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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 $MgCl_2$, 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,

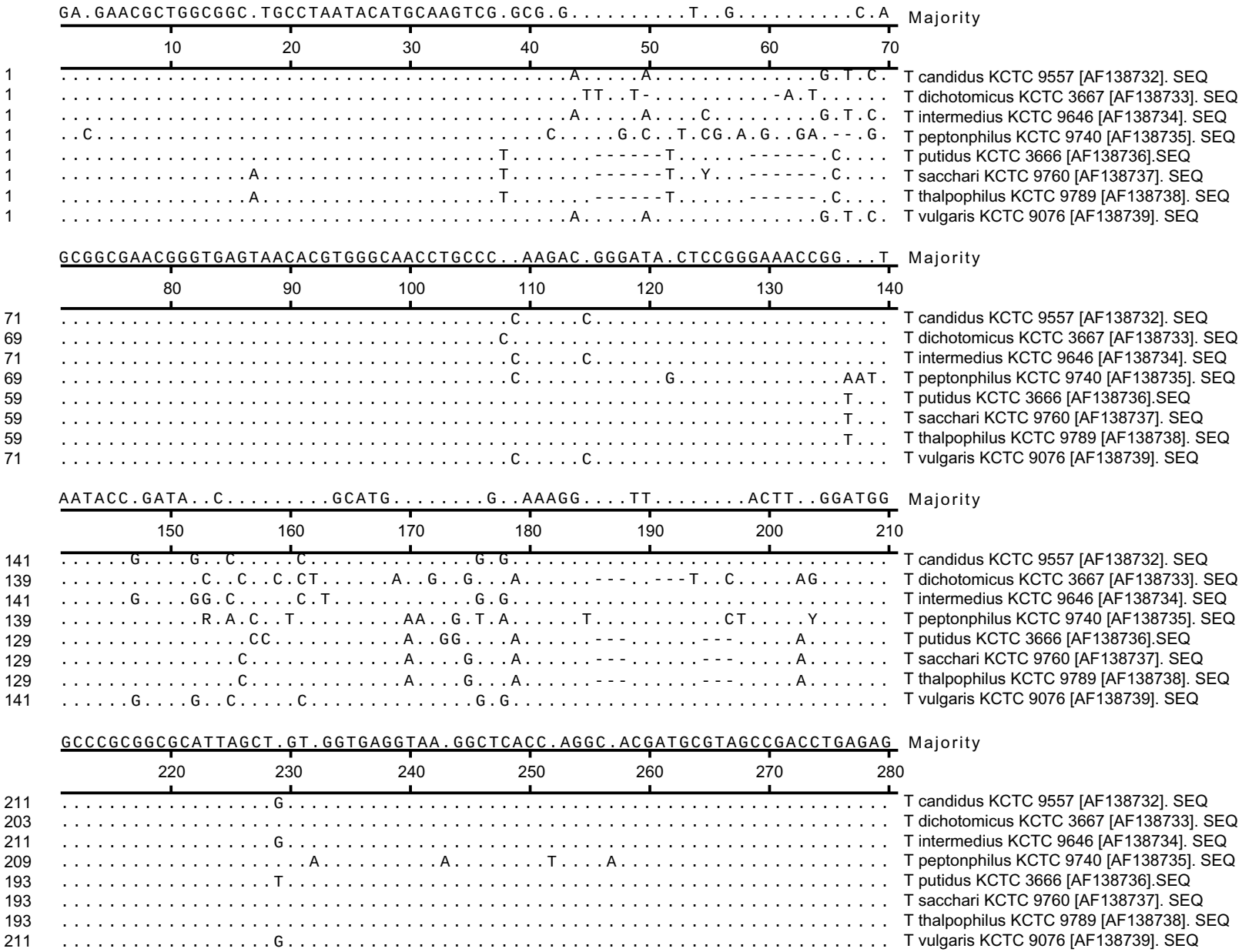

Fig. 1. Nucleotide alignments of part of the 16S rRNA gene of eight species of *Thermoactinomyces*. The consensus sequence is shown along the title bar and only differences are displayed.

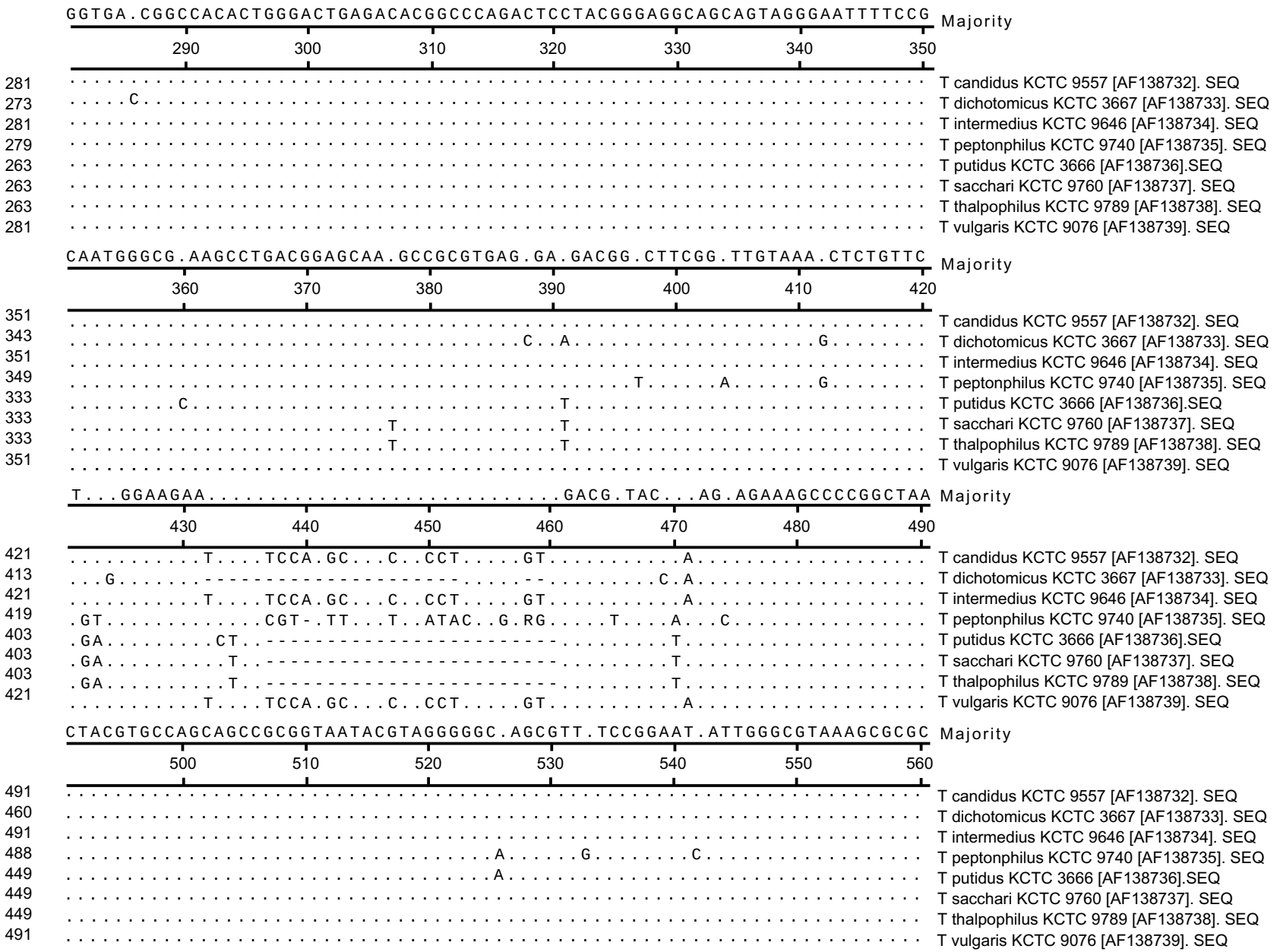

Fig. 1. (*continued*)

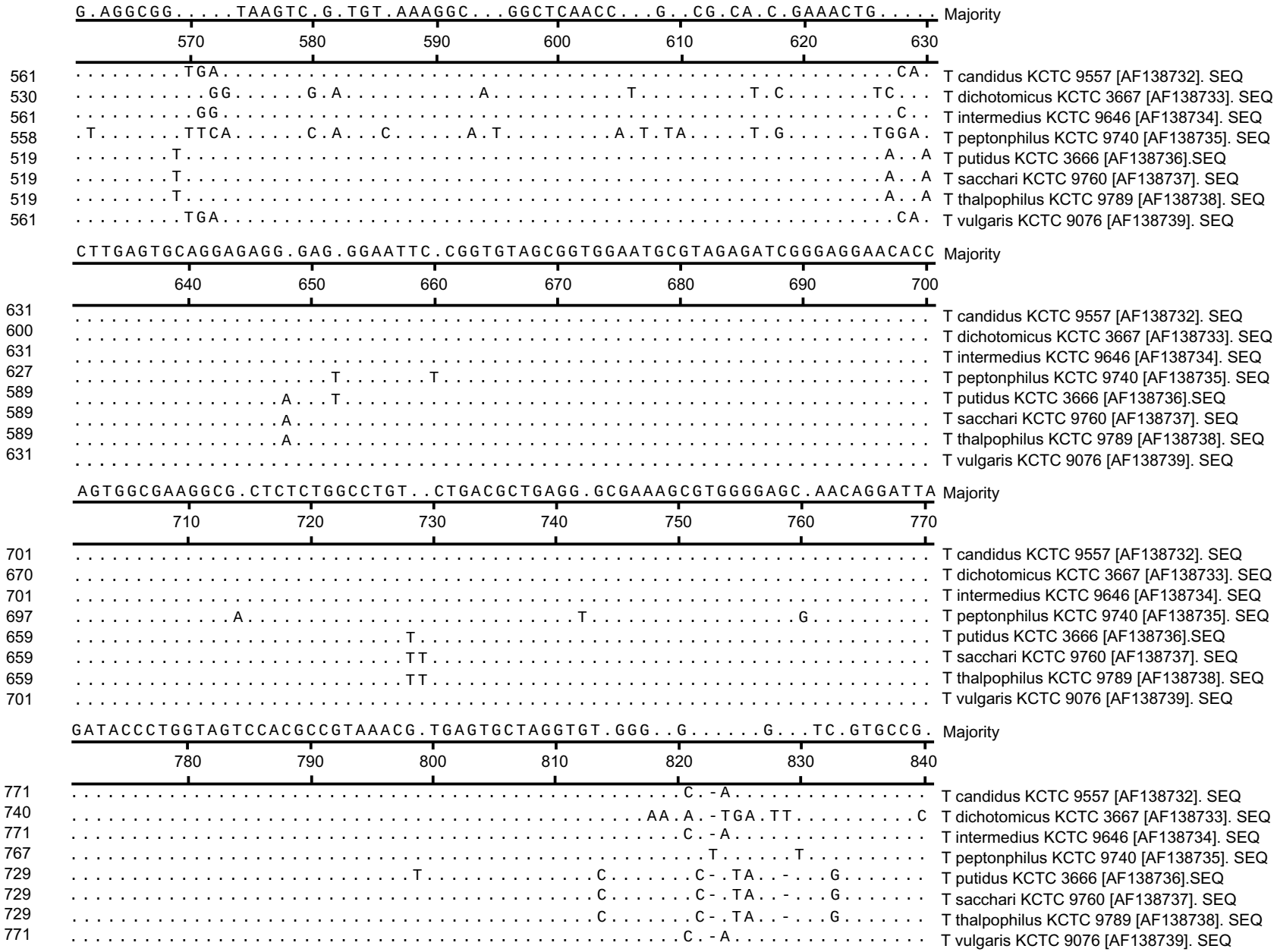

Fig. 1. (*continued*)

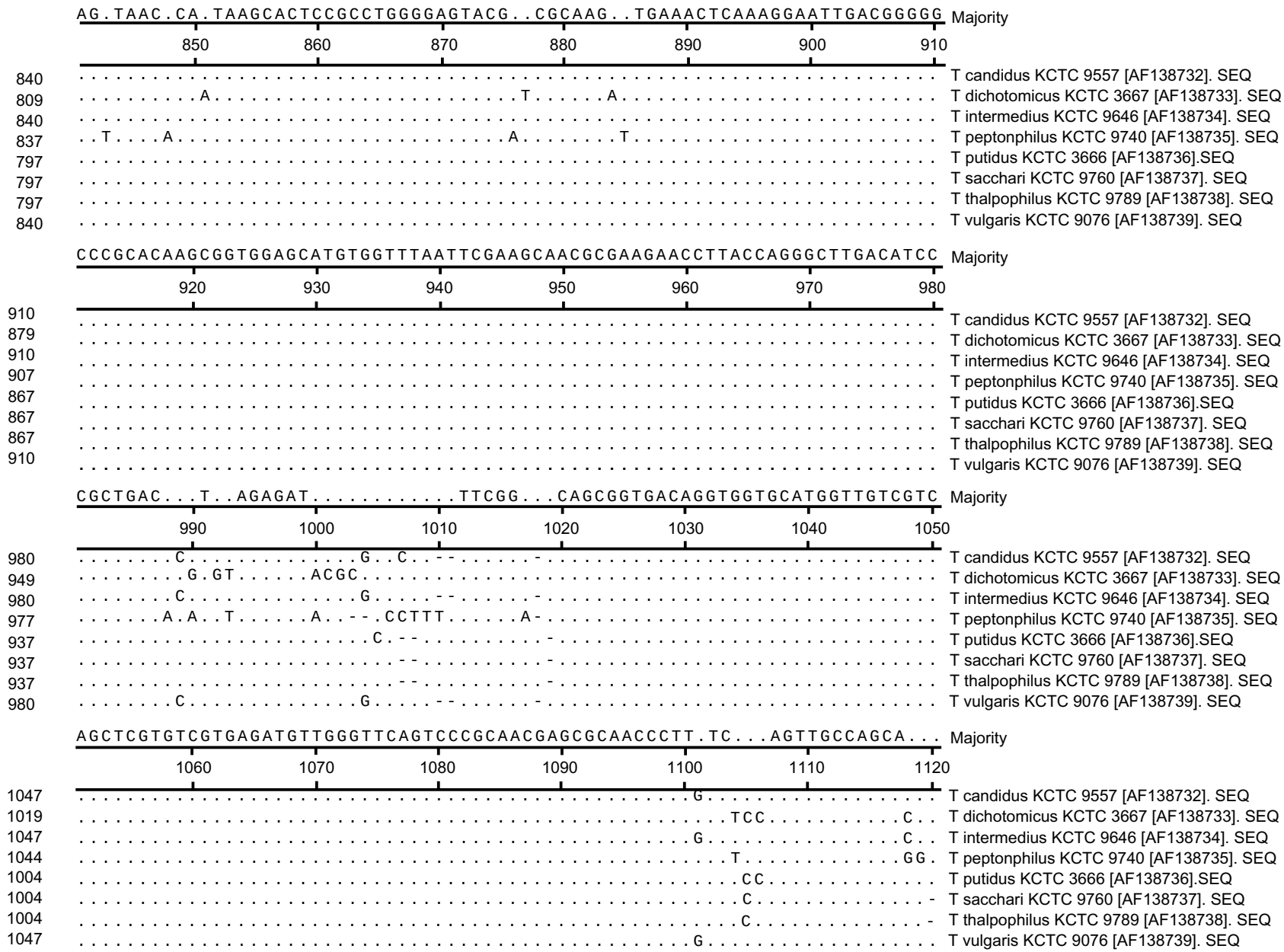

Fig. 1. (*continued*)

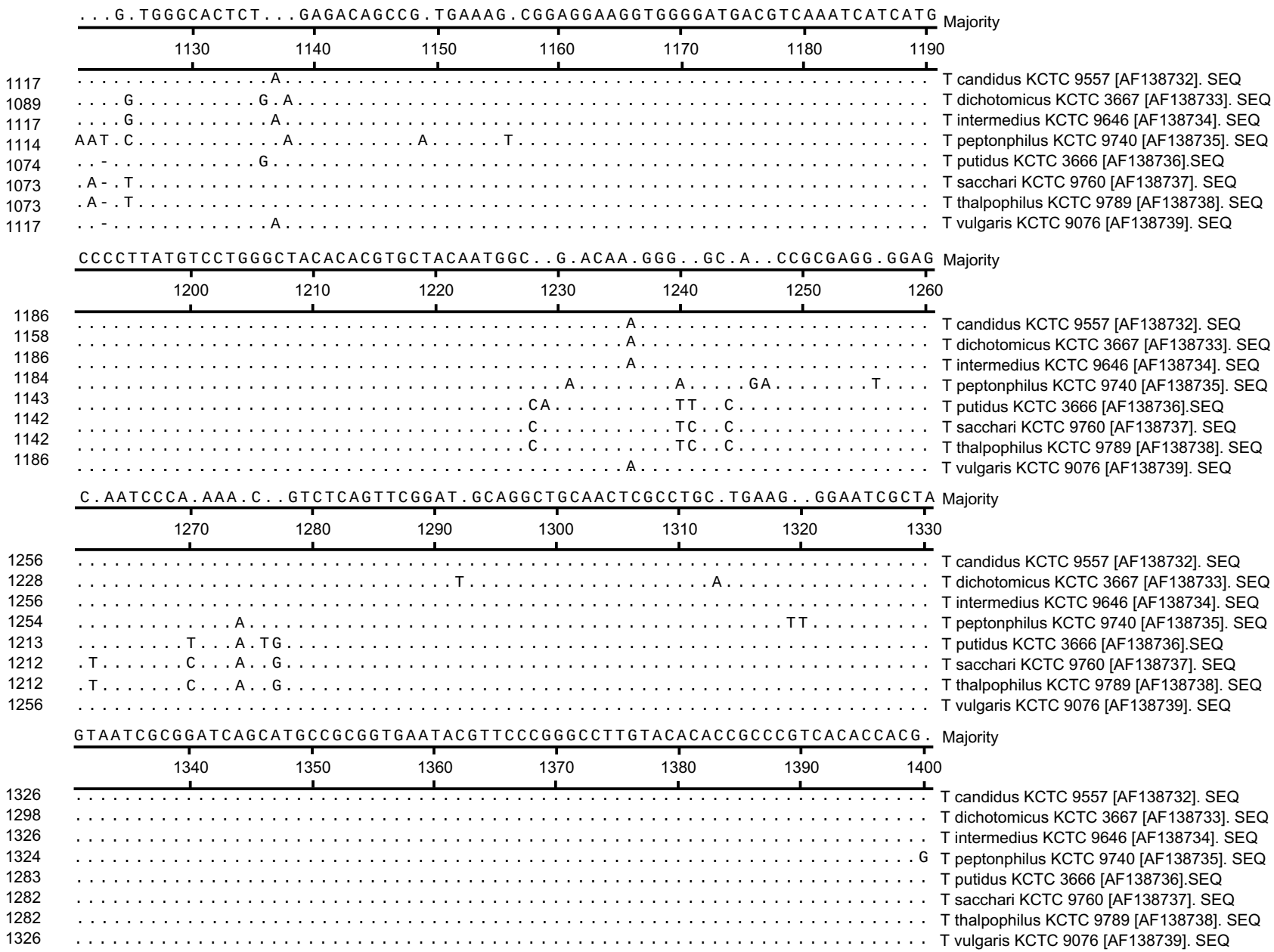

Fig. 1. (*continued*)

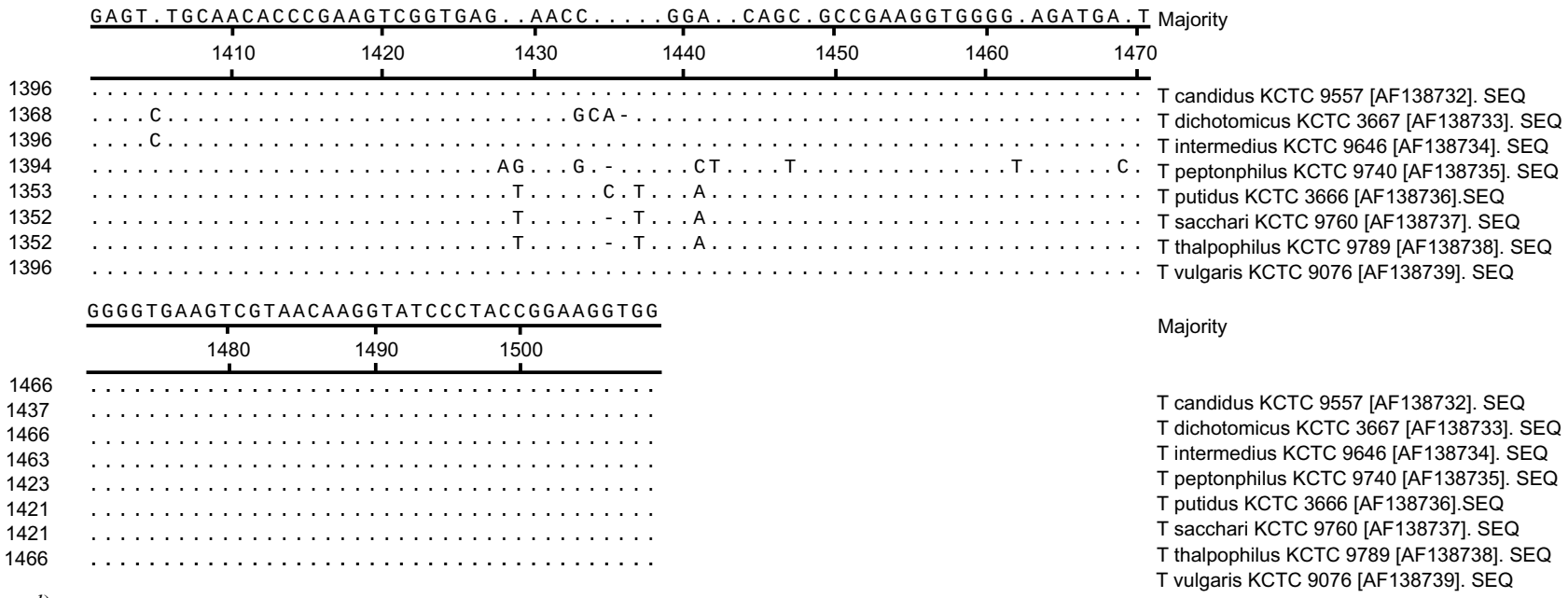

Fig. 1. (*continued*)

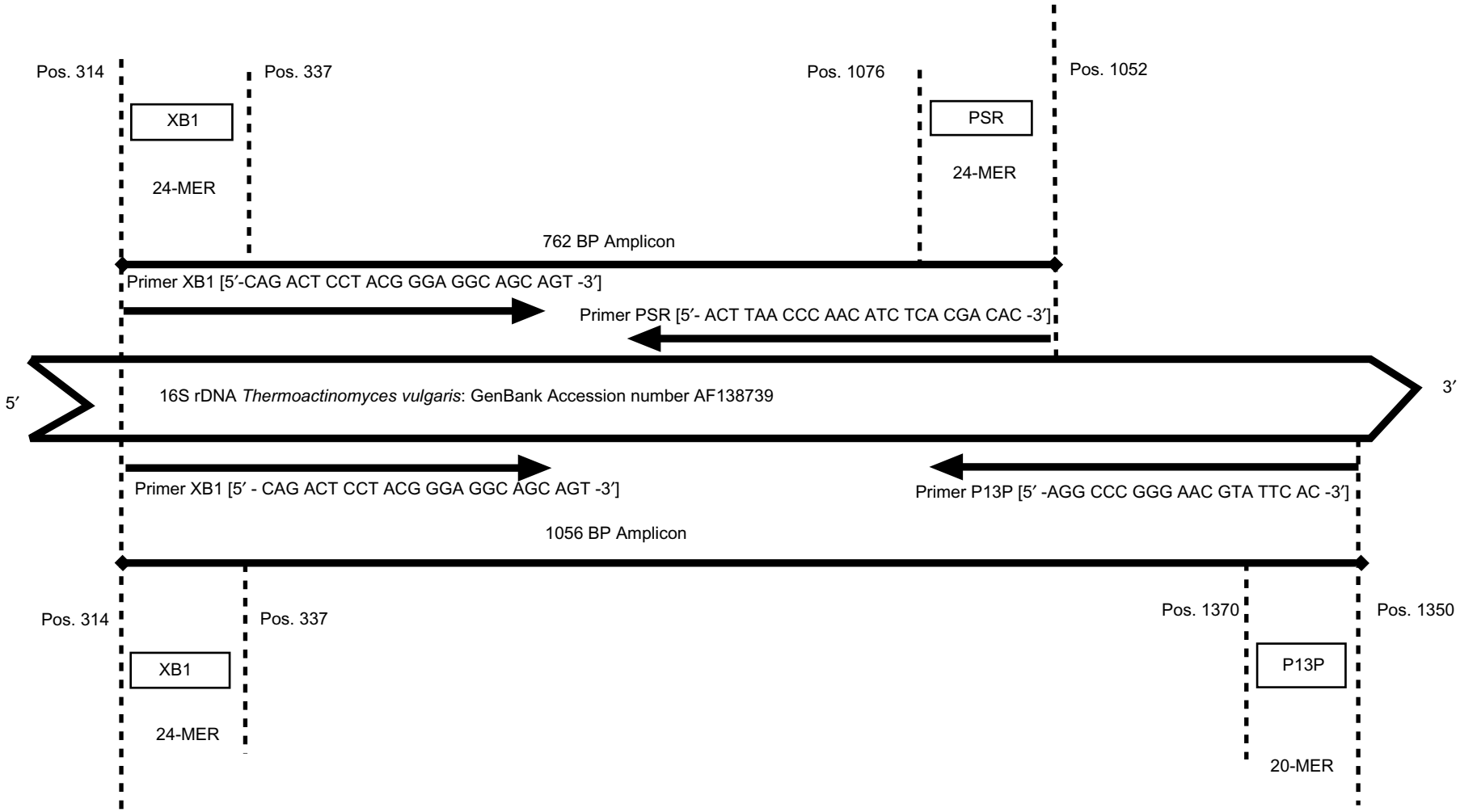

Fig. 2. Primer location, amplicon size and nucleotide sequence of the XB1/PSR and XB1/P13P primer combinations used.

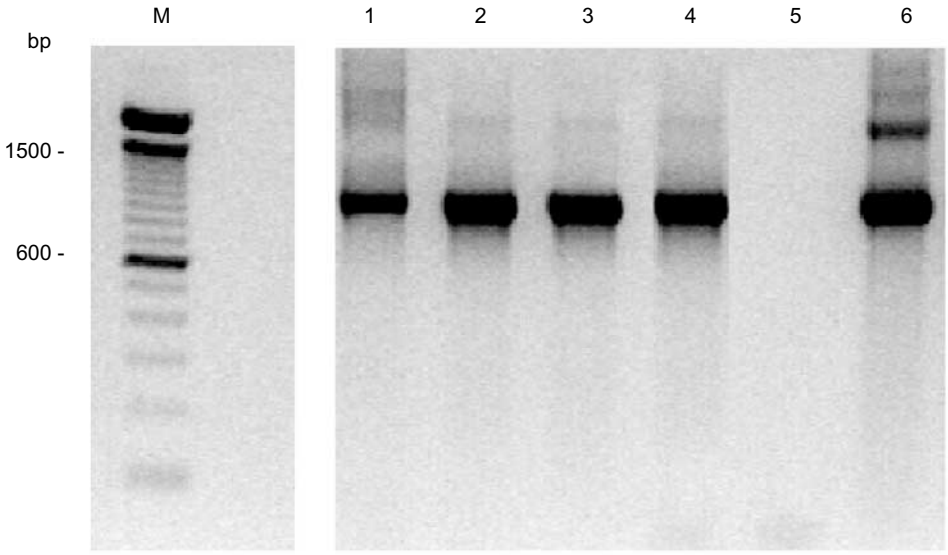

Fig. 3. PCR amplification of a partial 16S rDNA region (1056 bp) of four unidentified thermophilic organisms associated with mushroom worker's lung. Lanes **1–6**, XB1/P13P primer combination; **M**, 100-bp mol. wt marker (Life Technologies, Paisley, Scotland). Lane **1**, RVH210302; **2**, RVH210302B; **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	*T. vulgaris*	100	AY114167
		T. candidus	100	
RVH210302B	985	*T. sacchari*	100	AY114169
		T. thalpophilus	100	
RVH210302C	987	*T. sacchari*	100	AY114169
		T. thalpophilus	100	
RVH210302A	1001	*Saccharomonospora viridis*	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.

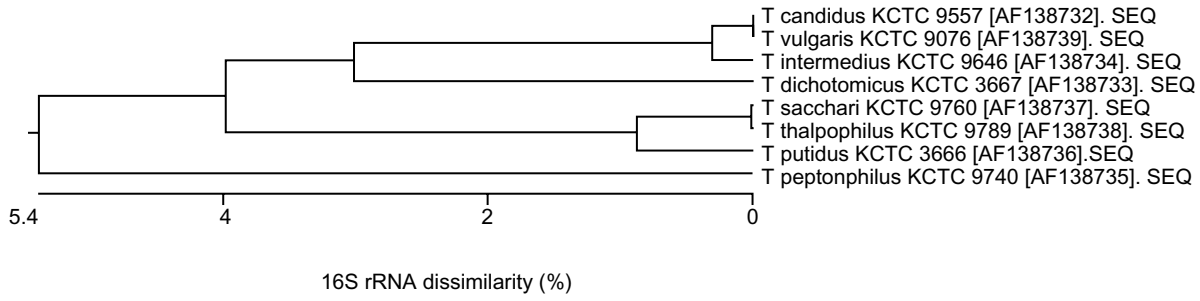

Fig. 4. Phylogenetic relationship of the eight described *Thermoactinomyces* spp. based on complete 16S rRNA gene sequences.

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