

Phylogenetic relationships among taxa in the Jahnulales inferred from 18S and 28S nuclear ribosomal DNA sequences

Jinx Campbell, Astrid Ferrer, Huzefa A. Raja, Somsak Sivichai, and Carol A. Shearer

Abstract: Jahnulales is an order of freshwater, lignicolous, bitunicate ascomycetes characterized by wide (10–40 µm), brown, septate hyphae, stalked and (or) sessile ascospores, ascomal walls of 2–6 layers of large cells, and 1-septate ascospores. A variety of ascospore modifications are represented among the species in the order, including wall roughening, gelatinous sheaths, appendages and (or) pads, and apical caps or spines. To clarify generic boundaries and phylogenetic relationships within the Jahnulales and to assess the taxonomic significance of various morphological characters, a molecular study was carried out using 18S and 28S rDNA sequence data from 15 species representing the four genera in the order. In addition, *Brachiosphaera tropicalis* Nawawi and *Xylomyces chlamydosporus* Goos, R.D. Brooks & Lamore, two mitosporic species that co-occur with *Jahnula* Kirschst., species and have wide (>10 µm), brown, septate hyphae were included in the study to determine whether these species are members of this order. Maximum likelihood analyses confirmed the monophyly of the Jahnulales and resolved four clades. Two robustly supported clades comprise the genera *Aliquandostipite* Inderb. and *Megalohypha* A. Ferrer & Shearer. A third well-supported clade encompassed species of *Brachiosphaera*, *Jahnula*, and *Xylomyces*. The fourth clade contained isolates of the type species of the genus *Jahnula*, *Jahnula aquatica* (Plötn. & Kirschst.) Kirschst., and two other members of this genus, but this clade was weakly supported. Our data suggest that the presence of very wide, brown, septate hyphae is an important character defining the Jahnulales. Based on molecular and morphological data, we propose the transfer of *Jahnula siamensiae* Sivichai & E.B.G. Jones and *Patescospora separans* Abdel-Wahab & El-Shar. to *Aliquandostipite* and emend the description of the Jahnulales.

Key words: Aliquandostipitaceae, ascomycetes, aquatic, Dothideomycetes, systematics.

Résumé : Les Jahnulales constituent un ordre d'Ascomycètes bituniqués caractérisés par des ascospores uniséptées, de larges hyphes (10–40 µm) bruns et septés, des parois ascospores avec 2–6 couches de cellules, et des ascospores uniséptées. On retrouve une variété de modifications des ascospores parmi les espèces de l'ordre, incluant une rugosité de la paroi, des enveloppes gélatineuses, des appendices et/ou des coussins, et des épines ou capuchons apicaux. Afin de délimiter les limites génériques et les relations phylogénétiques au sein des Jahnulales, et pour évaluer la signification taxonomique des divers caractères morphologiques, les auteurs ont conduit une étude moléculaire en utilisant les données de séquences des 18S et 28S rADN, provenant de 15 espèces et représentant quatre genres de l'ordre. De plus, on a inclus dans cette étude le *Brachiosphaera tropicalis* Nawawi et le *Xylomyces chlamydosporus* Goos, R.D. Brooks & Lamore, deux espèces mitosporiques qu'on retrouve avec les espèces de *Jahnula*, et qui possèdent des hyphes (>10 µm), bruns, et septés. Ces inclusions visent à déterminer si ces espèces appartiennent à l'ordre. Des analyses de probabilité maximale confirment la monophylie des Jahnulales et reconnaissent quatre clades. Deux clades robustement supportés comprennent les genres *Aliquandostipite* et *Megalohypha*. Un troisième clade bien étayé regroupe des espèces de *Brachiosphaera*, *Jahnula* et *Xylomyces*. Le quatrième clade contient des isolats de l'espèce type du genre *Jahnula*, le *Jahnula aquatica* (Plötn. & Kirschst.) Kirschst., et deux autres membres de ce genre, mais ce clade est faiblement supporté. Les données suggèrent que la présence d'hyphes bruns, très larges et septés constitue un caractère important pour définir les Jahnulales. Sur la base des données moléculaires et morphologiques, les auteurs proposent de transférer le *J. siamensiae* et le *Patescospora separans* Abdel-Wahab & El-Shar. au genre *Aliquandostipite* et éminent la description des Jahnulales.

Mots-clés : Aliquandostipitaceae, ascomycètes, aquatique, Dothidéomycètes, systématique.

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Introduction

The order Jahnulales Pang et al. (Dothideomycetes, Ascomycota), was established for species in the genera *Aliquandostipite* Inderb., *Jahnula* Kirschst., and *Patescospora* Abdel-Wahab & El-Shar., based on the analysis of 18S rDNA sequence data (Pang et al. 2002). The six species used in this study formed a well-supported clade, sister to a clade that included representatives of the Patellariales and Pleosporales (Pang et al. 2002). Two well-supported subclades were resolved within the Jahnulales. The first included species with obclavate asci containing an ocular chamber and ring at the ascus apex [*Jahnula sunyatsenii* (Inderb.) Pang et al., *Jahnula bipolaris* (K.D. Hyde) K.D. Hyde, and *Jahnula australiensis* K.D. Hyde]. The second included *Jahnula siamensiae* Sivichai & E.B.G. Jones, and two species with broadly clavate asci lacking an apical ring (*Aliquandostipite khaoyaiensis* Inderb. and *Patescospora separans* Abdel-Wahab & El-Shar.). The authors did not comment on the positioning of species of *Jahnula* in the two clades, and it remains unclear if one (or more) of the taxa included in their study had been assigned to the wrong genus.

Since the study by Pang et al. (2002), an additional new genus (Ferrer and Shearer 2007) and several new species (Raja et al. 2005; Raja and Shearer 2006, 2007) have been described in the Jahnulales. Currently, the order contains a single family, the Aliquandostipitaceae Inderb., and four genera, *Aliquandostipite* (four species), *Jahnula* (15 species), *Megalohypha* A. Ferrer & Shearer (one species), and *Patescospora* (one species). All of the species in the Jahnulales produce very wide (10–40 µm), brown, septate hyphae in culture and their ascomata are attached to one another and their substrates by wide (>10 µm), brown hyphae (Raja and Shearer 2006). In addition, the ascomal walls consist of large angular cells with large cell lumens, a feature considered to be adaptive to aquatic habitats (Hawksworth 1984). Ascospores of the Jahnulales species are 1-septate and hyaline, pale brown or dark brown. A variety of ascospore modifications are represented among the species in Jahnulales, including presence or absence and type of wall roughening, and presence or absence and morphology of gelatinous sheaths, appendages and pads, and apical caps and spines. The phylogenetic significance of these ascospore characters, however, is poorly understood.

To further clarify relationships among taxa in the Jahnulales, and to assess the phylogenetic significance of ascospore characters, we undertook a molecular and morphological study that involved more taxa and sequence data (28S rDNA in addition to 18S rDNA) than used in previous studies (Inderbitzin et al. 2001; Pang et al. 2002). In addition, we recently isolated two mitosporic fungi from submerged, decorticated woody debris with hyphal characteristics of the Jahnulales: *Brachiosphaera tropicalis* Nawawi and *Xylomyces chlamydosporus* Goos, R.D. Brooks & Lamore. We found both of these fungi growing in association with species of *Jahnula* and included them in our study to

determine whether they had phylogenetic affinities to this order.

In this study, we addressed the following questions: (i) Is the order Jahnulales monophyletic based on combined 18S and 28S rDNA sequences? (ii) Do molecular data support the morphological characters currently used to delineate genera in the Jahnulales? (iii) What are the phylogenetic relationships among the genera of Jahnulales? (iv) Are the freshwater lignicolous mitosporic fungi, *B. tropicalis* and *X. chlamydosporus* members of the Jahnulales?

Materials and methods

Fungal strains and morphological studies

All specimens isolated for this study were obtained from submerged woody debris collected from freshwater habitats according to the procedures of Shearer et al. (2004). Cultures are maintained at the American Type Culture Collection (ATCC) or the BIOTEC Culture Collection (Table 1). For the morphological study, herbarium specimens and fresh collections were examined where possible (see supplementary data²). Original and other published descriptions were consulted in the absence of available specimens. Collector's names are abbreviated: JLA (Jennifer L. Anderson), AF (Astrid Ferrer), NB (Nuttawut Boonyuen), CB (Christopher Brown), JLC (J.L. Crane), ANM (Andrew N. Miller), CMP (Cathy M. Pringle), NH (Nate Hamburger), HAR (Huzefa A. Raja), EBL (Edgar B. Lickey), CAS (Carol A. Shearer), SS (Somsak Sivichai), RW (Rebecca Wulffen). The procedures for sectioning, photographing and preserving specimens are outlined in Raja and Shearer (2006).

Sequence determination

The Assembling the Fungal Tree of Life project currently uses seven loci to resolve the phylogeny of the Kingdom Fungi at all taxonomic levels. In this study we used two of those loci, namely the small and large subunit RNA genes, as these are known to resolve phylogeny at the ordinal, familial, and genus level.

Fungal isolates were grown on peptone–yeast–glucose agar. Growth period ranged from two to four weeks. For extraction of genomic DNA, mycelia from axenic cultures were scraped from culture plates using a sterile scalpel and ground to a fine powder in liquid nitrogen with a mortar and pestle. About 400 µL of AP1 buffer from the DNAeasy Plant Mini Kit (Qiagen Inc., Valencia, Calif.) was added to the mycelial powder and DNA was extracted following the manufacturer's instructions. Total genomic DNA was observed on a 1% agarose gel stained with ethidium-bromide.

Fragments of partial small subunit (SSU) and large subunit (LSU) rDNA were amplified by the polymerase chain reaction (PCR) using puReTaq™ Ready-To-Go PCR beads (Amersham Biosciences Corp, Piscataway, N.Y.) according to Huhndorf et al. (2004). Primers NS1 and NS4 for SSU (White et al. 1990), and LROR and LR6 for LSU (Vilgalys and Hester 1990) were used for PCR reactions. PCR products were then purified to remove excess primers, dNTP's

²Supplementary data for this article are available on the journal Web site (<http://cjb.nrc.ca>) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5211. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

Table 1. Taxa included in this study (accession numbers are included in this table).

Taxon	Isolate No.	Accession No.	Locality	GenBank accession No.	
				18S nuc-rDNA	28S nuc-rDNA
<i>Aliquandostipite crystallinus</i> Raja, A. Ferrer & Shearer	R76-1	—	USA (TN)	EF175630	EF175651
	AF007	N/A	Panama	EF175631	EF175652
	A514-1	N/A	Costa Rica	EF175629	—
<i>A. khaoyaiensis</i> Inderb.	SS2961	BCC ^a 15577	Thailand	EF175626	EF175648
	SS3028	BCC 23986	Thailand	EF175627	EF175649
	SS3321	BCC 18283	Thailand	EF175628	EF175650
	F89-1	MYA ^b 4170	USA (FL)	EF175625	EF175647
<i>Brachiosphaera tropicalis</i> Nawawi	E192-1	N/A	Panama	—	EF175653
<i>Jahnula appendiculata</i> Pinruan et al.	SS2900	BCC 14536	Thailand	—	EF175654
<i>J. aquatica</i> (Plöttner & Kirschst.) Kirschst.	R68-1	MYA 4172	USA (IL)	EF175633	EF175655
	R68-2	—	USA (NC)	EF175632	—
<i>J. bipileata</i> Raja & Shearer	F49-1	MYA 4173	USA (FL)	EF175635	EF175657
	AF220-1	—	Ecuador	EF175634	EF175656
<i>J. bipolaris</i> (K.D. Hyde) K.D. Hyde	SS44	BCC 3390	Thailand	EF175637	EF175658
	A421	—	Costa Rica	EF175636	—
<i>J. granulosa</i> K.D. Hyde & S.W. Wong	SS1567	BCC 24222	Thailand	EF175638	EF175659
<i>J. rostrata</i> Raja & Shearer	F4-3	MYA 4176	USA (FL)	—	EF175660
<i>J. sangamonensis</i> Shearer & Raja	A482-1B	MYA 4174	USA (AR)	EF175640	EF175662
	A402-1B	—	USA (IL)	EF175639	EF175661
	F81-1	MYA 4175	USA (FL)	EF175641	EF175663
<i>J. seychellensis</i> K.D. Hyde & S.W. Wong	SS2113.1	BCC 14207	Thailand	EF175644	EF175665
	SS2113.2	BCC 12957	Thailand	EF175643	EF175664
	A492	N/A	Costa Rica	EF175642	—
<i>J. siamensis</i> Sivichai & E.B.G. Jones	SS81.02	BCC 3417	Thailand	EF175645	EF175666
<i>Megalohypha aqua-dulces</i> A. Ferrer & Shearer	AF005-2a	N/A	Panama	EF175646	EF175667
	AF005-2b	N/A	Panama	—	EF175668
<i>Xylomyces chlamydosporus</i> Goos et al.	H058-4	MYA 4177	USA (NC)	—	EF175669

^aBCC, Biotec Culture Collection, Thailand.

^bMYA, American Type Culture Collection.

and nonspecific amplification products using the Qiaquick PCR Purification Kit (Qiagen Inc.). Purified PCR products were used in 11 µL sequencing reactions utilizing BigDye[®] Terminators version 3.1 (Applied Biosystems, Foster City, Calif.) in combination with the SSU primers NS1, NS2, NS3, NS4 primers (White et al. 1990) and the LSU primers LR0R, LR3, LR3R, LR6, LARAM1, and LR5 primers (Vilgalys and Hester 1990; Rehner and Samuels 1995; Huhndorf et al. 2004) for LSU.

Taxon sampling

Species sequenced in this study and their isolate numbers and (or) ATCC or BCC numbers, collection localities, and GenBank accession numbers are listed in Table 1. The 41 additional species included in this study and their GenBank accession numbers are as follows: *Aliquandostipite*

khaoyaiensis AF201453; *Aureobasidium pullulans* (de Bary) G. Arnaud DQ471004, DQ470956; *Botryomyces caespitosus* de Hoog & C. Rubio Y18695; *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx U42476, AY928054; *Botryosphaeria ribis* Grossenb. & Duggar BRU42477, AY004336; *Candida valdiviana* Grimb. & Yarrow AB015910, U45835; *Capronia mansonii* (Schol-Schwarz) E. Müll. et al. X79318, AY004338; *Capronia pilosella* (P. Karst.) E. Müll. et al. U42473, AF279378; *Castanedomyces australiensis* Cano et al. AJ131786; *Ceramothyrium linnaeae* (Dearn.) S.J. Hughes AF022715; *Chromocleista cinnabarina* Yaguchi & Udagawa AB003952, AB047225; *Coccodinium bartschii* A. Massal. U77668; *Dothidea hippophaeos* (Passerini) Fuckel U42475; *Dothidea insculpta* Wallr. DQ247810, DQ247802; *Dothidea sambuci* (Pers.) Fr. AY544722, AY544681; *Elsinoë veneta* (Burkh.) Jenkins U43467,

U43484; *Hamigera avellanea* Stolk & Samson D14406, AB000620; *Helvella terrestris* (Velen.) Landvik AF046216; *Herpotrichia diffusa* (Schwein.) Ellis & Everh., U42484; *Herpotrichia juniperi* (Duby) Petr. U42483, DQ384093; *Hobsonia santessonii* Lowen & D. Hawksw. AF289658; *Jahnula australiensis* K.D. Hyde AF438182; *Jahnula siamensis* Sivichai & E.B.G. Jones AF438180; *Jahnula sunyatsenii* (Inderb.) K.L. Pang et al. AF201454; *Leptosphaeria doliolum* (Pers. ex Fr.) Ces. & de Not. U04205, U43475; *Leptosphaeria microscopica* P. Karst. U04235; *Letendraea helminthicola* (Berk. & Broome) Weese AY016345, AY016362; *Myriangium duriaei* Mont. & Berk. AF242266, AY016365; *Patescospora separans* Abdel-wahab & El. Shar. AF438179; *Pleospora herbarum* P. Karst. U05201, AF382386; *Preussia terricola* Cain AY544726, AY544686; *Pseudoamauroascus australiensis* Cano et al. AJ131788; *Pyrenophora tritici-repentis* (Died.) Dreschler U42486, AY544672; *Rhytidhysterium rufulum* (Spreng.) Speg. AF201452; *Rhytidhysterium rufulum* U20506; *Saccharomyces cerevisiae* Gasp. J01353, DQ674258; *Scorias spongiosa* (Schwein.) Fr. AF006726; *Talaromyces bacillisporus* (Swift) C.R. Benj., D14409; *Tubeufia helicoma* (W. Phillips & Plowr.) Piroz., AF201455, AY787939; *Tubeufia helicomyces* Höhn., AY856933, AY856887; *Wynnella silvicola* (Berk. ex Sacc.) Harmaja, U42655, U42682.

Phylogenetic analyses

Sequences were aligned with published sequence data using Clustal X (Thompson et al. 1997), and then refined manually in Se-Al (Rambaut 1996). Maximum parsimony and weighted parsimony analyses of the combined SSU and LSU rDNA sequence data were performed using PAUP*4.0b 10 (Swofford 2002). Gaps were treated as missing data. Owing to the dense taxonomic sampling of a number of the terminal clades, i.e., multiple representatives of several species, a two-step search approach was employed in an attempt to avoid local optima (Olmstead et al. 1993). Step one consisted of 100 heuristic replicates with random starting trees, random stepwise addition and tree-bisection-reconnection branch swapping with MulTrees off; a maximum of two trees was saved per replicate. All of the shortest trees from these initial runs were saved and then used as starting trees for the second step, which consisted of searches with MulTrees on. Weighted parsimony analyses, using the two-step approach as above, were performed using a step matrix to weight nucleotide transformations based on the reciprocal of the observed transition:transversion (ti/tv) ratio (Spatafora et al. 1998). Representatives from other orders of the Dothideomycetes were included to determine their affinities with the Jahnulales. Members of the Saccharomycotina (Saccharomycetales) were used as outgroups.

Maximum parsimony analyses were also performed on constrained topologies. The constraint tree included a clade of all *Jahnula* species. Heuristic searches were run with MulTrees off and repeated with MulTrees on, for 100 replicates using the same search criteria as for the unconstrained analyses and keeping trees only compatible with the constraint tree. A Shimodaira-Hasegawa (S-H) test (Shimodaira and Hasegawa 1999) was performed as implemented in

PAUP* to determine if the trees were significantly worse than the trees from the unconstrained analyses.

Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMCMC) analyses of the combined SSU and LSU rDNA sequence data were performed using MrBayes 3.0 (Huelsenbeck and Ronquist 2001). Searches were conducted for a total of 1 000 000 generations with phylogenetic trees sampled every 100 generations, employing the general time reversible model of substitution (Rodriguez et al. 1990) with invariant sites and gamma distribution (GTR+I+G). Four independent B-MCMCMC analyses were conducted to verify likelihood convergence and burn in parameter. The initial 1443 trees (144 300 generations) were identified as burn-in prior to the convergence of likelihoods and were excluded from post-run analyses. A majority rule consensus tree of 8557 trees was generated, along with average branch lengths and posterior probabilities.

The maximum likelihood model was selected using Modeltest (Posada and Crandall 1998), which selects the appropriate evolutionary model for the dataset. Analyses were then performed in PAUP* with heuristic searches and a tree-bisection-reconnection branch-swapping algorithm, and the evolutionary model set to the transition model: variable base frequencies, variable transitions, transversions equal, with invariant sites and gamma distribution (TIM+I+G).

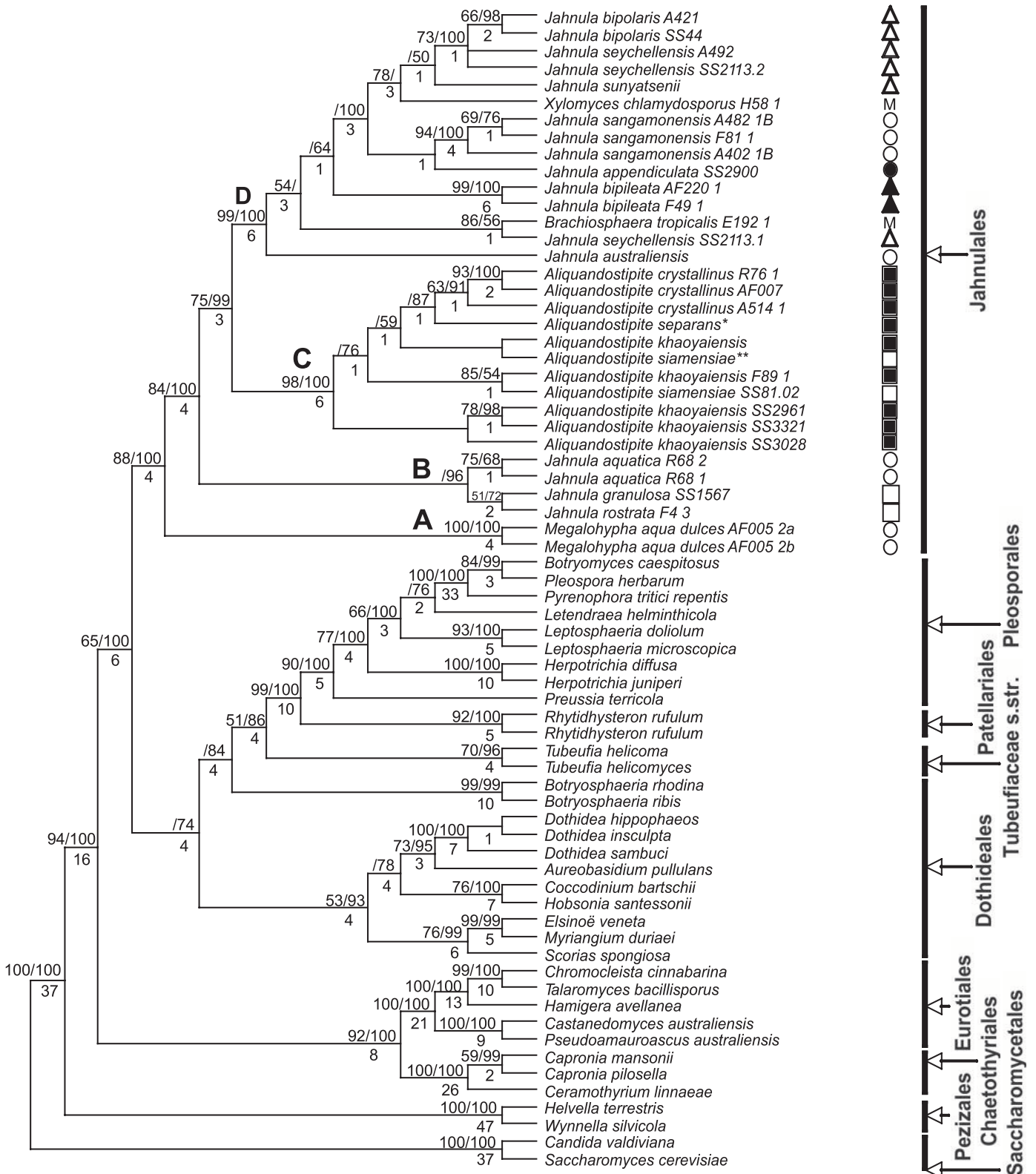
Bootstrap values (Felsenstein 1985) were calculated from 1000 replications using a heuristic search on 100 replicates with random starting trees, random stepwise addition and MulTrees off. Decay indices (Bremer 1988, 1994) were calculated in AutoDecay (Eriksson 1998).

The alternative tree topologies were tested in PAUP* using a Kishino-Hasegawa (K-H) test (Kishino and Hasegawa 1989), and an S-H test (Shimodaira and Hasegawa 1999).

Results

The K-H and S-H tests (results not shown) showed that the tree inferred in the maximum likelihood (ML) analyses (Fig. 1) was the best phylogenetic hypothesis for the data. The trees generated under maximum parsimony, weighted parsimony, and Bayesian analyses were significantly worse in the K-H test, but there was no significant difference ($P < 0.05$) among the trees in the S-H test. This result is not particularly surprising. From a statistical point of view, the inference of phylogenies is similar to the estimation of an unknown quantity in the presence of uncertainty. Given the intrinsic uncertainty in solving phylogenetic relationships from a limited number of samples, it is necessary to assume that phylogenetic estimates are subject to stochastic and systematic errors (Huelsenbeck et al. 2000). Consequently, the correct answer to a phylogenetic problem is not a single estimate of one topology that is optimal under the assumptions of a particular phylogenetic reconstruction method. Rather, it is more appropriate to derive a set of phylogenies that confine the uncertainty about the solution to the phylogenetic reconstruction problem from the available data (Czarna et al. 2006). Statistical tests of phylogenies based on maximum likelihood include the K-H and S-H tests. These tests can give contradictory results (Goldman et al. 2000; Strimmer and Rambaut 2002), which is due in part to the trees being tested. The K-H test is least biased when

Fig. 1. Cladogram of the best tree inferred from the maximum likelihood analyses of the combined 18S and 28S rDNA data. Bootstrap values and Bayesian posterior probabilities greater than 50%, respectively, are given above the corresponding nodes. Decay indices are indicated below corresponding nodes. *, formerly *Patescospora separans*. **, formerly *Jahnula siamensis*. ○, no sheath, pad or appendage; ●, elongating apical appendage and broad gelatinous sheath; △, gelatinous apical pads; ▲, caps; □, thin gelatinous sheath; ■, broad gelatinous sheath; M, mitosporic. The outgroup taxa are *Candida valdiviana*, and *Saccharomyces cerevisiae*.



the candidate trees have been fully specified a priori, based on independent evidence, and there is a large likelihood difference between them; the S–H test is least biased when the candidate trees have been selected based entirely on the data of interest, and are usually similar in likelihood. In our study, both tests indicated that the maximum likelihood tree was the “best” tree. The only discrepancies were that the S–H test found no difference in the remaining trees, whereas the K–H test determined that there were differences among the remaining trees. As our intention was only to find the “best” tree and both tests indicated it was the ML tree, we are confident that the tests are not biased. The tree topologies were similar in the ML tree and Bayesian tree. In the Bayesian tree, *Jahnula aquatica* (Plötn. & Kirschst.) Kirschst., *Jahnula granulosa* K.D. Hyde & S.W. Wong, and *Jahnula rostrata* Raja & Shearer were members of a monophyletic group, whereas in the maximum and weighted parsimony trees, *J. aquatica* was positioned in a clade by itself.

The maximum-likelihood tree (Fig. 1) is 2721 steps in length, two steps longer than the most parsimonious tree, with a Consistency Index (CI) of 0.55, Retention Index (RI) of 0.76, and Rescaled Consistency Index (RC) of 0.42. As indicated in previous studies with fewer taxa (Inderbitzin et al. 2001; Pang et al. 2002), Jahnulales is monophyletic with 88% bootstrap support and 100% posterior probability, and separate from other orders of Dothideomycetes included in the analyses. *Megalohypha aqua-dulces* occurs as a monophyletic clade (clade A; Fig. 1) with 100% bootstrap support and 100% posterior probability, basal to and sister to all other taxa in the Jahnulales clade. Isolates of *J. aquatica* form a monophyletic group (clade B; Fig. 1) and are members of a poorly supported clade (bootstrap support < 50% and posterior probability 96%), that includes *J. granulosa* and *J. rostrata*. *Aliquandostipite khaoyaiensis* is in a clade with *A. crystallinus*, *J. siamensiae*, and *P. separans* (clade C; Fig. 1) with 98% bootstrap support and 100% posterior probability, while *J. sangamonensis*, *J. appendiculata*, *J. bipileata*, *J. bipolaris*, *J. sunyatsenii*, *J. australiensis*, *J. seychellensis*, *X. chlamyosporus*, and *B. tropicalis* form a monophyletic clade (clade D; Fig. 1) with 99% bootstrap support and 100% posterior probability.

Maximum parsimony analysis on the constrained topology, which forced the monophyly of the *Jahnula* species, generated one tree of length 2758; 39 steps longer than the most parsimonious tree. An S–H test (data not shown) indicated that the constrained tree was significantly worse than all of the other trees generated in the unconstrained analyses ($P = 0.0001$).

Discussion

The wide, septate, subhyaline to brown hyphae that are attached to the peridial walls of the ascumata is a morphological character that appears to be unique to the Jahnulales. This type of hyphae is present in collections of all taxa currently described in the order and ranges from 10 to 40 μm wide. The widest hyphae (40 μm) form the hyphal stalks supporting ascumata in *A. khaoyaiensis* (Inderbitzin et al. 2001) and *M. aqua-dulces* (Ferrer and Shearer 2007). Wide hyphae are also produced in cultures of species of *Aliquandostipite* (Inderbitzin et al. 2001),

Jahnula (Raja and Shearer 2006), *Megalohypha* (Ferrer and Shearer 2007), *Patescospora* (Pang et al. 2002), and the mitosporic fungi, *B. tropicalis* and *X. chlamyosporus*. On natural substrates, the wide hyphae of many Jahnulales species are present before the ascumata appear and anchor the ascumatal bases to their substrates (Raja and Shearer 2006).

In most Jahnulales species, ascumata are sessile and partly to mostly immersed, often erupting through the softened wood at maturity leaving a small crater-like depression in the wood. In *A. khaoyaiensis*, *J. sunyatsenii*, *J. seychellensis*, and *M. aqua-dulces*, ascumata are sessile and (or) formed at the tips of long stalks (Inderbitzin et al. 2001; Ferrer and Shearer 2007). Whether the genetic capability to form stalked ascumata is common to all taxa in Jahnulales but expressed only under certain environmental conditions is not yet known.

All the taxa in the Jahnulales clade are also similar in having (i) globose to subglobose, ostiolate ascumata; (ii) peridial walls of a few (2–6) cell layers, the outer layers of which consist of large, polyhedral cells with a large lumen; (iii) cellular pseudoparaphyses; (iv) bitunicate, fissitunicate asci; and (v) 1-septate, multiguttulate, ascospores. Large peridial cells, although distinctive and present in all Jahnulales taxa, can be found in other orders and families within the Pezzizomycotina, such as the Hypocreales, Sordariales, and Tubeufiaceae s. str.

Asci in Jahnulales species range in shape from narrowly cylindrical, ovoid, obovate to cymbiform. The distribution of these character states does not correlate well with the molecular-based phylogeny. Narrow, cylindrical asci occur in species in the second to the most basal clade (clade B; Fig. 1) and in the most derived clade (clade D; Fig. 1), with various other forms in between.

Ascospores in Jahnulales are generally 1-septate, although additional septa may be produced in older or germinating ascospores. Other ascospore morphological characters, except for some appendage and (or) sheath characters, generally do not correlate well with the phylogeny inferred from analyses of rDNA sequences. An exception is the sulcate wall found only in *M. aqua-dulces* (Ferrer and Shearer 2007). This species forms a clade separate from other species in the order (Fig. 1). Species in the *Aliquandostipite* clade, with the exception of *J. siamensiae*, all have broad, slug-like gelatinous sheaths. Clade D (Fig. 1) encompasses species that differ in ascospore characters such as pigmentation, wall roughening, and the presence or absence of gelatinous pads, caps, appendages, or sheaths.

When the order Jahnulales was established, Pang et al. (2002) suggested that the presence of wide, brown hyphae might be an apomorphic character for the order. They did not, however, include this character in the ordinal description. Results from our study indicate that presence of wide, brown hyphae is common to all species in the Jahnulales clade. In addition, more species have been included in the order since it was established (Raja et al. 2005; Raja and Shearer 2006, 2007; Ferrer and Shearer 2007) and a wider variation in ascospore characters is now known. For these reasons, we are emending the description of Jahnulales to reflect this new information.

Jahnulales Pang et al. emended

Ascomata globose to subglobose, with a long, wide, brown, septate stalk or sessile and attached to substrate by wide (usually >10 µm), brown septate hyphae, immersed, partly immersed, or superficial, ostiolate, papillate, coriaceous to subcarbonaceous, hyaline, pale brown or black. Peridium thick, comprised of a few layers of relatively large cells. Hamathecium pseudoparaphysate, hyphal-like, filamentous, septate, unbranched between the asci, branching and anastomosing above the asci or septate, branched and persistent, separating to form cavities as asci separate from ascogenous hyphae to move upwards. Asci ovoid, cymbiform, saccate, clavate or cylindrical, thick-walled, bitunicate, fissitunicate, persistent or deliquescent. Ascospores ellipsoid–fusiform, 1-septate, becoming 3- or 4-septate at germination, apical cell slightly larger or equal in size to the basal cell, slightly or strongly constricted at the midseptum, smooth or rough-walled, with or without a gelatinous sheath, gelatinous pads, apical cellular appendages or elongated gelatinous apical appendages. Hyphae in culture wide (usually >10 µm), brown, septate unconstricted to strongly constricted at the septa. *Included family*: Aliquandostipitaceae Inderb. Am. J. Bot. (2001) 88: 54.

The most basal clade (clade A; Fig. 1) of the Jahnulales contains a single genus and species, *M. aqua-dulces*, a taxon distinguished from others in the order by ascospore and hyphal characters. The ascospores of this species are rough-walled, sulcate, brown to dark-brown, 1-septate, with a dark band about the mid-septum; both spore halves are similar in size and shape (Ferrer and Shearer 2007). Although other taxa in the Jahnulales have 1-septate, brown ascospores, the spore halves are usually not similar in size and none have a sulcate spore wall. The hyphae of *M. aqua-dulces*, including the ascomal stalk hyphae, are distinctly constricted at the septa — a feature not reported for other species in the Jahnulales.

The type species of *Jahnula*, *J. aquatica*, is located in a clade with two sister taxa, *J. granulosa* and *J. rostrata* (clade B; Fig. 1). In addition to the characters generally common to most members of the Jahnulales, all three species have brown to dark brown ascomata, cylindrical or clavate, pedicellate asci, and 1-septate, brown ascospores. *Jahnula granulosa* and *J. rostrata* both have rough-walled ascospores surrounded by a narrow gelatinous sheath. Presence of ascospores with rough walls and a gelatinous sheath were not reported for the type specimen and subsequent collections of *J. aquatica* (Hawksworth 1984; Hyde and Wong 1999), including the specimen of *J. aquatica* from which the sequences were obtained (Raja and Shearer 2006). Because it contains the type species of *Jahnula*, we consider taxa located in clade B to comprise *Jahnula* s. str. This clade, however, has only low bootstrap support, although posterior probability support is 96%.

Clade C (Fig. 1) is well supported statistically and contains species in the genera *Aliquandostipite*, *Jahnula*, and *Patescospora*. All of the species in clade C have the following characteristics that distinguish them from species in the other three clades: clavate to ovoid asci that may or may not separate readily from the ascogenous hyphae and cylindrical to fusiform, smooth-walled, and 1-septate ascospores surrounded by a gelatinous sheath. The sheath is large and

slug-like in *A. crystallinus*, *A. khaoyaiensis*, and *P. separans*, but narrow in *J. siamensiae*.

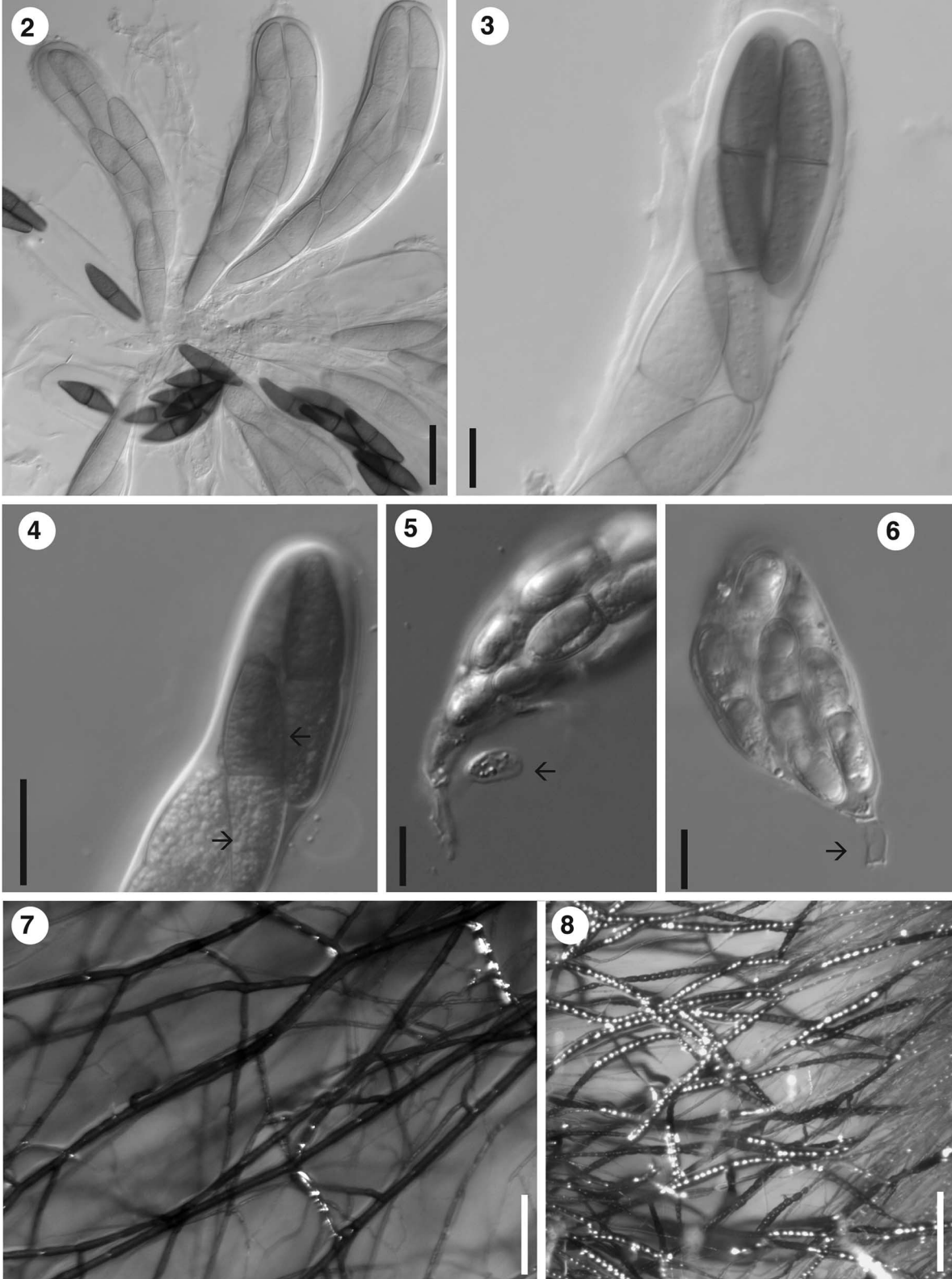
Jahnula siamensiae differs morphologically from the type species of *Jahnula* and more closely resembles members of the genus *Aliquandostipite*. This species shares a number of morphological features with *A. khaoyaiensis*, including its globose to subglobose, pale brown, transparent, papillate ascomata, clavate asci, and 1-septate, ovoid to ellipsoid, multi-guttulate, pale brown ascospores. In our analyses, *J. siamensiae* groups with isolates of *A. khaoyaiensis* (clade C; Fig. 1). Pang et al. (2002) distinguished *J. siamensiae* from the other members of this genus by its larger ascospores and noted that this species possesses ascospores in the same size range as those of *A. khaoyaiensis*. In the same paper (Pang et al. 2002), *J. siamensiae* was positioned as a sister taxon to *A. khaoyaiensis* in a phylogeny inferred from 18S rDNA sequences. Based on these data, we propose the combination *Aliquandostipite siamensiae* (Sivichai & E.B.G. Jones) J. Campb., Raja, A. Ferrer, Sivichai & Shearer, comb. nov. (basonym: *Jahnula siamensiae* Sivichai & E.B.G. Jones, Mycol. Res. 106: 1037, Pang et al 2002).

Aliquandostipite khaoyaiensis and *J. siamensiae* are morphologically distinct species. *Aliquandostipite khaoyaiensis* possesses asci that are apically thickened while those of *J. siamensiae* have an ocular chamber and faint ring (Pang et al. 2002). In addition, the ascospores of *A. khaoyaiensis* are large (50–75 µm × 13–22 µm), pale brown, and have a broad slug-like gelatinous sheath, while those of *J. siamensiae* are either small (33–45 µm × 10–13 µm), fusoid and dark brown or large (58–73 µm × 15–25 µm) and hyaline to pale brown in colour (Pang et al. 2002). We found both types of ascospores in a fresh collection of *J. siamensiae* from Florida (Fig. 2), but we also observed that the paler ascospores became smaller and darker as they matured. All stages from pale to dark brown could be seen in a single ascus (Fig. 3), as could the gradual darkening of individual ascospores (Fig. 4). Our examination of the type specimen of *J. siamensiae* also revealed ascospores within a single ascus that were at different stages of development. This type of spore maturation process is not unusual in ascomycetes (Webster 1970; Hawksworth and Booth 1976). Additional collections and fruiting cultures of *A. khaoyaiensis* and *J. siamensiae* should be examined to assess variation in their morphologies and to determine the closeness of their relationship.

The recognition of *Patescospora* as a separate genus is also not supported in our phylogeny (clade C; Fig. 1). This monotypic genus was established for *P. separans* and distinguished from *Jahnula* and *Aliquandostipite* by its immersed ascomata, ovoid–saccate to clavate asci, divided, loculate hamathecium, deeply constricted 1-septate ascospores that often separate at the midseptum, and a thick slug-like sheath (Pang et al. 2002).

Many of the characters used to circumscribe the genus *Patescospora* are actually common to most of the other taxa on clade C. These characters include ascus shape and presence of a broad slug-like, gelatinous ascospore sheath. Our examination of the type specimen of *P. separans* revealed that this species is not loculate in the traditional sense as presented by Ulloa and Hanlin (2000). The asci develop from ascogenous hyphae, and when the ascomata is opened

Figs. 2–8. Figs. 2–4. *Aliquandostipite siamensis* (F110-1). Fig. 2. Small, dark brown and large, pale brown ascospores. Fig. 3. An ascus containing two dark brown ascospores and a number of hyaline ascospores. Fig. 4. Maturing ascospores with a darkly coloured upper half and a lighter coloured lower half (arrows). Figs. 5 and 6. *Patescospora separans* (IM 386405, type specimen). Fig. 5. An immature ascus developing below a larger, mature ascus. Fig. 6. Ascus with thin-walled stalk separated from the ascogenous hyphae. Fig. 7. *Brachiosphaera tropicalis* (E192-1) hyphae in culture on corn meal agar culture. Fig. 8. *Xylomyces chlamydosporus* (H058-4) hyphae in culture on peptone yeast agar culture. Figs. 2–6 scale bars = 20 μ m. Figs. 7 and 8 scale bars = 0.5 mm.



with fine needles the asci come out of the ascomata as a connected group. Figure 5 shows a second ascus developing below a maturing ascus, both asci originating from the same ascogenous hypha. This feature is clearly shown in Figs. 5 and 8 of the protologue (Pang et al. 2002). Asci are attached to the ascogenous hyphae by a thin-walled stalk that separates easily from the ascogenous hyphae at either end of the stalk (Fig. 6). This feature is also seen in *A. crystallinus* whose asci separate from the ascogenous hyphae before ascospore release (see Figs. 6, 7, and 13 in Raja et al. 2005) and a new species of *Aliquandostipite* (Raja and Shearer 2007). In *P. separans*, as subsequent asci develop, the mature asci may be forced up into the hamathecium. In longitudinal section, the ascomata may appear to be loculate because the asci occur at different levels (See Fig. 1 in Pang et al. 2002), not because they are formed within individual locules throughout the centrum, but because they move upward within the ascoma. If one discounts this feature, the only remaining characters supporting the genus are the deliquescent asci and ascospores that separate into part spores. We did not observe these features in the type specimen but this could be because only a few, relatively young ascomata were present. Slides of sections or older material were not included with the type. We do not believe that these two character states warrant the recognition of a separate genus for this taxon. Since our molecular phylogeny positions *P. separans* within *Aliquandostipite* in a well-supported clade (clade C; Fig. 1), we propose the combination *Aliquandostipite separans* (Abdel-Wahab & El-Sharouney) J. Campb., Raja, A. Ferrer, Sivichai & Shearer, comb. nov. (basonym: *Patescospora separans* Abdel-Wahab & El-Sharouney, Mycol. Res. 106: 1033, 2002, Pang et al. 2002.).

The remaining species of *Jahnula* included in our analyses are members of a well-supported clade (clade D; Fig. 1) separate from *Jahnula* s. str. The complete range of morphological characters described for the Jahnulales, except for the presence of a large, slug-like sheath, are represented in this clade. Disparate morphologies among these species argue for more than a single genus, but more data are needed to resolve the relationships among the taxa. For example, the morphologically similar species *J. bipolaris*, *J. seychellensis*, and *J. sunyatsenii* possess ascospores equipped with gelatinous apical pads and may belong to the same genus (Hyde and Wong 1999; Inderbitzin et al. 2001; Raja and Shearer 2006).

Brachiosphaera tropicalis (Fig. 7) and *X. chlamyosporus* (Fig. 8) are also members of clade D. Both mitosporic species possess wide hyphae and occur on the same substrates in the same habitats as other members of the Jahnulales (Shearer and Ferrer, unpublished data, 2006). *Brachiosphaera tropicalis* was originally described from material collected in the tropics (Descals et al. 1976). *Xylomyces chlamyosporus* was originally described from temperate North America (Goos et al. 1977), but has since been reported from the paleo tropics several times (Goh et al. 1997; Hyde and Goh 1997, 1998; Fryar et al. 2004). Our findings suggest that it would be worthwhile to examine other species of freshwater, lignicolous, mitosporic fungi for the presence of broad hyphae and to investigate their possible relationship to the Jahnulales using sequence data.

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