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FULL PAPER

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Phylogenetic study of clavicipitaceous fungi using acetaldehyde dehydrogenase gene sequences

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Abstract *Aciculosporium* and *Heteroepichloë* (Clavicipitaceae) are characteristic bambusicolous fungi in east Asia. In this study, we examined their intergeneric relationships based on the *ALDH1-1* gene, which encodes a member of the aldehyde dehydrogenase family. In the clavicipitaceous fungi examined in this study, the nucleotide sequence of the third exon of *ALDH1-1* (Exon-3) is 889 bp in length and has no insertion/deletion. A phylogenetic tree based on Exon-3 indicated that the clavicipitaceous fungi could be divided into two large groups: *Cordyceps*, *Nomuraea*, and *Ustilaginoidea* species formed a paraphyletic group, and the other grass biotrophic species formed a monophyletic group. This monophyletic group was further divided into three groups with high bootstrap support: i.e., species with *Neotyphodium* anamorphs (e.g., *Epichloë*), species with *Ephelis* anamorphs (e.g., *Heteroepichloë*), and *Aciculosporium–Claviceps* species. We discuss the relationships among *Aciculosporium*, *Heteroepichloë*, and other clavicipitaceous fungi.

Key words *Aciculosporium* · Aldehyde dehydrogenase gene · Clavicipitaceae · *Heteroepichloë* · Phylogenetic study

Introduction

Fungi belonging to the family Clavicipitaceae (Hypocreales, Ascomycota) are biotrophs or pathogens of various hosts consisting predominantly of grasses and insects. Many clavicipitaceous species and their related anamorphs have been investigated for use as biological resources. For example, *Claviceps purpurea* (Fr.) Tul. causes severe ergotism, but produces ergot alkaloids of high pharmacological value (Tudzynski et al. 2001). *Neotyphodium* endophyte-infected tall fescue produces ergot alkaloids responsible for animal toxicosis, but some cultivars, which provide pest and drought resistance to the host grass, have been commercially introduced on pasturelands (Gunter and Beck 2004). Furthermore, entomopathogenic *Cordyceps* and related species are of potential value as medical and biocontrol agents (Shah and Pell 2003; Ng and Wang 2005). However, in contrast to the species that infect economically important grain crops or pasture grasses, the taxonomy, ecology, and potential utilization of many clavicipitaceous species have not been investigated in detail.

We have studied Asian clavicipitaceous fungi, including *Aciculosporium* and *Heteroepichloë* species, ecologically and phylogenetically (e.g., Tsuda et al. 1997; Tanaka et al. 2001, 2002). However, the relationships among *Aciculosporium*, *Heteroepichloë* species, and other clavicipitaceous fungi remain ambiguous, as described below. Diehl (1950) divided the Clavicipitaceae into three subfamilies: Clavicipitoideae, Oomycetoideae, and Cordycipitoideae. All Clavicipitoideae species are biotrophs of grasses (Poaceae) or sedge (Cyperaceae). The essential taxonomic characters in the Clavicipitoideae are conidiogenesis and conidiomata (Rykard et al. 1984). The results of molecular phylogenetic analysis based on 28*S* rDNA also supported the conclusion that the conidial characteristics, which represent the anamorphic genera (*Neotyphodium*, *Sphacelia*, and *Ephelis*), are of significance in the taxonomy of the Clavicipitaceae (Kuldau et al. 1997). *Aciculosporium* was originally classified in the subfamily Oomycetoideae (Diehl 1950). However, its anamorph, which produces holoblastic conidia, resembles the *Ephelis* anamorph associated with the tribe Balansieae in the Clavicipitoideae (Hino 1962). In contrast, a molecular phylogenetic study based on rDNA internal transcribed spacer (ITS) sequences showed *Aciculosporium* to be related to *Claviceps* (the tribe Clavicipiteae in the Clavicipitoideae) (Pazoutova et al. 2004). On the other hand,

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Table 1. Clavicipitaceous species and associated anamorphic fungi used in this phylogenetic study based on nucleotide sequences of the *ALDH1-1* Exon-3

Species	Strain	Voucher number	Host	Location	Nucleotide sequence GenBank Acc. no.
Aciculosporium take	*A1	FA-36792(CBM)/KYO-PRI-Cla-077	*Phyllostachys bambusoides*	Kyoto, Japan	AB257700
	A21	FA-36793(CBM)	*Semiarundinaria yoshi-matsumurae*	Kyoto, Japan	AB257701
Claviceps purpurea		FA-36794(CBM)	*Hordeum vulgare*	Yunnan, China	AB257702
	*Cla-Shi	FA-36795(CBM)	*Festuca arundinacea*	Shiga, Japan	AB257703
Cordyceps heteropoda	Osemi	FA-36796(CBM)	*Graptopsaltria nigrofuscata*	Kyoto, Japan	AB257704
Cordyceps ophioglossoides		FA-36797(CBM)	*Elaphomyces* sp. (False truffle)	Okinawa, Japan	AB257705
Cordyceps paradoxa	Umemura	FA-36798(CBM)	Cicadinae	Miyagi, Japan	AB257706
Cordyceps militaris	*SanagiK	FA-36799(CBM)	Lepidoptera	Kyoto, Japan	AB257707
	SanagiT	FA-36800(CBM)	Lepidoptera	Hokkaido, Japan	AB257708
Ephelis japonica	*Eph.Ska	FA-36801(CBM)/KYO-PRI-Cla-053	*Eragrostis ferruginea*	Kumamoto, Japan	AB257709
	C.paspalum	FA-36802(CBM)	*Paspalum thunbergii*	Jiangxi, China	AB257710
Epichloë typhina	*TM31	FA-36803(CBM)	*Phleum pratense*	Hokkaido, Japan	AB257711
Heteroepichloë bambusae		KYO-PRI-Cla-049	*Giganthochloa* sp.	Bandon, Indonesia	AB257712
Heteroepichloë sasae	H-sasaeA	FA-36804(CBM)/KYO-PRI-Cla-024	*Sasa palmata* (Sect. *Sasa*)	Kyoto, Japan	AB257713
Neotyphodium coenophialum	NZ1	FA-36805(CBM)	*Festuca arundinacea*	New Zealand	AB257714
Nomuraea atypicola	Kumo	FA-36806(CBM)	*Latouchia* sp. (Spider)	Kyoto, Japan	AB257715
Parepichloë cinerea		KYO-PRI-Cla-016	*Sporobolus* sp.	Ilam, Nepal	AB257716
Ustilaginoidea virens		FA-36807(CBM)	*Oryza sativa*	Yunnan, China	AB257717
	*TH91-1	FA-36808(CBM)	*Oryza sativa*	Tochigi, Japan	AB257718

Fungal specimens were deposited in the Kyoto University Museum (e.g., KYO-PRI-CLA-077) or the Natural History Museum and Institute, Chiba (e.g., FA-36792(CBM))
*These fungal isolates were used for Southern hybridization analysis (Fig. 3)

Heteroepichloë species were originally classified in the genus *Epichloë*. However, *Heteroepichloë* species possess *Ephelis* anamorphs (Tanaka et al. 2002), but not *Neotyphodium* anamorphs, which are commonly related to *Epichloë* species. The relationships among the *Heteroepichloë* species and *Ephelis* species were not clarified by molecular phylogenetic analysis based on rDNA ITS sequences (Tanaka et al. 2002). Consequently, previous phylogenetic analyses based on rDNA sequences were insufficient to assess the intergeneric relationships of the clavicipitaceous species.

To obtain more reliable results regarding the phylogenetic relationships among the clavicipitaceous species, we used the sequence of an aldehyde dehydrogenase (ALDH) gene. ALDHs comprise a superfamily of $NAD(P)^+$-dependent aldehyde dehydrogenases [aldehyde: $NAD(P)^+$ oxidoreductases, EC 1.2.1]. Some ALDHs are highly specific for a very limited range of substrates and others show broad substrate specificity. ALDHs are crucial enzymes that metabolize many toxic biologically important aldehydes. These ALDH superfamilies have been reviewed in *Arabidopsis*, human, and other organisms (Yoshida et al. 1998; Sophos and Vasiliou 2003; Kirch et al. 2004). However, fungal ALDH superfamilies have been characterized only in *Saccharomyces cerevisiae* (Navarro-Avino et al. 1999).

In this study, we first analyzed the filamentous fungal ALDH families. We then selected the most highly conserved ALDH gene (*ALDH1-1*) and cloned this gene from genomic DNA and cDNA of *Aciculosporium (Aci.) take* Miyake. Based on this nucleotide sequence, we cloned part of the *ALDH1-1* gene (Exon-3) from several clavicipitaceous fungi and related anamorphic species. Consequently, we discuss the relationships among *Aciculosporium*, *Heteroepichloë*, and other clavicipitaceous fungi based on the phylogenetic tree.

Materials and methods

Fungal materials

Clavicipitaceous species and associated anamorphic fungi used in this study are listed in Table 1. All species were identified by the authors. Of these fungal materials, *Aci. take*, *Ephelis (Eph.) japonica* Henn., *Heteroepichloë (Het.) bambusae* (Pat.) Tanaka et al., *Het. sasae* (Hara) Tanaka et al., and *Parepichloë cinerea* (Berk. & Broome) White & Reddy were used in our other studies (Tanaka et al. 2001, 2002, 2003). *Cordyceps (Cor.) militaris* (L.) Link, *Cor. ophioglossoides* (Ehrh.) Link, and *Cor. paradoxa* Kobayasi were provided by Dr. Noriko Kinjo. Fungal strains of *Aci. take*, *Claviceps* (*Cla.) purpurea*, *Cor. heteropoda* Kobayasi, *Eph. japonica*, *Epichloë (Epi.) typhina* (Pers.) Tul., *Het. sasae*, *Neotyphodium (Neo.) coenophialum* (Morgan-Jones & Gams) Glenn et al., *Nomuraea atypicola* (Yasuda) Samson, and *Ustilaginoidea virens* (Cooke) Takahashi (TH91-1) have been maintained in our laboratory by using complete agar medium [0.15% (w/v) $Ca(NO_3)_2{\cdot}4H_2O$, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, 0.04% (w/v) KH_2PO_4,

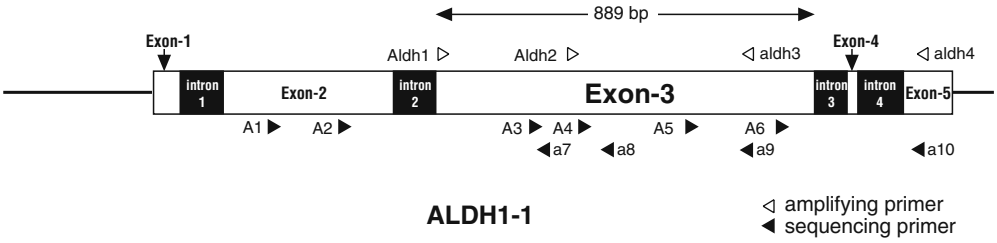


Fig. 1. Positions of primers on the *ALDH1-1* genes of *Aciculosporium (Aci.) take* used in this study. The *ALDH1-1* open reading frame of *Aci. take* is 1491 bp in length and Exon-3 is 889 bp in length. Clavicipitaceous fungi used in this study have the same insertion sites in Intron2, Intron3, and Intron4, although *Cordyceps* species, *Nomuraea atypicola*, and *Ustilaginoidea virens* have no Intron3

0.003% (w/v) K_2HPO_4, 0.1% (w/v) yeast extract, 0.1% (w/v) tryptone, 1% (w/v) glucose, and 1.5 % (w/v) agar].

Molecular biological methods

For cDNA library construction, total RNA of *Aci. take* strain A1 was extracted from liquid cultured mycelia ground in liquid nitrogen following the method of Takano et al. (1995). cDNA libraries were constructed with SMART PCR cDNA Synthesis and Library Construction Kits (Clontech Laboratories, Palo Alto, CA, USA). We designed degenerate primers to clone putative acetaldehyde dehydrogenase genes from the published ALDH genes of filamentous fungi, IADH gene of *Ustilago maydis* (DC.) Corda (GenBank acc. no. U74468), acetaldehyde dehydrogenase genes of *Aspergillus nidulans* (Eidam) Winter (GenBank acc. no. M16197), and *Cladosporium herbarum* (Pers.) Link (GenBank acc. no. X78228). The primers used for amplification were as follows: Aldh1, 5′-GTN TGY GGN CAG ATC ATC CCN TGG AA-3′; Aldh2, 5′-AAC CTS AAG AAG GTS ACN CTN GAG CT-3′; aldh3, 5′-CAC NAC NGG RCC GAA GAT CTC YTC-3′; aldh4, 5′-ACC GGA CTS CTT GWA NCC NCC GAA-3′. The positions of the primers are shown in Fig. 1. We performed nested polymerase chain reaction (PCR) with Aldh1-aldh4 and then Aldh2-aldh3 combinations from the *Aci. take* cDNA library. To obtain the complete cDNA and the regional genomic DNA sequences, we performed cassette-ligation-mediated PCR (Iwahana et al. 1994) with an LA PCR in vitro Cloning Kit (Takara, Otsu, Japan).

For phylogenetic analyses of clavicipitaceous fungi and associated anamorphic species, collected fruiting bodies or cultured mycelia were used (Table 1). The samples were freeze-dried, then ground with liquid nitrogen, and processed as described by Nakada et al. (1994). The extracted DNAs were stored in TE buffer at –20°C. The third exons of the *ALDH1-1* (Exon-3) of clavicipitaceous fungi were amplified and sequenced with the following primers: A1, 5′-GTY TGC GAA GCC ACC GAR AAG GAT GT-3′; A2, 5′-CAA CGG CAA GKC YAT CAC CAT GGC-3′; A3, 5′-GTG CTG CCA TCT CNT CYC ACA TGG A-3; A4, 5′-GGC AAR TCY CCC AAC ATY GTC TTC-3′; A5, 5′-CAG CTS CAG TAY GAC CGY ATC ATG-3′; A6, 5′-TAC GGY CTB GCB GCY GCY ATY CAC AC-3′; a7, 5′-TGA AGG CRA CCT TGT CRA TGT CCA T-3′; a8, 5′-AAG TAR ATK CCR AAG TTG ACC CA-3′; a9, 5′-AAG ATY TCT TCY TGC ATR ATC TTC AT-3′; a10, 5′-ACC GGA CTC CTT GAA GCC NCC GAA-3′. The positions of the primers are shown in Fig. 1. PCR was performed using KOD Dash polymerase (Toyobo, Osaka, Japan) on a PCR Thermal Cycler (iCycler; Bio-Rad, Hercules, CA, USA). These PCR products were cloned and sequenced by the Sanger method using a CEQ 2000 DNA Analysis System (Beckman Coulter, Fullerton, CA, USA). The sequencing reaction was completed with a CEQ DTCS-Quick Start sequencing kit (Beckman Coulter). The sequence data were edited with the software package DNAsis-Mac, version 3.0 (Hitachi Software Engineering, Tokyo, Japan). The determined nucleotide sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers listed in Table 1.

For Southern blotting analyses, genomic DNA were extracted from cultured mycelia of selected clavicipitaceous fungi indicated in Table 1. Southern blotting analyses were carried out using a partial DNA fragment of Exon-3 in the *Aci. take ALDH1-1* as a probe. The 722-bp fragment of the ALDH cDNA amplified with primers Aldh1 and a9 was labeled using Alkphos Direct Labeling Module (Amersham Pharmacia Biotech, Buckinghamshire, UK). Samples of total genomic DNA of *Aci. take* (~20 μg) were digested with *Eco*RI and *Eco*RV, or with *Bam*HI and *Hin*dIII. Furthermore, genomic DNAs of several clavicipitaceous fungi [*Aci. take*, *Cla. purpurea*, *Cor. militaris*, *Epi. typhina*, *Eph. japonica*, and *Ustilagoinoidea (Ust.) virens*; Table 1] were digested with *Bam*HI and *Eco*RI. The samples were electrophoresed on 0.7% (w/v) agarose gels and transferred onto nylon membranes (Hybond-N+). Filters were reacted with CDP-Star detection reagent (Amersham Pharmacia Biotech, Uppsala, Sweden).

Phylogenetic studies

To detect the fungal ALDH superfamily in filamentous fungi, we used the genome sequences of five filamentous fungal species: *Aspergillus (Asp.) nidulans*, *Neurospora crassa* Shear & Dodge, *Gibberella zeae* (Schwein.) Petch, *Magnaporthe grisea* (Hebert) Barr, and *Ustilago maydis*. A phylogenetic study was performed using amino acid sequences of hypothetical ALDH of the filamentous fungi and human ALDH family from NCBI RefSeq database (Table 2). We omitted truncated or duplicated sequences

Table 2. Aldehyde dehydrogenase (ALDH) families of five filamentous fungi and human with the NCBI RefSeq accession numbers database

ALDH family[a]	*Aspergillus nidulans*	*Gibberella zeae*	*Magnaporthe grisea*	*Neurospora crassa*	*Ustilago maydis*	Average similarity[b]	Human
ALDH1							NP_000681 NP_000684
ALDH1-1	XP_658158	XP_381155	XP_361426	XP_956862	XP_758655	70.4%	
ALDH1-2	XP_659293 XP_682303 XP_661730	XP_382449 XP_384370 XP_382336 XP_380315	XP_359769	XP_957264	XP_759670 XP_758913	58.5%	
ALDH2							NP_000680 NP_000683
ALDH3	XP_682254 XP_663248	XP_390136	XP_368525 XP_367986 XP_367345	XP_957628	XP_762570	39.1%	NP_000682 NP_000685 NP_000686 NP_000373
ALDH4	XP_659337 XP_682547 XP_663626	XP_383249 XP_381317	XP_369055	XP_964169	XP_757397	49.9%	NP_003739
ALDH5	XP_661433 XP_680584 XP_662424 XP_659189	XP_386928 XP_384372 XP_384112 XP_390849	XP_363304	XP_964762	XP_761757 XP_761747	48.7%	NP_001071
ALDH6	XP_661195	XP_382002 XP_380666	XP_363680	XP_964701	XP_756361	70.0%	NP_005580
ALDH9	XP_659034	XP_385551	XP_364470		XP_761554	48.9%	NP_000687
ALDH15	XP_664240	XP_388772	XP_366690	XP_964629	XP_759908	53.9%	
I	XP_661658 XP_663039	XP_382568	XP_370036	XP_960135		48.8%	
II	XP_660809 XP_682467 XP_658344	XP_382396 XP_391718 XP_387979	XP_360720	XP_958714 XP_956457	XP_759549	46.7%	
III	XP_662451		XP_365289	XP_964549	XP_760525	47.6%	
IV	XP_659145 XP_661654	XP_380894 XP_384846	XP_368592	XP_964012	XP_760193	47.7%	
V	XP_664745	XP_389938	XP_369650	XP_962018	XP_762117	57.8%	
Not grouped		XP_386007 XP_381935 XP_382472					

I, II, III, IV, and V are labeled as filamentous fungal ALDH families in this study
[a] Nomenclature of ALDH families (1, 2, 3, 4, 5, 6, 9, 15) refers to Sophos and Vasiliou (2003)
[b] Similarities were calculated using the PROTDIST program (PHYLIP, version 3.6; Felsenstein 2004)

from subsequent analyses. The amino acid sequences were aligned with Clustal W (Thompson et al. 1994). This alignment was deposited in TreeBASE (M3497). Amino acid sequence similarities were calculated using the PROTDIST program in the PHYLIP software package version 3.6 (Felsenstein 2004). A phylogenetic tree was constructed using the maximum-parsimony (MP) method with a heuristic search by PAUP*4.0b10 (Swofford 2002) and visualized with TreeView X (Page 1996). Bootstrap analysis was implemented using 1000 replicates of heuristic searches to determine the confidence levels of the inferred phylogenies (Felsenstein 1985).

To compare the *ALDH1-1* sequences of clavicipitaceous fungi and related anamorphic species (see Table 1), the Exon-3 nucleotide sequences were aligned manually. We added corresponding sequence regions from *Neu. crassa* (Sordariales; RefSeq acc. no. XM_951769) and *Gibberella zeae* (Hypocreales; RefSeq acc. no. XM_381155) as an outgroup. *Gibberella (Gib.) zeae* belongs to the family Nectriaceae, which is closely related to the family Clavicipitaceae (Spatafora and Blackwell 1993). The Exon-3 sequences had essentially no insertions or deletions. This alignment was deposited in TreeBASE (M3498). Phylogenetic analysis of the data was performed using the maximum-likelihood (ML) criterion as implemented with PAUP*4.0b10 using the GTR + I + G (general time reversible + gamma + proportion invariant) model as selected by hLRT (hierarchical likelihood ratio test). This model was determined by Modeltest version 3.7 (Posada and Crandall 1988). ML analyses with heuristic search resulted in a single tree. To assess the confidence of the branching patterns of the ML tree, 1000 bootstrap replicates of ML and MP with heuristic search, respectively, were performed (Felsenstein 1985). The bootstrap values are given on the ML tree (see Fig. 4).

Results

ALDH families of filamentous fungi

We constructed a molecular phylogenetic tree based on recorded hypothetical ALDH amino acid sequences of five

filamentous fungi [*Asp. nidulans*, *Gib. zeae*, *Magnaporthe (Mag.) grisea*, *Neu. crassa,* and *Ustilago maydis*] in addition to the amino acid sequences of 12 proteins belonging to the human ALDH family (Yoshida et al. 1998) (Fig. 2, Table 2). The ALDH phylogenetic tree was composed of at least 12 families. Compared to the ALDH families of humans and other organisms, the filamentous fungi possessed families 1, 3, 4, 5, 6, 9, and 15, in accordance with the ALDH gene nomenclature system (Sophos and Vasiliou 2003). ALDH1 of filamentous fungi was further divided into two groups designated here as ALDH1-1 and ALDH1-2. The other five families (I, II, III, IV, and V) corresponded to no relevant established families in eukaryotic organisms including human, although families II, IV, and V were similar to bacterial betaine aldehyde dehydrogenase, bacterial succinic semialdehyde dehydrogenase, and bacterial vanillin dehydrogenase, respectively. These five fungal ALDH families (I, II, III, IV, and V) are probably unique to these organisms.

Among these ALDH families, ALDH1-1 showed the lowest degree of diversity (see Table 2). The average similarity in the ALDH1-1 family was 70.4% and was highest compared to those in each other family (Table 2). In addition, the ALDH1-1 family contained a single copy of each fungal ALDH1-1, in contrast to other ALDH families (Table 2, Fig. 2). These results indicated that ALDH1-1 is the most stable enzyme among the families.

Cloning of the putative acetaldehyde dehydrogenase gene from *Aci. take*

To isolate the *ALDH1-1* gene, we constructed an *Aci. take* cDNA library and amplified the gene from the library using two sets of degenerate primers (Aldh1–aldh4 and Aldh2–aldh3), which were constructed based on conserved amino acid sequences within the *ALDH1-1* genes of five filamentous fungi. After nested PCR, a 385-bp fragment, which showed extensive homology with other *ALDH1-1* genes, was amplified from the cDNA library of *Aci. take.* The longest cDNA clone of the gene and its genomic sequence were also obtained using a cassette-ligation-mediated PCR method. The sequence of this gene (GenBank acc. no. AB259015) indicated the presence of a 1491-bp open reading frame (ORF) encoding a protein of 497 amino acids with a predicted molecular mass of 56 kDa. Four introns were found in the gene by comparison of the sequences of genomic DNA and cDNA (see Fig. 1). Comparison with sequences in the database indicated that the gene showed the highest degrees of similarity to the *ALDH1-1* genes from *Asp. nidulans* (Pickett et al. 1987), *Asp. niger* (O'Connell and Kelly 1988), *Cladosporium herbarum*, and *Alternaria (Alt.) alternata* (Achatz et al. 1995), and the IADH gene from *Ustilago maydis* (Basse et al. 1996) with 60%–70% identity. This protein seems to be a cytoplasmic enzyme, as it lacks any signal sequences or transmembrane regions. The hypothetical active site residues, Asn 165, Glu 263–270, and Cys 291–302 (numbers indicate the corresponding positions in *Aci. take ALDH1-1*), are conserved (Farrés et al. 1995; Wang and Weiner 1995).

Comparison of *ALDH1-1* gene sequences among clavicipitaceous fungi

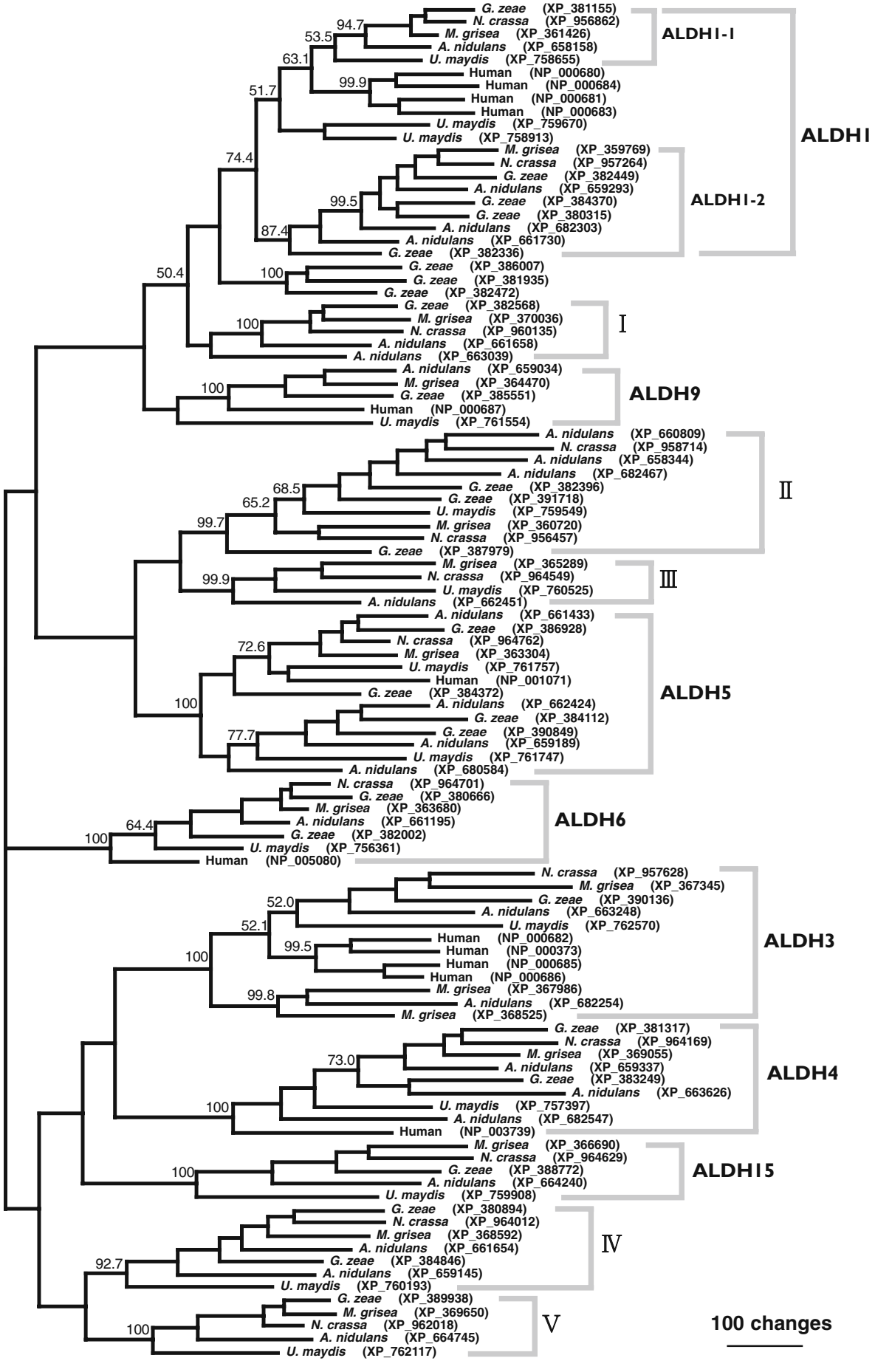
To compare the *ALDH1-1* gene sequences of clavicipitaceous fungi, we designed primers with reference to the *Aci. take ALDH1-1* gene sequence. These primers amplified the *ALDH1-1* gene in all clavicipitaceous fungi used in this study. DNA sequences analyses revealed that *Cla. purpurea*, *Eph. japonica*, *Het. sasae, Het. bambusae*, *Parepichloë (Par.) cinerea*, *Epi. typhina*, and *Neo. coenophialum* had four introns at the same sites as the *Aci. take* sequences (Fig. 1). However, *Cordyceps* species and *Ust. virens* had three introns in their *ALDH1-1*. The intervening sequence corresponding to *Aci. take ALDH1-1* Intron3 was missing in those fungi. For coding regions, no insertions or deletions of bases were found in the third exon (889 bp) of *Aci. take* and its corresponding regions of any other of the fungi examined in the present study. Therefore, we used this region (Exon-3) of those fungi for phylogenetic analysis, because there is no ambiguity in sequence alignment. The alignment data in this study were deposited in TreeBASE (M3498). The lowest level of sequence similarity in Exon-3 was 74.1% between those of *Eph. japonica* and *Cor. heteropoda*.

Southern blotting analyses were performed in order to show that the *ALDH1-1* paralogue does not exist in *Aci. take* and the other fungi used in this study (Fig. 3). The blots showed a very simple band pattern. Similar patterns were also obtained in the nonstringent hybridization condition (data not shown). These results indicated that each sequence for the phylogenetic study was a *ALDH1-1* orthologue.

Phylogenetic study using *ALDH1-1* Exon-3 sequences

ML analysis based on the 889-bp Exon-3 sequences of the *ALDH1-1* produced a distinct tree (Fig. 4). The following parameters were found: empirical base frequencies (freqA = 0.2208; freqC = 0.3264; freqG = 0.2531; freqT = 0.1997) and substitution rates ([A–C] = 1.2114; [A–G] = 2.6693; [A–T] = 0.6493; [C–G] = 0.7645; [C–T] = 5.9660; [G–T] = 1). The proportion of invariable sites was 0.4518, and the gamma distribution shape parameter (alpha) was 2.4384. The –lnL score was 6601.6665. The ML bootstrap values (1000 replicates; >50%) are indicated above the nodes in Fig. 4. MP analysis produced three trees. The strict consensus tree was topologically equivalent to the ML tree, and bootstrap values (1000 replicates; >50%) are shown below the nodes in Fig. 4.

The phylogenetic tree showed two distinct groups: a paraphyletic group including "*Cordyceps–Nomuraea–Ustilaginoidea*" species and another monophyletic group that included the other grass biotrophic species. The separation was supported by bootstrap values of 92% (ML) or 96% (MP). It is notable that *ALDH1-1* genes of the paraphyletic group have no intron sequences equivalent to the intron 3 in those of the monophyletic group. The monophyletic group was further divided into three distinct subgroups with high bootstrap support. The "*Aciculospo-*


G. zeae (XP_381155)
N. crassa (XP_956862)
M. grisea (XP_361426)
A. nidulans (XP_658158)
U. maydis (XP_758655)
Human (NP_000680)
Human (NP_000684)
Human (NP_000681)
Human (NP_000683)
U. maydis (XP_759670)
U. maydis (XP_758913)
M. grisea (XP_359769)
N. crassa (XP_957264)
G. zeae (XP_382449)
A. nidulans (XP_659293)
G. zeae (XP_384370)
G. zeae (XP_380315)
A. nidulans (XP_682303)
A. nidulans (XP_661730)
G. zeae (XP_382336)
G. zeae (XP_386007)
G. zeae (XP_381935)
G. zeae (XP_382472)
G. zeae (XP_382568)
M. grisea (XP_370036)
N. crassa (XP_960135)
A. nidulans (XP_661658)
A. nidulans (XP_663039)
A. nidulans (XP_659034)
M. grisea (XP_364470)
G. zeae (XP_385551)
Human (NP_000687)
U. maydis (XP_761554)
A. nidulans (XP_660809)
N. crassa (XP_958714)
A. nidulans (XP_658344)
A. nidulans (XP_682467)
G. zeae (XP_382396)
G. zeae (XP_391718)
U. maydis (XP_759549)
M. grisea (XP_360720)
N. crassa (XP_956457)
G. zeae (XP_387979)
M. grisea (XP_365289)
N. crassa (XP_964549)
U. maydis (XP_760525)
A. nidulans (XP_662451)
A. nidulans (XP_661433)
G. zeae (XP_386928)
N. crassa (XP_964762)
M. grisea (XP_363304)
U. maydis (XP_761757)
Human (NP_001071)
G. zeae (XP_384372)
A. nidulans (XP_662424)
G. zeae (XP_384112)
G. zeae (XP_390849)
A. nidulans (XP_659189)
U. maydis (XP_761747)
A. nidulans (XP_680584)
N. crassa (XP_964701)
G. zeae (XP_380666)
M. grisea (XP_363680)
A. nidulans (XP_661195)
G. zeae (XP_382002)
U. maydis (XP_756361)
Human (NP_005080)
N. crassa (XP_957628)
M. grisea (XP_367345)
G. zeae (XP_390136)
A. nidulans (XP_663248)
U. maydis (XP_762570)
Human (NP_000682)
Human (NP_000373)
Human (NP_000685)
Human (NP_000686)
M. grisea (XP_367986)
A. nidulans (XP_682254)
M. grisea (XP_368525)
G. zeae (XP_381317)
N. crassa (XP_964169)
M. grisea (XP_369055)
A. nidulans (XP_659337)
G. zeae (XP_383249)
A. nidulans (XP_663626)
U. maydis (XP_757397)
A. nidulans (XP_682547)
Human (NP_003739)
M. grisea (XP_366690)
N. crassa (XP_964629)
G. zeae (XP_388772)
A. nidulans (XP_664240)
U. maydis (XP_759908)
G. zeae (XP_380894)
N. crassa (XP_964012)
M. grisea (XP_368592)
A. nidulans (XP_661654)
G. zeae (XP_384846)
A. nidulans (XP_659145)
U. maydis (XP_760193)
G. zeae (XP_389938)
M. grisea (XP_369650)
N. crassa (XP_962018)
A. nidulans (XP_664745)
U. maydis (XP_762117)
94.7
53.5
63.1
99.9
51.7
74.4
99.5
87.4
50.4
100
100
100
100
68.5
65.2
99.7
99.9
72.6
100
77.7
64.4
100
52.0
52.1
100
99.5
99.8
73.0
100
100
92.7
100
ALDH1-1
ALDH1-2
ALDH1
I
ALDH9
II
III
ALDH5
ALDH6
ALDH3
ALDH4
ALDH15
IV
V
100 changes

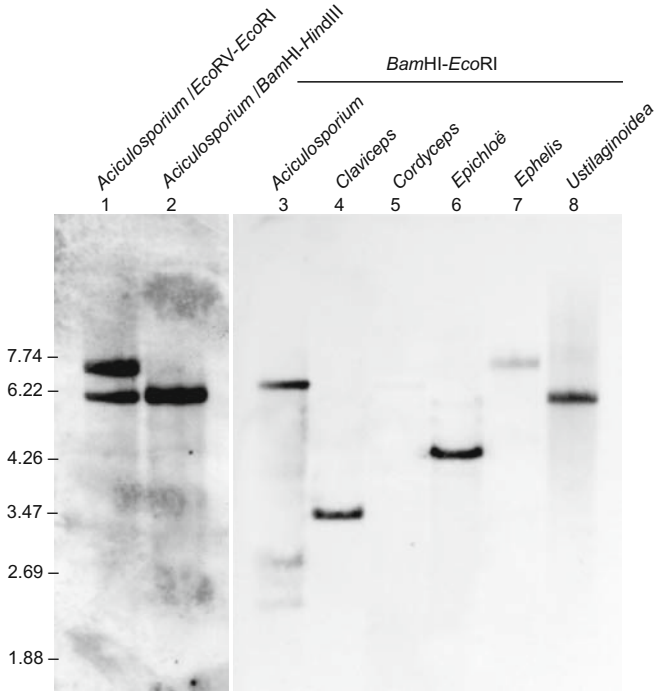


Fig. 3. Southern hybridization analysis of genomic DNA in clavicipitaceous fungi. The probe was a truncated *ALDH1-1* Exon-3 from *Aciculosporium take*. *Lane 1*, *Aci. take* digested with *Eco*RV and *Eco*RI; *lane 2*, *Aci. take* digested with *Bam*HI and *Hin*dIII; *lanes 3–8*, genomic DNA digested with *Bam*HI and *Eco*RI; *lane 3*, *Aci. take*; *lane 4*, *Claviceps purpurea*; *lane 5*, *Cordyceps militaris*; *lane 6*, *Epichloë typhina*; *lane 7*, *Ephelis japonica*; *lane 8*, *Ustilaginoidea virens*. *Eco*RV cut once within the *ALDH1-1* Exon-3 of *Aci. take*. *Bam*HI, *Eco*RI, and *Hin*dIII did not cut this region. Sizes of marker fragments are indicated at *left* in kilobase pairs. *Lane 5* showed a single weak signal at about 6.2 kilobase pairs. The very weak signals seen in *Epi. typhina* were false positive in view of the difference of signal intensity. This result showed that the *ALDH1-1* paralogue does not exist in these fungal genomes. The fungal isolates are listed in Table 1

rium–Claviceps" subgroup was supported by bootstrap values of 94% (ML) and 96% (MP). The "*Epichloë–Neotyphodium*" subgroup was supported by bootstrap values of 100% (both ML and MP) and formed by species with *Neptyphodium* anamorphs. The "*Parepichloë–Heteroepichloë–Ephelis*" subgroup was also supported by bootstrap values of 100% (both ML and MP) and formed by species with *Ephelis* anamorphs, except for *Parepichloë*.

Discussion

ALDH families of filamentous fungi

The filamentous fungi and human ALDH phylogenetic tree was composed of at least 12 families, five of which (I, II, III, IV, and V) could not be classified into any known ALDH families. Sophos and Vasiliou (2003) suggested that the characteristic grouping of ALDH families may be attributable to differences in endogenous substrates. Thus, the existence of ALDH families specific to filamentous fungi suggests that these organisms may have different endogenous substrates from other major taxonomic groups.

ALDH1-1 gene of filamentous fungi

ALDH1-1 amino acid sequences showed a high degree of conservation across filamentous fungi. The genomes of the clavicipitaceous fungi analyzed in the present study each had no paralogue of the *ALDH1-1* gene. In addition, the profiles of fungal ALDH1-1 enzymes have been studied in *Asp. nidulans*, *Asp. niger*, *Cladosporium fulvum*, *Alt. alternata*, and *Ustilago maydis* (Pickett et al. 1987; O'Connell and Kelly 1988; Achatz et al. 1995; Basse et al. 1996). Therefore, we considered this *ALDH1-1* gene appropriate for molecular phylogenetic study. The *Aci. take ALDH1-1* gene sequence showed conservation of active site residues, strongly suggesting that ALDH1-1 possesses enzymatic activity. Moreover, the 889-bp Exon-3 sequence had no insertions or deletions in the orthologous sequences of any of the clavicipitaceous fungi studied. In conclusion, we used Exon-3 nucleotide sequences of the *ALDH1-1* to determine the phylogenetic relationships among clavicipitaceous fungi.

Phylogenetic study

Based on the phylogenetic tree of the Exon-3 nucleotide sequences of *ALDH1-1*, the clavicipitaceous fungi were separated into two groups (Fig. 4). One paraphyletic group containing *Cordyceps* species, *Nomuraea (Nom.) atypicola*, and *Ust. virens*, and another monophyletic group containing grass-associated clavicipitaceous fungi, which was further divided into three groups with high boots-trap confidence: *Epichloë–Neotyphodium*, *Parepichloë–Heteroepichloë–Ephelis*, and *Claviceps–Aciculosporium*. Recent molecular phylogenetic studies revealed the paraphyly of *Cordyceps* species (Nikoh and Fukatsu 2000; Artjariyasripong et al. 2001; Sung et al. 2001; Stensrud et al. 2005). The paraphyly of *Cordyceps* species and the monophyly of grass-associated genera were also demonstrated based on multilocus (*LSU*, *TEF1*, and *RPB1*) sequences (Chaverri et al. 2005). Furthermore, the grass-associated genera possessing *Ephelis*, *Neotyphodium*, and *Sphacelia* anamorphs were separated monophyletically based on analyses of rDNA sequences (Kuldau et al. 1997; Sullivan et al. 2001; Bischoff et al. 2004). These studies support the reliability of our phylogenetic analysis and establish the effectiveness of the *ALDH1-1* Exon-3 sequences for analysis among the genera in the Clavicipitaceae. Our previous phylogenetic studies based on

Fig. 2. Parsimonious tree derived from amino acid sequences of ALDHs of five filamentous fungi and human (cf. Table 2). The tree is midpoint rooted at the *left edge*. Heuristic search found one parsimonious tree (length = 18164; CI = 0.379; RI = 0.535; RC = 0.203; HI = 0.621). Bootstrap values greater than 50% (1000 replicates) are given at the *nodes*. *Bar* represents 100 amino acid substitutions

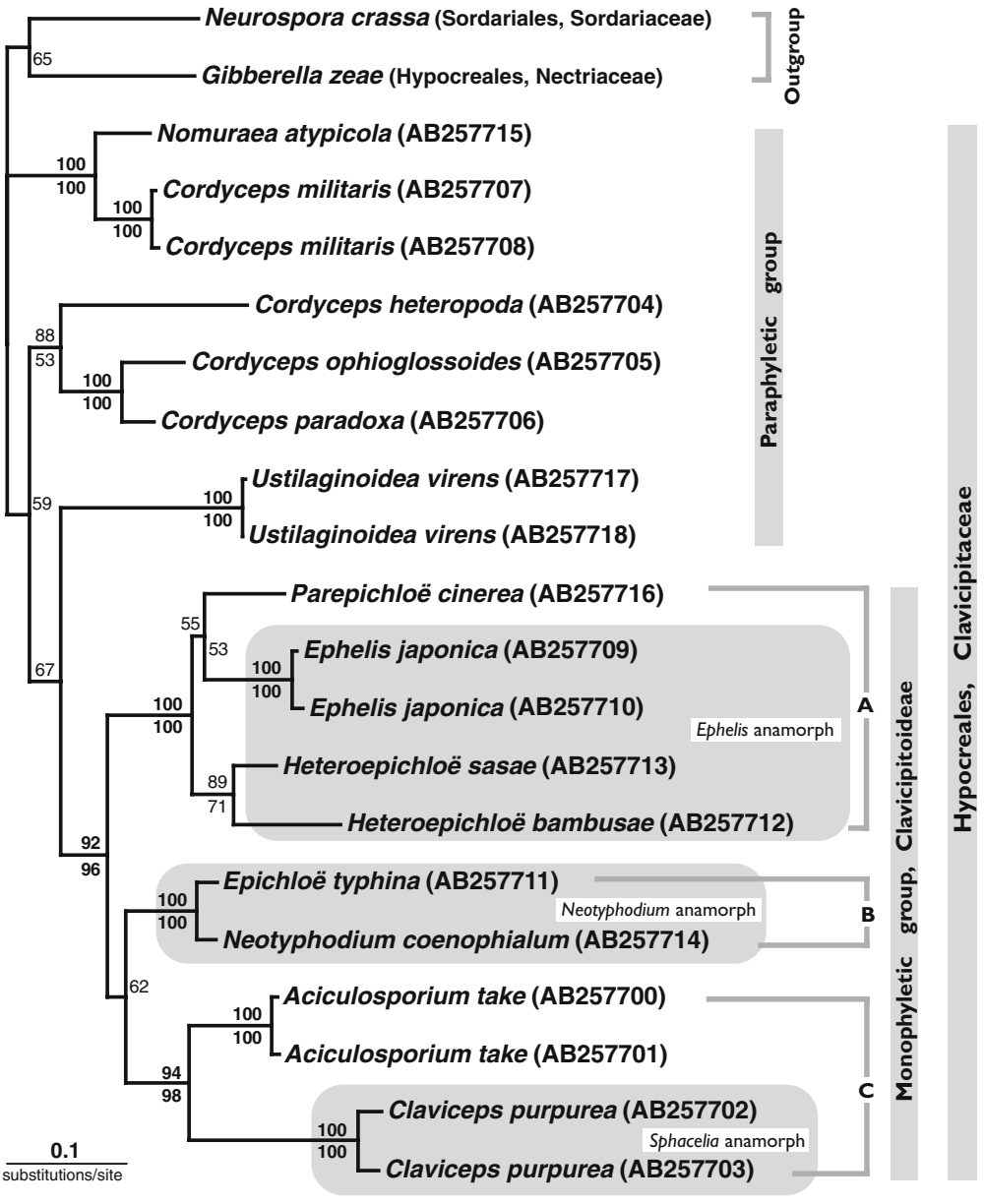


Fig. 4. Maximum-likelihood phylogram from the sequence alignment of Exon-3 of the *ALDH1-1*. The designated outgroup consists of *Gibberella zeae* (RefSeq acc. no. XM_381155) and *Neurospora crassa* (RefSeq acc. no. XM_951769). The *ln* likelihood is –6601.6665. Maximum likelihood bootstrap values greater than 50% (1000 replicates) are given (the values were rounded to the nearest whole number) *above the nodes*. Maximum parsimony bootstrap values greater than 50% (1000 replicates) are given below the nodes. The sample data are listed in Table 1. A–C groups: *A*, *Parepichloë–Heteroepichloë–Ephelis*; *B*, *Epichloë–Neotyphodium*; *C*, *Claviceps–Aciculosporium*. *Bar* represents a distance of 0.1 expected substitutions per site

rDNA sequences showed no apparent relationships between Asian bambusicolous *Aci. take* and *Heteroepichloë* species (Tanaka et al. 2002). Based on our results, we present a detailed discussion of the relationships among the clavicipitaceous fungi, in particular the Asian bambusicolous *Heteroepichloë* species and *Aci. take*, next.

Cordyceps-Nomuraea-Ustilaginoidea

The paraphyletic *Cordyceps-Nomuraea-Ustilaginoidea* group was separated into three groups: *Cor. militaris-Nom. atypicola* group, *Cor. ophioglossoides-Cor. paradoxa-Cor. heteropoda* group, and *Ust. virens*. The grouping of *Cor. militaris-Nom. atypicola* disagrees with other studies (Artjariyasripong et al. 2001; Stensrud et al. 2005). *Nomuraea atypicola* is considered the anamorph of *Cor. cylindrica* Petch (Petch 1937), and the connection was verified (Artjariyasripong et al. 2001). The grouping of *Cor. ophioglossoides-Cor. paradoxa-Cor. heteropoda* confirmed that *Cor. ophioglossoides* parasitizing false truffles (*Elaphomyces* spp., Basidiomycetes) is closely related to some parasites of cicadas (e.g., *Cor. paradoxa*) as reported elsewhere (Nikoh and Fukatsu 2000; Stensrud et al. 2005). The position of *Ust. virens* was intermediate between the *Cordyceps* species and the other grass-associated clavicipitaceous species. This result confirmed that *Ust. virens* on *Oryza sativa* is a member of the Clavicipitaceae. The teleomorph of *Ust. virens* has been treated as *Claviceps (Cla.) orizae-sativae* Hashioka (Hashioka 1971). However, our results indicated that *Ust. virens* has no affinity to the genus *Claviceps*. Bischoff et al. (2004) also confirmed that the *Ustilaginoidea* anamorph is distinct from *Claviceps*. Therefore, the teleomorph of *Ust. virens* should be transferred from

Claviceps to another teleomorphic genus in the family Clavicipitaceae.

Epichloë and *Neotyphodium*

Epichloë and *Neotyphodium* formed a monophyletic group, which was expected as *Neotyphodium* species have strong affinities to *Epichloë* species (Schardl et al. 1991). Some studies showed that *Neotyphodium* spp. possess multiple alleles (e.g., *tef1*, *tub2*), as they are thought to be of hybrid origin (Moon et al. 2002, 2004; Gentile et al. 2005). Nevertheless, there was no variation in *ALDH1-1* Exon-3 sequence of *Neo. coenophialum* in our study.

Heteroepichloë, *Parepichloë*, and *Ephelis*

Heteroepichloë-Parepichloë-Ephelis were placed in a single monophyletic group with high bootstrap support. These are *Balansia*-related species producing dark-colored stromata on culms, leaves, or inflorescences. *Heteroepichloë* species produce black ascostromata, which cover the lower face of the emerging flag leaves, and possess *Ephelis*-type conidia, similar to *Balansia* species (Tanaka et al. 2002). *Ephelis japonica*, which inhabits East Asia, produces dark-colored conidiostromata encircling the inflorescences of host warm-season grasses, and is closely related to *Balansia discoidea* and *Bal. andropogonis* (Tanaka et al. 2001). Although *Par. cinerea* has no anamorph stage, it shows morphological similarities to *Balansia* in the black ascostromata on inflorescence (White and Reddy 1998).

The genus *Heteroepichloë* is composed of two species, *Het. sasae* (synonyms: *Epichloë sasae*, *Parepichloë sasae*), which is distributed in Japan, and *Het. bambusae* (synonyms: *Epichloë bambusae*, *Parepichloë bambusae*), which is distributed in Southeast Asia. They were first treated as *Epichloë* species and then as *Parepichloë* species. However, Tanaka et al. (2002) reported that they differed from *Parepichloë* and *Epichloë* species. Our results based on analysis of the *ALDH1-1* Exon-3 sequences also indicated that *Heteroepichloë* species are separate from *Par. cinerea* and can be grouped with *Balansia*-related species. The relationships between *Heteroepichloë* and other species that possess *Ephelis*-type conidia (e.g., *Balansia*, *Myriogenospora*) have not been studied. The ascostromata of *Heteroepichloë* species are similar to those of *Myriogenospora* spp., which produce epibiotic black stromata and form regularly embedded perithecia. However, *Myriogenospora* species form fusiform part-spores, which distinguishes them from other species of the Balansieae (White and Glenn 1994). In regard to this character, *Heteroepichloë* species form dumbbell-like part-spores (Hino 1961), and they are obviously different from the others.

Aciculosporium and *Claviceps*

Based on the phylogenetic tree, *Aciculosporium* and *Claviceps* can be placed in a single group with high bootstrap support. This result shows that *Aciculosporium* is closely related to *Claviceps*. However, the characteristics of *Aci. take* are different from those of *Claviceps*. *Claviceps* species are characterized as follows: (1) typically possessing enteroblastic conidia (anamorph *Sphacelia*) associated with honeydew production and often possessing holoblastic secondary conidia (Pazoutova et al. 2004); (2) infecting florets of grasses and replacing the host ovules with a sclerotium; and (3) forming stipitate ascostromata arising from sclerotia. *Aciculosporium take* is characterized as follows: (1) possessing two types of conidia: three-celled filiform conidia with swollen ends, and two-celled holoblastic macroconidia with two dichotomously branched apical appendages that separate by breaking at the middle septum, with new germination occurring from the basal end (Tsuda et al. 1997); (2) living endophytically in vegetative shoots of bamboo (Poaceae) (Nozu and Yamamoto 1972); (3) forming conidiostromata and sessile ascostromata at the shoot of witches' broom twigs. These characteristics of *Aci. take* are similar to those of Balansieae species rather than those of *Claviceps*. Thus, *Aciculosporium* previously was suggested to belong to *Balansia* (Hino 1962). In recent years, two clavicipitaceous fungi producing apical appendaged conidia were reported: *Neoclaviceps monostipa* White et al. (Sullivan et al. 2001) and *Cepsiclava phalaridis* (Walker) Walker (Walker 2004). *Neoclaviceps monostipa* infects individual florets of grasses in Costa Rica; it replaces the host ovules and produces stipitate ascostromata from parasitized ovaries (Sullivan et al. 2001). *Cepsiclava phalaridis* (synonym: *Claviceps phalaridis*) is a systemic endophyte of pooid grasses in southeastern Australia. It produces sclerotia in florets of infected plants, produces stipitate ascostromata from sclerotia, and has two anamorphs, one of which produces holoblastic conidia with apical branched appendages (Walker 2004). Pazoutova et al. (2004) showed that these *Aciculosporium*, *Neoclaviceps*, and *Cepsiclava* species could be grouped together, and this group is related to *Claviceps* on the basis of ITS sequences. In conclusion, the results of the present study also strongly suggest that *Aciculosporium* is phylogenetically related to *Claviceps* rather than *Balansia*.

The appendaged conidia are distinguished from those of other fungi in Clavicipitoideae. *Sphacelia*-type conidia (anamorph of *Claviceps*) and *Neotyphodium*-type conidia (anamorph of *Epichloë*; accumulated in a drop at the tips of elongate attenuated *Acremonium*-type conidiogenous cells) are enteroblastic microconidia. The *Ephelis*-type conidia (anamorph of *Balansia*, *Myriogenospora*, etc.) is a holoblastic macroconidia that is narrowly cylindrical or linear, produced in moist mats on a hypothallus. Sullivan et al. (2001) reported that *Sphacelia* and *Neotyphodium* anamorphs were probably derived from ancestral fungi, because related families, such as Nectriaceae and Hypocreaceae, possess *Acremonium*-like microconidia. *Atkinsonella* species possess both *Ephelis* (macroconidia) and *Neotyphodium* synanamorphs (Diehl 1950; White 1994). Thus, the *Ephelis* anamorph is believed to have newly evolved in the Balansieae (Sullivan et al. 2001). As stated earlier, phylogenetic studies indicated that the species pro-

ducing appendaged macroconidia (e.g., *Aciculosporium*) were different from species with *Ephelis* anamorphs. *Cepsiclava phalalidis* also possesses microconidial and macroconidial synanamorphs (Walker 2004). Thus, the anamorph of *Aci. take* (appendaged macroconidia) is distinct from the *Ephelis* anamorph and presumably evolved independently in the Clavicipitoideae.

The characteristic conidial morphology of *Aci. take* may reflect its strategy of raindrop-mediated conidial transmission. Most of the related *Claviceps* species are transmitted by insects from the host flower to another host, which is reasonable because infection with these fungi occurs from the pistil. In contrast, bamboo species (hosts of *Aci. take*) rarely flower (e.g., *Phyllostachys bambusoides* flowers once at intervals of around 60 or 120 years) (Janzen 1976). However, the numerous twigs of witches' broom have conidiostromata at each apex. In addition, bamboo species generally reach heights of 10 m or more. Therefore, the conidia in raindrops shower down from the apexes of numerous twigs on the surrounding bamboo. The appendaged macroconidia of *Aci. take* are thought to be well adapted to its life strategy.

The ecology of clavicipitaceous fungi has attracted a great deal of interest. However, many clavicipitaceous species, including Asian species, remain to be studied. Examinations of the phylogenetic relationships of these will provide new insight to allow their utilization as biological resources.

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