



New *Ophiostoma* species associated with *Dryocoetes himalayensis* and decline of *Juglans regia* in Czechia

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Abstract

A new ophiostomatoid fungus involved in the decline of walnuts was discovered in Czechia. The fungus was isolated from necrotic wood surrounding the larval galleries of *Dryocoetes himalayensis*, the alien bark beetle attacking walnut trees. This insect species is rather unique within hardwood-associated bark beetle species for its aggressiveness and ability to attack apparently healthy trees. The isolated fungus, associated with an exotic insect vector is described as a new species, *Ophiostoma juglandis* sp. nov., based on its unique morphology and phylogenetic position. The pathogenicity tests confirmed the aggressiveness of the species to both *Juglans regia* and *J. nigra* plants, manifested by formation of extensive bleeding and necrotic lesions. The symbiotic relationship between *O. juglandis* and *D. himalayensis* is expected due to massive presence of the fungal spores on surface of adult beetles in pits forming the elytral striae. In the case of hardwood-associated ophiostomatoid fungal species, phytopathogenicity is very rarely observed. The described species is exceptional in this ability, which corresponds to the aggressive behaviour of its vector. The role of the fungus in walnut tree mortality caused by *D. himalayensis* is discussed.

Keywords Ophiostomatales · Scolytinae · Taxonomy · Walnut decline · Pest

Introduction

Juglans regia L. (Persian walnut, English walnut, or common walnut) is a tree species of probable origin in the Mediterranean region and central Asia but widely grown in suitable climate regions worldwide. This is particularly important for nuts production in food industry (Pollegioni et al. 2017). Although widely distributed and easy for regeneration, this tree species is affected by multiple pathogens and pests. Particularly important role in *J. regia* decline and dieback have pathogens like notorious Thousand Cankers Disease (TCD) caused by *Geosmithia morbida* Kolařík et

al. (Kolařík et al. 2011; Bracalini et al. 2023), *Phytophthora* spp. (Mircetich and Matheron 1983; Vettraino et al. 2003), or *Botryosphaeriaceae* (Gusella et al. 2021; Antony et al. 2023, 2024). Several other fungal species causing cankers, bark necrosis, blight, or decay and rot were also recorded on this host tree (Karadžić et al. 2016; Eichmeier et al. 2020). In addition, various insect species affect *J. regia* living trees and fruits (Khan and Kundoo 2018). Several harmful insects like bark beetles might have symbiotic relationships with pathogenic fungi (Six and Wingfield 2011; Six 2012), this phenomenon was demonstrated in the case of aggressive *G. morbida* being associated with walnut twig beetle *Pityophthorus juglandis* Blackman, 1928 (Kolařík et al. 2011; Montecchio et al. 2016). However, little is known about pathogenic fungi associated with another invasive bark beetle *Dryocoetes himalayensis* Strohmeyer, 1908 (Coleoptera: Curculionidae: Scolytinae), that was recorded on both *J. regia* and *J. nigra* in Europe (Foit et al. 2017; Kašák et al. 2023). The bark beetle *D. himalayensis*, originated from Himalayan regions and invaded to Europe in the 1970s, and is currently known from several European countries (Austria, Czech Republic, France, Germany, Slovakia, and Switzerland; (Procházka et al. 2018). Since 2009 the species

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occurs in Czechia (Knížek 2011) and is currently distributed over a substantial part of the country with distribution centre in lowlands with a warmer climate (Kašák et al. 2023). *D. himalayensis* is reported to develop in *J. regia* and *Pyrus lanata* D. Don in the region of its origin (Wood and Bright 1992), whereas *Juglans nigra* L. (black walnut) and *J. regia* have been confirmed as the host trees in Europe (Barnouin et al. 2020; Foit et al. 2017). *D. himalayensis* is associated with decline of *Juglans* spp. trees and can cause or at least contribute to a slow but gradual and possibly extensive dieback of walnuts (Kašák et al. 2023). This bark beetle prefers thick trees with larger diameter at breast height (dbh), but it has been found to develop even on thin trees (dbh = 60 mm; Kašák et al. 2023). Gallery systems of the beetle are typically located in the bark and phloem usually in the basal and middle parts of trunks. Gallery systems of *D. himalayensis* are often associated with dark brown (almost black) fluid oozing from entrance holes. These galleries are usually surrounded by dark brown to black necrotic zones, which were assumed to be caused by an associated fungus. This fungus possibly facilitates *D. himalayensis* to colonise more vital trees (Foit et al. 2017; Kašák et al. 2023). During the monitoring of *J. regia* decline in South Moravian region in Czechia, a strain of unidentified *Ophiostoma* sp. from necrotic wood around *D. himalayensis* larval galleries was obtained. The aims of this study were to: (i) perform morphological and phylogenetic analyses to characterize the isolated *Ophiostoma* sp., and (ii) test the aggressiveness of the isolated strain on *J. regia* and *J. nigra* through pathogenicity test.

Materials and methods

Study area and disease symptoms

Beetles and samples from infested common walnut were collected in Blučina (49.0476N, 16.65406E) in Czechia, South Moravian region. Locality represents an isolated patch of woodland with an area app. 25 ha in an intensive agricultural landscape. The forest is situated in the vegetation zone of mesic oak woodlands in the altitude of 250–300 m a.s.l. This is a characteristic mature broadleaf stand with close canopy and mixed tree composition. Dominant tree species are there *Quercus* spp., *Fraxinus excelsior* L., *Tilia* spp., and *Robinia pseudoacacia* L., but *Juglans regia* and *J. nigra* are remarkably widespread there. The main canopy of the stands forms an even-aged understorey composed of shrubs (dominant *Prunus spinosa* L. and *Sambucus nigra* L.), and common young trees. The forest stands are managed by clear-cutting system with prevailing artificial regeneration. During the monitoring of health status

of walnut trees in Blučina locality, trees displaying dieback symptoms were recorded. After the detailed inspection of trees, exit holes of the *Dryocoetes himalayensis* bark beetle at basal parts of the *J. regia* stems were noticed surrounded with dark exudates (Fig. 1A). After removing the bark and wood, necrotic dark brown tissues were recorded around the larval galleries (Fig. 1B).

Sampling, isolation, and morphological identification

The obviously declining living trees of *J. regia* were sampled by cutting ca. 10 × 20 cm fragments of dark brown necrotic wood surrounding gallery system of *D. himalayensis*. The samples were packed in plastic bags and stored under cold temperature (4–8 °C) before processing. The bark was removed from the wood sample with a knife and the surface was sterilized by the ethanol. Small pieces (ca. 0.5–1 cm) of wood were put on surface of Petri dishes with Potato dextrose agar (PDA; HiMedia, India) and incubated at 21 °C. The monosporic strain was deposited in the Culture Collection of Fungi (Dept. of Botany, Faculty of Sciences, Charles University, Prague, Czechia) under the number CCF 6633. Cultures were grown on 2% MEA (Oxoid) at 15 and 25 °C, with or without autoclaved host tree twigs to induce potential ascocarp or synnemata formation. Microscopic preparations were made in water using glass slides (Cole et al. 1969) and observed with an Axio Scope.A1 microscope (Carl Zeiss, Jena, Germany) under differential interference contrast (DIC) illumination. Measurements were taken from photos recorded with a ProgRes SpeedXTcore 5 digital camera (Jenoptik Optical Systems, Jena, Germany) using ProgRes image processing software. Fifty measurements were made for each taxonomically relevant structure. Averages, ranges, and standard deviations were calculated for the measurements, presented as '(min–)mean ± SD(–max)'. Growth characteristics were determined in three replicates after 7 days of cultivation at 5, 10, 15, 20, 25, 30, and 35 °C in the dark on 2% MEA. Agar disks (2 mm diam.) were cut from actively growing colony margins and placed in the center of the plate. Furthermore, the morphology of adult beetles was studied to identify structures for spore transmission. Both entire beetles and their parts (thorax, head, mandibles) were examined using scanning electron microscopy (SEM) according to Kolařík and Vohník (2018).

Molecular identification and phylogeny

Genomic DNA was isolated using the ArchivePure DNA Yeast & Gram+ Kit (5 PRIME, Hamburg, Germany). The nuclear ribosomal genes ITS (internal transcribed spacer), LSU (large subunit) and SSU (small subunit), and protein



Fig. 1 *Juglans regia*, locality Blučina (Czechia, South Moravian region). **A** Basal part of stem of living tree with exit holes of *D. himalayensis* and dark oozing. **B** Necrotic lesions of wood surrounding gallery of *D. himalayensis*. Photo Josef Kašák (19 May 2020)

coding genes CAL (calmodulin) and TEF-1 α (translation elongation factor 1 α) were amplified using the primers ITS1/ITS4 (ITS), NL1/LR3 (LSU), NS1/NS24 (SSU) (White et al. 1990), CMD5/CMD6 (Hong 2006; CAL), and EF-728F/EF2 (Carbone and Kohn 1999; O'Donnell et al. 1998; TEF-1 α). The amplification protocols followed Kolařík et al. (2017) for rDNA, TUB2 and TEF1- α and to Sklenář et al. (2022) for CAL. PCR products were sequenced at Macrogen Inc. (Amsterdam, the Netherlands) using the primers employed in the PCR. The sequences were compared with sequences retrieved from GenBank using the BLASTn algorithm (Altschul et al. 1990). Newly obtained sequences were deposited in NCBI GenBank database. The ITS was constructed using included best hits from NCBI GenBank obtained using BlastN search, then all sequences of the reference species in *Ophiostoma* s. s. and related genera based on the study of de Beer et al. (2022). Sequence alignment was done in MAFFT v. 7.222 (Katoh and Standley 2013) under the Q-INS-I strategy (considering rRNA secondary structure). Bayesian searches (MB) were conducted with MrBayes 3.0 (Ronquist and Huelsenbeck 2003) and 10 million replicates estimated together with burn-in value in Tracer v. 1.5 (Rambaut et al. 2018). Maximum likelihood (ML) searches were conducted in IQ-TREE v. 2.1.3 (Nguyen et al. 2015) with automated model selection using

ModelFinder (TIM2+F+I+G4) (Kalyaanamoorthy et al. 2017) with 10,000 replicates of SH-aLRT bootstrap. In the final alignment there were 89 sequences with 845 nucleotide sites, from which 464 were constant and 289 parsimony informative and 92 singleton sites. The ITS dataset is included in the Supplementary material.

Pathogenicity test

For the inoculum preparation, discs-shaped wood fragments containing wood and bark, ca. 2–3 mm high and 5 mm in diameter, were taken from living *J. nigra* branches using the metal cork borer. The collected wood discs were sterilized in the autoclave at 115 °C for 15 min. After cooling the fragments were plated on the surface of the Petri dishes with MEA medium. The dishes were then overgrown with *Ophiostoma* strain CCF 6633 after incubation at 20 °C in the dark for three weeks. Petri dishes with wood discs plated on the top of pure culture were incubated for an additional four weeks at 20 °C in the dark.

To assess the pathogenicity, two-year-old plants of *J. regia* and *J. nigra* plants were grown in 1-litre containers (mixture of peat, sand and perlite) under laboratory conditions at room temperature. The plants had a local origin. The average height and diameter of *J. regia* plants were

47.4±1.11 cm and 8.3±0.16 mm, respectively. In the case of *J. nigra*, tested plants averaged 67.5±13.67 cm and 4.9±0.14 mm, respectively. For the inoculation tests, the bark of plants was cleaned with cotton moist in 70% ethanol at 5–7 cm above the soil level, and pieces of bark 5 mm in diameter were removed using a sterilized metal cork borer. Wood discs overgrown with the strain CCF 6633 were placed, with wood-to-wood side, on the exposed tissue and sealed with parafilm. In total, 12 plants of each host species were inoculated. The control group consisted also of 12 plants and was mock-inoculated with sterilized wood discs. To test the ability of the fungus to penetrate healthy bark tissue, an additional 12 plants of each host species were inoculated without previous bark removal. Infected plants were incubated for eleven weeks at 22–25 °C in daylight laboratory conditions. Plants were watered regularly twice per week till the field capacity, and the symptoms of infection were monitored once per week. In the case of infected plants, reisolations were performed from the edges of the necrotic zones by plating wood pieces on MEA media upon the end of experiment. The authenticity of the reisolated strain was confirmed through micromorphological observations.

Statistical analysis

The data on necrosis length, width, and area of the wounds after inoculation were tested for normality and homogeneity of variance using the Shapiro–Wilk test and Levene's ANOVA test, respectively. Since the data on necrosis length and area did not pass both tests, nonparametric two-way PERMANOVAs were applied to evaluate the effects of host plant, inoculation, and their interactions on the wound characteristics. To reveal potential differences among specific experimental groups, one-way PERMANOVA and pairwise comparisons were applied. The data on necrosis width met the assumptions of normality and homogeneity of variance. Therefore, a two-way ANOVA was used to assess the effects of host plant, inoculation, and their interaction on necrosis width, followed by Fisher's LSD test to reveal differences among specific experimental groups.

Results

Fungal identification and taxonomy

A strain belonging to the genus *Ophiostoma* s.s., represented by the strain CCF 6633 was isolated as the main symbiotic fungus. When comparing the similarity of DNA markers of the strain CCF6633 with published sequences, no match indicating conspecificity was found. Specifically,

SSU had a best hit of 99% (1677/1689 bp) to *O. ulmi* ATCC 32437, M83261.1; ITS 92.8% (596/642 bp) to *O. ulmi* CBS 106.63, MH858227.1; LSU 98% (572/583 bp) to *O. pseudokarelicum* KFL9916RJTD, MH055687.1; TUB2 87.9% (395/449 bp) to *O. tsotsi* CMW41731, MG205705.1; TEF-1α 78.93% (547/693 bp) to *O. typographi* CMW44483, MH124337.1; CAM 83.60% (520/622 bp) to *O. araucariae* CBS114.68, KU184332. Based on the phylogenetic relatedness reconstructed using the ITS marker (Fig. 2), it forms a moderately statistically supported group (80/0.94) with species *O. triangulosporum*, *O. pehueninum*, and *O. araucariae*. This group clusters with strong support (90/1.00) with species from the *O. ulmi* species complex. Based on phylogeny, the strain CCF 6633 cannot be assigned to any known species complex. Considering the lack of significant genetic similarity to previously sequenced species and its unique host beetle association, we describe this fungus as a new species.

The study of *D. himalayensis* adults using SEM revealed that series of well-developed and deep pits forming the elytral striae in both sexes contain various organic particles. Among them, numerous fungal spores were identified. Conversely, shallow pits in the thoracic region did not contain fungal spores (Fig. 3).

Ophiostoma juglandis M. Kolařík, T. Májek, I. Milenković & Tomšovský, sp. nov. (Fig. 4).

Mycobank No. 851262.

Etymology. Refers to *Juglans*, the host tree.

Diagnosis. *O. juglandis* is phylogenetically distinct from all other sequenced species. It produces a hyalorhinocladia-like asexual morph similar to the related species *O. triangulosporum*, but differs by significant genetic differences in the barcoding genes.

Type. Necrosis around larval gallery of *Dryocoetes himalayensis* on *Juglans regia*, Czechia, South Moravian region, Blučina, 49.0476N, 16.65406E, 250–300 m a.s.l., isol. T. Májek as JR1, 18 May 2020, PRM 960094 (holotype, dried culture on MEA), PRM 960095, 960096 (isotypes), culture ex-type CCF 6633. GenBank accessions of the barcode genes: ITS OR946482, LSU rDNA OR946483, SSU rDNA OR946484, CAM OR973012, TUB2 OR973013, and TEF-1α OR973014.

Other material examined. Czechia, South Moravian region, Blučina, 49.0476N, 16.65406E, 250–300 m a.s.l., isol. T. Májek as JR3. *Juglans regia*. Morphologically and genetically identical to CCF 6633. TUB2 Genbank number: OR973015.

Description. Sexual morph not observed. Asexual state hyalorhinocladia-like. Conidiophores mononematous, micronematous, colourless, (5.0–)68.1±31.7 SD (–110.0) × (2.3–)2.5±0.33 SD(–3.5) µm. Conidia colourless, smooth, one-celled, clavate, obovoid to pyriform

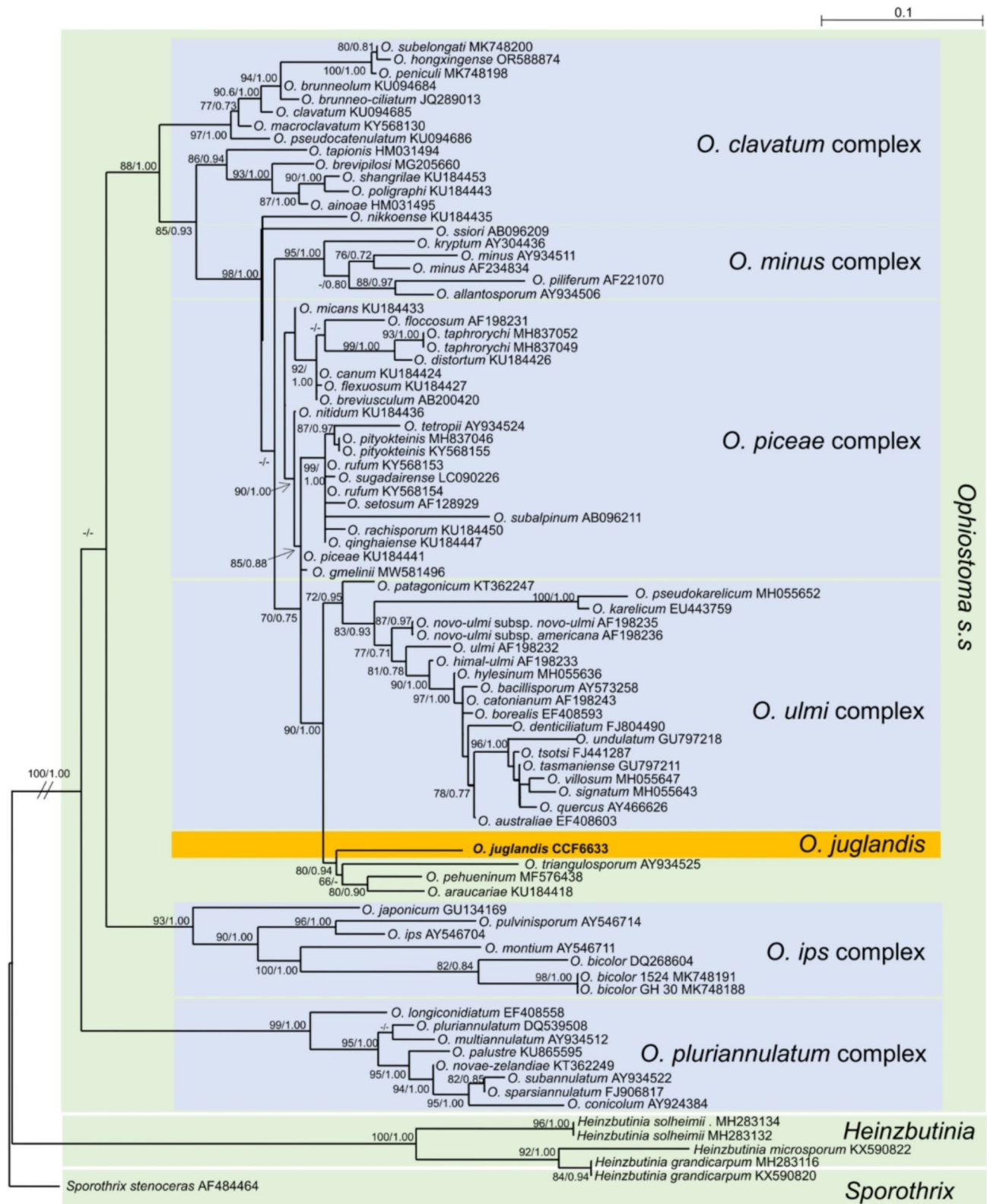


Fig. 2 Maximum likelihood phylogenetic tree showing position of *O. juglandis*. The SH-aLRT support followed by posterior probability values greater than 50/0.50 are shown. The branch leading to the root is 5× shortened. The tree is rooted with *Sporothrix*

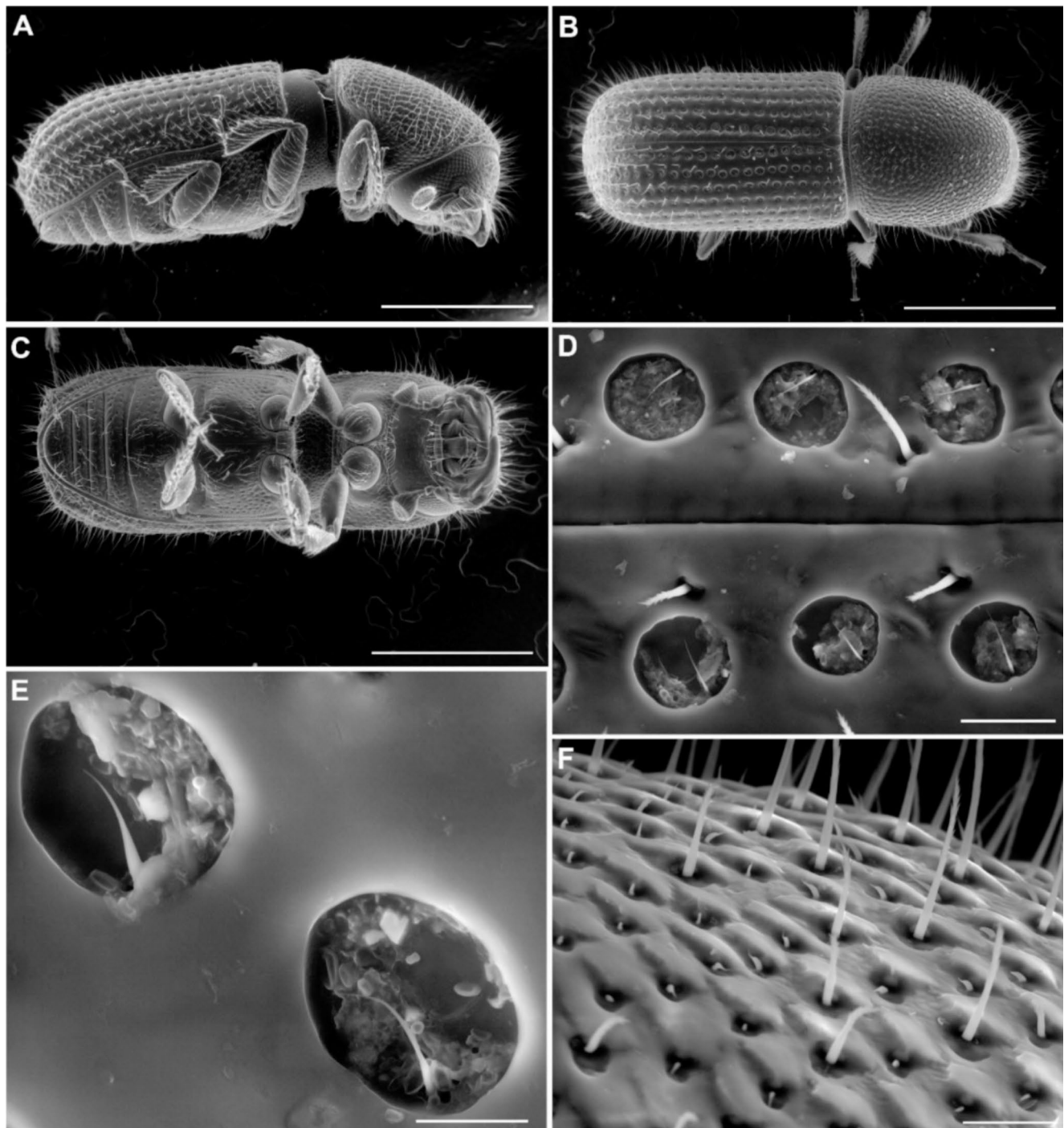


Fig. 3 Morphology of *D. himalayensis* (male or female) observed by SEM. **A** Lateral view. **B** Dorsal view. **C** Ventral view. **D**, **E** Pits in elytral striae with fungal spores. **F** Pronotal pits. Scale bar. **A–C** 10 μ m; **D**, **F** 50 μ m; **E** 20 μ m

(2.5–)5.1 $\pm$ 1.9 SD(–10.1), sometimes budding to one to three secondary conidia, which are formed directly from the primary conidium or on the small denticles. Hyphae colourless, but becoming melanised in age, smooth, submerged in the medium and aerial mycelium abundant, not constricted at the septa. Chlamydospore-like cells absent. **Cultural characteristics.** Colonies with optimal growth

at 25 °C. Does not growth at 30 °C. Growth at 5 °C does not occur after 7 d, but reach 16 mm diameter after 14 d. Colony diameters after 7 d at 10 °C were 19.3 mm, at 15 °C 37.3 mm, at 20 °C 47 mm, at 25 °C 50 mm. Colonies cream after 7–14 d, becoming grey to brown in age.

Notes. Phylogenetically, this species is closely related to three species known exclusively on *Araucaria* in South

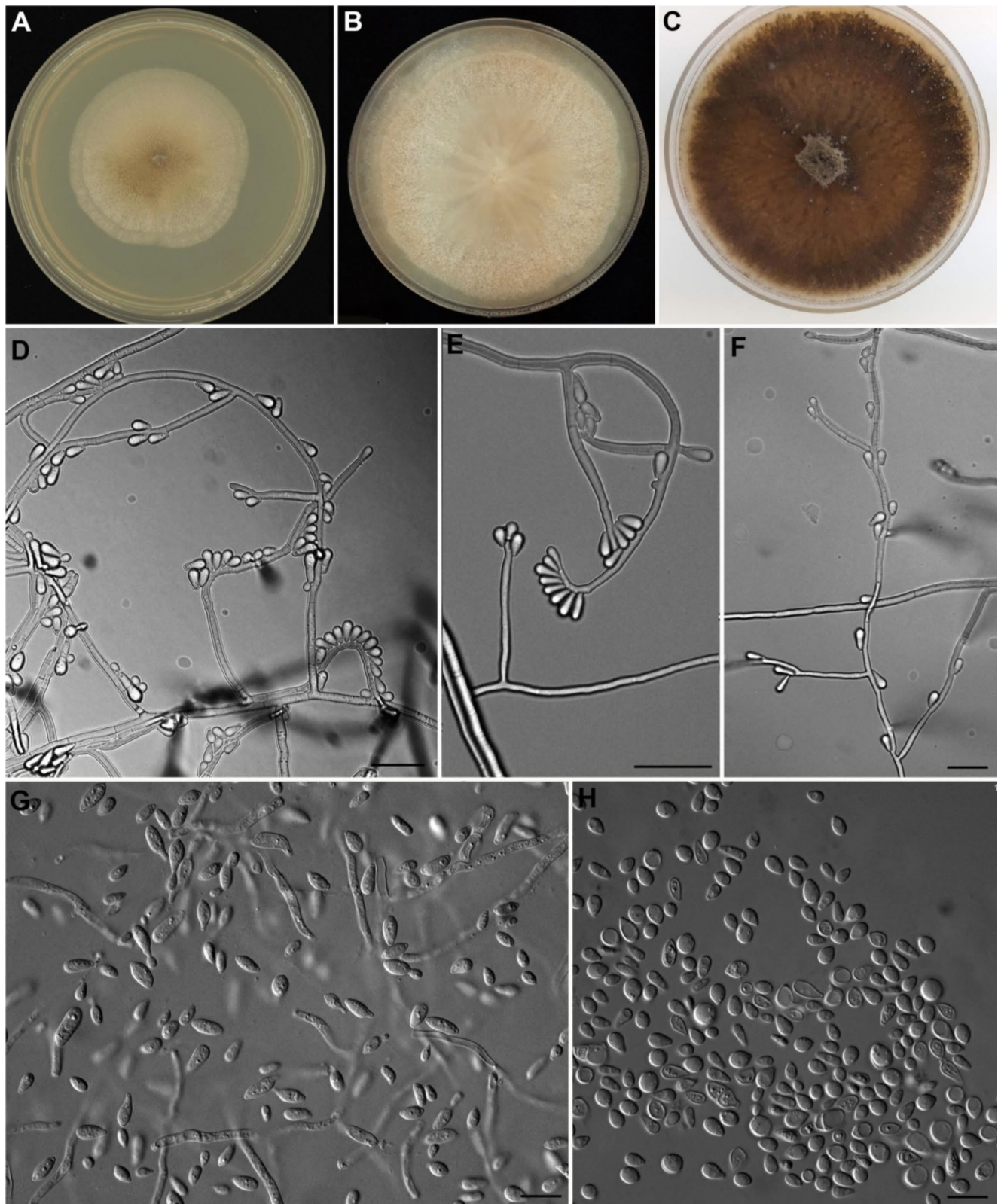


Fig. 4 Morphology of *O. juglandis*. Colony on MEA 25 °C. **A** after 7 days. **B** after 14 days. **C** after 31 days. **D–F** Macronematous and micronematous conidiophores with conidia. **G** Conidia, some are budding, forming a secondary conidium. **H** Conidia. Scale bar. **D, E, G, H** 10 μ m, **F** 20 μ m

America, specifically *O. triangulosporum* (Brazil), *O. araucariae*, and *O. pehueninum* (both from Chile) (De Beer et al. 2013). The anamorph of *O. juglandis* is partially similar to *O. triangulosporum*, which has raffaelea-to-hyalorhinocladiella-like morphology, with spores of similar shape and size ($4\text{--}9 \times 3.5\text{--}6 \mu\text{m}$; Butin 1978). The species *O. araucariae* has a *Pesotum*-like anamorph (Butin 1968). The last of the species, *O. pehueninum*, has a hyalorhinocladiella-like anamorph with distinctly round spores (Zapata et al. 2018). Species from the related *O. ulmi* complex differ by having *Pesotum*-like synnemata.

Host tree. Type strain isolated from *Juglans regia*, but potentially occurs on other host trees of its insect vector (*Juglans nigra*, *Pyrus*).

Insect vectors. *Dryocoetes himalayensis*.

Distribution. The fungus was described from Czechia, but the geographical range most probably follows the distribution of the host beetle, which is native Himalayan region and invaded to Europe – Austria, France, Germany, Slovakia, and Switzerland (Kašák et al. 2023).

Pathogenicity test

Eleven weeks after the inoculation, most of the inoculated *J. regia* plants exhibited extensive bleeding lesions on the outer bark (Fig. 5D), and the experiment was completed. The necroses were mostly ellipsoid in shape with visible active zone (Fig. 5E, F). Control group did not develop active lesions, only discoloration surrounded inoculation places (Fig. 5B). The average necrose dimensions of control plants were 9.3 ± 0.16 mm (length), 6.5 ± 0.37 mm (width), and 47.5 ± 3.12 mm² (necrosis area). The results of infection tests were influenced by the prior wounding of the host tree. Trees without bark removal exhibited no visible lesions and cankers (Fig. 5C). Average dimensions of the necroses in the *J. regia* inoculated treatment was 16.5 ± 0.95 mm (length), 9.1 ± 0.35 mm (width), and 118.4 ± 9.69 mm² (necrosis area). Reisolations from the edges of necrotic zones in the inoculated treatment were successful in 100%, while those from the control treatment were negative.

The reaction of *J. nigra* to infection was similar. The control group of *J. nigra* exhibited discoloration around inoculation places (Fig. 5G) but did not develop active lesions. In the control group, the average dimensions of necroses were 9 ± 0.36 mm (length), 5 ± 0.45 mm (width), and 34.4 ± 2.81 mm² (necrosis area). In the inoculated treatment *J. nigra* plants showed necroses (Fig. 5I) with average dimensions of 20.2 ± 0.45 mm (length), 6 ± 0.28 mm (widths) and 95.3 ± 4.56 mm² (necrosis area). In the *J. nigra* treatment without bark removal, no visible lesions and necroses were developed (Fig. 5H). Reisolations from the control treatment

were negative whereas in the inoculated treatment, reisolations were positive in 75% of plated fragments.

Two-way PERMANOVA revealed significant effects of the host plant and inoculation on necroses area, while necroses length was affected by inoculation and its interaction with the host plant (Fig. 6). Based on the pairwise test, significant differences in necroses lengths were observed between control and inoculated *J. regia* ($p < 0.001$) as well as in *J. nigra* ($p < 0.05$) plants, and between inoculated plants of the different walnut species ($p < 0.001$). Necroses area was significantly higher in inoculated compared to control plants of *J. regia* ($p < 0.001$), as well as in inoculated plants compared to the control group of *J. nigra* ($p < 0.001$). The two-way ANOVA revealed significant effects of both host plant and inoculation on necroses width (Fig. 6). Based on Fisher's LSD test, significant differences in necroses width were observed between control and inoculated *J. regia* seedlings ($p < 0.001$) and between inoculated plants of the different walnut species ($p < 0.001$), but no differences were observed between inoculated and control *J. nigra* plants ($p = 0.266$).

Discussion

The impact of already established non-native, insect pests and fungal pathogens on biodiversity and sustainability of European woodlands (forests, gardens, parks) is dramatically increasing (Santini et al. 2013; Woodward et al. 2022; Seebens et al. 2023). We revealed a new pathogenic ophiostomatoid fungus associated with the non-native but already established bark beetle *D. himalayensis* in Europe. Similar relationships of bark beetles with pathogenic fungi have been previously shown (Six and Wingfield 2011; Six 2012; Montecchio et al. 2016). In addition, our infection tests confirmed the negative impact of *O. juglandis* sp. nov. to both tested walnut species (*Juglans nigra* and *J. regia*) which is in agreement with observations by Kašák et al. (2023). The results of pathogenicity test revealed that necroses lengths and areas were statistically significantly different comparing to control in both tested walnut species, while necroses widths were significantly different only in *J. regia* plants (Fig. 6). Regarding necroses lengths, *J. nigra* showed slightly higher susceptibility being 1.2 times higher and significantly different comparing to *J. regia* plants, respectively (Fig. 6). On the contrary, necroses widths in *J. regia* were 1.5 times bigger and significantly different comparing to *J. nigra* plants (Fig. 6). Although being slightly bigger (1.2×) in *J. regia*, no significant difference was recorded between two host plants regarding necroses areas (Fig. 6).

Genetic differences among *O. juglandis* and sequenced *Ophiostoma* species are significant, its phylogenetic

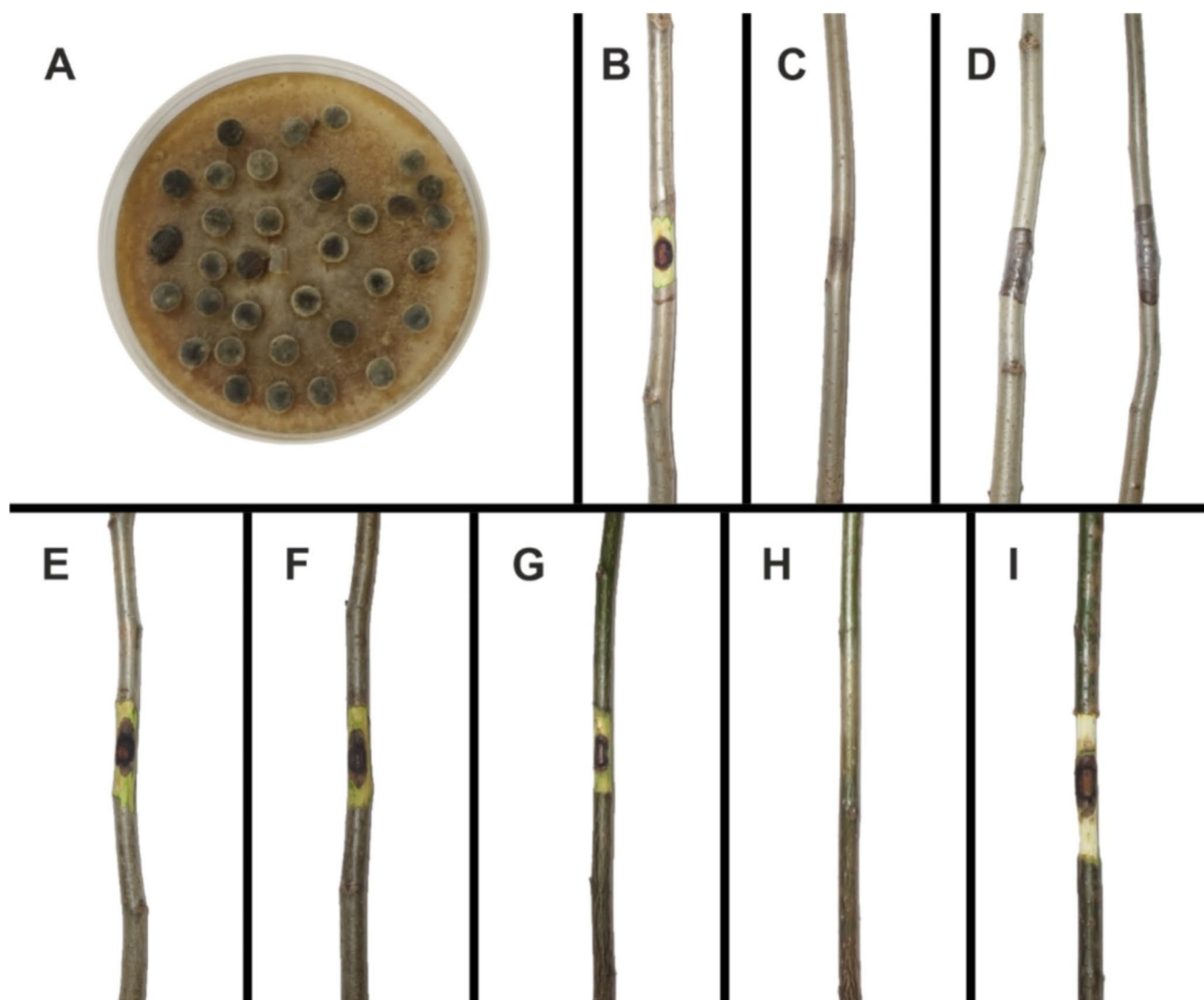


Fig. 5 Representative symptoms after 11 weeks of incubation on *Juglans regia* and *J. nigra* plants inoculated with *Ophiostoma juglandis*. **A** Wood fragments overgrown with *O. juglandis* pure culture after four weeks of incubation on MEA media at 20°C in the dark. *Juglans regia*: **B** Control plant with bark removal, **C** plant inoculated without bark removal, **D** bleeding necrosis on the stems with bark removal, **E**, **F** lesions on the stems of inoculated plants with bark removal. *Juglans nigra*: **G** control plant with bark removal, **H** plant inoculated without bark removal, **I** lesion on the stem of inoculated plant with bark removal

position within the genus *Ophiostoma* is rather isolated. We are not aware of any other *Ophiostoma* species that has not been sequenced and that is morphologically similar to *O. juglandis*. The sexual state of *O. juglandis* has not been observed. Morphologically, *O. juglandis* is characterised by hyalorhinocladia-like asexual morph, which is widespread among ophiostomatoid fungi (De Beer et al. 2013) and often resembles those of *O. juglandis* (e.g. *O. minus* (Hedgc.) Syd. & P. Syd.). These results also extended known diversity of fungal species associated with *J. regia* diseases in Europe. In the study of Belair et al. (2023), 71 different fungi were summarized as associated with *J. regia* diseases worldwide, whereas in the study of symptomatic *J. regia* branches in Czech Republic, 10 different species were

isolated and identified (Eichmeier et al. 2020). Also, in the study of Karadžić et al. (2016), 33 fungal species and two *Phytophthora* spp. were recorded on *J. regia* plants in Serbia solely, with majority (54%) being decay and rot fungi. All three studies showed no *Ophiostoma* spp. among the listed and identified organisms on *J. regia* plants.

The relationship between *O. juglandis* and the respective host *D. himalayensis* is likely to be symbiotic suggesting that they are forming ‘bark beetle holobiont’ described by Six (2013). Moreover, the SEM study of the adult beetles showed that pits in the elytral striae serve for the transmission and spread of fungal spores (Fig. 3D–E), most probably, *O. juglandis*. The genus *Dryocoetes* is characterized by the presence of elytral striae that are punctured in rows

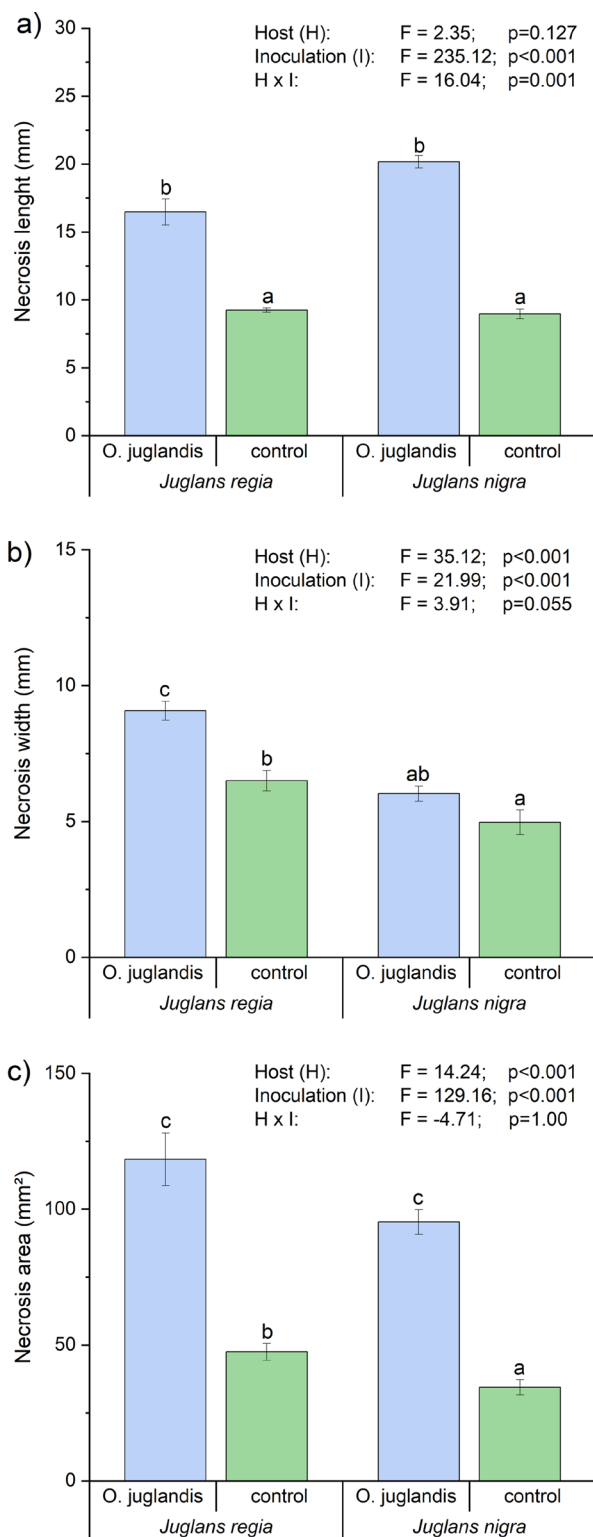


Fig. 6 Effect of host plant and inoculation by *O. juglandis* pathogen on the two walnut species seedlings wound characteristics: **a** necrosis length^{1,†}, **b** necrosis width^{2,‡}, and **c** necrosis area^{1,†}, with the results of the two-way PERMANOVA¹ and two-way ANOVA². Different letters above the bars indicate statistically significant difference ($p < 0.05$), based on the pairwise comparisons after one-way PERMANOVA[†], and Fisher's LSD test[‡]

(Wood 1992). Our study is likely to be the first to explore the function of these structures in the transmission of fungal symbionts in *Dryocoetes*, but this have been observed at *Dendroctonus pseudotsugae* Hopk. (Lewinsohn et al. 1994) or *Ips typographus* L. (Kandasamy et al. 2023). Nevertheless, the exclusivity in association of *O. juglandis* with *D. himalayensis* has to be verified by further research.

The majority of *Dryocoetes* spp. are secondary pests, attacking the humid phloem of the lower parts of dead trees. Only *D. betulae* Hopk., *D. confusus* Swaine, and *D. himalayensis* colonise vital trees (Furniss and Kegley 2006; Smith and Hulcr 2015; Kašák et al. 2023), what is a behaviour rarely found in hardwood associated species (Pfeffer 1955; Smith and Hulcr 2015). The genus *Dryocoetes* hosts a variety of ophiostomatoid fungi. Particularly, *D. autographus* Ratz., *D. alni* Georg, *D. hectographus* Reitt., and *D. villosus* Fabr. have been studied in Europe (Aas et al. 2018; Jacobs et al. 2010; Jankowiak et al. 2019; Kirisits 2004), *D. confusus* in North America, (Kendrick and Molnar 1965), and *D. baicalicus* Reitt. (Chung et al. 2006) in Japan. None of the detected fungi, including species newly described those from *Dryocoetes* (Aas et al. 2018; Chung et al. 2006; Jankowiak et al. 2020; Strzalka et al. 2020), were morphologically or genetically similar to *O. juglandis*. Fungal associates of *D. betulae* were not studied, even though formation of black discolorations of wood around its galleries resembling those of *D. himalayensis* were reported (Furniss and Kegley 2006). Systematic monitoring for early detection of disease symptoms (i.e. gallery system typical for *D. himalayensis* with fluid oozing, dark bleeding lesions, necroses, and canopy dieback) in walnut stands could be also combined with the monitoring of the invasive TCD caused by *G. morbida* associated with the walnut twig beetle *P. juglandis* (Montecchio et al. 2016). The future research will also increase the knowledge about associated mycobiota of *D. himalayensis*, distribution of *O. juglandis* and its role in decline of walnut trees.

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Author contributions All authors contributed to the study conception and design. Material preparation, samples collection and processing were performed by Tomáš Májek, Josef Kašák and Jiří Foit. DNA sequence analysis – Miroslav Kolařík and Michal Tomšovský. Phylogeny, SEM observation, and species description – Miroslav Kolařík. Pathogenicity tests – Ivan Milenković. The first draft of the manuscript was written by Michal Tomšovský. All authors have read and agreed to the published version of the manuscript.

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Data availability The DNA sequence data are available in the NCBI database (<https://www.ncbi.nlm.nih.gov/nucleotide/>).

Declarations

Conflict of interest The authors declare no conflict of interest.

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