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Occurrence and pathogenicity of *Graphium carbonarium* on elm trees in Iran

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Abstract: In a survey on species diversity and phylogeny of ophiostomatoid fungi associated with bark beetle galleries and decline symptoms on woody hosts in the northwestern region of Iran, nine synnematous fungal isolates with similar growth were isolated from the bark beetle galleries on declining elm trees in Mazandaran, East, and West Azerbaijan provinces. Based on the integration of molecular phylogeny (ITS-rDNA region and *tef1-α* gene sequences) with cultural and morphological characteristics, the isolates were identified as *Graphium carbonarium.* Pathogenicity test using an excised shoot assay revealed that the isolates are pathogenic on *Ulmus carpinifolia*. Our study is the first occurrence and pathogenicity of *G. carbonarium* from bark beetlae galleries on declining elm in the world.

Key words: Elm, *Graphium*, ITS, *tef1-α*

INTRODUCTION

Bark beetles (family *Curculionidae*) are among the most important and well-studied pests of elm trees. They may infest different parts of the plant but most species infest the inner bark or the xylem of trees, in which they spend the majority of their life cycle. During their feeding on the tree's inner bark, bark beetles carve galleries (Biedermann and Vega 2020). A diverse fungal flora grows within their galleries including ascomycetes, basidiomycetes, and zygomycetes. Ascomycetes represent the best-known fungal assemblages, associated with bark beetles (Linnakoksi et al. 2012). The ophiostomatoid fungi are well recognized for their symbiotic relationship with bark beetles. They are a polyphyletic group with similar morphology, ecology, and taxonomic history belonging to the phylum *Ascomycota*. The ophiostomatoid fungi are now either placed in the order *Ophiostomatales* (*Sordariomycetidae*), which includes one family (*Ophiostomataceae*), or in the order *Microascales* (*Hypocreomycetidae*), which includes four families (including *Microascaceae*, *Ceratocystidaceae*, *Gondwanamycetaceae*, and *Graphiaceae*). Identification of ophiostomatoid fungi based solely on morphological characteristics can be challenging since many similar characteristics have evolved independently in the various taxa, as a result, classification of ophiostomatoid fungi is complex and problematic. Presently, molecular sequence data are usually used to clarify the phylogeny of species belonging to this group (Aas 2007).

The genus *Graphium* (*Graphiaceae*, *Microascales*) was established by Corda (1837) with *G. penicilliaides* as the type species. Species in this genus are characterized by dark synnematous conidiophores that produce one-cell conidia in slimy masses at their apices. *Graphium* spp. has been reported from soil, plants, and bark beetle galleries (Jacobs et al. 2003).

Graphium carbonarium Paciura, Z.W. de Beer, X.D. Zhou & M.J. Wingf. was first identified associated with a *Pissodes* sp. on *Salix babylonica* (Paciura et al. 2010). *Graphium carbonarium* was isolated from *Ricinus communis* in China and *Acacia auriculiformis* trees in Vietnam (Lynch et al. 2016). This species has previously been reported as a pathogen of walnut trees (*Juglans regia*) in Iran (Sohrabi and Mohammadi 2020).

In this study, eight isolates of a *Graphium* species were isolated from elm trees in north and northwestern Iran. Thus, this work in here aimed to identify and characterize the pathogen associated with bark beetle galleries and decline symptoms using morphological studies, phylogenetic analyses based on ITS-rDNA sequence and (*tef1-α*) gene, and pathogenicity tests.

MATERIALS AND METHODS

Isolation and purification of fungi

In 2019, tree outer bark samples were collected from bark beetle galleries of elm trees (*Ulmus* sp.) showing

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general decline symptoms, with evident symptoms of bark beetle damage from Mazandaran, East, and West Azerbaijan provinces. For fungal isolation, bark samples were kept in a moist chamber for up to 48 hours and then inspected under a stereomicroscope. Aerial mycelia and or spore masses formed on synnemata were directly transferred onto PDA media (potato dextrose agar). In cases, fungal structures were absent, small samples of tree outer bark with galleries, approximately 0.5-1 cm were cut and, surface sterilized for 40–45 s in 70% ethanol and rinsed with sterile water three times and dried on sterile filter paper. Samples placed on a PDA culture medium supplemented with 100 mg l^{-1} Streptomycin sulfate and incubated at 25 °C for 2 weeks. Pure cultures were preserved on PDA in 2 ml microtube slants at 4 °C in the Culture Collection of Tabriz University (CCTU; Tabriz, Iran).

Morphological characterization

The morphological features of the isolates were determined following the protocol of Pacuira et al. (2010) and Jacobs et al. (2003). Fungal isolates were grown on malt-extract agar (MEA) (20 g Merck malt extract, 16 g agar, 1000 mL water) and incubated at 25 °C. The color description was made using Rayner's colour chart (1970). Microscopic features were determined using lactic acid mounts and photographed by Olympus digital camera system DP21 (Olympus Corporation, Japan) mounted on Olympus BX41 (Olympus Corporation). Thirty measurements were made for each structure where possible. Comprehensive morphological description and illustrations are provided for this species.

DNA extraction, PCR and Sequencing

Genomic DNA was extracted from the mycelia of fungal colonies grown on PDA (25 °C for 8–10 days), according to Moller et al. (1992). DNA sequencing data was obtained from the partial amplification and sequencing of two locus including the internal transcribed spacers (ITS) region of ribosomal DNA (White et al. 1990) and *tef1-α* gene (Jacobs et al. 2003), respectively based on ITS1/ITS4 and EF1-728F/EF2R primer pairs. The amplification was performed by Bio RAD-Mj Mini thermal cycler in a total volume of 25 µL. PCR mixture contained 12.5 µL of Taq DNA Pol (2x) Master Mix (Pishgam, Tehran), 0.2 µM of each forward and reverse primer, and 50–60 ng of DNA template. The cycling condition consisted of 5 min at 94 °C for primary denaturation, followed by 36 cycles of denaturation at 94 °C for 1 min, an annealing step at 52 °C and 56 °C, respectively, for ITS and *tef1-α* for 1 min, primer extension at 72 °C for 1 min and final extension at 72 °C for 7 min. PCR products were sent to Microsynth Company (The Swiss DNA Company, Bern, Switzerland) for sequencing.

Phylogenetic analysis

The dataset for ITS and *tef1-α* sequences was selected for phylogenetic analyses from GenBank and recent publications (Cruywagen et al. 2010). GenBank accession numbers for sequences used in the current research are presented in Supplementary Table 1. DNA Dragon v. 1.6.0 (Hepperle 2017) and BioEdit v. 5.0.6 (Hall 1999) software were used to create consensus sequences from the forward and reverse sequences. The collected sequences, together with sequences obtained in this study were aligned by MEGA 7 (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2016). The aligned sequences were concatenated with Mesquite v. 3.10 (Maddison & Maddison 2015). The significant evolutionary models were achieved for each gene partition using MrModeltest v. 2.3 (Nylander 2004). To determine the phylogenetic placement of this isolate in the genus, concatenated alignment was analyzed using Bayesian inference by MrBayes v. 3.2.1 software (Ronquist & Huelsenbeck 2003). using the GTR+G model with a heating parameter set at 0.15, and two MCMC running up to 1×10^7 generations and sampling trees every 1×10^3 generations. Satisfactory convergence was assessed using the standard deviation of split frequency. The first 25% of saved trees were discarded as the burn-in, and consensus trees and their posterior probabilities (PP) were determined from the remaining trees. The generated phylogenetic trees were examined using FIG TREE v. 1.3.1 (Rambaut 2009). *Scedosporium boydii* CBS 101.22 was used as an outgroup taxon in this analysis.

Pathogenicity test

Pathogenicity tests were conducted by using an excised twig method. The representative isolate was grown on PDA and incubated at 25°C in the dark for seven days before inoculation. Two-year-old woody branches were cut from healthy elm trees (*Ulmus carpinifolia* Borkh.) in the Tabriz region. Selected branches (approximately 30 to 35 cm in height) were surface-sterilized with 75% ethanol for 1 min and rinsed 3 times in sterile distilled water. Branches were subsequently left to dry under sterile conditions. Using a sterile scalpel, a 0.5 cm diameter incision was conducted at the mid-point of each twig. Each wound was inoculated by inserting a mycelial plug of the isolate and sterile PDA plugs were used to inoculate the control plants. All inoculated wounds wrapped with Parafilm (Pechiney Plastic Packaging) to prevent desiccation and kept in plastic containers containing a small beaker of sterile distilled water to keep the relative humidity high. One month after inoculation, twigs were checked and superficial bark surrounding the inoculated wounds was removed and the produced lesion lengths and extent of vascular discoloration were measured. The experiment was carried out using the representative fungal isolate in four replicates. Fungal re-isolations from inoculated branches were attempted by cutting small pieces of infected wood samples from the edge of each lesion and placing them on PDA. The identity of the re-isolated fungi was confirmed morphologically.

Table. 1. GenBank accession numbers of sequence data used in phylogenetic analysis**.**

Taxon	Strain	GenBank accession number		Reference
		ITS	*tef1-α*	
Graphium adansoniae	CMW 30620	GQ200613	HM630597	Cruywagen et al. 2010
Graphium basitruncatum	JCM 9300	AB038427	KJ131248	Sohrabi & Mohammadi 2020
Graphium carbonarium	CMW 12420	FJ434979	HM630603	Cruywagen et al. 2010
Graphium carbonarium	**P23I1**	**OQ916160**	**OQ944974**	**Present study**
Graphium euwallaceae	UCR 2308	KM592371	KM592363	Lynch et al. 2016
Graphium fimbriisporum	CMW 5605	AY148177	HM630590	Cruywagen et al. 2010
Graphium fimbriisporum	CMW 5606	AY148180	HM630591	Cruywagen et al. 2010
Graphium larics	CMW 5601	AY148183	HM630588	Cruywagen et al. 2010
Graphium larics	CMW 5603	AY148182	HM630589	Cruywagen et al. 2010
Graphium madagascariense	CMW 30628	GQ200619	HM630595	Cruywagen et al. 2010
Graphium madagascariense	CMW 30629	GQ200620	HM630594	Cruywagen et al. 2010
Graphium penicillioides	CMW 5292	HQ335310	HM630600	Cruywagen et al. 2010
Graphium penicillioides	CMW 5295	HQ335311	HM630601	Cruywagen et al. 2010
Graphium pseudormiticum	CMW 503	AY148186	HM630586	Cruywagen et al. 2010
Scedosporium boydii	CBS 101.22	AM887718	EF151369	Sohrabi & Mohammadi 2020

RESULTS

A total number of nine fungal isolates with the same growth pattern were isolated from bark beetle galleries on declining elm trees in the Tabriz region (five isolates), Mardanagom (East Azerbaijan province; one isolate), Firouragh (West Azerbaijan province; two isolates) and Sari (Mazandaran province; one isolate). The isolate (P23I1; collected from the Tabriz region) was selected for phylogenetic analysis.

In the phylogenetic analysis of *Graphium* spp., ITS-rDNA alignment consisted of 15 samples and 476 sites (including alignment gaps), and the *tef1-α* alignment consisted of 15 samples and 584 sites (including alignment gaps). The final concatenated alignment comprised 15 taxa and 1060 sites (locus boundaries: 1-476 and 477-1060 for ITS-rDNA and *tef1-α*, respectively). The Bayesian analysis started from a random tree topology and lasted in 96000 generations and a total of 1902 trees (Each file contained 951 trees). After discarding the first 25% of sampled trees, the remaining 1428 (75%) trees were used to calculate the consensus trees and posterior probabilities. In the phylogenetic assessments based on a combined ITS-rDNA and *tef1-α* sequence dataset, our isolate was nested in a well-supported clade together with the reference strain of *G. carbonarium* (CMW 12420) (Fig. 2).

TAXONOMY

Graphium carbonarium Paciura, Z.W. de Beer, X.D. Zhou & M.J. Wingf. Fungal Diversity 40 (1): 85 (2010)

Colonies on MEA (2%) attained a diameter of 35 mm after 14 days of incubation at 25 °C in the dark. Colonies on MEA, dark gray olivaceous, flat, and usually cottony with smooth aerial mycelium and a white, regular margin. The isolates developed abundant synnemata on MEA after two weeks. Synnemata were septate, erect, singly or in clusters, stalk dark brown, becoming hyaline at the end, and 80–220 µm in length and 6–20.5 µm in wide at the center and terminated in a slimy head of conidia by 19-65 µm wide. Conidia unicellular, hyaline, cylindrical with truncated bases, 3–7.5 × 1–2.5 µm that aggregate in a hyaline mucilaginous mass at the apices of the synnemata. *Scedosporium*-like

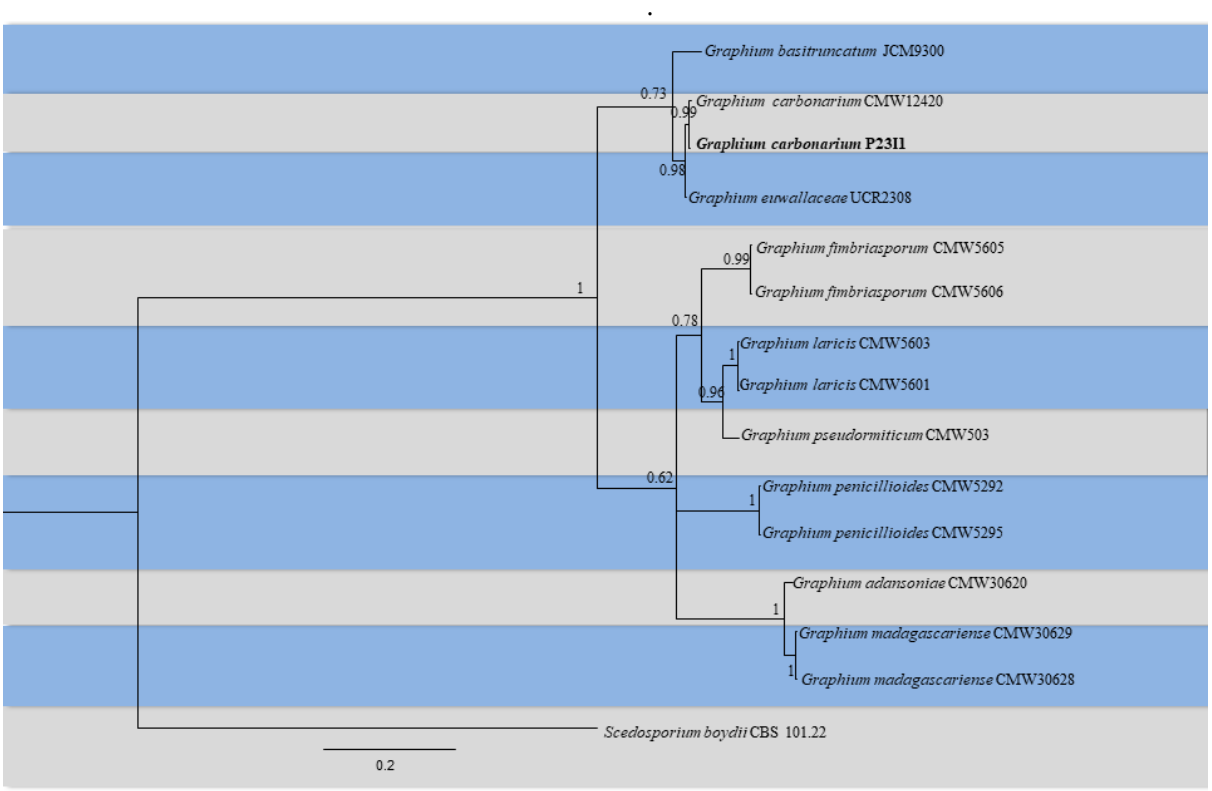

Fig. 2. Bayesian inference phylogenetic tree of *Graphium members* generated using concatenated sequences of ITS-rDNA and *tef1-α*. The values above branches show Bayesian posterior probability. The scale bar indicates the number of expected substitutions per site. *Scedosporium boydii* CBS 101.22 was used as the out-group. The sequence generated in this study is shown in boldface.

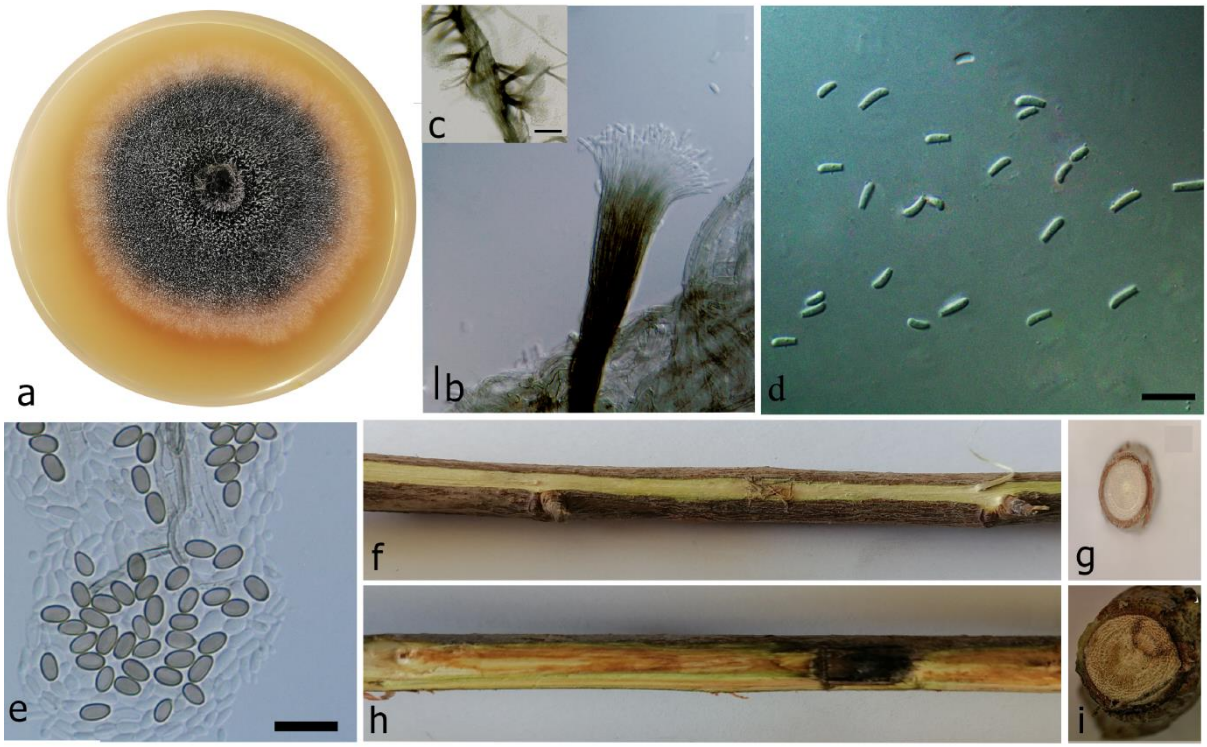

Fig. 3. *Graphium carbonarium*. a. Colony on 2% MEA b-c. Synnematous conidiomata. d. conidia. e. *Scedosporium*- like conidia. f-i. Pathogenicity assay using excided shoot method. Scale bars: b-d = 10 µm.

synanamorph with erect, simple, or branched conidiophores and conidia are obovoid, brown olivaceous, thick-walled, and measured 4–6 µm in length and 1.5–4.5 µm in wide. Sexual morph not observed.

Based on the integration of morphological characteristics with phylogenetic analysis, the isolates were identified as *G. carbonarium*, which is in full agreement with the original description of this species (Paciura et al. 2010). The results of the pathogenicity test showed that *G. carbonarium* is highly pathogenic on excised branches of *U. carpinifolia*. The representative isolate caused brown to black wood discoloration on the shoots. Necrosis and sapwood discoloration was found upward and downward the inoculation site, whereas twigs in the control set remained non-symptomatic. The average length of wood discoloration on the branches caused by tested isolate was measured at 8 ± 2 cm; while no disease symptoms were observed in controls (Fig. 3). The inoculated fungi were successfully re-isolated from inoculated branches. No fungal growth was observed in the control.

DISCUSSION

This study was initiated to characterize *Graphium* isolates associated with bark beetle galleries on declining elm trees in the north and northwestern zones of Iran. The genus *Graphium sensu lato* is a synnematous hyphomycete that comprises several species. We applied a phylogenetic approach (ITS-rDNA and *tef1-α* sequence) combined with morphology to identify *Graphium* isolates obtained in this study. Accordingly, our isolate was identified as *G. carbonarium.* No sexual stage was found, and descriptions were based on the asexual morph.

Graphium carbonarium was first identified and described by Paciura et al. (2010) associated with a *Pissodes* sp. on *Salix babylonica*. *Graphium carbonarium* is most closely related to *G. basitruncatum*; however, they also have minor morphological differences. This species develops larger synnemata and conidia compared to *G. basitruncatum*.

Pathogenicity tests revealed that this species can be pathogenic on elm trees in Iran. In our study, *G. carbonarium* isolate induced up to eight cm length of wood discoloration on excised shoots and significantly differed from the control inoculation (0.5 cm length). The plant pathogenic relevance of *Graphium* species has mainly remained obscure. A greenhouse assay on the pathogenicity of *G. penicillioides* associated with machete wounds on *Schizolobium parahyba* (Vell.) S.F.Blake in Ecuador resulted in very small lesions (with an average length of 12.1 mm) which were not significantly different from the control (Geldenhuis et al. 2004); hence, *G. penicillioides* was not considered as the primary cause of decline symptom on this host.

Our study is the first report of the occurrence and pathogenicity of *G. carbonarium* on elm in the world. However, greenhouse pathogenicity trials are necessary to further clarify the pathogenic potential of *G. carbonarium* on elm trees.

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وقوع و بیماریزایی *Graphium carbonarium* روی درختان نارون در ایران

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چکیده: در بررسی تنوع گونه‌ای و فیلوژنی قارچ‌های افیوستوماتوئید همراه با دالان‌های ناشی از تغذیه سوسک‌های پوست‌خوار و علایم زوال روی میزبان‌های چوبی در منطقه شمالغرب ایران، ۹ جدایه قارچی سینماتادار با الگوی رشدی مشابه از دالان‌های ناشی از تغذیه سوسک‌های پوست‌خوار از درختان نارون مازندران، آذربایجان شرقی وآذربایجان غربی جداسازی شد. بر اساس تلفیق فیلوژنی مولکولی و ویژگی‌های ریخت‌شناسی، هویت گونه *Graphium carbonarium* تعیین گردید. تست بیماریزایی بر اساس تکنیک شاخه بریده نشان داد که این جدایه بر روی نارون (*Ulmus carpinifolia*) بیماریزا است. این بررسی اولین وقوع و بیماریزایی قارچ *Graphium carbonarium* از دالان‌های سوسک‌های پوست‌خوار روی نارون در جهان است.

کلمات کلیدی: نارون، *Graphium*، ITS-rDNA، *tef1-α*

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