Phylogeny of *Ajellomyces*, *Polytolypa* and *Spiromastix* (*Onygenaceae*) inferred from rDNA sequence and non-molecular data.

W. A. Untereiner[1*], J. A. Scott[2,5], F. A. Naveau[3], R, S. Currah[4] and J. Bachewich[1]

[1] Department of Botany, Brandon University, Brandon, Manitoba R7A 6A9, Canada, [2] Department of Public Health Sciences, University of Toronto, Toronto, Ontario M5S 1A8, Canada, [3] Euroscreen, Brussels, B-1070 Belgium, [4] Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada, [5] SPOROMETRICS Inc., Toronto, Canada.

Abstract: Phylogenetic relationships within the *Onygenales* were inferred from maximum parsimony analyses of partial nuclear large ribosomal RNA subunit (nucLSU) sequences for 46 members of this order. The inferred phylogeny supports the division of the *Onygenales* into a number of separate lineages, two of which correspond to the *Arthrodermataceae* and *Gymnoascaceae*. The *Onygenaceae*, as circumscribed currently, is not monophyletic, and although the members of this family can be divided into a number of well-supported groups, the relationships among many of these taxa and their position relative to the *Gymnoascaceae* remain unresolved in our sequence-based phylogenies. *Shanorella* is more closely allied to the *Arthrodermataceae* than to the *Onygenaceae*. Our phylogenies provide additional evidence that a number of the morphological characters used to distinguish members of the *Onygenales* are of limited value for inferring phylogenetic relationships. Analysis of a data set that includes 12 non-molecular characters, partial nucLSU and mitochondrial small subunit RNA sequences (1406 bp) for a subset of eight taxa provides strong evidence for the close association of *Spiromastix grisea* and the dimorphic pathogen *Ajellomyces dermatitidis*. The new combination, *Ajellomyces grisea* (Currah & Locquin-Linard) Untereiner & Scott, is proposed.

Key words: anamorph, human-pathogenic fungi, molecular systematics, *Onygenales*, peridial appendages, ribosomal RNA gene sequences.

Introduction

As circumscribed by Currah (1985, 1994), the *Onygenaceae* encompasses ascomycetes with gymnothecial or cleistothecial ascomata, spherical, evanescent asci, pitted or punctate ascospores, and aleurio- or arthroconidial anamorphs. Keratinolytic ability, as demonstrated experimentally or inferred from the occurrence of these species on keratin-containing substrata, is a key character defining the *Onygenaceae* as well as the closely related family, *Arthrodermataceae*.

Morphological features considered to be of value in separating genera assigned to the *Onygenaceae* include the configuration of the ascomata, morphology of the peridium, surface ornamentation of ascospores, and the size and position of conidia (Currah 1985). Ascospore and peridial characters have proven to be particularly useful in defining the members of this family (Currah 1985). For example, ascospores within the *Onygenaceae* are always small and single-celled but range in shape from globose (*Ajellomyces* McDonough & Lewis) to oblate (*Auxarthron* Orr & Kuehn) or ellipsoidal-reniform (*Onygena* Persoon), and may be minutely pitted (*Shanorella* Benjamin), grooved (*Neogymnomyces* Orr) or punctate-reticulate (*Amauroascus* Schroeter) (Currah 1985). Ascospore surface ornamentation is a taxonomically useful character in the *Onygenaceae*, but this feature can be difficult to assess without the use of scanning electron microscopy or when the pits are very small and few in number (Currah 1997). Peridial appendages also vary considerably among the members of the *Onygenaceae* and may be lacking (*Aphanoascus* Zukal), pectinate (*Pectinotrichum* Varsavsky & Orr), curved (*Spiromastix* Kuehn & Orr) or helical (*Polytolypa* Scott & Malloch) (Cano & Guarro 1990; Currah 1985; Scott *et al.* 1993). Peridial characters are generally useful indicators of relationships at the genus level within the *Onygenaceae* (Currah 1997), but the members of this family cannot always be separated easily employing this feature. For example, species of *Amauroascus* and *Auxarthron* exhibit a continuum in the morphology of peridial hyphae that makes it difficult to differentiate these genera (Currah 1985, 1994).

Placement of species within the *Onygenaceae* can also be problematic in the absence of one or more definitive characters. For example, *Polytolypa hystricis* was thought to be more closely affiliated with the *Onygenaceae* than with the *Gymnoascaceae* on the basis of its anamorph and ascospore characters, but its closest relatives within the *Onygenaceae* could not be

*Corresponding author: Wendy A. Untereiner (e-mail: untereiner@brandonu.ca).

inferred from comparisons of morphological characters (Scott *et al.* 1993).

Comparisons of nucleotide sequences of chitin synthase (CHS) and nuclear ribosomal RNA (rRNA) genes have proven useful for examining relationships within *Onygenales* predicted on the basis of morphological and ecological characters (Bowman *et al*. 1996; Harmsen *et al*. 1995; Pan *et al.* 1994). Recently, phylogenetic analysis of nuclear small subunit rRNA gene (18S) sequences of pathogenic and saprobic *Onygenales* demonstrated that the family *Onygenaceae* as circumscribed currently is polyphyletic (Sugiyama *et al.* 1999). This finding has been corroborated in a study based on the analysis of partial nuclear large rRNA (nucLSU) gene sequences (Sugiyama & Mikawa 2001).

In the present investigation, we employed mitochondrial and nuclear rRNA gene sequences of an expanded set of taxa to infer phylogenetic relationships between the members of the *Onygenales* and within the *Onygenaceae*. And while one objective of this study was to clarify the phylogeny of the non-pathogenic members of the *Onygenaceae* employing molecular characters, we also used non-molecular characters to examine the phylogeny of a subset of closely related taxa that included species of *Ajellomyces*, *Polytolypa* and *Spiromastix*. Species used in this investigation included members of the families *Arthrodermataceae*, *Gymnoascaceae*, *Onygenaceae* (Order *Onygenales*) and *Trichocomaceae* (Order *Eurotiales*). Emphasis was placed on the inclusion of saprobic members of the *Onygenales*, and where possible, ex-type or authentic strains were used in comparisons.

Materials and methods

Fungal strains

Isolates employed in this study and their sources are listed in Table 1. All cultures were maintained at room temperature on modified Leonian's agar (MLA) (Malloch 1981a).

Temperature growth tests

Selected taxa were tested for their ability to grow at 35º C on MLA and on a basal medium (Scott & Untereiner 2002) supplemented with 1% glucose. Plates containing these media were inoculated with a single disk cut from the margin of colonies grown on MLA using a 4-mm cork-borer and the mycelium of the disk of inoculum was placed in contact with the surface of the agar. Inoculated test plates were sealed with Parafilm (American Can Co., Chicago, IL, USA), incubated in the dark in an incubator, and examined every 7 days for two weeks. Three replicates of each isolate were inoculated on both test media. Taxa were scored as positive for growth at 35º C if hyphae could be observed growing from the disk of inoculum or penetrating the agar medium below the disc using a dissecting microscope. Isolates capable of growing at 35º C were tested subsequently for their ability to grow at 37º C employing the same protocol.

DNA extraction, amplification and sequencing

Cultures used for DNA isolations were grown in modified Leonian's broth, harvested, and lyophilized as described previously (Untereiner *et al.* 1995). Total nucleic acids were extracted from ground, lyophilized cultures following the protocol of Lee & Taylor (1993). Precipitated DNA was pelleted by centrifugation, washed in 70% EtOH and dried in a vacuum centrifuge. Dried pellets of DNA were resuspended in 50 µL sterile, distilled water and the relative concentration of DNA was approximated by electrophoresis on a 0.7% agarose gel in 1X Tris-borate-EDTA buffer (pH 8.5). DNA was visualized by UV illumination following the staining of agarose gels in ethidium bromide.

A DNA fragment that extended from the nuclear 5.8S rRNA gene to approximately 1100-1200 base pair (bp) positions downstream of the 5' terminus of the nucLSU was amplified using the primers 5.8SR (5'-TCG-ATG-AAG-AAC-GCA-GCG-3') and LR7 (Vilgalys & Hester 1990) following the parameters described by Untereiner & Naveau (1999). The mitochondrial small subunit rRNA gene (mitSSU) was amplified using the primer pairs MS1 and MS2 (White *et al.* 1990) or MS1b (5'-GCA-GTG-AGG-AAT-ATT-GGT-CAA-TGG-3') and MS2b (5'-CAC-TAC-TGG-TTT-CAG-AAA-CGG-TC-3'). Residual primers, salts and unincorporated dNTPs were removed using a QIAquick PCR purification kit (Qiagen Ltd., Mississauga, ON, Canada) following the manufacturer's instructions.

Sequencing reactions were performed using a Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA, USA). Sequencing primers for the nucLSU included LR0R, LR2, LR3, LR3R, LR5, LR7 (Rehner & Samuels 1994; Vilgalys & Hester 1990) while MS1, MS1b, MS2, and MS2b were used to sequence the mitSSU. Excess dye terminators were removed by centrifugation using Centri-sep columns (Princeton Separations, Inc., Adelphia, NJ, USA) prior to analysis employing an Applied Biosystems 373A or 377 DNA sequencer.

Non-molecular data

A data set comprising morphological and physiological characters (Table 2) was compiled for eight members of the *Onygenaceae* and *Trichocomaceae* based on previously published descriptions of these taxa and the results of temperature growth tests.

Table 1. Substrates, sources and accession numbers of the isolates used in this study

Taxon	Substrate and locality	Source [a]	GenBank accession numbers	
			nucLSU	mitSSU
Arthrodermataceae				
Arthroderma curreyi Berkeley	not known	CBS 138.26	AY176726	---
A. gypseum (Nannizzi) Weitzman *et al.*	not known	ATCC 22925 T [b] (mt +) [c]	AY176727	---
A. incurvatum (Stockdale) Weitzman *et al.*	skin *ex H. sapiens*, United Kingdom	CBS 174.64 T	AY176738	---
A. otae (Hasegawa & Usui) McGinnis *et al.*	*ex* ringworm of *Felis domesticus* (cat), Japan	ATCC 28328 T (mt -)	AY176739	---
A. quadrifidum Dawson & Gentles	not known	ATCC 22954 T (mt +)	AY176728	---
A. silverae Currah *et al.*	*ex* dung of *Alopex lagopus* (arctic fox), Svalbard	UAMH 6715 T	AY176729	---
Chrysosporium vallenarense Oorschot & Piontelli	*ex* dung of *A. lagopus* (arctic fox), Svalbard	UAMH 6914	AY176732	---
Ctenomyces serratus Eidam	*ex* soil, Australia	CBS 187.61 NT [d]	AY176733	---
Epidermophyton floccosum (Harz) Langeron & Milochevitch	*ex Homo sapiens*, the Netherlands	CBS 553.84	AY176734	---
Microsporum canis Bodin	scraping and hair *ex* male *H. sapiens*, Canada	UAMH 2338	AY176735	---
M. cookei Ajello	*ex H. sapiens*, Canada	OMH H1-10	AY176736	---
M. persicolor (Sabouraud) Guiart & Grigorakis	*ex H. sapiens*, Canada	OMH, strain unnumbered	AY176737	---
Trichophyton krajdenii Kane *et al.*	*ex* skin lesion, *H. sapiens*, Canada	UAMH 3244 T	AY176740	---
T. mentagrophytes (Robin) Blanchard ("red" variant)	*ex H. sapiens*, Canada	OMH 607678	AY176741	---
T. mentagrophytes ("velvety" variant)	*ex H. sapiens*, Canada	OMH 566803	AY176742	---
T. raubitschekii Kane *et al.*	*ex H. sapiens*, Canada	OMH 6-1286	AY176743	---
T. rubrum (Castellani) Sabouraud	*ex* feet of *H. sapiens*, Canada	UAMH 2129	AY176744	---
T. simii (Pinoy) Stockdale *et al.*	*ex H. sapiens*, Canada	OMH 1585214	AY176745	---
Gymnoascaceae				
Arachniotus ruber (van Tieghem) Schroeter	*ex* soil, United Kingdom	CBS 352.90 NT	AY176746	---
Gymnascella aurantiaca Peck	*ex* soil, Russia	ATCC 22394 T	AY176747	---
Gymnoascoideus petalosporus Orr *et al.*	*ex* skin lesion of *H. sapiens*, India	ATCC 34351 T	AY176748	---
Gymnoascus reessii Baranetsky	*ex* soil, U.S.A.	CBS 410.72	AY176749	---
Onygenaceae				
Ajellomyces dermatitidis McDonough & Lewis	*ex H. sapiens*	ATCC 18187 T (mt A)	AY176704	AY176696
Amauroascus aureus (Eidam) von Arx	decayed wood, Japan	ATCC 18654 NT	AY176705	AY176701
A. niger Schroeter	*ex* soil, U.S.A.	ATCC 22339 NT	AY176706	---
A. purpureus Ito & Nakagiri	*ex* soil, Japan	IFO 32622 T	AY176707	---
Aphanoascus fulvescens (Cooke) Apinis	*ex* dung of *Ursus* sp. (bear), Canada	CBS 111.58	AY176708	---
A. mephitalis (Malloch & Cain) Cano & Guarro	carnivore dung, Canada	ATCC 22144 T	AY176725	---
A. terreum (Randhawa & Sandhu) Apinis	*ex* soil, India	ATCC 16413 T	AY176714	---
Apinisia graminicola La Touche	decomposing grass clippings, United Kingdom	CBS 721.68 T	AY176709	---
Ascocalvatia alveolata Malloch & Cain	carnivore dung, Canada	ATCC 22147 T	AY176710	---
Auxarthron californiense Orr & Kuehn	*ex* dung of *Neotoma* sp. (pack rat), U.S.A.	ATCC 15600 T	AY176711	---
A. zuffianum (Morini) Orr & Kuehn	*ex* lung of *Cynomys ludovicianus* (prairie dog), U.S.A.	CBS 219.58 NT	AY176712	---
Chrysosporium keratinophilum D. Frey *ex* Carmichael	*ex* soil, New Zealand	CBS 392.67 T	AY176730	---
C. tropicum Carmichael	*ex* woollen overcoat, Solomon Islands	MUCL 10068	AY176731	---
Coccidioides immitis Rixford & Gilchrist	not known	ATCC 7366	AY176713	---
Nannizziopsis vriesii (Apinis) Currah	*ex* skin and lungs of *Ameiva* sp. (Lizard), The Netherlands	ATCC 22444 T	AY176715	---
Neogymnomyces demonbreunii (Ajello & Cheng) Orr	*ex* soil, U.S.A.	ATCC 18394 NT	AY176716	---
Onygena equina (Wildenow) Persoon	hoof of *Bos taurus* (cow), Germany	ATCC 22731	AY176717	---

Polytolypa hystricis Scott & Malloch	dung of *Erethizon dorsatum* (American porcupine), Canada	UAMH 7299 T	AY176718	AY176700
Renispora flavissima Sigler *et al.*	*ex* bat guano and soil, U.S.A	ATCC 38503 T (mt +)	AY176719	---
Shanorella spirotricha Benjamin	feathers of a dead bird, U.S.A.	ATCC 12594 T	AY176720	---
Spiromastix grisea Currah & Locquin-Linard	dung of *Canis aureus* (jackal), Algeria	UAMH 6836	AY176721	AY176697
S. tentaculatum Guarro *et al.*	*ex* soil, Somalia	CBS 184.92 T	AY176722	AY176699
S. warcupii Kuehn & Orr	*ex* soil, Burundi	UAMH 7099	AY176723	AY176698
Unicinocarpus reesii Sigler & Orr	feathers, Australia	ATCC 34533 T (mt -)	AY176724	---
Trichocomaceae				
Byssochlamys nivea Westling	not known	CBS 100.11 T	AY176750	AY176703
Eurotium herbariorum (Wiggers ex Fr.) Link	unpainted board, U.S.A.	ATCC 16469 NT	AY176751	AY176702
Petromyces alliaceus Malloch & Cain	*ex* soil, Australia	ATCC 16891 T	AY176752	---

[a] Cultures were obtained from the following collections: ATCC, American Type Culture Collection, Manassas, VA, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; OMH, Ontario Ministry of Health, Toronto, ON, Canada; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada. [b] Strain derived from the type specimen. [c] Mating type. [d] Strain derived from the neotype specimen.

Table 2. Morphological characters and character states

Character	Character state [a]	Code
1. Colour of ascomata	white	0
	yellow-orange to tan	1
	greyish brown to brown	2
2. Diameter of ascomata	< 100 µm in diam.	0
	100-500 µm in diam.	1
	> 500 µm in diam.	2
3. Peridium	absent	0
	hyphal or mesh-like	1
	membranous or pseudoparenchymatous	2
4. Peridial appendages	absent	0
	wavy to slightly curved	1
	curved or helical	2
5. Ascospore colour	hyaline	0
	pigmented (yellow to pale brown)	1
6. Ascospore shape	globose	0
	ellipsoidal	1
	lenticular to oblate	2
7. Ascospore width	< 4.0 µm in diam.	0
	> 4.0 µm in diam.	1
8. Ascospore surface ornamentation	smooth to slightly roughened	0
	pitted to punctate	1
	echinulate-reticulate to reticulate-spiny	2
9. Ascospore equatorial furrow	absent	0
	present	1
10. Anamorph	absent	0
	aleurio- or arthroconidial	1
	phialoconidial	2
11. Growth at 37 C	absent	0
	present	1
12. Substrate	vertebrates	0
	plant material or soil	1
	dung	2

[a] Character states are based on descriptions provided by von Arx (1971), Currah (1985), Currah & Locquin-Linard (1988), Domsch *et al.* (1993), Guarro *et al.* (1993), Kuehn & Orr (1962), Kuehn *et al.* (1964), Malloch & Cain (1972), McDonough & Lewis (1968), Samson & Reenen-Hoekstra (1988) and Scott *et al.* (1993).

Table 3. Morphological character data matrix

Taxon	Character states											
	1	2	3	4	5	6	7	8	9	10	11	12
Ajellomyces dermatitidis	1	1	1	2	0	0	0	1	0	1	1	0,1
Amauroascus aureus	1	2	0,1	0	1	0	1	2	0	1	0	1
Byssochlamys nivea	0	1	1	0	1	1	0	0	0	2	1	1
Eurotium herbariorum	1	0	2	0	0	2	1	0	1	2	0	1
Polytolypa hystricis	2	1	1	2	1	1	0	1	0	1	0	2
Spiromastix grisea	2	1	1	2	0	2	0	1	0	0	1	2
S. tentaculatum	2	0	1	1	1	2	0	1	0	0	1	1
S. warcupii	2	0	1	2	1	2	0	1	0	0	1	1

The data matrix (Table 3) consisted of 12 unordered characters that were weighted equally in all analyses. Characters were treated as ambiguous for species exhibiting more than one character state

Data analysis

Sequences were edited and assembled into larger consensus sequences using Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, MI). Initial multiple alignments were produced using SeqPup version 0.6d (Gilbert 1996) and ClustalX version 1.7 (Thompson *et al.* 1994). The final multiple alignments were adjusted manually following visual inspection and the areas of sequence ambiguity were eliminated.

The first nucLSU data set (49 taxa, 892 bp) was analysed to examine the phylogenetic positions of pathogenic and saprobic members of the *Arthrodermataceae*, *Gymnoascaceae* and *Onygenaceae*. A second, smaller nucLSU data set (30 taxa, 897 bp) included sequences of 27 members of the *Onygenaceae*. The mitSSU (476 bp) and combined mitSSU-nucLSU (1406 bp) data sets contained the sequences of eight taxa. Taxa use as outgroups included members of the *Trichocomaceae* (*Byssochlamys nivea*, *Eurotium herbariorum* and *Petromyces alliaceus*). Gaps were defined as missing in analyses of all alignments.

Phylogenetic relationships were inferred from aligned sequences using the maximum parsimony (MP) method found in PAUP* (beta version 4.0b10) (Swofford 2002). Heuristic searches of the 49- and 30-taxon nucLSU data sets were performed employing tree bisection-reconstruction (TBR) branch swapping with the MulTrees and steepest descent options activated. Phylogenies inferred from the eight-taxon data sets were generated from exhaustive searches of the mitSSU, combined mitSSU-nucLSU and combined mitSSU-nucLSU-non-molecular data sets. Heuristic searches of the 30- and eight-taxon data sets for new optimal trees were conducted using 1000 random-addition-sequence replicates. Bremer support (Bremer 1994) was determined heuristically by searching for trees up to ten steps longer than the most parsimonious tree (MPT) and is given as the number of additional steps necessary for the collapse of a particular clade. For the smaller nucLSU data set (Fig. 2) the strict consensus of the first 50,000 trees from each search was compared to the MPT, whereas all trees up to 10 steps longer than the tree presented in Fig. 3 were examined. Bootstrap support (Felsenstein 1985) for internal branches was evaluated from 100 (larger nucLSU data set), 1000 (smaller nucLSU data set) or 10,000 (combined data sets) heuristic searches, and groups with a frequency of greater than 50% were retained in the bootstrap consensus trees. Congruence between the nucLSU, mitSSU and non-molecular data sets for eight taxa was measured based on 1000 searches using the partition-homogeneity test (PHT) (Farris *et al.* 1995) included in PAUP*. In this test, a $P < 0.05$ indicates incongruence of the data sets.

Results

Sequences employed in the molecular data sets ranged from 504 to 658 bp (mitSSU) and 941 to 952 bp (nucLSU) in length prior to the elimination of ambiguous or unalignable data (not shown). The larger nucLSU data set (49 taxa, 892 bp) included sequences of 46 members of the *Onygenales* and consisted of 159 phylogenetically informative characters. Parsimony analysis of this data set produced 60 MPTs, 697 steps in length (L) with a consistency index (CI) of 0.405 and a retention index (RI) of 0.691. The strict consensus of these trees (Fig. 1) included a large, well-supported clade (bootstrap support of 100%) that corresponds to the *Onygenales*. Well-supported lineages within this group (supported in >70% of 100 bootstrap replicates) included the *Aphanoascus fulvescens* - *Aph. mephitalis* - *Chrysosporium keratinophilum* - *Ch. tropicum* clade (83%), the *Auxarthron* clade (80%), the *Gymnoascaceae* (79%), the *Ascocalvatia alveolata* - *Onygena equina* clade (92%), the *Amauroascus purpureus* - *Neogymnomyces demonbreunii* - *Renispora flavissima* clade (81%), the *Arthrodermataceae* (70%), the *Spiromastix tentaculatum* - *S. warcupii* clade (88%), and a clade

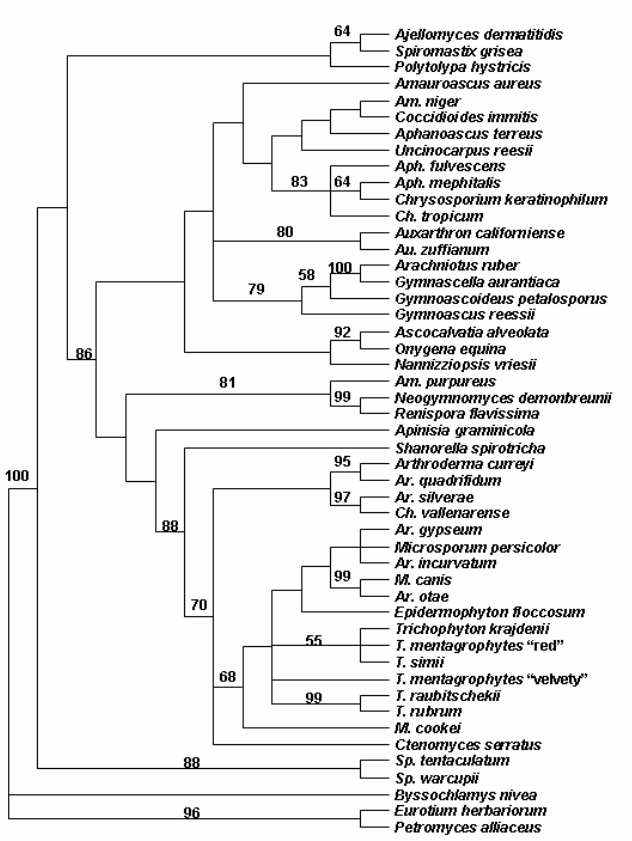

Fig. 1. Phylogenetic relationships of members of the *Onygenales* inferred from partial nucLSU sequence data. This is the strict consensus of 60 MPTs (L = 697) generated from an heuristic analysis of 892 bp for 49 taxa (CI = 0.405, RI = 0.691). Bootstrap values greater than 50% calculated from 100 replicates are given either above or adjacent to branches. The outgroup taxa are *Byssochlamys nivea*, *Eurotium herbariorum* and *Petromyces alliaceus*.

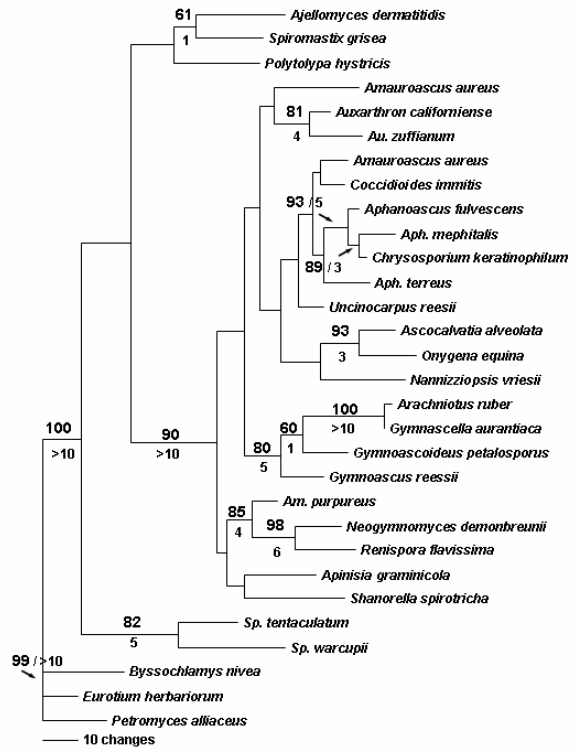

Fig. 2. Phylogenetic relationships of members of the *Onygenaceae* inferred from partial nucLSU sequence data. This is the single MPT (L = 608) generated from an heuristic analysis of 897 bp for 27 taxa (CI = 0.456, RI = 0.571). Bootstrap values greater than 50% calculated from 1000 replicates are given above branches or to left of the diagonal lines adjacent to branches. Bremer support is shown below branches or to the right of the diagonal lines adjacent to branches. The outgroup taxa are *Byssochlamys nivea*, *Eurotium herbariorum* and *Petromyces alliaceus*.

that encompasses all members of the *Onygenales* (86%) except *Ajellomyces dermatitidis*, *Polytolypa hystricis* and species of *Spiromastix*. Well-supported groups within the *Arthrodermataceae* included the *Arthroderma curreyi* - *Ar. quadrifidum* clade (95%), the *Ar. silverae* - *Ch. vallenarense* clade (97%), the *Microsporum canis* - *Arthroderma otae* clade (99%) and the *Trichophyton raubitschekii* - *T. rubrum* clade (99%). *Shanorella spirotricha* grouped with the *Arthrodermataceae* with a high level of support (88%).

Parsimony analysis of the data set for members of the *Onygenales* excluding the *Arthrodermataceae* (30 taxa, 897 bp, 149 phylogenetically informative characters) produced a single MPT (L = 608, CI = 0.456, RI = 0.571) (Fig. 2) that was similar in topology to the consensus tree inferred from sequences of 49 taxa. Shorter trees were not found in a search based on 1000 random-addition-sequence replicates. With the exception of the *Aphanoascus* - *Chrysosporium* clade, groups inferred from the larger nucLSU data set were also recovered with comparable levels of support (1000 bootstrap replicates). *Shanorella spirotricha* grouped with *Apinisia graminicola* (<50%) but the position of these species within the *Gymnoascaceae* - *Onygenaceae* clade (90%) was not resolved. *Ajellomyces*, *Polytolypa* and *Spiromastix* were again shown as not as closely allied to other members of the *Onygenaceae*.

Two MPTs (L = 414, CI = 0.746, RI = 0.488) were inferred in a heuristic search of the combined mitSSU-nucLSU data set (1406 bp, 129 parsimony informative characters) that included *A. dermatitidis*, *Am. aureus*, *P. hystricis*, three members of the genus *Spiromastix* and the outgroup taxa *B. nivea* and *E. herbariorum*. Data from these two rRNA gene regions were combined based on congruence demonstrated by the partition-homogeneity test (P = 0.486). Parsimony analysis of the combined mitSSU-nucLSU-non-molecular data set (1418 characters of which 139 are parsimony informative, P = 0.088) for the same subset of taxa generated 3 MPTs (L = 444, CI = 0.736, RI = 0.475). The strict consensus trees inferred from each of the combined data sets were identical (Fig. 3), as were the consensus trees inferred for each data set based on a search of 1000 of random-addition-sequence replicates. In these phylogenies, *A. dermatitidis* and *S. grisea* grouped together with a high level of support (93% and 91% of 10,000

bootstrap replicates, Bremer support of 7 and 6 for the molecular and combined molecular-morphological data sets, respectively) and formed a sister group to *P. hystricis*. Although this clade was not as strongly supported (61% and 58%), *A. dermatitidis*, *S. grisea* and *P. hystricis* formed a group that was distinct in all analyses from the clade that contained *S. tentaculatum* and *S. warcupii* (56% and 64%).

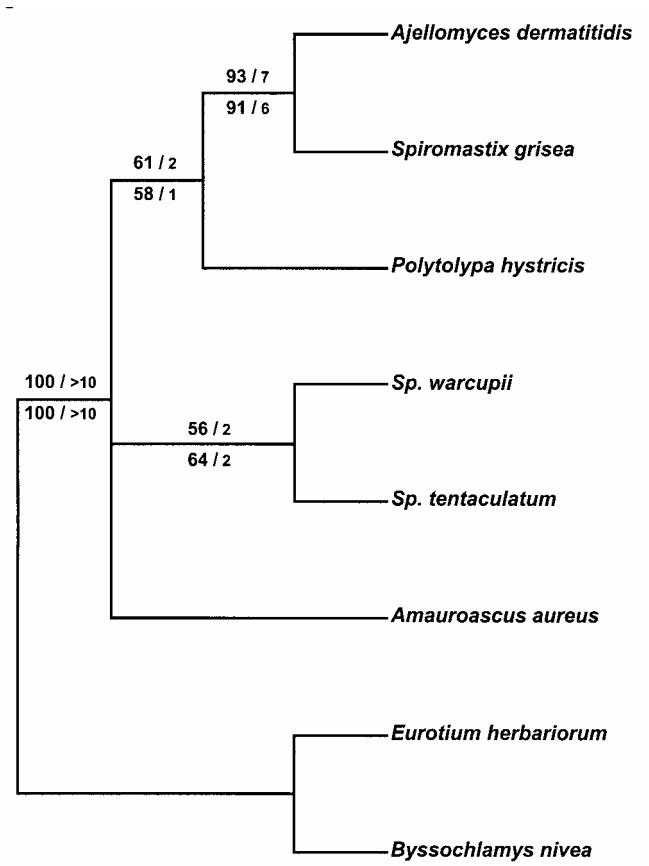

Fig. 3. Phylogenetic relationships of selected members of the *Onygenaceae* inferred from the combined data sets (mitSSU-nucLSU and mitSSU-nucLSU-non-molecular data) for eight taxa. The strict consensus trees inferred from exhaustive searches of these two data sets are identical. An exhaustive search of the mitSSU-nucLSU data set (1406 bp) generated 2 MPTs (L = 414, CI = 0.746, RI = 0.488) while an exhaustive search of the mitSSU-nucLSU-non-molecular data set (1418 characters) generated 3 MPTs (L = 444, CI = 0.736, RI = 0.475). Bootstrap values greater than 50% calculated from 10,000 replicates for the mitSSU-nucLSU data set are given to the left of the diagonal lines above the branches and to the left of the diagonal lines below the branches for the mitSSU-nucLSU-non-molecular data set. Bremer support for the mitSSU-nucLSU data set is shown to the right of the diagonal lines above the branches and to the right of the diagonal lines below the branches for the mitSSU-nucLSU-non-molecular data set. The outgroup taxa are *Byssochlamys nivea* and *Eurotium herbariorum*.

Discussion

Phylogenetic structure of the Onygenales

The revision of the *Onygenales* presented by Currah (1985) was the first comprehensive taxonomic treatment of this group and the first to propose the use of a suite of correlated ecological and morphological characters to delimit the families, genera and species placed in this order. With the exception of the *Myxotrichaceae*, the only markedly cellulolytic family assigned to the *Onygenales*, Currah's concept of this order is supported by the results of this investigation and other molecular systematic studies (Leclerc *et al.* 1994; Sugiyama & Mikawa 2001; Sugiyama *et al.* 1999). Members of the *Myxotrichaceae* are closely related phylogenetically (Hambleton *et al.* 1998; Sugiyama & Mikawa 2001; Sugiyama *et al.* 1999) and have been shown recently to be allied to the *Leotiales* (Sugiyama *et al.* 1999).

Species placed in the *Onygenales* are currently divided among the families *Arthrodermataceae*, *Gymnoascaceae* and *Onygenaceae*. The *Arthrodermataceae*, a group of keratinolytic and predominantly animal-associated taxa, represents a well-supported lineage that includes the dermatophytes (*Arthroderma* and related anamorphic taxa) and the saprobe, *Ctenomyces serratus*. The results of our study support previous sequenced-based phylogenies inferred from the analysis of rRNA gene regions that position these taxa within a well-supported clade that is sister to members of the *Onygenaceae* (Leclerc *et al.* 1995; Sugiyama & Mikawa 2001; Sugiyama *et al.* 1999). *Shanorella spirotricha* is allied to the *Arthrodermataceae* with a high level of support in our investigation (88% of bootstrap replicates, Fig. 1) and in the study of Sugiyama & Mikawa (2001). *Shanorella spirotricha* was placed originally in the *Onygenaceae* because it is keratinolytic and possesses pitted ascospores (Currah 1985), but we concur with Currah (1997) that this species can be accommodated in the *Arthrodermataceae*.

The *Gymnoascaceae* also comprises a monophyletic group that received significant support in our analyses of nucLSU sequences (Fig. 1, 2) and in two recently published sequence-based phylogenies (Sugiyama & Mikawa 2001; Sugiyama *et al.* 1999). Currah (1985, 1994) considered the *Gymnoascaceae* to be heterogeneous and thought that some of its members would be better placed in the *Arthrodermataceae* (in the case of *Gymnoascoideus petalosporus*) and *Eurotiales* (for *Arachniotus ruber* and species of *Gymnascella* Peck). Although the position of the *Gymnoascaceae* within the *Onygenales* is not well resolved in phylogenies inferred from rRNA genes, the available sequence data supports the suggestion of Malloch (1981b) that these fungi should be included in the *Onygenaceae*. Identifying the closest onygenalean relatives of the *Gymnoascaceae* will likely require the sequencing of additional gene regions and the analysis of combined data sets consisting of molecular and non-molecular characters.

Phylogenetic relationships within the Onygenaceae

The *Onygenaceae* has been shown previously to be polyphyletic in phylogenies inferred from the analyses of nuclear rRNA gene sequences (Sugiyama & Mikawa 2001; Sugiyama *et al.* 1999). Although the topology of the strict consensus presented in Fig. 1 is similar to the Neighbor-joining (NJ) tree of Sugiyama & Mikawa (2001) there are notable differences between these phylogenies with respect to the robustly supported clades that include members of the *Onygenaceae*. For example, a clade corresponding to the Amauroascaceae and including *Amauroascus kuehnii* von Arx, *Auxarthron californiense* and *Renispora flavissima* (Sugiyama & Mikawa 2001) was not resolved in our phylogeny. The lineage identified as '*Onygenaceae* 3' (Sugiyama & Mikawa 2001) probably corresponds to the *Aphanoascus* - *Chrysosporium* clade (Fig. 1, 2) but the close relationship of these taxa to *Aph. terreus*, *Coccidioides immitis* and *Uncinocarpus reesii* is not supported strongly in our phylogenies. *Aphanoascus* was enlarged by Cano & Guarro (1990) to include *Keratinophyton terreum* (Randhawa & Sandhu) Apinis and *Xynophila mephitalis*. Our phylogeny confirms the close relationship of the latter species to the type of the genus (*Aph. fulvescens*) but it does not clearly resolve the position of *Aph. terreus* (= *K. terreum*). Analysis of a more rapidly evolving gene region from a greater number of representatives of this genus would likely clarify the phylogeny of *Aphanoascus* and the relationship of these species to *C. immitis* and *U. reesii*.

The '*Onygenaceae* 2' lineage identified by Sugiyama & Mikawa (2001) as including *Nannizziopsis albicans* (Apinis) Guarro *et al.* and two species of *Apinisia* La Touche is sister to the *Arthrodermataceae*. Guarro *et al.* (1991) considered *Apinisia* to be closely related to *Ajellomyces*, but our own results are consistent with a phylogeny previously inferred from the analysis of 18S rRNA sequences (Sugiyama *et al.* 1999) that suggested this taxon is allied to the *Arthrodermataceae*. The position of the single representative of the genus *Nannizziopsis* Currah included in our investigation was not resolved (Fig. 1, 2). Curiously, *Shanorella spirotricha* was described as a member of the '*Onygenaceae* 2' by Sugiyama & Mikawa even though it was positioned within the *Arthrodermataceae* (see previous discussion). Finally, the '*Onygenaceae* 1' in the NJ tree of Sugiyama and Mikawa (2001) corresponds to the taxa positioned outside of the *Arthrodermataceae-Gymnoascaceae-Onygenaceae* clade in our analysis (Fig. 1). Although these taxa, including *Ajellomyces*, *Polytolypa* and *Spiromastix*, do not form a single well-supported clade in phylogenies inferred from the larger or smaller nucLSU data sets (Fig. 1, 2), they are not closely related to other members of the *Onygenaceae*.

Phylogeny of Ajellomyces, Polytolypa and Spiromastix

As circumscribed currently, *Spiromastix* encompasses species with ascomata bearing thick-walled, curved or scimitar-shaped to helical peridial appendages and oblate to globose ascospores that are pitted to punctate (Currah 1985, 1988; Currah & Locquin-Linard 1988; Guarro *et al.* 1993; Kuehn & Orr 1962; Udagawa & Uchiyama 1999; Uchiyama *et al.* 1995). The genus includes five species. All except *S. grisea* have been isolated from soil.

The position of *Spiromastix* within the *Onygenales* is controversial despite the similarity of these species to a number of the members of this order. Currah (1985) placed the genus in the *Onygenaceae* but later questioned its status as a member of this family because *Spiromastix* species lack anamorphs and were considered to be only weakly keratinolytic using the hair plate assay (Currah 1994). Guarro *et al.* (1993) treated *Spiromastix* as a member of the *Gymnoascaceae*. *Spiromastix grisea*, *S. tentaculatum* (as *Spiromastix* sp. UAMH 7098) and *S. warcupii* were shown subsequently to be incapable of degrading human hair *in vitro* (Scott *et al.* 1993).

The phylogeny of *Spiromastix* inferred from the combined mitSSU-nucLSU and mitSSU-nucLSU-non-molecular data sets (Fig. 3) conforms closely to the 13-taxon NJ tree presented by Sugiyama & Mikawa (2001). All trees show *Spiromastix* to be polyphyletic with *A. dermatitidis* and *S. grisea*, forming a well-supported clade (>90% bootstrap support) that is sister to the group that includes *S. warcupii* and *S. tentaculatum*. The thermally dimorphic pathogens *Ajellomyces capsulatus* (Kwon-Chung) McGinnis & Katz, *A. crescens* Sigler and *Paracoccidioides brasiliensis* (Splendore) Almeida are also members of the former clade in the 13-taxon NJ tree of Sugiyama & Mikawa (2001). The close phylogenetic relationship of *Ajellomyces*, *Paracoccidioides* Almeida and allied anamorphic taxa was demonstrated through the analyses of CHS and rRNA gene sequences (Bowman *et al.* 1996; Harmsen *et al.* 1995; Herr *et al.* 2001; Leclerc *et al.* 1994; Pan *et al.* 1994; Peterson *et al.* 1998) but *S. grisea*, a species described from the dung of gazelle, is the first saprobic taxon identified as a member of the clade comprising the pathogenic *Onygenaceae* (Sugiyama & Mikawa 2001).

Spiromastix grisea and species of *Ajellomyces* share a number of features including the production of ascomata with coiled appendages, small ascospores with faintly verrucose to punctate walls, limited or no keratinolytic activity and the ability to grow at 37º C (Currah & Locquin-Linard 1988; Scott et al. 1993; Scott & Untereiner 2002; Sigler 1996). *Spiromastix grisea* differs from *Ajellomyces* in possessing oblate ascospores and in lacking an anamorph and a yeast-like phase. Although *S. grisea* is not known as a pathogen of vertebrates, its occurrence on dung

suggests a reliance on animals for habitat formation and perhaps for dispersal.

On the basis of these criteria, we propose that *Spiromastix*, typified by *S. warcupii*, be restricted to species isolated from soil that possess oblate ascospores and peridial appendages that are wavy to curved or helical but with only 1-2 turns per helix. *Spiromastix tentaculatum* is allied closely to *S. warcupii*, but the phylogenetic positions of *S. saturnispora* Uchiyama *et al.* and *S. sphaerospora* Udagawa & Uchiyama remain to be examined by means of cladistic methods. *Spiromastix grisea* is transferred to *Ajellomyces*[1], the genus to which it is most closely related phylogenetically.

Species of *Ajellomyces* also resemble *Polytolypa hystricis*, a non-keratinolytic species known only from porcupine dung (Scott *et al.* 1993). The close affinity of *Polytolypa* to *Ajellomyces* was suggested by Sigler (1996) who noted that both taxa possess punctate-muricate ascospores and ascomata with coiled peridial appendages. *Polytolypa hystricis* is allied closely to *A. dermatitidis* and *A. grisea* in our phylogenies, but its position as sister to the clade that includes these species is more strongly supported in trees inferred from the combined molecular and molecular-non-molecular data sets (Fig. 3) than in trees derived from molecular analysis alone. *P. hystricis* can be separated from *Ajellomyces* and *Spiromastix* by its ellipsoidal ascospores and its production of alternate arthroconidia. Its phylogenetic position is insufficiently resolved to warrant its transfer to either genus.

Morphological and ecological characters of members of the Onygenales

Comparisons of nucleotide sequences of members of the *Onygenales* demonstrates that pathogenicity has arisen independently in several lineages within the order and provides strong evidence for the close relationship of taxa predicted on the basis of morphological criteria (Bowman *et al.* 1996; Pan *et al.* 1994). Molecular phylogenetic studies have also revealed connections among ecologically and morphologically dissimilar taxa. For example, *Renispora flavissima* has been shown to be more closely allied to species of *Auxarthron* and *Coccidioides* Stiles than to *Ajellomyces* even though it produces tuberculate conidia that resemble the anamorph of *A. capsulatus* (Bowman *et al.* 1996; Sugiyama *et al.* 1999). Similarly, the results of this investigation and the study of Sugiyama & Mikawa (2001) demonstrate that *Apinisia*, *Nannizziopsis* and *Shanorella* are more closely allied to the *Arthrodermataceae* than to other members of the *Onygenaceae*. The close connection between *Shanorella* and the *Arthrodermataceae* is reinforced by the occurrence of a number of the members of this family on dung (Currah 1985; Hubálek 2000) and by the similarity of the nodulose, contorted peridial hyphae of *Shanorella* to the ossiform cells of *Arthroderma*.

Molecular phylogenetic studies demonstrate ultimately that while the morphological characters used to separate families, genera and species within the *Onygenales* may be useful taxonomically, they may or may not be informative phylogenetically. The issue of the relative value of morphological features in the *Onygenales* has been discussed by Currah (1985, 1994, 1997) who noted that certain types of peridia and ascospores exhibit high levels of convergence that reflect common mechanisms of the dispersal rather than close phylogenetic relationships. Summerbell (2000) suggested that the helical peridial appendages of members of the *Onygenales* may function to deter arthropod grazing. Virulence, host- or substrate-specificity, sexual incompatibility system or the apparent loss of sexuality, thermotolerance and the production of keratinases are characters that reflect adaptations to specific environments and are also probably of limited taxonomic value within the *Onygenales* above the level of genus or species.

Because phylogenies of the *Onygenales* inferred from different regions of the nuclear rRNA cistron are not completely concordant, identifying the most recently diverged lineages within this order and determining the direction of the evolution of ecological and morphological characters will require the analysis of a greater number of genes and gene regions. However, we concur with Currah (1994, 1997) and Sugiyama & Mikawa (2001) that the accurate elucidation of lineages within the *Onygenales* necessitates an approach that considers chemical, ecological and morphological data as well as information gleaned from nucleotide sequences.

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[1] *Ajellomyces grisea* (Currah & Locquin-Linard) Untereiner & Scott comb. nov., basionym *Spiromastix grisea* Currah & Locquin-Linard, Canad. J. Bot. 66: 1135 (1988).

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