Shot hole disease on *Prunus laurocerasus* caused by *Neofusicoccum parvum* in Serbia

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Summary

Between 2010-2014 symptoms of a shot hole disease were observed on cherry laurel (*Prunus laurocerasus* L.) trees and shrubs in parks and other public plantings in Belgrade, Serbia. Ten symptomatic leaves were collected from each of the diseased plants and the associated fungus isolated and identified using multi-gene phylogenetic analyses and asexual morphological characters. The pathogen was identified as *Neofusicoccum parvum*. The same symptoms were produced when the pathogen was inoculated on test plants. To the best of our knowledge, this is the first report of *N. parvum* causing shot hole disease on *P. laurocerasus*.

1 Introduction

Cherry laurel (*Prunus laurocerasus* L.) is an evergreen plant with large, glossy, dark green leaves. It is a widely cultivated ornamental plant used for hedging and screening in public greens and homes in temperate regions worldwide. In Serbia, *P. laurocerasus* is a native plant that is commonly planted in the cities, propagated in ornamental nurseries and grows as an understory plant in a cherry laurel-beech forest (*Lauroceraso-Fagetum*) in Southeastern Serbia (Jovanović 1967).

During spring, summer and autumn of 2010-2014, a shot hole disease was observed on 30 *Prunus laurocerasus* plants at seven locations in the city of Belgrade, Serbia. Leaves of infected plants exhibited reddish-brown necrotic lesions (measuring 1−2.5 cm) often with concentric rings, surrounded by a reddish border or a light green halo (Fig. 1 b, d).With the development of the disease, black pycnidia formed on the lesion surface (Fig. 1c-d). Pycnidia contained conidia and spermatia. Conidia were one-celled, hyaline, fusiform to ellipsoid, smooth with fine granular content and measured 16.8 (14.8−18.3) × 6.4 (5.6−7.4) μm, resembling those of the *Neofusicoccum* spp.

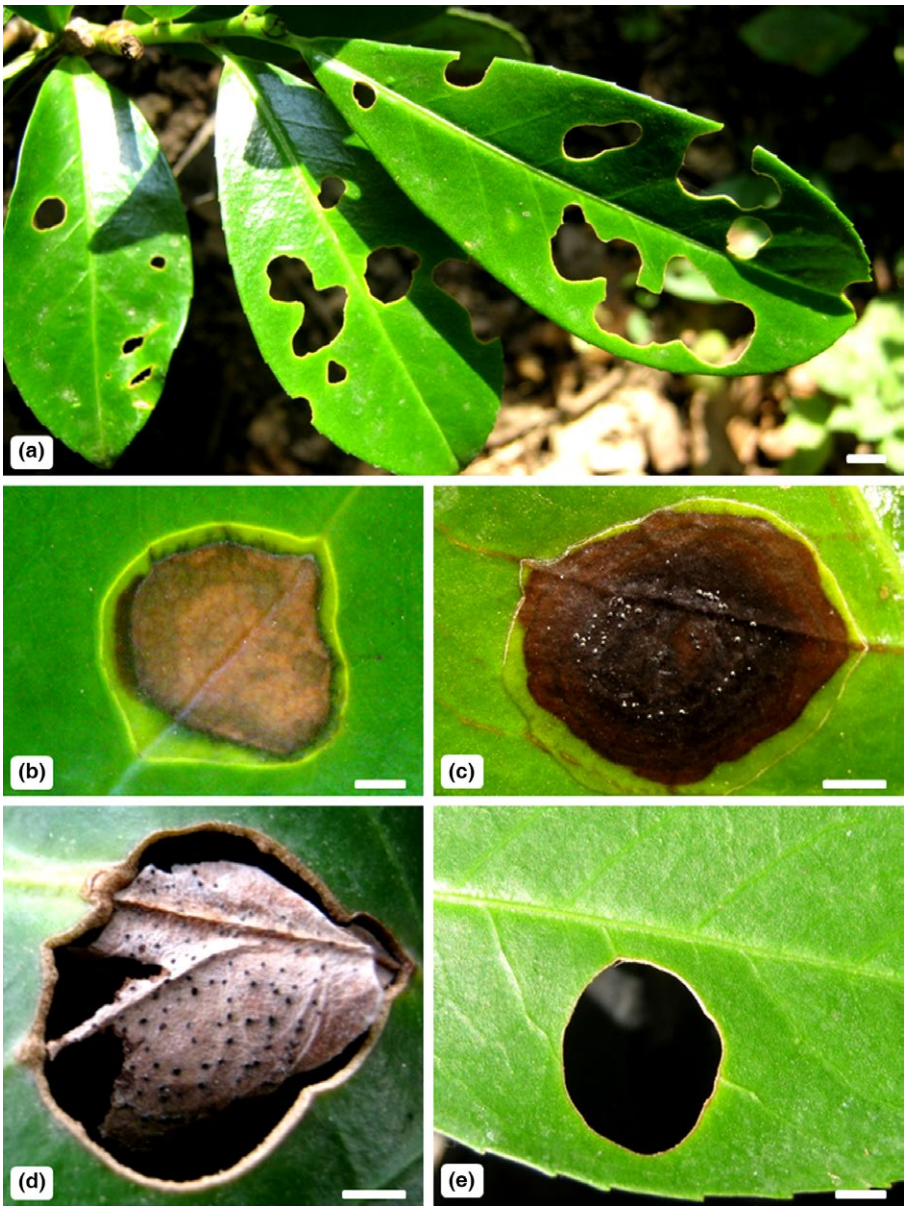

FIGURE 1 Symptoms of a shot hole disease caused by *Neofusicoccum parvum* on ornamental *Prunus laurocerasus* in Serbia. (a) Leaves with a shot hole appearance, (b) brown necrotic lesion surrounded by a light green halo, (c and d) necrotic lesion with *N. parvum* pycnidia developed on a lesion surface, (e) close up of leaf with hole. Bars: a = 1 cm, b–d = 0.3 cm

(Ascomycota: Botryosphaeriales). Spermatia were one-celled, hyaline, rod-shaped with rounded ends and measured 5.4 (2.4−5.7) × 2.8 (1.9−3.2) µm. In the final phase of disease development, necrotic lesions dropped out of the leaves, leaving holes in the leaf that resembeled damage from shot gun pellets ("shot-hole"appearance, Fig. 1 a, e). The aim of this study was to identify the causal agent of the shot hole disease on *P. laurocerasus* in Serbia using multi-gene phylogenetic analyses, spore morphology and pathogenicity tests.

2 Materials and methods

Ten symptomatic leaves were collected from each of the 30 diseased plants; pycnidia were removed with a sterile needle, examined using light microscopy and plated on malt extract agar (MEA) as described in Zlatković et al. (2016). One week later wooly, fast growing, grey fungal colonies had developed and these were purified using hyphal tip transfers. Two representative isolates, BOT 131 and BOT 275, each obtained from a different plant were further used in this study to identify the fungus and confirm its pathogenicity.

DNA was extracted from fungal mycelium of one-week-old cultures using PrepMan Ultra reagent (Applied Biosystems, Foster City, California) following the manufacturer's protocols. The internal transcribed spacer (ITS) region of rDNA operon was amplified using primers ITS-1 and ITS-4 (White et al. 1990); part of the translation elongation factor 1-alpha (TEF-1-α) gene was amplified using primers EF1-728F and EF1-986R (Carbone and Kohn 1999); the β-tubulin-2 (BT2) gene was amplified using primers Bt2a and Bt2b (Glass and Donaldson 1995) and part of the second largest subunit of RNA polymerase II (RPB2) gene was amplified using primers RPB2bot6F and RPB2bot7R (Sakalidis 2004). The conditions and procedures for PCR amplification and PCR sequencing were the same as those described in Zlatković et al. (2016).

The nucleotide sequences of both strands were examined and assembled with the CLC Main Workbench 6.6.1 (CLC Bio, Aarhus, Denmark). Sequence alignments were conducted following the method described by Zlatković et al. (2016). The phylogenetic analyses were performed using Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses. The MP analyses were performed in PAUP version 4.0b10 (Swofford 2003). The heuristic search function with 1000 random addition replicates and tree bisection and reconstruction (TBR) as branch swapping algorithm were selected. Gaps were treated as fifth characters and all characters were unordered and of equal weight. Branches of zero length were collapsed and all resulting equally parsimonious trees were saved. Measures such as consistency index (CI), retention index (RI) and tree length (TL) were recorded. ML analyses were run using an online version of

PhyML 3.0 (Guindon et al. 2010) and the best nucleotide substitution model was found using Jmodeltest v.0.1 (Posada 2008). Confidence levels were determined with 1000 bootstrap replications (Felsenstein 1985). Congruence between the different data sets was tested using the partition homogeneity test (PHT) in PAUP v. 4 (Farris et al. 1995). Phylogenetic trees were created with MEGA v.6. The DNA sequences of isolates BOT 131 and BOT 275 obtained in this study were deposited in GenBank (KF729040 and KF729068-ITS, KF729370 and KF729398- TEF-1-α, KF29330 and KF729358-β-tubulin).

A pathogenicity test was conducted using two of the collected isolates of *N. parvum* on 20 potted 2-year-old *P. laurocerasus* seedlings maintained in a nursery of the Faculty of Forestry, in Belgrade. Ten leaves per plant were lightly wounded with a sterile needle and one mycelium plug (3 mm diameter) harvested from the periphery of a 7-day-old colony was placed mycelial side down on the wound, covered with moist cotton wool and Parafilm (Pechiney, Chicago, USA). Plants were covered with transparent plastic bags to maintain a moist environment. In total ten plants were used for each isolate tested. Ten plants and ten leaves per plant were wounded and treated with sterile MEA plugs to serve as controls. The experiment was repeated once. Difference between the two isolates in mean lesion length was assessed using Student's t-test and Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA).

3 Results and discussion

The combined dataset contained 24 sequences including two sequences obtained in this study and 22 sequences retrieved from GenBank with *Pseudofusicoccum stromaticum* as an outgroup. The sequence dataset contained 1283 characters (416 parsimony informative, 867 parsimony uninformative, CI = 0.9, RI = 0.9, TL = 587). The results of the PHT test were not significant and showed that two loci can be combined (P = 0.1). The model TrN+G was chosen for the ML analyses (G = 0.2010). The MP and ML analyses yielded trees with the similar topology and the ML tree is shown (Fig. 2). Based on phylogenetic analyses, isolates considered in this study were identified as *N. parvum*.

Five days after inoculation, necrotic lesions (measuring 1-2.5 cm) were evident on all inoculated leaves and the Parafilm was removed from the leaves. There were no significant differences in mean lesion length between the two isolates. No lesions formed on controlinoculations. Two weeks after inoculation, pycnidia formed in all lesions, and all lesions dropped out of the leaves three weeks after inoculation producing symptoms similar to those seen under natural conditions. Successful re-isolations (100%) were made from all necrotic lesions, as well as from the pycnidia on the

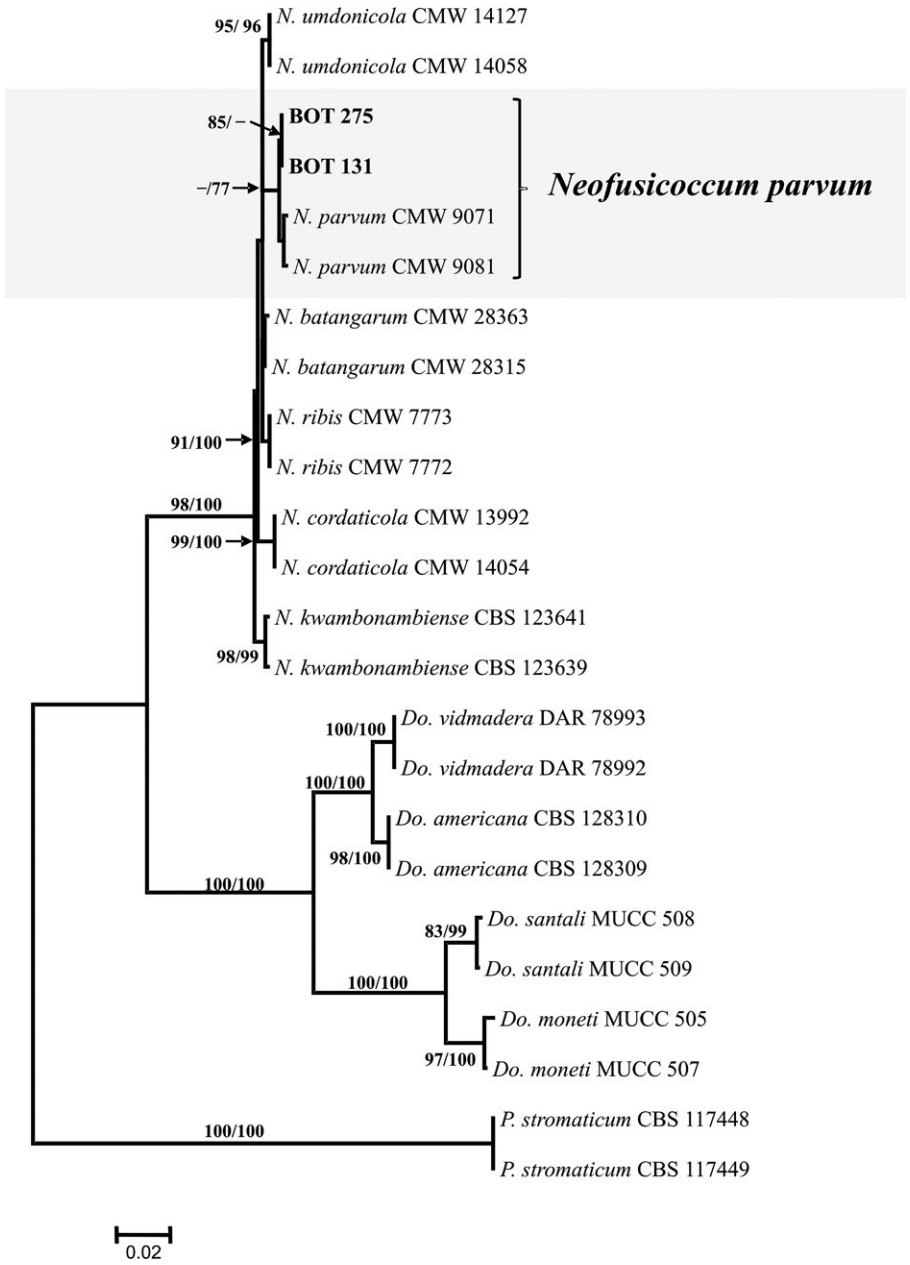

FIGURE 2 Maximum-likelihood (ML) tree resulting from ML analyses of the combined ITS, TEF-1-α and β-tub alignment and showing the phylogenetic position of *Neofusicoccum parvum* in relation to its closely related species and sister genus *Dothiorella*. The bootstrap support values (ML/MP ≥70%) are indicated at the nodes, and the scale bar represents the number of changes. The tree was rooted to *Pseudofusicoccum stromaticum*. Clade corresponding to *N. parvum* is highlighted

inoculated leaves. Morphological identification of the resulting isolates confirmed that the causal agent of the shot hole disease was *N. parvum.*

Shot hole disease has been found associated with stone fruits, ie. almonds, apricots, nectarines, peaches, and other *Prunus* species and represents a self-defence mechanism against fungal, bacterial or viral infection. The disease symptoms result from the development of an abscission layer around the infected tissue that causes the latter to drop out leaving holes in the leaf (Agrios 2005). Symptoms of a shot hole disease of *P. laurocerasus* have previously been reported associated with the bacterium *Xanthomonas arboricola* pv. *pruni* (Marchi et al. 2014) and fungi including *Stigmina carpophila*, *Sphaceloma* sp. and *Mycosphaerella* sp. (Butin 2003). To the best of our knowledge this is the first report of a shot hole disease on *P. laurocerasus* caused by *N. parvum.*

The discovery of *N. parvum* on *P. laurocerasus* in Serbia is not surprising, since the pathogen is a plurivorous species found on ornamental trees worldwide (Phillips et al. 2013). *N. parvum* has been recently isolated from branches of *Chamaecyparis lawsoniana* and the stem of *Aesculus hippocastanum* in Serbia and from branches of *P. laurocerasus* in the nearby Montenegro (Zlatković et al. 2016). Another *Botryosphaeriaceae* member, namely *Diplodia seriata* has been reported to cause twig cankers and die-back of *P. laurocerasus* in Italy (Quaglia et al. 2014).

The pathogenicity of *N. parvum* on *P. laurocerasus* is of special concern in Serbia because *P. laurocerasus* is a relict species endangered by extinction due to its limited population distribution (www.sepa.gov.rs). The species naturally occurs only on the Ostrozub mountain in Southeastern Serbia in a cherry laurel-beech forest (*Lauroceraso-Fagetum*, Jovanović 1967). It is not known if *P. laurocerasus* shrubs that grow in this forest have been affected by shot hole disease and this is a subject deserving further studies.

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