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Molecular identification of co-occurring *Cortinarius* and *Dermocybe* species from southeastern Australian sclerophyll forests

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Abstract The ability of restriction fragment length polymorphism (RFLP) analysis of the rDNA internal transcribed spacer (ITS) region to discriminate 10 co-occurring *Cortinarius* and *Dermocybe* species at a southeastern Australian sclerophyll forest site was assessed. Using the basidiomycete-specific primers ITS1F and ITS4B, some taxa were separated on the basis of individual RFLP patterns derived using the restriction endonucleases *Hae* III or *Hin*f I. Combined data from both endonucleases were, however, required to separate all taxa [*Dermocybe austro-veneta* Clel. (Moser & Horak), *C. rotundisporus* Clel. & Cheel, *C. archeri* Berk., *C. sinapicolor* Clel., *C. violaceus* (L.: Fr.) S.F.Gray, *C. radicatus* Clel. and four morphologically-distinct, but unidentified *Cortinarius* spp.]. ITS sequence comparisons confirmed that *D. austro-veneta* belongs in *Dermocybe*, that *C. rotundisporus* is correctly placed in subgenus *Phlegmacium*, and suggest that Australian *C. violaceus* collections are not conspecific with northern hemisphere *C. violaceus*.

Key words PCR · RFLP · ITS sequence analysis · Ectomycorrhizal fungi · *Cortinarius* taxonomy

Introduction

The genus *Cortinarius* has a widespread distribution, being regarded generally as an ectomycorrhizal (ECM) taxon (Liu et al. 1997). Although discriminating species of *Cortinarius* on the basis of sporocarp morphology is often difficult (Timonen et al. 1997), the genus has been commonly recorded in both northern and southern hemisphere forests, associated with coniferous and eucalypt hosts respectively (Allen et al. 1995). Indeed, sporocarp records indicate that *Cortinarius* is among the most frequently recorded ECM fungal genera in many European and North American conifer forests (eg. Alexander and Watling 1987; Villeneuve et al. 1989; Väre and Ohtonen 1996), along with Australian eucalypt forests (Bougher and Malajczuk 1986; Malajczuk et al. 1987) and tundra vegetation (Gardes and Dahlberg 1996).

Sporocarp data, however, represent only a minimum estimate of below-ground ECM fungal diversity and provide no reliable information on either the structure of ECM fungal communities in root systems (eg. Visser 1995; Gardes and Bruns 1996) or the relative importance of particular genera or species in a community. Thus, although the occurrence of sporocarps has been well documented in many forest systems, the importance of *Cortinarius* species in below-ground ECM fungal communities and their potential ecological roles are currently poorly understood. This may be explained partly by the acknowledged difficulties of isolating and maintaining axenic cultures of *Cortinarius* species in the laboratory (Brundrett et al. 1996). While ECM produced by a few *Cortinarius* species have been described using microscope-based ECM 'typing' methods (eg. Agerer 1988; Miller et al. 1991), the 'superficial' nature (namely a thin mantle) of the ECM produced by some *Cortinarius* species in combination with some host species (eg. Malajczuk et al. 1987) renders community analysis by conventional means difficult in some instances. Nonetheless, such typing methods suggest that *Cortinarius*-type ECM can constitute ca. 13% of the ECM fungal community on pine roots (Visser 1995).

Advances in molecular methods mean that 'difficult' fungal taxa such as *Cortinarius* can be investigated in detail and taxonomic relatedness inferred (Liu et al. 1997). Furthermore, accurate analysis of populations and communities of ECM fungi within root systems also can be undertaken, regardless of the ease with which the fungi can be isolated or positively identified by 'typing' procedures. While the rDNA internal transcribed spacer region (ITS) is conserved and is re-

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garded as being of little value for determining intraspecific variation in fungi (Erland et al. 1994; Pérez-Artés et al. 1995), restriction fragment length polymorphism (RFLP) analysis of the ITS region has proven useful for separating a range of ECM fungi at the interspecific level (eg. Gardes et al. 1991; Henrion et al. 1992; Erland et al. 1994; Henrion et al. 1994). In the particular case of *Cortinarius*, Pritsch et al. (1997) were able to discriminate three *Cortinarius* species from a German black alder stand on the basis of RFLP patterns produced following digestion with two restriction endonucleases. Kårén et al. (1997), however, were unable to discriminate several *Cortinarius* species from Scandinavian forests using three endonucleases.

ITS-RFLP studies of below-ground ECM fungal communities have shown that *Cortinarius* species occupy up to 14% of total infected roots in a Swedish Norway spruce forest (Kårén et al. 1996) and $<5\%$ of the community in North American pine forests (Gardes and Bruns 1996; Bradbury et al. 1998). To date, however, there has been no study of the importance of *Cortinarius* species in below-ground ECM fungal communities of southern hemisphere native forests. As a prelude to below-ground population and community studies of *Cortinarius* taxa in eastern Australian sclerophyll forests, we investigated the suitability of ITS-RFLP for separating co-occurring *Cortinarius* and *Dermocybe* species at a field site in New South Wales, Australia. We further compared ITS sequences of these fungi to those available for northern hemisphere Cortinariaceae taxa in the Genbank and EMBL nucleotide databases in order to examine relationships and clarify some taxonomic uncertainties in several Australian taxa.

Materials and methods

Sporocarp collection and DNA extraction

Sporocarps were collected from a sclerophyll forest site at the Lovers Jump Creek Reserve, Turramurra (New South Wales, Australia) in April/June 1996. Between two and four sporocarps were analysed for each species. Sporocarps of *D. austro-veneta* Clel.(Moser & Horak), *C. rotundisporus* Clel. & Cheel, *C. archeri* Berk., *C. sinapicolor* Clel., *C. violaceus* (L.: Fr.) S.F.Gray, *C. radicatus* Clel., along with those of four morphologically-distinct, but unidentified *Cortinarius* species were collected and stipe material frozen at –20 °C until use. DNA was extracted from stipe material using the method of Gardes and Bruns (1993). Where ITS amplification was unsuccessful, genomic DNA was purified using the Wizard DNA Clean-Up System (Promega).

ITS-RFLP

Amplifications were performed in a total volume of 50 μl containing approximately 100 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 25 pmol each primer [ITS1 and ITS4 (White et al. 1990) for sequencing, or ITS1F and ITS4B (Gardes and Bruns 1993) for ITS-RFLP], 2.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dTTP, dGTP, and 2.5 units *Taq* DNA polymerase (Promega). All amplifications were performed in a PTC-100 thermocycler (MJ Research) with 30 cycles of 94 °C for 60 s, 50 °C for 60 s, and 72 °C for 60 s, with a final extension of 72 °C for 5 min. Reactions were performed in duplicate for each isolate and negative controls (containing no DNA template) were included in every reaction. PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide and visualised under UV light.

PCR products were amplified as above using the primer pair ITS1F-ITS4B (Gardes and Bruns 1993). Restriction enzymes were chosen based on mapping of restriction sites in sequences of the six named species, using the program Map in the GCG version of the Wisconsin Package Version 8.1.0 (Rice 1996). Five units of each of the restriction enzymes *Hin*f I or *Hae* III (Promega) were used to digest approximately 1 μg PCR product following the manufacturer's instructions and the products were electrophoresed on 3% agarose gels. RFLP patterns were visualised as described above.

ITS sequence analysis

PCR products were cloned using the pGEM-T Easy vector system (Promega) and two or three clones of each ITS product were sequenced using an automated fluorescent DNA sequencer ABI model 373-A (Applied Biosystems, Inc.). The ITS sequences from the six species were analysed with the FASTA 3.0 program (Pearson and Lipman 1988) and sequences aligned using the Pileup and Pretty programs [in the EGCG extensions to the Wiconsin Package, Version 8.1.0 (Rice 1996)]. Sequence data from the six Australian isolates were analysed with 27 representative species of American and European *Cortinarius* and *Dermocybe* (Kårén et al. 1996; Liu et al. 1997). Nucleotides at the beginning of the Liu et al. (1997) sequences were removed (up to the end of the ITS 1 primer site) to allow for analysis of sequences of only the region within the ITS1-ITS4 primer sites. Neighbour-joining analysis of sequences was conducted using the beta version of PAUP (4.0b1), with bootstrap resampling (1000 replicates), with the ITS sequence for *Pisolithus* sp. (GenBank accession code AF004735) as an outgroup.

Results and Discussion

ITS-RFLP

For all 10 species, the ITS amplification products were 860–1080 bp in size. Although digestion with each endonuclease produced restriction patterns containing 2–4 fragments for each species, neither enzyme alone successfully separated all species (Table 1). A combination of enzymes was thus required to distinguish the 10 species. RFLP patterns generated using *Hae* III distinguished eight species, however species C and D were placed in the same RFLP grouping. *Hin*f I, while failing to distinguish some taxa, separated species C from species D. No intraspecific polymorphisms were detected in RFLP patterns for DNA extracted from replicate sporocarps for any of the four species investigated. ITS-RFLP typing using combined data from *Hin*f I and *Hae* III is thus a useful tool for separating mycelia of a range of co-occurring *Cortinarius* taxa from forests in southeastern Australian sclerophyll forests, allowing identification to species level. The use of the basidiomycete-specific primers ITS1F and ITS4B (Gardes and Bruns 1993) mean that the technique will allow identification of the 10 species by direct PCR amplification for ECM roots in future investigations of below-ground diversity.

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                    1                                                                                                                   100
D. austro-veneta    AAGGATCATT GTTGAAATAA ACCTGATG.G GTTGCTGCTG GTTCTCTAGG GAACA.TGTG CACACTTGTC GTCTTTATAT CT..CCACCT GTGCACCTTT
C. rotundisporus    AAGGATCATT ATTGAATTAA ACGTGATA.A GTTGCTGCTG GTTCTCTAGG GAACA.TGTG CACACTTGTC ATCTTTATAT CT..CCACCT GTGCACCTTT
  C. sinapicolor    AAGGATCATT ATTGAAATAA AACTGATG.G GTTGTTGCTG GCTCTCTGCG GAGCA.TGTG CACACTTGTC ATCTTTATAT CT..CCACCT GTGCACCTTG
       C. archeri   AAGGATCATT ATTGAAATAA ACTTGATG.A GTTGTTGCTG GCTCTCTAGG GAGCA.TGTG CACGCTTGTC CTCTTTATAT CT..CCACCT GTGCACCCTT
     C. violaceus   AAGGATCATT ATTGAAATAA ACCTGACGAG GTTGTTGCTG GCTCTCTAGG GAGTA.TGTG CACACTTGTC ATCTTTATAT CTTCCCACCT GTGCACCTTT
     C. radicatus   AAGGATCATT ATTGAAAATA AACCTGATGA GTTGCTGCTG GTTCTCTTGG GAACATTGTG CACGCTTATC ATCTTTATAT AATCCCCTGT GCACCTTTTG
        consensus   ********** _*****___* *_________ ****_***** *_*****__* **__*_**** ***_***_** _********* ____**___* *__*____*_

                    101                                                                                                                 200
D. austro-veneta    TGTAGACCTG GATATCTTTC TGAATG...C ACTAGCA... .TTCGGGTTT GAGGATTGAC T..TTCTGTC TTTCCTTACA TTTCCAGGCC TATGTTT..C
C. rotundisporus    TGTAGACTTG GATACTTTTC TGAATG...C TTTTTAAGCA TTTCGGGTTT GAGGATTGAC T.....TGCC TCTCCTTACA TTTCCAGGTC TATGTTT..C
  C. sinapicolor    TGTAGACCTG GATATCTTTT CCTAAA...T GCTTAGGCAT TCAGGTGTTG AGGACTGACC C..CGCGGTC TGTCCTTACA TTTCCAGGCC TATGTTT..C
       C. archeri   TGTAGACCTG GGAATGTTTG GGGATT...A ACTT...... .......... .......... .......... CTCCCTTGCG TTTCCGGGCC TATGTTT..A
     C. violaceus   TGTAGACCTG GATATCTTTC TGAAATGGCG GCTAGCCAAA TTCAGGTTTT GAGGGTTGAC TCCTTTTGTC TCTCTTTACA TTTCCAGGCC TATGTTTTCC
     C. radicatus   TAGATTCTGG GATAGCTCTC TGAATGCTTA TTTTGTAAGC AGACAGGTTT GAGGACTGAC ...ATTTGTC TTTCCTCGTC TTTCCAGGTC TATGTTTT.C
        consensus   *__*__*__* *__*__*_*_ ___*______ __*_______ __________ __________ __________ ___*_*____ *****_**_* *******___

                    201                                                                                                                 300
D. austro-veneta    TTCATATA.C CC.CATGTAT GTTATAGAAT GTAGT.A... ATGGGCCTTT GTGCCTATA. .AA.TCTATA CAACTTTCAG CAACGGATCT CTTGGCTCTC
C. rotundisporus    TTCATATA.C CCTAATGTAT GTTATAGAAT GTAATAA... ATGGGCCTTT GTGCCTATA. .AATTTTATA CAACTTTCAG CAACGGATCT CTTGGCTCTC
  C. sinapicolor    TTCATATA.C CCCAATATAT GTTTTAGAAT GTATTCA... ATGGGCTTTT ATGCCTATA. .AACTTTGTA CAACTTTCAG CAACGGATCT CTTGGCTCTC
       C. archeri   TTCATTTA.C CCCATTGTAT GTTATAGAAT GTAGTAA... TGGGCCTTTT ATGCCTATA. .AAGCTTATA CAACTTTCAG CAACGGATCT CTTGGCTCTC
     C. violaceus   TTCATATACC CCCAATCTAT GTTATAGAAT GTAATAATAT TTAGGCCTTT GTGTCTAT.. .AATCCTATA CAACTTTCAG CAACGGATCT CTTGGCTCTC
     C. radicatus   TTCATATACC CCAAATGTAT GTTACAGAAT GTAATCA.AA TGGGCCTTTT GTGCCTATAT GAAAAATGTA CAACTTTCAG CAACGGATCT CTTGGCTCTC
        consensus   *****_**_* **___*_*** ***__***** ***_*_*___ ___*_*_*** _**_****__ _**___*_** ********** ********** **********

                    301                                                                                                                 400
D. austro-veneta    GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATTGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CCTTGCGCT. CCTTGGTATT
C. rotundisporus    GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAA.TGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CCTTGCGCT. CCTTGGTATT
  C. sinapicolor    GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAA.TGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CCTTGCGCTC CCTTGGTATT
       C. archeri   GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAA.TGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CCTTGCGCT. CTTTGGTATT
     C. violaceus   GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAA.TGT GAATTGCAGA ATTCAGTGAA TCATCGAATT TTTGAACGCA CCTTGCGCT. CCTTGGTATT
     C. radicatus   GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAA.TGT GAATTGCAGA ATTCAGTGAA TCGTCGAATC TTTGAACGCA CCTTGCGCT. CCTTGGTATT
        consensus   ********** ********** ********** ******_*** ********** ********** **_******_ ********** *********_ *_********

                    401                                                                                                                 500
D. austro-veneta    CCGAGGAGCA TGCCTGTTTG AGTGTCATTA ATAT..ATCA ACCTCTTCAG C..TTTTGCT TGTTGAGTGT TGGA...... .TGTGGGGG. TATTCTTTTG
C. rotundisporus    CCGAGGAGCA TGCCTGTTTG AGTGTCATTA ATAT..ATCA ACTTCTTCAG CTTTTTTATT TGTTGAATGT TGGA...... .TGTGGGGG. T....TTTTG
  C. sinapicolor    CCGAGGAGCA TGCCTGTTTG AGTGTCATTA ATAT..ATCA ATCTCTTCAG C...TTTTGT TGTTGAGCAT TGGA...... .AGTGGGGGT TTTTCCTTTG
       C. archeri   CCGAAGAGCA TGCCTGTTTG AGTGTCATTA AAATA.ATCA ACCTCTTCGG C..TTTTGCT TGTCGAGTGT TGGA...... .TGTGGGGT CTTTTTTTTG
     C. violaceus   CCGAGGAGCA TGCCTGTTTG AGTGTCATTA ATATATATCA CTCTCTTCAA GCTTTTTTTT TTTTGCTTGC TTGAGTGTTG GATTTGGGGG TTTTTTTTTG
     C. radicatus   CCGAGGAGCA TGCCTGTTTG AGTGTCATTA ATAATATCAA CCTTGCTCG. .......... .TTTGAGTGT TGGA...... ...TGTGGGG GGATATTTGC
        consensus   ****_***** ********** ********** *_*______* ___*__**__ __________ __*_*_____ *_**______ ___*__***_ ______**__

                    501                                                                                                                 600
D. austro-veneta    CTGGTCTCT. TTCTGAGGTC GGCTCCCCTG AAATGCATTA GCGGAACAAT TTGTTGA.CC GTTCATTGGT GTGAT.AATT ATCTACGCTA TTGA.CGTGA
C. rotundisporus    CTGGCCT.T. TTCAAGGGTC AGCTCTCCTC AAATATATTA GCGAAACAAT CTGTTGA.CC GTTCATTGGT GTGAT.AACT ATCTACGCTA TTGATCGTGA
  C. sinapicolor    CTGGTCTCTC TTCTGAGGTC CGCTCCCCTG AAATGTATTA GCGGAACAAT TTGTTAA.CC TTTCATTGGT GTGAT.AACT ATCTACGCTA TTGGAATGGG
       C. archeri   CTGGGCT... TATCTAGCTC AGCTCCCCTG AAAAGCATTA GCGGAACAAA CTGTTGACCC GTTCATTGGC GTGAT.AATT ATCTATGCTA TTGACGGTGA
     C. violaceus   CTGGCCT.TT TCCGAGGGTC AGCTCCCCTG AAATGCATTA GTGGAACAAT TTGTTGA.AT GTTCATTGGT GTGATAAACT ATCTACGCTA TTGA.CGTGA
     C. radicatus   TGGTCTTTCT CTCTGAGGTC AGCTCCCCTG AAATACATTA GCTGGAACAA CTTGTTGATC GTTCATTGGT GTGAT.AACT ATCTACGCTA TTGA.CGTGA
        consensus   __*___*___ ______*_** _****_***_ ***___**** *____*__*_ _*__*_____ _********_ *****_**_* *****_**** ***_____*_

                    601                                                                                    690
D. austro-veneta    AGCAGGTTCA GCTTCTAACA GTCCATTGAC TTGGACAAAT TTTCA..TTA ATGTGACCTC AAATCAGGTA GGACTACCCG CTGAACTTAA
C. rotundisporus    GGCAAGTTTA GCTTCTAATA GTCCATTGAC TTGGACAACT TTTCAT.TTA ATGTGACCTC AAATCAGGTA GGACTACCCG CTGAACTTAA
  C. sinapicolor    AGCAG..TTA GCTTCTAATA GTCCATTGAC TTGGACAGAT AATCTTATTA ATGTGACCTC AAATCAGGTA GGACTACCCG CTGAACTTAA
       C. archeri   AGTAGTTCAG CTTCTAAATA GTCCATTGAC TTGGACAAAT TATCATCT.. ATGTGACCTC AAATCAGGTA GGACTACCCG CTGAACTTAA
     C. violaceus   AGCAGTTTCC GCTTCTAACA GTCCATTGAA TTGGACAAAT TTTCA..TTA ATGTGACCTC AAATCAGGTA GGACTACCCG CTGAACTTAA
     C. radicatus   AGCAG.TTCG GCTTCTAACA GTCCTT.... .........T TTTTCATTAA ATGTGACCTC AAATCAGGTA GGATTACCCG CTGAACTTAA
        consensus   _*_*______ __*___**_* ****_*____ _________* __*____*__ ********** ********** ***_****** **********
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Fig. 1 Pileup of rDNA ITS sequences of six Australian *Cortinarius* and *Dermocybe* species. * denotes bases identical across all sequences; ● denotes a gap resulting from insertion/deletion

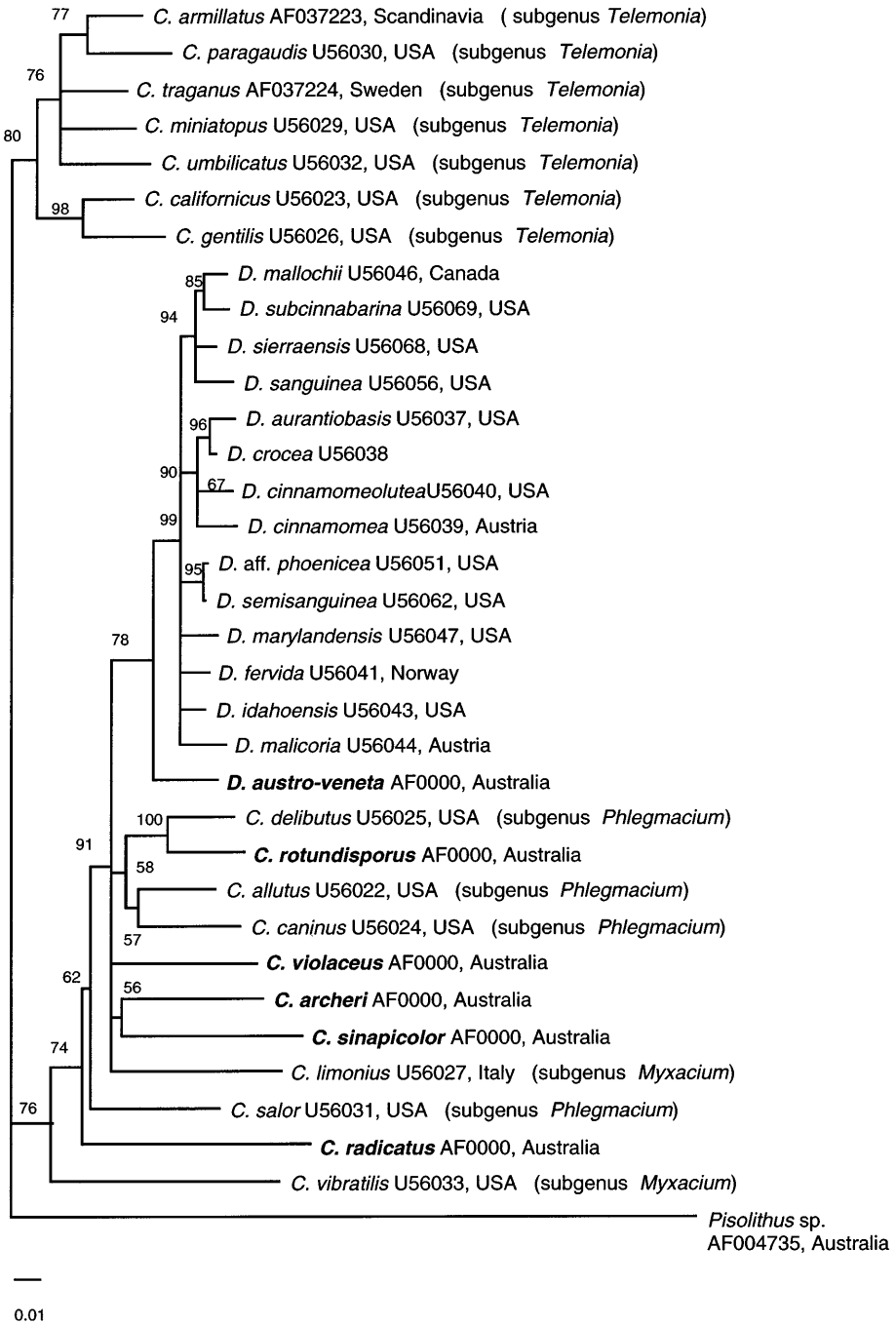

Fig. 2 Neighbour-joining tree of *Cortinarius* and *Dermocybe* species based on rDNA ITS sequence analysis using the beta version of PAUP (4.01b). Species for which sequences were obtained in the present study are shown in bold. *Numbers on branches* indicate bootstrap values (from 1000 replicates), and only those greater than 50% are shown. GenBank accession numbers for each sequence and country of origin are listed after each species name. The ITS sequence of *Pisolithus* sp. (AF004735) was used as an outgroup

It was shown previously that some *Cortinarius* taxa can be separated on the basis of RFLP patterns produced following digestion with two restriction endonucleases (Kårén et al. 1997; Pritsch et al. 1997). Kårén et al. (1997), however, were unable to separate 10 *Cortinarius* species on the basis of RFLP patterns generated using the three endonucleases *Cfo* I, *Hin*f I and *Mbo* I. In particular, closely-related species (generally within the same subgenus) were not separated. In the case of the two subgenus *Myxacium* species included in the present study (*C. archeri* and *C. sinapicolor*), individual RFLP patterns generated with either *Hinf* I or *Hae* III were sufficient to allow positive identification, suggesting that, although these species were grouped together by the neighbour-joining analysis (see below), considerable genetic distance exists between some taxa in this subgenus. Kårén et al. (1997) also observed intraspecific polymorphisms in ITS regions of some *Cortinarius* species collected throughout Fennoscandia. We found no evidence of such polymorphism in the sporocarps used in the present work. Perhaps more extensive sampling over a wider area would reveal intraspecific ITS polymorphisms in the taxa investigated.

Table 1 Restriction product sizes (in base pairs) of the rDNA ITS products of 10 species of *Cortinarius* and *Dermocybe* obtained using the restriction enzymes *Hin*f I and *Hae* III

	*Hin*f I	*Hae* III
Dermocybe austro-veneta	400, 350, 120	650, 220
Cortinarius rotundisporus	400, 350, 120	340, 300, 230
C. archeri	370, 360, 130	650, 210
C. sinapicolor	400, 350, 120	655, 215
C. violaceus	360, 220, 200, 80	350, 260, 250
C. radicatus	340, 260, 170, 130	600, 300
Cortinarius A	400, 350, 120	350, 250, 240
Cortinarius B	400, 350, 210, 120	650, 230, 160
Cortinarius C	400, 240, 140, 80	600, 290
Cortinarius D	400, 350, 120	600, 290

ITS sequence analysis

Sequences of the ITS region were obtained for the six named *Cortinarius* species (Fig. 1), and submitted to GenBank under the following accession codes: *C. archeri* (AF0000), *C. radicatus* (AF0000), *C. rotundisporus* (AF0000), *C. sinapicolor* (AF0000), *C. violaceus* (AF0000), *D. austro-veneta* (AF0000). Neighbour-joining analysis of these sequences and those obtained from the GenBank and EMBL nucleotide databases produced a tree comprising two major clades (Fig. 2). One clade contained all *Cortinarius* species belonging to the subgenus *Telemonia*, while the remaining *Cortinarius* and *Dermocybe* taxa were grouped in the second (major) clade. Within the major clade, *D. austro-veneta* was placed in a monophyletic group (91% bootstrap support) with the northern hemisphere *Dermocybe* species, although within this group it was placed separately on a strongly-supported individual (99% bootstrap) branch (Fig. 2). *D. austro-veneta* was originally described by Cleland (1928) as *Cortinarius austro-venetus* Clel., and subsequently as *C. subvenetus* and *C. cinnamomeus* by several authors (see May and Wood 1997). Although, based on morphological characters, *C. austro-venetus* was transferred to *D. austro-veneta* by Moser and Horak (1975), it has continued to be described by many authors using the earlier nomenclature (see May and Wood, 1997). The grouping of this taxon with other *Dermocybe* species in our neighbour-joining analysis supports it's placement in *Dermocybe*. It is interesting also that the *Dermocybe* clade in our neighbour-joining tree was nested within the *Cortinarius* taxa. This suggests that *Dermocybe* should be retained as a subgenus of *Cortinarius* rather than being treated as a separate species, as suggested by Liu et al. (1997).

The five remaining Australian isolates were grouped in the bottom half of the major clade. *C. rotundisporus* was placed in a monophyletic group with *C. delibutus*, *C. allutus* and *C. caninus* [all subgenus *Phlegmacium* as defined by Liu et al. (1997)], having 90–94% identity

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           1                                                    50
CVU56034   AAGGATCATT ATTGAAATAA ACCTGA.TAA GTTGCTGCTG GCTCTCTAGG
CVU56035   AAGGATCATT ATTGAAATAA ACCTGA.TAA GTTGCTGCTG GCTCTCTAGG
C. violaceus AAGGATCATT ATTGAAATAA ACCTGACGAG GTTGTTGCTG GCTCTCTAGG
consensus  ********** ********** ******--*- ****-***** **********

           51                                                   100
CVU56034   GAGCATGTGC ACACTTTCTC ATCTTTATAT CTT..CACAT GTGCACCTCT
CVU56035   GAGCATGTGC ACACTTTGTC ATCTTTATAT CTT..CACAT GTGCACCTCT
C. violaceus GAGTATGTGC ACAC.TTGTC ATCTTTATAT CTTCCCACCT GTGCACCTTT
consensus  ***-****** ****-**-** ********** ***--***-* ********-*

           101                                                  150
CVU56034   TGTAGACTTT GGATATCTTT CTGAA..... ......TGTA ATTCAGGTTT
CVU56035   TGTAGACTTT GGATATCTTT CTGAA..... ......TGTA ATTCAGGTTT
C. violaceus TGTAGAC.CT GGATATCTTT CTGAAATGGC GGCTAGCCAA ATTCAGGTTT
consensus  *******--* ********** *****----- ----------* **********

           151                                                  200
CVU56034   TTGAGGATTG ACTTTTTGGT CTCTCCTTAC ATTTCCAAAT CTATG..TTC
CVU56035   TTGAGGATTG ACTTTTTGGT CTCTCCTTAC ATTTCCAAAT CTATG..TTC
C. violaceus TGAGGGTTGA CTCCTTTTGT CTCTCTTTAC ATTTCCAGGC CTATGTTTTC
consensus  *---**-*-- ----***-** *****-**** *******--- *****--***

           201                                                  250
CVU56034   CTTCATATAC .....ACTTA TGTTATAGAA TGTAATAAAA TAGGCCTTTT
CVU56035   CTTCATATAC .....ACTTA TGTTATAGAA TGTAATAAAA TAGGCCTTTT
C. violaceus CTTCATATAC CCCCAATCTA TGTTATAGAA TGTAATAATA TTTAGGCCTT
consensus  ********** -----*--** ********** ********-* *-------**

           251                                                  300
CVU56034   TTGCCTTACA AAAACCTATA CAACTTTCAG CAACGGATCT CTTGGCTCTC
CVU56035   TTGCCTTACA AAAACCTATA CAACTTTCAG CAACGGATCT CTTGGCTCTC
C. violaceus T..GTGTCTA TAATCCTATA CAACTTTCAG CAACGGATCT CTTGGCTCTC
consensus  *-----*--* -**-****** ********** ********** **********
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Fig. 3 Pileup of rDNA ITS1 sequences of *C. violaceus* from southeastern Australia with two sequences of the same taxon from North America (Genbank accession codes CVU56034 and CVU56035). * denotes bases identical across all sequences; • denotes a gap resulting from insertion/deletion

with these taxa. There has been some debate regarding the *Cortinarius* subgenus within which *C. rotundisporus* belongs. Originally tentatively placed in subgenus *Phlegmacium* (Cleland and Cheel 1918), it was assigned to subgenus *Myxacium* by Horak and Wood (1990). In her recent review of the basidiomycete flora of South Australia, however, Grgurinovic (1997) suggests that, based on morphological traits, the original placement in subgenus *Phlegmacium* is more appropriate. The grouping of *C. rotundisporus* with other *Phlegmacium* taxa in the present study strongly supports placement in this subgenus.

With the exception of *C. radicatus* (subgenus *Phlegmacium*) and *C. violaceus*, the remaining taxa in the major clade belong to subgenera *Leprocybe* and *Myxacium* (Singer 1986; Grgurinovic 1997). Aside from the obvious placement of *C. rotundisporus* with other *Phlegmacium* species, no clear groupings could be discerned for the other Australian taxa, suggesting that wider sequence comparison is required. Although widely regarded as conspecific with *C. violaceus* (L.: Fr.) S.F.Gray (subgenus *Cortinarius*), the name has been applied to southern hemisphere *C. violaceus* collections with some doubt (see Bougher and Syme 1998). No full-length ITS sequences are currently available in the GenBank or EMBL databases for northern hemisphere *C. violaceus*. We were, thus, unable to include these in our neighbour-joining analysis. ITS1 sequences (296 nucleotides) are, however, available for northern hemisphere *C. violaceus* and a wide range of

Cortinariaceae, and these were compared using Fasta with the ITS1 sequence for putative *C. violaceus* sporocarps collected at our field site. While two North American collections of *C. violaceus* (GenBank accession codes CVU56034 and CVU56035) displayed >99% sequence identity with each other over the ITS1 region, the collections from our field site were <71% similar to these (Fig. 3). In addition to a number of nucleotide substitutions, several insertions, including an 11-nucleotide insertion, were evident in our *C. violaceus* collection compared to the North American sequences (Fig. 3), clearly suggesting that they belong to a separate taxon. This was further supported by the fact that the collections from our field site had highest sequence identities (82% over 238 nucleotides and 78% over 268 nucleotides) with *C. limonius* and *C. debilutus* [both recently assigned to subgenus *Myxacium*, Liu et al. (1997)]. A careful revision of Australian *C. violaceus* collections is clearly required.

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