British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Multi-gene analysis and morphology reveal novel *Ilyonectria* species associated with black foot disease of grapevines

Ana CABRAL[a], *Cecília REGO*[a], *Teresa NASCIMENTO*[a], *Helena OLIVEIRA*[a,*], *Johannes Z. GROENEWALD*[b], *Pedro W. CROUS*[b,c,d]

[a]CEER-Biosystems Engineering, Instituto Superior de Agronomia, Technical University of Lisbon, Lisboa, Portugal
[b]CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands
[c]Microbiology, Department of Biology, Utrecht University, Utrecht, The Netherlands
[d]Wageningen University and Research Centre (WUR), Laboratory of Phytopathology, Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 12 May 2011
Received in revised form
27 September 2011
Accepted 30 September 2011
Available online 11 October 2011
Corresponding Editor:
Joseph W. Spatafora

Keywords:
Cylindrocarpon macrodidymum
Ilyonectria
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*, *Ilyonectria liriodendri*, *Campylocarpon fasciculare*, and *Campylocarpon 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 multigene 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 (*Ilyonectria alcacerensis*, *Ilyonectria estremocensis*, *Ilyonectria novozelandica*, and *Ilyonectria 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 *Ilyonectria macrodidyma* (Halleen *et al.* 2004, 2006b), and two

* *Corresponding author*. CEER-Biosystems Engineering, Instituto Superior de Agronomia, Technical University of Lisbon, Tapada da Ajuda, 1349-017 Lisboa, Portugal. Tel.: +351213653100; fax: +351213653195.
E-mail address: heloliveira@isa.utl.pt
1878-6146/$ – see front matter
doi:10.1016/j.funbio.2011.09.010

Campylocarpon species, *Campylocarpon fasciculare*, and *Campylocarpon 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 (Halleen *et al.* 2004; Petit & Gubler 2005; Alaniz *et al.* 2007), 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.* in press), which was associated with diseased roots of *Vitis* spp. 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.* in press), 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*, *Cylindrocarpon cylindroides*, belongs to group 1 (microconidia present, mycelial chlamydospores lacking), while the type of the genus *Neonectria*, *Neonectria ramulariae*, which is the teleomorph of *Cylindrocarpon obtusiusculum* (=*Cylindrocarpon 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 *Cylindrocarpon destructans*, which is considered to be a species complex comprising various taxa, including *Cylindrocarpon macroconidialis*, *Cylindrocarpon coprosmae*, and *Cylindrocarpon liriodendri* (Seifert *et al.* 2003; Halleen *et al.* 2006b). Further studies recently led to the introduction of several novel *Ilyonectria* spp., including four species (*Ilyonectria europaea*, *Ilyonectria lusitanica*, *Ilyonectria pseudodestructans*, and *Ilyonectria robusta*) associated with grapevine black foot disease symptoms (Cabral *et al.* in press). 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 multilocus 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 *Ilyonectria macrodidyma*-like isolates from grapevine and 13 from other hosts (Table 1). Forty isolates were obtained in Portugal mainly from 1.5 to 4 y 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 °C 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, The 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 dNTP, 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 (Bio-Rad, USA) were 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 80 s, and a final elongation at 72 °C for 10 min. Primers were V9G (de Hoog & Gerrits van

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 y 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 y 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 y 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 y old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419074	AM419107	JF735616	JF735805
	CBS 129085; Cy145	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 y 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 y 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 y 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 y 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 y 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 y 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 y old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735326	JF735454	JF735623	JF735812
	Cy152	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5 y old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735327	JF735455	JF735624	JF735813
	Cy153	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5 y old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz	JF735328	JF735456	JF735625	JF735814
	Cy243	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 y 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
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 y 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*, roots of an asymptomatic nursery plant; scion Pinotage; rootstock 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*, roots, scion Chardonnay; rootstock 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
I. 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

(*continued on next page*)

Table 1 – (*continued*)

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
	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
	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
	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
	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 y 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 y 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 y old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira	JF735347	JF735476	JF735655	JF735844
	Cy258	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 y 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*, roots; scion Cabernet Sauvignon; rootstock 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*, roots of an asymptomatic plant; scion Sultana; rootstock 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
	Cy97	E. Halmschlager	*Quercus* sp., root	Austria, Patzmannsdorf	JF735353	JF735482	JF735669	JF735858
	Cy118; C07	W.D. Gubler	*Vitis vinifera*	USA, California	JF735354	JF735483	JF735670	JF735859
	Cy120; C12	W.D. Gubler	*Vitis vinifera*	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 y 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 y old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz	AM419070	JF735486	JF735674	JF735863

(*continued on next page*)

Table 1 – (*continued*)

Species	Strain number[a]	Collected/isolated by, Year	Isolated from	Location	GenBank accession numbers			
					ITS	TUB	H3	EF1
	Cy138	C. Rego and T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5 y 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 y 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 y 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 y 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
	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 y old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735366	JF735496	JF735685	JF735874
	Cy237	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 y old plant; scion Chardonnay; rootstock 110R	Portugal, Vidigueira	JF735367	JF735497	JF735686	JF735875
	Cy240	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 y old plant; scion Touriga Nacional; rootstock 140-RU	Portugal, Vidigueira	JF735368	JF735498	JF735687	JF735876
	Cy246	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 y old plant; scion Antão Vaz; rootstock 110R	Portugal, Vidigueira	JF735369	JF735499	JF735688	JF735877
	Cy260	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 y old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira	JF735370	JF735500	JF735689	JF735878
	Cy262	C. Rego, 2008	*Vitis vinifera*, basal end of a 2 y 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.

den Ende 1998) and ITS4 (White *et al.* 1990) for ITS, T1 (O'Donnell & Cigelnik 1997) and Bt-2b (Glass & 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.G., 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, 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 °C 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, The 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 °C 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 °C in 5 °C 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 & Correll 2001). The plates were incubated at 20 °C 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 model 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 *Ilyonectria torresensis* is basal to *Ilyonectria alcacerensis* and *Ilyonectria macrodidyma*. For TEF, all species could be resolved, except *Ilyonectria novozelandica*, which is divided into two separate groups. The ITS tree does not resolve any species. Neighbour-Joining (NJ) analyses using the three substitution

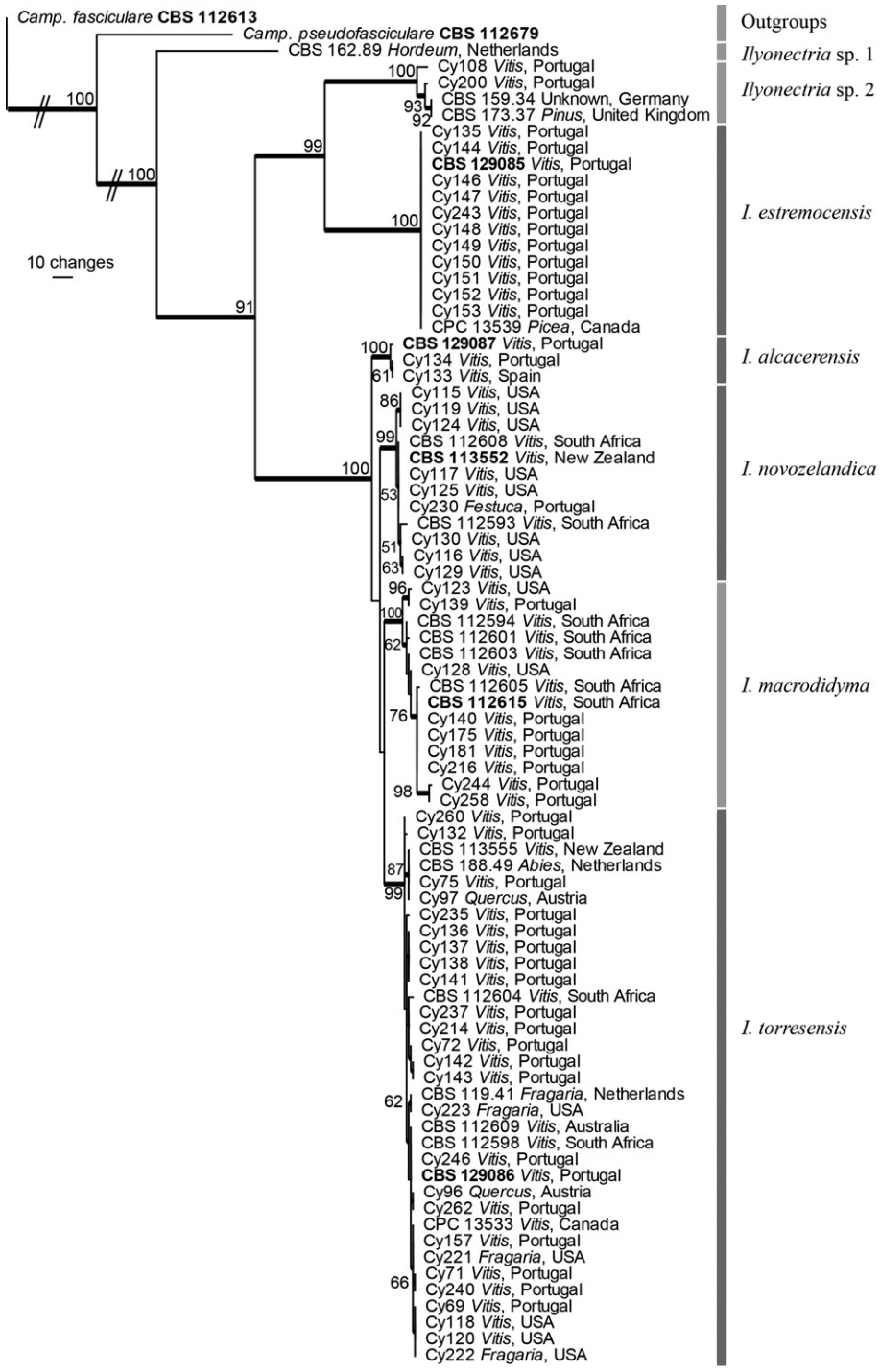

Fig 1 – The first of 455 equally most parsimonious trees obtained from the combined ITS, TUB, HIS, and TEF sequence alignment of *Ilyonectria* isolates and relatives with a heuristic search using PAUP v.4.0b10. The tree was rooted using *Campylocarpon* isolates as outgroup sequences and bootstrap support 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 dark grey boxes. Scale bar shows ten changes.

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 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.

Taxonomy

Based on the phylogenetic data derived in the present study, six new species could be distinguished in the *Ilyonectria 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; one-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); two-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 three-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,

Table 2 – Nucleotide differences for partial gene sequences of TUB, HIS, and 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

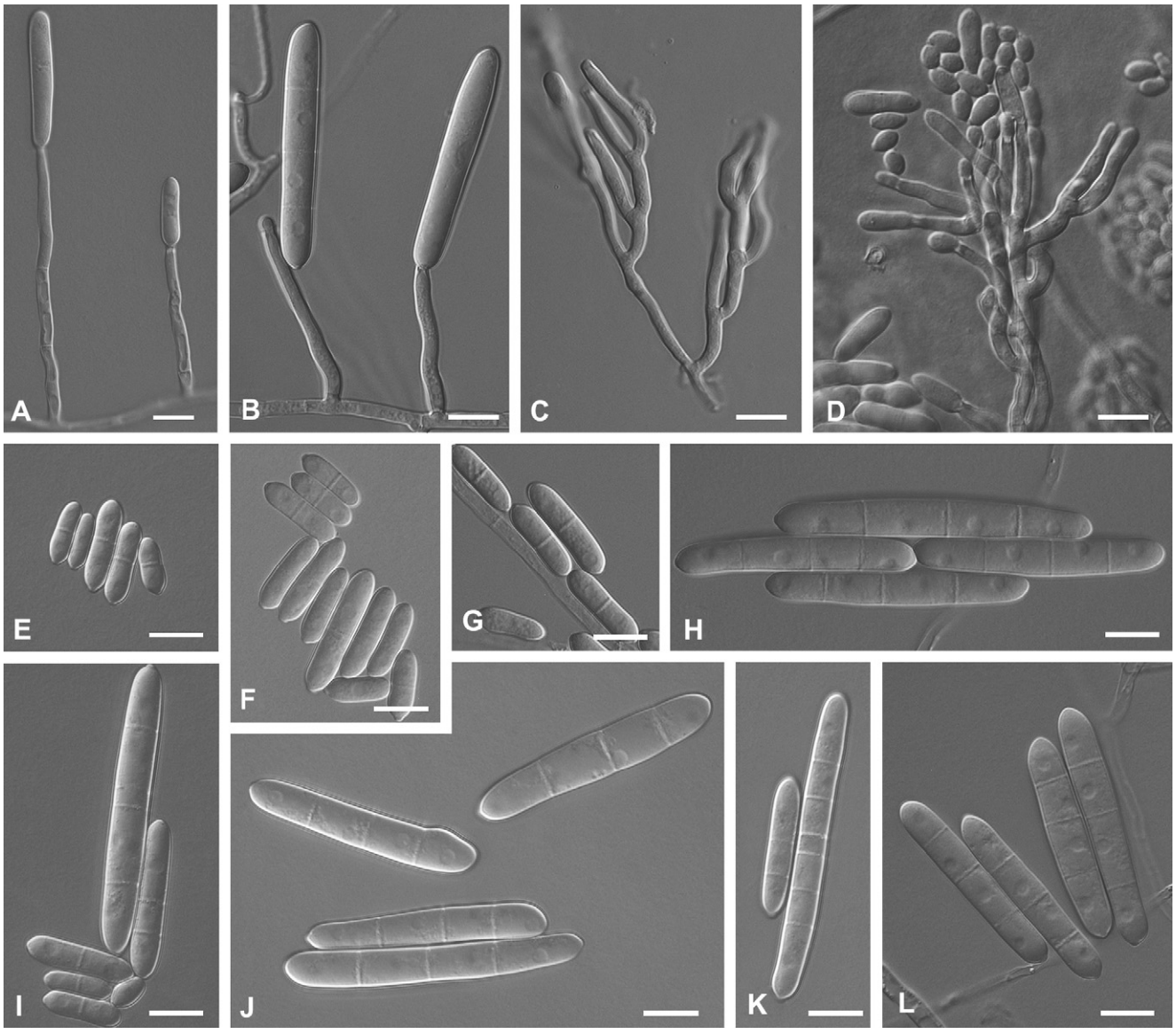

Fig 2 – *Ilyonectria alcacerensis* (A–C) Simple, sparsely branched conidiophores of the aerial mycelium. (D) Complex conidiophores. (F–L) Micro- and macroconidia. Bars = 10 μm. All from isolate CBS 129087.

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; one-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.

Holotype: **Portugal:** Alcácer do Sal, Torrão, *Vitis vinifera*, base of a 3 y 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 °C after 7 d. Optimum temperature between 20 and 25 °C, with colonies reaching 21–28 mm and 31–33 mm diam respectively. Maximum temperature around 30 °C, with colonies reaching 2–6 mm; no growth observed at 35 °C.

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: *V. 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 uni-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; one-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); two-septate

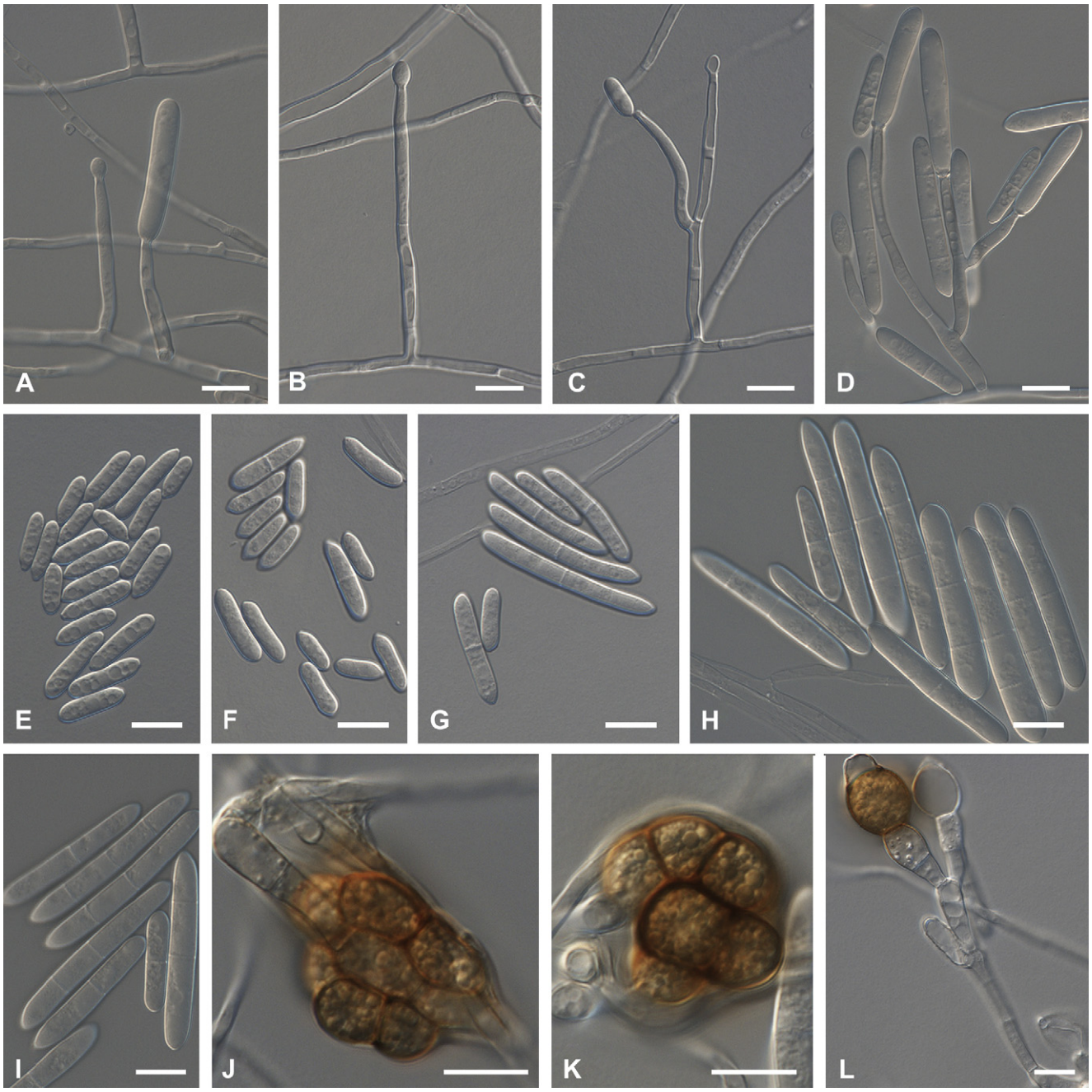

Fig 3 – *Ilyonectria estremocensis* (A–D) Simple, sparsely branched conidiophores of the aerial mycelium. (E–I) Micro- and macroconidia. (J–L) Chlamydospores. Bars = 10 μm. All from isolate CBS 129085.

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; three-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; zero-septate microconidia (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), one-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, *V. vinifera*, base of a 1.5 y 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 °C after 7 d colonies on PDA grew 5–8 mm. Optimum temperature between 20 and 25 °C, when colonies reached 33–41 mm, and 37–43 mm, respectively. For some isolates no growth was observed at 30 °C, while others grew 1–4 mm; no growth was observed at 35 °C.

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 *V. vinifera* (base and grafting zone) in Portugal.

Ilyonectria novozelandica A. Cabral & Crous, **sp. nov.** MycoBank 560154. Figs 4 and 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, eight-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; one-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); two-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 three-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; zero-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); one-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, *V. 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 °C after 7 d. Optimum temperature between 20 and 25 °C, when colonies reach 28–37 mm and 31–41 mm respectively. Maximum temperature around 30 °C, when colonies reach 3–8 mm; no growth was observed at 35 °C.

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

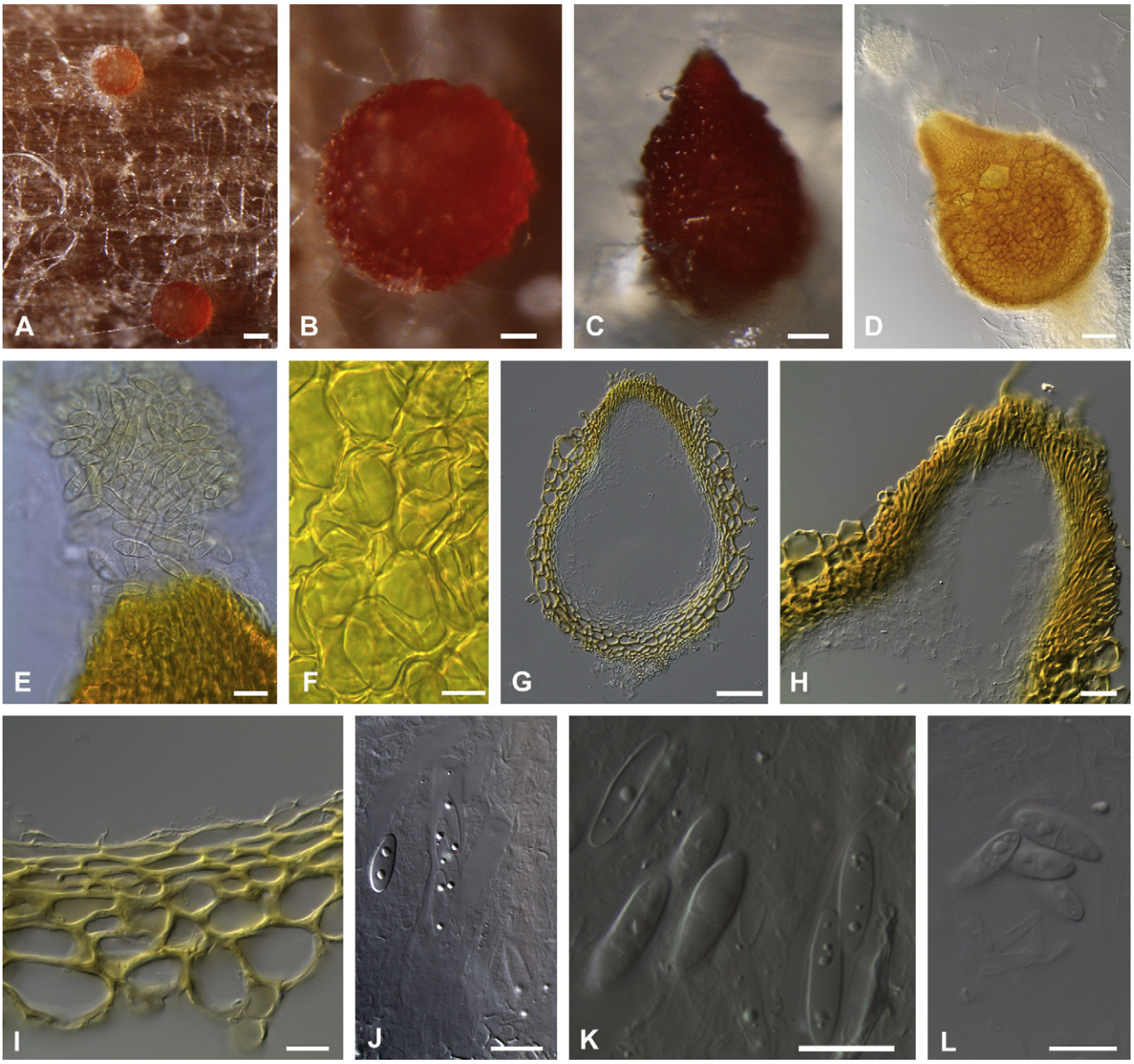

Fig 4 – *Ilyonectria novozelandica* (A–C) Development of perithecia on the surface of birch toothpick or agar. (D–F) Perithecium mounted in lactic acid. (E) Ostiolar area. (F) Surface view of perithecium wall region. (G–I) Longitudinal sections of perithecia showing details of ostiole and wall. (J) Asci and ascospores. (K–L) Ascospores. Bars: A = 100 μm; B–D, G = 50 μm; H = 20 μm; E, F, I–L = 10 μm. All from crossing of CBS 113552 × CBS 112593.

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), *V. vinifera* (New Zealand, South Africa, USA).

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

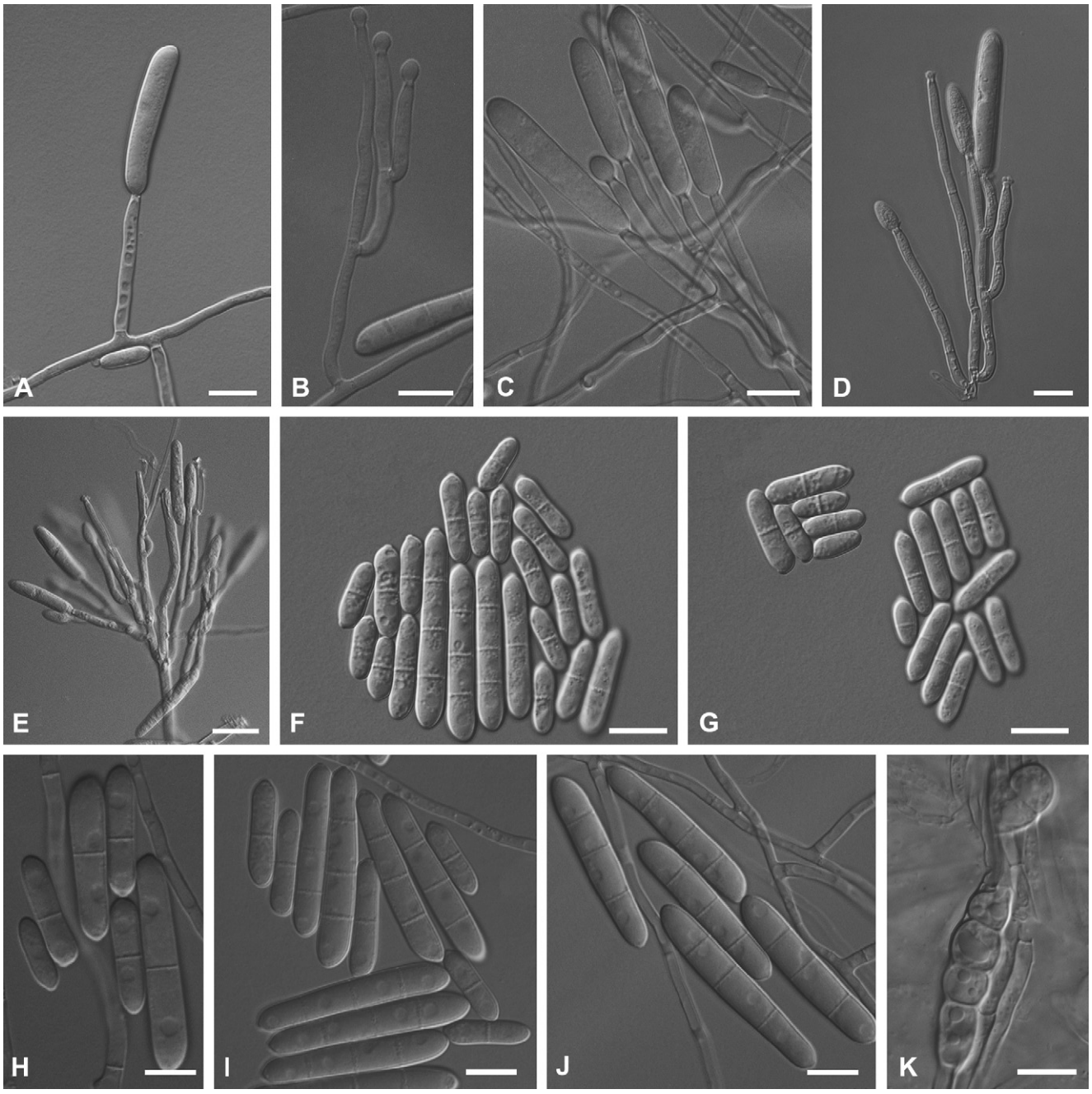

Fig 5 – ***Ilyonectria novozelandica*** **(A, B) Simple, sparsely branched conidiophores of the aerial mycelium. (C–E) Complex conidiophores. (F–J) Micro- and macroconidia. (K) Chlamydospores on mycelium. Bars = 10 μm. A, D–G from Cy130; B, C, H–K from CBS 113552.**

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, eight-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

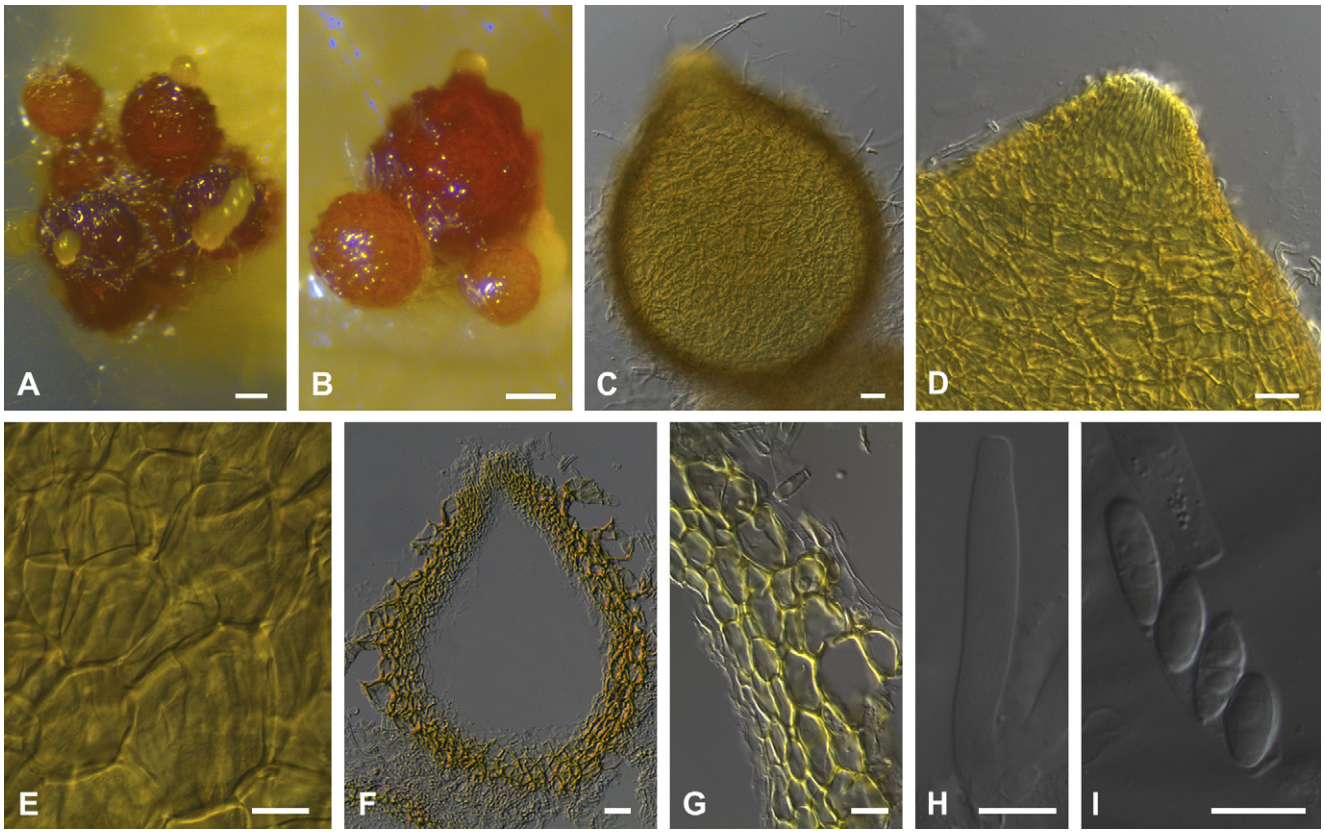

Fig 6 – *Ilyonectria torresensis* (A, B) Development of perithecia on the surface of birch toothpick. (C–E) Perithecium mounted in lactic acid. (D) Ostiolar area. (E) Surface view of perithecium wall region. (F, G) Longitudinal sections of perithecia showing detail of wall. (H) Asci. (I) Ascospores.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

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; one-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, two-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 three-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; zero-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, one-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, *V. 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.

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

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).

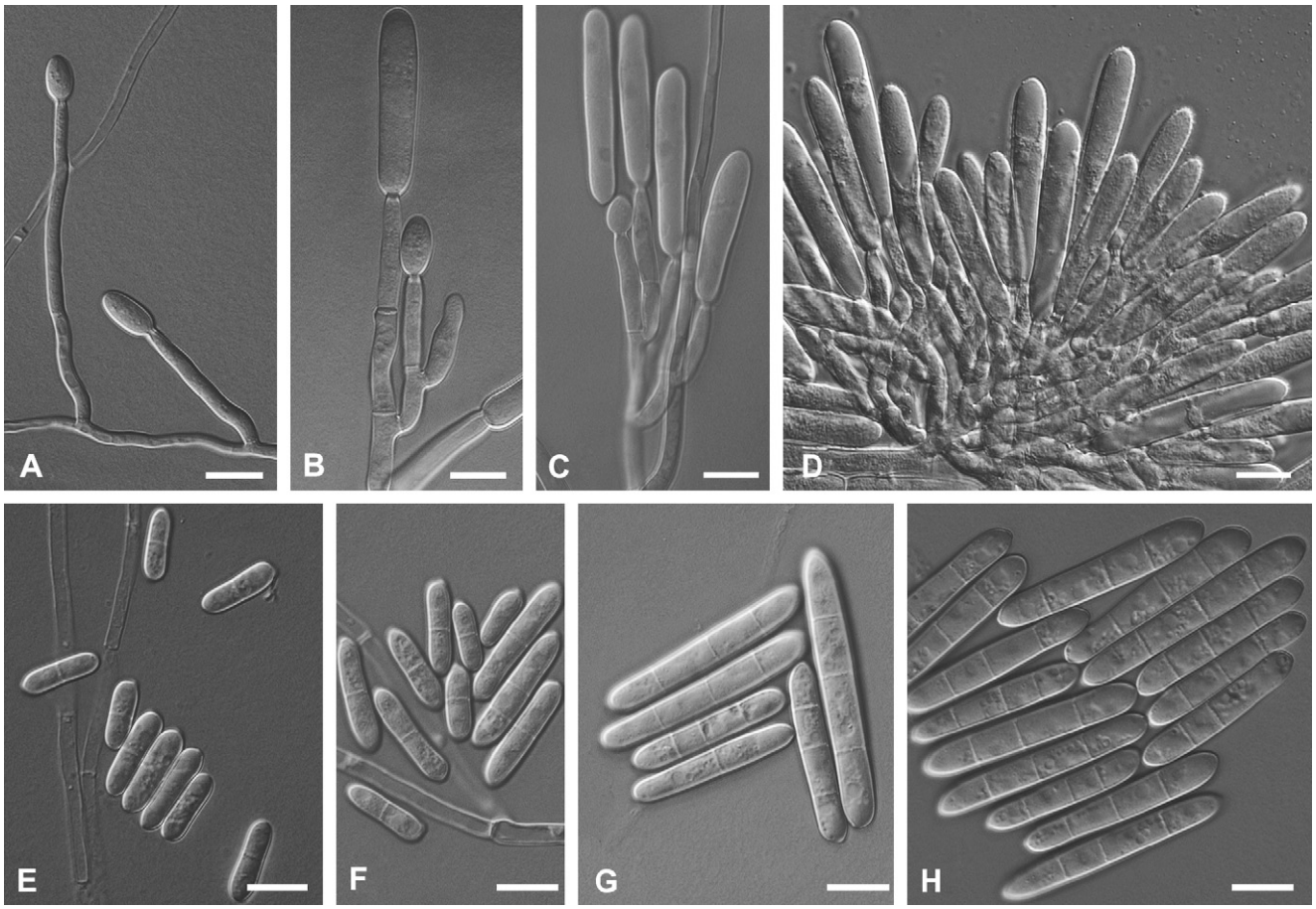

Fig 7 – *Ilyonectria torresensis* (A–C) Simple, sparsely branched conidiophores of the aerial mycelium. (D) Complex conidiophores. (E–H) Micro- and macroconidia. Bars = 10 μm. All from isolate CBS 129086.

Hosts and distribution: *Abies nordmanniana* (root) (Netherlands), *Fragaria* sp. (root) (Netherlands), *Fragaria* × *ananassa* (USA), *Quercus* sp. (root) (Austria), *V. 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 three-septate conidia). *Ilyonectria novozelandica* has slightly more elongated and shorter three-septate conidia, and less septate and shorter conidiophores than *I. torresensis*. The three-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; one-septate macroconidia are predominant, instead of three-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 *Ilyonectria liriodendri*, *I. macrodidyma* and '*Cylindrocarpon*' *pauciseptatum*. Since the first description of *Ilyonectria macrodidyma* as a new species (Halleen *et al.* 2004), several additional reports have implicated this pathogen as the causal agent of the disease (Petit & Gubler 2005; Rego *et al.* 2005; Alaniz *et al.* 2007; Auger *et al.* 2007; Abreo *et al.* 2010).

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 *Ilyonectria estremocensis*, *Ilyonectria torresensis*, *Ilyonectria alcacerensis*, and *Ilyonectria novozelandica*, while a further two *Ilyonectria* sp. 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 one-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 *V. vinifera* on the Iberian Peninsula. *Ilyonectria novozelandica* is mainly associated with *V. 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 three-septate conidia). *Ilyonectria torresensis* rarely produced conidia with four septa, and three-septate conidia are wider than *I. novozelandica*. A comparison to data from Halleen *et al.* (2004) shows that the three-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) 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).

REFERENCES

Abreo E, Martinez S, Bettucci L, Lupo S, 2010. A morphological and molecular characterization of *Campylocarpon* and *Cylindrocarpon* spp. associated with black foot disease of grapevines in Uruguay. *Australasian Plant Pathology* **39**: 446–452.

Alaniz S, León M, Vicent A, García-Jiménez J, Abad-Campos P, Armengol J, 2007. Characterization of *Cylindrocarpon* species associated with black foot disease of grapevine in Spain. *Plant Disease* **91**: 1187–1193.

Alaniz S, Armengol J, León M, García-Jiménez J, Abad-Campos P, 2009a. Analysis of genetic and virulence diversity of *Cylindrocarpon liriodendri* and *C. macrodidymum* associated with black foot disease of grapevine. *Mycological Research* **113**: 16–23.

Alaniz S, Armengol J, García-Jiménez J, Abad-Campos P, León M, 2009b. A multiplex PCR system for the specific detection of *Cylindrocarpon liriodendri*, *C. macrodidymum*, and *C. pauciseptatum* from grapevine. *Plant Disease* **93**: 821–825.

Auger J, Esterio M, Pérez I, 2007. First report of black foot disease of grapevine caused by *Cylindrocarpon macrodidymum* in Chile. *Plant Disease* **91**: 470.

Booth C, 1959. Studies of Pyrenomycetes IV. *Nectria* (part 1). *Mycological Papers* **73**: 1–115.

Booth C, 1966. The genus *Cylindrocarpon*. *Mycological Papers* **104**: 1–56.

Braun U, 1993. New genera of phytopathogenic Deuteromycetes. *Cryptogamic Botany* **4**: 107–114.

Brayford D, Honda BM, Mantiri FR, Samuels GJ, 2004. *Neonectria* and *Cylindrocarpon*: the *Nectria mammoidea* group and species lacking microconidia. *Mycologia* **96**: 572–597.

Brasier CM, 1997. Fungal species in practice: identifying species units in fungi. In: Claridge MF, Dawah HA, Wilson MR (eds),

Species: the units of biodiversity. Chapman and Hall, London, pp. 135–170.

Cabral A, Groenewald JZ, Rego C, Oliveira H, Crous PW. Cylindrocarpon root rot: multi-gene analysis reveals novel species within the *Ilyonectria radicicola* species complex. *Mycological Progress*, in press, doi:10.1007/s11557-011-0777-7

Chaverri P, Salgado C, Hirooka Y, Rossman AY, Samuels GJ, 2011. Delimitation of *Neonectria* and *Cylindrocarpon* (*Nectriaceae*, *Hypocreales*, *Ascomycota*) and related genera with *Cylindrocarpon*-like anamorphs. *Studies in Mycology* **68**: 57–78.

Crous PW, Gams W, Stalpers JA, Robert V, Stegehuis G, 2004a. MycoBank: an online initiative to launch mycology into the 21st century. *Studies in Mycology* **50**: 19–22.

Crous PW, Groenewald JZ, Risede JM, Hywel-Jones NL, 2004b. *Calonectria* species and their *Cylindrocladium* anamorphs: species with sphaeropedunculate vesicles. *Studies in Mycology* **50**: 415–429.

Crous PW, Verkleij GJM, Groenewald JZ, Samson RA (eds), 2009. *Fungal Biodiversity*. *CBS Laboratory Manual Series* 1. Centraalbureau voor Schimmelcultures, Utrecht.

Dettman JR, Jacobson DJ, Taylor JW, 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. *Evolution* **57**: 2703–2720.

Glass NL, Donaldson G, 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Guerber JC, Correll JC, 2001. Morphological description of *Glomerella acutata*, the teleomorph of *Colletotrichum acutatum*. *Mycologia* **93**: 216–229.

Halleen F, Fourie PH, Crous PW, 2006a. A review of black-foot disease of grapevines. *Phytopathologia Mediterranea* **45**: S55–S67.

Halleen F, Schroers H-J, Groenewald JZ, Rego C, Oliveira H, Crous PW, 2006b. *Neonectria liriodendri* sp. nov., the main causal agent of black foot disease of grapevines. *Studies in Mycology* **55**: 227–234.

Halleen F, Schroers H-J, Groenewald JZ, Crous PW, 2004. Novel species of *Cylindrocarpon (Neonectria)* and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (*Vitis* spp.). *Studies in Mycology* **50**: 431–455.

de Hoog GS, Gerrits van den Ende AHG, 1998. Molecular diagnostics of clinical strains of filamentous Basidiomycetes. *Mycoses* **41**: 183–189.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG, 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.

Lombard L, Crous PW, Wingfield BD, Wingfield MJ, 2010. Multigene phylogeny and mating tests reveal three cryptic species related to *Calonectria pauciramosa*. *Studies in Mycology* **66**: 15–30.

Mantiri FR, Samuels GJ, Rahe GE, Honda B, 2001. Phylogenetic relationships in *Neonectria* species having *Cylindrocarpon* anamorphs inferred from mitochondrial ribosomal DNA sequences. *Canadian Journal of Botany* **79**: 334–340.

Martín MT, Martín L, Cuesta MJ, García-Benavides P, 2011. First report of *Cylindrocarpon pauciseptatum* associated with grapevine decline from Castilla y León, Spain. *Plant Disease* **95**: 361.

Mayr E, 1963. *Animal Species and Evolution*. Harvard University Press, Cambridge.

Menkis A, Burokienė D, 2011. Distribution and genetic diversity of the root-rot pathogen *Neonectria macrodidyma* in a forest nursery. *Forest Pathology*. doi:10.1111/j.1439–0329.2011.00712.x.

Möller EM, Bahnweg G, Sandermann H, Geiger HH, 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research* **20**: 6115–6116.

Nirenberg H, 1976. Untersuchungen über die morphologische und biologische differenzierung in der Fusarium-Sektion Liseola. *Mitteilungen der Biologischen Bundesanstalt für Land- und Forstwirtschaft* **169**: 1–117.

Nylander JAA, 2004. *MrModeltest v2.2. Program Distributed by the Author*. Evolutionary Biology Centre, Uppsala University.

O'Donnell K, Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103–116.

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC, 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences of the USA* **95**: 2044–2049.

O'Donnell K, Ward TJ, Geiser DM, Corby Kistler H, Aoki T, 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**: 600–623.

Oliveira H, Rego C, Nascimento T, 2004. Decline of young grapevine caused by fungi. *Acta Horticulturae* **652**: 295–304.

Petit E, Gubler WD, 2005. Characterization of *Cylindrocarpon* species, the cause of black foot disease of grapevine in California. *Plant Disease* **89**: 1051–1059.

Rayner RW, 1970. *A Mycological Colour Chart*. British Mycological Society and CAB International Mycological Institute, Kew.

Rego C, Nascimento T, Cabral A, Oliveira H, 2005. Fungi associated with young vine decline in Portugal: results of nine years surveys. *OILB WPRS Bulletin* **29**: 123–126.

Rego C, Oliveira H, Carvalho A, Phillips A, 2000. Involvement of *Phaeoacremonium* spp. and *Cylindrocarpon destructans* with grapevine decline in Portugal. *Phytopathologia Mediterranea* **39**: 76–79.

Rossman AY, Samuels GJ, Rogerson CT, Lowen R, 1999. The genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). *Studies in Mycology* **42**: 1–248.

Schoch CL, Crous PW, Groenewald JZ, Boehm EWA, Burgess TI, Gruyter de J, Hoog de GS, Dixon LJ, Grube M, Gueidan C, Harada Y, Hatakeyama S, Hirayama K, Hosoya K, Hyde KD, Jones EBG, Kohlmeyer J, Li YM, Kruys Å, Lücking R, Lumbsch HT, Lutzoni F, Marvanová L, McVay AH, Mbatchou JS, Miller AN, Mugambi GK, Muggia L, Nelsen MP, Nelson P, Owensby CA, Li YM, Phillips AJL, Phongpaichit S, Pointing SB, Pujade-Renaud V, Raja HA, Rivas Plata E, Robbertse B, Ruibal C, Sakayaroj J, Sano T, Selbmann L, Shearer CA, Shirouzu T, Slippers B, Suetrong S, Tanaka K, Volkmann-Kohlmeyer B, Wingfield MJ, Wood AR, Woudenberg JHC, Yonezawa H, Zhang Y, Spatafora JW, 2009. A class-wide phylogenetic assessment of *Dothideomycetes*. *Studies in Mycology* **64**: 1–15.

Schroers H-J, Žerjav M, Munda A, Halleen F, Crous PW, 2008. *Cylindrocarpon pauciseptatum* sp. nov., with notes on *Cylindrocarpon* species with wide, predominantly 3-septate macroconidia. *Mycological Research* **112**: 82–92.

Seifert KA, McMullen CR, Yee D, Reeleder RD, Dobinson KF, 2003. Molecular differentiation and detection of ginseng-adapted isolates of the root rot fungus *Cylindrocarpon destructans*. *Phytopathology* **93**: 1533–1542.

Taylor JW, Jacobson DJ, Kroken SM, Kasuga T, Geiser DM, Hibbett DS, Fisher MC, 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21–32.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand D, Sninsky JJ, White TJ (eds), *PCR Protocols: a guide to methods and applications*. Academic Press, San Diego, pp. 315–322.