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Phylogeny of Tetracladium based on 18S rDNA

LILIYA G. NIKOLCHEVA and FELIX BÄRLOCHER

Department of Biology, Mt. Allison University
Sackville, N. B., E4L 1G7, Canada

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Complete sequences of 18S rDNA of seven strains of *Tetracladium* were determined. The following species were included: *T. apiense, T. furcatum, T. maxilliforme, T. setigerum* (one strain each) and *T. marchalianum* (3 strains). Sequence homology among the 7 strains was ≥98%. The closest published match (NCBI database) to the *Tetracladium* sequences is one by *Bulgaria inquinans* (homology 95–96%). Phylogenetic analysis placed the *Tetracladium* complex in the vicinity of the Ascomycete orders Onygenales, Erysiphales and Leotiales.

Key words: *Tetracladium*, 18S rDNA, Leotiales, Erysiphales, Onygenales

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Byly stanoveny kompletní sekvence 18S rDNA sedmi kmenů rodu *Tetracladium*. Následující druhy *T. apiense, T. furcatum, T. maxilliforme, T. setigerum* (od každého druhu jeden kmen) a *T. marchalianum* (3 kmeny). Homologie sekvencí mezi sedmi kmeny byla ≥98%. Nejbližší obdoba (NCBI databáze) k sekvencím rodu *Tetracladium* je sekvence druhu *Bulgaria inquinans* (homologie 95–96%). Fylogenetická analýza umístila komplex druhů rodu *Tetracladium* do blízkosti řádů vřeckatých hub a to Onygenales, Erysiphales a Leotiales.

INTRODUCTION

Aquatic hyphomycetes are the primary agents of leaf litter decay in streams and rivers (Bärlocher 1992). They 'condition' the substrate for consumption by detritus-feeding invertebrates and thus form an important trophic link in the food web (Bärlocher 1985, Suberkropp 1992). Aquatic hyphomycetes (also known as freshwater hyphomycetes, amphibious hyphomycetes, Ingoldian fungi) are not monophyletic, but are grouped together on the basis of morphological and ecological similarities (Bärlocher 1992). Their taxonomy is based on anamorph-genera (asexual or mitosporic states), which include species with conidia of similar development and morphology. These spores are typically tetraradiate or sigmoidal (Ingold 1975, Webster and Descals 1981). Since these shapes facilitate attachment to the substrate and provide a stable base for rapid germination (Read et al. 1992), they are believed to have evolved repeatedly and independently in fungi living in a similar habitat. This suggests that spore morphology provides little information on phylogenetic relationships (Seifert 1993, Dix and Webster 1995, Alexopoulos

et al. 1996). Studies connecting anamorphs to teleomorphs have shown that the majority of aquatic hyphomycetes belong to the Ascomycetes and only a few to the Basidiomycetes. They also revealed that several of the anamorph-genera are heterogeneous, i.e. include taxa of diverse relationship (Webster and Descals 1981). These findings support the assumption of convergent evolution and suggest that the current classification does not reliably reflect evolutionary relationships. An alternative, preferred approach is based on molecular data such as nucleotide sequences of selected genes (Hillis 1987, Olsen 1988, Smith, 1989). DNA sequences allow comparing groups at all taxonomic levels and are independent of an organism's stage in the life cycle or its reproductive phase. In particular, ribosomal genes have been an important source of phylogenetic information for many taxa, including fungi (Bruns et al. 1991, Hibett 1992). They are present in all organisms, highly conserved in form and function (White et al. 1990, Holst-Jensen et al. 1997, van de Peer and De Watcher 1997,) and are homologous (Brus et al. 1991). In eukaryotic organisms, the emphasis has been on 18S rDNA (small subunit nuclear rDNA; Berbee and Taylor 1993, Van de Peer and De Wachter 1997).

Tetracladium was one of the three genera of aquatic hyphomycetes with tetraradiate conidia that were discovered by de Wildeman (1893, 1894, 1895). He noticed the similarity between the newly described *Tetracladium marchalianum* and some old, doubtful or poorly defined genera of algae such as *Asterothrix* and *Cerasteria*. To complicate matters further, *T. marchalianum* was often confused with *T. setigerum* (first described as *Tridentaria setigera*; Grove 1912) or *T. maxilliforme* (described as *Titaea maxilliformis* by Rostrup 1894). The confusion dates back to de Wildeman (1893), whose drawings seem to include *T. marchalianum* and *T. setigerum*. A fourth species, *T. apiense*, was described by Sinclair and Eicker (1981), and *T. furcatum* was added two years later (Descals and Webster 1983). Based on pure culture studies, two more species, *T. breve* and *T. palmatum*, were defined by Roldán et al. (1989). Members of this genus have been reported from a wide range of geographic locations; however, conidial shapes of the various species show considerable overlap and identification from single conidia is problematic (Roldán et al. 1989). To date, no sexual state has been reported from any species.

The primary objective of this study was to determine 18S rDNA sequences of selected representatives of the genus *Tetracladium* and use them to establish its position within the Fungi. In addition, our goal was to determine if the molecular data confirm the monophyletic status of *Tetracladium*, as suggested by traditional, morphology-based taxonomy.

MATERIALS AND METHODS

Isolates examined in study

Pure cultures of 7 strains belonging to 5 species were obtained from the Czech Collection of Microorganisms (CCM). They are listed in Table 1.

Table 1. Fungal strains examined in this study. All were obtained from the Czech Collection of Microorganisms (CCM). Sequences were deposited in the GenBank database (NCBI).

Taxon	CCM Number	Origin	GenBank Accession Number
Tetracladium apiense	F-23199	Czech Republic	AF388575
T. furcatum	F-11883	Czech Republic	AF388578
T. marchalianum	F-11391	Czech Republic	AF388579
T. marchalianum	LH-89	Czech Republic	AF388580
T. marchalianum	F-312	Slovak Republic	AF388576
T. maxilliforme	F-14286	Czech Republic	AF388577
T. setigerum	F-20987	Canada, New Brunswick	AF388574

DNA extraction

Fungal mycelia were grown in 1% malt broth at 20 °C for 14–21 days. Mycelia were harvested on filters, freeze-dried overnight and ground in liquid nitrogen for up to 1 min. The crushed mycelia (150 mg) were placed in an Eppendorf tube together with 300 μl of lysis buffer (50 mM Tris-HCl, 50mM EDTA, 3% SDS, 1% 2-mercaptoethanol) and the sample was incubated at 75 °C for 10 min. Standard chloroform: phenol (1:1) extraction was performed with isopropanol precipitation of DNA. The supernatant was drained, the pellet was washed with ethanol, dried and resuspended in 100 μl of ddH_2O.

Amplification and purification of DNA

Partial nuclear SSU rDNA regions were amplified using combinations of primer pairs of NS1 to NS8 (White et al. 1990). Amplification was performed in 50 μl volumes containing 1 ng μl^{-1} template DNA, 2 U Taq polymerase (Pharmacia Biotech), 1X Taq polymerase buffer, 250 μM of each dNTP (Pharmacia Biotech), and 4 mM $MgCl_2$. The PCR was performed in a T-gradient Biometra thermocycler (Whatman). The program started with initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30s, annealing at 55 °C for 30s and extension at 72 °C for 2 min. The final extension was done at 72 °C for 5 min. The PCR product was purified from solution with a GFX DNA purification kit (Amersham Pharmacia Biotech). The product was eluted with 40 μl of ddH_2O. The

DNA was sequenced with the PCR amplification primers and a dideoxy terminator sequencing kit (as instructed by ABI Prism) at the Molecular Supercentre at the University of Guelph.

Sequence alignment and phylogeny construction

The 18S rDNA gene sequences of all seven *Tetracladium* isolates were submitted to the BLAST Search engine at NCBI. The sequences of the first hits together with other selected sequences were used for phylogeny construction. Altogether, 18S rDNA gene sequences of 42 fungal species were retrieved from GenBank for use in phylogenetic analyses. The selected species followed by accession number are: *Anamylospora pulcherrima* (AF119501), *Arthrocladiella mougeotii* (AB033477), *Arthroderma ciferrii* (AB015678), *Aspergillus sparsus* (AB008408), *Aureobasidium pullulans* (M55639), *Beauveria bassiana* (AB027336), *Blumeria graminis* (AB033476), *Bothryosphaeria ribis* (U42477), *Bulgaria inquinans* (AJ224362), *Byssoascus straitosporus* (AB015776), *Coccodinium bartschii* (U77668), *Comminutispora* sp. (Y18699), *Cryphonectria havanensis* (L42440), *Cyttaria darwinii* (U53369), *Erysiphe orontii* (AB133483), *Graphium silanum* (AB007661), *Helvella terrestris* (AF006306), *Lecidea fuscoatra* (AF088239), *Lepolichen coccophorus* (AF274110), *Leveillula taurica* (AB033479), *Monilinia laxa* (Y14210), *Microsphaera friestii* (AB033478), *Myxotrichum deflexum* (AB015777), *Nectria aureofulva* (AB013010), *Oidiodendron tenuissimum* (AB015787), *Ophiostoma piliferum* (AJ243295), *Peziza echinospora* (AF006309), *Phyllactina guttata* (AF021796), *Pseudogymnoascus roseus* (AB015778), *Psora decipherens* (AF184759), *Rhizina undulata* (U42664), *Rhizomucor michei* (AF192506), *Sarcoscypha austriaca* (AF006318), *Selenaspora guernisacii* (AF104667), *Sordaria fimicula* (X69851), *Spathularia flavida* (Z30239), *Sphaerotheca cucurbitae* (AB033477), *Stereocaulon paschale* (AF140236), *Taphrina maculans* (AB000953), *Tuber excavatum* (X98089), *Uncinula mori* (AB033484).

All sequences were aligned manually with Se-Al (Rambaut 1995). Regions with ambiguous alignment were excluded from the data set in order to increase bootstrap support for the branches. Phylogenetic trees were generated with PHYLIP (Felsenstein 1995) using the parsimony (DNAPARS) and neighbour-joining distance methods (DNADIST/NEIGHBOR) with equal weighing of the 1695 most conserved alignment positions. All trees were calculated with a random addition of taxa. Branching order stability was estimated by bootstrap analysis of 100 replicates. *Rhizomucor miehei* was used as an outgroup in all phylogenetic trees. For the Neighbour-Joining tree, dissimilarity values based on pairwise comparisons of sequences were transformed into distances according to the Kimura two-parameter correction and using a transition to transversion ratio of 2.

Results

Sequence alignment and BLAST search results

The sequences of the nuclear rDNA of the 7 *Tetracladium* strains were approximately 1780 bp long (all sequences were deposited in the GenBank database; accession numbers are listed in Table 1). Their alignment revealed very little variation in the 18S region: sequence homology was ≥98%; rare events often consisted of insertion/deletion or substitution of a single nucleotide.

When the 18S rDNA sequences of the seven isolates were aligned and submitted to the BLAST search engine on NCBI, the closest match was with *Bulgaria inquinans* (placed in Leotiales by Ainsworth et al. 1995, Alexopoulos et al. 1996). Sequence identity with *B. inquinans* varied between 95% (*T. apiense*) and 96% (*T. marchalianum* LH89). Other close matches were with species of the genera *Erysiphe,Blumeria* (Erysiphales); *Myxotrichum, Oidiodendron* (Onygenales); *Rhizina* (Pezizales).

Phylogeny of *Tetracladium* and its position in the fungal kingdom

A phylogeny of the aquatic hyphomycete genus *Tetracladium* inferred from the Neighbour-Joining distance method is presented in Fig. 1. Bootstrap values greater than 50% supporting the recovered branches in either the distance or the parsimony tree are placed at the internal nodes of the tree.

Parsimony analysis of the data set yielded 16 equally parsimonious trees, one of which is shown (Fig. 2). There were no major differences between the Parsimony tree and the Neighbour-Joining tree. *Tetracladium* species grouped together in 100% of the bootstrapped samples, suggesting that the genus is monophyletic. Within the *Tetracladium* genus there was no significant grouping. *T. setigerum* and *T. marchalianum* F-11391 grouped together in both trees but the branch was weakly supported. All members of the genus *Tetracladium* were closely related to species from the Ascomycete orders Onygenales, Erysiphales and Leotiales. In the Neighbour-Joining tree *Tetracladium* branched together with Onygenales and was more distantly related to Erysiphales. In the Parsimony tree this order was reversed. As seen in previous studies (Doering 1998), the 18S rDNA data were unable to give good resolution and bootstrap support for the relationship between many of the lineages within the Leotiales, Onygenales and Erysiphales. *Tetracladium* grouped significantly apart from Sordariales, Clavicipetales, Hypocreales, Diaporthales, Ophiostomatales and Sphaeriales. *Rhizomucor* was used as an outgroup and was significantly different from the other species in 100% of the analyses.

DISCUSSION

Sequence analysis of 18S genes clearly indicates that the *Tetracladium* strains available for this study are part of a monophyletic group, supporting traditional, morphology-based taxonomy. The seven species of the genus have been reported from many different geographic locations (Roldán et al. 1989). It is unlikely that this wide distribution could be based on long-range transport of the relatively fragile aquatic conidia (Bärlocher 1992). Instead, the original dispersal of the various species, or their persistence in widely separated areas, are more likely due to sexually produced spores. In aquatic hyphomycetes, such spores generally occur on moist substrates that are no longer covered in water; dispersal is through airborne propagules (Webster 1992). The evidence presented here clearly shows that this presumed teleomorph (or teleomorphs) belongs to the Ascomycota. The closest hit in the GenBank database is *Bulgaria inquinans*, placed in the Leotiaceae (Leotiales according to Ainsworth et al. 1995; Helotiales according to Alexopoulos et al. 1996). This species decomposes dead wood in terrestrial habitats (Alexopoulos et al. 1996). Other members of the same family are found on stems of annual plants, on cones and fruits as well as on living plants (Alexopoulos et al. 1996). Interestingly, roughly 50% of all established meiosporic states associated with aquatic hyphomycetes are members of the Leotiales (Webster 1992, Webster and Descals 1979).

However, it would be premature to accept members of the Leotiaceae as the closest relatives of *Tetracladium*. When other hits are included, and depending on the details of the analysis, the *Tetracladium* complex can be closest to the Leotiales, Onygenales or Erisyphales, without any clear pattern emerging. Although the order Onygenales contains many human pathogens, members of the family Myxotrichaceae (e.g., *Byssoascus, Oidiodendron, Myxotrichum*) are saprobic on cellulosic substrates and are typically psychrophilic (Alexopoulos et al. 1996). Both properties may be useful preadaptations to colonize leaves in streams (Bärlocher 1992). However, terrestrial conidia of the Onygenales secede rhexolytically (a trait rarely found outside this order); the aquatic conidia of *Tetracladium* are holoblastic, and conidiogenous cells are polyblastic with sympodial proliferation (Roldán et al. 1987, 1989).

Members of the Erysiphales are obligate biotrophs that cause plant diseases known as powdery mildews (Alexopoulos et al. 1996). It is unlikely that *Tetracladium* would have evolved from an obligate fungal pathogen. But Erysiphales and *Tetracladium* might have a common, relatively recent ancestor.

Onygenales, Erysiphales and Helotiales (or Leotiales) generally cluster closely together. In the NCBI database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) they are all placed within the Pezizamycotina; Onygenales are one of two defined orders in the Euratiomycetes, while Erysiphales

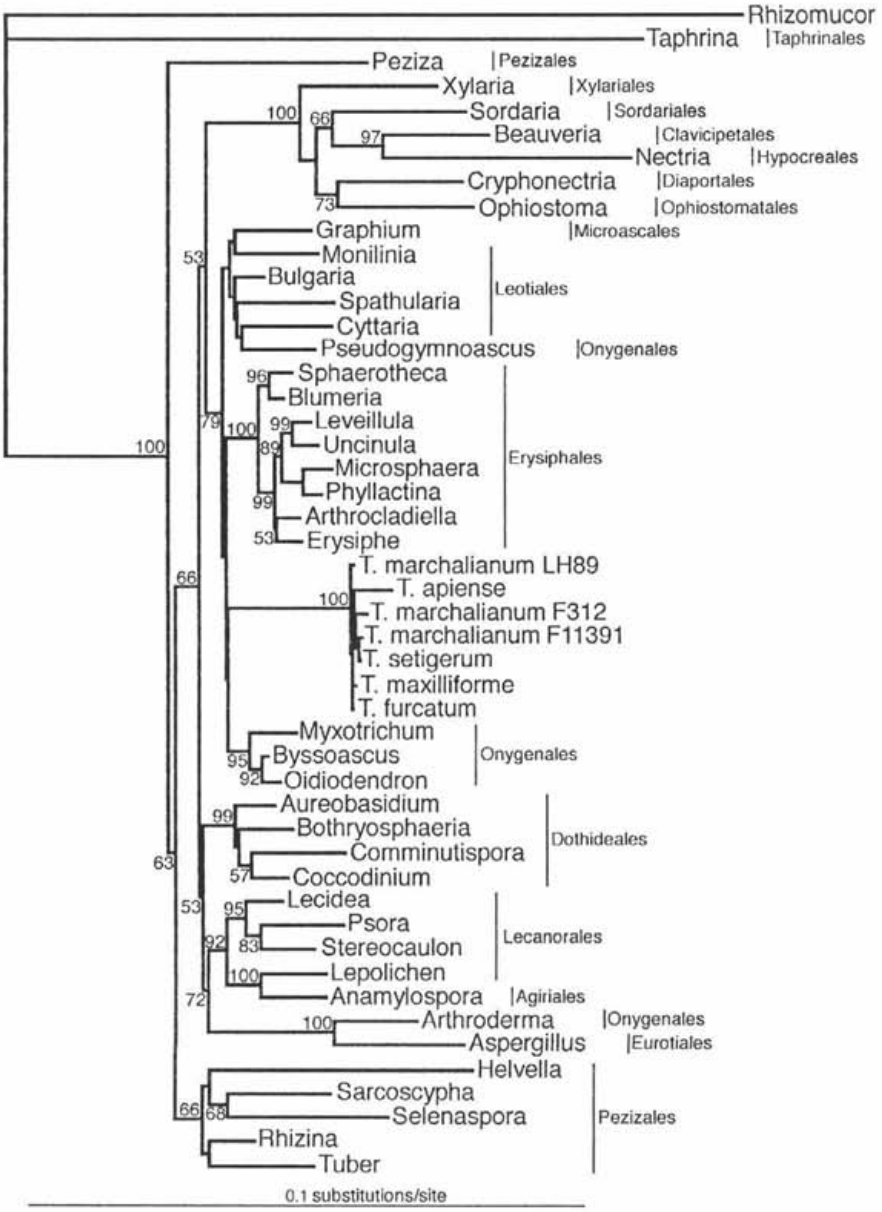

Fig. 1. Neighbour-joining tree based on 18S rDNA sequences *Tetracladium* and other selected fungal species. Distances were calculated using the aligned sequences and the PHYLIP program DNADIST with Kimura two-parameters method and random addition of taxa. *Rhizomucor* was used as an outgroup. The bar indicates a distance of 0.1 (10 bases change per 100 nucleotide positions). Bootstrap support of 50% or greater from 100 bootstrap replicates is shown in the internal nodes of the branches.

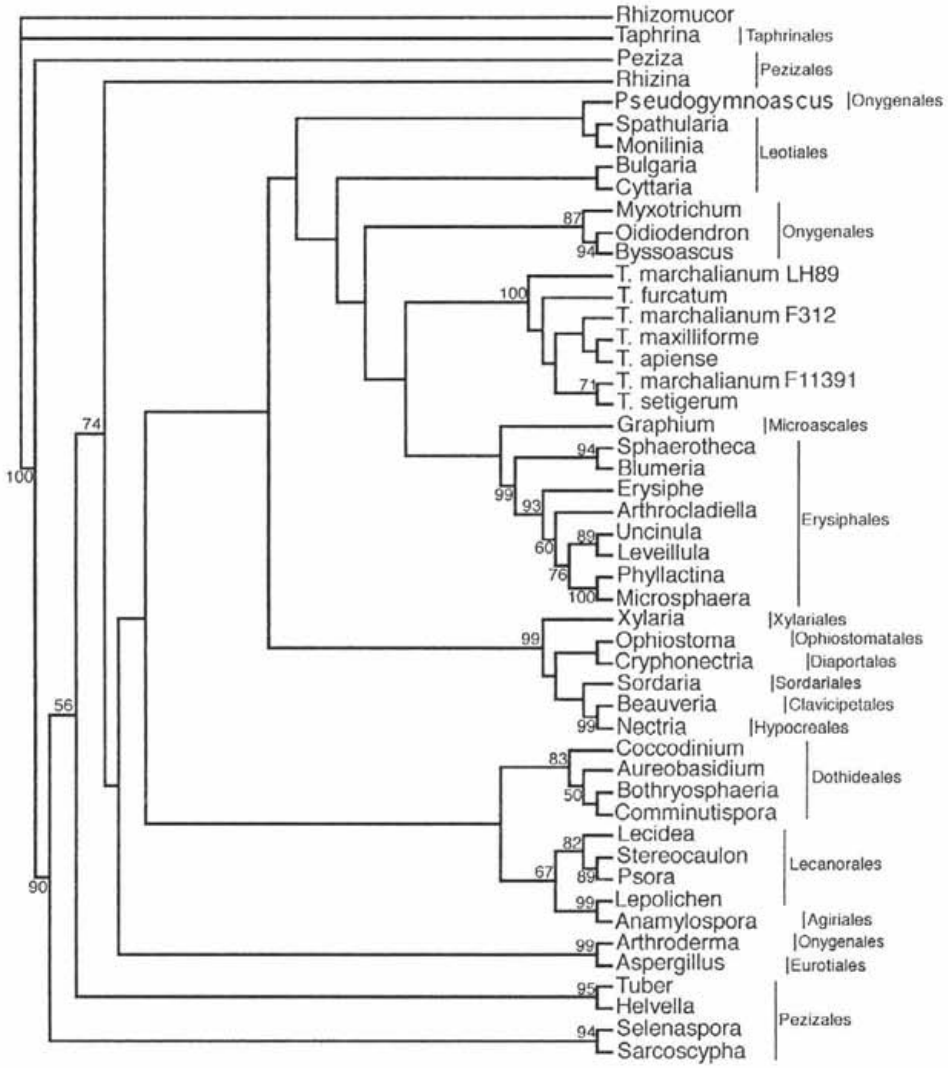

Fig. 2. One of 16 most parsimonious trees based on 18S rDNA sequences of *Tetracladium* and other selected fungal species. Branch topology was calculated using the aligned sequences and the PHYLIP program DNAPARS with random addition of taxa. *Rhizomucor* was used as an outgroup. Bootstrap support of 50% or greater from 100 bootstrap replicates is shown in the internal nodes of the branches.

and Helotiales represent two of the four defined orders in the Leotiomycetes. Our data suggest that the most common recent ancestors connects *Tetracladium* with one of these orders; without additional data, we cannot narrow it down any further. The major difficulty with establishing the phylogenetic relationship of fungal

species or genera is the presence of the large gaps in published databases. While rDNA sequences of pathogenic taxa are common, saprobic species are very much underrepresented (Cannon 1999). To our knowledge, our data on *Tetracladium* represent the first complete 18S rDNA sequences of any aquatic hyphomycete.

The evolutionary relationships among the members of the genus *Tetracladium* could not be further elucidated from our sequences. Although some strains, such as *T. setigerum* and *T. marchalianum* F-11391 consistently grouped together, the branching events were not significant. Also, there was considerable disparity among the results obtained when different criteria were used for the analysis. This was not surprising, since the sequences of the 18S rRNA gene evolve slowly and typically are not suited to comparisons between closely related taxa (Smith 1989). To elucidate such relationships, and to determine whether subdivision of *Tetracladium* into the seven described species (Roldán et al. 89) is justified, requires analysis of more variable regions of the fungal genome. Internal transcribed spacers (ITS) between the coding regions for the ribosomal subunits are generally more informative. In addition, a convincing analysis would require many more isolates of the various species, preferably from various geographic locations. Unfortunately, there are few strains of aquatic hyphomycetes available from culture collections. We were unable to obtain living cultures of *T. breve* or *T. palmatum*.

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