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Xylariaceae diversity in Thailand and Philippines, based on rDNA sequencing

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Abstract. Twenty three different Xylariaceae Tul. & C. Tul were isolated from samples collected from forest zones of Thailand and Philippines. The fungal samples were characterized based on morphological characteristics and nuclear ITS1-5.8S rDNA-ITS2 region sequences. Ten species of *Xylaria*, two species of *Hypoxylon, Biscogniauxia, Rosellinia* and one species of *Annulohypoxylon* and *Entonaema* were found. *Entonaema* the distinctive genus of Xylariaceae, isolated in the study from Thailand samples showed a close relationship with *Xylaria* in phylogenetic tree. Xylariaceous species identified at molecular level showed significant similarity of the morphological characters, such as stromal structure, ascal apex and the germ slit of ascospores. In addition, three species of *Arthrinium*, two species of *Pestalotiopsis* were also isolated and characterized in the study. A phylogenetic affinity of *Pestalotiopsis* with Xylariaceae was found.
Keywords Xylariaceae, isolation, molecular characterization, ITS region.

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Introduction

The classification of Xylariaceae Tul. & C. Tul is mainly based on macro morphological characters, such as size, color, texture, shape and color of the stroma and ascospores (Rogers 1979, Whalley & Edwards 1995). More than 85 genera and at least 1340 species belong to the family Xylariaceae and the central core of the genera are *Xylaria*, *Hypoxylon*, *Rosellinia*, *Poronia*, *Podosordaria*, *Hypocopra*, *Daldinia*, *Nummularia*, *Kretzschmaria*, *Camillea* and *Penzigia*; these are obviously related and the generic limits are arguable (Rogers 1979, Barr

1990, Eriksson & Hawksworth 1991, Laessoe 1994, Krik et al. 2008, Chen et al. 2013). Rogers (1979) reported that asci of xylariaceous fungi are usually 8 spored, unitunicate, cylindrical in outline and proximally attenuated to a short or long stipe and the apex of the ascus is characterized by an apical apparatus which usually stains blue with iodine and in many genera has a characteristic ring shape. Most Xylariaceae fungal spores are brown to almost black colored, with smooth or ornamented walls (Rogers 1975, 1979; Jong & Davis 1973, Whalley 1996).

Generally spore are present on dead wood and on plant remains in dung and soil (Rogers 1979, Whalley 1985, 1996). Accordingly, the Xylariaceae are mostly collected from dead wood of angiosperms. The distribution of Xylariaceae is mainly affected by temperature. Distributions of the Xylariaceae in tropical regions Thailand and Philippines are very diverse. However, very few publications are providing very detailed description of these fungi found in Thailand and Philippines (Vasilyeva et al. 2012, Okane et al. 2008, Osono et al. 2009, Seehanan & Petcharat 2011). Eriksson & Hawksworth (1993) reported that 75% genera of Xylariaceous belong to tropical regions. Many researchers reported the taxonomical distribution of Xylariaceous fungi from tropical regions based on the morphological characteristics (Dennis 1956, 1957, 1958, 1970, Martin 1967a,b, 1968, 1970, Taligoola & Whalley 1976, Whalley 1993). The ecology and classification of Xylariaceous fungi is difficult to understand due to several reasons. One of the main reasons is variations in stromata, so that, anamorphic characterization study of Xylariaceae is must essential to differentiate the closely related taxa (Callan & Rogers 1990, Lee et al. 2000). The developmental stages, locality and inherent variability of Xylariaceae play a main role in the variation of stromata (Lee et al. 2000). The taxonomic system of Xylariaceae is highly developed and the new genera and species are characterized based on the nature and appearance of subiculum, anamorph, stromatal characters and microscopic features of the ascus and ascospores. Previously, the identification and taxonomical distribution of Xylariaceae. Tul fungi were mainly carried out based on microscopic examination of morphological characters. However, precise identification of these fungi is necessary at molecular level and DNA sequence analyses are powerful tools for genus or species level identifications (Okane et al. 2012).

The molecular characterizations of Xylariaceae. Tul turned out to be a very important tool to determine the taxa, especially when studying Xylariaceae where telemorph and anamorph are often difficult to find in the nature and must be cultured in the laboratory.

The objective of the present research work was to characterize the Xylariaceae fungal species collected from Thailand and Philippines at a morphological and molecular level, in order to develop the taxonomy. Morphological observations were compared with the molecular identifications. The phylogenetic relationships among the isolated species of Xylariaceae. Tul were determined based on the sequence data from ITS1-5.8S-ITS2 regions. The resulting phylogenies were compared with sequences obtained from the GenBank. The results of the investigation are discussed and the widespread distribution and taxonomic classification of Xylariaceae in South East Asian Countries based on morphological and genetic characteristics is described.

Materials and methods

Sampling sites and isolation of Xylariaceae

The sample collection was made during September 2007 in Thailand and October 2007 in Philippines. Wood samples were collected from two sites in Thailand: (i) Tha Kum Forest Plantation, Amphor Muang, Trad, Thailand and (ii) Klong Trakaow Forest Plantation, Am-

phor That Ta Kiab, Cha Cherng Sao, Thailand. In Philippines, samples were collected from Freeport Services Corporation Amusement Park Subic Bay, Zambales, Philippines. All the samples were placed in sterilized containers and then taken into the laboratory.

The initial observations of surface and vertical section of stroma, ascus and ascospores were made using light microscope. Pure cultures were isolated from the ascospores by single-spore isolation technique (Choi et al. 1999). All microscopic observations were made in sterilized distilled water. Cultures were maintained in 2% MEA plates and subcultured on both MEA and PDA plates.

DNA extraction and analysis

Genomic DNA was isolated after 7 days from the actively growing fungal cultures in Potato Dextrose Broth by a modified method of Graham et al. (1994). DNA fragments were observed on a 0.8% agarose gel with a 100 bp ladder (TaKaRa, Japan). Amplicons of nuclear rDNA were obtained by PCR using primers ITS 1 (5-TCCGTAGGTGAACCTGCGC-3) and ITS 4 (5-TCCGTAGGTGAACCTGCGC-3). PCR reaction mixture consisting of 10X PCR buffer (50 mm/L KCI, 10 mm/L Tris-HCI, pH 8.3), 0.5 mm/L dNTPs, ITS forward and ITS reverse primers, each 0.5 mm/L, 2.0 mm/L $MgCI_2$, 1U AmpliTaq Gold DNA polymerase (Perkin-Elmer), 2 µL DNA sample, each mixture was made up to a final volume of 30 µL using deionized sterile distilled water. PCR amplification was carried out in the thermocycler (ESCO, Swift maxi thermal cycler) with following reaction conditions: denaturation (95ºC for 10 min.), annealing (58ºC for 30 sec.), and extension (72ºC for 1 min.), the cycle repeated 35 times and followed by final extension, 72ºC for 10 mins. After amplification, amplified fungal genomic DNA fragments were visualized on 1.5% agarose gel under UV light after EtBr staining.

PCR amplified fragments were cloned into a pGEM-T Easy Vector System (Promega, USA). Recombinant vectors were transformed into JM109 High Efficiency Competent Cells and transformants were selected based on the blue/white colony selection given in the manual (Promega, USA). Recombinant plasmids were isolated from the transformant cells using Wizard Plus Plasmid DNA purification kit (Promega, USA).

The cloned fungal gene fragments were custom sequenced using T7 promoter, with an automated DNA sequencer in MACROGEN sequencing company, South Korea and the sequences deposited in GenBank (Table 1). The sequences were analyzed using the softwares DNASIS and ClustalW (Thompson et al. 1997), the sequence alignments were manually corrected. GenBank BLASTn search was used to assign the sequence identity (Altschul et al. 1997). GenBank sequence data (Table 2) were used for the Neighbor-Joining (NJ) analysis of the current experimental data, analysis was conducted using ClustalW. The homologous regions of all species were aligned using the ClustalW multiple alignment program (Thompson et al. 1997). A phylogenetic tree was obtained based on the results of ClustalW and branch-supporting values of the parsimony tree differs from the tree constructed using the Molecular Evolutionary Genetic Analysis 2.1 Software (MEGA, Version 4), Neighbor-joining method (Saitou & Nei 1987). However, the Neighbor-joining tree was topologically identical to the parsimony tree. The fungal sequences obtained in the study were submitted to GenBank (Table 1).

Results

Xylariaceae fungal species were successfully isolated from the samples collected from Thailand and Philippines using a single-spore isolation technique. Pure cultures of isolated Xylariaceae Tul. & C. Tul fungal species were maintained on both PDA and

Table 1 The complete details of isolated endophytic fungal species and closest matched fungal species in GenBank databases with accession numbers and sequence similarity percentage

Name of isolated fungi	Sample reference no.	GenBank accession no.	Similar fungal species in GenBank Database with accession no.	Similarity (%)
Hypoxylon sp.	PP071013	AB462753	*Hypoxylon* sp. (DQ322109)	99.315
Xylaria venosula	PP071022	AB462754	*Xylaria* sp. (EU010000)	99.474
Arthrinium sp.	PP071021	AB462755	*Arthrinium* sp. (AY513945)	98.165
Biscogniauxia sp.	PP071018	AB462756	*Biscogniauxia* sp. (AB449095)	99.319
Xylaria venosula	PP071023	AB462757	*Xylaria venosula* isolate 9 (EF026149)	99.490
Arthrinium sp.	PP071024	AB462758	*Arthrinium* sp. Po8 (AY513945)	97.431
Annulohypoxylon sp.	PP071026	AB462759	*Annulohypoxylon moriforme* (EF026137)	99.187
Arthrinium sp.	PP071099	AB471012	*Arthrinium* sp. (AB462758)	97.863
Xylaria sp.	TI070901	AB449101	Fungal endophyte sp. (EU686052)	99.000
Xylaria sp.	TI070903	AB449096	*Xylaria bambusicola* (EF026123)	98.000
Xylaria sp.	TI070904	AB449092	Xylariaceae sp. (AB440092)	98.000
Rosellinia sp.	TI070906	AB449099	Xylariaceae sp. BCC 18796 (AB440092)	98.000
Rosellinia sp.	TI070907	AB449093	Fungal endophyte isolate 1595 (EU686908)	98.000
Xylaria sp.	TI070908	AB449100	*Entonaema cinnabarinum* (AM292043)	98.000
Xylaria sp.	TI070911	AB449094	*Xylaria* sp. PB30 (AB285482)	98.000
Biscogniauxia sp.	TI070912	AB449095	*Xylaria bambusicola* (EF026123)	98.000
Hypoxylon sp.	TI070913	AB449097	Fungal endophyte (EU686817)	98.000
Xylaria sp.	TI070914	AB449098	Fungal sp. ARIZ L525CLA (FJ612855)	99.000
Xylaria sp.	TI070916	AB495008	*Xylaria* sp. (AB449100)	99.828
Xylaria sp.	TI070923	AB495009	*Xylaria bambusicola* isolate (EF026123)	99.484
Entonaema sp.	TI070924	AB495010	*Entonaema cinnabarinum* (AM292043)	98.915
Pestalotiopsis sp.	GU071003	AB472079	*Pestalotiopsis cocculi* (EF055194)	100.000
Pestalotiopsis sp.	GU071004	AB472080	*Pestalotiopsis cocculi* (EF055194)	100.000

MEA plates. Common morphological features of Xylariaceous fungi were classified according to the following criteria: stromata variable, more or less upright, range from applanate to hemispherical and brightly pigmented or black surface. Exterior was woolly to woody, carbonaceous, with or without hair. Interior was white to dark, occasionally gelatinous. Ascomata were ostiolate, rarely cleistocarpic and with simple wall layer. Asci were unitunicate, persistent, cylindrical–clavate, stimpitate, apical apparatus well developed, rarely lacking, refractive, often large, commonly flared apically, mainly amyloid, with a narrow lumen. Ascospores were usually inequilateral, typically brown to dark brown, mainly one-celled with small hyaline, usually basal cell which is rarely persistent, germ slit usually present, varying in shape, length and position, spore wall-smooth or ornamented, without appendages, occasionally forming secondary appendages at the pole. Winter (1887) circumscribed the Xylariaceae family to include Pyrenomycetes based on the appearance of stroma, ascus and ascospores, Pyrenomycetes with predominately a dark stroma, dark-unicelled spores containing 5 genera: Nummularia, Hypoxylon Bull., Ustulina, Poronia Willd. and Xylaria Hill ex Schrank, and many more genera have been added to the family (Dennis 1961, Martin 1967a,b, Rogers 1979, Barr 1990, Eriksson & Hawksworth 1993).

Based on the molecular data, the analysis of the ITS sequences of 23 isolates showed that they belong to 8 genera (*Annulohypoxylon*, *Arthrinium*, *Biscogniauxia*, *Entonaema*, *Hypoxy-*

Table 2 List of endophytic fungal samples used in this study

Fungal samples	GenBank accession numbers
Hypoxylon sp.	AB462753
Xylaria venosula	AB462754
Arthrinium sp.	AB462755
Biscogniauxia sp.	AB462756
Xylaria venosula	AB462757
Arthrinium sp.	AB462758
Annulohypoxylon sp.	AB462759
Arthrinium sp.	AB471012
Xylaria sp.	AB449101
Xylaria sp.	AB449096
Xylaria sp.	AB449092
Rosellinia sp.	AB449099
Rosellinia sp.	AB449093
Xylaria sp.	AB449100
Xylaria sp.	AB449094
Biscogniauxia sp.	AB449095
Hypoxylon sp.	AB449097
Xylaria sp.	AB449098
Xylaria sp.	AB495008
Xylaria sp.	AB495009
Entonaema sp.	AB495010
Pestalotiopsis sp.	AB472079
Pestalotiopsis sp.	AB472080
Xylaria bambusicola	EF026123
Xylaria apiculata	AF163027
Nemania primolutea	EF026121
Xylaria sp.	AF153724
Nemania illita	EF026122
Xylariaceae sp.	AY315402
Rosellinia sp.	DQ322077
Xylaria sp.	AB285482
Xylaria multiplex	DQ322155
Xylaria sp.	AY315400
Xylaria sp.	AB255300
Hypoxylon monticulosum	DQ223749
Entonaema cinnabarinum	AM292043
Daldinia pyreaica	AM749927
Annulohypoxylon stygium	DQ223761
Hypoxylon stygium	AJ390409

lon, *Pestalotiopsis*, *Rosellinia* and *Xylaria*). The ITS sequence length of isolates ranged from 511 to 615 bp. The sequences of ITS region of isolates found to be variable between the genera and ITS region sequences of species of same genus showed close similarity. Phylogenetic trees were obtained using maximum parsimony and neighbor joining. The topologies were identical, but the bootstrap support of the clades differed (Figure 1). In this study, among the isolates, *Xylaria* species were representing >50%, *Biscogniauxia*, *Hypoxylon* and *Rosellinia* species were sharing 10% each and then other species were represented by *Annulohypoxylon* and *Entonaema*. The genus *Arthrinium* is now placed under Apiosporaceae family was representing 13% (Lumbsch & Huhndorf 2010) and the isolates belong to *Pestalotiopsis* genus is actually placed under Amphisphaeriaceae family (Jeewon et al. 2003, Jeewon et al. 2004). The length of *Pestalotiopsis* strains was 606 bp. The nucleotide sequences of two species of *Pestalotiopsis* were very closely related and they clearly belong to Amphisphaeriaceae within Xylariales. Both *Pestalotiopsis* species were sharing 606 nucleotides in length. Fungal species with sequences highly similar to our isolates were obtained using GenBank BLAST search (Table 1).

Discussion

The studies of Van der Gucht (1994 a,b,c) demonstrated that the Xylariaceae Tul. & C. Tul are well represented in tropical rain forests in South Pacific Islands. During Van der Gucht collection from Papua New Guinea, 9 species were referred to *Biscogniauxia*, 21 to *Hypoxylon* and 38 to *Xylaria*. Even though the Xylariaceae Tul. & C. Tul have been well documented from the tropics based on morphological characters (Dennis 1956, 1957, 1958, 1970, Martin 1967a,b, 1968, 1970), few studies have been carried out in South East Asia (Rogers et al. 1987, Whalley et al. 1994). In studies from North Sulawesi in Indonesia, 27 Xylaria species and 18 Hypoxylon species together with representatives of 5 other genera were recorded by Rogers et al. (1987), whereas from Ma-

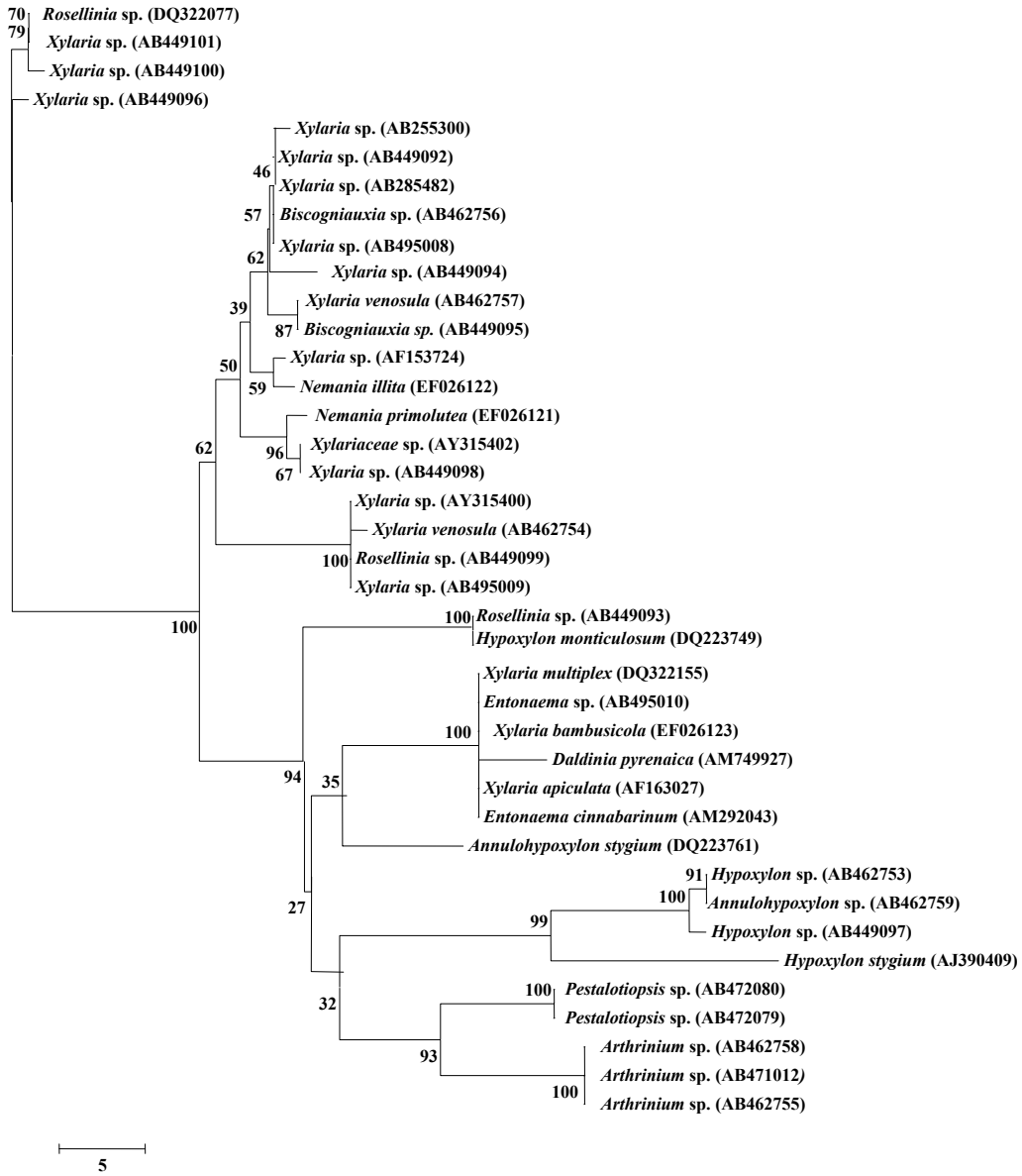

Figure 1 Neighbor-Joining (NJ) method phylogenetic tree of all Xylariaceae fungal samples used in this study (including samples obtained from GenBank)

laysia, 16 Hypoxylon species were reported by Whalley et al. (1994). However, most of the identification studies carried out were based on the morphological characters. Only a very few reports were published regarding the characterization and distribution of Xylariaceae from South East Asia, at a molecular level (Bahl et al. 2005, Johannesson et al. 2000, Mazzaglia et al. 2001, Okane et al. 2008, Osono et al. 2009, Pelaez et al. 2008, Seehanan & Petcharat 2011, Suwannasai et al. 2005, Vasilyeva et al. 2012,), but these reported a limited number of taxa.

Based on our results, the characterization and distribution of Xylariaceae at molecular level are very briefly explored and reviewed below.

The sequences of Xylariales samples used in the study (including those obtained from GenBank) formed nine clades. The *Xylaria* species AB449096, AB449100 and AB449101 belong to same group and were quite distant from *Daldinia pyrenaica* M. Stadler and Wollow. AM749927. *Arthrinium* species AB462755, AB462758, AB471012 and *Annulohypoxylon* sp. AB462759 were distant from *Annulohypoxylon stygium* Y.M. Ju, J.D. Rogers & H.M. Hsieh (DQ223761) and *Hypoxylon stygium* Ju & Rogers (AJ390409). From the phylogenetic tree (Figure 1), among 10 species of *Xylaria*, sequences of 7 species of *Xylaria* (except *Xylaria* species AB495009, AB462754 and AB449098) appeared to be very close and clustered together. Two *Xylaria venosula* Speg. (AB462754 and AB462757) isolated in the current study showed distant genetic relationship between each other's, whereas *Biscogniauxia* sp., shares close relation with *Xylaria venosula* Speg. (AB462757). Some species of *Daldinia*, especially *Daldinia vernicosa* Ces. & De Not. and *Daldinia concentrica* Ces. & De Not. can form a monophyletic group and can make another monophyletic group with *Daldinia* sp., this somewhat supported in turn with *Hypoxylon* sp. (Lee et al. 2000). Spatafora & Blackwell (1993) reported that *Xylaria* can form sister taxon with a group consisting of *Daldinia* and *Hypoxylon*. This report suggested that isolated *Arthrinium* (AB471012, AB462758, AB462755) species had highly homologous sequences and clustered as monophyletic group. The two *Biscogniauxia* species isolated in the study showed a distinct relationship between each other (Figure 1). *Biscogniauxia* isolated from Thailand showed a close relationship with *Xylaria* isolated from Thailand, whereas *Biscogniauxia* isolated from Philippines closely matched with *Xylaria venosula* Speg. isolated from Philippines (Figure 1). Similar type of result was obtained for *Hypoxylon* species isolated from Thailand and Philippines, the genetic relationship of both species was high when compared with *Xylaria*. According to the phylogenetic tree (Figure 1), the genetic affinity of *Rosellinia* species was relatively high with *Xylaria* species than *Nemania*.

Our results strongly supported the previous report of Pelaez et al. (2008) that *Xylaria* species were close together in a single clade in NJ analysis based on ribosomal DNA sequences. Lee et al. (2000) and Davis et al. (2003) also reported the phylogenetic analysis of *Xylaria*, the largest and oldest genera of the Xylariaceae Tul. & C. Tul family. However, all reports suggested that further studies are essential for the complete phylogenetic examination of *Xylaria* (Davis et al. 2003, Lee et al. 2000, Rogers et al. 2005). Because, the Amphisphaeriaceae group is closely related with clades of Xylarioidae and Hypoxyloideae (Tang et al. 2007a). Ribosomal DNA sequences of *Pestalotiopsis*, *Bartalinia* and *Seiridium* showed the most close affinity with *Xylaria*, *Annulohypoxylon*, *Hypoxylon* and *Daldinia* (Tang et al. 2007a). Our results also supported the previous reports (Davis et al. 2003, Pelaez et al. 2008, Tang et al. 2007a) regarding the phylogenetic relationship of Amphisphaeriaceae with the Xylariaceae Tul. & C. Tul clade. Rogers (1985) also suggested dividing *Xylaria* into at least four major sections. Roger reported on the identification and classification of *Xylaria* based on morphological characteristics. He classified the *Xylaria* mainly based on the polymorphic characters, such as appearance of stroma, ascospores structure and apical apparatus structure of asci.

Arthrinium species were identified in the study is now coming under Apiosporacease family (Lumbsch & Huhndorf 2010). The documentation of *Arthrinium* is not well recorded yet. *Arthrinium* is the most dominant fungi in bamboo. Only few reports exist regarding the distribution of *Arthrinium* on angiosperms, researchers mainly isolated and characterized

the *Arthrinium* from bamboo (Morakotkarn et al. 2006, Samuels et al. 1981). Samuels et al. (1981) characterized *Arthrinium* based on non-septate conidia, brown or dark brown in color. Morakotkarn et al. (2006) isolated 8 strains, phylogenetically related with *Arthrinium* sp. Strains isolated by Morakotkarn et al. (2006) shares 94-97% similarity with *Arthrinium* sp. AF393679 and *Arthrinium* sp. AJ279456. In accordance with our experimental results, 3 *Arthrinium* (AB471012, AB462758, AB462755) species have genetic affinity with *Annulohypoxylon* (AB462759), which might form a new group. Thus, our results supported the findings of previous molecular report (Morakotkarn et al. 2006) regarding the distribution of *Arthrinium*.

The unique genus *Rosellinia* is traditionally characterized by subiculum or the initial stage of stromatal development (Petrini 1992). *Rosellinia* can cause variety of plant diseases (Petrini 1992), which validates the importance of *Rosellinia* among other genera of Xylariaceae Tul. & C. Tul family. Bahl et al. (2005) characterized the novel species of *Rosellinia capetribulensis* sp. nov. and compared with the other Xylariaceae samples. According to ITS data of Bahl et al. (2005), *Rosellinia* was sharing a clade with *Astrocystis*, *Xylaria*, *Hypoxylon*, *Daldinia*, *Nemania* and *Entoleuca*. Miller (1928) also considered the genus *Rosellinia* close to genus *Hypoxylon*. Based on our molecular analysis results (Figure 1), *Rosellinia* strains share close affinity with *Hypoxylon monticulosum* Mont. DQ223749 and *Xylaria* sp. Pelaez et al. (2008) also reported that *Rosellinia* clades were occasionally intermingled with *Xylaria* sp. and also with *R. subiculata* (Schw.), *E. mammata* (Wahlenb.) J.D. rogers & Y.M. Ju. Thus, our results are exactly coincident with the previous reports published by Bahl et al. (2005), Miller (1928) & Pelaez et al. (2008). In addition our result showed that *Rosellinia* sp. AB449093 was phylogenetically distant from *Rosellinia* AB449099. *Rosellinia* AB449093 shows high affinity with *Hypoxylon*, whereas *Rosellinia* AB449099 shows close phylogenetic relationship with *Xylaria* sp. These results supported the diversity between taxons of *Rosellinia* and agreed with results of Pelaez et al. (2008). Pelaez et al. (2008) reported that *R. bambusae* Henn. was phylogenetically more related with *Xylaria* species and distant from other *Rosellinia* taxa used in the same study.

The genera *Annulohypoxylon*, *Biscogniauxia*, *Camillea*, *Creosphaeria* and *Whalleya* are closely related with *Hypoxylon*. *Biscogniauxia* and *Annulohypoxylon* were identified in our study. *Biscogniauxia* genus was individually separated from *Hypoxylon* genus by Pouzar (1986). *Annulohypoxylon* was formed from two genera *Hypoxylon* and *Annulata*. *Hypoxylon* s. str., was redefined by Ju and Rogers (1996) to establish a new highly defined genus *Annulohypoxylon*. *Hypoxylon*-related genera, *Annulohypoxylon*, *Hypoxylon* and *Daldinia*, could belong to the same clade. Many researchers reported the close phylogenetic affinity between the *Annulohypoxylon* and *Daldinia* (Pelaez et al. 2008, Tang et al. 2007a). *Annulohypoxylon* (AB462759) identified in this study shares >99% similarity (Table 1) with *Annulohypoxylon moriforme* (Henn.) Y.M. Ju, J.D. Rogers & H.M. Hsieh (EF026137). Based on the report of Pelaez et al. (2008), *Annulohypoxylon* was not grouped under a single monophyletic group. *Annulohypoxylon* merged either with *Hypoxylon* or *Daldinia* (Pelaez et al. 2008). Based on our NJ analysis of ITS sequences results, *Annulohypoxylon* (AB462759) was somewhat close to other *Hypoxylon* (AB449097 and AB462753) isolates. While one *Annulohypoxylon* isolate was not very distant from *Daldinia pyrenaica* M. Stadler & Wollw. This supports the previous reports that *Annulohypoxylon* closely shares molecular characteristics with *Hypoxylon* and *Daldinia* (Hsieh et al. 2005, Pelaez et al. 2008). According to current classification results, *Biscogniauxia* has a close affinity with *Xylaria* and *Nemania* (Tang et al. 2007a). The

same result was observed during phylogenetic analysis of our sequences, with *Biscogniauxia* isolates closely realted with *Xylaria* isolates (Figure 1). However, based on morphological data of the anamorphic stage, *Biscogniauxia* is more phylogenetically related to genera within *Hypoxylon* (Tang et al. 2007b).

Taxonomic classification of *Entonaema* was revised by Rogers (1981). The genus *Entonaema* consists of hollow, folded, lobed, irregular stromata filled with liquid. The occurrence and distribution of *Entonaema* is not well studied yet. Rogers (1981) reported that *Entonaema* are widely distributed in North, Central and South America, Africa, China and Thailand. The presence of *Entonaema* in Thailand was already confirmed by Sihanonth et al. (1998). Based on the teleomorphic and anamorphic characteristics *Entonaema* has a close relationship with *Daldinia* and with *Hypoxylon*. The strain *Entonaema* characterized in the present study also shares a very close affinity with *Daldinia pyrenaica* M. Stadler & Wollw (Figure 1). Stadler et al. (2008) reported the comparison and relationship of ribosomal DNA sequences of *Entonaema pallidum* G.W. Martin and *Xylaria* species. Stadler et al. (2008) described the sequence similarities between *E. pallidum* G.W. Martin and *Xylaria* species. Surprisingly they have not found any closest matches of *Entonaema*, *Daldinia* or *Hypoxylon* to their isolate *E. pallidum*. However, our results show the close affinity of *Entonaema cinnabarinum* (Cooke & Massee) LIoyd *Daldinia pyrenaica* M. Stadler & Wollw and *Xylaria* to our isolate *Entonaema* sp., AB495010. *Entonaema* species can produce unique or chemotaxonomically significant secondary metabolites, which are recently being utilized for the characterization of *Entonaema* (Stadler et al. 2008).

Morphologically undistinguished fungal strains *Pestalotiopsis* are grouped under Amphisphaeriaceae family within Xylariales (Jeewon et al. 2003, Jeewon et al. 2004). Jeewon et al. (2003) reported that *Pestalotiopsis* strains are producing a monophyletic clade and confirm the teleomorphic-anamorphic connections of some. Jeewon et al. (2004) also reported the phylogenetic relationship of *Pestalotiopsis* isolated from different hosts such as *Scaevola hainanensis*, *Leucospermum*, *Protea mellifera* and some other resources and he strongly suggested that assignment of new species in *Pestalotiopsis* must consider the morphological characters. Species relationship within this genus is quite complicated to determine because of the inadequate morphological characters available to differentiate the species. Many researchers have described a new species in *Pestalotiopsis* genus based on the host type interactions, *P. juncestris* Kohlm. & Volkm.-Kohlm, *P. embeliae* M.S. Patil & Theite, *P. chethallensis* Sohi & O. Prakash, *P. arborei* N.I. Singh and *P. acaciae* (Thum.) K. Yokoy. & S. Kaneko (Kohlmeyer & Kohlmeyer 2001, Patil & Thite 1977, Sohi & Prakash 1979, Singh 1981, Yuan 1996) were named based on host association. Some researchers already demonstrated the phylogenetic relationship of *Pestalotiopsis* within Xylariales (Tang et al. 2007a,b).

Conclusions

This investigation has contributed to knowledge about molecular characterization, classification, diversity, taxonomic and phylogenetic relationships among some genera of the Xylariaceae. Tul family distributed in Thailand and Philippines. Our present research work intensely discussed the relationship and affinities between the major genera of Xylariaceae family. This work has also been given notes on phylogenetic relationship between the Amphisphaeriaceae and Xylariaceae. During this study, presence of *Entonaema* in Thailand has also been reconfirmed and the molecular characteristics are discussed. Further sample collections and characterizations are essential to establish the complete distribution of Xy-

lariaceae. over South East Asia. The present study will be helpful to develop the taxonomy of Xylariaceae. at a molecular level, this can be useful for further development such as identification, characterization and comparison of unidentified Xylariaceae.

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