Phylogeny of the Yeast Genera *Hanseniaspora* (Anamorph *Kloeckera*), *Dekkera* (Anamorph *Brettanomyces*), and *Eeniella* as Inferred from Partial 26S Ribosomal DNA Nucleotide Sequences

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Partial 26S ribosomal DNA sequences of species assigned to the genera *Hanseniaspora*, *Kloeckera*, *Dekkera*, *Brettanomyces*, and *Eeniella* were determined. A phylogenetic analysis of the sequences showed that the genus *Eeniella* is derived within the genus *Brettanomyces* and that the genus *Hanseniaspora* (anamorph *Kloeckera*) is not closely related to the genus *Dekkera* (anamorph *Brettanomyces*). As a consequence, the name *Eeniella* is reduced to synonymy with the name *Brettanomyces*. In addition, our data do not support reassignment of certain *Hanseniaspora* species to the recently revived genus *Kloeckera*

**Materials and Methods**

Organisms and culture conditions. The strains which we studied are listed in Table 1. These organisms are maintained in the collections of the Yeast Division of the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research (Peoria, Ill.).

DNA isolation, PCR, and sequencing reactions. DNA was isolated for PCR by using a modified version of the sodium dodecyl sulfate (SDS) protocol of Raeder and Broda (18). Lyophilized cell pellets were pulverized in a 1.5-ml microcentrifuge tube with a pipette tip, resuspended in 1 ml of extraction buffer (200 mM Tris·HCl [pH 8.4], 200 mM NaCl, 25 mM EDTA [pH 8.0], 0.5% SDS), and extracted with phenol-chloroform and chloroform. The DNA was precipitated from the aqueous phase by adding 0.54 volume of isopropanol and was pelleted for ca. 120 s in a microcentrifuge at 55°C for 1 h to overnight. Dilute DNA samples for PCR

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were prepared by adding 4-μl portions of the genomic stock solutions to 1 ml of TE/10 buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA [pH 8.0]).

A divergent domain at the 5’ end of the 26S rDNA gene (6) was amplified by using primers NL 1 (5’-GCATATCAATAA GCAGGAAGAAC) and NL 4 (5’-GGTCGGTCTTCGACG) (16). Single-stranded DNA templates were prepared by using the asymmetric PCR method described by Klenkebock et al. (10), in which only one primer (40 pmol 100 μl−1) was used in each amplification reaction. Twenty PCR cycles were used. Annealing was performed at 50°C, extension was performed at 72°C for 2 min, and denaturation was performed at 94°C for 1 min. Amplified single-stranded DNA was visualized after electrophoresis in 2% NuSieve agarose supplemented with 1% agarose in 1× TPE (0.09 M Tris-phosphate, 0.02 M EDTA [pH 8.0]) by staining the preparation with ethidium bromide (8 × 10−5 μg μl−1). Prior to sequencing, the single-stranded DNA was purified with GeneClean II (Bio 101, La Jolla, Calif.) according to the manufacturer’s instructions.

Both strands of the rDNA regions compared were sequenced by using a chain-terminating dideoxynucleotide Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) and 33S-labeled dATP. Two sequencing reactions were performed for each strand by using 5 pmol of an external primer (NL 1 and NL 4) or an internal primer (NL 3 [5’-AGATGAAAAGAATTTGTTAGGAGAG] and NL 2 [5’-CTCT CTTTTCAAAAGTCTTTCTCTCT]) (16). The sequencing reaction mixtures were electrophoresed for approximately 3 h at 55 W and 2,500 V on 6% or 8% polyacrylamide wedge gels in 1× TBE (Bethesda Research Laboratories, Gaithersburg, Md.). After fixation, the gels were vacuum dried and exposed overnight on Kodak SB film.

Sequence data were read by using an IBM-compatible personal computer equipped with an IBI Pustell system (International Biotechnologies, Inc., New Haven, Conn.) and were visually aligned by using QEdit 2.15 (SemWare, Marietta, Ga.). The sequence of *Saccharomyces cerevisiae* was used as a reference. Phylogenetic relationships were determined by using the PAUP program (version 3.1) (27) and the branch-and-bound search option, followed by bootstrap analysis (1,000 replications). In an alternative analysis we used the heuristic search option of PAUP. The nucleotide sequences of the cytochrome oxidase subunit II gene from members of the genera *Brettanomyces*, *Dekkera*, and *Eeniella* (9) were reanalyzed in the manner described above.

**RESULTS**

The aligned sequences of the 5’ ends of the 26S rDNAs (*Saccharomyces cerevisiae* positions 83 to 653) of the type strains of *Dekkera anomala*, *Dekkera bruxellensis*, *Brettanomyces custersianus*, *Brettanomyces naardenensis*, *Eeniella nana*, *Hanseniaspora occidentalis*, *Hanseniaspora valbyensis*, *Eeniella nanu*, *Brettanomyces* species *D. bruxellensis*, and the type strain of *Kloeckera lindneri* (Kloecher) Janke are shown in Fig. 1. Of the 571 nucleotides sequenced, there were 290 variable sites, and 152 of these sites were phylogenetically informative. In a phylogenetic analysis performed by using the branch-and-bound option of PAUP we found four equally parsimonious trees of 454 steps. On the 50%-majority-rule consensus tree given by the branch-and-bound option, the ingroup tree topology was identical to the results of the bootstrap analysis (100%), as was the branch containing *D. bruxellensis* and *D. anomalus* (Fig. 2). However, the relationships of *B. naardenensis*, *E. nana*, and *B. custersianus* within the clade were weakly resolved, and placement of *B. custersianus* basal to other members of the clade was supported by a low bootstrap value (51%). Nonetheless, *B. custersianus* was basal in all four equally parsimonious trees. Either the alternate phylogenies placed *B. naardenensis* and *E. nana* on a branch adjacent to the *D. bruxellensis-D. anomalus* subclade or
FIG. 1. Alignment of 265 rDNA sequences from positions 83 to 653 for Saccharomyces, Dekkera, Brettanomyces, Eneiella, Hanseniaspora, and Kloekera species. The sequence of Saccharomyces cerevisiae was used as a reference. The dots indicate nucleotides identical to the nucleotides in the reference species, and the dashes indicate deletions. Abbreviations: Sac., Saccharomyces; Dek., Dekkera; Bre., Brettanomyces; Ene., Eneiella; Han., Hanseniaspora; Klo., Kloekera.
B. naardenensis was basal to E. nana. The 50%-majority-rule consensus tree that resulted from the general heuristic search option of PAUP was identical to the tree shown in Fig. 2 (454 steps; consistency index, 0.767; homoplasy index, 0.233; retention index, 0.740; rescaled consistency index, 0.567). Numbers indicate bootstrap values. Branch lengths are proportional to the number of steps. Abbreviations: Sac., Saccharomyces; Dek., Dekkera; Een., Eeniella; Han., Hanseniaspora; Klo., Kloekera. Saccharomyces cerevisiae was used as the designated outgroup in the analysis.

Our results showed that the genus Hanseniaspora is monophyletic and is composed of two subclades (Fig. 2). Subclade A includes H. occidentalis, H. osmophila, and H. vineae, while subclade B is made up of H. guillermondii, H. uvarum, H. valbyensis, and K. lindneri. Variability in the nucleotide sequences of strains of D. anomala and D. bruxellensis, the two species for which multiple strains were analyzed (Table 1), was limited to one or two substitutions. D. bruxellensis CBS 5512, the type strain of the facultative synonym B. bruxellensis, was the only exception. This strain exhibited six nucleotide differences compared with the type strain of D. bruxellensis (data not shown). E. nana exhibited a number of small deletions between nucleotide positions 475 and 556. Brettanomyces, Dekkera, and Eeniella species exhibited greater numbers of nucleotide substitutions than members of the genus Hanseniaspora (Fig. 1 and Table 2).

In the original description of the genus Eeniella (24), Smith et al. indicated that this genus shares phenotypic characteristics with the genera Brettanomyces (Dekkera) and Kloekera (Hanseniaspora). Our data show that the genus Eeniella is derived within the genus Brettanomyces and is not closely related to the genera Hanseniaspora and Kloekera.

**DISCUSSION**

Phylogenetic analyses based on rDNA or rRNA nucleotide sequences have proved to be a valuable tool in fungal systematics (1, 2, 5, 7, 11, 12, 17). We analyzed ca. 600 nucleotides near the 5' ends of the 26S rDNA genes of species belonging to the genera Dekkera (anamorph Brettanomyces), Hanseniaspora (anamorph Kloekera), and Eeniella to determine the phylogenetic relationships of these taxa. The current circumscription of these genera is based mainly on morphological and physiological characteristics, as discussed above. Our analysis showed that the genus Hanseniaspora is not closely related to the genera Dekkera, Brettanomyces, and Eeniella. This finding conflicts with previous suggestions that all of these genera are closely related (25, 29). Our data strongly support the proposal that the name Eeniella should be considered a synonym of the name Brettanomyces, an interpretation consistent with the physiological and biochemical characteristics that all of the species share (i.e., production of acetic acid, occurrence of a Cuff effect, and a similarity in the coenzyme Q systems). As a consequence, bipolar budding, which previously was considered a characteristic of prime taxonomic importance and was used to differentiate the genus Eeniella from the genus Brettanomyces (24, 25), is considered a homoplastic characteristic.

Clark-Walker et al. (3) and Hoeben et al. (9) presented a phylogenetic tree for the genera Brettanomyces and Eeniella that was inferred from sequences of the mitochondrially encoded cytochrome oxidase subunit II (COX2) gene. The 26S rDNA and COX2 gene trees are similar. This is particularly interesting because there are so few groups of yeasts for which relationships have been compared on the basis of two different gene sequences. B. custersianus, B. naardenensis, and E. nana were found to have the smallest mitochondrial genomes that shared the same gene order, and it was suggested that the mitochondrial genomes of these species are ancestral to those of B. anomala and B. bruxellensis (9, 13). This hypothesis is supported by our 26S rDNA sequence comparison data.

The high number (six) of ribosomal nucleotide substitutions in the type strain of B. custersii (CBS 5512), a facultative synonym of D. bruxellensis (25), compared with other strains of D. bruxellensis remains unexplained. The isozyme patterns of
The teleomorphic genus *Kloeckera*, which is characterized by bipolar budding, appears to be monophyletic (Fig. 2). Included in our comparison was *K. lindneri*, a nonascomycous species that proved to be most closely related to *H. valbyensis*. The members of the genus *Hanseniaspora* are heterogeneous with respect to ascospore morphology (23), G+C content (14), proton magnetic resonance spectra of cell wall mannans (26), and serology (28) (Table 3). Recently, Yamada and coworkers (33, 34) determined partial sequences of the 18S and 26S rRNAs of *Hanseniaspora* species and interpreted the resulting dichotomy as evidence that two genera were represented. These workers retained *H. valbyensis*, *H. guilliermondii*, and *H. uvarum* in the genus *Hanseniaspora* but transferred the remaining species to the genus *Kloeckeraspora*, a genus that they revived (34). According to the emended genus description of these authors, the genus *Hanseniaspora* is limited to species which produce hat-shaped ascospores that are released at maturity. However, spheroidal and ledged ascospores, which are not released at maturity, occur in *H. uvarum*. The genus *Kloeckeraspora* differs from the genus *Hanseniaspora* principally by the presence of spheroidal, warty ascospores, which are not released at maturity (33, 34), although smooth spheroidal ascospores with equatorial ledges may be present in *H. occidentalis* as well.

In several respects, *H. occidentalis* belongs in neither the genus *Hanseniaspora* nor the genus *Kloeckeraspora* sensu Yamada et al. The proton magnetic resonance spectra of its cell wall mannans are type 3 spectra. The characteristic spectrum of *Hanseniaspora* spp. is a type 1 spectrum, whereas *Kloeckeraspora* spp. produce type 2 spectra. The same heterogeneity is true for serological reactions (Table 3). For example, three strains of *H. occidentalis* investigated by Tsuchiya and coworkers (as *Kloeckera javanica*, *Kloeckera jensenii*, and *Kloeckera lafarii*) have antigens characteristic of both genera. Only *Kloeckera antillarum* IFO 0669 (= CBS 2578) (*H. occidentalis*) was found to have antigens unique to the genus *Kloeckeraspora*. Because of the lack of correlation between phenotypes, as well as the weak statistical support of the subclades observed in the phylogram of the genus *Hanseniaspora*, we do not accept the proposal of Yamada et al. (33, 34) to reinstate the genus *Kloeckeraspora*.


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REFERENCES


