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Typing of *Histoplasma capsulatum* strains by fatty acid profile analysis

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Abstract

The performance of fatty acid profiling for strain differentiation of *Histoplasma capsulatum* was assessed. Total fatty acids were isolated from the yeast-phase cells of seven stock and two previously unreported clinical strains of *H. capsulatum* var. *capsulatum*, as well as from one unreported clinical strain and one stock strain of *H. capsulatum* var. *duboisii*, and one strain of each of three other dimorphic zoopathogenic fungal species, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii*. Different colony morphology and pigmentation types of the *H. capsulatum* strains were also included. The most frequently occurring fatty acids were oleic, palmitic, stearic and linoleic acids. There were variations in the relative percentage fatty acid contents of *H. capsulatum* strains that could be used for strain identification and discrimination. Differentiation between *H. capsulatum* strains was achieved by the comparison of detected fatty acids accompanied by principal component analysis using calculated Varimax-rotated principal component loadings. Statistical analysis yielded three major principal components that explained over 94% of total variance in the data. All the strains of *H. capsulatum* var. *capsulatum* RFLP classes II and III were grouped into two distinct clusters: the heterogenic RFLP class I formed a large, but also well-defined group, whereas the outgroup strains of *H. capsulatum* var. *duboisii*, *B. dermatitidis*, *P. brasiliensis* and *S. schenckii* were shifted away. These data suggest that fatty acid profiling can be used in *H. capsulatum* strain classification and epidemiological studies that require strain differentiation at the intraspecies level.

Introduction

Histoplasma capsulatum is the aetiologic agent of histoplasmosis, the most common pulmonary mycosis of humans and other mammals. It is estimated that over 40 million people in the USA have become infected with *H. capsulatum*, with approximately 500 000 new cases each year. Approximately 95% of histoplasmosis cases are subclinical or self-limiting. Only a small percentage of chronic *H. capsulatum* infections may turn into a progressive, serious and often fatal systemic disease, especially in immunocompromised patients with AIDS or the lymphoma–leukaemia–Hodgkin's group of diseases, or those on steroid therapy or other immunosuppressive agents (Rippon, 1980; Wheat, 2003). Under these circumstances, *H. capsulatum* can cause frequent opportunistic infections, and for this reason, along with its worldwide distribution, this fungus is considered an important human pathogen.

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The accurate diagnosis of histoplasmosis has classically relied on direct visualization of *H. capsulatum* in tissue or body fluids and/or its isolation by culturing (Wheat, 2003). The efficiency and sensitivity of these approaches vary with the extent and severity of the infection, and many cases may be undetected or misidentified. These cases might in all probability be properly diagnosed using modern molecular biology techniques. *H. capsulatum* and other dimorphic fungi can be identified using various commercially available assays, including exoantigen reagents (DiSalvo *et al.*, 1980, 1981; Standard & Kaufman, 1982; Denys *et al.*, 1983; Sekhon *et al.*, 1984, 1986; Body *et al.*, 1988; Wheat *et al.*, 1989, 1991, 1992; Padhye *et al.*, 1992; Sandin *et al.*, 1993; Sutton *et al.*, 1997; Lacaz *et al.*, 1999) and acridinium ester-labelled chemiluminescent DNA probes (Accuprobe, Gen-Probe) (Hall *et al.*, 1992; Padhye *et al.*, 1992; Huffnagle & Gander, 1993; Sandin *et al.*, 1993; Sutton *et al.*, 1997; Chemaly *et al.*, 2001; Brandt *et al.*, 2005). These two methods are accurate and commonly used for *H. capsulatum* diagnosis (Wheat, 2003).

The use of genetic methods in the diagnosis of histoplasmosis offers not only precise identification, but also the ability to distinguish particular types of *H. capsulatum* strains. Because of the natural diversity and differences in relative virulence among strains, the application of various nucleic acid-based analytical techniques contributes to efficient strain typing, the determination of biology and infection mechanisms, and epidemiological studies. Initially, most applications were directed above the species level; however, this situation no longer applies, and advances in molecular biology make it possible to distinguish individual pathogenic fungi at the inter- and intrageneric levels. There are also several other strategies, such as grouping based on RFLP analysis of either mitochondrial DNA (Vincent *et al.*, 1986; Spitzer *et al.*, 1989) and rDNA (Vincent *et al.*, 1986; Spitzer *et al.*, 1989; Jiang et al., 2000; Kasuga *et al.*, 1999; Ueda *et al.*, 2003), or *Hc*-specific protein-coding genes such as *yps3* (Keath *et al.*, 1992), *arf*, ole, *tub1* (Kasuga *et al.*, 1999; Taylor *et al.*, 2005), *H-anti* (Kasuga *et al.*, 1999; Bracca *et al.*, 2003; Taylor *et al.*, 2005) and *M-anti* (Guedes *et al.*, 2003). *H. capsulatum* isolates of different types and origins can also be classified by PCR-based random amplification of polymorphic DNA (RAPD) (Kersulyte *et al.*, 1992; Poonwan *et al.*, 1998; Reyes-Montes *et al.*, 1999; Muniz *et al.*, 2001; Taylor *et al.*, 2000, 2005; Zancope-Oliveira *et al.*, 2005), a method sometimes combined with single-strand conformational polymorphism (SSCP) (Carter *et al.*, 1996, 1997), or by repetitive-sequence-based PCR (rep-PCR) (Pounder *et al.*, 2006), or finally by electrophoretic karyotyping and chromosome-length polymorphism analysis (Canteros *et al.*, 2005).

Despite the abundance of already-developed molecular techniques and those still being developed, not all these typing methods are equally effective, and some can lead to misinterpretation. In the case of *H. capsulatum* and other dimorphic fungal pathogens, no single approach has evolved as dominant over other methods, and for that reason various additional biotyping tests, such as carbohydrate assimilation/fermentation or isoenzyme analysis, are also concurrently in use (Soll, 2000). The chemical composition of cells is a characteristic trait that can also be exploited for the identification and differentiation of various microbial species. Fatty acid profiling [commercially available as the Microbial Identification System (MIS; MIDI, Inc.)] is one such method that is exploited in mycological laboratories, where it has successfully been used for the identification of various yeast species (e.g. Augustyn *et al.*, 1990; El Menyawi *et al.*, 2000; Hinton *et al.*, 2002; Jeennor *et al.*, 2006), including those of clinical importance (Gunasekaran & Hugh, 1980; Kellogg *et al.*, 1998; Peltroche-Llacsahuanga *et al.*, 2000). In comparison with bacteria, yeasts possess a relatively limited range of cellular fatty acids, but enough complexity for chemotaxonomic classification (El Menyawi *et al.*, 2000).

The current study was undertaken to examine total fatty acid patterns of a number of *H. capsulatum* var. *capsulatum* strains and to evaluate the usefulness of fatty acid analysis in the

differentiation of this pathogen at the intraspecies level. We also examined the fatty acid composition of *H. capsulatum* var. *duboisii* and three other dimorphic fungal species, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii*. This new approach provides not only an alternative tool for *H. capsulatum* strain polymorphism studies, but also a means to resolve questions concerning the epidemiology of *H. capsulatum* infections.

Methods

Fungal strains and growth conditions

The strains used in this study are listed in Table 1. Fungi were routinely grown in liquid *Histoplasma*-macrophage medium (HMM) (Woods *et al.*, 1998). Cultures were aerated by rotary shaking (150 r.p.m.) at 37 °C in a 5% CO_2/95% air atmosphere for 4 days in 20 ml HMM in 50 ml Erlenmeyer flasks. For all experiments, glass was initially treated with a solution of $CHCl_3$/MeOH (1 : 1, v/v), dried and then thoroughly washed with double-distilled water. The experiment was repeated five times for laboratory stock strains and three times for the clinical ones, and each culture was extracted as described below in the lipid-extraction section. In addition, to examine how fatty acid profiles vary during culture growth, six selected strains (Downs, UCLA 531S, G217B, G222B, G186AS, RV26821S) were grown for 3, 4 and 5 days in time-course experiments.

Lipid extraction

Yeast cells were harvested by centrifugation of 1 ml culture aliquots (3000 ***g***, 5 min) and washed twice with sterile distilled water. The pellet was resuspended in 0.5 ml 0.97% KCl, and transferred into a 2 ml screw-topped plastic tube containing 0.5 ml of acid- and chloroform-washed glass beads (106 mm and finer, Sigma). Cells were disrupted using a Mini-BeadBeater-8 (Biospec Products) for three 1 min periods interspersed with 1 min periods of cooling on ice. Afterwards, cell homogenates were transferred into glass tubes with Teflon seals and 1.5 ml of a $CHCl_3$/MeOH mixture (2 : 1, v/v) was added to each sample. Tubes were thoroughly vortexed for 3 min and lipids were extracted for 2 h at 4 °C. Next, 1.0 ml 0.97% KCl was added to each tube, mixed and then left to stand for 1 h. To separate organic and aqueous phases, the tubes were centrifuged (300 ***g***, 5 min) and the top aqueous layer and the interphase were removed with a Pasteur pipette. The organic layer containing extracted lipids was washed with 1.0 ml 0.97% KCl and the top-separated phase was again removed. In order to achieve complete extraction, the above procedure was repeated twice, and the extracts were pooled in a clean glass test tube and the solvent was completely removed under nitrogen. Fatty acids were converted into corresponding methyl ester derivatives in the presence of 14% BF_3 in MeOH (Sigma). Prepared fatty acid methyl esters (FAMEs) were extracted with *n*-hexane. All solvents contained 5 mg l^{-1} butylated hydroxytoluene (BHT) as antioxidant.

GC analysis

FAMEs were identified by GC using a Hewlett Packard 5890 gas chromatograph equipped with a capillary column coated with DB-225 (30 m length, 0.25 mm external diameter, 0.25 μm internal diameter; Agilent Technologies). Column temperature was kept at 70 °C for 1 min, increased to 180 °C at a rate of 20 °C min^{-1} and then to 220 °C at a rate of 3 °C min^{-1}. The temperature was kept at 220 °C for 15 min. Injector and detector temperature were set at 250 °C, and the injection port temperature was set at 300 °C. Peaks were identified by comparison of retention times with those of a set of authentic fatty acid standards (Supelco). The abundance of fatty acids was calculated from relative peak areas.

Statistical analysis

Six major fatty acids detected in these fungi [myristic (14 : 0), palmitic (16 : 0), palmitoleic (16 : 1), stearic (18 : 0), oleic (18 : 1) and linoleic (18 : 2) acids] were examined as descriptors for principal component analysis (PCA). PCA is a common technique for finding a set of weighted linear composites, principal components (PCs), of original variables such that each PC is uncorrelated with the others (Hotelling, 1933). The first PC (PC 1) is a weighted linear composite of the original variables with weights chosen such that the composite accounts for the maximum variation in the original data. The second PC (PC 2) accounts for the maximum variation that is not accounted for by the first. The third PC (PC 3) likewise accounts for the maximum, given the first two PCs, and so on. The technique is thus a useful device for representing a set of a large number of variables by a much smaller set of PCs that account for much of the variance among the set of original data. A more detailed description of the use of PCA is given elsewhere (Zarnowski *et al.*, 2004).

In this study, PCA was used to classify the strains into corresponding groups, and a dendrogram was constructed using calculated Varimax-rotated PC loadings. The proper matrices were constructed on the basis of total fatty acid composition in these fungal strains and the data were processed using Statistica for Windows version 5.1 (StatSoft).

Influence of prolonged culture growth on the fatty acid composition

To examine how fatty acid profiles vary during culture growth, six selected strains (Downs, UCLA 531S, G217B, G222B, G186AS, RV26821S) were grown for 3, 4 and 5 days in time-course experiments. Afterwards, fungal cells were harvested and processed for FAME analysis, as described above.

Results

Composition of fatty acids

Fatty acid patterns found in the fungi studied here are shown in Table 2. In addition, data for a set of selected strains are shown in Fig. 1. In all cases, at least six fatty acids could be detected. The most abundant fatty acids were palmitic (16 : 0), oleic (18 : 1) and linoleic (18 : 2) acids, which formed over 90% of the total fatty acid pool. Myristic (14 : 0), palmitoleic (16 : 1) and stearic (18 : 0) acids were present in considerably smaller amounts. Other fatty acids identified in this study, including linolenic (18 : 3), eicosanoic (20 : 0), eicosaenoic (20 : 1), eicosatetraenoic (20 : 4) and docosanoic (22 : 0) acids, were detected in spurious amounts only and for that reason were excluded from further statistical analyses as nonsignificant variables (data not shown). An example of a typical gas chromatogram obtained after chromatographic separation of fatty acids isolated from fungal cells is shown in Fig. 2. Despite this simplicity in fatty acid composition, certain variations between strains and species were detected.

In *H. capsulatum* var. *capsulatum* strains of RFLP class III, palmitic and linoleic acids were predominant, and were encountered in concentrations ranging between 35.4 and 37.7%, and 33.9 and 34.7%, respectively. Oleic acid was also detected in notable amounts in those strains, and its concentration varied between 23.4 and 24.8%. Strains of *H. capsulatum* RFLP class II contained higher amounts of linoleic acid (37.9–38.7%), whereas palmitic and oleic acids were present in slightly lower concentrations (34.0–34.5 and 22.4–23.1%, respectively). The greatest discrepancies were observed with respect to fatty acid patterns of *H. capsulatum* strains of RFLP class I. The level of palmitic acid in Downs and UCLA 531 variants was similar (30.6 versus 32.5–33.5%, respectively), but concentrations of oleic and linoleic acids varied in those strains considerably. Similar discrepancies were also observed in the content of palmitoleic and stearic acids (Fig. 1a, b).

The pattern of fatty acids isolated from *H. capsulatum* var. *duboisii* was substantially different from those observed for *H. capsulatum* var. *capsulatum*. In this case, the major fatty acids (palmitic and linoleic) formed over 80% of the total fatty acid pool, whereas the content of oleic acid was significantly reduced to only 10–12.1% (Fig. 1h).

Three previously unreported clinical specimens from human histoplasmosis cases were also subjected to fatty acid analysis. The UWclin01 strain contained equal amounts of palmitic and oleic acids (33.6% of each), whereas the level of linoleic acid was lower than those observed in RFLP classes II and III (only 27.4%) (Fig. 1e). The UWclin02 strain contained similar amounts of palmitic and linoleic acids (36.1 and 35.2%, respectively), but the concentration of oleic acid was considerably lower (24.1%) (Fig. 1f). The UWclin03 strain was found to possess a different pattern of fatty acids. In this case, the most abundant fatty acids were palmitic and linoleic acids, which were present in high amounts (41.6 and 42.0%, respectively) (Fig. 1g).

Individual strains of three other dimorphic fungi, *B. dermatitidis*, *P. brasiliensis* and *S. schenckii*, possessed significantly distinct fatty acid profiles. Interestingly, the content of linoleic acid in lipid extracts from *B. dermatitidis* cells was very high (65.8%), whereas oleic acid was considerably less abundant (only 7.6%) (Fig. 1i). In *P. brasiliensis* and *S. schenckii*, the level of stearic acid was elevated (7.4 and 5.6%, respectively) (Fig. 1j, k). The content of myristic acid in *S. schenckii* cells was also slightly higher (1.5%) than in other strains examined in this work, in which this acid was present in constant, but very small amounts, not exceeding 0.6%.

Statistical analysis

PCA yielded four PCs, of which the major three, PC1, PC2 and PC3, explained over 94% of total variance in the data. We calculated PCs in such a manner that the first PC accounted for as much of the variability in the original dataset as possible, the second PC accounted for as much of the remaining variability in the data as possible, and so on. The majority of this variance was explained by the first and second PCs (58.2 and 35.8%, respectively). Fig. 3 shows a delineation of the examined *H. capsulatum* strains based on the combination of the first three calculated PCs, which resulted in the formation of three RFLP-related clusters. The strains of *H. capsulatum* var. *capsulatum* RFLP classes II and III were grouped into two distinct clusters, whereas the heterogenic RFLP class I formed a large, but also well-defined group. As a result of different levels of major fatty acids as reflected by PC 1, *H. capsulatum* var. *duboisii*, together with the three other dimorphic fungal species, *B. dermatitidis*, *P. brasiliensis* and *S. schenckii* (included in this study as the outgroup), was shifted away from other strains of *H. capsulatum* var. *capsulatum*.

Overall, the classification pattern observed also resulted from differences in the contents of myristic and 18-carbon carboxylic acids (PC 2), and palmitic and stearic acids (PC 3).

The pattern of classification of the three clinical *H. capsulatum* isolates tested herein was found to be highly comparable to that of the RFLP-based classification. It is noteworthy that the clinical *H. capsulatum* strains were novel and had not previously been classified by any genetic method or fatty acid profiling. The latter approach that we describe in this work put UWclin01 into a broad cluster that grouped heterogeneous *H. capsulatum* var. *capsulatum* strains of RFLP class I. The UWclin02 strain was grouped within the *H. capsulatum* var. *capsulatum* RFLP class III cluster, and the UWclin03 strain was placed along with all strains of *H. capsulatum* var. *duboisii* (Fig. 3). The same strain-typing results were obtained by RFLP profiling after genomic DNA digestion with *Hin*dIII (data not shown).

Influence of prolonged culture growth on the fatty acid composition

As the differences observed in fatty acid patterns might have been the result of differences in culture and/or metabolism of the fungi at the time of harvest, we examined fatty acids isolated from cells of six selected fungal strains (Downs, UCLA 531S, G217B, G222B, G186AS, RV26821S) after 3, 4 and 5 days consecutive growth in HMM (data not shown). These time points roughly correspond to early exponential, exponential and stationary growth phases of the G217B strain. The variability in fatty acid patterns was mainly observed in young cultures, whereas cells in the exponential and stationary growth phases had highly comparable profiles (data not shown). The high stability in fatty acid patterns is probably due to an appropriately chosen culture medium. As HMM is a rich medium, fungi grown in this medium do not quickly face nutrient exhaustion. Based on these observations, we assume that the recommended incubation time for fatty acid profiling should be standardized to a minimum of 4 days of culture growth.

Discussion

Fatty acid profiling has been widely used in the identification of micro-organisms. Taxa are distinguishable by the fatty acids produced and their relative ratios. In comparison with bacteria, the spectrum of fatty acids produced by fungi is more limited, e.g. yeasts mainly produce fatty acids with 16 and 18 carbon atoms. For that reason, fungal fatty acids have been considered to have very little value in taxonomic studies of this group of organisms. Regardless of such simplicity in fatty acid patterns, this method has gained recognition as being suitable for the reliable identification of clinically and industrially important species. Indeed, many reports have shown that fatty acids can be successfully used to identify and delineate yeasts and yeast-like fungi at different taxonomical levels (Westhuizen et al., 1987; Brondz et al., 1989; Stahl & Klug, 1996; Kellogg et al., 1998; El Menyawi et al., 2000; Peltroche-Llacsahuanga et al., 2000). Many of these studies employed the automated Microbial Identification System (MIS; MIDI, Inc.), which is equipped with a database for the identification of certain micro-organisms. However, this system also has many limitations. A major drawback still is its inability to discriminate between various fungal species. Another important disadvantage of MIS is the fact that many clinically relevant species, such as dimorphic fungal pathogens, are not included in the databases.

This study was aimed at improving the identification and differentiation of the yeast phase of H. *capsulatum* var. capsulatum by the establishment of an appropriate fatty acid profile database. In this study, we have analysed the fatty acid composition of two clinical isolates and seven laboratory stock strains that included different colony and pigment types of *H. capsulatum*. One clinical and one laboratory stock strain of *H. capsulatum* var. *duboisii*, and one strain of each of three other dimorphic zoopathogenic fungal species, *B. dermatitidis*, *P. brasiliensis* and *S. schenckii*, were included in this work as the outgroup. The most frequently occurring fatty acids were oleic, palmitic, stearic and linoleic acids. There were certain variations in the relative percentage fatty acid content of *H. capsulatum* that could be successfully applied to discriminate this fungus at the intraspecies level. The differences in fatty acid patterns among the strains demonstrated here are in good agreement with other phenotypic and/or molecular studies. This indicates that fatty acid profiling is a reliable tool for the identification/differentiation of *H. capsulatum*. It is also noteworthy that to the best of our knowledge, this is the first comparative study of fatty acid profiles obtained from various *H. capsulatum* strains that is taxonomically relevant.

Our qualitative data are basically in agreement with those of Domer & Hamilton (1971), who demonstrated the presence of the same major fatty acid homologues in *H. capsulatum*. However, our data differ quite drastically when the relative percentage of fatty acids is compared. These discrepancies might be related to different culture conditions as well as to

different types of fatty acid isolation/preparation techniques. From a classification viewpoint, other investigations of *H. capsulatum* lipids provide limited data (Al-Doory, 1960; Nielsen, 1966; Domer & Hamilton, 1971).

We show a strong correlation between the fatty acid composition and the RFLP type of *H. capsulatum* var. *capsulatum* strains. In RFLP class III, the predominant fatty acids were palmitic (mean 36.6%) and linoleic (mean 34.3%) acids, followed by oleic acid (mean 24.1%). Strains of *H. capsulatum* RFLP class II contained higher amounts of linoleic acid (mean 38.3%), whereas palmitic and oleic acids were present in slightly lower concentrations (mean 34.3 and 22.8%, respectively). These two RFLP-related clusters are statistically different. This classification pattern, which is determined by the applied statistical algorithm, can be explained not only by differences in the abundance of the above-mentioned fatty acids, but also by their relative ratios, particularly between saturated and unsaturated fatty acids.

The greatest discrepancies were observed with the fatty acid patterns of *H. capsulatum* strains of RFLP class I. This class is highly heterogeneous and consists of strains that were obtained from miscellaneous and somewhat unusual patients. The Downs strain was isolated from an 80-year-old woman, who had disseminated disease with extensive joint and vaginal involvement (Gass & Kobayashi, 1969). UCLA 531R was isolated from a 13-year-old boy in a region of California, in which this fungus is not endemic (Stone *et al.*, 1990; Eissenberg *et al.*, 1991). RFLP class I isolates have been found to be less virulent than those of RFLP class II and III, and possess morphological and physiological traits substantially different from those of the other classes (Medoff *et al.*, 1986; Eissenberg & Goldman, 1991). In the RFLP class I strains studied in this work, the level of palmitic acid was relatively constant; however, concentrations of both oleic and linoleic acids varied considerably in these strains. Such discrepancies were also observed in the contents of palmitoleic and stearic acids. For that reason, the RFLP class I strains were sequestered from other *H. capsulatum* clusters.

The pattern of fatty acids isolated from *H. capsulatum* var. *duboisii* was substantially different from that observed for *H. capsulatum* var. *capsulatum*. In this case, the major fatty acids (palmitic and linoleic) formed over 80% of the total fatty acid pool, whereas the content of oleic acid was significantly reduced to a mean of only 11.1%.

One characteristic of *H. capsulatum* strains other than those of RFLP class II is the generation of spontaneous avirulent variants (Eissenberg *et al.*, 1991; Eissenberg & Goldman, 1991). When plated on solid medium, these smooth-colony type variants (S and E) can be easily distinguished from rough wild-type colonies (R). The major difference in this rough–smooth variation lies in the cell wall polysaccharides. From the data presented in Table 2, no differences in fatty acid patterns between these virulent (R) and avirulent (S and E) morphology type variants of *H. capsulatum* were detected. This observation concurs with an earlier hypothesis of Nielsen (1966), who suggested a lack of any relationship between extractable lipids and virulence.

There have also been relatively few published investigations of lipids and/or fatty acids of *B. dermatitidis* (DiSalvo & Denton, 1963; Domer & Hamilton, 1971), *P. brasiliensis* (Kanetsuna *et al.*, 1969; San-Blas *et al.*, 1977; Manocha, 1980) and *S. schenckii* (De Bieuvre & Mariat, 1975; Dart, 1976; Dart & Stretton, 1976; Stretton & Dart, 1976; Yamada, 1990). Published data show oleic acid as the predominant fatty acid and linoleic acid detected in negligible amounts or completely absent. In contrast, our study demonstrated the latter in substantially higher amounts in all examined species and strains. Linoleic acid contains two double bonds in its carbon side chain, and for that reason, this molecule is relatively unstable. However, linoleic acid (as well as other unsaturated compounds) can be protected from degradation by

adding small amounts of antioxidants. In this study, we successfully isolated linoleic acid using BHT, while no application of any antioxidants by other investigators is mentioned.

The ratios of major fatty acids in the three other dimorphic fungi involved in this study, *B. dermatitidis*, *P. brasiliensis* and *S. schenckii*, were substantially different from those found in both variants of *H. capsulatum*. Cells of *B. dermatitidis* contained elevated amounts of linoleic acid (over 65%), and this increase was compensated by decreases in the content of palmitic and oleic acids. In *P. brasiliensis* and *S. schenckii*, the level of stearic acid was elevated (~7.4 and 5.6%, respectively). Additionally, the content of myristic acid in *S. schenckii* cells was also slightly higher (1.5%), whereas in other strains this compound was present in constant but very small amounts, and its concentration did not exceed 0.6%.

We conclude that the high discriminative power of fatty acid profiling allows the differentiation of the yeast phase of *H. capsulatum* strains at the intraspecies level. Our preliminary results demonstrate fatty acid profiling to be a reliable method that could help to discriminate between *H. capsulatum* var. *capsulatum* and other variants within this species (such as *H. capsulatum* var. *duboisii*), or even between other closely related dimorphic fungal species. However, in this study, we examined only one strain each of *B. dermatitidis*, *P. brasiliensis* and *S. schenckii*, and more work is needed to test additional strains of these fungal species. To recapitulate, the proposed method is a valuable alternative tool that will be useful for further studies of *H. capsulatum* population biology and epidemiology, and possibly for other issues in fungal diagnostics and therapeutics.

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Abbreviations

BHT
: butylated hydroxytoluene

FAME
: fatty acid methyl ester

PC
: principal component

PCA
: principal component analysis

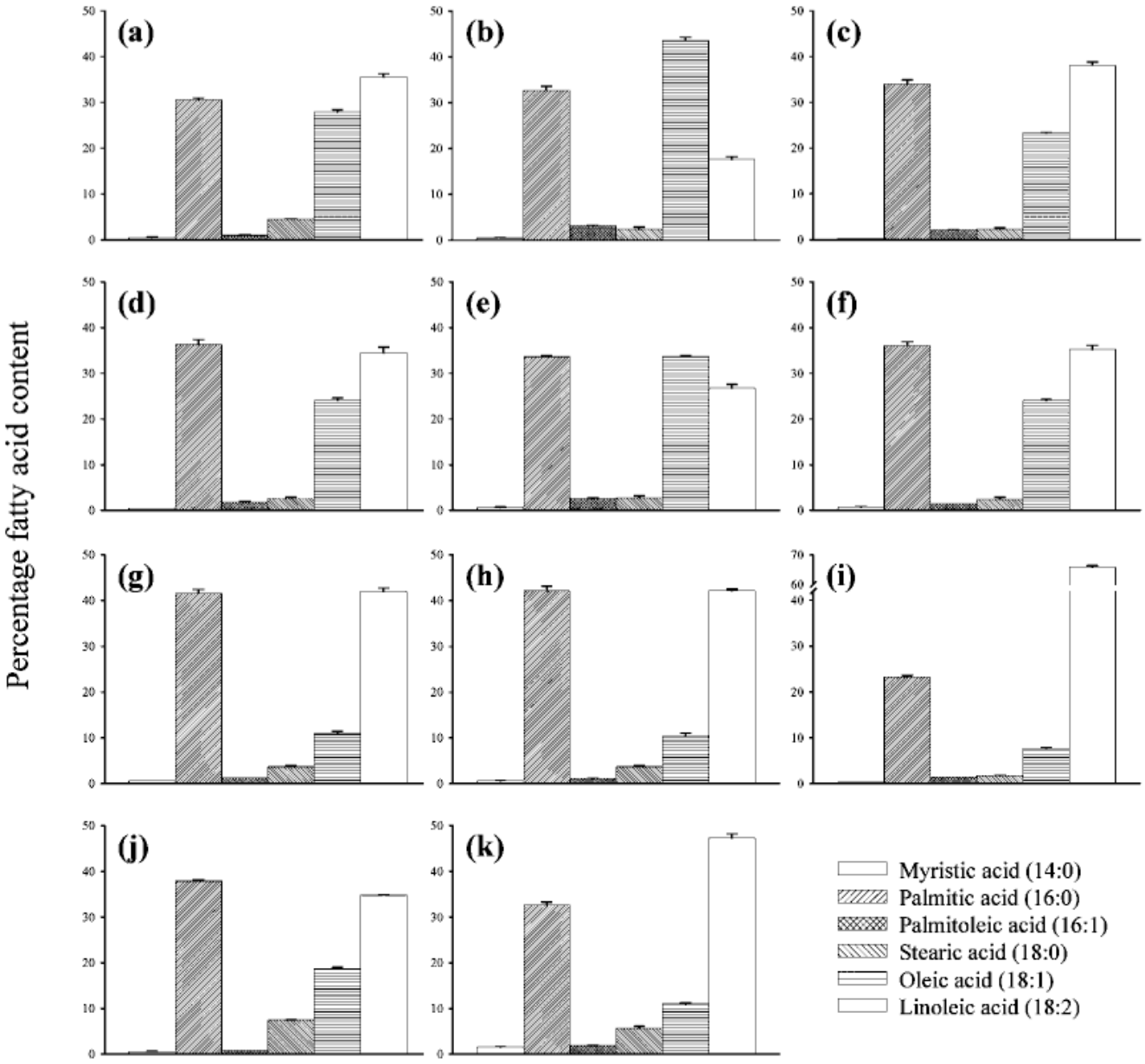

Fig. 1.

Fatty acid composition of lipid extracts from selected dimorphic fungi. (a–h) *H. capsulatum* strains: (a) Downs (class I); (b) UCLA 531S (class I); (c) G217B (class II); (d) G186B (class III); (e) UWclin01 (class I); (f) UWclin02 (class III); (g) UWclin03 (var. *duboisii*); (h) RV26821R (var. *duboisii*). (i–k) The remaining three dimorphic fungal species: (i) *B. dermatitidis*; (j) *P. brasiliensis*; (k) *S. schenckii*.

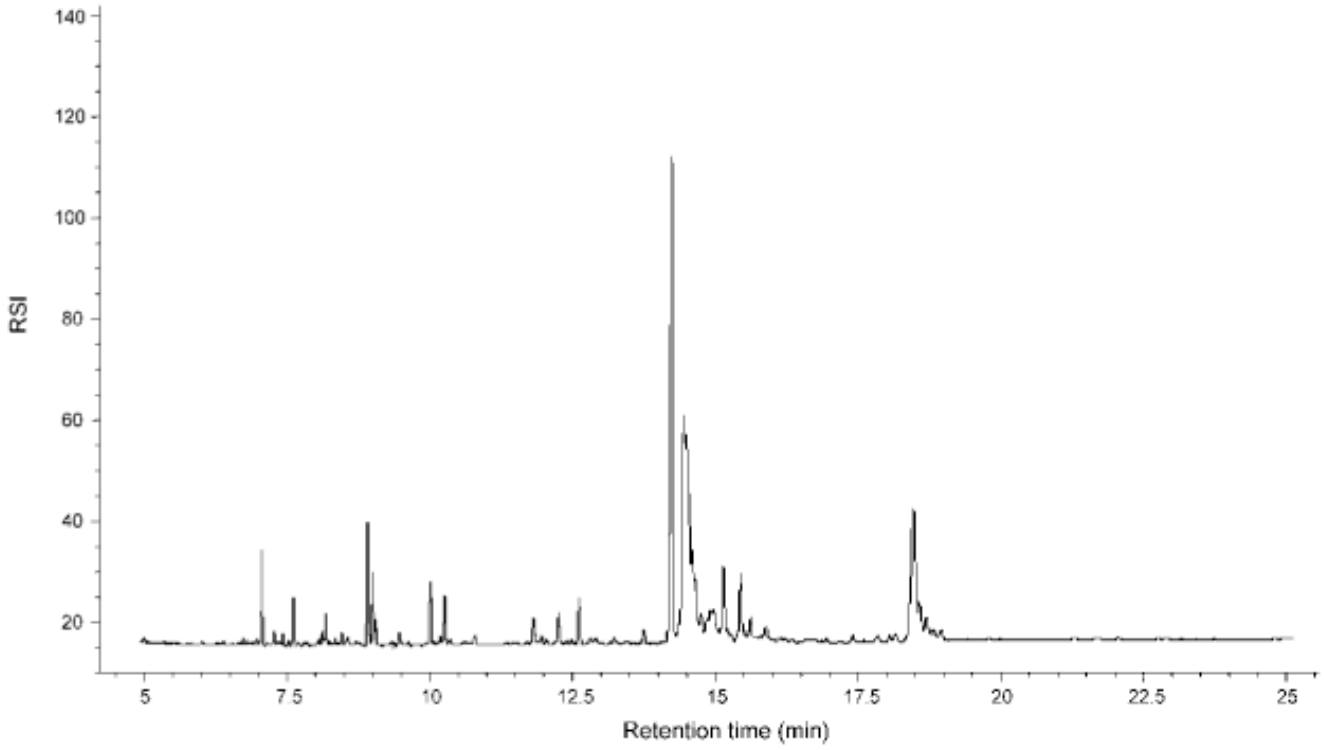

Fig. 2.
Typical gas chromatogram obtained after separation of total cellular fatty acids isolated from *H. capsulatum* cells. The conditions for fatty acid analysis were as described in Methods. RSI, relative signal intensity.

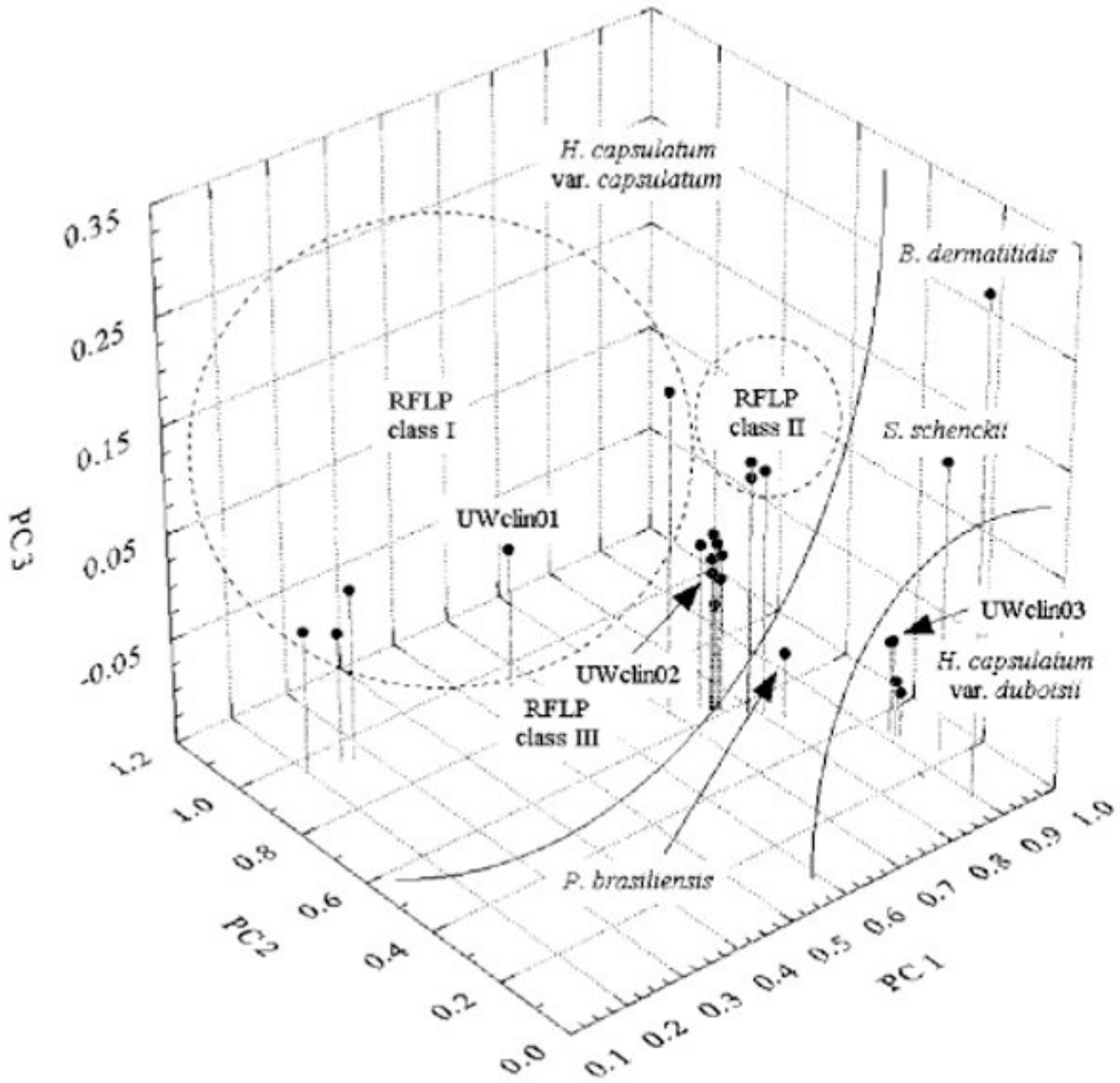

Fig. 3.

Total fatty acid profile-based grouping of *H. capsulatum* and other dimorphic fungi using PCA. The 3D score plot was created based on calculated Varimax-rotated PC loadings, PC 1, PC 2 and PC 3, that explained over 94% of total variance in the original dataset.

Table 1

Fungal strains used in this study

Strain*	RFLP class†
H. capsulatum	
var. *capsulatum*	
Downs (ATCC 38904)	I
UCLA 531S	I
UCLA 531E	I
UCLA 531R	I
G217A (ATCC 26031)	II
G217B (ATCC 26032)	II
G222B (ATCC 26034)	II
G184AS	III
G184AE	III
G184AR (ATCC 26027)	III
G186AS	III
G186AE	III
G186AR (ATCC 26029)	III
G186B (ATCC 26030)	III
var. *duboisii*	
RV26821S	
RV26821E	
RV26821R (ATCC 32281)	
Clinical isolates	
UWclin01‡	I
UWclin02‡	III
UWclin03‡	var. *duboisii*
Other dimorphic fungal species	
B. dermatitidis ATCC 26199	
P. brasiliensis ATCC 32071	
S. schenckii ATCC 10212	

*Morphological colony and pigment forms: R, rough (virulent); S, smooth (avirulent); E, epithelial variant (avirulent) (Eissenberg *et al.*, 1991); A, albino; B, brown (Berliner, 1973).

†Strain classification according to RFLP analysis (Spitzer *et al.*, 1989).

‡For the purpose of this study, the clinical isolates were identified based on RFLP profiles after digestion of genomic DNA with *Hin*dIII.

Table 2

Fatty acid composition in lipid extracts from dimorphic fungi
Values are mean±SD for five experimental repeats.

Strain	Fatty acid (percentage of total)					
	Myristic (14 : 0)	**Palmitic (16 : 0)**	**Palmitoleic (16 : 1)**	**Stearic (18 : 0)**	**Oleic (18 : 1)**	**Linoleic (18 : 2)**
***H. capsulatum*:**						
Downs	0.5 ± 0.1	30.6 ± 0.4	1.0 ± 0.1	4.5 ± 0.1	27.9 ± 0.5	35.5 ± 0.8
UCLA 531S	0.5 ± 0.1	32.7 ± 0.9	3.2 ± 0.1	2.4 ± 0.5	43.5 ± 0.8	17.6 ± 0.6
UCLA 531E	0.6 ± 0.1	32.5 ± 0.4	2.4 ± 0.0	2.5 ± 0.2	41.5 ± 0.4	20.4 ± 0.5
UCLA 531R	0.6 ± 0.0	33.5 ± 0.6	2.8 ± 0.2	2.5 ± 0.2	41.7 ± 0.3	19.0 ± 0.9
G217A	0.4 ± 0.1	34.5 ± 0.1	1.6 ± 0.1	2.5 ± 0.1	23.1 ± 0.1	37.9 ± 0.2
G217B	0.3 ± 0.0	34.0 ± 0.9	2.1 ± 0.1	2.3 ± 0.3	23.3 ± 0.8	38.1 ± 0.7
G222B	0.3 ± 0.0	34.4 ± 0.7	1.7 ± 0.1	2.4 ± 0.3	22.4 ± 0.8	38.7 ± 0.4
G184AS	0.5 ± 0.0	36.0 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	23.5 ± 0.1	34.7 ± 0.0
G184AE	0.5 ± 0.1	37.7 ± 0.5	1.8 ± 0.2	2.6 ± 0.3	23.4 ± 0.4	33.9 ± 0.3
G184AR	0.4 ± 0.0	36.4 ± 0.3	1.6 ± 0.0	2.5 ± 0.1	23.0 ± 0.5	34.0 ± 0.2
G186AS	0.5 ± 0.1	35.4 ± 0.2	2.3 ± 0.2	2.8 ± 0.4	24.2 ± 0.2	34.8 ± 0.4
G186AE	0.4 ± 0.0	36.8 ± 0.2	1.9 ± 0.2	2.6 ± 0.2	24.0 ± 0.7	34.3 ± 0.6
G186AR	0.5 ± 0.0	35.9 ± 0.7	2.1 ± 0.1	2.4 ± 0.2	24.8 ± 1.2	34.3 ± 0.5
G186B	0.5 ± 0.0	36.3 ± 1.1	1.9 ± 0.2	2.7 ± 0.2	24.1 ± 0.5	34.5 ± 1.2
UWclin01	0.6 ± 0.1	33.6 ± 0.3	2.6 ± 0.2	2.8 ± 0.4	33.6 ± 0.3	26.8 ± 0.8
UWclin02	0.7 ± 0.1	36.1 ± 0.8	1.5 ± 0.0	2.5 ± 0.4	24.1 ± 0.2	35.2 ± 0.9
UWclin03	0.6 ± 0.0	41.6 ± 0.8	1.2 ± 0.0	3.7 ± 0.2	10.9 + 0.5	42.0 ± 0.7
RV26821S	0.6 ± 0.1	40.2 ± 0.4	1.4 ± 0.1	3.4 ± 0.4	12.1 ± 0.3	42.3 ± 0.5
RV26821E	0.5 ± 0.1	40.2 ± 0.5	1.5 ± 0.0	3.3 ± 0.5	12.0 ± 1.0	42.5 ± 0.1
RV26821R	0.6 ± 0.1	42.1 ± 1.0	1.1 ± 0.1	3.7 ± 0.2	10.4 ± 0.6	42.1 ± 0.4
B. dermatitidis	0.3 ± 0.0	23.2 ± 0.4	1.4 ± 0.0	1.7 ± 0.1	7.6 ± 0.2	65.8 ± 0.5
P. brasiliensis	0.5 ± 0.1	37.9 ± 0.3	0.7 ± 0.0	7.4 ± 0.1	18.8 ± 0.2	34.8 ± 0.1
S. schenckii	1.5 ± 0.1	32.7 ± 0.6	1.8 ± 0.1	5.6 ± 0.4	11.1 ± 0.2	47.3 ± 0.9