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Characterization of *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* isolates associated with loquat decline in Spain, with description of *Cylindrodendrum alicantinum* sp. nov.

Carlos Agustí-Brisach · Ana Cabral · Elisa González-Domínguez · Ana Pérez-Sierra · Maela León · Paloma Abad-Campos · José García-Jiménez · Helena Oliveira · Josep Armengol

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Abstract Thirty-one loquat orchards (*Eriobotrya japonica* 'Algerie') with plants exhibiting decline symptoms were surveyed between 2004 and 2007 in the province of Alicante, Spain. Twenty-eight representative isolates with *Cylindrocarpon*-like asexual morphs recovered from affected roots were included in this study, with the objective to characterize them by means of phenotypical characterization, DNA analysis and pathogenicity tests. *Dactylonectria alcacerensis*, *D. torresensis* and *Ilyonectria robusta* were identified based on morphological and cultural characteristics as well as DNA sequence data for part of histone H3, with *D. torresensis* the most frequent species. All of them are reported for the first time on loquat, and *I. robusta* is reported for the first time in Spain. In addition, one species is newly described, *Cylindrodendrum alicantinum*. Pathogenicity tests with representative isolates showed that these species were able to induce typical root rot disease symptoms, affecting plant development or even leading to plant death. This research demonstrates the association of species belonging to the genera *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* with root rot of loquat and loquat decline in the province of Alicante (eastern Spain). This information should be considered for the improvement of the current management strategies against these soil-borne pathogens when establishing new loquat plantations or introducing new susceptible fruit crops in the region.

Keywords *Cylindrodendrum alicantinum* · *Dactylonectria alcacerensis* · *D. torresensis* · *Eriobotrya japonica* · *Ilyonectria robusta* · loquat decline

C. Agustí-Brisach · E. González-Domínguez · A. Pérez-Sierra · M. León · P. Abad-Campos · J. García-Jiménez · J. Armengol (✉)
Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain
e-mail: jarmengo@eaf.upv.es

A. Cabral · H. Oliveira
Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisbon, Portugal

Present Address:
E. González-Domínguez
Department of Sustainable Crop Production - DI.PRO.VE.S., Facoltà di Scienze agrarie, alimentari e ambientali, Università Cattolica del Sacro Cuore, Via Emilia Parmense, 84, 29122 Piacenza, Italy

Introduction

Loquat (*Eriobotrya japonica*) is a subtropical evergreen fruit tree species native to southern China that, under Mediterranean climatic conditions, flowers in autumn, develops fruits in winter, and ripens in spring (Janick 2011; Reig et al. 2012). It is mainly cultivated in Asia and the Mediterranean basin, being Spain, Israel, Italy and Turkey the main producing countries in this area (Calabrese 2006; Reig et al. 2012). Although there are no FAO statistics available for world loquat fruit production, Lin (2007) reported 550,000 tons for 2006, with a crop area exceeding 130,000 ha, with China

and Spain being the main producing countries. In Spain, one of the most important areas of loquat production is located in the province of Alicante (eastern Spain), in the valleys of the rivers Algar and Guadalest, which accounts for nearly 60 % of total Spanish loquat production. The crop is mostly located in family orchards, in high-density plantations, using drip irrigation. The cultivar Algerie and its mutations represent 98 % of the total production (Soler et al. 2007).

Loquat scab caused by *Fusicladium eriobotryae* is the main disease affecting this crop in Spain as well as in the whole Mediterranean basin (Soler et al. 2007; Sánchez-Torres et al. 2009; González-Domínguez et al. 2013, 2014). Nevertheless, since the late 1990s, an emerging problem of decline and death of loquat trees associated with *Armillaria mellea*, *Rosellinia necatrix* and *Phytophthora* spp. has been observed in the province of Alicante (González-Domínguez et al. 2008, 2009). Loquat trees infected by these soil-borne pathogens manifest two types of symptoms; those of the root system, and those of the aerial part of the plant arising as a consequence of the damaged roots. In some cases, very limited external symptoms of infections at ground level are observed, but in others, cankers are very noticeable. In this latter case, affected areas often include the lower trunk, root collar and large roots. As a consequence, affected trees present reduced plant vigour, chlorosis, small leaves and fruits, early leaf drop and dieback of twigs and branches. Dieback of young shoots has been observed when the crown area is severely diseased, presumably due to invasion by secondary pathogens. This condition is often referred as "tree decline", because it is a gradual loss of vigor, and the trees eventually die (González-Domínguez et al. 2008, 2009).

In addition to *A. mellea*, *R. necatrix* and *Phytophthora* spp., surveys of loquats showing the common symptomatology described before, conducted from 2004 to 2007 in the province of Alicante, lead to the recovery of abundant fungal isolates with *Cylindrocarpon*-like asexual morphs from rotted roots. It is well known that these are soil-borne fungi, which are generally regarded as pathogens and/or saprobes of various hosts and substrates, associated with a variety of disease symptoms that include rot of roots, stems and cuttings of agricultural, forestry and horticultural crops (Halleen et al. 2006; Schroers et al. 2008; Chaverri et al. 2011; Cabral et al. 2012b; Agustí-Brisach and Armengol 2013; Lombard et al. 2014). Recently, species belonging to this group of fungi have been isolated from fruit trees showing root rot symptoms, such as avocado (*Persea americana*) (Vitale et al. 2012), kiwifruit (*Actinidia chinensis*) (Erper et al. 2013) and Arecaceae palms (Aiello et al. 2014). However, species with *Cylindrocarpon*-like asexual morphs have never been described associated with root rot of loquat. Thus, the aim of this study was to characterize a collection of *Cylindrocarpon*-like isolates, which were obtained from the roots of loquat trees showing symptoms of decline in the province of Alicante, by means of phenotypical characterization, DNA analysis and pathogenicity tests.

Materials and methods

Fungal isolation

Thirty-one loquat orchards ('Algerie') exhibiting decline symptoms were surveyed between 2004 and 2007 in the province of Alicante. In each orchard, at least three loquat trees were examined carefully. Affected trees showed symptoms at ground level which included necrotic lesions on roots, with a reduction in root biomass and root hairs.

Affected roots were 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 discoloured tissues were placed on potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) amended with 0.5 g l^{-1} of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS). Plates were incubated for 5–10 days at 25 °C in the dark.

Twenty-eight representative isolates with *Cylindrocarpon*-like asexual morphs, obtained from nine orchards, were selected for further analysis (Table 1). These isolates were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair 1995). For long-term storage, cultures were transferred to Whatman no. 1 filter papers (Whatman International Ltd., Maidstone, England) overlaid on PDA, and after colonization, the filters were dried and stored at −20 °C (Petit and Gubler 2005).

Fungal identification

Morphological characterization

For morphological identification, single conidial cultures were grown for up to 5 weeks at 20 °C on synthetic nutrient-poor agar (SNA; Nirenberg 1976) with or

Table 1 *Campylocarpon*, *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* isolates used in this study

Species	Isolate	Host / substrate	Collector	Origin	GenBank Accession no.			
					ITS	TUB	H3	TEF
Campylocarpon fasciculare	**CBS 112613**; STE-U 3970; C 76	*Vitis vinifera*	F. Halleen	South Africa, Western Cape, Riebeeck Kasteel	AY677301	AY677221	JF735502	JF735691
Campylocarpon pseudofasciculare	**CBS 112679**; CPC5472; HJS-1227	*Vitis vinifera*	F. Halleen	South Africa, Western Cape, Wellington	AY677306	AY677214	JF735503	JF735692
Cylindrodendrum album	**CBS 301.83**; ATCC 46842; IMI 255534; TRTC 49165; UBC 8265	*Fucus distichus*	R.C. Summerbell	Canada, British Columbia, Vancouver, Wreck Beach	KM231764	KM532021	KM231484	KM231889
	CBS 110655; VC-51	Pine forest soil	F.X. Prenafeta-Boldú	The Netherlands, De Veluwe	KM231765	KM232022	KM231485	KM231890
Cylindrodendrum alicantinum	**CBS 139518**; Cyl-3[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	KP456014	KP400578	KP639555	KP452501
	Cyl-8[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	KP456015	KP400579	KP639556	KP452502
	Cyl-10[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	KP456016	KP400580	KP639557	KP452503
	Cyl-11[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	KP456017	KP400581	KP639558	KP452504
Cylindrodendrum hubeiense	CBS 129.97	*Viscum album*	W. Gams	France, Dép. Jura, Châtelneuf near St. Laurent	KM231766	KM232023	KM231486	KM231891
	CBS 124071; HMAS 98331, 5620	*Rhododendron*	W.P. Wu, W.Y. Zhuang & Y. Nong	China, Hubei	FJ560439	FJ860056	KR909093	HM054090
	CBS 949.70	*Castanea sativa*	W. Matheis	Switzerland	KR816357	KR816355	KP639560	KR816356
Dactylonectria alcacerensis	Cy134; IAFM Cy20-1	*Vitis vinifera*	J. Armengol	Spain, Ciudad Real, Villarrubia de los Ojos	JF735332	AM419104	JF735629	JF735818
	CBS 129087; Cy159	*Vitis vinifera*	A. Cabral & H. Oliveira	Portugal, Alcácer de Sol Torrão	JF735333	AM419111	JF735630	JF735819
	Cyl-5	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514071	–
	Cyl-7[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514072	–
	Cyl-9	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514073	–
	Cyl-13[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514074	–
	Cyl-18[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514075	–
	Cyl-20[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514076	–
	Cyl-25	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514077	–
Dactylonectria estremocensis	Cy135	*Vitis vinifera*	C. Rego & T. Nascimento	Portugal, Estremoz	AM419069	AM419105	JF735615	JF735804
	CBS 129085; Cy145	*Vitis vinifera*	C. Rego & T. Nascimento	Portugal, Estremoz	JF735320	JF735448	JF735617	JF735806

Table 1 (continued)

Species	Isolate	Host / substrate	Collector	Origin	GenBank Accession no.			
					ITS	TUB	H3	TEF
Dactylonectria hordeicola	**CBS 162.89**	*Hordeum vulgare*	M. Barth	Netherlands, Noordoostpolder, Marknesse, Lovinhhoeve	AM419060	AM419084	JF735610	JF735799
Dactylonectria macrodidyma	**CBS 112615** STE-U 3976; C98; CPC 20709	*Vitis vinifera*	F. Halleen	South Africa, Western Cape, Malmesbury, Jakkalsfontein	AY677290	AY677233	JF735647	JF735836
	Cy258	*Vitis vinifera*	C. Rego	Portugal, Vidigueira	JF735348	JF735477	JF735656	JF735845
Dactylonectria novozelandica	CBS 112608; STE-U 3987; C 62	*Vitis vinifera*	F. Halleen	South Africa, Western Cape, Citrusdal	AY677288	AY677235	JF735632	JF735821
	CBS 113552; STE-U 5713; HJS-1306; NZ C 41	*Vitis* sp.	R. Bonfiglioli	New Zealand, Candy P New Ground	JF735334	AY677237	JF735633	JF735822
Dactylonectria pinicola	Cy200	*Vitis vinifera*	N. Cruz	Portugal, Melgaço	JF735317	JF735445	JF735612	JF735801
	CBS 173.37; IMI 090176	*Pinus laricio*	T. R. Peace	UK, England, Devon, Haldon	JF735319	JF735447	JF735614	JF735803
Dactylonectria torresensis	CBS 113555; STE-U 5715; HJS-1309; NZ C 60	*Vitis* sp.	R. Bonfiglioli	New Zealand, Fiddlers Green	JF735350	AY677234	JF735661	JF735850
	CBS 129086; Cy218	*Vitis vinifera*	A. Cabral	Portugal, Torres Vedras	JF735362	JF735492	JF735681	JF735870
	Cyl-1	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514079	–
	Cyl-2	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514080	–
	Cyl-4	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514081	–
	Cyl-12	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514082	–
	Cyl-14	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514083	–
	Cyl-15[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514084	–
	Cyl-17	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514085	–
	Cyl-19	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514086	–
	Cyl-21	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514087	–
	Cyl-22	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514088	–
	Cyl-23	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514089	–
	Cyl-26[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514090	–
	Cyl-27	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià			KC514091	
	Cyl-28[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià			KC514092	

Table 1 (continued)

Species	Isolate	Host / substrate	Collector	Origin	GenBank Accession no.			
					ITS	TUB	H3	TEF
	Cyl-29[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià			KC514093	
	Cyl-30	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià			KC514094	
Ilyonectria capensis	**CBS 132815**; CPC 20695	*Protea* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231151	JX231103	JX231135	JX231119
	CBS 132816; CPC 20700	*Protea* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231160	JX231112	JX231144	JX231128
Ilyonectria coprosmae	CBS 119606; GJS 85-39	*Metrosideros* sp.	G. J. Samuels	Canada, Ontario	JF735260	JF735373	JF735505	JF735694
Ilyonectria crassa	**CBS 139.30**	*Lilium* sp.	W. F. van Hell	The Netherlands	JF735275	JF735393	JF735534	JF735723
	CBS 158.31; IMI 061536; NRRL 6149	*Narcissus* sp.	W. F. van Hell	The Netherlands	JF735276	JF735394	JF735535	JF735724
Ilyonectria cyclaminicola	**CBS 302.93**	*Cyclamen* sp.	M. Hooftman	The Netherlands, Roelofarendsveen	JF735304	JF735432	JF735581	JF735770
Ilyonectria destructans	**CBS 264.65**	*Cyclamen persicum*	L. Nilsson	Sweden, Skåne, Bjärred	AY677273	AY677256	JF735506	JF735695
Ilyonectria europaea	**CBS 129078**; Cy241	*Vitis vinifera*	C. Rego	Portugal, Vidigueira	JF735294	JF735421	JF735567	JF735756
	CBS 102892	*Phragmites australis*	W. Leibinger	Germany, Lake Constance	JF735295	JF735422	JF735569	JF735758
Ilyonectria gamsii	**CBS 940.97**	Soil	J. T. Poll	The Netherlands, Lelystad	AM419065	AM419089	JF735577	JF735766
Ilyonectria leucospermi	**CBS 132809**; CPC 20701	*Leucospermum* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231161	JX231113	JX231145	JX231129
	CBS 132810; CPC 20703	*Protea* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231162	JX231114	JX231146	JX231130
Ilyonectria liliigena	**CBS 189.49**; IMI 113882	*Lilium regale*	M.A.A. Schippers	The Netherlands, Hoorn	JF735297	JF735425	JF735573	JF735762
	CBS 732.74	*Lilium* sp.	G. J. Bollen	The Netherlands, Heemskerk	JF735298	JF735426	JF735574	JF735763
Ilyonectria liriodendri	**CBS 110.81**; IMI 303645	*Liriodendron tulipifera*	J.D. MacDonald & E.E.	USA, California	DQ178163	DQ178170	JF735507	JF735696
	CBS 117526; Cy68	*Vitis vinifera*	C. Rego	Portugal, Ribatejo e Oeste	DQ178164	DQ178171	JF735508	JF735697
Ilyonectria lusitanica	**CBS 129080**; Cy197	*Vitis vinifera*	N. Cruz	Portugal, Melgaço	JF735296	JF735423	JF735570	JF735759
Ilyonectria mors-panacis	**CBS 306.35**	*Panax quinquefolium*	A. A. Hildebrand	Canada, Ontario	JF735288	JF735414	JF735557	JF735746
	CBS 124662; NBRC 31881; SUF 811	*Panax ginseng*	Y. Myazawa	Japan, Nagano, Kitasaku-gun	JF735290	JF735416	JF735559	JF735748
Ilyonectria palmarum	CBS 135753; CPC 22088; DiGeSA-HF7	*Howea forsteriana*	G. Polizzi	Italy, Sicily, Catania province, Aci Castello	HF937432	HF922609	HF922621	HF922615
	CBS 135754; CPC 22087; DiGeSA-HF3	*Howea forsteriana*	G. Polizzi	Italy, Sicily, Catania province, Aci Castello	HF937431	HF922608	HF922620	HF922614
Ilyonectria panacis	**CBS 129079**; CDC-N-9a	*Panax quinquefolium*	K. F. Chang	Canada, Alberta	AY295316	JF735424	JF735572	JF735761
Ilyonectria protearum	**CBS 132811**; CPC 20707	*Protea* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231157	JX231109	JX231141	JX231125
	CBS 132812; CPC 20711	*Protea* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231165	JX231117	JX231149	JX231133

Table 1 (continued)

Species	Isolate	Host / substrate	Collector	Origin	GenBank Accession no.			
					ITS	TUB	H3	TEF
Ilyonectria pseudodestructans	**CBS 129081**; Cy20	*Vitis vinifera*	C. Rego	Portugal, Gouveia,São Paio	AJ875330	AM419091	JF735563	JF735752
	CBS 117824	*Quercus* sp.	E. Halmschlager	Austria, Patzmannsdorf	JF735292	JF735419	JF735562	JF735751
Ilyonectria robusta	**CBS 308.35**	*Panax quinquefolium*	A. A. Hildebrand	Canada, Ontario	JF735264	JF735377	JF735518	JF735707
	CBS 117815; IFFF 86	*Quercus* sp.	E. Halmschlager	Austria, Patsmannsdorf	JF735266	JF735380	JF735522	JF735711
	Cyl-16[b]	*Eriobotrya japonica*	J. Armengol	Spain, Alicante, Callosa d'En Sarrià	–	–	KC514078	–
Ilyonectria rufa	**CBS 153.37**	dune sand	F. Moreau	France	AY677271	AY677251	JF735540	JF735729
	CBS 156.47; IAM 14673; JCM 23100	*Azalea indica*	–	Belgium, Amandsberg	AY677272	AY677252	JF735541	JF735730
Ilyonectria venezuelensis	**CBS 102032**: ATCC 208837; AR2553	bark	A. Rossman	Venezuela, Amazonas, Cerro de la Neblina	AM419059	AY677255	JF735571	JF735760
Ilyonectria vredehoekensis	**CBS 132807**; CPC 20699	*Protea* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231155	JX231107	JX231139	JX231123
	CBS 132808; CPC 20697	*Protea* sp.	C. M. Bezuidenhout	South Africa, Western Cape, Stanford	JX231159	JX231111	JX231143	JX231127

[a] *AR* Amy Y. Rossman personal collection, *ATCC* American Type Culture Collection, USA, *CBS* CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, *CPC* Culture collection of Pedro Crous, housed at CBS, *Cy Cylindrocarpon* collection housed at Laboratório de Patologia Vegetal 'Veríssimo de Almeida' - ISA, Lisbon, Portugal, *DiGeSA* Dipartimento di Gestione dei Sistemi Agroalimentari e Ambientali, Catania, Italy, *GJS* Gary J. Samuels collection, *HJS* Hans-Josef Schroers collection, *HMAS* Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences, *IAFM* Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain, *IAM* Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan, *IFFF* Institute of Forest Entomology, Forest Pathology and Forest Protection, Austria, *IMI* International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K., *JCM* Japan Collection of Microorganisms, Japan, *NBRC* NITE Biological Resource Center, Japan, *NRRL* Agricultural Research Service Culture Collection, USA, *STE-U* Stellenbosch University, South Africa, *TRTC* Royal Ontario Museum Fungarium, Toronto, Ontario, Canada

[b] Isolates used in pathogenicity tests

Ex-type culture indicated in bold type

without the addition of two 1 × 1 cm pieces of filter paper to the medium surface, PDA, and oatmeal agar (OA; Crous et al. 2009) under continuous near-UV light (NUV; 400–315 nm; Sylvania Blacklight-Blue, The Netherlands). To induce perithecial formation, isolates were crossed as described by Cabral et al. (2012a).

Fungal structures were measured at a 1000× magnification using a Leica DM2500 and images were captured using a Leica DFC295 digital camera with the Leica Application Suite. For this purpose, an agar square was removed and placed on a microscope slide, to which a drop of water and a cover slip were added. For each isolate, 30 measurements were obtained for each structure. For conidial measurements, the 95 % confidence intervals were determined for the new species and the averages were calculated for the previously known species. The extremes of the conidial measurements are shown inside parenthesis. For the other structures only the extremes are presented. Detailed measurements were conducted for three isolates per species (once identified following morphological examination and DNA analyses), with the exception of *I. robusta* for which only one isolate was available for the study.

Culture characteristics (texture, density, colour, growth front, transparency and zonation) were described on PDA and OA after incubation at 20 °C in the dark for 14 days. 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 days in two orthogonal directions. Trials were conducted at 4 °C, 18–22 °C, 25° and 35 °C, with three replicate plates per strain at each temperature.

DNA isolation, sequencing and phylogenetic analysis

For DNA extraction, fungal mycelium and conidia from pure cultures grown on PDA for 2–3 weeks at 25 °C in the dark were scraped and mechanically 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, Doraville, USA) following manufacturer's instructions. DNA was visualized after electrophoresis on 0.7 % agarose gels stained with ethidium bromide and was stored at −20 °C

In order to identify the species involved, DNA of all isolates was amplified and sequenced for part of the histone H3 gene (HIS), that previously showed to be a very informative locus (Cabral et al. 2012a). Four isolates (Cyl-3, Cyl-8, Cyl-10 and Cyl-11) were additionally sequenced for the Internal Transcribed Spacer (ITS) region, and partial β-tubulin (TUB) and translation elongation factor 1-α (TEF) genes to better resolve their phylogenetic position. PCR amplifications were carried out using 1× PCR buffer, 1.25 mM $MgCl_2$, 80 μM of each dNTP, 0.2 μM of each primer, 0.7 U of *Taq* polymerase (Dominion MBL, Córdoba, Spain), and 1 μl of template DNA (20 ng μl^{-1}). The PCR reaction mix was adjusted to a final volume of 25 μl with ultrapure sterile water (Chromasolv Plus, Sigma-Aldrich, Steinheim, Germany). The cycle conditions in a Peltier Thermal Cycler-200 (MJ Research) were 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s, and a final extension at 72 °C for 10 min. Primers were CYLH3F and CYLH3R (Crous et al. 2004b) for HIS, ITS1F and ITS4 (Gardes and Bruns 1993) for ITS, T1 (O'Donnell and Cigelnik 1997) and Bt-2b (Glass and Donaldson 1995) for TUB, and CylEF-1 (5′- ATG GGT AAG GAV GAV AAG AC-3′; J.Z. Groenewald, unpublished) and CylEF-R2 (Crous et al. 2004b) for TEF. After confirmation by agarose gel electrophoresis, PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions by Macrogen Inc., Sequencing Center (Seoul, South Korea). The products were analyzed using Sequencer software v. 5.3 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were assembled and edited to resolve ambiguities using the program DNAman (Version 4.03, Lynnon BioSoft, Quebec, Canada), and consensus sequences for all isolates were compiled into a single file (Fasta format).

Phylogenetic analysis was first conducted on the HIS single-locus alignment for all isolates, and successively, the combined alignment of the four loci (HIS, ITS, TUB and TEF) was analyzed for inferring organismal phylogeny of Cyl-3, Cyl-8, Cyl-10 and Cyl-11 isolates. GenBank sequences (Table 1) from different species of *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* were selected based on their high similarity with our query sequences using MegaBLAST. These were added to the sequences obtained and aligned using CLUSTAL W v. 2.0.11 (Larkin et al. 2007). Phylogenetic analyses consisting of Maximum Parsimony were performed in MEGA 6.06 (Tamura et al. 2013) with the subtree-

pruning-regrafting algorithm, where gaps and missing data were treated as complete deletions. The robustness of the topology was evaluated by 1000 bootstrap replications (Felsenstein 1985). Measures for the maximum parsimony as tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were also calculated

Sequences derived in this study were lodged at GenBank, the alignment in TreeBASE (www.treebase.org), and taxonomic novelties in MycoBank (www.MycoBank.org) (Crous et al. 2004a). GenBank accession numbers of the strains collected during this study are listed in Table 1.

Pathogenicity tests

Thirteen fungal isolates representative of the different genera and species determined by phenotypical studies and HIS phylogeny, were selected to complete Koch's postulates on loquat (Tables 1 and 2).

Inoculum was produced on wheat (*Triticum aestivum*) seeds (Brayford 1993). Seeds were soaked for 12 h in distilled water; air dried, and transferred to 300 ml flasks. Each flask was autoclaved three times on 3 successive days at 120 °C during 1 h. Two fungal disks of a 2-week old culture of each isolate grown on PDA at 25 °C were placed aseptically in separate flasks. The flasks were incubated at 25 °C for 4 weeks, and shaken once a week to avoid clustering of inoculum.

Plastic pots (220 ml) were filled with a mixture of sterilized peat moss and 10 g of inoculum per pot. Seedlings of loquat 'Algerie' were planted individually in each pot at the two-true-leaf stage. Controls were inoculated with sterile uninoculated wheat seeds. Six replicates (each one in individual pots) for each isolate were used, with an equal number of control plants. After inoculation, plants were placed in a greenhouse at 25–30 °C in a completely randomized design and watered every 3 days or as needed.

Three months after inoculation, plants were observed for the development of foliar symptoms, and evaluated

Table 2 Pathogenicity of *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* species obtained from loquat to seedlings of the Algerie cultivar 3 months after inoculation

Species	Isolate	Shoot		Root	
		Disease severity[a]	Dry weight (g)	Disease severity[b]	Dry weight (g)
Cylindrodendrum alicantinum	Cyl-3	1.3±1.3 [c]	0.8±0.1	2.0±1.04*	0.3±0.06*
	Cyl-8	1.7±0.7	0.7±0.1	2.1±0.7*	0.3±0.06*
	Cyl-10	1.8±0.9	0.7±0.1	1.8±0.3*	0.5±0.06
	Cyl-11	1.2±0.8	0.7±0.1	2.2±0.9*	0.3±0.06*
Dactylonectria alcacerensis	Cyl-7	1.8±1.1	0.7±0.1	2.6±0.7*[d]	0.3±0.06*
	Cyl-13	1.5±1.1	0.6±0.1	2.6±0.7*	0.3±0.06*
	Cyl-18	1.8±0.9	0.7±0.1	2.3±0.8*	0.3±0.06*
	Cyl-20	2.0±1.3	0.8±0.2	2.6±1.1*	0.3±0.06*
D. torresensis	Cyl-15	1.9±1.0	0.4±0.1	2.4±0.7*	0.3±0.06*
	Cyl-26	1.6±0.8	0.6±0.1	2.6±0.8*	0.3±0.06*
	Cyl-28	1.9±1.1	0.6±0.1	2.3±1.0*	0.3±0.06*
	Cyl-29	1.4±0.6	0.5±0.1	2.7±0.8*	0.3±0.06*
Ilyonectria robusta	Cyl-16	2.1±1.1	0.6±0.1	2.3±1.1*	0.4±0.06*
Control	–	0.7±0.7	1.3±0.2	0.6±0.8	0.8±0.06

[a] Shoot symptoms were evaluated on the following scale: 0=no symptoms, 1=1 to 25 %, 2=26 to 50 %, 3=51 to 75 %, 4=76 to 100 % chlorotic and necrotic leaves with, eventually plant death

[b] Roots symptoms were evaluated on the following scale: 0=healthy with no lesions, 1=slight discoloration with 0 to 25 % of root mass reduction, 2=discoloration with 26 to 50 % of root mass reduction, 3=moderate discoloration with 51 to 75 % of root mass reduction, and 4=severe discoloration with >75 % of root mass reduction

[c] Values represent the means of 12 replications for each isolate; six per experiment±Standard error

[d] Asterisk denotes mean values significantly different from the control according to Dunnett's test (P=0.05)

using a 0 to 4 rating scale: 0=no symptoms, 1=1 to 25 %, 2=26 to 50 %, 3=51 to 75 %, and 4=76 to 100 % chlorotic and necrotic leaves (the latter including plant death). 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 25 % of root mass reduction, 2=discoloration with 26 to 50 % of root mass reduction, 3=moderate discoloration with 51 to 75 % of root mass reduction, and 4=severe discoloration with >75 % of root mass reduction. In addition, dry weights of shoot and root were recorded for each plant. Symptomatic roots were aseptically plated on PDAS in an attempt to re-isolate *Cylindrodendrum*, *Dactylonectria* or *Ilyonectria* and complete Koch's postulates. The experiment was repeated.

For all fungal isolates, analysis of variance (ANOVA) indicated that the data between the two repetitions were similar ($P>0.05$). Thus, data from both experiments were combined. ANOVA was performed on plant growth data (shoot and root dry weights), and disease severity and dry weight values were compared with those from control plants by the Dunnett's test. Data were analyzed using STATISTIX 9 (Analytical Software, Tallahassee, FL, USA).

Results

Morphological characterization and phylogenetic analyses

All isolates showed floccose to felted aerial mycelium which colour varied from white to yellow or light to dark brown on PDA. Colony margins were entire, slightly lobulated, or lobulated. In general, the isolates produced both microconidia and macroconidia, and chlamydospores were also present, generally intercalary, globose, single or in chains.

All isolates were amplified with the primers CYLH3F and CYLH3R. A PCR fragment of about 500 bp was obtained for all of them. Phylogenetic analysis on the HIS single-locus alignment contained a total of 49 ingroup taxa and two outgroup taxa (*Campylocarpon fasciculare* and *Campyl. pseudofasciculare*) resulting in a dataset of 531 characters, including alignment gaps, of which 346 were constant, 163 parsimony-informative, and 22 parsimony-uninformative. Parsimony analysis of 371 characters yielded three most parsimonious trees (TL=287 steps; CI=0.635; RI=0.918; and RC=0.583) which the first is shown in Fig. 1.

HIS region sequences of the isolates included in this study clustered into four groups with sequences from *Cylindrodendrum* (one group), *Dactyonectria* (two groups) and *Ilyonectria* (one group) species obtained from Genbank (Fig. 1).

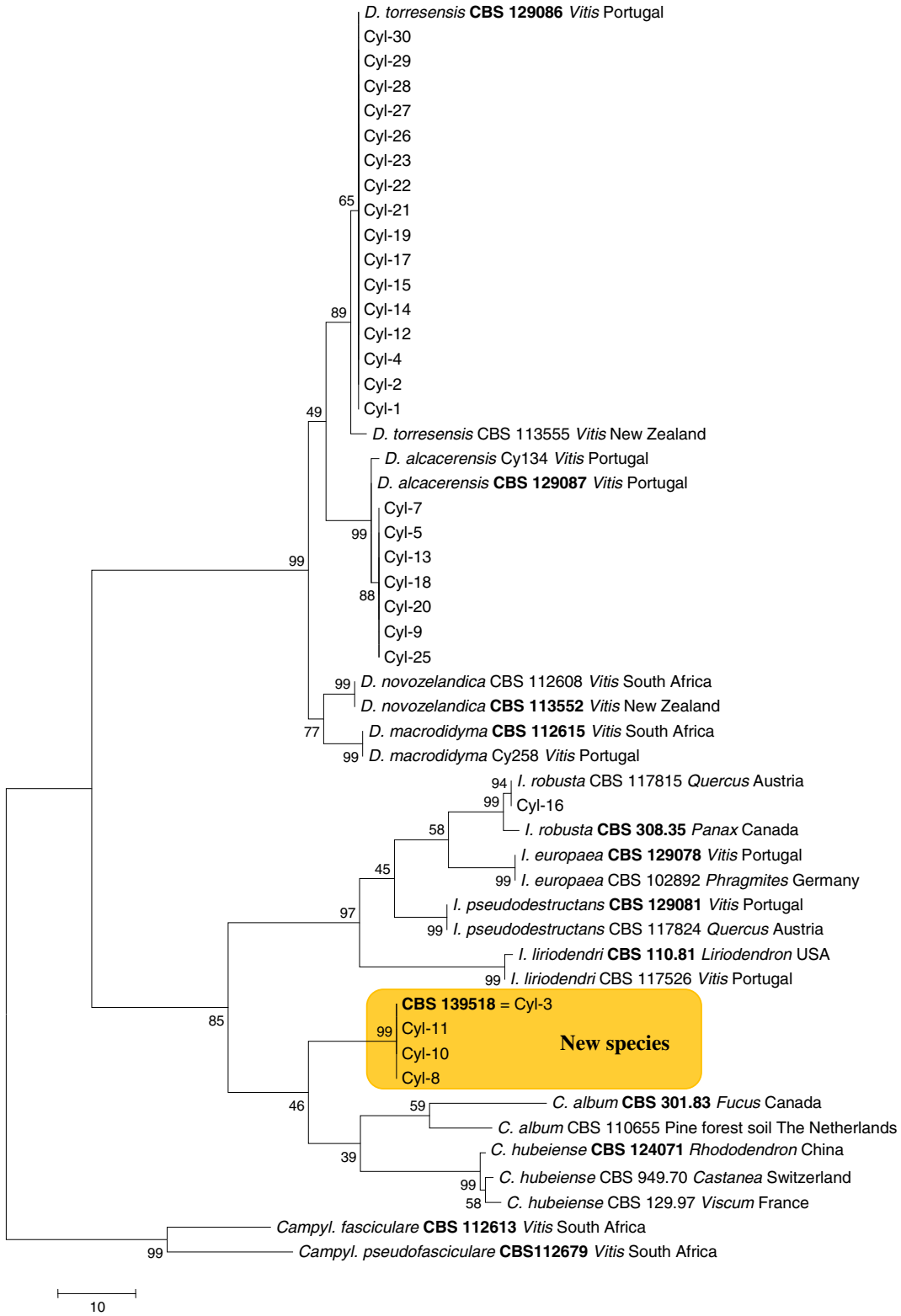
The first group, comprising seven isolates, clustered with high bootstrap support (99 %) with the ex-type culture of *D. alcacerensis* CBS 129087. These isolates produced straight, hyaline macroconidia with one-septum (14.25−) 21.48 (−34.45)×(2.92−) 4.41 (−6.33) μm, two-septa (31.16−) 36.74 (−41.74)×(4.82−) 6.14 (−7.36) μm and three-septa (29.39−) 40.26 (−48.62)×(4.26−) 6.13 (−7.45) μm; and oval to ellipsoidal microconidia with 0–septa measuring (7.44−) 11.14 (−14.85)×(2.55−) 3.54 (−5.38) μm.

The second group, comprising sixteen isolates, clustered (89 % bootstrap support) with the ex-type culture of *D. torresensis* CBS 129086. These isolates produced straight to slightly curved hyaline macroconidia with one-septum (17.37−) 28.16 (−38.78)×(3.22−) 5.44 (−7.89) μm, two-septa (31.32−) 37.12 (−42.67)×(4.71 −) 6.48 (−8.41) μm and three-septa (35.59−) 39.43 (−42.98)×(5.04−) 6.52 (−8.22) μm; and oval to ellipsoidal microconidia with 0–septa measuring (6.39−) 10.78 (−15.33)×(2.83−) 3.69 (−5.58) μm.

The third group, including only one isolate, formed a highly supported clade (99 % bootstrap support) with the ex-type culture of *I. robusta* CBS 129084. This isolate produced straight hyaline macroconidia with one-septum (11.33−) 15.86 (−24.12)×(3.2−) 4.66 (−6.16) μm; and oval to ellipsoidal microconidia with 0–septa measuring (5.56−) 9.03 (−11.73)×(2.51−) 3.59 (−4.77) μm.

The fourth group, comprising four isolates, formed a monophyletic clade with 99 % bootstrap support that is closely related with *Cylindrodendrum* species such as *C. album* or *C. hubeiense* (46 % bootstrap support). Therefore, this group was indicated as a novel phylogenetic species (yellow box in Fig. 1).

The combined alignment of ITS, TUB, HIS and TEF analyzed for inferring organismal phylogeny of the unknown species group (*Cylindrodendrum* sp. isolates Cyl-3, Cyl-8, Cyl-10 and Cyl-11) contained 57 taxa (including the two outgroups) and 2846 characters, including alignment gaps, of which 752 were constant, 857 parsimony-informative, and 147 parsimony-


D. torresensis CBS 129086 Vitis Portugal
Cyl-30
Cyl-29
Cyl-28
Cyl-27
Cyl-26
Cyl-23
Cyl-22
Cyl-21
Cyl-19
Cyl-17
Cyl-15
Cyl-14
Cyl-12
Cyl-4
Cyl-2
Cyl-1
D. torresensis CBS 113555 Vitis New Zealand
D. alcacerensis Cy134 Vitis Portugal
D. alcacerensis CBS 129087 Vitis Portugal
Cyl-7
Cyl-5
Cyl-13
Cyl-18
Cyl-20
Cyl-9
Cyl-25
D. novozelandica CBS 112608 Vitis South Africa
D. novozelandica CBS 113552 Vitis New Zealand
D. macrodidyma CBS 112615 Vitis South Africa
D. macrodidyma Cy258 Vitis Portugal
I. robusta CBS 117815 Quercus Austria
Cyl-16
I. robusta CBS 308.35 Panax Canada
I. europaea CBS 129078 Vitis Portugal
I. europaea CBS 102892 Phragmites Germany
I. pseudodestructans CBS 129081 Vitis Portugal
I. pseudodestructans CBS 117824 Quercus Austria
I. liriodendri CBS 110.81 Liriodendron USA
I. liriodendri CBS 117526 Vitis Portugal
CBS 139518 = Cyl-3
Cyl-11
Cyl-10
Cyl-8
New species
C. album CBS 301.83 Fucus Canada
C. album CBS 110655 Pine forest soil The Netherlands
C. hubeiense CBS 124071 Rhododendron China
C. hubeiense CBS 949.70 Castanea Switzerland
C. hubeiense CBS 129.97 Viscum France
Campyl. fasciculare CBS 112613 Vitis South Africa
Campyl. pseudofasciculare CBS112679 Vitis South Africa
10

◀ **Fig. 1** The first of three maximum parsimony trees obtained from the alignment of partial sequences of the histone H3 gene (HIS) of all isolates collected from loquat (Cyl isolates), and additional sequences of *Cylindrodendrum album* (KM231484 and KM231485), *C. hubeiense* (KR909093, KM231486 and KP639560), *Dactylonectria. alcacerensis* (JF735629 and JF735630), *D. macrodidyma* (JF735647 and JF735656), *D. novozelandica* (JF735632 and JF735633), *D. torresensis* (JF735661 and JF735681), *Ilyonectria europaea* (JF735567 and JF735569), *I. liriodendri* (JF735507 and JF735508), *I. pseudodestructans* (JF735562 and JF735563) and *I. robusta* (JF735518 and JF735522) obtained from GenBank. 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. New species is indicated by yellow boxes. Scale bar shows 10 changes

uninformative. Parsimony analysis of 1639 characters yielded seven most parsimonious trees (TL=1471 steps; CI=0.573; RI=0.877; and RC=0.503) the first of which is shown in Fig. 2. In this phylogenetic tree, combined sequences of the genera *Cylindrodendrum* (bootstrap= 94), *Ilyonectria* (BS=100) and *Dactylonectria* (BS= 100) formed three well-supported clades. The *Cylindrodendrum* clade incorporated representatives of *C. album* (CBS 110655 and ex-type CBS 301.83) and of *C. hubeiense* (CBS 949.70, CBS 129.97 and ex-type CBS 124071). Moreover, this clade also includes our unknown isolates previously classified inside *Cylindrodendrum* genus. These four strains (Cyl-3, Cyl-8, Cyl-10 and Cyl-11) grouped together in a monophyletic clade with 100 % bootstrap support (green box in Fig. 2), basal to the clades containing *C. album* and *C. hubeiense*, with no other closely related species, confirming them as a new *Cylindrodendrum* species.

Dactylonectria alcacerensis, *D. torresensis* and *I. robusta* were found in five, eight and one of the nine orchards where *Cylindrocarpon*-like asexual morphs were isolated, respectively. The new *Cylindrodendrum* species was found in only one orchard.

Taxonomy

Based on the DNA sequence analyses and morphological characters, one species of *Cylindrodendrum* proved to be distinct from all known species, and is newly described below. Sexual compatibility tests failed to induce perithecia among isolates.

Cylindrodendrum alicantinum C. Agustí-Brisach, J. Armengol & A. Cabral, *sp. nov.* — MycoBank MB 811663; Fig. 3.

Etymology. Named after the province of Alicante (Eastern Spain) where this fungus was first collected.

Conidiophores simple, branched or unbranched, bearing up to five phialides, 1-5- septate, frequently 3-septate, 70–170 μm long; phialides monophialidic, cylindrical to slightly subulate, 20–50 μm long, 1.5−3.3 μm wide at the base, 1.7−3.4 μm at widest point, 0.8−1.8 μm near the aperture. No sporodochial conidiophores were observed.

Microconidia (0-)1-septate, ellipsoid to subcylindrical, more or less straight, mostly without a visible hilum; 0-septate (5.8−)8.1−9.4(−13.1)×(1.9−)2.5−2.8(−4.0) μm (av. = 8.8×2.7 μm), with a length:width ratio of 2.0−4.9; 1-septate (7.7−)11−11.5(−15.9)×(2.1−)2.9−3(−4.2) μm (av. = 11.3×3.0 μm), with a length:width ratio of 2.4−5.6.

Macroconidia formed on simple conidiophores or agar surface, on SNA formed in flat domes of slimy masses, 1(−3)-septate, straight, cylindrical with both ends broadly rounded, mostly without a visible hilum; 1-septate, (12.0−)15.0−15.5(−19.9)×(1.7−)2.8−2.9(−3.7) μm (av. = 15.2×2.8 μm), with a length:width ratio of 3.9−8.3; 2-septate, (12.8−)17−18.5(−20.9)×(2.2−)2.9−3.2(−4.5) μm (av. = 17.7×3 μm), with a length:width ratio of 3.7−8.5; 3-septate, (14.6−)18.7−19.8(−29.7)×(2.4−)3.3−3.4(−4.3) μm (av. = 19.2×3.4 μm), with a length:width ratio of 3.9−9.0.

Chlamydospores subglobose to ellipsoidal, 8–19×6–10 μm, smooth but often appearing rough due to deposits, thick-walled, mainly in chains or in clumps, hyaline, becoming slightly brown in the outer wall.

Holotype: Spain: Alicante, *Eriobotrya japonica* 'Algerie' showing decline symptoms, coll./isol. J. Armengol CBS H-22113, culture ex-type CBS 139518=Cyl-3.

Culture characteristics: Mycelium felty with average to strong density. Surface on PDA umber, with aerial mycelium with dark saffron to cinnamon tufts in the centre, isabelline to buff towards the margin. Surface on OA sepia, with aerial mycelium buff to saffron, luteous to umber towards the margin. Reverse similar, except in colour, chestnut on PDA with luteous to orange margin, and umber on OA. Zonation absent, transparency homogeneous; entire margins. Colonies on PDA grow poorly (4–7 mm diam) at 4 °C after 7 days. Optimum temperature between 18 and 22 °C, when colonies reach 20–22 mm and 22–26 mm diam, respectively, after 7 days. Colony diam. was 21–25 mm at 25 ºC after 7 days. No growth was observed at 35 °C.

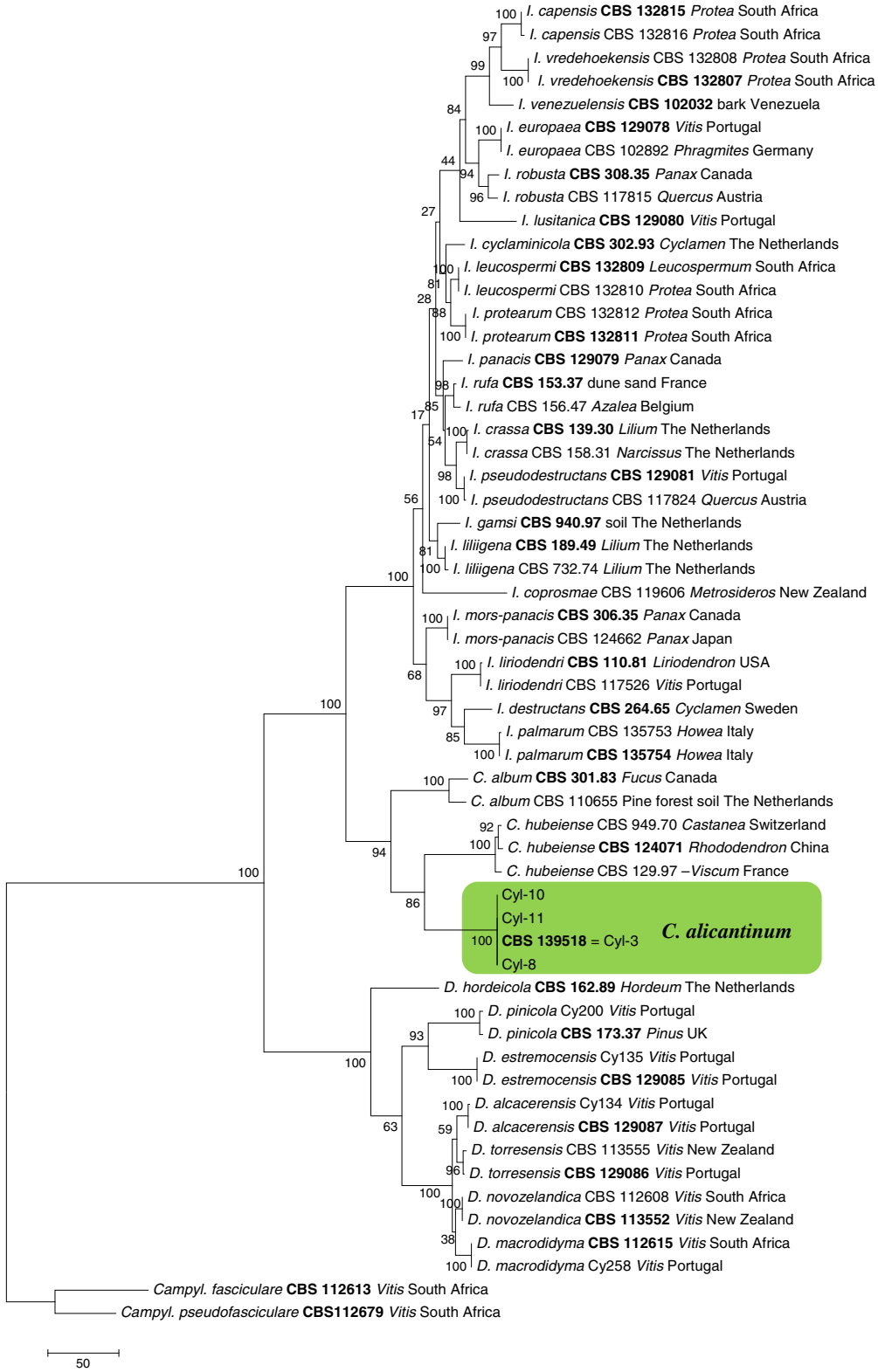

I. capensis CBS 132815 Protea South Africa
I. capensis CBS 132816 Protea South Africa
I. vredehoekensis CBS 132808 Protea South Africa
I. vredehoekensis CBS 132807 Protea South Africa
I. venezuelensis CBS 102032 bark Venezuela
I. europaea CBS 129078 Vitis Portugal
I. europaea CBS 102892 Phragmites Germany
I. robusta CBS 308.35 Panax Canada
I. robusta CBS 117815 Quercus Austria
I. lusitanica CBS 129080 Vitis Portugal
I. cyclaminicola CBS 302.93 Cyclamen The Netherlands
I. leucospermi CBS 132809 Leucospermum South Africa
I. leucospermi CBS 132810 Protea South Africa
I. protearum CBS 132812 Protea South Africa
I. protearum CBS 132811 Protea South Africa
I. panacis CBS 129079 Panax Canada
I. rufa CBS 153.37 dune sand France
I. rufa CBS 156.47 Azalea Belgium
I. crassa CBS 139.30 Lilium The Netherlands
I. crassa CBS 158.31 Narcissus The Netherlands
I. pseudodestructans CBS 129081 Vitis Portugal
I. pseudodestructans CBS 117824 Quercus Austria
I. gamsi CBS 940.97 soil The Netherlands
I. liliigena CBS 189.49 Lilium The Netherlands
I. liliigena CBS 732.74 Lilium The Netherlands
I. coprosmae CBS 119606 Metrosideros New Zealand
I. mors-panacis CBS 306.35 Panax Canada
I. mors-panacis CBS 124662 Panax Japan
I. liriodendri CBS 110.81 Liriodendron USA
I. liriodendri CBS 117526 Vitis Portugal
I. destructans CBS 264.65 Cyclamen Sweden
I. palmarum CBS 135753 Howea Italy
I. palmarum CBS 135754 Howea Italy
C. album CBS 301.83 Fucus Canada
C. album CBS 110655 Pine forest soil The Netherlands
C. hubeiense CBS 949.70 Castanea Switzerland
C. hubeiense CBS 124071 Rhododendron China
C. hubeiense CBS 129.97 –Viscum France
Cyl-10
Cyl-11
CBS 139518 = Cyl-3
Cyl-8
C. alicantinum
D. hordeicola CBS 162.89 Hordeum The Netherlands
D. pinicola Cy200 Vitis Portugal
D. pinicola CBS 173.37 Pinus UK
D. estremocensis Cy135 Vitis Portugal
D. estremocensis CBS 129085 Vitis Portugal
D. alcacerensis Cy134 Vitis Portugal
D. alcacerensis CBS 129087 Vitis Portugal
D. torresensis CBS 113555 Vitis New Zealand
D. torresensis CBS 129086 Vitis Portugal
D. novozelandica CBS 112608 Vitis South Africa
D. novozelandica CBS 113552 Vitis New Zealand
D. macrodidyma CBS 112615 Vitis South Africa
D. macrodidyma Cy258 Vitis Portugal
Campyl. fasciculare CBS 112613 Vitis South Africa
Campyl. pseudofasciculare CBS112679 Vitis South Africa
50

Fig. 2 The first of seven maximum parsimony trees obtained from the combined ITS, TUB, HIS and TEF sequence alignment of *Cylindrodendrum* sp. isolates (CBS 139518=Cyl-3, Cyl-8, Cyl-10 and Cyl-11), and additional sequences of *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* isolates. 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. Newly described species are indicated by green boxes. Scale bar shows 50 changes

Isolates studied: Cyl-3, Cyl-8, Cyl-10 and Cyl-11.

Host and distribution: *Eriobotrya japonica* (province of Alicante, Eastern Spain).

Notes: *Cylindrodendrum alicantinum* is the closest phylogenetic neighbour of *Cylindrodendrum hubeiense* (W.Y. Zhuang, Y. Nong & J. Luo) L. Lombard & Crous based on the phylogenetic analysis in this study. The phialides of *C. alicantinum* (20–50 μm long) are shorter than those of *C. hubeiense* (38–75 μm; Zhuang et al. 2007; Lombard et al. 2014). Also, the macroconidia (considering 1 to 3 septate, 12.1−29.7×1.7−4.5 μm) are wider than those of *C. hubeiense* (15–30×1.8−2.7 μm; Zhuang et al. 2007; Lombard et al. 2014). No reference is made for chlamydospores for *C. hubeiense* while they are abundant in *C. alicantinum*. Anastomoses were observed between hyphae (Read et al. 2009).

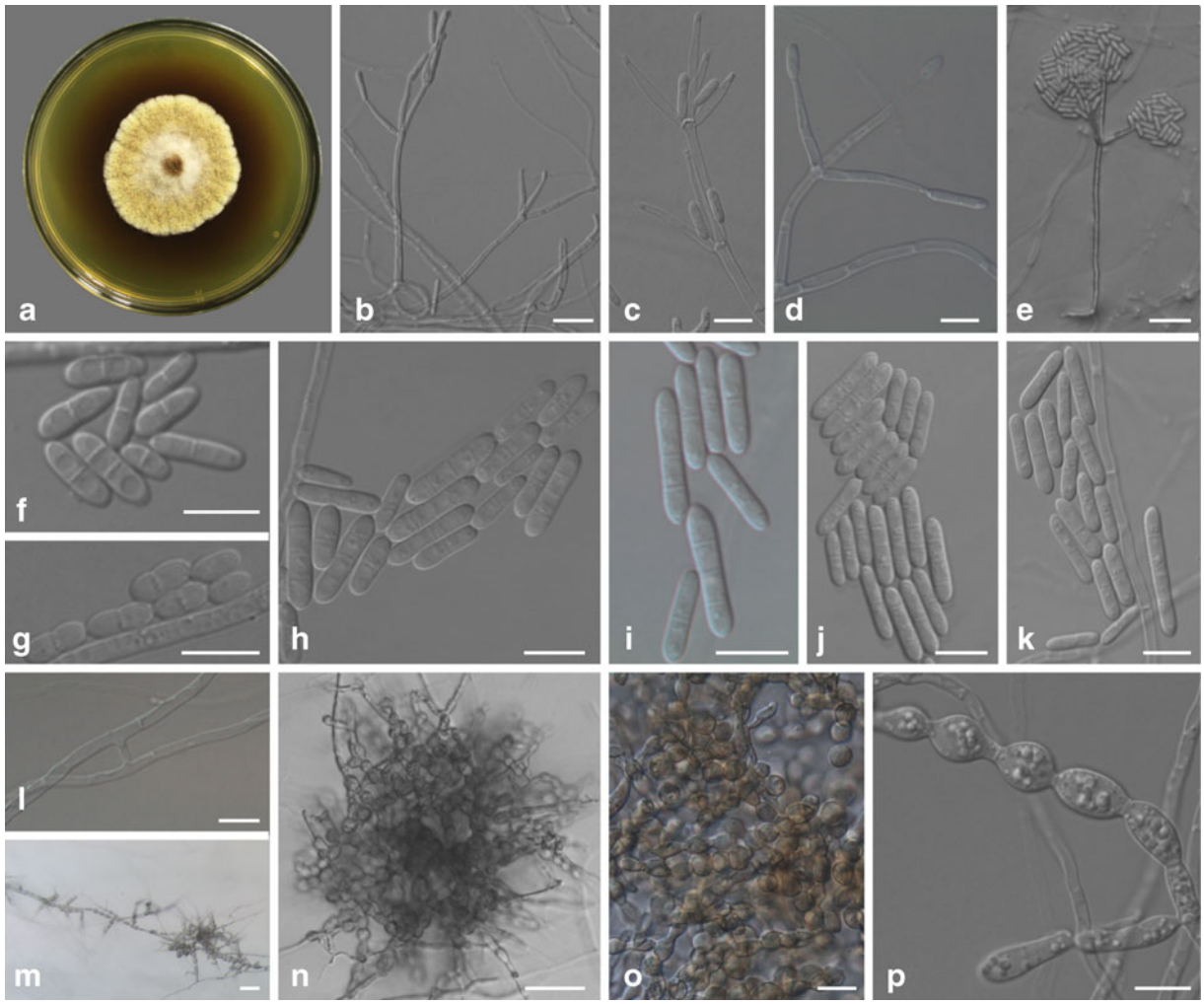


Fig. 3 *Cylindrodendrum alicantinum* (A) Fourteen-day-old colony grown on Potato Dextrose Agar at 20 °C in a 90 mm petri dish (B-D) Simple, sparsely branched conidiophores of the aerial mycelium (E) Phialides bearing microconidia in false heads (F-K) Micro- and macroconidia (L) Anastomosis in fungal hyphae (M-P) Chlamydospores. Scale bars: D, M, N - 50 μm; B, E, O - 20 μm; C-D, F-L and P - 10 μm; A, B, D, F and H-P from CBS 139518= Cyl-3 and C, E and G from Cyl-8

Pathogenicity tests

Symptoms developed in inoculated loquat seedlings after 3 months of inoculation, and consisted in reduced vigour, leaves with interveinal chlorosis and necrosis, and necrotic root lesions with a reduction in root biomass (Fig. 4).

The statistical analysis indicated significant differences from the control in root disease severity ($P<0.001$) and root dry weight ($P<0.001$), whereas shoot disease severity ($P=0.076$) and shoot dry weight ($P=0.088$) did not show significant differences. All isolates caused a significant increase of root disease severity and a significant reduction of root dry weight when compared to the uninoculated controls, with the exception of isolate Cyl-10, which was not significantly different for root dry weight (Table 2). All the isolates were re-isolated from root fragments of affected plants on PDAS (80 % to 100 % of isolation), confirming Koch's postulates.

Discussion

This research demonstrates the association of species belonging to the genera *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* with root rot of loquat and loquat decline in eastern Spain. To date, only *A. mellea*, *R. necatrix* and *Phytophthora* spp. had been described as causal agents of loquat decline in this region (González-Domínguez et al. 2008, 2009).

In our work, a collection of isolates with *Cylindrocarpon*-like asexual morphs obtained from diseased roots of loquat trees showing decline symptoms were characterized. Among them, two *Dactylonectria* species (*D. alcacerensis* and *D. torresensis*) and one *Ilyonectria* species (*I. robusta*) were identified based on the analysis of phenotypical characters and HIS data, with *D. torresensis* being the most frequent species. These three species are reported here for the first time on loquat and *I. robusta* is reported for the first time in Spain.

In addition, a group of four unidentified *Ilyonectria*-like isolates (Cyl-3, Cyl-8, Cyl-10 and Cyl-11) were also evaluated. According to their morphological characteristics, we hypothesized that they belonged to *Ilyonectria* or *Dactylonectria* genera (Booth 1966; Samuels and Brayford 1990; Halleen et al. 2004; Schroers et al. 2008; Chaverri et al. 2011; Cabral et al. 2012a, b; Lombard et al. 2014). Nevertheless, based on the multigene DNA analysis conducted of known species, these four isolates grouped together in a monophyletic clade closely to *Cylindrodendrum* spp., with no other closely *Ilyonectria* spp. or *Dactylonectria* spp. Thus, our results demonstrated that *Cylindrodendrum* isolates obtained from loquat orchards in Spain represent a novel species, described here as *C. alicantinum*.

Our study confirmed the pathogenicity of *C. alicantinum*, *D. alcacerensis*, *D. torresensis* and *I. robusta* to loquat. Root rot symptoms were reproduced on loquat seedlings and, although leaf yellowing and a shoot dry weight reduction were noticed, the statistical analysis showed that only root disease severity and root dry weight variables were significant when compared to the uninoculated controls. This could be due to the controlled conditions of incubation

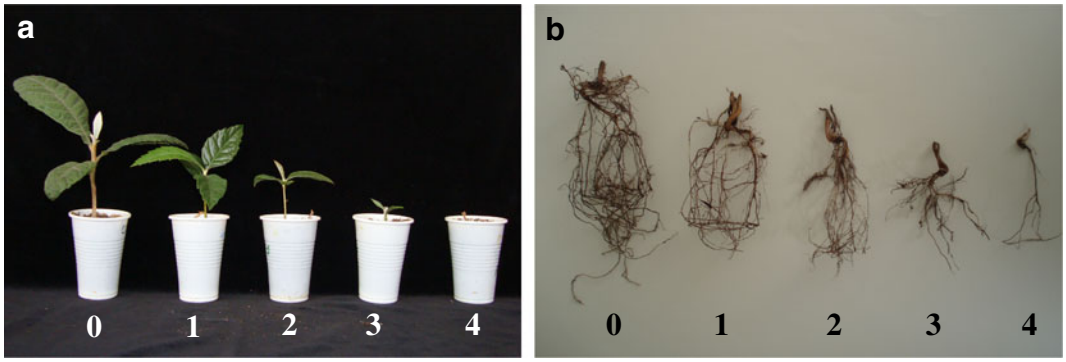


Fig. 4 Rating scale used for pathogenicity tests evaluation. **a** Foliar symptoms of individual plants were evaluated using a 0 to 4 rating scale: 0=no symptoms, 1=1 to 25 %, 2=26 to 50 %, 3=51 to 75 %, and 4=76 to 100 % chlorotic and necrotic leaves with, eventually plant death. **b** Root symptoms of individual plants were evaluated using a 0 to 4 rating scale: 0=healthy with no lesions, 1=slight discoloration with 0 to 25 % of root mass reduction, 2=discoloration with 26 to 50 % of root mass reduction, 3=moderate discoloration with 51 to 75 % of root mass reduction, and 4=severe discoloration with >75 % of root mass reduction

and the short period from inoculation to evaluation that were not enough to induce severe symptoms in the aerial part of loquat seedlings, which emerge as a consequence of root damage.

It has been demonstrated that *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* spp. cause root rot diseases on a range of diverse hosts worldwide (Halleen et al. 2006; Chaverri et al. 2011; Cabral et al. 2012a, b; Lombard et al. 2014). In fact, some *Dactylonectria* spp. such as *D. alcacerensis* and *D. torresensis*, which have been characterized in this study, were also isolated from grapevines in Spain (Agustí-Brisach et al. 2013a, b). In addition, Vitale et al. (2012) isolated fungal colonies belonging to *D. macrodidyma*-complex from avocado in Italy. Recently, Erper et al. (2013) also demonstrated the pathogenicity of *D. torresensis*, *I. europaea*, *I. liriodendri* and *I. robusta* to kiwifruit.

The simultaneous presence of *A. mellea*, *Phytophthora* spp., *R. necatrix* and the *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* species reported here in loquat orchards threatens the production of this fruit crop in eastern Spain. These pathogens severely affect mature and new plantations, as well as replanting (González-Domínguez et al. 2008, 2009). A similar scenario was reported in olive plantations (*Olea europaea* ssp. *europaea*) in southern Spain, where Sánchez-Hernández et al. (1998), reported the simultaneous presence of several soil-borne pathogens such as *Cylindrocarpon* spp., *Phytophthora* spp., *Pythium* spp. etc., inducing root and crown rot and/or dieback of twigs, resulting in severe economic losses, mainly in new plantations. In South Africa, *I. liriodendri*, *D. pauciseptata* and species belonging to the *D. macrodidyma*-complex were also associated with apple roots as causal agents of apple replant disease (Tewoldemedhin et al. 2011).

This information should be considered when establishing new loquat plantations or new susceptible fruit crops. In the area in which this research has been performed loquat and citrus are the main fruit crops. Currently, the production of citrus in this area is declining, due to the low price of this fruit. Thus, the farmers are encouraged to look for alternative fruits crops such as avocado, persimmon or kiwifruit. In this context, the susceptibility of these crops to the pathogens related here should be taken into account. Moreover, new research is needed focused on the improvement of the current management strategies against these soil-borne pathogens. This includes an evaluation of the different rootstocks proposed for loquat cultivation, such as *Cydonia oblonga*, *Eriobotrya deflexa* or *Photinia serrulata* (Lin 2007; Soler et al. 2007).

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