Emended descriptions of *Tilletiopsis washingtonensis*, *Tilletiopsis cremea* and *Tilletiopsis lilacina*

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For species discrimination, DNA relatedness, nuclear DNA (nDNA) base compositions and internal transcribed spacer (ITS) regions were studied in strains of the ‘washingtonensis group’ consisting of the type strains of *Tilletiopsis washingtonensis*, *Tilletiopsis cremea* and *Tilletiopsis lilacina* as well as nine strains previously identified as any one of the three species. *T. washingtonensis* is the neotype of the genus *Tilletiopsis* while *T. cremea* and *T. lilacina* are currently recognized as conspecific with *T. washingtonensis*. The G+C content of the DNA of the strains examined in this study was 66–68 mol%. The type strains of *T. washingtonensis*, *T. cremea* and *T. lilacina* were clearly discriminated on the basis of DNA complementarity. These genospecies should be treated as species. Moreover, an unrooted dendrogram constructed from ITS nucleotide sequences showed that the ‘washingtonensis group’ was divided into three clusters, corresponding to the three species *T. washingtonensis*, *T. cremea* and *T. lilacina*, respectively. The species descriptions of *T. washingtonensis*, *T. cremea* and *T. lilacina* are emended accordingly.

**Keywords:** *Tilletiopsis washingtonensis*, *Tilletiopsis cremea*, *Tilletiopsis lilacina*, *Tilletiopsis*, ballistoconidia-forming fungi

INTRODUCTION

The genus *Tilletiopsis* was suggested by Derx in 1930 for a ballistoconidia-forming fungus without budding cells and a Latin diagnosis was provided for *Tilletiopsis* ‘sp. 4’ as the type for this genus (Derx, 1948). However, this strain is no longer available. In 1950, Nyland described two species, *Tilletiopsis minor* and *Tilletiopsis washingtonensis*, and chose the latter species as the neotype for the genus *Tilletiopsis*. Tubaki (1952) described two new species, *Tilletiopsis cremea* and *Tilletiopsis lilacina*, as well as a variety of *T. minor* (*T. minor* var. *flava*), according to the consistency of colonies and the sizes of ballistoconidia. Gokhale (1972) made a comparative study of the assimilation of certain carbon and nitrogen compounds and of some other physiological properties, described three new species (*Tilletiopsis albescens*, *Tilletiopsis fulvescens* and *Tilletiopsis pallescens*) and proposed a key for the identification of the species of the genus based on their physiological and morphological characters. Yamazaki et al. (1985) studied the nutritional physiology and electrophoretic patterns of 10 enzymes of 17 strains in the genus *Tilletiopsis*. They concluded that *T. albescens*, *T. cremea*, *T. fulvescens*, *T. lilacina*, *T. minor* var. *flava*, *T. minor* var. *minor*, *T. pallescens* and *T. washingtonensis* were distinct species, but did not formally propose a species name for *T. minor* var. *flava*. Recently, Boekhout (1991) proposed that *T. minor* var. *flava* should be a separate species, i.e. *T. flava* (Tubaki) Boekhout comb. nov., on the basis of nutritional physiology, electrophoretic karyotype and nuclear base composition (Boekhout et al., 1992a, b), in addition to the electrophoretic enzyme patterns described earlier (Yamazaki et al., 1985). Moreover, Boekhout (1991) considered that *T. washingtonensis*, *T. cremea* and *T. lilacina* were conspecific according to the morphological, physiological and biochemical similarities, in spite of the low similarities of their electrophoretic enzyme patterns (Yamazaki et al.,

**Abbreviation:** ITS, internal transcribed spacer.

The DDBJ accession numbers for the internal transcribed spacer sequences are given in Table 1.
1985) and the considerable heterogeneity of their karyotype patterns (Boekhout et al., 1992a).

To define the taxonomic status of \( T \). \( \text{washingtonensis} \), \( T \). \( \text{cremea} \) and \( T \). \( \text{lilacina} \), we investigated nuclear DNA base composition, DNA relatedness and internal transcribed spacer (ITS) regions among the strains in the 'washingtonensis group'. This included the three type strains and nine strains previously identified as any one of the three species.

**METHODS**

**Strains used in this study.** Twelve strains of the 'washingtonensis group' used in this study were obtained from JCM (Japan Collection of Microorganisms, Saitama, Japan) (Table 1). All strains were grown at 17°C in yeast extract/malt extract broth or on malt extract agar (Difco).

**Assimilation test of carbon and nitrogen compounds.** Assimilation of carbon compounds was assessed with the method currently used in yeast taxonomy (van der Walt & Yarrow, 1984). Assimilation of nitrogen compounds was examined on solid media with starved inoculum, as described by Nakase & Suzuki (1986).

**Determination of DNA base composition and DNA relatedness.** DNA isolation, the determination of G+C content and DNA–DNA reassociation experiments were carried out as described previously (Hamamoto & Nakase, 1995).

**Nucleotide sequence analyses of ITS regions.** DNA extraction for the PCR was achieved as described in the following. One loop of cells was suspended in an equal volume of extraction buffer (200 mM Tris/HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, w/v) and one spoonful of aluminium oxide (50–70 mg). The suspended cells were disrupted by pestle (Kontes) on ice for 1–2 min. An equal volume of phenol saturated with TE buffer (10 mM Tris/HCl, pH 7.6, 1 mM EDTA, pH 8.0); chloroform (1:1, v/v) was added to the broken-cell suspension and mixed well. Following centrifugation, the aqueous upper layer was transferred to a new microtube. Nucleic acids were precipitated from the aqueous phase with 0.1 vol. 3 M sodium acetate (pH 5.2) and 2 vols 2-propanol held at —80°C for 10 min. Nucleic acids were recovered by centrifugation. The primers used for amplification and sequencing of the ITS region were those described by White et al. (1990). The PCR products were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Analyses of DNA sequence reactions were performed with an Applied Biosystems model 310 sequencer. Sequences were aligned using CLUSTAL W version 1.75 (Thompson et al., 1994) and were manually adjusted. Evolutionary distances were calculated using the PHYLIP version 3.57c program DNADIST (Felsenstein, 1995) with Kimura's two-parameter model; trees were constructed in NEIGHBOR by the neighbour-joining method (Saitou & Nei, 1987). The confidence values of branches were determined by performing a bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

The sequences determined were deposited in the DDBJ database under the accession numbers shown in Table 1.

**RESULTS**

**Physiological properties.**

We examined 12 strains of the 'washingtonensis group' for the assimilation of 36 carbon compounds and five nitrogen compounds. The assimilation pat-
**Table 2. DNA base composition and relatedness among *Tilletiopsis* strains**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>G + C content (mol%)</th>
<th>JCM 8362&lt;sup&gt;T&lt;/sup&gt;</th>
<th>JCM 7482</th>
<th>JCM 5184&lt;sup&gt;T&lt;/sup&gt;</th>
<th>JCM 5188&lt;sup&gt;T&lt;/sup&gt;</th>
<th>JCM 5189</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. washingtonensis</em></td>
<td>JCM 8362&lt;sup&gt;T&lt;/sup&gt;</td>
<td>66</td>
<td>100</td>
<td>87</td>
<td>27</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td><em>Tilletiopsis</em> sp.</td>
<td>JCM 7482</td>
<td>67</td>
<td>84</td>
<td>100</td>
<td>35</td>
<td>31</td>
<td>40</td>
</tr>
<tr>
<td><em>T. cremea</em></td>
<td>JCM 5184&lt;sup&gt;T&lt;/sup&gt;</td>
<td>67</td>
<td>28</td>
<td>26</td>
<td>100</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td><em>T. lilacina</em></td>
<td>JCM 5188&lt;sup&gt;T&lt;/sup&gt;</td>
<td>67</td>
<td>39</td>
<td>19</td>
<td>25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Tilletiopsis</em> sp.</td>
<td>JCM 5189</td>
<td>67</td>
<td>22</td>
<td>24</td>
<td>28</td>
<td>77</td>
<td>100</td>
</tr>
</tbody>
</table>

**DNA base composition**

The G + C contents of the DNA of the type strains of *T. washingtonensis*, *T. cremea* and *T. lilacina* were 66, 67 and 67 mol%, respectively. The values for the other nine strains of the 'washingtonensis group' ranged from 67 to 68 mol%: the value for strains JCM 2899, JCM 5190, JCM 5289, JCM 5736, JCM 7494, JCM 8501 and JCM 8502 was 68 mol% while that for strains JCM 5189 and JCM 7482 was 67 mol%.

**DNA–DNA hybridization among strains of *T. washingtonensis*, *T. cremea* and *T. lilacina* and two strains of the 'washingtonensis group'**

Table 2 shows the levels of DNA complementarity among the type strains of *T. washingtonensis*, *T. cremea* and *T. lilacina* and strains JCM 5189 and JCM 7482. The degrees of interspecific relative binding among the type strains of *T. washingtonensis*, *T. cremea* and *T. lilacina* ranged from 16 to 39%. Strain JCM 5189 showed high degrees of relative binding (77–100%) with the type strain of *T. lilacina* JCM 5188<sup>T</sup>, but low degrees (22–34%) with the type strains of *T. washingtonensis* and *T. cremea*. Strain JCM 7482 showed high degrees of relative binding (84–87%) with the type strain of *T. washingtonensis*, but low degrees (19–35%) with the type strains of *T. cremea* and *T. lilacina*. It was intended that strain JCM 10447, originally identified as *T. cremea* by Hoog and Boekhout [from the database of the Centraalbureau voor Schimmelcultures (CBS) Baarn/Delft, The Netherlands], would be tested. However, strain JCM 10447 was not included in the DNA–DNA reassociation experiments in this study because it had a G + C value of 58 mol%, which clearly showed it to be different from the type strains of the three species (*T. washingtonensis*, *T. cremea* and *T. lilacina*, which have G + C values of 66–67 mol%).

**Phylogenetic analysis**

The nucleotide sequences of ITS regions (ITS1 and ITS2) of the 12 strains shown in Table 1 were determined and analysed. The trees in Fig. 1 were constructed from the data sets aligned by CLUSTAL W version 1.75 on 156 ITS1 (Fig. 1a) and 259 ITS2 (Fig. 1b) sites. On the basis of the analysis, the strains were clearly divided into three clusters. The type strain of *T. washingtonensis* (JCM 8362<sup>T</sup>), strains JCM 2899, JCM
7482 and JCM 7494 formed a tight phylogenetic cluster in the ITS1 tree. The type strain of \textit{T. lilacina} (JCM 5188) and strains JCM 5189, JCM 5190, JCM 5289, JCM 5736, JCM 8501 and JCM 8502 formed a tight phylogenetic cluster in both the ITS1 and the ITS2 tree. The type strain of \textit{T. cremae} (JCM 5184) was placed independently in both the ITS1 and the ITS2 tree.

**DISCUSSION**

**Relationships among \textit{T. washingtonensis}, \textit{T. cremae} and \textit{T. lilacina}**

The differences in the G+C contents among the type strains of \textit{T. washingtonensis}, \textit{T. cremae} and \textit{T. lilacina} and the other nine strains of the ‘washingtonensis group’ examined in this study (within 2 mol%) were not significant enough to discriminate yeast species. This result is consistent with the result reported by Boekhout et al. (1992a).

As shown in Table 2, the low levels of DNA complementarity among the type strains of \textit{T. washingtonensis}, \textit{T. cremae} and \textit{T. lilacina} indicate that the three species should be treated as distinct species, as suggested by Yamazaki et al. (1985). In this study, strain JCM 5189, which has been identified as \textit{T. lilacina} mainly on the basis of its electrophoretic enzyme patterns (Yamazaki et al., 1985), was confirmed as \textit{T. lilacina} from the high levels of DNA reassociation values with the type strain of \textit{T. lilacina}. In addition, JCM 7482, which had been identified as \textit{T. lilacina} on the basis of its physiological similarities to the type strain of \textit{T. lilacina} (T. Nakase, unpublished results, 1986), was identified as \textit{T. washingtonensis} from the high levels of DNA reassociation with the type strain of \textit{T. washingtonensis}, as discussed in the next section (Physiological properties).

The ITS region has been found to be useful in resolving relationships among close taxa, because of its high substitution rate compared with that of 18S and 26S rDNA (Berbee et al., 1995; Waalwijk et al., 1996; James et al., 1996; Oda et al., 1997; Nagahama et al., 1999). To clarify the relationships within strains of the ‘washingtonensis group’ at the species level, the sequences of ITS1 and ITS2 among the 12 strains, including the type strains of \textit{T. washingtonensis}, \textit{T. cremae} and \textit{T. lilacina}, were determined and analysed. The trees derived from ITS1 and ITS2 placed the five strains (clearly separated into three distinct species, i.e. \textit{T. washingtonensis} (strains JCM 7482 and JCM 8362), \textit{T. cremae} (strain JCM 5184) and \textit{T. lilacina} (strains JCM 5188 and JCM 5189) based on the DNA complementarity in this study) into each species. From the result, it is clear that the three clusters in the ITS trees correspond with the three species, that strains JCM 2899 and JCM 7494 should be treated as \textit{T. washingtonensis} and that strains JCM 5190, JCM 5289, JCM 5736, JCM 8501 and JCM 8502 should be treated as \textit{T. lilacina}. Some differences in the topologies between the ITS1 and ITS2 trees may reflect the higher evolutionary rates of ITS1 relative to those of ITS2 (James et al., 1996).

**Physiological properties**

We compared the results of carbon- and nitrogen-assimilation tests from this study with those of Boekhout (1991), T. Nakase (unpublished results, 1986) and Yamazaki et al. (1985). The results for some carbon and nitrogen compounds differ between studies. For example, strain JCM 7482 (originally from strain NB 209 by T. Nakase) assimilated galactose, cellobiose, D-arabinose, ethanol and L-lysine in this study and that of Boekhout (1991) but did not do so in the study by T. Nakase (unpublished results, 1986). Strain JCM 8502 (originally from strain YK 817 = KG-3 by Yamazaki et al., 1985) assimilated melibiose, DL-lactic acid and L-lysine in this study and that of Boekhout (1991) but did not do so in the study by Yamazaki et al. (1985). A number of other differences were found. Differences in assimilation patterns for some carbon and nitrogen compounds in this study, by Boekhout (1991) and Yamazaki et al. (1985) were also observed for strains JCM 5184 (originally from NI 3113 by Tubaki, 1952), JCM 5188 (originally from NI 3114 by Tubaki, 1952) and JCM 5189 (originally from No. 84 by M. Yoshizawa) (data not shown). These differences might have been caused by difficulties in judging the assimilation reactions of some compounds. This indicates that the assimilation pattern of carbon and nitrogen compounds is not a sufficiently good criterion for species delimitation in the genus \textit{Tilletiopsis} and that species identification without molecular biological data are highly unreliable.

In \textit{The Yeasts, a Taxonomic Study}, Boekhout concluded that \textit{T. washingtonensis}, \textit{T. cremae} and \textit{T. lilacina} were considered conspecific on the basis of morphological, physiological and biochemical similarities (Boekhout, 1998); however, \textit{T. cremae} and \textit{T. lilacina} were revealed as distinct species in this study. Consequently, we propose the emendation of \textit{Tilletiopsis washingtonensis} Nyland, \textit{Tilletiopsis cremae} Tubaki and \textit{Tilletiopsis lilacina} Tubaki, as outlined below. A total of eight species, \textit{T. albescens} Gokhale, \textit{T. cremae} (Tubaki) Hamamoto & Nakase, \textit{T. flavus} (Tubaki) Boekhout, \textit{T. fulvescens} Gokhale, \textit{T. lilacina} (Tubaki) Hamamoto & Nakase, \textit{T. minor} Nyland, \textit{T. pallescens} Gokhale and \textit{T. washingtonensis} (Nyland) Hamamoto & Nakase, should be accepted in the genus \textit{Tilletiopsis}.

**Emended description of \textit{Tilletiopsis washingtonensis} Nyland**

Characteristics additional to the description of Nyland 1950 of this species are given below.

Assimilates D-glucose, galactose (variable), L-sorbose (variable), sucrose, maltose, cellobiose (variable), trehalose, melibiose (variable), raffinose, melezitose, soluble starch, D-xylene, L-arabinose, D-arabinose (vari-
Emended descriptions of three *Tilletiopsis* species

**Emended description of *Tilletiopsis cremea* Tubaki**

Characteristics additional to the description of Tubaki 1952 of this species are given below.

Assimilates D-glucose, galactose, sucrose, maltose, cellobiose (variable), trehalose, melibiose (variable), raffinose, melezitose, soluble starch, D-xyllose, L-arabinose, D-arabinose, D-ribose, ethanol (variable), glycerol, erythritol, ribitol (variable), D-mannitol, D-glucitol, methyl D-glucoside (variable), glucono-δ-lactone (variable), δ-glucoside (variable), gluconolactone, salicin, 2-ketogluconic acid, 5-ketogluconic acid, inositol and D-glucuronic acid. Assimilates nitrate, nitrite and t-lysine (variable). Does not assimilate ethylamine or cadaverine. Growth does not occur in vitamin-free medium. Thiamin is required for growth. Growth does not occur on 50% (w/w) glucose/yeast extract agar.

No starch-like substance is produced. Xylose is absent from whole-cell hydrolysates. Urease and Diazonium blue B reactions are positive. The major ubiquinone is Q-10. The G+C content of the DNA is 67–68 mol%, as determined by HPLC. The type strain is CBS 635.92T = CBS 603.83T = IFO 6832T.

**Emended description of *Tilletiopsis ilacina* Tubaki**

Characteristics additional to the description of Tubaki 1952 of this species are given below.

Assimilates D-glucose, galactose, L-sorbose (variable), sucrose, maltose, cellobiose (variable), trehalose, melibiose, raffinose, melezitose, soluble starch, D-xyllose, L-arabinose, D-arabinose (variable), D-ribose, ethanol (variable), glycerol, erythritol, ribitol (variable), D-mannitol, D-glucitol, methyl D-glucoside (variable), salicin (variable), glucono-δ-lactone (variable), δ-lactic acid (variable), succinic acid and citric acid. Does not assimilate lactose, inulin, L-rhamnose, galactitol, 2-ketogluconic acid, 5-ketogluconic acid, inositol or D-glucuronic acid. Assimilates nitrate, nitrite and l-lysine (variable). Does not assimilate ethylamine or cadaverine. Growth does not occur in vitamin-free medium. Thiamin is required for growth. Growth does not occur on 50% (w/w) glucose/yeast extract agar.

No starch-like substance is produced. Xylose is absent from whole-cell hydrolysates. Urease and Diazonium blue B reactions are positive. The major ubiquinone is Q-10. The G+C content of the DNA is 67–68 mol%, as determined by HPLC. The type strain is CBS 635.92T = CBS 603.83T = IFO 6832T = JCM 5184T.

**REFERENCES**


