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**Family placement of *Ascotaiwania* and *Ascolacicola* based on DNA sequences from the large subunit rRNA gene**

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Ranghoo, V.M., Hyde, K.D., Liew, E.C.Y. and Spatafora, J.W. (1999). Family placement of *Ascotaiwania* and *Ascolacicola* based on DNA sequences from the large subunit rRNA gene. *Fungal Diversity* 2: 159-168.

The family placement of *Ascotaiwania* and *Ascolacicola* have been analysed by using partial sequences of the large subunit ribosomal DNA (LSU rDNA). The 5' end of the LSU rDNA was included in the analysis, which consisted of species from the Diaporthales, Halosphaeriales, Hypocreales, Microascales, Sordariales and Xylariales. The resulting trees indicate that *Ascotaiwania* is unrelated to the Annulatascaceae. A close relationship between the anamorphs of the different species of *Ascotaiwania* were found and they were closely related to *Monotosporella setosa*, the type species of *Monotosporella*. Based on morphological characters, production of different anamorphs, and the sequence analysis, it was found that *Ascolacicola* differs from *Ascotaiwania*. *Ascolacicola* was most closely related to the Sordariaceae.

**Key words:** Annulatascaceae, *Ascolacicola*, *Ascotaiwania*, Lasiosphaeriaceae, LSU rDNA.

**Introduction**

*Ascotaiwania* Sivan. and H.S. Chang was established by Sivanesan and Chang (1992) for an ascomycete having dark-brown to black ascomata with a short to long, lateral neck, asci with a relatively massive, non-amyloid apical ring and 7-septate pigmented ascospores with hyaline end cells. *Ascotaiwania lignicola* Sivan. and H.S. Chang is the type species. *Ascotaiwania palmicola* K.D. Hyde which has 3-septate brown ascospores with hyaline end cells, was added by Hyde (1995). Chang *et al.* (1998) described *A. hsilio* H.S. Chang and Y.S. Hsieh, *A. lignicola* H.S. Chang and Y.S. Hsieh, *A. sawada* H.S. Chang and Y.S. Hsieh, *A. wulai* H.S. Chang and Y.S. Hsieh from Taiwan, while Ranghoo and Hyde (1998) described *A. mitriformis* V.M. Ranghoo and K.D. Hyde from Hong Kong. The anamorph of *Ascotaiwania*, *Monotosporella* S. Hughes has

been reported by Ranghoo and Hyde (1998). Sivichai, Hywel-Jones and Jones (1999) reported another anamorphic *Monotosporella* state for *Ascotaiwania* from the culture of *A. sawada*.

The family placement of *Ascotaiwania* has been widely debated. The relatively massive refractive apical ring was thought to be of the *Xylaria*-type (Sivanesan and Chang, 1992). Ascospores, however, are light to mid-brown and multiseptate, but not dark-brown and unicellular as in most xylariaceous species (Sivanesan and Chang, 1992). The early-deliquesting paraphyses and non-amyloid ascal ring are characteristic of the Diaporthales, but unlike species of the Diaporthales, asci do not become detached from the ascomata. *Ascotaiwania* was considered best placed in the Amphisphaeriaceae by Sivanesan and Chang (1992). However, ultrastructural work on *A. lignicola* (Chang *et al.*, 1998) indicated that *Ascotaiwania* was not amphisphaeriaceous, and Wong, Hyde and Jones (1998) transferred it to the Annulatascaceae based on the presence of the relatively massive and refractive apical rings of the asci, typical of *Annulatascus* K. D. Hyde and *Annulatascus*-like genera. At the ultrastructural level the ascal apical ring has the same bipartite structure as that in other genera in the Annulatascaceae, and they also share the same ontogeny of the ascal wall and apical ring (Wong *et al.*, 1998; Wong and Hyde, 1999; Hyde, Wong and Jones, 1998). *Ascotaiwania* is unusual in the Annulatascaceae, however, in having multiseptate, brown, ascospores with hyaline end cells. The placement of *Ascotaiwania* in the Annulatascaceae needs verification using molecular characters.

*Ascolacicola aquatica* V.M. Ranghoo and K.D. Hyde has also been reported from freshwater habitats (Ranghoo and Hyde, 1998). It shares several morphological characteristics with *Ascotaiwania*, especially its brown, septate, ascospores with hyaline end cells and cylindrical asci. The apical ring in the ascus of *Ascolacicola aquatica*, however differs from that of *Ascotaiwania*, in being discoid. It is similar to that in species of *Savoryella* E.B.G. Jones and R.A. Eaton, which is another genus having brown septate ascospores, with hyaline end-cells in cylindrical asci. Ascomatal shape and paraphyses structure in *Ascolacicola* are also atypical of *Ascotaiwania* and *Savoryella*. Another peculiarity of *Ascolacicola* is that it produces a *Triadelphia uniseptata* (Berk. and Broome) P.M. Kirk. anamorph (reported as *Trichocladium uniseptatum* Berk. and Broome in Ranghoo and Hyde (1998)). On the basis of these morphological characters, *Ascolacicola aquatica* was described as a new genus (Ranghoo and Hyde, 1998). Ranghoo and Hyde (1998) considered that *Ascolacicola aquatica* should be placed in the Lasiosphaeriaceae based on the

**Table 1.** Isolates and sequences used in this study.

Fungi used in the molecular study	Source	Genebank accession number
<i>Annulatascus velatisporus</i>	HKUCC 3701	AF132319
<i>Annulatascus hongkongensis</i> *	HKUCC 3702	AF132320
<i>Aquaticola hongkongensis</i> *	HKUCC 3703	AF132321
<i>Ascolacicola aquatica</i>	HKUCC 3704	AF132322
<i>Ascotaiwania sawada</i>	HKUCC 3705	AF132323
<i>Ascotaiwania mitriformis</i>	HKUCC 3706	AF132324
<i>Ascocollumdensa aquatica</i> *	HKUCC 3707	AF132325
<i>Ascobrunneispora aquatica</i> *	HKUCC 3708	AF132326
<i>Ascominuta lignicola</i>	HKUCC 3709	AF132335
<i>Cateractispora recepticuli</i> *	HKUCC 3710	AF132327
<i>Cercophora appalachiensis</i>	HKUCC 3711	AF132328
<i>Clohiesia corticola</i>	HKUCC 3712	AF132329
<i>Diaporthe phaseolarum</i>	Genebank	U47830
<i>Fluminicola coronata</i> *	HKUCC 3717	AF132332
<i>Hypocrea schweinitzii</i>	Genebank	U47833
<i>Microascus trigonosporus</i>	Genebank	U47835
<i>Monotosporella setosa</i>	HKUCC 3713	AF132334
<i>Sordaria fumicola</i>	HKUCC 3714	AF132330
<i>Verticicola confusa</i> *	HKUCC 3715	AF132331
<i>Xylaria hypoxylon</i>	HKUCC 3716	AF132333

\* = Presently undescribed ascomycete species belonging to the Annulatascaceae which have black perithecia with long necks, cylindrical asci with a relative large refractive apical ring and ascospores with appendages. They are included here with names for comparison.

presence of brown ascospores, asci with a distinct apical ring and perithecioid ascomata. However this needed verification.

The aims of the current study were to establish:

1. The relationship of *Ascotaiwania* with the Annulatascaceae.
2. The relationships between the anamorphs of the two *Ascotaiwania* species and the type species of *Monotosporella* using molecular data.
3. If *Ascolacicola* is related to *Ascotaiwania* and to determine its family placement.

## Materials and Methods

### Cultures

Twenty isolates representing 20 species of unitunicate ascomycetes were chosen for this study and are listed in Table 1.

### ***Culturing, DNA Isolation and Polymerase Chain Reaction***

Cultures were grown on Potato Dextrose Agar and the mycelium was directly harvested for DNA extraction. Genomic DNA was isolated directly from the mycelia using a modification of the DNA isolation procedure for small quantities of fresh leaf tissue (Doyle and Doyle, 1987). DNA was obtained from cultures with mostly aerial mycelia by scraping the superficial mycelia. In others, some agar was also included in the extraction, but the agar did not affect the efficiency of the DNA extraction since complex carbohydrates were discarded in the organic layer during extraction. In each extraction, 0.05 g of mycelia was mixed with 1g of sand (white quartz of mesh size 50 to 70) followed by addition of the extraction buffer.

### ***DNA sequencing***

DNA sequences were determined for taxa listed in Table 1. The PCR products (150  $\mu$ l each) amplified from the LSU and the ITS regions of the rDNA were purified using the Wizard PCR Preps DNA Purification System (Promega) to remove unincorporated primers and deoxynucleotides and used for DNA sequencing. DNA sequencing was conducted for both strands using Auto Cycle 200 Sequencing Kit (Pharmacia) with primers LROR, LR5, LR3, LR3R, ITS2, ITS3, ITS4, ITS5. The primers were CY5-labelled from Pharmacia Biotech. The amplified fragments were sequenced using the automated sequencer ALFexpress automated DNA sequencer AM V3.0 (Pharmacia Biotech).

### ***Sequence Data Analysis***

Crude DNA sequences were aligned using the computer programmes SeqApp and Clustal W. Minor adjustments of the alignment were carried out manually. Only unambiguously aligned sequences were used in the analyses. These data matrices were subjected to parsimony analysis independently using the heuristic and branch and bound search options in the computer programme PAUP 3.1 (Swofford, 1993). 100 replicates of each search was performed, with random addition of the first taxon and stepwise addition of subsequent taxa. Most parsimonious trees were also calculated by cladistic analysis of the DNA sequences using a simple heuristic search. Consistency, Homoplasy, Retention and Rescaled Consistency Indices were noted. If more than one tree was obtained then a consensus tree was calculated by a strict consensus. Bootstrap confidence levels were calculated using PAUP 3.1 with 500 bootstrap replications.



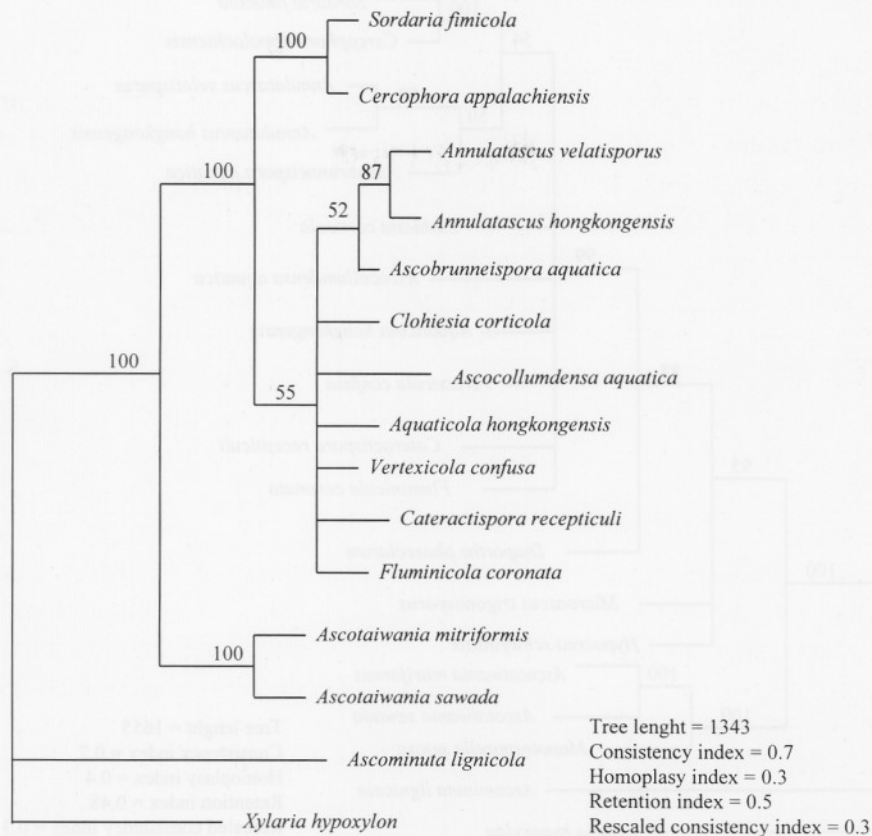


Fig. 1. The phylogenetic relationships between *Ascotaiwania* and the Annulatascaceae.

## Results

In order to establish if *Ascotaiwania* should be placed within the Annulatascaceae, sequences from *Ascotaiwania sawada* and *A. mitriformis* were aligned with the sequences of the genera in the Annulatascaceae and the aligned data were used in the parsimony analysis. The most parsimonious tree with a length of 1314, Consistency Index of 0.7, Homoplasy Index of 0.3, Retention Index of 0.5 and Rescaled Consistency Index of 0.3 was obtained (Fig. 1). *Ascotaiwania* species separated clearly from the Annulatascaceae and this monophyletic group was supported by a 100 % bootstrap support.

In order to establish if the anamorphs of *Ascotaiwania* were related to each other and to establish if *Ascotaiwania* belonged to any other orders, sequences from the type species of *Monotosporella* was also included in the sequence data. Representatives of the Diaporthales, Hypocreales, Microascales, Sordariales and Xylariales were also included in the sequence data and *Ascominuta lignicola* was used as the outgroup. The most parsimonious tree

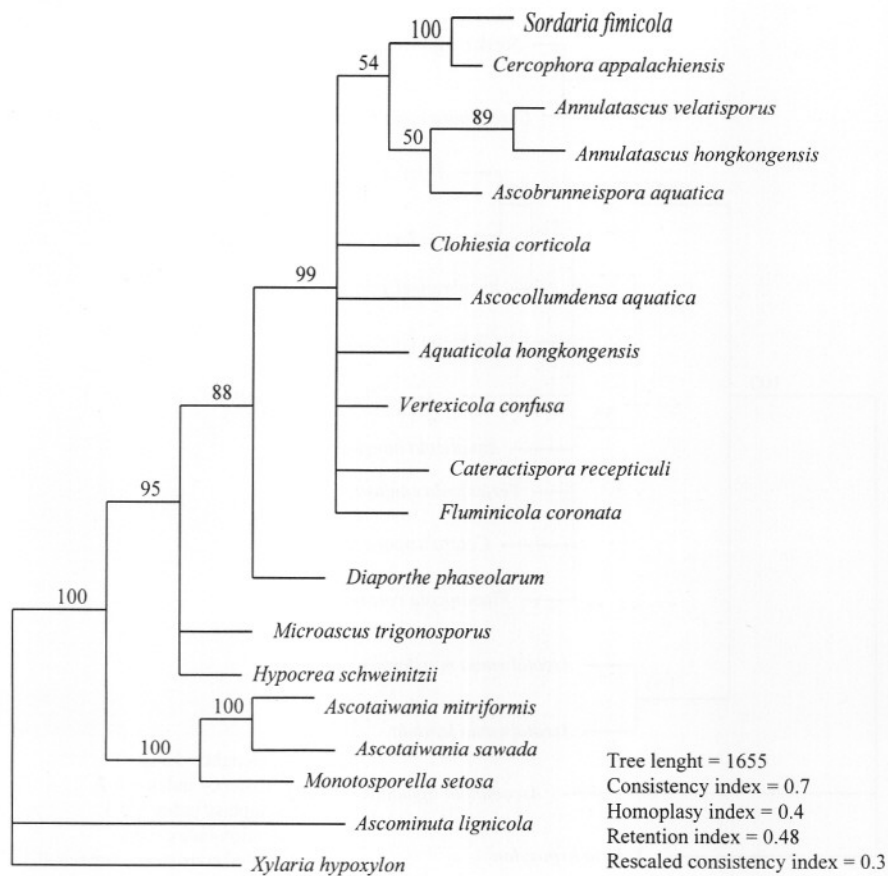


Fig. 2. The phylogenetic relationships between the anamorph of *Ascotaiwania* and *Monotosporella setosa*.

with a length of 1655, Consistency Index of 0.6, Homoplasy Index of 0.4, Retention Index of 0.48 and Rescaled Consistency Index of 0.3 was obtained (Fig. 2). *Ascotaiwania sawada*, *A. mitriformis* and *Monotosporella setosa* form a monophyletic group separate from the Annulatascaceae and Diaporthales, Hypocreales, Microascales, Sordariales and Xylariales with a 100 % bootstrap support.

In order to establish the placement of *Ascolacicola aquatica* at the family level and its relationship with *Ascotaiwania*, *A. aquatica* was analysed with sequences from representatives of Lasiosphaeriaceae, Sordariaceae, Diaporthales, Hypocreales and Microascales. The most parsimonious tree with a length of 827, Consistency Index of 0.6, Homoplasy index of 0.4, Retention Index of 0.51 and Rescaled Consistency Index of 0.3 was obtained (Fig. 3). Analysis of the 5' end of the LSU rDNA indicated that *Ascolacicola* is related

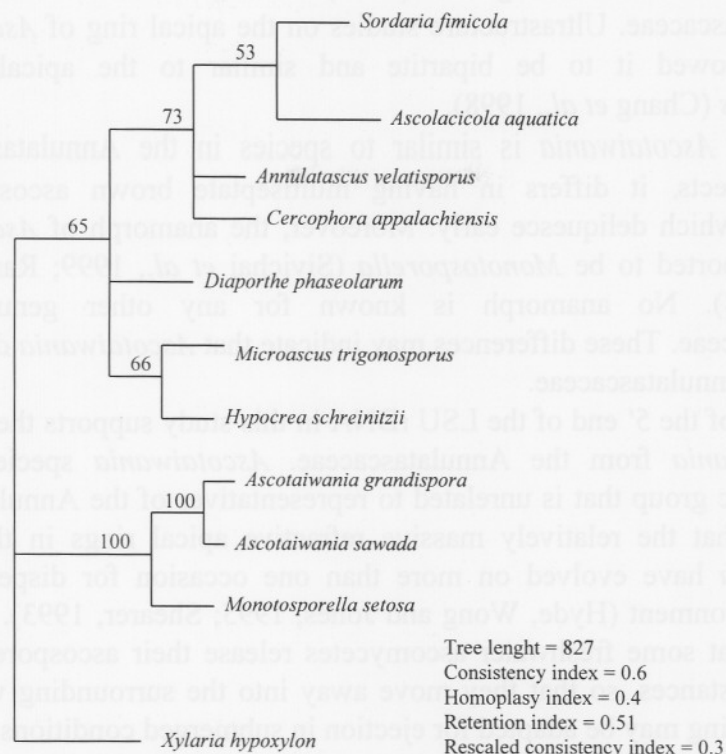


Fig. 3. The phylogenetic relationships of *Ascolacicola aquatica*.

to the sordariaceous species with a 73 % bootstrap support. *Ascolacicola* did not cluster with the representatives of *Ascotaiwania*.

## Discussion

### Family placement of *Ascotaiwania*

*Ascotaiwania* has a unique combination of morphological characters which include perithecioid ascomata, cylindrical asci with a relatively massive, refractive, apical ring and brown, septate ascospores with hyaline end cells (Sivanesan and Chang, 1992). However the asci deliquesce early in many species of the Diaporthales, but there is no evidence that the asci and paraphyses in *Ascotaiwania* separate from the subhymenium to float freely within the centrum at maturity as do those of other diaporthaceous taxa. *Ascotaiwania* most closely resembles *Annulatasascus* species, which also have perithecioid ascomata, cylindrical asci with relatively massive and refractive apical rings (Wong *et al.*, 1998). The massive apical ring was considered an

important character and Wong *et al.* (1998) therefore included *Ascotaiwania* in the Annulatascaceae. Ultrastructure studies on the apical ring of *Ascotaiwania lignicola* showed it to be bipartite and similar to the apical rings of *Annulatascus* (Chang *et al.*, 1998).

Although *Ascotaiwania* is similar to species in the Annulatascaceae in several respects, it differs in having multiseptate brown ascospores and paraphyses which deliquesce early. Moreover, the anamorph of *Ascotaiwania* has been reported to be *Monotosporella* (Sivichai *et al.*, 1999; Ranghoo and Hyde, 1998). No anamorph is known for any other genus in the Annulatascaceae. These differences may indicate that *Ascotaiwania* does not fit well in the Annulatascaceae.

Analysis of the 5' end of the LSU rDNA in this study supports the exclusion of *Ascotaiwania* from the Annulatascaceae. *Ascotaiwania* species form a monophyletic group that is unrelated to representatives of the Annulatascaceae suggesting that the relatively massive refractive apical rings in the asci of *Ascotaiwania* have evolved on more than one occasion for dispersal in an aquatic environment (Hyde, Wong and Jones, 1995; Shearer, 1993). It may be important that some freshwater ascomycetes release their ascospores actively over long distances, so that they move away into the surrounding water. The large apical ring may be adapted for ejection in submerged conditions.

It is however impossible to place *Ascotaiwania* in any families represented in the molecular analysis although it has morphological similarities with these families. The arrangement of the asci and interascal tissues is similar to the sordariaceous taxa in that they are basal in the centrum. Based on the characteristics of the centrum and other morphological characteristics, *Ascotaiwania* should be placed within the Annulatascaceae, but the molecular analysis does not support such inclusion.

#### ***Anamorph-teleomorph connection in Ascotaiwania***

*Monotosporella* was originally introduced by Hughes (1978) for a hyphomycete producing conidia on percurrently proliferating conidiophores on wood from a collection in New Zealand. It was only recently that Ranghoo and Hyde (1998) and Sivichai *et al.* (1999) noted the close similarities between the anamorphs of *Ascotaiwania* and *Monotosporella setosa* Berk. and M.A. Curtis. The *Monotosporella* states of *Ascotaiwania sawada* and *A. mitriformis* were thought to differ from *M. setosa* and were distinct species. In the analysis of the molecular data they separated as individual species, but clustered together. The current molecular analyses successfully verified the teleomorph-anamorph connection between *Ascotaiwania* and *Monotosporella*.



**Family placement of *Ascolacicola aquatica***

*Ascolacicola aquatica* was established by Ranghoo and Hyde (1998) for an ascomycete having brown ascospores with hyaline end cells and cylindrical asci with a relatively flattened apical ring. *Ascolacicola aquatica* had ascospores resembling those of *Ascotaiwania*, but differed in having a discoid apical ring instead of a massive refractive apical ring. *Ascolacicola aquatica*, also produced a *Triadelfia uniseptata* anamorph, while *Ascotaiwania* produced a *Monotosporella* anamorph and therefore a separate genus was established for the taxon. Of all the families within the Sordariales, *Ascolacicola*, is most closely related to the Annulatascaceae, Lasiosphaeriaceae and Sordariaceae on the basis of morphological characters.

Analysis of the 5' end of the LSU rDNA indicates that *Ascolacicola aquatica* is more closely related to sordariaceaeous representatives than it is to *Ascotaiwania*. *Ascolacicola aquatica* is neither related to the Annulatascaceae nor to the Lasiosphaeriaceae as suggested by Ranghoo and Hyde (1998), but is more closely related to the Sordariaceae. This may be supported by morphological characters, as, although some of the genera in the Lasiosphaeriaceae have cylindrical asci with a relatively discoid apical ring, the ascospores are hyaline with appendages e.g. *Lasio-sphaeria* species. Genera in the Sordariaceae, on the other hand, have cylindrical asci with relatively thickened apical ring and brown ascospores which may be ornamented or surrounded by mucilaginous sheaths (Barr, 1990). *Ascolacicola* is therefore more closely related with the Sordariaceae.

**Acknowledgements**

We are thankful to the Hong Kong Research Grants Council and The University of Hong Kong for grants to study freshwater fungi. V.M. Ranghoo is grateful to The University of Hong Kong for the award of a Postgraduate Studentship. A.Y.P. Lee and H.Y.M. Leung are thanked for photographic and technical assistance. Somsak Sivichai is thanked for supplying the culture of *Ascotaiwania sawada*.

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