

## A Molecular Phylogenetic Study of Selected in Goldian Species

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Taxonomic studies of Ingoldian fungi are chiefly based on morphological and ontogenetic traits. However, some of them need to be reclassified especially at the genus and species levels. Sivichai et al. (2003) proposed that the anamorph of *Hymenoscyphus varicosporoides* is a waiting to be described species of *Tricladium* rather than of the genus *Varicosporium* sp. as originally delineated by Tubaki (1966). Additionally, the two species, *H. varicosporoides* and *Cudoniella indica*, possess almost the same morphological characteristics except for differences in asci staining. Thus, we conducted a molecular phylogenetic analysis of complete ITS1-5.8S-ITS2 sequences of 37 selected Ingoldian taxa in Helotiaceae to clarify sexual-asexual links among these two conspecific genera. Significantly, phylogenetic data suggested that 37 selected genera formed a monophyletic group in Helotiaceae, Helotiales. The results indicate that both type species have an anamorph best assigned to *T. indicum* with a well-supported clade (82%) containing either teleomorph genera of *H. varicosporoides*, *C. indica*, or its anamorph. The molecular data suggest that the presence or absence of a staining reaction for the apical ring is not a phylogenetically reliable character. The highly significant identity 98-99.5% of ITS1-2 and 5.8S regions of *C. indica* (SS 708) and *H. varicosporoides* (SS 336, SS 76.01 and CBS 651.66) is valid evidence implying that the two genera are very closely related or synonymous. The inferred phylogenetic trees illustrate that a subclade of temperate strains isolated from the UK, India and Japan formed separately from a subclade of tropical strains isolated from Thailand. Molecular phylogenetic analysis that relies on ITS1-5.8S-ITS2 sequences is a useful tool for resolving the different genera or species and for integration of teleomorph-anamorph connections within these related Ingoldian taxa in Helotiaceae.

**Key words:** *Cudoniella indica*, *Hymenoscyphus varicosporoides*, Ingoldian fungi, molecular phylogeny

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### Introduction

Freshwater fungi play an important role in colonising and degrading organic matter in the aquatic habitat. In the last six years, over 400 species were collected adding to knowledge of diversity and ecology studies of freshwater fungi in Thailand. Of this number, most were new records for Thailand, while at least 20% are probably novel to science. Most of these freshwater fungi are ascomycetes or their mitosporic (asexual) states. Significantly, freshwater fungi comprise of three taxonomic groups. The first group is the “lower fungi” (e.g. Zygomycota) while the second is “higher fungi” (mostly Ascomycota with some Basidiomycota). The last group is not a strict taxonomic grouping but have been called Ingoldian fungi.

The Ingoldian fungi was named by Dr. Henry Descals in honour of Professor C.T. Ingold who drew attention to their significance in freshwater habitats over sixty years ago (Webster and Descals, 1981). This fungal group was referred to as the “aquatic hyphomycetes” with relatively large and modified conidia that adapted for staying in streams and damp environments. Most studies of Ingoldian fungi have been from northern temperate regions with few from the tropics (Descals, 1997). There are approximately 500 species currently known and hundreds await description especially on generic level (Descals, 1997). These are mostly mitosporic states of Ascomycota, although in most cases the teleomorph associations are unknown.

In Thailand, there were four published reports and a few unpublished surveys (Tubaki et al., 1983; Sivichai, 1999; Marvanova and Hywel-Jones, 2000; Sakayaroj, 2000; Sivichai and Boonyene, 2004). At present, this fungal group has been studied for a year with the results showing that over a hundred species were recognised and collected. However, many species of this fungal group are taxonomically problematic classification based on their morphological characteristics and ontogeny.

Current interest aspects of this study are the confusing names of its anamorph to one taxon in discomycete, in Leotiomycetes, Helotiales and Helotiaceae, which is referable to *Hymenoscyphus varicosporoides* Tubaki (Tubaki, 1966). Tubaki described an anamorph state of this fungus to *Varicosporium*. He also pointed out that the anamorph state *Varicosporium* could equally well be referred to *Polycladium* or *Tricladium*. Sati and Tiwari (1992) described a deuteromycetous fungus with appendage conidia as a new species –*T. indicum*– from India. In addition, other anamorphs have been described for *Hymenoscyphus* species: *Anguillospora*, *Articulospora*, *Dimorphospora*, *Geniculospora*, *Tricladium* while *Varicosporium* for Ingoldian hyphomycetes, and *Helicodendron* for aero-aquatic fungi (Tubaki, 1966; Abdullah et al., 1981; Fisher and Webster, 1983; Webster, 1992). For example, *Tricladium* has also been shown to have different teleomorphs: *T. splendens* with *H. splendens* (Leotiaceae), *T. chaetocladium* with *Hydrocina chaetocladia* (Hyaloscyphaceae) (Abdullah et al., 1981; Webster et al., 1990), and *T. indicum* with *Cudoniella indica* (Webster et al., 1995). However, these fungi are all genera with very different conidial morphologies. This species was collected in Thailand and identified to the same species described by Tubaki (1966) as *H. varicosporoides* in Sivichai et al. (2003).

Various anamorphs have been described for *Hymenoscyphus* species: *Anguillospora*, *Articulospora*, *Dimorphospora*, *Geniculospora*, *Tricladium*, and *Varicosporium* for Ingoldian hyphomycetes and *Helicodendron* for aero-aquatic fungi (Tubaki, 1966; Abdullah et al., 1981; Fisher and Webster, 1983; Webster, 1992). These are all genera with very different conidial morphologies. Importantly, *Tricladium* has also been shown to have different teleomorphs: *T. splendens* with *H. splendens* (Leotiaceae), *T. chaetocladium* with *Hydrocina chaetocladia* (Hyaloscyphaceae) (Abdullah et al., 1981; Webster et al., 1990), while Webster et al. (1995) described *C. indica* as the teleomorph of *T. indicum*.

Other genera considered by Tubaki (1966) were *Polycladium*, *Tricladium*, and *Tricladomyces*. *Polycladium* is ruled out, as the number of lateral branches is greater with tertiary laterals. Roldán et al. (1987) in describing *Dendrospora polymorpha* noted that the genera *Varicosporium*, *Tricladium*, and the *Dendrospora-Dendrosporomyces* complex ‘have become increasingly ill-defined in recent years’. Although the genus *Tricladium* is considered to have only primary branching (Roldán et al., 1987) there “are examples of frequent secondary branching in several *Tricladium* species” although ‘this is definitely not a characteristic of *T. splendens*, the type species. Sivichai et al. (2003) assigned a collection from Thailand to *Tricladium*, which is already an accepted anamorph for *Hymenoscyphus* species (Abdullah et al., 1981).

Both teleomorph genera of *H. varicosporoides* and *C. indica* are similar in morphology. However, the differences in the teleomorphs revolve around the staining of the asci: in *C. indica* asci are J- but J+ in *H. varicosporoides*, but the staining of the ascus tip in *Hymenoscyphus* is considered variable (Spooner pers. comm.).

With respect to the molecular work, this technique is useful for sorting out the problem to answer and address the confusion over the assignment of these fungi to teleomorph and anamorph genera. The following taxonomic issues need to be resolved:

- i. Is the anamorph described for *H. varicosporoides* best placed in *Varicosporium* as originally proposed by Tubaki (1966), or better placed in *Tricladium* as recommended by Sivichai et al. (2003) based on morphological characteristics?
- ii. Is the anamorph *T. indicum* conspecific of *H. varicosporoides* and *C. indica*?
- iii. Are the teleomorph genera of *Hymenoscyphus* and *Cudoniella* polyphyletic?

#### *Research Objectives:*

1. To use molecular techniques to determine whether teleomorphs *Hymenoscyphus* and *Cudoniella* even these two genera of freshwater species are belong to the same genus
2. To clarify anamorph-teleomorph relationships in these genera

3. To construct phylogenetic trees to examine evolutionary relationships of the selected genera and species

4. To isolate more species of Ingoldian fungi for future molecular studies and deposit in the BIOTEC Culture Collection (BCC) for future studies

## Methodology

Genomic DNA of selected fungi was extracted by Wang and colleague method (Wang et al., 1993). The internal transcribed spacer (ITS) regions of rDNA gene were amplified with universal primers ITS5 and ITS4 (White et al., 1990). Purified PCR products were submitted for sequence determination by BIOTEC Service Unit. Sequences were verified by employing the Blast program to identify matches in Genbank at NCBI database. DNA sequences were aligned using Clustal W 1.6 program (Thompson et al., 1994) bundled in BioEdit (Hall, 2001). Percent identity between *H. varicosporoides*, *C. indica* and other species pairs (*Tricladium* anamorph of *H. varicosporoides*, *T. splendens*) were also calculated. Analyses of all sequence alignments were performed using PAUP\* 4.0b8 (Swofford, 2003). The phylogenetic trees were generated using maximum parsimony criteria for analysis of nucleotides. Parsimony tree scores that are Tree length, CI, RI, RC, HI and Bootstrap were calculated and recorded in the phylogram figures.

## Results

The matrix table 1 was used to demonstrate percentage identity of the ITS1-5.8S-ITS2 sequences among *H. varicosporoides*, its anamorphic stages and two strains of *C. indica* (SS 708 and CBS 430.94). It showed that there was 97-100% identity among mentioned taxa. The *Tricladium* anamorph of *H. varicosporoides* (SS 472) and *H. varicosporoides* (SS 76.01) showed 100% identity while two strains of *T. varicosporoides* SS472 and SS451 possess 98% identity of ITS1-5.8S-ITS2 sequences. There were 97% identical nucleotide base pairs of ITS1-5.8S-ITS2 sequences between two strains of *C. indica* (SS 76.01) and *H. varicosporoides* (CBS 651.66). In addition, the matrix showed the highly significant identity 98-99% of ITS1-2, 5.8S regions of *C. indica* (SS 708) and *H. varicosporoides* (SS 336, SS 76.01 and CBS 651.66).

Table 1. Percentage identity of ITS1-2 and 5.8S rDNA sequences between *H. varicosporoides*, its anamorphic stages, its anamorphic stages and *C. indica* using pairwise alignment

Taxon pairs of ITS region	1	2	3	4	5	6	7
	SS 336	SS 76.01	CBS 651.66	SS 708	CBS 430.94	SS 451	SS 472
1 <i>H. varicosporoides</i> (SS 336)	100	100	98	98	98	98	100
2 <i>H. varicosporoides</i> (SS 76.01)	-	100	97	98	98	98	100
3 <i>H. varicosporoides</i> (CBS 651.66)	-	-	100	99	99.5	97	98
4 <i>Cudoniella indica</i> (SS 708)	-	-	-	100	99.5	97	97
5 <i>C. indica</i> (CBS 430.94)	-	-	-	-	100	97	98
6 <i>Tricladium</i> anamorph of <i>H. varicosporoides</i> (SS 451)	-	-	-	-	-	100	98
7 <i>Tricladium</i> anamorph of <i>H. varicosporoides</i> (SS 472)	-	-	-	-	-	-	100

The percentage of identity in the ITS1-5.8S-ITS2 regions of *Tricladium* and *Varicosporium* ranges between 71-98%. The highly significant identity of 96-98% was found in *Tricladium* anamorph of *H. varicosporoides* (SS 451, SS 472), *T. indicum* (CBS 430.94) and *T. splendens* (SS 2282, type species).

In addition, there is 96% identity of ITS1-5.8S rDNA-ITS2 sequences between strains of *T. splendens* (SS 2282, type species) and *Tricladium* anamorph of *H. varicosporoides* (SS 451, SS 472). Additionally, only 73-78% identity was compared between *H. varicosporoides* (*Tricladium* anamorph of *H. varicosporoides*) and *Varicosporium* anamorph (*V. delicatum* and *V. elodeae*) in table 2.

Table 2. Percentage of identity between *Tricladium* spp. and *Varicosporium* spp. by pairwise alignment on ITS1-2 and 5.8S gene

Taxon pairs of ITS region	1 SS 451	2 SS 472	3 SS 2282	4 CBS 430.94	5 CBS 697.73	6 CBS 249.90	7 AY 204611	8 CBS 508.71	9 CBS 541.92	10 CBS 574.95
1 <i>Tricladium</i> anamorph of <i>H. varicosporoides</i> (SS 451)	100	97	96	98	95	74.5	74	72.5	78	74
2 <i>Tricladium</i> anamorph of <i>H. varicosporoides</i> (SS 472)	-	100	96	97	94	73	74	72	78	73
3 <i>T. splendens</i> (SS 2282)	-	-	100	96	95	73	74	71	78	73
4 <i>T. indicum</i> (CBS 430.94)	-	-	-	100	94	74	74	71	78	73
5 <i>T. terrestre</i> (CBS 697.73)	-	-	-	-	100	73	73	71	77	73
6 <i>T. chaetocladium</i> (CBS 249.90)	-	-	-	-	-	100	70	77	72	89
7 <i>T. angulatum</i> (AY204611)	-	-	-	-	-	-	100	72	74	71
8 <i>T. giganteum</i> (CBS 508.71)	-	-	-	-	-	-	-	100	74	78
9 <i>Varicosporium elodeae</i> (CBS 541.92)	-	-	-	-	-	-	-	-	100	73
10 <i>V. delicatum</i> (CBS 574.95)	-	-	-	-	-	-	-	-	-	100

Phylogenetic trees were generated using the maximum parsimony analysis of ITS1-2, 5.8S rDNA data to clarify anamorph-teleomorph relationships in 37 selected species described in materials and methods. *Tuber mesentericum* and *T. aestivum* were outgroup taxa in Pezizales.

For the maximum parsimony analysis, the number of parsimony informative sites was 297 (34.61%). The data set yielded two equally parsimonious trees having the same MP scores (data not shown). The strict consensus tree was applied to these two trees resulting in the average tree smallest tree length (1191) with CI=0.623, RI=0.763, RC=0.476 and HI=0.377 (Fig. 1). All topologies were roughly separated into four clades that were A, B, C and D.

Clade A contained two sub-branches forming monophyletic group of the most heterogenous *Hymenoscyphus* spp. with a bootstrap value of 61%. These *Hymenoscyphus* species were recognized within GenBank strains with accession number. They were *H. serotinus* AY348592, *H. immutabilis* AY348584, *H. scutulus* AY348591, *H. cf. menthae* AY348588, *H. caudatus* AY348576, *H. fucatus* AY348583, *H. cf. fructigenus* AY348582, *H. fructigenus* AJ430396 (type species of the genus), *H. monotropae* AF169309, *H. lasiopodius* AY348587, *H. epiphyllus* AY348581 and *H. epiphyllus* AY348580. The bootstrap value of clade A and Clade B was 35%.

Clade B was composed of *Tricladium angulatum* AY204611, *T. angulatum* AY204608, *Anguillospora filiformis* AY148105, *A. filiformis* AY148104, and *V. elodeae* CBS 541.92 (type species of the genus) with bootstrap value of 35%. At this internode, there were two subgroups of *T. angulatum* AY204611 and *T. angulatum* AY204608 clustered together with the branch of *A. filiformis* AY148105, *A. filiformis* AY148104 and *Varicosporium elodeae* CBS 541.92 with bootstrap 100%.

Clade C was the most populated clade having *T. terrestre* CBS 697.73 (UK) and *C. acicularis* CBS 100273 (Denmark) at the base. And this clade was clustered by *Anguillospora crassa* AY204582 (anamorph of *Mollisia uda*), *A. crassa* (anamorph of *Mollisia uda*), *A. furtiva* AY148107

(anamorph of *Pezoloma* sp.), *Zalerion varium* AF169303 (marine habitat strain) and 10 sequences of representative Ingoldian taxa. The representative Ingoldian taxa included *T. splendens* AY204634 (type species of the genus and anamorph of *H. splendens*), *T. splendens* SS 2274 (anamorph of *H. splendens*, Spain), *T. splendens* SS 2282 (anamorph of *H. splendens*, Spain), *Cudoniella indica* SS 708 (teleomorph of *T. indicum*, UK), *T. indicum* CBS 430.94 (anamorph of *C. indica*, India), *H. varicosporoides* CBS 651.66 (Japan), *H. varicosporoides* SS 336 (Thailand), *Tricladium* anamorph of *H. varicosporoides* SS 472 (Thailand), *Tricladium* anamorph of *H. varicosporoides* SS 451 (Thailand) and *H. varicosporoides* SS 336 (Thailand). The bootstrap value of this main branch was 95%. The branch of 3 strains of *T. splendens* was the sister group of *A. crassa*, *A. furtiva* and *Z. varium* with 43% of bootstrap value. The highlight branches are formed by 2 internodes of *C. indica* SS 708 (teleomorph of *T. indicum*, UK), *T. indicum* CBS 430.94 (anamorph of *C. indica*, India) and *H. varicosporoides* CBS 651.66 (Japan), and *H. varicosporoides* SS 336 (Thailand), *Tricladium* anamorph of *H. varicosporoides* SS 472 (Thailand), *Tricladium* anamorph of *H. varicosporoides* SS 451 (Thailand) and *H. varicosporoides* SS 336 (Thailand) with 82% of bootstrap value. The geographical regions and climate zones of each Ingoldian fungal isolate were matched into these 2 highlight clades. The results reflected a correlation between geographical origin and climate for these two clades of the inferred MP tree.

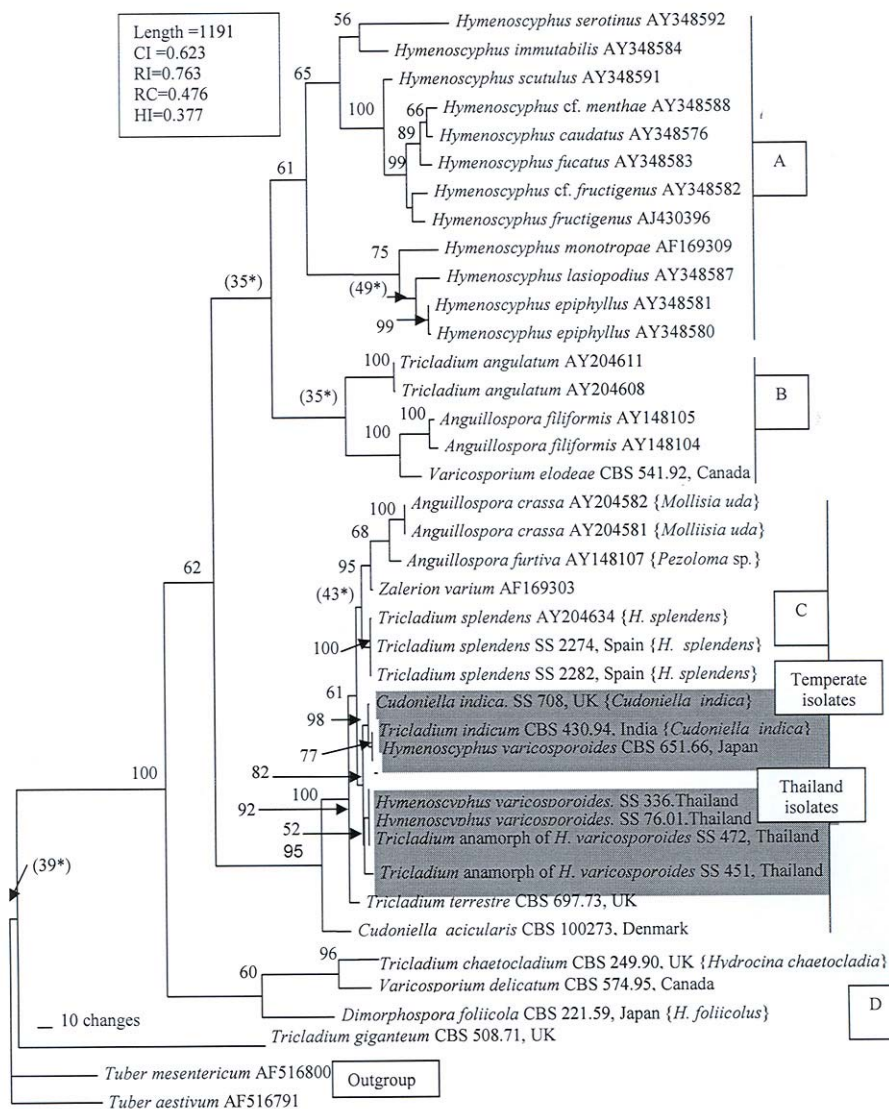


Figure 1. Strict consensus tree of 2 most parsimony trees calculated from ITS1-2, 5.8S rDNA sequence of 37 selected taxa in Helotiaceae, and *Tuber mesentericum* and *T. aestivum* as outgroup taxa in Pezizales. Bootstrap values over 50% and less than 50% were indicated at the base of the corresponding clade and in parenthesis with asterisk \*\*, respectively.

Clade D was formed by *T. chaetocladium* CBS 249.90, UK (anamorph of *Hydrocina chaetocladia*), *V. delicatum* CBS 574.95 (Canada), *Dimorphospora foliicola* CBS 221.59 (anamorph of *H. foliicola*, Japan) and *T. giganteum* CBS 508.71 (UK) at the base of connecting branch. A formed a single clade with a bootstrap value of 60%. *T. chaetocladium* CBS 249.90, *V. delicatum* CBS 574.95 were grouped together in the 96% supportive bootstrap branch.

## Discussion

The results of sequence analysis and phylogram strongly supported that the anamorph of *H. varicosporoides* should be classified under the species of *T. varicosporoide* as proposed by Sivichai et al. (2003) (Table 1 and Fig. 1). The molecular phylogenetic analysis indicated that the clade of *H. splendens* and *T. splendens* was grouped with *H. varicosporoides* with a bootstrap value of 61%, and held 96% identity of ITS1-5.8S-ITS2 sequences (Fig. 1). By contrast, the identity of the ITS1-5.8S-ITS2 regions between anamorphic stages of *Tricladium* spp. and *Varicosporium* spp. was only 71-96% (Table 2). *Varicosporium elodeae* CBS 541.92 was formed with *A. filiformis* AY148104, AY148105 in the clade B, and *V. delicatum* CBS574.95 was clustered with *T. chaetocladium* CBS249.90, *Dimorphospora foliicola* at the base of the phylogeny tree in the clade D (Fig. 1). *T. chaetocladium* was recorded as the anamorph of *Hydrocina chaetocladia* (Hyaloscyphaceae) (Abdullah et al., 1981; Webster et al., 1990). This result suggested that some anamorphs of *Tricladium*, *Varicosporium*, *Anguillospora* are probably polyphyletic corresponding to the conclusion of Zhang and Zhuang (2004). More species of *Tricladium*, *Anguillospora* and *Varicosporium* are needed for further study and revision.

It is interesting to note that these selected Ingoldian taxa in the clade C of MP inferred tree was delineated into 2 groups of *Tricladium* with primary branched conidia and *Anguillospora* with sigmoid conidia that somewhat consistent with classical classification based on shape of conidia. These molecular results are in accordance with Sivichai et al. (2003) classification based on branching characters of conidia. The asexual spores of *Tricladium* are primary branches whereas the conidia of *Varicosporium* are formed secondary branches (Sivichai et al., 2003). Additionally, Abdullah et al. (1981) also described that the teleomorph *H. splendens* is the species of *T. splendens*.

The inferred MP tree of ITS1-2 and 5.8S regions of *C. indica* (SS 708) and *H. varicosporoides* (SS 336, SS 76.01 and CBS 651.66) is a strong evidence showing that the two genera are very closely related or even synonymous fungi sharing a recent common ancestor (Fig.1 and Table 1). These results suggested that the presence or absence of a staining reaction is not a phylogenetically reliable character. Nonetheless, on the basis of 5.8S-ITS sequence data there is no support for confident combining *C. indica* (SS 708) and *H. varicosporoides* (SS 336, SS 76.01 and CBS 651.66) into the same species. The identity of 5.8S-ITS between *C. indica* SS 708, *T. indicum* CBS 430.94 were 99.5%, strongly support the idea that both of them are teleomorph and anamorph of each other while the identity of 5.8S-ITS between *C. indica* SS 708 and *H. varicosporoides* CBS 651.66 were 99.0%. In the same token identity of 5.8S-ITS sequences of *H. varicosporoides* strain SS 336, strain SS 76.01, strain SS 472, CBS 651.66 and strain SS 451 displayed 100%, 100%, 98% and 98%, respectively. The results of 5.8S-ITS divergences among strains of *H. varicosporoides* were 0-2% or 98-100% identity.

Notably, the identity of 5.8S-ITS sequences of *C. indica* SS 708 and *C. acicularis* CBS 100273 were only 88.85% (data not shown). That the greater difference is within the same genus excluded *C. acicularis* CBS 100273 to the base of clade C. Thus, the borderline of 5.8S-ITS divergences to delineate or to determine relationships at the species and within-species among this group of Ingoldian fungi from this study is still unclear. More evidences of other morphologies and other genetic makers are needed to clarify the division of *C. indica* SS 708 and *H. varicosporoides* SS 336, SS 76.01 and CBS 651.66. However, the previous molecular scientists who studied other fungi such as *Colletotrichum* spp. described that 0.4-0.8% divergences of 5.8S-ITS sequence were too low to confidently separate closely related *Colletotrichum* strains into different species (Martinez-Culebras et al., 2003).

Interestingly, the clade supporting *C. indica* and *H. varicosporoides* is a clade of species isolated from temperate regions in Spain, India and Japan grouped separately from species isolated from tropical forest in Thailand. Even though the *C. indica* (CBS 430.94) was the species from India, but it was collected from foam samples in the stream of Kumaun Himalaya, a high mountain with 1,850 m. above the sea level (Sati and Trivari, 1992). Moreover, both strain of *C. indica* SS708 and

*H. varicosporoides* CBS 651.66 are accommodated in the temperate zones of Japan and the United Kingdom. In addition, these two sub clades contain anamorphs representing the major development forms for fungi adapted to a lotic environment. Thus, the topology of this clade reflects a correlation of climate and habitats origins.

The clade A comprising of the ectomycorrhizae of the *Hymenoscyphus* spp. (Zhang and Zhuang, 2004) was included into the molecular phylogenetic tree construction as for the internal control of topology forming. And the results of topology clustering were the same as previously reported by Zhang and Zhuang, 2004. Interestingly, the topology of *Hymenoscyphus* (ectomycorrhizae) clade represented the unique nutritional mode of mutualistic relationships.

## Conclusions

1. The high significant identity 98-99.5% of ITS1-2 and 5.8S regions of *C. indica* (SS 708 and CBS 430.94) and *H. varicosporoides* (SS 336, SS 76.01 and CBS 651.66) is valid evidence showing that the two genera are very closely related or even synonymous.

2. Maximum parsimony tree (MP) of the completed ITS1-5.8S-ITS2 sequences can delineate selected Ingoldian genera in Helotiaceae in to four clades. Clade A is represented by *H. serotinus*, *H. immutabilis*, *H. scutulus*, *H. cf. menthae*, *H. caudatus*, *H. fucatus*, *H. cf. fructigenus*, *H. fructigenus* (type species of the genus), *H. monotropae*, *H. lasiopodius*, *H. epiphyllus* and *H. epiphyllus*, a monophyletic group for selected genera in this study. Clade B is composed of *T. angulatum*, *T. angulatum*, *A. filiformis*, *A. filiformis*, and *V. elodeae* CBS 541.92. Members in clade C are included *Anguillospora crassa*, *A. furtive*, *Zalerion varium*, *T. splendens* SS 2274, *T. splendens* SS 2282, *Cudoniella indica* SS 708, *T. indicum* CBS 430.94, *H. varicosporoides* CBS 651.66, *H. varicosporoides*, SS 336, *Tricladium* anamorph of *H. varicosporoides* SS 472, *Tricladium* anamorph of *H. varicosporoides* SS 451, *H. varicosporoides* SS 336, *T. terrestre* CBS 697.73, and *C. acicularis* CBS 100273. Clade D consists of *T. chaetocladium* CBS 249.90, *V. delicatum* CBS 574.95, *Dimorphospora foliicola* CBS 221.59 and *T. giganteum* CBS 508.71. The outgroup taxa are displayed by *Tuber mesentericum* and *T. aestivum* in Pezizales.

3. Phylogenetic analysis based on ITS1-5.8S-ITS2 data strongly supported that the anamorph of *H. varicosporoides* should be classified under the species of *T. varicosporoide* as proposed by Sivichai et al. (2003) rather than the genus of *Varicosporium* sp. as originally initiated by Tubaki (1966).

4. The molecular analysis suggested that the presence or absence of a staining reaction of the apical ring is not a phylogenetically reliable character used to distinguish two species of *C. indica* and *H. varicosporoides*. The high identity of the completed ITS1-5.8S-ITS2 sequences implied that *C. indica* and *H. varicosporoides* should be closely related or almost identical.

5. Phylogenetic analyses of completed ITS1-5.8S-ITS2 sequences provide strong evidence that *Hymenoscyphus* is a large and heterogeneous genus in both classical and molecular aspects. They have a wide range of substrata and different geographic origins from both terrestrial and aquatic habitats. However, the sequence data might be determined reflect for patterns in their anamorph and teleomorph characteristics as type of substrata/host, bio-geographic origins, climate habitats nutritional modes and historical evolution.

6. The significant identity between genera of anamorphic stages (*Tricladium* spp. and *Varicosporium* spp.) on the ITS1-5.8S-ITS2 regions has range 71-96%. Based on optimal criteria of MP analysis, the results of inferred phylogenetic tree revealed that the a number of lateral branches (two or three laterals) of *Tricladium* and *Varicosporium* anamorph are not a reliable morphological character. This taxon was described as the type species of the genus, in this stage, it is difficult to distinguish the grouping of these fungi especially *Tricladium* and *Varicosporium* anamorph as both genera are probably polyphyletic and need more study to clarify.

7. Phylogenetic analysis based on ITS1-5.8S-ITS2 DNA sequences by MP method suggested that 37 selected genera in Helotiaceae are a monophyletic group in the Helotiales. Molecular phylogeny analysis based on ITS1-5.8S-ITS2 regions is a useful tool for resolving the different genera or species and integration of teleomorph-anamorph connection within these related taxa in Helotiaceae.

## Concluding Remarks

For the further studies of selected Ingoldian fungi and their molecular phylogenetic many experiments should be performed:

1. Expanding the study to of nucleotide divergence of ITS1-5.8S-ITS2 from additional species of *Varicosporium* spp., *Tricladium* spp., *Cudoniella* spp. and *Hymenoscyphus* spp. will provide more useful data for clarifying the systematics of these Ingoldian fungi.
2. Other genetic makers are, for examples, the Beta-tubulin gene, which may be divergent enough to distinguish within sub-genotype level of the aquatic hyphomycete fungi.
3. The combination of molecular data from several genes and morphological characteristics including ultra structures, biochemical and ecological characters may give meaningful phylogenetic evidence for addressing evolutionary relationships of Ingoldian taxa.
4. Studying sequence data will be useful for not only the determination patterns in their anamorph and teleomorph characteristics as type of substrata/host range, bio-geographic origins, climate origin, habitats nutritional modes, historical evolution but also the metabolic fingerprinting of bioactive compounds.

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