Real-Time PCR-Based Monitoring of DNA Pools in the Tri-Trophic Interaction Between Norway Spruce, the Rust *Thekopsora areolata*, and an Opportunistic Ascomycetous *Phomopsis* sp.

Ari M. Hietala, Halvor Solheim, and Carl Gunnar Fossdal

Norwegian Forest and Landscape Institute, Pb. 115, NO-1431 Ås, Norway.
Accepted for publication 19 June 2007.

ABSTRACT

Hietala, A. M., Solheim, H., and Fossdal, C. G. 2008. Real-time PCR-based monitoring of DNA pools in the tri-trophic interaction between Norway spruce, the rust *Thekopsora areolata*, and an opportunistic ascomycetous *Phomopsis* sp. *Phytopathology* 98:51-58.

The difficulty in subculturing biotrophic fungi complicates etiological studies related to the associated plant diseases. By employing internal transcribed spacer rDNA-targeted quantitative real-time polymerase chain reaction, we now show that the heteroecious rust *Thekopsora areolata*, commonly associated in natural conditions to sapling shoots and cones of Norway spruce and leaves of wild bird cherry, frequently infects nursery-grown seedlings of the conifer. A spatial sampling scheme was used to investigate seedlings and saplings of Norway spruce showing phloem

necrosis: the highest concentration of DNA of *T. areolata* was recorded in the area with necrotic phloem. The separate analysis of bark and wood tissues suggested that the initial spread of the rust to healthy tissues neighboring the infection site takes place in the bark. A *Phomopsis* species found to coexist with *T. areolata* in several seedlings showed very high DNA levels in the upper part of the lesion, and even in the visually healthy proximal tissues above the lesions, which indicates that the ascomycete, most probably a secondary invader following primary infection by *T. areolata*, has a latent stage during early host colonization. We hypothesize that this hemibiotrophic mode of infection contributes to the successful coexistence of *Phomopsis* with a biotrophic rust.

Additional keywords: hemibiotrophy, infection, *Picea abies*.

Thekopsora areolata (Fr.) P. Magn. (*Pucciniastrum areolatum* (Fr.) Oth, *Pucciniastrum padi* (Schm. & Kunze) Diet.) is a Eurasian rust fungus recorded from England through the whole of Europe and from Russia to Kamtschatka and Japan (6,10,21). The fungus alternates between conifers and broadleaved trees in order to complete its life cycle with five distinct spore stages. Its main hosts are Norway spruce (*Picea abies* (L.) Karst.) and wild bird cherry (*Prunus padus* L.) (20).

T. areolata overwinters as telia in the leaves of wild bird cherry shed on the ground. In spring during rainy weather, the teliospores germinate and form basidiospores in synchrony with the flowering of Norway spruce. Female flowers of spruce, that eventually give rise to cones, are infected by basidiospores carried by air currents. Following the formation of pycnia on the outer sides of the cone scales and spermatization, dikaryotic hyphae form aecidia on both sides of the cone scales during the summer (6). The aecidia mature and open next spring and release aecidiospores, which infect cherry leaves. The scales of infected cones are characteristically spread apart from each other and no seeds are formed in infected cones, which often remain attached to shoots longer than the healthy ones. Basidiospores of *T. areolata* may also infect actively growing shoots of spruce, the fast-growing terminal shoots of spruce saplings being especially susceptible. Infected shoots usually become crooked, S-formed, with some dead tissue in the crooked part, and the shoots often die above the crook (13,19).

In a project focused on diseases of Norway spruce, we have been investigating the etiology of bark necrosis in nursery seed-

lings. Seedlings showing typical symptoms of *T. areolata* infection were often observed in forest nurseries, but no fruit bodies of the rust were observed in these seedlings. An ascomycete, a *Phomopsis* species, was now frequently codetected with *T. areolata* in these diseased shoots of Norway spruce. While necrotrophic fungi can trigger a hypersensitive response and programmed cell death in the host plant to feed subsequently as saprotrophs (7), rusts rely on haustoria in extracting nutrients from living host cells and enable their parasitic relationships with plants by long-term suppression of host defense responses (23). Due to this nutritional mode, biotrophic fungi such as rusts are difficult to culture on artificial media; however, the development of real-time polymerase chain reaction (PCR) technology has provided a powerful tool for quantitative detection of unculturable microbes in their natural substrates (9). To study the interaction of *T. areolata*, *Phomopsis* sp., and the hosting Norway spruce, we developed real-time PCR assays to quantify their DNA pools at the advancing margins of the lesions in diseased shoots.

MATERIALS AND METHODS

Sampling. A total of 100 2-year-old containerized nursery seedlings of Norway spruce that showed necrotic stem lesions with a slightly swollen bark area (Fig. 1) were collected from three different nurseries. To first screen the seedlings for the presence of candidate pathogens, a 5-mm-long bark sample was taken from the middle of the lesion from 50 randomly selected seedlings and processed for DNA analysis as described below. After this initial screen, 18 seedlings were sampled by taking 5-mm-long samples from the edges of the lesion area in order to obtain spatial colonization profiles. To examine tissue-specific colonization, the bark in the lesion area was separated from the wood with a scalpel in five seedlings, and the samples were processed separately for DNA isolation. In addition to diseased nursery seed-

Corresponding author: A. M. Hietala
E-mail address: Ari.Hietala@Skogoglandskap.no

lings, *T. areolata*-infected cones and shoots of naturally grown saplings of Norway spruce and the leaves of wild bird cherry were collected for DNA isolation/colonization profiling and internal transcribed spacer (ITS) rDNA sequence comparisons. These samples were collected from various localities in Norway, the sampling area stretching from Kristiansand in southern Norway to Stjørdal in central Norway covering a distance of 800 km (Table 1).

DNA isolation, cloning, and sequence analysis. For DNA isolation, infected bark and wood samples from Norway spruce were excised, frozen immediately in liquid N₂, and ground in

Fig. 1. The 2-year-old Norway spruce seedling Bu4 showing typical symptoms of *Thekopsora areolata* infection: crooked stem with dark brown, slightly swollen bark area with resin flow (bar = 1 cm).

liquid N₂-chilled containers for 2 min in an MM 300 mill (Retsch GmbH, Haan, Germany). Uredinia samples from *T. areolata*-infected leaves of wild bird cherry and aecidia samples from Norway spruce cones were ground with a pestle in an Eppendorf tube. To obtain negative references for testing the specificity of the real-time PCR assays used for quantifying Norway spruce and *T. areolata* DNA in diseased seedlings, the following strains of fungi commonly associated with diseased shoots of Norway spruce seedlings were cultured on cellophane-coated malt extract agar (2% malt extract and 1.5% agar) for DNA isolation: *Alternaria* sp. (strain 2006-210), *Botrytis cinerea* Pers. (strain 1983-145/1 Øa), *Epicoccum nigrum* Link (strain 1959-1898/1/1), *Fusarium* sp. (strain 2006-221), *Gremmeniella abietina* (Lagerberg) Morelet (strain 2002-26/2), *Herpotrichia juniperi* (Duby) Petrak (strain 1973-53), *Nectria fockeliana* Booth (strain 1973-74), *Phoma* sp. (strain 2006-229), *Phomopsis* sp. (strain 2002-62/1), *Rhizosphaera kalkhoffii* Bubak (strain 1997-1/6), *Sirococcus conigenus* (D.C.) P.F. Cannon & Minter (strain 2000-58/2), and *Tiarasporella parca* (Berk and Broome) Whitney (strain 1991-727/28) (all these isolates were obtained from the culture collection of the Norwegian Forest and Landscape Institute). In addition, aecidia samples of *Cronartium ribicola* (collection Ru29) and *C. flaccidum* (collection Ru30) were included as negative reference material. DNA isolation was performed with the Plant DNA Mini Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Amplification was carried out with ITS1-F and ITS4-B primers (5) in 25-µl reaction volumes using HotStarTaq DNA Polymerase (Qiagen) according to the manufacturer's instructions. After gel electrophoresis, the PCR products were purified with GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Little Chalfont, England), ligated into a cloning vector (pGEM-T Easy; Promega, Madison, WI), and transformed into *Escherichia coli* (DH5-α). For sequencing, plasmid DNA was isolated and purified using Wizard Plus Minipreps (Promega). Three distinct colonies were sequenced in both directions on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The alignments of complementary strands and the similarity studies within sequenced samples and other known published ITS regions in the NCBI GenBank sequence database were determined with Seqman software (Lasergene, DNASTAR Inc., Madison, WI).

TABLE 1. Rust samples sequenced for internal transcribed spacer rDNA region in the study

Code	Rust	Host	Source	Spore stage	Locality	Collector ^a	Accession no.
Bu4 2	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Seedling	None detected	Buskerud, Øvre Eiker, Hokksund	1	EF363336
Bu5 10	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Seedling	None detected	Buskerud, Øvre Eiker, Hokksund	1	DQ445891
LR 4B	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Seedling	None detected	Telemark, Sauherad, Gvarv	1	DQ445892
LR 14BA	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Seedling	None detected	Telemark, Sauherad, Gvarv	1	DQ445894
Sk1 10	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Seedling	None detected	Sør-Trøndelag, Melhus, Skjerdingsstad	1	DQ445901
Spl 1 5	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Seedling	None detected	Sør-Trøndelag, Melhus, Skjerdingsstad	1	DQ087229
Spl3 5	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Seedling	None detected	Sør-Trøndelag, Melhus, Skjerdingsstad	1	DQ087229
Thek 1	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Cone	Aecidia	Østfold, Eidsberg, Ramstad	1	DQ445895
Thek 2	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Cone	Aecidia	Østfold, Eidsberg, Ramstad	1	DQ087230
Thek 3	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Cone	Aecidia	Østfold, Eidsberg, Ramstad	1	DQ445896
Thek 4	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Cone	Aecidia	Hedmark, Trysil, Bekkvang	1	DQ445897
Thek 5	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Cone	Aecidia	Hedmark, Trysil, Bekkvang	1	DQ445898
Thek 7	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Cone	Aecidia	Nord-Trøndelag, Stjørdal, Hegra	2	DQ445899
Sta1	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Sapling	Aecidia	Hedmark, Stange, Tangen	1	DQ445902
Sta2	<i>Thekopsora areolata</i>	<i>Picea abies</i>	Sapling	None detected	Hedmark, Stange, Tangen	1	DQ445903
2A	<i>Thekopsora areolata</i>	<i>Prunus padus</i>	Leaf	Uredinia	Nord-Trøndelag, Stjørdal, Lånke	2	DQ445904
Ru4	<i>Thekopsora areolata</i>	<i>Prunus padus</i>	Leaf	Uredinia	Aust-Agder, Birkenes, Birkeland	1	DQ087231
Ru5	<i>Thekopsora areolata</i>	<i>Prunus padus</i>	Leaf	Uredinia	Vest-Agder, Kristiansand, Kroken	1	DQ445900
Hal1	<i>Thekopsora areolata</i>	<i>Prunus padus</i>	Leaf	Uredinia	Østfold, Halden, Lundene	1	DQ445905
Ru6	<i>Pucciniastrum ebllobii</i>	<i>Epilobium angustifolium</i>	Leaf	Uredinia	Vest-Agder, Kristiansand, Foss	1	DQ445906
Ru11	<i>Pucciniastrum ebllobii</i>	<i>Epilobium watsonii</i>	Leaf	Uredinia	Vest-Agder, Kristiansand, Foss	1	DQ445907
Ru29	<i>Cronartium ribicola</i>	<i>Pinus peuce</i>	Stem	Aecidia	Buskerud, Øvre Eiker, Hokksund	1	DQ445908

^a 1, H. Solheim; and 2, A. Hietala.

Oligonucleotide primers used for quantification of the host and pathogen. The real-time PCR primers used for monitoring *T. areolata* colonization in infected seedlings were designed with the Primer Express software 1.5a provided with Applied Biosystems real-time quantitative PCR systems (Applied Biosystems) by employing a conserved and species-specific sequence area in the ITS rDNA gene cluster. The amount of Norway spruce DNA in analyzed samples from infected nursery seedlings was estimated by using the polyubiquitin primer/probe set previously described (9). In addition, we monitored the presence of *G. abietina* and *Phomopsis* sp., pathogenic fungi commonly associated with necrotic stem lesions in Norway spruce seedlings, with primer/probe sets described by Børja et al. (3). The primer/probe sets used are shown in Table 2.

PCR conditions and specificity of primers. The real-time PCR detection of *T. areolata* DNA was performed in SYBR Green PCR Mastermix (P/N 4309155; Applied Biosystems), while amplification of Norway spruce, *G. abietina*, and *Phomopsis* DNA was performed with TaqMan Universal PCR Master Mix (P/N 4304437; Applied Biosystems). A primer concentration of 50 nM was chosen for the *T. areolata* primers, while the primer and probe concentrations of 150 and 333 nM (9), respectively, were used for detecting the DNA of Norway spruce. For *G. abietina* and *Phomopsis* sp., a primer concentration of 300 nM and a probe concentration of 400 nM were used (3). All PCR analyses were performed in singleplex conditions.

To construct standard curves for quantification of Norway spruce and pathogen DNA, DNA isolated from a visually healthy control seedling where DNA of *T. areolata* or *Phomopsis* sp. could not be detected by real-time PCR, from aecidia of *T. areolata* formed on a naturally infected cone of the sample Thek2 (Table 1), and from a pure culture of *Phomopsis* sp. (strain 2002-62/1) was quantified by a Versafluor fluorometer (Bio-Rad, Hercules, CA) and a PicoGreen DNA quantification kit (Molecular Probes, Eugene, Oregon) and then pooled together in known concentrations. The host DNA standard curve samples (100, 10, 1, and 0.1 ng of Norway spruce DNA) were all spiked with 1 ng of *T. areolata* and 1 ng of *Phomopsis* sp. DNA. The *T. areolata* DNA standard curve samples (100, 10, 1, 0.1, 0.01, and 0.001 pg of *T. areolata* DNA) were all spiked with 1 ng of Norway spruce and 1 ng of *Phomopsis* sp. DNA. The *Phomopsis* DNA standard curve samples (1,000, 100, 10, 1, and 0.1 pg of *Phomopsis* sp. DNA) were all spiked with 1 ng of Norway spruce and 1 ng of *T. areolata* DNA. All the standard curve samples were replicated three times within a single PCR run. To ensure that the cycle threshold (Ct) values (Ct determines the PCR cycle at which the reporter fluorescence exceeds that of the background) from the experimental samples fell within the standard curves and to investigate the presence of compounds inhibitory to PCR, 3- or

4-log dilution series were prepared for all the experimental samples. Each experimental sample had undiluted DNA as the most concentrated, and all log dilutions of a sample were used as templates in real-time PCR. For both of the series, the experimental and standard curve samples, 3 µl of the DNA solution was used as the template for each 25-µl PCR. For experimental samples, each reaction was repeated twice in a separate PCR run. PCR cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For *T. areolata*, the PCR cycling was followed by a melting curve analysis: after a denaturation step at 95°C for 15 s, the sample was cooled to 60°C for 1 min followed by a linear temperature increase to 95°C, while continuously monitoring the fluorescent signal. Fluorescence emissions were detected with an ABI Prism 7700 (Applied Biosystems). Data acquisition and analysis were performed with the Sequence Detection System software package (1.7a; Applied Biosystems). Genomic DNA from healthy Norway spruce and from the included reference fungi were used as negative controls for the *T. areolata* and *Phomopsis* markers, while fungal DNA served as a negative control for the host marker. To further verify the specificity of the real-time PCR data from experimental samples, selected DNA samples from seedlings were PCR amplified both by the basidiomycete-specific primer pair ITS1-F and ITS4-B and by the universal fungal-specific primer pair ITS1-F and ITS4 (5), followed by sequencing. For this purpose, the PCR products purified with MinElute PCR Purification Kit (Qiagen) were directly sequenced in both directions on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) by using the ITS primers as sequencing primers.

Calculation of fungal colonization levels. Standard curves for Norway spruce, *T. areolata*, and *Phomopsis* sp. were constructed based on the relationship of Ct values and known host and pathogen DNA concentrations: the Ct values were plotted against log-transformed DNA amounts, and linear regression equations were calculated for the quantification of DNA pools in unknown samples.

RESULTS

Alignment of *Thekopsora* sequences from different sources.

All the *T. areolata* samples studied yielded an 812-bp amplification product with the ITS1-F and ITS4-B primers employed. The corresponding ITS rDNA sequences comprised a 50-bp 3' end for the 18S rDNA gene, 210 bp in the ITS1 region, 162 bp for the 5.8S rDNA gene, 219 bp in the ITS2 region, and a 171-bp 5' end for the 28S rDNA gene. The ITS1-5.8S-ITS2 sequences from *T. areolata* samples from different hosts/tissue types showed little variation; the similarities among *T. areolata* sequences obtained from seedlings, saplings, and cones of Norway spruce and from

TABLE 2. Primers/probes used for monitoring pathogen colonization

Species detected	Primer/probe set ^a	Product size (bp)	Fluorescence label	Amplicon melt point
<i>Thekopsora areolata</i>	Forward: 5'-TTTGTGCATTGATGGACTTTAAATAAT-3' Reverse: 5'-CCAAGAGATCCATTGTTAAAGTTATATGAT-3'	72	SYBR Green	69.6
<i>Gremmeniella abietina</i>	Forward: 5'-CACCCGTGCCTATATTACTCTGTTG-3' Reverse: 5'-TATAGTACTCAGACGCGTAAATAATTCATAGGTT-3' Probe: 5'-TTCCCGGGCCTCAACCCCC-3'	90	FAM	NA ^b
<i>Phomopsis</i> sp.	Forward: 5'-GCACCCAGAAACCCCTTTGTG-3' Reverse: 5'-AAGAGTTGACTTGGCCGCC-3' Probe: 5'-CGGTAACGAGGAGCAGCCCGC-3'	116	FAM	NA
<i>Picea abies</i>	Forward: 5'-TGGTCGTACTCTGGCCGATTATA-3' Reverse: 5'-ACACCTAGCGGCACACAGTTAA-3' Probe: 5'-TGCTCCGTCTCCGTGGTGGCT-3'	96	VIC	NA

^a The primer set for detection of *Thekopsora areolata* DNA was designed in the present study, while the primer/probe sets for detection of the other two pathogens and host DNA were designed by Børja et al. (3) and by Hietala et al. (9), respectively.

^b Not applicable.

leaves of wild bird cherry were 99 to 100, 98 to 99, 98 to 100, and 98 to 99%, respectively, while the overall sequence similarity within this material was 98 to 100%. The conserved sequence was compared with sequences from the included reference rusts and with sequences available at the NCBI GenBank sequence database. Among all the sequences available, the rust fungi *Puccinia kuehnii* (W. Krüger) E.J. Butler, *C. ribicola* J.C. Fischer, *C. flaccidum* Alb. & Schwein, and *Peridermium harknessii* J.P. Moore

showed the greatest similarity to *T. areolata*, but in addition to numerous point mutations in the ITS1 and ITS2 regions, these reference species had a number of inserts in these regions (Fig. 2).

Specificity of the primer/probe sets used and the standard curves constructed. The primer pair now employed for quantifying *T. areolata* colonization in diseased Norway spruce seedlings amplified an 81-bp area between the 3' end of the ITS1 region and the 5' end of the 5.8S rDNA gene (Fig. 2). *Puccinia*

ITS 1		
<i>T. areolata</i>	GTAAAGGAGTGCACTTTATT--GTGACTCTGACACTTTTAAATAT---TAAACCATT--AAAC	59
<i>P. kuehnii</i>	.C.....--...G....AC.....ATT...C.....-A....	69
<i>C. ribicola</i>	.C.....--...G....A.AC.....ATT...C.....-A....	77
<i>P. harknessii</i>	.C.....A....-...G....AC.....ATT.C.C.....-A..A	77
<i>C. flaccidum</i>	.C.....TT..G....AC.....ATT...C.....TA....	125
ITS 1		
<i>T. areolata</i>	CCCCAAGCTGTTATGTGTGCCTTTTA---GGTATAGCATCTCAGTAGTA-CGTC-----	108
<i>P. kuehnii</i>	T.....TT--.....AC....-...TAACTTGTGTTGTG	133
<i>C. ribicola</i>	T.....TT--.....AC....-...TAACTTGTGTTGTG	141
<i>P. harknessii</i>	AT....T.....TTTT.....C....AT...ACCTTGTGCTGTA	144
<i>C. flaccidum</i>	T.....TT--.....AC....-...TAACTTGTGTTGTG	189
ITS 1		
<i>T. areolata</i>	-TTAACATTGTTTGTGACA-TTACCCCCCTTATAAGTGACCCCTTTTGTGCAATTGA-TGGACT	172
<i>P. kuehnii</i>	T..C....GA.C.C.....-.....T.....T.....T---.A.T.ACT..	196
<i>C. ribicola</i>	T..C....GA.C.C.....-.....T.....T.....T---.A.TA.ACT..	206
<i>P. harknessii</i>	T..C....AA.CCC....A.....T.....TT....GT.----A.A.ACT..	207
<i>C. flaccidum</i>	T..C....GA.C.C.....-.....T.....TTT....T.TA...A.T.ACT..	255
ITS 1 5.8S rDNA		
<i>T. areolata</i>	TTA-AATAAT GTT-TTAAGAATGTAAACCTAT----- AATCATATAACTTTTAAACAATGGATCT	230
<i>P. kuehnii</i>	...T..A.....-.....T.ATAAAAA...A.....	263
<i>C. ribicola</i>	...T..A.....T.....CT.TAAAA--A..AA.....	272
<i>P. harknessii</i>	...T..A.....T.....CTGTAAAA--...A.....	271
<i>C. flaccidum</i>	...T..A.....C.....AAAAAAACCCCTT.TAA.A.....	323
5.8S rDNA		
<i>T. areolata</i>	CTTGGCTCTC ACATCGATGAAGAACACAGTGAAATGTGTTAAGTAATGTGAATTGCAGATTGAGTGA	298
<i>P. kuehnii</i>	A.....	329
<i>C. ribicola</i>	A.....	340
<i>P. harknessii</i>	A.....	339
<i>C. flaccidum</i>	A.....	391
5.8S rDNA		
<i>T. areolata</i>	ATCATCGAATCTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTGAGTGTCA	365
<i>P. kuehnii</i>	TG.....	386
<i>C. ribicola</i>	TG.....	395
<i>P. harknessii</i>	CG.....	394
<i>C. flaccidum</i>	CA.....	446
ITS 2		
<i>T. areolata</i>	TGAAACCCTCTCATCTAATTCTT-TATTTGAT----ATAAGGAGTT---GGTATCGATGTTGAG	424
<i>P. kuehnii</i>	C.....-.....T..T---.....-GT.TA.....	459
<i>C. ribicola</i>	C.....-.....T..T---.....-GT.TA.....	467
<i>P. harknessii</i>	C.....-.....T..T---.....-GT.TA.....	466
<i>C. flaccidum</i>	C.....A....TT.TTTAT.....TTGT.TA.....	524
ITS 2		
<i>T. areolata</i>	TGTTGCCGTCATT-----GGCTCACTTTAAATACATAAGTACTTTTATCTTGAAAAATA--	476
<i>P. kuehnii</i>	T.....-.....A.....	511
<i>C. ribicola</i>	T.....-.....A.T-	520
<i>P. harknessii</i>	T.....-.....T..G.....AA	520
<i>C. flaccidum</i>	T.....TATATATATATA.....T.....-.....	589
ITS 2		
<i>T. areolata</i>	--AATGGAGAAATACTTGGTGTAATTTTAT--GTTTCATTAAGGAGTG TGG----TTTAATTAC	533
<i>P. kuehnii</i>	-A.....T.....--TA.....G.....A.....	569
<i>C. ribicola</i>	-A.....T.....--TA.....G.....A.....	578
<i>P. harknessii</i>	TA.....T.....--TA.....G.....A.....	579
<i>C. flaccidum</i>	-A.-.....T.....ATTA.....G.....GTAGTTT.....	653
ITS 2		
<i>T. areolata</i>	T-ACAGCCATTGTGTT--TTGGATAAATAGCTTCCTAACCTATTATA-TTTAACTTT	587
<i>P. kuehnii</i>	CA.....CCA.T---C.T..	606
<i>C. ribicola</i>	CA.....AC...T.--CA---.T..	628
<i>P. harknessii</i>	CA.....C...T..TC..C..T..	634
<i>C. flaccidum</i>	.T.....A..TT.CA.....T.....C.A.T.--CCATTTT...	709

Fig. 2. CLUSTAL W (1.82) multiple sequence alignment of a conserved internal transcribed spacer (ITS) rDNA sequence of *Thekopsora areolata* (identical sequences obtained from infected seedlings [sample Spl 3 5, accession no. DQ087229] and cones [sample Thek 2, DQ087230] of Norway spruce and from leaves of wild bird cherry [sample Ru4, DQ087231]) and its closest matches at the NCBI GenBank Sequence, *Puccinia kuehnii* (AJ406049), *Cronartium ribicola* (L76499), *Peridermium harknessii* (L76510), and *Cronartium flaccidum* (AY566270). The 3' end of 18S gene and the 5' end of 28S gene are not shown in alignment. The line symbol (–) indicates a gap in the alignment, a dot (.) denotes identical nucleotides between the sequences at the given positions. The real-time PCR primers used for quantification of the rust colonization levels in infected seedlings and saplings of Norway spruce are underlined and in bold.

kuehnii, *C. ribicola*, *C. flaccidum*, and *Peridermium harknessii* (Fig. 2), the included reference rusts and all the other fungal sequences available in the NCBI GenBank sequence database (data not shown), differed from *T. areolata* in several nucleotides in the binding sites of both primers. The primer set developed for monitoring *T. areolata* did not detect the DNA of Norway spruce, and the primer/probe set used for detecting DNA of Norway spruce did not detect the DNA of *T. areolata* (data not shown). In addition, these two sets did not detect the DNA of the included reference fungi when 1 ng of DNA from these was used as the template. In the standard curve samples constructed for Norway spruce, there was a linear relationship between the Ct values and the log-transformed amount of DNA in the range of 100 to 0.1 ng of host DNA (Fig. 3A). In the standard curves for *T. areolata* and *Phomopsis* sp., there was a linear relationship between the Ct values and the log-transformed amount of pathogen DNA in the range of 100 to 0.001 pg and 1,000 to 0.1 pg, respectively (Fig. 3B and C). The DNA amount standard curves for Norway spruce, *T. areolata*, and *Phomopsis* sp., based on the relationship of the log amount of template (x) generated from known host and pathogen DNA concentrations and their corresponding Ct values (y), were $y = 29.858 - 3.591x$, $y = 23.804 - 3.4951x$, and $y = 30.337 - 3.381x$, respectively. For the Norway spruce, *T. areolata*, and *Phomopsis* sp. standard curves, the PCR amplification efficiencies (E), calculated with the equation $E = (10^{(-1/\text{slope})} - 1) \times 100$, were 89.9, 93.2, and 97.6%, respectively. The melting curve analysis showed a single peak with a melt point of 69.6°C in the standard curve samples of *T. areolata*.

Symptoms of the disease and colonization profiles of *T. areolata* and other fungi monitored. The diseased seedlings and saplings of Norway spruce showed a few centimeter long, dark brown, slightly swollen necrotic bark area with resin flow, and many plants were crooked in the infected area (Fig. 1). On some occasions, the lesions were not distinct and the necrotic tissue was longer (not shown). In the middle of the lesions, both the outer bark and the phloem were dark and necrotic, while at proximal areas above and below this region, the phloem was light brown, eventually transitioning to a green color when examining more distal areas. The change in the phloem color from dark brown to light brown was abrupt, while the transition from light brown to green phloem was often gradual. Fruit bodies (aecidia, pycnia) were not observed in the examined seedlings. Similar symptoms as those observed in the nursery seedlings were also noted in the 5- to 10-m-long saplings included as reference material. Aecidia were observed in one of the leader shoots of these saplings.

T. areolata was detected by real-time PCR in all the seedlings and saplings showing the symptoms described above. As with the standard curve samples, a single peak with a melt point of 69.6°C was observed for experimental samples amplified with the *T. areolata* primer pair. To test for the presence of substances inhibitory to PCR, log-dilution series were prepared for all experimental samples. The differences in the corresponding Ct values between the undiluted and 10-fold diluted DNA samples were, particularly for *T. areolata*, often well below the expected difference of approximately 3.3 cycles (for example, the *Thekopsora* Ct values of 17.1, 18.3, and 21.7 were obtained for an undiluted, 10-fold, and 100-fold diluted sample from seedling Sk4, but only the latter two values are reliable as they gave the expected difference in the Ct value), which indicates the presence of substances inhibitory to PCR in the undiluted samples and/or excess levels of the template. In contrast, the differences in the corresponding Ct values between the 10- and 100-fold diluted DNA samples were in the range also observed between the log dilutions of the corresponding standard curves. This compatibility indicates that substances inhibitory to PCR amplification were not present in the diluted samples, and the ten- and hundred-fold diluted DNA samples were subsequently used for calculating the amounts of host and pathogen DNA present in the experimental samples. Within the

area colonized by *T. areolata*, the host DNA yields were in general the lowest in the middle of the lesion area with dark brown phloem, while the highest host DNA yields were invariably obtained in the lower lesion area with light brown phloem. When normalized against host DNA yield and expressed as fungal/host DNA amount (%), the highest levels of *T. areolata* DNA were recorded in the area with dark brown phloem having a relatively low yield of host DNA, whereas in the lesion margins, the DNA levels of the rust declined steeply (Fig. 4).

Out of the 18 seedlings examined along the lesion area with a spatial sampling scheme, *Phomopsis* sp. was codetected with *T. areolata* in five seedlings. Regarding the saplings analyzed as a reference material, *Phomopsis* sp. was codetected with *T. areolata*

Fig. 3. Standard curves for quantifying the DNA amount of Norway spruce, *Thekopsora areolata*, and *Phomopsis* sp; log-transformed DNA amounts were plotted against cycle threshold (Ct) values, and the linear regression equations obtained were used for calculating the amounts of the three DNA pools in experimental samples. The host DNA standard curve (A) samples (100, 10, 1, and 0.1 ng of Norway spruce DNA) were all spiked with 1 ng of *T. areolata* and 1 ng of *Phomopsis* sp. DNA, and analyzed by the primer/probe set targeting DNA of Norway spruce. The *T. areolata* DNA standard curve (B) samples (100, 10, 1, 0.1, 0.01, and 0.001 pg of DNA) were all spiked with 1 ng of Norway spruce and 1 ng of *Phomopsis* sp. DNA, and analyzed by the primer set targeting DNA of the rust fungus. The *Phomopsis* DNA standard curve (C) samples (1,000, 100, 10, 1 and 0.1 pg of *Phomopsis* DNA) were all spiked with 1 ng of Norway spruce and 1 ng of *T. areolata* DNA, and analyzed by the primer/probe set targeting DNA of *Phomopsis*. Three PCR replicates were run on each sample.

in one of the three saplings analyzed, but its presence was restricted to a single sampling position (Fig. 4A). In contrast to *T. areolata*, high levels of *Phomopsis* sp. DNA were observed particularly in the upper part of the lesion with light brown phloem and even in healthy-appearing bark with green phloem, and often there was no steep decline in its DNA levels even at the lesion margins (Fig. 4B, upper lesion). The third monitored pathogen, *G. abietina*, was not detected in any of the examined Norway spruce material. The ITS rDNA PCR and sequencing performed for verification of the real-time PCR data confirmed the presence of *T. areolata* and *Phomopsis* sp. in the seedlings analyzed (Fig. 4). While these *T. areolata* sequences obtained by direct sequencing of the PCR products showed 98 to 100% similarity to those obtained by cloning from seedlings, saplings, and cones of Norway spruce and from leaves of wild bird cherry the *Phomopsis* sp. sequences obtained here were 99 to 100% similar to those of the *Phomopsis* sp. recently characterized by Børja et al. (3).

In order to investigate fungal spread within different tissue types, five seedlings were sampled in such a way that the bark was separated from the wood, and these tissues were processed separately. For both *T. areolata* and *Phomopsis* sp., the colonization profiles, when using host DNA yield as the normalizer, were similar between the tissue types (Fig. 4C), but in the lesion margins, bark contained regularly higher normalized DNA levels of the rust than wood.

DISCUSSION

By amplifying the ITS1-5.8S-ITS2 region of *T. areolata* from DNA of diseased nursery-grown Norway spruce seedlings and comparing it to *T. areolata* sequences obtained from cones and saplings of Norway spruce and from leaves of wild bird cherry, we showed that this wide-spread pathogenic rust is commonly associated with stem lesions in container-grown 2-year-old seedlings of the conifer. The symptoms observed in these seedlings are similar to those observed in saplings infected frequently by the rust in forest conditions. In laboratory experiments published in 1900, Klebahn (11) was able to artificially inoculate shoots of Norway spruce seedlings with basidiospores of the pathogen; no fruit bodies were formed in those experiments, but the author noted the strong smell of sugary liquid exuded by pycnia. Based on fruit body observations, Roll-Hansen (19) in the 1940s showed the presence of *T. areolata* on 3- to 4-year-old nursery seedlings of Norway spruce. Otherwise there are no reports of young spruce seedlings hosting this rust. This is most likely due to the fact that the rust is difficult to culture in artificial media, and that the conspicuous acedia allowing conventional identification of the fungus are not formed in the currently produced 2-year-old Norway spruce seedlings. In Norway spruce saplings, acedia of *T. areolata* develop inside the bark and the poorly developed bark of young seedlings compared with that of saplings may explain the absence of fruit bodies in nursery seedlings. An alternative explanation is that mating, an event preceding acedia formation, does not commonly take place in nursery conditions.

Based on inoculation experiments (11,19,22), *T. areolata* is regarded to alternate between the conifer spruce (*Picea*) and broadleaved trees in the genus *Prunus* in order to complete its life cycle. Our comparison of ITS rDNA sequences of *T. areolata* from the different hosts/tissue types is in agreement with this concept, as ITS rDNA sequence variants specific to the hosts/tissue types were not detected. According to our preliminary observations and depending on annual weather conditions and locality of the nursery, up to four Norway spruce seedlings per a 96-seedling container tray can show symptoms characteristic of *T. areolata* infection. The affected seedlings are typically not located as a group but scattered within the nursery field and also within the container trays, which would be in accordance with an airborne inoculum, i.e., air-current spread of basidiospores formed on

fallen leaves of wild bird cherry. The incidence of *T. areolata* on sapling shoots of Norway spruce in forest conditions has been shown to depend on their distance to wild bird cherry (13). Thus, eradication of this alternate host from the immediate vicinity of affected Norway spruce nurseries should be recommended.

Based on a phylogenetic analysis of the 28S rDNA sequence, Maier et al. (14) concluded that the genus *Thekopsora* is polyphyletic and that *T. areolata* is closely related to *Cronartium* spp. This is compatible with our ITS sequence data as, among the deposits in the NCBI GenBank sequence database, the pine stem rust fungi *C. ribicola* and *C. flaccidum* showed high sequence similarity in the ITS regions to *T. areolata*. *Puccinia kuehnii* (a leaf rust of sugarcane) and *Peridermium harknessii* (a stem rust of pine), the other reference species with high sequence similarity to *T. areolata* in the ITS regions, were unfortunately not included in the study of Maier et al. (14).

The recently introduced real-time PCR procedure provides a robust tool for detecting and quantifying unculturable pathogens, such as rust fungi, in their natural substrates (2). For designing the real-time PCR assay, we employed an ITS1-5.8S-rDNA region that is species-specific to *T. areolata* to ensure specificity. Due to the high copy number of ITS rDNA gene cluster in the genome, the ITS-based detection is about 100 times more sensitive than an assay detecting a single copy gene (18). The lowest point in the standard curve for *T. areolata* was 0.001 pg of DNA, which equals 0.965 Mbp DNA (1 pg DNA = 965 Mbp [1]). The genome sizes of *T. areolata* and other *Thekopsora* species are not known, but as reviewed by Leonard and Szabo (12), in the rust genus *Puccinia*, the genome sizes range between 67 and 154 Mb. We thus conclude that our real-time PCR assay should be able to quantitatively detect even single nuclei of *T. areolata*.

There was a strong gradient in the amount of *T. areolata* DNA along the lesion length, with the highest levels being recorded in the area with necrotic phloem. Similar gradients in the amount of pathogen DNA have been observed in the Norway spruce-*Heterobasidion annosum* pathosystem for example, where pathogen DNA showed the highest levels next to the inoculation site in the bark and declined along with the distance away from this site (9). It seems obvious that the necrotic bark with high DNA levels of the rust represents initial infection sites from which *T. areolata* is spreading both upwards and downwards to the neighboring healthy tissues. The steep decline in DNA levels of *T. areolata* in the margin areas of the lesion coincided with the change of the phloem color from dark brown to light brown, indicating that the rust infection results in host response, leading eventually in host cell death and degradation of target DNA. The analysis of bark and wood tissues separately indicated that the rust is able to also colonize wood in the area with dark brown phloem, but its initial spread to healthy tissues neighboring the infection site presumably takes place within the bark.

In 5 out of the 18 seedlings examined in detail with a spatial sampling scheme, *T. areolata* was found to coexist with a *Phomopsis* sp. showing 99 to 100% ITS rDNA sequence similarity to the *Phomopsis* species recently characterized from Norway spruce seedlings (3). Compatible with our data, Cech and Perny (4), based on fruit body observations and fungal isolations, showed that *Phomopsis* spp. are commonly present in *T. areolata*-infected shoots of Norway spruce saplings in forest conditions. Based on pathogenicity tests, Børja et al. (3) concluded that this *Phomopsis* species is a weak pathogen depending on specific host-predisposing conditions such as abiotic stress or infection by other pathogens. Hahn (8) also describes *Phomopsis occulta* as a weak pathogen in conifers following injuries caused by frost, transplanting, drought, and parasitic fungi such as the white pine blister rust (*Cronartium ribicola*). We consider it highly likely that the *Phomopsis* sp. now codetected with *T. areolata* is a secondary invader benefiting from the primary infection by the rust. The fact that considerably higher maximal DNA levels were observed for *Phomopsis* sp. than

T. areolata can probably be explained by their differential karyotic condition; while the vegetative stage of *T. areolata* on Norway spruce is either uni- or binucleate depending on whether spermatization has taken place, the *Phomopsis* sp. has multinucleate cells in the vegetative stage (A. M. Hietala, H. Solheim, and C. G. Fossdal, unpublished observation).

Suppression of host defense responses, such as the hypersensitive response involving death of colonized cells, is essential for rust fungi, which rely on the success of haustoria, specialized hyphae for nutrient absorption, in extracting nutrients from living host cells (16). The fact that the *Phomopsis* sp. coexisting with *T. areolata* in the diseased seedlings showed very high DNA levels even in the healthy tissues above the lesions indicates that the fungus has a quiescent stage in the infection process. In several fruit diseases, *Phomopsis* species circumvent host defense responses by having a latent colonization stage during fruit development and by triggering the expression of pathogenicity factors after harvest (24). This transient type of biotrophy in which the fungus shows a biotrophic phase during early host colonization and then switches to a necrotrophic phase is referred to as hemibiotrophic (15). The initial penetration and invasion processes of a host by a fungal pathogen in the absence of a significant plant resistant response ensure successful colonization of host tissue and subsequent necrotrophic activities. Like hemibiotrophic fungi during their biotrophic stage, rusts have limited secretory activity, especially of hydrolytic enzymes, in order to minimize damages to host cells (15). It remains to be examined whether the detected *Phomopsis* species triggers the expression of cell-wall-degrading enzymes for example after the growing season. In any case, the mode of infection of the *Phomopsis* sp. resembles that of a biotroph as the fungus is apparently able to colonize spruce bark without triggering host cell death. This colonization mode undoubtedly contributes to the successful invasion of living host tissue by *Phomopsis* sp. and it may even facilitate its coexistence with a biotrophic rust.

Real-time PCR is currently the most sensitive quantification method for nucleic acids. Regarding quantification of infection in plants, the tool has so far been utilized for monitoring infection by singular pathogens. The multiplexing option provided by different fluorescent labels of the probe would allow simultaneous monitoring of several DNA pools in a single tube (9). Due to the high throughput nature of real-time PCR, we anticipate that the tool will become widely used also in ecological studies when monitoring events such as colonization of a common niche by several microorganisms. Besides monitoring colonization, real-time PCR can also be used for monitoring transcriptomes of the interacting host and fungi during early stages of infection (17), a situation still poorly understood in rust–host interaction.

ACKNOWLEDGMENTS

This project was financed by the Research Council of Norway (Project no. 156881/I10) and Skogforsk. The nurseries Buskerud Skogselskaps planteskole, Skogplanter Midt-Norge AS avd. Skjerdingsstad, Sønsterud planteskole AS and Telemark Skogplanteskule AS provided samples, mostly via the nursery consultants M. Andersen and A. Strømberg. C. Kierulf, Skogforsk, brought some samples from Skjerdingsstad. Parts of the laboratory work were performed by our technicians: O. Olsen did fungal isolations, while I. Heldal and L. Ljevo were responsible for cloning and sequencing.

LITERATURE CITED

- Bennett, M., and Smith, J. B. 1976. Nuclear DNA amounts in angiosperms. *Philos. Trans. R. Soc. London B* 274:227-274.
- Boyle, B., Hamelin, R. C., and Séguin, A. 2005. In vivo monitoring of obligate biotrophic pathogen growth by kinetic PCR. *Appl. Environ. Microbiol.* 71:1546-1552.
- Børja, I., Solheim, H., Hietala, A. M., and Fossdal, C. G. 2006. Etiology and real-time polymerase chain reaction-based detection of *Gremmeniella*- and *Phomopsis*-associated disease in Norway spruce seedlings. *Phytopathology* 96:1305-1314.
- Cech, T. L., and Perny, B. 1995. *Pucciniastrum areolatum* (Alb. Et Schw.) Liro (Uredinales) und andere Mikropilze im Zusammenhang mit Wipfelschäden an Jungfichten (*Picea abies* (L.) Karst.). *FBVA-Berichte* 88:5-28.
- Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes – Application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2:113-118.
- Gäumann, E. 1959. Die Rostpilze Mitteleuropas. Buchdruckerei Böhler, Bern.
- Govrin, E. M., and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10:751-757.
- Hahn, G. G. 1943. Taxonomy, distribution and pathology of *Phomopsis occulta* and *P. juniperivora*. *Mycologia* 35:112-129.
- Hietala, A. M., Eikenes, M., Kvaalen, H., Solheim, H., and Fossdal, C. G. 2003. Multiplex real-time PCR for monitoring *Heterobasidion annosum* colonization in Norway spruce clones that differ in disease resistance. *Appl. Environ. Microbiol.* 69:4413-4420.
- Hiratsuka, N. 1936. A monograph of the Pucciniastreae. *Mem. Tottori Agric. Coll.* 4:1-374.
- Klebahn, H. 1900. Kulturversuche mit Rostpilzen IX. *Jahrb. Wissensch. Bot.* 35: 660-710.
- Leonard, K. J., and Szabo, L. J. 2005. Pathogen Profile. Stem rust of small grains and grasses caused by *Puccinia graminis*. *Mol. Plant Pathol.* 6:99-111.
- Lilja, S. 1967. Tuomen merkityksestä kuusen tuomiruostesienien, *Pucciniastrum padi* (Kunze & Schm.) Diet., esiintymiselle kuusessa. (In Finnish.) *Silva Fenn.* 1:45-62.
- Maier, W., Begerow, D., Weiss, M., and Oberwinkler, F. 2003. Phylogeny of the rust fungi: An approach using nuclear large subunit ribosomal DNA sequences. *Can. J. Bot.* 81:12-23.
- Mendgen, K., and Hahn, M. 2002. Plant infection and the establishment of fungal biotrophy. *Trends Plant Sci.* 7:352-356.
- Mendgen, K., Struck, C., Voegelé, R. T., and Hahn, M. 2000. Biotrophy and rust haustoria. *Physiol. Mol. Plant Pathol.* 52:95-114.
- Mehli, L., Kjellsen, T. D., Dewey, F. M., and Hietala, A. M. 2005. A case study from the interaction of strawberry and *Botrytis cinerea* highlights the benefits of co-monitoring both partners at genomic and mRNA level. *New Phytol.* 168:465-474.
- Nielsen, K., Yohalem, D. S., and Jensen, D. F. 2002. PCR detection and RFLP differentiation of *Botrytis* species associated with neck rot of onion. *Plant Dis.* 86:682-686.
- Roll-Hansen, F. 1947. Nytt om lokkrusten (*Pucciniastrum padi*). (In Norwegian.) *Medd. Norske Skogforsk. Ves.* 9:503-510.
- Roll-Hansen, F. 1965. *Pucciniastrum areolatum* on *Picea engelmannii*. Identification by spermogonia. *Medd. Norske Skogforsk. Ves.* 20:389-397.
- Saho, H., and Takahashi, I. 1970. Notes on the Japanese rust fungi VI. Inoculation experiments of *Thekopsora areolata* (Fr.) Magnus, a cone rust of *Picea* spp. in Japan. *Trans. Mycol. Soc. Jpn.* 11:109-112.
- Tubeuf, C. 1900. Vorläufige Mitteilungen über Infektionsversuche mit *Aecidium strobilinum*. *Cbl. II Bact.* 6:428-429.
- Wiethölter, N., Graessner, B., Mierau, M., Mort, A. J., Moersbacher, B. M. 2003. Differences in the methyl ester distribution of homogalacturonans from near-isogenic wheat lines resistant and susceptible to the stem rust fungus. *Mol. Plant-Microbe Interact.* 16:945-952.
- Zhang, J. X., Bruton, B. D., and Biles, C. L. 1997. Polygalacturonase isozymes produced by *Phomopsis cucurbita* in relation to postharvest decay of cantaloupe fruit. *Phytopathology* 87:1020-1025.