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Phylogenetics of Saccharomycetales, the ascomycete yeasts

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Abstract: Ascomycete yeasts (phylum Ascomycota: subphylum Saccharomycotina: class Saccharomycetes: order Saccharomycetales) comprise a monophyletic lineage with a single order of about 1000 known species. These yeasts live as saprobes, often in association with plants, animals and their interfaces. A few species account for most human mycotic infections, and fewer than 10 species are plant pathogens. Yeasts are responsible for important industrial and biotechnological processes, including baking, brewing and synthesis of recombinant proteins. Species such as *Saccharomyces cerevisiae* are model organisms in research, some of which led to a Nobel Prize. Yeasts usually reproduce asexually by budding, and their sexual states are not enclosed in a fruiting body. The group also is well defined by synapomorphies visible at the ultrastructural level. Yeast identification and classification changed dramatically with the availability of DNA sequencing. Species identification now benefits from a constantly updated sequence database and no longer relies on ambiguous growth tests. A phylogeny based on single gene analyses has shown the order to be remarkably divergent despite morphological similarities among members. The limits of many previously described genera are not supported by sequence comparisons, and multigene phylogenetic studies are under way to provide a stable circumscription of genera, families and orders. One recent multigene study has resolved species of the Saccharomycetaceae into genera that differ markedly from those defined by analysis of morphology and growth responses, and similar changes are likely to occur in other branches of the yeast tree as additional sequences become available.

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INTRODUCTION

The subphylum Saccharomycotina contains a single order, the Saccharomycetales (FIG. 1, TABLE I). These ascomycetes have had a significant role in human activities for millennia. Records from the Middle East, as well as from China, depict brewing and bread making 8000–10 000 years ago (McGovern et al 2004, http://www.museum.upenn.edu/new/exhibits/online_exhibits/wine/wineintro.html). These early ''biotechnologists'' had no idea why bread dough rose or why beer fermented. The explanation awaited Antonie van Leeuwenhoek (1680), who showed with his microscope that small cells that were to become known as yeasts were present and further for Louis Pasteur (1857) to demonstrate conclusively that yeasts caused the fermentation of grape juice to wine.

What is a yeast?—Yeasts eventually were recognized as fungi, prompting description of the ''sugar fungus'' from beer to be named *Saccharomyces cerevisiae*. Yeasts usually grow as single cells with division by budding (FIGS. 2, 3) or less frequently by fission (FIG. 4), and asci and ascospores (FIGS. 5–13) are not produced in fruiting bodies as is common for many other kinds of ascomycetes. In addition both meiotic nuclear divisions occur within an intact nuclear envelope and the enveloping membrane system associated with each ascospore during delimitation has an independent origin. It initially was believed that all yeasts were ascomycetes. The presence of ballistoconidia in such yeasts as the asexual genus *Sporobolomyces* however led Kluyver and van Niel (1927) to suggest that some yeasts might be basidiomycetes. A clear connection was provided by Nyland's (1949) discovery of the teliospore-forming genus *Sporidiobolus*, which was followed by descriptions of teliosporic life cycles for a number of other genera (e.g. Banno 1967, Fell et al 1969, Kwon-Chung 1975, Boekhout et al 1991). In addition to ascomycete and basidiomycete yeasts, the term ''yeast-like'' also has been extended to the cellular phase of dimorphic members of the zygomycete genus *Mucor* (Flegel 1977), the ''black yeasts'' (de Hoog 1999), which comprise diverse pigmented ascomycete genera such as *Aureobasidium, Fonsecaea* and *Phaeococcomyces*, and even certain achlorophyllous algae in the genus *Prototheca* (Kurtzman and Fell 1998). Dimorphic (e.g. species of *Ajellomyces* and

Coccidioides immitis) (Bowmann et al 1992) and ''yeast-like'' fungi are ascomycetes (e.g. *Symbiotaphrina* and unnamed endosymbionts of plant-hoppers) that have a yeast growth form in their life history (Suh et al 2001). They are derived however from within several lineages of mycelial ascomycetes and now are excluded from the Saccharomycetales. One more striking finding on the way to defining a monophyletic group was the discovery that members of *Schizosaccharomyces* are not in the Saccharomycetales clade (see Taylor et al 1993).

In search of a monophyletic group.—From the moment that Banno (1967) demonstrated the formation of clamped hyphae and basidiospores by zygotes arising from crosses between strains of a *Rhodotorula* species, a growing list of deep-seated differences between ascomycete and basidiomycete yeasts gradually became obvious: (i) Cell wall polysaccharide composition is dominated by chitin in the basidiomycetes and β-glucans in the ascomycetes; (ii) nuclear DNA guanine + cytosine (G + C) composition tends to be higher than 50% in basidiomycetes and lower than 50% in ascomycetes (Kurtzman and Fell 1998); (iii) bud formation is typically enteroblastic in basidiomycetes and holoblastic in ascomycetes; (iv) ascomycete yeasts are generally more fermentative, more copiotrophic and at the same time specialized nutritionally, more fragrant and mostly hyaline. This is in contrast to basidiomycete species, which more often form mucoid colonies, display intense carotenoid pigments and tend to use a broader range of carbon compounds more efficiently at lower concentrations (Kurtzman and Fell 1998); (v) in terms of diagnostic tests, the diazonium blue B reaction is almost always positive in basidiomycete yeasts and negative in ascomycete yeasts (van der Walt and Hopsu-Havu 1976); and (vi) ascomycete yeasts are profoundly different ecologically and often found in specialized niches involving interactions with plants and insects or other invertebrate animals that they rely upon for dispersal. These niches tend to be liquid and rich in organic carbon. In contrast basidiomycete yeasts would seem to be adapted to the colonization of nutrient-poor, solid surfaces and might not rely to the same extent on animal vectors for their dispersal (Lachance and Starmer 1998).

The relationship of Saccharomycetales to other fungi has been the subject of many hypotheses. Because yeast cellular morphology is rather simple, it was believed that yeasts are primitive organisms. This idea gained some support because yeast genomes tend to be smaller than those of many other fungi and they have fewer introns in their gene sequences. The philosophy of yeast relationships changed in the 1970s (e.g. Cain 1972) with the proposal that yeasts represent morphologically reduced forms of filamentous fungi and, using this reasoning, some yeasts were classified into families with molds (Redhead and Malloch 1977). Gene sequence analyses have shown many of these ideas to be incorrect.

Identification of yeasts.—Earlier classifications of yeasts at lower taxonomic levels were based on presence or absence of a sexual state, type of cell division, presence or absence of hyphae and pseudohyphae, fermentation of simple sugars and growth on various carbon and nitrogen compounds. Traditional genetic crosses however showed that strains differing in morphological and metabolic characters could be members of the same species, which cast considerable doubt on the importance of these commonly used taxonomic and phylogenetic characters. These doubts prompted yeast taxonomists to turn to DNA-based methods for species delineation.

The transition from phenotypic identification of yeasts to molecular identification began with determination of the mol% G + C ratios of nuclear DNA. These analyses demonstrated that ascomycete yeasts have a range of ca. 28–50 mol% G + C whereas basidiomycete yeasts have a range of ca. 50–70 mol% G + C. Strains that differed by 1–2 mol% were recognized as separate species (Price et al 1978, Nakase and Komagata 1968). Quantitative assessment of genetic similarity between strains and species subsequently was determined by the technique of nuclear DNA re-association or hybridization (i.e. the extent of heteroduplex formation between the DNAs compared. DNA from the species pair of interest is sheared, made single-stranded, and the degree of heteroduplex formation between the pair is determined from the extent of re-association [Price et al 1978, Kurtzman 1993]). On the basis of shared phenotype, strains with 80% or greater re-association were proposed to represent members of the same yeast species (Martini and Phaff 1973, Price et al 1978). Correlation of this measure with the biological species concept has been examined from genetic crosses using both heterothallic and homothallic species. These results also lead to the conclusion that strains showing ca. 70% or greater heteroduplex formation are likely to be members of the same species (Kurtzman et al 1980a, b; Smith et al 2005).

Despite the remarkable impact that DNA reassociation experiments have had on yeast systematics, the re-association technique is slow and labor intensive and resolution does not extend beyond closely related species. Consequently DNA sequencing has been widely adopted to understand species

FIG. 1. Relationships of selected members of the Saccharomycetales. Consensus of 12 most parsimonious trees based on the combined dataset of SSU and LSU rDNA sequences with all missing and ambiguous characters deleted. Species of basal ascomycetes were used as outgroup taxa. The species shown in boldface are type species of each genus. The sequences of *Dekkera bruxellensis, Pichia kudriavzevii* and *Candida tropicalis* were combined with those from different strains of the species,

relationships because of its rapidity and resolution of both close and distant relationships (Kurtzman and Robnett 1998). Nonetheless DNA relatedness studies provided a strong foundation upon which to interpret sequence analyses.

MATERIALS AND METHODS

A total of 95 taxa were used in the analyses for this study, including three basal ascomycetes and five members of Pezizomycotina (SUPPLEMENTARY TABLE I). For the Saccharomycetales the type species of each genus listed in Kurtzman and Fell (TABLE I, 1998) was chosen (see FIG. 1 for further details). In addition ecologically important taxa that have appeared in monophyletic groups in other studies also were included in the analyses. DNA sequences at five loci (i.e. nuclear small subunit [SSU] ribosomal RNA gene [rDNA], D1/D2 region of nuclear large subunit [LSU] rDNA, elongation factor 1α gene (EF-1α), and the largest and the second largest subunits of RNA polymerase II gene [RPB1 and RPB2]) were obtained from GenBank and the AFTOL database. Based on the availability of sequence data all genes except LSU rDNA were compared with a limited number of taxa (i.e. 73 taxa for SSU rDNA, 27 taxa for RPB2, 30 taxa for EF-1α and 13 taxa for RPB1) (SUPPLEMENTARY TABLE I).

Initially DNA sequences were aligned with the multialignment program Clustal X (Thompson et al 1997) and were optimized visually. Phylogenetic analyses were conducted with parsimony, Bayesian and distance analyses with individual genes as well as concatenated datasets. Maximum parsimony analyses were performed with PAUP 4.0b10 (Swofford 2002). Heuristic tree searches were executed with the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. Bootstrap values for the most parsimonious tree were obtained from 1000 replications. Bayesian Markov chain Monte Carlo (B-MCMC) analyses were performed with MrBayes v3.0b4 (Ronquist and Huelsenbeck 2003). The analysis consisted of 1 000 000 generations of four chains sampled every 10 generations; the first 100 000 generations were discarded as burn-in, and the remaining trees were used to obtain a majority rule consensus tree for estimating the posterior probability of the branches. Neighbor joining analyses were conducted using PAUP 4.0b10 with the Kimura 2 parameter option.

RESULTS AND DISCUSSION

Phylogenetic relationships among yeasts.—The tree (FIG. 1) is based on a combined SSU rDNA and D1/D2 LSU rDNA dataset. The D1/D2 LSU rDNA region has been sequenced for almost all known yeasts as an identification tool and also to estimate phylogenetic relationships in the Saccharomycetales (Kurtzman and Robnett 1998). About 20 complete yeast genome sequences have been determined, but genome sequences for species in many basal clades, such as *Zygoascus, Trichomonascus* and *Kodamaea* (FIG. 11), are lacking. A stable tree based on phylogenetic analyses including representatives of the deep lineages is needed to provide a stable phylogenetic classification system. We will present information on 12 clades that are well supported in analyses based on rDNA sequences and discuss the support for the clades. Support for a few lineages is increased by sequences from protein-coding genes, but protein-coding genes are not available for clades 2, 3, 4, 5, 6, 9, 10, 11 and 12 (FIG. 1; SUPPLEMENTARY TABLE I).

As in many groups of fungi, the use of a morphological form concept has resulted in the circumscription of many genera and families that are not monophyletic. However by using phylogenetic analysis of a multigene dataset one clade is well supported (FIG. 1, clade 1) and corresponds with Saccharomycetaceae (TABLE I) (Kurtzman 2003, Kurtzman and Robnett 2003). In other cases groups are almost certainly polyphyletic and work is under way to redefine them and stabilize the nomenclature based on a concept of monophyly. For example the genus *Pichia* currently extends across the full phylogenetic spectrum of ascomycetous yeasts because species are characterized by budding cells that form hat-shaped or spherical ascospores, but relationships cannot be determined based on this simple, convergent phenotype. Thus separation of the new monophyletic genera on the basis of phenotype probably will not be possible. Several studies using multigene analyses soon will divide *Pichia* into about 20 genera. The *Pichia membranifaciens* clade (FIG. 1, clade 10), including species of *Issatchenkia*, will comprise the

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but the strains in each species were conspecific based on the sequences in the D1/D2 or ITS regions. Tree length = 3232; consistency index = 0.3583; homoplasy index = 0.6417; retention index = 0.5710; rescaled consistency index = 0.2046. Numbers above branches or to the left of slashes (/) indicate support above 50% in 1000 bootstrap replicates with parsimony analysis. Numbers below branches or to the right of slashes represent probability of nodes in Bayesian analysis. Clade numbers 1–12 in the tree correspond to those in the text. SSU, SSU rDNA; LSU, LSU rDNA; ^{T}, type strain; ^{NT}, neotype strain; ^{A}, authentic strain; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois.

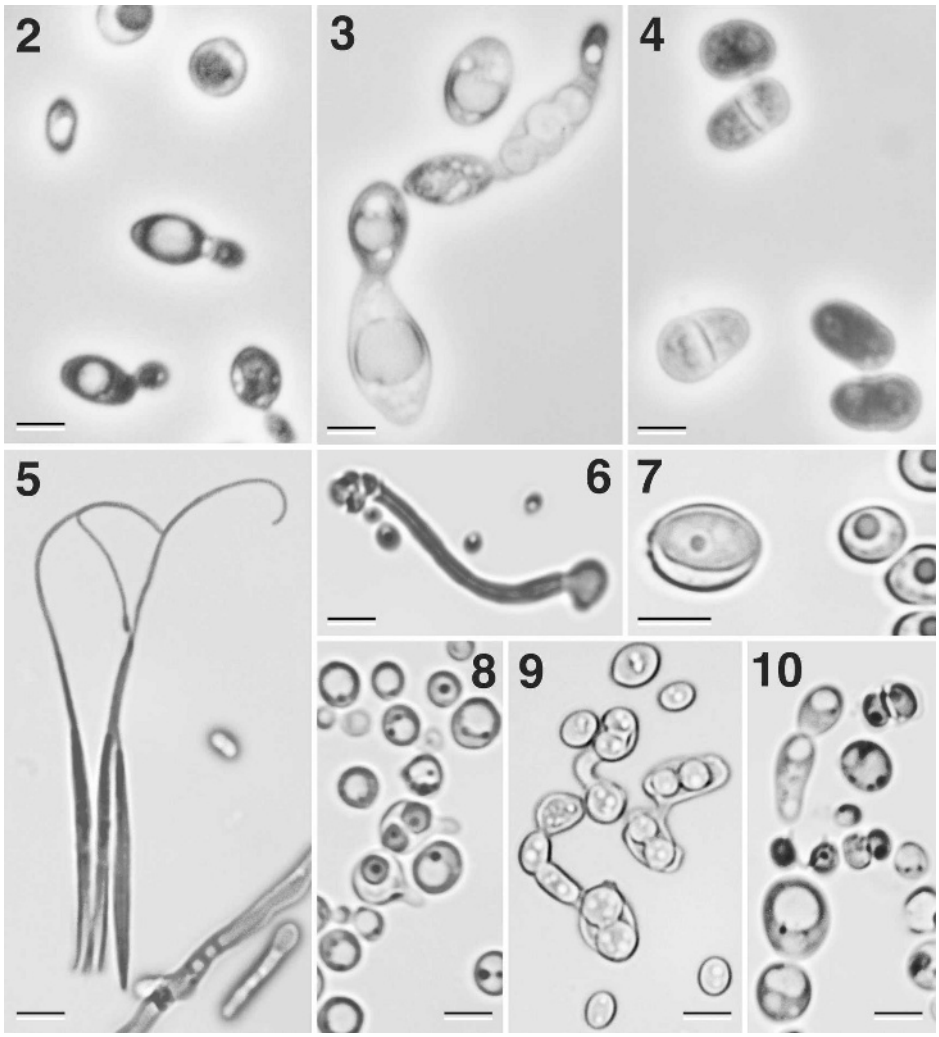
TABLE I. Currently recognized families and genera of the subphylum Saccharomycotina: class Saccharomycetes: order Saccharomycetales.[1,2] The classification provided is a revision of the current classification from ''Outline of Ascomycota—2006'' (Vol. 12, 22 Mar 2005) available at Myconet http://www.fieldmuseum.org/myconet/outline.asp#sub_sacch

- Saccharomycotina
  - Saccharomycetes
    - Saccharomycetales Kudryavtsev
      - Ascoideaceae J. Schröter
        - *Ascoidea* Brefeld & Lindau (T)
      - Cephaloascaceae L. R. Batra
        - *Cephaloascus* Hanawa (T)
      - Dipodascaceae Engler & E. Gilg
        - *Dipodascus* Lagerheim (T)
        - *Galactomyces* Redhead & Malloch (T)
        - *Geotrichum* Link:Fries (A)
      - Endomycetaceae J. Schröter
        - *Endomyces* Reess (T)
        - *Helicogonium* W. L. White (T)
        - *Myriogonium* Cain (T)
        - *Phialoascus* Redhead & Malloch (T)
      - Eremotheciaceae Kurtzman
        - *Coccidiascus* Chatton emend. Lushbaugh, Rowton & McGhee (T)
        - *Eremothecium* Borzi emend. Kurtzman (T)
      - Lipomycetaceae E. K. Novak & Zsolt
        - *Babjevia* van der Walt & M. Th. Smith (T)
        - *Dipodascopsis* Batra & Millner (T)
        - *Lipomyces* Lodder & Kreger van Rij (T)
        - *Myxozyma* van der Walt, Weijman & von Arx (A)
        - *Zygozyma* van der Walt & von Arx (T)
      - Metschnikowiaceae T. Kamienski
        - *Clavispora* Rodrigues de Miranda (T)
        - *Metschnikowia* T. Kamienski (T)
      - Pichiaceae Zender
        - *Brettanomyces* Kufferath & van Laer (A)
        - *Dekkera* van der Walt (T)
        - *Kregervanrija* Kurtzman (T)
        - *Pichia* Hansen (pro parte) (T)
        - *Saturnispora* Liu & Kurtzman (T)
      - Saccharomycetaceae G. Winter
        - *Kazachstania* Zubkova (T)
        - *Kluyveromyces* Kurtzman, Lachance, Nguyen & Prillinger (T)
        - *Lachancea* Kurtzman (T)
        - *Nakaseomyces* Kurtzman (T)
        - *Naumovia* Kurtzman (T)
        - *Saccharomyces* Mayen ex Reess (T)
        - *Tetrapisispora* Ueda-Nishimura & Mikata (T)
        - *Torulaspora* Lindner (T)
        - *Vanderwaltozyma* Kurtzman (T)
        - *Zygosaccharomyces* Barker (T)
        - *Zygotorulaspora* Kurtzman (T)
      - Saccharomycodaceae Kudryavtsev
        - *Hanseniaspora* Zikes (T)
        - *Kloeckera* Janke (A)
        - *Saccharomycodes* Hansen (T)
      - Saccharomycopsidaceae von Arx & van der Walt
        - *Saccharomycopsis* Schiönning (T)
      - Saccharomycetales incertae sedis
        - *Aciculoconidium* King & Jong (A)
        - *Ambrosiozyma* van der Walt (T)
        - *Ascobotryozyma* J. Kerrigan, M. Th. Smith & J. D. Rogers (T)
        - *Blastobotrys* von Klopotek (A)
        - *Botryozyma* Shann & M. Th. Smith (A)
        - *Candida* Berkhout (A)
        - *Citeromyces* Santa María (T)
        - *Cyniclomyces* van der Walt & Scott (T)
        - *Debaryomyces* Lodder & Kreger-van Rij (T)
        - *Hyphopichia* von Arx & van der Walt (T)
        - *Kodamaea* Y. Yamada, T. Suzuki, Matsuda & Mikata emend. Rosa, Lachance, Starmer, Barker, Bowles & Schlag-Edler (T)
        - *Komagataella* Y. Yamada, Matsuda, Maeda & Mikata (T)
        - *Kuraishia* Y. Yamada, Maeda & Mikata (T)
        - *Lodderomyces* van der Walt (T)
        - *Macrorhabdus* Tomaszewski, Logan, Snowden, Kurtzman & Phalen (A)
        - *Nadsonia* Sydow (T)
        - *Nakazawaea* Y. Yamada, Maeda & Mikata (T)
        - *Ogataea* Y. Yamada, Maeda & Mikata (T)
        - *Pachysolen* Boidin & Adzet (T)
        - *Phaffomyces* Y. Yamada, Higashi, S. Ando & Mikata (T)
        - *Schizoblastosporion* Ciferri (A)
        - *Sporopachydermia* Rodrigues de Miranda (T)
        - *Starmera* Y. Yamada, Higashi, S. Ando & Mikata (T)
        - *Starmerella* Rosa & Lachance (T)
        - *Sugiyamaella* Kurtzman & Robnett (T)
        - *Trichomonascus* Jackson (T)
        - *Trigonopsis* Schachner (A)
        - *Wickerhamia* Soneda (T)
        - *Wickerhamiella* van der Walt (T)
        - *Yamadazyma* Billon-Grand emend. M. Suzuki, Prasad & Kurtzman (T)
        - *Yarrowia* van der Walt & von Arx (T)
        - *Zygoascus* M. Th. Smith (T)

[1] (A) = anamorphic genus, (T) = teleomorphic genus.
[2] Phylogenetic relationships of many yeast genera are unclear and these are placed in Saccharomycetales incertae sedis until family relationships become known.

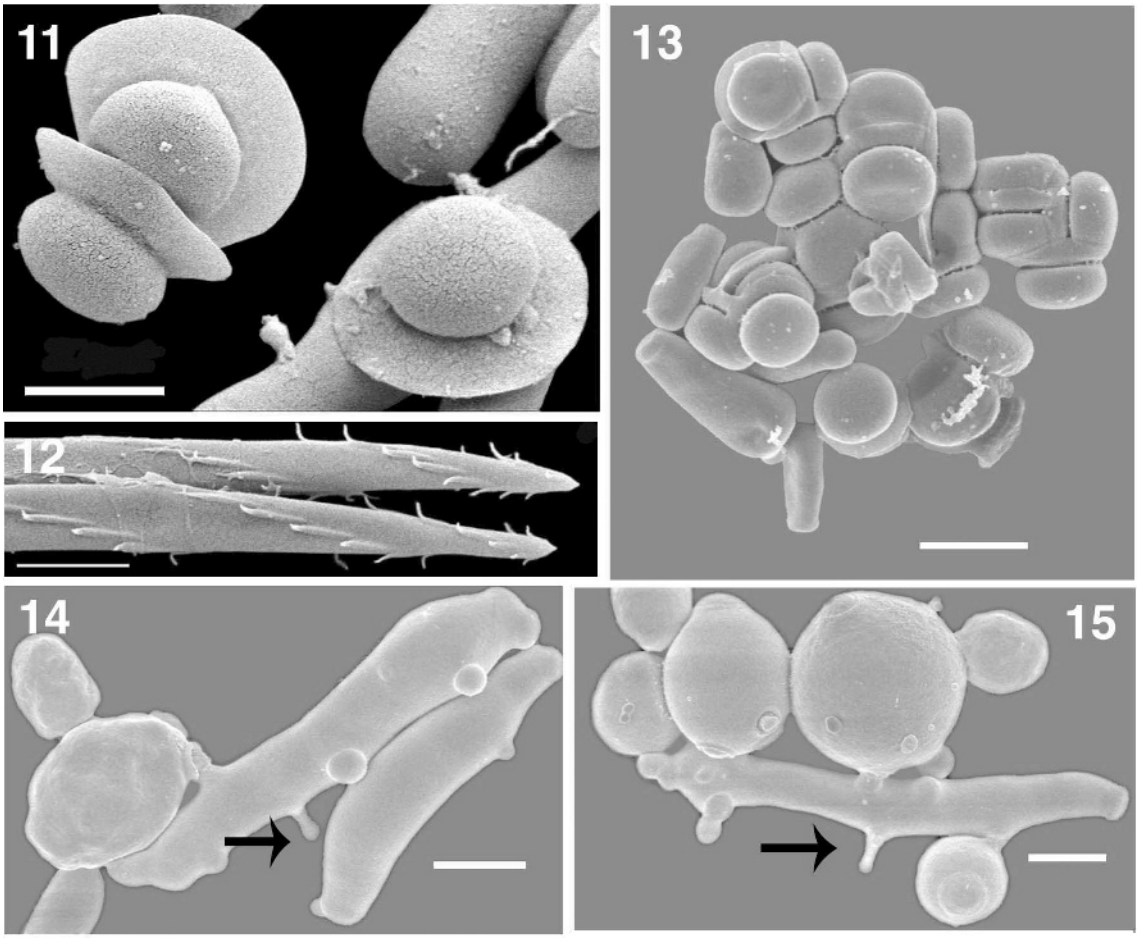
residual, much reduced *Pichia*. Other groups to be extracted from *Pichia* include the methanol-assimilating species (FIG. 1, clade 2), the *Pichia anomala* clade (FIG. 1, clade 3), the xylose-fermenting species in the *Pichia stipitis* clade (FIG. 1, clade 5) and numerous other smaller clades.

Another continuing source of disparity is the use of the genus *Candida* as a dumping ground for most



FIGS. 2–10. Ascomycete yeasts in pure culture. 2. *Saccharomyces cerevisiae*. Cells dividing by multilateral budding. 3. *Saccharomycodes ludwigii*. Cell division by bipolar budding. Note the ascus with four spherical ascospores above the dividing cell. 4. *Schizosaccharomyces pombe*. Cell division by fission. Once divided, the newly formed cells often will be morphologically indistinguishable from cells formed by budding. 5. *Eremothecium* (*Nematospora*) *coryli*. Free, needle-shaped ascospores with whip-like tails of extended wall material. Members of this group are some of the few yeasts that are plant pathogens. 6. *Pachysolen tannophilus*. A single ascus forms on the tip of an elongated refractile tube. The ascus wall becomes deliquescent and releases four hat-shaped ascospores. *P. tannophilus* was the first yeast discovered to ferment the pentose sugar D-xylose, a major component of hemicellulose from biomass. 7. *Lodderomyces elongisporus*. Persistent ascus with a single ellipsoidal ascospore. This is the only species of the clade, which includes *Candida albicans* and *C. tropicalis*, that is known to form ascospores. 8. *Torulaspora delbrueckii*. Asci with 1–2 spherical ascospores. Asci often form an elongated extension that may function as a bud conjugant. 9. *Zygosaccharomyces bailii*. Asci with spherical ascospores. Often there are two ascospores per conjugant giving rise to the term ''dumbbell-shaped'' asci. This species is one of the most aggressive food spoilage yeasts known. 10. *Pichia bispora*. Ascospores are hat-shaped and released from the asci at maturity. Hat-shaped ascospores are produced by species in a variety of different genera. FIGS. 2–4, phase contrast. FIGS. 5–10, bright field. Bars = 5 μm.

budding yeasts that do not form ascospores. Members of the current genus are found in essentially all teleomorphic clades. Division of *Candida* into a large number of monophyletic genera based on phylogenetic analysis has little appeal to taxonomists because most will be unrecognized from phenotype. Perhaps the best descriptor for these clades is to note their association with phylogenetically defined ascosporic genera, some of which also may be phenotypically inseparable. Although the yeast identification of the near future will rest on phylogenetic analysis of gene sequences rather than from phenotypic characters, this is not so different from some previous circumscriptions of taxa based heavily on metabolic data.



FIGS. 11–15. Scanning electron micrographs. 11. Ascospores of *Kodamaea anthophila*. Reproduced by permission from the Society for General Microbiology (Rosa et al 1999). 12. End of ascospores of *Metschnikowia borealis* released by treatment of the ascus with Mureinase. Reproduced by permission (Lachance et al 1998). 13. Agglutinated ascospores of *Saccharomycopsis synnaedendrus*. B. Schlag-Edler and M.-A. Lachance. 14, 15. Elongated predaceous cells of *Arthroascus schoenii* penetrating ovoid cells of *Saccharomyces cerevisiae* by means of narrow infection pegs (I). B. Schlag-Edler, A. Pupovac-Velikonja and M.-A. Lachance. Bars = 2 μm

Major yeast clades.—Although the basal branches of the ascomycete yeast tree are not yet well resolved due to lack of sampling of certain genes of some major groups, a number of clades however are well supported in most analyses. In this section we discuss members of the 12 clades and their habitats and substrates. It should be noted that because the rate of species discovery is so high many of the yeasts are poorly known and have been isolated only once or twice. The history of *Candida tanzawaensis* (see below) provides an example of this kind of problem. This species was isolated from a moss collected in the Tanzawa Mountains of Japan 22 y before it was described (Nakase et al 1988) and has never been recollected. It now is clear however that it is a member of a moderate-size clade of species that are common in associations with insects (see clade 6, below).

Saccharomycetales. Analyses of DNA usually provide strong support for the traditional view of Saccharomycetales as a monophyletic group (FIG. 1). These include trees based on (i) RPB2 with SSU and LSU (D1/D2 region) rDNA in a reduced dataset, (ii) SSU and LSU (D1/D2 region) rDNA and EF-1α; (iii) SSU and LSU (D1/D2 region) rDNA, EF-1α, and RPB2; (iv) SSU and LSU (D1/D2 region) rDNA, EF-1α, RPB1 and RPB2; and (v) RPB2 only. Support was somewhat lower for trees based on single gene datasets (e.g. SSU rDNA [79%] only and D1/D2 region of LSU rDNA [87%]) only.

Clade 1. For many the quintessential yeast or ζυμο is *Saccharomyces cerevisiae* (FIGS. 2, 3), a highly specialized, ethanol resistant species that contains an unusually high number of chromosomes. This clade contains most of the yeasts with known complete genome sequences, and it should be noted that the correct name for *Kluyveromyces waltii* is *Lachancea*

waltii, a member of a segregate genus from *Kluyveromyces*. Some members of clade 1, including *Saccharomyces cerevisiae*, underwent a genome duplication in the past (see *Genomics Contributions to Phylogenetics*, below). Of note the natural habitat of these well known model organisms remains to be established in spite of claims to the contrary (Pennisi 2005). *Saccharomyces* and several related genera once labeled ''*Saccharomyces sensu lato*'' are difficult to define ecologically. Most species are found only sporadically in nature, which might mean that we have yet to determine their principal habitats. One exception might be species of the basal genus *Hanseniaspora*, a predictable component of naturally fermenting fruit and other sugar-rich materials, including certain nectars and sap fluxes. These species use few carbon compounds but usually vigorously ferment the β-glucoside cellobiose (as well as glucose). A clear synapomorphy cannot be identified for the entire clade. The clade was supported by bootstrap analysis (80%) and posterior probability (100%) in analyses of SSU and LSU (D1/D2 region) rDNA (FIG. 1) but not when the RPB2 gene sequence was included with SSU and LSU rDNA in a reduced dataset. Analyses of other reduced datasets including SSU and LSU rDNA and EF-1α; SSU and LSU rDNA, EF-1α and RPB2 genes; SSU and LSU rDNA, EF-1α, RPB1 and RPB2 genes; and RPB2 gene only (analyses not shown) also were well supported.

Clade 2. This clade of ascomycete yeasts contains many species endowed with the interesting property of methanol assimilation (FIG. 1). These include *Candida boidinii, Komagatella (Pichia) pastoris, Ogataea (Hansenula) polymorpha* and related anamorphs. These species are intimately associated with the decaying wood of trees or the necrotic soft tissues of succulent plants and may serve as agents of detoxification for invertebrates that colonize these materials. *Komagatella pastoris* and *O. polymorpha* are widely used in biotechnology for expression of recombinant proteins.

Clade 3. This clade (FIG. 1), typified by *Pichia anomala*, contains many species that frequently are isolated from trees that suffer insect damage. Many of the species formerly were assigned to the genus *Hansenula* on the basis of nitrate use. The latter characteristic however cannot serve as a reliable synapomorphy.

Clade 4. The *Saccharomycopsis* clade (FIG. 1) groups a wide variety of morphologies and physiologies. Some species are purely cellular (*Arthroascus* spp.) and others are nearly exclusively hyphal (*Saccharomycopsis selenospora*). Some produce powerful extracellular hydrolases. All share a deficiency in sulfate uptake, which could afford them an accrued resistance to toxic ions that share the same transport pathway (Lachance et al 2000). Possibly related to these unusual properties is the widespread ability of the species to penetrate and kill other fungi by means of infection pegs (FIGS. 14, 15). Taken together these last two properties constitute a clear synapomorphy for the clade.

Clade 5. Xylose fermentation is a relatively rare trait among the yeasts that have been tested. The *Pichia stipitis* clade (FIG. 1) is of interest because many of these taxa have the ability to ferment xylose (Jeffries and Kurtzman 1994). Members of the clade have been found in wood, often in association with wood-ingesting beetles (Nguyen et al 2006, Suh et al 2003). It should be noted that other yeasts (e.g. *Pachysolen tannophilus*) have similar physiological profiles including the ability to ferment xylose although they are not members of the *P. stipitis* clade.

Clade 6. The relatively obscure *Candida tanzawaensis* clade (FIG. 1) has grown from a single described species (Nakase et al 1988) to a total of 23 (Kurtzman 2001, Suh et al 2004). The new taxa were isolated from a variety of mycophagous beetles, notably in the family Erotylidae, and other insects.

Clade 7. The notorious human commensal, *Candida albicans* combines extracellular lipase activity, the ability to form invasive hyphae and the ability to grow at 37 C, which may have earmarked this species to be the bane of many a human. Other members of the clade (FIG. 1) share these properties. This includes *C. tropicalis* isolated from clinical samples, soil, fodder, fermentation vats and rotten pineapples, and *Lodderomyces elongisporus* (FIG. 7) recovered from fingernails, baby cream and orange juice. Support for the clade was 76% with bootstrap analysis and 100% posterior probability in analyses of SSU and LSU (D1/D2 region) rDNA (FIG. 1). Bootstrap support with reduced datasets (12–27 taxa) including protein-coding genes was increased with SSU and LSU (D1/D2 region) rDNA and RPB2 gene; SSU and LSU rDNA, EF-1α and RPB2 genes; SSU and LSU rDNA, EF-1α, RPB1 and RPB2 genes. Support for reduced datasets including SSU and LSU rDNA, and EF-1α genes; and RPB2 gene only were essentially similar to SSU and LSU rDNA (not shown).

Clade 8. The large and diverse clade (FIG. 1) containing the genera *Metschnikowia* and *Clavispora* exhibits a remarkably uniform nutritional profile shared with certain less closely related species such as *Candida sake, Candida oleophila* and a few others. These species often are found in association with herbivorous invertebrates. The carbon compounds favored by these yeasts include plant sugars such as sucrose, maltose and other α-glucosides and β-glucosides, as well as sorbose, mannitol, glucitol, and *N*-acetyl-D-glucosamine. Lipolytic activity and the utiliza-

tion of alkanes are not unusual in the metabolic profile of the clade members. This clade contains a growing group of species associated with nitidulid beetles (Lachance et al 2000), which form unusually large ascospores ornamented with a helical array of barbs (FIG. 12). The well supported clade (FIG. 1) also obtained strong support with these reduced datasets: SSU and LSU (D1/D2 region) rDNA and RPB2 gene; SSU and LSU rDNA, EF-1α and RPB2 genes; SSU and LSU rDNA and EF-1α gene; and RPB2 gene only (not shown). No RPB1 data were available for inclusion.

Clade 9. The rapidly expanding *Starmerella* clade (FIG. 1) (Rosa et al 2003) consists of highly specialized, generally small yeasts that exhibit a clear association with bees of all sorts. Smaller somatic cells are characteristic of the *Wickerhamiella* clade with members that are isolated frequently from floricolous drosophilids. The remaining taxa shown in clade 9 represent a coherent assemblage of usually highly filamentous species (i.e. *Dipodascus* and *Galactomyces* spp.), in which unicellular growth is arthric and not blastic. The placement of these morphologically distinct members together within clade 9 probably is the result of long-branch attraction.

Clade 10. Pichia membranifaciens is widely known as an agent of spoilage of pickled vegetables. This and related species frequently are encountered in a number of substrates used by drosophilids as feeding and breeding sites. Many species in this clade (FIG. 1) are avid film formers and lie at an extreme in the spectrum of nutritional specialization, exhibiting a predilection for ethanol and simple organic acids as carbon sources and having poor fermentative power. Of interest, *Debaryomyces hansenii*, although not a member of the clade, is similar in that it also may be found in pickles and certain strains form films. It is a poor fermenter and can be found in a wide array of harsh natural substrates, including food in which salt is added for preservation, such as meats and cheeses in addition to pickles. There are many such guilds among yeasts that are examples of convergent evolution for life in specialized habitats.

Clade 11. This well supported clade (FIG. 1) was recognized relatively early as a monophyletic group coinciding with the family Lipomycetaceae and including *Dipodascopsis* and *Lipomyces* (van der Walt 1992). Members of the family are ecologically extremely diverse. The placement of the group in relation to other clades appears to be basal, but it is not well supported by bootstrap analysis. Many of the species use imidazole as a nitrogen source, although this trait is not exclusive to members of this clade. These yeasts frequently are isolated in soil, which may be favored by their production of an abundant slime capsule.

Clade 12. The species in this clade (FIG. 1) possess a wide variety of unusual metabolic and morphological traits, including assimilation of adenine as a carbon source and the formation of denticulate conidiogenous cells. This small group of yeasts includes species of *Trichomonascus* and *Blastobotrys* (Kurtzman and Robnett 2007) all of which exhibit a highly filamentous growth habit.

Contributions of genomics to phylogenetics.—A major effort of yeast systematists has been directed toward finding and describing new species and identifying new clades, and the discovery of entirely new lineages has had major effects on tree topology. At the same time species and clade discovery have been occurring, great advances also have been made in the field of comparative genomics of yeasts (Dujon et al 2004), principally because of the sequencing of about 20 yeast genomes. The comparison of genome sequences provides evidence of a past duplication of the entire genome in certain members of the Saccharomycetales (Wolfe and Shields 1997, Wong et al 2002, Langkjaer et al 2003).

The genome duplication of *Saccharomyces cerevisiae* is evident from comparisons with the complete genomes of other yeasts, particularly *Lachancea waltii,* and led to the conclusion that the duplication occurred in the *S. cerevisiae* lineage after the divergence of the *L. waltii* branch an estimated 10^8 y ago (Seoighe and Wolfe 1998). The haploid chromosome number in *S. cerevisiae* (16) is double that of *L. waltii* (8) and some paralogous genes are present in *S. cerevisiae.* The evidence also indicates that the duplication was unstable, and the duplicated genes in *S. cerevisiae* have largely been lost with only 12% of the genome retaining paired paralogs. In fact the comparison of genes in the duplicated versus nonduplicated genomes is not too disparate with about 5700 remaining genes in *S. cerevisiae* compared with 5200 in *L. waltii.* The pattern of losses shows small deletions without whole chromosome losses or deletions of large segments. Changes apparently were accelerated in only one of the paralogs from accumulated mutations, rearrangements and losses acted on by differences in selection pressure with the other paralog of a gene pair constrained (Kellis et al 2004, Langkjaer et al 2003). Comparison of *S. cerevisiae* with the *Eremothecium* (*Ashbya*) *gossypii* genome also has been used to support the *S. cerevisiae* genome duplication and subsequent loss of large numbers of genes (Dietrich et al 2004).

The duplication of an entire genome in yeast evolution calls attention to the possibility of misinterpretation of relationships within certain duplicated yeast lineages. For example questions concern-

ing paralogous genes might arise but the vast amount of data offers new unprecedented opportunities for phylogenetic analysis and the testing of methods among these yeasts. An analysis by Rokas and his colleagues (2003) used a dataset of 106 orthologous genes, analyzed singly and as concatenated databases to resolve the relationships of *S. cerevisiae* and its close relatives (*S. cerevisiae, S. paradoxus, S. mikatae, S. bayanus, S. kudriavzevii, N. castellii* and *L. kluyveri*) and the outgroup taxon (*C. albicans*). They obtained a single well resolved phylogenetic tree based on the 106-gene database. However these analyses, made possible by a small number of yeast species for which genomes have been sequenced, used much more data than usually are available for phylogenetic analysis. The 106-gene tree resulted in the same tree with similar support using as few as 20 unlinked concatenated genes; smaller datasets often supported incorrect relationships. The trees based on one or a few genes were not reliable in determining correct relationships or refuting proposed relationships, no matter how well supported by statistical measures. Although Rokas et al (2003) investigated factors that might cause phylogenetic error, no predictors of genes causing incongruence were identified. Other factors they considered to be important in analyses include taxon sampling, hybridization, variable rates of nucleotide site evolution and lineage sorting. They suggested that large datasets are especially important in resolving questions of speciation over shorter intervals of time.

Rapid yeast species identification using short DNA sequences.—Yeast identification has rapidly advanced from the requisite collection of tedious, labor-intensive and often equivocal physiological data to rapid identification with DNA sequences. Sequence-based approaches to yeast biodiversity have resulted in a twofold increase in the number of described species over the past decade, and a 100-fold increase is predicted in coming decades (Boekhout 2005). At a time when some taxonomists are discussing the merits of ''bar coding,'' the means for rapid yeast identification already have been accomplished. This is due largely to the work of Kurtzman and Robnett (1998), who sequenced the D1/D2 region of the LSU rDNA for more than 500 species from the ARS Culture Collection (NRRL). Most workers have followed their example and sequenced the corresponding region of all newly discovered yeast species. Yeast characterization by the D1/D2 region of 400–650 bp had begun before PCR because of the high copy number of the ribosomal repeat, but it also distinguished all known isolates, often at the established species level. It has been shown more recently that the region sometimes underestimates species (*Saccharomyces cerevisiae* and close relatives, Casaregola et al 2001), but nevertheless the dense database is extremely useful and lets a person distinguish yeasts rapidly at some level near species. It has been difficult to establish firm demarcation lines between some yeast species because often little is known about the natural variation among populations, breeding system and several species concepts that are in use. Two methods, BLAST searches and phylogenetic analysis, are used in the identification process by searching public databases for the closest known sequences to the unidentified yeast. It theoretically is possible that the two methods might give somewhat different answers, although this does not occur commonly. The Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (see below), database aids BLAST searches by providing for the pairwise identification of LSU and SSU rDNA, ITS and miscellaneous sequences.

Resources for the study of yeasts.—For many years the global community of yeast workers has come together to provide materials for the identification of both ascomycete and basidiomycete yeasts. Currently the 4th edition (Kurtzman and Fell 1998) is a direct descendant of a tradition begun by J. Lodder and N.J.W. Kreger-van Rij (1952), who wrote the first edition of *The Yeasts, A Taxonomic Study*. The current publication was the basis for the online database (see immediately below) that is updated regularly. A second publication (Barnett et al 2000) offers somewhat similar information about yeasts. The Centraalbureau voor Schimmelcultures (CBS) database http://www.cbs.knaw.nl/databases/index.htm is an invaluable one-stop site for both ascomycete and basidiomycete yeast workers to find descriptions, identification tools for morphological, metabolic and DNA characters, methods, nomenclature, photographs and literature. Myconet http://www.fieldmuseum.org/myconet/outline.asp#sub_sacch periodically has updated classifications of all ascomycetes, including Saccharomycetales.

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SUPPLEMENTARY TABLE I. Species and GenBank numbers of genes used in the analyses. Some AFTOL Project sequences are not yet submitted to GenBank and these sequences are listed by AFTOL numbers.

Species	SSU rDNA	LSU rDNA	EF−1a	RPB1	RPB2
Outgroup					
Aspergillus fumigatus	AB008401	AY660917	XM745295	XM747744	AY485610
Chromocleista malachitea	D88323	AB000621	—	—	—
Hamigera avellanea	D14406	AF454075	—	—	—
Hypocrea lutea	D14407	U00739	—	—	—
Neurospora crassa	AY046271	AY681158	XM329192	—	AF107789
Protomyces inouyei	AY548295	AY548294	—	—	—
Schizosaccharomyces pombe	X54866	Z19136	D82571	—	D13337
Taphrina deformans	AJ495826	AF492038	—	—	—
Saccharomycetes					
Aciculoconidium aculeatum	—	U40087	—	—	—
Ambrosiozyma monospora	—	U40106	—	—	—
Ascobotryozyma americana	—	AF538878	—	—	—
Ascoidea rubescens	—	U76195	—	—	—
Babjevia anomala	—	U76202	—	—	—
Blastobotrys nivea	—	U40110	—	—	—
Blastobotrys parvus	—	U40096	—	—	—
Blastobotrys terrestris	AB000663	U40103	—	—	—
Botryozyma nematodophila	—	U40105	—	—	—
Candida albicans	M60302	U45776	XM705052	XM714321	XM713346
Candida anneliseae	AY242149	AY242258	—	—	—
Candida boidinii	AB054551	U70242	—	—	—
Candida caryicola	AY488125	AY13717	—	—	—
Candida emberorum	AY242168	AY242277	—	—	—
Candida ergatensis	AB013524	U45746	—	—	—
Candida glabrata	AY198398	U44808	CR380957	CR380955	CR380958
Candida oleophila	AB013534	AF178047	—	—	—
Candida ovalis	AB054552	U70248	—	—	—
Candida picachoensis	AY452053	AY452039	—	—	—
Candida sake	AB013529	U45728	—	—	—
Candida shehatae var. insectosa	AB013583	U45773	—	—	—
Candida silvicultrix	AB054561	U69879	—	—	—
Candida sonorensis	AB054542	U70185	—	—	—
Candida tanzawaensis	AY227713	U44811	—	—	—
Candida tropicalis	M55527	U45749	CR382129	AAFN01000119	AY485615
Cephaloascus fragrans	—	U40091	—	—	—
Citeromyces matritensis	AB034908	U75959	—	—	—
Clavispora lusitaniae	M55526	AJ508071	AFTOL1318	AFTOL1318	AFTOL1318
Cyniclomyces guttulatus	—	U76196	—	—	—
Debaryomyces hansenii var. *hansenii*	AB013590	U45808	CR382138	CR382133	CR382139

SUPPLEMENTARY TABLE I. Continued

Species	SSU rDNA	LSU rDNA	EF−1a	RPB1	RPB2
Dekkera bruxellensis	X58052	U45738	—	—	—
Dipodascopsis uninucleata	—	U40137	—	—	—
Dipodascus albidus	X69840	U40081	—	—	—
Eremothecium gossypii	AE016820	AE016820	AE016817	AE016817	AE016819
Galactomyces geotrichum	AB000647	U40118	—	—	—
Hanseniaspora valbyensis	AY046254	U73596	AF402067	—	—
Kazachstania viticola	AY046234	AF398482	AF402026	—	AF527895
Kluyveromyces lactis	CR382124	CR382124	CR382122	CR382126	CR382122
Kluyveromyces marxianus	X89523	U94924	AF402085	—	—
Kodamaea ohmeri	AY520231	AY242300	—	—	—
Kuraishia capsulata	—	AY937231	—	—	—
Lachancea thermotolerans	X89526	U69581	AF402077	—	—
Lachancea waltii	X89527	U69582	AF402078	AADM01000294	AADM01000162
Lipomyces lipofer	X69848	U76533	—	—	—
Lodderomyces elongisporus	X78600	U45763	—	—	AY653539
Macrorhabdus orinthogaster	—	AF350243	—	—	—
Metschnikowia bicuspidata	AB023466	U44822	AFTOL1326	—	AFTOL1326
Metschnikowia pulcherrima	AB023473	U45736	—	—	—
Myxozyma melibiosi	—	U76344	—	—	—
Nadsonia fulvescens	—	U94942	—	—	—
Nakaseomyces delphensis	X83823	U69576	AF402030	—	AF527899
Naumovia castellii	Z75577	AY545582	AF402045	AACF01000092	AACF01000010
Naumovia dairenensis	Z75579	AY048168	AF402046	—	AF527908
Pachysolen tannophilus	AF132030	U76346	—	—	—
Phaffomyces opuntiae	AB017894	U76203	AFTOL1324	—	AFTOL1324
Pichia anomala	AB126679	U74592	—	—	—
Pichia guilliermondii	AB013587	U45709	AAFM01000070	AAFM01000028	AY485613
Pichia kudriavzevii	AB053239	U76347	—	—	AF107788
Pichia membranafaciens	X58055	U75725	—	—	AY497599
Pichia methanolica	AB018181	U75523	—	—	—
Pichia segobiensis	AB054288	U45742	—	—	—
Pichia stipitis	AB054280	U45741	—	—	—
Pichia sydowiorum	AJ508271	U74594	—	—	—
Saccharomyces cerevisiae	Z75578	U44806	X01638	X96876	M15693
Saccharomycodes ludwigii	X69843	U73601	AF402074	—	—
Saccharomycopsis capsularis	X69847	U40082	—	—	—
Saccharomycopsis fermentans	—	U73600	—	—	—
Saccharomycopsis fibuligera	X69841	U40088	—	—	—
Saccharomycopsis selenospora	—	U40099	—	—	—
Saturnispora dispora	AB053251	U94937	—	—	—

SUPPLEMENTARY TABLE I. Continued

Species	SSU rDNA	LSU rDNA	EF−1a	RPB1	RPB2
Schizoblastosporion starkeyihenricii	—	U40089	—	—	—
Sporopachydermia lactativora	—	U45851	—	—	—
Starmera amethionina	AB017897	U75424	—	—	—
Starmerella bombicola	AB013558	U45705	—	—	—
Tetrapisispora phaffii	AY046245	AY048166	AF402041	—	—
Torulaspora delbrueckii	X98120	U72156	AF402052	—	—
Trichomonascus ciferrii	AB000662	U40138	—	—	AY497613
Trigonopsis variabilis	—	U45827	—	—	—
Vanderwaltozyma polyspora	X83825	AY048169	AF402047	—	AF527909
Wickerhamia fluorescens	—	U45719	—	—	—
Wickerhamiella domercqiae	AB018157	U45847	—	—	—
Williopsis saturnus	—	U75958	—	—	—
Yarrowia lipolytica	AB018158	U40080	CR382129	CR382129	CR382130
Zygoascus hellenicus	AJ508278	U40125	—	—	—
Zygosaccharomyces rouxii	X90758	U72163	AF402054	—	—
Zygotorulaspora mrakii	AY046239	U72159	AF402033	—	AF527901
Zygozyma oligophaga	AFTOL1323	U45850	AFTOL1323	—	AFTOL1323