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Multiplex detection of *Phytophthora* spp. using the Fluidigm platform

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Abstract: The genus *Phytophthora* plays an important role not only in agriculture but also in forest ecosystems. As the number of known *Phytophthora* species continues to grow, identifying new isolates in this genus has become increasingly challenging even by DNA sequencing. Therefore, the development of proper techniques for detection and identification is crucial for monitoring and control of these pathogens in the forestry sector. In recent years, new molecular methods using innovative approaches have indeed been developed. However, the majority of these methods was designed to detect single *Phytophthora* species. Techniques that are able to target multiple species would offer advantages, especially for the assessment of Phytophthora diversity in the environment. This paper describes a multiplex assay for the identification of eight *Phytophthora* isolates, down to the species level, based on a Fluidigm platform employing pyrosequencing. The obtained results showed that for an accurate determination of the species, it is sufficient to know the sequence of two markers, ITS and COX1. The sensitivity of this test is sufficient to identify *Phytophthora* in a pure culture. Unfortunately, analysis based on a pyrosequencing platform does not provide enough data to simultaneous identify multiple *Phytophthora* species in samples collected in the field. This problem could be resolved in the future by sequencing using more efficient platforms like Illumina or IonTorrent.

Keywords: forest soil, oomycetes, oak dieback, pathogens, next generation sequencing

1. Introduction

For several years now, species of the genus *Phytophthora* have been the cause of significant losses in ornamental tree nurseries and forest stands. The pathogenic species of the genus *Phytophthora* belong to soil pathogens characterised by a high degree of parasitism in their host plants. They pose a significant threat to young, damaged and undamaged plant tissues (Oszako 2005; Oszako et al. 2007), causing rot of the shoot base and roots and shoot tip blight. An analysis of losses caused by *Phytophthora* species in the cultivation of plants in container nurseries showed that they can reach up to 80%. *Phytophthora* was the cause of root and shoot base or stem rot (Orlikowski, Ptaszek 2010; Orlikowski et al. 2012).

Pathogenic oomycetes of the genus *Phytophthora* constitute a great threat to broadly understood plant production, both agricultural and forest. Currently, 142 species of *Phytophthora* are formally described, and 43 of them have been given temporary names (Cook et al. 2000; Yang et al. 2017). Early detection and accurate pathogen identification is irreplaceable in effective plant protection, especially at the level of producing nursery material (Oszako et al. 2007). Preventive measures make it possible to avoid infection by selecting plants for crop rotation that are resistant or tolerate diseases caused by phytopathogens. Such a strategy is in line with the legal acts of the European Union on integrated plant protection: Directive 2009/128/EC of the European Parliament and of the Council (Directive 2009) and Regulation 1107/2009/EC of the European Parliament and of the Council (Regulation 2009).

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The results of many tests of field samples have shown that often more than one pathogenic species is present in a given sample; however, many of the DNA analysis techniques currently in use are unable to identify the *Phytophthora* species mix (Hulvey et al. 2010). Furthermore, molecular identification methods based solely on the polymerase chain reaction (PCR) have been developed mainly for the economically most important *Phytophthora* species such as: *Phytophthora infestans* (Mont.) de Bary, *Phytophthora ramorum* Werres, De Cock & Man in 't Veld, *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. (Martin, Tooley 2004; Schena et al. 2006), *Phytophthora megasperma* Drechsler, *Phytophthora plurivora* T. Jung and T.I. Burgess, *Phytophthora pseudosyringae* T. Jung & Delatour, *Phytophthora quercina* T. Jung and T.I. Burgess (Nowakowska et al. 2017), *Phytophthora multiformis* Brasier & S.A. Kirk, *Phytophthora hungarica* (Nowakowska et al. 2016), while there are still no specific detection methods for many other species. Due to the close relationship of many *Phytophthora* species, it is impossible to effectively identify these pathogens on the basis of only one sequence, e.g. the ITS fragment, which is a major methodological problem (Riddell et al. 2019). Analytical platforms such as microarrays (Sikora et al. 2012) or next-generation sequencing (NGS) (Vettraino et al. 2012; Mora-Sala et al. 2019; Morris et al. 2019) provide much greater possibilities for identification.

The aim of this study was to develop an effective method of *Phytophthora* species identification based on nuclear markers: ITS (internal transcribed spacer) and the elongation factor 1 α (TEF1), as well as mitochondrial markers: the gene of cytochrome oxidase subunit 1 (COX1) and the gene of dehydrogenase subunit 1 (NADH). The analysis was conducted using the Fluidigm platform, which, thanks to the addition of unique sequences, enables the simultaneous amplification of multiple markers in a mixture of different DNA samples, combined with pyrosequencing using the 454 platform.

2. Material and methods

Fragments of infected tissues (necrosis) were placed on V8-PARP selective medium, after which the Petri dishes were incubated for 2–3 days at 22°C in the dark (Table 1). Then the growing strands were transferred to V8A selective medium and incubated under the same conditions for another 7 days (Ivors 2015). After pure cultures were obtained, the pathogen strands were collected and inoculated into the V8 liquid culture medium, and after 5 days of culture, DNA was extracted from the obtained mycelium using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Waltham, MA, USA) according to manufacturer's instructions.

Soil samples were taken with a spade around trees showing symptoms of disease in the crowns (shoot dieback) at a distan-

Table 1. Results of pure culture sample analysis on Real Time PCR

Sample no	Provenance	Species	C_t value
1*	Konstantynowo	*Phytophthora cactorum*	20.00
2*	Piaski	*Phytophthora quercina*	19.77
3*	Konstantynowo	*Phytophthora alni*	23.18
4*	Brzeg	*Phytophthora gallica*	17.73
5*	Brzeg	*Phytophthora lacustris*	18.23
6*	Oborniki	*Phytophthora bilorbang*	25.66
7*	Legnica	*Phytophthora gonapodyides*	21.03
8*	Oborniki	*Phytophthora gonapodyides*	23.57
9	Oborniki	*Phytophthora lacustris*	23.42
10	Oborniki	*Phytophthora gonapodyides*	20.43
11	Oborniki	*Phytophthora* sp.	21.14
12	Oborniki	*Phytophthora bilorbang*	21.58
13	Oborniki	*Phytophthora gonapodyides*	21.34
14	Wołów	*Phytophthora bilorbang*	19.06
15	Oborniki	*Phytophthora* sp.	20.95
16	Brzeg	*Phytophthora* sp.	17.41
17	Krotoszyn	*Phytophthora quercina*	19.13
18	Piaski	*Phytophthora quercina*	24.79
19	Konstantynowo	*Phytophthora cactorum*	18.69
20	Konstantynowo	*Phytophthora plurivora*	21.69
21	Konstantynowo	*Phytophthora plurivora*	22.93
22	Konstantynowo	*Phytophthora quercina*	22.95
23	Konstantynowo	*Phytophthora cactorum*	19.91
24	Konstantynowo	*Phytophthora plurivora*	22.27

*samples selected for analysis on Fluidigm platform

Table 2. Results of soil sample analysis on Real Time PCR

Sample no	Provenance	Dominant tree species	C_t value
25*	Kościan	*Fraxinus excelsior*	35.27
26*	Kościan	*Fraxinus excelsior*	33.14
27*	Kościan	*Fraxinus excelsior*	36.10
28*	Karczma Borowa	*Quercus petraea*	39.42
29*	Karczma Borowa	*Quercus petraea*	39.67
30*	Karczma Borowa	*Quercus petraea*	37.25
31*	Karczma Borowa	*Quercus petraea*	39.26
32*	Krotoszyn	*Quercus robur*	39.07
33*	Krotoszyn	*Quercus robur*	38.56
34*	Krotoszyn	*Quercus robur*	38.48
35	Kościan	*Fraxinus excelsior*	38.18
36	Kościan	*Fraxinus excelsior*	36.16
37	Kościan	*Fraxinus excelsior*	35.75
38	Karczma Borowa	*Quercus petraea*	>40
39	Karczma Borowa	*Quercus petraea*	>40
40	Karczma Borowa	*Quercus petraea*	39.81
41	Karczma Borowa	*Quercus petraea*	>40
42	Karczma Borowa	*Quercus petraea*	39.55
43	Karczma Borowa	*Quercus petraea*	>40
44	Karczma Borowa	*Quercus petraea*	>40
45	Karczma Borowa	*Quercus petraea*	>40
46	Krotoszyn	*Quercus robur*	38.23
47	Krotoszyn	*Quercus robur*	>40
48	Krotoszyn	*Quercus robur*	38.84
49	Krotoszyn	*Quercus robur*	38.44
50	Szkółka	*Alnus glutinosa*	38.02
51	Szkółka	*Alnus glutinosa*	36.07

*samples selected for analysis on Fluidigm platform

ce of about 1 m from the tree trunks, from a depth of 20 cm in two places (about 0.5 kg) and mixed together (Table 2). The selective multiplication of pathogens from the soil was conducted in PB-PARP (PeaBroth PARP) selective medium with the addition of a mixture of antibiotics and PCNB according to the protocol described by Kubiak et al. (2012). DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, USA) according to the manufacturer's instructions. The DNA concentration was measured on the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Identification of *Phytophthora* with real-time PCR

The presence of *Phytophthora*'s genetic material in DNA samples extracted from the pure culture and soil samples was confirmed on the basis of real-time PCR. The reaction used the FITS_15Ph and RITS_279Ph universal primers for *Phytophthora* and the TaqMan-type All_Phytophthora molecular probe (Kox et al. 2007). The reaction mixture contained: 1 x reaction buffer (TaKaRa, Kusatsu, Shiga, Japan), 250 nM of each primer, 83 nM of probe, ROX reference dye and 1 ng DNA. The reaction thermal profile: pre-denaturation at 94°C, 3 min; amplification (40 cycles) – denaturation at 94°C, 5 s, primer annealing and amplification at 60°C, 1 min. fluorescence reading followed each amplification step. In order to determine the presence of the pathogen, a threshold cycle value (Ct) was determined, in which the fluorescence signal reaches the limit for its detection. This value is inversely proportional to the amount of DNA of the tested pathogen in the sample (Dorak 2007). Based on the TaqMan probe's fluorescence value (Ct), the presence of *Phytophthora* was confirmed and samples were selected for analysis using the Fluidigm platform.

Preparation of amplicon libraries for pyrosequencing

Libraries consisting of ITS, COX1, TEF and NADH1 amplicons were prepared for species composition analysis. For this purpose, a two-stage marker amplification was performed. In the first stage, markers with region-specific starters were amplified: ITS (White et al. 1990), COX1 (Martin et al. 2003), TEF and NADH1 (Kroon et al. 2004). In the second stage, the mixture of PCR products obtained in the first stage was amplified with barcoded primers, enabling sequences to be assigned to the sample and needed for the pyrosequencing: 5'-ACACTGACGACATGGTTCTACA-3' for the forward primers and 5'-TACGGTAGCAGAGACTTGGTCT-3' for the reverse primers. Amplification was performed in the Fluidigm Biomark HD (Fluidigm, San Francisco, CA, USA) at the Plant Research Institute, Wageningen, the Netherlands.

Pyrosequencing

Amplification products were mixed in equal proportions, taking 4 µl of each from the reaction. The mixture of amplicons

prepared in this way was separated in 1% agarose gel. PCR products were cut out from the agarose gel to a suitable length and cleaned with a set of QIAquick reagents (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified DNA was pyrosequenced at Greenomics Plant Research International BV, University of Wageningen in the Netherlands on a Roche/454 Titanium sequencer. The obtained sequences were analysed in the CLC Genomics Workbench program (Qiagen, Hilden, Germany). Species identification was performed on the basis of comparing the obtained DNA sequences with those deposited in the National Centre for Biotechnology Information (NCBI) – www.ncbi.nlm.nih.gov – and Q-bank – https://qbank.eppo.int (Bonants et al. 2013).

3. Results and discussion

Over the last two decades, the diversity of *Phytophthora* species has been extensively studied by analysing both nuclear and mitochondrial DNA. ITS and COX1 are among the most commonly used markers (Martin, Tooley 2004). β-tubulin (Villa et al. 2006), elongation factor 1 α (Van't Klooster et al. 2000) or NADH1 dehydrogenase (Kroon et al. 2004) are more rarely used in studies.

The analysis of the DNA extracted from the pure cultures showed the presence of *Phytophthora* in all 24 isolates (Table 1). The threshold cycle value (Ct) ranged from 18.23 to 25.66. The presence of *Phytophthora* was also found in 21 out of 28 analysed soil samples, but they had a much higher Ct value, from 33.14 to 39.67, which indicates less *Phytophtora*-derived DNA (Table 2).

Most PCR-based methods detect individual species in a sample and are not suitable for testing *Phytophthora* species diversity in field samples (Schena et al. 2008). NGS methods are promising. The pyrosequencing resulted in 1 to 475 sequences in the analysed samples (Table 3). Most sequences (2–475) were obtained for ITS, much less (1–36) for the remaining genes. This is particularly clear in the case of the soil sample analysis, where 17 to 474 sequences were obtained for ITS, 0 to 12 for NADH and 0 to 2 sequences for COX1. TEF sequencing of the soil samples was ineffective. The large number of obtained ITS sequences may be conditioned by the high number of ITS sequences in the *Phytophthora* genome. The *Phytophthora cactorum* genome has 376 copies of rDNA (Yang et al. 2018). It can be assumed that other *Phytophthora* species may also have a high number of rDNA copies, which includes ITS1 and ITS2.

The analysis of the obtained sequences in BLAST showed that ITS, COX1 or NADH can be used equally effectively to determine the species *Phytophthora cactorum* (the sequence was identical for > 97%). All obtained sequences of these genes were correctly assigned to the species. ITS and COX1 genes also allow for the correct identification of *Phytophthora quercina*, while only COX1 ensures the correct species assignment for *Phytophthora alni* Brasier & S.A.Kirk. COX1 and TEF genes ensure the correct identification of *Phytophthora bilorbang* and *Phytophthora gonapodyides* (H.E. Petersen) Buisman.

The ITS regions of nuclear ribosomal DNA (rDNA) are most often sequenced for *Phytophthora* because they have a high number of copies, high variability and primers are relatively easy to select for their amplification. Unfortunately, ITS variability is not always sufficient to identify *Phytophthora* species (Cooke et al. 2000; Kroon et al. 2004). It was insufficient to correctly assign sequences to the species *Phytophthora alni*, *Phytophthora bilorbang* and *Phy-*

Table 3. Number of obtained sequences after samples pyrosequencing

No of sample	Gene			
	ITS	COX1	TEF	NADH1
	Pure culture			
1	38	6	2	3
2	23	16	1	2
3	0	0	0	0
4	40	19	23	0
5	39	11	36	1
6	5	21	3	0
7	2	1	0	0
8	16	26	23	0
	Soil			
25	211	2	0	0
26	17	0	0	0
27	475	1	0	0
28	255	0	0	0
29	287	0	0	12
30	239	0	0	0
31	259	1	0	0
32	114	0	0	1
33	151	0	0	0
34	161	0	0	0

tophthora gonapodyides. For these species, COX1 was the effective gene. Analysis of the sequence of two markers, ITS and COX1, allowed the species in each sample to be determined. The results obtained are consistent with those of Yang and Hong (2018), who suggest sequencing two markers for *Phytophthora* species identification.

In the DNA samples extracted from the soil taken in the stands, pathogens belonging to the genera *Cryptococcus*, *Cylindrocarpon*, *Fusarium* and *Neonectria* were identified, as well as their antagonists from the genus *Trichoderma* and other saprotrophs that are not plant pathogens, such as *Mortierella*, the moulds *Mucor* and *Penicillium* or *Fibroporia* (from the family Fomitopsidaceae). Pyrosequencing did not detect the presence of *Phytophthora* in any soil sample, despite the positive result obtained in the real-time PCR. This indicates a lower sequencing sensitivity in the 454 platform compared to real-time PCR. The amount of *Phytophthora* compared to other organisms in the soil samples is lower. The difference in Ct values for DNA samples extracted from soil compared to pure culture samples was 7.48–21.34 PCR cycles. The amount of template in the sample is inversely proportional to the Ct value, and in each PCR cycle, the amount of amplicon approximately doubles, which means that the amount of *Phytophthora* was below 1% ($1/2^{7,478}$=0.056). The results obtained suggest that more efficient platforms are needed than the pyrosequencing platform, e.g. Illumina or Ion Torrent, to analyse the presence of *Phytophthora* in field samples (Catala et al. 2015; Aguayo et al. 2018; Burgess et al. 2018; Riddel et al. 2019).

Molecular DNA analyses are highly useful as early and rapid warning methods for dangerous fungi and oomycete species (*Phytophthora*, *Pythium*) in the soil and water of nurseries, crops and forest stands (Kox et al. 2007; Nowakowska et al. 2016; Nowakowska et al. 2017). In particular, verifying the health of seedlings planted for forest crops is essential for the sustainability and biodiversity of future forests.

4. Conclusions

Multiplex sequence analysis facilitates the identification of pathogens of the *Phytophthora* genus, especially in cases where close relationships prevent analysis on the basis of only one marker. It was found that the analysis of two markers: ITS and COX1 sufficed to identify species.

The sequencing efficiency of the Roche/454 Titanium platform is not sufficient for the multiplex identification of *Phytophthora* species in soil samples.

Conflict of interest

The authors declare the lack of potential conflicts of interest.

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Authors' contributions

K.S. – concept, experiment, analysis of results, text editing; T.O. – concept, text editing; K.K. – experiment, analysis of results, text editing; J.A.N. – concept, analysis of results, text editing; T.M. – analysis of results, text editing.