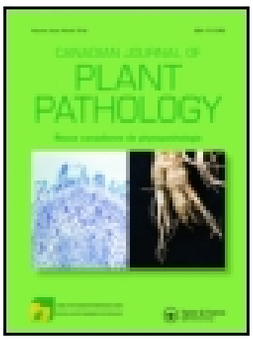

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A blight disease on highbush blueberry associated with *Macrophomina phaseolina* in Serbia

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Abstract: Unusual blight-like symptoms appeared on highbush blueberry plants in Serbia during August 2015 and infected plants showed browning and reddening of leaves, drying of foliage, and brown discoloration of internal vascular stem tissues. The objective of this study was to isolate and confirm a causal agent of the disease. Five diseased blueberry plants (2-yr-old), with visible brown discoloration in the wood, were collected for isolation on potato dextrose agar (PDA). Morphological analysis of the selected fungal isolates showed the presence of abundant black, round to oblong, or irregularly-shaped microsclerotia immersed in the PDA. Dark, globose pycnidia formed on water agar with an initially hyaline, granular content and single-celled conidia, indicating the presence of plant pathogenic fungus *Macrophomina phaseolina* associated with symptomatic plant tissues. Pathogenicity was confirmed on potted blueberry plants based on the initial symptoms of leaves turning yellowish to brown at the leaf edges, followed by the defoliation of leaves of the inoculated stems. Discoloration of vascular tissues was also observed on transverse sections of inoculated stems. The pathogen *M. phaseolina* was confirmed using molecular analysis of the ITS1-5.8S-ITS2 region of rDNA and a part of the TEF-1α gene region. This is the first report of *M. phaseolina* causing a blight disease on highbush blueberry in Serbia. The study should help in elucidating disease symptomatology and provide the knowledge on the risk which this fungus could pose in blueberry production.

Keywords: blueberry, blight, *Macrophomina phaseolina***,** identification, charcoal rot.

Résumé : En août 2015, des symptômes inhabituels ressemblant à ceux causés par la brûlure sont apparus sur des plants de bleuets en corymbe en Serbie. Les feuilles des plants infectés rougissaient ou brunissaient, le feuillage se desséchait et les tissus vasculaires internes des tiges

affichaient une coloration brunâtre. Le but de cette étude était d'isoler et d'identifier l'agent causal de la maladie. Cinq plants de bleuets infectés (vieux de deux ans), dont le bois présentait une coloration brunâtre, ont été collectés afin d'isoler l'agent pathogène sur de la gélose dextrosée à la pomme de terre. L'analyse morphologique des isolats fongiques sélectionnés a révélé de nombreux microsclérotes noirs, de forme ronde à oblongue ou irrégulière, ancrés dans le milieu de croissance. Des pycnides foncées et globulaires avec, initialement, un contenu hyalin et granulaire ainsi que des conidies unicellulaires, se sont développées sur de l'eau gélosée. Cela indiquait que le champignon pathogène *Macrophomina phaseolina* était responsable des symptômes associés aux tissus. La pathogénicité a été confirmée sur des plants de bleuets cultivés en pots en se basant sur les symptômes initiaux relatifs au jaunissement et au brunissement de la bordure des feuilles, suivis de la défoliation des tiges inoculées. Une coloration des tissus vasculaires a également été observée sur des sections transversales des tiges inoculées. L'identité de l'agent pathogène a été confirmée par analyse moléculaire de la région de l'ITS1-5.8S-ITS2 de l'ADNr et d'une partie de la région du gène du TEF-1α. Il s'agit de la première mention de *M. phaseolina* causant la brûlure sur des plants de bleuets en corymbe en Serbie. L'étude devrait aider à élucider la symptomatologie de la maladie et fournir de l'information quant au risque que ce champignon pourrait poser pour la production de bleuets.

Mots clés : Bleuet, brûlure, *Macrophomina phaseolina*, identification, pourriture noire.

Introduction

Blueberries (*Vaccinium* spp.) have gained popularity worldwide due to their economic value and nutritional and health benefits i.e. high antioxidant compounds such as anthocyanins, phenols and flavonols in fruit (Moyer et al. 2002). There are three types of blueberry in commercial production: lowbush (*Vaccinium angustifolium* Ait.), highbush (*V. corymbosum* L.) and rabbiteye (*V. ashei* Reade). Highbush is the most common, with different cultivars which vary in their response to exposure to low temperature periods and differ in their normal flowering and fruiting periods (DeFrancesco & Murray 2011).

Blueberries are cultivated commercially worldwide (Evans & Ballen 2014), mostly in the USA and Canada, but some production also occurs in a few European countries. In Serbia, blueberries are cultivated on an area of around 200 ha, with the biggest producers being located near urban areas. There has been a notable increase in blueberry production in Serbia over the last few years (Nikolić & Milivojević 2015), and 'Duke' is the most common cultivar grown in the region.

Blueberries are grown for 20-30 years, and are thus exposed to various fungal, oomycete, bacterial or viral diseases (Schilder & Miles 2008; Pscheidt & Weiland 2015). Botrytis blight, anthracnose and septoria leaf spot are prevalent diseases of blueberries in Serbia. In the summer of 2015 (at the end of August), in Pridvorica (Mačva region) in Serbia, unusual foliar blight symptoms (Fig. 1a) were observed on a 3-year-old highbush blueberry 'Duke' (1-year-old planted highbush blueberry from 2-year-old propagation material). The observed symptoms consisted of browning and reddening of leaves and drying of foliage, and brown discoloration of internal vascular tissues at the basal part of the bush. The disease incidence ranged from 2 to 5 %. The affected blueberry field had a long crop-rotation history, with corn and wheat being the last crops

sown in the season prior to the planting of blueberries. Following the planting of blueberries, the field was put under the conditions of intensive irrigation and fertilization. The disease symptoms were observed only on the part of the field where corn was the previous crop in the rotation system and where there was water retention in the soil. This study was carried out to identify and characterize the causative agent associated with the blight symptoms, and confirm its pathogenicity on highbush blueberry.

Materials and methods

Sampling and isolation of pathogens

A field survey, which included the 3-year-old highbush blueberry 'Duke' (i.e. one year after plantation), was carried out during 2015 to determine the incidence of blight symptom in a 2 ha commercial field located in Pridvorica (Mačva region), Serbia. Five diseased plants with visible brown discoloration in the wood tissue at the basal part of bush were randomly sampled (Fig 1b-c) and taken to the laboratory. Fungal isolation from the two-year-old stems was carried out as previously described by Xu et al. (2015) for blight disease on blueberry. Sections of vascular tissues (phloem and xylem) were cut with a sterile scalpel into 1 mm thick sections. These thin sections were surface-sterilized by immersing them in a 3% (v/v) bleach solution for 3 min, followed by dipping in 70% ethanol for 30 s and rinsing in sterile distilled water (SDW) three times, each time for 1 min. Five pieces were taken from each stem and placed onto potato dextrose agar, PDA (Difco, USA), amended with streptomycin sulfate (50 mg L^{-1}). Petri plates were incubated for 7 days at 24°C in the dark. The plates were examined daily to record the fungal colony growth. During the incubation process, with the exception of contaminations, only one fungal colony type, suspected to be pathogenic, was developed. Fungal colonies were purified by

transferring them onto new PDA plates. In total, 20 fungal isolates, TP1b – TP20b (four isolates representing one plant) were selected for further morphological study. Fungal isolates were maintained as stock cultures on PDA slants at 4°C.

Morphological characterization

All 20 purified fungal isolates were analyzed for colony morphology and conidial characteristics (Beas-Fernandez et al. 2006; Gupta et al. 2012; Kaur et al. 2012). The fungal isolates were incubated on PDA under a photoperiod of 10 h/14 h of light/dark, and cool, white fluorescent light, at 24°C for a period of 14 days. The colour and shape of the colonies were recorded on the seventh day. The shape, colour and size of at least 50 microsclerotia were observed under the optical microscope (Euinstruments WF10/20; Amscope FMA50 camera).

To observe the pycnidia, they were induced on 2% water agar (WA) with autoclaved pine needles as a substrate, after a 3-week incubation at 25 °C (Slippers et al. 2004). For each of the tested fungal isolates, length and width of 50 pycnidia and 100 conidia were measured using a compound microscope (Olympus BX51TF; Imaging Software; Olympus digital camera E-620). The mean value, standard deviation and 95% confidence limits were calculated for each fungal isolate. The colour of pycnidia and conidia was also recorded.

Based on the similar morphological results obtained for all of the tested fungal isolates, three representative fungal isolates, coded as TP1b, TP2b and TP3b, were selected for the purpose of the pathogenicity test, PCR and DNA sequencing.

Pathogenicity

Pathogenicity was conducted on 3-year-old potted blueberry 'Duke', according to previous pathogenic studies of *M. phaseolina* on other host plants (Almomani et al. 2013; Cummings & Bergstrom 2013; and Pavlović et al. 2015). Two-year-old stems of potted plants were first surface-disinfected with 70 % ethanol for 1 min, and cuts (5-8 mm in length, 2-3 mm in width) were made with a sterile scalpel through the bark to the cambium. Mycelial disks (5 mm in diameter) from 4-day-old fungal isolates (PDA) were applied behind a bark flap of the wounded site. To avoid drying, inoculation sites were wrapped with moist cotton and covered with aluminum foil. In the control plants, the wounds were treated with PDA disks. Three replicates were used for each of the three representative fungal isolates, TP1b, TP2b and TP3b. Inoculated plants were kept in a growth chamber at a temperature of 27±2 °C, 12h / 12h light-dark photoperiod and watered as necessary. The experiment was repeated twice.

The symptoms on inoculated plants were assessed over a period of two months. Two months after the inoculation, plants were transverse sectioned from the point of inoculation to observe the discoloration of the vascular tissue. To fulfill Koch's postulates, inoculated stem tissue with discoloration symptoms was used to reisolate the fungal pathogen onto PDA, as described above for the pathogen isolation. Three obtained re-isolates (RTP1b, RTP2b, RTP3b) were further identified based on morphological characteristics to confirm if they were the same as the original. Likewise, re-isolation from control blueberry stems was done in order to show that the healthy plants used for the pathogenicity test were not already infected.

Polymerase chain reaction (PCR) and DNA sequencing

Three fungal isolates selected for further molecular characterization (TP1b, TP2b and TP3b) were grown on PDA for 7 days at 24°C. Approximately 50 mg of dry weight of the mycelium

were collected and used for DNA extraction, following the manufacturer's instructions of Plant DNAesy Mini Kit (Qiagen, Valencia, CA, USA). For the identification of these isolates, amplification of internal transcribed spacer (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA), using universal primer pair ITS1/ITS4 (White et al. 1990), and a fragment of the TEF-1α gene region, using primer pair EF1-728F and EF1-986R (Carbone & Kohn 1999) were performed by polymerase chain reaction (PCR), following the procedures of White et al. (1990) for the ITS and Carbone & Kohn (1999) for the TEF-1α gene region. The resulting PCR products were separated by electrophoresis on a 1% agarose gel, and the bands were excised and purified (Purification Kit, Qiagen, USA) for sequencing (Macrogen, Seoul, Korea). The obtained fungal sequences were deposited into the NCBI GenBank database.

Phylogenetic analysis

Phylogenetic analysis was performed to determine the evolutionary relationship between the isolates from blueberry (TP1b, TP2b, TP3b) and the most closely related strains originating from other host plants. For the ITS-5.8S rDNA analysis, the isolates were identified as *Macrophomina phaseolina* based on sequences of *M. phaseolina* originating from different hosts from NCBI database (GenBank numbers for these strains are shown in Fig. 3a): *Helichrysum arenarium* (strain MP 1), hemp (strain MAC 81), *Phaseolus sp*. (strain MP 74), sesame (strain MP 219), soybean (strain MP 105), *Sorghum sp*. (strain MP 122), *Staevia rebaudiana* (strain 39R(3)), sugar beet (strain PM 19), sunflower (strain MAC 25) and *Zea mays* (strain CBS 227.33). *Botryosphaeria dothidea* strain PI565 was used as an out-group taxon.

The obtained TEF-1α sequences were compared with eight *M. phaseolina* sequences deposited into the NCBI database (GenBank numbers for these strains are shown in Fig. 3b),

originating from almond (strain PD112), *Eucalyptus sp.* (strain CBS 162.25), *Jatropha curcas* (strain 162), *Opuntia ficus-indica* (strain CPMM), *Phaseolus lunatus* (strain CPMM3543), *Ricinus communis* (strain CDA1108), strawberry (strain MAC-12) and *Zea mays* (strain 227.33). *B. dothidea* strain MFLUCC 16 0092 was used as an out-group taxon.

All sequences were aligned using ClustalW multiple sequence alignment implemented in BioEdit software (version 7.1.3), and phylogenetic trees were constructed in MEGA 7 (Kumar et al. 2016), using the Neighbor-joining method. Evolutionary distances were computed using the Kimura two-parameter nucleotide substitution model (Kimura 1980).

Results and discussion

Cultural and morphological characteristics

All fungal isolates from blueberry plants initially produced whitish colonies with concentric growth on PDA, which turned grey after 5 days, dark after 10 days, and black after 21 days (Fig. 2a). Black, round to oblong or irregularly-shaped microsclerotia also formed on PDA (Fig. 2b), and the size of microsclerotia in the 7-day-old culture ranged between 93.8-179±2.56 (length) × 54.3-111.36±2.8 (width) µm. Pycnidia which formed on pine needles WA medium were typically dark, globose, measuring 98.0-224±3.88 × 67.5-170±3.1 µm in size. The conidia were initially hyaline, with a granular content and single-celled, measuring, 13.7-26.6±1.2 (length) × 6.9-14.2±0.9 (width) µm (Fig. 2c). Based on these cultural and morphological characteristics, all fungal isolates were identified as *Macrophomina phaseolina*.

Pathogenicity

All tested *M. phaseolina* isolates produced disease symptoms on inoculated highbush blueberry 'Duke' plants (Fig 1d-g). The first symptom appeared 3 weeks after inoculation, when the edges of leaves turned yellowish to brown; later, the yellowish areas enlarged and turned brown and started to fall off, and almost all leaves dried out and had fallen after 60 days. The vascular tissues of the inoculated stems also showed internal discolouration. Following plating of vascular tissue sections from the inoculated stems, the developing fungal colonies on PDA were identical in colony morphology of the original *M. phaseolina* isolates from blueberry. Non-inoculated control plants developed no disease symptoms and were negative for fungal reisolation.

In this study, the fungus *M. phaseolina* was consistently isolated from infected blueberry plants showing blight symptoms and its pathogenicity was confirmed on potted healthy blueberry plants. Therefore, this study reports a new host for this pathogen from Serbia. Previously, only a short note, published by Zuckerman (1960) included *Macrophoma* sp. among the fungi isolated from diseased blueberry stems collected in Massachusetts. Following that initial report, there was no further data pertaining to this pathogen's specificity to this host. *M. phaseolina* affects more than 500 cultivated and wild plant species belonging to 75 families (Su et al. 2001) and has a wide geographic distribution. Cultivating blueberry mainly after susceptible hosts, as was the case with corn in the observed Serbian field, can further increase the importance of this pathogen for blueberry production. *M. phaseolina* causes a broad spectrum of symptoms, such as stem canker, seedling blight, charcoal rot, dry root rot, wilt, leaf and stem blight, and pre-emergence and post-emergence damping-off, root and stem rot (Babu et al. 2007; Kaur et al. 2012). Gupta et al. (2012) indicated that the reddish-brown discolouration of the vascular elements of roots and lower stem precedes the premature yellowing, as the fungus spreads up the stem during the season. Similarly, these symptoms could be observed for Serbian blueberries infected with *M.*

phaseolina. Wounding was conducted prior to inoculation of blueberry stems, which has been shown to be required for infection by *M. phaseolina*. Different methods including tissue wounding such as the toothpick inoculation and stem tape inoculation have been described to evaluate *M. phaseolina*, but these techniques do not remotely simulate the natural infection processes, in contrast to the soil inoculation method (Grezes-Besset et al. 1996). It has been reported that *M. phaseolina* indirectly penetrates through natural openings or wounds (Mayek-Pérez et al. 2002) and spreading over a larger area via conidia (Kaur et al. 2012). *M. phaseolina* is a primarily soil borne pathogen known to produce microsclerotia in the root and stem tissues of its infected hosts (Kaur et al. 2012).

Molecular characterization

ITS1 and ITS4 primers yielded a single 600-bp fragment from all three fungal isolates TP1b, TP2b, TP3b. The BLASTn analysis of these representative isolates showed a 100% identity with *M. phaseolina* isolate MAC17 from GenBank (Accession No. GU046807). The sequences of blueberry *M. phaseolina* isolates were deposited in GenBank (Acc. Nos. KU578311-KU578313). All isolates of *M. phaseolina* were grouped into the same cluster showing 100% similarity (Fig. 3a).

EF1-728F and EF1-986R primers amplified a 300-bp fragment from the three tested fungal isolates and BLASTn analysis showed a 99% identity with *M. phaseolina* strain MAC12 from GenBank (Acc. No. KX215103). The sequences of three representative blueberry *M. phaseolina* isolates were deposited in GenBank (Acc. Nos. KX400853-KX400855). The blueberry isolates were grouped together with the other compared *M. phaseolina* strains, showing 100% bootstrap value (Fig. 3b).

Although thus far no formae speciales, subspecies or physiological races of *M. phaseolina* have been reported, some researchers have found diversity among *M. phaseolina* isolates based on morphological, pathogenic and genetic studies (Vandemark et al. 2000; Mayek-Pérez et al. 2001; Su et al. 2001; Almeida et al. 2003; Beas-Fernandez et al. 2006; Purkayastha et al. 2006; Babu et al. 2007). It could be speculated that the genetic diversity of *M. phaseolina* could favour its survival and/or adaptation to variable environments or different hosts. Molecular tools used in this work were able to precisely identify the fungal isolates, but differences were not detected between the tested Serbian blueberry isolates and 10 comparative *M. phaseolina* strains originating from different hosts. Su et al. (2001) and Babu et al. (2007) reported high homogeneity in the ITS conserved sequences among the *M. phaseolina* isolates irrespective of their host specificity. Jana et al. (2003, 2005a, 2005b) reported random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs) or universal rice primer PCR (URP-PCR) for differentiation of *M. phaseolina* isolates on the basis of host or geographical origin. According to some studies (Vandemark et al. 2000; Almeida et al. 2003; Reyes-Franco et al. 2006; Sarr et al. 2014), there was no correlation between DNA polymorphisms and geographic locations or hosts.

In conclusion, this is the first report of *M. phaseolina* causing a blight disease on highbush blueberry in Serbia. The study should help in recognizing the disease symptomatology of *M. phaseolina* in blueberries, thus providing the basis for accurately predicting the risk which this fungus poses and could also serve as a strategy for devising future disease management.

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Figure Legends

Fig. 1. a) Blight-like disease on highbush blueberry 'Duke', due to natural infection by *M. phaseolina*. b, c) Diseased symptoms on highbush blueberry infected with *M. phaseolina* on which isolation was done. d, e, f, g) Symptoms on inoculated highbush blueberry stems ('Duke').

Fig. 2. a) Fungal colony of *M. phaseolina* isolates grown on PDA after 5 days (left), 10 days (middle), 21 days (right). b) Microsclerotia immersed in agar surface. c) Pycnidia (left), protruding conidia from pycnidia (middle), conidia (right).

Fig. 3. Phylogenetic tree constructed with the ITS-5.8S rDNA (a) and TEF-1α (b) sequences of three blueberry isolates of *M. phaseolina* (TP1b, TP2b, TP3b) and *M. phaseolina* sequences retrieved from NCBI GenBank originating from different hosts. The out-group taxon was *Botryosphaeria dothidea*.

Fig. 1.

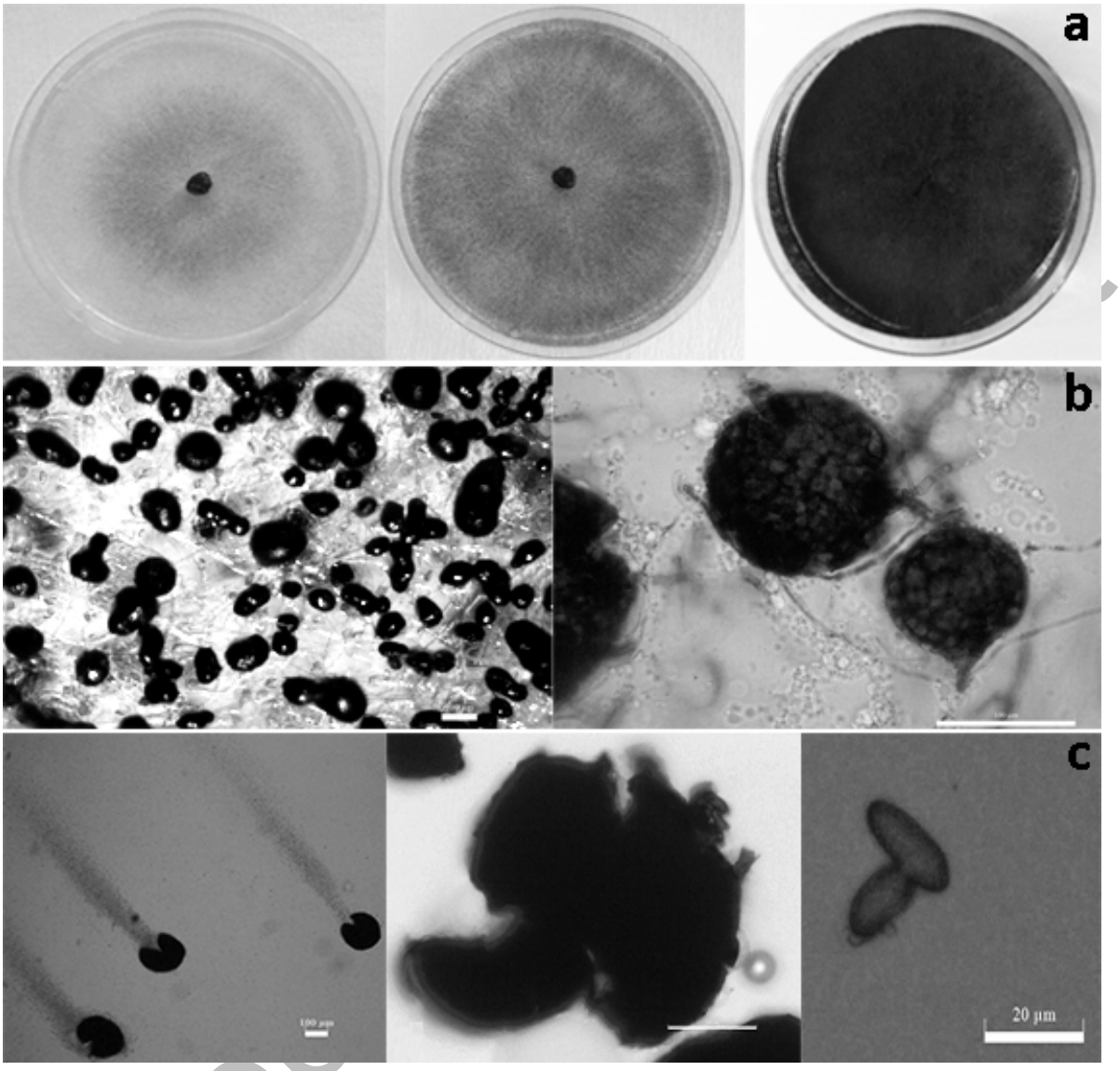

Fig. 2.

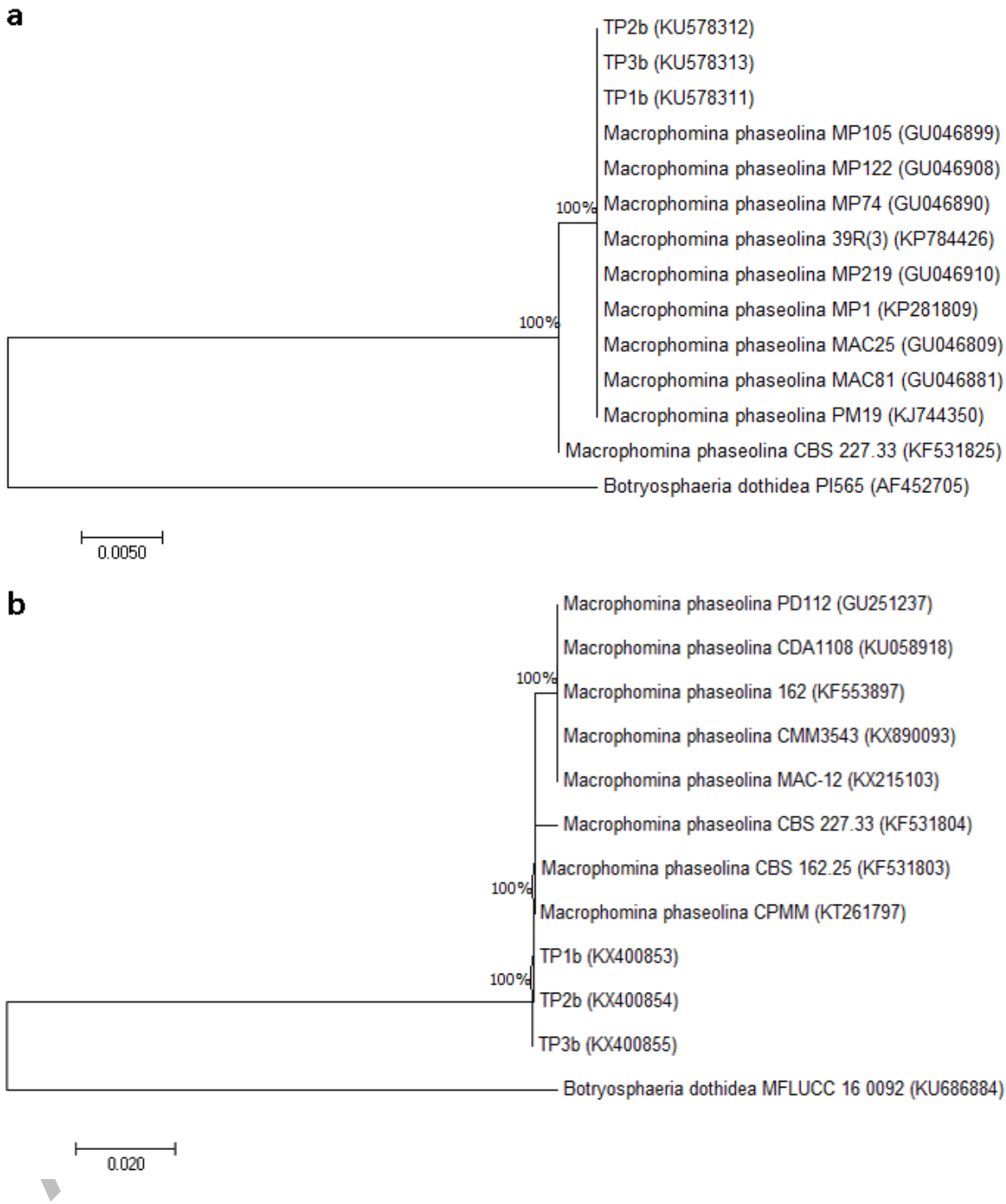
a
TP2b (KU578312)
TP3b (KU578313)
TP1b (KU578311)
Macrophomina phaseolina MP105 (GU046899)
Macrophomina phaseolina MP122 (GU046908)
100%
Macrophomina phaseolina MP74 (GU046890)
Macrophomina phaseolina 39R(3) (KP784426)
Macrophomina phaseolina MP219 (GU046910)
Macrophomina phaseolina MP1 (KP281809)
100%
Macrophomina phaseolina MAC25 (GU046809)
Macrophomina phaseolina MAC81 (GU046881)
Macrophomina phaseolina PM19 (KJ744350)
Macrophomina phaseolina CBS 227.33 (KF531825)
Botryosphaeria dothidea PI565 (AF452705)
0.0050
b
Macrophomina phaseolina PD112 (GU251237)
Macrophomina phaseolina CDA1108 (KU058918)
100%
Macrophomina phaseolina 162 (KF553897)
Macrophomina phaseolina CMM3543 (KX890093)
Macrophomina phaseolina MAC-12 (KX215103)
Macrophomina phaseolina CBS 227.33 (KF531804)
Macrophomina phaseolina CBS 162.25 (KF531803)
100%
Macrophomina phaseolina CPMM (KT261797)
TP1b (KX400853)
100%
TP2b (KX400854)
TP3b (KX400855)
Botryosphaeria dothidea MFLUCC 16 0092 (KU686884)
0.020

Fig. 3.