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ORIGINAL ARTICLE

Detection and quantification of *Cronartium pini* from Scots pine bark and wood with *Cronartium* spp.-specific quantitative PCR

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Abstract

The forest disease, Scots pine blister rust, is caused by the rust fungus *Cronartium pini*. This pathogen was previously known as the host-alternating type *C. flaccidum* and non-host-alternating type *Peridermium pini*. Recent epidemics of this disease in Northern European forests, especially young Scots pine forests in Sweden, caused significant economic and ecological losses. *Cronartium pini* can be identified based on the typical orange blister-like aecia in Scots pine in summer, but any molecular identification and quantification method has not been available for *Cronartium* spp. This study developed qPCR primers that are specific to *Cronartium* spp. and evaluated DNA extraction protocols from pine bark and wood to enable robust qPCR assays. As little as three *Cronartium* ITS copies can be detected with the protocol. Since only *C. pini* is known to infect Scots pine in Northern Europe, the protocols were applied to detect *C. pini* from Scots pine samples without typical symptoms and investigate the *C. pini* colonization in Scots pine branches from the forest. These results will aid the detection and quantification of *C. pini* in asymptomatic or symptomatic samples and monitoring Scots pine blister rust in the forest in northern Europe.

KEYWORDS
Cronartium, fungal pathogen detection, scots pine blister rust, stem and branch cankers

1 | INTRODUCTION

Conifer trees dominate the forests in Northern Europe. In Sweden, 63% of the land is covered by forests, and Scots pine (*Pinus sylvestris* L.) is one of the most common species, which forms 39.8% of the standing volume in Swedish forests (SLU National Forest Inventory, 2023). The growth of Scots pine can be severely damaged by rust diseases such as Scots pine blister rust. For instance, Martinsson & Nilsson (1987) reported that the radial stem increment of Scots pine was reduced by 40%–70% and 20%–40% by severe and minor attacks of Scots pine blister rust, respectively. Aggressive attacks by the disease in the stem can girdle stems and result in the death of tops or entire trees (Hansen, 1997).

Scots pine blister rust is caused by the rust fungus *Cronartium pini*. This species is previously known as *C. flaccidum* and *Peridermium pini*; the two synonyms describe the heteroecious and autoecious types of this species, respectively (Hantula et al., 2002). The heteroecious type requires host alteration to fulfill its life cycle. Many two-needle pine species are the aecial host, such as Scots pine (*P. sylvestris*), bog pine (*P. mugo*) and Italian stone pine (*P. pinea*) (Kaitera & Nuorteva, 2008; Raddi & Fagnani, 1978). Its alternate hosts/telial hosts include many plant genera such as *Melampyrum*, *Loasa*, *Nemesia*, *Paeonia*, *Pedicularis*, *Tropaeolum* and *Vincetoxicum* (Kaitera et al., 1999a; Kaitera et al., 2015). Aeciospores of *C. pini* are produced in the orange blister-like aecia in lesions on pine trees, these spores infect the alternate hosts, and then basidiospores are produced from

the alternate hosts to infect pine. The aeciospores of the heteroecious type cannot re-infect pine. However, the aeciospores from the autoecious type only re-infect pine trees; its life cycle only involves one host and one spore type (Kaitera & Nuorteva, 2008; Pei & Brodie, 1995).

Besides their differences in life cycles, the two different types of *C. pini* cannot be distinguished by their morphological characters or disease symptoms (Kaitera et al., 1999b; Van Der Kamp, 1969). Genetic variation studies indicated that the two types resemble a single species; the autoecious type might originate as asexual or self-fertilizing host range mutants of the heteroecious type (Hantula et al., 2002). Recent studies suggested using *C. pini* (Willd.) Jørst. to describe both heteroecious and autoecious types (Wijesinghe et al., 2019).

During the sporulation season, *C. pini* produces orange-yellow blister-like aecia with orange-yellow spores on Scots pine branches and stems (Butin, 1995); this is the most typical symptom for identification and diagnosis. However, identifying *C. pini* could still be complicated and ambiguous, especially in the field. At the early stages of *C. pini* infection, infected pine needles have no symptoms except yellow-red spots after a latent period of several months (Ragazzi, 1989). The growth of the pathogen from the needle to the branches causes the branch to swell and deform, but the lesions with typical aecia are not produced until 1 or 2 years later (Greig, 1987). These typical aecia are only visible for approximately 1 month in summer (Samils & Stenlid, 2022). Active lesions during other times of the year or non-active lesions appear to be swelling and blackened with flaky bark (Figure S1); correct diagnosis of them requires experience and re-visiting the site, which cannot always be achieved in the forest survey.

Quantitative polymerase chain reaction (qPCR) has been widely applied to the detection of many fungal plant pathogens (Chandelier et al., 2021; Hu et al., 2014; Luchi et al., 2020) for its optimal accuracy, sensitivity, and the possibility to quantify the DNA (Smith & Osborn, 2008). A sensitive qPCR protocol will allow researchers to detect *C. pini* from samples when the symptom is latent, or the typical symptom is absent. In addition, recent epidemics of Scots pine blister rust caused major damage in northern Sweden, especially in young pine forests (Normark, 2019), the qPCR technique will enable the monitoring of *C. pini* spore dispersal and provide us with more information on the epidemiology of *C. pini* in the forest.

Cronartium pini mainly colonizes the cambium, inner bark/phloem, and outer bark, and active hyphae also grow into the xylem (Van Der Kamp, 1969). Its colonization can promote the phenolic compounds synthesis in Scots pine as a defence mechanism (Hyll et al., 2022). For example, monoterpenes and resin acids at high concentrations have been found in *C. pini*-infected Scots pine (Kaitera et al., 2021). The phenolic structures of these compounds may cause DNA polymerase inhibition in qPCR assays (Albers et al., 2013). Furthermore, healthy pine bark also contains high concentrations of polyphenolics and polysaccharides (Fradinho et al., 2002) as qPCR inhibitors, extensive purification process after DNA or RNA extraction is often needed for downstream processes such as PCR or next-generation sequencing (Broberg & McDonald, 2019). A simple and reliable DNA extraction method is necessary for robust *C. pini* qPCR detection to avoid false-negative results.

Our objectives are (i) to develop and validate sensitive and reliable qPCR protocols for *C. pini* detection and quantification, (ii) to compare and optimize pine bark and wood DNA extraction methods for pathogen detection and (iii) to apply the DNA extraction and qPCR protocols and detect *C. pini* from field samples.

2 | MATERIALS AND METHODS

2.1 | Sample collection and fungal DNA extraction

Species included in the qPCR assay development were listed in Table 1. Four *C. pini* isolates were used as positive controls in the qPCR assay; these aeciospore samples were collected from Scots pine trees with typical Scots pine blister rust lesions from four different locations in Jämtland and Norrbotten, Sweden. Three *C. ribicola* DNA samples from western white pine were provided by Canadian collaborators. Other rust species included in this study are *Melampsora larci-epitea*, *M. piniqorqua* (synonym *M. populnea*), *Gymnosporangium* sp., *Puccinia triticina*, *P. graminis Coleosporium asterism* and *Thekopsora areolata*. These samples represent rust species common in Sweden and rust species infecting Scots pine or conifer trees. Endophytic fungal species were isolated from Scots pine branch samples from pine forest stands in Jämtland, Sweden. These species include *Sydowia polyspora*, *Sarea coeloplata*, *Aequabiliella palatina* and *Cladosporium* sp. All fungal samples were identified based on morphology and ITS sequences. Scots pine and lodgepole pine were included to confirm that pine DNA causes no complication in qPCR assay.

Aeciospores were used in DNA extractions from rust fungi, except *Puccinia* spp. (urediniospores) and *Gymnosporangium* sp. (telial horns). Endophytic fungal species were cultured on PDA (potato dextrose agar) plates for 7 days, and then the mycelia were collected and lyophilized overnight. Spore, telial horns, and lyophilized samples were transferred to individual tubes and homogenized with glass beads and tissue homogenizer (Bertin Technologies). Fungal DNA samples were extracted with DNeasy Plant Kit (Qiagen) following the manufacture manual.

2.2 | qPCR primer design

ITS (internal transcribed spacer) sequences of *C. pini* isolates were selected as the target in the qPCR primer design since ITS has high copy numbers in the fungal genomes and the most entries in Genbank compared to other genes in *Cronartium*. In addition, there is often a positive correlation between ITS copy number in qPCR and fungal dry weight and spore counts (Lwin et al., 2011; Zhang et al., 2022). The 479 entries of *Cronartium* ITS in Genbnk are dominated by

TABLE 1 List of fungal and other materials tested in the qPCR assay development.

Species	Host	Location	Taxonomy	qPCR detection (+ positive, − negative)
Cronartium pini (autoecious)	Scots pine (*Pinus sylvestris*)	Jämtland, Sweden	Pucciniales, Cronartiaceae	+
C. pini (autoecious)	Scots pine (*P. sylvestris*)	Jämtland, Sweden	Pucciniales, Cronartiaceae	+
C. pini (heteroecious)	Scots pine (*P. sylvestris*)	Norrbotten, Sweden	Pucciniales, Cronartiaceae	+
C. pini (heteroecious)	Scots pine (*P. sylvestris*)	Jämtland, Sweden	Pucciniales, Cronartiaceae	+
C. ribicola	Western white pine (*P. monticola*)	British Columbia, Canada	Pucciniales, Cronartiaceae	+
C. ribicola	Western white pine (*P. monticola*)	British Columbia, Canada	Pucciniales, Cronartiaceae	+
C. ribicola	Western white pine (*P. monticola*)	British Columbia, Canada	Pucciniales, Cronartiaceae	+
Coleosporium sp.	Scots pine (*P. sylvestris*)	Jämtland, Sweden	Pucciniales, Coleosporiaceae	−
Gymnosporangium sp.	Juniper (*Juniperus* sp.)	Uppland, Sweden	Pucciniales, Pucciniaceae	−
Melampsora larci-epitea	Willow (*Salix* sp)	Sweden	Pucciniales, Melampsoraceae	−
Melampsora larci-epitea	Willow (*Salix* sp)	Uppland, Sweden	Pucciniales, Melampsoraceae	−
Melampsora pinitorqua	Scots pine (*P. sylvestris*)	Sweden	Pucciniales, Melampsoraceae	−
Puccinia triticina	Wheat (*Triticum aestivum*)	Sweden	Pucciniales, Pucciniaceae	−
Puccinia graminis	Wheat (*Triticum aestivum*)	Sweden	Pucciniales, Pucciniaceae	−
Thekopsora areolata	Norway spruce (*Picea abies*)	Uppsala, Sweden	Pucciniales, Pucciniastraceae	−
Thekopsora areolata	Norway spruce (*Picea abies*)	Lillpite, Sweden	Pucciniales, Pucciniastraceae	−
Aequabiliella palatina	Scots pine (*P. sylvestris*)	Jämtland, Sweden	Phaeomoniellales	−
Cladosporium sp.	Scots pine (*P. sylvestris*)	Jämtland, Sweden	Cladosporiales, Cladosporiaceae	−
Sarea coeloplata	Scots pine (*P. sylvestris*)	Jämtland, Sweden	Sareales, Zythiaceae	−
Sydowia polyspora	Scots pine (*P. sylvestris*)	Jämtland, Sweden	Dothideales, Dothioraceae	−
Pinus sylvestris (Scots pine)	n.a	Uppsala, Sweden	Plant (host)	−
Pinus contorta (lodgepole pine)	n.a.	Norrbotten, Sweden	Plant (non-host)	−

Note: The fungal isolates were identified by ITS sequencing.
Abbreviation: qPCR, quantitative polymerase chain reaction.

C. ribicola (147 entries), *C. pini* (110 entries), *C. comandrae* (44 entries) and *C. quercuum* (24 entries) (accessed by 2023-08-08). Only *C. appalachianum*, *C. arizonicum*, *C. coleosporioides*, *C. comandrae*, *C. comptoniae*, *C. pini*, *C. strobilinum*, *C. ribicola*, *C. quercuum*, *C. occidentale*, *C. harknessii* and some hybrid species have the complete ITS1-5.8S-ITS2 region. These species are all included in the reference sequences. In total, full ITS sequences of 10 isolates of *C. pini*, 25 isolates of other *Cronartium* spp., 11 isolates of *Melampsora* spp. and six isolates of other rust species (*Puccinia gramini*, Thekopsora areolata, *Chrysomyxa zhuoniensis* and *Coleosporium plumeriae*) were retrieved from GenBank (Table S1). The ClustalW alignment was finished in BioEdit and then manually adjusted. Four pairs of forward and reverse primers based on the SYBR green method and three sets of primers and probes based on the TaqMan method were designed with the assistance of PrimerQuest™ Tool (Intergrated DNA Technologies). These oligos target sequence regions specific to *C. pini* or *Cronartium* spp. The primer and probe oligos were synthesized by Eurofins Scientific.

After initial tests of all primers and probes, the optimization of qPCR mixture and conditions with the best-performed primers Cr26F and Cr199R (Table 2), the qPCR protocol is determined as below: the 20 μL reaction system includes 10 μL 2× SsoFast EvaGreen Supermix (Bio-Rad) (1× in the final mixture), 1 μL 10 μM each of forward and reverse primers (0.5 μM in the final mixture), 5 μL DNA extraction and 3 μL water. PCR cycling program is 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by 0.5°C per 5 s temperature increment from 60 to 95°C to generate the melt curve.

TABLE 2 QPCR primers developed for *Cronartium* detection.

Primer	Type	Sequences (5′–3′)	Amplicon length (bp)
Cr26F	Forward	AACCTGCGGAAGGATCATTATTA	174
Cr199R	Reverse	TCATGTGAAACAACAACACAAGT	

2.3 | qPCR assay validation

To prepare standard samples, *C. pini* genomic DNA was amplified with Cr26F and Cr199R (Table 2) in standard PCR. The quantity of the 174 bp amplicon was quantified with a Nanodrop spectrometer after purification with E.Z.N.A.® Cycle Pure Kit (Omega Bio-tek). The amplicon concentration was transformed from ng/μL to ITS copies/μL based on the average molecular mass of the 174 bp amplicon. The purified amplicon was serial diluted to make standard samples for sensitivity assays and standard curves to quantify unknown samples from the field later.

In sensitivity assays, 5 μL standard samples with concentrations from 0.6 copies/μL to 6×10^7copies/μL and 5 μL water as non-template control were loaded onto a 96-well plate. Each standard sample and control sample has five technical replicates on the plate. The experiment was repeated twice.

The primers were tested for their specificity using DNA extracted from *C pini* aeciospores (4 isolates), *C. ribicola* aeciospores (3 isolates), other rust fungi and endophytes and pines as described in the sample collection (Table 1). Each sample was loaded twice on the plate. The experiment was repeated twice. Amplicons were purified and sequenced at Macrogen Europe for BLAST search in Genbank.

2.4 | Protocol of DNA extraction from scots pine wood and bark

Young Scots pine branch sections (ca. 1 cm in diam.) with Scots pine blister rust symptoms was collected from the forest (Jämtland, Sweden) in June 2021, the sections close to the rust lesions but without rust pustules were used in DNA extraction tests. Branch samples were rinsed with detergent and water, then surface sterilized with 1% sodium hypochlorite for 1 min and 70% ethanol for 1 min, and then rinsed with sterilized water three times. The bark (including periderm, phloem and cambium) and wood (including sapwood and heartwood) were separated and divided into small pieces with a sterilized scalpel. The bark and wood fragments were lyophilized for 24 h, then ground in 10 mL cylinders with a mixer mill (5000 r.p.m., 30–60 s) (Retsch) until a fine powder texture was achieved.

The pine wood and bark powder were aliquoted into 1.5 mL centrifuge tubes. Each tube contains 20 mg of wood or bark powders. Five wood samples and five bark samples, as technical replicates, were extracted with the following DNA extraction kits following the manufacturers' manual: Macherey–Nagel NucleoSpin Soil, Macherey–Nagel NucleoSpin Plant II, Qiagen DNeasy PowerSoil, Qiagen DNeasy Plant, Qiagen DNeasy Plant Pro. In total, 25 wood samples and 25 bark samples were extracted with the five extraction kits. The final elution volumes are 100 μL for each sample.

The total DNA yield (ng) and DNA quality (A260/A280 and A260/A230) of the extractions were tested with a Nanodrop spectrophotometer. The qPCR assay of the 50 unknown samples and seven standard samples (3×10^1 to 3×10^7 *C. pini* ITS copies) were processed as described above. Each sample was loaded twice. In summary, there are five technical replicates for each DNA extraction method and two technical repeats in qPCR for each of the five samples. The average Ct (threshold cycle) value and *C. pini* ITS copy number were calculated from the 10 reactions (5 extraction repeats × 2 qPCR repeats), and the average RFU at each cycle was calculated from the 10 reactions as well to represent the average amplification curve of each sample type and DNA extraction method.

2.5 | *Cronartium* DNA detection from scots pine

Fieldwork was done in June 2021 in Jämtland, Sweden, where Scots pine blister rust is present. Thirty Scots pine branch samples were collected, among which 24 have cankers or swellings caused by unknown factors (Figure S1). These samples are used for diagnostic purposes. Another six samples have typical Scots pine blister rust symptoms (new lesions with rust pustules or/and old lesions without rust pustules) (Figure 4a, Figure S1). These samples were used to study the colonization of *C. pini* in pine.

For the 24 branches with symptoms caused by unknown factors, small pieces (ca. $1\,cm^2\times1$–5 mm) of bark and wood samples at the age of the canker or lesions were collected. From the six *C. pini*-positive branches, small pieces (ca. $1\,cm^2\times1$–5 mm) of bark and wood samples were cut from the centre of the lesions, the edge of the lesion, 3, 6, 9 and 12 cm away from the lesions, lateral branches and needles (example in Figure 4a). The sizes of the six branch samples vary. Therefore, different numbers of samples were collected from each branch. In total, 149 samples (13 needle samples, 68 bark samples and 68 wood samples) were collected.

The wood, bark and needle samples were lyophilized and homogenized as described above, and the DNA from each sample was extracted with the Macherey–Nagel NucleoSpin Plant II kit. *C. pini* DNA in the bark and wood was quantified with the qPCR protocol described above. The *C. pini* DNA concentrations in the samples (ITS copies/mg pine samples) were calculated based on the qPCR results and the weight of the homogenized sample used in DNA extraction.

3 | RESULTS

3.1 | qPCR primer development

The alignment of the partial 18S rRNA—ITS1 region of *Cronartium* spp. and selected reference sequences and the primer pair with the best amplification performance, Cr26F and Cr199R, is shown in Figure 1. Limited species-specific variations exist among *C. pini* and other *Cronartium* spp. Therefore, the primer pair Cr26F and Cr199R is a compromise between robust amplification and specificity. Based on the alignment, Cr26F is located in the 18S rRNA region which is relatively conserved among various genera, and Cr199R is located in the ITS 1 region which is conserved in most *Cronartium* spp. in Figure 1. Cr26F and Cr199R are *Cronartium* spp. specific, they can amplify *C. pini* and other *Cronartium* spp. except for *C. quercuum* and *C. harknessii*. Other rust fungi genera, such as *Thekopsora*, *Coleosporium*, *Melampsora* and *Puccinia*, in Figure 1 will not be amplified. The in silico blast of Cr26F and Cr199R in NCBI Genbank

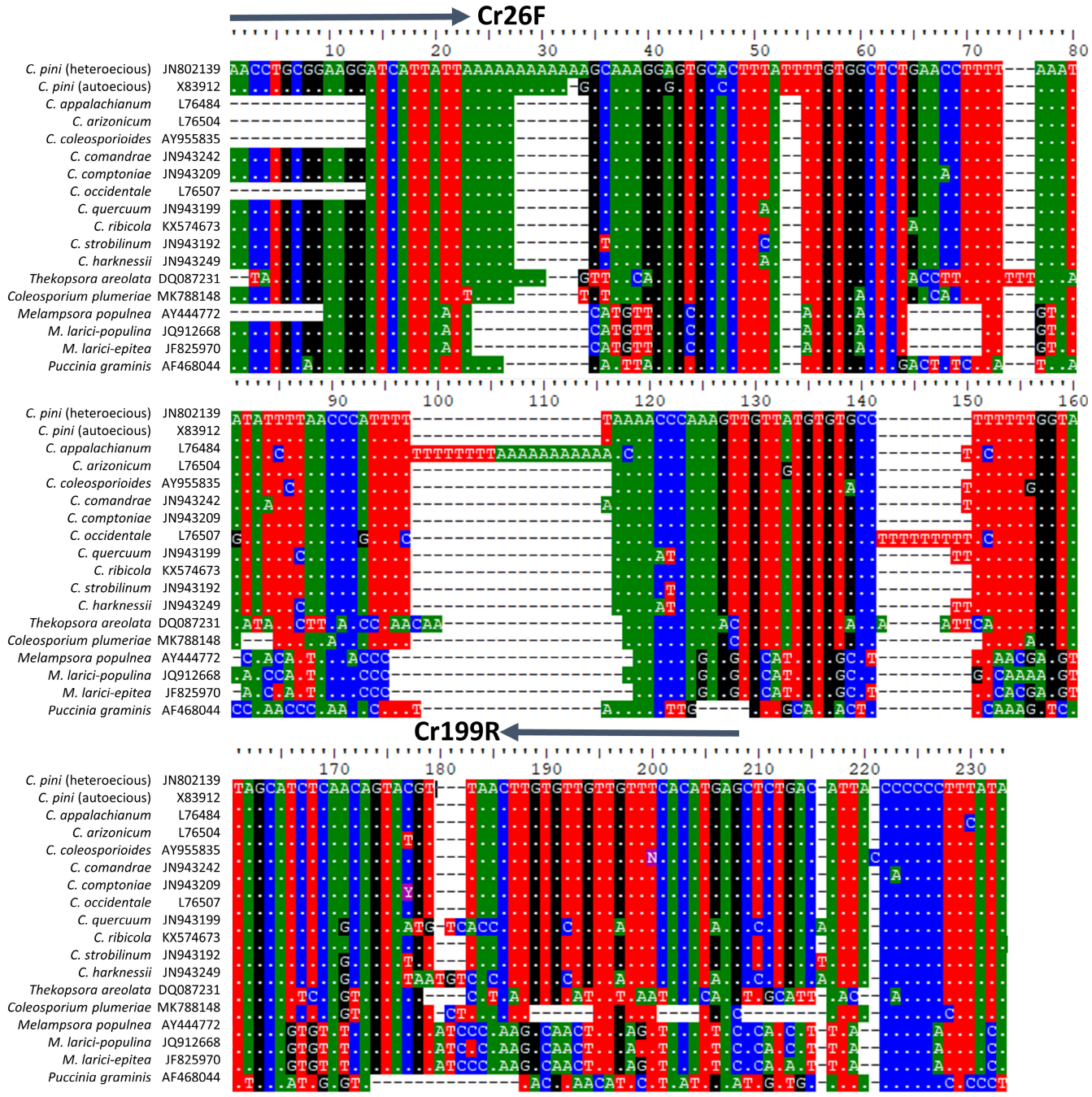


FIGURE 1 Alignment of internal transcribed spacer 1 (ITS1) sequences of *Cronartium* spp. and other rust species. Partial 18S ribosomal RNA: 1–23; partial ITS1: 24–234. Dots indicate the same nucleotides as *C. pini* JN802139. Dashes indicate missing data in the 18S ribosomal RNA region and deletions in the ITS1 region. Written out ATCG indicates mismatching nucleotides except in the *C. pini* JN802139.

returned no sequence without mismatch on any primer other than *Cronartium* spp. ITS sequences. The result suggested that Cr26F and Cr199R have good specificity for *Cronartium* spp.

3.2 | qPCR validation

In sensitivity assay, the start quantities of standard samples are from 3 to 3×10^8 ITS copies. Non-template samples have no amplification. The standard curve generated by the Bio-rad CFX manager is shown in Figure 2. Samples with 3×10^8 ITS copies have a Ct value of 10.26 ± 0.159, and samples with three copies of ITS copies have a Ct value of 37.62 ± 0.492. The standard curve has a high correlation coefficient ($R^2=0.999$) and high amplification efficiency ($E=95.3\%$). The melt curves of all standard samples are uniform, with melt curve temperatures at 76.0–76.5°C (Figure S2). This suggested that the targeted sequences were amplified from all samples. In summary, the qPCR protocol is robust with high sensitivity.

In specificity assay, DNA samples from *C. pini* aeciospores, *C. ribicola* aeciospores and Scots pine infected by *C. pini* have positive amplification signals. All other samples, which include common pathogen and endophyte from Scots pine, other common rust fungi genera, and host and non-host of *C. pini*, gave no amplification signal (Table 1). Sequences of the amplicons obtained from *C. pini* and *C. ribicola* were almost identical with *C. pini* and *C. ribicola* ITS sequences in Genbank respectively, and *C. pini* sequences have typical 10–12 repeats of adenine (Table S2, Figure 1).

3.3 | DNA extraction protocol

The five DNA extraction kits perform differently when processing 20 mg of homogenized pine bark or wood powders (Table 3). Macherey–Nagel NucleoSpin Plant II kit extracted most total DNA with good quality from both bark and wood. Qiagen DNeasy Plant pro and Qiagen DNeasy PowerSoil kits extracted less total DNA with less ideal quality. QPCR assay detected the most *C. pini* DNA from samples extracted by the Macherey–Nagel NucleoSpin Plant II kit. The kits included in this study have different efficiencies in extracting *C. pini* DNA and other DNA; this result is represented by the ratio of *C. pini* DNA quantified in qPCR and total DNA quantified by

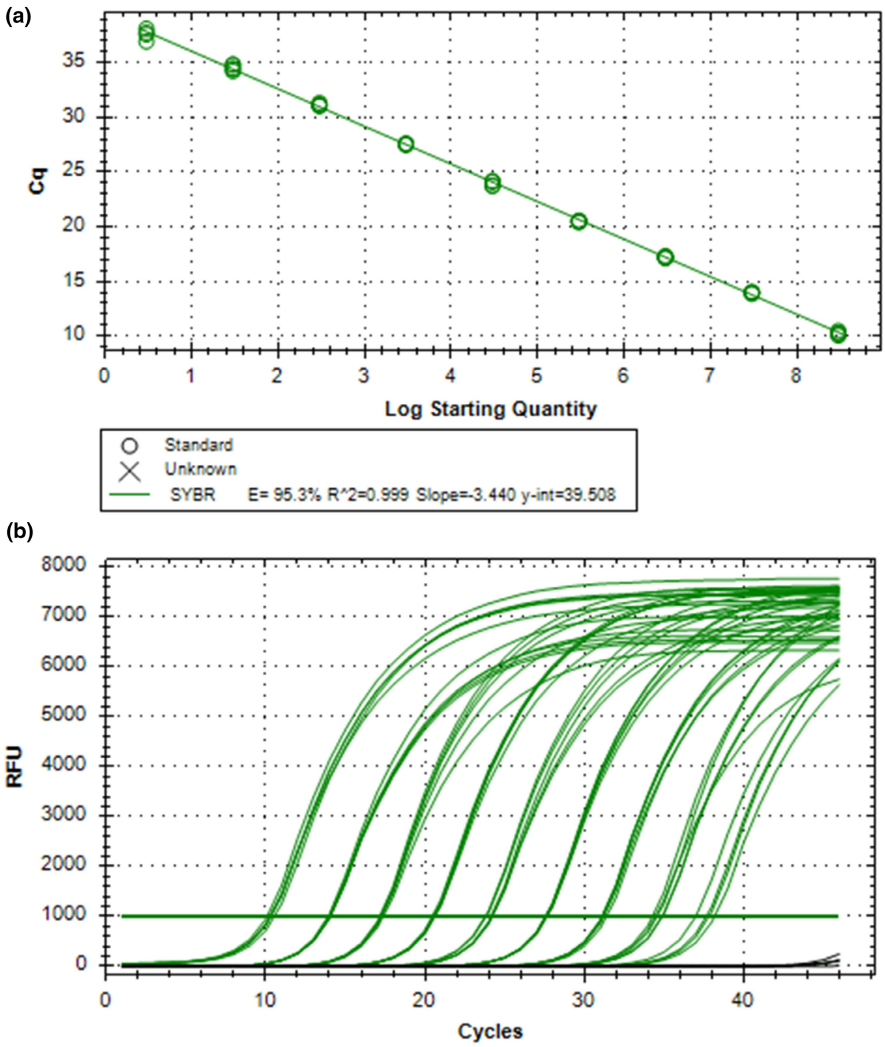


FIGURE 2 Standard curve (a) and amplification curves (b) of qPCR sensitivity test with serial diluted amplicons of the targeted region of *Cronartium pini* ITS. Cq, quantification cycle; RFU, relative fluorescence units.

TABLE 3 Yield and quality of total DNA and *Cronartium pini* DNA from Scots pine samples extracted with various commercial DNA extraction kits.

Extraction method	Sample type	DNA yield (ng)	DNA quality		Ct value	*Cronartium* ITS copy number ($\times 10^7$ copies)	*C. pini* DNA concentration ($\times 10^4$ copies/ng of total DNA yield)
			260/280	260/230			
Macherey–Nagel NucleoSpin Soil	Bark	2042 ± 596	1.96 ± 0.08	1.99 ± 0.46	17.05 ± 0.53	3.14 ± 1.11	1.54
	Wood	1560 ± 76	2.03 ± 0.17	1.99 ± 0.53	16.93 ± 0.10	3.26 ± 0.25	2.09
Qiagen DNeasy PowerSoil	Bark	872 ± 370	2.25 ± 0.45	0.90 ± 0.10	18.50 ± 0.37	1.08 ± 0.28	1.24
	Wood	992 ± 223	2.07 ± 0.22	1.45 ± 0.30	18.07 ± 0.26	1.45 ± 0.26	1.46
Macherey–Nagel NucleoSpin Plant II	**Bark**	**4746 ± 234**	**1.89 ± 0.02**	**1.76 ± 0.06**	**13.90 ± 0.61**	**31.0 ± 10.8**	**6.53**
	Wood	**3206 ± 523**	**1.96 ± 0.06**	**2.01 ± 0.07**	**15.77 ± 0.40**	**8.63 ± 1.62**	**2.69**
Qiagen DNeasy Plant	Bark	2048 ± 141	1.75 ± 0.04	1.26 ± 0.04	16.71 ± 0.59	4.09 ± 1.68	2.00
	Wood	2524 ± 314	1.86 ± 0.08	1.75 ± 0.17	17.08 ± 0.15	2.95 ± 0.35	1.17
Qiagen DNeasy Plant pro	Bark	1634 ± 354	1.63 ± 0.14	0.46 ± 0.18	16.40 ± 0.32	4.68 ± 1.16	2.68
	Wood	384 ± 77	2.17 ± 0.49	0.46 ± 0.15	19.05 ± 0.33	0.72 ± 0.18	1.88

Note: Data were summarized from five replicates of 20 mg lyophilized *P. sylvestris* samples infected by *C. pini*. All 25 samples are homogenized. The kit with the best performance is labelled in bold.

Nanodrop. Macherey–Nagel NucleoSpin Plant II kit has the best efficiency in extracting *C. pini* DNA from pine bark and wood samples.

The qPCR assay of all samples extracted from wood showed optimal amplification curves and the exponential phase of the reaction (Figure 3). This may result from less inhibitor in wood compared to bark. The amplification curve and exponential phase of the reaction of bark samples are less optimal than wood samples. The exponential phase of bark samples extracted by the Qiagen DNeasy PowerSoil kit is the closest to standard samples, which suggests the kit performed better in inhibitor removal than the other methods. The amplification curve of samples extracted by MN NucleoSpin Plant II is less optimal, but the lowest Ct value indicated that more *C. pini* DNA was extracted from the same material with this method.

3.4 | *Cronartium pini* colonization in pine branches

For the 24 Scots pine branches without typical blister rust symptoms (Figure S2), no *C. pini* was detected in the qPCR assay.

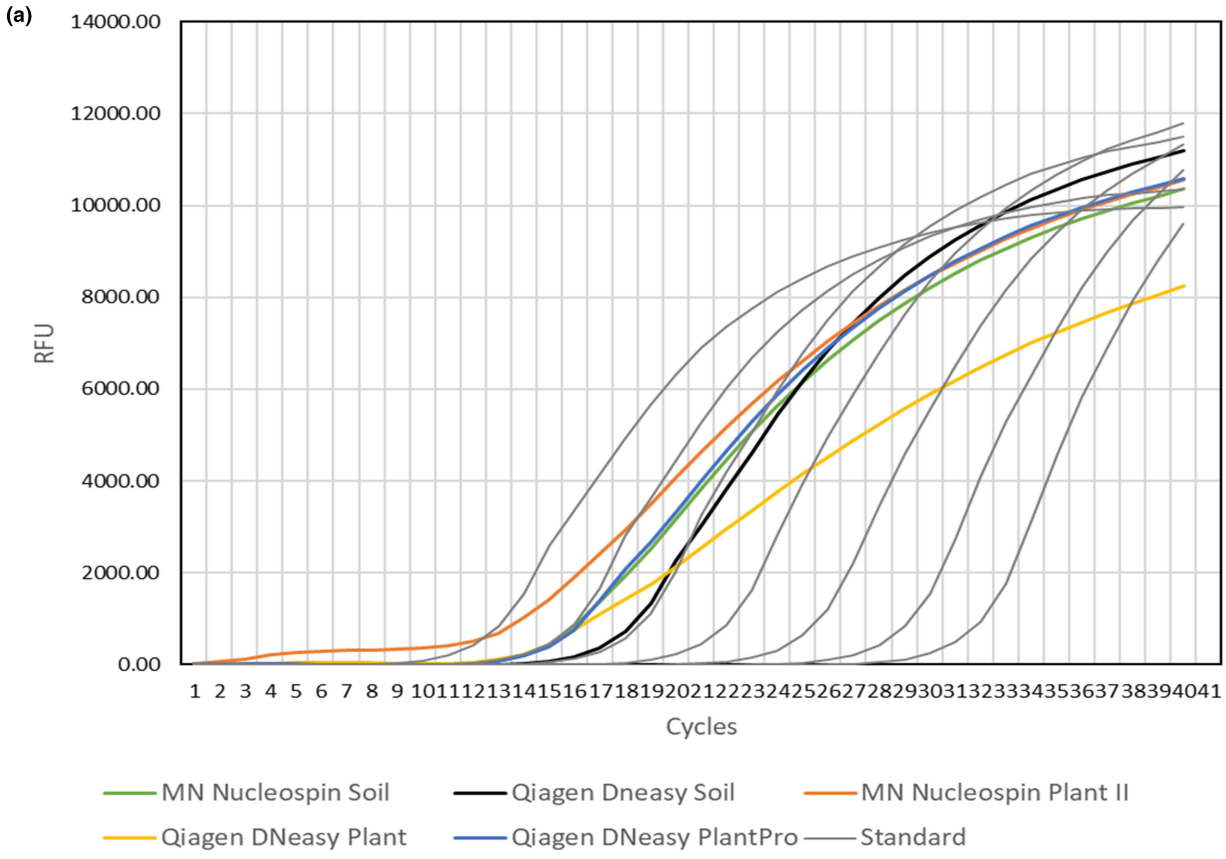
DNA of *C. pini* can always be detected from bark and wood samples collected within the blister-containing lesions (positive rate = 100%), and the average concentration is the highest compared to other groups of samples (Figure 4b,c). Within this group, samples from active lesions with new blisters have the highest *C. pini* concentration (Figure S3). The concentrations of samples collected from the centre of old non-active lesions are the lowest, but the concentration increased at the edge of old lesions (Figure S3).

Cronartium pini DNA was detected from 91.67% and 83.33% of the bark and wood samples collected 3 cm away from the lesions, where no swelling or aecia formation occurred (Figure 4b). The positive detection rate and *C. pini* DNA concentration decreased as the distance between the sampling area and lesion edge increased (Figure 4b,c). The result suggested that *C. pini* can colonize branch sections 3–6 cm away from the lesions without showing symptoms.

When *C. pini* can be detected from the pair of bark–wood samples collected from the same area, the bark sample is always positive, whereas wood samples may be negative, and *C. pini* DNA concentrations are usually higher in the bark than in the wood (Figure 4c, Figure S3). Among the 13 needle samples, only one sample from dead needles attached to the *C. pini* lesion (Figure 4b, Figure S4) has a low amount of *C. pini* DNA; all other dead or living needle samples have negative results in qPCR assay. The results suggested that bark is the advisable sample for *C. pini* detection.

4 | DISCUSSION

In this study, we developed a qPCR protocol with high sensitivity to detect *Cronartium* spp. from various types of samples and compared the impact of using different DNA extraction kits on *C. pini* DNA quantification. The optimal DNA extraction method and the qPCR protocol were applied to detect *C. pini* samples from the field and investigate the colonization of *C. pini* in the pine branches.



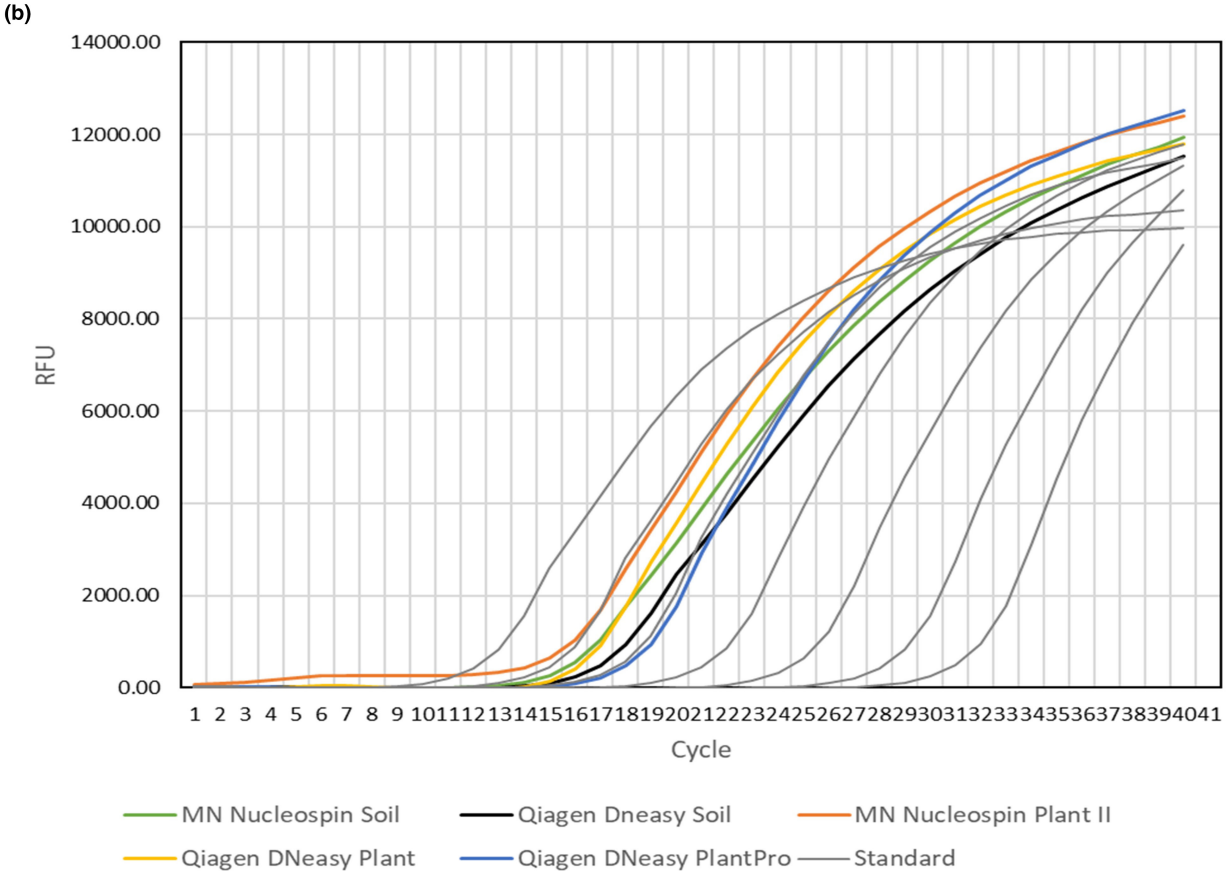


FIGURE 3 QPCR amplification curve of *C. pini-positive* Scots pine bark (a) and wood (b) samples extracted with commercial DNA extraction kit. The curve of each kit is plotted based on the average RFU (relative fluorescence units) of 10 reactions (five DNA extraction replicates times two qPCR replicates).

ITS sequences were selected as the amplification target for the following reasons: (i) this locus is most commonly sequenced and used as a universal fungal barcoding marker (Schoch et al., 2012), so we have the best knowledge about its conserved and variable regions in various *Cronartium* species. The primer development process included all *Cronartium* spp. with full ITS1-5.8S-ITS2 sequences in Genbank. (ii) ITS copy number in a single genome is abundant in fungi. For example, the estimation of ITS copy numbers in genomes is 241 and 232 in *C. quercuum* and *Puccinia striiformis* (Lofgren et al., 2019). Therefore, this ITS-based protocol has a high chance of detecting a trace amount of *C. pini* cells in the samples.

The forward primer Cr26F targets the end of 18S rRNA region which is more conserved in various rust genera, and the reverse primer targets the ITS 1 region which is conserved in many *Cronartium* spp. (Figure 1). This assay is rather *Cronartium* specific than *C. pini* specific. Based on the ITS alignment of *Cronartium* spp. and other rust species, the qPCR assay set will not amplify other rust genera, but several *Cronartium* spp., such as *C. pini* and *C. ribicola* in the specificity test (Table 1) and *C. appalachianum*, *C. arizonicum*, *C. coleosporiodes*, *C. comandrae*, *C. comptoniae*, *C. occidentale* and *C. strobilinum* in Figure 1, will be detected. This protocol is valuable for numerous purposes. For example, spore inoculation tests are essential to study the pine resistance against *C. pini*, but the experiments can last for months and years due to the long latent period of this disease (Greig, 1987; Ragazzi, 1989). During the early stages of *C. pini* inoculation tests on Scots pine seedlings, we applied this qPCR protocol to provide information on the infection rate (data not shown). Complication caused by other *Cronartium* spp. is not a concern since they do not exist in the experiment setting.

Only two *Cronartium* spp., *C. pini* and *C. ribicola*, have been reported from Sweden (Farr & Rossman, 2023). The two species have different aecial host specificities. *C. ribicola* only infects five-needle pines (subgenus *Strobus*) such as western white pine (*Pinus monticola*) and sugar pine (*P. lambertiana*), *C. pini* only infects two- or three-needle pines (subgenus *Pinus*) such as Scots pine (*P. sylvestris*) and stone pine (*P. pinea*) (Hunt, 1984; Kaitera & Nuorteva, 2006; McDonald et al., 2006; Raddi & Fagnani, 1978). Natural forests and managed forests in Northern Europe are dominated by Scots pine, Norway spruce and silver birch (SLU National Forest Inventory, 2023); *C. pini* is the major pathogen that causes the epidemic in Swedish pine forests. Therefore, this qPCR protocol can be used to diagnose and confirm *C. pini* in field samples and monitor the spore dynamics in the Scots pine forests in Sweden. *Melampyrum* spp. are the most important alternate (telial) hosts for *C. pini* epidemics in northern Europe (Kaitera et al., 2005); and *C. ribicola* mainly spreads via *Ribes* as the alternate host (Kaitera & Nuorteva, 2006). However, both species can infect *Bartsia*, *Castilleja* and *Tropaeolum* (Kaitera et al., 2015). This qPCR protocol can be used to confirm *Cronartium* spp. infection in potential alternate host or pine especially in inoculation test. To identify the *Cronartium* species, amplicons from the qPCR assay that represents the ITS1 region (Figure 1), can be sequenced and compared with *Cronartium* records in Genbank (Table S2). The ITS1 region of *C. pini* in the amplicon has 10–12 repeats of adenine (Figure 1, Table S2), which can be identified easily from the sequences. This protocol can detect as little as three copies of *Cronartium* ITS (Figure 3). If the ITS copy number in the *C. pini* genome is similar to that in the *C. quercuum* genome (ITS copy = 242), the theoretical detection limit of this protocol is less than one *C. pini* cell. However, the result can be influenced by other factors in practical application: (i) qPCR inhibitors such as polyphenolics and polysaccharides (Fradinho et al., 2002) can cause false-negative results; (ii) different DNA extraction methods vary in DNA yields and quality (Verbylaitė et al., 2010); (iii) special types of samples require additional treatment, for example, spores need to be dislodged from petroleum gel spore traps (Quesada et al., 2018) or rinsed down from filter paper spore traps (Zhang et al., 2022). These processes will reduce the final DNA yield. DNA extraction with commercial kits is convenient to process, and sometimes the kits are made to remove potential PCR inhibitors in specific types of samples, but no kit is the most optimal for all types of samples (Claassen et al., 2013; Hu et al., 2014; Verbylaitė et al., 2010). In our tests, the Macherey–Nagel NucleoSpin Plant II kit performs best in DNA extraction from Scots pine for *C. pini* identification and quantification (Figure 4, Table 3).

We applied the DNA extraction and qPCR protocol to investigate if *C. pini* caused the unknown symptoms among some field samples (Figure S1). No positive amplification was found among samples without typical *C. pini* lesions included in this study, while *C. pini* can be regularly detected from other Scots pine samples with signs of the rust. Since pine bark samples have higher positive detection rates and higher *C. pini* DNA concentrations (Figure 4b,c) and are easier to sample than wood, it is the most suitable tissue type for diagnosis and pathogen colonization monitoring.

Cronartium colonization studies are mainly focused on *C. ribicola* and *C. quercuum* in pine needles, seedlings or stems (Hudgins et al., 2005; Hunt et al., 2010; Lundquist & Miller, 1984; Sweeney, 2013), except Longo et al. studied the early colonization of *C. pini* in *P. pinea* needles (Longo et al., 2012). In *P. monticola* stem colonized by *C. ribicola*, the pathogen hyphae were commonly found in the cortex, secondary phloem and xylem rays (Hudgins et al., 2005). Our qPCR test confirmed that *C. pini* could be detected from both bark and wood tissues (Figure 4). Knowledge about how far *Cronartium* hyphae can grow latently along the branch was not available previously. This information can be of practical value for disease management strategies such as removing infected branches but retaining as much as healthy branches in

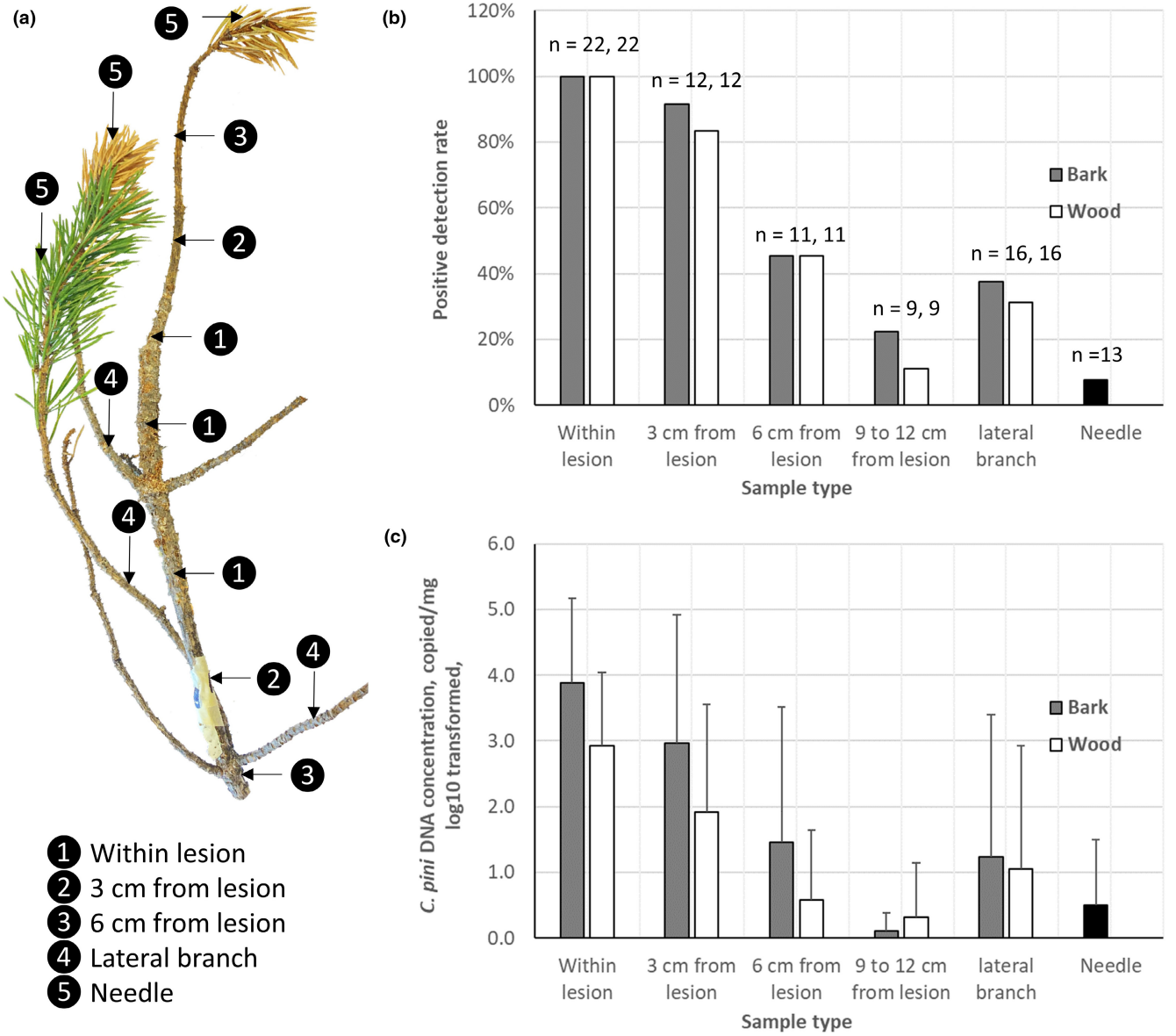


FIGURE 4 Colonization of *Cronartium pini* in Scots pine branches. (a) An example of sampling locations in a Scots pine branch. (b) Positive detection rates of *Cronartium pini* from each sample type, the numbers on top of the bars indicate the number of bark and wood samples. (c) Average *C. pini* DNA concentration in different parts of Scots pine branches. The same numbers of samples as in B were summarized.

pruning (Kim et al., 2022; Samils & Stenlid, 2022). Among samples tested in this study, *C. pini* hyphae can usually grow at least 3 cm from the edge of the visible lesion, some of them can extend to 6 cm, but they seldom reach 9–12 cm (Figure 4b,c).

In summary, this qPCR protocol provided a sensitive tool to detect and quantify *C. pini* in Scots pine, and *Cronartium* spp. from other type of samples. With a good awareness of its proposition and limitations, it can also be used for other purposes. This protocol can test the growth of *Cronartium* spp. growth in inoculated pine and other plant in the greenhouse, and monitor *Cronartium* spp. spore release in managed forests, since the target pathogen species is already known in these scenarios. This protocol can be used for preliminary screening of potential alternate hosts of *Cronartium* spp. in the forest, but more information such as pine species that existed in the forest is required to draw the conclusion, since some different *Cronartium* spp. may have the same alternate hosts (Kaitera et al., 2015). For quarantine and phytosanitary purposes requiring accurate identification of specific species, a follow-up sequencing of the amplicon can be used for identification based on ITS1. Furthermore, species-specific primers and probes need to be developed for each species.

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CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Albers, C. N., Jensen, A., Bælum, J., & Jacobsen, C. S. (2013). Inhibition of DNA polymerases used in Q-PCR by structurally different soil-derived humic substances. *Geomicrobiology Journal*, *30*, 675–681.

Butin, H. H. (1995). *Tree diseases and disorders: Causes, biology, and control in forest and amenity trees*. Oxford University Press.

Broberg, M., & McDonald, J. E. (2019). Extraction of Microbial and Host DNA, RNA, and Proteins from Oak Bark Tissue. *Methods and Protocols*, *2*(1), 15. https://doi.org/10.3390/mps2010015

Chandelier, A., Hulin, J., Martin, G. S., Debode, F., & Massart, S. (2021). Comparison of qPCR and metabarcoding methods as tools for the detection of airborne inoculum of forest fungal pathogens. *Phytopathology*, *111*, 570–581.

Claassen, S., du Toit, E., Kaba, M., Moodley, C., Zar, H. J., & Nicol, M. P. (2013). A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. *Journal of Microbiological Methods*, *94*, 103–110. https://doi.org/10.1016/J.MIMET.2013.05.008

Farr, D. F., & Rossman, A. Y. (2023). *Fungal Databases*. U.S. National Fungus Collections, ARS, USDA. Retrieved February 9, 2023, from https://nt.ars-grin.gov/fungaldatabases/

Fradinho, D. M., Neto, C. P., Evtuguin, D., Jorge, F. C., Irle, M. A., Gil, M. H., & Pedrosa de Jesus, J. (2002). Chemical characterisation of bark and of alkaline bark extracts from maritime pine grown in Portugal. *Industrial Crops and Products*, *16*(1), 23–32. https://doi.org/10.1016/s0926-6690(02)00004-3

Greig, B. J. W. (1987). History of *Peridermium* stem rust of Scots pine (*Pinus sylvestris* L.) in Thetford Forest, East Anglia. *Forestry: An International Journal of Forest Research*, *60*, 193–202. https://doi.org/10.1093/FORESTRY/60.2.193

Hansen, E. M., & Lewis, K. J. (1997). *Compendium of Conifer Diseases*. *American Phytopathological Society*. Minnesota, St. Paul.

Hantula, J., Kasanen, R., Kaitera, J., & Moricca, S. (2002). Analyses of genetic variation suggest that pine rusts Cronartium flaccidum and Peridermium pini belong to the same species. *Mycological Research*, *106*(2), 203–209. https://doi.org/10.1017/s0953756201005457

Hu, J., Johnson, E. G., Wang, N.-Y., Davoglio, T., & Dewdney, M. M. (2014). qPCR quantification of pathogenic *Guignardia citricarpa* and nonpathogenic *G. mangiferae* in citrus. *Plant Disease*, *98*, 112–120. https://doi.org/10.1094/PDIS-04-13-0465-RE

Hudgins, J. W., McDonald, G. I., Zambino, P. J., Klopfenstein, N. B., & Franceschi, V. R. (2005). Anatomical and cellular responses of *Pinus monticola* stem tissues to invasion by *Cronartium ribicola*. *Forest Pathology*, *35*, 423–443. https://doi.org/10.1111/J.1439-0329.2005.00425.X

Hunt, R. S. (1984). Inoculations of Scrophulariaceae with *Cronartium ribicola*. *Canadian Journal of Botany*, *62*, 2523–2524. https://doi.org/10.1139/B84-343

Hunt, R. S., Roke, G., & Jensen, G. D. (2010). Initiation of stem infection in western white pine by blister rust [*Cronartium ribicola*]. *Canadian Journal of Plant Pathology*, *29*, 18–24. https://doi.org/10.1080/07060660709507433

Hyll, K., Joevenller, S., Svennerstam, H., Nordström, M., Broman, O., Oja, J., & Sandberg, D. (2022). X-ray computed tomography for the detection of damage in scots pine trunks caused by blister-rust fungus *Cronartium pini* (Willd.). *Wood Material Science & Engineering*, *17*, 1022–1024. https://doi.org/10.1080/17480272.2022.2120826

Kaitera, J., Hitunen, R., Samils, B., & Hantula, J. (2015). *Cronartium* rusts sporulate on a wide range of alternate hosts in northern Europe. *Journal of Forest Research*, *28*, 746–751.

Kaitera, J., & Nuorteva, H. (2006). Finnish *Cronartium ribicola* does not infect alternate hosts of *Cronartium flaccidum*. *Forest Pathology*, *36*, 247–252. https://doi.org/10.1111/J.1439-0329.2006.00451.X

Kaitera, J., & Nuorteva, H. (2008). Inoculations of eight *Pinus* species with *Cronartium* and *Peridermium* stem rusts. *Forest Ecology and Management*, *255*, 973–981. https://doi.org/10.1016/J.FORECO.2007.10.005

Kaitera, J., Nuorteva, H., & Hantula, J. (2005). Distribution and frequency of *Cronartium flaccidum* on *Melampyrum* spp. in Finland. *Canadian Journal of Forest Research*, *35*, 229–234.

Kaitera, J., Piispanen, J., & Bergmann, U. (2021). Terpene and resin acid contents in scots pine stem lesions colonised by the rust fungus *Cronartium pini*. *Forest Pathology*, *51*, e12700.

Kaitera, J., SeitamÄKi, L., Hantula, J., Jalkanen, R., & Kurkela, T. (1999a). Inoculation of known and potential alternate hosts with *Peridermium pini* and *Cronartium flaccidum* aeciospores. *Mycological Research*, *103*(2), 235–241. https://doi.org/10.1017/s0953756298006947

Kaitera, J., SeitamÄKi, L., Hantula, J., Jalkanen, R., & Kurkela, T. (1999b). Morphological variation of *Peridermium pini* and *Cronartium flaccidum* aeciospores. *Mycological Research*, *103*(6), 677–683. https://doi.org/10.1017/s0953756298007503

Kim, M.-S., Hantula, J., Kaitera, J., Zambino, P. J., Woodward, S., Richardson, B. A., Stewart, J. E., Spaine, P., Shaw, D. C., Takeuchi, Y., & Klopfenstein, N. B. (2022). Recovery plan for scots pine blister rust caused by *Cronartium pini*. *Plant Health Progress*, *23*, 105–130. https://doi.org/10.1094/PHP-06-21-0099-RP

Lofgren, L. A., Uehling, J. K., Branco, S., Bruns, T. D., Martin, F., & Kennedy, P. G. (2019). Genome-based estimates of fungal rDNA copy number variation across phylogenetic scales and ecological lifestyles. *Molecular Ecology*, *28*, 721–730. https://doi.org/10.1111/MEC.14995

Longo, N., Poggiolesi, S., Naldini, B., & Tani, G. (2012). Penetration and early colonization in basidiosporederived infection on needles of *Pinus pinea* L. by *Cronartium flaccidum* (Alb. et Schw.). Caryologia, 9–29. https://doi.org/10.1080/00087114.2000.10589177

Luchi, N., Ioos, R., & Santini, A. (2020). Fast and reliable molecular methods to detect fungal pathogens in woody plants. *Applied Microbiology and Biotechnology*, *104*, 2453–2468. https://doi.org/10.1007/S00253-020-10395-4

Lundquist, J. E., & Miller, T. (1984). Development of stem lesions on slash pine seedlings infected by *Cronartium quercuum* f. sp. *fusiforme*. *Phytopathology*, *74*, 514–518.

Lwin, K. O., Hayakawa, M., Ban-Tokuda, T., & Matsui, H. (2011). Real-time PCR assays for monitoring anaerobic fungal biomass and population size in the rumen. *Current Microbiology*, *62*, 1147–1151. https://doi.org/10.1007/S00284-010-9843-7/FIGURES/1

Martinsson, O., & Nilsson, B. (1987). The impact of Cronartium flaccidum on the growth of Pinus sylvestris. *Scandinavian Journal of Forest Research*, *2*(1–4), 349–357. https://doi.org/10.1080/02827588709382472

McDonald, G. I., Richardson, B. A., Zambino, P. J., Klopfenstein, N. B., & Kim, M. S. (2006). *Pedicularis* and *Castilleja* are natural hosts of *Cronartium ribicola* in North America: A first report. *Forest*

Pathology, *36*, 73–82. https://doi.org/10.1111/J.1439-0329.2006.00432.X

Normark, E. (2019). Multiskadad ungskog i Västerbottens-och Norrbottens län-Möjliga åtgärder för att mildra problemen. Skogsstyrelsen Rapport, 10. https://www.skogsstyrelsen.se/globalassets/om-oss/rapporter/rapporter-20222021202020192018/rapport-2019-10-multiskadad-ungskog-i-vasterbottens-och-norrbottens-lan.pdf

Pei, M. H., & Brodie, J. (1995). Inoculation of young pine seedlings with *Peridermium pini* from north-East Scotland. *European Journal of Forest Pathology*, *25*, 24–30. https://doi.org/10.1111/J.1439-0329.1995.TB01068.X

Quesada, T., Hughes, J., Smith, K., Shin, V. K., James, P., & Smith, J. (2018). A low-cost spore trap allows collection and real-time PCR quantification of airborne *Fusarium circinatum* spores. *Forests*, *9*, 586. https://doi.org/10.3390/f9100586

Raddi, P., & Fagnani, A. (1978). Relative susceptibility to blister rust caused by *Cronartium flaccidum* of several species of pine. *European Journal of Forest Pathology*, *8*, 58–61. https://doi.org/10.1111/J.1439-0329.1978.TB00616.X

Ragazzi, A. (1989). Past, present, and future of *Cronartium flaccidum* in Italy. *Phytopathologia Mediterranea*, *28*, 5–9.

Samils, B., & Stenlid, J. (2022). A review of biology, epidemiology and management of *Cronartium pini* with emphasis on northern Europe. *Scandinavian Journal of Forest Research*, *37*, 153–171. https://doi.org/10.1080/02827581.2022.2085322

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Bolchacova, E., Voigt, K., Crous, P. W., Miller, A. N., Wingfield, M. J., Aime, M. C., An, K. D., Bai, F. Y., Barreto, R. W., Begerow, D., Bergeron, M. J., Blackwell, M., … Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 6241–6246. https://doi.org/10.1073/PNAS.1117018109/-/DCSUPPLEMENTAL

SLU National Forest Inventory. (2023). Riksskogs Taxeringen. Officiell statistik om de svenska skogarna. Retrieved March 30, 2023, from https://skogsstatistik.slu.se/pxweb/sv/OffStat/

Smith, C. J., & Osborn, A. M. (2008). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiology Ecology*, *67*, 6–20. https://doi.org/10.1111/j.1574-6941.2008.00629.x

Sweeney, K. (2013). Histological comparisons of needle tissues of four species of white pine infected by *Cronartium ribicola*. [Master thesis, Oregon State University].

Van Der Kamp, B. J. (1969). *Peridermium pini* (pers.) lev. And the resin-top disease of scots pine: II. Lesion Anatomy. *Forestry*, *42*, 185–201. https://doi.org/10.1093/forestry/42.2.185

Verbylaitė, R., Beišys, P., Rimas, V., & Kuusienė, S. (2010). Comparison of ten DNA extraction protocols from wood of European aspen (*Populus tremula* L.). *Baltic Forestry*, *16*, 35–42.

Wijesinghe, S. (2019). The Genus *Cronartium* Revisited. *Plant Pathology & Quarantine*, *9*(1), 219–238. https://doi.org/10.5943/ppq/9/1/20

Zhang, K., Kaitera, J., Samils, B., & Olson, Å. (2022). Temporal and spatial dispersal of *Thekopsora areolata* basidiospores, aeciospores, and urediniospores. *Plant Pathology*, *71*, 668–683. https://doi.org/10.1111/PPA.13510

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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