Bot. Bull. Acad. Sin. (2004) **45:** 325-330

Phylogenetic relationships of *Monascus* species inferred from the ITS and the partial β-tubulin gene

Houng G. PARK*, Elena K. STAMENOVA, and Shung-Chang JONG

American Type Culture Collection, P.O. Box 1549, Manassas VA 20108, USA

(Received February 16, 2004; Accepted June 14, 2004)

Abstract. ITS and partial β-tubulin genes of 17 ATCC reference strains of *Monascus* species were PCR amplified and sequenced. *Monascus pilosus* and *M. ruber* could not be differentiated with these sequences, suggesting a synonymy. In maximum parsimony analyses on both data sets, *M. ruber*, *M. pilosus*, *M. purpureus*, and *M. sanguineus* were placed into the same clade. ITS sequence alignment revealed a number of gaps in ITS1 and ITS2 of *M. pallens*, *M. lunisporas*, and *M. eremophilus* compared to *M. purpureus*, *M. ruber*, and *M. pilosus*. Accordingly, analyses with the ITS sequences placed these species into clades, incongruent with the analyses using the partial β-tubulin genes and the previous results with the partial large subunit rRNA genes. The phylogenetic relationship derived from the partial β-tubulin genes was similar to those postulated by the 5'-partial LSU rRNA genes. This finding strongly suggests that evolutionary or phylogenetic classification with ITS sequence information should be performed with caution. In the phylogenetic trees with the ITS sequences, *M. lunisporas* was distantly associated with *Aspergillus ustus*; *M. pallens* was placed in a clade that shares a common node with *A. versicolor*; and *M. eremophilus* was placed on a branch separate from the *M. purpureus*, *M. ruber*, and *M. pilosus* group while *M. pallens* and *M. lunisporas* were placed into the related clades sharing a common node in the tree derived from the partial β-tubulin gene. Each of the phylogenetic analyses with the partial β-tubulin genes, the ITS, or the 5'-end of the LSU rRNA, as previously carried out, placed *M. eremophilus* into a different lineage. Molecular analyses with these molecular targets generated three different topologies for *M. eremophilus*, indicating a unique and unpredictable genetic combination for this species. It might reflect extreme environmental stress on this species and subsequent genetic changes.

Keywords: β-tubulin; ITS; LSU rRNA; *Monascus* and phylogeny.

Introduction

Since the time Hawksworth and Pitt (1983) recognized three species of *Monascus* (*M. pilosus* K. Sato, *M. ruber* van Tieghem, and *M. purpureus* Went) based on physiological and morphological characteristics, several new species have been described. Barnard and Cannon (1987) described *M. floridanus* Cannon & Barnard, isolated from the roots of sand pine trees in Florida. Hocking and Pitt (1988) described a xerophilic species, *M. eremophilus* Hocking & Pitt, which differed from the other species by its slow growth rate, lack of an anamorph, and requirement for extremely dry conditions. Cannon et al. (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 activity tests using APIZYM strip tests (BioMerieux Vitek, Inc., Hazelwood, MO). In addition, Udagawa and Baba (1998) described *M. lunisporas* Udagawa & Baba, unique for its lunate ascospores and dark, olive-brown ascomata.

*Corresponding author. Tel 703-365-2700 Ext 2562; Fax: 703-365-2730; E-mail: tohoungpark@yahoo.com

In 2003, phylogenetic relationships among the species were determined by sequences of the D1/D2 region of the large subunit (LSU) rRNA genes by Park and Jong. *Monascus ruber* and *M. pilosus* could not be differentiated. *Monascus ruber*, *M. pilosus*, and *M. purpureus* were closely related and clustered into the same subgroup.

We have carried out a further phylogenetic characterization using the ITS and partial β-tubulin genes in search of a better molecular differentiation marker and have evaluated the integrity and consistency of molecular phylogenetic relationships postulated by different molecular markers related to different biological functions.

Materials and Methods

Cultivation of the Strains

Seventeen strains of *Monascus* (Table 1) were obtained from cryopreserved material at ATCC. The strains were cultivated using one of four agar and broth media at 25°C or 30°C for approximately seven days. 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 glu-

Table 1. Strains studied and GenBank accession numbers for the ITS and β-tubulin genes.

Strain	Species[a]	Medium[b]	Temperature (°C)	ITS	β-tubulin
ATCC 16246	*Monascus ruber*	336	25	AY498574	AY498589
ATCC 16363	*Monascus pilosus,* Type	325	25	AY498581	AY498596
ATCC 16366	*Monascus rube*	28	25	AY498571	AY498587
ATCC 16368	*Monascus pilosus*	28	25	AY498582	AY498597
ATCC 16369	*Monascus pilosus*	325	30	AY498575	AY498595
ATCC 16370	*Monascus* sp.	325	30	AY498576	AY498590
ATCC 16371	*Monascus ruber*	28	30	AY498572	AY498588
ATCC 16379	*Monascus purpureus*	325	30	AY498573	AY498598
ATCC 16384	*Monascus ruber*	325	25	AY498570	AY498592
ATCC 16773	*Monascus* sp.	325	30		AY498591
ATCC 36113	*Monascus purpureus*	336	25	AY498577	AY498599
ATCC 36114	*Monascus purpureus*	336	25	AY498578	AY498600
ATCC 58358	*Monascus ruber*	28	25	AY498580	AY498593
ATCC 62925	*Monascus eremophilus,* Type	319	25	AY498584	AY498603
ATCC 200612	*Monascus pallens,* Type	325	25	AY498585	AY498601
ATCC 200613	*Monascus sanguineus,* Type	325	25	AY498586	AY498602
ATCC 204397	*Monascus lunisporas,* Type	319	25	AY498583	AY498604
ATCC 200942	*Amorphotheca resinae*	325	25	AF393726	

[a]"Type" indicates type strain.
[b]Numbers indicate ATCC medium designations (Jong and Edwards, 1996).

cose broth 30 g [Difco 0382] and agar 20 g per liter); and Harrold's M40Y (ATCC medium 319: malt extract 20 g, yeast extract 5 g, sucrose 400 g, and agar 20 g per liter). The species identity of each strain was confirmed by observing the size, shape, and pigmentation of conidia, conidiophores, ascospores, and ascomata.

Isolation of Genomic DNA

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

PCR

Two primers, NS7 (gaggcaataacaggtctgtgatgc) and LR3 (ccgtgtttcaagacggg), were used to sequence ITS regions of the rDNAs (Vilgalys and Gonzalez, 1990). One forward primer (caactgggctaagggtcatt) and a reverse primer (gtgaactccatctcgtccata) were used for PCR amplification of the partial β-tubulin genes (Wu et al., 1996). Each of the 50-µl PCR reaction mixtures consisted of two "Ready-to-go" PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ), 4 µl of template genomic DNA (20 ng), 1 µl of each primer (10 pmol), and 44 µl of deionized H_2O. The amplifications were carried out using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) according to the following steps: initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°C for 2 min, 72°C for 2 min, and an additional cycle at 72°C for 5 min prior to maintaining the mixture at 4°C (O'Donnell, 1993). DNA molecules of about 1.1 kb were amplified for ITS and about 1.0 kb for β-tubulin. The PCR products were cleaned with a Qiaex II gel extraction kit following the manufacturer's protocol (Qiagen Inc., Chatsworth, CA).

DNA Sequencing

The cycle sequencing reactions were carried out using a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). The reaction mixtures consisted of 4 µl (50 ng) of DNA template, 0.5 µl of primer (5 pmol), 8 µl of Big Dye terminator, and 7.5 µl of 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 cycle at 60°C for 7 min prior to storing the sample at 4°C. The extension products were purified with CentriSep spin columns (Princeton Separations, Adelphia, NJ) prior to being loaded onto an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA).

The sequencing gel (5% acrylamide) was cast with a Long Ranger Singel pack (BioWhittaker Molecular Applications, Rockland, ME). The sequences were tracked and extracted with the ABI Prism 377-96 data collection software. Primers ITS1 (tccgtaggtgaacctgcgg) and ITS4 (tcctccgcttattgatatgc) were used for sequencing the ITS

region (Gardes and Bruns, 1993). Two primers (one primer in the 5' direction, caagatccgtgaggagt and another in the 3'-direction, gtgaactccatctcgtccata) were used for sequencing the partial β-tubulin genes. Sequence information was submitted to GenBank, National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

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 at 15 and 30, respectively. For the estimation of the phylogenetic relationship among the strains, all nucleotides were unordered with equal weights. Gaps were considered as missing bases, 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.

Maximum likelihood analyses on the same data set were carried out using a heuristic search with empirical nucleotide frequency. The transition/transversion ratio was estimated via maximum likelihood from the minimum evolution tree and tree-bisection-reconnection branch swapping, and the starting branch length obtained with the Rogers-Swofford approximation method. Rates for variable sites were assumed to be equal. No molecular clock was enforced.

Results

Maximum parsimony on the dataset of the ITS sequences placed *M. lunisporas*, *M. pallens*, and *M. eremophilus* into separate branches (Figure 1), different from the clusterings postulated by the D1/D2 sequences of the LSU rRNA genes (Park and Jong, 2003). Maximum likelihood analysis on the same data set also inferred similar topologies among the species (tree not shown). *Monascus lunisporas* was distantly clustered with *A. ustus*. *Monascus pallens* was also distantly clustered with *A. versicolor*, and *M. eremophilus* was placed in a separate clade from *M. pilosus*, *M. ruber*, and *M. purpureus*. *Monascus ruber* and *M. pilosus* were identical in ITS sequence.

The same analyses on the dataset of the partial β-tubulin genes clustered the species in a similar manner (Figure 2) with the 5'-end of the LSU rRNA genes (Park and Jong, 2003). Again *M. ruber* and *M. pilosus* were identical. *Monascus lunisporas* and *M. pallens* were clustered into clades sharing a common node with a strong bootstrap support although they diverged significantly from the common node. Phylogenetic relationships for *M. eremophilus* with the ITS and the partial β-tubulin genes obtained in this study were different from each other, and both results are also different from the previous one obtained with the D1/D2 region of the LSU rRNA gene.

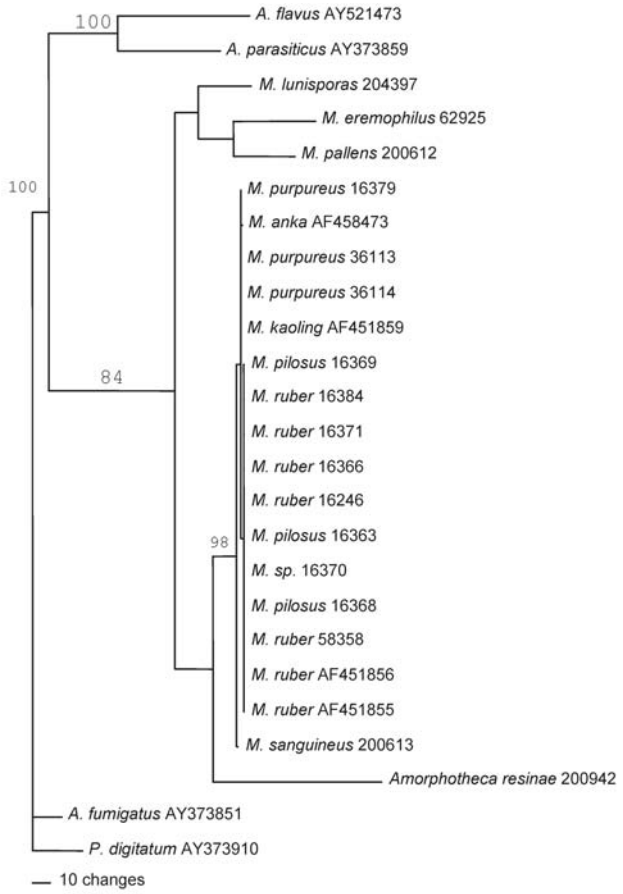

Figure 1. Most parsimonious tree postulated with the ITS sequences. Total numbers of ingroup taxa and outgroup were 24 and one (*Penicillium digitatum* AY373851), respectively. Out of 672 characters, 399 were constant, 131 variable characters were parsimony-uninformative, and 142 variable characters were parsimony-informative. At each node, a bootstrap value larger than 50 percent from 1000 replicates is shown. Three sequences, *Aspergillus fumigatus* (AY373851), *A. flavus* (AY521473), and *A. parasiticus* (AY373859), and four sequences of *Monascus* (AF451856, AF451855, AF451859, and AF458473) from GenBank were incorporated into the database. ATCC strain numbers or GenBank accession numbers for the sequences obtained from GenBank are specified after the species name.

Discussion

While the phylogenetic relationship inferred from the partial β-tubulin sequences (Figure 2) was congruent with the relationship obtained with D1/D2 region of LSU rRNA genes (Park and Jong, 2003), those postulated by the ITS sequences were incongruent in regard to *M. pallens*, *M. eremophilus*, and *M. lunisporas* (Figure 1). As Bruns pointed out in his short communication (2001), ITS sequences are often not unambiguously alignable among different genera because of insertions and deletions, commonly noted through personal communications between scientists. Alignment of the sequences of this genus include many insertions and deletions, as

demonstrated in Figure 3. These insertions and deletions could lead to an inconsistent phylogenetic relationship with other targets. This finding strongly suggests that phylogenetic classification with ITS sequence information should be performed cautiously. The relationships established with ITS sequences for this genus should be considered along with others based on biologically functional genes as stressed by Bruns.

The alignment of the ITS sequences demonstrates that the different clades in the trees carry a number of gaps that might be caused by repeated deletions and insertions while the partial SSU, 5.8S, and LSU rRNA genes in the alignment carry few gaps (Figure 3). A separate alignment with the partial β-tubulin genes showed fewer gaps (not shown). Individual BLAST searches with the sequences that had caused gaps in the alignment in the ITS1 and ITS2 regions against the GenBank database, including the whole genome sequences, produced no meaningful similarity hits, indicating these additional nucleotides are not associated with the currently known sequences. These results strongly suggest that insertions or deletions might have occurred independently without recombinations with already known, existing sequences. In regard to *M. ruber* and *M. pilosus*, both the ITS and partial β-tubulin genes were identical. Although these two species have been recognized as separate, molecular information consistently indicates that they are the same.

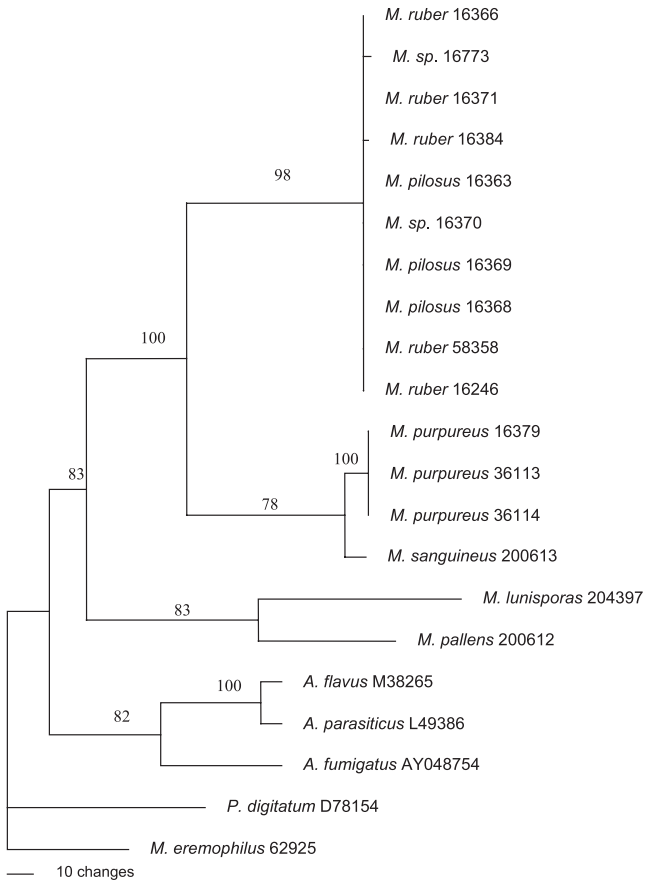

Figure 2. Most parsimonious tree inferred from analysis with partial β-tubulin genes. Total number of ingroup taxa was 20, and total number of outgroup taxa was one (*Penicillium digitatum* D78154). Out of 852 characters, 549 were constant, 119 variable characters were parsimony-uninformative, and 184 variable characters were parsimony-informative. At each node, a bootstrap value larger than 50 percent from 1000 replicates is shown. Bootstrap values less than 50 percent are not shown. Three sequences, *Aspergillus flavus* (M38265), *A. parasiticus* (L49386), and *P. fumigatus* (AY048754) from GenBank were incorporated into the database. ATCC strain numbers or GenBank accession numbers for the sequences obtained from GenBank are specified after the species name.

It is also very interesting to note the phylogenetic relationships between *M. eremophilus* and other *Monascus* species, based on the information of the three different molecular targets. The three different systems placed *M. eremophilus* into three different clades, indicating a unique and unpredictable genetic combination for this species. It might reflect enormous and extreme environmental stress and subsequent drastic genetic changes to adapt to extremely dry conditions. It has been known that environmental stress enhances mutational changes (Kishony and Leibler, 2003), and temperature extremes could influence genetic variations (Sgrò and Hoffmann, 1998).

Acknowledgments. The authors thank David Chalkley and Jane Edwards for critically reading the manuscript. This research was supported in part by the National Science Foundation Grants BIR-9420750 and DBI-9982243 to SCJ.

Literature Cited

Barnard, E.L. and P.F. Cannon. 1987. A new species of *Monascus* from pine tissues in Florida. Mycologia **79:** 479-484.

Bruns, T.D. 2001. ITS reality. Inoculum **52:** 2-3.

Cannon, P.F., S.K. Abdullah, and B.A. Abbas. 1995. Two new species of *Monascus* from Iraq, with a key to known species of the genus. Mycol. Res. **99:** 659-662.

Cenis, J.L. 1992. Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res. **20:** 2380.

Gardes, M. and T.D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol. Ecol. **2:** 113-118.

Hawksworth, D.L. and J.I. Pitt. 1983. A new taxonomy for *Monascus* based on cultural and microscopical characters. Aust. J. Bot. **31:** 51-61.

Hocking, A.D. and J.I. Pitt. 1988. Two new species of xerophilic fungi and a further record of *Eurotium halophilicum*. Mycologia **80:** 82-88.

Jong, S.C. and M.J. Edwards. (eds.) 1996. ATCC Catalogue of Filamentous Fungi, 19th edition. American Type Culture Collection, Rockville, Maryland.

Kishony, R. and S. Leibler. 2003. Environmental stresses can alleviate the average deleterious effect of mutations. J. Biol. **2:** 1-10.

O'Donnell, K. 1993. *Fusarium* and its near relatives. *In* D. R. Reynolds and J. W. Taylor (eds.), The Fungal Holomorph:

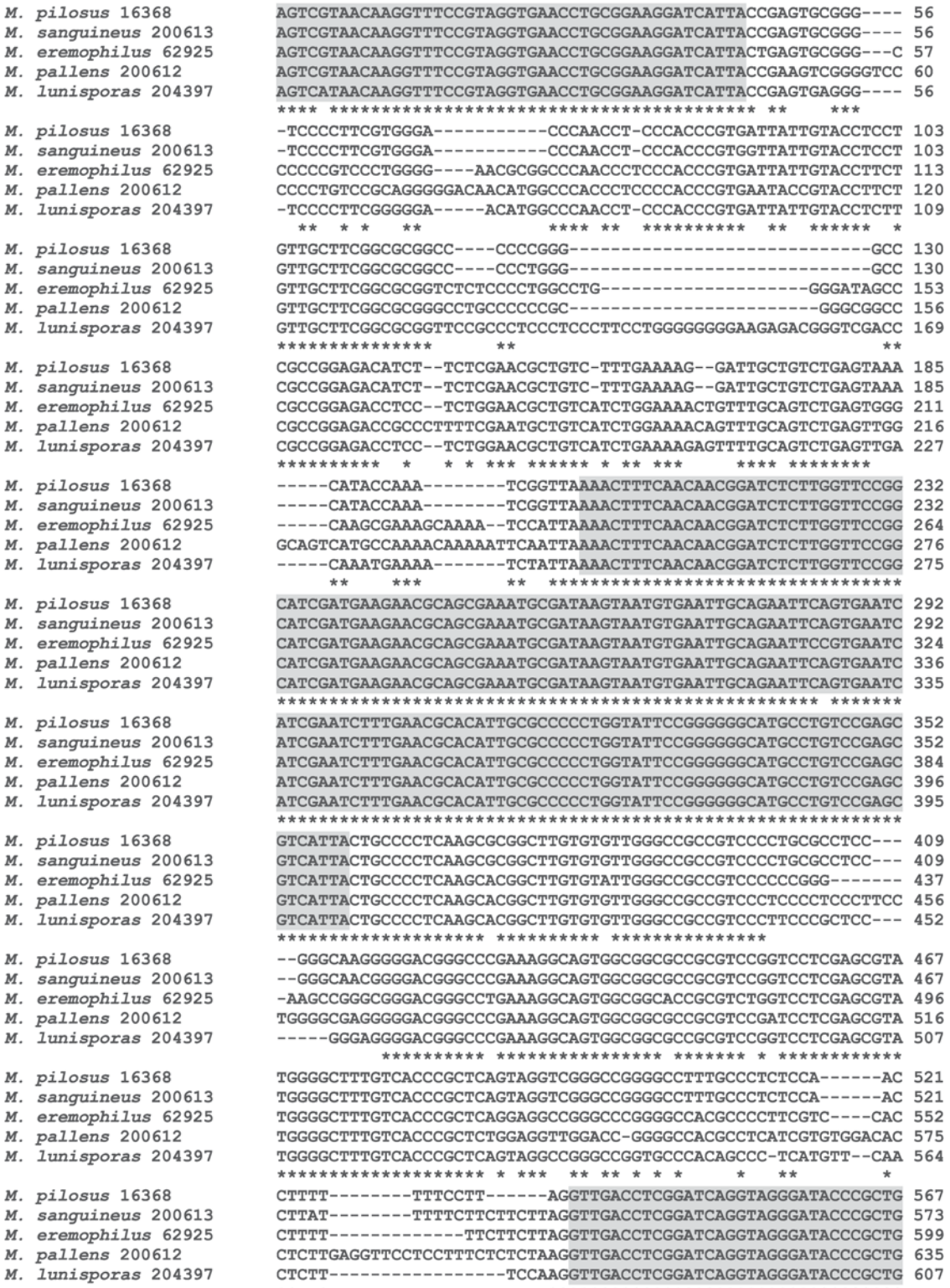

Figure 3. Multiple sequence alignment with Clustal W. The first gray block is the 3'-end of the SSU rRNA genes, the second is 5.8S rRNA genes, and the third is the 5'-end of the LSU rRNA genes. The unshaded blocks represent ITS1 and ITS2, respectively. ITS1 and ITS2 regions carry a number of gaps. Asterisks underneath the alignment indicate conserved nucleotides among the species.

Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics. CAB International, Wallingford, United Kingdom, pp. 225-233.

Park, H.G. and S.-C. Jong. 2003. Molecular characterization of *Monascus* strains based on the D1/D2 regions of LSU rRNA genes. Mycoscience **44:** 25-32.

Sgrò, C.M. and A.A. Hoffmann. 1998. Effects of temperature extremes on genetic variances for life history traits in *Drosophila melanogaster* as determined from parent-offspring comparisons. J. Evol. Biol. **11:** 1-20.

Swofford, D.L. 2000. PAUP: Phylogenetic Analysis Using Parsimony, Version 4.0b4a Sinauer Associates, Sunderland, MA.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:** 4876-4882.

Udagawa, S.-I. and H. Baba. 1998. *Monascus lunisporas*: a new species isolated from mouldy feeds. Cryptogamie Mycol. **19:** 269-276.

Vilgalys, R. and D. Gonzalez. 1990. Organization of ribosomal DNA in the basidiomycete *Thanatephorus praticola*. Curr. Genet. **18:** 277-280.

Wu, T.S., C.D. Skory, J.S. Horng, and J.E. Linz. 1996. Cloning and functional analysis of a beta-tubulin gene from a benomyl resistant mutant of *Aspergillus parasiticus*. Gene **182:** 7-12 .

從 ITS 及部份 β-tubulin 基因推演出之 *Monascus* 屬之各種間之親緣關係

Houng G. PARK, Elena K. STAMENOVA, and Shung-Chang JONG

American Type Culture Collection, PO Box 1549, Manassas VA 20108, USA

Monascus 屬下種之 17 個 ATCC 參考株的 ITS 及部份 β-tubulin 基因以 PCR 放大且定序。*M. pilosus* 及 *M. ruber* 無法區分，可能是異名同物。”maximum parsimony analyses” 顯示 *M. ruber*, *M. pilosus*, *M. purpureus* 及 *M. sanguineus* 歸屬於同一節（clade）。ITS 序列排位顯示 *M. pallens*, *M. lunisporas* 及 *M. eremophilus* 之 ITS1 及 ITS2 當和 *M. purpureus*, *M. ruber* 及 *M. pilosus* 相比對時有若干缺口存在。因此，如果用ITS序列做分析會把這些種放在同支，如此一來就和以部份 β-tubulin 基因及先前用部份 rRNA 大次單元基因之所得之結果不一致。以部份 β-tubulin 基因所導出之親緣關係和先前用 5'-部份 rRNA 大次單元之基因所推演者相似。此發現強烈地暗示無論是演化或親緣關係之推演當使用 ITS 序列資訊時要非常小心。以 ITS 序列所推出之親緣樹， *M. lunisporas* 和 *Aspergillus ustus* 相隔很遠；*M. pallens* 所在之節（clade)與 *A. versicolor* 共屬一支（node）；而 *M. eremophilus* 被置於有別於 *M. purpureus*, *M. ruber* 及 *M. pilosus* 所形成group 之一分支（branch）。但以 β-tubulin 基因所得之親緣樹卻把 *M. pallens* 及 *M. lunisporas* 歸屬於共享一支 （node）之相關兩個節（clades）。前述三種數據所推出之親緣樹把 *M. eremophilus* 分別歸屬在不同之 lineage。這些分子層次之分析顯示 *M. eremophilus* 之獨特的，不可預測的基因組合。這可能反映環境對此物種之極端逆境效應及以後之遺傳改變。

關鍵詞： β-tubulin；ITS；rRNA 之大次單元；*Monascus* 及分類。