Mycoscience 42: 413–421, 2001

Phylogenetic analysis of the non-pathogenic genus *Spiromastix* (Onygenaceae) and related onygenalean taxa based on large subunit ribosomal DNA sequences

Masato Sugiyama and Takashi Mikawa

MCC-Group Science and Technology Research Center, Mitsubishi Chemical Corp., 1000, Kamoshida-cho, Aoba-ku, Yokohama, Kanagawa, 227–8502, Japan

Received 21 July 2000
Accepted for publication 11 July 2001

The phylogenetic positioning of the non-pathogenic genus *Spiromastix* in the Onygenales was studied based on large subunit rDNA (LSU rDNA) partial sequences (ca. 570 bp.). Four *Spiromastix* species and 28 representative taxa of the Onygenales were newly sequenced. Phylogenetic trees were constructed by the neighbor-joining (NJ) method and evaluated by the maximum parsimony (MP) method with the data of 13 taxa retrieved from DNA databases. *Spiromastix* and dimorphic systemic pathogens, *Ajellomyces* and *Paracoccidioides*, appear to be a monophyletic group with 74% bootstrap probability (BP) in the NJ tree constructed with the representative taxa of the Onygenales. The tree topology was concordant with the NJ tree based on SSU rDNA sequences of our previous work and corresponded to the classification system of the Onygenales by Currah (1985) and its minor modification by Udagawa (1997) with the exception of the classification of the Onygenaceae. The Onygeneceae sensu Udagawa may still be polyphyletic, since three independent lineages were recognized. The taxa forming helicoid peridial appendages were localized to two clades on the tree. The topology of the NJ tree constructed with *Spiromastix* and its close relatives suggested that the helicoid peridial appendages were apomorphic and acquired independently in the two clades of the Onygenales.

Key Words——large subunit rDNA; Onygenales; phylogeny; *Spiromastix*.

In our previous paper (Sugiyama et al., 1999), we analyzed the phylogenetic relationships of 22 onygenalean taxa based on their SSU rDNA sequences. Although the obtained neighbor-joining (NJ) tree revealed the phylogenetic structure of the Onygenales, *Spiromastix warcupii* was not included in any clade, but appeared as an independent branch on the NJ tree. Thus, the actual phylogenetic position of this genus remains uncertain.

Spiromastix Kuehn & Orr (Kuehn and Orr, 1962) is an onygenalean genus, members of which have been isolated from tropical and temperate regions. It is characterized by brown ascomata with hyphal peridia, thick-walled and helicoid or curved peridial appendages, and oblate ascospores with minutely pitted surface, and no anamorph has yet been demonstrated. Currently, five species are classified in this genus: *S. warcupii, S. tentaculatum, S. grisea, S. saturnispora* Uchiyama, Udagawa & Kamiya and *S. sphaerospora* Udagawa & Uchiyama. These species are distinguished by morphological differences in their peridial appendages, ascospore shape and ornamentation (Table 1).

In the Onygenales, *Spiromastix* was classified into the Onygenaceae based on its punctate ascospores and keratinolytic ability (Currah, 1985). However, Scott et al. (1993) reported that three species of *Spiromastix* (*S. grisea, S. tentaculatum* and *S. warcupii*) did not decompose hair in vitro. Recently, Udagawa (1997) redefined the Amauroascaceae von Arx (von Arx, 1987) as a member of the Onygenales based on its possession of a Q-10 (H_2) ubiquinone system and relatively low karitonolytic ability. He classified *Spiromastix* into the family with *Amauroascus* J. Schroet, *Amaurascopsis* Guarro, Gené & de Vroey and *Auxarthron* Orr & Kuehn.

The purpose of this study is to investigate the phylogenetic position of *Spiromastix* by using a molecular technique. We adopted LSU rDNA partial sequences that contained D1 and D2 regions, which are known to be suitable for phylogenetic analysis at the genus or species level in the Onygenales (LeClerc et al., 1994; Guého et al., 1997), and re-evaluated the phylogenetic structure of the Onygenales based on the SSU rDNA sequences (Sugiyama et al., 1999). Thirty-two onygenalean taxa including four species of *Spiromastix* were examined to clarify their phylogenetic relationships.

Materials and Methods

Strains examined The LSU rDNA sequences of representative strains of 32 species in 25 genera were determined in this study (Table 2). Twenty-seven of these strains were supplied from CBS (Centraalbureau voor Schimmelcultures, Baarn, the Netherlands). All of the strains whose SSU rDNA sequence was determined by Sugiyama et al. (1999) were also used in this study.

Table 1. Comparison of of *Spiromastix* species with JCM11276.

Species	Ascoma (μm)	Peridial appendage	Ascus (μm)	Ascospore (μm)	Source/Habitat	
S. grisea[a]	50–200	helidoid (2–8 turns) smooth-walled	6–9×5–7	2.8–4.0×2–2.5 lenticular to oblate pitted along equatorial zone	gazelle dung	Africa
S. saturnispora[b]	100–240	curved or sinuous smooth-walled	8–14.5×5.5–9	3.2–4.8×2.5–3 oblate with an equatorial rim pitted*	soil	Indonesia
S. sphaerospora[c]	(75–)150–250	slightly curved or wavy coarsely roughened	9–13.5×8–10	4–5×3.5–5 globose reticulate to punctate	soil	Japan
S. tentaculatum[d]	45–70	slightly curved or wavy, inflated at ends smooth-walled	7.5–8×5–6	2.5–3.5×2–2.2 oblate irregulary pitted	soil	Africa
S. warcupii[e]	50–100	curved or helicoid (1–2 turns) smooth-walled	7–7.7×5–5.5	2.5–2.9×2–2.5 oblate pitted with ditches on polar region*	soil	Australia, Malaysia, Indonesia, Africa
Spiromastix sp. JCM11276[f]	40–120	curved or wavy smooth-walled	6.1–6.8×4.7–5.0	2.1–3.1×1.8–2.4 oblate regularly pitted*	soil	U.S.A.

Morphological data were cited from: [a] Currah and Locquin-Linard, 1988; [b] Uchiyama et al., 1995; [c] Udagawa and Uchiyama, 1999; [d] Guarro et al., 1993; [e] Kuehn and Orr, 1962; [f] Morphological descriptions and measurements were done by the present authors.
*: SEM observation was done by the present authors.

Four *Spiromastix* species, *S. grisea*, *S. tentaculatum*, *S. warcupii* and *Spiromastix* sp. JCM 11276 were used to confirm the phylogenetic position of the genus. The strain JCM 11276 was isolated by the authors from a soil sample collected in Florida, U.S.A. and identified as a *Spiromastix* species based on the generic key of the Onygenales (Currah, 1988). The ascospore ornamentation of JCM 11276 is distinguishable from that of the ex-type strain of *S. warcupii* CBS 576.63 under SEM observation, although the other morphological data of this strain were within the range of the original description of *S. warcupii* (Table 1). *Arthroderma incurvatum* JCM 11274 was isolated from soil collected in Kanagawa prefecture, Japan and identified by reference to the description of Otani (1998). *Coccidioides immits* 3257 was isolated and identified based on its morphology on a Petri dish by Dr. R. Talbot in Kern County Health Department, Bakersfield, California, U.S.A. and used in the genetic analysis of an epidemic population structure of *C. immitis* by Fisher et al. (2000). *Malbranchea* sp. JCM 11275 was isolated as a contaminant of the strain of *Histoplasma capsulatum* Darling (EH366) from a bat intestine by Taylor et al. (1999), and we received it from Dr. T. Kasuga of Roche Molecular Systems, California, U.S.A. The sequence of this strain was determined and suggested that the strain was a close relative of *S. warcupii*. However, the culture of JCM 11275 produced only arthroconidia, and we identified the strain as a species of the genus *Malbranchea* by use of the generic key of Sigler and Carmichael (1976) and Oorschot (1980).

Extraction, PCR amplification, purification and sequencing of LSU rDNA genes The cultivation of fungal strains and the extraction and purification of DNA from their mycelia were performed as described by Sugiyama et al. (1999). The total DNA samples extracted from *Uncinocarpus reesii*, *Coccidioides immitis* and *Malbranchea* sp. JCM 11275 were provided by Dr. T. Kasuga.

The LSU rDNA sequences were determined by gene amplification using polymerase chain reaction (PCR). The PCR conditions were identical to those of Sugiyama et al. (1999) and the following primer pairs were used, as in O'Donnell (1993): NL1 [5'-GCATATCAATAAGCGGAGGAAAAG-3'] and NL4 [5'-GGTCCGTGTTTCAAGACGG-3'].

Amplicons corresponding to positions 153-767 of the complete LSU rDNA sequence data of *Saccharomyces cerevisiae* Meyen ex E. C. Hansen (Georgiev et al., 1981, Accession No. J01355) were purified by using a QIAquick PCR purification kit (QIAgen, Hilden, Germany). Automated DNA sequencing reactions were performed using ABI PRISM™ Cycle Sequencing Kits (Perkin Elmer Applied Biosystems, Foster, CA, U.S.A.) with 1 or 5 μl of amplicons and each primer for PCR, then processed and analyzed by ABI PRISM® 377 automated sequencers (PE Applied Biosystems). Obtained sequence data were checked, and complementary sequence data were connected by using the sequence editing software Genetyx-SV/R ver. 4.0 for Windows.

Phylogenetic analyses Data on 13 LSU rDNA sequences were retrieved from the nucleotide sequence databases (GenBank/EMBL/DDBJ) to complement the sequence data obtained here (Table 3). For all data, less reliable regions of both ends of rDNA fragments were excluded manually, and a ca. 570- bp region of each sequence was adopted for phylogenetic analyses. Multi-

Table 2. Species and strains of *Spiromastix* and other onygenalean taxa sequenced in this study.

Families	Species[a]	Strain No.[b]	Accession No.
Amauroascaceae	*Amauroascus kuehnii* von Arx	CBS 539.72^{T}	AB040691
	Auxarthron compactum Orr & Plunket	CBS 200.64^{T}	AB040692
	Spiromastix grisea Currah & Locquin-Linard	CBS 128.88^{T}	AB040677
	Spiromastix tentaculatum Guarro Gené & de Vroey	CBS 184.92^{T}	AB040678
	Spiromastix warcupii Kuehn & Orr	CBS 576.63^{T}	AB040679
	Spiromastix sp.	JCM 11276	AB040680
Arthrodermataceae	*Arthroderma ciferrii* Varsavsky & Ajello[a] (*Chrysosporium georgiae*)	CBS 272.66^{T}	AB040681
	Arthroderma incurvatum (Dawson & Gentles) Weitzman, McGinnis, Padhye & Ajello	JCM 11274	AB040682
	Ctenomyces serratus Eidam	CBS 187.61^{NT}	AB040683
Gymnoascaceae	*Gymnascella aurantiaca* Peck[a] (*Arachniotus verruculosus*)	CBS 655.71^{T}	AB040684
	Gymnoascoideus petalosporus Orr, Roy & Ghosh[a] (*Gymnoascus petalosporus*)	CBS 252.72	AB040685
	Gymnoascus reessii Baranetzky	CBS 410.72	AB040686
	Rollandina hyalinospora (Kuehn, Orr & Ghosh) Roy, Orr & Ghosh[a] (*Gymnascella hyalinospora*)	CBS 548.72	AB040687
Myxotrichaceae	*Byssoascus striatosporus* (Barron & Booth) von Arx	CBS 642.66^{T}	AB040688
	Myxotrichum deflexum Berk.	CBS 228.61^{NT}	AB040689
	Pseudogymnoascus roseus Raillo var. *roseus*	CBS 395.65^{NT}	AB040690
Onygenaceae	*Arachnomyces nodosetosus* Sigler & Abbott[a] (*Onychocola canadensis*)	CBS 313.90	AB053452
	Aphanoascus mephitalis (Malloch & Cain) Cano & Guarro[a] (*Neoxenophila foetida*)	CBS 453.75	AB040693
	Aphanoascus terreus (Randhawa & Sandhu) Apinis[a] (*Chrysosporium indicum*)	CBS 342.64^{T}	AB040694
	Apinisia graminicola La Touche	CBS 721.68^{T}	AB040695
	Apinisia racovitzae (Lagarde) Guarro, Cano & Vroey[a] (*Arachnotheca albicans*)	CBS 156.77	AB040696
	Ascocalvatia alveolata Malloch & Cain	CBS 777.70	AB040697
	Pectinotrichum llanense Varsavsky & Orr	CBS 882.71^{T}	AB040698
	Renispora flavissima Sigler, Gaur, Lichtwardt & Carmichael	CBS 708.79	AB040699
	Shanorella spirotricha Benjamin	CBS 305.56	AB040700
	Uncinocarpus reesii Sigler & Orr	UAMH 2002[c]	AB040701
	Xanthothecium peruvianum (Cain) von Arx & Samson	CBS 112.54	AB053453
Mitosporic fungi related to Onygenales	*Coccidioides immitis* Stiles	3257[c]	AB040702
	Geomyces pannorum (Link) Sigler & Carmichael var. *pannorum*	CBS 108.14	AB040703
	Malbranchea aurantiaca Sigler & Carmichael	CBS 127.77^{T}	AB040704
	Malbranchea sp.	JCM 11275[d]	AB040705
	Oidiodendron tenuissimum (Peck) Hughes	CBS 238.31	AB040706

[a] Teleomorph names are shown. Species names used in CBS catalogue are indicated in parenthesis.
[b] Cultures were derived from: CBS, Centraalbureau voor Schimmelcultures, Baarn, the Netherlands; UAMH, University of Alberta Mold Herbarium and Culture Collection; JCM 11274 and JCM 11276 were isolated by the present authors and deposited in JCM, Japan Collection of Microorganisms (RIKEN). T, ex-type; NT, ex-neotype.
[c] DNA sample was derived from Dr. T. Kasuga.
[d] Culture and DNA sample were derived from Dr. T. Kasuga. Culture was deposited in JCM.

ple alignment of all sequences, and construction of the neighbor-joining tree (Saitou and Nei, 1987) based on Kimura's two-parameter method (Kimura, 1980) were performed using the programs Clustal X ver. 1.8 for Windows 95/98, the GUI version of Clustal W (Thompson et al., 1994). Bootstrap analysis (Felsenstein, 1985) was also performed by Clustal X for evaluating NJ tree topology with 1,000 random samplings. For the maximum parsimony (MP) analysis, PAUP ver 3.1.1 (Swofford, 1993) was used for heuristic search and bootstrap analy-

Table 3. Reference LSU rDNA sequences derived from the databases.

Order	Family	Species	Accession No.
Onygenales	Arthrodermataceae	*Arthroderma benhamiae* Ajello & Cheng	AF038359
	Gymnoascaceae	*Gymnascella citrina* (Massee & Salmon) Orr, Ghosh & Roy	U17915
	Onygenaceae	*Ajellomyces capsulatus* (Kwon-Chung) McGinnis & Katz	AF071950
		Ajellomyces crescens Sigler[a] (*Emmonsia crescens*)	AF071864
		Ajellomyces dermatitidis McDonough & Lewis	AF038358
		Aphanoascus fulvescens (Cooke) Apinis	AF038357
		Auxarthron californiense Orr & Kuehn	AF038352
		Nannizziopsis albicans (Apinis) Guarro, Cano & de Vroey[a] (*Amauroascus albicans*)	U17914
Mitosporic fungus related to Onygenales		*Paracoccidioides brasiliensis* (Splendore) Almeida	U81263
Eurotiales	Trichocomaceae	*Emericella rugluosa* (Thom & Raper) Benjamin	U29680
		Eupenicillium inusitatum Scott	AF033431
		Talaromyces helicus (Raper & Fennell) C. R. Benjam. var. *helicus*	AF033396
Mitosporic fungus related to Eurotiales		*Aspergillus flavus* Link	AF027863

[a] Species names used in DNA databases are indicated in parenthesis.

sis. This search was repeated several times from different random starting points to make certain the most parsimonious tree was found. In both phylogenetic analyses for all onygenalean taxa, species in the Myxotrichaceae were employed as an outgroup, since the previous SSU and LSU rDNA sequence analysis showed that their phylogenetic position was much closer to those of the Leotiales or the Erysiphales rather than to the other onygenalean taxa (Mori et al., 2000; Sugiyama et al., 1999).

Results

Figure 1 shows the phylogenetic tree constructed with the LSU rDNA sequence data of 42 representative taxa of the Onygenales and the Eurotiales. In the multiple alignment of these data, 238 sites were variable and 195 sites were parsimony-informative in 571 sites. The topology of the MP tree was congruent with that of the NJ tree, and thus only the bootstrap probability (BP) values of the MP tree are indicated on the NJ tree. In the phylogenetic tree, plectomycetous taxa were divided into two major clades, I and II. Clade I corresponded to the Eurotiales. The Onygenales except for the Myxotrichaceae constitute a recognizable monophyletic group as clade II (BP=74% in NJ; BP=54% in MP).

Two subclades (IIa and IIb) were distinguished in clade II. Clade IIa consisted of two genera of the Onygenales, *Ajellomyces* McDonough & Lewis, *Spiromastix*, and related mitosporic genera, *Malbranchea* Saccardo and *Paracoccidioides* Almeida. The BP of the clade IIa was 74% in the NJ tree and 56% in the MP analysis.

Three families of the Onygenales (Gymnoascaceae, Amauroascaceae, and Arthrodermataceae) corresponded to the subclades in clade IIb (Fig. 1), although the BP value of each subclade was less than 86% in the NJ tree and much weaker in the MP analysis.

In the case of the Amauroascaceae, the observed monophyly of this lineage agreed well with the results of the SSU rDNA sequence analysis by Sugiyama et al. (1999). *Renispora flavissima* was included in this lineage, although it was classified into the Onygenaceae (Currah, 1985). This result was also consistent with that of Sugiyama et al. (1999).

The taxa in the Onygenaceae were separated into two independent subclades in clade IIb. *Apinisia graminicola, A. racovitzae, Nannizziopsis albicans* and *Shanorella spirotricha* formed an independent clade (Onygenaceae 2) with the Arthrodermataceae apart from the other onygenaceous taxa (Onygenaceae 3), although the bootstrap support of the clade was weak (BP=67% in NJ and 56% in MP).

Coccidioides immitis, another known dimorphic pathogen, was included in the clade of Onygenaceae 2 with *Uncinocarpus reesii*. This result was coincident with the analysis of the SSU rDNA sequences by Pan et al. (1994) and with the morphological examination by Sigler et al. (1998).

Pectinotrichum llanense was also included in the clade of Onygenaceae 2, as in the results of the previous SSU rDNA sequences analysis (Sugiyama et al., 1999), although it was transferred to *Auxarthron* by Currah (1994).

Figure 2 shows the phylogenetic tree constructed with 13 selected taxa to focus on the phylogenetic relationships among *Spiromastix* species and related taxa. *Ajellomyces crescens, Arachnomyces nodosetosus* and *Xanthothecium peruvianum* were newly added to the data set, since they were included in clade IIa in the preliminary phylogenetic analysis (Sugiyama, unpublished data). In the multiple alignment of 565 sites in total, 148 sites were variable and 97 sites were parsimony-informative. The topologies of the NJ and the MP trees were congruent, and the BP values of the bootstrap consensus of the MP trees are shown in the same

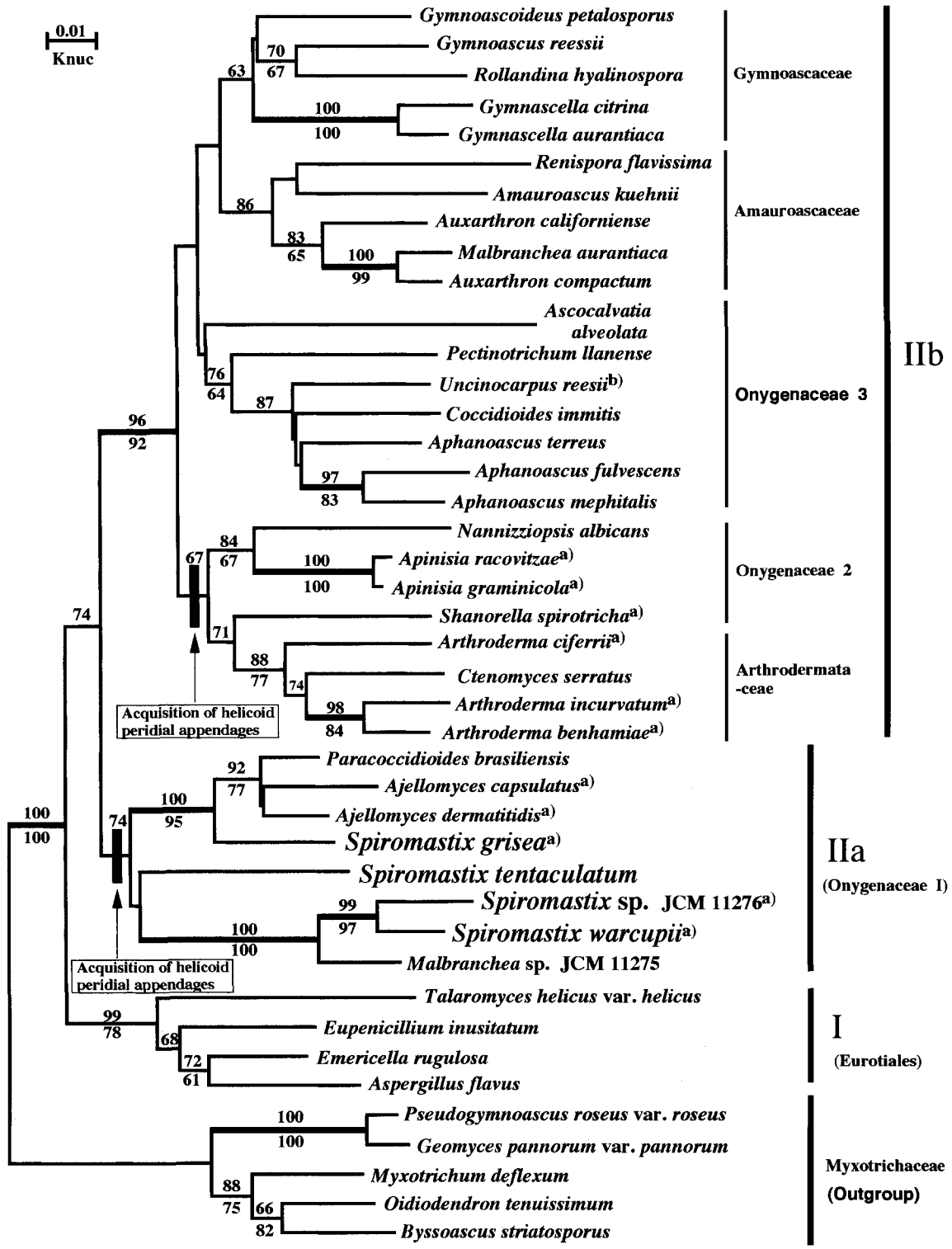

Fig. 1. Phylogenetic tree inferred from LSU rDNA partial sequence data. NJ tree topology and branch lengths are shown. A more than 60% bootstrap probability (1,000 replicates) is indicated from both the NJ analysis (above internodes) and the full heuristic search of maximum parsimony (MP) analysis (below internodes). Darkened branches indicate 80% or greater bootstrap support in both analyses. The tree statistics for the MP analysis are as follows: tree length of bootstrap consensus = 1014 steps; Consistency Index = 0.356; Retention Index = 0.604; Rescaled Consistency Index = 0.215. a) Taxon forming helicoid or loose spiral peridial appendages; b) Taxon forming uncinate peridial appendages.

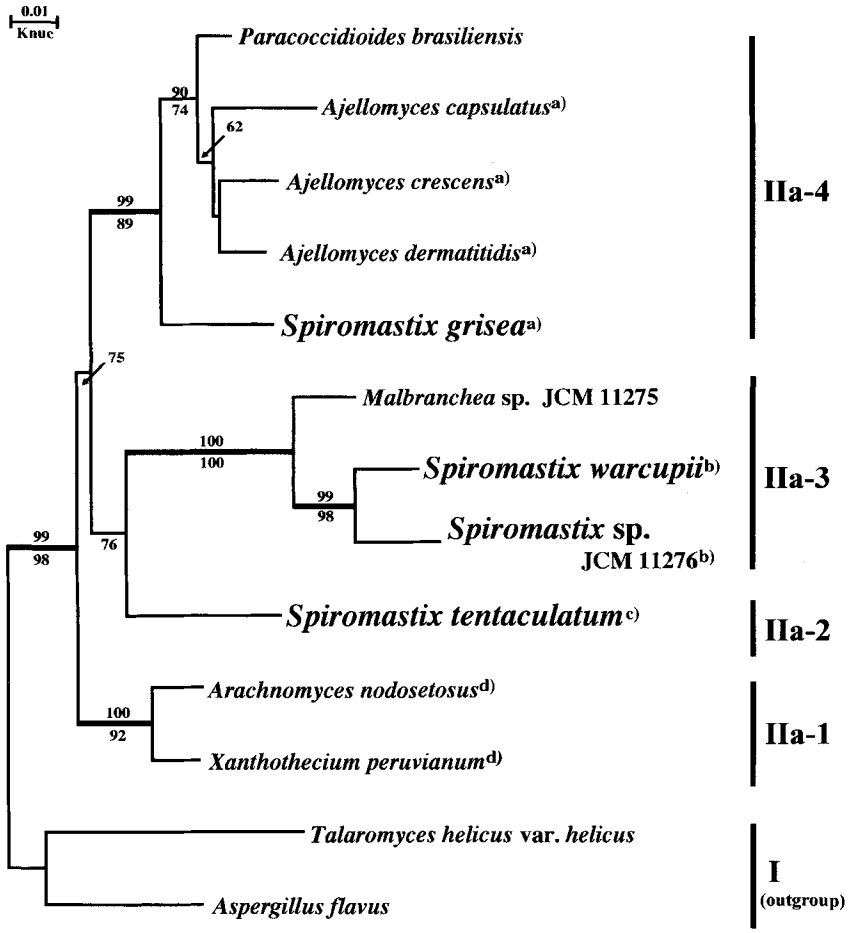

Fig. 2. Phylogenetic tree based on LSU rDNA sequences of *Spiromastix* and nine related taxa. Tree topology and BP values are indicated in the same manner as in Fig. 1. The tree statistics for the MP analysis are as follows: tree length of bootstrap consensus=270 steps; Consistency Index=0.670; Retention Index=0.656; Rescaled Consistency Index=0.440. a) Taxon forming spiral peridial appendages; b) Taxon forming loose spiral to wavy peridial appendages; c) Taxon forming curved peridial appendages with inflated ends; d) Taxon forming long, slender peridial appendages.

manner as in Fig. 1.

In Fig. 2, the monophyly of clade IIa from the Eurotiales was well supported by both the NJ and the MP bootstrap analyses. Four subclades were recognizable in clade IIa. Subclade IIa-1 consisted of *Arachnomyces nodosetosus*, which causes toenail and skin infection, and a non-pathogenic species, *Xanthothecium peruvianum*. *Spiromastix tentaculatum* was distinguishable from the other *Spriomastix* species (subclade IIa-2). Subclade IIa-3 included *S. warcupii*, *Spiromastix* sp. JCM 11276 and *Malbranchea* sp. JCM 11275. *Spiromastix warcupii* and *Spiromastix* sp. JCM 11276, with a sequence similarity of 96.8%, were clearly distinguishable on the phylogenetic tree. Subclade IIa-4 included three *Ajellomyces* species, *S. grisea* and *Paracocidioides brasiliensis*.

Discussion

In this study, LSU rDNA sequence analyses revealed the phylogenetic positioning of *Spiromastix* (Figs. 1, 2). The tree topology was consistent with the phylogenetic trees shown in previous molecular studies (LeClerc et al., 1994; Pan et al., 1994; Sugiyama et al., 1999). In addition, the results provided some interesting new data on the phylogenetic relationships in this order.

Phylogenetic relationships among *Spiromastix* and related taxa The dimorphic systemic pathogens, such as *Ajellomyces* and *Paracoccidioides*, are known to be phylogenetically distinct from the other onygenalean taxa (LeClerc et al., 1994; Bowman et al., 1996; Sugiyama et al., 1999). However, our present results indicated that non-pathogenic *Spiromastix* species are also included in the clade of *Ajellomyces* and *Paracoccidioides*. Similarly, another pathogenic taxon, *Arachnomyces nodosetosus* (=*Onychocola canadensis*), a known agent of Onychomycosis, and a non-pathogenic taxon, *Xanthothecium peruvianum*, were included in clade IIa (Fig. 2). Therefore, each major lineage in the Onygenales (IIa and IIb) contains skin (or nail) pathogens, dimorphic pathogens and non-pathogenic taxa.

Table 4 compares the phenotypic characters of *Ajellomyces* and *Spiromastix*. Both genera were similar in the morphology of ascospores and the helicoid peridial

Table 4. Comparison of morphological and other characteristics of *Ajellomyces* and *Spiromastix*.

Character		*Ajellomyces*[a]	*Spiromastix*[b]
ascomata	color	white to yellowish brown	brown to grayish brown or reddish brown
	size	$<350\ \mu m$	$<240\ \mu m$
	peridial appendages	helicoid	helicoid to curved or wavy
ascospores	color	hyaline	pale brown to brown or hyaline
	shape	spherical	discoid to lenticulate or spherical
	size	$<2\ \mu m$	$2–5\ \mu m$
	ornamentation	minutely pitted or muriculate	pitted
anamorph		*Blastomyces* *Histoplasma* *Emmonsia*	lacking
sexuality		heterothallic	probably homothallic
dimorphism		+	−
pathogenicity		+	−

[a] Unified data from the references of *A. crescens* (Sigler, 1996), *A. capsulatus* (Kwon-Chung, 1972) and *A. dermatitidis* (Kwon-Chung, 1975).
[b] Unified data from the reference listed in Table 1.

appendages of ascomata. However, dimorphism (yeast phase growth at 37°C) and pathogenicity to human or animals were not reported in any species of *Spiromastix*. The phenotypic differences between *Spiromastix* and the dimorphic pathogens suggest two hypotheses: 1) *Ajellomyces* is a highly specialized taxon that acts as a human pathogen; or 2) many intermediate taxa remain to be discovered or analyzed.

Figures 1 and 2 demonstrate that *Malbranchea* sp. JCM 11275 is a close relative of *S. warcupii* and *Spiromastix* sp. JCM 11276. This is noteworthy because no species with anamorphs has yet been reported in *Spiromastix*. This fact supports the latter hypothesis of the phylogenetic structure of clade IIa. More comprehensive exploration of unknown taxa and phylogenetic analysis incorporating their sequence data are necessary for understanding the phylogenetic structure of clade IIa.

On the other hand, *Malbranchea*-type anamorphs are commonly produced by the species in the Amauroascaceae (Currah, 1985; Udagawa, 1977), and the fact that *Malbranchea* species formed a clade with *Amauroascus* and *Auxarthron* in both of SSU and LSU rDNA trees (Sugiyama et al., 1999; Fig. 1) supports their phylogenetic relationships. Recently, Vidal et al. (2000) reviewed the genus *Chrysosporium*, which is also known as a major anamorphic genus in the Onygenales. They constructed a phylogenetic tree based on ITS1-5.8S-ITS2 rDNA sequences with their teleomophic taxa and reported the existence of several independent lineages in the genus. A similar study on *Malbranchea* and its teleomorphs will be needed to clarify their phylogenetic structure.

Udagawa (1997) treated *Spiromastix* as a member of the Amauroascaceae based on its Q-10 (H_2) ubiquinone system and weak kelatinophylic ability, although the data of individual species were not shown. We confirmed that the ubiquinone of *S. warcupii* was Q-10 (H_2) (Sugiyama, unpublished data). Interestingly, *Ajellomyces capsulatus* and *A. crescens* (=*Emmonsia parva*) possess Q-10 (H_2) (Fukushima et al., 1993; Takizawa et al., 1994), but *P. brasiliensis* and *Ajellomyces dermatitidis* have Q-10 (Fukushima et al., 1991). This diversity means that the ubiquinone system cannot be used as a common character of members of clade IIa.

Phylogenetic structure of the Onygenales Current classification systems of the Onygenales by Currah (1985) and Udagawa (1997) are based on comprehensive studies of phenotypic characters, such as ascospore ornamentation, substratum preferences, morphology of anamorph and ubiquinone system. In Fig. 1, each subclade corresponds to a family of the Onygenales, except for the Onygenaceae. It is remarkable that, despite their morphological differences, *Apinisia, Nannizziopsis* and *Shanorella* in the Onygenaceae (Onygenaceae 2) are close relatives of the Arthrodermataceae. The arthrodermataceous taxa are characterized by their smooth surface ascospores and multiseptate macroconidia, and they are easily distinguishable from the onygenaceous taxa. This clade (Onygenaceae 2) is the third lineage to be newly found in the Onygenaceae by molecular techniques. Re-evaluation of the phenotypic characters of these clades is therefore required for the phylogenetic classification of the onygenaceous taxa.

LSU and SSU rDNA analyses showed different tree topologies related to the Gymnoascaceae. The phylogenetic tree derived by LSU rDNA analysis indicated that the Gymnoascaceae is the latest diversified lineage in the Onygenales, whereas SSU rDNA analysis indicated that it is the earliest (Sugiyama et al., 1999). The BP values for clade IIb were more reliable in the LSU rDNA analysis (96% in NJ; 92% in MP) than in the SSU rDNA analysis (less than 50%). However, the BP values of the nodes between the Gymnoascaceae, the Amauroascaceae and the Onygenaceae (Onygenaceae 3) were still insufficient in this study. Therefore, further phylogenetic studies based on other genes or alternate analysis

methods are needed to confirm the time course of their diversification.

As already mentioned, Vidal et al. (2000) analyzed the phylogenetic relationships of the genus *Chrysosporium* with major onygenalean taxa by ITS1-5.8S-ITS2 rDNA sequences. Their phylogenetic trees exhibited good correspondence with the trees based on the SSU rDNA sequences (Sugiyama et al., 1999) and the LSU rDNA sequences in this study, with the exception of the positions of *Nannizziopsis albicans* and *Pectinotrichum llanense*. Their result for *P. llanense* was particularly interesting, because the taxon was clustered with the arthrodermataceous taxa. We are now examining the rDNA sequences and morphology of two other strains of *P. llanense* and *Nanniziopsis albicans* NRRL 5441 in order to confirm their phylogenetic positions.

Phylogenetic relationships among the onygenalean taxa with helicoid peridial appendages Several taxa in the Onygenales are known to produce helicoid peridial appendages on their ascomata similar to *Ajellomyces* and *Spiromastix*. These are *Arthroderma* Currey ex Berkeley emend. Weitzman, McGinnis, Padhye & Ajello, *Apinisia* La Touche, *Polytolypa* Scott & Malloch, *Shanorella* Benjamin and *Uncinocarpus* Sigler & Orr (only *U. queenslandicus* (Apinis & R. G. Rees) Sigler). These taxa have been discussed together (Orr, 1976; Currah, 1988; Guarro and Cano, 1991) or with the taxa forming uncinate peridial appendages (Scott et al., 1993).

Our tree showed that the taxa forming helicoid peridial appendages were localized to clade IIa and the subclade that included the Onygenaceae 3 and the Arthrodermataceae (Fig. 1). The tree also suggested that clade IIa was the earliest differentiated lineage from the rest of the Onygenales. We can therefore presume that, in the Onygenales, these appendages are plesiomorphic. However, Currah (1985) stated that reduced or mesh-like peridia were found in all four families of the Onygenales, and proposed that these structures were developed independently as an adaptation to similar environmental conditions, such as animal-dependent dispersal. If this is so, the helicoid peridial appendages are also derived from convergence evolution, although their nutritional strategy and habitats of these taxa are diversified (non-pathogenic, dermatophytic, or systemic to animal organs).

In Fig. 2, each subclade is characterized by morphological differences in the peridial appendages. The taxa in the clade IIa-1 form long, slender appendages that are not coiled, but that sometimes contain apical curves. *Spiromastix tentaculatum* forms wider appendages than those of clade IIa-1. The taxa in clade IIa-3 form long, wavy or loosely helical appendages. Typical spiral appendages are characteristic in clade IIa-4. Thus, the peridial appendages in clade IIa show a series of developments that reflects its phylogenetic structure. The topology of the tree also suggests that the long, slender appendages in clade IIa-1 are the most ancestral form. Consequently, helicoid peridial appendages produced on the ascomata of the onygenalean taxa like *Spiromastix* are apomorphic in clade IIa and acquired independently in both clade IIa and clade IIb after these clades were diversified.

In conclusion, LSU rDNA sequence analyses of the onygenalean taxa showed a strong association between the non-pathogenic genus *Spiromastix* and the pathogenic genera *Ajellomyces* and *Paracoccidioides*. It is also suggested that the helicoid peridial appendages are apomorphic in the Onygenales and evolved independently in clade IIa and clade IIb. The phylogenetic structure of this order is coincident with current classification systems of the Onygenales by Currah (1985) and Udagawa (1997) except for the Onygenaceae. The onygenaceous taxa are polyphyletic, and at least three independent lineages are recognized. Further new species exploration, phylogenetic studies based on other genes and the cladistic re-evaluation of their phenotypic characters will be needed to establish a new classification system in the Onygenales.

Acknowledgements——The authors are grateful to Dr. Seiji Tokumasu, University of Tsukuba, Dr. Shun-ichi Udagawa, Nodai Research Institute, Tokyo University of Agriculture, and an anonymous reviewer for their detailed review of the manuscript and their many helpful suggestions. Dr. S. Udagawa also kindly gave us advice on identification of the strains JCM 11275 and JCM 11276. We also thank Dr. Takao Kasuga, Roche Molecular Systems, and Dr. John W. Taylor and Dr. Matthew C. Fisher, University of California at Berkeley, for their kind help in supplying DNA samples of pathogenic onygenalean species and the living strain of JCM 11275. Finally, thanks are due to Centraalbureau voor Schimmelcultures, Baarn, the Netherlands, for providing the strains listed in Table 2.

Literature cited

Arx, J. A. von. 1987. A re-evaluation of the Eurotiales. Persoonia **13**: 273–300.

Bowman, B. H., White, T. J. and Taylor, J. W. 1996. Human pathogenic fungi and their close nonpathogenic relatives. Mol. Phylogenet. Evol. **6**: 89–96.

Currah, R. S. 1985. Taxonomy of the Onygenales: Arthrodermataceae, Gymnoascaceae, Myxotrichaceae and Onygenaceae. Mycotaxon **24**: 1–216.

Currah, R. S. 1988. An annotated key to the genera of the Onygenales. Syst. Ascomycet. **7**: 1–12.

Currah, R. S. 1994. Peridial morphology and evolution in the prototunicate ascomycetes. In: Ascomycete systematics: Problems and perspectives in the nineties, (ed. by Hawksworth, D. L.), pp. 281–293. Plenum Press, New York.

Currah, R. S. and Locquin-Linard, M. 1988. *Spiromastix grisea* sp. nov. and its relationship to other Onygenaceae with helical appendages. Can. J. Bot. **66**: 1135–1137.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**: 783–791.

Fisher, M. C., Koenig, G. L., White, T. J. and Taylor, J. W. 2000. Pahthogenic clones versus environmentally driven population increase: Analysis of an epidemic of the human fungal pathogen *Coccidioides immitis*. J. Clin. Microbiol. **38**: 807–813.

Fukushima, K., Nishimura, K., Takizawa, K., Sano, A., Takaki, G. M. C., Tateno, S., Takeo, K. and Miyaji, M. 1991. Ubiquinone systems of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*. Jpn. J. Med. Mycol. **32**: 1–4.

Fukushima, K., Takizawa, K., Okada, K. Maebayashi, Y., Nishimura, K., and Miyaji, M. 1993. Suitability of sterilization methods for ubiquinone analysis of pathogenic fungi. Trans. Mycol. Soc. Japan **34**: 473–480.

Guarro, J., Cano, J. and de Vroey, Ch. 1991. *Nannizziopsis* (Ascomycotina) and related genera. Mycotaxon **42**: 193–200.

Guarro, J., Gené, J. and de Vroey, Ch. 1993. A new species of *Spiromastix* from Africa. Mycotaxon **44**: 307–313.

Guého, E., LeClerc, M. C., de Hoog, G. S. and Dupount, B. 1997. Molecular taxonomy and epidemiology of *Blastomyces* and *Histoplasma* species. Mycoses **40**: 69–81.

Georgiev, O. I., Nikolaev, N., Hadjiolov, A. A., Skryabin, K. G., Zakharyev, V. M. and Bayev, A. A. 1981. The structures of the yeast ribosomal RNA genes 4. Complete sequence of the 25S rRNA gene from *Saccharomyces cerevisiae*. Nucl. Acids Res. **9**: 6953–6958.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequence. J. Mol. Evol. **16**: 111–120.

Kuehn, H. H. and Orr, G. F. 1962. A new genus of Gymnoascaceae. Mycologia **54**: 160–167.

Kwon-Chung, K. J. 1972. *Emmonsiella capsulata*: Perfect state of *Histoplasma capsulatum*. Science **177**: 368–369.

Kwon-Chung, K. J. 1975. Perfect state (*Emmonsiella capsulata*) of the fungus causing large-form African Histoplasmosis. Mycologia **67**: 980–990.

LeClerc, M. C., Philippe, H. and Guèho, E. 1994. Phylogeny of dermatophytes and dimorphic fungi based on large subunit ribosomal RNA sequence comparisons. J. Med. Vet. Mycol. **32**: 331–341.

Mori, Y., Sato, Y. and Takamatsu, S. 2000. Molecular phylogeny and radiation time of Erysiphales inferred from the nuclear ribosomal DNA sequences. Mycoscience **41**: 437–447.

Oorschot, C. A. N. 1980. A revision of *Chrysosporium* and allied genera. Stud. Mycol. **20**: 8.

O'Donnell, K. 1993. *Fusarium* and its near relatives. In: The fungal holomorph: Mitotic, meiotic and pleomorphic speciation in fungal systematics, (ed. by Reynolds, D. R. and Taylor, J. W.), pp. 225–233. CAB International, Wallingford.

Orr, G. F. 1976. *Kuehniella*, a new genus of the Gymnoascaceae. Mycotaxon **4**: 171–178.

Otani, Y. 1988. Seiya Ito's Mycological Flora of Japan. Vol. III. Ascomycotina No. 2. pp. 26–33. Yokendo, Ltd., Tokyo. (In Japanese.)

Pan, S., Sigler, L. and Cole, G. T. 1994. Evidence for a phylogenetic connection between *Coccidioides immitis* and *Uncinocarpus reesii* (Onygenaceae). Microbiology **140**: 1481–1494.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.

Scott, J. A., Malloch, D. W. and Gloer, J. B. 1993. *Polytolypa*, an undescribed genus in the Onygenales. Mycologia **85**: 503–508.

Sigler, L. 1996. *Ajellomyces crescens* sp. nov., taxonomy of *Emmonsia* spp., and relatedness with *Blastomyces dermatitidis* (teleomorph *Ajellomyces dermatitidis*). J. Med. Vet. Mycol. **34**: 303–314.

Sigler, L. and Carmichael, J. W. 1976. Taxonomy of *Malbranchea* and some other hyphomycetes with arthroconidia. Mycotaxon **4**: 349–488.

Sigler, L., Flis, A. L. and Carmichael, J. W. 1998. The genus *Uncinocarpus* (Onygenaceae) and its synonym *Brunneospora*: new concepts, combinations and connections to anamorphs in *Chrysosporium*, and further evidence of relationship with *Coccidioides immitis*. Can. J. Bot. **76**: 1624–1636.

Sugiyama, M., Ohara, A. and Mikawa, T. 1999. Molecular phylogeny of onygenalean fungi based on small subunit ribosomal DNA (SSU rDNA) sequences. Mycoscience **40**: 251–258.

Swofford, D. L. 1993. Phylogenetic analysis using parsimony (PAUP version 3.1.1.). Illinois Natural History Survey, Champaign, Illinois.

Takizawa, K., Okada, K., Maebayashi, Y., Nishimura, K., Miyaji, M. and Fukushima, K. 1994. Ubiquinone system of the form-genus *Chrysosporium*. Mycoscience **35**: 327–330.

Taylor, M. L., Chávez-Tapia, C. B., Vargas-Yañez, R., Rodríguez-Arellans, G., Peña-Sandoval, G. R., Toriello, C., Pérez, A. and Reyes-Montes, M. R. 1999. Environmental conditions favoring bat infection with *Histoplasma capsulatum* in Mexican shelters. Am. J. Trop. Med. Hyg. **61**: 914–919.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673–4680.

Uchiyama, S., Kamiya, S. and Udagawa, S. 1995. *Spiromastix saturnispora*, a new species from Indonesian soil. Mycoscience **36**: 353–357.

Udagawa, S. 1997. Taxonomic studies on Plectomycetes (Cleistothecial ascomycetes). Nippon Kingakukai Kaiho **38**: 143–157. (In Japanese.)

Udagawa, S. and Uchiyama, S. 1999. Taxonomic studies on new or critical fungi of non-pathogenic Onygenales 2. Mycoscience **40**: 291–305.

Vidal, P., Vinuesa, M. de los A., Sánchez-Puelles, J. M. and Guarro, J. 2000. Phylogeny of the anamorphic genus *Chrysosporium* and related taxa based on rDNA internal transcribed spacer sequences. Rev. Iberoam Micol. **17**: 22–29.