Validation of the Species Concept in the Genus *Dekkera* by Restriction Analysis of Genes Coding for rRNA

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The gene coding for the small-subunit rRNA of 11 type strains belonging to the genus *Dekkera* and its anamorph, *Brettanomyces*, was amplified by using the polymerase chain reaction and subjected to digestion with a series of restriction endonucleases. Similarity coefficients were calculated from the number of shared and unique fragments, and a cluster analysis yielded four distinct groups with the following ascosporegenous states: *Dekkera anomala*, *Dekkera bruxellensis*, *Dekkera custersiana*, and *Dekkera naardenensis*. Results correlate with evidence from isoenzyme electrophoresis and DNA homology analysis. They also confirm previously reported anamorph-teleomorph connections and recently proposed synonymies within the genus.

Most classification schemes for the genus *Dekkera*, the ascosporegenous genus established by van der Walt in 1964 (20) to accommodate the sexual state of the genus *Brettanomyces*, are based on morphological and physiological characteristics (2, 20, 21). In their recent account of the genus, Barnett and coworkers (2) recognized four species: *Dekkera anomala* (syn. *Dekkera clausenii*), *Dekkera bruxellensis* (syn. *Dekkera abstinens*, *Dekkera intermedia*, and *Dekkera lambica*), *Dekkera custersiana*, and *Dekkera naardenensis*. Anamorph-teleomorph connections between this genus and the imperfect form, *Brettanomyces*, have been reported (7, 9–11).

Although molecular characteristics are increasingly being used in classification for a better understanding of relationships among yeasts, taxonomic affinities among *Dekkera* species are not yet clearly established, and various groupings have been proposed (4, 5, 19–21). Restriction fragment length polymorphisms (RFLPs) and sequencing of the genes coding for rRNAs, which have been used successfully to demonstrate inter- and intraspecific differences in yeasts (8, 12, 13, 22), may provide further bases for species delineation within this genus. In this study, we employed the polymerase chain reaction (PCR) to amplify the gene coding for the small-subunit rRNA in 11 type strains of *Dekkera* and its anamorph, *Brettanomyces*, and used RFLP analysis to determine their degree of relatedness.

MATERIALS AND METHODS

Strains and total DNA extraction. The strains of *Dekkera* used in this study are listed in Table 1. Overnight cultures were harvested by centrifugation at $1,750 \times g$ and incubated for 2 to 3 h in 5 ml of 1.0 M sorbitol–0.1 M EDTA (pH 7.5) containing 5 mg of zymolyase in 20 mM KH_2PO_4 buffer (pH 6.0). The protoplasts formed were centrifuged at $3,000 \times g$ for 5 min and resuspended in 500 μl of TE buffer (50 mM Tris-HCl and 20 mM EDTA). A $\frac{1}{10}$ volume of 10% sodium dodecyl sulfate was added, and the mixture was incubated for 1 h at 65°C. The DNA was precipitated from the suspension by the addition of a $\frac{1}{10}$ volume of 3.0 M sodium acetate and 550 μl of ice-cold 2-propanol. The DNA was pelleted by centrifugation at $10,000 \times g$ for 30 min, dried in a HetoVac for 10 min, and resuspended in 100 μl of distilled

water. It was then treated with 1 μl of 20-mg/ml RNase, incubated at 37°C for 1 h, and reprecipitated prior to its use as an amplification target.

Riboprinting. Prior to amplification with PCR, DNA preparations were adjusted to a concentration of 1 to 50 ng/ μl . PCR was then performed according to the manufacturer's instructions (Perkin-Elmer Cetus) on a PTC-100 thermal cycler (MJ Research) with primers designed to amplify the small-subunit rRNA gene. The primer sequences were as follows: A, 5'-AACCTGGTTGATCCTGCCAGT, and B, 3'-CATCCACTTGGACGTCTTCCTAGT. The amplification program consisted of predenaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min; and a final incubation at 94°C for 5 min to complete the last extension. PCR products were purified with a Gene Clean II kit (Bio 101) and then digested with a series of 10 endonucleases. The restriction enzymes were *Bst*UI, *Dde*I, *Hae*III, *Hin*FI, *Msp*I, *Rma*I, *Rsa*I, *Sau*3AI, *Scr*FI, and *Taq*AI. Restriction fragments were electrophoresed on 2.6% NuSieve 3:1 agarose (FMC Bioproducts) in 0.5 $\times$ Tris-borate buffer with PGEM and *Eco*RI-*Hind*III λ markers (Promega). A matrix constructed by scoring for the presence (scored as 1) or absence (scored as 0) of bands was used as input for the SIMQUAL module of the NTSYS-pc program (15) with the similarity coefficient set to Jaccard. Clustering and tree construction were performed with the SAHN/UPGMA feature. A cophenetic value matrix was constructed (16), and the MXCOMP program was used to compare it with the original matrix in order to test the goodness of fit of the clustering to the data set.

RESULTS

The small-subunit rRNA gene was successfully amplified in all of the strains, and the size of the PCR products, estimated by comparing them with the *Eco*RI-*Hind*III λ marker, was approximately 1.8 kbp. Comigration of the bands indicated similar molecular weights.

Two restriction digests are shown in Fig. 1. Most of the enzymes gave distinctive restriction profiles for *D. custersiana* and *D. naardenensis*. Either *Dde*I (Fig. 1A) or *Rsa*I could be used to discriminate between *D. naardenensis* and the other species. However, *Hae*III and *Bst*UI were non-diagnostic, for they yielded identical patterns. When PCR products were digested with *Rma*I or *Scr*FI, strains in the

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TABLE 1. Species and strains examined^a

Original name	ATCC no.	Other designation(s)
<i>Brettanomyces</i> Kufferath et van Laer (1921)		
<i>B. abstinens</i> Yarrow et Ahearn (1971)	22341 ^T	CBS 6055
<i>B. anomalus</i> Custers (1940)	10559 ^T	CBS 77 ^T , NRRL Y-1415 ^T
<i>B. bruxellensis</i> Kufferath et van Laer (1921)	10560 ^T	CBS 72 ^T , NRRL Y-1411 ^T
<i>B. clausenii</i> Custers (1940)	10562 ^T	CBS 76 ^T , NRRL Y-1414 ^T
<i>B. custersianus</i> van der Walt (1961)	34446 ^T	CBS 4805 ^T , IFO 1585 ^T
<i>B. intermedius</i> Krumbholz et Tauschanoff (1933)	34448 ^T	CBS 73 ^T , IFO 1586 ^T
<i>B. lambicus</i> Kufferath et van Laer (1921)	10563 ^T	CBS 75 ^T , NRRL Y-1413 ^T
<i>B. naardenensis</i> Kolschoten et Yarrow (1970)	22075 ^T	CBS 6042 ^T , IFO 1588 ^T
<i>Dekkera</i> van der Walt (1964)		
<i>D. anomala</i> Smith et van Grinsven (1984)	58985 ^T	CBS 8139 ^T
<i>D. bruxellensis</i> van der Walt (1964)	36234 ^T	CBS 74 ^T , IFO 1590 ^T
<i>D. intermedia</i> van der Walt (1964)	36235 ^T	CBS 4914 ^T

^a ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; IFO, Institute for Fermentation, Osaka, Japan; NRRL, Northern Regional Research Laboratory.

"*anomalus*" and "*bruxellensis*" groups could not be distinguished from each other. Four types of restriction patterns could be recognized among the strains with the enzyme *MspI* (Fig. 1B).

The phenogram generated from cluster analysis of the data set showed two major groups (Fig. 2). Two species of *Dekkera*, *D. bruxellensis* ATCC 36234 and *D. intermedia* ATCC 36235, and the anamorphs *Brettanomyces abstinens* ATCC 22341, *Brettanomyces bruxellensis* ATCC 10560, *Brettanomyces intermedius* ATCC 34448, and *Brettanomyces lambicus* ATCC 10563 formed a single group equivalent to *D. bruxellensis* van der Walt. The second group, consist-

ing of *D. anomala* ATCC 58985, *Brettanomyces anomalus* ATCC 10559, and *Brettanomyces clausenii* ATCC 10562, corresponded to *D. anomala* Smith et van Grinsven. Within each cluster, strains had a Jaccard similarity coefficient of 1.0. *Brettanomyces naardenensis* ATCC 22075 and *Brettanomyces custersianus* ATCC 34446 occurred at separate branches of the tree, with *B. custersianus* showing the greatest divergence from the other strains. Its similarity to the other taxa ranged from 32 to 39%. The cophenetic correlation coefficient (*r*) of 0.99 indicated a very good fit for the cluster analysis (16).

DISCUSSION

The identification of species in the genus *Dekkera* is based mainly on physiological characteristics. However, there is evidence suggesting that assimilative properties may not always be adequate for species delimitation in yeasts, and interstrain variability has been observed (2, 6, 14, 16). Molecular approaches have therefore been used for a better understanding of the taxonomic relationships within this genus (19). We have used restriction analysis of PCR-amplified genes coding for rRNA (rDNA) to validate the species concept in the genus *Dekkera*. The method has a distinct advantage over classical RFLP studies that make use of labeled probes: the need for performing a Southern transfer and subsequent hybridization with a labeled probe is eliminated.

The groupings we obtained from rDNA restriction analysis correlate with the isoenzyme and DNA reassociation data reported previously by Smith et al. (19). They observed that strains in the "*bruxellensis*" group exhibited an 85 to 100%

FIG. 1. Comparison of restriction digests. PCR-amplified small-subunit rDNA was digested with 10 restriction enzymes, and the resulting fragments were electrophoresed on 2.6% NuSieve 3:1 agarose. (A) *DdeI* digests; (B) *MspI* digests. Lanes: MW Marker, PGEM molecular weight standards (molecular weights [in base pairs] are on the left); 22341ab, *B. abstinens* ATCC 22341^T; 10559an, *B. anomalus* ATCC 10559^T; 58985an, *D. anomala* ATCC 58985^T; 10560br, *B. bruxellensis* ATCC 10560^T; 36234br, *D. bruxellensis* ATCC 36234^T; 10562cl, *B. clausenii* ATCC 10562^T; 34446cu, *B. custersianus* ATCC 34446^T; 34448in, *B. intermedius* ATCC 34448^T; 36235in, *D. intermedia* ATCC 36235^T; 10563la, *B. lambicus* ATCC 10563^T; 22075na, *B. naardenensis* ATCC 22075^T.

FIG. 2. Phenogram produced by unweighted pair-group arithmetic average clustering of Jaccard coefficients of similarity between strains. The strain designations are American Type Culture Collection numbers.

similarity in their enzyme pattern and high DNA reassociation values (98 to 100%). Likewise, strains in the "anomala" cluster shared similar isoenzyme profiles and equally high rates of DNA reassociation. There was a sharp decline in reassociation rates when DNA from *D. bruxellensis* was mixed with that from *D. anomala* (19). Within the "bruxellensis" group, *D. bruxellensis* and *D. intermedia* have been found to share antigenic properties (1). Thus, it appears that the two clusters are genetically distinct. The low values for DNA relatedness between members of these groups and either *B. custersianus* or *B. naardenensis* are consistent with the occurrence of these taxa in separate branches of our phenogram. Restriction patterns from the enzyme *MspI* can be used to distinguish between these groups.

Although there is good agreement between our rDNA RFLP analysis and previously reported DNA homology data (19), our results did not correlate consistently with groupings based on morphological and physiological characters. A numerical analysis of standard descriptions has shown both *B. custersianus* and *B. naardenensis* to be valid species (4); this conclusion is supported by our rDNA RFLP data. *B. naardenensis* also appears to be the only species that has coenzyme Q-6 as a minor ubiquinone (3). In contrast, although we found that the remaining strains separated into the "bruxellensis" and "anomala" groups, the overlaps obtained by numerical analysis were so extensive that the two groups were considered conspecific (4). These inconsistencies can be attributed to the use of different character sets: morphological and physiological properties are based on the phenotypic expression, whereas rDNA RFLP analysis and DNA reassociation studies are based on DNA sequences.

The ascosporeogenous genus *Dekkera* is clearly related to the anamorphic genus *Brettanomyces*. Members of these genera produce copious amounts of acetic acid resulting from an incomplete oxybiontic carbohydrate dissimilation (17). They also exhibit a negative Pasteur effect, or Custers effect—i.e., fermentation is stimulated by molecular oxygen and acetoin (18). Furthermore, their coenzyme Q system is of the Q-9 type (3, 23). Mapping of mitochondrial DNA genes in this group has also shown that the sequences for the small rRNA-cytochrome *b* and the large rRNA-cytochrome oxidase subunit 3 are constantly juxtaposed, indicating a close taxonomic affinity (5). Lastly, relationships between the ascosporeogenous and the asporogenous states have been reported previously (7, 8, 10, 11). The results of our rDNA

studies provide further evidence for these anamorph-teleomorph relationships.

On the basis of rDNA restriction patterns and previously reported molecular data (19), we recognize four species in the genus *Dekkera*: (i) *D. bruxellensis* and its facultative synonyms *D. abstinens*, *D. lambica*, and *D. intermedia*; (ii) *D. anomala* and its facultative synonym *D. clausenii*; (iii) *D. naardenensis*; and (iv) *D. custersiana*. These species correspond to the four teleomorphic taxa accepted by Barnett et al. (2). The following modification of the key provided by Smith and coworkers (19) may be used for routine identification:

Key to the species of *Dekkera*

1a. Sucrose fermented	2
1b. Sucrose not fermented	4
2a. Lactose fermented	<i>D. anomala</i>
2b. Lactose not fermented	3
3a. Growth on succinic acid	<i>D. anomala</i>
3b. No growth on succinic acid	<i>D. bruxellensis</i>
4a. Growth on xylose	<i>D. naardenensis</i>
4b. No growth on xylose	5
5a. Growth on galactose	<i>D. bruxellensis</i>
5b. No growth on galactose	<i>D. custersiana</i>

The extent of interstrain variability in rDNA restriction polymorphisms remains to be investigated in the genus *Dekkera*. We are presently examining nontype strains to address this issue.

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