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Environmental barcoding of the ectomycorrhizal fungal genus *Cortinarius*

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Abstract Understanding the role of ectomycorrhizal fungi in plant communities is hampered by a lack of knowledge about fungal diversity. DNA barcoding of the ectomycorrhizal fungal genus *Cortinarius* was used to compare fungal diversity in soil from four plant communities: (i) *Nothofagus* forest (where *Cortinarius* is common and diverse), (ii) *Kunzea* forest (where *Cortinarius* is present but with low diversity), (iii) a *Pinus radiata* plantation (*Cortinarius* is not thought to be present) and (iv) a sub-Antarctic island (where known ectomycorrhizal hosts are absent). PCR primers specific for the ITS region of *Cortinarius* species were developed. Specificity was tested in vitro and in silico against DNA from basidiocarps of *Cortinarius* and non-*Cortinarius* species. The primers were tested for their ability to amplify *Cortinarius* DNA in soil from forests of the three ectomycorrhizal forest communities and a range of soils from the ectomycorrhiza-free subantarctic Campbell Island. High diversity of *Cortinarius* was associated with soil of all three ectomycorrhizal communities, despite *Cortinarius* being previously unrecorded from *Pinus*. Soil from all three communities share some ectomycorrhizal fungi (including fungi shared between native and exotic hosts), having implications for community succession, introduction of exotic fungi and biodiversity assessment. No *Cortinarius* was detected from Campbell Island samples. The validated molecular protocol assessed species diversity in a rapid and cost effective way. Baseline biodiversity assessment based on DNA barcoding is more effective at detecting diversity than traditional methods, but requires careful consideration of the difference between ectomycorrhizal fungal diversity in soil versus root-tips.

Keywords *Cortinarius* · Internal transcribed spacer region · Ectomycorrhizal ecology · Environmental DNA · Barcoding · Taxon-specific primers

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Introduction

Ectomycorrhizal fungi are essential for the growth and survival of many important forest tree species (Smith and Read 2008) but the subterranean, cryptic lifestyle of the fungi presents unique challenges for research (Horton and Bruns 2001; Anderson and Cairney 2004). The assessment of ectomycorrhizal fungal diversity is a prerequisite to understanding the functional significance of these complex communities (Jonsson et al. 2001; Leake 2001; Baxter and Dighton 2005). Molecular methods recruited to the study of ectomycorrhizal fungi include DNA fingerprinting/profiling by terminal restriction fragment length polymorphism (TRFLP) analysis (Dickie and FitzJohn 2007) or denaturing gradient gel electrophoresis (DGGE) analysis (Anderson et al. 2003; Smit et al. 2003), and DNA sequence analysis/barcoding by conventional (e.g. Edwards and Zak 2010) or high-throughput (e.g. Tedersoo et al. 2008) methods. Amplification of total soil DNA using specific primers cannot target ectomycorrhizal fungi, as the ectomycorrhizal trait has been independently derived many times, both in the Ascomycota (Tedersoo et al. 2006) and Basidiomycota (Horton and Bruns 2001). The use of hyphal ingrowth bags does allow enrichment of ectomycorrhizal fungi in soil

DNA, but does not exclude saprotrophic fungi completely (Bastias et al. 2006; Wallander et al. 2010).

Phylogenetic analyses of environmental clones are revealing a great number of previously unknown fungal phylotypes with no close sequence matches on open resources such as GenBank (Taylor et al. 2000; Ryberg et al. 2008). Giving species names to environmental sequences can be difficult, and confounded by the problem that there is no guarantee all named sequences on open resources are identified correctly (Bridge et al. 2003). Environmental DNA derived from soil can only be analyzed with one gene at a time, making resolution of phylogenies poor; this creates further difficulty when discovering described and new species from environmental clone sequences. To circumvent the issue of identifying clone sequences to species levels, authors use the terms operational taxonomic units (OTUs) or molecular operational taxonomic units (MOTUs) when referring to clone sequences grouped together on the basis of percentage similarity or phylogenetic analysis (Anderson et al. 2003; O'Brien et al. 2005; Li and Godzik 2006; Porras-Alfaro et al. 2007; Buée et al. 2009). Integrating taxonomic diversity based on sequences from named sporocarp collections with molecular diversity from soil or ectomycorrhizal root tips has the potential to unify these different approaches for studying ectomycorrhizal communities.

The use of taxon-specific primers is commonly practiced in molecular amplification of soil fungal communities, with fungal specific (Gardes and Bruns 1993), basidiomycete and ascomycete specific (Gardes and Bruns 1993; Tedersoo et al. 2008) and a variety of genus-specific (Tedersoo et al. 2008) primers currently in use. Genus-specific primers reduce complexity in environmental samples, facilitating a more direct focus on taxa of interest. This would be valuable for common, ecologically important but difficult to culture genera such as *Cortinarius* (Pers.) Gray and *Russula* Pers. (Brundrett et al. 1996; Cairney and Chambers 1999). The use of specific primers would be less expensive on a small scale than next generation sequencing and is more immediate than using hyphal in-growth bags. Environmental DNA can be screened with genus-specific primers and diversity measured by the analysis of DNA fingerprinting/profiling or MOTUs as defined by cluster analysis.

The fungal genus *Cortinarius* is one of the larger genera of agaricoid basidiomycetes (Peintner et al. 2001; Danks et al. 2010) and the largest genus of ectomycorrhizal fungi (Høiland and Holst-Jensen 2000; Kirk et al. 2001). With ~2000 named species, *Cortinarius* represents over a third of all named ectomycorrhizal fungi (Høiland and Holst-Jensen 2000; Kirk et al. 2001). The generic concept is expanding with a number of sequestrate genera (e.g., *Thaxterogaster*, *Protoglossum* and some *Hymenogaster*) and agaricoid genera (e.g., *Cuphocybe*, *Dermocybe* and *Rozites*) shown to be taxonomic synonyms of *Cortinarius* (Peintner et al. 2001, 2002a, b, 2004; Garnica et al. 2005). *Cortinarius* is widely distributed in both hemispheres, throughout mainly temperate to subarctic-alpine climates (Peintner et al. 2001, 2004; Danks et al. 2010), although some species are described from the tropics (Peintner et al. 2003). The large number of species in the genus, combined with the wide range of ecologically and economically important host species (Smith and Read 2008) makes *Cortinarius* a valuable target genus for further study.

This paper describes the development and testing of a PCR-based protocol to amplify *Cortinarius* DNA in environmental samples using genus-specific primers. To determine primer specificity, the primers were tested on DNA extracted from basidiocarps of ectomycorrhizal species from within and outwith the genus *Cortinarius*. To determine the effectiveness of the protocol, we attempted to detect *Cortinarius*, and determine its diversity, in environmental DNA extracted from soil collected in three temperate forest types where ectomycorrhizal hosts are present, and on a sub-Antarctic island where no ectomycorrhizal hosts are found.

Materials and methods

Primer design

Genus-specific primers were developed with the program PRIMER-BLAST from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) using the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA cistron as the target region (Fig. 1). Primers were designed against the template *Cortinarius rotundisporus* NZ8501 (GenBank AF389127) and their specificity was checked against the nr database, with an optimum primer melting temperature of 60 °C and minimum amplified sequence length of 200 base pairs. The specificity check returned 20 matches from the nr database that would be amplified by the primer pair, and we selected primers that returned only *Cortinarius* sequences in this list. Six primer pairs were used for preliminary screening against herbarium collections. The primer pair "cort4" ($^{5'}$CGTGATGAGTTGCTGCTGGTTCT$^{3'}$) and "cort5" ($^{5'}$GGGCCAGCAAAACCCCCACAT$^{3'}$) (Fig. 1) were found to amplify *Cortinarius* and were selected for further testing.

Testing with sporocarp DNA

To test the specificity of the cort4/cort5 primer pair, the primers were tested on genomic DNA extracted from the herbarium sporocarps of 20 *Cortinarius* species (including two with names only in *Dermocybe*) and twenty species from other ectomycorrhizal genera (Table 1). Genomic

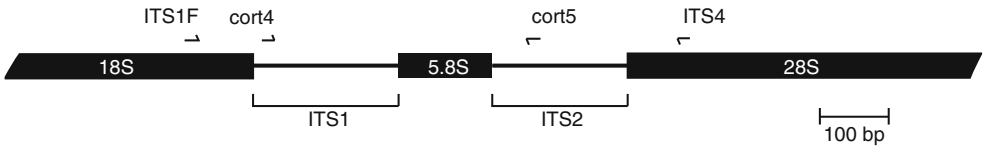

Fig. 1 Schematic diagram of the 18S–ITS1–5.8S–ITS2–28S tandem repeat region. The approximate annealing sites for the genus specific primers cort4 (*forward*) and cort5 (*reverse*), the fungal specific primer ITS1F (*forward*) and universal primer ITS4 (*reverse*) are indicated

Table 1 Ectomycorrhizal species used to test the specificity of the primer pair cort4/cort5. Voucher numbers refer to collections housed in the University of Otago Herbarium (OTA) or the New Zealand Fungal Herbarium (PDD)

Species	GenBank	Voucher
Austropaxillus nothofagi (McNabb) Bresinsky and Jarosch	JX178626	OTA61949
Cortinarius alboroseus (R. Heim) Peintner, E. Horak, M.M. Moser and Vilgalys	JX178620	OTA60230
Cortinarius austrovenetus Cleland	JX178608	OTA60167
Cortinarius caryotis Soop	JX178613	OTA60229
Cortinarius chalybaeus Soop	JX178617	OTA61433
Cortinarius collybianus Soop	JX178623	OTA60149
Cortinarius cycneus E. Horak	JX178610	OTA60178
Cortinarius dulciolens E. Horak, M.M. Moser, Peintner and Vilgalys	JX178605	OTA60170
Cortinarius indotatus (E. Horak) G. Garnier	n/a	OTA60293
Cortinarius indotatus (E. Horak) G. Garnier	GU222322	PDD92040
Cortinarius magellanicus Speg.	JX178611	OTA60291
Cortinarius mariae (E. Horak) E. Horak, Peintner, M.M. Moser and Vilgalys	JX178622	OTA61920
Cortinarius porphyroideus (G. Cunn.) Peintner and M.M. Moser	JX178612	OTA61406
Cortinarius sp. aff. *peraurantiacus* Peintner and M.M. Moser	JX178606	OTA61413
Cortinarius sp. aff. *perelegans* Soop	JX178615	OTA60285
Cortinarius sp. aff. *taylorianus* E. Horak	JX178616	OTA60233
Cortinarius sp. aff. *xenosma* Soop	JX178619	OTA60162
Cortinarius sp. OTA60292	JX178607	OTA60292
Cortinarius sp. OTA61427	JX178618	OTA61427
Cortinarius subcalyptrosporus Moser	JX178614	OTA60199
Dermocybe cardinalis E. Horak	JX178609	OTA61428
Dermocybe kula Grgur.	JX178621	OTA60156
Descolea phlebophora E. Horak	JX178627	OTA60177
Descolea recedens (Sacc.) Singer	JX178628	OTA60312
Gallacea eburnea Castellano and Beever	JX178634	OTA61972
Hebeloma sacchariolens Quél	JX178629	OTA60226
Inocybe calamistratoides E. Horak	JX178624	OTA61416
Inocybe leptospermi E. Horak	JX178625	OTA61938
Laccaria fibrillosa McNabb	JX178631	OTA61885
Laccaria glabripes McNabb	JX178632	OTA61989
Lactarius hauroko J.A. Cooper & P. Leonard ined.	JQ279512	OTA62000
Leotia lubrica (Scop.) Pers.	JX178635	OTA61895
Rossbeevera pachyderma (Zeller and C.W. Dodge) T. Lebel	JX178494	OTA61896
Russula atroviridis Buyck	JX178493	OTA61381
Russula pseudoareolata McNabb	JX178488	OTA61991
Russula roseopileata McNabb	JX178489	OTA61927
Russula tawai McNabb	JX178491	OTA61981
Russula tricholomopsis McNabb	JX178492	OTA61928
Russula umerensis McNabb	JX178490	OTA61987
Tricholoma elegans G. Stev.	JX178633	OTA61947
Tricholoma viridiolivaceum G. Stev.	JX178630	OTA61887

DNA was extracted using either the DNeasy Plant Mini Kit (QIAGEN) or the Genomic DNA Mini Kit (Plant) (Geneaid) following the manufacturer's protocols. Concentration of DNA was quantified using a spectrophotometer (Nanodrop ND-1000) and adjusted to 0.5 ng/μl. To ensure the DNA extraction had been successful, all forty specimens were amplified using the universal fungal primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990).

All PCR reactions were carried out in a volume of 20 μl, and used 17 μl of 1.1× ReddyMix PCR Master Mix (Thermo Scientific) 10 pmol of each primer and 0.5 ng of template. Negative controls used sterile purified water instead of the template. All reactions were conducted in a Mastercycler gradient PCR machine (Eppendorf AG, Germany). The protocol for amplification using the cort4/cort5 primer pair was: 95 °C for 5 min, followed by 30 cycles of: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; then a final elongation step of 72 °C for 10 min. The PCR conditions for amplification using ITS1F/ITS4 were: 95 °C for 5 min, followed by 35 cycles of: 94 °C for 45 s, 45 °C for 45 s, and 72 °C for 45 s; then a final elongation step of 10 min at 72 °C. Gel electrophoresis was used to determine the success of amplification; the DNA was observed by UV transillumination and images captured using a Gel Logic Image System (Kodak, USA). The ITS region was sequenced using the ITS1F/ITS4 amplification products. All sequencing used BigDye® Terminator Version 3.1 (Applied Biosystems Inc., Scoresby, Victoria, Australia) on an ABI3730 DNA Analyser (Applied Biosystems Inc.) at the Genetic Analysis Service, Department of Anatomy and Structural Biology, University of Otago, Dunedin, New Zealand.

Sequences were assembled using GENEIOUS PRO V5.3 (Drummond et al. 2010) and the cort4/cort5 primer attachment sites were located in an alignment of all sequences amplified. Sequences were aligned in the program GENEIOUS PRO V5.3 with the MAFFT (Katoh et al. 2002) plugin using the L-INS-i algorithm, scoring matrix 200PAM/k=2 and a gap open penalty of 1.53; obvious misalignments (due to poor alignment of short and long sequences) were then corrected manually. The alignment is available on TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S12824). Sequences were tested with the program AMPLIFY 3 version 3.1.4 (Engels 2005), which uses sequence data to test primer compatibility and ability to amplify *in silico*. The sporocarp sequences from amplification with the primer pair ITS1F/ITS4 were analysed with AMPLIFY 3, along with the primer sequences for cort4 and cort5. All sequences were analysed with the PCR option and default settings to test for the ability to be amplified by the pair. For the species *Cortinarius indotatus* (OTA60293), multiple intragenomic, polymorphic indels prevented successful reading of a direct sequencing reaction, and a clean sequence was not obtained. Therefore, a sequence of this species retrieved from GenBank (*C. indotatus* (as *D. indotata*) GU222322) was used to locate the primer attachment sites and for AMPLIFY 3 analysis.

The *Cortinarius* sequence amplicons obtained from in vitro amplification with primers ITS1F/ITS4 and in silico amplification with primers cort4/cort5 were used in two separate clustering analyses to compare MOTU formations of the entire ITS region against the region amplified by the specific primers. Full-length ITS and trimmed cort4/cort5 sequences were uploaded to the online program CD-HIT Suite: Biological Sequence Clustering and Comparison (http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit-est). Both analyses used the identity cutoff threshold of 0.975 as described below for "Detection range of *Cortinarius*".

Testing on soil DNA

To determine if the cort4/cort5 primer pair could amplify *Cortinarius* DNA from soil, the primer pair was tested on DNA extracted from soil collected in forests of known ectomycorrhizal hosts and soil collected from a sub-Antarctic island, where no ectomycorrhizal hosts were present.

For the ectomycorrhizal host sites, an approximately 214 ha forest block privately owned by A and J Knight at Tuapeka West, South Otago, New Zealand was selected. The block contains a planted stand of *Pinus radiata* D. Don at its highest eastern end, descending through several stands of *Kunzea ericoides* (A.Rich.) Joy Thomps., to a mixed stand of *Nothofagus menziesii* (Hook.f) Oerst. and *N. solandri* var. *cliffortioides* (Hook.f) Poole. All of these species are known to form ectomycorrhizal associations (Chu-Chou and Grace 1988; McKenzie et al. 2000; Orlovich and Cairney 2004; McKenzie et al. 2006). During March 2010, two sites were chosen within each of three forest types, with 10 samples collected per site: *Pinus radiata* (Samples 1–20), *Kunzea ericoides* (Samples 21–40) and *Nothofagus menziesii* and *N. solandri* var. *cliffortioides* (Samples 41–60). Samples were collected within a 10 × 10 m plot using a random sampling plan. Each site was at least 50 m away from the edge of the stand and from the other site within the same forest type. For each sample, a 50 ml soil core was taken using a clean and sterile 50 ml polypropylene centrifuge tube. The tubes were capped to prevent cross contamination. Samples were homogenized by grinding briefly with a clean, sterile mortar and pestle; large visible roots (>2 mm thick) were removed by hand. 0.25 g of each soil sample (including root fragments <2 mm thick) was weighed, placed in a collection tube (MoBio PowerBead Tubes, MoBio Laboratories, Inc; Solana Beach, CA) and stored at 4 °C until extraction.

For the non-ectomycorrhizal sites, New Zealand's southernmost sub-Antarctic territory, Campbell Island, 660 km south of Bluff, New Zealand was selected. Two soil samples were collected approximately 5–10 m apart from each of five locations around the island in November 2007. The sites cover a range of vegetation types, all expected to be comprised of non-ECM hosts, including *Poa litorosa* Cheesman, *Bulbinella* and *Chionochloa* grassland (Samples 1 and 1a, S5231.72 E16908.24 206 m.a.s.l.), mixed *Chionochloa* and *Dracophyllum* (Samples 3 and 3a 160 m.a.s.l.), *Dracophyllum* shrubland (Samples 6 and 6a, S5233.08 E16908.45, 8.5 m.a.s.l.), amongst *Pleurophyllum speciosum* (Samples 8 and 8a, S5232.91 E16906.65 191 m.a.s.l.), and a *P. litorosa* and *Dracophyllum* heath (Samples 9 and 9a, S5235.63 E16908.67 23 m.a.s.l.). The ten soil samples were collected from the top 5–10 cm of soil using individual, new, clean, wooden ice-cream sticks. The approximate weight of each sample was 0.1–0.25 g. Soil was added directly to the MoBio PowerBead tubes and stored at 4 °C until extraction.

Soil DNA extraction

Extractions were done using the PowerSoil DNA Isolation Kit (MoBio) following the manufacturer's protocol, except at step 5 where samples were placed in a bead beater (Retsch Mixer Mill MM301) at maximum frequency (30.0 cycles/s) for 2 min. DNA was stored at −20 °C.

Isolation of *Cortinarius* clones from soil samples

The cort4/cort5 primer pair was tested on the DNA extracted from soil samples using the same PCR protocol used for the sporocarp amplifications. The samples were also amplified using the fungal-specific primers ITS1F and ITS4 as a positive control, as described above. The successfully amplified cort4/cort5 PCR products from the soil samples were ligated into plasmid vector pCR2.1, transformed into competent *E. coli* (strain: Top10), selecting 10 clones for each sample using the TA Cloning Kit (Invitrogen). The clones were screened with the restriction enzymes *Alu*I (Roche), *Hae*III (Roche) and *Hinf*I (Roche) and the unique RFLP types for each site were then sequenced with the primers M13f and M13r, as described previously.

Detection range of *Cortinarius*

The program CD-HIT-EST (Li and Godzik 2006) was used to define molecular operational taxonomic units (MOTUs) from the soil clone sequences. Previous studies have used a range of cut-offs (90–97 %) when conducting cluster analysis of ITS sequence data as a proxy for species boundaries between fungal genera (O'Brian et al. 2005; Higgins et al. 2007). The sequence data were analysed using a range of percentage identity thresholds (90–99.9 %) to determine a suitable cut-off point for the maximum number of MOTUs. CD-HIT-EST cluster analysis used a greedy incremental clustering algorithm. If the similarity with a representative is equal to or greater than the set threshold (90–99.9 %), the sequence is grouped into that cluster (Li and Godzik 2006). Similarities below the threshold create new clusters. The representative for each MOTU was blasted using SEQUENCE SEARCH MEGABLAST against the GenBank nucleotide (nr) database within GENEIOUS PRO version 5.3, recording the top five blast hits as ordered by the bit-score.

To place the representative sequence from each MOTU into a systematic framework for the genus *Cortinarius*, the alignment of Peintner et al. (2001) was aligned with the MOTU representative sequences and all sequences found on GenBank using the keyword search 'Cortinarius, New Zealand, Internal' to produce a phylogeny of the genus. The data set comprised of 244 in-group taxa and three out-group taxa from the related genus *Hebeloma* (Peintner et al. 2001).

Sequences were aligned as described previously for basidiocarp sequences; resulting alignments were then manually edited. A Bayesian phylogeny was created using MRBAYES version 3.2.1 (Ronquist and Huelsenbeck 2003) with a randomly selected starting tree, run for 50 million generations with every 500th tree stored. The burn-in value was equal to 25 % of the sampled generations (25,000). The 18S and 28S genes were excluded from the Bayesian analysis.

Cortinarius diversity literature survey

The host-associations of *Cortinarius* in New Zealand were recorded from the following sources: Chu-Chou and Grace 1988, 1990; McKenzie et al. 2000, 2006; Segedin and Pennycook 2001; Soop 2001, 2002, 2005, 2012; Orlovich and Oliver 2002; Orlovich and Cairney 2004; Gasparini and Soop 2008; Walbert et al. 2010a, b.

Results

Primer development and testing

The developed primer pair cort4 and cort5 amplify the majority of ITS1, all of the 5.8S gene and the 5′ end of ITS2 (Fig. 1). All species used for the primer test were successfully amplified with the universal fungal primer pair ITS1F/ITS4 (Fig. 2). The cort4/cort5 primer pair successfully amplified 19 of the 20 *Cortinarius* test specimens and failed to amplify 18 out of 20 species from other genera (Fig. 2). The primer pair was unable to amplify positive test species *Cortinarius subcalyptrosporus* and did amplify

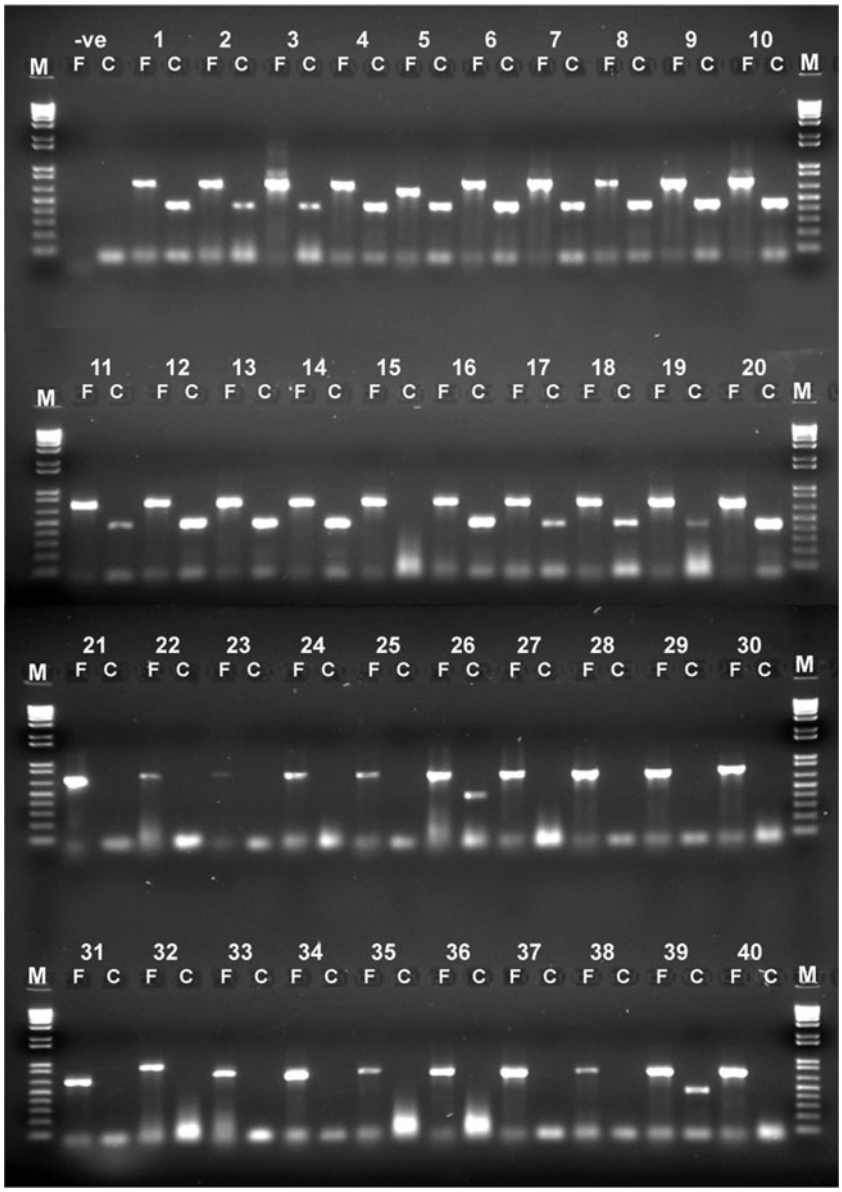

Fig. 2 Gel image of amplification products from sporocarp samples amplified using primer pairs ITS1F/ITS4 and cort4/cort5. *Individual lanes* are labeled for primer pairs ITS1F/ITS4 (F) and cort4/cort5 (C). Samples 1–20: *Cortinarius*/*Dermocybe* species; samples 21–40: non-*Cortinarius* species. −ve: negative controls; 1: *Cortinarius alboroseus*; 2: *C. austrovenetus*; 3. *C. caryotis*; 4: *C. chalybaeus*; 5: *C. collybianus*; 6: *C. cycneus*; 7: *C. dulciolens*; 8: *C. magellanicus*; 9: *C. mariae*; 10: *C.* sp. aff. *peraurantiacus*; 11: *C.* sp. OTA60292; 12: *C.* sp. aff. *perelegans*; 13: *C. porphyroideus*; 14: *C.* sp. aff. *tayloria-nus*; 15: *C. subcalyptrosporus*; 16: *C.* sp. OTA61427; 17: *C.* sp. aff. *xenosma*; 18: *Dermocybe cardinalis*; 19: *C. indotatus*; 20: *D. kula*; 21: *Austropaxillus nothofagi*; 22: *Descolea recedens*; 23: *D. phlebophora*; 24: *Gallacea eburnea*; 25: *Hebeloma sacchariolens*; 26: *Inocybe calamistratoides*; 27: *I. leptospermi*; 28: *Laccaria fibrillosa*; 29: *L. glabripes*; 30: *Lactarius hauroko* ined.; 31: *Leotia lubrica*; 32: *Rossbeevera pachyderma*; 33: *Russula tawai*; 34: *R. pseudoareolata*; 35: *R. atroviridis*; 36: *R. umerensis*; 37: *R. roseopileata*; 38: *R. tricholomopsis*; 39: *Tricholoma elegans*; 40. *T. viridiolivaceum*. 1 kb plus ladder is indicated with 'M'

negative test species *Inocybe calamistratoides* and *Tricholoma elegans* (Fig. 2).

An alignment of the sequences obtained from amplification of the complete ITS region in all test species was used to show the attachment sites of the cort4/cort5 primer pair (Fig. 3). Those test species that amplified in vitro had good base pairing at the 3′ end of both genus specific primers, whereas overall those that did not amplify in vitro had poor base pairing at the 3′ end of one or both primers (Fig. 2 and Fig. 3). All species amplified in vitro using the PCR protocol were also analysed using the program AMPLIFY3. The species *Hebeloma sacchariolens*, *Laccaria glabripes* and *Tricholoma viridolivaceum* did not amplify in vitro (Fig. 2), however, binding sites cort5 were found with the program AMPLIFY3 (Fig. 3). Overall, the *Cortinarius* species tested displayed good matches to the cort4 and cort5 primers at the 3′ ends of both primers. Both *Cortinarius subcalyptrosporus* and *C. collybianus* displayed poorer matches to the primer sequences at the 3′ end of cort5, but *C. subcalyptrosporus* also did not match well at the 3′ end of cort4 and was similarly not amplified in silico (Fig. 3). In the two non-target species that did amplify in vitro and *in silico*, *Inocybe calamistratoides* and *Tricholoma elegans*, attachment sites for both primers cort4 and cort5 show few mismatches at the 3′ end (Fig. 3).

The clustering analysis determined 19 unique MOTUs from the 20 full-length ITS *Cortinarius* sequences and 18 unique MOTUs from the 20 trimmed cort4/cort5 sequences (Online Resource 1). These results show that using a 97.5 % identity cutoff is 94.7 % accurate at determining species diversity using the full-length ITS region, and that the narrower cort4/cort5 amplicon detects 89.5 % of known

	5' [cort4] 3'	3' [cort5] 5'	AMPLIFY		GEL
Primer sequence	**CGTGATGAGTTGCTGCTGGTTCT**	**ATGTGGGGGTTTTGCTGGCCC**	cort4	cort5	
Cortinarius abloroseus	-C----------T------C---	---------G-A-TT-TTTG-	+	+	+
Cortinarius austrovenetus	-C-----G---------------	----------A--CT-TTG-T	+	+	+
Cortinarius caryotis	-C----------------------	---------G-CATT-TTGGT	+	-	+
Cortinarius chalybaeus	-C-----------------C---	-------------CT-TTG-T	+	+	+
Cortinarius collybianus	-TGAT-A-----T----------	--T-AT---GG--TGCT-GA-	+	-	+
Cortinarius cycenus	AC----------------------	----------C--TT-TTG-T	+	+	+
Cortinarius dulciolens	-C----------------------	----------C--C--TTTG-	+	+	+
Cortinarius indotatus GU222322	-C----------------------	---------C---TT--CTGG	+	+	+1
Cortinarius magellanicus	-C----------------------	----------C--TT-T--TG	+	+	+
Cortinarius mariae	-CC----------------C---	---------G---TT-TTTTG	+	+	+
Cortinarius porphyroideus	-C----------------------	---------G-T-TT-TTTG-	+	+	+
Cortinarius sp. aff. *peraurantiacus*	-C----------------------	-C---------C-TT-T-TTG	+	-	+
Cortinarius sp. aff. *perelegans*	-C----------------------	---------G-C-TT-TTTG-	+	+	+
Cortinarius sp. aff. *taylorianus*	-C------------------C---	-----------GCTGGTCT-T	+	+	+
Cortinarius sp. aff. *xenosma*	-C----------------------	---------G---CT-TTTTT	+	+	+
Cortinarius sp. OTA60292	-C----------------------	----------A-AAT-TATTT	+	+	+
Cortinarius sp. OTA61427	-C----------------------	-----------A-TT-T---G	+	+	+
Cortinarius subcalyptrosporus	-C----A--C--T----------	-C-G------G-GCTG-T-T-	-	-	-
Dermocybe cardinalis	-C----AG---------------	--------K----TT--CTGG	+	+	+
Dermocybe kula	-C-----G-----------C---	----------A--TTGCTGGT	+	+	+
Austropaxillus nothofagi	TTCTA-T--G-TG------CCT-	-----C-A----G-A-A-TGG	-	+	-
Descolea phlebophora	-T-----G----TA-----C---	-CT---------GCGG-CTT-	+	-	-
Descolea recedens	-T-----G----TA-----C---	-CT------A---AT-T--AG	+	-	-
Gallacea eburnea	G-G-T-AGAACCGA-----CC-C	-CT---AC---GCCG-CTTTG	-	-	-
Hebeloma sacchariolens	TTG-TGTG-T--T------CCT-	----------C-ATT-T--TG	-	+	-
Inocybe calamistratoides	TTGA-CAG-C--T-------C--	--A------GA--T---TTTG	+	+	+
Inocybe leptospermi	TTGA-CAG-C--T------CC-C	T-A----------TT-T--TG	-	-	-
Laccaria fibrillosa	-TGATGTG----TA-----C-T-	GGA--------GC-GGCTT-A	-	-	-
Laccaria glabripes	-TGATGTG----TA-------T-	-----------GC-GGCTT-A	-	+	-
Lactarius hauroko ined.	ATGC-A-G-C--T----GAC-T-	-CT-T--A-GCC-TTGCTGG-	-	-	-
Leotia lubrica	--A-TGCT-CCT---GGCAAC--	G-----CCCCGA-CACT-GGG	-	-	-
Rosbeevera pachyderma	-AG-A-TGT-GCTG---A-A-TC	-GT----AA--GCTGGTCTTA	-	-	-
Russula atroviridis	GTGCGA-G-C--TC----ACCT-	-C-G--A--A-AGRAAT--TT	-	-	-
Russula pseudoareolata	GTGCGA-G-C--TC----ACC--	-CT-T--A-G--ATTGCTGGT	-	-	-
Russula roseopileata	GTGCGA-G-C--TC----ACCT-	-CT---A--CC--TTGCTGGT	-	-	-
Russula tawai	TTGT-A-G-C--T----AACCT-	-CT---A--T--AATGCTTG-	-	-	-
Russula tricholomopsis	TTGT-A-G-C--T----GACCTC	-CT---A-----AATGCTTG-	-	-	-
Russula umerensis	GTGTGA-G-C--TC----ACC-C	-CT---A----CAATGCT-G-	-	-	-
Tricholoma elegans	TTG-T--G----TC-----C---	-------A----GCGG-CTT-	+	+	+
Tricholoma viridolivaceum	TTG-T--G----T------C-T-	------------GCTG-CTT-	-	+	-
Primer sequence	**CGTGATGAGTTGCTGCTGGTTCT**	**ATGTGGGGGTTTTGCTGGCCC**			
	5' [cort4] 3'	3' [cort5] 5'			

Fig. 3 Alignment of the cort4/cort5 primer sequences with each species used to test for primer specificity. Expected amplification of each species in silico with AMPLIY3X, is indicated as positive (+) or negative (−) attachment for each primer, and amplification success in vitro as analysed by gel electrophoresis is indicated as positive (+) or negative (−). [1]*Cortinarius indotatus* OTA60293 was used for in vitro amplification

species diversity in the pool tested here. These results are to some degree dependent on the relatedness of the species in the sample pool, so these percentages are not likely to be directly transferrable to other studies.

Isolation of *Cortinarius* clones from soil samples

Fungal DNA was successfully amplified from all soil samples using the universal primer pair ITS1F/ITS4 (Fig. 4). The primer pair cort4/cort5 successfully amplify DNA from soil samples collected beneath the ectomycorrhizal hosts *K. ericoides*, *Nothofagus* spp. and *P. radiata* (Fig. 4a). The cort4/cort5 primer pair did not amplify any of the soil DNA samples collected from Campbell Island (Fig. 4b). A total of 145 soil-clones were sequenced from the cort4/cort5 primer pair amplifications of soil samples collected from the ectomycorrhizal host forests. 53 soil-clones were amplified from the *Nothofagus* spp. stand, 57 from the *K. ericoides* stand and 36 from the *P. radiata* stand (Online Resource 2).

Phylogenetic analyses of soil clone MOTUs

An optimal similarity index was determined by plotting the number of sequence clusters obtained at a range of thresholds, from 90 to 99.9 %. The point at which the sequences stop forming close-paired groupings was considered to be the best estimation of the number of total MOTUs. The cluster analysis determined an optimal similarity index of 31 unique MOTUs at 0.975 sequence identity (Fig. 5). Of the total 31 MOTUs, three were shared between all forest types, with 21 MOTUs found from the *Kunzea* sampling sites, 16 from the *Pinus* sites and 11 from the *Nothofagus* sites (Fig. 6a). Four MOTUs were shared between *P. radiata* and *Nothofagus*, 12 MOTUs shared between *P. radiata* and

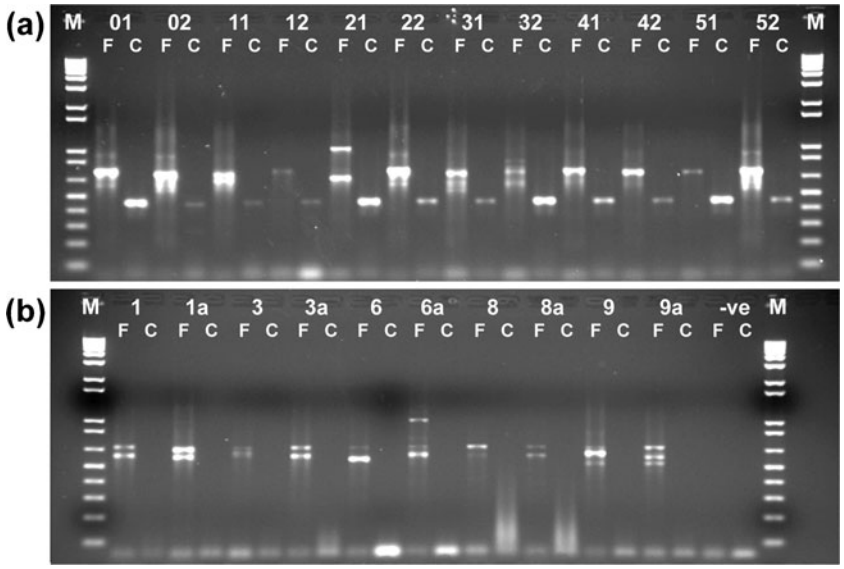

Fig. 4 Gel image of soil samples testing for amplification of fungal DNA (ITS1F/ITS4) and *Cortinarius* DNA (cort4/cort5). *Individual lanes* are labeled for primer pairs ITS1F/ITS4 (F) and cort4/cort5 (C). **a**. Amplification products from representative soil samples chosen from the Tuapeka West sites (samples 01–12: *Pinus*; 21–32: *Kunzea*: 41–52: *Nothofagus*). **b**. Amplification products from soil samples from Campbell Island (1–1a: *Poa litorosa*, *Bulbinella* and *Chionochloa* grassland; 3–3a: mixed *Chionochloa* and *Dracophyllum*; 6–6a: *Dracophyllum* shrubland; 8–8a: megaherb field; 9–9a: *P. litorosa* with a *Dracophyllum* heath). 1 kb plus ladder is indicated with an 'M'

K. ericoides, and four shared between *K. ericoides* and *Nothofagus*. The MEGABLAST hits for representatives of the MOTUs are all from the genus *Cortinarius* (Online Resource 3). In both the *K. ericoides* and *P. radiata* forest sites, there were a higher number of MOTUs than the number of species that have been recorded for either host throughout New Zealand (Fig. 6b, Online Resource 5).

Phylogenetic analysis of the representative sequences from each cluster (Online Resource 2) showed that the soil-clones are related to a range of species in the genus *Cortinarius* with 20 MOTUs related to 11 clades of Northern and Southern Hemisphere species, supported by the Bayesian posterior probabilities >0.97 (Online Resource 4).

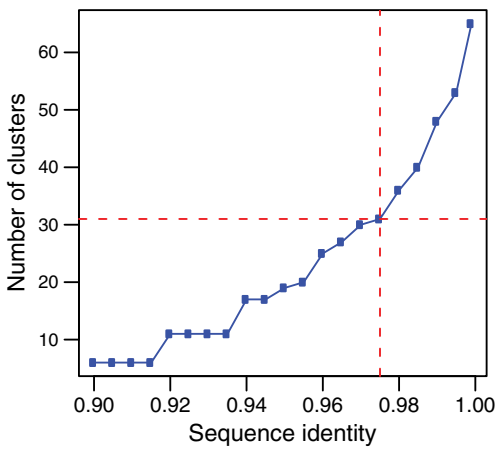

Fig. 5 Plot of the number of clusters (MOTUs) formed using sequence similarity threshholds from 0.9 to 0.99. The *dotted lines* indicate the intersection of the plot at a sequence identity of 0.975, corresponding to 31 MOTUs

Discussion

Through the use of genus-specific primers, we have successfully measured the soil diversity of the ectomycorrhizal fungal genus *Cortinarius* in three ectomycorrhizal forest types and soil on Campbell Island. The diversity detected in soil is likely to be a superset of diversity on ectomycorrhizal root tips, since we cannot exclude the possibility that some of the MOTUs detected in the present study were from spores in soil, and not from ectomycorrhizas with the host in that soil. This could explain why the molecular diversity of *Cortinarius* shared between the three forest types was greater than expected. We found unexpectedly high molecular diversity of *Cortinarius* in forest soil associated with *Kunzea* and *Pinus*, including MOTUs restricted to soil of just one host. The molecular diversity of *Cortinarius* shared between the three forest types was also greater than expected. The range of detection indicates the potential for use of these primers to investigate directly, the molecular ecology of this speciose (~ 2000 described species), but understudied genus. This PCR-based protocol creates the opportunity to focus research on the taxonomic diversity of *Cortinarius* from different forest communities. The importance of knowing and understanding baseline diversity is crucial where host species are strongly reliant on mycorrhizal associations, such as New Zealand *Nothofagus* species

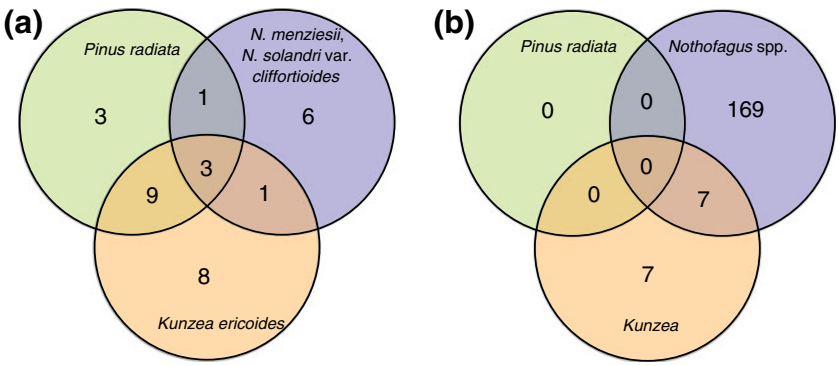

Fig. 6 Venn diagrams illustrating the diversity of *Cortinarius*. **a**. Diversity based on numbers of MOTUs from the forest soil sampled in the present study. **b**. Diversity based on taxonomic descriptions of species recorded in New Zealand (see Online Resource 5 for details) from *Pinus radiata*, *Nothofagus* and *Kunzea*. *Kunzea* includes species described pre-1989 from *Leptospermum*, when *K. ericoides* was *L. ericoides*, where no newer information is available

(Baylis 1967). While the detection of *Cortinarius* DNA in soil does not necessarily mean that it is forming ectomycorrhizas with that host, it may indicate range of dispersion of these fungi and the availability and diversity of potential inoculum.

This study records the first molecular detection of *Cortinarius* through amplification of soil DNA in association with ectomycorrhizal host species *P. radiata*. The cort4/cort5 primer pair successfully amplified *Cortinarius* in all twenty samples from the two *P. radiata* sites with 16 unique MOTUs identified within the genus *Cortinarius.* There are currently three unidentified collections of *Cortinarius* basidiocarps from *P. radiata* in New Zealand (from the mid-Canterbury region, voucher: PDD87791, PDD87792 and PDD95864; New Zealand Fungal Herbarium) and no published record of *Cortinarius* ectomycorrhizas in association with *P. radiata* in New Zealand (Chu-Chou and Grace 1988, 1990; Walbert et al. 2010a, b). Two of the three unidentified *Cortinarius* sporocarps have been sequenced (available on GenBank KC153997 and KC153998) and preliminary phylogenetic analyses (not shown) indicated that these new sequences are not closely related to any of our soil clone sequences, nor were they related to collections of named *Cortinarius* species. We cannot confirm that *Cortinarius* forms ectomycorrhizas with *Pinus radiata*, and indeed its absence in previous studies does indicate that it is rare at best in production forests. Nonetheless, it is intriguing that the soil of the *Pinus radiata* forest harbored unique *Cortinarius* MOTUs, and when considered with the basidiocarp collections from *Pinus radiata* forests elsewhere in New Zealand, does indicate a strong possibility that such associations may occur, albeit rarely, and would be worthy of further study.

The similarity of molecular diversity for *Cortinarius* in the three forest types is in sharp contrast to the diversity based on sporocarp-based descriptions. *Nothofagus* is known to support a greater ectomycorrhizal fungal diversity than any other New Zealand host (Orlovich and Cairney 2004), with 176 species of *Cortinarius* described in association with *Nothofagus* spp. (Online Resource 5), However, the number of MOTUs detected in the present study was relatively even, indicating that the *Pinus* and *Kunzea* communities may be taxonomically under-sampled if these MOTUs are indeed ectomycorrhizal with these host trees. While these differences may reflect a taxonomic sampling bias in favor of *Nothofagus* forests, the high diversity of MOTUs detected in the present study, despite a relatively small study area, indicates that amplification of soil DNA has great potential for the study of the diversity of *Cortinarius* or other genera.

Although there is poor resolution in the *Cortinarius* phylogeny a number of the soil-clone MOTUs are related to named species. The ITS region is frequently used for fungal species identification (Bruns et al. 1991; Gardes and Bruns 1993; Peintner et al. 2003; Tedersoo et al. 2003; Schoch et al. 2012). This is because of the high variation found within the internal transcribed spacers, which are subject to faster evolutionary change in comparison to the 18S, 5.8S and 28S genes. The short length of the soil-clone sequences (350–450 bp) may have limited the resolution of the phylogeny. Short variable sequences are sufficient for species identification, DNA barcoding and separation within a phylogeny, but do not necessarily contain enough sequence data or gene coverage to resolve deeper evolutionary relationships. Whilst this is a limiting factor in using the cort4/cort5 primer pair to construct a phylogeny, this is not a limitation for DNA barcoding. Truncating the ITS sequences of the basidiocarps sequenced in this study to the cort4/cort5 region only resulted in one less MOTU cluster, indicating that using a shorter sequence had a minimal effect on the diversity recovered.

The coverage of the ITS region by the cort4/cort5 primer pair has the benefit of including the majority of ITS1, which is often used for sequence identification to species level and is more variable on average in most fungi (Nilsson et al. 2008). Although testing of the primers on ectomycorrhizal

basidiocarps revealed some non-specific amplification, no non-*Cortinarius* sequences were amplified from the soil-DNA. This indicates that while some non-specific amplification of species is possible in single species amplifications, this was not a practical problem in the analysis of the soil samples. While wider sampling of *Cortinarius* species was beyond the scope of this study, it would be a valuable exercise to further validate the primer pair in regions of different diversity.

The high specificity and sensitivity of the primers to *Cortinarius* on DNA extracted from soil means that a negative PCR reaction using the PCR-based protocol is indicative of the absence of the target genus. Further in silico and in vitro validation would clarify the boundaries of utility of this primer pair, given the very large phylogenetic diversity of the genus *Cortinarius*. The absence of *Cortinarius* in Campbell Island soil is not surprising, since there are no ectomycorrhizal host species present on the island. The cost effectiveness of this PCR-based protocol allows for the assessment of diversity across a wide range of locations or forest types. For example, Peintner et al. (2003) made the first record of *Cortinarius* from India; the use of specific primers would aid in screening this and other underexplored regions for biodiversity. Microhabitats like canopy soil (Nadkarni 1981; Vance and Nadkarni 1996; Hertel 2010) are known in some cases to support ectomycorrhizal communities (Hertel 2010; Orlovich et al. 2012), but there is little information on the diversity of fungal species in these habitats. The primers developed here could be used to screen soil DNA from such unique soil systems for comparison with terrestrial soils. Surveying *Cortinarius* in forests of different host trees would be facilitated by using the primers developed here if used on root-tip-extracted DNA, enabling questions of host specificity and local endemism to be addressed. *Cortinarius* is one of the most commonly occurring and diverse genera on *Nothofagus* roots in New Zealand (Dickie et al. 2010) but little is known about specificity of *Cortinarius* to different *Nothofagus* species, nor to the other two native ectomycorrhizal host genera in New Zealand, *Leptospermum* and *Kunzea*. Using genus-specific primers like those described here will permit rapid, cost effective surveys that would inform taxonomic and systematic studies of *Cortinarius* and other important ectomycorrhizal genera. Our strategy will also be useful in validating next generation sequencing results of ectomycorrhizal soil communities.

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