IDENTIFYING PINE-INHABITING LOPHODERMIUM SPECIES USING PCR-RFLP*

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ABSTRACT

Three species of *Lophodermium* are reported from pine in New Zealand—the previously recorded *L. conigenum* (Brunaud) Hilitzer and *L. pinastri* (Schrad.) Chevall., and the newly reported *L. molitoris* Minter. All are saprobes with an initial endophytic phase. The North American species *L. seditiosum* Minter, Staley & Millar is considered a potential threat to New Zealand's plantation forests. Because these fungi are difficult to distinguish morphologically, a simple molecular method was developed to distinguish the four species. After PCR amplification, the ITS* region is digested using *Hae*III and *Hpa*II in a single reaction, resulting in unique RFLP banding patterns for each of the species treated. A phylogenetic analysis based on ITS sequences revealed two genetically distinct groups within *L. pinastri*, one North American and one European. All the New Zealand isolates of *L. pinastri* tested were identical to the North American group. Further development of the method described here, so that fungal DNA is targeted directly from infected plant tissue, will provide an effective quarantine tool for New Zealand's forestry industry.

Keywords: quarantine; Rhytismataceae; fungi; *Elytroderma*; *Lophodermium conigenum*; *Lophodermium molitoris*; *Lophodermium pinastri*; *Lophodermium seditiosum*.

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* PCR = polymerase chain reaction
RFLP = restriction length fragment polymorphism
ITS = internal transcribed spacer region of the ribosomal DNA

INTRODUCTION

Lophodermium Chevall. is a large and complex genus in the family Rhytismataceae, with more than 100 species described from different host plants (Kirk *et al.* 2001). The genus is especially diverse on the needles of pine (*Pinus* spp). In his classic monograph of *Lophodermium* on pines, Minter (1981) reported 16 species, and since then a further 16 pine-inhabiting *Lophodermium* species have been described, mostly from China. Most of the pine-inhabiting species are saprobes, with an endophytic phase to their life-cycle (Minter 1981). They are some of the most common endophytes isolated from symptomless needles of pine (Stone *et al.* 2000). Only *L. seditiosum* is known to be a major pathogen (Minter & Millar 1980; Sinclair *et al.* 1987). However, three of the recently described Asian species were reported to be associated with disease symptoms (*L. iwatense* Tudeshi Sakuyama (Sakuyama 1993), *L. maximum* Bing Zhang He & D.Q.Yang, and *L. parasiticum* Bing Zhang He & D.Q.Yang (He *et al.* 1986)), but these species are poorly known, and their significance to New Zealand as pathogens is unknown.

Pennycook (1989) listed two species of *Lophodermium* on pine in New Zealand — *L. pinastri* and *L. conigenum*. Both are widely distributed throughout New Zealand, on several pine species, and may be found together in a single stand, or even on the same needle. Both species were introduced to New Zealand, and are widely distributed on pines throughout the world (Minter 1981). They have an endophytic phase to their life-cycle, infecting living needles apparently without symptoms, and developing fruiting bodies on the dead needles after the needles have fallen naturally, or after the branch to which they are attached has been broken or cut from the tree. They may also fruit on dead portions of otherwise-living needles still attached to the tree — for example, on the tips of needles killed by frost or *Dothistroma pini* Hulbary (P. Gadgil, pers. comm.). Neither species is known to have any detrimental effect on pine in New Zealand.

Before the studies of Minter and others in the late 1970s (e.g., Minter *et al.* 1978), many collections of *Lophodermium* on pine needles were referred uncritically to *L. pinastri*. Minter (1981) distinguished the pine-inhabiting *Lophodermium* species by morphological features such as the position at which the ascomata develop within needles, the degree to which the lower wall of the ascoma develops, the occurrence of zone lines on needles, and the presence or absence of anamorph conidiomata. These features can be difficult to determine for non-specialists and, apart from a few well-studied species, there remains limited understanding of the significance of morphological variation between collections, especially those taken from different hosts. For example, the position at which the ascomata develop in relation to host tissue layers can be difficult to assess, and consideration needs to be given to how differences in needle anatomy between pine species might influence ascomatal development. The ascomatal lower wall becomes differentiated late in the development of the ascoma (Johnston 1988), suggesting this feature could be influenced by ascomatal maturity or minor genetic variation. One of the New Zealand species, *L. conigenum*, is morphologically very similar to *L. australe* Dearn., and Minter (1981) noted that differentiation of these species is on the basis of the degree of development of the lower wall of the ascoma, but that this is unsatisfactory, and that "the two species may intergrade."

Accurate and rapid differentiation of the saprobic pine-inhabiting *Lophodermium* species in New Zealand from the exotic pathogenic *L. seditiosum* is an important quarantine

issue. Because of the difficulty in using morphology to distinguish the pine-inhabiting *Lophodermium* species, a simple molecular tool has been devised to distinguish isolates of these species with certainty. In addition, data gathered during this study provided some insight into the genetic distinctness of taxa recognised on the basis of the morphological characters used in current *Lophodermium* taxonomy.

METHODS

Collection of Isolates

Collections from New Zealand pine plantations were made from widely scattered sites. Dead needles with open, fresh, *Lophodermium* ascomata were collected from branches and litter, placed in plastic bags, and transported to the laboratory. Collections of needles with *Lophodermium* ascomata were also made from trees with needle-cast disease symptoms from Christmas tree farms in the northern United States in Michigan and Wisconsin. Selected ascomata were excised from the needles, and placed above water agar in petri dishes to allow ascospores to drop. Germinating ascospores were transferred to 2% malt extract agar (MEA, Difco) plates. After 20 days colonies were examined and described, and growth rate was measured. After identification, subcultures were made on to MEA and allowed to grow for 1–2 weeks at 20°C; 5-mm agar plugs were then removed from the margins of actively growing colonies and placed in 10% glycerol in short pieces of plastic drinking straws. The straws were heat sealed and stored under liquid nitrogen in ICMP (International Collection of Micro-organisms from Plants, Landcare Research, Mt Albert). Vouchers of most collections from which isolations were made are stored in PPD (New Zealand Fungal Herbarium, Landcare Research, Mt Albert) or NZFRI (New Zealand Forest Research Institute) (herbarium abbreviations follow Holmgren *et al.* 1990). Isolates studied are listed in Table 1.

Extraction and Amplification of DNA

Cultures were grown in liquid 2% Difco malt extract medium in petri dishes in the dark at 18–20°C until copious mycelial growth was observed (about 3–4 weeks). The cultures were filtered through Miracloth (Calbiochem), and the mycelium was dried between paper tissues. The mycelium was freeze-dried overnight, then ground with liquid nitrogen in a mortar and pestle. The genomic DNA isolation protocol was based on Kim *et al.* (1990), with the following modifications: 1 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4) was added to 50 mg ground, dried mycelium, 2 µl Proteinase K (20 mg/ml) was added and mixed, and 20% SDS solution was added to a final concentration of 2%. After incubation at 65°C for 30 min., the material was centrifuged at 13 000 rpm for 15 min. To the supernatant was added sodium chloride to a final concentration of 1.4 M, and 1/10 volume 10% CTAB buffer, followed by thorough mixing and incubation at 65°C for 30 min. After a phenol-chloroform extraction step, the genomic DNA was ethanol precipitated, dried, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR reactions were carried out as described by White *et al.* (1990), with FastStart Taq enzyme (Roche) on a GeneAmp PCR system 9700 (Applied Biosystems). The complete ITS region (ITS1, ITS2, and 5.8S) was amplified using the primers ITS1 and ITS4, which

TABLE 1–Details of live cultures studied, and additional ITS sequences analysed.

Taxon number (*see* Fig. 1 and 3)	Taxon	Source	Reference	Host geographic distribution	Culture collection number* († indicates isolates for which RFLP banding patterns were obtained)	GenBank Accession Number (‡ indicates sequences obtained during this study)
17	*Elytroderma deformans* (Weir) Darker	Gernandt & Stone		*P. ponderosa* Lawson & C. Lawson, Montana, USA	CBS 181.68	AF203469
8	*Lophodermium australe*	Ortiz-García *et al.*		*P. pseudostrobus* Lindl., Mexico		AY100647
9	*L. australe*	Gernandt *et al.*		*P. palustris* (Engl.) Miller, Florida, USA		U92308
26	*L. baculiferum* Mayr	Ortiz-García *et al.*		*P. ponderosa* Oregon, USA		AY100655
27	*L. baculiferum* (*Leptostroma decipiens* Petr.)	Ortiz-García *et al.*		*P. ponderosa* Oregon, USA		AY100653
28	*L. baculiferum* (*Leptostroma decipiens*)	Ortiz-García *et al.*		*P. ponderosa* Oregon, USA		AY100654
29	*L. baculiferum*	Ortiz-García *et al.*		*P. montezumae* Lambert, Nuevo Leon, Mexico		AY100656
30	*L. baculiferum*	Ortiz-García *et al.*		*P. montezumae* Morelos, Mexico		AY100657
	L. conigenum	Forest Research	NZFS690	*P. radiata* D. Don, Bay of Plenty, NZ	†ICMP 14835	
	L. conigenum	Forest Research	NZFS691	*P. radiata* Bay of Plenty, NZ	†ICMP 14836	
	L. conigenum	Forest Research	NZFS693	*P. radiata*, Auckland, NZ	†ICMP 14837	
10	*L. conigenum*	Forest Research	NZFS709	*P. radiata* Waikato, NZ	†ICMP 14838	‡AY247751
	L. conigenum	Forest Research	NZFS711	*P. radiata* Taupo, NZ	†ICMP 14839	
	L. conigenum	Forest Research	NZFS712	*P. radiata* Taupo, NZ	†ICMP 14840	
	L. conigenum	Forest Research	NZFS742	*P. contorta* Loudon, Taupo, NZ	†ICMP 14841	
	L. conigenum	Forest Research	NZFS743	*P. contorta* Taupo, NZ	†ICMP 14842	
	L. conigenum	Forest Research	NZFS756	*P. radiata* Auckland, NZ	†ICMP 14843	
	L. conigenum	Forest Research	NZFS757	*P. radiata* Auckland, NZ	†ICMP 14844	

TABLE 1–cont.

Taxon number (*see* Fig. 1 and 3)	Taxon	Source	Reference	Host geographic distribution	Culture collection number* († indicates isolates for which RFLP banding patterns were obtained)	GenBank Accession Number (‡ indicates sequences obtained during this study)
	L. conigenum	Forest Research	NZFS778	*P. radiata* Taupo, NZ	†ICMP 14845	
	L. conigenum	Forest Research	NZFS781	*P. radiata* Taupo, NZ	†ICMP 14846	
	L. conigenum	Forest Research	NZFS782	*P. radiata* Taupo, NZ	†ICMP 14847	
	L. conigenum	Forest Research	NZFS786	*P. radiata* Taupo, NZ	†ICMP 14848	
	L. conigenum	Forest Research	NZFS790	*P. radiata* Taupo, NZ	†ICMP 14849	
	L. conigenum	Forest Research	NZFS798	*P. radiata* Westland, NZ	†ICMP 14850	
11	*L. conigenum*	Ortiz-García *et al*.		*P. radiata* Westland, NZ	ICMP 13979	AY100645
13	*L. indianum*	Ortiz-García *et al*.		*P. greggii* Mexico		AY100642
14	*L. indianum*	Ortiz-García *et al*.		*P. hartwegii* Mexico		AY100641
32	*L. molitoris*	Forest Research	NZFS789	*P. radiata* Auckland, NZ	†ICMP 14900	‡AY247752
31	*L. molitoris*	Ortiz-García *et al*.		*P. taeda* L. North Carolina, USA	CBS 597.84	AY100659
37	*L. nitens* Darker	Deckert *et al*.		*P. strobus* L. Nova Scotia, Canada		AF426057
38	*L. nitens*	Deckert *et al*.		*P. strobus* Nova Scotia, Canada		AF426059
34	*L. piceae*	Gernandt & Stone		*Picea abies* (L.) H. Karst., Germany		AF203471
	L. pinastri	Landcare Research	R886	*P. radiata* Southland, NZ	†ICMP 14997	
	L. pinastri	Forest Research	NZFS754	*P. pinaster* Aiton Wellington, NZ	†ICMP 14851	
	L. pinastri	Forest Research	NZFS755	*P. pinaster* Wellington, NZ	†ICMP 14852	
	L. pinastri	Forest Research	NZFS776	*P. roxburghii* Sargent, Wellington, NZ	†ICMP 14853	
	L. pinastri	Forest Research	NZFS779	*P. roxburghii* Wellington, NZ	†ICMP 14854	

TABLE 1–cont.

Taxon number (*see* Fig. 1 and 3)	Taxon	Source	Reference	Host geographic distribution	Culture collection number* († indicates isolates for which RFLP banding patterns were obtained)	GenBank Accession Number (‡ indicates sequences obtained during this study)
	L. pinastri	Forest Research	NZFS780	*P. roxburghii* Wellington, NZ	†ICMP 14855	
	L. pinastri	Forest Research	NZFS783	*P. muricata* D. Don, Wellington, NZ	†ICMP 14856	
20	*L. pinastri*	Forest Research	NZFS785	*P. pinaster* Wellington, NZ	†ICMP 14857	‡AY247753
	L. pinastri	Forest Research	NZFS788	*P. muricata* Wellington, NZ	†ICMP 14858	
	L. pinastri	Forest Research	NZFS796	*P. pinaster* Wellington, NZ	†ICMP 14859	
	L. pinastri	Forest Research	NZFS797	*P. sargentii* Wellington, NZ	†ICMP 14860	
	L. pinastri	Forest Research	NZFS800	*P. densiflora* Taranaki, NZ	†ICMP 14861	
	L. pinastri	Forest Research	NZFS802	*P. densiflora* Taranaki, NZ	†ICMP 14862	
	L. pinastri	Forest Research	NZFS803	*P. palustris* Taranaki, NZ	†ICMP 14863	
	L. pinastri	Forest Research	NZFS804	*P. palustris* Taranaki, NZ	†ICMP 14864	
	L. pinastri	Forest Research	NZFS820	*P. muricata* Wellington, NZ	†ICMP 14865	
18	*L. pinastri*	Ortiz-García *et al*.		*P. ponderosa* Oregon, USA		AY100649
21	*L. pinastri*	Stenstrom & Ihrmark		Sweden		AF473557
22	*L. pinastri*	Catal & Adams		*P. sylvestris* L. Scotland	ATCC 28347	AF013224.
23	*L. pinastri*	Catal & Adams		*P. nigra* Vancouver, Canada		AF462434
24	*L. pinastri*	Stenstrom & Ihrmark		Sweden		AF473555
25	*L. pinastri*	Ortiz-García *et al*.		*P. sylvestris* Germany		AY100650
	L. seditiosum	Landcare Research		*P. sylvestris* Cadillac, Michigan, USA	†ICMP 14656	
	L. seditiosum	Landcare Research		*P. sylvestris* Cadillac, Michigan, USA	†ICMP 14662	

TABLE 1–cont.

Taxon number (*see* Fig. 1 and 3)	Taxon	Source	Reference	Host geographic distribution	Culture collection number* († indicates isolates for which RFLP banding patterns were obtained)	GenBank Accession Number (‡ indicates sequences obtained during this study)
	L. seditiosum	Landcare Research		*P. sylvestris* Kalamazoo, Michigan, USA	†ICMP 14666	
	L. seditiosum	Landcare Research		*P. sylvestris* vic. Saxeville, Wisconsin, USA	†ICMP 14674	
	L. seditiosum	Landcare Research		*P. sylvestris* vic. Saxeville, Wisconsin, USA	†ICMP 14677	
	L. seditiosum	Landcare Research		*P. sylvestris* vic. Saxeville, Wisconsin, USA	†ICMP 14678	
	L. seditiosum	Landcare Research		*P. sylvestris* vic. Saxeville, Wisconsin, USA	†ICMP 14679	
	L. seditiosum	Landcare Research		*P. sylvestris* vic. Saxeville, Wisconsin, USA	†ICMP 14681	
	L. seditiosum	Landcare Research		*P. sylvestris* vic. Saxeville, Wisconsin, USA	†ICMP 14689	
	L. seditiosum	Landcare Research		*P. sylvestris* vic. Saxeville, Wisconsin, USA	†ICMP 14690	
	L. seditiosum	H. Butin	L225	*P. sylvestris* Germany	†DSMZ 5029	
1	*L. seditiosum*	Catal & Adams		*P. sylvestris* Scotland	ATCC 28345	AF462435
2	*L. seditiosum*	Ihrmark		*P. sylvestris* Sweden		AF473551
3	*L. seditiosum*	Stenstrom & Ihrmark		*P. sylvestris* Sweden		AF473552
4	*L. seditiosum*	Stenstrom & Ihrmark		*P. sylvestris* Sweden		AF473554
5	*L. seditiosum*	Gernandt & Stone				AF203468
6	*L. seditiosum*	Stenstrom & Ihrmark		*P. sylvestris* Sweden		AF473553
7	*L. seditiosum* & Ihrmark	Stenstrom		*P. sylvestris* Sweden		AF473550
15	*Lophodermium* sp.1	Ortiz-García *et al*.		*P. ayacahuite* Mexico		AY100643
16	*Lophodermium* sp.2	Ortiz-García *et al*.		*P. douglasiana* Mexico		AY100644

TABLE 1–cont.

Taxon number (*see* Fig. 1 and 3)	Taxon	Source	Reference	Host geographic distribution	Culture collection number* († indicates isolates for which RFLP banding patterns were obtained)	GenBank Accession Number (‡ indicates sequences obtained during this study)
12	*Lophodermium* sp.3	Ortiz-García *et al.*		*P. montezumae* Mexico		AY100648
35	*Lophodermium* sp.4	Ortiz-García *et al.*		*P. chiapensis* (Martínez) Andreson, Mexico		AY100651
36	*Lophodermium* sp.4 (*Leptostroma* sp.)	Ortiz-García *et al.*		*P. ayacahuite* Mexico		AY100652
33	*Lophodermium* sp.5	Ortiz-García *et al.*		*P. montezumae* Mexico		AY100660

* ATCC, American Type Culture Collection.
CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.
DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.
ICMP, International Collection of Micro-organisms from Plants, Landcare Research, Auckland.

anneal to the flanking small subunit and large subunit nrDNA, respectively (White *et al.* 1990). PCR conditions were 1 cycle at 95°C for 4 min, 30 cycles at 94°C for 1 min., 45°C for 1 min., 72°C for 1 min., and a final cycle at 72°C for 7 min. PCR product was purified with High Pure PCR Product Purification kit (Roche).

Sequencing reactions were performed with ABI PRISM BigDye Terminator Ready Reaction Kit (Applied Biosystems) and run on an Applied Biosystems ABI prism 310 Genetic Analyser. Sequence data were analysed and edited with Sequencher v3.1 software.

Five µl of the ITS PCR products were digested with 2 units of *Hae*III (Roche) and 2 units of *Hpa*II (Roche) in supplied buffers for 4 hours at 37°C. For electrophoresis, 2 µl 6X loading dye was added to the restriction digests, and the sample and 1 kb ladder (Invitrogen, USA) were loaded on to 4% NuSieve GTG agarose (BMA), run 2 hours at 5 V/cm in TBE buffer, stained with EtBr (0.5 µg/ml) for 30 min, destained with water for 30 min., and photographed with a Bio-Rad gel documentation system running Quantity One software.

Sequence Analysis

Sequences obtained during this study, and sequences obtained from an earlier study on pine-inhabiting *Lophodermium* species (Ortiz-García *et al.* in press, listed in Table 1), were edited to include the full sequence for both PCR primers. Sequence alignments were performed using Clustal W (Thompson *et al.* 1994) and adjusted manually (alignment deposited in TreeBase, Study accession number S944. Phylogenetic analyses were performed with PAUP* 4.0b10 (Swofford 2002) using a branch and bound parsimony analysis. Support for branches in the phylogenetic tree was tested using 100 bootstrap replicates.

Lophodermium picea (Fuckel) Höhn. was included as an outgroup. GeneDoc (Nicholas & Nicholas 1997) was used to calculate virtual restriction band sizes using the ITS sequences.

RESULTS

Isolates collected from New Zealand were initially identified on the basis of cultural appearance. *Lophodermium pinastri* has white, compact, slow-growing colonies, 10–20 mm in diameter after 4 weeks, with a sharply defined margin and irregular black patches developing across the colony surface; growth ceases before the edge of the plate is reached. *Lophodermium conigenum* has colonies that are initially white, the agar becoming orange to brown (usually in a flecked or feathery pattern), mycelium mealy in appearance; growth is fast, 55–85 mm in diameter after 4 weeks, with colonies usually reaching the edge of the plate. Molecular analyses consistently confirmed that these distinct growth patterns related to the two common New Zealand species, *L. conigenum* and *L. pinastri*. The culture subsequently identified as *L. molitoris* on the basis of DNA sequences, was slow-growing, 20–40 mm in diameter after 4 weeks, with agar dark brown in colour, aerial mycelium sparse, margin feathery. North American collections included two species, *L. pinastri* and *L. seditiosum*, identified on the basis of morphological descriptions of ascomata by Minter (1981); only isolates derived from *L. seditiosum* ascomata were included in this study.

GeneDoc analysis of ITS sequences suggested that a double digest of the ITS region using the restriction enzymes *Hae*III and *Hpa*II would most effectively distinguish the species. In all species tested (*L. conigenum*, *L. pinastri* (from New Zealand), *L. seditiosum*, and *L. molitoris*) the banding patterns predicted by GeneDoc from ITS sequences (Fig. 1) matched the actual band sizes on the gels (in Fig. 2 can be seen a gel with *L. conigenum*, *L. pinastri*, and *L. seditiosum*). Bands down to 40 bp could be visualised reliably on the gels. Three species were sampled intensively on a gel — *L. conigenum* (16 isolates, all from New Zealand), *L. pinastri* (16 isolates, all from New Zealand), and *L. seditiosum* (one isolate from Europe, seven from North America). There was no variation in ITS-RFLP band sizes between isolates within each of these species (Fig. 2).

A phylogenetic analysis divided the 15 putative species for which ITS sequences were available into eight distinct clades (Fig. 3 and 4). Each of these clades could be distinguished from the ITS-RFLP *Hae*III/*Hpa*II banding pattern predicted by GeneDoc (Fig. 1). Two clades contained isolates with different ITS-RFLP banding patterns. In both, this variation resulted from the loss of a single restriction site. In Clade 3, a single base change at position 420 in isolate 38 resulted in the loss of a *Hpa*II restriction site — a 100-bp band and a 62-bp band combined to produce a single 162-bp band. In Clade 6, a single base pair change at position 359 in isolate 7 resulted in the loss of a *Hae*III restriction site — a 76-bp band and a 22-bp band combined to give a 98-bp band. Other minor differences in the band sizes were due to scattered deletions or insertions in the sequences. The variation in ITS sequences may reflect either sequencing errors, or variation within the population.

Several clades contain more than one distinct morpho-species. These taxa were distinguished using features traditionally considered important in the taxonomy of these species (Minter 1981), such as depth to which the ascoma is embedded in the host leaf

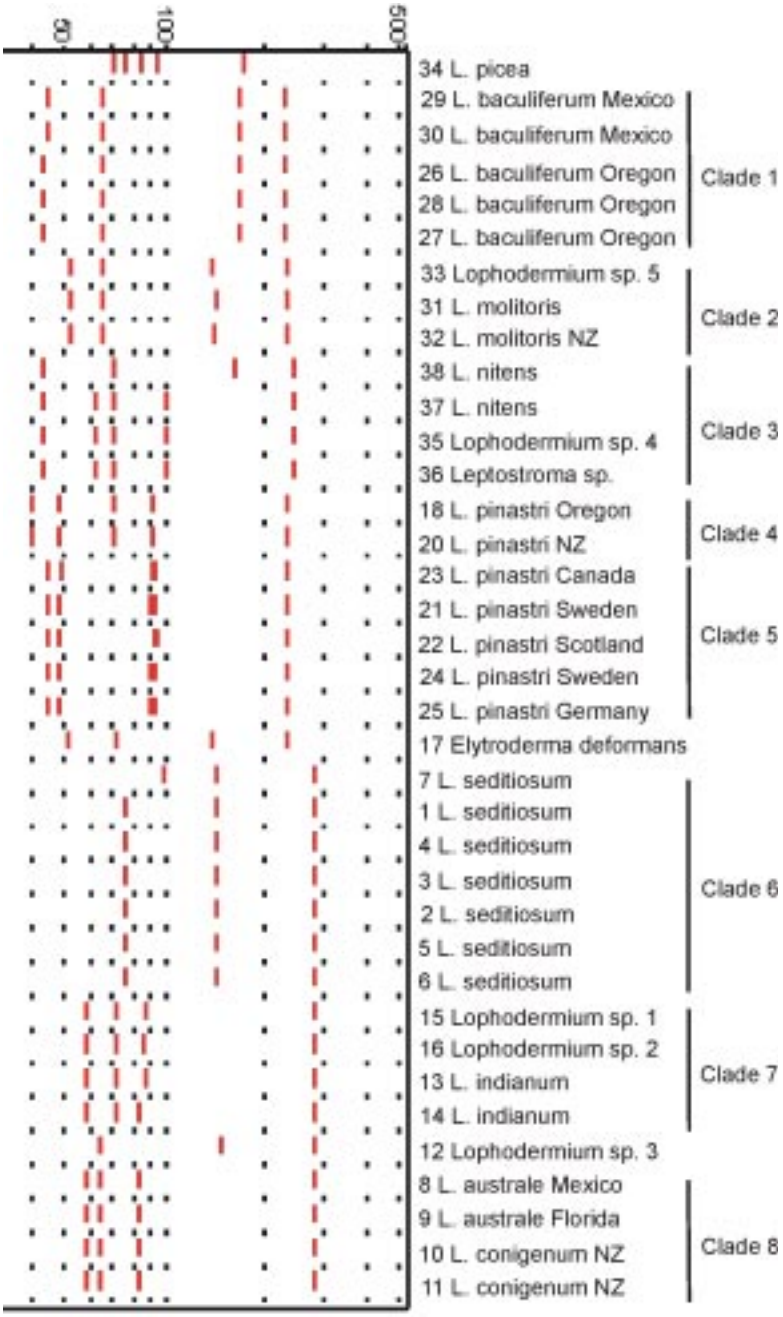

FIG. 1–Banding patterns predicted from GeneDoc, based on ITS sequences, in a double digest with *Hae*III and *Hpa*II. The clades indicated are the same as those in Fig. 3. Numbers in front of taxon names relate to column 1 in Table 1. Figures across the top are band sizes. Bands down to about 40 bp can be reliably visualised on a gel, so only bands 40 bp and larger have been included here.

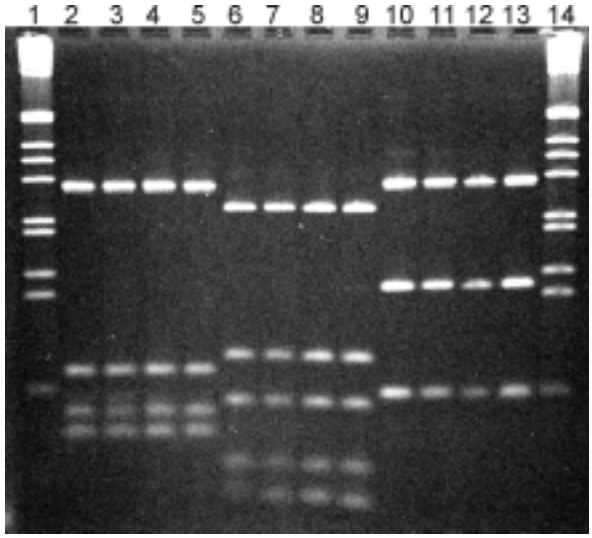

FIG. 2–RFLP banding patterns generated from a double digest of the ITS region with *Hae*III and *Hpa*II. Lanes 2–5 are four of the 16 New Zealand isolates of *L. conigenum* tested. Lanes 6–9 are four of 14 New Zealand isolates of *L. pinastri* tested. Lanes 10–13 are one European and three North American isolates of *L. seditiosum*. Lanes 1 and 14 contain a 1-kb ladder.

tissue, pattern of displacement of host epidermal cells as the ascoma develops, and the degree of development of the lower wall of the ascoma.

Collections morphologically typical of *L. pinastri* sensu Minter (1981) formed two sister groups. One group contained four isolates from Europe and Canada (isolated from the introduced European *Pinus nigra* J.F.Arnold), the other contained isolates from Oregon and New Zealand.

DISCUSSION

The PCR-RFLP method described here targeting the ITS region is effective for distinguishing the pine-inhabiting *Lophodermium* species occurring in New Zealand (*L. conigenum*, *L. pinastri*, and *L. molitoris*) from the species considered a potential risk, *L. seditiosum*. Although this study has revealed unresolved uncertainty around species limits of the pine-inhabiting *Lophodermium* species, it is likely that the method will be useful for distinguishing most, if not all, of these species (or at least closely related species groups). The RFLP banding patterns of *Cyclaneusma* spp. (a genus of Rhytismataceae common as a pathogen of pine needles in New Zealand) and fungi such as *Penicillium* spp. and *Paecilomyces* spp. (sometimes isolated as saprobes from dead pine needles infected with *Lophodermium*) were all distinct from those found for *Lophodermium* spp. (unpubl. data).

For this method to be a truly effective quarantine tool for New Zealand, there is a need to develop it further so that it can be used to target fungal mycelium within the host leaf

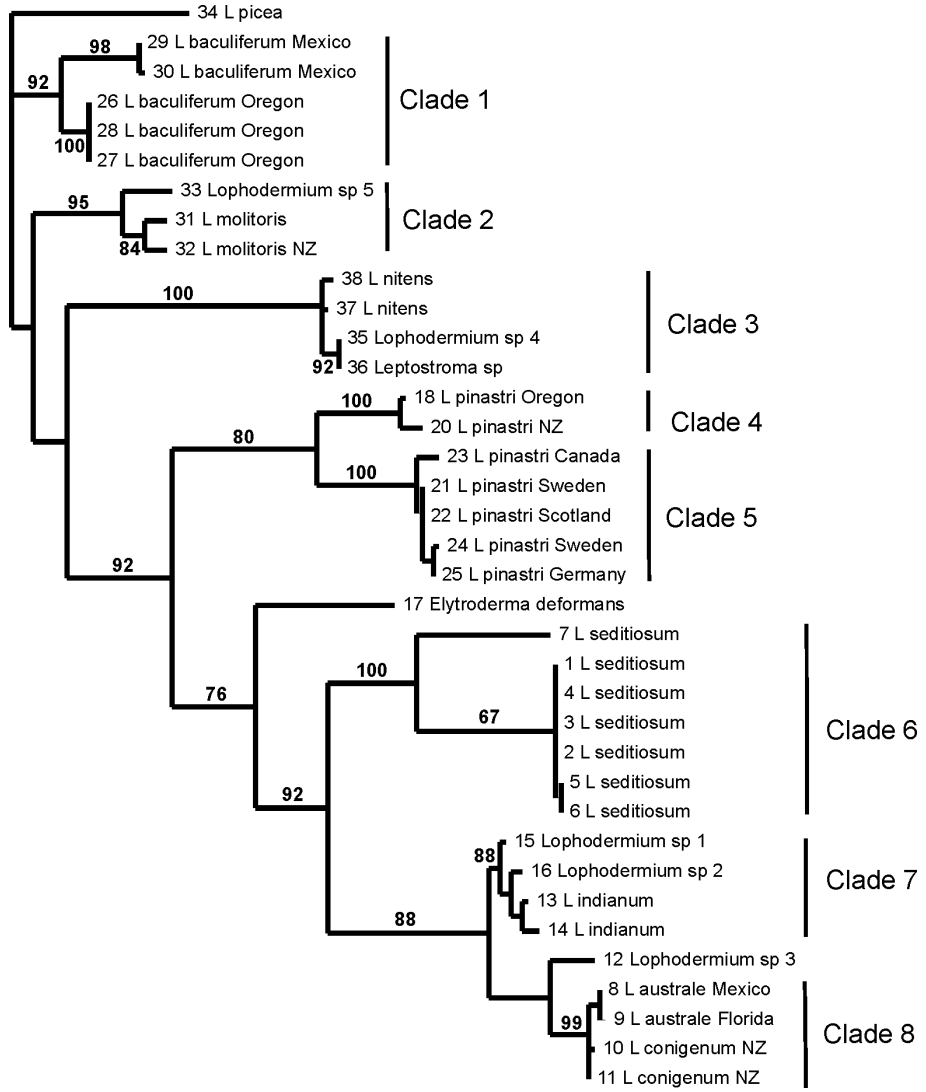

FIG. 3–One of the 126 most parsimonious trees of 345 steps based on ITS sequences, with bootstrap values indicated when above 50%. Numbers in front of taxon names relate to column 1 in Table 1. Well-supported clades indicated on the right share the same PCR/RFLP banding pattern (*see* Fig. 1).

tissue. Relying on extraction of DNA after isolation of the fungus into culture could take up to 4 weeks from the time disease symptoms were first noticed until there was a confirmed identification.

Lophodermium molitoris is known in New Zealand from culture only, the voucher specimen from which it was isolated having been lost. Despite this, the ITS data clearly group the New Zealand isolate with an isolate (CBS 597.84, IMI 286884) identified as

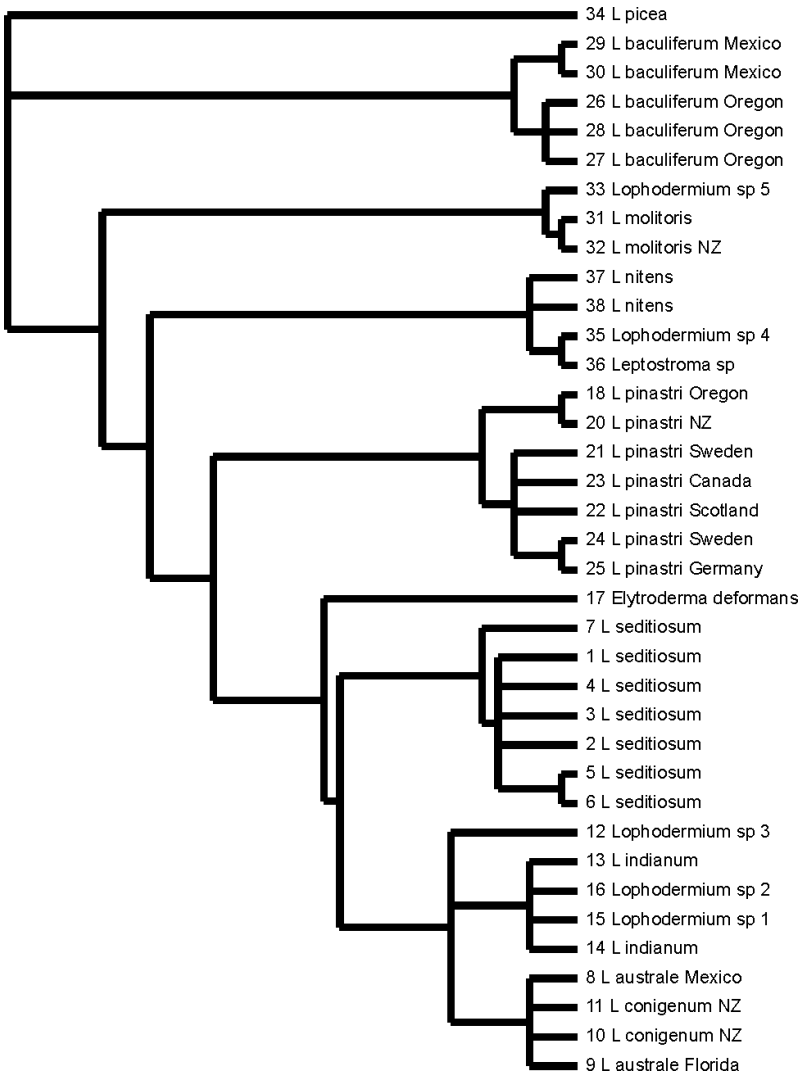

FIG. 4–Strict consensus of the 126 most parsimonious trees.

L. molitoris by experts on the taxonomy of these fungi, C.Millar and D.W.Minter. Morphologically, *L. molitoris* differs from *L. pinastri* and *L. conigenum* in having darker, more deeply embedded ascomata, and dark-walled conidiomata (Minter 1981). It is not known how common this species is in New Zealand, or how long it has been in the country. Biologically it is similar to the two largely endophytic species already known for New Zealand, and its presence will have little impact on forestry disease management.

Collections morphologically typical of *L. pinastri* sensu Minter (1981) formed two sister groups. One group contained four isolates from Europe and one from Canada (isolated from an introduced European pine species); the other group included one isolate

from Oregon and another from New Zealand. RFLP banding patterns showed all *L. pinastri* isolates tested from New Zealand fell into the same clade, suggesting the New Zealand *L. pinastri* population may have originated from the western United States. If detailed morphological examination of collections from each *L. pinastri* clade reveals as-yet-unrecognised morphological characters useful for distinguishing these groups, then establishment of an additional pine-inhabiting species might be justified.

Isolates identified as *L. conigenum* and as *L. australe* formed a single clade based on ITS sequences (Fig. 3), and gave a single RFLP banding pattern (Fig. 1 and 2). Assuming identification of the specimens sequenced was accurate, this suggests Minter's (1981) doubts about the distinctness of these two species may have been justified. However, any formal proposal to synonymise these species must await more complete molecular sampling, with this ideally including the type specimens of both species.

The results of this study and an earlier study (Ortiz-García *et al.* in press) suggest some of the characters used at present to distinguish pine-inhabiting *Lophodermium* species may deserve re-evaluation. For example, if a single species is able to colonise hosts with different needle anatomies, then differences in host anatomy may be reflected in the location of the fruiting body in relation to tissue layers within the needle (Ortiz-García *et al.* in press). This may be the case for Clade 7 (*L. indianum* S. Singh & Minter and two undescribed putative species of *Lophodermium*), found on *Pinus greggii* Engelm., *P. hartwegii* Lindl., *P. ayacahuite* Ehrenb., and *P. douglasiana* Martínez. *Pinus hartwegii* and *P. greggii* have two to three layers of hypodermal cells while *P. ayacahuite* has only one. This anatomical difference limits the sites available for ascocarp insertion in *P. ayacahuite* and similar soft pines, and thus constrains the potential morphological characters related to the embedding of the ascocarp (Ortiz-García *et al.* in press), one of the features considered important to distinguish pine-inhabiting *Lophodermium* species (Minter 1981). Whether groups such as Clade 7 contain sets of closely related taxa difficult to distinguish with ITS sequences alone, or whether the morphological characters used to define taxa within clades such as this are misleading, awaits further more detailed molecular and morphological studies of these fungi.

Elytroderma deformans (Weir) Darker was included in the phylogenetic and RFLP analyses, based on a single ITS sequence from GenBank. This species causes a serious needle-cast disease of pines (Funk 1985), and is another species of Rhytismataceae of potential threat to New Zealand. Based on the single sequence available, the RFLP method described here also distinguishes this species from the other species of Rhytismataceae known on pines in New Zealand. However, its position amongst the *Lophodermium* species in the phylogenetic analysis throws doubt on the identity of the isolate from which the sequence was obtained. This result should be confirmed when authentic material of *E. deformans* becomes available.

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