

Short communication

Some new DNA barcodes of aquatic hyphomycete species

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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/phyloids/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 $28.9 \pm 1.5\%$. 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 ($\geq 75\%$, Fig. 2). New ITS barcodes of *A. acuminata* diverged $3.0 \pm 0.7\%$ from *A. acuminata sensu lato* AY204591 and *A. acuminata sensu stricto* AY204587, *V. delicatum* isolates differed $1.3 \pm 0.4\%$ from the isolate DQ202520, and *A. pulchella* new isolates diverged $0.5 \pm 0.2\%$ 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 $0.2 \pm 0.2\%$ 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: $0.3 \pm 0.07\%$ for *Fontanospora*, $1.3 \pm 0.4\%$ for *Lemonniera* and $0.3 \pm 0.08\%$ 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 $33.9 \pm 2.2\%$ 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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References

- Bärlocher F, 2005. Freshwater fungal communities. In: Deighton J, Oudemans P, White J (eds), *The fungal community: its organization and role in the ecosystem*, 3rd edn. Taylor and Francis, CRC Press, Boca Raton, pp 39–59.
- Baschien C, Marvanová L, Szewzyk U, 2006. Phylogeny of selected aquatic hyphomycetes based on morphological and molecular data. *Nova Hedwigia* 83: 311–352.
- Baschien C, Tsui CK-M, Gulis V, Szewzyk U, Marvanová L, 2013. The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes. *Fungal Biology* 117: 660–672.
- Belliveau M, Bärlocher F, 2005. Molecular evidence confirms multiple origin of aquatic hyphomycetes. *Mycological Research* 109: 1407–1417.
- Duarte S, Seena S, Bärlocher F, Cássio F, Pascoal C, 2012. Preliminary insights into the phylogeography of six aquatic hyphomycete species. *PLoS ONE* 7: e45289.
- Duarte S, Seena S, Bärlocher F, Pascoal C, Cássio F, 2013. A decade's perspective on the impact of DNA sequencing on aquatic hyphomycete research. *Fungal Biology Reviews* 27: 19–24.
- Gulis V, Baschien C, Marvanová L, 2012. Two new *Tricladium* species from streams in Alaska. *Mycologia* 104: 1510–1516.
- Gulis V, Marvanová L, Descals E, 2005. An illustrated key to the common temperate species of aquatic hyphomycetes. In: Graça MAS, Bärlocher F, Gessner MO (eds), *Methods to study litter decomposition: a practical guide*. Springer, Dordrecht, pp 153–168.
- Hebert PDN, Cywinska A, Ball SL, de Waard JR, 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B – Biological Sciences* 270: 313–321.
- Joly S, Davies TJ, Archambault A, Bruneau A, Derry A, Kembel S, Peres-Neto P, Vamosi J, Wheeler T, 2013. Ecology in the age of DNA barcoding: the resource, the promise, and the challenges ahead. *Molecular Ecology Resources*; doi: 10.1111/1755-0998.12173.
- Kimura M, 1980. A simple method of estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.
- Nikolcheva LG, Cockshutt AM, Bärlocher F, 2003. Determining diversity of freshwater fungi on decomposing leaves: comparison of traditional and molecular approaches. *Applied and Environmental Microbiology* 69: 2548–2554.

- Nilsson RH, Ryberg M, Abarenkov K, Sjökvist E, Kristiansson E, 2009. The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. *FEMS Microbiology Letters* 296: 97–101.
- Pascoal C, Marvanová L, Cássio F, 2005. Aquatic hyphomycete diversity in streams of Northwest Portugal. *Fungal Diversity* 19: 109–128.
- Saitou N, Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406–425.
- Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America* 109: 6241–6246.
- Seena S, Pascoal C, Marvanová L, Cássio F, 2010. DNA barcoding of fungi: a case study using ITS sequences for identifying aquatic hyphomycete species. *Fungal Diversity* 44: 77–87.
- Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596–1599.
- White TJ, Bruns T, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR protocols: a guide to methods and applications*. Academic Press, New York, pp 315–322.

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 |
|---|-------------------|------------------------------|-----------|--------------------------------|--------------------------|
| <i>Alatospora acuminata</i> Ingold | UMB-743.11 | Me (51°49'N 6°17'E) | Holland | L | KF730795 |
| | UMB-744.11 | Me (51°49'N 6°17'E) | Holland | L | KF730796 |
| | UMB-745.11 | Me (51°49'N 6°17'E) | Holland | L | KF730797 |
| <i>A. pulchella</i> Marvanová | UMB-907.12 | Can (40°08'N 8°20'W) | Portugal | L | KF730798 |
| | UMB-520.10 | Can (40°08'N 8°20'W) | Portugal | L | KF730799 |
| | UMB-559.10 | Br (42°07'N 12°13'E) | Italy | L | KF730800 |
| | UMB-560.10 | Br (42°07'N 12°13'E) | Italy | L | KF730801 |
| | UMB-581.10 | Br (42°07'N 12°13'E) | Italy | L | KF730802 |
| | UMB-647.10 | Ho (30°14'S 153°9'E) | Australia | L | KF730803 |
| | UMB-740.11 | Me (51°49'N 6°17'E) | Holland | L | KF730804 |
| | UMB-903.12 | Can (40°08'N 8°20'W) | Portugal | L | KF730805 |
| | UMB-911.12 | Can (40°08'N 8°20'W) | Portugal | L | KF730806 |
| | UMB-912.12 | Can (40°08'N 8°20'W) | Portugal | L | KF730807 |
| <i>Clavatospora longibrachiata</i> (Ingold) Sv. Nilsson | UMB-1109.13 | An (41°57'N 8.18'W) | Portugal | L | KF730808 |
| | UMB-1200.13 | An (41°57'N 8.18'W) | Portugal | L | KF730809 |
| <i>Dendrospora tenella</i> Descals & J. Webster | UMB-891.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730810 |
| | UMB-913.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730811 |
| <i>Fontanospora eccentrica</i> (R.H. Petersen) Dyko | UMB-881.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730812 |
| | UMB-882.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730813 |
| | UMB-883.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730814 |
| | UMB-885.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730815 |
| | UMB-886.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730816 |
| | UMB-888.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730817 |
| | UMB-896.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730818 |

| | | | | | |
|--|-------------|-------------------------|-------------|---|----------|
| <i>Lemonniera aquatica</i> De Wild. | UMB-448.09 | Re (47°43'N 8°46'E) | Switzerland | L | KF730819 |
| | UMB-449.09 | Re (47°43'N 8°46'E) | Switzerland | L | KF730820 |
| | UMB-451.09 | Re (47°43'N 8°46'E) | Switzerland | L | KF730821 |
| | UMB-458.10 | Be (46°16'N 3°39'E) | France | L | KF730822 |
| | UMB-512.09 | Re (47°43'N 8°46'E) | Switzerland | L | KF730823 |
| <i>Lemonniera cf. alabamensis</i> R.C. Sinclair & Morgan-Jones | UMB-550.10 | Ta (41°23'N 7°69'W) | Portugal | L | KF730824 |
| | UMB-551.10 | Ta (41°23'N 7°69'W) | Portugal | L | KF730825 |
| <i>Lunulospora cymbiformis</i> K. Miura | UMB-660.10 | NN1 (30°23'S 152°53'E) | Australia | L | KF730826 |
| | UMB-664.10 | Le (30°43'S 152°8'E) | Australia | L | KF730827 |
| | UMB-665.10 | Le (30°43'S 152°8'E) | Australia | L | KF730828 |
| | UMB-715.10 | NN1 (30°23'S 152°53'E) | Australia | L | KF730829 |
| <i>Tricladium angulatum</i> Ingold | UMB-436.09 | Re (47°43'N 8°46'E) | Switzerland | F | KF730830 |
| | UMB-437.09 | Re (47°43'N 8°46'E) | Switzerland | F | KF730831 |
| | UMB-438.09 | Re (47°43'N 8°46'E) | Switzerland | F | KF730832 |
| | UMB-443.09 | Re (47°43'N 8°46'E) | Switzerland | F | KF730833 |
| <i>Tricladium attenuatum</i> S. H. Iqbal | UMB-1201.11 | Mac (41°45'N 8°08'W) | Portugal | F | KF730834 |
| <i>Triscelophorus cf. acuminatus</i> Nawawi | UMB-558.10 | Br (42°07'N 12°13'E) | Italy | L | KF730835 |
| | UMB-608.10 | Ma (40°33'N 3°32'E) | Spain | L | KF730836 |
| | UMB-875.12 | Can (40°08'N 8°20'W) | Portugal | L | KF730837 |
| | UMB-901.12 | Can (40°08'N 8°20'W) | Portugal | L | KF730838 |
| | UMB-1118.13 | An (41°57'N 8.18'W) | Portugal | L | KF730839 |
| <i>Triscelophorus monosporus</i> Ingold | UMB-709.10 | Le (30°43'S 152°8'E) | Australia | L | KF730840 |
| <i>Triscelophorus</i> sp1. | UMB-724.11 | NN2 (30°22'S 152°54'E) | Australia | L | KF730841 |
| <i>Triscelophorus</i> sp2. | UMB-949.12 | He10 (64°03'N 021°18'W) | Iceland | S | KF730842 |
| | UMB-956.12 | He10 (64°03'N 021°18'W) | Iceland | S | KF730843 |
| | UMB-958.12 | He10 (64°03'N 021°18'W) | Iceland | S | KF730844 |

| | | | | | |
|--|------------|----------------------------|----------|---|----------|
| | UMB-959.12 | He10 (64°03'N 021°18'W) | Iceland | S | KF730845 |
| | UMB-960.12 | He10 (64°03'N 021°18'W) | Iceland | S | KF730846 |
| | UMB-962.12 | He10 (64°03'N 021°18'W) | Iceland | S | KF730847 |
| <i>Varicosporium delicatum</i> 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 |

^aStream 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).

^bSampled 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: *Lemmoniera 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 μ m, B-G: 50 μ m.

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

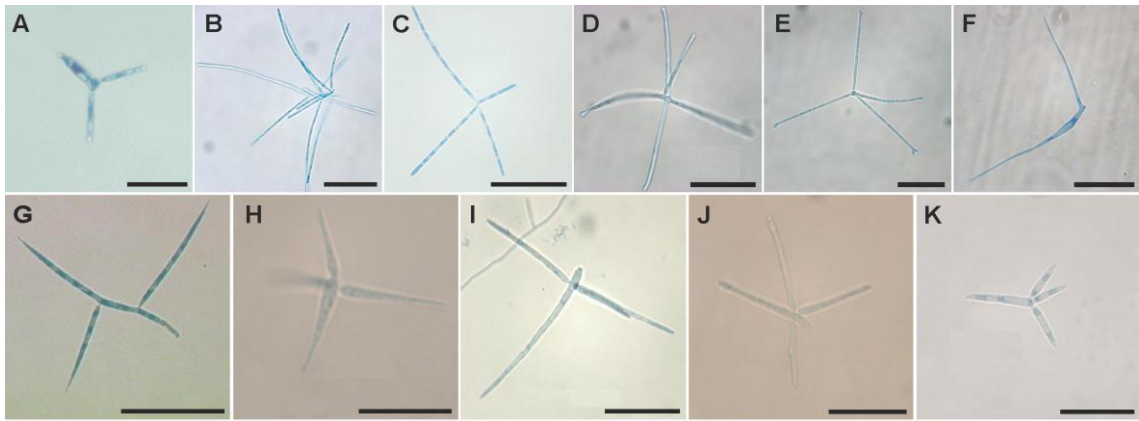
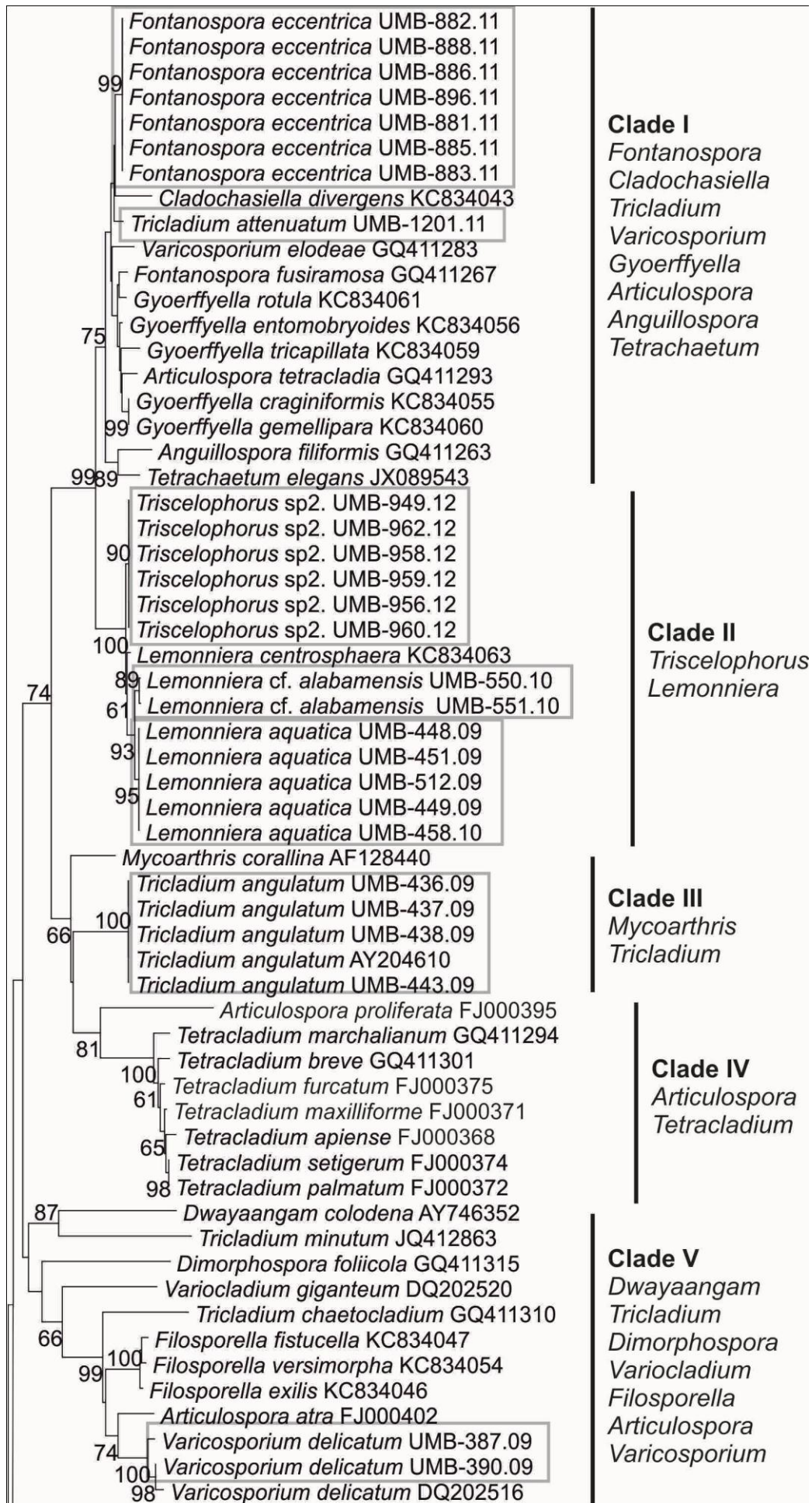


Figure 1



0.05

