Persoonia 21, 2008: 119–134
www.persoonia.org

RESEARCH ARTICLE

doi:10.3767/003158508X374385

Molecular and phenotypic characterisation of novel *Phaeoacremonium* species isolated from esca diseased grapevines

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Key words

actin
β-tubulin
esca
morphology
Phaeoacremonium
phylogeny

Abstract Petri disease and esca are very destructive grapevine decline diseases that occur in most countries where grapevine (*Vitis vinifera*) is cultivated. *Phaeoacremonium* species are among the principal hyphomycetes associated with symptoms of the two diseases, producing a range of enzymes and phytotoxic metabolites. The present study compared the phylogeny of a global collection of 118 *Phaeoacremonium* isolates from grapevines, in order to gain a better understanding of their involvement in Petri disease and esca. Phylogenetic analyses of combined DNA sequence datasets of actin and β-tubulin genes revealed the presence of 13 species of *Phaeoacremonium* isolated from esca diseased grapevines. *Phaeoacremonium aleophilum* was the most frequently isolated species with an incidence up to 80 % of all isolates investigated. Species previously described mainly as human pathogenic species, namely *Pm. alvesii*, *Pm. griseorubrum* and *Pm. rubrigenum* are newly reported on grapevine from Turkey, Italy and Croatia, respectively. *Phaeoacremonium viticola* and *Pm. scotyli* represent new records for Italy, as well as *Pm. mortoniae* for Hungary and Croatia. In addition, four new species of *Phaeoacremonium,* namely *Pm. croatiense*, *Pm. hungaricum*, *Pm. sicilianum* and *Pm. tuscanum* are newly described from grapevine based on morphology, cultural characteristics, as well as molecular phylogeny.

Article info Received: 15 July 2008; Accepted: 26 September 2008; Published: 7 October 2008.

INTRODUCTION

Phaeoacremonium species are well-known vascular plant pathogens causing wilt and dieback of woody hosts. In grapevine, the two principal diseases in which they are involved are Petri disease and esca, the latter of which comprises young esca and esca proper according to the nomenclature of esca diseases proposed by Graniti et al. (2000). Petri disease causes stunted growth and dieback of young grapevines. It often occurs in 1–5 yr old grapevines and causes significant losses in newly planted vineyards (Mugnai et al. 1999, Morton 2000, Pascoe & Cottral 2000, Edwards & Pascoe 2004, Surico et al. 2006). Internal symptoms can normally be seen when transverse or longitudinal cuts are made in the rootstock. These include black spots and dark brown to black streaking of the xylem tissues. Esca can be typically identified by various types of internal wood deterioration as well as symptoms on leaves and berries. Vines with typical symptoms on the leaves show interveinal areas of chlorotic tissue that turn yellow-brown or red-brown and finally necrotic, an appearance that can also be described as 'tiger stripes' (Larignon & Dubos 1997, Mugnai et al. 1999, Edwards et al. 2001, Calzarano & Di Marco 2007) (Fig. 1). In the USA, esca has been referred to as 'black measles' because of the small, dark-brown to purple spots that can develop on the berries (Fig. 2) (Vasquez et al. 2007). When a transverse cut is made in the trunk and main shoots, black spots (black streaking in longitudinal section) appear in the wood as in the case of Petri disease, but in young esca also pink-brown or dark red-brown areas can be found, occasionally with other wood discolorations (Mugnai et al. 1999). Esca proper (Surico 2001) differs from young esca by the presence of wood decay (Mugnai et al. 1999, Fischer 2002, Surico et al. 2006) (Fig. 3).

Foliar and fruit symptoms do not necessarily appear on the same diseased plant every year (Mugnai et al. 1999, Marchi et al. 2006), and often infected vines remain asymptomatic (Surico et al. 2006). In severe cases 'apoplexy' can occur when vines or vine-parts suddenly wilt during hot, dry conditions in the summer.

Fungi that have been associated with esca symptoms in Europe include the wood-rotting basidiomycete *Fomitiporia mediterranea*, and occasionally *Stereum hirsutum* (Larignon & Dubos 1997, Fischer 2006), while *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* are the principal hyphomycetes associated with black streaking and brown-red wood (Larignon & Dubos 1997, Mugnai et al. 1999, Crous & Gams 2000). It is the combination of these fungi that causes 'esca proper', affecting mostly vines older than 15 yr, while vines showing esca foliar symptoms, wood black streaking and necrosis are mainly infected with *Phaeomoniella chlamydospora* and/or *Phaeoacremonium* species.

Species of *Phaeoacremonium* mainly involved in Petri disease and esca are *Pm. aleophilum*, *Pm. angustius*, *Pm. mortoniae* and *Pm. parasiticum* (Eskalen et al. 2005a, Mostert et al. 2006a, Martin & Cobos 2007), but the degree of involvement of other *Phaeoacremonium* species remains uncertain. Furthermore, the identity of fungi associated with esca symptoms in many grapevine-growing areas, especially from the area where

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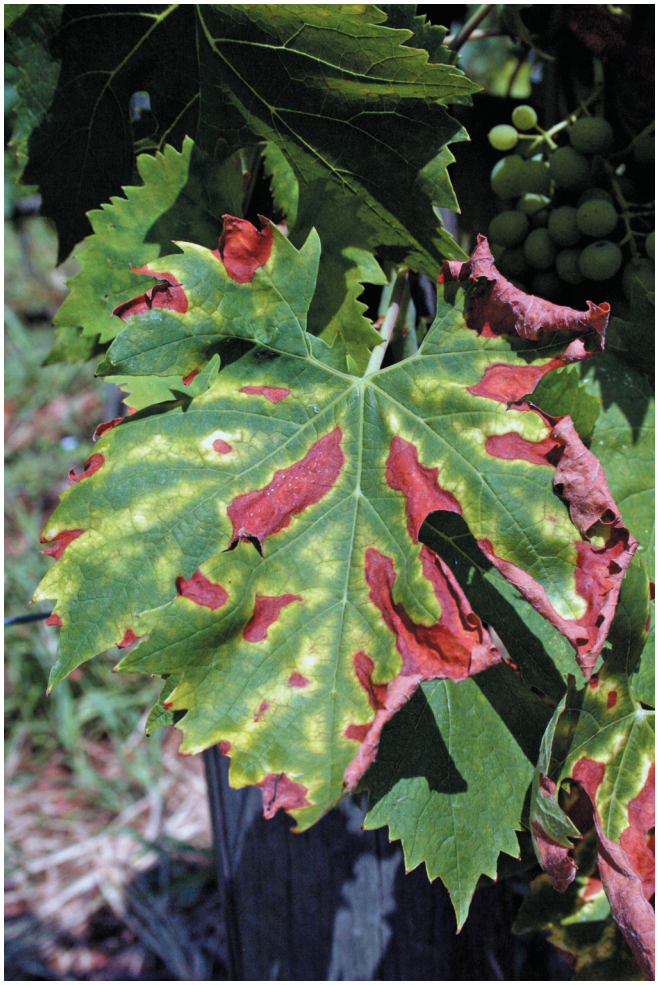


Fig. 1 Symptoms associated with esca of grapevine: chlorosis and necrosis on the leaves showing typical 'tiger-like' pattern.

Fig. 2 Symptoms associated with esca of grapevine: black measles on the berries.

Fig. 3 Typical wood symptoms in a vine affected by esca: brown-red wood, black streaking, and central white decay.

grapevine has originated, and several isolated regions have not yet been studied, and therefore many elusive aspects remain to be clarified.

The present study investigates the identity of a group of 118 *Phaeoacremonium* isolates from grapevine, collected mainly from very old vines, in isolated locations in Italy and other countries. Knowledge pertaining to the involvement of *Phaeoacremonium* species in esca and Petri disease should shed light on the epidemiology of these destructive diseases of grapevine, with the final aim of helping in refining control strategies, since there are no effective curative chemicals for Petri disease and esca.

MATERIAL AND METHODS

Fungal isolates

Branches and trunks of *Vitis vinifera* showing esca symptoms in wood, including brown and black streakings, brown-red wood, necrosis and white rot (Fig. 4) and in some cases also foliar symptoms of esca, were collected from different regions of Italy, primarily from isolated locations and very old vineyards (80–100 yr old), from different vineyards within the same region and from different positions on the same vine. Other strains, collected from different countries (Croatia, Greece, Hungary, Israel, Turkey and the USA), were also investigated in this study.

Trunk and shoots of diseased grapevines were cut into disks and the surface was sterilised. Small pieces of tissue were cut from just below the surface, around and in the darkened vascular tissues, and plated onto malt extract agar (MEA; 2 % malt extract, Oxoid Ltd., Basingstoke, Hampshire, England; 1.5 % agar, Difco, Detroit, Michigan, USA) and incubated at 25 °C in the dark for 2–3 wk until cultures sporulated. Single conidial isolations were established from emerging colonies identified as species of *Phaeoacremonium*. Isolates were maintained at the Dipartimento di Biotecnologie Agrarie, Sezione di Patologia Vegetale, University of Florence, and representative strains lodged at the CBS Fungal Biodiversity Centre, Utrecht, Netherlands. Isolates used for morphological and sequence analysis are presented in Table 1.

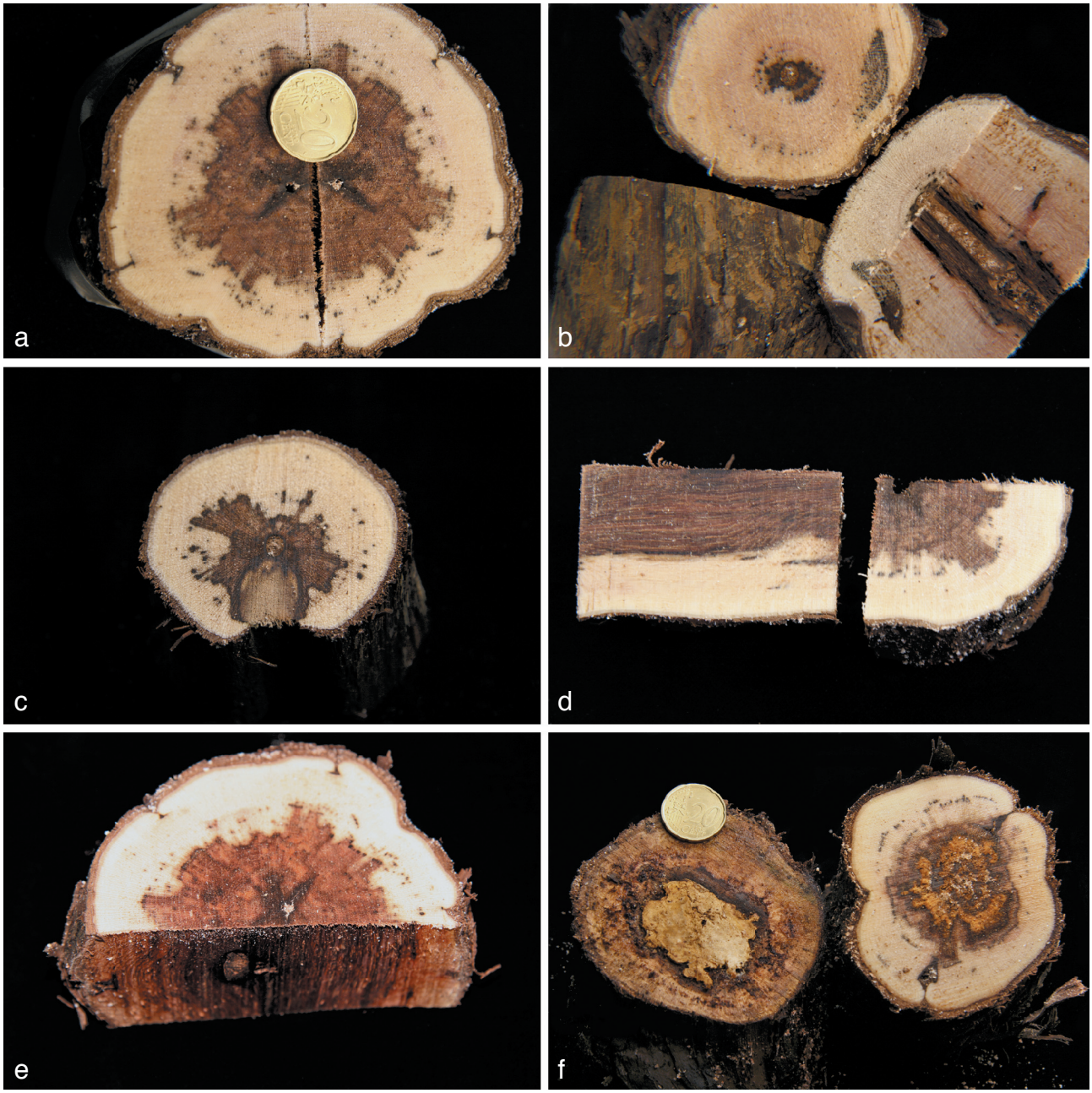


Fig. 4 Internal symptoms seen when transversal or longitudinal cuts were made in the trunk or cordon of vines used for fungal isolation. a, b. Black spots and dark brown to black streaking of the xylem tissues; c. cross section showing sectorial necrosis; d. longitudinal section showing wood discoloration; e. central brown-red necrosis; f. cross section showing a central white rot surrounded by brown-red wood.

DNA isolation and amplification

Genomic DNA was extracted from 118 strains identified as *Phaeoacremonium* using approximately 300 mg mycelium with the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 600 bp of the 5' end of the β-tubulin (TUB) and approximately 300 bp of the 5' end of the actin (ACT) genes were amplified for the strains identified as *Phaeoacremonium* as described by Mostert et al. (2006b) using primer sets T1 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) and ACT-512F and ACT-783R (Carbone & Kohn 1999), respectively.

Amplicons were sequenced using both PCR primers with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and sequences were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA, USA). A consensus sequence was computed from the forward and reverse sequences with the SeqMan from the Lasergene package (DNA star, Madison, WI, USA).

Table 1 Names, GenBank accession numbers and collection details of *Phaeoacremonium* isolates studied. *Phaeoacremonium aleophilum* sequence types based on ACT and TUB respectively are indicated between round brackets (superscript in isolate number).

Phaeoacremonium species	Isolate number	Location	GenBank accession numbers	
			ACT	β-tubulin
Pm. aleophilum	4.ss2Pal (1/1)	Tuscany, Italy	EU863496	EU863464
	146Pal (1/1)	Abruzzo, Italy		
	32Pal (1/1)	Marche, Italy		
	59Pal, 69Pal, 75Pal, 76.ss1Pal (1/1)	Sicily, Italy		
	4ss1Pal, 23Pal (1/1)	Tuscany, Italy		
	140Pal (2/1)	Friuli-Venezia Giulia, Italy		
	33Pal (2/1)	Marche, Italy		
	38Pal, 39Pal (2/1)	Sardinia, Italy		
	44ss1Pal, 51Pal, 52a.ss1Pal, 52ass2 Pal, 52-bPal, 53Pal, 64Pal, 70Pal, 71Pal, 72Pal, 77Pal, 80Pal (2/1)	Sicily, Italy		
	30Pal (2/1)	Trentino-Alto Adige, Italy		
	17Pal, 20Pal (2/1)	Tuscany, Italy		
	143ss2Pal (2/1)	Umbria, Italy		
	124Pal, 125ss1Pal, 126Pal, 127Pal (2/1)	Turkey		
	60Pal, 61Pal, 62Pal, 73Pal, 74Pal, 78Pal, 79Pal (3/1)	Sicily, Italy		
	142Pal (3/1)	Tuscany, Italy		
	145Pal (4/1)	Abruzzo, Italy		
	148Pal (4/1)	Apulia, Italy		
	100Pal, 101Pal, 103Pal, 104Pal (4/1)	Hungary		
	130Pal, 131Pal, 133Pal (6/1)	Israel		
	81Pal (2/2)	Sicily, Italy	EU863497	EU863465
	168Pal (2/3)	Trentino-Alto Adige, Italy	EU863498	EU863466
	56Pal (1/3)	Sicily, Italy		
	31Pal (1/3)	Trentino-Alto Adige, Italy		
	158Pal (1/3)	Tuscany, Italy		
	139Pal (2/3)	Lombardy, Italy		
	47Pal, 57Pal, 58Pal, 65Pal, 66Pal, 67Pal, 68Pal (2/3)	Sicily, Italy		
	167Pal, 171Pal (2/3)	Trentino-Alto Adige, Italy		
	13Pal, 14Pal, 22Pal, 24Pal, 25Pal, 28Pal, 152Pal, 153Pal (2/3)	Tuscany, Italy		
	115Pal, 116Pal (2/3)	Croatia		
	120Pal, 121Pal, 122Pal, 123Pal, 128Pal, 129Pal (2/3)	Turkey		
	159Pal, 161Pal (2/3)	U.S.A		
	137Pal, 138ss1Pal (3/4)	Lombardy, Italy	EU863500	EU863468
	156Pal (5/4)	Tuscany, Italy	EU863499	EU863467
	84Pal, 85Pal (1/4)	Greece		
	117Pal, 118Pal (2/4)	Croatia		
	98Pal (3/5)	Hungary	EU863501	EU863469
	21Pal (4/6)	Tuscany, Italy	EU863502	EU863470
	144Pal (1/7)	Abruzzo, Italy	EU863503	EU863471
	132Pal (6/8)	Israel	EU863504	EU863472
Pm. alvesii	125ss2 Pal	Turkey	EU883991	EU883990
Pm. croatiense sp.nov.	CBS 123037	Croatia	EU863514	EU863482
Pm. hungaricum sp.nov.	CBS 123036	Hungary	EU863515	EU863483
Pm. iranianum	2Pal	Tuscany, Italy	EU863491	EU863459
	3Pal	Tuscany, Italy	EU863492	EU863460
	6Pal	Tuscany, Italy	EU863493	EU863461
	7Pal	Tuscany, Italy	EU863494	EU863462
	163Pal	Piedmont, Italy	EU863495	EU863463
Pm. griseorubrum	42Pal	Sicily, Italy	EU863517	EU863485
Pm. mortoniae	110bss1Pal	Hungary	EU863507	EU863475
	110.ss2Pal	Hungary	EU863508	EU863476
	111Pal	Hungary	EU863509	EU863477
	112ss1Pal	Hungary	EU863510	EU863478
	110bss2Pal	Hungary	EU863511	EU863479
	114Pal	Hungary	EU863512	EU863480
	94Pal	Croatia	EU863513	EU863481
Pm. parasiticum	40ss2Pal	Sicily, Italy	EU863519	EU863487
Pm. rubigenum	CBS 123038	Croatia	EU863516	EU863484
Pm. scolyti	109Pal	Tuscany, Italy	EU863518	EU863486
Pm. sicilianum sp.nov.	CBS 123034	Sicily, Italy	EU863520	EU863488
	CBS 123035	Sicily, Italy	EU863521	EU863489
Pm. tuscanum sp.nov.	CBS 123033	Tuscany, Italy	EU863490	EU863458
Pm. viticola	40ss1Pal	Sicily, Italy	EU863505	EU863473
	41Pal	Sicily, Italy	EU863506	EU863474

Phylogenetic analysis

Sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Se-Al; Rambaut 2002) by inserting gaps, and additional reference sequences were obtained from GenBank and added to the alignment. The TUB and ACT alignments were concatenated to make it possible to perform combined analyses. Phylogenetic analyses of the aligned sequence data were performed with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) and consisted of neighbour-joining analyses with the uncorrected ('p'), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analyses, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with 100 random simple taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page 1996). New sequences were lodged in GenBank and the alignment and phylogenetic tree in TreeBASE (www.treebase.org). *Pleurostomophora richardsiae* (CBS 270.33; GenBank ACT = AY579271, TUB = AY579334) was used as outgroup in the phylogenetic analyses.

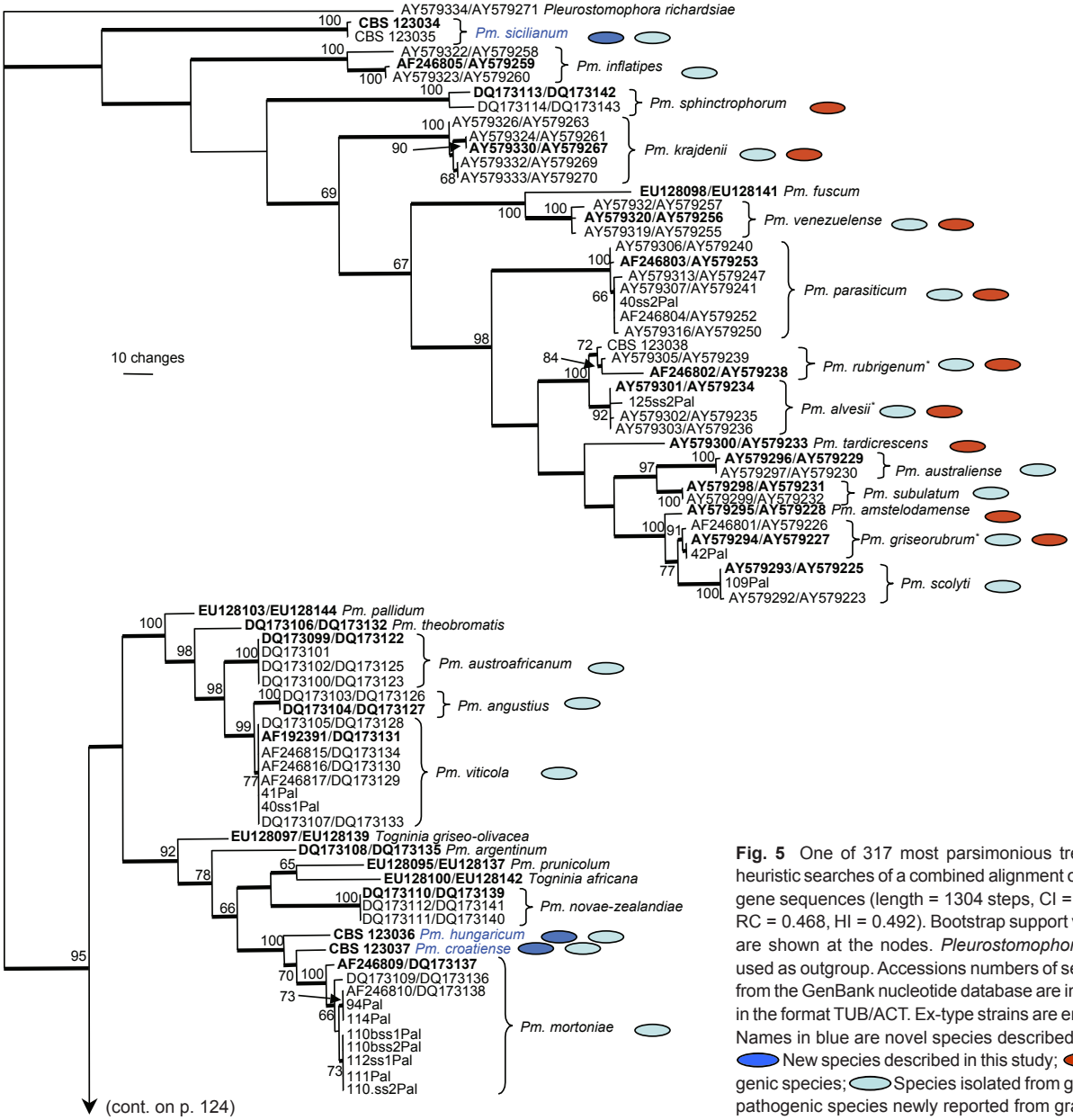
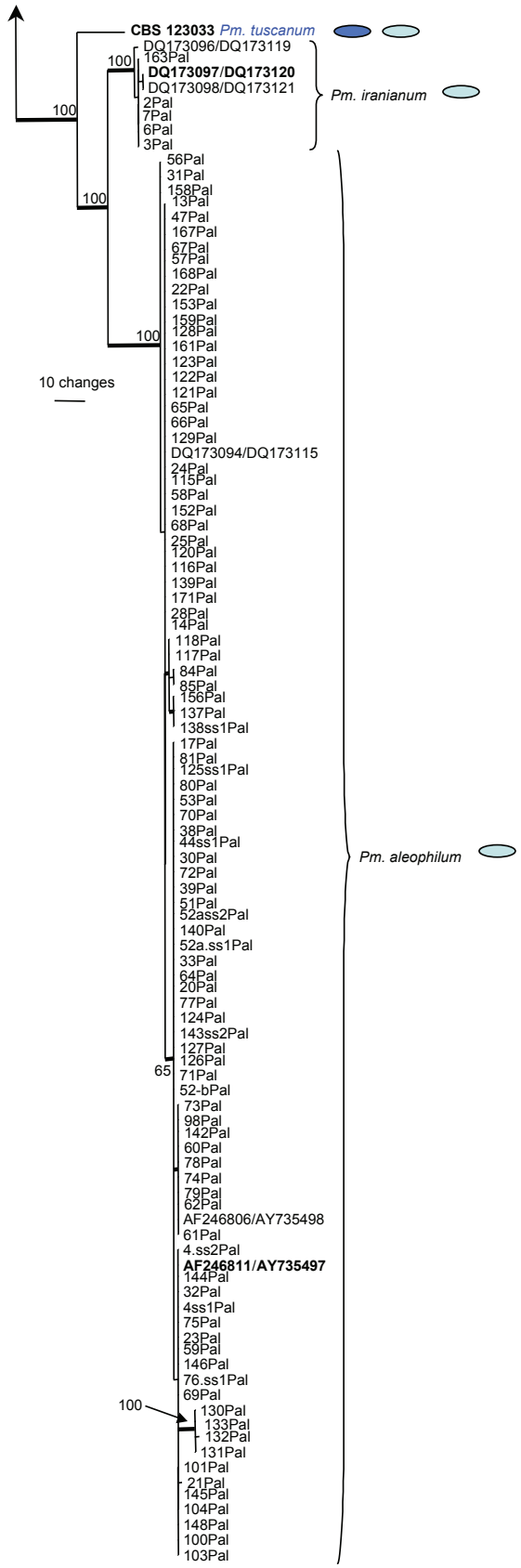


Fig. 5 One of 317 most parsimonious trees obtained from heuristic searches of a combined alignment of the TUB and ACT gene sequences (length = 1304 steps, CI = 0.508, RI = 0.922, RC = 0.468, HI = 0.492). Bootstrap support values above 64 % are shown at the nodes. *Pleurostomophora richardsiae* was used as outgroup. Accessions numbers of sequences obtained from the GenBank nucleotide database are indicated on the tree in the format TUB/ACT. Ex-type strains are emphasised in **bold**. Names in blue are novel species described in this study. New species described in this study; Human pathogenic species; Species isolated from grapevine; * Human pathogenic species newly reported from grapevine.

Fig. 5 (cont.)



Morphology

Morphological characters used in distinguishing species included conidiophore morphology, phialide type and shape, size of hyphal warts, and to a lesser extent, conidial size and shape. Cultural characters that were investigated included the colour of colonies on MEA, the production of yellow pigment on potato-dextrose agar (PDA; 3.9 % potato-dextrose agar, Difco) and oat meal agar (OA, 30 g oats; 8 g Roko agar, La Coruña, Spain; 1 000 mL water) (Gams et al. 2007), the growth rate of colonies at 25 °C and the maximum temperature for growth in vitro.

Microscopic observations were made from aerial mycelium of colonies cultivated on MEA or by using the transparent tape or slide culture technique, as respectively explained by Schubert et al. (2007) and Arzanlou et al. (2007). Photos were captured by means of a Nikon camera system (Digital Sight DS-5M, Nikon Corporation, Japan). Structures were mounted in lactic acid, and 30 measurements (× 1 000 magnification) were determined. The 5th and 95th percentiles were defined for all measurements with the extremes given in parentheses.

Cardinal temperatures for growth were determined by incubating inoculated MEA plates in the dark at temperatures ranging from 6 to 40 °C. Radial growth was measured after 8 d at 25 °C. Colony colours were defined after 16 d from the same plates according to the colour charts of Rayner (1970).

RESULTS

Phylogenetic analyses

The combined alignment consisted of 184 sequences including the outgroup sequence and included 473 characters and alignment gaps (number of included characters: TUB = 266 and ACT = 207) that were subjected to the phylogenetic analyses. Of these, 275 were parsimony informative, 47 were variable and parsimony uninformative and 151 were constant. The small number of characters included for the TUB is due to the inclusion of GenBank accession AF192391, which represents the TUB sequence of the type strain of *Pm. viticola* and which is missing more than 250 characters on the 5' end when compared to the other sequences in the alignment. Parsimony analyses yielded 317 equally most parsimonious trees that mainly differed in the order of taxa at the terminal nodes; one of the trees is presented in Fig. 5 (TL = 1304; CI = 0.508; RI = 0.922; RC = 0.468; HI = 0.492). Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. The phylogenetic tree clustered some isolates obtained in this study with previously published species and indicated that others did not match any sequences available in GenBank. The second group of sequences represents unknown species which are described below.

Analyses of the individual loci did not reveal any significant deviation from the topology obtained from analyses of the combined alignment and 72 equally most parsimonious trees were obtained for both loci (data not shown). For the TUB data, the 266 characters including alignment gaps consisted of 136 parsimony informative, 31 variable and parsimony uninformative

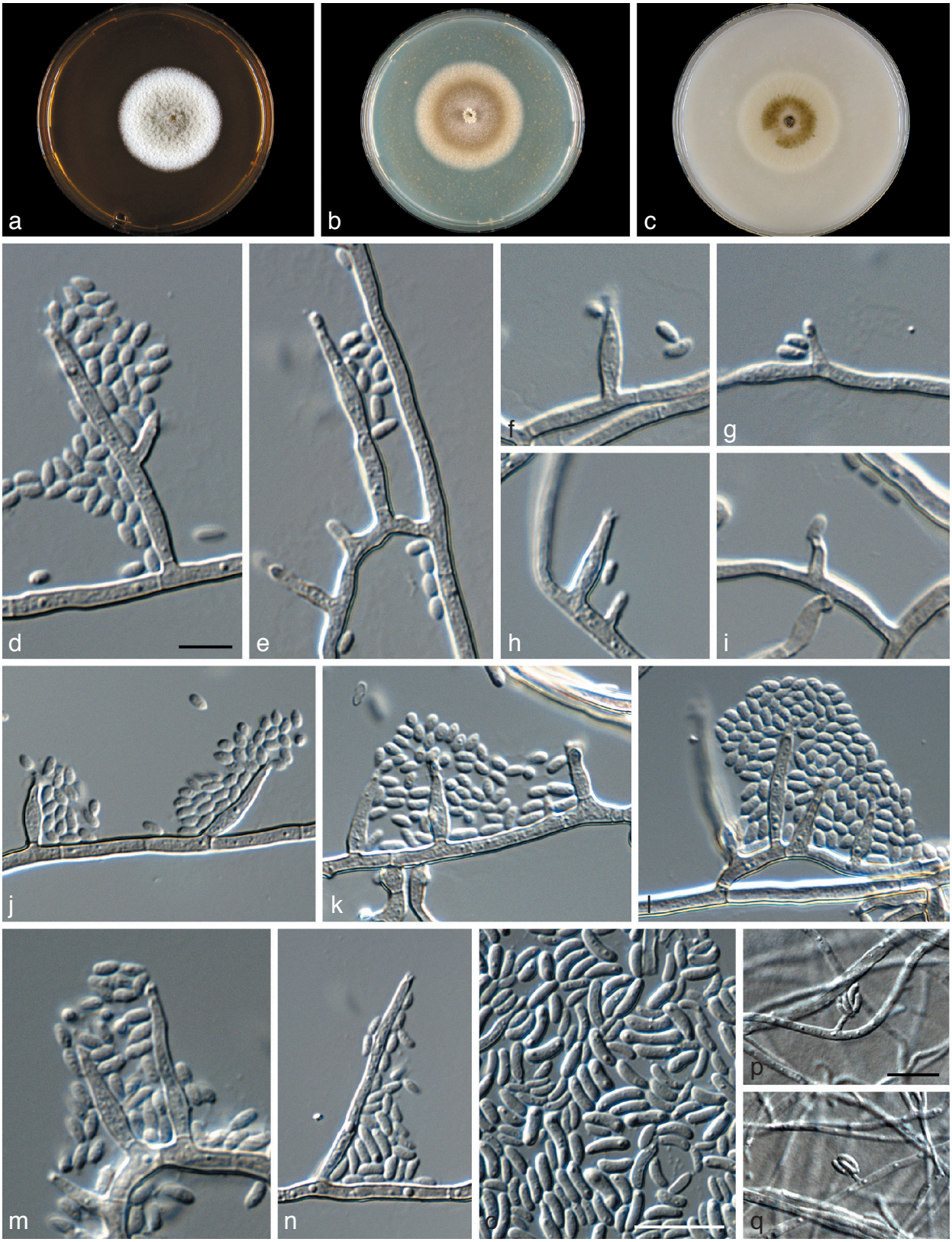


Fig. 6 *Phaeoacremonium croatiense*. a–c. Sixteen days old colonies on 2 % MEA (a), PDA (b) and OA (c). — d–o. Aerial structures on 2 % MEA; d, e. conidiophores; f–i. type I phialide; j–l. type II phialide; m, n. type III phialide; o. conidia. — p–q. Structures on the surface of and in 2 % MEA: adelophialides with conidia; all from CBS H-20120 (holotype); d–q: DIC. — Scale bars: d–p = 10 µm; scale bar for d applies to e–n and q.

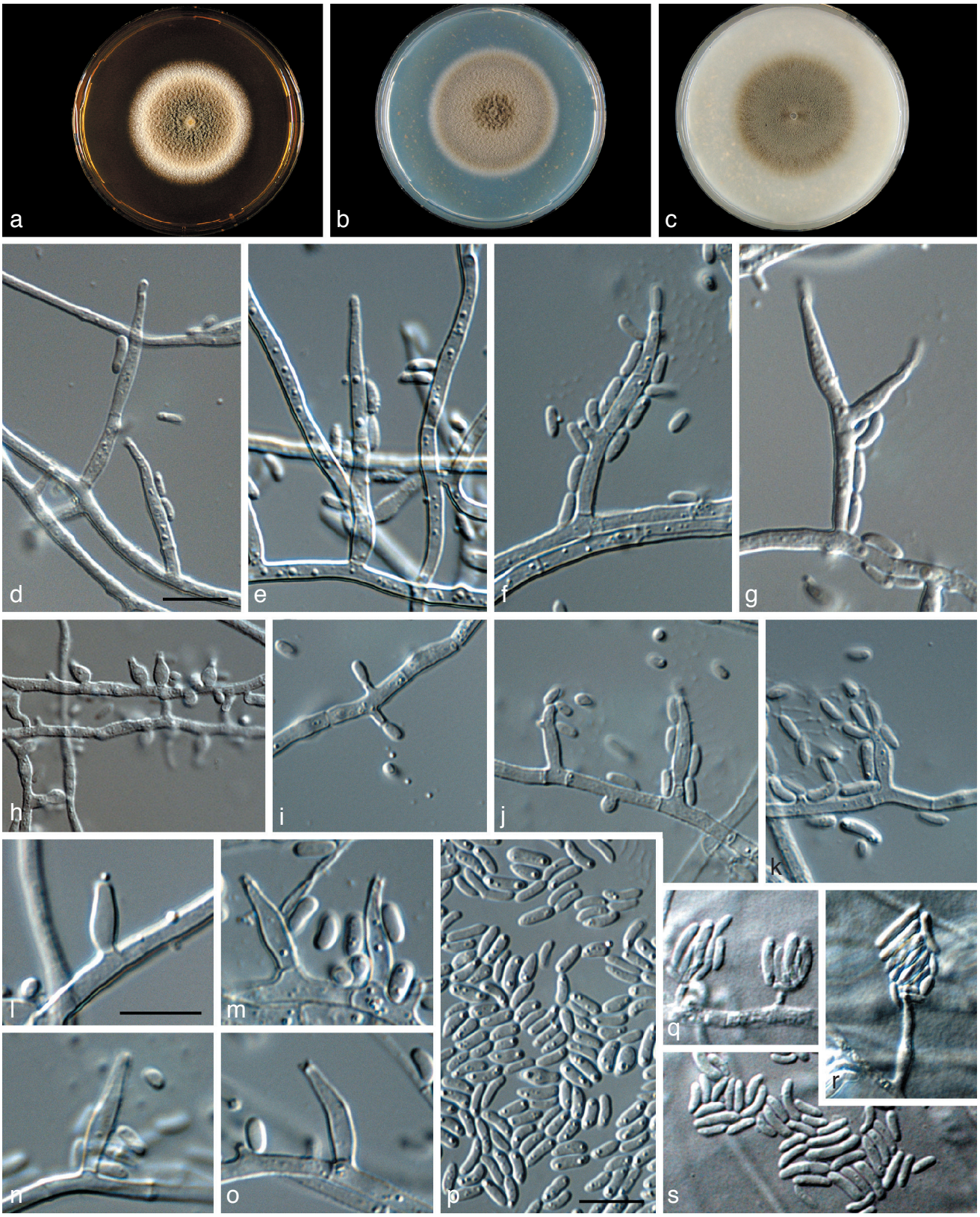


Fig. 7 *Phaeoacremonium hungaricum*. a–c. Sixteen days old colonies on 2 % MEA (a), PDA (b) and OA (c). — d–p. Aerial structures on 2 % MEA. d–g. conidiophores; h–k. type I phialide; i–o. type II phialide; p. conidia. — q–s. Structures on the surface of and in 2 % MEA; q–r. adelophialides with conidia; s. conidia; all from CBS H-20119 (holotype); d–s: DIC. — Scale bars: d–s = 10 µm; scale bar for d applies to e–k and q–s; bar for l applies to m–o.

and 99 constant characters; for the ACT data the 207 characters including alignment gaps consisted of 139 parsimony informative, 16 variable and parsimony uninformative and 52 constant characters.

Taxonomy

According to DNA sequence analyses and morphological characters, the 118 strains isolated from wood of *Vitis vinifera* showing Petri disease or esca symptoms could be assigned to 13 different species of *Phaeoacremonium*. Four taxa proved to be distinct from known species and are described in this study. In addition to the novel species, the morphologically variant form seen in *Pm. rubrigenum* isolate CBS 123038 was discussed in contrast to the isolates described by Mostert et al. (2006b).

Phaeoacremonium croatiense Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506947; Fig. 6

Phaeoacremonio mortoniae phylogenetice simile, sed coloniis olivaceo-griseis in agaro MEA, sine pigmento flavido in agaro OA.

Etymology. Named after Croatia, where this species was collected.

Aerial structures in vitro on MEA: *Mycelium* consisting of branched, septate hyphae that occur singly or in bundles of up to 4; tuberculate with warts up to 0.5 µm wide, subhyaline to pale brown, smooth to verruculose, 2–3 µm wide. *Conidiophores* mostly of medium length, usually unbranched, arising from aerial or submerged hyphae, erect to flexuous, up to 5-septate, often ending in a single terminal phialide, subhyaline to pale brown, paler towards the tip, smooth to verruculose, (10–)16–23(–48) (av. 19) µm long and (2–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, hyaline to subhyaline, collarettes, 1.5–2 µm long, 1–1.5 µm wide; type I phialides predominant, mostly cylindrical to subcylindrical, or elongated ampulliform, attenuated at the base, (6–)11–13(–15) × (1.5–)2.5(–3) (av. 12 × 2.5) µm; type II phialides mostly subulate, some navicular, tapering towards the apex, (10–)15–19(–20) × (2–)2.5(–3) (av. 17 × 2.5) µm; type III phialides subcylindrical, subulate, (20–)23–27(–28) × 2(–2.5) (av. 24 × 2) µm. *Conidia* hyaline, mostly subcylindrical or allantoid, some cylindrical or ellipsoidal, (2–)3–4.5(–7) × (1–)1.5(–2) (av. 4 × 1.5) µm, L/W = 2.6. On surface or submerged in the agar: *Phialides* hyaline, mostly cylindrical to subcylindrical, (2–)5–8(–12) × (1.5–)2(–3) (av. 7 × 2) µm. *Conidia* hyaline, subcylindrical, or allantoid, (4–)6–7(–9) × (1–)2 (av. 6.5 × 2) µm, L/W = 3.25.

Cultural characteristics — Colonies reaching a radius of 10 mm after 8 d at 25 °C. Minimum temperature for growth 12 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, cottony, with entire margin; after 16 d, pale olivaceous-grey to whitish above, orange to yellowish white in reverse. Colonies on PDA flat, short woolly to felty, with entire edge, after 16 d, colonies smoke-grey to pale grey-olivaceous above, white towards the margin above, brownish grey in reverse. Colonies on OA flat, felty, with entire margin, after 16 d, grey-olivaceous to whitish towards the edge above, pale olivaceous-grey, yellowish white towards the edge in reverse.

Substrate — *Vitis vinifera*.

Known distribution — Croatia.

Specimen examined. Croatia, Moslavina, Voloder, isolated from *Vitis vinifera* (cv. Škrlet) cutting showing necrosis and black streakings, June 2007, *B. Cvjetković*, holotype CBS H-20120, culture ex-type CBS 123037.

Notes — DNA sequence analyses revealed *Pm. croatiense* to be closely related to *Pm. mortoniae*. It can, however, be distinguished based on its olivaceous-grey colonies on MEA, as well as by the absence of yellow pigment production on OA.

Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506948; Fig. 7

Phaeoacremonio mortoniae phylogenetice simile, sed structuris coremioidibus fertilibus in agaro MEA et phialidibus plerumque typi II.

Etymology. Named after Hungary, where this species was collected.

Aerial structures in vitro on MEA: *Mycelium* composed of branched, septate hyphae that occur singly or in bundles of up to 14, subhyaline to medium brown, smooth, occasionally verruculose, 1–3.5 µm wide. *Conidiophores* mostly short, usually unbranched, arising from aerial or submerged hyphae, erect, simple, up to 2-septate, often ending in a single terminal phialide, subhyaline to pale brown, paler towards the tip, smooth to verruculose, (20–)26–30(–36) (av. 27) µm long and (2–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, mostly subhyaline, sometimes pale brown, collarettes, 1 µm long, 1.5 µm wide; type I phialides most predominant, elongated ampulliform, attenuated at the base, or constricted, some cylindrical, (3–)7–12(–15) × (1–)2.5(–3) (av. 7 × 2.5) µm; type II phialides navicular or subulate, subcylindrical, tapering towards the apex, (9–)12–15(–20) × (1.5–)2.5(–3) (av. 13 × 2.5) µm. *Conidia* hyaline, mostly, subcylindrical or cylindrical, often allantoid, (3–)4–5(–6) × (1.5–)2 (av. 4.5 × 2) µm, L/W = 2.25. On surface or submerged in the agar: *Phialides* hyaline, cylindrical to subcylindrical, occasionally navicular, (2–)7–11(–15) × (1.5–)2(–3) (av. 9 × 2) µm. *Conidia* hyaline, cylindrical, subcylindrical or allantoid (3–)5–7.5(–12) × (1–)2.5(–3) (av. 6.5 × 2) µm, L/W = 3.75.

Cultural characteristics — Colonies reaching a radius of 10 mm after 8 d at 25 °C. Minimum temperature for growth 10 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, woolly, with entire margin; after 16 d, whitish yellow to whitish above, dark brown to pale orange in reverse. Colonies on PDA flat, felty, with entire edge, after 16 d, colonies beige to whitish grey-olivaceous, white towards the margin above. Colonies on OA flat, felty, with entire margin, after 16 d, greenish olivaceous to white towards the edge; greenish olivaceous above, olivaceous-grey in reverse.

Substrate — *Vitis vinifera*.

Known distribution — Hungary.

Specimen examined. Hungary, Mád, Tokaj, isolated from *Vitis vinifera* (cv. Hárslevelű) showing external esca symptoms, wood necrosis and black streaking, Feb. 2007, *B.T. Dula*, holotype CBS H-20119, culture ex-type CBS 123036.

Notes — Phylogenetically, this species clusters as a sister clade to *Pm. mortoniae*. However, it can be distinguished by its conidiophores which are mostly reduced to phialides. The aerial mycelium has an abundant number of phialides, which are elongated ampulliform in shape.

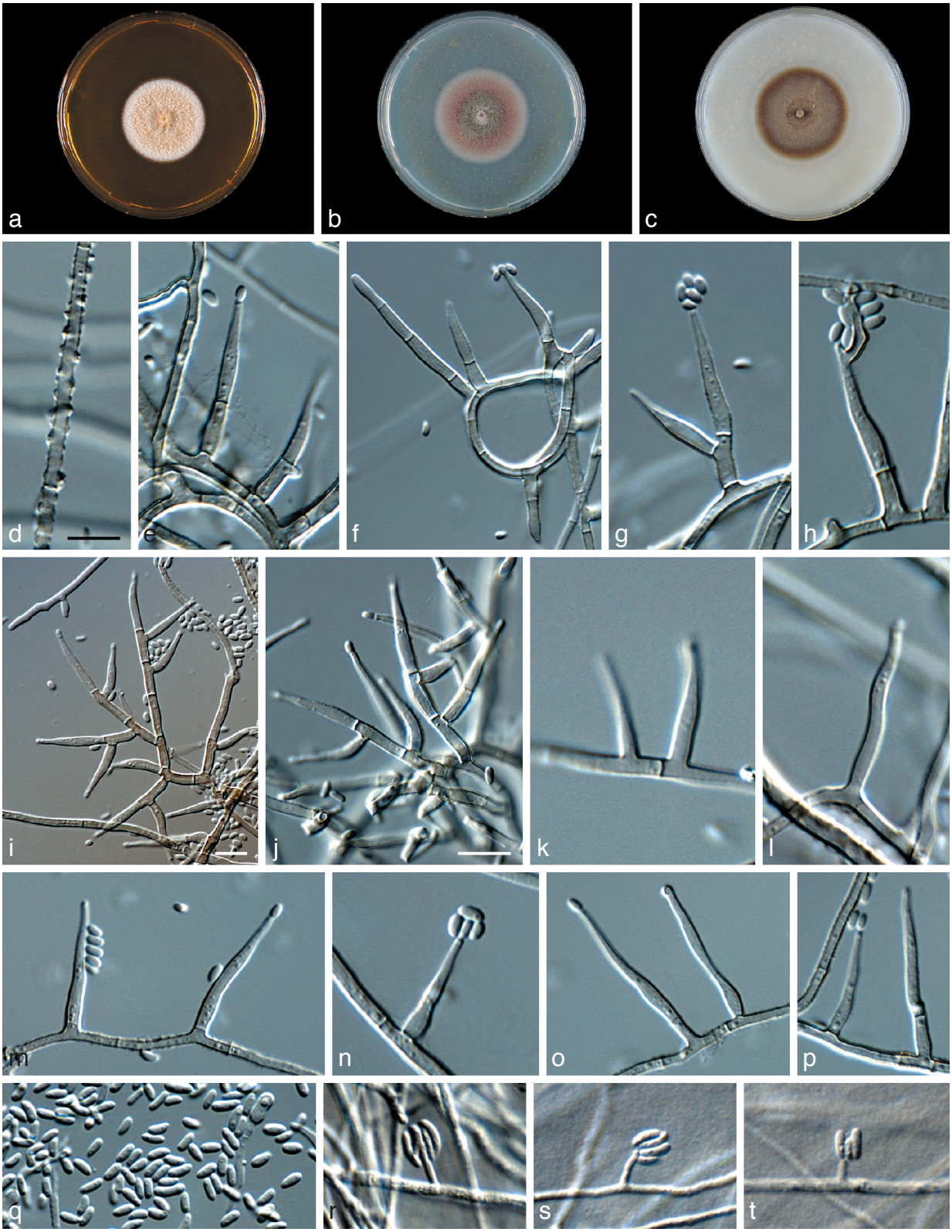


Fig. 8 *Phaeoacremonium rubrigenum*. a–c. Sixteen days old colonies on 2 % MEA (a), PDA (b) and OA (c). — d–q. Aerial structures on 2 % MEA; d. mycelium showing prominent exudate droplets observed as warts; e–h. single conidiophores; i, j. branched conidiophores; k, l. type I phialide; m, n. type II phialide; o, p. type III phialide; q. conidia. — r–t. Structures on the surface of and in 2 % MEA: adelophialides with conidia; all from H-20121 (holotype); d–t: DIC. — Scale bars: d = 10 µm; scale bar for d applies to i–k and k–t.

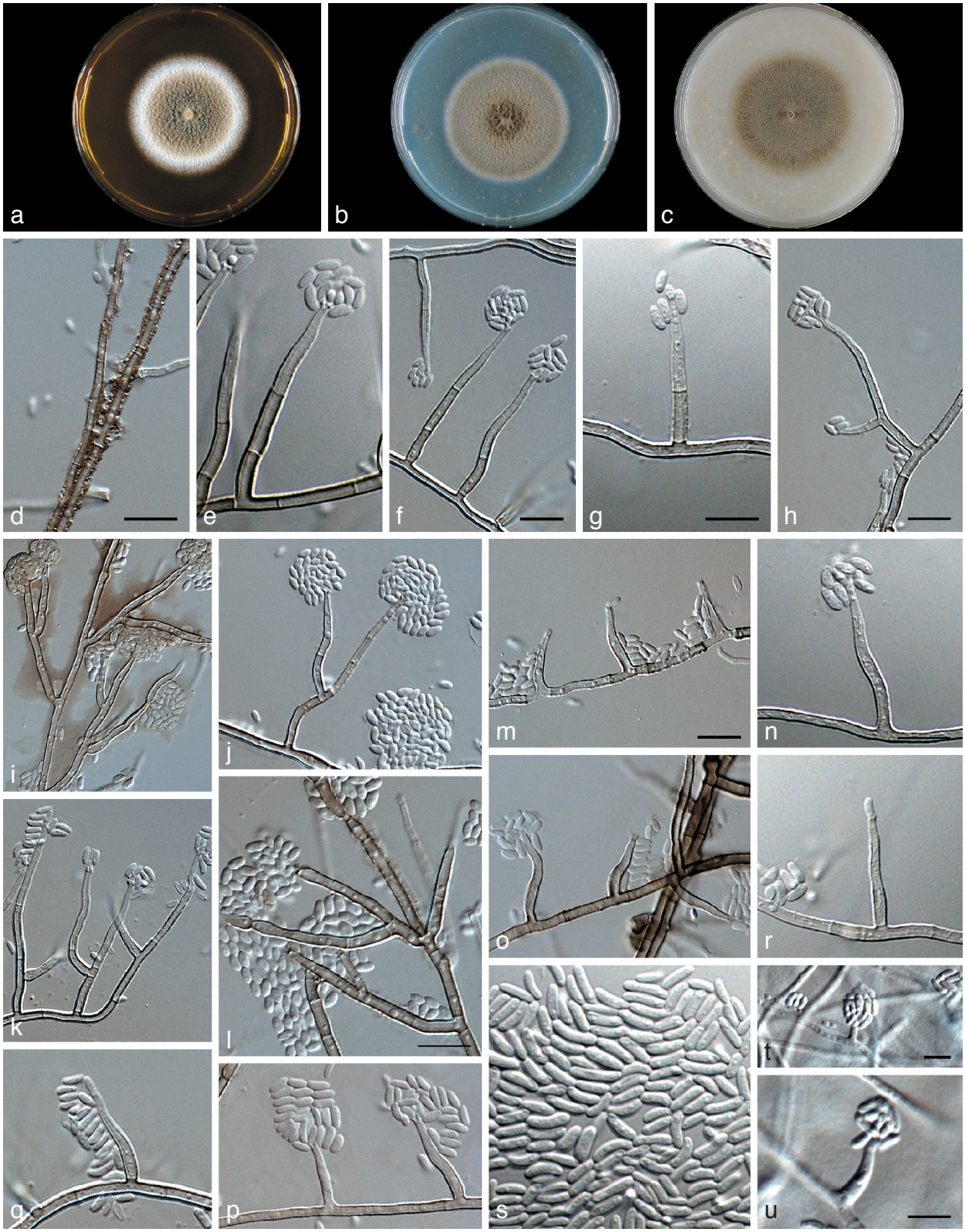


Fig. 9 *Phaeoacremonium sicilianum*. a–c. Sixteen days old colonies on 2 % MEA (a), PDA (b) and OA (c). — d–s. Aerial structures on 2 % MEA; d. mycelium showing prominent exudate droplets observed as warts; e–h. single conidiophores; i–l. branched conidiophores; m–p. type I phialide; q. type II phialide; r. type III phialide; s. conidia. — t–u. Structures on the surface of and in 2 % MEA: adelophialides with conidia; all from CBS H-20118 (holotype); d–u: DIC. — Scale bars: d–u = 10 µm; scale bar for d applies to e and i–k; bar for n applies to o–r.

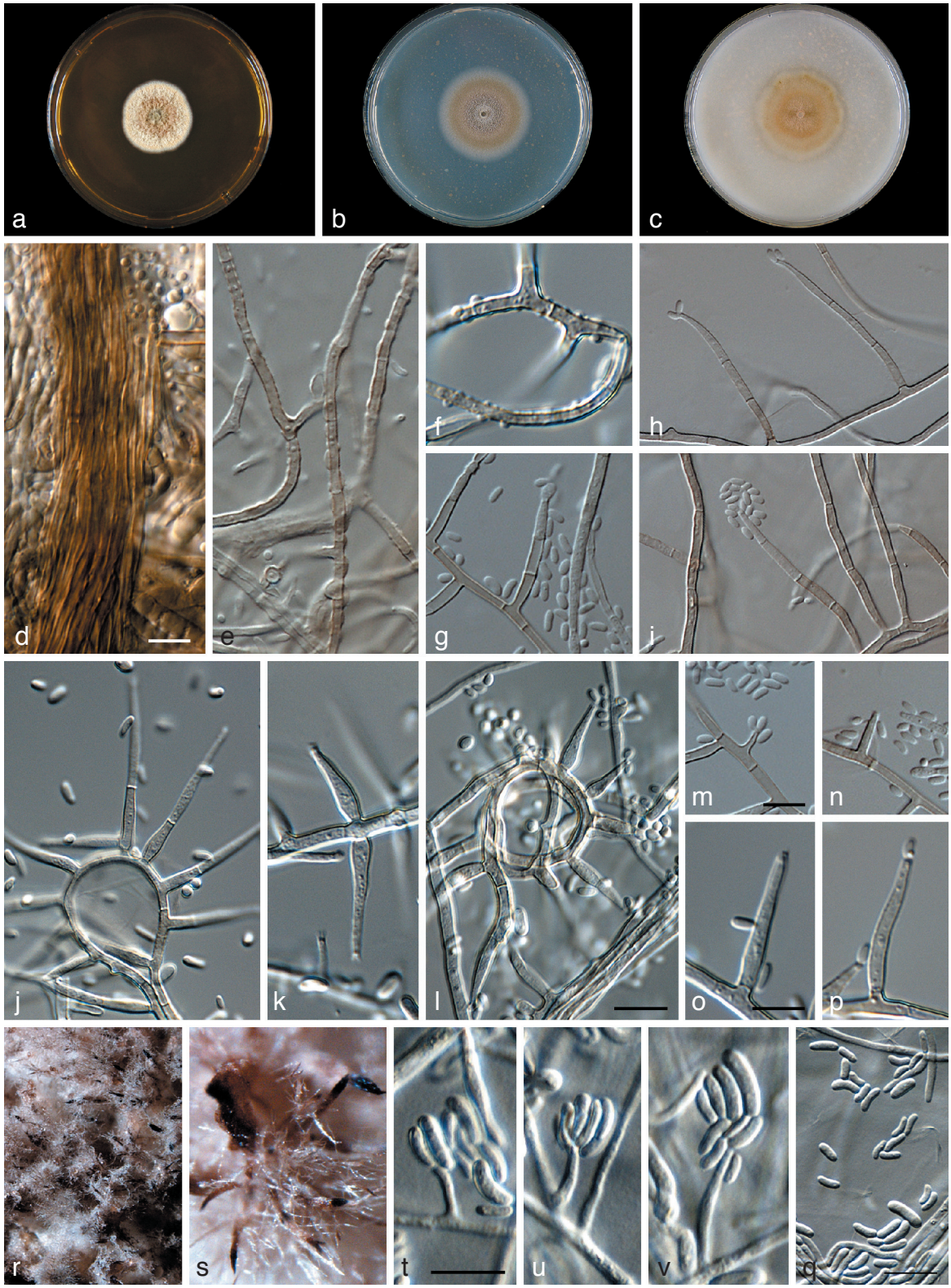


Fig. 10 *Phaeoacremonium tuscanum*. a–c. Sixteen days old colonies on 2 % MEA (a), PDA (b) and OA (c). — d–q. Aerial structures on 2 % MEA; d. mycelium occurring in bundles of up to nine; e, f. mycelium showing prominent exudate droplets observed as warts; g–j. single conidiophores; k, l. type II phialide; m, n. type I phialide; o, p. type III phialide; q. conidia. — r–v. Structures on the surface of and in 2 % MEA; r, s. coremium-like structures; t–v. adelophialides with conidia; all from CBS H-20118 (holotype); r, s: DM; d–q: DIC. — Scale bars: d–v = 10 µm; scale bar for d applies to e–k and p–s; bar for m applies to n; bar for t applies to u–v.

Phaeoacremonium rubrigenum W. Gams, Crous & M.J. Wingf., Mycologia 88: 795. 1996. — Fig. 8

Aerial structures in vitro on MEA: *Mycelium* consisting of branched, septate hyphae that occur singly or in bundles of up to 8; tuberculate with warts up to 1 µm wide, subhyaline to pale brown, smooth to verrucose, 1.5–2.5 µm wide. *Conidiophores* mostly of medium length, arising from aerial or submerged hyphae, branched, occasionally unbranched, each branched conidiophores often ending in a single terminal phialide, occasionally also with lateral phialides; erect, up to 6-septate, subhyaline to pale brown, paler towards the tip, smooth to verrucose, (18–)27–32(–48) (av. 29) µm long and (1.5–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, pale brown to subhyaline; collarettes, 2–3 µm long, 1–1.5 µm wide; type II phialides most common, type I phialides subcylindrical, or elongated ampulliform, attenuated at the base, or constricted, (5–)8–11(–15) × (1–)2(–2.5) (av. 9 × 2) µm; type II phialides mostly subulate, some navicular, tapering towards the apex, (10–)15–18(–20) × (2–)2.5(–3) (av. 17 × 2.5) µm; type III phialides rarely present, subulate, tapering towards the apex, (25–)26(–28) × (2–)2.5(–3) (av. 26 × 2.5) µm. *Conidia* hyaline, mostly ellipsoidal, some cylindrical, (3–)3.5–4(–6.5) × (1–)1.5(–2) (av. 4 × 1.5) µm, L/W = 2.6. On surface or submerged in the agar: *Phialides* hyaline, mostly navicular, tapering towards the apex, some cylindrical, (2–)5–8(–12) × (1.5–)2(–3) (av. 9 × 2) µm. *Conidia* hyaline, allantoid or subcylindrical, (3–)4.5–5.5(–8) × 1.5(–2) (av. 5 × 1.5) µm, L/W = 3.3.

Cultural characteristics — Colonies reaching a radius of 9 mm after 8 d at 25 °C. Minimum temperature for growth 12 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, cottony to woolly, with entire margin; after 16 d, beige, whitish towards the margin above, brown to orange in reverse. Colonies on PDA flat, appressed, woolly to powdery, with entire edge, after 16 d, colonies brown to vinaceous-white towards the margin above, brown to violet in reverse. Colonies on OA flat, felty, with entire margin, after 16 d, brown to greyish sepia above, pale purplish grey in reverse.

Substrate — Human, *Vitis vinifera*.

Known distribution — USA, Croatia.

Specimen examined. Croatia, Šibenik, isolated from *Vitis vinifera* (cv. Lasina), showing necrosis and black streakings, June 2007, *B. Cvjetković*, CBS H-20121, culture ex-type CBS 123038.

Notes — Phylogenetically this isolate clusters with other strains of *Pm. rubrigenum*. It is morphologically different, however, in its predominance of branched conidiophores and in its beige colonies on MEA. In contrast, Mostert et al. (2006b) described colonies of *Pm. rubrigenum* as having usually unbranched conidiophores and being pink to purplish on MEA.

Phaeoacremonium sicilianum Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506949; Fig. 9

Phaeoacremonio parasitico et *P. inflatipedi* simile. Differt a *P. parasitico* hyphis magnis sine verrucis, et a *P. inflatipedi* phialidibus plerumque typorum I et II.

Etymology. Named after the island of Sicily, from where the species was collected.

Aerial structures in vitro on MEA: *Mycelium* consisting of branched, septate hyphae that occur singly or in bundles of up to 5; tuberculate with warts up to 1 µm diam, smooth to verruculose, medium to pale brown, 1.5–3 µm wide. *Conidiophores* mostly short and branched, occasionally unbranched, arising from aerial or submerged hyphae, erect, up to 4-septate, often bearing a terminal phialide and an additional lateral one, pale brown to subhyaline, paler towards the tip, smooth to verruculose, (15–)22–47(–68) (av. 35) µm long and (1.5–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, mostly subhyaline, occasionally pale brown, collarettes, 1.5–3 µm long, 1–2 µm wide; type I phialides, cylindrical to subcylindrical, tapering towards the apex and often widened at the base, (4–)9–12(–17) × (1–)2(–3) (av. 9 × 2) µm; type II phialides subulate, subcylindrical, tapering towards the apex, (9–)15–18(–23) × (1.5–)2.5(–3) (av. 18 × 2.5) µm; type III phialides subcylindrical, navicular, (20–)23–27(–28) × 2(–2.5) (av. 25 × 2) µm. *Conidia* hyaline, mostly allantoid, subcylindrical (3–)4–6(–10) × 1.5–2(–2.5) (av. 5 × 2) µm, L/W = 2.5. On surface or submerged in the agar: *Phialides* hyaline, cylindrical to subcylindrical, (3–)4–13(–17) × 2.5 (av. 6 × 2) µm. *Conidia* hyaline, mainly allantoid, some subcylindrical (3.5–)6–8(–11) × 1.5(–2) (av. 7 × 2) µm, L/W = 3.5.

Cultural characteristics — Colonies attained a radius of 12 mm after 8 d at 25 °C. Minimum temperature for growth 15 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, cottony, with entire margin; after 16 d, pale greyish sepia to beige towards the edge above, brown to pale orange in reverse. Colonies on PDA flat, cottony to woolly, with entire edge, after 16 d, colonies pale brown to smoke-grey above, olivaceous-grey in reverse. Colonies on OA flat, felty to powdery, with entire margin; after 16 d, smoke-grey to pale olivaceous above, olivaceous-grey to pale mouse-grey in reverse.

Substrate — *Vitis vinifera*.

Known distribution — Italy.

Specimen examined. Italy, Sicily, Messina, San Filippo del Mela, isolated from the necrotic margins and brown to black streakings of branches and trunk of very old *Vitis vinifera* vines showing wood esca symptoms, May 2007, *L. Mugnai*, holotype CBS H-20118, culture ex-type CBS 123034.

Notes — DNA sequence analysis revealed this species to be basal to other species of *Phaeoacremonium.* Nevertheless, in terms of morphological characters, *Pm. parasiticum* and *Pm. inflatipes* are similar to *Pm. sicilianum* in the predominance of branched conidiophores. *Phaeoacremonium parasiticum* is distinct from *Pm. sicilianum* by virtue of its dark brown hyphae, and large hyphal warts of up to 3 µm diam, while *Pm. sicilianum* can be distinguished from *Pm. inflatipes* by the predominance of type I and II phialides, in comparison with the predominance of phialide type III in *Pm. inflatipes*. Differences in colony colour also distinguish *Pm. sicilianum* from *Pm. inflatipes.*

Phaeoacremonium tuscanum Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506950; Fig. 10

Phaeoacremonio iraniano phylogenetice simile, sed structuris coremioidibus fertilibus in agaro MEA et phialidibus plerumque typi II.

Etymology. Named after Tuscany, Italy, where this fungus was collected.

Aerial structures in vitro on MEA: *Mycelium* composed of branched, septate hyphae that occur singly or in bundles of up to 22; tuberculate with warts up to 1 µm diam, smooth to verruculose, medium brown to subhyaline and 1.5–2.5 µm wide. *Conidiophores* mostly short and usually unbranched, occasionally branched, arising from aerial or submerged hyphae, straight, simple, up to 3-septate, usually bearing one terminal phialide, pale brown to subhyaline, paler towards the tip, smooth to verruculose, (13–)25–30(–40) (av. 28) µm long and (1.5–)2(–2.5) (av. 2) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, pale brown to hyaline, collarettes, 1.5–3 µm long, 1–1.5 µm wide; type II phialides predominant, type I phialides subcylindrical, occasionally widened at the base, (4–)9–11(–17) × (1.5–)2(–2.5) (av. 10.5 × 2) µm; type II phialides subulate, navicular, or subcylindrical, attenuated at the base and tapering towards the apex, (8–)13–15(–20) × 1.5–2(–3) (av. 14 × 2) µm; type III phialides subcylindrical, subulate (20–)21–23(–25) × 2(–2.5) (av. 22 × 2) µm. *Conidia* hyaline, mostly allantoid, subcylindrical or cylindrical, ellipsoidal (2.5–)4(–5.5) × (1.5–)2 (av. 4 × 2) µm, L/W = 2. On surface or submerged in the agar: *Phialides* hyaline, subcylindrical, 4–9(–14) × 1(–2) (av. 6 × 1) µm. *Conidia* hyaline, subcylindrical or allantoid, 2.5–5(–8.5) × (1–)2(–3) (av. 5 × 2) µm, L/W = 2.5.

Cultural characteristics — Colonies attaining a radius of 8 mm after 8 d at 25 °C. Minimum temperature for growth 12 °C, optimum 33 °C, maximum 37 °C. Colonies on MEA flat, cottony, with entire margin; after 16 d, colonies pale brown to beige towards the edge above, pale orange in reverse. Colonies on PDA flat, short, woolly to felty, with entire edge, after 16 d colonies brown to beige, pale greyish orange and whitish towards the margin above, pale greyish sepia in reverse, becoming whitish towards the edge. Colonies on OA flat, felty, with entire margin; after 16 d pale orange to yellow towards the margin above, same in reverse, producing yellow pigmentation in the agar.

Substrate — *Vitis vinifera*.

Known distribution — Italy.

Specimen examined. Italy, Tuscany, San Gimignano, isolated from the margin of necrosis in the trunk of *Vitis vinifera* sampled from an about 100 yr old vineyard that showed wood esca symptoms, March 2007, *L. Mugnai*, holotype CBS H-20118, culture ex-type CBS 123033.

Notes — According to the phylogenetic analysis, *Pm. tuscanum* can be considered as a sister clade to *Pm. iranianum.* However, it can be distinguished from it by the production of coremium-like structures on MEA. These are fertile, erect hyphal bundles up to 2 mm tall and 1 mm wide, dark to pale brown, composed of conidiophores bearing conidia at the apex. Type II phialides are predominant in *Pm. tuscanum*. By comparison, *Pm. iranianum* lacks these structures and has a predominance of type III phialides.

DISCUSSION

The correct identification of fungi involved in diseases within the esca complex is a crucial precondition for the conduct of meaningful studies on the epidemiology of these destructive diseases of grapevine. Epidemiological studies will be especially important in the design of control strategies, since no fully effective chemical or biological control measures exist for this disease complex.

Several species of *Phaeoacremonium* have already been attributed to the grapevine diseases within the esca complex worldwide. However, the identity, distribution and frequency of the *Phaeoacremonium* species involved in many grapevine-growing areas, especially the area where *Vitis vinifera* evolved, have not yet been studied. The present study has included a wide collection of *Phaeoacremonium* isolates from Italy, mainly from isolated locations like Sardinia and Sicily where farmers grow local grape varieties. However, we also included a diverse set of isolates from other countries.

Integration of morphology, cultural characters and DNA sequence data revealed the presence of 13 *Phaeoacremonium* species in the areas sampled. Phylogenetic analyses of ACT and TUB sequences revealed that four of these species were novel. It is of interest to notice that the four new species here described were isolated from very old vines growing in Italy, Hungary and Croatia. Old vines were included in this study as a source of esca tracheomycotic fungi with the specific objective of gathering a population of both genera, *Phaeomoniella* and *Phaeoacremonium*, showing an as wide as possible variability within the population of the two fungi. Here are reported the results so far obtained in *Phaeoacremonium*.

Morphological traits such as presence or absence of hyphal warts, size of warts, conidiophore structure and cultural characteristics have been shown to be useful in species identification. For example, *Pm. parasiticum* can easily be distinguished from other species based on the occurrence of hyphal warts that are up to 3 µm diam (Mostert et al. 2006b). Some other species such as *Pm. inflatipes* and *Pm. sphinctrophorum* have frequently branched conidiophores, which can be used to distinguish them from species with short and infrequently branched or unbranched conidiophores. Nevertheless, species delimitation in this genus solely based on morphological and cultural characteristics has proven to be difficult. This difficulty is mainly inherent to the overlapping nature of morphological characters among the different species. Hence, DNA sequence data analysis remains of the utmost importance for complete and reliable species delineation. Mostert et al. (2006b) developed a multiple-entry polyphasic identification key for *Phaeoacremonium* species. This tool combines DNA sequence data, with different morphological and cultural characters to identify up to 22 *Phaeoacremonium* species. The four new species described in this study can be distinguished from the existing *Phaeoacremonium* species based on a combined cultural, morphological and DNA sequence dataset. *Phaeoacremonium tuscanum* clusters as sister clade to *Pm. iranianum.* However, it can be distinguished from *Pm. iranianum* by the production of coremium-like structures on MEA, consisting of erect, fertile hyphal bundles up to 2 mm high and 1 mm wide.

Two of the other new species described in this study, *Pm. hungaricum* and *Pm. croatiense*, cluster as sister clade to *Pm. mortoniae* and can only be distinguished by minute morphological differences. *Phaeoacremonium hungaricum* is distinct from *Pm. mortoniae* by the rare presence of conidio-

phores which are mostly reduced to conidiogenous cells or phialides. In fact, the aerial mycelium is composed of mostly elongated, ampulliform phialides, whereas *Pm. croatiense* can be differentiated from *Pm. mortoniae* based on cultural characters such as its olivaceous-grey colonies on MEA and the absence of yellow pigment production on OA. It is not surprising that these three species have a similar morphology as they have a close phylogenetic affinity, suggesting that they have evolved from a common ancestor.

The newly described *Pm. sicilianum* is markedly distinct from the other known *Phaeoacremonium* species. DNA sequence analysis showed that this clade is basal to the others, even though the species overlaps morphologically with *Pm. parasiticum* and *Pm. inflatipes* in its predominance of branched conidiophores. *Phaeoacremonium parasiticum* differs from *Pm. sicilianum* in its dark brown hyphae and large hyphal warts up to 3 µm diam. *Phaeoacremonium sicilianum* differs from *Pm. inflatipes* in its predominance of type I and II phialides and in its colony colour.

Growth temperature studies carried out for new species described in this study showed that *Pm. tuscanum* has a maximum growth temperature at 37–40 °C, reaching a radius of 7 mm after 8 d. This finding is quite interesting in relation to the ecology of *Phaeoacremonium* species, as several thermotolerant *Phaeoacremonium* species are associated with phaeohyphomycosis in humans (Mostert et al. 2005). *Phaeoacremonium parasiticum*, *Pm. rubrigenum* and *Pm. inflatipes* (later identified as *Pm. alvesii*), were initially reported from human phaeohyphomycosis (Padhye et al. 1998, Guarro et al. 2003). *Phaeoacremonium krajdenii*, *Pm. parasiticum* and *Pm. venezuelense* cause phaeohyphomycosis and have also been isolated from grapevines showing esca symptoms (Mostert et al. 2005). Four *Phaeoacremonium* species have heretofore been isolated only from human infections: *Pm. amstelodamense*, *Pm. griseorubrum*, *Pm. rubrigenum* and *Pm. tardicrescens* (Mostert et al. 2005). The present study revealed that *Pm. alvesii*, *griseorubrum* and *Pm. rubrigenum* are also associated with grapevine. This finding further supports the hypothesis that human pathogenic *Phaeoacremonium* species may have originated from woody host plants. The same hypothesis has been proposed for some other groups of human opportunistic pathogens such as the black yeasts (de Hoog et al. 2007). To confirm this hypothesis, studies are needed inoculating strains from woody hosts into an animal model, and strains from human cases into woody plants.

Although several species of *Phaeoacremonium* are associated with necrosis and discolorations in grapevine wood, *Pm. aleophilum* is the most common species associated with foliar symptoms. It appears also to be the most widely distributed species (Crous et al. 1996, Larignon & Dubos 1997, Mugnai et al. 1999, Tegli et al. 2000, Mostert et al. 2006a) in grapevine. Our data were consistent with this pattern. *Phaeoacremonium aleophilum* was the most frequently isolated species with an incidence up to 80 % in all the samples examined. Inoculation studies have proven this species to be pathogenic on grapevines, showing that *Pm. aleophilum* can cause brown wood streaking (Adalat et al. 2000, Sparapano et al. 2000b, Feliciano et al. 2004, Halleen et al. 2005) and reduced shoot growth (Gubler et al. 2001), as well as esca symptoms on leaves and berries (Sparapano et al. 2000a, Feliciano et al. 2004).

A recent study has shown the occurrence of several novel *Phaeoacremonium* species on other woody hosts such as species in the genus *Prunus* (Damm et al. 2008). However, on grapevine the pathological relevance of the other *Phaeoacremonium* species as well as of the novel species described in this study remains to be determined.

The prevalence of the other 12 *Phaeoacremonium* species differs among grapevine growing areas. *Phaeoacremonium griseorubrum*, *Pm. scolyti* and *Pm. viticola* represent new records for Italy, *Pm. mortoniae* and *Pm. rubrigenum* for Croatia, *Pm. alvesii* for Turkey and *Pm. mortoniae* for Hungary. Other species that have also been isolated in relatively high frequencies from the different grapevine growing areas studied here include *Pm. iranianum* and *Pm. mortoniae*.

Where known, *Phaeoacremonium* species have teleomorphs in the genus *Togninia* (Diaporthales, Togniniaceae). Several researchers have induced the teleomorph of *Phaeoacremonium* species by crossing complementary strains in vitro (Mostert et al. 2005, 2006b, Damm et al. 2008). Our attempts to induce the teleomorph for the species described in this paper were unsuccessful.

Given the occurrence of various *Phaeoacremonium* species on grapevine and the involvement of some of those species in human infections, accurate identification is critical. Since, as mentioned, morphological identification is not always reliable, robust molecular-based detection tools are of great help in species detection and identification. As mentioned, an existing multiplex PCR assay based on use of species-specific TUB and ACT primers can identify 22 species of *Phaeoacremonium* (Mostert et al. 2006b). It is necessary to test these primer sets on DNA from the new species described here to determine if target specificity remains intact. The ACT and TUB gene barcodes generated in the present study can be used to develop species-specific molecular detection tools that will facilitate ecological and epidemiological studies.

Acknowledgements The work of Salwa Essakhi, PhD student of the University of Florence, was in part financially supported by the European Commission's Research Infrastructure Action via the SYNTHESYS Project (NL-TAF-4099), which is gratefully acknowledged. For providing material, we warmly acknowledge Salvatorica Serra from Sardinia, Giovanni Raiti, Pietro Rinaldi and Filippo Trovato from Sicily, Terézia Dula Bencene from Hungary, Bodgan Cvjetković from Croatia, Pietro Tsicopoulos and Dimitri Dimou from Greece, Saygili Hikmet from Turkey and Lisa van der Water from the USA. The first author thanks Dr Mahdi Arzanlou for technical help and scientific discussions. We also thank Tamara Cinelli for helping in the fungal isolation, Marjan Vermaas for preparing the photographic plates and Arien van Iperen for helping with the drying of culture specimens. The Italian part of the research study was commissioned from ARSIA-Toscana (Regional Agency for Development and Innovation in Agriculture and Forestry) by 14 administrative regions and one autonomous province, and financed with funds provided by the Ministero per le Politiche Agricole e Forestali (Ministry for Agriculture and Forestry Policy) to implement the inter-Regional Project 'Grapevine esca: research and experiment in the nursery and in the field for prevention and cure.'

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