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Original Article

New records from *Botryosphaeriaceae* (*Ascomycota*) for mycobiota of Iran

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Abstract: A large collection of *Botryosphaeriaceae* isolates obtained from fruit and forest trees with fruit rot, canker and dieback disease symptoms in northern provinces of Iran were examined in this study. Based on morphology and sequence data (ITS and *EF1-α*), two species, *Diplodia mutila* and *Spencermartinsia viticola* are illustrated and described as new records for mycobiota of Iran. Furthermore, *D. sapinea* morphotype A is determined here for the first time from Iran.

Key words: *Diplodia*, *Spencermartinsia*, taxonomy, phylogeny

INTRODUCTION

Species of the *Botryosphaeriaceae* are cosmopolitan and occur on a wide range of woody plants (von Arx & Müller 1954, Barr 1987). They are well-known plant pathogens and saprobes and found as endophytes in symptomless tissues (Denman et al. 2000, Slippers & Wingfield 2007). Thus far, 17 genera have been recognized in this family including *Diplodia* Fr. and *Spencermartinsia* A.J.L. Phillips, A. Alves & Crous (Phillips et al. 2013). Species of *Diplodia* are associated with different disease symptoms such as twig blight, canker, die-back, gummosis and fruit rot (Lazzizera et al. 2008). Some *Diplodia* species such as *D. mutila* and *D. seriata* have a wide host range while, the others; *D. africana*, *D. agrifolia*, *D. alatafructa*, *D. allocellula*, *D. bulgarica*, *D. corticola*, *D. cupressi*, *D. intermedia*, *D. malorum*, *D. olivarum*, *D. pseudoseriata*, *D. quercivora*, *D. rosulata*, *D. sapinea*, *D. scrobiculata* and *D. tsugae* have been reported from very limited plant species and host specificity has apparently been occurred (Phillips et al. 2012). Taxonomy of the genus based on host association in the past resulted in description of more than 1000 species and Index Fungorum lists 1246 names (Oct. 2013; www.indexfungorum.org). Most of these names are probably synonymous because it is now clear that host association is not an important taxonomic character in the family *Botryosphaeriaceae* (Slippers et al. 2004). Phillips et al. (2005) provided an amended description for *Diplodia* based on the type species (*D. mutila*). Two morphologically distinct groups are recognized in this genus (Phillips et al. 2013). In the first group containing the type species (*D. mutila*), conidia are hyaline, thick-walled, becoming brown usually after discharge from conidiomata, often the coloration is delayed or never occurs, occasionally becoming septate. Phylogenetically this group is divided to two completely distinct sub-clades. In the second morphological group consisting *D. alatafructa*, *D. allocellula* *D. intermedia*, *D. pseudoseriata*, *D. scrobiculata*, *D. sapinea* and *D. seriata* conidia becoming brown within pycnidial cavity before discharge from conidiomata and rarely becoming septate. The latter group is supported as a single clade by phylogenetic analyses. During the past decade, more than 10 species have been described and thus far 18 species have been studied properly based on molecular and morphological data.

Spencermartinsia was established as a monotypic genus by Phillips et al. (2008) to accommodate *Dothiorella viticola*. *Spencermartinsia* is similar to *Dothiorella* in asexual morph but, is differed from *Dothiorella* in sexual morph by the presence of an apiculus at either ends of brown and 1-septate ascospores. Recently phylogenetic analyses confirmed these two as distinct genera in the *Botryosphaeriaceae* (Liu et al. 2012, Phillips et al. 2013, Abdollahzadeh et al. In press). In a recent phylogenetic study based on morphology and sequences data (ITS and *EF1-α*), Abdollahzadeh et al. (In press) described three new species in *Spencermartinsia* and bringing the total number of species to four.

The history of taxonomic studies on *Botryosphaeriaceae* in Iran has been discussed by Abdollahzadeh et al. (2013). Before 2009, all *Botryosphaeriaceae* species reported from Iran have morphologically been characterized. Thus, some of these reports are doubtful. As stressed by different researchers species characterization based on

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morphological features has resulted in mis-identification and chaos in the taxonomy of *Botryosphaeriaceae* (Crous et al. 2006, Alves et al. 2007, Phillips et al. 2008, 2013). Therefore, it is suggested as needed to use molecular methods for precise identification of species in this family.

Extensive sampling in the past five years especially in the north of Iran, has led to characterization of more than 20 species in the *Botryosphaeriaceae*. Of these, 14 have been formally described and published as new taxa (Abdollahzadeh et al. 2009, 2010, 2013, In press, Phillips et al. 2012). *Lasiodiplodia pseudotheobromae* (Abdollahzadeh et al. 2010) and *Neofusicoccum mediterraneum* (Abdollahzadeh et al. 2013) were reported as new records for Iran. Six species, *Botryosphaeria dothidea*, *Diplodia seriata*, *D. sapinea*, *Dothiorella sarmentorum*, *L. theobromae* and *N. parvum*, have already been reported from Iran (Ershad 2009).

In this paper, *D. mutila* and *S. viticola* are described and illustrated as new records and *D. sapinea* morphotype A is characterized for the first time from Iran.

MATERIALS AND METHODS

Fungal isolates

Isolations were made by transferring conidia to potato-dextrose agar (PDA; Difco Laboratories) or directly plating out pieces of tissue taken from the junction of the diseased and healthy areas of the samples, after surface sterilization (1–4 min in 70 % ethanol), plated on PDA supplemented with 100 mg/L chloramphenicol. Representative isolates were deposited at the culture collection of Iranian Research Institute of Plant Protection ('IRAN', Tehran, Iran).

Morphology

To induce sporulation, isolates were transferred 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. The conidiogenous layer was dissected from conidiomata formed in culture. Structures were mounted in 100% lactic acid and digital images were recorded with a DP72 camera on an Olympus BX51 or a Leica DFC320 camera on a Leica DMR HC microscope. 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 parentheses. Dimensions of other fungal structures are given as the range of at least 20 measurements. Colony morphology, color (Rayner 1970), and growth rates were determined on 2% malt extract agar (MEA; Difco Laboratories) in the dark at 25˚C.

DNA isolation, PCR amplification and sequencing

DNA was extracted by modified method of Raeder & Broda (1985) method as described by Abdollahzadeh et al. (2009). 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 synthesized by STAB Vida Lda. 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. Sequences of both DNA regions of additional isolates were retrieved from GenBank.

Phylogenetic analyses

The nucleotide sequences were aligned with ClustalX v1.83 (Thompson et al. 1997). Alignments were checked and manual adjustments were made where necessary. Phylogenetic information contained in indels (insertions/deletions) was incorporated into the phylogenetic analyses using simple indel coding as implemented by GapCoder (Young & Healy 2003). Phylogenetic analyses were carried out using PAUP v4.0b10 (Swofford 2003). Trees were rooted to *Neofusicoccum luteum* and visualized with TreeView (Page 1996). The K2P nucleotide substitution model (Kimura 1980) was used for Neighbor-joining (NJ) analysis. All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 NJ bootstrap replicates.

Maximum - parsimony (MP) analysis was performed using the heuristic search option with 1000 random taxon additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 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).

RESULTS AND DISCUSSION

DNA phylogeny

A partition homogeneity test in PAUP was not significant (P = 0.46) indicating that the individual datasets were congruent and produced trees with the same topology. Therefore the two datasets were combined in a single analysis. The ITS and EF1-α sequences for the 7 isolates studied were combined and aligned with 47 sequences of 25 taxa. The combined dataset after alignment consisted of 1086 characters including alignment gaps, of which 607 were constant, 140 were excluded and 14 were variable and parsimony-uninformative. Maximum parsimony analysis of the remaining 325 parsimony-informative characters resulted in 795 most parsimonious trees of 529 steps (HI = 0.22, RI=0.95, CI = 0.78). NJ analysis resulted in a tree with the same topology as the MP tree. One of the 795 MP trees is shown with bootstrap values of MP on the nodes (Fig. 1). According to the phylogenetic analyses, our isolates were resided in two genera in three distinct clades representatives of three different species; *Diplodia mutila*, *D. sapinea* (morphotype A) and *Spencermartinsia viticola*. Of these, *D. mutila* and *S. viticola* are new records for mycobiota of Iran. Although in a morphological study *D. mutila* has previously been reported from Iran (Abdollahzadeh et al. 2007), but it was actually a misidentification and all of those isolates phylogenetically were later characterized as a new species named *Phaeobotryon cupressi* (Abdollahzadeh et al. 2009). Furthermore, *D. sapinea* morphotype A is determined in this study.

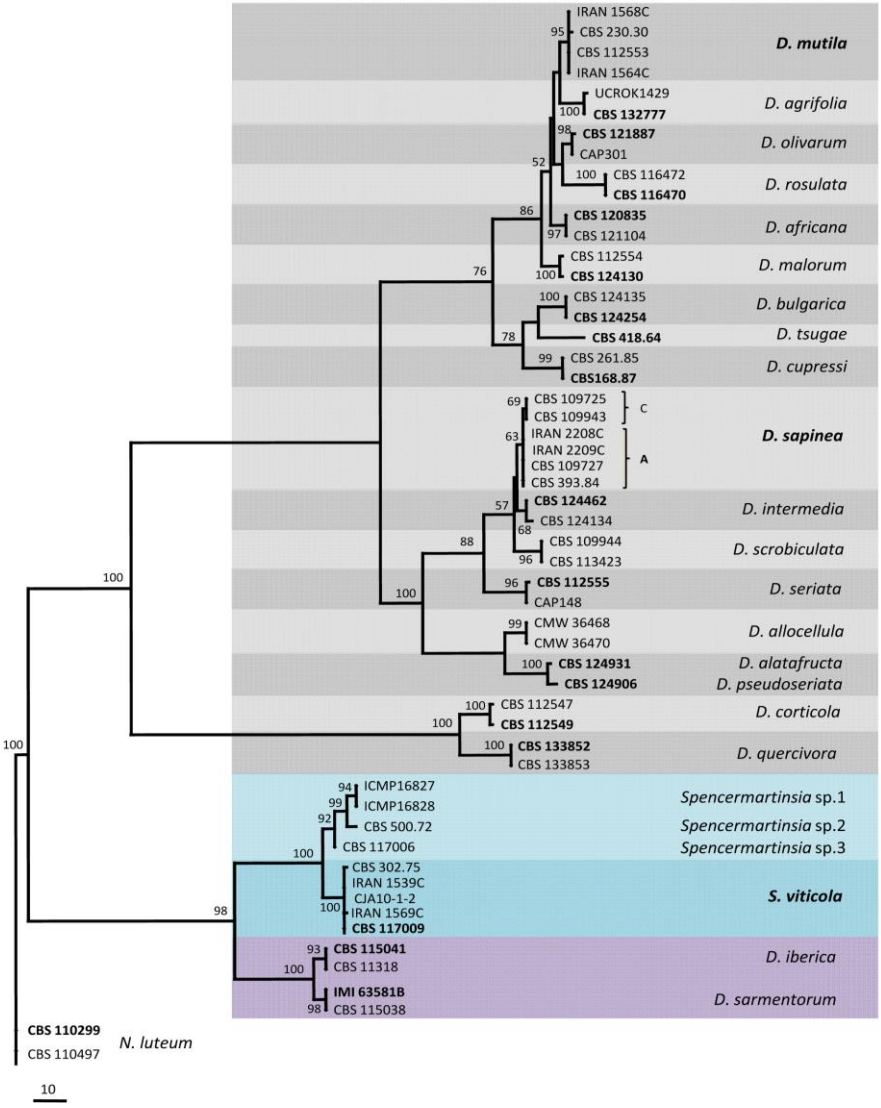

Fig. 1. One of 795 most parsimonious trees obtained from combined ITS and *EF1-α* sequence data. MP bootstrap values are given based on 1000 pseudoreplicates on the nodes. The tree is rooted to *Neofusicoccum luteum* (CBS 110299, CBS 110497). The bar represents 10 changes. Species characterized in this study and ex-type strains are in bold typeface.

Morphology

All three species identified by phylogenetic analyses in this study could be distinguished from their relatives on account of their conidial size and shape. All identified species are described and illustrated here.

Diplodia mutila (Fr.) Mont., Annales des Sciences Naturelles Botanique 1: 302. 1834. MycoBank MB201741. Fig. 2.

Basionym: *Sphaeria mutila* Fr., Syst. Mycol. (Lundae) 2: 424. 1823.

≡ *Physalospora mutila* (Fr.) N.E. Stevens, Mycologia 28: 333. 1936.

= *Botryosphaeria stevensii* Shoemaker, Canad. J. Bot. 42: 1299. 1964.

Further synonyms are given by Stevens (1933).

Conidiomata stromatic, pycnidial, produced on pine needles on WA within 1–2 wk, superficial or partially immersed, dark-brown to black, mostly unilocular, up to 500 µm diam., individual or aggregated, thick-walled, ostiolate. Ostiole central, circular, sometimes papillate. Conidiophores absent. Conidiogenous cells hyaline, smooth, thin-walled, cylindrical, discrete, (5–) 8–14 (–16) × 3–5 µm, holoblastic, determinate, proliferating at the same level giving rise to periclinal thickening. Conidia hyaline, guttulate or sometimes with a large central guttule, smooth, thick walled, aseptate, ellipsoid to oblong, ends broadly rounded, rarely becoming brown and septate with age, (18.9–) 23–29 (–32.4) × (9.9–) 11–15 (–16.2) µm, 95 % confidence limits = 26.6–27 × 13.4–13.6 µm, mean ± S.D. = 26.8 ± 2.2 × 13.4 ± 1.1 µm, l/w ratio = 2 ± 0.2. *Microconidiomata* immersed or partially immersed. *Microconidia* thin-walled, hyaline, smooth, rod shape, rounded ends, aseptate, 3–5 × 1.5 µm (Fig. 2).

Cultural characteristics – Colonies appressed and without aerial mycelium or with abundant aerial mycelium, becoming pale smoke grey (21’’’’f) to olivaceous black (27’’’’m) or pale olivaceous grey (21’’’’’d) to iron black (23’’’’’k) at the surface, and greenish olivaceous (23’’’i) to olivaceous black (27’’’’m) in reverse after 2 wk in the dark at 25 °C. Colonies on MEA reaching 60–90 mm diam. after 4 d in the dark at 25 °C.

Specimens examined: IRAN, GUILAN, Asalem, Gisoom Coast, *Alnus* sp., Jul. 2006, A. Eskandari, IRAN1564C, IRAN1542C; Lahijan to Anzali, unknown woody plant, Jun. 2007, J. Abdollahzadeh/A. Javadi, IRAN1540C, CJA81; Rahimabad, Garmab-doost, unknown woody plant, Jun. 2007, J. Abdollahzadeh/A. Javadi, IRAN1568C, IRAN1562, CJA33; MAZANDARAN, Babolsar to Babol, *Salix* sp., Jun. 2007, A. Javadi, CJA126. Notes: *Diplodia mutila* is an important and common canker and dieback pathogen and reported as the causal agent of oak dieback and canker in Hungary (Vajna 1986) and death of oak trees in northeastern Spain (Luque & Girbal 1989). It has been isolated from different forest and fruit trees and more than 50 hosts are listed for *D. mutila* (Farr & Rossman 2013).

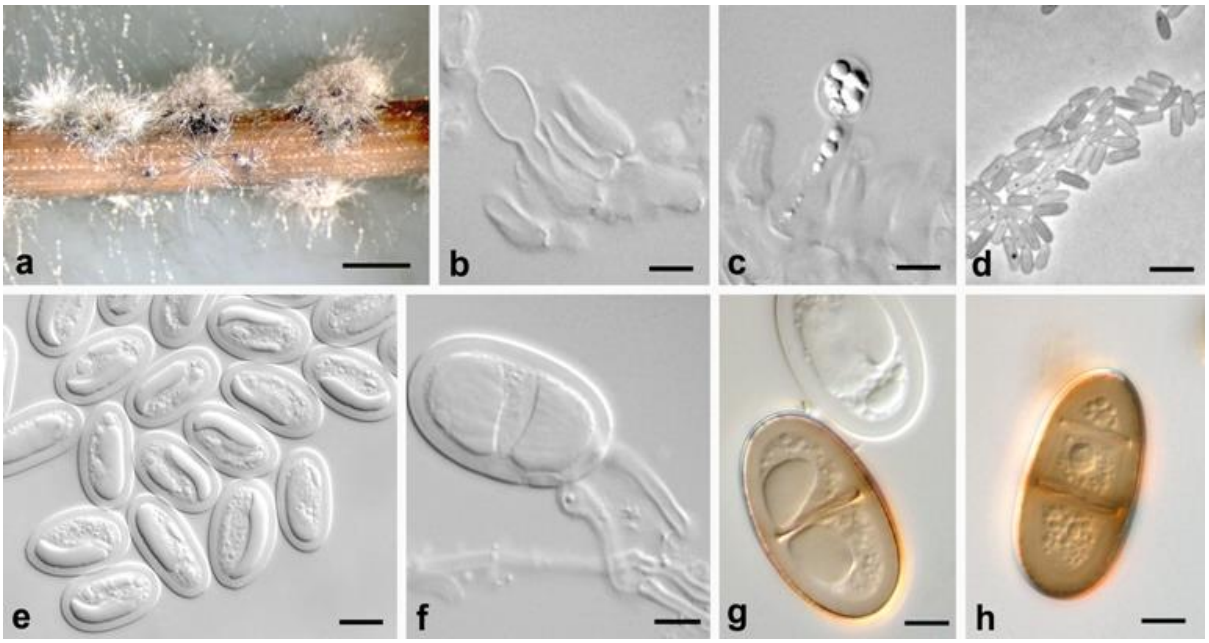

Fig. 2. *Diplodia mutila* (**a**) Conidiomata on pine needles in culture, (**b**), (**c**) Hyaline immature conidia developing on conidiogenous cells; (**d**) Microconidia; (**e**) Hyaline aseptate conidia; (**f**) Germinated conidia; (**g**), (**h**) Brown and septate conidia. Scale bars: **a** = 1000 µm; **b**-**d**, **f**-**h** = 5 µm: **e** = 10 µm;.

It can be distinguished from closely related species such as *D. corticola*, *D. olivarum*, *D. affricana*, *D. cupressi*, *D. bulgarica* and *D. rosulata* based on conidial size, host plants and phylogenetic data. Morphologically *D. mutila* is completely similar to *Phaeobotryon cupressi* and the only difference between these two is the presence of pycnidial paraphyses in *P. cupressi*. Sometimes it is difficult to distinguish paraphyses from conidiophores or conidiogenous cells in the *Botryosphaeriaceae*. Thus, it is likely that some errors have been occurred in identification of these two taxa in the past. Although Abdollahzadeh et al. (2007) have previously misidentified *D. mutila* from Iran, that species what later described and named as a new species, *P. cupressi*. Thus, this is the first report of *D. mutila* from Iran. The taxonomic history of *D. mutila* is explained by Sutton (1980), Alves et al. (2004) and Phillips et al. (2013).

Diplodia sapinea (Fr.) Fuckel, Jahrbücher des Nassauischen Vereins für Naturkunde 23–24: 393. 1870. MycoBank MB146913. Fig. 3.

Basionym: *Sphaeria sapinea* Fr., Syst. Mycol. 2: 491. 1823.

Synonyms are given by Sutton & Dyko (1989).

Conidiomata stromatic, pycnidial, produced on pine needles on WA within 1–2 wk, superficial, dark-brown to black, mostly unilocular and up to 570 µm diam., individual or aggregated, thick-walled, ostiolate. Ostiole central, circular, sometimes papillate. Conidiophores absent. Conidiogenous cells hyaline, smooth, thin-walled, lageniform, swollen at the base, discrete, (8.7–) 11–18 (–20.4) × 3–5 µm, holoblastic, determinate or indeterminate, proliferating at the same level giving rise to periclinal thickening or proliferating percurrently. Conidia initially hyaline, becoming dark brown, moderately thick-walled (ca. 0.5 µm), wall externally smooth and internally roughened, aseptate (very rarely septate), ovoid to clavate, apex rounded, base truncate or rounded, (24.3–) 35–39 (–45) × (11–) 12–18 (–18.9) µm, 95 % confidence limits = 34.7–38.1 × 13.2–15.3 µm, mean ± S.D. = 38.2 ± 3.7 × 14 ± 1.2 µm, l/w ratio = 2.6 ± 0.3 (Fig. 3).

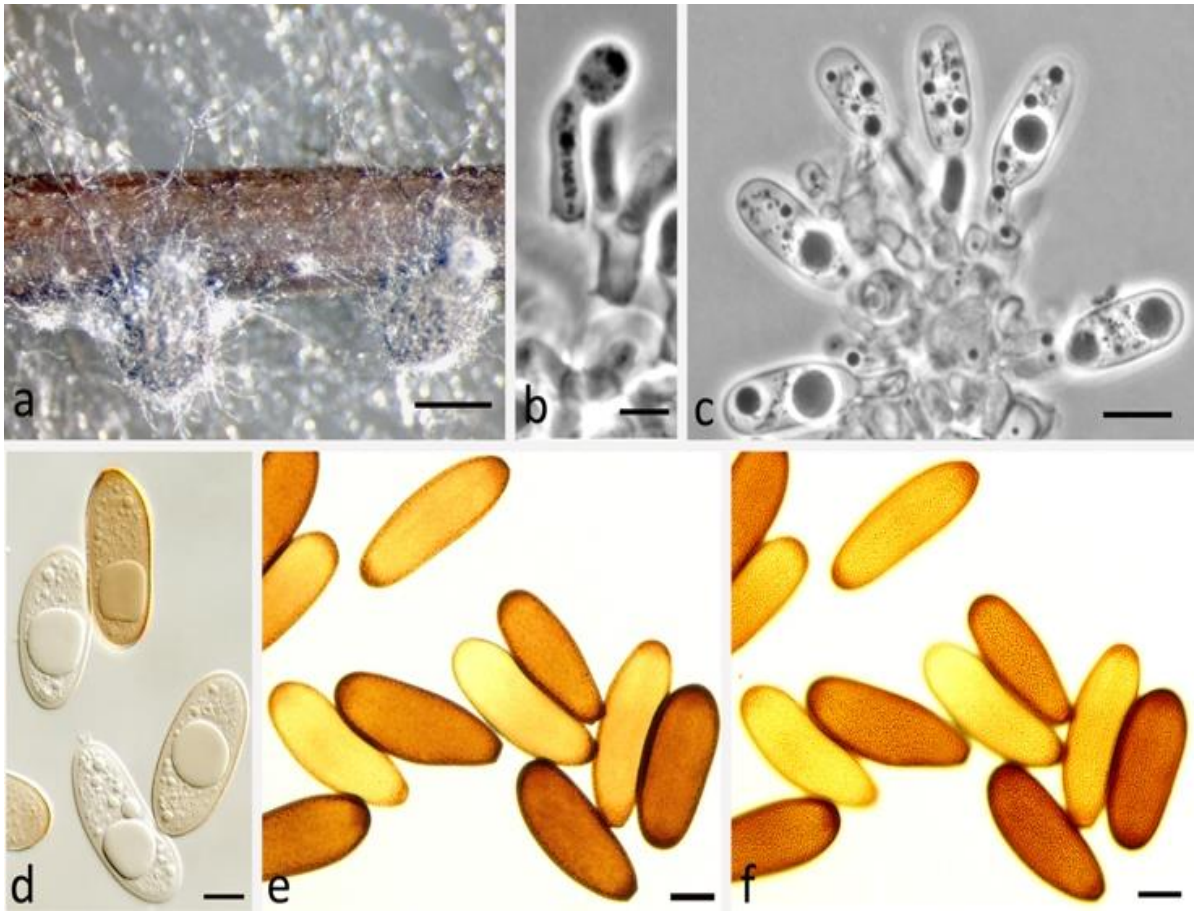

Fig. 3. *Diplodia sapinea* A. (**a**) Conidiomata on pine needles in culture, (**b**), (**c**) Hyaline immature conidia developing on conidiogenous cells, (**d**) Hyaline and brown aseptate conidia; (**e**), (**f**) Mature conidia in two different focal planes. Scale bars: **a** = 500 µm; **b** = 5 µm; **c-f** = 10 µm.

Cultural characteristics– Colonies with aerial mycelium, becoming grey olivaceous (21’’’’i) to greenish-black (33’’’’’k) at the surface, and grey olivaceous (21’’’’i) to olivaceous black (27’’’’m) or greenish grey (33’’’’i) to olivaceous black (27’’’’m) in reverse after 2 wk in the dark at 25 °C. Colonies on MEA reaching 90 mm diam. after 4 d in the dark at 25 °C.

Specimens examined: IRAN, GUILAN, Rasht, Saravan, forest park, *Pinus* sp., Jun. 2007, J. Abdollahzadeh/A. Javadi, IRAN 2208C; MAZANDARAN, Marzanabad, *Pinus* sp., Jun. 2009, A. Javadi, IRAN 2209C.

Notes: *Diplodia sapinea* (= *D. pinea*) is known as a latent and opportunistic pathogen of conifers with worldwide distribution (Swart & Wingfield 1991) and usually associated with trees under stress conditions and causes intensive losses especially on susceptible cultivars of *Pinus* (Zwolinski et al. 1990). More than 150 hosts are listed for *D. sapinea* mostly from conifers (Farr & Rossman 2013). It consists of two morphotypes A and C that can be separated on account of larger conidial size of C morphotype (De Wet et al. 2000).

D. sapinea is clearly distinguishable from closely related species such as *D. seriata*, *D. scrobiculata* and *D. intermedia* based on conidial size and phylogenetic data. It has been reported previously from Iran as *D. pinea* on *Pinus* without morphotype detection (Viennot-Bourgin et al. 1970).

The taxonomic history of *D. sapinea* has been explained in detail by Sutton & Dyko (1989) and Phillips et al. (2013).

Spencermartinsia viticola (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous, Persoonia 21: 51. 2008. MycoBank MB511763. Fig. 4.
Basionym: *Dothiorella viticola* A.J.L. Phillips & J. Luque, Mycologia 97: 1116. 2005.
= *Botryosphaeria viticola* A.J.L. Phillips & J. Luque, Mycologia 97: 1116. 2005.

Conidiomata stromatic, pycnidial, produced on poplar twigs on WA within 1–2 wk, solitary or aggregated, individual conidiomata globose, up to 420 µm diam., superficial or semi-immersed, covered with hyphal hairs, uniloculate, thick-walled, non-papillate with a central ostiole. Conidiophores absent. Conidiogenous *cells* cylindrical to lageniform, discrete or integrated, holoblastic, indeterminate, proliferating at the same level giving rise to periclinal thickenings, hyaline, thin-walled, smooth, (3.6–) 8–12 (–12.4) × 2–4 µm. Conidia oblong to subcylindrical, brown, 1-septate even while they are still attached to the conidiogenous cells, occasionally slightly constricted at septum, moderately thick-walled, externally smooth, internally finely verruculose, ends truncate, (16–) 18–23 (–26) × (7–) 8–12 (–15.3) µm, 95% confidence limits = 20.7–20.9 × 9.9–10 µm (av. ± S.D. = 20.8 ± 1.7 × 10 ± 1.3 µm, l/w ratio = 2.1 ± 0.2).

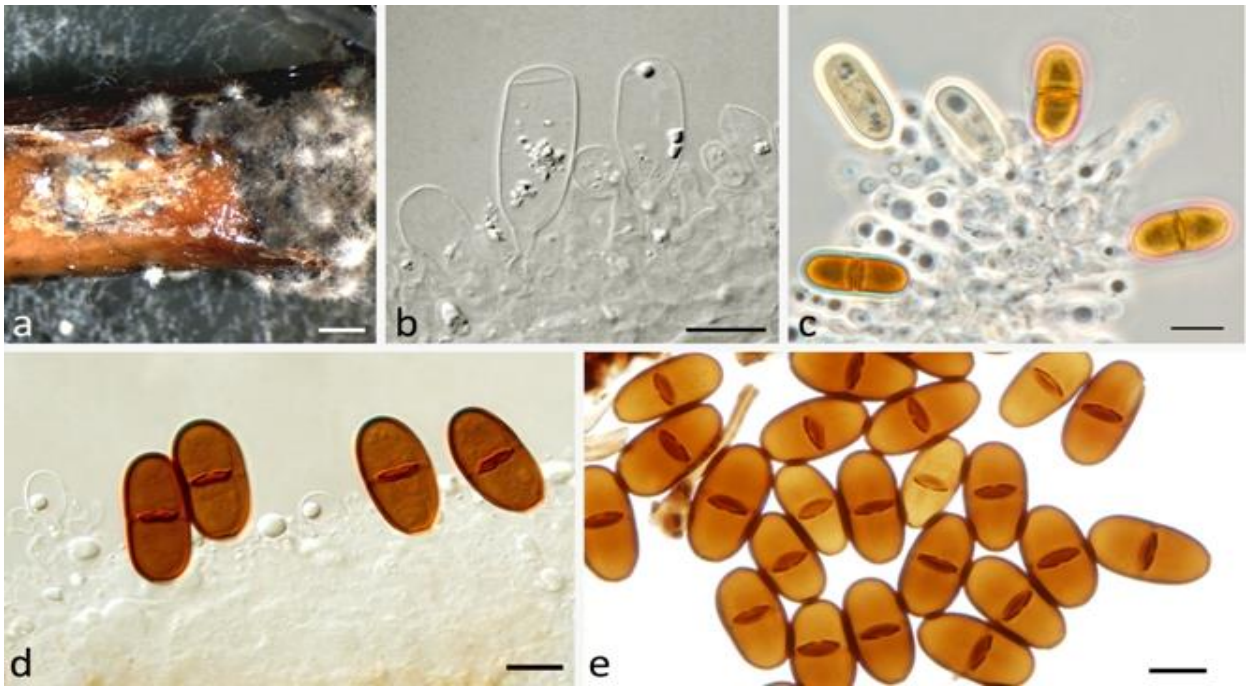

Fig. 4. *Spencermartinsia viticola*. (**a**) Conidiomata on poplar twigs in culture, (**b**) Hyaline immature conidia developing on conidiogenous cells, (**c**), (**d**) Brown and septate conidia on conidiogenous cells, (**e**) Brown and septate conidia. Scale bars: **a** = 1000 µm; **b**-**e** = 10 µm.

Cultural characteristics – Colonies cottony with dense aerial mycelium, aerial mycelium becoming pale smoke grey (21’’’’f) to iron black (23’’’’’k) at the surface and grey olivaceous (21’’’’i) to olivaceous black (27’’’’m) at the reverse after 2 wk in the dark at 25 ºC. Colonies reaching 70–80 mm on MEA after 4 days in the dark at 25 ºC. Cardinal temperatures for growth; min ≤5 ºC, max ≥35 ºC, opt 25 ºC.

Specimens examined: IRAN, GUILAN, Chaboksar, *Citrus* sp., Jun. 2007, J. Abdollahzadeh/A. Javadi, IRAN 1539C; GOLESTAN, Gorgan, City Park, *Cupressus sempervirens*, Aug. 2006, M. A. Aghajani, IRAN 1569C; Gorgan, Touskestan Forest, *Prunus* sp., Jun 2009, A. Javadi, CJA10–1–2.

Notes: *Spencermartinsia viticola* is reported here for the first time from Iran. Three isolates were found on *Citrus* sp., *Cupressus sempervirens* and *Prunus* sp. This species has never been isolated from *C. sempervirens*. So far it has been found on six hosts from six different countries. For more detail see Phillips et al. (2013).

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گزارش های جدید از (*Ascomycota*) *Botryosphaeriaceae* برای میکوبیوتای ایران

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چکیده: مجموعه بزرگی از جدایه های *Botryosphaeriaceae* از روی درختان میوه و جنگلی با نشانه های پوسیدگی میوه، شانکر و زوال در استان های شمالی جمع آوری و مورد بررسی قرار گرفت. بر اساس مطالعات ریخت شناسی و تعیین توالی نواحی ITS و *EF1-α* دو گونه *Diplodia mutila*، *Spencermartinsia viticola*، شناسایی و برای اولین بار برای میکوبیوتای ایران تشریح شدند. علاوه بر این، مرفوتیپ A از *Diplodia mutila*، در اینجا برای اولین بار از ایران گزارش می شود.

واژه های کلیدی: *Diplodia*، *Spencermartinsia*، رده بندی، فیلوژنی

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