RESEARCH ARTICLE

# Multilocus sequence typing confirms synonymy but highlights differences between *Candida albicans* and *Candida stellatoidea*

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## Abstract

We used multi-locus sequence typing (MLST) to investigate 35 yeast isolates representing the two genome-sequenced strains plus the type strain of *Candida albicans*, four isolates originally identified as *Candida stellatoidea* type I and 28 representing type strains of other species now regarded as synonymous with *C. albicans*. DNA from all 32 *C. albicans* synonyms readily formed PCR products with the *C. albicans* MLST primer sets. Their sequences placed all of them within the existing *C. albicans* clade structure, represented by 1516 isolates. One isolate, originally received as *Mycotorula sinensis*, was resistant to flucytosine, but no other unusual susceptibilities were found to polyene, azole or echinocandin antifungal agents. The four isolates of *C. stellatoidea* type I coclustered with two other sucrose-negative isolates, originally identified as examples of *Candida africana*, in a group of strains highly distinct from the majority of *C. albicans*. Our results not only confirm the synonymity of all the isolates with *C. albicans* but also confirm an obvious genotypic difference in the case of *C. stellatoidea* type I.

## Introduction

*Candida albicans* is the *Candida* species most commonly associated with human infections, and the species most intensively studied among the more than 160 accepted species within the genus *Candida* (Meyer *et al*., 1998). In the late 19th and early 20th centuries, the fungus now known as *C. albicans* was rediscovered many times. In her Ph.D. thesis, Berkhout (1923) studied the many conflicting accounts of species that had been proposed in the genera *Monilia, Oidium, Oospora* and *Torula*, examined the phenotypic properties of the published isolates and proposed a classification that erected the genus *Candida* with an isolate of *Candida tropicalis* (originally designated *Candida vulgaris*) as the type strain. The species *C. albicans* was named to accommodate several yeasts with identical characteristics, which were thus synonyms of *C. albicans*. The genus name *Candida* was accepted and, by 1930, recommended by others in the field (Ciferri & Redaelli, 1929; Ashford, 1930). *Candida* was adopted by the Eighth Botanical Congress in 1954 as a *nomen conservandum*.

In the most recent edition of the major taxonomic resource, *The Yeasts, A Taxonomic Study*, 163 species or varietal names are listed as synonyms of *C. albicans* (Meyer *et al*., 1998). Of these, 95 were first published after 1930, and 10 since 1960. Most of these species names have received no further attention after they were reclassified as *C. albicans* synonyms on the basis of morphologies and physiologies indistinguishable from the species defined by Berkhout. A notable exception is *Candida stellatoidea*. This was first published in 1938 as a new species, *Monilia stellatoidea*, which did not utilize sucrose as a carbon source, formed few chlamydospores on corn meal agar and was nonpathogenic for rabbits (Jones & Martin, 1938). It was formally renamed as *C. stellatoidea* the following year (Langeron & Guerra, 1939). In 1979, DNA reassociation studies showed clearly that isolates of *C. stellatoidea* were conspecific with *C. albicans* (Meyer, 1979), a finding supported by confirmatory DNA reassociation data (Kamiyama *et al*., 1989) and by antigenic comparisons (Montrocher, 1980). However, many publications continued to list *C. stellatoidea* at least as a variety, if not still a species separate from *C. albicans*.

Kwon-Chung *et al*. (1988) showed that isolates of *C. stellatoidea* could be divided into two subtypes on the basis of electrophoretic karyotype profiles. Type I isolates constituted authentic examples of *C. stellatoidea sensu strictu*: negative for sucrose assimilation and with low mouse virulence, whereas type II isolates were essentially sucrose-

negative variants of *C. albicans*. The identity of *C. stellatoidea* type II with *C. albicans* was confirmed by the demonstration that type II isolates could be induced to revert to growth on sucrose (Kwon-Chung *et al.*, 1990). However, it was subsequently found that reversion to sucrose assimilation could also be induced in type I *C. stellatoidea*, a shift of phenotype that resulted from chromosomal rearrangements (Wickes *et al.*, 1991). This observation finally laid to rest any lingering doubts concerning the conspecificity of *C. stellatoidea* and *C. albicans*.

Notwithstanding the acknowledged conspecificity of *C. stellatoidea* type I with *C. albicans*, several studies showed that the former were notably distinct from other *C. albicans* isolates at the DNA level, as shown by cytochrome *b* sequences (Biswas *et al.*, 2001), multi-locus enzyme electrophoresis (Pujol *et al.*, 1997), restriction fragment length polymorphisms (Magee *et al.*, 1987), electrophoretic karyotyping (Kwon-Chung *et al.*, 1988, 1989) and electrophoretic pattern of tRNAs (Santos *et al.*, 1994).

Multi-locus sequence typing (MLST), in which strain differences in single nucleotide polymorphisms are determined for fragments of six or seven housekeeping genes, has now been extended from its original applications in bacteriology (Maiden, 2006) to provide highly discriminatory strain typing schemes for several pathogenic fungi, including *C. albicans* (Bougnoux *et al.*, 2002, 2003; Tavanti *et al.*, 2003). The MLST approach has so far been applied mainly to fresh clinical isolates of *C. albicans* and has been used to provide information on the epidemiology (Bougnoux *et al.*, 2004, 2006; Odds *et al.*, 2006) and phylogenetics (Tavanti *et al.*, 2004, 2005; Aliyu *et al.*, 2006; Chen *et al.*, 2006) of the species. In this study, we have used MLST to type authentic isolates of strains now regarded as *C. albicans* but originally described as distinct species, including four isolates originally identified as *C. stellatoidea*.

## Materials and methods

### Yeast isolates

The material for this study comprised 35 yeast isolates (Table 1). They included *C. albicans* SC5314 and WO-1, the two strains that have been used for whole genome sequencing, *C. albicans* CBS562, the type strain of the species, and 32 other isolates representing yeasts that were originally reported as species other than *C. albicans*, but which are currently regarded as synonyms of *C. albicans*. Three strains identified as *C. stellatoidea* type I were the kind gift of Dr K.J. Kwon-Chung. The remaining isolates, including the type strains for *C. stellatoidea* and *Candida claussenii*, came from the collection of the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands. A further five isolates from our *C. albicans* collection were tested for sucrose assimilation. These were IHEM17984, HK03M120736, AM2005/0411, JIMS500006 and AM2005/0411. The first four of these were also included in a previous publication on MLST (Odds *et al.*, 2007).

### Strain typing and susceptibility testing

The yeasts were submitted to typing by MLST and for homozygosity at the mating-type locus (MTL) as previously described (Bougnoux *et al.*, 2003; Tavanti *et al.*, 2003). The result of MLST was expressed as a diploid sequence type (DST) and DSTs were assigned to clades according to criteria defined previously (Odds *et al.*, 2007). MTL typing led to designation of strains as *a*/α, *a*/*a* or α/α according to their heterozygosity or homozygosity at the MTL. ABC typing by PCR (McCullough *et al.*, 1999) was used to detect the presence or absence of an intron in the *ITS1* region of DNA encoding rRNA. Strains without the intron in both alleles are designated type A; those with the intron in both alleles are type B and strains with a heterozygous distribution of the intron are type C. This PCR would also have detected any strains that were examples of *Candida dubliniensis* rather than *C. albicans* (McCullough *et al.*, 1999) but none were found. The susceptibility of the yeast panel was determined to amphotericin B, fluconazole, itraconazole, posaconazole, voriconazole, flucytosine and caspofungin by the EUCAST microdilution method (Cuenca-Estrella *et al.*, 2007) with spectrophotometric endpoints read at 24 and 48 h.

### Assimilation tests

Sucrose assimilation by yeast isolates was tested by inoculation of 5 mL volumes of yeast nitrogen base with added 1% sucrose or glucose, sterilized by membrane filtration. The inoculated tubes were incubated at 30 °C with constant rotation at 20 r.p.m. at an angle of 4° from the horizontal, to provide continuous turbulence. Tubes were inspected after 24 and 48 h. Sucrose assimilation was recorded as negative when growth in the tube containing glucose was apparent as strong turbidity but minimal or no turbidity was evident in the tube containing sucrose.

### Statistical analysis

A dendrogram for 1516 *C. albicans* isolates from different sources or from the same source but representing different DSTs was constructed from the database of MLST results (http://test1.mlst.net/) in August 2007. The 1516 isolates included the 35 in the present study. The dendrogram was based on the unweighted pair-group method using arithmetic averages (UPGMA) and determined by *p*-distance as implemented by MEGA3 software (Kumar *et al.*, 2004).

**Table 1.** Details of yeast strains typed and results of MLST, ABC typing and MTL typing

| Reference no. | Details/alternative names | Original source/ country of origin | Strain typing data | | | |
|---|---|---|---|---|---|---|
| | | | DST | ABC | MTL | Clade |
| B4257 | *Candida stellatoidea*; ≡ CBS 8190, ATCC 36232 | USA | 1031 | B | het | S |
| B4404 | *Candida stellatoidea* | WS Riggsby collection | 1031 | B | het | S |
| B4406 | *Candida stellatoidea* | WS Riggsby collection | 1031 | B | het | S |
| CBS562 | Type strain of *Candida albicans* (Robin) Berkhout; ≡ ATCC18804 | Interdigital lesion | 1030 | B | het | 5 |
| CBS1899 | Type strain of *Candida truncata* Vanbreuseghem | Skin/Republic of Congo | 124 | B | het | 4 |
| CBS1905 | Neotype of *Candida stellatoidea* (Jones & Martin) Diddens & Lodder; ≡ ATCC11006 | Vagina/USA | 1031 | B | het | S |
| CBS1912 | Type strain of *Candida langeronii* Dietrichson ex van Uden & Buckley | Sputum/Norway | 119 | A | het | 2 |
| CBS1949 | Type strain of *Candida claussenii* Lodder & Kreger-van Rij; type strain of *Syringospora claussenii* Van der Walt; ≡ ATCC18814 | | 1048 | B | het | 5 |
| CBS2312 | Received as *Monilia butantanensis*; ≡ ATCC28776 | Lung | 1032 | B | het | 3 |
| CBS2689 | Type strain of *Mycelorrhizoides gruetzii* Ota | Interdigital/Germany | 1033 | C | het | 7 |
| CBS2690 | Type strain of *Cryptococcus copellii* Froilano de Mello | Tongue | 1049 | B | het | 3 |
| CBS2691 | Type strain of *Monilia tumefaciens-albus* Fullerton; formerly named *Saccharomyces tumefaciens-albus* | Pharyngitis | 1034 | A | α/α | 1 |
| CBS2692 | Type strain of *Myceloblastanon favrei* Ota | Dermatitis/Germany | 1035 | B | α/α | 3 |
| CBS2695 | Syntype of *Monilia psilosis* Ashford | Sprue/Puerto Rico | 1036 | A | het | 2 |
| CBS2696 | Type strain of *Mycotoruloides ovalis* Langeron & Talice | Oropharynx/Germany | 170 | A | het | 1 |
| CBS2697 | Type strain of *Mycotoruloides triadis* Langeron & Talice | Sputum/France | 170 | A | het | 1 |
| CBS2698 | Type strain of *Blastodendrion erectum* Langeron & Talice; *Endomyces albicans* Sabouraud | Oral thrush/France | 1037 | C | het | 5 |
| CBS2700 | Type strain of *Monilia aldoi* Pereira; also named *Mycotoruloides aldoi; Candida aldoi* | Tongue/Brazil | 1038 | A | het | 15 |
| CBS2702 | Type strain of *Candida desidiosa* Ciferri & Redaelli | Pigeon droppings/Italy | 1039 | A | het | 1 |
| CBS2703 | Type strain of *Candida mycotoruloidea* Redaelli & Ciferri | Throat/Italy | 1040 | A | *a/a* | 1 |
| CBS2704 | Type strain of *Cryptococcus pinoysimilis* Castellani; earlier called *Mycocandida pinoysimilis* | Skin lesion | 69 | A | het | 1 |
| CBS2705 | Type strain of *Mycotorula verticillata* Redaelli & Ciferri | Dermatitis/Italy | 840 | A | *a/a* | 7 |
| CBS2706 | Type strain of *Monilia periunguealis* Niño | Nail/Argentina | 1041 | A | het | 2 |
| CBS2707 | Type strain of *Monilia alvarezsotoi* Mazza & Niño | Skin | 1071 | A | het | 15 |
| CBS2710 | Type strain of *Blastodendrion oosporoides* Zach | Nail/Austria | 1042 | C | het | 5 |
| CBS2712 | Type strain of *Mycotorula sinensis* Reiss | Sputum/China | 1072 | A | het | 13 |
| CBS5137 | Type strain of *Syringospora stellatoidea* Van der Walt; ≡ ATCC32077 | Sputum/Netherlands | 69 | A | het | 1 |
| CBS5144 | Type strain of Candida intestinalis Batista & Silveira | Faeces/Brazil | 1044 | B | het | 3 |
| CBS5145 | Type strain of *Candida biliaria* Batista & Silveira | Bile/Brazil | 344 | B | het | 3 |
| CBS5703 | Types strain of *Procandida grubyii* Novák & Vitéz | Sputum/Hungary | 1043 | A | het | 7 |
| CBS5736 | Neotype of *Syringospora albicans* (Robin) Dodge (designated Van der Walt, 1970) | Vagina/South Africa | 918 | A | het | 9 |
| CBS6552 | Type strain of *Candida nouvelii* Saëz | Pharynx of *Cephalophus dorsalis*/France | 1045 | A | *a/a* | 15 |
| CBS8781 | Type strain of *Candida africana* Tietz | Balanitis/Germany | 182 | A | het | 13 |
| SC5314 | *Candida albicans* strain used for whole genome sequencing | Generalized *Candida* infection/USA | 52 | A | het | 1 |
| WO-1 | *Candida albicans* white–opaque switcher, used for whole genome sequencing | Blood isolate/USA | 383 | A | α/α | 6 |

## Results

### Typing of *C. albicans* synonym isolates

Detailed strain typing results for the panel of 35 yeasts are given in Table 1. The 35 yeasts represented 30 different DSTs and 10 different clades. Several of the DSTs were novel and details have been added to the online MLST database (http://test1.mlst.net/). By ABC typing, 20 isolates in the panel were type A, 12 were type B and three were type C. Six of the yeasts were homozygous at the MTL: three were *a/a* and three were α/α. All the isolates were susceptible to all the

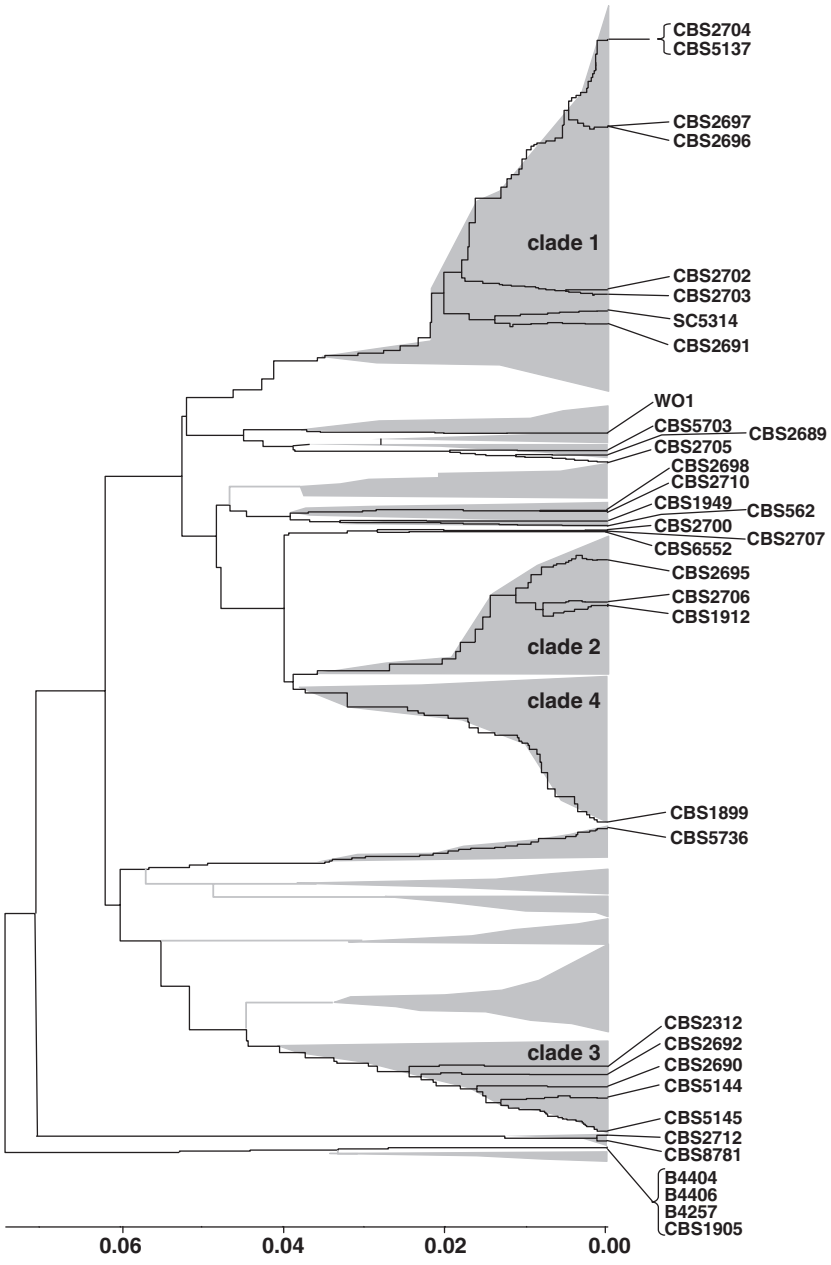


**Fig. 1.** UPGMA dendrogram for 1516 *Candida albicans* isolates. The paths highlighted are for the 35 yeasts listed in Table 1: the shape of the rest of the diagram is sketched in gray shadow. The position of the four major *C. albicans* clades is indicated.

antifungal agents tested, with the exception of CBS2712, which had intermediate flucytosine susceptibility (MIC = 16 μg mL$^{-1}$).

The relationship of the yeasts to other *C. albicans* strains was demonstrated by the UPGMA dendrogram (Fig. 1). Seven of the *C. albicans* synonyms and strain SC5314 were members of clade 1: CBS2704 and CBS5137 were DST 69, the most common DST for *C. albicans* isolates globally (Odds *et al.*, 2007). Three of the synonyms were members of clade 2, five of clade 3 and one belonged to clade 4 (Table 1 and Fig. 1). None of the strains fell into clade 11, the fifth most populous clade and one which is dominated by European isolates of *C. albicans* (Odds *et al.*, 2007). The *C. albicans* type strain CBS562 and three other isolates coclustered in clade 5. Three isolates clustered in minor clade 15. One isolate, CBS2712, coclustered with the *Candida africana* type strain MYA-2669 in clade 13.

The four isolates of *C. stellatoidea*, including the species type strain, formed a cluster of indistinguishable strains with DST 1031. In the UPGMA dendrogram, they were well separated from most other *C. albicans* strains (Fig. 1). Their closest neighbors in the full 1516-isolate UPGMA dendrogram (Fig. 2) were isolates P2216 and P2246, originally identified as *C. africana*. Only these six isolates coclustered

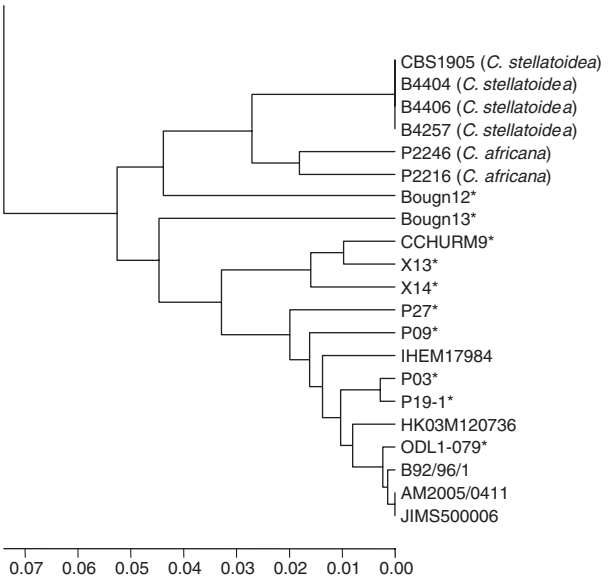


**Fig. 2.** Detail for the portion of the dendrogram at the bottom of Fig. 1. This shows the positions of the six sucrose-negative isolates (designated *Candida stellatoidea* or *Candida africana*) and the other *Candida albicans* isolates within this region of the dendrogram. An asterisk indicates the isolate was not available to us for study: MLST data were provided from other laboratories or were determined by our laboratory from DNA samples only.

at a similarity with *p*-distance $< 0.04$, the previous arbitrary cut-off for clade distinctions (Odds *et al.*, 2007). All six isolates in this small cluster were negative in tests for sucrose assimilation. As shown in Fig. 2, a further 15 isolates coclustered with the six sucrose-negative isolates beyond the limit of *p*-distance $< 0.04$. For all but five of these isolates, the MLST data came from other laboratories or from our own laboratory on the basis of a DNA sample, not the live isolate; hence we had no material for conducting a sucrose assimilation test. For the five isolates in our collection, sucrose assimilation was positive. CBS5137, nominally the type strain of *Syringospora stellatoidea*, considered by Van der Walt (1970) to be the teleomorph of *C. stellatoidea*, was also negative for sucrose assimilation, but was DST 69, the most common *C. albicans* type. We have tested sucrose assimilation for two other randomly chosen DST 69 isolates: both were sucrose-positive.

## Discussion

Our study confirms the existing phenotypic and genetic data that reclassified the diverse putative novel yeast species we studied as synonyms of *C. albicans* (Robin) Berkhout. All 32 synonymous isolates were typeable with *C. albicans* MLST PCR primers and coclustered with existing *C. albicans* isolates in the UPGMA dendrogram based on MLST results. We have tested four isolates of *C. dubliniensis* with the same set of PCR primers (unpublished data). All four formed PCR products readily with the *ADP1* primers and, with prolongation of the PCR cycles, with the *MPIb* primers. Reactions were weak or nonexistent with the other five sets of primers. Although the MLST primer sets were designed for typing strains within a single species and not for species identification, if isolates of such a closely related species as *C. dubliniensis* (Fitzpatrick *et al.*, 2006) react only with a subset of those primers, it seems unlikely that isolates of any species other than *C. albicans* would readily form PCR products with the full primer set. Our results therefore lend no support to any possibility of reclassifications for the alternative species names represented in our isolate panel.

Our study reveals that the species type strain for *C. albicans*, CBS562, is neither a member of the most populous *C. albicans* clade (clade 1) nor the most commonly encountered ABC type (type A). With the DNA sequence information now available, a more rational choice for a *C. albicans* type strain would be an example of DST 69, type A, to represent the most common *C. albicans* strain type known (Odds *et al.*, 2007). Of course, once designated in a Latin description, strain types retain their authority as representatives of the phenotypic properties of a species: however, the level of detail to which DNA from a single isolate can now be scrutinized means that isolates other than phenotypically designated strain types are more important tools for experimental investigation. The two *C. albicans* strains that have so far been the subject of whole genome sequencing were chosen over and above the species strain type.

Isolates at one time designated as *C. africana* have been described in several publications concerned with phenotypically atypical strains of *C. albicans* (Tietz *et al.*, 1995, 2001; Forche *et al.*, 1999). Most such isolates have already been shown by MLST to cocluster at a high level of similarity away from the majority of *C. albicans* strains (Odds *et al.*, 2007). However, two isolates we received as examples of *C. africana* coclustered in this study with the four isolates originally classed as *C. stellatoidea* type I (Fig. 2) and, like the *C. stellatoidea*, failed to grow in broths with sucrose as the sole carbon source – the main phenotypic property that differentiated *C. stellatoidea* from *C. albicans* (Kwon-Chung *et al.*, 1989). The portion of the dendrogram shown in Fig. 2 seems to include two strain subclusters on the basis of the sucrose assimilation properties of the isolates (but with the limitation that the majority of these isolates were not available to us for testing). The fate of most *C. albicans* isolates that were first purported to be new species was to become synonyms of *C. albicans* with some rapidity. However, *C. stellatoidea* retained its separate status from *C. albicans* for 40 years until 1979 when Meyer first proposed conspecificity on the basis of DNA reassociation studies (Meyer, 1979) and was still referred to at least as a variety for many years afterwards. Our data confirm beyond any

question that *C. stellatoidea* is a synonym of *C. albicans* – the sucrose-negative isolates are unequivocally coclustered with sucrose-positive *C. albicans* isolates. However, our data extend the previous studies showing a marked genetic difference between *C. stellatoidea* type I isolates and other *C. albicans* (Magee *et al.*, 1987; Kwon-Chung *et al.*, 1988, 1989; Santos *et al.*, 1994; Pujol *et al.*, 1997; Biswas *et al.*, 2001). It is, perhaps, most impressive overall that many isolates, earlier regarded by investigators equipped only with experience and phenotypic data as sufficiently different from *C. albicans* to constitute a separate species, still emerge as a separate strain cluster with high dissimilarity from most of the other *C. albicans* types. However, the typing of the sucrose-negative isolate originally named as *S. stellatoidea* as *C. albicans* DST 69 suggests that this isolate is an example of *C. stellatoidea* type II, rather than the better demarcated subcluster represented by the six sucrose-negative isolates CBS1905, B4404, B4406, B4257, P2216 and P2246 (Fig. 2).

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