Studies on the Amphisphaeriales 1. Amphisphaeriaceae (*sensu stricto*) and its phylogenetic relationships inferred from 5.8S rDNA and ITS2 sequences

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The Amphisphaeriaceae (*sensu lato*) presently includes 36 genera and 23 synonyms and is placed in the Xylariales. It is a relatively large and complicated family of ascomycetes. Molecular studies based on DNA sequence data of the 5.8S rRNA gene and internal transcribed spacer ITS2 of seventeen amphisphaeriaceous or related taxa are reported. A phylogenetic tree, using *Penicillium marneffei* as an outgroup, was derived from 435 sites which have been aligned. Based on the topology of the dendrogram and teleomorph-anamorph connections, the family Amphisphaeriaceae (*sensu stricto*) are restricted to *Amphisphaeria*, *Discostroma*, *Ellurema*, *Lepteutypa*, *Pestalosphaeria* and other genera possessing *Pestalotia*-like anamorphs. The Clypeosphaeriaceae are retained to include *Apioclypea*, *Capsulospora*, *Clypeosphaeria*, *Oxydothis* and other related genera. *Atrotorquata* which segregates from the Amphisphaeriaceae in the phylogenetic tree and morphologically resembles *Cainia* in having ascus apparatus comprising a series of rings and brown bicelled ascospores with longitudinal germ slits is placed in the Cainiaceae which are revived. *Xylaria* and *Hypoxylon* (Xylariales) are from another lineage which are well separated from the Amphisphaeriaceae (*sensu stricto*), Clypeosphaeriaceae and Cainiaceae. It is not appropriate to place these families in the Xylariales. The Order Amphisphaeriales are therefore revived to accommodate these families.

Introduction

This is part of a series of papers on the Amphisphaeriales which analyses the Amphisphaeriaceae (*sensu lato*). In this paper, results of molecular studies are used to reestablish the Amphisphaeriales and include within it three families, the Amphisphaeriaceae G. Winter (*sensu* Kang, Hyde and Kong, 1998a), Clypeosphaeriaceae G. Winter (*sensu* Kang, Hyde and Kong, 1998b) and Cainiaceae J.C. Krug (*sensu* Kang, Hyde and Kong, 1998c).

The Amphisphaeriaceae was originally established (as Amphisphaerieae) to accommodate *Amphisphaeria* Ces. and De Not., *Caryospora* De Not., *Ohleria* Fuckel, *Strickeria* Körb., *Trematosphaeria* Fuckel, and *Winteria* Rehm (Winter, 1887). The name then appeared to have become lost in the literature, however, Müller and Arx (1962) reintroduced the family, Amphisphaeriaceae, to accommodate *Amphisphaeria*, *Apiorhynchostoma* Petr., *Apiospora* Sacc., *Apiothyrium* Petr., *Cainia* Arx and E. Müll., *Cainiella* E. Müll., *Ceriophora* Höhn., *Ceriospora* Niessl, *Chaetapiospora* Petr., *Leiosphaerella* Höhn., *Oxydothis* Penz. and Sacc., *Pseudomassaria* Jacz., *Roussöella* Sacc., and *Seynesia* Sacc. The basis was the presence of a small iodine positive ring or disc in the ascus apex and ascomata which were immersed under a clypeus. The Amphisphaeriaceae were previously placed in the Order Sphaeriales (Müller and Arx, 1973). However, Eriksson (1983) suggested the Amphisphaeriales as a provisional Order to accommodate the Amphisphaeriaceae, Cainiaceae, Clypeosphaeriaceae and Hyponectriaceae Petr. This was then formally introduced by Hawksworth and Eriksson (1986). Thereafter based on studies on the anamorphs of *Collodiscula* I. Hino and Katum., *Induratia* Samuels, E. Müll. and Petrini and *Iodosphaeria* Samuels, E. Müll. and Petrini, Eriksson and Hawksworth (1987) combined the Cainiaceae with the Amphisphaeriaceae and considered them Xylariaceous. The Amphisphaeriaceae was then placed in the Xylariales (Eriksson and Hawksworth, 1993).

Currently the Amphisphaeriaceae (*sensu lato*) is a relatively large and complicated family of ascomycetes including 36 genera and 23 synonyms and placed in the Xylariales, Ascomycotina (Hawksworth *et al.*, 1995). The amphisphaeriaceous taxa are presently understood to have erumpent or immersed, clypeate, ostiolate, typically globose ascomata. The asci are unitunicate, cylindrical or clavate with a relatively simple apical ring which is usually amyloid or occasionally nonamyloid. Ascospores are mostly radially symmetric, hyaline or brown, usually transversely septate, and often have germ pores (Müller and Arx, 1962; Barr, 1990, 1994). One of the problems in the Amphisphaeriaceae is that most genera including the type have not been linked with anamorphs. However, Nag Raj (1977) observed pycnidia of *Pestalotia*-like *Bleptosporium pleurochaetum* (Speg.) Sutton associated with the ascomata of *Amphisphaeria argentinensis* Nag Raj, which he considered to be closely related to the type species of *Amphisphaeria*, *A. umbrina*. Samuels, Müller and Petrini (1987) therefore suggested that the group of amphisphaeriaceous genera linked to *Pestalotia*-like anamorphs, i.e. *Amphisphaeria*, *Broomella* Sacc., *Discostroma* Clem., *Lepteutypa* Petr. and *Pestalosphaeria* M.E. Barr should be separated from the other genera in the family and defined as Amphisphaeriaceae

(*sensu stricto*). The other genera (e.g. *Cainia* Arx and E. Müll. and *Oxydothis* Penz. and Sacc.) should be placed in other families.

Winter (1887) introduced the Clypeosphaeriaceae G. Winter to include *Anthostomella* Sacc., *Clypeosphaeria* Fuckel, *Hypospila* Sacc., *Linospora* Fuckel and *Trabutia* Sacc. and Roum. However, Miller (1949) and Munk (1957) placed the type genus *Clypeosphaeria* in the Xylariaceae Tul. and C. Tul. Subsequently *Clypeosphaeria* was included in the Amphisphaeriaceae (Dennis, 1978; Hawksworth, Sutton and Ainsworth, 1983; Eriksson and Hawksworth, 1987). Barr (1989) revived the Clypeosphaeriaceae to accommodate *Apiorhynchostoma* Petr., *Clypeosphaeria* Fuckel, *Endoxyla* Fuckel, *Melomastia* Nitschke and Sacc., *Pseudovalsaria* Spooner, *Saccardoella* Speg., and *Urosporella* G.F. Atk. which are related and morphologically similar to the Amphisphaeriaceae. Hawksworth *et al.* (1995) accepted *Apiorhynchostoma*, *Ceratostomella* Sacc., *Clypeosphaeria*, *Crassoascus* Checa, Barrasa and A.T. Martínez, *Duradens* Samuels and Rogerson, *Frondicola* K.D. Hyde, *Jobellisia* (Höhn.) M.E. Barr, *Melomastia* and *Pseudovalsaria* in the Clypeosphaeriaceae.

The Cainiaceae were originally introduced to accommodate the genus *Cainia* Arx and E. Müll. (Krug, 1977). *Cainia* has longitudinal germ slits in the ascospores and a complex ascus apparatus comprising a series of rings. The features were considered significant and distinguished the Cainiaceae from the Amphisphaeriaceae. However not all authors agreed with Krug (1977) and presently *Cainia* is in the Amphisphaeriaceae (*sensu lato*) (Hawksworth *et al.*, 1995).

To further understand and substantiate the phylogenetic relationships between the genera in the Amphisphaeriaceae (*sensu lato*), molecular studies of amphisphaeriaceous or related taxa were carried out. The phylogenetic relationships of various fungal taxa have been reconstructed using ribosomal RNA genes and spacer regions (Bruns, White and Taylor, 1991). The 5.8S rRNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) have been successfully used to investigate phylogenies of Pezizales (Momol and Kimbrough, 1994), *Leptosphaeria* Ces. and De Not. (Morales, Pelcher and Taylor, 1993; Morales *et al.*, 1995) and *Alternaria* Nees (Jasalavich *et al.*, 1995). In the present study, we have analysed the DNA sequences of the 5.8S rRNA gene and internal transcribed spacer ITS2 of selected amphisphaeriaceous and related taxa to infer their phylogenies.

Materials and methods

Seventeen amphisphaeriaceous and related fungal species representing seventeen different genera were used in the study (Table 1). Fungi were cultured

Table 1. List of taxa, their sources and GenBank accession number.

Species	Source	GenBank accession number
Amphisphaeria umbrina	HKUCC3175	AF009805
Apioclypea livistonae	HKUCC 3267	AF009804
Atrotorquata lineata	HKUCC 3263	AF009807
Capsulospora sp.	HKUCC 3998	AF009819
Clypeosphaeria mamillana	HKUCC 3264	AF009808
Cytoplea hysterioides	HKUCC 2096	AF009811
Discostroma tosta	HKUCC 1004	AF009814
Ellurema indica	IMI 136542	AF009816
Hypoxylon fragiforme	HKUCC 3265	AF009810
Lepteutypa cupressi	IMI 052255	AF009817
Myelosperma tumidum	HKUCC 2057	AF009813
Oxydothis frondicola	HKUCC 3173	AF009803
Pestalosphaeria elaeidis	IMI 061175	AF009815
Pestalotia palmarum	ATCC 10085	AF009818
Roussoëlla hysterioides	HKUCC 2041	AF009812
Roussoëlla sp.	HKUCC 1874	AF009806
Xylaria hypoxylon	HKUCC 3266	AF009809

HKUCC = The University of Hong Kong Culture Collection.
IMI = International Mycological Institute.
ATCC = American Type Culture Collection.

in Bacto Sabouraud Dextrose broth (1 % Neopeptone, 2 % Bacto Dextrose, Difco) for DNA isolation. The primers ITS1, ITS2, ITS3, ITS4 and ITS5 (for sequences and code names of primers, see White *et al.*, 1990) used for PCR amplification and DNA sequencing were obtained from University of Portsmouth.

PCR amplification and purification of the products

Genomic DNA was isolated from lyophilized fungal mycelia using the isolation protocol of Lee and Taylor (1990). Template DNA (200 μg) was amplified in a 100 μL PCR reaction mixture consisting of 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 6 mM $MgSO_4$, O.1 % Triton X-100, 500 μM each of dATP, dCTP, dGTP, and dTTP, with 56 pmols ITS5 and 62 pmols ITS4 primers, and 5 units of VENT (Biolabs) or Taq (Promega) DNA polymerase. The reaction was set up as follows: initial denaturation at 95 C for 2.5 min, followed by 35 cycles of denaturation at 95 C for 30 s, annealing at 50 C for 1 min, extension at 72 C for 1.5 min, and final extension at 72 C for 10 min in a Gene Cycler (BIO-RAD). A negative control using water instead of template DNA was set up for each experiment. PCR products were analysed by

electrophoresis at 75 V for 2 h in a 0.8 % (w/v) agarose gel in 1 × TAE buffer (0.4 M Tris, 0.05 M NaAc, 0.01 M EDTA, pH 7.85) and visualized under UV light in a transilluminator (TFX-35C, Vilber Lourmat) following ethidium bromide staining.

PCR products were purified from gel using the PREP-A-GENE matrix (BIO-RAD). Briefly, PCR bands were excised from the gel following estimation of DNA concentration by comparison to a band of a known amount of DNA on gel. For each microgram of DNA, 5 μL of prep-A-gene matrix was used. Based on the volume of the gel slice obtained, 3 volumes of binding buffer (6 M sodium perchlorate; 50 mM Tris-HCl, pH 8; 10 mM EDTA) were added to the gel slice and incubated at 37 C for 20 min to dissolve the gel. The matrix was added to the mixture and incubated at 37 C for 10 min. The matrix containing DNA was pelleted by brief centrifugation and the supernatant was removed. The pellet was washed twice in 1 ml of binding buffer and three times with 1 ml of wash buffer (20 mM Tris-HCl, pH 7.5; 2 mM EDTA; 50 % ethanol). To elute the bound DNA, the pellet was resuspended in an equal volume of elution buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) and incubated at 37 C for 10 min. The matrix was then pelleted by centrifugation at 13000 rpm for 2 min and the supernatant containing DNA was transferred to a sterile tube and stored at -20 C until use.

Sequencing

Purified PCR products were directly sequenced using the T7 sequencing kit (Pharmacia) with modification. PCR products (10 μL containing 1 μg DNA templates) were mixed with 2 μL annealing buffer(1 M Tris-HCl, pH 7.6, 100 mM $MgCl_2$ and 160 mM DTT) and 2 μL sequencing primer (30 pmol). The mixture was boiled for 3 min and immediately plunged into liquid nitrogen for 3 min and then thawed on ice. Three microlitres of labelling Mix-dATP (1.375 μM each dCTP, dGTP and dTTP and 333.5 mM NaCl), 10 μCi of [α-^{35}S]dATP, and 4 units T7 polymerase were added to the annealed primer:template mixture and incubated at room temperature for 5 min. The resulting mixture (4.5 μL each) was dispersed into four tubes containing 2.5 μL of the appropriate termination mixture (G-short, A-short, T-short, C-short) and incubated at 37 C for 5 min. The reactions were terminated by adding 5 μL of the stop solution (0.3 % each Bromophenl Blue and Xylene Cyanol FF; 10 mM EDTA, pH 7.5, and 97.5 % deionized formamide). The products of the sequencing reaction were separated by electrophoresis in 6 % (w/v) polyacrylamide, 7 M urea sequencing gels at 2000 volts for 3-6 h. The gels were fixed for 15 min in a 10 % methanol/acetic acid mixture and dried in a gel drier (Bio-Rad) and autoradiographed on

Amersham or Fuji X-ray film. The film was developed in Kodak GBX developer.

Phylogenetic analysis

The sequences of this study and sequence of *Penicillium marneffei* G. Segretain, M. Capponi and P. Sureau obtained from GenBank (L37406) as outgroup were stored as sequence files manually using the SEQED programme of the University of Wisconsin Genetics Computer Group (GCG) software package (Devereux, Haeberli and Smithies, 1984). Alignments of the sequence files were conducted using the CLUSTAL W software (Thompson, Higgins and Gibson, 1994). Phylogenetic trees were generated by two different methods: (i) Using the neighbor-joining method (phenetic approach; Saitou and Nei, 1987), 100 bootstrap data sets were generated by the programme SEQBOOT to evaluate the robustness of the tree. The bootstrap data sets were processed by DNADIST with 100 replications to generate distance matrices of pair-wise genetic distance between pairs of sequence data sets using the Kimura two-parameter model. Distance matrices were then calculated by the neighbor-joining method with 100 replications to generate phylogenetic trees. The majority rule consensus tree was derived from the trees of the neighbor-joining methods by the programme CONSENSE. (ii) Using the maximum likelihood method (cladistic approach) (Felsenstein, 1993), the tree was calculated by the DNAML programme with 10 randomizations of sequence input order of the original data set and global rearrangement of the tree.

Results and Discussion

Alignment of nucleotide sequences

For each species, about 530 bases of the 5.8S rRNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) have been determined. The algorithmic alignment of the nucleotide sequences produced a consensus length of 726 sites for the 18 fungal species. Manual editing of the algorithmically aligned sequences was not attempted. In the variable regions (ITS1 and ITS2) of the alignment, a number of domains which contain highly conserved base sequence were observed amongst closely related taxa e.g. *Amphisphaeria umbrina* (Fr.) De Not., *Discostroma tosta* (Berk. and Broome) Brockmann, *Ellurema indica* (Punith.) Nag Raj and Kendr., *Lepteutypa cupressi* (Nattrass, Booth and Sutton) Swart, *Pestalosphaeria elaeidis* (C. Booth and J.S. Robertson) H.A. van der Aa, *Pestalotia palmarum* Cooke, and also amongst all the *Roussoëlla* Sacc. species (data not shown). Because sequences in the ITS1 region show big variations both in terms of base substitution and length

polymorphism, they were excluded from the data set for phylogenetic tree construction. Certain amount of variations were observed within the 5.8S rDNA which are accountable for the phylogenetic relationships of the taxa above genus level in this study. Therefore the 5.8S rDNA and ITS2 sequences including 435 sites were used for the phylogenetic analyses.

Phylogenetic relationships of the Amphisphaeriaceae* (sensu stricto) *and related taxa

The neighbor-joining and maximum likelihood methods produced two dendrograms which are almost identical. The only difference is that the neighbor-joining tree forms a terminal cluster comprising *Ellurema indica* and *Lepteutypa cupressi*, while the maximum likelihood tree forms a terminal cluster comprising *Pestalotia palmarum* and *Discostroma tosta*. Since *Ellurema indica* and *Lepteutypa cupressi* are closely related with morphological similarities (Nag Raj and Kendrick, 1985) and the cluster receives quite a high bootstrap support (82 %) in the neighbor-joining tree, this cluster is therefore accepted. The topology of the dendrogram (Fig. 1) shows two groups. The major group is composed of the broadly defined amphisphaeriaceous taxa, while the two xylariaceous taxa comprise the other group with *Penicillium marneffei* as an outgroup. In the major group, *Amphisphaeria umbrina*, *Discostroma tosta*, *Ellurema indica*, *Lepteutypa cupressi*, *Pestalosphaeria elaeidis*, *Pestalotia palmarum* and form a monophyletic clade supported by a high bootstrap value (93 %). Relatively high bootstrap values (81-88 %) are also observed at the terminal nodes. This supports the hypothesis of Samuels *et al*. (1987) that the Amphisphaeriaceae (*sensu stricto*) should be restricted to the genera in the Amphisphaeriaceae (*sensu lato*) which produce *Pestalotia*-like anamorphs (Kang *et al*., 1998a).

The relatively high bootstrap value at the node of *Atrotorquata lineata* Kohlm. and Volkm.-Kohlm. (86 %) indicates that *Atrotorquata lineata*, *Apioclypea livistonae* K.D. Hyde, *Clypeosphaeria mamillana* (Fr.) Lamb., *Capsulospora* sp. and *Oxydothis frondicola* K.D. Hyde are related to the Amphisphaeriaceae (*sensu stricto*). Although the phylogenies of these genera require further investigation, the Clypeosphaeriaceae is retained as a polyphyletic family to include *Apioclypea livistonae* K.D. Hyde, *Capsulospora* sp., *Clypeosphaeria mamillana* (Fr.) Lamb., *Oxydothis frondicola* K.D. Hyde and other related genera which are related to the Amphisphaeriaceae (*sensu stricto*; Barr, 1990, 1993; Kang *et al*., 1998b). *Atrotorquata* Kohlm. and Volkm.-Kohlm. flanks the Amphisphaeriaceae (*sensu stricto*). This genus has certain morphological resemblance with *Cainia* (Kohlmeyer and Volkmann- Kohlmeyer,

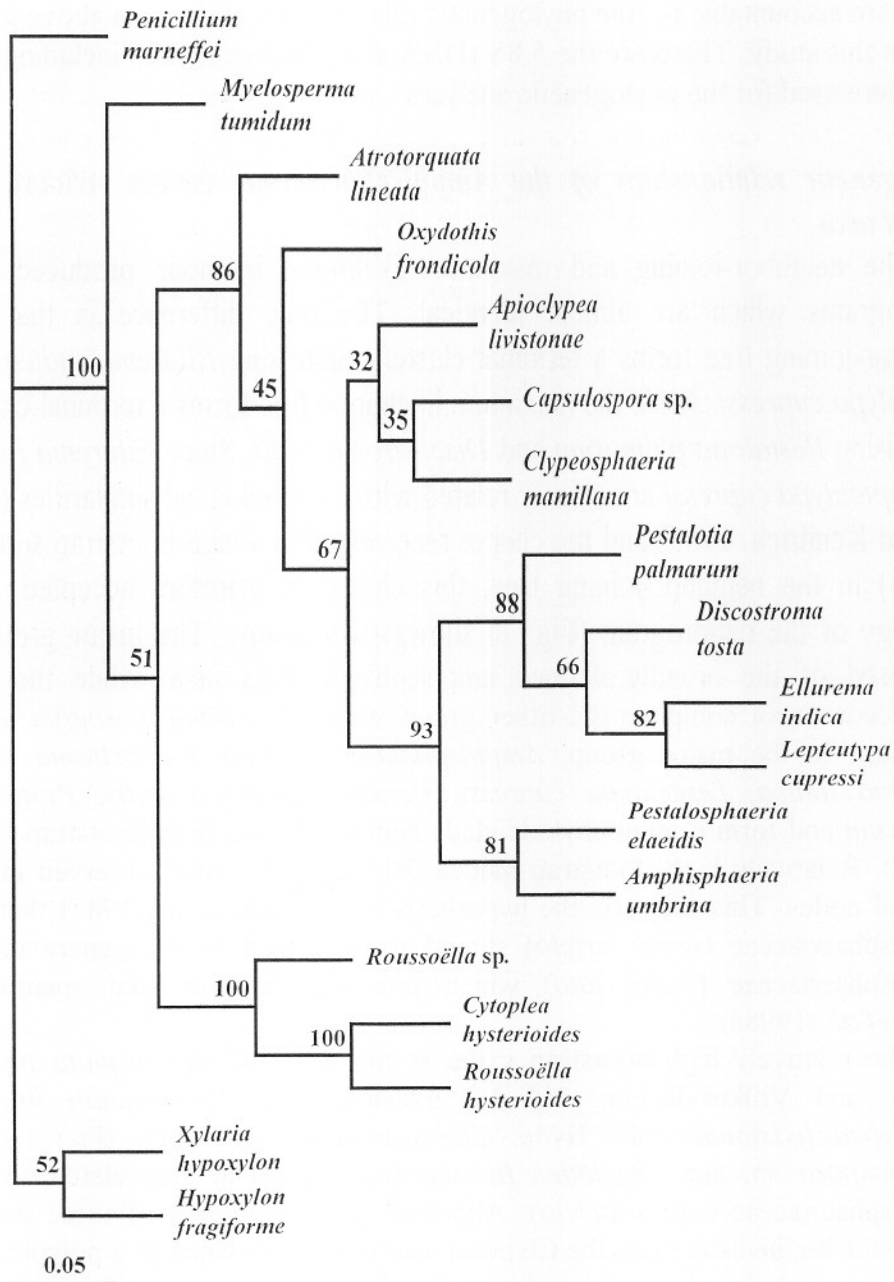

Fig. 1. The majority rule consensus tree derived from the alignment of the 5.8S rDNA and ITS2 spacer of 18 taxa. The tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap values derived from 100 samples are indicated on each branch. The bar indicates 5 substitutions per 100 nucleotides.

1993). The Cainiaceae J.C. Krug, based on the characters of ascus apparatus comprising a series of rings and brown bicelled ascospores with longitudinal germ slits (Krug, 1977), is revived to include *Atrotorquata*, *Cainia* and other related genera with morphological similarities (Kang *et al.*, 1998c).

Myelosperma tumidum Syd. and P. Syd. separates from the above taxa and its phylogenetic relationships remain uncertain. *Roussoëlla hysterioides* (Ces.) Höhn., its anamorph *Cytoplea hysterioides* K.D. Hyde and the other *Roussoëlla* sp. form a monophyletic clade with high bootstrap value support (100 %) which may represent another family. Recently Hyde, Eriksson and Yue (1996), Aptroot (1995) and Ju, Rogers and Huhndorf (1996) transferred *Roussoëlla* into the Didymosphaeriaceae Munk by recognizing its bitunicate asci. The phylogenetic relationships of *Roussoëlla* and Didymosphaeriaceae are pending further research. The xylariaceous taxa, *Xylaria hypoxylon* (L.: Fr.) Grev. and *Hypoxylon fragiforme* (Pers.: Fr.) Kickx fall into another group and is well separated from all the above taxa with a high bootstrap value support (100 %), which indicate that the Xylariaceae Tul. and C. Tul. and Amphisphaeriaceae (*sensu stricto*) are from two different phylogenetic lineages. However the low bootstrap value at the node shows that *Xylaria* Hill ex Schrank and *Hypoxylon* Bull. may be only distantly related and the phylogenetic relationships of these and other xylariaceous taxa require further studies.

The phylogenetic analysis based on the 5.8S rRNA gene and ITS2 spacer indicates that at least two major lineages exist in the ascomycetes examined. *Xylaria* and *Hypoxylon*, representing the Xylariales are from one lineage. These genera have a hyphomycetous anamorphs which have the conidia simply generated from the hypha, e.g. *Geniculosporium* Chesters and Greenh. and *Nodulisporium* Preuss (Carmichael *et al.*, 1980). On the other hand the Amphisphaeriaceae (*sensu stricto*) which have coelomycetous anamorphs with conidia formed within a conidiomata comprising fungal hypha and host tissue, e.g. *Pestalotia* De Not. and *Hyalotiopsis* Punith. (Nag Raj, 1993), represent another lineage. Furthermore the Clypeosphaeriaceae and the Cainiaceae are more closely related to the Amphisphaeriaceae (*sensu stricto*), than to the Xylariaceae. The Amphisphaeriales (Hawksworth and Eriksson, 1986) is therefore revived to include the Amphisphaeriaceae (*sensu stricto*), the Clypeosphaeriaceae and the Cainiaceae.

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