Characterization of *Cylindrocarpon* Species Associated with Black Foot Disease of Grapevine in Spain

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ABSTRACT

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In this work, 82 *Cylindrocarpon* isolates associated with black foot disease of grapevines in Spain were studied by means of phenotypical characterization, DNA analyses, and pathogenicity tests. Partial sequences of the beta-tubulin (BT) gene, BT1, were amplified using primers BT1a and BT1b. A unique and conserved 52-bp insertion in the BT1 sequence, which is a specific marker for *C. macrodidymum*, was found in 56 of the isolates. The rest of the isolates (26) were identified as *C. liriodendri*. The BT phylogeny grouped all the isolates of each species into two well-supported clades. Phenotypical data were subjected to multivariate factorial analysis. According to this study, the isolates were clearly separated into two groups which were in agreement with BT species identification. *C. macrodidymum* isolates were differentiated from *C. liriodendri* by producing fewer conidia, presenting longer and wider macroconidia, and lower growth rate at 5 and 10°C. Selected isolates of each species inoculated onto rooted cuttings of grapevine rootstock cv. 110 R caused typical black foot disease symptoms. This is the first report of *C. liriodendri* and *C. macrodidymum* causing black foot disease of grapevines in Spain.

Additional keywords: grapevine nurseries, *Vitis*, young vineyards

Over the last decade, the incidence of fungal grapevine trunk diseases has increased significantly in the different grapevine production areas around the world, particularly in grapevine nurseries and young vineyards. Consequently, there is a renewed interest in the research about these diseases and their causal agents; among them, black foot disease of grapevines caused by *Cylindrocarpon* spp. (13).

Vines affected by *Cylindrocarpon* spp. show sunken necrotic root lesions with a reduction in root biomass and root hairs. Removal of rootstock bark reveals black discoloration and necrosis of wood tissues which develops from the base of the rootstock. Other symptoms include reduced vigor, shortened internodes, sparse foliage, and small leaves with interveinal chlorosis and necrosis, frequently leading to death of the plants (11,13,19,25,29). Characteristic black discoloration of affected rootstocks has given the disease the name of black foot (12,19).

Two species of *Cylindrocarpon* had traditionally been reported as the causal agents of black foot disease of grapevines:

C. destructans (Zinssmeister) Scholten and *C. obtusisporum* (Cooke & Harkn.) Wollenweb. *C. destructans* was first reported on grapevine in France in 1961 (19), and since then, it was isolated from diseased vines in Italy (11), Portugal (25), Argentina (9), Germany (7), New Zealand (14), South Africa (14), and Brazil (8). *C. obtusisporum* was identified as the causal agent of this disease in Sicily (12) and in California (29).

In 2004, Halleen et al. (14) characterized phylogenetically a collection of *Cylindrocarpon* isolates obtained from grapevine plants in South Africa, New Zealand, Australia, and France, and described a new species: *C. macrodidymum* Halleen, Schroers, & Crous. Later, this species was reported in California (22) and Chile (3). Recently, Halleen et al. (15) compared *C. destructans* isolates obtained from diseased grapevines and other herbaceous and woody hosts with other species in the genus *Cylindrocarpon*. The results revealed that the French, Portuguese, and South African grapevine isolates previously identified as *C. destructans* or *C. liriodendri* J.D. MacDonald & E.E. Butler had identical morphological characters, internal transcribed spacer (ITS), and partial beta-tubulin (BT) gene sequences. *C. liriodendri* was first reported associated with root rot of tulip poplar (*Liriodendron tulipifera* L.) (18); consequently, Halleen et al. (15) proposed that all *C. destructans* isolates from grapevine included in this study must

be reidentified as *C. liriodendri*. Petit and Gubler (23) reached the same conclusion in relation to *C. destructans* isolates from California.

Cylindrocarpon spp. also have been isolated from young declining vines with basal rot or root necrosis in several grapevine-growing countries such as Spain, Greece, or Australia (1,6,26), but to date, no taxonomic studies have been conducted in these countries to further identify the species present.

In Spain, surveys carried out in recent years in grapevine nurseries and young vineyards have confirmed the importance of *Cylindrocarpon* spp. affecting this crop. These pathogens were found in grapevine nurseries very early in the planting material production process, in grapevine plants ready to be planted and in young vineyards showing decline symptoms. In all cases, *Cylindrocarpon* spp. were mostly isolated from rootstocks, especially from the basal end (2,10).

The objective of this study was to identify the species of *Cylindrocarpon* causing black foot disease of grapevine in Spain. In order to do this, isolates from grapevines representative of different locations in Spain were studied by means of phenotypical characterization, DNA analyses, and pathogenicity tests.

MATERIALS AND METHODS

Fungal isolates. In this study we used 82 *Cylindrocarpon* isolates representative of different locations and scion/rootstock combinations, obtained from grapevine nurseries and young vineyards in Spain between 2001 and 2004. Additionally, reference isolates of the species *C. liriodendri* (CBS 117640, CBS 117526, Cy 13 Portugal, and Cy 15 Portugal) and *C. macrodidymum* (CBS 112609) were obtained from the collection of the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (CBS), and from Portugal provided by C. Rego (Laboratório de Patologia Vegetal "Veríssimo de Almeida", Lisboa, Portugal) (Table 1).

Isolates were recovered from roots and the basal end of rootstocks from grapevines exhibiting symptoms of black foot. Sections were cut from affected areas, washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces of discolored tissues were plated on malt

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extract agar (MEA) (Oxoid Ltd., Basingstoke, Hants, England) supplemented with 0.5 g liter⁻¹ of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (MEAS). Plates were incubated for 5 to 10 days at 25°C in the dark. Isolates were transferred to potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France), incubated as previously described, and identified as *Cylindrocarpon* spp. based on colony morphology and conidial characteristics (4,28).

The isolates were single-spored prior to use by means of the serial dilution method (5) and stored in 15% glycerol solution at -80°C in 1.5-ml cryovials.

Phenotypical characterization. All *Cylindrocarpon* isolates were grown at 25°C in darkness or under NUV + fluorescent illumination with a 12-h photoperiod (Philips TLD18W/33) for 10 days. Media

used were PDA and Spezieller Nährstoffarmer agar (SNA) with the addition of 1 × 1 cm piece of filter paper to the colony surface. Macroscopic characters such as colony texture, color, and the type of growing margin were evaluated on PDA. Colonies grown on PDA were further incubated during 20 days to determine the presence/absence of chlamydospores. The diameter of 20 chlamydospores per isolate was measured. Length and width of 50 conidia (microconidia and one-, two-, and three-septate macroconidia) per isolate were measured on SNA at ×400 magnification. Reverse colony color was determined as described by Petit and Gubler (22); for this, all isolates were grown at 25°C for 6 days in the light conditions described above on 2% MEA (Difco, Sparks, MD, USA).

To determine the effect of temperature on radial colony growth, all isolates were

maintained and grown on PDA plates at 25°C. Plugs of agar, 5 mm in diameter, were cut from the leading edge of 7-day-old colonies and placed in the center of PDA plates that were then incubated in the dark at 5, 10, 15, 20, 25, 30, and 35°C. There were four replicates for each isolate and temperature combination. After 10 days, colony diameter was measured along two perpendicular axes, and data were converted to radial growth in millimeters per day. The experiment was repeated twice.

The number of conidia produced on mycelia from agar plugs was measured following the method described by Whiting et al. (31). Each isolate was grown on four PDA plates and incubated in darkness at 25°C. After 7 days of incubation, two plugs of agar with mycelia and spores, 4 mm diameter, were cut from the growing

Table 1. *Cylindrocarpon* isolates studied

Species ^x Isolate ^y	Year	Geographical origin		Scion/rootstock
		Town ^z	Province	
<i>C. liriodendri</i>				
Cy 18	2002	Daimiel	Ciudad Real	Tempranillo/110 R
Cy 21	2002	Ladero	La Rioja	Tempranillo/110 R
Cy 23	2002	Campo de Criptana	Ciudad Real	Cencibel/110 R
Cy 36	2002	Iniesta	Cuenca	Cencibel/1103 P
Cy 37	2002	Villanueva de Alcardete	Toledo	Cencibel/110 R
Cy 38	2002	Villanueva de Alcardete	Toledo	Cencibel/110 R
Cy 39	2002	Herencia	Ciudad Real	Tempranillo/110 R
Cy 58	2003	Cózar	Ciudad Real	Cencibel/110 R
Cy 59	2003	Tarazona	Albacete	Cencibel/1103 P
Cy 64	2003	Badajoz	Badajoz	Pardino/n/d
Cy 66	2003	Villarobledo	Albacete	Cencibel/110 R
Cy 67	2003	Tarazona	Albacete	Tempranillo/110 R
Cy 69	2003	Almaguer	Toledo	Tempranillo/41-B M
Cy 72	2003	Xàtiva	Valencia	Monastrell/110 R
Cy 80	2003	Aielo de Malferit	Valencia	n/d
Cy 85	2003	Fontaneres	Valencia	Tempranillo/161-49 C
Cy 87	2004	Aielo de Malferit	Valencia	Macabeo/110 R
Cy 88	2004	Aielo de Malferit	Valencia	Macabeo/110 R
Cy 89	2004	Aielo de Malferit	Valencia	Garnacha/110 R
Cy 90	2004	Aielo de Malferit	Valencia	Garnacha/110 R
Cy 91	2004	Tarazona de la Mancha	Albacete	Cabernet /1103 P
Cy 93	2004	Villamayor de Santiago	Cuenca	Cencibel/110 R
Cy 94	2004	Calzada de Calatrava	Ciudad Real	Tempranillo/110 R
Cy 97	2004	Haro	La Rioja	Tempranillo/110 R
Cy 99	2004	Monóvar	Alicante	Syrah/1103 P
Cy 100	2004	Alesanco	La Rioja	Garnacha/110 R
Cy 13 Portugal	1995		Portugal	99 R
Cy 15 Portugal	1995		Portugal	99 R
CBS 117640	1992		Portugal	Seara Nova /99 R
CBS 117526	1999		Portugal	99 R
<i>C. macrodidymum</i>				
Cy 1	2001	n/d	Alicante	Aledo/1103 P
Cy 2	2001	Autol	La Rioja	Tempranillo/110 R
Cy 3	2001	Autol	La Rioja	Tempranillo/110 R
Cy 9	2001	L'Alcudia	Valencia	n/d
Cy 12	2002	Villafranca de Caballeros	Toledo	Cencibel/110 R
Cy 14	2002	n/d	Burgos	Tempranillo/110 R
Cy 15	2002	n/d	Ciudad Real	Tempranillo/110 R
Cy 16	2002	n/d	Ciudad Real	Tempranillo/110 R
Cy 17	2002	La Solana	Ciudad Real	Tempranillo/1103 P
Cy 19	2002	Villarubia de los Ojos	Ciudad Real	Tempranillo/110 R

(continued on next page)

^x Species of Spanish isolates were determined by means of beta-tubulin analysis and multivariate factorial analysis of phenotypical data.

^y Reference isolates of the species *C. liriodendri* (CBS117640, CBS 117526, Cy 13 Portugal, and Cy 15 Portugal) and *C. macrodidymum* (CBS 112609) were obtained from the collection of the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (CBS), and from Portugal provided by C. Rego (Laboratório de Patologia Vegetal "Veríssimo de Almeida" Lisboa, Portugal).

^z n/d: not determined.

edge of the colonies, and each was placed in a 1.5-ml Eppendorf vial containing 1 ml of sterile water. Vials with plugs were vortexed 5 s, and the number of conidia per ml was counted using a hemacytometer. The experiment was repeated twice.

Phenotypical data were analyzed by multivariate factorial analysis (21) using the Statgraphics Plus 5.1 software (Manugistics Inc., Rockville, MD, USA). Sixteen variables were included in this analysis: mean length and width of microconidia and one-, two-, and three-septate macroconidia, mean chlamydospore diameter, mean daily growth rate at all temperatures studied, and mean log₁₀ of conidia per ml.

DNA extraction, polymerase chain reaction (PCR), sequencing, and BT phylogeny. Fungal mycelium and conidia from pure cultures grown on PDA for 2 weeks at 25°C in the dark were scraped and me-

chanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, USA) following the manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and was stored at -20°C.

The specific primers Dest1 and Dest4 for *C. destructans*, which amplify one region of the ITS (16), were used for direct PCR amplification as described by Nascimento et al. (20). In addition, partial sequences of the BT gene, BT1, were amplified using primers BT1a and BT1b (22). Each PCR reaction contained 1× PCR buffer, 2.5 mM MgCl₂, 200 μM each dNTP, 0.4 μM of each primer, 1 U of DNA *Taq* polymerase (Dominion MBL, Córdoba, Spain), and 1 μl of template DNA.

The PCR reaction mix was adjusted to a final volume of 25 μl with water (Chromasolv Plus, Sigma-Aldrich, Steinheim, Germany). PCR amplifications were performed on a Peltier Thermal Cycler-200 (MJ Research). The program consisted of an initial step of 4 min at 94°C, followed by 30 cycles (35 cycles for BT) of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and an elongation at 72°C for 1.5 min. A final extension was performed at 72°C for 10 min. The PCR products were visualized in 1.5% agarose gels (agarose D-1 Low EEO, Conda, Madrid, Spain). A 100-bp ladder was used as a molecular weight marker (Dominion MBL).

PCR products of BT1 were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Germany) and sequenced in both directions by the DNA Sequencing Service of the Universidad

Table 1. (continued from previous page)

Species ^x Isolate ^y	Year	Geographical origin		Scion/rootstock
		Town ^z	Province	
<i>C. macrodidymum</i> (continued)				
Cy 22	2002	Tomelloso	Ciudad Real	Tempranillo/140 Ru
Cy 25	2002	n/d	Murcia	n/d
Cy 28	2002	Monóvar	Alicante	Monastrell/n/d
Cy 29	2002	Monóvar	Alicante	Monastrell/n/d
Cy 32	2002	Daimiel	Ciudad Real	n/d
Cy 33	2002	Daimiel	Ciudad Real	n/d
Cy 34	2002	Granja de Iniesta	Cuenca	Cabernet Sauvignon/110 R
Cy 40	2002	Carrión de Calatrava	Ciudad Real	Cencibel/110 R
Cy 41	2002	Carrión de Calatrava	Ciudad Real	Cencibel/110 R
Cy 42	2002	Villar	Valencia	Garnacha/161-49 C
Cy 43	2002	Ejea de los Caballeros	Zaragoza	n/d
Cy 45	2002	Herencia	Ciudad Real	Tempranillo/110 R
Cy 47	2003	Mollina	Málaga	Pedro Ximénez/1103 P
Cy 48	2003	Mollina	Málaga	Pedro Ximénez/140 Ru
Cy 49	2003	Mollina	Málaga	Pedro Ximénez/140 Ru
Cy 57	2003	Mollina	Málaga	Cabernet Franc/n/d
Cy 60	2003	Campo de Criptana	Ciudad Real	Tempranillo/n/d
Cy 61	2003	Pozuelo de Calatrava	Ciudad Real	Tempranillo/161-49 C
Cy 62	2003	Quero	Toledo	Tempranillo/110 R
Cy 63	2003	Daimiel	Ciudad Real	Cencibel/110 R
Cy 65	2003	Membrilla	Ciudad Real	Cencibel/110 R
Cy 68	2003	Almaguer	Toledo	Tempranillo/41-B M
Cy 70	2003	Quero	Toledo	161-49 C
Cy 71	2003	Beniganim	Valencia	161-49 C
Cy 73	2003	Fuente de la Higuera	Valencia	41-B M
Cy 74	2003	Anna	Valencia	Tempranillo/41-B M
Cy 75	2003	Xàtiva	Valencia	Tempranillo/41-B M
Cy 76	2003	Anna	Valencia	1103 P
Cy 77	2003	Aielo de Malferit	Valencia	161-49 C
Cy 78	2003	Alfarrasí	Valencia	1103 P
Cy 79	2003	Albaida	Valencia	140 Ru
Cy 81	2003	Beneixama	Alicante	Tempranillo/161-49 C
Cy 82	2003	Xàtiva	Valencia	Syrah/110 R
Cy 83	2003	Fontaneres	Valencia	Tempranillo/161-49 C
Cy 84	2003	Aielo de Malferit	Valencia	140 R
Cy 86	2004	Mollina	Málaga	Pedro Ximénez/110 R
Cy 92	2004	Alcázar de San Juan	Ciudad Real	Cencibel/161-49 C
Cy 95	2004	Alcázar de San Juan	Toledo	Moscatel/Gravesac
Cy 96	2004	Lillo	Toledo	1103 P
Cy 98	2004	Turís	Valencia	Monastrell/161-49 C
Cy 101	2004	Alesanco	La Rioja	Tempranillo/110 R
Cy 102	2004	Haro	La Rioja	Tempranillo/110 R
Cy 103	2004	Villar	Valencia	Merseguera/n/d
Cy 104	2004	Fuendejalón	Zaragoza	Cabernet Sauvignon/n/d
Cy 105	2004	Fuendejalón	Zaragoza	Merlot/110 R
Cy 106	2004	Almansa	Albacete	Tempranillo/n/d
CBS 112609	1979		Australia	Cabernet Sauvignon/n/d

Politécnica de Valencia-CSIC. Sequences were edited using the program DNAMAN (Version 4.03, Lynnon BioSoft, Quebec, Canada). Sequences were aligned using the CLUSTAL W program (30). The genetic distances were calculated using the Kimura 2-parameter model. For phylogenetic inference, the neighbor-joining (NJ) method was used (27). The NJ tree and the statistical confidence of a particular group of sequences in the tree, evaluated by bootstrap test (1,000 pseudoreplicates), were performed using the computer program MEGA version 3.1 (17). Additional sequences of *C. liriodendri* from California (AY997601 and AY997602) and France (AY997587), and *C. macrodidymum* from California (AY997608 and AY997619) and Chile (AY997604) were obtained from GenBank and added to the alignment.

Pathogenicity. The pathogenicity of selected isolates (Cy 1, Cy 14, Cy 32, Cy 36,

Cy 58, and Cy 88) representative of species previously determined by phenotypical studies and BT phylogeny was evaluated. Isolates were grown on PDA for 1 month at 25°C prior to inoculation.

Inoculum was prepared for each isolate by flooding the agar surface with 10 ml of sterile distilled water (SDW) and scraping with a spatula. The resulting spore suspension was filtered through two layers of cheesecloth. The filtrate was diluted with SDW and conidial concentration was adjusted with a hemacytometer to 10^6 conidia per ml.

Eight-month-old dormant rooted cuttings of grapevine rootstock cv. 110 R were used for pathogenicity tests. Before inoculation, roots were trimmed and disinfested by immersion for 2 min in a 1.5% sodium hypochlorite solution, and washed twice with SDW. For each *Cylindrocarpon* isolate, eight plants were inoculated by dipping their roots for 30 min in the conid-

ial suspension. Control plants were dipped in SDW. Rooted cuttings were then planted individually in 15-cm-diameter plastic pots containing sterilized peat moss and placed in a greenhouse at 25 to 30°C in a completely randomized design. One month later, each plant was drench inoculated with 40 ml of the corresponding spore solution (10^6 conidia per ml) to guarantee root infection or SDW. After 4 months of incubation, all plants were gently uprooted and washed free of soil. Root symptoms of individual plants were evaluated on the following scale: 0 = healthy with no lesions, 1 = slight discoloration with 0 to 10% root mass reduction, 2 = slight discoloration with 10 to 25% root mass reduction, 3 = moderate discoloration with 25 to 50% root mass reduction, 4 = severe discoloration with >50% root mass reduction, 5 = dead plant. In addition, dry weights of shoot and root were recorded for each plant. Symptomatic roots were aseptically plated on MEAS to reisolate the fungus, as described above. Pathogenicity tests were conducted twice.

Root disease severity data were analyzed with the GENMOD procedure using the multinomial distribution and the cumulative logit as link function in SAS (SAS Institute Inc., Cary, NC, USA). Mean values were separated by chi-square test at $P < 0.05$. Analyses of variance (ANOVA) were conducted on root and shoot dry weights to determine the effects of different *Cylindrocarpon* isolates on grapevine rootstock cv. 110 R, using the Statgraphics Plus 5.1 software (Manugistics Inc., Rockville, MD, USA). Mean values were separated by Student's least significant difference test at $P < 0.05$.

RESULTS

Phenotypical studies. Colony morphology on PDA was very heterogeneous. Aerial mycelium was floccose to felted, and the color varied from white to yellow or light to dark brown. Colony margins were entire, slightly lobulated, or lobulated. All isolates produced both microconidia and macroconidia, the latter having one to three septa, and chlamydospores, which were intercalary or terminal in the mycelium, single or occasionally in chains. The types of conidia and chlamydospores and their dimensions agreed with those described for the genus *Cylindrocarpon* (4,28). Approximately 50% of the isolates had a buff reverse on 2% MEA, whereas 50% had an orange-dark brown reverse.

All isolates grew at temperatures of 5, 10, 15, 20, 25, and 30°C but not at 35°C. The optimal temperature was between 20 and 25°C. The sporulation of the isolates also showed a broad range of variation (from 13.0 to 2.2×10^6 conidia per ml).

In the multivariate factorial analysis of the phenotypical data, the two factors that presented the highest eigenvalues (7.03 and 2.28, respectively) were extracted.

Table 2. Parameters for factors 1 and 2 extracted from the multivariate factorial analysis

Variables analyzed	Factor 1	Factor 2
Log ₁₀ conidia per ml	0.809333 ^z	-0.069914
Daily growth rate at 5°C	0.899077 ^z	-0.248582
Daily growth rate at 10°C	0.913733 ^z	-0.212563
Daily growth rate at 15°C	0.674998	-0.234995
Daily growth rate at 20°C	0.241948	-0.183932
Daily growth rate at 25°C	-0.132501	0.044135
Daily growth rate at 30°C	0.505865	0.127111
Length of microconidia	-0.734763	0.393431
Width of microconidia	-0.733337	0.432814
Length of one-septate macroconidia	-0.203174	0.804342 ^z
Width of one-septate macroconidia	-0.152505	0.732897 ^z
Length of two-septate macroconidia	-0.457782	0.730430 ^z
Width of two-septate macroconidia	-0.071949	0.877877 ^z
Length of three-septate macroconidia	-0.504006	0.654497 ^z
Width of three-septate macroconidia	-0.123738	0.837912 ^z
Diameter of chlamydospores	0.435739	0.047171

^z Main contributing variables for factors 1 and 2.

Fig. 1. Projection of *Cylindrocarpon* isolates on the plane of factors 1 and 2 from multivariate factorial analysis.

They accounted for approximately 58.3% of the total variance (44.0 and 14.3% for factors 1 and 2, respectively). The main contributing variables for factor 1 were $\log_{10}$ of conidia per ml and the daily growth rate at 5 and 10°C, and the main contributing variables for factor 2 were the length and width of one-, two-, and three-septate macroconidia (Table 2). The projection of all *Cylindrocarpon* isolates on the plane of factor 1 and 2 clearly separated them into two groups (Fig. 1). One of them clustered 56 of the Spanish *Cylindrocarpon* isolates and the reference isolate of *C. macrodidymum*, while the other clustered the remaining 26 Spanish *Cylindrocarpon* isolates and the four reference isolates of *C. liriodendri*. Mean and range values for these dominating phenotypical variables used to characterize both species among Spanish *Cylindrocarpon* isolates are listed in Table 3.

PCR, sequencing, and BT phylogeny. All Spanish and reference *Cylindrocarpon* isolates were amplified with the primers Dest1 and Dest4. A PCR fragment of about 400 bp was obtained for all of them.

Using PCR with the primers BT1a and BT1b, a fragment of about 520 bp was obtained for 56 of the Spanish isolates, and a fragment of about 470 bp was obtained for the remaining 26 isolates. When sequences obtained from these PCR products were aligned using the CLUSTAL W program, a 52-bp insertion in the BT1 sequence was observed in those of greatest size.

Of the 423 nucleotides included in the BT1 phylogeny, 31 were variable. The NJ analysis identified two well-supported (100%) clades (Fig. 2). One clade comprised the 56 Spanish *Cylindrocarpon* isolates with the 52-bp insertion, the reference isolate of *C. macrodidymum*, and the sequences of *C. macrodidymum* (AY997620, AY997619, AY997608, AY997604), all with quasi-similar sequences. The other clade comprised the other 26 Spanish *Cylindrocarpon* isolates, the four reference isolates of *C. liriodendri*, and the sequences of *C. liriodendri* (AY997602, AY997587, AY997601), all with quasi-similar sequences.

Pathogenicity. Results of pathogenicity tests 4 months after plantation are shown in Table 4. A preliminary analysis of the data showed no significant trial-treatment interactions; therefore, the data from the two trials were combined. All Spanish *Cylindrocarpon* isolates tested were pathogenic to grapevine rootstock cv. 110 R. *P* values indicated a significant effect of the isolates on root severity index ($P < 0.0001$), root weight ($P = 0.0003$), and shoot weight ($P < 0.0001$). The root severity index ranged from 0.93 to 2.15 with a complete gradation among the isolates. All isolates significantly decreased the root and shoot dry weight, with the exception of isolate Cy 36, from which the average

shoot dry weight was not significantly different compared with the control. The isolates were reisolated from affected plants on MEAS as described above, confirming Koch's postulates.

DISCUSSION

This is the first study of a wide group of *Cylindrocarpon* isolates obtained from grapevine plants showing symptoms of black foot in grapevine nurseries and young vineyards in Spain. Based on the analysis of phenotypical characters and BT data, two species have been identified: *C. liriodendri* and *C. macrodidymum*. This result agrees with those obtained in South Africa and California, where these species have also been described as the main causal agents of black foot disease of grapevine (14,15,22,23).

The species-specific primers Dest1 and Dest4 were designed by Hamelin et al. (16) to specifically detect *C. destructans* in the roots of conifer seedlings. In previous studies, Nascimento et al. (20) showed that these primers also amplified two other *Cylindrocarpon* species: *C. liriodendri* (previously identified as *C. destructans*) obtained from grapevines in Portugal (24) and *C. obtusisporum* obtained from grapevines in California. Here we confirmed that these primers also amplified all *C. liriodendri* and *C. macrodidymum* isolates nonspecifically. Thus, these primers are not useful to specifically recognize *C. destructans*.

Analysis of the BT1 sequence data, which has been used by other researchers to identify *Cylindrocarpon* species on grapevine (14,22), allowed the identification of *C. macrodidymum* or *C. liriodendri* among the Spanish isolates. The existence of a 52-bp insertion in the BT1 sequence is considered to be a specific marker for *C. macrodidymum* (22). Here this insertion was found in the reference isolate for this species and 56 of the Spanish isolates,

whereas it was not present in the reference isolates for *C. liriodendri* and the rest of the isolates (26 in total), which were identified as *C. liriodendri*. Moreover, the BT phylogeny grouped all the isolates of each species into two well-supported clades, which included the reference isolates and the additional sequences of *C. macrodidymum* and *C. liriodendri*. Strikingly, we found that *Cylindrocarpon* isolates of each species from diverse Spanish geographical origins and isolates from other origins including Australia, California, Chile, France, and Portugal had quasi-similar sequences.

In concordance with the published results obtained by other authors, phenotypical characteristics of the Spanish isolates were very variable (14,22,24). *Cylindrocarpon* colonies showed different colors and textures. Conidial dimensions were also heterogeneous, and sporulation showed a broad range of variation. Thus, it was not possible to establish a clear concordance with these results and the description of each species. Halleen et al. (14) used the sizes of macroconidia to separate *C. macrodidymum* from *C. liriodendri*, indicating that the first produces larger and wider macroconidia than the second. Nevertheless, in our collection of *Cylindrocarpon* isolates this characteristic overlapped because some of the isolates, which were identified as *C. macrodidymum*, had lower mean dimensions of macroconidia than isolates identified as *C. liriodendri*. Petit and Gubler (22) noted that *C. macrodidymum* isolates produced relatively more chlamydospores than *C. liriodendri*, although they considered this characteristic too inconsistent to be used for identification purposes. These authors indicated that it is possible to differentiate *C. liriodendri* from *C. macrodidymum* on the basis of reverse color on 2% MEA. *C. liriodendri* isolates had a buff reverse, whereas *C. macrodidymum* isolates had an

Table 3. Mean and range values for the main contributing phenotypical variables in the multivariate factorial analysis used to characterize Spanish isolates of *Cylindrocarpon*

Phenotypic characters	Species ^{y,z}	
	<i>C. liriodendri</i>	<i>C. macrodidymum</i>
Log ₁₀ of conidia per mL	(5.3) 5.7 (6.4)	(1.1) 3.5 (5.5)
Daily growth rate (mm)		
5°C	(0.19) 0.32 (0.46)	(0.01) 0.06 (0.12)
10°C	(0.46) 0.72 (0.81)	(0.11) 0.28 (0.50)
Macroconidia (μm)		
One-septate		
Length	(11.6) 16.1 (21.6)	(12.7) 18.6 (26.9)
Width	(3.7) 4.4 (5.0)	(3.8) 4.6 (5.3)
Two-septate		
Length	(21.5) 23.4 (25.6)	(27.2) 30.5 (33.7)
Width	(4.8) 5.0 (5.3)	(5.0) 5.4 (5.7)
Three-septate		
Length	(24.6) 27.5 (29.7)	(33.1) 35.9 (39.0)
Width	(5.0) 5.3 (5.6)	(5.5) 5.8 (6.2)

^y Species of Spanish isolates were determined by means of beta-tubulin analysis and multivariate factorial analysis of phenotypical data.

^z Values represent the mean of each phenotypic character (range—minimum and maximum—in parentheses).

Fig. 2. Neighbor-joining phylogeny based on nucleotide divergences estimated according to the Kimura 2-parameter model from partial sequences of the beta-tubulin (BT) gene, BT1 of *Cy lindrocarpon* isolates listed in Table 1, and additional sequences of *C. liri dendri* from California (AY997601 and AY997602) and France (AY997587), and *C. macrodidymum* from California (AY997608 and AY997619) and Chile (AY997604) obtained from GenBank. Numbers on nodes are the percent frequency with which a cluster appears in a bootstrap test of 1,000 runs.

orange–dark brown reverse. Nevertheless, although both types of colors were observed when our isolates were grown on 2% MEA, they did not correspond with the species identification because they were produced both by *C. liri dendri* and *C. macrodidymum* isolates.

In our study, the phenotypical differentiation between both species was achieved by multivariate factorial analysis. The two factors selected in this analysis accounted for approximately 60% of the total variance. The projection of Spanish *Cy lindrocarpon* isolates on the plane of these factors clearly clustered them into two groups of 56 and 26 isolates, which corresponded to the species *C. macrodidymum* and *C. liri dendri*, respectively. The species identification performed using this analysis coincided exactly with the results obtained with the study of the sequences of the BT1 gene. Log₁₀ of conidia per ml, the daily growth rate at 5 and 10°C, and the length and width of one-, two-, and three-septate macroconidia were the main contributing variables to separate both groups. Consequently, *C. macrodidymum* can be differentiated from *C. liri dendri* into a collection of isolates by producing fewer conidia, presenting longer and wider macroconidia, and lower growth rate at 5 and 10°C.

Pathogenicity tests showed that Spanish isolates of both species were able to induce typical black foot disease symptoms and caused shoot and root mass reduction, confirming their pathogenicity to grapevine rootstock cv. 110 R, in agreement with previous results (14,22).

C. obtusisporum has been one of the species associated in the past with black foot disease of grapevine. Nevertheless, in our study this species was not found among Spanish *Cy lindrocarpon* isolates. Halleen et al. (14), who did not find this species, discussed the possibility that isolates previously identified as *C. obtusisporum* were in fact *C. macrodidymum*. Petit and Gubler (22) indicated that *C. obtusisporum* isolates described associated to black foot in California (29) also might be misidentified.

In conclusion, *C. macrodidymum* and *C. liri dendri* have been identified associated with young vines in Spain. *C. macrodidymum* is the predominating species, representing two-thirds of the studied isolates, although both *Cy lindrocarpon* species are present in all grapevine growing regions in Spain. This is the first report of *C. liri dendri* and *C. macrodidymum* causing black foot disease of grapevine in Spain.

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Table 4. Pathogenicity of Spanish *Cylindrocarpon* isolates to grapevine rootstock cv. 110 R 4 months after plantation

Species Isolate	Root disease severity ^w	Root dry weight (g)	Shoot dry weight (g)
<i>C. liriodendri</i>			
Cy 36	1.20 ^x a ^y	4.09 c ^z	11.21 d ^z
Cy 59	2.15 a	3.35 ab	9.20 bc
Cy 88	1.27 a	4.09 c	9.85 bc
<i>C. macrodidymum</i>			
Cy 1	1.50 a	3.63 abc	8.88 ab
Cy 14	2.00 a	3.22 a	7.64 a
Cy 32	0.93 a	4.07 bc	9.34 bc
Control	0.00 b	4.92 d	10.25 cd

^w Root symptoms were rated using the following scale: 0 = healthy with no lesions, 1 = slight discoloration with 0 to 10% root mass reduction, 2 = slight discoloration with 10 to 25% root mass reduction, 3 = moderate discoloration with 25 to 50% root mass reduction, 4 = severe discoloration with >50% root mass reduction, 5 = dead plant.

^x Values represent the mean of 16 replications for each isolate.

^y GENMOD. Means in a column followed by the same letter are not significantly different according to Chi-square test at $P < 0.05$.

^z ANOVA. Means in a column followed by the same letter are not significantly different according to Student's least significant difference test at $P < 0.05$.

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