Molecular phylogenetic studies within the *Xylariaceae* based on ribosomal DNA sequences

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The *Xylariaceae* (*Xylariales*, *Ascomycota*) are considered one of the largest families of filamentous *Ascomycetes*. To infer the evolutionary relationships of some genera considered within the *Xylariaceae*, the 5.8S rRNA gene and ITS2 sequences of 100 isolates covering 15 genera and 62 taxa, were analysed phylogenetically. To obtain an accurate view on the evolutionary relationships of genera within *Xylariaceae*, four different sequence analysis methods (*i.e.* Parsimony, Neighbor-joining, Maximum-likelihood and Bayesian analyses) were employed, and a consensus phylogram was obtained to integrate data from all these mentioned approaches. Rates of congruence between topologies of the trees generated were also estimated by different methods. The phylogenetic reconstructions showed a reasonable degree of correlation between the sequence data and the proposed morphological classification schemes only for some genera within the family. The *Hypoxylon*-related genera included in the study (*i.e*. *Hypoxylon*, *Annulohypoxylon*, *Biscogniauxia*, *Camillea*, *Creosphaeria*, *Whalleya* and *Daldinia*) appeared closely related within a large clade in all the trees. *Nemania* always clusters apart from this clade of *Hypoxylon*-related genera, often found at the base of the tree. Phylogenetic reconstruction supported a polyphyletic origin for the genera *Xylaria* and *Rosellinia*, suggesting that these must be considered as large and complex genera, made up of a mixture of weakly related species. *Kretzschmaria* and *Stilbohypoxylon* appeared to be highly related to some *Xylaria* species. Finally, *Entoleuca* seems to be closely related to *Rosellinia*. In summary, this study suggests the need for further revision of the generic concepts and diagnostic characters within the *Xylariaceae*.

Key words: *Ascomycetes*, Bayesian analysis, ITS phylogeny, Maximum-likelihood analysis, Neighbor-Joining analysis, Parsimony analysis, systematics, *Xylariales*

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Introduction

The *Xylariaceae* (*Xylariales*, *Ascomycotina*) constitutes a large family of filamenttous fungi, with at least 73 accepted genera to date (Eriksson, 2006). Most of them occur on a wide range of living or dead angiosperms (more rarely on gymnosperms), being considered in general terms as saprotrophs or weak parasites, although some species from this family are known to be serious plant pathogens (Whalley, 1996). Members of this family can also be found in litter, soil, dung, and associated with insects (Rogers, 1979, 1993; Whalley, 1985, 1993, 1996). In addition, taxa from the *Xylariaceae* have been reported living as endophytes within many plants (Petrini and Petrini, 1985; Promputtha *et al*., 2005; Wang *et al*., 2005) and even lichens (Li *et al*., 2007). Most of these endophytic xylariaceous fungi are usually isolated as hyphomycetous anamorphs from a wide variety of plants, although teleomorphs develop on a narrower range of hosts (Bills and Peláez, 1996; Collado *et al*., 2001; Petrini and Petrini, 1985). Many members of this family have been reported as producers of secondary metabolites (Huang and Kaneko, 1996; Stadler and Hellwig, 2005; Whalley and Edwards, 1987, 1995), some of them with interesting

biological activities, such as nodulisporic acid, a potent insecticide isolated from an endophytic pantropical *Nodulisporium* sp. (Polishook *et al*., 2001); the antifungal lipodepsipeptide LL-15G256γ isolated from *Halorosellinia oceanica* (S. Schatz) Whalley, E.B.G. Jones, K.D. Hyde & Laessøe or the sordarin analogs produced by *Hypoxylon croceum* J.H. Mill. and *Xylaria* sp. (Stadler and Hellwig, 2005).

Morphological characters widely accepted to define the limits of the family *Xylariaceae* include the presence of stromata, type of centrum, structure of the apical apparatus of the asci (bluing in Melzer's iodine reagent), and the number, arrangement and morphology of ascospores (Martin, 1967a; Ju and Rogers, 1996). The number of genera accepted within the *Xylariaceae* is still open to debate (Whalley, 1996), and several generic schemes have been proposed based mainly on morphological characters (Whalley and Edwards, 1987). Main characters used to delimitate genera within the family *Xylariaceae* include the morphology of asci, ascospores and stromata (unipartite, bipartite, uniperitheciate, multiperitheciate, etc.), the presence of extractable stromatal pigments in KOH and the different anamorphs exhibited. These have been traditionally classified (Laessøe, 1994; Ju and Rogers, 1996) as *Nodulisporium*-like (includeing *Xylocladium*, *Periconiella*, *Virgariella* and more simple anamorphs with *Sporothrix*-like branching patterns), *Geniculosporium*-like (including *Acanthodochium*, *Dematophora*, *Dicyma* and *Xylocoremium*), *Libertella* and *Lindquistia*. Although there is no universally accepted system of classification for these teleomorphic genera, due to overlap in morphology and poorly resolved phylogeny, the view most widely accepted in recent times distributes most of the genera of the familiy in two large groups, the *Hypoxyloideae*, characterized by the production of stromatal pigments in KOH and *Nodulisporium*-like anamorphs (*e.g*. *Hypoxylon*, *Biscogniauxia*, *Camillea*, *Daldinia*), and the *Xylarioideae*, which do not yield stromatal pigments in KOH and produce *Geniculosporium*-like anamorphs (*e.g*. *Xylaria*, *Rosellinia*, *Nemania*) (Stadler and Hellwig, 2005; Tang *et al*., 2007).

Likewise, the relationships of the *Xylariaceae* with other *Pyrenomycetes* remain uncertain, although the family is considered to be closer to the *Diatrypaceae* than to other families in the *Sordariomycetes* (Rogers, 1979; Ju *et al*., 1993). In a recently revised outline of Ascomycete classification, Eriksson (2006) arranged the *Xylariaceae* together with the *Diatrypaceae* within order *Xylariales*, subclass *Xylariomycetidae*, class *Sordariomycetes*. Some recent phylogenetic studies (Kang *et al*., 2002; Jeewon *et al*., 2003), have suggested the existence of a close relationship between the *Xylariaceae* and members of the *Amphisphaeriaceae s. str*., traditionally included in the order *Amphisphaeriales*, together with the *Cainiaceae*, *Clypeosphaeriaceae* and *Hyponectriaceae*. A recent study (Smith *et al*., 2003) based on 28S and 18S rDNA sequences, including members of several families traditionally related to the *Xylariales*, suggested that the order, including both the *Diatrypaceae* and the *Amphisphaeriaceae*, could be considered as a monophyletic taxon, subsequently refuting the ordinal status for the two latter groups suggested by some authors (Barr, 1990; Eriksson and Hawksworth, 1998; Kang *et al*., 2002).

The *Xylariaceae* have been previously subjected to some phylogenetic studies based on ribosomal DNA or other gene sequences, but the number of taxa and taxonomic scope covered in those reports has usually been limited (*e.g*. Bahl *et al*., 2005 on *Rosellinia*; Johannesson *et al*., 2000 on *Daldinia*; Lee *et al*., 2000 on *Xylaria*; Mazzaglia *et al*., 2001 on *Biscogniauxia*; Suwannasai *et al*., 2005 on *Hypoxylon*; Sánchez-Ballesteros *et al*., 2000 on *Hypoxylon*-related genera). More ambitious endeavors include the works from Hsieh *et al*. (2005) and Triebel *et al*. (2005), on *Hypoxylon*-related genera; and the more recent work from Tang *et al*. (2007), which covers the whole family.

In this work, the phylogenetic relationships among 100 isolates representing 15 genera and 62 species and varieties from the *Xylariaceae* were explored on the basis of the comparison of the complete sequences of the ITS2 regions and the 5.8 rRNA gene. Results obtained from the molecular analysis are discussed and compared with morphology-

based classification schemes reported for the group.

Materials and methods

Fungal isolates and culture conditions

The isolates used in this work were either isolated by the authors, purchased from the American Type Culture Collection (ATCC, Rockville, Maryland), the Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands), provided by Prof. Jack Rogers (Washington State University) or by the University of Alcalá de Henares, Madrid, Spain. Voucher specimens for strains prefixed GB or JP are deposited at the National Fungus Collection (BPI), Beltsville, Maryland, and cultures are maintained in the Merck Microbial Resources Culture Collection, Merck Research Laboratories, Rahway, New Jersey. Cultures from strains prefixed as F are maintained in the CIBE Culture Collection, Merck, Sharp & Dohme de España. Strains purchased from collections had been deposited or identified by well-known specialists in this fungal group to minimize the risk of including misidentified strains in the analysis. However, incorporating deposited culture samples has been hampered by the fact that some materials were deposited long time ago and were subsequently identified using rather outdated species and generic concepts. In addition, some sequences representing xylariaceous taxa were also retrieved from Genbank and incorporated to the analyses. The selection of sequences from GenBank was focused on broadening the number of species available for certain critical genera, as well as including some molecular data obtained by our group in previous studies. Data concerning species name, original substrates and geographical origins are listed in Table 1. The most recent accepted names in Index Fungorum (http:// www.index fungorum. org/) and Mycobank (http://www. mycobank. org/) have been used throughout, unless otherwise specified in Table 1.

Isolates were grown in liquid complete media (5 g/l of each malt extract, yeast extract and glucose) in Petri dishes at 26°C for up to 3 weeks, and maintained on plates at 4°C on potato dextrose agar (Oxoid, CM139, Hampshire, UK).

DNA procedures

All the procedures used in this study for DNA purification and amplification have been previously described (Sánchez-Ballesteros *et al*., 2000). Asymmetric PCR amplification, used to synthesize ssDNA, was done with a 50:1 molar ratio between the two primers (Gyllenstein and Erlich, 1988). The primers used for amplification of the ITS regions were ITS4 (White *et al*., 1990), as concentrated primer, and ITS1F (Gardes and Bruns, 1993) for one strand, and ITS1F, as concentrated primer, and ITS4A, primer specific for *Ascomycetes* (Larena *et al*., 1999), for the other strand. An automated thermal cycler (Perkin Elmer Cetus Corp. model 480, Norwalk, Connecticut) was used for the amplification reaction. The cycling parameters and the electrophoretic analysis of the PCR products were the same as previously described (Sánchez-Ballesteros *et al* 2000). The amplified products were purified using Geneclean II kit (Bio101 Inc., Vista, California) and sequenced using an ABI PRISM™ Dye Terminator Cycle sequencing kit (Perkin Elmer Cetus). All samples were sequenced in both directions. The primers used for sequencing the ITS regions were ITS3 and ITS5 (White *et al*., 1990), when ITS4/ITS1F 50:1 were used for amplification, and ITS2 (White *et al*., 1990) and ITS4, when ITS1F/ITS4A 50:1 were used for amplification. The sequences were deposited in GenBank (see Table 1 for accession numbers).

Sequence analysis

Sequences from each strain were assembled to obtain the consensus sequence of the entire ITS regions using the GCG Fragment Assembly System (GCG Wisconsin Package version 10.1, Madison, Wisconsin). Alignments of the homologous regions of the different strains were performed using the multiple alignment program ClustalW (Thompson *et al*., 1994).

Phylogenetic analysis

Phylogenetic reconstructions of the aligned sequences were subjected to four different methods of phylogenetic analyses: Maximum Parsimony, Maximum Likelihood, Neighbor-Joining and Bayesian analysis.

Table 1. Isolates and sequences used in this study. Entries labeled with (*) are new sequences obtained for the present paper.

Species	Genbank accesion number	Strain code	Substrate	Geographic origin
Annulohypoxylon annulatum (Schwein.) Y.M. Ju, J.D. Rogers & H.M. Hsieh	AJ390395	GB 5659	*Quercus* sp.	New Jersey
Annulohypoxylon annulatum	AY909026*	F-160,849	Unknown	Unknown
Annulohypoxylon atroroseum (J.D. Rogers) Y.M. Ju, J.D. Rogers & H.M. Hsieh	AF201712	ATCC76081	Unknown	Ille de Reunion
Annulohypoxylon cohaerens (Pers.) Y.M. Ju, J.D. Rogers & H.M. Hsieh	AY909025*	F-160,842	Unknown	Unknown
Annulohypoxylon minutellum (Syd. & P. Syd.) Y.M. Ju, J.D. Rogers & H.M. Hsieh	AJ390399	ATCC38977	*Quercus* sp.	France
Annulohypoxylon multiforme (Fr.) Y.M. Ju, J.D. Rogers & H.M. Hsieh	AF201717	ATCC36665	*Betula* sp.	Unknown
Annulohypoxylon multiforme[1]	AY354245	olrim319	*Betula pendula*	Lithuania
Annulohypoxylon multiforme	AY909003*	F-160,843	Unknown	Unknown
Annulohypoxylon truncatum (Schwein.) Y.M. Ju, J.D. Rogers & H.M. Hsieh	AF201716	ATCC38991	Unknown	Florida
Anthostomella sepelibilis (Berk. & M.A. Curtis) Sacc.	AY908989*	F-160,798	*Smilax* sp. (dead stems)	USA
Anthostomella sepelibilis	AY908990*	F-160,797	*Smilax* sp. (dead stems)	USA
Biscogniauxia atropunctata (Schwein.) Pouzar	AJ390411	ATCC13359	*Quercus robur*	Florida
Biscogniauxia atropunctata	AF201705	ATCC38987	Southern red oak	Florida
Biscogniauxia atropunctata var. *intermedia* Y.M. Ju, J. D. Rogers & F. San Martín	AJ390412	GB 4796	Dead *Quercus* sp.	Costa Rica
Biscogniauxia bartholomaei (Peck) Lar.N. Vassiljeva	AF201719		Unknown	Unknown
Biscogniauxia marginata (Fr.) Pouzar	AJ390417	ATCC62608	*Quercus* sp.	Pennsylvania
Biscogniauxia mediterranea (De Not.) Kuntze	AJ390413	CBS 280.61	Unknown	USA
Biscogniauxia mediterranea	AJ390414	GB 5664	*Quercus rubra*	New Jersey
Biscogniauxia nummularia (Bull.) Kuntze	AJ390415	CBS 969.70	*Fagus sylvatica*	England
Biscogniauxia repanda (Fr.) Kuntze	AJ390418	ATCC62606	Unknown	Unknown
Camarops ustulinoides (Henn.) Nannf.	AY908991*	F-999,001	*Dacryodes excelsa*	Puerto Rico
Camillea obularia (Fr.) Laessøe, J.D. Rogers & Lodge	AJ390423	ATCC28093	Rotten wood	Puerto Rico
Camillea tinctor (Berk.) Laessøe, J.D. Rogers & Whalley	AJ390421	CBS 203.56	Dead *Sassafras* sp.	USA
Camillea tinctor	AJ390422	GB 4624	*Robinia pseudoacacia*	New Jersey
Creosphaeria sassafras (Schwein.) Y.M. Ju, F. San Martín & J.D. Rogers	AJ390424	GB 4588	*Lindera benzoin*	New Jersey
Creosphaeria sassafras	AJ390425	GB 4591	*Sassafras albidum*	New Jersey
Daldinia concentrica (Bolton) Ces. & De Not.	AF176958	Wat Herb 13965	*Fraxinus* sp.	United Kingdom
Daldinia cf. *fissa* Lloyd	AF176981	P.D. Rabenborg	*Malus* sp.	Denmark
Daldinia loculata (Lév.) Sacc.	AF176969	HJ 107	*Sorbus* sp.	Sweden
Daldinia loculatoides Wollw. & M. Stadler	AF176982	B.J. Coppins 8630	*Fagus* sp.	United Kingdom
Daldinia petriniae Y.M. Ju, J.D. Rogers & F. San Martín	AF176975	HJ 103	*Alnus* sp.	Sweden
Dicyma funiculosa Guarro & Calvo	AY908992*	CBS 323.86	Forest soil	Spain
Dicyma funiculosa	AY908994*	CBS 324.86	Soil	Spain
Dicyma pulvinata (Berk. & M.A. Curt.) Arx	AY908993*	CBS 194.56	*Prunus persica*	Italy
Entoleuca mammata (Wahlenb.) J.D. Rogers & Y.M. Ju	AF201713	ATCC58108	*Populus tremuloides*	Michigan
Hypoxylon cinnabarinum (Henn.) Y.M. Ju & J.D. Rogers	AJ390398	F-108,404	Unknown	Taiwan
Hypoxylon fendleri Berk. ex Cooke	AJ390400	F-108,405	Unknown	Mexico

Table 1 (continued). Isolates and sequences used in this study. Entries labeled with (*) are new sequences obtained for the present paper.

Species	Genbank accesion number	Strain code	Substrate	Geographic origin
Hypoxylon fragiforme (Pers.) J. Kickx f.	AF201709	ATCC36662	*Fagus*	Unknown
Hypoxylon fragiforme	AJ390401	CBS 206.31	Unknown	Germany
Hypoxylon fragiforme	AJ390402	CBS 204.32	*Fagus sylvatica*	Denmark
Hypoxylon fragiforme	AJ390403	GB 4503	*Fagus grandifolia*	New Jersey
Hypoxylon fragiforme	AY618235	olrim777	*Picea abies*	Sweden
Hypoxylon fuscum (Pers.) Fr.	AJ390404	F-076,920	Unidentified plant	Italy
Hypoxylon fuscum	AJ390405	ATCC36663	*Corylus* sp.	Unknown
Hypoxylon intermedium (Schwein.) Y.M. Ju & J.D. Rogers	AJ390396	ATCC38986	*Fraxinus* sp.	Wales
Hypoxylon ochraceum Henn.	AJ390406	F-108,406	Unknown	Guadeloupe
Hypoxylon papillatum Ellis & Everh.	AF201710	ATCC58729	Unknown	West Virginia
Hypoxylon perforatum (Schwein.) Fr.	AJ390407	F-108,407	Unknown	USA
Hypoxylon rickii Y.M. Ju & J.D. Rogers	AJ390408	F-108,408	Unknown	Mexico
Kretzschmaria clavus (Fr.) Sacc.	AJ390434	JP 3113	*Guarea guidonia*	Puerto Rico
Kretzschmaria deusta (Hoffm.) P.M.D. Martin	AJ390435	CBS 826.72	*Fagus sylvatica*	Belgium
Kretzschmaria deusta	AJ390437	CBS 288.30	Unknown	England
Kretzschmaria deusta [2]	AF201718		Unknown	Unknown
Nemania aenea (Nitschke) Pouzar	AF201720		Unknown	Unknown
Nemania aenea	AJ390426	ATCC60818	*Salix alba*	Czechoslo-vakia
Nemania aenea var. *aureolutea* (L.E. Petrini & J.D. Rogers) Y.M. Ju & J.D. Rogers	AJ390427	CBS 680.86	*Quercus* sp.	Switzerland
Nemania aenea var. *aureolutea*	AJ390428	ATCC60819	*Quercus* sp.	Switzerland
Nemania aenea var.*aureolutea*	AF201704	ATCC60819	*Quercus* sp.	Switzerland
Nemania bipapillata (Berk. & M.A. Curtis) Pouzar	AJ390429	JP 3034	*Guarea guidonia*	Puerto Rico
Nemania bipapillata	AY541610	CL8	*Cinnamomum* sp.	Thailand
Nemania chestersii (J.D. Rogers & Whalley) Pouzar	AJ390430	ATCC38988	*Fraxinus* sp.	Wales
Nemania serpens (Pers.) Gray	AJ390431	ATCC16078	Soil	Canada
Nemania serpens	AJ390432	CBS 533.72	*Corylus avellana*	Netherlands
Nemania serpens	AJ390436	CBS 659.70	Soil from *Populus tremuloides* forest	Canada
Nemania serpens var. *macrospora* (J.H. Mill.) Pouzar	AF201707	ATCC60823	Unknown	Unknown
Nemania serpens var. *macrospora*	AJ390433	ATCC60822	*Quercus* sp.	California
Rosellinia arcuata Petch	AB017660	CBS 347.29	Unknown	Unknown
Rosellinia australis Sacc. & Trotter	AY908997*	AH 24323	*Nicotiana glauca*	Spain
Rosellinia bambusae Henn.[3]	AY908998*	ATCC66430	*Dendrocalamus latiflorus*	Taiwan
Rosellinia buxi Fabre	AY909000*	ATCC32869	Unknown	England
Rosellinia corticium (Schwein.) Sacc.	AY908999*	F-160,845	Unknown	Unknown
Rosellinia necatrix Berl. ex Prill.	AB017657	R-4-1	Unknown	Unknown
Rosellinia necatrix	AB017658	R-24-1	Unknown	Unknown
Rosellinia necatrix	AY909001*	CBS 349.36	*Malus sylvestris*	Argentina
Rosellinia pepo Pat.	AB017659	CBS350.36	*Theobroma cacao*	Trinidad
Rosellinia quercina R. Hartig	AB017661	ATCC36702	*Quercus pubescens*	France
Rosellinia subiculata (Schwein.) Sacc.	AY909002*	ATCC58850	Wood	Illinois
Stilbohypoxylon quisquiliarum (Mont.) J.D. Rogers & Y.M. Ju	AY909023*	F-999,004	Unknown	Puerto Rico
Whalleya microplaca (Berk. & M.A. Curtis) J.D. Rogers, Y.M. Ju & F. San Martín	AJ390419	GB 4514	*Sassafras albidum*	New Jersey
Whalleya microplaca	AJ390420	GB 5078	*Sassafras albidum*	New Jersey
Xylaria allantoidea (Berk.) Fr.	AY909005*	F-165,173	Leaf litter	Puerto Rico
Xylaria arbuscula Sacc.	AY183369	WARMI	*Pseudotsuga menziesii* var. *glauca*	Unknown

Table 1 (continued). Isolates and sequences used in this study. Entries labeled with (*) are new sequences obtained for the present paper.

Species	Genbank accesion number	Strain no.	Original Substrate	Origin
Xylaria digitata (L.) Grev.	AY909006*	CBS 161.22	Unknown	Unknown
Xylaria globosa (Spreng.) Mont.	AY909007*	F-999,002	*Guarea guidonia*	Puerto Rico
Xylaria globosa	AY909008*	F-165,169	Unknown	Puerto Rico
Xylaria guareae Laessøe & Lodge	AY909009*	F-999,003	Unknown	Puerto Rico
Xylaria hypoxylon (L.) Grev.	AY327477	ATCC42768	Unknown	Unknown
Xylaria hypoxylon	AY909010*	CBS 589.72	Angiosperm trunk	Netherlands
Xylaria hypoxylon	AY909011*	CBS 868.72	*Fagus sylvatica*	Netherlands
Xylaria hypoxylon	AY909012*	CBS 590.72	*Picea abies*	Netherlands
Xylaria longipes Nitschke	AY909013*	CBS 148.73	*Fagus sylvatica*	Germany
Xylaria longipes	AY909014*	CBS 347.37	Unknown	Unknown
Xylaria longipes	AY909015*	CBS 580.88	Unknown	Germany
Xylaria longipes	AY909016*	F-160,846	Unknown	Unknown
Xylaria longipes	AY909017*	CBS 147.73	Angiosperm wood	Germany
Xylaria multiplex (Kunze) Fr.	AY909018*	F-165,170	Wood	Puerto Rico
Xylaria multiplex	AY909019*	F-165,171	Unknown	Puerto Rico
Xylaria multiplex	AY909020*	F-165,172	Wood	Puerto Rico
Xylaria liquidambaris J.D. Rogers, Y.M. Ju & F. San Martín [4]	AY909021*	ATCC42766	*Liquidambar styraciflua*	Georgia
Xylaria liquidambaris	AY909022*	F-165,174	*Liquidambar* sp.	New Jersey

[1]Strain codes others than ATCC, CBS, JP, GB and F (explained in Materials and Methods) correspond to sequences retrieved from GenBank, deposited by other groups.
[2]This sequence appears in GenBank as *Ustulina deusta* (Hoffm.) Lind., although the currently accepted name in Index Fungorum and Mycobank is *Kretzschmaria deusta*.
[3]This strain is labeled as *Rosellinia bambusase* in ATCC, although the currently accepted name in Index Fungorum and Mycobank is *Astrocystis bambusae*.
[4]This strain is labeled as *X. persicaria* in ATCC, but it has been renamed as *X. liquidambar* (=*X. liquidambaris* in Index Fungorum and Mycobank) by Rogers *et al*. (2002).

Maximum Parsimony analysis (MP) was carried out with a Heuristic search with PAUP 4.0 (Swofford 2001) under constraint of simple addition of sequences and tree bisection-reconnection (TBR) branch swapping, with MaxTrees set to 100. All characters were unordered and equally weighted, with gaps treated as missing data. The confidence of the branches was measured by bootstrap analysis with 1000 bootstrap replicates using heuristic search (Felsenstein, 1985). Gaps were treated as missing data. The trees were visualized with the application Treeview 1.5 (Page, 1996).

For the Maximum Likelihood analyses (ML), MODELTEST (Posada and Crandall, 1998) was used to identify the model of DNA substitution that best fits. MODELTEST analyses selected the GTR+G model with the estimation of nucleotide frequencies (A = 0.19374 C = 0.29498 G = 0.21926 T = 0.29203) with the shape parameter of the gamma distribution (a = 0.291131) to accommodate rate variations among sites. Maximum likelihood analysis was performed with PAUP 4.0. The starting tree was obtained via neighbor-joining with TBR as branch-swapping algorithm.

Neighbor-Joining analysis (NJ) was conducted with the uncorrected distance (p) model. Branch-swapping algorithm was TBR, steepest descent option not in effect, multrees option in effect. Support for internal nodes was estimated by 1000 bootstrap replicates under the same model settings (Swofford, 2001).

Bayesian analysis (BY) based on Markov Monte Carlo chain approach was run as implemented in the computer program MrBayes 3.01 (Huelsenbeck *et al*., 2002). To improve mixing of the chain, four incrementtally heated simultaneous Monte Carlo Markov chains were run over 2,000,000 generations,

using the GTR model of DNA substitution with gamma-distributed substitution rates. Trees were sampled every 100 generations, resulting in an overall sampling of 20,000 trees. The initial 1000 trees were not used for the posterior analysis. From those trees that were sampled after the process had reached stationarity, a 50% majority-rule consensus tree was computed to get estimates for clade credibilities.

To integrate data from the four phylogenetic approaches, a phylogram representing the 50% majority rule consensus tree of the phylograms obtained by MP, ML, NJ and BY was also performed. The congruence among the topologies of the trees generated by the four phylogenetic approaches was tested by the Kishino Hanegawa (KH) test (Kishino, 1989) with full optimization and the two tailed test, and the Shimodaira Hasegawa (SH) test (Shimodaira, 1999) with full optimization and one tail, performed with PAUP 4.0. (Kim *et al*, 2005).

As a second estimate of the congruence of the trees generated by the different methods of phylogenetic analysis, the trees were compared pairwise, and the number of common nodes between every two trees was obtained by using PEST software (Zujko-Miller and Miller, 2004). A measure of the congruence between each pair of trees was obtained applying the Dice coefficient [(2 × nº of common nodes/(nodes tree A+ nodes of tree B)].

All the phylogenetic trees were rooted using *Neurospora crassa* as outgroup (GenBank accession number M13906).

Results

Sequence analysis

Molecular data have been derived from the analysis of the ITS sequences of 100 isolates belonging to 62 taxa within 15 genera of the *Xylariaceae* (*Annulohypoxylon*, *Anthostomella*, *Biscogniauxia*, *Camillea*, *Creosphaeria*, *Daldinia*, *Dicyma*, *Entoleuca*, *Hypoxylon*, *Kretzschmaria*, *Nemania*, *Rosellinia*, *Stilbohypoxylon*, *Whalleya* and *Xylaria*), and *Camarops*, actually placed in the *Boliniaceae* (*Boliniales*). The length of the entire ITS regions ranged from 436 bp to 595 bp. The length of ITS1 ranged from 129 bp to 279 bp, whereas the length of ITS2 varied from 152 bp to 178 bp. For most of the isolates, ITS2 length was shorter and more homogeneous than ITS1 length. The length of the 5.8 rRNA gene was 155 bp for all the strains in this study (including *Camarops ustulinoides*). The length of the complete ITS regions was found to be very variable between different species of the same genus, but was usually conserved between conspecific strains or closely related taxa. Some exceptions were found for several strains of *Biscogniauxia atropunctata*, *Nemania aenea*, *N. serpens*, *Xylaria globosa* and specially *X. hypoxylon* (variation in length among ATCC42768 strain and the other *X. hypoxylon* isolates was 91 bp). The sequenced regions did not contain any introns.

In a previous work (Platas *et al*., 2001), we reported the presence of simple tandem repeats (STR) in the ITS1 region of some strains of *Hypoxylon* and other genera (*Biscogniauxia*, *Camillea*, *Creosphaeria*, *Kretzschmaria*, *Nemania* and *Whalleya*). These STR motifs were also present in the ITS1 region of other strains belonging to genera *Dicyma*, *Rosellinia* and *Xylaria* (Sánchez-Ballesteros, 2001). The number and distribution of these motifs were found to be very variable, even among different species in the same genus. The presence of STR, together with the high rates of nucleotide divergence, suggested the convenience of removing the ITS1 fragment from the phylogenetic analyses. The total alignment obtained for the 5.8S-ITS2 fragment was of 374 bp, being 170 of these characters constant.

Tree Congruence

The topology of the trees obtained from the four different phylogenetic approaches was apparently different. To check if the differences among the trees were significantly greater than expected from random sampling error, both the Kishino Hasegawa and the Shimodara Hasegawa tests were applied using PAUP, with bootstrap analysis with full optimization and two tailed test. The results of the analysis are shown in Table 2. The tree with the highest likelihood score was the one obtained by BY inference, suggesting that it would more accurately reflect the evolutionary

patterns for these sequence datasets. Nevertheless, all the remaining trees were also coherent with the present datasets ($P > 0.05$). Another measure of the congruence between the trees was obtained by calculating Dice coefficients from the number of nodes in the trees (Table 3). The comparison showed that all the trees shared about two thirds of their nodes when compared pairwise, suggesting that all of them were similarly divergent. A consensus tree (Fig. 5) was generated applying the 50% majority rule to the four phylogenetic trees obtained with the different methods.

Table 2. Results of Kishino Hasegawa and Shimodara Hasegawa tests on the trees generated by the four different phylogenetic approaches compared. P is the probability of getting a more extreme T value under the null hypothesis of no difference between the two trees, with significance at $P < 0.05$.

Tree	-ln L	Diff -ln L	KH-test P	SH-test P
BY	7319.75592	(best)		
ML	7331.09424	11.33832	0.702	0.701
MP	7341.68564	21.92972	0.536	0.545
NJ	7339.39109	19.63518	0.526	0.589

Table 3. Congruence rates between the phylogenetic trees generated by different methods, calculated by means of Dice coefficients applied to the number of common nodes.

	N[a] total Nodes	MP	BY	NJ	ML
MP	90	1			
BY	99	0.61	1		
NJ	85	0.64	0.68	1	
ML	88	0.69	0.67	0.69	1

Phylogenetic analysis based on the 5.8S-ITS2 region

Phylogenetic analyses of the whole 5.8S-ITS2 region showed a reasonable degree of correlation with the previously proposed morphological classification schemes of genera within the family with some exceptions, as discussed below. After the comparison and critical review of the different phylogenetic reconstruction methods applied to this sequence data set, some general trends conserved in most or all of the phylogenies performed can be defined. These trends can be better outlined from the consensus phylogram generated by the 50% Majority Rule (Fig. 5), although some of the deviant results found by each analysis method used will be discussed individually.

In general terms, the consensus phylogram (Fig. 5) showed that many of the groupings at the tips of the branches were common to the phylograms resolved by the four methods, but very few of the large clades were consistently supported. As a rule, a large clade containing *Hypoxylon*-related genera was supported by all the molecular analysis. This clade contained sequences from *Hypoxylon* and genera *Annulohypoxylon*, *Biscogniauxia*, *Camillea*, *Creosphaeria* and *Whalleya*. An even closer relationship between *Daldinia*, *Hypoxylon* and *Annulohypoxylon* was also supported by three out of the four phylogenetic methods employed (node represented by dashed line in Fig. 5). *Nemania* sequences appeared generally in a basal node with respect to the rest of xylariaceous taxa studied. *Xylaria* and *Rosellinia* species appeared usually related to some extent, although some differences were found in the relationships of these two complex taxa, depending on the method of analysis. The existence of separate and sometimes distant nodes including groups of *Xylaria* species was supported by three out of the four methods employed. Similar results were observed for *Rosellinia*, with two main clades containing sequences representing this genus supported by three out of the four analyses performed. Finally, *Camarops* appeared consistently as external to the remaining taxa studied.

The most parsimonious tree derived from the MP analysis of the 5.8S-ITS2 region is shown in Fig. 1. As expected, a basal branch segregated *Camarops ustilinoides* from the rest of strains belonging to the *Xylariaceae*, grouped in a clade with high bootstrap support. Within the xylariaceous clade, a first basal node contained two strains representing *Nemania bipapillata*, excluded from the rest of taxa of this genus. The remaining *Nemania* isolates appeared as a monophyletic group (68% bootstrap support) just below the *N. bipapillata* node. The next branch grouped a number of *Rosellinia* species (*R. subiculata*,

Fig. 1. Best phylogenetic tree obtained by MP. Tree length: 1348 steps. CI= 0.298; RC= 0.214; HI= 0.702. Number of parsimony informative characters = 123. Bootstrap support values above 50% are indicated at the branches.

R. australis, *R. pepo* and *R. corticium*), plus one sequence representing *Entoleuca mammata* in a clade with low bootstrap support (57%). Most of the *Xylaria* sequences were grouped in a clade without bootstrap support, together with the strains of *Kretzschmaria* and *Stilbohypoxylon*, as well as some sequences from *Rosellinia* (*R. buxi*, *R. necatrix* and *R. arcuata*). One sequence of *X. globosa* (AY909008) was placed out of this large clade, as well as three *X. hypoxylon* sequences, which grouped together in a clade separate from the large *Xylaria* clade, near *Rosellinia quercina* and *R. bambusae*, which appeared in individual branches at that level. Below this branch, a node containing taxa from *Anthostomella*

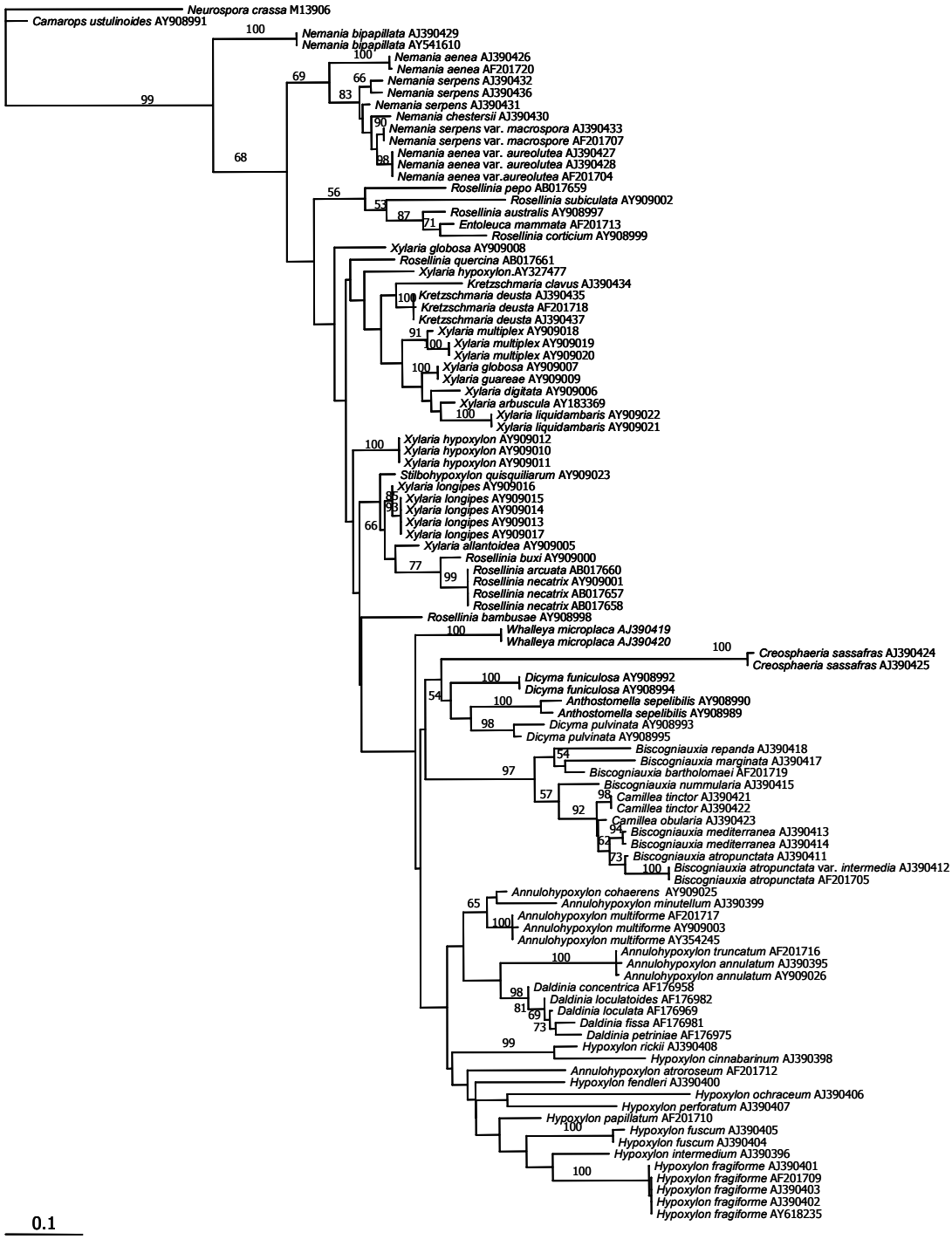

Fig. 2. The most likely phylogenetic tree obtained by ML analysis under the GTR+G model with a Ln likelihood = -7331.09424. Tree length: 1366 steps. Bootstrap support values above 50% are indicated at the branches.

and *Dicyma* was resolved. Finally, a well supported clade containing the sequences representing genus *Daldinia* was resolved just above a large node containing the remaining *Hypoxylon*-like genera included in the phylogenetic reconstruction (*i.e*. *Annulohypoxylon*, *Creosphaeria*, *Biscogniauxia*, *Camillea* and *Whalleya*). Within this clade, which lacked statistical support, there was only good bootstrap support for the branch containing the sequences of *Biscogniauxia* and *Camillea*. The *Hypoxylon* isolates were not arranged as a monophyletic group.

The ML phylogram (Fig. 2), shared several features of the topology resolved in MP analysis, such as the position of the clades

containing *Nemania* or *Rosellinia/Entoleuca* species, as well as the large clade containing all the *Hypoxylon*-like genera and the robust branch of *Biscogniauxia/Camillea*, but some differences were identified. Thus, several separate clades containing *Xylaria* species could be outlined. One of these nodes contained the sequences from *X. longipes*, *X. allantoidea* and *S. quisquiliarum* together with the remaining *Rosellinia* sequences, whereas the other branch grouped most of the remaining *Xylaria* species together with *Kretzschmaria*. The *X. hypoxylon* strains were separate from those two main clades, same as *X. globosa*. This tree grouped all the *Annulohypoxylon*, *Hypoxylon* and *Daldinia* isolates together in a clade, although without bootstrap support. Interestingly, this clade shows some segregation between the species of *Annulohypoxylon* and *Hypoxylon*, supporting the former as a separate genus within the *Hypoxylon* complex. It also suggests that *Annulohypoxylon* would be closer to *Daldinia* than *Hypoxylon*.

The tree generated by NJ analysis (Fig. 3) also showed several differences, the most relevant being the segregation of *Nemania* species from *N. bipapillata* and clusters in a separate node with taxa belonging to *Rosellinia*, *Xylaria* and *Kretzschmaria*. In addition, taxa from *Biscogniauxia* and *Camillea* clustered together in a basal node just below two strains of *N. bipapillata*, and distant from the rest of taxa, including the other *Hypoxylon*-like genera. This was the only phylogenetic tree that grouped all the *Xylaria* isolates together as a monophyletic group (although including *Kretzschmaria*, *Stilbohypoxylon* and *Rosellinia bambusae*). Likewise, all the *Rosellinia* species (except for *R. bambusae*) were included in a single clade, together with *Entoleuca mammata*.

The phylogenetic reconstruction obtained by BY analysis (Fig. 4) also showed some differences. The *Rosellinia* strains were placed basal to the rest of sequences, although divided into essentially the same two groups observed in Figs 1 and 2. The *Xylaria* sequences appeared distributed in two main clades. The node containing *S. quisquiliarum*, *X. longipes* and *X. allantoidea*, appeared right after the *Rosellinia* sequences, in a basal position with respect to the remaining taxa. The rest of *Xylaria* species were grouped together with *Kretschmaria* and *Nemania* in a branch without statistical support. This tree provided very good support for the large clade containing all the *Hypoxylon* related genera (including also *Dicyma* and *Anthostomella*). Likewise, it provided high support for the *Biscogniauxia/Camillea* clade and weak support for a monophyletic group containing *Hypoxylon*, *Annulohypoxylon* and *Daldinia*, suggesting also a closer relationship between the latter two genera, which is in agreement with the ML analysis.

Discussion

In previous work we documented the existence of a high variability within the ITS1 of *Xylariaceae* in size, number, sequence and distribution of simple tandem repeats (Platas *et al*., 2001, 2002, 2004). Unlike the *Diatrypaceae* (Acero *et al*., 2004), sequence variability found in *Xylariaceae* was higher in ITS1 than in ITS2 (Platas *et al*., 2001). The presence of these repeated motifs has created some controversy on the use of the ITS region to provide insight on the evolutionary relationships among the members of the *Xylariaceae*. Molecular phylogenies based on the analysis of rDNA ITS sequences have been repeatedly performed in *Xylariaceae* and adjacent families to infer evolutionary relationships (e.g. Johannesson *et al*., 2000; Sánchez-Ballesteros *et al*., 2000; Kang *et al*., 2002; Triebel *et al*., 2005; Tang *et al*., 2007), although some authors (e.g. Hsieh *et al*., 2005) have pointed that these insertions made the ITS region not suitable to resolve systematic questions in some genera of the family like *Hypoxylon*.

Due to the high number of taxa studied and consequently the high variability encountered in the ITS1 size, only the 5.8S gene and the ITS2 internal transcribed spacer were used for this work. The short size of this alignment could make it unreliable to infer the phylogenetic relationships among the *Xylariaceae* using just one single phylogenetic approach. Thus, we decided to use the four most widely used methods for molecular phylogenetic analysis (ML, BY, NJ and MP) to check for clades that were more consistently supported by the sequence analysis. Nevertheless the

Fig. 3. Phylogenetic tree obtained by NJ analysis using the uncorrected distance (p) model. Tree length: 1369 steps. Bootstrap support values above 50% are indicated at the branches.

Dice congruence coefficient showed that only about two thirds of the nodes were constant in each of the pairwise comparisons of the trees. To identify which were the most conserved clades a consensus tree was obtained.

The combination of results from MP, ML, NJ and BY analyses appeared suitable for answering some classical systematic questions around genera usually considered under the concept of the *Xylariaceae*. Some of these topics included the polyphyletic origin of genera *Xylaria* and *Rosellinia*, or the taxonomic placement of *Nemania*, as well as the differentiation and evolutionary relationships

Fig. 4. Phylogenetic tree generated by BY analysis. Tree length: 1364 steps. Posterior probability values are indicated at the branches.

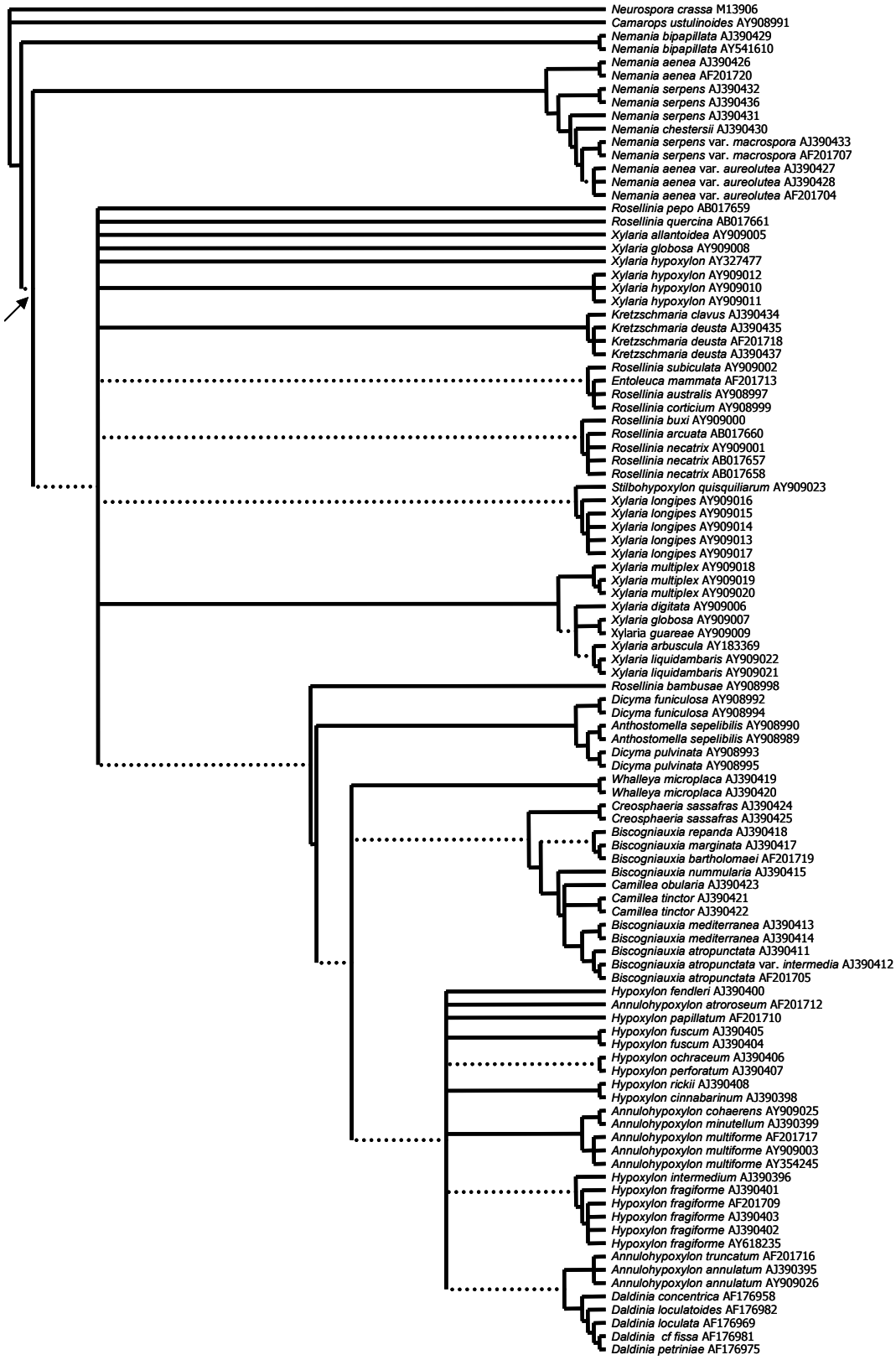

Fig. 5. Phylogram of the 50% majority rule consensus tree based on the phylograms obtained by MP, BY, NJ and ML. Dashed lines represent clades supported by 3 out of the 4 techniques applied. Solid lines represent clades supported by the four techniques applied.

of genera closely related with *Hypoxylon*, such as *Annulohypoxylon*, *Camillea*, *Biscogniauxia* or *Whalleya*. These and other issues are discussed below.

Camarops

Our phylogenetic reconstruction supported the segregation of genus *Camarops* out of the *Xylariaceae*, as largely suggested by several authors. Species included in this genus have been considered in ancient taxonomic studies (Martin, 1969a) as belonging to the *Xylariaceae* because of their stromatal affinities with *Hypoxylon* and related genera. Nannfeldt (1972), however, considered this affinity as superficial and, based in the differences in morphology of asci and ascospores, placed *Camarops* in a new family, the *Boliniaceae*, now included in the *Boliniales* (Eriksson, 2006). This conclusion has received further support from recent molecular studies based on SSU and LSU rDNA sequences (Smith *et al*., 2003; Huhndorf *et al*., 2004).

Hypoxylon-related genera

Hypoxylon is one of the largest genera of the *Xylariaceae*, although definitions and delimitations of the genus have been under discussion (Martin, 1967b, 1968, 1969a). Miller (1961) included as *Hypoxylon* many species that were later segregated in different genera, like *Biscogniauxia* (Pouzar, 1986), *Camillea* (Laessøe *et al*., 1989), *Whalleya* and *Jumillera* (Rogers *et al*., 1997), *Nemania* (Pouzar, 1985a,b) and others. Ju and Rogers (1996) redefined *Hypoxylon s*. *str*., establishing a more restrictive concept for the genus, with only two sections, *Hypoxylon* and *Annulata*. More recently, Hsieh *et al*. (2005) erected *Annulohypoxylon* to include taxa from section *Annulata* based on phylogeny of β-tubulin and α-actin gene sequences.

The monophyly of the *Hypoxyloideae* was supported in most of the phylogenetic trees, as previously suggested by other molecular studies based on ITS and RBP2 datasets (Tang *et al*., 2007). We found also good support for the segregation of all the above mentioned genera from *Hypoxylon*, in agreement with previous molecular studies based on ITS sequences (Sánchez-Ballesteros *et al*., 2000; Mazzaglia *et al*., 2001a; Triebel *et al*., 2005; Tang *et al*., 2007) and other genes (Hsieh *et al*., 2005). In addition, the relative position of *Daldinia* and *Annulohypoxylon* sequences in the phylograms would suggest the genus *Hypoxylon* as paraphyletic, in agreement with previous data (Hsieh *et al*., 2005; Triebel *et al*., 2005; Tang *et al*., 2007).

Two of the phylogenetic trees in particular (ML and BY) provided some support to the segregation of the taxa from section *Annulata* as suggested by Hsieh *et al*. (2005). However, the species transferred to *Annulohypoxylon* did not constitute one single monophyletic group, in contrast with the data from these authors. Rather, those species were consistently divided in two monophyletic groups which intermingled with *Daldinia* or *Hypoxylon* sequences, depending on the analysis method. One of the groups included *A*. *annulatum* and *A*. *truncatum*, two morphologically similar species. The second group contained *A*. *cohaerens*, *A*. *minutellum* (formerly *H*. *cohaerens* var. *microsporum* J.D. Rogers & Cand.) and *A*. *multiforme*. These groupings were totally in agreement with the data from Triebel *et al*. (2005) and Hsieh *et al*. (2005), although in the latter case the new genus appeared as a monophyletic taxon. Interestingly, *A*. *atroroseum*, a species not included by these authors in their study, appeared always separate from those two groups, intermingled with other *Hypoxylon* species. These data would suggest that the new genus *Annulohypoxylon*, likewise *Hypoxylon*, might also be paraphyletic, and probably not all the species from section *Annulata* should be transferred. Interestingly, previous work on ITS sequences from other species of these two genera have provided only partial support to the segregation of the species in section *Annulata* out of *Hypoxylon* (Suwannasai *et al*., 2005; Triebel *et al*., 2005: Tang *et al*., 2007).

The sequences from five *Daldinia* species appeared clustered together as a monophyletic group in all the phylogenetic trees. The *Daldinia* clade appeared intermingled with the *Hypoxylon* and *Annulohypoxylon* species within a large branch in all the trees except in the MP analysis, where it appeared at the base of the clade containing the *Hypoxylon* related genera (Fig 1). These data suggest a close relationship between *Daldinia* and *Hypoxylon*,

as reported in other molecular studies (Triebel *et al*., 2005; Hsieh *et al*., 2005, Tang *et al*., 2007). The relationship between these two genera had been previously concluded from their similar morphological features, such as the pigmented stroma, *Nodulisporium*-like anamorphs and the similar ascus ring morphology (Rogers, 1982). Ju *et al*. (1997) have suggested that *Daldinia* could be derived from *Hypoxylon*, since fruitbodies habit and the concentric ring structure of the stroma in the former could represent a modern evolutionary adaptation to dry environment. Other authors have considered *Daldinia* and *Hypoxylon* as synonyms (*e.g*. Laessøe, 1994). However, their different lifestyles have provided a justification to keep these two genera separate. Furthermore, recent molecular studies have supported this view (Hsieh *et al*., 2005; Triebel *et al*., 2005; Tang *et al*., 2007). Hsieh *et al*. (2005) considered *Daldinia* as an independent taxon, and suggested a recent evolutionary origin for the genus, based on the anatomical uniformity exhibited by most of *Daldinia* species. Likewise, Stadler and Hellwig (2005), in an extensive study on the chemotaxonomy of the *Xylariaceae*, demonstrated the convenience of maintaining *Daldinia* as an independent genus from *Hypoxylon*, based on their different secondary metabolite profiles detected in both cultures and stromata. Our own results are consistent with these reports, suggesting that *Daldinia* is a monophyletic genus that should be regarded as a *Hypoxylon*-related genus, and not a synonym.

Interestingly, *Daldinia* appeared to be closer to *Annulohypoxylon* (particularly to the *A. truncatum* clade) than to *Hypoxylon* in our phylogenetic analysis. This is in disagreement with the topology of the trees reported by Hsieh *et al*. (2005), where *Daldinia* was placed near *Hypoxylon* and far less related with *Annulohypoxylon*. Whether this inconsistency is due to the different genes sequenced (β-tubulin and α-actin *vs*. 5.8S-ITS2) or the different species selected (or both) remains to be clarified.

Biscogniauxia, *Camillea*, *Creosphaeria* and *Whalleya*, formerly included under the concept of *Hypoxylon* s. l., were found close to *Hypoxylon* but constituted independent clades. This is essentially consistent with previous data based on ITS sequences (Sánchez-Ballesteros *et al*., 2000; Triebel *et al*., 2005). The position of *Biscogniauxia* in our trees was fully consistent with the morphological and chemotaxonomic characters that assimilate this genus to the *Hypoxyloideae*, in contrast with the work by Tang *et al*. (2007), who found it to be apparently closer to *Nemania*. *Biscogniauxia* and *Camillea* are usually considered to be very closely related genera (if not synonyms), characterized by bipartite stromata (whereas in *Hypoxylon* this is unipartite). The separation between *Biscogniauxia* and *Camillea*, based on morphological characters of ascospores as well as in the anamorphic state, is not supported by our analysis, as previously discussed (Sánchez-Ballesteros *et al*., 2000). *Creosphaeria* and *Whalleya* also include species formerly considered as *Hypoxylon*, characterized by the presence of *Libertella*-like anamorphs, bearing scolecosporous conidia, similar to those of the *Diatrypaceae* (Ju *et al*., 1993; Rogers *et al*., 1997). Strains of both genera were clearly separated from *Hypoxylon* in our analysis, always clustering in separate branches within the *Hypoxyloideae* clade. Interestingly, Tang *et al*. (2007) found *Craeosphaeria* to be closer to the *Xylarioideae* (although external to the clade) than to the *Hypoxyloideae* based on RPB2 sequences, but closer to the *Hypoxyloideae* based on ITS sequences. However, the statistical support for these relationships was very weak or non-existant. On the other hand, Triebel *et al*. (2005) found *Craeosphaeria* to be close to the *Hyponectriaceae*, although with little statistical support.

Nemania

Nemania was used by Pouzar (1985a,b) to include the species-complex *Hypoxylon serpens* and several related taxa, previously classified by Miller (1961) into *Hypoxylon* section *Papillata* subsection *Primocinerea*. Pouzar (1985a) considered *Nemania* as more related to *Xylaria* and *Kretzschmaria*. More recent monographies on *Nemania* have confirmed its segregation from *Hypoxylon* based on morphological data (Granmo *et al*., 1999; Ju and Rogers, 2002). Our data support this conclusion, in line with previous molecu-

lar studies (Granmo *et al*., 1999; Sánchez-Ballesteros *et al*., 2000; Tang *et al*., 2007).

Two of the analysis methods (BY and NJ) suggested a relationship of *Nemania* with the other *Xylarioideae* taxa (*Xylaria*, *Rosellinia*) but without statistical support, whereas the other two (ML and MP) placed *Nemania* rooted at the base of the tree. Tang *et al*. (2007) found *Nemania* consistently associated to *Xylaria* species, although their study included a lower number of xylariaceous species.

Nemania bipapillata (formerly *Hypoxylon bipapillatum* Berk. & M.A. Curtis) was usually accepted as *Hypoxylon unitum* (Fr.) Nitschke before it became considered under *Hypoxylon serpens* by Miller (1961). However, Petrini and Rogers (1986) considered it as a taxon separated from *H. serpens*, due to its dark-colored ascospores with long germ slits, and its different cultural features. In this and previous analysis (Sánchez-Ballesteros *et al*., 2000), *N. bipapillata* appeared in a basal position with respect to the other *Nemania* strains. In fact, the two sequences representing *N. bipapillata* clustered out of the rest of xylariaceous taxa in three of the four phylogenetic trees, suggesting a peripheral position for this taxon with respect to the rest of genera under study.

As previously reported (Sánchez-Ballesteros *et al*., 2000), no molecular support was found to distinguish between the rest of *Nemania* species and their respective varieties included in the analyses (*i.e. N. chestersii*, *N. aenea* and *N. serpens*). Thus, *N. aenea* var. *aureolutea* seems to be closely related with sequences representing *N. serpens* (including both the type variety and the var. *macrospora*) and *N. chestersii*, clustering apart from the two sequences that represented *N. aenea* var. *aenea*.

Dicyma and Anthostomella

These genera are closely associated to the clade of *Hypoxylon*-related genera. The name *Dicyma* designates the anamorph of *Ascotricha*, a genus with a confusing taxonomic position. *Ascotricha* has been accepted by some authors as a member of the *Xylariaceae*, mainly due to the amyloid ascus plug and the *Dicyma* anamorph, close to *Geniculosporium* and *Nodulisporium* (Udagawa *et al*., 1994; Stchigel and Guarro, 1998). However, *Dicyma funiculosa* differs from other *Dicyma* species in the conidiogenous cell morphology (Guarro and Calvo, 1983). Our data confirm the position of *Dicyma* to the *Xylariaceae*. However, the molecular analysis did not support its monophyletic origin, since the sequences from the two species included in the study (*D. funiculosa* and *D. pulvinata*) clustered with *Anthostomella sepelibilis*. Since only two species of this genus are represented in this study and, in addition, no molecular data are presently available for neither *Ascotricha chartarum* Berk. nor *Dicyma ampullifera* B. de Lesd., the type species of the genus and its anamorph, no conclusions on the phylogenetic placement of this genus can be made.

Anthostomella is another heterogeneous and poorly known genus, whose anamorphs have been referred to mostly as *Geniculosporium*, *Nodulisporium* and *Virgariella* (Martin, 1969b; Francis *et al*., 1980; Laessøe, 1994), although *Libertella*-like anamorphs has been also detected in some taxa still included under the concept of *Anthostomella* (Ju and Rogers, 1996). Rappaz (1995) adopted a more restrictive concept than other authors, including only species with ascospores bearing cellular appendages at early stages of development. Such structures have been also observed in several species of *Biscogniauxia* and *Nemania*, but many other morphological characters distinguish *Anthostomella* from those two genera. Accordingly, our molecular phylogenetic analysis did not support any relationship between *A. sepelibilis* and *Nemania* or *Biscogniauxia*, but it suggested the existence of a close relationship with *Dicyma*.

Xylaria

This is one of the largest genera of the family, and the oldest one (Martin, 1970). *Xylaria* is considered to be a complex genus that needs to be constantly under revision. Unfortunately, a world monograph of the genus is still lacking, and much of the controversial taxonomy of the group could be due to the fact that a critical comparison between the several Northern temperate *Xylaria* spp. is not available, and different species concepts are actually being used among European and non-European specialists. Traditionally, *Xylaria*

species have been defined on the basis of the presence of vertical stromata. However, this can be considered a variable feature, since many taxa exhibit horizontal stromata, in the same way as other genera in the family (Rogers, 1979). The genus has been traditionally divided in sections based on the morphological features of the stroma (Martin, 1970; Rogers, 1985). However, these characters are usually polymorphic, showing gradual differences among individuals of the same species. This makes convenient the adoption of criteria based on more stable features, such as ascospore morphology or the structure of the apical apparatus of the asci (Rogers, 1985). Likewise, this author has pointed out the taxonomic significance of anamorphs, with special reference to their position in the biological cycle of the different species, or the morphology and development of the conodiogenous apparatus. From these data, Rogers (1985) proposed to split the genus in at least four sections, updating Martin's ancient division of *Xylaria* in two sections; *Xylorugosa* and *Xyloglossa* (Martin, 1970). However, the scheme proposed by Rogers can be considered as preliminary, since a number of taxa can not be assigned to any of these sections.

Our results suggest that *Xylaria* species form a large complex of paraphyletic origin, as already suggested by other authors based on morphological and ITS sequence data (Rogers, 1985; Lee *et al*., 2000). Thus, the species of *Xylaria* were positioned in several nodes, usually associated with other genera such as *Kretzschmaria*, *Stilbohypoxylon*, *Rosellinia* or even *Nemania*. Only the NJ analysis grouped all the *Xylaria* species together in a single clade (Fig. 3). In general, the topologies of the different trees recognized one main clade of sequences that could represent taxa of *Xylaria s. str*. This "core" node would include *X. multiplex*, *X. liquidambaris*, *X. arbuscula*, *X. digitata* and *X. guareae* (a likely misidentification of a sequence labeled as *X. globosa* is discussed below). Another minor clade of *Xylaria* species included *X. longipes* and the only sequence of genus *Stilbohypoxylon* included in the study, *S. quisquiliarum*, and occasionally *X. allantoidea*. This clade appeared near the first main clade only in the MP (Fig. 1) and NJ analysis (Fig 3). Furthermore, three sequences representing *X. hypoxylon* grouped together in a clade with an unclear position with respect to the other two clades. Finally, *X. globosa* and another strain of *X. hypoxylon* were usually found apart from any of those clades.

Given that most of the taxa studied belonged to section *Xylorugosa*, it was impossible to establish the correlation between the sections usually proposed for *Xylaria* and the grouping of species observed in the phylogenetic trees. Our molecular results showed that *X. allantoidea*, included by Rogers (1985) within section *Xyloglossa*, appeared associated with the sequences representing *X. longipes* and *S. quisquiliarum* in two of the phylogenies performed (NJ and BY analyses) although with low bootstrap support. However, in the other two methods of sequence analysis this taxon appeared related to *Rosellinia* (Fig. 2) or closer to the main core clade of *Xylaria* species (Fig. 1). Thus, the systematic relationships of *X. allantoidea*, the only member of section *Xyloglossa* included in our study, with the rest of members of *Xylaria* remain unclear. Other phylogenetic studies of *Xylaria* (Lee *et al*., 2000) have not found any relationship between *X. longipes* and *X. cubensis*, another member of section *Xyloglossa* akin to *X. allantoidea*.

Although conspecific strains usually clustered together in well supported clades, there were a couple of exceptions. For instance two *X. globosa* strains did not cluster together. Surprisingly, the sequence AY909007 (*X. globosa* F-999,002) was identical to that of *X. guareae* F-999,003 (AY909009), both isolated from specimens collected in Puerto Rico. Interestingly, the strain F-999,002 was recovered from *Guarea guidonia* (L.) Sleumer, the typical host of *X. guareae* (Laessøe and Lodge, 1994). This could suggest that this is most likely a case of misidentification or mislabeling. Unfortunately, no voucher specimen of the teleomorph was available to double check the identity of this strain. The second case was that of the type species of the genus, *X. hypoxylon* ATCC 42768 (AY327477), a strain deposited by Rogers and Chacko, which clustered far apart from other three strains of *X. hypoxylon* that showed almost identical sequences. A similar strain was identified by Dennis (1958) as a variety of *X.*

hypoxylon that would be better classified as *X. fastigiata* Fr., a species that later was synonymized with *X. multiplex* by Dennis (1961). Chacko and Rogers (1981) maintained the culture as a peculiar variety within the *X. hypoxylon* complex. Our data strongly suggest that *Xylaria hypoxylon* ATCC 42768 is not conspecific with the rest of strains of *X. hypoxylon* included in our work, and it should be classified as a different species. These findings suggest the convenience of a broad taxonomic re-assessment of the strains of this genus deposited in public collections, comparing with authentic material derived from stromata containing the teleomorph.

Stilbohypoxylon

The genus *Stilbohypoxylon*, as originally described by Hennings (1902), included taxa from the ancient section *Stilbohypoxylon* (Henn.) P. Martin of genus *Kretzschmaria*. Hennings' genus *Stilbohypoxylon* has been recently resurrected by Rogers and Ju (1997), to include a few xylariaceous taxa of assorted origin (Petrini, 2004). *Stilbohypoxylon* is characterized by the diagnostic presence of one or more spinose, conical synnemata arising directly from the incipient, inmature stroma. Conidial production typically stops as stromatal perithecia develop, and old synnemata usually remain as sharp protuberances on the surface of mature stromata. In this study, we have included one ITS sequence representing *S. quisquiliarum* (= *Hypoxylon quisquiliarum*), a very common pantropical or subtropical species (Petrini, 2004), transferred to this genus by Rogers and Ju (1997). Interestingly, these same authors questioned the inclusion of *S. quisquiliarum* in *Stilbohypoxylon*, based on the absence of synnematal remnants in mature fructifications, although this could be due to the fact that such remnants probably deteriorate rapidly and could be easily overlooked. Our molecular analysis showed that *S. quisquiliarum* clustered consistently as a sister group to *X. longipes*, although no relationship has been previously reported between these two taxa. Other molecular studies (Tang *et al*., 2007) have shown *S. quisquiliarum* as a sister group of a *Xylaria–Kretschmaria* clade. Obviously, more in-depth studies including additional *Stilbohypoxylon* species are required to define the limits and evolutionary affinities of this genus.

Kretzschmaria

This genus is characterized by short branched stromata ending in clavate swellings containing completely immersed perithecia, and it has been considered closely related to *Xylaria* (Dennis, 1957; Martin, 1970; Laessøe, 1994). Dennis (1957) suggested that many typical species of *Kretzschmaria* are so intimately allied to individual species of *Xylaria* that may be just varieties or forms of these *Xylaria* species. This affinity has been supported by cytological and developmental studies, as well as by the anamorph morphology (Rogers and Ju, 1998). On the other hand, these authors also postulated a relationship between *Kretzschmaria* and *Nemania* as emended by Pouzar (1985a,b), although they considered both genera as clearly distinct, especially regarding the conidiophores development. In our analysis, *Kretzschmaria* appeared consistently as an internal branch within the clade of *Xylaria*, suggesting a close kinship between these two genera and no affinities with *Nemania*, in agreement with previous ITS data (Tang *et al*., 2007). *Ustulina deusta* is an old synonym of *K. deusta* (Ko *et al*., 1982; Rogers and Ju, 1998). Our current and previous data (Sánchez-Ballesteros *et al*., 2000) clearly support this synonymy.

Rosellinia

The members of genus *Rosellinia* are characterized by the presence of a definite stroma surrounding the perithecia. Another character traditionally used to delimitate *Rosellinia* is the presence of a subiculum, at least in the initial stages of stromatal development (Saccardo, 1883; Petrini, 1992). Nevertheless, the concept and taxonomic value of the subiculum is debatable among authors. According to Laessøe and Spooner (1994), the stromata of most *Rosellinia* species are placed directly on the substrate and surrounded by more or less compacted hyphae considered as a "false subiculum". Petrini (1992) included the presence of a more or less persistent hyphal mat (a "false subiculum") as a diagnostic character at least for two of the subgenera of the genus, *Rosellinia* and *Calomastia*. Another

delimitating feature is the presence of one or few (less than five) perithecia per stroma, and the superficial nature of the stromata, not embedded in the host plant tissues (Petrini and Müller, 1986). Miller (1928) considered this genus as close to *Hypoxylon*, section *Papillata* subsection *Primocinerea*, a relationship later supported by numerical taxonomy (Whalley, 1976). Nevertheless, other authors (Dennis, 1968; Müller and von Arx, 1973; Dargan and Thind, 1979) considered both genera as independent. For Laessøe and Spooner (1994), *Rosellinia* was closely related to *Nemania*.

According to our results, those morphological features would not be reliable diagnostic characters to delimitate *Rosellinia*. The genus appeared as polyphyletic except in NJ phylogenies (Fig. 3). *Rosellinia* species grouped in two clades well separated from each other. One of them contained *R. necatrix*, *R. arcuata* and *R. buxi*, usually intermingled with *Xylaria* spp. The second clade included *R. subiculata*, *R. australis*, *R. corticium* and *E. mammata*, and occasionally *R. pepo* (Figs 1 and 2); in a more or less basal position with respect to the rest of xylariaceous fungi (only *Nemania* occupied a more basal position in the trees). *Rosellinia bambusae* was always phylogenetically distant; whereas *R. quercina* grouped with other *Rosellinia* spp. only in NJ phylogenies (Fig. 3).

Rosellinia bambusae has been renamed by Laessøe and Spooner (1994) as *Astrocystis bambusae* (Henn.) Laessøe & Spooner. The genus *Astrocystis* (Diehl, 1925) was originally used to accommodate *Rosellinia*-like taxa lacking any type of subiculum but with erumpent stromata sometimes with a carbonaceous extension at the base, together with the presence of an *Acanthodochium* anamorph. Our results show the sequence of *R. bambusae* is distant from the rest of *Rosellinia* taxa, and more related with to *Xylaria* species. This would support the exclusion of this taxon from *Rosellinia*, which is in agreement with results from other authors (e.g Bahl *et al*., 2005).

Another taxon with a controversial systematic placement in our study is *R. quercina*. It shares close affinities to *Kretzschmaria* and *Xylaria* species, except in the NJ analysis. *Rosellinia quercina* was previously considered as a *Hypoxylon* (*H. quercinum* (R. Hartig) P.M.D. Martin, a *nom. illeg*.) and it is currently proposed to be a synonym of *R. desmazieresii* (Berk. & Broome) Sacc. (Petrini, 1992). It is an infrequently reported taxon, only known from Europe (Petrini, 1992; Francis, 1985), occurring on both coniferous and dycotiledons. This species was proposed by Petrini (1992) to typify the subgenus *Corru-gata*, one of the three subgenera of *Rosellinia*. Having only one sequence from this section in our study makes it impossible to elucidate with confidence the relationships of this group of taxa with the rest of *Rosellinia* species studied, most of them belonging to subgenus *Rosellinia*.

Rosellinia subiculata was previously considered as a synonym of *Hypoxylon chrysoconium* Berk. & Broome (Martin, 1968). Other authors prefered to retain these taxa as different, despite the affinities between their anamorphs (Miller, 1961; Rogers, 1985). The species was retained in *Rosellinia* by Petrini (1992), but the presence of a non-persistent subiculum prompted her to include it in the heterogeneous section *Calomastia*. Later, Laessøe and Spooner (1994) suggested that those features could justify its exclusion from *Rosellinia*, and a new genus might be necessary to accommodate this taxon. It has also been suggested (Laessøe and Spooner, 1994; Ju and Rogers, 1996) that this species could have affinities with *Nemania*, based on its *Geniculosporium*-like anamorph (Rogers, 1985; Petrini, 1992). Our study did not support a systematic placement of this taxon apart from the rest of *Rosellinia* species, as it consistently clustered with *R. australis* and *R. corticium*, and with no particular relationship with *Nemania*.

Rosellinia necatrix, well known as an important plant pathogen, has an unclear status, due to the lack of a type specimen and the difficulties of finding the teleomorphic state in nature (Petrini, 1992). *Rosellinia necatrix* and *R. buxi* grouped together in all our phylogenetic trees, confirming the relationship observed by Petrini (1992) and Bahl *et al*. (2005) based on morphology. Both taxa share some morphological characters such as relatively long ascospores, with germ slits shorter than the spore length, ascus apical structure with an angular rim in outline, and

the presence a *Dematophora* anamorph (Petrini and Petrini, 2005). *Rosellinia necatrix* is also closely related to *R. arcuata*, as previously shown by Bahl *et al*. (2005), and this is in agreement with the morphological analysis from Petrini and Petrini (2005).

Entoleuca

Our results also confirmed a close relationship between *Entoleuca mammata*, previously included in *Hypoxylon* [as *H. mammatum* (Wahlenb.) P. Karst.] and members of *Rosellinia*, in agreement with Bahl *et al*. (2005). This could suggest the convenience of considering *E. mammata* as a member of *Rosellinia*, and as a consequence, the diagnostic uniperitheciate character of *Rosellinia* should be revised to accommodate *E. mammata*, which is multiperitheciate (Bahl *et al*. 2005). However, considering the polyphyletic origin of *Rosellinia*, broadening the generic concept to include *E. mammata* may not be recommendable, since it would just add more confusion to an already sufficiently confusing genus.

In summary, this work represents a contribution to the knowledge of some aspects of the taxonomy and phylogenetic relationships among several genera of the family *Xylariaceae*, from a molecular point of view. Our results are consistent with the monophyletic character of the family, providing support to some taxonomic questions, such as the affinities of the genera close to *Xylaria* or to *Hypoxylon*. Nevertheless, delimitation of some of those genera requires further studies. One example is the systematic placement of genera *Rosellinia* and *Xylaria*, whose polyphyletic nature is quite clear, and that would require a more in-depth revision. Some questions regarding particular species and strains are also pending of further studies. In addition to its contribution to the study of the taxonomy of *Xylariaceae*, this work can be useful to design of probes in the identification of new isolates from environmental samples, or as a database for comparing sequences of unidentified fungi.

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