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FULL PAPER

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Molecular characterization of *Monascus* strains based on the D1/D2 regions of LSU rRNA genes

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Abstract The D1/D2 regions of the large subunit (LSU) rRNA genes of 65 strains of *Monascus* and *Xeromyces* were PCR amplified and sequenced in both directions. Maximum-parsimony analysis produced five most parsimonious trees. The strict consensus tree of these five parsimonious trees clustered *M. eremophilus*, *M. ruber*, *M. pilosus*, *M. purpureus*, and *M. sanguineus* in the same clade, reflecting high sequence similarity. *M. sanguineus*, *M. purpureus*, *M. ruber*, and *M. pilosus* differed in one or two nucleotides. The sequence of *M. eremophilus* ATCC 62925 isolated from a xerophilic environment differed from *M. purpureus* in only one nucleotide, despite pronounced morphological and ecological differences when compared with the other species. *M. lunisporas*, *M. floridanus*, *M. pallens*, and *X. bisporus* were each placed in a separate branch, confirming their taxonomic descriptions as individual species. Maximum-likelihood analysis on the same data set generated a single tree and grouped the species of the first clade in the parsimony analysis into a single clade but placed the rest of the *Monascus* species and *Xeromyces bisporus* on different branches. The trees inferred from both analyses revealed a monophyletic relationship between *Monascus* and *Xeromyces*, when compared with other related cleistothecial or imperfect genera.

Key words D1/D2 region · Large subunit (LSU) rRNA gene · *Monascus*.

Introduction

Strains of the genus *Monascus* van Tieghem have been used in the preparation of various types of oriental fermented foods and are important in the production of food coloring. They are also a source of biologically active compounds (Endo et al. 1986; Juzlova et al. 1996).

Based on physiological and morphological characteristics, Hawksworth and Pitt (1983) recognized three species of *Monascus*: *M. pilosus* K. Sato, *M. ruber* van Tieghem, and *M. purpureus* Went. *Monascus ruber* is distinguished from *M. pilosus* by the presence of a brownish pigment in its cleistothecial walls and conidia. These structures are hyaline in *M. pilosus*. *Monascus purpureus* was differentiated from *M. pilosus* by much slower growth on certain types of agar.

Barnard and Cannon (1987) described *M. floridanus* Cannon & Barnard, isolated from the roots of sand pine trees in Florida. This species shares many features with *M. ruber*: neither produces soluble pigments, but both have brown-pigmented ascomatal walls and conidia. *Monascus floridanus* differs from the other species by its slower growth rate and smaller ascospores and conidia. Hocking and Pitt (1988) described a xerophilic species, *M. eremophilus* Hocking & Pitt, which differed from the other species in its slow growth rate, the absence of an anamorph, and the requirement for extremely dry conditions. However, its morphology and biogenesis of ascospores were consistent with the description of the genus. Cannon and coworkers (1995) reported two additional species, *M. pallens* Cannon, Abdullah & Abbas and *M. sanguineus* Cannon, Abdullah & Abbas, based on the size of ascospores and colonies, pigmentation, and enzymatic profiles. Udagawa and Baba (1998) described *M. lunisporas* Udagawa & Baba, unique for lunate ascospores and dark olive-brown ascomata. Although most of these species are considered members of the genus, the taxonomic classification of *Xeromyces bisporus* Frazer, which requires a very dry environment ($0.61a_w$), has been very controversial. It was once considered a distinct species of *Monascus* (von Arx 1970) but was later classified as a closely related but separate genus (Pitt and Hocking 1982). *Xeromyces bisporus* is distinguished from *Monascus* species by the presence of colorless and sessile ascomata and two-spored asci.

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The current criteria for the species delimitation and description of *Monascus* and *Xeromyces* consist of morphological and physiological characteristics. However, because the pigmentation of cultures and the shape and the size of spores, which are major features for species delimitation, can be influenced by slight environmental differences and are subject to mutation, the species boundaries dictated by morphological and physiological features need to be reexamined using molecular information.

Comparative analyses of the nucleotide sequences of the D1/D2 regions of the large subunit (LSU) rRNA genes were carried out to determine the suitability of the D1/D2 region as a genetic marker to differentiate species and the inter- and intraspecies variability among these sequences.

Materials and methods

Cultivation of strains

Sixty-four strains of *Monascus* and 2 strains of *Xeromyces* were obtained from cryopreserved material at ATCC. The strains were cultivated using one of six agar or broth media at 25° or 30°C for approximately 7 days (Table 1). The media formulations included Blakeslee's formula (ATCC medium 325: malt extract 20 g, glucose 20 g, peptone 1 g, and agar 20 g per liter); PDA (ATCC medium 336: diced potatoes 300 g, glucose 20 g, and agar 15 g per liter); Emmon's modification of Sabouraud's agar [ATCC medium 28: Sabouraud's glucose broth 30 g (Difco 0382) and agar 20 g per liter]; Harrold's M40Y (ATCC medium 319: malt extract 20 g, yeast extract 5 g, sucrose 400 g, and agar 20 g per liter); malt agar (ATCC medium 323: Difco 0024); and malt extract agar (ATCC medium 324: malt extract 20 g, peptone 5 g, and agar 15 g per liter).

The species identity of each strain was confirmed by observing the size, shape, and pigmentation of conidia, conidiophores, or ascomata. Growth rates were measured 7 days after inoculation on malt extract agar at 25°C. The production of soluble pigments, the size of colonies, and the pigmentation of mycelia were noted (Hawksworth and Pitt 1983; Barnard and Cannon 1987; Cannon et al. 1995) (Table 2). Synonyms acknowledged by Hawksworth and Pitt in 1983 are in parentheses.

Isolation of genomic DNA

Mycelia were harvested by centrifugation at $13\,800 \times g$ for 5 min with a microfuge. Genomic DNAs were isolated according to the method of Cenis (1992) with some modification. Mycelia were placed in yeast lysis matrix tubes (Bio101, Vista, CA, USA) and subjected to vigorous agitation in a FastPrep FP120 shaker (Bio101) for two 40-s intervals at a setting of 4.0. To 50 µl DNA solution, 0.25 µl (0.5 µg/µl) RNase (Boehringer Mannheim, Indianapolis, IN, USA) was added, and the mixture was incubated for 30 min at 30°C. Genomic DNAs were used for PCR. The concentration of genomic DNA was determined by comparing band intensity with a molecular weight standard on an agarose gel and UV absorbency at 260 nm measured by a GeneQuant Pro RNA/DNA calculator (Biochrom, Cambridge UK). Isolated genomic DNA was stored in a −80°C mechanical freezer.

Polymerase chain reaction

The D1/D2 regions of the LSU rRNA genes, positions 63 through 633 relative to the 5′-end of the *Saccharomyces cerevisiae* LSU rRNA gene (Georgiev et al. 1981), were amplified by polymerase chain reaction (PCR) from the genomic DNA using the following two primers (O'Donnell 1993): F63, GCTGAACTTAAGCATATCAATAAGC GGAGGAAA, and R635, TAGACTCCTTGGTCCG TGTTTCAAGACGGGC. Each of the 50-µl PCR reaction mixtures consisted of two ready-to-go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 4 µl template genomic DNA (20 ng), 1 µl each primer (10 pmol), and 44 µl deionized H_2O. The amplifications were carried out using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) according to the following steps: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 2 min, 72°C for 2 min, and an additional 72°C for 5 min before maintaining the mixture at 4°C (O'Donnell 1993). DNA molecules of about 600 nucleotides were amplified, and the PCR products were cleaned with Qiaex reagents following the manufacturer's protocol (Qiagen, Chatsworth, CA, USA).

Sequencing

The cycle sequencing reactions were carried out using a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems). The reaction mixtures consisted of 4 µl (50 ng) DNA template, 0.5 µl primer (5 pmol), 8 µl Big Dye terminator, and 7.5 µl deionized H_2O for a total volume of 20 µl. The cycle sequencing program was as follows: initial denaturation at 95°C for 5 min, 25 cycles at 95°C for 30 s, 50°C for 30 s, 60°C for 4 min, and an additional 60°C for 7 min before storing the sample at 4°C. The extension products were purified with Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA) before being loaded onto an ABI 377 automated sequencer (Applied Biosystems).

The sequencing gel (5% acrylamide) was cast with a LongRanger Singel pack (BioWhittaker Molecular Applications, Rockland, ME, USA). The sequences were tracked and extracted with the ABI Prism 377–96 data collection software. Primers F63, R635, NL2 (CTCTCTTTTCAAA GTTCTTTTCATCT), and NL3 (AGATGAAAAGAAC TTTGAAAAGAGAG) were used for sequencing (O'Donnell 1993). Sequence information was submitted to GenBank, National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

Table 1. Strains studied and GenBank accession numbers for the D1/D2 regions of the genes

Strain	Species[a]	Medium[b]	Temperature (°C)	GenBank accession no.
ATCC 64205	*Monascus floridanus*, type	336	25	AF364960
ATCC 64206	*Monascus floridanus*	324	25	AF364961
ATCC 66695	*Monascus floridanus*	324	25	AF364962
ATCC 66696	*Monascus floridanus*	324	25	AF364963
ATCC 15670	*Monascus ruber*, type	336	25	AF364964
ATCC 16363	*Monascus pilosus*, type	325	25	AF364965
ATCC 16365	*Monascus purpureus*, type	325	30	AF364966
ATCC 200612	*Monascus pallens*, type	325	25	AF364967
ATCC 200613	*Monascus sanguineus*, type	325	25	AF364968
ATCC 204397	*Monascus lunisporas*, type	319	25	AF364969
ATCC 46597	*Monascus purpureus* (*kaoliang*)	336	30	AF364970
ATCC 46592	*Monascus purpureus* (*kaoliang*)	336	30	AF364971
ATCC 46593	*Monascus purpureus* (*kaoliang*)	336	30	AF364972
ATCC 46594	*Monascus purpureus* (*kaoliang*)	336	30	AF364973
ATCC 46595	*Monascus purpureus* (*kaoliang*)	336	30	AF364974
ATCC 46596	*Monascus purpureus* (*kaoliang*)	336	30	AF364975
ATCC 62949	*Monascus pilosus*	336	25	AF364976
ATCC 16368	*Monascus pilosus* (*rubropunctatus*)	28	25	AF364977
ATCC 16433	*Monascus* sp.	325	30	AF364978
ATCC 16434	*Monascus* sp.	325	30	AF364979
ATCC 16772	*Monascus* sp.	325	30	AF364980
ATCC 16774	*Monascus* sp.	325	30	AF364981
ATCC 16775	*Monascus* sp.	325	30	AF364982
ATCC 16966	*Monascus sp.* (*barkeri*)	325	30	AF364983
ATCC 96218	*Monascus ruber*	336	30	AF364984
ATCC 16367	*Monascus purpureus* (*rubiginosus*)	325	25	AF364985
ATCC 16362	*Monascus purpureus* (*major*)	325	25	AF364986
ATCC 16361	*Monascus purpureus* (*araneosus*)	325	30	AF364987
ATCC 58358	*Monascus ruber*	28	25	AF364988
ATCC 13692	*Monascus ruber*	28	25	AF364989
ATCC 48162	*Monascus purpureus*	336	30	AF364990
ATCC 6405	*Monascus purpureus*	336	25	AF364991
ATCC 16358	*Monascus purpureus* (*albidus var. glaber*)	325	30	AF364992
ATCC 16360	*Monascus purpureus* (*anka*)	325	30	AF364993
ATCC 16436	*Monascus purpureus*	325	30	AF364994
ATCC 16366	*Monascus ruber*	28	25	AF364995
ATCC 16371	*Monascus ruber* (*vitreus*)	28	30	AF364996
ATCC 66229	*Monascus ruber*	336	25	AF364997
ATCC 34892	*Monascus purpureus*	336	25	AF364998
ATCC 22080	*Monascus ruber* (*paxii*)	336	25	AF364999
ATCC 20657	*Monascus ruber*	336	25	AF365000
ATCC 16386	*Monascus ruber*	325	25	AF365001
ATCC 16385	*Monascus ruber*	325	25	AF365002
ATCC 16369	*Monascus pilosus* (*serorubescens*)	325	30	AF365003
ATCC 16246	*Monascus ruber*	336	25	AF365004
ATCC 16370	*Monascus* sp.	325	30	AF365005
ATCC 16384	*Monascus ruber*	325	30	AF365006
ATCC 36113	*Monascus purpureus*	336	25	AF365007
ATCC 36114	*Monascus purpureus*	336	25	AF365008
ATCC 36928	*Monascus purpureus* (*anka*)	336	25	AF365009
ATCC 46598	*Monascus purpureus*	336	30	AF365010
ATCC 16379	*Monascus purpureus* (*anka var. rubellus*)	325	30	AF365011
ATCC 16357	*Monascus purpureus* (*albidus*)	323	30	AF365012
ATCC 16378	*Monascus ruber* (*fuliginosus*)	325	30	AF365013
ATCC 16427	*Monascus purpureus*	325	25	AF365014
ATCC 16435	*Monascus* sp.	325	30	AF365015
ATCC 16437	*Monascus* sp.	325	30	AF365016
ATCC 16666	*Monascus* sp.	325	30	AF365017
ATCC 16773	*Monascus* sp.	325	30	AF365018
ATCC 18199	*Monascus ruber*	336	25	AF365019
ATCC 28298	*Xeromyces bisporus*	319	25	AF365020
ATCC 34570	*Monascus* sp.	336	30	AF365021
ATCC 36964	*Xeromyces bisporus*	319	25	AF365022
ATCC 62925	*Monascus eremophilus*, type	319	25	AF365023
ATCC 26264	*Monascus purpureus*	336	25	AF365024

[a] Species names in parentheses are synonyms; Type, type strain
[b] Numbers indicate ATCC medium designations (Jong and Edwards 1996)

Table 2. Morphological and cultural characteristics of the type strains of the recognized *Monascus* species and *Xeromyces bisporus*

Strain and colony size after 7 days	Species	Color of mycelium	Pigment secreted	Shape of ascospores	Size of ascospores	Color of ascomata
ATCC 64205 15 mm	*M. floridanus*	Olive-green	No pigment	No ascospores		
ATCC 15670 35 mm	*M. ruber*	White	No pigment	Ellipsoidal	5–7 × 3–4.5 μm	Dark orange
ATCC 16363 30 mm	*M. pilosus*	White	No pigment	No ascospores		
ATCC 16365 9 mm	*M. purpureus*	Strong orange	Orange	No ascospores		
ATCC 200612 11 mm	*M. pallens*	Light gray	No pigment	Subglobose to ellipsoidal	3.7–4.7 × 3.0–3.3 μm	Hyaline
ATCC 200613 9 mm	*M. sanguineus*	Grayish-red	Strong orange	Ellipsoidal	5–7 × 4–6 μm	Orange-brown
ATCC 204397 12 mm	*M. lunisporas*	Olive-brown	No pigment	Lunate	5–6 × 2.7–3 μm	Orange-brown
ATCC 36964 N/A	*Xeromyces bisporus*	Dark yellow	No pigment	Globose to subglobose	4.5–6 × 3–6 μm	Hyaline
ATCC 62925 N/A	*Monascus eremophilus*	White	No pigment	Subglobose to ellipsoidal	5–7 × 5.5–6.3 μm	Hyaline to orange-brown

All measurements and observations were made after 7 days growth on malt extract agar at 25°C, except xerophilic species

Sequence alignment and phylogenetic analysis

The sequences were aligned with CLUSTALX (Thompson et al. 1997). Phylogenetic relationships among the strains were estimated with PAUP 4.0b4a (Swofford 2000). For alignments with CLUSTALX, the gap opening cost and the gap extension cost were set between 2 and 16 in increments of 2. For estimation of the phylogenetic relationships among the strains, all nucleotides were unordered with equal weights. Gaps were considered as a fifth state, and a heuristic search was carried out with the branch swapping option using the tree-bisection-reconnection algorithm. Starting trees were obtained via stepwise addition, and branches of maximum length zero were allowed to collapse yielding polytomies.

The sequences (GenBank accession no. in parentheses) of *Aspergillus anthodesmis* NRRL 22884 (U17916), *Penicillium inflatum* NRRL 5179 (AF033393), *Cephalotheca sulfurea* (AF096188), *Albertiniella polyporicola* (AF096185), and *Aporothielavia leptoderma* (AF096186) were used as outgroup because they belong to the same order (Eurotiales) as the genus *Monascus*.

Maximum-likelihood analysis on the same data set was carried out using a heuristic search with empirical nucleotide frequency, transition/transversion ratio estimated via maximum likelihood from the minimum evolution tree and tree-bisection-reconnection branch swapping, and starting branch lengths obtained with the Rogers–Swofford approximation method. Rates for variable sites were assumed to be equal. Nucleotide frequencies were estimated from the dataset where A = 0.21, C = 0.25, G = 0.34, T = 0.20. No molecular clock was enforced.

Results

Genomic DNAs of 66 strains of *Monascus* and *Xeromyces* were isolated. ATCC 16364, deposited as *M. pilosus*, exhibited strong DNase activity in the crude extract. Accordingly, only its RNAs were isolated, and this strain was excluded from the study. The sequences of the D1/D2 regions of the LSU rRNA genes of 65 strains were determined and aligned, and their evolutionary relationship was estimated with PAUP 4.0b4a (Fig. 1). The alignments with CLUSTALX with varying settings of gap opening cost and gap extension cost were identical. All sequence data were registered with the accession numbers AF364960 through AF365024 (see Table 1).

Sequence comparisons and comparative morphological observations determined the species identity for the unidentified strains. Three strains deposited as *Monascus* sp. (ATCC 16435, ATCC 16437, and ATCC 34570) were confirmed as *Monascus purpureus.* The other nine strains (ATCC 16772, ATCC 16773, ATCC 16774, ATCC 16775, ATCC 16433, ATCC 16434, ATCC 16370, ATCC 16966, and ATCC 16666) were identified as *M. ruber*, based on ascospore size, ascomata and conidia pigmentation, and molecular information on the D1/D2 region of the LSU

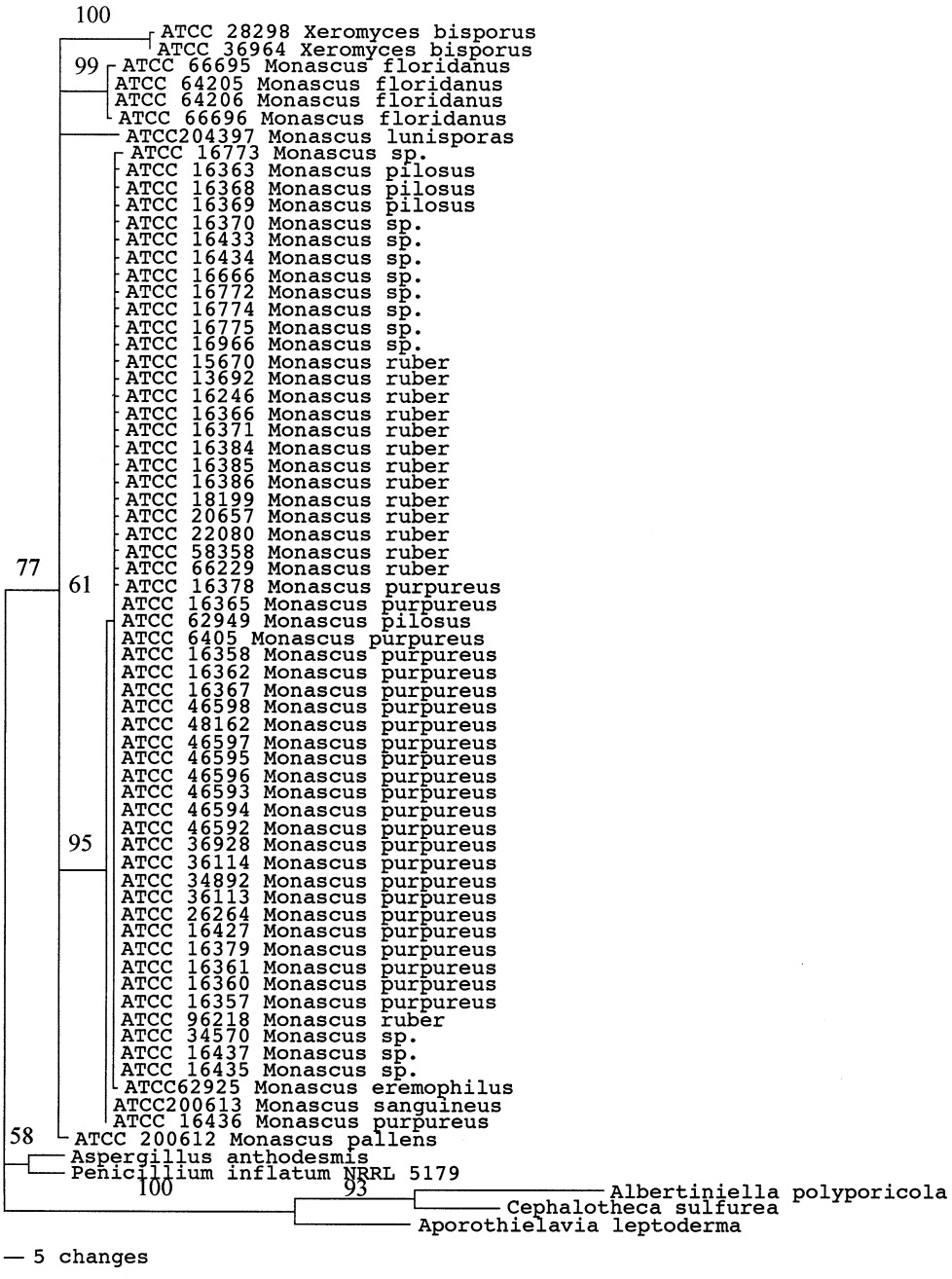

Fig. 1. A strict consensus phylogenetic tree obtained from five parsimonious trees of all 65 strains studied. The sequences (GenBank accession no. in parentheses) of *Aspergillus anthodesmis* NRRL 22884 (U17916), *Penicillium inflatum* NRRL 5179 (AF033393), *Cephalotheca sulfurea* (AF096188), *Albertiniella polyporicola* (AF096185), and *Aporothielavia leptoderma* (AF096186) were used as outgroup because they are closely related taxonomically with the genus *Monascus*. The *number at the left side of each tree node* is a bootstrap value based on a heuristic search of the data with 1000 bootstrap replications; it was 95% for the tree node of *M. sanguineus*, *M. purpureus*, *M. ruber*, *M. eremophilus*, and *M. pilosus*. Bootstrap values less than 50% are not shown. The consistency index, retention index, rescaled consistency index, and homoplasy index for this tree were 0.811, 0.854, 0.693, and 0.189, respectively

rRNA genes. ATCC 13692, ATCC 16371, ATCC 16384, and ATCC 20657 were identified as *M. pilosus*. All strains of *M. kaoliang* (ATCC 46592, ATCC 46593, ATCC 46594, ATCC 46595, ATCC 46596, and ATCC 46597) had identical sequences with the type strain of *M. purpureus* (ATCC 16365). The sequences of ATCC 62949 and ATCC 96218 deposited as *M. pilosus* and *M. ruber*, respectively, were identical to *M. purpureus.* Furthermore, the color of the pigments excreted into the medium resembled *M. purpureus.* ATCC 16378 was reidentified as *M. ruber* on the basis of sequence homology and cultural characteristics. The sequence of ATCC 16436 deposited as *M. purpureus* matched that of *M. sanguineus*. This strain had colorless conidia and ascomata, and the size and pigmentation of the ascospores were similar to *M. purpureus*. *M. sanguineus* and *M. purpureus* differed by two nucleotides in the D1/D2 regions. The nucleotide sequence of *M. eremophilus* ATCC 62925, which grows in extremely dry conditions, differed from *M. purpureus* by only one nucleotide, implying its relatedness with *M. purpureus*, although a significant ecological separation and morphological and physiological differences exist between these two species.

The maximum-parsimony analyses of the 65 strains generated five most parsimonious trees. The strict consensus tree of these five clustered *M. ruber*, *M. pilosus*, *M. purpureus*, *M. eremophilus*, and *M. sanguineus* as one clade,

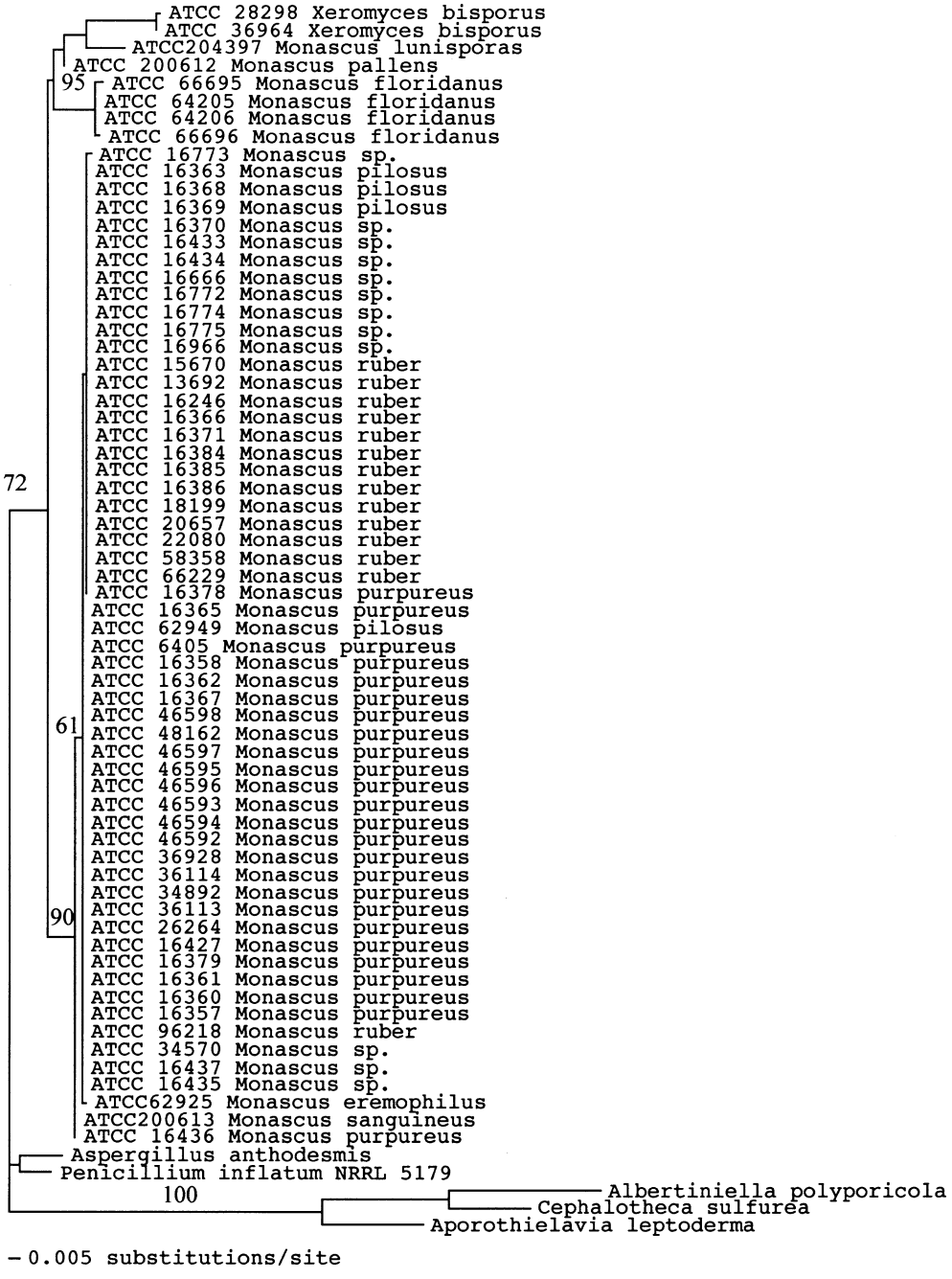

Fig. 2. Maximum-likelihood tree inferred from the same sequence data set used for the maximum-parsimony analysis. The tree was obtained using a heuristic search with empirical nucleotide frequency, transition/transversion ratio estimated via maximum likelihood from the minimum evolution tree (the transition/transversion ratio = 1.96) and tree-bisection-reconnection branch swapping (TBR), and starting branch lengths obtained using the Rogers–Swofford approximation method. The probability of nucleotide substitution per site is indicated by the *scale bar*. The *numbers above branches* are the frequencies of occurrence in 100 bootstrap replicates. The bootstrap value of the branch for *Albertiniella polyporicola* and *Cephalotheca selfurea* is 94%; values less than 50% are not shown

Aspergillus anthodesmis and *Penicillum inflatum* as a second clade, and *Albertiniella polyporicola*, *Cephalotheca sulfurea*, and *Aporothielavia leptoderma* as a third clade. Also, this tree placed *Xeromyces bisporus*, *M. floridanus*, and *M. lunisporas* at separate branches. In this analysis 378 of the 570 nucleotides were constant, and 140 nucleotides were parsimony informative; 52 variable nucleotides were parsimony uninformative.

The maximum-likelihood analysis using the same data set created a single phylogenetic tree (Fig. 2). It placed *M. pilosus*, *M. purpureus*, *M. ruber*, *M. eremophilus*, and *M. sanguineus* in one clade and the other *Monascus* species and *Xeromyces bisporus* in another.

Discussion

A combination of sequence comparison and morphological analysis confirms the synonyms recognized by Hawksworth and Pitt (1983) based on morphological and cultural characters. *M. kaoliang*, *M. rubiginosus*, *M. anka*, *M. albidus*, *M. araneosus*, and *M. major* are synonyms of *M. purpureus*; *M. pubigerus*, *M. rubropunctatus*, and *M. serorubescens* are synonyms of *M. pilosus*; and *M. paxii*, *M. vitreus*, and *M. fuliginosus* are synonyms of *M. ruber*. Although *M. ruber* and *M. pilosus* have been widely accepted as distinct species, they have an identical sequence on the D1/D2 region

of the LSU rRNA genes, indicating they are closely related. We observed remarkable differences between these two species in production of ascospores and conidia on malt extract agar, in extracellular pigment production, and in pigmentation on ascomata. On malt extract agar, *M. pilosus* produced few ascospores and conidia while *M. ruber* produced abundant spores. Ascomata of *M. pilosus* are hyaline and those of *M. ruber* are pigmented. Other characters at the molecular level may be useful to resolve the further distinction of these two species.

This investigation revealed unique nucleotides for specific species. The sequence comparison of *M. floridanus* and *M. pallens* detected three unique nucleotides common only to these two species. Counting from the 5′-end, we found an additional G as the 59th nucleotide instead of a gap, G as the 100th nucleotide instead of A, and G as the 557th nucleotide instead of A. This common molecular nucleotide information is the basis of the parsimonious phylogenetic relationship that places *M. pallens* and *M. floridanus* in the same clade in two of the five most parsimonious trees. *Xeromyces bisporus* also has five unique nucleotides with *M. lunisporas*. In addition, this investigation distinguished closely related species and expedited taxonomic identification of *M. sanguineus*, *M. purpureus*, *M. eremophilus*, *M. ruber*, and *M. pilosus*, which differed in one or two nucleotides. Otherwise, the sequence of ATCC 16436 was identical to that of *M. sanguineus*, whereas its colorless conidia, ascomata, and ascospore size were consistent with the description of *M. purpureus.*

Maximum-parsimony and maximum-likelihood analyses on the aligned data set suggest that the two genera, *Monascus* and *Xeromyces*, are monophyletic, justifying placement of both genera in the same family. The bootstrap value for the branch of *Monascus* and *Xeromyces* in Fig. 1 was 77%. However, *Xeromyces bisporus* is closely related to *M. lunisporas*, sharing five variable nucleotides. The issue of a separate genus for *Xeromyces bisporus* requires further investigation. These analyses have also placed *M. lunisporas*, *M. floridanus*, *M. pallens*, and *X. bisporus* in well-separated branches, confirming their taxonomic status as distinct species.

A further analysis of the ITS sequences of *M. purpureus* (cited as *M. anka*) IF0 30873 (AF458473), *M. purpureus* ATCC 16365 (AF458472), *M. ruber* ATCC 22080 (AF458470), *M. purpureus* (cited as *M. kaoliang*) ATCC 46596 (AF451859), and *M. pilosus* ATCC 16364 (AF451856) from GenBank also suggested a similar phylogenetic relationship among these strains. The similarity of ITS and D1/D2 sequences placed ATCC 22080 of *M. ruber* and ATCC 16364 of *M. pilosus* in one group and all three *M. purpureus* strains (IFO 30873, ATCC 16365, and ATCC 46596) in another. The former group differs from the latter in 2 of 502 nucleotides in the ITS sequence and in 1 nucleotide in the D1/D2 sequence.

In 1999, the phylogenetic relationship of 25 strains of *Monascus* isolated from red rice and sofu was estimated using random amplified polymorphic DNA (RAPD) with multiple markers (Lakrod et al. 1999). Species identity of most of these strains was not determined. Four ATCC strains of *M. purpureus* included in this analysis were further divided into two groups. Results indicated that ATCC 16360 and ATCC 16365 were of one lineage and ATCC 26264 and ATCC 16427 belonged to another, although the D1/D2 sequences of these four strains of *M. purpureus* were identical in this study. Recently, Chaisrisook (2002) reported the presence of compatibility groups among the isolates of *M. purpureus*. However, this classification in regard to the compatibility group for the four ATCC strains was not consistent with the RAPD subgroups of Lakrod and coworkers (1999).

In this study, comparative analyses of the D1/D2 regions of the LSU rRNA genes and subsequent analyses of maximum parsimony and maximum likelihood on the data set have shed light on a number of issues concerning taxonomic and phylogenetic relationships, including identification, among strains and species of the genus *Monascus*. As we enter the era of multigene phylogenies, other sequence analyses at protein gene targets such as translation elongation factor 1-alpha, beta tubulin, RNA polymerases, chitinase subunits, and others need to be examined.

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