



Hidden *Phytophthora* diversity unveiled in tree nurseries of the Czech Republic with traditional and metabarcoding techniques

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Accepted: 17 May 2024 / Published online: 17 June 2024
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Abstract *Phytophthora* diversity was examined in eight forest and ornamental nurseries in the Czech Republic. A leaf baiting isolation technique and, in two nurseries, also Illumina DNA metabarcoding were used to reveal the diversity of *Phytophthora* in soil and irrigation water and compare the efficacy of both approaches. In total, baiting revealed the occurrence of 12 *Phytophthora* taxa in 59.4% of soil samples from seven (87.5%) nurseries. Additional baiting of compost was carried out in two nurseries and two *Phytophthora* species were recovered. Irrigation water was examined in three nurseries by baiting or by direct isolation from partially decomposed floating leaves collected from the water source, and two *Phytophthora* species were obtained. Illumina sequencing of soil and water samples was done in two and one nurseries, respectively. *Phytophthora* reads were

identified as 45 *Phytophthora* taxa, 15 of them previously unknown taxa from Clades 6, 7, 8 and 9. Another 11 taxa belonged to known or undescribed species of the oomycete genera *Globisporangium*, *Hyaloperonospora*, *Nothophytophthora*, *Peronospora* and *Plasmopara*. Overall, with both techniques 50 *Phytophthora* taxa were detected with five taxa (*P. organica*, *P. plurivora*, *P. rosacearum*, *P. syringae* and *P. transitoria*) being exclusively detected by baiting and 38 only by DNA metabarcoding. Particularly common records in DNA barcoding were *P. cinnamomi* and *P. lateralis* which were not isolated by baiting. Only seven species were detected by both techniques. It is recommended to use the combination of both techniques to determine true diversity of *Phytophthora* in managed or natural ecosystems and reveal the presence of rare or unknown *Phytophthora* taxa.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-024-02886-1>.

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Keywords Oomycetes · Metabarcoding · Baiting · Infestation · Soil microbiome

Introduction

Phytophthora (Oomycota, Peronosporales) is a genus of mainly soilborne or aerial plant pathogens causing a range of diseases of woody plants like collar and root rots, aerial bleeding cankers on trunks, discolorations and small size of leaves or needles, and eventually dieback and mortality (Chen et al., 2022;

Ho, 2018; Jung et al., 2013a, 2018a;). *Phytophthora* pathogens cause 66% of diseases affecting fine roots and over 90% of collar rot diseases of woody plants (Tsao, 1990). The worldwide count of significant *Phytophthora* diseases of forest trees has surged dramatically since the 1960s, rising from four to currently 41 (Brasier et al., 2022). Many species such as *P. cactorum*, *P. cinnamomi*, *P. cryptogea*, *P. nicotianae*, *P. palmivora*, *P. ramorum* and *P. ×cambivora* have very wide host ranges encompassing various taxa of woody plants (Erwin & Ribeiro, 1996; Oßwald et al., 2014). In nursery plants, *Phytophthora* pathogens cause root and collar rots leading to mortality and significant economic losses. Moreover, if the infection is suppressed by fungicides or fungistatic chemicals such as phosphites the infected plants often remain asymptomatic and after being planted in the wider environment cause significant losses in afforestations, reforestations, horticultural plantations, road-side plantings, parks, and private gardens (Frankel et al., 2020; Jung et al., 2016; Migliorini et al., 2015; Pérez-Sierra & Jung, 2013). Denman et al. (2009) described *P. ramorum* and *P. kernoviae* causing asymptomatic infections and sporulation on rhododendron and holm oak (*Quercus ilex*) leaves. Despite absence of necroses, both pathogens were regularly obtained from leaves using baiting tests. These findings are significant for phytosanitary services which base the regulation of these pathogens on visual inspection of plants (Denman et al., 2009).

Soilborne *Phytophthora* pathogens infect host plants primarily via the fine root system. In most cases, a successful infection begins with the chemotactical attraction of biflagellate zoospores to host roots. (Khew & Zentmyer, 1973). The zoospores adhere to the root surface where they encyst, secreting an adhesive substance that attaches them to the root epidermis (Hardham, 2001). A germ tube grows from the cyst, expands, and develops into hyphae that penetrate the root surface (Oh & Hansen, 2007). Initially, hyphae grow intercellularly, later intracellular hyphae are formed to obtain nutrition from living host cells.

Plants grown in greenhouses and nurseries play a significant role in spreading invasive pathogens and pests, which have a detrimental impact on ornamental plants, agricultural crops, and forest trees (Jung et al., 2016; Migliorini et al., 2015; Moralejo et al., 2009; Pérez-Sierra & Jung, 2013; Prospero et al., 2013).

Phytophthora is often imported into nurseries with infected nursery stock, substrate, potting media or water used for irrigation. After being established in a nursery, inoculum is frequently disseminated through splashing irrigation water and horticultural techniques such as pruning, potting and moving plants. In some nurseries, the irrigation water is recycled and thus a constant circulation of the inoculum occurs (Bush et al., 2003, 2006; MacDonald et al., 1994; Pérez-Sierra & Jung, 2013; Themann et al., 2002).

Irrigation water is often taken from local surface water sources such as lakes, ponds, and rivers which have been demonstrated globally as important reservoirs of a broad spectrum of *Phytophthora* species and other oomycetes (Jung et al., 2017a, 2018b, 2019, 2020, 2022; Orlikowski et al., 2010, 2011, 2012; Shrestha et al., 2013). Research of plant diseases in commercial nurseries revealed that *Phytophthora* species can persist within the water recycling systems all year round (Bush et al., 2003; MacDonald et al., 1994; Themann et al., 2002). Thus, the use of effective techniques to disinfect irrigation water in commercial nurseries is crucial (Ufer et al., 2008).

People have engaged in trading and relocation of live plants for thousands of years (Maxwell et al., 2024). However, global trade is associated with risk of invasive alien species. The introduction of non-native pathogens into new environments is currently considered as the second most significant factor contributing to the decline in biodiversity, habitat destructions and rising economic costs (Brasier et al., 2022; Perrings et al., 2005; Pimentel et al., 2005).

Most – if not all – exotic invasive *Phytophthora* spp. causing epidemics of non-coevolved native vegetation were introduced via the international trade in living plants and subsequently spread with nursery stock (Brasier, 2008; Brasier et al., 2022; Chen et al., 2022; Jung et al., 2016, 2018a, 2021). For example, the devastating ‘Sudden oak death’ (*P. ramorum*) was imported to North America with nursery plants. In the United Kingdom *P. ramorum* has transitioned from rhododendrons to forest plantations of *Larix kaempferi* (Brasier et al., 2010, 2012). *P. lateralis*, first recorded in Seattle horticultural nurseries in the 1920s, has spread to the natural habitat of *Chamaecyparis lawsoniana* in Oregon and Northern California (Hansen, 2011; Hansen et al., 2000; Parke et al., 2014). The pathogen is now also responsible for decline among *Chamaecyparis* trees in Brittany

(France) and Scotland. This epidemic was caused by the planting of trees from infected nurseries (Brasier et al., 2012; Green et al., 2020; Robin et al., 2011). *Phytophthora xalni* in Europe, *P. austrocedri* in Chile and Argentina, and *P. kernoviae* in the United Kingdom are other *Phytophthora* spp. causing devastating forest epidemics with probable introduction by the nursery stock trade (Brasier et al., 2004, 2005; Greslebin et al., 2007; Hansen et al., 2012; Jung et al., 2016, 2018a, 2022; Jung & Blaschke, 2004; Parke et al., 2014).

A serious decline and dieback of European beech (*Fagus sylvatica*) stands caused by widespread fine root losses, root rot and aerial bleeding cankers has been observed across Europe in recent decades and 17 *Phytophthora* species have been obtained from affected European beech forests including several invasive species driving the disease, i.e. *P. cactorum*, *P. plurivora* and *P. xambivora* (Cacciola et al., 2005; Corcobado et al., 2020; Jung, 2009; Jung et al., 2005, 2013a, 2018a, 2019; Schmitz et al., 2009; Stepniewska & Dłuszyński, 2010; Telfer et al., 2015). In several countries, including Bavaria (Germany) and Austria, the initiation of the *Phytophthora* outbreak on beech was found associated with the alternation between extensive and long-lasting heavy rain and severe droughts during the vegetation period (Corcobado et al., 2020; Jung, 2009). Also, with the extensive decline of oak (*Quercus* spp.) forests across Europe an involvement of in total 22 *Phytophthora* taxa has been demonstrated with *P. cinnamomi*, *P. plurivora*, *P. quercina* and *P. xambivora* being most widespread and most aggressive to oak roots (Brasier et al., 1993; Jung et al., 1996, 2013a, 2018a, 2019, 2000; Seddaiu et al., 2020). In Southern Europe, the UK and the Czech Republic, *P. cinnamomi* and *P. xambivora* are causing, partly since the 1930s, the destructive ink disease of chestnut (*Castanea sativa*) trees (Day, 1938; Jung et al., 2018a; Vettraiño et al., 2005). A study that took place in almost 2000 nursery stands and more than 2500 plantings across Europe showed almost omnipresent infestations of nurseries and young plantings of beech, chestnut, and oaks with a heterogenous mixture of *Phytophthora* species including those driving the declines of mature forest stands, i.e. *P. cactorum*, *P. cinnamomi*, *P. plurivora*, *P. quercina* and *P. xambivora* which were most common (Jung et al., 2016). In forest nurseries, *P. cactorum* is also causing

damping-off of beech seedlings (Stepniewska, 2005; VÚLHM, 2007).

Surveys of *Phytophthora* infestation and diversity in both natural ecosystems and managed environments such as nurseries and plantations can be performed using baiting methods or metabarcoding approaches. Leaf baiting is a method that is widely used to detect *Phytophthora* pathogens. A soil sample is flooded with distilled water and young leaves (e.g. oak or beech) are placed on the water surface ca 2 cm above the soil. Zoospores released from sporangia produced in the the soil sample infect the floating leaves and cause necrosis. *Phytophthora* is then isolated from the necrotic tissue using a selective agar medium (Jung et al., 2000, 2013b, 2020). Baiting methods have long been used for isolation of *Phytophthora* from rhizosphere soil samples and occasionally also from root samples or bark cankers (Corcobado et al., 2020; Jung, 2009; Jung et al., 2013b; Pérez-Sierra et al., 2022). It helps to monitor *Phytophthora* diversity in natural or managed ecosystems (Balci & Halmschlager, 2003a; Balci & Halmschlager, 2003b; Jung et al., 2000, 2013b, 2017a, b, 2018b, 2019, 2020; Vettraiño et al., 2002) or nurseries (Jung et al., 2016; Moralejo et al., 2009; Simamora et al., 2018). The advantage of baiting is the isolation of cultures, which are essential for taxonomic and genomic studies (Jung et al., 2016; La Spada et al., 2022). The success of baiting is impacted by many factors including the season and climatic conditions (Vannini et al., 2013); the interaction with other soil microorganisms, in particular the occurrence of faster-growing oomycetes like *Pythium* or *Phytophythium* spp. or antagonistic bacteria and fungi (Bose et al., 2018; Erwin & Ribeiro, 1996; Jung et al., 1996, 2000, 2017a, 2020; Pérez-Sierra et al., 2022); and quality of the baiting procedure and experience and skills of the research team. The compatibility of the *Phytophthora* species with the host bait leaves is also important (Sarker et al., 2023a, b) although *Fagaceae* leaves have proven effective in the isolation of far more than 100 known and many previously unknown *Phytophthora* taxa (Corcobado et al., 2020; Jung et al., 2000, 2016, 2017a, b, 2019; Seddaiu et al., 2020; Vettraiño et al., 2002). Finally, the presence of dead or resting propagules cannot be detected by baiting (Catalá et al., 2015; La Spada et al., 2022). *Phytophthora* species exhibit differences in the timing and quantity of sporangia and zoospore production

following baiting, and species that produce sporangia slowly may be challenging to identify because faster-sporulating species compete with them. In addition, *Phytophthora* species with slow growing mycelia, especially on selective agar media, can be overgrown by faster growing *Pythium*, *Phytophythium* or *Phytophthora* species (Jung et al., 2000, 2016, 2017a, 2018b, 2019, 2020; Pérez-Sierra et al., 2022; Sarker et al., 2021), hence getting pure cultures of slower growing *Phytophthora* species requires experience and special skills (Pérez-Sierra et al., 2022).

Metabarcoding studies are valuable for monitoring of invasive pathogens and diversity studies (Burgess et al., 2017; Catalá et al., 2015; Riddell et al., 2019). This method uses a highly specific PCR assay followed by high throughput DNA sequencing in order to distinguish every species within a specified group (here *Phytophthora* and related oomycetes) present within any given environmental sample (Green et al., 2020; Mendoza et al., 2015). Studies conducted in various countries, including France, Ireland, Italy, Spain, Sweden and Australia (Burgess et al., 2017; Caballol et al., 2024; Catalá et al., 2015; La Spada et al., 2022; Redondo et al., 2018; Vannini et al., 2013) have proven the effectiveness of metabarcoding in the *Phytophthora* diversity studies in both soil and water. In all these studies, primers amplifying parts of the nuclear region spanning the internal transcribed spacer (ITS1–5.8S–ITS2) region of the ribosomal DNA were used since the ITS is one of the barcode regions of *Phytophthora*. However, metabarcoding detection of environmental DNA (eDNA) only confirms the existence of *Phytophthora* DNA within a specific environment. It does not provide information about whether viable individuals of each identified species are actually present (Burgess et al., 2017). Because smaller amount of soil is used for metabarcoding than for baiting, this may result in omission of some species which are present in the ecosystem but not in the soil sample tested (Sarker et al., 2023a). In contrast, metabarcoding of baiting leaves without symptoms of infection can be a useful additional technique for the monitoring of *Phytophthora* diversity (Sarker et al., 2023b).

In studies that used both baiting and metabarcoding techniques, many more species were detected by metabarcoding compared with baiting (Catalá et al., 2015; Bose et al., 2018; Riddell et al., 2019; Landa et al., 2021; Sarker et al., 2023a; La Spada et al.,

2022). However, to a lesser extent, metabarcoding approaches have also failed to detect *Phytophthora* species obtained by baiting (Sarker et al. 2023a).

In this study a survey of *Phytophthora* infestations of nurseries producing wide range of plant material (forest nurseries, nurseries producing large amenity trees and trees for landscape plantings and ornamental nurseries) in the Czech Republic was performed using and comparing baiting and metabarcoding methods.

Material and methods

Study areas and sampling of rhizosphere soil, compost, and irrigation water

A total of eight forest and ornamental nurseries were sampled across the Czech Republic (Table 1).

Forest nurseries produce only plants of forest trees for the state forestry and forestry enterprises in Czech Republic exclusively using seeds from the Czech Republic. No plants are imported from abroad. In contrast, ornamental nurseries produce a very wide spectrum of tree and shrub species, both native and non-native to the Czech Republic, for planting activities in private gardens and urban green areas. Plants are often imported from other European countries. Many ornamental nurseries also produce amenity trees which are grown for more years and to larger sizes. They are most often used for public parks and road-side plantings.

Sampling for baiting method

Soil samples were taken in all eight nurseries from trees and shrubs known to be susceptible to *Phytophthora*. Samples of compost, necrotic leaves floating in water ponds, and irrigation water were taken from selected sites (Table 2) because most nurseries did not have their own compost or did not have access to a surface water source.

At least three soil samples of each tree species per nursery were bulked and examined. Both symptomatic and asymptomatic plants were examined. In the case of bare root plants or containerised/potted plants older than five years, 3–5 soil samples were taken from opposing sides around the plant at a depth of 10 to 20 cm, depending on the age and

Table 1 Nurseries sampled in the Czech Republic

Nursery no.	Type of nursery	Samples	Sampling period
1	Forest	Leaves from pond, soil	April 2017
2	Ornamental+forest	Soil	April 2017
3	Ornamental+amenity trees	River water, filtered water, soil	April 2017 May 2021
4	Ornamental	Compost, soil	May 2017
5	Ornamental	Soil	May 2017
6	Ornamental	Soil	May 2017
7	Forest	Compost, soil	May 2017
8	Forest	Leaves from pond, soil	September 2019

size of the plant. Fine roots were also included in the soil sample, but coarser roots were avoided. The soil sub-samples for each individual plant were combined and mixed to create one bulked soil sample with a volume of approximately 0.5–1.0 L. In case of containerised plants younger than five years, 3–5 plants were removed from their pots and their rhizosphere soil pooled and mixed in a 1 L plastic bag.

If the nursery was using compost for preparing their soil substrate, samples were also taken from 3 to 5 different sites of a compost pile and pooled to a bulked compost sample of ca 0.5–1.0 L volume.

Irrigation water was sampled in nursery no. 3. Since this nursery was using both river and a reservoir as water sources and sand water filters, water samples of 10 L per sample were taken directly from the river and the reservoir and also after the filtration treatment.

Furthermore, in nurseries nos. 1, 3 and 8 fallen necrotic leaves floating on irrigation water reservoirs were sampled for detection of *Phytophthora* presence (natural baiting) according to Jung et al. (2017a, b, c). The collected leaves were immediately processed in the laboratory as described below for baiting leaves without the need to carry out baiting with fresh leaves.

Sampling for direct DNA extraction and metabarcoding

For detailed analyses by DNA metabarcoding two nurseries were selected with expected high (nursery no. 3) and low (nursery no. 8) *Phytophthora* diversity, respectively, according to different management practices. Nursery no. 8 is a forest nursery propagating

nursery stock of local origin and not importing plants from abroad. In contrast, nursery no. 3 is focused on the propagation and sale of ornamental tree species, often not native to the Czech Republic, which are regularly imported from various European countries. Moreover, nursery no. 3 occupies a larger area than nursery no. 8.

NGS was only performed with water samples from nursery no. 3. Since this nursery has a water filter system, samples were taken before (water from river) and after the filtration. One sample was taken before filtration (from the river) and four samples from different parts of irrigation system after the filtration. The four samples were not mixed.

Isolation by baiting

Baiting commenced on either the day of sampling or within 24 hours of sampling and was therefore carried out with moist soil samples. Each soil or compost sample was placed in a plastic box 20 × 15 × 30 cm and flooded with distilled water to a depth of 3–4 cm above the soil surface. Litter and debris floating to the surface of the water was removed using a paper towel. After ca 2 hours of sedimentation, young soft leaves (without cuticle) of *F. sylvatica* and *Q. suber* were floated on the water and the baiting trays were incubated in a room with daylight at 18–20 °C (Jung, 2009; Pérez-Sierra et al., 2022).

Leaves becoming blackish or showing necrotic spots were briefly dried on a paper towel and small 2–3 mm pieces cut from the necroses were plated onto selective PARPNH – agar (Jung et al., 2017a) and incubated at 20 °C in the dark.

Baiting of irrigation water was conducted similarly. The irrigation water from the nursery was poured into

Table 2 *Phytophthora* taxa detected by baiting in rhizosphere soil samples

Nursery no.	Host species	Number of bulked soil samples taken	Baited <i>Phytophthora</i> taxa (no. of positive samples)	Number of isolates
1	<i>Fagus sylvatica</i>	4	<i>P. plurivora</i> (3)	3
			<i>P. ×cambivora</i> (1)	5
			<i>P. cactorum</i> (1)	2
	<i>Quercus robur</i>	3	<i>P. plurivora</i> (3)	3
	<i>Picea abies</i>	1	–	–
<i>Larix decidua</i>	1	–	–	
2	<i>Pinus aristata</i>	1	<i>P. cryptogea</i>	4
			<i>P. ×cambivora</i>	1
	<i>Picea abies</i>	2	<i>P. cryptogea</i> (1)	1
	<i>Quercus robur</i>	2	<i>P. cryptogea</i> (1)	1
			<i>P. plurivora</i> (1)	1
	<i>Quercus</i> sp.	2	<i>P. gonapodyides</i> (1)	3
			<i>P. quercina</i> (1)	2
			<i>P. cryptogea</i> (1)	1
	<i>Viburnum × pragense</i>	1	<i>P. rosacearum</i>	1
			<i>P. t. organica</i>	6
			<i>P. cryptogea</i>	11
			<i>P. chlamydospora</i>	3
	<i>Picea omorika</i>	1	–	–
	<i>Fagus sylvatica</i>	2	–	–
	<i>Acer pseudoplatanus</i>	1	–	–
<i>Rhododendron</i> sp.	1	–	–	
3	<i>Quercus robur</i>	1	<i>P. transitoria</i>	1
	<i>Berberis</i> sp.	1	<i>P. plurivora</i>	3
	<i>Chamaecyparis pisifera</i>	1	<i>P. occultans</i> / <i>P. citrophthora</i>	2
	<i>Euonymus</i> sp.	1	<i>P. occultans</i> / <i>P. citrophthora</i>	2
	<i>Buxus</i> sp.	1	<i>P. plurivora</i>	3
	<i>Quercus robur</i>	1	–	–
	<i>Fagus sylvatica</i>	1	–	–
	<i>Abies concolor</i>	1	–	–
	<i>Abies koreana</i>	1	–	–
	<i>Aesculus hippocastanum</i>	1	–	–
	<i>Acer platanoides</i>	1	–	–
	4	<i>Quercus</i> sp.	2	<i>P. chlamydospora</i> (1)
			<i>P. cryptogea</i>	3
<i>Acer palmatum</i>		1	<i>P. ×cambivora</i>	1
<i>Viburnum burkwoodii</i>		2	<i>P. plurivora</i> (1)	2
			<i>P. cactorum</i> (2)	1
<i>Picea abies</i>		1	–	–
<i>Pinus nigra</i>		1	–	–
<i>Fagus sylvatica</i>	1	–	–	

Table 2 (continued)

Nursery no.	Host species	Number of bulked soil samples taken	Baited <i>Phytophthora</i> taxa (no. of positive samples)	Number of isolates
5	<i>Alnus</i> sp.	3	<i>P. plurivora</i> (3)	4
	<i>Magnolia</i> sp.	1	<i>P. plurivora</i>	2
	<i>Quercus</i> sp.	2	–	–
	<i>Euonymus</i> sp.	1	–	–
	<i>Cornus alba</i>	1	–	–
6	<i>Abies</i> sp.	1	–	–
	<i>Carpinus</i> sp.	1	–	–
	<i>Fagus</i> sp.	1	–	–
7	<i>Fagus sylvatica</i>	5	<i>P. cryptogea</i> (5)	1
	<i>Ulmus glabra</i>	2	<i>P. cryptogea</i> (1)	4
	<i>Picea abies</i>	2	–	–
	<i>Acer pseudoplatanus</i>	1	–	–
	<i>Abies nordmanniana</i>	1	–	–
8	<i>Fagus sylvatica</i>	1	<i>P. plurivora</i>	3
			<i>P. syringae</i>	3
			<i>P. cactorum</i>	2
	<i>Quercus robur</i>	1	<i>P. plurivora</i>	2

a plastic box 20 × 15 × 30 cm and the water surface was covered with young *Fagus* and *Quercus* leaves as in the soil baiting. Leaves developing necrotic lesions were processed as described before.

Naturally fallen necrotic leaves floating in irrigation water reservoirs were used as natural baits and taken to the laboratory, where they were briefly dried and processed as described before (Jung et al., 2020).

PARPNH – Petri dishes with plated leaf pieces were examined daily under stereomicroscope at ×20 magnification for mycelial growth. When *Phytophthora* hyphae were observed an agar plug with a few hyphae from the growing margin of the colony was cut out and transferred onto vegetable juice agar (V8A) or carrot juice agar (CA) (Jung et al., 1996, 2017a; Pérez-Sierra et al., 2022).

All oomycete isolates were deposited in the culture collection of the Phytophthora Research Centre at Mendel University in Brno.

DNA extraction from mycelia and identification of *Phytophthora* isolates

DNA from mycelia of clean isolates obtained from baiting (or by cultivating naturally fallen necrotic leaves

on selective agar) was extracted according to a modified protocol of Lamour and Finley (2006) optimised by Jung et al. (2023). DNA was then purified using the Monarch Genomic DNA Purification Kit (New England Biolabs, Ipswich, USA) and treated with RNase A following the manufacturer's protocol for tissue samples. DNA was eluted with 100 µL of pre-warmed elution buffer and preserved at –80 °C for long term storage.

Identification of *Phytophthora* cultures by sequencing of the nuclear region spanning the internal transcribed spacer (ITS1–5.8S–ITS2) region of the ribosomal DNA and the mitochondrial *Cox1* gene was performed according to Jung et al. (2020). The ITS region was amplified using primer-pairs ITS1/ITS4 or ITS6/ITS4 (Cooke et al., 2000; White et al., 1990). The *Cox1* gene was amplified with both primer-pairs COXF4N/COXR4N and FM84/FM83 (Kroon et al., 2004; Martin & Tooley, 2003). The PCR reaction mixture and the amplification conditions for ITS and *Cox1* were according to Cooke et al. (2000), Martin and Tooley (2003) and Kroon et al. (2004). All amplicons were purified and sequenced in both directions by Eurofins Genomics GmbH (Cologne and Ebersberg, Germany) using the amplification primers.

For species identification, consensus sequences were blasted against GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) and a local database containing sequences of ex-type isolates and reference isolates from published studies. Isolates were assigned to a species when their sequences were at least 99% identical to a reference isolate.

DNA extraction from rhizosphere soil and water samples

DNA from soil was extracted using DNeasy PowerLyzer PowerSoil Kit (QIAGEN). Three replicates per soil or water sample were processed. Each sample was divided into three homogenisation tubes included in the kit and the kit protocol was followed without modifications.

After the extraction, the DNA was purified using Monarch Genomic DNA Purification Kit (New England Biolabs, Ipswich, USA).

This DNA was subsequently used for nested PCR and DNA metabarcoding.

PCR

For DNA metabarcoding a nested PCR assay to amplify the ITS1 region was conducted in accordance with the protocol of Riddell et al. (2019) also used in La Spada et al. (2022) using the primer pairs 18Ph2F (5'-GGATAGACTGTTGCAATTTTCAGT-3') and 5.8S-1R (5'-GCARRGACTTTCGTC CCYRC-3') (Scibetta et al., 2012) in the first round and ITS-6 (5'-GAAGGTGAAGTCGTAACAAGG-3') (Cooke et al., 2000) and 5.8S-1R in the second round. The second-round primers were modified with overhang adapters for compatibility to the Illumina index and sequencing adapters. These were: forward overhang; 5'-TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAG-3' (ITS-6) and reverse overhang; 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAG-3' (5.8S-1R) (Illumina Inc., 2013). In each reaction, hi-fidelity KAPA HiFi DNA Polymerase (Roche SA, Basel, Switzerland) was used. Control reactions were included that comprised a mix of four synthetic DNA oligonucleotides with an equivalent length to the *Phytophthora* ITS1 region and amplification primers (ITS-6 and 5.8S-1R) included at each end (GenBank accession numbers PP407413 to PP407416). The amplicons obtained were then run

on 1.5% agarose gels and all oomycete-positive PCR products were selected for downstream processing.

Illumina sequencing library preparation and sequencing

Library preparation was done according to La Spada et al. (2022). In brief, PCR products were prepared for Illumina metabarcode sequencing following the instructions reported in the protocol 16S Metagenomic Sequencing Library Preparation (Illumina Inc., 2013). The obtained amplicons were subjected to a PCR clean-up using the Agencourt® Ampure® XP beads kit (Agencourt Bioscience, Beverly, MA, USA) and then moved to Index PCR using the Nextera XT Index Kit (Epicentre, Madison, WI, USA) to attach dual indices and Illumina sequencing adapters to each amplicon. This step was to allow the association of each unique index and amplicon to a single sample after the sequencing run. A second PCR clean-up was then run, as above, and the final PCR products from each sample were visualized on a 1.5% agarose gel. All the products were then quantified by fluorometry with the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen™, Waltham, MA, USA) and pooled to a single library that was adjusted to a concentration of 4 nM. The libraries were sequenced at the James Hutton Institute (Dundee, United Kingdom) using the MiSeq version 2 chemistry with 500 cycles (reagent kit MS-102-2003, Illumina, San Diego, CA, USA). The obtained FASTQ files containing barcode reads for each sample were then exported for bioinformatic analysis.

Analyses of Illumina data

The Illumina paired end reads were processed using version 0.12.1 of the Tree Health and Plant Biosecurity Initiative *Phytophthora* ITS1 Classifier Tool THAPBI PICT open-source software pipeline for metabarcoding analysis (Cock et al., 2023). The core of the analysis involves data reduction to unique marker sequences termed amplicon sequence variants (ASVs) which are then processed against dynamic thresholds based on control samples on the Illumina flow cell. Then ASVs are classified against a curated reference database of THAPBI PICT *Phytophthora* ITS1 and a local *Phytophthora* Research Centre database containing sequences of ex-type or key isolates

from published studies (Aghighi et al., 2012; Cooke et al., 2000; Jung et al., 2019, 2020; Riolo et al., 2020; Safaiefarahani et al., 2015; Santilli et al., 2020; Scanu et al., 2014) using BioEdit. ASVs were assigned to a species when their sequences were at least 99% identical to a reference sequence. Any sequences not assigned to a species from the reference databases were classified at the genus or clade level. The phylogenetic relationship of aligned dataset of detected sequences was analysed by means of the maximum likelihood algorithm carried out using Phylogeny.fr (<http://phylogeny.lirmm.fr/phylo.cgi/index.cgi>) the “A la Carte” Mode (PhyML, GBlock disabled, GTR evolutionary model, bootstrapping procedure with 100 replicates).

Results

Species detected by baiting isolation and ITS sequencing from soil

Using a baiting technique *Phytophthora* spp. were isolated from 38 of the 64 rhizosphere soil samples (59.4%) tested from seven of the eight nurseries sampled. In nursery no. 6 no *Phytophthora* isolates were recovered. Overall, 94 *Phytophthora* isolates were obtained which, according to ITS sequence analysis, belonged to 12 *Phytophthora* taxa (Table 2). *P. plurivora* was most commonly isolated (26 isolates, 7 host species, 6 nurseries) followed by *P. cryptogea* (26 isolates, 6 host species, 3 nurseries). *Phytophthora plurivora* was isolated regularly from *Fagus* and *Quercus*, but also from *Berberis*, *Buxus*, *Viburnum*, *Alnus* and *Magnolia*, whereas *P. cryptogea* was found on *Fagus*, *Quercus*, *Viburnum*, *Ulmus* and the conifers *Picea* and *Pinus*. Interestingly, these two species never co-occurred in the same soil sample.

A previously unknown *Phytophthora* species from phylogenetic Clade 13 was obtained during this survey from seedlings of *Quercus robur* in nursery no. 3 and described in 2022 as *Phytophthora transitoria* (Abad et al., 2023; Chen et al., 2022).

Compost

Compost was tested in nurseries no. 4 and no. 7 and *P. cryptogea* and *P. gonapodyides* were isolated from both nurseries (Table 3).

Irrigation water

Irrigation water was tested in three nurseries by baiting or by direct isolation from necrotic leaves collected from the water source. Only *P. lacustris* and *P. gonapodyides* were isolated from 3 and 2 nurseries, respectively (Table 3).

Species detected by Illumina sequencing from eDNA

Illumina metabarcoding was completed on samples from nurseries nos. 3 and 8 with a total of 14 eDNA samples (five from water, nine from soil) which produced positive nested PCR amplicons suitable for metabarcoding. A plate threshold of 422 reads was established on the basis of control samples, meaning only sample ASVs with read numbers above 422 were considered in the following results.

Overall, ITS1 barcodes corresponding to 45 *Phytophthora* taxa were detected from soil and water samples, 15 of them undescribed *Phytophthora* taxa from Clades 6, 7, 8, 9. Another 11 taxa belonged to both known and unknown species of *Globisporangium*, *Hyaloperonospora*, *Nothophytophthora*, *Peronospora* and *Plasmopara*.

Soil samples

Abundance, i.e. the number of samples in which a particular species was detected and read abundance, i.e. the number of metabarcode reads of a specific species, are shown in Figs. 1 and 2, respectively.

Table 3 *Phytophthora* taxa detected in compost by baiting and in water samples by baiting and plating of naturally floating leaves; n.t. = not tested

Nursery no.	<i>Phytophthora</i> taxa from water	<i>Phytophthora</i> taxa from compost
1	<i>P. lacustris</i> , <i>P. gonapodyides</i>	n.t.
2	n.t.	n.t.
3	<i>P. lacustris</i> , <i>P. gonapodyides</i>	n.t.
4	n.t.	<i>P. gonapodyides</i>
5	n.t.	n.t.
6	n.t.	n.t.
7	n.t.	<i>P. cryptogea</i>
8	<i>P. lacustris</i>	n.t.

Barcodes matching a total of 34 *Phytophthora* taxa were detected in soil samples from nurseries 3 and 8 (Figs. 1, 2 and 3; Table 4). In nursery no. 8, four bulked soil samples were tested. Two were from *Pinus sylvestris* plants of different ages and two from *Fagus sylvatica* plants of different ages. In this nursery very low diversity of *Phytophthora* species was found. Only ASVs associated with *P. ×cambivora* on *Pinus sylvestris* and *P. aleatoria/alpina/cactorum* on *Fagus sylvatica* were found. In contrast, with 32 *Phytophthora* taxa, diversity was much higher in nursery no. 3. In this nursery, five bulked soil samples were taken from the species *Berberis* sp., *Chamaecyparis pisifera*, *Buxus* sp., and two samples from *Euonymus* sp. with different ages. With 19 *Phytophthora* species the highest diversity was found in soil from the rhizosphere of symptomatic *Berberis* plants. The *Phytophthora* species with the highest abundance in nursery no. 3 was *P. cinnamomi* (Figs. 1, 2 and 3), recorded from soil of all tested plants and all samples. Barcodes matching *P. lateralis*, a species previously

not recorded from Czech Republic, was discovered with the second highest abundance in four soil samples from *Chamaecyparis*, *Berberis*, *Buxus*, and *Euonymus*. Two species, *P. virginiana* and *P. uniformis* were detected with the same abundance as *P. lateralis* in four out of five samples from this nursery. Another 20 known species or informally designated taxa of *Phytophthora* were found in nursery 3 including the globally distributed wide-host range pathogens *P. citrophthora* and *P. megasperma* and *P. ×multiformis*, together with *P. uniformis* one of the drivers of the devastating *Alnus* dieback along rivers in Europe. The findings of *P. kwongonina*, *P. mississippiiae*, *P. taxon sulawesiensis* (exact match with GenBank accession no. EF590257), *P. ×stagnum* (all from Clade 6) and *P. taxon AUS9C* (exact match with GenBank accession no. KY110356.1) from Clade 9d are first records for Europe whereas *P. condilina*, *P. ornamentata* and *P. sansomeana* are for the first time reported from Czech Republic (Fig. 1). The relatively short ITS1 sequence produced by the metabarcoding

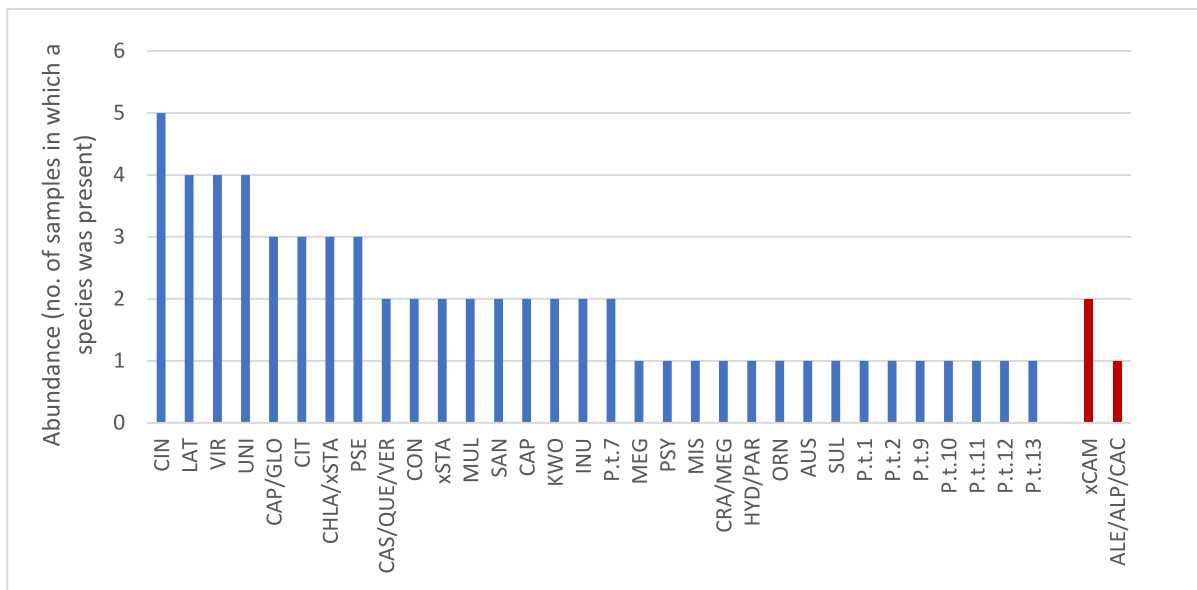


Fig. 1 Abundance of *Phytophthora* taxa identified in soil samples from two nurseries (blue=ornamental, red=forest) by DNA metabarcoding. ALE/ALP/CAC=*P. aleatoria/P. alpina/P. cactorum*; AUS=*P. t. AUS9C*; ×CAM=*P. ×cambivora*; CAP=*P. aff. capsici*; CAP/GLO=*P. capsici/P. glovera*; CAS/QUE/VER=*P. castanetorum/P. quercinal/P. versiformis*; CIN=*P. cinnamomi*; CIT=*P. citrophthora*; CON=*P. condilina*; CRA/MEG=*P. crassamura/P. megasperma*;

HYD/PAR=*P. hydropathical/P. parsiana*; CHL/×STA=*P. chlamydospora/P. ×stagnum*; INU=*P. inundata*; KWO=*P. kwongonina*; LAT=*P. lateralis*; MIS=*P. mississippiiae*; MEG=*P. megasperma*; ×MUL=*P. ×multiformis*; ORN=*P. ornamentata*; PSE=*P. pseudosyringae*; PSY=*P. psychrophila*; P.t. 1–13=*Phytophthora* taxon 1–13 (novel taxons); SAN=*P. sansomeana*; ×STA=*P. ×stagnum*; SUL=*P. sulawesiensis*; UNI=*P. uniformis*; VIR=*P. virginiana*

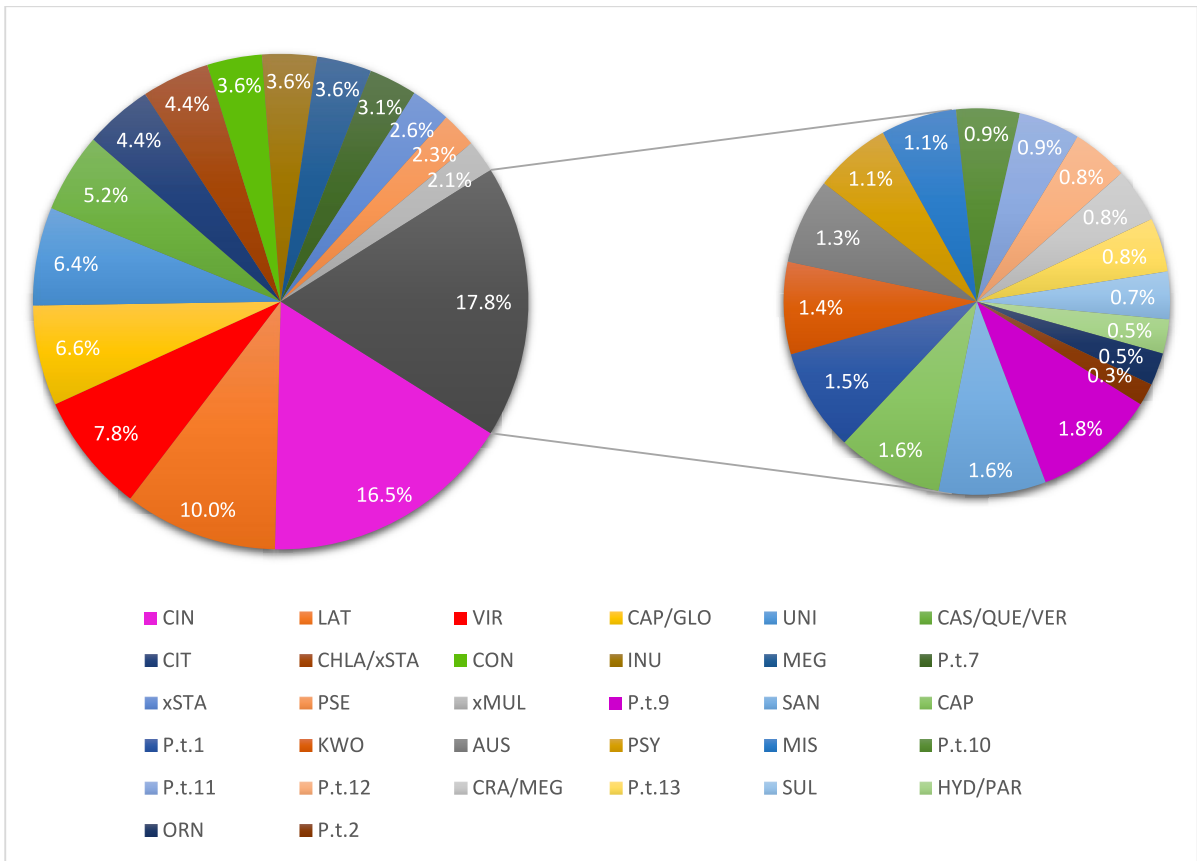


Fig. 2 Read abundance of *Phytophthora* taxa identified in soil samples from ornamental nursery no. 3 by DNA metabarcoding. ALE/ALP/CAC = *P. aleatoria/P. alpina/P. cactorum*; AUS = *P. t. AUS9C*; xCAM = *P. xCambivora*; CAP = *P. aff. capsici*; CAP/GLO = *P. capsici/P. glovera*; CAS/QUE/VERS = *P. castanetorum/P. quercinal/P. versiformis*; CIN = *P. cinnamomi*; CIT = *P. citrophthora*; CON = *P. condilina*; CRA/MEG = *P. crassamura/P. megasperma*; HYD/PAR = *P.*

hydropathical/P. parsiana; CHL/xSTA = *P. chlamydospora/P. xstagnum*; INU = *P. inundata*; KWO = *P. kwongonina*; LAT = *P. lateralis*; MIS = *P. mississippiae*; MEG = *P. megasperma*; xMUL = *P. xmultiformis*; ORN = *P. ornamentata*; PSE = *P. pseudosyringae*; PSY = *P. psychrophila*; P.t. 1–13 = *Phytophthora* taxon 1–13 (novel taxon); SAN = *P. sansomeana*; xSTA = *P. xstagnum*; SUL = *P. sulawesiensis*; UNI = *P. uniformis*; VIR = *P. virginiana*

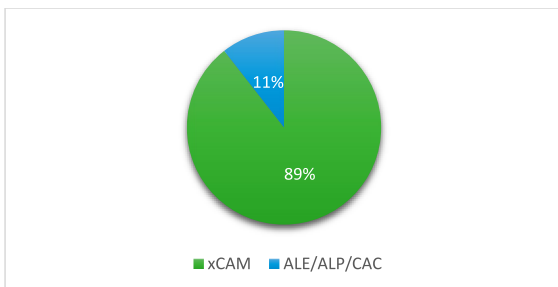


Fig. 3 Read abundance of *Phytophthora* taxa identified in soil samples from forest nursery by DNA metabarcoding. ALE/ALP/CAC = *P. aleatoria/P. alpina/P. cactorum*; xCAM = *P. xCambivora*

primers could not discriminate taxa from closely related species complexes to the species level, i.e. *P. aleatoria/P. alpina/P. cactorum*, *P. capsici/P. glovera*, *P. castanetorum/P. quercinal/P. versiformis*, *P. chlamydospora/P. xstagnum*, *P. crassamura/P. megasperma* and *P. hydropathical/P. parsiana* (Fig. 1). Barcodes of an additional eight novel *Phytophthora* taxa from Clades 6, 7, 8 and 9 (Fig. 7), *Nothophytophthora intricata* and the downy mildew (DM) species *Peronospora variabilis* and *Pe. cf. fagopyri* were detected in nursery 3 (Table 4), whereas in nursery 8 three known *Peronospora* species, i.e. *Pe. variabilis*, *Pe. conglomerata* and *Pe. mayorii*, and three and two novel taxa of the DM genera *Hyaloperonospora* and

Table 4 Previously undescribed *Phytophthora* taxa and taxa from other oomycete genera detected by DNA metabarcoding; n.a. = not applicable

Taxon	Phylogenetic Clade	Nursery	Source
<i>Phytophthora</i> t. 1	Novel, Clade 7	no.3	Soil and Filtered Water
<i>Phytophthora</i> t. 2	Novel, Clade 9	no.3	Soil
<i>Phytophthora</i> t. 3	Novel, Clade 9	no.3	Filtered Water
<i>Phytophthora</i> t. 4	Novel, Clade 9	no.3	Filtered Water
<i>Phytophthora</i> t. 5	Novel, Clade 9	no.3	River Water
<i>Phytophthora</i> t. 6	Novel, Clade 9	no.3	Filtered Water
<i>Phytophthora</i> t. 7	Novel, Clade 9	no.3	Soil
<i>Phytophthora</i> t. 8	Novel, Clade 9	no.3	Filtered Water
<i>Phytophthora</i> t. 9	Novel, Clade 9	no.3	Soil
<i>Phytophthora</i> t. 10	Novel, Clade 8	no.3	Soil
<i>Phytophthora</i> t. 11	Novel, Clade 9	no.3	Soil
<i>Phytophthora</i> t. 12	Novel, Clade 6	no.3	Soil
<i>Phytophthora</i> t. 13	Novel, Clade 6	no.3	Soil
<i>Phytophthora</i> t. 14	Novel, Clade 6	no.3	River Water
<i>Phytophthora</i> t. 15	Novel, Clade 9	no. 3	River and Filtered Water
<i>Hyaloperonospora</i> t. 1	Novel	no.8	Soil
<i>Hyaloperonospora</i> t. 2	Novel	no.8	Soil
<i>Hyaloperonospora</i> t. 3	Novel	no.8	Soil
<i>Peronospora variabilis</i>	n.a.	no.3 and 8	Soil
<i>Peronospora conglomerata</i>	n.a.	no.8	Soil
<i>Peronospora</i> cf. <i>fagopyri</i>	n.a.	no.3	Soil
<i>Peronospora mayorii</i>	n.a.	no.8	Soil
<i>Plasmopara</i> t. 1	Novel	no.8	Soil
<i>Plasmopara</i> t. 2	Novel	no.8	Soil
<i>Nothophytophthora intricata</i>	n.a.	no.3	Soil
<i>Globisporangium</i> t. 1	Novel	no.8	Soil

Plasmopara, respectively, were detected (Table 4). The finding of *N. intricata*, originally described from the rhizosphere of a riparian *Aesculus hippocastanum* in Germany (Jung et al., 2017c), is the second record of this species and the first report of a *Nothophytophthora* species from a nursery environment. Table 4 describes differences in the species diversity in soil between nurseries no. 3 and no. 8.

Water samples

Abundance and read abundance are shown in Figs. 4, 5 and 6.

With nine species the *Phytophthora* diversity was lower in the unfiltered natural river water (Fig. 4). Most common (highest number of reads) was *P. cryptogea* followed by *P. ×multiformis*, *P. chlamydospora/P. ×stagnum* and *P. uniformis* (Fig. 6). Interestingly, *P. cinnamomi* was also present in the river water.

In contrast, 21 known and 6 novel *Phytophthora* taxa were detected in the filtered water (Fig. 4). At least 15 *Phytophthora* taxa were present in each of the four tested water samples. The most common species was *P. cinnamomi* which appeared in all replicates (Fig. 4) with the highest total number of 38,435 reads (Fig. 5) followed by *P. lateralis* with 29,579 reads (Figs. 4 and 5). Overall, 14 *Phytophthora* taxa were present in all four replicates (Fig. 4). *Phytophthora uniformis* and *P. castanetorum/P. quercinal/P. versiformis* had more than 15,000 reads whereas *P. chlamydospora/P. ×stagnum* and *P. virginiana* had more than 10,000 reads. The relatively short ITS1 sequence produced by the metagenomic primers did not allow the discrimination to species level for several taxa from closely related species complexes, i.e. *P. chlamydospora/P. ×stagnum*, *P. capsici/P. glovera*, *P. castanetorum/P. quercinal/P. versiformis*, *P. gibbosa/P. gregata* and *P. hydropathica/P. parsiana*.

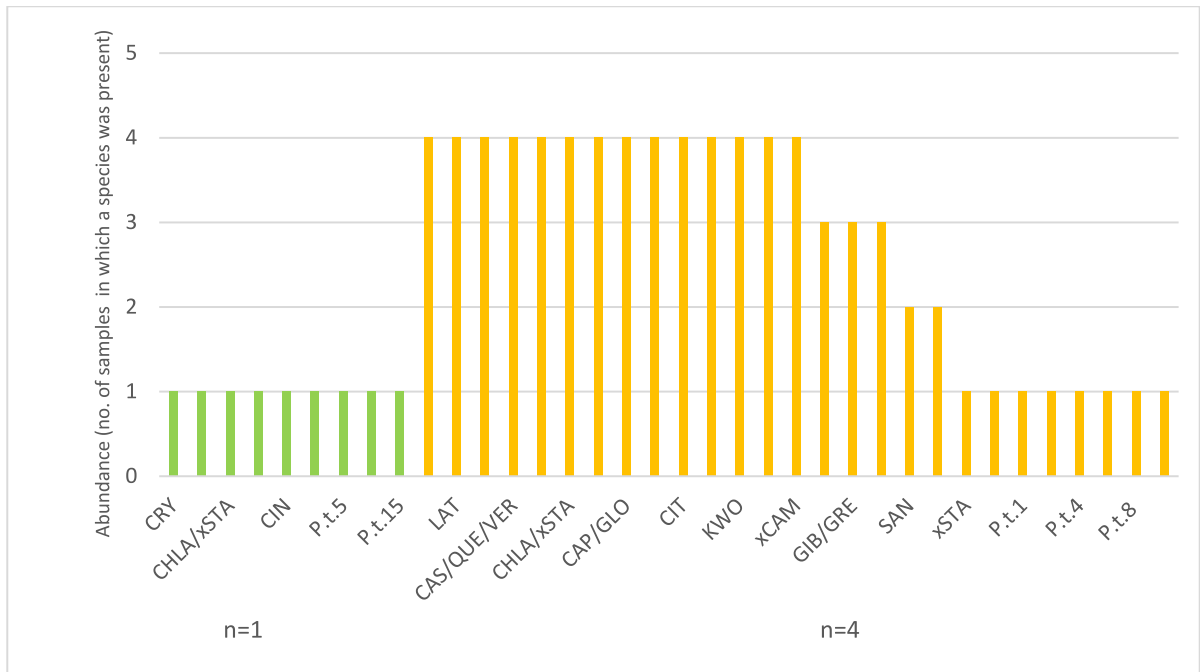


Fig. 4 Abundance of *Phytophthora* taxa detected by DNA metabarcoding in water samples (green=river water before filtration, yellow=after filtration) from nursery no.3. ×CAM = *P. ×cambivora*; CAP/GLO = *P. capsici/P. glovera*; aff CAP = *P. aff. capsici*; CAS/QUE/VERS = *P. castanetorum/P. quercina/P. versiformis*; CIN = *P. cinnamomi*; CIT = *P. citrophthora*; CON = *P. condilina*; CRY = *P. cryptogea*; GIB/

GRE = *P. gibbosa/P. gregata*; GON = *P. gonapodyides*; CHL/×STA = *P. chlamydospora/P. ×stagnum*; INU = *P. inundata*; KWO = *P. kwongonina*; LAT = *P. lateralis*; MEG = *P. megasperma*; ×MUL = *P. ×multiformis*; PSE = *P. pseudosyringae*; Pt. 1–15 = *Phytophthora* taxa 1–15 (novel taxa); SAN = *P. sansomeana*; ×STA = *P. ×stagnum*; UNI = *P. unifornis*; VIR = *P. virginiana*

Surprisingly, no other oomycete taxa were detected in any water sample (Fig. 7).

Comparison of results from baiting and DNA metabarcoding

Overall, 50 *Phytophthora* species were detected with both techniques in this study from soil and water samples of eight and three nurseries, respectively. Soil samples from nurseries no. 3 and 8 and water samples from nursery 3 were tested by both baiting and DNA metabarcoding permitting some comparisons between both techniques.

Soil samples

Nursery no. 8 showed low *Phytophthora* diversity with both methods but, it was noteworthy that no

species overlap was detected and more species, i.e. *P. plurivora*, *P. syringae* and *P. cactorum*, were detected with baiting than with DNA metabarcoding, i.e. *P. cactorum* (or *P. aleatoria* or *P. alpina*) and *P. ×cambivora*. Nine known and unknown taxa from other oomycete genera *Globisporangium*, *Hyaloperonospora*, *Nothophytophthora*, *Peronospora* and *Plasmopara* were also detected in this nursery (Table 4) but only by metabarcoding and not by baiting.

In contrast, in nursery no. 3 the diversity detected by DNA metabarcoding was much higher compared to baiting. While baiting revealed only *P. plurivora*, *P. transitoria* and *P. citrophthora*, DNA metabarcoding detected 32 *Phytophthora* taxa including eight novel ones plus three known and unknown taxa from the oomycete genera *Nothophytophthora* and *Peronospora*.

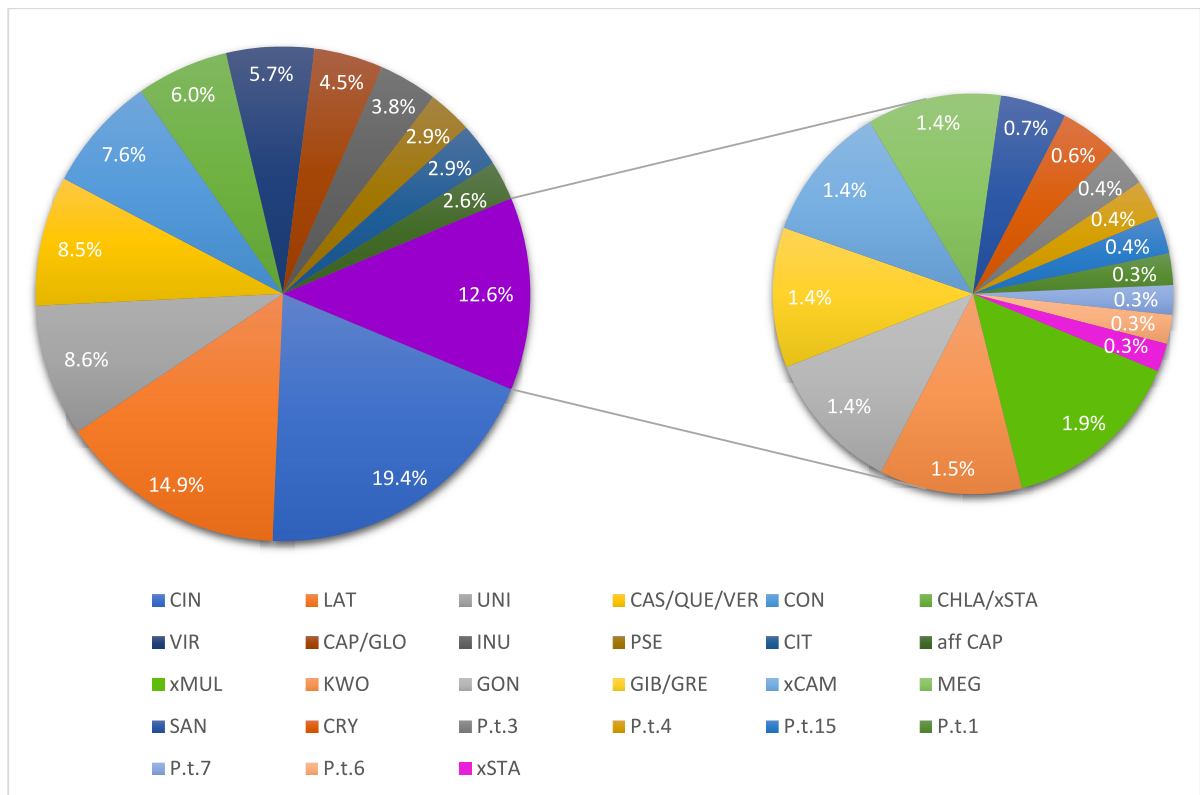


Fig. 5 Read abundance of *Phytophthora* taxa detected by DNA metabarcoding in filtered water samples from nursery no.3. xCAM = *P. xambivora*; CAP/GLO = *P. capsici*/*P. glovera*; aff CAP = *P. aff. capsici*; CAS/QUE/VERS = *P. castanetorum*/*P. quercina*/*P. versiformis*; CIN = *P. cinnamomi*; CIT = *P. citrophthora*; CON = *P. condilina*; CRY = *P. cryptogea*; GIB/GRE = *P. gibbosa*/*P. gregata*; GON = *P. gonapody-*

ides; CHL/xSTA = *P. chlamydospora*/*P. xstagnum*; INU = *P. inundata*; KWO = *P. kwongonina*; LAT = *P. lateralis*; MEG = *P. megasperma*; xMUL = *P. xmultiformis*; PSE = *P. pseudosyringae*; P.t. 1–15 = *Phytophthora* taxa 1–15 (novel taxa); SAN = *P. sansomeana*; xSTA = *P. xstagnum*; UNI = *P. uniformis*; VIR = *P. virginiana*

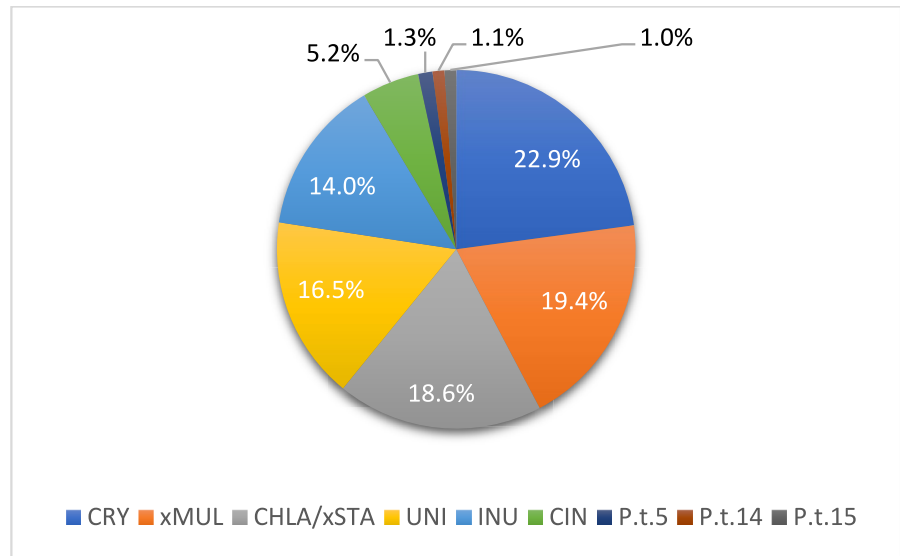
Water samples

While baiting detected only *P. gonapodyides* and *P. lacustris*, both primarily aquatic species with almost ubiquitous distribution in European waterways, DNA metabarcoding unveiled a rich community of 22 known and eight previously unknown taxa of *Phytophthora*. *Phytophthora gonapodyides* was the only species detected with both techniques. Surprisingly, neither *P. lacustris* nor any other oomycete genus were detected by metabarcoding of water samples.

Discussion

Previous studies have demonstrated a high diversity of *Phytophthora* populations in both natural and managed ecosystems across Europe (Catalá et al., 2015; Corcobado et al., 2023; Green et al., 2020; Jung, 2009; Jung et al., 1996, 2000, 2016, 2019; La Spada et al., 2022; Moralejo et al., 2009; Mora-Sala et al., 2022; Vannini et al., 2013; Vettraino et al., 2002). Since planting of infested nursery stock is the primary pathway of *Phytophthora* pathogens to

Fig. 6 Read abundance of *Phytophthora* taxa detected by DNA metabarcoding in unfiltered water samples from nursery no. 3. CIN = *P. cinnamomi*; CRY = *P. cryptogea*; CHL/×STA = *P. chlamydospora*/*P. ×stagnum*; INU = *P. inundata*; ×MUL = *P. ×multiformis*; P.t. 5–15 = *Phytophthora* taxon 5–15 (novel taxon); UNI = *P. uniformis*



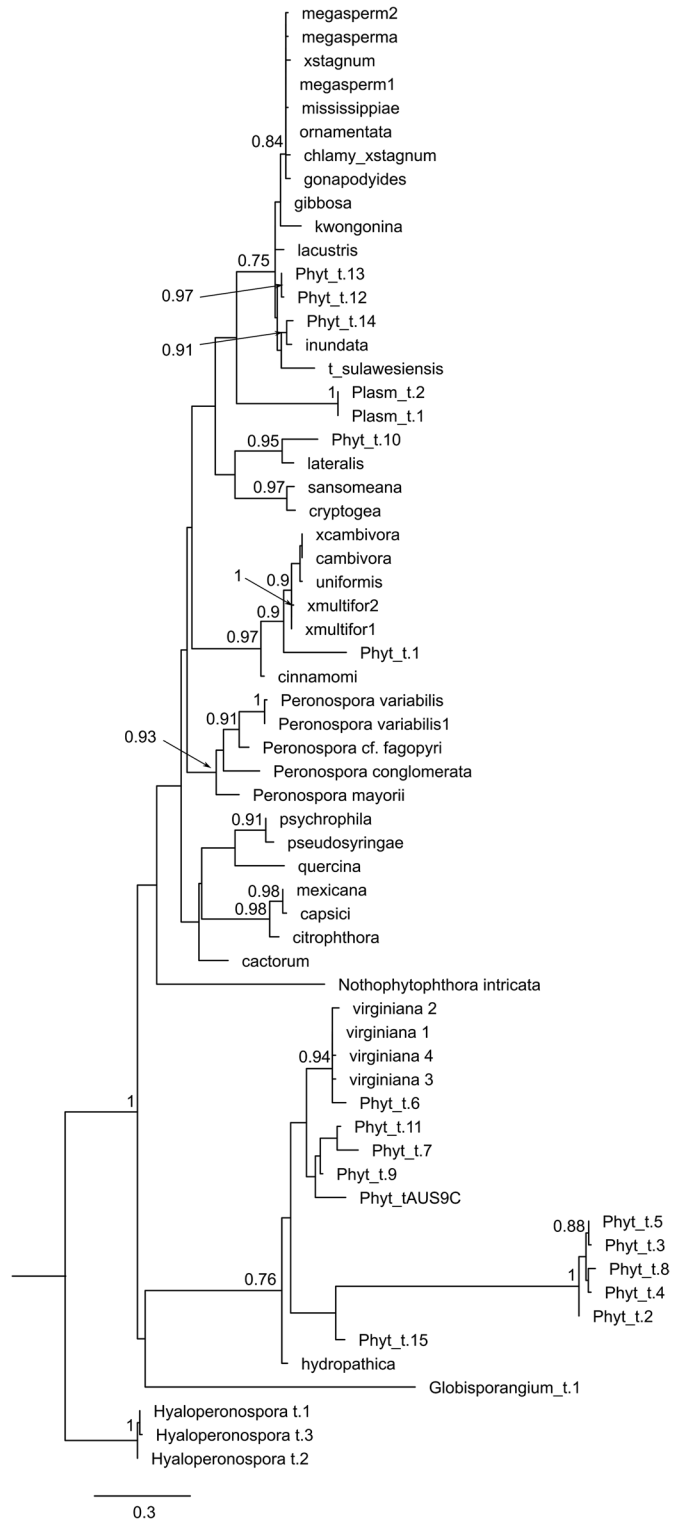
the wider environment (Brasier et al., 2022; Frankel et al., 2020; Jung et al., 2016, 2018a; Pérez-Sierra & Jung, 2013) this study was focused on forest and ornamental nurseries in Czech Republic.

Using both traditional and metagenomic approaches, in total 50 *Phytophthora* taxa and many taxa from other oomycete genera were found in soil and water samples in seven of the eight nurseries tested. Many of the known *Phytophthora* and other oomycete taxa detected in this study, including the wide-host range pathogens *P. cactorum*, *P. cryptogea*, *P. multivora*, *P. plurivora*, and *P. ×cambivora*, were expected as they had been reported previously in Czech Republic and many European countries. For example, *P. ×cambivora* has been known to occur in the Czech Republic since 2008 (Černý et al., 2008). While Redondo et al. (2018) reported this species to be more common in nurseries than forests in Sweden, where the pathogen prefers managed (anthropogenic) forests, it is one of the main drivers of the devastating oak, beech and chestnut declines in Austria, Germany, Italy and other European countries (Cech & Jung, 2005; Corcobado et al., 2020; Jung, 2009; Jung et al., 2000, 2013a, 2018a, 2019; Milenković et al., 2012; Schmitz et al., 2009; Stepniewska & Dłuszyński, 2010; Telfer et al., 2015; Vettraino et al., 2002, 2005). Due to its presence in Czech nurseries, outbreaks of the diseases caused by *P. ×cambivora* can be expected also in the Czech Republic. Also *P. plurivora*, a wide-host range pathogen involved in European

oak and beech declines with a widespread distribution in European nurseries (Corcobado et al., 2020, 2023; Jung et al., 2016, 2018a, 2019, 2024; Jung & Burgess, 2009; Mora-Sala et al., 2022), has previously been reported from Czech Republic (Mrázková et al., 2011, 2013). *Phytophthora cinnamomi*, arguably the most dangerous invasive *Phytophthora* pathogen with a global host range of more than 5000 plant species (Hardham & Blackman, 2018), was very prominent in our NGS study. It has occasionally been reported in the Czech Republic, mostly from *Rhododendron* and *Vaccinium* in ornamental nurseries and gardens (Černý et al., 2011, 2020; Mrázková et al., 2011). Common occurrence of the pathogen in irrigation water is probably a consequence of these records especially when filtration of contaminated water is not applied or not fully functional in the nurseries. In this study, *P. cinnamomi* was detected in both, non-filtered and filtered water. Model calculations indicate that ongoing climate changes will enable this pathogen to increase its activity in Central Europe (Burgess et al., 2017). This is also supported by recent outbreaks of *P. cinnamomi* in chestnut stands and blueberry plantations in Germany (Nechwatal & Jung, 2021; Peters et al., 2019).

However, the finding of the new species *P. transitoria* (Chen et al., 2022) and ITS1 DNA barcodes consistent with 15 novel *Phytophthora* taxa, five novel downy mildew species from the genera *Hyaloperonospora* and *Plasmopara* and several

Fig. 7 Phylogenetic position of sequences of *Phytophthora* taxa and other oomycete genera detected in this study. Numbers at branches indicate maximum likelihood bootstrap ≥ 0.75 , the bar indicates the number of expected substitutions per position



Phytophthora taxa with unknown, uncommon or geographically limited occurrence in Europe, i.e. *P. lateralis*, *P. virginiana*, *P. sansomeana*, *P. kwongonina*, *P. mississippiiae*, *P. gibbosa*/*P. gregata* and *P. taxon sulawesiensis*, was unexpected. This study constitutes the first report of *P. lateralis* in the Czech Republic, albeit based only on DNA barcode data. The species was detected with the second highest number of reads in NGS in four of the five tested plant species and water samples from nursery no. 3. The dominant hosts of *P. lateralis*, *Chamaecyparis* spp. and other *Cupressaceae*, are popular ornamentals in the Czech Republic and elsewhere in Europe. The pathogen was first reported in Europe from a nursery in France in 1999 where it was reported as having been eradicated (Hansen et al., 1999). However, ten years later severe outbreaks of root and collar rot and aerial infections of *Chamaecyparis* trees occurred in Brittany, France, the Netherlands and the UK (EPPO, 2011; Green et al., 2013; Jung et al., 2018a; Robin et al., 2011). The distribution of *P. lateralis* in the rest of Europe is unknown. The explanation for the absence of *P. lateralis* in our soil baiting tests may be the intolerance of the pathogen to the generally hot and dry continental climatic conditions in the summer. *Phytophthora virginiana* and several closely related taxa were detected in rivers and waterways in Portugal (Jung et al., 2023, unpublished data). The species was probably introduced to Europe from Asia, where it is widespread. *Phytophthora virginiana* was involved in hybridisation processes resulting in several hybrids with *P. virginiana* as one parent (Jung et al., 2017a, 2020). This species is classified as tolerant to high temperatures (Yang & Hong, 2013) which may provide an advantage for the summer temperatures in Czech Republic. *Phytophthora sansomeana* was reported previously from Europe in Croatia and Germany (Jung et al., 2016). Grígel et al. (2019) detected *P. sansomeana* also in a fruit orchard in Czech Republic. The species *P. gibbosa* and *P. gregata* are sister taxa of subclade 6b described from Western Australia (Jung et al., 2011). They cannot be discriminated using the ITS1 sequences but to date *P. gibbosa* has never been found outside Western Australia whereas *P. gregata* has been reported from the Czech Republic by Černý et al. (2011) and Grígel et al. (2019). Known only from waterbodies in the USA, *P. mississippiiae* is closely related to *P. ornamentata* described by Scanu et al. (2015) in Sardinia.

Similarly, *P. kwongonina* which was first reported from Australia (Burgess et al., 2018) is closely related to *P. rosacearum* that was already reported from the Czech Republic by Grígel et al. (2019). Both species are closely related and have identical or highly similar ITS1 sequences, hence they cannot be distinguished (Burgess et al., 2017) by the ITS1 barcode used in this study. The informally named *P. taxon sulawesiensis* from Clade 6 was identified in only one soil sample from *Chamaecyparis* in this study. This species was abundantly detected in Indonesia and Japan (T. Jung, M. Horta Jung and I. Milenković, unpublished results) but it has not yet been detected in the Czech Republic or elsewhere in Europe. The presence of *P. capsici* or *P. glovera*, which also cannot be discriminated using the ITS1 barcode, was surprising. They are soilborne pathogens associated with vegetable crops, mainly *Capsicum* spp. and tobacco (Jung et al., 2024). Although not reported in forest nurseries, the ITS1 barcode of *P. capsici*/*P. glovera* has been reported from UK forests (Landa et al., 2021) and it cannot be excluded that an unknown sister taxon of *P. capsici* and *P. glovera* is occurring in European habitats.

The records of both known and novel non-native *Phytophthora* species in Czech nurseries in this study further illustrate problems of European plant biosecurity (Brasier, 2008; Jung et al., 2016, 2018a, 2024). The European plant biosecurity system primarily relies on visual inspection of plants for the presence of symptoms caused by listed quarantine organisms. However, the widespread use of fungicides and fungistatic chemicals in nurseries suppresses the development of disease symptoms making the detection of asymptomatic pathogens by visual inspections impossible (Brasier, 2008; Jung et al., 2016; Jung & Blaschke, 2004; Pérez-Sierra & Jung, 2013). Nursery no. 3 imports most of its plants from foreign nurseries and the consequences of this practice are manifested by a high diversity of *Phytophthora* spp. identified in this study. Other nursery surveys demonstrated *Phytophthora* presence in visually healthy trees with EU plant passports obtained from producers in Netherlands or Germany (Jung et al., 2016; Rossmann et al., 2021). Husson et al. (2007) also demonstrated that plants infected by *P. ramorum* were imported to France from the Netherlands while Ginetti et al. (2015) reported *P. pachypleura* from *Aucuba japonica* plants imported by Italian nurseries from France. The

potential solution of these problems could involve regulation of pathogen routes and employing risk-based inspection procedures carried out by a sufficient number of trained personnel using advanced molecular detection techniques (Jung et al., 2016). These protocols are recommended for implementation both at entry points to countries and in nurseries to decrease the risks associated with the introduction and spread of both known and unknown potential pathogens to Europe (Jung et al., 2016).

DNA metabarcoding is an increasingly popular technique in current microbial diversity research (Taberlet et al., 2012) and also used in freshwater habitats (Matsuoka et al., 2022). In this study, the spectrum of *Phytophthora* spp. detected by both baiting and barcoding techniques differed considerably which is in common to other similar studies (Catalá et al., 2015; Bose et al., 2018; Riddell et al., 2019; Landa et al., 2021; Sarker et al., 2023a). Sarker et al. (2023a) and Burgess et al. (2017) mention that metabarcoding typically reveals a higher diversity of species in an environmental sample compared to that identified by isolation methods. This difference was also confirmed in this study. This is certainly caused by the fact that species with low levels of inoculum are outcompeted by those with higher levels. In addition, the environmental conditions during baiting are likely to bias the detection rate of all species present (Redekar et al., 2019). This may explain why there were generally fewer species detected by soil and water baiting than by DNA metabarcoding. However, occasionally baiting tests reveal the presence of *Phytophthora* taxa not detected with metagenomic approaches (La Spada et al., 2022; Sarker et al., 2023a). In this study, *P. plurivora* was the most common *Phytophthora* species isolated from soil samples by baiting but was not detected by metabarcoding of soil. Also *P. cactorum*, *P. lacustris* and *P. syringae* were not detected by DNA metabarcoding. There is no single clear explanation for this discrepancy but uneven scattered distribution of inoculum in soil in combination with the – compared to baiting – low volume of soil used for metabarcoding is a likely explanation. Ruiz Gómez et al., 2019 detected *P. plurivora* as the most common *Phytophthora* species in holm oak stands in Andalusia (Spain) using a similar NGS method. Also, Rossmann et al. (2021) detected *P. plurivora* in the soil of nursery plants using the same methodology of DNA extraction and DNA

metabarcoding. Therefore, the failure to detect *P. plurivora* using NGS in our study is probably not caused by any methodological failing such as PCR primer specificity. In this study, the absence of *P. lacustris* in the DNA barcoding data from water samples was particularly unexpected given its frequent detection with baits and the reported detection of this species in similar NGS studies (Catalá et al., 2015; Landa et al., 2021). It appears that in our metabarcoding test competition within the PCR with other highly abundant taxa such as *P. cinnamomi* and *P. lateralis* may have outcompeted amplification of *P. lacustris* barcodes in the water samples.

Phytophthora species with resting spores (oospores, chlamydospores) that germinate slowly or remain dormant are difficult to isolate using baiting tests, but their DNA is available for detection using metabarcoding (Sarker et al., 2023a). However, the detection of eDNA by metabarcoding confirms the presence of an organism but its viability remains unclear since DNA of a dead organism can also be detected (Burgess et al., 2017). In contrast to metabarcoding approaches, traditional isolation methods produce living isolates confirming that an organism is alive and enabling further studies on the taxonomy, ecology, pathogenicity and control of *Phytophthora* pathogens (Sarker et al., 2023a). Overall, although baiting is a slower and less effective method, it is still necessary to use it together with NGS methods due to the potential detection of taxa not found with NGS, the availability of living isolates for further studies and the knowledge that detected taxa are alive.

Water sourced from rivers, reservoirs and ponds can pose a substantial threat for pathogen dispersal to nurseries. (Pettitt, 2017). Numerous studies have demonstrated an ubiquitous high diversity of *Phytophthora* species and other oomycetes in rivers and streams in Australia, the Americas, Asia, Africa and Europe (e.g. Caballol et al., 2024; Catalá et al., 2015; Corcobado et al., 2023; Hüberli et al., 2013; Jung et al., 2017a, 2018b, 2019, 2020, 2022, 2024; Oh et al., 2013; Reeser et al., 2011; Seddaiu et al., 2020; Shrestha et al., 2013). Previous research confirmed the presence of *Phytophthora* species in nursery irrigation water using baiting tests. Themann et al. (2002) found a wide range of *Phytophthora* species in recirculation systems of ornamental nurseries in Germany. Rytönen et al. (2008) reported detection of

P. cactorum in Finnish nurseries by baiting with *Rhododendron* leaves. Mrázková et al. (2011) detected *P. gonapodyides* by baiting from irrigation water in an ornamental nursery of Czech Republic. However, single sampling by baiting cannot reveal the total diversity of oomycetes. In metagenomic approaches, filtering of water samples is often the first step before the DNA extraction and since it can allow a larger volume of water to be examined than from baiting it has been shown to enable detection of a wider range of species diversity (Marčiulynas et al., 2020, Rodríguez-Padrón et al., 2019, Matsuoka et al., 2022).

Slow sand filters appear to be a promising method for decreasing the numbers of pathogen propagules in irrigation water (Kubiak et al., 2015; Ufer et al., 2008). However, our results suggested that in the tested nursery the filtration of water using sand filters does not eliminate oomycetes sufficiently from infested irrigation water. Interestingly, we detected higher diversity of oomycete species (27 species) in the water after filtration than in the river (9 species) which is the source of the irrigation water. Although there were four samples taken after filtration and only one sample taken from the river, at least 15 species were detected in each of the filtered water samples. Therefore, the efficiency of these filters to prevent dispersal of *Phytophthora* pathogens is questionable. More detailed investigations are required but it seems probable that the sand filters in this nursery contain a population of *Phytophthora* propagules and are not sufficient for removing *Phytophthora* spores from the water. Because surplus irrigation water is recycled and reused after watering, the greater diversity of pathogens in the nursery irrigation system is not entirely surprising. In agriculture and horticulture, slow sand filters are used commonly to efficiently eliminate pathogen propagules from water more economically in comparison to other techniques (e.g. ozonation, thermal and chemical treatments or UV radiation) (Runia et al., 1997; Ufer et al., 2008; Van Os et al., 2000). The crucial factor for the functionality of slow sand filters is, however, the cleaning and regular maintenance of these filters. Therefore, it seems possible that the staff of the respective nursery did not clean and maintain the filters as recommended by the scientific literature.

In this study, both methods (DNA metabarcoding and soil baiting) confirmed that the forest nursery (nursery no. 8) had much lower *Phytophthora*

diversity (2 taxa) than the ornamental nursery (nursery no. 3; 32 taxa) which regularly imports a high volume of plant material. The import of a host species, the transport via transport vector, or natural expansion from adjacent regions are recognized as three principal ways of non-native species introduction (Hulme et al., 2008). Therefore, native or long-established introduced *Phytophthora* species are expected to be dominant in forest nurseries with local production of plant material whereas non-native *Phytophthora* species and sometimes even novel *Phytophthora* species are more likely to occur in ornamental nurseries with regular import of nursery stock. Liebhold et al. (2012) noted that ca 70% of problematic forest pests and pathogens occurring in the US between 1860 and 2006 are probably associated with imported live plants. Similarly, Jung et al. (2016) hypothesized that the import of living plants for planting from overseas is responsible for most, if not all, introductions of exotic *Phytophthora* species to Europe and suggested a pathway regulation approach and phytosanitary inspections based on modern high-throughput metagenomic approaches instead of visual inspections for potential symptoms of listed quarantine organisms.

Conclusion

This study demonstrates the widespread presence of 50 *Phytophthora* taxa and other oomycete genera in plant nursery stock in the Czech Republic. Only 24% of the detected *Phytophthora* species were recovered by baiting while 90% were detected by eDNA metabarcoding. Only 7 species were detected by both techniques but usually from different samples. *Phytophthora cinnamomi* and *P. lateralis* were particularly common as revealed by DNA metabarcoding but they were not obtained by baiting. Therefore, both tested detection methods complement each other. Some species can be more easily isolated by baiting, but they do not necessarily have to be the most abundant species. DNA metabarcoding revealed that an ornamental nursery with regular imports of plants had much higher diversity of *Phytophthora* (32 taxa) than a forest nursery with exclusively local production (2 taxa) highlighting the biosecurity risk posed by plant imports. Results revealed that water filtration using sand filters does not prevent the occurrence of oomycetes in

irrigation water if these filters are not maintained properly. The results of metabarcoding revealed numerous unexpected and undescribed oomycete species. It is evident that in plant biosecurity the use of both traditional and modern metabarcoding techniques is necessary to detect all known and unknown pathogens.

Acknowledgements The authors are grateful to all nursery owners for their collaboration in collecting soil and water samples and providing information about nurseries. Matěj Pánek (Institute of Microbiology, Czech Academy of Sciences, Prague) and Miloň Dvořák (Mendel University in Brno) are acknowledged for supplying several soil samples for this research. The authors acknowledge the Research/Scientific Computing teams at The James Hutton Institute and NIAB for providing computational resources and technical support for the “UK’s Crop Diversity Bioinformatics HPC” (BBSRC grant BB/S019669/1), use of which has contributed to the results reported within this paper. The authors are grateful to Peter Hedley and Jenny Morris at The James Hutton Institute for sequencing services.

Author contributions All authors contributed to the study conception and design. Material preparation and samples collection and processing were performed by A. Bačová, I. Milenkovič, T. Májek, Z. Nagy and T. Corcobado. Sample Analysis - A. Bačová, Z. Nagy, E. Randall, B. Keillor, M. Horta Jung, and M. Tomšovský. Data analysis, software, data curation - P. Cock and D. Cooke. Writing - review and editing - M. Tomšovský, D. Cooke, and T. Jung. The first draft of the manuscript was written by A. Bačová, and all authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding Open access publishing supported by the National Technical Library in Prague. The work of the team from Mendel University in Brno (MENDELU) was supported by the European Regional Development Fund, Project *Phytophthora-Research Centre Reg. No. CZ.02.1.01/0.0/0.0/15_003/0000453 and the internal MENDELU project IGA LDF_VP_2019019. Staff at The James Hutton Institute acknowledge funding from The Rural and Environment Science and Analytical Services Division of the Scottish Government.*

Data availability Data are available in a publicly accessible repository. The raw Illumina data presented in this study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.10000121> as FASTQ files.

Declarations

Conflict of interest The authors declare no conflict of interest.

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