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Survey of potential sapstain fungi on *Pinus radiata* in New Zealand

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Abstract A nationwide survey of New Zealand sapstain fungi on *Pinus radiata* was undertaken between 1996 and 1998 with collections of 1958 samples of material from 869 sites in the North and South Islands. Material was collected from mills, ports, forest plantations of native, exotic, or *P. radiata*, nurseries, farms, and urban areas. Material collected included branches, twigs, needles or leaves, cones, logs, wood chips, timber, and veneer. From these collections, 2154 potential sapstain fungi, representing 14 known species plus a number of unidentified species, were isolated. The predominant sapstain fungi isolated were *Sphaeropsis sapinea*, *Ophiostoma ips*, *O. flocccosum*, *O. piliferum*, *Leptographium procerum*, and *O. querci*. *S. sapinea* was isolated from all material sampled including collections from the forest floor (including branches, twigs, needles, leaves, cones, and logs) as well as from logs and timber. In contrast *Ophiostoma* species were mainly found on logs, timber, and wood chips.

Keywords *Sphaeropsis sapinea*; *Ophiostoma*; *Pesotum*; *Sporothrix*; *Leptographium*; *Pinus radiata*; sapstain

INTRODUCTION

Sapstain is the discoloration of sapwood caused by pigmented hyphae of several taxonomic groups of fungi including *Botryosphaeria* species, *Sphaeropsis sapinea*, *Ophiostoma* species, *Ceratocystis* species, *Leptographium* species, and *Ceratocystiopsis* species. The cosmetic damage to the wood causes no appreciable loss of strength, but it affects domestic and export earnings for the forest industries (Schirp et al. 2003a,b). In New Zealand, there are around 1.7 million hectares of plantation forestry, and of this approximately 1.6 million hectares is planted with *Pinus radiata*. The fast growth and subsequent high proportion of sapwood of *P. radiata* imparts greater susceptibility to detrimental fungi including sapstain fungi. The prevention of fungal contamination during the export of wood and wood products is crucial due to concerns with lengthy transport times, the concomitant changes in climate occurring while crossing the Equator, and increased biosecurity issues. An efficacious, economic, and environmentally sound method of sapstain prevention is currently being sought by the New Zealand forest industry.

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Historically, there was limited research on the sapstain fungi affecting *P. radiata*. Major sapstain problems were linked with *Sphaeropsis sapinea* (Birch 1936; Butcher 1968a). Other minor sapstain species were isolated, including members of the Ophiostomataceae family (Butcher 1968b). Hutchison & Reid (1988a,b) sampled wood from exotic and indigenous trees in six locations in the North Island and found a number of *Ophiostoma* species associated with sapstained wood.

The majority of research on *S. sapinea* was dedicated to latent infections caused by this endophytic fungus. *S. sapinea* grows as a saprophyte in dead bark, wood, needles, cones, and forest debris (Birch 1936). Three morphotypes, A, B, and C, are now described for *S. sapinea* (de Wet et al. 2002). The A morphotype is common and has a wide distribution in southern hemisphere countries including New Zealand (Swart et al. 1991; Burgess et al. 2001).

The large and diverse ascomycete genus *Ophiostoma* includes insect-associated species that are commonly found colonising sapwood. *Leptographium*, *Pesotum*, *Sporothrix*, and other anamorphic genera are known to have *Ophiostoma* teleomorphs or are thought to be closely related to *Ophiostoma* species based on DNA sequence analyses or tolerance to the antibiotic cycloheximide (Harrington 1981; Hausner et al. 1993, 2000; Harrington et al. 2001; Jacobs et al. 2001). The taxonomy of *Ophiostoma* outside of the *O. piceae* complex is poorly known, and accurate identification of species is difficult. Many of the recognised species are complexes of morphologically similar sister species. Therefore, mating studies and DNA sequence analyses are often necessary to separate these species (Harrington et al. 2001). Species of *Ophiostoma* are also frequently isolated in mixtures, further complicating identification.

The present study was intended to broadly survey potential sapstain fungi in New Zealand. The main objectives were firstly to isolate and identify any potential sapstain fungi and, secondly, to establish the most commonly occurring species.

MATERIALS AND METHODS

A survey extending from southern hemisphere spring 1996 to autumn 1998 was conducted throughout New Zealand. In total, 1958 samples of material were collected from 869 different sites. Material was collected from mills, ports, forest plantations of native, exotic, or *P. radiata* plantations, nurseries, farms, and urban areas during spring (Sep–Nov), summer (Dec–Feb), autumn (Mar–May), and winter (Jun–Aug) for the period between spring 1996 and autumn 1998. Approximately 95% of the collection was from *P. radiata* sites, and the rest within native forest areas or non-radiata plantations. Some sites were re-visited at different times of the year, but the majority of sites were visited only once during the survey.

From each site, at least one targeted material was collected (Table 1). Branches, twigs, needles or leaves, and cones were collected from the forest floor. From recently felled trees (logs), cross sectional discs at least 50 mm thick, including both sapwood and bark, were obtained using a chain saw. From mill sites, logs, wood chips, timber, and veneer (peeled from the logs before being made into plywood) were collected. All material was placed in clean plastic bags, sealed, and sampled within 24 h for culture isolations in the laboratory.

Culture isolation

All collected material was surface sterilised (to eliminate surface inhabiting micro-organisms) by soaking in 5% hypochlorite for 2 min followed by rinsing twice in sterile water. Slivers were taken aseptically with a sterile scalpel and placed on two selective media. The first medium consisted of MYEA (0.2% yeast extract, 1.5% malt extract, 2% bacteriological agar) supplemented with 200 μg/ml chloramphenicol and 100 μg/ml streptomycin sulphate to suppress bacterial growth, which allowed for the growth of all potential sapstain fungi, including *S. sapinea* and *Ceratocystis* species. The second medium, selective for *Ophiostoma* species, was modified slightly from that used by Harrington (1981) and consisted of MYEA supplemented with 200 μg/ml chloramphenicol, 100 μg/ml streptomycin sulphate, and 400 μg/ml cycloheximide.

Plates were incubated in a darkened growth chamber at 25°C for up to 30 days. Any potential sapstain fungal colonies were aseptically transferred onto fresh agar plates with and without cycloheximide. The presence or absence of a species was recorded at each site. The occurrence of a species at a site was scored as a single record, regardless of the number of times it was isolated from that particular site or the number of colonies developing on the two media types.

Sphaeropsis sapinea was identified on the basis of morphology. Potential *S. sapinea* cultures were inoculated onto sterile *P. radiata* needles and grown under ultraviolet light at ambient temperatures for 2 weeks; following this they were examined

microscopically in sterile water for the presence of pycnidia and subsequent spore release.

For identification, cultures of *Ophiostoma*-type fungi were grown on MEA (1.5% malt extract, 2.0% agar) at room temperature (21–24°C) and lighting for 7–14 days and initially grouped into putative species based on similarity of mycelium colour and texture, growth rate, smell, and presence of anamorph and perithecial characters. Pairings of heterothallic species for sexual compatibility tests were done by placing mycelial plugs of two isolates on MEA or by spermatising mycelia with conidial suspensions (Harrington et al. 2001). Representative isolates of the morphological species or intersterility groups were used for sequencing of the internal transcribed spacer regions (ITS) of the rDNA (Harrington et al. 2001) (Table 2). For most species, the ITS-1 and ITS-2 regions, with the intervening 5.8S gene, were amplified using primers ITS1-F and ITS4 (White et al. 1990; Gardes & Bruns 1993). Either extracted DNA (DeScenzo & Harrington 1994) at 10–100 ng per reaction or scraped mycelium with spores (Harrington & Wingfield 1995) were used as template for the polymerase chain reaction (PCR). The reaction mixture and cycling conditions for PCR are described in Harrington et al. (2001). Amplicons were sequenced using the primers ITS1-F and ITS4 and a ABI PRISM 377 Genetic Analyzer (Perkin-Elmer Inc., USA) at the DNA Synthesis and Sequencing Facility (Iowa State University, Ames, Iowa) after purification using QIAquick PCR purification Kits (Qiagen Inc., USA) or Microcon-100 Microconcentrators (Amicon, Inc., USA). Sequences of isolates were aligned manually and analysed using PAUP 4.0 (Swofford 1998). However, there were many insertions and deletions in the data set, especially in the ITS-1 region, and sequences needed to be grouped according to similarity and analysed separately.

Representative cultures are maintained in the Mycology Collection of the Department of Biological Sciences, University of Waikato, Hamilton, New Zealand, and at Iowa State University, Ames, Iowa, USA.

RESULTS

In total, 1958 samples of material were collected from 869 sites during this study and 2154 independent isolates of potential sapstain fungi were obtained. *S. sapinea*, 14 known species of *Ophiostoma* or *Leptographium*, and 2 unidentified *Sporothrix* anamorphs were isolated (Table 3). For each species, the number and proportion of isolations as well as the substrates from which they were isolated are shown in Table 2. Overall, the most commonly found sapstain fungus was *S. sapinea*, isolated from 38.5% of all material collected. The predominant *Ophiostoma* species isolated were *Ophiostoma ips*, *O. floccosum*, *O. piliferum*, *L. procerum*, and *O. querci*.

A number of unidentified isolates (248) showed recognisable features of the Ophiostomataceae: *Pesostum*, *Leptographium*, *Sporothrix* (*Ophiostoma* anamorphs), or perithecia (*Ophiostoma* teleomorphs). These isolates grew on cycloheximide, but were not fully identified as they could not be subcultured from original plates without contamination.

Most of the *Ophiostoma* isolates were readily grouped into morphological species based on mycelial characteristics and anamorph states. Many of the species were similar to common *Ophiostoma* or *Leptographium* species from North America or Europe, and representative Northern Hemisphere isolates of these species were compared with New Zealand isolates by sexual compatibility testing and by their ITS sequences (Table 2). Twelve of the

Table 1 Total number of samples taken from each site type.

Material sampled	Exotic plantations	Farm	Mill	Native forest	Nursery	*P. radiata* plantation	Port	Urban
Bark	14	3	1	14	0	73	4	5
Cone	11	3	1	5	0	96	0	6
Log	19	0	106	4	0	694	111	15
Needles/leaves	17	5	1	14	0	92	1	6
Seedling	0	0	0	0	17	1	0	0
Stump	0	1	0	0	0	8	0	0
Timber	0	0	91	0	0	4	0	0
Twigs/branches	35	19	3	61	0	200	3	11
Veneer	0	0	18	0	0	0	0	0
Wood chip	0	0	165	0	0	0	0	0

Table 2 Fungal isolates included in this study.

Species	Isolate	GenBank	Other no./Collector or supplier	Substrate	Country of origin
Ophiostoma coronatum	C1647	DQ062969	IMI 176533, WIN(M)71-26/Reid	*Pinus banksiana*	Canada
O. floccosum	C1086	AF198231	CBS 799.73/Käärik	unknown	Sweden
	C988	= C1086	CBS 102361, 103/Farrell	*Pinus radiata*	New Zealand
	C989	= C1086	CBS 102362, 2NZ-35/Farrell	*Pinus radiata*	New Zealand
O. galeiforme	C1101	DQ062979	NZ-413-12/Farrell	*Pinus radiata*	New Zealand
O. huntii	C12	NA	Harrington	*Pinus ponderosa*	USA
	C139	NA	Harrington	*Pinus ponderosa*	USA
	C774	NA	CBS 153-65, MUCL 9970, CMW 455, LMW 31	*Pinus contorta*	Canada
	C1533	NA	NZ-259/Farrell	*Pinus radiata*	New Zealand
O. ips	C944	DQ062980	NZ-5/Farrell	*Pinus radiata*	New Zealand
O. nicrocarpum-like	C1649	NA	NZ-1131/Farrell	*Pinus radiata*	New Zealand
	C1142	DQ062978	NZ-493/Farrell	*Pinus radiata*	New Zealand
O. perfectum	C1104	DQ062970	CBS 636.66 (AUT for type)		
O. piceae	C1087	AF198226	CBS 108.21/Münch	Unknown	Germany
	C1246	AF198217	CBS 102356/Worrall	*Pseudotsuga*	New York, USA
	C993	= C1087	4NZ-125/Farrell	*Pinus radiata*	New Zealand
	C992	= C1246	3NZ-S501-5/Farrell	*Pinus radiata*	New Zealand
O. pluriannulatum	C1033	DQ062971	NZ-150/Farrell	*Pinus radiata*	New Zealand
	C102	= C1033	RWD 799/Davidson	unknown	USA
	C705	= C1033	CMW 1251/Wingfield	*Eucalyptus grandis*	South Africa
	C1628	= C1033	NZ-1422/Farrell	*Pinus radiata*	New Zealand
	C691	= C1033	RB92-1/Blanchette	*Populus* sp.	USA
	C1257	= C1033	TAB 394	*Populus tremuloides*	USA
	C1567	DQ062972	UAMH 9559, WIN(M)869/Reid (*Ceratocystis novae-zelandiae*)	*Podocarpus* sp.	New Zealand
	C1300	DQ062973	Hofstra	*Pinus radiata*	California, USA
	C1258	= C1300	TAB 397/Blanchette	wood	California, USA
Ophiostoma sp. E.	C1097	DQ062974	NZ-432-1/Farrell	*Pinus radiata*	New Zealand
close to *O. pluriannulatum*	C1098	= C1097	NZ-432-6/Farrell	*Pinus radiata*	New Zealand
	C1626	DQ062975	AU57/Uzunovic	*Pinus contorta*	Canada

O. querci	C969	AF198238	CBS 102352, H1042/Scard and Webber	*Quercus*	United Kingdom
	C970	AF198239	CBS 102353, H1039/Scard and Webber	*Quercus*	United Kingdom
	C984	= C970	2NZ-26/Farrell	*Pinus radiata*	New Zealand
	C936	= C969	109/Blanchette	*Pinus radiata*	New Zealand
O. setosum	C1194	AF198230	CBS 102358	*Pseudotsuga*	Washington, USA
	C985	= C1194	3NZ-S509	*Pinus radiata*	New Zealand
O. stenoceras	C966	AF484455	NZ-38b-3/Farrell	*Pinus radiata*	New Zealand
Pesotum fragans	C990	DQ062976	NZ-35/Farrell	*Pinus radiata*	New Zealand
	C991	= C990	NZS515/Farrell	*Pinus radiata*	New Zealand
	C1224	AF198248	CBS 279.54, ATCC 24590/Kaarik	*Pinus*	Sweden
Pesotum sp. near *P. fragrans*	C1496	DQ062971	A1-50/Farrell	*Pinus radiata*	Australia
	C1560	= C1496	A2-64/Farrell	*Pinus radiata*	Australia
	C1561	= C1496	NZ-2372/Farrell	*Pinus radiata*	New Zealand

species were separated into three main groupings based on their anamorphs: species with *Pesotum* and *Sporothrix* synanamorphs (*O. piceae*, *O. querci*, *O. floccosum*, and *O. setosum*); species with *Leptographium* anamorph (*O. galeiforme*, *O. huntii*, *L. procerum*, and *L. truncatum*); and species with only a *Sporothrix* anamorph (*O. piliferum*, *Ophiostoma* species E, *O. pluriannulatum*, *O. nigrocarpum*-like, and *O. stenoceras*).

The most common *Ophiostoma* species of the group with only a *Sporothrix* anamorph was *O. piliferum*, and this species was identified by its anamorph. It produces *Sporothrix* conidiophores with denticles forming a distinct rachis up to 30 µm long, bearing terminal conidia and ramoconidia. This extended rachis distinguished *O. piliferum* from the other *Sporothrix*-producing species. No ITS sequence could be obtained for *O. piliferum* isolates.

The New Zealand isolates of *Ophiostoma* sp. E cultures (C1097, C1098) were fully compatible with the Canadian isolate (C1626) (Uzunovic et al. 1999). The ITS sequences of *Ophiostoma* sp. E isolates from New Zealand and Canada were identical to each other and slightly different from the isolates of other species in the *Sporothrix* only complex. This species may have been the fungus identified as *O. coronatum* by Hutchison & Reid (1988a). However, the ITS sequence of the culture from the holotype of *O. coronatum* (C1647) was similar to that of *O. nigrocarpum* and different from that of *Ophiostoma* sp. E, and pairings between the Canadian isolate of *O. coronatum* (C1647) and isolates of *Ophiostoma* sp. E from Canada and New Zealand failed to produce perithecia.

On MEA, cultures of *O. pluriannulatum* were generally white, but there were darker areas of mycelium if perithecia were produced. *Sporothrix* conidiophores produce terminal conidia and ramoconidia. Perithecial necks usually proliferated, that is, produced ostiolar hyphae and exuded ascospores, then repeatedly extended, producing more ostiolar hyphae and exuding more ascospores, leaving whorls of ostiolar hyphae and ascospore masses at each annulation along the length of the neck. Sterile perithecia, which have shorter necks and no annulations, were commonly seen in unmated cultures. These annulations and the generally less pigmented mycelium helped distinguish *O. pluriannulatum* from *O. piliferum*. Our ITS analysis (unpublished) showed five main groups or putative species in the *O. pluriannulatum* complex, similar to the analysis of the large subunit (LSU) data analysis (Hausner et al. 1993). One species, *O. perfectum* (C1104), from

the USA had perithecia without annulations. Two isolates from South America (C960 and C1495) had long perithecial necks with annulations, were heterothallic, and were reproductively isolated from each other and the other members of the *Sporothrix*-only complex, indicating that C960 and C1495 were probably two distinct species. Two other isolates (C1258 and C1300) from California were morphologically similar to *O. californicum* (DeVay et al. 1968), were heterothallic, and had an ITS sequence similar to C1604 from Trinidad, which is homothallic. The two California isolates were sexually compatible with each other but were incompatible with other isolates in the complex. Finally, isolates of two intersterility groups had identical ITS sequences. One of these intersterility groups (represented by C102, C705, C1033, C1567, and C1628) appeared to be *O. pluriannulatum* sens. str., and isolates C691 and C1257 from *Populus* spp. may be *O. populinum* (Hinds & Davidson 1975). The two sets of isolates were heterothallic but reproductively isolated and exhibit some morphological differences. The New Zealand isolates (C1033 and C1628) were interfertile with and had the ITS sequences typical of *O. pluriannulatum* sens. str.

The absence of ramoconidia, prolific perithecia production, and ITS sequence separated *O. stenoceras* from the other *Sporothrix*-forming species. *O. stenoceras* is homothallic and forms abundant perithecia on MEA, with the perithecia forming in distinct concentric rings.

Two other unidentified *Sporothrix* species (C1649 and C1142), both similar to *O. nigrocarpum*, were each isolated once from *P. radiata*. Neither produced perithecia in culture, and their ITS sequences were distinct from each other and dissimilar to other examined species of *Ophiostoma*. We assumed that they were the anamorphs of *Ophiostoma* species, as they tolerated cycloheximide (Harrington 1981).

The *Leptographium* group was comprised of species forming conidiophores with pigmented, mononematous stipes, ending in penicillately branched conidiogenous cells bearing hyaline, unicellular conidia, forming a white mass at the tip. The most common *Leptographium* species was *L. procerum*. On MEA, *L. procerum* was easily identified by the production of distinct concentric rings of conidiophores and a sweet smell, which distinguished it from the other species with a *Leptographium* anamorph. No ITS sequence could be obtained for

Table 3 Potential sapstain species isolated from all material sampled.

Potential sapstain species	Number of isolates from all material	Wood type
Leptographium procerum (Kendrick) Wingfield	95	*Pinus radiata*, *Pinus taeda*
L. truncatum (Wingfield & Marasas) Wingfield	4	*P. radiata*
Ophiostoma sp. E	16	*P. radiata*
O. floccosum Mathiesen	293	*P. radiata*
O. galeiforme (Bakshi) Math-Käärik	11	*P. radiata*
O. huntii (Robinson-Jeffrey) de Hoog & Scheffer	35	*P. radiata*, *P. taeda*
O. ips (Rumbold) C.Moreau	311	*P. radiata*, *Acer negundo*, *P. taeda*
O. nigrocarpum-like	2	*P. radiata*
O. piceae (Münch) H. & P.Sydow	62	*P. radiata*, *Pinus maritima*
O. piliferum (Fries) H. & P.Sydow	154	*P. radiata*, *P. taeda*, *Liriodendron tulipifera*
O. pluriannulatum (Hedgecock) H. & P.Sydow	13	*P. radiata*, *P. taeda*, *Eucalyptus* sp., *Nothofagus solandri* var. *solandri*
O. querci (Georgévitch) Nannf.	90	*P. radiata*, *Eucalyptus* sp., *Pseudotsuga menziesii*
O. setosum Uzuonovic, Seifert, Kim & Breuil	70	*P. radiata*, *P. menziesii*, *P. taeda*
O. stenoceras (Robak) Melin & Nannf.	3	*P. radiata*
Pesotum fragans (Math-Käärik) Okada & Seifert	1	*P. radiata*
Sphaeropsis sapinea (Fr.Fr.) Dyko & Sutton	756	*P. radiata*, *A. negundo*, *Cordyline australis*, *P. menziesii*, *Eucalyptus* sp., *Liriodendron tulipifera*, *Cypressus lusitanica*, *P. maritima*, *Populus* sp., *Chamaecyparis lawsonia*, *Magnolia grandiflora*

Table 4 Percentage of each species isolated from materials sampled. Mill samples are all samples from logs, timber, wood chips, and veneer taken at mills. Data from these individual groups also presented separately.

Species	Bark ($n = 114$)	Cone ($n = 122$)	Log ($n = 949$)	Needles /leaves ($n = 136$)	Seedling ($n = 18$)	Stump ($n = 9$)	Timber ($n = 95$)	Twig/ branch ($n = 332$)	Veneer ($n = 18$)	Wood chip ($n = 165$)	Mill samples ($n = 386$)	Total ($n = 1598$)
Leptographium procerum	0.9	0.8	8.6	0.7	0.0	0.0	3.2	1.2	0.0	1.8	1.6	4.9
L. truncatum	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.8	0.2
Ophiostoma sp. E	0.0	0.8	1.4	0.0	5.6	0.0	0.0	0.3	0.0	0.0	0.0	0.8
O. floccosum	2.6	0.0	22.6	1.5	0.0	11.1	27.4	3.6	0.0	21.2	23.6	15.0
O. galeiforme	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6
O. huntii	0.0	0.0	3.0	0.7	0.0	0.0	2.1	0.0	5.6	1.8	1.6	1.8
O. ips	0.9	0.8	28.6	0.0	0.0	0.0	14.7	1.2	0.0	12.1	15.0	15.9
O. nigrocarpum-like	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
O. piceae	0.9	0.0	4.4	1.5	0.0	0.0	6.3	0.9	0.0	4.8	6.5	3.2
O. piliferum	0.9	0.0	15.2	0.0	0.0	0.0	5.3	0.6	0.0	1.2	3.1	7.9
O. pluriannulatum	0.9	0.0	0.8	0.7	0.0	0.0	0.0	0.6	0.0	0.6	0.3	0.7
O. querci	0.9	0.8	7.5	0.0	0.0	0.0	5.3	0.9	5.6	4.8	9.3	4.6
O. setosum	0.0	0.0	4.4	0.0	0.0	11.1	10.5	0.6	5.6	8.5	8.8	3.6
O. stenoceras	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.2
Pesotum fragans	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.3	0.1
Sphaeropsis sapinea	37.7	46.7	44.9	44.1	16.7	22.2	25.3	39.5	16.7	4.2	19.2	38.6

isolates of *L. procerum*. In contrast, on MEA, *O. huntii* will not readily form its *Leptographium* conidiophores but produced distinct serpentine surface hyphae, which distinguished *O. huntii* from *L. procerum* and *L. truncatum*. The perithecia and ascospores of *O. huntii* were similar in size and shape to *O. galeiforme*. The ascospores were cucullate in side view and triangular in end view. The ITS sequences of *O. huntii* isolates from New Zealand (C1533) and the USA (C12, C139) were identical to each other but slightly different from the sequence of the culture from the Canadian holotype (C774).

The anamorph of *O. galeiforme* exhibited a range of morphologies from *Leptographium*-like conidiophores to *Pesotum*-like conidiophores, with the synnema appearing to be a loose aggregation of *Leptographium*-like conidiophores. This range of conidiophore types differentiated *O. galeiforme* from the other *Ophiostoma* and *Leptographium* species. The ITS sequences of New Zealand isolates of *O. galeiforme* were identical to those of European and South African isolates (Zhou et al. 2004).

Leptographium truncatum was distinguished from *L. procerum* by the absence of concentric rings and a distinctive aroma. The conidia of *L. truncatum* had a broad truncate end at the point of abscission from the conidiogenous cell, while the conidia of *L. procerum* were not as distinctly truncate. *L. truncatum* can be distinguished from *O. huntii* by the lack of serpentine hyphae and from *O. galeiforme* by the lack of synnemata. Our New Zealand isolates of *L. truncatum* appeared to be conspecific with C8 (PREM 45699) from the paratype of *L. truncatum* collected in New Zealand and C59 (= ATCC 22735) from Sweden. No ITS sequence could be obtained from *L. truncatum* isolates.

Morphologically, *O. ips* and *P. fragrans* did not fit well into one of the three main anamorph groups. They produced synnemata but also simple, hyaline, mononematous conidiophores other than *Leptographium* or *Sporothix*. *O. ips* was easily distinguished from other *Ophiostoma* species by its brown colour on MEA and ascospores with a rectangular sheath.

Pesotum fragrans may be comprised of two closely related species as indicated from the ITS sequence of the Swedish (C1224) and other cultures isolated from wood of Pinaceae from New Zealand (C990, C991, C1561), Australia (C1496, C1560), and the Pacific Northwest,

USA (C1202, C1348). Two New Zealand isolates (C990, C991) showed slight sequence differences from C1224 and C1202 but were morphologically similar, and we consider them to be *P. fragrans*. The other group in this complex is comprised of isolates from New Zealand (C1561) and Australia (C1560 and C1496). The isolates in this group produced black stalked synnemata up to 1 mm in length and simple, hyaline conidiophores similar to *P. fragrans*, but produced no distinct odour, and no yellow colour was produced when grown on MEA. With these differences, along with sequence differences, we felt this group was a distinct species from *P. fragrans*. Though we have seen no perithecia associated with any of the isolates, and all pairings among the isolates have failed to produce perithecia, ITS sequences suggest that *P. fragrans* is an anamorph of an *Ophiostoma* species.

The proportion of each species according to the type of material isolated is shown in Table 4. *Sphaeropsis sapinea* was isolated from all material sampled including collections from the forest floor (including branches, twigs, needles, leaves, cones, and logs) as well as from logs and timber. In contrast *Ophiostoma* species were mainly found on logs, timber, and wood chips. There were no apparent differences between the species according to season or location within New Zealand (data not shown).

DISCUSSION

There are few detailed surveys of geographical, seasonal, and substrate distribution of sapstain fungi worldwide (Uzunovic et al. 1999). There are two main reasons for this. Firstly, knowledge about the taxonomy of one of the main genera, *Ophiostoma*, is complicated and complex morphological studies are required to adequately identify the fungi to species. With molecular analysis and increased knowledge of the *Ophiostoma* species within New Zealand, identification is possible (Harrington et al. 2001). Secondly, a survey of the sapstain fungi within a country requires extensive and organised collection of material (Uzunovic et al. 1999).

Two types of media were used in this study. MYEA supplemented with streptomycin sulphate and chloramphenicol was used to isolate all sapstain fungi including *S. sapinea* and *Ceratocystis* species. The second medium, MYEA supplemented with streptomycin sulphate, chloramphenicol, and cycloheximide, was used to isolate only *Ophiostoma* species. Prior to establishment of this survey, an unpublished study found that a higher concentration of cycloheximide than that used by Harrington (1981) reduced the amount of contaminating fungi such as *Trichoderma* species and *Penicillium* species without effecting the growth of *Ophiostoma* species and their anamorphs.

This study found that *S. sapinea* was the most widely distributed and main cause of sapstain of wood, supporting the results of Birch (1936) and Butcher (1968a). All the *S. sapinea* isolates were the type A morphotype. Type A isolates were described as having fluffy white to grey-green mycelia and smooth-walled conidia, lacking microconidia, and being pathogenic to both wounded and unwounded hosts (Swart et al. 1991). The second most dominant sapstain fungus in this survey was *O. ips*. The predominance of *S. sapinea* and *O. ips* raises serious forest health issues, as both are known pathogens of *Pinus* species (Mathre 1964; Chou 1984; Rane & Tatter 1987). These were the only known sapstaining plant pathogens to be isolated in this survey. There has been some discussion that the human pathogen *Sporothrix schenckii* may be the anamorph of *O. stenoceras* (Summerbell et al. 1993). The two species are similar in the *Sporothrix* morphology, but they have different ITS sequences (de Beer et al. 2003). The ITS sequences of isolates of *O. stenoceras* from around the world are identical, while the *S. schenckii* sequences comprise three well-supported, closely related clades, which may represent separate species.

Prior to this survey, Hutchison & Reid (1988a,b) described a limited number of *Ophiostoma* species in New Zealand. Detailed descriptions of the synanamorphs of *O. ips* were given by Hutchison & Reid (1988a). Hutchison & Reid (1988a) reported *O. piliferum*, but they described and illustrated the necks of perithecia with annulations, similar to the characteristic annulations along the necks of *O. pluriannulatum*. Our isolates of *O. piliferum* did not produce perithecial necks with annulations, and it is likely that some of the isolates of *O. piliferum* examined by Hutchison & Reid (1988a) were *O. pluriannulatum*, which they did not report. In addition, we believe that their newly described species, *Ceratocystis novae-zelandiae*, is a synonym of *O. pluriannulatum*. They reported that some isolates of *C. novae-zelandiae* rarely formed synnemata, but none of their four isolates (C1562, C1563, C1566, C1567 = WIN(M)863, WIN(M)864, WIN(M)865, and WIN(M)869, respectively) that we examined did so. Furthermore, their cultures were sexually compatible with our tester strains of

O. pluriannulatum and had ITS sequences identical to *O. pluriannulatum*. The LSU sequences (Hausner et al. 1993) also showed *C. novae-zelandiae* to be similar to *O. pluriannulatum*. We suspect that the description (Hutchison & Reid 1988a) of *C. novae-zelandiae* was based on a mixed culture of *O. pluriannulatum* and *O. piceae* or *O. querci*, and that *C. novae-zelandiae* is, therefore, a synonym of *O. pluriannulatum*. No *Ceratocystis* species or *Ceratocystiopsis* species were isolated in this survey.

Morphologically, *Ophiostoma* sp. E resembled the description of *O. coronatum* by Hutchison & Reid (1988a), but they expressed doubts about the identification of their fungus as *O. coronatum*. Based on morphology and LSU rDNA data (Hausner et al. 1993), *Ophiostoma* sp. E may be *O. longirostellatum* or *O. coniculim*. Hausner et al. (1993) found that *O. pluriannulatum* (= *C. novae-zelandiae*), *O. californicum*, *O. populinum*, *O. longirostellatum*, and *O. coniculim* have similar LSU sequences.

Hutchison & Reid (1988a) also reported *O. piceaperdum* from *P. nigra*, *P. radiata*, and *P. taeda*. *O. huntii* is very similar morphologically to *O. piceaperdum* (= *O. europhioides*) and their isolates were probably *O. huntii* and not *O. piceaperdum*, which is known only from North America and Europe (Jacobs et al. 1998). The ITS sequences of the two species were shown to be quite distinct, and, in contrast to *O. huntii*, *O. piceaperdum* is homothallic.

Hutchison & Reid (1988b) described *Hyalopesotum pini* as an anamorphic fungus, associated with bark beetle galleries in *P. radiata* and *P. taeda*. We compared their isolates of *H. pini* (82–87b, 82–88b; supplied by J. Reid) to our cultures of *O. galeiforme* and concluded that they are morphologically the same. Perithecia of *O. galeiforme* were slow to develop in culture, usually only after two compatible isolates were paired on media with pine twigs. Thus, it is not surprising that perithecia were not found in the material of *H. pini* (Hutchison & Reid 1988b). Okada et al. (1998) transferred *H. pini* to *Pesotum pini*, but we (Harrington et al. 2001) had used *Pesotum* in a stricter sense, to include only those synnema-forming fungi with *Sporothrix* synanamorphs. Kim et al. (2005) described the teleomorph of *H. pini* as *O. radiaticola*, which they reported to have an ITS sequence that differed slightly from that of *O. galeiforme*. However, our New Zealand isolates of *H. pini* have the identical ITS sequence as those of *O. galeiforme* (Zhou et al. 2004), and we consider *O. radiaticola* to be a synonym of *O. galeiforme*.

Although Hutchison & Reid (1988b) did not report *Leptographium procerum*, they reported isolating a *Leptographium* sp. from *Larix* sp., *P. nigra*, and *P. radiata*, and their description of this unidentified species resembled *L. procerum*. Jacobs et al. (2001) described several new species that were morphologically very similar to *L. procerum*, including *L. euphyes*. However, we have seen uniformity among all of our isolates of *L. procerum*, and we were unable to clearly distinguish their new species from our concept of *L. procerum* except that *L. procerum* isolates consistently showed annual rings of sporulation when cultured on PDA and other media. Our isolate C51 (= PREM 45703 = CMW 107) was reported to be from the paratype of *L. euphyes*, but it had the concentric rings of sporulation and sweet smell typical of *L. procerum*. It is possible that this culture is not representative of the holotype of *L. euphyes*. The description of *L. euphyes* appeared to us to be more similar to *L. truncatum* than to *L. procerum*.

Leptographium truncatum was reduced to synonymy with *L. lundbergii* by Strydom et al. (1997), designating CBS 352.29 as the neotype as there is no type material for *L. lundbergii* and the original description was vague. The status of this species remains confused. Until the concept of *L. lundbergii* is more clearly defined, we prefer to maintain *L. truncatum* as a distinct species.

Ophiostoma piceae has been used in the broad sense by earlier investigators. Hutchison & Reid (1988a) listed *O. floccosum* and *O. querci* as a synonym of *O. piceae*. However, it is not clear if their cultures were indeed *O. piceae*, and it is likely that many were *O. floccosum*, *O. querci*, or *O. setosum*. Isolates of one or perhaps two morphologically unidentified species similar to *O. querci* were also reported from *P. radiata* and a species of *Nothofagus* in New Zealand (Harrington et al. 2001). These isolates were not interfertile with *O. querci* tester strains and had slightly different ITS sequences.

Reay et al. (2002) described the significant relationship between sub-lethal attack of seedlings by the introduced bark beetle *Hylastes ater* and the subsequent invasion by many sapstain fungi in New Zealand. *O. querci*, *O. piceae*, *L. procerum*, *L. truncatum*, *O. huntii*, and *O. galeiforme* were isolated from living *P. radiata* seedlings damaged by *H. ater* (Reay et al. 2002). In this study, only one species, *O. ips*, was isolated from *H. ater* beetles. In a subsequent study, Reay et al. (2005) isolated *O. galeiforme*, *O. huntii*, *O. floccosum*, *O. setosum*, *L. procerum*, and *L. truncatum* from *P. radiata* stumps,

living seedlings damaged by *H. ater*, and *H. ater* beetles. *O. querci* was isolated from stumps and beetles and *O. stenoceras* was isolated from damaged seedlings. *H. ater* was confirmed as a vector of many *Ophiostoma* species to *P. radiata* seedlings in laboratory experiments (Reay et al. 2005). This beetle has been suggested as the mechanism by which a number of species of sapstain fungi were introduced to New Zealand (Wingfield & Gibbs 1991).

The spores of the rain-splash dispersed *S. sapinea* are associated with cones and needles (Palmer et al. 1988) that are found more commonly on the forest floor. The inoculum density of *S. sapinea* is higher, therefore, in the forest environment. *Ophiostoma* species were isolated more in wood chips, timber, and logs than *S. sapinea*. The sticky spores on the synnema and perithecial stalks of *Ophiostoma* species are disseminated primarily by insect vectors, and are commonly found on logs and timber (Dowding 1970).

Not all *Ophiostoma* species growing on wood impart a stain to the wood. The lightly pigmented species are often found growing ubiquitously with other darker sapstain fungi. *O. stenoceras* is not associated with sapstain but is a common saprophyte on wood in Europe and North America (de Beer et al. 2003).

Knowledge of the biology, ecology, and taxonomy of sapstain fungi obtained by this survey provides important information that documents the species present in New Zealand. This information is essential if effective control methods for stain reduction are to be realised. Biological control methods with albino technology using colourless strains of the most common sapstain fungi in New Zealand are also currently being evaluated (Held et al. 2003).

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REFERENCES

Birch TTC 1936. *Diplodia pinea* in New Zealand. New Zealand Forest Service Bulletin 8. 32 p.

Burgess T, Wingfield BD, Wingfield MJ 2001. Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. Mycological Research 105: 1331–1339.

Butcher JA 1968a. The ecology of fungi infecting untreated sapwood of *Pinus radiata*. Canadian Journal of Botany 46: 1577–1589.

Butcher JA 1968b. The causes of sapstain in red beech. New Zealand Journal of Botany 6: 376–385.

Chou CKS 1984. Diplodia leader dieback, Diplodia crown wilt, Diplodia whorl canker. Forest Pathology in New Zealand 7. 2 p.

de Beer ZW, Harrington TC, Vismer HF, Wingfield BD, Wingfield MJ 2003. Phylogeny of the *Ophiostoma stenoceras-Sporothrix schenckii* complex. Mycologia 95: 434–441.

DeScenzo RA, Harrington TC 1994. Use of $(CAT)_5$ as a DNA fingerprinting probe for fungi. Phytopathology 84: 534–540.

DeVay JE, Davidson RW, Moller WJ 1968. New species of *Ceratocystis* associated with bark injuries on deciduous fruit trees. Mycologia 60: 635–641.

De Wet J, Wingfield MJ, Coutinho T, Wingfield BD 2002. Characterization of the 'C' morphotype of the pine pathogen *Sphaeropsis sapinea*. Forest Ecology and Management 161: 181–188.

Dowding P 1970. Colonization of freshly bared pine sapwood surfaces by staining fungi. Transactions of the British Mycological Society 55: 399–412.

Gardes M, Bruns TD 1993. ITS primers with enhanced specificity for basidomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113–118.

Harrington TC 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia 73: 1123–1129.

Harrington TC, Wingfield BD 1995. A PCR-based identification method for species of *Armillaria*. Mycologia 87: 280–288.

Harrington T, McNew D, Steimel J, Hofstra D, Farrell R 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. Mycologia 93: 111–136.

Hausner G, Reid J, Klassen GR 1993. On the phylogeny of *Ophiostoma*, *Ceratocystis* s.s., and *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. Canadian Journal of Botany 71: 1249–1265.

Hausner G, Reid J, Klassen GR 2000. On the phylogeny of members of *Ceratocystis* s.s. and *Ophiostoma* that possess different anamorphic states, with emphasis on the anamorph genus *Leptographium*, based on partial ribosomal DNA sequences. Canadian Journal of Botany 78: 903–916.

Held BW, Thwaites JM, Farrell RL, Blanchette RA 2003. Albino strains of *Ophiostoma* species for biological control of sapstaining fungi. Holzforschung 57: 237–242.

Hinds TE, Davidson RW 1975. Two new species of *Ceratocystis*. Mycologia 67: 715–721.

Hutchison LJ, Reid J 1988a. Taxonomy of some potential wood-staining fungi from New Zealand. 1. Ophiostomataceae. New Zealand Journal of Botany 26: 63–81.

Hutchison LJ, Reid J 1988b. Taxonomy of some potential wood-staining fungi from New Zealand 2. Pyrenomycetes, Coelomycetes and Hyphomycetes. New Zealand Journal of Botany 26: 83–98.

Jacobs K, Wingfield MJ, Wingfield BD, Yamaoka Y 1998. Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *O. aenigmaticum* sp. nov. Mycological Research 102: 289–294.

Jacobs K, Wingfield MJ, Wingfield BD 2001. Phylogenetic relationships in *Leptographium* based on morphological and molecular characters. Canadian Journal of Botany 79: 719–732.

Kim JJ, Lim YW, Seifert KA, Kim SH, Breuil C, Kim GH 2005. Taxonomy of *Ophiostoma radiaticola* sp. nov. (Ophisotomatales, Ascomyctes), the teleomorph of *Pesotum pini*, isolated from logs of *Pinus radiata*. Mycotaxon 91: 481–496.

Mathre DE 1964. Pathogenicity of *Ceratocystis ips* and *Ceratocystis minor* to *Pinus ponderosa*. Contributions from Boyce Thompson Institute 22: 363–388.

Okada G, Seifert KA, Takematsu A, Yamaoka Y, Miyazaki S, Tubaki K 1998. A molecular phylogenetic reappraisal of the *Graphium* complex based on 18 rDNA sequences. Canadian Journal of Botany 76: 1495–1506.

Palmer MA, McRoberts RE, Nicholls TH 1988. Sources of inoculum of *Sphaeropsis sapinea* in forest tree nurseries. Phytopathology 78: 831–835.

Rane K, Tatter TA 1987. Pathogenicity of blue-stain fungi associated with *Dendroctonus terebrans*. Plant Disease 71: 879–883.

Reay SD, Walsh PJ, Ram A, Farrell RL 2002. The invasion of *Pinus radiata* seedlings by sapstain fungi, following attack by the Black Pine Bark Beetle, *Hylastes ater* (Coleoptera: Scolytidae). Forest Ecology and Management 165: 47–56.

Reay SD, Thwaites JM, Farrell RL 2005. A survey of *Ophiostoma* species vectored by *Hylastes ater* to pine seedlings in New Zealand. Forest Pathology 35: 105–113.

Schirp A, Farrell RL, Kreber B 2003a. Effects of New Zealand sapstaining fungi on structural integrity of unseasoned radiata pine. Holz als Rohund Werkstoff 61: 369–376.

Schirp A, Farrell R, Kreber B, Singh AP 2003b. Advances in understanding the ability of sapstaining fungi to produce cell-wall degrading enzymes. Wood and Fiber Science 35: 434–444.

Strydom RC, Wingfield BD, Wingfield MJ 1997. Ribosomal DNA sequence comparison of *Leptographium lundbergii* and *L. truncatum* and neotypification of *L. lundbergii*. Systematic and Applied Microbiology 20: 295–300.

Summerbell RC, Kane J, Krajden S, Duke EE 1993. Medically important *Sporothrix* species and related Ophiostomatoid fungi. In: Wingfield MJ, Seifert KA, Webber JJ ed. *Ceratocystis* and *Ophiostoma*: Taxonomy, ecology and pathogenicity. St Paul, APS Press. Pp. 185–192.

Swart WJ, Wingfield MJ, Palmer MA, Blanchette RA 1991. Variation among South African isolates of *Sphaeropsis sapinea*. Phytopathology 81: 489–493.

Swofford DL 1998. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Uzunovic A, Yang D, Gagne P, Breuil C, Bernier L, Byrne A, Gignac M, Kim SH 1999. Fungi that cause sapstain in Canadian softwood. Canadian Journal of Microbiology 45: 914–922.

White TJ, Bruns T, Lee S, Taylor J 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ ed. PCR protocols: a guide to methods and application. San Diego, Academic Press. Pp. 315–322.

Wingfield MJ, Gibbs JN 1991. *Leptographium* and *Graphium* species associated with pine-infesting bark beetle in England. Mycological Research 95: 1257–1260.

Zhou X, de Beer ZW, Harrington TC, McNew D, Kiristis T, Wingfield MJ 2004. Epitypification of *Ophiostoma galeiforme* and phylogeny of species in the *O. galeiforme* complex. Mycologia 96: 1306–1315.