Short communication

Some new DNA barcodes of aquatic hyphomycete species

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Text 6 pages; Tables 1; Figures 2

Abstract

Aquatic hyphomycetes play a key role in organic matter processing in freshwaters. Traditionally, species have been identified through the morphology of their conidia, but mycelia can fail to sporulate, making aquatic hyphomycetes a group where DNA barcoding is crucial. We generated ITS barcodes for 9 aquatic hyphomycetes without published ITS sequences that, together with all published sequences, were used to construct a neighbor-joining tree. In general, the new barcoded species grouped with species of the same genus, but *Tricladium* and *Triscelophorus* species were interspersed among different clades of the tree, confirming the multiple origins of aquatic hyphomycetes.

Keywords

Freshwaters, Litter-decomposing fungi, Molecular identification

DNA barcoding is a diagnostic approach in which short DNA sequences (DNA barcodes) are used for species identification in a rapid and inexpensive manner (Hebert et al. 2003). Fungi play pivotal roles in ecosystems and the adoption of culture-independent methods, based on nucleic acids extracted directly from environmental samples, has been increasingly used to examine their diversity (e.g. Nikolcheva et al. 2003, Nilsson et al. 2009). Aquatic hyphomycetes are a polyphyletic group of fungi that play a key role in organic matter processing in freshwaters (Bärlocher 2005). The internal transcribed spacer (ITS) region from rRNA gene was recently designated as the most suitable barcode for fungal identification (Schoch et al. 2012). To date, over 300 aquatic hyphomycete species have been described based on conidium morphology and conidiogenesis (e.g. Gulis et al. 2005), but less than 50 species have published ITS sequences in the International Nucleotide Sequence Database (http://www.ncbi.nlm.nih.gov/) (Duarte et al. 2013). Here, we generated ITS barcodes for 9 species, which were not previously recorded in public databases. At least one complete ITS sequence of all aquatic hyphomycete species present in GenBank was retrieved and a neighbor-joining tree constructed to visualize the clustering of species with known sequences.

New DNA barcodes for 9 aquatic hyphomycete species

Fungi were isolated from single spores (Pascoal et al. 2005) and deposited in the culture collection of the Department of Biology, University of Minho (Braga, Portugal) (UMB). The strains isolated in the current study are available at UMB under request for scientific purposes. All cultures were grown at room temperature on 1% malt agar extract for 15 days before DNA extraction.

DNA was extracted from cultures with the UltraClean® Soil DNA Isolation kit (MoBio Laboratories, Solana Beach, CA, US) according to the manufacturer's instructions. PCR reactions were conducted according to Duarte et al. (2012). The PCR products were cleaned with PureLink™ PCR purification kit according to the manufacturer's instructions (Invitrogen, Life Technologies, CA, US) and the amplicons sequenced at StabVida (Oeiras, Portugal), using ITS1 and ITS4 primers (White et al. 1990).

Our complete dataset consisted of 71 sequences retrieved from NCBI and 55 ITS sequences obtained in our study (Table 1), belonging to 79 aquatic fungal species. Twenty-nine ITS barcodes belonging to 9 aquatic hyphomycete species were obtained for the first time - *Clavatospora longibrachiata*, *Dendrospora tenella*, *Fontanospora eccentrica*, *Lemonniera aquatica*, *Lemonniera* cf. *alabamensis*, *Lunulospora cymbiformis*, *Tricladium attenuatum*, *Triscelophorus* cf. *acuminatus* and *Triscelophorus monosporus*. An additional 7 ITS barcodes were obtained for two unidentified species, *Triscelophorus* sp.1 and *Triscelophorus* sp.2. Conidia of these aquatic hyphomycete species are shown in Fig. 1.

New barcodes for *Alatospora acuminata*, *A. pulchella*, *Tricladium angulatum* and *Varicosporium delicatum* were also generated, and compared with those already in

GenBank. These new barcodes and all ITS sequences for aquatic hyphomycetes published so far were used to construct a neighbor-joining (NJ) tree, using Kimura 2-parameter distance.

Consensus sequences of the new ITS regions were obtained with CodonCode Aligner 2.0.6 (Codon Co., USA). For aquatic hyphomycete species with ITS barcodes deposited in NCBI, at least one sequence belonging to each species was retrieved and used to construct the phylogenetic tree (Fig. 2). Sequences were aligned using ClustalW, divergence was analyzed using Kimura 2-parameter (K2P) distance (Kimura 1980) and the dendrogram was generated with Neighbor-joining (NJ) method, using MEGA4 software (Saitou and Nei 1987), after checking data suitability using the average Jukes-Cantor (JC) distance. Branch support was assessed with bootstrap analysis (1000 replicates) (Tamura et al. 2007). Sequence data obtained during this study were deposited in GenBank (Table 1) and the alignment in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S15040).

Phylogenetic analysis

All fungal species included in this analysis have different ITS sequences. The average sequence divergence using JC distance was 0.288 indicating that our DNA dataset is suitable for making a NJ tree (<1). Using K2P, the average overall sequence divergence (number of base substitutions per site from averaging overall sequence pairs) between the 79 aquatic hyphomycete species was 28.9 ± 1.5%. The 119 ITS sequences were placed in 11 clades within the NJ tree and most of the species belonging to the same genus were dispersed among several clades (Fig. 2).

The ITS barcodes of *Alatospora acuminata*, *A. pulchella*, *Tricladium angulatum* and *Varicosporium delicatum* exhibited taxonomic cohesiveness with those retrieved from NCBI grouping into the same clades and were generally supported by high bootstrap values (≥75%, Fig. 2). New ITS barcodes of *A. acuminata* diverged 3.0 ± 0.7% from *A. acuminata sensu lato* AY204591 and *A. acuminata sensu stricto* AY204587, *V. delicatum* isolates differed 1.3 ± 0.4% from the isolate DQ202520, and *A. pulchella* new isolates diverged 0.5 ± 0.2% from the isolate KC834039. On the other hand, no divergence was found between the new isolates of *T. angulatum* and the AY204610 isolate (Fig. 2).

In the newly sequenced species, ITS barcodes ranged from 511 to 648 bp, for the species *Lemonniera aquatica* and *Clavatospora longibrachiata*, respectively. Isolates belonging to the same species also showed taxonomic cohesiveness grouping in the same clades (Fig. 2). Intraspecific divergence of 0.2 ± 0.2% was found for *L.* cf. *alabamensis* and *Dendrospora tenella* (both comparisons), while no divergence was found within isolates of *L. aquatica*, *Lunulospora cymbiformis*, *Fontanospora eccentrica*, *Triscelophorus* cf. *acuminatus* and *Triscelophorus* sp.2.

Isolates of *F. eccentrica*, *L. aquatica*, *L.* cf. *alabamensis* and *L. cymbiformis* grouped into the clades containing other species of the same genera, i.e., *F. fusiramosa* (clade I), *L. centrosphaera* (clade II) and *L. curvula* (clade IX), respectively, whose

sequences were retrieved from NCBI (Fig. 2). These groups were supported by the low average evolutionary divergence found between the species belonging to the same genera: 0.3 ± 0.07% for *Fontanospora*, 1.3 ± 0.4% for *Lemonniera* and 0.3 ± 0.08% for *Lunulospora*, respectively. On the other hand, the *Triscelophorus* species *T.* cf. *acuminatus*, *T. monosporus* and *Triscelophorus* sp.1 grouped into the same clade as *C. longibrachiata* (clade X) (Fig. 2), but not in the same clade as *Triscelophorus* sp.2 (clade II) and a high average evolutionary divergence of 33.9 ± 2.2% was also found among ITS sequences belonging to species from *Triscelophorus* genus. Based on conidial morphology, it appears that the two unidentified species in this study belong to *Triscelophorus*, but a detailed description is required to determine if these taxa have already been described or are new to science, and if they in fact belong to that genus. In addition to data from conidium morphology and conidiogenesis, barcoding will inform a decision where to place newly isolated aquatic fungal species (e.g. Gulis et al. 2012).

The species *Tricladium attenuatum* grouped with species from clade I, the most diverse clade with respect to the number of genera - *Fontanospora*, *Cladochasiella*, *Varicosporium*, *Gyoerffyella*, *Articulospora*, *Anguillospora* and *Tetrachaetum* - and distant from the remaining *Tricladium* species (Fig. 2). In addition, we also inferred that species of the genera *Articulospora*, *Filosporella*, *Flagellospora*, *Anguillospora* and *Varicosporium* are interspersed among different clades in the NJ tree (Fig. 2). Similar conclusions were reached for species of these genera, using a combination of ITS and partial large subunit rRNA gene regions (Baschien et al. 2013), and for species belonging to the *Tricladium* genus, using ITS barcodes (Seena et al. 2010).

Our study supports the multiple origins of aquatic hyphomycetes (e.g. Belliveau and Bärlocher 2005; Baschien et al. 2006, 2013; Seena et al. 2010) and adds new information on ITS barcodes of aquatic hyphomycetes on NCBI. The enlargement of DNA datasets provides an opportunity to accurately assess biodiversity, and further examine the phylogeny and evolution of aquatic hyphomycetes (Joly et al. 2013). In particular, assessing the identity of fungal species through emerging next-generation sequencing techniques will ultimately depend on a comprehensive reference library of sequences from described species. Its construction with respect to aquatic hyphomycetes is still at an early stage.

Disclosure

The authors declare no conflict of interest.

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Tables

Table 1 – Aquatic hyphomycete species, isolate reference, country of stream location, sampled substrate and GenBank accession number of sequenced isolates in the current study. Isolates are deposited in the culture collection of the Department of Biology, University of Minho (Braga, Portugal) (UMB).

Species	Isolate reference	Stream location[a]	Country	Sampled substrate [b]	GenBank accession number
Alatospora acuminata Ingold	UMB-743.11	Me (51º49'N 6º17'E)	Holland	L	KF730795
	UMB-744.11	Me (51º49'N 6º17'E)	Holland	L	KF730796
	UMB-745.11	Me (51º49'N 6º17'E)	Holland	L	KF730797
	UMB-907.12	Can (40º08'N 8º20'W)	Portugal	L	KF730798
A. pulchella Marvanová	UMB-520.10	Can (40º08'N 8º20'W)	Portugal	L	KF730799
	UMB-559.10	Br (42º07'N 12º13'E)	Italy	L	KF730800
	UMB-560.10	Br (42º07'N 12º13'E)	Italy	L	KF730801
	UMB-581.10	Br (42º07'N 12º13'E)	Italy	L	KF730802
	UMB-647.10	Ho (30º14'S 153º9'E)	Australia	L	KF730803
	UMB-740.11	Me (51º49'N 6º17'E)	Holland	L	KF730804
	UMB-903.12	Can (40º08'N 8º20'W)	Portugal	L	KF730805
	UMB-911.12	Can (40º08'N 8º20'W)	Portugal	L	KF730806
	UMB-912.12	Can (40º08'N 8º20'W)	Portugal	L	KF730807
Clavatospora longibrachiata (Ingold) Sv. Nilsson	UMB-1109.13	An (41º57'N 8.18'W)	Portugal	L	KF730808
	UMB-1200.13	An (41º57'N 8.18'W)	Portugal	L	KF730809
Dendrospora tenella Descals & J. Webster	UMB-891.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730810
	UMB-913.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730811
Fontanospora eccentrica (R.H. Petersen) Dyko	UMB-881.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730812
	UMB-882.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730813
	UMB-883.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730814
	UMB-885.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730815
	UMB-886.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730816
	UMB-888.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730817
	UMB-896.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730818

Lemonniera aquatica De Wild.	UMB-448.09	Re (47º43'N 8º46'E)	Switzerland	L	KF730819
	UMB-449.09	Re (47º43'N 8º46'E)	Switzerland	L	KF730820
	UMB-451.09	Re (47º43'N 8º46'E)	Switzerland	L	KF730821
	UMB-458.10	Be (46º16'N 3º39'E)	France	L	KF730822
	UMB-512.09	Re (47º43'N 8º46'E)	Switzerland	L	KF730823
Lemonniera cf. *alabamensis* R.C. Sinclair & Morgan-Jones	UMB-550.10	Ta (41º23'N 7º69'W)	Portugal	L	KF730824
	UMB-551.10	Ta (41º23'N 7º69'W)	Portugal	L	KF730825
Lunulospora cymbiformis K. Miura	UMB-660.10	NN1 (30º23'S 152º53'E)	Australia	L	KF730826
	UMB-664.10	Le (30º43'S 152º8'E)	Australia	L	KF730827
	UMB-665.10	Le (30º43'S 152º8'E)	Australia	L	KF730828
	UMB-715.10	NN1 (30º23'S 152º53'E)	Australia	L	KF730829
Tricladium angulatum Ingold	UMB-436.09	Re (47º43'N 8º46'E)	Switzerland	F	KF730830
	UMB-437.09	Re (47º43'N 8º46'E)	Switzerland	F	KF730831
	UMB-438.09	Re (47º43'N 8º46'E)	Switzerland	F	KF730832
	UMB-443.09	Re (47º43'N 8º46'E)	Switzerland	F	KF730833
Tricladium attenuatum S. H. Iqbal	UMB-1201.11	Mac (41º45'N 8º08'W)	Portugal	F	KF730834
Triscelophorus cf. *acuminatus* Nawawi	UMB-558.10	Br (42º07'N 12º13'E)	Italy	L	KF730835
	UMB-608.10	Ma (40º33'N 3º32'E)	Spain	L	KF730836
	UMB-875.12	Can (40º08'N 8º20'W)	Portugal	L	KF730837
	UMB-901.12	Can (40º08'N 8º20'W)	Portugal	L	KF730838
	UMB-1118.13	An (41º57'N 8.18'W)	Portugal	L	KF730839
Triscelophorus monosporus Ingold	UMB-709.10	Le (30º43'S 152º8'E)	Australia	L	KF730840
Triscelophorus sp1.	UMB-724.11	NN2 (30º22'S 152º54'E)	Australia	L	KF730841
Triscelophorus sp2.	UMB-949.12	He10 (64º03'N 021º18'W)	Iceland	S	KF730842
	UMB-956.12	He10 (64º03'N 021º18'W)	Iceland	S	KF730843
	UMB-958.12	He10 (64º03'N 021º18'W)	Iceland	S	KF730844

	UMB-959.12	He10 (64º03'N 021º18'W)	Iceland	S	KF730845
	UMB-960.12	He10 (64º03'N 021º18'W)	Iceland	S	KF730846
	UMB-962.12	He10 (64º03'N 021º18'W)	Iceland	S	KF730847
Varicosporium delicatum S.H. Iqbal	UMB-387.09	Ca (41°38'N 8°19'W)	Portugal	F	KF730848
	UMB-390.09	Ca (41°38'N 8°19'W)	Portugal	F	KF730849

[a]Stream locations were: Andorinhas stream (An), Bellinger River (Ho and Le sites), Beron stream (Be), lake Bracciano (Br), Cávado River (Ca), Candal Stream (Can), geothermal Hengill area, site 10 (He10), Maceira Stream (Mac), Madrid Stream (Ma), Meuse River (Me), Never Never River (NN1 and NN2 sites), Resgendorf Stream (Re) and Tanha Stream (Ta).

[b]Sampled substrates were: foam (F), leaves (L) and shirley test cloth (S).

Figure legends

Fig. 1 - Conidia of aquatic hyphomycete species whose ITS sequences were obtained for the first time in this study. A: *Clavatospora longibrachiata* UMB-1200.13, B: *Dendrospora tenella* UMB-891.11, C: *Fontanospora eccentrica* UMB-888.11, D: *Lemonniera* cf. *alabamensis* UMB-550.10, E: *Lemmoniera aquatica* UMB-458.10, F: *Lunulospora cymbiformis* UMB-665.10, G: *Tricladium attenuatum* UMB-1201.11, H: *Triscelophorus* cf. *acuminatus* UMB-558.10, I: *Triscelophorus monosporus* UMB-709.10, J: *Triscelophorus* sp.1 UMB-724.11, K: *Triscelophorus* sp.2 UMB-958.12. Scale bars: A, H-K: 20 µm, B-G: 50 µm.

Fig. 2 - Neighbor joining tree based on ITS sequences belonging to 79 aquatic hyphomycete species, using Kimura 2-parameter distances. Bootstrap values above 50% calculated from 1000 full heuristic replicates are shown at the nodes. Scale bar indicates one base change per 100 nucleotide positions. Sequences obtained in the current study are bordered with grey squares.

Figures

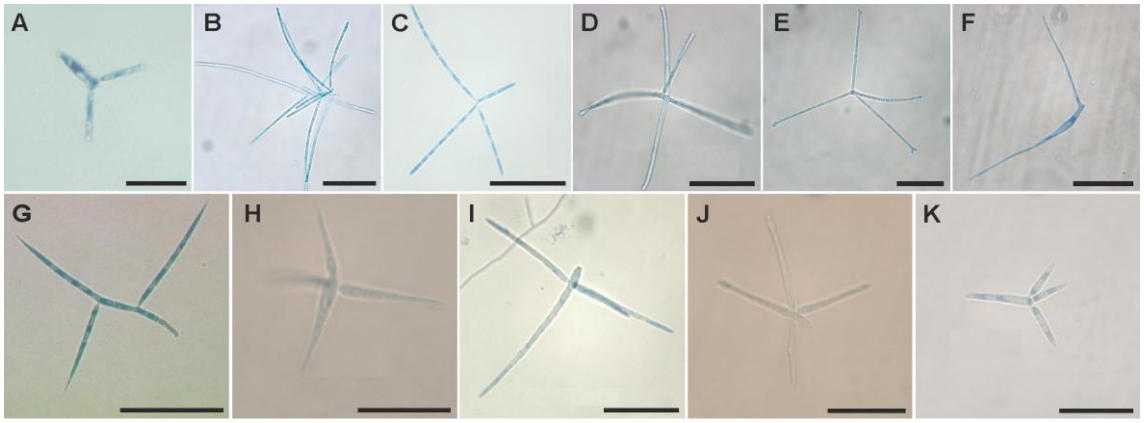
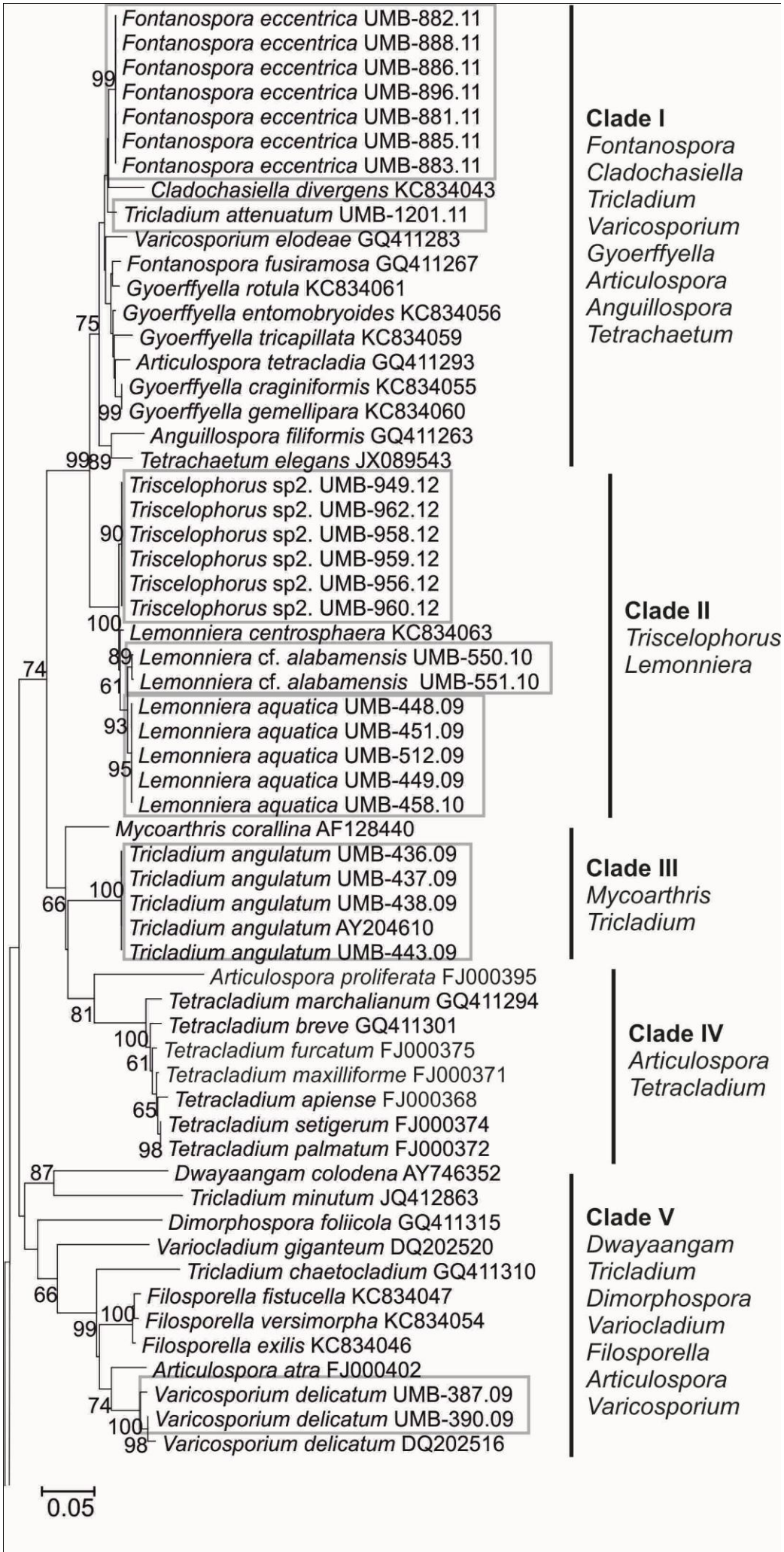


Figure 1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Fontanospora eccentrica UMB-896.11
Fontanospora eccentrica UMB-881.11
Fontanospora eccentrica UMB-885.11
Fontanospora eccentrica UMB-883.11
Cladochasiella divergens KC834043
Tricladium attenuatum UMB-1201.11
Varicosporium elodeae GQ411283
Fontanospora fusiramosa GQ411267
Gyoerffyella rotula KC834061
Gyoerffyella entomobryoides KC834056
Gyoerffyella tricapillata KC834059
Articulospora tetracladia GQ411293
Gyoerffyella craginiformis KC834055
Gyoerffyella gemellipara KC834060
Anguillospora filiformis GQ411263
Tetrachaetum elegans JX089543
Triscelophorus sp2. UMB-949.12
Triscelophorus sp2. UMB-962.12
Triscelophorus sp2. UMB-958.12
Triscelophorus sp2. UMB-959.12
Triscelophorus sp2. UMB-956.12
Triscelophorus sp2. UMB-960.12
Lemonniera centrosphaera KC834063
Lemonniera cf. alabamensis UMB-550.10
Lemonniera cf. alabamensis UMB-551.10
Lemonniera aquatica UMB-448.09
Lemonniera aquatica UMB-451.09
Lemonniera aquatica UMB-512.09
Lemonniera aquatica UMB-449.09
Lemonniera aquatica UMB-458.10
Mycoarthris corallina AF128440
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Tricladium angulatum UMB-437.09
Tricladium angulatum UMB-438.09
Tricladium angulatum AY204610
Tricladium angulatum UMB-443.09
Articulospora proliferata FJ000395
Tetracladium marchalianum GQ411294
Tetracladium breve GQ411301
Tetracladium furcatum FJ000375
Tetracladium maxilliforme FJ000371
Tetracladium apiense FJ000368
Tetracladium setigerum FJ000374
Tetracladium palmatum FJ000372
Dwayaangam colodena AY746352
Tricladium minutum JQ412863
Dimorphospora foliicola GQ411315
Variocladium giganteum DQ202520
Tricladium chaetocladium GQ411310
Filosporella fistucella KC834047
Filosporella versimorpha KC834054
Filosporella exilis KC834046
Articulospora atra FJ000402
Varicosporium delicatum UMB-387.09
Varicosporium delicatum UMB-390.09
Varicosporium delicatum DQ202516
99
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74
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93
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66
100
81
100
61
65
98
87
66
100
99
74
100
98
0.05
Clade I
Fontanospora
Cladochasiella
Tricladium
Varicosporium
Gyoerffyella
Articulospora
Anguillospora
Tetrachaetum
Clade II
Triscelophorus
Lemonniera
Clade III
Mycoarthris
Tricladium
Clade IV
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Variocladium
Filosporella
Articulospora
Varicosporium

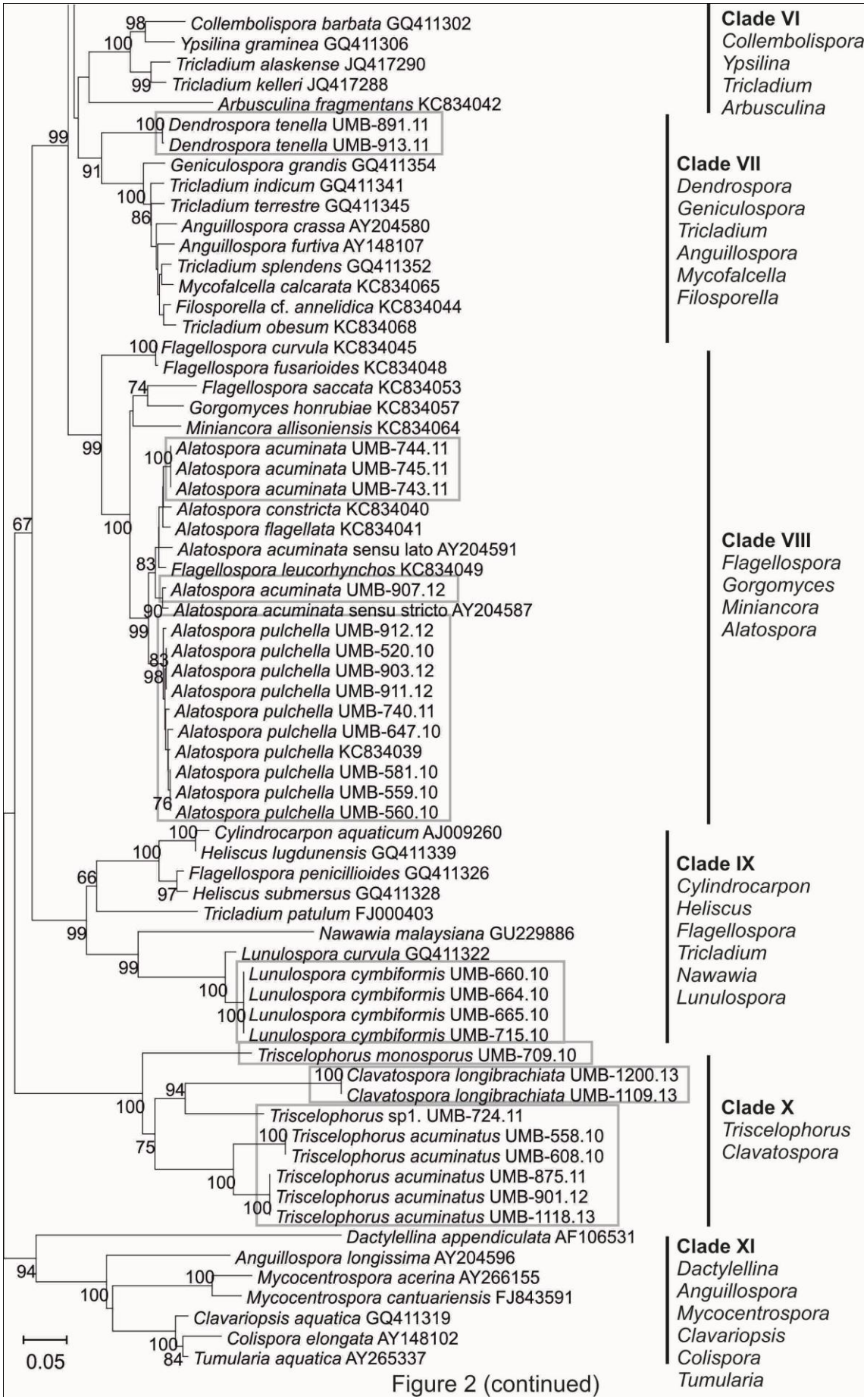

Figure 2 (continued)