Final Report

Contributions towards a *Hypocrella* Monograph

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Contributions towards a *Hypocrella* Monograph

By

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Abstracts

The genus Hypocrella is poorly understood and in need of major revision. Although only having one known anamorph (Aschersonia) it is likely that Hypocrella will prove to consist of four separate genera. Modern molecular techniques – in parallel with traditional morphology - offer a means to determine these separations. To begin this work a re-assessment of whole-spored Hypocrella species was initiated. Collections of Hypocrella and Aschersonia from natural forest resulted in sixty pure cultures being sent to the culture collection. Specifically, Hypocrella badia appears to include three separate species. These were collected, isolated and characterised using standard morphological criteria. From a morphological basis there are consistent characteristics within each taxa that suggest the validity of making species splits. Ten isolates (representing seven species) were sent to Oregon State University. A preliminary tree demonstrates that two of these species are a recent evolution from plant-pathogenic Clavicipitaceae. The preliminary results significantly suggest the need to develop a large dataset of Hypocrella/Aschersonia sequences for the further characterisation of the group.

Background

The genus *Hypocrella* was monographed by Petch (1921). Apart from a few studies on species of *Aschersonia* (the anamorph) which have been assessed as potential biocontrol agents little further work has been done. However, within the last ten years the largest collection of these species in the world has been established at the BIOTEC Mycology Laboratory. Screening of these isolates has demonstrated that these are highly active in a range of screens and produce new metabolites – especially ones with activity against insect cell lines. There is therefore a great need to more fully resolve the species separations within this genus.

Twelve years work on *Hypocrella* in Thailand has resulted in many records and isolates being added to the BIOTEC culture collection and database. However, major taxonomic problems are present. Four species included in the BIOTEC database are invalidly named species. These need to be formally described in publications. One species is a complex of at least two and possibly three closely related species. As these cultures are being used in fermentation and screening studies it is necessary to have correct names available. A re-assessment of whole-spored *Hypocrella* species is overdue.

Benefits

1. The pure culture of *Hypocrella* and *Aschersonia* is expected to increase since these genera have proved to be of interest to a number of groups: for instance, cytoxicity, fermentation and novel metabolites.
2. New species are likely to be named for Thailand and the World.
3. This basic information consolidates a long overdue (the last was 1921) monograph of *Hypocrella*.

Objectives

- Survey, collection and isolation of *Hypocrella* and *Aschersonia*.
- Characterise the morphology from fresh specimens and pure culture by separating in to 3 groups as following
    - The light brown stromal form (AL74)
    - The very dark brown stromal form (AL98)
    - The green-brown with an individual cushion in the same stromal form (AL114)
- Molecular study- DNA marker and sequencing are to be used in order to compare the results from the morphology characterisation.

Results

1. The survey and collection was as detailed below,

    - Jet Kot waterfall, Sam Lan National Park, 30 Aug. 2000
    - Khao Soi Dao National Park, Chantaburi, 18-20 Sep. 2000
    - Kaeng Krachan National Park, Phetchaburi, 25-29 Sep. 2000
    - Khao Yai National Park, 17 Oct. 2000
    - Nam Nao National Park, Phetchabun, 25-27 Oct. 2000
    - Ton Nga Chang Waterfall, Songkhla, 20-24 Nov. 2000
    - Ko Chang, Trat, 18-20 Dec. 2000
    - Khao Sok, Surat Thani, 8-12 Jan. 2000

A total of 284 specimens were collected and added to the BIOTEC herbarium. These samples were broadly identified as follows:

- *Hypocrella raciborski*
    - *Aschersonia placenta*

- *Hypocrella oxystoma*
    - *Aschersonia oxystoma*

- *Hypocrella badia*
    - *Aschersonia badia*

- *Hypocrella tamurai*
    - *Aschersonia tamurai*

- *Hypocrella tubulata*
    - *Aschersonia tubulata*

- *Hypocrella discoidea*
    - *Aschersonia samoensis*
    - *Aschersonia cf samoensis*

- *Hypocrella hypocreoidea*
    - *Aschersonia hypocreoidea*

- *Aschersonia coffeae?*

The indented *Aschersonia*'s are the accepted anamorphs of the *Hypocrella* forms identified above.

Some of these identifications are still, however, questionable. They do not fit with any of the descriptions given by Petch (1921). Consequently, they are either new species or are species previously reported but included by Petch in other species names.

- *Hypocrella* sp.1
  - *Aschersonia* sp. 1

- *Hypocrella* sp.2
  - *Aschersonia* sp.2

- *Hypocrella* sp.3
  - *Aschersonia* sp.3

- *Hypocrella* sp.4
  - *Aschersonia* sp.4

It is important to note that these identifications are based on the assessments made by N.L. Hywel-Jones of the Petch Monograph of 1921. These assessments face the problems of altered terminology and attitudes to systematics and nomenclature in the ensuing 80 years. They face the problem that the last authority to fully study the group died ten years before the current world authority (N.L. Hywel-Jones) was born. There has been no continuity for this work. This work holds to the concept of the Ghost's Apprentice as considered by Malloch (2000, pers. comm.).

- Ninety five pure cultures were isolated from the 284 specimens collected and were preserved on slopes of PDA at the Mycology Laboratory, BIOTEC.

- Sixty pure cultures were sent to the culture collection.

- The morphological characteristics were then studied and notes on these are presented below.

Natural Habitat

Hypocrella and *Aschersonia* spp. are pathogenic to scale insects (Homoptera) which are usually found on the underside of leaves from shrubs, and trees (especially in the genus *Mangifera* (Dicotylidinae) and famiiy Zingiberaceae (Monocotylidinae) or on fallen leaves in tropical forest that is not too exposed to sun light.

Morphological considerations

Hywel-Jones (unpubl. obs.) has acquired over ten years of field experience in observation and identification of *Hypocrella* and *Aschersonia*. Within the 'whole-ascospored' group an apparent complex of superficially brown species has been identified. This is based on the Type Species of *Hypocrella discoidea* which was recognised by Hywel-Jones & Evans (1993) to produce and discharge whole ascospores. This observation was contrary to the conclusions made by Petch (1921) and demonstrated the value of working with fresh, living material rather than dried herbarium material. Petch's observation of whole ascospores in asci were assumed to be immature. Hywel-Jones & Evans (1921) proved otherwise. The brown-stromal complex was chosen to make a preliminary determination of whether this represents variations on a theme within a species or whether these represent a complex of related, but discrete, species.

Aschersonia badia AL 74

Characteristics: The stroma is light brown to light grey, velvety, hypothallus (rough mycelium) is white and formed around the stroma, round- shaped. The pycnidia are scattered and irregular in cushion. The cushion is 0.3 up to 0.8 mm high and 0.7-2 mm diameter. In culture (single isolation), round-shaped, colony is yellow-brown, pycnidia produced scattered on the colony, conidial mass is cream, under colony is brown, growth rate about 3 mm within 13 days.

Pycnidia: The shape is cup-liked, 275-300 μm diameter, 300-380 μm long, paraphyses in the range of 90-115 μm.

Conidia: The shape is fusiform, sharp-ended. Size are 12.5-15 x 2-2.5 μm and in the culture (PDA at RT) showed 12.5-16.3 x 2-2.5 μm

Aschersonia badia AL 98

Characteristics: The stroma is very dark brown, hypothallus is white formed around the stroma, round- shaped, velvety. The pycnidia scattered irregular in cushion. The cushion is 0.3 up to 0.5 mm high and 0.5-1 mm diameter. In culture (single isolation), colony is brown, round-shaped, pycnidia were produced

scattered on colony, conidial mass is cream, under colony is brown, growth rate about 9 mm within 18 days.

Pycnidia: The shape is cup-liked, long neck, 300 up to 450 μm diameter and 325 up to 375 μm, paraphyses in a range of 60-125 μm.

Conidia: The shape is fusiform, sharp-ended. Size are 10-13.8x 2.0-2.5 μm and in the culture (PDA at RT) showed 12.5-15x 2-2.5 μm.

Aschersonia badia AL 114

Characteristics: The stroma is yellow-brown and become to dark brown when old, hypothallus is yellow formed around the stroma, velvety. Each pycnidium is contained inside an individual cushion and form around one stroma. The cushion is 0.5 up to 0.7 mm high and 1-2 mm diameter. In culture (single isolation), colony is light-brown, round-shaped, in each pycnidia were produced inside an individual cushion and form around one stroma (seemed to be copy from original shape). Conidial mass is cream and more darker when old, under colony is dark brown, growth rate about 26 mm within 140 days.

Pycnidia: The shape is cup-liked, short neck 210 up to 325 μm diameter and 375 up to 425 μm, paraphyses in a range of 60-125 μm.

Conidia: The shape is fusiform, sharp-ended, short and fat in the center. Size are 3.2-4.7x 1.0-1.7 μm and in the culture (PDA at RT) showed 3.3-4.8x 1-1.8 μm..

Hywel-Jones (unpubl. obs.) began to acquire information that the brown-stromal *Hypocrella/Aschersonia* species in Thailand may be separate species. Based on the descriptions given above there is good evidence to suggest this might be so. Especially, *badia*-AL114 stands out as a result of its much smaller conidia. All of Petch's description describe conidia that are 8-15 μm long with one exception. *Hypocrella palmicola* was described from Madagascar with conidia of an un-named *Aschersonia* being 4-6 μm. This also had a brown stroma. The Thai material comes close but has enough differences to make the association with *H. palmicola* questionable.

Molecular Studies

Introduction

Any gene sequence has the potential to be used in evolutionary studies, however, if the sequence is to be used to determine the history of the gene within a number of taxa certain criteria must be fulfilled. The gene in question must;

- be found in all the test organisms
- evolve at the same rate, or have regions that evolve at the same rate
- be present as a single copy or behave like single copy
- have the same function in all taxa under investigation.

An increasing number of genes are now being examined for phylogenetics studies. In the early days work was mostly with 18S, 28S and the ITS-5.8S region. Increasingly, other gene sequences are being considered. Especially, for members of the Hypocreales the β-tubulin gene and Elongation Factor 1-α are being studied.

Genes that have been used in phylogenetic studies where these criteria apply include the ribosomal RNA gene cluster (which behave like a single copy gene), the genes coding for cytochromes, ATPase and translation elongation factors. The most widely used set is the ribosomal RNA genes and sequences for these genes are available for a range of fungi. For this reason thesesgenes were chosen for this study.

Ribosomal RNA gene cluster

The ribosomal gene of prokaryotic and eukaryotic cells consists of a large number of protein subunits and 3-4 'species' of RNA. The RNA molecules play an important role in the initiation of translation and in giving the ribosome stability. In fungi there are four main species of ribosomal RNA characterized by size:

1. the 28S molecule,
2. the 18S molecule,
3. the 5.8S molecule,
4. the 5S molecule

In this study I studied the 28S molecule and the ITS regions for the above described *Aschersonia badia* cluster.

Objective

Determine at the molecular level the three different morphologies of *Aschersonia badia* (as considered above).

Method

- The 3 different *Aschersonia badia* were grown in 50 ml. PDB (Potato Dextrose Broth) by shaking at room temperature.
- Harvest cell mass was done after 30 days and dried by lyophilisation.
- Grinding cell mass for extraction of DNA.
- Increase DNA by using PCR technique.

Material

1. Reagent for PCR

H_2O= 19

10x buffer= 2.5

$MgCl_2$= 1.25

dNTP= 0.5

Primer 1= 0.5 (for 28S= JS1 for ITS= ITS1)

Primer 2= 0.5 (for 28S= JS8 for ITS= ITS4)

DNA= 0.25

Taq polymerase= 0.25

Total volume= 25

Conditions for PCR for 28S

The DNA was denatured at 94^{o}C for 1 minute, followed by 35 cycles consisting of:

- denaturation at 94 ^{o}C for 1 minute
- primer annealing at 55 ^{o}C for 1.5 minutes
- primer extension at 72 ^{o}C for 2.5 minutes

The final step was extension at 72 ^{o}C for 10 minutes.

Condition for PCR for ITS

The DNA was denatured at 96°C for 2 minute, followed by 35 cycles consisting of:

- denaturation at 96 °C for 1 minute.
- primer anneal at 55 °C for 1 minutes
- primer extension at 72 °C for 2 minutes

The final step was extension at 72 °C for 7 minutes.

Sequencing was done after getting a PCR product.

Results

1. The pictures below demonstrate the 3 different morphologies of *Aschersonia badia* in PDB (Potato Dextrose Broth) by shaking at 25°C

AL74  *Aschersonia badia*

AL98  *Aschersonia badia*

AL114  *Aschersonia badia*

2. The 3 different morphology were extracted and sequencing as following,

Sequencing Collaboration with Joey Spatafora (Oregon State University)

For this work to compete adequately on the 'world stage' many species and isolates have got to be isolated. Not just that, but many gene regions must also be sequenced to build a library of sequences that can be used. This work has merely scratched the surface of what has the potential to be a most valuable study. Already, evidence (albeit not yet conclusive, still less publishable) has been acquired to support the contention that the *badia*-group is a complex of similar but separate species. In the spirit of international collaboration that is promoted by N.L. Hywel-Jones the following isolates were also sent to Joey Spatafora of Oregon State University for sequencing and incorporation in to his large library.

Code no.	Species	Site	Collection date
AL027	*Aschersonia tamurai*	Sam Lan Waterfall; Sara Buri	30/8/00
AL046	*Aschersonia tamurai*	Khao Soi Dao; Chantaburi	19/9/00
AL167	*Hypocrella* sp.	Khao Yai	3/11/00
AL038	*Aschersonia placenta*	Doi Inthanon; Chiang Mai	22/7/00
AL067	*Aschersonia placenta*	Kaeng Krachan; Petchaburi	28/9/00
AL115	*Aschersonia placenta*	Pha Deo Dai Khao Yai	17/10/00
AL031	*Aschersonia oxystoma*	Jet Kot Waterfall; Sara Buri	30/8/00
AL230	*Aschersonia hypocreoidea*	Khao Sok; Surat Thani	10/1/01
AL237	*Aschersonia badia*	Khao Sok; Surat Thani	10/1/01
SSC13	*Hypocrella scutata*	Phu Dho Deang; Narathiwat	18/4/01

The aim of this was to determine if there could be a separation of *Hypocrella* into those that produced whole ascospores and those that produce part-spores. Again, the preliminary results of this form the basis of a recommendation that the genus is far more complex than originally considered by Petch. There is increasing evidence that *Aschersonia placenta* is also a complex of similarly confused species. *Aschersonia hypocreoidea* also falls within this complex but has enough morphological separations to make its identity acceptable.

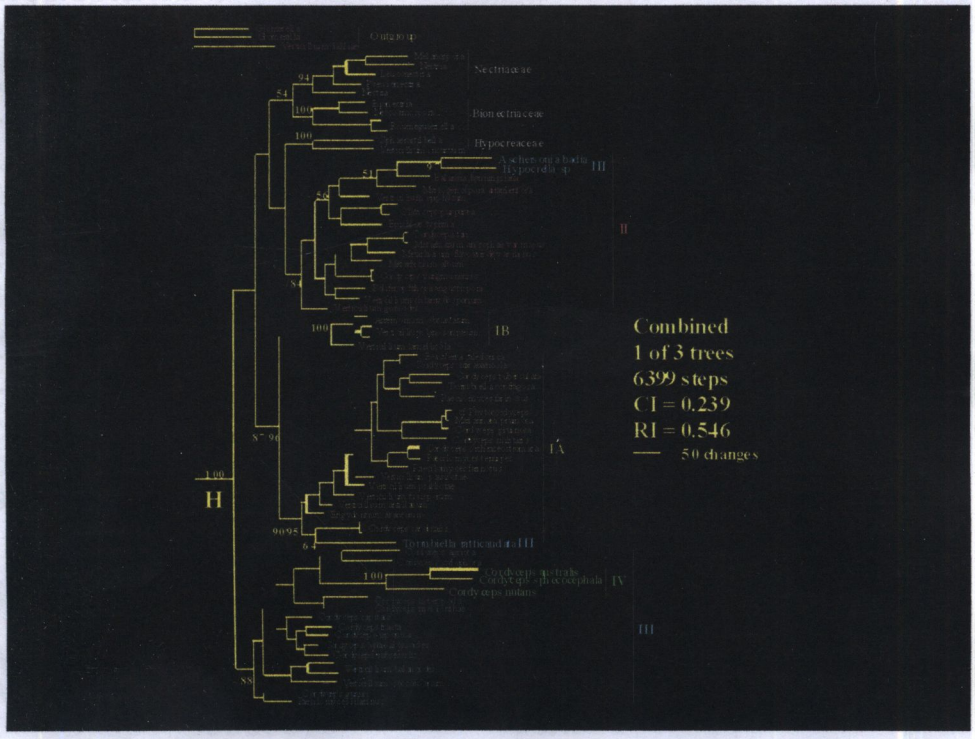
A preliminary tree featuring *Aschersonia badia* – AL237 and *Hypocrella* sp. – AL167 places these within a clade of plant-pathogenic Clavicipitaceae (Tree 1). The Major Clade II of the Spatafora tree can be broken into two sub-clades. One clade is predominantly

insect-pathogenic in nature and includes the *Metarhizium* isolates as well as associated *Cordyceps* species (e.g. *Cordyceps taii* which is the sexual state of a *Metarhizium*).

Significantly, the second sub-clade contains plant-pathogenic genera such as *Claviceps*, *Epichloë* and *Balansia*. The evidence is increasing that while plant-pathogenic Clavicipitaceae appear to have evolved out of *Cordyceps* the insect-pathogenic *Hypocrella* appear to be a more recent evolution back into insect from a purported plant-pathogenic Clavicipitaceae. This is hinted at by the placement of *Aschersonia badia* within a group of plant-pathogens.

The genus is clearly an interesting one. Hywel-Jones & Samuels[1] (1998) reported on the apparent use that *Hypocrella schizostachyi* was able to make of phloem nutrients supplied by the stylet of the dead scale insect. Observations also indicate that *Hypocrella scutata* can also complete its development using nutrients from the insect stylet. Especially, Sullivan *et al.*[2] (2000) have described further examples of an epibiont ability in an insect fungus on plants. The close link between plant, insect host and insect-pathogen is providing novel research on tri-trophic interactions. The genus *Hypocrella* is basic to answering these questions.

Tree 1: Unpublished tree supplied by Dr J. Spatafora – Oregon State Univeristy



[1] **Hywel-Jones, N.L.** & Samuels, G.J. (1998). Three species of *Hypocrella* with large stromata pathogenic on scale insects. *Mycologia* **90**: 36-46

[2] Sullivan, R.F., Bills, G.F., **Hywel-Jones, N.L.** & White, J.F. (2000). *Hyperdermium*: a new clavicipitalean genus for some tropical epibionts of dicotyledonous plants. *Mycologia* **92**: 908-918

Conclusions

After one year, a very small start has been made at understanding the phylogenetic relationships within *Hypocrella*. That there are at least two species complexes (*badia* and *placenta*) is increasingly apparent. But the few isolates that have been sequenced to date is merely a beginning. The work of Jennifer Luangsa-ard with *Paecilomyces* gives a strong indication of where this work must in the future develop if any meaningful (and publishable) conclusions are to be drawn. Continued research on Thai *Hypocrella* and *Hyperdermium* has the potential to continue introducing to the scientific community novel concepts on tri-trophic relations between insect, host-plant and insect-fungus.

Appendix of Sequences

AL96 the region is 28S

GAGACCAACAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAA
TCTGGCCCCTCCCCCGGGGGGGCCCGAGTTGTAATTTGCAGAGGATGCTTCTGGCGAGGT
GCCTTCCGAGTTCCCTGGAACGGGACGCCGCAGAGGGTGAGAGCCCCGTCTGGTCGGACA
CCGAGCCTCTGTGAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAATGCTGCTCTAAACGG
GAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTG
ATCGAAAGATGAAAAGCACTTTGAAAAGAGGGTTAAACAGTACGTGAAATTGTTGAAAGG
GAAGCGCTCACGACCAGACCTGGTCCCGGCGAATCACCCGGCGTTCTCGCCGGTGCACTT
CGACGGGCTTCCAGGCCAGCATCAGTCCGCGCCGGGGGACAAAGGCGGCGGGAACGTGGC
TCCCCAGGGAGTGTTATAGCCCGCCGCGCAATGCCCCGGGGGCGGACTGAGGACCGCGCG
TCACCGCAAGGATGCTGGCGTAATGGTCGTCAGCGACCCGTCTTGAAACACGGACCAAGG
AGTCGTCCTCGTATGCGAGTGTTGGGGCGTCAAACCCCCCGCGCGTAATGAAAGTGAACG
GCAGGTGAGAGCCCTCGCGGGCGCATCATCGACCGATCCTGATGTTCTCGGATGGATTTG
AGTAGGAGCATACGGGGCCGGACCCGAAAGAAGGTTATGCCTTTTTTAGGGGAAGCCAGA
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CGCATACTTGCCTTCATCCATTCTCAAACTTTAAATATGTAAGAAGCCCTTGTTGCTTAG
CTGAACGTGGGCATTCGAATGTATCAGCACTAGTGGGCCATTTTTGGTAAGCAGAACTGG
CGATGCGGGATGAACCGAACGCGAGGTTAAGGTGCCGGAGTGGACGCTCATCANACACCA
CAAAAGGTGTTAGTACATCTTGACAGCAGGACGGTGGCCATGGAAGTCGGAATCCGCTAA
GGACTGTGTNACAACTCACCTGCCGAATGTACTANCCCTGAAAATGTGG

ITS/5.8S regions

AL98 ITS/5.8
AGGATATTACCGAGTGCGGGCCCCTNGGGGCCCAACCTCCACCCGTGTTGCCCGAACCTA
TGTTGCCTCGGCGGGCCCCGCGCCCGCCGACGGCCCCCCTGAACGCTGTCTGAAGTTGCA
GTCTGAGACCTATAACGAAATTAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCA
TCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAGTCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTCCGAGCGT
CATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCCCCCGCCGGGGGGACGG
GCCCCAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCACCCG

CTCTAGTAGGCCCGGCCGGNGCCAGCCGACCCCCAACCTTTAATTATCTCAGGTTGACCT
CGGATCAGGNAGGGATACCCGCTGAACTTAAGCATATCAATAGCGGGG

AL114 ITS1/5.8
GGGTNATTACCGAGTTTCGCAACTCCTAANCCNCCTGTGNACCGCTACCCAGAACGTTGC
CTCGGCGGGCTCGCCCCCGCCGGAGGACGCCCGCAAACTCTTCTCCAGCGGTCCTCGCCG
AAAGCCAGGCCCCCCCGGAAGGGCGGGCGGGCAGAGGCGCAGGGCCCCCTCCCTCTGAGT
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CTGAACTTAAGCATATCAATNACCAGGA

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Aschersonia badia (AL114 and AL119)

A. Stroma from natural habitat

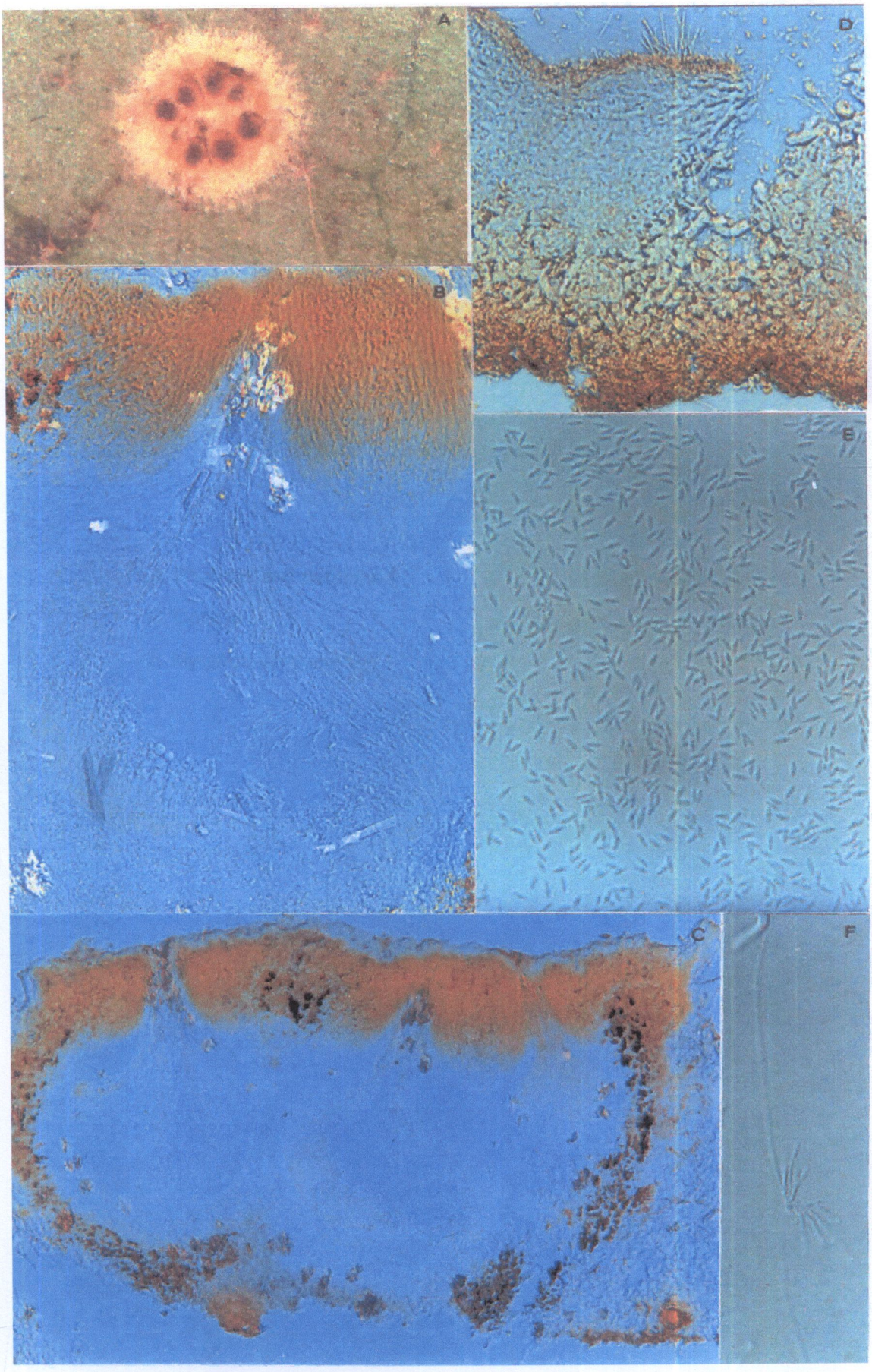
B. Pycnidia shape

C. Section whole stromata

D. Mycelium characteristic

E. Conidia

F. Phialides and paraphysis


A
D
B
E
C
F

Aschersonia badia (AL71 and AL96)

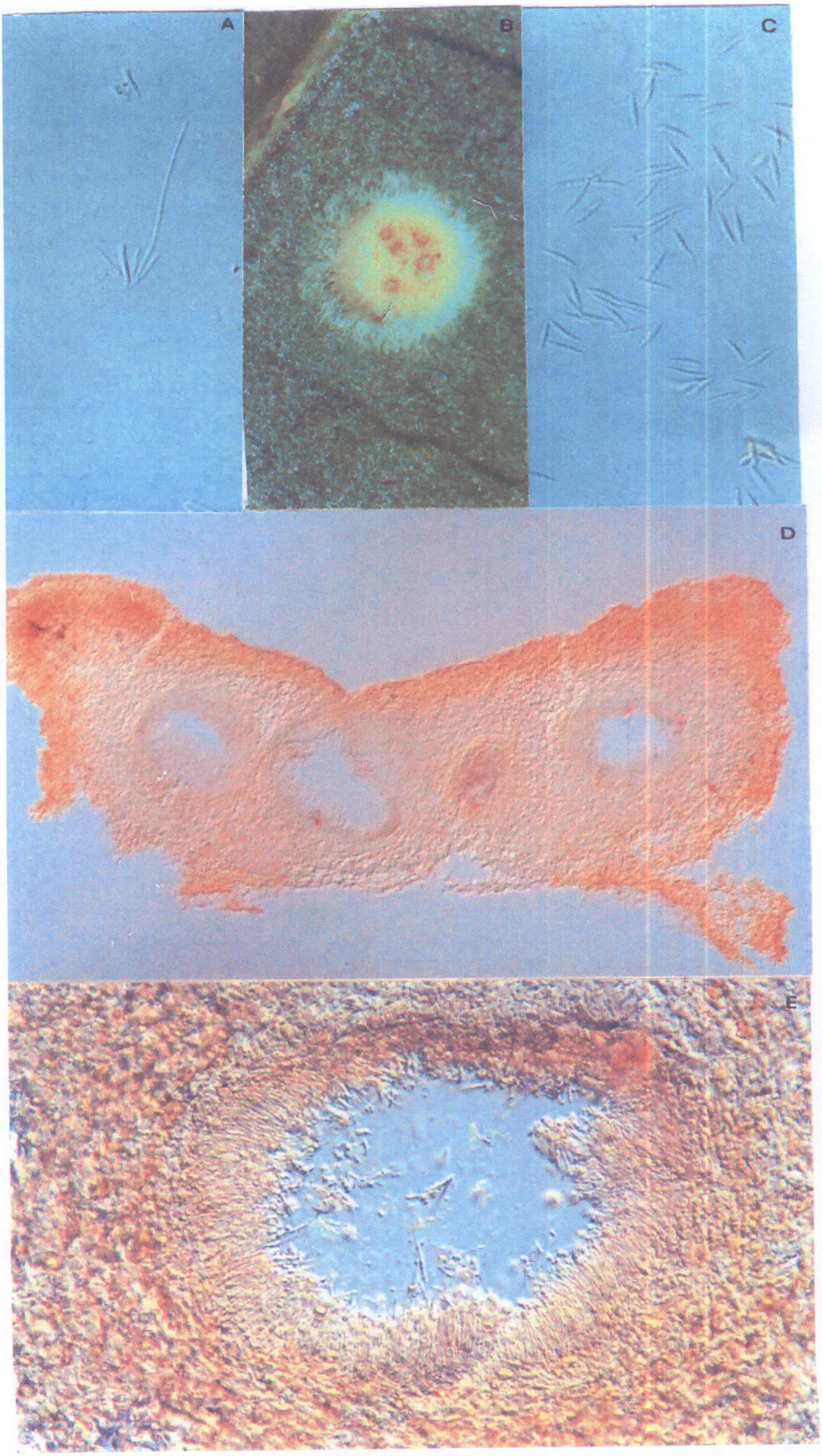
A. Phialides and paraphysis

B. Stroma from natural habitat

C. Conidia

D. Section whole stromata

E. Mycelium characteristic


A
B
C
D
E