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ORIGINAL ARTICLE

Didymella corylicola sp. nov., a new fungus associated with hazelnut fruit development in Italy

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Abstract

A new fungal species constantly associated with hazelnut (*Corylus avellana*) fructification starting from its primordia is described. The fungus is associated with hazelnut fruit during all their developmental stages, being consistently more present in spring (March–June). A 4-year survey has been conducted, from young fruit formation to full kernel maturity including also the post-harvest phase, to collect fungi associated with damaged/discoloured kernels. A collection of 60 isolates of a new species has been obtained in this study, which is here described as *Didymella corylicola* sp. nov. Multi-locus phylogenies based on four genomic loci (nuITS and LSU rDNA, *RPB2* and *TUB2*) in combination with morphological data confirmed the fungus to represent a new species of *Didymella* (Didymellaceae). The occurrence of *D. corylicola* sp. nov. might have an impact on the quality of hazelnut production by contributing to kernel defects.

Keywords *Corylus avellana* · Kernel defects · Nut disease · *Phoma* · Pleosporales · 1 new species

Introduction

Hazelnut (*Corylus avellana* L.) is native to Europe and Western Asia where it is widely distributed (Olsen 2013; Enescu et al. 2016). The main hazelnut producing countries are Turkey, Italy and the USA. Turkey produces over 60% of the world total, followed by Italy, Azerbaijan and the United States (FAOSTAT; data for 2017 retrieved from http://www.fao.org/faostat/en/#data/QC). Commercial hazelnut orchards have expanded significantly in the last years due to an increasing demand for direct consumption of fruit as well as often in combination with chocolate. Nearly 90% of the harvested yield is destined to processing companies, whereas fresh consumption represents the residual 10%. Hazelnut cultivation is dispersed all over Italy, from north to south, with main production located in Piedmont (26%), Latium (32%), Campania (28%) and Sicily (9%) regions (ISTAT, http://dati.istat.it/). These percentages just give an indication since production may vary from year to year. Hazelnut is characterized by good rusticity which makes it an adaptable fruit tree species compared to other nut species such as almond, walnut and pistachio. Nevertheless, it is affected by several diseases and fungal pathogens which can be particularly harmful in altering the kernel and consequently reducing harvest quality and yield. Hazelnut represents a high value product suffering kernel defects which might be climate dependent, inducing production fluctuations. In Piedmont and in Campania, gleosporiosis (purple necrotic spots on female flowers, petioles, nut bracts and husk), caused by *Elsinoe coryli* (syn. *Sphaceloma coryli*), has been reported (Minutolo et al. 2016), while in central Italy (Latium, Viterbo province) and in Campania, *Alternaria* spp., *Colletotrichum* spp., *Fusarium* spp. and *Diaporthe* spp. have

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also been isolated (Librandi et al. 2006). From the year 2000 onwards, special attention has been given to identify the causal agent of hazelnut fruit drop, known as nut grey necrosis (NGN) disease, which was identified as *Fusarium lateritium* (Vitale et al. 2011). In this extensive survey, an unknown fungus with typical deeply red-pigmented colonies was isolated in every phenological phase, particularly in the early stages of hazelnut fruit formation. This fungus was also isolated in the surveys from 2016 onwards, which were addressed to identify the fungi associated with kernel damage/discoloration and the potential causal agents of kernel defects.

The aims of this study were to provide morphological, taxonomic and phylogenetic data for the red-pigmented fungus which proved to be an undescribed species of the genus *Didymella*. Experimental evidence on its relation with hazelnut kernel defects is given.

Materials and methods

Field surveys and isolations

In past years, numerous surveys were carried out, mainly in Latium region, on the causal agent of the nut grey necrosis (NGN) disease, which caused heavy losses for about 10 years, starting from its outbreak in 2000 (Vitale et al. 2011). More recently, surveys of a total of 9 hazelnut orchards were conducted from 2016 to 2019 in Campania, as well as in 7 hazelnut orchards located in Piedmont. Except for 2016, in which the survey started at fruit yield, sampling started from the end of March onwards, considering several phenological stages, namely, bud break/initial fruit formation (March–April), nut development (May–June), embryo and kernel development (late June–July), ripening nuts-harvest phase (August–September), and post-harvest sampling (September–October). About 300 fruits were sampled per orchard. Samples were subjected to surface disinfection in 10% sodium hypochlorite for 60 s, rinsed in sterile water for 60 s, and dried on sterile filter paper in a laminar flow. Tissue fragments were placed onto potato dextrose agar (PDA) Petri dishes at 25 °C in the dark, and daily observed for mycelial growth. Numerous red-pigmented cultures were obtained, and single-conidial isolations were performed with conidia collected from pycnidia produced on those cultures within 1 month of incubation at room temperature under daylight. More than 60 single-spore isolates were obtained from symptomatic tissue isolation. Amongst these, 10 isolates representative of the three main colony morphologies were characterized by molecular data and phylogenetic analyses (Table 1), and the three isolates CREADC-F2281, CREADC-F2402 and CREADC-F2403 were subjected to detailed morphological, taxonomic, phylogenetic and pathogenic studies.

Morphological characterization

For culture characteristics, colonies were grown on 2% (w/v) malt extract agar (MEA, VWR), potato dextrose agar (PDA, Sigma-Aldrich), corn meal agar (CMA, Sigma-Aldrich) supplemented with 2% w/v dextrose (CMD), oatmeal agar (OA, Sigma-Aldrich) and hazelnut extract agar (HEA; 25 g/l fresh chopped hazel twigs, autoclaved in 2% agar). Colony diameters were measured after 7 days, and colony morphologies were determined after 14 days of incubation at room temperature (22 °C) and daylight. To promote pycnidial formation, cultures were grown on HEA and hazel twig agar (HTA; autoclaved split hazel twigs placed on 2% agar plates, shortly before solidification). The isolates used in this study are maintained in the culture collection of the CREA-DC (ex CREA-PAV). The ex-holotype isolate (CREADC-F2403) of the new hazelnut pathogen was deposited at the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and the holotype specimen in the fungarium of the Department of Botany and Biodiversity Research, University of Vienna (WU).

Microscopic observations were made in tap water except where noted in the figure legend. Sections of 8 μm thickness of pycnidia were prepared with a Leica FrigoCut 2700 freezing microtome. Methods of microscopy included stereomicroscopy using a Nikon SMZ 1500 equipped with a Nikon DS-U2 digital camera, and Nomarski differential interference contrast (DIC) using a Zeiss Axio Imager.A1 compound microscope equipped with a Zeiss Axiocam 506 colour digital camera. Images and data were gathered using the NIS-Elements D v. 3.22.15 or Zeiss ZEN Blue Edition software packages. Measurements are reported as maxima and minima in parentheses and the range representing the mean plus and minus the standard deviation of a number of measurements given in parentheses.

Temperature-growth relationships

For investigations of temperature-growth relationships of the new hazelnut fungus, the ex-holotype isolate CREADC-F2403, and the isolates CREADC-F2281 and CREADC-F2402 were used. Agar plugs (5 mm diameter) were taken from the edge of actively growing cultures on PDA and transferred onto the centre of 9-cm PDA Petri dishes. Three replicate plates were incubated at 5, 10, 15, 20, 25, 30 and 35 °C in the dark and measurements were taken after 7 days at right angles along two lines intersecting the centre of the inoculum and the mean growth rates plus and minus the standard deviation (± SD) were calculated.

Table 1 Isolates and accession numbers of sequences used in the phylogenetic analyses. Isolates and sequences in bold were obtained in the present study

Species	Strain number[1]	GenBank accession numbers[2]			
		LSU	ITS	*RPB2*	*TUB2*
Didymella acetosellae	CBS 179.97	GU238034	GU237793	KP330415	GU237575
D. aeria	LC 8120	KY742052	KY742206	KY742138	KY742294
	CGMCC 3.18353	KY742051	KY742205	KY742137	KY742293
D. aliena	CBS 379.93; PD 82/945	GU238037	GU237851	KP330416	GU237578
D. americana	CBS 185.85; PD 80/1191	GU237990	FJ426972	KT389594	FJ427088
	CBS 568.97; ATCC 44494; PD 94/1544	GU237991	FJ426974	–	FJ427090
D. anserina	CBS 253.80	KT389715	KT389498	KT389595	KT389795
	CBS 285.29	KT389716	KT389499	–	KT389796
	CBS 360.84	GU237993	GU237839	KT389596	GU237551
	CBS 397.65	KT389717	KT389500	KT389597	KT389797
D. aquatica	CGMCC 3.18349	KY742055	KY742209	KY742140	KY742297
	LC 5555	KY742056	KY742210	KY742141	KY742298
D. arachidicola	CBS 333.75; ATCC 28333; IMI 386092; PREM 44889	GU237996	GU237833	KT389598	GU237554
D. aurea	CBS 269.93; PD 78/1087	GU237999	GU237818	KT389599	GU237557
D. bellidis	CBS 714.85; PD 74/265	GU238046	GU237904	KP330417	GU237586
	PD 94/886	GU238047	GU237923	–	GU237587
D. boeremae	CBS 109942; PD 84/402	GU238048	FJ426982	KT389600	FJ427097
D. brunneospora	CBS 115.58; DSM 62044	KT389723	KT389505	KT389625	KT389802
D. calidophila	CBS 448.83	GU238052	FJ427059	–	FJ427168
	PD 84/109	GU238053	FJ427060	–	FJ427169
D. chenopodii	CBS 128.93; PD 79/140	GU238055	GU237775	KT389602	GU237591
D. chloroguttulata	CGMCC 3.18351	KY742057	KY742211	KY742142	KY742299
	LC 8122	KY742058	KY742212	KY742143	KY742300
D. coffeae-arabicae	CBS 123380; PD 84/1013	GU238005	FJ426993	KT389603	FJ427104
D. corylicola	**CREADC-F2281**	**MN954299**	**MN954288**	**MN958321**	***MN958331***
	CREADC-F2402	**MN954300**	**MN954289**	**MN958322**	**MN958332**
	CBS 146357; CREADC-F2403	**MN954301**	**MN954290**	**MN958323**	**MN958333**
	CREADC-F2404	**MN954302**[3]	**MN954291**[3]	–	–
	CREADC-F2405	**MN954303**	**MN954292**	**MN958324**	**MN958334**
	CREADC-F2406	**MN954304**	**MN954293**	**MN958325**	**MN958335**
	CREADC-F2407	**MN954305**	**MN954294**	**MN958326**	**MN958336**
	CREADC-F2408	**MN954306**	**MN954295**	**MN958327**	**MN958337**
	CREADC-F2409	**MN954307**	**MN954296**	**MN958328**	**MN958338**
	CREADC-F2410	**MN954308**	**MN954297**	**MN958329**	**MN958339**
	CREADC-F2411	**MN954309**	**MN954298**	**MN958330**	**MN958340**
D. curtisii	CBS 251.92; PD 86/1145	GU238013	FJ427038	–	FJ427148
	PD 92/1460	GU238012	FJ427041	KT389604	FJ427151
D. dactylidis	CBS 124513; PD 73/1414	GU238061	GU237766	–	GU237599
D. dimorpha	CBS 346.82	GU238068	GU237835	–	GU237606
D. ellipsoidea	CGMCC 3.18350	KY742060	KY742214	KY742145	KY742302
	LC 8123	KY742061	KY742215	KY742146	KY742303
D. eucalyptica	CBS 377.91; PD 79/210	GU238007	GU237846	KT389605	GU237562
D. exigua	CBS 183.55	EU754155	GU237794	EU874850	GU237525
D. gardeniae	CBS 626.68; IMI 108771	GQ387595	FJ427003	KT389606	FJ427114
D. glomerata	CBS 133.72	KT389718	FJ427004	–	FJ427115
	CBS 528.66; PD 63/590	EU754184	FJ427013	GU371781	FJ427124

Table 1 (continued)

Species	Strain number[1]	GenBank accession numbers[2]			
		LSU	ITS	*RPB2*	*TUB2*
D. heteroderae	CBS 109.92; PD 73/1405	GU238002	FJ426983	KT389601	FJ427098
D. ilicicola	CGMCC 3.18355	KY742065	KY742219	KY742150	KY742307
	LC 8127	KY742066	KY742220	KY742151	KY742308
D. infuscatispora	CGMCC 3.18356	KY742067	KY742221	KY742152	KY742309
	LC 8129	KY742068	KY742222	–	KY742310
D. keratinophila	CBS 143032; UTHSC:DI16–200; FMR 13690	LT592901	LN907343	LT593039	LT592970
	UTHSC:DI16–228; FMR 13718	LT592915	LN907371	LT593053	LT592984
	UTHSC:DI16–282; FMR 13774	LT592938	LN907425	LT593077	LT593007
D. lethalis	CBS 103.25	GU238010	GU237729	KT389607	GU237564
D. longicolla	CBS 124514; PD 80/1189	GU238095	GU237767	–	GU237622
D. macrophylla	CGMCC 3.18357	KY742070	KY742224	KY742154	KY742312
	LC 8132	KY742071	KY742225	KY742155	KY742313
D. macrostoma	CBS 482.95	GU238099	GU237869	KT389609	GU237626
	CBS 529.66; PD 66/521	GU238098	GU237885	–	GU237625
	CBS 223.69	GU238096	GU237801	KT389608	GU237623
	CBS 247.38	KT389719	KT389501	–	KT389798
D. maydis	CBS 588.69	EU754192	FJ427086	GU371782	FJ427190
D. microchlamydospora	CBS 105.95	GU238104	FJ427028	KP330424	FJ427138
D. molleriana	CBS 229.79; LEV 7660	GU238067	GU237802	KP330418	GU237605
	CBS 109179; PD 90/835–1	GU238066	GU237744	–	GU237604
D. musae	CBS 463.69	GU238011	FJ427026	LT623248	FJ427136
D. negriana	CBS 358.71	GU238116	GU237838	KT389610	GU237635
D. nigricans	CBS 444.81; PDDCC 6546	GU238000	GU237867	–	GU237558
	PD 77/919	GU238001	GU237915	KT389611	GU237559
D. ocimicola	CGMCC 3.18358	KY742078	KY742232	–	KY742320
	LC 8138	KY742079	KY742233	–	KY742321
D. pedeiae	CBS 124517; PD 92/612A	GU238127	GU237770	KT389612	GU237642
D. pinodella	CBS 318.90; PD 81/729	GU238016	FJ427051	–	FJ427161
	CBS 531.66	GU238017	FJ427052	KT389613	FJ427162
D. pinodes	CBS 525.77	GU238023	GU237883	KT389614	GU237572
D. pomorum	CBS 285.76; ATCC 26241; IMI 176742; VKM F-1843	GU238025	FJ427053	KT389615	FJ427163
	CBS 388.80	GU238027	FJ427055	KT389617	FJ427165
	CBS 539.66; ATCC 16791; IMI 122266; PD 64/914	GU238028	FJ427056	KT389618	FJ427166
	CBS 354.52	KT389720	KT389502	KT389616	KT389799
D. protuberans	CBS 132.96; PD 93/853	GU237989	GU237778	–	GU237550
	CBS 377.93; PD 80/976	GU238014	GU237847	KT389619	GU237565
	CBS 391.93; PD 80/87	GU238015	GU237858	KT389621	GU237566
	CBS 381.96; PD 71/706	GU238029	GU237853	KT389620	GU237574
D. pteridis	CBS 379.96	KT389722	KT389504	KT389624	KT389801
D. rhei	CBS 109177; LEV 15165; PD 2000/9941	GU238139	GU237743	KP330428	GU237653
D. rosea	BRIP 50788	KT287003	KT338640	–	KT286945
D. rumicicola	CBS 683.79; LEV 15094	KT389721	KT389503	KT389622	KT389800
D. sancta	CBS 281.83	GU238030	FJ427063	KT389623	FJ427170
D. segeticola	CGMCC 3.17489	KP330443	KP330455	KP330414	KP330399
	CGMCC 3.17498	KP330442	KP330454	KP330413	KP330398
D. senecionicola	CBS 160.78; LEV 11451	GU238143	GU237787	–	GU237657
D. sinensis	LC 8142	KY742087	KY742241	KY742166	KY742329

Table 1 (continued)

Species	Strain number[1]	GenBank accession numbers[2]			
		LSU	ITS	*RPB2*	*TUB2*
	LC 8143	KY742088	KY742242	KY742167	KY742330
D. subglomerata	CBS 110.92; PD 76/1010	GU238032	FJ427080	KT389626	FJ427186
D. subherbarum	CBS 249.92; PD 78/1088	GU238144	GU237808	–	GU237658
	CBS 250.92; DAOM 171914; PD 92/371	GU238145	GU237809	–	GU237659
D. suiyangensis	CGMCC 3.18352	KY742089	KY742243	KY742168	KY742331
	LC 8144	KY742090	KY742244	KY742169	KY742332
D. tanaceti	BRIP 50785	KT287022	KT338641	–	KT286974
D. viburnicola	CBS 523.73; PD 69/800	GU238155	GU237879	KP330430	GU237667
Macroventuria anomochaeta	CBS 525.71	GU237984	GU237881	GU456346	GU237544
M. wentii	CBS 526.71	GU237986	GU237884	KT389642	GU237546
Paraboeremia adianticola	CBS 187.83; PD 82/128	GU238035	GU237796	KP330401	GU237576
	CBS 260.92; PD 86/1103	KT389752	KT389534	–	KT389832
P. putaminum	CBS 130.69; CECT 20054; IMI 331916	GU238138	GU237777	LT623254	GU237652
P. selaginellae	CBS 122.93; PD 77/1049	GU238142	GU237762	LT623255	GU237656

[1] *ATCC*, American Type Culture Collection, Virginia, USA; *BRIP*, Queensland Plant Pathology Herbarium, Brisbane, Australia; *CBS*: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; *CECT*, Colección Española de Cultivos Tipo, Valencia University, Spain; *CGMCC*, China General Microbiological Culture Collection, Beijing, China; *CREADC*, Consiglio per la Ricerca in Agricoltura e l'analisi dell'economia agraria, Centro di ricerca per la Difesa e la Certificazione, Roma, Italy; *DAOM*, Canadian Collection of Fungal Cultures, Ottawa, Canada; *DSM*, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; *IMI*, International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; *LC*, L. Cai personal collection housed at the Chinese Academy of Sciences (CAS), China; *LEV*, Plant Health and Diagnostic Station, Auckland, New Zealand; *PD*, Plant Protection Service, Wageningen, the Netherlands; *PDDCC*, Plant Diseases Division Culture Collection, Auckland, New Zealand; *PREM*, National Collection of Fungi: Culture Collection, Pretoria, South Africa; *UTHSC*, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA; *VKM*, All-Russian Collection of Microorganisms, Pushchino, Russia

[2] *ITS*, internal transcribed spacer regions 1 and 2 including 5.8S nrDNA gene; *LSU*, 28S large subunit of the nrRNA gene; *RPB2*, RNA polymerase II second largest subunit; *TUB2*, ß-tubulin

[3] ITS-LSU sequences not included in the phylogenetic analyses, as they were identical to other accessions of *D. corylicola* sequenced

DNA extraction, PCR amplification and sequencing

The extraction of genomic DNA from pure cultures was performed as reported in previous studies (Voglmayr and Jaklitsch 2011; Vitale et al. 2018) by using the DNeasy Plant Mini Kit (QIAgen GmbH, Hilden, Germany) or the Wizard Genomic DNA Purification Kit (Promega Corporation, WI, USA). For the ex-holotype strain of the new species, the complete internal transcribed spacer region (ITS1-5.8S-ITS2) and a ca. 0.9 kb fragment of the large subunit nuclear ribosomal DNA (nLSU rDNA) were amplified and sequenced as a single fragment with primers V9G (de Hoog and Gerrits van den Ende 1998) and LR5 (Vilgalys and Hester 1990); the complete ITS region of the other strains was amplified with primers ITS5 and ITS4 (White et al. 1990); the RNA polymerase II subunit 2 (*RPB2*) gene was amplified with primers fRPB2-5F2 and fRPB2-7cR (Liu et al. 1999, Sung et al. 2007) or dRPB2-5f and dRPB2-7r (Voglmayr et al. 2016); and the beta-tubulin (*TUB2*) gene with primer pairs T1 and T22 or TUB2Fd and TUB4Rd (O'Donnell and Cigelnik 1997; Aveskamp et al. 2009). The PCR product was purified using an enzymatic PCR cleanup (Werle et al. 1994) as described in Voglmayr and Jaklitsch (2008). DNA was cycle-sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington, UK) with the same primers as in PCR; in addition, primers ITS4, LR2R-A (Voglmayr et al. 2012) and LR3 (Vilgalys and Hester 1990) were used for the ITS-LSU fragment. Sequencing was performed on an automated DNA sequencer (3730xl Genetic Analyser, Applied Biosystems).

Phylogenetic analyses

The newly generated sequences were aligned with selected sequences of Chen et al. (2017) and complemented with a few recent additions from GenBank. The GenBank accession numbers of sequences used in the analyses are given in Table 1. A combined matrix of the three loci (partial nuITS-LSU rDNA, *RPB2* and *TUB2*) was produced for phylogenetic analyses, with two species of *Macroventuria* (*M. anomochaeta*, *M. wentii*) and three species of *Paraboeremia* (*P. adianticola*, *P. putaminum* and *P. selaginellae*) added as the outgroup taxa according to the results of the phylogenetic analyses of Chen et al. (2017). Sequence alignments were produced with the server

version of MAFFT (http://mafft.cbrc.jp/ alignment/server/), checked and refined using BioEdit v. 7.2.6 (Hall 1999). The combined data matrix contained 3010 characters; viz. 493 nucleotides of ITS, 1327 nucleotides of the LSU, 846 nucleotides of *RPB2* and 344 nucleotides of *TUB2*.

Maximum parsimony (MP) analyses were performed with PAUP v. 4.0a166 (Swofford 2002). All molecular characters were unordered and given equal weight; analyses were performed with gaps treated as missing data; the COLLAPSE command was set to MINBRLEN. MP analysis of the combined multi-locus matrix was done using 1000 replicates of heuristic search with random addition of sequences and subsequent TBR branch swapping (MULTREES option in effect, steepest descent option not in effect). Bootstrap analyses with 1000 replicates were performed in the same way, but using 5 rounds of random sequence addition and subsequent branch swapping during each bootstrap replicate.

Maximum likelihood (ML) analyses were performed with RAxML (Stamatakis 2006) as implemented in raxmlGUI 1.3 (Silvestro and Michalak 2012), using the ML + rapid bootstrap setting and the GTRGAMMA substitution model with 1000 bootstrap replicates. The matrix was partitioned for the different gene regions. In the "Results" and "Discussion" sections, bootstrap values below 70% are considered low, between 70 and 90% medium and above 90% high.

Pathogenicity

Pathogenicity tests with the three fungal strains of the undescribed hazelnut pathogen were performed to fulfil Koch's postulates. For this, conidial suspensions were prepared by washing the conidia from actively sporulating agar cultures with sterile distilled water; the conidial concentration was determined using a haemocytometer, and the concentration adjusted with sterile distilled water to 1×10^6 conidia/ml. The isolates were tested either by inoculating the apex of fresh and mature hazelnuts after making a little hole with a sterile needle and introducing 25 μl of conidial suspension into each kernel cavity (Scarpari et al. 2018) or a drop (10 μl) of conidial suspension was adjusted at the centre of halved hazelnut kernels. The inoculated nuts (twenty replicates for each isolate) and negative controls (ten replicates) treated with sterile water only were all incubated in the dark at 25 °C in a humid growth chamber for the first 72 h. After 2 weeks, the results of inoculations were checked by halving the whole nuts or directly on the halved kernels.

Results

Isolations and morphological characterization

In the recent surveys carried out from 2016 to 2019, the undescribed fungal species associated with hazelnut fructifications was only isolated in Campania, during the whole vegetative season (from March to September) and occasionally from post-harvest nuts. Until now, the novel *Corylus* fungus has never been isolated in Piedmont. In the previous investigations of 2000–2010, colonies of this fungus were obtained in surveys conducted on hazelnut orchards in Viterbo province (Latium Region).

Culture images of three strains (CREADC-F2281, CREADC-F2402, CREADC-F2403) grown on OA, PDA, CMD and HEA for 2 weeks at room temperature are shown in Fig. 1. Detailed descriptions of morphological traits are given in the "Taxonomy" section below.

Temperature-growth relationships

The growth rate experiments (Fig. 2) revealed 20 °C as optimal temperature for all the three isolates with an evidently better growth of culture CREADC-F2281 (10.54 ± 0.25 mm/day), originating from Latium region, compared to the ex-holotype culture CREADC-F2403 with 7.50 ± 1.07 mm/day. Conversely, the ex-holotype culture showed a faster growth at 25 °C in comparison with the other two isolates. No growth was recorded at 35 °C, while all three isolates were able to grow at 5 °C, at which the ex-holotype culture performed best (1.55 ± 0.1 mm/day).

Phylogenetic analyses

Of the 3010 characters included in the phylogenetic analyses, 449 were parsimony informative (41 from the ITS, 20 from the LSU, 256 from *RPB2*, 132 from *TUB2*). MP analyses revealed 114 MP trees 2199 steps long, one of which is shown as Fig. 3. The tree backbone was identical in all MP trees, except for topologies of several deeper nodes of the *Peyronellaea* clade marked by an asterisk in Fig. 3. The best ML tree (lnL = −14,674.1908) revealed by RAxML was largely compatible with the MP tree shown in Fig. 3, except for differences in some deeper unsupported nodes (not shown). In the MP and ML analyses, the novel *Corylus* fungus was placed within a clade containing *D. pedeiae*, *D. ilicicola* and *D. subherbarum* with maximum support (group G of Aveskamp et al. 2010), but it remained unresolved whether *D. ilicicola* or *D. subherbarum* is its closest relative (Fig. 3).

Pathogenicity

Necrotic lesions were evident on the halved kernels while rather limited on the whole nuts. Re-isolations from the margin of lesions cultured on PDA gave colonies with the same morphological characters as those used for inoculation, thus confirming Koch's postulates.

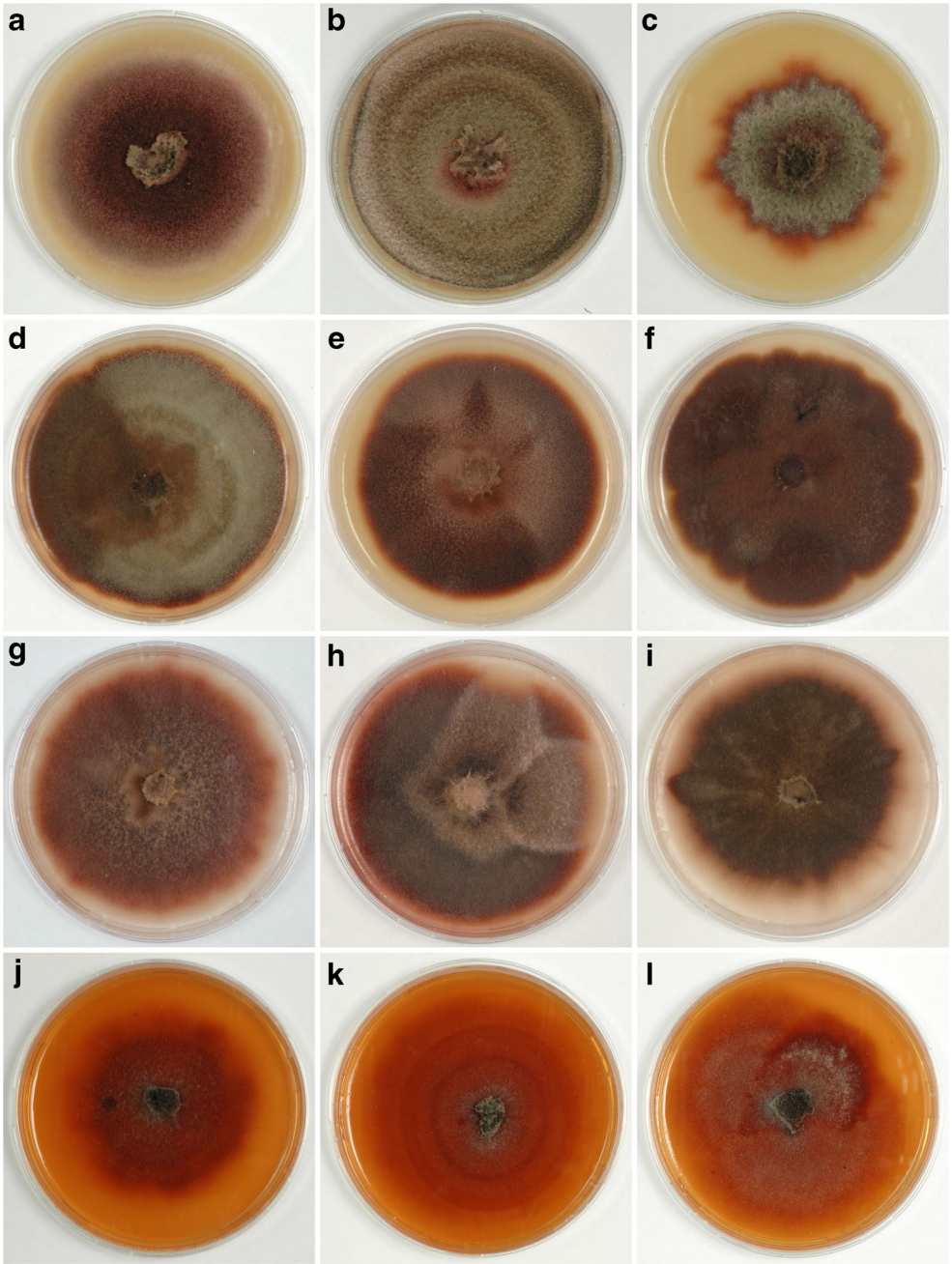

Fig. 1 *Didymella corylicola* cultures after 14 days at 22 °C on OA (**a**–**c**), PDA (**d**–**f**), CMD (**g**–**i**) and HEA (**j**–**l**). **a**, **d**, **g**, **j** Strain CREADC-F2402. **b**, **e**, **h**, **k** Strain CBS 146357 = CREADC-F2403 (ex-holotype). **c**, **f**, **i**, **l** Strain CREADC-F2281

Taxonomy

Didymella corylicola Voglmayr, Scarpari, Di Giambattista, Vitale and Luongo sp. nov. Figs 1 and 4.

MycoBank: MB 833929

Etymology: *corylicola*, referring to its occurrence on *Corylus* fructifications.

Diagnosis: *Didymella corylicola* is recognized by colonies producing bright red diffusible pigments in pure culture.

Description: *Pycnidia* (46–)55–82(–110) µm diameter ($n = 100$), solitary, produced within the agar, on the agar surface or on the aerial mycelium, (sub-)globose to broadly pyriform, glabrous, brown, with 1 (rarely 2) conspicuous, non-papillate ostioles (8–)11–17(−22) µm wide ($n = 32$). *Pycnidial wall* (4–)4.5–6.5(–7.5) µm thick ($n = 31$), brown, pseudoparenchymatous, composed of 1–3 layers of isodiametric to oblong cells (4–)6–10(–16) × (3–)4–8(–11) µm ($n = 66$). *Conidiogenous cells* (4.3–)4.5–7.8(–10.0) × (2.8–)3.5–5.0(–5.8) µm ($n = 18$), phialidic, hyaline, simple, smooth, globose to flask-shaped. *Conidia* (3.2–)3.8–4.5(–5.3) × (1.4–)1.6–1.9(–2.1) µm, l/w = (1.7–)2.1–2.6(–3.1) ($n = 221$), ellipsoidal to oblong, commonly slightly allantoid, thin-walled, smooth, hyaline, aseptate, with 0–2 tiny guttules. *Conidial matrix* white.

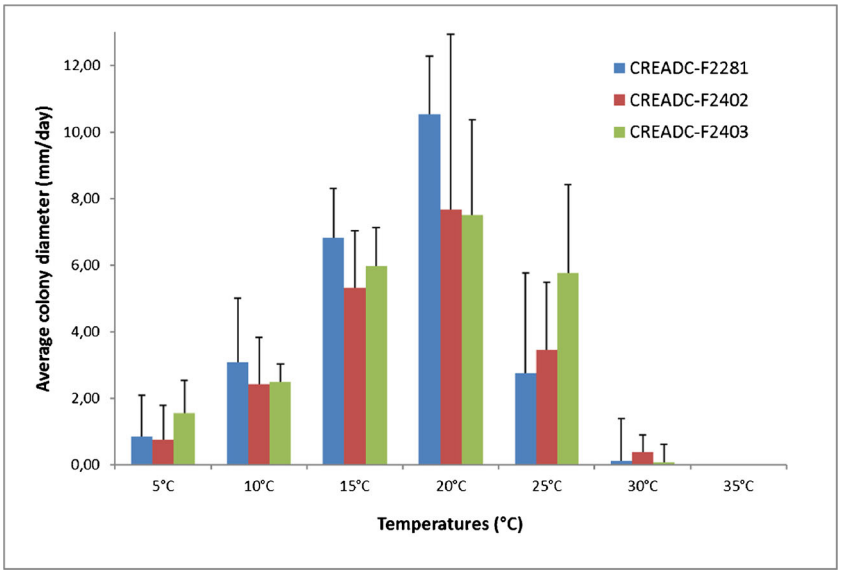

Fig. 2 Temperature-growth relationships of the ex-holotype strain CBS 146357 = CREADC-F2403 compared to two other strains of *Didymella corylicola* on PDA. Daily mean growth rate (mm/day) ± SD calculated on three replicates after 7 days of incubation are shown

Culture characteristics: Colonies on PDA 47–62 mm diameter after 7 days at 22 °C, margin regular or irregular, covered by floccose aerial mycelium, grey brown to dark vinaceous brown, at least in the centre becoming dark vinaceous brown with age, sometimes with concentric rings; reverse blackish with bright red brown margins. Colonies on MEA 53–58 mm diameter after 7 days at 22 °C, with culture characteristics similar to those on PDA. Colonies on CMD 67–72 mm diameter after 7 days at 22 °C, with culture characteristics similar to those on PDA. Colonies on OA with regular or irregular margin, covered by floccose aerial mycelium, olivaceous brown, grey brown to dark vinaceous brown, at least in the centre becoming dark vinaceous brown with age, sometimes with concentric rings; reverse buff to olivaceous brown with a dark vinaceous brown centre. Colonies on HEA with sparse aerial mycelium, bright orange red, becoming dark carmine red in the centre, sometimes with darker concentric rings, producing diffusible pigments staining the agar bright orange; reverse orange red to dark carmine red. Immersed hyphae with age containing rosy to carmine red pigments in all media tested. NaOH test negative.

Holotype: Italy, Campania region, Caserta province, Teano, from kernel of *Corylus avellana* (Betulaceae) at harvest phase, August 2017 (WU 40039; ex-holotype culture CBS 146357 = CREADC-F2403).

Other specimens examined (all from kernels of *Corylus avellana*): Italy, Campania, Caserta province, Teano, August 2017 (WU 40040, culture CREADC-F2402); Latium, Viterbo province, Ronciglione, August 2006 (WU 40041, culture CREADC-F2281).

Notes: *Didymella corylicola* is phylogenetically closely related to *D. pedeiae*, *D. ilicicola* and *D. subherbarum* (Fig. 3), from which it differs in 1, 1 and 2 nucleotides, respectively, in the ITS; in 25, 3 and ? (no sequence available for *D. subherbarum*) nucleotides, respectively, in *RPB2*; and in 14–15, 6–7 and 11–12 nucleotides, respectively, in *TUB2*. However, *D. corylicola* differs significantly from *D. pedeiae*, *D. ilicicola* and *D. subherbarum* in its bright red pigment produced in agar cultures and by its specific host, *Corylus avellana*. Morphologically, *D. corylicola* has conidial sizes similar to those of *D. ilicicola*, *D. pedeiae* and *D. subherbarum* (*D. corylicola*: 3.2–5.3 × 1.4–2.1 μm; *D. ilicicola*: 3–4 × 1.5–2.5 μm (Chen et al. 2017); *D. pedeiae* 3–4.5 × 1.5–2.5 μm (Aveskamp et al. 2010); *D. subherbarum*: 4–6.4 × 1.6–2.2 μm (de Gruyter et al. 1993)). However, in contrast to its closest relatives which all have straight ellipsoid to oblong conidia, the conidia of *D. corylicola* are commonly slightly allantoid.

Discussion

The Didymellaceae in general and the genus *Didymella* in particular are a species-rich lineage within Pleosporales, containing numerous plant pathogens (Chen et al. 2015, 2017). The taxonomy of the group is complex and challenging, as it contains, amongst others, highly speciose sexual (*Didymella*, *Ascochyta*) as well as asexual (e.g. *Epicoccum*, *Phoma*) morph genera. Until recently, the simple morphology of both sexual and asexual morphs was a serious obstacle for reliable genus and species circumscriptions as well as for species identification. However, with the application of multigene phylogenies, substantial progress in genus and species delimitation

Fig. 3 Phylogram showing one of 114 MP trees 2199 steps long revealed ▶ by PAUP from an analysis of the combined ITS-LSU-*RPB2*-*TUB2* matrix of *Didymella*, showing the phylogenetic position of *D. corylicola* (bold red). MP and ML bootstrap support above 50% are given above or below the branches. Nodes marked by an asterisk (*) collapsed in the strict consensus of all 114 MP trees. The ex-holotype strain of *D. corylicola* is marked by a superscript HT.


Didymella pomorum CBS 388.80
Didymella pomorum CBS 539.66
Didymella pomorum CBS 354.52
Didymella pomorum CBS 285.76
Didymella sinensis LC 8142
Didymella sinensis LC 8143
Didymella aeria LC 8120
Didymella aeria CGMCC 3.18353
Didymella curtisii PD 92/1460
Didymella curtisii CBS 251.92
Didymella keratinophila CBS 143032
Didymella keratinophila FMR 13718
Didymella keratinophila FMR 13774
Didymella sancta CBS 281.83
Didymella coffeae-arabicae CBS 123380
Didymella eucalyptica CBS 377.91
Didymella protuberans CBS 132.96
Didymella protuberans CBS 377.93
Didymella protuberans CBS 391.93
Didymella protuberans CBS 381.96
Didymella nigricans CBS 444.81
Didymella nigricans PD 77/919
Didymella lethalis CBS 103.25
Didymella pinodes CBS 525.77
Didymella pinodella CBS 318.90
Didymella pinodella CBS 531.66
Didymella arachidicola CBS 333.75
Didymella glomerata CBS 133.72
Didymella glomerata CBS 528.66
Didymella aurea CBS 269.93
Didymella musae CBS 463.69
Didymella anserina CBS 253.80
Didymella anserina CBS 285.29
Didymella anserina CBS 360.84
Didymella anserina CBS 397.65
Didymella americana CBS 185.85
Didymella americana CBS 568.97
Didymella maydis CBS 588.69
Didymella subglomerata CBS 110.92
Didymella gardeniae CBS 626.68
Didymella heteroderae CBS 109.92
Didymella corylicola CREADC-F2281
Didymella corylicola CREADC-F2402
Didymella corylicola CREADC-F2405
Didymella corylicola CREADC-F2406
Didymella corylicola CREADC-F2408
Didymella corylicola CREADC-F2407
Didymella corylicola CREADC-F2410
Didymella corylicola CREADC-F2411
Didymella corylicola CBS 146357HT
Didymella corylicola CREADC-F2409
Didymella ilicicola CGMCC 3.18355
Didymella ilicicola LC 8127
Didymella subherbarum CBS 249.92
Didymella subherbarum CBS 250.92
Didymella pedeiae CBS 124517
Didymella boeremae CBS 109942
Didymella dimorpha CBS 346.82
Didymella longicolla CBS 124514
Didymella calidophila CBS 448.83
Didymella calidophila PD 84/109
Didymella acetosellae CBS 179.97
Didymella rumicicola CBS 683.79
Didymella exigua CBS 183.55
Didymella aliena CBS 379.93
Didymella bellidis CBS 714.85
Didymella bellidis PD 94/886
Didymella segeticola CGMCC 3.17489
Didymella segeticola CGMCC 3.17498
Didymella suiyangensis CGMCC 3.18352
Didymella suiyangensis LC 8144
Didymella ocimicola CGMCC 3.18358
Didymella ocimicola LC 8138
Didymella chenopodii CBS 128.93
Didymella senecionicola CBS 160.78
Didymella aquatica LC 5555
Didymella aquatica CGMCC 3.18349
Didymella macrophylla CGMCC 3.18357
Didymella macrophylla LC 8132
Didymella infuscatispora CGMCC 3.18356
Didymella infuscatispora LC 8129
Didymella tanaceti BRIP 50785
Didymella brunneospora CBS 115.58
Didymella rosea BRIP 50788
Didymella molleriana CBS 229.79
Didymella molleriana CBS 109179
Didymella negriana CBS 358.71
Didymella macrostoma CBS 482.95
Didymella macrostoma CBS 529.66
Didymella macrostoma CBS 223.69
Didymella macrostoma CBS 247.38
Didymella viburnicola CBS 523.73
Didymella pteridis CBS 379.96
Didymella ellipsoidea CGMCC 3.18350
Didymella ellipsoidea LC 8123
Didymella dactylidis CBS 124513
Didymella rhei CBS 109177
Didymella microchlamydospora CBS 105.95
Didymella chloroguttulata CGMCC 3.18351
Didymella chloroguttulata LC 8122
Paraboeremia putaminum CBS 130.69
Paraboeremia selaginellae CBS 122.93
Paraboeremia adianticola CBS 187.83
Paraboeremia adianticola CBS 260.92
Macroventuria anomochaeta CBS 525.71
Macroventuria wentii CBS 526.71
Peyronellaea - clade
10 changes

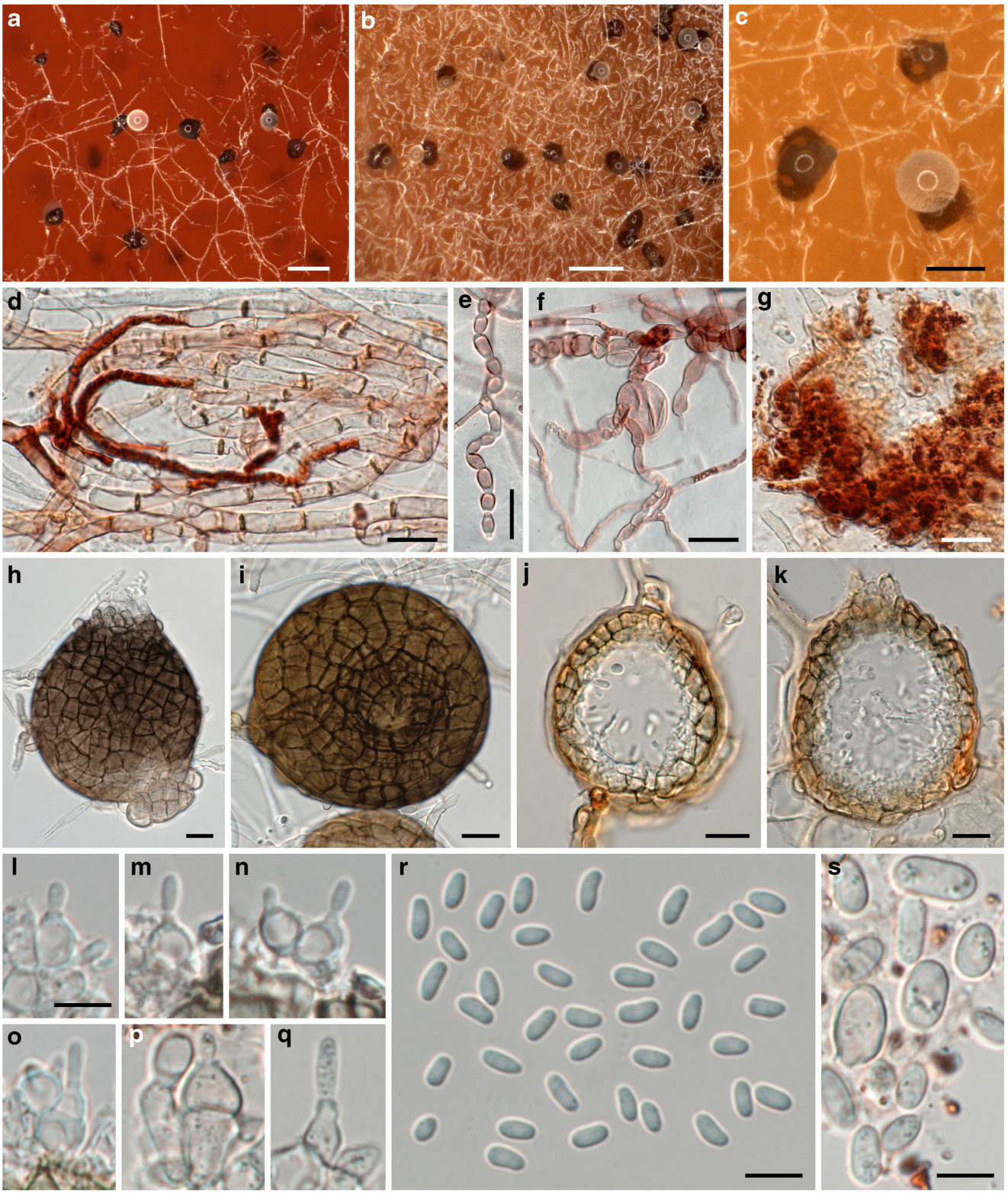

Fig. 4 *Didymella corylicola*. **a**–**c** Pycnidia in face view. **d** Substrate hyphae. **e**, **f** Torulose hyphae. **g** Amorphous red pigments in agar. **h**, **i** Pycnidia in side (**h**) and face (**i**) view. **j**, **k** Pycnidia in section. **l**–**q** Conidiogenous cells (phialides). **r**, **s** Conidia (**s** ejected and swollen, with amorphous red pigments in-between). All in water, except **h**, **i** in 3% KOH. Media: **a** HEA; **b**–**d**, **g**–**s** HTA; **e**, **f** MEA. Sources: **a**–**c**, **e**, **f**, **h**–**o**, **r** ex-holotype strain CBS 146357 = CREADC-F2403; **d**, **g**, **p**, **q**, **s** CREADC-F2402. Scale bars **a**, **b** 200 μm; **c** 100 μm; **d**–**k** 10 μm; **l**–**s** 5 μm

within Didymellaceae has been achieved in recent years (Aveskamp et al. 2010; Chen et al. 2015, 2017), in particular for the large genera *Ascochyta*, *Didymella* and *Phoma*. Several new genera were established, the genus *Phoma* was restricted to the type species, *P. herbarum*, and numerous species of *Phoma* were transferred to the genera *Allophoma*, *Ascochyta*, *Boeremia*, *Calophoma*, *Didymella*, *Epicoccum*, *Heterophoma*, *Neoascochyta*, *Neodidymelliopsis*, *Nothophoma*, *Paraboeremia*, *Stagonosporopsis* and *Xenodidymella* (Chen et al. 2015). In addition, new species of *Didymella* have been described (e.g. Chen et al. 2015, 2017; Valenzuela-Lopez et al. 2018), highlighting that the species diversity of *Didymella* is still insufficiently known.

In the molecular phylogenetic analyses, *D. corylicola* forms a highly supported clade with *D. pedeiae*, *D. ilicicola* and *D. subherbarum*, which corresponds to *Phoma* group G of Aveskamp et al. (2010). In contrast to *D. corylicola*, which is, as far known, host specific to *Corylus*, *D. pedeiae* and *D. subherbarum* are plurivorous (Aveskamp et al. 2010), while *D. ilicicola* is so far only known from seedlings of *Ilex chinensis* (Chen et al. 2017). All species of the clade share a negative NaOH reaction of the colonies, and while conidial sizes are overlapping with its closest relatives, *D. corylicola* is well characterised by the bright red pigment produced in agar cultures and its specific host. In addition, the genetic differences of *D. corylicola* justify its status as a distinct species.

Didymella corylicola represents a fungal species associated with hazelnut fructification from the primordial to post-harvest phase. Its abundance in the early stages of fruit development may be facilitated by the psychrophilic nature of this fungus which is able to grow at 5 °C. In addition, it seems to be associated only with hazelnut, since it has never been isolated from other common nut species such as walnut and pistachio, which were intensely surveyed in the past years by the authors (Belisario et al. 2002; Vitale et al. 2007, 2018; Scotton et al. 2015), and it has never been reported from almond. Although weak pathogenicity was revealed in the inoculation experiments, the impact of *D. corylicola* on the development of hazelnut fruit is yet unclear. Ongoing studies might show whether this fungus plays a direct role in the emergence of kernel defects, or whether it has an indirect effect by an interaction with known hazelnut fruit pathogens such as *Colletotrichum* spp., *Diaporthe* spp. or *Fusarium* spp.

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