A re-evaluation of the evolutionary relationships within the *Xylariaceae* based on ribosomal and protein-coding gene sequences

Tang, A.M.C.[1*], Jeewon, R.[2], and Hyde, K.D.[3,4]

[1]Kwong Living Trust Food Safety & Analysis Laboratory, Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, PR China
[2]Division of Microbiology, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, PR China
[3]International Fungal Research & Development Centre, The Research Institute of Resource Insects, Chinese Academy of Forestry, Kunming 650224, PR China
[4]School of Science, Mae Fah Luang University, Chiang Rai, Thailand

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Previous phylogenetic analyses of the *Xylariaceae* have mostly utilized ITS-5.8S gene sequences and there is no multi-gene study that have addressed evolutionary relationships. We conducted phylogenetic analyses with separate and combined sequences of ITS-5.8S rDNA, LSU rDNA, RPB2 and β-tubulin genes to re-evaluate the generic relationships within the *Xylariaceae*. All phylogenies inferred from individual and combined datasets suggest that *Xylariaceae* has two main lineages, namely Hypoxyloideae and Xylarioideae. This result is generally concordant with taxon chemotaxonomy and anamorph characters. The phylogenetic position and relationship of *Biscogniauxia* and *Camillea* with other xylariaceous genera are still obscure as they nested together and form a separate lineage. The phylogenetic significance of morphological affinities within Hypoxyloideae and Xylarioideae are discussed and phylogenetic positions of selected genera are re-evaluated.

Key words: ascomycetes, molecular systematics, ITS rDNA, LSU rDNA, RPB2, β-tubulin

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*Corresponding author: Tang, A.M.C.; e-mail: alvtangmc@yahoo.com.hk

Introduction

Xylariaceae is the type and largest family of the *Xylariales* with at least 75 genera and a total of 800 or more species (Kirk *et al*., 2001; Eriksson, 2007). The family is characterized by perithecial ascomata embedded in more or less well-developed dark-coloured stromata; cylindrical asci with an amyloid apical ring; ascospores which are mainly pigmented with germ slits or pores; and an anamorph producing conidia holoblastically from a sympodially, or occasionally percurrently, proliferating conidiogenous region (Whalley, 1996; Rogers, 2000; Thienhirun and Whalley, 2004). *Xyliaceous* species are typically saprobes, but are also commonly isolated as endophytes and some species are pathogens (Pinruan *et al*., 2007; Sánchez Márquez *et al*., 2007, 2008; Kodsueb *et al*., 2008a,b; Oses *et al*, 2008).

Xylariaceae has been studied extensively for bioactive properties (Daferner *et al*., 1999; Singh *et al*., 1999; Isaka *et al*., 2000; Quang *et al*., 2006), chemistry (Stadler *et al*., 2005; Hellwig *et al*., 2005; Stadler and Hellwig, 2005; Stadler *et al*., 2006; Bitzer *et al*., 2008), ecology (Lee, 1997), and taxonomy (Ju and Rogers, 1996, 2002; Petrini, 1992; Ju *et al*., 1997, 1998; Laessøe and Spooner, 1994; Rogers and Ju, 1997; Lu and Hyde, 2000). Sixteen genera of the *Xylariaceae* have been monographed or revised, including *Anthostomella*, *Ascovirgaria*, *Biscogniauxia*, *Creosphaeria*, *Daldinia*, *Discoxylaria*, *Entoleuca*, *Hypoxylon*, *Jumillera*, *Kretzschmaria*, *Kretzschmariella*, *Nemania*, *Rosellinia*, *Stilbohypoxylon*, *Vivantia* and *Whalleya* (Petrini, 1992; Laessøe and Spooner, 1994; Ju and Rogers,

1996, 2002; Rogers and Ju, 1996; Ju *et al*., 1997, 1998; Lu and Hyde, 2000).

The current classification of this family is mainly based on a combination of characters such as anamorphic type, form of stroma, extractable stromatal pigments, and other teleomorphic characters (Rogers, 1979; Laessøe and Spooner, 1994). It is generally accepted that *Xylariaceae* can be divided into two major groups, Hypoxyloideae and Xylarioideae, based on their respective anamorphic types (*Nodulisporium*-type or *Geniculosporium*-type), their stromatal pigments (KOH +ve or –ve), and other secondary metabolites (Stadler *et al*., 2005; Stadler and Hellwig, 2005).

Molecular techniques are commonly used to overcome taxonomic problems posed by the limitation of morphological characters or in cases where morphological characters are in conflict, ambiguous or missing (Bruns *et al*., 1991; Hibbett, 1992, Hillis, 1987). Nuclear ribosomal DNA and ITS-5.8S are commonly used gene regions for inferring phylogenetic relationships. There are only a few published studies regarding inter-generic relationships within *Xylariaceae* and most of these utilized ITS-5.8S gene sequences. Lee *et al*. (2000) investigated the phylogenetic relationships of *Xylaria* and found that stromatal structure, ascal apex, ectostromal surface, perithecial structure, stipe differentiation and germ slit of ascospores were phylogenetically significant characters in grouping *Xylaria* species. Sánchez-Ballesteros *et al*. (2000) investigated the phylogenetic relationships of *Hypoxylon* and six other genera formerly classified in Hypoxylon sensu Miller (1961). Their results supported the recent segregation of some allied genera (*Biscogniauxia*, *Camillea*, *Whalleya*, *Creosphaeria*, *Nemania* and *Kretzschmaria*) from *Hypoxylon* sensu Miller (Miller, 1961; Pouzar, 1985a,b; Ju *et al*., 1998; Ju and Rogers, 1996). Sánchez-Ballesteros *et al*. (2000) however, did not include many other taxonomically important genera within *Xylariaceae* such as *Anthostomella*, *Astrocystis*, *Daldinia* and *Xylaria*. Other molecular studies with sparse taxon sampling only provided limited information on the phylogeny among xylariaceaous genera (Granmo *et al*., 1999; Johannesson *et al*., 2000; Mazzaglia *et al*., 2001). Recently, the phylogeny of several xylariaceous genera was evaluated using protein-coding genes, such as β-tubulin and α-actin genes (Hsieh *et al*., 2005). These gene regions were found to be particular useful since limited success has been achieved in delineating genera and resolving generic relationships based on ribosomal DNA genes (Sánchez-Ballesteros *et al*., 2000, Smith *et al*., 2003, Triebel *et al*., 2005; Peláez *et al*., 2008).

In this study, DNA sequences from ITS-5.8S rDNA, LSU rDNA, RPB2 and β-tubulin genes were analyzed separately and in combination to re-evaluate the evolutionary relationships of the Xylariaceae. Phylogenetic significance of morphological characters is also assessed and morphological affinities within two generic groups Hypoxyloideae and Xylarioideae are explored.

Materials and methods

Collection, observation, cultures

Wood samples were collected from forests in Guizhou (China), Hong Kong (China) and Chiang Mai (Thailand). Microscopic observations were made in water. Melzer's reagent was used to test the amyloidity of apical rings; aqueous Congo Red, Blue Black Waterman ink or Phloxine for measurements of ascal stipes, and 10% KOH for testing the dehiscence of perispores. Measurements were taken at ×1000 magnification on samples of 30 ascospores, at ×400 on 20 asci and 10 perithecia. Single spore cultures were obtained from a single spore isolation method (Choi *et al*., 1999). Cultures were grown on malt extract agar (MEA). The external stromatal colors were recorded and coded after Rayner (1970). Fungal cultures and GenBank sequences used in this study are listed in Table 1. Taxa that were collected and identified by Jacques Fournier and Alvin M. C. Tang were coded with JF and AT respectively.

DNA extraction and PCR

Genomic DNA was extracted from mycelia grown on malt extract agar (MEA) culture following the protocol as outlined by Jeewon *et al*. (2002, 2004) and Cai *et al*. (2005), otherwise extracted directly from ascomata using a DNA extraction kit

Table 1. DNA sequences used in the phylogenetic analyses, their specimen voucher and GenBank accession numbers.

Taxon names	Specimen code	GenBank Accession No. ITS	LSU	RPB2	β-tubulin
Order Boliniales					
Boliniaceae					
Camarops amorpha	SMH 1450	–	AY780054	AY780156	–
Camarops petersii	JM 1655	–	AY346265	–	–
Camarops microspora	CBS 649.92	–	AY083821	–	–
Camarops tubulina	SMH 4614(a)	–	AY346266	AY780157	–
Order Coniochaetales					
Coniochaetaceae					
Coniochaeta discoidea	SANK12878	–	–	AY780191	–
Coniochaetidium savoryi	TRTC 51980	–	–	AY780174	–
Coniochaeta ligniaria	CBS 178.75	–	AF353582	DQ631958	–
Coniochaeta velutina	IFO 9439	–	AF353594	DQ631959	–
Order Diaporthales					
Diaporthaceae					
Diaporthe phaseolorum	NRRL 13736	AY705851	U47830	AY641036	–
Gnomoniaceae					
Cryptodiaporthe corni	CBS 245.90	–	AF408343	AF277149	–
Discula destructiva	ATCC76230	–	AF362568	AF277147	–
Gnomonia ribicola	CBS 115443	–	DQ368626	DQ368642	–
Plagiostoma euphorbiae	CBS 340.78	–	AF277131	DQ368643	–
Valsaceae					
Amphiporthe castanea	CBS 392.93	–	AF277128	DQ368644	–
Apioplagiostoma aceriferum	CBS 781.79		AF277129	–	–
Diaporthales *Insertae sedis*					
Cryphonectria parasitica	SA713/ CP 155	–	AF277132	AY485619	–
Order Halosphaeriales:					
Halosphaeriaceae					
Aniptodera chesapeakensis	ATCC 32818		U46882	–	–
Corollospora maritima	CBS 264.59	–	AF491260	DQ368632	–
Halosphaeria appendiculata	CBS 197.60	–	U46885	–	–
Lignincola laevis	JK 5180A	–	U46890	–	–
Nais inornata	ATCC 200453	–	AF539476	–	–
Neptunella longirostris	HK AT-2061	–	AF539473	DQ368633	–
Order Hypocreales:					
Bionectriaceae					
Bionectria pityrodes	BCS246.78/ GJS 95-26	AF210673	AY489728	–	–
Myrothecium inundatum	CBS582.93/ IMI 158855	AY254152	AY489731	–	–
Valsonectria pulchella	SMH 1193	–	AY346304	–	–
Ceratostomataceae					
Melanospora zamiae	CBS 421.87/ ATCC 12340	–	U17405	DQ368634	–
Melanospora zamiae	CBS 421.87/ ATCC 12340	–	U17405	DQ368634	–
Clavicipitaceae					
Beauveria caledonica	CCRC32867/ Arsef 2567	AY245625	AF339520	–	–
Cordyceps militaris	CBS178.59	AF163020	AF327374	AY545732	–
Hypocreaceae					
Hypocrea pallida	GJS 89-83	–	U00740	AY015636	–
Hypocrea schweinitzii	CBS 243.63/ ICMP5421	–	U47833	DQ368635	–

Table 1 (continued). DNA sequences used in the phylogenetic analyses, their specimen voucher and GenBank accession numbers.

Taxon names	Specimen code	GenBank Accession No. ITS	LSU	RPB2	β-tubulin
Hypomyces polyporinus	CBS 168.89/ ATCC 76479	–	AF543793	DQ368636	–
Hydropisphaera erubescens	ATCC 36093	–	–	AY545731	–
Nectriaceae					
Cosmospora coccinea	AR 2741	–	AY489734	–	–
Gibberella moniliformis	ATCC 38932	–	–	AY533830	–
Order Lulworthiales					
Lulworthiaceae					
Lulworthia uniseptata	CBS16760	AF169305	AY878991	–	–
Order Microascales					
Chadefaudiellaceae					
Faurelina elongata	CBS 126.78	–	DQ368625	DQ368639	–
Microascaceae					
Microascus trigonosporus	RSA1942/IFO3222	–	U47835	AF107792	
Petriella setifera	CBS 110344	–	U48421	DQ368640	–
Microascales inc. sed.					
Ceratocystis fimbriata	CBS 374.83	–	AF221009	DQ368641	–
Order Ophiostomatales					
Ophiostomataceae					
Ophiostoma piliferum	Unknown	–	AY281094	–	–
Ophiostoma ulmi	CBS 298.87	–	DQ368627	–	–
Order Sordariales					
Cephalothecaceae					
Cepthalotheca sulfurea	CBS 135.34	–	AF431950	–	–
Cryptendoxyla hypophloia	FR 58	–	AF222499	–	–
Chaetomiaceae					
Chaetomium elatum	ATCC 42780	–	–	AF107791	–
Zopfiella ebriosa	CBS 111.75	–	–	AY780200	–
Chaetosphaeriaceae					
Melanochaeta hemipsila	SMH 2125	–	–	AY780184	–
Chaetosphaeria ovoidea	SMH 2605	–	–	AY780173	–
Lasiosphaeriaceae					
Bombardia bombarda	SMH 4821	–	AY80053	AY780154	–
Cercophora caudata	CBS 606.72	AY999135	AY999113	DQ368646	–
Lasiosphaeria ovina	SMH 1538/ SMH 3286	AY587931	AF064643	AY600292	–
Podospora fimbriata	CBS 144.54	–	–	AY780189	–
Schizothecium curvisporum	ATCC 36709	–	–	AY780192	–
Sordariaceae					
Gelasinospora tetrasperma	ATCC 96230	–	–	AY780177	–
Neurospora crassa	ICMP 6360/ FGSC 2489	AY681193	AY681158	AF107789	–
Sordaria fimicola	CBS 723.96/ HKUCC 3714	AY681188	AF132330	DQ368647	DQ840087
Sordaria macrospora	Buck s.n.	–	–	AY780195	–
Order Xylariales					
Amphisphaeriaceae					
Bartalinia robillardoides	BRIP 14180	AF405301	AF382366	DQ368653	–
Discostroma botan	HHUF 4642	–	DQ368629	DQ368648	–
Monochaetia monochaeta	CBS115004/AF382370	AY853243	AF382370	–	–
Pestalotiopsis maculans	CBS322.76	AF405296	AF382354		
Pestalotiopsis versicolor	BRIP 14534	AF409993	AF382357	DQ368654	–
Seiridium cardinale	ICMP 7323	AF409995	AF382377	DQ368655	–
Seiridium eucalypti	CBS 343.97	–	DQ414533	DQ368656	–
Diatrypaceae					

Table 1 (continued). DNA sequences used in the phylogenetic analyses, their specimen voucher and GenBank accession numbers.

Taxon names	Specimen code	GenBank Accession No. ITS	LSU	RPB2	β-tubulin
Cryptosphaeria eunomia	CBS 216.97	AJ787693	AY083826	–	–
Diatrype disciformis	F-091,971/ CBS 197.49	AJ390410	U47829	–	–
Diatrype spilomea	D17C	AJ302433	–	–	–
Eutypa lejoplaca	CBS 248.87	DQ006922	–	–	–
Eutypa leptoplaca	CBS 287.87	DQ006924	–	–	–
Eutypa maura	CBS 219.87	DQ006926	–	–	–
Eutypa tetragona	CBS 284.87	DQ006923	–	–	–
Eutypa sp.	HKUCC 337	–	AY083825	–	–
Eutypa sp.	SMH 3580	–	AY346280	–	–
Apiosporaceae					
Apiospora tintinnabula	ICMP 6889	–	DQ810217	DQ368649	–
Apiospora setosa	ICMP 4207	–	DQ368631	DQ368650	–
Xylariaceae					
Annulohypoxylon annulatum	GB 5659	AJ390395	–	–	–
Annulohypoxylon atroroseum	ATCC 76081	AJ390397	DQ840060	–	–
Annulohypoxylon bovei	YMJ 90081914	–	–	–	AY951654
Annulohypoxylon cohaerens	YMJ 310	–	–	–	AY951655
Annulohypoxylon ilanense	YMJ 37	–	–	–	AY951657
Annulohypoxylon minutellum	YMJ 316	–	–	–	AY951658
Annulohypoxylon moriforme	YMJ 90080807	–	DQ840057	–	AY951660
Annulohypoxylon moriforme var. microdiscum	JF TH-28-01	DQ631935	DQ840061	DQ631960	DQ840095
Annulohypoxylon moriforme var. microdiscum	JF TH-28-01	DQ631935	DQ840061	DQ631960	DQ840095
Annulohypoxylon moriforme var. *microdiscum*	AT-GZ-M77	DQ983229	DQ840059	DQ983230	DQ840093
Annulohypoxylon multiforme	YMJ 317	–		–	AY951661
Annulohypoxylon nitens	ST2313/ YMJ 91022108	DQ223751	DQ840063	–	AY951663
Annulohypoxylon stygium	CM AT-010	AJ390409	DQ840064	DQ631962	–
Astrocystis cocoes	Unknown	AY862571	–	–	–
Biscogniauxia anceps	YMJ 123	–	–	–	AY951671
Biscogniauxia arima	YMJ 122	–	–	–	AY951672
Biscogniauxia atropunctata	ATCC 13359A/ YMJ 108	AJ390411		–	AY951673
Biscogniauxia capnodes	CM AT-015/ YMJ 142	DQ631933	DQ840055	–	AY951674
Biscogniauxia cylindrispora	YMJ 89092701	–	–	–	AY951679
Biscogniauxia formosana	YMJ 89032201	–	–	–	AY951680
Biscogniauxia granmo	YMJ 135	–	–	–	AY951681
Biscogniauxia mediterranea	CBS 280.61	AJ390413	–	–	AY951684
Biscogniauxia simplicior	B73C/YMJ 136	AJ390416	–	–	AY951686
Biscogniauxia uniapiculata	YMJ 90080608	–	–	–	AY951687
Biscogniauxia sp.	JF 06-05	DQ631932	DQ840054	–	–
Camillea tinctor	C83C	AJ390421			
Camillea tinctor	C84M	AJ390422			
Creosphaeria sassafras	CM AT-018	DQ631934	DQ840056	DQ631964	–
Creosphaeria sassafras	Cr90M	AJ390424	–	–	–
Creosphaeria sassafras	Cr91M	AJ390425	–	–	–
Daldinia bambusicola	YMJ 107	–	–	–	AY951688
Daldinia caldariorum	YMJ 104	–	–	–	AY951689
Daldinia childiae	YMJ 91010202	–	–	–	AY951692
Daldinia concentrica	M-0066225	AY616681	–	DQ368651	–
Daldinia concentrica	CBS 139.73	AF163021	–	–	–

Table 1 (continued). DNA sequences used in the phylogenetic analyses, their specimen voucher and GenBank accession numbers.

Taxon names	Specimen code	GenBank Accession No. ITS	LSU	RPB2	β-tubulin
Daldinia concentrica	ATCC 36659	–	U47828	–	–
Daldinia eschscholzii	YMJ 101	–	–	–	AY951695
Daldinia fissa	Lind & Andreasen s.n.	AF176976	–	–	AY951697
Daldinia fissa	CBS 157.32	AF163022	–	–	–
Daldinia loculata	HJ108	AF176959	–	–	AY951698
Daldinia loculatiodes	BJ Coppins 8630	AF176982	–	–	–
Daldinia petriniae	H Knudsen s.n.	AF176970	–	–	AY951699
Entoleuca mammata	E.MAMM3	AJ246235	–	–	–
Entoleuca mammata	ATCC 58108	AF201713	–	–	–
Entoleuca mammata	E.MAMM1	AJ246232	–	–	–
Euepixylon udum	FR-AT-115	–	DQ840065	–	–
Hypoxylon fendleri	F-108, 405	AJ390400	–	–	–
Hypoxylon fragiforme	agrD459/ HKUCC 1022	AY616690	AY083829	–	–
Hypoxylon fuscum	YMJ 77	–	–	–	AY951722
Hypoxylon hypomiltum	YMJ 88113016	–	–	–	AY951729
Hypoxylon macrosporum	YMJ 47	–	–	–	AY951736
Hypoxylon monticulosum (TH)	CM AT-04	DQ631938	DQ840066	–	–
Hypoxylon monticulosum (HK)	HK AT-PTC015	DQ631939	–	DQ631950	DQ840096
Hypoxylon monticulosum (GZ)	GZ AT-M050	DQ631936	DQ840067	DQ631955	–
Hypoxylon munkii	YMJ 90080403	–	–	–	AY951738
Hypoxylon notatum	YMJ250	–	–	–	AY951739
Hypoxylon ochraceum	H17R	AJ390406	–	–	–
Hypoxylon perforatum	H18R	AJ390407	–	–	–
Hypoxylon pilgerianum	YMJ 92042505	–	–	–	AY951744
Hypoxylon shearii	YMJ 29	–	–	–	AY951753
Hypoxylon submonticulosum	YMJ 351	–	–	–	AY951756
Hypoxylon ulmophilum	YMJ 350	–	–	–	AY951760
Kretzschmaria clavus	JP 3113	AJ390434	–	–	–
Kretzschmaria deusta	CBS 826.72	AJ390435	–	–	–
Kretzschmaria deusta	K171C	AJ390437	–	–	–
Kretzschmaria deusta	JF 05154	–	DQ840077	–	–
Muscodor albus	Unknown	AF324336	–	–	–
Muscodor vitigenus	Unknown	AY100022	–	–	–
Nemania aenea	ATCC 68818	AJ390426	–	–	–
Nemania aenea	JF 02118	–	DQ840070	DQ631951	DQ840085
Nemania aenea	CBS 680.86	AJ390427	–	–	–
Nemania aenea var. *aureolutea*	ATCC 60819	AJ390428	–	–	–
Nemania aenea var. *macrospora*	ATCC 60822	AJ390433	–	–	–
Nemania bipapillata	JP 3034	AJ390429	–	–	–
Nemania chestersii	ATCC 38988	AJ390430	–	–	–
Nemania chestersii	JF 04024	–	DQ840072	DQ631949	DQ840089
Nemania diffusa (FR)	FR AT-113	DQ658238	DQ840073	DQ631947	DQ840088
Nemania diffusa (GZ)	GZ AT-F006	FJ438909	DQ840076	DQ631957	–
Nemania maritima	JF04055	DQ631941	DQ840074	DQ631946	–
Nemania plumbea	JF TH-04-01	DQ641634	DQ840071	DQ631952	–
Nemania serpens	ATCC 16078	AJ390431	–	–	–
Nemania serpens	FR AT-114	DQ631942	DQ840075	DQ631948	DQ840086
Rosellinia bambusae	Unknown	AY862573	–	–	–
Rosellinia capetribulensis	HKUM 17499	AY862570	–	–	–
Rosellinia corticium	GZ-AT-F004	DQ631940	DQ840078	–	DQ840091
Rosellinia mirabilis	Unknown	AY862572		–	–
Stilbohypoxylon quisquiliarum	CM AT-016	DQ631937	DQ840079	–	–
Xylaria acuta	ATCC 56487	–	AY544676	–	–
Xylaria bambusicola	YMJ 205	–	–	–	AY951762
Xylaria berteri	YMJ 90112623	–	–	–	AY951763

Table 1 (continued). DNA sequences used in the phylogenetic analyses, their specimen voucher and GenBank accession numbers.

Taxon names	Specimen code	GenBank Accession No. ITS	LSU	RPB2	β-tubulin
Xylaria curta	Unknown	–	U47840	–	–
Xylaria sp.	XT09003	DQ631945	DQ840080	DQ631953	–
Xylaria grammica	XT09009	DQ631944	DQ840081	DQ631956	DQ840090
Xylaria cornu damae	CBS 724.69	AF163031	–	–	–
Xylaria hypoxylon	CBS 499.80	AJ309350	U47841	DQ368652	–
Xylaria mali	CBS 385.35	AF163040	–	–	–
Xylariaceae sp.	JF TH-06-04	DQ631943	DQ840069	DQ631954	DQ840097
Whalleya microplaca	W81M	AJ390420	–	–	–
Whalleya microplaca	W80M	AJ390419	–	–	–
Sordariomycetes *insertae sedis*					
Thyridiaceae					
Sinosphaeria bambusicola	SMH1999	–	–	AY780193	–
Order Pleosporales					
Pleosporaceae					
Pleospora herbarum	EGS04-188C	–	–	AF107804	–

(E.Z.N.A.® Forensic DNA kit, D3591-01,Omega Bio-Tek). Target regions of the ITS-5.8S rDNA, LSU rDNA, RPB2 and β-tubulin regions were amplified using fungal specific primers. ITS4 and ITS5 were used to amplify ITS-5.8S rDNA gene, LROR and LR5 for partial LSU rDNA gene (Vilgalys and Hester, 1990), fRPB2-5F and fRPB2-7cR for partial RPB2 gene (Liu *et al*., 1999), T1, T2, T12, T22 for partial β-tubulin gene (Glass and Donaldson, 1995). PCR products were purified with DNA and Gel Band Purification Kit (Amersham Biosciences, Catalog no. 27-9602-01). Sequencing reactions were carried out using the same primers as mentioned above in an Applied Biosystem 3730 DNA Analyzer at the Genome Research Centre (The University of Hong Kong).

Phylogenetic Analysis

DNA sequences were aligned using BioEdit (Hall, 1999) and ClustalX 1.83 (Thompson *et al*., 1997). Manual gap adjustments were made to improve the alignment. Phylogenetic analyses were conducted using PAUP* version 4.0b10 (Swofford, 2002) and MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). Weighted parsimony analyses were performed using a symmetric step matrix generated with the program STMatrix version 2.2 (Francois Lutzoni and Stefan Zoller, Department of Biology, Duke University), by which the relative frequencies of nucleotide substitutions were calculated and converted into costs of changes. Un-weighted maximum parsimony trees were found using 1000 heuristic search and including parsimony-informative characters in stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Max-trees were set to 5,000 branches of zero length were collapsed and all parsimonious trees were saved. Branch support for all parsimony analyses was estimated by performing 1,000 bootstrap replicates (Felsenstein, 1985).

Maximum likelihood best-fit models were estimated using MrModelTest 2.2 (Posada and Crandall, 1998, Nylander, 2004). Independent substitution models were obtained from the test for Bayesian analyses in MrBayes 3.0b4. The Bayesian analyses were conducted with the Markov chains run for 1,000,000 generations and trees were sampled every 100th generation resulting in 10,000 trees. The first 1000 trees, which represented the burn-in phase of the analysis, were discarded, and the remaining 9000 trees were used for calculating posterior probabilities in the consensus tree. Descriptive tree statistics tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for all trees generated under different optimality criteria. Trees were figured in Treeview (Page, 1996). Confident branch support is defined as bootstrap values ≥70% and Bayesian posterior probabilities ≥0.95 (Alfaro *et al*., 2003).

Table 2. Comparison of datasets in weighted parsimony analyses and trees generated from un eighted and weighted parsimony.

	ITS	**LSU**	**RPB2**	**β-tubulin**	**ITS+LSU**	**ITS+RPB2**	**ITS+β-tub**	**LSU+RPB2**	**ITS+LSU+RPB2**
Total sites	613	945	970	2016	1668	1573	2696	1844	2604
Taxa used	51	56	61	45	34	20	26	52	22
No. of excluded sites	282	111	136	914	542	303	1269	239	630
No. of included sites	331	834	834	1102	1126	1270	1427	1605	1974
No. of constant sites	160	463	291	459	573	592	649	766	1204
No. of uninformative sites	30	87	31	234	207	117	274	123	108
No. of informative sites	143	284	512	409	346	561	504	716	662
% Informative sites	43.2%	34.1%	61.4%	37.1%	30.7%	44.2%	35.3%	43.0%	33.5%
Length of WP trees	871	2809	10914	2528	3622	5377	5399	11384	5811
Consistency index	0.436	0.437	0.202	0.373	0.531	0.477	0.520	0.269	0.455
Retention index	0.746	0.748	0.544	0.563	0.648	0.549	0.553	0.567	0.536
Rescaled consistency index	0.325	0.327	0.110	0.210	0.344	0.262	0.287	0.152	0.244
Homoplasy index	0.564	0.563	0.798	0.627	0.469	0.523	0.480	0.731	0.545
No. of UP tree*	2	3	3	4	12	2	1	2	1
No. of WP tree*	1	1	1	1	1	1	1	1	1

* No. of trees generated from UP (unweighted parsimony analyses) or WP (weighted parsimony analyses)

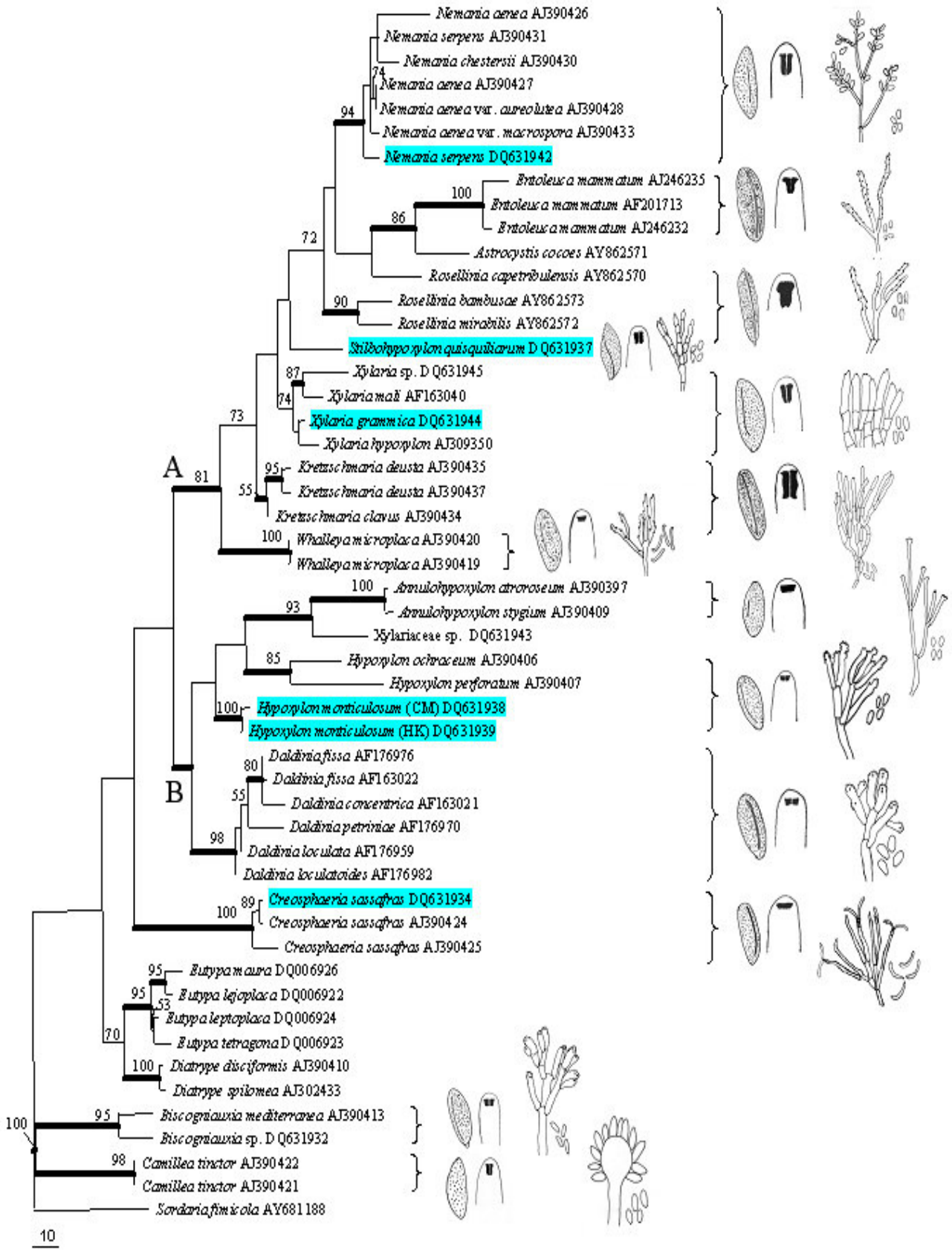

Fig. 1. The tree generated from weighted parsimony analysis based on ITS-5.8S rDNA gene dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions. (Zhang *et al*., 2008)

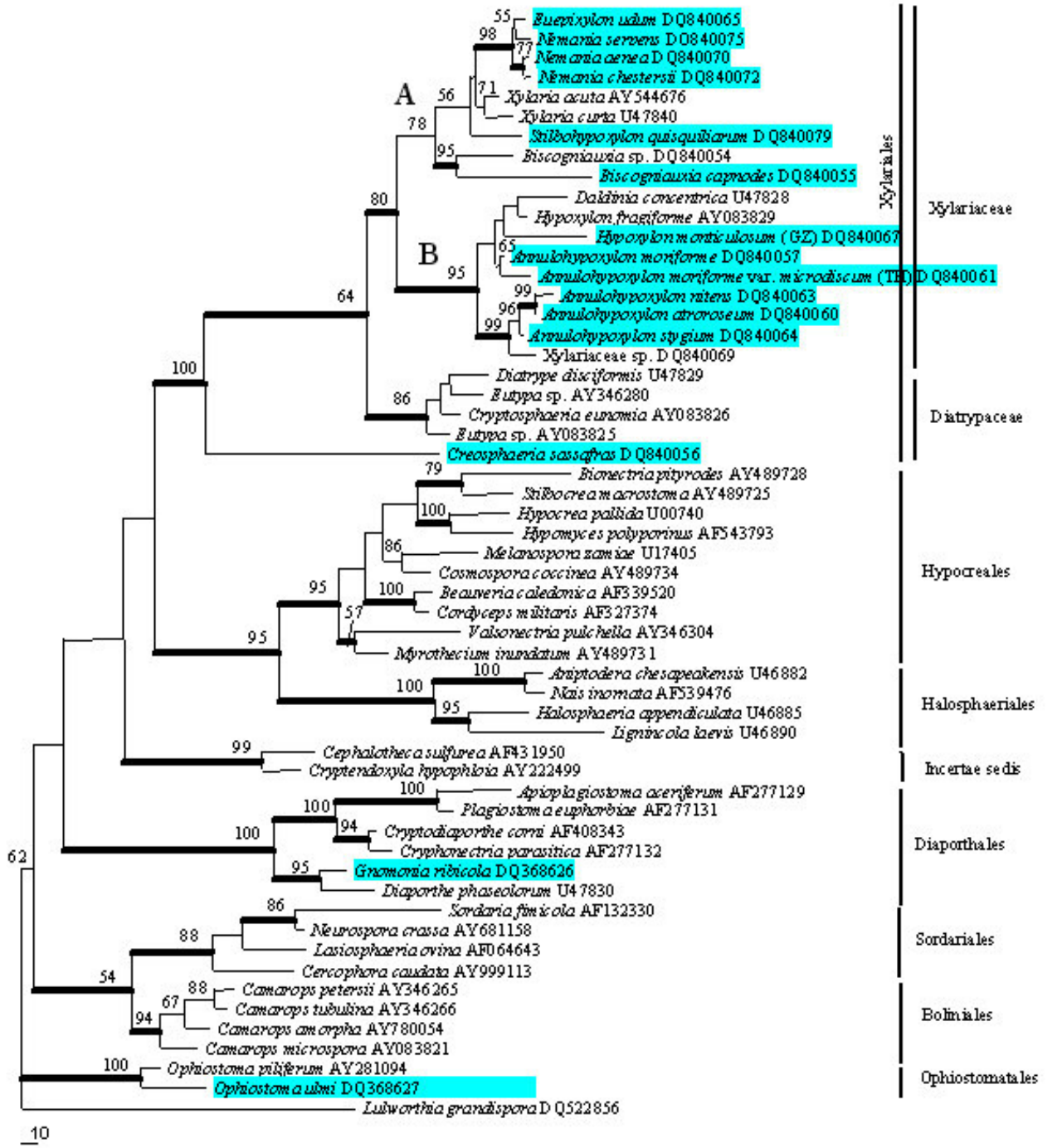

Fig. 2. The tree generated from weighted parsimony analysis based on LSU rDNA gene dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions. (Zhang *et al*., 2008)

Results

ITS gene based phylogenies

The ITS-5.8S rDNA gene dataset contained 51 taxa with 613 characters. Two-hundred and eighty-two base pairs of ambiguously aligned regions were excluded from parsimony, weighted parsimony and Bayesian analyses. Of the remaining 331 unambiguously aligned characters, 160 were constant, 28 were parsimony uninformative and 143 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=2.25, A↔G=2.21, A↔T=2.26, C↔T=2.14 C↔G=2.30, G↔T=2.31, A↔gap=2.39, C↔gap=2.37, G↔gap=2.44,

T↔gap=2.39) in weighted parsimony analyses, the number of parsimony uninformative and informative characters were 30 and 143 respectively. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated twelve equally parsimonious trees, while one tree was obtained from weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModeltest was SYM+I+G. Fig. 1 shows the tree generated from weighted parsimony (TL=871, CI=0.436, RI=0.746, RC=0.325, HI=0.564).

Genera of *Xylariaceae* cluster into two main clades (Fig. 1). Clade A consists of *Astrocystis*, *Entoleuca*, *Kretzschmaria*, *Nemania*, *Rosellinia*, *Stilbohypoxylon*, *Whalleya* and *Xylaria* with high branch support (MP=81%, BP≥95%); while Clade B consists of *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and *Xylariaceae* sp. with (MP<50%, BP≥95%). *Creosphaeria* appears to be phylogenetically distinct from the two main clades and formed a separate lineage with excellent bootstrap and Bayesian support (MP=100%, BP≥95%). *Biscogniauxia* and *Camillea* formed a clade basal to all other genera of *Xylariaceae* and *Diatrypaceae*. Genera producing similar anamorphs and types of amyloid apical apparatus nested together in the same clade. Most genera from Clade A produce *Geniculosporium*-like anamorphs (except *Whalleya*) and have an urn-shaped or inverted hat-shaped apical apparatus, while all genera from Clade B produce *Nodulisporum*-like anamorphs and have a discoid or capital-shaped apical apparatus.

LSU gene based phylogenies

The LSU gene dataset contained 56 taxa with 945 characters. One-hundred and eleven base pairs were excluded in all analyses. Of the remaining 834 unambiguously aligned characters, 463 were constant, 87 were parsimony uninformative and 284 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=2.25, A↔G=2.03, A↔T=2.19, C↔T=1.70, C↔G=2.07, G↔T=2.14, A↔gap=3.22, C↔gap=2.68, G↔gap=2.86, T↔gap=2.83) in weighted parsimony analyses, the number of parsimony uninformative and informative characters were 85 and 286, respectively. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated three equally parsimonious trees, while one tree was obtained from weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModelTest was GTR+I+G. Fig. 2 shows the tree generated from the weighted parsimony analyses (TL=2809, CI=0.437, RI=0.748, RC=0.327, HI=0.563).

Genera within *Xylariaceae*, except for *Creosphaeria*, constitute a well supported (both bootstrap and Bayesian) clade and appear to be phylogenetically distinct from *Diatrypaceae* (Fig. 2). Within the *Xylariaceae*, genera are clustered into two clades. Clade A comprises *Biscogniauxia*, *Euepixylon*, *Nemania*, *Stilbohypoxylon* and *Xylaria*; while Clade B comprises *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and *Xylariaceae* sp. Clade B received strong bootstrap and Bayesian, while Clade A only has confident bootstrap support (>70%) but not Bayesian.

RPB2 gene based phylogenies

The RPB2 gene dataset contained 61 taxa with 970 characters. One hundred and thirty-six base pairs were excluded from all analyses. Of the remaining 834 unambiguously aligned characters, 291 were constant, 31 were parsimony uninformative and 512 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=1.89, A↔G=1.82, A↔T=2.03, C↔T=1.92, C↔G=1.97, G↔T=2.10, A↔gap=3.20, C↔gap=3.25, G↔gap=3.33, T↔gap=3.44) in weighted parsimony analyses, the number of parsimony uninformative and informative characters were 28 and 575, respectively. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated three equally parsimonious trees, while one tree was obtained from weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModelTest was GTR+I+G. Fig. 3 shows the tree generated from the weighted parsimony analyses (TL=10914, CI=0.202, RI=0.544, RC=0.110, HI=0.798).

The family *Xylariaceae* is highly supported as a monophyletic clade. Genera of *Xylariaceae* clustered into two well-supported (Bayesian and bootstrap) monophyletic groups (Fig. 3). *Nemania* and *Xylaria* constitute Clade

A, while *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and Xylariaceae sp. constitute Clade B. Six *Nemania* species clustered into the same clade with high bootstrap support but poor Bayesian support. However, *N. maritima* was separated and located basal to most *Nemania* and *Xylaria* species.

β-tubulin gene based phylogenies

The β-tubulin gene dataset contained 45 taxa with 2016 characters. Nine hundred and fourteen base pairs were excluded from all analyses. Of the remaining 1102 unambiguously aligned characters, 512 were constant, 204 were parsimony uninformative and 386 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=2.25, A↔G=2.03, A↔T=2.19, C↔T=1.70, C↔G=2.07, G↔T=2.14, A↔gap=3.22, C↔gap=2.68, G↔gap=2.86, T↔gap=2.83) in weighted parsimony analyses, the number of constant sites was 459 parsimony uninformative was 234 and informative characters was 409. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated four equally parsimonious trees, while one tree was obtained from weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModelTest was GTR+I+G. Fig. 4 shows the tree generated from the weighted parsimony analyses (TL=2528, CI=0.373, RI=0.563, RC=0.210, HI=0.627).

Most genera of *Xylariaceae* clustered into two clades. However, the clades do not receive acceptable branch support in either Bayesian or bootstrap analyses (Fig. 4). Clade A receives good Bayesian support but poor bootstrap support; Clade B receives acceptable bootstrap support but poor Bayesian support. Within Clade A, four species of *Nemania* formed a strongly supported subclade, while *Rosellinia corticium* clustered within the *Xylaria* subclade. Within Clade B, *Annulohypoxylon* appears as a distinct clade to *Hypoxylon*. Two *Hypoxylon* spp. share the same subclade with *Daldinia*. Xylariaceae sp. Clustered within the *Annulohypoxylon* subclade. *Biscogniauxia* appeared as a distinct genus and basal to the two major clades.

Combined ITS-5.8 and LSU rDNA genes based phylogenies

The combined ITS-5.8 and LSU rDNA gene dataset contained 34 taxa with 1668 characters. Five hundred and forty-two base pairs were excluded from all analyses. Of the remaining 1126 unambiguously aligned characters, 606 were constant, 198 were parsimony uninformative and 322 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=2.28, A↔G=2.21, A↔T=2.28 C↔T=2.08, C↔G=2.29, G↔T=2.28, A↔gap=2.45, C↔gap=2.37, G↔gap=2.43, T↔gap=2.42) in weighted parsimony analyses, the number of constant sites was 573 parsimony uninformative was 207 and informative characters was 346. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated twelve equally parsimonious trees, while one tree was obtained from weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModelTest was SYM+I+G. Fig. 5 shows the tree generated from the weighted parsimony analyses (TL=3622 CI=0.531, RI=0.648, RC=0.344, HI=0.469).

Kretzschmaria, *Nemania*, *Stilbohypoxylon* and *Xylaria* clustered together in Clade A with high support, while *Annulohypoxylon*, *Hypoxylon* and Xylariaceae sp.1 clustered together in Clade B (Fig. 5). However, Clade B does not receive acceptable Bayesian and bootstrap support and *Annulohypoxylon* and *Hypoxylon* do not resolve into two subclades. *Biscogniauxia* spp. appear as a sister group to Clade A, but this affinity do not get enough branch support. The family *Xylariaceae* receives an acceptable bootstrap support but poor support from Bayesian analysis. The basal branch *Xylariales* is strongly supported in Bayesian and bootstrap analyses.

Combined ITS-5.8 rDNA and RPB2 genes based phylogenies

The combined ITS-5.8S rDNA and RPB2 gene datasets comprised 20 taxa with 1573 characters. Three hundred and three base pairs were excluded from all analyses. Of the remaining 1270 unambiguously aligned characters, 592 were constant, 117 were parsimony uninformative and 561 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix A↔C=2.09, A↔G=1.98, A↔T=2.20,

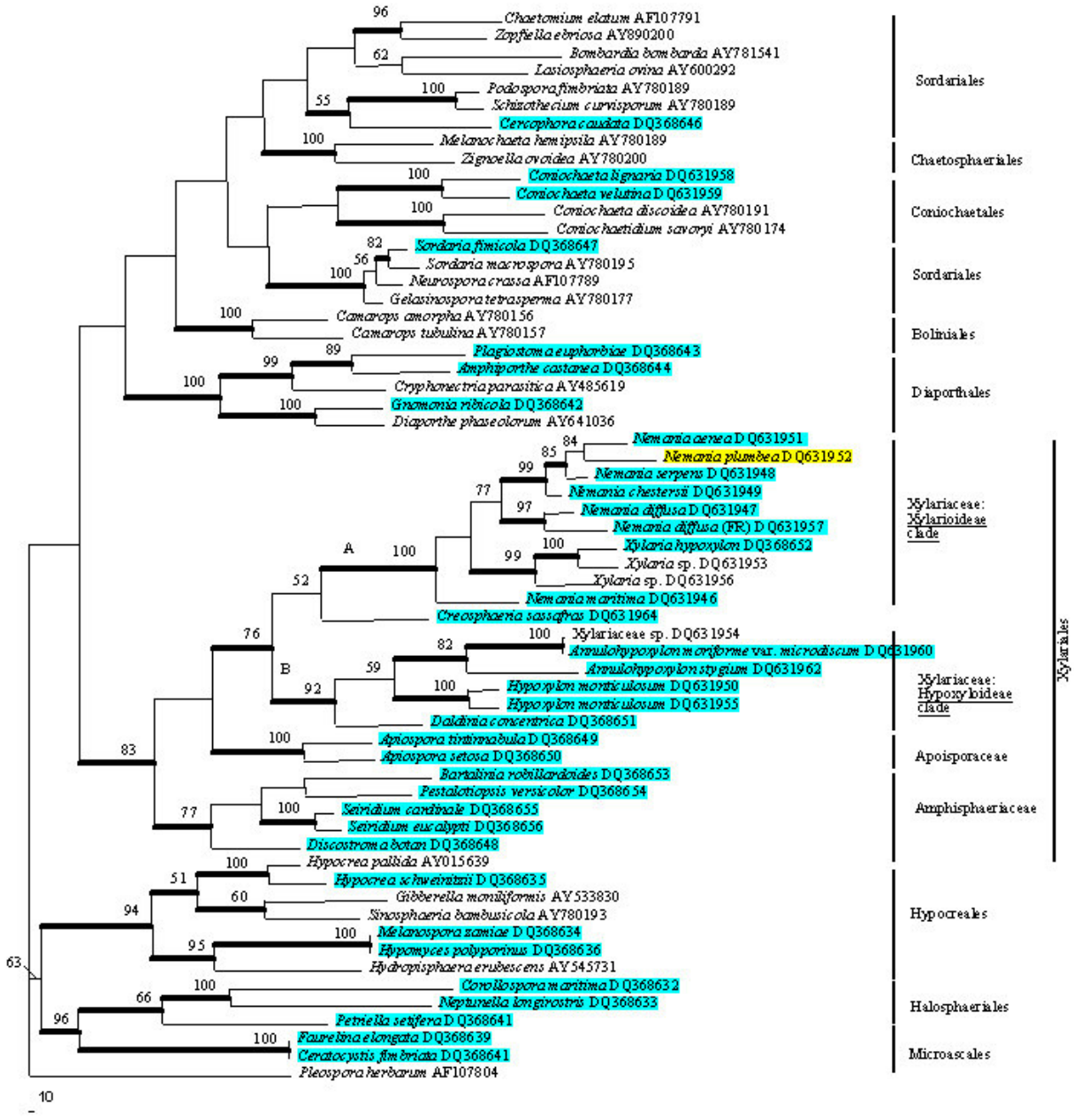

Fig. 3. The tree generated from weighted parsimony analysis based on RPB2 gene dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Yellow highlight means type strain, Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions (Zhang *et al*., 2008).

C↔T=1.86, C↔G=2.12, G↔T=2.23, A↔gap=2.80, C↔gap=2.77, G↔gap=2.81, T↔gap=2.80) in weighted parsimony analyses, the number of parsimony uninformative and informative characters were101 and 577, respectively. Gap was treated as fifth character for the analyses. Unweighted parsimony ana-lysis generated two equally parsimonious trees, while one tree was obtained from the weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModeltest was GTR+I+G. Fig. 6 shows the tree generated from the weighted parsimony analyses (TL=5377, CI=0.477, RI=0.549, RC=0.262, HI=0.523).

Six *Nemania* species formed a well supported monophyletic clade and are related to *Xylaria*. *N. aenea*, *N. plumbea*, *N. serpens* and *N. chestersii* formed a group with high bootstrap and Bayesian support (Fig. 6). *Nemania maritima* appeared to be a basal taxon to all *Nemania* species analysed. The seven

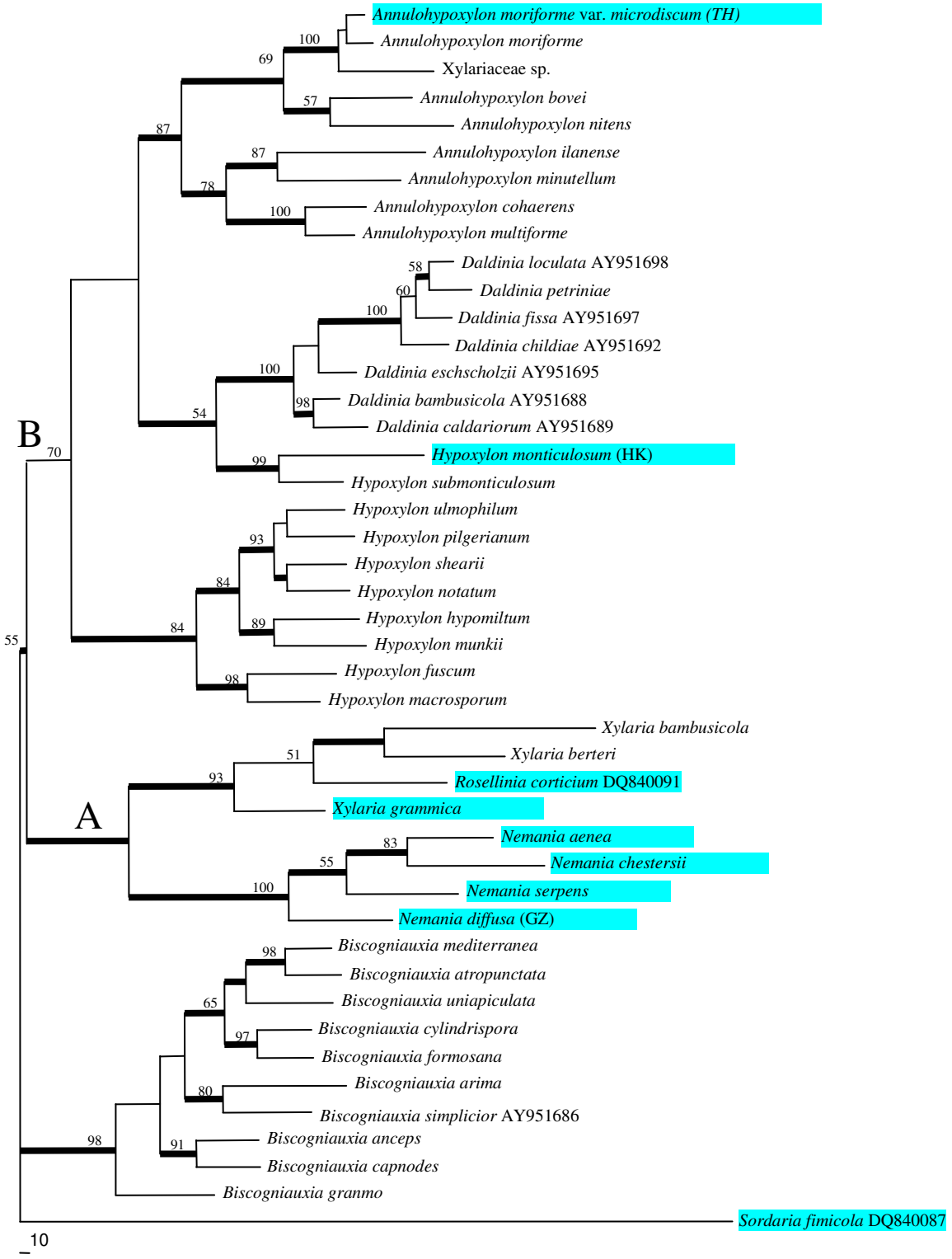

Fig. 4. The tree generated from weighted parsimony analysis based on β-tubulin gene dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions. (Zhang *et al*., 2008)

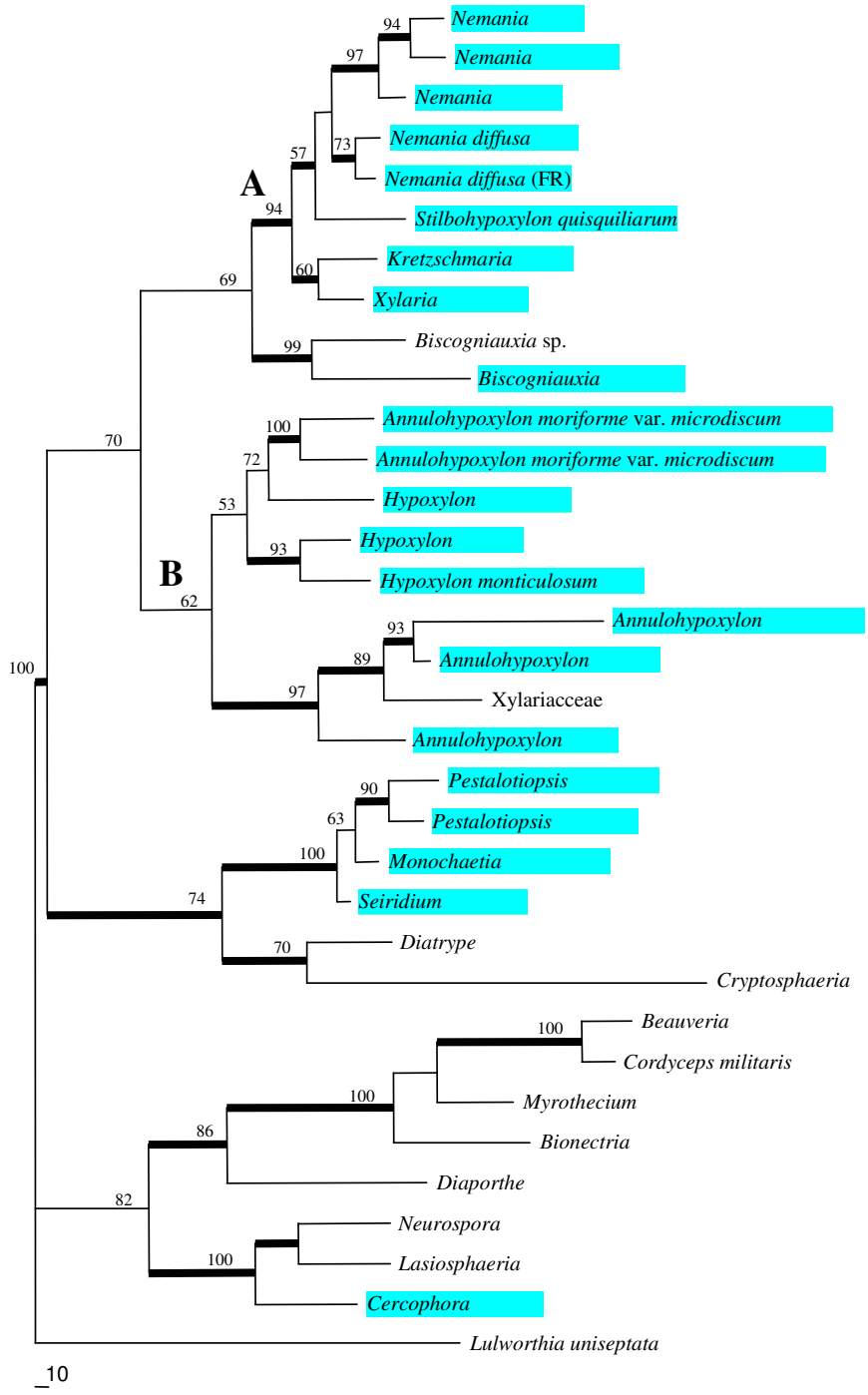

Fig. 5. The tree generated from weighted parsimony analysis based on combined ITS-5.8S and LSU rDNA genes dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions. (Zhang *et al*., 2008)

genera analysed in this dataset formed two main well supported clades, the Hypoxyloideae (Clade A) and Xylarioideae clade (Clade B). Xylarioideae clade (Clade A) comprises *Nemania* and *Xylaria*, while Hypoxyloideae clade (Clade B) comprises *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and Xylariaceae sp.1. This is similar to the phylogeny generated from RPB2 gene dataset.

Combined ITS-5.8 rDNA and β-tubulin genes based phylogenies

The combined ITS-5.8 rDNA and β-tubulin genes dataset contained 26 taxa with 2696 characters. One thousand, two hundred and sixty-nine base pairs were excluded from all analyses. Of the remaining 1427 unambiguously aligned characters, 703 were

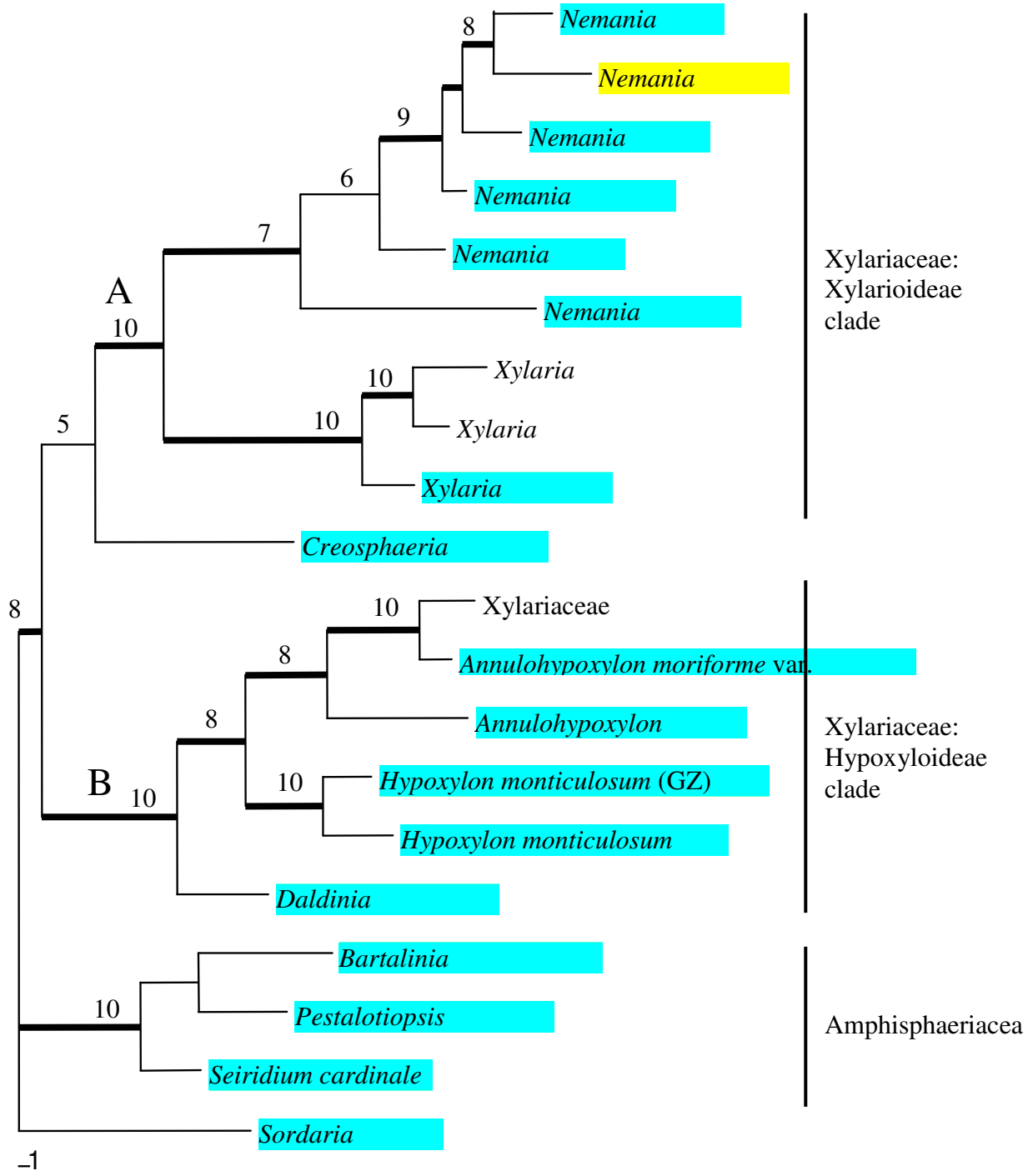

Fig. 6. The tree generated from weighted parsimony analysis based on combined ITS-5.8S rDNA and RPB2 genes dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Yellow highlight means type strain, Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions (Zhang *et al*., 2008).

constant, 254 were parsimony uninformative and 468 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=2.27, A↔G=2.35, A↔T=2.30, C↔T=2.04, C↔G=2.37, G↔T=2.40, A↔gap=2.35, C↔gap=2.26, G↔gap=2.44, T↔gap=2.29) in weighted parsimony analysis, the number of constant sites was 649 parsimony uninformative was 274 and informative characters was 504. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated one equally parsimonious tree, while one tree was obtained from weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModelTest was GTR+I+G. Fig. 7 shows the tree generated from the weighted parsimony analyses (TL=5399, CI=0.520,

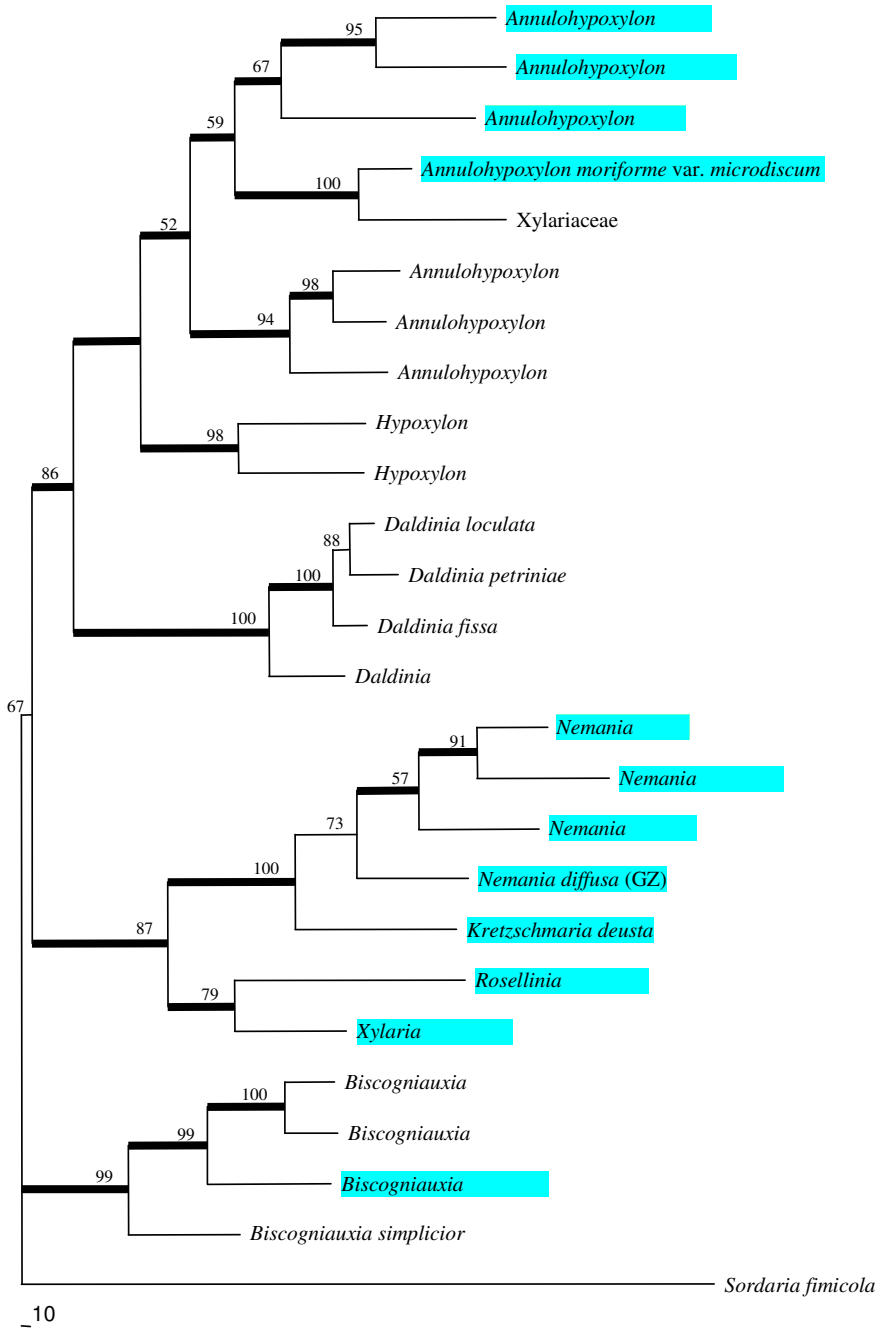

Fig. 7. The tree generated from weighted parsimony analysis based on combined ITS-5.8S rDNA and β-tubulin genes dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions. (Zhang *et al.*, 2008)

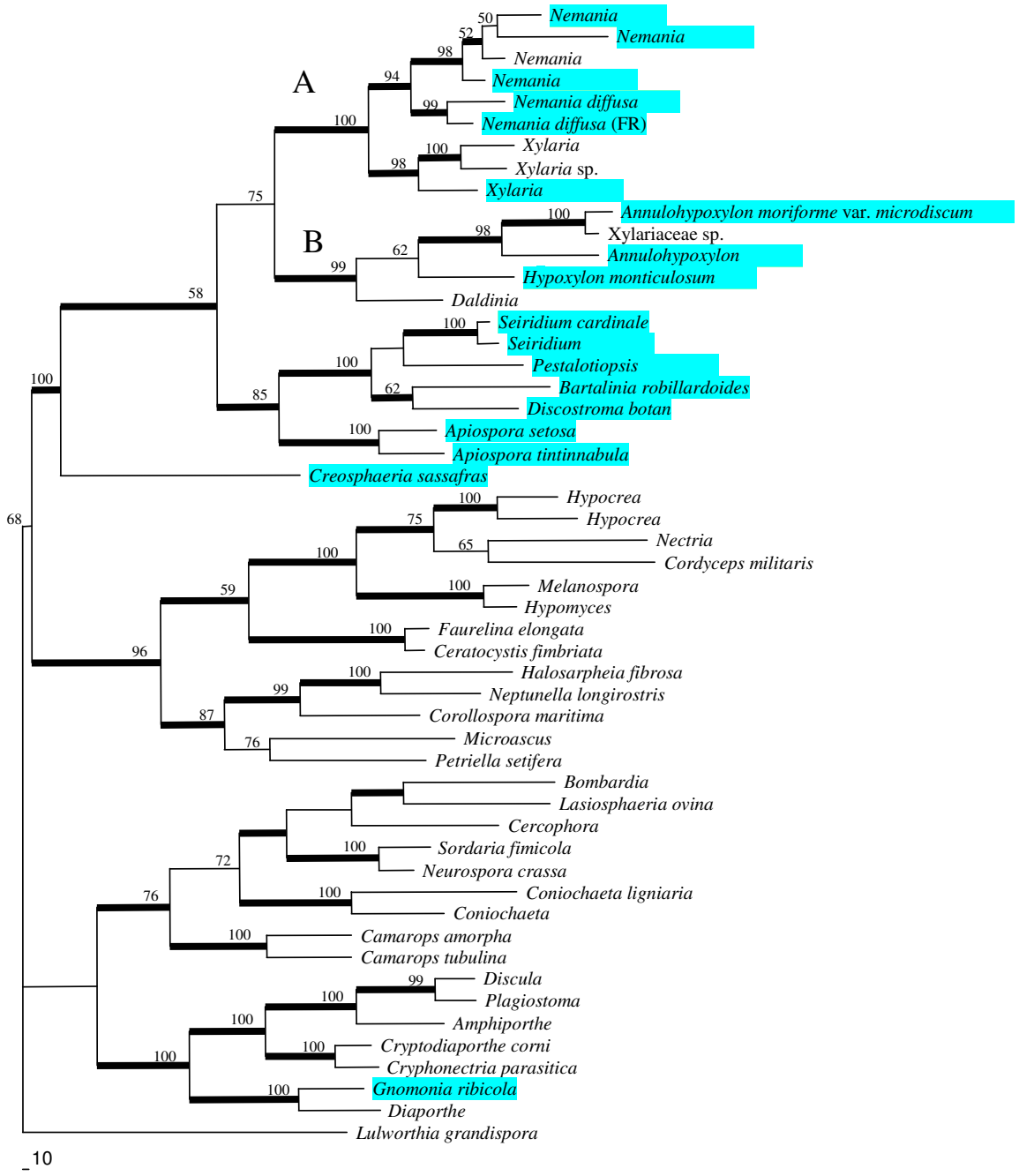

Fig. 8. The tree generated from weighted parsimony analysis based on combined LSU rDNA and RPB2 genes dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions. (Zhang *et al*., 2008)

RI=0.553, RC=0.287, HI=0.480). *Kretzschmaria*, *Nemania*, *Rosellinia* and *Xylaria* clustered together in Clade A with high support, while *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and Xylariaceae sp.1 clustered together in Clade B (Fig. 7). Both clades receivehigh Bayesian and bootstrap support. *Biscogni-auxia* was a distinct genus basal to the two major clades.

Combined LSU rDNA and RPB2 genes based phylogenies

The combined LSU rDNA and RPB2 gene dataset contained 52 taxa with 1844 characters. Two hundred and thirty-nine base pairs were excluded from all analyses. Of the remaining1605 unambiguously aligned characters, 779 were constant, 123 were excluded from all analyses. Of the remaining1605 unambiguously aligned characters, 779 were

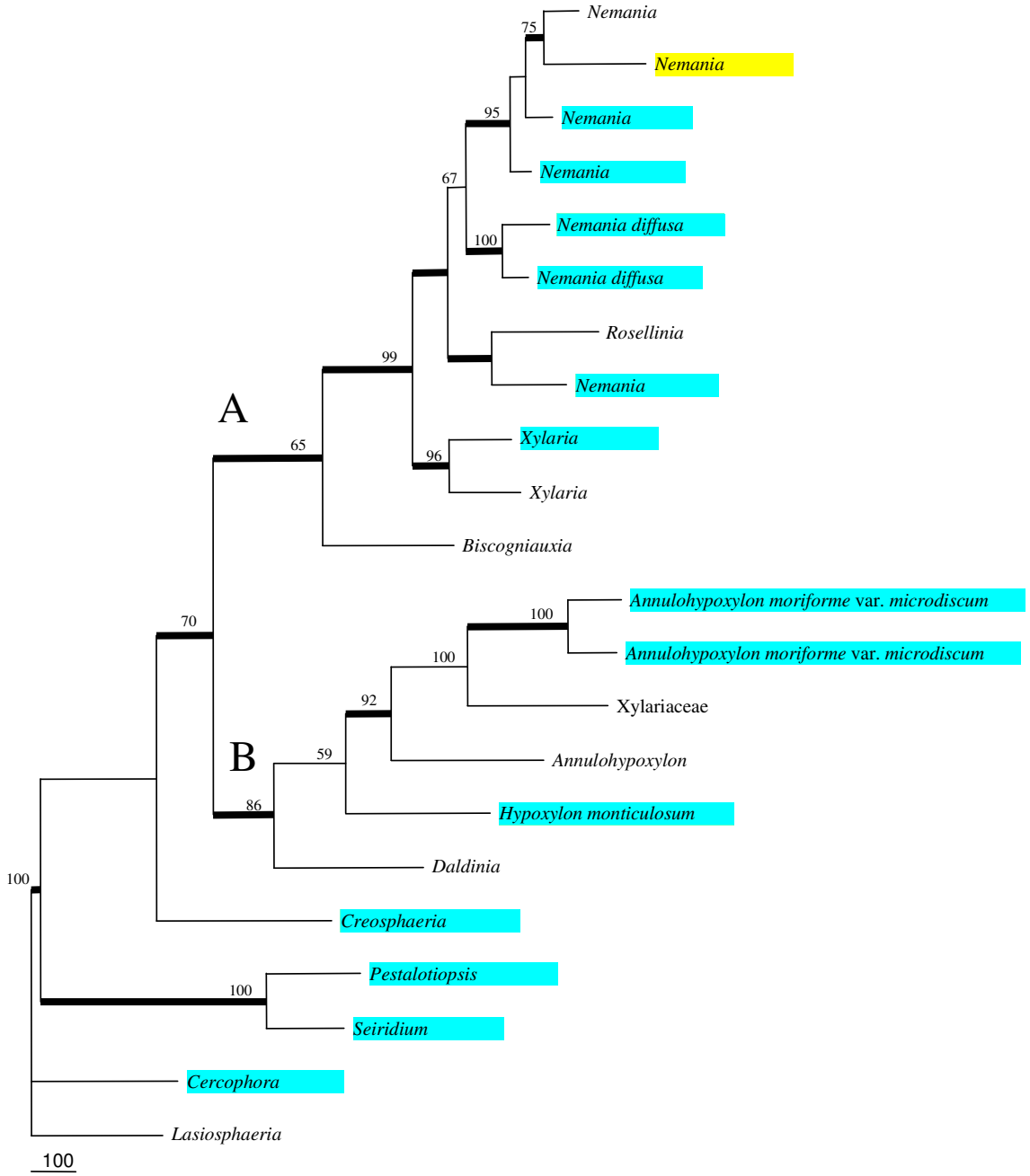

Fig. 9. The tree generated from weighted parsimony analysis based on combined ITS-5.8S, LSU rDNA and RPB2 genes dataset. Bootstrap values ≥50% are shown above the branches and Bayesian posterior probabilities ≥95% are indicated as thickened branches. Yellow highlight means type strain, Blue highlight means the collection was verified morphologically in previous or this study and no highlight means unverified GenBank accessions (Zhang *et al*., 2008).

constant, 123 were parsimony unin-formative and 703 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=1.91, A↔G=1.83, A↔T=2.00, C↔T=1.76, C↔G=1.95, G↔T=2.05, A↔gap=2.84, C↔gap=3.45, G↔gap=3.47, T↔gap=3.51) in weighted parsimony analyses, the number of constant sites was 766 parsimony uninformative was 123 and informative characters was 716. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated two equally parsimonious trees, while one tree was obtained from the weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModelTest was GTR+I+G. Fig. 8 shows the tree generated from the weighted parsimony analyses (TL=11384, CI=0.269, RI=0.567, RC=0.152, HI=0.731).

All genera of *Xylariaceae*, except *Creosphaeria*, clustered into two groups and both groups received high Bayesian and

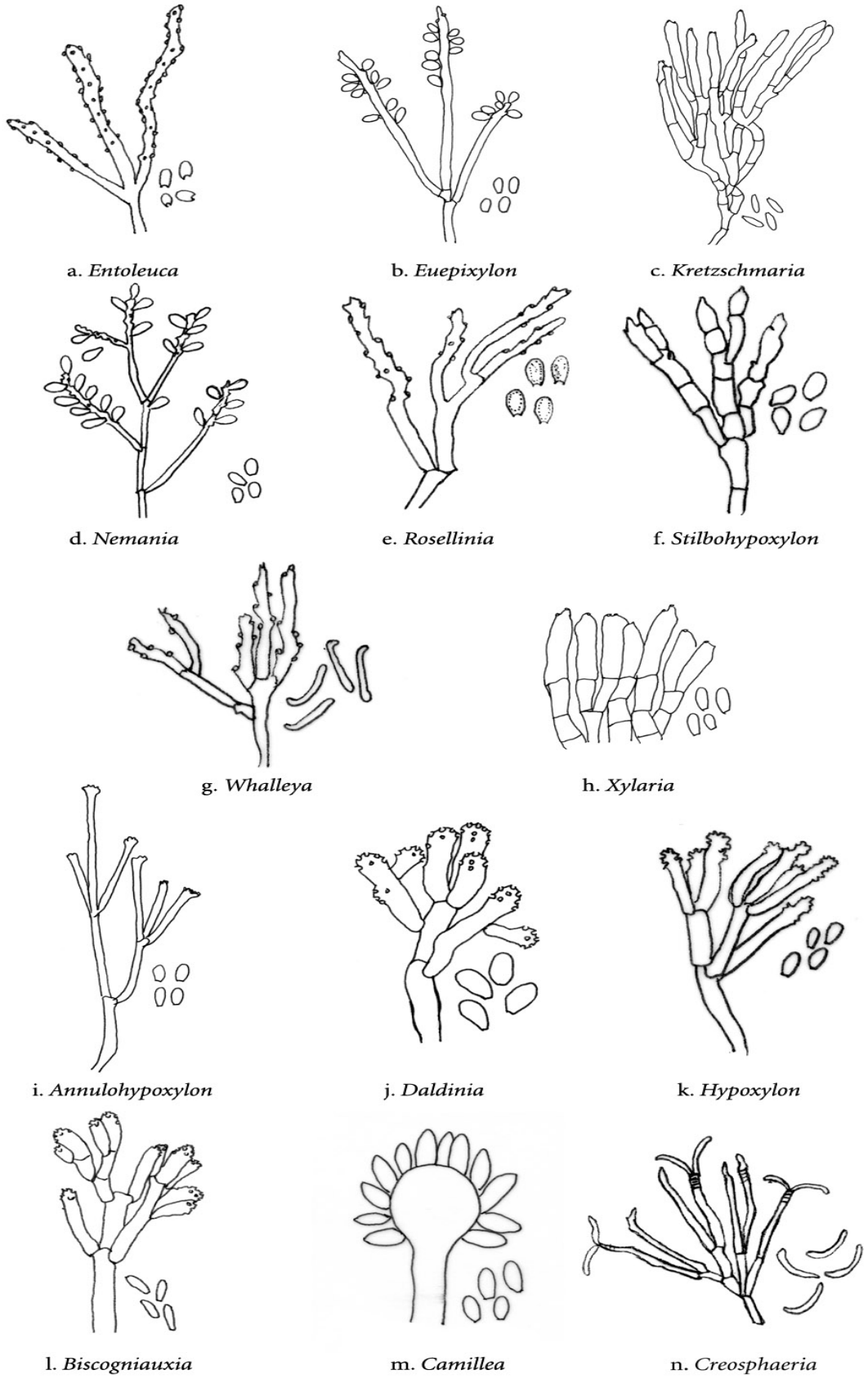

Fig. 10. Anamorphs of Xylariaceae. a-h. *Geniculosporium*-like anamorphs of Xylariaceae genera. a. *Entoleuca*; b. *Euepixylon*; c. *Kretzschmaria*; d. *Nemania*; e. *Rosellinia*; f. *Stilbohypoxylon*; g. *Whalleya*; h. *Xylaria*. i-l. *Nodulisporium*-like anamorphs. i. *Annulohypoxylon*; j. *Daldinia*; k. *Hypoxylon*; l. *Biscogniauxia*. m. *Xylocladium* anamorph of *Camillea*. n. *Libertella*–like anamorph of *Creosphaeria*. (Drawing by Alvin M.C. Tang)

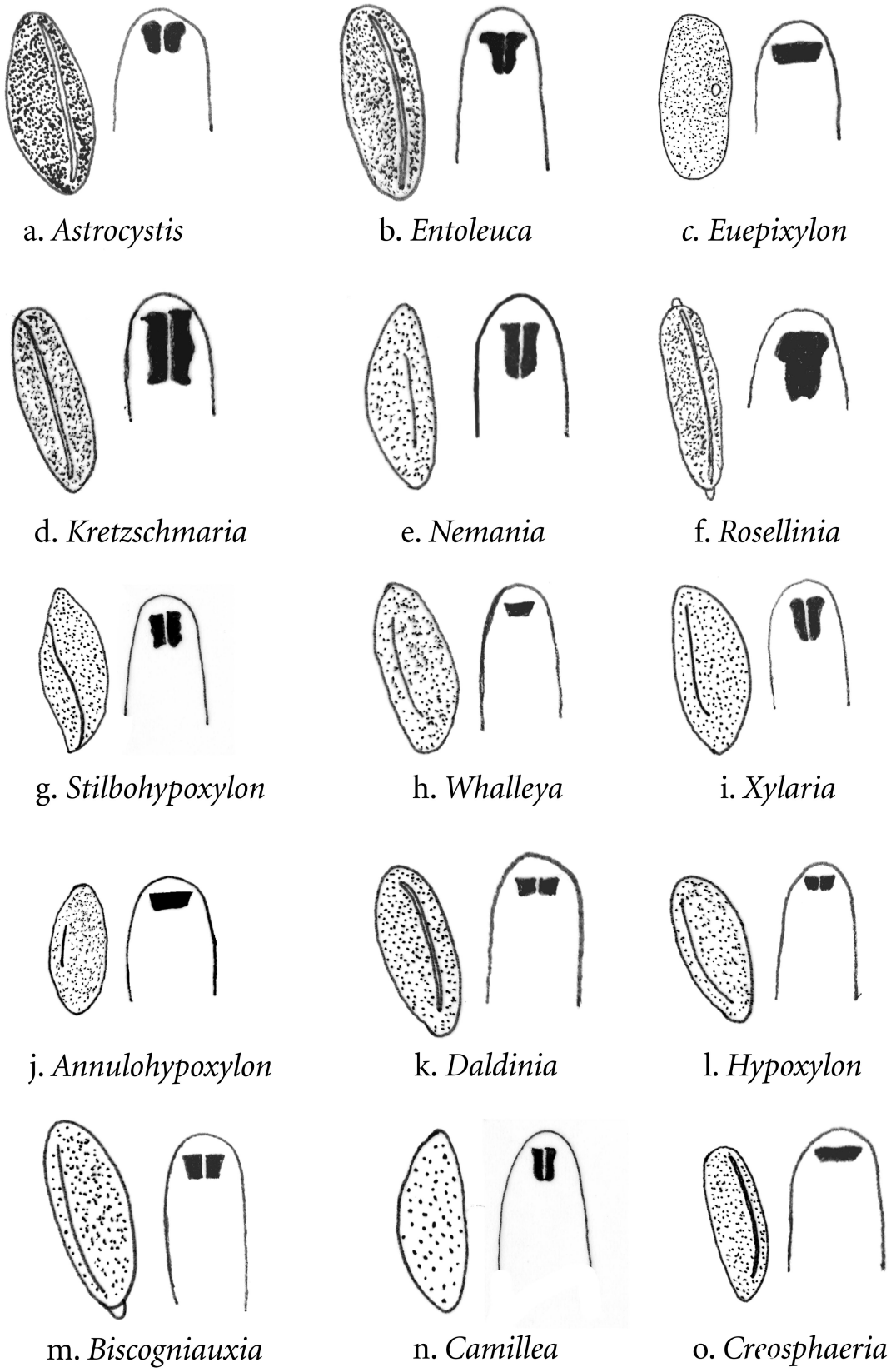

Fig. 11. Diversity of amyloid apical apparatus and ascospores of Xylariaceae. a-i. Xylarioideae group. a. *Astrocystis*; b. *Entoleuca*; c. *Euepixylon*; d. *Kretzschmaria*; e. *Nemania*; f. *Rosellinia*; g. *Stilbohypoxylon*; h. *Whalleya*; i. *Xylaria*. j-l. Hypoxyloideae group. j. *Annulohypoxylon*. k. *Daldinia*; l. *Hypoxylon*. m. *Biscogniauxia*. n. *Camillea*. o. *Creosphaeria* (Drawing by Alvin M.C. Tang).

bootstrap support (Fig. 8). *Nemania* and *Xylaria* constitute Clade A, while *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and Xylariaceae sp.1 constitute Clade B. *Creosphaeria* appears as a distinct lineage basal to *Xylariaceae* and *Amphisphaeriaceae*. The *Xylariales* is highly supported as a monophyletic clade.

Combined ITS-5.8, LSU rDNA and RPB2 genes based phylogenies

The combined ITS-5.8, LSU rDNA and RPB2 genes dataset contained 22 taxa with 2604 characters. Six hundred and thirty base pairs were excluded from all analyses. Of the remaining 1974 unambiguously aligned characters, 1216 were constant, 117 were parsimony uninformative and 641 were parsimony informative (Table 2). For the same dataset, with the use of step-matrix (A↔C=1.91, A↔G=1.83, A↔T=2.00, C↔T=1.76, C↔G=1.95, G↔T=2.05, A↔gap=2.84, C↔gap=3.45, G↔gap=3.47, T↔gap=3.51) in weighted parsimony analyses, the number of constant sites was 1204, parsimony uninformative was 108 and informative characters was 662. Gap was treated as fifth character for the analyses. Unweighted parsimony analysis generated one equally parsimonious tree, while one tree was obtained from weighted parsimony analysis. The best-fit model selected for Bayesian analysis by MrModelTest was SYM+I+G. Fig. 9 shows the tree generated from the weighted parsimony analyses (TL=5811, CI=0.455, RI=0.536, RC=0.244, HI=0.545).

Nemania, *Rosellinia* and *Xylaria* constitute Clade A, while *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and Xylariaceae sp. constitute Clade B (Fig. 9). Both clades receive high branch support. *Creosphaeria* appears as a separate lineage to the main clades, while *Biscogniauxia* also appears as a distinct genus as its grouping with Clade A is not strongly supported.

Discussion

Phylogenetic relationships of Xylariaceae based on molecular data

This study presents a multigene approach to clarify the taxonomic confusion that surrounds the grouping of genera within the *Xylariaceae*. Multigene approaches has proven to be highly useful in resolving evolutionary relationships (O'Donnell *et al*., 1998; Baker *et al*., 2001; Buckley *et al*., 2002; Slippers *et al*., 2004; Thell *et al*., 2004; Lumbsch *et al*., 2005; Miller and Huhndorf, 2005). This approach not only increases the phylogenetic resolution of the target groups, but also substantially increases phylogenetic support that can never be recovered from analyses restricted to single genes (Lutzoni *et al*., 2004).

In the present study, protein-coding genes are found to be more phylogenetically reliable than ribosomal genes. Phylogeny inferred from RPB2 gene appear to be highly resolved. This gene also provided the highest proportion of informative characters (61.4%). In addition, when RPB2 gene sequences were analyzed in combination with other sequence datasets, such as with ITS-5.8S or LSU rDNA genes, phylogenies obtained were more resolved with better statistical support for most clades (Figs 6, 8). β-tubulin gene also yielded promising results in increasing resolution within the *Xylariaceae* (Fig. 4). Only β-tubulin dataset or the combined datasets with β-tubulin was significant in resolving and separating *Annulohypoxylon* and *Hypoxylon sensu stricto* (Hsieh *et al*., 2005). Within the single genes, LSU gene dataset appears to be the least robust although the number of characters analysed is the same as in RPB2 gene dataset (Fig. 2; Table 2).

Use of only ITS-5.8S rDNA gene sequences to infer phylogenetic relationships of *Xylariaceae* was found to be inappropriate (Granmo *et al*., 1999; Johannesson *et al*., 2000; Lee *et al*. 2000; Sánchez-Ballesteros *et al*. 2000; Mazzaglia *et al*., 2001; Smith *et al*., 2003; Suwannasai *et al*. 2005; Triebel *et al*., 2005). Inclusion of more taxa did not result in better phylogenetic and genetic resolution. For example, when ITS sequences from 93 taxa were analysed, *Diatrype* failed to separate from *Eutypa* and *Eutypella*; *Nemania*, *Rosellinia* and *Xylaria* species were distributed in only 1 or 2 clades that were totally unresolved (Acero *et al*., 2004).

The separation of Hypoxyloideae and Xylarioideae lineages in the past was mainly based on anamorphic type and chemotaxonomy (Rogers, 1979; Sánchez-Ballesteros *et al*. 2000, Smith *et al*., 2003, Triebel *et al*., 2005). There

was not much information from DNA sequence data to validate these taxonomic schemes and most of the existing studies were based upon ribosomal DNA genes data (Sánchez-Ballesteros *et al*., 2000, Smith *et al*., 2003, Triebel *et al*., 2005). The current study strongly supports the distinction of the two lineages and generic relationships are discussed below.

The Xylarioideae clade (Clade A) was recovered with strong support (bootstrap and Bayesian) in RPB2 gene, combined ITS-5.8S + RPB2 genes, and combined LSU rDNA + RPB2 genes phylogenies (Figs 3, 6, 8). Phylogeny inferred from the combined LSU rDNA + RPB2 gene datasets appeared to be the best in resolving internal branches as well as the basal branches (Fig. 8). *Nemania* and *Xylaria* are within two distinct subclades as sister groups. This was highly supported in most phylogenies. The phylogenetic relationships of other genera such as *Astrocystis*, *Entoleuca*, *Kretzschmaria*, *Rosellinia*, *Whalleya* to *Xylaria* and *Nemania* are still obscure at present due to difficulties in obtaining DNA from specimens and amplifying different gene regions. *Whalleya* with scolecosporous conidium but with *Geniculosporium*-like conidiogenesis clustered within the Xylarioideae, contrary to previous phylogenies by Sánchez-Ballesteros *et al*. (2000) (see below). *Rosellinia* is paraphyletic and its relationships with *Astrocystis* and *Entoleuca* are still ambiguous. *Stilbohypoxylon* is related with *Xylaria* (perhaps *Astrocystis* or *Rosellinia*). This is concordant with the type of conidiogenesis and development of anamorphic stage on stromata references. Interspecies relationships among *Xylaria* are still unresolved as inclusion of more taxa in the ITS sequence analyses resulted in different topologies with poor statistical support (results not shown). It is therefore difficult to reproduce or confirm the three groups of *Xylaria* as reported by Lee *et al*. (2000).

The Hypoxyloideae clade (Clade B) was recovered with strong support (bootstrap and Bayesian) in combined ITS-5.8S + RPB2 genes, and combined LSU rDNA + RPB2 genes phylogenies (Figs 6, 8). There were differences in statistical support and resolution of the internal clades among phylogenies derived from different genes. This study demonstrates that rDNA genes alone, both single and combined datasets, are not suitable to distinguish the genera *Annulohypoxylon* and *Hypoxylon*. Protein-coding genes, either RPB2 or β-tubulin gene, seem to be more appropriate (Figs 3, 4). With the use of protein-coding genes and different combinations of datasets, our results confirm that *Annulohypoxylon* and *Hypoxylon* are two distinct genera. Xylariaceae sp. is closely related to *Annulohypoxylon*. β-tubulin gene phylogeny indicates that *Hypoxylon* is paraphyletic with *Daldinia* as sister taxa This is similar to the results obtained by Hsieh *et al*. (2005) that *Hypoxylon* clustered within the *Daldinia* clade.

The phylogenetic position and the relationships of *Biscogniauxia* and *Camillea* with other genera are interesting but rather ambiguous. There were some topological differentces in different gene trees. In the ITS-5.8S and β-tubulin gene phylogeny, these genera formed a basal clade to all other genera (Figs 1, 4). A similar topology based on β-tubulin and α-actin genes was reported by Hsieh *et al*. (2005). However, in the LSU rDNA gene alone (Fig. 2) and combined ITS-5.8S + LSU rDNA gene dataset (Fig. 5), *Biscogniauxia* appears as a sister group to the Xylarioideae clade (Clade A), although this relationships did not receive acceptable branch support. At this time, it is difficult to infer appropriate phylogenetic affinities for this genus. The separation of *Biscogniauxia* and *Camillea* from the two main clades (Hypoxyloideae and Xylarioideae) and their uniqueness amongst the *Xylariaceae* is clear. Both genera have been classified in the Hypoxyloideae clade as they are characterized as having *Nodulisporium*-like anamorphs (Ju and Rogers, 1996). *Biscogniauxia* and *Camillea* are however, different from other members of Hypoxyloideae in having bipartite stromata and lack of KOH-extractable stromatal pigments (Ju *et al*., 1998). Ascospores of *Biscogniauxia* bear a hyaline cellular appendage in some species (Fig. 11) while those of *Camillea* are intricately ornamented and produce a *Xylocladium* anamorph. Their basal position in the ITS-5.8S and β-tubulin gene datasets may indicate that the Hypoxyloideae and Xylarioideae clades were once derived from *Biscogniauxia* or *Camillea*, and some *Biscogniauxia*-like forms are likely to constitute the basic

forms of both the xylarioid and the hypoxyloid clade. It was also reported that *Xylaria* is linked to *Biscognauxia* as some *Xylaria* spp. were found to contain traces of mellein dihydroi-socoumarins (see Stadler & Foruneir 2006).

Correlation of gene phylogenies to morphological and chemical properties

The separation of Hypoxyloideae from Xylarioideae clade in all phylogenies generated in this study is generally concordant with the distinction of xylariaceous fungi based upon chemotaxonomy and their specific anamorphs (Sánchez-Ballesteros *et al*. 2000, Smith *et al*., 2003, Triebel *et al*., 2005). Clade A (Xylarioideae clade) represents the genera that do not yield stromatal pigments in potassium hydroxide and produce a *Geniculosporium*-like anamorph, including *Entoleuca*, *Euepixylon*, *Kretzschmaria*, *Nemania*, *Rosellinia*, *Stilbohypoxylon*, *Whalleya* and *Xylaria* (Figs 10a-h). Clade B (Hypoxyloideae clade) represents the genera that can yield stromatal pigments in potassium hydroxide and produce *Nodulisporium*-like anamorph, including *Annulohypoxylon*, *Daldinia* and *Hypoxylon* (Figs 10i-k) (Ju and Rogers, 1996; Stadler *et al*., 2001; Stadler *et al*., 2004; Stadler and Hellwig, 2005; Stadler *et al*., 2006, 2008; Bitzer *et al*., 2008). *Creosphaeria* appears to be phylogene-tically distinct from the two clades. This genus is characterized by having a *Libertella*-like scolecosporous anamorph.

Types of amyloid apical apparatus are also generally consistent (for most genera) with the result of this study (Martin, 1967, 1968a,b; Van der Gucht, 1994). Members of Xylarioideae generally bear an urn-shaped or inverted hat-shaped apical apparatus (Figs 11a-i). This is found in most species of *Astrocystis*, *Entoleuca*, *Kretzschmaria*, *Nemania*, *Rosellinia*, *Xylaria* and some species of *Biscogniauxia*. Members of Hypoxyloideae generally bear discoid or capital-shaped apical apparatus. Most species of *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and some species of *Biscogniauxia* possess this character (Figs 11j-m). Nonetheless, there are exceptions, such as *Euepixylon*, *Whalleya*, which has a discoid apical apparatus, and are members of Xylarioideae (Figs 11c,h); the mysterious xylariaceous taxa such as *Phylacia*, *Pulveria* and *Pyrenomyxa* that lack any apical apparatus and have been recently classified as members of Hypoxyloideae based on chemotaxonomy (Stadler *et al*., 2005; Bitzer *et al*., 2008). According to recent chemotaxonomic data by Bitzer (2008), Pyrenomyxa is similar with Hypoxylon rubiginosum complex, while Phylacia is related to Daldinia and Entonaema by particular marker metabolites.

Secondary metabolites from stromata and cultures correspond well with molecular data. This chemical data provides constant and reliable classification for the main groups (Hypoxyloideae and Xylarioideae), as well as intra- and inter- generic associations (Whalley and Edwards, 1995; Hellwig *et al*., 2005; Quang *et al*., 2005; Stadler and Hellwig, 2005). Dihydroisocoumarins are present only in Hypoxyloideae, while Butyrolactones, succinic acid derivatives, punctaporonins and griseofulvin are present only in Xylarioideae (Whalley and Edwards, 1995). Within the Hypoxyloideae group, chemical data resolved the relationship of *Annulohypoxylon* and *Hypoxylon*, supporting the view that they should be recognized as separate genera. Several metabolite classes, such as mitorubrins, rubiginosins, macrocarpones, hypomiltin and daldinal are present only in *Hypoxylon*; while binaphthalene and its derivatives, the benzo[*j*]fluoranthenes daldinone A and truncatone are present only in *Annulohypoxylon* (Quang *et al*., 2005).

Evolutionary relationships within *Xylariaceae*

Annulohypoxylon, *Daldinia*, *Entonaema* and *Hypoxylon* appear to be very closely related. It has been suggested that the zonate ring structure of *Daldinia* were derived from the stroma of *Hypoxylon* (Ju *et al*., 1997). The ring structure of *Daldinia* is basically for water storage, and a similar structure can be observed in *Entonaema* which has hollow, turgid, liquid-filled stromata. The close relationship of *Daldinia* and *Entonaema* is further indicated by the presence of 8-Methoxy-1-naphthol and 5-hydroxy-2-mythylchromone in both (Bitzer et al., 2008).

It has been suggested that there are morphological similarities between *Entoleuca*, *Euepixylon*, *Kretzschmaria*, *Nemania*, *Roselli-*

nia, *Stilbohypoxylon* and *Xylaria*. For instance, the immature ascospores of *Entoleuca*, *Nemania* and *Xylaria* bear evanescent cellular appendages (Rogers, 1979, Rogers and Ju, 1996, 1997; Ju and Rogers, 2002). The white stromatal flesh of *Entoleuca*, *Kretzschmaria*, *Nemania*, *Rosellinia* and *Stilbohypoxylon* encases the perithecia during the development of perithecia. During maturity, this white flesh darkens or deteriorates in most taxa (Rogers and Ju, 1996). Similar type of conidiogenesis and development of anamorphic stage on stromata can be found in *Astrocystis*, *Stilbohypoxylon* and *Xylaria*. Remnants of anamorphs usually adhere to mature stromata in some species (Rogers and Ju, 1997).

Annulohypoxylon

Annulohypoxylon was introduced by Hsieh *et al*. (2005) to accommodate *Hypoxylon* sect. *Annulata sensu* Ju and Rogers (1996). This includes taxa in *Hypoxylon* sect. *Annulata sensu* Miller (1961) and several taxa in *Hypoxylon* sect. *Papillata sensu* Miller (1961) (including *Hypoxylon cohaerens* and its var. *microsporum* (now known as *H. minutellum*), and *H. multiforme* and its var. *alaskense*). This genus is characterized by its carbonaceous stromatal layer discretely enclosing each perithecium and ostioles that are always higher than the level of surrounding stromatal surface (Ju and Rogers, 1996). The results of the current study support the separation of *Annulohypoxylon* from *Hypoxylon* in the β-tubulin and RPB2 genes datasets (Figs 3, 4). Chemotaxonomy also support the generic separation based on the presence of unique metabolite classes (Quang *et al*., 2005). There are two subclades within the genus *Annulohypoxylon*. However, there is no obvious correlation between the subclades with ostiolar disk formation (*truncatum*-type and *bovei*-type).

Hypoxylon

Hypoxylon was recently re-defined by Hsieh *et al*. (2005) and is now restricted to include only those taxa in *Hypoxylon* sect. *Hypoxylon sensu* Ju and Rogers (1996). Similar to the results in Hsieh *et al*. (2005), the phylogeny obtained from the β-tubulin gene dataset in this study indicates that *Hypoxylon* is paraphyletic as two taxa (two samples of *Hypoxylon monticulosum*) share the same clade with *Daldinia*. In the RPB2 phylogeny, these two strains of *Hypoxylon monticulosum* constitute a clade with low Bayesian and bootstrap support. From morphological features and secondary metabolite profiles, *H. monticulosum* is generally regarded as basal in *Hypoxylon* owing to the derived or reduced characters. There are some species that are possibly the intermediates between *Daldinia* and *Hypoxylon*, such as *D. placentiformis* and *H. polyporus*, have been gradually re-defined according to more available chemotaxonomic and molecular data (Hsieh et al., 2005; Bitzer et al., 2008). Recent data have indicated that *Daldinia*, *Entonaema sensu stricto*, *Phylacia*, *Rhopalostroma* and probably *Thanmomyces* are a group that may have been derived from *Hypoxylon (*Bitzer et al., 2008).

Nemania

Most *Nemania* species in this study clustered within one clade. They appear as a distinct genus with high Bayesian and bootstrap support within Xylarioideae. Historically, the type species of *Nemania*, *N. serpens*, has long been recognized as *Hypoxylon serpens* under *Hypoxylon* section *Papillata*: subsection *Primocinerea* (Miller, 1961). *Nemania* was later given generic status by Pouzar (1985a,b) and later by Petrini and Rogers (1986). Detailed morphological examination of *Nemania* revealed that it should be classified as xylarioid – absence of colored granules and do not release color pigments in 10% potassium hydroxide, asci bluing upon treatment with Melzer's reagent and production of *Geniculosporium*-like anamorphs (Pouzar, 1985a,b; Ju and Rogers, 2002). Of the *Nemania* species studied, *N. diffusa* and *N. maritima* were found to be divergent from the others. Addition of *N. diffusa* and *N. maritima* to the ITS-5.8S or LSU rDNA gene dataset resulted in a drastic topological difference and poor phylogenetic resolution. They were then excluded from two single gene analyses. Analyses with ITS-5.8S gene sequences revealed that *N. maritima* is related to *Astrocystis*, *Entoleuca* and *Rosellinia* (tree not shown). This affinity was recovered again in the combined ITS-5.8S + LSU rDNA + RPB2 genes dataset, where *N. maritima*

grouped with *R. corticium* (Fig. 9). Morphologically, *N. maritima* is characterized by scattered stromata with one to only few perithecia enclosed, immersed or half-erumpent when young; while typical *Nemania* species have superficial and effused stromata (Ju and Rogers, 2002).

The deviation of *N. diffusa* from the main group was also tested by using two samples from two different localities, Guizhou (China) and France. In RPB2, combined LSU rDNA + RPB2 genes, and combined ITS-5.8 + LSU rDNA + RPB2 genes datasets, two strains of *N. diffusa* appear as sister taxa to the main *Nemania* group (Figs 8-9). Similar results were also reported by Sánchez-Ballesteros *et al.* (2000) where *N. bipapillata* (= *N. diffusa*) was phylogenetically apart and basal to the main clade of *Nemania*. Morphologically, *N. diffusa* is different from the main group of *Nemania* by having darker ascospores and a longer, more conspicuous and straight germ slit (Petrini and Rogers, 1986; Van der Gucht, 1995).

Whalleya

Two strains of *Whalleya microplaca* were the only available sequences in the GenBank deposited by Sánchez-Ballesteros *et al*. (2000). They found that these two sequences were phylogenetically distinct but their affinities to other xylariaceous genera were unclear since there was no support for a reliable phylogenetic placement. In the current analyses, *Whalleya* appears to be a member of the Xylarioideae clade and basal to other genera that produce *Geniculosporium*-like anamorphs (Fig. 1). This grouping is highly supported by Bayesian and bootstrap analyses. Morphologically, *Whalleya* deviates from other typical members within the Xylarioideae clade. *Whalleya* possesses bipartite stromatal layers, while typical members of the group possess unipartite stromatal layer (Roger *et al*., 1997). The scolecosporous conidia (with *Geniculosporium*-like conidiogenesis) (Fig. 10g) of *Whalleya* are atypical of other members of the Xylarioideae clade. The deviations of *Whalleya* from typical members of Xylarioideae is interesting from an evolutionary point of view as this could be suspected to be the ancestral characters of the group (Tribel et al., 2005).

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