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Description of the sexual morph of *Seimatosporium vitis*

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Abstract – *Seimatosporium vitis* was recently described based on the collection of its coelomycetous asexual morph on *Vitis vinifera* in Italy. In this study *Seimatosporium vitis* is introduced for the first time from grapevine in Iran. The sexual morph is illustrated and a full description is provided. The connection between two different morphs was proved in culture and based on ITS sequence data.

Iran / Pestalotioid fungi / Phylogeny / *Vitis* sp.

INTRODUCTION

Seimatosporium Corda is a member of family *Discosiaceae* in *Amphisphaeriales* (Senanayake *et al.*, 2015) and typified by *S. rosae* (Corda, 1833). The genus is defined primarily based on conidial characteristics including size, septation, pigmentation, and presence or absence of appendages (Sutton, 1980; Nag Raj, 1993). Its members are considered so-called “pestalotioid fungi”, and their life styles range from pathogens to saprobes (Tanaka *et al.*, 2011). This genus currently contains numerous species with more than 86 names recorded to date (Index Fungorum; <http://www.indexfungorum.org/Names/Names.asp>) and detailed descriptions of *Seimatosporium* spp. have been presented by several authors (Shoemaker, 1964; Pirozynski & Shoemaker, 1970; Sutton, 1980; Nag Raj, 1993; Hatakeyama & Harada, 2004; Tanaka *et al.*, 2011; Barber *et al.*, 2011; Norphanphoun *et al.*, 2015; Senanayake *et al.*, 2015; Goonasekara *et al.*, 2016; Perera *et al.*, 2016).

Tanaka *et al.* (2011) in a phylogenetic study based on LSU and ITS sequence data confirmed *Discostroma* Clem. (1909) as sexual morph of *Seimatosporium* species, while other related genera, including *Sporocadus*, *Sarcostroma*, *Diploceras* and *Vermisporium*, which lack sexual morphs, clustered in *Seimatosporium sensu stricto*, suggesting that they should be placed under the genus *Seimatosporium*. (Tanaka *et al.*, 2011; Barber *et al.*, 2011). Réblová *et al.*, (2016) proposed to use *Seimatosporium* over *Discostroma*, because the former is the oldest name, has the greater number of species, and is more commonly used.

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There are very few publications on pestalotioid fungi from Iran. Arzanlou *et al.* (2012) have provided a check list for the known pestalotioid fungi from Iran, listing 3 *Seimatosporium* species, viz. *S. fusisporum* H.J. Swart & D.A. Griffiths (Aminae & Ershad, 2008), *S. lonicerae* (Cooke) Shoemaker (Gräfenhan, 2006) and *S. lichenicola* (Corda) Shoemaker & Müll (Aghapour *et al.*, 2010). More recently, Crous *et al.* (2014) described *S. pistaciae* from Iran. As mentioned by Norphanphoun *et al.* (2015) this species is similar to *S. rosea* based on phylogenetic analysis of LSU and ITS sequence data, but morphologically it appears to be distinct from *S. rosea* by having larger conidia, but sequence data from protein-coding genes may shed more light on the possible synonymy of these two species. More recently Ayoubi & Soleimani (2016) characterized a new species *Neopestalotiopsis iranensis* and *N. mesopotamica* on strawberry fruits from Kurdistan Province, Iran.

Through our work on the taxonomy of Diatrypaceae on trees native to the Arasbaran forest in Iran, *Seimatosporium vitis* was found as a new record for Iran. In this paper it is characterized morphologically and phylogenetically and a detailed description and illustration for both sexual and asexual morphs is provided.

MATERIALS AND METHODS

Morphological characterization. Samples were collected from Arasbaran forest in the East Azerbaijan on dead branch of *Vitis* sp. Isolation were made from single ascospores (IRAN2454C and IRAN2455C) and conidia (IRAN2427C). For microscopic examinations sections were cut freehand under an Olympus SZH stereo microscope. A Nikon Eclipse 80i light microscope with a Canon digital camera was used to capture micromorphological images. The measurements for each structure are in parentheses. Colony color were determined on potato dextrose agar (PDA) and malt extract agar (MEA) at 24°C using a color chart (Rayner, 1970). Dry specimens and fresh cultures were deposited in the herbarium and culture collection of the Iranian Research Institute of Plant Protection (IRAN, Tehran, Iran).

DNA extraction and sequencing. Isolates were grown in malt extract broth (1.5 % MEB) at room temperature for 14 d. Genomic DNA was extracted with an initial step of grinding the mycelia in liquid nitrogen as described by Liu *et al.* (2000). Polymerase chain reactions (PCR) were carried out using primer pairs of ITS1/ITS4 (White *et al.*, 1990) to amplify the internal transcribed spacers (ITS) and LROR/LR7 (Rehner & Samuels, 1994; Vilgalys & Hester, 1990) to amplify part of large subunit rDNA (LSU). The PCR reaction mixtures 25 μ L contained 2.5 μ L 10x high yield PCR buffer (Jena Bioscience, Germany), 1 μ L MgCl₂ (25 mM), 0.5 μ L dNTPs (10 mM), 1 μ L of each primer (10 pmol/ μ L, Takapouzist Inc.), 1.5 unit *Taq* polymerase (Jena Bioscience, Germany) and 1 μ L genomic DNA (~30 ng/ μ L). PCR reactions were run on a PC-320 PCR System (ASTEC Co., Japan) machine with an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 35 s at 58°C/57°C (ITS/LSU), 90 s at 72°C, and a final extension of 10 min at 72°C. PCR products were visualized in 1% agarose gel in 1xTBE buffer. Purification and sequencing of PCR products were performed by Macrogen (South Korea).

Phylogenetic analysis. The new sequences obtained in the present study were read and edited with FinchTV v. 1.4.0 (Geospiza Inc.). The sequences were compared with those in the GenBank database using the Megablast algorithm. Additional sequences were selected from Norphanphoun *et al.* (2015) and Senanayake

et al. (2015). *Pseudoestalotiopsis coccus* and *Pestalotiopsis hollandica* were included as outgroup taxa. The ITS and LSU sequence data (Table 1), were aligned with ClustalX with default settings (Thompson *et al.*, 1997). 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 performed with neighbor joining (NJ) and maximum parsimony (MP) as implemented in PAUP* v4.0b10 (Swofford, 2003). The neighbour-joining analysis was performed using Kimura-2-parameter nucleotide substitution model (Kimura, 1980). All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 NJ bootstrap replicates. In MP analysis characters were equally weighted, and gaps were treated as missing data. Trees were inferred with the heuristic search option with TBR branch swapping and 1000 random sequence additions. Branches of zero length were collapsed and all parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis & Bull, 1993). Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI), Homoplasy index (HI). New sequences have been deposited in GenBank (Table 1).

RESULTS

Phylogenetic analyses

The LSU and ITS sequences for 30 isolates of 27 taxa were combined and aligned. Incomplete portions at the ends of the sequences were excluded from the analyses. The dataset consisted of 2120 characters after alignment, of which 1015 were excluded, 930 were constant, 58 were variable and parsimony uninformative and 117 were parsimony informative. A heuristic search of the 117 parsimony informative characters resulted in nine most parsimonious trees (TL = 290, CI = 0.69, RI = 0.77, HI = 0.31). NJ analysis produced a tree with the same topology as the MP trees. One of the MP trees with bootstrap support values is shown in Fig. 1. Isolates sequenced in this study were placed in a clade representative of *Seimatosporium vitis*.

Taxonomy

Seimatosporium vitis Y.P. Xiao, Camporesi & K.D. Hyde, *Fungal Diversity* 73: 103. 2015 (Fig. 2)

Lignicolous. **Sexual morph:** Ascomata perithecial, solitary, partly to completely immersed in the host tissue, globose to subglobose, blackish brown to black, 200-400 μm in diameter, 100-250 μm long. Paraphyses filiform, hyaline, unbranched, septate. Asci unitunicate, cylindrical, apex rounded, short stalked, 8-spored, 70-110 \times 9-11.5 μm (\bar{x} = 92.5 \times 10.4 μm , n = 20). Ascospores uniseriate, elliptic to fusiform, hyaline, 2-celled, very rarely 3-4 celled, smooth, constricted at the septa when mature, (11.5-)14-20(-21) \times 5-7(-7.5) μm (\bar{x} = 17.3 \times 6.5 μm , n = 20). **Asexual morph:** Conidiomata acervular, solitary or gregarious, immersed or erumpent, circular or irregular, dark brown to black, opening by a split in the host

Table 1. Isolates used for the phylogenetic analyses

Species	Host	Location	Culture no.	LSU	GenBank ^{1,2}	ITS
<i>Discostrroma fuscillum</i>	<i>Rosa canina</i>	Japan	NBRC 32625	AB593726	AB594794	
<i>D. stonaeae</i>	–	Japan	NBRC 32690	AB593729	AB594797	
<i>D. fostium</i>	–	Japan	NBRC 32626	AB593727	AB594795	
<i>Pestalotiopsis hollandica</i>	<i>Sciadopitys verticillata</i>	Netherlands	CBS 265.33	KM116228	KM199328	
<i>Pseudopezizomorphopsis coccos</i>	<i>Cocos nucifera</i>	Indonesia: Java	CBS 272.29	KM116276	KM199378	
<i>Seimatopsporium biseptatum</i>	<i>Eucalyptus oresbia</i>	Australia	CPC 13584	JN871208	JN871199	
<i>S. botan</i>	<i>Paeonia stufifruitcosa</i>	Japan	H4619	AB593731	AB594799	
<i>S. cornii</i>	<i>Cornus</i> sp.	Italy	MFLUCC 14-0467	KR559739	KT162918	
<i>S. discosporioides</i>	<i>Punica granatum</i>	Japan	H 4621	AB593732	AB594800	
<i>S. elegans</i>	<i>Melaleuca ericifolia</i>	Japan	NBRC 32674	AB593733	AB594801	
<i>S. eucalypti</i>	<i>Eucalyptus smithii</i>	South Africa	CBS 115131	JN871209	JN871200	
<i>S. falcatum</i>	<i>Eucalyptus alligatrix</i>	Australia	CPC 13580	JN871214	JN871205	
<i>S. ficeae</i>	<i>Ficus</i> sp.	China	MFLUCC 15-0519	KR920686	KR092800	
<i>S. foliicola</i>	<i>Juniperus phoenicea</i>	Japan	NBRC 32676	AB593734	AB594802	
<i>S. glandigenum</i>	<i>Fagus sylvatica</i>	Japan	NBRC 32677	AB593735	AB594803	
<i>S. grevilleae</i>	<i>Protea</i> sp.	South Africa	ICMP 10981	AF382372	AF405304	
<i>S. hakeae</i>	<i>Pteridium aquilinum</i>	Japan	NBRC 32678	AB593736	AB594804	
<i>S. hypericinum</i>	<i>Hypericum</i> sp.	Japan	NBRC 32647	AB593737	AB594805	
<i>S. mariae</i>	<i>Correa reflexa</i>	Japan	NBRC 32681	AB593740	AB594807	
<i>S. obtusum</i>	<i>Corymbia henryi</i>	Australia	CPC 12935	JN871215	JN871206	
<i>S. parastiticum</i>	<i>Physocarpus amurensis</i>	Japan	NBRC 32682	AB593741	AB594808	
<i>S. pistocarp</i>	<i>Physocarpus opulifolius</i>	Russia	MFLUCC 14-0625	KT198723	KT198722	
<i>S. pistaciae</i>	<i>Pistacia vera</i>	Iran	CBS 138865	KP004491	KP004463	
<i>S. rhombisporium</i>	<i>Vaccinium myrtillus</i>	Italy	MFLUCC 15-0543	KR092780	KR092792	
<i>S. rosae</i>	<i>Rosa kalmiussica</i>	Russia	MFLUCC 14-0621	KT198727	KT198726	
<i>S. vitis</i>	<i>Vitis</i> sp.	Iran	IRAN2427C	KU162942	KU162941	
<i>S. vitis</i>	<i>Vitis</i> sp.	Iran	IRAN2454C	n.s.	KU648401	
<i>S. vitis</i>	<i>Vitis</i> sp.	Iran	IRAN2455C	n.s.	KU648402	
<i>S. vitis</i>	<i>Vitis vinifera</i>	Italy	MFLUCC 14-0051	KR920362	KR920363	
<i>S. walkeri</i>	<i>Eucalyptus</i> sp.	Australia	CPC 17644	JN871216	JN871207	

¹ Sequences in bold face were generated during the present study.² n.s.: Not sequenced.

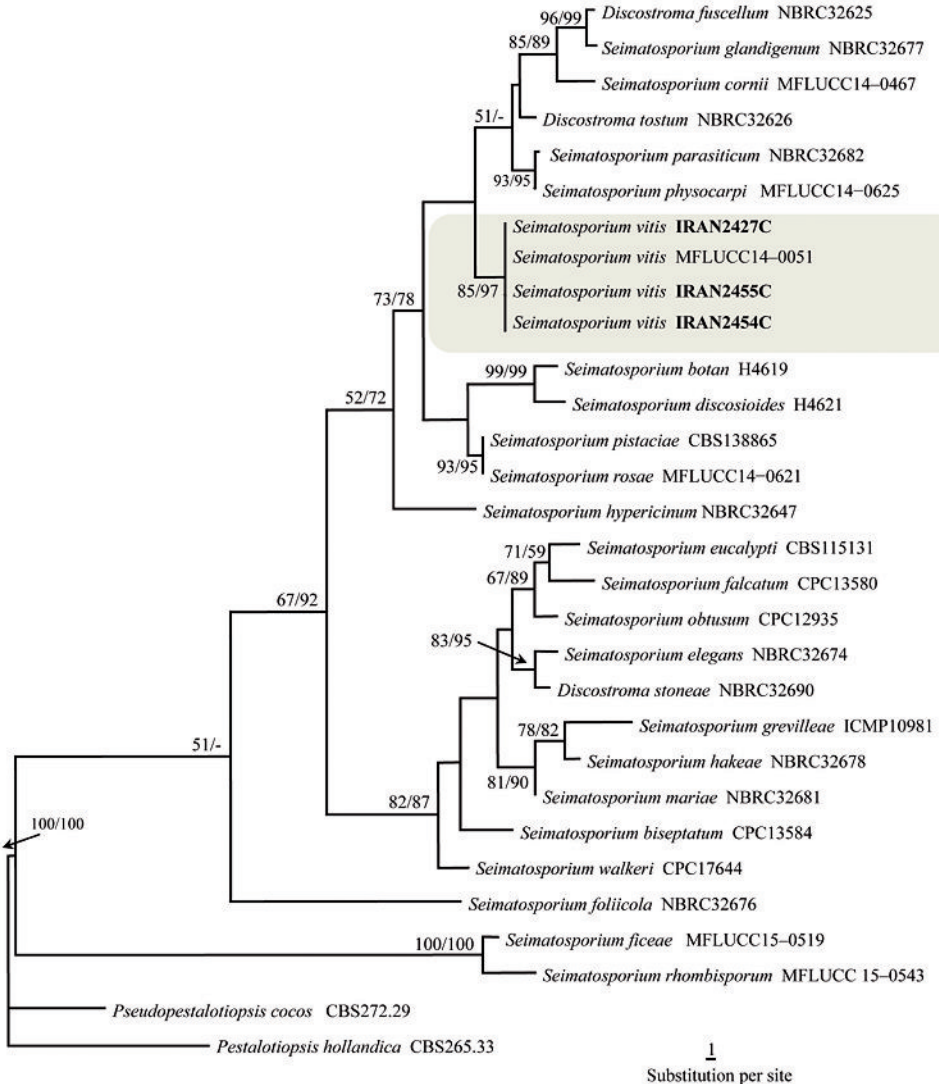


Fig. 1. One of the nine most parsimonious trees for *Seimatosporium* species based on a combined dataset of LSU and ITS sequence data. MP/NJ bootstrap support values from 1000 replicates higher than 50% are given at the nodes. Isolates from Iran are in boldface.

tissue surface, 100-600 μm diameter. Conidiomata wall multi-layered, outer wall thick, composed of brown cells of *textura angularis*, inner wall thin, hyaline. Conidiophore reduced to conidiogenous cells, arising at the base of the cavity of the conidiomata, sometimes branched at the base. Conidiogenous cells hyaline, smooth, cylindrical, $15\text{-}44 \times 1\text{-}1.5 \mu\text{m}$ long. Conidia ellipsoid to fusiform, straight to slightly curved, 3-septate (on MEA and PDA 3(4-6)-septate), slightly constricted at the

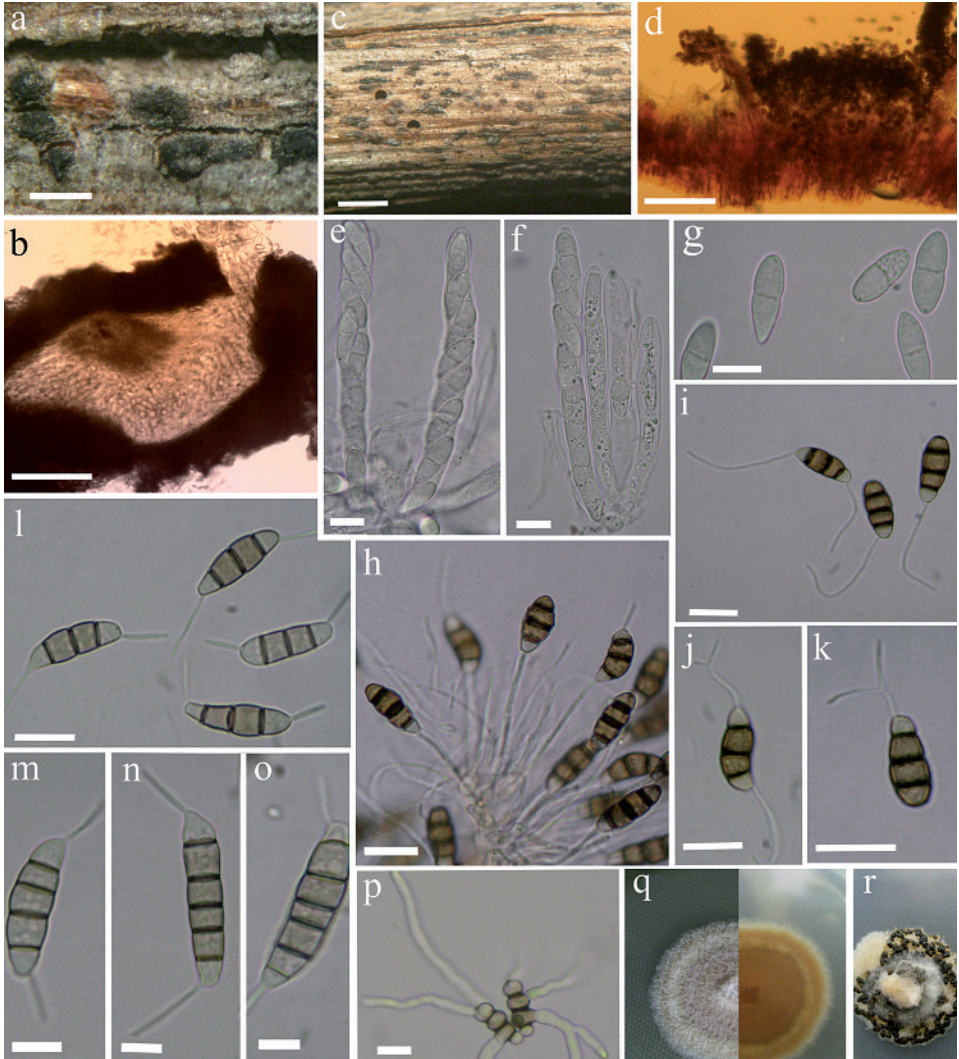


Fig. 2. *Seimatosporium vitis* from *Vitis* sp.: **a.** Habit of ascomata on bark; **b.** ASCOMA in longitudinal section; **c.** Habit of conidiomata on bark; **d.** CONIDIOMA in longitudinal section; **e-f.** Asci; **g.** Ascospore; **h.** Developing conidia attach to conidiogenous cells on natural substrate; **i-k.** Conidia on natural substrate; **l-o.** Conidia on MEA; **p.** Germinating spore on MEA; **q.** Colony morphology on MEA from above(left) and below(right); **r.** Colony morphology on PDA. Bars: a = 500 μm . b,d = 100 μm . c = 1 mm. e-l = 10 μm . m-o = 5 μm . p = 10 μm .

septa, (11-)13-16(-17) \times 5-6 (\bar{x} = 14.7 \times 5.6, n=30) μm on natural substrate (on MEA; 15-25 \times 4.5-5.5 μm , \bar{x} = 18.4 \times 4.8 μm , n=20), basal cell obconic with a truncate base, with an appendage, hyaline to subhyaline, 2.5-3.7 (\bar{x} = 3.1) μm long; 2 median cells subcylindrical to doliiform, slightly thick-walled, smooth, brown to dark brown, with septa darker than the rest of the cell, together 7.3-9.8 (\bar{x} = 8.6) μm long, (the second cell from the base 3.3-5 (\bar{x} = 3.7) μm long, the third cell 3.4-5.8 (\bar{x} = 4.2) μm long); the apical cell conical with a rounded or acute apex, hyaline to subhyaline or concolorous with the central cells, 3-4 (\bar{x} = 3.6) μm long; with or without 1 tubular apical filiform appendage (on MEA and PDA always present), sometimes branched, arising from the apex of the apical cell, 10-22 (\bar{x} = 16) μm long, basal appendage present, filiform, tubular, single, sometime branched, eccentric, 7-30 (\bar{x} = 19.7) μm long.

Cultural characteristics: On MEA circular with regular margin, white to primrose (23''b), cottony, reverse sienna (13i), reaching 20 cm in 7 days at 24°C. On PDA white to primrose (23''b) from above, white to primrose (23''b) from below, circular, cottony, with abundant black acervuli, reaching 10 cm in 7 days at 24°C.

Specimen examined: IRAN, East Azerbaijan Province, Arasbaran, on dead branches of *Vitis* sp., 11 July 2015, M. Mehrabi, IRAN 16717F, IRAN 2427C, IRAN 2454C, IRAN 2455C.

DISCUSSION

Based on phylogenetic analysis of LSU and ITS sequence data our isolates were placed in a strongly supported clade containing the type strain of *Seimatosporium vitis*. *Seimatosporium vitis* was recently described from *Vitis* plants in Italy based solely on the asexual morph, but although sequence data were produced, the species was not included in the phylogenetic analyses based on LSU and ITS sequence data (Senanayake *et al.* 2015). In terms of phylogeny we show here that it is completely distinct from all other *Seimatosporium* species (Fig. 1). *Seimatosporium vitis* resembles *D. ficicola* by having ellipsoid and hyaline ascospores of similar size and with one septum. *D. ficicola* was described based on sexual morph on leaves of *Ficus pleurocarpa* from Australia (Paulus *et al.*, 2006). Since there is no information on the asexual morph and no DNA sequence data for *D. ficicola*, it is not known whether they are conspecific. According to Paulus *et al.* (2006), *S. vitis* is also similar to *D. hyperboreum* in terms of ascospore size. However the latter has wider ascospores (14-17 \times 7-8 μm vs. 14-20 \times 5-7 μm).

In pestalotioid fungi conidial dimension, number of septa, shape and color and presence and morphology of the appendages are the most important characters to differentiate the isolates at the species level. In the absence of a standard procedure for morphological studies, it is important to realize that morphology is affected by the nature of substrates and environmental factors (Hatakeyama & Harada, 2004). Senanayake *et al.* (2015) have described *S. vitis* based on fungal structures on natural substrate. Dimensions of conidia on natural substrate in our isolates are distinctly smaller (13-16 \times 5-6 μm) compared to the conidial dimensions (34-40 \times 14-17 μm) measured by Senanayake *et al.* (2015). But, we strongly recommend checking the measures presented by Senanayake *et al.* (2015) because there is no compliance between scales and conidial size. Moreover they have mentioned that conidia bear a single appendage at the basal cell. In our isolates we observed both apical and basal appendages both on conidia from natural substrate and conidia on MEA and

PDA culture media, even though we observed rare conidia from natural substrate without any apical appendage. On MEA and PDA at 24°C, the fungus produced pale brown conidia with mostly 3 (rarely 4-6) septa, whereas on natural substrate conidia only had 3 septa. The conidia produced on artificial media were also longer than those on host tissue (15-25 × 4.5-5.5 µm vs. 13-16 × 5-6 µm). Our results suggest therefore that the number of septa and size of conidia are affected by the substrate (see Figs 2 i-o).

As mentioned by Senanayake *et al.* (2015) this species is a first *Sematosporium* member reported on *Vitis* and to the best of our knowledge this is the second report of this species on *Vitis* sp. and its first report from Iran. In this survey we have found the isolates as saprophyte on dead branches of *Vitis* sp., but it is important to examine possible pathogenicity of this species in future works.

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