



## A new species and a new record of Diatrypaceae from Iran

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### Abstract

Two species of Diatrypaceae (Xylariales) are described and illustrate from Iran. *Diatrypella iranensis* from dead branches of *Quercus brantii* is described as a new species based on both morphology and molecular sequence data. It differs from other members of the genus on the basis of stroma morphology and ascus and ascospore sizes. Molecular data of the ITS rDNA region show that the new species is a sister taxon of *Diatrypella quercina*. *Cryptovalsa ampelina* is described from dead branches of *Juglans regia* and is a new record from Iran. This study is the first in a series that investigate the diversity of Diatrypaceae from Iran.

**Key word** – *Cryptovalsa* – *Diatrypella* – Iran – Taxonomy

### Introduction

According to the Dictionary of fungi (Kirk et al. 2008), the Diatrypaceae is a family of the Xylariales order within the Ascomycota phylum. The family contains 13 genera and 229 species and the most common diatrypaceous genera consist of *Cryptosphaeria* Ces. & De Not. *Cryptovalsa* (Ces. & De Not.), *Diatrype* Fr., *Diatrypella* (Ces. & De Not.) De Not., *Eutypa* Tul. & C. Tul., and *Eutypella* (Nitschke) Sacc. Morphologically, the member of Diatrypaceae are characterized by perithecial ascomata, long stalked asci and allantoid ascospores (Glawe & Rogers 1984).

Several species in this family have been shown to cause plant diseases. *Eutypa lata* (Pers.) Tul. & C. Tul. (syn: *E. armeniacae* Hansf. & Carter), the causal agent of *Eutypa* dieback, is the best known plant pathogen in this family. This fungus has a cosmopolitan distribution and occurs on many woody plant species including cultivated plants such as grapevine, apricots and cherries (Carter 1991, Trouillas & Gubler 2004). Other diatrypaceous taxa, such as *Cryptosphaeria lignyota* (Fr.) Auersw. (Hinds 1981), *Cryptosphaeria pullmanensis* Glawe (Trouillas & Gubler 2010), *Cryptovalsa ampelina* (Nitschke) Fuckel (Mostert et al. 2004, Luque et al. 2006, Martín et al. 2009, Trouillas & Gubler, 2010), *Eutypa leptoplaca* (Mont.) Rappaz (Trouillas & Gubler 2004, 2010), *Eutypella parasitica* R.W. Davidson & R.C. Lorenz (Davidson & Lorenz 1938) and *Eutypella vitis* (Schwein.) Ellis & Everh. (Catal et al. 2007, Jordan & Schilder 2007, Úrbez-Torres et al. 2009, 2011) also were shown to cause canker diseases and wood decay in various woody plants.

A recent review on Diatrypaceae was provided by Trouillas et al. (2011). Additional detailed reviews on the taxonomy of the family and the generic concepts are given by Tiffany & Gilman (1965), Glawe & Rogers (1984), Rappaz (1987), Vasilyeva & Stephenson (2004, 2005) and Carmarán et al. (2006). Rappaz (1987) revised the taxonomy of octosporous diatrypaceous fungi

and eight genera were recognized. Glawe & Rogers (1984) and Tiffany & Gilman (1965) studied this family in North America and reported various species of the Diatrypaceae in this area. Vasilyeva & Stephenson (2004, 2005, 2006, 2009) described various species of the Diatrypaceae from the Great Smoky Mountains National Park in the eastern United States, as well as Arkansas and Texas. Acero et al. (2004) presented the first phylogenetic classification of these fungi based on ITS rDNA sequence data. Parsimony analysis established the existence of nine phylogenetic groups and none of the five main diatrypaceous genera including *Cryptosphaeria*, *Diatrype*, *Diatrypella*, *Eutypa* and *Eutypella* were found to be monophyletic. Also, the phylogenetic relationships also did not correlate well with classical taxonomic schemes. Romero & Carmaran (2003) and Carmaran et al. (2009) provided new records as well as new species of the Diatrypaceae from Argentina. Grassi et al. (2014) investigated the extracellular production of ligninolytic enzymes by xylariaceous and diatrypaceous species from Argentina and described a new species of *Eutypa* genus. Trouillas & Gubler (2004) and Trouillas et al. (2010a,b, 2011) also reported and described many other diatrypaceous species occurring on *Vitis vinifera* and other woody plants in California and Australia based on both morphological studies and DNA sequence analyses. In Panama, Chacon et al. (2013) reported and described a new species and a new record of the Diatrypaceae. In north-eastern China, Vasilyeva & Ma (2014) described 13 species belonging to the genera *Diatrype* and *Cryptosphaeria*.

There are few publications on the Diatrypaceae from Iran. Records of fungal species known in Iran have been compiled by Ershad (2009) who listed 4 species in the third edition of "Fungi of Iran". These included *Cryptovalsa mori* (Nitschke) Lar. N. Vassiljeva, *Diatrype stigma* (Hoffm.) Fr., *Eutypa lata* and *Eutypella iranica* Petr. Occurrence of anamorphic states of this family in Iran was reported by Mehrabi & Hemmati (2012). Overall, little information is available on the occurrence and diversity of Diatrypaceae in Iran. This study is the first in a series to investigate the diversity of the Diatrypaceae in Iran using morphological and molecular data.

## **Materials & methods**

### **Fungal isolates**

During April and May 2013, two diatrypaceous species were collected on dead branches of *Quercus brantii* Lindl. and *Juglans regia* L. from Iran. Isolates were grown from a single ascospore using the method of Trouillas et al. (2010a). Dry specimens were deposited in the herbarium of Iranian Research Institute of Plant Protection (IRAN...F), and fresh cultures were deposited at the Iranian Fungal Culture Collection (IRAN...C) at the same institute.

### **Identification and morphological studies**

To identify the diatrypaceous species collected in this study, we compared the morphology of our specimens with the descriptions of Glawe & Rogers (1984), Croxall (1950), Vasilyeva & Stephenson (2005) and Trouillas et al. (2010a). Identifications of species were based on morphological features of the sexual morph fruiting bodies produced on infected plant tissues. For this purpose, thin cross sections were prepared by hand from fruiting bodies. Morphological features of sexual morph such as size of stromata, size and number of perithecia, presence or absence of black zone, size of asci and ascospores, were used for the identification of fungi. Microscopic analyses and photography were conducted using standard light microscopy with a Nikon 80i microscope and a Canon digital camera. Stromal and perithecial characteristics were examined using an Olympus (SZH) stereo microscope. Examinations and measurements of microscopic characters were carried out in water. Thirty ascospores, twenty asci and thirty perithecia of each species isolate were measured.

### **DNA extraction and sequencing**

Isolates were obtained from single ascospore and pure cultures were grown on potato-dextrose agar at 25° C. To proceed with DNA extraction, isolates were grown in liquid cultures of potato dextrose broth for 7-14 days at 25 C on a shaker at 120 rpm. Mycelia were then vacuum

filtered and subsequently stored at -20° C. Genomic DNA for PCR was isolated by method of Liu et al. (2000). Sequences of the nuclear internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) were obtained following amplification by polymerase chain reaction (PCR) using primers ITS1 and ITS4 (White et al. 1990). All reactions were performed on a PC-320 PCR System (ASTECCo., Japan), following the temperature profiles described in Stewart et al. (2006) for the ITS region. The PCR products were purified and sent out for sequencing in both directions (Macrogen company, South Korea).

### Sequencing alignment and phylogenetic analysis

The newly obtained nucleotide sequences were proofread and edited with FinchTV v. 1.4.0 (Geospiza Inc.). The sequences obtained were compared with those in the GenBank databases using the BLAST program. Sequences retrieved from GenBank and those in this study were consequently manually aligned in the program Molecular Evolutionary Genetic Analysis (MEGA) v5 (Tamura et al. 2011). DNA sequences were aligned with Clustal W (Thompson et al. 1994), within the MEGA5. Phylogenetic analyses of the aligned dataset were performed with neighbor joining (NJ) and maximum likelihood (ML) analyses, also within the MEGA5 package. Based on the Bayesian information criterion of MEGA 5, Kimura 2-parameter model with gamma distribution (K2 + G) was selected for the NJ and ML analysis. The NJ and ML trees were obtained using the default settings with “partial deletion” for gaps/missing data treatment. A bootstrap analysis was conducted with 1000 replication. *Xylaria hypoxylon* was used as the outgroup. The sequences from our study were deposited in GenBank under the accession number KJ767718 for *Cryptovalsa ampelina* and KM245033 for *Diatrypella iranensis*.

**Table 1** Isolates used in this study with the GenBank accession numbers of the ITS rDNA sequences\*

Species	Strain no.	GenBank accession no.
<i>Cryptosphaeria ligniota</i> (Fr.) Auersw.	C2C	AJ302418
<i>Cryptosphaeria pullmanensis</i> Glawe	UCD2373NV	GQ293967
<i>Cryptosphaeria subcutanea</i> (Wahlenb.) Rappaz	A56B	JQ781795
<i>Cryptovalsa ampelina</i> (Nitschke) Fuckel	A001	GQ293901
<i>Cryptovalsa ampelina</i> (Nitschke) Fuckel	CBS 117485	AY920391
<b><i>Cryptovalsa ampelina</i> (Nitschke) Fuckel</b>	<b>KHJ20</b>	<b>KJ767718</b>
<i>Cryptovalsa rabenhorstii</i> (Nitschke) Sacc.	WA08CB	HQ692619
<i>Diatrype bullata</i> (Hoffm. : Fr.) Fr.	CBS 215.87	AJ302422
<i>Diatrype disciformis</i> (Hoffm. : Fr.) Fr.	D8M	AJ302437
<i>Diatrype prominens</i> Cooke & Harkn.	ATCC MYA-4410	FJ430594
<i>Diatrype spilomea</i> Syd.	D17C	AJ302433
<i>Diatrype stigma</i> (Hoffm. : Fr.) Fr.	F-091,970	AJ302435
<i>Diatrype undulata</i> (Pers. : Fr.) Fr.	CBS 271.87	AJ302436
<b><i>Diatrypella iranensis</i></b>	<b>KDQ18</b>	<b>KM245033</b>
<i>Diatrypella favacea</i> (Fr.) Ces. & De Not.	R191	JN689955
<i>Diatrypella frostii</i> Peck	ATCC 52484	AJ302441
<i>Diatrypella prominens</i> (Howe) Ellis & Everh.	DL28A	AJ302442
<i>Diatrypella pulvinata</i> Nitschke	DL29C	AJ302443
<i>Diatrypella quercina</i> (Pers.) Cooke	DL30M	AJ302444
<i>Diatrypella verruciformis</i> (Ehrh.: Fr.) Nitschke	UCROK856	JX144788
<i>Diatrypella vulgaris</i> Trouillas, W. M. Pitt & Gubler	HVGRF03	HQ692590
<i>Eutypa armeniaca</i> Hansf. & Carter	ATCC 28120	DQ006948
<i>Eutypa consobrina</i> (Mont.) Rappaz	CBS:122678	EU552125
<i>Eutypa laevata</i> (Nitschke) Sacc.	OHCS1	KF453548
<i>Eutypa lata</i> (Pers.) Tul. & C. Tul.	PA4	AY462563
<i>Eutypella caricae</i> (De Not.) Berl.	GL08362	JX241652
<i>Xylaria hypoxylon</i> (L.) Grev.	ATCC 42768	AY327477

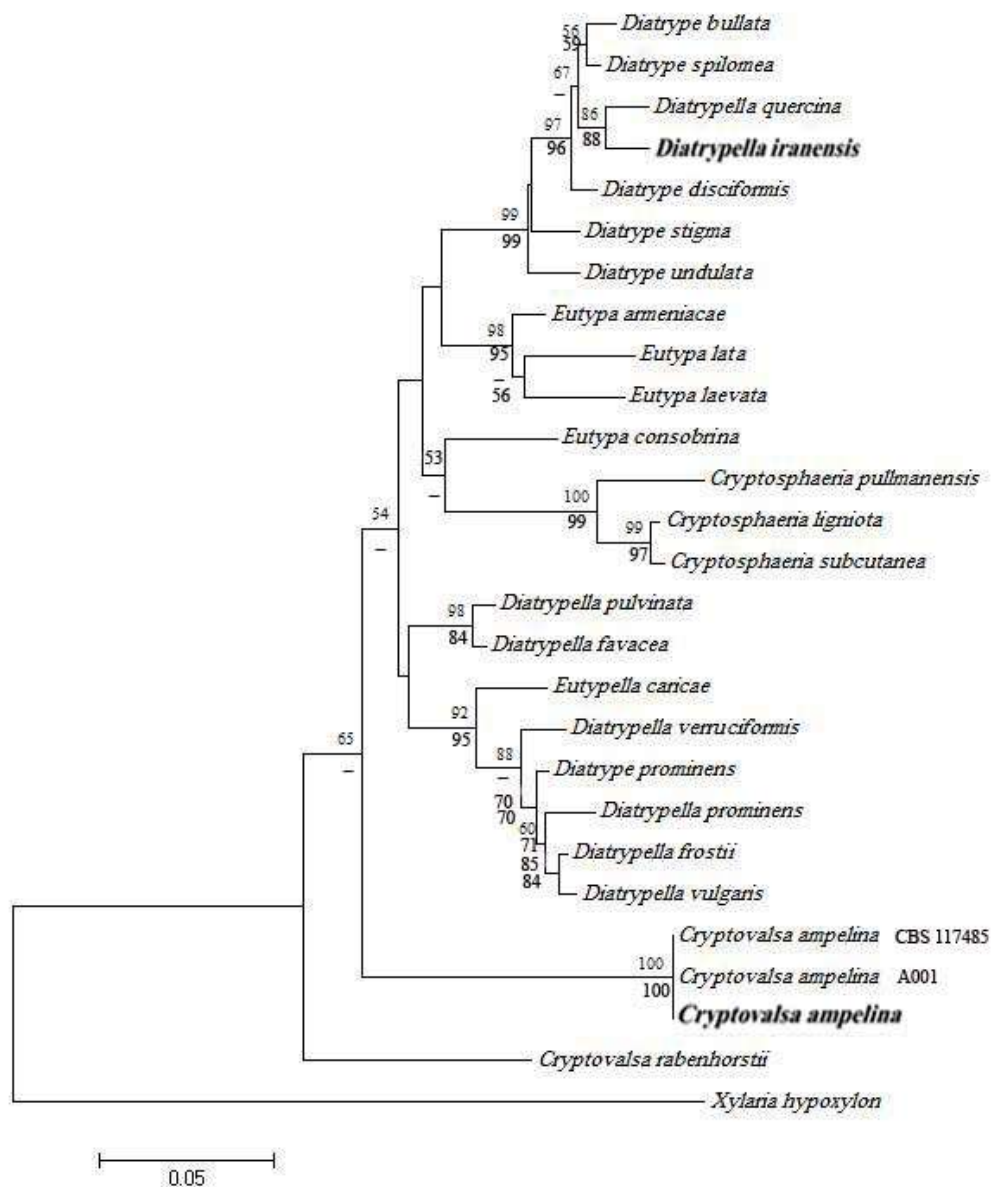
\*Sequences obtained in this study are given in bold.

## Results

### Molecular phylogeny

Sequences of the ITS1-5.8S-ITS2 region were obtained by using primers ITS1 and ITS4 from the specimens of new species and *Cryptovalsa ampelina*. These sequences data were aligned together with sequences available in GenBank (Table 1). The final ITS dataset included 25 taxa and 726 positions, of which 282 were constant. Size of our sequences were 571 bp for the new species and 543 bp for *Cryptovalsa ampelina*. On the phylogenetic tree, the undescribed species was a sister taxon of *Diatrypella quercina* supported with high bootstrap value (Fig. 1).

The neighbor joining and maximum likelihood trees using the ITS region presented similar topologies, and only the neighbor joining tree is presented (Fig. 1). *Diatrypella quercina* and *Diatrypella iranensis* formed an individual clade with *Diatrype* species. Based on both morphology and molecular sequence data, the occurrence of *Cryptovalsa ampelina* in Iran was confirmed with 100% bootstrap values.



**Fig. 1** – Phylogenetic tree for selected species of the Diatrypaceae obtained by neighbor joining analysis based on an alignment of ITS1-5.8S-ITS2 sequence data. Bootstrap values greater than 50% are indicated at the nodes. The new sequences obtained in this study are indicated in boldface. Bootstrap values (in %) above branches are from the neighbor joining analysis (1000 bootstraps) and under branches from maximum likelihood analyses (1000 bootstraps).

## Taxonomy

*Diatrypella iranensis* Mehrabi & Hemmati, **sp. nov.**

Figs 2–6

Mycobank MB 809719

Etymology – Refers to the country where the specimen was found.

Stromata immersed in the bark of dead branches (3 mm diameter), erumpent, circular to ovoid, surface black, finely rugose due to the ostioles of the perithecia, 2–5(2.9) mm diam, delimited by a black zone in the host tissues, with groups of 4–57 perithecia, arranged on a unique layer in the bark, circular to ovoid, 280–650 µm diam, ostioles dark brown, at the same level of the stroma surface or slightly above, 28–100 µm diam, perithecia dark brown, circinate arranged, crowded, globoid to subgloboid, surrounded by whitish yellow entostroma. Asci elongate, subcylindrical to clavate, long-stipitate, (55–)70–110(–130) × 6–9(–10) µm, polyspored; Ascospores allantoids, subhyaline, yellowish in mass, aseptate, (5–)6–7(–8) × 1–1.3(–1.5) µm. Paraphyses elongate, filiform, septate. Colonies white on PDA, cottony, reverse of colonies pale yellow, covering 85 mm Petri dish after 8 days at 24°C.

Anamorph on *Quercus* twig: Acervuli immersed in bark, 500–700 µm diam, flat to subconical, hymenium labyrinthiform, yellow to black, with golden yellow stratum of spores. Spores slender and strongly arcuate, 18–38(–45) × 0.6–0.8 µm

Known distribution – Southwestern Iran

Type – Iran, Sisakht city, on dead branches of *Quercus brantii* Lindl., 9 April 2013, coll. M. Mehrabi, KDQ18 (Holotype, IRAN 16600 F) – Ex-type culture IRAN 2280 C.

ITS sequence – KM245033.

Notes – The genus *Diatrypella* is one of the largest genera of Diatrypaceae and is represented by about 33 species worldwide (Kirk et al. 2008). The delineation of the diatrypaceous genera is often problematic because the characters of the stromata are extremely variable within the same species (Vasilyeva and Stephenson, 2004). The presence of conical or truncate stromata, cushion-like or discoid, usually delimited by a black zone in host tissues, umbilicate or sulcate ostioles with polysporous asci assigned this fungus to the genus *Diatrypella* (Vasilyeva & Stephenson 2005). The new species belongs to the genus *Diatrypella* based on the features mentioned above and resembles in many aspects earlier description of *D. quercina* and *D. pulvinata* (Croxall 1950, Glawe & Rogers 1984). However, the spores of *D. quercina* are longer (8–12 × 2–3 µm vs. 6–7 × 1–1.3 µm) (Croxall, 1950) than those of the new species. In addition, *D. quercina* differs from the new species by marked curvature of the ascospores and wider asci, with sporiferous parts of 64–160 × 8–12 µm. The new species also differs from *D. pulvinata* (Glawe & Rogers 1984) on the basis of stromatal morphology and ascus size. Despite resemblance in stroma morphology, *Diatrypella iranensis* could be clearly distinguished from *D. favacea* because of the unique 6-sulcate ostioles of this last species (Croxall 1950, Vasilyeva & Stephenson 2005).

Sequence data of the ITS region confirm the position of *Diatrypella iranensis* within the genus *Diatrypella* (Fig. 1). Also, phylogenetic analysis of the ITS data set showed that *D. iranensis* formed a unique lineage that differed from all *Diatrypella* species included in the study. In the ITS analysis, *D. iranensis* and *D. quercina* were shown to be sister species, which was supported by moderate bootstrap value (86% in NJ, 88% in ML, Fig.1). *D. iranensis* and *D. quercina* also grouped with other *Diatrype* spp. including *D. bullata*, *D. disciformis* and *D. spilomea*. Previous studies have suggested that *Diatrypella* is the polysporous counterpart of *Diatrype* (Vasilyeva & Stephenson 2005) and the multisporate asci character may have appeared several times during the evolution of the family (Acero et al. 2004). Molecular phylogenetic studies that include more representative taxa and more gene regions may help better resolve such taxonomic relationships.

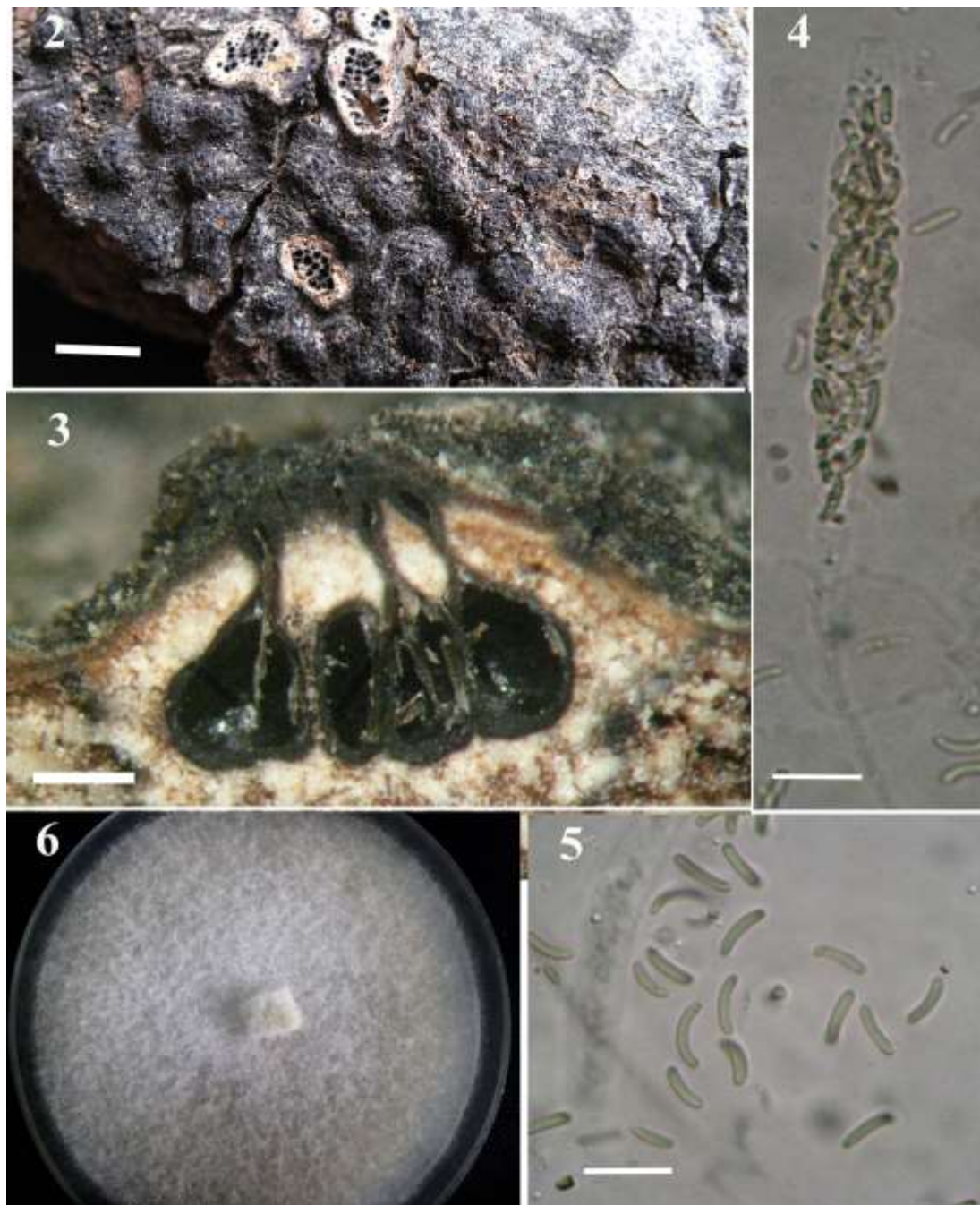
*Cryptovalsa ampelina* (Nitschke) Fuckel, Symbolae Mycologicae, p 212. 1870.

Figs 7–11

Basionym: *Valsa ampelina* Nitschke, Pyrenomycetes Germanici 1, p. 156, 1867.

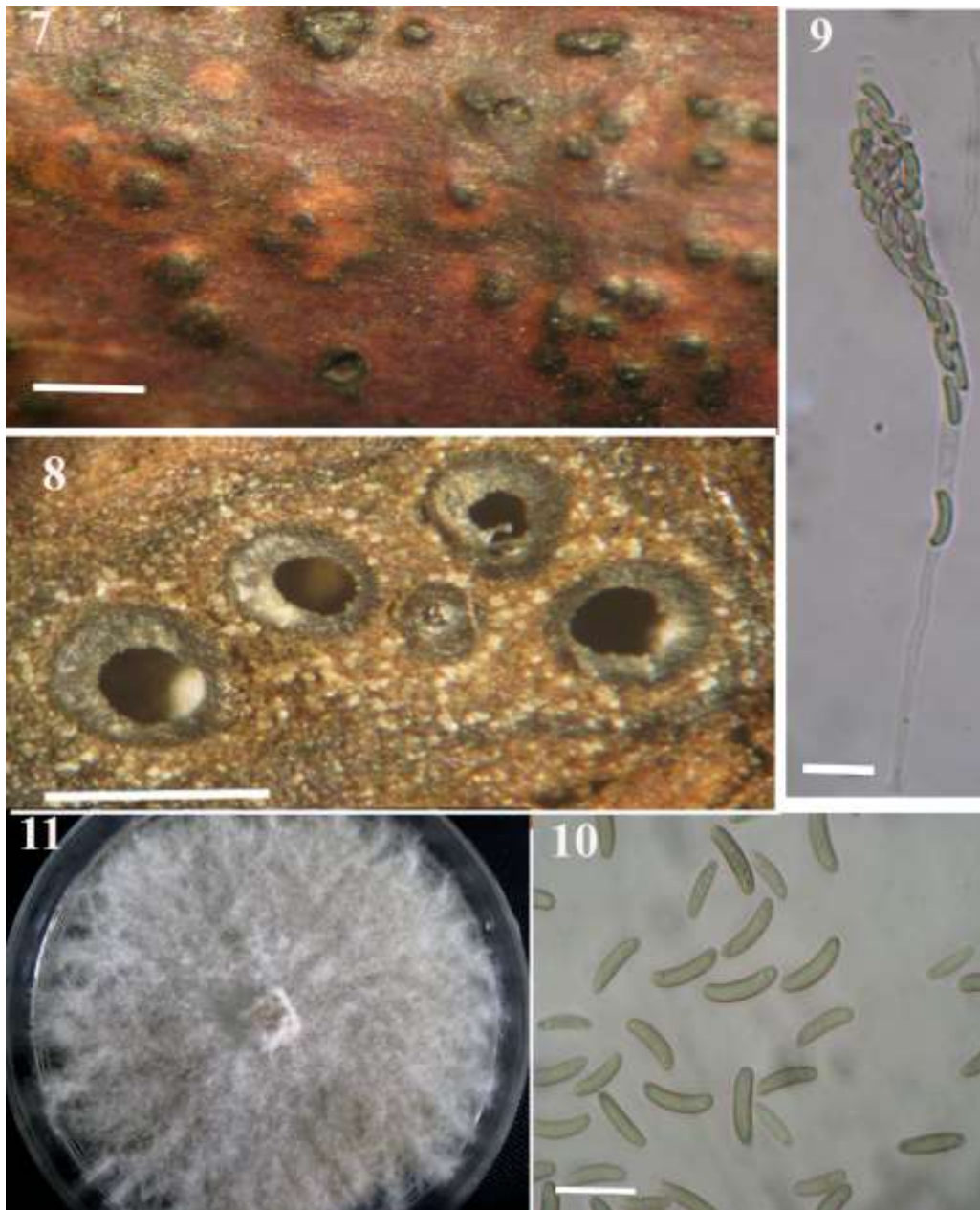
On dead branches 0.5 mm diameter. Stromata immersed in bark, poorly developed, dotted at the surface with visible black zone on the bark surface, circular to ovoid, 0.3–1.5(0.5) mm diam,





**Figs 2–6** – Morphology of *Diatrypella iranensis* from *Quercus brantii*. 2 Habit of ascostromata on bark. 3 Longitudinal section through the stroma shows ostioles and perithecia surrounded in whitish yellow entostroma and ascoma delimited by black zone. 4 Ascus with ascospores. 5 Allantoids ascospores. 6 Colony morphology on PDA. – Bars 2 = 5 mm. 3 = 500  $\mu$ m. 4,5= 10  $\mu$ m.

containing 1–6 perithecia arranged at different depth in the bark, 280–630  $\mu$ m diam, delimited by distinct black zone, discs dark brown to gray, 0.1–0.3 mm diam, ostioles at the same level as the discs or slightly above, dark brown, 28–70  $\mu$ m diam, perithecia dark brown, circinate arranged, globoid to subgloboid, surrounded by yellow to grey entostroma. Asci elongate, subcylindrical to clavate, long-stipitate, (60–)80–120(–140)  $\times$  6–9(–10)  $\mu$ m, polyspored; Ascospores allantoid, rounded at the apex, subhyaline to yellowish brown, aseptate, (6–)8–10(–12)  $\times$  1.8–3  $\mu$ m. Paraphyses absent. Colonies white on PDA, cottony, with diffuse margins, covering 85 mm Petri dish after 7 days at 24°C, reverse later (>20 days) turning dark, beginning from the center of the colony.



**Figs 7–11** – Morphology of *Cryptovalsa ampelina* from *Juglans regia*. 7 Habit of ascostromata on bark with visible black zone on the bark surface. 8 Transverse section through the ascoma shows globose perithecia surrounded in entostroma and ascoma delimited by black zone. 9 Ascus with ascospores. 10 Allantoid ascospores. 11 Colony morphology on PDA. – Bars 7 = 1 mm. 8 = 500  $\mu$ m. 9,10 = 10  $\mu$ m.

Specimen examined – Iran, Kermanshah provinces, Paveh city, on dead branches of *Juglans regia* L., 13 May 2013, M. Mehrabi, KHJ20(IRAN 16599 F, IRAN 2281 C).

Notes – This species is morphologically typical members of the genus *Cryptovalsa* (poorly developed stroma, polysporous asci) and resembles closely with *C. ampelina* (Trouillas et al. 2010a). However, this fungus has a visible black zone on the bark surface that surrounded the globose perithecia inner the host tissue, and also asci are narrower in width. The genus *Cryptovalsa* is similar to *Cryptosphaeria* in stromal morphology (poorly developed) but differs from it by its polysporous asci. This species previously was reported from *Juglans regia* in California, and its pathogenicity to grapevine has been confirmed (Trouillas et al. 2010a, Trouillas & Gubler 2010), although some authors suggested a low virulence (Luque et al. 2006, Mostert et al. 2004). The present record is the first report of this species in Iran.

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