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Novel *Phaeoacremonium* species associated with Petri disease and esca of grapevine in Iran and Spain

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Abstract: Eight *Phaeoacremonium* (*Pm.*) isolates from grapevines in Iran and Spain were studied with morphological and cultural characteristics as well as phylogenetic analyses of combined DNA sequences of the actin and β-tubulin genes. Two new species are described. *Pm. cinereum* was isolated from a young vine in Spain and from older vines in Iran and can be identified by its distinct gray colonies on malt extract agar, an optimum growth temperature of 25 C and subulate type III phialides. *Pm. hispanicum* was isolated only once from a young vine in Spain and can be identified by the common occurrence of percurrently rejuvenating phialides, an optimum growth temperature of 20 C and predominant type II phialides.

Key words: actin, β-tubulin

INTRODUCTION

The anamorph genus *Phaeoacremonium* W. Gams, Crous & M. J. Wingf. was established by Crous et al (1996) with *Phaeoacremonium parasiticum* W. Gams, Crous & M.J. Wingf (syn. *Phialophora parasitica* Ajello, Georg & C.J.K. Wang) designated as the type of the genus. Since 1996 a total of 29 species of *Phaeoacremonium* have been described (Crous et al 1996; Dupont et al 2000; Groenewald et al 2001; Mostert et al 2005, 2006; Réblová and Mostert 2007; Damm et al 2008; Essakhi et al 2008), of which 12 have been linked to *Togninia* teleomorphs (Mostert et al 2006, Réblová and Mostert 2007, Damm et al 2008).

Molecular characters have played an important role in the detection and identification of *Phaeoacremonium* species (Tegli et al 2000; Dupont et al 2002; Overton et al 2004, 2005; Mostert et al 2006; Aroca and Raposo 2007; Aroca et al 2008a). Species-specific primers based on the β-tubulin and actin genes have been developed (Mostert et al 2006) and can be used in multiplex polymerase chain reactions (PCR) for the identification of unknown isolates. A nested PCR as well as a real-time PCR were developed for the rapid detection and identification of some of the *Phaeoacremonium* species (Overton et al 2004, 2005; Aroca and Raposo 2007; Aroca et al 2008a).

Phaeoacremonium species have been associated with decline and dieback diseases of woody plants as well as infections in humans (Ajello et al 1974, Rumbos 1986, Guarro et al 2003, Di Marco et al 2004, Kubátová et al 2004, Hemashettar et al 2006). *Phaeoacremonium* strains from grapevines (*Vitis vinifera* L.) have been studied intensively because of the involvement of these taxa in two complex fungal diseases, namely Petri disease in young vines and esca in adult vines (Mostert et al 2006, Aroca et al 2008a). External symptoms of Petri disease include chlorotic foliage with necrotic margins, stunted shoot growth, retarded sprouting, absence of sprouting, wilting and dieback. Internal wood symptoms range from brown to black vascular streaking, visible in cross sections as spots or circular discoloration of the xylem tissue (Mostert et al 2006). Esca shows a range of symptoms from severe to mild or chronic. The severe form, known as apoplexy, is characterized by sudden wilting and shedding of the leaves from all or part of the infected vine and shriveling of all fruit (Mugnai et al 1999). Symptoms on chronically affected fruit appear as superficial brown to purple spots scattered over the surface of the berries (Eskalen et al 2007). Symptoms on leaves consist of interveinal regions of chlorotic and yellowish tissue that turn yellow-brown or red-brown, which also have been described as tiger stripes (Essakhi et al 2008).

To date 20 species of *Phaeoacremonium* have been isolated from grapevines. Four species were described based on morphological and cultural characteristics by Crous et al (1996), namely *Pm. aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai, *Pm. angustius* W. Gams, Crous & M.J. Wingf., *Pm. inflatipes* W. Gams, Crous & M.J. Wingf. and *Pm. parasiticum* (Crous et al 1996). Two additional species of *Phaeoacremonium*,

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Pm. viticola J. Dupont and *Pm. mortoniae* Crous & W. Gams, were described based on phenotypic characters, the internal transcribed spacer (ITS) regions 1 and 2, the 5.8S rDNA (Dupont et al 2000) and the β-tubulin gene (Groenewald et al 2001). Subsequent studies included the actin and calmodulin gene regions and led to the description of seven additional *Phaeoacremonium* species from grapevines that included *Pm. australiense* L. Mostert, Summerb. & Crous, *Pm. austroafricanum* L. Mostert, W. Gams & Crous, *Pm. iranianum* L. Mostert, Gräf., W. Gams & Crous, *Pm. krajdenii* L. Mostert, Summerb. & Crous, *Pm. scolyti* L. Mostert, Summerb. & Crous, *Pm. subulatum* L. Mostert, Summerb. & Crous and *Pm. venezuelense* L. Mostert, Summerb. & Crous (Mostert et al 2005, 2006). Four *Phaeoacremonium* species from grapevines recently were described and include *Pm. croatiense* Essakhi, Mugnai, Surico & Crous, *Pm. hungaricum* Essakhi, Mugnai, Surico & Crous, *Pm. sicilianum* Essakhi, Mugnai, Surico & Crous and *Pm. tuscanum* Essakhi, Mugnai, Surico & Crous (Essakhi et al 2008). Essakhi et al (2008) also first reported *Pm. alvesii* L. Mostert, Summerb. & Crous, *Pm. griseorubrum* L. Mostert, Summerb. & Crous and *Pm. rubrigenum* W. Gams, Crous & M.J. Wingf. on grapevines, previously known only from humans. Of the species occurring on grapevines *Pm. aleophilum* is the most common and widely distributed species (Crous et al 1996, Larignon and Dubos 1997, Mugnai et al 1999) and *Pm. parasiticum* is encountered frequently (Dupont et al 2002, Mostert et al 2006). The degree of involvement of most of these species in Petri disease and esca remains uncertain. Their pathological relevance on grapevines needs to be determined.

The aim of the present study was to characterize isolates of *Phaeoacremonium* collected from young and old vines in Iran and Spain that appeared to be morphologically and genetically different from known *Phaeoacremonium* species.

MATERIALS AND METHODS

Isolation.—Trunks and shoots of diseased grapevines in Iran and Spain were cut in sections (10 cm long), washed under running tap water, surface sterilized 1 min in 1.5% sodium hypochlorite solution and washed twice with sterile distilled water. Sections were split longitudinally, and small pieces of discolored tissues were plated on malt extract agar (MEA; 2% malt extract, Oxoid Ltd., England; 1.5% agar, Difco, USA) supplemented with 0.5 gL^{-1} streptomycin sulphate (Sigma-Aldrich, St Louis, Missouri) (MEAS). Cultures were incubated at 25–26 C in the dark 14–21 d, and all colonies were transferred to potato dextrose agar (2% PDA; Biokar-Diagnostics, Zac de Ther, France). *Phaeoacremonium* isolates were single-spored by means of the serial dilution method (Dhingra and Sinclair 1995) and stored in 15% glycerol solution at −80 C in 1.5 mL cryovials. Eight strains of *Phaeoacremonium* were obtained from young grapevine plants (< 7 y old), showing Petri disease symptoms in Spain, and from older vineyards (25–30 y), showing esca symptoms in Iran and were used in this study (TABLE I).

Isolates are deposited at Centraalbureau voor Schimmelcultures (CBS).

Morphological characterization.—Characters observed included conidiophore morphology, phialide type and shape, size of hyphal warts, conidial size and shape. Colony characters and pigment production on MEA, PDA (2% PDA; Biolab, Midrand, South Africa) and oatmeal agar (OA; 60 g oatmeal; 12.5 g agar; Difco, France) (Gams et al 2007) incubated at 25 C were noted after 8 and 16 d. Colony colors were determined with the color charts of Rayner (1970). Cardinal temperatures for growth were determined by incubating MEA plates in the dark at 5–40 C in 5 C intervals, also including 37 C, human body temperature. Radial growth was measured after 8 d at 25 C.

Microscopic observations were made from aerial mycelium of colonies cultivated on MEA or by using slide culture technique, as explained by Arzanlou et al (2007). Photos were captured with a Nikon camera system (Digital Sight DXM 1200, Nikon Corp., Japan). Structures were mounted in lactic acid, and 30 measurements (1000× magnification) were determined. The 5th and 95th percentiles were defined for all measurements with the extremes given in parentheses.

DNA isolation and amplification.—Fungal mycelium and conidia from pure cultures grown on PDA 2 wk at 25 C in the dark were scraped and mechanically disrupted by grinding to a fine powder under liquid nitrogen with a mortar and pestle. Total DNA was extracted with the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, USA) following the manufacturer's instructions. DNA was viewed on 0.7% agarose gels stained with ethidium bromide and stored at −20 C.

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 as described by Mostert et al (2006) using respectively primer sets T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) and ACT-512F and ACT-783R (Carbone and Kohn 1999).

PCR products of both primers were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Germany) and sequenced in both directions by the DNA Sequencing Service of the Universidad Politécnica de Valencia-CSIC. Sequences were edited with DNAman 4.03 (Lynnon BioSoft, Québec, Canada).

Phylogenetic analysis.—The sequences were aligned with MAFFT 6 sequence alignment program (Katoh and Toh 2008) followed by manual adjustments of the alignments in Sequence alignment editor 2.0a11 (Rambaut 2002). Reference sequences representing the known *Phaeoacremonium* species were obtained from GenBank and added to the alignment. The TUB and ACT alignments were concate-

TABLE I. Names, accession numbers and collection details of *Phaeoacremonium* isolates studied

Species	Accession number[1]	Location	*Vitis vinifera*	Age (years)	Collector, year of collection	GenBank accession numbers	
						TUB	ACT
Pm. cinereum sp. nov.	Pm-1	Abadeh, Fars, Iran	Askari[2]	25–30	H. Mohammadi, 2007	FJ517157	FJ517149
	Pm-2	Abadeh, Fars, Iran	Askari	25–30	H. Mohammadi, 2007	FJ517158	FJ517150
	Pm-3	Bavanat, Fars, Iran	Askari	25–30	H. Mohammadi, 2007	FJ517159	FJ517151
	Pm-4	Bavanat, Fars, Iran	Askari	25–30	H. Mohammadi, 2007	FJ517160	FJ517152
	Pm-5, CBS 123909	Kavar, Fars, Iran	Askari	25–30	H. Mohammadi, 2004	FJ517161	FJ517153
	Pm-6	Kavar, Fars, Iran	Askari	25–30	H. Mohammadi, 2004	FJ517162	FJ517154
	Pm-7	Olivenza, Badajoz, Spain	Syrah[3]	6	D. Gramaje, 2007	FJ517163	FJ517155
Pm. hispanicum sp. nov	Pm-8, CBS 123910	Yecla, Murcia, Spain	Monastrell[3]	5	D. Gramaje, 2008	FJ517164	FJ517156

[1] CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Diversity Centre, Utrecht, The Netherlands.
[2] Rootstock cultivar.
[3] Scion cultivar.

nated to make it possible to perform a combined analysis. Phylogenetic analyses of the aligned sequence data were performed with PAUP (phylogenetic analysis using parsimony) 4.0b10 (Swofford 2003). Alignment gaps were treated as missing data, and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. In parsimony analyses alignment gaps were treated as missing data and all characters were unordered and of equal weight.

Maximum parsimony analysis was performed with the heuristic search option with 10 random simple taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm with the option of saving no more than 10 trees with a score greater than or equal to 5 (Harrison and Langdale 2006). Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis and 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 1.6.6 (Page 1996). Sequences derived in this study were lodged at GenBank (TABLE I) and the alignment and phylogenetic tree in TreeBASE (www.treebase.org). *Pleurostomophora richardsiae* (ACT = AY579271, TUB = AY579334) and *Wuestneia molokaiensis* (ACT = AY579272, TUB = AY579335) were used as outgroups in phylogenetic analyses.

Bayesian analyses was conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The program MrModeltest (J.J.A. Nylander, available at www.ebc.uu.se/systzoo/staff/nylander.html) was used for selecting the optimal model of sequence evolution for each clade alignment. The likelihood and prior settings were changed in MrBayes according to the models found with MrModeltest for each partition. Markov chains were initiated from a random tree and run 2 000 000 generations, keeping one out of every 100 generations. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. The average deviation of split frequencies was 0.0173, and the potential scale reduction factors (PSRF) were one for each of the parameters. The first 50 000 generations (burn-in) were discarded. The remaining samples were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches.

RESULTS

Phylogenetic analysis.—The partition homogeneity test (*p*-value = 0.368) led us to combine the TUB and ACT datasets (528 characters in dataset 1260 in dataset 2). Eight isolates were used for phylogenetic analysis with 66 further sequences added from GenBank. The data contained 905 characters including gaps, of which 496 were parsimony informative, 128 were variable and parsimony uninformative and 281 were constant. After a heuristic search 42 most parsimonious trees with the same overall topology were retained (Length = 2477 steps, CI = 0.491, RI = 0.834, RC = 0.410) (one of which is shown in FIG.1). Isolates Pm-1, Pm-2, Pm-3, Pm-4, Pm-5, Pm-6 and Pm-7 formed a well supported clade with 100% bootstrap support and probability of 1.00. Isolate Pm-8 clustered with *Togninia vibratilis* (100% bootstrap

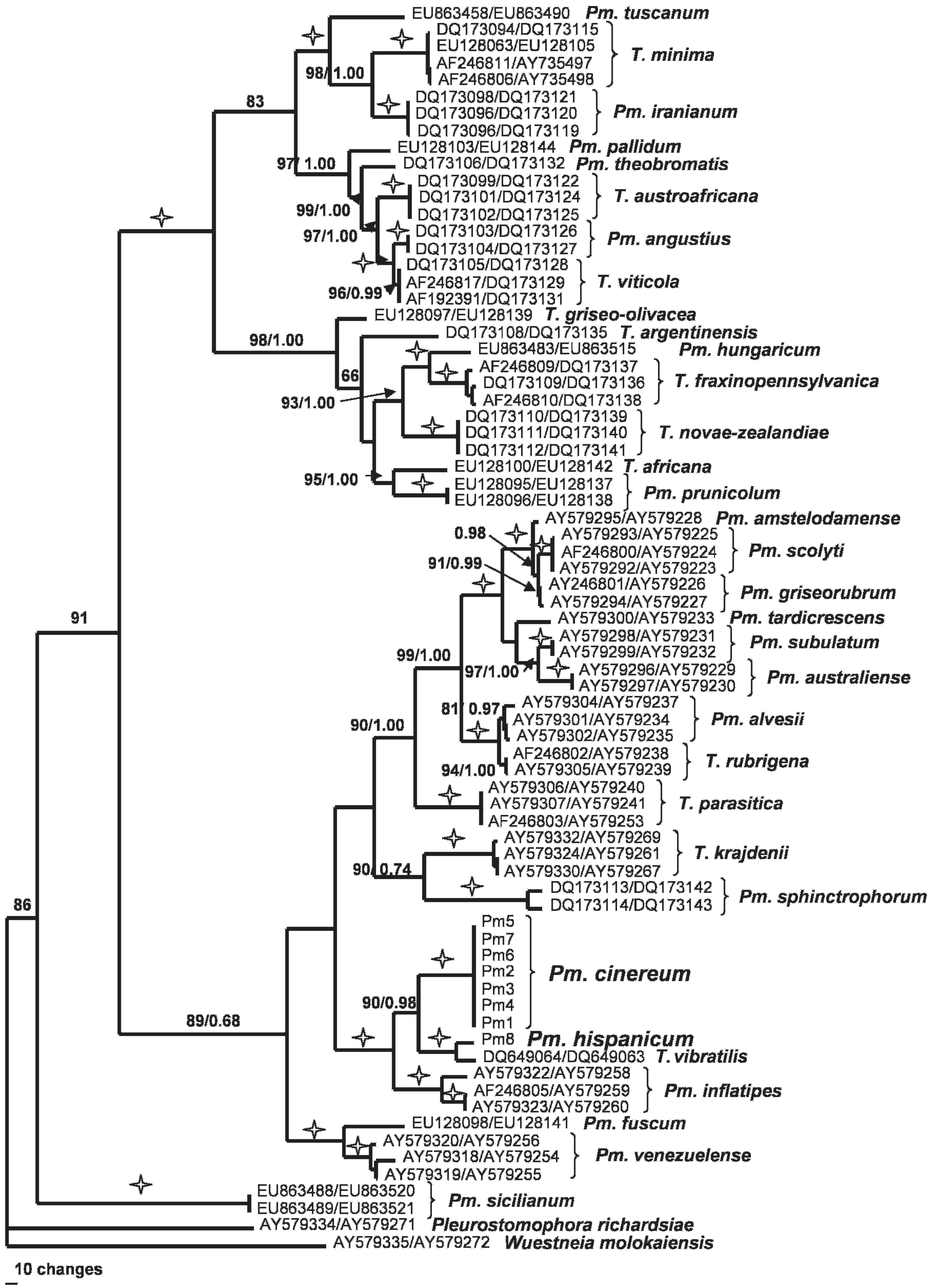

FIG. 1. One of 42 most parsimonious trees obtained from heuristic searches of a combined alignment of the TUB and ACT gene sequences (Length = 2477 steps, CI = 0.491, RI = 0.834, RC = 0.410). Bootstrap support values above 65% are shown at the nodes as well as the probability values. *Pleurostomophora richardsiae* and *Wuestneia molokaiensis* were used as outgroups. Accessions numbers of sequences obtained from the GenBank nucleotide database are indicated on the tree in the format TUB/ACT. The star-like symbol indicates bootstrap support values of 100% and posterior probability values of 1.00 obtained for a node.

support and 1.00 probability) but was different from it with 19 nucleotides in the beta tubulin gene and seven nucleotides in the actin gene. Both these clades clustered well with *Pm. inflatipes* (100% bootstrap support and 1.00 probability).

TAXONOMY

The eight strains of *Phaeoacremonium* fell into two distinct new taxa, according to DNA sequence analyses and morphological characters, and are described below. Mostert et al (2006) developed a polyphasic, online identification system for species recognition (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx). This key has been updated to include the two new taxa described in this study.

Phaeoacremonium cinereum D. Gramaje, H. Mohammadi, Z. Banihashemi, J. Armengol & L. Mostert, sp. nov. FIG. 2
MycoBank 513286

Phaeoacremonio fusco simile, sed lentius crescens, phialidibus praecipue typi III formatis et coloniis olivaceo-bubalinis in agaro farina avenae confecto differt.

Etymology. Named after the smoke gray colony of this species on MEA.

Aerial structures in vitro on MEA.—*Mycelium* consisting of branched, septate hyphae that occurs singly or in bundles of up to 8; hyphae tuberculate with warts up to 2 µm diam, verruculose to smooth, medium brown to pale brown and 2–3 µm wide. *Conidiophores* mostly short, usually unbranched, arising from aerial or submerged hyphae, erect to flexuous, up to 6-septate, in addition to the terminal phialide a second one at the apical septum, medium brown to pale brown, verruculose on the lower part, (17–)22–48(–71) ($\bar{x}$ = 34) µm long and (2–)2.5–4($\bar{x}$ = 2.9) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, hyaline, collarettes, 1.5–2.5 µm long, 1–1.5 µm wide; type I phialides mostly navicular, some elongate-ampulliform and attenuated at the base, 2–7.5(–8) × 1.5–3($\bar{x}$ = 5 × 2) µm; type II phialides cylindrical to subcylindrical, 10–15 × 2–3(–3.5) ($\bar{x}$ = 13 × 2.5) µm; type III phialides most predominant, mostly subcylindrical, some subulate, 16–26(–29) × 2–3.5(–4) ($\bar{x}$ = 20.5 × 2.5) µm. *Conidia* hyaline, mostly oblong ellipsoidal, (3.5–)4–6 × 1.5–3 ($\bar{x}$ = 5.5 × 2) µm, L/W = 2.6.

On surface or submerged in the agar.—*Phialides* hyaline, mostly cylindrical, (3–)3.5–10 × 1–1.5 ($\bar{x}$ = 5.5 × 1) µm. *Conidia* hyaline, mostly allantoid, few reniform, 4–8(–10) × 1–1.5(–2) ($\bar{x}$ = 6 × 1) µm, L/W = 4.7.

Cultural characteristics.—Colonies reaching a radius of 8.5–11 mm after 8 d at 25 C. Minimum temperature for growth 15 C, optimum 25 C, maximum 35–37 C. Colonies on MEA flat, felt-like, with entire margin; after 8 d, smoke gray (21′′′′f) above, olivaceous black (21′′′′m) to gray olivaceous (21′′′′i) in reverse; after 16 d pale olivaceous gray (21′′′′′f) to smoke gray (21′′′′d) above, olivaceous black (21′′′′m) to gray olivaceous (21′′′′i) in reverse. Colonies on PDA flat, felt-like, with entire margin; after 8 d, white to buff (19′′d) above, honey to buff (19′′d) in reverse; after 16 d buff (21′′d) to olivaceous buff (19′′′d) above, olivaceous buff (21′′′d) to greenish olivaceous (23′′′b) in reverse. Colonies on OA flat, felt-like with few woolly tufts near the center, with entire margin; after 8 d, greenish gray (33′′′′b) to glaucous gray (31′′′′d); after 16 d buff (21′′d) to olivaceous buff (19′′d) above.

Substrate.—*Vitis vinifera.*

Known distribution.—Iran, Spain.

HOLOTYPE: IRAN, FARS, Kavar, isolated from Askari rootstock of *Vitis vinifera* showing esca symptoms in 2004, *H. Mohammadi*, Pm-5, herb. CBS H-20216 dried MEA colony (ex-type culture CBS 123909).

Specimens examined: IRAN, FARS, Abadeh, Bavanat and Kavar, isolated from Askari rootstock of *Vitis vinifera* showing esca symptoms in 2004 and 2007, *H. Mohammadi*, Pm-1 herb. CBS H-20212, Pm-2 herb. CBS H-20213, Pm-3 herb. CBS H-20214, Pm-4 herb. CBS H-20215, Pm-6 herb. CBS H-20217. SPAIN, BADAJOZ, Olivenza, isolated from black streaks in shoots of *Vitis vinifera* showing Petri disease symptoms, Sep 2007, *D. Gramaje*, Pm-7, herb. CBS H-20218.

Notes.—*Pm. cinereum* can be distinguished by the distinct gray colonies on MEA. Other *Phaeoacremonium* species that form similar colonies of similar color include *Pm. fuscum* (dark mouse gray), *Pm. prunicolum* (olivaceous gray) and *T. griseo-olivac*ea (mouse gray). *Pm. fuscum* grows faster (13.5–14 mm after 8 d) than *Pm. cinereum*, and *Pm. prunicolum* and *T. griseo-olivac*ea have lower minimum growth temperature (10 C) than *Pm. cinereum. Pm. cinereum* also can be identified by its subulate type III phialides, similar to those found in *Pm. subulatum.*

Phaeoacremonium hispanicum D. Gramaje, J. Armengol & L. Mostert, sp. nov. FIG. 3
MycoBank 513287

Togninae vibratili affine, sed conidiophoris identidem percurrenter proliferentibus, tribus typis phialidum formatis, et temperature maxima crescentiae 37 C differ.

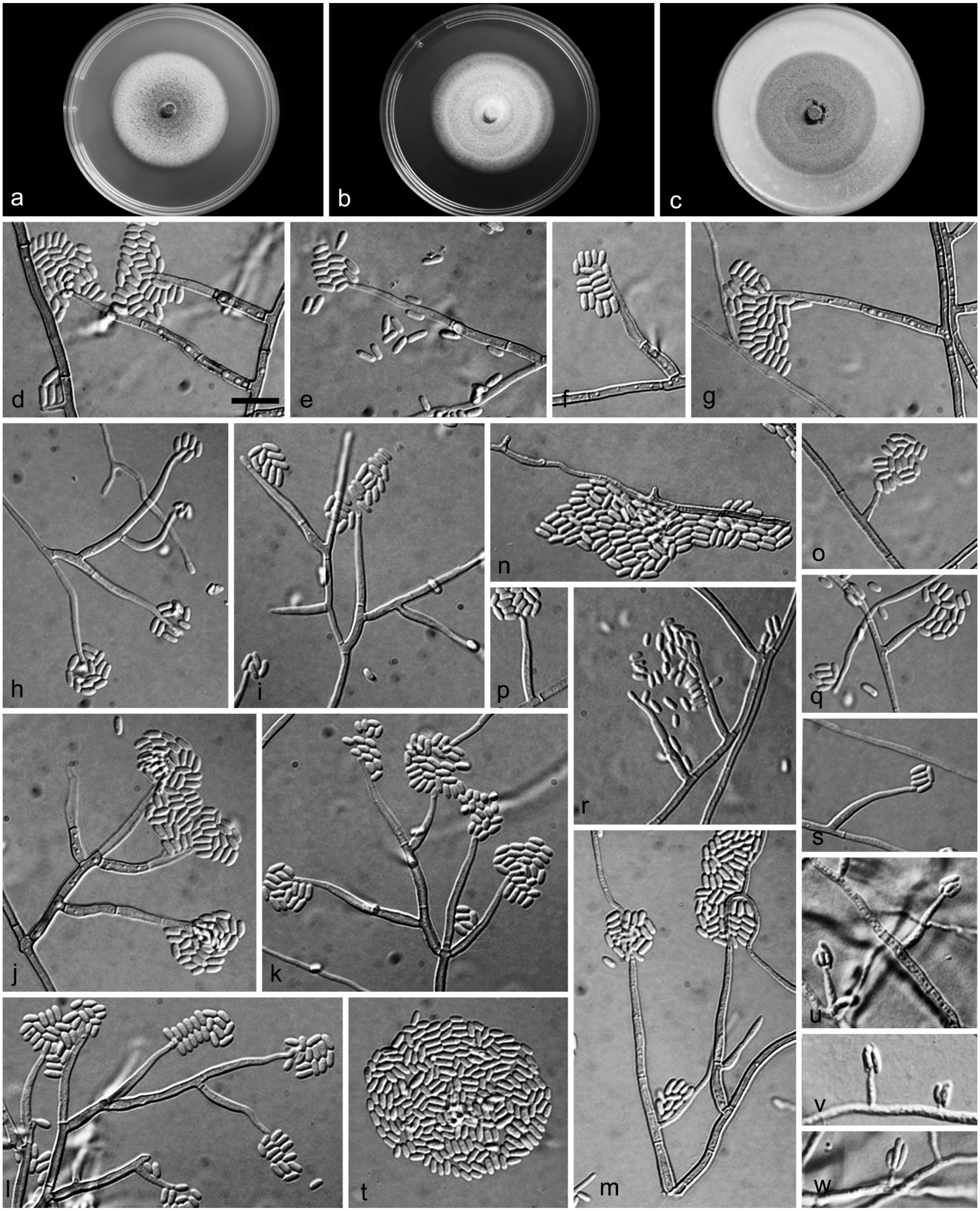

FIG. 2. *Phaeoacremonium cinereum.* a–c. Sixteen-day-old colonies on MEA at 25 C (a), PDA (b) and OA (c). d–t. Aerial structures on MEA; d–g. Single conidiophores; h–m. Branched conidiophores; n–o. Type I phialides; p–q. Type II phialides; r–s. Type III phialides; t. Conidia. u–w. Structures on the surface of and in MEA; phialides and adelophialides with conidia. Bars: d–w = 10 μm.

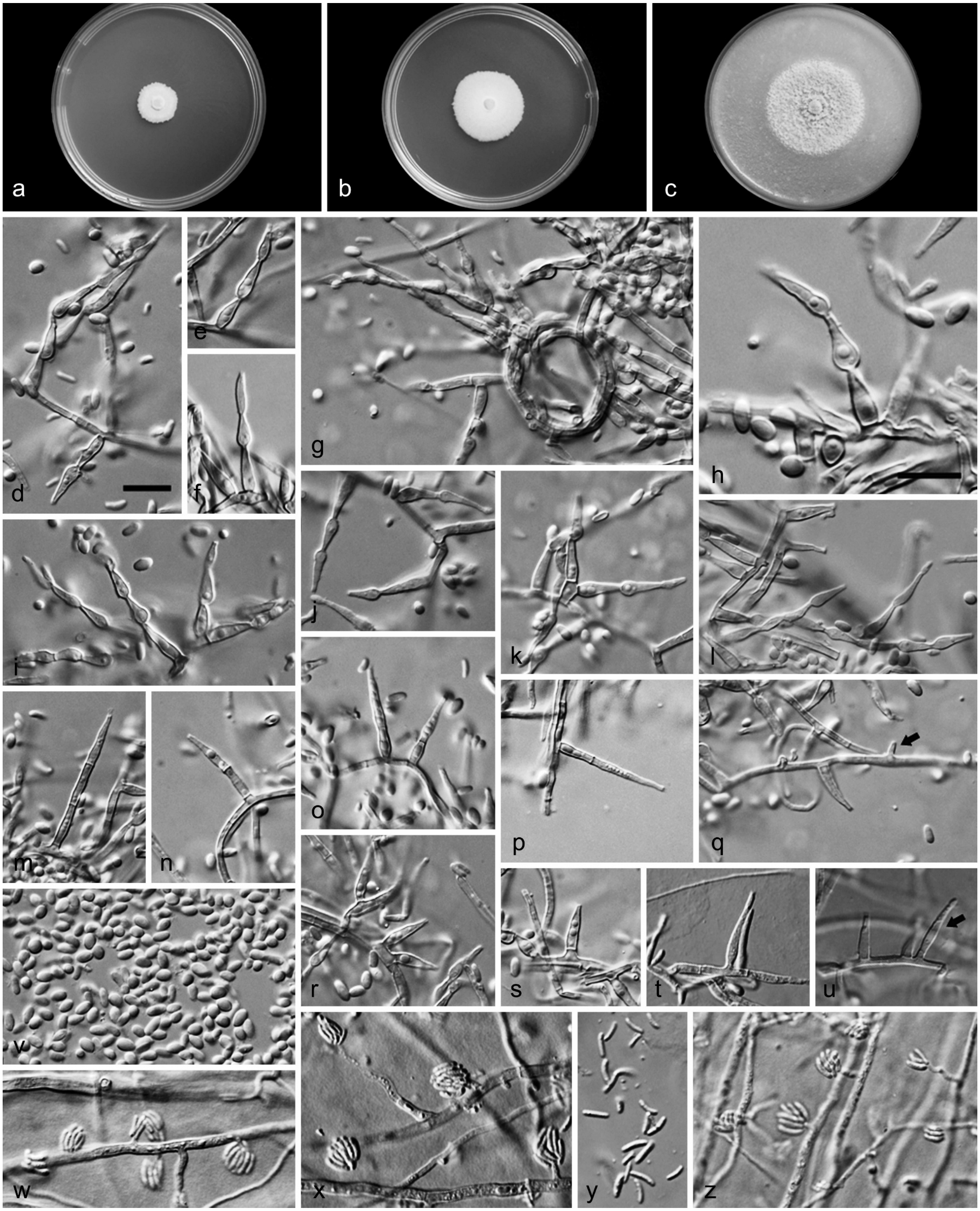

FIG. 3. *Phaeoacremonium hispanicum.* a–c. Sixteen-day-old colonies on MEA at 25 C (a), PDA (b) and OA (c). d–v. Aerial structures on MEA; d–l. Conidiophores showing percurrent rejuvenation; m–p. Single conidiophores; q. Type I phialide (indicated by arrow); r–s. Type II phialides; t–u. Type III phialides (indicated by arrow); v. Conidia. w–z. Structures on the surface of and in MEA; phialides and adelophialides with conidia. Bars: d–z = 10 μm.

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

Aerial structures in vitro on MEA.—Mycelium consisting of branched, septate hyphae that occurs singly or in bundles of up to 7; tuberculate with warts up to 1 μm diam, pale orange-brown, mostly smooth, 2–3 μm wide. *Conidiophores* mostly short, usually unbranched, arising from aerial or submerged hyphae, erect to slightly flexuous, up to 6-septate, often inflated at the base, light brown, smooth, percurrent rejuvenation common, (17–)19.5–45(–49) ($\bar{x}$ = 31) μm long and (2–)2.5–4 ($\bar{x}$ = 3.2) μm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, hyaline, collarettes, 1.5–2 μm long, 1–1.5 μm wide; type I phialides cylindrical occasionally widened at the base, some ampulliform, (2.5–)3–7.5(–8) × (1.5–)2–4 ($\bar{x}$ = 5.5 × 3) μm; type II phialides most predominant, elongate-ampulliform constricted at the base, 9–13(–14.5) × (2–)2.5–4 ($\bar{x}$ = 11.5 × 3) μm; type III phialides mostly subcylindrical or subulate, few navicular, (14–)15–23(–28) × (2–)2.5–3.5 ($\bar{x}$ = 18.5 × 3) μm. *Conidia* hyaline, oblong ellipsoidal or obovoid, 4–6(–7.5) × 1.5–3 ($\bar{x}$ = 5 × 2.5) μm, L/W = 2.3.

On surface or submerged in the agar.—Phialides hyaline, mostly cylindrical, some ampulliform, 3–8 × 1–2 ($\bar{x}$ = 4.5 × 1.5) μm. *Conidia* hyaline, mostly allantoid, few reniform, 5–7.5(–9) × 1–2 ($\bar{x}$ = 6.5 × 1.5) μm, L/W = 4.

Cultural characteristics.—Colonies reaching a radius of 2.5–7 mm after 8 d at 25 C. Minimum temperature for growth 15 C, optimum 20 C, maximum 37 C. Colonies on MEA flat, felt-like, with entire margin after 8 d and with crenated to entire margin after 16 d; after 8 d and 16 d white to buff (19′′f) above, buff (19′′f) in reverse. Colonies on PDA flat, felt-like, with entire margin after 8 d and with crenated to entire margin after 16 d; after 8 d and 16 d white to buff (19′′f) above, buff (19′′f) in reverse. Colonies on OA flat, woolly, with entire margin; after 8 d and 16 d white above.

Substrate.—Vitis vinifera.

Known distribution.—Spain.

HOLOTYPE. SPAIN, MURCIA, Yecla, isolated from black streaks in shoots of *Vitis vinifera* showing Petri disease symptoms, Mar 2008, *D. Gramaje*, Pm-8, herb. CBS H-20219 dried MEA colony (ex-type culture CBS 123910).

Notes.—Pm. hispanicum can be distinguished by the abundant percurrently rejuvenating conidiophores. Other species of *Phaeoacremonium* with percurrently rejuvenating conidiophores are *Pm. amstelodamense*, *Pm. argentinense*, *Pm. fuscum*, *Pm. krajdenii* and the anamorph of *Togninia africana* (Mostert et al 2006, Damm et al 2008). *Pm. hispanicum* can be distinguished from these species based on the white to buff colonies and slower growth after 8 d at 25 C (2.5–7 mm) on MEA and the production of conidiophores with inflated bases. Phylogenetically this species clusters with *Togninia vibratilis*. *Pm. hispanicum* can be distinguished from *Togninia vibratilis* by the higher minimum (15 C) and maximum (37 C) growth temperatures and the presence of conidiophores with percurrent rejuvenation in the former. *Pm. hispanicum* produces all three phialide types in comparison with *Togninia vibratilis* that produces only type I and type II phialides (Réblová and Mostert 2007). Only *Togninia vibratilis* and *Pm. pallidum* have an optimum growth temperature of 20 C, similar to *Pm. hispanicum* (Réblová and Mostert 2007, Damm et al 2008). *Pm. hispanicum* can be distinguished from *Pm. pallidum* by the predominance of the type II phialides, the presence of conidiophores showing percurrent rejuvenation and the slow growth on MEA compared to *Pm. pallidum*, which produces predominantly type I phialides and grows faster.

DISCUSSION

In the present study two novel species of *Phaeoacremonium* were found on grapevine, bringing the total number of species of *Phaeoacremonium* isolated from grapevines to 22. New *Phaeoacremonium* species often are found during surveys of grapevine trunk pathogens in other grapevine-growing countries (Mostert et al 2006, Essakhi et al 2008). *Pm. cinereum* was isolated from diseased vines in Spain and Iran. The presence of a taxon in two geographically separated countries has been found with other *Phaeoacremonium* species. Dispersal through infected planting material from different grapevine production areas and countries could explain the wide distribution associated with several species of *Phaeoacremonium.*

Micromorphological characters, such as conidiophore morphology, phialide type and shape, size of hyphal warts, and cultural characters are useful in distinguishing *Phaeacremonium* species (Mostert et al 2005). *Pm. cinereum* could be identified by its distinct gray colonies on malt extract agar, an optimum growth temperature of 25 C and subulate type III phialides. Distinct features of *Pm. hispanicum* include the abundant percurrently rejuvenating conidiophores, predominance of type II phialides and an optimum growth temperature of 20 C. The abundance of percurrently rejuvenating conidiophores has not been observed in other species.

Growth temperature studies showed that three isolates of *Pm. cinereum* and the one isolate of *Pm. hispanicum* had a maximum growth temperature of 37 C, suggesting that they have the potential to survive at human body temperature. Several thermotolerant *Phaeoacremonium* species, such as *Pm. krajdenii*, *Pm. parasiticum* and *Pm. venezuelense*, are associated with phaeohyphomycosis in humans and also have been isolated from grapevines with esca symptoms (Mostert et al 2005). Essakhi et al (2008) isolated *Pm. alvesii*, *Pm. griseorubrum* and *Pm. rubrigenum*, previously associated with human infections (Padhye et al 1998, Guarro et al 2003, Mostert et al 2005), from branches and trunks of *Vitis vinifera* with esca symptoms. These researchers propose that human pathogenic *Phaeoacremonium* species might have originated from woody host plants. However further studies are needed to confirm this hypothesis.

Genus *Togninia* was confirmed as teleomorph of *Phaeoacremonium* (Mostert et al 2003) and several researchers have induced the production of the teleomorph of *Phaeoacremonium* species by crossing complementary strains in vitro (Mostert et al 2005, 2006; Damm et al 2008). We were unsuccessful in our attempts to induce teleomorph production in the two novel species described here.

The detection of *Phaeoacremonium* species in Spain has increased in recent years, mainly due to the development of new techniques for the detection and identification of these pathogens (Overton et al 2004, 2005; Mostert et al 2006; Aroca and Raposo 2007; Aroca et al 2008a). Petri disease is considered a major cause of death of vines in nurseries and young vineyards in Spain (Aroca et al 2008b). The occurrence of new species of *Phaeoacremonium* on grapevines makes it necessary to expand the existing molecular-based detection tools because morphological identification is not always conclusive due to overlapping phenotypic characters. The ability to rapidly and accurately identify pathogens that cause Petri disease and esca is the crucial first step for epidemiological studies and for a better understanding of the distribution and importance of individual species. Rapid and accurate identification also can greatly assist growers' efforts to reduce losses due to these destructive diseases.

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