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Identification and pathogenicity of *Nigrospora shadeganensis* on oak trees in Zagros forests, Iran

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Abstract: In a study on taxonomy and pathology of fungal species associated with oak trees showing canker, defoliation, dieback, gummosis, wilting, and decline symptoms in Zagros forests, we found 12 isolates morphologically resemble *Nigrospora* spp., with the same ISSR fingerprinting patterns generated by M13 primer. Thus, a representative isolate IRAN 4332C was selected for phylogenetic analyses based on ITS, *tef1,* and *tub2* DNA sequence data. Multigene phylogenetic analyses characterized isolate IRAN 4332C as *Nigrospora shadeganensis*. Weak leaf necrosis and weak irregular wood necrosis were recorded in pathogenicity tests performed on leaves and stems of two-year-old *Quercus brantii* seedlings *in vitro* and under greenhouse conditions.

Keywords: *Apiosporaceae, Dieback, Phylogeny, Quercus brantii*

INTRODUCTION

The Zagros oak forests, dating back to 5500 years, cover the country from northwest to southwest (Eskandari et al. 2020). In recent decades, various biotic and abiotic stresses such as reduced rainfall, increasing temperatures, drought, soil erosion, fire, air pollution and dust, human activities, pests, and pathogens have threatened the existence of these forests (Hosseini 2012, Amir Ahmadi et al. 2015, Mehri et al. 2024). Fungi are an important biological factor associated with oak trees showing a complex of disease symptoms including dieback, canker, gummosis, defoliation, and branch necrosis (Safaee et al. 2017, Alidadi et al. 2019, Sabernasab et al. 2019, Di Lecce et al. 2020, Bashiri et al. 2020a, b, 2022, Bashiri and Abdollahzadeh 2024).

In an extensive study, we identified 24 fungal species corresponding to 19 fungal genera which we recently presented and discussed their identity and pathogenicity (Bashiri and Abdollahzadeh 2024). In this research, we isolated 462 fungal isolates of which 12 isolates, with similar ISSR fingerprinting patterns, were morphologically placed in the genus *Nigrospora*. Based on the phylogenetic analyses of ITS, *tef1,* and *tub2* sequence data the representative isolate IRAN 4332C was identified as *Nigrospora* sp., a candidate of a new species (Bashiri and Abdollahzadeh 2024). In a most recent study, this species was introduced as *Nigrospora shadeganensis* isolated as an endophytic fungal species from grasses and shrubs in Iran (Safi et al. 2024). The genus *Nigrospora* was introduced by Zimmerman (1902) based on the type species *N. panici*. It was revised by Seifert et al. (2011) and placed in *Apiosporaceae* by Wang et al. (2017) based on morphology and multi-gene phylogeny. So far, some 50 species have been listed in Index Fungorum, Species Fungorum, and MycoBank databases. Of these, four species *N. chinensis*, *N. oryzae*, *N. osmanthi,* and *N. sphaerica* have been isolated from different oak species in China, Italy, Mexico, and the United States (Lynch et al. 2014, Wang et al. 2017, Raza et al. 2019, Pinna et al. 2019, Ghasemi-Esfahlan et al. 2019). *Nigrospora* is a ubiquitous genus found in association with a broad spectrum of plants as endophyte, saprobe, and pathogen (Wang et al. 2017). *Nigrospora oryzae* is the causal agent of stem blight on *Brassica juncea* (Sharma et al. 2013), *N. sphaerica* causes leaf blight on *Camellia sinensis* (Liu et al. 2015), and *N. musae* causes 'squirter' disease on bananas (Jones and Stover 2000). To date, no species in *Nigrospora* has been reported as pathogenic on *Quercus* species. To our knowledge, in addition to *N. shadeganensis*, the most recently introduced species, *N. oryzae* is the only species reported from Iran. *Nigrospora oryzae* has been reported as the causal agent of leaf spot on rosemary (Moshrefi et al. 2014), pearl millet (Hashemian Kalati et al. 2014), and peppermint (Farid et al. 2020). Here, we focused on molecular identification and pathogenicity of *N. shadeganensis* on oak trees for the first time.

MATERIALS AND METHODS

Sampling, fungal isolation, and purification

From July to November 2017, samples were collected from oak trees showing external disease symptoms such as canker, dieback, discoloration, and crack of bark tissue in twigs, branches, and trunks. To isolate fungi, small sections of infected woody tissues were disinfected using 70% ethanol for 3 minutes, transferred to potato dextrose agar (PDA) complemented with 100 mg/L streptomycin sulfate and ampicillin and incubated at 20-25°C. Fungal

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isolates were purified on Water Agar (WA 2%, Quelab) using the hyphal tip technique.

Morphological examinations

Morphological and cultural characteristics were examined on PDA and Synthetic low-nutrient Agar (0.2 g Glucose, 0.2 g Sucrose, 1 g KH2PO4, 1 g KNO3, 0.5 g MgSO4 7H2O, 0.5 g KCl, 15 g Agar, 1 L distilled water) at room temperature in the darkness after 7 and 15 days. Fungal structures were examined in 100% lactic acid or distilled water using an Olympus BX51 microscope equipped with an Olympus DP72 camera and a Cell Sense Entry measurement module. Dimensions of fungal structure were estimated based on at least 30 microscopic measurements.

Molecular examinations

For molecular studies, the genomic DNA of pure cultures was extracted according to the modified Raeder and Broda (1985) method as described by Abdollahzadeh et al. (2009).

To reduce the number of isolates for sequencing, ISSR fingerprinting patterns generated by M13 primer were analyzed as described by Alves et al. (2007). A representative isolate from each cluster was selected for sequencing and phylogenetic analyses. For molecular identification, the ITS region (ITS1-5.8S-ITS2) of ribosomal DNA, partial β-tubulin gene (*tub2*), and partial translation elongation factor 1-alpha (*tef1*) gene were amplified and sequenced using the primer pairs ITS5/ITS4 for ITS (White et al. 1990), EF1-728F/EF1-986R for *tef1* (Vilgalys and Hester, 1990), and T1/Bt2b for *tub2* (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997). We followed Bashiri and Abdollahzadeh (2024) for PCR reaction mixtures, PCR conditions, and sequencing. Generated sequences in this research together with those recovered from GenBank (http://www.ncbi.nlm.nih.gov) and the outgroup species *Arthrinium malaysianum* CBS 102053 were aligned using MAFFT v. 7 online tools (http://mafft.cbrc.jp/alignment/server/index.html). If necessary, alignment was manually edited in BioEdit v. 7.0.0. (Hall 2004). Each locus was aligned separately and the alignments were concatenated with Mesquite 2.75 (Maddison and Maddison 2023). Phylogenetic analyses were carried out using Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) methods. PAUP v. 4.0b10 (Swofford 2003) used for MP performance. ML and BI were carried out through the CIPRES Science Gateway portal (https://www.phylo.org/) (Miller et al. 2012) using RAxML-HPC BlackBox v. 8.2.10 (Stamatakis 2014) and MrBayes v. 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), respectively. Newly generated sequences were submitted to GenBank. Characterized isolates were deposited at the culture collection (IRAN) of the Iranian Research Institute of Plant Protection (Tehran, Iran).

Pathogenicity trails

Pathogenicity tests were conducted on leaves in Petri plates and stems of two-year-old oak (*Q. brantii*) seedlings under laboratory and greenhouse conditions. Sterile PDA plugs were placed on leaves and stems as negative control. To assay the phytotoxic activity of the fungal isolates, inoculated leaves were incubated at 25 °C for 72 hours. The inoculated seedling wounds wrapped with Parafilm incubated in greenhouse conditions (22–28 °C) and watered as needed. After 2 months, the external symptoms of inoculated seedlings were recorded and the extent of vascular discoloration (lesion length) was measured. To confirm the pathogenicity of fungal isolates, re-isolation of inoculated fungi from pieces of inoculated tissues on PDA at 25 °C followed according to Koch's postulates. To finalize pathogenicity tests, colony and microscopic features of re-isolated fungi were examined.

RESULTS AND DISCUSSION

Sampling

We collected 12 fungal isolates that morphologically resemble *Nigrospora* species. These isolates were obtained from trunks and branches showing wood necrosis symptoms (Fig. 1).

Molecular identification

Similar ISSR fingerprinting patterns (Fig. 2) were generated with M13 for all 12 isolates of *Nigrospora*. Thus, we selected isolate IRAN 4332C as a representative for phylogenetic studies. The analysis of concatenated sequences ITS, *tef1*, and *tub2* was done to elucidate the taxonomic position of our *Nigrospora* isolate (IRAN 4332C). The combined alignment of ITS (532), *tef1* (465), and *tub2* (430) were subjected to the Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). After alignment, the concatenated dataset consisted of 1429 characters including alignment gaps. Of these, 876 were constant, 145 were variable and parsimony-uninformative and 408 were parsimony-informative. MP analysis of the remaining 408 parsimony-informative characters resulted in 9 most parsimonious trees (TL = 1980, CI = 0.47, RI = 0.62, HI = 0.52). IQ-TREE best tree (log-likelihood -11037.998) was found after 20 iterations. The best evolutionary model selected by ModelFinder in IQ-TREE is TN+F+I+G4. The Bayesian analyses of the concatenated alignments of three loci generated 4322 trees from which 1080 trees were discarded as burn-in. The consensus tree and posterior probability values (PP) were calculated from the remaining 3242 trees. The average standard deviation of split frequencies was 0.009751 at the end of the run. Based on multigene phylogenetic

analyses, isolate IRAN 4332C (GenBank accession no.: ITS: OR478184, *tef1*: OR482439, *tub2*: OR482438) placed in a well-supported clade containing *Nigrospora shadeganensis* ex-type strain IRAN 4958C (Fig. 3).

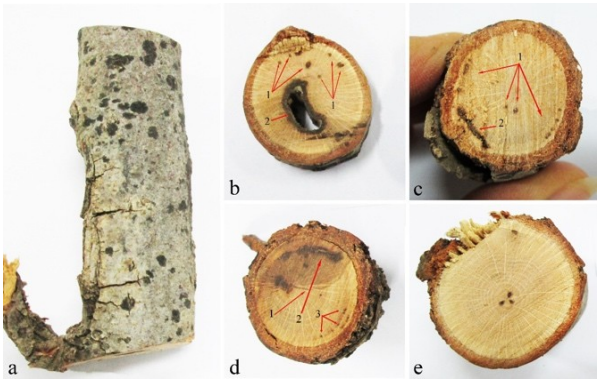

Fig. 1. External and internal wood symptoms in cross-sections of branches of oak trees. a. Cankers on branches; b. Co-occurrence of black spots (1) and borer holes (2); c. Co-occurrence of black spots (1) and black wood streaking; d. Co-occurrence of irregular necrosis (1), black wood streaking (2), and black spots (3); e. Black spots.

Morphology

Nigrospora shadeganensis Safi, MehrabiKoushki & Arzanlou, Antonie van Leeuwenhoek 117:77 (2024); Fig. 4

Colonies on PDA reaching 90 mm diam. after 7 days at room temperature in dark, flat with aerial mycelium, initially white, becoming grayish with age at the surface and reverse. On SNA, colonies flat with sparse aerial mycelium, light brown to grey olivaceous at the surface, dark brown at the surface, and at the reverse, sporulation slow. *Hyphae* branched, septate, hyaline to pale brown. *Conidiophores* mostly reduced to conidiogenous cells. *Conidiogenous cells* monoblastic, discrete, solitary, determinate, at first hyaline then turning to pale brown, ampulliform to cylindrical, subspherical, (3.8–) 5–25 (–26.9) × (1.47–) 2–3.5 (–4.4) µm (av. ± S.D. = 13.2 ± 2.2 × 1.8 ± 0.2 µm). *Conidia* solitary, globose or subglobose, black, shiny, smooth, aseptate, (7.1–) 8–10 (–11) µm (av. ± S.D. = 9.1 ± 0.1 µm) diam.

Specimens examined: Iran, Ilam Province, Ilam (33°35'40.4"N 46°26'30.4"E), from trunk of *Quercus brantii*, 13 September 2016, S. Bashiri, CJASB422; Kurdistan Province, Baneh (36°05'01.1"N 45°40'19.7"E), from trunk of *Quercus infectoria*, 22 August 2016, S. Bashiri, IRAN 4841C = CBS 149777; Kurdistan Province, Sarvabad (35°09'52.3"N 46°32'25.4"E), from branch of *Quercus libani*, 16 August 2016, S. Bashiri, CJASB424; Kurdistan Province, Sarvabad (35°06'53.8"N 46°32'59.6"E), from branch of *Quercus brantii*, 16 August 2016, S. Bashiri, CJASB425; Kurdistan Province, Marivan (35°36'58.8"N 46°01'57.4"E), from branch of *Quercus infectoria*, 16 August 2016, S. Bashiri, CJASB426; Kurdistan Province, Marivan (35°38'34.4"N 46°06'31.4"E), from branch of *Quercus libani*, 14 August 2016, S. Bashiri, CJASB427; Kermanshah Province, Gilan-e Gharb (34°01'15.1"N 46°24'53.2"E), from branch of *Quercus brantii*, 9 October 2016, S. Bashiri, CJASB428, CJASB429; Lorestan Province, Kuhdasht (33°30'47.3"N 48°11'37.7"E), from branch of *Quercus brantii*, 24 September 2016, S. Bashiri, CJASB430, CJASB431; Ilam Province, Eyvan (33°50'50.9"N 46°12'12.6"E), from branch of *Quercus brantii*, 15 September 2016, S. Bashiri, IRAN 4332C = CBS 149776, CJASB421.

Notes: No differences were detected in ITS and tef1 sequences of our isolate (IRAN 4332C) with the ex-type strain *N. shadeganensis*, but one nucleotide difference (substitution) in tub2 sequence was detected. Morphologically our isolate differed from the ex-type strain by having longer and narrower conidiogenous cells (av. ± SD: 13.2 ± 2.2 × 1.8 ± 0.2 vs. 9±1.7 × 6.5 ± 0.9 µm) and smaller conidia (av. ± SD: 9.1 ± 0.1 vs. 14 ± 1.2 µm). Moreover, conidiogenous cells in our isolate were observed as discrete, but in the ex-type strain both discrete and aggregated have been recorded.

Pathogenicity

In this study, Nigrospora shadeganensis showed weak phytotoxic activity on leaves during 72 hours. After two months, weak necrosis appeared on the leaves of inoculated seedlings. Small brown lesions measured on the stem internodes were significantly different from the control (Fig. 5, Bashiri and Abdollahzadeh 2024/Table 3). According to our findings, N. shadeganensis showed weak pathogenicity on Q. brantii, but more experiments are needed to develop a precise and reliable interpretation on the pathogenicity of this species on oak trees. More sampling and studies on various plants would contribute to a better understanding of *N. shadeganensis* lifestyle, host range, geographic distribution, ecology, and pathology.

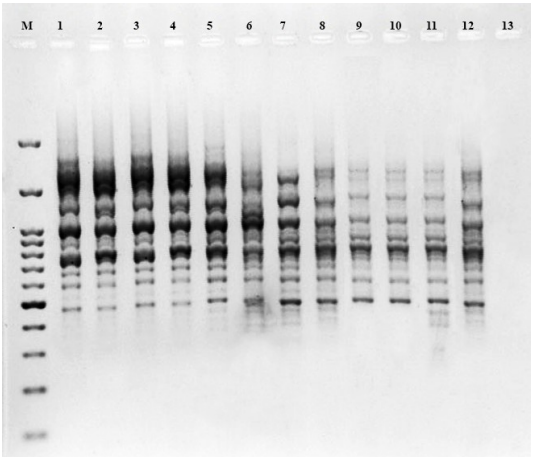

Fig. 2. DNA fingerprinting patterns generated with primer M13 for all strains. Lanes 1–12. *Nigrospora shadeganensis* isolates; lane 13. Negative Control; M. GeneRuler DNA Ladder Mix.

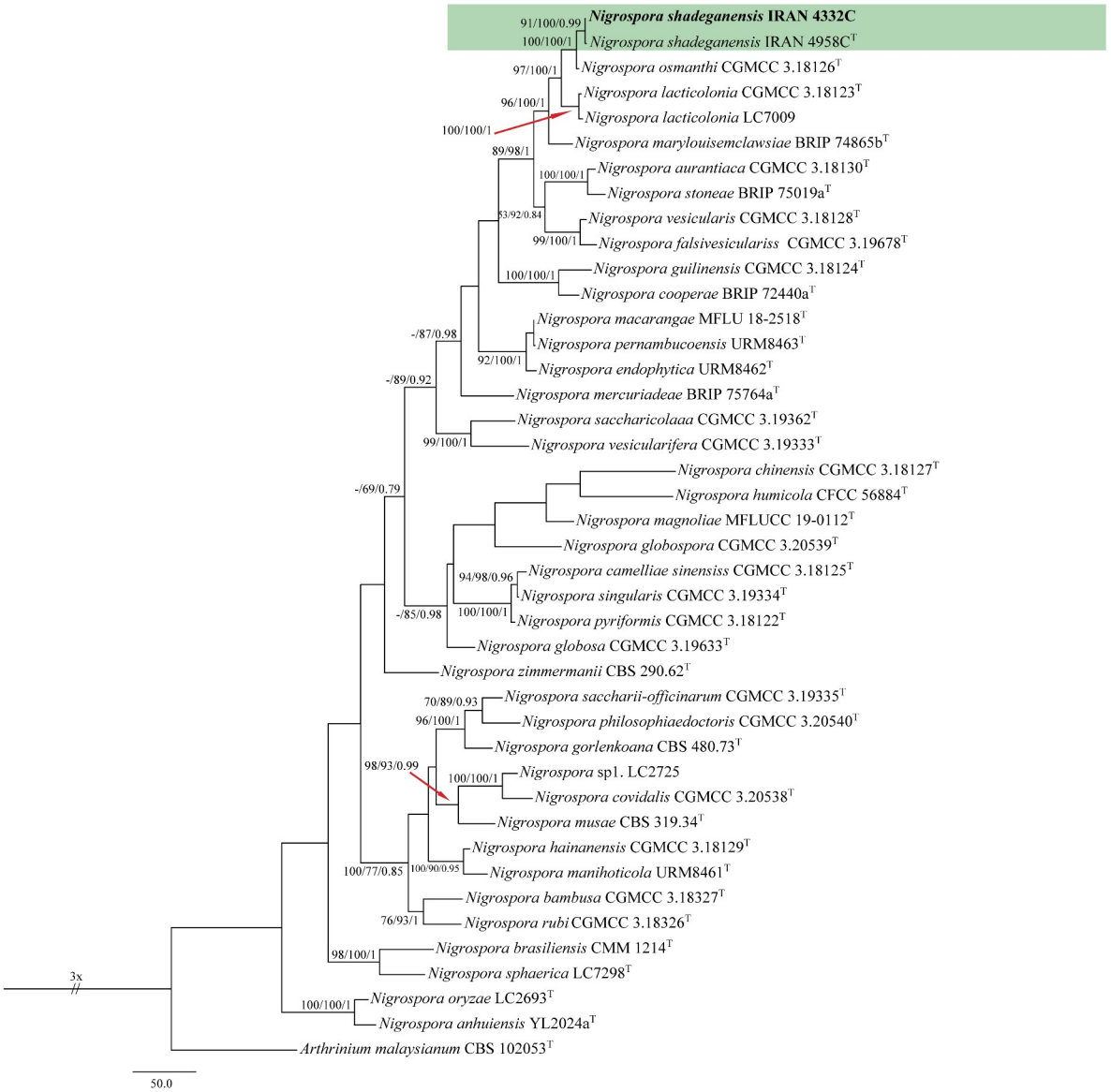

Fig. 3. One of the nine equally most parsimonious trees generated based on combined ITS, *tef1,* and *tub2* sequence data of *Nigrospora* species. The tree is rooted to *Arthrinium malaysianum* (CBS 102053). The scale bar represents the expected number of changes per site. Maximum Parsimony (MP), Maximum Likelihood (ML) bootstrap values, and Bayesian Inference (BI) posterior probabilities are indicated at the nodes (MP-BS/ML-BS/BI-PP). ^{T} Ex-type.

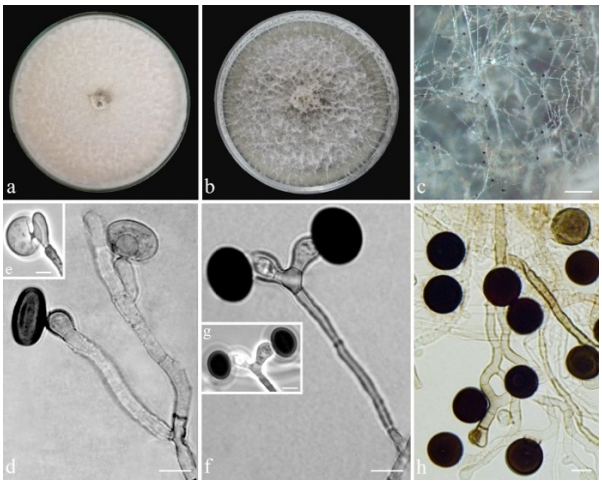

Fig. 4. *Nigrospora shadeganensis*. a, b. Colonies on PDA and SNA at room temperature, respectively; c. sporulation on SNA; d–g. Conidiophores, conidiogenous cells, mature and young conidia developing on conidiogenous cells; h. Mature black conidia. Scale bars: c = 200 µm, d, f, h = 5 µm, e, g = 2.5 µm.

Fig. 5. Symptoms caused by *N. shadeganensis* on oak seedlings in greenhouse and leaves under *in vitro* conditions. a. Inoculated plant after 2 months; b. Negative control; c. Necrotic lesion on the stem internodes; d. Necrotic spot on leaves; e. Negative control; f. *N. shadeganensis* colony re-isolated from inoculated seedlings; g. Stem cross-sections of inoculated seedlings.

Briefly, *N. shadeganensis* was recently introduced as a new endophytic fungal species associated with *Aeluropus lagopoides*, *Phragmites australis*, *Seidlitzia rosmarinus, Halocnemum strobilaceum,* and *Tamarix passerinoides* from Iran (Safi et al., 2024). It is the first time this species is isolated from declined oak trees. We collected 12 isolates of *N. shadeganensis* from various oak species (*Q. brantii*/*Q. infectoria*/*Q. libani*) in different parts of Zagros forests located in Ilam, Kermanshah, Kurdistan, and Lorestan provinces. Here we characterized *N. shadeganensis* based on DNA sequences (ITS, *tef1* and *tub2*) and morphology and presented our result on pathogenicity tests together with host and geographic distribution in detail.

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شناسایی و بیماری‌زایی گونه *Nigrospora shadeganensis* روی درختان بلوط در جنگل‌های زاگرس ایران

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چکیده: در یک مطالعه روی تاکسونومی و بیماری‌زایی گونه‌های قارچی همراه با درختان بلـوط جنگل‌های زاگرس دارای علائم شـانکر، ریزش برگ، سرخشکیدگی، گموز، پژمردگی و زوال، ۱۲ جدایه قارچی از نظر خصوصیات ریختی شبیه گونه های جنس *Nigrospora* با الگوی انگشت‌نگاری ISSR (ایجاد شده با آغازگر M13) یکسان جمع آوری شد. از این رو، جدایه IRAN 4332C به عنوان نماینده جهت بررسی تحلیل‌های تبارزایی براساس توالی DNA نواحی ITS و بخشی از ژن‌های *tef1* و *tub2* انتخـاب شـد. نتـایج تحلیل‌های تبارزایی نشان داد که این جدایه به گونه *Nigrospora shadeganensis* تعلق دارد. در آزمون های بیماری‌زایی روی برگ در شرایط درون شیشه ای و نهال ۲ ساله گونه *Quercus brantii* در شرایط گلخانه علائمی شامل نکروز ضعیف برگ و نکروز نامنظم ضـعیف چـوب بـه ترتیب مشاهده و ثبت گردید.

کلمات کلیدی: زوال، تبارزایی، تیره *Apiosporaceae*، *Quercus brantii*.

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