Recognition of Yeast Species from Gene Sequence Comparisons

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Abstract: This review discusses recognition of yeast species from gene sequence comparisons, which have been responsible for doubling the number of known yeasts over the past decade. The resolution provided by various single gene sequences is examined for both ascomycetous and basidiomycetous species, and the greater resolution of species provided by multigene analyses is discussed. Various methods are presented for rapid species identification using gene sequences.

Keywords: Yeasts, yeast identification, gene sequence analysis.

INTRODUCTION

DNA sequence analyses have transformed the way in which yeasts are identified, and the focus of this review will be on the genes analyzed and the interpretation of what constitutes a species as measured from sequence divergence. Prior to the availability of molecular methods, yeasts were identified from their fermentation reactions on various sugars, their growth reactions on various carbon and nitrogen sources and the morphology of their sexual and asexual states. Not surprisingly, there is a certain degree of ambiguity for identifications determined from phenotypic methods.

Initial molecular studies were technologically limited to determination of the mol% guanine + cytosine (G+C) content of DNA. From this work, it was seen that ascomycetous yeasts had a nuclear DNA content of ca. 28-50 mol%, whereas basidiomycetous yeasts had a noticeably higher range of 50-70 mol% [1, 2]. These studies suggested that strains differing by 1-2 mol% were likely to represent different species, thus providing a means for excluding some strains that were incorrectly assigned to a particular species. Methods for determination of G+C content were reported by Kurtzman [3], Price *et al.* [2] and Tamaoka and Komagata [4].

Quantitation of genome similarity between strains became possible with the development of DNA reassociation techniques that measure the extent of pairing of nucleotide sequences when DNA is made single-stranded and allowed to re-pair as a double strand. Two methods are commonly used. One is 'free-solution' in which both DNAs of the test pair react while dissolved in a buffer solution, whereas the second method relies on binding of single-stranded DNA from one member of the pair to a matrix, such as a nitrocellulose filter, while the other DNA as single strands, is free to react with the bound DNA in the buffer solution that surrounds the filter. Free solution assays may be done spectrophotometrically [5, 6] or with the use of radioisotopes [2]. If a filter or other binding method is employed, the extent of reassociation is determined by measuring the radioactivity bound to the DNA on the filter [7]. Measurements of DNA complementarity are commonly expressed as percent relatedness, which provides an approximation of overall genome similarity between two organisms.

An interpretation of DNA reassociation data was provided by Martini and Phaff [8] and Price *et al*. [2], who suggested that on the basis of shared phenotype, strains that showed 80% or greater nuclear DNA relatedness, as measured by reassociation, are members of the same species. Correlation of DNA relatedness with the biological species concept has been examined from genetic crosses utilizing both heterothallic and homothallic species [5, 9-14]. In general, these studies support the idea that strains showing 70-80% or greater DNA complementarity are conspecific. However, results from genetic crosses are not always clear, which adds uncertainty when using these data to interpret the meaning of sequence analyses. For example, the following two heterothallic species pairs showed 25% nuclear DNA relatedness as measured by reassociation. In the first comparison, *Wickerhamomyces amylophilus* and *W. mississippiensis* mated but formed no viable ascospores [5]. In contrast, mating between *Pichia exigua* and P. *scutulata* gave abundant asci with viable ascospores. However, F_1 and F_2 crosses showed reduced fertility and backcrosses to parentals showed even less fertility, suggesting that the two taxa represent closely related but separate biological species [11].

RECOGNITION OF SPECIES FROM SINGLE GENE SEQUENCE ANALYSIS

A limitation of DNA reassociation experiments has been that genetic resolution extends no further than to closely related species. In contrast, gene sequence comparisons offer the opportunity to resolve closely related species, as well as more distantly related taxa, and a database of sequences can be developed and continually expanded as new species become available. Nonetheless, data from DNA reassociation experiments provided some of the initial

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reference points for interpretation of gene sequence comparisons.

The variable domain 2 (D2) from nuclear large subunit ribosomal RNA (LSU rRNA) was initially examined and found to resolve closely related species that had been circumscribed from genetic crosses and DNA reassociation experiments [15]. This work of Peterson and Kurtzman [15] was expanded by Kurtzman and Robnett [16] to include domains 1 and 2 (D1/D2) and applied to all described species of ascomycetous yeasts, resulting in a diagnostic database (barcode) for rapid species identification. Fell *et al*. [17] developed a complementary D1/D2 database for known basidiomycetous yeasts. By comparing divergence among ascomycetous strain pairs with previously determined nuclear DNA reassociation values, it appeared that conspecific strains differed by no more than three nucleotides among the 500-600 nucleotides of the D1/D2 domains, whereas differences of 6 or more nucleotides (1%) indicated that the strains were different species [16].

The preceding guidelines developed by Kurtzman and Robnett [16] were treated as a prediction because exceptions had been found earlier. DNA reassociation studies revealed *Saccharomyces pastorianus* to have intermediate relatedness with *S. cerevisiae* (57%) and *S. bayanus* (72%) [14], and LSU rRNA sequence analysis showed *S. bayanus* and *S. pastorianus* to have identical D2 sequences [15]. These results were interpreted to mean that *S. pastorianus* was a hybrid of *S. bayanus* and *S. cerevisiae* and that *S. pastorianus* received its D2 LSU rRNA sequence from *S. bayanus*. Later, *S. pastorianus* and *S. bayanus* were shown to share the entire rRNA repeat [18]; consequently, neither D1/D2 rRNA, SSU rRNA or ITS would separate these two sister species. The problem of resolving hybrids was further illustrated by Groth *et al.* [19] from the discovery that *Saccharomyces* sp. strain CID1 was actually a triparental hybrid with DNA from *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii* (Fig. **1**).

The presence of indels further complicates the estimation of relatedness among strains when using single gene analyses. Liu and Kurtzman [20] found 4-6 deletions in the D2 domain of LSU rRNA among strains of *Barnettozyma* (*Williopsis*) *californica* (Table **1**). The strains were believed to be conspecific because they showed 94-100% nuclear DNA relatedness as determined from reassociation [21]. The deletions were contiguous and may represent a single evolutionary event. Consequently, in the predictive guidelines offered by Kurtzman and Robnett [16],

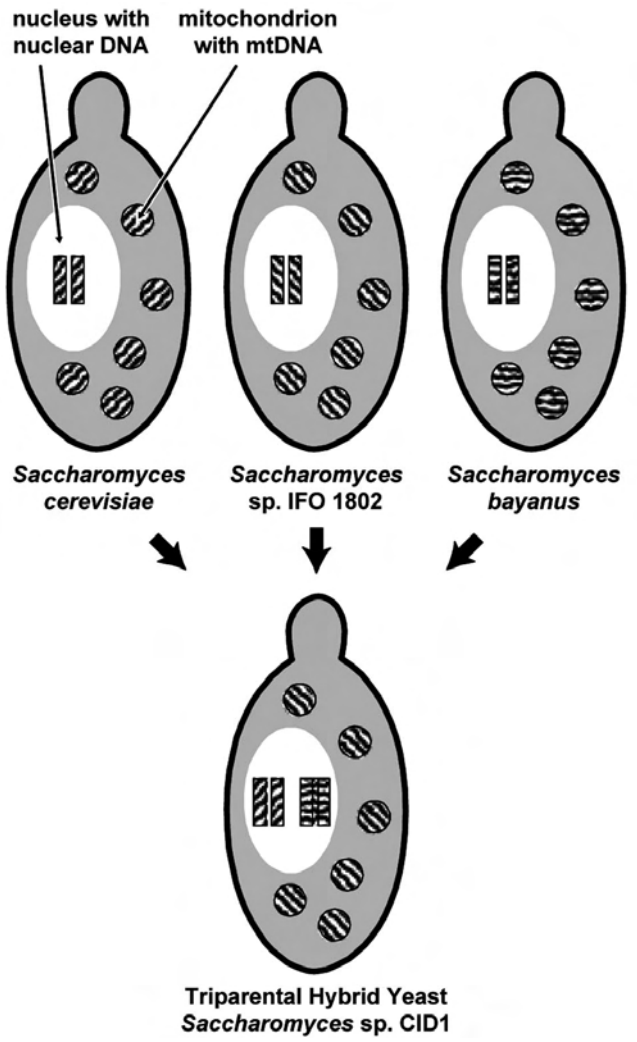

Fig. (1). *Saccharomyces* sp. CID1 is a triparental hybrid with mitochondria from *S. kudriavzevii* (IFO 1802) and nuclear DNA from *S. cerevisiae* and *S. bayanus*. Redrawn from Groth *et al.* [19]. As discussed in the text, multiple diagnostic genes are required to detect hybrids.

Table 1. Nucleotide Heterogeneity Among Strains of *Barnettozyma* (*Williopsis*) *californica* in the D2 Domain of Large Subunit Ribosomal RNA[1,2]

Strain (NRRL)	Nucleotide No.														
	423		**425**	**585**											**599**
Y-1680	U	U	A	C	A	A	A	U	U	U	A	U	U	U	U
Y-5863	U	U	A	C	A	A	A	U	U	U	A	U	U	U	U
Y-6420	U	G	A	C	A	A	-	-	-	-	U	U	U	U	U
Y-6421	U	G	A	C	A	A	-	-	-	-	U	U	U	U	U
YB-3239	U	U	A	C	A	A	A	-	-	-	-	-	-	U	U

[1]From Liu and Kurtzman [20].
[2]Strains showed 94-100% nuclear DNA relatedness as measured from reassociation [21].

contiguous deletions were treated as a single event and weighted as one nucleotide substitution. Similarly, Lachance *et al*. [22] found certain strains of *Clavispora lusitaniae* to be highly polymorphic in the D1/D2 domains of the LSU rRNA gene (Fig. **2**). The polymorphic strains, which have similar actin-1 sequences, mated and formed ascospores, although viability of the ascospores was not tested. Further work is needed to understand the *C. lusitaniae* polymorphisms, and this would include comparing additional gene sequences as well as determining if there are multiple alleles of the LSU rRNA gene.

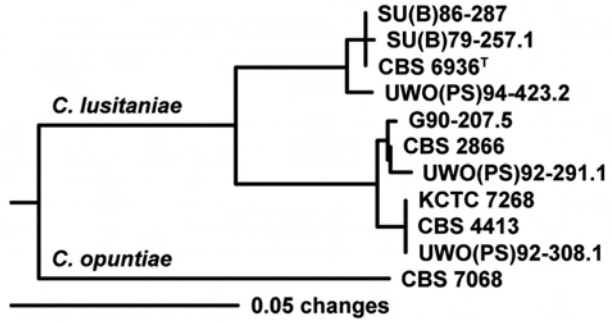

Fig. (2). D1/D2 LSU rRNA gene tree demonstrating the sequence polymorphisms that were found among strains of *Clavispora lusitaniae*. Adapted from Lachance *et al*. [22].

Daniel and Meyer [23] and Daniel *et al*. [24] compared species of *Candida* from their divergence in the gene sequences of D1/D2 LSU rRNA and actin-1. Divergence in actin-1 was greater than in D1/D2, thereby potentially providing greater resolution among closely related taxa. However, as for D1/D2, actin-1 sequences were sufficiently variable in nucleotide substitutions among apparently conspecific strains that it was sometimes difficult to resolve closely related species. A comparison of *Saccharomyces* species from substitutions in ITS1-5.8S-ITS2 and the genes coding for D1/D2 LSU rRNA, translation elongation factor-1α, mitochondrial SSU rRNA and cytochrome oxidase II provided similar resolution, although some species showed greater divergence when compared from cytochrome oxidase II sequences [18]. Actin-1 was not included in the preceding study, but the results presented for *Candida* species suggest that this gene sequence may provide greater resolution among closely related species than obtained from other genes that were compared.

Another factor influencing species resolution is the apparent difference in substitution rates among lineages for the diagnostic gene being used. For example, the closely related species *Meyerozyma guilliermondii*, *M. caribbica* and *Candida carpophila* differ from one another by 1-3 nucleotides in D1/D2 and will not be recognized as separate species using the guideline that 0-3 substitutions indicate conspecificity (Table **2**). Examples of apparent lineage-specific differences in extent of substitutions for D1/D2 and other genes are given in Table **3**.

The internal transcribed spacers 1 and 2 of the rDNA, which are located between the SSU and LSU rRNA genes of the rDNA repeat and separated by the 5.8S rRNA gene, are commonly used to resolve species, often in conjunction with sequences from the D1/D2 LSU rRNA gene. The resolution provided by ITS sometimes exceeds that of D1/D2, but the reverse is also true. For species of *Bensingtonia* and *Kondoa*, ITS clearly provides greater resolution of species (Fig. **3**), but species of *Trichosporon* were less well resolved by ITS [32] (Fig. **4**).

Of rDNA regions used for species identification, the intergenic spacer (IGS) appears the most substituted and offers the greatest resolution of closely related species and subspecific lineages. The IGS is comprised of two regions, IGS1 and IGS2, which are often separated by the 5S rRNA gene. IGS sequences have been used to resolve lineages within *Cryptococcus neoformans* and closely related taxa [33-36]. The IGS has also been employed for resolution of closely related species of *Trichosporon* [37, 38], *Mrakia* [39] and *Xanthophyllomyces* [40, 41]. A characteristic of IGS is the diversity of length polymorphisms. Sugita *et al*. [38] reported that the IGS1 region ranged in length from 195-704 nucleotides among *Trichosporon* species. The IGS region often includes a series of multiple repeat units with numerous deletions and insertions (indels). These repeat units and indels may provide characteristics for defining strains and species, and may delineate geographical strain distributions [41, 42], but in some cases interpretation is uncertain. Intragenomic sequence heterogeneity is another factor to consider for IGS analysis. Fell *et al*. [41] reported sequence heterogeneity in the ITS and IGS regions among certain strains of *Xanthophyllomyces*, which required cloning prior to sequence analysis. Intragenomic variation in the rDNA spacer regions is not uncommon among fungi and reports include ITS variation in *Fusarium* [43], IGS variability in hybrids of *Cryptococcus neoformans* [44] and possible divergence among the multiple copies of D1/D2 in certain species of *Metschnikowia* [C. P. Kurtzman, unpublished data]. The presence of these variants may be used as a tracking tool for investigations of the origin and distribution of strains and species. A significant advantage to

Table 2. Percent Nuclear DNA Relatedness and LSU D1/D2 Nucleotide Divergence Among Closely Related Species of the *Meyerozyma guilliermondii* Clade[1]

	% Nuclear DNA Relatedness and D1/D2 Nucleotide Substitutions					
	M. guilliermondii		***M. caribbica***		***C. carpophila***	
Species	**%DNA**	**D1/D2**	**%DNA**	**D1/D2**	**%DNA**	**D1/D2**
M. guilliermondii	100	0	37	3	55	1
M. caribbica			100	0	68	2
Candida carpophila					100	0

[1]Data from Vaughan-Martini *et al*. [25]. DNA reassociation values are an average from 5 strain pairs of each species. All strains of each species had the same D1/D2 sequence.

Table 3. Extent of Nuclear DNA Reassociation and Gene Sequence Divergence between Closely Related Species in Several Ascomycetous Genera

Species Pair[1]	Percent[2] DNA Reassoc.	Genes (Substitutions - Indels)[3]			
		D1/D2	SSU	EF-1α	MtSm
Lindnera (*Pichia*) *amylophila* - *L.* (*P.*) *mississippiensis*	25	2 - 2	4 - 0	19 - 0	
L. amylophila - *L.* (*P.*) *fabianii*		9 - 2	19 - 2	39 - 0	
L. mississippiensis - *L. fabianii*		7 - 0	15 - 2	51 - 0	
Lindnera (*Pichia*) *americana* - *L.* (*P.*) *bimundalis*	21	2 - 0	0 - 0	22 - 0	
Lindnera (*Williopsis*) *saturnus* - *L.* (*W.*) *mrakii*	52	1 - 0	0 - 0	12 - 0	
L. mrakii - *L.* (*W.*) *subsufficiens*	44	4 - 0	0 - 0	12 - 0	
L. saturnus - *L. subsufficiens*	56	5 - 0	0 - 0	12 - 0	
Pichia cactophila - *P. pseudocactophila*	34	11 - 8	1 - 3	14 - 0	0 - 0
P. cactophila - *Candida inconspicua*[4]		1 - 1	0 - 0	0 - 0	0 - 0
Pichia kluyveri - *P. eremophila*	66	7 - 1	5 - 0	26 - 0	1 - 0
P. kluyveri - *P. cephalocereana*	72	3 - 0	2 - 0	11 - 0	1 - 0
P. eremophila - *P. cephalocereana*	69	7 - 1	4 - 0	25 - 0	1 - 0
Pichia (*Issatchenkia*) *scutulata* - *P.* (*I.*) *exigua*	25	20 - 7	7 - 1	36 - 0	10 - 4
Starmera (*Pichia*) *amethionina* - *S.* (*P.*) *pachycereana*	65	8 - 10	5 - 3	12 - 0	
S. amethionina - *S.* (*P.*) *caribaea*	40	21 - 2	8 - 3	11 - 0	
S. pachycereana - *S. caribaea*	37	17 - 5	9 - 5	6 - 0	

[1]Species pairs are type strains. Genus names in parentheses were those used in the publication describing these results.
[2]Data are from Holzschu *et al.* [26], Kurtzman [9, 21], Kurtzman *et al.* [5, 11], Phaff *et al.* [27-29], Shen and Lachance [30].
[3]D1/D2 = domains 1 and 2, LSU rRNA; SSU = small subunit rRNA, EF-1α = translation elongation factor-1α; MtSm = mitochondrial SSU rRNA [31].
[4]From these data, *Pichia cactophila* and *Candida inconspicua* are considered to be conspecific.

the use of rRNA gene sequences is that ribosomes have a common evolutionary history, and within the sequences, there are highly conserved regions between the variable regions that serve for pan-specific primer attachment for PCR amplification and sequencing. In contrast, protein coding genes tend to be variable across the entire gene, often making primer design difficult.

RECOGNITION OF SPECIES FROM MULTIGENE PHYLOGENETIC ANALYSIS

In the examples presented, determination of whether strains are conspecific or members of separate species can be confused by hybridization events (Fig. **1**), by unexplained sequence polymorphisms (Fig. **2**), and by differences in nucleotide substitution rates (Figs. **3**, **4**). Multigene analyses offer a means for detecting these changes, which would be signalled by lack of congruence for a particular gene tree. This approach was recommended by Goodman [45] for vertebrates, for bacteria by Dykhuizen and Green [46], and for fungi by O'Donnell *et al.* [47] and Taylor *et al.* [48]. The paper by Taylor *et al.* [48] provides an inclusive review of species concepts, and the term Genealogical Concordance Phylogenetic Species Recognition (GCPSR) was introduced to describe the concept of multigene analysis for species recognition. An example of GCPSR is found in a multigene sequence analysis of the *Kazachstania* (*Arxiozyma*) *telluris* species complex. D1/D2 LSU rRNA gene sequence analysis resolved the complex into five species as did analysis of mitochondrial SSU rRNA gene sequences. However,

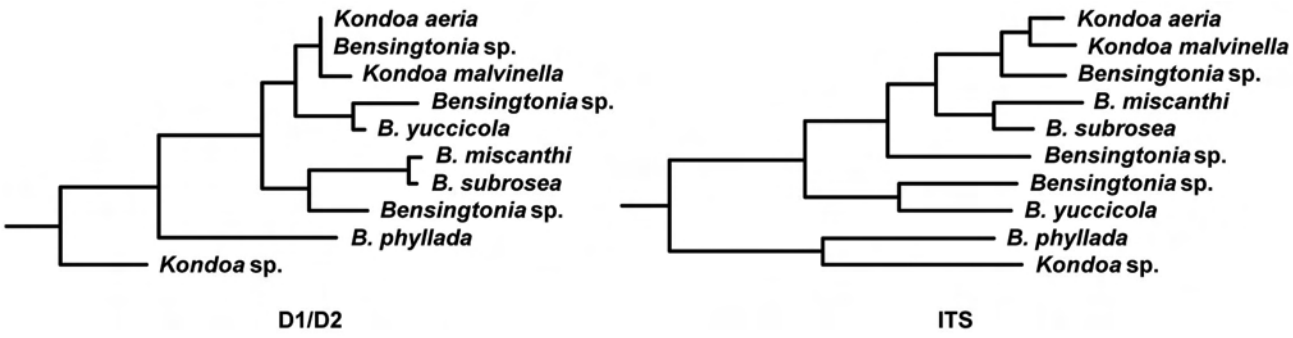

Fig. (3). Contrasting resolution of *Bensingtonia* and *Kondoa* species when analyzed from gene sequences of D1/D2 LSU rRNA and from sequences of ITS. Lineages in this clade are more highly resolved from ITS sequences than from D1/D2. Adapted from Scorzetti *et al.* [32].

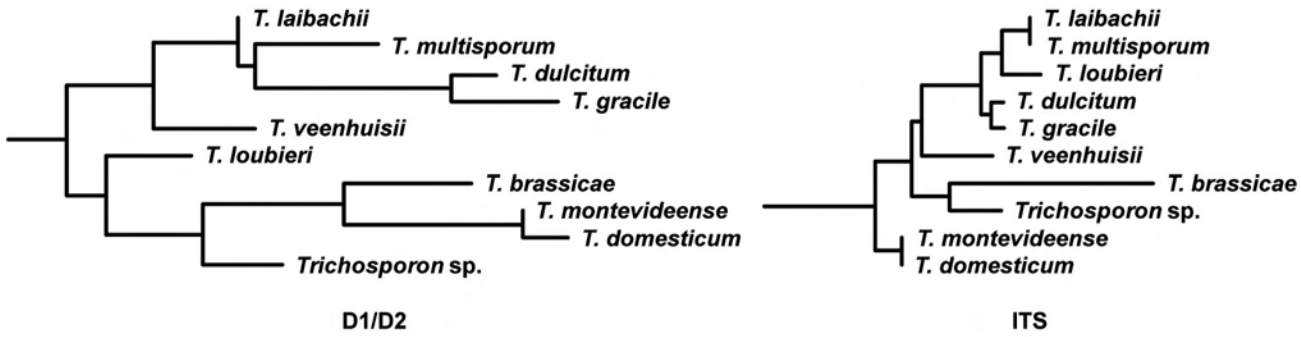

Fig. (4). Resolution of *Trichosporon* species from analysis of D1/D2 LSU rRNA gene sequences and from ITS sequences. Greater species resolution is provided by D1/D2 than from ITS, which is in contrast to resolution of species in the *Kondoa* clade (Fig. **3**). Adapted from Scorzetti *et al*. [32].

analysis of RNA polymerase II (Fig. **5**) detected four species because *K. pintolopesii* and *K. heterogenica* have nearly identical sequences for this third gene [49]. The preceding analyses suggest that *K. heterogenica* may have arisen as a hybrid between *K. pintolopesii* and an undescribed species of *Kazachstania* because lateral gene transfer does not appear common among fungi, but this hypothesis needs further testing (Fig. **5**). From the examples presented, it is apparent that single gene analyses can lead to incorrect interpretations. Consequently, in addition to D1/D2 and / or ITS, one or more protein coding gene sequences should be utilized as well. This would also apply to rapid molecular detection methods that are now being widely adopted.

Multilocus sequence typing (MLST) has become an important application of GCPSR for the study of genetic structure in a number of clinically significant yeast species, such as *Candida albicans*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *Cryptococcus neoformans* [50-56]. In addition to the preceding species, MLST schemes have been developed for *Saccharomyces cerevisiae* [57].

As discussed, recognition of species from gene sequence analysis can be subjective and better analytical methods need to be developed. The rationale for interpreting D1/D2 sequence divergence was discussed above along with examples of closely related species that would not be resolved using current guidelines. For the taxa shown in Figs. (**3**, **4**), we need to ask whether the lineages resolved by the two contrasted gene sequences represent species or subspecies. This problem is also illustrated by the species pairs listed in Table **3**, in which pairs having similar DNA reassociation values may show noticeably different numbers of nucleotide substitutions in certain genes. Consequently, our current practice of simply counting nucleotide differences between strain pairs to determine if the strains are the same or different species is somewhat subjective. A better approach is to examine multiple gene trees to estimate genetic separation. It is clear that multiple strains of a large number of species need to be compared to provide a stronger basis for data interpretation. An additional approach would be to identify species from whole genome sequence comparisons. Whether this proves practical for rapid diagnostics is uncertain, but it is a concept worth testing because of the ease of determining whole genome sequences for microorganisms. This approach would be especially revealing for species of such genera as *Saccharomyces* because the species are heterothallic and appear to interbreed, thus it would contribute to our understanding of the fate of interspecific hybrids and their role in the speciation process.

RAPID MOLECULAR METHODS FOR SPECIES IDENTIFICATION AND QUANTITATION

Rapid molecular-based methods commonly used for species identification include species-specific primer pairs

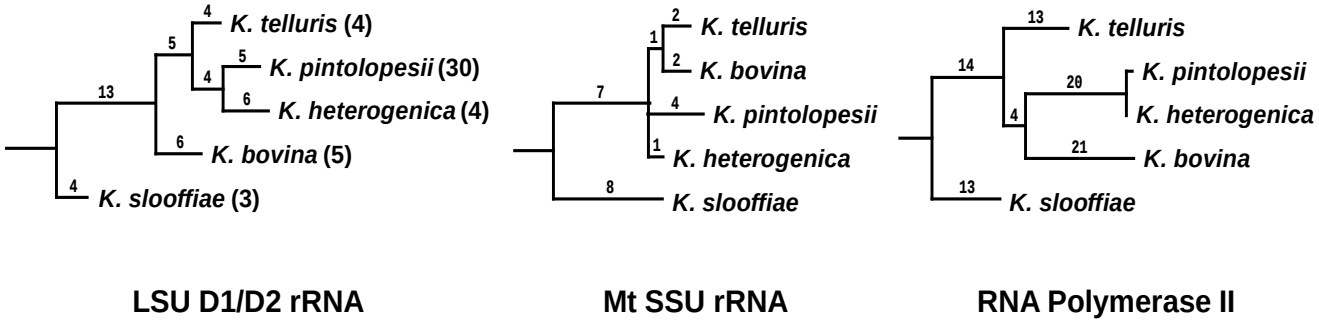

Fig. (5). Maximum parsimony analyses of gene sequences for D1/D2 LSU rRNA, mitochondrial SSU rRNA and RNA polymerase II from *Kazachstania* species. The gene trees show overall congruence, but for RNA polymerase II, *K. pintolopesii* and *K. heterogenica* are nearly unresolved, suggesting that the latter species is a hybrid. The mitochondrial SSU rRNA tree shows *K. telluris* and *K. bovina* to be more closely related than is indicated by the other two gene trees, again suggesting an interspecific hybridization event. Numbers above branches reflect nucleotide substitutions and numbers in parentheses are the number of strains sequenced for each species for all three genes. Adapted from Kurtzman *et al*. [49].

and probes, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP) and karyotyping. In this section, some of the commonly used methods are described.

Species-specific primers. The use of species-specific primer pairs is effective when used for PCR-based identifications involving a small number of species or when a particular species is the subject of the search [58-60] (Fig. **6**). However, PCR mixtures that contain large numbers of species-specific primer pairs may lead to uncertain banding patterns and possible misidentification.

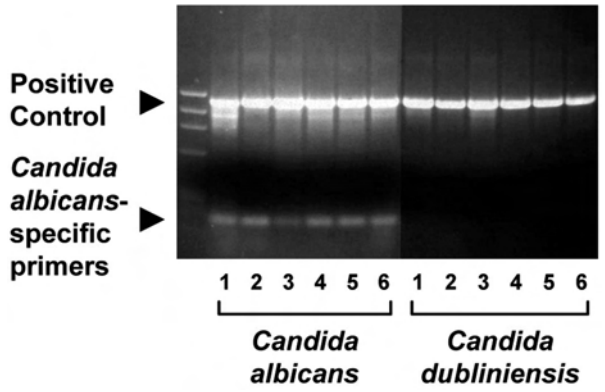

Fig. (6). Use of species-specific PCR primer pairs for rapid identification of a target species. In this example, the primer pairs were designed to detect *Candida albicans* but not the closely related species *C. dubliniensis.* The PCR mixture included species-specific D1/D2 primers and pan-specific SSU primers to serve as a positive control [60].

PNA. Peptide nucleic acid (PNA) probes offer a means for detection and quantitation of species in clinical samples and food products through fluorescence *in situ* hybridization (FISH). PNA probes have a peptide backbone to which is attached nucleotides complementary to a species-specific target sequence, and a fluorescent label is added for detection by fluorescence microscopy [61]. If probes are complementary to rRNA, the whole cell of the target species will 'glow' when visualized, which will also allow quantitation by cell counts. An advantage is that a sample can be diluted and directly probed. One disadvantage is that probes must be developed for each species of interest, a problem common to most probe technology. PNA technology has been effective for detection of *Dekkera* (*Brettanomyces*) *bruxellensis* in spoiled wine [61]) and for detection of *Candida albicans* in blood samples [62].

RAPD/AFLP. Microsatellite-primed RAPDs [63] and AFLP fingerprints [64] have been effectively used for rapid preliminary identification of large numbers of isolates, and the pattern-based identification is then often followed by gene sequencing of representative strains from each group that has a unique pattern. RAPD analysis has also been used to resolve populations in various species, such as *S. cerevisiae, S. bayanus* var. *uvarum, C. albicans* and *C. glabrata* [65-72]. One concern in using pattern-based identification techniques is reproducibility between laboratories, because small differences in PCR conditions may impact the species-specific patterns that serve as reference. Karyotyping with pulse field electrophoresis and RAPD on mitochondrial DNA can also serve in the initial characterization and identification of yeast species, but the interpretation of chromosomal banding patterns and mitochondrial restriction fragments for taxonomic purposes is complicated by a high degree of polymorphisms, such as chromosomal rearrangements within some yeast taxa [73].

Real time PCR. The technique of real time PCR has been widely studied for applications in medical mycology, especially those aiming to detect and quantify loads of *C. albicans*. In typical assays, 5 cfu ml^{-1} could be detected. Furthermore, clinically relevant *Candida* and *Cryptococcus* species can be identified. An advantage of this method is the potential early detection of the pathogen, thus contributing to the early start of treatment. Most commonly used primers are based on sequences of the rDNA repeat, such as ITS 1 and 2, or the SSU rRNA gene (Bergman *et al.* 2007 [74-78]. This technique is also becoming widely employed in food and beverage analyses and has been used for detection and quantitation of spoilage yeasts in orange juice [79] as well as in wine fermentations [80].

DGGE. Denaturing gradient gel electrophoresis (DGGE) is a promising technique that has been used for species identification and quantitation of yeast populations in foods and beverages. The technique is based on separation of DNA fragments of differing nucleotide sequences (e.g., species-specific) through decreased electrophoretic mobility of partially melted double-stranded DNA amplicons in a polyacrylamide gel containing a linear gradient of DNA denaturants (i.e., a mixture of urea and formamide). A related technique is temperature gradient gel electrophoresis (TGGE), in which the gel gradient of DGGE is replaced by a temperature gradient [81]. Recent applications of DGGE include identification and population dynamics of yeasts in sourdough bread [82], in coffee fermentations [83] and on wine grapes [84]. Levels of detection are often around 10^3 cfu ml^{-1}, but 10^2 cfu ml^{-1} have been reported, which compares favorably with standard plate count methods. Prakitchaiwattana *et al*. [84] provided information on mixed species populations, noting that when the ratio of species is not greater than 10-100-fold, detection of individual species was possible, but if the ratio exceeds 100-fold, the low population species will not be detected. Masoud *et al*. [83] and Prakitchaiwattana *et al*. [84] reported detection of species by DGGE that were not recovered by plating, suggesting that some yeasts may establish significant populations in a product and then die.

Flow cytometry. High throughput probe hybridization methods are available for detection of multiple species in multiple samples. One method that is effective for yeasts [37, 85] is an adaptation of the Luminex xMAP technology (Luminex Corp), which consists of a combination of 100 different sets of fluorescent beads covalently bound to species-specific DNA capture probes. Upon hybridization, the beads bearing the target amplicons are classified in a flow cytometer by their spectral addresses with a 635 nm laser. The hybridized biotinylated amplicon is quantitated by fluorescent detection with a 532 nm laser. The multiplex

assay is specific and fast; species that differ by 1 nucleotide often can be discriminated and the assay can be performed, after amplification, in less than 50 min in a 96-well format with as many as 100 different species-specific probes per well. The advantage of this method for clinical, food quality and ecology laboratories is that multiple species can be identified quickly from multiple samples.

Luminex technology was employed by Diaz and Fell [37] to develop a direct hybridization assay for high-throughput detection of *Trichosporon* spp. The assay used a set of 48 species-specific probes and three sets of primers, which were designed to amplify three different target regions of the rRNA repeat: D1/D2, ITS and IGS. The assay was specific and enabled detection of 10^2 genome copies. This report, which was the first to adapt the technology for the detection of pathogenic yeasts, was expanded to an eight-plex hybridization array for the detection of the varieties and genotypes within the *Cryptococcus neoformans* species complex [35]. The method, which employed a direct hybridization assay format, allowed discrimination of 1 bp mismatch with no apparent cross-reactivity, permitted the detection of 10^1 to 10^3 genome copies and allowed simultaneous detection of multiple target sequences. Also, the assay can be carried out directly with yeast cells or from isolated DNA. The described assay format was validated with a collection of environmental and clinical isolates [86]. The suspension array correctly identified the isolates at species and subspecies level. The results confirmed the identification of hybrid isolates that according to flow cytometric profiles and cloning experiments were classified as diploids or partial diploids. These hybrid isolates, which were found to contain two IGS 1 alleles, belonged to *Cr. neoformans* serotype AD or BD. Further adaptation of the technology was also documented for the detection of *Malassezia* species [87]. In this particular study the authors designed an array comprising 16 sets of species-specific and multi-species-specific probes to identify recognized and new emergent species within the genus.

The molecular detection methods just discussed have provided some remarkable capabilities for yeast identification, but a number of factors affect detection and quantitation. These include: 1) cellular copy number of the gene to be used, 2) whether the gene is sufficiently conserved to be PCR amplified by 'universal' primers that will detect all species of interest, 3) efficiency of DNA extraction from cells in the sample, 4) efficiency of DNA recovery from the sample, 5) sample components that may interfere with DNA recovery or PCR amplification, and 6) level of cell population detectable.

CONCLUSIONS

Rapid detection, accurate identification and quantitation of yeasts is now possible through use of a variety of molecular methods. Increased application of these methods will bring a greater degree of clarity to all questions in yeast microbiology, which previously was not possible when yeasts were identified from phenotype. Not included in this review has been the impact of multigene phylogenetic analyses on circumscription of yeast genera and families. The following references will provide an introduction to the rapid changes occurring in ascomycete yeast classification: Kurtzman and Robnett [18, 88], Kurtzman and Suzuki [89] and Kurtzman *et al*. [31, 49, 90]. The paper of Rokas *et al*. [91] should also be consulted because it addresses the issue of minimum number of gene sequences needed for an accurate species phylogeny within genera. By inference, data from this study can be used to examine the question of whether a large number of genes is needed for accurate identification of species.

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CONFLICT OF INTEREST

None declared.

REFERENCES

[1] T. Nakase, and K. Komagata, "Taxonomic significance of base composition of yeast DNA", *J. Gen. Appl. Microbiol.*, vol. 14, pp. 345-357, 1968.

[2] C. W. Price, G. B. Fuson, and H. J. Phaff, "Genome comparison in yeast systematics: delimitation of species within the genera *Schwanniomyces*, *Saccharomyces*, *Debaryomyces* and *Pichia"*, *Microbiol. Rev.*, vol. 42, pp.161-193, 1978.

[3] C. P. Kurtzman, "DNA-DNA hybridization approaches to species identification in small genome organisms," in *Methods in Enzymology*, Vol. 224, E. A. Zimmer, T. J. White, R. L. Cann, and A. C. Wilson, Eds., New York: Academic Press, 1993, pp. 335-348.

[4] M. Tamaoka, and K. Komagata, "Determination of DNA base composition by reversed phase high-performance liquid chromatography", *FEMS Microbiol. Lett.* vol. 25, pp. 125-128, 1984.

[5] C. P. Kurtzman, M. J. Smiley, C. J. Johnson, L. J Wickerham, and G. B. Fuson, "Two new and closely related heterothallic species, *Pichia amylophila* and *Pichia mississippiensis*: characterization by hybridization and deoxyribonucleic acid reassociation", *Int. J. Syst. Bacteriol.*, vol. 30, pp. 208-216, 1980.

[6] R. J. Seidler, and M. Mandel, "Quantitative aspects of deoxyribonucleic acid renaturation: base composition, state of chromosome replication, and polynucleotide homologies", *J. Bacteriol*., vol. 106, pp. 608-614, 1971.

[7] S. A. Meyer, K. Anderson, R. E. Brown, M. T. Smith, D. Yarrow, G. Mitchell, and D. G. Ahearn, "Physiological and DNA characterization of *Candida maltosa*, a hydrocarbon-utilizing yeast", *Arch. Microbiol*., vol. 104, pp. 225-231, 1975.

[8] A. Martini, and H. J. Phaff "The optical determination of DNA-DNA homologies in yeasts", *Ann. Microbiol.*, vol. 23, pp. 59-68, 1973.

[9] C. P. Kurtzman, "Resolution of varietal relationships within the species *Hansenula anomala*, *Hansenula bimundalis* and *Pichia nakazawae* through comparisons of DNA relatedness", *Mycotaxon*, vol. 19, pp. 271-279, 1984.

[10] C. P. Kurtzman, "Prediction of biological relatedness among yeasts from comparisons of nuclear DNA complementarity", in *The Expanding Realm of Yeast-like Fungi*, G. S. de Hoog, M. Th. Smith, and A. C. M. Weijman, Eds., Amsterdam: Elsevier, 1987, pp. 459-468.

[11] C. P. Kurtzman, M. J. Smiley, and C. J. Johnson, "Emendation of the genus *Issatchenkia* Kudriavzev and comparison of species by deoxyribonucleic acid reassociation, mating reaction, and ascospore ultrastructure", *Int. J. Syst. Bacteriol*., vol. 30, pp. 503-513, 1980.

[12] G. I. Naumov, S. A. James, E. S. Naumova, E. J. Louis, and I. N. Roberts, " Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae"*, *Int. J. Syst. Evol. Microbiol*., vol. 50, pp. 1931-1942, 2000.

[13] M. Th. Smith, V. Robert, G. A. Poot, W. Epping, and A. W. A. M. de Cock, "Taxonomy and phylogeny of the ascomycetous yeast genus *Zygoascus*, with proposal of *Zygoascus meyerae* sp. nov. and related anamorphic varieties", *Int. J. Syst. Evol. Microbiol.*, vol. 55, pp. 1353-1363, 2005.

[14] A. Vaughan-Martini, and C. P. Kurtzman, " Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces* sensu stricto", *Int. J. Syst. Bacteriol.*, vol. 35, pp. 508-511, 1985.

[15] S. W. Peterson, and C. P. Kurtzman, " Ribosomal RNA sequence divergence among sibling species of yeasts", *Syst. Appl. Microbiol.* vol. 14, pp. 124-129, 1991.

[16] C. P. Kurtzman, and C. J. Robnett, " Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences", *Antonie van Leeuwenhoek*, vol. 73, pp. 331-371, 1998.

[17] J. W. Fell, T. Boekhout, A. Fonseca, G. Scorzetti, and A. Statzell-Tallman, " Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis", *Int. J. Syst. Evol. Microbiol.*, vol. 50, pp. 1351-1371, 2000.

[18] C. P. Kurtzman, and C. J. Robnett, "Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses", *FEMS Yeast Res*., vol. 3, pp. 417-432, 2003.

[19] C. Groth, J. Hansen, and J. Piškur, "A natural chimeric yeast containing genetic material from three species", *Int. J. Syst. Bacteriol*., vol. 49, pp. 1933-1938, 1999.

[20] Z. Liu, and C. P. Kurtzman, "Phylogenetic relationships among species of *Williopsis* and *Saturnospora* gen. nov. as determined from partial rRNA sequences", *Antonie van Leeuwenhoek*, vol. 60, pp. 21-30, 1991.

[21] C. P. Kurtzman, "DNA relatedness among saturn-spored yeasts assigned to the genera *Williopsis* and *Pichia", Antonie van Leeuwenhoek*, vol. 60, pp. 13-19, 1991.

[22] M. A. Lachance, H. M. Daniel, W. Meyer, G. S. Prasad, S. P. Gautam, and K. Boundy-Mills, "The D1/D2 domain of the large-subunit rDNA of the yeast species *Clavispora lusitaniae* is unusually polymorphic", *FEMS Yeast Res.*, vol. 4, pp. 253-258, 2003.

[23] H.-M. Daniel, and W. Meyer, "Evaluation of ribosomal RNA and actin gene sequences for the identification of ascomycetous yeasts", *Int. J. Food Microbiol*., vol 86, pp. 71-78, 2003.

[24] H.-M. Daniel, T. C. Sorrell, and W. Meyer, "Partial sequence analysis of the actin gene and its potential for studying the phylogeny of *Candida* species and their teleomorphs", *Int. J. Syst. Evol. Microbiol., vol*. 51, pp. 1593-1606, 2001.

[25] A. Vaughan-Martini, C. P. Kurtzman, S. A. Meyer, and E. B. O'Neill, "Two new species in the *Pichia guilliermondii* clade: *Pichia caribbica* sp. nov., the ascosporic state of *Candida fermentati*, and *Candida carpophila* comb. nov.", *FEMS Yeast Res*., vol. 5, pp. 463-469, 2005.

[26] D. L. Holzschu, H. J. Phaff, J. Tredick, and D. Hedgecock, "*Pichia pseudocactophila*, a new species of yeast occurring in necrotic tissue of columnar cacti in the North American Sonoran Desert", *Can. J. Microbiol*., vol. 29, pp. 1314-1322. 1983.

[27] H. J. Phaff, M. W. Miller, and M. Miranda, "*Pichia scutulata*, a new species from tree exudates", *Int. J. Syst. Bacteriol.*, vol. 26, pp. 326-331, 1976.

[28] H. J. Phaff, W. T. Starmer, and J. Tredick-Kline, "*Pichia kluyveri* sensu lato - a proposal for two new varieties and a new anamorph", in *The Expanding Realm of Yeast-like Fungi*, G. S. de Hoog, M. Th. Smith, and A. C. M. Weijman, Eds., Amsterdam: Elsevier, 1987, pp. 403-414.

[29] H. J. Phaff, W. T. Starmer, M. A. Lachance, V. Aberdeen, and J. Tredick-Kline, "*Pichia caribaea*, a new species of yeast occurring in necrotic tissue of cacti in the Caribbean area", *Int. J. Syst. Bacteriol*., vol. 42, pp. 459-462, 1992.

[30] R. Shen, and M.-A. Lachance, "Phylogenetic study of ribosomal DNA of cactophilic *Pichia* species by restriction mapping", *Yeast*, vol. 9, pp. 315-330, 1993.

[31] C. P. Kurtzman, C. J. Robnett, and E. Basehoar-Powers, "Phylogenetic relationships among species of *Pichia*, *Issatchenkia* and *Williopsis* determined from multigene phylogenetic analysis, and the proposal of *Barnettozyma* gen. nov., *Lindnera* gen. nov. and *Wickerhamomyces* gen. nov.", *FEMS Yeast Res*., vol. 8, pp. 939-954, 2008.

[32] G. Scorzetti, J. W. Fell, A. Fonseca, and A. Statzell-Tallman, "Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions", *FEMS Yeast Res.*, vol. 2, pp. 495-517, 2002.

[33] M. R. Diaz, and J. W. Fell, " Rapid identification of the varieties and genotypes of *Cryptococcus neoformans* species complex using a high-throughput suspension array", *J. Clin. Microbiol.*, vol. 43, pp. 2662-2672, 2005.

[34] M. R. Diaz, T. Boekhout, B. Theelen, and J. W. Fell, "Molecular sequence analysis of the intergenic spacer (IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans", Syst. Appl. Microbiol.*, vol. 23, pp. 535-545, 2000.

[35] M. R. Diaz, T. Boekhout, T. Kiesling, and J. W. Fell, "Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans", FEMS Yeast Res.*, vol. 5, pp. 1129-1140, 2005.

[36] T. Sugita, R. Ikeda, and T. Shinoda, "Diversity among strains of *Cryptococcus neoformans* var. *gattii* as revealed by sequence analysis of multiple genes and chemotype analysis of capsular polysaccharide", *Microbiol. Immunol*., vol. 45, pp. 757-768, 2001.

[37] M. R. Diaz, and J. W. Fell, "High-throughput detection of pathogenic yeasts of the genus *Trichosporon", J. Clin. Microbiol*., vol. 42, pp. 3696-3706, 2004.

[38] T. Sugita, M. Nakajima, R. Ikeda, T. Matsushima, and T. Shinoda, "Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species". *J. Clin. Microbiol*., vol. 40, pp. 1826-1830, 2002.

[39] M. R. Diaz, and J. W. Fell, "Molecular analysis of ITS and IGS rDNA regions of the psychrophilic yeasts in the genus *Mrakia", Antonie van Leeuwenhoek*, vol. 77, pp. 7-12, 2000.

[40] J. W. Fell and G. M. Blatt, "Separation of strains of the yeasts *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* based on rDNA IGS and ITS sequence analysis", *J. Ind. Microbiol. Biotechnol.*, vol. 23, pp. 677-681, 1999.

[41] J. W. Fell, G. Scorzetti, A. Statzell-Tallman, and K. Boundy-Mills, "Molecular diversity and intragenomic variability in the yeast genus *Xanthophyllomyces*: the origin of *Phaffia rhodozyma*?", *FEMS Yeast Res.*, vol. 7, pp. 1399-1408, 2007.

[42] D. Libkind, A. Ruffin, A. van Brook, L. Alves, and J. P. Sampaio, "Biogeography, host specificity and molecular phylogeny of the basidiomycetous yeast *Phaffia rhodozyma* and its sexual form, *Xanthophyllomyces dendrorhous", Appl. Environ. Microbiol.*, vol. 73, pp. 1120-1125, 2007.

[43] K. O'Donnell, and E. Cignelnik, "Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous", *Mol. Phylogenet. Evol*., vol. 7, pp. 103-116, 1997.

[44] M. Bovers, F. Hagen, E. E. Kuramae, M. R. Diaz, L. Spanjaard, F. Dromer, H. L. Hoogveld, and T. Boekhout, "Unique hybrids between fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii", FEMS Yeast Res*., vol. 6, pp. 599-607, 2006.

[45] M. Goodman, "Protein sequences in phylogeny", in *Molecular Evolution*, F.J. Ayala, Ed., Sunderland, Massachusetts: Sinauer Assoc., 1976, pp. 141-159.

[46] D. E. Dykhuizen, and L. Green, "Recombination in *Escherichia coli* and the definition of biological species", *J. Bacteriol*., vol. 173, pp. 7257-7268, 1991.

[47] K. O'Donnell, H. C. Kistler, B. K. Tacke, and H. H. Casper, "Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab", *Proc. Natl. Acad. Sci. USA*, vol. 97, pp.7905-7910, 2000.

[48] J. W. Taylor, D. J. Jacobson, S. Kroken, T. Kasuga, D. M. Geiser, D. S. Hibbett, and M. C. Fisher, "Phylogenetic species recognition and species concepts in fungi", *Fungal Genet. Biol*., vol. 31, pp. 21-32, 2000.

[49] C. P. Kurtzman, C. J. Robnett, J. N. Ward, C. Brayton, P. Gorelick and T. J. Walsh, "Multigene phylogenetic analysis of pathogenic *Candida* species in the *Kazachstania* (*Arxiozyma*) *telluris* complex and description of their ascosporic states as *Kazachstania bovina* sp. nov., *K. heterogenica* sp. nov., *K. pintolopesii* sp. nov., and *K. slooffiae* sp. nov.", *J. Clin. Microbiol*., vol. 43, pp. 101-111, 2005.

[50] M. E. Bougnoux, A. Tavanti, C. Bouchier, N. A. Gow, A. Magnier, A. D. Davidson, M. C. Maiden, C. D'Enfert, and F. C. Odds, "Collaborative consensus for optimized multilocus sequence typing

of *Candida albicans", J. Clin. Microbiol*., vol. 41, pp. 5265-5266, 2003.

[51] A. R. Dodgson, C. Pujol, D. W. Denning, D. R. Soll, and A. J. Fox, "Multilocus sequence typing of *Candida glabrata* reveals geographically enriched clades", *J. Clin. Microbiol.* vol. 41, pp. 5709-5717, 2003.

[52] M. D. Jacobsen, N. A. Gow, M. C. Maiden, D. J. Shaw, and F. C. Odds, "Strain typing and determination of population structure of *Candida krusei* by multilocus sequence typing", *J. Clin. Microbiol*., vol. 45, pp. 317-323, 2007.

[53] B. A. McManus, D. C. Coleman, G. Moran, E. Pinjon, D. Diogo, M. E. Bougnoux, S. Borecká-Melkusova, H. Bujdákova, P. Murphy, C. d'Enfert, and D. J. Sullivan, "Multilocus sequence typing reveals that the population structure of *Candida dubliniensis* is significantly less divergent than that of *Candida albicans", J. Clin. Microbiol*., vol. 46, pp. 652-664, 2008.

[54] F. C. Odds, and M. D. Jacobsen, "Multilocus sequence typing of pathogenic *Candida* species", *Eukaryot. Cell*, vol. 7, pp. 1075-1084, 2008.

[55] A. Tavanti, A. D. Davidson, E. M. Johnson, M. C. Maiden, D. J. Shaw, N. A. Gow, and F. C. Odds, "Multilocus sequence typing for differentiation of strains of *Candida tropicalis", J. Clin. Microbiol.*, vol. 43, pp. 5593-5600, 2005.

[56] W. Meyer, D. M. Aanensen, T. Boekhout, M. Cogliati, M. R. Diaz, M. Carmela Esposto, M. Fisher, F. Gilgado, F. Hagen, S. Kaocharoen, A. P. Litvintseva, T. G. Mitchell, S. P. Simwami, L. Trilles, M. A. Viviani, and K. J. Kwon-Chung, "Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii", Med. Mycol*., vol. 47, pp. 561-570, 2009.

[57] M. J. Ayoub, J. L. Legras, R. Saliba, and C. Gaillardin, "Application of multi locus sequence typing to the analysis of the biodiversity of indigenous *Saccharomyces cerevisiae* wine yeasts from Lebanon", *J. Appl. Microbiol*., vol. 100, pp. 699-711, 2006.

[58] D. D. Chapman, D. L. Abercrombie, C. J. Douady, E. K. Pikitch, M. J. Stanhopen, and M. S. Shivji, "A streamlined, bi-organelle, multiplex PCR approach to species identification: Application to global conservation and trade monitoring of the great white shark", *Cons. Genet*., vol. 4, pp. 415-425, 2003.

[59] J. W. Fell, "Rapid identification of yeast species using three primers in a polymerase chain reaction", *Mol. Mar. Biol. Biotechnol*., vol. 2, pp. 174-180, 1993.

[60] B. M. Mannarelli, and C. P. Kurtzman, "Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR", *J. Clin. Microbiol*., vol. 36, pp. 1634-1641, 1998.

[61] H. Stender, C. Kurtzman, J. J. Hyldig-Nielsen, D. Sørensen, A. Broomer, K. Oliveira, H. Perry-O'Keefe, A. Sage, B. Young, and J. Coull, "Identification of *Dekkera bruxellensis* (*Brettanomyces*) from wine by fluorescence in situ hybridization using peptide nucleic acid probes", *Appl. Environ. Microbiol.*, vol. 67, pp. 938-941, 2001.

[62] S. Rigby, G. W. Procop, G. Haase, D. Wilson, G. Hall, C. Kurtzman, K. Oliveira, S. Von Oy, J. J. Hyldig-Nielsen, J. Coull, and H. Stender, "Fluorescence in situ hybridization with peptide nucleic acid probes for rapid identification of *Candida albicans* directly from blood culture bottles", *J. Clin. Microbiol*., vol. 40, pp. 2182-2186, 2002.

[63] M. Gadanho, J. M. Almeida, and J. P. Sampaio, "Assessment of yeast diversity in a marine environment in the south of Portugal by microsatellite-primed PCR". *Antonie van Leeuwenhoek*, vol. 84, pp. 217-227, 2003.

[64] M. de Barros Lopes, S. Rainiere, P. A. Henschje, and P. Langridge, "AFLP fingerprinting for analysis of yeast genetic variation", *Int. J. Syst. Bacteriol*., vol. 49, pp. 915-924, 1999.

[65] F. Botterel, C. Desterke, C. Costa, and S. Bretagne, "Analysis of microsatellite markers of *Candida albicans* used for rapid typing", *J. Clin. Microbiol.*, vol. 39, pp. 4076-4081, 2001.

[66] S. R. Fan, F. Y. Bai, Q. P. Liao, Z. H. Liu, J. Li, and X. P. Liu, "Genotype distribution of *Candida albicans* strains associated with different conditions of vulvovaginal candidiasis, as revealed by microsatellite typing", *Sex. Transm. Infect.*, vol. 84, pp. 103-106, 2008.

[67] F. Foulet, N. Nicolas, O. Eloy, F. Botterel, J. C. Gantier, J. M. Costa, and S. Bretagne, "Microsatellite marker analysis as a typing system for *Candida glabrata". J. Clin. Microbiol*., vol. 43, pp. 4574-4579, 2005.

[68] D. Garcia-Hermoso, O. Cabaret, G. Lecellier, M. Desnos-Ollivier, D. Hoinard, D. Raoux, J. M. Costa, F. Dromer, and S. Bretagne, "Comparison of microsatellite length polymorphism and multilocus sequence typing for DNA-Based typing of *Candida albicans", J. Clin. Microbiol*., vol. 45, pp. 3958-3963, 2007.

[69] C. Hennequin, A. Thierry, G. F. Richard, G. Lecointre, H. V. Nguyen, C. Gaillardin, and B. Dujon, "Microsatellite typing as a new tool for identification of *Saccharomyces cerevisiae* strains", *J. Clin. Microbiol*., vol. 39, pp. 551-559, 2001.

[70] J. L. Legras, O. Ruh, D. Merdinoglu, and F. Karst, "Selection of hypervariable microsatellite loci for the characterization of *Saccharomyces cerevisiae* strains", *Int. J. Food Microbiol*., vol. 102, pp. 73-83, 2005.

[71] J. Y. Malgoire, S. Bertout, F. Renaud, J. M. Bastide, and M. Mallié, "Typing of *Saccharomyces cerevisiae* clinical strains by using microsatellite sequence polymorphism", *J. Clin. Microbiol*., vol. 43, pp. 1133-1137, 2005.

[72] I. Masneuf-Pomarède, C. Le Jeune, P. Durrens, M. Lollier, M. Aigle, and D. Dubourdieu, "Molecular typing of wine yeast strains *Saccharomyces bayanus* var. *uvarum* using microsatellite markers", *Syst. Appl. Microbiol*., vol. 30, pp. 75-82, 2007.

[73] M. Špírek, J. Yang, C. Groth, R. F. Petersen, R. B. Langkjær, E. S. Naumova, P. Sulo, G. I. Naumov, and J. Piškur, "*Saccharomyces* sensu lato chromosomes are highly dynamic", *FEMS Yeast Res*., vol. 3, pp. 363-373, 2003.

[74] A. Bergman, V. Fernandez, K. O. Holmström, B. E. Claesson, and H. Enroth, "Rapid identification of pathogenic yeast isolates by real-time PCR and two-dimensional melting-point analysis", *Eur. J. Clin. Microbiol. Infect. Dis*., vol. 26, pp. 813-818, 2007.

[75] M. Khlif, C. Mary, H. Sellami, A. Sellami, H. Dumon, A. Ayadi, and S. Ranque, "Evaluation of nested and real-time PCR assays in the diagnosis of candidaemia", *Clin. Microbiol. Infect.*, vol. 15, pp. 656-661, 2009.

[76] L. Klingspor, and S. Jalal, "Molecular detection and identification of *Candida* and *Aspergillus* spp. from clinical samples using real-time PCR", *Clin. Microbiol. Infect*., vol. 12, pp. 745-753, 2006.

[77] J. Loeffler, N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele, "Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system", *J. Clin. Microbiol*., vol. 38, pp. 586-590, 2000.

[78] N. Wellinghausen, D. Siegel, J. Winter, and S. Gebert, "Rapid diagnosis of candidaemia by real-time PCR detection of *Candida* DNA in blood samples", *J. Med. Microbiol*., vol. 58, pp. 1106-1111, 2009.

[79] G. C. Casey, and D. W. Dobson, "Potential of using real-time PCR-based detection of spoilage yeast in fruit juice - a preliminary study", *Int. J. Food Microbiol*., vol. 91, pp. 327-335, 2004.

[80] L. Cocolin, A. Heisey, and D. A. Mills, "Direct identification of the indigenous yeasts in commercial wine fermentations", *Am. J. Enol. Viti*., vol. 52, pp. 49-53, 2001.

[81] G. Muyzer, and K. Smalla, "Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology", *Antonie van Leeuwenhoek*, vol. 73, pp. 127-141, 1998.

[82] C. B. Meroth, W. P. Hammes, and C. Hertel, "Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis", *Appl. Environ. Microbiol.*, vol. 69, pp. 7453-7461, 2003.

[83] W. Masoud, L. B. Cesar, L. Jespersen, and M. Jakobsen, "Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturing gradient gel electrophoresis", *Yeast*, vol. 21, pp. 549-556, 2004.

[84] C. J. Prakitchaiwattana, G. H. Fleet, and G. M. Heard, "Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes", *FEMS Yeast Res.*, vol. 4, pp. 865-877, 2004.

[85] B. T. Page, and C. P. Kurtzman, "Rapid identification of *Candida* and other clinically important yeast species by flow cytometry", *J. Clin. Microbiol*., vol. 43, pp. 4507-4514, 2005.

[86] M. Bovers, M. R. Diaz, F. Hagen, L. Spanjaard, B. Duim, C. E. Visser, H. L. Hoogveld, J. Scharringa, I. M. Hoepelman, J. W. Fell, and T. Boekhout, "Identification of genotypically diverse *Cryptococcus neoformans* and *Cryptococcus gattii* isolates using Luminex xMAP technology", *J. Clin. Microbiol*., vol. 4, pp. 1874-1883, 2007.

[87] M. R. Diaz, T. Boekhout, M. Bovers, F. J. Cabanes, and J. W. Fell, "Microcoding and flow cytometry as a high-throughput fungal identification system for *Malassezia* species", *J. Med Microbiol*., vol. 55, pp. 1197-1209, 2006.

[88] C. P. Kurtzman, and C.J. Robnett, "Multigene phylogenetic analysis of the *Trichomonascus*, *Wickerhamiella* and *Zygoascus* yeast clades, and the proposal of *Sugiyamaella* gen. nov. and 14 new species combinations", *FEMS Yeast Res*., vol. 7, pp. 141-151, 2007.

[89] C. P. Kurtzman, and M. Suzuki, "Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces"*, *Mycoscience*, vol. 51, pp. 2-14, 2010.

[90] C. P. Kurtzman, J. Albertyn, and E. Basehoar-Powers, "Multigene phylogenetic analysis of the Lipomycetaceae and the proposed transfer of *Zygozyma* species to *Lipomyces* and *Babjevia anomala* to *Dipodascopsis"*, *FEMS Yeast Res*., vol. 7, pp. 1027-1034, 2007.

[91] A. Rokas, B. L. Williams, N. King, and S. B. Carroll, "Genome-scale approaches to resolving incongruence in molecular phylogenies", *Nature*, vol. 425, pp. 798-804, 2003.

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