available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/mycres

Molecular Systematics of *Zopfiella* and allied genera: evidence from multi-gene sequence analyses

Lei CAI, Rajesh JEEWON, Kevin D. HYDE*

Centre for Research in Fungal Diversity, Department of Ecology and Biodiversity, The University of Hong Kong, Pokfulam Rd, Hong Kong SAR, PR China

ARTICLE INFO

Article history:
Received 22 June 2005
Received in revised form
10 December 2005
Accepted 4 January 2006
Published online 20 March 2006
Corresponding Editor:
David L. Hawksworth

Keywords:
Ascomycota
Lasiosphaeriaceae
Molecular phylogenetics
Sordariales
β-Tubulin

ABSTRACT

This study aims to reveal the phylogenetic relationships of *Zopfiella* and allied genera in the *Sordariales*. Multiple gene sequences (partial 28 S rDNA, ITS/5.8 S rDNA and partial β-tubulin) were analysed using MP and Bayesian analyses. Analyses of different gene datasets were performed individually and then combined to infer phylogenies. Phylogenetic analyses show that currently recognised *Zopfiella* species are polyphyletic. Based on sequence analyses and morphology, it appears that *Zopfiella* should be restricted to species having ascospores with a septum in the dark cell. Our molecular analysis also shows that *Zopfiella* should be placed in *Lasiosphaeriaceae* rather than *Chaetomiaceae*. *Cercophora* and *Podospora* are also polyphyletic, which is in agreement with previous studies. Our analyses show that species possessing a *Cladorrhinum* anamorph are phylogenetically closely related. In addition, there are several strongly supported clades, characterised by species possessing divergent morphological characters. It is difficult to predict which characters are phylogenetically informative for delimiting these clades.

© 2006 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.

Introduction

Zopfiella, a teleomorphic genus in the family *Lasiosphaeriaceae* (*Sordariales*, *Ascomycota*), was first established by Winter (1884) to accommodate two species, *Z. tabulata* and *Z. curvata*. *Zopfiella* species have been characterised by non-ostiolate ascomata, clavate to cylindrical, usually evanescent asci lacking an apical ring. Ascospores are ellipsoidal, dark brown, transversely septate, with a hyaline pedicel which often collapses (Guarro *et al.* 1991). The anamorphs of most *Zopfiella* species are unknown, with *Z. latipes* forming a *Humicola*-like anamorph in culture (Guarro *et al.* 1991). There have been considerable confusion and controversies about the phylogenetic relationships of *Zopfiella* and other morphologically similar genera such as *Podospora* and *Cercophora* (Guarro *et al.* 1991).

Podospora species generally differ from those of *Zopfiella* in that their ascospores are larger, with gelatinous appendages, and pedicels that are longer and non-collapsed (Guarro *et al.* 1991). The immature ascospores of *Podospora* are usually clavate rather than ellipsoidal as found in *Zopfiella*. *Zopfiella* has also traditionally been distinguished from *Podospora* by the presence of non-ostiolate ascomata. This morphological character, however, has been regarded as unreliable based on cultural studies (e.g. von Arx 1973). Although the above distinctions have been widely used in the classification of this group of fungi, the division between *Podospora* and *Zopfiella* is still problematical. Khan and Krug (1990) have suggested that *Z. tabulata*, the type species of *Zopfiella* belongs in *Podospora*. Guarro *et al.* (1991) was of the opinion that many species of *Zopfiella* should be transferred to *Podospora*.

* Corresponding author. Fax: +852-25176082.
E-mail address: leicai@hkusua.hku.hk

0953-7562/$ – see front matter © 2006 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2006.01.007

Tripterospora is another genus bearing morphological similarities to *Zopfiella* and *Podospora*. This genus was established by Cain (1956) based on cleistothecial ascomata and lack of gelatinous appendages. *Tripterospora* can be distinguished from *Zopfiella* by the lack of septum in the dark cell of ascospores (Cain 1956). The taxonomic relationships between *Tripterospora* and *Zopfiella* has been widely debated. Malloch and Cain (1971) and Khan and Krug (1990) preferred to separate the two genera. Lundqvist (1969, 1972) however, suggested that *Tripterospora* and *Zopfiella* should be better treated as congeneric. Guarro *et al.* (1991) agreed with Lundqvist that the separation of the two genera was artificial. Similar taxonomic views were shared by von Arx (1973) and Udagawa and Furuya (1974). Most recently, *Tripterospora* has been treated as a synonym of *Zopfiella* (Guarro *et al.* 1991; Kirk *et al.* 2001). Nevertheless, whether *Tripterospora* and *Zopfiella* are distinct or congeneric is a matter of personal opinion.

Cercophora is also similar to *Zopfiella* and *Podospora* in many morphological features. *Cercophora* resembles *Podospora* in having gelatinous appendages attached to either the dark cell and/or the pedicel (Lundqvist 1972). There are only some minor distinctions in ascospore shape currently applied in their taxonomy. The anamorph of *Cercophora* is similar to that of *Podospora*, being *Cladorrhinum* or *Phialophora* (Kendrick & Dicosmo 1979; Udagawa & Muroi 1979; Mouchacca & Gams 1993). *Cercophora* has been suggested as being polyphyletic (Lundqvist 1972; Miller & Huhndorf 2001), and its relationships with other genera such as *Bombardia*, *Lasiosphaeria*, *Podospora* and *Zopfiella* are also unclear (Lundqvist 1972).

In order to investigate the phylogenetic relationships of *Zopfiella* and other allied genera, a number of fungi that exhibit a broad range of ascomatal and ascospore morphologies were sampled. Phylogenetic analyses were conducted based on partial 28 S rDNA, ITS/5.8 S rDNA and partial β-tubulin sequences using MP and bayesian analyses. The objectives of this study were: (1) to examine the phylogenetic relationships of *Zopfiella* and its allies; and (2) to provide an overview of the phylogenetic significance of morphologies in the delineation of these closely related genera.

Materials and methods

DNA extraction, PCR, and sequencing

Cultures were obtained from culture collections CBS (Utrecht) and NITE (Tsukuba) (Table 1). Isolates were grown on potato dextrose agar (PDA) for two to four weeks and total genomic DNA was extracted from fresh mycelium using the protocol as outlined by Jeewon *et al.* (2003) and Lacap *et al.* (2003). Partial 28 S rDNA, complete ITS/5.8 S rDNA and partial β-tubulin were amplified using fungal specific primers LROR and LR5 (Vilgalys & Hester 1990), ITS5 and ITS4 (White *et al.* 1990) and Bt2A and Bt2B (Glass & Donaldson 1995), respectively. The PCR thermal cycle were same as that of Cai *et al.* (2006). PCR products were checked on 1 % agarose electrophoresis gels stained with ethidium bromide.

PCR products were then purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham Bioscience, UK, product code 27-9602-01). DNA sequencing was performed using the primers mentioned above in an Applied Biosystem 3730 DNA Analyzer at the Genome Research Centre (University of Hong Kong).

Sequence alignment and phylogenetic analysis

For each fungal strain, sequences obtained from pair primers were aligned to obtain an assembled sequence using Bioedit (Hall 1999). In total four datasets were analysed: 28 S rDNA dataset, ITS/5.8 S rDNA dataset, β-tubulin dataset, and a combined dataset. Novel sequences generated from this study were submitted to GenBank (Table 1). Sequences for each strain, together with reference sequences obtained from GenBank (Table 2), were aligned using Clustal X (Thomson *et al.* 1997). Alignment was manually adjusted to allow maximum alignment and minimise gaps.

Phylogenetic analyses were performed by using PAUP* 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded from all analyses. Unweighted parsimony (UP) and weighted parsimony (WP) analyses were performed with gaps treated as missing data. WP analyses were performed using a symmetric step matrix generated with the program STMatrix version 2.2 (François Lutzoni & Stefan Zoller, Department of Biology, Duke University), by which the relative frequencies of nucleotide substitutions were calculated and converted into costs of changes. Trees were inferred using the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all parsimonious trees were saved. Descriptive tree statistics such as tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for trees generated under different optimality criteria. Clade stability was assessed in a bootstrap (BS) analysis with 1000 replicates, each with ten replicates of random stepwise addition of taxa. Kishino–Hasegawa tests (KH Test) (Kishino & Hasegawa 1989) and Templeton test (Templeton 1983) were performed in order to determine whether trees were significantly different. Trees were viewed in Treeview (Page 1996).

A model of evolution was estimated by using Modeltest 3.06 (Posada & Crandall 1998). Posterior probabilities (PP) (Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002) were determined by MCMC sampling in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001), using above estimated model of evolution. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generations (resulting in 10,000 total trees). The first 2000 trees that represented the burn-in phase of the analyses were discarded. The remaining 8000 trees were used for calculating PP in the majority rule consensus tree.

Results

The 28 S rDNA dataset comprised 868 sites, of which four ambiguous regions were excluded in the analysis. There were 228 parsimony informative characters (PIC) in those included regions. Two hundred and eighty trees were generated from UP, while WP resulted in a single tree. KH test ($0.3784 \leq P \leq 0.8900$) and Templeton tests ($0.2351 \leq P \leq 0.7230$)

Table 1 – Sequences generated from this study

Species	Isolate code[a]	Origins	GenBank accessing No.		
			28 S rDNA	ITS rDNA	β-tubulin
Achaetomium strumarium	CBS 333.67	Soil, India, isotype	AY681170	AY681204	AY681238
Apodus deciduus	CBS 506.70	Dung, USA, type	AY681165	AY681199	AY681233
A. oryzae	CBS 376.74	Straw, Italy, type	AY681166	AY681200	AY681234
Cercophora ambigua	CBS 215.60	Twig, Canada	AY999114	AY999137	AY999147
C. caudata	CBS 606.72	Soil, Netherlands	AY999113	AY999135	AY999151
C. coprophila	NITE 32091	Burnt soil, Japan	AY999112	AY999136	AY999141
C. samala	CBS 109.93	Dung, Japan	AY999111	AY999134	AY999140
Diplogelasinospora inaequalis	CBS 436.74	Soil, Papua New Guinea, type	AY681167	AY681201	AY681235
D. grovesii	CBS 340.73	Soil, Japan, type	AY681168	AY681202	AY681236
Gelasinospora calospora	NITE 32008	Burnt soil	AY681155	AY681190	AY681223
G. tetrasperma	CBS 178.33	Dung, Canada, type	AY681144	AY681178	AY681212
Lasiosphaeris hispida	CBS 955.72	Wood, Germany	AY681169	AY681203	AY681237
Neurospora tetrasperma	NITE 32011	Burnt soil	AY681159	AY681194	AY681227
Podospora cupiformis	CBS 246.71	Dung, Africa, type	AY999102	AY999125	AY999149
P. didyma	CBS 232.78	Dung, Canada	AY999100	AY999127	AY999142
P. appendiculata	NITE 8549	Dung, Japan	AY999103	AY999126	AY999144
P. austroamericana	CBS 724.68	Flower, India, isotype	AY999101	AY999124	AY999138
P. cochleariformis	CBS 249.71	Dung, Africa	AY999098	AY999123	AY999145
P. curvicolla	NITE 8548	Dung, Japan	AY999099	AY999122	AY999148
P. intestinacea	CBS 113106	Dung, New Zealand	AY999104	AY999121	AY999152
Schizothecium aloides	CBS 879.72	Soil, Netherlands	AY999097	AY999120	AY999159
S. curvisporum	CBS 507.50	Carrot Canada	AY999096	AY999119	AY999155
S. fimbriatum	CBS 144.54	Dung	AY999092	AY999115	AY999156
S. glutinans	CBS 134.83	Bearberry, Switzerland	AY999093	AY999116	AY999157
Sordaria fimicola	CBS 508.50	Dung, Canada	AY681160	AY681188	AY681228
S. lappae	CBS 154.97	Soil, Hungary	AY681137	AY681171	AY681205
Zopfiella karachiensis	NITE 32902	Soil, Japan	AY999106	AY999128	AY999153
Z. latipes	NITE 9826	Soil, Japan	AY999107	AY999129	AY999146
Z. longicaudata	NITE 30296	Soil, Japan	AY999109	AY999131	—
Z. tabulata	CBS 230.78	Dung, Canada	AY999105	AY999132	AY999143
Z. tetraspora	NITE 32904	Soil	AY999108	AY999130	AY999139
Z. erostrata	CBS 255.71	Dung, Africa	AY999110	AY999133	AY999150

a Abbreviations: CBS, Centraalbureau voor Schimmelcultures, Utrecht, NITE, National Institute of Technology and Evaluation, Tsukuba, Japan.

showed that these trees were not significantly different. The single tree generated from WP (TL = 1956.61, CI = 0.399, RI = 0.662, RC = 0.264, HI = 0.601) is shown in Fig 1.

ITS/5.8 S dataset had 48 taxa with 715 characters, of which 12 ambiguous regions were excluded in the analysis. There were 103 PIC in this dataset. UP generated 261 trees, while WP resulted in three trees. These 264 trees were not significantly different based on KH test ($0.2904 \leq P \leq 1.0000$) and Templeton tests ($0.1797 \leq P \leq 1.0000$). One of the three trees generated from the WP (TL = 756.00, CI = 0.523, RI = 0.717, RC = 0.375, HI = 0.477) is shown in Fig 2.

The β-tubulin dataset comprised 31 taxa, and 566 sites. Five ambiguous regions were excluded in all analyses. There were 74 PIC in this dataset. Two trees and one tree, respectively, were generated from UP and WP. KH test ($0.6923 \leq P \leq 0.7020$) and Templeton tests ($0.8271 \leq P \leq 0.9903$) revealed no significant difference among these trees. The single tree generated from the WP (TL = 438.39, CI = 0.442, RI = 0.583, RC = 0.258, HI = 0.558) is shown in Fig 3.

The combined dataset with 31 taxa, has 2039 characters. Sixteen ambiguous regions were excluded in all analyses, and there were 239 PIC in those included regions. Ten trees and one tree, respectively, were generated from UP and WP. KH test ($0.6024 \leq P \leq 1.0000$) and Templeton tests ($0.3227 \leq P \leq 1.0000$) revealed that these trees were not significantly different. The single tree generated from the WP (TL = 1511.22, CI = 0.517, RI = 0.680, RC = 0.352, HI = 0.483) is shown in Fig 4.

Although there were different taxon sampling between datasets, phylogenies generated were essentially similar in species groupings (Figs 1–4). To discuss tree outputs, phylograms were divided into several clades (A, B, C, Figs 1–4). Among them, clades A and C are strongly supported by 92 % BS/ 99 % PP and 92 % BS/ 100 % PP respectively in the 28 S rDNA tree (Fig 1). In the combined gene tree, the support for these two clades was even higher (Fig 4). Clade B received moderate support (e.g. 85 % BS in 28 S rDNA tree; Fig 1). In addition, clade stabilities are relatively low in the trees generated from ITS/5.8 S rDNA and β-tubulin dataset (Figs 2–3), owing to the lower number of PIC in these datasets. The β-tubulin dataset and the combined dataset, consisting of the same 31 taxa, generated trees that are essentially similar in the main groupings (Figs 3–4). However, the tree generated from the combined dataset is much better resolved. For instance, in the combined gene tree, 21 subclades are supported by >50 % BS and 21 subclades are supported by >95 % PP

Table 2 – Additional sequences used in analyses (obtained from GenBank)

Species	GenBank no.
Amphisphaeria umbrina	AF452029[b]
Apiosordaria verruculosa	AY346258[b]
Aporothielavia leptoderma	AF096186[b]
Barrina polyspora	AY346261[b]
Bombardia bombarda	AY346263[b]
Bombardioidea anartia	AY346264[b]
Cercophora appalachianensis	AF132328[b]
C. areolata	AY587936[b]
C. areolata	AY587911[c]
C. atropurpurea	AY780057[b]
C. costaricensis	AY780059[b]
C. mirabilis	AY346271[b]
C. newfieldiana	AF064642[b]
C. septentrionalis	U47823[b]
C. scortea	AY780063[b]
C. striata	AY780065[b]
C. sulphurella	AY587938[b]
C. sulphurella	AY587913[c]
Chaetomium globosum	AY545729[b]
C. globosum	AY429056[c]
Chaetosphaeria innumera	AY017375[b]
Coniochaetidium savoryi	AY346276[b]
Corynascus kuwaitiensis	AJ715483[c]
Diaporthe pustulata	AF408358[b]
Halosphaeria appendiculata	U46885[b]
Immersiella caudata	AY436407[b]
I. immersa	AY436408[b]
Jugulospora rotula	AY346287[b]
Lasiosphaeria ovina	AY436413[b]
L. rugulosa	AY436414[b]
Lasiosphaeris hirsuta	AY436417[b]
Lignincola laevis	U46890[b]
Melanochaeta hemipsila	AY346292[b]
Pestalotiopsis versicolor	AF409993[c]
Podospora anserina	AY525771[c]
P. austrohemisphaerica	AY026939[c]
P. bicolor	AF443848[c]
P. comata	AY780072[b]
P. comata	AF443849[c]
P. decidua	AF443851[c]
P. decipiens	AY780073[b]
P. decipiens	AY515359[c]
P. ellisiana	AY515360[c]
P. fibrinocaudata	AY780074[b]
P. fimiseda	AY346296[b]
P. fimiseda	AY515361[c]
P. petrogale	AY071831[c]
P. pleiospora	AY515364[c]
P. setosa	AF443852[c]
Strattonia carbonaria	AY346302[b]
Thielavia cephalothecoides	AF286413[b]
T. coactilis	AJ271585[c]
T. fraqilis	AJ271578[c]
Triangularia mangenotii	AY346303[b]
Valsella salicis	AF408389[b]
Xylaria hypoxylon	U47841[b]
Zygopleurage zygospora	AY346306[b]

b 28 S rDNA.
c ITS/5.8 S rDNA.

(Fig 4). Conversely, there were only 13 and ten subclades supported by BS and PP, respectively, in the β-tubulin tree (Fig 3). The inclusion of more genes thus, resulted in a better statistical support for most of the clades.

Results generated from this study showed: (1) *Zopfiella* is polyphyletic, with six different species clustered in five different clades as shown in Fig 1. *Zopfiella* species are interspersed in the trees and show association with a variety of genera such as *Apiosordaria*, *Apodus*, *Cercophora*, *Podospora* and *Triangularia*; (2) *Zopfiella* species clustered with lasiosphaeriaceous species rather than chaetomiaceous species; (3) *Cercophora* and *Podospora* are also polyphyletic, which is in agreement with previous studies (Miller & Huhndorf 2005; Cai *et al.* 2005); (4) Species possessing a *Cladorrhinum* anamorph grouped in clade A, indicating their close phylogenetic relationships; and (5) Other highly supported clades, such as clades A and C, include species having diverse morphological characters.

Discussion

Phylogenetic relationships of Zopfiella

Phylogenetic analyses indicate that *Zopfiella* species, as currently circumscribed, do not constitute a monophyletic group (Figs 1–4). The non-ostiolate ascomata, and the absence of gelatinous appendages have been considered important in distinguishing *Zopfiella* from *Podospora* (Guarro *et al.* 1991). However, both of these characters are shown to be unreliable in understanding phylogenetic relationships. That Non-ostiolate *Zopfiella* species interspersed in different clades in the trees (Figs 1–4) suggests multiple origins of this morphological character, and this conclusion is in agreement with previous studies (e.g. Berbee & Taylor 1992; Rehner & Samuels 1995; Suh & Blackwell 1999; Cai *et al.* 2006). In addition, the presence or absence of gelatinous appendages, as shown here, are phylogenetically less informative. As shown in the phylogenetic trees, *Zopfiella* species grouped in different clades that also include many species possessing elaborate gelatinous appendages. Similar results pertaining to *Schizothecium* have been reported (Cai *et al.* 2005), in which strongly supported *Schizothecium* clade included species with or without gelatinous appendages. Although the presence of the gelatinous appendages have been suggested to be an adaptation for those coprophilous species, this morphology appears to have arisen independently in several different lineages.

Tripterospora has been treated as a synonym of *Zopfiella* (Kirk *et al.* 2001; Eriksson *et al.* 2004). So-called *Tripterospora* species are morphologically similar to other *Zopfiella* species but differ in lacking a septum in the brown cell of the ascospores. Molecular analyses in our study showed that these species did not cluster together. *Z. erostrata*, *Z. tetraspora*, *Z. longicaudata* and *Z. latipes* have ascospores lacking a septum in the dark cell and had been placed in *Tripterospora*. However, these species are found to cluster in different distantly related clades. (Figs 1–4). *Tripterospora* is therefore, an artificial taxonomic arrangement. Cain (1956) established a new family *Tripterosporaceae* based on several species of *Tripterospora*, but this familial circumscription clearly does not reflect a natural grouping.

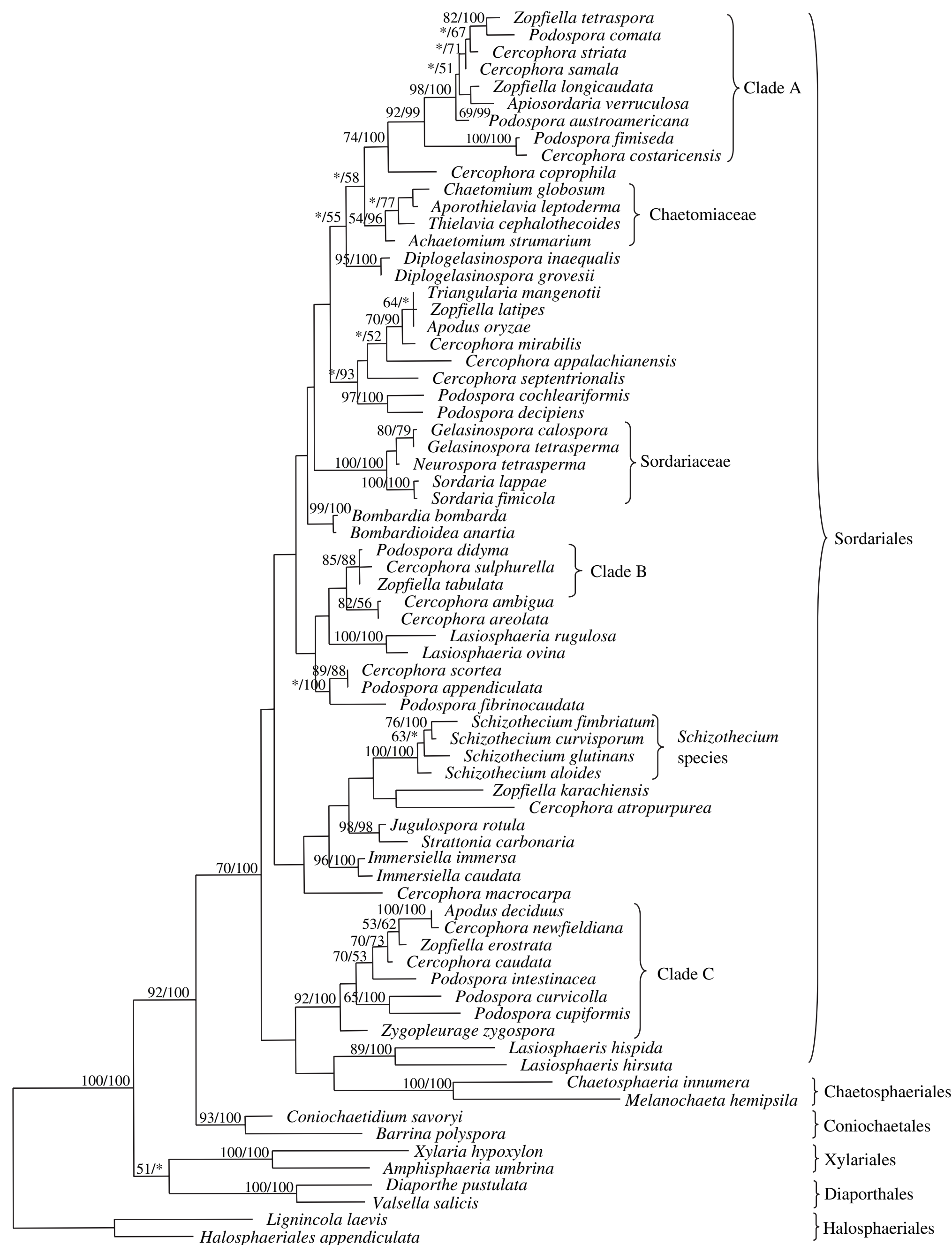

Fig 1 – Phylogram of single tree generated from parsimony analysis based on 28 S rDNA sequences (TL = 1956.61, CI = 0.399, RI = 0.662, RC = 0.264, HI = 0.601). Data were analysed with random addition sequence, WP and treating gaps as missing data. Values before the backslash are parsimony BS (above 50 %) while after are Bayesian posterior probabilities (above 50 %). The tree is rooted with *Lignincola laevis* and *Halosphaeria appendiculata*. *Clades that receive less than 50 % support.

As the current concept of *Zopfiella* does not reflect natural relationships, taxonomic changes will be necessary. Typical *Zopfiella* species, such as the type species *Z. tabulata*, have ascospores with a septum in the dark cell. In the present study, *Z. tabulata* nested in clade B in all the trees (Figs 1–4). Other species grouped in clade B include *Podospora didyma* and *Cercophora sulphurella*. It is interesting that both *P. didyma* and *C. sulphurella* also produce one septum in the dark cell (Mirza & Cain 1969; Hilber & Hilber 1979). A possible explanation for this is that these species are united by their morphological similarity (ascospores with septate dark cell), and this character, can be phylogenetically informative. In addition, *C. ambigua* and *C. areolata* appear close to the above species in clade B (resolved as a sister clade to clade B, Fig 1). In these two species, a septum in the dark cell of the ascospore has also been observed (Hilber & Hilber 1979; Udagawa & Muroi 1979). Cain (1956)

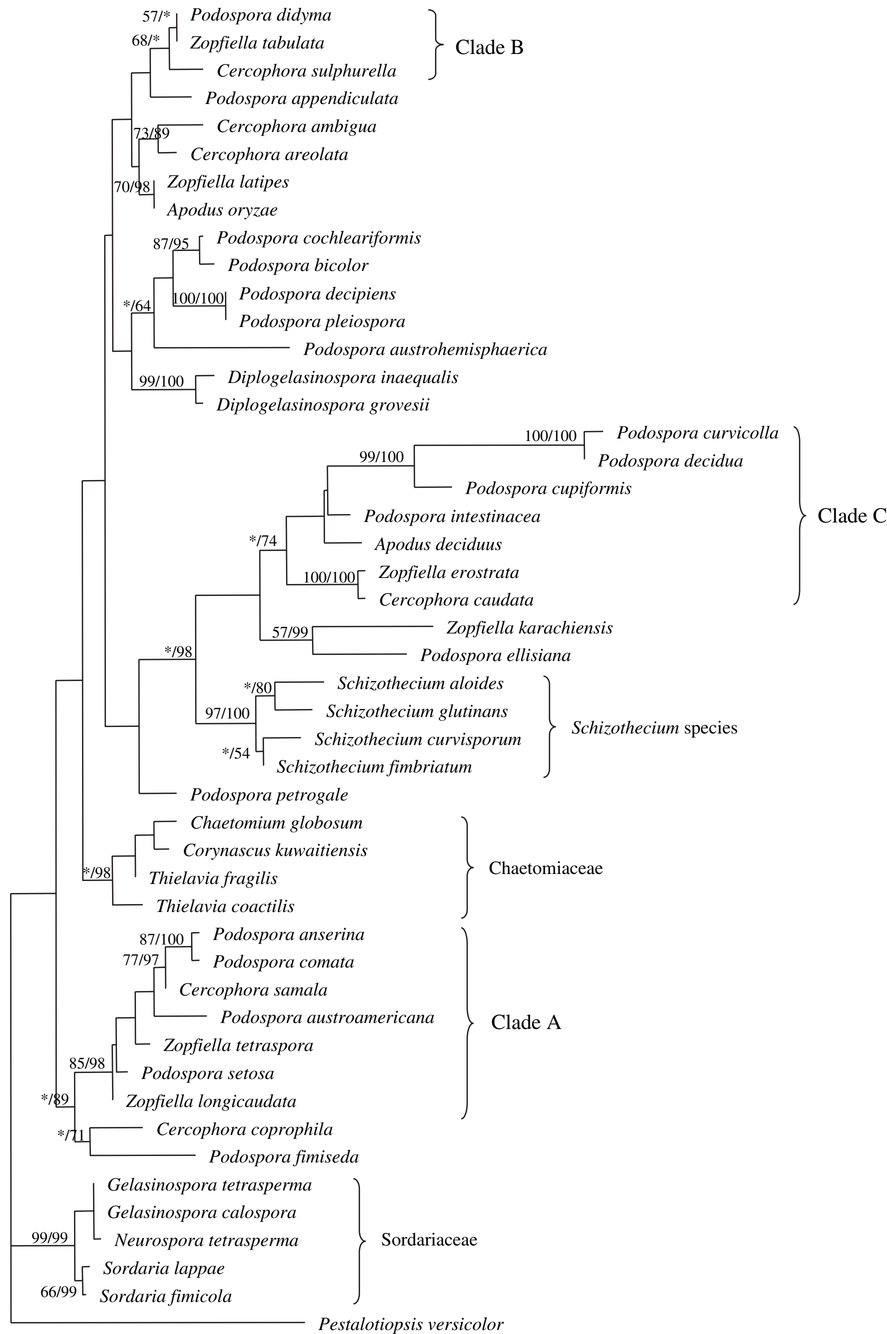

Fig 2 – Phylogram of one of three trees generated from parsimony analysis based on ITS/5.8 S rDNA sequences (TL = 756.00, CI = 0.523, RI = 0.717, RC = 0.375, HI = 0.477). Data were analysed with random addition sequence, WP and treating gaps as newstate. Values before the backslash are parsimony BS (above 50 %) while after are Bayesian posterior probabilities (above 50 %). The tree is rooted with *Pestalotiopsis versicolor*.*Clades that receive less than 50 % support.

suggested that the presence of a septum in the dark cell might represent side branches in the evolution in lasiosphaeriaceous species. A similar view was shared by Mirza & Cain (1969). Our molecular results are generally congruent with Cain's (1956) postulate as these species are phylogenetically closely related. The present study is in agreement with Cai *et al.* (2006), who found that the spore septum is useful in understanding the phylogenetic relationship amongst the families *Lasiosphaeriaceae* and *Sordariaceae*. We would therefore suggest that *Zopfiella* should be restricted to species with a septum in the dark cell as *Zopfiella* species that lack a spore septum are not related. It appears highly plausible that *P. didyma* and *C. sulphurella* in clade B should be transferred to *Zopfiella*, but there are some indications that do not favour this amendment. In particular, the septum in the dark cell of the ascospores seems has arisen independently

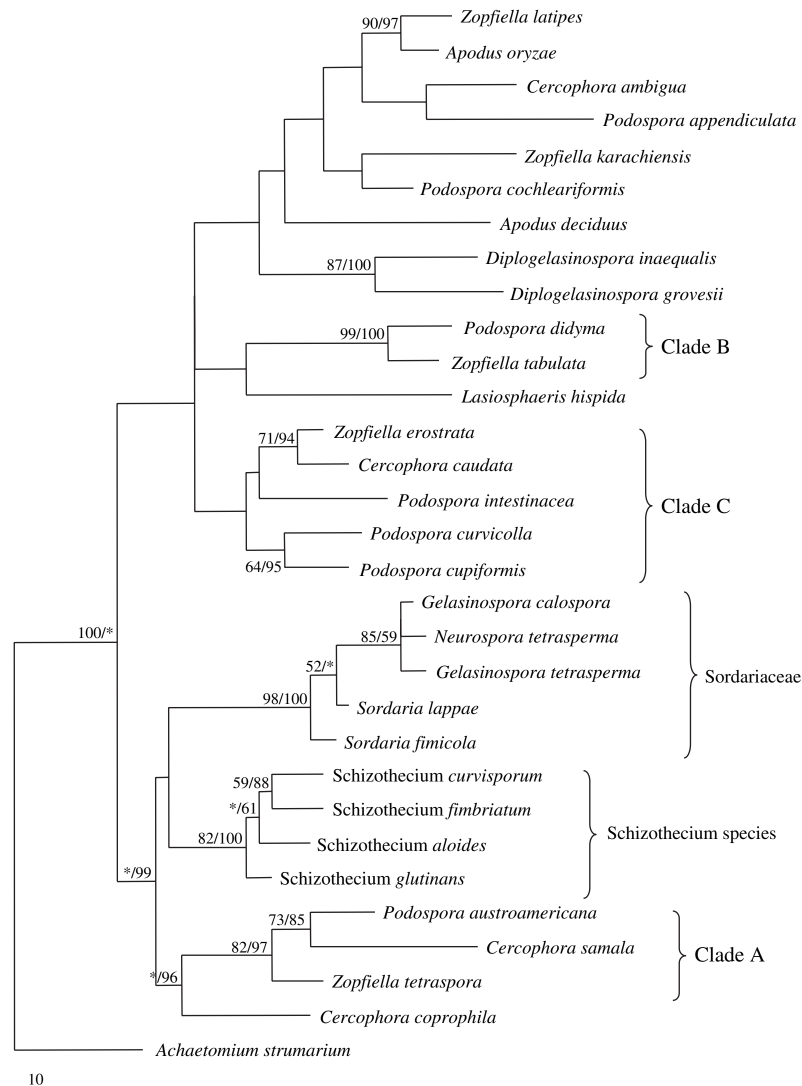

Fig 3 – Phylogram of the single tree generated from parsimony analysis based on β-tubulin sequences (TL = 438.39, CI = 0.442, RI = 0.583, RC = 0.258, HI = 0.558). Data were analysed with random addition sequence, WP and treating gaps as missing data. Values before the backslash are parsimony BS (above 50 %) while after are Bayesian posterior probabilities (above 50 %). The tree is rooted with *Achaetomium strumarium*. *Clades that receive less than 50 % support.

in a few more distantly related fungi. For example, *C. newfieldiana* is also characterised by a similar spore septum in the dark cell of the ascospore (Hilber & Hilber 1979), but it is more closely related to *Apodus deciduus*, *Z. erostrata*, and *C. caudata* (clade C).

Huhndorf *et al.* (2004) found that *Z. ebriosa* clustered with chaetomiaceous species rather than lasiosphaeriaceous species and based on this they suggested the transfer of *Zopfiella* from *Lasiosphaeriaceae* into *Chaetomiaceae*. The inclusion of six different *Zopfiella* species in this study reveals that *Zopfiella* is more closely related to other lasiosphaeriaceous species rather than chaetomiaceous species. The affinity of *Z. ebriosa* (AY346305) was also tested and as reported by Huhndorf *et al.* (2004), it clustered with chaetomiaceous species (result not shown). However, *Z. ebriosa* as used by Huhndorf *et al.* is an atypical species of *Zopfiella*. As first described by Guarro *et al.* (1991), *Z. ebriosa* is markedly different from all other *Zopfiella* species in lacking a spore germ pore. Furthermore, the cylindrical asci and the ostiolate tendency of this fungus is also different from other *Zopfiella* species. Therefore, Huhndorf *et al.*'s familial replacement of *Zopfiella* in the family *Chaetomiaceae* based on only one and an atypical species is not accepted here.

The association of *Cercophora* and *Podospora* with a variety of genera in our phylogenetic trees shows that the current circumscription of them does not accurately reflect natural relationships. It is therefore, inappropriate to discuss and make any conclusive taxonomic statement of *Cercophora* and *Podospora*. Nomenclatural changes to classify them into natural groupings are left for future studies. The present study is in agreement with previous studies that the traditionally used ascospore morphology is highly homoplasious.

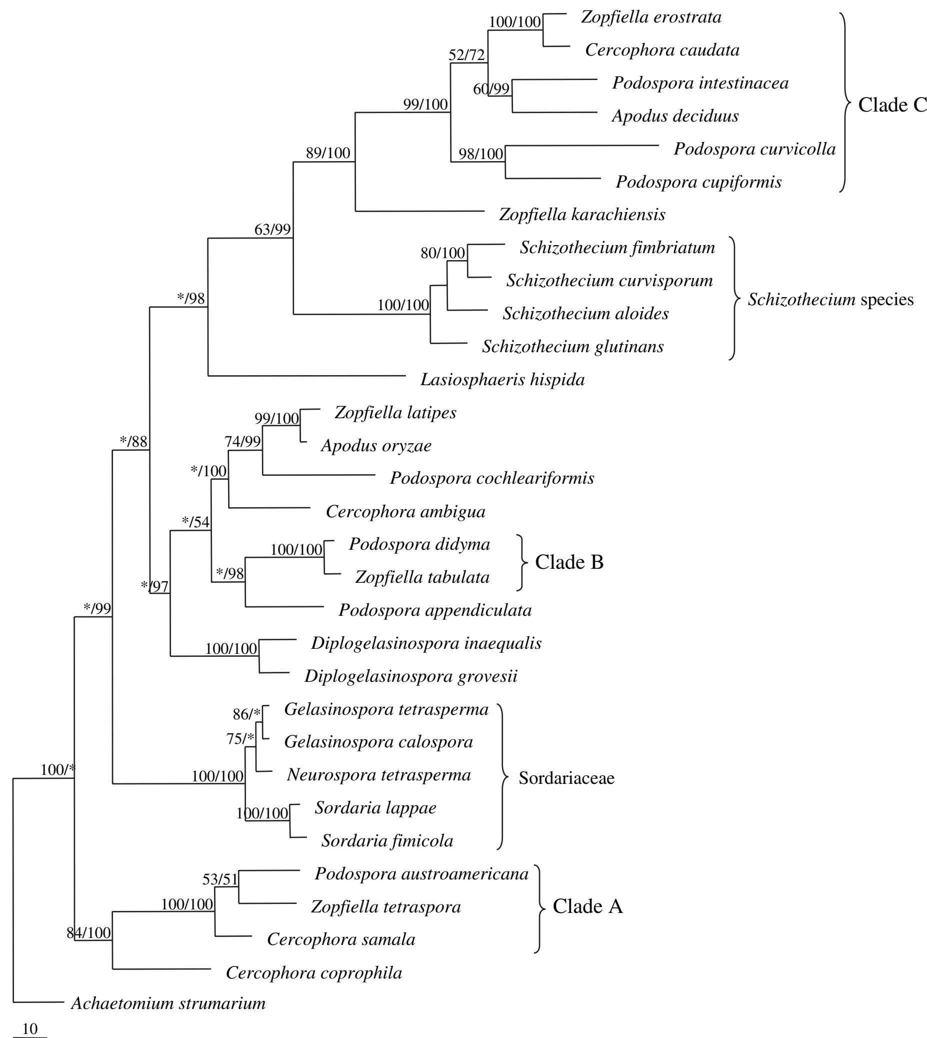

Fig 4 – Phylogram of one of the single tree generated from parsimony analysis based on combined β-tubulin, ITS rDNA and 28 S rDNA sequences (TL = 1511.22, CI = 0.517, RI = 0.680, RC = 0.352, HI = 0.483). Data were analysed with random addition sequence, WP and treating gaps as missing data. Values before the backslash are parsimony BS (above 50 %) while after are Bayesian posterior probabilities (above 50 %). The tree is rooted with *Achaetomium strumarium*. *Clades that receive less than 50 % support.

Phylogenetic relationships of species possessing a Cladorrhinum *anamorph*

Phylogenies based on sequence analyses have been shown to be important in the classification of many anamorphs and their integration with teleomorphs (Taylor 1995; Rehner & Samuels 1995; Jacobs & Rehner 1998). Most of the known anamorphs of *Podospora* species have been reported as *Phialophora*, except *Podospora fimiseda* which produces a *Cladorrhinum* anamorph (Bell & Mahoney 1997; Lundqvist *et al.* 1999). The anamorphs of *Cercophora* are similar, mostly *Cladorrhinum* and *Phialophora* (Mouchacca & Gams 1993; Udagawa & Muroi 1979; Miller & Huhndorf 2001). *Phialophora* has been shown to be a heterogeneous assemblage of anamorphs of unrelated ascomycetes in various studies (Domsch *et al.* 1980; Gams 2000). *Cladorrhinum* species, however, have been reported to be anamorphs of several species, including *Apiosordaria verruculosa* (von Arx & Gams 1967), *Cercophora samala* (Udagawa & Muroi 1979), *Cercophora striata* (Miller & Huhndorf 2001) and *Podospora fimiseda* (Bell & Mahoney 1997). Interestingly, all of the above species clustered together in clade A (Fig 1). *Podospora austroamericana* (syn. *P. castorinospora*), another species clustered in clade A, produces an anamorph characterised by very short, peg-like phialides scattered on the mycelium (Cain 1962; Mirza & Cain 1969). Although a proper anamorphic connection has not been established by above authors, these anamorphic characters are mostly restricted to *Cladorrhinum*. Our results therefore, indicate that species possessing a *Cladorrhinum* anamorph are phylogenetically closely related, despite the anamorphs of several other species in clade A not being known and requiring further investigation.

Implications of several supported clades

In our phylogenetic trees, there are several strongly supported clades, such as clades A and C. However, species in these

groupings have diverse morphological characters. For instance, in clade A, although they have similar anamorphs, their teleomorph morphologies are quite divergent. Besides the highly diverse ascospore morphology, taxa in clade A are also diverse in characters, such as ascomata being non-ostiolate or ostiolate, glabrous or adorned with hairs, asci being 4- or 8-spored, cylindrical or clavate, and peridia of different types. As these morphological characters also occur in several other taxa throughout the phylogram, it is presently unclear which characters are phylogenetically the most informative for delimiting these clades. Even for the ascomatal morphology, which has been regarded as a more reliable phylogenetic predictor (Miller & Huhndorf 2005), our study suggests a conservative application of it. For instance, *P. fimiseda* and *C. costaricensis* clustered in clade A (Fig 1). These two species are characterised by a typical pseudo-bombardioid peridium (a kind of non-stromatic peridium wall with a gelatinized layer consisting of interwoven, thin-walled hyphae) which has been regarded as phylogenetically important (Miller & Huhndorf 2001, 2005). However, with a broader taxonomic sampling in our study, species in clade A is shown to have various types of peridia. For instance, *C. striata* has peridium consisting of flattened, elongate or polygonal cells (Miller & Huhndorf 2001); *C. samala* has a peridium of '*textura angularis*', consisting brown, polygonal cells (Udagawa & Muroi 1979); *Z. tetraspora* and *Z. longicaudata* have ascomata that are cleistothecial and consist of compressed, elongated cells, and they are polygonal or irregular in surface view (Cain 1956; Rai *et al.* 1963).

Clade C, with high bootstrap and posterior probabilities support contained a group of species from *Apodus*, *Cercophora*, *Zopfiella*, *Podospora* and *Zygopleurage*. All sampled species in clade C possess ascomata that are covered by flexuous, long or short, brown hairs. Nevertheless, these hairs show variations among different species. For example, the ascomata of *A. deciduus* and *Zopfiella erostrata* are covered with very long, flexuous, somewhat rigid, septate, brown hairs (Malloch & Cain 1970; Guarro *et al.* 1991); *P. intestinacea* has ascomata covered with short, flexuous, soft, septate, ramified hairs (Lundqvist 1972); *P. curvicolla* has ascomata covered by short, flexuous, light brown, septate hairs and on the neck covered by long, slender, tapering tufts of rigid, cylindrical, septate hairs (Mirza & Cain 1969).

Zygopleurage is morphologically interesting as it produces ascospores that are elongate, biseptate and provided with swollen, pigmented end cells and a long, vermiform, hyaline intercalary middle cell. This is abnormal in lasiosphaeriaceous species and is not stable in some cases. For instance, in *P. trichomanes*, some ascospores may become biseptate with a hyaline middle cell similar to those of *Zygopleurage* species. This may indicate a close affinity between some *Podospora* species and *Zygopleurage* species. Lundqvist (1972) has suggested that *Zygopleurage* may share a common ancestry and evolved in parallel with some *Podospora* species. *Zygopleurage* might be closely related to several species such as *P. curvicolla*, *P. cupiformis*, and *P. intestinacea*, which also clustered in clade C.

In this study, several well-supported clades in which species are characterised by divergent morphological characters are conservatively treated. It may be that a combination of morphological characters unites these clades. Based on our present understanding of this group of fungi, it is not possible to establish which character or character combination unites above supported monophyletic clades. Other authors may have different notion on the taxonomic treatment of these taxa. Regardless of the subjectivity in taxonomic practice, the important implication of this study are that phylogenetically defined groups of individuals exist, and the relationships among them can and should be considered when these species are included in further studies of evolutionary biology.

Acknowledgements

This study was funded by the Hong Kong Research Grants Council (HKU 7320/02M), National Natural Science Foundation of China (NSFC3026002) and International Cooperation Research Foundation, Yunnan Province (2000C002). The University of Hong Kong is acknowledged for providing L.C. with a postgraduate scholarship. We are grateful to CBS, NITE and ICMP for providing cultures. Helen Leung, Keith Cheung and Heidi Kong are thanked for technical assistance.

REFERENCES

von Arx JA, 1973. Ostilate and non-ostiolate pyrenomycetes. *Proceedings of the Koninklijke Nederlandse Akdemie van Wetenschappen, Amsterdam, Series C* **76**: 289–296.

von Arx JA, Gams W, 1967. Űber *Pleurage verruculosa* und die zugehörige *Cladorrhinum*-Konidienform. *Nova Hedwigia* **13**: 199–208.

Bell A, Mahoney DP, 1997. Coprophilous fungi in New Zealand. II. *Podospora* species with coriaceous perithecia. *Mycologia* **89**: 908–915.

Berbee ML, Taylor JW, 1992. Convergence in ascospore discharge mechanism among pyrenomycete fungi based on 18 S ribosomal RNA gene sequences. *Molecular Phylogenetics and Evolution* **1**: 59–71.

Cai L, Jeewon R, Hyde KD, 2005. Phylogenetic evaluation and taxonomic revision of *Schizothecium* based on ribosomal DNA and protein coding genes. *Fungal Diversity* **19**: 1–21.

Cai L, Jeewon R, Hyde KD, 2006. Phylogenetic investigations of *Sordariaceae* based on multiple gene sequences and morphology. *Mycological Research* **110**: 137–150.

Cain RF, 1956. Studies of coprophilous ascomycetes IV. *Tripterospora*, a new cleistocarpous genus in a new family. *Canadian Journal of Botany* **34**: 699–710.

Cain RF, 1962. Studies of *coprophilous Ascomycetes* VIII. New species of *podospora*. *Canadian Journal of Botany* **40**: 447–490.

Domsch KH, Gams W, Anderson TH, 1980. *Compendium of Soil Fungi*, Vol. I, Academic Press, London.

Eriksson OE, Baral HO, Currah RS, Hansen K, Kurtzman CP, Rambold G, Laessøe T, 2004. Outline of Ascomycota 2004. *Myconet* **10**: 1–99.

Gams W, 2000. *Phialophora* and some similar morphologically little-differentiated anamorphs of divergent ascomycetes. *Studies in Mycology* **45**: 187–199.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Guarro J, Cannon PF, van der AAA, 1991. A synopsis of the genus *Zopfiella* (ascomycetes, *Lasiosphaeriaceae*). *Systema Ascomycetum* **10**: 79–112.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hilber R, Hilber O, 1979. Einige anmerkungen zu der gattung *Cercophora* Fuckel (*Lasiosphaeriaceae*). *Zeitschrift fur Mykologie* **45**: 209–233.

Huelsenbeck JP, Ronquist FR, 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Biometrics* **17**: 754–755.

Huhndorf SM, Miller AN, Fernández FA, 2004. Molecular systematics of the Sordariales: the order and the family *Lasiosphaeriaceae* redefined. *Mycologia* **96**: 368–387.

Jacobs KA, Rehner SA, 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia* **90**: 601–610.

Jeewon R, Liew ECY, Simpson JA, Hodgkiss IJ, Hyde KD, 2003. Phylogenetic significance of morphological characters in taxonomy of *Pestalotiopsis* species. *Molecular Phylogenetics and Evolution* **27**: 372–383.

Kendrick B, Dicosmo F, 1979. Teleomorph–anamorph connections in ascomycetes. In: Kendrick B (ed), *The Whole Fungus*, Vol. 1. National Museums of Canada, Ottawa, pp. 283–410.

Khan RS, Krug JC, 1990. New records of the Sordariaceae from East Africa. *Mycologia* **81**: 862–869.

Kirk PM, Cannon PF, David JC, Stalpers JA, 2001. *Ainsworth & Bisby's Dictionary of the Fungi*. CABI Publishing, Wallingford, UK.

Kishino H, Hasegawa M, 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of *Hominoidea*. *Journal of Molecular Evolution* **29**: 170–179.

Lacap DC, Hyde KD, Liew ECY, 2003. An evaluation of the fungal 'morphotype' concept based on ribosome DNA sequences. *Fungal Diversity* **12**: 53–66.

Lundqvist N, 1969. *Tripterospora* (*Sordariaceae s. lat.*, *Pyrenomycetes*). *Botaniska Notiser* **122**: 589–603.

Lundqvist N, 1972. Nordic *Sordariaceae s. lat. Symbolae Botanicae Upsalienses* **20**: 1–374.

Lundqvist N, Mahoney DP, Bell A, Lorenzo LE, 1999. *Podospora austrohemisphaericai*, a new heterothallic ascomycete from dung. *Mycologia* **91**: 405–415.

Miller AN, Huhndorf SM, 2001. Neotropical ascomycetes 10. New and interesting *Cercophora* species. *Sydowia* **53**: 211–226.

Miller AN, Huhndorf SM, 2005. Multi-gene phylogenies indicate ascomal wall morphology is a better predictor of phylogenetic relationships than ascospore morphology in the *Sordariales* (*Ascomycota*, *Fungi*). *Molecular Phylogenetics and Evolution* **35**: 60–75.

Malloch D, Cain RF, 1970. Five new genera in the new family *Pseudeurotiaceae*. *Canadian Journal of Botany* **48**: 1815–1825.

Malloch D, Cain RF, 1971. New cleistothecial *Sordariaceae* and a new family, *Coniochaetaceae*. *Canadian Journal of Botany* **49**: 869–880.

Mirza JH, Cain RF, 1969. Revision of the genus *Podospora*. *Canadian Journal of Botany* **47**: 1999–2048.

Mouchacca J, Gams W, 1993. The hyphomycete genus *Cladorrhinum* and its teleomorph connection. *Mycotaxon* **48**: 415–440.

Page RDM, 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357–358.

Posada D, Crandall KA, 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **49**: 817–818.

Rai JN, Mukerji KG, Tewari JP, 1963. *Tripterospora tetraspora* spec. nov., a new cleistothecial ascomycete. *Canadian Journal of Botany* **41**: 327–330.

Rannala B, Yang Z, 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. *Journal of Molecular Evolution* **43**: 304–311.

Rehner SA, Samuels GJ, 1995. Molecular systematics of the *Hypocreales*: a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* **73**: S816–S823.

Suh SO, Blackwell M, 1999. Molecular phylogeny of the cleistothecial fungi placed in *Cephalothecaceae* and *Pseudeurotiaceae*. *Mycologia* **91**: 836–848.

Swofford DL, 2002. *PAUP*: phylogenetic analysis using parsimony (*and other methods)*. Version 4b10. Sinauer Associates, Sunderland, Massachusetts, USA.

Taylor JW, 1995. Making the Deuteromycota redundant: A practical integration of mitosporic and meiosporic fungi. *Canadian Journal of Botany* **73**: S754–S759.

Templeton AR, 1983. Phylogenetic inference from restriction endonuclease cleavage sites maps with particular reference to the evolution of humans and the apes. *Evolution* **37**: 269–285.

Thomson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.

Udagawa S, Furuya K, 1974. Notes on some Japanese ascomycetes XIII. *Transactions of the Mycological Society of Japan* **15**: 206–214.

Udagawa S, Muroi T, 1979. Coprophilous pyrenomycetes from Japan V. *Transactions of the Mycological Society of Japan* **20**: 453–468.

Vilgalys R, Hester M, 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, pp. 315–322.

Winter G, 1884. Ascomyceten: Gymnoasceen und Pyrenomyceten. *Rabenhorst's Kryptogamenflora von Deutschland, Öesterreich und der Schweiz* **1**: 1–64.

Zhaxybayeva O, Gogarten JP, 2002. Bootstrap, Bayesian probability and maximum likelihood mapping: exploring new tools for comparative genome analyses. *Genomics* **3**: 1–15.