Molecular identification of phytopathogenic fungi of forest-forming species in the Central Black Soil Region: English oak (*Quercus robur* L.) and Scots pine (*Pinus sylvestris* L.)

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Abstract. The classical assessment method of trees infestation by phytopathogenic fungi is the collection of infected plant specimens and their morphological description. However, this method captures pathogenic fungi by the presence of fruiting bodies, which are not formed neither at the beginning of infection nor annually. This makes difficult the early pathogen detection. Molecular methods make it possible to accurately determine the genus (and in some cases - the species) of the fungus that infects the plant, even in the early stages of invasion. The aim of this study was to evaluate the application of molecular analysis methods on phytopathogenic fungi of *Pinus sylvestris* L. and *Quercus robur* L. for monitoring the pathogen contamination of tree plantations of natural and urban systems in the Central European part of Russia. We isolated eukaryotic microorganisms from infected leaves and wood of *P. sylvestris* L. and *Q. robur* L. and performed DNA barcoding of these microorganisms. *Fusarium* sp., *Hyphodontia pallidula*, and *Gibellulopis nigrescens* were identified in *P. sylvestris*. During the molecular identification of eukaryotic microorganisms isolated from *Q. robur* we found that in 90% of the cases the fungus *Rhizopus oryzae* was detected, in 10% of the cases - *Sordaria* sp. In this paper, we discuss the possibility of identification of plant pathogens by classical methods in combination with molecular methods.

1. Introduction

Suburban natural and plantation forests often experience high anthropogenic stress that can weaken them. These forest communities are characterized by a high activity and rapid spread of wood-decay fungi [1]. It can significantly differ in the extent of damage by phytopathogens [2]. Belov [3] also notes that an increase in the degree of exposure to the urbanized environment leads to an increase in the spread rate of tar cancer pathogens in the plantations due to their weakness. The manifestation of the disease in infected plants depends on the species of the fungal pathogen, but, as a rule, it is already observed at the stages of fungal reproduction and plant inhibition. Wood-destroying basidial fungi are an integral part of forest ecosystems. They perform the functions of structural rebuilding of phytocenoses in their evolutionary development. If we consider the effect of xylotrophic fungi in its pure form, without the intervention of biotic and abiotic factors, the fungi of this group should ensure
a constant loss of tree part from the stand, the volume of which should be comparable to the growth of new trees, taking into account succession [4]. It is important to note that phytopathogenic fungi cause rot and destruction of weakened trees, which leads to decay from the stand of the least resistant trees [5].

The active development of plant introduction indirectly leads to the appearance of new pathogen types. This is due to the contamination of planting material imported from other regions and countries, and the appearance of missing links in the life cycles of fungi. Thus, the weakening of plantations, the appearance of new species of phytopathogens, as well as the change in climatic parameters can lead to the intensive development of mycotic diseases and, as a result, mortality of the tree stand.

The classical method for assessing tree infestation by phytopathogenic fungi involves collection of specimens and morphological description of the pathogens. However, the collected samples are fruit bodies, which are not formed neither at the beginning of infection nor annually. Often, pathogen identification requires additional microbiological and molecular studies. It should be also mentioned that some diseases could be caused by several fungal species that could belong to the different genera. For example, powdery mildew and false powdery mildew have similar manifestations and affect a large number of flowering plants. However, these diseases are caused by a dozen different fungal species belonging to diverse genera [6, 7]. It is possible that the methods chosen for fighting the disease might be inefficient toward some of the pathogens, which will result in plant mortality. Another serious problem is the ambiguity (insufficient accuracy) of morphological description, when the infection could be attributed to a wrong pathogen [8].

Molecular methods make it possible to accurately determine the genus (and in some cases - the species) of the fungus already at the early stages of invasion, which facilitates efficient and timely selection of appropriate methods for disease treatment and prophylaxis, as well as accurate monitoring of the infection spread in the forest, landscape, and agricultural communities [9].

The studied area (the city of Voronezh) is surrounded by planted and natural forests that have the protected zone status. Voronezh also has communal green zones, including two large parks and two arboreta. The green zones provide the ecosystem services [10]. This is why monitoring the state of suburban forests is essential for their preservation and development. The major forest-forming species in the Voronezh region are Scots pine and English oak that form evergreen forests and oak-woods, respectively. The accompanying species are silver birch, aspen, and maple.

The aim of our study was the identification of phytopathogenic fungi infecting English oak and Scots pine trees by molecular methods for the purpose of further monitoring tree infections in natural and urban ecosystems.

2. Materials and Methods

Sample collection. The specimens were collected by the route collection method. We collected the leaves with white mildew from the city population of trees in the vicinity of Voronezh State University of Forestry and Technologies named after G.F. Morozov (geographical coordinates: 51.721060726712; 39.22422766854866) to identify the powdery mildew pathogen on the common oak. Number of collected damaged leaves was 10 for each tree (in general – 20 trees). Then powdery mildew plaque at each leave was cultivated on plates.

Bark and wood samples were collected in urban pine stand near the 9th km of the Zadonskoe highway (geographical coordinates: 51.76412159528861; 39.190807342529304) from the near-root zone and at a height of 2 meters from the ground from trees with visible symptoms of suppression (falling-off bark, presence of fruiting bodies of xylotrophic fungi, yellowing needles, white mildew on the roots) to identify phytopathogens of the Scots pine in an amount 15 samples. The number of collected fruit bodies of the fungus from pine trees was 5. Then the mycelium from the cortex and spores from fruiting bodies were cultivated on plates.
Microbiological analysis was performed after washing off the microorganisms from the leaves, bark, and wood of the infected trees and plating them on antibiotic-containing Czapek medium [11]. The grown colonies were studied morphologically and used for DNA isolation.

DNA was isolated from the isolated eukaryotic microorganisms using a Proba-GS kit (DNK-technology, Russia). DNA concentration was determined at 260 nm with a Hitachi F-7000 spectrophotometer. The purity of the isolated DNA was estimated from the A260/A280 ratio.

Polymerase chain reaction (PCR). The following primers were used to amplify the ITS1 and ITS4 DNA regions of eukaryotic microorganisms:

1. Forward ITS1 (5'-TCCGTAGTGAACCTGCGG-3')
2. Reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

The PCR program was as follows: denaturation at 95°C for 5 min; 37 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and elongation at 72°C for 45 s; and final elongation at 72°C for 5 min. The reaction mixture contained 1 μl of 10 μM of forward primer, 1 μl of 10 μM of reverse primer, 2 μl of DNA template, 5 μl of 5× ScreenMix-HS buffer (Evrogen, Russia), and 16 μl of distilled water.

Electrophoresis in agarose gel and purification of PCR products. PCR products were analyzed by electrophoresis in 2% agarose gel and visualized at 312 nm with a transilluminator. DNA-ladder 100 bp+ (Evrogen, Russia) was used to determine the length of the PCR products. DNA fragments of the required size were cut out of the gel and purified with a Cleanup Standard kit (Evrogen, Russia).

PCR product sequencing and microorganism identification. Purified PCR products were sequenced with an Applied Biosystems 3500 Genetic Analyzer and a BigDye Terminator v3.1 Cycle Sequencing Kit. Microorganisms were identified using the NCBI GenBank database with the BLAST tool [12] and the BOLDSystems database [13].

3. Results and Discussion

3.1. Plant pathogens on the Scots pine

Three morphological types for the pathogen of Scots pine and 2 morphological types for powdery mildew of oak leaves were obtained by during microbiological investigation.

Morphological type 1 for the pathogen of Scots pine: the mycelium hyphas are white, rising above the surface of the plate by 1.5 - 2 cm, the ends of the hyphae are thickened, with time it turns black.

Morphological type 2 for the pathogen of Scots pine: the mycelium hyphas are white with yellow tint, rising above the surface of the plate by 1.0 - 2 cm, the ends of the hyphae are thickened, with time it turns black.

Morphological type 3 for the pathogen of Scots pine: white homogeneous layer on plates without well visible distinguishable hyphas.

Morphological type 1 for the pathogen of Oak: at first white homogeneous layer on plates without well visible distinguishable hyphas, then in central part appeared rising above the surface of the plate by 1.5 cm with time it turns brown.

Morphological type 2 for the pathogen of Oak: the mycelium hyphas are white, rising above the surface of the plate by 1 cm, with time it turns black.

Search for the sequenced DNA fragments in the NCBI GenBank revealed that the morphological type of the 1st colony (bark samples) belonged to species of the genus Fusarium (GenBank KX219597.1, KU671036.1, KY425717.1, etc.). The identity of the obtained sequences to the sequences deposited in the GenBank and BOLDSystems databases was 99.68 and 98.49%, respectively (table 1).

It should be noted that the obtained nucleotide sequences most closely resembled the sequences of Fusarium oxysporum and Fusarium chlamydosporum from the GenBank database and F. chlamydosporum and Fusarium equiseti from the BOLDSystems database.
Table 1. Fungi identified in *P. sylvestris*.

| Morphological type | Colony | Match in NCBI GenBank | Similarity in NCBI GenBank, % | Match in BOLDSystems | Similarity in BOLDSystems, % |
|--------------------|--------|-----------------------|-------------------------------|----------------------|------------------------------|
| Type 1             | 1      | *Fusarium oxysporum*  | 99.68                         | *F. chlamydosporum*  | 98.49                         |
|                    |        |                       |                               |                      |                               |
|                    | 2      | *F. oxysporum*        | 99.68                         | *F. equiseti*        | 98.30                         |
|                    |        |                       |                               |                      |                               |
|                    | 3      | *F. oxysporum*        | 99.68                         | *F. chlamydosporum*  | 98.49                         |
| Type 2             | 1      | *Verticillium sp.*    | 99.00                         | *G. nigrescens*      | 97.80                         |
|                    |        |                       |                               |                      |                               |
|                    | 2      | *Verticillium sp.*    | 99.00                         | *G. nigrescens*      | 97.80                         |
|                    |        |                       |                               |                      |                               |
|                    | 3      | *Verticillium sp.*    | 99.00                         | *G. nigrescens*      | 97.80                         |
| Type 3             | 1      | *Hyphodontia pallidula* | 99.66                       | *Hyphodontia pallidula* | 100.00                       |
|                    |        |                       |                               |                      |                               |
|                    | 2      | *Hyphodontia pallidula* | 99.66                       | *Hyphodontia pallidula* | 100.00                       |

The search for the nucleotide sequences isolated from the morphological type 2 colonies (Scots pine bark) revealed that the colonies belonged to species of the genus *Verticillium* (GenBank HG935577.1, HG935558.1, EU754977.1, etc.). The identity of the obtained sequences with the sequences deposited in the NCBI GenBank and BOLDSystems databases was 99.00 and 97.61%, respectively (table 1). Analysis of the obtained nucleotide sequence using the BOLDSystems database revealed that it belonged to the eukaryotic microorganism *Gibellulopis nigrescens*, formerly known as *Verticillium nigrescens* [14].

The search for the nucleotide sequences number 3 (eukaryotic colonies grown from the white mildew on wood samples) in the NCBI GenBank database revealed that they belonged to the fungus *Hyphodontia pallidula* (GenBank KP814392.1, KP814354.1, KP814341.1, etc.). The identity of the obtained sequences to the sequences deposited in the NCBI GenBank and BOLDSystems databases was 99.66 and 100%, respectively (table 1). The isolated microorganism was identified as *H. pallidula* in both NCBI GenBank and BOLDSystems databases. Therefore, phytopathogens isolated from the infected Scots pine trees were identified as *Fusarium sp.*, *Hyphodontia pallidula*, and *Gibellulopis nigrescens*, formerly known as *Verticillium nigrescens*. It is interesting that microbiological manifestation of the *F. chlamydosporum* infection is similar to that of *Heterobasidion annosum*, a
basidiomycete fungus that causes “annosum root rot” common for the Central Black Soil region (figure 1). Earlier, Bertoni-Mann et al [15] isolated and identified a new Fusarium fujikuroi strain infecting P. sylvestris, which confirms the possibility of fusariosis in Pinus sylvestris.

The growth of H. pallidula on the trunk, roots, and damaged areas of pine trees is manifested as the appearance of white mildew on the plant surface. Widely occurring phytopathogens of the Fusarium and Verticillium genera can cause extreme damage that has been especially well characterized for agricultural plants [15]. Hadj Taieb et al [16] found that propagation of phytopathogenic fungi is promoted by mature specimens of pistachio bark beetle (Chaetopterus vestitus). In the washes from these insects, 41 isolates of fungi were obtained, some of which are represented by species of the genus Fusarium, exhibiting high pathogenic activity not only on pine, but also on other plants.

3.2. Plant pathogens on the common oak
Phytopathogens of English oak isolated from leaves with visible white mildew were identified by the above methods used for identification of pathogens on the Scots pine. We found that 90% of the isolated colonies were Rhizopus oryzae and 10% were Sordaria sp. (table 2). No fungi of the genus Microsphaera were identified. R. oryzae is a mold fungus from the Mucoraceae family. Due to morphological similarity, it can be easily confused with black head molds, including those from the Mucor genus. R. oryzae exhibits high enzymatic activity and produces various organic acids, so it has found wide practical application [17].

It is believed that the major agents of powdery mildew on oaks are ascomycetes of the Microsphaera genus that form easily identifiable fruiting bodies [18, 19]. The presence of these fungi in the leaf-covering mildew has been confirmed by numerous microscopic studies during the previous decades. Commonly used methods for evaluation of oak infection with powdery mildew are based on the presence/absence of mildew formed by the fungal mycelium, since the fruiting bodies form only during the second half of June [20]. Our microscopic studies of the fungal fruiting bodies performed during the last few years gave no results.

No formation of fruiting bodies typical of Microsphaera and Rhizopus fungi were observed after plating the samples washed from the oak leaves on the Czapek medium, although the white mildew on the leaves was well pronounced.
Table 2. Fungi identified in *Q. robur*.

| Morphological type | Colony | Match in NCBI Genbank | Similarity in NCBI Genbank, % | Match in BOLDSystems | Similarity in BOLDSystems, % |
|--------------------|--------|------------------------|------------------------------|----------------------|-----------------------------|
| Type 1             | 1      | *Rhizopus oryzae*      | 100.00                       | *Rhizopus oryzae*    | 100.00                      |
|                    | 2      | *Rhizopus oryzae*      | 100.00                       | *Rhizopus oryzae*    | 100.00                      |
|                    | 3      | *Rhizopus oryzae*      | 100.00                       | *Rhizopus oryzae*    | 100.00                      |
| Type 2             | 1      | *Sordaria alcina*      | 99.00                        | *Sordaria alcina*    | 99.00                       |
|                    |        | *Sordaria fimicola*    | 100.00                       | *Sordaria fimicola*  | 100.00                      |
|                    | 2      | *Sordaria alcina*      | 99.00                        | *Sordaria alcina*    | 99.00                       |
|                    |        | *Sordaria fimicola*    | 100.00                       | *Sordaria fimicola*  | 100.00                      |
|                    | 3      | *Sordaria alcina*      | 99.00                        | *Sordaria alcina*    | 99.00                       |
|                    |        | *Sordaria fimicola*    | 100.00                       | *Sordaria fimicola*  | 100.00                      |

Molecular genetic analysis of phytopathogens of the Scots pine and English oak revealed the presence of atypical fungal pathogens. The symptoms of infection by these pathogens closely resemble the symptoms of powdery mildew and root rot caused by *H. annosum* and *Microsphaera* sp. Such discrepancies in the results of morphological, microbiological, and molecular genetic studies have been demonstrated for the Schutte disease (needle cast) of conifers caused by *Lophodermium pinastri*, while molecular genetic methods revealed that pine seedlings at the nursery were infected by the *Phoma* sp. fungus [8].

The results of our study need to be further verified by using larger samples collected from different populations of the English oak and Scots pine. Molecular genetic analysis will make it possible to more precisely define morphological and microbiological characteristics of the most important pathogens, to identify sources and distribution pathways of infection, as well as to restrict spreading of infection. New methods based on post-genomic technologies will ensure the “smart approach” to controlling infection by using natural signalization pathways between the host plants and bacterial pathogens [21], as well as fungal phytopathogens.

4. Conclusion

Using two typical fungal diseases (root rot and powdery mildew) as examples, we demonstrated that despite typical morphological manifestations, plant infections could be caused by pathogens other than the common agents of these diseases, as determined by molecular genetic identification of infecting microorganisms. Precise identification of phytopathogens in both natural and urban ecosystems requires a combined use of several methods that would refine and complement each other. The severity of a disease and the rate of its development can differ significantly depending on the type of pathogenic fungus (wood-decay polypores, head molds, other molds), and each host plant-mycopathogen system will require its own methods for the infection detection and control.
Molecular diagnostic methods will allow extensive and rapid monitoring of suburban forests for the infection with mycopathogens and identification of most weakened plantations requiring sanitary measures.

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