RESEARCH ARTICLE

Barriopsis iraniana and *Phaeobotryon cupressi*: two new species of the *Botryosphaeriaceae* from trees in Iran

J. Abdollahzadeh[1], E. Mohammadi Goltapeh[1], A. Javadi[2], M. Shams-bakhsh[1], R. Zare[2], A.J.L. Phillips[3]

Abstract Species in the *Botryosphaeriaceae* are well known as pathogens and saprobes of woody hosts, but little is known about the species that occur in Iran. In a recent survey of this family in Iran two fungi with diplodia-like anamorphs were isolated from various tree hosts. These two fungi were fully characterised in terms of morphology of the anamorphs in culture, and sequences of the ITS1/ITS2 regions of the ribosomal DNA operon and partial sequences of the translation elongation factor 1-α. Phylogenetic analyses placed them within a clade consisting of *Barriopsis* and *Phaeobotryon* species, but they were clearly distinct from known species in these genera. Therefore, they are described here as two new species, namely *Barriopsis iraniana* on *Citrus*, *Mangifera* and *Olea*, and *Phaeobotryon cupressi* on *Cupressus sempervirens*.

INTRODUCTION

Species of the *Botryosphaeriaceae* are cosmopolitan and occur on a wide range of plant hosts (von Arx & Müller 1954, Barr 1987). They can be primary pathogens, opportunists, endophytes or saprobes (Denman et al. 2000, Slippers & Wingfield 2007). While the family is well circumscribed, segregation of genera within the *Botryosphaeriaceae* has proven to be problematic. Von Arx & Müller (1954) made extensive synonymies under *Botryosphaeria* and included several genera known to have pigmented ascospores. In this way they effectively broadened the concept of *Botryosphaeria* to include species with hyaline ascospores, brown, aseptate ascospores, and brown, 1-septate ascospores. At least 18 anamorph genera have been associated with *Botryosphaeria*, including *Diplodia*, *Lasiodiplodia*, *Fusicoccum* and *Sphaeropsis*. Of these, only *Fusicoccum* and *Diplodia* were recognised by Denman et al. (2000), and this was supported by several studies (Jacobs & Rehner 1998, Zhou & Stanosz 2001, Alves et al. 2004). Pavlic et al. (2004) employed morphological and phylogenetic data to separate *Lasiodiplodia* from *Diplodia*. Later, Phillips et al. (2005) further broadened the concept by including *Dothiorella* within *Botryosphaeria*.

In a phylogenetic study based on 28S rDNA sequence data, Crous et al. (2006) recognised 10 lineages within *Botryosphaeria* corresponding to individual genera. A further lineage representing *Aplosporella* was subsequently added (Damm et al. 2007), and Phillips et al. (2008) recognised a further five genera bringing the total to 16.

The *Botryosphaeriaceae* has been the subject of numerous critical studies on the species associated with different hosts including grapevines (van Niekerk et al. 2004), *Eucalyptus* (Slippers et al. 2004), *Olea* (Lazzizera et al. 2008), *Prunus* (Slippers et al. 2007, Damm et al. 2007) and *Protea* (Denman et al. 2003, Marincowitz et al. 2008). Such studies have yielded several new species, thus revealing the diversity within this family. Furthermore, intensive sampling in different regions of the world has also revealed many new species (Pavlic et al. 2008, Taylor et al. 2009). Despite the importance attributed to the species in this family, there have been no studies on the *Botryosphaeriaceae* in Iran.

In the course of a survey of *Botryosphaeriaceae* in Iran during 2005–2007, besides some 14 known species, two new species with diplodia-like conidia were encountered. The aim of the present study was to characterise the species and to describe them based on DNA sequence data and morphology.

MATERIALS AND METHODS

Isolates and isolation

Infected branches, fruits and leaves with various disease symptoms, including dieback, canker, rot and necrosis, were collected from *Cupressus sempervirens*, *Mangifera indica*, *Citrus* sp. and *Olea* sp. in northern and southern provinces of Iran. Isolations were made by transferring conidia to potato-dextrose agar (PDA; Difco Laboratories). After incubating at 25 °C for 12 h, single germinating conidia were transferred to fresh PDA plates. Some isolates were obtained by plating pieces of tissue taken from the junction of the diseased and healthy areas of the samples, after surface sterilisation (1–4 min in 70 % ethanol), on PDA supplemented with 100 mg chloramphenicol. Representative isolates were deposited at the Iranian Research Institute of Plant Protection (IRAN, Tehran, Iran) and the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands).

Morphology

Sporulation was induced by culturing the isolates on 2 % tap water agar bearing pieces of double-autoclaved, halved poplar twigs or pine needles under near-ultraviolet light in a 12 h light-dark regime for 2–6 wk at 25 °C. Vertical sections through

[1] Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modarres University, P.O. Box 14115-336, Tehran, Iran.
[2] Department of Botany, Iranian Research Institute of Plant Protection, P.O. Box 1454, Tehran 19395, Iran.
[3] Centro de Recursos Microbiológicos, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal; corresponding author e-mail: alp@fct.unl.pt.

Table 1   Isolates included in the phylogenetic study.

Species	Culture no.[1]	Substrate	Locality	Collector	GenBank[2] ITS	GenBank[2] EF
Barriopsis fusca	CBS 174.26	*Citrus sp*	Cuba	N.E. Stevens	EU673330	EU673296
Barriopsis iraniana	IRAN1448C	*Mangifera indica*	Minab, Iran	J. Abdollahzadeh & A. Javadi	FJ919663	FJ919652
	IRAN1449C	*Olea sp.*	Rodan, Iran	J. Abdollahzadeh & A. Javadi	FJ919665	FJ919654
	IRAN1450C	*Citrus sp.*	Minab, Iran	J. Abdollahzadeh & A. Javadi	FJ919667	FJ919656
	IRAN1451C	*Citrus sp.*	Minab, Iran	J. Abdollahzadeh & A. Javadi	FJ919668	FJ919657
	IRAN1452C	*Citrus sp.*	Minab, Iran	J. Abdollahzadeh & A. Javadi	FJ919666	FJ919655
	IRAN1453C	*Mangifera indica*	Minab, Iran	J. Abdollahzadeh & A. Javadi	FJ919664	FJ919653
Botryosphaeria corticis	CBS119047	*Vaccinium corymbosum*	USA	P.V. Oudemans	DQ299245	EU017539
	ATCC22927	*Vaccinium sp.*	USA	R.D. Millholland	DQ299247	EU673291
Botryosphaeria dothidea	CBS 110302	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259092	AY573218
	CMW 8000	*Prunus sp.*	Switzerland	B. Slippers	AY236949	AY236898
Diplodia corticola	CBS 112549	*Quercus suber*	Portugal	A. Alves	AY259100	AY573227
	CBS 112546	*Quercus ilex*	Spain	M.E. Sánchez & A. Trapero	AY259090	EU673310
Diplodia mutila	CBS 112553	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259093	AY573219
	CBS 230.30	*Phoenix dactylifera*	USA	L.L. Huillier	DQ458886	DQ458869
Diplodia seriata	CBS 112555	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259094	AY573220
	CBS 119049	*Vitis sp.*	Italy	L. Mugnai	DQ458889	DQ458874
Dothiorella iberica	CBS 115041	*Quercus ilex*	Spain	J. Luque	AY573202	AY573222
	CBS 113188	*Quercus suber*	Spain	M.E. Sánchez & A. Trapero	AY573198	EU673278
Dothiorella sarmentorum	IMI 63581b	*Ulmus sp.*	UK	E.A. Ellis	AY573212	AY573235
	CBS 115038	*Malus pumila*	Netherlands	A.J.L. Phillips	AY573206	AY573206
Lasiodiplodia gonubiensis	CBS 115812	*Syzygium cordatum*	South Africa	D. Pavlic	DQ458892	DQ458877
Lasiodiplodia parva	CBS 494.78	Cassava-field soil	Colombia	O. Rangel	EF622084	EF622064
	CBS 456.78	Cassava-field soil	Colombia	O. Rangel	EF622083	EF622063
Lasiodiplodia pseudotheobromae	CBS 116459	*Gmelina arborea*	Costa Rica	J. Carranza-Velásquez	EF622077	EF622057
	CBS 374.54	*Coffea sp.*	Zaire	Unknown	EF622080	EF622059
Neodeightonia phoenicum	CBS 169.34	*Phoenix dactylifera*	USA	H.S. Fawcett	EU673338	EU673307
	CBS 122528	*Phoenix dactylifera*	Spain	F. Garcia	EU673340	EU673309
Neodeightonia subglobosa	CBS 448.91	keratomycosis in eye	UK	Unknown	EU673337	EU673306
Neofusicoccum luteum	CBS 110299	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259091	AY573217
	CBS 110497	*Vitis vinifera*	Portugal	A.J.L. Phillips	EU673311	EU673277
Neofusicoccum parvum	CMW 9081	*Populus nigra*	New Zealand	G.J. Samuels	AY236943	AY236888
	CBS 110301	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259098	AY573221
Phaeobotryon cupressi	IRAN1454C	*Cupressus sempervirens*	Gorgan, Iran	M.A. Aghajani	FJ919673	FJ919662
	IRAN1455C	*Cupressus sempervirens*	Gorgan, Iran	M.A. Aghajani	FJ919672	FJ919661
	IRAN1456C	*Cupressus sempervirens*	Gorgan, Iran	M.A. Aghajani	FJ919670	FJ919659
	IRAN1457C	*Cupressus sempervirens*	Gorgan, Iran	M.A. Aghajani	FJ919669	FJ919658
	IRAN1458C	*Cupressus sempervirens*	Gorgan, Iran	M.A. Aghajani	FJ919671	FJ919660
Phaeobotryon mamane	CPC 12440	*Sophora chrysophylla*	Hawaii	W. Gams	EU673332	EU673298
	CPC 12442	*Sophora chrysophylla*	Hawaii	W. Gams	EU673333	EU673299
Phaeobotryosphaeria citrigena	ICMP 16812	*Citrus sinensis*	New Zealand	S.R. Pennycook, P.R. Johnston & B.C. Paulus	EU673328	EU673294
	ICMP 16818	*Citrus sinensis*	New Zealand	S.R. Pennycook, P.R. Johnston & B.C. Paulus	EU673329	EU673295
Phaeobotryosphaeria porosa	CBS 110496	*Vitis vinifera*	South Africa	J.M. van Niekerk	AY343379	AY343340
Phaeobotryosphaeria visci	CBS 100163	*Viscum album*	Luxembourg	H.A. van der Aa	EU673324	EU673292
	CBS 186.97	*Viscum album*	Germany	T. Graefenhan	EU673325	EU673293
Spencermartinsia viticola	CBS 117009	*Vitis vinifera*	Spain	J. Luque & S. Martos	AY905554	AY905559
	CBS 117006	*Vitis vinifera*	Spain	J. Luque & S. Martos	AY905555	AY905562

[1] ATCC: American Type Culture Collection; CBS: Centraalbureau voor Schimmelcultures; CMW: M.J. Wingfield, FABI, University of Pretoria, South Africa; CPC: Collection of Pedro Crous housed at CBS; ICMP: International Collection of Micro-organisms from Plants, Landcare Research, New Zealand; IMI: CABI Bioscience, Egham, UK; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran.

[2] ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; EF: Translation elongation factor 1-α partial sequence.

conidiomata were made with a Leica CM1100 cryostat micro-tome. The conidiogenous layer was dissected from conidiomata formed in culture. Structures were mounted in 100 % lactic acid and digital images were recorded with a Leica DFC320 camera on a Leica DMR HC microscope. Measurements were made with the Leica IM500 measurement module. For each isolate the mean, standard deviation and 95 % confidence interval were calculated from measurements of at least 50 conidia. Dimensions are presented as a range with extremes in paren-theses. Dimensions of other fungal structures are given as the range of at least 20 measurements. Colony morphology, colour (Rayner 1970), and growth rates between 5 and 35 °C in 5 °C intervals, were determined on 2 % malt extract agar (MEA; Difco Laboratories) in the dark.

DNA extraction, PCR amplification and sequencing

Isolates were grown on 2 % malt extract broth (MEB) and incu-bated at room temperature for 4–7 d. Mycelium was collected by filtration. Mycelial mats were washed with sterile distilled water and freeze-dried with an Edward MicroModulyo 1.5K System (England) freeze drier. Genomic DNA was obtained by a modification of the method described by Reader & Broda (1985). The mycelium was ground in liquid nitrogen in 1.5 mL microtubes. Five hundred microlitres of extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 1 % SDS) was added, the mixture thoroughly vortexed, and incubated at 65 °C for 1 h. Subsequently 500 µL of chloroform was added. The mixture was shaken gently and centrifuged at 13 000 rpm for 1 h at 4 °C. The supernatant was transferred to a new microtube and template DNA was precipitated overnight at -20 °C with 0.54 volume of ice-cold isopropanol and 3 M sodium acetate (0.1 volume). The DNA was pelleted at 13 000 rpm for 10 min at 4 °C. The resulting pellets were washed with 100 µL of cold 70 % ethanol and dried at room temperature. The dried pellets of template DNA were re-suspended in 100 µL of distilled water and incubated at 65 °C for 1 h. DNA concentrations were determined with a NanoDrop® ND-1000 spectrophotometer and DNA was stored at -80 °C.

The PCR reactions were carried out with Taq DNA polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania), and PCR reaction mixtures were prepared according to Alves et al. (2004), with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. All primers used were synthesised by STAB Vida Lda. (Portugal). The ITS plus D1/D2 region of the LSU and the translation elongation factor 1-α (EF-1α) were amplified using the primer pairs ITS1 (White et al. 1990) /NL4 (O'Donnell 1993) and EF1-688F/EF1-1251R, respectively, as described by Alves et al. (2008). Nucleotide sequences of the ITS and EF-1α regions were determined using the primers ITS1/ITS4 (White et al. 1990) and EF1-688F/EF1-1251R (Alves et al. 2008). Both strands of the PCR products were sequenced by STAB Vida Lda (Por-tugal). Sequences of both DNA regions of additional isolates were retrieved from GenBank (Table 1). New sequences were deposited in GenBank (Table 1) and the alignment and trees in TreeBase (study accession number S2392, matrix accession number M4535).

Phylogenetic analyses

The nucleotide sequences were aligned with ClustalX v1.83 (Thompson et al. 1997), using the following parameters: pair-wise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and manual ad-justments were made where necessary. Phylogenetic analyses were carried out using PAUP v4.0b10 (Swofford 2003) for maxi-mum-parsimony (MP) analysis and MrBayes v 3.0b4 (Ronquist & Huelsenbeck 2003) for the Bayesian analysis. Trees were visualised with TreeView (Page 1996). Maximum-parsimony analysis was performed using the heuristic search option with 1 000 random taxon additions and tree bisection and recon-nection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as fifth character. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robust-ness of the most parsimonious trees was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI). A partition homogeneity test was done to determine the possibility of combining the ITS and EF1-α datasets (Farris et al. 1995, Huelsenbeck et al. 1996).

Bayesian analyses employing a Markov Chain Monte Carlo (MCMC) method were performed. The general time-reversible model of evolution (Rodriguez et al. 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+I+Γ) was used. Four MCMC chains were run simultaneously, starting from random trees, for 10^6 generations. Trees were sampled every 100th generation for a total of 10^4 trees. The first 10^3 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala & Yang 1996) were determined from a majority-rule consensus tree generated from the remaining 9 000 trees. The analysis was repeated three times starting from different random trees to ensure trees from the same tree space were being sampled during each analysis.

RESULTS

DNA phylogeny

The ITS and EF1-α sequences for the 11 isolates studied were combined and aligned with 41 sequences of 22 taxa retrieved from GenBank, representing a selection of genera and species in the *Botryosphaeriaceae*. Incomplete portions at the ends of the sequences were excluded from the analyses. The combined dataset after alignment consisted of 941 characters including alignment gaps. A partition homogeneity test in PAUP was not significant (P = 0.52) indicating that the individual datasets were congruent and produced trees with the same topology. There-fore the two datasets were combined in a single analysis. Of the 941 characters, 446 were constant, while 10 were variable and parsimony-uninformative. Maximum parsimony analysis of the remaining 485 parsimony-informative characters resulted in a single tree of 1 340 steps (HI = 0.343). The overall topology of the 50 % majority rule consensus tree of 9 000 trees sampled in the Bayesian analysis had a similar topology as the MP tree (TreeBase S2392), which is presented in Fig. 1.

Ten clades were identified, each corresponding to a separate genus. Isolates obtained in this study clustered in clades 1 and 2, corresponding to *Phaeobotryon* and *Barriopsis*. The five iso-lates from *C. sempervirens* clustered in a subclade of clade 1, separate from *P. mamane*. The remaining six isolates from this study formed a subclade within the *Barriopsis* clade (clade 2) separate from *B. fusca*. In both cases bootstrap support for the subclades was high.

Morphology

All isolates studied here produced pycnidia on pine needles and *Populus* twigs on WA within 2–3 wk. No ascomata were seen either on the host or in culture. Based on morphology and phylogenetic positions, these isolates were separated into two species, one in *Barriopsis* and the other in *Phaeobotryon*. On account of their unique morphology and phylogeny they are described here as two new species.

Taxonomy

Barriopsis iraniana Abdollahzadeh, Zare & A.J.L. Phillips,
sp. nov. — MycoBank MB513235; Fig. 2

Teleomorph. Unknown.

Conidiomata brunnea vel nigra, uni- vel multilocularia, globosa. Cellulae conidiogenae hyalinae, cylindricae, conidio primo holoblastico, posteriora phialidica, proliferatione in eodem plano periclinaliter incrassatae. Conidia (22.7–)27–27.4(–37.7) × (12.8–)16.2–16.6(–21.5) µm, ovoidea, utrinque rotundata, primum hyalina, longitudinaliter striata, maturitate brunnea et 1–3 septa formantia.

Typus. IRAN 13939F.

Etymology. The name refers to Iran where the fungus was discovered.

Conidiomata stromatic, pycnidial, superficial, dark brown to black, covered with dense mycelium, on pine needles mainly unilocular and up to 600 µm diam; on *Populus* twigs mostly multilocular, individual or aggregated, thick-walled, ostiolate. *Ostiole* central, circular, non-papillate. *Paraphyses* arising from the conidiogenous layer, extending above the level of developing conidia, up to 70 µm long, 3.5 µm wide, thin-walled, hyaline, usually aseptate, sometimes becoming up to 2–3-septate, not constricted at the septa, tip rounded, occasionally branched. *Conidiophores* absent. *Conidiogenous cells* 7–12 × 3–5 µm, hyaline, thin-walled, smooth, cylindrical, holoblastic, proliferating at the same level, with visible periclinal thickening. *Conidia* thick-walled, initially hyaline, aseptate with longitudinal striations, striations visible on hyaline conidia even while attached to

Fig. 1 Single most parsimonious tree resulting from maximum parsimony analysis of combined ITS and EF1-α sequence data. MP bootstrap values are given at the nodes. The tree was rooted to two isolates of *Pseudofusicoccum stromaticum*. The new species are in **bold** face.

conidiogenous cells; oval, both ends broadly rounded, becoming brown, aseptate or 1–3-septate, with prominent longitudinal striations, wall smooth, (22.7–)24–30 × (12.8–)14–18(–21.5) µm, 95 % confidence limits = 27–27.4 × 16.2–16.6 µm (av. ± S.D. = 27.2 ± 1.8 × 16.4 ± 1.3 µm, l/w ratio = 1.7 ± 0.16). *Chlamydospores* catenate, intercalary, brown, smooth, thick-walled, formed within the agar medium.

Culture characteristics — Colonies with appressed mycelial mat and fluffy aerial mycelium in the middle, becoming dull green to olivaceous-black at the surface, and dull green to grey-olivaceous at the reverse after 2 wk in the dark at 25 °C. Colonies reaching 45–50 mm diam on MEA after 4 d in the dark at 25 °C. Cardinal temperatures for growth; min 5 °C, max > 35 °C, opt 25–30 °C.

Substrates — Endophytic in stems of *Citrus* sp., *Mangifera indica*, *Olea* sp.

Known distribution — Hormozgan Province, Iran.

Specimens examined. Iran. Hormozgan Province, Minab, Hajikhademi, on twigs of *Mangifera indica*, 27 Feb. 2007, *J. Abdollahzadeh & A. Javadi*, holotype IRAN 13939F, culture ex-type IRAN 1448C = CBS 124698. Other isolates are listed in Table 1.

Notes — Conidia of *Barriopsis iraniana* are significantly larger than those of *B. fusca*, the only other species known in this genus. The only available culture of *B. fusca* (CBS 174.26, ex-type) has lost its ability to sporulate. According to Stevens (1926) the anamorph is lasiodiplodia-like with hyaline conidia that become dark-brown and septate with irregular longitudinal striations. These characters of the anamorphs of *Barriopsis* are confirmed in the present study. Furthermore, we have shown that, in contrast to *Lasiodiplodia*, the conidia of *Barriopsis* are striate at a very early stage of development and the striations are clearly visible in young, hyaline conidia (Fig. 1d–i). This is an unusual character not found in any other genus of the *Botryosphaeriaceae*. We did not encounter the teleomorph of *B. iraniana* and it did not form in culture.
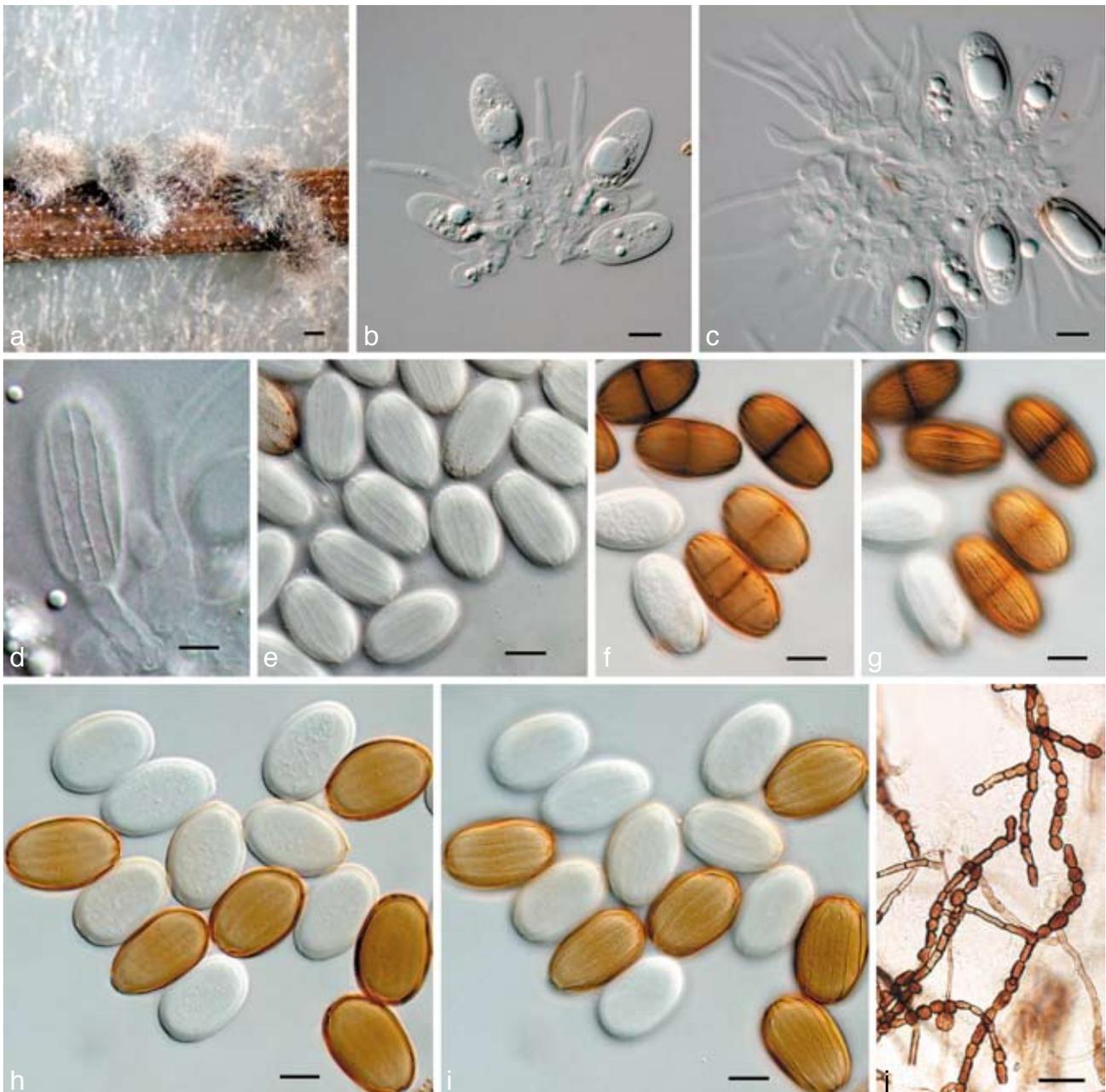

Fig 2 *Barriopsis iraniana* holotype. a. Conidiomata on pine needles in culture; b, c. conidia developing on conidiogenous cells between paraphyses; d. young conidium showing longitudinal striations while attached to a conidiogenous cell; e. hyaline, striate conidia; f–i. hyaline and brown, striate conidia, 1- and 3-septate conidia can be seen in f and g; j. catenulate chlamydospores. — Scale bars: a = 250 µm; b, c, e–i = 10 µm; d = 5 µm; j = 40 µm.

Phaeobotryon cupressi Abdollahzadeh, Zare & A.J.L. Phillips, *sp. nov.* — MycoBank MB513236; Fig. 3

Teleomorph. Unknown

Conidiomata brunnea vel nigra, uni- vel multilocularia, globosa. Cellulae conidiogenae hyalinae, cylindricae, holoblasticae, conidio primo holoblastico, posteriora phialidica, proliferatione in eodem plano periclinaliter incrassatae. Conidia (19.8–)24.6–25(–30) × (10.2–)12.2–12.5(–17) μm, ovoidea, utrinque rotundata, hyalina, aseptata.

Typus. IRAN 13940F.

Etymology. Name refers to *Cupressus*, the host genus on which the fungus was discovered.

Conidiomata stromatic, pycnidial, superficial, dark-brown to black, mostly unilocular on pine needles and up to 650 μm diam, mostly multilocular on *Populus* twigs, individual or aggregated, thick-walled, ostiolate. *Ostiole* central, circular, non-papillate. *Paraphyses* hyaline, thin-walled, arising from the conidiogenous layer, extending above the level of developing conidia, up to 42 μm long, 4.8 μm wide, usually aseptate, sometimes becoming up to 2-septate, tip rounded, occasionally branched. *Conidiophores* absent. *Conidiogenous cells* hyaline, smooth, thin-walled, cylindrical, 7–14 × 2–5 μm, holoblastic, phialidic, proliferating internally with visible periclinal thickening. *Conidia* thick-walled, initially hyaline, oval, both ends broadly rounded, aseptate, (19.8–)21–28(–30) × (10.2–)11–15(–17) μm, 95 % confidence limits = 24.1–25 × 12.2–12.5 μm ($\overline{x}$ ± S.D. = 24.8 ± 1.9 × 12.4 ± 1.3 μm, l/w ratio = 2 ± 0.3), forming a single septum at germination, rarely becoming brown and 1-septate, internally verruculose when aged. *Microconidiomata* globose, dark-brown to black, superficial, occasionally immersed in pine needle or *Populus* tissue. *Microconidiophores* cylindrical, 7–13 × 1.5–2.5 μm, hyaline, aseptate becoming 1–2-septate, branched. *Microconidiogenous cells* hyaline, thin-walled, phialidic, proliferating internally, giving rise to periclinal thickening, 6–10 × 1–2 μm. *Microconidia* oval, thin-walled, hyaline, aseptate 2–4 × 1–2. *Chlamydospores* intercalary, brown, smooth, thick-walled, formed within the agar medium.

Cultural characteristics — Colonies with abundant aerial mycelium towards periphery, appressed in the centre, becoming grey-olivaceous to olivaceous-grey at the surface, and grey-olivaceous in reverse after 2 wk in the dark at 25 °C. Colonies on MEA reaching 46–53 mm diam after 4 d in the dark at 25 °C. Cardinal temperatures for growth; min 5 °C, max > 35 °C, opt 25 °C.

Substrate — Endophytic in stems of *Cupressus sempervirens*.

Known distribution — Golestan Province, Iran.

Specimens examined. Iran, Golestan Province, Gorgan, City Park, on twigs of *Cupressus sempervirens*, 15 Aug. 2006, *M.A. Aghajani*, holotype IRAN 13940F, culture ex-type IRAN 1455C = CBS 124700. Other isolates are listed in Table 1.

Notes — This species differs from *P. quercicola* and *P. mamane* in its smaller conidia, and has thus far only been collected from *Cupressus* species. The hyaline, aseptate conidia of *P. cupressi* are superficially similar to those of other *Diplodia* species with hyaline conidia. Furthermore, conidial dimensions of *P. cupressi* are similar to those of *Diplodia cupressi* (21.5–30.5 × 12–16) as reported by Alves et al. (2006). Microconidia have been reported for *P. quercicola* (Phillips et al. 2005), *P. mamane* (Phillips et al. 2008) and *P. cupressi* (this paper). They have also been reported in *D. cupressi* (Alves et al. 2006), but not in other *Diplodia* species (Alves et al. 2004, Damm et al. 2007, Phillips et al. 2007, Lazzizera et al. 2008). Thus it is possible that *P. cupressi* has been mistaken for *D. cupressi* in the past. Pycnidial paraphyses in *Phaeobotryon* clearly distinguish this genus from *Diplodia*.

DISCUSSION

This paper forms part of a larger study of the *Botryosphaeriaceae* from Iran, and is the first attempt to characterise the species present in this country. Two new species are described, one in *Barriopsis* and another in *Phaeobotryon*. These species could be distinguished based on their DNA sequence data and unique morphological characteristics. Only a few species are thus far known from these genera, and confirmed reports have been infrequent.

Barriopsis was introduced when Phillips et al. (2008) transferred *Physalospora fusca* to *Barriopsis fusca*. Stevens (1926) originally placed this species in *Physalospora*, but was obviously hesitant to do so on account of its brown ascospores. Petrak & Deighton (1952) then transferred it to *Phaeobotryosphaeria* as *Phaeobotryosphaeria fusca*. Although von Arx & Müller (1954) considered *Phaeobotryosphaeria* a synonym of *Botryosphaeria*, Phillips et al. (2008) showed that it is morphologically and phylogenetically distinct from other genera in the *Botryosphaeriaceae*. However, the fungus considered by Stevens (1926) and Petrak & Deighton (1952) does not have apiculi on its ascospores, and thus does not fall within the concept of *Phaeobotryosphaeria*, which has small, hyaline apiculi on the ascospores. It was for this reason that Phillips et al. (2008) introduced the genus *Barriopsis*. *Barriopsis iraniana* is only the second species to be described in this genus. The new data on morphology of the anamorph, as reported in this paper, reveal further distinctions from other genera in the *Botryosphaeriaceae*, namely the striations visible on conidia at an early stage of development.

Phaeobotryon was introduced by Theissen & Sydow (1915) to accommodate *Dothidea cercidis*. In the original description of *D. cercidis* the ascospores were reported to be hyaline. However, Theissen & Sydow (1914) observed them to become brown with age and subsequently (Theissen & Sydow 1915) introduced the genus *Phaeobotryon*. Von Arx & Müller (1954, 1975) placed *Phaeobotryon* in synonymy with *Botryosphaeria* in their broader concept of this genus. However, Phillips et al. (2008) considered *Phaeobotryon* as morphologically and phylogenetically distinct from other genera in the *Botryosphaeriaceae* and thus reinstated the name. The genus at present consists of four species (*P. cercidis*, *P. cupressi*, *P. mamane* and *P. quercicola*), while the status of *P. disruptum* and *P. euganeum* remains uncertain. Cultures are available for only two of these, *P. mamane* and *P. cupressi*, and therefore these were the only two for which DNA sequence data are available. Phillips et al. (2008) did not observe conidia of *P. cercidis*, but they reported the conidia of *P. mamane* as brown and 1–2-septate. Phillips et al. (2005) found hyaline, aseptate conidia associated with *P. quercicola*, and considered these to be the anamorph. *Phaeobotryon cupressi* has smaller conidia than both *P. mamane* and *P. quercicola*, and the three species can be distinguished easily on the basis of conidial dimensions. No information is available regarding conidial characters of the remaining *Phaeobotryon* species, namely *P. cercidis*, *P. disruptum* and *P. euganeum*, since they were described based on features of the teleomorphs only and no anamorphic characters were considered.

Barriopsis appears to be confined to regions with tropical or subtropical climates. The type species, *B. fusca*, was originally collected from *Citrus* and an unknown woody host in Cuba (Stevens 1926). A search of the Systematic Mycology and Microbiology Laboratory Fungal Database (April 2009; http://nt.ars-grin.gov/fungaldatabases) revealed that the majority of reports of this species are from warmer climates. In the present study, *B. iraniana* was found only in the subtropical southern part of Iran. In contrast to *Barriopsis*, *Phaeobotryon* appears

Fig. 3 *Phaeobotryon cupressi* holotype. a. Conidiomata formed on pine needles in culture; b. sectioned conidioma; c. paraphyses and developing conidia; d, e. microconidiogenous cells; f. microconidia; g. conidia and conidiogenous cells; h. hyaline conidia; i, j. brown, aseptate conidia; k. germinating conidia; l. chlamydospores and a hyaline conidium. — Scale bars: a = 250 µm; b = 100 µm; c, h, i, k, l = 10 µm; d, e = 2.5 µm; f, g, j = 5 µm.

to have a wider distribution, and species have been reported from Germany, USA (Carolina and Hawaii) and the northern, temperate regions of Iran.

Both *B. iraniana* and *P. cupressi* were isolated from diseased plants. However, pathogenicity was not tested for either species and their role as causal agents of plant diseases is not known. Furthermore, as far as we are aware, pathogenicity of none of the other species in *Barriopsis* and *Phaeobotryon* is known.

REFERENCES

Alves A, Correia A, Luque J, Phillips AJL. 2004. Botryosphaeria corticola, sp. nov. on Quercus species, with notes and description of Botryosphaeria stevensii and its anamorph, Diplodia mutila. Mycologia 96: 598–613.

Alves A, Correia A, Phillips AJL. 2006. Multi-gene genealogies and morphological data support Diplodia cupressi sp. nov., previously recognized as D. pinea f. sp. cupressi, as a distinct species. Fungal Diversity 23: 1–15.

Alves A, Crous PW, Correia A, Phillips AJL. 2008. Morphological and molecular data reveal cryptic species in Lasiodiplodia theobromae. Fungal Diversity 28: 1–13.

Arx JA von, Müller E. 1954. Die Gattungen der amerosporen Pyrenomyceten. Beiträge zur Kryptogamenflora der Schweiz 11, 1: 1–434.

Arx JA von, Müller E. 1975. A re-evaluation of the bitunicate ascomycetes with keys to families and genera. Studies in Mycology 9: 1–159.

Barr ME. 1987. Prodromus to class Loculoascomycetes. Amherst, Massachusetts: Published by the author.

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Phillips AJL, Alves A, Burgess T, Barber P, Groenewald JZ. 2006. Phylogenetic lineages in the Botryosphaeriaceae. Studies in Mycology 55: 235–253.

Damm U, Crous PW, Fourie PH. 2007. Botryosphaeriaceae as potential pathogens of Prunus in South Africa, with descriptions of Diplodia africana and Lasiodiplodia plurivora sp. nov. Mycologia 99: 664–680.

Denman S, Crous PW, Groenewald JZ, Slippers B, Wingfield BD, Wingfield MJ. 2003. Circumscription of Botryosphaeria species associated with Proteaceae based on morphology and DNA sequence data. Mycologia 95: 294–307.

Denman S, Crous PW, Taylor JE, Kang J-C, Pascoe I, Wingfield MJ. 2000. An overview of the taxonomic history of Botryosphaeria, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. Studies in Mycology 45: 129–140.

Farris JS, Kallersjo M, Kluge AG, Bult C. 1995. Testing significance of incongruence. Cladistics 10: 315–319.

Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42: 182–192.

Huelsenbeck JP, Bull JJ, Cunningham CV. 1996. Combining data in phylogenetic analysis. Trends in Ecology & Evolution 11: 152–158.

Jacobs KA, Rehner SA. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of Botryosphaeria and related taxa. Mycologia 90: 601–610.

Lazzizera C, Frisullo S, Alves A, Phillips AJL. 2008. Morphology, phylogeny and pathogenicity of Botryosphaeria and Neofusicoccum species associated with drupe rot of olives in Southern Italy. Plant Pathology 57: 948–956.

Marincowitz S, Groenewald JZ, Wingfield MJ, Crous PW. 2008. Species of Botryosphaeriaceae occurring on Proteaceae. Persoonia 21: 111–118.

Niekerk JM van, Crous PW, Groenewald JZ, Fourie PH, Halleen F. 2004. DNA phylogeny, morphology and pathogenicity of Botryosphaeria species on grapevines. Mycologia 96: 781–798.

O'Donnell K. 1993. Fusarium and its near relatives. In: Reynolds DR, Taylor JW (eds), The fungal holomorph: Mitotic, meiotic and pleomorphic speciation in fungal systematics: 225–233. CAB International, Wallingford, UK.

Page RD. 1996. TreeView: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357–358.

Pavlic D, Slippers B, Coutinho TA, Gryzenhout M, Wingfield MJ. 2004. Lasiodiplodia gonubiensis sp. nov., a new Botryosphaeria anamorph from native Syzygium cordatum in South Africa. Studies in Mycology 50: 313–322.

Pavlic D, Wingfield MJ, Barber P, Slippers B, Hardy GESJ, Burgess TI. 2008. Seven new species of the Botryosphaeriaceae from baobab and other native trees in Western Australia. Mycologia 100: 851–866.

Petrak F, Deighton FC. 1952. Beiträge zur Pilzeflora von Sierra Leone. Sydowia 6: 309–322.

Phillips AJL, Alves A, Correia A, Luque J. 2005. Two new species of Botryosphaeria with brown, 1-septate ascospores and Dothiorella anamorphs. Mycologia 97: 513–529.

Phillips AJL, Alves A, Pennycook SR, Johnston PR, Ramaley A, Akulov A, Crous PW. 2008. Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the Botryosphaeriaceae. Persoonia 21: 29–55.

Phillips AJL, Crous PW, Alves A. 2007. Diplodia seriata, the anamorph of "Botryosphaeria" obtusa. Fungal Diversity 25: 141–155.

Rannala B, Yang Z. 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. Journal of Molecular Evolution 43: 304–311.

Rayner RW. 1970. A mycological colour chart. Kew, Surrey, UK: CMI and British Mycological Society.

Reader U, Broda P. 1985. Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology 1: 17–20.

Rodriguez F, Oliver JF, Marin A, Medina JR. 1990. The general stochastic model of nucleotide substitutions. Journal of Theoretical Biology 142: 485–501.

Ronquist F, Huelsenbeck JP. 2003. MrBayes3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Slippers B, Fourie G, Crous PW, Coutinho TA, Wingfield BD, Carnegie AJ, Wingfield MJ. 2004. Speciation and distribution of Botryosphaeria spp. on native and introduced Eucalyptus trees in Australia and South Africa. Studies in Mycology 50: 343–358.

Slippers B, Smit WA, Crous PW, Coutinho TA, Wingfield BD, Wingfield MJ. 2007. Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world. Plant Pathology 56: 128–139.

Slippers B, Wingfield MJ. 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. Fungal Biology Reviews 21: 90–106.

Stevens NE. 1926. Two species of Physalospora on Citrus and other hosts. Mycologia 18: 206–217.

Swofford DL. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods) v4. Sinauer Associates, Sunderland, Massachusetts.

Taylor K, Barber PA, Hardy GESJ, Burgess TI. 2009. Botryosphaeriaceae from tuart (Eucalyptus gomphocephala) woodland, including the description of four new species. Mycological Research 113: 337–353.

Theissen F, Sydow H. 1914. Dothideazeen studien II. Annales Mycologici 12: 268–281.

Theissen F, Sydow H. 1915. Die Dothideales. Annales Mycologici 13: 149–746.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleaic Acids Research 25: 4876–4882.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), PCR Protocols: a guide to methods and applications: 315–322. Academic Press, San Diego, California, USA.

Zhou S, Stanosz GR. 2001. Relationships among Botryosphaeria species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. Mycologia 93: 516–527.