing black foot disease of grapevine, different patterns of distribution around the world were reported (e.g., *I. torresensis* was reported from various countries, *I. alcacerensis* was found in the Iberian Peninsula, *I. novozelandica* was reported from New Zealand, South Africa and USA, and *I. estremocensis*, *I. europaea*, *I. lusitanica*, *I. pseudodestructans*, *I. robusta* and *I. vitis* were only reported from Portugal). Over the next few years, as this new taxonomic framework is applied to grapevine black foot pathogens around the world, it will be interesting to analyse how geographically restricted or widespread each of these species are. Also, this analysis revealed histone H3 nucleotide sequences as the most phylogenetically informative, resolving the same number of

species as the combined dataset spanning the four genes, highlighting its importance for future works on the taxonomy of the Nectriaceae. In fact, histone H3 proved even more informative than the mating type genes for phylogenetic purposes.

The structure and complete sequence of the mating type idiomorphs of *I. liriodendri* and several species from the macrodidyma species complex was obtained and validated by comparison to sequences from closely related species. Most species/isolates analysed were either *MAT1-1* (containing *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*) or *MAT1-2* (containing *MAT1-2-1*) and could therefore be inferred as heterothallic.

The organization of the mating-type locus in *I. liriodendri* was completely different. Two types, A and B, could be distinguished. Both types contained *MAT1-2-1* and *MAT1-1-1*, but the type B also contained *MAT1-1-2* and *MAT1-1-3* making it genetically homothallic. Nevertheless, all *I. liriodendri* isolates (either type A or type B) are self-sterile, therefore functionally heterothallic, suggesting a phenomenon of pseudo-heterothallism, which could represent an intermediate evolutionary step from heterothallism to homothallism in *I. liriodendri*. Sequence information generated here enabled the design of specific PCR primers that were employed for the analysis of the population structure of 238 isolates representing 23 species, showing strong variations in frequencies of each mating type across those species. Among grapevine isolates collected in Portugal during 1992-2008, *MAT1-2* was predominant in *C. pauciseptatum*, *I. estremocensis*, *I. macrodidyma* and *I. robusta*, while similar proportions between *MAT1-1* and *MAT1-2* were recorded in *I. europaea*, *I. pseudodestructans* and *I. torresensis*. Similarly, *I. liriodendri* type A predominated over type B. Moreover, both idiomorphs were recorded in *I. torresensis* and *I. liriodendri* in the same year, region or even vineyard, often at similar proportions. This is a strong indication of active sexual recombination in these pathogens, which is a route for a rise in genetic diversity and consequently an increased threat to grapevine plants. The analysis of the new taxonomic framework proposed here, combined with the increased virulence of isolates clustering in mating types that were not present earlier (e.g., type B of *I. liriodendri*, *MAT1-1* in *I. macrodidyma*, *MAT1-2* in *I. estremocensis* and *I. europaea*), together with the increased frequency of isolation of species that were absent during the 1990's (e.g., type B of *I. liriodendri* and *I. torresensis*), suggests that the appearance of new genotypes of black foot pathogens may be responsible for the increased incidence and severity of the disease.

In this work, the virulence of the 12 *Ilyonectria* species associated with black foot disease of grapevine was compared, revealing *I. lusitanica*, *I. estremocensis* and *I. europaea* to be more virulent to grapevine than the species previously accepted as the main causal agents of black foot, such as *I. liriodendri* and *I. macrodidyma*, a result of utmost importance to understand

black foot incidence and severity. This further validates the need for the above-mentioned taxonomic dissection of this group of pathogens. Besides determining the virulence of these species to grapevine, this work further showed most isolates obtained from non-grapevine hosts to be as virulent to grapevines as isolates obtained from grapevine, underlying the cross-infection potential of these pathogens.

The complex epidemiology and histopathology of black foot disease of grapevines led to the need to develop experimental tools enabling future genetic transformation studies. For such, a stable genetic transformation protocol was tested and optimised for *I. liriodendri*, based on transformation of protoplasts tagged with the green fluorescent protein (GFP) gene. The success of transformation was subsequently confirmed by PCR amplification of the transgene and microscopic observation of GFP-tagged mycelium. Several variations to an *Agrobacterium tumefaciens*-mediated transformation protocol proved unsuccessful for the transformation of *I. liriondendri*, suggesting that this fungus may not be amenable for *Agrobacterium* transformation. The protocol described here enables the stable insertion of genes of interest in the *I. liriodendri* genome, opening routes for tagged mutagenesis, gene knock-out or heterologous expression studies. Furthermore, strains expressing the GFP may be used for histopathology studies, enabling detailed insights into the complex infection and colonization mechanisms of *I. liriodendri*.

Results from the taxonomy and diversity studies enabled the design of PCR primers capable of amplifying DNA for the ITS region from *Ilyonectria* pathogens responsible for black foot disease of grapevines, and not amplifying DNA from fungi responsible for other grapevine diseases (including *Campylocarpon* spp.). These were used to perform a quantitative comparison of *Ilyonectria* spp. in different nursery and vineyard soils by quantitative PCR, revealing the effect of rotation in the reduction of levels of the black foot pathogens in nursery soils and indicating that the levels of *Ilyonectria* spp. infestation in grapevine soils are lower than in nursery or mother-plant soils. An extreme situation stood out from these results, showing that a field that was abandoned due to rooting problems yielded the highest *Ilyonectria* DNA content, estimated as 87 $ng.g^{-1}$ of soil. The DNA extraction protocol tested here, together with the serial dilutions both of soil DNA and of pure culture *Ilyonectria* spp. DNA, enabled the establishment of a window of linearity for qPCR amplifications of 1 ng to 1 pg of genomic DNA, a limit of detection of 100 fg DNA, the choice of 1000x dilutions of soil samples to avoid the inhibitory effect of soil components and primer efficiencies of 84 to 102%. These parameters show that the qPCR protocol and primers presented here can be employed for epidemiological studies on black foot disease.

Over all, the work reported here aims to improve our knowledge on black foot pathology and biology of its causal agents by detailing its taxonomy, virulence and population structure of mating types, generating tools for the analysis of the dynamics of the pathogen in soil and for stable genetic transformation. It is expected that this work may promote downstream research focused on:

-the study of population frequencies and their variation through time and space, considering the new taxonomic framework presented here as well as the mating type structure;

-the monitoring of black foot pathogens in nursery soils throughout the rotation;

-the histopathological analysis of the infection process, using the green fluorescent protein-tagged strains;

-functional genetic studies, using the transformation protocol optimized here to silence genes putatively involved in key steps of the pathogenic process;

-the investigation of the functional significance of the unusual structure of mating type loci in *I. liriodendri* and the role of the putative ORF found in *MAT1-2* idiomorphs of the macrodidyma species complex and in type A isolates of *I. liriodendri*.