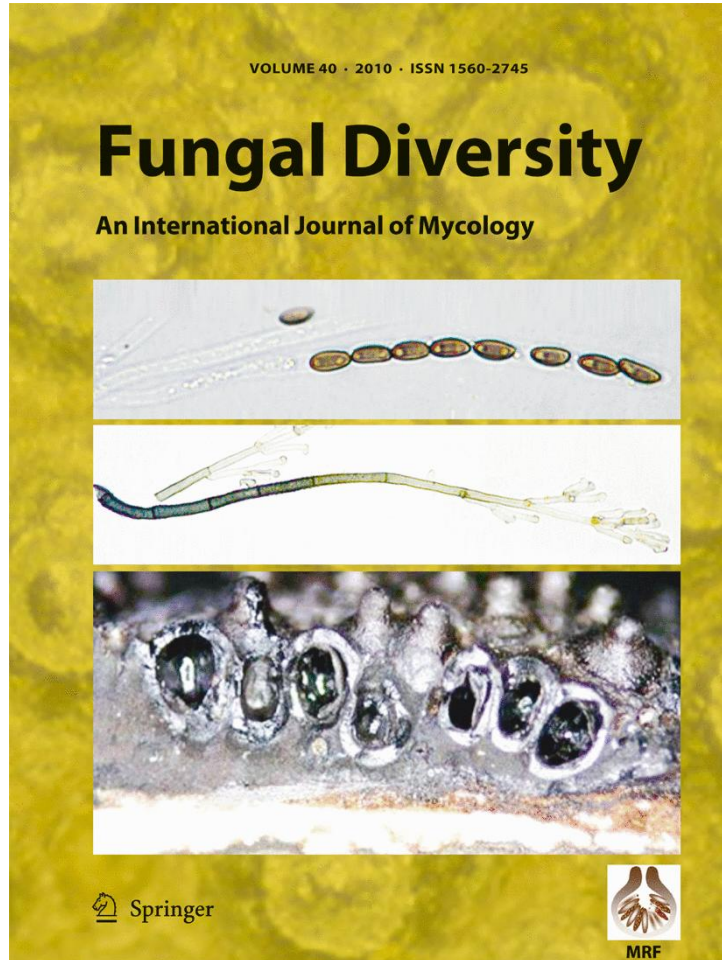


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Potential use of barcoding to identify aquatic hyphomycetes

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Abstract *Tetracladium* is a common aquatic hyphomycete genus, whose taxonomy has been based on the morphology and development of asexual spores. Ecological surveys have relied almost exclusively on spore morphology. Since selective pressures have resulted in convergent shapes, misidentifications are a concern. To supplement morphological information, we determined COX1, ITS and D1/D2 sequences as potential barcodes on 21 strains belonging to 7 described *Tetracladium* species and an unidentified strain. Attempts to amplify the IGS region were unsuccessful. The ratio of intraspecific to interspecific variability was optimal with ITS, which also provided the intuitively most acceptable cladogram. Typical conidia and their variability for the seven described species are illustrated. Internal node reliabil-

ity depended less on total sequence length and more on the mixture of conserved and variable regions used to build cladograms. This finding can be exploited for quickly increasing phylogenetic accuracy without greatly increasing the amount of amplification and sequencing. The results have important implications for identifying freshwater hyphomycetes in the field but further work is required to establish if this method works with plant pathogens.

Keywords *Tetracladium* · Barcode · ITS · COX1 · D/D2

Introduction

There is growing concern that anthropogenically induced species loss may threaten ecosystem function and services (Kinzig et al. 2001; Loreau et al. 2002). To investigate these connections in nature requires accurate estimates of species diversity. Classical morphology-based identification is suffering from a increasing shortage of trained taxonomists, and phenotypic plasticity and genetic variability can lead to misinterpretations. An alternative is DNA barcoding. It is based on comparing a short DNA sequence from a specified region of the genome to provide a unique barcode for each species (Hebert et al. 2003). Several regions of the genome have been explored. Criteria for species resolution are a relatively short region of genomic DNA that can be reliably extracted and amplified, and is of low intraspecific and sufficient interspecific variability. For animals, a 648 BP fragment at the 5' end of the mitochondrial cytochrome c oxidase 1 gene (COX1 or CO1) has been recommended due to its high variability, low intron interference and robust amplification ability (Hebert et al. 2003). The Barcode Of Life Database (BOLD) was created with the goal of identifying all known taxa using molecular data

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Table 1 *Tetracladium* strains analysed with their geographic provenance. Strain numbers refer to the Czech Collection of Microorganisms at Masaryk University

Species	Strain	Abbreviation	Provenance
<i>T. apiense</i>	CCM F-23099	T.api1	Gran Canaria (Spain)
	CCM F-23199	T.api2	Gran Canaria (Spain)
	CCM F-23299	T.api3	Gran Canaria (Spain)
	CCM F-23399	T.api4	Gran Canaria (Spain)
<i>T. breve</i>	CCM F-12505	T.bre1	Portugal
	CCM F-10501	T.bre2	Portugal
<i>T. furcatum</i>	CCM F-06983	T.fur1	UK
	CCM F-11883	T.fur2	Czech Republic
<i>T. marchalianum</i>	CCM F-11001	T.mar1	UK
	CCM F-26299	T.mar2	Czech Republic
	CCM F-26399	T.mar3	Czech Republic
	CCM F-312	T.mar4	Slovak Republic
	CCM F-11391	T.mar5	Czech Republic
	CCM F-19399	T.mar6	Czech Republic
<i>T. maxilliforme</i>	CCM F-529	T.max1	Czech Republic
	CCM F-13186	T.max2	Czech Republic
<i>T. palmatum</i>	CCM F-10001	T.pal	Portugal
<i>T. setigerum</i>	CCM F. 19499	T.set1	Czech Republic
	CCM F-20987	T.set2	Canada
	CCM F-10186	T.set3	Czech Republic
<i>Tetracladium</i> sp.	CCM F-10008	T.sp	Malaysia

through COX1 sequences (Ratnasingham and Hebert 2007). However, in several groups of higher fungi, this is problematic due to the common occurrence of mobile introns and because of the rapid evolution of the priming sites, which makes the approach less suitable for environmental surveys (Summerbell et al. 2005; Rossman 2007; Seifert et al. 2007). As pointed out by Summerbell et al. (2005), other sequences were used for this purpose long before the term barcoding was introduced. This includes the variable D1/D2 domains of LSU rDNA for yeasts (Kurtzman 1994; Kurtzman and Robnett 1998; Fell et al. 2000), the partial elongation factor 1 α (EF- α ; Geiser et al. 2004), partial β -tubulin (BenA; Samson et al. 2004) and, most commonly, partial or entire ITS sequences (O'Donnell et al. 1998; Pryce et al. 2003; Druzhinina et al. 2005; Kõljalg et al. 2005; Huang et al. 2009; Tang et al. 2009; Wannathes et al. 2009). The consensus of the first meeting of the All Fungi Barcode Initiative (FBI) recommends the entire ITS sequence as a first step for species identification, with a second sequence added for more precise identification and phylogenetic placement (Rossman 2007).

Aquatic hyphomycetes are a phylogenetically heterogeneous group with worldwide distribution (Bärlocher 1992a, b; Dix and Webster 1995; Tsui and Hyde 2003; Cai et al. 2006). Both traditional (morphology-based) and molecular techniques have placed most of them within the Ascomycota (Belliveau and Bärlocher 2005; Baschien et al. 2006;

Campbell et al. 2006). They are the major fungal group involved in the decomposition of leaf detritus in running waters (Bärlocher 2005; Gessner et al. 2007), though the presence of other fungal taxa has been demonstrated by molecular means (Nikolcheva and Bärlocher 2004; Seena et al. 2008, Das et al. 2008). The success of aquatic hyphomycetes in running water has been attributed to the prolific release of conidia (mitospores) that are typically multiradiate or sigmoid, which facilitates their attachment to leaf surfaces. Shape and development of these conidia have formed the basis for classifying and identifying genera and species of aquatic hyphomycetes. Currently, close to 300 species have been described. However, unequivocal identification often requires access to a pure culture (Marvanová and Bärlocher 2001), and some mycelia may be present on decaying leaves without releasing conidia (Nikolcheva et al. 2003). The availability of a molecular barcode would bypass some of these complications. In the current study, we investigated the variability of three sequences (ITS, COX1, D1/D2) and their potential use as barcodes for *Tetracladium*. This anamorph genus with 7 species (Roldán et al. 1989) has not been connected to any teleomorph, but 18S rDNA analyses have placed it in the vicinity of the Leotiomycetes (Ascomycota) (Nikolcheva and Bärlocher 2002; Baschien et al. 2006). The recognized species are: *T. marchalianum* De Wild. (type of the genus), *T. apiense* R.C. Sinclair & Eicker, *T. breve* A. Roldán, *T.*

Table 2 Primers evaluated in this study. The primers chosen for the study are marked by *. References: 1, Arora et al. (1996); 2, M. Beaton and J. Ferrell (Mt. Allison, unpubl.); 3, Seifert et al. (2007); 4, Takano and Horiguchi (2005); 5, Website by R. Vilgalys, <http://www.biology.duke.edu/fungi/mycolab/primers.htm>

Name	Direction	Sequence	Section	Reference
*ITS 1	Forward	TCCGTAGGTGAACCTGCGG	ITS	5
*ITS 4	Reverse	TCCTCCGCTTATTGATATGC	ITS	5
SR6R	Forward	AAGWAAAAGTCGTAACAAGG	ITS/SSU	5
*NS1	Forward	GTAGTCATATGCTTGTCTC	SSU	5
NS2	Reverse	GGCTGCTGGCACCAGACTTGC	SSU	5
*NS3	Forward	GCAAGTCTGGTGCCAGCAGCC	SSU	5
*NS4	Reverse	CTTCCGTCAATTCCTTTAAG	SSU	5
*NS5	Forward	AACTTAAAGGAATTGACGGAAG	SSU	5
NS7	Reverse	GAGGCAATAACAGGTCTGTGATGC	SSU	5
*LR0R	Forward	ACCCGCTGAACTTAAGC	LSU	5
*LR3R	Forward	GTCTTGAAACACGGACC	LSU	5
*LR5	Reverse	TCCTGAGGGAAACTTCG	LSU	5
LR7	Reverse	TACTACCACCAAAGATCT	LSU	5
LR12R	Forward	CTGAACGCCTCTAAGTCAGAA	LSU/IGS	5
*LR16	Reverse	TTCCACCCAAACACTCG	LSU	5
invSR1R	Reverse	ACTGGCAGAATCAACCAGGTA	LSU/IGS	5
5S RNA	Reverse	ATCAGACGGGATGCGGT	LSU/IGS	5
5S RNAr	Forward	ACQGCATCCCGTCTGAT	LSU/IGS	5
PenF1	Forward	GACAAGAAAGGTGATTTTTATCTTC	COX1	3
PenR1	Reverse	GGTAAAGATAATAATAAACACTGCTG	COX1	3
AspR1	Reverse	GGTAATGATAATAATAATACAGCTG	COX1	3
PenF2	Forward	TWAGTTTCTGATTATTAGTACCTAGTTT	COX1	3
PenR2	Reverse	AACTAGGTACTAATAATCAGAACTWA	COX1	3
DinoCO1F1	Forward	AAAAATTGTAATCATAAACGCTTAGG	COX1	4
DinoCO1R2	Reverse	TGGATCAAAGAAAAGTGTATTA	COX1	4
DinoCO1F2	Forward	AGACTATGCCATTATTCCTTGG	COX1	2
DinoCO1R1	Reverse	CAAAATGAAAATGAGCTACAACAT	COX1	4
DinoCO1R2T	Reverse	TGGATCATAGAAAAGTGTATT	COX1	4
LR7R	Forward	GCAGATCTTGGTGGTAG	LSU	5
*LR8R	Forward	AGCAGGTCTCCAAGGTG	LSU	5
*ITS2	Reverse	GCTGCGTTCTTCATCGATGC	ITS	5
*ITS3	Forward	GCATCGATGAAGAACGCAGC	ITS	5
*AHyFU-F	Forward	CTTAGTGGGCCAGGAGTTCAATA	COX1	3
*AHyFU-R	Reverse	ACCTCAGGGTGTCCGAAGAAT	COX1	3
CNL12	Forward	CTGAACGCCTCTAAGTCAG	IGS	1

furcatum Descals, *T. maxilliforme* (Rostr.) Ingold, *T. palmatum* A. Roldán and *T. setigerum* (Grove) Ingold. The genus is rather homogeneous in terms of cultural characters and conidiogenesis. The present species concept is based on the morphology of conidia. Ontogenetically they consist of an axis and primary and secondary branches, but the mature detached conidia appear like a bunch of variously shaped elements (branches plus distal part of the axis) arranged on a stalk. There are two fundamentally different types of conidial elements: (1) broad with rounded apices (globose, short clavate, digitiform or fusoid) and (2) narrow with pointed apices (obclavate and attenuated to acute ends or appearing filiform when extremely narrow).

Although there is a considerable variation in conidial size and configuration among and within species, identifications at the genus level on the basis of single detached conidia in environmental samples are reliable. However, due to the presence of more or less frequent intermediates, identifications at the species level from single conidia may be ambiguous. In pure culture, colonies are medium to fast growing, off-white, yellowish, rosy or pale orange. The reverse is usually of the same colour; aerial mycelium is mostly abundant over the entire colony, often finely funiculose or tufted; chlamydospores have been described (as “metasclerotia” cf. Descals and Webster 1983) only in *T. furcatum*. Conidiophores are terminal or lateral, simple or

Table 3 GenBank accession numbers for 21 *Tetracladium* sp. strains for the COX1, ITS and combined 18S, ITS1, 5.8S, ITS2 and 26S sequences

Strain	COX1	ITS	5,205bp Sequence
CCM F-11001	EU883393	FJ000357	EU883413
CCM F-26299	EU883392	FJ000358	EU883414
CCM F-26399	EU883394	FJ000359	EU883415
CCM F-312	EU883395	FJ000360	EU883416
CCM F-11391	EU883396	FJ000361	EU883417
CCM F-19399	EU883402	FJ000370	EU883423
CCM F-529	EU883406	FJ000362	EU883429
CCM F-13186	EU883409	FJ000371	EU883430
CCM F-19499	EU883407	FJ000363	EU883426
CCM F-20987	EU883408	FJ000365	EU883425
CCM F-10186	EU883405	FJ000374	EU883427
CCM F-23099	EU883398	FJ000366	EU883419
CCM F-23199	EU883399	FJ000367	EU883420
CCM F-23299	EU883400	FJ000368	EU883422
CCM F-23399	EU883401	FJ000369	EU883421
CCM F-12505	EU883410	FJ000405	EU883431
CCM F-10501	EU883397	FJ000364	EU883418
CCM F-10001	EU883403	FJ000372	EU883424
CCM F-06983	EU883404	FJ000373	EU883428
CCM F-11883	EU883411	FJ000375	EU883432
CCM F-10008	EU883412	FJ000376	EU883433

sparsely branched. Conidiogenous cells are thalloblastic (Hennebert and Sutton 1994), with sympodial proliferations. Conidia appear usually in close sequence and often group in various stages of development on the conidiophore apex.

Most of the data on *Tetracladium* in the literature are based on detached conidia identified by stream ecologists. Little work has been done on pure cultures and only a few strains of *Tetracladium* species are available from public culture collections. Hence the phenotypical variability within individual species remains unknown. Differences known in conidial size and morphology (missing or

additional branches) among isolates (Roldán et al. 1989) suggest the possible existence of intraspecific taxa or cryptic species. Unfortunately, like in many aquatic hyphomycetes, ex-type (or ex-neotype) strains, which “fix the undisputed application of a fungal name” (<http://www.cbs.knaw.nl/research/collection.asp>), are not available in any registered culture collection (http://wcdm.nig.ac.jp/simple_search.html). In the absence of such strains, isolates whose identity is ensured by a specialist, may constitute the preferred material to generate barcodes (<http://www.cbs.knaw.nl/research/collection.asp>). Some of the isolates from the Czech Collection of Microorganisms are here pointed out as having conidia which conform with those of the type or neotype specimens of the respective species.

In the current study, we analyzed DNA sequences from all 7 known *Tetracladium* species and from one strain which could not be assigned to a described species. We were interested in three aspects: 1. Which of the three sequences is most suitable as barcode? 2. Is there any intraspecific variation? 3. How long does the sequence have to be to provide reliable phylogenetic separation? Does the length of the sequence or degree of variability matter more for internal node reliability (Min and Hickey 2007)?

Materials and methods

Isolates and morphology

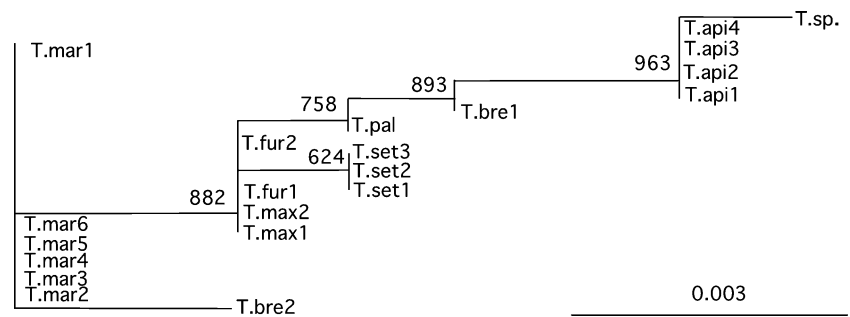
A total of 22 *Tetracladium* strains, all isolated from single spores, were obtained from the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic. We were unable to extract amplifiable DNA from one of the strains. All analyses are based on the remaining 21 strains listed in Table 1.

Cultures were grown at room temperature on malt extract medium (1.5% malt extract, 1.5% agar, 0.01% chloramphenicol). After 1 week, DNA was extracted with the MO BIO Ultraclean™ Soil DNA Isolation Kit using the manufacturer's alternative protocol for maximum

Table 4 Percentage sequence differences within and between species. For species with more than 2 strains, and for interspecies differences, minima and maxima are given in parentheses

	Strain	D1/D2	COX1	ITS
within	<i>T. apiense</i>	0 (0, 0)	0	0
	<i>T. breve</i>	0.74	3.58	1.57
	<i>T. furcatum</i>	0	1.02	0
	<i>T. marchalianum</i>	0 (0,0)	0	0
	<i>T. maxilliforme</i>	0 (0,0)	0	0
	<i>T. setigerum</i>	0 (0,0)	0.11 (0, 0.17)	0
	Average	0.03	0.61	0.05
	between		0.20 (0, 1.10)	3.28 (1.02, 5.29)

Fig. 1 D1/D2 phylogram of 21 strains of *Tetracladium* (Neighbor Joining, Bootstrap 1000; CLC DNA Workbench; Knudsen et al. 2008)



yield. The extract was stored at -20°C for a maximum of 10 days before further analysis.

The primers that were investigated for compatibility with *Tetracladium* ribosomal and COX1 genes are listed in Table 2. Melting temperatures (T_m) were calculated with the program available at <http://www.basic.northwestern.edu/biotools/oligocalc.html>.

PCR solutions contained 8.0 μl DEPC water (Invitrogen), 1.5 μl DNA extract, 1.0 μl of forward and 1.0 μl of reverse primer solution and 14.0 μl of GoTaq[®] Green Master Mix (Promega). Amplifications were conducted in Progene 0.2 ml strip tubes with ABgene Flat Cap Strip on a BIO RAD MyCycler[™] Thermal Cycler.

The initial separation temperature was set at 94°C for 2 min, the cycle separation temperature at 94°C for 45 s, the elongation temperature at 73°C for 90 s and the post cycle elongation temperature at 73°C for 10 min.

To check homogeneity and size of amplicons, they were run on 1.3% agarose gels (BIO-RAD Molecular Biology Agarose) in 0.5X TBE running buffer, produced from NOVEX[®]5X stock solution (Invitrogen). A BIO-RAD PowerPac Basic[™] gel electrophoresis instrument set at 180 V for 10 min ran gels.

Six replicates from each amplicon (3 forward, 3 reverse of independent extracts and amplifications) were sent to McGill University and Genome Quebec Innovation Centre for Sequencing. Sequencing was performed on 3,730 \times 1 DNA Analyzer system from Applied Biosystems. Dow-

loaded sequences were aligned with BioEdit (Hall 1999) and consensus sequences were created. They were trimmed at the 5' and 3' ends and exported in FASTA format. They were converted into the Nexus format using Sequence Alignment Editor v2.0a11 (Rambaut 1996). Consensus sequences were submitted to National Center for Biotechnology Information at gb-admin@ncbi.nlm.nih.gov. Accession numbers are listed in Table 3.

Alignment and subsequent joining of the ribosomal gene segments provided an edited sequence of 5,205 bp. The D1/D2 region was edited to be 817 bp, the COX1 5' end was edited to 586 bp and the ITS region was edited to 512 bp. Editing the 5,205 bp sequence by removing 2,205 bp from the 3' end created a 3,000 bp sequence. The 1,400 bp extended variable region was the ITS region combined with D1/D2 giving a 1,392 bp sequence. Also, the first 1,400 bp from the 5,205 bp sequences were selected as a control for the extended variable region.

Sequences were analyzed with CLC DNA Workbench (Knudsen et al. 2008) for pairwise comparisons.

Nexus files were imported into Phylogenetic Analysis Under Parsimony (PAUP) beta version 4.0 (Swofford 1998) and phylogenetic analysis was performed using multiple algorithms. Tree graphics were exported from PAUP as cladograms and compared for internal node confidence. Internal node confidence levels were set to 70% (1000 bootstrap replicates).

Fig. 2 COX1 phylogram of 21 strains of *Tetracladium* (Neighbor Joining, Bootstrap 1000; CLC DNA Workbench; Knudsen et al. 2008)

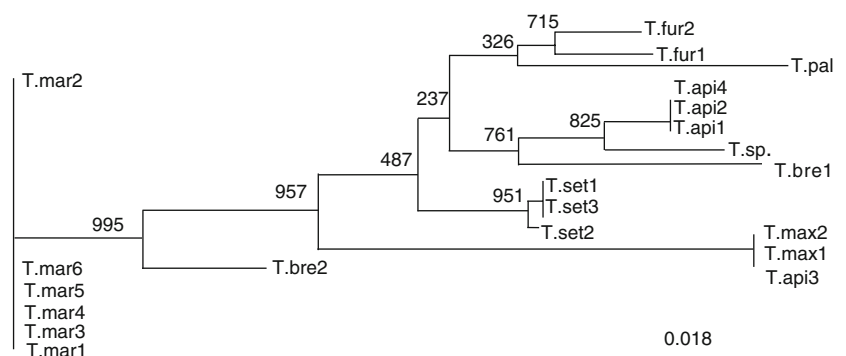
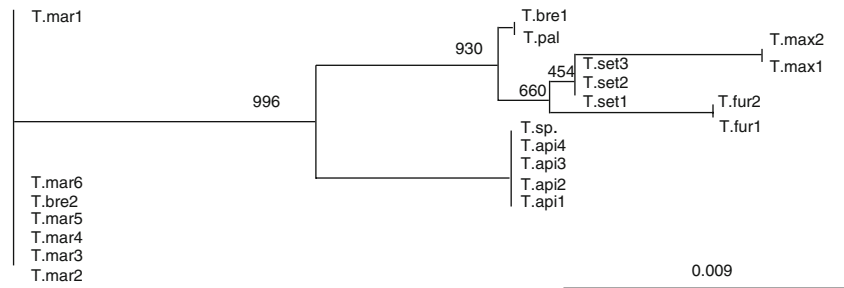


Fig. 3 ITS phylogram of 21 strains of *Tetracladium* (Neighbor Joining, Bootstrap 1000; CLC DNA Workbench; Knudsen et al. 2008)



Results

Successful primer pairs

The sizes of all successful amplicons were evaluated by gel electrophoresis. Blast results confirmed the presence of the desired gene sequence.

Attempts to amplify the IGS region gave unreliable and inconsistent results during many attempts with the following primer pairs: LR12R/5sRNA, LR20R/5sRNA, LR3R/5sRNA, CNL12/5sRNA, InvisR1R/5sRNAr, 5sRNAr/NS2 (for complete list of tested primers, see Table 2).

The COX1 region was successfully amplified with the primer pair AHyFu-F and AHyFu-R.

The entire ribosomal gene amplification required 8 overlapping amplicons from the 5' end of the SSU to the 3' end of the LSU (NS1/NS4; NS3/ITS2; NS5/ITS2; ITS1/ITS4; ITS3/LR16; LR0R/LR5; LR3R/LR10; LR8R/LR12). Gel electrophoresis gave high confidence for the presence of the desired amplicon. Blast results reinforced the presence of ribosomal gene segments.

Sequence comparisons

Similarities of pairwise alignments of the three amplified regions D1/D2, COX1 and ITS were computed. Differences among the D1/D2 sequences were negligible for the 21 strains (average of 0.03% within species and of 0.20% between species; Table 4). They were greater for COX1 (0.61 and 3.28%, respectively) and intermediate for the ITS region (0.05 and 1.50%, respectively).

Phylogenetic analyses based on D1/D2, COX1, and ITS sequences are shown in Figs. 1, 2 and 3. All grouped the *T. marchalianum* and *T. setigerum* strains together, and separated the two *T. breve* strains. The two *T. maxilliforme* remained grouped with ITS, and were joined by *T. furcatum* (D1/D2) or *T. apiense* (COX1).

Cladogram analysis with all 5,205 bp by PAUP 4.0 (distance method Jukes-Cantor, Neighbor Joining, 1000 bootstrap replicates; Swofford 1998) placed the strains of *T. marchalianum*, *T. setigerum*, *T. furcatum* and *T. maxilliforme* in separate clusters (Fig. 4). Three strains of *T. apiense* also formed a distinct cluster in close proximity to

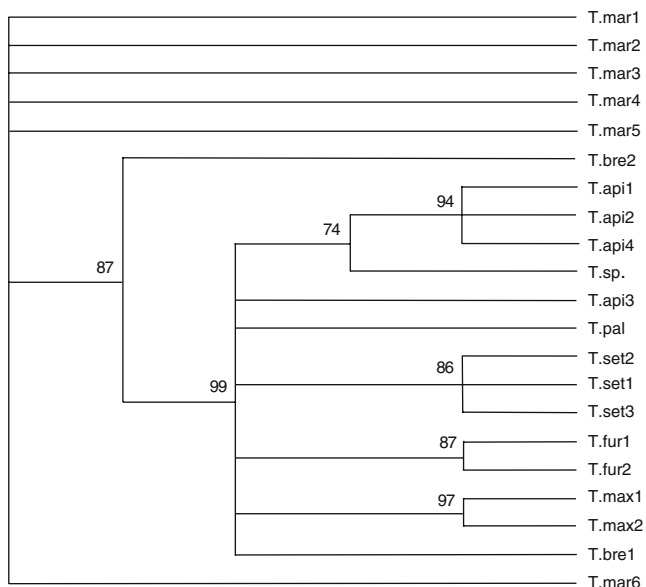


Fig. 4 Cladogram created from 5205 bp (Distance method Jukes-Cantor, Neighbor Joining, Bootstrap 1000; Swofford 1998)

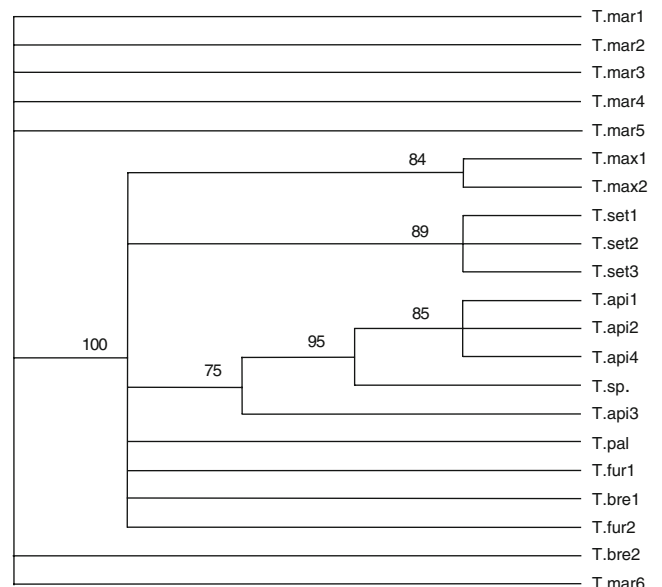
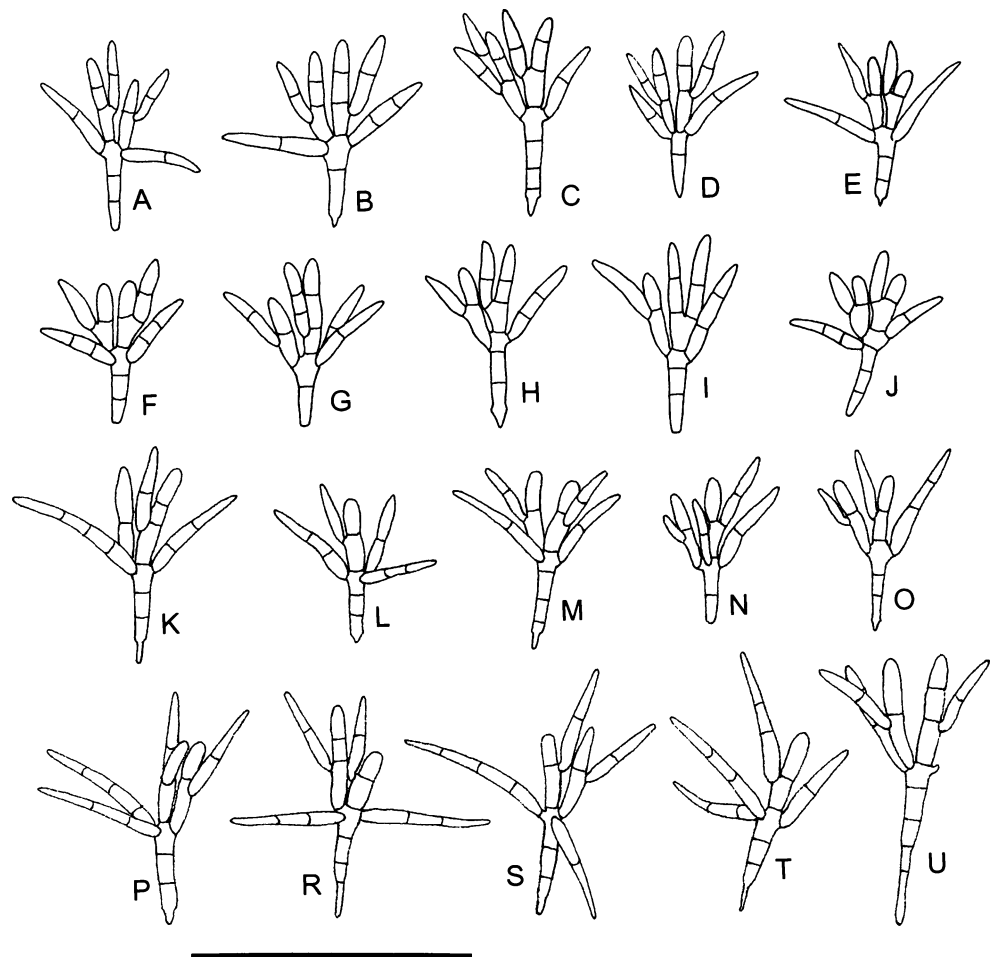


Fig. 5 Cladogram created from ITS+D1/D2 (Distance method Jukes-Cantor, Neighbor Joining, Bootstrap 1000; Swofford 1998)

Fig. 6 *Tetracladium apiense*, conidia. A–E, from CCM F-23099; F–J, from CCM F-23199; K–O, from CCM F-23399; P–U, from CCM F-23299. Scale bar=50µm



the unidentified *Tetracladium* sp. CCM F-10001, but a fourth strain was outside of this primary cluster and was not separated from the second *T. breve* and the *T. palmatum* strain.

Internal node reliability (Min and Hickey 2007) was reduced from 7 to 6 nodes at >70% reliability when the sequence was edited from 5,205 to 3000 bp by removing 2,205 bp from the 3'; the ITS sequence by itself reduced to

Fig. 7 *Tetracladium breve*, conidia. A–H, from CCM F-12505; I–P, from CCM F-10501. Scale bar=50µm

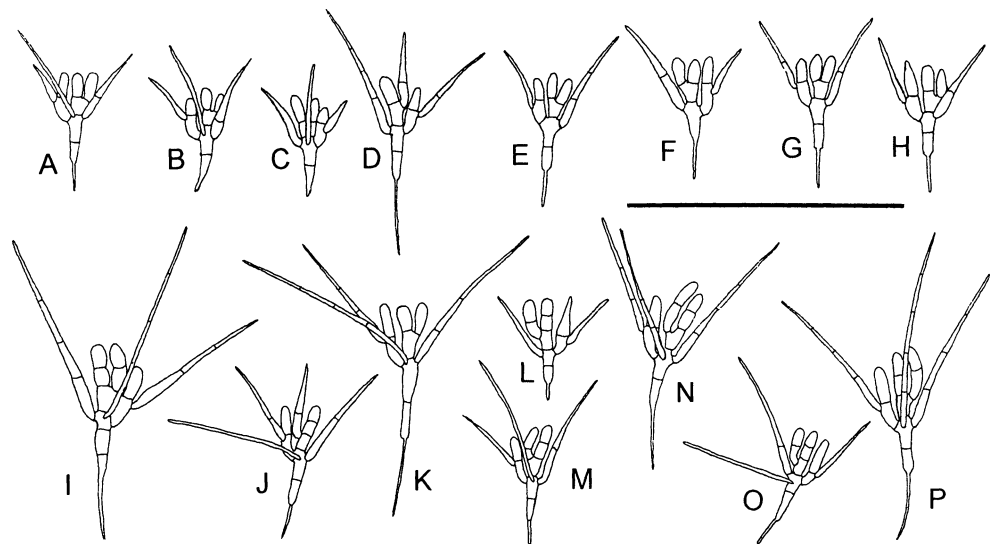
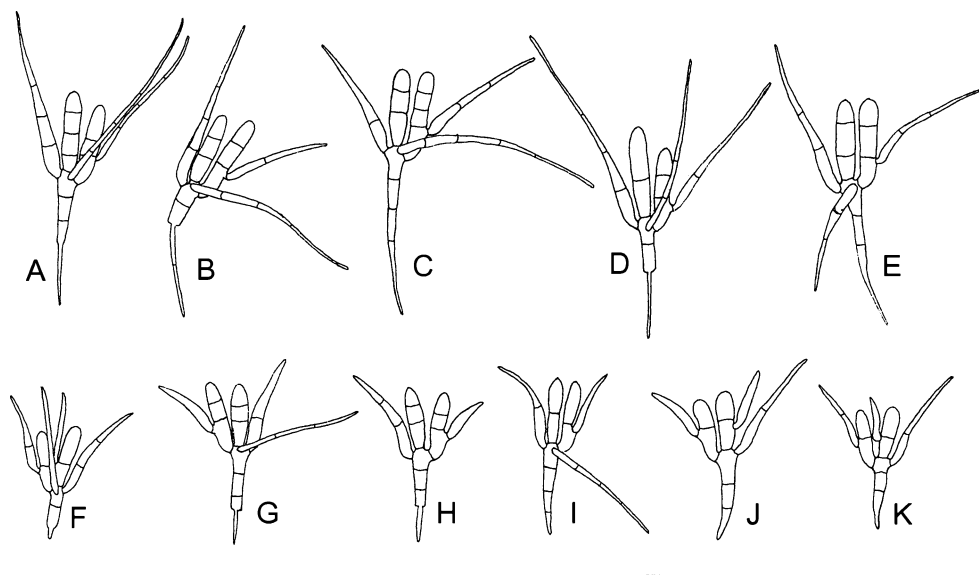


Fig. 8 *Tetracladium furcatum*, conidia. A–E, from CCM F-11883; F–K, from CCM F-06983. Scale bar=50 μ m



5 internal nodes. With the extended variable region (ITS plus D1/D2), internal node reliability was similar to the one based on 5,205 bp (Fig. 5). The 1,400 bp control region had no internal nodes with >70% reliability (not shown). Five methods of parameter calculations (HKY85, LogDet/Paralinear, K2P, Jukes-Cantor, F81) gave the same results.

Morphological comparisons

Tetracladium apiense is characterised by two furcate-digitiform and two simple fusoid branches with more or less rounded apices, supported by a clavate stalk (Fig. 6). Thin obclavate or filiform branches are absent. Our four isolates originated from the same small stream, from one piece of a submerged unidentified dicot twig. Conidia of two strains (CCM F-23099 and CCM F-23199) conform well to the illustrations of the type (Sinclair and Eicker 1981, Fig. 1 A, B) but are less plump than drawn from the type material by Roldán et al. (1987a, Fig. 2 G–W). CCM F-23399 often has one of the furcate branch missing. CCM F-23299 (Fig. 6, P–U) differs mostly by larger conidia with rather slender axis and branches.

The conidial configuration of *T. breve* is similar to that in *T. setigerum*, i.e. three digitiform elements plus two narrow obclavate plus one filiform element on a stalk (Fig. 7). Both strains were isolated in Portugal. Although morphologically similar, they differ markedly in the variability of the conidial size: in CCM F-12505 the conidia agree well with the illustration of the type (Roldán et al. 1989, Fig. 5), while conidia of CCM F-10501 often exceed the upper limit of the dimensions given in the protologue. The thin pointed branches are often conspicuously longer and the upper digitiform branch on the axis usually tends to be somewhat

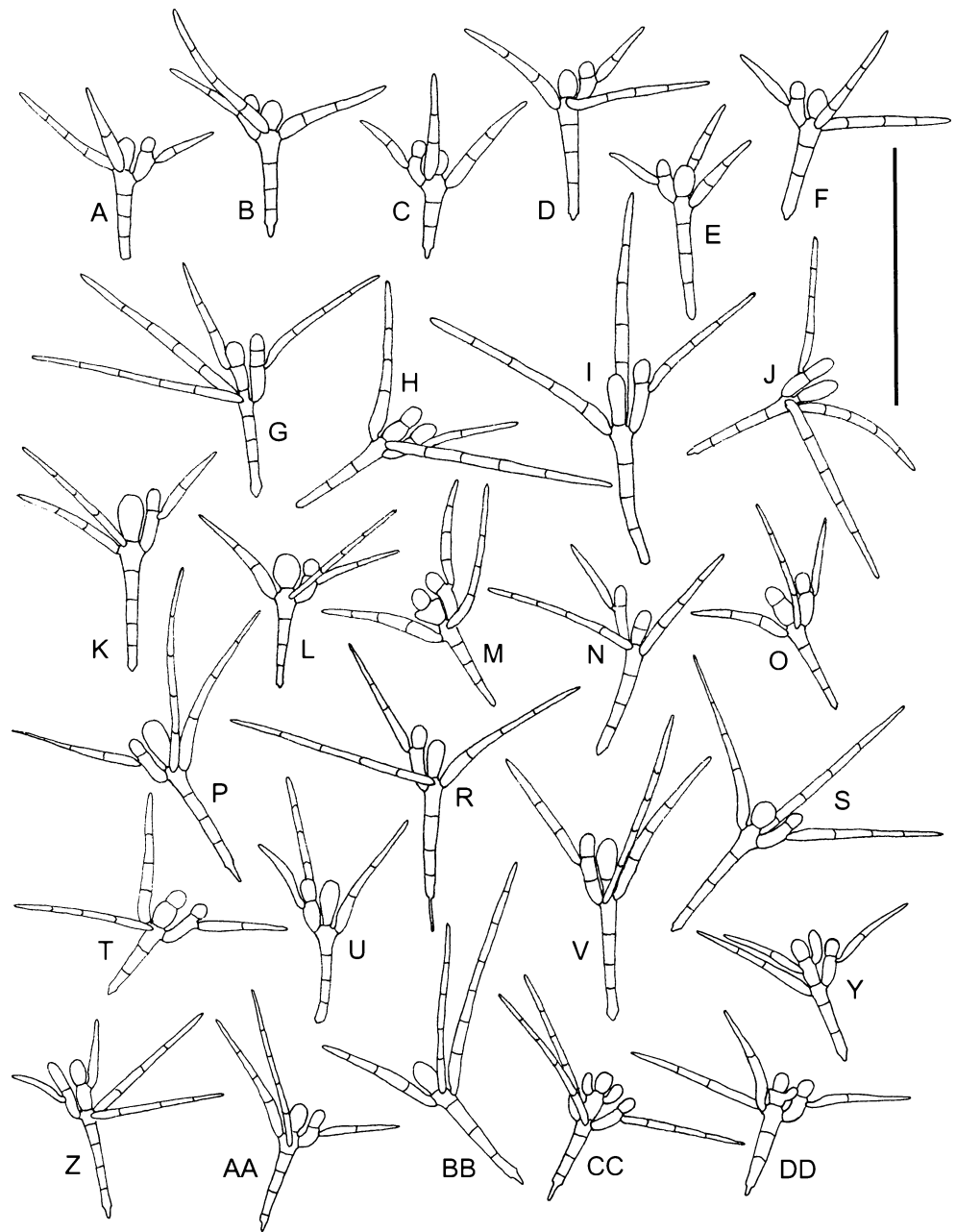
curved adaxially (Fig. 7 I, M, N, P), which makes them even more similar to small conidia of *T. setigerum*.

Tetracladium furcatum (Fig. 8) is characterised by conidia with two usually slightly diverging digitiform elements, two pointed narrow obclavate branches and one filiform branch. Morphologically, the conidia resemble aberrant conidia of *T. setigerum* with one digitiform element missing, or conidia of *T. maxilliforme*. Our two isolates originated from foam collected in softwater streams. Chlamydospores with slightly darkish cell walls reported in the protologue (Descals and Webster 1983) were seen only in CCM F-11883 (Fig. 8 A–E).

Tetracladium marchalianum is well known for the extreme variability of its conidia in nature, which may also appear in pure cultures (Fig. 9). Although the terminal cell of the axis is generally described as globose, it is in fact often short to longer clavate or ellipsoid (e.g., Fig. 9 I, K). In some isolates, this element is bicellular, and constricted at the septum (Fig. 9 O, T), as seen in pure culture (e.g. Roldán et al. 1989; Fig. 11) and in stream foam samples (Roldán et al. 1987b; Fig. 3A). In some conidia, the cell underneath is broadened (Fig. 9 M), sometimes developing one or two short branches on either side of the ultimate cell of the axis (Fig. 9 Y, CC, DD), as also illustrated in several conidia by Matsushima (1983; Fig. 240) in an isolate from leaves in a Canadian pond. Such conidia were relatively frequent in CCM F-11001 from the UK. The isolate CCM F-11391 has conidia similar to those of the neotype (Roldán et al. 1989; Fig. 3).

Tetracladium maxilliforme (Fig. 10, A–I) has conidia similar to those of *T. furcatum*, but the two digitiform elements are parallel or slightly converging. The two narrow obclavate, pointed branches, parallel to the axis in their lower half, typically curve outwards in the upper half. Percurrent

Fig. 9 *Tetracladium marchalianum*, conidia. **A–F**, from CCM F-11391; **G–J**, from CCM F-312; **K–O**, from CCM F-26299; **P–S**, from CCM F-26399; **T–V**, from CCM F-19399; **Y–DD** from CCM F-11001. Scale bar=50µm



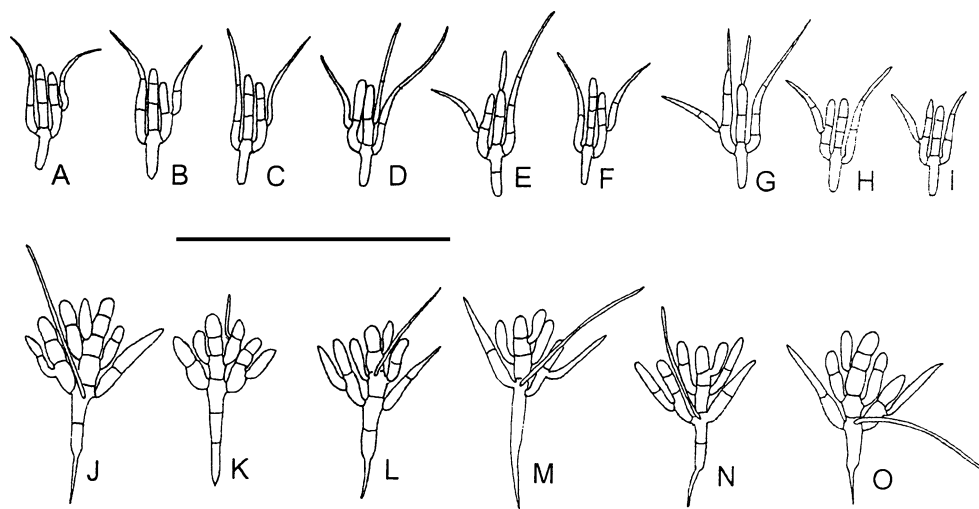
basal extension is absent. The conidia are described laterally flattened in the neotype (Roldán et al. 1989), but in CCM F-526 some conidia bear a diverging thin pointed branch on the axis. This is in accordance with Ingold's later interpretation of the species (Ingold and Ellis 1952). The strain CCM F-13186 produces hyaline inflated cells in culture, which may indicate some relationship to *T. furcatum*.

Tetracladium palmatum has conidia typically with 4–5 digitiform elements plus two narrow obclavate and one filiform branches. The branches arise on three levels of the axis (Fig. 10, F–K). Owing to the capacity of *Tetracladium* species of irregular branching of conidia (instability of branching levels, some branches doubled), there may be

confusion with other species (e.g. Domsch et al. 2007; Fig. 339, one conidium among *T. setigerum* conidia, source not quoted). Conidia of our isolate from Portugal agree well with those of the type as illustrated and described from Spain (Roldán et al. 1989; Fig. 8).

Tetracladium setigerum (Fig. 11, A–O) is characterised by conidia with three digitiform and three narrow obclavate elements. The upper digitiform branch on the axis is often somewhat curved adaxially (Fig. 11). It is represented by three isolates, two from the Czech Republic (CCM F-10186, CCM F-19499) and one from Canada (CCM F-20987), whose conidia resemble those of the neotype by their rather long, pointed branches. Conidia of the two

Fig. 10 *Tetracladium maxilliforme*, conidia. A–F, from CCM F-529; G–T, CCM F-13186. *Tetracladium palmatum*, conidia. J–O from CCM F-10001. Scale bar=50µm



Czech isolates are smaller, similar to each other, with the thin branches shorter and less pointed.

Tetracladium sp. is an isolate from Malaysia. The conidial configuration resembles that of the small-spored Czech isolates of *T. setigerum*, while the conidial size is closer to that of *T. breve*. However, the adaxial curving of the upper digitiform branch is lacking and the diverging filiform branch is rare. At least two reports on similar conidia appeared in the literature: Matsushima (1981; Fig. 19, as *T. setigerum*) depicts conidia of an isolate from Alabama (USA) very similar to those of the Malaysian strain. A different point of view is expressed by Descals (1987, Fig 3, K–O), who, in spite of the occurrence of some pointed branches, sees similarity of such conidia collected in foam in Spain with those of *T. apiense*.

Discussion

Ideally, barcodes are short sequences that can be obtained in a single amplification, are flanked by conserved regions to allow universal primers, and whose interspecific differences exceed intraspecific distances. Identification is straightforward when a sequence is constant within one species and does not occur anywhere else (Hebert et al. 2003). Failing this, intraspecific differences have to be much lower than those among species. Based on many studies in various animal groups, ranging from invertebrates to fish to birds, Hebert et al. (2004) suggest a threshold of a factor of 10 between sequence variation within and among species. In our study, this was only achieved with the ITS sequence. A rigid threshold, however, may overlook species with a short evolutionary history or species exposed to recent hybridization. In addition, an effective test of sequences as potential barcodes requires access to many individuals

from the whole range of its geographic distribution. This is a perennial problem when working with aquatic hyphomycetes. Since the establishment of pure cultures is time-consuming, almost all taxonomic and biogeographical work has been based on spore morphologies or on a limited number of cultures. Our conclusions are therefore preliminary.

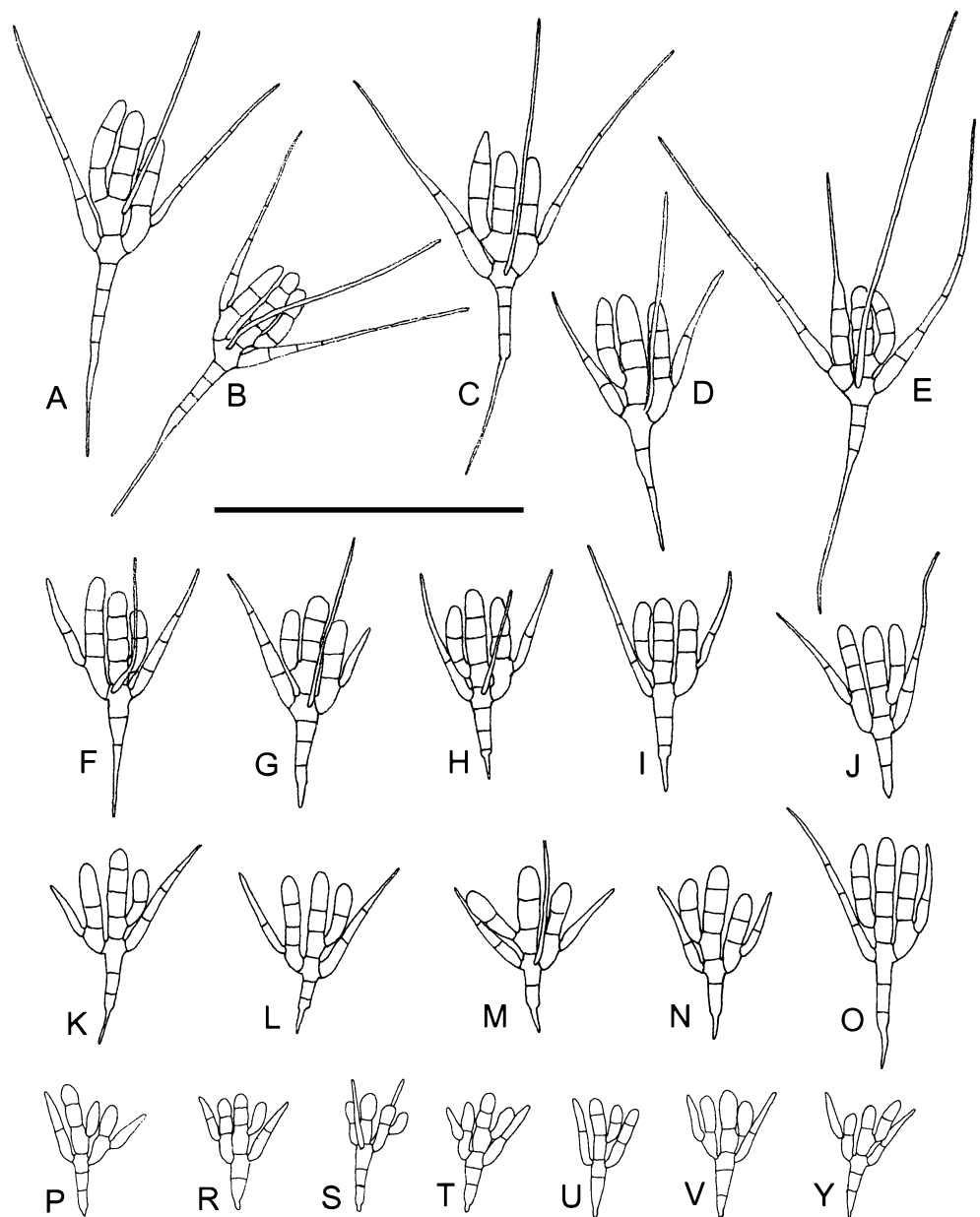
The intergenic spacer region (IGS) amplified poorly, and multiple amplicons were produced, which did not produce well supported consensus sequences. This could be due to the presence of multiple copies of the 5 S region. Or, the primers lacked stringency and annealed to multiple sites. The extreme length variation in this area also supported the conclusion that the IGS region is not suitable for identification, and it was therefore eliminated from further consideration.

Of the remaining three sequences, the most intuitively appealing resolution congruent with conidial morphology is provided by the ITS sequence. *Tetracladium marchalianum* and *T. apiense* are well separated and clearly morphologically distinct from the other species. *Tetracladium marchalianum* is unique with the two short clavate conidial elements (often called “knobs”). Our six isolates appear in one cluster somewhat distantly from the rest of species in all trees.

Tetracladium apiense with its 5–6 similar distal conidial elements and its absence of pointed branches differs clearly from all other members of the genus. A possible intraspecific taxon (CCM F-23299) is separated from the rest of isolates only in the COX1 phylogram, where it clusters with the morphologically dissimilar *T. maxilliforme*.

Three clades of species with 2–3 digitiform and several pointed conidial elements (*T. furcatum*, *T. maxilliforme* and *T. setigerum*) together form a small, well separated cluster in the ITS phylogram. A less distinct clustering

Fig. 11 *Tetracladium setigerum*, conidia. **A–E**, from CCM F-20987; **F–J**, from CCM F-19499; **K–O**, from CCM F-10186. *Tetracladium* sp., conidia. **P–Y**, from CCM F-10008. Scale bar=50 μ m



also appears in the D1/D2 phylogram and in the cladogram. In the COX1 phylogram they are mixed up with other species.

The placement of *T. breve* is variable in all trees. Our two isolates, though morphologically similar to the group of *T. furcatum*, *T. maxilliforme* and *T. setigerum*, are not aligned with each other in any of the phylograms. In two scenarios (D1/D2, ITS), the variable CCM F-10501 appears among or near *T. marchalianum* isolates.

In *T. palmatum*, the relationship to other *Tetracladium* species is also unclear: based on D1/D2, it is isolated; in the COX1 phylogram, it forms a sister clade to both *T. furcatum* strains and based on ITS, it appears in one clade with *T. breve* CCM F-12505.

Tetracladium palmatum and *T. breve* were described from Spain, each from a single location (Roldán et al. 1989), but were later reported as conidia from foam from several other sites in Spain (*T. palmatum*) and also from Portugal (*T. breve*, Descals and Rodríguez Pérez 2002). Our single isolate of *T. palmatum* and two of *T. breve* originate from the Iberian Peninsula. Hence a broader geographical basis for evaluation of morphological intraspecific or interspecific variation is lacking.

Tetracladium sp. appears close to or on a sister branch with *T. apiense* in all trees except in those based on COX1. As explained above, similar conidia were identified as *T. setigerum* (Matsushima 1981) or compared to *T. apiense* (Descals 1987). Unlike *T. setigerum*, *T. apiense* typically

appears in warm climates. The geographical origin of CCM F-20008, of Matsushima's strain No. 9724 from Alabama and of the conidia reported by Descals (1987) from Spain point to the placement of such taxa near *T. apiense* as its marginal variant or as a closely related new species.

To reliably evaluate intraspecific morphological variation in aquatic hyphomycetes will require many monoklonal isolates of the same species, preferably from geographically distant localities and sporulating at standard laboratory conditions. However, in some taxa (notably among staurosporous aquatic hyphomycetes), the morphology of conidia under laboratory conditions tends to differ from that in natural populations (e.g. *Culicidospora grandidata*, *Varicosporium delicatum*). Various methods of inducing sporulation should be tried (Descals 1997). In any case, the number of isolates in our study was too low for detecting intraspecific taxa with certainty.

Phylogenetics

The length of sequences is often coupled to the reliability of trees (Min and Hickey 2007; Zhang et al. 2008; Cai et al. 2009; Tang et al. 2009). The more of the genome is included in the analysis, the greater is the reliability of the tree. Reliability reaches a maximum when the entire genome is examined, which is often the case with viruses (ref). Examination of a 5,000 bp ribosomal gene segment gave a base tree with seven internal nodes of reliability greater than 70% (Fig. 4). Reduction in the length of the sequence to 1,400 bp of SSU decreased internal node reliability to 0, which is comparable to results by Min and Hickey (2007). With the extension of the ITS region into the D1/D2 region, also forming a 1,400 bp sequence, much of the internal node reliability was recovered (Fig. 5) and exceeded the reliability of the 3,000 bp sequence (not shown). Therefore, extending a variable region, such as ITS in this study or COX1 in Min and Hickey (2007), increases internal node reliability. This finding can be exploited for quickly increasing phylogenetic accuracy without greatly increasing the amount of amplification and sequencing. Trees can later be confirmed or refined with additional sequences.

Future work

Clearly, the molecular approach complements classical morphology. Molecular data should therefore be determined and stored for easy access in identification scenarios. Several new techniques may facilitate this development. Standard DNA extraction from pure culture was used for this study but the ultimate goal is to extract DNA from a few or even single aquatic hyphomycete conidia and use that minimal amount of DNA for

identification. This should become feasible as a routine technique in the near future (Bärlocher et al. 2009). It is also important to apply such an approach to taxa other than aquatic hyphomycetes, especially to plant pathogenic genera such as *Colletotrichum* (Hyde et al. 2009a, b; Cai et al. 2009); *Fusarium* (Kvas et al. 2009), *Phoma* (Aveskamp et al. 2008), *Phomopsis* (Santos and Phillips 2009) *Pestalotiopsis* (Jeewon et al. 2002, 2003), and *Phyllosticta* (Wulanderi et al. 2009).

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