DEVELOPMENT OF A DNA SAMPLING KIT TO DETECT PATHOGENIC, SAPROTROPHIC, AND STAN FUNGI IN SAPWOOD OF DECLINING RED PINE (*PINUS RESINOSA*) IN THE UPPER MIDWEST

M.T. Banik[1], D.L. Lindner[1], J. Juzwik[2], and J.A. Glaeser[1]

ABSTRACT

An inexpensive kit was developed to collect wood samples for molecular detection of pathogenic, saprotrophic and stain fungi in declining *Pinus resinosa* in the Upper Midwest. The kit contained materials for "clean" collection of sapwood drill shavings, which were then subjected to PCR of the rDNA ITS region with fungal-specific primers, followed by cloning and sequencing. Twenty-seven stands with declining *P. resinosa* lacking obvious fungal fruiting bodies were sampled by natural resources personnel throughout MN, WI, and MI. No single mortality agent predominated and causal agent(s) likely differed by location. A complex interaction of abiotic and biotic agents is likely involved in the symptomatic stands. None of the selected trees tested positive for *Heterobasidion irregulare* with either a pathogen-specific primer or more general ITS primers. Root rot fungi that were detected in the declining and dead trees included *Armillaria solidipes* (= *A. ostoyae*), *Scytinostroma* sp., and two species of *Leptographium*, the genus associated with Red Pine Pocket Mortality. The brown rot fungi *Coniophora arida* and *C. puteana*, known to cause root and butt rots in conifers in the western U.S., were found on both living, declining trees and dead snags. Many ophiostomoid sapstain fungi were present and were usually associated with signs of insect activity. Saprotrophic decay fungi were prevalent with white rot fungi predominating, including *Amylostereum chailletii*, *Hyphoderma setigerum*, *Hypholoma fasiculare*, and *Trichaptum fuscoviolaceum*. Numerous ascomycetes, including endophytic fungi and yeasts, were recovered in great quantities; many of these were uncommon and site specific. Thirty-two unique sequences from basidiomycetes and 129 from ascomycetes had no species, genus, or family identification in GenBank, illustrating the complex and largely unstudied community of fungi found in declining *P. resinosa*. The procedures presented here can be used to address questions of fungal diversity and ecology as well as forest pathology, and the technique can be easily adapted to screening with species specific primers when screening for individual pathogens, thus reducing cost and labor. **Key words:** Heterobasidion irregulare, Armillaria Root Rot, Red Pine Pocket Mortality, ITS (internal transcribed spacer), root rot fungi, white rot fungi, brown rot fungi, sapstain, Pinus resinosa.

INTRODUCTION

Red pine, *Pinus resinosa* Ait., is the most widely planted tree species in the Lake States with nearly 1.9 million total acres in Michigan, Minnesota and Wisconsin (Gilmore and Palik 2006). Approximately 44 percent of the stands are on private land and 56 percent on public. Many plantings occurred in the 1930s and 1950s with most trees now in the pole and saw-timber size classes. The species is generally resistant to many insects and diseases and is typically unaffected by ice, snow or wind (Gilmore and Palik 2006).

In the past three decades, foresters and natural resource personnel in the Upper Midwest have observed unexplained thinning and discoloration of foliage in crowns of red pine and pockets of tree mortality in plantations. In Wisconsin, many of these symptoms are associated with a complex of insects and fungi termed "Red Pine Pocket Mortality (RPPM)" (Klepzig et al. 1991). The major biotic agents involved with this decline include the fungi *Leptographium terrebrantis* and *L. procerum* that are vectored by a variety of insects, including the red turpentine beetle, *Dendroctonus valens*, root collar weevil (*Hylobius radices)*, pales weevil (*H. pales*), pitch-eating weevil (*Pachylobius picivorus*), and the bark beetle *Hylastes porculus* (Klepzig et al. 1991). The beetles feed on freshly cut stumps as well as the lower stems and roots of red pines, spreading the fungi from tree to tree. The fungi grow within the insect galleries and through root

In: Browning, J. Comp. Proceedingsof the 60th Annual Western International Forest Disease Work Conference; 2012 October 8-12; Tahoe City, CA. [1]U.S. Forest Service, Northern Research Station, Center for Forest Mycology Research, Madison, WI. [2]U.S. Forest Service, Northern Research Station, St. Paul, MN.

grafts to healthy trees, which are then stressed by decreased water conduction by the damaged roots. The stressed trees attract additional beetles and may ultimately be killed by the pine engraver beetle, *Ips pini*, and its fungal associate, *Ophiostoma ips* (Klepzig et al. 1991; Wisc. DNR 2011).

Other unexplained pockets of mortality have been attributed to Armillaria root disease, caused primarily by *Armillaria solidipes* (= *A. ostoyae*) (Kromroy 2004). In the eastern and upper midwestern U.S., this pathogen is usually associated with trees that are stressed by various biotic or abiotic factors, including drought, defoliation by insects or frost, foliage diseases, soil compaction, and flooding (Wargo and Shaw 1985). In some cases, the fungus acts as a primary pathogen, particularly in young conifer stands previously planted in areas dominated by hardwoods (Sinclair and Lyon 2005).

A third significant disease that causes crown fading and tree death is Heterobasidion root disease (HRD) caused by the fungus *Heterobasidion irregulare*. Prior to the early 1990s, HRD was not thought to be major disease problem in *P. resinosa* plantations of the Upper Midwest and was rarely reported in this region. In 1993, a fruiting body of *H. irregulare* was first collected by Dr. Glen Stanosz, University of Wisconsin – Madison, near Coloma, WI in Adams County on a *P. resinosa* stump with incipient root decay. Before this, records from the herbarium of the U.S. Forest Service Center for Forest Mycology Research (CFMR) in Madison, WI show that only two collections had been made in Minnesota and none in Wisconsin despite intensive collecting by noted mycologists H.H. Burdsall, Jr., Frances Lombard, M.J. Larsen and R. L Gilbertson. The fungus had been observed more frequently in Michigan (J. O'Brien, personal communication; Stong and Lemmien 1964), including a collection by C.H. Kaufman from Houghton, MI that was placed in the University of Michigan Herbarium in 1908. By the end of 2011, HRD had been detected in 23 counties within Wisconsin (Scanlon 2011; Wisc. DNR 2011). Because species of *Heterobasidion* are considered endemic on susceptible hosts in the North Temperate Zone in North America, Europe and Asia, the pathogen may have been present at low levels on scattered coniferous and hardwood hosts in the region and not recognized until recently. The irregular and often infrequent fruiting of the fungus may also help to explain the small number of historical detection reports in the Lake States. The increase in occurrence and severity of the disease over the past two decades may be attributable to the widespread planting of monocultures of red pine and subsequent thinning of those plantations. However, it is also possible that the pathogen is spreading into new regions of the Upper Midwest (Woodward et al. 1998).

Traditional diagnosis of HRD requires careful site inspection and laborious sampling methods. One common sampling technique involves excavation of two main roots on opposite sides of the suspect tree (Alexander and Skelly 1974). A short root segment is removed and submitted to a diagnostic laboratory for pathogen assay (e.g. incubation in a moist chamber and observation of the asexual reproductive structures of the fungus). In the past decade, drill shavings taken from trees and processed using molecular techniques have been used to identify fungi in forest stands and in urban settings (Guglielmo et al. 2010; Lindner et al. 2011). Thus, we proposed to modify such sampling protocols and laboratory methodologies to identify the fungi associated with unexplained crown decline and tree mortality in red pine stands in the Upper Midwest. Accurate diagnosis of a disease is necessary before appropriate preventive measures and control actions can be prescribed for such situations. The objectives of the study were: 1) to develop a protocol for use by state forest health specialists to sample red pines exhibiting unexplained crown thinning and discoloration, 2) to use primers (ITS1F/ITS4) that detect a broad range of fungal species to identify the fungi associated with declining trees, and 3) to use a primer specific for *Heterobasidion irregulare* to screen samples as a verification of the presence of this fungus in the region.

MATERIALS AND METHODS

Sampling Kit Composition and Distribution

Sampling kits were constructed for field personnel consisting of the following: fourteen sterile 1/8 in. diameter drill bits, fourteen 1.5 ml bottomless tubes ("microfunnels"), fourteen 1.5 or 2.0 ml sample collection tubes labeled with sample number, 1 larger tube of CTAB DNA extraction solution (Lindner et al. 2011), 3 disposable plastic transfer pipettes, 1 sample collection sheet with sample numbers matched to sample collection tube numbers, 3 sealed alcohol wipes,

1 small plastic bag for used items, and instructions on sampling procedure. Drill bits were sterilized by soaking in a 20 percent dilution (v/v) of 6 percent sodium hypochlorite bleach to remove all cross-contaminating DNA; all other plasticware and the extraction buffer were sterile. Each kit contained enough materials to sample a stand for two symptomatic trees, one control healthy tree, and an air sample control.

Figure 1. Sampling a freshly prepared wood surface after removing bark to cambium, taking care not to touch the exposed sampling surface.

Sampling Procedure

Field personnel were instructed to sample living, symptomatic trees that had no obvious fungal fruiting bodies. In each stand, two symptomatic trees were selected that had the general appearance of decline, including dieback and dead branches, but did not have fungal fruiting bodies. In some cases, stumps or snags were sampled rather than living trees. Symptomatic trees, stumps or snags were sampled at 4 points, representing the 4 quadrants of the lower stem, as recommended by Guglielmo et al. (2010). In addition, air samples and drill shavings from healthy trees were collected to serve as controls. These were collected before sampling of symptomatic trees to minimize the risk of cross contamination. Air sampling was conducted by holding two sample tubes open in the air for one minute; the tubes were agitated to ensure good airflow into the tube. Tubes were then capped and labeled as "air controls." One "healthy," non-symptomatic tree was chosen in each stand as an additional control. Control trees were sampled at two points near the base of the tree using the same procedure as that described for symptomatic trees.

In each sampling, the blade of a hatchet or knife was initially wiped with 70 percent ethanol to prevent cross contamination from previous sampling. A "clean" surface on the stem of the tree was then prepared, about 6 in. above the soil surface (Figure 1) by using the knife or hand axe to remove the bark down to the cambium on a small section, taking care not to touch the exposed sampling surface. This area was then sampled using a sterile drill bit. Shavings were collected by placing the small end of a bottomless tube ("microfunnel") against the clean exposed surface of the bole, with the long axis of tube parallel to the ground. The drill bit was placed through the microfunnel so that it rested against the sample point (Figure 2). Shavings were collected by drilling horizontally into the stem, "pumping" the drill in and out of the hole as the shavings collected in the microfunnel. When the microfunnel was full of shavings, the drill bit and tube were removed from the substrate, the drill bit being kept in the microfunnel to act as a plug. Shavings were then transferred to the sample collection tube by using the drill bit to force the shavings down the microfunnel into the tube (Figure 3). The procedure was repeated until approximately 0.5 ml of shavings were collected at the bottom of the collection tube. Sterile, new drill bits and fresh tubes were used for each sampling point. After leaving the field and in a cleaner environment (i.e. kitchen, laboratory, office, etc.), but within 4 hours of sample collection, the sterile transfer pipette was used to add enough CTAB DNA extraction buffer (Lindner et al. 2011) to the sample tubes to completely cover the shavings (approximately 1 ml). Samples that were kept more than a few days before shipping were frozen. Thawed samples were sent to the Center for Forest Mycology Research in Madison, WI with collection information that included date, sample location, site number, collector, host, site information (including species composition, stand age/size class, soil type, etc.), and any observed symptoms.

Sampling Development and Verification

Using the techniques described above, three known positive trees with fruiting bodies of *H. irregulare* were sampled from a *P. resinosa* stand in Wild Rose, WI. Four samples were drilled at the base of the trees and

two samples were drilled along major roots exposed by digging. In one tree, 14 samples were collected at regular intervals along two roots exposed by excavation. Samples were assayed as described below.

Figure 2. Inserting the drill bit through the microfunnel before drilling into the sapwood.

Figure 3. Transferring the collected wood shavings from the microfunnel to the sample collection tube.

Sampling Locations

Forest stands were sampled by natural resources personnel in MI, MN, and WI. Site information is listed in Table 1 and shown in Figure 4. Collection information, including stand description, is presented in Table 1.

DNA Extraction, Cloning and Identification of Taxa

Samples received at CFMR were stored at -80° C until processed. For DNA extraction the 1.5 ml tubes containing the samples were thawed at 65° C for 1- 2 hours and then centrifuged at 16.1 rcf for 5 min at room temperature with 100 µL of the supernatant transferred to strip tubes. DNA was then extracted from samples using the technique of Lindner and Banik (2009) modified for use with 200 µL strip tubes as per Lorch et al. (2013). Amplification of the resulting DNA was accomplished using the fungal specific primer pair ITS1F/ITS4 following the technique of Lindner and Banik (2009) as was cloning of the resulting amplicons. Eight clones were chosen from each sample for reamplification and sequencing. Fungal identifications are based on the nearest BLAST match in GenBank using similarities of 297 percent to denote species identification and 90 – 97 percent for genus identification. Similarities of less than 90 percent were tentatively identified to higher level taxa that provided the best match (i.e., order or family). In addition, all samples underwent PCR with an *H. irregulare* specific primer (HA2, TACCCCACGGCGTAGACA) paired with ITS1F. This primer was tested against the previously described diluted *H. irregulare* positive samples to verify its efficacy.

RESULTS AND DISCUSSION

A molecular testing kit was developed to sample declining *Pinus resinosa* trees for pathogenic, saprotrophic, and stain fungi using a “clean” drilling procedure followed by PCR, cloning and sequencing of the ITS region of DNA. The technique was refined using known positive trees. The methodology was initially tested on replicate samples taken at the base of the stem and along major roots of trees that had fruiting bodies of *H. irregulare* at their base. As all of these samples tested positive regardless of sampling position, a final protocol was developed in which four drill samples were taken from the base of the trunk in all 4 quadrants for suspected trees, following the procedure recommended by Guglielmo et al. (2010). The test kits were used to sample trees at 27 stands in MN, WI and MI during 2011. These were stands that appeared to be in a state of decline but in which no fungal fruiting bodies had been observed.

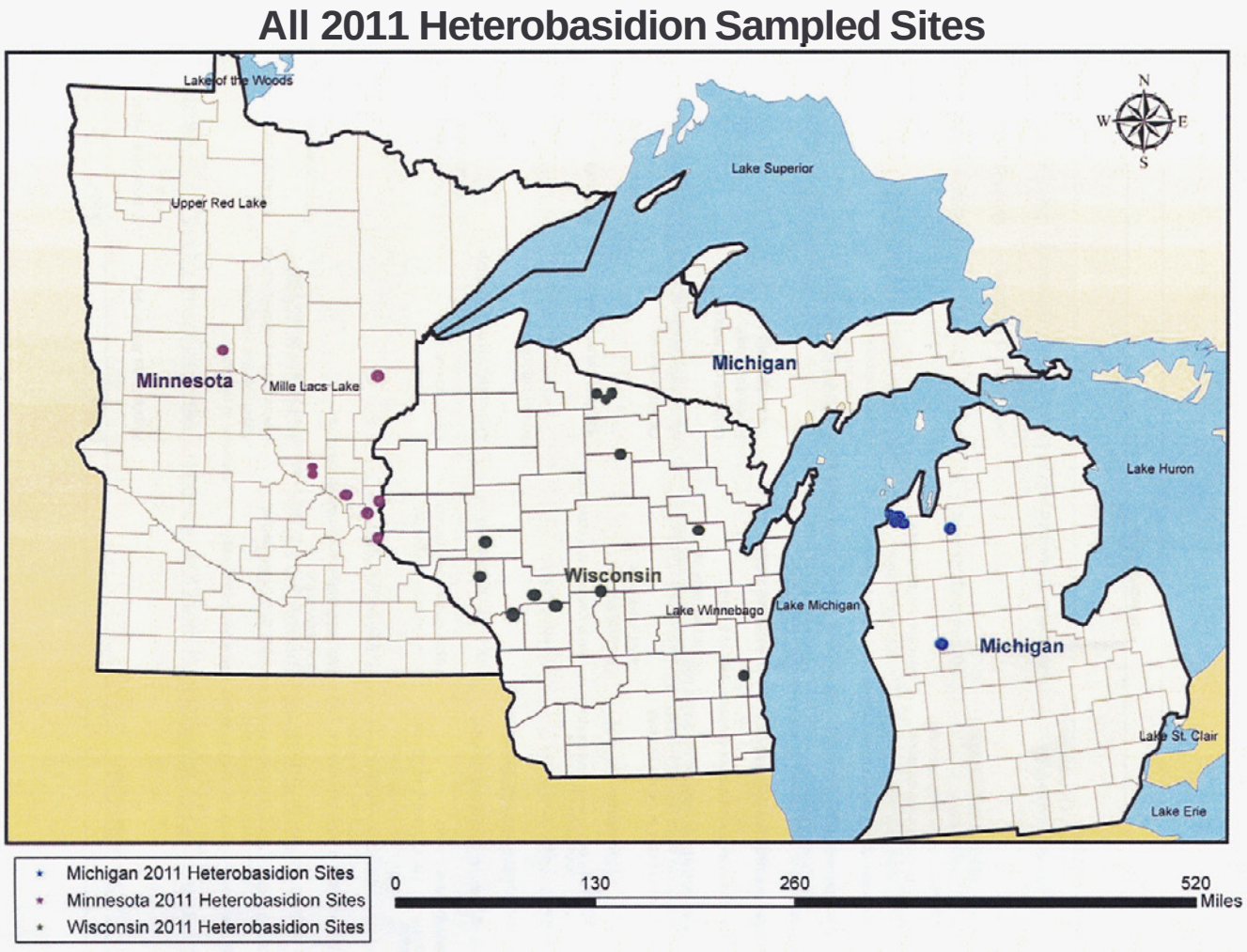

Figure 4. Location of sampling sites.

None of the selected trees tested positive for *Heterobasidion* either with the pathogen-specific primer or the more general, fungal-specific primers ITS1F/ITS4. The use of nonspecific primers made it possible to inventory the range of fungi present in declining trees (Table 2). Several other root rot fungi were detected in the declining and dead trees, including the white rot fungi *Armillaria solidipes* (= *A. ostoyae*) in Stands 14 and 26 and a species of *Scytinostroma* sp. – presumably *S. galactinum* - in Stands 22 and 25. The brown rotters, *Coniophora arida* (Stands 21, 22, 24) and *C. puteana* (Stands 17, 20), which cause brown cubical rot in the roots and bases of living conifers throughout the West (Rocky Mountain Region, Forest Health Protection 2010), were found on both living, declining trees and dead snags. Saprotrophic decay fungi were prevalent with white-rot species predominating. These included *Hypholoma fasiculare* (Stand 7), the agaric "sulfur top" mushroom that is prolific on dead wood of both conifers and hardwoods (Miller and Miller 2006). Other white rot fungi were *Hyphoderma setigerum* (Stands 8, 9, 13), *Trichaptum fuscoviolaceum* (Stand 11), and *Amylostereum chailletii* (Stand 18). These were all found on living, declining trees, with or without signs of insect colonization.

Many ophiostomoid sapstain ("blue stain") fungi were detected, as expected for declining pines. These included *Ophiostoma pulvinisporum* (Stands 8, 9), *O. minus* (Stands 14, 22), *Leptographium lundbergii* (Stands 10), *Leptographium guttulatum* (Stand 10), *Graphium penicillioides* (Stand 30), and unknown species of *Ophiostoma* (Stands 8, 13, 18), *Ceratocystis* (Stands 3, 12), and *Graphium* (Stand 30). Most of these fungi were associated with dead trees (Stands 12, 22, 30) or signs of insect activity (Stands 10, 12). These fungal genera are frequently vectored by bark beetles and may be opportunistic pathogens (Gibbs 1993; Harrington 1993).

Table 1. Sampling site information.

Stand #	Date Sampled	State	County	Symptomatic tree #	DBH (")	Site Description
1	6/28/2011	Michigan	Kalkaska	1	12.8	Possible trulling bodies
2	11/5/2011	Michigan	Leelanau	2	13.2	
3	11/5/2011	Michigan	Benzie	1	8.5	Pockets of decline clearcut; no dead trees
4	11/5/2011	Michigan	Benzie	2	13.7	
5	11/5/2011	Michigan	Leelanau	1	10.2	Pocket started about 20 years ago; spread from 10-15 trees to several acres. Some dead trees very old and rotten.
6	11/17/2011	Michigan	Mecosta	2	7.2	
7	11/17/2011	Michigan	Mecosta	1	10.2	No information.
8	5/23/2011	Minnesota	Anoka	2	10.4	
9	6/14/2011	Minnesota	Washington	1	6.8	Pockets of decline. Dead trees clearcut to prevent spread.
10	6/16/2011	Minnesota	Sherburne	2	7.2	
11	6/16/2011	Minnesota	Sherburne	1	11.8	Large stand with 2 dead pockets.
12	6/17/2011	Minnesota	Washington	2	10.8	
13	6/17/2011	Minnesota	Washington	1	11.4	Large stand with 2 dead pockets.
14	6/24/2011	Minnesota	Pine	2	11.3	
17	11/4/2011	Wisconsin	Wood	1	8.3	Stand of remnant pine on suburban lot adjacent to road. Several dead and dying trees.
18	11/15/2011	Wisconsin	Lincoln	2	8.8	
20	11/17/2011	Wisconsin	La Crosse	1	11.6	Stand was located just off the east side of the parking lot of nature center building. Some wilting and dead red pine trees present.
21	11/17/2011	Wisconsin	Jackson	2	10	
22	11/17/2011	Wisconsin	Shawano	1	10.1	Tress with dead needles and frass at base located on south side of a country road.
23	12/1/2011	Wisconsin	Eau Claire	2	14.1	
24	12/1/2011	Wisconsin	Trempealeau	1	10	*Ips* and red turpentine beetle present in stand located south of paved road.
25	12/2/2011	Wisconsin	Vilas	2	11.7	
26	12/2/2011	Wisconsin	Vilas	1	6.9	Pockets of dead and dying trees located northeast of entrance building to park.
27	12/2/2011	Wisconsin	Vilas	2	6.2	
28	12/6/2011	Wisconsin	Monroe	1	14.9	Dead and dying trees located northeast of entrance building to park.
29	12/6/2011	Wisconsin	Monroe	2	10.7	
30	12/8/2011	Wisconsin	Washington	1	10	Dead trees found stand located just east of a state highway.
				2	11.3	
				1	5.5	Non-thinned plantation
				2	6.1	
				1	10.2	Clump of 13 symptomatic trees with thin crowns and mortality. *Armillaria* rhizomorphs observed.
				2	9.7	
				1	12.3	Thinning crowns and dead trees approx. 60 years old.
				2	11.1	
				1	12.1	Horseshoe-shaped pocket; numerous trees with thinned crown.
				2	15.1	
				1	14	Established pocket of dead trees, on ground and standing.
				2	10	
				1	16	Dead trees and trees with thinning crowns.
				2	12	
				1	14	Stand of small saw timber.
				2	14	
				1	13	Large pocket of about 2 acres. Pre-salvaged ring of asymptomatic trees around symptomatic pines in 2009.
				2	14.2	
				1	10.3	Approx. 20 extremely thin-crowned at edge of plantation. *Leptographium, Armillaria* and *Ips* sp. observed.
				2	10.5	
				1	7.5	9 scattered pockets of mortality. Bark beetles, *Armillaria, Leptographium* observed. Pocket sampled had 14 symptomatic trees.
				2	10.7	
				1	9.7	
				2	7.3	
				1	5.6	Red/white pine plantation approx. 50 years old. White pine healthy and regenerating.
				2	6.1	
				1	9.5	Red/white pine plantation approx. 50 years old. Dead, declining trees with tufted foliage. WP scattered in overstory.
						Red/white pine plantation approx. 40 years old. Scattered dead and declining trees, rapid mortality. Southern Pine Engraver and *Ips grandicollis* suspected.

Table 2. Potential pathogens, decay and sapstain fungi recovered by location[A].

Stand #	County	State	Tree # or Control	Symptoms / Signs Observed	*Drill 1*	Drill 2	Drill 3	Drill 4
3	Benzie	MI	2	Dead with bark still attached	*Ceratocystis sp.*			
7	Mecosta	MI	1	Recently dead tree near pocket edge			*Hypholoma fasciculare*	*Hypholoma fasciculare*
8	Anoka	MN	1	Thinning crown				*Valsa sp.*
			2	Thinning crown	*Ophiostoma pulvinisporum*			*Valsa sp.*
					Ophiostoma sp. B			*Phoma sp.*
					Hyphoderma setigerum			
9	Washington	MN	1	Wilted needles attached	*Hyphoderma setigerum*		*Hyphoderma setigerum*	
			2	Wilted needles attached		*Hyphoderma setigerum*	*Ophiostoma pulvinisporum*	*Hyphoderma setigerum*
10	Sherburne	MN	1	Dead needles with frass at base	*Leptographium lundbergii*	*Leptographium lundburgii*	*Leptographium guttulatum*	
11	Sherburne	MN	1	*Cryptoporus* and red turpentine beetle		*Botryobasidium A*		*Trichaptum fuscoviolaceum*
				Woodpecker damage, *Ips* sp.		*Botryobasidium subcoronatum*		
12	Washington	MN	C	"Healthy" control tree	*Daedalopsis confragosa*			
			1	Dead, Ips sp.	*Nectria nigrescens*	*Ceratocystis sp. B*		
			2	*Ips* sp. holes, wilting needles	*Nectria nigrescens*			
13	Washington	MN	1	*Ips* sp. holes, *Armillaria*, red turpentine beetle	*Hyphoderma setigerum*	*Hyphoderma setigerum*		
			2	*Ips* sp. holes, *Armillaria*, red turpentine beetle		*Hyphoderma setigerum*	*Ophiostoma sp.*	*Hyphoderma setigerum*
14	Pine	MN	1	Root collar weevil, resinous wood				*Ophiostoma minus*
			2	Discolored wood		*Armillaria solidipes*	*Leptographium guttulatum*	
15	Wadena	MN	1	Dead	*Nectria mariannaeae*			
			2	Sawdust at base indicating borers			*Nectria mariannaeae*	
17	Wood	WI	1	Thin crown, tufted needle tips, chlorotic needles		*Coniophora sp.*		
			2	Thin crown, tufted needle tips, chlorotic needles	*Coniophora puteana*			
18	Lincoln	WI	1	Dead top, 40% necrotic needles & thin crown, Ips			*Amylostereum chailletii*	*Parasola sp.*
			2	Thin crown	*Amylostereum chiantii*	*Ophiostoma sp.*		
						Ophiostoma sp. A		*Ophiostoma sp.*
							Ophiostoma sp.	
20	LaCrosse	WI	2	Very thin crown, tufted needles	*Coniophora puteana*	*Coniophora puteana*	*Coniophora puteana*	*Coniophora puteana*
22	Shawano	WI	C	"Healthy" control tree		*Scytinostroma sp.*		
			1	Dead			*Ophiostoma minus*	
							Scytinostroma sp.	
			2	Dead	*Scytinostroma sp.*	*Coniophora arida*	*Scytinostroma sp.*	
						Scyinostroma sp.		
24	Trempealeau	WI	1	Thin, tufted foliage	*Coniophora arida*	*Coniophora arida*		
25	Vilas	WI	1	Stump with bark attached			*Scytinostroma sp. A*	
							Scytinostroma sp. B	
							Scytinostroma sp. C	
26	Vilas	WI	1	Dead	*Armillaria solidipes*	*Armillaria solidipes*	*Armillaria solidipes*	
27	Vilas	WI	2	Dead	*Nectria mariannaeae*	*Nectria mariannaeae*		
28	Monroe	WI	C	"Healthy" control tree	*Sistotrema sernanderi*			
			2	Thin, tufted foliage	*Polyporales*	*Mycena sanguinolenta*		*Mycena sanguinolenta*
29	Monroe	WI	2	Thinning crown, tufting foliage	*Mycena sanguinolenta*			
30	Washington	WI	1	Dead			*Graphium penicillioides*	*Graphium sp.*
							Graphium sp.	
			2	Bark beetle	*Nectria flavoviridis*	*Graphium sp.*	*Botryobasidium botryosum*	*Graphium sp.*
					Hebeloma mesophaeum			

[A] Stands with no identified fungi are excluded from this table.

Three species of *Nectria,* the genus responsible for Nectria canker and Nectria dieback, were also common on the dead and declining *P. resinosa* (Stands 12, 15, 27, 30). *Nectria mariannaea, N. nigrescens,* and *N. flavoviridis* are saprotrophs or endophytes not associated with disease that are commonly found on bark and woody substrates of numerous hosts, sometimes only in their imperfect state (Hirooka et al. 2011; Samuels and Seifert 1991). Identification to exact species based on ITS data is not possible, however. *Nectria nigrescens,* which has been associated exclusively with hardwoods, is part of the *Nectria cinnabarina* species complex (NCSC). Other members of the NCSC have been collected from conifers including the Pinaceae (Hirooka et al. 2011) and may be difficult to differentiate using only ITS primers. *Nectria mariannaea* has only been reported from French Guiana and Venezuela, but its anamorph, *Stilbella aciculosa,* is distributed widely throughout Europe, North America, Asia and the tropics (Samuels and Seifert 1991). *Nectria flavoviridis* is often a mycoparasite (Ellis and Ellis 1988) so it could be colonizing other ascomycetous saprotrophs.

Numerous ascomycetes, including endophytic fungi and yeasts, were recovered in great quantities (data not shown). Many of these were uncommon and site specific. Thirty-two unique sequences from basidiomycetes and 129 from ascomycetes had no species, genus, or family identification in GenBank, thus illustrating the complex and largely unstudied community of fungi found in declining pines.

Fungal DNA was not detected in any of the air samples. Control "healthy" trees contained large numbers of nonpathogenic fungi, including endophytes, yeasts, and discomycetes, as well as many "unknown" ascomycetes that have not been identified in GenBank. Two control trees contained DNA associated with decay fungi. In Stand 12, the white rotter *Daedalopsis confragosa* was detected in one drill sample. This was unexpected because the fungus is found only rarely on conifers (Gilbertson & Ryvarden 1986) and the tree was asymptomatic. In Stand 22, one drill sample from the asymptomatic control tree contained DNA from *Scytinostroma* sp., a root rotting white-rot fungus that was prevalent in the sampled trees from that plot.

Field personnel were surveyed to determine their opinions regarding the drilling technique. Many saw its value for research but felt that it was somewhat time-consuming compared to normal field sampling techniques. This observation may have been due to individuals' unfamiliarity with the technique and the fact that each individual only collected a small number of samples. Interestingly, many recognized the value of being able to identify a wide range of fungal pathogens in sites where fruiting bodies could not be located.

No single fungal pathogen was found to dominate samples in sites where obvious fruiting bodies were not present. *Armillaria solidipes* (= *A. ostoyae*) could be a contributor to tree decline in certain stands but is usually associated with trees that are already stressed by other agents in the Northeast and Midwest (Sinclair and Lyon 2005). Another possible mortality agent is *Scytinostroma galactinum.* This fungus is frequently associated with ash, apples, and other hardwoods but has also been known to cause root rot, butt rot, and heart rot of conifer species, including *P. resinosa* and *P. banksiana,* in Wisconsin and Ontario (Krebill 1963; Basham and Morawski 1964). Reports of its pathogenicity are summarized in Lentz and Burdsall (1973). The prevalence of this fungus and its role in the unexpected dying of red pine in the Lake States needs to be examined further.

Two species of *Leptographium, L. lundbergii* and *L. guttulatum,* were detected in the survey, but these are not the species typically associated with RPPM (Klepzig et al. 1991). They are sapstain fungi, however, and are likely vectored to stressed trees by beetles. Many of the sampled trees exhibited signs of insect activity – frass, emergence holes, and beetle galleries. *Ophiostoma ips* is a major contributor to RPPM and is often responsible for ultimate tree death along with its insect associate (Klepzig et al. 1991). Although *O. ips* was not identified, other species of *Ophiostoma* were found, including *O. minor* which has been associated with RPPM (Klepzig et al. 1991). Other isolates of *Ophiostoma, Graphium,* and *Ceratocystis* could not be identified to species without the use of additional primers and may be species associated with RPPM. The role of abiotic factors, including drought and poor soil types, and the attraction of insects to weakened trees are also possible contributors to the observed declines.

As we were assaying with both *H. irregulare*-specific and nonspecific fungal primers, the laboratory analysis was quite time-consuming but resulted in much additional information on fungi associated with dying *Pinus resinosa* in affected stands. The procedures presented here can be used to address questions of fungal diversity and ecology as well as forest pathology. The technique can also be easily adapted to screening with species-specific primers. Sampling fungal DNA directly from wood removes the extensive labor and biases associated with culturing. The sensitivity of the assay would allow drill samplings from a tree or even an entire stand to be pooled, as recommended by Guglielmo et al. (2010), and thus greatly speed identification and decrease laboratory costs. These procedures have many potential applications in forest pathology for both applied survey work and basic research studies.

ACKNOWLEDGEMENTS

This research was partially funded by the USDA Forest Service Special Technology Development Program (project number NA-2009-02).

REFERENCES

Alexander, S.A.; Skelley, J.M. 1974. A comparison of isolation methods for determining the incidence of *Fomes annosus* in living loblolly pine. European Journal of Plant Pathology. 4:33-38.

Basham, J.T.; Morawski, Z.J.R. 1964. Cull studies, the defects and associated basidiomycete fungi in the heartwood of living trees in the forests of Ontario. Publication 1075. Canadian Department of Forestry.

Ellis, M.B.; Ellis, J.P. 1988. Microfungi on Miscellaneous Substrates. New York: Springer Publishing.

Gibbs, J.N. 1993. The biology of ophiostomatoid fungi casuing sapstain in trees and freshly cut logs. Pages 153-160 *In:* Wingfield, M.J. et al (eds) *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology, and Pathogenicity. St. Paul, MN: APS Press.

Gilbertson, R.L.; Ryvarden, L. 1986. North American Polypores. Oslo, Norway: Fungiflora.

Gilmore, D.W.; Palik, B.J. 2006. A revised manager's handbook for red pine in the North Central Region. Gen. Tech. Rep. NC-264. St. Paul, MN: USDA, Forest Service, North Central Research Station.

Guglielmo, F.; Gonthier, P.; Garbelotto, M.; Nicolotti, G. 2010. Optimization of sampling procedures for DNA-based diagnosis of wood decay fungi in standing trees. Letters in Applied Microbiology. 51:90-97.

Harrington, T.C. 1993. Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. Pages 161-172 *In:* Wingfield, M.J. et al. (eds) *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology, and Pathogenicity. St. Paul, MN: APS Press.

Hirooka, Y.; Rossman, A.Y.; Chaverri, P. 2011. A morphological and phylogenetic revision of the *Nectria cinnabarina* species complex. Studies in Mycology. 68:35-56.

Krebill, R.G. 1963. Etiology of a jack and red pine plantation decline characterized by root weevil injury and fungal deterioration. Dissertation Abstracts. 23:1861.

Klepzig, K.D.; Raffa, K.F.; Smalley, E.B. 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. Forest Science. 37:1119-1139.

Kromroy, K.W. 2004. Identification of *Armillaria* species in the Chequamegon-Nicolet National Forest. Res. Note NC-388. St. Paul, MN: USDA, Forest Service, North Central Research Station.

Lentz, P.L.; Burdsall, H.H., Jr. 1973. *Scytinostroma galactinum* as a pathogen of woody plants. Mycopathologia et Mycologia Applicata. 49:289-305.

Lindner, D.L.; Banik, M.T. 2009. Effects of cloning and root-tip size on observations of fungal ITS sequences from *Picea glauca* roots. Mycologia. 101:157-165.

Lindner, D.L.; Vasaitis, R.; Kubartova, A. and others. 2011. Initial fungal colonizer affects mass loss and fungal community development in *Picea abies* logs 6 year after inoculation. Fungal Ecology. 4:449-460.

Lorch, J.M.; Lindner, D.L.; Gargas, A. and others. 2013. A culture-based survey of fungi in soil from bat hibernacula in the eastern United States and its implications for detection of *Geomyces destructans*, the causal agent of bat white-nose syndrome. Mycologia. 105(2):237-52.

Miller, O.K. Jr.; Miller, H.H. 2006. North American mushrooms. Helena, MT: Globe Pequot Press.

Rocky Mountain Region, Forest Health Protection. 2010. Field guide to diseases & insects of the Rocky Mountain Region. Gen. Tech. Rep. RMRS-GTR-241 Fort Collins, CO: USDA, Forest Service, Rocky Mountain Research Station.

Samuels, G.J.; Seifert, K.A. 1991. Two new species of *Nectria* with *Stilbella* and *Mariannaea* anamorphs. Sydowia. 43:249-263.

Scanlon, K. 2011. Annosum root rot: biology, symptoms and prevention. Madison, WI: Wisconsin Department of Natural Resources, Forest Health Protection. http://dnr.wi.gov/topic/ForestHealth/ /documents/Annosum–Factsheet.pdf(accessed 08 22 2012).

Sinclair, W.A.; Lyon, H.H. 2005. Disease of trees and shrubs. Ithaca, NY: Comstock Publishing Associates.

Strong, F.C.; Lemmien, W.A. 1964. *Fomes annosus* in southwestern Michigan. Plant Disease Reporter. 48: 110.

Wargo, P.M.; Shaw, C.G. III. 1985. *Armillaria* root rot: the puzzle is being solved. Plant Disease. 69:826-832.

Wisconsin Department of Natural Resources (Wisc. DNR), Division of Forestry, Forest Health Protection. 201 1. Annosum root rot and red pine pocket mortality in Wisconsin: Biology and management. http://drn. wi.gov/topic/foresthealth/documents/annosum_red_pine _pocket_mortality.pdf (assessed 01/29/2013).

Woodward, S.; Stenlid, J.; Karjalainen, R.; Hüttermann, A. 1998. *Heterobasidion annosum*: Biology, ecology, impact, and control. New York: CAB International.

Proceedings of the 60th Annual Western International Forest Disease Work Conference

October 8-12, 2012
Granlibakken
Tahoe City, California, U.S.

Compiled by:

John Browning
Weyerhaeuser Forestry Research, Centralia, WA

and

Patsy Palacios
S.J. and Jessie E. Quinney Natural Resource Research Library
College of Natural Resources
Utah State University, Logan, UT

Papers are formatted and have minor editing for language, and style, but otherwise are printed as they were submitted. The authors are responsible for content.