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RESEARCH PAPER

Diversity of *Ilyonectria* species in a young vineyard affected by black foot disease

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Summary. Fungi of the *Ilyonectria* genus are the main causal agents of black foot disease of grapevine. These pathogens cause necrosis in the basal end of the rootstock, leading to the early decline and the death of vines in nurseries and young vineyards. In the present study a collection of isolates of the genus *Ilyonectria* obtained from a vineyard located in the Alentejo region, Portugal, was characterised. This vineyard was established with planting material originating from three different nurseries. To assess the inter- and intra-specific variability among isolates, morphological, cultural and biomolecular characteristics were evaluated. Morpho-cultural and molecular data (RAPD and ISSR markers and histone H3 nucleotide sequence) identified *I. estremocensis*, *I. europaea*, *I. liriodendri*, *I. macrodidyma*, *I. torresensis*, *I. vitis* and "*Cylindrocarpon*" *pauciseptatum*. *Ilyonectria torresensis* was the most common species found in the survey, representing more than 50% of the isolates.

Key words: black foot disease, "*Cylindrocarpon*" species, young vineyards, *Vitis vinifera*.

Introduction

Black foot of grapevine is an important disease caused primarily by fungi of the *Ilyonectria* genus. These pathogens cause necrosis at the basal end of the rootstock, leading to the early decline and the death of vines in nurseries and young vineyards (Halleen *et al*., 2004; Oliveira *et al*., 2004). This disease was first described in 1961 (Grasso and Magnano Di San Lio, 1975) and over the last decade its incidence has increased significantly in different grapevine growing areas around the world (Rego *et al*., 2000; Halleen *et al*., 2004; Petit and Gubler, 2005; Alaniz *et al*., 2007). Vines affected by black foot disease show sunken necrotic root lesions and reduced root biomass. By removing the bark, black discoloration and necrosis of wood tissues can be observed extending from the base of the rootstock. Other symptoms include reduced vigour, shortened internodes, sparse foliage and small leaves with interveinal chlorosis and necrosis frequently leading to the death of the plants (Grasso, 1984; Maluta and Larignon, 1991; Scheck *et al*., 1998; Rego *et al*., 2000; Halleen *et al*., 2006a; Alaniz *et al*., 2007).

The genus *Ilyonectria* represents one of several newly established genera of fungi with *Cylindrocarpon*-like anamorphs (Chaverri *et al*., 2011). Previously, Booth (1966) had segregated the genus *Cylindrocarpon* into four groups, based on the presence or absence of microconidia and chlamydospores. Most of the teleomorphs of *Cylindrocarpon* (groups 1, 2 and 4; Booth, 1966) have been classified into the genus *Neonectria*. Recently, Chaverri *et al.* (2011), based on molecular phylogenetic analyses and morphological characters, demonstrated that *Neonectria* comprises at least four different genera: *Neonectria*/*Cylindrocarpon sensu stricto* (Booth's groups 1 and 4), *Rugonectria*, *Thelonectria* (group 2) and *Ilyonectria* (group 3).

Black foot disease of grapevine has been associated with four causal agents, *Ilyonectria liriodendri*

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and *Ilyonectria macrodydima* (Halleen *et al*., 2004; 2006b) and two *Campylocarpon* species, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare* (Halleen *et al*., 2004). A fifth species, "*Cylindrocarpon*" *pauciseptatum*, associated with diseased roots of *Vitis* spp. in Slovenia and New Zealand was reported by Schroers *et al.* (2008). The role of "*C.*" *pauciseptatum* as a black foot pathogen was hypothesized by Alaniz *et al.* (2009a) and later confirmed by Cabral *et al.* (2012b). Since its first association with black foot disease, "*C.*" *pauciseptatum* has also been reported in Uruguay (Abreo *et al*., 2010), Spain (Martín *et al*., 2011) and Portugal (Cabral *et al*., 2012a).

Since *I. macrodidyma* was first reported as a new species (Halleen *et al*., 2004), several additional reports have associated this pathogen with grapevine black foot disease (Petit and Gubler, 2005; Rego *et al*., 2006; Alaniz *et al*., 2007; Abreo *et al*., 2010; Cabral *et al*., 2012c), which appears to be more virulent than *I. liriodendri* (Alaniz *et al*., 2009b). However, diversity among groups of *I. macrodidyma* was detected by Inter-Simple Sequence Repeat (ISSR) markers and pathogenicity tests (Alaniz *et al*., 2009b). Later, Cabral *et al.* (2012c) recognised six new species within *I. macrodidyma* (*I. alcacerensis*, *I. estremocensis*, *I. novozelandica*, *I. torresensis*, *Ilyonectria* sp1 and *Ilyonectria* sp2), thus demonstrating that *I. macrodidyma* was a species complex. Further, *I. europaea*, *I. lusitanica*, *I. pseudodestructans*, *I. robusta* and *I. vitis*, formerly included in the *I. radicicola* complex (Cabral *et al*., 2012a), were found to be associated with black foot of grapevine (Cabral *et al*., 2012b).

With the recent changes in the taxonomy of the causal agents of the grapevine black foot disease, namely in the *I. radicicola* and *I. macrodydima* complexes, the aim of the current study was to assess the extent of diversity of *Ilyonectria*-like fungi occurring in a single young vineyard. To this end, we studied a collection of isolates obtained from one vineyard showing symptoms of early decline that was established with propagating material from three different nurseries. Isolates were characterised using morphological, cultural and molecular methods (Inter-Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers and histone H3 (HIS) nucleotide sequence).

Material and methods

Isolates

This study included 33 isolates of *Ilyonectria*-like fungi obtained from a 2-year-old vineyard located in the southern region of Portugal (Vidigueira, Alentejo), that was showing severe symptoms of early decline (Table 1). The total vineyard area was 60 ha and the planting material was obtained from three independent commercial nurseries, two located in Portugal (nurseries A and B), and the third located abroad (nursery C).

Sample vines were washed with abundant water and small pieces of blackened rootstock woody tissues were collected from 2–4 cm above the rootstock base. Tissue pieces were disinfected by immer-

Table 1. *Ilyonectria*-like isolates obtained from a young vineyard showing symptoms of early decline: species identified, isolate, grapevine cultivar and rootstock, nursery origin (A and B, Portugal; C, abroad) and GenBank accession number.

Species and isolate	Cultivar / Rootstock	Nursery origin	GenBank accession H3
"Cylindrocarpon" pauciseptatum			
Cy238	Petit Verdot / 110R	B	JF735591
Cy239	Cabernet Sauvignon / 110R	B	…
Ilyonectria estremocensis			
Cy243	Touriga Nacional / 110R	B	JF735626
I. europaea			
Cy241	Petit Verdot / 110R	C	JF735567

(*Continued*)

Table 1. *Continues.*

Species and isolate	Cultivar / Rootstock	Nursery origin	GenBank accession H3
I. liriodendri			
Cy252	Petit Verdot / 110R	C	…
Cy253	Petit Verdot / 110R	B	KC119445
Cy254	Touriga Nacional / 110R	B	…
Cy255	Touriga Nacional / 110R	B	…
Cy256	Petit Verdot / 110R	A	…
Cy257	Petit Verdot / 110R	A	KC119446
I. macrodidyma			
Cy244	Petit Verdot / 110R	A	JF735655
Cy250	Chardonnay / 110R	B	KC119443
Cy258	Cabernet Sauvignon / 110R	C	JF735656
Cy264	Petit Verdot / 110R	B	KC119449
I. torresensis			
Cy234	Chardonnay / 110R	B	KC119438
Cy235	Cabernet Sauvignon / 110R	C	JF735685
Cy236	Cabernet Sauvignon / 110R	C	…
Cy237	Chardonnay / 110R	C	JF735686
Cy240	Touriga Nacional / 140RU	A	JF735687
Cy242	Touriga Nacional / 110R	A	KC119439
Cy245	Petit Verdot / 110R	A	…
Cy246	Antão Vaz / 110R	B	JF735688
Cy247	Cabernet Sauvignon / 110R	B	KC119440
Cy248	Touriga Nacional / 110R	B	KC119441
Cy249	Touriga Nacional / 110R	B	KC119441
Cy251	Chardonnay / 110R	A	KC119444
Cy259	Touriga Nacional / 110R	B	KC119447
Cy260	Cabernet Sauvignon / 110R	B	JF735689
Cy261	Touriga Nacional / 110R	B	…
Cy262	Cabernet Sauvignon / 110R	A	JF735690
Cy263	Touriga Nacional / 110R	B	KC119448
Cy265	Touriga Nacional / 110R	A	KC119450
I. vitis			
Cy233	Touriga Nacional / 110R	A	JF735580

sion for 1 min in a solution of sodium hypochlorite (0.35% w/w active chlorine), rinsed with sterile distilled water (sdw), dried on filter paper and placed in Petri dishes (90 mm diam.) containing potato dextrose agar (PDA, Difco, BD, Sparks, MD, USA) amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppliChem, Darmstadt, Germany). Inoculated dishes were incubated in darkness at 20°C for 12 to 15 days and all *Ilyonectria*-like cultures were single-spored and stored in the collection of the Laboratório de Patologia Vegetal "Veríssimo de Almeida" (LPVVA), ISA, Lisbon, Portugal. Representative isolates were obtained from either the LPVVA collection or Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) (Table 2).

Cultural and morphological variability

Cultural characteristics (density, growing margin, zonation, texture, transparency) of all isolates were evaluated on PDA and oatmeal agar (OA, Difco, BD, Sparks, MD, USA) after incubation at 20°C, in the dark for 12 days (Samuels and Brayford, 1990). Colony colour (surface and reverse) was determined using Rayner's colour chart (1970).

In order to study the morphological characters, isolates were grown for 10 days on synthetic nutrient agar (SNA) (Nirenberg, 1976) under 12h light (Philips TL 15W/33), at 20°C. Measurements were made by removing 1 cm^2 squares of agar and placing them on microscope slides, to which a drop of water was added and a cover slip laid (Brayford, 1992). For each isolate, 20 measurements were obtained for each type of conidia at a 1000 × magnification, using a Leica DM2500 microscope. Minimum, average, and maximum conidial measurements were determined.

Molecular characterisation

DNA was extracted according to a protocol of Cenis (1992), modified by Nascimento *et al.* (2001). DNA analysis was based on ISSR and RAPD markers, as well as HIS nucleotide sequences. Four ISSR primers were tested: $HVH(TG)_7$ (Gilbert *et al.*, 1999), $(AG)_8YT$ (Fang and Rose, 1997), $(TCC)_5$ and MR (Bridge *et al.*, 1997). PCR amplifications were performed using 1 × PCR buffer, 3 mM $MgCl_2$ (Fermentas, Vilnius, Lithuania), 200 μM of each dNTP, 0.5 μM of each primer, 0.5 units of *Taq* DNA Polymerase (recombinant) (Fermentas) and 2.5 μL of diluted gDNA, in a final volume of 12.5 μL. The cycle conditions in a Biometra T-Gradient termocycler were 94°C for 1 min, followed by 40 cycles of 94°C for 30s, 50°C for 45s, 72°C for 2 min, and a final elongation of 10 min at 72°C. In the case of the $HVH(TG)_7$ and $(AG)_8YT$ primers, there was a slight change in the hybridization temperature from 50°C to 52°C (Talhinhas *et al.*, 2003). Four RAPD markers were also used: OPA-09, OPA-10, OPB-01 and OPD-13 (previously validated for black foot pathogens by Rego, 2004). Each PCR amplification was performed using 1 × PCR buffer, 3.5 mM $MgCl_2$ (Fermentas), 200 μM of each dNTP, 0.6 μM of each primer, 1 unit of *Taq* DNA Polymerase (recombinant) (Fermentas), and 2 μL of diluted gDNA, in a final volume of 20 μL. The cycle conditions, also in a Biometra T-Gradient termocycler were 94°C for 5 min, followed by 35 cycles of 94°C for 2 min, 37°C for 1 min, 72°C for 2 min, and a final elongation at 72°C for 15 min.

PCR products were separated by electrophoresis on 2% agarose gels in 0.5 × TBE buffer for 18 h at 40V. The gels were stained with ethidium bromide, visualized under ultraviolet light and photographed.

Gel images were analyzed using the software GelCompar II version 5.10 (Applied Maths NV, Sint-Martens-Latem, Belgium). Molecular weights were assigned to each band using a 1 Kb Plus ladder marker (Invitrogen, Gaithersburg, MD, USA) and DNA polymorphic fragments were scored automatically and rectified manually as present (1) or absent (0), generating a binary matrix. Clustering was performed using Dice's similarity coefficients and the unweighted pair group method (UPGMA). Internal branch support was evaluated by bootstrap analysis with 2,000 replicates.

Sequencing of part of the HIS gene was performed by STAB Vida, Lda. (Monte de Caparica, Portugal), after PCR amplification as described by Cabral *et al.* (2012a). Sequences were assembled and edited to resolve ambiguities, using the SeqMan module of the Lasergene software package (DNAStar, Madison, WI, USA). Consensus sequences for all isolates were compiled into a single file (Fasta format). The sequences obtained were then blasted in GenBank against the corresponding sequences from the epitype strains for *Ilyonectria* spp. to confirm the identity of the isolates.

Table 2. Details of *Ilyonectria* spp. and "*Cylindrocarpon*" spp. isolates used in phylogenetic studies.

Species and isolate[a]	Collected (year)	Location	Host (cultivar/rootstock)
"*Cylindrocarpon*" sp.			
Cy228	2003	Lisbon, Portugal	*Ficus* sp.
"*C.*" *pauciseptatum*			
Cy217	2007	Torres Vedras, Portugal	*V. vinifera* (Gouveio/-)
Ilyonectria alcacerensis			
Cy134; IAFM Cy20-1	…	Ciudad Real, Villarubia de los Ojos, Spain	*V. vinifera*
CBS 129087; Cy159	2004	Alcácer do Sal, Torrão, Portugal	*V. vinifera* (Sangiovese/1103P)
I. anthuriicola			
CBS 564.95	1995	Bleiswijk , The Netherlands	*Anthurium* sp.
I. estremocensis			
Cy135	2003	Estremoz, Portugal	*V. vinifera* (Aragonez/3309C)
CBS 129085; Cy145	2003	Estremoz, Portugal	*V. vinifera* (Aragonez/3309C)
I. europaea			
Cy155	2004	Alter do Chão, Portugal	*V. vinifera* (Alfrocheiro/SO4)
CBS 537.92	1992	Liège, Belgium	*Aesculus hippocastanum*
I. liriodendri			
CBS 117640; IMI 357400; Cy1	1992	Dois Portos, Torres Vedras, Portugal	*V. vinifera* (Seara Nova/99R)
CBS117526; Cy68	1999	Ribatejo e Oeste, Portugal	*Vitis* sp. (99R)
macrodidyma			
CBS 112603	1999	Darling, Western Cape, South Africa	*V. vinifera*
I. novozelandica			
Cy117	…	Califórnia, EUA	*Vitis* sp.
CBS 113552	2003	Candy P, New Ground, New Zealand	*Vitis* sp.
I. pseudodestructans			
CBS 129081; Cy20	1996	São Paio, Gouveia, Portugal	*V. vinifera* (Malvasia Fina/1103P)
Cy22	1996	Silgueiros, Viseu, Portugal	*Vitis* (Aragonez/99R)
I.robusta			
Cy23	1997	Ribatejo e Oeste, Portugal	*Vitis* sp. (99R)
CBS 773.83	…	Utrecht, The Netherlands	water
I. torresensis			
Cy214	2007	Torres Vedras, Portugal	*V. vinifera* (Grenache/-)

[a] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; Cy, *Cylindrocarpon* collection housed at Laboratório de Patologia Vegetal Veríssimo de Almeida, ISA, Lisbon, Portugal; IAFM, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia, Spain; IMI, International Mycological Institute, CABI-Bioscience, Egham, UK.

Results

Morpho-cultural characteristics

Only 21 out 33 isolates produced abundant conidia on SNA, predominantly (1−)3(−4)-septate macroconidia, straight or minutely curved, cylindrical with round extremities, sometimes widening towards the tip. The values obtained from the analysis of conidia size were within those described for species of *Ilyonectria* and "*C.*" *pauciseptatum* associated with black foot disease. Seven groups were created according to macro- and microconidia measurements, as well as conidial features (Table 3).

Isolates Cy252, Cy253, Cy254 and Cy256 were within the range described for *I. liriodendri*. They formed 1−3-septate macroconidia, straight or slightly curved, cylindrical, base mostly with a visible, centrally located or laterally displaced hilum. The microconidia were formed in heads or on the agar surface; 0−1-septate, ellipsoidal, cylindrical or ovoid, more or less straight, with a laterally displaced hilum.

Conidia morphology and size for the isolate Cy241 were within those described for *I. europaea*. Macroconidia were 1−3-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, occasionally narrow towards the tip and mostly without a visible hilum. Microconidia were 0−1-septate, ellipsoid to ovoid, more or less straight and without a visible hilum.

Isolates Cy244 and Cy258 were within the range described for *I. macrodydima*. Macroconidia usually 1−3(4−)-septate, mostly straight, sometimes slightly curved and apically rounded. The typical apical cell was slightly bent to one side with a laterally displaced hilum. Microconidia were usually 0−1-septate, ellipsoid or ovoid, more or less straight and with a laterally displaced hilum.

For isolates Cy238 and Cy239 the values recorded were within those described for "*C.*" *pauciseptatum*. Macroconidia were predominately 3-septate, straight or slightly curved, more or less cylindrical, with both ends rounded. Usually no hilum was visible. Conidia morphology and size of isolates Cy237, Cy240, Cy242, Cy246, Cy247, Cy249, Cy259, Cy261, Cy262 and Cy263 were within those described for *I. torresensis*. Macroconidia were predominately, (1−)3(−4)-septate, straight or minutely curved, cylindrical, or with minute widening towards the tip and appearing rather clavate, particularly when still attached to the phialide. The apex or apical cell was typically slightly bent to one side and minutely beaked. The base typically had a visible, centrally located or laterally displaced hilum. The microconidia were 0−1-septate, ellipsoidal to ovoid, more or less straight, with a minutely or clearly laterally displaced hilum and a constriction at the septum.

For isolate Cy243, the values recorded were within those described for *I. estremocensis*. Macroconidia predominated, and were formed on simple conidiophores. On SNA macroconidia formed in flat domes or slimy masses, 1(3)-septate, straight or slightly curved, cylindrical, but typically with a slight widening towards the apex and appearing somewhat clavate. The apex was obtuse and the base mostly with a visible, centrally located or laterally displaced hilum. Microconidia were 0−1-septate, cylindrical, more or less straight and with a minutely or clearly laterally displaced hilum.

Finally, isolate Cy233 was slightly different from all others and fitted the characteristics described for *I. vitis*. Macroconidia were predominantly 3-septate, commonly 4−5-septate, but rarely 1−2-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, mostly without a visible hilum. Microconidia formed in heads and were aseptate, subglobose to ovoid, mostly with a centrally located or slightly laterally displaced hilum.

Morpho-cultural differences among isolates are reported in Table 4, revealing nine different groups of isolates, mainly based on mycelium coloration (surface and reverse) and growing margin. Although seven different types of mycelium coloration have been observed on PDA, differences on OA medium and/or differences between the growing margins resulted in the identification of the nine groups referred to above (Table 4). Only isolates of *I. torresensis* and *I. macrodidyma* could not be clearly distinguished from each other by morphological features in culture and were scattered over four groups rather than two as would be expected.

Molecular characterisation

The combined analysis of RAPD and ISSR fingerprints enabled the clustering of the *Ilyonectria*-like isolates into three major groups (Figure 1). Group A, which includes species from *I. radicicola* complex and contains isolates belonging to *I. europaea*, *I. liriodendri*, *I. pseudodestructans* and *I. robusta.* Group B includes isolates belonging to *I. alcacerensis*, *I. macrodidyma*, *I.*

Table 3. Average conidia size for 21 isolates of *Ilyonectria* spp. and "*Cylindrocarpon*" *pauciseptatum* in SNA medium, after 20 days incubation (20°C, 12 h light).

Species and isolate	Microconidia (µm)		Macroconidia (µm)	
	0-1-septate	1-septate	2-septate	3-septate
"*Cylindrocarpon*" *pauciseptatum*				
Cy238, Cy239	…	(21.2-)31.2(-43.4) × …	(25.6-)41.7(-55.4) × (6.4-)7.9(-9.3)	(38.9-)45.0(-53.9) × (6.7-)8.2(-9.3)
		(3.1-)7.0(-9.3)		
Ilyonectria estremocensis				
Cy243	(12.0-)16.6(-20.0) × (4.0-)4.4(-5.0)	(27.3-)30.9(-34.1) × (4.1-)4.9(-5.4)	(26.4-)34.4(-42.6) × (4.7-)5.5(-6.3)	(34.0-)39.0(-43.1) × (4.8-)5.6(-6.5)
I. europaea				
Cy241	(3.0-)9.1(-17.0) × (1.7-)3.4(-5.0)	(16.4-)22.6(-34.0) × (4.0-)5.4(-7.8)	(22.0-)27.2-(34.0) × (4.4-)6.1(-8.0)	(22.0-)30.6(-40.0) × (5.0-)6.7(-8.6)
I. liriodendri				
Cy252, Cy253, Cy254, Cy256	(5.7-)9.2(-14.3) × (1.4-)3.5(-5.7)	(12.9-)16.7(-20.0) × (2.9-)4.15(-5.7)	(11.4-)21.1(-28.6) × (2.9-)4.1(-7.1)	(20.0-)23.6(-30.0) × (2.9-)5.4(-7,1)
I. macrodidyma				
Cy244, Cy258	(7.1-)9.4(-11.4) × (2.9-)3.6(-5.7)	(17.2-)20.1(-28.6) × (4.0-)5.0(-8.9)	(18.6-)26.6(-34.3) × (4.3-)5.8(-7.1)	(20.0-)29.1(-38.6) × (5.6-)6.6(-8.6)
I. torresensis				
Cy237, Cy240, Cy242, Cy246, Cy247, Cy249, Cy259, Cy261, Cy262, Cy263	(5.7-)10.7(-14.3) × (1.4-)3.7(-4.3)	(12.8-)22.3(-34.1) × (2.9-)4.7(-6.4)	(15.7-)27.9(-42.6) × (4.3-)5.4(-8.6)	(20.0-)35.2(- 48.6) × (5.6-)6.1(-8.6)
I. vitis				
Cy233	(3.7-)5.1(-6.7) × (3.2-)3.8(- 4.6)	(23.-)34.3(-46.0) × (5.4-)7.5(-9.1)	(37.0-)42.5(-47.7) × (7.5-)8.1(-9.0)	(43.7-)45.0(-53.7) × (7.6-)8.6(-9.1)

novozelandica and *I. torresensis* that were formerly included in the *I. macrodidyma* complex. Finally, group C that included isolates belonging to *I. anthuriicola*, "*Cylindrocarpon*" sp., *I. vitis* and "*C.*" *pauciseptatum*. This combined analysis of ISSR and RAPD fingerprints enabled the clustering of *Ilyonectria*-like isolates belonging to the same species together (bootstrap value of 100%) at a similarity greater than 65% (Figure 1). These results were also corroborated by DNA sequence analysis of part of the HIS gene, with the isolates in the study assigned to seven species (Table 1).

The analysis of the frequency of the species within the collection revealed the predominance of *I. torresensis* (55%), followed by *I. liriodendri* (18%) and *I. macrodidyma* (12%). The isolates of these species were recovered from plants obtained from all three nursery suppliers. The two isolates of "*C.*" *pauciseptatum* were isolated from plants supplied by nursery B. The remaining species, *I. estremocensis*, *I. europaea* and *I. vitis*, were each represented by only one isolate; each one originating from a vine from a different nursery supplier (Table 1).

Discussion

Until recently, the *Ilyonectria* species most widely associated with black foot disease of grapevine

Table 4. Cultural characteristics of 33 *Ilyonectria* spp. and "*Cylindrocarpon*" *pauciseptatum* isolates grown on PDA and OA, after 10 days incubation (20°C in the dark).

Isolate	Texture	Density	Colour	Growing margin	Transparency	Zonation	Reverse
Cy235, Cy242, Cy247, Cy249	Felty	Average	PDA: chestnut with aerial mycelium buff to cinnamon; OA: cinnamon to dark sienna, mycelium buff to luteous	Uneven	Homogeneous on PDA	Concentric on PDA, sometimes absent on OA	Same as surface, except for the colour (PDA: sienna to chestnut; OA: light cinnamon to dark fulvous)
Cy237, Cy240, Cy244, Cy250, Cy259, Cy262, Cy263, Cy245, Cy260, Cy261, Cy264, Cy265	Felty	Average	PDA: chestnut with aerial mycelium buff to cinnamon; OA: cinnamon to dark sienna, mycelium buff to luteous	Even	Homogeneous on PDA	Concentric on PDA, sometimes absent on OA	Same as surface, except for the colour (PDA: sienna to chestnut; OA: light cinnamon to dark fulvous)
Cy251	Felty	Average	PDA: chestnut with aerial mycelium buff to cinnamon; OA: chestnut, mycelium buff	Even	Homogeneous on PDA	Concentric on PDA, sometimes absent on OA	Same as surface, except for the colour (PDA: sienna to chestnut; OA: light chestnut)
Cy234, Cy236, Cy246, Cy248, Cy258	Cottony on PDA, felty on OA	Average to strong	PDA: dark sienna to chestnut with aerial mycelium buff to luteous; OA: light sienna to chestnut with aerial mycelium buff to luteous and growing margin saffron to luteous	Uneven sometimes even on OA	Homogeneous on PDA	Concentric or absent	Same as surface, except for the colour (PDA: chestnut to dark chestnut; OA: light cinnamon to light chestnut)
Cy238, Cy239	Felty to cottony	Average on PDA, average to weak on OA	PDA: orange to sienna with aerial mycelium saffron, growing margin buff to pale luteous; OA: pale luteous to luteous with growing margin luteous	Even	Homogeneous on PDA	Concentric on PDA, absent on OA	Same as surface, except for the colour (PDA: dark orange to sienna; OA: luteous to orange)
Cy243	Cottony	Average to strong	PDA: sienna with dark luteous to sienna mycelium and growing margin buff to luteous; OA: buff to saffron to cinnamon and growing margin amber to pure yellow	Even	Homogeneous on PDA	Absent	Same as surface, except for the colour (PDA: chestnut; OA: light sienna)

(*Continued*)

Table 4. *Continues.*

Isolate	Texture	Density	Colour	Growing margin	Transparency	Zonation	Reverse
Cy233	Felty	Weak to average	PDA: chestnut with aerial mycelium sienna and growing margin luteous; OA: sienna with aerial mycelium saffron and growing margin luteous	Even	Homogeneous on PDA	Concentric on PDA, absent on OA	Same as surface, except for the colour (PDA: chestnut to umber, OA: sienna to saffron
Cy241	Felty	Average	PDA: sienna to saffron with aerial mycelium luteous; OA: chestnut with aerial mycelium saffron	Even	Homogeneous on PDA	Concentric on PDA	Same as surface, except for the colour (PDA: chestnut to umber; OA: sepia)
Cy252, Cy253, Cy254, Cy255, Cy256, Cy257	Felty to cottony	Average to strong on pda, weak to average on OA	PDA: dark saffron to cinnamon with aerial mycelium buff to light luteous; OA: sienna with aerial mycelium buff	Even	Homogeneous on PDA	Absent	Same as surface, except for the colour (PDA: ochreous to fulvous; OA: ochreous to light fulvous)

were *I. liriodendri*, *I. macrodydima* (Halleen *et al*., 2004, 2006a) and, to a lesser extent, "*C.*" *pauciseptatum* (Schroers *et al*., 2008). Latterly, Cabral *et al.* (2012c) demonstrated that *I. macrodidyma* was a species complex encompassing six different species, as well as the *I. radicicola* complex (Cabral *et al*., 2012a). From this complex, at least *I. europaea*, *I. lusitanica*, *I. pseudodestructans* and *I. robusta* were recognized as causal agents of black foot disease of grapevine, as well as *I. vitis* (Cabral *et al*., 2012b).

In the present study, a young vineyard showing symptoms of early decline was surveyed and *Ilyonectria*-like fungi were consistently isolated from declining vines. The variation in morpho-cultural characteristics of the collected isolates clearly indicated that several species were present in the collection. Conidial morphology, especially septation, shape and size, was the most informative phenotypic characteristic and enabled the clustering of isolates into seven distinct groups. Results of conidia measurements were in accordance with those described for *I. liriondendri* (Halleen *et al*., 2006b), *I. macrodydima* (Halleen *et al*., 2004), *I. europaea* and *I. vitis* (Cabral *et al*., 2012a), *I. estremocensis* and *I. torresensis* (Cabral *et al*., 2012c), and "*C*". *pauciseptatum* (Schroers *et al*., 2008). However, this grouping did not match that determined by cultural characteristics, thus revealing that morpho-cultural characteristics alone are not a reliable means of identifying cryptic species.

RAPD and ISSR molecular markers were used to first disentangle species within the *Ilyonectria*-like fungi collection, followed by sequencing part of the HIS gene. Although the establishment of species boundaries from DNA polymorphisms should be done carefully (Menzies *et al*., 2003), the combined use of RAPD and ISSR analyses revealed its usefulness for disentangling species. Cluster analysis based on RAPD and ISSR marker data were further supported by results of HIS gene sequencing and were mostly in agreement with results of morpho-cultural characters. Several clusters were obtained and a predominance of one particular species, *I. torresensis*, was revealed, followed by *I. liriodendri*, *I. macrodidyma* and "*C.*" *pauciseptatum*. The remaining species namely *I. estremocensis*, *I. europaea* and *I. vitis*, were only represented by one isolate each. There was no clear relationship between most isolates and the nursery of origin. However, the three species represented by one isolate each came from vines from three different nurseries.

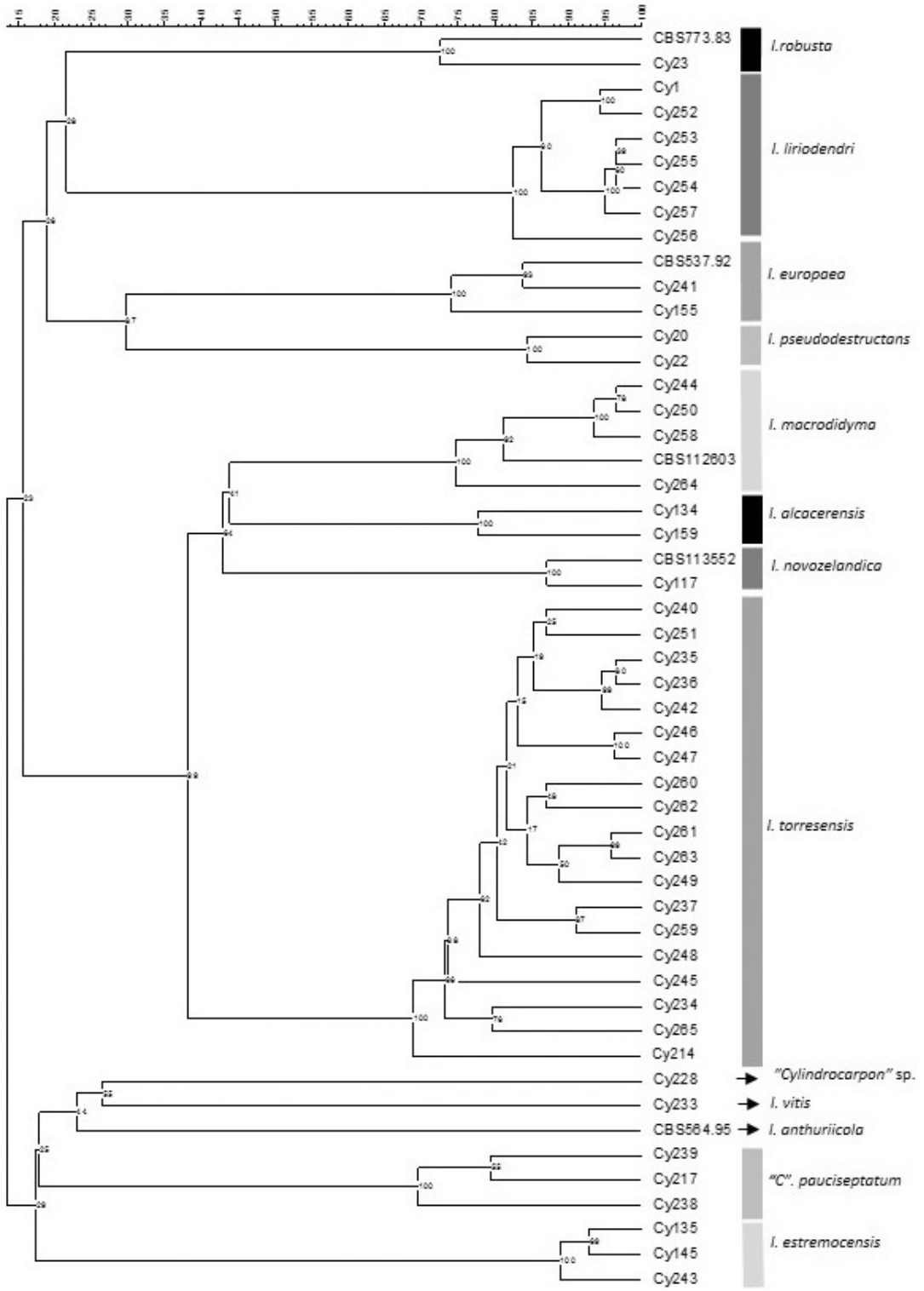

Figure 1. Dendrogram showing the diversity and relationships among *Ilyonectria* spp. and "*Cylindrocarpon*" spp. isolates based on cluster analysis (UPGMA) of a similarity matrix (Dice) generated from ISSR-PCR and RAPD-PCR profile data with four primers each. A total of 2,000 bootstrap replicates were used.

All *Ilyonectria* spp. identified during the present study, as well as "*C.*" *pauciseptatum* are pathogenic to grapevine and potentially to other hosts. Representatives of each species found during this survey were previously tested for pathogenicity on the grapevine rootstock 1103P (Cabral *et al*., 2012b). The results showed high virulence of *I. estremocensis* and *I. europaea* to grapevine followed by *I. liriodendri* and *I. macrodidyma* or *I. torresensis*, ranging over the positions of intermediate virulence.

The present study revealed the predominance of *I. torresensis* within the collection, and available data indicate that this pathogen is well established in at least some Mediterranean countries. In fact, in addition to Portugal, *I. torresensis* has been recently reported as one of the most frequent pathogens associated with black foot disease of grapevine in Spain (Agustí-Brisach *et al*., 2013a, 2013b) and with a root rot disease of kiwifruit in the Black Sea Region of Turkey (Erper *et al*., 2013). It is also very probable that previous records of *I. macrodidyma* from grapevine and other host plants may correspond in part to *I. torresensis*.

During the course of this study six *Ilyonectria* species and "*C.*" *pauciseptatum* were recovered. A maximum of five *Ilyonectria*-like species were collected from material coming from one nursery, thus revealing the primary role of propagation material movement in spreading black foot pathogens. The presence of these pathogens in grapevine propagation material is well documented, as a result of infections that occur in grapevine mother-fields, at different stages of the grapevine propagation process or during the rooting phase in nursery fields (Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013).

Ilyonectria species found from this survey are not grapevine specific and the potential for cross-infection from other host species was recently demonstrated (Tewoldemedhin *et al*., 2011; Agustí-Brisach *et al*., 2011; Cabral *et al*., 2012b). Furthermore, "*C.*" *pauciseptatum* is also not grapevine-specific and this pathogen has been recovered from apple orchards affected by apple replant disease (Tewoldemedhin *et al*., 2011) and from kiwifruit trees (Erper *et al*., 2013).

Overall, our results reinforce the potential of grapevine propagating material to carry a multitude of *Ilyonectria*-like fungi to new growing areas, thus contributing to the establishment of foreign pathogens in the soil, and potentially compromising not only grapevine but also other plant hosts.

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