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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 *Kloeckeraspora*.

Taxonomic separation of the ascomycetous yeast genera *Dekkera* (anamorph *Brettanomyces*) and *Hanseniaspora* (anamorph *Kloeckera*) and the anamorphic ascomycetous genus *Eeniella* is based mainly on the following characteristics: cellular morphology, type of conidiogenesis, comparative physiology, type of coenzyme Q, G+C content, and similarities of DNA and isozymes (14, 24, 25, 35).

The genus *Dekkera* and its anamorph, the genus *Brettanomyces*, are characterized by multipolar budding (25, 30, 31), formation of acetic acid (4), the presence of a negative Pasteur effect (also called the Custers effect) (19–22), and a coenzyme Q that contains nine isoprene units (35). Species have been defined on the basis of nuclear DNA relatedness data and the results of electrophoretic comparisons of cellular enzymes (25) and by ribotyping (15). Currently, four *Brettanomyces* species are distinguished: these species are *Brettanomyces anomalus*, *Brettanomyces bruxellensis*, *Brettanomyces custersianus*, and *Brettanomyces naardenensis*. Teleomorphs are known for two of these species, *Dekkera anomala* (anamorph *B. anomalus*) and *Dekkera bruxellensis* (anamorph *B. bruxellensis*) (25).

The genus *Eeniella* shares some characteristics with the genus *Brettanomyces*, including acetic acid formation, the presence of a Custers effect (20, 24), a coenzyme Q which contains nine isoprene units (35), and a similar mitochondrial DNA gene order (8, 9). However, in current taxonomic treatments, the genus *Eeniella* is recognized as a genus distinct from the genus *Brettanomyces* because it exhibits enteroblastic bipolar budding similar to that observed in members of the genus *Hanseniaspora* (24, 25).

The genus *Hanseniaspora* is characterized by apiculate cells with percurrent, bipolar budding (23) and a coenzyme Q which contains six isoprene units (33, 34). Species are defined on the basis of levels of nuclear DNA relatedness (14), and the species have been supported by the results of partial 18S and 26S rRNA nucleotide sequence analyses (33, 34). However, the genus *Hanseniaspora* is heterogeneous with respect to ascospore morphology (23), serology (28), and proton magnetic resonance spectra of cell wall mannans (26). In their study of partial 18S and 26S rRNA nucleotide sequences of the six recognized species belonging to the genus *Hanseniaspora*, Yamada et al. (33, 34) observed that the species could be divided into two clusters. Because of this dichotomy within the genus, these authors proposed that *Hanseniaspora occidentalis*, *Hanseniaspora osmophila*, and *Hanseniaspora vineae* should be transferred to the genus *Kloeckeraspora*, which they revived.

In this study, we determined the partial nucleotide sequences of a divergent domain near the 5′ end of the 26S ribosomal DNA (rDNA) gene. On the basis of our data, we reanalyzed the phylogenetic relationships among the genera *Eeniella*, *Dekkera* (anamorph *Brettanomyces*), and *Hanseniaspora* (anamorph *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.). Cells of each strain were grown for approximately 48 h at 25°C in 50 ml of yeast extract-malt extract broth (32) on a rotary shaker at 200 rpm and were harvested by centrifugation. After the cells were washed with distilled water, they were resuspended in 2 ml of distilled water, and 1.5 ml of each suspension was pipetted into a microcentrifuge tube. After centrifugation, the excess water was decanted, and the packed cells were lyophilized overnight and stored in a freezer until they were used.

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 10,000 rpm. The pellet was washed gently with 70% ethanol, resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and dissolved by incubating the preparation at 55°C for 1 h to overnight. Dilute DNA samples for PCR

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TABLE 1. *Dekkera*, *Brettanomyces*, *Eeniella*, *Hanseniaspora*, and *Kloeckera* strains studied

Species	Synonym	Strain no.[a]	
		CBS	NRRL
Dekkera anomala M. T. Smith et van Grinsven	*Brettanomyces anomalus* Custers	77^T	Y-1415^T
	Brettanomyces claussenii Custers	76	Y-1414
	Torulopsis cylindrica Walters	1947	YB-4241
Dekkera bruxellensis van der Walt		8139	Y-17522
		74^T	Y-12961^T
	Brettanomyces bruxellensis Kufferath et van Laer	72	Y-1411
	Mycotorula intermedia Krumbholz et Tauschanoff	73	Y-17534
	Dekkera intermedia van der Walt	4914	Y-17524
	Brettanomyces custersii Florenzano	5512	Y-17535
	Brettanomyces abstinens Yarrow et Ahearn	6055	Y-17525
Brettanomyces custersianus van der Walt		4805^T	Y-6653^T
Brettanomyces naardenensis Kolfschoten et Yarrow		6042^T	Y-17526^T
Eeniella nana M. T. Smith et al.		1945^T	Y-17527^T
Hanseniaspora guilliermondii Pijper		465^T	Y-1625^T
Hanseniaspora occidentalis M. T. Smith		2592^T	Y-7946^T
Hanseniaspora osmophila (Niehaus) Phaff et al.		313^T	Y-1613^T
Hanseniaspora uvarum (Niehaus) Shehata et al.		314^T	Y-1614^T
Hanseniaspora valbyensis Klöcker		479^T	Y-1626^T
Hanseniaspora vineae van der Walt et Tscheuschner		2171^T	Y-17529^T
Kloeckera lindneri (Klöcker) Janke		285^T	Y-17531^T
Saccharomyces cerevisiae Meyen ex Hansen		1171^{Tb}	Y-12632^{Tb}

[a] CBS, Centraalbureau voor Schimmelcultures Yeast Division; NRRL, National Center for Agricultural Utilization Research.

[b] Neotype strain.

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 GCGGAGGAAAAG) and NL 4 (5′-GGTCCGTGTTTCAA GACGG) (16). Single-stranded DNA templates were prepared by using the asymmetric PCR method described by Kaltenboeck 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 ^{35}S-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′-AGA TGAAAAGAACTTTGAAAAGAGAG] and NL 2 [5′-CTCT CTTTTCAAAGTTCTTTTCATCT]) (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 vineae*, *Hanseniaspora osmophila*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Hanseniaspora valbyensis*, and *Kloeckera lindneri* 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 genus *Eeniella* is derived within the genus *Dekkera* (anamorph *Brettanomyces*). The ingroup tree topology was identical whether we used *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* as the outgroup. Monophyly of the *Dekkera-Brettanomyces-Eeniella* clade was strongly supported by the results of the bootstrap analysis (100%), as was the branch containing *D. bruxellensis* and *D. anomala* (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. anomala* subclade or

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(83-182)
Sac. cerevisiae     AGTAACGGCG AGTGAAGCGG CAAAAGCTCA AATTTGAAAT CTGGTACCTT CGGTGCCCGA GTTGTAATTT GGAGAGGGCA ACTTTGGGGC CGTTCCT-T-
Dek. bruxellensis   .....T.... .A........ ...G...C.. .......... .G..C.A--- ------.... .......... .....C..G. CAC.A.A.AG GAGGAAGG-C
Dek. anomala        .....T.... .A........ ...G...C.. .......... .A..C-..-. ..CG..TT.. .......... .....C..G. TAC.A.A.A- GAGGGAGGGC
Bre. custersianus   .....T.... .AC....... ...C...C.. .......... ....--.-G. -A-A...... .......... ....GTA.AC TA......-T G-.CTG.G.C
Bre. naardenensis   .......... .......... .........G .......... .C--.-.-.. ...A.---.. .......... .A..CT..TT CT..A...A- GT..TG.T.T
Een. nana           .......... .......... .........G .......... .AA..--T.. .-----TT.. A......... .A...A.CGT CGCAA..--T G.A-GT-G--
Han. occidentalis   .......... .......... .......... .......... ....C..T.. .A...T.... .......... .T...A.TAG TT..G..ACT G..C.T.---
Han. vineae         .......... .......... .......... .......... ....C..TC. .A...T.... .......... .T...A.TAG TT..G...CT G..C.T.---
Han. osmophila      .......... .......... .......... .......... ....C..T.. .A...T.... .......... .T...A.TAG TT..G...CT G..C.T.---
Han. uvarum         .......... .......... T......... .......... .......T.. .A........ .......... .T...ATTTG T.....ATTA G..C.T.---
Han. guilliermondii .......... .......... T......... .......... .......T.. .A........ .......... .T...ATTTG T.....ATTA G..C.T.---
Han. valbyensis     .......... .......... T......... .......... .......T.. .A........ .......... .T...ATTTG T.....ATTT G..C.T.---
Klo. lindneri       .......... .......... T......... .......... .......T.. .A........ .......... .T...ATTTG T.....ATTT G..C.TC---

(183-282)
Sac. cerevisiae     -GT-CT---- ATGTTCCTTG GAACAGGACG TCATAGAGGG TGAGAATCCC GTGTGGCGAG GAGTGC-GGT TCTTTGTAAA GTGCCTTCGA AGAGTCGAGT
Dek. bruxellensis   -.-AT.---- .A..G..... .......CT. C.G....... .....GC... ...AAT..CT .GAG---ACC GA.CAA.T.G TGC..GC... ..........
Dek. anomala        -.-A..---- .A..G..... .......CT. C.G....... .....GC... ...A.T..C. TGAA---CT. GA.CAA.T.G TGC..GC... ..........
Bre. custersianus   -A-A..---- .A..G..... ...T...C.. C.G....... .....GC... ...A.TT..- CTAGC--A.. .A..CAGTTT ..-.T.C... C.........
Bre. naardenensis   T.-G..GCGG .A..G..... .......C.. C.G.G..... .....GC... ..A.C.T.-. CCAGCAA.A. .T.CGTAT.. .G..TAG... ..........
Een. nana           -A.AG.GC-- .A..G..... .......C.. C..AG..... .....GC... ...ACA.T.- CCACCGACAC CG.CA.CG.T .--...-... ..........
Han. occidentalis   -A.-..---- .....T.... .......... .......... ......C... ..A..AT... .CCCC.-A.. C......... AC..T..... ..........
Han. vineae         -..-..---- .......... .......... .......... .......... .......... ..TCC.-A.. .......... AC..T..... ..........
Han. osmophila      -..-T.---- .......... .......... .......... .......... .......... ..TCC.-A.. C......... AC..T..... ..........
Han. uvarum         -..-..---- .......... .......... .......... .......... ..T....... ..TAC.-TT. ...C.....G ACTTT..... ..........
Han. guilliermondii -..-..---- .......... .......... .......... .......... ..T....... ..TAC.-TT. ...C.....G ACTTT..... ..........
Han. valbyensis     -..-..---- .......... .......... .......... .......... ..T....... ..TAC.-AT. .........G ACTTT..... ..........
Klo. lindneri       -..-..---- .......... .......... .......... .......... ..T....... ..TAC.-AT. .........G ACTTT..... ..........

(283-382)
Sac. cerevisiae     TGTTTGGGAA TGCAGCTCTA AGTGGGTGGT AAATTCCATC TAAAGCTAAA TATTGGCGAG AGACCGATAG CGAACAAGTA CAGTGATGGA AAGATGAAAA
Dek. bruxellensis   .......... .......... .......... .T........ ...G...... ....A..... .......... .A........ .......... ..........
Dek. anomala        .......... .......... .......... .T........ ...G...... ....A..... .......... .A........ .......... ..........
Bre. custersianus   .........T .......... .......... .T........ ...G...... ..A.A..... .......... .A........ .......... ..........
Bre. naardenensis   .......... .......... .......... .T........ ...G...... ...G...... .......... .A........ .......... ..........
Een. nana           .......... .......... ..C....... .T........ ...G...... .......... .......... .A........ .......... ..........
Han. occidentalis   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Han. vineae         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Han. osmophila      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Han. uvarum         .......... ........A. .......... .......... .......... .......... .......... .......... .......... ..........
Han. guilliermondii .......... ........A. .......... .......... .......... .......... .......... .......... .......... ..........
Han. valbyensis     .......... ........A. .......... .......... .......... .......... .......... .......... .......... ..........
Klo. lindneri       .......... ........A. .......... .......... .......... .......... .......... .......... .......... ..........

(383-482)
Sac. cerevisiae     GAACTTTGAA AAGAGAGTGA AAAAGTACGT GAAATTGTTG AAAGGGAAGG GCATTTGATC AGACATGGTG TTTT--G--T G-CCCTCTGC TCCTTGTGGG
Dek. bruxellensis   .......... .......... ..T....... .......... .......... .T........ C......... ...--A.CA- .-.GGC.C.T ....C....A
Dek. anomala        .......... .......... ..T....... .......... .......... .T........ C......... ...--A.CA- .-.GGC.C.T ....C....A
Bre. custersianus   .......... .......... ..T....... .......... .......... .T..C..... C...G....A ...--A.-A. .T.G.CT-.. C.T.-....-
Bre. naardenensis   .......... .......... .......... .......... .......... .T........ C........A ...--A.-A. .T.G..T-.. C..C-....-
Een. nana           .......... .......... ..T....... .......... .......... .T........ C....-.ACC ....-A--A- ---------- -------..-
Han. occidentalis   .......... .......... .......... .......... .......... .......... .......... ....--.C-- .-....T... ..........
Han. vineae         .......... .......... .......... .......... .......... .......... .......... ...--G.-AC .-....GC.. ..........
Han. osmophila      .......... .......... .......... .......... .......... .......... .......... ...--G.-AC .-....GC.. ..........
Han. uvarum         .......... .......... .......... .......... .......... .......... .......... ....TT.CA. .-.A..-C.. CT..C.....
Han. guilliermondii .......... .......... .......... .......... .......... .......... ........A. ....TT.CA. .-.A..-C.. CT..C.....
Han. valbyensis     .......... .......... .......... .......... .......... .......... .......... ....TT.CA. .-.A..-C.. CT..C.....
Klo. lindneri       .......... .......... .......... .......... .......... .......... .......... ....TT.CA. .-.A..-C.. CT........

(483-582)
Sac. cerevisiae     TAGGGGAATC TCGC--ATTT CACTGGGCCA GCATCAGTTT TGGTGGCAGG ATAAA----- TCCATAGGAA TGTAG--CTT -G---CC-TC -GGTAA---G
Dek. bruxellensis   .G..T.C.C. .G.---T..A .......... .....G...C ...GA..C-- ...T.CGGGG .T.G.--... ...G.CC... C.ATT.TG.. -..AGG---.
Dek. anomala        .G..T.C.C. .G.---T..A .......... .....G...C ...GA..C-- ...T.CGGGG CAGGC--... ...G.CC... C.ATT.TG.. -..AGG---.
Bre. custersianus   CG-.T.CTC. -GATTTT... -......... ..G.G....- -A.AA.-G.. ..G..AA-GG CGGGA.C... .....C-... CC-TC..T.T G..AG.GGG.
Bre. naardenensis   CG-.C.CTC. --ATCTT... -......... .....G..GC ...---.G.. .C.GGA--GG .TTC.GT... .....CC... C--------- G..A-.----
Een. nana           .G-.T.---- ---------- -------... .....G...G GC.AA.A... .....---GG .GT--G.C.. ...-.CC... C--------- -..A------
Han. occidentalis   CG.A...TA. G.----.GC. .......... A......... ...GA..... .....----- ..TG...... C...--.... ----T.T-.. G..A-.---.
Han. vineae         .GCA...CA. G.----T... .......... A...G..... ...CA..... .....----- ..GT...... C...--..- -.--C.-.. G...-.---.
Han. osmophila      .GCA...CA. G.----T... .......... A......... ...CA..... .....----- ..GCC..... C...--..- -.--C.-.. G...-.---.
Han. uvarum         CTT...CC.. ..AAAA.... .......... A.....A..C ...CA..... .....----- ..AT..A... .....--..- --A--.T-.. -...-.---.
Han. guilliermondii CTT...CC.. ..AAAA.... .......... A........C ...CA..... .....----- ..AT..A... .....--..- --A--.-.. -...-.---.
Han. valbyensis     CTT...CC.. ..AAAA.... .......... A......... ...C..T... .....----- ..AT...... C...--.... -----.T-.. G..--.---.
Klo. lindneri       CTT...CC.. ..AAAA.... .......... A......... ...C...... .....----- ..AT...... ...G.--... -----.T-.. G..--.---.

(583-653)
Sac. cerevisiae     TATTATAGCC TGTGGG-AAT AC-TG-CCAG CTGGGACTGA GGACTGCGA- --CGTAAGT- --------CA A
Dek. bruxellensis   .G.......G C.GAC--.TC TTG..G.T.. .C.....C.G ........GT GA.T.--..C A------C.. .
Dek. anomala        .G.......G CCA.C--C.G ..G..G.T.T .C.....C.G ........GA TT.TA---.C G------C.. .
Bre. custersianus   .G.....A.A .TCCT.-TT. .-G.--T.CT T.CT...... ..T.C...G- ---T.T-T.T ATTA---C.. .
Bre. naardenensis   C-......AA CAG.ATTC.. ..G.CT.TCC .C..T..C.. ........GG AA-------- A------C.. .
Een. nana           -G......-G CC-.C.-C.C .GACTT.TCT TG.C...C.. ........GA AGTAGTGT.T ACTGCTTC.. .
Han. occidentalis   .G........ .A....-... ..-.-G.T.C .......... ........T- --.T.T--.G A-------.. .
Han. vineae         .G...C.... ..GC..-... ..-.-G.T.. .C........ ........T- --.T.T--.G A-------.. .
Han. osmophila      .G...C.... ..GT..-... ..-.-G.T.. .C........ ........T- --.T.T--.G A-------.. .
Han. uvarum         .G.......T .T.T..-... ..-.-GTT.. .C....T... ........-- --.T.C-.-G --------.. .
Han. guilliermondii .G.......T .A.T..-... ..-.-G.T.. ......T... ........-- --.T.C-.-G --------.. .
Han. valbyensis     .G........ .AGT..-... ..-.-AT... .C........ ........-- --.T.T-A.G --------.. .
Klo. lindneri       .G........ .T.T..-... ..-.-GT... .C........ ........-- --.T.T--.G --------.. .
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FIG. 1. Alignment of 26S rDNA sequences from positions 83 to 653 for *Saccharomyces*, *Dekkera*, *Brettanomyces*, *Eeniella*, *Hanseniaspora*, and *Kloeckera* 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*; *Een.*, *Eeniella*; *Han.*, *Hanseniaspora*; *Klo.*, *Kloeckera*.

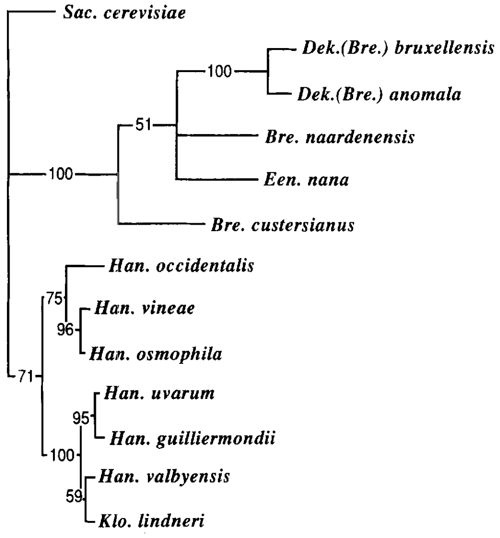

FIG. 2. Fifty-percent-majority-rule branch-and-bound consensus tree of four equally parsimonious phylograms obtained from an analysis of the sequences shown in Fig. 1 (454 steps; consistency index, 0.767; homoplasy index, 0.233; retention index, 0.740; rescaled consistency index, 0.567). Numbers indicate bootstrap values. Branch lengths are proportional to the number of steps. Abbreviations: *Sac.*, *Saccharomyces*; *Dek.*, *Dekkera*; Bre., *Brettanomyces*; *Een.*, *Eeniella*; *Han.*, *Hanseniaspora*; *Klo.*, *Kloeckera*. *Saccharomyces cerevisiae* was used as the designated outgroup in the analysis.

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). In another comparison, deletions (only one of a contiguous group) were coded as a fifth state, and the sequences were reanalyzed by the branch-and-bound option (539 steps; consistency index, 0.764; homoplasy index, 0.236; retention index, 0.733; rescaled consistency index, 0.560). In this analysis, *B. custersianus* was located midway between the branches that supported the species pairs *D. bruxellensis* plus *D. anomala* and *B. naardenensis* plus *E. nana*.

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. guilliermondii*, *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. custersii*, 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 *Kloeckera* (*Hanseniaspora*). Our data show that the genus *Eeniella* is derived within the genus *Brettanomyces* and is not closely related to the genera *Hanseniaspora* and *Kloeckera*.

TABLE 2. Matrix showing numbers of differences for *Brettanomyces*, *Dekkera*, *Eeniella*, *Hanseniaspora*, and *Kloeckera* species and *Saccharomyces cerevisiae* for 26S rDNA nucleotides 83 to 653

Species	No. of nucleotide differences											
	Saccharomyces cerevisiae	*D. bruxellensis*	*D. anomala*	*B. custersianus*	*B. naardenensis*	*E. nana*	*H. occidentalis*	*H. vineae*	*H. osmophila*	*H. uvarum*	*H. guilliermondii*	*H. valbyensis*
D. bruxellensis	153											
D. anomala	150	44										
B. custersianus	165	152	149									
B. naardenensis	159	159	153	151								
E. nana	196	179	175	191	164							
H. occidentalis	72	158	155	160	157	183						
H. vineae	67	151	151	158	159	190	39					
H. osmophila	68	152	152	160	161	189	38	7				
H. uvarum	80	163	159	173	167	204	75	66	67			
H. guilliermondii	77	164	160	173	166	202	73	65	66	6		
H. valbyensis	75	167	164	165	166	201	65	58	59	22	24	
K. lindneri	73	165	160	165	167	200	64	58	59	20	24	9

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 *Kloeckera*), 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*, and this interpretation is consistent with the physiological and biochemical characteristics that all of the species share (i.e., production of acetic acid, occurrence of a Custers 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 homoplasic 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

TABLE 3. Some characteristics of *Hanseniaspora* and *Hanseniaspora* (*Kloeckeraspora*) species

Species	G+C content (mol%)[a]	Coenzyme Q	No. of ascospores[b]	Ascospore morphology[b]	Ledged ascospores	Proton magnetic resonance spectrum type[c]	Antigens[d]
Hanseniaspora (*Kloeckeraspora*) *occidentalis*	36	6	1-2	Spherical, smooth	+	3	1, 2, 3, 5, 6, 7, 8, 10, 28, 40
Hanseniaspora (*Kloeckeraspora*) *vineae*	40	6	1-2	Spherical, warty	−	2	1, 2, 3, 5, 6, 7, 40
Hanseniaspora (*Kloeckeraspora*) *osmophila*	40	6	1-2	Spherical, warty	−	2	1, 2, 3, 5, 6, 7, 40
Hanseniaspora uvarum	34	6	1-2	Spherical, warty	+	1	1, 8, 10, 28
Hanseniaspora guilliermondii	33	6	1-4	Hat-shaped, smooth	+	?	?
Hanseniaspora valbyensis	29	6	1-4(2)	Hat-shaped, smooth	+	1	1, 8, 10, 28

[a] Value to nearest whole number. Data from reference 14.

[b] Data from reference 23.

[c] Data from reference 26.

[d] Data from reference 28.

CBS 5512^T (T = type strain) and CBS 74, the type strain of *D. bruxellensis*, exhibit low levels of similarity (range, 40 to 59%); however, DNA reassociation data (88% complementarity) support the hypothesis that *B. custersii* and *D. bruxellensis* are conspecific (25).

The teleomorphic genus *Hanseniaspora*, which is characterized by bipolar budding, appears to be monophyletic (Fig. 2). Included in our comparison was *K. lindneri*, a nonascosporous 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*.

Because 26S rDNA sequence comparisons support incorporation of the genus *Eeniella* into the genus *Brettanomyces*, we propose the following new combination: *Brettanomyces nanus* (Smith, Batenburg-van der Vegte, et Scheffers) Smith, Boekhout, Kurtzman, et O'Donnell comb. nov. (Basionym: *Eeniella nana* Smith, Batenburg-van der Vegte, et Scheffers. Int. J. Syst. Bacteriol. 31:202, 1981).

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