

## Evidence for a phylogenetic connection between *Coccidioides immitis* and *Uncinocarpus reesii* (Onygenaceae)

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***Coccidioides immitis* is an anomaly amongst the human systemic fungal pathogens. Its unique parasitic cycle has contributed to confusion over its taxonomy. Early investigators mistakenly suggested that the pathogen is a protist, while others agreed it to be a fungus but placed it in four different divisions of the Eumycota. The taxonomy of *C. immitis* is still unresolved. Ultrastructural examinations of its parasitic and saprobic phases have revealed features that are diagnostic of the ascomycetous fungi. Moreover, striking similarities between the kind of asexual reproduction (i.e. arthroconidium formation) of this pathogen and certain anamorphic and teleomorphic members of the genus *Malbrancheda* have suggested a close relationship. Teleomorphs of these *Malbrancheda* species are members of the Onygenaceae (Order, Onygenales). This family also includes teleomorphs of two human respiratory pathogens, *Histoplasma capsulatum* and *Blastomyces dermatitidis*. Although the 18S rRNA gene sequences (1713 bp) of these two pathogenic forms differ from that of *C. immitis* by only 35 and 33 substitutions, respectively, their mode of conidiogenesis is characterized by production of solitary aleuroconidia rather than alternate arthroconidia. In this study we have used characters derived from biochemical, immunological and molecular analyses to compare relatedness between *C. immitis*, *H. capsulatum*, *B. dermatitidis*, and six non-pathogenic species of *Malbrancheda* (the *Malbrancheda* states of *Uncinocarpus reesii* and *Auxarthron zuffianum*, as well as *M. absolutea*, *M. dendritica*, *M. filamentosa* and *M. gypsea*). Evidence is presented which supports inclusion of *C. immitis* in the Onygenaceae, and indicates that a close phylogenetic relationship exists between the *Malbrancheda* state of *U. reesii* and this respiratory pathogen.**

**Keywords:** *Coccidioides immitis*, *Malbrancheda*, Onygenaceae, phylogeny

### INTRODUCTION

*Coccidioides immitis* is a soil-inhabiting, filamentous fungus and causative agent of a human respiratory disease known as coccidioidomycosis, or San Joaquin Valley fever. Upon inhalation of its air-dispersed propagules, *C. immitis*

**Abbreviations:** CHEF, contour-clamped homogeneous electric field; CHS, chitin synthase; ConA, concanavalin A; F+L, mycelial culture filtrate plus toluene lysate; ID-TP, immunodiffusion-tube precipitin; ITS, internal transcribed spacer; pNP-β-D-Glc, p-nitrophenol β-D-glucopyranoside; RFLP, restriction fragment length polymorphism.

The GenBank accession numbers for the nucleotide sequences determined in this work are given in the text.

undergoes a process of morphogenesis in lung tissue which is unique amongst the systemic fungal pathogens (Cole & Sun, 1985). Early workers considered this microbe a member of the protozoa (Wernicke, 1892), and C. W. Stiles (in Rixford & Gilchrist, 1896) coined the generic name to indicate its similarity to coccidia. The mycotic affinity of *C. immitis* was established by Ophuls & Moffitt (1900). The mycelia produce asexual, airborne arthroconidia by simple fragmentation of hyphal elements (Cole & Kirkland, 1991). The conidia are small enough to pass down the respiratory tree and reach the alveoli of the host. Initiation of the parasitic cycle is signalled by rounding-up of the cylindrical arthroconidia (approx. 3–6 × 2–4 µm). Continued isotropic growth of these cells

results in formation of coenocytes, called spherules, which can grow to 60 µm or more in diameter. The spherules subsequently undergo a process of segmentation which involves progressive centripetal growth of the innermost layer of the cell envelope and results in differentiation of a myriad of endospores from the compartmentalized cells. The spherule envelope stretches and eventually ruptures to release the already enlarging endospores, which give rise to the second generation of spherules.

This process of progressive cleavage of spherules observed under the light microscope first led some mycologists to align *C. immitis* with the chytrids (Ciferri & Redaelli, 1936), and later provided the basis for its accommodation in the Zygomycota (Baker *et al.*, 1943). Several morphogenetic features of *C. immitis*, on the other hand, indicate its relationship to the Ascomycota. These include the mode of conidium development and the formation of simple septal pores with Woronin bodies (Cole & Sun, 1985). The arthroconidia of *C. immitis* are spaced apart from each other by separating cells which undergo lysis. The mechanism of conidiogenesis in *C. immitis* has been described as enteroarthroconidium formation (Cole & Samson, 1979). Emmons (1954, 1967) reported that some soil ascomycetous fungi which belong to the Gymnoascaceae have common habitat preferences with *C. immitis*, demonstrate similar modes of conidiogenesis, and can survive passage through animals. Sigler & Carmichael (1976) reintroduced the genus *Malbranchea* for some soil saprobes with arthroconidia which form by a process identical to that of *C. immitis*. They used the name *Malbranchea* state of *C. immitis* for the saprobic phase of the respiratory pathogen and described *M. dendritica* and *M. gypsea* for isolates which had been considered atypical variants of *C. immitis*. Emmons (1954, 1967) described a fungus that produces barrel-shaped arthroconidia which is highly reminiscent of *C. immitis*. However, the fungus also produces appendages associated with a teleomorphic phase of development and was named *Uncinocarpus reesii* (Sigler & Carmichael, 1976). Although formerly a member of the Gymnoascaceae, this heterothallic fungus, together with teleomorphs of other morphologically similar *Malbranchea* species, are now classified in the family Onygenaceae (order Onygenales; Currah, 1985).

Many of the true fungal pathogens of humans are accommodated in the Onygenales, including agents of cutaneous infection in the Arthrodermataceae (*Trichophyton* and *Microsporum*), and human respiratory pathogens in the Onygenaceae (*Histoplasma* and *Blastomyces*) (Currah, 1985; Sigler, 1993). Currah (1985) has argued that these two families include natural groups of related ascomycetous fungi characterized by ascospore cell walls which are smooth (Arthrodermataceae), or punctate-reticulate (Onygenaceae), conidia with lytic dehiscence mechanisms, and mycelia which have the ability to degrade keratin. Although strong morphological evidence points to a close relationship between *C. immitis* and certain members of the Onygenaceae (Sigler & Carmichael, 1976; Currah, 1985), confirmatory evidence

has been lacking. Bowman *et al.* (1992) showed that the 1713 nucleotide sequence of the 18S rRNA gene of *C. immitis* differed from that of *Histoplasma capsulatum* and *Blastomyces dermatitidis* (Onygenaceae) by only 35 and 33 substitutions, respectively. However, these two latter species produce solitary aleuroconidia rather than enteroarthroconidia. Follow-up studies of a broader group of onygenalean fungi by these and other investigators (Bowman *et al.*, 1992; McGinnis *et al.*, 1992; Bowman & Taylor, 1993) showed that non-pathogens were interspersed with pathogens throughout the Onygenales and confirmed that some *Malbranchea* species demonstrated a close relationship to *C. immitis*. In this study we compared the degree of relatedness between *C. immitis* and six non-pathogenic species of *Malbranchea* using characters derived from biochemical, immunological and molecular analyses. The respiratory pathogens *H. capsulatum* and *B. dermatitidis* were also included for comparison.

## METHODS

**Test fungi.** The two clinical strains of *C. immitis*, which have been previously characterized in our laboratory (Cole *et al.*, 1991, 1992; Kirkland *et al.*, 1991; Kruse & Cole, 1992; Pan & Cole, 1992), are designated C634 and C735. Isolates of *Malbranchea* examined in this study have been reported (Sigler & Carmichael, 1976; Currah, 1985), and were obtained from the University of Alberta Microfungus Collection and Herbarium. The isolates include *Malbranchea* states of *Uncinocarpus reesii* (UAMH 160) and *Auxarthron zuffianum* (UAMH 4098), *M. dendritica* (UAMH 2731, ex-type), *M. filamentosa* (UAMH 4097, ex-type), *M. absolutea* (UAMH 2846, ex-type), and *M. gypsea* (UAMH 1841). *Histoplasma capsulatum* (CDC A-811) and *B. dermatitidis* (CDC 1442) were obtained from the Centers for Disease Control, Atlanta, GA, USA.

**Cultivation.** The mycelial phases of *C. immitis*, *U. reesii*, *A. zuffianum* and *Malbranchea* spp. were grown in glucose/yeast extract (GYE) liquid medium (Cole *et al.*, 1989) in a shaking incubator at 30 °C. Cultures were incubated for 4 d, except *M. gypsea*, which was incubated for 7 d. These same fungi were also grown on GYE agar plates for 3–4 weeks to produce arthroconidia. The mycelial phases of *H. capsulatum* and *B. dermatitidis* were cultured in yeast extract/phosphate liquid medium (Smith & Goodman, 1975) in a shaking incubator at 24 °C for 10 d and 15 d, respectively.

**Examination of pigmented exudates.** Amber-coloured liquid exudates produced by the mycelial mat of *C. immitis* and *U. reesii* when grown on agar plates after incubation for 15–30 d were collected separately with a micropipette. The mycelial exudates were centrifuged (10 000 g, 15 min) to remove particulate material, and lyophilized. Equal dry weights of each sample were resolubilized in light petroleum (b.p. 35–60 °C) and the absorption spectra were compared using a Hewlett-Packard diode array spectrophotometer (model HP 8452A) set in the range of 210–550 nm. β-Carotene (Sigma) solubilized in light petroleum was used as a standard.

**Immunoassay and immunoblot analysis.** Crude antigenic preparations were obtained from liquid cultures of each test fungus by identical steps. These preparations included the mycelial culture filtrate plus toluene lysate (F+L) and the ConA-bound, antigenic fraction of each F+L sample, which were obtained by methods previously reported (Cole *et al.*, 1991). Immunodiffusion (ID) assays for detection of

coccidioidomycosis patient tube precipitin (TP) antibody reactivity with each ConA-bound fraction were performed by the method of Huppert & Bailey (1965). The reference antigen and human test serum employed were the same as reported earlier (Kruse & Cole, 1990). Immunoblot (Western blot) analysis of the ConA-bound fraction of each fungus was conducted using a polyclonal antibody preparation raised in guinea pigs against a purified 120 kDa  $\beta$ -glucanase of *C. immitis* (Kruse & Cole, 1992). The procedures used for SDS-PAGE separation of the ConA-bound fractions, electrotransfer of gel-separated components to nitrocellulose, and incubation with either the anti-120 kDa test serum or control (preimmune) guinea pig serum have been described (Yuan *et al.*, 1988; Kruse & Cole, 1992).

**GC-MS.** The monosaccharide content of each ConA-bound fraction of the F+L preparations obtained from the nine fungi examined in this study was determined by GC-MS with authenticated, spectroscopic-grade sugar standards as previously described (Cole *et al.*, 1990). The 3-O-methyl-D-mannose standard was synthesized and kindly provided by M. B. Goren, National Jewish Hospital, Denver, CO, USA.

**Identification of  $\beta$ -glucanase activity.** Alkaline 1,3- $\beta$ -glucanase activity of the ConA-bound fractions of test fungi which were positive in the immunoblot assays (i.e. *U. reesii*, *M. filamentosa* and *M. dendritica*) were determined and compared to the  $\beta$ -glucanase activity of *C. immitis* ConA-bound fractions as described previously (Kruse & Cole, 1992). The reaction mixture consisted of substrate (pNP- $\beta$ -D-Glc) in enzyme buffer to which 0.3–10  $\mu$ g of the ConA-bound fraction dissolved in 10  $\mu$ l of distilled water was added. Substrate and enzyme were mixed in wells of 96-well microtitre plates, incubated for 2 h at 37 °C, and the change in absorbance per minute at 405 nm was determined with a Titertek Twinreader Plus kinetic ELISA reader (ICN Biomedicals). The control mixtures contained substrate in enzyme buffer without the ConA-bound fraction, or enzyme buffer plus the ConA-bound fraction in the absence of pNP- $\beta$ -D-Glc.

**DNA extraction.** Mycelia from liquid cultures were collected by filtration and washed three times with buffer containing 0.9 M sorbitol plus 0.1 M EDTA (pH 7.5). Approximately 10 g (wet wt) of sorbitol-washed mycelia was resuspended in 30 ml of the same buffer and then incubated with 100 mg DTT and 60  $\mu$ l 2-mercaptoethanol at 37 °C for 1.5 h. This was followed by the addition of 60 mg each of dried preparations of Zymolyase 5000 (5000 units of activity g<sup>-1</sup>; Seikagaku Kogyo Co.) and chitinase (450 units of activity g<sup>-1</sup>; Sigma). The suspension was mixed by gentle vortexing, incubated at 37 °C for 2 h, and centrifuged at 2500 g, 4 °C, for 10 min. The pellet was resuspended in 5 ml 50 mM Tris/HCl plus 20 mM EDTA (pH 7.5), to which 0.5 ml of 10% (w/v) SDS and 0.3 ml proteinase K (10 mg ml<sup>-1</sup> stock; Sigma) were added. The mixture was incubated at 65 °C for 30 min. The protein was precipitated by addition of 1.5 ml 5 M potassium acetate followed by incubation on ice for 60 min. The suspension was then centrifuged at 10000 g, 4 °C, for 20 min. The DNA in the supernatant was precipitated with 2 vols of ethanol, centrifuged (10000 g), and the pellet was dissolved in 5 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) with the addition of 50  $\mu$ l RNase (10 mg ml<sup>-1</sup> stock; Sigma). The mixture was incubated at 37 °C for 1 h and then extracted with phenol and chloroform. The DNA was precipitated with an equal volume of 2-propanol during storage at -20 °C for 1 h. The suspension was centrifuged (10000 g); the pellet was washed with 70% (v/v) ethanol, air dried, and then resuspended in TE buffer.

**PCR.** Amplification of chitin synthase (CHS) gene sequences,

nuclear small (18S) ribosomal RNA (rDNA) genes, mitochondrial small rDNA genes, and the internal transcribed spacer regions (ITS1, 5.8S rDNA and ITS2) was conducted by PCR using appropriate nucleotide primers reported by Bowen *et al.* (1992) for CHS, and White *et al.* (1990) for ribosomal genes. Primers were synthesized by Operon Technologies (Alameda, CA, USA). PCR amplification was performed using a model PTC-150 Minicycler (MJ Research) and the Perkin-Elmer/Cetus GeneAmp kit. Thirty to forty cycles were conducted for amplification of genomic DNA of each test fungus. For the CHS gene fragment, each run consisted of a 94 °C (1 min) melting step, 50 °C (1 min) annealing step, and 72 °C (1 min) extension. The PCR products were digested with *Hind*III and *Xba*I (Promega Corp.), isolated using a Geneclean II kit (Bio 101) according to the manufacturer's protocol, and inserted into the *Hind*III and *Xba*I sites of pBluescript II KS plasmid (Stratagene). The latter was used as a sequencing vector. Each insert (approx. 600 bp) was sequenced as described below. The PCR amplification protocols for the 18S rDNA and ITS rDNA of all fungi tested consisted of a 94 °C (1 min) melting step, 55 °C (1 min) annealing step, and 72 °C (1 min) extension. Mitochondrial small rDNAs from most fungi tested were amplified as above, except for *C. immitis* and *B. dermatitidis*, for which the protocol included an annealing step performed at 37 °C (1 min) instead of 55 °C. PCR products derived from amplification of rDNA were separated by low-melting-point agarose electrophoresis, isolated using a Geneclean II kit, and subjected to direct sequence analysis as described below. The final PCR products of each test fungus, which were either ligated to the sequencing vector (in the case of partial CHS genes) or directly sequenced (in the case of ribosomal genes), consisted of a pool of at least four separate reactions to control for PCR errors.

**Analysis of RFLPs.** PCR-amplified, double-stranded DNA products (mitochondrial small and ITS ribosomal RNA genes) of each fungus were separately digested using four 4-base-specific endonucleases (*Apa*I, *Hae*III, *Mbo*I and *Rsa*I), which were purchased from Promega. Each reaction tube contained 5  $\mu$ l DNA, 10 units restriction enzyme, and 1  $\mu$ l 10 × reaction buffer (Promega) diluted with distilled water to a final volume of 10  $\mu$ l. Control tubes had no enzyme. Reactions were conducted at 37 °C for 12 h. This incubation condition was determined optimal for activity of the selected endonucleases. Ethidium-bromide-stained minigels (White *et al.*, 1990) were electrophoresed at approximately 8 V cm<sup>-1</sup> for 45 min under conditions previously described (Mitchell *et al.*, 1992).

**DNA sequencing.** The CHS gene fragments were sequenced by the method of Sanger *et al.* (1977). The reactions were conducted with the SK and reverse primers of the cloning vector (pBluescript) using [<sup>35</sup>S]dATP $\alpha$ S and a United States Biochemical (USB) Sequenase kit as described in the manufacturer's protocol. The GenBank accession numbers for the CHS1 gene sequences determined in this study are as follows: *C. immitis*, L28067; *Malbranchea* state of *A. zuffmanum*, L28068; *Malbranchea* state of *U. reesii*, L28069; *M. albolutea*, L28070; *M. dendritica*, L28071; *M. filamentosa*, L28072; *M. gypsea*, L28073. The 18S rDNA PCR product of each test fungus was directly sequenced using a USB Sequenase kit. The reaction mixture (10  $\mu$ l) consisted of 300 ng template DNA (PCR product isolated using Geneclean kit), 2  $\mu$ l 5 × Sequenase reaction buffer (USB), and 1.0 pmol primer (NS1, or NS8; White *et al.*, 1990) solubilized in sterile distilled water. For the annealing step, the reaction mixture was heated to 100 °C for 3 min and then immediately transferred to an ice-bath. The labelling and termination reactions were performed according to the manufacturer's protocol, except that the termination temperature was 45 °C. The complete 18S rDNA sequence of *C. immitis* (GenBank

accession no. X58571), *Malbranchea* states of *U. reesii* (L27991) and *A. zuffianum* (L28062), *M. filamentosa* (L28065), *M. dendritica* (L28064), *M. absolutea* (L28063) and *M. gypsea* (L28066) were obtained using multiple internal primers identified in Fig. 6.

**Sequence alignment and phylogenetic analyses.** Amino acid and DNA sequences were edited using the Eyeball Sequence Editor (ESEE 1.09; Cabot & Beckenbach, 1989) and aligned using the CLUSTAL V program (Higgins & Sharp, 1988) with gap adjustments. Parsimony analyses for construction of phylogenetic trees were performed with Wagener parsimony using PAUP (phylogenetic analysis using parsimony, version 3.1.1; Swofford, 1993) on a Macintosh Quadra 700 computer. Bootstrap analyses (Felsenstein, 1985) were conducted using 1000 replicates.

**Pulsed-field gel electrophoresis.** Preparation of intact chromosomal DNA was performed by the agarose spheroplast procedure (Pan & Cole, 1992), except that lyticase (Sigma) was used instead of chitinase. The lyticase was added to the mycelial suspension at a concentration of 5 mg ml<sup>-1</sup> (720 units of activity mg<sup>-1</sup>). CHEF gel electrophoresis was performed with a model DRII (Bio-Rad) gel apparatus and power supply using the methodology described by Pan & Cole (1992). The optimal electrophoresis conditions determined for *U. reesii*, *A. zuffianum* and all *Malbranchea* spp. were 40 V, switch interval of 30 min, and 140 h duration of run. The conditions used for *C. immitis* and *H. capsulatum* have been described (Pan & Cole, 1992, and Steele *et al.*, 1989, respectively). The running conditions used for *B. dermatitidis* were 40 V, switch interval of 75 min, and total run duration of 144 h. Southern hybridization between the separated chromosomal DNA and selected DNA probes was conducted as reported for *C. immitis* to facilitate estimations of chromosome number (Pan & Cole, 1992). A conserved ribosomal gene probe provided by K.-J. Kwon-Chung (National Institutes of Health, Bethesda, MD, USA; Restrepo & Barbour, 1989), and the conserved 600 bp PCR-amplified fragment of the CHS gene of *C. immitis* derived from this study were used as DNA probes. The hybridization protocol was performed as previously described (Pan & Cole, 1992).

**DNA dot-blot hybridization.** Total RNA was isolated from *C. immitis* mycelial cultures (3 d, 30 °C) using a RNeasy total RNA isolation kit (Promega) by the method of Cisar & TeBeest (1992). Reverse transcription was performed using a GeneAmp RNA PCR kit (Perkin-Elmer-Cetus) with the oligo-d(T) 16-mer primer supplied by the manufacturer. *C. immitis* cDNA was labelled by the random hexamer primer method of Feinberg & Vogelstein (1983) with [ $\alpha$ -<sup>32</sup>P]dATP (> 3000 Ci mmol<sup>-1</sup>, > 111 TBq mmol<sup>-1</sup>; ICN). The concentration of genomic DNA prepared from each test fungus obtained as described above was calculated by absorbance at multiple wavelengths using a nucleic acid Soft-Pac module attached to a Beckman DU series 60 spectrophotometer, and by comparison to a purchased *Saccharomyces cerevisiae* standard DNA preparation (0.5 µg µl<sup>-1</sup>; Clontech Laboratories). The genomic DNAs were diluted with 0.4 M NaOH plus 10 mM EDTA to the same initial concentration, and serially diluted with equal volumes applied to a Zeta-Probe GT blotting membrane (Bio-Rad) using a Bio-Dot microfiltration apparatus (Bio-Rad). The filters were hybridized with the labelled *C. immitis* cDNA probe as previously described (Pan & Cole, 1992), except that the washing temperature was 60 °C. Three separate genomic DNA preparations from each test fungus were used for comparison of levels of hybridization with the cDNA probe of *C. immitis*. Control genomic DNA

preparations were obtained from *Sacch. cerevisiae* and *Homo sapiens* (Clontech) and treated as above.

## RESULTS

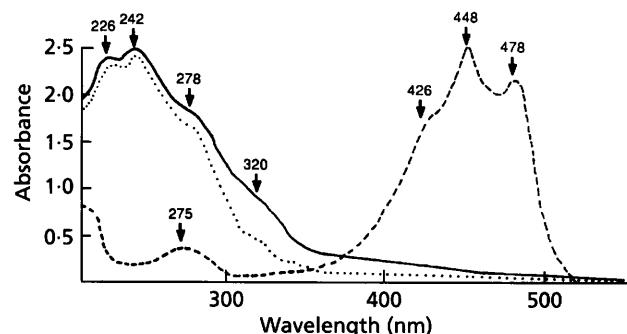
### Absorption spectra of pigmented exudates

Of the fungi examined in this study, only *C. immitis* and *U. reesii* released an amber-coloured exudate during mycelial growth on agar plates. The UV/visible absorption spectra of the *C. immitis* and *U. reesii* exudates are shown in Fig. 1. The two spectra are essentially identical.  $\beta$ -Carotene, which was chosen as a standard because of the amber colour of the fungal exudates, showed a characteristic spectrum with three defined peaks at approximately 275, 448 and 478 nm (Vetter *et al.*, 1971). The spectra of the mycelial exudate samples showed no obvious similarity to the  $\beta$ -carotene spectrum.

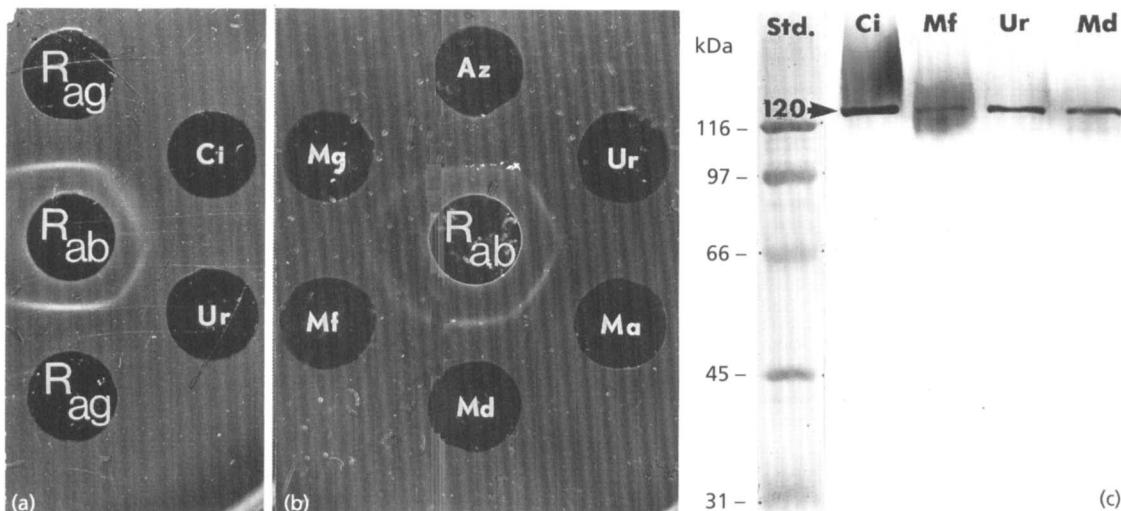
### Immunoidentification of common epitopes

Reactivity of the patient reference serum ( $R_{ab}$ ) with the *C. immitis* reference antigen ( $R_{ag}$ ) in the upper and lower wells of the ID-TP plate in Fig. 2(a) resulted in formation of characteristic precipitin bands. Fusion of the reference band with the precipitin produced by reaction of the same human serum with the test antigen from *U. reesii* in Fig. 2(a) indicates that the antigenic preparations of *C. immitis* and *U. reesii* have common epitopes. In Fig. 2(b), the fused precipitin ring indicates presence of a common TP antigen in the ConA-bound fractions (Az, Ur, Ma, Md, Mf and Mg) of all the fungi tested. On the other hand, no precipitin bands were visible in the ID-TP assays of ConA-bound fractions obtained from *H. capsulatum* and *B. dermatitidis* mycelial cultures (data not shown).

Immunoblot analysis of these same ConA-bound fractions obtained from each of the test fungi was performed using antiserum raised against the purified 120 kDa  $\beta$ -glucanase of *C. immitis* (Kruse & Cole, 1992). A 120 kDa band was visible only on immunoblots of the *C. immitis*, *M. filamentosa*, *M. dendritica* and *U. reesii* fractions (Fig. 2c).



**Fig. 1.** UV/visible absorption spectra of mycelial exudates of *U. reesii* (—) and *C. immitis* (····), and of crystalline  $\beta$ -carotene from carrot (----). All samples were solubilized in light petroleum.



**Fig. 2.** (a, b) Comparison of immunoreactivity by ID of ConA-bound fractions of mycelial F + L preparations obtained from *C. immitis* (Ci), *U. reesii* (Ur), *A. zuffianum* (Az), *M. albolutea* (Ma), *M. dendritica* (Md), *M. filamentosa* (Mf) and *M. gypseia* (Mg).  $R_{ab}$ , human reference antibody;  $R_{ag}$ , *C. immitis* TP reference antigen. (c) Immunoblots of SDS-PAGE separations of ConA-bound fractions of *C. immitis*, *M. filamentosa*, *U. reesii* and *M. dendritica* reacted with antisera raised in guinea pigs against the purified 120 kDa  $\beta$ -glucanase from *C. immitis* (Kruse & Cole, 1992).

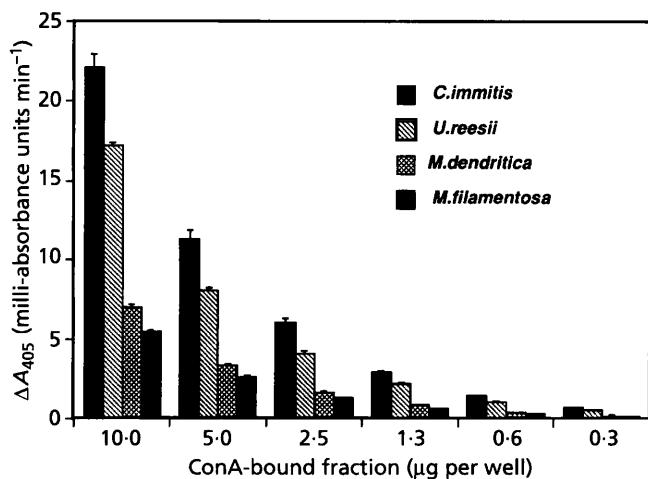
The polyclonal antibody recognized common epitope(s) in macromolecules of identical size produced by these four fungi.

#### GC-MS

We had previously shown that the ConA-bound fraction of *C. immitis* mycelial F + L material contains 3-O-methyl-D-mannose residues, which are at least partly responsible for reactivity of coccidioidomycosis patient immunoglobulin M precipitin antibody with this antigenic preparation in the ID-TP assay (Cole *et al.*, 1990). Analysis of the monosaccharide composition of ConA-bound fractions of *U. reesii*, *A. zuffianum* and the four *Malbranchea* species, which showed positive reactivity in the ID-TP assay (Fig. 2b), also revealed 3-O-methyl-D-mannose (data not shown). The *C. immitis* and *U. reesii* fractions showed similar GC profiles. Previously, we reported that ConA-bound fractions of *H. capsulatum* and *B. dermatitidis* contain no 3-O-methyl-D-mannose (Cole & Sun, 1985).

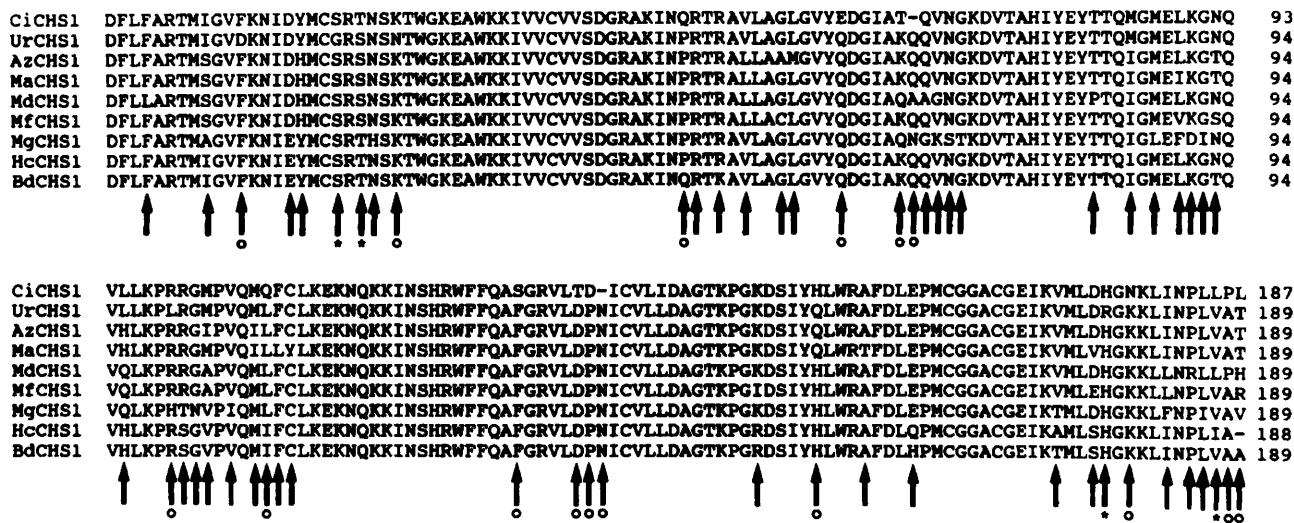
#### Comparison of alkaline $\beta$ -glucanase activity

Digestion of pNP- $\beta$ -D-Glc was observed during incubation at pH 8.0 with ConA-bound fractions of either *C. immitis*, *U. reesii*, *M. dendritica* or *M. filamentosa*. Glucanase activity in these reaction mixtures was determined by the rate of change in  $A_{405}$  of the mixture over a 2 h incubation period (Fig. 3). A range of concentration of each enzyme-containing ConA-bound fraction was tested in the reaction mixture. The  $\beta$ -glucanase activities of *U. reesii* were most comparable to those of *C. immitis*. Only a single alkaline  $\beta$ -glucanase was detected in each ConA-bound fraction. This was determined by protein separation of the



**Fig. 3.** Comparison of  $\beta$ -glucanase activity in ConA-bound fractions of mycelial F + L preparations obtained from four test fungi. Activity was measured as change in  $A_{405}$  (milli-absorbance units  $\text{min}^{-1}$ ) of the reaction mixture incubated at 37 °C using a range of concentrations of the  $\beta$ -glucanase-containing fractions, and a fixed concentration (10 mM) of the substrate, pNP- $\beta$ -D-Glc. The error bars indicate SD ( $n = 3$ ).

ConA-bound fractions in non-reducing SDS-PAGE gels and incubation of gel strips with cellulose filter paper saturated with pNP- $\beta$ -D-Glc as previously described for *C. immitis* (Kruse & Cole, 1992). As in the case of *C. immitis*, a single enzymically-reactive band of molecular mass approximately 120 kDa was visible in the ConA-bound fractions of *U. reesii*, *M. dendritica* and *M. filamentosa* (data not shown).



**Fig. 4.** Amino acid sequence alignment of CHS gene fragments of nine taxa as derived by CLUSTAL. The taxa examined were: *C. immitis* (Ci), *U. reesii* (Ur), *A. zuffianum* (Az), *M. albolutea* (Ma), *M. dendritica* (Md), *M. filamentosa* (Mf), *M. gypsea* (Mg), *H. capsulatum* (Hc) and *B. dermatitidis* (Bd). The arrows locate differences between the nine sequences. The asterisks below the arrows indicate conservative substitutions for *U. reesii* and *C. immitis* only (as defined by Higgins & Sharp, 1988). The circles indicate non-conservative residue substitutions between *U. reesii* and *C. immitis* only.

### CHS sequence comparison and phylogenetic analysis

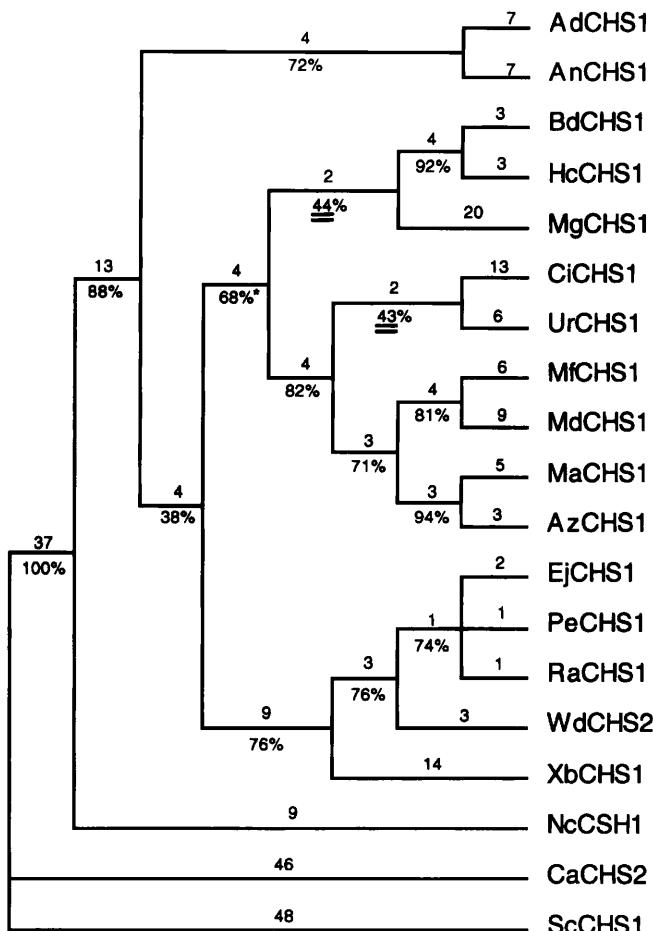
We further tested the relatedness of *C. immitis* to *U. reesii*, *A. zuffianum* and selected *Malbranchea* species by comparison of partial amino acid sequences of CHS. Approximately 600 bp CHS gene fragments were obtained by PCR amplification of genomic DNA of the test fungi using reported primers (Bowen *et al.*, 1992). The deduced amino acid sequences shown in Fig. 4 are designated CHS1, since this is the first chitin synthase reported for each species. Earlier reported sequences of CHS1 obtained from *H. capsulatum* (Hc) and *B. dermatitidis* (Bd) were included for comparison. The nine translated sequences were aligned using the CLUSTAL V program. Two gaps were inserted in the *C. immitis* sequence (CiCHS1) during alignment. It is evident from visual analysis that close similarity exists between the nine sequences. It was also determined that these sequences are all accommodated in CHS class I on the basis of their comparison to the current CHS amino acid sequence database (Bowen *et al.*, 1992; L. Mendoza & P. J. Szaniszlo, University of Texas, Austin, personal communication).

These nine sequences, together with ten previously reported class I CHS sequences (Bowen *et al.*, 1992), were compared, and the data used for construction of a phylogenetic tree based on the principle of maximum parsimony (Camin & Sokal, 1965). Wagener parsimony analysis of the data from the 19 representative fungi yielded a single parsimonious tree (Fig. 5). The tree length is 192 steps, based on 67 autapomorphic and 56 synapomorphic positions. The tree has a consistency index of 0.719 (excluding autapomorphies) and a retention index of 0.713 (Farris, 1989). Branch lengths are proportional to the inferred number of substitutions. The phylogenetic analysis program (PAUP 3.1.1) permits measurement of

skewness (g1) of the distribution of data used in construction of trees to determine whether there is phylogenetic information or mainly noise in the data set (Bowman *et al.*, 1992). If the g1 value is strongly negative, it indicates a left skewing of the tree distribution and, thus, a high degree of confidence that the results of character analysis fall near the most parsimonious tree in the distribution (Hillis & Huelsenbeck, 1992). The g1 value for the tree in Fig. 5 is -0.753, which exceeds the critical value for significance at the 99% level (Hillis, 1991). The internal consistency of the tree was not offset by internal noise. The tree supports a monophyletic branch for the nine members of the Onygenaceae (68% confidence value at the internode indicated by an asterisk). The phylogenetic tree also suggests that the onygenaceous fungi evolved as a group separate from the ten other taxa, at least based on CHS sequence comparisons. Within the nine taxa of the Onygenaceae, *U. reesii* and *C. immitis* form a monophyletic branch, while *B. dermatitidis*, *H. capsulatum* and *M. gypsea* are separated as a sister polyphyletic branch. However, the confidence values at the internodes of these two branches are relatively low (43% and 44%, respectively). Sequence comparison of the conserved CHS protein, therefore, did not clarify the phylogenetic relationships among these fungi and it was necessary to extend our molecular analyses to include rRNA genes.

### 18S rDNA sequence comparison and phylogenetic analysis

Seven 18S rDNA sequences, obtained from *C. immitis*, *U. reesii*, *A. zuffianum* and four selected *Malbranchea* species, were PCR-amplified, sequenced, and aligned (Fig. 6). Based on this comparison, *U. reesii* is the closest relative to *C. immitis* since it differs at only five positions within the



**Fig. 5.** Phylogenetic tree showing the relationships between 19 taxa based on comparison of amino acid sequences of their CHS gene fragments provided in Fig. 4 and reported by Bowen *et al.* (1992). The numbers above the lines indicate numbers of changes supporting each node. The numbers below the lines represent the bootstrap percentages (confidence values) derived from 1000 replicates. Nodes with confidence values of < 50% are not supported (double underlined values). The CHS sequences of the following species were reported by Bowen *et al.* (1992): *Aspergillus nidulans* (AdCHS1), *A. niger* (AnCHS1), *Exophiala jeanselmei* (EjCHS1), *Phaeococcomyces exophialae* (PeCHS1), *Rhinocladiella atriorens* (RaCHS1), *Wangiella dermatitidis* (WdCHS2), *Xylohypha bantiana* (XbCHS1), *Neospora crassa* (NcCHS1), *Candida albicans* (CaCHS2) and *Saccharomyces cerevisiae* (ScCHS1).

1713 bases sequenced. Wagener parsimony analysis of these seven sequences was performed together with that of 18S rDNA sequences of nine additional pathogenic and non-pathogenic fungi obtained from the GenBank database. A strict consensus cladogram from eight equally parsimonious trees was constructed (Fig. 7). The phylogenetic tree has a length of 782 steps based on 198 autapomorphic and 241 synapomorphic positions, a consistency index of 0·720 (excluding autapomorphies), a retention index of 0·603, and was strongly skewed to the left with a g1 value of -1·395. The branches uniting *C. immitis* with *U. reesii*, *M. filamentosa* with *M. dendritica*, and *H. capsulatum* with *B. dermatitidis* are strongly supported at

93%, 94% and 84% confidence values, respectively. However, the other branches are less strongly supported. The 18S rDNA sequence of *M. gypsea* revealed the highest number of base substitutions compared to *C. immitis*, and was separated from other members of the Onygenaceae. *Cryptococcus neoformans*, a basidiomycetous fungus, was shown to be an outgroup, but selection of outgroups (*Cryp. neoformans* alone, or *Cryp. neoformans*, *Sporothrix schenckii*, *Sacch. cerevisiae* and *Candida albicans*) did not affect the monophyly of the pairs of taxa cited above (i.e. *C. immitis* with *U. reesii*, *M. filamentosa* with *M. dendritica*, and *H. capsulatum* with *B. dermatitidis*). The tree in Fig. 7 is based on 241 phylogenetically informative positions in the alignable regions of the 18S rDNA sequences of all 16 taxa. We deleted 44 unalignable positions before tree construction. However, the tree topology was unchanged if the unalignable regions were not deleted. A pairwise analysis of all characters for the 16 taxa is provided in Table 1. These data are presented without deletion of unalignable sequences because such regions of the 18S rDNA include phylogenetically informative sites.

#### Phylogenetic analysis of combined sequence data

The amino acid sequence data derived from the translated CHS gene sequences and the 18S rDNA nucleotide sequence data for the nine test fungi were subjected to Wagener parsimony analysis. *Sacch. cerevisiae* was included as an outgroup. The phylogenetic tree derived from analysis of the combined data is shown in Fig. 8. The structure of the combined tree is more strongly influenced by the 18S rDNA than the CHS sequence analysis because of the much larger number of characters used in the former. The tree length is 474 steps, based on 258 autapomorphic and 82 synapomorphic positions. The tree has a consistency index of 0·869 (excluding autapomorphies), a retention index of 0·634, and a g1 value of -1·089. The monophyletic of *C. immitis* and *U. reesii* is most strongly supported in this tree. Monophyletic of *M. dendritica* and *M. filamentosa*, and that of *B. dermatitidis* and *H. capsulatum*, is supported by 100% confidence values. The combined tree suggests that *M. gypsea* is more closely aligned with *B. dermatitidis* and *H. capsulatum* than other members of the Onygenaceae examined in this study.

#### rRNA gene restriction patterns

Our aim at this stage of the study was to further test the degree of relatedness between *C. immitis* and *U. reesii* by comparison of RFLP patterns. Mitochondrial small rRNAs isolated from the nine members of the Onygenaceae were each digested with four restriction enzymes (*Alu*I, *Hae*III, *Mbo*I and *Rsa*I) and the digestion products were then separated by agarose gel electrophoresis (Fig. 9). Visual examination of the gels showed identical banding patterns for *C. immitis* and *U. reesii* using all four endonucleases. Differences in RFLPs were clearly apparent between *H. capsulatum*, *B. dermatitidis* and *C. immitis*. Digestion with *Alu*I and *Mbo*I (Fig. 9a and c,

Ci	AAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTGACGGTAAACTGCGAATGGCTCATTAATCAGTTATGTTTATTTGATAGTACCTTACTACATGGATACCGTGGTAATT	120
Ur	.....C...A...C.....	0
Az	.....	5
Ma	.....	2
Md	.....A...C.....	4
Mf	.....A...C.....	4
Mg	.....T...A.....A...T.....C.....	6
Ci	TAGAGCTAAATACATGCTAAAAACCTCGACTTCGAAGGGGTATTTATTAGATAAAAAACCAATGCCCTCGGGCTCTTGCTGATTCAATAAACCTTGCGAATCGCATGCCCTGC	240
Ur	.....	0
Az	.....T.....	6
Ma	.....T.....	8
Md	.....C.....	6
Mf	.....C.....	6
Mg	.....C.....A.....C.....	9
Ci	GCTGGCGATGGTTCATCAAATTCTGCCCTATCAACTTCTGATGGTAGGATAGTGGCCT ACCATGGTGGCAACGGGTAACGGGAATTAGGGTTGATTCCGGAGAGGGAGCCTGAGAA	360
Ur	.....	0
Az	.....C.....	7
Ma	.....	8
Md	.....C.....	7
Mf	.....C.....	7
Mg	.....G.....	10
Ci	ACGGCTACCACATCCAAGGAAGGCAGCAGGGCGCAAATTACCCAATCCCACACGGGGAGGTAGTGACAATAAACTGATACAGGGCTCTTCCGGGTCTTGTAAATTGAAATGAGTACA	480
Ur	.....	0
Az	.....T.....	11
Ma	.....G.T.....	10
Md	.....T.....	10
Mf	.....G.....	11
Mg	.....G.T.....T.....C.G.....A.....C.G.....C.....A.....	19
Ci	ATCTAAATCCCTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCAGCAGCCGGTAA TTCCAGCTCAAATAGCGTATATTAAAGTTGAGTAAAGCTCGTAGTTGAACCTT	600
Ur	.....	0
Az	.....T.....	12
Ma	.....	10
Md	.....T.....AC.....	13
Mf	.....T.....	12
Mg	.....	19
Ci	GGGTCTGGCTGGCCGGTCCGGCTTCGGCGTGCACGGTCCGGTGGACCTTCTCTG GGGAACCCATGGCCTTCACTGGCTGTAGGGGAACCAGGACTTTACTGTGAAAAAATT	720
Ur	.....T.....	4
Az	.....C.....G.....	18
Ma	.....A.C.....	16
Md	.....C.....C.....C.G.....	21
Mf	.....C.....T.C.....C.....GT.G.....C.....	17
Mg	.....T.....C.A.C.....T.....A.....C.....G.....	27
Ci	AGAGTGTCAAAGCAGGCCCTTGCTGGATACATTAGCATGAAATAAGAATAGGACGTGTGGTTCTATTGTTGAGGACCGCCGTAATGATAATAGGATAGTCGGGGC	840
Ur	.....	4
Az	.....	18
Ma	.....	17
Md	.....	21
Mf	.....	18
Mg	.....	28
Ci	GTCAGTATTGGCTGTCAGAGGTAAATTCTGGATTGCTGAAAGACTAACTACTGCGAAAGCATTGCCAAGGATGTTTCAATTACTGAAAGCTAGGGGATCGAACAGAT	960
Ur	.....	4
Az	.....	18
Ma	.....	17
Md	.....T.....	22
Mf	.....	18
Mg	.....A.....T.....	30
Ci	CAGATACCGTCGACTCTAACATAACTATGCCGACTAGGGATCGGACGGCACTTGAATAACCGCTGGCACCTTACGAGAAATCAAAGTTTGGTTCTGGGGGGATGG	1080
Ur	.....	4
Az	.....T.....	19
Ma	.....T.....	18
Md	.....T.....	23
Mf	.....GTTT.....T.....G.....	19
Mg	.....	36
Ci	TCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAAGGCGTGGAGGCTGGCGCTTAATTGACTCACACGGGAAACTCACCAAGGTCCAGACAAAATAAGGATTGACAGAT	1200
Ur	.....	4
Az	.....	19
Ma	.....	18
Md	.....	25
Mf	.....C.....CT.....	22
Mg	.....A.....	37

**Fig. 6.** For legend see facing page.

Ci	TGAGAGCTCTTCTTGATCTTTGGATGGTGGTGCATGGCGTCTTAGTTGGAGTGAATTGTCGTGCTTAATTGCGATAACGAAACGAGACCTAACCTGCTAAATAGCCGACCCAC	1320
Ur	.	G. 5
Az	.	GT. 21
Ma	A.	GT. 22
Md	T.	GT. 28
Mf	T.	GT. 25
Mg	.	GT. 39
P4		
Ci	GTGGTGGGCCGCTGGCTCTTAGAGGACTATCGGCTCAAGCCGATGGAAGTTGAGCC AATAACAGGCTGTGATGCCCTTAGATGTTCTGGGCCACCGCGCTACACTGACAGGG	1440
Ur	.	5
Az	.	21
Ma	.	22
Md	.	28
Mf	.	25
Mg	.	39
Ci	CCAGCGAGTACATCACCTTGGCCGAGAGGTCTGGTAATCTTGTAAACCCCTGTCGTGCTGGGATAGAGCATTGCAATTATTGCTCTCAACGAGGAATGCCAGTAGGACAAGTCAT	1560
Ur	.	5
Az	.C.	A. 23
Ma	.	22
Md	.C.	A. 30
Mf	.C.	A. 27
Mg	.	39
Ci	CAGCTGTGCCGATTACGTCCCTGCCCTTGTACACACCGCCGTCGCTACTACCGATTGAAATGGCTCAGTGAGGCCCTCGGACTGGCTCAGGGAGGTTGGCAACGCCAGAGCCG	1680
Ur	.	5
Az	.	28
Ma	.	23
Md	.	33
Mf	.	30
Mg	.	44
Ci	GAAAGTTGGTCAAACTGGTCATTTAGAGGAAG	1713
Ur	.	5
Az	.	28
Ma	.	23
Md	.	33
Mf	.	30
Mg	.	44

**Fig. 6.** Alignment of 18S rDNA sequences. Abbreviations for taxa are defined in the legend to Fig. 4. A dot (.) indicates a base that is the same as the top reference sequence (Ci). The values to the right represent the numbers of base substitutions compared to the reference sequence. The internal primers used to obtain the 18S rDNA sequence of the test fungi are labelled P1, P2, P3 and P4.

respectively) yielded fragment patterns which distinguished *C. immitis* from *A. zuffianum*, *M. albolutea*, *M. dendritica*, *M. filamentosa* and *M. gypsea*.

ITS rDNAs were isolated from these same fungi, digested with identical restriction enzymes and separated by agarose gel electrophoresis. Distinct RFLP patterns for the nine taxa were obtained (data not shown). Only the two clinical isolates of *C. immitis* (C634 and C735) showed fragments of the same size after digestion with each restriction enzyme.

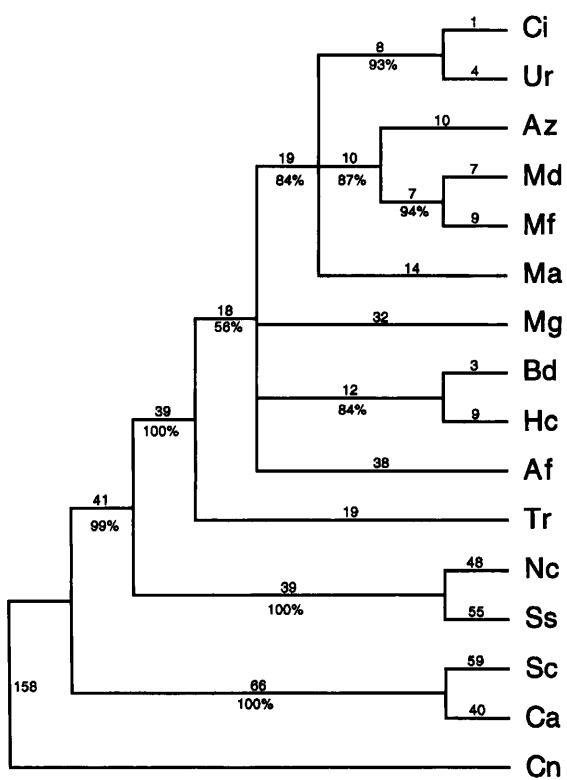
#### Chromosome number and estimated genome size

The CHEF method of pulsed-field gel electrophoresis was used to estimate chromosome number and genome size of the test fungi. At least three separate chromosomal DNA preparations were analysed for the fungi listed in Table 2 whose electrophoretic karyotype had not been previously reported. A standard curve was constructed for calculation of the molecular size of individual chromosomes of each species as previously described (Pan & Cole, 1992). The total DNA content derived from addition of the average molecular sizes of chromosomes represented the approximate genome size of each fungus. *Coccidioides immitis*, *U. reesii*, *M. gypsea* and *B. dermatitidis* have the same chromosome number and all differed from that

reported for the Downs strain of *H. capsulatum* (Steele *et al.*, 1989).

#### *C. immitis* cDNA hybridization with genomic DNA of test fungi

We examined the degree of hybridization between labelled cDNA derived from total mRNA of the mycelial phase of *C. immitis* (C634), and titrated genomic DNA preparations from *C. immitis* strains C634 and C735, six test fungi, and two control organisms (*Sacch. cerevisiae* and *Homo sapiens*). The results shown in Fig. 10 are representative of three separate hybridization experiments. The starting concentration of each genomic DNA preparation was near-identical and the same dilutions were performed for each sample. The genome sizes of all test fungi examined by DNA dot-blot hybridization were within the approximate range of 23–29 Mb (Table 2). Disparity in genome size, therefore, was probably not a significant factor influencing *C. immitis* cDNA hybridization with genomic DNA of the test fungi. Visual inspection of the dilution series in Fig. 10 indicates that the level of hybridization between the *C. immitis* cDNA probe and *U. reesii* genomic DNA was almost equal to that between the cDNA probe and two homologous strains (C634 and C735) of the pathogen. Lower but approximately equal amounts of hybridization occurred between the cDNA probe and genomic DNA of



**Fig. 7.** Single most parsimonious tree showing phylogenetic relationships between 16 taxa based on comparison of 18S rDNA gene sequences. The sequences and accession numbers for the following fungi were obtained from the GenBank database: *Aspergillus fumigatus* (Af), M55626, *Trichophyton rubrum* (Tr), X58570, *Neurospora crassa* (Nc), X04971, *Sporothrix schenckii* (Ss), M85054, *Saccharomyces cerevisiae* (Sc), J01353, *Candida albicans* (Ca), X53497 and *Cryptococcus neoformans* (Cn), X60183. The numbers above the lines indicate the number of characters supporting each node. The numbers below the lines represent bootstrap percentages (confidence values) derived from 1000 replicates. The phylogenetic tree is rooted with *Cryp. neoformans* (a basidiomycetous fungus) as the outgroup.

the other test fungi. Little to no hybridization was visible between the *C. immitis* cDNA and genomic DNA of *Sacch. cerevisiae* (Sc) and *Homo sapiens* (Hu).

## DISCUSSION

Striking similarities between the kind of arthroconidium formation of *C. immitis* and certain anamorphic and teleomorphic members of *Malbranchea* led to Sigler & Carmichael's (1976) proposal to accommodate the saprobic phase of the human respiratory pathogen in this genus of the Onygenaceae. Our results, obtained from comparative biochemical, immunological and molecular studies, have shown that *U. reesii* is the closest relative of *C. immitis* among the Onygenaceae so far examined, and provides strong support for the contention (Currah, 1985) that the teleomorph of *Coccidioides*, if it exists, would be found in this family of Ascomycetes.

Among the test fungi examined in this study, only *C. immitis* and *U. reesii* released an amber-coloured exudate

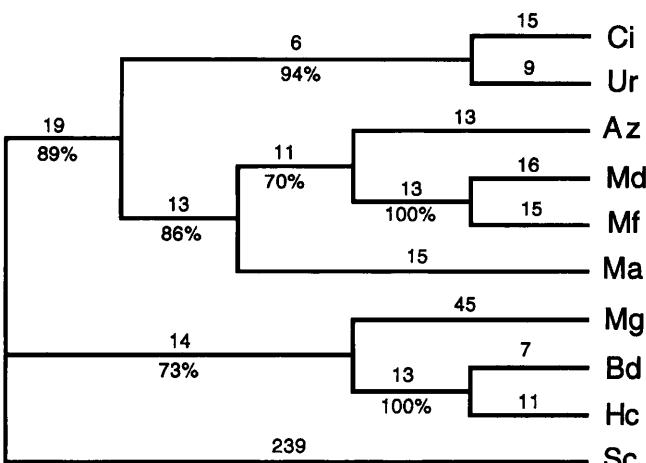
from aerial mycelia when grown on GYE agar. The UV/visible absorption spectra of the preparations obtained from the exudates were essentially identical. The nature of the pigment is unknown. Our immunological comparison showed a close antigenic relationship between all test fungi and *C. immitis*. Reactivity between human sera obtained from patients with coccidioidomycosis and antigenic, mycelial-derived fractions of *C. immitis* and all *Malbranchea* species indicated common epitopes. The positive TP antibody reaction shown in the immunodiffusion assays of *C. immitis* and *Malbranchea* antigens is the same as that reported to be serodiagnostic of early coccidioidal infection (Zimmer & Pappagianis, 1989). We have previously demonstrated that a 120 kDa glycoprotein component of the ConA-bound fraction of *C. immitis* used in the ID-TP assay was at least partly responsible for the TP antibody reaction (Cole *et al.*, 1990). We also showed that patient antibody reacts with the glycosylated portion of this 120 kDa macromolecule, and more specifically with 3-O-methyl-D-mannose residues, which are probably the immunodominant epitopes of the TP antigen (Cole *et al.*, 1992). Finally, we demonstrated that the 120 kDa precipitin antibody-reactive macromolecule of *C. immitis* is an alkaline  $\beta$ -glucanase (Kruse & Cole, 1992). This study showed that three of the test fungi, *U. reesii*, *M. filamentosa* and *M. dendritica*, produce a 120 kDa glycoprotein with alkaline  $\beta$ -glucanase activity, that the ConA-bound fraction contains 3-O-methyl-D-mannose as detected by GC-MS, and that this fraction contains epitopes reactive with patient anti-*Coccidioides* TP antibody as detected by the ID-TP assays. These data contradict an earlier report that the ID-TP assay can be used to distinguish *C. immitis* and *U. reesii* (Kaufman *et al.*, 1983). Our comparative study of the alkaline  $\beta$ -glucanase in *C. immitis*, *U. reesii*, *M. filamentosa* and *M. dendritica* revealed that only *U. reesii* had levels of glucanase activity which were comparable to that of *C. immitis*, based on equal dry weights of the  $\beta$ -glucanase-containing ConA-bound fractions. Together, these biochemical and immunological studies suggested a close relationship between *C. immitis* and *U. reesii*.

Additional compelling evidence for a close relationship between these two taxa was derived from molecular analyses. Aligned sequences of the 600 bp fragments of the conserved CHS gene were subjected to Wagener parsimony analysis. As demonstrated by Bowen *et al.* (1992) in their Fitch analysis of CHS sequence data, *Aspergillus niger* and *Emericella* (*Aspergillus*) *nidulans* were separated as a monophyletic pair, while the dematiaceous taxa (*Exophiala jeanselmei*, *Phaeoconzymyces exophialae*, *Rhino-cladiella atrovirens*, *Wangiella dermatitidis* and *Xylohypha bantiana*) formed a separate polyphyletic group. *Neurospora crassa*, *Candida albicans* and *Sacch. cerevisiae* terminated separate branches of the phylogenetic tree. The derived phylogenetic tree separates the taxa which are accommodated in the Onygenaceae as a group, but suggests that a high degree of polyphyly exists within the family. The data also support earlier reports of phylogenetic analyses of this family which indicate that non-pathogens are interspersed among the pathogenic genera, and that

**Table 1.** Number of pairwise differences in 1713 aligned and unaligned sequence positions for the 16 taxa examined

The numbers above the diagonal are the percentage difference in pairwise comparison. The numbers below the diagonal are number of differences detected in sequence comparison. The fungi included in the comparison are the same as those listed in the legends to Figs 4 and 7.

	Ci	Ur	Az	Ma	Md	Mf	Mg	Bd	Hc	Af	Tr	Nc	Sc	Ca	Ss	Cn
1 Ci	—	0.3	1.6	1.3	1.9	1.8	2.6	1.9	2.0	3.4	2.8	8.5	10.7	9.5	8.9	12.5
2 Ur	5	—	1.8	1.5	2.1	2.0	2.9	1.8	2.1	3.5	2.9	8.6	10.7	9.5	8.9	12.5
3 Az	28	31	—	1.7	1.4	1.5	2.8	2.3	2.5	3.6	3.1	8.6	11.4	9.9	8.9	12.5
4 Ma	23	36	29	—	1.8	1.9	2.9	2.3	2.5	3.7	3.1	8.8	11.1	9.7	9.2	12.8
5 Md	33	36	24	30	—	0.9	3.4	2.6	2.7	4.0	3.2	8.3	11.3	9.8	8.6	12.8
6 Mf	30	34	25	33	16	—	3.4	2.7	2.9	4.0	3.0	8.6	11.4	10.0	8.7	12.3
7 Mg	44	49	48	50	58	59	—	2.2	2.5	3.5	3.7	9.1	11.5	10.2	9.2	12.9
8 Bd	33	30	40	39	45	47	37	—	0.7	2.7	2.9	8.3	10.7	9.0	8.2	12.2
9 Hc	35	36	42	43	47	49	42	12	—	3.0	3.0	8.6	11.0	9.5	8.5	12.4
10 Af	59	60	62	64	69	69	60	47	51	—	4.0	9.2	11.4	10.2	9.2	12.5
11 Tr	48	49	53	53	54	52	64	49	52	68	—	8.1	11.6	9.9	8.5	12.4
12 Nc	140	141	141	145	136	141	150	136	141	152	133	—	12.6	11.4	6.3	13.9
13 Sc	182	183	195	190	193	195	197	182	188	194	197	206	—	5.8	12.4	13.8
14 Ca	161	161	167	164	166	169	172	153	160	173	167	186	99	—	10.8	12.2
15 Ss	151	151	151	156	147	148	156	139	145	157	144	103	210	183	—	12.1
16 Cn	213	213	212	218	218	210	219	208	211	212	211	228	234	205	206	—



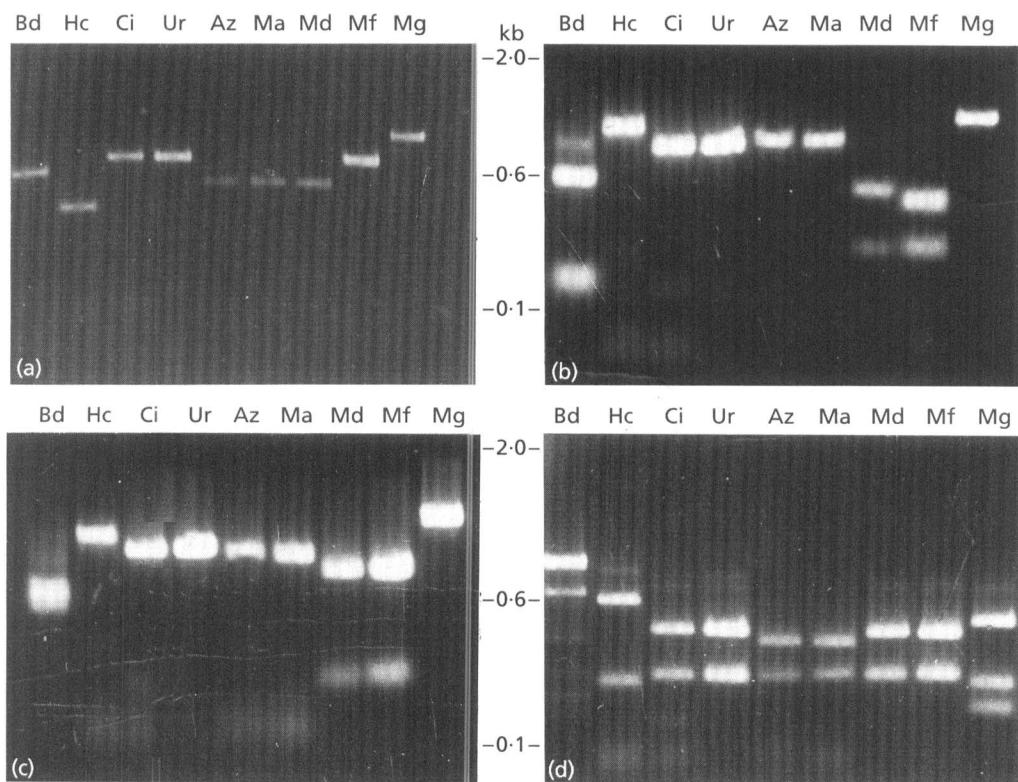
**Fig. 8.** Single most parsimonious tree showing phylogenetic relationships between nine members of the Onygenaceae examined in this study based on combined analysis of CHS and 18S rDNA sequence data. Abbreviations for taxa are defined in the legend to Fig. 4. *Sacch. cerevisiae* (Sc) is shown as an outgroup. The numbers above the lines indicate the number of characters supporting each node. The numbers below the lines represent bootstrap percentages (confidence values) derived from 1000 replicates.

the pathogens do not form a monophyletic group (Bowman *et al.*, 1992; Bowman & Taylor, 1993). Our analysis of the CHS translated amino acid sequence data suggested that *B. dermatitidis* and *H. capsulatum* together with *M. gypseum* comprise a distinct monophyletic subgroup, while *C. immitis* and *U. reesii* are separated as a second monophyletic pair. However, the confidence values for these two branches in the bootstrap analysis

were too low to provide conclusive evidence for relationships between these five taxa.

The maximum parsimony tree derived from nucleotide sequence comparison of 18S rDNA, on the other hand, strongly supported *C. immitis* and *U. reesii* as close relatives and their distinction as a monophyletic pair separated from *B. dermatitidis*, *H. capsulatum* and other members of the Onygenaceae tested in this analysis. The 1713 bp sequence of *U. reesii* differs from that of *C. immitis* by only five substitutions. The predicted branch nodes of the 18S rDNA-derived phylogenetic tree were based on high confidence values determined by bootstrap analysis. The phylogenetic tree derived from the combined analysis of CHS and 18S rDNA sequence data provided further support for the relatedness of *C. immitis* and *U. reesii*. The apparent similarity between mitochondrial small rRNA sequences of *C. immitis* and *U. reesii* based on identical RFLPs using four separate 4-base-specific endonucleases provides evidence for an even closer phylogenetic relationship than suggested by 18S rDNA sequence comparison (Bruns *et al.*, 1991). Our method of DNA dot-blot hybridization utilized a mycelial-phase-specific cDNA probe of *C. immitis* and genomic DNAs from the test fungi. Differences in levels of heterologous DNA hybridization was between the *C. immitis* cDNA probe and genomic DNA of *U. reesii*.

Although these findings together provide strong evidence of relatedness between *C. immitis* and *U. reesii*, no data are so far available to suggest any degree of pathogenicity for *U. reesii*. Sigler & Carmichael (1976) have stated that *U. reesii* is a 'transient inhabitant of man and animals and can survive for extended periods in tissue'. We showed that



**Fig. 9.** RFLPs of PCR-amplified and isolated mitochondrial small rRNAs of nine members of the Onygenaceae. The DNA preparations were digested separately with (a) *AuI*, (b) *HaeIII*, (c) *MboI* and (d) *RsaI*. Abbreviations for taxa are defined in the legend to Fig. 4.

**Table 2.** Estimates of fungal chromosome number and genome size based on electrophoretic karyotypes

Fungus examined	Chromosome number*	Average genome size and range (Mb)†	Reference
<i>C. immitis</i>	4	29 ± 3.0‡	Pan & Cole (1992)
<i>U. reesii</i>	4	24 ± 3.0	This study
<i>M. filamentosa</i>	5	24 ± 1.5	This study
<i>M. dendritica</i>	5	23 ± 2.0	This study
<i>A. zuffianum</i>	5	27 ± 2.5	This study
<i>M. albolutea</i>	5	23 ± 2.0	This study
<i>M. gypseum</i>	4	25 ± 3.0	This study
<i>B. dermatitidis</i>	4	28 ± 2.0	This study
<i>H. capsulatum</i> (Downs)§	7	30 ± 2.0	Steele <i>et al.</i> (1989)

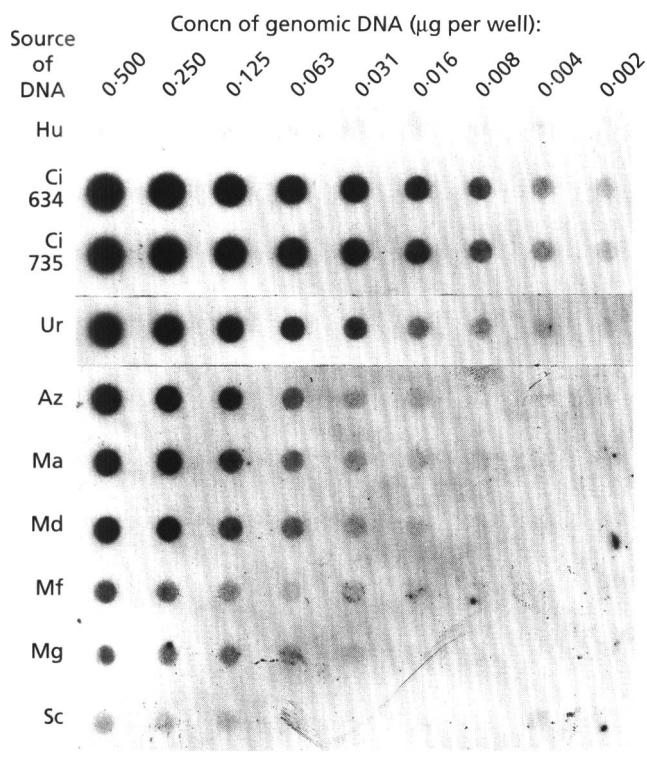
\* Chromosome numbers estimated from ethidium-bromide-stained, pulsed-field gels as well as Southern blots of electrophoresed chromosomal DNA using conserved DNA probes (Pan & Cole, 1992).

†,‡ Variation in genome size was observed due to differences in mobility of chromosomal DNA under altered electrophoresis running conditions, and to differences between strains in the case of *C. immitis*.

§ Chromosome number and genome size based only on results of examination of the Downs strain of *H. capsulatum*.

the fungus was unable to grow in a glucose/salts medium (Cole & Sun, 1985) under incubation conditions (39 °C, 20 % CO<sub>2</sub>) employed for culturing the parasitic phase of *C. immitis*. In an unpublished study, five Balb/c mice (23 g, male) were each inoculated intranasally with a

suspension of 1.2 × 10<sup>4</sup> conidia of *U. reesii* in phosphate-buffered saline (pH 7.4) by the method previously reported (Sun *et al.*, 1986). No animal deaths were recorded at 4 weeks post-inoculation, in contrast to 100% mortality after 10–15 d when mice were inoculated with



**Fig. 10.** DNA dot-blot hybridization between *C. immitis* cDNA probe, derived from total mRNA of the mycelial phase, and genomic DNA from selected fungi (0.500–0.002 µg per well). Abbreviations for taxa are defined in the legend to Fig. 4. Genomic DNA preparations from *Homo sapiens* (Hu) and *Saccharomyces cerevisiae* (Sc) were used as controls.

an equal number of *C. immitis* arthroconidia. However, *U. reesii* could be reisolated from the lungs of intranasally-challenged animals, thus confirming Sigler & Carmichael's (1976) statement and Emmon's (1954) observation that some members of the 'Gymnoascaceae' were able to survive mouse passage.

We have demonstrated complementarity and consensus between data sets derived from molecular and non-molecular analyses of *C. immitis* and *U. reesii*. Although statistical analyses were applied only to the CHS and 18S rRNA sequence data, the congruence of results obtained from the multiple comparison of the two taxa in this study is strong evidence that the proposed phylogenetic relationships are valid (Hillis, 1987; Doyle, 1992).

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