The *Xylariales*: a monophyletic order containing 7 families

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A number of often conflicting, morphology-based classifications have been suggested for the *Xylariales*. However, no attempt has previously been made to test these classifications using molecular data. Phylogenetic relationships of 6 accepted families of the *Xylariales* (*Amphisphaeriaceae*, *Clypeosphaeriaceae*, *Diatrypaceae*, *Graphostromataceae*, *Hyponectriaceae* and *Xylariaceae*) plus members of the *Apiosporaceae*, were investigated using individual and combined analyses of 28S and 18S rDNA gene fragments. Analyses were conducted using maximum and weighted parsimony, and likelihood criteria. The *Xylariales* was found to be a monophyletic order containing the above 7 families. However, the 28S and 18S rDNA data proved to be inadequate in determining the familial relationships within the order. This finding is contrary to most other studies in ascomycete systematics using these particular genes.

Key words: phylogeny, *Amphisphaeriaceae*, *Apiosporaceae*, *Clypeosphaeriaceae*, *Diatrypaceae*, *Graphostromataceae*, *Hyponectriaceae*, *Xylariaceae*.

Introduction

The *Xylariales* is a large order of unitunicate, perithecial ascomycetes that contains over 92 genera and 795 species. Delimitation of the *Xylariales*, and the families within the order, has long been problematic. A number of different classification systems, based predominantly on morphological characters, have been proposed that accept anywhere from three to 11 families within the order (Munk, 1953; Müller and von Arx, 1962, 1973; Wehmeyer, 1975; Barr, 1990; Hawksworth *et al*., 1995; Eriksson *et al*., 2001, 2003). Differences between these classifications have largely arisen due to greater or lesser emphasis being placed on certain characters, while often completely ignoring other information.

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There are, however, features generally agreed upon that unite members of the *Xylariales*. Briefly, these include production of well-developed stromata, perithecial ascomata with thick walls, eight-spored unitunicate asci with a J+ apical apparatus. Paraphyses are apically free and develop from a hymenial layer, while ascospores, usually pigmented, possess germ pores or germ slits, and may or may not be transversely septate, or have a mucilaginous sheath (Barr, 1990; Hawksworth *et al*., 1995). Anamorphs of the *Xylariales* are usually hyphomycetous, with holoblastic conidial production (Rogers, 1979; Whalley, 1996).

The most recent classifications of the *Xylariales* are provided by Eriksson *et al*. (2003), in their "Outline of Ascomycota - 2003" and by Kirk *et al*. (2001) in the ninth edition of the "Dictionary of the Fungi". Eriksson *et al*. (2003) accept six families in the order, *Amphisphaeriaceae*, *Clypeosphaeriaceae*, *Diatrypaceae*, *Graphostromataceae*, *Hyponectriaceae* and *Xylariaceae*, while Kirk *et al*. (2001) include two additional families, the *Cainiaceae* and *Myelospermataceae*, that latter containing the single genus *Myelosperma*.

Evidence for the classification of Eriksson *et al*. (2003) comes, in part, from small subunit ribosomal DNA (18S rDNA) sequences. However, this molecular evidence has resulted indirectly from studies of other taxonomic groups that have used only a few species from 1 or 2 families of the *Xylariales* as outgroup or reference taxa (e.g. Spatafora *et al*., 1998; Winka and Eriksson, 2000; Réblová and Winka, 2001) and the scheme is still mostly based on morphological data. The only significant molecular taxonomy that has taken place on higher-level relationships within the *Xylariales* has revolved around the work of Kang *et al*. (1998, 1999a,b,c,d, 2002) and Jeewon (2002) that deal with the *Amphisphaeriaceae* and other families thought to be related.

In addition to the 6 families accepted by Eriksson *et al*. (2003), the *Apiosporaceae* is thought to have affinities with the *Xylariales*. The *Apiosporaceae* was erected by Hyde *et al*. (1998) with *Apiospora* as the type genus, and also tentatively including *Appendicospora*. Hyde *et al*. (1998) provide a discussion on the placement of *Apiospora* which has been of uncertain taxonomic placement for many years, but they do not make a suggestion as to where the *Apiosporaceae* should be placed, although all other families with apiospores discussed belong to families from the *Xylariales*. It has also been suggested that the *Apiosporaceae* may be related to the *Xylariales*, based on the type of anamorph (Wang and Hyde, 1999). Kang *et al*. (2002) included a single sequence of an *Arthrinium* species (the anamorph of *Apiospora*) in an ITS rDNA analysis that indicated affinities between the *Apiosporaceae* and the *Xylariales*. Jeewon (2002) and Kang *et al*. (2002) both

accept the *Cainiaceae* as a family within the *Xylariales*, while Eriksson *et al*. (2003) retain those taxa within the *Amphisphaeriaceae*, although Eriksson (2000, 2002) suggests it may deserve familial rank, due to the lack of conclusive molecular data. Taxa from the *Cainiacaeae sensu* Kang *et al*. (1999b), which are placed in the *Amphisphaeriaceae* by Eriksson *et al*. (2003), have been included in the current study to determine the status of this group.

While much has been written about various aspects of the classification of the *Xylariales*, no significant attempt has been made to investigate the evolutionary relationships of the order using molecular techniques, either with respect to other groups of fungi or with regard intra-ordinal organisation. Given this situation this study was undertaken to test if the *Xylariales* (*sensu* Eriksson *et al*., 2003) represents a monophyletic group, and to attempt to elucidate familial relationships within the *Xylariales*.

Materials and methods

Cultures and DNA sequences

A total of 23 taxa from the *Xylariales*, representing the 6 families, and an additional 3 taxa from the *Apiosporaceae* were included in this study. A further 16 taxa were included as reference taxa in addition to the outgroup taxon. Reference taxa chosen represent each of the three sub-classes, all of the orders (except the *Microascales*) and 8 families from Class Sordariomycetes (Eriksson *et al*., 2003). An additional 4 reference taxa from Classes Dothideomycetes and Pezizomycetes (from the same subphylum Pezizomycotina as Sordariomycetes) were included. A basal ascomycete, *Saccharomyces cerevisiae* (Subphylum Saccharomycotina, Class Saccharomycetes) was included as an outgroup. In all cases an attempt to include the type species of each of the families under investigation was made, however the type species of the *Clypeosphaeriaceae* was not included.

Fungal strains and GenBank sequences used in this study are listed in Table 1. GenBank sequences for 4 taxa were from different strains for the 28S and 18S rDNA respectively: *Lasiosphaeria ovina*, *Nectria cinnabarina*, *Pleospora herbarum* and *Sordaria fimicola* (Table 1). Sequences of *Saccharomyces cerevisiae* were obtained from the Saccharomyces Genome Database (SGD) (Cherry *et al*., 2001).

Cultures were obtained from a number of sources: CABI Bioscience (IMI), Centraalbureau voor Schimmelcultures (CBS), Hong Kong University Culture Collection (HKUCC) and Uppsala University Culture Collection (UPSC). All cultures were grown on potato dextrose agar (PDA) and incubated

Table 1. Fungal taxa and their GenBank accession numbers.

Species	Culture source [a]	GenBank accession number		Family *sensu* Eriksson *et al*. (2003)	Order *sensu* Eriksson *et al*. (2003)
		28S rDNA	18S rDNA		
Amphisphaeria umbrina	HKUCC 994	AF452029	AY083811	*Amphisphaeriaceae*	*Xylariales*
Arecophila bambusae	HKUCC 4794	AF452038	AY083802	*Amphisphaeriaceae*	*Xylariales*
Cainia graminis	CBS 136.62	AF452033	AY083801	*Amphisphaeriaceae*	*Xylariales*
Discostroma tosta	HKUCC 1004	AF382380	AY083814	*Amphisphaeriaceae*	*Xylariales*
Lepteutypa cupressi	IMI 052255	AF382379	AY083813	*Amphisphaeriaceae*	*Xylariales*
Pestalosphaeria sp.	HKUCC 8677	AF452031	AF104356	*Amphisphaeriaceae*	*Xylariales*
Apiospora sinensis	HKUCC 3143	AY083831	AY083815	*Apiosporaceae*	*Incertae sedis*
Appendicospora sp.	HKUCC 1120	AY083833	AY083817	*Apiosporaceae*	*Incertae sedis*
Arthrinium phaeospermum	HKUCC 3395	AY083832	AY083816	*Apiosporaceae*	*Incertae sedis*
Camarops microspora	CBS 649.92	AY083821	AY083800	*Bolinaceae*	*Boliniales*
Apioclypea sp.	HKUCC 6269	AY083836	AY083819	*Clypeosphaeriaceae*	*Xylariales*
Clypeosphaeria uniseptata	HKUCC 6349	AY083830	AY083812	*Clypeosphaeriaceae*	*Xylariales*
Oxydothis frondicola	HKUCC 1001	AY083835	AY083818	*Clypeosphaeriaceae*	*Xylariales*
Diaporthe phaseolorum	-	U47830	L36985	*Valsaceae*	*Diaporthales*
Cryptosphaeria eunomia var. *eunomia*	CBS 216.87	AY083826	AY083807	*Diatrypaceae*	*Xylariales*
Diatrype disciformis	CBS 205.87	U47829	U32403	*Diatrypaceae*	*Xylariales*
Eutypa sp.	HKUCC 337	AY083825	AY083806	*Diatrypaceae*	*Xylariales*
Graphostroma platystoma	CBS 270.87	AY083827	AY083808	*Graphostromataceae*	*Xylariales*
Aniptodera chesapeakensis	-	U46882	U46870	*Halosphaeriaceae*	*Halosphaeriales*
Ceriosporopsis halima	-	U47844	U47843	*Halosphaeriaceae*	*Halosphaeriales*
Halosphaeria appendiculata	-	U46885	U46872	*Halosphaeriaceae*	*Halosphaeriales*
Ophiodeira monosemeia	-	U46894	U46879	*Halosphaeriaceae*	*Halosphaeriales*

Table 1. (continued).

Species	Culture source [a]	GenBank accession number		Family *sensu* Eriksson *et al*. (2003)	Order *sensu* Eriksson *et al*. (2003)
		28S rDNA	18S rDNA		
Hypocrea schweinitzii	-	U47833	L36986	*Hypocreaceae*	*Hypocreales*
Glomerella cingulata	HKUCC 9036	AY083820	AY083798	*Incertae sedis*	*Incertae sedis*
Hyponectria buxi [b]	UME 31430 herbarium specimen	AY083834	AF130976	*Hyponectriaceae*	*Xylariales*
Monographella nivalis	UPSC 3273	AF452030	AF064049	*Hyponectriaceae*	*Xylariales*
Lulworthia medusa	-	AF195637	AF195636	*Lulworthiaceae*	*Lulworthiales*
Ophiostoma piliferum	-	U47837	U20377	*Ophiostomataceae*	*Ophiostomatales*
Lasiosphaeria ovina[d]	CBS 958.72	AF064643	AY083799	*Lasiosphaeriaceae*	*Sordariales*
Sordaria fimicola [d]	-	AF132330	X69851	*Sordariaceae*	*Sordariales*
Astrocystis cocoes	HKUCC 3441	AY083823	AY083804	*Xylariaceae*	*Xylariales*
Daldinia concentrica	-	U47828	U32402	*Xylariaceae*	*Xylariales*
Fasciatispora petrakii	HKUCC 207	AY083828	AY083809	*Xylariaceae*	*Xylariales*
Halorosellinia oceanicum	HKUCC 5548	AY083822	AY083803	*Xylariaceae*	*Xylariales*
Hypoxylon fragiforme	HKUCC 1022	AY083829	AY083810	*Xylariaceae*	*Xylariales*
Rosellinia necatrix	HKUCC 9037	AY083824	AY083805	*Xylariaceae*	*Xylariales*
Xylaria curta	-	U47840	U32417	*Xylariaceae*	*Xylariales*
Xylaria hypoxylon	-	U47841	U20378	*Xylariaceae*	*Xylariales*
Morchella esculenta [c]	-	U42669 + AY016364	U42642	*Morchellaceae*	*Pezizales*
Pleospora herbarum [d]	-	AF382386	U05201	*Pleosporaceae*	*Pleosporales*
Dothidea ribesia	-	AY016360	AY016343	*Dothideaceae*	*Dothideales*
Wilcoxina mikolae	-	AF156926	U62014	*Pyronemataceae*	*Pezizales*
Saccharomyces cerevisiae	SGD	-	-	*Saccharomycetaceae*	*Saccharomycetales*

[a]Cultures were obtained for new sequences only. [b] Direct PCR from herbarium material. [c] 28S rDNA assembled from 2 GenBank sequences for the same strain

[d]28S and 18S rDNA from two different strains

at 25°C for 5-10 days. Prior to DNA extraction, each culture was purified by making a single hyphal tip isolation onto PDA and incubated as above.

DNA extraction

Extraction of DNA followed a modified protocol of Doyle and Doyle (1987) used by Liew *et al*. (2000) and Jeewon (2002). Fresh fungal mycelia were scraped off culture plates and placed in a 1.5 ml centrifuge tube with approx. 300 mg of white quartz sand (Sigma) and 500 µl of 2x CTAB buffer [2% (w/v) CTAB; 100 mM Tris-HCl; 1.4 M NaCl; 20 mM EDTA, pH 8.0]. The mixture was ground with a glass or plastic pestle and incubated, with occasional mixing, at 60°C for 1 hour. The mixture was subjected to multiple phenol:chloroform:isoamyl alcohol (25:24:1) extractions, with the upper aqueous phase retained each time. DNA was precipitated with 2 volumes of 100% ethanol (EtOH) and kept at -20ºC overnight, then washed twice with 70% EtOH and dried in a vacuum centrifuge for 10 minutes. The DNA pellet was then resuspended in 100 µl TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH8) and treated with RNase A (1 mg ml^{-1}). Extracted DNA was visualised by gel electrophoresis [1% agarose in 1x TAE buffer (40 mM Tris-acetate; 1 mM EDTA; pH 8.0)], stained with ethidium bromide (EtBr, 10 mg ml^{-1}), before storing at -20ºC. In the case of heavily pigmented specimens, if the final DNA suspension was heavily coloured, an additional extraction was performed after 400 µl of TE was added to the suspended DNA, using the method of Cubero *et al*. (1998).

DNA fragment amplification

Partial 28S and 18S rDNA sequences were symmetrically amplified using primers LROR and LR5 (Vilgalys and Hester, 1990) and primers NS1 and NS4 (White *et al*., 1990) respectively. One to 5 µl of genomic DNA was used in a 50 µl polymerase chain reaction (PCR) mixture (1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each primer pair, and 1.0 U of *Taq* DNA polymerase). The same thermal cycling conditions were used for both gene fragments. After an initial 3 minutes denaturation at 94°C all samples were amplified as follows: 94°C for 1 minute, 52°C for 50 seconds, 72°C for 1 minute; followed by an extension period of 72°C for 10 minutes. To check product purity and size 5 µl of amplified product was visualised by gel electrophoresis (as above). PCR products were purified using the Wizard® Preps PCR DNA Purification System (Promega), and the purified product

checked for purity and sufficient concentration by gel electrophoresis as described.

The 28S rDNA of *Hyponectria buxi* was amplified directly from dried herbarium material (see Table 1). Approximately 0.1 g of hamathecial tissue was removed from the surrounding leaf material under a dissecting microscope (Leica MZ12) and placed in a 0.5 ml centrifuge tube with 50 µl of PCR mixture. Subsequent procedures were identical to those described above.

DNA sequencing

The amplified 28S and 18S rDNA fragments were directly sequenced using the ALFexpress™ II Automated DNA sequencer (Amersham Pharmacia Biotech). Sequencing reactions were conducted using the AutoCycle™ 200 Sequencing Kit (Amersham Pharmacia Biotech) and according to the manufacturer's instructions. Four CY-5™ labelled sequencing primers were used for each gene fragment, allowing both strands of the DNA to be sequenced. Primers LROR, LR3R, LR5 and LR3 (Vilgalys and Hester, 1990) were used for the 28S rDNA, while primers NS1, NS2, NS3 and NS4 (White *et al*., 1990) were used for the 18S rDNA.

Sequence assembly and alignment

Four separate sequences obtained for each primer were spliced into a consensus sequence using ALFwin™ Sequence Analyser v2.10 software (Amersham Pharmacia Biotech). During assembly individual bases from each sequence were checked against the original fluorescence signal. Consensus sequences and those obtained from GenBank were aligned using ClustalX (Thompson *et al*., 1997) before being aligned manually. A major insertion of 73 bp was excluded from the *Clypeosphaeria uniseptata* 28S rDNA, while areas of ambiguous alignment were removed from the alignment by using the default options for coding DNA in the Gblocks computer program (Castresana, 2000).

Phylogenetic analysis

Sequence alignments were analysed individually and as a combined dataset (28S, 18S and 28S+18S). Phylogenetic analyses were conducted using PAUP* 4.0b8 (Swofford, 2001). The 3 datasets were subjected to 3 methods of phylogenetic analysis: maximum parsimony (MP), weighted parsimony (WP) and maximum likelihood (ML). Rooting of trees was determined by the

inclusion of reference taxa and by assigning *Saccharomyces cerevisiae* as the outgroup prior to analysis.

Most parsimonious trees were obtained using heuristic searches of 1000 replicates with random stepwise sequence addition, and tree bisection-reconnection (TBR) branch swapping. All characters were treated as unordered. Gaps were treated as a both missing data and as a fifth character state. Nonparametric bootstrap support (Felsenstein, 1985; Sanderson, 1989) was calculated for each internal node with a simple stepwise sequence addition, TBR branch swapping and 1000 replicates. Consistency index (CI), homoplasy index (HI), retention index (RI), rescaled consistency index (RC) and tree length (TL) were calculated for all parsimony trees generated. Phylogenetic trees were drawn using TREEVIEW (Page, 1996).

During WP analysis, most parsimonious trees, and bootstrap support, were generated as for MP described above. Rates of nucleotide substitution (transition:transversion (Ti:Tv) substitution matrix, or stepmatrix) used in WP were determined from maximum likelihood estimates (RMATRIX option in PAUP*). The Ti:Tv stepmatrix was included in the analyses in the ASSUMPTIONS block of the input file. In the case of WP when gaps were treated as a fifth state, they were defined as such using the FORMAT SYMBOLS option in the CHARACTER block of the PAUP* input file. In this situation, gaps were only able to be weighted at half the maximum cost of a transversion in order to meet the criterion of a balanced stepmatrix.

The programs MODELTEST (Posada and Crandall, 1998) and WinMODELTEST (Patti, 2001) were used to identify the model of sequence evolution that best fit the data by hierarchical likelihood ratio tests (hLRTs). Once the appropriate model was determined, ML trees were generated for each dataset from a MP starting tree using a heuristic search and TBR branch swapping. Gaps were treated as missing data. No bootstrap support was calculated for the ML analysis due to the large computational times involved.

Congruence of the combined 28S and 18S rDNA dataset was tested using the partition homogeneity test ($P > 0.01$; PHT), as implemented in PAUP*, after excluding all invariant characters (Farris *et al*., 1995; Cunningham, 1997). Congruence testing was conducted for MP and WP analyses. Only combined datasets that were found to be congruent were subjected to further analysis. Kishino-Hasegawa (Kishino and Hasegawa, 1989) and Templeton tests (Templeton, 1983) were conducted to compare trees generated from different methods and to determine if they were significantly different.

Results

Phylogenetic analyses of the 28S rDNA

The final 28S rDNA alignment, after exclusion of areas of ambiguous alignment and the single 73 bp insertion in *C. uniseptata*, included 673 bp that contained 187 (27.8%) parsimony informative positions.

MP analysis yielded 12 most parsimonious trees 989 steps in length (CI = 0.425; RI = 0.559; RC = 0.238; HI = 0.575). No differences in tree topology or tree statistics were present between gaps being treated as missing data or as a fifth state. The strict consensus of the most parsimonious trees with bootstrap values is shown in Fig 1a.

MP analysis of the 28S rDNA data groups all taxa from the *Xylariales* as a monophyletic group but with no bootstrap support (Fig. 1a). There is 100% bootstrap support for the monophyly of the *Xylariales* and reference taxa from the Sordariomycetes, and 82% bootstrap support at the base of sordariomycetous taxa, and strong support for relationships within this clade (Fig. 1a). There is little other bootstrap support for other relationships in Fig. 1a. The 3 taxa of the *Diatrypaceae* are well-supported as a monophyletic group, but there is no support for the position of the clade. The same applies to the 2 taxa of the *Cainiaceae* that form a monophyletic group at with 74% bootstrap support. All other bootstrap support in Fig. 1a is usually at the terminal nodes supporting the relationship of 2 taxa. There are three broad groups of taxa, all paraphyletic and unsupported by bootstrapping, present in the strict consensus (Fig. 1a). These groups are defined only to assist in discussion of results and the taxa in each group change between different analyses. Group A contains members from 3 families - *Amphisphaeriaceae* (*Discostroma*, *Lepteutypa* and *Pestalosphaeria*), *Clypeosphaeriaceae* (*Oxydothis*) and *Hyponectriaceae* (*Hyponectria* and *Monographella*). A second group (group B) consists of *Amphisphaeria*, two taxa from the *Clypeosphaeriaceae* (*Clypeosphaeria* and *Apioclypea*), the monophyletic *Diatrypaceae*, and *Daldinia* and *Hypoxylon* from the *Xylariaceae*. The remaining 9 taxa form group C which includes *Graphostroma*, the remaining 6 members of the *Xylariaceae* plus *Arecophila* and *Cainia*.

Maximum likelihood estimates of rates of nucleotide substitution indicated that 3 categories of nucleotide substitution were present. The resulting 3 category stepmatrix weighed CT transitions (most common) 1, AG transitions (common) 2.5, and all transversions (least common) 5. Gaps, when treated as a fifth state, were weighted 2.5.

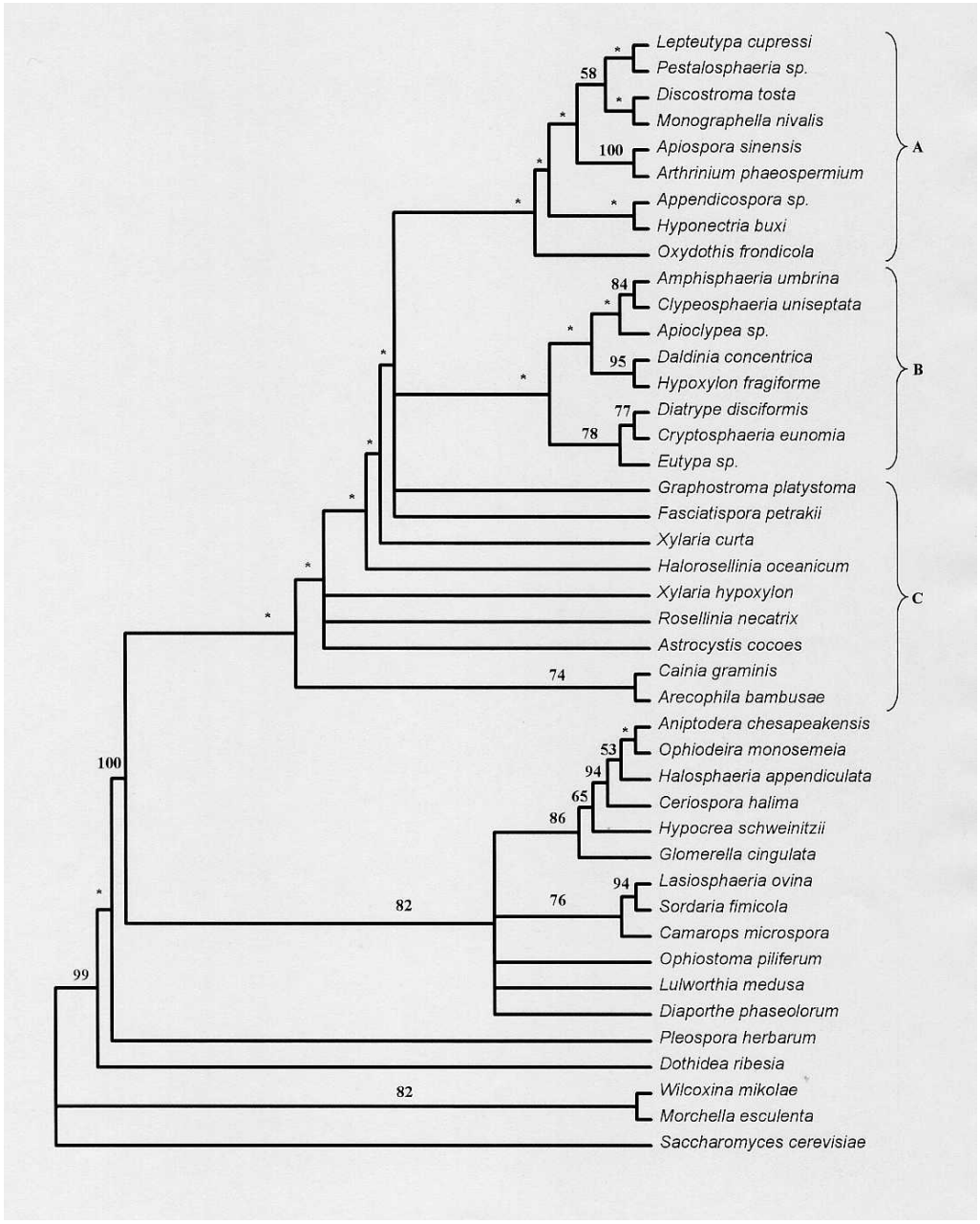

Fig. 1a. Strict consensus of 12 equally most parsimonious trees produced using maximum parsimony analysis of the 28S rDNA. *Saccharomyces cerevisiae* is the designated outgroup. Numbers above the nodes indicate bootstrap values from 1000 bootstrap replicates. * indicates nodes with <50% bootstrap support. Tree length = 1013, CI = 0.415, RI = 0.541, RC = 0.224, HI = 0.568.

Using the 3 category stepmatrix with gaps treated as missing data, WP yielded 2 most parsimonious trees of length 2902 (CI = 0.432; RI = 0.574; RC = 0.248; HI = 0.568). The same analysis with gaps treated as a fifth state yielded 3 most parsimonious trees 28995 steps long with similar tree statistics to gaps treated as missing. Some minor differences in tree topology between trees generated using differing gap treatments were present. However, Templeton tests revealed no significant differences between the trees (Table 2). The strict consensus of the 2 most parsimonious trees (gaps treated as missing) with bootstrap values is shown in Fig 1b. The monophyly of the *Xylariales* with 7 families is supported with 56% bootstrap support. The *Diatrypaceae* (*Cryptosphaeria*, *Diatrype* and *Eutypa*) are again shown as monophyletic with 77% bootstrap support, while there is no support for the monophyly, or otherwise, of the remaining families within the *Xylariales*. The same paraphyletic groups present in the MP analysis (Fig. 1a) are found, with some differences, in the WP analysis, although the relations between these groups vary between these analyses. Groups A and C are now sister groups while group B is at the base of the *Xylariales* clade (Fig. 1b). Group A contains the same taxa but with the loss of *Monographella* and inclusion of *Fasciatispora*. Group B is still present but with different branching patterns and with the addition of *Monographella* (Fig. 1b). Taxa in group C are the same as for MP, except that *Fasciatispora* is now placed in group A (Fig. 1b).

Table 2. Results of Tempelton tests for different gap treatment of the 28S rDNA using WP.

Gap treatment	**Tree scores and *P* values** [a]	
	Missing (2 trees)	Fifth state (3 trees)
Missing	2902	2903
	-	$P = 1.0000$
Fifth state	2900	2899
	$P = 0.9191$	-

[a] Significant difference at $P < 0.05$.

Hierarchical LRT tests indicated that the Tamura-Nei model with equal base frequencies (TrNef) was the model of best fit (Table 3). This model has 3 base substitution types, CT transitions, AG transitions and all transversions, from most to least common. The model chosen also incorporated an estimated proportion of invariable sites (I = 0.447) and a gamma distribution with shape parameter $\Gamma = 0.618$ for rates of nucleotide substitution among variable sites. This model yielded a tree with a log likelihood (lnL) of -5680.45, a phylogram of which is shown in Fig. 1c. The ML tree topology is completely different from those of the other two methods, with the Sordariomycete reference taxa

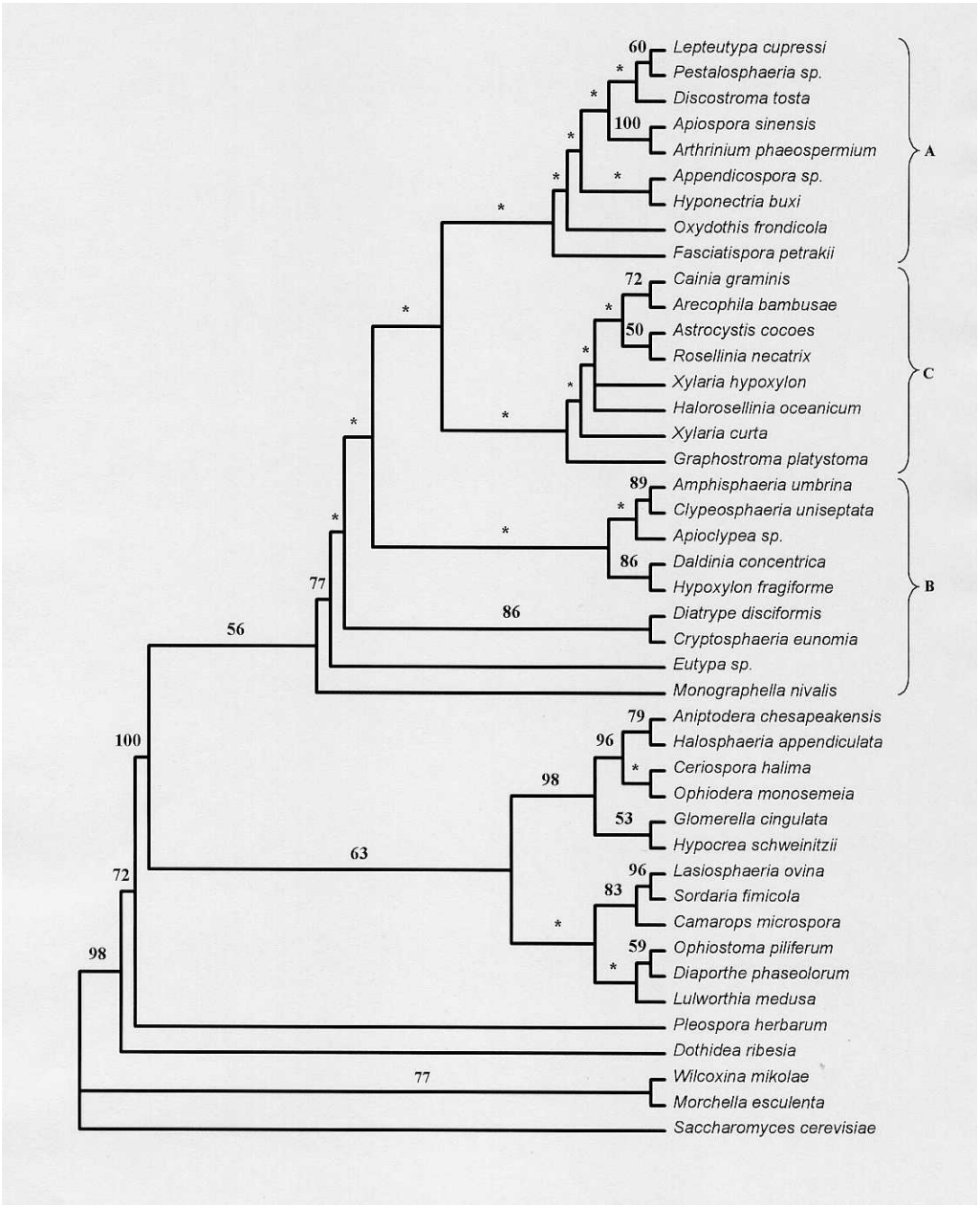

Fig. 1b. Strict consensus of 2 equally most parsimonious trees produced using weighted parsmony analysis of the 28S rDNA with gaps treated as missing. *Saccharomyces cerevisiae* is the designated outgroup. Numbers above the nodes indicate bootstrap values from 1000 bootstrap replicates. * indicates nodes with <50% bootstrap support. Tree length = 2902, CI = 0.432, RI = 0.574, RC = 0.248, 0.568.

no longer basal to the *Xylariales* but as a sister group to members of group A from the previous analyses (Fig. 1c). As the reference taxa are mixed with the ingroup, this topology is obviously incorrect. Also, *Oxydothis* and *Apioclypea* are included at the base of this reference taxa clade. Despite this radical change in topology, the same broad patterns are still visible, with the 3 groups mentioned above still present, again with changes in the taxa included in each (Fig 1c). Members of groups A and C are similar across the 3 methods while group B members are frequently repositioned (Figs. 1a, b, c). Figure 1c also demonstrates the shortness of the branches in the *Xylariales* clade, both in an absolute sense and with reference to the remaining Sordariomycetes.

Table 3. Best fit sequence evolution models as determined by MODELTEST.

Dataset	Model	I [a]	Γ [b]	Base frequencies	Substitution model
28S rDNA	TrNef +I+Γ	0.4468	0.6176	Equal	[A-C] = 1.0000 [A-G] = 2.1482 [A-T] = 1.0000 [C-G] = 1.0000 [C-T] = 4.6159 [G-T] = 1.0000
18S rDNA	TrN +I +Γ[d]	0.4560	0.6549	A = 0.2822 C = 0.2005 G = 0.2509 T = 0.2664	[A-C] = 1.0000 [A-G] = 2.5944 [A-T] = 1.0000 [C-G] = 1.0000 [C-T] = 4.4035 [G-T] = 1.0000
Two genes combined	GTR +I +Γ[e]	0.4567	0.5350	A = 0.2811 C = 0.1961 G = 0.2726 T = 0.2502	[A-C] = 1.0000 [A-G] = 2.3819 [A-T] = 1.3823 [C-G] = 1.3823 [C-T] = 6.2038 [G-T] = 1.0000

[a]I = proportion of invariable sites (estimated via ML); [b]Γ= shape parameter of the gamma distribution (estimated via ML). [c] Tamura and Nei (1993)
3 substitution category model with equal base frequencies (TrNef). [d] Tamura and Nei (1993) 3 substitution category model with unequal base frequencies (TrN).
[e]General time-reversible model (Lanave *et al*., 1984; Rodriquez *et al*., 1990) 4 substitution category model with unequal base frequencies (GTR).

Kishino-Hasegawa and Templeton tests indicated that for the 28S rDNA data there was no significant difference between the 3 methods of analysis (Table 4) despite there being drastic topological differences between trees generated in each method.

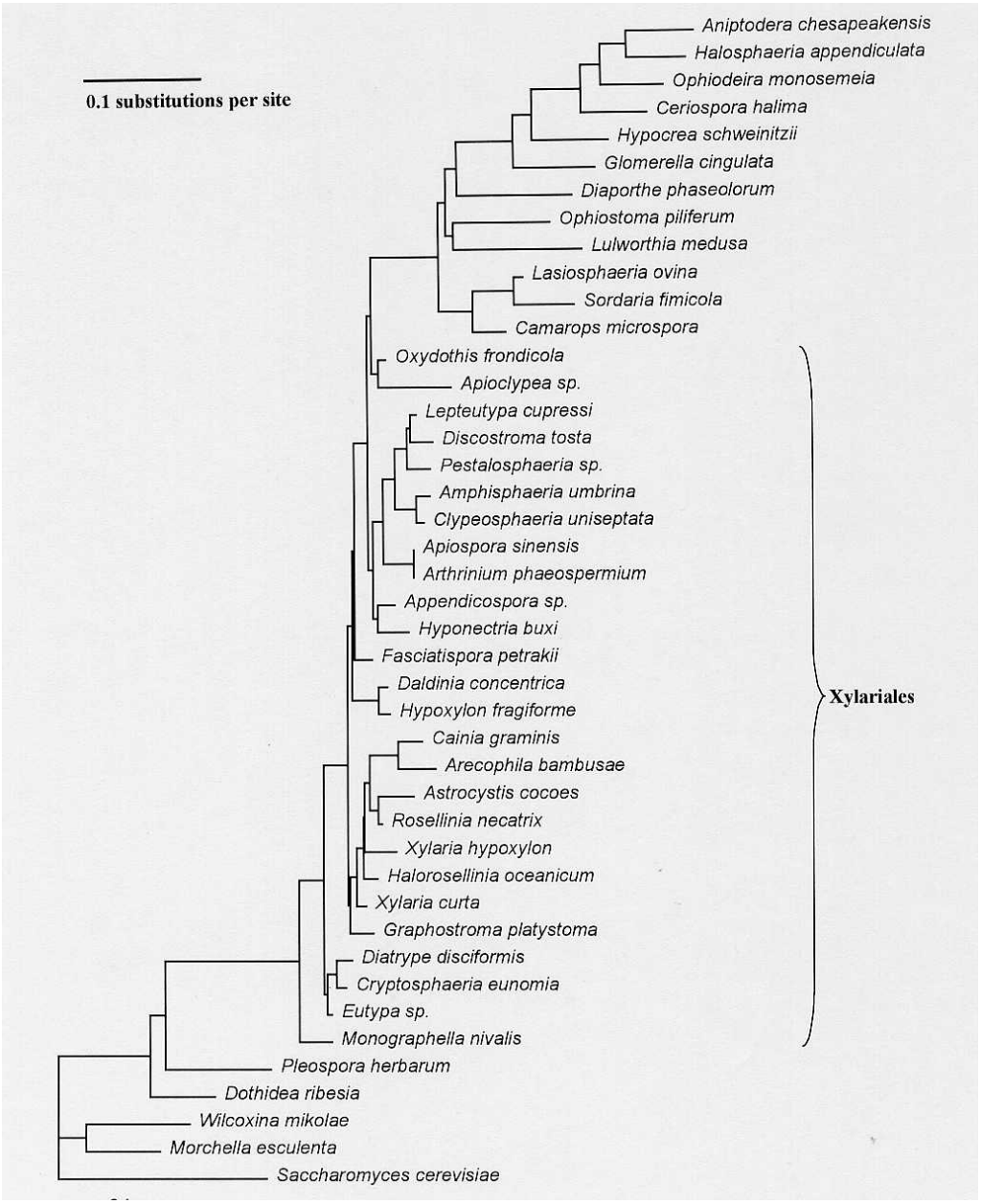

Fig. 1c. Phylogram produced using maximum likelihood analysis (model = TrNef +I +Γ) of the 28S rDNA with gaps treated as missing. *Saccharomyces cerevisiae* is the designated outgroup. Log likelihood (lnL) = -5680.45.

Phylogenetic analyses of the 18S rDNA

The final 18S rDNA alignment, after exclusion of areas of ambiguous alignment, included 934 bp that contained 131 (14.0%) parsimony informative positions.

MP analysis yielded 4 most parsimonious trees 620 steps in length (CI = 0.644; RI = 0.590; RC = 0.380; HI = 0.410). No differences in tree topology or tree statistics were present between gaps being treated as missing data or as a fifth state. A strict consensus of the most parsimonious trees with bootstrap support is shown in Fig. 2a. The *Xylariales* are maintained as a monophyletic group but with no bootstrap support, which is lower across the entire cladogram than the support in the analyses of the 28S rDNA data. Two of the arbitrary groups described previously are not maintained in this analysis. Groups A and B have been broken and joined with parts of the other, while forming a distinct clade from group C. For example, from group B, *Daldinia* and *Hypoxylon* are no longer associated with *Amphisphaeria*, *Apioclypea* and *Clypeosphaeria* but with members of group A (Figs. 1a,b). *Apioclypea* is now a sister taxon to parts of group A, and *Cryptosphaeria* is now associated with *Arecophila* and *Cainia* in group C. The subgroups A1 and B1 form a separate clade that includes the *Amphisphaeriaceae* and members of the *Clypeosphaeriaceae* and *Diatrypaceae*. None of these relationships have any bootstrap support. Subgroup A1, which consists of all the *Amphisphaeriaceae* taxa minus *Amphisphaeria*, is well supported (66%) as monophyletic. Subgroup A2 does not form a clade and consists of taxa from the *Apiosporaceae*, *Clypeosphaeriaceae* and *Hyponectria*. Group C forms a separate clade and contains similar taxa as the previous analyses - members of the *Xylariaceae*, excluding *Daldinia* and *Hypoxylon*, *Arecophila* and *Cainia* plus the addition of *Cryptosphaeria* (Fig. 2a). *Arecophila* and *Cainia* group together with 57% bootstrap support. The relationship between the *Xylariales* and the Sordariomycete reference taxa has also changed. A section of these reference taxa (Fig. 1b, group D) now groups at the base of the *Xylariales* clade and not with the other reference taxa. This relationship has no bootstrap support and group D is separated from the *Xylariales* with 64% bootstrap support (Fig. 2a).

Maximum likelihood estimates of rates of nucleotide substitution indicated that 3 categories of nucleotide substitution were present. The resulting 3 category stepmatrix is the same as that for the 28S rDNA. Using the 3 category stepmatrix with gaps treated as missing or a fifth state, WP yielded 2 most parsimonious trees of length 1798 (CI = 0.633; RI = 0.670; RC = 0.424;

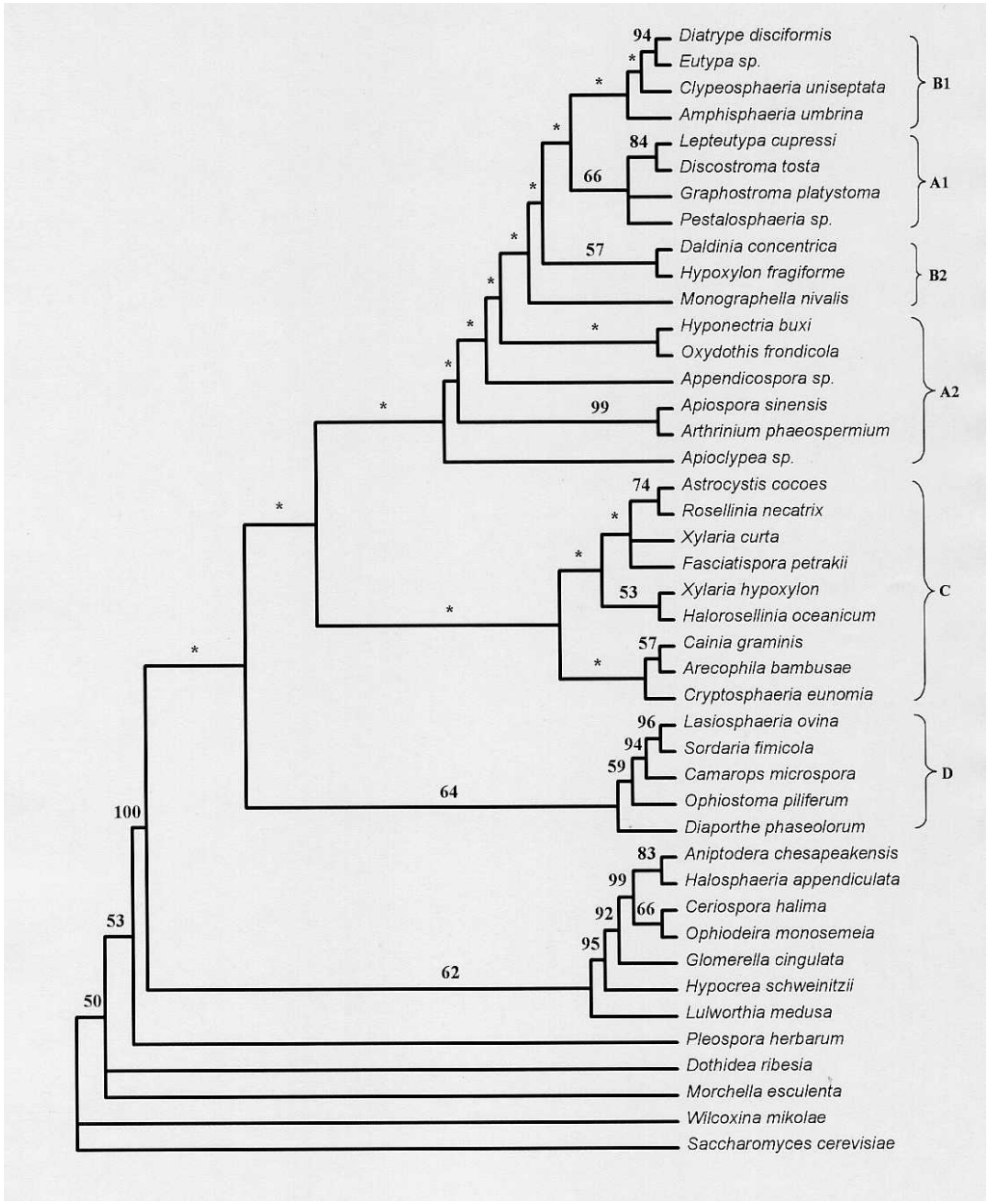

Fig. 2a. Strict consensus of 4 equally most parsimonious trees produced using maximum parsmony analysis of the 18S rDNA. *Saccharomyces cerevisiae* is the designated outgroup. Numbers above the nodes indicate bootstrap values from 1000 bootstrap replicates. * indicates nodes with <50% bootstrap support. Tree length = 625, CI = 0.586, RI = 0.637, RC = 0.373, HI = 0.414.

HI = 0.367). A strict consensus of the most parsimonious trees with bootstrap support is shown in Fig. 2b. The *Xylariales* is present as an unsupported monophyletic group, while the family relations are confused and mostly unsupported. The members of subgroup A1, with 79% bootstrap support, are now joined with *Amphisphaeria* and *Clypeosphaeria* (group B) forming a separate paraphyletic clade of the *Amphisphaeriaceae* plus *Clypeosphaeria* (Fig. 2b). The remaining group B taxa are a sister group to this clade and also includes *Monographella*. The subgroup A2 taxa and topology are the same as for Fig. 2a. The members of group C no longer form a separate clade but they still group together (Fig. 2b). *Arecophila*, *Cainia* and *Cryptosphaeria* are grouped together as a separate clade with 63% support. The relationship between the *Xylariales* and part of the reference taxa (group D) is the same as in the MP analysis (Fig. 2b).

Hierarchical LRT tests indicated that the Tamura-Nei model (TrN) was the model of best fit (Table 3). This model differs from the TrNef model in having variable base frequencies (A = 0.282, C = 0.200, G = 0.251, and T = 0.267). Proportion of invariable sites was estimated at I = 0.456 and a gamma distribution with shape parameter $\Gamma = 0.655$ for rates of nucleotide substitution among variable sites. This model yielded a tree with a log likelihood (lnL) of -4835.39 which is shown in Fig. 2c. The *Xylariales* is not present as a monophyletic group with *Apioclypea*, *Diatrype*, *Eutypa* and the 3 *Apiosporaceae* placed as a sister group to the Sordariomycete reference taxa (Fig. 2c). The remaining *Xylariales* form a separate group with 3 clades. One consists of the group C taxa with the addition of *Daldinia* and *Hypoxylon*, although the *Xylariaceae* is not monophyletic. A second clade includes all the *Amphisphaeriaceae* plus *Clypeosphaeria*, while *Monographella* is sister to both these clades. The third clade contains *Hyponectria* and *Oxydothis*. The branch lengths in the ML tree are very short for the *Xylariales* taxa when compared to the reference taxa (Fig. 2c).

Kishino-Hasegawa and Templeton tests indicate that there is no significant difference between the 3 tree building methods using the 18S rDNA data (Table 4).

Phylogenetic analyses of the combined dataset

The PHT results indicated that the two datasets are combinable but only under certain criteria (Table 5). Weighted parsimony using a 4 category stepmatrix with the following weightings - CT transitions (most common) 1,

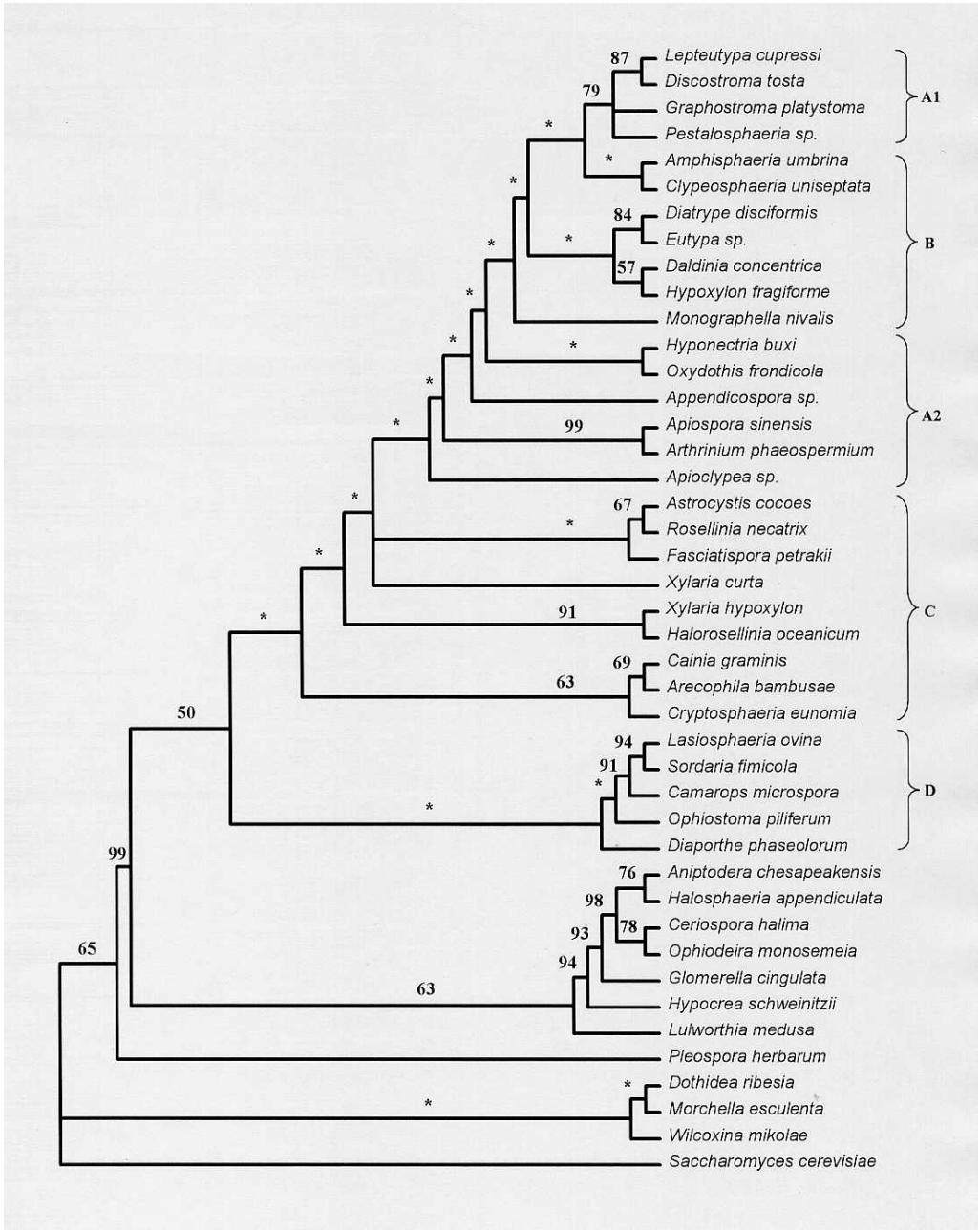

Fig. 2b. Strict consensus of 2 equally most parsimonious trees produced using weighted parsmony analysis of the 18S rDNA with gaps treated as missing. *Saccharomyces cerevisiae* is the designated outgroup. Numbers above the nodes indicate bootstrap values from 1000 bootstrap replicates. * indicates nodes with <50% bootstrap support. Tree length = 1798, CI = 0.633, RI = 0.670, RC = 0.424, HI = 0.367.

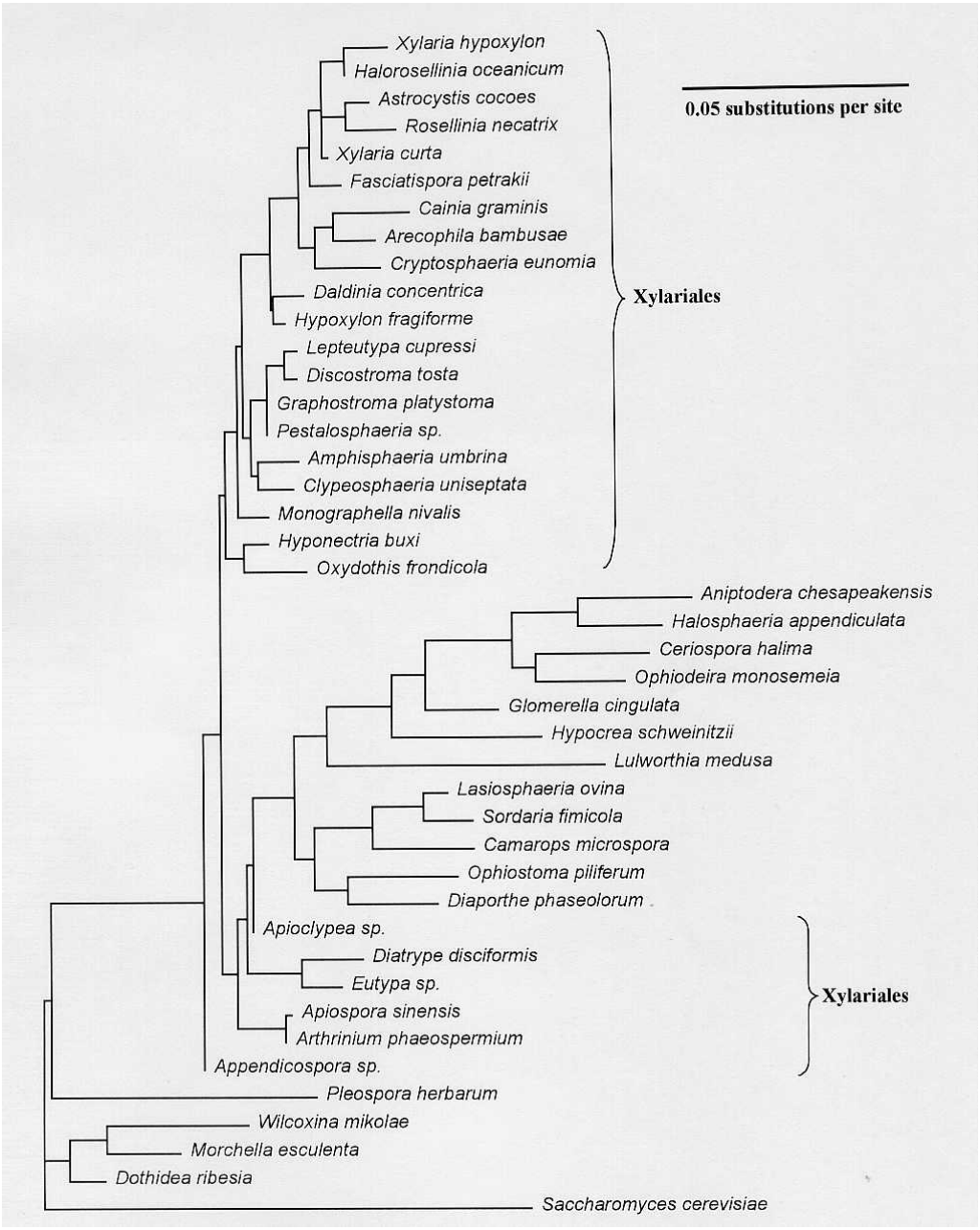

Fig. 2c. Phylogram produced using maximum likelihood analysis (model = TrN +I +Γ) of the 18S rDNA with gaps treated as missing. *Saccharomyces cerevisiae* is the designated outgroup. Log likelihood (lnL) = -4835.39.

Table 4. Results of Templeton and Kishino-Hasegawa tests for tree topology congruence

		Tree scores and *P* values [a]					
Optimality criterion	**Dataset**	**MP - 28S rDNA (12 trees)**	**WP - 28S rDNA (2 trees)**	**ML - 28S rDNA (1 tree)**	**MP - 18S rDNA (4 trees)**	**WP - 18S rDNA (2 trees)**	**ML - 18S rDNA (1 tree)**
MP [b]	28S rDNA	989	996	1001	1075-1089	1084	1085
		-	$P > 0.3662$	$P = 0.7199$	$P < 0.0001$**	$P < 0.0001$**	$P < 0.0001$**
	18S rDNA	678-690	675	676	620	624	629
		$P < 0.0001$**	$P < 0.0001$**	$P < 0.0001$**	-	$P = 0.3323$	$P = 0.4990$
WP [b]	28S rDNA	2934-2950	2902	2932	3138-3171	3155	3152
		$P > 0.4094$	-	$P = 0.5713$	$P < 0.0001$*	$P < 0.0001$*	$P < 0.0001$*
	18S rDNA	1951-1985	1943	1958	1803-1810	1798	1830
		$P < 0.0001$**	$P = 0.0001$**	$P = 0.0003$**	$P > 0.6291$	-	$P = 0.5716$
ML [c]	28S rDNA	5694.03-5700.14	5695.28-5695.31	5680.45	5869.18-5905.10	5889.93	5865.19
		$P > 0.5144$	$P > 0.5034$	-	$P < 0.0001$**	$P < 0.0001$**	$P < 0.0001$**
	18S rDNA	50004.07-5028.46	4997.48	4977.81	4840.62-4846.33	4847.78	4835.39
		$P < 0.0001$**	$P < 0.0001$**	$P < 0.0001$**	$P = 0.5051$	$P = 0.5071$	-

[a] Significant difference at $P < 0.05$. [b] Templeton test (Templeton, 1983). [c] Kishino-Hasegawa Test (Kishino and Hasegawa, 1989).

AG transitions (common) 2, AT and CG transversions (less common), and AC and GT transversion (least common) 4; and WP using the same 3 category stepmatrix as previous. Gaps treated as a fifth state had higher support than gaps as missing data, while support was higher for the 3 category stepmatrix and marginal for the 4 category stepmatrix (Table 5). Therefore, analyses were conducted only under these 4 criteria and ML.

Table 5. Results of partition homogeneity tests for congruence of the 18S and 28S rDNA datasets.

Optimality criterion	Gap treatment	*P* values [a]
	-	$P = 0.002$*
WP - 3 category	Missing	$P = 0.049$
stepmatrix	fifth state	$P = 0.070$
WP - 4 category	Missing	$P = 0.011$
stepmatrix	fifth state	$P = 0.019$

[a] Significant difference at $P < 0.010$.

Maximum likelihood estimates of the combined dataset indicated that 4 categories of rates of nucleotide substitution were present (weights given above). However, when compared with the 3 category model using Templeton tests, trees generated using the 4 category stepmatrix were found to be significantly worse (data not shown). Therefore the 3 category stepmatrix was used for the WP analysis of the combined dataset.

Using the 3 category stepmatrix with gaps treated as missing, WP yielded 3 most parsimonious trees of length 4797 (CI = 0.499; RI = 0.590; RC = 0.295; HI = 0.501). A strict consensus of the most parsimonious trees with bootstrap support is shown in Fig. 3a. The *Xylariales* forms a monophyletic group with 79% bootstrap support. Support for relationships within the *Xylariales* is very low. There are 3 main groups present in the strict consensus. The first (group X) contains 2 subgroups. Subgroup X1 consists of *Arecophila* and *Cainia* (97% support) and the *Xylariaceae* minus *Daldinia* and *Hypoxylon*, while subgroup X2 contains the monophyletic *Diatrypaceae* (74% support) with *Graphostroma* forming a sister group. This clade is well-resolved with no polytomies but support within this group is low (Fig. 3a). This group forms a distinct clade from the rest of the *Xylariales*, but this separation does not have any bootstrap support (Fig. 3a). The second group (Y) is a sister group to group X and contains the *Amphisphaeriaceae* (minus *Amphisphaeria*), the *Apiosporaceae*, 2 *Clypeosphaeriaceae* (*Apioclypea* and *Oxydothis*) and the *Hyponectriaceae*. Group Y also forms a distinct clade, again with no bootstrap support. Apart from the 3 *Amphisphaeriaceae* taxa which group with 71% support and *Apiospora* and *Arthrinium* (100% support), the clade collapses to a

polytomy (Fig. 3a). The final groups consist of group Z1 containing *Amphisphaeria* and *Clypeosphaeria* (95% support), which is sister to groups X1, X2 and Y. *Daldinia* and *Hypoxylon* (94% support) form group Z2 which is in turn the sister to all other groups (Fig. 3a).

WP with gaps treated as a fifth state yielded a single most parsimonious tree of length 4792 (CI = 0.499; RI = 0.591; RC = 0.295; HI = 0.501). The topology and support levels of the generated tree (Fig. 3b) is similar to Fig. 3a but with the polytomy in group Y now fully resolved, and some minor differences. From the taxa in group Y two clades are formed. Subgroup Y1, as a sister group to group X, contains *Apiospora*, *Arthrinium*, *Apioclypea* and *Fasciatispora* which previously was placed in group X. Subgroup Y2 contains the remaining taxa from group Y in Fig. 3a. Apart from the movement of *Fasciatispora* group X remains unchanged, while groups Z1 and Z2 are also the same (Fig. 3b). Figure 3c is a phylogram of Fig. 3b, and demonstrates the short branch lengths present within the *Xylariales* clade in comparison to the reference taxa.

Hierarchical LRT tests indicated that a general time-reversible (GTR) model (Lanave *et al*., 1984; Rodriquez *et al*., 1990) with unequal base frequencies (A = 0.281, C = 0.196, G = 0.273, and T = 0.250) and 4 substitution types. Proportion of invariable sites was estimated at I = 0.457 and a gamma distribution with shape parameter $\Gamma = 0.535$ was estimated for rates of nucleotide substitution among variable sites. This model yielded a tree with a log likelihood (lnL) of -10718.88 (Fig. 3d). The ML tree has a much different topology to the parsimony analyses. Subgroup X1 is maintained as a separate clade, with *Fasciatispora* included, and with the addition of *Daldinia* and *Hypoxylon* (group Z2). Another clade is formed by taxa of group X2, Z1 and parts of Y2. This clade itself contains 2 groups, one with all the *Amphisphaeriaceae*, plus *Clypeosphaeria* and *Graphostroma*. While the second consists of the *Diatrypaceae* with *Monographella*. Group Y1 is placed as a sister group to the above taxa, instead of group X, while *Oxydothis* (from group Y2) is sister group to Y1. *Appendicospora* and *Hyponectria* (group Y2) are then the sister group to all the above taxa (Fig. 3d).

Discussion

The results of this investigation clearly shows that the *Xylariales* is a monophyletic order containing 7 families - *Amphisphaeriaceae*, *Apiosporaceae*, *Clypeosphaeriaceae*, *Diatrypaceae*, *Graphostromataceae*, *Hyponectriaceae* and *Xylariaceae*. This arrangement is supported with 79%

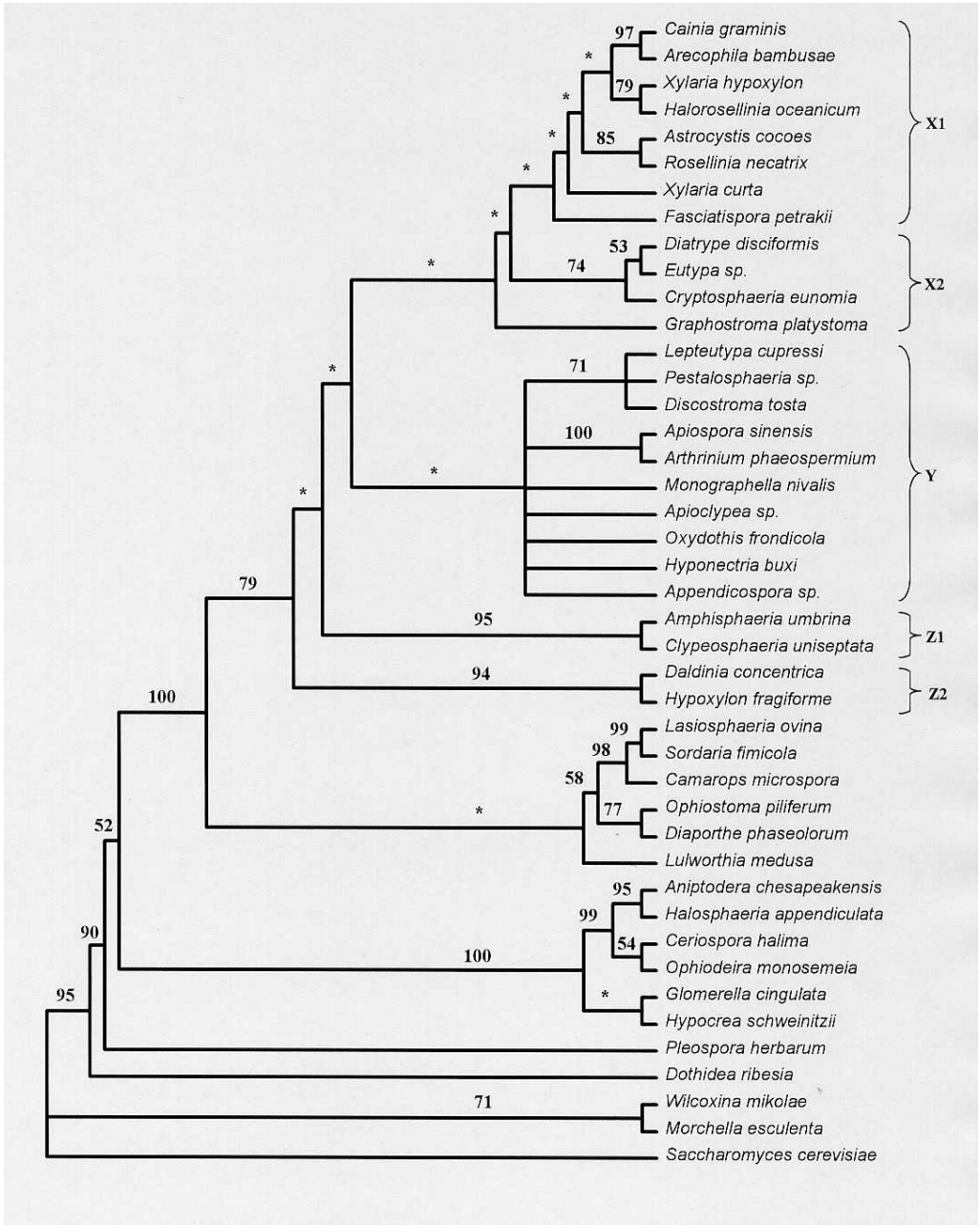

Fig. 3a. Strict consensus of 3 equally most parsimonious trees produced using weighted parsimony analysis of the combined 28S and 18S rDNA with gaps treated as missing. *Saccharomyces cerevisiae* is the designated outgroup. Numbers above the nodes indicate bootstrap values from 1000 bootstrap replicates. * indicates nodes with <50% bootstrap support. Tree length = 4876, CI = 0.491, RI = 0.577, RC = 0.283, HI = 0.509.

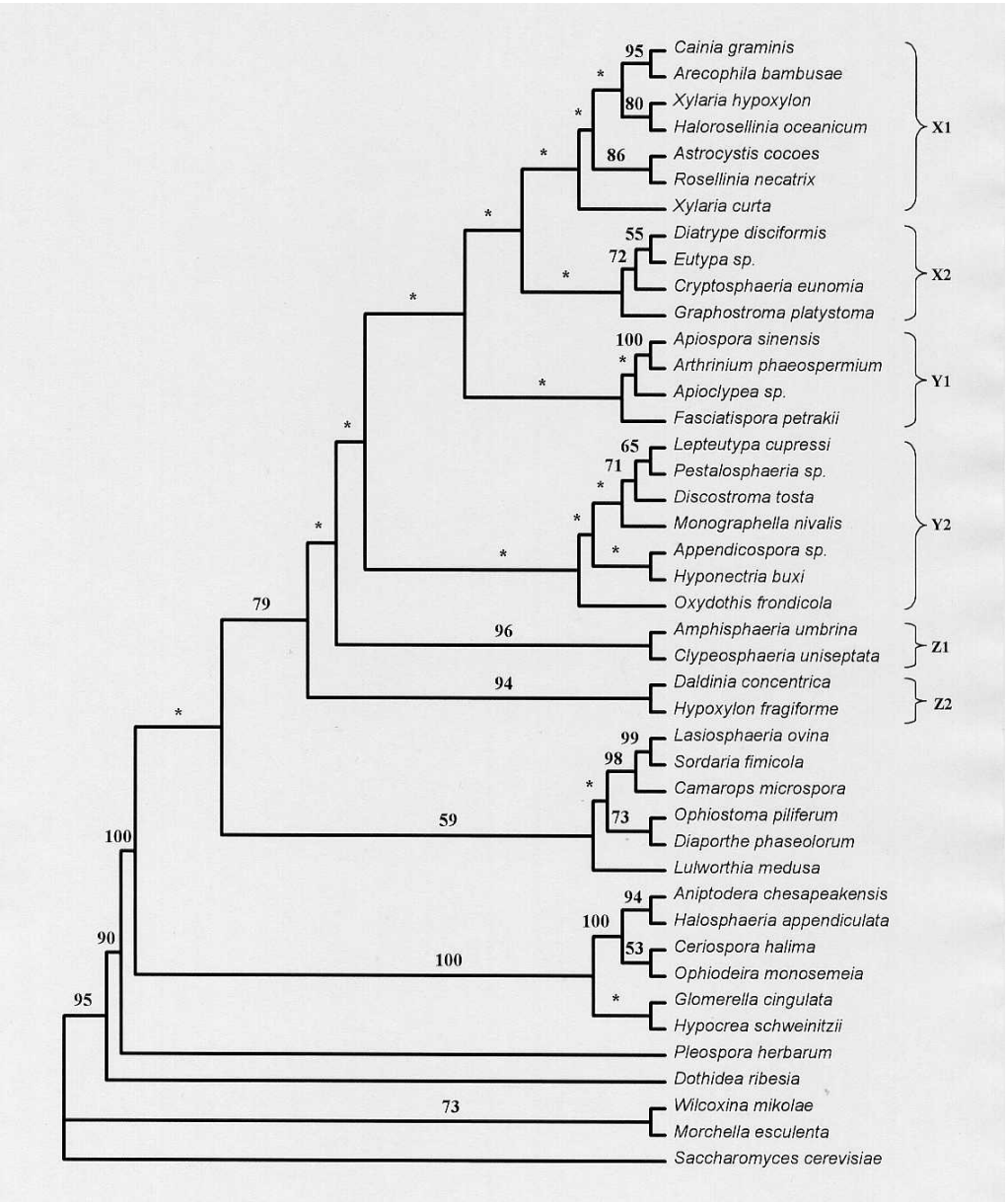

Fig. 3b. Cladogram of the single most parsimonious tree produced using weighted parsimony analysis of the combined 28S and 18S rDNA with gaps treated as fifth state. *Saccharomyces cerevisiae* is the designated outgroup. Numbers above the nodes indicate bootstrap values from 1000 bootstrap replicates. * indicates nodes with <50% bootstrap support. Tree length = 4792, CI = 0.499, RI = 0.591, RC = 0.295, HI = 0.501.

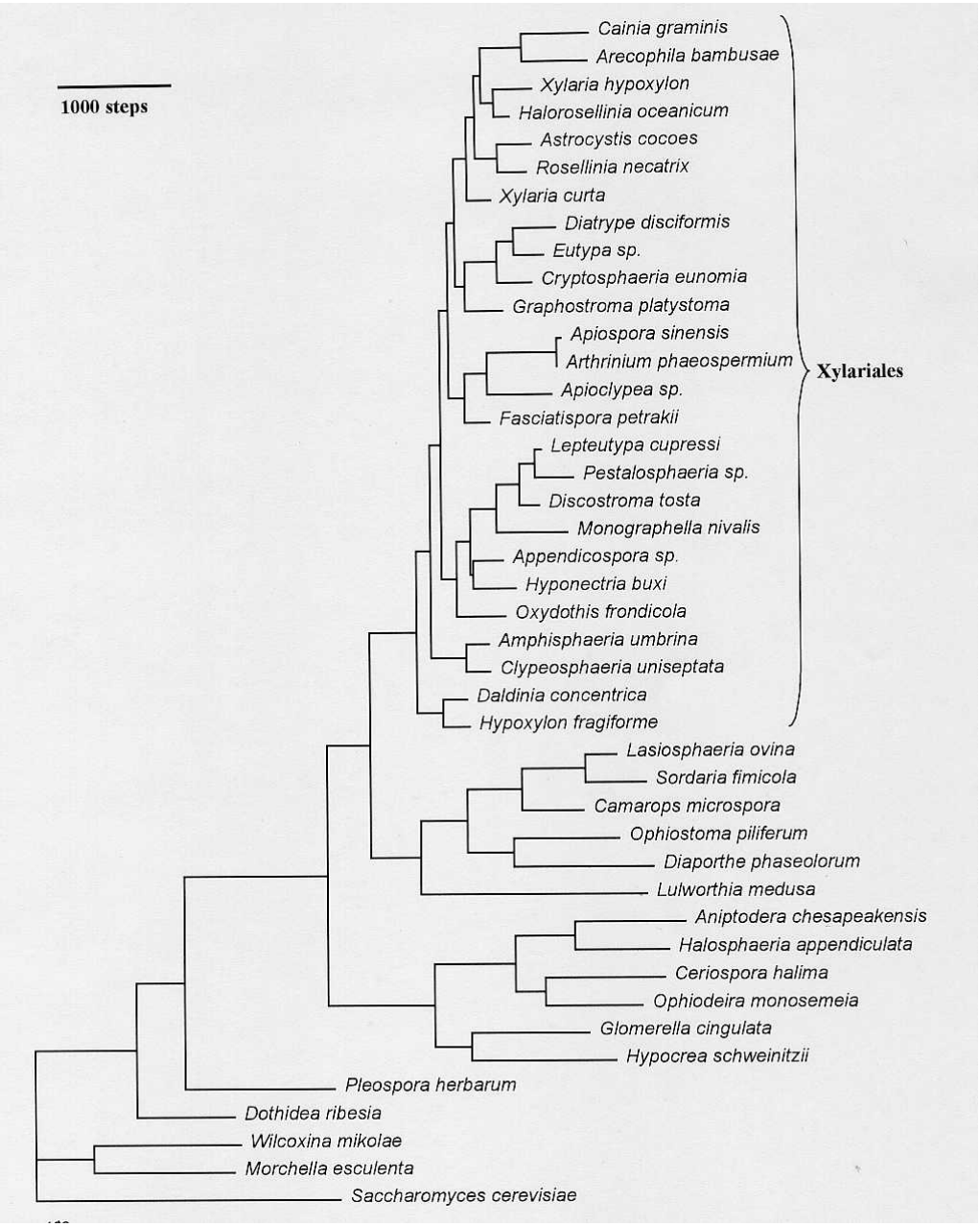

Fig. 3c. Phylogram of the same data as shown in Fig. 3b.

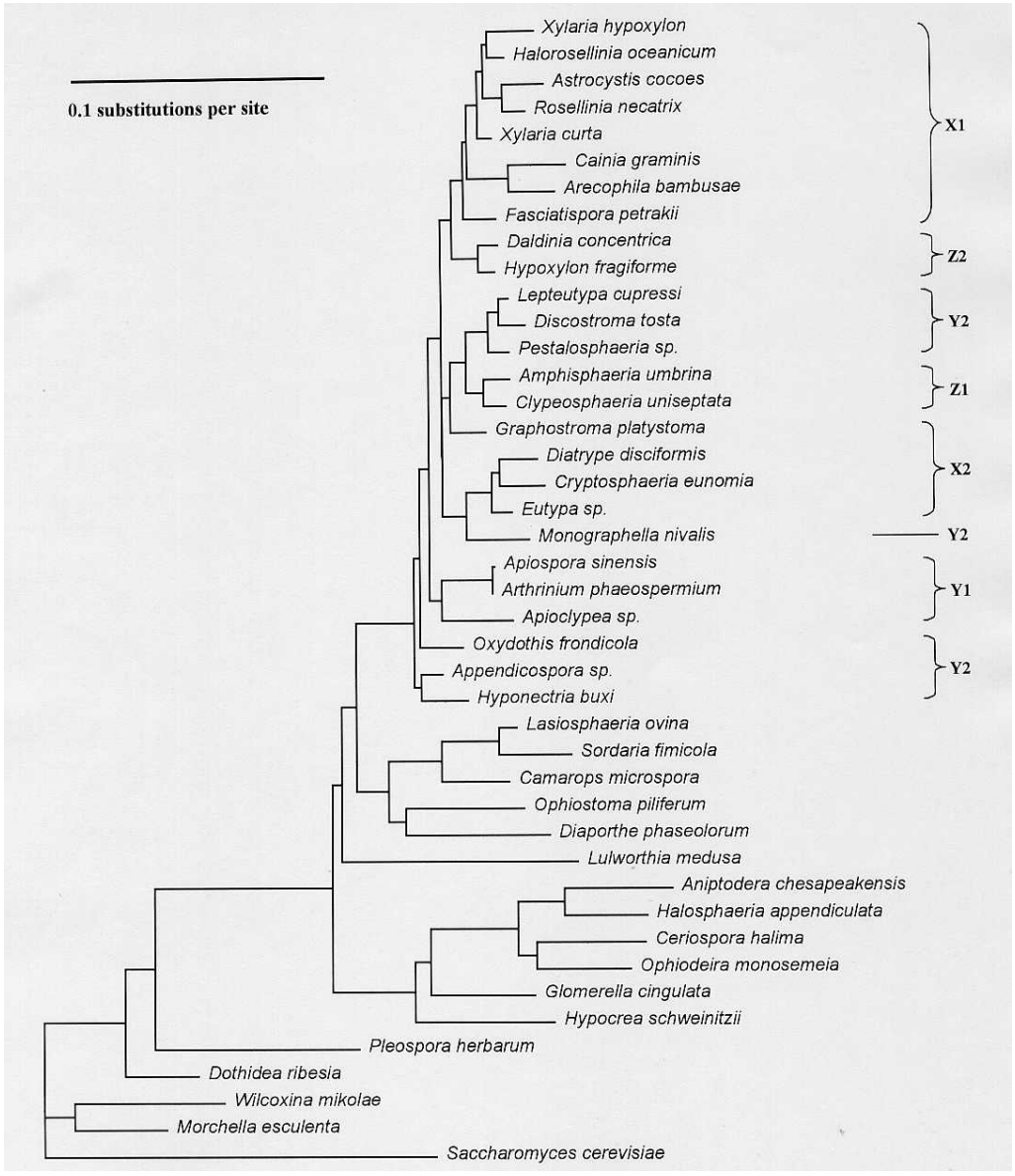

Fig. 3d. Phylogram produced using maximum likelihood analysis (model = GTR +I +Γ) of the combined 28S and 18S rDNA with gaps treated as missing. *Saccharomyces cerevisiae* is the designated outgroup. Log likelihood (lnL) = -10718.88.

bootstrap support in the analysis of the combined datasets. This agrees with the classification provided by Eriksson *et al*. (2003) and Kirk *et al*. (2001), with the exception of the inclusion of the *Apiosporaceae* and that Kirk *et al*. (2001) include the *Myelospermataceae* within the order. This concept of the *Xylariales* also agrees with the results of Kang *et al*. (2002) that showed a member of the *Apiosporaceae* to group within the *Xylariales* using ITS rDNA data.

The inclusion of other families has support from Jeewon (2002) and Kang *et al*. (2002). Although concerned primarily with the *Amphisphaeriaceae*, using 28S rDNA data, Jeewon (2002) demonstrated a close relationship between the *Amphisphaeriaceae*, *Cainiaceae*, *Clypeosphaeriaceae* and *Hyponectriaceae*. However, there was insufficient sequence data for reference taxa from outside the order, and sequences from the *Apiosporaceae*, *Graphostromataceae* and *Xylariaceae* were not included in the study. This was also the case with Kang *et al*. (2002) who included ITS rDNA sequences from all families, except the *Graphostromataceae*, with all members of the *Xylariales* forming a monophyletic clade with 81% bootstrap support. Again there was a lack of reference taxa and most families were only represented by a single specimen (Kang *et al*. 2002). It could also be argued that the use of the ITS rDNA is inappropriate for the inference of ordinal level relationships which will be discussed later.

The results of this study also clearly refute the use of the orders *Amphisphaeriales* (Kang *et al*., 1998, 1999a,b,c,d) and Diatrypales (Barr, 1990; Rogers, 1994; Alexopolous *et al*., 1996; Eriksson and Hawksworth, 1998).

Unfortunately, the status of the *Myelospermataceae* with regard to the *Xylariales* could not be investigated due to a lack of available sequences and cultures. Hyde and Wong (1999) had earlier described the *Myelospermaceae*, an invalid name under the International Code of Botanical Nomenclature, which was corrected to *Myelospermataceae* in the Index of Fungi 7(1) January 2001. Hyde and Wong (1999) investigated the ultrastructure of asci and ascospores in species of *Myelosperma* and concluded that the family was best placed within the *Diaporthales sensu* Barr (1990). Kang *et al*. (1998) included one *Myelosperma tumidum* in a phylogenetic analysis of taxa from the *Xylariales*. The results of this experiment were inconclusive and doubtful due to the lack of suitable reference taxa and the use of an inappropriate gene fragment for analysis (Eriksson 1999; Jeewon *et. al*., 2002; Kang *et al*., 2002). In addition, the sequence of *Myelosperma* used by Kang *et al*. (1998) was found to either contain ambiguities or to be contaminated and has been withdrawn from GenBank (Kang *et al*., 2002). Therefore, the placement of the

Myelospermataceae by Kirk *et al*. (2001) within the *Xylariales*, based on no new data is questionable. Currently, Eriksson *et al*. (2003) place *Myelosperma* within the *Lasiosphaeriaceae*. Until a more thorough molecular investigation of *Myelospermataceae* is undertaken, it would be prudent to either retain the genus in the *Lasiosphaeriaceae* or consider it *incertae sedis* within the Sordariomycetes.

Recently, Réblová and Winka (2001) have shown a possible relationship between the *Xylariales* and four taxa from the *Annulatascaceae* and *Trichosphaeriaceae* that form a sister group to the *Xylariales*. However there was no bootstrap support (<50%) for the relationship and a lack of suitable reference taxa from the *Xylariales*, hence a more comprehensive study of both groups is required before any conclusions may be drawn.

The most striking result of this study is the almost complete failure of the ribosomal DNA sequence data, whether analysed singularly or in combination, to provide support for familial relationships within the order. This was a most unexpected result and contrary to almost all other published studies using these two molecules in ascomycete phylogenetics (e.g. Kohlmeyer *et al*., 2000; Réblová and Winka, 2001; Jeewon, 2002). The level of variation shown by the 28S and 18S rDNA sequences within the *Xylariales* is low compared to demonstrated variation in other studies. Typically, in similar studies investigating ordinal/familial level relationships, the 28S rDNA has around 35% parsimony informative characters while the 18S rDNA has 25% (eg. Kohlmeyer *et al*., 2000), *ca*. 10% higher than encountered in this study. This may either indicate the *Xylariales* to be a more recent group in phylogenetic terms, or that it has undergone a slower rate of evolution, relative to other taxonomic groups.

Interestingly, while the partition homogeneity test indicated that the two datasets could be combined under certain circumstances (Table 5), the tests of congruence showed that the tree topologies produced from the individual datasets to be entirely incongruent (Table 4), even though trees were generated from the separate data under the exact conditions as those prescribed by the acceptable congruence test. This can be due to differences in the phylogenetic histories of the two molecules, variation in modes and rates of evolution or differences in the phylogenetic resolution offered by each molecule (Doyle, 1992; Bull *et al*., 1993; Moncalvo *et al*., 2000). The combination of the two genes would only be invalid if they had different phylogenetic histories. There is no evidence that this is the case as the conflicting branches in the trees generated all have low bootstrap support and the same branches show high support whatever the analysis (de Queiroz, 1992; Moncalvo *et al*., 2000). While the other 2 factors were not investigated, the MODELTEST (Posada and

Crandall, 1998) results indicate that the mode of evolution is similar for the 18S and 28S rDNA (Table 3), and the most probable reason for incongruence amongst the tree topologies is the weak phylogenetic signal present in these genes. This lack of signal is demonstrated in the phylograms presented (Figs. 3c and 3d), as indicated by the shortness of the branch lengths within the *Xylariales* relative to those of the reference taxa. Likewise, within the *Xylariales* many of the internal branches are too short to receive any bootstrap support (some less than 1 step for MP). It has been shown that increasing the number of characters also increases the level of support and phylogenetic accuracy of an analysis (Huelsenbeck, 1995; Poe and Swofford, 1999; Moncalvo *et al*., 2000; Rosenberg and Kumar, 2001), which is clear from the results presented here as the combined analysis has higher support, and is more stable, than the individual analyses. Also, increasing the number of taxa does not necessarily give more resolved results compared to increasing the number of characters (Poe and Swofford, 1999; Rosenberg and Kumar, 2001).

The results for various analyses produced highly variable results with regard to the internal organisation of the *Xylariales*. These arrangements typically have no bootstrap support and it is not possible to determine familial relationships within the order with any certainty. There are, however, broad patterns visible within the trees generated that suggest that the current disposition of taxa within families may be incorrect. As the analyses of the individual datasets have lower support than the combined analysis, and because the well-supported branches are similar in each, the following discussion will be restricted to the results from the combined data.

Jeewon (2002) has dealt extensively with the molecular phylogenetics of the *Amphisphaeriaceae*. The MP results from this study agree on the close association of *Monographella* with the *Amphisphaeriaceae*, however the position of *Amphisphaeria umbrina* changes dramatically between MP and ML and it is impossible to make any inferences about its phylogenetic position (Jeewon, 2002). The remaining members of the family consistently grouped together and it is likely the family is monophyletic as demonstrated by Jeewon (2002) and Kang *et al*. (2002).

The genera from the *Cainiaceae sensu* Kang *et al*. (1999b) have been thought to be closely related to the *Amphisphaeriaceae* (Kang *et al*. 1998, 1999b), however these results indicate that the *Cainiaceae* is more closely related to the *Xylariaceae* and that it probably does not deserve familial rank. This association with the *Xylariaceae* agrees with Jeewon (2002) who also found the *Cainiaceae* taxa to be close to the *Xylariaceae*. Kang *et al*. (1998, 1999b), using a single representative, found the *Cainiaceae* to be related to the *Amphisphaeriaceae*, but subsequently showed the family to be a basal group of

the *Xylariales* (Kang *et al*., 2002) and not closely allied with the *Amphisphaeriaceae*. Given the present study it is highly unlikely that the *Cainiaceae sensu* Kang *et al*. (1999b) is a valid family and that the members should be transferred from the *Amphisphaeriaceae sensu* Eriksson *et al*. (2003) to the *Xylariaceae*. However, before this can be done further molecular evidence with stronger bootstrap support needs to be gathered.

No bootstrap support is available for the *Apiosporaceae* in all analyses *Apiospora* and its anamorph consistently grouped together with *Apioclypea*, which is currently in the *Clypeosphaeriaceae*. Although morphological differences exist between *Apioclypea* and the *Apiosporaceae* (Hyde *et al*., 1998) it is possible the two could be closely related and this warrants further investigation. *Appendicospora* consistently grouped with *Hyponectria*, again with no bootstrap support. There are morphological similarities between the two genera and it may be that *Appendicospora* is best placed within the *Hyponectriaceae* (Hyde *et al*., 1998; Wang and Hyde, 1999) although further work is also needed to confirm this.

Members of the *Clypeosphaeriaceae* are spread among different groups and, although there is a lack of bootstrap support, it seems likely that this family is polyphyletic, which has previously been suggested (Kang *et al*., 1999c). The relation of these taxa to other families is far from clear in the current analysis and conflicts with other findings (Jeewon, 2002; Kang *et al*., 2002). Further study is obviously necessary before any conclusions can be made about the status of this family.

Strong support was present for the monophyly of the *Diatrypaceae* (>72%), however, the relationship of the *Diatrypaceae* to the remaining families is unclear as it is a sister group to the *Xylariaceae* in the MP analyses but is found as a sister group to the *Amphisphaeriaceae* in the ML analysis. Previous work has placed *Diatrype disciformis* within the *Xylariaceae*, suggesting a close relationship but these studies have lacked taxa from other families of the *Xylariales* (Spatafora *et al*., 1998; Réblová and Winka, 2001) and additional work with other genes needs to be conducted before any conclusions can be made as to the position of this family.

The position of the *Graphostromataceae* has not been clarified at all, as in the MP analysis *Graphostroma* groups with the *Diatrypaceae* while, in the ML analysis, with the *Amphisphaeriaceae*. Morphologically, *Graphostroma* has a *Nodulosporium* anamorph in common with the *Xylariaceae* but has allantoid to oblong spores and diatrypoid ascomata similar to the *Diatrypaceae* (Barr, 1990; Barr *et al*., 1993).

Only two members of the *Hyponectriaceae* were included in this study. *Monographella* has been shown by Jeewon (2002) to be more closely related to

the *Amphisphaeriaceae* and suggest that it is better accommodated in that family. *Hyponectria* grouped with *Appendicospora*, as discussed previously, and also with *Oxydothis*, although none of these relationships have any support. The position of this family cannot be determined from this study.

The position of taxa from the *Xylariaceae* were also variable in these analyses. *Xylaria hypoxylon* did not group with *X. curta* but with *Halorosellinia*, suggesting that *Xylaria* may be polyphyletic. *Astrocystis* and *Rosellinia* grouped with high bootstrap support, as did *Daldinia* and *Hypoxylon* which were either basal to the family (ML) or removed from it completely (MP). Although there is no bootstrap support for the arrangements, the results suggest that the *Xylariaceae* is unlikely to be monophyletic given the inclusion of *Arecophila* and *Cainia* within the clade.

Molecular phylogenetics of the *Xylariales*, using 28S and 18S rDNA, was able to show that the order *sensu* Eriksson *et al*. (2003) is paraphyletic as the *Xylariales* was found to contain an additional family, the *Apiosporaceae*. The analyses conducted in this study demonstrated that the use of ribosomal DNA sequences is inadequate to elucidate familial relationships within the *Xylariales*, an unexpected and unusual result. Typically, the 18S rDNA is able to resolve familial relationships within an order. That the 28S rDNA also did not provide any degree of phylogenetic resolution is even more surprising, as it is less conserved than the 18S rDNA. The phylogenetic relationships within the Xylariales needs further investigation with more variable genes that have evolved independently of the rDNA genes.

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