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Relationship of the genus *Cordyceps* and related genera, based on parsimony and spectral analysis of partial 18S and 28S ribosomal gene sequences

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A molecular phylogenetic study of selected species of three sub-genera of the genus *Cordyceps* was undertaken, along with representatives of the genera *Akanthomyces*, *Aschersonia*, *Gibellula*, *Hymenostilbe*, *Hypocrella*, *Nomuraea*, and *Torrubiella*, to examine their inter-relationship. Phylogenetic analyses of the data indicated that the Clavicipitaceae form a monophyletic group within the Hypocreales, while the monophyly of *Cordyceps* was not supported. Four clades were identified: *Cor. militaris/Cor. pseudomilitaris*; *Cor. irangiensis/Cor. sphecocephala*; *Cor. intermedia/Cor. capitata*; and *Cor. cylindrica/Nom. atypicola*. The sub-genus *Neocordyceps* was shown to be monophyletic while the sub-genera *Eucordyceps* and *Ophiocordyceps* do not form monophyletic groups. The genus *Hypocrella* appeared monophyletic, and radiated after the formation of the genera *Cordyceps*, and *Torrubiella*. *Akanthomyces arachnophilus* and *Gi. pulchra*, anamorphs of *Torrubiella* species, formed a distinct clade that was separate from one formed by the scale insect pathogens, *To. luteorostrata* and *Paecilomyces cinnamomeus*, suggesting that this genus may be polyphyletic.

Key Words——18S and 28S rRNA; Clavicipitaceae; entomopathogens; phylogeny.

Within the Ascomycota, the Hypocreales is an order of many genera and species that are important pathogens of plants and animals. Of three families: Hypocreaceae de Not., Niessliaceae Kirscht. and Clavicipitaceae (Lindau) O. Eriksson, it is the latter which contains the most numerous invertebrate pathogens. Clavicipitaceous fungi contain ca 500 species (Hawksworth et al., 1995; Hywel-Jones, unpublished observations), with about 75% of these pathogenic on invertebrates: most often on Insecta (including Coleoptera, Homoptera, Lepidoptera, Hymenoptera, Diptera, Isoptera, Heteroptera, Orthoptera and Odonata) and Arachnida (spiders, mites and ticks). As well as the invertebrate pathogens, the Clavicipitaceae also contains important plant-pathogenic genera (*Epichloë* (Fr.) Tul. & C. Tul., *Balansia* Speg. and *Claviceps* Tul.) and pathogens of fungi (*Cordyceps* (Fr.) Link hosts: e.g. Elaphomycetales, *Elaphomyces* Nees). Originally, Nannfeldt (1932) treated the clavicipitalean fungi as an order, the Clavicipitales. Miller (1949), however, placed these fungi as a family, Clavicipitaceae, within the Sphaeriales, although he did recognise the possible relationship with the Hypocreales. Rogerson (1970) proposed that the Clavicipitaceae was distinct from the Hypocreales, and that it should be considered as a separate hypocrealean-like order. This has not been supported by recent molecular studies (Spatafora and Blackwell, 1993), where members of the genera *Cordyceps*, *Balansia*, *Claviceps* and *Epichloë* were considered to form a family within the Hypocreales. Rehner and Samuels (1995) presented a phylogeny of hypocrealean fungi based on partial 28S rRNA gene sequences. They reported that the hypocrealean and clavicipitaceous species sampled formed monophyletic groups. These studies, however, did not include species from the genus *Torrubiella* Boud. and only one from *Hypocrella* Sacc. Furthermore, the number of *Cordyceps* species, the genus containing the most number of species within this group, was limited to mainly fungal pathogens.

Our study on the molecular biology of selected invertebrate pathogenic species is concerned with the genera *Cordyceps*, *Hypocrella* and *Torrubiella*. *Cordyceps* contains more than 300 species pathogenic to a large number of insect orders, spiders and other fungi. In contrast, the genus *Hypocrella* contains less than fifty species and is known to only infect the immature stages of the insect order Homoptera and is further confined within that order to only two families of scale insects, Aleyrodidae and Coccidae (Evans and Hywel-Jones, 1990). Species representing *Torrubiella* number over fifty and these are found mainly on Homoptera (including scale insects and hoppers, but not cicadas), and spiders.

Cordyceps species are separated into four subgenera: *Ophiocordyceps* (Petch) Kobayasi, *Eucordyceps* Kobayasi, *Neocordyceps* Kobayasi (Kobayasi, 1982) and *Bolacordyceps* O. Eriksson (Eriksson, 1986) based largely on ascospore morphology. The sub-genus *Bolacordyceps* was created as a placement for the genus *Phytocordyceps* C. H. Su & H. H. Wang. No species

from this sub-genus were included in this analysis. The characteristics describing the separation of the sub-genera *Eucordyceps*, *Ophiocordyceps* and *Neocordyceps* are listed in Table 1, and the examples given are those to be studied in this investigation.

These entomopathogenic fungi, with their various hosts, pose several questions with respect to their evolution. Did they co-evolve with these insects? Did they evolve separately to their hosts from plant pathogens? Have different anamorphs evolved along different routes? Rossman (1993) stated that over 40% of hypocrealean teleomorphs are known to have anamorphs. However, within the genus *Cordyceps* this figure is more than 80% (Hywel-Jones, unpublished observations), with a wide range of anamorphic forms. The relationships between anamorphs and species of *Cordyceps* are often not clear because some species, which produce anamorphs readily in nature, do not in culture (Hywel-Jones, 1995a, b, 1996), while others, which do not produce anamorphs in nature do so in culture (Hywel-Jones, 1994). It is not surprising, therefore, that there are many examples where anamorphic species are known from nature but which have not been linked with teleomorphs (Hywel-Jones, 1995c; Hywel-Jones et al, 1998). By contrast, the genus *Hypocrella* has fewer species and a limited number of anamorph forms, principally from the genus *Aschersonia*.

Tropical and sub-tropical forests are rich in *Cordyceps* species (Tzean et al., 1997) and a large collection of known and new undescribed species have been collected and isolated in Thailand. These species have occurred on a variety of insects and spider hosts, and provide a valuable resource for study at the molecular level. Host switiching was observed by Nikoh and Fukatsu (2000). The objective was, therefore, to determine the origin and evolution of the genus *Cordyceps*, its cohesiveness as a genus and the validity and significance of the three sub-genera: *Eucordyceps*, *Neocordyceps* and *Ophiocordyceps*; and its relationship to other clavicipitaceous genera including *Hypocrella* and *Torrubiella*.

Materials and Methods

Isolates studied The taxa sampled for this study were selected based upon their occurrence on different insect hosts and isolated from material collected in Thailand including one from England, and maintained in the BIOTEC Culture Collection (Table 2).

Growth media Cultures were maintained on malt extract agar and sub-cultured at regular intervals. For DNA extraction, fungi were grown in 100 ml malt extract broth (Oxoid malt extract 17 gl^{-1}, peptone, 3 gl^{-1}, in distilled water) in Erlenmeyer flasks (250 ml). The medium was inoculated with a mycelial suspension and incubated on a rotary shaker (200 rpm) at 20°C for 3–7 d. For slow growing species the incubation period was extended to 2–3 wk. Contamination of cultures was checked by microscopic examination of growth on PDA agar. The biomass was harvested by vacuum filtration, washed with distilled water, frozen in liquid nitrogen and lyophilised.

DNA extraction Large-scale extraction was performed using the method of Lee and Taylor (1990). Frozen lyophilised mycelial pellets (0.3 g) were ground with a mortar and pestle with dry ice, or liquid nitrogen, for 10–15 min. Lysis buffer (50 mM Tris-HCl pH 7.2, 50 mM EDTA, 3% w/v SDS and 1% v/v β-mercaptoethanol) was added to the mortar, and the mixture extracted repeatedly with phenol: chloroform: isoamyl alcohol (25 : 24 : 1) mix. The nucleic acids were recovered by ethanol precipitation and re-suspended in a low salt Tris-HCl, EDTA buffer (TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). RNA and proteins were removed by digestion with Rnase A (Sigma) and proteinase K (Boehringer, Mannheim).

PCR amplification and DNA sequencing The primers NS1 and NS6 or NS8 (White et al., 1990) were used to amplify the 18S rDNA. The 28S rDNA was amplified using primers LROR and LR7 (Bunyard et al., 1994). PCR reactions were performed in a 100 μl solution containing 1–200 ng DNA, 100 ng of each primer, 10 μl of each dNTP (2 mM), 3 μl of nM $MgCl_2$, 10 μl of 10x NH_4-reaction buffer and 2.5 units of BIOTAQ™ DNA polymerase (BioRad). The amplification cycle for 18S rDNA consisted of an initial denaturation step of 95°C for 5 minutes followed by 35 cycles of (1) denaturation (94°C for 1 min), (2) annealing (52–55°C for 1 min), and (3) elongation (72°C for 1 min), and finally a 5 minutes elonga-

Table 1. Ascospore morphology in sub-genera of *Cordyceps* (after Kobayasi, 1982).

Sub-genera	Ascospore	Examples
Ophiocordyceps	Filiform; septate; not separating into part-spores; discharge whole spores.	*Cor. khaoyaiensis* *Cor. pseudomilitaris*
Eucordyceps	Cylindrical; septate; separate into truncated part-spores; discharge part-spores	*Cor. militaris* *Cor. cylindrica*
Neocordyceps	Filiform; multiseptate; separate into fusoid or truncated part-spores.	*Cor. irangiensis* *Cor. sphecocephala*

Table 2. Species used in this study and the BIOTEC identification code.

Species	Code	Host	Reference
Cordyceps			
Cordyceps militaris (L.: Fr.) Link		Lepidoptera	Link, 1833
Cordyceps pseudomilitaris Hywel-Jones & Sivichai	945.02	Lepidoptera	Hywel-Jones, 1994
Cordyceps khaoyaiensis Hywel-Jones	885.01	Lepidoptera	Hywel-Jones, 1994
Cordyceps cylindrica Petch	2347	Spider	Petch, 1937; Kobayasi, 1982
Cordyceps irangiensis Moureau	3938	Ant	Kobayasi, 1982
Cordyceps sphecocephala f. *oxycephala* (Penz & Sacc.) Kobayasi	4018	Wasp	Kobayasi, 1982
Anamorph species			
Hymenostilbe aurantiaca Hywel-Jones	3450	Ant	Hywel-Jones, 1996
Hymenostilbe state of *Cor. sphecocephala*	4169	Wasp	Hywel-Jones, 1995b
Akanthomyces pistillariiformis (Pat.) Samson & Evans	4996	Lepidoptera	Samson and Evans, 1974
Nomuraea atypicola (Yasuda) Samson	3076	Spider	Samson, 1974
Hypocrella			
Hypocrella discoidea (Berk. & Br.) Sacc.	4093	Scale insect	Hywel-Jones and Evans, 1993
Hypocrella raciborski Zimm.	739.03	Scale insect	Parkin, 1906
Anamorph species			
Aschersonia samoensis P. Henn.	2630	Scale insect	Hywel-Jones and Evans, 1993
Aschersonia badia Patouillard	1431	Scale insect	Patouillard, 1897
Aschersonia placenta Berk. & Br.	4293	Scale insect	Ibrahim and Low, 1993
Torrubiella			
Torrubiella luteorostrata Zimm.	555	Scale insect	Hywel-Jones, 1993
Anamorph species			
Paecilomyces cinnamomeus (Petch) Samson & W. Gams	2692	Scale insect	Hywel-Jones, 1993
Gibellula pulchra (Sacc.) Cavara	2956	Spider	Samson and Evans, 1973
Incertae sedis			
Akanthomyces novoguineensis Samson & Brady	4314	Spider	Samson and Brady, 1982
Akanthomyces arachnophilus (Petch) Samson & Evans	5125	Spider	Samson and Evans, 1974

tion step at 72°C. The amplification cycle for 28S rDNA consisted of denaturation at 96°C for 2 min, followed by 35 cycles of (1) dentaturation (96°C for 1 min), (2) annealing (55°C for 1 min) and (3) elongation (72°C for 2 min) and a final elongation step at 72°C for 10 min. PCR products were separated by agarose gel electrophoresis.

PCR products to be sequenced were amplified using a biotinylated NS1, or LROR primer to facilitate the separation of single-strands, which was achieved using dynabeads and M-280 streptavidin columns (Hultman et al., 1989). Each strand of the template was sequenced using the chain termination method of Sanger et al. (1977) and radiolabelled with [α-^{32}P]dATP (Amersham). The sequencing primers used for this study were the internal PCR primers shown in Table 3. The reaction products were separated by PAGE. After electrophoresis, the acrylamide gels were fixed in methanol/acetic acid and dried prior to X-ray film exposure. Both strands of the amplification products were sequenced from a minimum of four replicate reactions. All sequences were submitted to Genbank, NCBI database (http://www.ncbi.nlm.nih.gov/), and the accession numbers for the 18S and 28S rRNA genes are shown in Table 4.

DNA sequence alignment Partial DNA sequences were assembled using GGC Sequence Analysis software, providing a consensus sequence for each strain from primer positions NS1 to NS6 (approximately 1000 nucleotides), and from LROR to LR7 (approximately 1000 nucleotides). A multiple alignment of these sequences with other fungal 18S and 28S rDNA genes, retrieved from the NCBI database (Table 4), was made using CLUSTAL W (Thompson et al., 1994). Final adjustments to the multiple alignments were made manually in Se-Al (Rambault, 1995) and the sequence editor of PAUP 4.b.2 (Swofford, 1998). In all cases length mutations were included in the alignments.

Phylogenetic analysis Maximum parsimony and bootstrap analyses were performed using PAUP 4.b.2 (Swofford, 1998). Heuristic searches were performed on a stepwise starting tree with random sequence addition on 10 replicas and a tree-bisection-reconnection branch-swapping algorithm. Support for the inferred clades within the trees was obtained by bootstrap analysis (Felsenstein, 1985) from re-sampling of the data set and calculation of decay indices (Bremer, 1988). Trees generated from heuristic searches were compared and maximum likelihood values calculated using the Kishino and Hasegawa test (Kishino and Hasegawa, 1989)

Table 3. Primers used to amplify and sequence fungal ribosomal RNA genes[a].

Primer	Sequence	Reference
Nuclear 18S rRNA gene		
NS1*	(5'-)GTAGTCATATGCTTGTCTC(-3')	White et al., 1990
NS2	(5'-)GGCTGCTGGCACCAGACTTGC(-3')	White et al., 1990
NS3*	(5'-)GCAAGTCTGGTGCCAGCAGCC(-3')	White et al., 1990
NS20UCB	(5'-)TGTCCCTATTAATCATTACG(-3')	Gargas et al., 1992
NS20UCBR*	(5'-)CGTAATGATTAATAGGGACA(-3')	Gargas et al., 1992
NS4	(5'-)CTTCCGTCAATTCCTTTAAG(-3')	White et al., 1990
NS5*	(5'-)AACTTAAAGGAATTGACGGAAG(-3')	White et al., 1990
NS23UCBR	(5'-)GAGTTTCCCCGTGTTGAGTC(-3')	Gargas et al., 1992
NS22UCBR*	(5'-)AGTGATTTGTCTGCTTAATT(-3')	Gargas et al., 1992
NS6	(5'-)GCATCACAGACCTGTTATTGCCTC(-3')	White et al., 1990
NS7*	(5'-)GAGGCAATAACAGGTCTGTGATGC(-3')	White et al., 1990
NS8	(5'-)TCCGCAGGTTCACCTACGGA(-3')	White et al., 1990
Nuclear 28S rRNA gene		
LROR*	(5'-)ACCCGCTGAACTTAAGC(-3')	Bunyard et al., 1994
NL1	(5'-)GCATATCAATAAGCGGAGGAAAAG(-3')	O'Donnell, 1993
NL2	(5'-)CTCTCTTTTCAAAGTTCTTTTCATCT(-3')	O'Donnell, 1993
NL3*	(5'-)AGATGAAAAGAACTTTGAAAAGAGAG(-3')	O'Donnell, 1993
NL4	(5'-)GGTCCGTGTTTCAAGACGG(-3')	O'Donnell, 1993
NL4R*	(5'-)CCGTCTTGAAACACGGACC(-3')	O'Donnell, 1993
LR7	(5'-)TACTACCACCAAGATCT(-3')	Bunyard et al., 1994

a) NS1, ITS1, LROR and NL1 were modified with Biotin at 5' end.
The primers with * are the upstream primers. The other primers are downstream primers.

present in PAUP 4.b.2. Only trees where there were no *a priori* reasons to suspect one tree was better than any other were used in this test. Once tree topologies had been discovered after heuristic searches, character weighting using successive approximation was employed (Farris, 1969). Transversion parsimony was performed by including a cost matrix in the Nexus file where transversion : transition weightings of 2 : 1 and 3 : 1 could be applied to the data.

Spectral analysis Spectral analysis (Hendy and Penny, 1993; Charleston and Page, 1999) was performed on the sequence data for 18–19 taxa using Spectrum ver 2.0 (Charleston, 1998). This programme can not handle more than 20 taxa in an analysis, and so the analyses were confined to taxa within the Clavicipitaceae.

Results and Discussion

PCR amplification of the 18S and 28S rRNA genes The 18S and 28S rDNA were PCR amplified from *Cordyceps militaris* (L.:Fr.) Link, *Cor. pseudomilitaris* Hywel-Jones & Sivichai, *Cor. irangiensis* Moureau, *Cor. sphecocephala* (Klotzsch) Sacc., *Cor. khaoyaiensis* Hywel-Jones, *Cor. cylindrica* Petch, *Akanthomyces pistillariiformis* (Pat.) Samson & Evans, *Nomuraea atypicola* (Yasuda) Samson, *Torrubiella luteorostrata* Zimm., and *Gibellula pulchra* (Sacc.) Cavara. using primers NS1/NS6 for the 18S RNA gene (White et al., 1990), and LROR and LR7 for the 28S rRNA gene (Bunyard et al., 1994). In addition to this, the 28S rRNA gene was amplified from *Hypocrella discoidea* (Berk. & Br.) Sacc., *Aschersonia badia* Pat., *As. samoensis* P.Henn, *Hymenostilbe aurantiaca* Hywel-Jones, *Hymenostilbe* state of *Cor. sphecocephala* (Hywel-Jones, 1995b), *Akanthomyces novoguineensis* Samson & Brady, *Aka. arachnophilus* (Petch) Samson & Evans and *As. placenta* Berk. & Br. The size of the fragments varied from 1238 to 2897 base pairs. Variation was observed in the fragments amplified for the 18S rRNA gene. The majority of species amplified fragments between 1238 to 1318 bp for the region NS1 to NS6 except *Cor. militaris*, *Aka. pistillariiformis* and *Cor. pseudomilitaris*, which produced fragments of 1771, 1662 and 2897 bp respectively. Subsequent sequence analysis revealed the presence of substantial length mutations (391 bp for *Cor. militaris*; 388 bp for *Aka. pistillariiformis* and 1515 bp for *Cor. pseudomilitaris*) in the gene, occurring after primer NS5, for these species. In contrast, very little variation was observed in the size of fragments amplified for the 28S rRNA gene using primers LROR and LR7, where all species amplified a product of approximately 1423 bp.

Identification of phylogenetic signals using spectral analysis Spectral analysis and split decomposition are useful techniques to explore phylogenetic signals in a data set. Spectral analysis was performed on the 18S and 28S rRNA gene sequences to identify phylogenetic signals linking taxa and conflicting signals that might influence parsimony analysis. Spectral analysis visualises all the phylogenetic signals as bipartitions, or splits, in the nucleotide alignment without imposing a tree on it, and shows the frequencies for all possible splits, which

Table 4. Species used in this study: Accession numbers from the NCBI database.

	Accession Number			Accession number	
	18S	28S		18S	28S
DIAPORTHALES			*Akanthomyces arachnophilus*		AF327385*
Valsaceae			*Nomuraea atypicola*	AF327400*	AF327382*
Diaporthe phaseolorum	L36985	U47830	*Gibellula pulchra*	AF327399*	AF327391*
HALOSPHAERIALES			Hypocreaceae		
Halosphaeriaceae			*Aphysiostroma stercoanium*	U32398	U47820
Aniptodera chesapeakensis	U46870	U46882	*Hypocrea gelatinosa*		U00738
Corollospora maritima	U46871	U46884	*Hypocrea lutea*	U32407	U00739
Halosphaeria appendiculata	U46872	U46885	*Hypocrea schweinitzii*	L36986	U47833
Halosarpheia fibrosa		U46886	Nectriaceae		
Nimbospora effusa	U46877	U46894	*Nectria aureofulva*	AB013010	U88123
Nohea umiumi	U46878	U46893	*Nectria ochroleuca*	AB003950	U00752
Ophiodeira monosemeia	U46879	U46894	*Neocosmospora endophytica*		U17411
Mitosporic Halosphaeriales			*Neocosmospora diparietospora*		U17413
Varicosporina ramulosa	U43846	U44092	Mitosporic Nectriaceae		
HYPOCREALES			*Cylindrocladium scoparium*		U17409
Clavicipitaceae			*Cylindrocladium floridanum*		U17408
Atkinsonella sp.		U17397	Bionectriaceae		
Balansia aristidae	U44035	U57677	*Heleococcum japonicum*		U17429
Balansia henningsiana		U57678	*Roumegueriella rufula*		U00754
Balansia obtecta		U17395	Melanosporaceae		
Balansia sclerotica	U32399	U47821	*Melanospora zamiae*		U17404
Balansia strangulans	U44038	U57679	*Melanospora fallax*		U17405
Claviceps fusiformis		U17402	LULWORTHIALES		
Claviceps paspali	U32401	U17398	Lulworthiaceae		
Claviceps purpurea	U44040	U57085	*Lanspora coronata*	U48424	U46889
Cordyceps capitata	AB027318	U57086	MICROASCALES		
Cordyceps intermedia	U46881	U47827	*Microascus trigonosporus*	L36987	U47835
Cordyceps ophioglossoides	U46881	U47827	*Petriella setifera*	U32421	U48421
Cordyceps militaris	AF327392*	AF327374*	Incertae sedis		
Cordyceps pseudomilitaris	AF327394*	AF327376*	*Ceratocystis virescens*	U32418	U17401
Cordyceps khaoyaiensis	AF327393*	AF327275*	*Ceratocystis fimbriata*	U32419	U47824
Cordyceps cylindrica	AF327395*	AF327377*	PHYLLACORALES		
Cordyceps irangiensis	AF327396*	AF327378*	Phyllachoraceae		
Cordyceps sphecocephala	AF327397*	AF327379*	*Glomerella cingulata*		Z18999
Torrubiella luteorostrata	AF327398*	AF327380*	SACCHAROMYCETALES		
Hypocrella discoidea		AF327381*	Saccharomycetaceae		
Hypocrella sp GJS 89-104	U32409	U47832	*Saccharomyces cerevisiae*	M27607	SAC
Epichloë amarillans		U57680	SORDARIALES		
Epichloë typhina	U32405	U17396	Chaetomiaceae		
Mitosporic Clavicipitaceae			*Chaetomium globosum*	U20379	U47825
Paecilomyces tenuipes	AB027334	D85136	Lasiosphaeriaceae		
Paecilomyces cinnamomeus		AF327388*	*Cercophora septenrionales*	U32400	U47823
Hymenostilbe aurantiaca		AF327389*	Diatrypaceae		
Hymenostilbe sp.[a]		AF327390*	*Diatrype disciformis*	U32403	U32403
Aschersonia badia		AF327386*	Xylariaceae		
Aschersonia samoensis		AF327387*	*Daldinia concentrica*	U32402	U47828
Aschersonia placenta			*Xylaria curta*	U32417	U47840
Akanthomyces pistillariiformis	AF327401*	AF327384*	*Xylaria hypoxylon*	U20378	U47841
Akanthomyces novoguineensis		AF327383*			

a) *Hymenostilbe* state of *Cor. sphecocephala*.
* Sequenced in this study.

can be used as a measure of support. The first part of this analysis is to produce a bipartition spectrum of the data where each nucleotide position in the alignment is partitioned into a two-way split according to sequence similarity amongst the taxa. The support, or conflict, of different splits can be visualised using a Lento plot. The spectrum is transformed using a Hadamard conjugation (Hendy and Charleston, 1982), which corrects for multiple state changes under the symmetric two-state (one parameter) model of Cavender (Cavender, 1978). In this case the two-state model consisted of purine/pyrimidine, and pyrimidine/purine, changes. The commonest splits can be represented as a tree by constructing a Manhattan tree, which has a spectrum closest to the one observed. Support from internal edges of the tree can be obtained using the nearest neighbour interchange (NNI). This alters the internal edges of the tree for each perturbation. Each edge in the tree has two possible re-arrangements that can generate adjacent trees. The nearest neighbour interchange measures the support for the edge in the tree and its two possible rearrangements. Figure 1 shows the Manhattan tree and the NNI splits for 19 taxa that belong to the Clavicipitaceae. This analysis used 1670 character states out of a total of 2185 from the combined 18S and 28S rRNA gene sequences. The Manhattan tree (Fig. 1a) shows the commonest splits, and their number, in the data, and the support for each edge is shown in the NNI splits histogram (Fig. 1b). The analysis revealed 12 splits that received support. The split with the greatest frequency was split 384, which grouped the *Neocordyceps* species of *Cor. irangiensis* and *Cor. sphecocephala* from all other taxa. The *Neocordyceps* species also were grouped with *Cor. militaris* and *Cor. pseudomilitaris* by split 260695. This split also included *Gi. pulchra* and *Hypocrella* spp. indicating that there was a phylogenetic signal linking these species to the genus *Cordyceps*. Other frequent splits containing species of *Cordyceps* included split 2112 (*Cor. cylindrica* and *Nom. atypicola*) and split 65540 (*Cor. ophioglossoides* (Ehrh.) Fr. and *Cor. intermedia* Imai). There was, however, no bipartition that linked these groups to the main *Cordyceps* group of *Cor. militaris*, *Cor. pseudomilitaris*, *Cor. irangiensis* and *Cor. sphecocephala* in a split. Conflict was present along some of the edges in this tree; the splits 12288 (*Balansia aristidae* (Atk.) Diehl and *Bal. strangulans* (Montagne) Diehl), 49152 (*Claviceps purpurea* (Fr.) Tulasne and *Cor. capitata* (Fr.) Link) and 61440 (*Bal. aristidae*, *Bal. strangulans*, *Cla. purpurea* and *Cor. capitata*) all had alternative edges that occurred frequently, and were therefore not particularly well supported. The presence of this conflict in the data might explain the poorly supported nodes observed in the parsimony cladogram (clade B) shown in Fig. 3.

Spectral analysis was also performed on 18 clavicipitaceous taxa for the 28S rRNA gene alone. The data set used in this analysis included species where the 18S rRNA gene sequences were not available for study, and allowed the relationships of the genera *Torrubiella* and *Hypocrella* to be investigated. The analysis used 770 character sites out of a total of 1032. The Manhattan tree and NNI splits histogram (Fig. 2a, b) identified 10 splits in the data that occurred frequently with no conflicting edges. The most frequent was split 28864, which partitioned *Cor. militaris*, *Aka. pistillariiformis*, *Cor. pseudomilitaris*, *Aka. novoguineensis* and *Aka. arachnophilus* from the other taxa. This suggested that there was a phylogenetic signal in the data linking the spider-infecting *Akanthomyces* species with the genus *Cordyceps*. These *Akanthomyces* species were grouped together in split 20480 without conflict. Interestingly, split 31168 grouped all of the *Cordyceps* species in the data set with these *Akanthomyces* species, although split 2304 clearly separated the spider-infecting *Cor. cylindrica* with its anamorph *Nom. atypicola*. The other splits bipartitioned *Hyp. discoidea* with its anamorph *As. samoensis* (split 66560) and with *As. badia* (split 99328), and with the plant pathogen *Cla. purpurea* (split 99328). The plant pathogens received only one common split, number 59 (*Bal. aristidae*, *Bal. obtecta* Diehl, *Epichloë typhina* (Per.: Fr.) Tulasne, *Claviceps paspali* F. Stevens & J. G. Hall and *Ep. amarillans* White). Split 3 grouped the two *Balansia* species together. In this analysis, the *Hypocrella* and related species showed a greater affinity for the plant pathogens than *Cordyceps* species, while *To. luteorostrata*, pathogenic on scale insects, showed little relationship to *Aka. arachnophilus*, a spider pathogen with affiliations to *Torrubiella*.

Parsimony analysis of 18S and 28S rRNA gene sequences The complete data set for analysis included 43 taxa where 1196 nucleotides of the 18S rRNA genes were combined with 1069 nucleotides from the 28S rRNA genes to produce an alignment of 2265 nucleotides. *Saccharomyces cerevisiae* E. Hansen was included in this set as the outgroup taxon. An heuristic search of the data with random sequence addition on 10 replicates produced three tree islands containing trees of length 2504, 2505 and 2509 steps with 490 parsimony-informative sites. The island with the smallest tree length contained 4 trees with a consistency index (CI) of 0.474, a retention index (RI) of 0.582, a re-scaled consistency index (RC) of 0.276 and a homoplasy index (HI) of 0.526. A strict consensus of these trees with bootstrap supports is shown in Fig. 3. The Clavicipitaceae species form a single clade with a bootstrap support of 89%, and are separate from other hypocrealean species. The clade contains all species of *Cordyceps*, *Balansia*, *Claviceps*, *Epichloë* and members of *Torrubiella* and *Hypocrella*. The representatives of the genus *Cordyceps* do not form a monophyletic group in this clade. *Cordyceps militaris*, *Cor. pseudomilitaris*, and the mitosporic species *Paecilomyces tenuipes* (Peck) Samson and *Aka. pistillariiformis* form a strongly supported clade (94% bootstrap support) within clade A. The species within this clade are pathogenic on lepidopteran hosts, and are distinct from other *Cordyceps* clades. The *Neocordyceps* species (*Cor. irangiensis* and *Cor. sphecocephala*) form a strongly supported (100% bootstrap support) group within clade A, as do the *Cordyceps* species that infect *Elaphomyces* species. The other species in

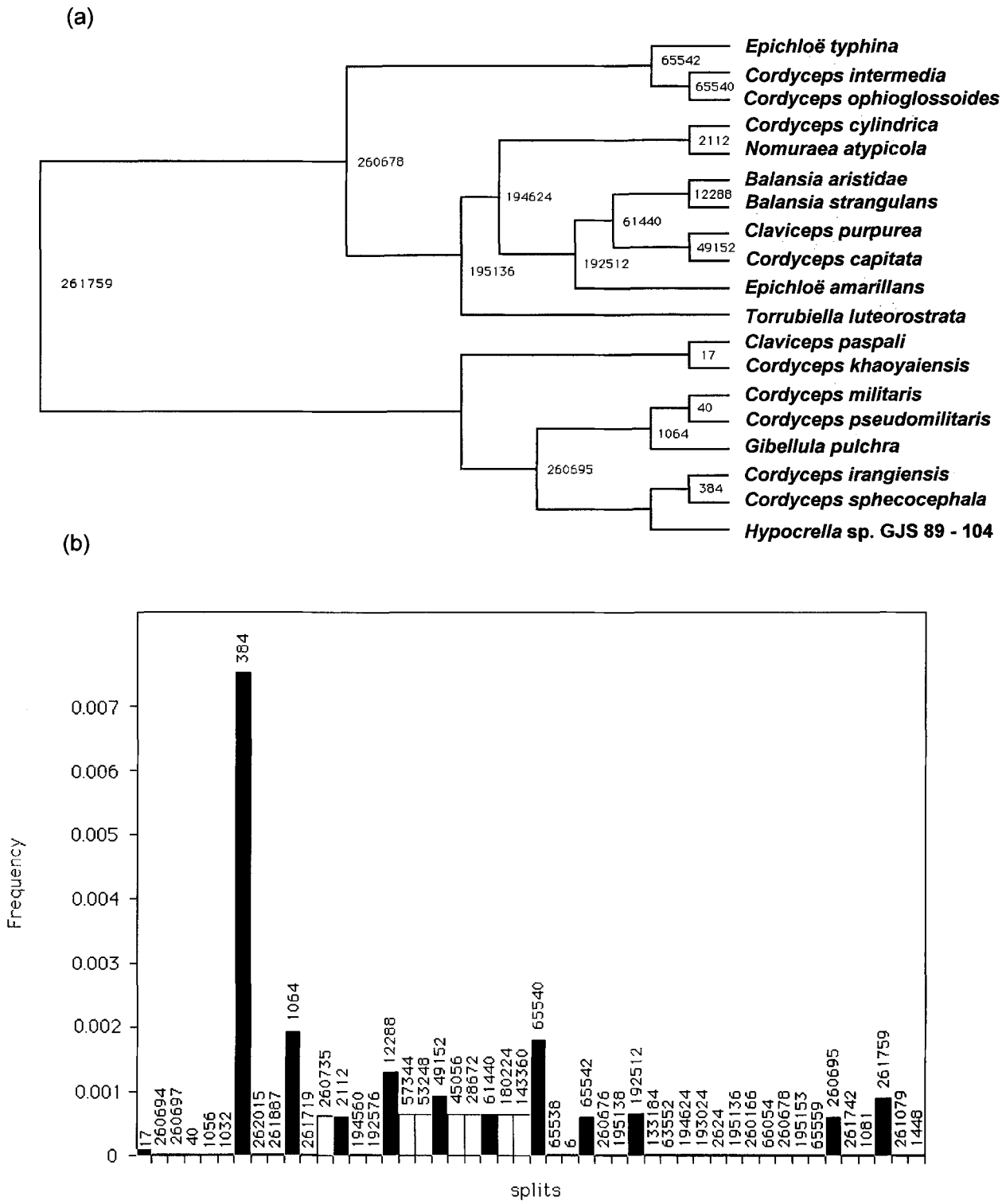

Fig. 1. Manhattan tree (a) and NNI splits histogram (b) generated from a spectral analysis of 19 taxa for 18S and 28S rRNA gene sequences. The numbers at the nodes of the tree and in the histogram are the split number assignations representing a bipartition in the data. The darkly shaded bars in (b) represent splits present in the Manhattan tree. The white bars represent alternative splits not present in the tree.

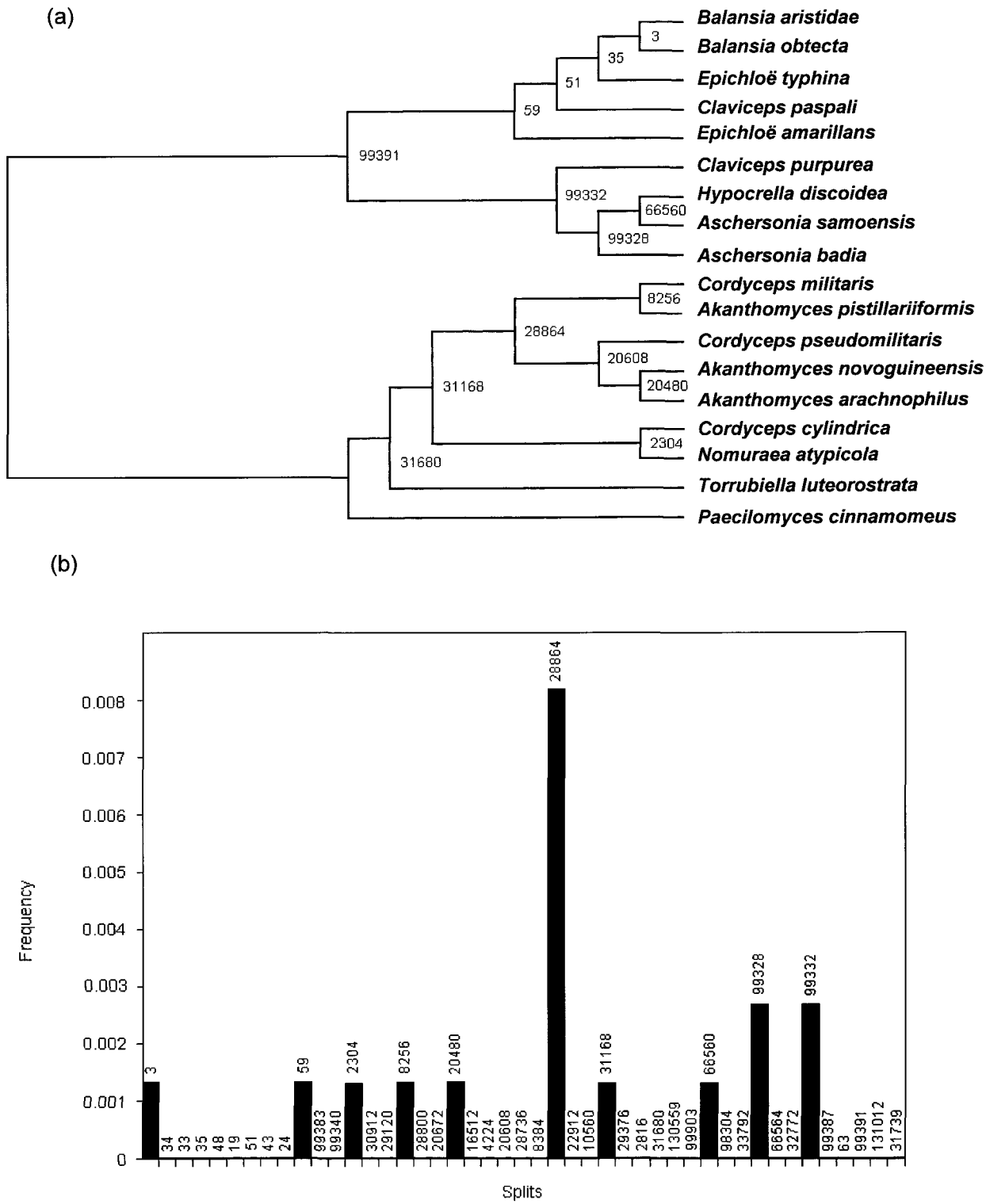

Fig. 2. Manhattan tree (a) and NNI splits histogram (b) generated from a spectral analysis of 18 taxa for 28S rRNA gene sequences. The numbers at the nodes of the tree and in the histogram are the split number assignations representing a bipartition in the data. The darkly shaded bars in (b) represent splits present in the Manhattan tree. The white bars represent alternative splits not present in the tree.

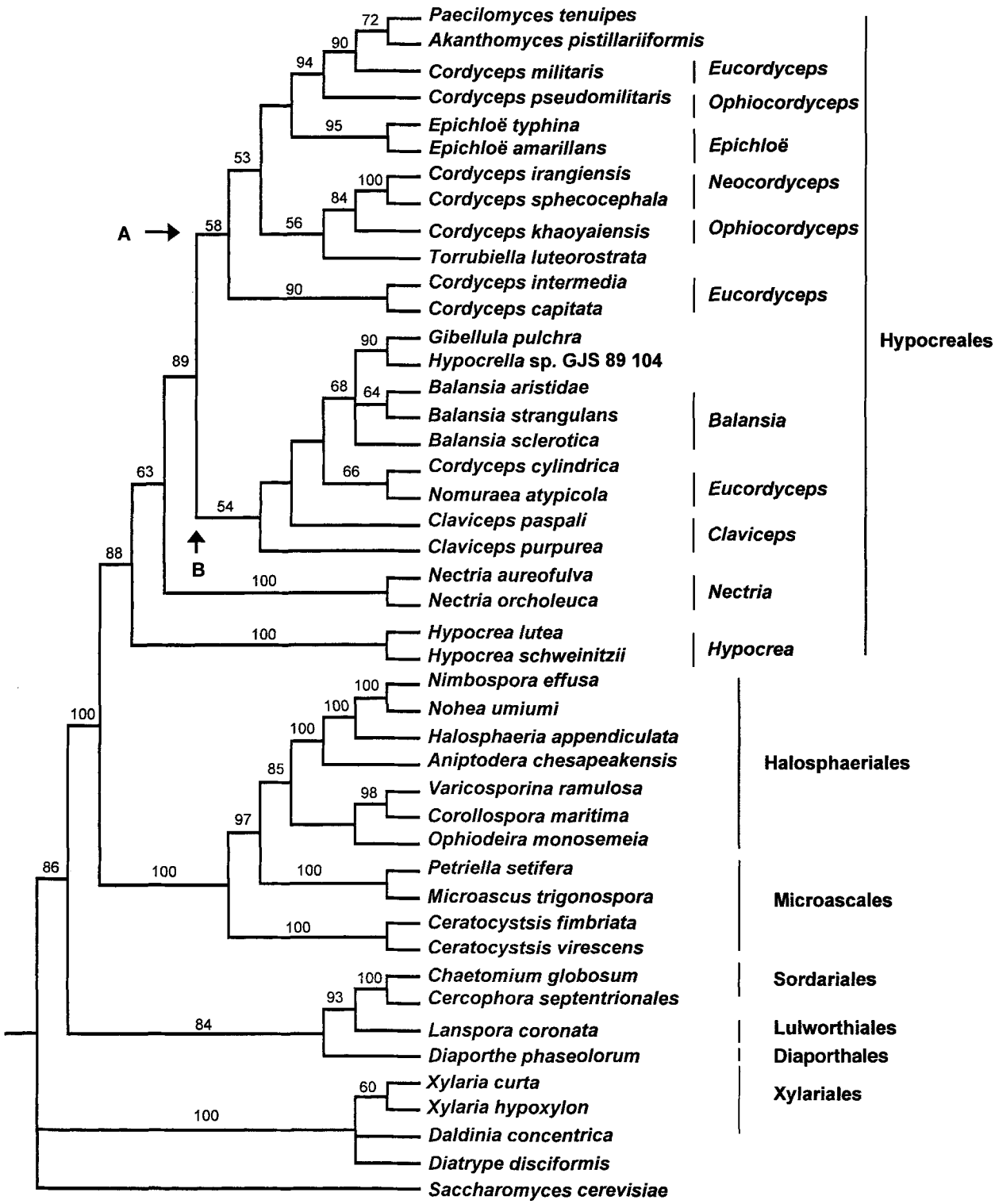

Fig. 3. The strict consensus cladogram of 4 most parsimonious trees generated using 18S and 28S rRNA gene sequences for 45 taxa. The 4 trees had a tree length of 2504 steps, a CI value of 0.474, a RI value of 0.582, a RC value of 0.276 and an HI value of 0.526. Bootstrap values are shown above the branches. Arrows indicate the nodes for clade A and clade B.

clade A include *To. luteorostrata* and the *Epichloë* species, the latter of which form a strongly supported clade. These species are interspersed between the main *Cordyceps* clades, but with little support. The second main clavicipitaceous clade (clade B) contains the other plant pathogens of the group (the genera *Claviceps* and *Balansia*), *Hypocrella* sp. GJS 89–104, *Gi. pulchra* (an anamorphic form of *To. arachnophila*) and *Cor. cylindrica*

and its anamorph *Nom. atypicola*. The internal supports for nodes within this clade are weak, making it difficult to determine the relationships described within it, but it is clear that this clade contains other lineages of the genus *Cordyceps*. The data set contained some homoplasy (HI=0.526), particularly related to the plant pathogens as identified in the spectral analysis, and this reduces the resolution of the tree.

A second heuristic search was performed with a data set of 24 taxa containing 379 parsimony-informative sites. This analysis produced a single most parsimonious tree of 1335 steps from a single tree island with a CI of 0.647, a RI of 0.637, a RC of 0.412 and an HI of 0.353. The resulting cladogram is shown in Fig. 4 with bootstrap supports and decay indices. In this tree the *Cor. militaris* clade and the *Neocordyceps* clade form at the base of the clavicipitaceous clade. These basal *Cordyceps* clades are separated from the other *Cordyceps* clades (*Cor. intermedia* Imai and *Cor. capitata*) and (*Cor. cylindrica* and *Nom. atypicola*) by the plant pathogens belonging to *Epichloë*. The other strongly supported clade within this tree contains the plant pathogens of *Claviceps* and *Balansia*, and the insect pathogens, *Cor. cylindrica* and *Hypocrella* sp. GJS 89–104, an isolate from South America that has an *Aschersonia* anamorph stage (J. Spatafora, pers. commun.)

The relationships detected using a combined data set of 18S and 28S rDNA show that the clavicipitaceous species form a monophyletic clade within the Hypocreales with bootstrap values of 89% (Fig. 3) and 99% (Fig. 4). The hypocreaceous fungi form a basal group to the Clavicipitaceae, suggesting that the latter were derived from a hypocreaceous ancestor. This is in agreement with the observations of Spatafora and Blackwell (1993), Rossman (1993), Glen et al. (1996) and Spatafora et al. (2000), who regarded the clavicipitaceous species as a family, the Clavicipitaceae, within the Hypocreales. One of the primary objectives of this study was to examine the relationships of species assigned to the three sub-genera of *Cordyceps*: *Eucordyceps*, *Ophiocordyceps* and *Neocordyceps*. Of the three sub-genera, only species of *Neocordyceps* form well-defined lineages, while the other species do not form lineages corresponding solely to their classification as *Ophiocordyceps* or *Eucordyceps*. There is also evidence that the groups may be more related with respect to their hosts. The two lepidopteran pathogens of *Cor. militaris* (*Eucordyceps*) and *Cor. pseudomilitaris* (*Ophiocordyceps*) form a strongly supported clade in Figs. 3 & 4. This clade also contains *Aka. pistillariiformis* and *Pae. tenuipes*, which also infect lepidopteran hosts (Hywel-Jones, 1994). The other *Ophiocordyceps* species studied, *Cor. khaoyaiensis*, that also infects lepidopteran hosts does not group with other *Cordyceps* species and may represent another distinct clade. The fungal-pathogens of *Eucordyceps* (*Cor. capitata* and *Cor. intermedia*) also form a strongly supported clade that is positioned differently from the other *Eucordyceps* species that infect spiders. The position of *Cordyceps* species in these trees suggests that the genus is polyphyletic within the Clavicipitaceae. This observation is supported by the spectral analysis of the 18 and 28S rDNA, which indicates that there is no clear phylogenetic signal linking all the *Cordyceps* species together. These results are in agreement with those of Spatafora et al. (2000) who suggested that *Cordyceps* is polyphyletic with three recognised clades: (1) *Cordyceps militaris* group consisting of morphologically similar teleomorphs that produce brightly coloured stromata, superficial to immersed, with fleshy ascomata; (2) *Neocordyceps* group, consisting of *Cor. australis* and *Cor. nutans* Pat. that have oblique ascomata with a faster rate of rDNA nucleotide substitution; and (3) a group consisting of species with a diverse range of ascomatal and stromatal morphology (e.g. *Cor. capitata* and *Cor. gunnii*). Nikoh and Fukatsu (2000) also believed that the genus is polyphyletic. However, their trees only contained *Cordyceps* species, with no representatives from the other clavicipitaceous genera. They distinguish a number of clades: truffle-cicada clade e.g. *Cor. ophioglossoides*; cicada-clade A and B, e.g. *Cor. prolifica* and *Cor. sobolifera* (Hill) Berk.; respectively; scale-insect clade e.g. *Cor. cochildiicola*; and a moth clade e.g. *Cor. militaris*. Although the *Cordyceps* lineages seem clear in these trees, little can be said about their relationships with the genera *Hypocrella* and *Torrubiella*. To answer these questions, a separate analysis was performed using 28S rDNA only.

Parsimony analysis of the 28S rRNA gene sequences A maximum parsimony analysis was performed on the 28S rRNA gene sequences for 58 taxa in order to investigate the relationships of the genera *Hypocrella* and *Torrubiella*, as the 18S rDNA sequences for many taxa were not available for study. 1026 nucleotides were aligned for the 58 taxa with *Saccharomyces cerevisiae* as the outgroup taxon. The data contained 272 parsimony-informative sites. An heuristic search with random sequence addition produced a single most parsimonious tree from four tree islands with tree lengths 1597, 1598, 1599 and 1600 for each island. The single most parsimonious tree (Fig. 5) came from the smallest island and had a CI of 0.425, a RI of 0.603, a RC of 0.256 and an HI of 0.575. The species representing the Clavicipitaceae formed a single clade, with the members of the Hypocreaceae forming a sister clade. *Cordyceps militaris*, *Cor. pseudomilitaris*, *Aka. pistillariiformis* and *As. placenta* form a strongly supported clade with *Cor. capitata* and *Cor. intermedia* at the base of the other clavicipitaceous species. The placement of *As. placenta* in this *Cordyceps* clade is probably doubtful. The teleomorph of *As. placenta* is *Hypocrella raciborski* Zimm. and would be expected in the *Hypocrella* clade. Work is being undertaken to confirm the identity of this isolate. The next *Cordyceps* clade occurs after the plant pathogens. This consisted of *Cor. cylindrica* and its anamorph *Nom. atypicola*. The *Hypocrella* clade of *Hyp. discoidea*, *As. badia* and *As. samoensis* forms after this, with the spider-associated *Akanthomyces* group as a sister clade. This placement is not in agreement with the spectral analysis of the 28S rDNA (Fig. 2), which showed that there were phylogenetic signals linking this *Akanthomyces* group

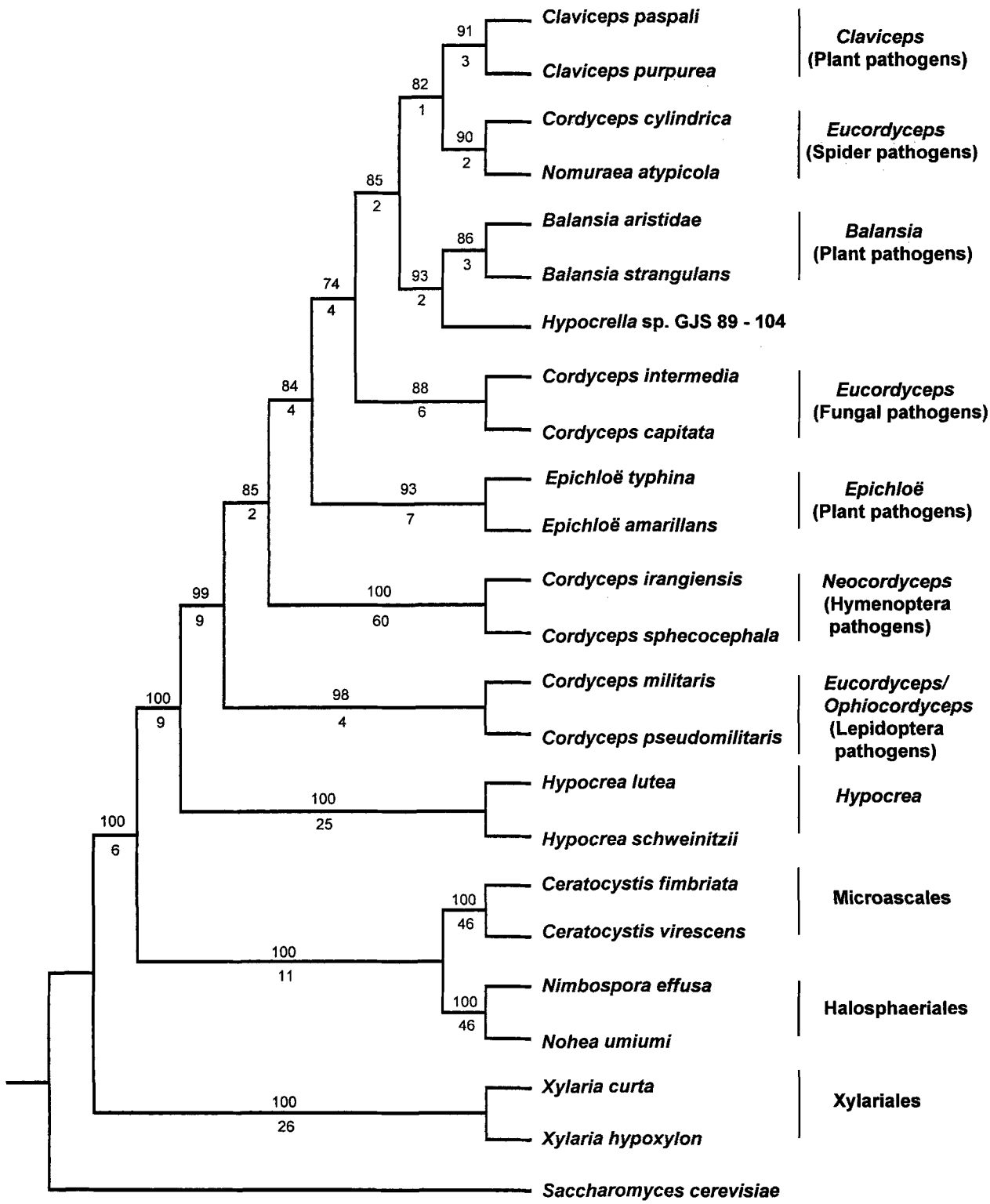

Fig. 4. Cladogram of a single most parsimonious tree generated from 18S and 28S rRNA gene sequences for 24 taxa. The tree had a length of 1335 steps, a CI value of 0.647, a RI value of 0.637, a RC value of 0.412 and an HI of 0.353. Bootstrap values are shown above and decay indices below the branches.

with a *Cordyceps* clade. The topology of this tree is similar to the ones generated for the 18S and 28S rDNA sequences; however there is considerable homoplasy in the data (HI=0.575) and this reduces the support for internal nodes in the tree.

The identification of a phylogenetic signal linking the

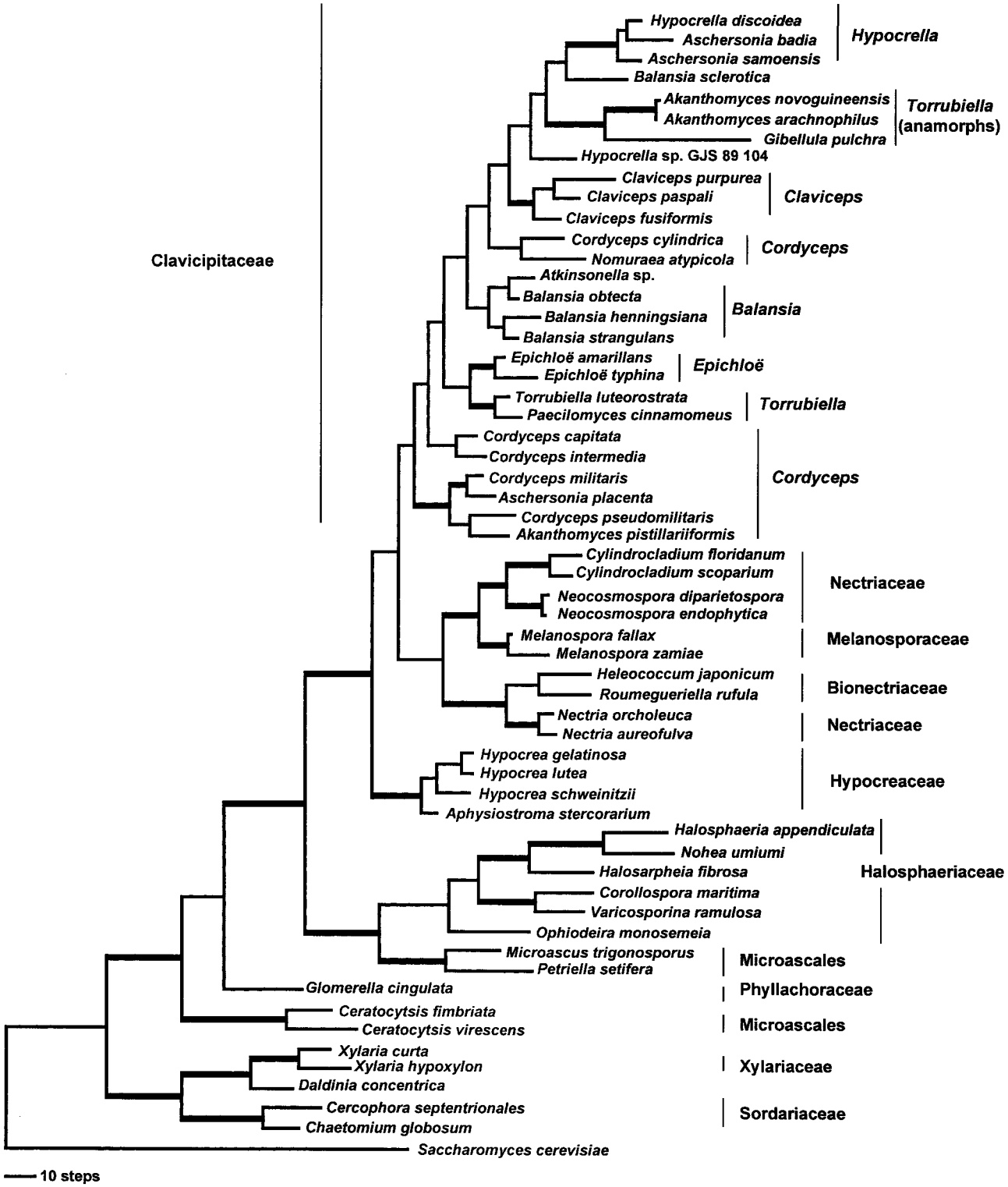

Fig. 5. Phylogram of a single most parsimonious tree for 58 taxa generated from 28S rRNA gene sequences. The tree had a length of 1597 steps, a CI value of 0.425, a RI value of 0.603, a RC value of 0.256 and an HI of 0.575. The darkly shaded branches represent those that received a bootstrap value above 90%.

spider-infecting *Akanthomyces* with the *Cor. militaris* group by spectral analysis was further investigated by performing a transversion parsimony analysis, where transversions were given a greater weight. An heuristic search was performed on a data set of 26 taxa using a cost matrix with a transversion: transition weighting of

2 : 1, and with *Xylaria curta* Fr. as the outgroup taxon. The search was performed with random sequence addition on 10 replicas and a tree-bisection-reconnection branch-swapping algorithm. This produced a single most parsimonious tree (Fig. 6) from a single tree island with 1127 steps (CI=0.636; RI=0.728; RC=0.463; HI=0.364). The phylogram shown in Fig. 6 identifies a number of well-supported clades. The *Neocordyceps* species (*Cor. irangiensis*, *Cor. sphecocephala*, *Hym. aurantiaca* and *Hymenostilbe* state of *Cor. sphecocephala*) form a strongly supported (100% bootstrap) clade at the base of the Clavicipitaceae. These species have a faster rate of rDNA nucleotide substitutions and characteristic sequence patterns that might be used as molecular signals for identification. One of these is found at nucleotide position 111–112 after the end of the NL1 primer. This sequence, 5'-AC(A/G)GCAC(T/C)GAACA-3', is not found in any other clavicipitaceous species examined in this study. The spider-infecting anamorphs of *Torrubiella* (*Aka. novoguineensis*, *Aka. arachnophilus* and *Gi. pulchra*) also form a strongly supported clade (94% bootstrap), which is sister to the *Cor. militaris* clade (98% bootstrap). This is in agreement with the spectral analysis for this genetic region. *Gibellula pulchra* and *A. arachnophilus* have known affiliations with the genus *Torrubiella* (*Gi. pulchra* is the anamorph of

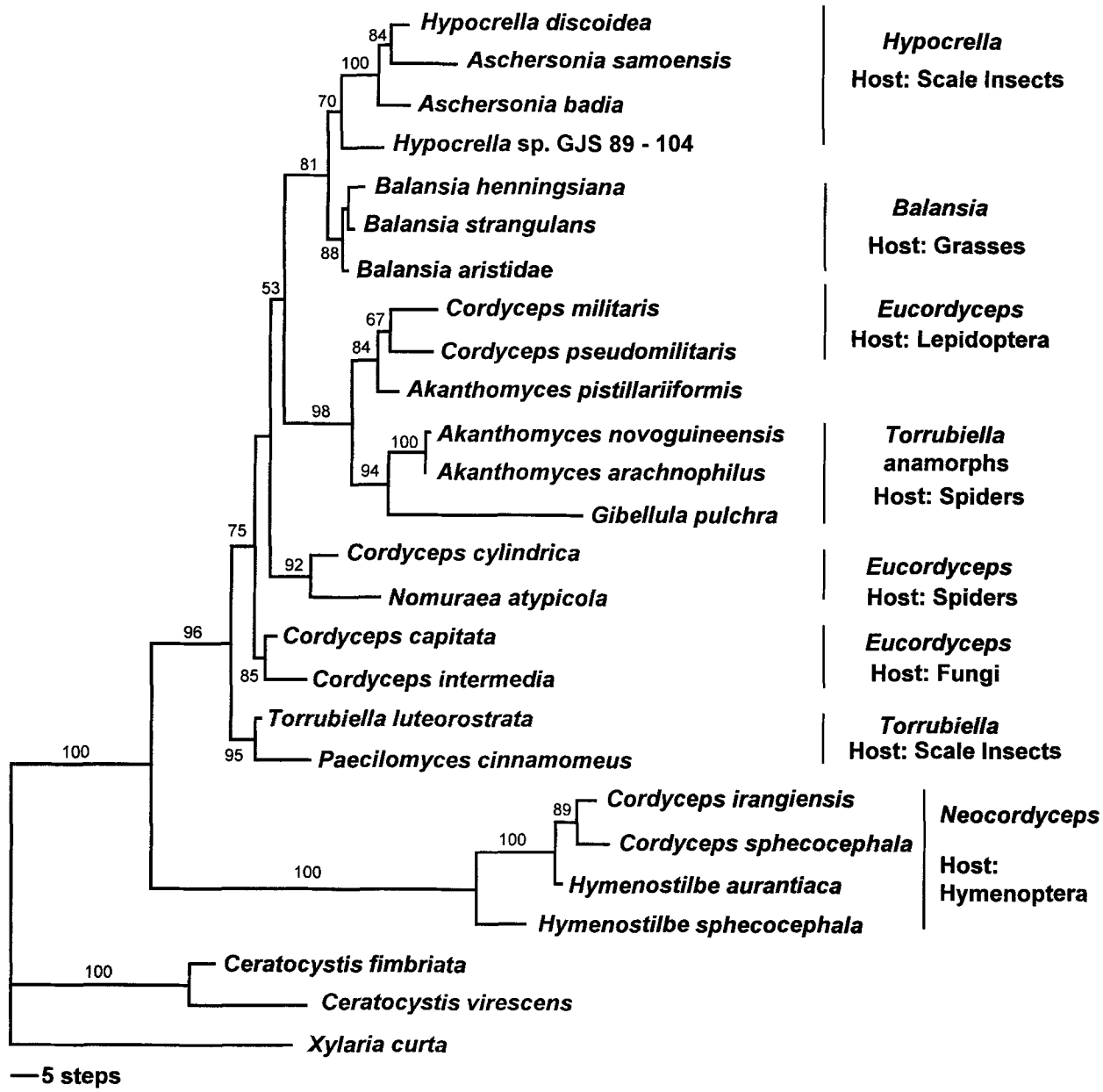

Fig. 6. Phylogram of a single most parsimonious tree for 26 taxa generated from 28S rRNA gene sequences. The data contained 221 parsimony-informative sites and was weighed with a cost matrix giving a transversion: transition ratio of 2 : 1. (Tree length=1127; CI=0.636; RI=0.728; RC=0.463; HI=0.364). Bootstrap values above 50% are shown on the tree.

To. arachnophilus (Johnston) Mains and *Aka. arachnophilus* is the possible anamorph of *To. flava* Petch (Samson and Evans, 1973, 1974). This clade is separate from the second main *Torrubiella* clade (*To. luteorostrata* and *Pae. cinnamomeus*), which infect scale insects (Hywel-Jones, 1993). This indicates that the genus *Torrubiella* may be polyphyletic. In contrast, the genus *Hypocrella* appears to be monophyletic, according to the species used in this study. This genus was represented by four isolates. Three were isolates from Thailand while the fourth was from S. America. The three isolates from Thailand grouped together well and could be identified with species that routinely have ascospores that do not separate into part-spores. These form the *Hyp. discoidea* clade (100% bootstrap support) of *Hyp. discoidea* and its anamorph *As. samonensis*, and *As. badia*. *Hypocrella* sp. GJS 89–104 originated from S. America and has cultural characteristics that fit with *Aschersonia* species that have teleomorphs with part-spores (Spatafora pers. comm.). This species joined the *Hyp. discoidea* clade with a bootstrap support of 70%.

In conclusion, this study confirms the placement of clavicipitaceous genera within the family Clavicipitaceae, order Hypocreales. It seemed logical to expect that the ancestor of the Clavicipitaceae would be a plant pathogen, considering the origins of this group, making a host switch to insects causing subsequent radiation throughout the Insecta. However, our work clearly demonstrates that a *Cordyceps* insect pathogen is basal to all the clades that we discuss with the plant-pathogenic Clavicipitaceae derived within these clades.

The ancestral *Cordyceps* has radiated into many host groups, including plants, from a possible pre-nectrian ancestor. It is therefore to be expected, when considering phylogenetic relationships and co-evolution, that radiations would occur within particular host groups leading to clades exhibiting a host specificity (Evans, 1988) but with clear jumps to other host groups. It is clear from the results presented in this study that the *Cordyceps* form host group clades, rather than clades based on ascospore morphology, but there is little evidence of host-switching within the clades described in this report. However, when further species are included the anticipated widespread nature of host-switching will become more apparent, as in the work described by Nikoh and Fukatsu (2000).

Of the invertebrate-associated genera, *Torrubiella* does not show the host range of *Cordyceps* but it appears to be polyphyletic and has clearly radiated into a wider group of hosts (spiders, scale insects and fungi) than *Hypocrella*. Significantly, *Hypocrella* appears to be the most recently derived genus. It is monophyletic, with a limited host range (scale insects) and also, compared to the other two genera, a limited number of anamorphs.

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