Molecular Phylogeny of *Cercophora*, *Podospora*, and *Schizothecium* (Lasiosphaeriaceae, Pyrenomycetes)

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ABSTRACT: Pyrenomycete species of *Cercophora* and *Podospora* are common on the dung of herbivores. *Schizothecium* was differentiated from *Podospora* based on morphological characters and further supported by molecular phylogenetic analyses. This study focused on phylogenetic relationships of *Cercophora* and *Podospora* including species of *Schizothecium*. Two gene sequences, ribosomal DNA internal transcribed spacers (ITS/5.8S rDNA) and a fragment of glyceraldehyde-3-phosphate dehydrogenase (GPD), were amplified and examined using maximum-parsimony and Bayesian analyses. In all analyses, *Cercophora* and *Podospora* were found to be polyphyletic, each consisting of a group of morphologically heterogeneous and phylogenetically distant species. Species of *Schizothecium* were all located in one clade and two *Cercophora* species with similar perithecial hair structures formed a sister group to it. The recognition of *Shizotheium* is reconfirmed. The phylogeny indicated a relevant relationship with the perithecial morphology in the Lasiosphariaceae.

KEY WORDS: *Cercophora*, Lasiosphaeriaceae, Phylogeny, *Podospora*, *Schizothecium*, Systematics.

INTRODUCTION

Species of *Cercophora* and *Podospora* are common pyrenomycetes growing on the dung of herbivores. These two genera are similar in having large dark perithecia and are differentiated by the morphology of the ascospores. *Podospora* is characterized by dark-colored perithecia and ascospores provided with an apical germpore, a basal hyaline pedicel, and gelatinous appendages. The genus has a worldwide distribution with over 80 species (Kirk et al., 2001; Stchigel et al., 2002; Chang and Wang, 2005).

Lundqvist (1972) divided *Podospora* into two genera: *Podospora* and *Schizothecium*. The latter is mainly characterized by perithecia adorned with swollen, agglutinated hairs or prominent protruding perithecial cells, and with minor characters such as the lack of interascal filiform paraphyses, ascospores becoming septate at an early stage of development, and pedicels being plasma-filled and persistent. This arrangement is not accepted by many mycologists (Bell and Mahoney, 1995, 1996; Lorenzo and Havrylenko, 2001). Recently, Cai et al. (2005) corroborated the generic status of *Schizothecium* by sequence analyses.

The main objective of this study was to examine the phylogeny of species of *Cercophora, Podopspora*, and *Schizothecium.* A number of fungi that exhibit a broad range of perithecial and ascospore morphologies were sampled. Phylogenetic analyses were constructed based on ITS/5.8S rDNA and GPD sequences using maximum-parsimony analyses (MP) and Bayesian analyses (BA).

MATERIALS AND METHODS

Fungal strains, culture conditions, and DNA isolates

Specimens examined and cultures isolated from Taiwan were deposited at the herbarium of National Museum of Natural Science (TNM). Isolates initiated in this study are listed in Table 1. Isolation of individual ascospores was initially inoculated on complete-medium (Bos, 1996) agar plates containing 30 μg/mL streptomycin sulfate and 100 units/ml penicillin at room temperature. Pure cultures were then transferred to 100 ml liquid MEA (20 g malt extract, 20 g agar in 1 L of sterile water) medium and were grown with shaking for 2 weeks at 25 °C for extraction of total DNA. DNA was extracted using a Plant Genomic DNA Extraction Miniprep System Kit (Viogene, Taiwan) and stored at -20 °C.

PCR amplification and sequencing

The 100 μL PCR mix contained 10x buffer (Protech, HP^{TM}: 100 mM Tris-HCl, 500 mM KCl, and 30 mM $MgCl_2$), 2.5 mM dNTPs, 50 μM of each primer, 5 U/μL *Taq* polymerase, and 100 ng template DNA. The thermal cycling consisted of initial denaturing for 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C; this was followed by a final extension at 72 °C for 10 min. For amplification, primers ITS5 (or ITS1) and ITS4 for the ITS/5.8S rDNA region were used to yield the complete ITS region (both ITS1 and ITS2 and 5.8S rDNA) (White et al., 1990), and primers N-gpd and C-gpd (Pöggeler, 1999) were used for the GPD region. PCR products were purified with the

Table 1. Isolates sequenced in this study.

Taxa	Strain no.	GenBank accession no.	
		ITS	GPD
Arnium macrotheca	Jong 3	EF197065	EF197089
Cercophora acanthigera	Wang 9819	EF197066	EF197090
C. coprophila	Jong 46	EF197067	EF197091
C. coronata	Jong 43	EF197068	EF197092
C. mirabilis	Jong 20	EF197069	EF197093
C. tuberculata	Jong 19	EF197070	EF197094
Neurospora intermedia	BCRC 32682	EF197071	EF197095
Podospora anserina	Wang 9827	EF197072	EF197096
P. araneosa	Jong 8	EF197073	EF197097
P. communis	Wang 9801	EF197074	EF197098
P. decipiens	Wang 9616	EF197076	EF197100
P. fimiseda	Wang 9727	EF197077	EF197101
P. immersa	Jong 4	EF197079	EF197103
P. inflatula	Wang 2002	EF197080	EF197104
P. myriaspora	Jong 45	EF197083	EF197107
P. pleiospora	Jong 21	EF197084	EF197108
P. prethopodalis	Jong 38	EF197085	EF197109
P. setosa	Jong 22	EF197086	EF197110
Schizothecium curvuloides	Wang 9805	EF197075	EF197099
Sch. formosanum	Wang 825	EF197078	EF197102
Sch. multipilosum	Jong 24	EF197082	EF197106
Sordaria lappae	Jong 6	EF197087	EF197111
Zygopleurage zygospora	Jong 33	EF197088	EF197112

Table 2. Sequences of ITS/5.8S rDNA obtained from GenBank.

Taxa	Accession No.	Taxa	Accession No.
Apodus deciduus	AY681199	*P. pleiospora*	AY515364
Amphiporthe leiphaemia	AJ293882	*P. setosa*	AF443852
Cercophora ambigua	AY999137	*Schizothecium aloides*	AY999120
C. areolata	AY587911	*Sch. carpinicola*	AY999118
C. caudata	AY999135	*Sch. conicum*	AY515356
C. coprophila	AY999136	*Sch. curvisporum*	AF443850
C. samala	AY999134	*Sch. curvisporum*	AY999119
C. sparsa	AY587912	*Sch. curvuloides*	AY515357
C. sulphurella	AY587913	*Sch. dakotense*	AY515358
Diaporthe helianthi	DQ221692	*Sch. fimbriatum*	AY999115
Neurospora intermedia	AY681192	*Sch. glutinans*	AY999116
Podospora anserina	AY525771	*Sch. inaequale*	AY999117
P. appendiculata	AY999126	*Sch. miniglutinans*	AY515362
P. austro-americana	AY999124	*Sch. vesticola*	AY515365
P. austrohemisphaerica	AY026939	*Sordaria lappae*	AY681171
P. cochleariformis	AY999123	*Strattonia insignis*	AY277912
P. comata	AF443849	*Zopfiella erostrata*	AY999133
P. cupiformis	AY999125	*Z. karachiensis*	AY999128
P. didyma	AY999127	*Z. latipes*	AY999129
P. ellisiana	AY515360	*Z. tabulata*	AY999132
P. fimiseda	AY515361	*Z. tetraspora*	AY999130
P. intestinacea	AY515363		

PCR-M Clean Up System Kit (Viogene, Taiwan). Sequencing of the cleaned PCR products was carried out using the DNA sequencing service of Tri-I Mission Biotech (Taipei, Taiwan).

Molecular phylogenetic analyses

Forty-four sequences of taxa from GenBank were obtained in this study (Table 2) in addition to the sequences obtained from cultures isolated from the Taiwanese specimens. Sequences were aligned using Clustal X (Thomson et al., 1997). The alignment was manually refined with the BioEdit (Hall, 1999), and ambiguously aligned sites were removed from the dataset. The data were then converted into the NEXUS format and analyzed by using PAUP vers. 4.0b10 (Swofford, 2002). Phylogenetic trees were constructed for each gene separately. MP was used for the analyses, and a bootstrapping analysis was performed with 1000 replications of heuristic search algorithm. We conducted 100 replicated heuristic searches with the addition of taxon sequences in a random order and tree-bisection-reconnection (TBR) branch swapping. All sites were treated as unordered and unweighted, with gaps treated as missing data.

BA was performed with MrBayes 3.0B4 (Huelsenbeck and Ronquist, 2001; http://morphbank.edc.uu.se/mrbayes3). The best-fit model was determined by Modeltest vers. 3.5 (Posada and Ceandall, 1998). The model of evolution was implemented and 3 x 10^6 generations were sampled every 1000th generation resulting in 3000 trees. The first 750 trees were considered the burn-in period and the remaining 2250 trees were used for calculating posterior probabilities in the consensus tree.

RESULTS

Phylogenetic analyses

The ITS dataset consisted of 66 taxa, of which 12 are members of *Cercophora* and 39 are members of *Podospora*. Each aligned sequence in the data set contained 647 characters, of which 80 sites were variable and 282 sites were phylogenetically informative. The MP found 1691 most parsimonious trees (with a consistency index (CI) of 0.4098, a retention index (RI) of 0.6937, and a rescaled consistency index (RC) of 0.2843). In the BA, the most suitable model was selected using Modeltest. The GTR+I+G model, with a gamma-distributed rate heterogeneity model (with a gamma Shape (G) of 0.7257) and an estimated proportion of invariable sites (of 0.3136) was chosen with Akaike Information Criterion (AIC). The BA consensus tree is shown in Figure 1.

Species of *Cercophora* and *Podospora* were dispersed in different clades scattered throughout the BA consensus tree based on the ITS dataset. The best-supported clade (Fig. 1, clade C) was composed of *Cercophora* and *Schizothecium* species which possess an outer perithecial wall with swollen agglutinated hairs. The other three well-supported clades (Fig. 1, clades B, D, and E) contained species of *Arnium* that clustered with species of *Cercophora, Podospora* and *Zopfiella* taxa which possess ascospore morphologies that do not occur along the putative evolutionary transition. *Podospora decipens*, *P. myriaspora,* and *P. pleiospora,* which are characterized by ascospores having lyre-shaped upper gelatinous appendages, and *P. cochleariformis*, which has a different ascospore morphology, were placed within a single clade (Fig. 1, clade F).

The GPD dataset consists sequences of 23 taxa, of which 5 are members of *Cercophora,* 11 are members of *Podopspora,* and 3 are members of *Schizothecium*. The dataset consists of 505 characters, where 34 sites were variable and 200 sites were phylogenetically informative. The MP found 832 most-parsimonious trees (with a CI of 0.4567, a RI of 0.5795, and a RC of 0.2647). In BA, the most suitable model was selected by using Modeltest. The TIM+I+G model, with a gamma-distributed rate heterogeneity model (gamma Shape (G) = 0.9668) and an estimated proportion of invariable sites (0.4415) was chosen with (AIC). The BA consensus tree is shown in Figure 2.

Species of *Cercophora* and *Podospora* also occurred in different clades scattered throughout the BA consensus tree based on the GPD dataset. The best-supported clade (Fig. 2, clade B) was composed of species of *Cercophora* and *Schizothecium*. Analyses revealed groupings of *Zygopleurage zygospora* with *P. communis* (Fig. 2, clade A), and *Arnium macrotheca* with certain *Cercophora* and *Schizothecium* species. The fourth clade (Fig. 2, clade D) contained three species of *Podospora* with similar lyre-shaped upper gelatinous appendages. This clade did not contain *P. cochleariformis*, because it lacks the GPD sequence.

The constrained tree topology based on the overall molecular analyses showed that *Cercophora* and *Podopspora* are polyphyletic. All of the *Schizothecium* species constitute a monophyletic clade, and two *Cercophora* species with similar perithecial structures formed a sister group next to this clade (Figs. 1 & 2).

DISCUSSION

These results are in agreement with previous studies (Miller and Huhndrof, 2005; Cai et al., 2006) in that the taxa with a similar perithecial wall morphology share a close relationship in certain clades in the Lasiosphaeriaceae. *Cercophora* and *Podospora* were found to be polyphyletic, consisting of a group of morphologically heterogeneous and phylogenetically distant species.

Cai et al. (2005) analysed gene sequences of 28S rDNA, ITS/5.8S rDNA and partial β-tubulin and indicated that perithecial morphology is more informative than ascospore characters and habitat association. The separation of *Schizothecium* from *Podospora* as a distinct genus was supported by their phylogenetic analyses. Thus, they accepted the generic status of *Schizothecium,* where 24 species were included.

Added with 14 fragments of ITS/5.8 S rDNA and GDP sequences in these analyses, our study showed a similar topology of all *Schizothecium* species being clustered in a single monophyletic clade. In addition, two *Cercophora* species, *C. acanthigera* and *C. coronata* were in a sister group to them. The perithecial structures of these two *Cercophora* species are similar to those of *Schizothecium*. In *C. acanthigera,* the perithecial wall is composed of large claw-like cells, and *C. coronata* has tufted perithecial hairs (Udagawa and Muroi, 1979).

The morphology of ascospores is highly variable within the Lasiosphariaceae and is traditionally used for delimiting genera. All *Cercophora* species have

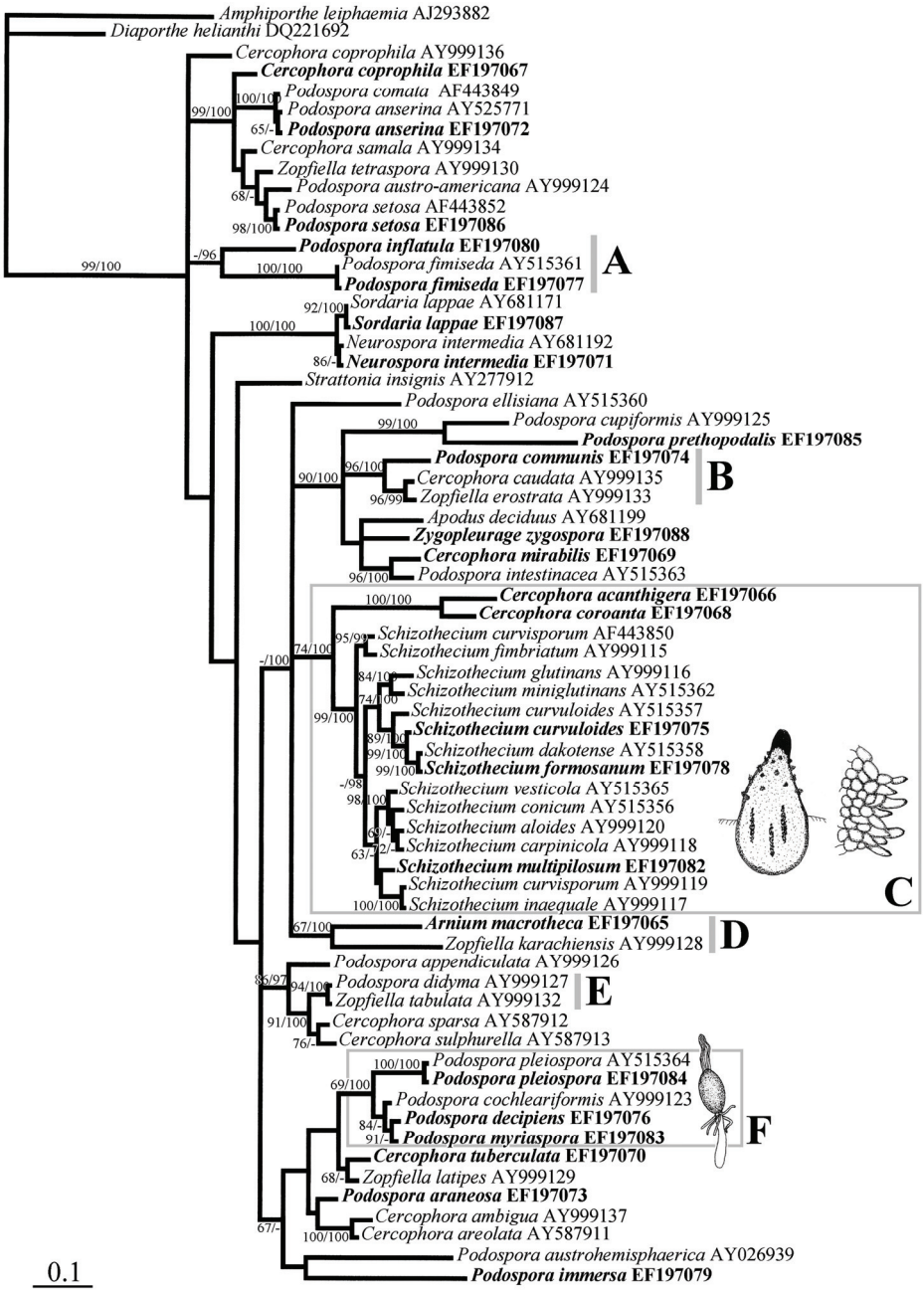

Fig. 1. Consensus tree derived from the Bayesian phylogenetic analysis based on the ITS/5.8S rDNA dataset of 66 taxa. Topology based on the Bayesian analysis was estimated using the GTR+I+G model with the transformation parameters of [A-C] = 2.5327, [A-G] = 2.6862, [A-T] = 2.1495, [C-G] = 1.3401, [C-T] = 3.655, and [G-T] = 1.00. Values before the slash are parsimony bootstrapping values of >60% while those after the slash are Bayesian posterior probabilities (of > 95%).

cylindrical young ascospores which become ellipsoid and dark brown with a long pedicel when mature, thus well segregating them from *Podospora* and *Schizotheium*. But in our analyses, *Cercophora* species were intermixed with those of *Podospora* and *Schizothecium* (Figs. 1 & 2). In the analyses using the ITS dataset, *P. decipiens*, *P. myriaspora*, and *P. pleiospora* with similar ascospores were grouped together with *P. cochleariformis* which has another kind of ascospores (Figs. 1F & 2D). *Podospora fimiseda* and *P. inflatula* were also placed together (Figs. 1A & 2C). But the upper and basal gelatinous appendages on the ascospores of *P. inflatula* are transversely striated, and those of *P. fimiseda* are longitudinally striated

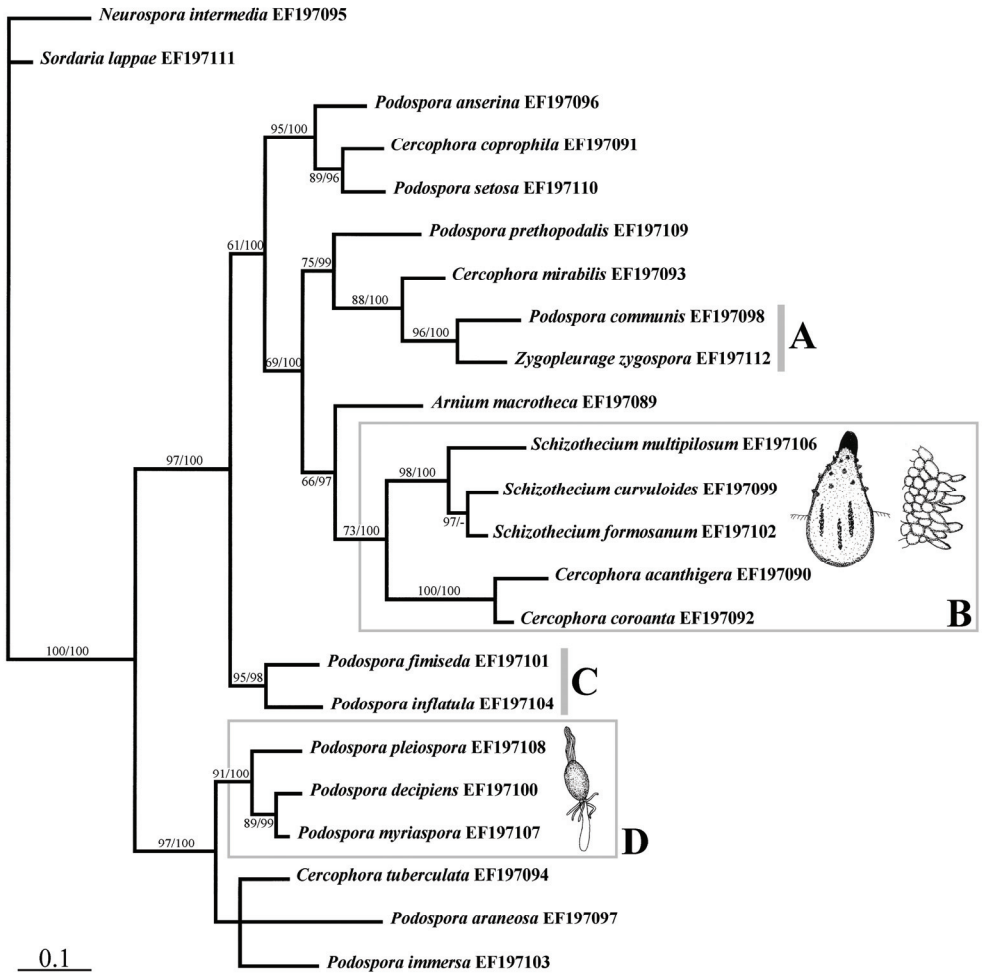

Fig. 2. Consensus tree derived from the Bayesian phylogenetic analysis based on the GPD dataset of 23 taxa. The topology based on the Bayesian analysis was estimated using the TIM+I+G model with the transformation parameters of [A-C] = 1.00, [A-G] = 2.6086, [A-T] = 1.8004, [C-G] = 1.8004, [C-T] = 4.3707, and [G-T] = 1.00. Values in before the slash are parsimony bootstrapping values of > 60%, while those after the slash are Bayesian posterior probabilities (of > 95%).

(Lundqvist, 1972; Wang, 2000). In the phylogenetic tree using the GPD dataset, *P. communis* and *Zyg. zygospora* were located in one clade (Fig. 2A). *Podospora communis* possesses some morphological characters close to *Zyg. zygospora*, as obpyriform perithecia and the end cells of ascospores tipped with four lash-like gelatinous appendages. *Zyg. zygospora* is easily distinguished from *P. communis* by its up to 200-µm-long ascospores and the two dark cells connected by a hyaline cord.

Many studies on the Lasiosphaeriaceae revealed that the phylogeny can not be inferred with ascospore morphology (Miller and Huhndorf, 2004, 2005; Cai et al., 2005, 2006) which however, remain essential for identification purposes.

Our study supports the recognition of the genus *Schizothecium*, to which *Podospora multipilosa* is transferred.

TAXONOMIC TREATMENT

Schizothecium multipilosum (J. H. Chang & Y. Z. Wang) J. H. Chang & Y. Z. Wang, comb. nov. MycoBank, MB 515504.

Podospora multipilosa J.H. Chang & Y.Z. Wang, Bot. Bull. Acad. Sin. 46: 169. 2005.

This species is characterized by multiple large tufted perithcial hairs composed of swollen cells and ascospores with a slender pedicel. In our phylogenetic analyses, it clustered with other S*chizothecium* species in a monophyletic clade (Figs. 1 & 2).

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毛球殼科尾柄孢殼屬、柄孢殼屬與裂殼屬間的分子親緣關係

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摘要：尾柄孢殼屬 (*Cercophora*) 和柄孢殼屬 (*Podospora*) 均為在草食性動物糞便上的常見真菌。以往依據形態特徵，將柄孢殼屬另分出一個裂殼屬 (*Schizothecium*)，近來以分子親緣分析，也證實這屬的成立。本研究是以細胞核 DNA 分子序列來探討尾柄孢殼屬和柄孢殼屬包含裂殼屬成員間的親緣關係。先以聚合酶鏈結增幅反應 (polymerase chain reaction, PCR) 的方法，增幅目標菌種的 ITS/5.8S rDNA 及 GPD (甘油醛-3-磷酸脫氫酶；glyceraldehyde-3-phosphate dehydrogenase) 基因序列。並以 ITS/5.8S rDNA 與 GPD 序列，利用最大簡約法 (maximum parsimony) 與貝貽理論 (Bayesian Inference) 找到最佳的親緣關係樹。由分析結果顯示，尾柄孢殼屬和柄孢殼屬均為多親緣性(polyphyletic)，裂殼屬成員與柄孢殼屬分開另成一群，並與具有相似子囊殼毛形態的二個尾柄孢殼屬成員為姐妹群，本研究認同以往的研究，裂殼屬可獨立成一屬，而親緣分析顯示子囊殼的形態在毛球殼科中是具有關連性的。

關鍵詞：尾柄孢殼屬、毛球殼科、親緣關係、柄孢殼屬、裂殼屬、系統分類學。