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ITS and Alpha-actin Sequence Analysis of some Complex Species in *Annulohypoxylon* from Thailand

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Abstract

Forty fungal collections of *Annulohypoxylon* species, being closely related in morphological characters, were identified by using nucleotide sequences of two loci. The internal transcribed spacer (ITS) regions and alpha-actin gene were amplified, sequenced and analysed for species identification and phylogenetic construction. Blast results of two loci exhibited low % similarity (less than 93%) of six different samples studied against GenBank database. The phylogenetic trees also revealed clear separation of them from other known species. These results showed the variation in terms of genotypes within the genus complex, which contains *Annulohypoxylon bovei* var. *microspora*, *A. moriforme*, *A. nitens* and *A. stygium* var. *annulatum*. In addition, their morphological and anamorph characters were carefully observed in these complex species for species delimitation. Moreover, multiple gene analysis including chemotaxonomy will be then applied in a further study.

Keyword: *Annulohypoxylon*, Xylariaceae, ITS, Alpha-actin gene, Thailand

Introduction

Annulohypoxylon Y.-M. Ju, J.D. Rogers & H.-M. Hsieh is a fungus genus in family Xylariaceae and is widely distributed in tropical and subtropical regions. This genus contains more than 40 known species worldwide (Ju & Rogers 1996, Hsieh et al. 2005, Stadler & Fournier 2006). It was previously described as a section in genus *Hypoxylon* (Ju & Rogers 1996). Recently, *Hypoxylon* sect. *Annulata* has been elevated to a new genus based on the combination of morphological and molecular data of alpha-actin and beta-tubulin genes (Hsieh et al. 2005). *Annulohypoxylon* is then restricted in having a carbonaceous stromatal layer discretely surrounding each perithecium, ostioles always higher than the surrounding stromatal surface and usually encircled with a distinct or

sometimes a highly reduced annulate disc, and ascospore perispores, when dehiscing, with a thickened area visible at the position of ca. 1/3 ascospore length.

Therefore, *Hypoxylon* s. str. is now restricted to only sect. *Hypoxylon*, which contains four main characters of unipartite stromata, solid and homogeneous stromatal tissue below the perithecial layer, stromata not upright, and *Nodulisporium*-like anamorphs (Hsieh et al. 2005).

In Thailand, only a few studies of *Annulohypoxylon* and *Hypoxylon* species have been reported (Schumacher 1982, Whalley et al. 1995, Thienhirun 1997, Thienhirun & Whalley 2004, Suwannasai et al. 2005, Phosri et al. 2008, Vasilyeva et al. 2012), but recently many of them were described as new species such as *Annulohypoxylon bahnpadengense*, *A. maeteangense*, *Hypoxylon juventuterubrum*, *H. lienhwaheense*, *H. pseudoretzela*, *H. rubroargillaceum*, and *H. sepipigmentum* (Fournier et al. 2010, Vasilyeva et al. 2012). Due to these studies, many samples are still noted as unidentified and/or are complex species.

Nowadays, molecular methods such as nucleotide sequencing provide a potential method for fungal identification and there have been extensive studies. Ribosomal nuclear sequences, the internal transcribed spacer (ITS) regions, and protein coding genes such as alpha-actin and beta-tubulin have been proven as good alternative choices for fungal problematic identification (Hsieh et al. 2005, Suwannasai et al. 2005, Stadler & Fournier 2006, Tang et al. 2007). Therefore, the purpose of this study was to examine some selected problem species of *Annulohypoxylon* from Thailand by combining the morphological and molecular data.

Experimental and Methods

Fungal collection and Morphological Identification

The teleomorph stage of *Annulohypoxylon* species were collected from forests in Phitsanulok and Chaiyaphum provinces, Thailand. They were then isolated as pure cultures on potato dextrose agar (PDA) and transferred to oat meal agar (OA) for anamorph study. The cultures were incubated at 30°C until anamorphs developed. All fungal stromata were classified and identified to species level based on their morphological and anamorph characters (Miller 1961, Rayner 1970, Ju & Rogers 1996, Thienhirun 1997, Hsieh et al. 2005, Suwannasai et al. 2005, Vasilyeva et al. 2012). Then, the stromata were dried and preserved in the fungal herbarium at SWU. Pure cultures of fungal mycelium were recovered and kept in 1.5 mL microcentrifuge tubes at -20°C until DNA extraction.

Molecular identification and phylogenetic analysis

The frozen fungal mycelium was ground and extracted for DNA by using Plant genomic DNA extraction kit (FavorPrep™, Favorgen) according to the manufacturer's recommendation. ITS regions and the alpha-actin gene were amplified by using primer set of ITS5/ITS4 (White et al. 1990) and ACT512F/ACT783R (Carbone & Kohn 1999), respectively. The reaction mixture for PCR (total volume of 25 µl) contained 10-100 nM of template DNA, 0.5 mM of each primer, 10 mM of dNTP, 1.25 Unit of KAPA Taq DNA

polymerase and 1x PCR buffer containing 1.5 mM MgCl₂. The samples were run in DNA Thermocycler (Eppendorf, Germany) according to the following cycling parameters: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 50-52°C (48-50°C for alpha-actin) and extension for 1.30 min at 72°C. The cycling was ended by an extension phase for 10 min at 72°C. All PCR products were checked using gel electrophoresis with 1% agarose gel in 0.5x TBE buffer, followed by staining in an ethidium bromide, and observed under UV light. A single band of PCR product was purified using GEL/PCR purification mini kit (FavorPrep™, Favorgen) and then sequenced. In case of unreadable sequences, the purified PCR fragments were cloned using TA cloning kit (RBC Bioscience Corp., Taiwan) before sequenced.

Nucleotide sequences obtained were analysed by BioEdit programme (Hall 1999), and compared to nucleotide sequences in GenBank database by running MEGABLAST tool. All complete sequences examined were aligned and the phylogenetic trees constructed using neighbor-joining (NJ) and maximum parsimony (MP) methods with PHYLIP and PAUP*4.0 Beta software (Felsenstein 1993, Swofford 2002).

Results and Discussion

Forty samples of *Annulohypoxylon* species collected from Chiyaphum and Phitsanulok provinces, having greenish olivaceous in 10% KOH extractable pigments, were basically identified based on their morphological and anamorph characters. They were at least six species *A. atroseum*, *A. bovei* var. *microspora*, *A. moriforme*, *A. moriforme* var. *microdiscus*, *A. nitens* and *A. stygium*. Due to the high variation of ascospore sizes and minor characters, some of them were noted as unidentified species i.e. *Annulohypoxylon* sp. H130, *Annulohypoxylon* sp. H255, *Annulohypoxylon* sp. PK10006, *Annulohypoxylon* sp. PK10007, *Annulohypoxylon* sp. PK07001 and *Annulohypoxylon* sp. PK07019 (Table 1). Then, two loci of ITS regions and the alpha-actin gene were sequenced and analysed. The phylogenetic trees were constructed using two methods of NJ and MP with all identified *Annulohypoxylon* sequences obtained from GenBank database. Most of the major clades in phylogenetic trees from both methods were similar. Then, the phylogenetic trees of two loci constructed by NJ method were selected (Figs 1 and 2).

Table 1 Blast results of *Annulohypoxylon* problematic species observed in this study

Taxon	Blast result	Accession No.	Similarity (%)
Alpha-actin gene analysis			
Annulohypoxylon sp. H130	<i>A. thouarsianum</i> var. <i>macrosporum</i> voucher YMJ 48	AY9 51780	92%
Annulohypoxylon sp. H255	<i>A. stygium</i> var. <i>annulatum</i> voucher YMJ 91042205	AY9 51778	88%
Annulohypoxylon sp. PK10006 ^a	<i>A. bovei</i> var. <i>microspora</i> voucher YMJ 90081914	AY9 51765	90%
Annulohypoxylon sp. PK10007	<i>A. stygium</i> var. <i>annulatum</i> voucher YMJ 91042205	AY9 51778	88%
Annulohypoxylon sp. PK07019	<i>A. bovei</i> var. <i>microspora</i> voucher YMJ 90081914	AY9 51765	89%
ITS sequence analysis			
Annulohypoxylon sp. H130	<i>A. multifforme</i> strain F-160	AY9 09003	92%
Annulohypoxylon sp. H255	<i>A. multifforme</i> isolate olrim319	AY3 54245	88%
Annulohypoxylon sp. PK10007	<i>A. stygium</i> isolate XH16	AB4 40107	92%
Annulohypoxylon sp. PK07001 ^b	<i>A. stygium</i> isolate 105F	FJ00 8986	93%
Annulohypoxylon sp. PK07019	<i>A. bovei</i> var. <i>microspora</i> isolate 90081914	EF02 6141	89%

^a Sample has only alpha-actin sequences. ^b Sample has only ITS sequences.

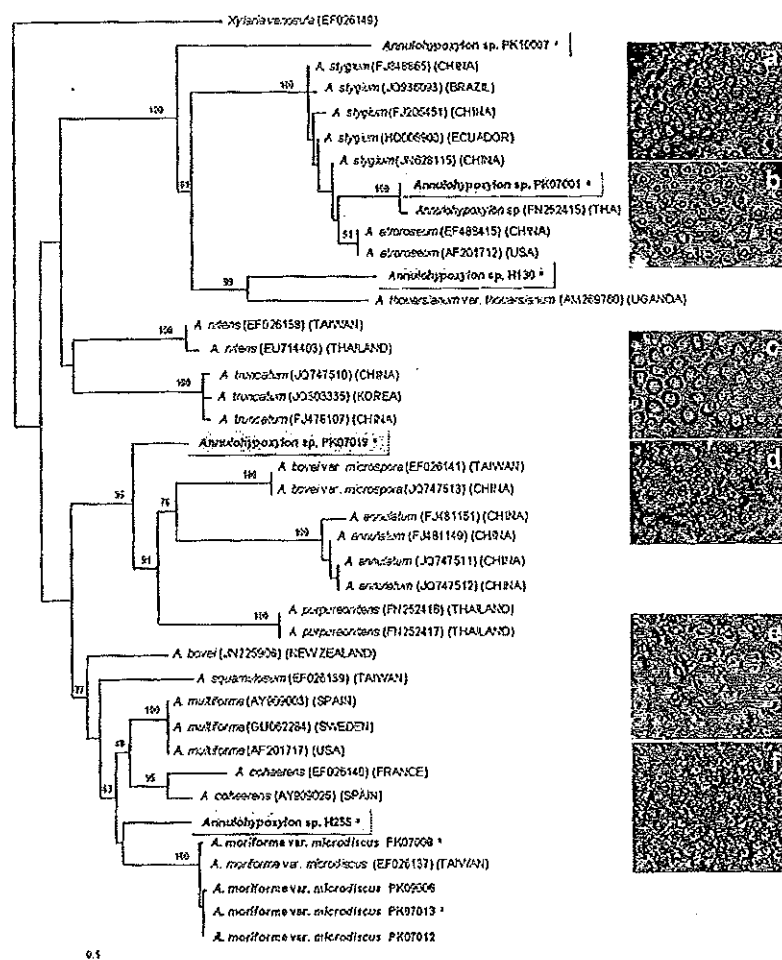


Fig.1 Phylogenetic tree of *Annulohypoxyton* species based on ITS sequences constructed by using neighbour-joining method. The numbers above branches indicate the bootstrap value from 1000 replications. *Xylaria venosula* (EF026149) is the outgroup. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. a) *Annulohypoxyton* sp. PK10007, b) *Annulohypoxyton* sp. PK07001, c) *Annulohypoxyton* sp. H130, d) *Annulohypoxyton* sp. PK07019, e) *Annulohypoxyton* sp. H255 and f) *A. moriforme* var. *microdiscus* PK07008 (* means sample has alpha-actin sequences.)

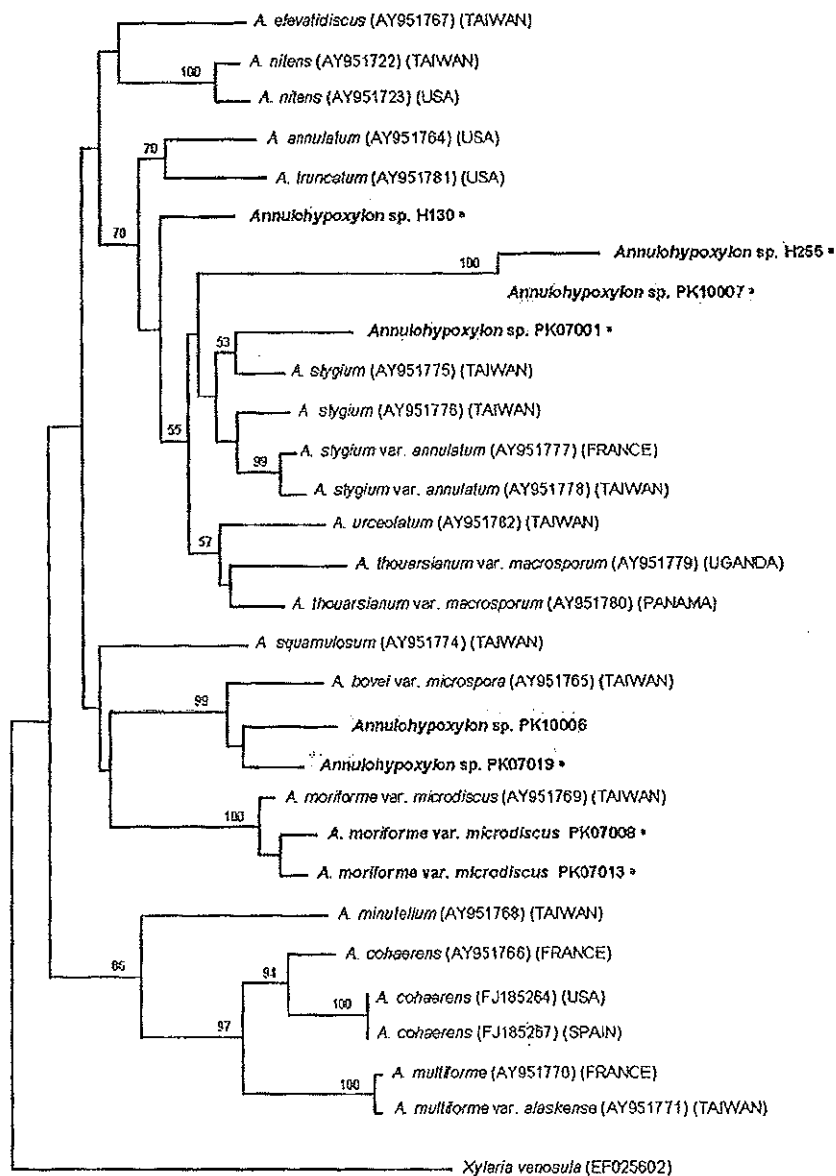


Fig.2 Phylogenetic tree of *Annulohypoxyton* species based on alpha-actin sequences constructed by using neighbour-joining method. The numbers above branches indicate the bootstrap value from 1000 replications. *Xylaria venosula* (EF026149) is the outgroup. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. (*) means sample has ITS sequences.)

The phylogenetic tree based on ITS sequences revealed a clear separation of five samples (PK10007, PK07001, PK07019, H130 and H255) from other known species with high bootstrap support (Fig 1). This result was similar to alpha-actin analysis except for some samples of PK07001 and PK07019 showed lower bootstrap values (Fig 2). This might be because of high variable regions within ITS sequences. Even though, alpha-actin gene analysis is commonly used to classify at genus level, most reference strains of *Annulohypoxylon* species available in GenBank belong to alpha-actin and beta-tubulin genes (Hsieh et al. 2005). In addition, blast results of five problematic *Annulohypoxylon* samples exhibited low % similarity to nucleotide sequences in GenBank database (Table 1).

Therefore, data analyses of both morphological characters and molecular data were then combined. Among these samples there are two types of ostiolar discs. Four samples of H130, H255, PK10007 and PK07001 have *truncatum*-type, while two samples of PK10006 and PK07019 have *bovei*-type.

The morphological features of H255 and PK10007 samples are similar to *A. stygium* (Lév.) Sacc. var. *annulatum* (Rehm) Y.-M. Ju & J.D. Rogers (Ju & Rogers 1996) by having small ascospores ($5.5-6.8 \times 2.4-3.7 \mu\text{M}$ and $5.6-6.7 \times 2.4-3.3 \mu\text{M}$, respectively) and larger ostiolar discs (0.3-0.4 mm in diameter) with of the *truncatum*-type. Their nucleotide sequences are not only different from each other but are also different from *A. stygium* var. *annulatum* (AY951777 and AY951778) in GenBank database (Table 1, Fig 2). *Annulohypoxylon* sp. H130 and PK07001 are close to *A. stygium* var. *annulatum* except for large ascospore sizes ($5.8-8.7(-9.7) \times 2-2.9(-4.8) \mu\text{M}$ and $7.6-10.1 \times 2.3-4 \mu\text{M}$, respectively). The results of phylogenetic analysis revealed the relationships of both samples to *A. stygium* and *A. atroroseum* clade. The nucleotide sequence results of this clade contain large fragments of ITS regions up to 900 bp (data not shown).

Annulohypoxylon sp. PK07019 and PK10006 samples are similar to *A. bovei* var. *microspora* as described by Ju & Rogers (1996). The sizes of ascospores ($6.9-10.5 \times 2.4-4.3 \mu\text{M}$) and ostiolar discs (0.3-0.4 mm) of PK07019 are slightly narrow than the species description ($8.5-10.5 \times 4-5 \mu\text{M}$ and 0.3-0.7 mm, respectively). For PK10006, the size of ascospores ($6.8-9.5 \times 3-4.4 \mu\text{M}$) is the same length as PK07019 sample, while ostiolar discs (0.2-0.3 mm) are small in size. The phylogenetic analysis of these samples grouped them together with the *A. bovei* var. *microspora* clade and with 89% similarity to GenBank database.

Conclusions

Data combination of morphological characters and phylogenetic analyses of *Annulohypoxylon* samples revealed six complex species with high bootstrap support. Even though their morphological characters are slightly different from described species the molecular results demonstrated clear separation from known species in GenBank database. These results can be explained by genetic variation of *Annulohypoxylon* species found in Thailand based on ITS and alpha-actin sequences. However, more samples examined and

multiple gene analysis, and chemotaxonomic investigations are required in further studies for clarifying these complex species.

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