CHAPTER 4

RESULTS AND DISCUSSION

4.1 Collection of specimens

4.1.1 Survey sites:

The collecting trips were to the Sirindhorn Peat Swamp Forest, Narathiwat; Bala-Hala Wildlife Sanctuary, Narathiwat; Khao Luang National Park, Nakhon Srithammarat; Nam Nao National Park, Petchabun; Ton Nga Chang Wildlife Sanctuary, Songkhla; Khao Yai National Park, Saraburi and Doi Inthanon National Park, Chiang Mai between May, 2001 and February, 2003. The survey sites were shown in Figure 4.1.

4.1.2 Sample collection:

Two hundred and twenty five *H. scutata* specimens were collected from Sirindhorn Peat Swamp Forest, Narathiwat and one specimen was collected from Bala-Hala Wildlife Sanctuary, Narathiwat. *H. schizostachyi* was not found from any other survey sites.

H. scutata was found only at Sirindhorn Peat Swamp forest, and Bala-Hala Wildlife Sanctuary, Narathiwat. The first record of *H. scutata* was observed in 1999 by Sivichai and Hywel-Jones from Sirindhorn Peat Swamp Forest (Hywel-Jones unpubl. pers. comm.). This first observation occurred on the second floor of the Visitor Centre of Sirindhorn Peat Swamp Forest on the top of *Syzygium* spp leaves. Hywel-Jones and Samuels (1998) noted that *H.*

schizostachyi was a rarely found species. *H. schizostachyi* was not found from any of the survey sites during my work.

4.1.3 Isolation of fungi

Forty five *H. scutata* isolates were isolated from ninety seven fresh mature specimens (46.4%). Most of the isolates were from the ascospore ejection isolation method. The contamination by airborne fungi is the main problem in the isolation because *H. scutata* grows very slowly. The contaminants can grow over them overnight. The *H. scutata* stroma usually contaminated with the other fungi which grew over them as a hyperparasite. This is the first record for the isolation of *H. scutata*. A *H. schizostachyi* NHJ 4547 isolate was received from Dr. Nigel L. Hywel-Jones (Hywel-Jones and Samuels, 1998.

At BIOTEC Thailand, three isolates of *H. discoidea* (NHJ 4031, NHJ 5567, NHJ 5256) and one isolate of *H. schizostachyi* NHJ 4547 were deposited in 1997-1999.

4.2 Morphological studies

4.2.1 Macroscopic structures

The fungal stromata were observed with the naked eye and under a stereomicroscope and were photographed (Figure 4.2). The mature stroma size of *H. scutata* were in the range of 0.5-1.2 cm. The specimens collected have all the characteristics as described by Petch (1921) in Table 4.1. The characteristics of *H. schizostachyi* were described clearly by Hywel-Jones and Samuels (1998).

The specimens of *H. scutata* were always found on the upper sides of leaves. This is rare for species of *Hypocrella*. Often they were surrounded by a black sooty mould. This feature is often associated with honeydew produced by sap-sucking insects especially aphids. Petch (1921) noted that specimens were resinous and fractured like glass. Hywel-Jones and Samuels (1998) speculated that large stromatal *Hypocrella* species could only reach such size by making use of the phloem sap that would come up the dead insects stylet by the action of phloem pressure. In fresh specimens of *H. scutata* in the field the central raised part of the stroma often had a sticky substance which I assumed to be sap oozing up from the leaf as suggested by Hywel-Jones and Samuels (1998).

Figure 4.1 Survey sites

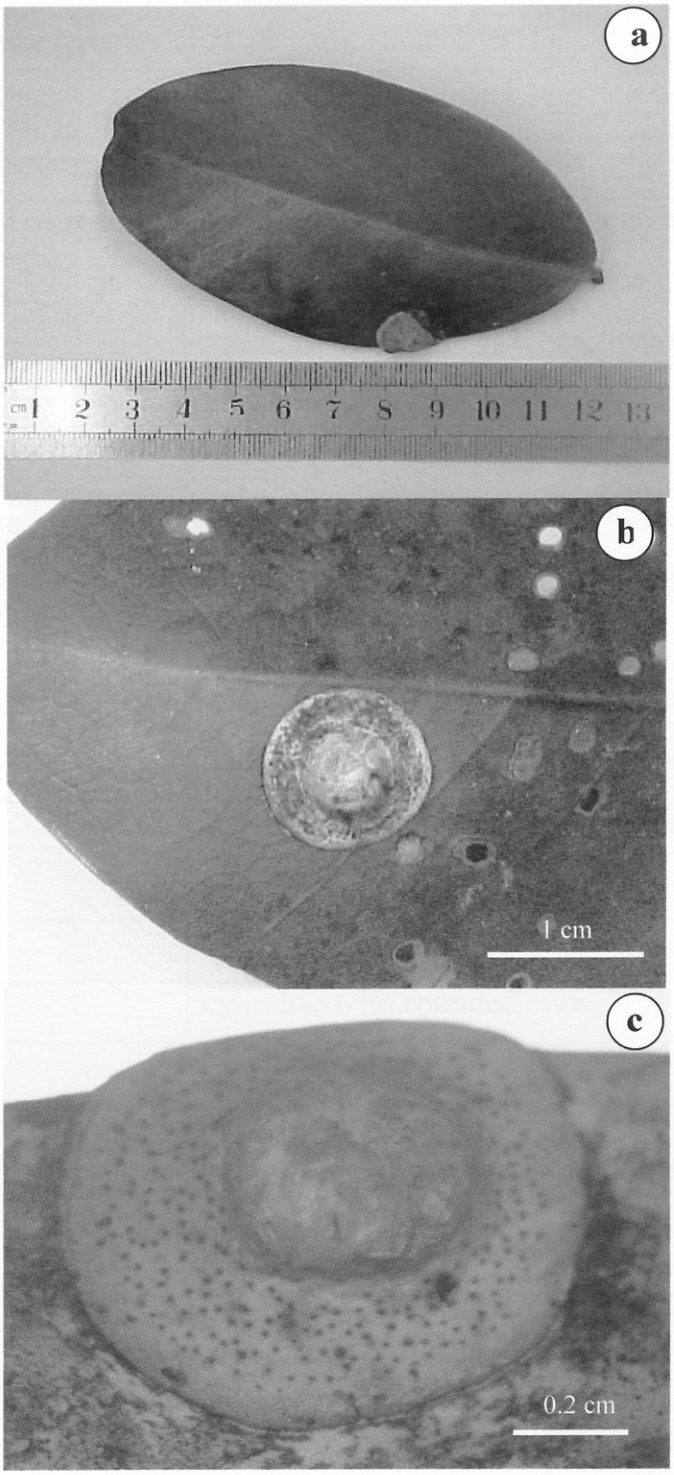

Figure 4.2 The macroscopic appearance of mature *H. scutata*, a) actual size, b) and c) with higher magnification

Table 4.1 The characteristics of *H. scutata*. (Petch, 1921)

Characteristics	Details
Shape and texture of stroma	flattened convex, up to 2 mm thick in centre, margin acute or obtuse, surface even, glabrous, resinous, fractures vitreous, hypothallus (when present), membranous, translucent; lower surface flat.
Colour of stroma	bright ochraceous orange when fresh pale brown, red-brown ostioles to dark brown with aging. Lower surface translucent yellow-brown with central yellowish opaque spot with radiating anastomosing yellow lines.
Perithecia	rather deeply sunk, flask-shaped or laterally compressed, up to 0.8 mm deep.
Asci	cylindric, 400-500x8-10μm.
Part-spores	cylindric with rounded ends, 3-6x1.5μm.
Distribution	Malaysia, Singapore, Philippines, Thailand.
Synonyms	*Hypocrea scutata.*
Conidia	Irregular shape, 7-12x1.5μm.

4.2.2 Microscopic structures

Mature fungal stromata were sectioned using a Microtome. The morphological structures were observed and photographed using light and scanning electron microscopes (SEM).

Light microscopy study shows that flask-shaped perithecia are deeply sunk in the stromata (Fig 4.3a). Periphyses are present as shown in Fig 4.3b. The ascus is typical of the family Clavicipitaceae in being long and cylindric, 700-800 x 40-50 μm which is larger than that described by Petch (1921). Each ascus contains many part-spores (Fig.4.3c). These part-spores are cylindric with rounded ends, 1x3 μm (Fig. 4.3d). The producing conidia stroma (in culture) and the non-producing conidial stroma were shown in Fig. 4.4a and 4.4b respectively. The conidia are irregular, rounded end, 5x10 μm (Fig. 4.4c).

Scanning electron microscopy study shows the long cylindric asci (Fig 4.5a). The ascus contained the ascospores, which were arranged inside them (Fig. 4.5b). The part ascospore connected to each other (Fig. 4.5c). The ascospore is septate (Fig 4.5d). The SEM pictures show that the conidia of *H. discoidea*'s anamorph are fusiform, 7-12 μm (Fig. 4.6) while the conidia of the anamorph of *H. schizostachyi* and the anamorph of *H. scutata* are irregular with rounded ends (Fig 4.7, 4.8). This feature alone separates the anamorph from the genus *Aschersonia,* which is an acknowledged anamorph of true *Hypocrella* species. All known species of *Aschersonia* have fusoid conidia tapering to a point. None have been described with rounded ends (Petch, 1921). This indicates that the anamorphs of *H. scutata* and *H. schizostachyi* are morphologically different from the anamorph of *H. discoidea,* which is the type species for the genus. This is morphological support for *H. scutata* and *H.*

schizostachyi being separated from *Hypocrella* and suggests these should be classified into a new genus.

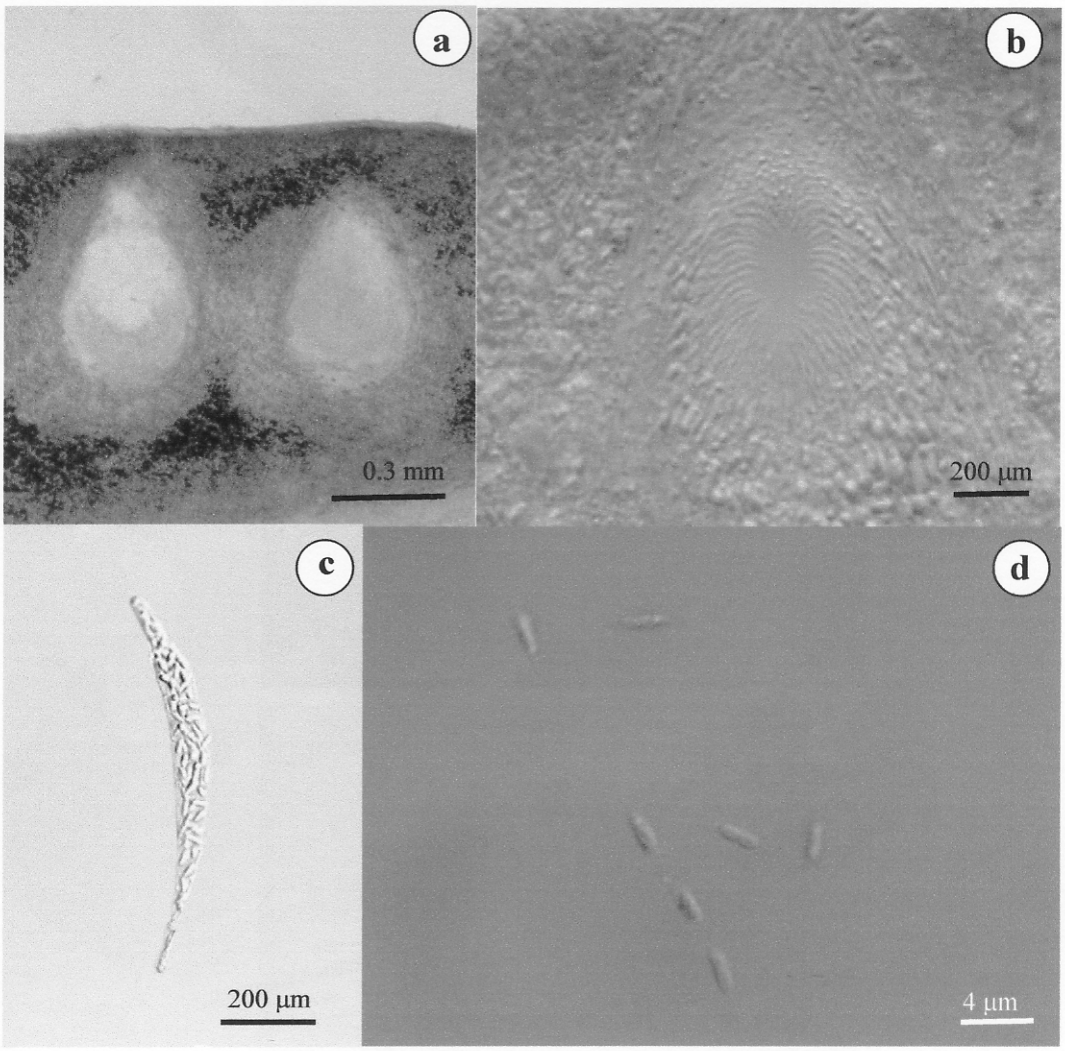

Figure 4.3 Light microscopy study of *H. scutata*, a) the perithecia, b) the periphyses, c) the ascus with part- spores, d) the ascospores

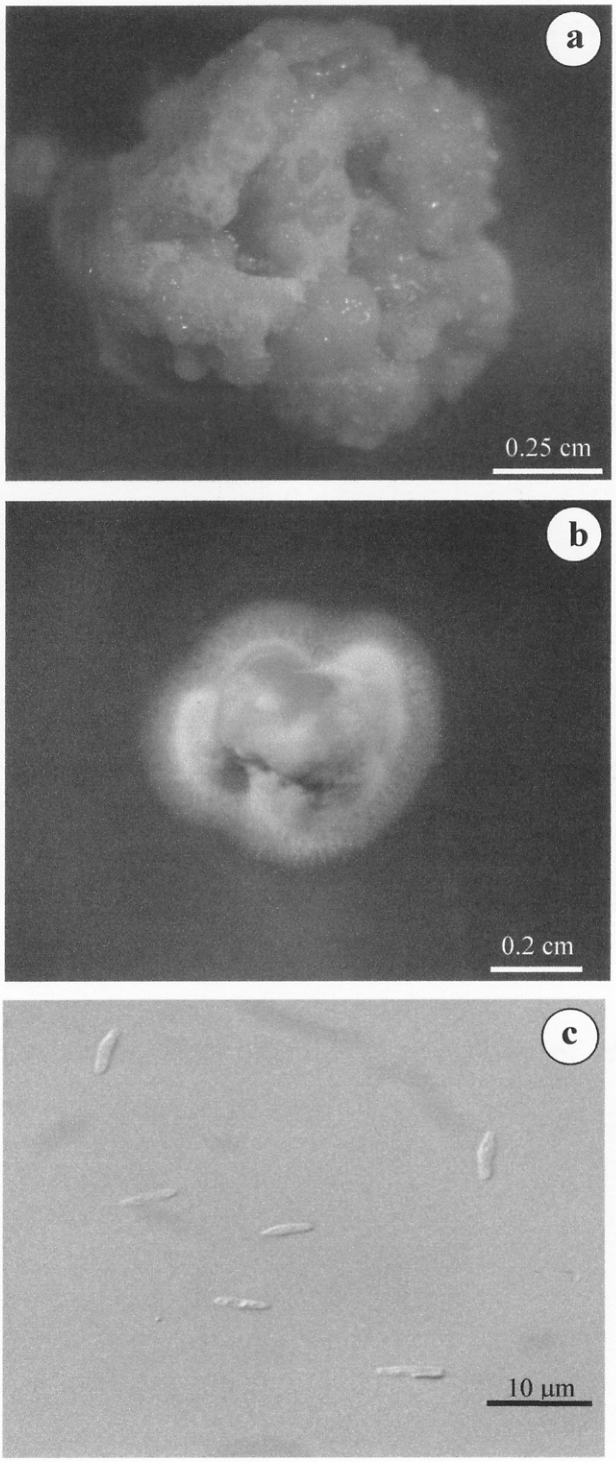

Figure 4.4 The anamorph of *H. scutata* on PDA, a) producing conidia on the stroma, b) the non-producing conidial stroma, c) the conidia

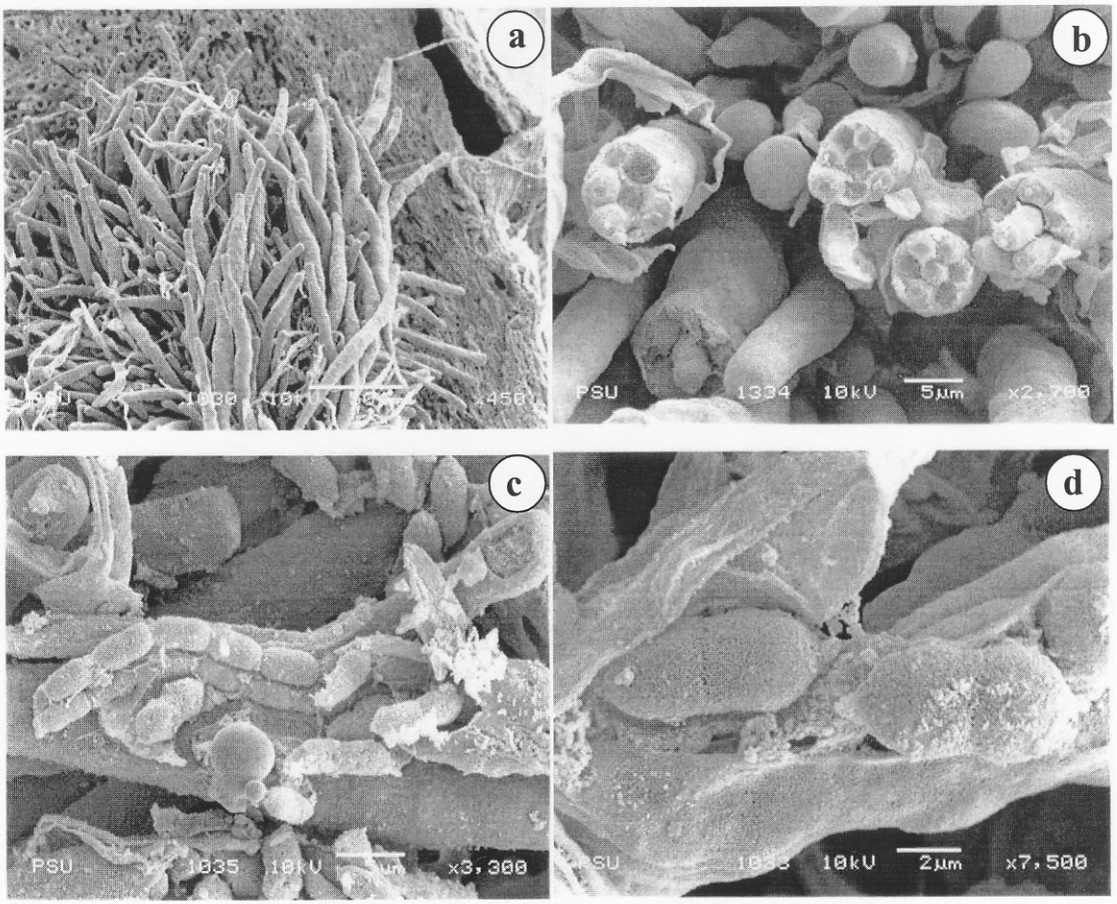

Figure 4.5 The scanning electron microscopy study of *H. scutata*, a) and b) asci, c) and d) ascospores

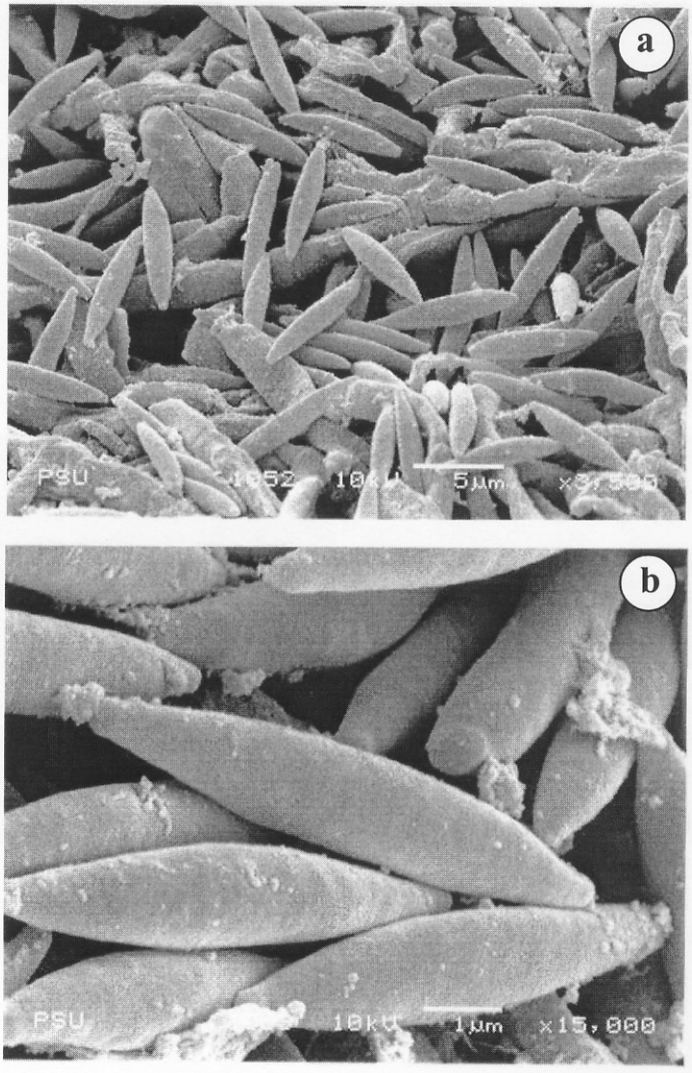

Figure 4.6 The conidia of the anamorph of *H. discoidea* NHJ 5004 (type species)

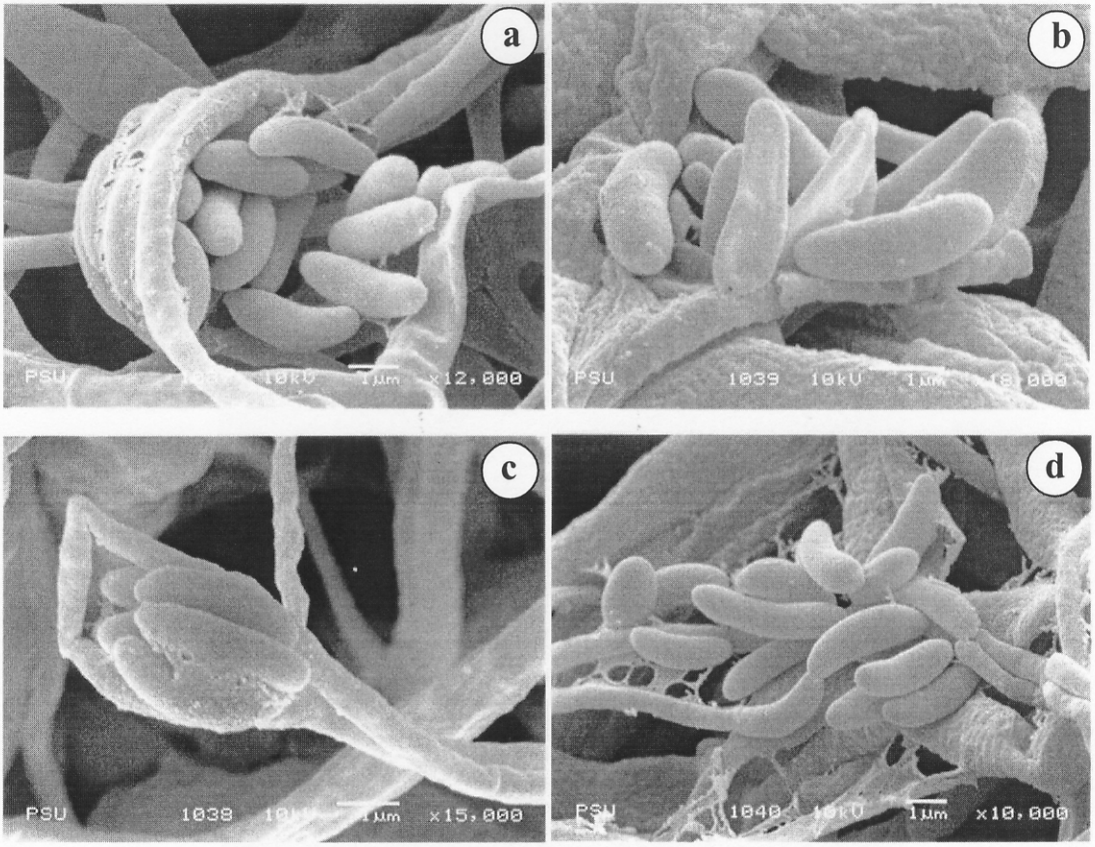

Figure 4.7 The conidia of the anamorph of *H. schizostachyi* NHJ 4547

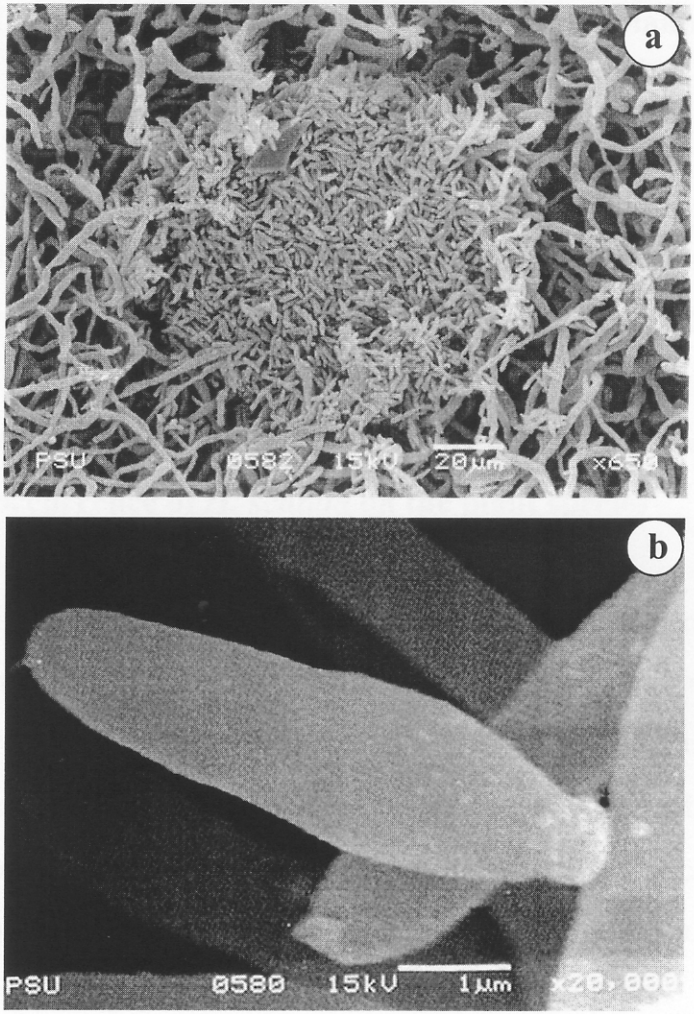

Figure 4.8 The conidia of the anamorph of *H. scutata* P32

4.3 Distribution of *H. scutata* in an experimental *Syzygium* plantation

The *H. scutata* stromata were found only on the upper surface of leaves of *Syzygium tumida* and *Syzygium oblatum*. The relationship between plant species and the level of branch to the occurrence of *H. scutata* stromata were analysed by statistical method (χ^2 test), SPSS program version 11.

4.3.1 The relationship of plant species, *H. scutata* occurrence and the proportion of the occurrence of *H. scutata* on the plant species

The statistical analysis was performed to determine the relationship of plant species and the *H. scutata* occurrence. The total numbers of the sampled plants were 140 trees (shown in Table 4.2). The χ^2 test was shown in Table 4.3. The p-value for a 2x2 table from the χ^2 test is 0.003 which is less than 0.05 (significant level (α) = 0.05). The results of the statistical analysis suggest that the plant species influenced the occurrence of *H. scutata*.

Table 4.4 and 4.5 show the statistics of the proportion of the *H. scutata* occurrence on each plant species (*S. tumida* and *S. oblatum*). The p-value is 0.006 which is less than 0.05 (significant level (α) = 0.05). Therefore I conclude that the proportion of the *H. scutata* occurrence on each plant species (*S. tumida* and *S. oblatum*) is significantly different. This shows that the *H. scutata* occurrence on *S. tumida* is higher than *S. oblatum*.

Table 4.2 Cross-tabulation of the number of sampled plants (*S. tumida* and *S. oblatum*) and *H. scutata* occurrence

			H. scutata occurrence		Total
			Not found	Found	
Plant species	*S. tumida*	Count	42.0	28.0	70.0
		Expected Count	50.5	19.5	70.0
	S. oblatum	Count	59.0	11.0	70.0
		Expected Count	50.5	19.5	70.0
Total		Count	101.0	39.0	140.0
		Expected Count	101.0	39.0	140.0

Significant level (α) = 0.05

Table 4.3 χ^2 tests of the number of sampled plants (*S. tumida* and *S. oblatum*) and *H. scutata* occurrence

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson χ^2	10.272[b]	1	0.001		
Continuity Correction[a]	9.099	1	0.003		
Likelihood Ratio	10.540	1	0.001		
Fisher's Exact Test				0.002	0.001
Linear-by-Linear Association	10.198	1	0.001		
N of Valid Cases	140				

a. Computed only for a 2x2 table

b. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 19.50.

Table 4.4 Frequencies of the number of sampled plants (*S. tumida* and *S. oblatum*) and *H. scutata* occurrence

Plant species	Observed No.	Expected No.	Residual
S. tumida	28.0	19.5	8.5
S. oblatum	11.0	19.5	-8.5
Total	39.0		

Table 4.5 Test statistics of the number of sampled plants (*S. tumida* and *S. oblatum*) and *H. scutata* occurrence

	Plant species
χ^2	7.410 [a]
df	1
Asymp. Sig.	0.006

a. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 19.5

4.3.2 The relationship between the level of branch on the plants and the *H. scutata* occurrence

4.3.2.1 The relationship between the level of branch on the *S. tumida* and the occurrence of *H. scutata*

The relationship between the position of the branch on *S. tumida* and *H. scutata* occurrence was analysed at the 95% significance. The total number of sampled plants were 70 trees (shown in Table 4.6). The χ^2 test was shown in Table 4.7. The p-value for a 2x2 table from the χ^2 test is 0.806 which is more than 0.05 (significant level (α) = 0.05). From this analysis I conclude that the level of branch on *S. tumida* does not influence the occurrence of *H. scutata*.

Tables 4.8 and 4.9 show the statistics for determining the proportion of the *H. scutata* occurrence in each branch level of *S. tumida*. The p-value is 0.846 which more than 0.05 (significant level (α) = 0.05). Therefore I conclude that the proportion of the *H. scutata* occurrence on each branch level of *S. tumida* is not significantly different. This shows that the *H. scutata* occurrence on *S. tumida* does not depend on the level of branch.

Table 4.6 Cross-tabulation of the number of sampled plants (*S. tumida*) and *H. scutata* occurrence

			H. scutata occurrence on *S. tumida*		Total
			Not found	Found	
Branch level	Upper	Count	58.0	12.0	70.0
		Expected Count	56.7	13.3	70.0
	Middle	Count	57.0	13.0	70.0
		Expected Count	56.7	13.3	70.0
	Lower	Count	55.0	15.0	70.0
		Expected Count	56.7	13.3	70.0
Total		Count	170.0	40.0	210.0
		Expected Count	170.0	40.0	210.0

Significant level (α) = 0.05

Table 4.7 χ^2 tests of the number of sampled plants (*S. tumida*) and *H. scutata* occurrence

	Value	df	Asymp. Sig. (2-sided)
Pearson χ^2	0.432[a]	2	0.806
Likelihood Ratio	0.429	2	0.807
Linear-by-Linear Association	0.415	1	0.519
N of Valid Cases	210.0		

a 0 cells (0.0%) have expected count less than 5. The minimum expected count is 13.33.

Table 4.8 Frequencies of the number of sampled plants (*S. tumida*) and *H. scutata* occurrence

Branch level	Observed N	Expected N	Residual
Upper	17.0	18.0	-1.0
Middle	17.0	18.0	-1.0
Lower	20.0	18.0	2.0
Total	54.0		

Table 4.9 Test statistics of the number of sampled plants (*S. tumida*) and *H. scutata* occurrence

Statistics	The number of *H. scutata* found on each branch level of *S. tumida*
χ^{2a}	0.333
df	2
Asymp. Sig.	0.846

a. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 18.0.

4.3.2.2 The relationship between the level of branch on the *S. oblatum* and the *H. scutata* occurrence

The relationship between the position of the branch on *S. oblatum* and *H. scutata* occurrence was analysed at the 95% significance. The total number of sampled plants were 70 trees (shown in Table 4.10). The χ^2 test was shown in Table 4.11. The p-value from the χ^2 test is 0.247 which is more than 0.05 (significant level (α) = 0.05). Therefore the level of branch on *S. oblatum* does not influence the occurrence of *H. scutata*.

Tables 4.12 and 4.13 show the statistics for determining the proportion of the *H. scutata* occurrence in each branch level of *S. oblatum*. The p-value is 0.047 which less than 0.05 (significant level (α) = 0.05). So the proportion of the *H. scutata* occurrence on each branch level of *S. oblatum* is significantly different. This shows that the *H. scutata* occurrence on *S. oblatum* depended on the level of branch. The occurrence of *H. scutata* on the high level branches is higher than the others.

Table 4.10 Cross-tabulation of the number of sampled plants (*S. oblatum*) and *H. scutata* occurrence

			H. scutata occurrence on *S. oblatum*		Total
			Not found	Found	
Branch level	Upper	Count	64.0	6.0	70.0
		Expected Count	66.0	4.0	70.0
	Middle	Count	65.0	5.0	70.0
		Expected Count	66.0	4.0	70.0
	Lower	Count	69.0	1.0	70.0
		Expected Count	66.0	4.0	70.0
Total		Count	198.0	12.0	210.0
		Expected Count	198.0	12.0	210.0

Significant level (α) = 0.05

Table 4.11 χ^2 Tests of the number of sampled plants (*S. oblatum*) and *H. scutata* occurrence

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson χ^2	3.712[a]	2	0.156	0.247		
Likelihood Ratio	4.535	2	0.104	0.162		
Fisher's Exact Test	3.986			0.162		
Linear-by-Linear Association	3.299[b]	1	0.069	0.101	0.050	0.029
N of Valid Cases	210.0					

a. 3 cells (50.0%) have expected count less than 5. The minimum expected count is 4.00.
b. The standardised statistic is -1.816.

Table 4.12 Frequencies of the number of sampled plants (*S. oblatum*) and *H. scutata* occurrence

Branch level	Observed N	Expected N	Residual
Upper	9.0	5.3	3.7
Middle	6.0	5.3	.7
Lower	1.0	5.3	-4.3
Total	16.0		

Table 4.13 Test statistics of the number of sampled plants (*S. oblatum*) and *H. scutata* occurrence

	The number of *H. scutata* found on each branch level of *S. oblatum*
$\chi^{2\,a}$	6.125
df	2
Asymp. Sig.	0.047

a. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 5.3.

4.4 Effect of temperature and media on the growth of fungi

Entomopathogenic fungi can be grown on artificial media. Good growth of the mycelium is related to the temperature and nutrients supplied. Lilly (1965) noted that fungi require different nutrients and that no one medium or substrate will be suitable for all species. Another important factor for fungal growth is temperature. Fungi can grow over a wide range of temperatures depending on their habitat origin.

This study of the growth of *H. scutata* and *H. schizostachyi* was performed to determine their optimal growth on selected media over a range of temperature. This data will be useful to determine the optimal conditions for biomass production for molecular studies. The data may be useful biotechnologically in bioactive compound production.

Three isolates of *H. scutata* (SSC 32, SSC 33 and SSC 47) and one isolate of *H. schizostachyi* NHJ 4547 were cultured on four different media and incubated at 20, 25, 30 and 35°C. Growth data of each species are shown in Figures 4.9, 4.10, 4.11 and 4.12. All isolates grew slowly at 20, 25 and 30°C within 4 weeks. They did not grow at all at 35°C on any of the media. The opimal growth of H. schizostachyi NHJ 4547 was on PDA at 25°C, 2±0.1cm diameter at 4 weeks (Fig 4.13). The optimal growth of H. scutata SSC 32 was also on PDA at 25°C, 1.2-1.3 cm diameter at 4 weeks (Fig. 4.14, Appendix 4)

The optimal temperature for growth of *H. scutata* and *H. schizostachyi* was between 20-25°C. Smith and Onions (1994) recommended the temperature between 20-25°C for the growth of fungi on MA and PDA. The result from my study is the same. Gorgre (1991) reported temperature for growth of the entomopathogenic fungi is in the range 15-30°C.

Artjariyasripong (1999) reported that *H. discoidea* and *A. samoensis* grew very slowly at 30°C and the optimal growth was at 25°C. *H. scutata* and *H. schizostachyi* also grew slowly at 30°C. *H. scutata* and *H. schizostachyi* grew better at 20°C than at 25°C. They did not grow at 35°C.

H. scutata and *H. schizostachyi* were able to grow on all media. Altman and Dittmer (1974) and Onions *et al.* (1981) reported that fungi required thiamine, nicotinic acid and pyridoxine for growth. These growth factors are found in PDA, MA, GYA and CMA. *H. scutata* and *H. schizostachyi* grew on PDA quicker than MA, GYA and CMA. This was affected by the quantity of glucose in the media as a carbon source. The glucose level of PDA, MA, GYA and CMA are 20g/litre, 3.247g/litre, 10g/litre and 0g/litre, respectively. Brachet and Mirsky (1964) and Jennings (1995) reported that fungi used glucose for growth more than any other sugar.

The optimal growth of *H. scutata* and *H. schizostachyi* was at 25°C on PDA. The data is the same as previous studies (Artjariyasripong, 1999). Fig. 4.13 and Fig 4.14 show the growth of *H. schizostachyi* NHJ 4547 and *H. scutata* SSC 32 on different media at 25°C, respectively. The growth of insect fungi depends on both media and temperatire used in culturation.

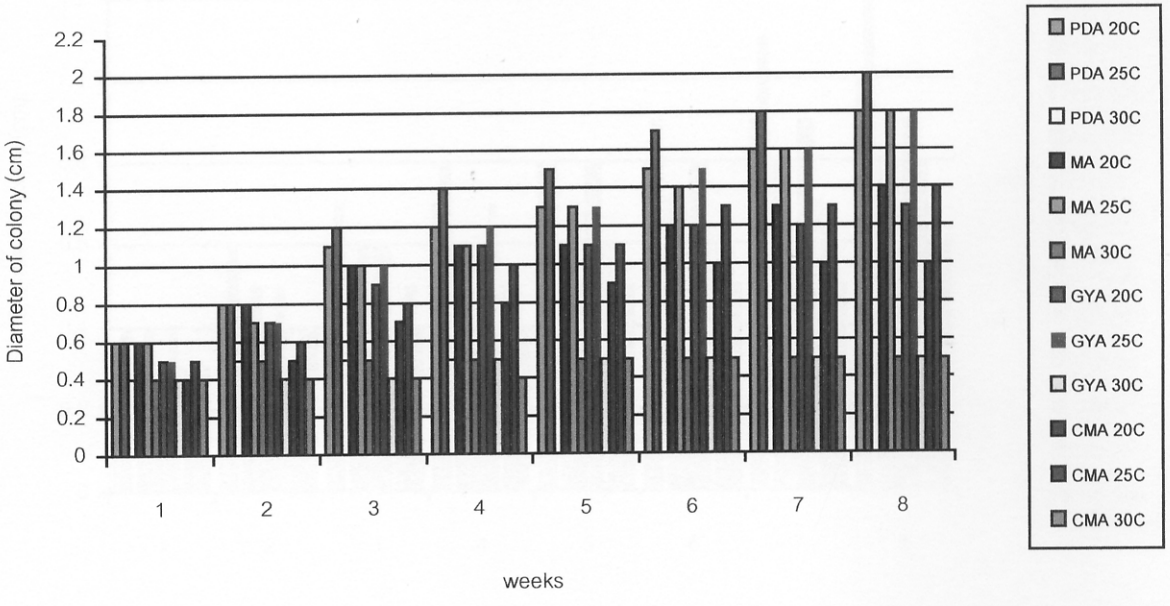

Figure 4.9 Growth of *H. schizostachyi* NHJ 4547 on four different media at 20, 25, 30°C (PDA, potato dextrose agar; MA, malt extract agar; GYA, glucose yeast extract agar; CMA, corn meal agar)

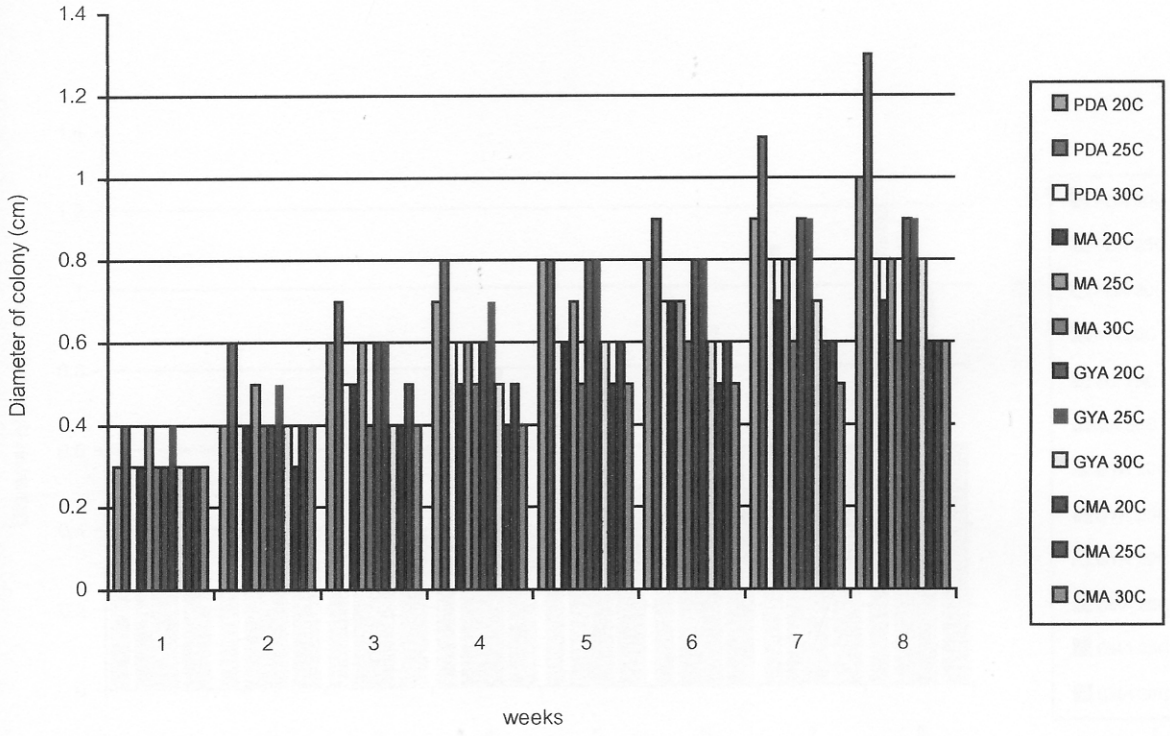

Figure 4.10 Growth of *H. scutata* SSC32 on four different media at 20, 25 and 30°C (PDA, potato dextrose agar; MA, malt extract agar; GYA, glucose yeast extract agar; CMA, corn meal agar)

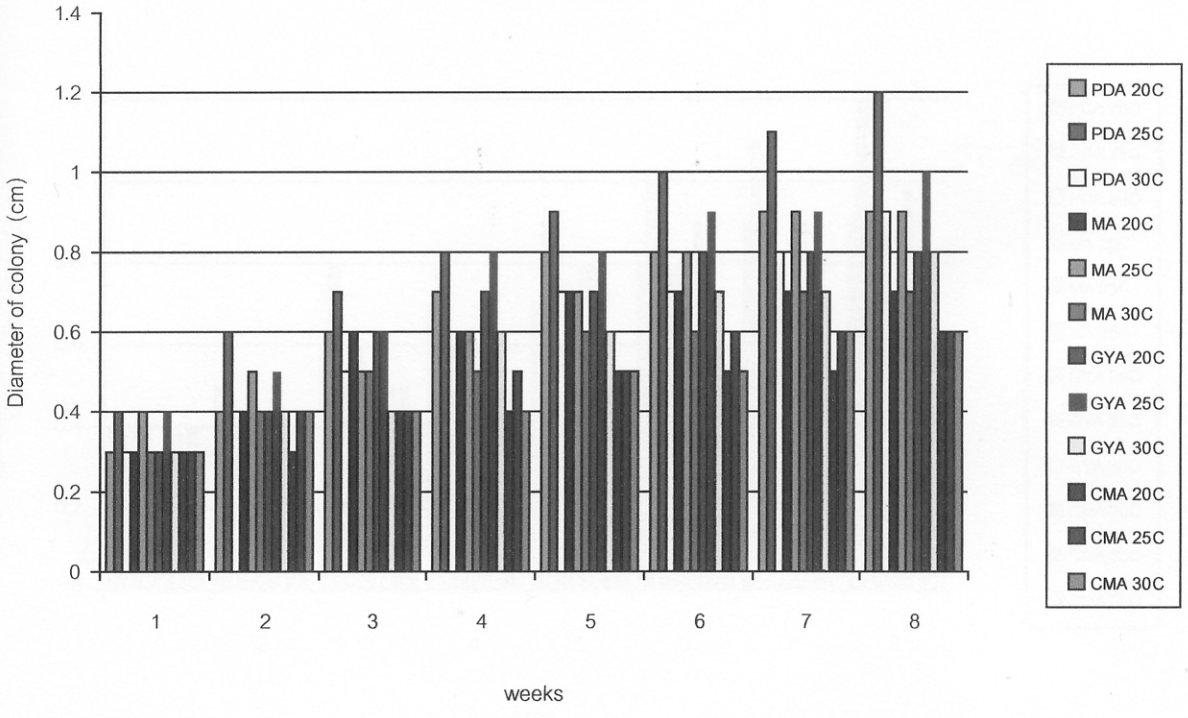

Figure 4.11 Growth of *H. scutata* SSC33 on four different media at 20, 25, 30°C (PDA, potato dextrose agar; MA, malt extract agar; GYA, glucose yeast extract agar; CMA, corn meal agar)

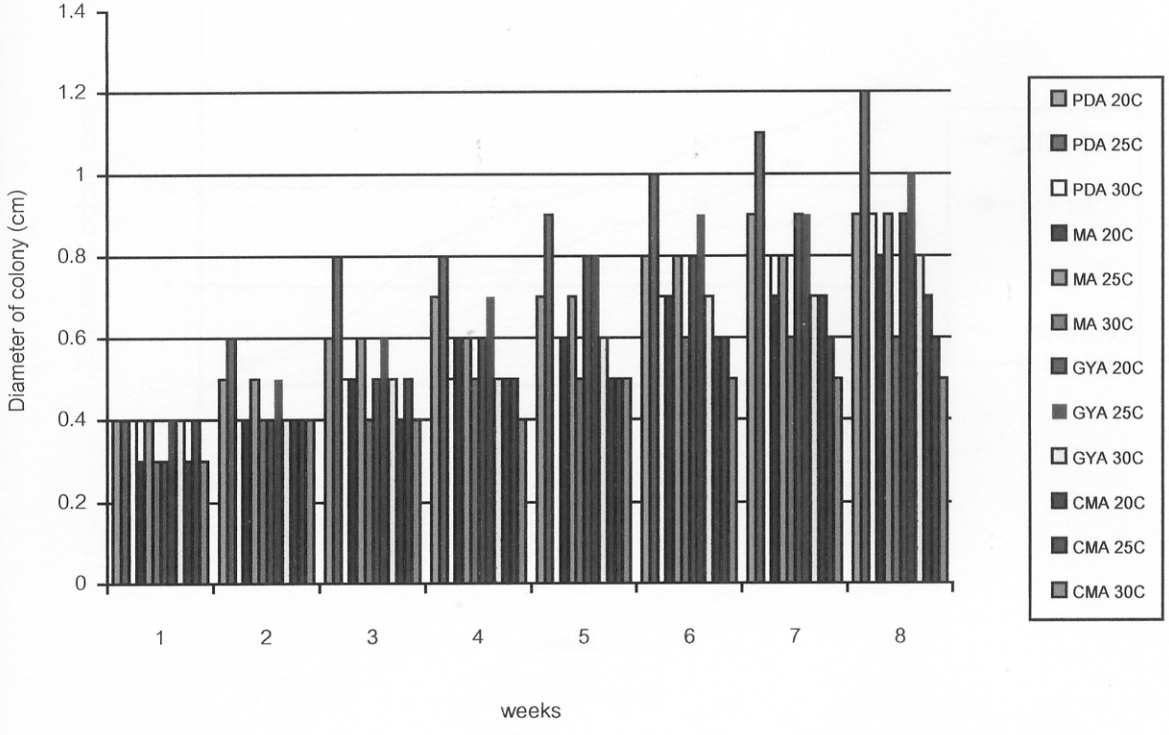

Figure 4.12 Growth of *H. scutata* SSC47 on four different media at 20, 25, 30°C (PDA, potato dextrose agar; MA, malt extract agar; GYA, glucose yeast extract agar; CMA, corn meal agar)

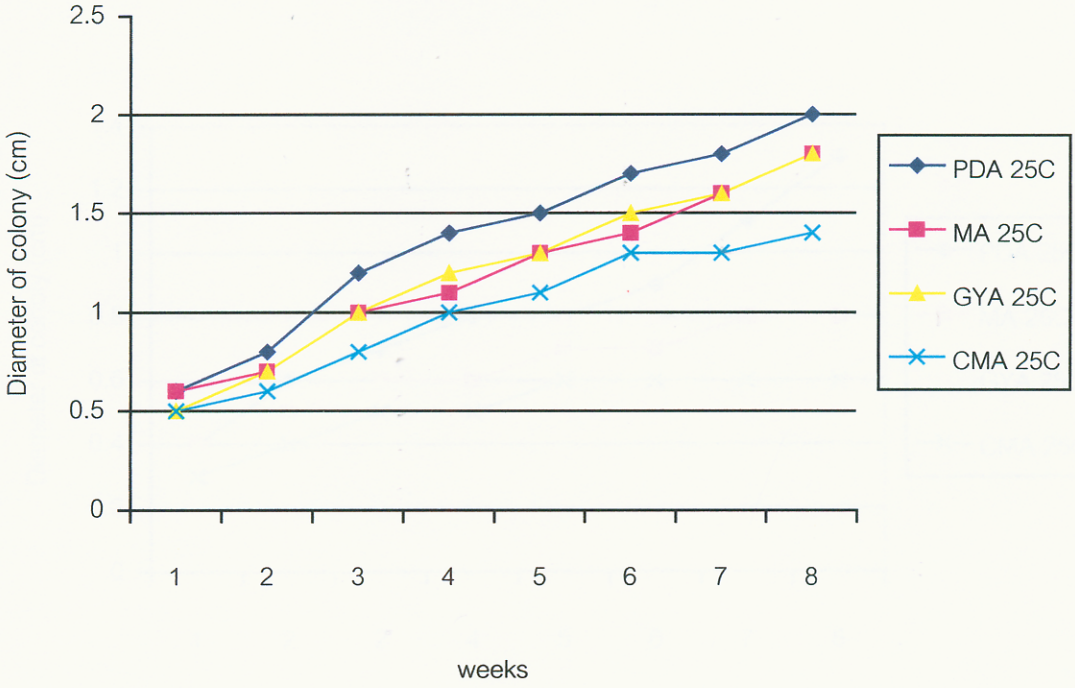

Figure 4.13 Growth of *H. schizostachyi* NHJ 4547 on four different media at 25°C (PDA, potato dextrose agar; MA, malt extract agar; GYA, glucose yeast extract agar; CMA, corn meal agar)

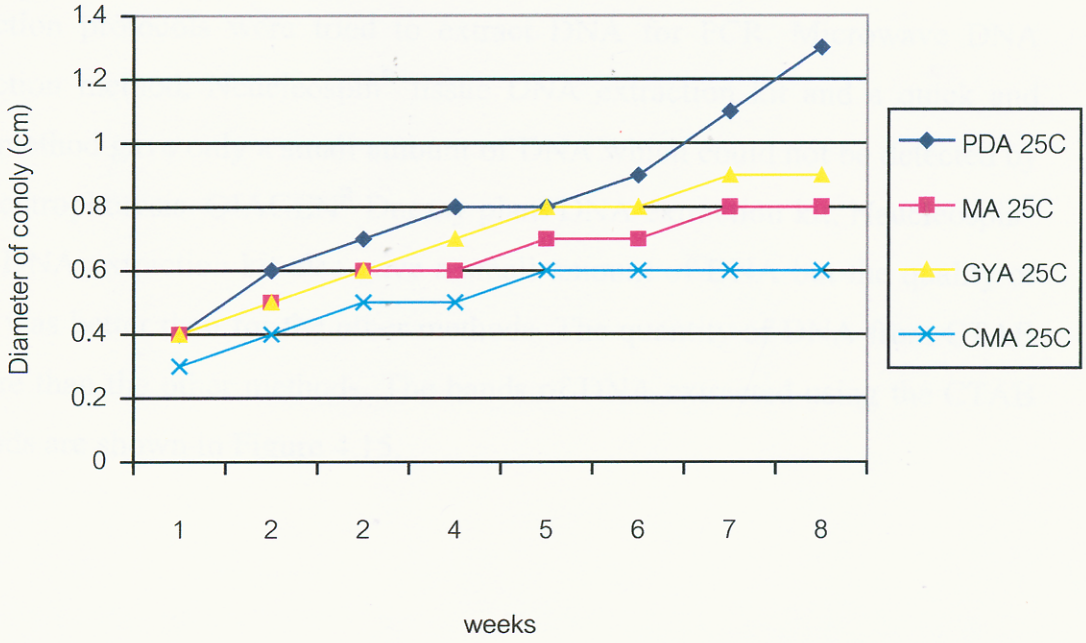

Figure 4.14 Growth of *H. scutata* SSC32 on four different media at 25°C (PDA, potato dextrose agar; MA, malt extract agar; GYA, glucose yeast extract agar; CMA, corn meal agar)

4.5 DNA extraction

Artjariyasripong (1999) and more recently Spatafora (Hywel-Jones, pers. comm.) experienced difficulties in securing PCR products of *H. schizostachyi*. In my study, I experienced the difficulties as well. The fungal mycelium was harvested from 3-4 week old culture in PDB. Many DNA extraction protocols were tried to extract DNA for PCR. Microwave DNA extraction method, Neucleospin^{R} tissue DNA extraction kit and a quick and dirty method gave only a small amount of DNA which could not be detected by gel electrophoresis. QIAGEN^{R} Dneasy plant DNA extraction kit, Neucleospin^{R} plant DNA extraction kit also gave a small amount of DNA but the quality of DNA was better than for the other methods. The quantity of DNA from CTAB is more than the other methods. The bands of DNA extracted using the CTAB methods are shown in Figure 4.15.

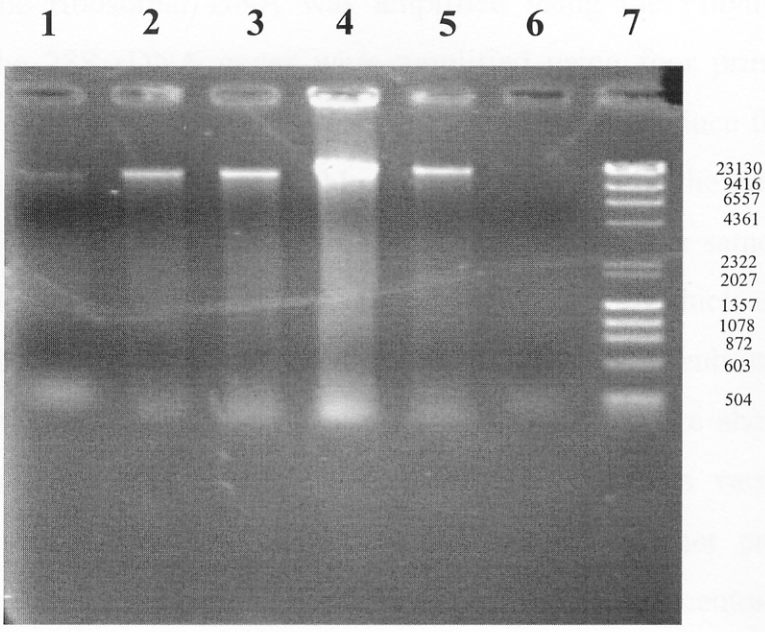

Figure 4.15 The genomic DNA from CTAB DNA extraction method

Lane 1: *H. discoidea* NHJ 5004

Lane 2: *H. schizostachyi* NHJ 4547

Lane 3: *H. scutata* SSC 46

Lane 4: *H. scutata* SSC 47

Lane 5: *H. scutata* SSC 57

Lane 6: *H. scutata* (Microwave method directly from stroma)

Lane 7: DNA marker (Finnzymes)

4.6 Fungal rDNA amplification

The ribosomal DNA was amplified using the Polymerase Chain Reaction. The 28S rDNA genes were amplified using four primers that are LROR, LR7, JS1 and JS8. The 5' end region was studied since this has more variable regions than the more highly conserved 3' end of the gene (White *et al.*, 1990). The molecular size of this PCR product was the same (1353 base pairs) for all taxa used in my study. The bands of PCR products are presented in Figure 4.16. The PCR primer used to amplify the nuclear sub-unit ribosomal rDNA genes (the 18S, ITS1-5.8S-ITS2, and 28S rDNA) are shown in Table 3.2. The conditions for amplification using these primers varied for each primer. The most important difference between the primer pairs was the temperature used for annealing (White *et al.*, 1990). The concentration of DNA did not have much effect on amplification because PCR has high sensitivity. The genes were successfully amplified using between 0.1-550 ng of template DNA. All the reactions performed in this study used the lower concentrations of template DNA. The high concentration of template DNA caused many reactions to fail may be due to the contamination of polysaccharides (Artjariyasripong, 1999). *H. scutata* and *H. schizostachyi* produced high levels of polysaccharides which are difficult to remove in the media. High levels of polysaccharides are known to inhibit PCR amplification from genomic DNA because it binds to the DNA and prevents good annealing of the primers (Foster *et al.*, 1993; Landvik, 1996). One way to avoid the production of polysaccharides is to culture these two species on the solid media where it is easy to harvest mycelium, but agar complicates DNA extraction. Siefert (2003) introduced a novel method of growing fungi for DNA extraction using reverse agar (BASF puronic polyol F-127). The reverse agar is solid at normal room temperature, but liquid at 4°C. This properties allow the seperation of mycelium and medium by simply placing a mature culture in a refrigerator.

The other way to reduce polysaccharide production is to harvest the mycelium quickly before the polysaccharides are produced in quantity. Washing the mycelium with hot water removes some polysaccharides and is a useful step to include prior to DNA extraction. The reagents have many effects on PCR amplification, especially the concentration of primers, magnesium, deoxynucleotide triphosphates, and *Taq* polymerase (Innis and Gelfand, 1990). The conditions of use for all these reagents were optimised for each primer pair.

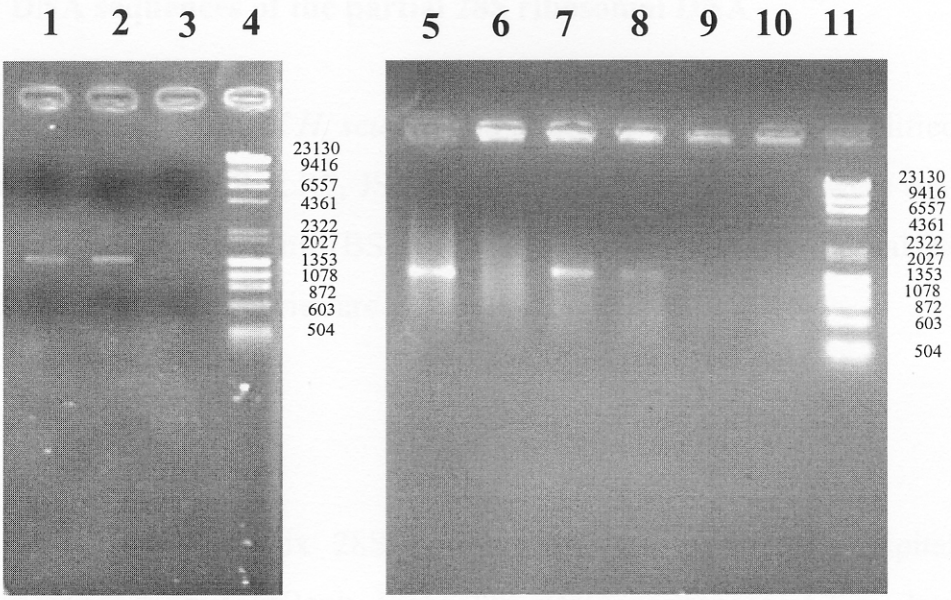

Figure 4.16: PCR-amplified 28S rDNA fragments of *H. scutata* SSC 57 and *H. schizostachyi* NHJ 4547.

Lane 1: *H. discoidea* NHJ 5004

Lane 2: *H. schizostachyi* NHJ 4547

Lane 3: *H. scutata* SSC 57

Lane 4, 11: DNA marker (Finnzymes

Lane 5: *H. discoidea* NHJ 5004

Lane 6: *H. scutata* SSC 46

Lane 7: *H. scutata* SSC 57

Lane 8: *H. schizostachyi* NHJ 4547

Lane 9: *H. scutata* SSC 46 (1)

Lane 10: *H. scutata* SSC 46 (2)

4.7 DNA sequences of the partial 28S ribosomal DNA

The DNA of *H. scutata* and *H. schizostachyi* were amplified by PCR using primers LROR, LR7, JS1 and JS8. The PCR products were sent directly to BIOTEC Service Unit (BSU) for DNA sequencing. The partial 28S rDNA sequences of these species are shown in Appendix 5.

4.8 Alignment

The thirty-six 28S rDNA sequences of the Clavicipitales were downloaded from GenBank. Eight 28S rDNA sequences of the Clavicipitales were kindly provided by Dr. Julian Mitchell, Portmounth University (Appendix 6). The nucleotide sequences were aligned using the BioEdit multiple sequences alignment programme.

The alignment of 46 Hypocreales as show below

```
46 1454

Hyp dis    ---------- ---------- -----CTGAA CTTAAGCATA TCAATAAGCG
Asc bad    ---------- ---------- -----CTGAA CTTAAGCATA TCAATAAGCG
Asc sam    ---------- ---------- -----CTGAA CTTAAGCATA TCAATAAGCG
Hyp sch    ---------- ---------- ---------- --------TA TCAATAAGCG
Hyp scu    ---------- ---------- ---------- ---------- -----CAATC
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------

Hyp dis    GAGGAAAAGA AACCAACAGG -ATTGCCCCA GTAACGGCGA GTGAA-----
Asc bad    GAGGAAAAGA AACCAACAGG GATTGCCCCA GTAACGGCGA GTGAA-----
Asc sam    GAGGAAAAGA AACCAACAGG GATTGCCCCA GTAACGGCGA GTGAA-----
Hyp sch    GAGGAAAAGA AACCAACAGG GATTGCCCCA GTAACGGCGA GTGAA-----
Hyp scu    GCGGGGAAAT ACTCCACACG GATTGCCCTA GTCACGGTGA GTGAA-----
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
```

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Asc hyp   ---------- ---------- ---------- ---------- ----------
Cor myr   ---------- ---------- ---------- ---------- ----------
Cor uni   ---------- ---------- ---------- ----------
Gib lei   ---------- ---------- ---------- ---------- ----------
Asc oxy   ---------- ---------- ---------- ---------- ---------T

Hyp dis   ------GCGG CAACAGCTCA AATTTG-AAA TCTGCCC--- TCC-----GG
Asc bad   ------GCGG CAACAGCTCA AATTTG-AAA TCTGCCC--- TCC-----GG
Asc sam   ------GCGG CAACAGCTCA AATTTG-AAA TCTGCC---- TCG-----GG
Hyp sch   ------GCGG CAGCAGCTCA AATTTG-AAA TCTGGCG--- CCC---CCCG
Hyp scu   ------GCGG TAACAGCTCA AATTTG-AAA TCTGGC---- TCTTT----C
Cor com   ---------- -----CTTTC ATATTG-ATA TCCGGT---- CCACC-----
Cor bru   ---------- ---------- ---------- ---------- -------CGC
Cor ira   ---------- ---------- ---------- ---------- ----------
Asc hyp   ---------- -AACTTCTCC TACCTG-ATA TATGT----- TCCCTCCACG
Cor myr   ---------- ---------- ---------- ---------- ----------
Cor uni   -AAACCGTGN AACTTA-TCA TACCTGGATA CCTGGC---- GC--------
Gib lei   ----TTATGC TACCCGCTTA TGTTAAGCTA GGCGGT---- G---------
Asc oxy   GAACTTAAGC TACCCGCTGA ACTTAAGCTA CCCGGT---- G---------

Hyp dis   GGGGGCCCGA GT-TGTAATT TGCA-GAGGA TGCT-TCTGG CGAGGTGCCT
Asc bad   GGGGGCCCGA GT-TGTAATT TGCA-GAGGA TGCT-TCTGG CGAGGTGCCT
Asc sam   GGGGGCCCGA GT-TGTAATT TGCA-GAGGA TGCT-TCTGG CGAGGTGCCT
Hyp sch   GGGAGCCCGA GT-TGTAGTT TGCA-GAGGA TGCT-TTTGG CGAGGCGCCT
Hyp scu   A-GGGTCCGA GT-TGTAATT TGCA-GAGGG CGCT-CTGGC TTTGGCACGC
Cor com   -GCGGCCCGA GT-TGTAATC TGCA-GGGGA TGCT-TCTGG CGACGCGCCT
Cor bru   GGTGGCCCTT GT-TGTAATT TGCA-GAGGA TGCT-TTTGG CGCGGCGCCC
Cor ira   --CCAGCCCG AT-TGTAATT TGCA-GAGGA TGCT-TTTGG CGACGCGCCT
Asc hyp   GTGGGCCCGA GT-TGTAATT TGCAACANGA TGCT-TTTGG CGAGGTGCCT
Cor myr   --AGGGCCCG AT-TGTAATT TGTA-GAGGA TGCT-TTTGG CGAGGCGCCT
Cor uni   --AAGCCCGG AT-TGTAATT TGCAGCAGGA TGCTTCTGGG CAGCGGCCGT
Gib lei   --ACCTTGA- -T-TGTGATC TGCAACAGGA TGCC-CTGGG CGCGGT-CCT
Asc oxy   --GGGTTAAG CT-AGTAGTT TGCC-TTGGA TGCT-TCTGG CAAGGTGCCT


Hyp dis   --TCCGAGTT CCCTGGAACG GGACGCCACA GAGGGTGAGA GCCCCGTCT-
Asc bad   --TCCGAGTT CCCTGGAACG GGACGCCATA GAGGGTGAGA GCCCCGTCT-
Asc sam   --TCCGAGAT CCCTGGAACG GGACGCCACA GAGGGTGAGA GCCCCGTCT-
Hyp sch   --TCCGAGTT CCCTGGAACG GGACGCCGCA GAGGGTGAGA GCCCCGTCT-
Hyp scu   GGTCCAAGTT CCTTGGAACA GGACGTCACA GAGGGTGAGA ATCCCGTACG
Cor com   --TCCGAGTT CCCTGGAACG GGACGCCATA GAGGGTGAGA GCCCCGTCC-
Cor bru   --TCCGAGTT CCCTGGAACG GGACGCCGGA AAGGGTGAGA GCCCCGTAC-
Cor ira   --TCCGAGTT CCCTGGAACG GGACGCCATA GAGGGTGAGA GCCCCGTCT-
Asc hyp   --TCCGA-TT CCCTGGA-CG GGACGCCACA GAGGGTGAGA -CCCCGTCT-
Cor myr   --TCCGAGTT CCCTGGAACG GGACGCCACA GAGGGTGAGA GCCCCGTCT-
Cor uni   --TCTAAGTT CCTTGGAACA GGACGTCATA GAGGGTGAGA ATCCCGTATG
Gib lei   --TCCGAGTT CCCTGGAACG GGACGCCACA GAGGGTGAGA GCCCCGTCCG
Asc oxy   --TCCGAGTT CCCTGGAACG GGACGCCGCA AAGGGTGAGA GCCCCGTCTG

Hyp dis   --GGTCGGAC A-CCGAGCCT CTGTG--AAG CTCC-TTCGA CGAGTC-GAG
Asc bad   --GGTCGGAC AACCGAGCCT CTGTG--AAG CCCC-TTCGA CGAGTC-GAG
Asc sam   --GGTCGGAC A-CCGAGCCT CTGTG--AAG CTCC-TTCGA CGAGTC-GAG
Hyp sch   --GGTCGGAC G-CCGAGCCT CTGTA--AAG CTCC-CTCGA CGAGTC-GAG
Hyp scu   -TGGTCGC-T AGCTATTGCC GCGTA--AAG CCCCCTTCTA CGAGTCCGAG
Cor com   --GGTCGGAC G-CCAAGCCT CTGTG--AAG CTCC-TCCGA CGAGTC-GAG
Cor bru   --GGTGGGAC G-CCTA-CCT CTGTA--AAG CTCC-TTCGA CGAGTC-GAG
Cor ira   --GGTTGGAT G-CCGA-CCT CTGTA--AAG CTCC-TTCGA CGAGTC-GAG
Asc hyp   --GGTCGGAC G-CCGA-CCT CTGTA--AAG CTCC-TTCGA CGAGTC-GAG
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```
Cor myr   --GGTCGGAT G-CCAAGCCT ATGTA--AAG CTCC-TTCGA CGAGTC-GAG
Cor uni   T--GACCGGC G-CGCACCCT CCACG--TAG CTCC-TTCGA CGAGTC-GAG
Gib lei   ---GTCGGAC G-CCATGCCC GTGTA--GGG TTCC-TTCGA CGAGTC-GAG
Asc oxy   ---GTCGGTC A-CCGAGCCT CTGTG--AAG CTCC-TTCGA CGAGTC-GAG

Hyp dis   TAGTTTGGGA ATGCTGCTCT AAATGGGAGG TATATGTCTT CTAAAGCTAA
Asc bad   TAGTTTGGGA ATGCTGCTCT AAATGGGAGG TATATGTCTT CTAAAGCTAA
Asc sam   TAGTTTGGGA ATGCTGCTCT AAATGGGAGG TATATGTCTT CTAAAGCTAA
Hyp sch   TAGTTTGGGA ATGCTGCTCT AAACGGGAGG TATATGTCTT CTAAAGCTAA
Hyp scu   TTGTTTGGGA ATGCAGCTCT AAATGGGAGG TAAATTTCTT CTAAAGCTAA
Cor com   TAGTTTGGGA ATGCTGCTCA AAGCGGGAGG TGTATGTCTT CTAAAGCTAA
Cor bru   TAGTTTGGGA ATGCTGCTCA AAACGGGAGG TATATGTCTT CTAAAGCTAA
Cor ira   TAGTTTGGGA ATGCTGCTCT AAATGGGAGG TATATGTCTT CTAAAGCTAA
Asc hyp   TAGTTTGGGA ATGCTGCTCT AAATGGGAGG TATATGTCTT CTAAAGCTAA
Cor myr   TAGTTTGGGA ATGCTGCTCA AAATGGGAGG TATATGTCTT CTAAAGCTAA
Cor uni   TTGTTTGGGA ATGCAGCTCT AAATGGGAGG TAAATTTCTT CTAAAGCTAA
Gib lei   TAGTTTGGGA ATGCTGCTCA AAGTGGGAGG TACACGTCTT CTAAAGCTAA
Asc oxy   TAGTTTGGGA ATGCTGCTCT AAATGGGAGG TATATGTCTT CTAAAGCTAA

Hyp dis   ATACCGGCCA G-AGACCGAT AGCGCACA-G TAGAGTGATC GAAAGATGAA
Asc bad   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Asc sam   ATATTGGCCA CCAGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Hyp sch   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Hyp scu   ATATTGGCCA CCAGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Cor com   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Cor bru   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Cor ira   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Asc hyp   AT-CCCGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Cor myr   ATATTGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Cor uni   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Gib lei   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA
Asc oxy   ATACCGGCCA G-AGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA


Hyp dis   AAGCACTTTG AAAAGAGGGT TAAACAGTAC GTGAAATTGT TGAAAGGG-A
Asc bad   AAGCACTTTG AAAAGAGGGT TAAACAGTAC GTGAAATTGT TGAAAGGG-A
Asc sam   AAGCACTTTG AAAAGAGGGT TAAACAGTAC GTGAAATTGT TGAAAGGG-A
Hyp sch   AAGCACTTTG GAAAGAGGGT TAAACAGTAC GTGAAATTGT TGAAAGGG-A
Hyp scu   AAGCACTTTG GAAAGAGAGT CAAACAGCAC GTGAAATTGT TGAAAGGG-A
Cor com   AAGCACTTTG GAAAGAGGGT TAAACAGTAC GTGAAATTGT TGAAAGGG-A
Cor bru   AAGCACTTTG AAAAGAGGGT TAAACAGTAC GTGAAATTGT TGAAAGGG-A
Cor ira   AAGCACTTTG AAAAGAGGGT TAAATAGTAC GTGAAATTGT TGAAAGGG-A
Asc hyp   AAGCACTTTG AAAAGGGGGT -AAACAGTAC GTGNAANTGT TGAAAGGGGA
Cor myr   AAGCACTTTG AAAAGAGGGT TAAAAAGTAC GTGAAATTGT TGAAAGGG-A
Cor uni   AAGCACTTTG GAAAGAGAGT TAAAAAGCAC GTGAAATTGT TGAAAGGG-A
Gib lei   AAGCACTTTG GAAAGAGGGT TAAAGAGCAC GTGAAATTGC TGAAAGGG-A
Asc oxy   AAGCACTTTG AAAAGAGGGT TAAACAGTAC GTGAAATTGT TGAAAGGG-A

Hyp dis   AGCGCTCACG ACCAGACCTG GT-CCCGGCG AATCAGC--- ---CGTTCTC
Asc bad   AGCGCTCATG ACCAGACCTG GT-CCCGGCG AATCAGC--- ---CGTTCTC
Asc sam   AGCGCTCACG ACCAGACCTG GT-CCCGGCG AATCACG--- ---CGTTCTC
Hyp sch   AGCGCTCGTG ACCAGACTCG GG-CGCGGCG GATCATCTCG GCGCCACGCG
Hyp scu   AGCGCTTGCA GCCAGACTT- GCTTGCAGTT GCTCATCCGG --GCTTT-TG
Cor com   AGCGCTCGTG ACCAGACTC- GGGCGCGGCG GATCA-CCGG ---CGTTCTC
Cor bru   AGCGCTCGTG ACCAGACTC- GGCCCCGGCG GATCATCCGG ---CGTTCTC
Cor ira   AGCGCTTATG ACCAGACGT- GGGCTCGGCG GATCATCCG- ---CGTTCTC
Asc hyp   AGCGCTTGCG AACAGACTCC GGGCCTTGCG GATCATNCCG ---CGTTCTC
Cor myr   AGCGCCTATT ACCAGACTT- GGGCCCGGTG AATCATCCA- ---CGTTCTC
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Cor uni   AGCGCTTGCA ACCAGACTC- CGCGGCGGTG TTCCGCC--- -GGTCTTCTG
Gib lei   A-CGCCCGCG ACCAGACTT- GGGCCCGGCA GGTCACCCA- -GGCTCTCGG
Asc oxy   AGCGCTCACG ACCAGACCT- GGTCCCGGCG AATCACCC-- -GGCGTTCTC

Hyp dis   GCC-GGTGCA C--TTCGACG GGCT-TCCAG GCCAGCATCA GTCCGCGCCG
Asc bad   GCC-GGTGCA C--TTCGACG GGCT-TCCAG GCCAGCATCA GTCCGCGCCG
Asc sam   GCC-GGTGCA C--TTCGACG GGCT-TCCAG GCCAGCATCA GTCCGCGCCG
Hyp sch   CCC-GGCGCA C--TTCGCCG GGCC---CGG GCCAGCATCG GTTCGCGCCC
Hyp scu   CCC-GGTGCA C--TCTTCTG T---AGGCAG GCCAGCATCA GTTTGGGCGG
Cor com   GCC-GGCGCA C--TCCGCCG C---GCCCGG GCCAGCGTCN GTTCCGGCG-
Cor bru   GCC-GGTGCA CT-CCCGCCC GGCG--CCNG GCCA-CATCG GTTCCCCCGC
Cor ira   CCC-G-TGCA CT-TC--CCC GGT---CCAG GCCA-CATCG GTTCCCCCG-
Asc hyp   GCC-NGTGCA C--TTCGGCG C----CCCGG GCCA-CATTA ACTTCCCCTT
Cor myr   GCT-GGTGCA CT-TT-GCCG GGC---ACAG CCAA-CATCA GTTTGGC-GC
Cor uni   ACC-GGTCCA CTCGCCGCCG TG------GG GCCAACATCG TCTGGGGCCG
Gib lei   GCCAGGGGCA CTCTGCCGGG CG-----CAG GCCAGCATCG GCTCGGCGCG
Asc oxy   GCC-GGTGCA CTTCGACGGG CTT---CCAG GCCAGCATCA GTCCGCGCCG

Hyp dis   -GGGGACAAA GGCGGC-GGG AACGTG--GC TCCCCAGGGA G--TG-TTAT
Asc bad   -GGGGACAAA GGCGGC-GGG AACGTG--GC TCCCCAGGGA G--TG-TTAT
Asc sam   -GGGGACAAA GGCGGC-GGG AACGTG--GC TCCCCAGGGA G--TG-TTAT
Hyp sch   TGGGGACAAA GGCGGC-GGG AACGTG--GT CCCCCAGGGG GGTTA-TAGC
Hyp scu   -TGGGATAAA GGTCTC-TGT CACGTACCTC TCTTCGGGGA GG-CC-TTAT
Cor com   -GGGGATAAA GGCCCCTGGA AACGTG--GC TTCCCAGGGA G--TG-TTAT
Cor bru   GGGGGATAAA GGCGCC-CGG AACGTG--GC TTCCCCGGGA G--TG-TTAT
Cor ira   -GGGGAGAAA GGCGTC-GGG AACCTG--GC TTCCTCGGAG G--GG-T-AT
Asc hyp   GGGGGAAAAA GGCCGC-NGG AATTTGG-CT TCCTTAGGGA G---GGTTAT
Cor myr   GGGGGATAAA AGGTTC-GGG AACCTG--GC TTCCTCNGGA G--TG-GTAT
Cor uni   -CCGGATAAG ACCCG--AGG AATGTA--GC TCATTGA--- ---TG-TTAT
Gib lei   -GGGGACAAA GGCGGC-GGG AACGTG--GC TTCTCAGGGA G--TGCTTAT
Asc oxy   -GGGGACAAA AGCGGC-TGG AACGTG--GC TTCCCANGGA G--TG-TTAT

Hyp dis   AGC-CCGCCG CGCAATGCCC CGGGGGCGGA CTGAGGACCG -CGCGTCACC
Asc bad   AGC-CCGCCG CGCAATGCCC CGGGGGCGGA CTGAGGCCCG -CGCGTCACC
Asc sam   AGC-CCGCCG CGCAATGCCC CGGG---CGA CTGAGGACCG --GCGTCACC
Hyp sch   CCCGCCGCCG CCATGCCCCC GGGGC-TGGG CCCAGGTTTT --CCCCTCCA
Hyp scu   AGG--GGAGA CGACATACCA CCAGCCTAGA CTGAGGTCCG -CGCAT----
Cor com   ACC--CGGCG CGCAATGCCC CGCGG-GGGA CCGAGGCCCG -CGCATTCC-
Cor bru   ACC--CGGCG CGCAAT-GCC CGCGGC-GGA CCGAGGTTCG -CGCACAC--
Cor ira   AGC--CCGTC CACAAT-CCC TTGGGC-GGA C-GAGGTTC- -CGCATCT--
Asc hyp   AGC--CGCCC NCCATGGCCC CCGGGCNGGC TTAAGCCTTG CCAATTTT--
Cor myr   ACC--CGTTG CGTAATACCC TGCCCC-CGA CTGANGTCC- --CCCTCC--
Cor uni   ACC-TCNGGG GATGCNGCGC C----NCCNG GCGAGGTCCG CG-CTTCN--
Gib lei   AGC-CCGCCG CGCCATGCCC C-GTGCCGGG CCGAAGCACG CG--CACC--
Asc oxy   AGC-CCGCCG GGCAATGCCC CCGGGGCNGA CTGAGGACCG TCGTCACC--

Hyp dis   GCAA--GGAT GCTGGCGTAA TGGTCTTCAG CGA-CCCGTC TTGAAACACG
Asc bad   GCAA--G-AT GCTGGCGTAA TGGTCATCAG CGA-CCCGTC TTGAAACACG
Asc sam   GCAA--G-AT GCTGGCGTAA TGGACTTCAG CGA-CCCGTC TTGAAACACG
Hyp sch   CCAC--GGAT GCTGGCCTTA ACGG------ ---------- ----------
Hyp scu   --GCTGATA- ---------- ---------- ---------- ----------
Cor com   ---CAAAGAC CCTTGCGTAA TGGTCACCAA CGA-CCCGTC TTGAAACACG
Cor bru   ---CCAAGGT TCTTGCGTTA TGGTCACCA- CGA-CCC-TC TTGNAACACG
Cor ira   ---TCAAGGA TCTGGCGTNA TGGTCATCAA CGA-CCG-TC TTGAAACACG
Asc hyp   ---CAANGGA TCTTGCCTNA TGGTCCCCAA GAA-CCCTTT TTGAA-CACG
Cor myr   ---GCAAGGA TCTTGCGTNA TGGTCATCAG CGA-CCCCTC TTTGAACACG
Cor uni   --GCAANGAT TTNGGCAGNA TGGNTGTCAT CGG-CC-GTC TNGAA-CACT
Gib lei   --GCAAGGAT GCTTGCGTAA TGGTCGCCGG CGA-CCCGTC TTGAAACACG
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Asc oxy    --GCAAGGAT GCTGGCGTAA TGGTCGTCAG CGA-CCCGTC TTGAAACACG

Hyp dis    GACCAAGGAG T-CGTCCTCG TATGCGAGTG TTGGGGCGTC AAACCCCCCG
Asc bad    GACCAAGGAG T-CGTCTTCG TATGCGAGTG TTGGGGCGTC AAACCCCC-G
Asc sam    -ACCAAGGAG T--GTCTTCG TATGCGA-TG TTGGGGCGTC AAACCCCC-G
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    GACCAANGAG T-CGTCCTCG TATGCGAGTG TTCGGGTGTC AA--CCCCTC
Cor bru    GAC-AAGGAA T-CGTCTTTT A-TGC-AATG TTCGGGCGTC AAACCCT--T
Cor ira    GCC-CAGGAG T-CTCTTCTT T-TGC-AATG GTCGGGTGTC AAACCCCC-T
Asc hyp    GACCAAGGAT --CGGCTTTC TATTCNAATG TTCNGGTTTA AAA-CCCT--
Cor myr    GAC-AAGGAG T-CGTCTTCG T-TGCCAATG TTCGGGTGTA AAANCCCT-A
Cor uni    GACCANGGAN TTTAACCATC TATGCNAAN- ---------- ----------
Gib lei    GACCAAGGAG --TCGTCCTC TATGCCAATG TTGGGGTGGC CAACCCC---
Asc oxy    GCCCAAGGA- --TCGTCCTC GATGCCAATG TTGGG-CGTA AACCCCC---

Hyp dis    TGCGAAA-TG AAA-GTGAAG --CTAG-GTG AGAGCCTGTT ACAGGGTGCA
Asc bad    CGCG-AA-TG AAA-GTGAAC --GCAG-GTG AGAGCTTCGG -----CCGGA
Asc sam    CGCG-AA-TG AAA-GTGAAC --G-CAGGTG AGAGCCTT-C G-CGGCCGGA
Hyp sch    CGCGTAA-TG AAA-GTGAAC --G-CGGGTG AGAGCTT--- ---CGGCGCA
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    CCCCCAATGA AAGTGAACCT ----T--GTG AAACCTT--- ----GGGGCA
Cor ira    CGCGCAAT-G AAAGT-AACT ----AGGTNG G-AGCTTTGG GG----GGCA
Asc hyp    CCCGTTATTG AAAGTGAACG ----CAGGTG A-AACTTTTG G------GCA
Cor myr    TCCCGGAATG AAATGAACCT T------GTN AAACTTT--- ------GGGC
Cor uni    CCCCTTATTN AAGTGNACCG ----CAGGTG AGAACTTTNG G------GCA
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    TACCCCATGN AAGCGAACGC -----AGGTT AAAGCCCAAA C-TTCGCGCA

Hyp dis    TCATCGACCG ATCCTTGATG TTCTCGGATG GATTTGAG-T AAGAGCATAC
Asc bad    TCATCGACCG ATCCT-GATG TTCTCGGATG GATTTGAG-T AGGAGCATAC
Asc sam    TCATCGACCG ATCCT-GATG TTCTCGGATG GATTTGAG-T AAGAGCATAC
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    TCACCGACCG ATCCC--GAT TTTTCGGACG GATTTAAT-- -AGGACATAC
Cor bru    CCATCGACCG ATC--TGATG TTTTCGGATG G-ATTGAG-T AAAA-CATAC
Cor ira    TCATCGACCG ATCC-TGATG TTTTTGGATG GGATTGAG-T AAAAACATAC
Asc hyp    ATATTNACCG ACCTATGTTT TGGATGGATT TATTGGACTT CGGGCCGCCC
Cor myr    TCATCGACCG ATCCTTGATG TTCTTGGATG G-ATTGAG-T AAAAACATAC
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    TCATTGACCG ATCT---GAT GTTTTGGATG GATTTAAT-- AGGAGCATAC
Asc oxy    TTAT-GACCG ATCT---GAT GTCCCGGATG GATTGAGT-- NGGAACTACN


Hyp dis    GGGGCCGGAC CCGAAAGAAG GTGAAC--TA TGCCTGTGTA GGGTGAAGTC
Asc bad    GGGGCCGGAC CCGAAAGAAG GTGAAC--TA TGCCTGTGTA GGGTGAAG-C
Asc sam    GGGGCCGGAC CCGTTTGAAG GTGAAC--TA TGCCTGTGTA GGGTGAAG-C
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    GGGGGCCGAC CCCAAA-AAA GTGAAC--TA TTCCTGCT-A NGGTGAAA-C
Cor bru    NGGGGCCGGA CCCCAAAGAA GGGAAC--TT TTCTTCCT-- AGGTGAAA-C
Cor ira    NGGGCCGGGA CCCCAAAAAA GGTAACCATT TNCTTTTTTA GGGTGAAA-C
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    CGGGGCCGGA CCCCAAAAAA GGGAAC--TA TGCCTGTTTT NGGGGAAA-C
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    GGGGCCGACC CCAAAA--AA GTGAAC--TT TGCCTTTTTA GGTGAAAC--
Asc oxy    GGGGCCGGAC CCAAAA--AA GGGA----CT TGCCTTNTTG GGGGAAAC--
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Hyp dis    CAGAGGAAAC TCTGGTGGAG GCTCGCAGCG GTTCTGACGT GCAAATTGAT
Asc bad    CAGAGGAAAC TCTGGTGGAG GCTCGCAGCG GTTCTGACGT GCAAATCGAT
Asc sam    CAGAGGAAAC TCTGGTGGAG GCTCGCAGCG GTTCTGACGT GCAAATCGAT
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    CCNAAGAAAC TTTGGGGGAG C-TTNCCACG GTTCT--ACT GCAAATGGTN
Cor bru    CCAAAGAAAC TTTTGTGGAG G-TCCCAACG GTTTT-ACTT C-AAATT-AT
Cor ira    CCAAGGAAAA CTTTGTGGAG GGTTTCAACG GTTTTTACTT CCAAATTGAT
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    CCAA-GAAAC TCTGGNGGAG G-TTTCNANG GGTTTTACTG C-GAATT-AT
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    CCAA-GGAAA TCTTGTGGAG GTTCCAACNG GTTCT-TACT GCGAATTGAT
Asc oxy    CCA--GGAAA CCTTGTGGAG GTCTCCAAGG GTTTT-AANG GCAAATGGTT

Hyp dis    CGTCAAACA- GGGCATGGGG GCGAAAGACT AATCGAACCT TCTAGTAGCT
Asc bad    CGTCGGGCAT GGGCATGGGG GCGAAAGACT AATCGAACCT TCTAGTAGCT
Asc sam    CGTCAAACAT GGGCATGGGG GCGAAAGACT AATCGAACCT TCTAGTAGCT
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    CGNAAACTNG GNNT------ ---------- ---------- ----------
Cor bru    TTTCAA-ACT TGGCTTNGGG GCGA------ ---------- ----------
Cor ira    TTTCAACAAT TGGCTTNGGG GGGNAAAAAA AAAN------ ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    CGTCAA-AAT TGGCTTGGGG GGNAAGAATA ATCNN----- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    GTCAAAATT- -GGCCTTGGG GGCAAAAACT TATTGAACCT TTTAAAACTG
Asc oxy    CGCAACAT-- -GGCCTTGGG GGNAAAA-CT TATTGAACCT TTTATA-CTG

Hyp dis    GGTTTCCGCC GAAGTTTCC- TCAGGATAGC AGTGCTG-AG CTCAGTTTTA
Asc bad    GGTTTCCGCC GAAGTTTCCC TCAGGATAGC AGTGTTG-AG CTCAGTTTTA
Asc sam    GGTTTCCGCC GAAGTTTCC- TCAGGATAGC AGTGCTG-AG CTCAGTTTTA
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    GGTTCCCCCG AAATTTCCTN ---------- ---------- ----------
Asc oxy    GGTN------ ---------- ---------- ---------- ----------

Hyp dis    TGAGGTAAAG CGAATGATTA GGGACCCGGG GGCGCATACT T-GCCTTCAT
Asc bad    TGAGGTAAAG CGAATGATTA GGGACCCGGG GGCGCATACT T-GCCTTCAT
Asc sam    TGAGGTAAAG CGAATGATTA GGGACCCGGG GGCGCATACT T-GCCTTCAT
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------
```

```
Hyp dis    CCATTCTCAA ACTTTAAATA TGTAAGAAGC CCTTGTTACT TAGCTGAACG
Asc bad    CCATTCTCAA ACTTTAAATA TGTAAGAAGC CCTTGTTGCT TGGCTGAACG
Asc sam    CCATTCTCAA ACTTTAAATA TGTAAGAAGC CCTTGTTACT TAGCTGAACG
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------

Hyp dis    TGGGCATTCG AATGTATCAG CACTAGTGGG CCATTTTTGG TAAGCAGAAC
Asc bad    TGGGCATTCG AATGTATCAA CACTAGTGGG CCATTTTTGG TAAGCAGA-C
Asc sam    TGGGCATTCG AATGTATCAG CACTAGTGGG CCATTTTTGG TAAGCAGA-C
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------

Hyp dis    TGGCGATGCG GGATGAACCG AACGCGAGGT TAAGGTGCCG GAGTGGACGC
Asc bad    TGGCGATGCG GGATGAACCG AACGCGAGGT TAAGGTGCCG GAGCGGACGC
Asc sam    TGGCGATGCG GGATGAACCG AACGCGAG-T TAAGGTGCCG GAGTGGACGC
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------

Hyp dis    TCATCA-GAC ACCACAAAAG GTGTTAGTAC ATCTTGACAG CAGGACGGTG
Asc bad    TCATCA-GAC ACCACAAAAG GTGTTAGTAC ATCTTGACAG CAGGACGGTG
Asc sam    TCATCA-GAC ACCACAAAAG GTGTTAGTAC ATCTTGACAG CAGGACGGTG
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------
```

```
Hyp dis    ---------- ---------- ---------- ---------- ----------
Asc bad    GCCATGGAAG TCGGAATCCG CTAAGGACTG TGTAACAACT CACCAGCCGA
Asc sam    GCCATGGAAG TCGGAATCCG CTAAGGACTG TGTAACAACT CACCTGCCGA
Hyp sch    GCCATGGAAG TCGGAATCCG CTAAGGACTG TGTAACAACT CACCTGCCGA
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------

Hyp dis    ATGTACTAGC CCTGAAAATG GATGGGCCTC AAGCGTCCCA CCCATACCTG
Asc bad    ATGTACTAGC CCTGAAAATG GATGGGCCTC AAGCGTCCCA CCCATACCTC
Asc sam    ATGTACTAGC CCTGAAAATG GATGGCGCTC AAGCGTCCCA CCCATACCT-
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------

Hyp dis    GCCCTCGGGG CAGGATCGAG GCCCCGAGGA GTAGGCGGAC GTGG------
Asc bad    GCC-TCGGGG CAG-ATCGAG GCCCCGCGGC GTAGGCGGAC GTGG------
Asc sam    ---------- ---------- ---------- ---------- ----------
Gib pul    ---------- ---------- ---------- ---------- ----------
Hyp sch    ---------- ---------- ---------- ---------- ----------
Hyp scu    ---------- ---------- ---------- ---------- ----------
Cor com    ---------- ---------- ---------- ---------- ----------
Cor bru    ---------- ---------- ---------- ---------- ----------
Cor ira    ---------- ---------- ---------- ---------- ----------
Asc hyp    ---------- ---------- ---------- ---------- ----------
Cor myr    ---------- ---------- ---------- ---------- ----------
Cor uni    ---------- ---------- ---------- ---------- ----------
Gib lei    ---------- ---------- ---------- ---------- ----------
Asc oxy    ---------- ---------- ---------- ---------- ----------
```

4.9 Phylogenetic relationship of *H. scutata* and *H. schizostachyi* and other true *Hypocrella* based on partial 28S rDNA sequences

A maximum parsimony analysis was performed on the 28S rRNA gene sequences for 46 taxa to investigate the relationship of *H. scutata* and *H. schizostachyi* and the true *Hypocrella* (*H. discoidea*). The DNA sequences obtained for two taxa (*H. scutata* and *H. schizostachyi*) were aligned with 1454 nucleotides representing the 28S rDNA from 44 other taxa. The combined data set contained 46 taxa including representations from the *Hypocreales* (*Clavicipitaceae* and *Hypocreaceae*). An initial heuristic maximum parsimony analysis on this data set produced three trees representing eleven tree islands. An heuristic search with random sequence addition on 100 replicates produced eleven tree islands with 478 parsimony-informative sites. The island with the smallest tree length contained three trees with a consistency index (CI) of 0.461, a retention index (RI) of 0.625, a re-scaled consistency index (RC) of 0.288 and a homoplasy index (HI) of 0.539. These trees are shown in Figure 4.17, 4.18 with the bootstrap supports. In order to reduce the number of trees certain taxa were removed from this data set. The removal of taxa was done systematically by identifying OTU's that did not form strong clades. This resulted in the removal of sixteen taxa including *Trichoderma koningii*, *T. strictipilis*, *Hypocrella* sp.GJS 89-104, *Aschersonia aleyrodis*, *Aschersonia goldiana*, *Aschersonia insperata*, *Aschersonia turbinata*, *Hypocrea lutea*, *Hypocrea gelatinosa*, *Aschersonia* sp. Ap isolate A28, *Aschersonia* sp. Ap isolate A31, *Gibellula leiopus*, *Aschersonia hypocreoidea*, *Aschersonia oxystoma*, *Cordyceps irangiensis* and *Cordyceps myrmecophila*. A maximum parsimony analysis, using a heuristic search with random sequence addition and 100 replicates, was then performed on the new data set of 30 taxa with *Gibberella* species as the outgroup. One most parsimonious tree, forming one tree island contained four trees with a consistency index (CI) of 0.584, a retention index (RI) of 0.684, a re-scaled consistency index (RC) of 0.399 and a

homoplasy index (HI) of 0.416, was obtained from this analysis. These trees are shown in Figure 4.19, 4.20 with the bootstrap supports. In the

The next analysis the *Cordyceps tuberculata*, *Cordyceps pseudomilitaris*, *Cylindrocladium floridanum* and *Nectria rigidiuscula* sequences were removed because in previous analysis they did not form a strongly supported clade. An heuristic search with random sequence addition on 100 replicates produced two tree islands with 478 parsimony-informative sites. The island with the smallest tree length contained seven trees with a consistency index (CI) of 0.614, a retention index (RI) of 0.686, a re-scaled consistency index (RC) of 0.421 and a homoplasy index (HI) of 0.386. These trees are shown in Figure 4.21, 4.22 with the bootstrap supports.

The next analysis was performed by removing *Hyperdermium bertonii* and *Hyperdermium pulvinatum*. One most parsimonious tree, forming one tree island contained four trees with a consistency index (CI) of 0.653, a retention index (RI) of 0.699, a re-scaled consistency index (RC) of 0.456 and a homoplasy index (HI) of 0.347, was obtained from this analysis. These trees are shown in Figure 4.23, 4.24 with the bootstrap supports. This resulted in the best tree in which *H. discoidea* and *Aschersonia samoensis* showed their strong teleomorph-anamorph relationship. *H. scutata* grouped with a mixed *Cordyceps* clade while *H. schizostachyi* grouped with an *Akanthomyces* group. Significantly, in all analyses they did not place in the same clade as the true *Hypocrella*. However, the other genes (18S, ITS1-5.8S-ITS2 rDNA gene) should be studied to support that *H. scutata* and *H. schizostachyi* are not placed in the genus *Hypocrella*.

Figure 4.17 Phylogram of 46 taxa of the Hypocreales

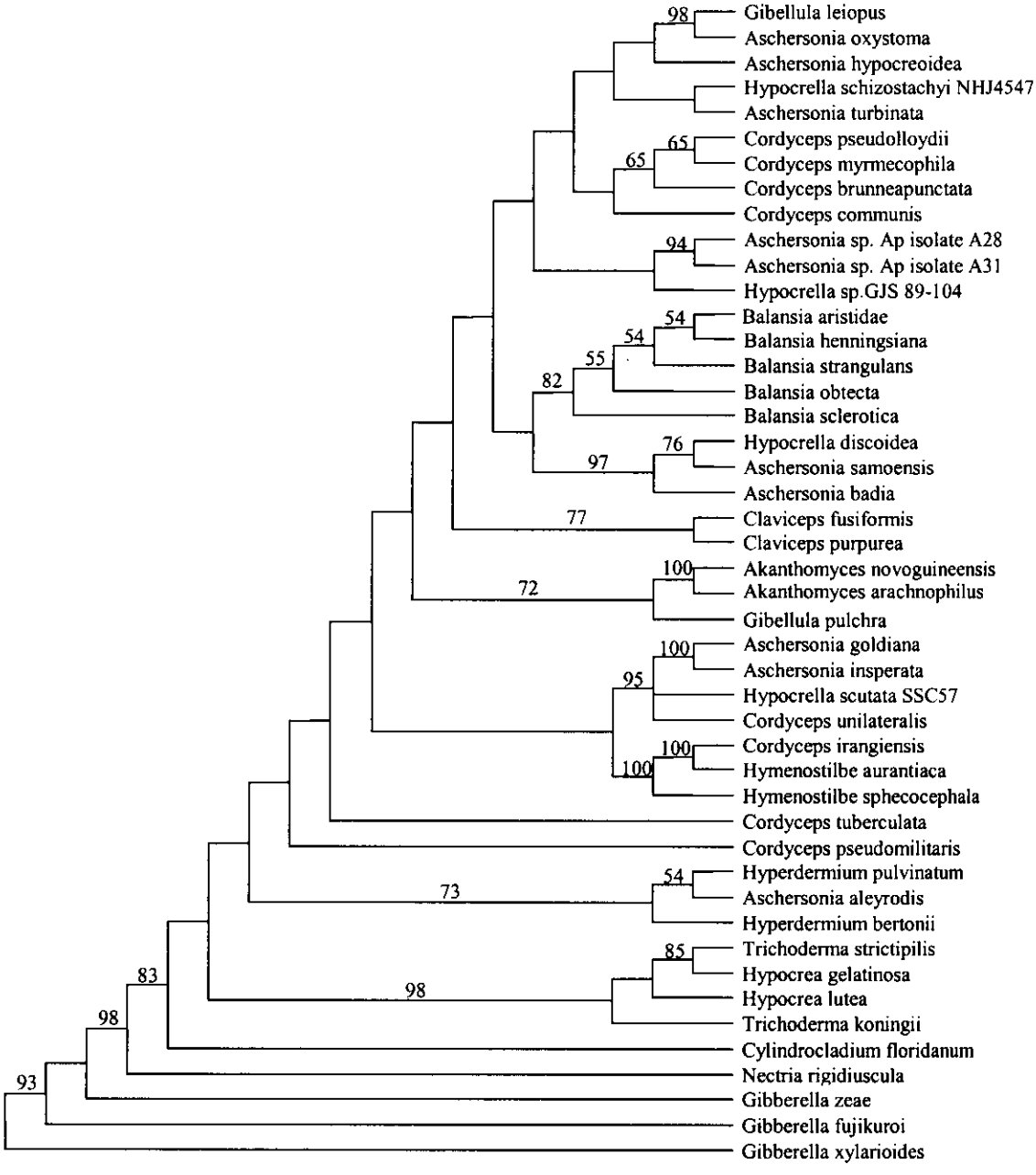

Figure 4.18 Strict consensus of 3 most parsimony phylogenetic tree with heuristic search based on partial 28S rDNA sequences from 46 taxa of Hypocreales. Bootstrap percentages based on 100 replications are shown over branches

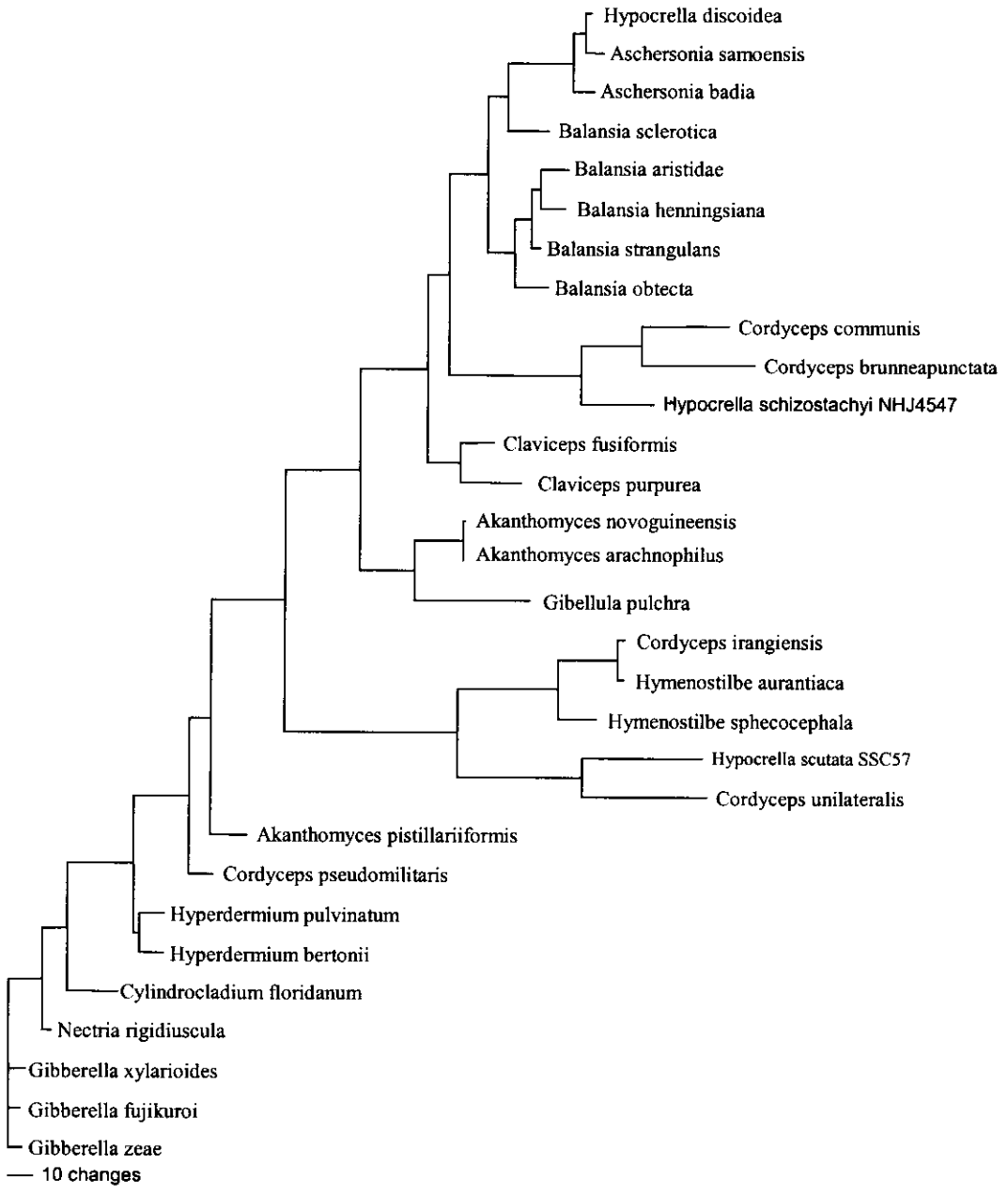

Figure 4.19 Phylogram of 30 taxa of Hypocreales

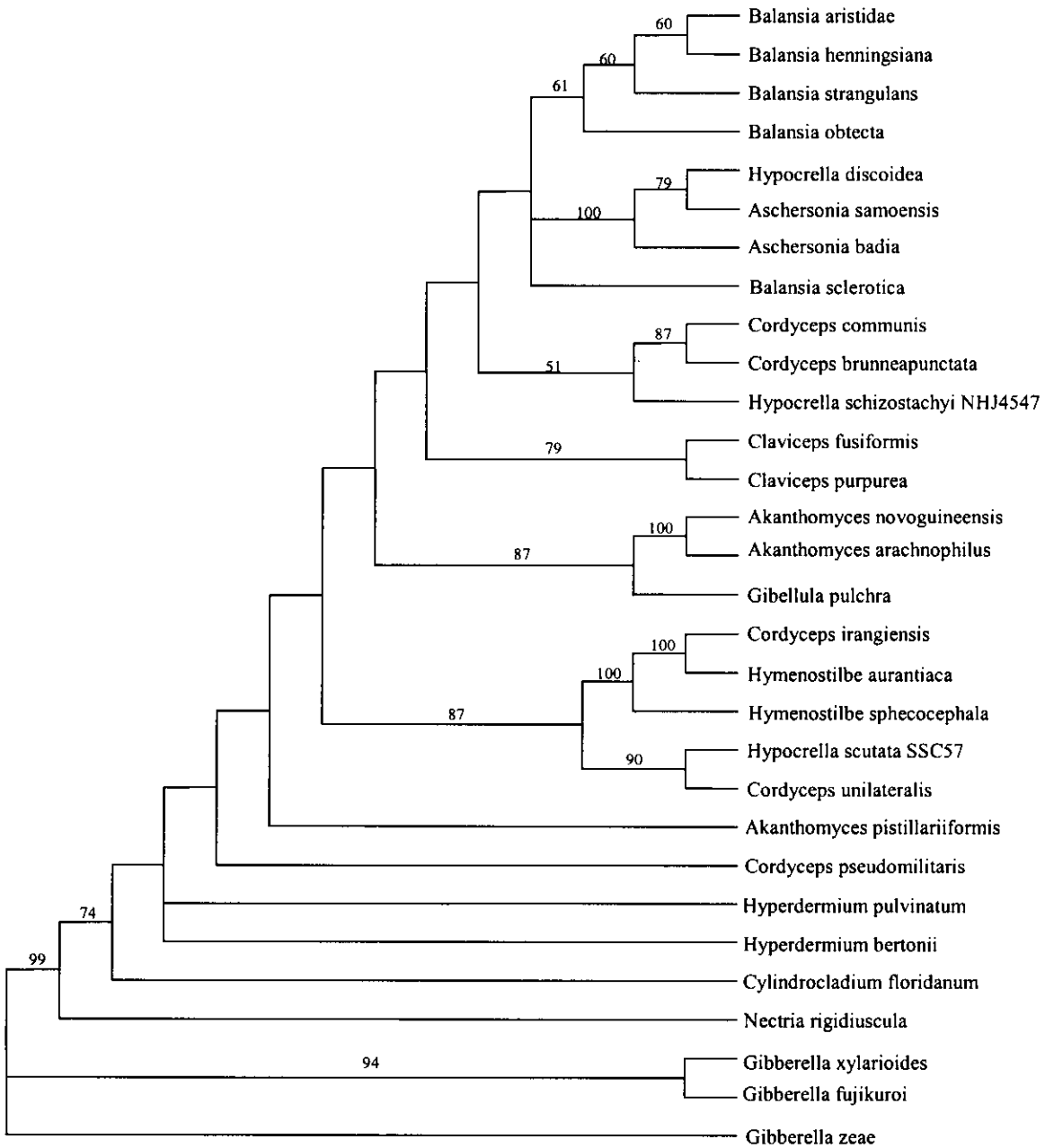

Figure 4.20 Strict consensus of 4 most parsimony phylogenetic tree with heuristic search based on partial 28S rDNA sequences from 30taxa of Hypocreales. Bootstrap percentages based on 100 replications are shown over branches

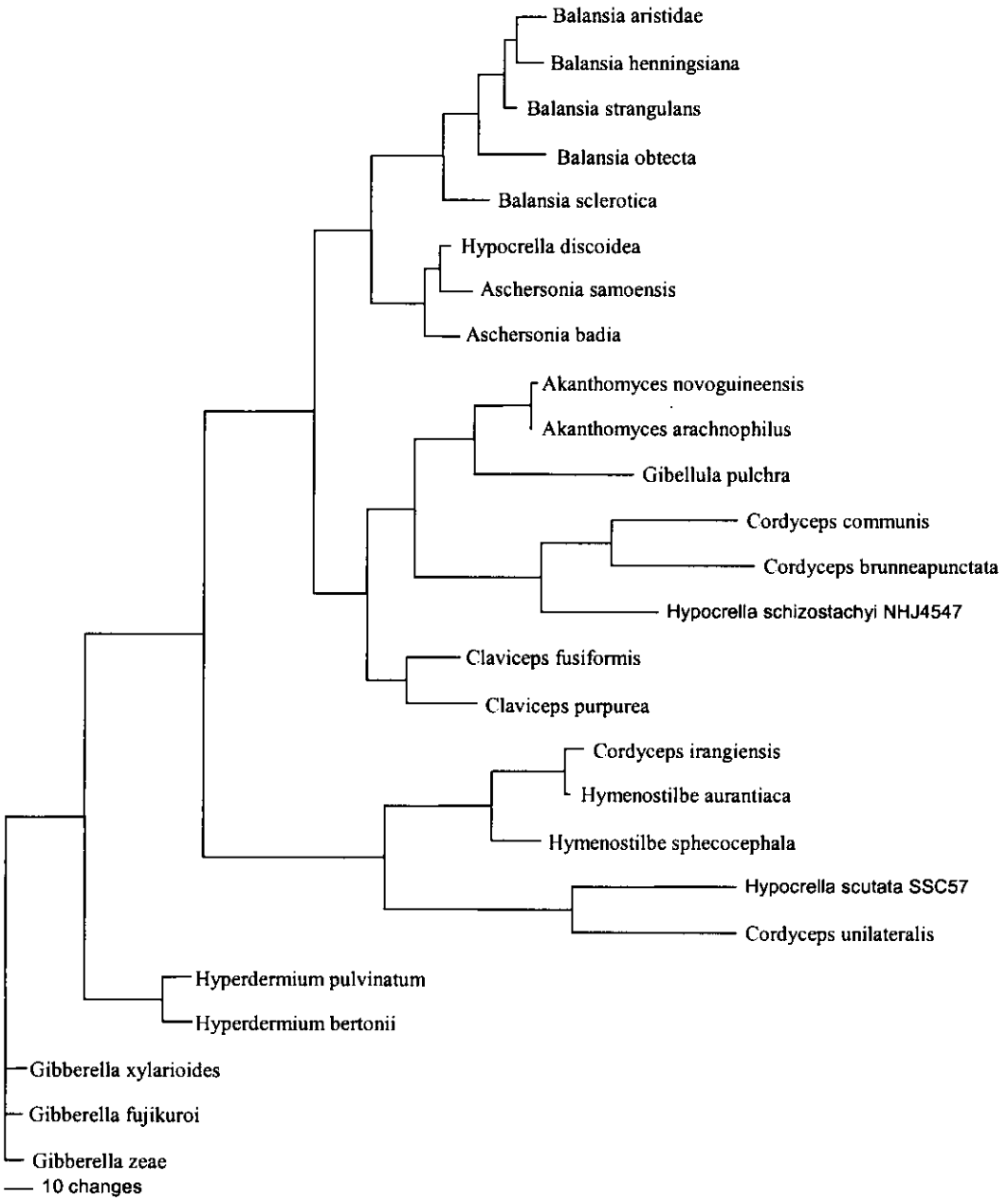

Figure 4.21 Phylogram of 26 taxa of Hypocreales

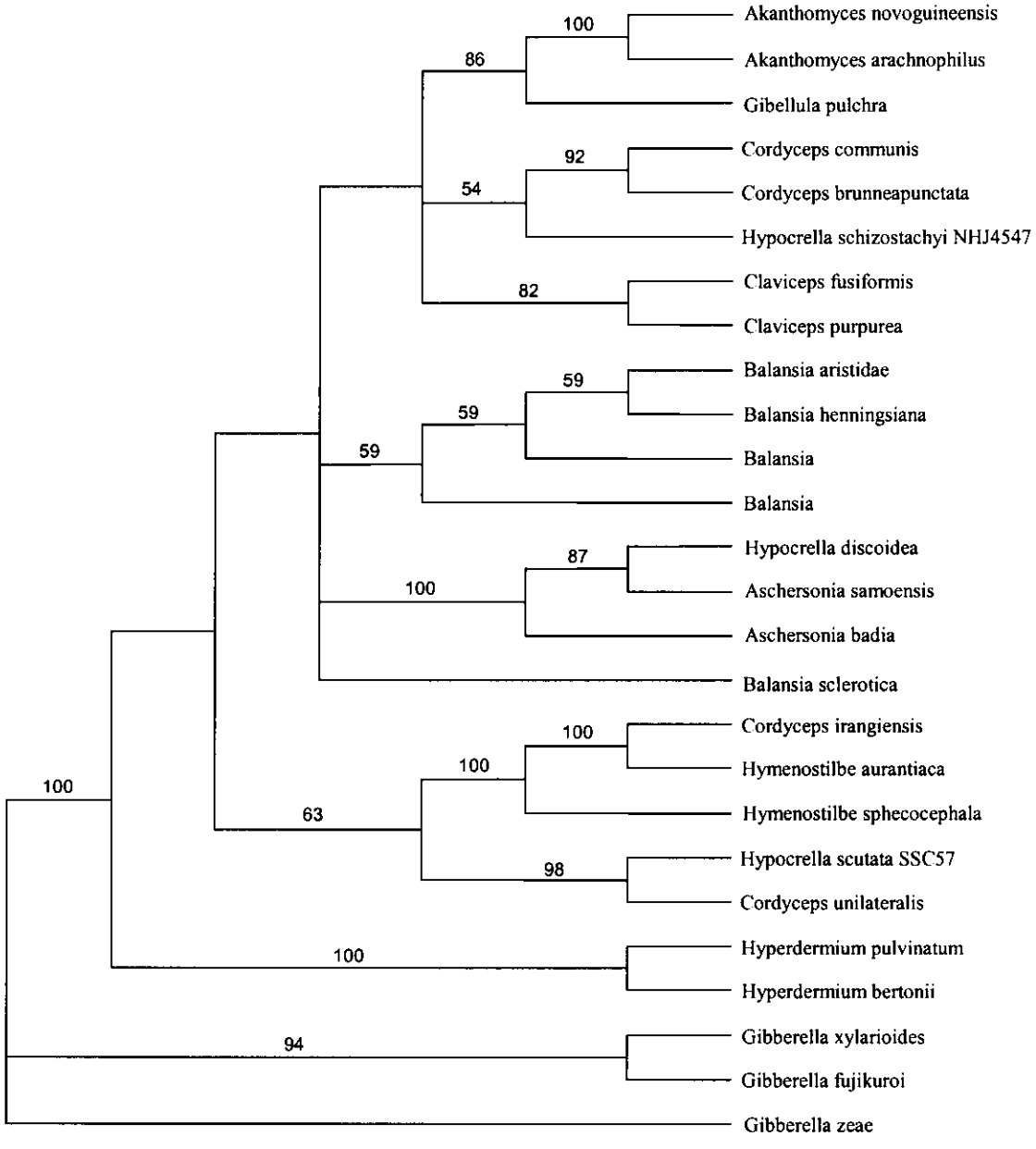

Figure 4.22 Strict consensus of 7 most parsimony phylogenetic tree with heuristic search based on partial 28S rDNA sequences from 26 taxa of Hypocreales. Bootstrap percentages based on 100 replications are shown over branches

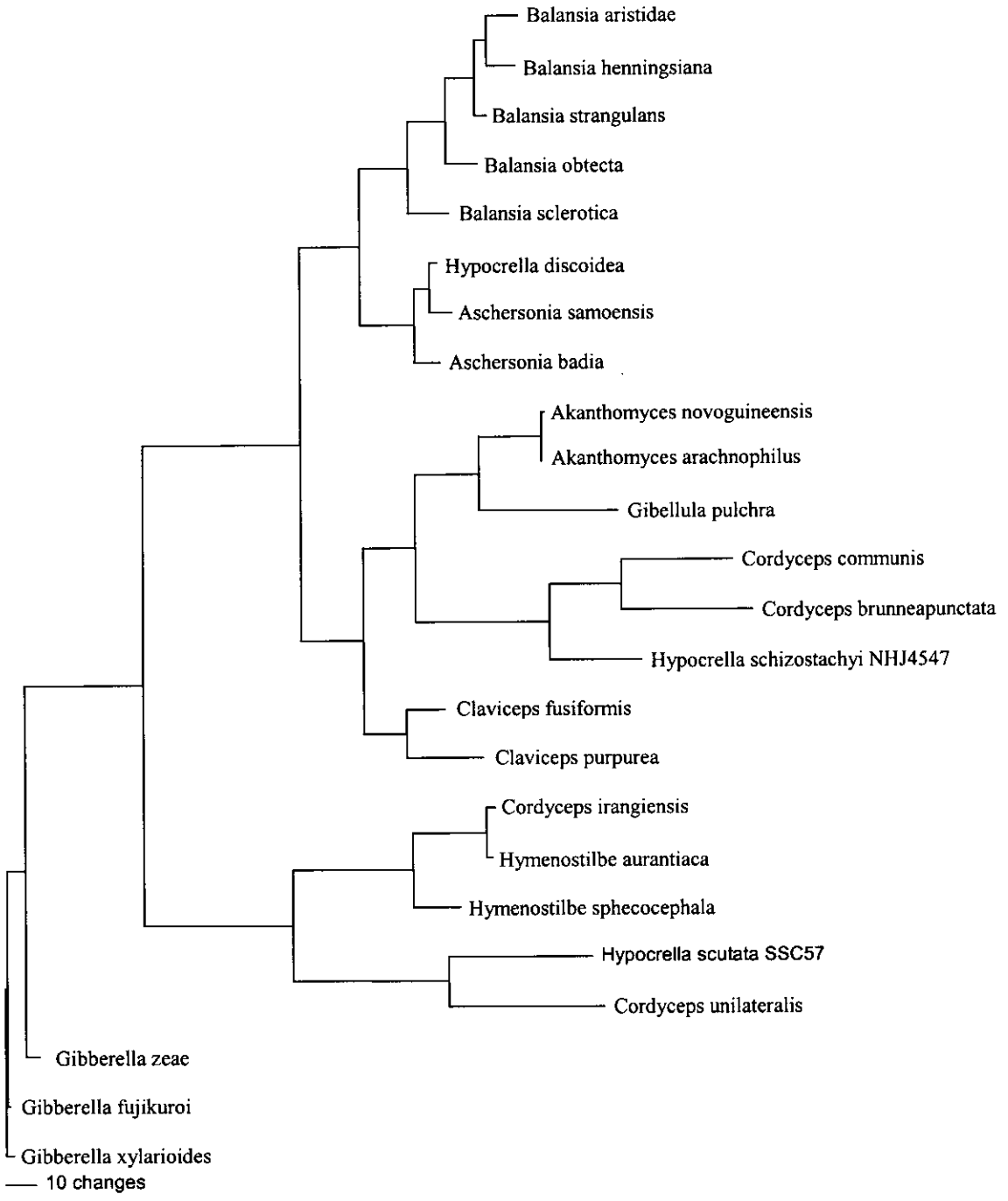

Figure 4.23 Phylogram of 24 taxa of Hypocreales

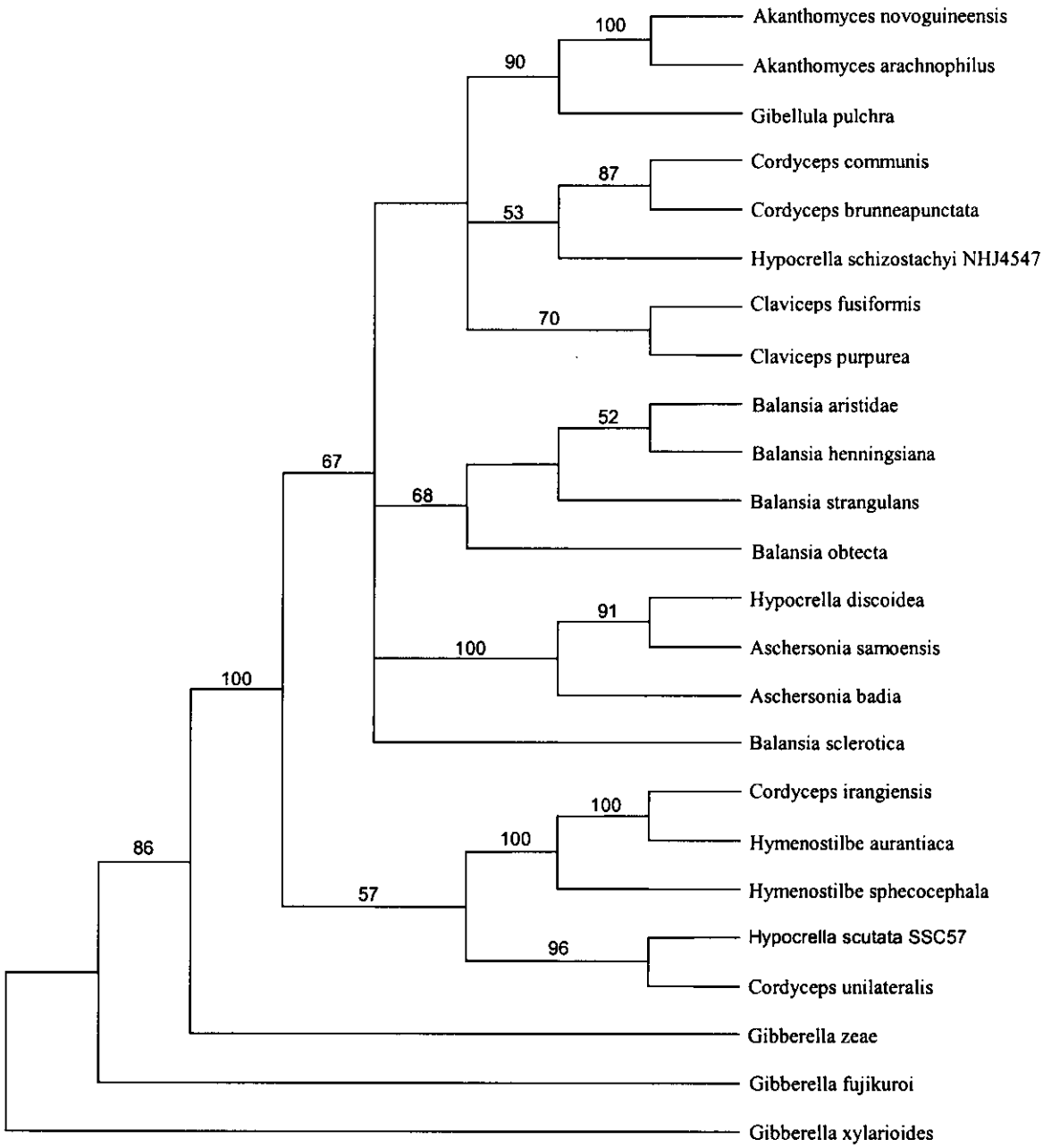

Figure 4.24 Strict consensus of 4 most parsimony phylogenetic tree with heuristic search based on partial 28S rDNA sequences from 24 taxa of Hypocreales. Bootstrap percentages based on 100 replications are shown over branches

level of branch. The high *H. scutata* occurrence on *S. tumida* may result in finding *H. scutata* at all branch levels. The level of branch on *S. oblatum* does not influence the occurrence of *H. scutata* because the numbers of trees which *H. scutata* occurred on was lower than those of which *H. scutata* does not occur. In the trees which *H. scutata* occurred, the level of branch influenced occurrence as the test of proportion of the *H. scutata* occurrence on each branch level was significantly different. The occurrence of *H. scutata* on high branch level is higher than the other levels. This result is the guideline for further survey of *H. scutata* in Sirindhorn Peat Swamp Forest.

The study of the growth of *H. scutata* and *H. schizostachyi* was performed to determine their optimum growth on selected media over a range of temperature. Optimum temperature for growth of *H. scutata* and *H schizostachyi* was between 20-25°C. *H. scutata* and *H. schizostachyi* grew on PDA quicker than MA, GYA and CMA. The medium for optimum growth depends on the temperature used. PDA composed high quantity of glucose in the media. Fungi can grow well on PDA and produce the secondary metabolites during their growth. When we used the PDB for biomass production, the polysaccharides were produced because fungi utilise glucose. The polysaccharides can inhibit the PCR. One way to solve this problem is to grow fungal mycelium in low glucose broth such as MA.

The relationship between the suspected species with known ones is still unclear. One way to improve the identification of these known and unknown isolates is to study the molecular variation in conserved DNA sequences. The phylogenetic studies indicated that *H. scutata* and *H. schizostachyi* did not place in the same clade as the true *Hypocrella*. *H. discoidea* and *Aschersonia samoensis* showed their strong teleomorph-anamorph relationship. The other genes (18S, ITS1-5.8S-ITS2 rDNA gene)

should be studied to support that *H. scutata* and *H. schizostachyi* are not placed in the genus *Hypocrella*.

The classical taxonomy showed that the conidial morphology of the anamorph of *H. scuata* and *H. schizostachyi* are definitely different from *H. discoidea*. Moreover, the preliminary phylogenetic studies showed that *H. scutata* and *H. schizostachyi* did not place in the same clade as *H. discoidea*. This supports the conclusion that *H. scutata* and *H. schizostachyi* should be classified to a new genus.