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New record of boxwood volutella blight fungal agents in Iran

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Abstract: During the study on boxwood blight from 2016 to 2017, some isolates of *Pseudonectria* were isolated from boxwood leaves and branches. Fungi were isolated and purified using conventional methods. According to the morphological characteristics and ITS rDNA sequences, two species of *Pseudonectria* viz. *P. buxi* and *P. foliicola* were identified. Moreover, the pathogenicity of these two species under laboratory and greenhouse conditions were examined. None of the species were able to produce disease on the healthy plant leaves, and leaf wounds were necessary to establish the infection. According to our knowledge, this is the first report of *Pseudonectria* species on boxwood from Iran.

Keywords: *Buxus sempervirens*, *Pseudonectria buxi*, *Pseudonectria foliicola*, pathogenicity

INTRODUCTION

Buxus sempervirens subsp. *hyrcana* is one of the most important evergreen species of Hyrcanian forests which is listed as an endangered plant species by the International Union for Conservation of Nature (IUCN). This species grows best in the north of Iran at the height of 200-400 meters above sea level; however, evergreen shrubs have been observed up to 1200 meters in this region (Sabeti 2008). Different parts of boxwood, including leaves, root, bud, seed and bloom, are considered to have industrial, decorative medical and pharmaceutical application.

Buxus plant could be infected in various stages of development with several fungal pathogens such as Macrophomina leaf spot, root rot and blight caused by *Calonectria pseudonaviculata* and *Pseudonectria* spp. Shi & Hsiang 2014a). One of the major boxwood diseases caused by *C. pseudonaviculata*, which is referred to as boxwood blight, while blight disease caused by *Pseudonectria* (previously known as *Volutella*) species is usually known as Volutella leaf and stem blight.

The symptoms of Volutella blight varied from yellowing leaves to completely dead branches. Additionally, the reproductive structures of the fungus are produced on the undersurface of leaves and branches as cushion-like structures in cream to pale pink color. The leaves turn from dark green to orange or bronze (Shi & Hsiang 2014a). The boxwood Volutella blight has been recorded in different regions in the world such as the USA (Hepting & Toole 1950), Canada (Shi and Hsiang 2014b, Elmhirst and Auxier 2013), Turkey (Simsek et al. 2019), Czech Republic (Spetik et al. 2019, Safrankova et al. 2012), Brazil (Andrade et al. 2017), China (Shi and Hsiang 2014a, Wang et al. 2017), and Italy (Rivera et al. 2018, Garibaldi et al. 2016). The recent outbreak of Buxus blight caused by *C. pseudonaviculata* has been reported in Iran (Rezaee et al. 2013, Mirabolfathy et al. 2013), although the identity of the causal agent of the Volutella blight in Iran still requires further investigation. Therefore, the objectives of this study were to characterize the causal agent associated with boxwood shrubs showing blight using morphological and molecular characteristics and examine the pathogenicity of the related fungal species.

MATERIALS AND METHODS

Sample collection and fungal isolation

During 2016-2017, infected plants with typical symptoms of Volutella blight were collected and transferred to the laboratory. Branches and leaves were disinfected for one minute with 70 % ethanol and washed three times with sterile distilled water. Surface sterilized leaves and branches were transferred to 9 cm Petri dishes lined with wet filter paper and incubated at 25°C until the growth of sporodochia on the leaves and branches surface. Sporodochia developed after three days, followed by yellowing and browning of tissues.

Isolation and purification of fungi were conducted based on the single-spore method (Ho & Ko 1997) on

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WA 2 %. The isolates were subcultured on potato-dextrose agar (PDA) supplemented with ampicillin 500 (32 mg/L), rifampicin 300 (32 mg/L) and nystatin 100 (30 mg/L) and incubated at 25°C under a 12:12 light-dark regime.

Morphological and molecular studies

In order to identify fungal species, macroscopic and microscopic characteristics were analyzed. The fungal structures were mounted on slides in 50 % Lactic acid and examined with the Olympus BH2 microscope. Microscopic images were provided by a Leica microscope equipped with a Canon digital camera. For measurement of fungal structures, at least 30 structures in each case were measured (Bezerra 1963, Rossman et al. 1993, Lombard et al. 2015).

For the molecular study, genomic DNA was extracted from mycelia grown on PDA using 5 % Chelex (Hirata and Takamatsu 1966; Walsh et al. 1991). The internal transcribed spacers (ITS1, ITS2 and 5.8S) regions were amplified using primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G -3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') (White et al. 1990). The PCR product of each isolate was submitted to Bioneer Company (South Korea) for sequencing. All the sequences were edited in MEGA7.0 (Kumar et al. 2016) and compared with sequences available at the GenBank nucleotide database using a BLAST search (Altschul et al. 1990). Several reliable sequences with high similarity to our sequences were selected for phylogenetic analysis. The phylogenetic tree was inferred using the Minimum Evolution method in MEGA7.0 (Kumar et al. 2016). The evolutionary distances were computed using the Kimura 2-parameter method. The rate variation among sites was modeled with a gamma distribution (shape parameter =1). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. All ambiguous positions were removed for each sequence pair. There was a total of 549 positions in the final dataset.

Pathogenicity tests

The inoculum preparation was conducted based on the Shi and Hsiang (2014b) protocol. Seven days after incubation of *P. buxi* and *P. foliicola* on PDA at 25 °C, the colonies were washed with sterilized water and the spore suspension with mycelia was passed through multilayer sterilized gauze. Subsequently, a suspension of 1×10^6 (spores/mL) was used for inoculation. For the pathogenicity test on detached leaves, the leaves were disinfected for one minute with 70 % ethanol, then washed three times with sterile distilled water. They were then immersed in spore suspension for one minute. The number of four detached leaves was placed in 8-cm-diameter Petri dishes lined with filter paper. Two milliliters of sterile distilled water were added to each Petri dish to maintain high humidity during the test. All of the cultures were sealed with parafilm and placed at 25 °C under a 12:12 light-dark regime. These experiments were repeated at least two times. Sterile distilled water was used for negative control. The leaves were observed after 7 days.

Additionally, two-year-old healthy potted plants of *Buxus sempervirens* subsp. *hyrcana* were used for pathogenicity test according to Garibaldi et al. (2016). Some leaves of healthy plants were cut about by half before the inoculation and whole bushes inoculated by spraying with spore suspension of 1×10^6 (spores/ml) concentration. Control plants were inoculated with sterile water, then were covered with plastic bags for 5 days and maintained in a growth chamber at 25±4 °C (Fig 3).

Following inoculation, the pathogenicity of two isolates on detached leaves and progress of symptoms were scored according to Table 1.

Table 1. Scoring the virulence of two *Pseudonectria* species isolates on *Buxus sempervirens* subsp. *hyrcana* on detached leaves and greenhouse experiments.

Leaf spot size	Disease grade
No symptom	0
About 2 % of leaf surface	1
About 4 % of leaf surface	2
8 % of leaf surface	3
16 % of leaf surface	4
32 % of leaf surface with sporodochia	5
64 % of leaf surface with sporodochia	6
100 % of leaf surface infected and orange surface of the substrate with sporodochia	7

The disease severity index (DSI) was calculated based on the following formula (Wheeler 1969):

$$\text{DSI} = \frac{\sum(\text{Number of leaves}\times\text{Disease score})}{\text{Number of leaves observed}\times\text{Maximum disease score}}$$

Statistical analysis was made for comparison of pathogenicity of *Pseudonectria* species on the detached leaves and plants using disease severity. The analysis was performed on the Arcsine transformations of severity values using Excel 2016 software. Comparisons of the means were performed by Student's *t*-test using Tukey test at a probability level of (P<0·01) using SAS® software. The Microsoft Excel software (2016) was used to draw plot charts.

RESULTS AND DISCUSSION

Morphological and molecular identification

According to morphological and molecular studies, two species, including *P. buxi* and *P. foliicola* were identified. Voucher specimens are deposited in the mycology herbarium of the University of Guilan (GUM).

***Pseudonectria buxi* (DC.) Seifert, Gräfenhan & Schroers, in Gräfenhan et al., Stud. Mycol. 68: 107 (2011)**

Sporodochia nonstromatic, variable in size, 25–150 × 25–175 µm, pink-red, with hyaline septate setae, 80–180 × 3–5.5 µm, distributed on recently decayed leaves and easily removed from the surface, conidia fusiform,

7–14 × 2–3 µm, hyaline, aseptate and smooth (Fig 1). Colonies on PDA, 8–9 mm in diam. after 5 days, with scattered white mycelium, became pale as a result of slimy conidia production. Conidiophores simple, as single monophialides or in sporodochia.

Specimen examined. IRAN, Guilan Province, Rasht, 10 Aug. 2018, S.A. Mousavi; rDNA ITS GenBank: MW330393; (GUM1592).

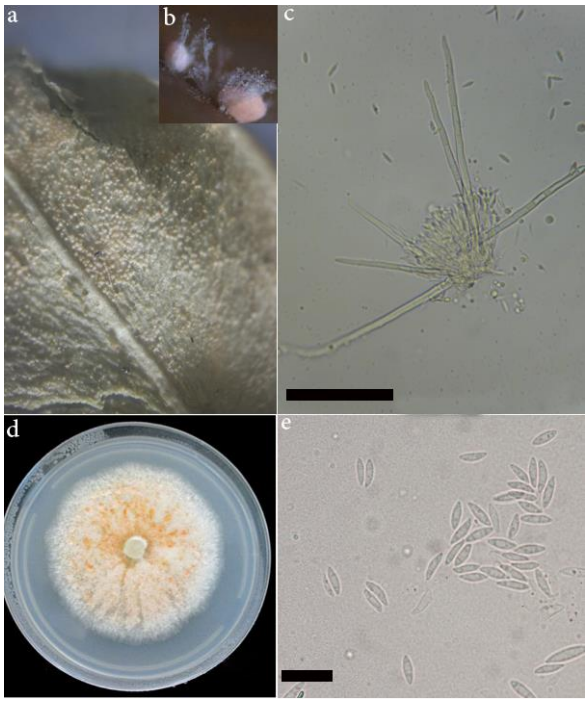

Fig. 1. *Pseudonectria buxi.* a. Sporodochia on leaf surface; b. a close-up of a sporodochia bearing setae on leaf surface; c. sporodochia with setae; d. colony on PDA after 10 days; e. conidia. — Scale bars: c=100 µm; e=10 µm.

***Pseudonectria foliicola* L. Lombard & Crous, in Lombard, et al. Stud. Myco. 80: 219 (2015)**

Sporodochia similar to *P. buxi* but without setae (Fig 2), variable size, 62.5–275 × 75–125 µm on the leaf surface, conidia smaller, fusiform to ellipsoid, 5–8 × 2–3 µm, colonies on PDA 17–30 mm diam. after 5 days (GUM 1593, GenBank: MW349711).

Specimen examined: IRAN, Guilan Province, Rasht, 10 Jan. 2018, S.A. Mousavi; rDNA ITS GenBank: MW349711; (GUM1593)

Based on a BLAST search, rDNA ITS sequences of *P. buxi* and *P. foliicola* showed 100 % and 99.81 % similarities to *P. buxi* (CBS: 125483, HQ897800, 534/534 bp) and *P. foliicola* strain P1 (CBS 123190, NR164229, type material, 534/535), respectively. In the phylogenetic analysis, these two species were clearly separated based on ITS sequences in two distinct clades with high bootstrap support (Fig.3). According to the available literature data, this is the first report of the existence of *Pseudonectria* species in Iran.

Because of similarities in symptoms and some morphological characteristics, ITS sequence is a good candidate for DNA barcoding of these two closely related species.

Pathogenicity tests

During the detached leaf test for both species, the first symptoms of the disease, including brown spots containing sporodochia of both species, appeared on the leaves two days after inoculation (Fig. 4). After two days, the mean disease severity for *P. buxi* and *P. foliicola* was 0.92 % and 43.44 %, respectively. In both species, spots developed from the tips of the detached leaves to the entire leaf surface, and at the same time sporodochia formed on the underside of the leaves and petioles.

On potted plants (under greenhouse conditions), sporodochia were visible three days after inoculation in the lower surface of the leaves for *P. foliicola* and boxwood leaves fall was observed at 4 days post-inoculation, but in *P. buxi*, sporodochia were not visible to the naked eye under greenhouse conditions. By development of the disease, brown spots appeared on the leaves and the first leaf fall occurred after ten days. Under greenhouse conditions and on healthy potted plants, the disease was clearly developed on cut leaves and abundant sporodochia were produced on the underside of the leaves, while non-wounded leaves remained healthy. Five days after inoculation in the greenhouse, the average of disease severity in the two species was 1.5 % and 40 %, respectively. For each case described above, the pathogen was re-isolated to successfully meet Koch's postulates.

The student's t-test was used to compare mean performance of two fungal species in pathogenicity on detached leaves under laboratory and greenhouse conditions. The test was two-tailed and $P<0.05$ was considered statistically significant (Tables 2 and 3).

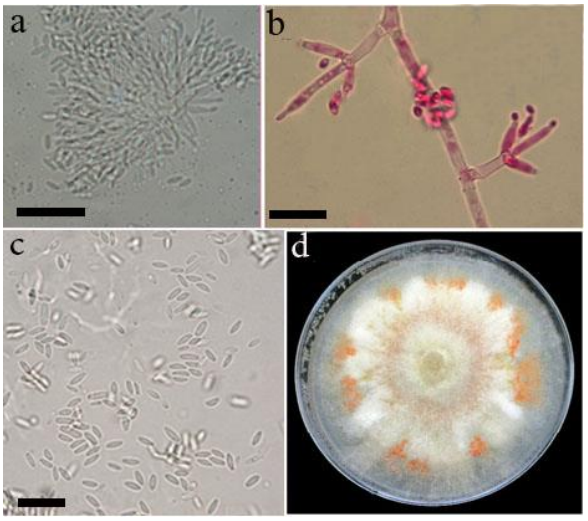

Fig. 2. *Pseudonectria foliicola.* a. sporodochia; b. conidiophores and phialides; c. conidia; d. colony of *Pseudonectria foliicola* on PDA after 10 days. — Scale bars: a=50 µm, b=20 µm, c=10 µm.

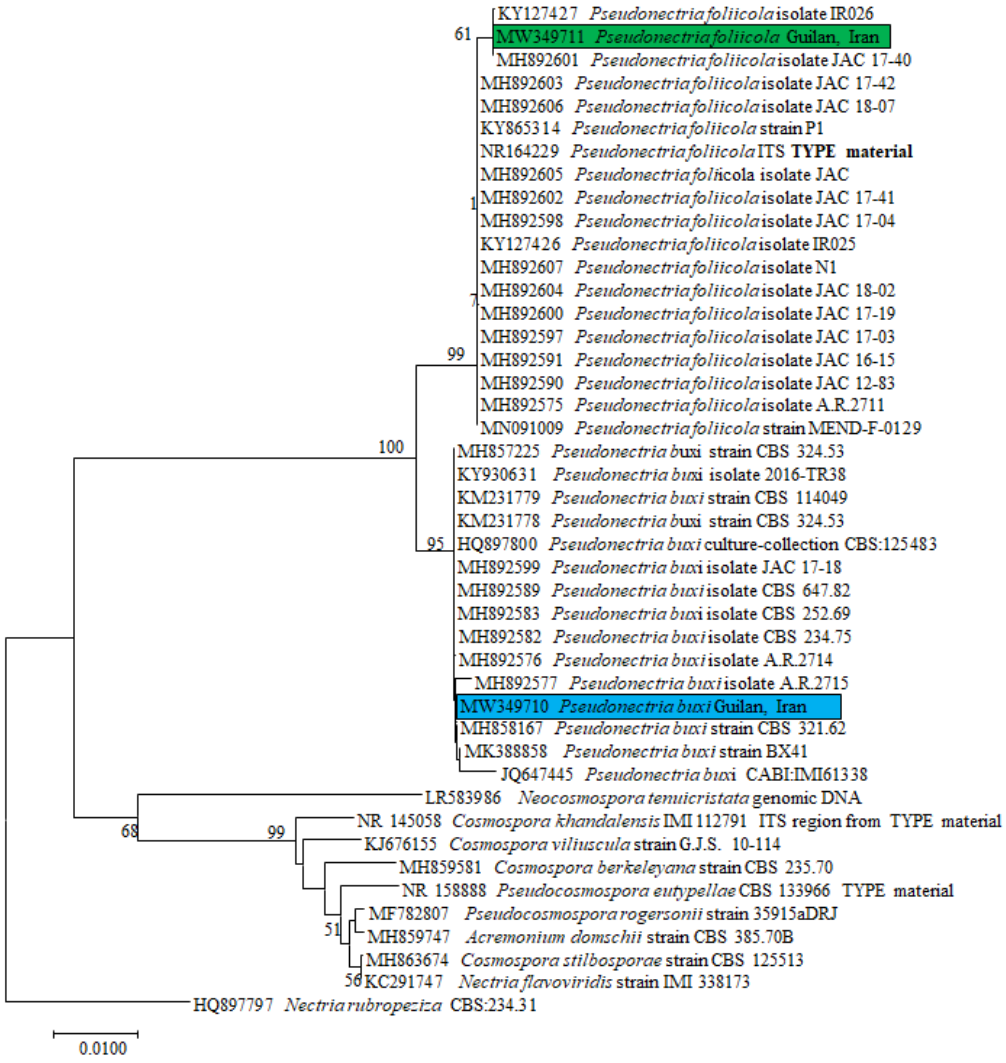

Fig 3. A Minimum Evolution tree based on ITS sequences for 44 sequences of *Pseudonectria* and some related fungi from Hypocreales. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method. All ambiguous positions were removed for each sequence pair. The numbers above the branches represent branch support using 1000 bootstrap replications (Bootstrap values below 50 % are not shown). Evolutionary analyses were conducted in MEGA7.0

Table 2. Mean comparison of the disease severity on detached leaves in *Pseudonectria* species by t-test.

Fungus	Mean±SE	Difference	t	df	P
P. buxi	58.33±9.39	-38.1	-3.95	5.58	0.009
P. foliicola	96.43±2.26				

Table 3. Mean comparison of the disease severity in greenhouse conditions in *Pseudonectria* species by t-test.

Fungus	Mean±SE	Difference	t	df	P
P. buxi	19.29±3.39	-54.05	-7.01	10	0.0001
P. foliicola	73.33±6.74				

The difference between the performance of the two species on detached leaves was obtained 38.1, that was statistically significant (diff=-38.1; t=-3.95; P=0.009).

The difference between the performance of the two fungi under greenhouse conditions was obtained 54.05, that was statistically significant (diff=-54.05; t=-7.01; P=0.0.0001).

As shown in this study, both species could not cause disease on the healthy leaves of the plant, but they can cause disease only in the presence of wounds on the plant. During recent years, boxwood tree in the north of Iran has threatened by two major pests including the invasive fungus *C. pseudonaviculata* and box tree moth (*Cydalima perspectalis*) and considerable natural scope were destroyed and disappeared. Although *Pseudonectria* species penetrate the plant through the wounds, such wounds are usually available in nature as a point of infection for these fungi. When the fungus enters the leaves, disease development is much faster than one may expect; the leaves begin to yellow and detach from branches. Unfortunately, under humid conditions, which is available in northern Iran, the fungi further grow and produce abundant sporodochia on fallen leaves; therefore, the whole surface of leaves is covered by the fungus reproductive structures. Hence, occurring of these fungi, along with two above-mentioned pests on box trees, can promote death and destruction of the trees scope in the northern forests of Iran.

Fig. 4. Pathogenicity test of two *Pseudonectria* species. a. and c. symptoms produced by *Pseudonectria buxi*; b. and d. symptoms created by *Pseudonectria foliicola*.

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گزارش جدید از عوامل قارچی بلایت ولوتلایی شمشاد جنگلی از ایران

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چکیده: طی یک مطالعه روی بلایت شمشاد جنگلی طی سالهای ۲۰۱۶ تا ۲۰۱۷ جدایه‌هایی از *Pseudonectria* از برگ و سرشاخه های شمشاد جنگلی به دست آمد. جداسازی و خالص‌سازی قارچها مطابق روشهای معمول انجام شد. بر اساس ویژگیهای شکل‌شناسی و مولکولی مبتنی بر توالی ناحیه ITS rDNA دو گونه *Pseudonectria buxi* و *Pseudonectria foliicola* شناسایی شدند. بیماریزایی این دو گونه در شرایط آزمایشگاهی و گلخانه‌ای بررسی شد. هیچ یک از گونه ها قادر به ایجاد بیماری روی برگهای سالم گیاه نبودند و زخم برای آلودگی لازم بود. بر اساس یافته‌های ما این اولین گزارش از وجود گونه‌های *Pseudonectria* روی شمشاد جنگلی در ایران است.

کلمات کلیدی: بیماریزایی، *Buxus sempervirens*، *Pseudonectria buxi*، *Pseudonectria foliicola*

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