Investigations for the Scientific Description of New *Nothophytophthora* Species

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INTRODUCTION

During various recent surveys of *Phytophthora* diversity in Germany, Portugal, Chile and Vietnam slow growing oomycete isolates were obtained from rhizosphere soil samples and small streams in natural and planted forest stands. Multigene phylogenetic analyses revealed they belong to six new species of a new genus, *Nothophytophthora* gen. nov., which constitutes a monophyletic sister group of the genus *Phytophthora*. Two new *Nothophytophthora* species have since been described from Ireland and unknown taxa were also detected in Czech Republic, Slovakia, Norway, Scotland, Spain, Sweden, New Zealand, Japan and the USA. *Nothophytophthora* species share numerous morphological characters with *Phytophthora* and can be differentiated from the latter by the presence of a conspicuous, opaque plug inside the sporangiophore close to the base of most mature sporangia and intraspecific co-occurrence of sporangial caducity and nonpapillate sporangia with internal nested and extended proliferation in several *Nothophytophthora* species. The lifestyle of *Nothophytophthora* species is still unknown. Their widespread occurrence in watercourses suggests a saprophytic lifestyle as decomposers of leaf litter. However, production of caducous sporangia by five of the eight described *Nothophytophthora* species suggests a partially aerial lifestyle as adaptation to humid habitats, potentially as leaf pathogens.

MATERIALS AND METHODS

DNA isolation, amplification and sequencing

For all isolates of the five new *Nothophytophthora* species DNA will be extracted from fresh pure cultures on vegetable juice agar (V8A). Five nuclear loci, i.e. the internal transcribed spacer region (ITS) and the 5’ terminal domain of the large subunit (LSU) of the nuclear ribosomal RNA gene, the partial heat shock protein 90 (*hsp90*), β-tubulin (*btub*) and *tigA* genes, and three mitochondrial loci, i.e. cytochrome c oxidase subunit 1 (*cox1*), NADH dehydrogenase subunit 1 (*nadh1*) genes and partial *rps10* genes, will be amplified using standardized primer pairs and PCR conditions (White et al. 1990; Moncalvo et al. 1995; Cooke et al. 2000; Martin and Tooley 2003; Kroon et al. 2004; Riethmüller et al. 2002; Blair et al. 2008; oomycetedb.cgrb.oregonstate.edu/protocols.html). All sequences derived from this study will be deposited at GenBank.

Phylogenetic analyses

To clarify the phylogenetic position of the five new *Nothophytophthora* species within the genus *Nothophytophthora* a 5-partition dataset of the nuclear loci ITS, LSU, *btub*, *hsp90* and *tigA* and a 3-partition dataset of the mtDNA genes *cox1*, *nadh1* and *rps10* will be established. Bayesian (BI) analyses will be performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Hülsenbeck 2003) into partitions with the best-fitting model. Maximum-Likelihood (ML) analyses will be carried out using the raxmlGUI v. 2.0 (Edler et al. 2020) implementation of RAxML (Stamatakis 2014) with the best-fitting nucleotide substitution model. Phylogenetic trees will be visualized in MEGA X (Kumar et al. 2018) and edited in figure editor programs. Datasets presented and trees deriving from BI and ML analyses will be made available at TreeBASE.

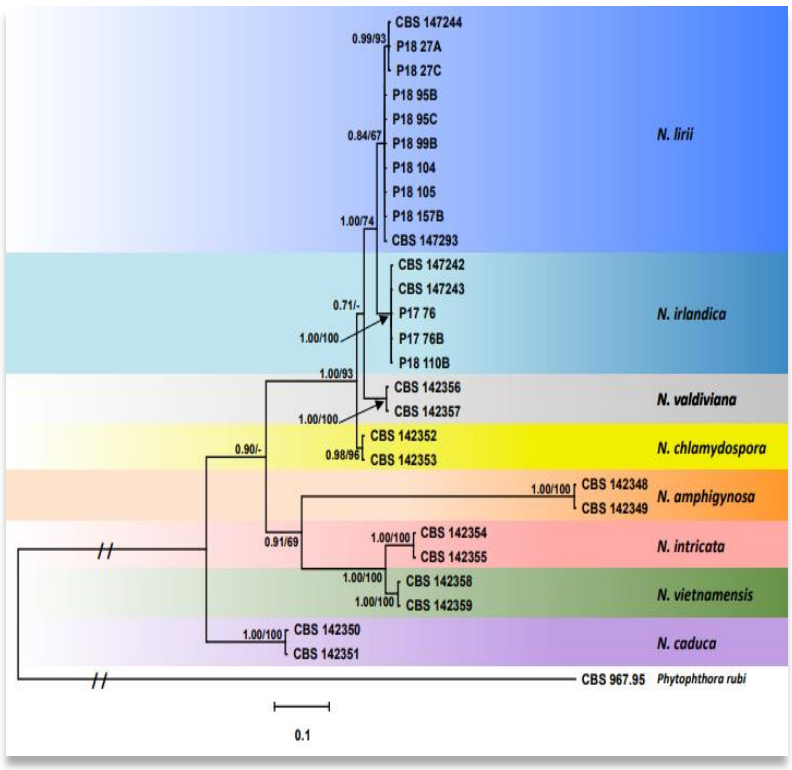

Fig.1 *Nuclear Phylogenetic Tree of Nothophytophthora Species (O´Hanlon et al. 2021).*

Assessments of colony morphology, growth rates, cardinal temperatures

Colony growth patterns of all five *Nothophytophthora* species will be described from 10-d-old cultures grown at 20 ° C in the dark in 90 mm plates on CA, V8A, and potato dextrose agar (Jung et al. 2017). For temperature-growth relationships, representative isolates of the five *Nothophytophthora* species will be subcultured onto 90 mm V8A plates and three replicate plates per isolate will be incubated at 10, 15, 20, 22.5, 25, 26, 27, 28, 29 and 30 ° C. Radial growth will be recorded after 8 d and mean growth rates (mm/d) and cardinal temperatures will be calculated.

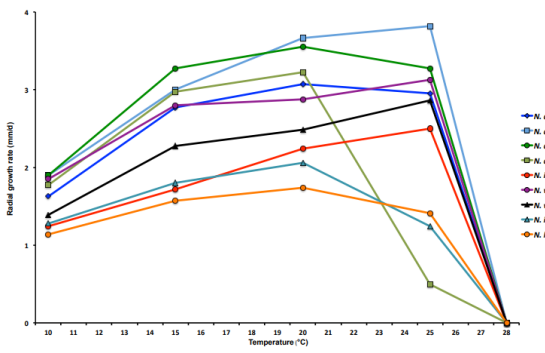

Fig. 2 *Mean radial growth rates of Nothophytophthora spp. at different temperatures (O´Hanlon et al. 2021).*

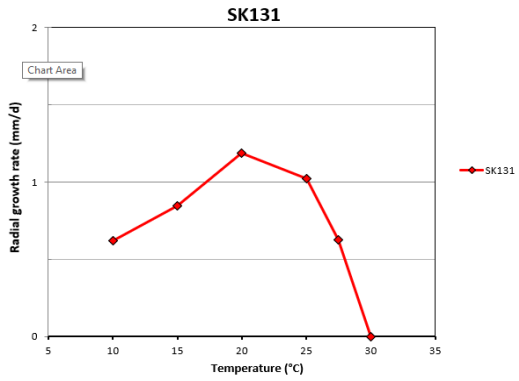

Fig. 3 *Mean radial growth rate of a new Nothophytophthora species from Slovakia*

Morphological studies of sexual and asexual structures

Morphological features of sporangia, oogonia, oospores, antheridia, chlamydospores, hyphal swellings and aggregations of the five new species will be compared with each other and with all described *Nothophytophthora* species.

Formation of sporangia will be induced by submersing discs cut from the growing edge of a 3–7-d-old V8A colony in non-sterile soil extract (Jung *et al.* 1996, 2017a). The Petri dishes will be incubated at 20 ° C in natural light. Shape, type of apex, caducity and special features of sporangia and the formation of hyphal swellings and aggregations will be recorded after 24–48 h. The formation of chlamydospores and hyphal swellings (on V8A) and of gametangia (oogonia and antheridia; on carrot agar (CA)) and their characteristic features will be examined after 21–30 d growth at 20 ° C in the dark. For each isolate each 40 sporangia, oogonia, oospores, antheridia, chlamydospores and hyphal swellings will be measured at ×400 using a compound microscope (Zeiss Imager.Z2), a digital camera (Zeiss Axiocam ICc3) and a biometric software (Zeiss ZEN).

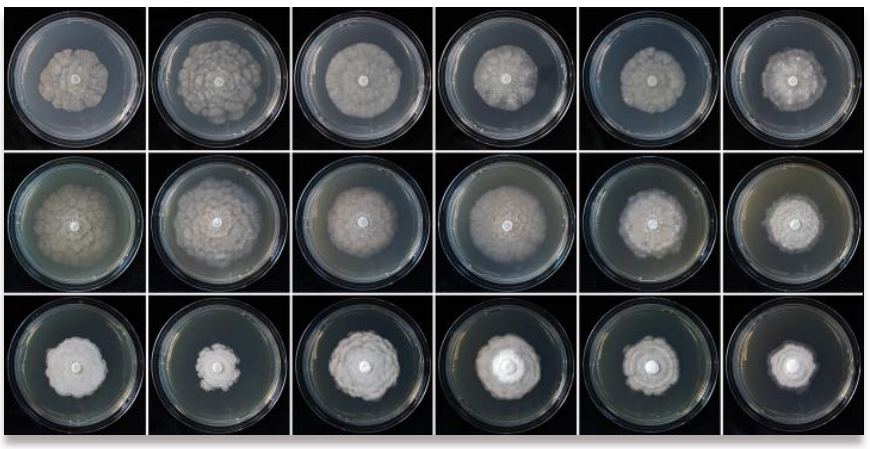

Fig. 4 *Colony morphology of N. lirii and N. irlandica isolates grown at 20℃ on V8 agar, carrot agar and potato-dextrose agar (from top to bottom) (O´Hanlon et al. 2021).*

RESULTS

The preliminary results showed for **temperature-growth tests** a need for prolongation of (1) the time needed for the onset of growth of the isolates at the beginning from 24 hours to 48 hours, and (2) the duration of the experiment from 6 to 8 days. Also, an additional temperature setting at 22.5° C was added to determine the temperature optimum more precisely.

For the assessment of **colony morphologies**, Malt Extract Agar was excluded and all new *Nothophytophthora* species together with all described species are currently being examined on Potato Dextrose Agar (PDA), V8A and CA. Growth of the colonies was prolongated from 10 to 12 days due to slow growth of the isolates.

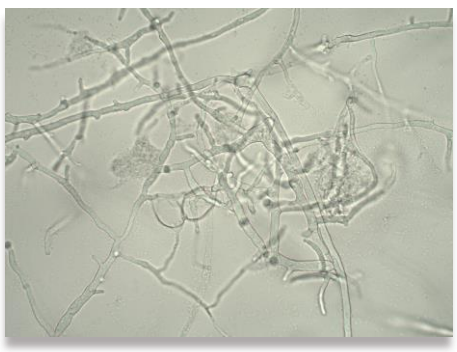

Fig. 5 *Hyphae and empty sporangia of a new Nothophytophthora species from Moravia (×400)*

Fig. 6 *Sporangium of a new Nothophytophthora species from Slovakia (×400)*

Blair JE, Coffey MD, Park S-Y, et al. 2008. A multi-locus phylogeny for Phytophthora utilizing markers derived from complete genome sequences. Fungal Genetics and Biology 45: 266–277.
Cooke DEL, DrenthA, Duncan JM, et al. 2000.Amolecular phylogeny of Phytophthora and related oomycetes. Fungal Genetics and Biology 30: 17–32
Edler D, Klein J, Antonelli A, Silvestro D (2020) raxmlGUI 2.0: A graphical interface and toolkit for phylogenetic analyses using RAxML. Methods Ecol Evol 00: 1–5
Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755
Jung T, Scanu B, Bakonyi J, Seress D, Kova´cs GM, Dura´n A, et al. (2017a) Nothophytophthora gen. nov., a new sister genus of Phytophthora from natural and semi–natural ecosystems. Persoonia 39: 143–174.
Kroon LPNM, Bakker FT, Van den Bosch GBM, et al. 2004. Phylogenetic analysis of Phytophthora species based on mitochondrial and nuclear DNA sequences. Fungal Genetics and Biology 41: 766–782.
Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35: 1547–1549. https://doi.org/10.1093/molbev/msy096 PMID: 29722887
Martin FN, Tooley PW. 2003. Phylogenetic relationships among Phytophthora species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia 95: 269–284.
Moncalvo JM, Wang HH, Hseu RS. 1995. Phylogenetic relationships in Ganoderma inferred from the internal transcribed spacers yYYand 25S ribosomal DNA sequences. Mycologia 87: 223–238.
O'Hanlon R, Destefanis M, Milenković I, Toms˘ovsky´ M, Janous˘ek J, Bellgard SE, et al. (2021) Two new Nothophytophthora species from streams in Ireland and Northern Ireland: Nothophytophthora irlandica and N. lirii sp. nov. PLoS ONE 16(5): e0250527.
Riethmüller A, Voglmayr H, Göker M, et al. 2002. Phylogenetic relationships of the downy mildews Peronosporales and related groups based on nuclear large subunit ribosomal DNA sequences. Mycologia 94: 834–849.
Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.
White TJ, Bruns T, Lee S, et al. 1990. Amplification and direct sequencing of fungal ribosomal RNAgenes for phylogenetics. In: Gelfand DH, Sninsky JJ, Innes MA, et al. (eds), PCR protocols: a guide to methods and applications: 315–322. Academic Press, San Diego, California, USA.

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