Phylogenetic Evidence that Two Submerged-Habitat Fungal Species, *Speiropsis pedatospora* and *Xylomyces chlamydosporus,* Belong to the Order Jahnulales *Insertae Sedis* Dothideomycetes

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The genera *Speiropsis* and *Xylomyces* are anomorph fungi. The taxonomic address for the fungi has been unclear. In this study, observation of morphological traits indicates that they have a unique pattern of mycelia with dark-brown to black colour and thick-walled hyphae. The same culture patterns of certain fungi isolated from freshwater habitats in Thailand were selected from BIOTEC Culture Collection (BCC, Thailand), while more species were added from Centraalbureau voor Schimmelcultures (CBS, Netherlands). These fungi were composed of *Jahnula* spp. (2-celled ascospores), *Brachiosphaera tropicalis* (hyaline and 4-5 armed conidia), *S. pedatospora* (hyaline and branches conidia) and *Xylomyces* sp. (dematiaceous and fusiform chlamydospores). This study was undertaken to confirm the taxonomic address for *S. pedatospora* and *Xylomyces* based on phylogenetics relationships as inferred from their ITS rDNA sequence data by using MP (unweighted and successive weighted MP), NJ, ML and Bayesian analysis. Phylogenic analysis revealed that isolates of *S. pedatospora* (2 strains) was a member of the Order Jahnulales and clustered with *Jahnula* spp. (5 strains) and *B. tropicalis* (4 strains) with >82% bootstrap support and 100% posterior probabilities. Four isolates of *X. chlamydosporus, X. elegans* and *X. aquaticus* were shown to be polyphyletic within the *Jahnulales* and *Pleoporales*. The MP and NJ showed the same topology as in the Jahnulales clade obtained by ML analysis.

Key words: ITS rDNA, *Speiropsis pedatospora*, *Xylomyces*

The genus *Speiropsis* are possessed with erect, simple, straight, septate, mononematous, aggregates in fascicles or synnematous to sporodochial conidiophores with discrete, denticulate, polyblastic conidiogenous cells and catenate conidia in branched or unbranched chains (Barbosa and Gusmao 2005). *S. pedatospora* is a mitosporic fungus found in submerged leaves in freshwater bodies (Barbosa and Gusmao 2005) and also a leaf pathogen in *Eucalyptus saligna*.

Additionally, the genus *Xylomyces* a mitosporic fungus, has different nodes of ontogeny which produces large, dematiaceous, thick-walled, multiseptate and fusiform chlamydospores. This anamorph fungus is mainly found in freshwater-submerged-wood (Goh *et al.* 1997). The taxonomic position of *Xylomyces* and *S. pedatospora* are still unclear.

Furthermore, *Brachiosphaera,* another freshwater fungus, characterized by producing round-shaped conidia with conidial arms, mostly 4-5 each with 1-4 septa. Its colonies are characterized by effuse, septate mycelia, mostly submerged in culture media. Initially it is hyaline and turns into olivaceous brown when older. A few segments of hyphae become slightly constricted at the septa, and cells are enlarged, with ellipsoidal to round conidia in their clustering (Chang 1994).

All *Jahnula* sp., a group of fungi that inhabit wood submerged in freshwater, are characterized by hyaline to blackish-translucent-membranous ascomata with subtending, wide-septate-brown-spreading hyphae, large angular cells of peridia, septate pseudoparaphyses, hamathecium, eight-spored, clavate to cylindrical asci, one-septate, broad fusiform, brown and multiguttulate ascospores. Moreover, *Jahnula* colonies on potato dextrose agar (PDA) grew slowly having dark-brown to black, effuse hyphae which are thick-walled, septate, constricted at the septa and cells of the hyphae are cylindrical to subglobose (Raja and Shearer 2006). These cultural traits of *Jahnula* spp. were similar to *Xylomyces* sp., *S. pedatospora* and *Brachiosphaera tropicalis* (Boonyuen and Sivichai, personal observation).

Based on those similarities, these fungi were suspected to have same taxonomic address. Since *Jahnula* sp. and *B. tropicalis.* were in order *Jahnulales* (Campbell *et al.* 2007), the same order was suspected to be the taxonomic address for *S. pedatospora* and *Xylomyces* sp. However, the limited of morphological traits of these fungi made it impossible to determine if they belonged to the same order. The molecular phylogenetics approach therefore offers the opportunity to elucidate the taxonomic position of these fungi.

The two objectives of this study are: (i) to address the taxonomic position of *S. pedatospora* and *Xylomyces* sp. based on ITS sequence data and (ii) to compare the phylogenic topologies of the order *Jahnulales* calculated on the Maximum Parsimony, Distance Matrix method and Maximum Likelihood analysis.

MATERIALS AND METHODS

Fungal Isolates. We studied a total 15 strains obtained from the BIOTEC Culture Collection (BCC, Thailand) and the Centraalbureau voor Schimmelcultures (CBS, Netherlands). All strains were maintained on PDA. Approximately 100 mg of mycelium was used for each DNA extraction.

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DNA Extraction. Fungal mycelium was harvested to the 10 ml tubes and added by sterile sand with 300 ìl 0.5N NaOH and griund into a fine powder. The harvested tubes were centrifuged at 11 000 g for 3 min. Then 5 ìl supernatant was dissolved in 195 μl Tris HCl pH 8.0. These samples were used directly as templates in PCR (modified from Wang *et al.* 1993).

DNA Amplification, Purification and Sequencing. The ITS region (ITS1, 5.8S and ITS2) was amplified using the pair of primers ITS 1 and ITS4 (White *et al.* 1990). A single 50 μl PCR reaction contained: 34 μl sterile water, 5 μl 10 times PCR buffer (final concentration 1X), 2.5 μl 50 mM $MgCl_2$ (final concentration 2.5 mM), 1 μl 10 mM dNTP mix (final concentration 0.2 mM), 1 μl 10 μM each of the primers (final concentration 0.2 μl), 0.5 μl Taq polymerase and 4 μl DNA template. The PCR thermal cycling profile for the primer ITS 1 and ITS 4 included a first denaturation step at 94°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension step at 72°C for 2 min. Analysis of the PCR products were performed on a 0.8% agarose gels stained with ethidium bromide under a 1000 v illuminator. The PCR products were purified using Nucleosprin Plant DNA Purification Kit (Macherey-Nagel). Sequencing of the amplified purified PCR product was performed by the BIOTEC Research Unit (BSU). The sequences were then submited to Genbank (www.ncbi.com) to obtain genbank accession numbers.

Phylogenetics Analysis. Multiple alignments were performed on the sequences generated in this study with those obtained from GenBank (shown with GenBank accession numbers) using the Clustal W1.6 incorporated in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were adjusted manually to minimize the gap. The data set was exported as a nexus file for maximum parsimony, maximum likelihood and distance matrix method analyses in PAUP 4.0b10 (Swofford 2002). The phylogenetic trees were generated using optimality criteria: maximum parsimony (MP), distance matrix method (Neighbor Joining/ NJ), and maximum likelihood (ML) to generate trees with robust support.

Maximum parsimony analyses were performed by using heuristic methods with the character first defined as unordered and given equal weighting. Successive weighted parsimony was executed to select the more consistent characters. Relative support for resulting trees was obtained from bootstrap analysis using 1000 heuristic searches by taking a 50% or greater frequency in the consensus trees. All heuristic searches were performed by tree-bisection-reconstruction branch swapping with 100 random sequences. Successive weighting was performed to ensure the stability of the weight and to evaluate the consistency of the characters. Successive weighting was performed based on the maximum value of the rescaled consistency index for each character for all most parsimonious trees (Farris 1989). The robustness test of the each branch was calculated by the consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI). The Kishino-Hasegawa test (KHT) was performed in order to determine the best tree fit (Kishino and Hasegawa 1989).

Anisomeridium polyori and *Pyrenula pseudobufonia* were used as the out-group.

An evolutionary model was constructed by using the Modeltest 3.06 (Posada and Crandall 1998). Bayesian posterior probabilities were determined by the Markov Chain Monte Carlo sampling in MrBayes 3.0b4 (Huelsanbeck and Ronqvist 2001), using an estimated model of evolution. Markov chains were run for 2 m generation and trees were sampled every 100th generation (resulting 20 000 total trees). The first 2000 trees were used for the burn in phase of the analyses and were discarded. The remaining 18 000 (post-burning) trees were used to generate a majority-rule consensus tree.

Sequences used for phylogenetic analysis (accession numbers in parentheses): *Aliquandostipitate khaoyaensis* (AF201728), *Jahnula sunyatsenii* (AF201727), *Capnodium coffea* (DQ491515), *Capnodium salicinum* (AJ244240), *Dothidea hippophaeos* (AF027763), *D. sambuci* (DQ491505), *D. insculpta* (AF027763), *Westerdykella dispersa* (DQ468031), *W. cylindrical* (AY943056), *Elsinoe ampelina* (AY826764), *E. proteae* (AF097578), *Mycosphaerella fijiensis* (EF666077), *M. graminicola* (DQ019341), *M. punctiformis* (AY152594), *Alternaria alternate* (EF192234), *Pyrenophora phaeocom* (DQ491507), *Setosphaeria rostrata* (AF071342), *Leptosphaeria maculans* (DQ133891), *L. biglobosa* (DQ133893), *Lophiostoma arundinis* (AJ496633), *A. polypori* (DQ782838), *P. pseudobufonia* (DQ782845).

RESULTS

Sequences partial 18S, ITS1 and ITS2 regions were aligned along with the intervening 5.8S rDNA and partial 28S regions. The average size of this region was 495 bp, of which the shortest sequence was *X. acuaticus* CBS636.91 (465 bp) and the longest was *Jahnula* sp. SS3792 (536 bp). The partial 18S region ranged from 5 to 49 bp, ITS1 region from 137 to 172 bp, ITS2 region from 160 to 189 bp, partial 28S region from 5 to 8 bp, while 5.8 region was conserved (156 bp). Overall, *S. pedatospora* displayed a sequence of 486 to 487 bp, *Xylomyces* sp. 465 to 502 bp, *B. tropicalis* 485 to 488 bp, *Jahnula* sp. 487 to 536 bp, and *T. aristata* 487 bp. The gene position of 15 new sequences generated in this study are presented in Table 1. The matrix was process to produce phylogenies based on MP (Fig 1), NJ (Fig 2) and ML (Fig 3).

DISCUSSION

The phylogenies were placed within the *Dothideomycetes*, which is based on *A. polypori* and *P. pseudobufonia* (both species belong to the *Pyrenulales*, *Eurotiomycetes*). Two strains of *S. pedatospora* and *X. chlamydosporus* were placed in the *Jahnulales,* together with all strains of *B. tropicalis* and *Jahnula* sp. Meanwhile, other strains, including *X. elegans, X. aquaticus* and *T. aristata* were clade in Pleosporales.

The order *Jahnulales*, *Dothideomycetes* was introduced by Pang *et al.* (2002), characterized by stalked/sessile, dimorphic ascomata, hyphal-stalk-cell of approximately

Table 1 Gene position in nuclear partial 18S, ITS 1, 2 regions, 5.8 rDNA, and partial 28S rDNA sequence

| Fungal species	Accession no.	Sequence length (bp)	Partial 18S	Position of each region on the sequence			
				ITS1	5.8S	ITS2	Partial 28S
Jahnula appendiculata SS3028	FJ887914	511	1-5	6-161	162-317	318-506	507-511
Jahnula australiensis SS3613	FJ887915	487	1-5	6-158	159-314	315-482	483-487
Jahnula sp. SS3792	FJ887916	536	1-49	50-186	187-342	343-531	532-536
Jahnula granulosa SS3815	FJ887917	523	1-5	6-177	178-333	334-518	519-523
Xylomyces chlamydosporus SS0807	FJ887918	502	1-5	6-162	163-318	319-497	498-502
Xylomyces chlamydosporus SS2917	FJ887919	497	1-5	6-159	160-315	316-492	493-497
Xylomyces elegans SS1077	FJ887920	494	1-5	6-145	146-301	302-476	477-494
Xylomyces aquaticus CBS636.91	FJ887921	465	1-5	6-144	145-300	301-460	461-465
Brachiosphaera tropicalis SS2522	FJ887922	485	1-5	6-156	157-312	313-480	481-485
Brachiosphaera tropicalis SS2523	FJ887923	488	1-5	6-159	160-315	316-483	484-488
Brachiosphaera tropicalis SS2724	FJ887924	487	1-5	6-158	159-314	315-482	483-487
Brachiosphaera tropicalis SS2944	FJ887925	486	1-5	6-157	158-313	314-481	482-486
Speiropsis pedatospora SS2229	FJ887926	487	1-5	6-158	159-314	315-482	483-487
Speiropsis pedatospora SS2236	FJ887927	486	1-5	6-157	158-313	314-481	482-486
Tetraploa aristata CBS996.70	FJ887928	487	1-5	6-166	167-322	323-482	483-487
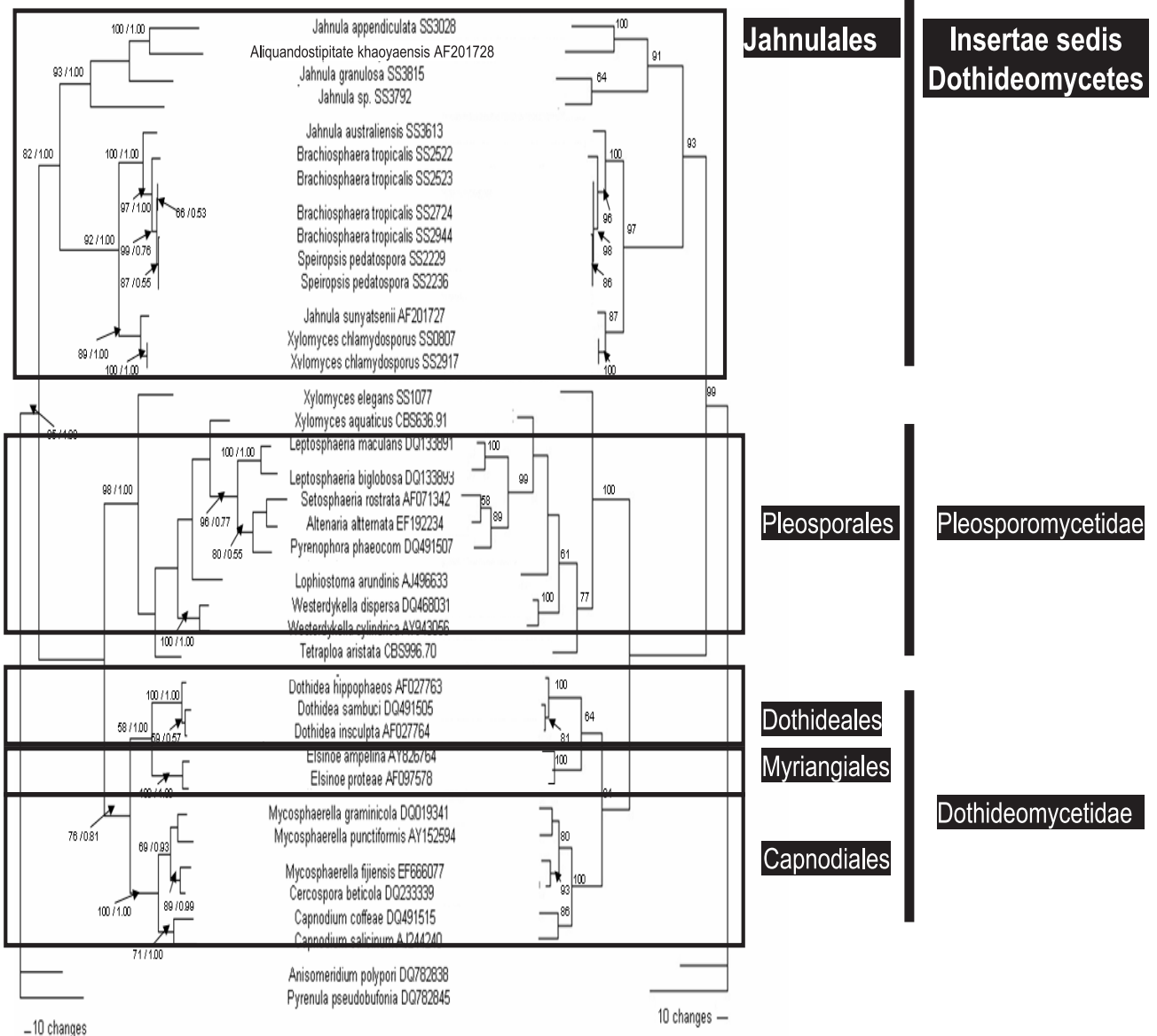
Fig 1 Phylogenetics relationship of *Speiropsis pedatospora*, *Xylomyces* spp., *Brachiosphaera tropicalis* and *Jahnula* spp. based on ITS and 5.8S rDNA sequences. The phylogram represented were the best trees obtain from Kishino-Hasegawa Test in PAUP* 4b10 based on unweighted parsimony (left) and successive weighted parsimony (right). Bootstrap support >50% for both trees were shown above the branches. Posterior probabilities were demonstrated above the branches of unweighted parsimony tree after the bootstrap values. The unweighted parsimony yielded 2 MPTs with 2204 steps, CI = 0.468, RI = 0.667, RC = 0.312 and HI = 0.532. The successive weighted parsimony analysis yielded a phylogeny with 1031 steps, CI = 0.564, RI = 0.712, RC = 0.402 and HI 0.436.
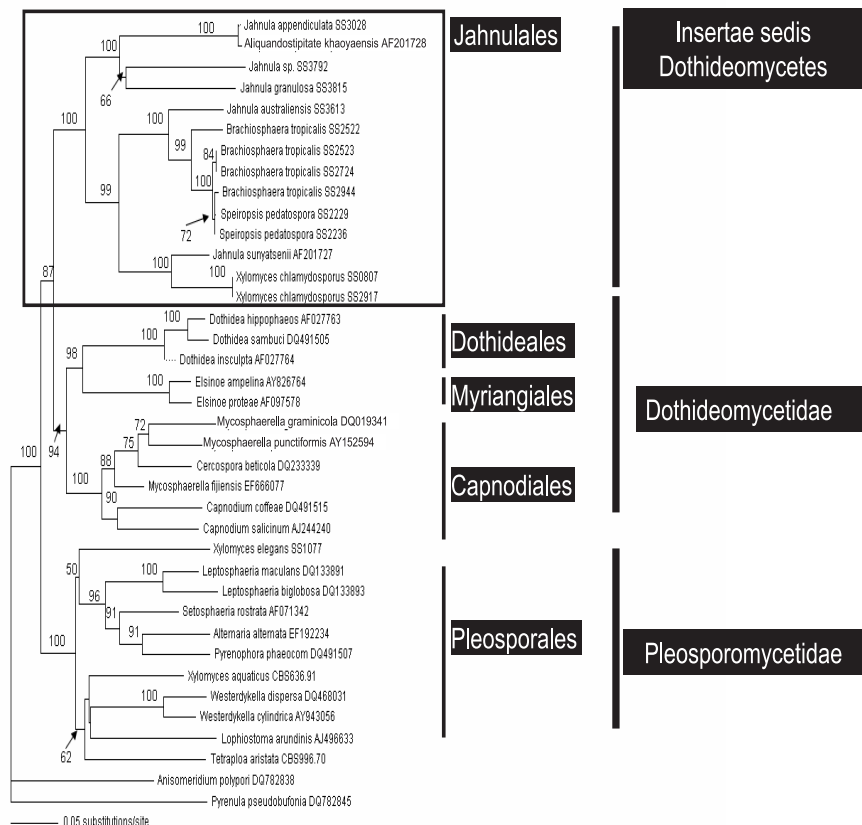
Fig 2   Phylogenetics relationship of *Speiropsis pedatospora, Xylomyces* spp.*, Brachiosphaera tropicalis* and *Jahnula* spp. based on ITS and 5.8S rDNA sequences. The phylogram calculated on the neighbor joining analysis in PAUP* 4b10. Bootstrap support >50% for neighbor joining trees were shown above the branches.
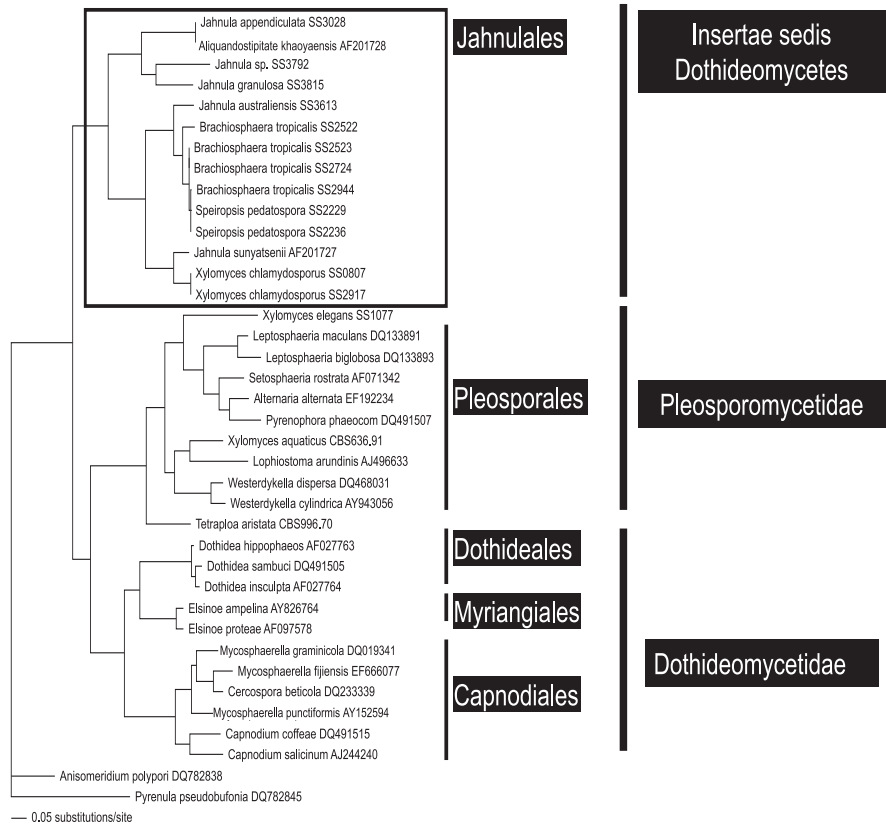

Fig 3   Phylogenetics relationship of *Speiropsis pedatospora, Xylomyces, Brachiosphaera tropicalis* and *Jahnula* spp. based on ITS and 5.8S rDNA sequences. The phylogram calculated on the maximum likelihood analyses in PAUP* 4b10. The likelihood value = -9229.03443, base frequencies were estimated as follow: A = 0.22546, C = 0.27760, G = 0.24967 and T = 0.24727, the estimated value of the proportion as invariable sites = 0.225, and the gamma shape parameter = 1.536. The maximum likelihood analysis yielded a phylogeny with 2223 steps, CI = 0.466, RI = 0.661, RC = 0.307 and HI = 0.536.

40 μm wide, and ascospores composed of 2-cells or without appendages (Pang *et al.* 2002). The order comprised a single family, the *Aliquandostipitaceae*. *Jahnulaes* are widely distributed from temperate to tropical regions. Mostly the species of this order are to be found in freshwater habitats on lignicolous materials. This ecological name refers to their habitats being submerged wood (Pang *et al.* 2002; Raja and Shearer 2006).

Moreover, phylogeny analysis confirms that *S. pedatospora* is closely related to *B. tropicalis,* with strong boostrap support (98%) and high posterior probabilities (76%). The hyline conidia could be the apomorphic character, since both *S. pedatospora* and *B. tropicalis* possessed this morphology.

The phylogenies revealed that *Xylomyces* strains comprised a polyphyletic genus. *Xylomyces chlamydosporus* is placed in the Jahnulales while *X. elegans* and *X. aquaticus* are both accommodated in the Pleoporales. Even though the genus *Xylomyces* was created based on morphology and ontogeny (Goh *et al.* 1997), their phylogenies did not support that placement. This might result from convergent evolution among the *Xylomyces* species. Convergent evolution appears as a result of ecological equivalents. Since all *Xylomeces* share an aquatic habitat, this scheme indicates that chlamydospore-morphology in this genus results from adaptation to their habitat. Molecular phylogenetic studies also have confirmed the polyphyly of many anamorphic genera and species (Shenoy *et al.* 2007), such as *Chalara* (Paulin-Mahady *et al.* 2002), *Galerina* (Gulden *et al.* 2005) and *Zopfiella* (Cai *et al.* 2006).

Furthermore, *Jahnulales* has been divided into three subclades, each one with strong bootstrap support (>95%). The first subclade is composed of *J. appendiculata, A. khaoyaensis, Jahnula* sp. SS3792 while *J. granulosa, B. tropicalis* and *S. pedatospora* are grouped in the second subclade. The third subclade is comprised of two species being *J. sunyatsenii* and *X. chlamydosporus.*

All of trees based on MP (unweighted and successive weighted parsimony), NJ and ML analyses showed similar topology. Those strains were placed in the three big clades, with clustering in the Jahnulales, Pleosporomycetidae and Dothideomycetidae. The NJ tree has a different branch pattern with MP and ML trees of the three big clades. The NJ tree showed Dothideomycetidae as a sister group of *Insertae sedis* Dothideomycetidae (Jahnulales), while in other trees, Dothideomycetidae appears as sister group of Pleoporomycetidae.

The phylogenic tree analyses obtained agrees with result of Schoch *et al.* (2006) that the Dothideomycetes was segregated into two subclasses comprised of the Dothideomycetidae (Dothideales, Myriangiales, Capnodiales) and the Pleosporomycetidae (Pleosporales). However several classes belonged to *Insertae sedis* Dothideomycetes. Based on these phylogenies, the Jahnulales is a sister group of either the Pleoporomycetidae or the Dothideomycetidae. We conclude that the Jahnulales was belongs to the *Insertae sedis* Dothideomycetes with a 100% bootstrap support and 100% posterior probability.

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