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review

Yeast Systematics – from Phenotype to Genotype

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Summary

Yeast systematics is in transition from reliance on phenotypic characters to application of molecular comparisons. Measurements of molecular divergence represent a quantitative means for assessing genetic relationships among species, and these molecular comparisons have revealed that the standard phenotypic tests used in yeast taxonomy are often poor indicators of relatedness. The impact of molecular comparisons on yeast systematics is discussed and examples of application of these data for rapid species identification are given.

Keywords: yeast taxonomy, molecular systematics, rRNA/rDNA, gene sequences

Introduction

Methods for yeast identification often include characterization of cellular morphology from microscopic examination and the determination of diagnostic responses on a variety of fermentation and assimilation tests. Yeast taxonomists worldwide have relied on these standard tests for species identification as well as for systematic placement of taxa, and many of the tests are widely used in kit form in clinical laboratories. With the advent of molecular biology, the opportunity to determine the reliability of these standard methods for resolution of species and genera has come. This review will examine some of the molecular methods now being adopted for identification and systematic placement of yeasts and will compare these results with those from traditional phenotypic tests.

Species Identification

Molecular methods commonly used for systematics and species identification are listed in Table 1. Nuclear DNA (nDNA) reassociation is the first of the quantitative molecular techniques used for assessing relatedness among taxa, and the different means for measuring reassociation have been discussed by Kurtzman *(1)*. Results from studies of nDNA relatedness have had a profound impact on yeast taxonomy. Presence or absence of pseudohyphae and true hyphae were often used to define species and genera, but results from nDNA reassociation showed that strains of a single species could differentially exhibit these characters. This is also true for the assimilation of many sugars and for nitrate (Table 2). Indeed, the nDNA comparisons of Vaughan-Martini and Kurtzman *(2)* showed that 15 commonly accepted phenotypic species of *Saccharomyces* were synonyms of *Sacch. cerevisiae*.

Table 1. Molecular comparisons used for species identification

Electrophoretic mobilities of isozymes *(31)*
Nuclear DNA reassociation *(1)*
Restriction fragment length polymorphisms (RFLPs) *(32)*
Amplified fragment length polymorphisms (AFLPs) *(33)*
Randomly amplified polymorphic DNA (RAPD) *(34)*
Gene length polymorphisms *(10)*
Gene sequencing *(6,13)*

The extent of genetic resolution afforded by nDNA reassociation appears to extend no further than the level of closely related species, as assessed from genetic crosses (Table 3). Other techniques for recognizing species,

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Table 2. Nuclear DNA relatedness (DNA rel.) between yeast species differing in traditional taxonomic characteristics

Species[1]	Characteristic	+ or –	DNA rel. %	Ref.
Candida slooffii	Pseudohyphae	+	80	*(35)*
Torulopsis pintolopesii		–		
Hansenula wingei	True Hyphae	+	78	*(36)*
H. canadensis		–		
Debaryomyces formicarius	Glucose ferm.	+	96	*(37)*
D. vanriji		–		
Schwanniomyces castellii	Lactose assim.	+	97	*(37)*
S. occidentalis		–		
Hansenula minuta	Nitrate assim.	+	75	*(38)*
Pichia lindneri		–		
Sterigmatomyces halophilus	Nitrate assim.	+	100	*(39)*
S. indicus		–		

[1] The names used are those appearing in the original publications

Table 3. Species resolution from nDNA relatedness *(1)*

Percent nDNA relatedness	Taxonomic designation	Expected fertility
70–100	Same species	High
40–70	Variety or sister species	Low or none
0–40	Different species	None

such as electrophoretic mobilities of isozymes, RFLPs, AFLPs, RAPDs and gene length polymorphisms, provide essentially the same degree of resolution as nDNA reassociation. Of these, RAPD patterns are sometimes difficult to reproduce between laboratories.

Gene sequence comparisons offer the opportunity for species detection as well as for genetic resolution beyond the level of close species. The question has been which genes to sequence. Some of the genes commonly compared are listed in Table 4. Early work focused on 5S ribosomal RNA (rRNA) because it appeared to have a common origin for all species compared and it was sufficiently small (ca. 120 nucleotides) to be easily sequenced by the methods of the time. This molecule does not allow resolution of close relationships, but it does show that the basidiomycetes separate into large groups that correlate with septal pore structure and that the ascomycetes are comprised of three major lineages, *i.e.*, fission yeasts, budding yeasts and the filamentous fungi or euascomycetes *(3,4)*. Because of their greater informational content, as well as the development of improved sequencing techniques, interest soon shifted to small (18S) and large (26S) subunit rRNA/rDNA. Phylogenetic analysis of these gene sequences has shown the same overall relationships first predicted from 5S sequences, but in far greater detail.

Table 4. Genes commonly sequenced for species identification and for phylogenetics[1]

Nuclear small subunit (18S) rDNA
Nuclear large subunit (26S) rDNA
5S rDNA
ITS1/5.8/ITS2
Intergenic spacer (IGS)
Mitochondrial small subunit rDNA
Cytochrome oxidase II
Transfer elongation factor
β-tubulin
Calmodulin

[1] See *(6,10,40)* and references therein

What of species level resolution? Peterson and Kurtzman *(5)* examined the variable 5' end of 26S rRNA (domain D2) and showed that it was sufficiently divergent to differentiate closely related species of heterothallic ascomycetous yeasts. Later, on the basis of several hundred additional determinations in which strain pairs were compared from both nDNA reassociation and divergence in the 600-nucleotide D1/D2 domains of 26S rDNA, it was shown that conspecific strains usually have 0–3 nucleotide differences, whereas 6 or more nucleotide differences signal that strains are not members of the same species (Table 5). Despite the impressive predictiveness demonstrated from these determinations, a few exceptions have been found. For example, *Pichia toletana* and *P. xylosa* show 29% nDNA relatedness and 1 D1/D2 nucleotide difference (Table 6). Similarly, *Candida shehatae* and its variety *lignosa* exhibit 46% nDNA relatedness but no D1/D2 nucleotide divergence. Consequently, a difference of 6 or more nucleotides in domain D1/D2 strongly indicates strains to be different species, but strains with 0–5 nucleotide differences may not always be conspecific, showing that predictions of close relatedness should be verified by nDNA reassociation or other means. From D1/D2 nucleotide comparisons, Kurtzman and Robnett *(6–8)* predicted that 55 species from all currently accepted ascomycetous yeasts are likely to represent synonyms of earlier described species. The work of Fell *et al. (9)* with basidiomycetous yeasts suggests that closely related species may be worsely resolved from divergence in domain D1/D2 than it is seen for ascomycetous yeasts.

Table 5. Correlation of nDNA relatedness and LSU D1/D2 divergence among ascomycetous yeasts[1]

Strain pairs	nDNA rel. %	D1/D2 nucleotide differences
70 conspecific pairs	70–100	0–3
Ca. 200 unrelated pairs	0–20	6~250

[1] Kurtzman and Robnett *(6–8)*

Table 6. Extent of nDNA complementarity and LSU D1/D2 divergence between selected closely related yeasts[1]

Taxa	nDNA reassoc. %	D1/D2 divergence
Candida shehatae var. *shehatae*		
var. *insectosa*	49	1
var. *lignosa*	46	0
Pichia amylophila		
Pichia mississippiensis	27	4
Pichia toletana		
Pichia xylosa	29	1
Torulaspora delbrueckii		
Torulaspora pretoriensis	13	5

[1] Kurtzman and Robnett *(7,8)*

Other gene sequences may offer resolution comparable to or perhaps greater than the D1/D2 domain of 26S rDNA. The work of James *et al. (10)* demonstrated that ITS1 sequence divergence is adequate for separation of closely related species in the genera *Torulaspora* and *Zygosaccharomyces*. For species of the Saccharomycetaceae, mitochondrial small subunit rDNA (Kurtzman and Robnett, unpublished data), transfer elongation factor (Kurtzman and Robnett, unpublished data) and cytochrome oxidase II *(11)* gave resolution similar to 26S D1/D2. In contrast, 18S rDNA is too conserved to resolve close species relationships *(10)*.

Phylogenetic Relationships

Ascomycetous yeasts

The impact of molecular phylogenetics on yeast systematics has been profound. The relationship of ascomycetous yeasts to filamentous species has been vigorously debated with theories ranging from considering the yeasts as a distinct evolutionary group to viewing them as reduced forms of many filamentous taxa. Phylogenetic analysis of 18S and 26S rRNA/rDNA sequences has shown the ascosporogenous yeasts, with the exception of *Schizosaccharomyces*, to form a monophyletic group distinct from the filamentous species *(3,12–20)*.

This work demonstrated that the yeasts, as well as yeastlike genera such as *Ascoidea* and *Cephaloascus* comprise a clade sister to the "filamentous" ascomycetes (euascomycetes). *Schizosaccharomyces, Taphrina, Protomyces* and *Saitoella* form a divergent clade basal to the yeast--euascomycete branch. *Eremascus*, which forms asci unenclosed in a fruiting body, aligned with the euascomycete clade. These results substantiate the long-held observation that yeasts cannot be recognized solely on the basis of presence or absence of budding. Such members of the yeast clade as *Ascoidea, Eremothecium* (and its synonym *Ashbya*) show no typical budding, whereas *Aureobasidium, Phialophora* and certain other genera of euascomycetes are usually dimorphic. Budding is also a common mode of vegetative reproduction among many basidiomycetous genera. Similarly, vegetative reproduction by fission is shared by *Dipodascus* and *Galactomyces*, members of the yeast clade, as well as by the distantly related genus *Schizosaccharomyces*. Sexual states of all members of the yeast clade are characterized by asci unenclosed in a fruiting body. This feature is shared by only a few taxa outside the yeast clade such as *Eremascus* and *Schizosaccharomyces*.

Basidiomycetous yeasts

Two morphologically distinct teleomorph states are found among the basidiomycetous yeasts *(21)*. In the first, teliospores are formed and germinate to produce a basidium (metabasidium) that bears basidiospores. This type of sexual cycle shows considerable similarity to the rust and smut fungi. The second type of sexual state lacks teliospores, and basidia develop on hyphae or yeast cells and give rise to basidiospores in a manner similar to the Tremellales (jelly fungi). Additionally, taxa have been defined from presence or absence of carotenoids, ballistoconidia, type of hyphal septum (simple or

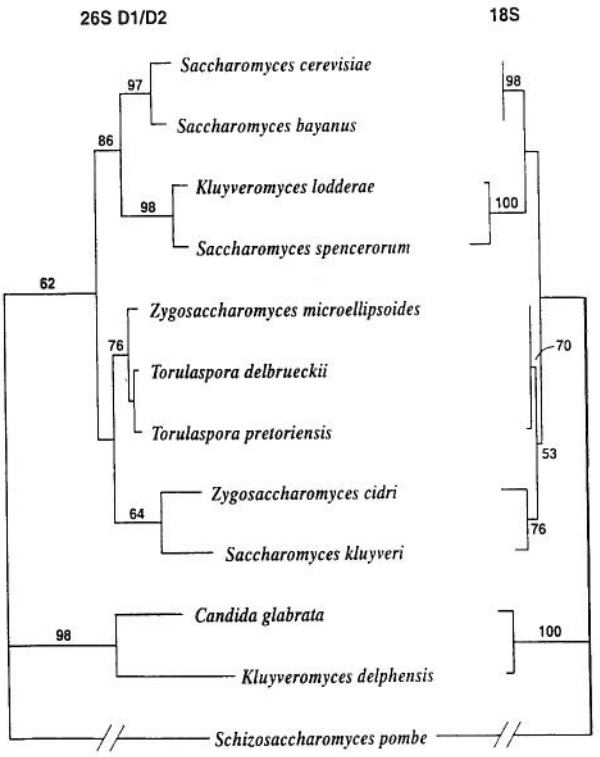

Fig. 1. Phylogenetic trees derived from maximum parsimony analysis depicting relationships among type strains of selected species of the *Saccharomyces* clade analyzed from 26S domain D1/D2 rDNA and from 18S rDNA. Branch lengths are proportional to nucleotide differences, and the numbers given at nodes are the percentage of frequencies with which a given branch appeared in 1000 bootstrap replications. Frequencies under 50% are not given. The branch for outgroup species *Schizosaccharomyces pombe* is one half of the actual length in both trees. These two gene trees are concordant, but this is often not the case when branches are weakly supported. Sequence data from Kurtzman and Robnett *(8)* and James *et al. (10)*.

the ultrastructurally more complex dolipore), and cellular xylose, which evidently arises from extracellular polysaccharides *(22)*.

Guého *et al. (23)* presented an overview of the phylogeny of basidiomycetous yeasts from divergence among partial sequences of large and small subunit rRNAs. In this study, three groups were resolved: (1) teliospore formers with hyphae having simple septal pores, (2) teliospore formers with hyphae having dolipore septa and, (3) non-teliospore formers with hyphae having dolipore septa. Fell *et al. (9)* analyzed relationships among 117 basidiomycetous species assigned to 23 genera from divergence in a 247-nucleotide segment in the 26S D2 domain. Although this region resolves closely related species, it has too few phylogenetically informative sites to accurately assess more distant relationships. Nonetheless, the analysis generally supports the concept that taxa assigned to the Tremellales are characterized by dolipore septa and cellular xylose, whereas taxa placed in the Ustilaginales form teliospores, have simple septal pores, and lack cellular xylose. Some exceptions

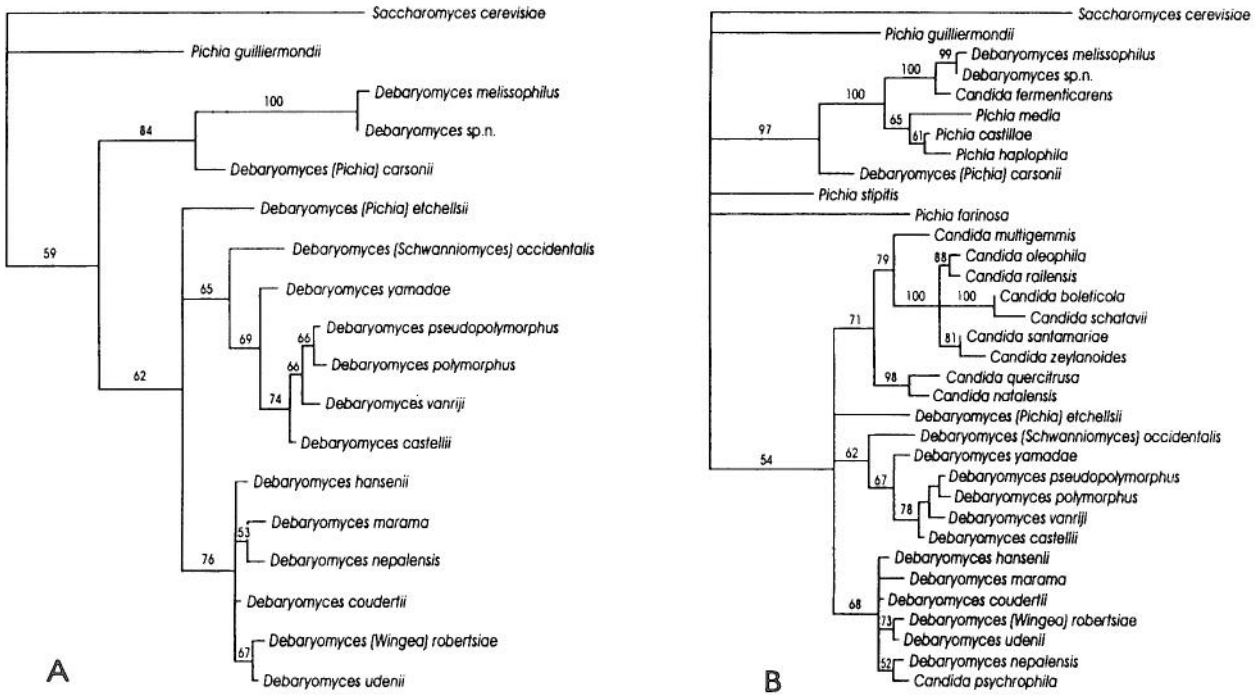

Fig. 2. Phylogenetic analyses of relationships among species of *Debaryomyces* and reference taxa. The trees are derived from maximum parsimony (PAUP) analysis of ca. 600 nucleotides from the 5' end of 26S rDNAs. Branch lengths are proportional to nucleotide differences, and the numbers given on branches are the percentage of frequencies with which a given branch appeared in 100 bootstrap replications. Values less than 50% are not given. A. Phylogenetic tree comprised of describe *Debaryomyces* species and two reference taxa. B. Phylogenetic tree as in A, but with eighteen reference taxa. *D. carsonii*, *D. melissophilus* and *Debaryomyces* sp. n. are separated from other members of *Debaryomyces* in this analysis [analyzed from the data of Kurtzman and Robnett *(7)*].

were noted. The teleomorphic genus *Erythrobasidium* does not form teliospores as do other members of the clade, but *Cystofilobasidium* and *Mrakia*, both apparently members of the Tremellales, do form teliospores.

Swann and Taylor *(24)* examined relationships among 35 basidiomycetous species selected to represent major taxonomic groups. Their analysis detected three major lineages, which they recognized as the classes Urediniomycetes, Ustilaginomycetes and Hymenomycetes. Each of these lineages had some yeasts or yeastlike taxa. Prillinger *et al. (25)* determined the composition of neutral sugars from cell walls of a large variety of basidiomycetous yeasts, and these analyses correspond well with phylogenies from rDNA divergence.

Phylogeny and systematics

The goal of systematics is to define taxa on the basis of their natural or evolutionary relationships, and phylogenetic analysis of gene sequences provides the opportunity to achieve this objective. At present, most phylogenetic trees are based on ribosomal DNA gene sequences, so the phylogenies are really rDNA gene trees. Corroboration of the relationships predicted from rDNA need to come from other gene comparisons, though at present, there appear to be no significant conflicts between rDNA and the few other gene trees generated.

An example of gene tree comparisons is shown in Fig. 1. In this selected group of species from the Saccharomycetaceae, relationships from 26S D1/D2 analysis are concordant with those from analysis of the 18S gene. This is also the case for trees generated from sequences of mitochondrial small subunit rDNA and translation elongation factor (Kurtzman and Robnett, unpublished data). The D1/D2 tree gives greater resolution of terminal branches than does 18S rDNA, thus allowing separation of species. Surprisingly, basal resolution for both trees is similar, as shown from bootstrap values. Consequently, this analysis demonstrates that neither gene tree strongly resolves basal lineages, thus precluding reassignment of species to phylogenetically defined genera. From this, it is clear that more robust datasets are needed before most genera can be phylogenetically circumscribed.

Another factor influencing circumscription of genera is the issue of missing taxa. Taxa may be excluded either because they are extinct or they have not been recognized in nature. An indication of missing taxa is the presence of long branches on phylogenetic trees, assuming rates of nucleotide substitutions are reasonably constant for all species compared. The taxonomy of *Debaryomyces* is an example of the problems encountered when there is incomplete knowledge of species. On the basis of nucleotide similarities, Kurtzman and Robnett *(26,27)* proposed transfer of *Schwanniomyces occidentalis* and *Wingea robertsiae* to *Debaryomyces*, and Yamada *et al. (28)* further enlarged the genus with the transfer of *Pichia carsonii* and *P. etchellsii*. A phylogenetic analysis that included the preceding four species with previously described *Debaryomyces* species suggests that *Debaryomyces* is monophyletic (Fig. 2A). When additional species are included in the analysis, *D. carsonii*, *D. melissophilus* and

Debaryomyces sp. n. are no longer closely allied with other species in the genus (Fig. 2B). If the circumscription of genera is to be based on monophyly of their assigned species, the preceding three species and other members of their clade represent a new genus. Before such taxonomic changes are made, all known yeasts need to be compared from multiple gene sequences, taking into account taxa located on the long branches of phylogenetic trees and their possible effect on genus circumscription.

Conclusions

Molecular comparisons have provided a powerful genetic means for recognizing yeast species, and these studies have demonstrated that many of the standard growth tests and morphological criteria used for yeast identification are unreliable predictors of relatedness. Furthermore, analyses of gene sequences indicate that most genera are not circumscribed along phylogenetic lines. However, before genera can be resolved phylogenetically, more robust sequence data are needed.

A practical aspect of molecular comparisons is the development of rapid methods for yeast identification. This may be done using an automated sequencer with a library of species-specific sequences such as those of domain D1/D2 from 26S rDNA *(8)*. Species-specific oligonucleotide probes have also been effective *(29,30)*, and still other methods will be developed.

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Sistematika kvasaca – od fenotipa do genotipa

Sažetak

Sistematika je kvasaca danas na prijelazu od određivanja prema fenotipskim značajkama do primjene molekularnih poredbi. Mjerenje molekularnih razlika je kvantitativni način određivanja srodnosti među vrstama, a te su molekularne usporedbe otkrile da su standardni fenotipski testovi, primijenjeni u taksonomiji kvasaca, često slabi pokazatelji srodnosti. Razmatran je utjecaj molekularnih poredbi na sistematiku kvasaca, a izneseni su i primjeri primjene tih podataka za brzu identifikaciju vrsta.