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Juglanconis juglandina, a new record for the mycobiota of Iran

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Abstract: During the study of fungal species associated with canker and dieback diseases of walnut trees (*Juglans regia* L.) in East Azerbaijan province, Iran, eight fungal isolates with similar characteristics resembling asexual stage of the genus *Juglanconis* were isolated from trees showing canker, dieback, and dead skin symptoms in Osko and Horand regions. Conidiomata on the host tissue were acervular and stromatic, 1 – 2.5 mm diam, blackish, scattered or confluent, covered with black conidial masses when mature. Conidiophores were unbranched or rarely branched at the base. Conidiogenous cells were cylindrical and annellidic. Conidia elliptic to ovate, truncate with distinct scar at the base, thick-walled and with distinct ornamentation on the inner side of the wall consisting of irregular confluent verrucae, hyaline when immature, brown to blackish when mature, covered with gelatinous sheath, (11) 14 – 16 (– 18) × (15 –) 22 – 25 (– 28) µm. Fungal isolates were identified as *J. juglandina* based on morphological characteristics and host association. Identification of the species was further confirmed by sequence analysis of the elongation factor (*tef1-α*) gene. Present study is the first report of *J. juglandina* for the mycobiota of Iran.

Key words: acervular, *Juglanconis juglandina*, *Juglans regia*, morphology, *tef1-α* gene

INTRODUCTION

The order Diaporthales (Ascomycota, Sordariomycetes) comprises major economically significant plant pathogens. However, species diversity, host range and phylogeny of many important plant pathogenic species in this order remain partly unresolved (Voglmayr et al. 2019). The members of this order are characterized by perithecia with a long neck, which are often formed in stromal tissues (Voglmayr et al. 2017). Several species in this order represent significant pathogens on walnut causing canker and dieback diseases including *Diaporthe rostrata* C.M. Tian, X.L. Fan & K.D. Hyde, *Cytospora chrysosperma* (Pers.) Fr. and *Juglanconis* species namely *J. juglandina* (Kunze) Voglmayr & Jaklitsch. and *J. oblonga* (Berk.) Voglmayr & Jaklitsch. (Voglmayr et al. 2017, Fan et al. 2018, Ma et al. 2019).

The genus *Juglanconis* was recently established based on *J. juglandina*, as the type species, in the new family *Juglanconidaceae* to accommodate *Melnaconis* species associated with the *Juglandaceae* family based on their unique phylogenetic placement in the order Diaporthales (Voglmayr et al. 2019). In a recent phylogenetic analysis, *Melanconis* species on *Juglans* and *Pterocarya* clustered in a highly supported lineage distinct from *Melanconis sensu stricto*, which required a new genus and new family as well (Voglmayr et al. 2017).

Morphologically, ascomata of the members of this family are perithecia, and have octosporous asci, with an apical ring, ascospores hyaline, bicellular, with or without gelatinous appendages, and conidiomata acervular with brown conidia with gelatinous sheaths (Voglmayr et al. 2017, Fan et al. 2018).

In the past, taxonomy of *Melanconis* species (currently treated in *Juglanconis*) on *Juglandaceae* have been studied in detail, considering their economic significance on walnuts. *Melanconis* species have been known to cause walnut black pustular dieback disease (Graves 1923, Belisario 1999). In this regard, Graves (1923) provided a detailed study on the description of the asexual and sexual morphs of *M. oblongum* in pure culture and also on the pathogenicity of this specie. He combined the sexual morph, *Diaporthe juglandis*, in *Melanconis* as *J. juglandis*, in analogy to the European *M. carthusiana*. In his study the North American isolates of *M. oblongum* was also considered to be morphologically different from the European *M. juglandinum*. Pathogenic nature of this species on *Juglans cinerea* was confirmed by inoculation

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experiments, which resulted in serious disease symptoms (Voglmayr et al. 2017). Based on the detailed cultural and morphological studies, *M. juglandis* was recorded and described from Japan (Kobayashi 1970), representing that the Japanese collections agreed well with North American material (Voglmayr et al. 2017).

The novel genus *Juglanconis* was recently described by Voglmayr et al. (2017) for four *Melanconis* species on hosts of the tribe *Juglandinae*, *Juglanconis juglandina*, *J. appendiculata*, and *J. oblonga* on various *Juglans* species and *J. pterocaryae* on *Pterocarya* spp. *Juglanconis juglandina* has been reported as the causal agent of black pustular dieback disease of *J. regia* (Belisario 1999) and lived on the dead corticated twigs and branches of *Juglans* spp. (Voglmayr et al. 2017, Fan et al. 2018).

In the years 2017-2018, symptoms of canker, dieback, truncation of trunks and branches as well as vessel browning of walnut trees were observed in Osko and Horand regions located in East Azerbaijan province in north-western Iran. Therefore, this study was initiated to identify the causative agent of these symptoms on walnut trees in Iran based on morphological characteristics and sequence data of *tef1-α* gene.

MATERIALS AND METHODS

Sample collection and fungal isolation

During 2017-2018, walnut trees (*Juglans regia* L.) in Osko and Horand regions of East Azerbaijan province were surveyed for fungal species associated with canker and dieback diseases. Symptomatic branches (1-4 samples from each tree) were collected randomly in aforementioned regions. For fungal isolation, small marginal wood fragments (5 mm^3) (between healthy and affected parts) were cut and surface disinfected by immersion in 1.5 % sodium hypochlorite (NaOCl) for 1 min and ethanol 70 % for 2 min and rinsed twice in sterile distilled water. Then, they were dried under sterile airflow in the laminar hood and were placed on Petri dishes containing potato dextrose agar (PDA) supplemented with 2 % lactic acid. Petri dishes were incubated at 25°C for 5-15 days. In most cases, cankers and twigs with dieback symptoms were covered with black conidiomata (acervuli). Fungal isolations were also made from conidiomata formed on cankers and twigs. Obtained single-spore cultures were kept in 2 ml tubes containing potato carrot agar (PCA) (Crous et al. 2009) at 4°C. Pine needles and segments of walnut tree woods were used in water-agar (WA) medium to facilitate the formation of asexual structures.

Morphological identification

Examination of morphological characteristics was conducted both on the natural substrate and under *in vitro* conditions. Colony color (surface and reverse) and growth rates of all fungal isolates were checked on malt extract agar (MEA) after seven days of incubation at 25°C in darkness. Microscopic characteristics including the shape and size of conidia and conidiophores were also examined on MEA culture medium. For all microscopic observations, sterile water was used as a mounting medium. In case of possibility, a minimum of 25 measurements were made per structure with extreme values given in parentheses. Photographs of microscopic fungal structures were taken by Olympus digital camera system (DP 25) mounted on an Olympus BX41 light microscope.

Molecular identification and phylogeny

Single-conidium isolates were prepared and grown on MEA. Genomic DNA was extracted from fungal mycelia using Moller et al. (1992) protocol. The elongation factor 1-alpha (*tef1-α*) gene was amplified and sequenced using EF1-728F and EF2 primer pairs (O' Donnel et al. 2004). The reaction mixture was the same as described in Torbati et al. (2018). PCR amplification was carried out in a thermocycler with following program: 94 ºC for 3 min initial denaturation, 35 cycles of 94 ºC for 3 min denaturation, 52 ºC for 35 s annealing, and 72 ºC for 60 s extension. Final extension was for 10 min at 72 ºC. Amplicons were resolved in 1% agarose gel. SeqManTMII (DNASTAR) was used to refine the obtained sequences and to generate the consensus sequences. The identity of the representative isolate was confirmed by conducting a BLAST search against the GenBank nucleotide database.

Sequences of reference isolates were obtained from the GenBank (Voglmayr et al. 2019) and *Melanconis stilbostoma* (KY427225; Voglmayr et al. 2019) was also used as out group taxon. All sequences were aligned together with the sequence of our isolate using ClustalW algorithm implemented in MEGA7 (Kumar et al. 2016). Obtained alignments were visually checked and in case of necessity they were improved manually. Phylogenetic analysis was conducted using MrBayes version 3.2.6 (Ronquist & Huelsenbeck 2003). The generated phylogenetic tree was visualized in FigTree version 1.3.1.

RESULTS AND DISCUSSION

Eight fungal isolates with similar cultural and morphological features were obtained from collected specimens in Horand and Osko regions. In natural condition, fungal structures were seen as black and fine structures on the branches of walnut trees. Under *in vitro* conditions, colonies on MEA reached 45 mm in diameter after seven days of incubation. Colonies at 25°C and dark were yellowish white; near the center white aerial mycelia were seen as growth rings. Colonies were embossed with irregular margins (Fig. 1a-b). Stromatic and acervular conidiomata developed on pine needle and pieces of walnut wood on WA after 1-2 weeks, 1-2 mm in diameter, black, scattered, covered by black discharged conidial masses (Fig. 1c). Conidiophores branched only at the base,

smooth, mostly aseptate, sometimes few-celled, subhyaline to pale brown, size (20 –) 29 – 35 (– 60) × (4 –) 5 – 6.5 (– 8) µm (Fig. 1e-f). Conidiogenous cells cylindrical and annellidic with distinct annellations, forming a single conidium at the tip (Fig. 1g-i). Conidia (15 –) 22 – 25 (– 28) × (11 –) 14 – 16.5 (– 18) µm, unicellular, hyaline when immature, brown to blackish when mature, elliptic to ovoid, truncate with distinct scar at the base, and covered with gelatinous sheath (Fig. 1i). They were seen as black masses on the acervul. Cultural and morphological characteristics of the isolates were in full agreement with *J. juglandina* species as described by Voglmayr et al. (2017).

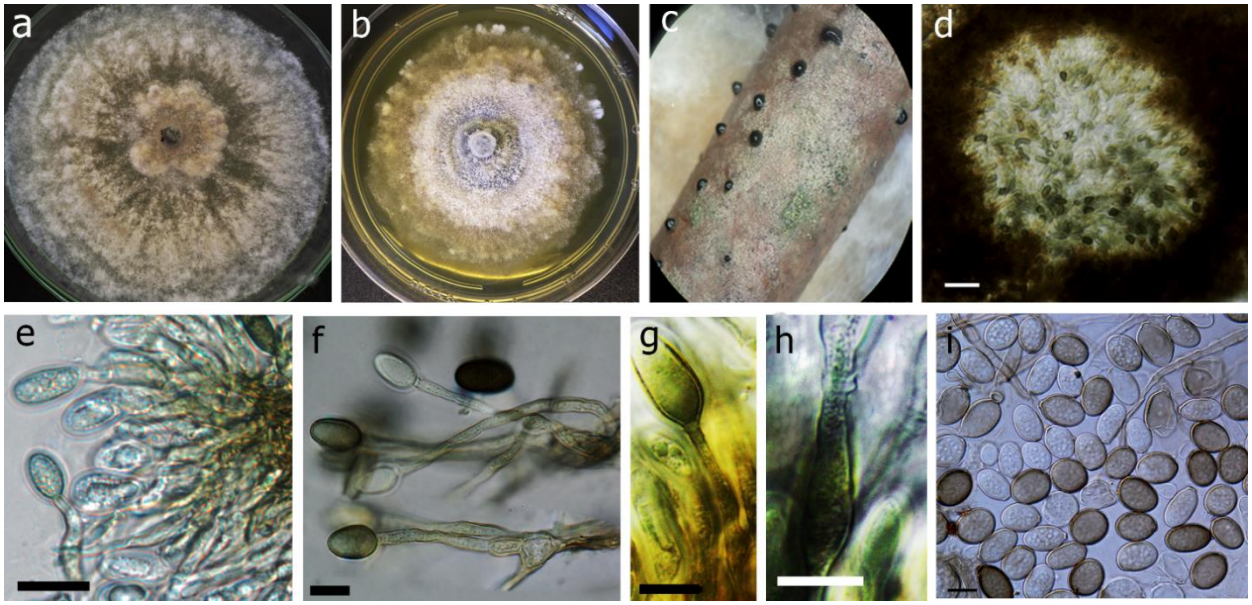

Fig. 1. *Juglanconis juglandina*. a. 14-day-old colony on PDA; b. 14-day-old colony on MEA; c. Conidiomata on host tissue; d. Conidiomata cross-section; e-f. Conidiophores and conidia; g-h. Conidiogenous cells; i. Conidia. - Scale bars = d. 50 µm; e. 20 µm; g-i. 10 µm.

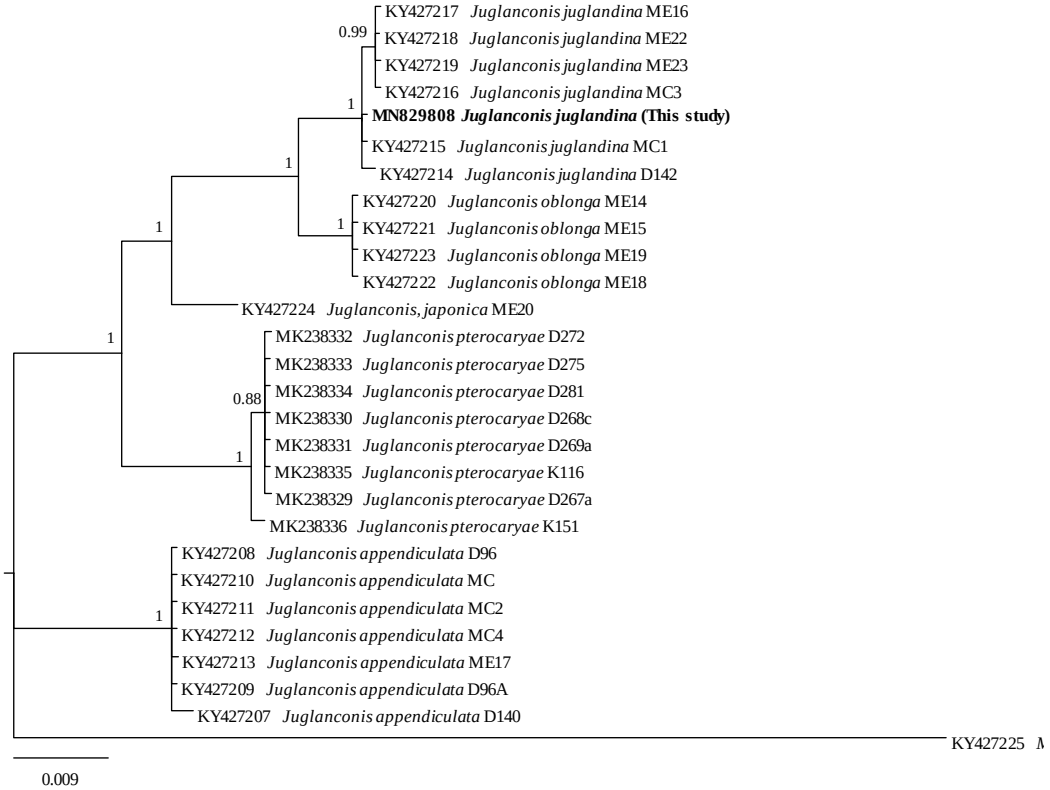

Fig. 2. Phylogram generated by Bayesian analysis of the *tef1-α* gene sequence alignment using MrBayes v. 3.2.6 of *Juglanconis* species. The scale bar indicates 0.009 expected changes per site. The tree was rooted to *Melanconis stilbostoma* (KY427225).

PCR amplification of the *tef1-α* gene of a representative isolate (CCTUHR16) in this study was followed by sequencing of the fragments and on the basis of BLAST analysis, our isolate was identified as *J. juglandina* with 99 % homology to other published sequences of *J. juglandina* isolates available in the GenBank (Acc. nos. KY427214, KY427215, KY427216, KY427217, KY427218, KY427219; Voglmayr et al. 2019). Our results showed that isolate obtained in the current study (Acc. no. MN829808) clustered with *J. juglandina* with highly supported value (Fig. 2). Living culture of the representative fungal isolate was deposited in the Iranian Fungal Culture Collection with accession number IRAN 3919C.

So far, three species of this genus have been reported on walnut trees by Voglmayr et al. (2017). *Juglanconis appendiculata* and *J. juglandina* have been reported on European walnut in Europe and black walnut (*J. nigra* L.) in North America. *Juglanconis oblonga* has been reported from *J. cinerea* and *J. nigra* in North America and from *J. ailanthifolia* in Japan. *Juglanconis juglandina* also has been reported from *J. regia* in China (Ma et al. 2019). To the best of knowledge, this is the first report of *J. juglandina* for the mycobiota of Iran.

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اولین گزارش از گونه *Juglanconis juglandina* برای مایکوبیوتای ایران

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چکیده: در طی مطالعه گونه‌های قارچی مرتبط با بیماری‌های شانکر و سرخشکیدگی گردو (*Juglans regia* L.) در استان آذربایجان شرقی در سال‌های ۹۶-۹۵، تعداد هشت جدایه قارچی از شاخه‌های دارای علایم شانکر، سرخشکیدگی و پوست‌های مرده درختان گردو در مناطق اسکو و هوراند با ویژگی‌های مشابه مرحله غیرجنسی *Juglanconis* جداسازی گردید. کنیدیوماتا روی بافت میزبان آسروولی و استروماتیک به قطر ۲/۵-۱ میلی‌متر، سیاه رنگ، پراکنده یا مجتمع که در زمان بلوغ توسط توده‌های کنیدیومی سیاه پوشیده شدند. کنیدیوفورها بدون انشعاب یا در پایه منشعب، سلول‌های کنیدیوم‌زایی استوانه‌ای و آنلیدیک، کنیدیوم‌ها بیضوی تا تخم مرغی، پایه بریده با زخم متمایز در پایه، با دیواره ضخیم و برجستگی‌های نامنظم در سطح داخلی دیواره، با قطرات چربی متراکم، که در ابتدا شفاف و به‌تدریج به رنگ قهوه‌ای روشن تا تیره تغییر رنگ داده و با غلاف ژلاتینی پوشیده شده‌اند. اندازه کنیدیوم‌ها (۱۸-)۱۶-۱۴(-۱۱) × (۲۸-)۲۵-۲۲(-۱۵) میکرومتر بودند. جدایه‌های قارچی بر اساس ویژگی‌های ریخت شناختی و رابطه میزبانی *J. juglandina* شناسایی شدند. هویت گونه با استفاده از داده‌های توالی ژن *tef1-α* مورد تایید قرار گرفت. تحقیق حاضر اولین گزارش از گونه *Juglanconis juglandina* در ایران می‌باشد.

واژه‌گان کلیدی: آسروول دار، *Juglanconis juglandina* ، درخت گردو، ریخت شناسی، ژن *tef1-α*

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