A MOLECULAR PHYLOGENETIC STUDY OF SELECTED INGOLDIAN SPECIES

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Thesis
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ABSTRACT

Taxonomic studies of Ingoldian fungi are mainly based on morphological characters and ontogeny of sexual and asexual spore formation. However, some of them need revision and reclassification especially at the genus and species levels because of misleading of teleomorph and anamorph connections. Sivichai *et al*. (2003) proposed that the anamorph of *H. varicosporoides* a species of *Tricladium* rather than *Varicosporium* sp. as originally initiated by Tubaki (1966). In addition, the two species *H. varicosporoides* and *Cudoniella indica* possess almost the same morphological characteristics except for differences in the ascus staining. Thus, we conducted a molecular phylogenetic analysis of the complete ITS1-5.8S-ITS2 DNA sequences of 37 selected Ingoldian taxa in the Helotiaceae to clarify teleomorph-anamorph connections for these two conspecific genera. The phylogenetic analysis suggested that the 37 selected taxa comprised a monophyletic group in Helotiaceae, Helotiales. The results indicated that both type species have an anamorph best assigned to *Tricladium indicum* with a well-supported clade (82%) containing both of the teleomorph genera *H. varicosporoides* and *C. indica,* and their anamorphs. The molecular data suggested that the presence or absence of a staining reaction for the apical ring was 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 evidence that the two genera are very closely related or synonymous. The inferred phylogenetic trees illustrate that temperate strains isolated from the UK, India and Japan formed the subclade separate from subclade of tropical strains isolated from Thailand. Molecular phylogeny analysis based on ITS1-5.8S-ITS2 DNA sequences is a useful tool for resolving regarding teleomorph-anamorph connections within related Ingoldian taxa.

KEY WORDS: PHYLOGENETIC/ MOLECULAR/ INGOLDIAN FUNGI/ *VARICOSPORIUM* spp./ *HYMENOSCYPHUS VARICOSPOROIDES*/ *CUDONIELLA INDICA*/ *TRICLADIUM VARICOSPOROIDES*

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การศึกษาความสัมพันธ์ทางวิวัฒนาการระดับโมเลกุลของราน้ำ ในกลุ่ม อินโกเดียน
(A MOLECULAR PHYLOGENETIC STUDY OF SELECTED INGOLDIAN SPECIES)

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บทคัดย่อ

การศึกษาอนุกรมวิธานของราน้ำในกลุ่มอินโกเดียนส่วนใหญ่อาศัยสัณฐานวิทยาและรูปร่างของสปอร์ที่เกิดจากการสืบพันธุ์แบบอาศัยเพศและไม่อาศัยเพศ อย่างไรก็ตามราน้ำในกลุ่มอินโกเดียนบางกลุ่มโดยเฉพาะในระดับสกุลและสปีชีส์จำเป็นต้องมีการทบทวนการจำแนกใหม่ ศิวิชัยและคณะ (2546) เสนอว่าราน้ำ *H. varicosporoides* ควรมีชื่อในช่วงการสืบพันธุ์แบบไม่อาศัยเพศเป็นราน้ำสกุล *Tricladium* มากกว่าที่จะเป็นสกุล *Varicosporium* ตามที่ถูกเสนอโดยทาบูกิ (2509) นอกจากนี้ราน้ำสองสปีชีส์คือ *H. varicosporoides* และ *C. indica* มีรูปร่างสัณฐานวิทยาเกือบเหมือนกัน ยกเว้นลักษณะความแตกต่างของการย้อมสีที่ปลายบนถุงสปอร์แอสคัส ดังนั้นการศึกษาวิวัฒนาการระดับโมเลกุลด้วยเทคนิคการวิเคราะห์ดีเอนเอของลำดับเบสในบริเวณ ITS1-2 และ 5.8S rDNA ถูกนำมาใช้เพื่อที่จะตอบปัญหาความสัมพันธ์ในช่วงของการสืบพันธุ์อาศัยเพศและไม่อาศัยเพศของราน้ำสองสายพันธุ์ดังกล่าวที่ใกล้ชิดกันมาก รวมทั้งตัวอย่างราน้ำในกลุ่มอินโกเดียนทั้งหมด 37สายพันธุ์ในแฟมมีลี่ Helotiaceae ผลการศึกษาทางวิวัฒนาการระดับโมเลกุลพบว่าราน้ำในกลุ่มอินโกเดียนทั้ง 37 สายพันธุ์มีวิวัฒนาการมาจากบรรพรุษเดียวกันจากวงศ์ Helotiales ผลจากการศึกษายังบ่งชี้ได้ว่าในช่วงการสืบพันธุ์อาศัยเพศราน้ำสายพันธุ์ *H. varicosporoides* และ *C. indica* และช่วงการสืบพันธุ์อาศัยเพศมีค่าความเชื่อมั่นอย่างมีนัยสำคัญ 82 เปอร์เซ็นต์ในความสัมพันธ์เชิงวิวัฒนาการจากบรรพรุษเดียวกัน ดังนั้นลักษณะของการย้อมติดสีและไม่ติดสีที่ปลายบนถุงสปอร์แอสคัสของราน้ำทั้งสอง สปีชีส์ ถือว่าไม่ใช่ลักษณะรูปร่างที่สำคัญที่จะนำมาใช้ในการแยกของราในสองสปีชีส์นี้ ผลการวิเคราะห์ลำดับนิวคลีโอไทด์บน ITS1-5.8S rDNA-ITS2 ใน*C. indica* SS708; *H. varicosporoides* SS76.01; SS336 และ CBS 651.66 พบว่าการเรียงตัวของลำดับนิวคลีโอไทด์ของราดังกล่าวมีความเหมือนกันอย่างมีนัยสำคัญถึง 98-99.5 เปอร์เซ็นต์ ซึ่งเป็นหลักฐานที่กล่าวเป็นนัยได้ว่า *H. varicosporoides* และ *C. indica* มีความใกล้ชิดกันมากหรือเกือบเป็นสายพันธุ์ เดียวกัน ต้นไม้ความสัมพันธ์เชิงวิวัฒนาการยังแสดงให้เห็นว่าสายพันธุ์ *C. indica* และ *H. varicosporoides* ที่แยกมาจากสหราชอาณาจักร อินเดีย และ ญี่ปุ่นจับกลุ่มแยกจากสายพันธุ์ที่แยกมาจากเขตร้อนชื้นในประเทศไทย ดังนั้นการศึกษาความสัมพันธ์ทางวิวัฒนาการระดับโมเลกุลของลำดับนิวคลีโอไทด์บริเวณ ITS1-5.8S-ITS2 ถือว่าเป็นเครื่องมือที่สามารถแก้ปัญหาความความสัมพันธ์ของราน้ำในกลุ่มอินโกเดียนในช่วงการสืบพันธุ์อาศัยเพศและไม่อาศัยเพศที่ได้

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LIST OF ABBREVIATIONS

/	Per
%	Percent
A	Adenine
ATCC	American Type Culture Collection
acc. no.	GenBank accession number
BCC	BIOTEC Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
C	Cytosine
Y	Number of taxa present in both samples being compared
^{o}C	Degree celcius
ca.	Approximately (latin)
CBS	Centraal Bureau voor Schimmel cultures, Utrecht, The Netherlands
CI	Consistency Index
cm.	Centrimeter
CMA	Corn Meal Agar
CMB	Corn Meal Broth
Cont.	Continued
D	Simpson index
D'	Margalef *D* index
DNA	Deoxynucleic acid
E_H	Equitability or Species evenness (E_H)
e.g.	For example
et al.	*et. alii* (Latin), and others
etc.	For et cetera (latin)
ETS	Externally transcribed spacer
Fig.	Figure (s)

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LIST OF ABBREVIATIONS (cont.)

G	Guanine
g	Gram
H^1	Shannon diversity index (H^1) or Shannon Weaver index
HI	Homoplasy Index
hr	Hour (s)
H_2O	Water
HCl	Hydrochloric acid
i.e.	Meaning of (latin)/ *id est* (latin), that is
IGS	Intergenic spacer
ITS	Internal transcribed spacer
J+	Chemical reactions reaction with Melzer's reagent presence (Blue color at ascus tip)
J-	Chemical reactions reaction with Melzer's reagent absence (Colorless at ascus tip)
jN	The lower of the two sums of all abundance in each of the sites
k	Kilo (10^3)
kb	Kilobase (=1000 bp)
kg	Kilogram
km.	Kilometer
L	Litre
Ln likelihood	Logarithmic likelihood
LSU	Large subunit rRNA gene or 28S rRNA gene
M	Molar
m	Meter
mg.	Milligram
min.	Minute
ml	Milliliter

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LIST OF ABBREVIATIONS (cont.)

mol	Mole
mm	Millimeter
mM	Millimolar
No.	Number
NTS	Non transcribed spacer
ML	Maximum Likelihood (treeing method)
MP	Maximum Parsimony (treeing method)
ms	Milliseconds (10^{-3} s)
mt	Mitochondrial DNA
n	Nano (10^{-9})
n_i	The number of species with i individuals (The information measure is nits for base e and bits per individual for base 2 logarithms)
N	The number of individuals in the *i*th species
N_i	The number of individuals
N_1	Total number of taxa present in first samples but absent in the second samples
N_2	Total number of taxa present in second samples but absent in the first samples
N_T	The total individuals in the sample
NA	Numerical aperture
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining (treeing method)
NNI	Nearest neighbour interchange
NP.	National Park
NTP	Nucleotide triphosphate
nr	Nuclear Ribosomal

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LIST OF ABBREVIATIONS (cont.)

OD	Optical density
p_i	The proportion of individuals in the i^{th} species; or in terms of species abundance; The proportion of S_{obs} made up of the i^{th} species
PAUP*	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
ppm	Parts per million
U	Unit
UV	Ultraviolet radiation
RC	Rescaled Consistency Index
RI	Retention Index
RNA	Ribonucleic acid
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
rDNA	Ribosomal deoxynucleic acid
S/ sec	Second(s)
S_{obs}	Total number of species in the community (species richness) $H^1_{max} = \ln S_{obs}$
sp./spp.	Species
SS	Somsak Sivichai (Original name)
SSU	Small subunit rRNA gene or 18S rRNA gene
T	Thymine
TBE	Tris-Borate Ethylene diamine tetraacetic acid
TE	Tris- Ethylene diamine tetraacetic acid
Tris	Tris-(hydroxymethyl)-aminomethane
T_m	Melting temperature

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LIST OF ABBREVIATIONS (cont.)

U	Uracil
U.	Unit
µ	Micro (10^{-6})
µg	Microgram (10^{-6})
UK	United Kingdom
µl	Microlitre (10^{-6})
µm	Micrometre
V	Volt
v	Volume
v/v	Volume/volume
w	Weight
w/v	Weight/volume
X	Time(s) of Concentration

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CHAPTER I
INTRODUCTION

Fungi play an important role in colonising and degrading organic matters in the freshwater habitats. In the last six years, over 400 species have been collected adding to knowledge of diversity and ecology of freshwater fungi in Thailand (Tubaki *et al*., 1983; Marvanova and Hywel-Jones, 2000; Sakayaroj, 2000; Sivichai and Boonyuen, 2004). Of this number, most are new records in Thailand and at least 20% are probably new to science (Tubaki *et al*., 1983; Marvanova and Hywel-Jones, 2000; Sakayaroj, 2000; Sivichai and Boonyuen, 2004). Most of these freshwater fungi are ascomycetes or their mitosporic states. Freshwater fungi comprise of four taxonomic groups. The first phylum group are lower fungi (i.e. phylum Chytridiomycota and phylum Zygomycota) while the second are "higher fungi" included under phylum Ascomycota and phylum Basidiomycota. The third group are the mitosporic states of Ascomycota and Basidiomycota. The last group under is not a formed taxonomic grouping have been called Ingoldian fungi (Hawksworth *et al*., 1995).

The Ingoldian fungus group was named by Dr. Henry Descals in honour of Professor Cecil Terence Ingold who drew attention to their significance in freshwater habitats more than sixty years ago (Webster and Descals, 1981). Ingoldian fungi are also referred to as the "aquatic hyphomycetes" that are distinguished by relatively large, modified conidia adapted for dispersal in streams and humid habitats. Most studies of Ingoldian Fungi have been carried out in northern temperate regions and few in the tropics (Descals, 1997). There are approximately 500 species currently known and hundreds await description, especially at the generic level (Descals, 1997). They are mostly mitosporic states of Ascomycota, and in most cases their teleomorph associations are unknown.

In Thailand, there have been four published reports and a few unpublished surveys are (Tubaki *et al*., 1983; Marvanova and Hywel-Jones, 2000; Sakayaroj, 2000; Sivichai and Boonyune, 2004). At present, over a hundred species have been

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recognized and collected. However, many of them fungal group are taxonomically problematic with classification based on their morphological characteristics and ontogeny.

This study focused on confusing reports regarding the anamorph of the discomycete, in the class Leotiomycetes, order Helotiales and family Helotiaceae. *Hymenoscyphus varicosporoides* (Tubaki, 1966). Tubaki described the anamorph of this fungus as *Varicosporium*. He also pointed out that *Varicosporium* could equally well be referred to as *Polycladium* or *Tricladium*. Later, Sati and Tiwari (1992) described a mitosporic fungi with appendaged conidia as a new species - *Tricladium indicum* - from India. In addition, other anamorphs have been described for *Hymenoscyphus* species: including *Anguillospora*, *Articulospora*, *Dimorphospora*, *Geniculospora*, *Tricladium*, *Varicosporium* and *Helicodendron* (Tubaki, 1966; Abdullah *et al.*, 1981; Fisher and Webster, 1983; Webster, 1992). Further, *Tricladium* has been reported to have teleomorphs in different groups such as *T. splendens* with *Hymenoscyphus 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). All have very different conidial morphologies. One collection from Thailand was identified *Hymenoscyphus varicosporoides* in Sivichai *et al*. (2003) according to the descriptive described by Tubaki (1966).

Genera considered by Tubaki (1966) as possible anamorphs for *Hymenoscyphus varicosporoides* were *Polycladium*, *Tricladium*, and *Tricladiomyces*. *Polycladium* was ruled out, as the number of lateral branches was greater than tertiary laterals. Roldán *et al*. (1987) in describing *Dendrospora polymorpha* noted that the genera *Varicosporium*, *Tricladium*, and *Dendrospora-Dendrosporomyces* complex 'had become increasingly ill-defined in.' 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).

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The teleomorph genera *Hymenoscyphus varicosporoides* and *Cudoniella indica* are similar in morphology. However, differences revolve around staining of the asci: in *C. indica* asci are reported to be J- (chemical reactions reaction with Melzer's reagent absence) but in *H. varicosporoides*, J+ (chemical reactions reaction with Melzer's reagent presence). On the other hand, staining of the ascus tip in *Hymenoscyphus* to be variable (Spooner pers. comm.).

Molecular Phylogenetics can be very useful in sorting out the confusion over the assignment of fungi to teleomorph and anamorph genera. The following taxonomic issues need to be resolved:

i. Is the anamorph described for *Hymenoscyphus 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 Are *Cudoniella* and *Hymenoscyphus* conspecific genera as *Tricladium* is the anamorph of these both teleomorph genera ?

ii. Are the teleomorph genera of *Hymenoscyphus* and *Cudoniella* polyphyletic?

The first chapter briefly introduces the fungal world and the groups of “Freshwater fungi” and “Ingoldian fungi”. Chapter II reviews the current state of knowledge regarding fungal taxonomy, morphology and methods employed in the study. Chapter III presents the Materials and Methods. Chapter IV describes results. Chapter V and Chapter VI include the discussion and conclusions, respectively. The sequences, alignments and some inferred phylogeny trees are placed in the appendix.

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Objectives:

1. To isolate and deposit more species of Ingoldian fungi in the BIOTEC Culture Collection for future molecular study.
2. To clarify problematic anamorph-teleomorph relationships in the genera of *Hymenoscyphus* and *Cudoniella* using molecular techniques.
3. To examine evolutionary relationships among selected genera and species of Ingoldian fungi using molecular phylogeny.

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CHAPTER II
LITERATURE REVIEW

1. Freshwater Fungi

1.1 Definition of Freshwater Fungi

There are several versions for the meaning of freshwater fungi. In the broadest sense, "freshwater fungi" or "aquatic fungi" includes fungi that may be present transiently in water, to release spores and be dispersed in water, or that live entirely within water (from http://bugs.bio.usyd.edu.au/Mycology/January/2004). For this definition, the term water means transient pools after rain, swamps, estuaries, permanent rivers and oceans. In another sense, freshwater fungi include any fungi regardless of their classified phylum that are found in the water bodies (Goh and Hyde, 1996; Wong *et. al*., 1998). These water bodies can be sediments of sand, gravel, silt, mud, wood, leaves and other organic matters pooling on the floor of water habitats. The freshwater fungi have been classified into three main groups as Ingoldian fungi, aquatic ascomycetes, and chytrids and oomycetes (Goh and Hyde, 1996; Wong *et al*., 1998). The Ingoldian fungi are fungi found on decaying leaves and litter in streams and lakes whereas aquatic ascomycetes and hyphomycetes are present on submerged woody material. Chytrids and oomycetes or non-Ingoldian hyphomycetes comprise fungi that generally cause disease of plants and animals (Fuller and Jaworski, 1987; Barr, 1988; Powell, 1993).

1.2 Taxonomy and Habitats of Freshwater Fungi

More than 600 described species of freshwater fungi include *ca*. ascomycetes, 300 anamorphic fungi and a number of chytrids and oomycetes. A greater number is novel to science and awaits identification (Schoenlein-Crusius and Grandi, 2003). Their distribution is cosmopolitan, restricted to temperate regions to tropical and warm areas. Each species is observed differently based upon two freshwater habitats, lentic habitat (lacking a flow of water) and lotic habitat (continuous flowing water).

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The lentic habitats define any natural aquatic environment lacking a flow of water, such as ponds, dams, lakes, swamps and canals whereas the lotic habitats refer to continuous flowing water, such as streams, rivers, brooks and creeks (Thomas, 1996). Over 100 freshwater ascomycetes, esp. discomycetes, and their anamorph have been observed in lentic habitats in both temperate and tropical regions (Wong, *et al*., 1998). In contrast, almost one-third of the freshwater ascomycetes have been reported in lotic habitats (Wong, *et al*., 1998).

2. Aquatic Hyphomycetes

2.1 Definition of Aquatic Hyphomycetes

The aquatic hyphomycetes comprise of fungi producing conidia mainly in the aquatic environment or in terrestrial water among soil particles, such as streams, reservoirs and lakes with different kinds or levels of pollution (Ingold, 1975; Schoenlein-Crusius and Grandi, 2003). However, this fungal group has also been called “tetraradiate fungi” according to the shape of their conidia with a divergent radiate or star-like (3-4) arms or branches built by a central part. These distinctive conidial forms may facilitate trapping in foam, dispersal in water, or floating on water surfaces for the chance of colonization by landing on organic substrates such as each leaf litter and twigs.

Based on their form and life–cycle, Goh and Hyde (1996) divided the aquatic hyphomycetes into four groups: Ingoldian hyphomycetes, aero-aquatic hyphomycetes, terrestrial-aquatic hyphomycetes and submerged-aquatic hyphomycetes. Ingoldian hyphomycetes are fungi whose hydrodynamic conidia are reproduced mainly in the aquatic environment, while the aero-aquatic hyphomycetes are fungi that reproduce out of the aquatic environment. In contrast to Ingoldian and aero-aquatic hyphomycetes, terrestrial-aquatic hyphomycetes and submerged-aquatic hyphomycetes are fungi that are facultative in both aquatic and terrestrial ecosystems. However, Goh (1997) revised the aquatic hyphomycetes into three groups: Ingoldian fungi, aero-aquatic fungi and lignicolous fungi. In addition, in term of nutritional mode Shearer (1993) divided the teleomorphic freshwater hyphomycetes

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(Ascomycota) into parasites and endophytes of aquatic macrophytes and algae, and saprophytes of submerged vegetation.

3. Ingoldian Fungi

3.1 Historical Perspective

From 1893 to 1895, De Wildeman isolated "aquatic hyphomycetes" from decaying plant materials, *Hippuris vulgaris*. He described and reported four new fungal species, named *Lemonniera aquatica, Tetracladium marchlianum, Clavariopsis aquatica* and *Anguillospora longisima* (Wildeman, 1893, 1894, 1895). These four species were also found in a small lake or pond. In 1938, Cecil Terence Ingold, a young professor in Leicester, isolated a group of aquatic hyphomycetes on decaying alder leaves in a stream in Southern England.

3.2 Definition of Ingoldian Fungi

In 1981 Webster and Descals proposed that among the aquatic hyphomycete, some of them should be named "Ingoldian fungi" to honor Professor Ingold (Cecil Terence Ingold), who was the first pioneer studying this group of fungi. The term "Ingoldian fungi" was described by Schoenlein-Crusius and Grandi, (2003) as aquatic hyphomycete species that produce sigmoid, fusiform, coiled and spherical conidal and are restricted to aquatic environments to complete their life cycle.

3.3 Morphological Features of the Ingoldian Conidia

Spores of Ingoldian fungi are very unique and distinctive from other fungal spore forms. They are invariably two- to several- armed, variously branched (usually tetraradiate) and sigmoid shapes (Fig. 2.1). These special appendages characterize their scolecoform or stauroform conidia that are released mostly from submerged conidiophores.

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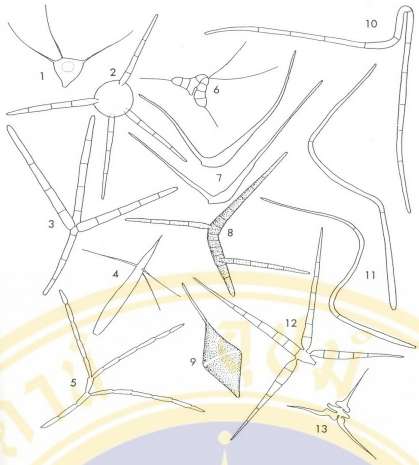

Figure 2.1 Different conidial types of Ingoldian spores commonly found in water (Goh, 1997).

The shapes of appendage are presumably adapted to anchorage and dispersal in aquatic environments, since these branches are easily trapped on the surface of air bubbles (Ingold, 1942, 1975; Barlocher, 1982). The presence of viscous and sticky modification on appendages may enable the conidia to stick onto substrates in moving water. Helical spore forms are adapted for attachment and dispersal in aquatic environments (Ingold, 1975 and Webster, 1992). The sigmoidal conidial form appears to have evolved in several different evolutionary ways for anchorage and dispersal in the aquatic environments (Webster, 1992).

3.4 Taxonomy of Ingoldian Fungi

From the taxonomical point of view, the Ingoldian fungi are an artificial, heterogeneous group, constituting mostly of anamorphs of discomycetes in Ascomycota (Goh, 1997). In 1995, Webster, Eicker and Spooner collected and isolated *Tricladium indicum* from a foam sample in South Africa. *Tricladium indicum* could produce a sexual state called *Cudoniella indica* in sterile water in the laboratory. (Webster, Eicker and Spooner, 1995). It was the first report for connection of a *Tricladium* with this genus. Furthermore, conidia of *T. indicum* were found developing from cultures of *Cudoniella* ascospores.

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3.5 Teleomorph/ Anamorph Connections of Ingoldian Fungi

The confusion regarding teleomorph and anamorph connections for Ingoldian fungi results from by the occurrence of sexual and asexual states in different places and at different periods of time. Some anamorphic genera have teleomorphs in various genera and vice versa some teleomorph genera have a wide range of anamorph stages. For example, *Tricladium* species are reported as anamorphs of *Cudoniella*, *Hydrocina* and *Hymenoscyphus*. While *Anguillospora*, *Clavariopsis* and *Tumularia* are repeated as the anamorph of *Massarina* species. Some species can be observed more abundant in cold or cool temperature climates. These Ingoldian fungi are *Hymenoscyphus varicosporoides*, *Massarina aquatica* and *Ascotaiwania hughesii* that have the teleomorph forms of *Varicosporium* sp, *Tumularia aquatica* and *Helicoon farinosum*, respectively (Table 2.1).

Webster (1992) reviewed the teleomorph/anamorph relationships in Ingoldian fungi (Table 2.1). Approximately 27 telemorphs are known (Webster, 1992; Descals, 1997). In table 2.1 *Hymenoscyphus* has anamorphs in various genera, such as *Hymenoscysphus splendens* (with *Tricladium splendens*), *Hymenoscyphus foliicolus* (with *Dimophospora folicala*), *Hymenoscyphus inberbis* (with *Anguillospora fustiformis*), *Hymenoscyphus paradoxus* (with *Helicodendron paradoxum*), *Hymenoscyphus africanus* (with *Geniculospora grandis*), *Hymenoscyphus tetracladius* (with *Articulospora tetracladia*). On the other hand, some anamorphic genera have teleomorphs in various genera, such as, *Tricladium* spp and *Anguillospora*.

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Table 2.1 Teleomorph-anamorph stages of aquatic Ingoldian fungi (From http://fm5web.life.uiuc.edu: 23523 /ascomycete/2004/August).

Teleomorph	Anamorph
Cudoniella indica Webster, Eicker, and Spooner	*Tricladium indicum* S. C. Sati and N. Trivari Webster, J., A. Eicker and B. M. Spooner. 1995
Hymenoscyphus varicosporoides Tubaki	*Varicosporium* sp. Tubaki, K. 1966
Hymenoscyphus splendens Abdullah, Descals and Webster	*Tricladium splendens* Ingold Abdullah, S. K., E. Descals and J. Webster. 1981
Hymenoscyphus africanus Descals, Fisher and Webster	*Geniculospora grandis* (Greathead ex Nolan) *Nilsson* Descals, E., J. Fisher and J. Webster. 1984
Hymenoscyphus foliicola Abdullah, Descals and *Webster*	*Dimorphospora foliicola Tubaki* Abdullah, S. K., E. Descals and J. Webster. 1981
Hymenoscyphus inberbis Descals and Marvanová Unpublished	*Anguillospora fustiformis*
Hymenoscyphus malawienis Fisher and Spooner	Unnamed hyphomycete
Hymenoscyphus paradoxus Fisher and Webster	*Helicodendron paradoxum* Peyronel Fisher, P. J. and J. Webster. 1983
Hymenoscyphus tetracladius Abdullah, Descals and Webster	*Articulospora tetracladia* Ingold Abdullah, S. K., E. Descals and J. Webster
Massarina sp.	*Anguillospora longissima* (Sacc. and Syd.) Ingold Willoughby, L. G. and J. F. Archer. 1973.
Massarina sp.	*Clavariopsis aquatica de Wild.* Webster, J. and E. Descals. 1979
Massarina aquatica Webster	*Tumularia aquatica* (Ingold) Descals & Marv. (sub Pyricularia aquatica Ingold.) Webster, J. 1965
Mollisa uda (Pers.: Fr.) Gill	*Anguillospora crassa* Ingold Webster, J. 1961
Mollisia casaresiae (Ascomycetes) Webster, Shearer and *Spooner*	*Casaresia sphagnorum* Fragoso Webster, J., C. A. Shearer & B. M. Spooner. 1993
Mollisia gigantea Fisher and Webster	*Helicodendron giganteum* Glenn-Bott Fisher, P. J. and J. Webster. 1983
Ascotaiwania hughesii Fallah, Crane and Shearer	*Helicoon farinosum* Linder Fallah, P., J. L. Crane and C. A. Shearer. 1999
Hydrocina chaetocladia Scheuer	*Tricladium chaetocladium* Ingold Webster, J., C. Scheuer and S. O. Khattab. 1991
Lambertella tubulosa Abdullah and Webster	*Heliocodendron tubulosum* (Reiss) Linder Abdullah, S. K. and J. Webster. 1981
Loramyces juncicola Weston	*Anguillospora* sp. Digby, S. and R. D. Goos. 1987
Miladina lechithina (Cooke) Svrcek	*Actinospora megalospora* Ingold Descals, E. and J. Webster. 1978

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3.6 Diversity and Geographical Distribution of Ingoldian Fungi

After aquatic hyphomycetes fungi were reported by De Wildeman in 1893,1894 and 1895 and Professor C. T. Ingold (1942) reported on the hyphomycete flora of decaying alder leaves in streams in 1938, a large number of Ingoldian fungi have been observed. A large number of papers were written by Ingold (Ingold, 1942, 1943, 1944, 1951, 1952, 1954, 1955, 1956, 1958a-b, 1959a-c, 1960a-b, 1961, 1965, 1966, 1967, 1971, 1975, 1976; Ingold and Chapman, 1952; Ingold and Webster, 1973). The number of studies on tropical species has also increased dramatically. (Tubaki *et al*., 1983; Sindhar *et al*., 1992; Sivichai, 1999; Ho, 1998; Marvanova and Hywel-Jones, 2000; Sakayaroj, 2000; Chan *et al*., 2000a; 2000b; Sivichai and Boonyuen, 2004). Aquatic Ingoldian fungi are abundant in cold temperate areas, but appear to be less frequency in tropical or warm climates (Chan *et al*., 2000a).

3.7 Roles of Ingoldian Fungi in the Aquatic Ecosystem

Aquatic Ingoldian hyphomycetes are the predominant microorganisms that colonize leaves in streams. Therefore, they may be considered the primary decomposers of leaves in streams (Chauvet and Suberkropp, 1998). The biomass of fungi associated with leaf detritus throughout decomposition causing changes in leaf litter. Ingoldian fungi can also transform leaf litter into a more palatable and nutritious food source for invertebrate detritivores (decomposing invertebrates), indicating that they are also important intermediaries of energy flow between the leaf detritus and animals (Suberkropp, 2001). These Ingoldian decomposers include *Articulospora tetracladia, Tricladium chaetocladium, Anguillospora filiformis, Anguillospora longissima, Tetrachaetum elegans, Flagellospora curvula* (Charcosset and Chauvet, 2001; Suberkropp, 2001).

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3.8 Applications of Ingoldian Fungi

The potential applications from Ingoldian fungi are secondary metabolites and their enzymes. Some Ingoldian aquatic fungi are capable of producing extracellular and hydrolytic enzymes (amylase, cellulase, protease, lipase, phenoloxidase, xylanase, ligninase, pecticnase) that degrade plant constituents such as polysaccharide, cellulose, starch, lipids, tannic acid and protein. These Ingoldian enzymatic producers are *Helicodendron intestinale*, *Anguillospora longissima*, *A. crassa*, *Lunulospora curvula, Lemonniera aquaticus*, *Tetracladium setigerum*, *Heliscus lugdunensis*, *Dactylella aquatica* and *Varicosporium elodeae* (Zemek *et al*., 1985; Shearer and Zare-Maivan, 1988). For the secondary metabolites, Anguillosporal, ($C_{15}H_{22}O_3$), a new biologically active natural product with antibacterial and antifungal activates against (*Staphylococcus aureus*) and (*Candida albican*), in which isolated from *Anguillospora longissima* in a freshwater stream (Harrigan *et al*., 1995).

3.9 Taxonomy of selected *Hymenoscyphus* and *Cudoniella*

This study focused on the two genera of *Hymenoscyphus* and *Cudoniella* whose taxonomic ranks were shown bellow while their morphological characteristics were compared in Table 2.2.

Domain Eukayota
Kingdom Eumycota
Phylum Ascomycota
Subphylum Pezizomycotina
Class Leotiomycetes
Order Helotiales
Family Helotiaceae
Genus *Hymenoscyphus* and *Cudoniella*

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***Hymenoscyphus varicosporoides*:** Sivichai *et al*., 2003.

Apothecia generally scattered or occasionally aggregated, superficial, disk and stalk originally white, becoming pale brown when mature and dry, disks circular, slightly convex, and up to 380 µm diam. Receptacle smooth, soft, white, stalk cylindrical, length variable (up to 1400 µm long), and pale brown at the base. Flesh thin-walled, compact with slightly interwoven, hyaline hyphae becoming parallel toward the excipulum. Asci persistent, cylindric-clavate, 70-100 x 10-12.5 µm, 8-spored, apex round, pedicellate, unitunicate and J+. Ascospores 14-20 x 5-7.5 µm, hyaline, biseriate or partly biseriate, elliptic-fusiform, equally or unequally 1-septate, with ends rounded, sometimes continuous, and often slightly curved. Paraphyses numerous, unbranched, filamentous, non-septate, with rounded apex 1.2 µm wide and similar in length to the asci. The anamorph appeared on test blocks after seven days of incubation, while apothecia appeared after 6-10 days, and were grew together with the anamorph (Figure 2.2).

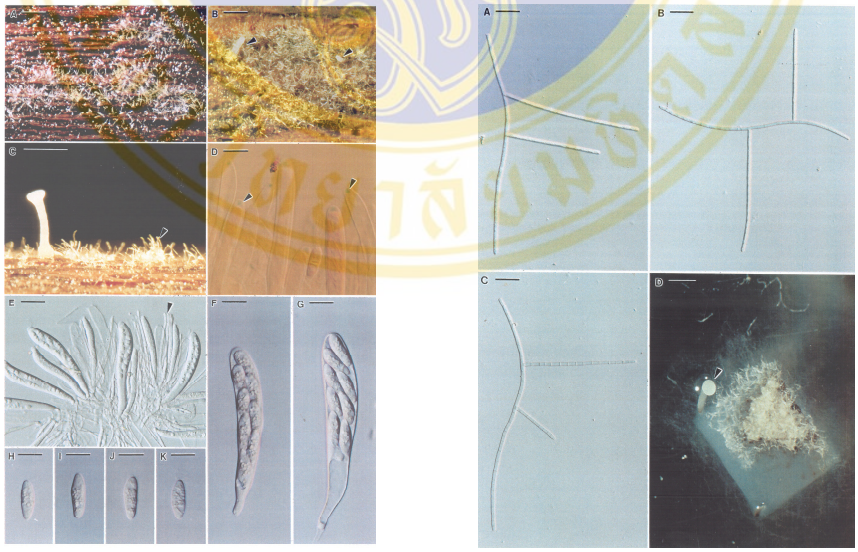

Figure 2.2 *Hymenoscyphus varicosporoides* and *Tricladium* anamorph (From Sivichai *et al*., 2003).

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Tricladium *anamorph*. (*T. varicosporoides*) Sivichai *et al*., 2003.

Colonies up to 20 mm long, and 6 mm wide appeared on the wood after 6-10 days of incubation and were originally white, becoming pale brown when mature and dry. Conidiophores were simple, bearing branched conidia. Conidia were hyaline, with a main axis, 300-600 μm long, and 5-7.5 μm wide, with one to three laterals of the same width usually developing on one or both sides of the main axis, up to 220-500 μm long. Conidia typically had two lateral branches which were positioned on the same side of the main axis and in the same plane. Occasionally the two laterals were on opposite sides of the conidial axis. Rarely, three branches were seen, which could either all be on the same side or on opposite sides. In the typical form the distance between the laterals was consistently between 60-80 μm. No tertiary branches were observed either from material in culture or from field material. The main axis and laterals are multiseptate, each cell *ca*. 10-20 μm with the lateral arms joined to the main axis by a narrow isthmus (Figure 2.2).

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3.10 Genera concept of *Hymenoscyphus* and *Cudoniella*

3.10.1 *Hymenoscyphus* (S.F. Gray)

Ascomata an apothecium, up to 4 mm diam., solitary or clustered, apothecium convex, flat or cupulate, stipitate, with stipe short or long, white, pink, yellow to brownish, ectal expipulum composed of parallel, thin-walled cells, medullary expipulum of loosely interwoven hyphae. Paraphyses cylindrical, sometimes slightly inflated at apex. Asci unitunicate, cylindric-clavate, pore blueing in iodine, 8 –spored. Ascospores hyaline, 1 celled or occasionally 2 celled, elliptical to fusiform.

3.10.2 *Cudoniella* (Saccardo, 1889)

Ascomata hemisphere, soft, smooth margin, stripe, fragile. Asci with paraphyses, 8 ascospore. Ascospores, hyaline, 2-4 cell, fusiform, 1-3 septate.

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Table 2.2 Comparison of *Hymenoscyphus varicosporoides* (type isolates and Thailand isolates) and *Cudoniella indica* (Modified from Sivichai *et al*., 2003).

Taxonomy	***Hymenoscyphus varicosporoides*** (Sivichai *et al*., 2003)	***Hymenoscyphus varicosporoides*** (Tubaki, 1966)	***Cudoniella indica*** (Webster *et al*., 1995)
Kingdom	Fungi		
Phylum	Ascomycota		
Sub-phylum	Pezizomycotina		
Class	Leotiomycetes		
Order	Helotiales		
Family	Helotiaceae		
Genus	*Hymenoscyphus*		*Cudoniella*
Species	*varicosporoides*		*indica*
Types species	*Hymenoscyphus fructigenus* (Bull, 1821)		*Cudoniella queletii* (Sacc, 1889)
Country origin	Thailand	Japan	South Africa
Citation	Sivichai *et al*., 2003	Tubaki, 1966	Webster *et al*., 1995
Habitat	Lotic	Lentic	Lotic (river)
Substrate origin	Test Blocks	Woody (twigs)	Foam samples
Anamorph stage	*Tricladium* anamorph of *Hymenoscyphus varicosporoides*	*Varicosporium* sp. (Tubaki, 1966)	*Tricladium indicum* (Sati *et al*., 1992; Webster *et al*., 1995)
Ascomata	Up to 380 um. (Sivichai *et al*., 2003)	2 mm. (Tubaki, 1966)	Up to 2 mm. (Webster *et al*., 1995)
Stipe	1400 um. (Sivichai *et al*., 2003)	0.2-0.7 mm. (Tubaki, 1966)	Up to 6 mm. (Webster *et al*., 1995)
Paraphyses	Present (Tubaki, 1966; Webster *et al*., 1995; Sivichai *et al*., 2003)		

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Table 2.2 Comparison of *Hymenoscyphus varicosporoides* (type isolates and Thailand isolates) and *Cudoniella indica* (Modified from Sivichai *et al*., 2003). (Cont.)

Taxonomy	***Hymenoscyphus varicosporoides*** (Sivichai *et al*., 2003)	***Hymenoscyphus varicosporoides*** (Tubaki, 1966)	***Cudoniella indica*** (Webster *et al*., 1995)
Ascospores	14-19x 5-7.5 (Sivichai *et al*., 2003)	(10)12 -15(19)x 4-5(7) (Tubaki, 1966)	15-20x5-7.5 (Webster *et al*., 1995)
Septation	1 septa (Sivichai *et al*., 2003)	1 septa (Tubaki, 1966)	0-1 septa (Webster *et al*., 1995)
Asci	70-100x10-12 (Sivichai *et al*., 2003)	70-85x10-12 (Tubaki, 1966)	87-95 x10-11
Staining of asci	J + (Blue color)		J – (Color less)
Anamorph name	*Tricladium* anamorph of *Hymenoscyphus varicosporoides*) (Sivichai *et al*., 2003)	*Varicosporium* spp. (Tubaki, 1966)	*Tricladium indicum* (Sati and Trivari, 1992; Webster *et al*., 1995)
Types species of anamorph name	*Tricladium splendens* (Webster *et al*., 1981)	*Varicosporium elodeae* (Kegel, 1906)	*Tricladium splendens* (Webster *et al*., 1981)

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Table 2.3 Comparison of anamorphic stages between *Tricladium* spp and *Varicosporium* spp (Modified from Sivichai *et al*., 2003).

Species of anamorph	Length x width		Number of laterals
	Main axis (um.)	Lateral branches (um.)	
Tricladium anamorph of *H. varicosporoides*	300-600x5-7.5	200-500x5-7.5	1-3
Tricladium indicum	150-580(800) x10-12	80-315x10-11	1-4
Tricladim splendens	60-120x6-7	30-80x7.6-7	6-7
Tricladim chatocladdium	160-260x4-5	60-140x4	2
Tricladim terrestre	120-280x4-6	24-200x3-4	1-5
Tricladim giganteum	200-300x4-4.7	90-180x44.6	1-2
Tricladim malaysianum	230-328x 4.5-6.5	34-106x3.6-6	2-4
Tricladim brunneum	68-108x5-8	30-84x3-4	1-2
Varicosporium delicatum	110-300x1.5-2	50-300x1.5	2-3
Varicosporium macrosporum	197-322x2-2.5	Up to 260	2-4

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4. Diversity Measurement

Hawksworth *et al.* (1991, 1995) estimated that global fungal diversity, ranged from 0.5-9.9 million species. Mycologists have currently classified and identified approx. 72,000 species, representing only 5% of the estimated 1.5 million species. Fungi are found in almost every environment. In terms of measures of diversity, Whittaker (1960) can be divided diversity indexes into three types:

(i) Alpha diversity is a diversity within a particular sample, e.g., Shannon-Wiener function index, Simpson index (D), Species number, Margalef (D') index, Equitability (or Evenness diversity) and Species richness. Most analyses for conservation purposes have focused only on species diversity.

(ii) Beta diversity is a diversity associated with changes in sample composition along environmental gradients such as Whitaker' index, Cody's index, Routedge's index and Wilson and Schimida index. Beta diversity reflects not only differences in abundance, but also differences in species composition.

(iii) Gamma diversity is a diversity due to differences among samples when they are combined into a single sample. For diversity purposes (and for many others). Gamma diversity is offer ignored because it reflects about how samples are collected, not about communities in nature.

5. Systematics and Molecular Phylogeny

Traditionally, systematics and phylogeny are studies of classification, genealogical relationships and historical patterns of groups of organisms. The ideal approach to infer evolutionary history of organisms is to use the fossil record. However, fossil records are usually incomplete and fragmented. Since Linnaeus, fungal systematics and phylogeny have been based on morphological characters. With the advance of molecular techniques in recent years, molecular data are now gathered and used for systematics and phylogenetic evaluation.

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5.1 Morphological Techniques

In the past, morphological characters were used for identification. The most important characters for classification and identification of mitosporic fungi are asexual spores and mode of conidium formation. In most cases, separation of species within a family is focused on host-specialization, fruiting body type, perithecial, ontogeny and ascus/ascospore characters (White *et al.,* 2000). The taxonomic identification of filamentous ascomycete fungi is based mainly on micro- and macro-morphological characteristics. These include cultural morphologies such as colony and color characteristics on specific culture media; size, shape and development of sexual spores and asextual spores and spore forming structures, and/or physiological characteristics (Glass and Donaldson, 1995). However, these characters are offer either too variable or relatively few. Thus, evolutionary change of morphological characters does not give a clear-cut picture of evolutionary relationships. The evolutionary change of morphological characters is extremely complicated, even for a short evolutionary period. Besides, some fungi cannot be cultured or lack spore production and may exist only as hyphae in the substratum. Thus, they do not have enough characters to construct a phylogenetic tree. The majority of the diagnostic features, e.g. size and shape of fungal spores, are not discrete characters and cannot be used in phylogenetic analysis (Harrington, 1999). According to Pipenbring *et al.* (1999), phylogenetic trees based only on morphological data were not good. Though systematics and phylogeny based on morphological characters are more seldom used, morphological data still has some advantages. These are:

(i) greater applicability to museum specimens because characters can be analyzed quickly and cheaply for a large number of individuals; and

(ii) greater applicability to fossil species since paleontology is primarily restricted to morphological analysis.

Therefore, the use of molecular data for systematics can be used where morphological characters are conflicting, ambiguous or missing (Ota *et al*., 2000). In addition, morphological characters can be used for analysis together with molecular characters to infer the phylogeny of fungi and other organisms.

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5.2 Molecular Techniques

The application of molecular biology methods to mycology has become a widespread, almost routine process. As with prokaryotic taxonomy, fungal systematics has traditionally been based on observations of various morphological features and physiological properties. Molecular techniques now permit the analysis of genetic markers to establish the identity of individual microorganisms, as well as taxonomic and phylogenetic relationships between organisms. Thus, they play a major role in modern taxonomy (Hillis and Moritz, 1990). Molecular data used in systematic studies are always useful in cases where morphological characters alone are insufficient for the delineation of clear taxonomic groups. These methods are mainly based on the analysis of chromosomes, genes, and their translation products, i.e. proteins. Early studies were based on DNA re-association (Bak and Stenderup, 1969), and this technique has been used in several mycological studies (Kurtzman, 1989). Depending on the aim of study in taxonomy, phylogeny or identification of organisms, various methods can be applied.

5.2.1 Molecular Phylogeny and Nuclear Ribosomal RNA Genes

The molecular phylogenetic techniques and molecular characters have been important to infer evolutionary history and test morphological development characters. The most widely used region of DNA in mycology is the ribosomal RNA (rRNA) gene cluster, the region of DNA that codes for the production of the ribosomes. (Hillis and Dixon, 1991; Hibbett, 1992).

The rRNA cluster has been used extensively in studies on evolution and systematics because they are present in most genomes as multiple copies and for the development of molecular diagnostics in medicine, agriculture, and ecology (Bruns *et al*., 1991; Bowman *et al*., 1992; Gardes and Bruns, 1993; Swann and Taylor, 1993; Sreenivasaprasad *et al*., 1996). The rRNA gene cluster is featured in many research papers and so this description is limited to only a few major factors. The region consists of three major genes that code for the large (LSU nrDNA), small (SSU nrDNA) and 5.8S ribosomal subunits (Figure 2.2).

Generally, SSU nrDNA is used to resolve phylogenetic relationships at the family, ordinal, or higher levels because it is conservative and evolves at a slow rate.

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LSU nrDNA has a higher evolutionary rate and has been used for the species, generic, and family level phylogenic studies in fungi (Berbee and Taylor, 1992, 1995; Spatafora, 1992; Spatafora and Blackwell, 1993, 1994a,b; Rehner and Samuels, 1995). Sequence data of the internal transcribed and intergenic spacers of the ribosomal RNA gene (ITS and IGS) accumulate changes rapidly and are useful in generic or lower taxonomic level studies.

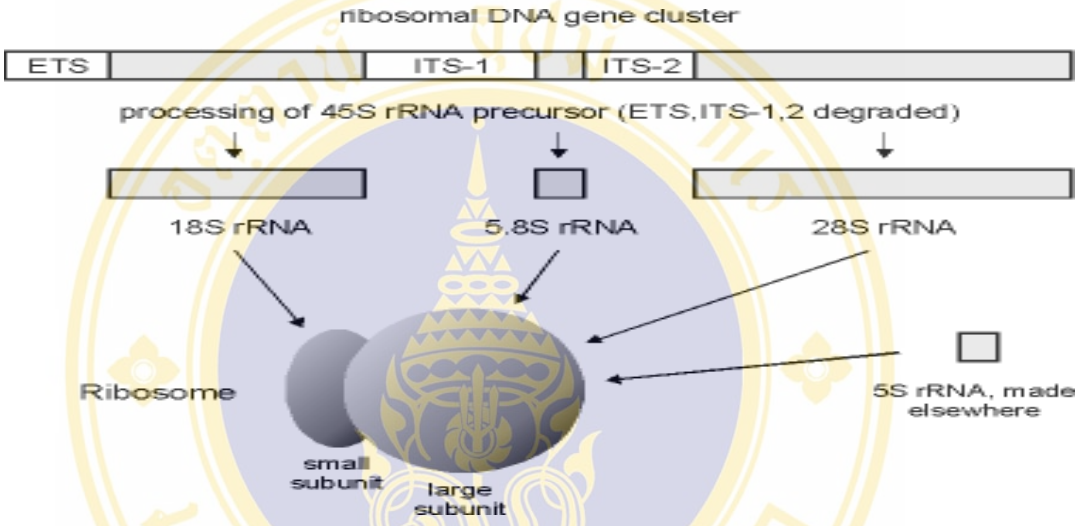

Figure 2.3 Ribosomal RNA gene cluster of the eukaryotic cell (From http://www.rzuser.uni-heidelberg.de/~bu6/Introduction11.html/2004/April).

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CHAPTER III

MATERIALS AND METHODS

1. Studied Sites

Natural foam samples were collected from six sites at Bala Hala Wildlife Sanctuary in Narathiwat province, Nam Nao National Park in Petchabun province, Khao Yai National Park in Nakorn Ratchassima province, Tone Nga Chang Wildlife Sanctuary in Songkra province, Kaeng Kranchan National Park in Petchaburi province and Doi Inthanon National Park in Chaingmai province (Figure 3.1). Each site was divided into sub study sites as described below.

	Studied Sites	Date of collection
1.	Hala Bala Wildlife Sanctuary	11-15/07/ 03
2.	Nam Nao National Park	22-23 /09/ 03
3.	Khao Yai National Park	10-12 /08/03
4.	Tone Nga Chang Wildlife Sanctuary	29/01/04
5.	Kaeng Krachan National Park	17-20 /05/ 04
6.	Doi Inthanon National Park	9-11/08/04

Figure 3.1 Six studied sites in different regions of Thailand for collecting foam samples (From http://www.dnp.go.th/parkreserve/2005/January).

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1.1 Bala Hala Wildlife Sanctuary

Bala Hala Wildlife Sanctuary covers the Sangalakiri mountain range in Narathiwat province, and extending to the border of Malaysia. The fertile evergreen forest is supported by a density of huge dipterocarp trees (From http://www.dnp.go.th/conserv/wildlife/sanctuary/sanc40.htm/2005/January). The two sub studied sites were:

1.1 Nam Tok Klong I Ga Ding

1.2 Nam Tok Sirindhorn

1.2 KhaoYai National Park

In Nakorn Ratchassima province, this collecting site consists of complicated mountains, the height about 802 –1300 meters above sea level. Khao Yai has three main seasons, with an annual mean temperature of 23°C, though this varies greatly with the season. During the rainy season in May-October, it rains almost all days, resulting in stunning waterfalls. The atmosphere is humid, with average daytime temperatures of 27°C. In the humidity, flora and fauna flourish and after the rain, there is clean air and clear visibility. In the winter season in November- February, the temperature during day time averages 22°C. The temperature can drop to 10°C at night. During the hot season in March-April, even daytime temperatures can reach between a high 30°C to a low 20°C. It is dry and often windy during this season. Waterfalls can be dry by April. A tropical moist evergreen forest covers the central area of Khao Yai National Park with dry evergreen forests, dry deciduous forests, tropical moist evergreen forest, hill evergreen forests and grasslands, (From http://www.dnp.go.th/parkreserve/2005/January). Three sub studied sites were:

2.1 Nam Tok Wang Jum Pee

2.2 Nam Tok Tanratana

2.3 Nam Tok Heaw Narok

1.3 Nam Nao National Park

In Petchabun province, Nam Nao National Park covers the border between northeastern and northern Thailand. It including particularly part of the Phetchabun mountainous range running through Chaiyaphum and Phetchabun provinces. The

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forest of Nam Nao contains many different forest types, including dipterocarp, deciduous, evergreen, hill evergreen and pine forest (From http://www.dnp.go.th/parkreserve/2005/January). Two studied sites were:

1.3.1 Nam Tok Haew Sai

1.3.2 Khod Hin stream

1.4 Tone Nga Chang Wildlife Sanctuary

This is located in Songkha province, at about 932 meters above sea level. The waterfall is located in Amphoe Rattaphum on highway 406 (Hat Yai-Satun) at Km.34-35 (From http://www.dnp.go.th/parkreserve/2005/January). One sub studied site was:

1.4.1 Stream in Tone Nga Chang Wildlife Sanctuary

1.5 Kaeng Krachan National Park

In Thailand, Kaeng Krachan National Park is a forest where is the original of Phetchaburi and Pranburi rivers originate. The mountain range is approximately 500 meters above sea level. The highest peak is 1,200 meters above sea level. Most of the park is covered in deep, steep forest. Over three-quarters of the area has slopes greater than 30% while 85% of the terrain is evergreen rainforest, another 10% is mixed deciduous forest (From http://www.dnp.go.th/parkreserve/2005/January). Two sub studied sites were:

1.5.1 Stream at Bankrang trail

1.5.2 Stream at km.18.

1.6 DoiInthanon National Park

In Chaingmai province, the geographical features of this National Park consist of high mountains, inclding the highest mountaintop in Thailand (2,565 meters above the sea level). Doi Inthanon National Park has cold weather and high humidity throughout the year, particularly on the top of the National Park. The forests include moist evergreen forest, pine forest and mixed deciduous forest (From http://www.dnp.go.th/parkreserve/2005/January). Two sub studied sites were:

1.6.1 Stream at Aar-Ka trail

1.6.2 Stream at Siri-Phum

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2. Sampling and Isolation of Freshwater Fungi

Natural foam bubble samples were collected from six sites of Bala Hala Wildlife Sanctuary, Khao Yai National Park, Nam Nao National Park, Tone Nga Chang Wildlife Sanctuary, Kaeng Kranchan National Park and Doi Inthanon National Park (Figure 3.1). Foam samples from each stream were placed in bottles and spread over corn meal agar (CMA) media with antibiotics for 8-10 plates/sites in the field. Plates were then returned to the laboratory and incubated at 20°C in plastic boxes on sterile tissue paper. Samples were examined with a stereomicroscope and compound microscope for evidence of Ingoldian spore germination.

Figure 3.2 Natural foam bubble samples in freshwater stream at Tone Nga Chang Wildlife Sanctuary (Left) and (Right).

Single spore isolations were made after morphological examination. They were grown on CMA plates, incubated at 20°C in a cabinet with cool white fluorescent light and examined with a stereomicroscope and compound microscope each day for signs of spore germination. Spores of individual species were transferred to new plates and incubated in the same cabinet.

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3. Fungal Identification Based on Morphological Characteristics

To identify Ingoldian fungi, foam samples were prepared by placing a few drops of the foam sample on glass microscope slides. A cover slip was put on top and tissue paper was used to absorb excess water. The materials were examined, measured and photographed with a Nomarski Differential Interference Contrast microscope. Permanent slides were prepared by allowing a drop of lactophenol cotton blue to replace water on the slide, and then the cover slip was sealed with polyvinyl alcohol. Voucher slides are kept in the BIOTEC Herbarium, Pathumthanee, Thailand. Some Ingoldian fungi collected in this study were fully identified while others had to await further identification and description. Ingoldian fungi were induced to sporulate using fungal sporulation techniques under sterile, aerated water. Fungal identification was based on used by morphological features and spore ontogeny. All abbreviations of each test were shown in table 3.1.

4. Analysis of Fungal Biodiversity

Analysis of fungal biodiversity in different sites was carried out using the Shanon diversity Index in the program named "Species diversity and richness" version 2.2 program (Henderson, 1998) to calculated species richness, abundance or evenness, changed into a single value. The species diversity of each sampling site was calculated using Shannon Weaver index (H^1) (Begon *et al*., 1998).

4.1 Shannon Diversity Index (H^1) or Shannon Weaver Index

A function devised to determine the amount of information in a code or signal, and defined. The Shannon Diversity index is commonly used to characterize species diversity in a community (Begon *et al*., 1998). The resulting product is summed across species, and multipled by –1.

The Shannon diversity index (H^1) or Shannon Weaver index is calculated using:

$$H^1 = - \sum_{i=1}^{S\,obs} p_i \log_e p_i$$

$$H^1 = - \sum_{i=1}^{S\,obs} p_i \ln p_i$$

$$H^1 = -\Sigma (N_i/N) \log^2(N_i/N)$$

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$$H^{l} = \log_e N-(1/N) \sum_{i=1}^{\infty} (p_i \log_e p_i) n_i$$

4.2 Equitability (E_H) or Species Evenness

This value refers to the pattern of distribution of individuals between species. It assumes a value between 0 and 1, with 1 being complete evenness, when the community structure is perfectly even. For example, all taxa are found in an equal number of samples.

On the other hand, when the E_H value is equal to 0 or less than 0, the community structure is extremely of uneven, for example when only one taxon is present.

Equitability (E_H) can be calculated by dividing H^l *by* H^l_{max} (here $H^l_{max} = \ln S_{obs}$). These indexes are

$$E_H = H^l / H^l_{max}$$

$$E_H = H^l / \ln S_{obs}$$

$$E_H = H^l / \log S_{obs}$$

4.3 Margalef's Index (D')

This index provides a measure of species richness (Independent of sample size) that is roughly normalized for sample size without using more complex rarefaction techniques (Margalef, 1958). This index is calculated by the following formula:

$$D' = (S_{obs} -1)/\ln N$$

4.4 Simpson Index (D)

This diversity index was proposed by Simpson (1949), to describe the probability that a second individual drawn from population will be the same species as the first. This index is calculated by the following formula:

$$D = 1/C$$

The statistic, Y is give by: $Y = - \sum_{i=1}^{S\ obs} p_i^{\ 2}$

$p_i^{\ 2} = \{N_i(N_i-1)\}/ (N_T-1)$, usually approximated as: $p_i = \{ (N_i/ N_i) \}^2$

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4.5 Percent Similarity

Percent similarity can be calculated by two formula as Sorenson's similarity or Jaccard's similarity index. A similarity index used to compare species composition of different ecosystems (McCracken *et al*., 2001).

Sorenson's similarity and Jaccard's similarity index are:

$$\text{Sorenson's similarity} = 2(jN)/(N_1+N_2)$$

$$\text{Jaccard's similarity index} = (jN)/(N_1-N_2-(jN))$$

The values for Sorenson's similarity and Jaccard's similarity indexes fall between 0 (0%) and 1 (100%). When the values equal to 0, it means that fungal taxa from both sites are totally different. When the value equal to 1, it mean that fungal taxa from both sites are identical.

Table 3.1 Fungal biodiversity variable Abbreviations.

Variables	Where:
H^1	Shannon diversity index (H^1) or Shannon Weaver index
S_{obs}	Total number of species in the community (species richness) $H^1_{max} = \ln S_{obs}$
p_i	The proportion of individuals in the i^{th} species; or in terms of species abundance; The proportion of S_{obs} made up of the i^{th} species
N	The number of individuals
N_i	The number of individuals in the i^{th} species.
n_i	The number of species with i individuals (The information measure is nits for base e and bits per individual for base 2 logarithms)
E_H	Equitability (E_H) (species evenness)
N_1	Total number of taxa present in first samples but absent in the second samples
N_2	Total number of taxa present in second samples but absent in the first samples
C or *Y*	Number of taxa present in both samples being compared
D	Simpson index
D'	Margalef index
N_T	The total individuals in the sample
jN	The lower of the two sums of all abundance in each of the sites

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5. Molecular Study of Selected Ingoldian Species

A total of sixteen isolates of Ingoldian fungi and twenty-three strains for phylogenetic analysis from GenBank were selected and sequenced (Table 3.2 and Table 3.3) on the ITS/5.8S region. ITS4 and ITS5 primers were used to amplify fragments from Thai isolates and other international fungal isolates via various collaborations, especially for type strains.

5.1 Fungal Isolates

The Ingoldian fungal isolates in studies were isolates of *Hymenoscyphus varicosporoides* and *Tricladium* anamorph of *H. varicosporoides*. Both isolates were isolated from wood, in Khao Yai National Park, Nakorn Ratchassima, Thailand by Somsak Sivichai while, *Cudoniella indica* was isolated from foam samples in England. And the overseas isolates *Tricladium splendens* (Type species) was isolated from the Genil river, in Spain (Table 3.2). Other fungal isolates considered in this study were obtained from the Centraalbureau voor Schimmelcultures (CBS) as type isolates of related genera and species. These are shown with original numbers, locations, and substrates in table 3.2.

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Table 3.2 Selected Ingoldian fungi from BCC and CBS culture collection for sequencing, including type strains, original code, substrate and location origins.

No.	Fungal identification	Original code	Substrate origin	Location origin
1	*Cudoniella indica*	SS 708	Foam bubble samples	UK
2	*Hymenoscyphus varicosporoides*	SS 76.01	Hard wood	Khao Yai National Park, Thailand
3	*H.*varicosporoides	SS 336	*Anisoptera oblonga*	Khao Yai National Park, Thailand
4	*Tricladium* anamorph of *H. varicosporoides*	SS 451	*Dipterocarpus alatus*	Khao Yai National Park, Thailand
5	*Tricladium* anamorph of *H. varicosporoides*	SS 472	*Dipterocarpus alatus*	Khao Yai National Park, Thailand
6	*T. splendens*	SS 2274	Drifted	Genil river, Spain
7	*T. splendens*	SS 2282	Drifted	Genil river, Spain
8	*C. acicularis*	CBS 100273	Rotting wood of *Fagus sylvatica*	Denmark, near Honsnap Skov
9	*C. indica* (*T. indicum*)	CBS 430.94	-	India, Kumaun
10	*H.varicosporoides*	651.66	Decaying twig	Japan, Kyoto
11	*T. chaetocladium* (*Hydrocina chaetocladia*)	CBS 249.90	Submerged twig	UK, Devon
12	*Varicosporium delicatum*	CBS 574.95	Foam bubble samples	Canada, Nova Scotia
13	*V. elodeae*	CBS 541.92	Submerged leaf of *Betula* sp.	Canada, Ontario
14	*Dimorphospora foliicola*	CBS 221.59	Decaying leaf of *Castanea* sp.	Japan, Tokyo, Shakuju Park
15	*T. terrestre*	CBS 697.73	Leaf litter of *Quercus* sp. and *Prunus* sp.	UK, Belfast, Chlorine Gardens
16	*T. giganteum*	CBS 508.71	Decaying submerged leaf of *Crataegus monogyna*	UK, Derbyshire, Edale Valley

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Table 3.3 Additional species and genera for phylogenetic analysis of ITS1, 5.8S and ITS2 regions from the National Center for Biotechnology Information (NCBI) with GenBank accession number and their reference.

No.	Fungal identification	Accession number	Reference
1	*Hymenoscyphus serotinus*	AY348592	Zhang and Zhuang, 2004
2	*H.* cf. *menthae*	AY348588	Zhang and Zhuang, 2004
3	*H. immutabilis*	AY348584	Zhang and Zhuang, 2004
4	*H. scutulus*	AY348591	Zhang and Zhuang, 2004
5	*H. caudatus*	AY348576	Zhang and Zhuang, 2004
6	*H. fucatus*	AY348583	Zhang and Zhuang, 2004
7	*H.* cf. *fructigenus*	AY348582	Zhang and Zhuang, 2004
8	*H. fructigenus*	AJ430396	Vralstad *et al*., 2002
9	*H. monotropae*	AF169309	Bills *et al*., 1999
10	*H. lasiopodius*	AY348587	Zhang and Zhuang, 2004
11	*H. epiphyllus*	AY348581	Zhang and Zhuang, 2004
12	*H. epiphyllus*	AY348580	Zhang and Zhuang, 2004
13	*Tricladium splendens*	AY204634	Baschien *et al*., unpublished
14	*T. angulatum*	AY204611	Baschien *et al*., unpublished
15	*T. angulatum*	AY204608	Baschien *et al*., unpublished
16	*Anguillospora filiformis*	AY148104	Nikolcheva and Baerlocher., unpublished
17	*A. filiformis*	AY148105	Nikolcheva and Baerlocher., unpublished
18	*A. crassa*	AY204581	Baschien, *et al*., unpublished
19	*A. crassa*	AY204582	Baschien, *et al*., unpublished
20	*A. furtiva*	AY148107	Nikolcheva and Baerlocher., unpublished
21	*Zalerion varium*	AF169303	Bills *et al*., 1999
22	*Tuber mesentericum*	AF516800	Paolocci *et al*., 2004
23	*T. aestivum*	AF516791	Paolocci *et al*., 2004

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5.2 Growth of Fungi and DNA Extraction

This protocol was modified from Hong *et al*., 1993. It included rapid extraction of genomic DNA (within 1 hour) and an inexpensive method for phylogenetic analysis. Fungal cultures were examined and grown on corn meal agar or potato dextrose agar for 3-4 weeks at room temperature. Fresh fungal mycelium was harvested into a micro-centrifuge tube. Approximately 50-100 mg of fungal mycelium was transfer to a new microcentrifuge containing 20-60 µl of 0.5 N NaOH and mixed thoroughly with a pipette tip. Two µl of liquid suspension was transferred into a new microcentrifuge tube and 198 µl of Tris HCl was added. Finally, 3-4 µl of this solution was used for the PCR reactions.

Figure 3.3 Cultivation of Ingoldian fungi in CMA and CMB

5.3 PCR Amplification of ITS1-5.8S-ITS2 Regions

The internal transcribed spacer (ITS) regions and 5.8S of rDNA genes were amplified with universal primers ITS5 and ITS4. The ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TATG C 3') following White *et al*, (1990). The PCR reaction was performed in a single 50 µl PCR reaction mixture containing 39 µl sterile deionized water, 5 µl of 10X PCR buffer, 2.5 µl of 50 mM $MgCl_2$ (final concentration 2.5 mM), 1 µl of 10 mM dNTP mix (final concentration 0.2 mM), 1 µl of 10 uM of each primer (final concentration 0.2 um), 0.5 µl of Taq polymerase (1 unit/ul) and 1 µl genomic DNA (approximately 10 ng). The PCR thermal cycling profile for amplifications of the 5.8S rDNA and flanking ITS regions consisted of denaturation at 95^{o}C for 2 minutes followed by 35 cycles 95^{o}C for 1 minute 55^{o}C for 1 minute, 72^{o}C for 2 minutes and the final step of 72^{o}C for 7 minutes (Modified from Ka-Lai *et al*, 2002). The PCR product was observed by gel

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electrophoresis (1.5% agar) and observed under UV-light after staining in ethidium bromide (0.5 μg/ml) for 15 minutes.

5.4 PCR Product Purification and DNA Sequencing

The PCR products were purified from agarose gels with the commercial kit Nucleospin^{R} Plant DNA purification kit (Macherey-Nagel). Purified PCR products were submitted for sequence determination by BIOTEC Service Unit.

5.5 Analysis of PCR-Induced Sequence Errors and Sequencing Analysis

Sequences were verified by employing the Blast program to find matches in GenBank at NCBI database (From http://www.ncbi.nlm.nih.gov/BLASn). DNA sequences were aligned visually using Clustal W1.6 of Bioedit version 5.0.6 (Hall, 2001), and manually adjusted using Se-Al v1.0al. (Rambout, 1966).

This work focused on pairwise alignment of sequences between species. The total number of different base pairs is reported as percentage of base difference in each region of ITS1-5.8S-ITS2. Percent identity between *Hymenoscyphus varicosporoides, Cudoniella indica* and other species pairs (*Tricladium* anamorph of *H. varicosporoides, Tricladium splendens*) are also calculated.

5.6 Phylogenetic Analysis

The sequences of ITS1, 5.8S and ITS2 regions from all strains including those from CBS were re-analysed along with others obtained from the GenBank database at http://www.ncbi.nlm.nih.gov/, with suitable outgroup taxa. All sequences were aligned using Clustal W 1.6 program (Thompson, *et al*., 1994) bundled in BioEdit, then visually and manually verified using Se-Al v1. Oa1 program (Rambout, 1996) under the BioEdit version no. 5.06 (Hall, 2001). Phylogenetic analysis of all sequence alignments were performed using PAUP* 4.0b8 (Swofford, 2003). The phylogenetic trees were generated using maximum parsimony criteria for analysis of ITS1-2 and 5.8S nucleotide alignments. A maximum parsimony (MP) tree was produced with support for clades using a full heuristic search to find the MP tree.

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5.6.1 Parsimony Analyses, Confident Statistics and Tree Scores

Bootstrap analysis using the heuristic search option of PAUP* were performed to calculate the robustness of each branch. The analysis was set as the following parameter: 1000 bootstrap replicates, with gaps treated as missing data, tree-bisection-reconnection branch-swapping algorithm and random sequence addition (Felsenstein, 1985). All characters were weighted equally. The final step were the calculation of the percentages of bootstrap replicates performed for testing the confirmation of each clade. Parsimony tree scores that are tree length, CI, RI, RC and HI were calculated and recorded in the phylogram figures.

5.6.2 Distance Analyses

In contrast to parsimony analyses, distance analyses cluster taxa due to character similarity without expecting a molecular clock. Based on the principle of minimum increase in overall distance in the tree. The Neighbor-joining (NJ) tree building algorithm was employed for all distance based tree building and calculations of bootstrap for the branching topologies.

5.6.3 Maximum Likelihood Analyses

The most parsimonious tree from the data set with gaps treated as missing data was used to estimate the rate of transition to transversion, proportion of invarible sites, gamma shape parameter, and the based frequencies employing the Hasegawa *et al*., (1985) model for weighed Maximum likelihood analyses. The Maximum likelihood analysis was carried out on the original data set a heuristic search (starting branch length using the Rogers Swofford approximation method, as-is stepwise addition of sequence, TBR) with the estimated parameters.

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CHAPTER 4
RESULTS

1. Diversity of Ingoldian Fungi

In order to determine the diversity of Ingoldian fungi in Thai rain forest, six National Parks in each region of Thailand were selected. These studied sites were Bala Hala Wildlife Sanctuary, KhaoYai National Park, Nam Nao National Park, Tone Nga Chang Wildlife Sanctuary, Kaeng Krachan National Park and Doi Inthanon National Park. Each major studying site was divided into 2-3 sub-studying areas, except for the Tone Nga Chang Wildlife Sanctuary (Table 4.1).

Five hundred and eighty six specimens were collected for Ingoldian spore investigation from all six major rain forests. There were 123, 92, 117, 34, 144 and 76 foam samples from Bala Hala Wildlife Sanctuary, KhaoYai National Park, Nam Nao National Park, Tone Nga Chang Wildlife Sanctuary, Kaeng Krachan National Park and Doi Inthanon National Park, respectively (Table 4.1).

Table 4.1 Number of foam specimens from different sites in Thailand.

Geographical Origins											
Bala Hala Wildlife Sanctuary		**KhaoYai National Park**			**Nam Nao National Park**		**Tone Nga Chang Wildlife Sanctuary**	**Kaeng Krachan National Park**		**Doi Inthanon National Park**	
KID	SRD	WJP	TRN	HNR	Haew Sai	Khod Hin	Stream in Tone Nga Chang	Bankrang trail	Km. 18	Aaka	SRP
99	24	48	8	36	88	29	34	70	44	62	14
123		92			117		34	144		76	

Klong I Ga Ding (K.I.D); Sirindhorn (SRD); Wang Jum Pee (WJP); Tanratana (TRN); Heaw Narok (HNR); Siriphum (SRP).

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Table 4.2 shown the number of Ingoldian specimens, species and species per number of specimen from 6 studied forests. There were 12, 37, 28, 21, 22 and 10 Ingoldian species that were found from 34, 123, 92, 117, 144 and 76 specimens collected from Tone Nga Chang Wildlife Sanctuary, Bala Hala Wildlife Sanctuary, Khao Yai National Park, Nam Nao National Park, Kaeng Krachan National Park, Tone Nga Chang Wildlife Sanctuary and Doi Inthanon National Park, respectively. The highest species per number of specimens (0.35) was from Tone Nga Chang Wildlife Sanctuary while the lowest species per number of specimens (0.18) was found in Doi Inthanon National Park. Fungal diversity accumulation on foam, however, did not reach a maximum at all samples sites (Figure 4.1).

Table 4.2 Number of Ingoldian specimens, species and species per number of specimens from different sites.

Geographical Origin	Province	Part of Thailand	No. of Specimens	No. of Ingoldian Species	No. of Ingoldian Species per no. of Specimen
Tone Nga Chang Wildlife Sanctuary	Songha	Southern	34	12	0.35
Bala Hala Wildlife Sanctuary	Narathiwat	Southern	123	37	0.30
KhaoYai National Park	Nakorn Ratchassima	North-eastern	92	28	0.30
Nam Nao National Park	Petchaboon	Central	117	21	0.18
Kaeng Krachan National Park	Petchaburi	Western	144	22	0.15
Doi Inthanon National Park	Chaingmai	Northern	76	10	0.13

Eighty-four species of Ingoldian fungi were collected from a total of 586 foam samples at six collecting sites (Table 4.3 and 4.4). Nineteen fungi and 26 fungi were identified at the species and genus levels, respectively. There were 39 fungi that were unidentified, and awaiting further identification.

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Table 4.3 Number of identified species, genus, and unidentified species of Ingoldian fungi in 6 studied sites.

No.	Geographical Origin	No. of fungi at species level	No. of fungi at genus level	No. of fungi unidentified	Total
1	Bala Hala Wildlife Sanctuary	13	11	13	37
2	KhaoYai National Park	13	7	8	28
3	Nam Nao National Park	8	7	6	21
4	Tone Nga Chang Wildlife Sanctuary	6	3	3	12
5	Kaeng Krachan National Park	7	7	8	22
6	Doi Inthanon National Park	2	4	4	10

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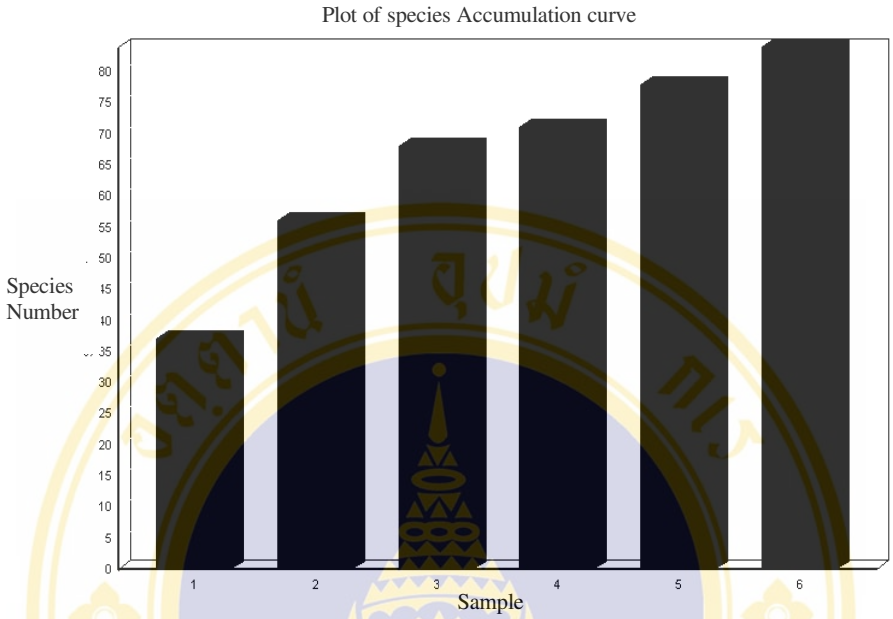

Figure 4.1 Species accumulation curve for six collecting sites (Bala Hala Wildlife Sanctuary, KhaoYai National Park, Nam Nao National Park, Tone Nga Chang Wildlife Sanctuary, Kaeng Krachan National Park and Doi Inthanon National Park)

2. Species Frequency

Sixty-one species in this study were found once at each collecting site. However three species of *Brachiosphaera tropicalis*, *Flabellospora verticillata* and *Triscelophorus monosporus* were not found in Doi Inthanon National Park. The common Ingoldian fungi *Brachiosphaera tropicalis*, *Flabellospora verticillata*, *Helicomyces* sp.01, *Speiropsis pedatospora*, *Triscelophorus acuminatus* and *Triscelophorus monosporus* appeared frequently at more than 3 streams/trials (Figure 4.2).

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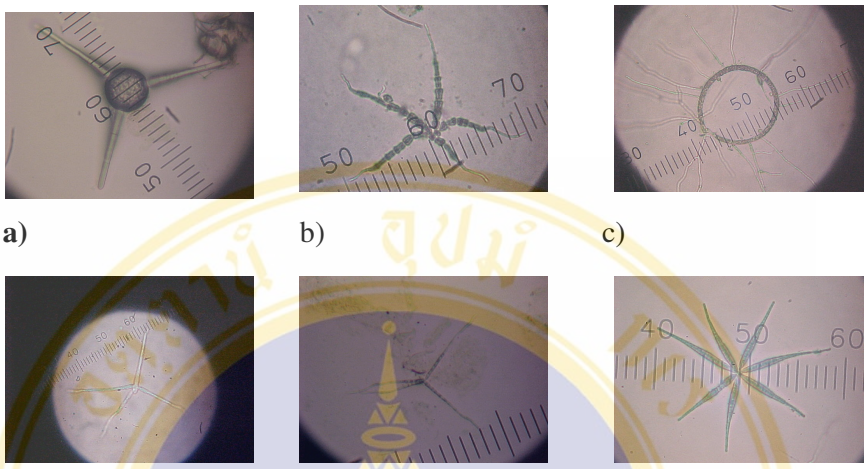

Figure 4.2 Common Ingoldian species found in this studied: a) *Brachiosphaera tropicalis*, b) *Speiropsis pedatospora,* c) *Helicomyces* sp.01, d) *Triscelophorus monosporus*, e) *Triscelophorus acuminatus* and f) *Flabellospora verticillata*.

No Ingoldian species was found at every study area. Species were found at only one selected National Park of all 6 sites were considered rare species. The strained numbers of each isolate were assigned in the parenthesis. These Ingoldian fungi were *Anguillospora crassa, Anguillospora* sp.(03), *Anguillospora* sp.(04), *Anguillospora* sp.(05), *Anguillospora* sp.(06), *Beltrania* sp.(01), *Clavariopsis* sp.(01), *Dendrospora* sp.(01), *Dendrospora* sp.(02), *Dendrospora* sp.(03), *Diplocladiella scalaroides, Geniculospora grandis, Helicoma* sp.(01), *Helicoma* sp.(02), *Isthmotricladia* sp.(01), *Lateriramulosa uniinflata, Phalangispora* sp.(01), *Tetraploa aristata, Tricladium* sp.(01), *Tricladium* sp.(02), *Tricladium* sp.(03), *Tricladium* sp.(04), *Triscelophorus septatus,* unidentified Hyphomycete sp.(01)- sp.(39), *Varicosporium* sp.(01) and *Varicosporium* sp.(02).

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3. Comparison between Sites

The number of species found in streams was similar and there was no significant difference in species diversity (H^1) of Ingoldian fungi between sites. The results in Table 4.4 indicated that the highest Shannon diversity index (H^1) was from Bala Hala Wildlife Sanctuary (3.13), while Khao Yai National Park, Nam Nao National Park, Tone Nga Chang Wildlife Sanctuary, Kaeng Krachan National Park and Doi Inthanon National Park yielded 2.75, 2.50, 2.21, 2.54 and 1.83 respectively. Thus implied a higher level of diversity of Ingoldian fungi at Bala Hala Wildlife Sanctuary. The results are shown in Table 4.4 and graphically in Figure 4.3.

Table 4.4 Frequency of occurrence of Ingoldian fungi at each site.

Species No.	Original code	Fungal identification	Site locations for collecting specimens					
			Bala Hala Wildlife Sanctuary	Khao Yai National Park	Nam Nao National Park	Tone Nga Chang Wildlife Sanctuary	Kaeng Kranchan National Park	Doi Inthanon National Park
1	SS 2555	*Anguillospora crassa*	0.81	-	-	-	-	-
2	SS 3243	*Anguillospora* sp.01	-	1.08	-	-	1.38	1.31
3	SS 2677	*Anguillospora* sp.02	-	1.08	-	-	1.38	-
4	SS 2812	*Anguillospora* sp.03	-	-	15.38	-	-	-
5	SS 2564	*Anguillospora* sp.04	8.13	-	-	-	-	-
6	SS 2831	*Anguillospora* sp.05	-	-	0.85	-	-	-
7	SS 2981	*Anguillospora* sp.06	-	-	-	11.76	-	-
8	SS 2651	*Beltrania* sp.01	-	1.08	-	-	-	-
9	SS 3093	*Brachiosphaera tropicalis*	12.19	5.43	11.96	23.52	7.63	-
10	SS 3105	*Campylospora chaetocladia*	0.81	-	-	-	10.41	-
11	SS 3078	*Campylospora* sp.01	9.75	-	5.12	-	2.08	-
12	SS 2603	*Clavariopsis* sp.01	0.81	-	-	-	-	-
13	SS 2696	*Condylospora spumigena*	0.81	2.17	0.85	-	-	-
14	SS 2719	*Dendrospora* sp.01	-	11.95	-	-	-	-
15	SS 2554	*Dendrospora* sp.02	0.81	-	-	-	-	-
16	SS 3240	*Dendrospora* sp.03	-	-	-	-	-	27.63

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Table 4.4 Frequency of occurrence of Ingoldian fungi at each site. (cont.).

Species No.	Original code	Fungal identification	Site locations for collecting specimens					
			Bala Hala Wildlife Sanctuary	Khao Yai National Park	Nam Nao National Park	Tone Nga Chang Wildlife Sanctuary	Kaeng Kranchan National Park	Doi Inthanon National Park
17	SS 2750	*Diplocladiella appendiculata*	1.62	-	3.41	-	-	-
18	SS 2615	*Diplocladiella scalaroides*	1.62	-	-	-	-	-
19	SS 3182	*Flabellocladia* sp.01	4.06	-	-	-	0.69	-
20	SS 2973	*Flabellocladia tetracladia*	-	1.08	-	17.64	-	-
21	SS 3280	*Flabellospora acuminata*	-	-	-	-	4.86	3.94
22	SS 3183	*Flabellospora crassa*	1.62	2.17	-	-	22.91	-
23	SS 2979	*Flabellospora multiradiata*	4.87	4.34	-	-	-	-
24	SS 2985	*Flabellospora verticillata*	7.31	3.26	14.52	5.88	0.69	-
25	SS 3115	*Geniculospora grandis*	-	1.08	-	-	-	-
26	SS 2659	*Helicoma* sp.01	-	1.08	-	-	-	-
27	SS 2559	*Helicoma* sp.02	0.81	-	-	-	-	-
28	SS 3289	*Helicomyces* sp.01	-	2.17	1.70	-	3.47	6.57
29	SS 3136	*Helicomyces* sp.02	1.62	-	-	2.94	2.08	-
30	SS 2974	*Ingoldiella hamata*	0.81	1.08	-	8.82	-	-
31	SS 2558	*Isthmotricladia* sp.01	3.25	-	-	-	-	-
32	SS 2612	*Isthmotricladia* sp.02	0.81	-	-	-	0.69	-
33	SS 2776	*Lateriramulosa uniinflata*	-	-	0.85	-	-	-
34	SS 2601	*Phalangispora* sp.01	1.62	-	-	-	-	-
35	SS 3104	*Speiropsis pedatospora*	3.25	2.17	2.56	-	1.38	-
36	SS 2670	*Tetraploa aristata*	-	1.08	-	-	-	-
37	SS 2662	*Tricladium* sp.01	-	1.08	-	-	-	-
38	SS 2531	*Tricladium* sp.02	-	-	7.69	-	-	-
39	SS 3144	*Tricladium* sp.03	4.06	-	-	-	-	-
40	SS 3241	*Tricladium* sp.04	-	-	-	-	-	19.73
41	SS 3280	*Triscelophorus acuminatus*	1.62	2.17	12.82	2.94	-	9.21
42	SS 3113	*Triscelophorus monosporus*	15.40	9.78	12.82	5.88	9.02	-
43	SS 2819	*Triscelophorus septatus*	-	1.08	-	-	-	-

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Table 4.4 Frequency of occurrence of Ingoldian fungi at each site. (cont.).

Species No.	Original code	Fungal identification	Site locations for collecting specimens					
			Bala Hala Wildlife Sanctuary	Khao Yai National Park	Nam Nao National Park	Tone Nga Chang Wildlife Sanctuary	Kaeng Kranchan National Park	Doi Inthanon National Park
44	SS 2653	Unidentified Hyphomycete sp.01	-	2.17	-	-	-	-
45	SS 2663	Unidentified Hyphomycete sp.02	-	3.26	-	-	-	-
46	SS 2664	Unidentified Hyphomycete sp.03	-	2.17	-	-	-	-
47	SS 2667	Unidentified Hyphomycete sp.04	-	4.34	-	-	-	-
48	SS 2672	Unidentified Hyphomycete sp.05	-	25	-	-	-	-
49	SS 2648	Unidentified Hyphomycete sp.06	-	1.08	-	-	-	-
50	SS 3080	Unidentified Hyphomycete sp.07	-	2.17	-	2.94	2.08	-
51	SS 2685	Unidentified Hyphomycete sp.08	-	3.26	-	-		-
52	SS 2743	Unidentified Hyphomycete sp.09	-	-	0.85	-	-	-
53	SS 2772	Unidentified Hyphomycete sp.10	-	-	0.85	-	2.08	-
54	SS 2778	Unidentified Hyphomycete sp.11	-	-	0.85	-	-	-
55	SS 2780	Unidentified Hyphomycete sp.12	-	-	0.85	-	-	-
56	SS 2795	Unidentified Hyphomycete sp.13	-	-	0.85	-	-	-
57	SS 2799	Unidentified Hyphomycete sp.14	-	-	0.85	-	-	-
58	SS 2801	Unidentified Hyphomycete sp.15	0.81	-	-	-	-	-
59	SS 3191	Unidentified Hyphomycete sp.16	1.62	-	-	-	-	-
60	SS 2534	Unidentified Hyphomycete sp.17	0.81	-	-	-	-	-
61	SS 2978	Unidentified Hyphomycete sp.18	0.81	-	-	2.94	-	-
62	SS 2571	Unidentified Hyphomycete sp.19	0.81	-	-	-	-	-
63	SS 2572	Unidentified Hyphomycete sp.20	0.81	-	-	-	-	-
64	SS 2598	Unidentified Hyphomycete sp.21	0.81	-	-	-	-	-
65	SS 2600	Unidentified Hyphomycete sp.22	0.81	-	-	-	-	-
66	SS 2602	Unidentified Hyphomycete sp.23	0.81	-	-	-	-	-

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Table 4.4 Frequency of occurrence of Ingoldian fungi at each site. (cont.).

Species No.	Original code	Fungal identification	Site locations for collecting specimens					
			Bala Hala Wildlife Sanctuary	Khao Yai National Park	Nam Nao National Park	Tone Nga Chang Wildlife Sanctuary	Kaeng Kranchan National Park	Doi Inthanon National Park
67	SS 2613	Unidentified Hyphomycete sp.24	0.81	-	-	-	-	-
68	SS 2614	Unidentified Hyphomycete sp.25	0.81	-	-	-	-	-
72	SS 2993	Unidentified Hyphomycete sp.29	-	-	-	2.94	-	-
73	SS 3002	Unidentified Hyphomycete sp.30	-	-	-	-	0.69	-
74	SS 3083	Unidentified Hyphomycete sp 31	-	-	-	-	0.69	-
75	SS 3096	Unidentified Hyphomycete sp 32	-	-	-	-	0.69	-
76	SS 3153	Unidentified Hyphomycete sp 33	-	-	-	-	2.08	-
77	SS 3128	Unidentified Hyphomycete sp 34	-	-	-	-	2.08	-
78	SS 3131	Unidentified Hyphomycete sp 35	-	-	-	-	0.69	-
79	SS 3230	Unidentified Hyphomycete sp 36	-	-	-	-	-	23.68
80	SS 3234	Unidentified Hyphomycete sp 37	-	-	-	-	-	1.31
81	SS 3235	Unidentified Hyphomycete sp 38	-	-	-	-	-	5.26
82	SS 3239	Unidentified Hyphomycete sp 39	-	-	-	-	-	1.31
83	SS 2741	*Varicosporium* sp.01	-	-	0.85	-	-	-
84	SS 2761	*Varicosporium* sp.02	-	-	0.85	-	-	-
Total species of Ingoldian fungi at each site			37	28	21	12	22	10
Total of samples of Ingoldian fungi at each site			123	92	117	34	144	76
Number species per samples			0.3	0.3	0.17	0.35	0.15	0.13
Shannon diversity index (H^1) (Shannon Weaver index)			3.13	2.75	2.50	2.21	2.54	1.83
Shanon simpson index (D)			18.2	10.13	10.38	8.02	8.6	5.32
Margalef's index (D')			7.73	5.91	4.39	2.37	4.75	1.95
Equitability (E_H) or Eveness (J^1)			0.70	0.62	0.56	0.50	0.57	0.41

Margalef's index (D') showed that the highest number of species (7.73) was collected from Bala Hala Wildlife Sanctuary and the lowest (1.95) from Doi Inthanon National Park (Figure 4.3). The Margalef's indexes (D') of Ingoldian fungi in Khao

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Yai National Park, Nam Nao National Park, Tone Nga Chang Wildlife Sanctuary, Kaeng Krachan National Park and Doi Inthanon National Park were 5.91, 4.39, 2.37, and 4.75, respectively.

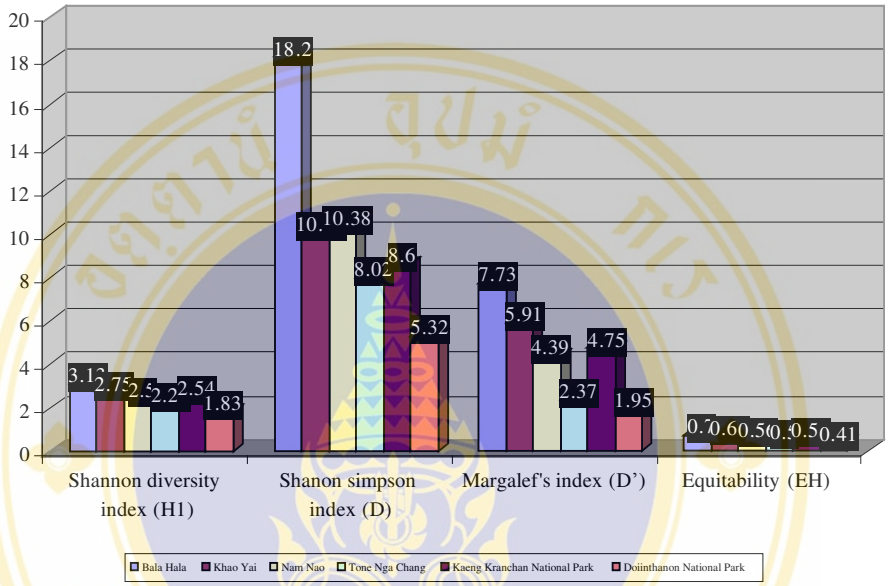

Figure 4.3 The comparison of Shannon diversity index, Shanon Simpson index, Margalef's index and Equitability values of all studied sites.

The Equitability values of Ingoldian fungi in Bala Hala Wildlife Sanctuary, Khao Yai National Park, Nam Nao National Park, Tone Nga Chang Wildlife Sanctuary, Kaeng Krachan National Park and Doi Inthanon National Park were 0.7, 0.62, 0.56, 0.5, 0.57 and 0.41.

The highest similarity in species composition (11.90%) was found between Kaeng Kranchan National Park and BalaHala Wildlife Sanctuary (Table 4.5). In contrast, the lowest similarity in species composition (1.19%) was between the Doi Inthanon National Park and BalaHala Wildlife Sanctuary, and the Doi Inthanon National Park and Tone Nga Chang Wildlife Sanctuary.

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Table 4.5 Percentage of similar taxa found among 6 different sites.

% Similarity among different sites		1 Bala Hala Wildlife Sanctuary	2 Khao Yai National Park	3 Nam Nao National Park	4 Tone Nga Chang Wildlife Sanctuary	5 Kaeng Kranchan National Park	6 Doi Inthanon National Park
1	Bala Hala Wildlife Sanctuary	100	10.71	8.33	7.14	11.90	1.19
2	Khao Yai National Park	-	100	8.33	7.14	10.70	3.57
3	Nam Nao National Park	-	-	100	4.76	8.33	2.38
4	Tone Nga Chang Wildlife Sanctuary	-	-	-	100	5.95	1.19
5	Kaeng Kranchan National Park	-	-	-	-	100	3.57
6	Doi Inthanon National Park	-	-	-	-	-	100

4. Molecular Study of Selected Ingoldian Species

For molecular comparison, the size of the PCR product amplified by universal ITS primers ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') was approximately 500-600 bps. All of the edited complete sequences of from both forward and reverse sequences are shown in the appendix. Based on Blast search results, all the selected sequences belonged to the phylum Ascomycota, subphylum Pezizomycotina, class Leotiomycetes, order Helotiales, and family Helotiaceae.

4.1 Sequence Lengths and Positions of rRNA Genes in 16 Ingoldian Fungi

Sequence analysis of flanking ITS1, ITS2 regions and 5.8S rDNA was carried out for 16 species shown in Table 4.6. The size of ITS regions ranged from 522 to 662 base pairs (Table 4.6). The positions of ITS1, ITS2 and 5.8S rRNA in each completed sequence are also shown in Table 4.6.

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Table 4.6 Gene position in partial 18S, ITS 1-2 regions, 5.8S rDNA and partial 28S rDNA sequence.

Fungal species	Total sequence length (bp)	Position of each gene on the sequence				
		Partial 18S	ITS1	5.8S	ITS2	Partial 28S
Cudoniella indica (SS 708)	561	1-37	38-203	204-361	362-510	511-561
Hymenoscyphus varicosporoides (SS 76.01)	522	1-25	26-191	192-349	350-495	496-522
H. varicosporoides (SS 336)	573	1-55	56-221	222-379	380-525	526-573
Tricladium splendens (SS 2274)	575	1-58	59-225	226-383	384-530	531-575
T. splendens (SS 2282)	539	1-32	33-199	200-357	358-504	505-539
Tricladium anamorph of *H.varicosporoides* (SS 451)	542	1-35	36-201	202-359	360-507	508-542
Tricladium anamorph of *H.varicosporoides* (SS 472)	573	1-56	57-222	223-380	381-525	526-573
Cudoniella acicularis (CBS 100273)	566	1-44	45-215	216-373	374-524	525-566
Cudoniella indica (CBS 430.94)	573	1-52	53-218	219-376	377-525	526-573
H. varicospoides (CBS 651.66)	565	1-45	46-211	212-369	370-521	522-565
Tricladium chaetocladium (CBS 249.90)	621	1-44	45-199	200-366	367-506	507-621
Varicosporium delicatum (CBS 574.95)	566	1-46	47-211	212-375	376-464	465-566
Varicosporium elodeae (CBS 541.92)	584	1-56	57-215	216-373	374-523	524-584
Dimorphospora foliicola (CBS 221.59)	662	1-55	56-224	225-382	383-528	529-662
Tricladium terrestre (CBS 697.73)	578	1-56	57-223	224--381	382-530	531-578
Tricladium giganteum (CBS 508.71)	555	1-44	45-195	196-354	355-500	501-555

4.2 The Total Base Pairs and Variation within the ITS-5.8S-ITS2 Region

The entire length of the ITS-5.8S-ITS2 region for all 16 studied taxa was between 418-480 base pairs. ITS1 varied in length from 151–170 base pairs while those of ITS2 varied from 88-151 base pairs. The ITS2 region, thus, was shorter than ITS1 by 20-64 base pairs for all 16 species selected. The 5.8S rDNA sequences from 14 samples in this study was 158 base pairs. Exceptions were *Tricladium chaetocladium* (CBS 249.90) and *Varicosporium delicatum* (CBS 574.95), at 166 and 163 base pairs, respectively.

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Table 4.7 Length of ITS 1-2-and 5.8 rDNA regions of 16 Inglodian fungi.

Taxa	Total base pairs completed sequence (bp)	Total base pairs ITS1-5.8S-ITS2 (bp)	Total base pairs (bp)		
			ITS1 (bp)	5.8S (bp)	ITS2 (bp)
Cudoniella indica (SS 708)	561	473	166	158	149
Hymenoscyphus varicosporoides (SS 76.01)	522	470	166	158	146
H. varicosporoides (SS 336)	573	470	166	158	146
Tricladium splendens (SS 2274)	575	472	167	158	147
T. splendens (SS 2282)	539	472	167	158	147
Tricladium anamorph of *H.varicosporoides* (SS 451)	542	472	166	158	148
Tricladium anamorph of *H.varicosporoides* (SS 472)	573	469	166	158	145
Cudoniella acicularis (CBS 100273)	566	480	170	158	150
Cudoniella indica (CBS 430.94)	573	473	166	158	148
H. varicosporoides (CBS 651.66)	565	476	166	158	151
T. chaetocladium (CBS 249.90)	621	462	156	166	139
Varicosporium delicatum (CBS 574.95)	566	418	165	163	88
V.elodeae (CBS 541.92)	584	467	158	158	149
Dimorphospora foliicola (CBS 221.59)	662	472	169	158	145
T. terrestre (CBS 697.73)	578	474	167	158	148
T. giganteum (CBS 508.71)	555	456	151	158	145

4.3 Comparison of ITS1-5.8S-ITS2 Sequences between *C. indica* (SS 708) and Other Selected Species

Comparison of the ITS1-5.8S-ITS2 region from *C. indica* (SS 708) to other selected species is recorded in Table 4.8. There was low sequence variation between two isolates of *C. indica* SS 708 (UK isolate) and *C. indica* (CBS 430.94), which were 0.21% or 0.60% in ITS1, and 0.67% in ITS2. The comparison between *C. indica* (SS 708) and *H. varicosporoides* isolate numbers SS 76.01 and SS 336 revealed 2.11-2.14% differences (Table 4.8). There was no variation in the whole region of the 5.8S rDNA for these three species. A 3.80% difference in the ITS regions and 5.8S rDNA was found when the sequence of *C. indica* SS 708 was compared with the sequence of two isolates of *T. splendens*, SS 2274 and SS 2284. *Tricladium*, the anamorph of *H. varicosporoides* (SS 451, SS 472) displayed 2.32 and 2.53% differences in the ITS regions and 5.8S rDNA sequence, respectively, when compared with *C. indica* SS 708. Considering each region separately, percentage divergence between *C. indica* (SS

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708) and previously mentioned species ranged from 0.42%- 4.19% in ITS1, and from 0.67%-9.39% in ITS2, but there was no nucleotide divergence (0 %) in 5.8S regions.

Divergence percentages in the ITS1-5.8S-ITS2 regions between *C. indica* (SS 708) versus *C. acicularis* (CBS 100273) and *Tricladium giganteum* (CBS 508.71) were 11.45% (20% in ITS1 and 11.76% in ITS2) and 22.19% (37.95% in ITS1 and 30.87% in ITS2), respectively. It is interesting to note that there was 1.26% nucleotide divergence in 5.8S rDNA between *C. indica* (SS 708) vs. *C. acicularis* (CBS 100273), and 1.89% nucleotide divergence in 5.8S rDNA between *Cudoniella indica* (SS 708) vs. *Tricladium giganteum* (CBS 508.71).

Table 4.8 Base pair differences in the nuclear ITS regions and 5.8S rDNA, between *Cudoniella indica* (SS 708) and other selected species.

Taxon pairs of ITS1-5.8S-ITS2	**Total (bp)**	**No. of different bp**	**% difference**	**Nucleotide divergence (%)**		
				ITS1 (166-167 bp)	**5.8S (158 bp)**	**ITS2 (145-149 bp)**
Cudoniella indica (SS 708) vs. *H. varicosporoides* (SS 76.01)	473, 470	10	2.11	0.42	0	1.69
Cudoniella indica (SS 708) vs. *H. varicosporoides* (SS 336)	473, 470	10	2.14	0.42	0	1.69
Cudoniella indica (SS 708) vs. *Tricladium splendens* (SS 2274)	473, 472	18	3.80	1.90	0	1.90
Cudoniella indica (SS 708) vs. *T. splendens* (SS 2282)	473, 472	18	3.80	1.90	0	1.90
Cudoniella indica (SS 708) vs. *Tricladium* anamorph of *H.varicosporoides* (SS 451)	473, 472	12	2.53	1.26	0	1.26
Cudoniella indica (SS 708) vs. *Tricladium* anamorph of *H.varicosporoides* (SS 472)	473, 469	11	2.32	0.42	0	1.90
Cudoniella indica (SS 708) vs. *Cudoniella indica* (CBS 430.94)	473, 473	1	0.21	0.60	0	0.67
Cudoniella indica (SS 708) vs. *Cudoniella acicularis* (CBS 100273)	473, 480	55	11.45	20	1.26	11.76
Cudoniella indica (SS 708) vs. *Tricladium terrestre* (CBS 697.73)	473, 474	19	4.00	4.19	0	9.39
Cudoniella indica (SS 708) vs. *Tricladium giganteum* (CBS508.71)	473, 456	105	22.19	37.95	1.89	30.87

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4.4 Comparison of ITS1-5.8S-ITS2 Sequences between *Hymenoscyphus varicosporoides* and Various *Tricladium*

The results of ITS1-5.8S-ITS2 sequence comparison showed that there was no variation between *H. varicosporoides* (SS 76.01, Thailand) the *H. varicosporoides* (SS 336, Thailand). They were thus confirmed as different isolates of *H. varicosporoides*. By contrast, comparison between *H. varicosporoides* (SS 76.01, SS 336 from Thailand) and *H. varicosporoides* (CBS 651.66 from Japan) revealed nucleotide variation of 0.60% in ITS1 and 6.62% in ITS2.

Nucleotide variation in the ITS1-5.8S-ITS2 region for the three strains of *H. varicosporoides* (SS 76.01, SS 336 and CBS 651.66) and two isolates of *T. splendens* (SS 2274, SS 2282) ranged between 3.81-4.02% while comparison of *H. varicosporoides* (SS 76.01, SS 336 and CBS 651.66) with two isolates of the *Tricladium* anamorph of *H. varicosporoides* (SS 451, SS 472) showed variation between 0.21 to 1.90%. Overall, the percentage of nucleotide divergence in ITS1 ranged from 0% to 37.95%. The ITS2 region also showned 0% to 32.19% nucleotide divergence, while the 5.8S region were highly conserved. Comparison of *H. varicosporoides* (SS 336) and *Tricladium giganteum* (CBS 508.71) revealed 1.89% nucleotide divergence in the 5.8S region.

Table 4.9 Base pair differences in the nuclear ITS regions and 5.8S rDNA, compared between *Hymenoscyphus varicosporoides* (SS 76.01, SS 336 and CBS 651.66) and *Tricladium* spp.

Taxon pairs of ITS1-5.8S-ITS2	Total (bp)	No. of different bp	% difference	Nucleotide divergence (%) ITS1 (166-167 bp)	5.8S (158 bp)	ITS2 (145-149 bp)
H. varicosporoides (SS 76.01) vs. *H. varicosporoides* (SS 336)	470,470	0	0	0	0	0
H. varicosporoides (SS 76.01) vs. *H. varicosporoides* (CBS 651.66)	470, 476	10	2.10	0.62	0	6.62
H. varicosporoides (SS 76.01) vs. *Tricladium splendens* (SS 2274)	470,472	19	4.02	1.69	0	2.33
H. varicosporoides (SS 76.01) vs. *T. splendens* (SS 2282)	470,472	19	4.02	1.69	0	2.33
H. varicosporoides (SS 76.01) vs. *T. varicosporoides* (SS 451)	470,472	8	1.69	0.84	0	0.84

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Table 4.9 Base pair differences in the nuclear ITS regions and 5.8S rDNA, compared between *Hymenoscyphus varicosporoides* (SS 76.01, SS 336 and CBS 651.66) and *Tricladium* spp. (cont.).

Taxon pairs of ITS1-5.8S-ITS2	Total (bp)	No. of different bp	% difference	Nucleotide divergence (%) ITS1 (166-167 bp)	5.8S (158 bp)	ITS2 (145-149 bp)
H. varicosporoides (SS 76.01) vs. *T .varicosporoides* (SS 472)	470,469	1	0.21	0	0	0.21
H. varicosporoides (SS 336) vs.*T. splendens* (SS 2274)	470,472	19	4.02	1.69	0	2.33
H. varicosporoides (SS 336) vs.*T. splendens*(SS 2282)	470,472	18	3.81	1.48	0	2.33
H. varicosporoides (SS 336) vs. *T. varicosporoides* (SS 451)	470,472	9	1.90	0.84	0	1.05
H. varicosporoides (SS 336) vs. *T. varicosporoides* (SS 472)	470,469	1	0.21	0	0	0.21
H. varicosporoides (SS 336) vs. *Cudoniella indica* (CBS 430.94)	470, 473	10	2.11	0.60	0	6.08
H. varicosporoides (SS 336) vs. *Tricladium terrestre* (CBS 697.73)	470, 474	22	4.64	2.99	0	13.51
H. varicosporoides (SS 336) vs. *Tricladium giganteum* (CBS 508.71)	470, 456	125	26.60	37.95	1.89	32.19
H. varicosporoides (SS 336) vs. *H. varicosporoides* (CBS 651.66)	470, 476	10	2.10	0.60	0	6.62

4.5 Comparison of ITS1-5.8S-ITS2 Sequences between *Tricladium splendens* (SS 2274, SS 2282) and *Tricladium* anamorph of *H. varicosporoides* (SS 451, SS 472)

ITS1-5.8S-ITS2 sequence comparison for *Tricladium splendens* (SS 2274, SS 2282) and *Tricladium* anamorph of *H. varicosporoides* (SS 451, SS 472) revealed 1.69-2.33% variation in ITS1 and 1.90-2.11% variation in ITS2 while *T. splendens* (SS 2274) and *T. splendens* (SS 2282) showed no nucleotide variation in the ITS1-5.8S-ITS2 sequence.

Between *T. splendens* and the *Tricladium* anamorph of *H. varicosporoides*, i there was a small percentage difference of 0.84-2.33% and 1.27-2.11% in the ITS1 and ITS2 regions, respectively. Comparison of *Tricladium splendens* (SS 2274, SS 2282) and *Tricladium indicum* (CBS 430.94) or *Cudoniella indica* (CBS 430.94) revealed 5.38% difference in ITS1 and 6.08% in ITS2.

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The highest base divergence was between *Tricladium splendens* (SS 2274) and *Tricladium giganteum* (CBS 508.71) at 40.71% in ITS1 and 33.33% in ITS2.

Table 4.10 Base pair differences in the nuclear ITS regions and 5.8 rDNA between *Tricladium splendens* (SS 2274, SS 2282) and the *Tricladium* anamorph of *H. varicosporoides* (SS 451, SS 472).

Taxon pairs of ITS1-5.8S-ITS2	**Total (bp)**	**No. of different bp**	**% difference**	**Nucleotide divergence (%)**		
				ITS1 (166-167 bp)	**5.8S (158 bp)**	**ITS2 (145-149 bp)**
Tricladium splendens (SS 2274) vs. *T. splendens* (SS 2282)	472, 472	0	0	0	0	0
T. splendens (SS 2274) vs. *Tricladium* anamorph of *H.varicosporoides* (SS 451)	472, 472	20	4.23	2.33	0	1.90
T. splendens (SS 2274) vs.*Tricladium* anamorph of *H.varicosporoides* (SS 472)	472, 469	18	3.81	1.69	0	2.11
T.splendens (SS 2282) vs. *Tricladium* anamorph of *H.varicosporoides* (SS 451)	472, 472	21	4.44	2.33	0	1.90
T. splendens (SS 2282) vs. *Tricladium* anamorph of *H.varicosporoides* (SS 472)	472, 469	18	3.81	1.69	0	2.11

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Table 4.10 Base pair differences in the nuclear ITS regions and 5.8 rDNA between *Tricladium splendens* (SS 2274, SS 2282) and the *Tricladium* anamorph of *H. varicosporoides* (SS 451, SS 472). (cont.)

Taxon pairs of ITS1-5.8S-ITS2	Total (bp)	Total (bp)	% difference	% difference		
				ITS1 (166-167 bp)	5.8S (158 bp)	ITS2 (145-149 bp)
Tricladium anamorph of *H.varicosporoides* (SS 451) vs. *Tricladium* anamorph of *H.varicosporoides* (SS 472)	472, 469	10	2.11	0.84	0	1.27
Tricladium splendens (SS 2274) vs. *Cudoniella indica* (CBS 430.94)	472, 473	18	3.80	5.38	0	6.08
Tricladium splendens (SS 2274) vs. *Tricladium terrestre* (CBS 697.73)	472, 474	19	4.00	4.79	0	7.43
Tricladium splendens (SS 2274) vs. *Tricladium giganteum* (CBS 508.71)	472, 456	102	21.61	40.71	1.89	33.33

4.6 Percentage Identity for ITS1-5.8S-ITS2 Regions among *H. varicosporoides*, Anamorphic Stages and Two Isolates of *C. indica* (SS 76.01 and CBS 651.66)

A matrix table was used to demonstate percentage similarity for ITS1-5.8S-ITS2 sequences among *H. varicosporoides*, its anamorphic stages and two isolates of *C. indica* (SS 76.01 and CBS 651.66) (Table 4.11). There was 97-100% identity among the taxa. The *Tricladium* anamorphs of *H. varicosporoides* (SS 472) and *H. varicosporoides* (SS 76.01) showed 100% identity while two isolates of *T. varicosporoides* (SS472 and SS451) possess 98% identity. There was 97% identity between two isolates of *C. indica* (SS 76.01) and *H. varicosporoides* (CBS 651.66). In addition, the matrix showed high identity (98-99%) between *Cudoniella indica* (SS 708) and *H. varicosporoides* (SS 336, SS 76.01 and CBS 651.66).

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Table 4.11 Percentage identity of ITS1-2 and 5.8S rDNA sequences between *H. varicosporoides*, its anamorphic stages and *Cudoniella indica.*

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

The percentage of identity for ITS1-5.8S-ITS2 regions of *Tricladium* and *Varicosporium* ranged from 71-98%. High identity of 94-98% was found between the *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 the *Tricladium* anamorph of *H.varicosporoides* (SS 451, SS 472). By contrast, there was 73-78% identity between *H. varicosporoides* or the *Tricladium* anamorph of *H. varicosporoides* and the anamorph of *Varicosporium* (*V. delicatum* and *V. elodeae*) in (table 4.12).

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Table 4.12 Percentage identity between *Tricladium* species and *Varicosporium* species of ITS1-2 and the 5.8S sequences.

	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	*Tricladium* anamorph of *H. varicosporoides* (SS 451)	-	97	96	98	95	74.5	74	72.5	78	74
2	*Tricladium* anamorph of *H. varicosporoides* (SS 472)	-	-	96	97	94	73	74	72	78	73
3	*Tricladium splendens* (SS 2282)	-	-	-	96	95	73	74	71	78	73
4	*Tricladium indicum* (CBS 430.94)	-	-	-	-	94	74	74	71	78	73
5	*Tricladium terrestre* (CBS 697.73)	-	-	-	-	-	73	73	71	77	73
6	*Tricladium chaetocladium* (CBS 249.90)	-	-	-	-	-	-	70	77	72	89
7	*Tricladium angulatum* (AY204611)	-	-	-	-	-	-	-	72	74	71
8	*Tricladium giganteum* (CBS 508.71)	-	-	-	-	-	-	-	-	74	78
9	*Varicsporium elodeae* (CBS 541.92)	-	-	-	-	-	-	-	-	-	73
10	*Varicosporium delicatum* (CBS 574.95)	-	-	-	-	-	-	-	-	-	-

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5. Molecular Phylogenetic Analysis

Phylogenetic trees were generated using the maximum parsimony, neighbor-joining and maximum likelihood analyses of ITS1-2, 5.8S rDNA data to clarify anamorph-teleomorph relationships in 37 selected strains described in the materials and methods chapter. *Tuber mesentericum* AF516800 and *T. aestivum* AF516791 were outgroup taxa in the Pezizales. Inferred trees from all three methods showed the same pattern of evolutionary phylograms of selected Ingoldian fungi (Figure 4.4-4.6).

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, smallest tree length (1191) with CI=0.623, RI=0.763, RC=0.476 and HI=0.377 (Figure 4.4). All topologies were roughly separated into four clades that were A, B, C and D.

Clade A contained two sub branches forming a monophyletic group of the most heterogenous *Hymenoscyphus* species with a bootstrap value of 61%. These *Hymenoscyphus* species were obtained from 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 *Varicosporium elodeae* CBS 541.92 (type species of the genus) supported by a 35% of bootstrap value. 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 a bootstrap value of 100%.

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

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Mollisia uda), *A. furtiva* AY148107 (anamorph of *Pezoloma* sp.), *Zalerion varium* AF169303 (a marine fungus) and 10 sequences of representative Ingoldian taxa. The representative Ingoldian taxa included *Tricladium 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 a 43% bootstrap value. The highlighted branches significantly 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 a bootstrap value of 82%. The geographical regions and climate zones of each Ingoldian fungal isolate matched into these 2 highlighted clades. The results reflected a correlation between geographical origin and climate for these two clades of the inferred maximum parsimony tree.

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

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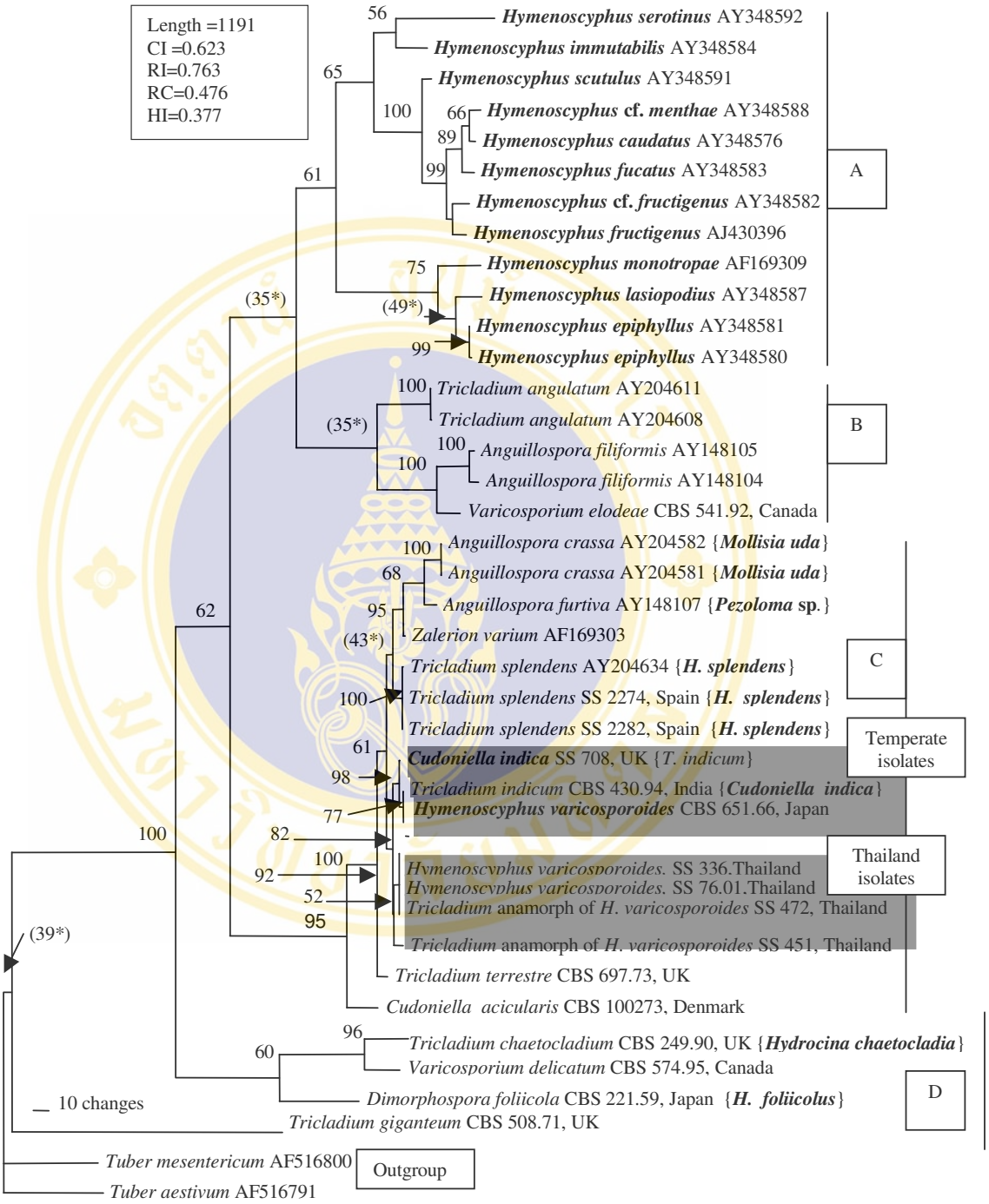

Figure 4.4 Strict consensus tree of 2 most parsimonious trees calculated from ITS1-2, 5.8S rDNA sequences of 37 selected taxa in the Helotiaceae, with *Tuber mesentericum* and *T. aestivum* were outgroup taxa in the Pezizales. Bootstrap values (over 50% and less than 50%) are indicated at the base of the corresponding branches and in parenthesis with asterisk **, respectively.

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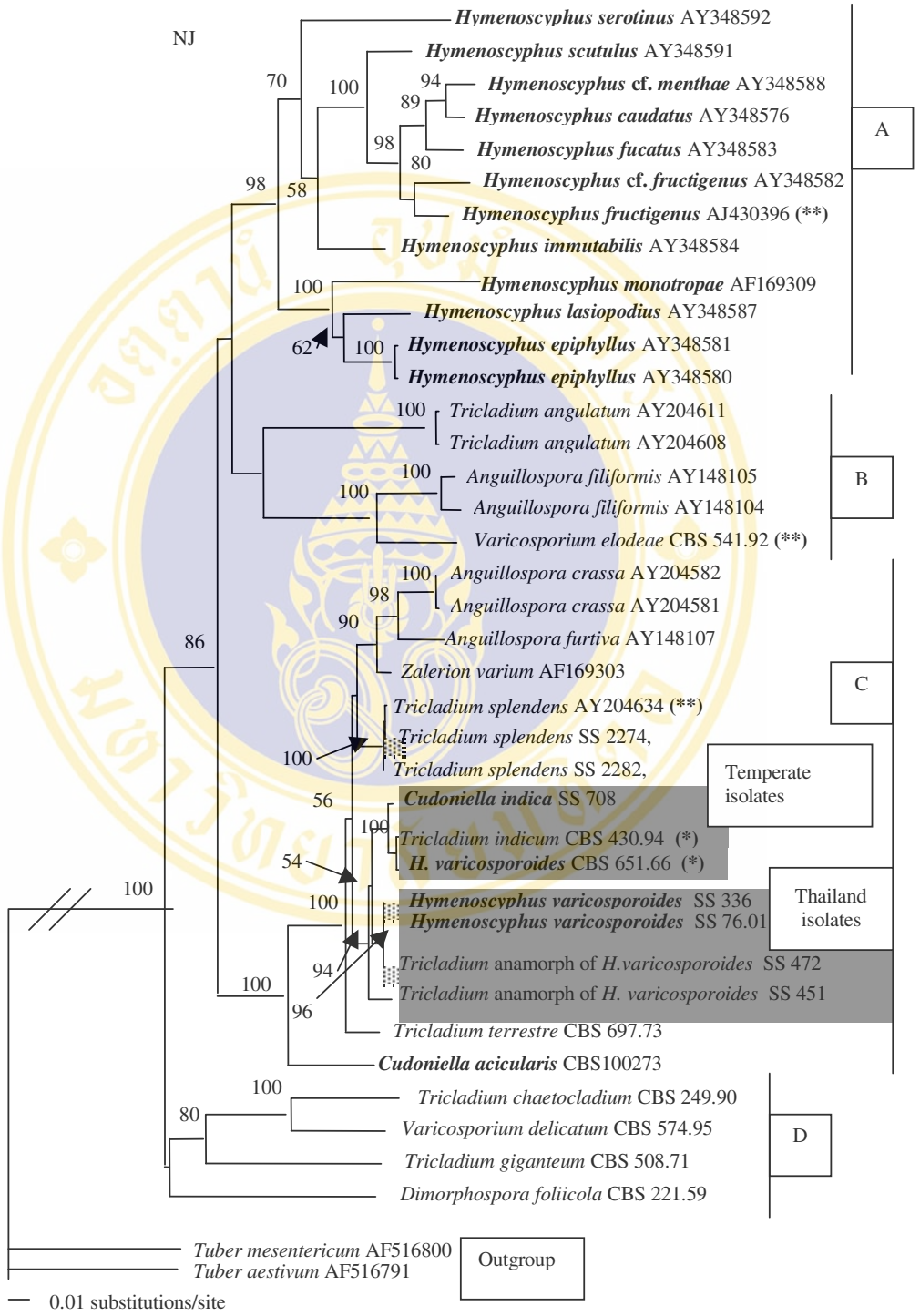

Figure 4.5 NJ tree calculated from ITS1-2, 5.8S rDNA sequence of 37 selected taxa in Helotiaceae, with *Tuber mesentericum* and *T. aestivum* were outgroup taxa in the Pezizales. Type strain and types species in parenthesis with asterisk *,**, respectively.

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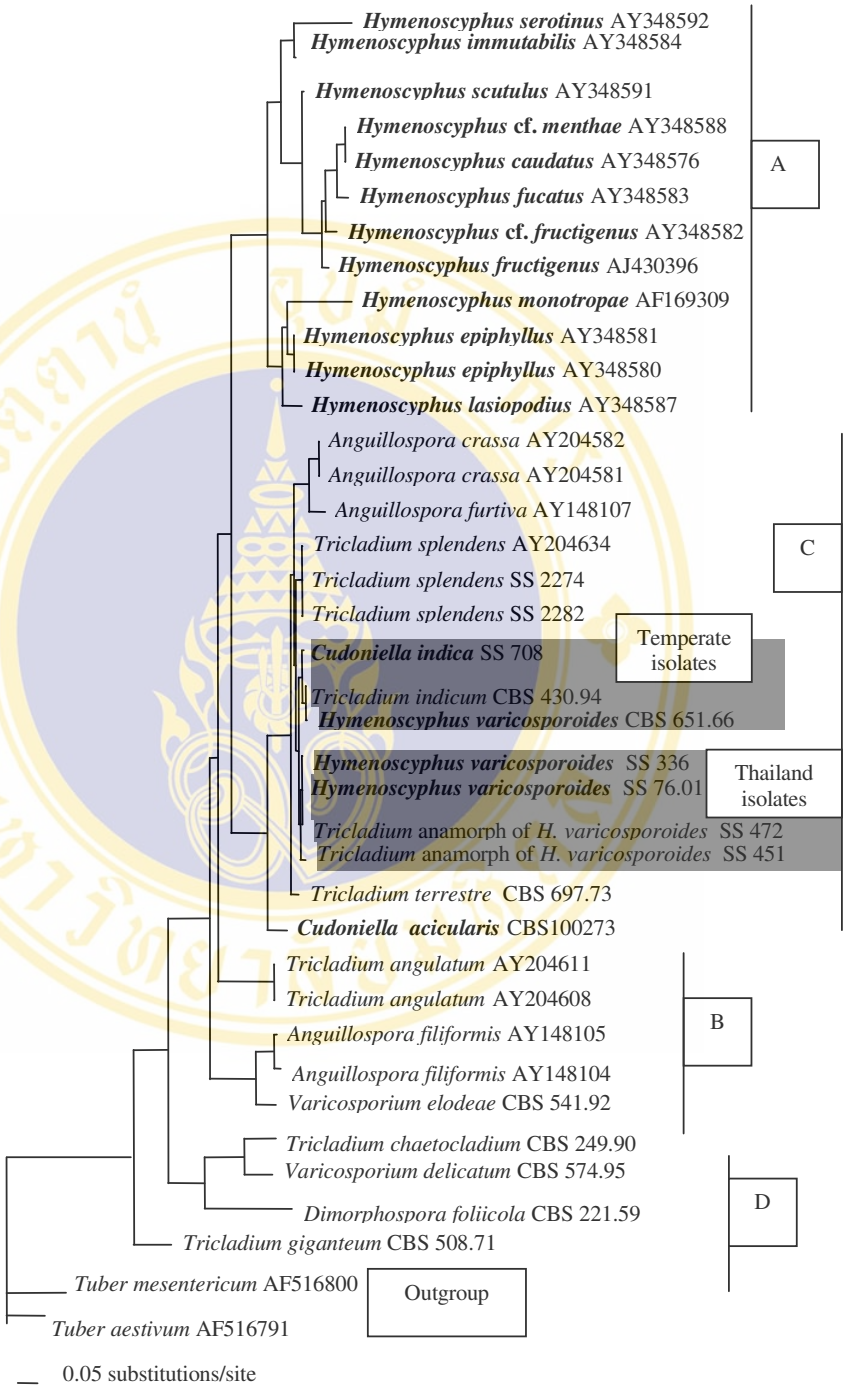

Figure 4.6 ML tree (-ln likelihood=6123.30537) with parameters estimated from the most parsimonious trees from a dataset gaps treated with as missing data and with characters equally weighted. Estimated base frequency: A, 0.22212; C, 0.26378; G, 0.26236; and T, 0.25174; estimated ti/tv ration= 1.001320; estimated value of

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proportion of invarible sites= 0.154484; and estimated value of gamma shape parameter= 0.859396.

Neighbor-joining analysis of the same alignment resulted in a tree that upheld the topology of the four main clades resolved in the parsimony tree. The internal topology of the strongly supported branches resolved by NJ analysis was also identically maintained by ML analysis (Figure 4.5 and 4.6).

Maximum likelihood analysis was carried out on the original data set using a heuristic search (stating using as-is stepwise addition of sequence, TBR) with estimated parameters (Figure 4.6). The inferred ML tree displayed a similar topology to the maximum parsimony tree and NJ tree. The only minor difference was the swapping of clade B from clustering with clade A to the former topologies of clade C. Then, followed by clade D that contained *Tricladium chaetocladium* CBS 249.90, UK (anamorph of *Hydrocina chaetocladia*), *Varicosporium delicatum* CBS 574.95 (Canada), *Dimorphospora foliicola* CBS 221.59 (anamorph of *Hymenoscyphus foliicola*, Japan) and *T. giganteum* CBS 508.71 (UK).

Additional, MP, NJ and ML trees of 31 taxa, excluding taxa of *Anguillospora filiformis*, *A. crassa*, *A. furtiva* and *Zalerion varium*, and 19 selected taxa, excluding taxa of *Anguillospora filiformis*, *A. crassa*, *A. furtive, Zalerion varium* and *Hymenoscyphus* species. (terrestrial strains) are shown in the appendix (Figure 4.7-4.12).

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CHAPTER 5
DISCUSSION

1. Analysis of The Ingoldian Fungi from Various Sites

Ingoldian fungi are an intensively documented group worldwide from freshwater habitats, mostly from temperate regions but hundreds await description (Descals, 1997). Now the number of tropical studies has increased dramatically (Chan *et al*., 2001a; Chan *et al*., 2001b; Marvanova and Hywel-Jones, 2000; Sakayaroj, 2000; Sindhar *et al*., 1992; Sivichai, 1999; Sivichai and Boonyune, 2004; Tubaki *et al*., 1983;). We have now extensively studied the diversity and distribution of Ingoldian fungi in Thailand.

A total of 84 species of Ingoldian fungi isolated from six collecting sites were identified based on spore ontogeny and morphological characters. According to the Shannon diversity index (H^1), Shanon simpson index (D), Margalef's index (D') and Equitability (E_H) index, the highest diversity was found at Bala Hala Wildlife Sanctuary (37 species). The values from all 4 indexes for each site were not significantly different. This result may have been caused by the different time frame of collecting samples (Figure 3.1). The time frame for collecting samples at Bala Hala Wildlife Sanctuary was during the rainy season in July. In addition, this forest is a huge rain forest having high humidity throughout the year (Table 4.4). By contrast, foam samples at Tone Nga Chang Wildlife Sanctuary were collected in January during the dry season. This observation was consistent with results previously reported by Willoughby and Archer (1973). They found that conidia of Ingoldian fungi were more abundant during wet than dry periods. Nonetheless, collecting samples during the rainy seasons at Doi Inthanon National Park did not give a lot of Ingoldian fungi possibly because there was flooding at Siri-Phum stream, resulting in disappearance of foam bubbles.

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Brachiosphaera tropicalis, *Flabellospora verticillata* and *Triscelophorus monosporus* were the most common Ingoldian fungi found in streams at the 6 study sites. *Brachiosphaera tropicalis* and *Triscelophorus monosporus* produce spores with appendages and tetraradiate, respectively while *Flabellospora verticillata* spores being multi-tetraradiate. Spores with these shapes are well adapted for dispersal in the aquatic environment (Ingold, 1976 and Barlocher, 1992). Another explanation for the abundance of species producing tetraradiate conidia is that the shape of the spores might facilitate the dispersal in an aqueous film layer (Wong, 1998). Furthermore, the four-armed conidial shape is a configuration that aids attachment and colonizing of organic substrates (Wong, 1998). Read *et al*. (1992) showed that tetraradiate conidia were more efficient in securing attachment to substrates than sigmoid or ovoid forms. However, other species of Ingoldian fungi posses sigmoid, coiled and spherical conidia and are also dependent of the aquatic environment to complete their life cycle (Goh and Hyde, 1996; Marvanova, 1997).

Ingoldian genera recently recorded from different parts of Thailand included *Alastospora*, *Anguillospora*, *Campylospora*, *Condylospora, Flabellocladia, Flabellospora, Ingoldiella*, *Isthmortricladia*, *Tricladium*, *Triscelophorus* and *Varicosporium* (Sakayaroj, 2000; Sivichai and Boonyune, 2004). Three genera commonly found are *Anguillospora*, *Tricladium* and *Triscelophorus*. By contrast, Ingoldian species rarely found from foam samples in Thailand are *Actinospora megalospora, Ariticulospora, tetracladia, Campylospora chaetocladia, Clavariopsis aquatica, Clavatospora longbrachiata* and *Lemonniera aquatica*. These later Ingoldian fungi have been reported from Argentina, South America by Schoenlein-Crusius, 2003.

Our results are in accordance with those obtained by many authors from Hong Kong who have carried out biodiversity studies using different sampling techniques for foam collection (Chan *et al*., 2000a,b; Ho, 1998). A total of 51 species of Ingoldian fungi have been reported from Hong Kong. The overlapping species between Hong Kong and Thailand are *Anguillospora crassa, Brachiosphaera tropicalis, Condylospora spumigena, Diplocladiella scalaroides, Flabellospora acuminata, Flabellospora crassa, Flabellospora verticillata, Triscelophorus*

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acuminatus and *Triscelophorus monosporus*. Overlapping species from foam of temperate in Irish rivers were *Anguillospora crassa, Triscelophorus acuminatus, Triscelophorus monosporus* (Harrington, 1997).

Different in diversity of Ingoldian fungi between temperate and tropical regions may be due to seasonal and geographical distribution, riparian vegetation-type, composition of water, water temperature, pH, turbidity, oxygen availability, types of substrate, physical changes in the stream and complex interactions among several climate and ecological factors (Barlocher, 1992; Ingold,1942; Iqbal and Webster, 1977; Marvarnova, 1977; Sridhar and Barlocher, 2000).

2. Sequence Alignment and Phylogenetic Analysis of Selected Ingoldian Fungi

Sivichai *et al*. (2003) proposed that the anamorph of *H. varicosporoides* was an undescribed species of *Tricladium* rather than the a species of genus *Varicosporium* sp. as originally proposed by Tubaki (1966). Based on morphological characteristics, the major distinction between *H. varicosporoides* and *C. indica* is a staining reaction of the apical ring of the ascus (Sati and Trivari, 1992; Webster *et al*., 1995). It has been proposed that the anamorph of *C. indica* is *T. indicum*. These confusing classifications call into question the taxonomic relationships. Therefore, the molecular phylogenetic approach was used to clarify the anamorph and teleomorph connections of *H. varicosporoides*. Both asexual and sexual states of selected Ingoldian fungi were included in the phylogenetic analysis. The inferred Maximum likelihood tree best elucidated evolutionary relationships indicating that *H. varicosporoides* and its 8 closely related taxa in clade C were monophyletic evolving from *T. terrestre* CBS 697.73 and *C. acicularis* CBS 100273 (Figure 4.5). Each clade of inferred ML tree was dissected into four major clades and was discussed start from clade C, the core studied taxa to the minor clades A, B and D in the following paragraphs.

The results of sequence analysis and the phylogram strongly supported that the anamorph of *H. varicosporoides* should be classified under the species of *Tricladium varicosporoides* as proposed by Sivichai *et al*. (2003) (Table 4.11 and Figures 4.5). The molecular phylogenetic analysis showed that the clade of *H. splendens* and *T.*

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splendens was grouped with *H. varicosporoides* with a bootstrap value of 61%. They also showed 96% identity of ITS1-5.8S-ITS2 sequences (Figures 4.5 and Table 4.12). By contrast, the identity of the ITS1-5.8S-ITS2 regions between anamorphic stages of *Tricladium* species and *Varicosporium* species was only 71-96% (Table 4.12). *V. elodeae* CBS 541.92 were aligned with *A. filiformis* AY148104 and AY148105 in the clade B, and *V. delicatum* CBS574.95 clustered with *T. chaetocladium* CBS249.90, *Dimorphospora foliicola* at the base of the phylogenetic tree in clade D (Figures 4.5). *T. chaetocladium* was recorded as the anamorph of *Hydrocina chaetocladia* (Hyaloscyphaceae) (Abdullah *et al*. 1981; Webster *et al*. 1990). The results suggested that the anamorphic genus of *Tricladium*, *Varicosporium* and *Anguillospora* are probably polyphyletic corresponding to the conclusion of Kirk *et al*. and Zhang and Zhuang (2001 and 2004). More species of *Tricladium*, *Anguillospora* and *Varicosporium* are needed for further study and revision.

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

The inferred ML tree for 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 evidence showing that the two genera are very closely related or even synonymous, sharing a recent common ancestor (Figures 4.5 and Table 4.11). These results suggest that the presence or absence of a staining reaction at the ascus apex is not a phylogenetically reliable character. Nonetheless, on the basis of 5.8S-ITS sequence data there is still insufficient support for confident combining of *C. indica* (SS 708) and *H. varicosporoides* (SS 336, SS 76.01 and CBS 651.66) into the single species. The sequence identity of 5.8S-ITS between *C. indica* SS 708, *T. indicum* CBS 430.94 were 99.5%, which strongly supported the confusion that they constitute a teleomorph and

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anamorph pairs while the identity of 5.8S–ITS between *C. indica* SS 708 and *H. varicosporoides* CBS 651.66 was 99.0%. By the same token sequence identity for *H. varicosporoides* strain SS 336, strain SS 76.01, strain SS 472, CBS 651.66 and strain SS 451 was 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 between *C. indica* SS 708 and *C. acicularis* CBS 100273 was only 88.85%. This the great difference within the same genus placed *C. acicularis* CBS 100273 at the base of clade C. Thus, the borderline of 5.8S–ITS divergences needed to delineate relationships at the species and within-species level among this group of Ingoldian fungi is still unclear. Other genetic makers are needed to clarify the relationships among of *C. indica* SS 708 and *H. varicosporoides* SS 336, SS 76.01 and CBS 651.66. However, previous molecular scientists studying other fungi such as *Colletotrichum* species proposed that 0.4-0.8% divergence in 5.8S–ITS sequences was 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* represents a group of isolates from temperate regions in Spain, India and Japan that separate from species isolated from tropical forests in Thailand. Although the *C. indica* (CBS 430.94) was a species from India, it was collected from foam samples in a stream of Kumaun Himalaya, at 1,850 m. above sea level (Sati and Trivari, 1992). Moreover, *C. indica* SS708 and *H. varicosporoides* CBS 651.66 are from temperate zones of Japan and the United Kingdom. In addition, these two sub clades contain anamorphs representing major developmental forms for fungi adapted to a lotic environment. Thus, the topology of this clade reflects a correlation of climate and habitat.

Clade A comprising of the ectomycorhizal of the *Hymenoscyphus* species. (Zhang and Zhuang, 2004) was included in the molecular phylogenetic tree for internal control of topology forming, and the results of topology clustering was 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.

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For the further studies of selected Ingoldian fungi and their molecular phylogeny the following may be considered:

1. Study of nucleotide divergence of ITS1-5.8S-ITS2 from additional species of *Varicosporium* spp., *Tricladium* spp., *Cudoniella* spp. and *Hymenoscyphus* spp. to provide more useful data for clarifying the systematics of these Ingoldian fungi.

2. Other genetic makers are, such as the Beta-tubulin gene, which may be divergent enough to better distinguish within subgenotype level of the aquatic hyphomycete fungi.

3. Combination of molecular data from several genes with morphological characteristics including ultrastructures, biochemical and ecological characters may give more meaningful information for addressing evolutionary relationships among Ingoldian taxa.

4. Study of sequence data not only to determine anamorph and teleomorph connections but also 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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CHAPTER 6
CONCLUSION

In my molecular study and phylogenetic analysis based on ITS1-5.8S-ITS2 DNA sequences by MP, NJ and ML methods, it cloud be concluded that:

1. All 37 selected strains (28 species) in Helotiaceae are a monophyletic group. However, the genus of *Cudoniella*, *Hymenoscyphus* and their anamorph genera were proved to be polyphyletic.

2. The significant identity 98-99.5% and molecular phylogeny analysis of ITS1-2 and 5.8S regions strongly suggested that *Cudoniella indica* and *H. varicosporoides* are synonymous fungi sharing a recent common ancestor. Therefore, the name of *Cudoniella indica* should be changed to *Hymenoscyphus indica* (teleomorph of *Tricladium indicum*) based on molecular systematic evidence and their generic concept.

3. The presence or absence of a staining reaction of the apical ring in the genus *Cudoniella indica* and *Hymenoscyphus varicosporoides* is not a phylogenetically reliable character used to distinguish these two species.

4. The combination of molecular data based on ITS1-5.8S-ITS2 and morphological characteristics strongly supported that the anamorph of *Hymenoscyphus 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).

5. The results of inferred phylogenetic tree and the identity of the ITS1-5.8S-ITS2 regions also revealed that these two genus should be reclassified. The number of lateral branches of *Tricladium* and *Varicosporium* anamorph may not be a reliable morphological character. However, more taxa of those species are needed for further study of teleomorph-anamorph connection of the Ingoldian fungi.

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BIBLIOGRAPHY

Abdullah, S. K, Descals, E., and Webster, J. 1981. Teleomorphs of three aquatic hyphomycetes. Trans Br Mycol Soc. 77: 475-483.

Bärlocher, F. 1982. On the ecology of Ingoldian fungi. Bioscience. 32: 581-586.

Bärlocher, F.1992. Community organization. In: Bärlocher, F. editor. The Ecology of Aquatic Hyphomycetes. Springer-Verlag, Heidelberg, Berlin, pp. 38-76.

Bills,G.F., Platas,G., Pelaez,F. and Masurekar,P. 1999. Reclassification of a pneumocandin-producing anamorph, *Glarea lozoyensis* gen. et sp. nov, previously identified as *Zalerion arboricola*. Mycol. Res. 103: 179-192.

Begon, M., Harper, J.L., and Townsend, C.R. 1998. Ecology–Individuals, Populations, and Communities, 3rd edition. Sinauer, Sunderland.

Bruns, T.D., White, T.J., and Taylor, J.W.1991. Fungal molecular systematics. Annu Rev Ecol Syst. 22: 525-564.

Bak, A.L., Stenderup, A.1969. Deoxyribonucleic acid homology in yeasts. Genetic relatedness within the genus Candida. J. Gen. Micro. 59: 21–30.

Barr, D.J.S. 1988. How modern systematics relates to the rumen fungi. Bio Systems, 21: 351–356.

Berbee, M.L. and Taylor, J.W. 1992. Two ancomycete classes based on fruiting-body characters and ribosomal DNA sequence. Mol. Biol. Evol. 9: 278-284.

Berbee, M.L. and Taylor, J.W. 1995. From 18S ribosomal sequence data to evolution of morphology among the fungi. Can. J. Bot. 73 (Suppl. 1): S677-S683.

Bowman, J.L., Sakai, H., Jack, T., Weigel, D., Mayer, U. and Meyerowitz, E. M. 1992. SUPERMAN, a regulator of floral homeotic genes in *Arabidopsis*. Development. 114: 599-615.

Chang, S.Y., Goh, T.K. and Hyde, K.D. 2000a. Ingoldian fungi of Hong Kong. Fung. Div. 5: 89-107.

Chang, S. Y., Goh, T.K. and Hyde, K.D. 2000b. Ingoldian fungi in Lam Tsuen River and Tai Po Kau Forest Stream, Hong Kong. Fung. Div. 5: 109-118.

Copyright by Mahidol University

Chauvet, E. and Suberkropp, K. 1998. Temperature and sporulation of aquatic hyphomycetes. Appl. Environ. Microbiol. 64: 1522-1525.

Charcosset, J.Y., and Chauvet, E. 2001. Effect of Culture Conditions on Ergosterol as an Indicator of Biomass in the Aquatic Hyphomycetes. Appl. Environ. Microbiol. 67: 2051–2055.

De Wildeman, E. 1893. Notes mycologiques. Fasc. 2. Annales de la Societe Belge de Microbiologique Bruxelles. 17: 35-68.

De Wildeman, E. 1894. Notes mycologiques. Fasc. 3. Annales de la Societe Belge de Microbiologique Bruxelles.18: 135-161.

De Wildeman, E. 1895. Notes mycologiques. Fasc. 6. Annales de la Societe Belge de Microbiologique Bruxelles. 19: 193-206.

Descals, E. 1997. Ingoldian Fungi: some field and laboratory techniques. Boll. Soc. Hist. Nat. Balears. 40: 169-221.

Fisher, P.J., Webster, J. 1983. The teleomorphs of *Helicodendron giganteum* and *H. paradoxum*. Trans Br Mycol Soc. 81: 646-659.

Felsenstein, J. 1985. Confidence intervals on phylogenies: an approach using the bootstrap. Evolution. 39: 83-791.

Fuller, M. S. and Jaworski, A. 1987. Zoosporic fungi in teaching and research. Southeastern Pub. Corp., thens, Georgia.

Glass, N.L and Donaldson, G. 1995. Development of primer sets designed for use with the PCR to amplify conserved filamentous ascomycetes. Appl. Environ. Microbiol. 61: 323-1330.

Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2: 113-118.

Gray, S.F. 1997. In: Hanlin, R.T. editor. Ilustrated Genera of Ascomycetes. APS, Minnesota, USA.

Goh, T.K., Hyde, K.D. 1996. Biodiversity of freshwater fungi. J. Ind. Microbiol.17: 28-345.

Goh, T.K. 1997. Tropical Freshwater Hyphomycetes. In: Hyde, K.D. editor. Biodiversity of Tropical Microfungi, Hong Kong University Press, Hong Kong, pp.189-227.

Copyright by Mahidol University

Hall, T. 2001. Bioedit version 5.06, Department of Microbiology, North Carolina State University.

Hasegawa, M., Kishino, H., and Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22: 60–174.

Hawksworth, D.L. 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol. Res. 95: 641-655.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C., and Pegler, D.M. 1995. Ainsworth and Bisby's Dictionary of the fungi. 8th ed. Egham, UK, CAB International. pp. 616

Harrigan, G.G., Armentrout, B.L, Gloer, J.B., and Shearer, C.A. 1995. Anguillosporal, a new antibacterial and antifungal metabolite from the freshwater fungus *Anguillospora longissima*. J. Nat. Prod 58: 1467–1469.

Harrington, T. C. and Rizzo, D. M. 1999. Defining species in the fungi. In: Worrall, J.J. editor. Structure and Dynamics of Fungal Populaitons. Kluwer Press, Dordrecht, The Netherlands. pp. 43-70.

Henderson, P.A, and Seaby, R.M.H. 1998. Species Diversity and Richness version Version 2.2. PISCES Conservation Ltd, Pennington, Lymington, United Kingdom.

Hillis, D.M., and Moritz, C. 1990. An overview of applications in molecular systematics. In: Hillis, D.M. and Moritz, C. editors. Molecular Systematics. Sinauer Associates, Sunderland, MA. pp. 502-515.

Hillis, D.M., and Dixon, M.T. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. Quart. Rev. Biol. 66: 411-453.

Hibbett, D.S. 1992. Ribosomal RNA and fungal systematics. Trans. Mycol. Soc. J. pn. 33: 533-556.

Ho, W.H. 1998. Biodiversity, ecological and ultrastructural studies in freshwater fungi on wood submerged in tropical streams. [Ph.D. Thesis in Microbiology]. Hong Kong University. 229 pp.

Hong, W., Meiqing, Q., and Adrian, J.C. 1993. A simple method of preparing plant samples for PCR. Nuc. Res. 20: 4153-4154.

Ingold, C.T. 1942. Aquatic hyphomycetes of decaying alder leaves. Trans Brit Mycol Soc. 25: 339-417.

Copyright by Mahidol University

Ingold, C.T. 1943. Further observations on aquatic hyphomycetes of decaying alder leaves. Trans Brit Mycol Soc. 26:104-115.

Ingold, C.T. 1944. Some new aquatic hyphomycetes. Trans Brit Mycol Soc. 27: 34-47.

Ingold, C.T. 1951. Aquatic Ascomycetes: *Ceriospora caudae-suis* new. sp. and *Ophiobolus typhae*. Trans Brit Mycol Soc. 34: 210-215.

Ingold, C.T. 1952. *Actinospora megalospora* new.sp., an aquatic hyphomycete. Trans Brit Mycol Soc. 35: 66-70.

Ingold, C.T. and Chapman, B. 1952. Aquatic Ascomycetes: *Loramyces juncicola* Weston and *L. macrospora* new. sp. Trans Brit Mycol Soc. 35: 268-272.

Ingold, C.T. 1954. Aquatic Ascomycetes: Discomycetes from lakes. Trans Brit Mycol Soc. 37: 1-18.

Ingold, C.T. 1955. Aquatic Ascomycetes: further species from the English Lake District. Trans Brit Mycol Soc. 37:157-168.

Ingold, C. T. 1956. Stream spora in Nigeria. Trans Brit Mycol Soc. 39: 106-110.

Ingold, C.T. 1958a. Aquatic hyphomycetes from Uganda and Rhodesia. Trans Brit Mycol Soc. 41: 109-114.

Ingold, C.T. 1958b. New aquatic hyphomycetes: *Lemonniera brachycladia, Anguillospora crassa* and *Fluminospora ovalis*. Trans Brit Mycol Soc. 41: 365-372.

Ingold, C.T. 1959a. Aquatic spora of Omo forest, Nigeria. Trans Brit Mycol Soc. 42: 479-485.

Ingold, C.T. 1959b. Polycladium equiseti gen. nov., sp. nov.: an aquatic hyphomycete on Equisetum fluviatile. Trans Brit Mycol Soc. 42: 112-114.

Ingold, C.T. 1959c. Submerged aquatic hyphomycetes. J Que Micro Seri. 4 5: 115-130.

Ingold, C.T. 1960a. Aquatic hyphomycetes from Canada. Can. J. Bot. 38: 803-806.

Ingold, C.T. 1960b. Aquatic hyphomycetes in Southern Rhodesia. Proceedings Rhodesian Science Association. 43: 49-53.

Ingold, C.T. 1961. Another aquatic spore-type with clamp-connexions. Trans Brit Mycol Soc. 44: 27-30.

Ingold, C.T. 1965. Hyphomycete spores from mountain torrents. Trans Brit Mycol Soc. 48: 453-458.

Copyright by Mahidol University

Ingold, C. T. 1966. The tetraradiate aquatic fungal spore. Mycologia. 58: 43-56.

Ingold, C.T. 1967. Spores from foam. Bull Brit Myco Soc. 1: 60-63.

Ingold, C.T. 1971. Fungal Spores, their Liberation and Dispersal, Clarendon Press, Oxford.

Ingold, C.T. and Webster, J. 1973. Some aquatic hyphomycetes from India. Kavaka. 1: 4-9.

Ingold, C.T. 1975. An Illustrated Guide to Aquatic and Water-Borne Hyphomycetes (Fungi Imperfecti) with notes on their Biology. Freshwater Biological Association, Science Publication. 30: 1-96.

Ingold, C.T. 1976. The morphology and biology of freshwater fungi excluding phycomycetes. In: Jones, E.B.G. editor. Recent Advances in Aquatic Mycology, Elek Science, London. pp. 335-358.

Iqbal, S.H. and Webster, J. 1977. Aquatic hyphomycete spora of some Dartmoor streams. Trans Brit Mycol Soc. 69: 233-241.

Iqbal, S.H. and Webster, J. 1973. The trapping of aquatic hyphomycetes spores by air bubbles. Trans Brit Mycol Soc. 60: 37-48.

Ka-Lai, P., Abdel-Wahab, M.A., Sivichai, S., El-Sharouney, H.M. and Jones, E.B.G. (2002). *Jahnulales* (*Dothideomycetes, Ascomycota*): a new order of lignicolous freshwater ascomycotes. Mycological Research. 106 (9): 1031-1042.

Kirk, P.M., Cannon, P.F., David, J.C. and Stalpers, J.A. 2001. Dictionary of the fungi. 9th edition. CAB International Press, Wallingford, UK.

Margalef, R. 1958. Information theory in ecology. General Systematics 3: 36-71.

Martinez-Culebras, P.V.A., Querol, M.B., Suarez-Fernandez, M.D., Garcia-Lopez and Barrio, E. 2003. Phylogenetic Relationships Among Colletotrichum Pathogens of Strawberry and Design of PCR Primers for their Identification. J. Phyt. 151: 135–143.

Marvanova, L. Freshwater Hyphomycetes.1997. A survey with remarks on tropical taxa. In: Janardhanan, K.K., Rajendran, C., Natarajan, K., Hawksworth, D.L. editors. Tropical Mycology, Science Publishers, Inc, 1997. pp.169-226.

Copyright by Mahidol University

Marvanova, L and Hywel-Jones, N.L. 2000. *Sigmoidea contorta* sp. nov. and two rare species from waters in Thailand. Cry Mykolo. 21:13-26.

McCracken, V.J., Simpson, J.M., Mackie, R.I., and Gaskins, H.R. 2001. Molecular ecological anlaysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. J. Nutr. 131: 1862–1870.

Paolocci,F., Rubini,A., Riccioni,C., Topini,F. and Arcioni,S. 2004.*Tuber aestivum* and *Tuber uncinatum*: two morphotypes or two species. FEMS Microbiol. Lett. 235 (1): 109-115

Powell, M.J. 1993. Looking at mycology with a Janus face: a glimpse at Chytridiomycetes active in the environment. Mycologia. 85: 1–20.

Rambout, A.1996. Se-Al. Department of zoology, University of Oxford. Oxford, OX1 4JD, UK.

Rehner, S.A., and Samuels, G.J. 1995. Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. Can. J. Bot. 73 (Suppl 1). S816-S823.

Roldán, A., Descals, E., Honrubioa, M. 1987. *Dendrospora polymorpha* sp. nov. a new hyphomycete from Spanish streams. Mycotaxon. 29: 21-27.

Saccardo, P.A. 1889. *Syll. fung*. (Abellini) 8: 41. In: http://www.indexfungorum.org /Names/namesrecord.asp?RecordID=229857/March/2005.

Sakayajoj, J. 2000. A survey and study of the Aquatic Fungi at Tone Nga Chang Wildlife-Sactuary, Songkhla Province. [M.Sc. Thesis in Microbiology], Prince of Songkhla University, Thailand.

Sati, S.C and Tiwari, N. 1992. A new species of *Tricladium* from Kumaum Himalaya, India. Mycol. Res. 96: 229-232.

Schoenlein-Crusius, I.H. and Grandi, R.A.P. 2003. The diversity of aquatic Hyphomycetes in South America. Bra J Micro. 34: 183-193.

Shearer, C.A, Zare-Maivan, H. 1988. *In vitro* hyphal interactions among wood- and leaf-inhabiting ascomycetes and fungi imperfecti from freshwater habitats. Mycologia. 80: 31–37.

Shearer, C. A. 1993. The freshwater Ascomycetes. Nova Hedwigia. 56: 1-33.

Simpson, E.H. 1949. Measurement of diversity. Nature: 163-688.

Copyright by Mahidol University

Sindhar, K.R., Chandrashekar, K.R. and Kaveriappa, K.R. 1992. Research on the Ingoldian sub continent. In: Barlocher, F. editor. The Ecology of Aquatic Hyphomycetes. Springer-Verlag, Berlin. pp.182-211.

Sindhar, K.R., Chandrashekar, K.R., and Kaveriappa, K.R. 1992. Research on the Ingoldian sub continent. In: The Ecology of Aquatic Hyphomycetes (ed. F. Barlocher). Springer-Verlag, Berlin. pp.182-211.

Sivichai, S. 1999. Tropical Freshwater Fungi: Their Taxonomy and Ecology. [Ph.D, Thesis in Microbiology], Portsmouth University, UK.

Sivichai, S and Boonyuen, N. 2004. Freshwater fungi. In: Jones, E.B.G., Tanticharoen, M., and Hyde, K.D. editors. Thai Fungal Diversity. BIOTEC, Thailand. pp. 95-106.

Sivichai, S., Jones, E.B.G., and Hywel-Jones, N.L. 2003. *Hymenoscyphus varicosporoides* and its *Tricladium* anamorph Lignicolous freshwater Ascomycota from Thailand: *Hymenoscyphus varicosporoides* and its *Tricladium* anamorph. Mycologia. 95(2): 340-346.

Sparrow, F.K. 1968. Ecology of freshwater fungi. In: Ainsworth, G.C and Sussmann, A.S. editors. The Fungi, Vol 3. Academic Press, London. pp. 41-93.

Spatafora, J.W. 1992. The Molecular Systematics of Unitunicate, Perithecial Ascomycetes. Louisiana State University and Agricultural and Mechanical College. Louisiana.

Spatafora, J.W., and Blackwell, M. 1993. Molecular systematics of unitunicate perithecial ascomycetes: the Clavicipitales-Hypocreales connection. Mycologia. 85: 912-922.

Spatafora, J.W. and Blackwell, M. 1994a. Cladistic analysis of partial ssrDNA sequences among unitunicate perithecial ascomycetes and its implications on the evolution of centrum development. In: Hawksworth, D.L. editor. Ascomycete Systematics: Problems and Perspectives in the nineties. Plenum Press, New York. pp. 233-242.

Spatafora, J.W. and Blackwell, M. 1994b. The polyphyletic origins of ophiostomatoid fungi. Mycol. Res. 98: 1-9.

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Sreenivasaprasad, S., Mills, P.R, Meehan, B.M., and Brown, A.E. 1996. Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. Genome. 39: 499–512.

Sridhar, K.R., Bärlocher, F. 2000. Initial colonization, nutrient supply, and fungal activity on leaves decaying in streams. Appl. Environ. Microbio. 66(3): 1114-1119

Suberkropp, K. 2001. Fungal growth, production, and sporulation during leaf decomposition in two streams. Appl. Environ. Microbiol. 67: 5063-5068.

Swann, E.C and Taylor, J.W. 1993. Higher taxa of basidiomycetes: an 18S rRNA gene perspective. Mycologia. 85: 923-936.

Swofford, D.L. 2003. PAUP, Phylogenetic analysis using parsimony version 4.6.2. Laboratory of molecular systematic, Smithsonian Institution, Washington, D.C, U.SA.

Thomas, K. 1996. Freshwater fungi. In: Orhard, A. E. editor. Fungi of Australia. Vol. 1.B. Introduction-Fungi in the Environment, ABRS/CSIRO, Australia. pp. 1-37.

Thompson, J.D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through weighting, positions-specific gap penalties and weight matrix choice. Nuc. Res. 22: 4673-4680.

Tubaki, K. 1966. An undescribed species *of Hymenoscyphus,* a perfect stage of *Varicosporium*. Trans Brit Mycol Soc. 49: 345-349.

Tubaki, K., Watanabe, K. and Manoch, L. 1983. Aquatic hyphomycetes from Thailand. Trans Brit Mycol Soc. of Japan. 6: 11-16.

Vralstad,T., Myhre,E. and Schumacher,T. 2002. Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. New Phytol. 155: 131-148.

Webster, J. and Scheuer, Ch. Om Khaltoum Khattab. S. 1990. *Hydrocina chaetocladia* gen. nov., the teleomorph of *Tricladium chaetocladium*. Nova Hedwigia. 52: 65-72.

Copyright by Mahidol University

Webster, J. 1992. Anamorph-teleomorphs relationships. In: Barlocher, F. editor. The ecology of aquatic hyphomycetes. Springer-Verlag, Berlin Press. pp. 99-117.

Webster J.R., Wallace J.B. and Benfield E.F. 1995. Organic processes in streams of the eastern United States. In: Cushing, C.E., Cummins, K.W., and Minshall, G.W editors. River and Stream Ecosystems. Elsevier Science BV, Amsterdam. pp. 117-187.

Webster, J., Eicker, A and Spooner, B.M. 1995. *Cudoniella indica* sp. nov. (Ascomycetes, Leotiales), the teleomorph of *Tricladium indicum*, an aquatic fungus isolated from a South African river. Nova Hedwigia. 60: 493-498.

Webster, J. 1977. Seasonal observations on aquatic hyphomycetes on oak leaves on the ground. Trans Brit Mycol Soc. 68: 108-111.

Whittaker, R.H. 1960. Vegetation of the Siskiyou Mountains, Oregon and California. Ecological Monographs. 30(3): 279-338.

White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. editors. PCR protocols. A guide to methods and applications. Academic Press, San Diego. pp. 315-322.

Willoughby, L.G and Archer, J.F. 1973. The fungal spora of a freshwater stream and its colonization pattern on wood. Freshwater Biol. 3: 219-239.

Wong, M.K.M., Goh, T.K., Hodgkiss, I.J., Hyde, K.D., Ranghoo, V.M., Tsui, C.M. Ho, W.H., Wong, W.S.W., and Yuen, T.K. 1998. Role of fungi in freshwater ecosystems. Biod and Cons 7: 1187-1206.

Zemek, J., Marvanova, L., Kuniak, L. and Kadlecıkova, B. 1985. Hydrolytic enzymes in aquatic hyphomycetes. Folia Microbiol. 30: 363–372.

Zhang, Y.H. and Zhuang, W.Y. 2004. Phylogenetic relationships of some members in the genus *Hymenoscyphus* (Ascomycetes, Helotiales). Nov Hed.78: 475-484.

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APPENDIX

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Tree Scores of Maximum Parsimony

Tree Length:

The number of characters in data set and number of the states of the characters or actual of evolutionary steps on the observed cladogram.

Consistency Index (CI):

To measure of the amount of homoplasy exhibited by a character or set of characters on a tree, the folmular (range 0 to 1, 1 means “no homoplasy).

The statistic is give by: CI = <minimum possible tree length> / <Tree length>.

Retention Index (RI):

This value used to separates between synapomorphic and apomorphic charaters (range 0 to 1, 1 means “no homoplasy).

This value calculated using: RI ={<maximum possible tree length>-<Tree length>}/ {<maximum possible tree length>-<minimum possible tree length>}.

Rescaled Consistency Index (RC):

This value used to assess the congruency and fit of character within a given tree A higher value of *RC* indicates that the characters in the data set are more congruent with each other and the given tree (range 0 to 1, 1 means “no homoplasy)
This tree score can be calculated by: RC= CI x RI.

Homoplasy Index (HI):

To accounts for the amount of homoplastic charaters in a given data set.

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Tree Scores of Maximum Parsimony

Data set	Number of the most parsimony	Tree Scores				
		Length	CI	RI	RC	HI
Figure 4.4	2	1191	0.623	0.763	0.476	0.377
Figure 4.7	12	1115	0.644	0.750	0.483	0.356
Figure 4.10	2	806	0.768	0.747	0.574	0.232

Character status of all characters in the Figure 4.3, Figure 4.6 and Figure 4.9

Data set	Total number of characters	Constant		Varible (parsimony-uninformative)		Informative	
		Number	%	Number	%	Number	%
Figure 4.4	858	410	47.78	151	17.59	297	34.61
Figure 4.7	858	418	48.71	155	18.06	285	33.21
Figure 4.10	858	458	53.37	146	17.01	254	29.60

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MP, NJ and ML trees of 31 and 19 selected taxa analysis, exclude taxa of *Anguillospora* spp., *Zalerion varium* and *Hymenoscyphus* spp.

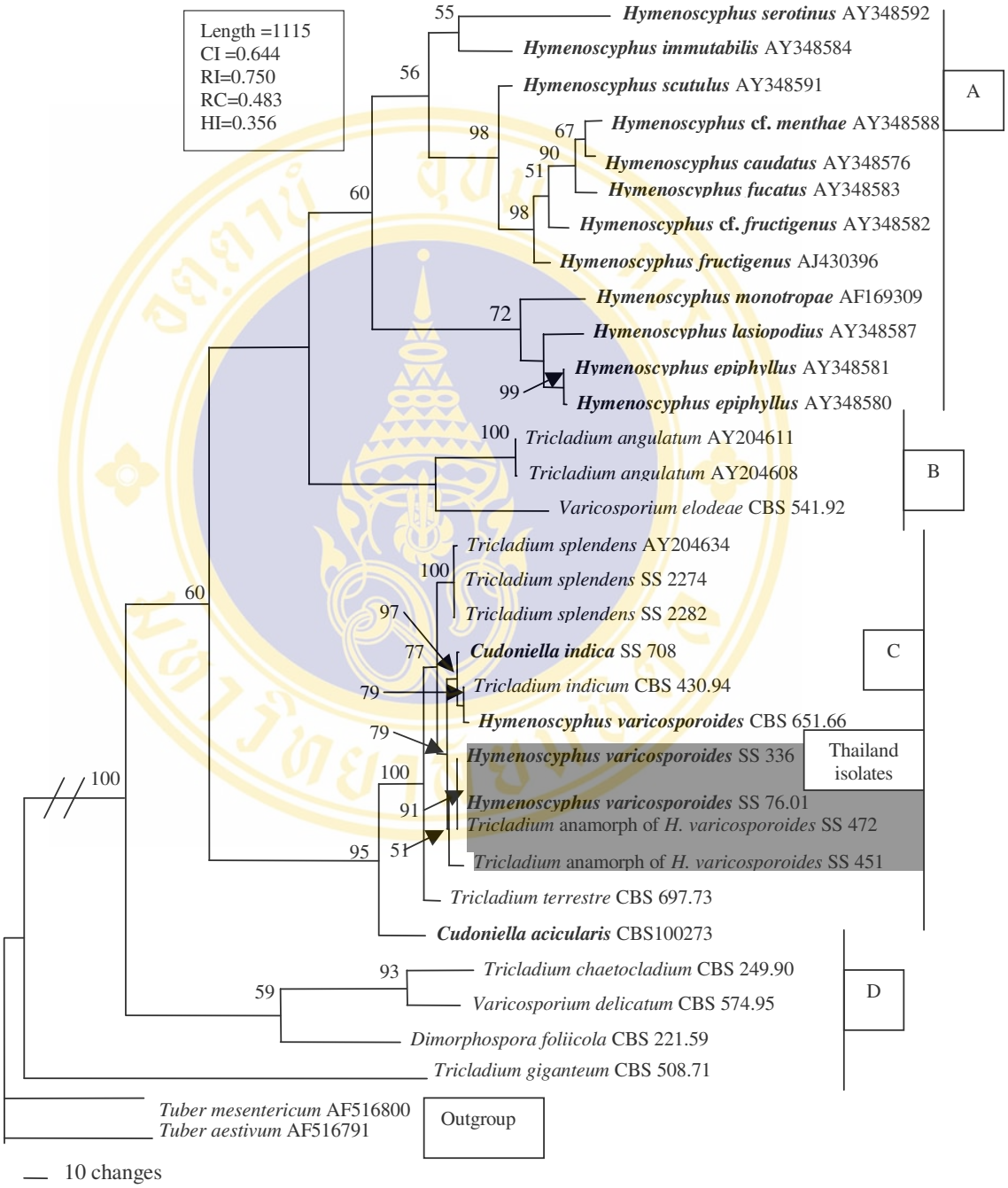

Figure 4.7 Strict consensus tree of 12 most parsimony trees calculated from ITS1-2, 5.8S rDNA sequences of 31 selected taxa in Helotiaceae, excluding taxa of *Anguillospora filiformis*, *A. crassa*, *A. furtiva* and *Zalerion varium*. *Tuber mesentericum* and *T. aestivum* as outgroup taxa in Pezizales. Bootstrap values over 50% were indicated at the base of the corresponding clade.

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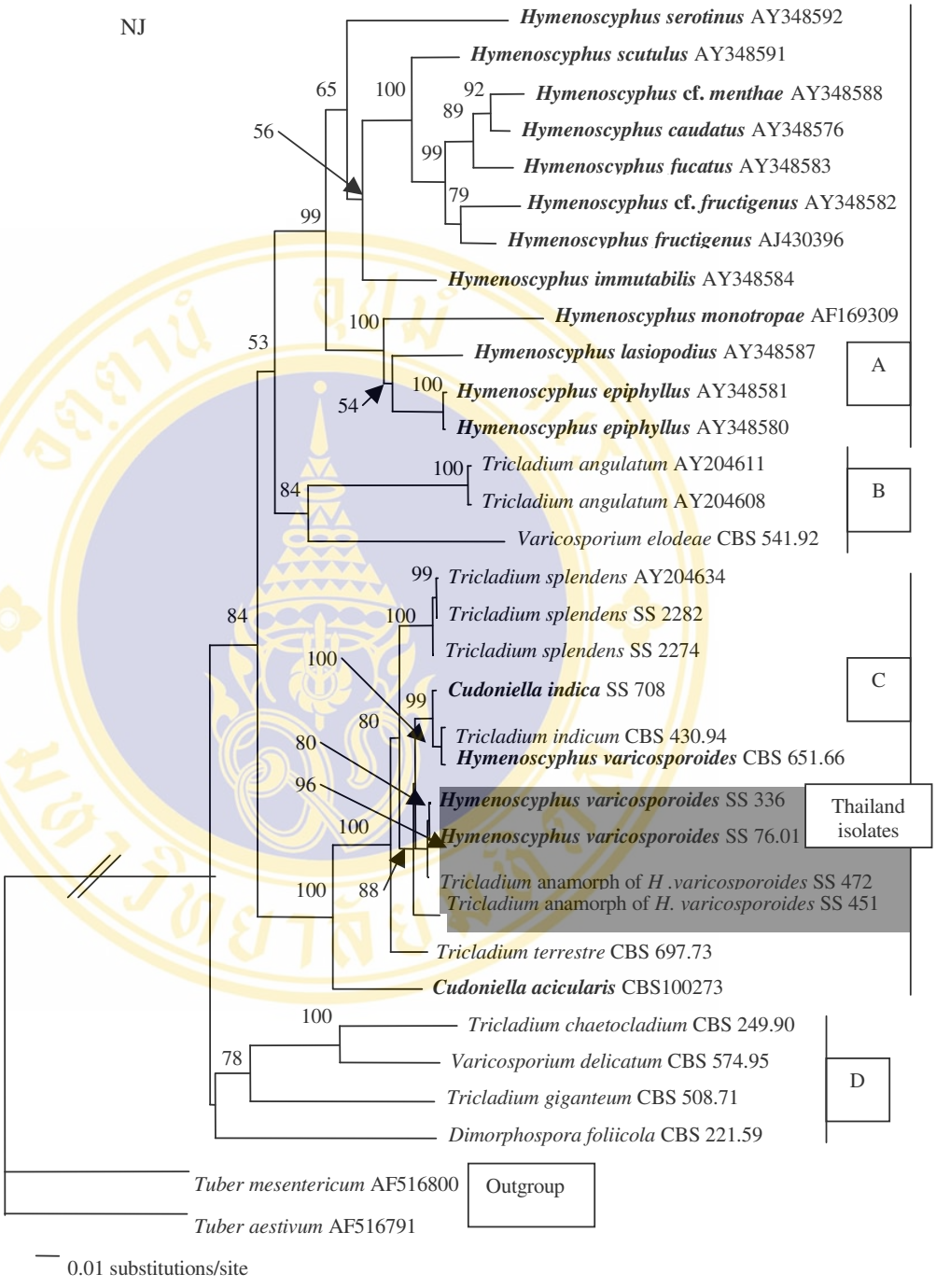

Figure 4.8 NJ trees calculated from ITS1-2, 5.8S rDNA sequence of 31 selected taxa in Helotiaceae, excluding taxa of *Anguillospora filiformis*, *A. crassa*, *A. furtive* and *Zalerion varium*. *Tuber mesentericum* and *T. aestivum* were outgroup taxa in Pezizales.

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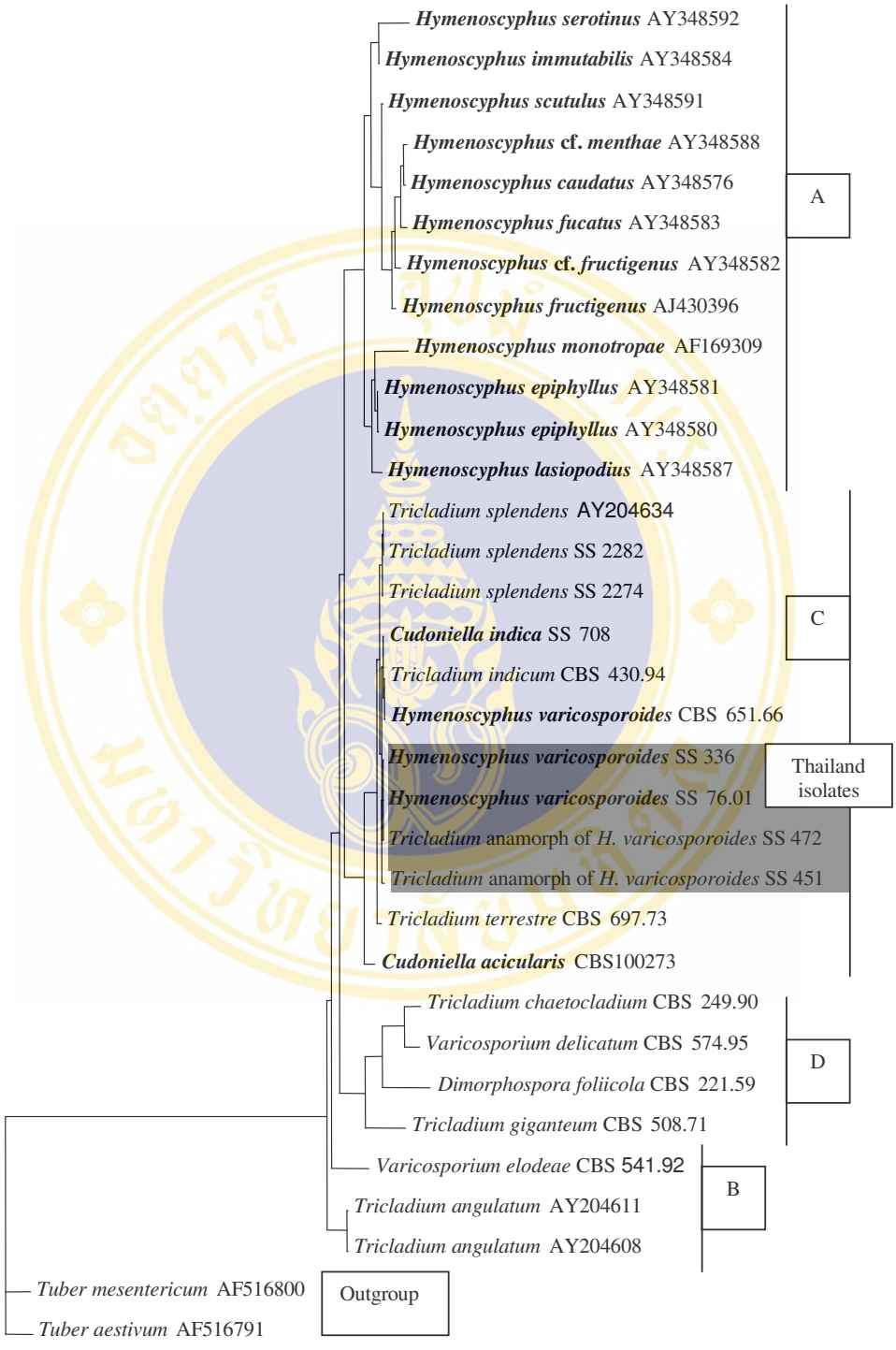

Figure 4.9 ML trees (-ln likelihood=5683.3825) with the parameters estimated from the most parsimony trees from dataset treated gaps as missing data with equally weighted characters. Estimated base frequency: A, 0.222165; C, 0.26557; G, 0.26220; and T, 0.25059; estimated ti/tv ration= 1.034186; estimated value of proportion of invarible sites= 0.144510; and estimated value of gamma shape parameter= 0.788461.

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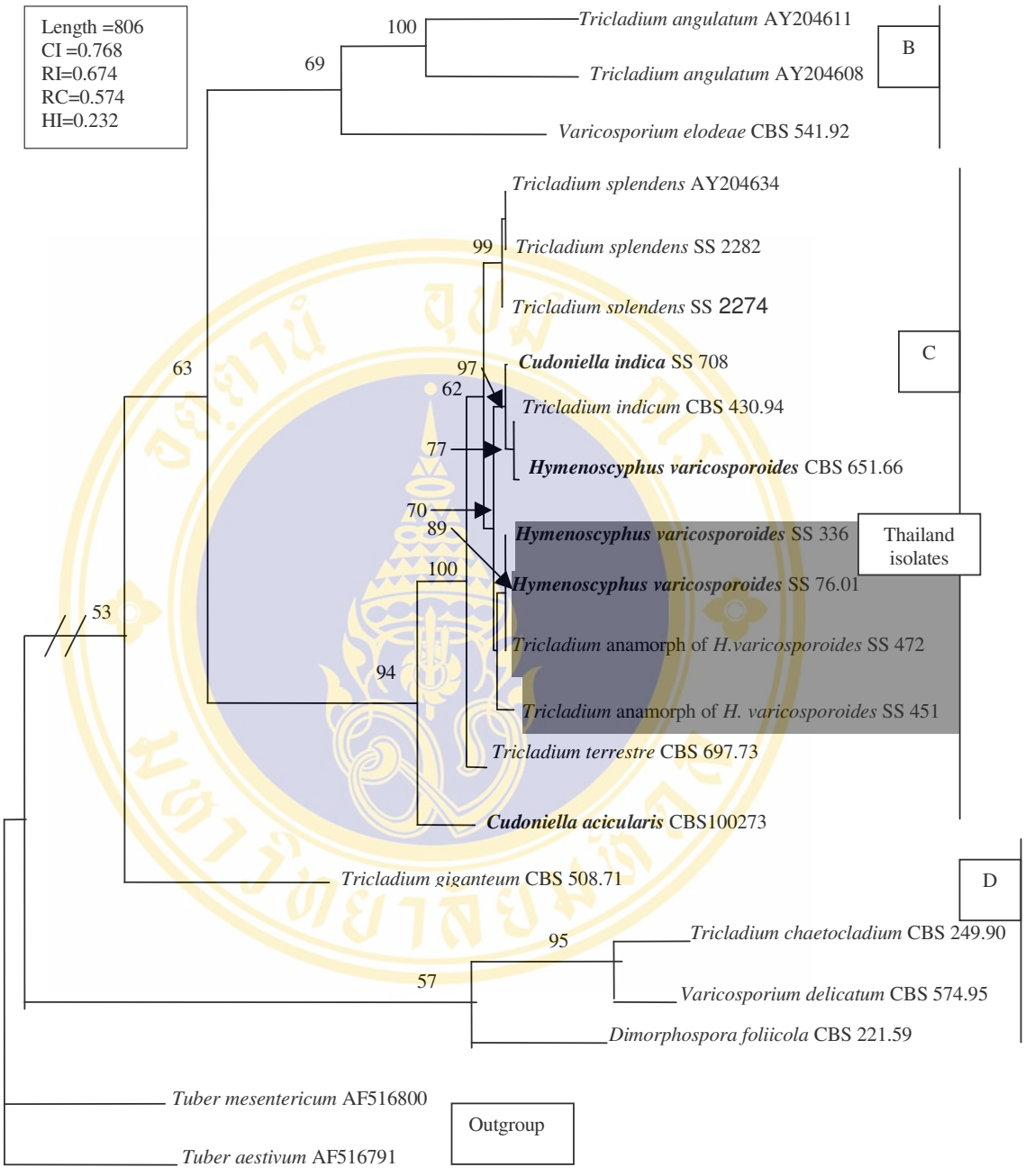

Figure 4.10 Strict consensus tree of 2 most parsimony trees calculated from ITS1-2, 5.8S rDNA sequences of 19 selected taxa in Helotiaceae. Taxa of *Anguillospora filiformis*, *A. crassa*, *A. furtive, Zalerion varium* and *Hymenoscyphus* spp. (terrestrial strains) were exclude. *Tuber mesentericum* and *T. aestivum* were outgroup taxa in Pezizales. Bootstrap values over 50% was indicated at the base of the corresponding clade.

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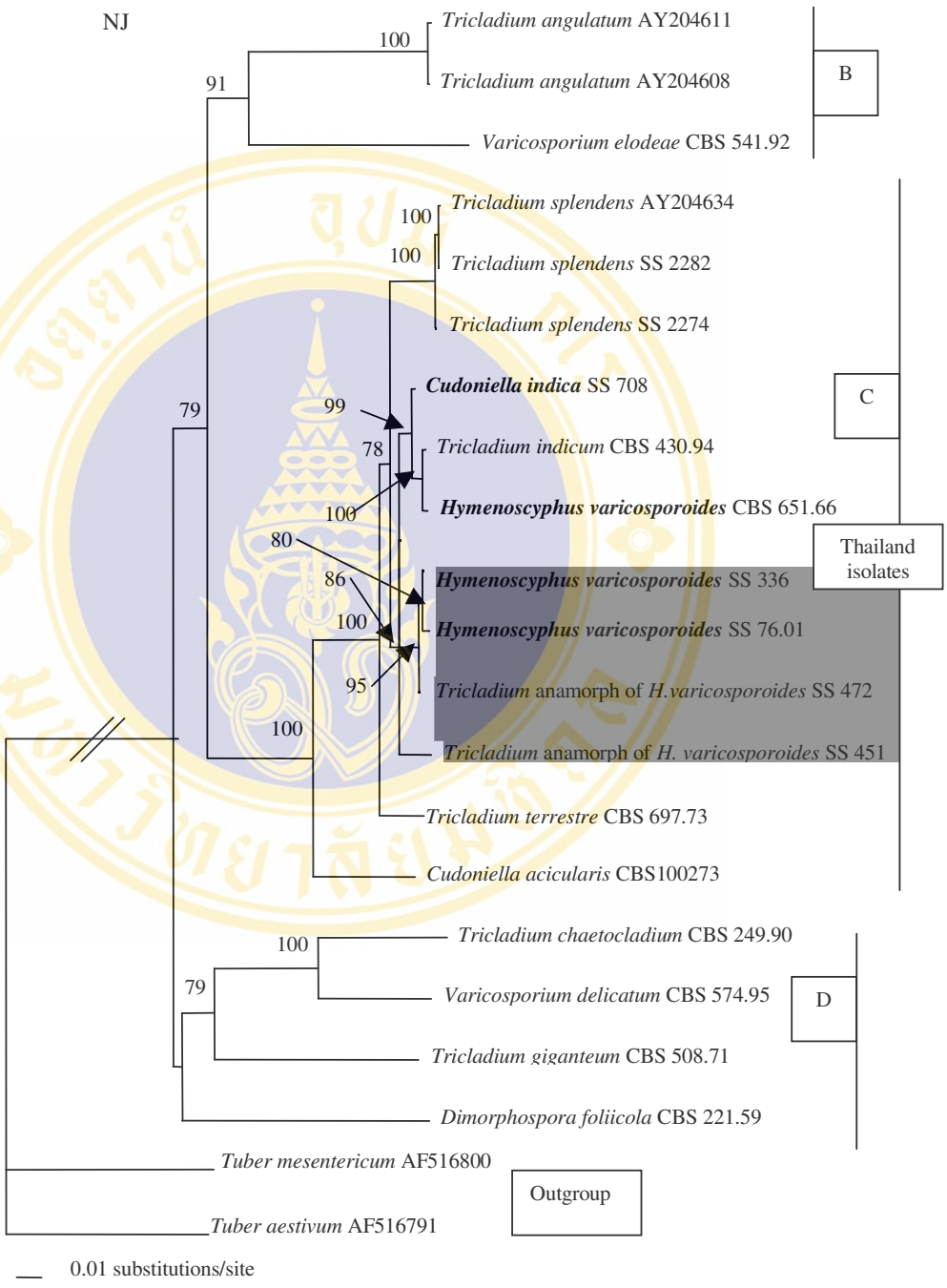

Figure 4.11 NJ trees calculated from ITS1-2, 5.8S rDNA sequence of 19 selected taxa in Helotiaceae, Taxa of *Anguillospora filiformis*, *A. crassa*, *A. furtive, Zalerion varium* and *Hymenoscyphus* spp. (terrestrial strains) were exclude. *Tuber mesentericum* and *T. aestivum* were outgroup taxa in Pezizales.

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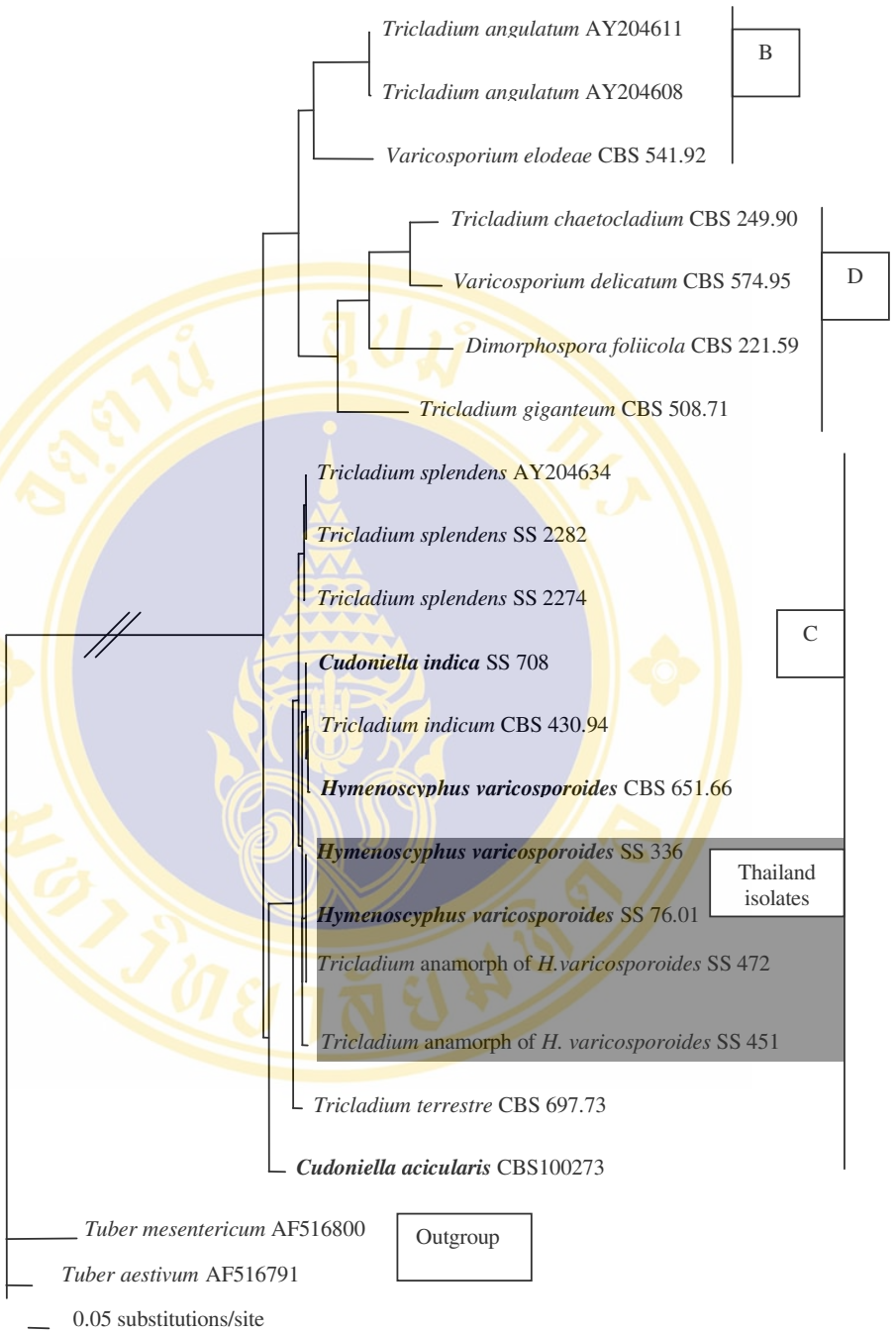

Figure 4.12 ML trees (-ln likelihood=4235.76598) with the parameters estimated from the most parsimony trees from dataset treated gaps as missing data with characters equally weighted. Estimated base frequency: A, 0.21781; C, 0.27125; G, 0.27111; and T, 0.23983; estimated ti/tv ration= 0.891732; estimated value of proportion of invarible sites= 0.265252; and estimated value of gamma shape parameter= 1.134883.

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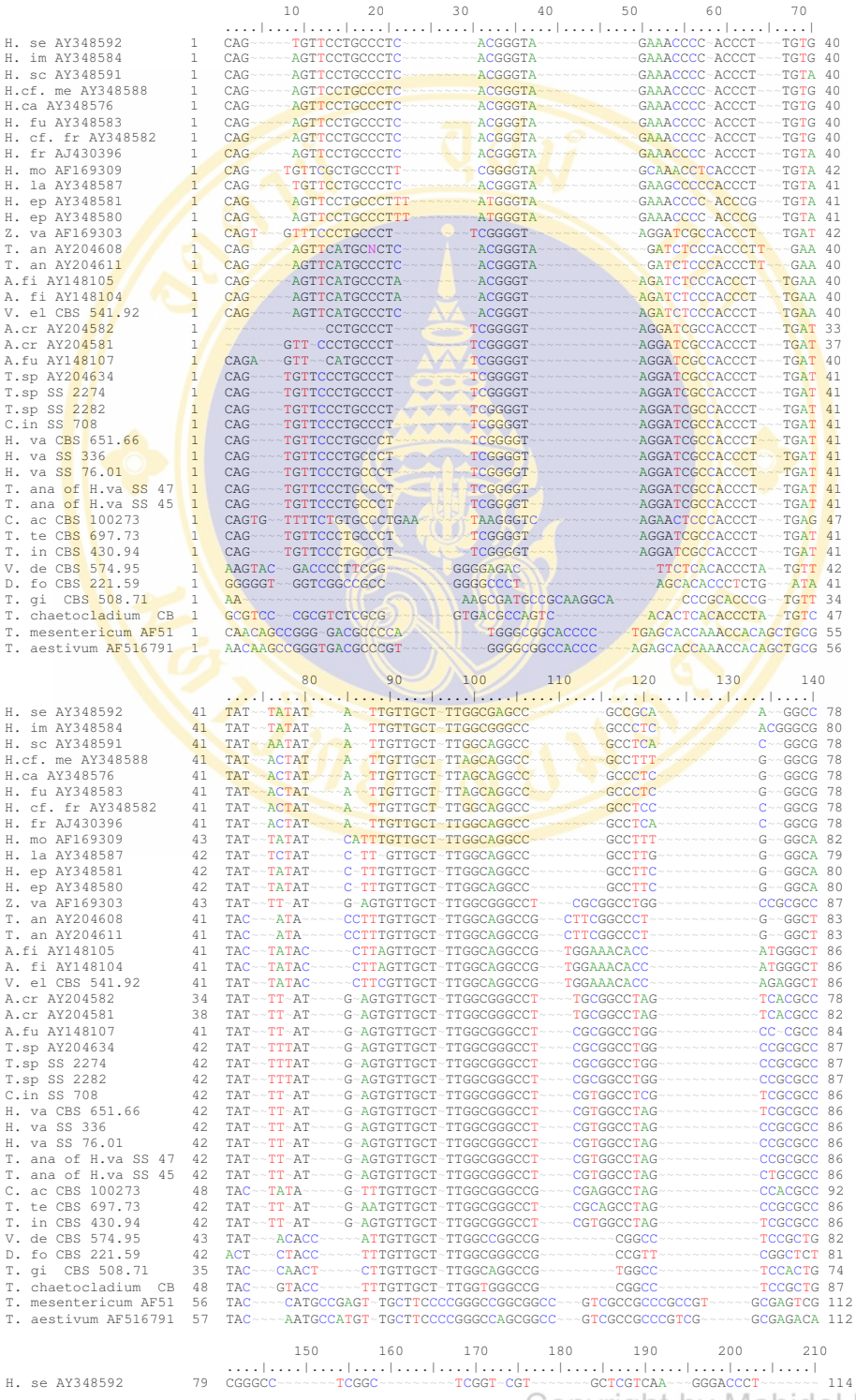
Appendix

Nucleotide sequences of from 37 selected Ingoldian species on ITS1-2, 5.8S rDNA



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H. im AY348584          81 CTGGCT------CACGC---------TGGCT-CGC------GCTCGCCAA---AGGACCCC-------- 116
H. sc AY348591          79 TCGGCT------CACGC---------TGGCT-CGT------GCCTGCCAG---AGGACCCT-------- 114
H.cf. me AY348588       79 TCGGCT------CACGC---------TGAC--CGT------GCCTGCTAG---AGGACCCT-------- 113
H.ca AY348576           79 TCGGCT------CACGC---------TGAC--CGT------GCCTGCTAG---AGGACCCT-------- 113
H. fu AY348583          79 TCGGCG------CACGC---------TGAC--CGT------GCCTGCTAG---AGGACCCT-------- 113
H. cf. fr AY348582      79 TCGGCT------CACGC---------TGAC--CGT------GCCTGCCAG---AGAACCCT-------- 113
H. fr AJ430396          79 TTGGCT------CACGC---------TGAC--TGT------GCCTGCCAG---AGGACCCT-------- 113
H. mo AF169309          83 CCGGCT------TCGGC---------TGGAC-CGC------GCCTGCCAG---AGAACCCCT------- 119
H. la AY348587          80 CCGGCT------CGCGC---------CGGCT-CGT------GCCTGCCAG---AGGACCCC-------- 115
H. ep AY348581          81 CCGGCA------CACGC---------TGGAT-CGT------GCCTGCCAG---AGGACCCC-------- 116
H. ep AY348580          81 CCGGCA------CACGC---------TGGAT-CGT------GCCTGCCAG---AGGACCCC-------- 116
Z. va AF169303          88 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----C 125
T. an AY204608          84 TCGGCT-------------------------CGGGCGT------GCCTGCCAG---AGG-ACCCC----- 113
T. an AY204611          84 TCGGCT-------------------------CGGGCGT------GCCTGCCAG---AGG-ACCCC----- 113
A.fi AY148105           87 CCGGCT------TATGC-------------------GT------GCCTGCCAG---GGGAATAAA----- 117
A. fi AY148104          87 CCGGCT------TATGT-------------------GT------GCCTGCCAG---GGGAATCAA----- 117
V. el CBS 541.92        87 CCGGCT------GATGC-------------------GT------GCCTGCCAG---AGGAAACAC----- 117
A.cr AY204582           79 CCGGCT------TCGG----------TGGGGGAGC------GCCCGCCAG---AGGATTCTA-----C 116
A.cr AY204581           83 CCGGCT------TCGG----------TGGGGGAGC------GCCCGCCAG---AGGATTCTA-----C 120
A.fu AY148107           85 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG----GGATTCTA-----C 121
T.sp AY204634           88 CCGGCT------CCGG----------CGGGGGAGC------GCCCGCCAG---AGGCTTCTA-----C 125
T.sp SS 2274            88 CCGGCT------CCGG----------CGGGGGAGC------GCCCGCCAG---AGGCTTCTA-----C 125
T.sp SS 2282            88 CCGGCT------CCGG----------CGGGGGAGC------GCCCGCCAG---AGGCTTCTA-----C 125
C.in SS 708             87 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----T 124
H. va CBS 651.66        87 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----T 124
H. va SS 336            87 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----T 124
H. va SS 76.01          87 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----T 124
T. ana of H.va SS 47    87 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----T 124
T. ana of H.va SS 45    87 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----T 124
C. ac CBS 100273        93 CCGGCT------TCGG----------CGGGGGAGT------GCCCGCCAG---AGGACCCCA------ 129
T. te CBS 697.73        87 CCGGCT------TCGGA---------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----C 125
T. in CBS 430.94        87 CCGGCT------TCGG----------CGGGGGAGC------GCCCGCCAG---AGGATTCTA-----T 124
V. de CBS 574.95        83 CGGGC-------TCAC----------GCTCGCACGT------GCCGGCCAG---AGGACCC------- 117
D. fo CBS 221.59        82 CGGCC-------TCGT----------GCTGGGACGC------GCCCGCCAG---AGGACCC------- 116
T. gi  CBS 508.71       75 TGGGC-------TTAA----------GCCTGCACGT------GCCTGCCAG---AGGACCA------- 109
T. chaetocladium  CB    88 CGGGCC------TCGC----------GCTCGCACGT------GCCCGCCAG---AGAACCC------- 123
T. mesentericum AF51   113 CCATGTTGGCTCGCCCGGCGGAGGCGCCCGGGGGTGGTTGGGGTCCAGCCCCGACGTGAAAAACGCTTTG 182
T. aestivum AF516791   113 CAGGCGTGGATCGCCGCGCGGAGGCGCCCGGGGGTGG---GGCTCCCGCCCCT-CGAAACTAAGACGTCG 178

                                220       230       240       250       260       270       280
                           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
H. se AY348592         115 AAACGC---TG-TCT---TATAGT---GT---C-GTCTGAGTA-----CTA---TTTAA---TAGTTAA 158
H. im AY348584         117 AAACTC---TG-AAG---TATAGT---GT---C-GTCTGAGTA-----CTA---TTTAA---TAGTTAA 160
H. sc AY348591         115 AAACTC---TG-AAA---TACAGT---GT---C-GTCTGAGTA-----CTA---TTTAA---TAGTTAA 158
H.cf. me AY348588      114 AAACTT---TG-AAA---TACAGT---GT---C-GTCTGAGTA-----CTA---TTCAA---TAGTTAA 157
H.ca AY348576          114 AAACTC---TG-AAA---TACAGT---GT---C-GTCTGAGTA-----CTA---TTTAA---TAGTTAA 157
H. fu AY348583         114 AAACTC---TG-AAA---TATCGT---GT---C-GTCTGAGTA-----CTA---TTTAA---TAGTTAA 157
H. cf. fr AY348582     114 CAACTC---TG-TAA---TACAGT---GT---C-GTCTGAGTA-----CTA---TTTAA---TAGTTAA 157
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H. ep AY348581         117 AAATTC---TG-AAT---GTTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 160
H. ep AY348580         117 AAATTC---TG-AAT---GTTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 160
Z. va AF169303         126 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 168
T. an AY204608         114 AAACTC---TG-AATT--AC-AGT---GT---C-GTCTGAGTAC----TA---TATAA---TAGTTAA 157
T. an AY204611         114 AAACTC---TA-AATT--AC-AGT---GT---C-GTCTGAGTAC----TA---TATAA---TAGTTAA 157
A.fi AY148105          118 AAACTC---TG---TT---TTTAGT---GA---T-GTCTGAGTA-----CTA---TATAA---TAGTTAA 160
A. fi AY148104         118 AA-TTC---TG---TT---TTTAGT---GA---T-GTCTGAGTA-----CTA---TATAA---TAGTTAA 159
V. el CBS 541.92       118 AAACTC---TG---TT---TTTAGT---GA---T-GTCTGAGTA-----CTA---TATAA---TAGTTAA 160
A.cr AY204582          117 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 159
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A.fu AY148107          122 AAACC-----TG-AGT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TACTTAA 164
T.sp AY204634          126 AAACC-----TG-TGT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 168
T.sp SS 2274           126 AAACC-----TG-TGT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 168
T.sp SS 2282           126 AAACC-----TG-TGT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 168
C.in SS 708            125 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 167
H. va CBS 651.66       125 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 167
H. va SS 336           125 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 167
H. va SS 76.01         125 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 167
T. ana of H.va SS 47   125 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 167
T. ana of H.va SS 45   125 AAACC-----TG-ACT---GTGAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 167
C. ac CBS 100273       130 AACCC-----TG-AAT---GTTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 172
T. te CBS 697.73       126 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 168
T. in CBS 430.94       125 AAACC-----TG-ATT---ATTAGT---GT---C-GTCTGAGTA-----CTA---TATAA---TAGTTAA 167
V. de CBS 574.95       117 -AACTCT---TGATT---T-TAGT---GA---T-GTCTGAGTA-----CTA---TTAAA---TAGTT-A 159
D. fo CBS 221.59       116 -AACTCT---TGATT---T-TAGT---GA---C-GTCCGAGTA-----CTA---TATAA---TAGTT-A 158
T. gi  CBS 508.71      109 -AACTC----TGAAA---TTTAGT---GA---T-GTCTGAGTA-----CTA---TATAA---TAGTT-A 151
T. chaetocladium  CB   123 -AACTCT---TGATT---T-TAGT---GA---T-GTCTGAGTA-----CTATA-TTTAA---TAGTT-A 167
T. mesentericum AF51   183 GACGGATAACG---AGAAGTCTGAACCCGGCGCC-CTCTTGGGGC-----CGCCAACCTGA--AAAGTTAA 242
T. aestivum AF516791   179 GGAGCGTGGAC---TGT-GTCTGACCTGGGCCAC-ATTGGGCCGC-----CGAAAACTTGA--CAAGTTAA 237

                                290       300       310       320       330       340       350
                           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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H. im AY348584         161 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 224
H. sc AY348591         159 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 222
H.cf. me AY348588      158 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 221
H.ca AY348576          158 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 221
H. fu AY348583         158 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 221
H. cf. fr AY348582     158 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 221
H. fr AJ430396         158 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 221
H. mo AF169309         164 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 227
H. la AY348587         160 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 223
H. ep AY348581         161 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 224
H. ep AY348580         161 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 224
Z. va AF169303         169 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 232
T. an AY204608         158 AA-CTTTCAACAACGG-ATCTCTTG-GTTCTG-GCAT-CGATGAAGAACGCAGCGAAATGCGAT-AAGTA 221
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A.fi AY148105           161 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGATTAAGTA 225
A. fi AY148104          160 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCACGCAAATGCGATTAAGTA 224
V. el CBS 541.92        161 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 224
A.cr AY204582           160 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 223
A.cr AY204581           164 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 227
A.fu AY148107           165 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 228
T.sp AY204634           169 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 232
T.sp SS 2274            169 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 232
T.sp SS 2282            169 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 232
C.in SS 708             168 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 231
H. va CBS 651.66        168 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 231
H. va SS 336            168 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 231
H. va SS 76.01          168 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 231
T. ana of H.va SS 47    168 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 231
T. ana of H.va SS 45    168 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 231
C. ac CBS 100273        173 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 236
T. te CBS 697.73        169 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 232
T. in CBS 430.94        168 AA CTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 231
V. de CBS 574.95        160 AAACTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 224
D. fo CBS 221.59        159 AAACTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 223
T. gi  CBS 508.71       152 AAACTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 216
T. chaetocladium  CB    168 AAACTTTCAACAACGG ATCTCTTG GTTCTG GCAT CGATGAAGAACGCAGCGAAATGCGAT AAGTA 232
T. mesentericum AF51    243 AA CTTTCAACAACGG ATCTCTTG GCTCTC GCAT CGATGAAGAACGCAGCGAAATGCGAT AGGTA 306
T. aestivum AF516791    238 AA CTTTCAACAACGG ATCTCTTG GCTCTC GCAT CGATGAAGAACGCAGCGAAATGCGAT AGGTA 301

                                 360       370       380       390       400       410       420
                            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
H. se AY348592          223 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 290
H. im AY348584          225 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 292
H. sc AY348591          223 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 290
H.cf. me AY348588       222 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 289
H.ca AY348576           222 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 289
H. fu AY348583          222 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 289
H. cf. fr AY348582      222 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 289
H. fr AJ430396          222 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 289
H. mo AF169309          228 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCGGTGGTATTCCGCC 295
H. la AY348587          224 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 291
H. ep AY348581          225 ATGTGAATT GCAGAATTTAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 292
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Z. va AF169303          233 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 300
T. an AY204608          222 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGGG 289
T. an AY204611          222 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGGG 289
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A. fi AY148104          225 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCC GTGGTATTCCGCG 291
V. el CBS 541.92        225 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 292
A.cr AY204582           224 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 291
A.cr AY204581           228 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 295
A.fu AY148107           229 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTAT CCGCG 295
T.sp AY204634           233 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 300
T.sp SS 2274            233 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 300
T.sp SS 2282            233 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 300
C.in SS 708             232 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 299
H. va CBS 651.66        232 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 299
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T. ana of H.va SS 47    232 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 299
T. ana of H.va SS 45    232 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 299
C. ac CBS 100273        237 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCTTGGTATTCCGAG 304
T. te CBS 697.73        233 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 300
T. in CBS 430.94        232 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 299
V. de CBS 574.95        225 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCGCTGGTATTCCGGC 292
D. fo CBS 221.59        224 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCCGTGGTATTCCGCG 291
T. gi  CBS 508.71       217 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGTCCCTGTGGTATTCCGCA 285
T. chaetocladium  CB    233 ATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCGCTGGTATTCCGGC 300
T. mesentericum AF51    307 ATGTGAATT GCAGAACTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCTGGGGCATTCCTCA 374
T. aestivum AF516791    302 ATGTGAATT GCAGAACTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCTGGGGCATTCCTCA 369

                                 430       440       450       460       470       480       490
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H. se AY348592          291 GGGCATGCCTGTTCGAGCGTCA TGTACGCCAACTCCCG      CTCCG               GCG   336
H. im AY348584          293 GGGCATGCCTGTTCGAGCGTCA TATTAACCA CTCCCG      CTT                 GCG   335
H. sc AY348591          291 GGGCATGCCTGTTCGAGCGTCA TTTTGACCAACTCCTA      CCTCG               GTA   336
H.cf. me AY348588       290 GGGCATGCCTGTTCGAGCGTCA TTTATACCAACTCCT       CTGCA               AAGA  335
H.ca AY348576           290 GGGCATGCCTGTTCGAGCGTCA TTTATACCAACTCCT       CTCTA               TAGA  335
H. fu AY348583          290 GGGCATGCCTGTTCGAGCGTCA TTTATACCAACTCCT       CTTCA               TAGA  335
H. cf. fr AY348582      290 GGGCATGCCTGTTCGAGCGTCA TATATACCAACTCCTA      TTCT                ATA   334
H. fr AJ430396          290 GGGCATGCCTGTTCGAGCGTCA TTTATACCAACTCCTA      CTCTC               AGTA  336
H. mo AF169309          296 GGGCATGCCTGTTCGAGCGTCA TTTAAACCAA TCCAG      CTT                 GCT   338
H. la AY348587          292 GGGCATGCCTGTTCGAGCGTCA TTTAAACCAA TCTAG      CTT                 GCT   334
H. ep AY348581          293 GGGCATGCCTGTTCGAGCGTCA TTTAAACCAA TCCAG      CTT                 GCT   335
H. ep AY348580          293 GGGCATGCCTGTTCGAGCGTCA TTTAAACCAA TCCAG      CTT                 GCT   335
Z. va AF169303          301 GGGCATGCCTGTTCGAGCGTCA TTATGACCAATCCC G      CTC                 GCG   343
T. an AY204608          290 GGGCATGCCTGTTCGAGCGTCA TCAA AAACCCTCAAG      CCT                 AGCT  333
T. an AY204611          290 GGGCATGCCTGTTCGAGCGTCA TCAA AAACCCTCAAG      CCT                 AGCT  333
A.fi AY148105           293 GGGCATGCCTGTTCGAGCGTCA TTTC AACCAATCAAGC     CTC                 GGCT  337
A. fi AY148104          292 GGGCATGCCTGTTCGAGCGTCA TTTC AACCAATCAAGC     CTC                 GGCT  336
V. el CBS 541.92        293 GGGCATGCCTGTTCGAGCGTCA TTTC AACCCATCATGC     TTC                 GGCA  337
A.cr AY204582           292 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCC G      TTT                 ACG   334
A.cr AY204581           296 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCC G      TTT                 ACG   338
A.fu AY148107           296 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCCCG      CTC                 GCG   339
T.sp AY204634           301 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCA G      CTC                 GCT   343
T.sp SS 2274            301 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCA G      CTC                 GCT   343
T.sp SS 2282            301 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCA G      CTC                 GCT   343
C.in SS 708             300 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCA G      CTC                 GCT   342
H. va CBS 651.66        300 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCA G      CTC                 GCT   342
H. va SS 336            300 GGGCATGCCTGTTCGAGCGTCA TTATAACCAATCCA G      CTC                 GCT   342
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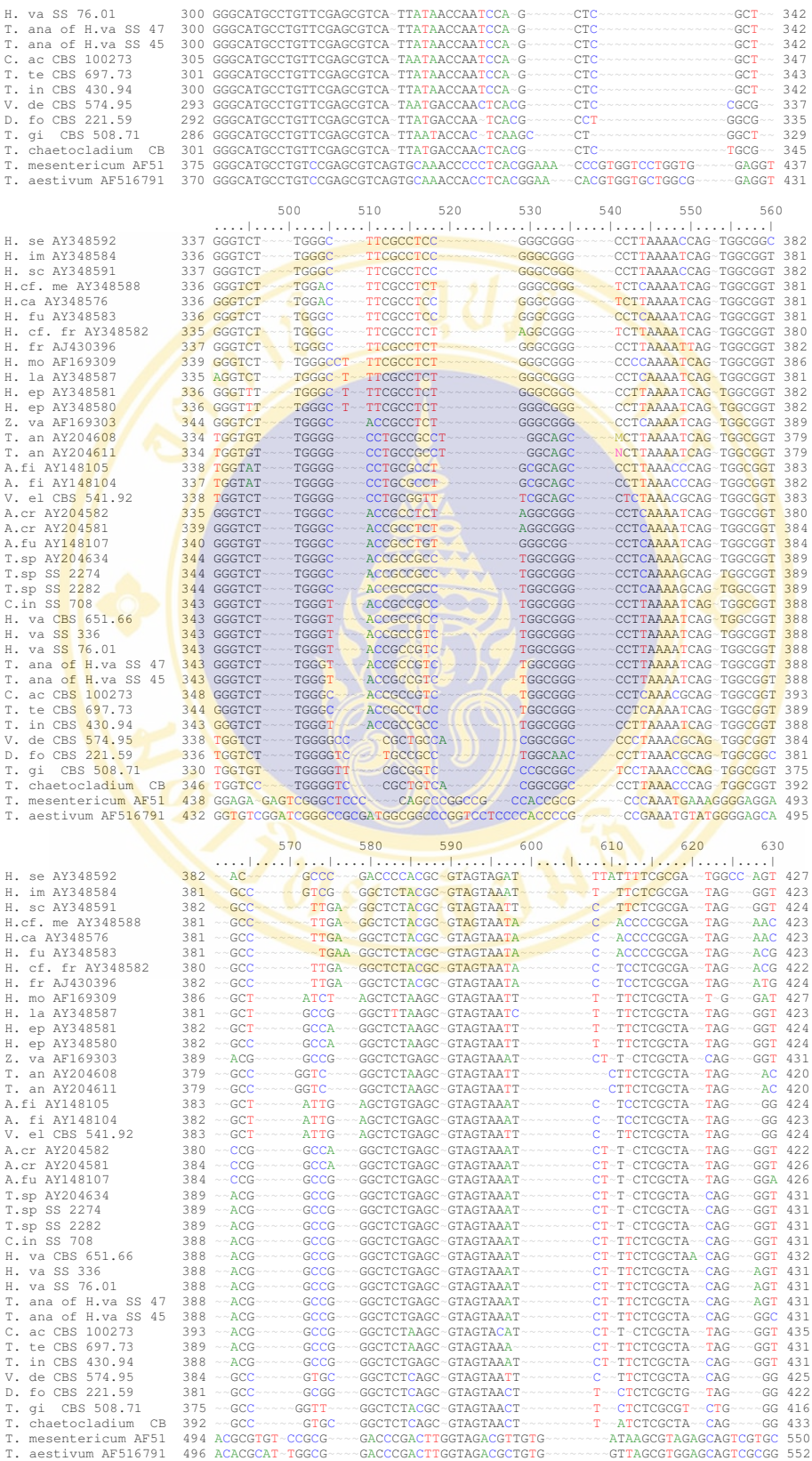

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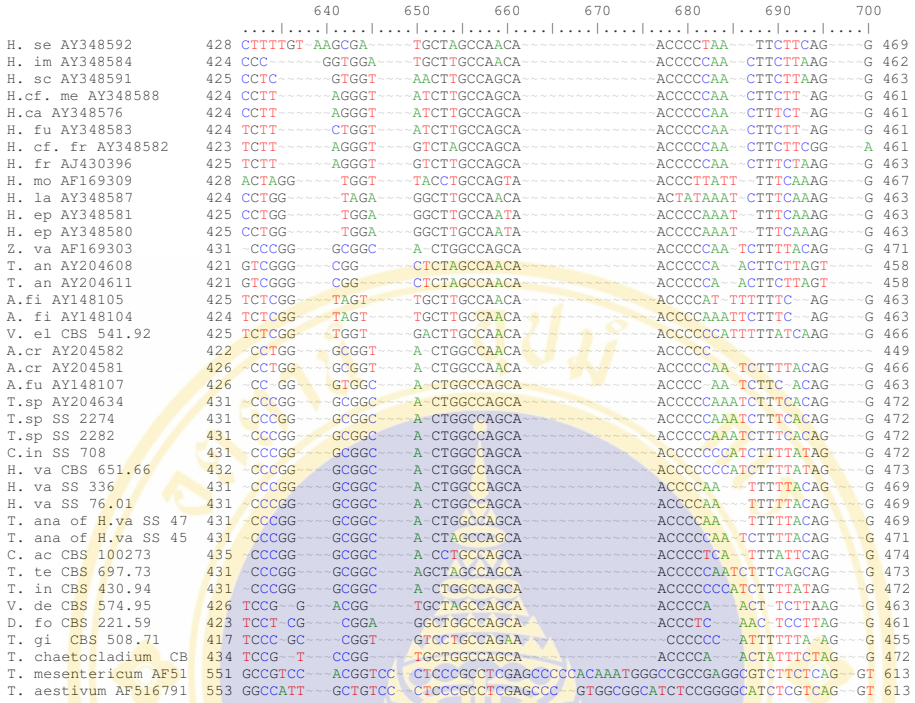

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