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FULL PAPER

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Molecular phylogeny of four selected species of the strictly anamorphic genus *Thysanophora* using nuclear ribosomal DNA sequences

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Abstract To estimate the phylogenetic position of the strictly anamorphic genus *Thysanophora* among the class Ascomycetes *sensu* Kirk et al. and to examine the phylogenetic relationships among *T. penicillioides* and other *Thysanophora* species, 18S and 28S rDNA (D1 and D2 regions) sequences of 22 strains of four known and two unidentified *Thysanophora* species were determined and phylogenetically analyzed. The 18S rDNA analysis suggested that all *Thysanophora* species examined were members of Eurotiomycetidae, Eurotiales, Trichocomaceae. The 28S rDNA analysis indicated that these species were clustered together with *Chromocleista*, *Eupenicillium, Geosmithia*, and *Penicillium* assignable to three subgenera – *Aspergilloides*, *Furcatum*, and *Penicillium*. In the *Eupenicillium* lineage, a monophyly of *T. penicillioides*, *T. longispora*, *T. taxi*, *T. canadensis*, and *T.* cf. *canadensis* was supported by comparatively high bootstrap values. However, the ex-type strain and two strains of *T. longispora* isolated in Japan were of different phylogenetic positions. *Thysanophora* sp. was positioned at the base of the *Thysanophora* clade, although it was not supported by significant bootstrap values. From the results of this study, we consider that two anamorphic genera, *Penicillium* and *Thysanophora*, are clearly distinct in morphology but that they are not phylogenetically separable.

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Introduction

The strictly anamorphic genus *Thysanophora* was erected by Kendrick (1961) based on the basionym *Haplographium penicillioides* Roum. It is still a relatively small genus, consisting of eight accepted species. Kendrick (1961) characterized the genus as producing dark and stout conidiophores with *Penicillium*-like penicillus with long chains of dry amerospores; also, the subapical proliferation that of ten occur in the stipe: secondary growth of the stipe by means of the proliferation after the production of an apical penicillus. Kendrick described two species, *T. penicillioides* (Roum.) W.B. Kendr. as the type species of the genus and *T. longispora* W.B. Kendr. Stolk and Hennebert (1968) described *T. canadensis* Stolk & Hennebert and *T. taxi* (R. Schneid.) Stolk & Hennebert that agreed well with the definition of the genus *Thysanophora*. In 1970, Barron and Cooke (1970) described *T. striatispora* G.L. Barron & W.B. Cooke. They noted that subapical proliferation occurs only exceptionally in the species. Subsequently, three species have been added or transferred from other genera to the genus *Thysanophora*: *T. asymmetrica* Subram. & Sudha (1985), *T. verrucosa* Mercado, Gené & Guarro (1998), and *T. taiwanensis* (Matsush.) Mercado, Gené & Guarro (1998). However, these three species lack the subapical proliferation of the stipes that is part of the generic definition by Kendrick (1961).

The afore mentioned eight *Thysanophora* species can be divided into three groups according to the presence or absence of the subapical proliferation of stipes, namely, species that usually proliferate, species that only occasionally proliferate, and species that do not show apical proliferation. The species of the first group, including the type species, are morphologically close to each other. Thus, they are usually distinguished by the dimensions of conidia and phialides and by the type of conidiophore branching such as

monoverticillate or biverticillate penicilli. In addition, some strains produce sclerotia in all the species. In contrast, the respective species of the second and the third groups have a highly individualistic morphology, i.e., they have striated conidia (*T. striatispora*), asymmetrical penicilli and smooth, pale conidia (*T. asymmetrica*), verrucose conidiophores and phialides, and dark conidia (*T. verrucosa*) and dark, cylindrical-doliform conidia (*T. taiwanensis*). Because there are clear differences among the three groups, we have held some doubts about a monophyly of this genus. However, among eight known species of *Thysanophora*, *T. asymmetrica* and *T. verrucosum* were not cultured, and the strains of *T. striatispora* and *T. taiwanensis* were not available for our study.

In this study, we analyzed nuclear small subunit (18S) rDNA sequences and nuclear large subunit (28S) rDNA sequences to address two problems of the genus *Thysanophora*. First, we estimated the phylogenetic position of *Thysanophora* species among the class Ascomycetes sensu Kirk et al. (2001) to consider the teleomorphs of *Thysanophora* species that have not yet been discovered. Second, we attempted to examine the phylogenetic relationships among *T. penicillioides* and other available *Thysanophora* species. For these purposes, we determined 18S and 28S (D1 and D2 regions) rDNA sequences of four known *Thysanophora* species including ex-type strains of three species, two unidentified *Thysanophora* strains that we had isolated, and two strains of *Penicillium arenicola* Chalab.

Materials and methods

Fungal strains and cultivation

Fungal strains used to determine rDNA sequences in this study are shown in Table 1. *Thysanophora longispora* UAMH 1460, *T. taxi* CBS 206.57, and *T. canadensis* CBS 334.68 were ex-type strains. We also examined two Japanese strains of *T. longispora* and *T. canadensis*. In *T. penicillioides*, for which living culture from the type material was not available, we used seven strains of CBS including CBS 292.60, which Kendrick (1961) examined, and five strains isolated in Japan. All Japanese strains except for IFO 8842 (Tubaki 1969) were isolated by the authors, and the nine representative strains were deposited in IFO or MAFF. The isolates were identified by referring to the original descriptions of *Thysanophora* species (Kendrick 1961; Stolk and Hennebert 1968). We isolated two unidentified *Thysanophora* species, *T.* cf. *canadensis* and *Thysanophora* sp., and examined them. Of two unidentified *Thysanophora* species, *T.* cf. *canadensis*, which occurred on decaying needles of *Taxus cuspidata* Sieb. & Zucc., resembles *T. canadensis* or *T. taxi* but has larger conidia than the former and smaller conidia than the latter. *Thysanophora* sp., which occurred on the decaying needles of *Abies mariesii* Masters, distinctly differs from already-described *Thysanophora* species in morphology and rarely has subapical proliferation of the stipes.

All strains were incubated on corn meal agar (Nissui, Tokyo, Japan) plates at 20°C.

PCR amplification

Each DNA fragment was amplified by a method modified from the direct polymerase chain reaction (PCR) method described by Suyama et al. (1996) for pollen DNA. The conidial mass on the plate, replacing the pollen grains of Suyama et al. (1996), was picked using a pipette tip and suspended in the PCR tube with sterile distilled water. Polymerase chain reactions were performed using a HotStarTaq Master Mix (Qiagen, Hilden, Germany). Each PCR tube contained a total 50 µl mixture (24 µl distilled water with conidial mass, 25 µl master mix, and 0.5 µl each primer (final, 0.25 µM)). 18S rDNA partial fragments were amplified using originally designed primers STP1 (5′-GAAACTGCGA ATGGCTCAT-3′) and STP6 (5′-GTTCACCTACGG AAACCTTGT-3′). 28S rDNA (D1 and D2 regions) fragments were amplified using published primers D1 (Peterson 2000) and NL4 (O'Donnell 1993). Amplification of each DNA fragment was performed using a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) under this thermal cycling condition: an initial denaturation at 95°C for 15 min, 45 cycles of denaturation at 94°C for 20 s, annealing at 56°C or 58°C (18S rDNA)/60°C (28S rDNA) for 1.5 min, a final extension period at 72°C for 10 min, and a 4°C soak. PCR products were purified with a QiAquick PCR Purification Kit (Qiagen).

DNA sequencing

The primers STP1, STP6, SST3 (5′-AATTGGAGGGCAA GTCTGGTG-3′), SST4 (5′-CACGCCTTGTGGTGCCC TTCC-3′), NS2, and NS5 (White et al. 1990) for 18S rDNA, and D1 and NL4 for 28S rDNA were used for direct sequencing of the amplified fragments. Sequencing reactions were performed using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and GeneAmp PCR System 2400 (Applied Biosystems) according to the manufacturer's instructions. Sequencing reaction products were purified with a DyeEx Spin Kit (Qiagen). DNA sequences from both strands were read on an ABI PRISM 377 DNA Sequencing System (Applied Biosystems).

Alignment and phylogenetic analysis

Sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ); their serial accession numbers are shown in Table 1. In the case that sequences of some strains in one species were identical, the sequence of one representative strain was added to the data set.

For 18S rDNA analysis, the aligned data set was downloaded from "The rRNA WWW Server" at the University of Antwerp (URL: http://rrna.uia.ac.be/) (Van de Peer et al. 1997). Sequences of our sequenced taxa and additional taxa from GenBank were individually added to the aligned data

Table 1. *Thysanophora* and *Penicillium* species sequenced in this study

Species	Strain number[a]	18S rDNA[b]	18S rDNA sequence type[c]	28S rDNA[b]	28S rDNA sequence type[c]	Habitat	Origin
T. penicillioides	CBS 292.60	AB075428	a	AB075444	m	*Coprinus micaceus*	France
T. penicillioides	CBS 314.56	AB075429	b	AB075445	n	Burnt soil	Canada, Ontario
T. penicillioides	CBS 344.33	AB075430	c	AB075446	o	–	–
T. penicillioides	CBS 345.33	AB075431	c	AB075447	o	–	Sweden
T. penicillioides	CBS 348.64	AB069699	c	AB069705	o	Soil	Belgium, near Louvain
T. penicillioides	CBS 553.86	AB075432	c	AB075448	o	Root of *Pseudotsuga menziesii*	Netherlands
T. penicillioides	CBS 576.68	AB075433	c	AB075449	o	–	Russia, Moscow
T. penicillioides	WC 1098 (MAFF 238385)	AB075434	d	AB075450	p	*Abies mariesii* decaying needle	Japan, Niigata
T. penicillioides	WC 1104 (MAFF 238391)	AB075435	e	AB075451	q	*Abies mariesii* decaying needle	Japan, Niigata
T. penicillioides	WC 1109 (MAFF 238396)	AB075436	f	AB075452	o	*Abies mariesii* decaying needle	Japan, Miyagi
T. penicillioides	WC 1111 (MAFF 238398)	AB075437	f	AB075453	o	*Abies mariesii* decaying needle	Japan, Iwate
T. penicillioides	WC 1112 (MAFF 238399)	AB075438	f	AB075454	o	*Abies mariesii* decaying needle	Japan, Fukushima
T. longispora	UAMH 1460[d]	AB075439	g	AB075455	r	*Tsuga canadensis* needle on a decaying fruit-body of *Russula xerampelina*	Canada, Quebec
T. longispora	IFO 8842	AB075440	h	AB075456	s		Japan, Mie
T. longispora	WC 0738 (IFO 33255)	AB069700	h	AB069706	s	*Abies firma* decaying needle	Japan, Hiroshima
T. taxi	CBS 206.57[d]	AB069701	i	AB069707	t	*Taxus baccata* needle	Germany
T. canadensis	CBS 334.68[d]	AB069702	j	AB069708	u	*Tsuga canadensis* needle	Canada, Ontario
T. canadensis	WC 1084 (IFO 33254)	AB075441	k	AB075457	u	*Abies mariesii* decaying needle	Japan, Fukushima
T. canadensis	WC 1086	AB075442	k	AB075458	u	*Abies mariesii* decaying needle	Japan, Iwate
T. cf. *canadensis*	WC 0946 (IFO 33257)	AB069703	j	AB069709	u	*Taxus cuspidata* decaying needle	Japan, Nagano
T. cf. *canadensis*	WC 1113	AB075443	j	AB075459	u	*Taxus cuspidata* decaying needle	Japan, Hokkaido
Thysanophora sp.	WC 0944 (IFO 33256)	AB069704	l	AB069710	v	*Abies mariesii* decaying needle	Japan, Fukushima
P. arenicola	JCM 9929[d,e]	–	–	AB069711	w	Soil in a pine forest	Russia, near Kiev
P. arenicola	IFO 7739[f]	–	–	AB069712	w	Soil from *Cedrus* sp. community	–

[a] Culture collection sources of strains: Centraalbureau voor Schimmelcultures (CBS); Genebank, Genome and Biodiversity Research Center, National Institute of Agrobiological Sciences (MAFF); University of Alberta Microfungus Collection & Herbarium (UAMH); Institute for Fermentation, Osaka (IFO); Japan Collection of Microorganisms (JCM); the representative strains that the authors isolated (WC) were deposited in IFO or MAFF

[b] Accession number for DNA sequence

[c] The strains with the same alphabetical character have the identical sequence

[d] Ex-type strain

[e] JCM 9929 = CBS 220.66 = IMI 117658

[f] IFO 7739 = IMI 95493; this strain was first identified as *P. canadense* by Christensen and Backus (1961)

Table 2. Additional taxa selected for the 18S rDNA analysis

Classification[a]				
Subclass	Order	Family	Species	Accession no.
Chaetothyriomycetidae	Chaetothyriales	Chaetothyriaceae	*Ceramothyrium linnaeae*	AF022715
		Anamorphic Herpotrichiellaceae	*Exophiala dermatitidis*	X79312
Eurotiomycetidae	Elaphomycetales	Elaphomycetaceae	*Elaphomyces leveillei*	U45441
			E. maculatus	U45440
	Eurotiales	Trichocomaceae	*Byssochlamys nivea*	M83256
			Chromocleista malachitea	D88323
			Eupenicillium crustaceum	D88324
			E. javanicum	U21298
			Eurotium rubrum	U00970
			Hamigera avellanea	D14406
			Neosartorya fischeri	U21299
			Talaromyces bacillisporus	D14409
			Talaromyces flavus	M83262
			Thermoascus crustaceus	M83263
		Anamorphic Trichocomaceae	*Geosmithia namyslowskii*	AB028190
			Penicillium chrysogenum	M55628
	Onygenales	Arthrodermataceae	*Ctenomyces serratus*	U29391
		Gymnoascaceae	*Gymnoascoideus petalosporus*	U29392
		Onygenaceae	*Onygena equina*	U45442
	Anamorphic Onygenales		*Histoplasma capsulatum*	X58572
	Incertae sedis	Ascosphaeraceae	*Ascosphaera apis*	X69849
		Eremascaceae	*Eremascus albus*	M83258
		Monascaceae	*Monascus purpureus*	M83260

[a] Classification adopted by Kirk et al. (2001)

set through a profile alignment process by Clustal W, version 1.71 (Thompson et al. 1994). Our preliminary phylogenetic analysis indicated that all *Thysanophora* strains were related to the Eurotiomycetidae among the Ascomycetes *sensu* Kirk et al. (2001). Therefore, we selected members of the Eurotiomycetidae as shown in Table 2 and *Ceramothyrium linnaeae* (Dearn.) S. Hughes and *Exophiala dermatitidis* (Kano) de Hoog (i.e., an anamorphic *Capronia*) in the Chaetothyriomycetidae as outgroups in addition to 13 *Thysanophora* strains for 18S rDNA analysis. This data set, composed of 36 taxa, was aligned and gap positions were removed. A data set that included 1637 sites was prepared.

For 28S rDNA analysis, sequences from this study and GenBank were aligned with Clustal W and refined by eye because there were a few gaps in the data set. The data set included six teleomorphic genera classified in the Trichocomaceae, *Chromocleista*, *Eupenicillium*, *Eurotium*, *Hamigera*, *Neosartorya*, and *Sclerocleista*; *Monascus*, classified in the Monascaceae; and the strictly anamorphic species of *Geosmithia* and *Penicillium* (Table 3). In *Eupenicillium* and *Penicillium*, several species were chosen from *Eupenicillium* and subgenera *Aspergilloides*, *Furcatum*, and *Penicillium* of *Penicillium* defined by Pitt (1979a), respectively (Table 3). Recently, Peterson (2000) studied a phylogenetic analysis based on ITS-28S rDNA combined sequences of a large number of species of *Eupenicillium* and *Penicillium* including subgenera *Aspergilloides*, *Furcatum*, and *Penicillium*. He showed that these taxa formed six phylogenetic groups. In this study, some species from each of his six groups were selected (see Table 3). Eleven *Thysanophora* strains and two *P. arenicola* strains were sequenced in this study, and *Talaromyces luteus* (Zukal) C.R. Benj. and *T. bacillisporus* (Swift) C.R. Benj. (anamorph: *Geosmithia swiftii* Pitt) as outgroups were added to the data set. Sequences of these 41 taxa were aligned and gap positions were removed. The remained 570 sites were analyzed.

Phylogenetic analyses were performed from the aligned sequences for each data set by the neighbor-joining (NJ) method (Saitou and Nei 1987) and the maximum parsimony (MP) method using PAUP 4.0b8 (Swofford 2000). NJ trees were constructed with the HKY 85 model (Hasegawa et al. 1985). MP trees were constructed using the heuristic search option. Bootstrap analysis (Felsenstein 1985) was based on 1000 bootstrap replicates using the NJ option for NJ trees and the heuristic search option for MP trees.

Results

18S rDNA sequence type

18S rDNA fragment lengths amplified using the primer set STP1–STP6 were either 1659 or 1660 bp in all strains. The length of *T. penicillioides*, *T. taxi*, *T. canadensis*, and *T.* cf. *canadensis* was 1659 bp in all strains. Three strains of *T. longispora* were separated into two categories by length; length was 1659 bp in UAMH 1460 and 1660 bp in both IFO 8842 and WC 0738. *Thysanophora* sp. had a 1660-bp sequence length.

Table 1 shows 18S rDNA sequence types of respective strains of *Thysanophora* species, whereas Table 4 shows the

Table 3. Additional taxa selected for the 28S rDNA analysis

Species	Subgenus[a]	Group[b]	Accession no.
Teleomorphic genera having *Aspergillus* anamorph			
Eurotium rubrum	*Aspergillus*		U29544
Neosartorya fischeri	*Fumigati*		U28467
Sclerocleista ornata	*Ornati*		U29818
Eupenicillium and *Penicillium*			
Eupenicillium crustaceum		Group 6	AF033466
E. hirayamae		Group 3	AF033418
E. javanicum		–	
E. katangense		Group 4	AF033458
E. lapidosum		Group 2	AF033409
E. pinetorum		Group 2	AF033411
E. shearii		Group 1	AF033420
E. stolkiae		Group 5	AF033444
Penicillium lividum	*Aspergilloides*	Group 2	AF033406
P. glabrum	*Aspergilloides*	Group 2	AF033407
P. adametzii	*Aspergilloides*	Group 3	AF033401
P. vinaceum	*Aspergilloides*	Group 4	AF033461
P. implicatum	*Aspergilloides*	Group 5	AF033428
P. herquei	*Furcatum*	Group 3	AF033405
P. raistrickii	*Furcatum*	Group 6	AF033491
P. miczynskii	*Furcatum*	Group 1	AF033416
P. paxilli	*Furcatum*	Group 1	AF033426
P. janthinellum	*Furcatum*	Group 5	AF033434
P. chrysogenum	*Penicillium*	Group 6	AF034451
P. expansum	*Penicillium*	Group 6	AF033479
Related genera			
Chromocleista malachitea			AB000621
Geosmithia namyslowskii			AB000487
Hamigera avellanea			AB047216
Monascus purpureus			AF033394
Outgroup			
Talaromyces bacillisporus			AF033388
Talaromyces luteus			AB047227

[a] The subgenera of *Aspergillus* are based on Gams et al. (1985); the subgenera of *Penicillium* are based on Pitt (1979a)
[b] The phylogenetic groups of *Penicillium* that Peterson (2000) showed based on ITS-28S rDNA sequences are quoted

Table 4. The number of differential positions among 18S rDNA seqence types of *Thysanophora* species[a]

Sequence type[b]	a	b	c	d	e	f	g	h	i	j	k
b	6										
c	5	1									
d	6	4	3								
e	6	3	3	3							
f	4	2	1	2	2						
g	7	5	4	5	5	3					
h	15	13	12	13	13	11	10				
i	7	4	4	5	3	3	2	10			
j	7	3	2	5	5	3	2	10	2		
k	8	4	3	6	6	4	3	11	3	1	
l	23	21	20	21	21	19	18	12	18	18	19

[a] The number of differential positions included gap sites
[b] Sequence types: a, *T. penicillioides* CBS 292.60; b, *T. penicillioides* CBS 314.56; c, *T. penicillioides* CBS 344.33, CBS 345.33, CBS 348.64, CBS 553.86, CBS 576.68; d, *T. penicillioides* WC 1098; e, *T. penicillioides* WC 1104; f, *T. penicillioides* WC 1109, WC 1111, 1112; g, *T. longispora* UAMH 1460; h, *T. longispora* WC 0738, IFO 8842; i, *T. taxi* CBS 206.57; j, *T. canadensis* CBS 334.68, *T* cf. *canadensis* WC 0946, WC 1113; k, *T. canadensis* WC 1084, 1086; l, *Thysanophora* sp. WC 0944

number of differential positions among 18S rDNA sequence types of *Thysanophora* species. Five 18S rDNA sequence types (sequence types a–f) were obtained from ten strains of *T. penicillioides*, and nucleotide substitutions were observed in a total of ten positions among them. Sequence type c was observed in five CBS strains and sequence type f in three Japanese strains. Both sequences of *T. longispora* WC 0738 and IFO 8842 were identical (h).

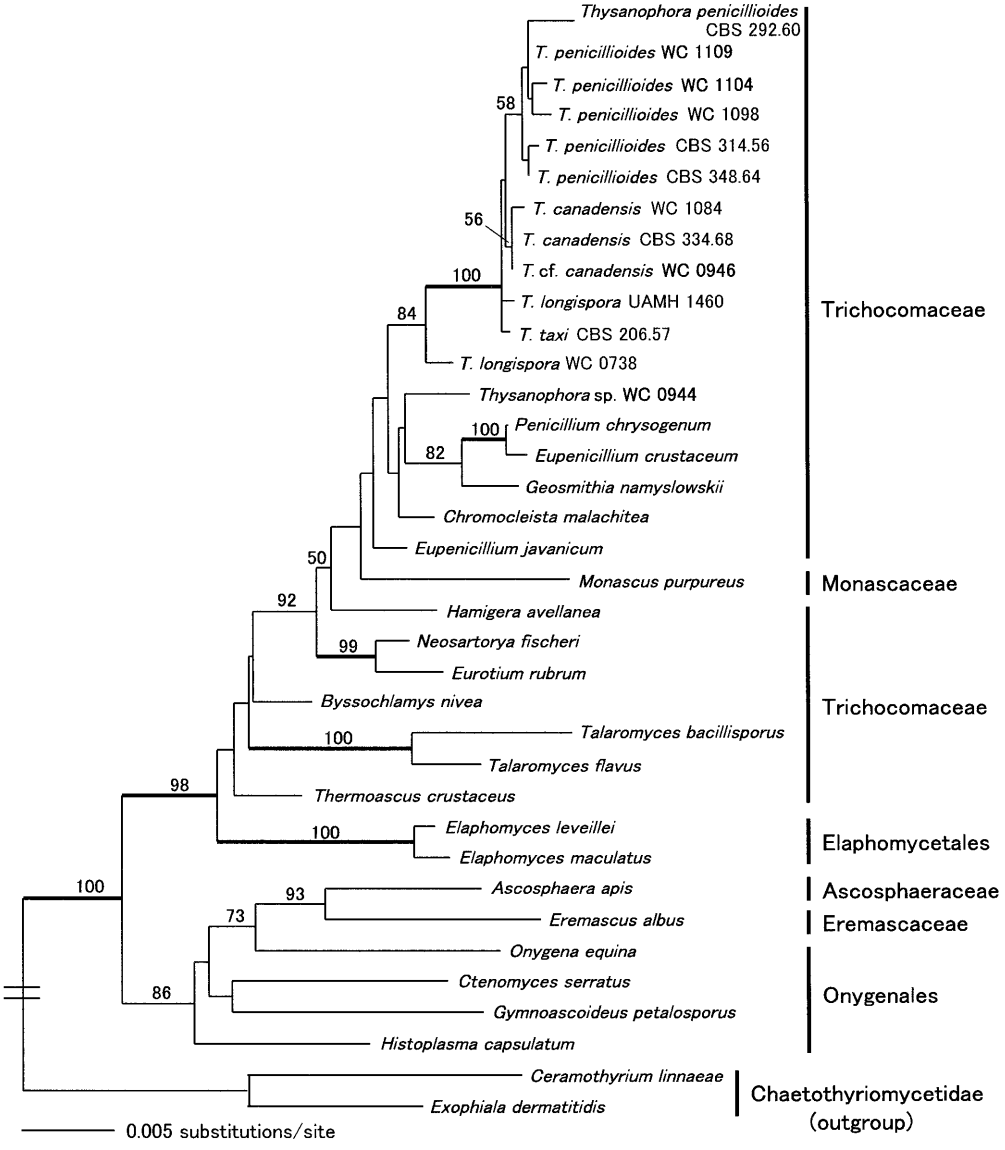

Fig. 1. Phylogenetic positions of *Thysanophora* species among the Ascomycetes inferred from the neighbor-joining (NJ) analysis of 18S rDNA sequences. Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. Branches significantly supported by bootstrap value above 95% are shown with *thick lines*

However, ten positions were different between these two strains and UAMH 1460 (g). *Thysanophora canadensis* CBS 334.68 (j) and two Japanese strains (k), WC 1084 and WC 1086, exhibited only one nucleotide difference. Two strains of *T.* cf. *canadensis* showed the same sequence as *T. canadensis* CBS 334.68 (j).

Molecular phylogeny based on 18S rDNA data

The NJ tree showed that *Thysanophora* species were included in the Eurotiomycetidae clade with 100% bootstrap support in the tree (Fig. 1). This clade was separated into two subclades, consisting of the Onygenales lineage including Ascosphaeraceae and Eremascaceae and the Eurotiales-Elaphomycetales lineage including Monascaceae. *Thysanophora* species belonged to the latter subclade with 98% bootstrap support. Moreover, in the Eurotiales-Elaphomycetales subclade, *Thysanophora* species were clustered together with three teleomorphic genera having an *Aspergillus* anamorph, *Chromocleista malachitea* Yaguchi & Udagawa (anamorph: *Geosmithia malachitea* Yaguchi & Udagawa), *Eupenicillium crustaceum* F. Ludw., *E. javanicum* (J.F.H. Beyma) Stolk & D.B. Scott, *Hamigera avellanea* (Thom & Turesson) Stolk & Samson [anamorph: *Merimbla ingelheimensis* (J.F.H. Beyma) Pitt], *Penicillium chrysogenum* Thom and *Geosmithia namyslowskii* (K.M. Zalessky) Pitt classified in the Trichocomaceae, and *Monascus purpureus* Went classified in the Monascaceae with 92% bootstrap support. *Thysanophora penicillioides*, *T. longispora, T. taxi*, *T. canadensis*, and *T.* cf. *canadensis* formed a monophyletic group with 84% bootstrap support. However, the phylogenetic positions of *T. longispora* UAMH 1460 and *T. longispora* WC 0738 were different. This tree also showed that *T. penicillioides*, *T. longispora* UAMH 1460,

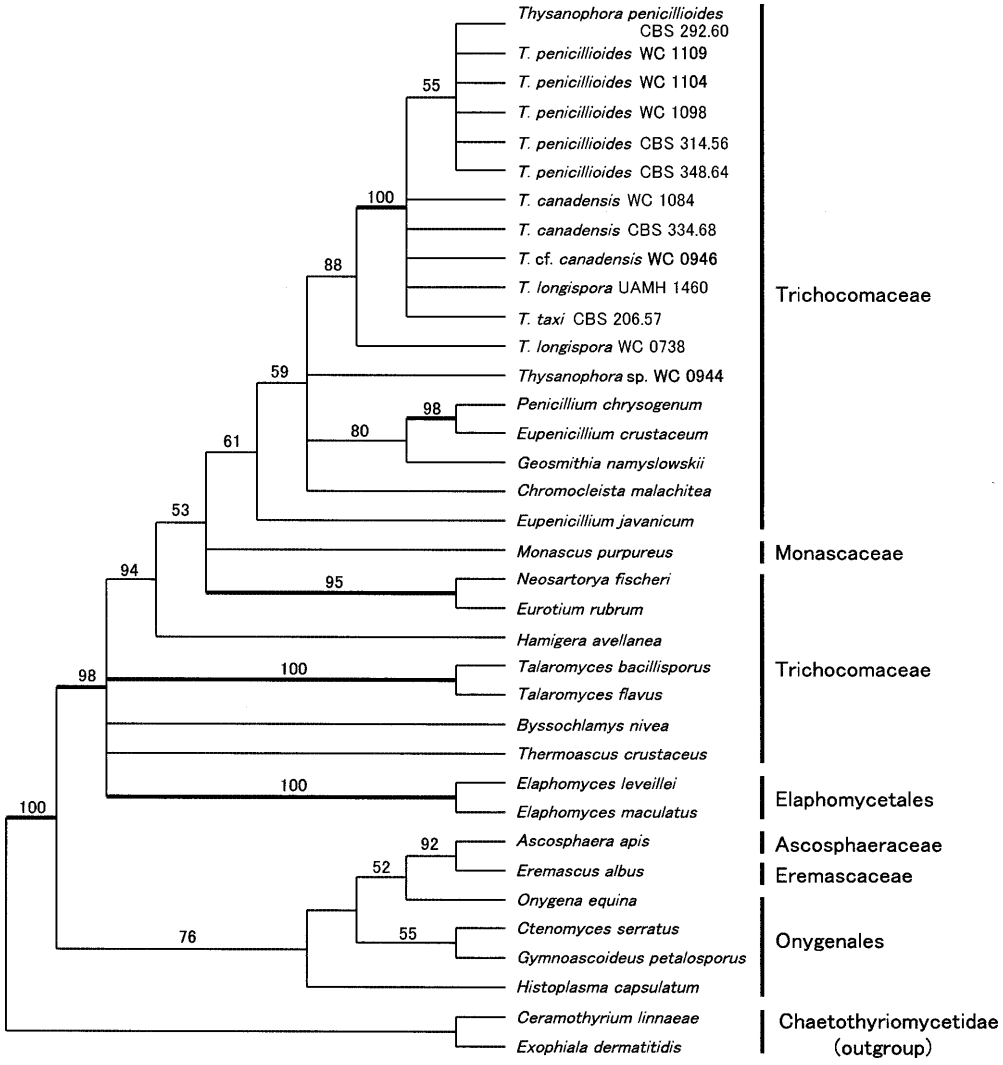

Fig. 2. The strict consensus tree of 560 maximum parsimony (MP) trees from 18S rDNA sequences (length = 461 steps, CI = 0.648, RI = 0.775, RC = 0.502). Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. Branches significantly supported by bootstrap value above 95% are shown with *thick lines*

T. taxi, *T. canadensis*, and *T.* cf. *canadensis* formed a statistically well supported clade (100% bootstrap support). However, the phylogenetic relationships among these *Thysanophora* species remain unresolved. Six strains of *T. penicillioides*, and two strains of *T. canadensis* and *T.* cf. *canadensis* formed one cluster with 59% and 57% bootstrap support, respectively.

The MP analysis using the same data set as the NJ analysis consisted of 1637 sites (172 parsimony-informative sites) that produced 560 MP trees [length = 461 steps, consistency index (CI) = 0.648, retention index (RI) = 0.775, and rescaled consistency index (RC) = 0.502]. The consensus tree of 560 trees (Fig. 2) indicated that all *Thysanophora* species were included in the Eurotiomycetidae clade and the Eurotiales subclade with 100% and 98% bootstrap support, respectively. The MP analysis indicated that *Thysanophora* species nested in the *Eupenicillium* lineage with 61% bootstrap support (Fig. 2). All *Thysanophora* strains except for *Thysanophora* sp. WC 0944 formed a monophyletic group with comparatively high bootstrap support (88%) as well as the NJ tree.

28S rDNA sequence type

28S rDNA fragment lengths amplified using the primer set D1–NL4 were 575 bp in all strains. Each 28S rDNA sequence type of examined strains and the number of differential positions among 28S rDNA sequence types of *Thysanophora* species are shown in Table 1 and Table 5, respectively. Ten *T. penicillioides* strains were divided into five sequence types and nucleotide substitutions were observed in 11 positions among them. In the comparison of 28S rDNA sequences of three *T. longispora* strains, strains WC 0738 and IFO 8842 were identical, and 29 nucleotide sites were different between both strains and UAMH 1460. All strains of *T. canadensis* and *T.* cf. *canadensis* showed the same sequence type. 28S rDNA sequences of two *P. arenicola* strains were identical.

Molecular phylogeny based on 28S rDNA data

To estimate the taxa more related to *Thysanophora* species, the data set of 28S rDNA D1–D2 region sequences were

Table 5. The number of differential positions among 28S rDNA seqence types of *Thysanophora* species

Sequence type[a]	m	n	o	p	q	r	s	t	u
n	6								
o	5	1							
p	7	7	6						
q	7	3	2	6					
r	8	4	3	3	3				
s	31	31	30	28	28	29			
t	13	7	8	14	10	11	34		
u	7	3	2	8	4	5	28	6	
v	30	26	25	27	23	24	17	31	25

[a] Sequence types: m, *T. penicillioides* CBS 292.60; n, *T. penicillioides* CBS 314.56; o, *T. penicillioides* CBS 344.33, CBS 345.33, CBS 348.64, CBS 553.86, CBS 576.68, WC 1109, WC 1111, WC 1112; p, *T. penicillioides* WC 1098; q, *T. penicillioides* WC 1104; r, *T. longispora* UAMH 1460; s, *T. longispora* WC 0738, IFO 8842; t, *T. taxi* CBS 206.57; u, *T. canadensis* CBS 334.68, WC 1084, 1086, *T.* cf. *canadensis* WC 0946, WC 1113; v, *Thysanophora* sp. WC 0944

analyzed. In the phylogenetic tree estimated by the NJ method (Fig. 3), *Chromocleista*, *Eupenicillium*, *Geosmithia*, and *Penicillium* formed one clade (i.e., the *Eupenicillium* lineage) with 87% bootstrap support and all *Thysanophora* species were included in the same clade. In this clade, seven subclades were generated and six subclades that consisted of members of *Chromocleista*, *Eupenicillium*, *Geosmithia*, and *Penicillium* corresponded to six groups defined by Peterson (2000), whereas *Thysanophora* species formed one subclade. *Thysanophora penicillioides, T. longispora, T. taxi, T. canadensis*, and *T.* cf. *canadensis* appeared as a monophyletic group with 89% bootstrap support. Moreover, the aforementioned members of *Thysanophora*, except for *T. longispora* WC 0738, formed a strongly monophyletic group with 100% bootstrap support. NJ analysis using 28S rDNA sequences also showed that *T. longispora* was not one lineage as well as the 18S rDNA analysis. In the *Thysanophora* group, *T. penicillioides* diverged and did not cluster, only appearing itself. *Thysanophora penicillioides* WC 1098 and *T. longispora* UAMH 1460 clustered with 60% bootstrap support. The bootstrap analysis did not support *Thysanophora* sp. to be in the clade together with other *Thysanophora* species.

Penicillium arenicola clustered with *Sclerocleista ornata* (Raper & al.) Subram. (anamorph: *Aspergillus ornatulus* Samson & W. Gams) with 66% bootstrap support. It was shown that this species was phylogenetically distant from the genus *Thysanophora*.

The MP analysis (108 parsimony-informative sites of 570 sites in the data set) resulted in 128 MP trees (length = 378 steps, CI = 0.511, RI = 0.731, RC = 0.373). The consensus tree of 128 trees (Fig. 4) indicated that five subclades of groups 1 to 4 and 6 in the *Eupenicillium* lineage were supported at 83%–99% bootstrap levels, but the subclade of group 5 did not reappear in the analysis of the parsimony method. *Thysanophora penicillioides* clustered with *T. longispora* UAMH 1460, *T. taxi*, *T. canadensis*, and *T.* cf. *canadensis* with high bootstrap value (100%) as indicated in other trees (Figs. 1–3). The MP analysis for the 28S rDNA data set also showed that *T. longispora* WC 0738 was clearly separated from *T. longispora* UAMH 1460.

Discussion

Molecular phylogenetic positions of *Thysanophora* species

The phylogenetic trees (Figs. 1–4) support that the *Thysanophora* species that we examined belong to Eurotiomycetidae, Trichocomaceae, Eurotiales. Up to the present the teleomorphs of the genus have not been discovered, and its taxonomic position in the class Ascomycetes is still unknown (Kirk et al. 2001). Consequently, our results may provide us with new and interesting information.

In the analysis of 18S rDNA sequences, *Thysanophora* species had the relatedness of the teleomorphic genera with an *Aspergillus* anamorph and *Eupenicillium* in Trichocomaceae (Figs. 1,2). Moreover, the analysis of 28S rDNA obviously indicated that *Thysanophora* species were closely related to the genus *Penicillium* and its teleomorphic genus *Eupenicillium* but were not related to the genus *Talaromyces*, another teleomorphic genus with a *Penicillium* anamorph (Figs. 3,4). Berbee et al. (1995) showed that two teleomorphic genera with a *Penicillium* anamorph, *Talaromyces* and *Eupenicillium*, were phylogenetically different using 18S, 5.8S, and ITS rDNA sequence data. The lineage of *Talaromyces* is involved with the species classified in the subgenus *Biverticillium*, and the *Eupenicillium* lineage is composed of *Penicillium* species belonging to other subgenera – *Aspergilloides*, *Furcatum*, and *Penicillium* (Pitt 1995). Therefore, *Thysanophora* species were considered to be the group closely related to the three subgenera of *Penicillium*.

Ogawa et al. (1997) and Ogawa and Sugiyama (2000) have analyzed 18S rDNA of *Chromocleista malachitea*, *Eupenicillium crustaceum*, *E. javanicum*, *Geosmithia namyslowskii*, and *Penicillium chrysogenum*. Our analyses indicated that *Thysanophora* species joined to the clades consisted of the five taxa shown by Ogawa et al. (1997) and Ogawa and Sugiyama (2000) with 82% and 93% bootstrap support in the NJ analysis, respectively. In the 28S rDNA analyses, this lineage was supported by relatively high bootstrap values (NJ tree, 87%; MP tree, 77%). These analyses

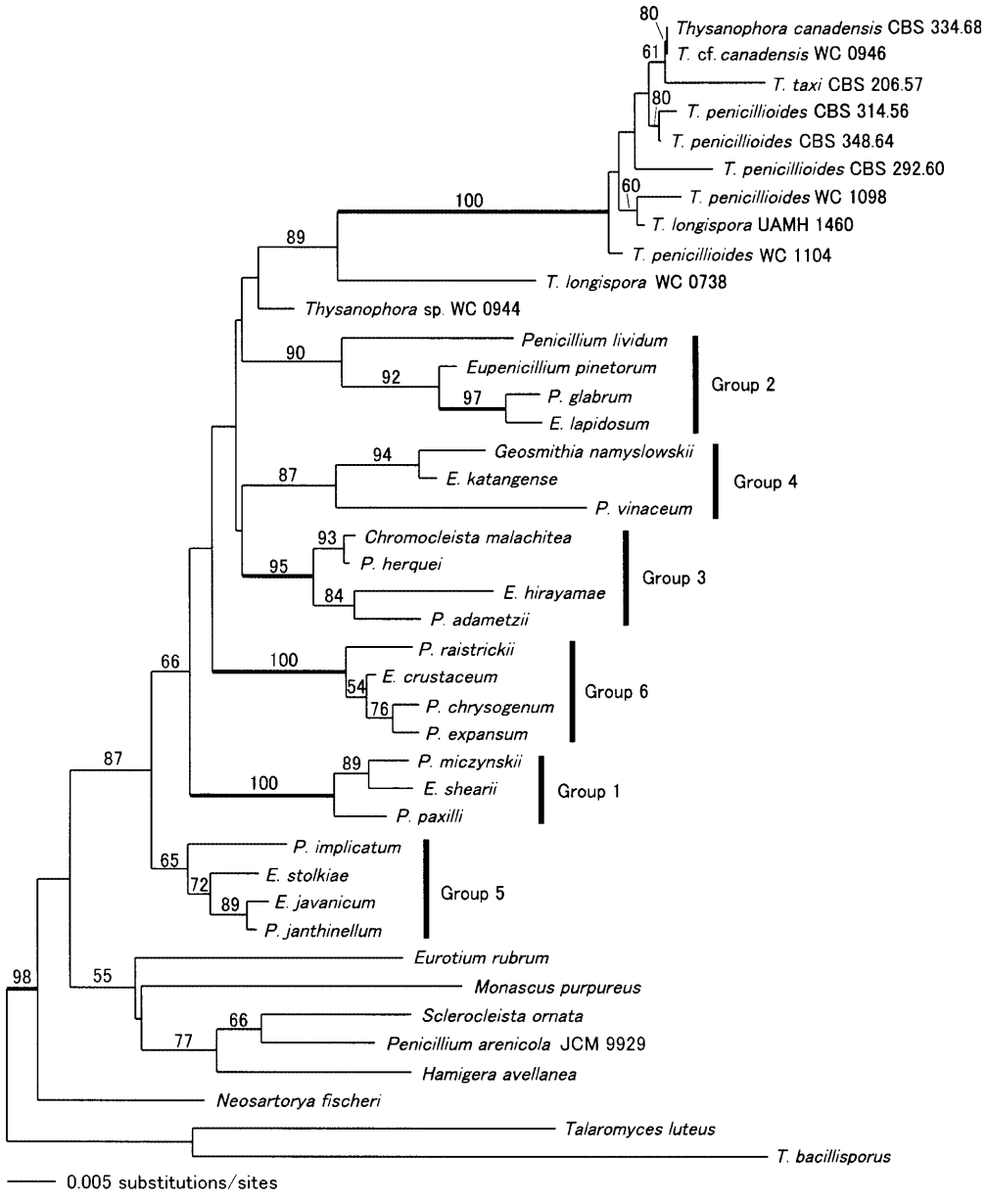

Fig. 3. Phylogenetic relationships among the species of *Thysanophora* and related genera in Trichocomaceae inferred from the NJ analysis of 28S rDNA (D1–D2 region) sequences. Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. Branches significantly supported by bootstrap value above 95% are shown with *thick lines*. Groups 1–6 are correspond to the phylogenetic groups of *Penicillium* indicated by Peterson (2000)

suggested that the anamorphic genera included in the *Eupenicillium* lineage are not only *Penicillium* with greenish conidial masses, penicilli with smooth elements, and hyaline conidiophores but also *Geosmithia* with conidial masses of greenish-gray (e.g., *G. namyslowskii*) and penicilli with rough elements (Pitt 1979b), and *Thysanophora* with conidial masses of grayish-olive (e.g., *T. penicillioides*) and dark-pigmented conidiophores (Kendrick 1961). Moreover, *Hemicarpenteles paradoxus* A.K. Sarbhoy & Elphick (anamorph: *Aspergillus paradoxus* Fennell & Raper), *A. malodoratus* Kwon-Chung & Fennell, and *A. crystallinus* Kwon-Chung & Fennell indicated the relatedness of the genus *Eupenicillium* by the ITS-28S rDNA analysis of Peterson (2000) and the 18S–28S rDNA analysis of Tamura et al. (2000). *Penicillium arenicola* was classified in the subgenus *Penicillium*, section *Inordinate* by Pitt (1979a), phylogenetically positioned with *Sclerocleista ornata* having an *Aspergillus* anamorph and having an affinity with neither *Thysanophora* nor *Eupenicillium*.

As already mentioned, our results indicated that the genus *Thysanophora* is closely related to *Eupenicillium* and its related *Penicillium* species. Molecular phylogenetic studies in relation to the *Eupenicillium* lineage have been done by Peterson (1993), Boysen et al. (1996), Peterson et al. (1999), Skouboe et al. (1999), Seifert and Louis-Seize (2000), and Tuthill et al. (2001). However, there have been few studies covering a broad range of the *Eupenicillium* lineage. Peterson (2000) analyzed many species of the lineage based on ITS-28S rDNA. He indicated that the subgeneric classification by Pitt (1979a) was not reflected in phylogeny and that approximately six clades over the subgeneric criteria were present. In this study, the data set of 28S rDNA was made of selected species from species used by Peterson (2000). The phylogenetic tree by the NJ

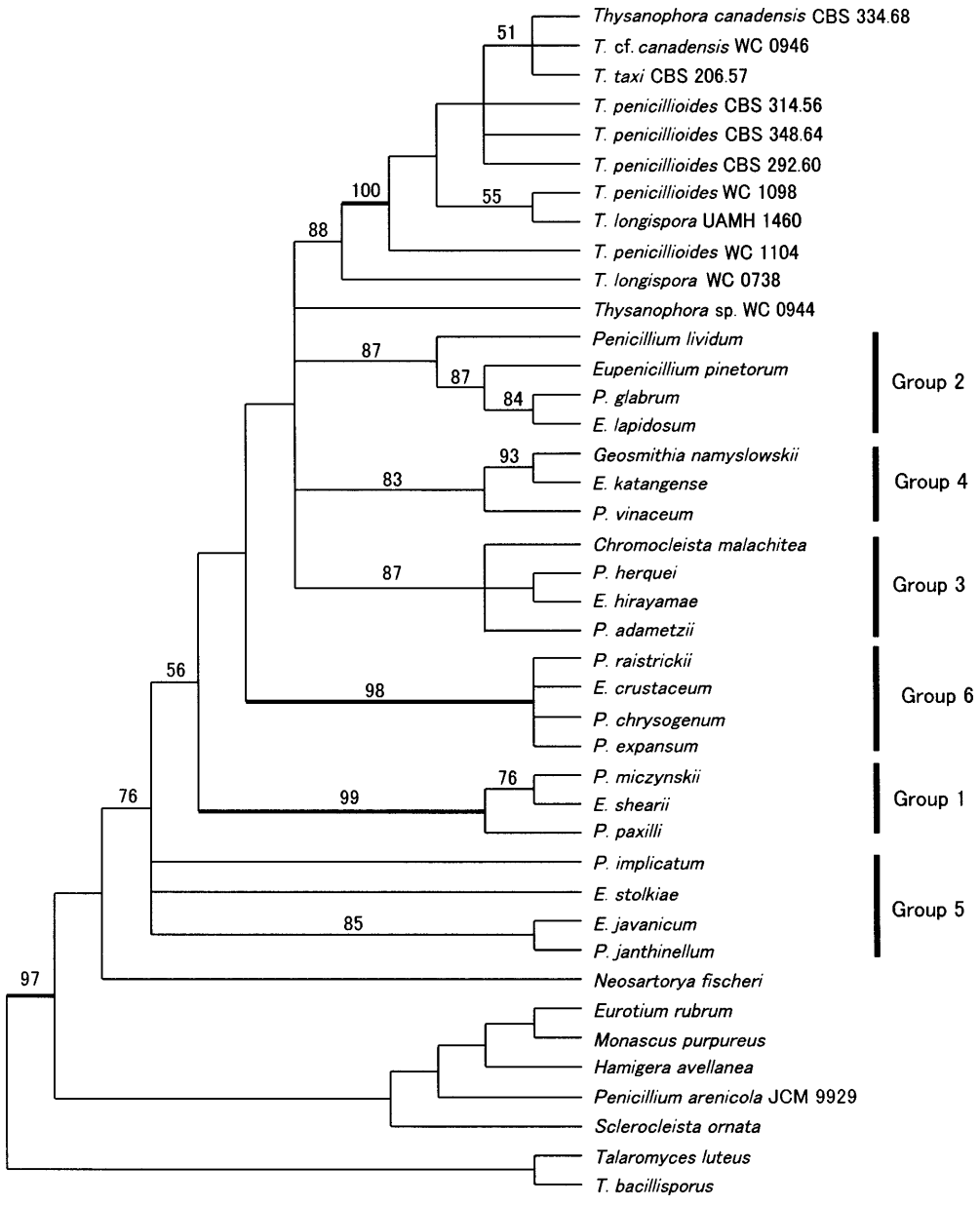

Fig. 4. The strict consensus tree of 128 MP trees from 28S rDNA (D1–D2 region) sequences (length = 378 steps, CI = 0.511, RI = 0.731, RC = 0.373). Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. Branches significantly supported by bootstrap value above 95% are shown with *thick lines*. Groups 1–6 correspond to the phylogenetic groups of *Penicillium* indicated by Peterson (2000)

analysis also showed that the *Eupenicillium* lineage was composed of six groups as shown by Peterson (2000). However, *T. penicillioides* and the other four *Thysanophora* species did not join to any group and formed a seventh group with high bootstrap support (Figs. 3,4). In the NJ analysis, the *Thysanophora* clade formed a sister group with group 2 [*E. lapidosum* D.B. Scott & Stolk, *E. pinetorum* Stolk, *P. glabrum* (Wehmer) Westling, *P. lividum* Westling]. However, this relationship between the *Thysanophora* clade and group 2 was not supported by a significant bootstrap value. To clarify the phylogenetic relationships of these seven groups, we need further analyses of other gene regions.

The relationship between the genera *Thysanophora* and *Penicillium* has been already discussed by Pitt (1979a) and Simpson (1993). In particular, a common coniferous litter fungus, *T. penicillioides*, had been sometimes confused with *Penicillium* before Kendrick (1961), and *T. taxi* was also described as a species of *Penicillium* (Schneider 1956) at first because of the *Penicillium*-like penicilli of the genus. In addition, some strains of *Thysanophora* species produce sclerotia resembling several *Penicillium* and *Aspergillus* spp.

Phylogenetic relationships among *Thysanophora* species

Our results clearly showed that the first group of *Thysanophora* described in the 1960s was a monophyletic group with high bootstrap support and that these species were phylogenetically close (Figs. 3,4). Morphologically, these species were distinguished by the monoverticillate or

biverticillate penicilli of conidial heads and the dimensions of the conidia and phialides (the key to *Thysanophora* species by Mercado-Sierra et al. 1998). We recognized intraspecific variations of 18S and 28S rDNA sequences of the *T. penicillioides* strains we examined (Tables 4,5). In the 28S rDNA analysis, these strains did not form one cluster by themselves. However, all strains have common morphological characteristics of *T. penicillioides*, i.e., biverticillate penicillus and small conidia, and were easily distinguishable from *T. longispora*, *T. canadensis*, and *T. taxi*.

In this study, it was shown that the *T. longispora* strains examined were separated into two lineages. *Thysanophora longispora* UAMH 1460, the type strain isolated from Canada, was closely related to *T. penicillioides* and relatives, but two Japanese strains, which were identical in the 18S and 28S rDNA sequences, were phylogenetically distant from them. We consider that these two strains are phylogenetically different species from *T. longispora* described by Kendrick (1961) and that they should undergo detailed morphological analysis. *Thysanophora canadensis* CBS 334.68 (ex-type strain) and two Japanese strains, WC 1084 and 1086, showed an identical sequence of the 28S rDNA sequence and one nucleotide substitution in the 18S rDNA sequence.

It is known that *T. taxi* appears on decaying needles of *Taxus* with high frequency (Stolk and Hennebert 1968; Ellis and Ellis 1997). However, *T.* cf. *canadensis*, which was different from *T. taxi* in morphology, appeared on decaying needles of *Taxus* in some locations in Japan. 18S and 28S rDNA sequences of two *T.* cf. *canadensis* strains were identical with *T. canadensis* CBS 334.68 rather than *T. taxi* CBS 206.57. It is very interesting that *T.* cf. *canadensis* indicated a high preference for *Taxus* needles in Japan when we think about the substrate preference of saprophytic fungi. In this study, *Thysanophora* sp. did not form a monophyletic group with the first group. *Thysanophora* sp. has several different morphological characteristics from *T. penicillioides*, so that we can understand the results of the molecular analysis. 28S rDNA analyses indicated two *Geosmithia* species, *Geosmithia namyslowskii* and *Chromocleista malachitea* with a *Geosmithia* anamorph, were polyphyletic in the *Eupenicillium* lineage. We need further analyses to consider the polyphyly of taxa with dark conidiophores in the *Eupenicillium* lineage.

In conclusion, we showed that the strictly anamorphic genus *Thysanophora* has an affinity with *Penicillium* and its related teleomorphic genus *Eupenicillium* by the evidence of 18S and 28S rDNA sequence data. Although *Thysanophora* had been traditionally classified in the Dematiaceae and *Penicillium* in the Moniliaceae of hyphomycetes, many characteristics of the genus *Thysanophora* are common to *Penicillium* except for producing dark conidiophores and the subapical proliferation of stipes. Therefore, we can draw the conclusion from the morphological and molecular similarities of both genera that the genus *Thysanophora* is involved with the genus *Penicillium*. We consider that both genera are clearly distinct in morphology but that they are not phylogenetically separable.

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