Molecular analysis of *Phytophthora* species found in Poland

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**Abstract**

Pathogens of *Phytophthora* genus are common not only in forest nurseries and stands, but also in water courses. Species of *Phytophthora* spread with plants for plantings (and soil attached to them) and with water courses as well, attacking the plants growing in riparian ecosystems. Several specialized organisms damaging only one tree species were identified like *P. alni* on alders or *P. quercina* on oaks. Some *Phytophthora* species can develop on several hosts like *P. plurivora* and *P. cactorum* on oaks, beeches, alders, ashes and horse chestnuts. Other oomycetes like *P. gallica* species was found for the first time in Poland in water used for plant watering in forest nursery. Species *P. lacustris* and *P. gonapodyides* were found in superficial water. *Phytophthora* species *P. polonica* was identified in the declining alder stands for the first time in the world, and *P. taxon hungarica* and *P. megasperma* were found in the rhizosphere of seriously damaged ash stands for the first time in Poland. The most often isolated species were *P. plurivora* (clade 2) with frequency 37% and *P. lacustris* with frequency 33% (clade 6). The best represented clade 6 revealed the occurrence of 6 species: *P. gonapodyides*, *P. lacustris*, *P. megasperma*, *P. sp. raspberry*, *P. taxon hungarica* and *P. taxon oak soil*.

**Key words**

fine root pathogens, sequencing DNA, alien, invasive, emerging

**Introduction**

An increase in trade of plants and globalization pose a risk of plant disease epidemics, resulting from introductions of exotic plant pathogens. An associated risk that accelerates pathogen evolution may occur as a consequence of genetic exchange between introduced or introduced and resident pathogens (Brasier et al. 1999). There is a likelihood of such evolutionary events occurring in Poland, as well. On the other hand, new diagnostic methods based on molecular tools are currently sufficiently sensitive to allow detection of new phytopathogens. Recently, in forestry, emerging diseases are caused by invasive, alien oomycetes, which are soil-borne fine root pathogens, sometimes specializing in damage of certain forest tree species as their host. As established in
Central Europe, *Phytophthora quercina* is often recovered from declining oaks proved to be more pathogenic to European oaks *Q. robur* than any other *Phytophthora* species (Jung et al. 1999). The common species *Phytophthora plurivora* occurs all over Italy, while *P. quercina* is the species significantly associated with declining of oak trees (Vetraino et al. 2002). In Italy, eleven soil-borne species of *Phytophthora* were detected in oak forests with 35% as the frequency of isolations, being also correlated with soil pH and longitude of the sites. *P. cactorum* was recovered from sites in central and southern Italy, whereas *P. quercina* was isolated in the northern and central part of the country. In Denmark, several species of *Phytophthora* were found in the rhizosphere of declining ash trees (Orlikowski et al. 2011); earlier, they were also found in nurseries (Jung et al. 2016).

Since pathogens from genus *Phytophthora* are responsible for serious diseases world-wide and can occur on a wide range of hosts, in the present study, we concentrated on an assessment of the occurrence of these pathogens in the Polish forest nurseries and stands.

**MATERIAL AND METHODS**

Soil, together with the root system, was sampled in plastic bags weighing 0.5 kg each and isolation tests were performed using rhododendron, oak or beech leaves as baits. Water was collected with 1.5 l plastic bottles, which were sterilized with 70% ethanol and washed with distilled water. The sampled water was filtered in the lab, using the Millipore vacuum pump with nylon filters of 5 µm pore-size. Filters with biological sediment as well as the fragments of discoloured bait tissues were placed on selective PARPNH medium (potato dextrose agar amended with 10 µg ml⁻¹ pimaricin, 200 µg ml⁻¹ ampicillin, 10 µg ml⁻¹ rifampicin, 25 µg ml⁻¹ pentachloronitrobenzene (PCNB), 50 µg ml⁻¹ nystatin, and 50 µg ml⁻¹ hymexazol).

Pure cultures of *Phytophthora* sp. isolates obtained from the water and soil samples were grown in the liquid V8 media (100 ml clarified V8 juice in 900 ml distilled water, amended with 2 g of CaCO₃ for 3–5 days in the dark at 22–25°C. The mycelium was subsequently rinsed in sterile distilled water, dried and disrupted in liquid nitrogen prior to the DNA extraction. Total DNA was extracted from mycelium by using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich® GmbH, Germany), following the manufacturer’s protocol. Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using primers ITS6 and ITS4 (White et al. 1990; Cooke et al. 2000) in a 50 µl reaction containing 50–100 ng genomic DNA, 250 nM of each primer, 200 µM of each dNTP, 1 mM MgCl₂, 1U Taq polymerase, 1xQ solution and 1xPCR buffer (Qiagen Ltd., Valencia, CA, USA). The reaction was performed in a PTC-200Ô Programmable Thermal Controller (MJ Research, Inc.) for 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 50 s, with initial denaturation of 3 min at 94°C before cycling and a final extension of 10 min at 72°C after cycling. The PCR product was purified using the Clean-up kit (A&A Biotechnology), following the manufacturer’s protocol. Sequencing was conducted on a CEQ™8000 9.0.25 automated sequencer, (Beckman Coulter®, Fullerton, USA). Forward and reverse sequences were linked in BioEdit software and the resulting sequences were aligned with NCBI Nucleotide collection.

All the collected sequences were compared in ITS1 region by using the ClustalW algorithm provided in the BioEdit software; further phylogenetic analysis was performed using MEGA5. The Maximum Likelihood method based on the Tamura-Nei model was used. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Joining and BIONJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 117 nucleotide sequences (116 *Phytophthora* sequences and *Pythium sterilum* JX271797 sequence as an outgroup).

**RESULTS**

As given in the table below, the *Phytophthora* isolates were identified to species on the basis of sequence alignment with NCBI database (Tab. 1). Among the list
of identified \textit{Phytophthora} isolates, there is a new species in Poland – \textit{Phytophthora gallica}, which is considered to be moderately aggressive to \textit{Alnus glutinosa} and \textit{Fagus sylvatica}, weakly aggressive to \textit{Quercus robur} and \textit{Salix alba} and non-pathogenic to \textit{Fraxinus excelsior} (Jung and Nechwatal 2008). The origin of \textit{P. gallica} and its ecological role in wet ecosystems remain unclear.

\textbf{Table 1.} List of \textit{Phytophthora} isolates used in the study

<table>
<thead>
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<th>NCBI №</th>
<th>Isolate</th>
<th>Country</th>
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<td>Poland</td>
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<td>IBL/2011/9/1/2</td>
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</table>
As many as 12 species of *Phytophthora* belonging to 7 different clades were found based on the sequence analysis (Tab. 2). The most abundant clades present in Poland are clade 6 (43.1%) and 2 (37.1%). *Phytophthora* species in clade 6 have non-papillate sporangia and are Mostly infectious to roots or present in the rhizosphere. *Phytophthora plurivora*, the only representative of clade 2, is considered to be the cause of several devastating declines and diebacks of major forest tree species.

**Table 2.** *Phytophthora* species found in Poland

<table>
<thead>
<tr>
<th>Species</th>
<th>Clade</th>
<th>N</th>
<th>%</th>
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<tbody>
<tr>
<td><em>P. alni</em></td>
<td>7a</td>
<td>5</td>
<td>4.31</td>
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<tr>
<td><em>P. cactorum</em></td>
<td>1a</td>
<td>11</td>
<td>9.48</td>
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<tr>
<td><em>P. gallica</em></td>
<td>10</td>
<td>1</td>
<td>0.86</td>
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<tr>
<td><em>P. gonapodyides</em></td>
<td>6</td>
<td>4</td>
<td>3.45</td>
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<tr>
<td><em>P. lacustris</em></td>
<td>6</td>
<td>38</td>
<td>32.76</td>
</tr>
<tr>
<td><em>P. megasperma</em></td>
<td>6</td>
<td>1</td>
<td>0.86</td>
</tr>
<tr>
<td><em>P. plurivora</em></td>
<td>2</td>
<td>43</td>
<td>37.67</td>
</tr>
<tr>
<td><em>P. polonica</em></td>
<td>9</td>
<td>4</td>
<td>3.45</td>
</tr>
<tr>
<td><em>P. quercina</em></td>
<td>4</td>
<td>2</td>
<td>1.72</td>
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<tr>
<td><em>P. sp. raspberry</em></td>
<td>6</td>
<td>1</td>
<td>0.86</td>
</tr>
<tr>
<td><em>P. taxon hungarica</em></td>
<td>6</td>
<td>2</td>
<td>1.72</td>
</tr>
<tr>
<td><em>P. taxon oaksoil</em></td>
<td>6</td>
<td>4</td>
<td>3.45</td>
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</table>

As shown, many of the discovered *Phytophthora* species were found on different hosts, including important forest tree species (Tab. 3). Also, there was a diversity in the age of host species – *Phytophthora* species were found in mature forest stands and on tree seedlings in forest nurseries. The identification of species like *P. gallica, P. lacustris, P. gonapodyides* and *P. alni* in riparian ecosystems was possible due to the use of water filtration techniques, plating and DNA (ITS) analysis.

Since 2000, an increasing decline and dieback of alders has been observed in Poland. Ten different species of obtained *Phytophthora* isolates, including those shown in Table 3, originated from diseased trunks and from rhizosphere (Trzewik et al. 2015). Phylogeny of Polish *Phytophthora* species is shown on the dendrogram created based on Maximum Likelihood method (Fig. 1). The new for knowledge oomycete species *P. polonica* was found in declining alder stands along the river Ner (Belbahri et al. 2006).

**Table 3.** Host range of *Phytophthora* species fund in Poland

<table>
<thead>
<tr>
<th>Species</th>
<th>Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. alni</em></td>
<td><em>Aesculus hippocastanum</em></td>
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<td><em>P. cactorum</em></td>
<td><em>Fagus sylvatica</em></td>
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<td><em>Fraxinus excelsior</em></td>
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<tr>
<td></td>
<td><em>Quercus robur</em></td>
</tr>
<tr>
<td><em>P. gonapodyides</em></td>
<td><em>Alnus glutinosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Quercus robur</em></td>
</tr>
<tr>
<td><em>P. lacustris</em></td>
<td><em>Acer pseudoplatanus</em></td>
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<tr>
<td><em>P. megasperma</em></td>
<td><em>Alnus glutinosa</em></td>
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<tr>
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<td><em>Fraxinus excelsior</em></td>
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<td><em>P. plurivora</em></td>
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<td><em>Fagus sylvatica</em></td>
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<tr>
<td></td>
<td><em>Fraxinus excelsior</em></td>
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<tr>
<td><em>P. quercina</em></td>
<td><em>Pyrus sp.</em></td>
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<tr>
<td><em>P. polonica</em></td>
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<tr>
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</tr>
<tr>
<td><em>P. taxon oaksoil</em></td>
<td><em>Quercus robur</em></td>
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**Figure 1.** Phylogeny of *Phytophthora* isolates (ML method, bootstrap = 1000)
Conclusions

1. Pathogens of Phytophthora genus are common not only in nurseries and forest stands, but also in parks and orchards.

2. Species of Phytophthora spread with plants for plantings (and soil attached to them) and with water along water courses as well, attacking the plant associations or shelterbelts of the riparian ecosystems, especially alders.

3. Several specialized organisms damaging only one tree species were identified like P. alni on alders or P. quercina on oaks.

4. Some Phytophthora species can develop on several hosts like P. plurivora and P. cactorum on oaks, beeches, ashes and horse chestnuts.

5. Other oomycetes like P. gallica species was found for the first time in Poland in water used for plant watering in the Kiejsze nursery (Kolo Forest District).

6. In water ecosystems, species like P. lacustris and P. gonapodyides were found. The pathogenicity of these species is not fully recognized yet.

7. For the first time in the world, the new Phytophthora species P. polonica was identified in the declining alder stands (Kolo FD); and for the first time in Poland, two other species P. taxon hungarica and P. megasperma were found in the rhizosphere of seriously damaged ash stands (showing ash dieback).

8. The most often isolated species were P. plurivora (clade 2) with frequency 37% and P. lacustris with frequency 33% (clade 6).


References


