

MYCOLOGY

Improved molecular identification of *Thermoactinomyces* spp. associated with mushroom worker's lung by 16S rDNA sequence typing

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Mushroom worker's lung (MWL) is a hypersensitivity pneumonitis or allergic alveolitis caused by a type III IgG-mediated immunopathogenic inflammatory reaction in the host due to the inhalation of several thermophilic organisms, including *Thermoactinomyces* spp. It is difficult to distinguish phenotypically the eight species of this genus; therefore, this study sought to develop an improved molecular means of identifying *Thermoactinomyces* spp. associated with MWL by partial 16S rDNA PCR amplification and direct sequencing. Hypervariable regions within the 16S rRNA gene, which could be employed as signature sequences of the eight individual species, were identified and employed with highly conserved flanking primers to allow initial PCR amplification, before direct DNA sequencing of the 16S rDNA amplicons. A novel 24-mer 16S rDNA oligonucleotide upstream primer was designed from *in silico* alignments of all *Thermoactinomyces* spp. and was employed in combination with downstream (reverse) 16S rDNA primers. This permitted the successful identification of all four isolates associated with mushroom workers' lung. The method may be useful in the identification of *Thermoactinomyces* spp. associated with allergic alveolitis or pneumonitis associated with occupational exposure in agricultural and horticultural environments.

Introduction

Mushroom worker's lung (MWL) is a hypersensitivity pneumonitis or allergic alveolitis caused by a type III IgG-mediated immunopathogenic inflammatory reaction in the host due to the inhalation of several thermophilic organisms, including members of the genera *Thermoactinomyces*, *Saccharomonospora* and *Micropolyspora faeni* [1]. Of these, *Thermoactinomyces* spp., including *T. vulgaris*, have been associated frequently with the disease [2, 3]. The condition, as the name suggests, is prevalent in workers involved in

the mushroom industry, particularly in those whose occupation involves working with phase II compost or in the spawning function of mushroom production, because of exposure to high concentrations of aerial mycelia and endospores.

Laboratory culture of causal agents of MWL may be performed from clinical specimens, including sputum and broncho-alveolar lavage (BAL) fluid, as well as from the occupational areas with greatest mycelial and spore loading, by selective culture at elevated temperature (56°C for 4–5 days), although serological detection is most commonly employed. However, reliable identification and, in particular, speciation within the *Thermoactinomyces* genus, may be troublesome because of the lack of well-defined phenotypic assays to

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differentiate between the eight species currently described as belonging to this genus [4]. Furthermore, correct identification is important for epidemiological reporting, as well as for identifying strain types that may be employed for subsequent antigen extraction for serological precipitin testing in routine diagnostic laboratories.

Therefore, the aim of this study was to examine partial 16S rDNA PCR amplification and direct sequencing as an improved molecular means of identification of *Thermoactinomyces* spp. associated with MWL.

Materials and methods

PCR primer selection

The complete 16S rDNA sequences of the eight species of *Thermoactinomyces* [4], including the following organisms: *T. candidus* (GenBank accession no. AF138732), *T. dichotomicus* (AF138733), *T. intermedius* (AF138734), *T. peptonophilus* (AF138735), *T. putidus* (AF138736), *T. sacchari* (AF138737), *T. thalpophilus* (AF138738) and *T. vulgaris* (AF138739), were aligned by employing the Clustal method software (DNASTar, Wisconsin, USA) as shown in Fig. 1. Universal 16S rDNA PCR primers were selected to span at least two-thirds of the 16S rDNA gene, to include most regions of hypervariability between the species lying between position 314 and 1370, and included the reverse primers PSR [5] and P13P [6], as described in relation to *T. vulgaris* (AF138739) (Fig. 2). A novel forward primer, XB1, with the sequence 5'-CAG ACT CCT ACG GGA GGC AGC AGT-3' was designed from the complete 16S rDNA alignment of the eight species and was selected at position 314–337 (*T. vulgaris* AF138739) which represented 100% sequence homology among all eight species (Fig. 2).

Extraction and PCR amplification of microbial DNA

Four unidentified isolates associated with MWL were examined. All were purified on Colombia agar base (CM331, Oxoid) supplemented with defibrinated horse blood (Oxoid) 5% v/v and D-glucose 1% w/v and were incubated at 56°C for 4–5 days. Genomic DNA was extracted from a single colony with the Roche High Purity PCR Template kit (Roche Diagnostics), in accordance with the manufacturer's instructions. All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the amplification and post-PCR room to minimise contamination, in accordance with the laboratory guidelines of Millar *et al.* [7]. PCR reaction mixes (50 µl) contained: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 200 µM (each) dATP, dCTP, dGTP and dTTP, 1.25 U of *Taq* DNA polymerase (AmpliTaq; Perkin Elmer), 0.2 µM (each) of the 16S rRNA primers, as detailed (Fig. 2) and 4 µl of DNA template

containing c. 50 ng of DNA/ml of extract. Following a 'hot start', the reaction mixtures were subjected to the following thermal cycling conditions in a Perkin Elmer 2400 thermocycler: 96°C for 3 min followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. During each run, molecular grade water (Biowhittaker) instead of DNA was included randomly as a negative control and *Staphylococcus aureus* DNA was included as a positive control. After amplification, portions (15 µl) were removed, electrophoresed (80 V, 45 min) in agarose (Gibco) gels 2% w/v in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and stained with ethidium bromide (5 µg/100 ml). Gels were visualised under UV illumination with a gel image analysis system (UVP Products) and all images were archived as digital (*.bmp) graphic files.

Sequencing of amplicons and analysis of sequence data

Amplicons for sequencing were purified with the QIAquick PCR purification kit (Qiagen) and eluted in Tris-HCl (10 mM, pH 8.5) before sequencing. Cy-5'-labelled primer, XB1, was prepared and used for sequencing in the forward direction with the ALF Express II (Amersham-Pharmacia, Bucks) employing the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech; no. RPN 2438) (96°C for 1 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 5 s, followed by a 4°C hold). The sequences obtained were compared with those stored in the GenBank data system with BLAST alignment software (<http://www.blast.genome.ad.jp/>). Sequence homology identity was determined in accordance with the criteria described previously [8].

Results and discussion

All four unknown thermophilic isolates examined generated PCR products of the expected size of c. 762 bp and 1056 bp for primer combinations XB1/PSR and XB1/P13P, respectively (Fig. 3). Subsequent sequence analysis of the 1056-bp amplicon identified the isolates as shown in Table 1 and the sequences have been deposited in GenBank, as detailed (Table 1).

In this study, the initial objective was to identify a highly conserved upstream region of the 16S rRNA gene in *Thermoactinomyces* spp. that could act as the forward primer in a PCR reaction, in combination with a highly conserved downstream 16S rDNA primer. The approach adopted was to identify hypervariable regions within the 16S rRNA gene, which could be employed as signature sequences of the eight individual species within this genus and to employ highly conserved flanking primers to allow initial PCR amplification,

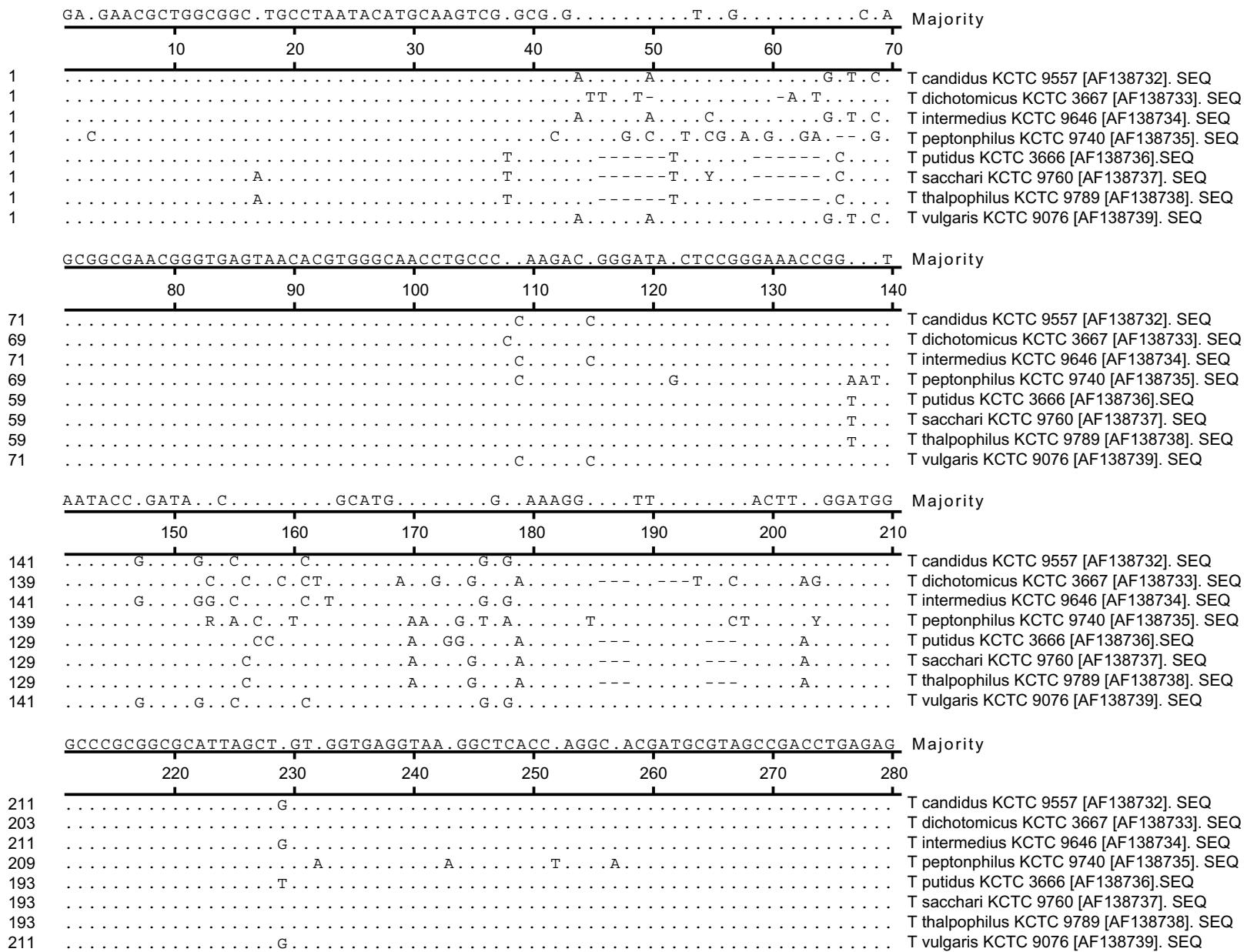


Fig. 1. Nucleotide alignments of part of the 16S rRNA gene of eight species of *Thermoactinomyces*. The consensus sequence is shown along the title bar and only differences are displayed.

GGTGA . CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGAAATTTCG Majority
 290 300 310 320 330 340 350

281 C
 273
 281
 279
 263
 263
 263
 281

T candidus KCTC 9557 [AF138732]. SEQ
 T dichotomicus KCTC 3667 [AF138733]. SEQ
 T intermedius KCTC 9646 [AF138734]. SEQ
 T peptonphilus KCTC 9740 [AF138735]. SEQ
 T putidus KCTC 3666 [AF138736].SEQ
 T sacchari KCTC 9760 [AF138737]. SEQ
 T thalpophilus KCTC 9789 [AF138738]. SEQ
 T vulgaris KCTC 9076 [AF138739]. SEQ

CAATGGGCG . AAGCCTGACGGAGCAA . GCCGCGTGAG . GA . GACGG . CTTCGG . TTGTAAA . CTCTGTT Majority
 360 370 380 390 400 410 420

351
 343
 351 C . A G
 349
 333 T A G
 333 C T
 333 T T
 333 T T
 351

T candidus KCTC 9557 [AF138732]. SEQ
 T dichotomicus KCTC 3667 [AF138733]. SEQ
 T intermedius KCTC 9646 [AF138734]. SEQ
 T peptonphilus KCTC 9740 [AF138735]. SEQ
 T putidus KCTC 3666 [AF138736].SEQ
 T sacchari KCTC 9760 [AF138737]. SEQ
 T thalpophilus KCTC 9789 [AF138738]. SEQ
 T vulgaris KCTC 9076 [AF138739]. SEQ

T . . . GGAAGAA GACG . TAC . . AG . AGAAAGCCCCGGCTAA Majority
 430 440 450 460 470 480 490

421 T . . . TCCA . GC . . . C . CCT . . . GT . . . A . . .
 413 G .
 421 T . . . TCCA . GC . . . C . CCT . . . GT . . . A . . . C . . .
 419 GT .
 403 CGT - . TT . . . T . ATAC . G . RG . . . T . . . A . . . C . . .
 403 GA .
 403 GA .
 421 GA .
 421 T . . . TCCA . GC . . . C . CCT . . . GT . . . A . . .

T candidus KCTC 9557 [AF138732]. SEQ
 T dichotomicus KCTC 3667 [AF138733]. SEQ
 T intermedius KCTC 9646 [AF138734]. SEQ
 T peptonphilus KCTC 9740 [AF138735]. SEQ
 T putidus KCTC 3666 [AF138736].SEQ
 T sacchari KCTC 9760 [AF138737]. SEQ
 T thalpophilus KCTC 9789 [AF138738]. SEQ
 T vulgaris KCTC 9076 [AF138739]. SEQ

CTACGTGCCAGCCAGCGCGGTAATACTGTAGGGGGC . AGCGTT . TCCGGAAT . ATTGGGCGTAAAGCGCGC Majority
 500 510 520 530 540 550 560

491
 460
 491
 488 A . . . G . . . C . . .
 449 A . . .
 449
 449
 491

T candidus KCTC 9557 [AF138732]. SEQ
 T dichotomicus KCTC 3667 [AF138733]. SEQ
 T intermedius KCTC 9646 [AF138734]. SEQ
 T peptonphilus KCTC 9740 [AF138735]. SEQ
 T putidus KCTC 3666 [AF138736].SEQ
 T sacchari KCTC 9760 [AF138737]. SEQ
 T thalpophilus KCTC 9789 [AF138738]. SEQ
 T vulgaris KCTC 9076 [AF138739]. SEQ

Fig. 1. (continued)

	G . AGGCGG TAAGTC . G . TGT . AAAGGC . . . GGCTCAACC . . . G . CG . CA . C . GAAACTG . . .	Majority
	570 580 590 600 610 620 630	
561TGA.....CA.....
530GG.....G.A.....A.....T.....T.C.....TC.....	T candidus KCTC 9557 [AF138732]. SEQ
561GG.....	T dichotomius KCTC 3667 [AF138733]. SEQ
558T.....TTCA.....C.A.....C.....A.T.....A.T.TA.....T.G.....TGGA.....	T intermedius KCTC 9646 [AF138734]. SEQ
519T.....	T peptonphilus KCTC 9740 [AF138735]. SEQ
519T.....	T putidus KCTC 3666 [AF138736]. SEQ
519T.....	T sacchari KCTC 9760 [AF138737]. SEQ
561TGA.....	T thalpophilus KCTC 9789 [AF138738]. SEQ
CA.....	T vulgaris KCTC 9076 [AF138739]. SEQ
	CTTGAGTGCAGGAGAGG . GAG . GGAATT C . CGGTGTAGCGGTGGAATGCGTAGAGATCGGGAGGAACACC	Majority
	640 650 660 670 680 690 700	
631	T candidus KCTC 9557 [AF138732]. SEQ
600	T dichotomius KCTC 3667 [AF138733]. SEQ
631	T intermedius KCTC 9646 [AF138734]. SEQ
627	T peptonphilus KCTC 9740 [AF138735]. SEQ
589T.....T.....	T putidus KCTC 3666 [AF138736]. SEQ
589A.....T.....	T sacchari KCTC 9760 [AF138737]. SEQ
589A.....	T thalpophilus KCTC 9789 [AF138738]. SEQ
631A.....	T vulgaris KCTC 9076 [AF138739]. SEQ
	AGTGGCGAAGGCG . CTCTCTGGCCTGT . . CTGACGCTGAGG . GCGAAAGCGTGGGGAGC . AACAGGATTA	Majority
	710 720 730 740 750 760 770	
701	T candidus KCTC 9557 [AF138732]. SEQ
670	T dichotomius KCTC 3667 [AF138733]. SEQ
701	T intermedius KCTC 9646 [AF138734]. SEQ
697A.....	T peptonphilus KCTC 9740 [AF138735]. SEQ
659T.....	T putidus KCTC 3666 [AF138736]. SEQ
659TT.....	T sacchari KCTC 9760 [AF138737]. SEQ
659TT.....	T thalpophilus KCTC 9789 [AF138738]. SEQ
701	T vulgaris KCTC 9076 [AF138739]. SEQ
	GATAACCCTGGTAGTCCACGCCGTAAACG . TGAGTGCTAGGTGT . GGG . G . . . G . . . TC . GTGCCG .	Majority
	780 790 800 810 820 830 840	
771C-A.....	T candidus KCTC 9557 [AF138732]. SEQ
740AA.A.-TGA.TT.....	T dichotomius KCTC 3667 [AF138733]. SEQ
771C-A.....	T intermedius KCTC 9646 [AF138734]. SEQ
767T.....T.....	T peptonphilus KCTC 9740 [AF138735]. SEQ
729T.....	T putidus KCTC 3666 [AF138736]. SEQ
729C.....C-TA---G.....	T sacchari KCTC 9760 [AF138737]. SEQ
729C.....C-TA---G.....	T thalpophilus KCTC 9789 [AF138738]. SEQ
771C-A.....	T vulgaris KCTC 9076 [AF138739]. SEQ

Fig. 1. (continued)

	AG . TAAC . CA . TAAGCACTCCGCCTGGGGAGTACG .. CGCAAG .. TGAAACTCAAAGGAATTGACGGGGG	Majority
	850 860 870 880 890 900 910	
840 A ..	T candidus KCTC 9557 [AF138732]. SEQ
809 T ..	T dichotomicus KCTC 3667 [AF138733]. SEQ
840 A ..	T intermedius KCTC 9646 [AF138734]. SEQ
837 A ..	T peptonphilus KCTC 9740 [AF138735]. SEQ
797 A ..	T putidus KCTC 3666 [AF138736].SEQ
797 A ..	T sacchari KCTC 9760 [AF138737]. SEQ
797 A ..	T thalpophilus KCTC 9789 [AF138738]. SEQ
840 A ..	T vulgaris KCTC 9076 [AF138739]. SEQ
	CCCCCACAGCGGTGGAGCATGTGGTTAATTCGAAGCAACGCGAAGAACCTTACCAAGGGCTTGACATCC	Majority
	920 930 940 950 960 970 980	
910 A ..	T candidus KCTC 9557 [AF138732]. SEQ
879 T ..	T dichotomicus KCTC 3667 [AF138733]. SEQ
910 T ..	T intermedius KCTC 9646 [AF138734]. SEQ
907 T ..	T peptonphilus KCTC 9740 [AF138735]. SEQ
867 T ..	T putidus KCTC 3666 [AF138736].SEQ
867 T ..	T sacchari KCTC 9760 [AF138737]. SEQ
867 T ..	T thalpophilus KCTC 9789 [AF138738]. SEQ
910 T ..	T vulgaris KCTC 9076 [AF138739]. SEQ
	CGCTGAC .. T .. AGAGAT .. TTCGG .. CAGCGGTGACAGGTGGTGCATGGTTGTCGTC	Majority
	990 1000 1010 1020 1030 1040 1050	
980 C ..	T candidus KCTC 9557 [AF138732]. SEQ
949 G .. GT .. ACGC ..	T dichotomicus KCTC 3667 [AF138733]. SEQ
980 C ..	T intermedius KCTC 9646 [AF138734]. SEQ
977 A .. A .. T .. A .. - - - CCTTT .. A ..	T peptonphilus KCTC 9740 [AF138735]. SEQ
937 C ..	T putidus KCTC 3666 [AF138736].SEQ
937 C ..	T sacchari KCTC 9760 [AF138737]. SEQ
937 C ..	T thalpophilus KCTC 9789 [AF138738]. SEQ
980 C ..	T vulgaris KCTC 9076 [AF138739]. SEQ
	AGCTCGTGTGCGTAGATGTTGGGTTCA GTCCC GCAACGAGCGCAACCC TT .. TC .. AGTTGCCAGCA ..	Majority
	1060 1070 1080 1090 1100 1110 1120	
1047 G ..	T candidus KCTC 9557 [AF138732]. SEQ
1019 TCC ..	T dichotomicus KCTC 3667 [AF138733]. SEQ
1047 G ..	T intermedius KCTC 9646 [AF138734]. SEQ
1044 T ..	T peptonphilus KCTC 9740 [AF138735]. SEQ
1004 CC ..	T putidus KCTC 3666 [AF138736].SEQ
1004 C ..	T sacchari KCTC 9760 [AF138737]. SEQ
1004 C ..	T thalpophilus KCTC 9789 [AF138738]. SEQ
1047 G ..	T vulgaris KCTC 9076 [AF138739]. SEQ

Fig. 1. (continued)

	... G . TGGGC ACTCT . . . GAGACAGCCG . TGAAAG . CGGAGGAAGGTGGGGATGACGTCAAATCATCATG	Majority
	1130 1140 1150 1160 1170 1180 1190	
1117 A ..	T candidus KCTC 9557 [AF138732]. SEQ
1089 G G . A ..	T dichotomicus KCTC 3667 [AF138733]. SEQ
1117 G	T intermedius KCTC 9646 [AF138734]. SEQ
1114	AAT . C	T peptonphilus KCTC 9740 [AF138735]. SEQ
1074	... -	T putidus KCTC 3666 [AF138736]. SEQ
1073	. A - . T	T sacchari KCTC 9760 [AF138737]. SEQ
1073	. A - . T	T thalpophilus KCTC 9789 [AF138738]. SEQ
1117	... -	T vulgaris KCTC 9076 [AF138739]. SEQ
	CCCCTTATGTCCTGGGCTACACACGTGCTACAATGGC .. G . ACAA . GGG .. GC . A .. CCGCGAGG . GGAG	Majority
	1200 1210 1220 1230 1240 1250 1260	
1186	T candidus KCTC 9557 [AF138732]. SEQ
1158	T dichotomicus KCTC 3667 [AF138733]. SEQ
1186	T intermedius KCTC 9646 [AF138734]. SEQ
1184 A	T peptonphilus KCTC 9740 [AF138735]. SEQ
1143 CA	T putidus KCTC 3666 [AF138736]. SEQ
1142 C	T sacchari KCTC 9760 [AF138737]. SEQ
1142 C	T thalpophilus KCTC 9789 [AF138738]. SEQ
1186 A	T vulgaris KCTC 9076 [AF138739]. SEQ
	C . AATCCCCA . AAA . C .. GTCTCAGTTCGGAT . GCAGGGCTGCAACTCGCCTGC . TGAAG .. GGAATCGCTA	Majority
	1270 1280 1290 1300 1310 1320 1330	
1256	T candidus KCTC 9557 [AF138732]. SEQ
1228 T	T dichotomicus KCTC 3667 [AF138733]. SEQ
1256	T intermedius KCTC 9646 [AF138734]. SEQ
1254 A	T peptonphilus KCTC 9740 [AF138735]. SEQ
1213 T .. A . TG ..	T putidus KCTC 3666 [AF138736]. SEQ
1212 T .. C .. A .. G ..	T sacchari KCTC 9760 [AF138737]. SEQ
1212 T .. C .. A .. G ..	T thalpophilus KCTC 9789 [AF138738]. SEQ
1256	T vulgaris KCTC 9076 [AF138739]. SEQ
	GTAATCGGGATCAGCATGCCGGGTGAATACGTTCCCGGGCTTGTACACACCGCCCGTCACACCACG .	Majority
	1340 1350 1360 1370 1380 1390 1400	
1326	T candidus KCTC 9557 [AF138732]. SEQ
1298	T dichotomicus KCTC 3667 [AF138733]. SEQ
1326	T intermedius KCTC 9646 [AF138734]. SEQ
1324 G	T peptonphilus KCTC 9740 [AF138735]. SEQ
1283 T	T putidus KCTC 3666 [AF138736]. SEQ
1282 T	T sacchari KCTC 9760 [AF138737]. SEQ
1282 T	T thalpophilus KCTC 9789 [AF138738]. SEQ
1326	T vulgaris KCTC 9076 [AF138739]. SEQ

Fig. 1. (continued)

	GAGT . TGCAACACCCGAAGTCGGTGAG .. AACC .. GGA .. CAGC . GCCGAAGGTGGG . AGATGA . T Majority						
	<hr/> 1410 1420 1430 1440 1450 1460 1470						
1396						
1368 C						
1396 C						
1394 AG .. G - .. CT .. T .. T .. C ..						
1353 T .. C .. T .. A ..						
1352 T .. - T .. A ..						
1352 T .. - T .. A ..						
1396						
	GGGGTGAAGTCGTAACAAGGTATCCCTACCGGAAGGTGG						
	<hr/> 1480 1490 1500						
1466						
1437						
1466						
1463						
1423						
1421						
1421						
1466						
	Majority						
	T candidus KCTC 9557 [AF138732]. SEQ						
	T dichotomius KCTC 3667 [AF138733]. SEQ						
	T intermedius KCTC 9646 [AF138734]. SEQ						
	T peptonphilus KCTC 9740 [AF138735]. SEQ						
	T putidus KCTC 3666 [AF138736]. SEQ						
	T sacchari KCTC 9760 [AF138737]. SEQ						
	T thalpophilus KCTC 9789 [AF138738]. SEQ						
	T vulgaris KCTC 9076 [AF138739]. SEQ						

Fig. 1. (continued)

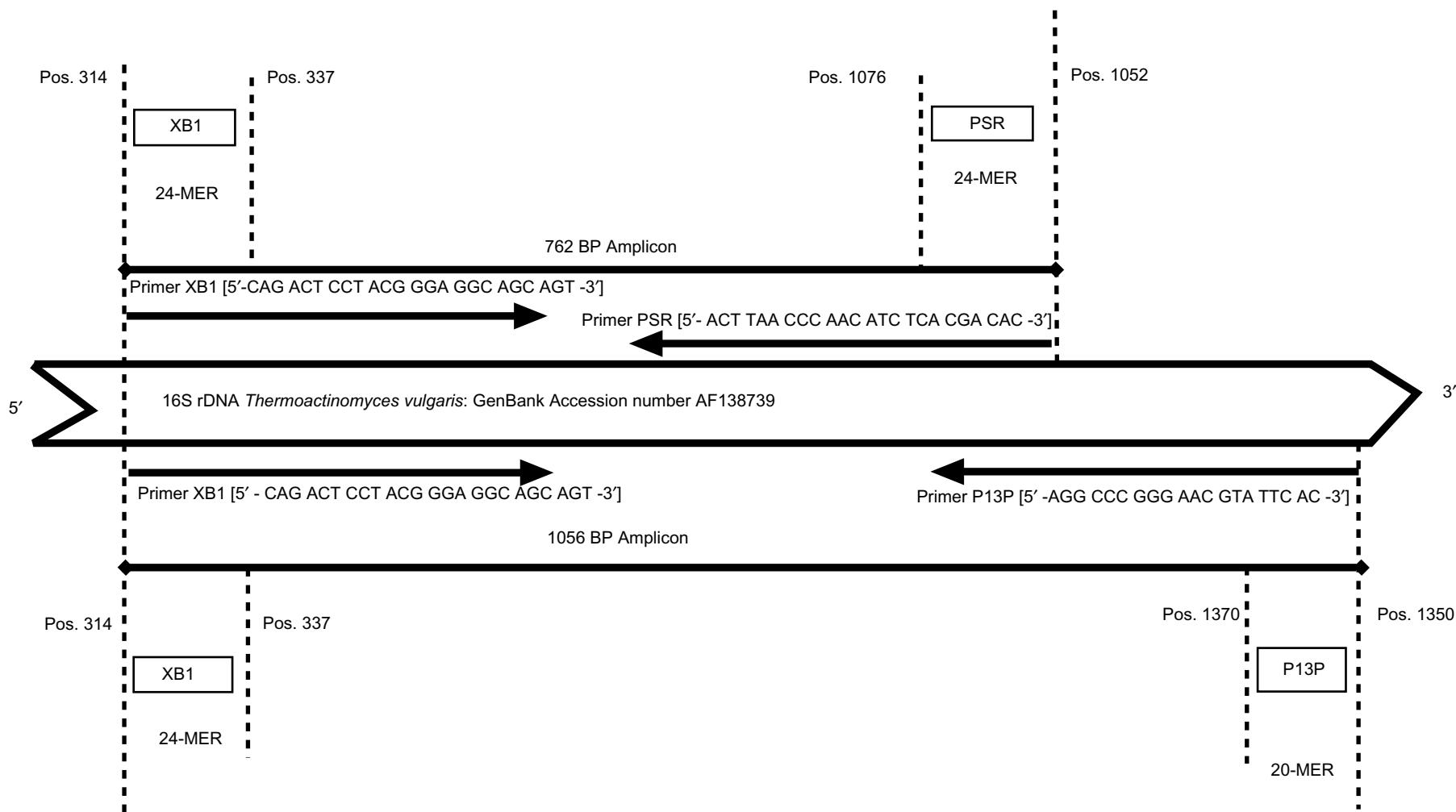


Fig. 2. Primer location, amplicon size and nucleotide sequence of the XB1/PSR and XB1/P13P primer combinations used.

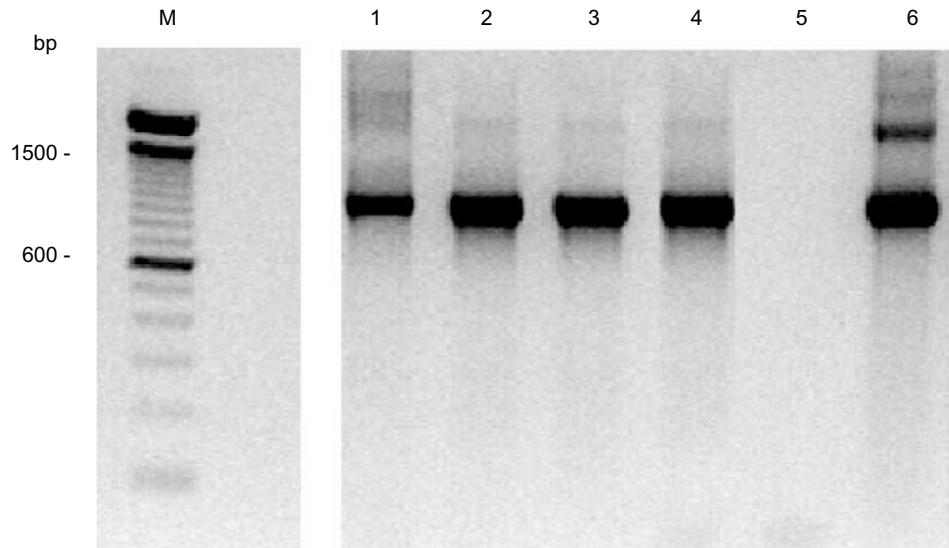


Fig. 3. PCR amplification of a partial 16S rDNA region (1056 bp) of four unidentified thermophilic organisms associated with mushroom worker's lung. Lanes 1–6, XB1/P13P primer combination; M, 100-bp mol. wt marker (Life Technologies, Paisley, Scotland). Lane 1, RVH210302; 2, RVH210302B; 3, RVH210302C; 4, RVH210302A; 5, negative control; 6, positive PCR control (*S. aureus*).

Table 1. Identification of four unidentified thermophilic isolates associated with mushroom worker's lung by PCR amplification and direct sequencing of partial regions of 16S rDNA

Isolate identifier	Number of bases called	Identification	Percentage homology	Submitted GenBank accession no.
RVH210302	1015	<i>T. vulgaris</i>	100	AY114167
		<i>T. candidus</i>	100	
RVH210302B	985	<i>T. sacchari</i>	100	AY114169
		<i>T. thalpophilus</i>	100	
RVH210302C	987	<i>T. sacchari</i>	100	AY114169
		<i>T. thalpophilus</i>	100	
RVH210302A	1001	<i>Saccharomonospora viridis</i>	100	AY114168

before direct DNA sequencing of the 16S rDNA amplicon.

Several universal 16S rDNA primers have been described previously including PSR [5] and P13P [6]. The sequence regions they detect were also highly conserved within *Thermoactinomyces* spp. and hence were suitable for PCR detection of this genus. Examination of the 16S rRNA gene sequence between the eight species demonstrated several hypervariable and conserved regions (Fig. 1). In particular, four hypervariable regions, at positions 400–450, 810–840, 1100–1150 and 1220–1250, where base diversity in the 16S rRNA gene was highest, were noted. To exploit this heterogeneity for identification purposes, a conserved primer site was sought upstream of position 400. The primer XB1, a 24-mer oligonucleotide, was identified at position 314–337, with reference to *T. vulgaris* AF138739, which was totally conserved within the other seven *Thermoactinomyces* spp. This primer was combined separately with the reverse primers PSR and P13P, as shown in Fig. 3, with the

successful generation of PCR amplicons of the expected sizes. Identification of the MWL isolates was subsequently achieved by direct sequencing of the PCR amplicons with the forward primer (XB1) and gave the results shown in Table 1. BLAST alignment was unable to separate *T. candidus* from *T. vulgaris*, or *T. thalpophilus* from *T. sacchari*. However, recent studies by Yoon *et al.* [4] based on DNA–DNA hybridisation and 16S rDNA homology have demonstrated that *T. candidus* is a synonym of *T. vulgaris* and *T. thalpophilus* is a synonym of *T. sacchari* (Fig. 4).

Overall, the use of the XB1/P13P primer combination is recommended, as this will generate a longer PCR amplicon than that generated by the XB1/PSR primer combination and, more importantly, contains a further two hypervariable regions at base positions 1100–1150 and 1220–1250. In conclusion, this study has identified 16S rDNA PCR primer pairs that may be useful in the identification of *Thermoactinomyces* spp. associated with allergic alveolitis or pneumonitis.

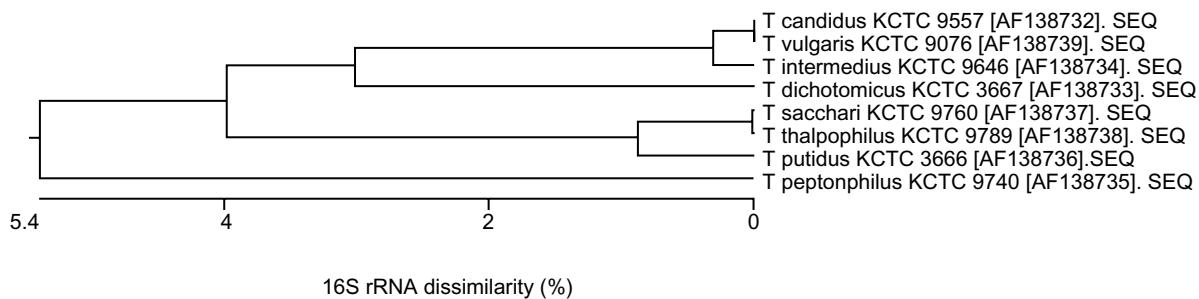


Fig. 4. Phylogenetic relationship of the eight described *Thermoactinomyces* spp. based on complete 16S rRNA gene sequences.

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