



Validation of a metabarcoding-based screening method for detecting fungal pathogens in conifer seeds using Illumina MiSeq and the FungiSearch pipeline

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Received: 22 June 2025 / Accepted: 8 May 2026
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Abstract To detect pathogenic fungi in conifer seed lots from different continents, we applied DNA metabarcoding targeting the fungal ITS2 region using the MiSeq platform and analyzed the sequences with the FungiSearch pipeline developed in a previous study. Analysis of a mock community comprising twelve fungal species showed that the method had a detection limit comparable to that of a species-specific PCR. All twelve species were detected with high repeatability. However, the method proved to be non-quantitative, as relative abundances were under- or over-estimated depending on the fungal species. Analysis of seed batches showed that the number of biological replicates influenced fungal community diversity.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-026-03249-8>.

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Several pathogenic fungal species were detected: some were known to be associated with conifer seeds, whereas others were not. Pathogenic fungi emerging in Europe were also detected. Specific molecular tests (PCR or qPCR) were performed on DNA from different seed batches to target eight of these pathogens. All the pathogens detected by high-throughput sequencing were also detected by specific molecular tests, although detection rates varied slightly between the two methods. Based on these results, the high-throughput sequencing method validated in this study could be proposed as a screening test detecting fungal pathogens in conifer seeds. Positive detection should be confirmed with specific tests, particularly for fungi not previously reported in Europe, or with limited distribution.

Keywords Biosecurity · Diagnostic · Emergent disease · Forest · FungiSearch · Illumina

Introduction

In northern Europe, coniferous forests are largely regenerated using seedlings produced in nurseries. In many forest tree species, seed production shows strong year-to-year variability influenced by climatic cues, and climate change is expected to alter these cues, increasing the frequency of years with low or failed seed production (Pearse et al., 2017). Consequently, local seed supplies may not always

be reliable. Additionally, assisted migration of provenances is considered a climate change adaptation strategy (Twardek et al., 2023), indirectly promoting the supply of seeds from non-local populations better adapted to changing conditions. Foresters are also evaluating non-native conifers that are more drought-resistant and for which local seed sources are unavailable. Finally, the ornamental sector drives demand for exotic conifer seeds. Consequently, international trade in conifer seeds is expanding and becoming increasingly diversified (Franic et al., 2023). Tree seeds are generally considered to pose a low risk of fungal pathogen spread, and phytosanitary control of this material remains limited (Franic et al., 2024). This underestimation is apparent in the current European phytosanitary framework, notably in Regulation (EU) 2019/2072 implementing Regulation (EU) 2016/2031, which establishes protective measures against organisms harmful to plants but only includes very limited specific measures regarding pathogens associated with conifer seeds (European Commission, 2019). However, several studies have demonstrated that conifer seeds can transmit fungal pathogens (Brodal et al., 2020; Carey et al., 2005; Talgo et al., 2010; Zamora-Ballesteros et al., 2019). Therefore, the risk of introducing invasive fungal pathogens into new geographical areas through international trade in tree seeds is real and warrants greater attention to protect this valuable forestry resource.

As described in ISPM 38 (FAO, 2021), seed-borne fungal pathogens can infect seeds either internally or externally. They can be vertically transmitted to the plant growing from the seed or released into the environment, potentially infecting new plants (Martin et al., 2022). In addition to damping-off, which can cause seedling emergence failure or decay of young seedlings, damage can also occur at later stages of plant development (Wingfield et al., 2008; Zamora-Ballesteros et al., 2019). Beyond the biosecurity risk, the economic impact of seed-borne fungal pathogens is therefore a concern for seed suppliers, nursery workers, and foresters.

Several fungal pathogens can be present in the same batch of seeds, and infected seeds are generally symptomless (Franic et al., 2019; Mancini et al., 2016). For these reasons, specific molecular tests (conventional or real-time PCR), which generally detect only one known target at a time, are not appropriate. Non-specific diagnostic methods capable

of simultaneously detecting a wide range of pathogens are therefore preferred. Culture-based isolation techniques remain the gold standard for identifying seed-borne fungi. However, they require considerable taxonomic expertise. These methods are also time-consuming and labor-intensive, as seeds must be analyzed individually, and hundreds of seeds are required for a representative sample (FAO, 2008; ISTA, 2020). In addition, some pathogens require specific media for effective isolation (Decourcelle et al., 2015) while others, such as obligate biotrophs, cannot be cultivated. Finally, because some fungal pathogens are slow-growing or present at very low concentrations, their detection is often masked by fast-growing saprophytic fungi, resulting in a high risk of false negatives.

High-throughput sequencing (HTS), particularly metabarcoding, which can theoretically detect all the fungi in a sample, offers a means to overcome the limitations of culture-based methods. Recent studies using Illumina MiSeq have demonstrated its effectiveness for detecting fungi associated with tree seeds (Cleary et al., 2019; Franic et al., 2019; Mittelstrass et al., 2025). However, HTS is not routinely applied for the detection of plant pathogenic fungi. A major challenge is that the quality of HTS results depends on reference databases, which are not regularly updated and may lack sequences from newly described pathogens or contain non-curated sequences (Winand et al., 2025). Another challenge is the choice of metabarcoding primers which are not always specific enough—leading to co-amplification of plant DNA—or may provide insufficient coverage, failing to detect some fungal species. The selected marker, typically the ITS region for fungi, can also lack specificity as identical sequences may correspond to different fungal species (Aragona et al., 2022; Piombo et al., 2021; Tedersoo et al., 2019). Finally, processing the large volumes of sequence data generated often requires powerful computing resources and bioinformatics expertise (Olmos et al., 2018).

The aim of this study was to evaluate a metabarcoding approach using Illumina MiSeq technology and the FungiSearch pipeline, as developed by Chandelier et al. (2021), for detecting fungal pathogens associated with conifer seeds. Two main questions were addressed: (1) whether the observed relative abundance of fungal species within a sample is influenced by its composition, and (2) whether the method

can be proposed as a pre-screening tool for detecting fungal pathogens absent from Europe or with limited distribution.

Materials and methods

Conifer seeds

Twenty-nine seed batches were collected from various locations in Europe and other continents (Asia, North America) (Table 1). The seed lots were purchased from the four main suppliers of conifer seeds used by Belgian nursery growers. They were selected

to represent a diversity of botanical genera and geographical origins. Upon receipt, the seeds were stored at 2–8 °C. All seed lots were analyzed using HTS without prior surface disinfection, to account for the potential presence of pathogenic fungal species on the seed surface, as described for *Fusarium circinatum* (Evira-Recuenco et al., 2015), a species regulated at the European level (Regulation (EU) 2019/2072) .

Preparation of DNA from conifer seeds

There were three biological replicates of 100 seeds per sample and two DNA extractions per replicate. DNA extractions were obtained from ~50 mg ground

Table 1 List of seed lots used in this study

#	Origin	Conifer species	Supplier	Year of harvest
1	Turkey	<i>Abies nordmanniana</i> <i>var. bornmuelleriana</i>	1	2021
2	Spain	<i>Abies cephalonica</i>	2	na
3	USA	<i>Abies concolor</i>	2	na
4	USA	<i>Abies fraseri</i>	1	2019
5	USA	<i>Abies grandis</i>	2	na
6	USA	<i>Abies lasiocarpa</i>	1	1997
7	Russia	<i>Abies nordmanniana</i>	1	2020
8	Russia	<i>Abies sibirica</i>	1	2016
9	USA	<i>Calocedrus decurrens</i>	2	na
10	France	<i>Cedrus atlantica</i>	2	na
11	India	<i>Cedrus deodara</i>	2	na
12	Turkey	<i>Cedrus libani</i>	1	2020
13	Denmark	<i>Chamaecyparis lawsoniana</i>	1	2009
14	Asia	<i>Cryptomeria japonica</i>	3	na
15	Italy	<i>Hesperocyparis arizonica</i>	2	na
16	USA	<i>Metasequoia glyptostroboides</i>	2	na
17	USA	<i>Picea engelmannii</i>	1	2013
18	Denmark	<i>Picea omorika</i>	1	1990
19	Spain	<i>Picea orientalis</i>	2	na
20	USA	<i>Picea pungens</i>	1	2014
21	Denmark	<i>Picea sitchensis</i>	1	2019
22	France	<i>Picea sitchensis</i>	4	na
23	France	<i>Pinus brutia</i>	4	na
24	Serbia	<i>Pinus heldreichii</i>	1	2019
25	Bulgaria	<i>Pinus sylvestris</i>	2	na
26	Asia	<i>Pinus thunbergii</i>	3	na
27	USA	<i>Sequoia sempervirens</i>	2	na
28	Denmark	<i>Thuja occidentalis</i>	1	2019
29	Denmark	<i>Thuja plicata</i>	1	2021

Supplier indicates a number corresponding to a specific seed supplier from whom the seed lots were obtained. *na* not available

seeds using the DNeasy Plant Pro Kit (QIAGEN) as recommended by Franic et al. (2019). To evaluate the quality of the DNA (i.e., absence of degradation), 5 µl of DNA extract was analyzed by electrophoresis (0.9% agarose gel in TAE buffer). DNA quantification and purity were determined at 260 nm and 280 nm using a Biospectrometer (Eppendorf).

Mock communities of fungal DNA for HTS

Twelve fungal isolates were used to prepare the mock communities (MCs) serving as positive and negative controls for HTS. Ten of these isolates corresponded to species commonly associated with conifer seeds, while two additional fungal isolates are not present in Belgium (quarantine organisms in Europe) and are not associated with conifer seeds (Table 2). These two isolates, *Phyllosticta citricarpa* and *Elsinoe australis*, which infect *Citrus* species, were used as alien controls as recommended by Massart et al. (2022). To confirm the identity of the isolates used in the MCs, Sanger sequencing was done with primers ITS5 and ITS4 (White et al., 1990) on DNA extracted from pure culture. DNA yield and purity were determined by measuring the absorbance at 260 nm and 280 nm. The different DNA extracts were mixed at a final concentration of 100 pg per PCR except for five “target” fungal isolates (*Fusarium circinatum*, *Neonectria neomacrospora*, *Diplodia sapinea*, *E. australis*, and *P. citricarpa*) for which four DNA concentrations

were tested (1 ng, 100 pg, 10 pg, and 1 pg/PCR) to evaluate the analytical sensitivity of the HTS method and its ability to provide relative abundance of fungal species within a sample (Table 2). The choice of working on variable concentrations for 5 of the 12 targets in the community (and not on all 12 targets) was considered to mimic real-life conditions where targets are present in variable concentrations in the sample.

PCR with high coverage primers and HTS analysis

PCR reactions were carried out in a final volume of 25 µl using 5 µl DNA at 10 ng/µl with a proofreading DNA polymerase (Kapa HiFi HotStart ReadyMix, Roche Diagnostics) and the high coverage primers 58A2F and ITS4 targeting ITS2 (Toju et al., 2012) linked to Illumina adapters (NGS_58A2F: TCGTCG GCAGCGTCAGATGTGTATAAGAGACAGATC GATGAAGAACGCAG; NGS_ITS4: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GT CCT CCG CTT ATT GAT ATG C) used for 300 nM. The ITS region was chosen because it offers numerous advantages as a DNA barcode compared to other regions of the genome (Kausserud, 2023). The ITS2 region was preferred over the ITS1 region for its greater accuracy in fungal taxonomic assignment using Illumina sequencing (Winand et al., 2025). The thermal regime consisted of a first denaturation at 95 °C for 3 min, followed by 25 cycles at 98 °C for 20 s, 60 °C for 20 s, and 72 °C for 40 s. A final

Table 2 Amounts of fungal species DNA (in pg/PCR) used in the four mock communities (MC1 to MC4)

Fungal species	Order	MC1	MC2	MC3	MC4
<i>Fusarium circinatum</i> ^a	Hypocreales	1000	100	10	1
<i>Neonectria neomacrospora</i> ^b	Hypocreales	1000	100	10	1
<i>Diplodia sapinea</i> ^b	<i>Incertae sedis</i>	1000	100	10	1
<i>Sclerotinia sclerotiorum</i> ^b	Helotiales	100	100	100	100
<i>Sydowia polyspora</i> ^b	Dothideales	100	100	100	100
<i>Sirococcus conigenus</i> ^b	Diaporthales	100	100	100	100
<i>Epicoccum nigrum</i> ^b	Pleosporales	100	100	100	100
<i>Trichoderma paraviridescens</i> ^b	Hypocreales	100	100	100	100
<i>Chaetomium globosum</i> ^b	Sordariales	100	100	100	100
<i>Trichothecium roseum</i> ^b	Hypocreales	100	100	100	100
<i>Phyllosticta citricarpa</i> ^c	Botryosphaeriales	1000	100	10	1
<i>Elsinoe australis</i> ^c	Myrangiiales	1000	100	10	1

^a = Professor Matteo Garbelotto (UC Berkeley, USA); ^b = CRAW collection (Belgium); ^c = CBS-KNAW collection (The Netherlands)

Species highlighted in grey were included at different concentrations to evaluate the analytical sensitivity of the HTS method

extension was carried out at 72 °C for 5 min. The PCRs were carried out on a T100 thermocycler (Bio-Rad Laboratories N.V.). A negative control (sterile water) was introduced in all PCRs, and contamination was verified by the absence of bands in the negative control after electrophoresis on a 1.5% agarose gel. One PCR was carried out on each DNA extract, and the two PCRs from the same biological replicate were pooled. The pooled PCR products were purified using the PureLink PCR purification kit (Thermo Fisher Scientific). The purified PCR products were then analyzed using a 4150 Tape Station and the D1000 kit (Agilent Technologies) to determine their concentration and size. Library construction and Illumina sequencing (96 reactions per HTS run) were carried out at Macrogen company using the Illumina MiSeq platform with the v3 chemistry (2×300 pb).

Bioinformatic analysis of the data

FastQ files were analyzed on a personal computer with the FungiSearch pipeline (Chandelier et al., 2021) based on USEARCH (Edgar, 2010). Paired-end reads were merged with USEARCH *-fastq_merge-pairs*. Part of the primers corresponding to the first 17 bp were removed with the USEARCH *-fastx_truncate*. Quality filtering, searches for unique read sequences, and clustering into amplicon sequence variants (ASVs) were carried out with the USEARCH *-fastq_filter*, *-fastx_unique*, and *-unoise3* algorithms (Edgar, 2016; Edgar & Flyvbjerg, 2015). One sample corresponding to one of the three biological replicates of lot 19 (Table 1) yielded too few reads and was excluded from the analysis. After excluding this sample, the number of filtered reads ranged from 14,043 to 105,790 depending on the seed sample (average = $62,958 \pm 22,303$). Rarefaction curves of the number of Amplicon Sequence Variants (ASVs) against the number of reads reached the plateau phase for all mock communities and seed samples, indicating a sufficient sequencing depth for all samples (data not shown). For the taxonomic assignment, a normalized sequencing depth of 10,000 reads and a read cut-off of 0.05% were applied to all samples in the FungiSearch pipeline. A BLAST analysis was carried out on ASV files using a reference database created on October 9, 2022 (which consisted of fungal ITS DNA sequences corresponding to rDNA extracted from NCBI) and the options of BLAST+*perc-identity*

(percentage identity)=99.5%; *Qcov-hsp-perc* (query coverage high-scoring sequence pair percent)=85%; *Max-target-seqs* (maximum number of aligned sequences to keep)=1000. Two parameters calculated by the FungiSearch pipeline for each fungal species identified were used for the analysis: the percentage of reads and the Hit Proportion (HP value). This last parameter corresponds to the ratio between the number of accessions for the assigned fungal species and the total number of accessions for the DNA sequence with a percentage of identity $\geq 99.5\%$ and a query coverage $\geq 85\%$ in the reference database (Chandelier et al., 2021). The best BLAST+hit (lowest E-score) is used to assign the species, and the HP value can be used to evaluate the quality of this assignment. The programs of the FungiSearch pipeline (version 2 with the reference database created on October 9, 2022, and an updated version of the EPPO list used to alert on presence of fungi placed on EPPO A1, A2, or Alert lists) and the procedures to use the pipeline are provided as a public figshare repository (https://figshare.com/articles/online_resource/FungiSearch_V2/28130825). Raw HTS data generated in this study were deposited in the NCBI Sequence Read Archive database under Bioproject PRJNA1190857.

Statistical analysis of the data

All statistical analyses were carried out using R software (R Core Team, 2024) and the packages Vegan (Oksanen et al., 2022), Phyloseq (McMurdie & Holmes, 2013), ggplot2 (Wickham, 2016) and dplyr (Wickham et al., 2023). To evaluate the limit of detection and the linearity of the HTS method, expected read percentages were calculated from the DNA concentrations put in each MC. Expected and observed quantities were log-transformed and compared using scatterplots and linear regression. The repeatability of the HTS method between biological replicates was evaluated by fitting a random effect model with the fungal species, the expected percentage of reads (“treatment”), and their interaction as random effects and by computing the intra-class correlation coefficient (ICC) as the ratio of the sum of the variance explained by each component and the total variance. The impact of sampling effort on fungal diversity detected by HTS was evaluated by quantifying how observed species richness increased with the number of biological replicates. Although

species accumulation curves and rarefaction/extrapolation frameworks (e.g. iNEXT) are standard for estimating asymptotic richness and optimal sample size, the availability of only three biological replicates per seed lot prevented robust model fitting. Therefore, the analysis focused on the marginal gains in detected diversity. For 28 of the 29 seed lots, three biological replicates (R1, R2, R3) were available. Because the order in which replicates are analysed is arbitrary, all unique replicate order permutations (R1–R2–R3, R2–R3–R1, and R3–R1–R2) were considered. For each permutation, cumulative fungal species richness was calculated after one replicate (first replicate in the permutation), after two replicates (cumulative richness of the first two replicates), and after three replicates (cumulative richness across all three replicates). Species richness values for one and two replicates were then averaged across permutations, while richness for three replicates was identical across permutations. For each seed lot, differences in observed species richness between replicate numbers were computed, and the null hypothesis that the median within-lot difference equals zero was tested using one-sided paired Wilcoxon signed-rank tests. Based on a literature study, all fungal species identified by HTS analysis were manually categorized as conifer pathogen, plant pathogen, or other (non-plant pathogenic species or species with unknown function). A heat map showing the detection frequency with HTS across different biological replicates of each seed lot was created using data extracted for conifer pathogens.

Specific PCR tests

Molecular tests described in the literature were applied on positive samples obtained with the HTS method for the species *Fusarium circinatum* (Ioos et al., 2009), *Phyllosticta citricarpa* (Van Gent-Pelzer et al., 2007), *Elsinoe australis* (Ahmed et al., 2019), *Botrytis cinerea* (Belen Suarez et al., 2005), *Diplodia sapinea* (Smith & Stanosz, 2006), *Fomes fomentarius* (Pirronitto et al., 2024), *Neonectria neomacrospora* (Nielsen et al., 2019), *Rhizosphaera kalkhoffii* (Kihara et al., 2015), and *Sirococcus conigenus* (Smith & Stanosz, 2008). These tests are described in supplementary information S1. New tests were developed for the emergent species *Lasiodiplodia pseudotheobromae* and *Fusarium brachygibbosum*, as

specific molecular tests were not available for these fungi (EFSA et al., 2021; EFSA et al., 2023). Validation data for these two tests are available in supplementary information S2. Specific tests were carried out on the two DNA extracts from the three biological replicates used in HTS. The status of the seed lot (positive/negative for the specific fungal target) was defined regardless of the number of positive replicates with the specific molecular test. The PCRs were carried out at a volume of 25 µl using 5 µl of DNA from the extracts used for HTS. The GoTaq G2 Flexi DNA Polymerase (Promega) and a T100 thermocycler (Bio-Rad) were used for conventional PCRs. The FastGene 2×probe mix (Nippon Genetics Europe) and a StepOne Plus device (Thermo Fisher Scientific) were used for qPCRs.

Results

Evaluation of the HTS method on mock communities of fungi

Four mock communities (MC1, MC2, MC3 and MC4) consisting of twelve fungal species served as controls for the HTS experiment. Five of these species (“target species”) were used at four concentrations while seven species (“companion species”) were used at the same concentration in the four MCs. All twelve fungal species were detected at the species level. The expected and observed read percentages were compared by considering the proportion of DNA from each of the fungal species relative to the total DNA content of the MC. Regarding the five “target” species used for four DNA concentrations (Fig. 1a), a linear increase between the expected and observed read percentages was observed for only three species. For *P. citricarpa* and *E. australis*, the mean read percentages were higher in MC2 (100 pg in each PCR) than in MC1, despite MC1 containing a higher amount of DNA (1000 pg, see Fig. 1a). As a result, at the highest DNA amount (MC1), a tenfold difference in read percentages was observed between *Diplodia sapinea* (28.9%) and *Elsinoe australis* (3.2%), despite both being present in equal quantities (1000 pg of DNA, i.e., 17.54%) in this mock community. All species except *E. australis* were detected in all MCs, although read counts were very low in MC4, which contained the lowest DNA amount (1 pg per PCR).

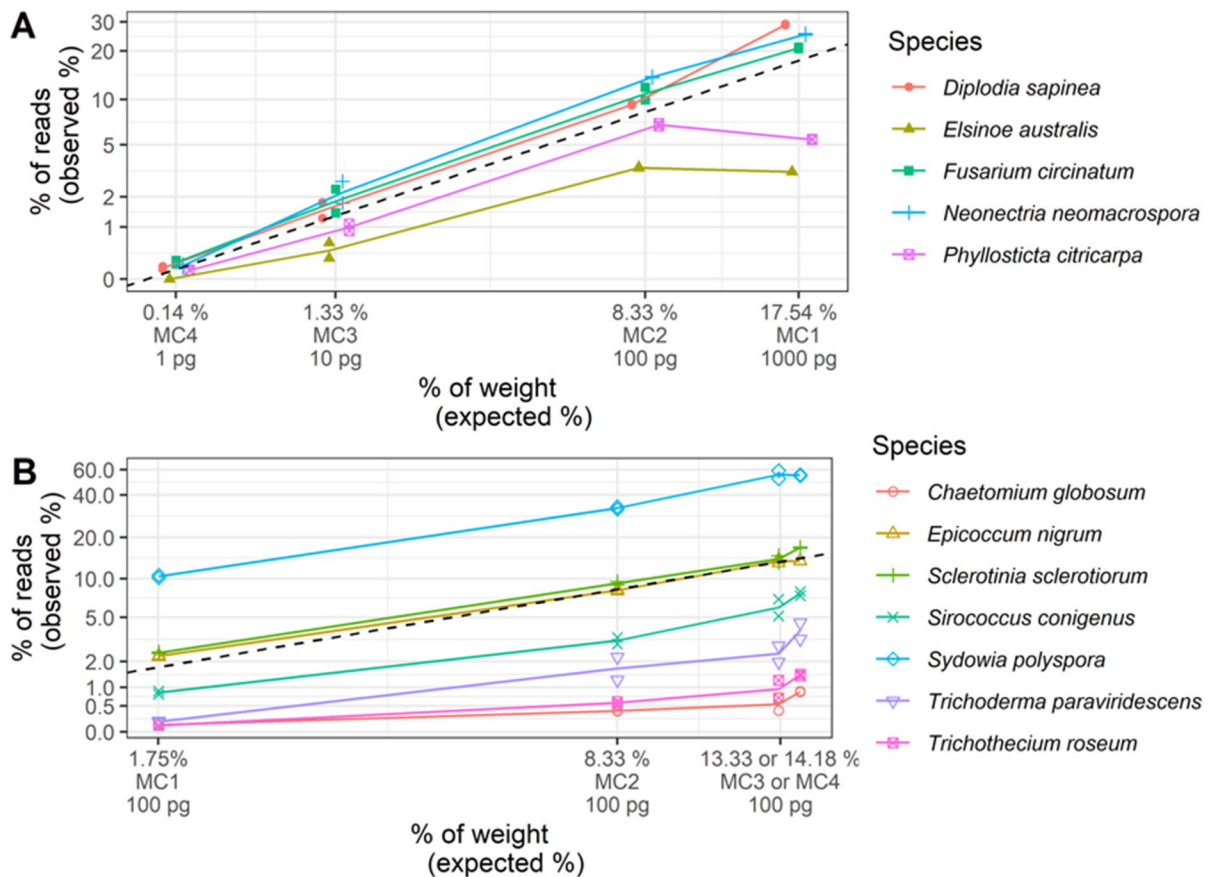


Fig. 1 Comparison of observed and expected mean read percentages (two replicates, log-transformed) obtained with Illumina MiSeq sequencing of DNA from 12 fungal species. **A:** results for the five “target” species diluted in mock communities (MC) at four concentrations. MC1, 1000 pg/PCR; MC2, 100 pg/PCR; MC3, 10 pg/PCR; MC4, 1 pg/PCR. **B:** results for

the seven “companion” species in the MC at 100 pg/PCR. The dashed line represents the 1:1 relationship between expected and observed values. Percentages on the X-axis indicate the proportion of DNA from each species relative to the total DNA content of the MC

Regarding the seven “companion” species used at 100 pg in the four MCs, the differences between species were even more striking, with some species being systematically under- or over-represented (Fig. 1b).

The two replicates were highly similar across the different fungal species and the four MCs (Fig. 1a and b). Comparing the read percentages between the replicates for each fungal species and each MC, revealed a strong correlation ($R^2=0.999$). In a random effects model including fungal species, expected read percentage, and their interaction, the percentage of explained variance yielded an intraclass correlation coefficient (ICC) of 0.996 (95% Confidence Interval,

0.991–0.998), indicating that read percentages were highly repeatable across replicates.

Specific PCR tests were also performed on the DNA extracts of the “target” species in the four MCs, which contained variable amounts of DNA for five fungal species (Table 2). All five species were detected in all cases with Cq values exceeding the fluorescence threshold for real-time PCR, and visible signal on gel for conventional PCR (data not shown). The detection limits of the five specific PCRs using DNA from pure cultures were 10 fg per PCR for *Fusarium circinatum* and *Phyllosticta citricarpa*, and 1 pg per PCR for *Elsinoe australis*, *Neonectria neomacrospora*, and *Diplodia sapinea*.

Application of the HTS method on conifer seed lots of different origins

Twenty-nine conifer seed lots were analysed using HTS, considering three biological replicates per lot except for one lot (lot 19) for which only two replicates were available. A total of 484 fungal species were detected, including 76.2% Ascomycota, 22.1% Basidiomycota, 1.4% Mucoromycota, and 0.2% Zygomycota. Ten classes of Ascomycota (Sordariomycetes, 34.4%; Dothideomycetes, 24.8%; Eurotiomycetes, 22.8%; Leotiomycetes, 8.7%; Saccharomycetes, 6.8%; Pezizomycetes, 0.8%; Chaetothyriomycetes, 0.8%; Lecanoromycetes, 0.5%; Orbiliomycetes, 0.3%; Xylonomycetes, 0.3%) and seven classes of Basidiomycota (Tremellomycetes, 45.8%; Agaricomycetes, 18.7%; Microbotryomycetes, 14%; Cystobasidiomycetes, 9.3%; Exobasidiomycetes, 5.6%; Wallemiomycetes, 3.7%; Ustilaginomycetes, 2.8%) were represented (supplementary information S3).

The fungal species richness increased monotonically with the number of biological replicates (Fig. 2a). On average, two replicates yielded 32.9% more species than a single replicate, while three replicates yielded 69.6% more species than one replicate. The addition of a third replicate further increased observed richness by 21.4% relative to two replicates, with gains observed in all seed lots (minimum increase = 10.7%, maximum = 34.6%; Fig. 2b). These within-lot increases in observed species richness were highly significant for all comparisons

(one-sided Wilcoxon signed-rank tests; $p < 2 \times 10^{-6}$). The total number of fungal species detected per seed lot ranged from 7 (*Abies lasiocarpa*) to 113 (*Sequoia sempervirens*).

Of the fungi detected, forty-eight were conifer pathogens. Their HP values ranged from 0.25 (25% of sequences corresponding to the assigned species in the reference database among all sequences with at least 99.5% identity and 85% coverage) to 1 (100% of sequences corresponding to the assigned species) (Table 3). For some species of the genera *Pestalotiopsis* or *Fusarium*, HP values were relatively low. Different HP values were sometimes observed for the same fungal species, either in the same sample or in different samples. For example, two different ITS sequences assigned to the species *Diplodia sapinea* were detected in *Pinus thunbergii* seed lot originating from Asia, one with a HP value of 1.00 and the other one with a HP value of 0.72. In contrast, only one sequence (the one with HP value = 0.72) was detected in the other positive seed lots for this pathogen. Two ITS sequences were also detected in the three seed lots infected with *Caloscypha fulgens*, both with a HP value = 1.00. One was present in seeds of *Abies lasiocarpa* and was assigned to the species *C. fulgens* f. sp. f. *caesia* in the reference database (accession number MK010996.1), while the other was present in the two other samples (*Abies nordmanniana* seed lot from Denmark, accession number OP178082.1).

A heatmap illustrating the fungal pathogens of conifers detected in the 29 seed lots analyzed was

Fig. 2 **A** Increase in the average total number of fungal species detected with the number of biological replicates, using Illumina MiSeq sequencing of DNA from conifer seed lots (100 seeds per replicate). Each line represents one seed lot. **B** Increase in detected species richness expressed as a percentage according to the number of biological replicates compared (3 versus 1, 2 versus 1 and 3 versus 2 biological replicates)

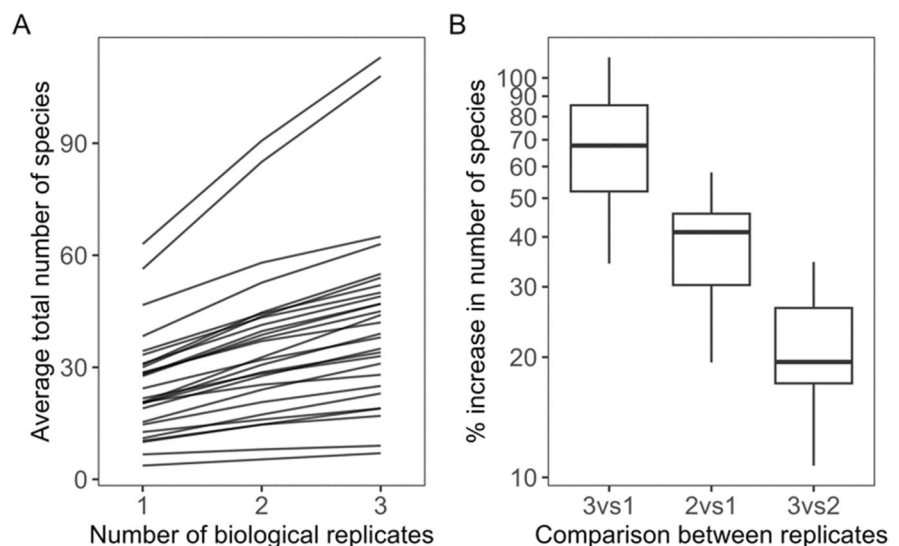


Table 3 HP values for the fungal species identified as conifer pathogens

Species	HP values				
	No. 1	No. 2	No. 3	No. 4	No. 5
<i>Allantophomopsiella pseudotsugae</i>	1				
<i>Botryosphaeria dothidea</i>	0.93				
<i>Botrytis cinerea</i>	0.90				
<i>Caloscypha fulgens</i>	1	1			
<i>Curvularia spicifera</i>	0.61				
<i>Cyclaneusma minus</i>	1				
<i>Cytospora piceae</i>	1				
<i>Cytospora cedri</i>	1	0.4			
<i>Diaporthe eres</i>	0.95	0.74	0.71	0.69	0.57
<i>Diplodia sapinea</i>	1	0.72			
<i>Diplodia seriata</i>	0.98				
<i>Dothiorella gregaria</i>	0.64				
<i>Fomes fomentarius</i>	1				
<i>Fusarium avenaceum</i>	0.51	0.43			
<i>Fusarium lateritium</i>	0.51				
<i>Fusarium oxysporum</i>	0.98				
<i>Fusarium sambucinum</i>	0.98				
<i>Fusarium solani</i>	0.91	0.77			
<i>Herpotrichia juniperi</i>	1				
<i>Heterotruncatella spartii</i>	0.50				
<i>Juxtiphoma eupyrena</i>	0.57				
<i>Lasiodiplodia pseudotheobromae</i>	0.61				
<i>Lophodermium piceae</i>	1				
<i>Lophodermium pinastri</i>	1				
<i>Lophodermium pini-excelsae</i>	1				
<i>Lophodermium seditiosum</i>	1				
<i>Nectria balsamea</i>	1				
<i>Neocatenulostroma germanicum</i>	1				
<i>Neocucurbitaria cava</i>	0.65				
<i>Neonectria neomacrospora</i>	0.67				
<i>Paecilomyces maximus</i>	0.83				
<i>Paraphoma fimeti</i>	1				
<i>Pestalotiopsis funerea</i>	0.33				
<i>Pestalotiopsis hollandica</i>	0.33				
<i>Pestalotiopsis microspora</i>	1	0.25			
<i>Pestalotiopsis neglecta</i>	0.83	0.33			
<i>Pestalotiopsis pini</i>	0.35				
<i>Racodium therryanum</i>	1				
<i>Rhizoctonia butinii</i>	1				
<i>Rhizoctonia solani</i>	0.87				
<i>Rhizosphaera kalkhoffii</i>	1				
<i>Schizophyllum commune</i>	0.94				
<i>Seiridium ceratosporum</i>	1				
<i>Seiridium podocarpi</i>	1				
<i>Sirococcus conigenus</i>	1				

Table 3 (continued)

Species	HP values				
	No. 1	No. 2	No. 3	No. 4	No. 5
<i>Sydowia polyspora</i>	1	0.95	0.84		
<i>Thyronectria pinicola</i>	0.8				
<i>Valsa friesii</i>	1				

The number of HP values (No.) indicates the number of corresponding ITS sequences in the database while the HP value (value between 0 and 1) reflects the proportion of sequences in the database that correspond to the assigned species

constructed. As shown in Fig. 3, some species, such as *Sydowia polyspora*, *Botrytis cinerea* or *Diplodia sapinea* were found across different conifer genera. In contrast, other fungal species were restricted to specific conifer genera, for example *Caloscypha fulgens* was found in three lots of *Abies* spp., and *Sirococcus conigenus* in three lots of *Picea* spp. The number of conifer pathogens identified per seed batch ranged from 1 to 13, depending on the batch. Seed lots of *Pinus brutia* and *P. thunbergii* were the most heavily colonized, with 10 and 11 conifer fungal pathogens, respectively.

Fungal pathogens not associated with conifer hosts were also detected. These include *Aureobasidium lini* (in seed lot 19, HP value = 1), *Monilinia laxa* (in seed lot 2, HP value = 1), *Nigrospora oryzae* (in seed lots 19, 26 and 27, HP values = 1/0.98) or *Stemphylium vesicarium* (in seed lots 17, 20, 21 and 23, HP value = 0.94). There were two species categorized as potential Union Quarantine Pest based on EU legislation, *Fusarium brachygibbosum* (in seed lot 2, HP value = 0.73), and *Lasiodiplodia pseudotheobromae* (in seed lot 26, HP value = 0.61).

Confirmation of HTS results with specific molecular tests (PCR/qPCR)

Specific molecular tests were applied to the seed lots that tested positive with the HTS method for eight fungal pathogens. As shown in Table 4, all the pathogens were detected using these specific assays. For *Botrytis cinerea*, six of the eight HTS-positive seed lots were confirmed by specific qPCR; the pathogen was not detected in seed lots 20 and 26. For *Diplodia sapinea*, ten samples were positive by HTS, and nine were confirmed by specific PCR, with seed lot 4 testing negative. For *Rhizosphaera kalkhoffii*, the pathogen was confirmed in seed lot 26 but not in seed

lot 19. The presence of the two emerging species *F. brachygibbosum* (seed lot 2) and *N. neomacrospora* (seed lots 1, 7, 8, 13, 21) was confirmed by specific PCR tests, except in seed lot 21 for *N. neomacrospora*. For *L. pseudotheobromae*, Cq values close to the end of the PCR run were observed in DNA extracts from two of the three biological replicates of seed lot 26. The pathogen *Sirococcus conigenus* was confirmed in seed lots 17 and 22 but not in seed lot 18, whereas the wood decay fungus *F. fomentarius* was confirmed in the two samples that tested positive with HTS (seed lots 26 and 27). Among the six PCR-negative but HTS-positive combinations (*B. cinerea* in lots 20 and 26, *D. sapinea* in lot 4, *N. neomacrospora* in lot 21, *R. kalkhoffii* in lot 19 and *S. conigenus* in lot 18, Table 4), only one biological replicate was positive in HTS. Relative accuracy, defined as the proportion of results that were identical between the two methods (HTS versus specific molecular method) out of the total number of results, varied from 63% for *B. cinerea* to 100% for *F. fomentarius* and *F. brachygibbosum* (Table 4).

Discussion

Analysis of mock communities is needed to evaluate the performance of the metabarcoding method

The detection method described in this study, based on ITS2 sequencing using MiSeq technology followed by analysis with the FungiSearch pipeline and a high percentage of identity (99.5%), was evaluated on DNA from a MC of twelve fungi. All twelve fungi were detected at the species level, and no additional fungal species were identified, indicating the absence of contamination in these samples. Moreover, none of the alien targets present in

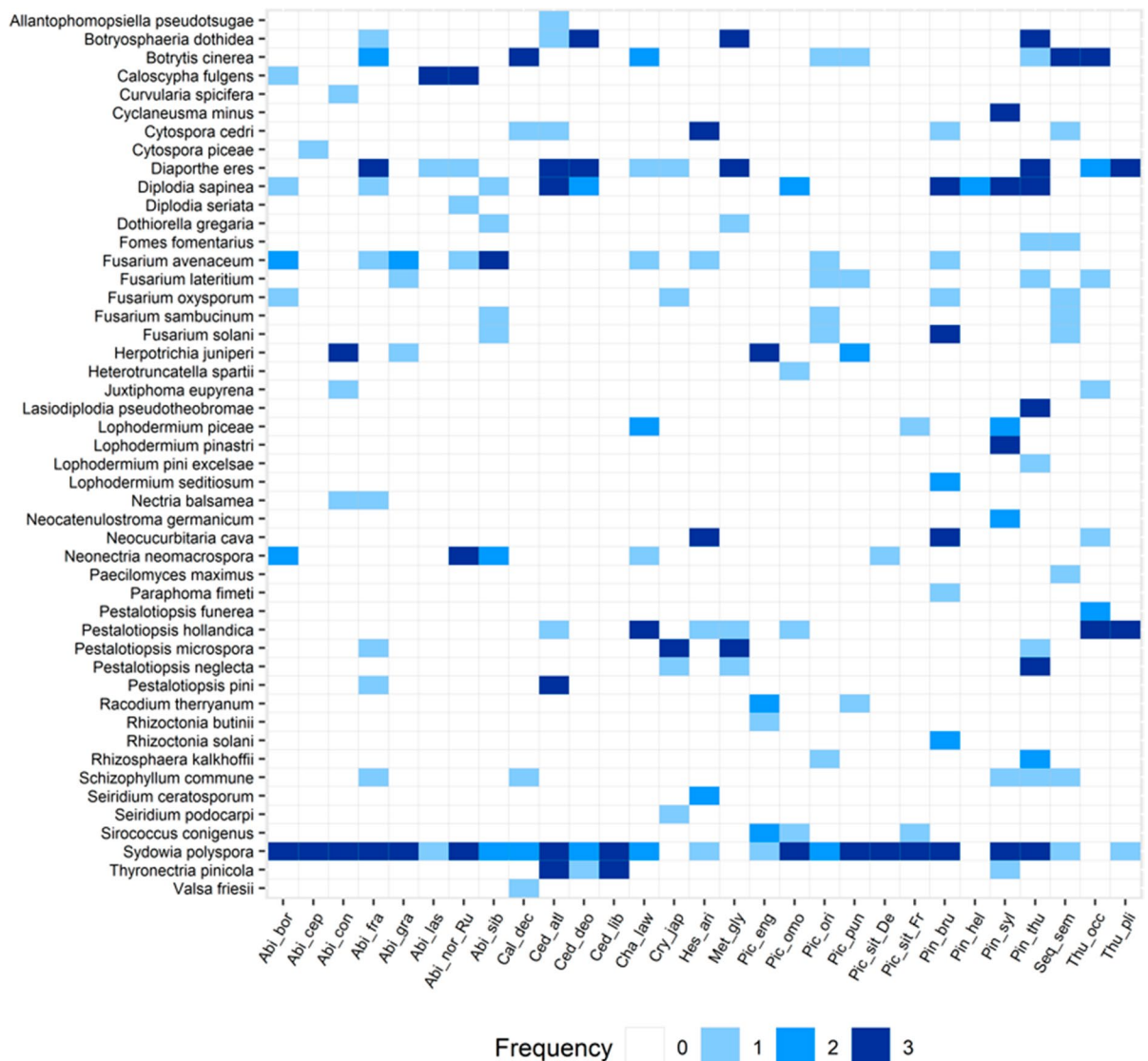


Fig. 3 Heat map of conifer fungal pathogens detected by HTS (Illumina MiSeq, FungiSearch pipeline) showing the frequency of occurrence across biological replicates in 29 seed lots from different origins. Abi_bor=*Abies nordmanniana* var. *bornmuelleriana*; Abi_cep=*A. cephalonica*; Abi_con=*Abies concolor*; Abi_fra=*Abies fraseri*; Abi_gra=*Abies grandis*; Abi_las=*Abies lasiocarpa*; Abi_nor=*Abies nordmanniana*; Abi_sib=*Abies sibirica*; Cal_dec=*Calocedrus decurrens*; Ced_atl=*Cedrus atlantica*; Ced_deo=*Cedrus deodara*; Ced_lib=*Cedrus libani*; Cha_law=*Chamaecyparis lawso-*

niana; Cry_jap=*Cryptomeria japonica*; Hes_ari=*Hesperocyparis arizonica*; Met_gly=*Metasequoia glyptostroboides*; Pic_eng=*Picea engelmannii*; Pic_omo=*Picea omorika*; Pic_ori=*Picea orientalis*; Pic_pun=*Picea pungens*; Pic_sit=*Picea sitchensis*; Pin_bru=*Pinus brutia*; Pin_hel=*Pinus heldreichii*; Pin_syl=*Pinus sylvestris*; Pin_thu=*Pinus thunbergii*; Seq_sem=*Sequoia sempervirens*; Thu_occ=*Thuja occidentalis*; Thu_pli=*Thuja plicata*. Fr=France; De=Denmark

the MCs were detected in the samples. For four of the five fungal species for which standard curves were generated ("target" species), a detection limit of approximately 1 pg per PCR was achieved which

is comparable to conventional PCR assays. This result was obtained despite the use of a limited number of PCR cycles in the first PCR (25 cycles), an approach recommended to reduce the risk of

Table 4 Comparison of HTS and specific molecular tests (SMT; PCR or qPCR) for detecting eight fungal pathogens in conifer seed lots

Fungal pathogen	HP value	Lots + HTS	Lots + SMT ^a	Comparison HTS & SMT (Replicates)				Total ^b
				HTS + SMT +	HTS - SMT -	HTS + SMT -	HTS - SMT +	
<i>Botrytis cinerea</i>	0.9	8/29	6/8	11	6	5	2	24
<i>Diplodia sapinea</i>	1/0.72	10/29	9/10	13	7	8	2	30
<i>Fomes fomentarius</i>	1	2/29	2/2	2	4	0	0	6
<i>Fusarium brachygibbosum</i>	0.73	1/29	1/1	1	2	0	0	3
<i>Lasiodiplodia pseudotheobromae</i>	0.61	1/29	1/1	2	0	1	0	3
<i>Neonectria neomacrospora</i>	0.67	5/29	4/5	8	4	1	2	15
<i>Rhizosphaera kalkhoffii</i>	1	2/29	1/2	2	3	1	0	6
<i>Sirococcus conigenus</i>	1	3/29	2/3	3	5	1	0	9

^aThe proportion was calculated based on the total number of positive lots detected using the HTS method

^bThe value shown represents the total number of HTS-positive lots multiplied by the number of biological replicates per lot

A total of 29 seed lots were analysed using HTS, with three biological replicates per lot

chimera formation (Lahr & Katz, 2009) and to maintain balanced amplicon representation among fungal species in the sample (Tedersoo et al., 2022). This strategy differs from other HTS studies in which the number of PCR cycles was similar to that used in species specific PCR assays (Cleary et al., 2019; Franic et al., 2019).

However, analysis of the four MCs containing variable DNA amounts for five of the twelve fungal species revealed that read percentages sometimes deviated substantially from expected values. Two situations occurred: over- or underestimation depending on the fungal species and loss in linearity between expected and observed values. These deviations may partly result from differences in PCR efficiency (e.g., GC content or amplicon length) as well as variation in ITS copy number among fungal species, as described by Kauserud (2023). However, the non-linearity observed for *P. citricarpa* and *E. australis*, with lower read proportions in MC1 than in MC2, despite higher fungal DNA amounts (Fig. 1) was not consistent with the corresponding specific tests (qPCR for *P. citricarpa* and PCR for *E. australis*). This suggests that fungal community richness and composition (i.e.,

the number and identity of fungal species present and their respective concentrations) may also influence read percentages. Because the diversity of fungal communities in field samples is unknown at the time of testing, subsequent analyses were performed using occurrence data, with the number of positive biological replicates used as an indicator of relative species abundance in a seed lot. The use of presence-absence data across multiple subsamples has been proposed to infer abundance from DNA-based datasets (Luo et al., 2023).

Sampling strategy impacts the diversity of fungal species in seeds

Sampling is a crucial step in seed health testing (Vis-hunavat et al., 2023). Our study showed that analyzing three biological replicates of 100 seeds separately substantially increased the number of species detected. This finding is consistent with the study by Oskay et al. (2022), who highlighted the effect of sample size on the diversity of the fungal communities identified by HTS. Our findings further suggest that most detectable diversity is captured with three replicates, and

that additional replication would likely provide diminishing returns (Fig. 2b). The probability of detecting fungal species present at very low concentrations in a sample—common for seed-borne fungal pathogens (Mancini et al., 2016)—can be unreliable due to stochastic PCR amplification and random sampling during Illumina MiSeq sequencing (Leray & Knowlton, 2017). To address this, two DNA extractions were performed per biological replicate, resulting in a total of six PCRs per seed lot (three biological replicates, each with two DNA extracts and one PCR per extract). This approach was necessary because, when specific PCR tests were performed on the two DNA extracts from a single biological replicate, both extracts did not always yield positive results.

The HP value can help in identifying potential incorrect taxonomic assignment

Among sequences available in reference databases, the ITS region offers several advantages. First, its multi-copy nature enables amplification from samples with low DNA concentrations. Second, conserved flanking sequences allow the design of “high coverage” PCR primers capable of targeting a broad range of fungal species (Taylor et al., 2016; Toju et al., 2012). Third, the ITS region is well represented in reference databases compared with other genomic regions, providing a rich resource for taxonomic assignment. However, different studies have shown that this DNA marker does not always provide species-level resolution (Kausserud, 2023; Piombo et al., 2021; Tedersoo et al., 2022; Yang et al., 2018).

In this study, we used the FungiSearch pipeline, which provides taxonomic assignment for each identified sequence (when a reference sequence with 99.5% identity and a coverage of 85% exists in the database), and reports one or several HP values depending on the species detected. The number of HP values indicates the number of corresponding ITS sequences in the database while each HP value reflects the proportion of sequences in the database that corresponds to the assigned species (ranging from 0 and 1). Analysis of HP values for forty-eight fungal pathogens (Table 3) showed that most (81%) displayed only one HP value, suggesting no intraspecific or intragenomic variation of the ITS region for these species in the reference database. Among the nine fungal species with more than one HP value,

Diaporthe eres (Udayanga et al., 2014) and *Fusarium solani* (Coleman, 2016) have been reported as species complexes. For twenty-four species (50%), the HP value was 1, indicating a single corresponding sequence in the reference database. Most species with low HP value (< 0.6) belong to two genera (*Fusarium* and *Pestalotiopsis*), for which the ITS region is not recommended as a barcoding marker (Maharachchikumbura et al., 2011; O'Donnell et al., 2015). Based on this information, results with a HP value of 1 can be considered positive, whereas results with a lower HP value should be confirmed using a specific method. This conclusion applies only if the reference database, which is continually enriched with new accessions, is kept up to date. Updating the reference database is one of the procedures described in the FungiSearch pipeline.

Fungal pathogens not yet reported in Europe or with a very limited distribution can occur in conifer seeds from intercontinental trade

Our study revealed a wide range of fungal pathogens associated with conifer seeds, including some species not previously reported in Europe or with a limited distribution. This is the case for *Nectria balsamea* (HP=1) described as a canker agent on *Abies balsamea* in the USA but absent in Europe according to the Global Biodiversity Information Facility database (<https://www.gbif.org/what-is-gbif>). Similarly, *Cytospora piceae* (HP=1) was recently reported in China (Pan et al., 2018). *Pestalotiopsis pini*, an emerging species on *Pinus pinea* in Portugal (Silva et al., 2020) was also detected; however, the HP value was very low (HP=0.33) indicating that a specific test should be used to confirm this result. The case of *L. pseudotheobromae* (HP=0.61) and *F. brachigibbosum* (HP=0.73) are of particular interest for Europe as European Food Safety Authority (EFSA) risk assessments based on multiple criteria concluded that these species meet the European Union's quarantine criteria (EFSA et al., 2021; EFSA et al., 2023). These criteria include, among others, the presence of host plants in Europe, a limited distribution of the pathogen within Europe, a suitable climatic environment, known introduction pathways, and substantial economic or environmental impacts. The presence of these two species was confirmed by specific qPCR tests targeting another genomic region (Elongation Factor 1- α).

Fusarium brachygibbosum is not reported as a conifer pathogen (EFSA, 2021). Other pathogens not known to infect conifers were also detected in different seed lots, and some of these displayed an HP value of 1. These findings suggest that conifer seeds could serve as a potential pathway for the introduction of fungal pathogens affecting other plant species.

Conclusion

Our approach, which enables species-level detection with an associated risk of error (HP value) is promising for the surveillance of seed-borne pathogens, at least as a screening method. However, the use of appropriate controls is strongly recommended to ensure the reliability of results. Mock communities of fungal species serve as essential positive/negative controls, allowing the monitoring of cross-contamination, assessment of test sensitivity and validation of the method at the intra-laboratory level. Alien controls, targeting organisms from the same taxonomic group as the group under study but that cannot be present in the sample (Massart et al., 2022), are also useful for evaluating contamination risk. In addition, criteria for assessing the reliability of taxonomic assignments are essential. In this study, we used the HP parameter which provides diagnosticians with an indication of the risk of taxonomic misassignment and highlights whether confirmation via specific molecular assays is necessary. From a risk perspective, although our findings raise concern, they should be interpreted with caution. The detected fungi may be non-viable or may not be transmitted to seedlings or the environment. Moreover, their establishment could be limited by restricted host availability in the new environment, as demonstrated by Franic et al. (2020). For these reasons, further research, including pest risk analyses, is needed to assess the actual risk of introducing emerging fungal pathogens through international conifer seed trade.

Acknowledgements The authors wish to thank Florence Paquet, Mélanie Gourgue, Antoine Ghesquière, and Vincent Gaucet for their technical assistance.

Author contributions Anne Chandelier designed the study. Sophie Schmitz coordinated the selection of samples (seed lots and fungal strains used for the preparation of mock communities). Lab work and bio-informatic analyses of the data were

done by Anne Chandelier. Gilles San Martin carried out the statistical analyses and produced Figs. 1 and 2. Anne Chandelier produced Fig. 3 and wrote the manuscript with the support of Benjamin Dubois for the part dealing with Illumina metabarcoding. All co-authors participated to the review of the manuscript.

Funding The study was carried out as part of the ALERT-SEED project funded by the Belgian Federal Public Service for Public Health, Food Chain Safety and Environment (project no. RF 21/6344).

Data availability The data that support the findings of this study are openly available in figshare at https://figshare.com/articles/online_resource/FungiSearch_V2/28130825 for the pipeline FUNGISEARCH, and at NCBI for the FASTQ files (Bioproject PRJNA1190857).

Declarations

Conflict of interest The authors declare no conflict of interest.

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