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Characterization of *Gnomoniopsis idaeicola*, the Causal Agent of Canker and Wilting of Blackberry in Serbia

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Abstract

Blackberry cane diseases with the symptoms of necrosis, canker, and wilting are caused by several fungi worldwide. Surveys conducted from 2013 to 2016 in Serbia revealed the occurrence of *Gnomoniopsis idaeicola*, the causal agent of cane canker and wilting, which was found to be distributed in almost half of the surveyed orchards, in three blackberry cultivars, and with disease incidence of up to 80%. Wide distribution and high disease incidence suggest that *G. idaeicola* has been present in Serbia for some time. Out of 427 samples, a total of 65 *G. idaeicola* isolates were obtained (isolation rate of 34.19%). Reference isolates, originating from different localities, were conventionally and molecularly identified and characterized. *G. idaeicola* was detected in single and mixed infections with fungi from genera *Paraconiothyrium*, *Colletotrichum*, *Diaporthe*, *Botryosphaeria*, *Botrytis*, *Septoria*, *Neofusicoccum*, and *Discostroma*, and no diagnostically specific symptoms could be related directly to the *G. idaeicola* infection. In orchards solely infected with *G. idaeicola*, blackberry plant mortality was up to 40%, and yield loses were estimated at 50%. *G. idaeicola* isolates included in this study demonstrated intraspecies diversity in morphological, biological, pathogenic, and molecular features, which indicates that population in Serbia may be of different origin. This is the first record of a massive outbreak of *G. idaeicola* infection, illustrating its capability of harmful influence on blackberry production. This study represents the initial step in studying *G. idaeicola* as a new blackberry pathogen in Serbia, aiming at developing efficient control measures.

Blackberries (*Rubus* L. subgenus *Rubus* Watson) have long been readily consumed wild fruits, known to be rich in anthocyanins and antioxidants, with beneficial effects on human health and the immune system (Pellegrini et al. 2003; Reyes-Carmona et al. 2005). The first commercial blackberry cultivars were introduced in the mid-1980s in the United States, and modern cultivars are mainly hybrids derived from two or more species, including European blackberry, *Rubus fruticosus* L. (= *R. plicatus* Weihe & Nees), as well as *R. laciniatus* Willdenow, *R. procerus* P. J. Muell., *R. allegheniensis* Porter, *R. argutus* Link., and *R. trivales* Michaux (Clark and Finn 2011). As for the plant architecture, blackberry cultivars are usually described as erect, semierect, and trailing and almost all are characterized by having perennial roots and crowns and biennial canes. In the first year, canes are vegetative (primocanes), and they enter reproductive stage (floricane) the next spring, producing short lateral branches on which flowers and fruits are formed (Strik et al. 2007).

The world production of blackberries is organized on approximately 20,000 ha, with Europe being the world's largest producer (Strik et al. 2007). Serbia is the fourth largest producer in the world (Strik et al. 2007), with blackberry grown on around 5,300 ha in several regions with suitable climate conditions (Nikolić and Milivojević 2015) and participating in the world's annual production with approximately 18% (Strik et al. 2007). Numerous blackberry cultivars are commercially available worldwide. Semierect cultivar Čačanska bestrna, which is popular and well accepted by the growers in Serbia (Nikolić and Milivojević 2015), is currently grown on more than 5% of blackberry production area in the world (Strik et al. 2007).

Fungal family Gnomoniaceae (Diaporthales, Sordariomycetes, Sordariomycetidae) is morphologically and molecularly well-established (Castlebury et al. 2002) and comprises the well-studied genus *Gnomonia* and seven additional segregated genera, including *Gnomoniopsis* (Sogonov et al. 2008). Some members of the Gnomoniaceae family are well-known plant pathogens (Rossman et al. 2007), but some of them are commonly present as endophytes on the leaves and overwintering plant organs (Danti et al. 2002). The genus *Gnomoniopsis* currently contains 13 recognized species (Walker et al. 2010), including several economically important pathogens of plants mainly belonging to the families Rosaceae, Fagaceae, and Tiliaceae (Sogonov et al. 2008).

Apart from *Gnomoniopsis idaeicola* (P. Karst.) D.M. Walker, there are at least 21 different fungal species that cause cane and foliar diseases of blackberry, excluding rusts, as well as 16 additional pathogenic fungi that cause root and crown diseases described worldwide (Martin et al. 2017). The first data on *G. idaeicola* (initially referred to as *Calosphaeria idaeicola* P. Karst.) originate from 1886, when the holotype was collected on raspberry (*Rubus idaeus* L.) in Finland. Subsequent isolations were recorded in 2008 from *Rubus* sp. (France and United States [California]), *R. armeniacus* Focke (United States [Oregon and Washington]), and *R. pedatus* Banks & Sol. ex Lowe (United States [Oregon]) (Walker et al. 2010) and in 2011 in Australia (Cunnington et al. 2011). In Iran, Mirhosseini et al. (2015) isolated *Gnomoniopsis* sp., which caused leaf spots on *R. fruticosus* and, on the basis of its morphology and internal transcribed spacer (ITS) sequencing, designated it as probably *G. idaeicola*. Although *G. idaeicola* has been described as having a narrow host range, limited to *Rubus* spp. (Walker et al. 2010), there are some unpublished data on its isolation from *Actinidia deliciosa* in New Zealand (A. Bulajić, personal communication), wheat in France (Comby et al. 2016), and *Myrtus communis* in Spain (Vaz 2012), but with no trace data on how the identification was performed and if pathogenicity was confirmed. Considering that the number of *G. idaeicola* isolates described and characterized so far is limited, its epidemiology may only be deduced from a broad range of common characteristics described for the *Gnomoniopsis* spp. (Sogonov et al. 2008; Walker et al. 2010).

In Serbia, the presence of several fungi that cause blackberry cane diseases, including *Gnomonia rostellata* (Fries) Wehmeyer (Arsenijević and Veselić 1995), *Seimatosporium lichenicola* (Fuckel) Shoemaker & E. Muller (Arsenijević et al. 1999), *Phomopsis* sp. (Arsenijević 2005), *Septocyta ruborum* (Lib.) Petr., *Didymella applanata* (Niessl) Sacc. (Arsenijević 2006a), *Botryosphaeria dothidea* (Moug.: Fr.) Cesati & De Notaris, *B. obtusa* (Schwein.)

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Shoemaker, *Coniothyrium fuckelii* Saccardo, and *Sphaceloma necator* (Ellis & Everhart) Jenkins & Shear (Arsenijević 2006b), have been reported so far. Owing to the upward trend of blackberry production level in Serbia, cane diseases have become widely spread in certain production regions, causing significant yield reductions (Nikolić and Milivojević 2015). Currently available data on fungi that cause blackberry cane diseases in Serbia are limited, mainly out of date or based on only symptomatology and conventional identification.

In this paper we report the first results of the 4-year study of blackberry cane diseases in Serbia and the occurrence of *G. idaeicola*, the causal agent of cane canker and wilting. The main objectives of this study were (i) to determine distribution and incidence of *G. idaeicola*, (ii) to investigate symptomatology and morphological and pathogenic variability of Serbian isolates originating from different blackberry production regions, and (iii) to establish relationships among Serbian isolates and their position within the genus *Gnomoniopsis* by sequencing of four genetic markers of ribosomal DNA and protein coding regions, including *β-tubulin*, *tef-1α* (translation elongation factor 1α), FG1093 gene (60S ribosomal protein L37), and the ITS region (ITS1, 5.8S rDNA, and ITS2 regions).

Materials and Methods

Sampling and isolate collection. To determine the presence and distribution of *G. idaeicola* in Serbia, a 4-year survey of blackberry cane diseases was conducted in 2013 to 2016. Field inspection and collection of samples were performed in March and April of each year. A total of 24 fields of blackberry crops were surveyed at 20 localities in 13 administrative districts of major production areas. A total of 427 samples were collected, including overwintering blackberry canes with the symptoms of canker and bark necrosis. The survey included the five most commonly grown blackberry cultivars in Serbia: Čačanska bestrna, Loch Ness, Thornfree, Chester Thornless, and Triple Crown, commercially grown in 16, 4, 2, 1, and 1 orchards, respectively. Inspected orchards were of different sizes (0.03 to 1.6 ha) and of different ages (2 to 15 years). Before sampling, the disease incidence was estimated by randomly counting and rating 100 plants after zigzag walking throughout the orchards. Different numbers of samples (5 to 30) were collected from each orchard, depending on its size and types of symptoms. Each sample consisted of two symptomatic canes from one plant. Sampled canes were cut into 30- to 40-cm-long fragments, sealed in plastic bags, stored at 5°C, and processed within 24 h after being brought to the laboratory.

Two 5-cm-long fragments were cut off from each sampled cane, washed with tap water for 2 h, and surface sterilized for 2 min with commercial bleach (0.5% sodium hypochlorite). Four small pieces from different parts of the border between necrotic and healthy tissue of each fragment (16 per sample) were aseptically excised, air dried for 30 min, and placed on potato dextrose agar (PDA; 200 g of potato, 20 g of dextrose, 17 g of agar, and 1 liter of distilled H_2O). Plates were incubated at 24°C for 5 days, and developed colonies were transferred onto fresh PDA. Pure cultures of fungal isolates obtained from each sample were grouped according to colony appearance and were used for obtaining single-spore isolates. All morphospecies obtained from each sample were preliminarily identified to the genus level according to the morphology and sequencing of the ITS region of rDNA. Isolates of *Gnomoniopsis* sp., originating from different localities, were maintained on PDA slants at 4°C and used for further characterization. The overall isolation frequency and the isolation frequency per field were calculated (Saleemi et al. 2012).

Morphological identification. Eleven single-spore isolates of *G. idaeicola*, one from each locality, were selected as representative and were used for conventional identification based on macroscopic and microscopic characteristics. Colony appearance, radial growth rate, and conidiomata presence, formation pattern, and abundance were assessed on PDA plates 10, 20, and 40 days postinoculation (dpi), incubated at 24°C and a cycle of 12 h of light/12 h of darkness. The appearance and formation pattern of perithecia, asci, and ascospores were assessed directly on the overwintered canes of cultivar Čačanska bestrna (locality of Miokus). Isolated conidiomata and perithecia were placed on a glass slide in a drop of sterile water and directly observed using a stereo (EU Instruments SM-2TXX/10) or a compound microscope (Olympus CX41), and photos were taken using microscope eyepiece camera (10 MP Aptina Color CMOS, MU1000). In all measurements, an ocular micrometer was used for measuring 50 (conidiomata and perithecia) or 100 (conidia, asci, and ascospores) microstructures per isolate. Colony growth rate was estimated by measuring two perpendicular colony diameters on five plates per isolate and calculating the average value for each isolate. Measurements of all morphological features were analyzed by one-way analysis of variance at the 5% probability level after testing the normality (Shapiro–Wilk test) and fulfilling the homogeneity assumptions, and the individual pairwise comparisons were made using Tukey's test (Sokal and Rohlf 1995). Data on colony growth were analyzed using the Kruskal–Wallis test because data were not homogeneous. All statistical analyses were performed using XLSTAT software.

DNA amplification and sequencing. Molecular identification and characterization of Serbian isolates were performed by sequencing four selected genomic fragments: nuclear ribosomal ITS region rDNA and protein-coding genes *β-tubulin*, *tef-1α* (translation elongation factor 1α), and FG1093 (60 S ribosomal protein L37). Selected genomic fragments were amplified using the following primers: ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for the ITS region, T1 and T2 (O'Donnell and Cigelnik 1997) for the *β-tubulin* gene, EF1-728F/EF1-1199R and EF1-983F/EF1-1567R (Carbone and Kohn 1999; Rehner and Buckley 2005; Walker et al. 2010) for the *tef-1α* gene, and FG1093 E1F1/E3R1 (Walker et al. 2012) for the FG1093 gene (Table 1). Total genomic DNA was extracted from 100 mg of dry mycelium weighed from 10-day-old cultures, using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) in a total reaction volume of 25 μl, consisting of 12.5 μl of 2× PCR Master mix (K071, Fermentas, Lithuania), 9 μl of RNase-free water, 1.25 μl of both forward and reverse primers (100 pmol/μl, Metabion International, Planegg/Steinkirchen, Germany), and 1 μl of template DNA. Amplification conditions were as follows: initial denaturation step of 10 min at

Table 1. DNA primers used in this study

Primer name	Primer sequence (5′–3′)	References
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn (1999)
EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	Rehner and Buckley (2005)
EF1-1567R	ACHGTRCCRATACCACCRATCTT	Rehner and Buckley (2005)
EF1-1199R	GGGAAGTACCMGTGATCATGT	Walker et al. (2010)
T1	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik (1997)
T2	TAGTGACCCTTGGCCCAGTTG	
E1F1	GCGCCACAMCAAGWCSCACRC	Walker et al. (2012)
E3R1	TTCTBCGCTTGGCCTTCTCRS	

95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, with a final extension period of 10 min at 72°C. Amplification conditions for the FG1093 gene were as follows: 2 min at 95°C, followed by 10 cycles of 1 min at 95°C, 30 s at 65 to 55°C (decreasing by 1°C in each cycle), and 1 min at 72°C, followed by 35 cycles of 1 min at 95°C, 30 s at 59°C, and 1 min at 72°C. Amplified products were stained with Midori Green DNA Stain (Nippon Genetics), analyzed by 1% agarose gel electrophoresis, and visualized under an ultraviolet transilluminator.

The PCR products of all genomic regions were sequenced in both directions in an automated sequencer (ABI 3730XL Automatic Sequencer, Macrogen, Seoul, South Korea), using the same primers as for the amplification. Consensus sequences were computed using ClustalW (Thompson et al. 1994), integrated in MEGA6 software

Table 2. Isolates of the *Gnomoniopsis* spp. and *Gnomoniopsis*-related species used in this study

Species	Strain/isolate	Host	Country	GenBank accessions			
				ITS	*β-tubulin*	*tef-1α*	FG1093
Apiognomonia veneta	CBS 342.86	*Platanus acerifolia*	France	DQ313531	…	…	…
Gnomoniopsis alderdunense	CBS 125679	*Rubus pedatus*	U.S.A. (OR)	GU320826	…	…	…
G. alderdunense	CBS 125680	*R. parviflorus*	U.S.A. (OR)	GU320825	GU320787	GU320801	JF274653
G. alderdunense	CBS 125681	*R. parviflorus*	U.S.A. (OR)	GU320827	…	…	…
G. chamaemori	CBS 804.79	*R. chamaemorus*	Finland	GU320817	GU320777	GU320809	JF274646
G. clavulata	AR 4313; CBS 121255	*Quercus falcata*	U.S.A. (MD)	EU254818	EU219211	GU320807	JF274644
G. comari	CBS 806.79	*Comarum palustre*	Finland	EU254821	EU219156	GU320810	JF274647
G. comari	CBS 807.79	*C. palustre*	Finland	EU254822	…	…	…
G. comari	CBS 809.79	*C. palustre*	Switzerland	EU254823	…	…	…
G. fructicola	AR 4275; CBS 121226	*Fragaria vesca*	U.S.A. (MD)	EU254824	…	…	…
G. fructicola	CBS 208.34	*Fragaria* sp.	France	EU254826	EU219149	GU320808	JF274645
G. fructicola	CBS 125671	*Fragaria* sp.	U.S.A. (NJ)	GU320816	…	…	…
G. guttulata	BPI 877452A	*Agrimonia eupatoria*	Bulgaria	EU254812	…	…	…
G. idaeicola	CBS 125672	*Rubus* sp.	U.S.A. (CA)	GU320823	…	…	…
G. idaeicola	CBS 125673	*R. pedatus*	U.S.A. (OR)	GU320824	…	…	…
G. idaeicola	CBS 125674	*Rubus* sp.	France	GU320820	…	…	…
G. idaeicola	CBS 125675	*R. armeniacus*	U.S.A. (OR)	GU320822	…	…	…
G. idaeicola	CBS 125676	*R. armeniacus*	U.S.A. (WA)	GU320821	GU320784	GU320811	JF274654
G. idaeicola	V1EG6	wheat plant	France	KT692597	…	…	…
G. idaeicola	155e	*Myrtus communis*	Spain	KC959208	…	…	…
G. idaeicola	ICMP:11546	*Actinidia deliciosa*	New Zealand	KC145891	…	…	…
G. idaeicola	ICMP:10075	*R. fruticosus*	New Zealand	KC145872	…	…	…
G. idaeicola	KMS4-14	*R. fruticosus*	Serbia	MF537338	MG860499	MG755816	MG860489
G. idaeicola	KVR2-14	*R. fruticosus*	Serbia	MF537339	MG860500	MG773585	MG860490
G. idaeicola	KRU9-15	*R. fruticosus*	Serbia	MF537340	MG860501	MG773586	MG860491
G. idaeicola	KNEI7-15	*R. fruticosus*	Serbia	MF537341	MG860502	MG773587	MG860492
G. idaeicola	KZAI1-15	*R. fruticosus*	Serbia	MF537342	MG860503	MG773588	MG860493
G. idaeicola	KMI5-15	*R. fruticosus*	Serbia	MF537337	MG860504	MG773589	MG860494
G. idaeicola	KDK28-16	*R. fruticosus*	Serbia	MF537333	MG860505	MG773590	MG860495
G. idaeicola	KPK10-16	*R. fruticosus*	Serbia	MF537334	MG860506	MG773591	MG860496
G. idaeicola	KAR2-16	*R. fruticosus*	Serbia	MF537335	MG860507	MG773592	MG860497
G. idaeicola	KKR7-16	*R. fruticosus*	Serbia	MF537336	MG860508	MG773593	MG860498
G. idaeicola	KSV1-16	*R. fruticosus*	Serbia	MG878401	MG878402	MG878403	MG878404
G. idaeicola	KMS2-14	*R. fruticosus*	Serbia	MG893859	…	…	…
G. idaeicola	KMS6-14	*R. fruticosus*	Serbia	MG893860	…	…	…
G. idaeicola	KMS12-14	*R. fruticosus*	Serbia	MG893861	…	…	…
G. idaeicola	KVR1-14	*R. fruticosus*	Serbia	MG893862	…	…	…
G. idaeicola	KMI8-15	*R. fruticosus*	Serbia	MG893863	…	…	…
G. idaeicola	KRU7-15	*R. fruticosus*	Serbia	MG893864	…	…	…
G. idaeicola	KZAIII3-15	*R. fruticosus*	Serbia	MG893865	…	…	…
G. macounii	AR 3866; CBS 121468	*Spiraea* sp.	U.S.A. (NY)	EU254762	EU219126	GU320804	JF274641
G. occulta	CBS 125677	*Potentilla* sp.	U.S.A. (OR)	GU320828	…	…	…
G. occulta	CBS 125678	*Potentilla* sp.	U.S.A. (OR)	GU320829	GU320786	GU320800	JF274650
G. occulta	BPI 877455	*Potentilla anserina*	Russia	EU254811	…	…	…
G. paraclavulata	CBS 123202	*Quercus alba*	U.S.A. (MD)	GU320830	GU320775	GU320815	JF274642
G. racemula	CBS 121469; AR 3892	*Epilobium angustifolium*	U.S.A. (MN)	EU254841	…	…	…
G. sanguisorbae	CBS 858.79	*Sanguisorba minor*	Switzerland	GU320818	GU320790	GU320805	JF274648
G. sanguisorbae	CBS 125299	*R. parviflorus*	U.S.A. (OR)	GU320819	…	…	…
G. tormentillae	CBS 904.79	*Potentilla erecta*	Switzerland	EU254856	EU219165	GU320795	JF274649
Ophiognomonia setacea	CBS 128354	*Quercus* sp.	U.S.A. (NJ)	JF514847	JF514839	JF514823	JF274652
Plagiostoma euphorbiae	CBS 340.78	*Euphorbia palustris*	Netherlands	EU199198	…	…	…
Plagiostoma sp.	CBS 128351	*Acer* sp.	U.S.A. (NY)	JF514852	JF514836	JF514833	JF274651
Sirococcus conigenus	CBS 113.75	*Picea pungens*	Germany	EF512482	EU219129	EF512544	JF274643
S. piceicola	CBS 119620	*Picea sitchensis*	Canada	EF512480	…	…	…
S. tsugae	CBS 119627	*Cedrus deodara*	U.S.A. (OR)	EF512478	…	…	…
S. tsugae	CBS 128356	*Tsuga canadensis*	U.S.A. (ME)	JF514853	JF514844	JF514834	JF274655
Gnomoniopsis sp.	GiM	*R. fruticosus*	Iran	KJ563296	…	…	…

(Tamura et al. 2013), and deposited in GenBank (Table 2). All generated sequences were compared with each other by calculating nucleotide (nt) identities, as well as with previously deposited *Gnomoniopsis* spp. isolates available in GenBank, using the similarity search tool BLAST.

Phylogenetic analysis. Newly generated ITS, *β-tubulin*, *tef-1α*, and FG1093 sequences were analyzed with all *G. idaeicola* sequences available in NCBI and previously listed type-derived sequences of *Gnomoniopsis* spp. (Walker et al. 2010, 2012). The analyses of individual ITS gene alignment for identification of Serbian isolates and the analyses of concatenated four-gene combined alignment for in-depth characterization were performed using MEGA6 software (Tamura et al. 2013). ITS region alignment consisted of sequences of 18 Serbian and 31 reference isolates of *Gnomoniopsis* spp., three isolates of *Sirococcus* spp., and two outgroup taxa: *Apiognomonia veneta* (Sacc. & Speg.) Höhn and *Plagiostoma euphorbiae* (Fuckel) Fuckel from the Gnomoniaceae, retrieved from the GenBank (Table 2). A multilocus phylogenetic analysis based on combined sequences of ITS, *β-tubulin*, *tef-1α*, and FG1093 genes included sequences of 11 Serbian and 11 reference isolates of *Gnomoniopsis* spp. and two species of *Sirococcus*, compiled with the outgroup taxa: *Plagiostoma* sp. and *Ophiognomonia setacea* (Pers.) Sogonov (Table 2).

Phylogenetic trees were inferred using the maximum likelihood implemented in MEGA version 6.0 software (Tamura et al. 2013) on individual ITS as well as combined data sets for the ITS, *β-tubulin*, *tef-1α*, and FG1093 gene sequences. The gamma-distributed Tamura–Nei model determined by Modeltest implemented in MEGA6 was used as the best fitting model of nt substitution. The reliability of the obtained trees was evaluated using 1,000 bootstrap replicates, and bootstrap confidence values <70% were omitted.

Pathogenicity on blackberry canes. Ten representative isolates of *G. idaeicola*, sampled in different orchards and years of the survey, were tested for pathogenicity by artificial wound inoculations of primocanes of blackberry cultivar Čačanska bestrna. During September 2017, well-developed primocanes (approximately 15 mm in diameter) were removed from symptomless blackberry plants in an orchard with no history of cane diseases, transported to the laboratory, and inoculated on the same day. Primocanes were shortened to the length of 40 cm, the foliage and lateral shoots removed, the upper ends sealed with moist cotton wool and Parafilm, and the primocanes placed in 15-cm pots containing sterilized moist sand. For the purpose of inoculation, primocanes were wounded by making clear cuts, approximately 10 mm long, without damaging the cambial tissue underneath. Mycelial plugs (5 mm in diameter) from the edge of actively growing 15-day-old PDA culture were placed under the bark (mycelial surface face down), and the wound was sealed with sterilized moist cotton wool and Parafilm. As a negative control, primocanes were inoculated with sterile PDA plugs. Each isolate was inoculated into five primocanes, and the experiment was repeated twice. Inoculated primocanes were incubated at 25°C, cycle of 12 h light/12 h darkness, and high humidity. The pathogenicity of isolates was assessed 14 dpi according to the severity of developed symptoms. For the purpose of rating, the following 0 to 5 scale was established: 0 = no visible symptoms; 1 = necrosis only on the wounding point; 2 = necrotic tissue exceeding the wounding point up to 2 cm; 3 = necrotic tissue exceeding the wounding point up to 4 cm; 4 = necrotic tissue exceeding the wounding point up to 6 cm; and 5 = visible necrosis longer than 6 cm around the point of inoculation. All data were analyzed by Kruskal–Wallis test at the 5% probability level, with individual pairwise comparisons made using Dunn's test. Statistical analyses were performed using XLSTAT software.

Fig. 1. Geographic distribution of localities in Serbia included in the surveys. Arrows indicate the localities where *Gnomoniopsis idaeicola* was detected.

Results

Presence and distribution. During the 4-year survey, the presence of *G. idaeicola* in Serbia was confirmed at 11 out of 24 localities (Fig. 1, Table 3), in both single (localities of Vrbovac, Miokus, and Svilajnac) and mixed infections. On the basis of their morphology and ITS sequencing (data not presented), different morphospecies, belonging to the genera *Paraconiothyrium*, *Colletotrichum*, *Diaporthe*, *Botryosphaeria*, *Botrytis*, *Septoria*, *Neofusicoccum*, and *Discostroma*, were also detected from orchards with and without *G. idaeicola* presence. The overall isolation rate of *G. idaeicola* was 34.19% (146 positive out of 427 samples), or 66.06% (146 positive out of 221 samples) after excluding localities without its presence (Table 3). During 2013, *G. idaeicola* was not detected, whereas during 2014 to 2016 overall isolation rates were 75.75, 51.85, and 26.75%, respectively (or 75.75, 65.88, and 63.11%, respectively, after excluding localities without its presence). Mostly, only one pathogenic fungus was isolated from each sample. Mixed infections with *G. idaeicola* and additional pathogenic fungi in the same sample were recorded in 17 out of 221 samples (7.69%).

In all surveyed orchards, overall cane disease incidence was 5 to 80% (average 37.71%) (Table 3), whereas in *G. idaeicola*-positive orchards it was in the range of 10 to 80% (average 44.09%). In the localities of Svilajnac and Miokus, where *G. idaeicola* was the only detected pathogen, symptoms were present in a very high incidence of 70 and 80%, respectively, with a high intensity of leaf necrosis and cane wilting (Fig. 2A).

In orchards with Serbian cultivar Čačanska bestrna, *G. idaeicola* was widely distributed (in 8 out of 16 surveyed orchards, disease incidence up to 80%). The presence of *G. idaeicola* was also detected in orchards with cultivars Loch Ness (locality of Svilajnac, incidence of 70%) and Thornfree (localities of Mišar and Donja Kamenica, incidences of 35 and 25%, respectively). Cultivars Chester Thornless and Triple Crown, represented in the survey in only one orchard each, were not infected with *G. idaeicola*.

Symptomatology and impact. In all surveyed orchards, particularly those infected solely with *G. idaeicola*, the first visible symptoms on blackberry primocanes were conspicuous, smaller or larger, mostly elliptic necrotic lesions. Lesions were commonly present on nodes and internodes and had brownish-red, dark red, or grayish-black discoloration and a prominent purple border. Over time, lesions enlarged and coalesced, forming large necrotic areas and cankers, usually girdling the canes, and the bark within the diseased area became pale, grayish to silver (Fig. 2B). Larger cankers often split longitudinally, revealing discolored and disorganized necrotic pith. Numerous small black fruiting subepidermal conidiomata, utterly scattered with erumpent ostioles, were frequently recorded on necrotic areas of the canes (Fig. 2K). In the spring, heavily infected overwintered floricanes exhibited reduced lateral growth, with leaves becoming chlorotic followed by necrosis, prominent wilting, and decay. Fruits on such canes either failed to develop or developed to a reduced size, or even ceased to develop prematurely. All of that caused significant yield reduction. Yield losses

Table 3. Geographic distribution of *Gnomoniopsis idaeicola* isolates collected in Serbia in the survey conducted from 2013 to 2016

Year	Locality	Blackberry cultivar	No. of samples/ *G. idaeicola* positive samples	Incidence of cane disease symptoms (%)	Additional fungi isolated[v]	No. with mixed infection	*G. idaeicola* isolates	
							Culture	PCR
2013	Leskovac	Čačanska bestrna	15/0	55[w]	*Paraconiothyrium* spp.	…	0	0
	Dragojevac	Čačanska bestrna	28/0	40	*Colletotrichum* spp. *Paraconiothyrium* spp. *Diaporthe* spp. *Botryosphaeria* spp.	…	0	0
2014	Vrbovac	Čačanska bestrna	10/10	15	…	…	3[x]	2[y]
	Mišar	Thornfree	23/15	35	*Paraconiothyrium* spp. *Diaporthe* spp.	4[z]	6	4
2015	Miokus	Chester Thornless	5/0	70	*Paraconiothyrium* spp. *Botryosphaeria* spp.	…	0	0
		Čačanska bestrna	8/8	80	…	…	2	2
	Dobrić	Čačanska bestrna	18/0	40	*Botrytis* spp. *Botryosphaeria* spp.	…	0	0
	Ruma	Čačanska bestrna	25/16	25	*Botryosphaeria* spp. *Botrytis* spp.	6	9	2
	Negotin	Čačanska bestrna	26/13	45	*Septoria* spp. *Botryosphaeria* spp. *Diaporthe* spp.	1	10	1
	Zaječar	Čačanska bestrna	26/19	60	*Paraconiothyrium* spp. *Neofusicoccum* spp.	4	11	2
2016	Boljevac	Čačanska bestrna	17/0	20	*Botryosphaeria* spp. *Paraconiothyrium* spp. *Septoria* spp.	…	0	0
	Knjaževac	Čačanska bestrna	5/0	5	*Septoria* spp.	…	0	0
	Medveđa	Čačanska bestrna	23/0	30	*Septoria* spp. *Paraconiothyrium* spp.	…	0	0
	Donja Kamenica	Thornfree	17/6	25	*Paraconiothyrium* spp. *Botryosphaeria* spp.	2	3	1
		Triple Crown	5/0	5	*Discostroma* spp.	…	0	0
		Loch Ness	23/0	40	*Septoria* spp.	…	0	0
	Ivanjica	Čačanska bestrna	12/0	30	*Septoria* spp.	…	0	0
		Loch Ness	9/0	25	*Septoria* spp.	…	0	0
	Arilje	Čačanska bestrna	5/3	10	*Paraconiothyrium* spp.	…	1	1
	Čačak	Loch Ness	29/0	20	*Septoria* spp. *Colletotrichum* spp.	…	0	0
	Kragujevac	Čačanska bestrna	25/11	70	*Paraconiothyrium* spp. *Botryosphaeria* spp. *Colletotrichum* spp.	…	3	1
	Prokuplje	Čačanska bestrna	26/15	50	*Paraconiothyrium* spp. *Septoria* spp.	…	7	1
	Brus	Čačanska bestrna	17/0	40	*Paraconiothyrium* spp. *Septoria* spp. *Botrytis* spp.	…	0	0
	Svilajnac	Loch Ness	30/30	70	…	…	10	1

[v] Isolated pathogenic morphospecies identified to the genera level on the basis of morphology and ITS sequencing.
[w] Disease incidence estimated by randomly counting and rating 100 plants.
[x] Number of single-spore isolates in the isolate collection.
[y] Number of isolates characterized by sequencing of ITS or of ITS, *β-tubulin*, *tef-1α*, and FG1093.
[z] Number of samples in which *G. idaeicola* was present in mixed infections.

could not be estimated in all localities and investigated years, but at the locality of Svilajnac, in the orchard in the third year of orchard exploitation, where *G. idaeicola* was the only isolated pathogen, a plant death rate of over 40% and yield loses estimated at 50% were recorded in 2016.

Morphological characterization. All 11 single-spore isolates of *G. idaeicola*, which were morphologically characterized, formed cottony, white, and fast-growing colonies on PDA, with entire or slightly undulate margins and moderate to dense aerial mycelia. After 10 days of incubation, isolates exhibited a different type of growth, forming smooth (KRU9-15, KMS4-14, KDK28-16, and KSV1-16), curled (KMI5-15, KVR2-14, KZAI1-15, and KKR7-16), or rosaceous (KNEI7-15, KPK10-16, and KAR2-16) colonies (Fig. 2E to G, Table 4), all with no visible conidiomata. Most isolates formed conidiomata at 20 dpi, and the colonies became beige to pale brown.

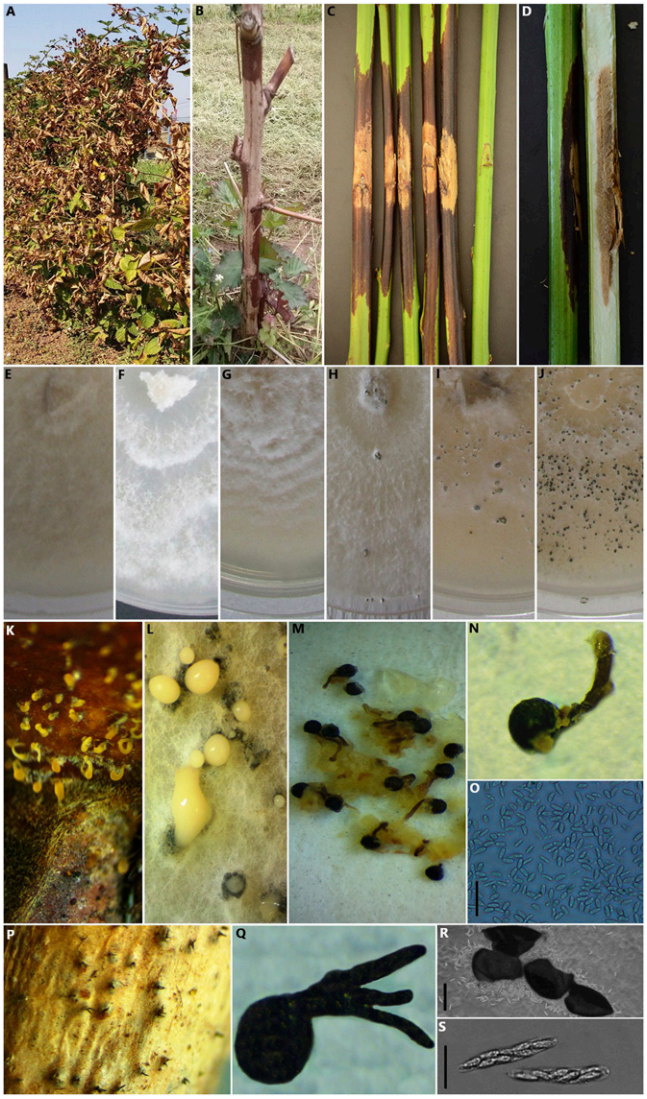

Fig. 2. Symptomatology and morphology of *Gnomoniopsis idaeicola* isolates from Serbia: **A,** blackberry leaf necrosis and wilting; **B,** canker and bark silvering; **C,** artificially inoculated primocanes (left) and negative control (right); **D,** bark and pith necrosis of artificially inoculated primocanes; **E to G,** smooth, rosaceous, and curled types of colonies on potato dextrose agar (PDA), respectively; **H to J,** weak, moderate, and intensive conidiomata formation on PDA, respectively; **K,** conidiomata immersed in the blackberry bark; **L,** conidial mass releasing on PDA; **M,** stromatic structure containing pycnidia; **N,** isolated pycnidium; **O,** one-celled conidia; **P,** perithecia immersed in the blackberry bark; **Q,** isolated perithecium with secondary branched neck; **R,** crushed perithecia and asci; and **S,** asci containing ascospores. Scale bars of conidia and asci containing ascospores = 20 μm. Scale bar of crushed perithecia and asci = 200 μm.

Conidiomata were present in the colonies of all the isolates 40 dpi, but with differences in sporulation intensity. Delayed conidiomata formation (absent in 20-day-old cultures) followed by low sporulation intensity was exhibited by the isolates KMI5-15, KMS4-14, KDK28-16, and KAR2-16. This group of isolates formed only a few individual conidiomata per colony at 40 dpi (Fig. 2H). The isolates KVR2-14, KPK10-16, and KSV1-16 exhibited moderate sporulation intensity (Fig. 2I), whereas the isolates KRU9-15, KNEI7-15, KZAI1-15, and KKR7-16 formed numerous conidiomata, densely distributed all over the colonies (Fig. 2J). The growth rate of all 11 representative isolates on PDA was 2.1 to 3.0 mm/day (on average 2.66 mm/day), with no significant differences ($P = 0.313$) (Table 4).

Conidiomata of all 11 isolates (Fig. 2L to O) had uniform features and were immersed in yellowish-brown stromatic structures, each containing 3 to 50 subglobular black pycnidia (125 to 250 μm in diameter, 193.82 μm on average). Pycnidia had long necks (371 to 480 μm, on average 421.09 μm), and conidia were hyaline, one-celled, ellipsoid to fusiform, occasionally slightly curved, and 4.5 to 7.25 × 2.15 to 3.02 μm (average 5.84 × 2.55 μm) in size. Beige drops of conidial mass, exuding from pycnidia, were visible on the surface of stromatic structures at 20 to 40 dpi. There were no significant differences between the isolates in terms of the appearance and dimension of any asexual structure ($P > 0.05$).

None of the isolates formed perithecia on PDA during 5 months of cultivation, and the morphology of sexual structures was studied after field observation. During the survey, the presence of perithecia, protruding through host epidermis, was observed on overwintered canes only in one orchard of cultivar Čačanska bestrna (locality of Miokus) (Fig. 2P to S). The identity of sexual structures was confirmed by ITS sequencing. Perithecia were solitary, numerous, black, and subglobose (195 to 300 μm in diameter, on average 275 μm), having a central neck with secondary branching (341 to 461 μm, on average 421 μm). Asci were numerous, hyaline, and fusiform (33 to 44.9 × 6.61 to 9.56 μm, on average 40.54 × 8.24 μm), containing six to eight hyaline, two-celled obovoid ascospores (6.77 to 11.4 × 2.62 to 3.55 μm, on average 8.65 × 3.08 μm).

Molecular identification and sequence analysis. BLAST analyses of all the four targeted genome regions (ITS, *β-tubulin*, *tef-1α*, and FG1093) supported conventional identification of selected Serbian *G. idaeicola* isolates. All 18 Serbian ITS sequences shared the similarity of 99.3 to 100% (4 bp differences), and BLAST analysis revealed the highest nt identities of 99 to 100% (100% query coverage) with two sequences of *G. idaeicola* from New Zealand (KC145872 and KC145891), two from France (KT692597 and NR145281), and one from Spain (KC959208). The *β-tubulin* sequences of the 11 Serbian *G. idaeicola* isolates shared similarity of 98.9 to 100% (8 bp differences), and BLAST results revealed nt identity of 98 to 100% with *G. idaeicola* isolates from the United States and France (GU320781, GU320780, GU320783, and GU320784). The *tef-1α* sequences of Serbian isolates showed nt identities of 99.4 to 100% (7 bp differences), and BLAST analysis confirmed the highest nt homology of 99% with four *G. idaeicola* isolates from the United States (GU320796 to GU320799) and one from France (GU320796). The FG1093 sequences of all the 11 Serbian *G. idaeicola* isolates proved to be 100% identical at the nt level, as well as with the only available *G. idaeicola* isolate from *Rubus armeniacus* from the United States (JF274654).

Molecular characterization and phylogeny. Maximum likelihood analyses of the ITS sequence alignment of 54 sequences of 534 nt each, including the outgroup taxa, resulted in a phylogenetic tree with established resolution and topology for *Gnomoniopsis* spp. (Fig. 3). All *G. idaeicola* isolates grouped together in one main clade with high bootstrap support (98%), containing all Serbian blackberry isolates as well as nine *G. idaeicola* isolates from different hosts and of different geographic origin (United States, Spain, France, and New Zealand). Blackberry isolate *Gnomoniopsis* sp. from Iran (GiM) was also included in *G. idaeicola* clade but on a separate branch (99% bootstrap support).

Multilocus analysis, based on the Tamura–Nei model assuming gamma distribution (Tamura et al. 2013), resulted in a maximum

likelihood tree for the four concatenated loci. The ITS, *β-tubulin*, *tef-1α*, and FG1093 sequence alignments consisted of 530, 788, 1,123, and 426 nt, respectively (2,867 nt in total), of 26 different isolates, including the outgroup taxa. At the *Gnomoniopsis* genus level, the phylogenetic tree with the combined data set (Fig. 4) shared similar topology with the ITS single locus tree obtained in this study. Better resolution was obtained within the species-specific *G. idaeicola* clade, which contained almost exclusively Serbian isolates (except for the isolate CBS 125676 from the United States). Within the clade, *G. idaeicola* isolates were, with great confidence, separated into two subgroups (bootstrap support of 100%). The first branch included the single Serbian isolate KSV1-16, whereas the remaining 10 Serbian isolates and one isolate from the United States grouped in the second branch, showing an additional subgrouping of still unclear significance.

Pathogenicity. All 10 reference *G. idaeicola* isolates caused development of prominent symptoms on inoculated blackberry primocanes at 14 dpi, confirming their pathogenicity and satisfying Koch's postulates. The symptoms were visible as dark brown to gray bark discoloration, with numerous black conidiomata around the point of inoculation (Fig. 2C). The intensity of developed symptoms and sporulation were different with each *G. idaeicola* isolate (Table 4). Silvering of bark near the inoculation point was noticeable after inoculation with almost all the isolates, resembling the symptoms of natural infection. Regardless of the intensity of bark necrosis, all the isolates colonized internal tissue, causing brown discoloration and partial pith disintegration (Fig. 2D). In all symptomatic primocanes, the presence of *G. idaeicola* was confirmed by reisolation and morphological comparison with a respective isolate. There were no visible symptoms and no pathogen isolated from negative control primocanes inoculated with sterile PDA plugs.

Differences in virulence among the Serbian *G. idaeicola* isolates were determined by comparing the symptom intensity (Table 4). Three isolates, with the highest and the lowest virulence, were significantly different ($P < 0.01$) in terms of the size of necrotic area, the size of bark silvering area, and the number of conidiomata. The most virulent was the isolate KMI5-15 (median rate 5), which caused formation of large dark gray lesions (up to 12 cm long), bark silvering over 3 cm long, and numerous pycnidia. As the opposite, the lowest virulence was exhibited by the isolates KDK28-16 and KKR7-16 (median rates 3 and 2, respectively), which caused necrosis only on wounded bark and formed only a small number of pycnidia (and for isolate KKR7-16 not in all repetitions). The remaining seven isolates also showed a trend of slightly different virulence, but with no significant distinctions ($P > 0.05$).

Discussion

In this paper we described the first results of a 4-year survey on the presence and distribution of *G. idaeicola*, a new pathogen of blackberry in Serbia. To the best of our knowledge, this is the first record of a massive outbreak of *G. idaeicola* in a blackberry production area in Europe, as well as in the world. Generally, *G. idaeicola* was found to be distributed in almost half of the surveyed orchards, in three blackberry cultivars, with medium to high disease incidence (up to 80%). Such distribution and incidence suggest that the presence of *G. idaeicola* in Serbia is not the result of a recent introduction and that the pathogen has already been established on the territory of Serbia. There are at least two main reasons why the pathogen's presence could have remained undetected. First, no diagnostically specific symptoms caused by *G. idaeicola* were observed on the blackberry leaves and canes. It can be assumed that the symptoms of *G. idaeicola* infection were noticed but were attributed to other causal agents. Cankers caused by *G. idaeicola* observed during this study greatly resembled the previous descriptions of *Leptosphaeria* and *Botryosphaeria* infections on blackberry in Serbia and worldwide (Arsenijević 2006b; Martin et al. 2017) and could not be distinguished solely on the basis of symptomatology. On the other hand, blackberry production in Serbia has expanded quickly recently and, during the period 1997 to 2005, blackberry growing areas and yields tripled. At the same time, the composition of cultivars has been changed, and domestic cultivar Čačanska bestrna became prevalent, occupying approximately 60% of the growing area (Nikolić and Milivojević 2015). During that dynamic period, a number of orchards were established with planting material of uncontrolled and uncertain health status.

Although there are no data on economic significance and impact of *G. idaeicola* in comparison with other pathogens (Martin et al. 2017),

Table 4. Morphological and pathogenic characterization of *Gnomoniopsis idaeicola* isolates from blackberry in Serbia after inoculation of primocanes of blackberry cultivar Čačanska bestrna

Isolate	Year of isolation	Locality	Cultivar	Mean growth rate	Colony appearance	Sporulation in vitro[w]	Median virulence rate	Sporulation in vivo[x]
KMI5-15	2015	Miokus	Čačanska bestrna	2.8 a[y]	Curled	Weak	5[z] a[y]	Intensive
KRU9-15	2015	Ruma	Čačanska bestrna	2.1 a	Smooth	Intensive	4 ab	Intensive
KZAI1-15	2015	Zaječar	Čačanska bestrna	2.8 a	Curled	Intensive	4 ab	Intensive
KPK10-16	2016	Prokuplje	Čačanska bestrna	2.5 a	Rosaceous	Moderate	4 ab	Moderate
KNEI7-15	2015	Negotin	Čačanska bestrna	2.6 a	Rosaceous	Intensive	3 ab	Moderate
KAR2-16	2016	Arilje	Čačanska bestrna	2.8 a	Rosaceous	Weak	3 ab	Moderate
KMS4-14	2014	Mišar	Thornfree	2.7 a	Smooth	Weak	3 ab	Weak
KVR2-14	2014	Vrbovac	Čačanska bestrna	2.6 a	Curled	Moderate	3 ab	Weak
KDK28-16	2016	Donja Kamenica	Thornfree	3.0 a	Smooth	Weak	3 b	Weak
KKR7-16	2016	Kragujevac	Čačanska bestrna	2.6 a	Curled	Intensive	2 b	Weak
KSV1-16	2016	Svilajnac	Loch Ness	2.8 a	Smooth	Moderate	…	…

[w] Sporulation intensity estimated from the number of conidiomata formed on potato dextrose agar 40 days postinoculation: intensive = numerous conidiomata distributed all over the colony surface; moderate = ≤100 conidiomata per colony; and weak = a few individual conidiomata per colony.

[x] Sporulation intensity estimated from the number of conidiomata formed on artificially inoculated blackberry canes 14 days postinoculation: intensive = numerous conidiomata distributed all over necrotic lesions; moderate = medium number of conidiomata formed near the inoculation point; and weak = conidiomata not present or only a few conidiomata formed within the lesions.

[y] Values followed by a common letter are not significantly different ($P > 0.05$).

[z] Median rates are sorted in decreasing order.

our results showed its capacity to have substantial influence on blackberry production in Serbia and elsewhere. The only previously published estimation of blackberry yield loses that could be related to *G. idaeicola* originates from Iran (Mirhosseini et al. 2015), although the authors referred to the causal agent as *Gnomoniopsis* sp. In Iran, blackberry leaf necrosis and blighting reached an incidence of 80%, with 30% dead plants and yield loses estimated at 50 to 80%. A similar situation was recorded in Serbia, with detected disease incidence caused by *G. idaeicola* of up to 80%. Several producers claimed that they were experiencing substantial loses. The most extreme situation was recorded in a young blackberry orchard of the cultivar Loch Ness (locality of Svilajnac), where the plant death rate reached 40% and yield loses were estimated at 50%. Considering the importance of blackberry production in Serbia, *G. idaeicola* has clearly demonstrated the potential to act as a harmful pathogen.

During this study, *G. idaeicola* was detected on three out of five blackberry cultivars grown in Serbia: domestic cultivar Čačanska bestrna and two international cultivars, Loch Ness and Thornfree. Available data on the host range and on cultivar reactions to infections with *Gnomoniopsis* spp. and *G. idaeicola* in particular were limited and with no details (Sogonov et al. 2008; Walker et al. 2010). This study provides the first data on differences between *G. idaeicola* infections in various blackberry cultivars. Nevertheless, because blackberry cultivars are not equally represented in the production in Serbia and they were consequently represented with different number of samples in our survey, only some of the aspects could be discussed. Additionally, there have been no reports in the literature on the correlation between *G. idaeicola* disease incidence and the age of an orchard. Based on field observation, it seems that neither the cultivar type nor the duration of commercial exploitation of an orchard has crucial influence on the level of *G. idaeicola* infection frequency in Serbia. In orchards with both domestic (Čačanska bestrna) and international cultivars (Loch Ness), the incidence of *G. idaeicola* infection was high. Similarly, in both young (locality of Svilajnac) and older orchards (locality of Miokus) diseases caused by *G. idaeicola* were also present at a high incidence. Contrary to that, a certain number of inspected blackberry orchards were with no or with little presence of *G. idaeicola*, regardless of the cultivar type and duration of orchard exploitation. It can be assumed that introduction of *G. idaeicola* into new production areas was carried out via infected blackberry planting material, whereas short-distance dissemination from plant to plant occurred via water-dispersing conidia. For the comprehensive understanding of complicated aspects of the pathogen epidemiology, such as plant age and cultivar predisposition, as well as dissemination strategies, an additional insight into *G. idaeicola* biology and reports from other blackberry production areas worldwide would be necessary.

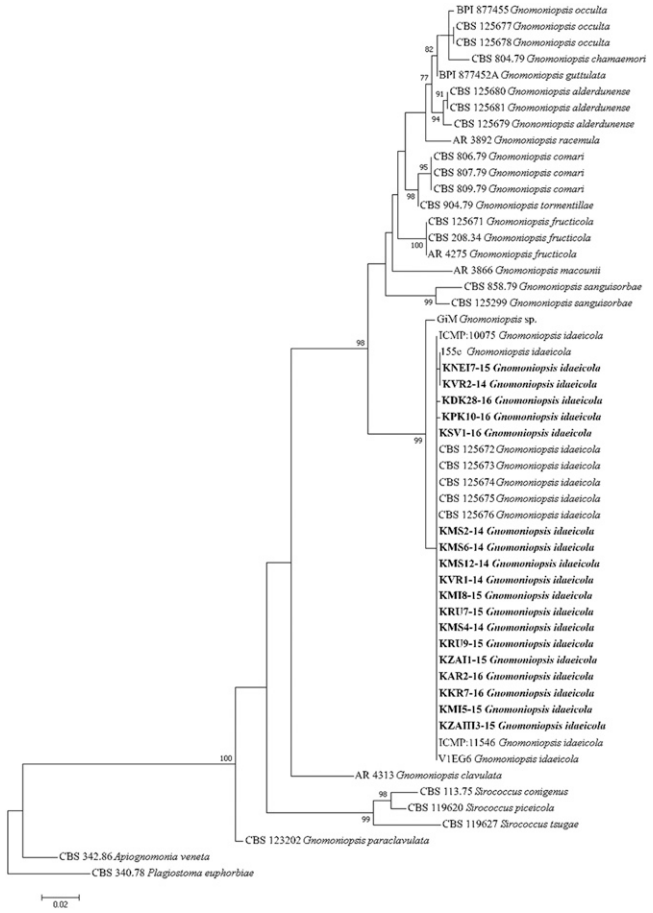

Fig. 3. Maximum likelihood phylogenetic tree of internal transcribed spacer rDNA sequences of 18 Serbian and 31 reference isolates of *Gnomoniopsis* spp., three isolates of *Sirococcus* spp., and two outgroup taxa (*Apiognomonia veneta* and *Plagiostoma euphorbiae*). The phylogram was generated with MEGA6 using the gamma-distributed Tamura–Nei model (Tamura et al. 2013). Bootstrap analysis was performed with 1,000 replicates, and bootstrap values (>70%) are shown next to relevant branches. The Serbian *Gnomoniopsis idaeicola* isolates are bolded.

Morphological features of the majority of *G. idaeicola* isolates from this study are in agreement with previous descriptions (Mirhosseini et al. 2015; Walker et al. 2010). However, morphological characterization of Serbian *G. idaeicola* isolates, originating from different localities, revealed that colony appearance and sporulation capacity varied substantially among the isolates. *G. idaeicola* colony color was described as a variable feature among isolates of the same or different origin (Mirhosseini et al. 2015; Walker et al. 2010). All isolates examined in this study formed white to beige colonies, which appeared to be a stable characteristic of Serbian *G. idaeicola* isolates. On the other hand, Serbian isolates demonstrated different growth patterns, particularly in early stages of colony development, forming either smooth, rosaceous, or curled colonies. Isolates from Serbia also varied substantially in regard to in vitro sporulation capacity. Variability in growth pattern had not been reported previously, as well as variability in sporulation intensity. The only available description of *Gnomoniopsis* spp. (Sogonov et al. 2008) and, particularly, *G. idaeicola* sporulation capacity (Mirhosseini et al. 2015; Walker et al. 2010) was that several characterized isolates from France, the United States, and Iran exhibited abundant sporulation.

Sexual reproduction was not found to be a common feature among *G. idaeicola* isolates in Serbia. None of the isolates, originating from different blackberry orchards, produced perithecia in vitro on PDA, although it was expected and previously described (Walker et al. 2010). The presence of perithecia was recorded only in one out of 11 orchards with *G. idaeicola* blackberry infection. The morphology and dimensions of perithecia, asci, and ascospores were similar to previous descriptions of *G. idaeicola* (Walker et al. 2010), which supported the conventional identification based on the appearance of asexual structure. Data on *G. idaeicola* epidemiology, especially on the ways of overwintering and the significance of different sources of inoculum in the world, are limited (Walker et al. 2010). Nevertheless, based on field observation, it seems that perithecia have limited importance in overwintering and as the source of inoculum, at least in Serbia. A completely different dissemination strategy was described for a pathogenic species from the same genera, *G. smithogilvyi*, on chestnut, in which ascospores dominated as the source of inoculum, whereas conidia had limited or no impact at all (Shuttleworth and Guest 2017). Taking into account that *G. idaeicola* is widely distributed on blackberry in Serbia, further epidemiological studies would be necessary, especially on the sources of inoculum and the role of wind in disseminating ascospores. This is emphasized by recent records of *G. idaeicola* as a pathogen on host plants other than *Rubus* spp. in New Zealand (A. Bulajić, personal communication) and as an endophyte in different plants, such as wheat and *M. communis* (Comby et al. 2016; Vaz 2012).

Molecular identification and phylogeny supported the conventional identification of Serbian *G. idaeicola* isolates. Applying the proposed concept of backbone phylogenetic trees (Hyde et al. 2014), we used a phylogenetic tree with defined and sufficient

resolution within the genus *Gnomoniopsis* (Walker et al. 2010). The position of Serbian isolates and all the others from different hosts and parts of the world (Spain, France, and New Zealand) within the *G. idaeicola* branch additionally supported the taxonomy of this well-defined and separated species-specific branch. The *G. idaeicola* clade also included a *Gnomoniopsis* sp. isolate from Iran on a slightly distant and well-supported position (Mirhosseini et al. 2015). Two additional *Rubus* spp. pathogens from the genus *Gnomoniopsis*, *G. alderdunense* and *G. chamaemori* (Walker et al. 2010), exhibited clearly distant topology from all *G. idaeicola* isolates included in the ITS-based phylogenetic tree, additionally confirming the identification of Serbian isolates. According to our results, the ITS rDNA sequence is a confident delineation tool for species-specific *G. idaeicola* identification.

In multilocus phylogenetic analyses, aiming at further characterization of the Serbian *G. idaeicola* population, together with the proposed tree loci ITS, *β-tubulin*, and *tef-1α* (Walker et al. 2010), we included the additional molecular marker FG1093, which codes for the 60S ribosomal protein L37. FG1093 was described as an attractive genetic marker for species-level phylogenetic studies within the *Gnomoniopsis* genus (Walker et al. 2012). In our study, the FG1093 gene proved to be highly conserved at a species level for *G. idaeicola*, but a multilocus phylogenetic tree based on combined data of all four genetic markers provided additional information and represented the first insight into possible intraspecies variability, based on subgrouping within a well-supported branch of *G. idaeicola*. One Serbian isolate (KSV1-16) positioned separately from all the other Serbian isolates, as well as from the only available *G. idaeicola* isolate from the United States, which indicated the possibility of its different origin. Considering the frequent import of blackberry planting material into Serbia, it can be expected that some of the Serbian isolates may be of different geographic origin and related to the isolates from different blackberry production areas. Much more data and more characterized isolates are needed for a comprehensive understanding of the biological meaning and phylogenetic relationships among the isolates of *G. idaeicola*.

Generally, *G. idaeicola* isolates characterized in this study exhibited strong virulence and caused prominent external and internal cane necrosis after a short incubation period. Moreover, the majority of the isolates formed >50 conidiomata on inoculated primocanes only 14 dpi, demonstrating the potential for at least several secondary infections and the fast-spreading nature. Interestingly, some of the isolates exhibited an unusual discrepancy in sporulation intensity on host tissue and in culture. The largest number of pycnidia on inoculated primocanes was noticeable after inoculation with the most virulent isolate, KMI5-15, whereas at the same time it exhibited delayed and scarce sporulation on PDA. The least virulent isolate, KKR7-16, exhibited the opposite behavior, with scarce or no sporulation on inoculated primocanes and abundant sporulation on PDA. This suggested that sporulation of some *G. idaeicola* isolates is mediated by multiple factors and more data are necessary to fully understand the process. Differences in virulence among the species from *Gnomoniopsis* spp. have rarely been reported. Only Shuttleworth and Guest (2017) reported that isolates of *G. smithogilvyi* originating from Australia and New Zealand exhibited different virulence after artificial inoculations, compared with the isolates originating from Italy. Because the selected Serbian *G. idaeicola* isolates also demonstrated different virulence, from mild to severe, their comparison with isolates originating from different parts of the world could give an answer to the question of whether virulence of a certain isolate can be related to its geographic origin.

The results of this study contribute to the research of etiology of blackberry cane diseases and highlight the significance of *G. idaeicola*, not only as a new pathogen in Serbia but also as a pathogen capable of causing devastating blackberry diseases worldwide. Its wide distribution in different areas and cultivars in Serbia, as well as occasional high disease incidences, indicate that *G. idaeicola* has not been introduced recently and that it has been well established. At the same time, intraspecies variability in morphological, biological, pathogenic, and molecular features implies that the *G. idaeicola* population in Serbia may be of different origin, and probably the result of more than one introduction. This study represents the first initial step in the study of *G. idaeicola*, as a new blackberry pathogen in Serbia, and many aspects of its biology and, above all, epidemiology are still unknown. Taking into account its demonstrated capability to cause substantial yield loses and to limit blackberry production, there is a

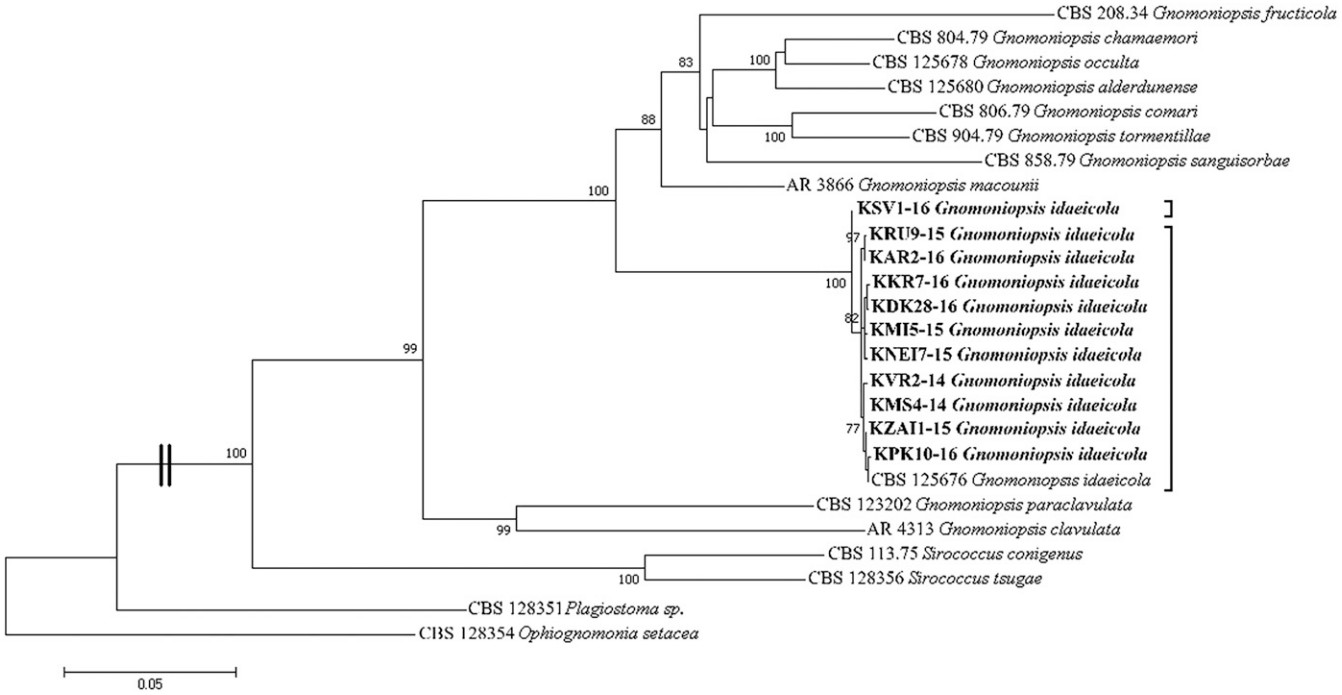

Fig. 4. Maximum likelihood phylogenetic tree inferred from concatenated internal transcribed spacer rDNA, *β-tubulin*, *tef-1α*, and FG1093 sequences of 11 Serbian and 11 reference isolates of *Gnomoniopsis* spp., two species in *Sirococcus*, and two outgroup taxa (*Plagiostoma* sp. and *Ophiognomonia setacea*). The phylogram was generated with MEGA 6 using the gamma-distributed Tamura–Nei model (Tamura et al. 2013). Bootstrap analysis was performed with 1,000 replicates, and bootstrap values (>70%) are shown next to relevant branches. The Serbian *Gnomoniopsis idaeicola* isolates are bolded.

strong need to include *G. idaeicola* into a phytosanitary scheme in the production of pathogen-free planting material, as the first step in developing efficient control measures.

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