THE PHYLOGENETIC POSITION OF *TILLETIA NIGRIFACIENS*

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Abstract

In order to determine the phylogenetic position of the unusual smut fungus *Tilletia nigrifaciens*, the ribosomal DNA Internal Transcribed Spacer region from two specimens (one holotypic) was obtained and compared with other species of *Tilletia*. *Tilletia nigrifaciens* was found to be affiliated with those species of *Tilletia* sometimes referred to the genus *Neovossia*. Methods for obtaining ITS sequences from small amounts of herbarium material are provided, along with PCR primers with increased specificity for the ITS region of the Ustilaginomycetes.

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Introduction

Tilletia nigrifaciens Langdon & Boughton is known only from Australia on the leaves of *Phragmites australis* (Cav.) Trin. ex Steud. It is not very common, restricted to collections from Logan River and Broadbeach in south-eastern Queensland and Kiama in New South Wales.

Although Langdon & Boughton (1978) were not able to germinate spores of *T. nigrifaciens*, they placed this smut in *Tilletia* based on the mode of sporulation, the presence of sterile cells in the sorus and spores encased with gelatinous sheaths. However, *T. nigrifaciens* is unique for a species of *Tilletia* as it produces external sori on the leaves. External sori are typical for the smut genera *Orphanomyces*, *Clintamra* and also some species of *Ustilago* (Vánky 1991). Vánky (1991) examined the type of *T. nigrifaciens* and noted that its morphology fitted well with the characters of a *Tilletia*. Despite this he concluded that the fungus was an ascomycete based on an unpublished ultrastructural study that showed the septal pore was simple and Woronin bodies were present.

In October 2000, smut infected leaves of *Phragmites australis* growing in a drain adjacent to a sugarcane field near the Logan River were submitted to the DPI Plant Pathology Herbarium (BRIP) by a concerned farmer. The smut was identified as *T. nigrifaciens* and a specimen was deposited as BRIP 28242. Attempts to germinate the spores were unsuccessful. This material provided an opportunity to obtain ribosomal DNA Internal Transcribed Spacer (ITS) region sequences for *T. nigrifaciens* in order to confirm whether it is a smut fungus as well as to determine its phylogenetic position amongst other *Tilletia* species. ITS sequences have been used previously in the taxonomy and classification of smut fungi (Boyd & Carris 1997, Boyd *et al.* 1998).

Materials and Methods

Two specimens were used, BRIP 6484 (BRIU 2533), which is the holotype, collected 18 May 1974 and BRIP 28242 collected 1 October 2000. DNA was extracted by grinding a small amount of spores (1 mm^3) in 50 μL of 5% Chelex-100 (Biorad). The material was spun down briefly in a microcentrifuge.

The holotype of *T. nigrifaciens* is in poor condition and contaminated with other microfungi. In order to specifically amplify the rDNA ITS region from the smut fungus a reverse ITS primer for the Ustilaginomycetes was designed. Ribosomal DNA large subunit (LSU) sequences (obtained from GenBank) from all orders of Ustilaginomycetes were compared with rDNA LSU sequences from plants, ascomycetes, Hymenomycetes and

Urediniomycetes. A primer was chosen for increased specificity to the Ustilaginomycetes, with the exception of the Entorrhizomycetidae, approximately 400 bases downstream from the start of the large subunit region. This reverse ITS primer for the Ustilaginomycetes was designated ITSUR (TGTTCGCTATCGGTCTCTCC).

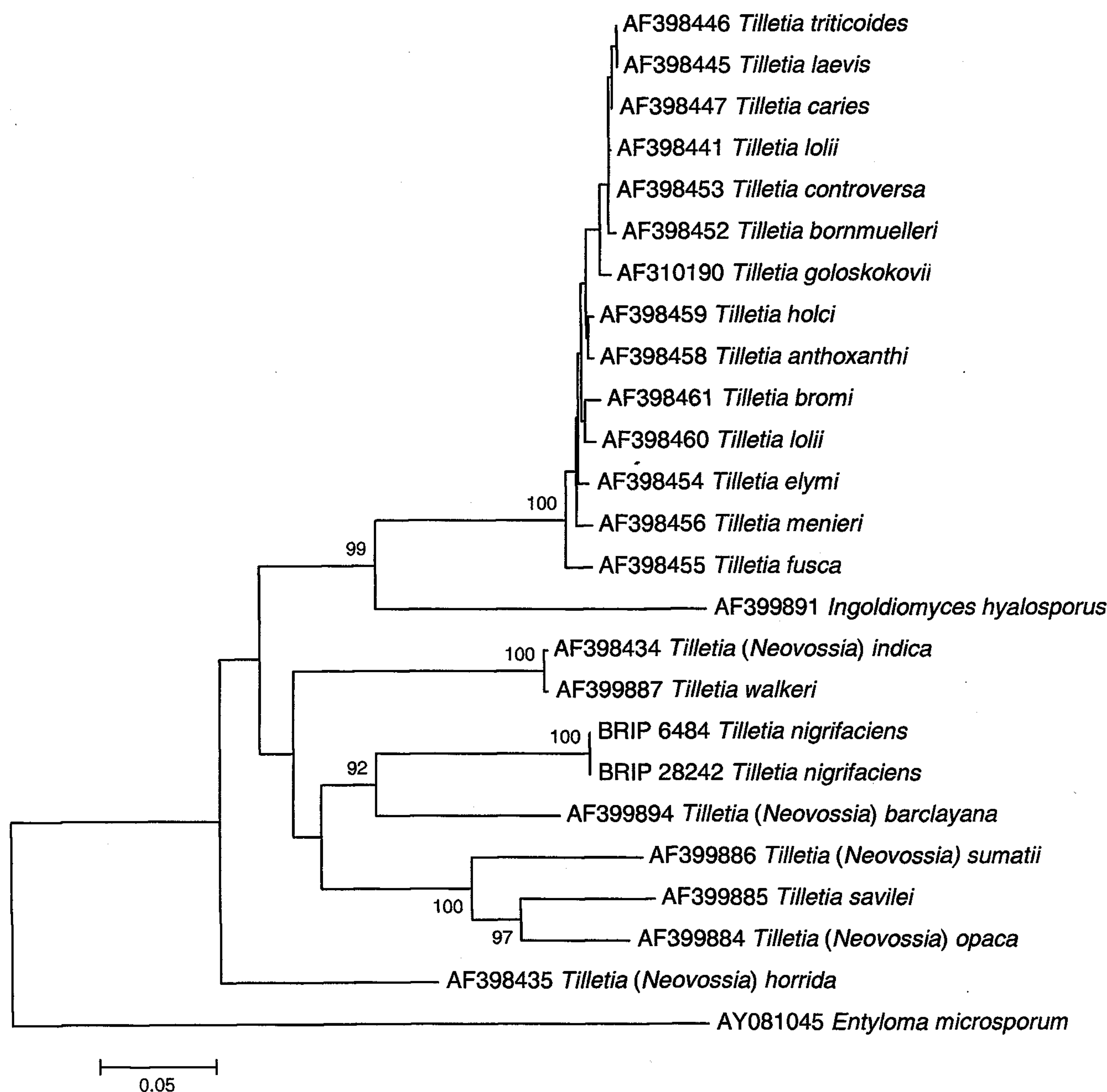

Figure 1. Phylogenetic position of *T. nigrifaciens* based on rDNA ITS sequences. GenBank numbers are indicated and only bootstrap values above 90% are shown. Those species previously placed in *Neovossia* are indicated.

The initial PCR was performed in 25 µL containing 1 µL DNA extract, 200 µM of each dNTP (Pharmacia Biotech), 1.5 mM $MgCl_2$, 2.5 µL 10× buffer, 4 ng each of primers ITSF1 (Gardes & Bruns 1993) and ITSUR, and 0.5 units of Hotstar Taq (Qiagen). Reaction cycle was 15 min at 95°C, 35 cycles of: 30 sec at 94°C, 30 sec at 50°C, 1 min at 72°C. A nested PCR was performed in 25 µL as outlined above, but using primers ITS5 (White *et*

al. 1990) and ITSUR using 1 μL of the first round PCR product as template. PCR products were detected by running 4 μL on a 1.4% agarose gel in TBE buffer.

Nested PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced directly using primers ITS5 (White *et al.* 1990) and ITS4, with an ABI PRISM® BIGDYE™ Terminator Cycle Sequencing Kit (Perkin-Elmer) according to the manufacturers instructions.

The forward and reverse sequences for each specimen were aligned to obtain a final sequence for each. These sequences were aligned with a wide range of ITS sequences for other *Tilletia* species (obtained from GenBank) using ClustalX (Thompson *et al.* 1997). The ITS sequence for *Entyloma microsporum* was included as an outgroup. A neighbour-joining tree was created using the Kimura-2-paramater method with a complete deletion of gaps using MEGA (Kumar *et al.* 2001). 1000 bootstrap replicates were performed.

Results

Neither specimen yielded a visible PCR product from the first round PCR; however, both produced a single band approximately 1 Kb in length from the nested PCR. The ITS sequences for both specimens were found to be identical and the sequence for the holotype has been lodged in GenBank under accession AY309481. The two sequences were readily aligned with those of the other *Tilletia* species. The phylogenetic tree revealed that *T. nigrifaciens* does belong in *Tilletia* and that it is most closely allied to several species of *Tilletia* previously place in *Neovossia* (Figure 1).

Discussion

Successful amplification of the ITS region using primer ITSUR indicated that *T. nigrifaciens* belongs in the Ustilaginomycetes. Phylogenetic analysis clearly shows the two specimens examined belong to *Tilletia* in the Tilletiaceae. There are two interpretations for the conflicting ultrastructural evidence presented by Vánky (1991). The first interpretation is that *T. nigrifaciens* is a good member of the Tilletiaceae that forms simple septa and Woronin bodies. Moore (1972) reported that *Tilletia horrida* Tak. exhibited an ascomycete-type septum. He suggested that this inexplicable micromorphology could indicate that the isolate examined was not actually a culture of *T. horrida* (Moore 1987), that *T. horrida*, so-called, was an ascomycete mimicking *T. barclayana* (a similar species according to Durán & Fischer (1961) on the basis of morphology and inoculation studies), or least likely, that it was a good *Tilletia* with ascomycetous septa. Our results show that *T. horrida* must be a primitive member of the Tilletiales as it clusters by itself at the base of Figure 1. Thus it is possible that it exhibits an atypical septal ultrastructure. There is a need for further research in this area to study the diversity of septal ultrastructure in this group that includes both *T. horrida* and *T. nigrifaciens*.

The second, and in our view most likely, interpretation is that more than one fungus was present in the host material examined by Vánky (1991) and, as such, the septa of a contaminating ascomycete were viewed. Bauer *et al.* (1997) examined *Tilletia barclayana* (found in Figure 1 to be a close relative of *T. nigrifaciens*) and found it to produce dolipore septa. As septal ultrastructure is used as a character for high level classification (Bauer *et al.* 1997), it seems unlikely that two such similar species of *Tilletia* would have different septal types.

It is interesting to note that the phylogenetic analysis showed that *T. nigrifaciens*, which has spores with reticulate surfaces, did not cluster with the other thirteen analysed species of *Tilletia* that have reticulate spore surfaces. *Tilletia nigrifaciens* clustered with seven other species that have spores with verrucose surfaces; many of which have at one time or another have been referred to *Neovossia*. Vánky (2002) currently restricts *Neovossia* to a single species, *N. moliniae* (Thüm.) Körn. Vánky (2002) further noted that the differences between *Neovossia* and *Tilletia* were not sharp, several characters transgressed and that a revision of these two genera was in progress.

The methods developed here to amplify and sequence the ITS region from small amounts of contaminated herbarium material of *T. nigrifaciens*, should be applicable to other groups of Ustilaginomycetes. We have successfully obtained ITS sequences from several herbarium specimens of *Sporisorium* species (data not shown) using primer ITSUR. Hopefully these methods will aid in future molecular taxonomic studies where the species are poorly known, or known only from the holotype.

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