Systematics of *Hanseniaspora* Zikes and *Kloeckera* Janke

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The physiological and morphological characteristics of eighty-two strains of *Hanseniaspora* and *Kloeckera*, representing twenty-nine described species, were examined. These results along with DNA base composition and DNA/DNA reassociation experiments revealed that the genus *Hanseniaspora* comprises six distinct species, viz. *H. valbyensis*, *H. uvarum*, *H. guilliermondii*, *H. occidentalis*, *H. osmophila* and *H. vineae*, with *K. japonica*, *K. apiculata*, *K. apis*, *K. javanica*, *K. corticis* and *K. africana*, respectively, as their imperfect states.

INTRODUCTION

The genus *Hanseniaspora* Zikes and its imperfect counterpart *Kloeckera* Janke were studied extensively by Miller and Phaff (1958). These authors recognized three species in the genus *Hanseniaspora*, viz. *H. osmophila* (Niehaus) Phaff, Miller et Shifrine, *H. uvarum* (Niehaus) Shehata, Mrak et Phaff and *H. valbyensis* Klöcker. The species *H. guilliermondii* Pijper, *H. melligeri* Lodder and *H. apuliensis* Castelli were taken as synonyms of *H. valbyensis*. The species *H. vineae* van der Walt et Tscheuschner was placed in synonymy with *H. osmophila*.

In the imperfect genus *Kloeckera*, Miller and Phaff recognized four species, viz. *K. africana* (Klöcker) Janke, *K. apiculata* (Reess emend. Klöcker) Janke, *K. corticis* (Klöcker) Janke and *K. javanica* (Klöcker) Janke. Correlating the perfect with the imperfect state, Miller and Phaff suggested *H. uvarum* and *H.*

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valbyensis as perfect forms of *K. apiculata*, and *H. osmophila* as the perfect stage of *K. corticis*. Two imperfect species, *K. africana* and *K. javanica*, could not be identified with any perfect apiculate yeast species.

Nakase and Komagata (1970) in their study on the significance of DNA base composition in the classification of the yeast genera *Hanseniaspora* and *Kloeckera*, recognized four groups. Considering relationships between the perfect and imperfect states, they suggested *H. guilliermondii* and *H. uvarum* as the perfect state of *K. apiculata*. *K. japonica* Saito et Ohtani (1931), a synonym of *K. apiculata* (Lodder and Kreger-van Rij, 1952) was provisionally considered as the imperfect state of *H. valbyensis* because not all synonyms of *K. apiculata* as listed by Lodder and Kreger-van Rij (1952) were examined. *H. osmophila* was taken as the perfect state of *K. africana*. The species *K. javanica* could not be related to a perfect apiculate species. Nakase and Komagata tentatively recognized *H. vineae* as a synonym of *H. osmophila* insofar as both species exhibited the same GC content and the same physiological characteristics. However, these authors suggested that a closer study was necessary to evaluate the status of *H. vineae*, in view of the fact that Novak and Zsolt (1961) considered this species a representative of a new genus, *Vanderwaltia*, based on a difference in ascospore morphology. The species *K. corticis* was placed in synonymy with *K. africana* since Nakase and Komagata considered it unreasonable to separate these species on the basis of the assimilation of sucrose as Miller and Phaff did. Nakase and Komagata found this characteristic to be variable.

Smith (1974) described *H. occidentalis* and regarded this species the perfect form of *K. javanica*.

Using DNA reassociation techniques, Meyer et al. (1977) demonstrated *H. guilliermondii* to be a species separate from *H. valbyensis*. *H. melligeri* was placed in synonymy with *H. guilliermondii*; the species *H. apuliensis* was not considered.

On the basis of DNA reassociation studies, Smith et al. (1977) described *K. apis* as the imperfect form of *H. guilliermondii*. In this study, *K. apiculata* was considered the imperfect state of *H. uvarum*, based on physiological characteristics.

The relationships of *Hanseniaspora* to *Kloeckera* species as proposed by the different authors are presented in Table 1.

To evaluate the status of the different *Hanseniaspora* species and to determine the correct relationship between them and their *Kloeckera* states, the physiological and morphological properties and the DNA relatedness were examined in this study.

Table 1. Some systems on the relationships of *Hanseniaspora* to *Kloeckera*

Authors	Perfect	Imperfect
Miller and Phaff (1958)	*H. uvarum* *H. valbyensis*	*K. apiculata*
	?	*K. africana*
	H. osmophila	*K. corticis*
	?	*K. javanica*
Nakase and Komagata (1970)	*H. guilliermondii* *H. uvarum*	*K. apiculata*
	H. valbyensis	*K. japonica* ?
	H. osmophila *H. vineae* ?	*K. africana*
	?	*K. javanica*
Smith (1974)	*H. occidentalis*	*K. javanica*
Smith et al. (1977)	*H. guilliermondii* *H. uvarum*	*K. apis* *K. apiculata*

MATERIALS AND METHODS

Organisms and physiological and morphological characteristics. Eighty-two cultures were examined (Table 2). These cultures include the type strains of all the *Hanseniaspora* species and synonyms as listed by Phaff (1970*a*), the type strains of all the *Kloeckera* species and synonyms as listed by Phaff (1970*b*), except the type strain of *K. lindneri*, and all species which were not considered by Phaff viz. *H. occidentalis* Smith (1974), *K. japonica* Saito and Ohtani (1931), *K. brevis* var. *rohrbachense* Szilvinyi and Kaulich (1948), *K. apis* Lavie ex Smith et al. (1977) and *K. corticis* var. *pulquensis* Ulloa and Herrera (1973) which was placed in synonymy with *K. apiculata* by von Arx et al. (1977). The physiological properties were investigated according to the methods given in "The Yeasts – a taxonomic study" (Lodder, 1970). The cultures used in the assimilation tests were incubated on a shaker for 21 days. Ascosporulation was induced on 5% malt-extract agar. Ascospores were measured according to the method used by Barnett and Buhagiar (1971), i.e. the longest axis and the widest part at right angles to it were measured.

Table 2. List of cultures

Organisms and strain designation	Source
H. guilliermondii	
CBS 465, ATCC 10630	Diseased nail. Type strain.
CBS 95	Fermenting tomatoes.
CBS 466	Dates. Type strain of *H. melligeri*
CBS 1972	Grape juice. Type strain of *H. apuliensis*
CBS 2567	Grape must
CBS 2574	Grape juice
CBS 4378	Caecum of baboon
CBS 5060	Culture contaminant
CBS 6619	Received from T. Nakase as AJ 5176
CBS 6707	Received from T. Nakase as AJ 5175
H. occidentalis	
CBS 2592, ATCC 32053	Soil in France. Type strain
CBS 2569	*Drosophila* species
CBS 6782	Orange juice
CBS 6783	Orange juice
H. osmophila	
CBS 313, ATCC 24231	Grapes. Type strain
H. uvarum	
CBS 314	Grapes. Type strain
CBS 276, ATCC 10634	Soil in Denmark
CBS 279	Type strain of *K. brevis*
CBS 312	Fermenting cacao
CBS 2566	*Drosophila obscura*
CBS 2570	*Drosophila* species
CBS 2579	Soil in Austria. Type strain of *K. austriaca*
CBS 2581	Unknown
CBS 2582	Throat
CBS 2583	Fermenting cucumber brine
CBS 2584, ATCC 9774	P. R. Burkholder 188 (*K. brevis*)
CBS 2585	Baker's yeast. Type strain of *K. lodderi*
CBS 2586	Caterpillar
CBS 2587	Fruit must. Type strain of *K. brevis* var. *rohrbachense*
CBS 2589	Grape must
CBS 5072	Intertidal bathing area
CBS 5073	Grapes
CBS 5074	Apple must
CBS 5450	Sea water
CBS 5914, ATCC 18212	NCYC 245 (*K. brevis* strain B-768)
CBS 5934	Cider
CBS 6617	Received from T. Nakase as AJ 4800
ATCC 18859	Lake Champlain, LCA 40
ATCC 34535	Lake Champlain, LCA 42
ATCC 34536	Lake Champlain, LCA 43
ATCC 34537	North Sea, NS 1057
ATCC 34538	North Sea, NS 1065

H. valbyensis	
CBS 479, ATCC 10631	Soil in Denmark. Type strain
CBS 311	Beer
CBS 480	Obtained from O. Winge (Copenhagen)
CBS 481, ATCC 2108	Received from the ATCC via W. L. Miller (Canada)
CBS 6618	Tomato. Received from T. Nakase as AJ 4810
H. vineae	
CBS 2171	Soil in South Africa. Type strain
CBS 2568	*Drosophila persimilis*
CBS 2827	Soil in Sardinia
CBS 5068	Obtained from O. Verona
CBS 6555, ATCC 20109 ATCC 20131	Takeda Chem. Ind. Ltd
ATCC 10632	Sour Calimyrma fig, San Joaquin Valley
K. africana	
CBS 277, ATCC 24232	Soil in Algeria. Type strain
CBS 6706	Obtained from T. Nakase as AJ 5174
ATCC 16512	Received from Kyowa Ferm. Ind. Co., Ltd.
K. apiculata	
CBS 104	Obtained from O. Winge. Type strain
CBS 286, ATCC 10639	Soil. Type strain of *K. lindneri* var. *pelliculosa*
CBS 287	Soil. Type strain of *K. muelleri*
CBS 2580	Soil. Type strain of *K. germanica*
NCYC 588	Rotting strawberries
K. apis	
CBS 2591	Bee. Type strain
K. corticis	
CBS 106, ATCC 10635	Bark and moss. Type strain
CBS 105, ATCC 10640	Grapes. Type strain of *K. magna*
CBS 1999	Soil in France. Type strain of *K. santacruzensis*
CBS 4266	Cider
CBS 6554, ATCC 20111	Received from Takeda Chem. Ind., Ltd.
CBS 6622	Received from IFO as IFO 0670
CBS 6704	Obtained from T. Nakase as AJ 5172
K. japonica	
CBS 281	Exudate of tree. Type strain
CBS 2590	Draught beer
CBS 6558	Pulque. Type strain of K. *corticis* var. *pulquensis*
NCYC 468	Spoiled beer
NCYC 766	Isolated by R. Davenport
K. javanica	
CBS 282, ATCC 24234	Soil in Java. Type strain
CBS 280	Soil in West Indies. Type strain of *K. antillarum*
CBS 283, ATCC 10637	Soil in Java. Type strain of *K. jensenii*
CBS 284, ATCC 24174	Soil in Java. Type strain of *K. javanica* var. *lafarii*
CBS 2335	Soil in Himalayas. Type strain of *K. indica*
CBS 2578	Soil in West Indies. Type strain of *K. willi*
CBS 6623	Obtained from T. Nakase as AJ 5195
CBS 6624	Obtained from T. Nakase as AJ 5197
ATCC 20110	Takeda Chem. Ind., Ltd. (IFO 1095) Production of steroids (U.S. Pat. 3,616,225)

DNA base composition and DNA reassociation. Yeasts were grown in a liquid medium composed of glucose (4%), peptone (0.5%), and yeast extract (0.5%) for approximately 24 h on a gyrotary shaker at room temperature. Cells were harvested by centrifugation and washed twice with saline – EDTA (0.15 M sodium chloride and 0.01 M sodium ethylenediamine tetraacetate, pH 8.0). DNA was isolated and purified according to the methods previously described (Meyer and Phaff, 1969). DNA base composition (expressed as the mean molar percent of the guanine and cytosine content, % GC) was determined by the thermal denaturation method and formula, % GC = (Tm – 69.3)/0.41, of Marmur and Doty (1962). DNA from the type culture of *Candida parapsilosis* (ATCC 22019) was used as the standard DNA (Tm = 85.9 C). The DNA filter reassociation technique (Denhardt, 1966) was employed with modifications: DNA was extracted from cultures grown for 17 to 27 h in 5% glucose in Yeast Nitrogen Base (Difco) supplemented with 1.2 or 2.0 μCi/ml of both uracil-6-^{3}H and adenine-8-^{3}H. DNA was sheared by passing the sample twice through a French mini-pressure cell at 20000 psi. Vials containing one 1-cm filter with approximately 25 μg of immobilized single-stranded DNA were incubated with preincubation medium (PM) at 64 $\pm$ 1 C for 2.5 to 2.75 h. The PM was removed and 0.8 to 1.0 μg of fragmented, labeled DNA in 100 μl of 2 $\times$ SSC (SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0 $\pm$ 0.2) was added to each vial. Incubation was continued at 64C in a reciprocal water bath at ca. 20 strokes per min for 13 to 15 h. The filters were washed in 2 $\times$ SSC at 65C and allowed to dry before addition of scintillation fluid (Spectrafluor PPO-POPOP Amersham/Searle; prepared in toluene-Triton X-100 2:1 vol/vol). Relatedness was calculated from the following equation: (disintegrations per min (dpm) of bound ^{3}H-DNA in the heterologous reaction)/(dpm of bound ^{3}H-DNA in the homologous reaction) $\times$ 100 = percentage of genetic relatedness.

RESULTS

Physiological and morphological characteristics. All *Hanseniaspora* and *Kloeckera* cultures fermented glucose and assimilated glucose, cellobiose, salicin, arbutin and glucono-δ-lactone. Salient differences were found in the fermentation of sucrose and the assimilation of sucrose, maltose, glycerol and 2-keto-gluconate and in maximum temperature of growth. The sporogenous *Hanseniaspora* cultures showed differences in number per ascus and shape of ascospores produced. These salient physiological and morphological characteristics are presented in Table 3. Based on these characteristics, the *Hanseniaspora* and *Kloeckera* strains could be divided into 6 groups.

The *H. valbyensis* group represents five sporulating strains (CBS 311, CBS 480, CBS 481, CBS 6618 and the type strain of *H. valbyensis* CBS 479) and five

Table 3. Salient characteristics to separate *Hanseniaspora* and *Kloeckera* species

Perfect	Imperfect	No. of strains[1]	Fermentation sucrose	Assimilation sucrose	Assimilation maltose	Assimilation glycerol	Assimilation 2-ketogluconate	Growth at 34 C	Growth at 37 C	No. and form of ascospores	Mean + standard deviation of ascospores in μm Length	Mean + standard deviation of ascospores in μm Breadth
H. valbyensis		4	–	–	–	–	–		–	1–2 hat	2.3 ± 0.3	1.7 ± 0.2
	K. japonica	5	–	–	–	–	–		–			
CBS 311		1	–	–	–	–	–		–	1–4 hat	1.6 ± 0.2	1.2 ± 0.2
H. uvarum		27	–	–	–	–	+		–	1–2 round, warty and/or smooth, with equatorial ledge		nd
	K. apiculata	5	–	–	–	–	+		–			
H. guilliermondii		10	–	–	–	–	+		+	1–4 hat	1.8 ± 0.2	1.3 ± 0.2
	K. apis	1	–	–	–	–	+		+			
H. occidentalis		4	+	+	–	+	–		–	1–2 round, smooth with equatorial ledge		nd
	K. javanica	8	+	+	–	+	–		–			
H. osmophila		1	–	–	+	–	–	–	–	1–2 round, warty		nd
	K. corticis	7	–	v	+	–	–	–	–			
H. vineae		6	–	v	+	–	–	+	–	1–2 round, warty		nd
	K. africana	3	–	v	+	–	–	+	–			

[1] Including the type strain of the species; v = variable; nd = not determined.

non-sporulating strains (CBS 2590, NCYC 468, NCYC 766 and the type strains of *K. japonica* CBS 281 and *K. corticis* var. *pulquensis* CBS 6558). These cultures failed to assimilate sucrose, maltose, glycerol and 2-keto-gluconate and to grow at 37C. Four sporulating cultures produced 1–2, mostly 2, hat-shaped ascospores per ascus ($2.3 \pm 0.3 \times 1.7 \pm 0.2$ μm). One strain CBS 311 produced 1–4, mostly 4, hat-shaped ascospores per ascus ($1.6 \pm 0.2 \times 1.2 \pm 0.2$ μm).

The *H. uvarum* group represents twenty-seven sporogenous strains including the type strains of *H. uvarum* CBS 314, *K. austriaca* CBS 2579, *K. brevis* CBS 279, *K. brevis* var. *rohrbachense* CBS 2587 and *K. lodderi* CBS 2585 and five non-sporulating cultures (NCYC 588 and the type strains of *K. apiculata* CBS 104, *K. lindneri* var. *pelliculosa* CBS 286, *K. muelleri* CBS 287 and *K. germanica* CBS 2580). These cultures failed to assimilate sucrose, maltose and glycerol and to grow at 37C. They assimilated 2-keto-gluconate. Sporulating cultures produced 1–2 round, warty and/or smooth ascospores per ascus with an equatorial or subequatorial ledge.

The *H. guilliermondii* group represents ten sporulating cultures including the type cultures of *H. guilliermondii* CBS 465, *H. melligeri* CBS 466 and *H. apuliensis* CBS 1972, and one non-sporulating strain, the type strain of *K. apis* CBS 2591. These cultures failed to assimilate sucrose, maltose and glycerol. They assimilated 2-keto-gluconate and grew at 37C. Sporulating strains produced 1–4, mostly 4, hat-shaped ascospores per ascus ($1.8 \pm 0.2 \times 1.3 \pm 0.2$ μm).

The *H. occidentalis* group represents four sporulating cultures (CBS 2569, CBS 6782, CBS 6783 and the type strain of *H. occidentalis* CBS 2592) and eight non-sporulating strains including the type cultures of *K. javanica* var. *javanica* CBS 282, *K. javanica* var. *lafarii* CBS 284, *K. antillarum* CBS 280, *K. jensenii* CBS 283, *K. indica* CBS 2335 and *K. willi* CBS 2578. These cultures fermented sucrose and assimilated sucrose and glycerol. They failed to assimilate maltose and 2-keto-gluconate. Sporulating strains produced 1–2 ascospores per ascus which were smooth and round with an equatorial ledge.

The *H. osmophila* group represents one sporulating culture, the type strain of *H. osmophila* CBS 313 and seven non-sporulating strains including the type strains of *K. corticis* CBS 106, *K. magna* CBS 105 and *K. santacruzensis* CBS 1999. These cultures assimilated maltose and failed to assimilate glycerol and 2-keto-gluconate. Assimilation of sucrose was variable. Growth at 34C was negative. The sporulating culture produced 1–2 ascospores per ascus which were round and warty.

The *H. vineae* group represents six sporulating cultures (CBS 2568, CBS 2827 CBS 5068, CBS 6555, ATCC 10632 and the type culture of *H. vineae* CBS 2171) and three non-sporulating cultures (CBS 6706, ATCC 16512 and the type strain of *K. africana* CBS 277). These cultures were characterized by assimilation of maltose and growth at 34C. Assimilation of sucrose was variable and assimilation of glycerol and 2-keto-gluconate was negative. The sporulating

cultures produced 1–2 round and warty ascospores per ascus.

DNA base composition. The DNA base composition was determined for 67 of the *Hanseniaspora* and *Kloeckera* strains (Table 4). The GC values spanned a range of 28.8 to 40.7% with *H. valbyensis* group at the lower end and *H. osmophila* and *H. vineae* group at the upper limits. The average and median GC percentages, respectively, of the groups were:

H. valbyensis group	29.2%, 29.2%
H. guilliermondii group	33.4%, 33.3%
H. uvarum group	34.1%, 34.4%
H. occidentalis group	35.5%, 35.6%
H. osmophila group	40.3%, 40.5%
H. vineae group	40.2%, 40.5%

DNA relatedness within Hanseniaspora and Kloeckera

A. *H. valbyensis, H. uvarum, H. guilliermondii and their imperfect counterparts.*

The results of the DNA reassociation studies on *H. valbyensis, H. uvarum, H. guilliermondii* and their imperfect counterparts are presented in Table 5. A high degree of reassociation (81–100%) was demonstrated between the DNA from the type culture of *H. valbyensis* (ATCC 10631) and the DNA from the strains physiologically identical with *H. valbyensis* and *K. japonica.* Insignificant DNA reassociation was exhibited with strains of the other five groups.

Because of the unique properties of strain CBS 311, physiologically like *H. valbyensis* and morphologically like *H. guilliermondii*, additional DNA reassociation experiments were performed to verify the relatedness of this strain and the strains grouped in *H. valbyensis* and *K. japonica.* These results are presented in Table 6. Strain CBS 311 revealed significant DNA reassociation with strains of *H. valbyensis* and *K. japonica* and little DNA reassociation with *H. guilliermondii, K. apis, H. uvarum* and *K. apiculata.*

A high degree of reassociation (82–100%) was demonstrated between the DNA from the type culture of *H. uvarum* (CBS 314) and the DNA of strains physiologically identical with *H. uvarum* and *K. apiculata.* A low degree of DNA reassociation was revealed with strains of the other five groups.

Reassociation between 91–100% was demonstrated between the DNA from the type culture of *H. guilliermondii* (ATCC 10630) and the DNA of strains physiologically identified as *H. guilliermondii* and *K. apis.* A low degree of DNA reassociation was evident with strains of the other five groups.

B. *H. occidentalis, H. osmophila, H. vineae and their imperfect counterparts.*

The results of the DNA reassociation studies on *K. javanica, H. osmophila, H. vineae* and their counterparts are presented in Table 8. In these experiments labeled DNA of *K. javanica* was used instead of *H. occidentalis.*

A high degree of reassociation (75–100%) was demonstrated between the

Table 4. DNA base composition of selected strains of various *Hanseniaspora* and *Kloeckera* species

Perfect	Imperfect	Tm + standard deviation[a]	% GC
H. valbyensis			
CBS 479[b]		81.6 ± 0.03	30.0
CBS 311		81.1 ± 0.13	28.8
CBS 480		81.4 ± 0.13	29.5
CBS 481		81.4 ± 0.09	29.5
CBS 6618		81.4 ± 0.12	29.5
	K. japonica		
	CBS 281[b]	81.2 ± 0.17	29.0
	NCYC 766	81.1 ± 0.12	28.8
	CBS 2590	81.2 ± 0.10	29.0
	CBS 6558	81.2 ± 0.06	29.0
	NCYC 468	81.3 ± 0.21	29.3
H. uvarum			
CBS 314[b]		83.2 ± 0.12	33.9
CBS 2585		82.8 ± 0.10	32.9
CBS 6617		83.0 ± 0.10	33.4
CBS 2570		83.1 ± 0.12	33.7
ATCC 18859		83.1 ± 0.20	33.7
CBS 5914		83.2 ± 0.07	33.9
CBS 276		83.3 ± 0.12	34.1
CBS 2579		83.3 ± 0.10	34.1
CBS 279		83.4 ± 0.15	34.4
CBS 2581		83.4 ± 0.29	34.4
CBS 2584		83.4 ± 0.15	34.4
ATCC 34536		83.4 ± 0.10	34.4
ATCC 34535		83.6 ± 0.05	34.9
ATCC 34537		83.6 ± 0.05	34.9
ATCC 34538		83.6 ± 0.10	34.9
CBS 2587		83.7 ± 0.09	35.1
	K. apiculata		
	CBS 104[b]	82.7 ± 0.10	32.7
	CBS 286	82.9 ± 0.10	33.2
	CBS 2580	83.4 ± 0.13	34.4
	CBS 287	83.5 ± 0.15	34.6
H. guilliermondii			
CBS 465[b]		82.9 ± 0.16	33.2
CBS 5060		82.8 ± 0.17	32.9
CBS 6619		82.9 ± 0.10	33.2
CBS 2567		82.9 ± 0.10	33.2
CBS 4378		82.9 ± 0.11	33.2
CBS 95		83.0 ± 0.19	33.4
CBS 466		83.0 ± 0.14	33.4
CBS 1972		83.2 ± 0.21	33.9
CBS 6707		83.2 ± 0.26	33.9
CBS 2574		83.3 ± 0.10	34.2
	K. apis		
	CBS 2591[b]	83.2 ± 0.11	33.9

Perfect	Imperfect	Tm + standard deviation[a]	%GC
H. occidentalis			
CBS 2592[b]		83.6 ± 0.15	34.9
CBS 6782		83.9 ± 0.07	35.6
CBS 6783		84.0 ± 0.19	35.9
	K. javanica		
	CBS 282[b]	83.6 ± 0.16	34.9
	CBS 284	83.6 ± 0.15	34.9
	CBS 2578	83.7 ± 0.17	35.1
	CBS 2335	83.8 ± 0.15	35.4
	CBS 283	83.9 ± 0.05	35.6
	CBS 6623	83.9 ± 0.16	35.6
	CBS 6624	83.9 ± 0.12	35.6
H. osmophila			
CBS 313[b]		85.9 ± 0.07	40.5
	K. corticis		
	CBS 106[b]	85.9 ± 0.16	40.5
	CBS 105	85.6 ± 0.09	39.8
	CBS 4266	85.8 ± 0.23	40.2
	CBS 6704	85.8 ± 0.13	40.2
	CBS 1999	85.9 ± 0.12	40.5
	CBS 6622	85.9 ± 0.05	40.5
H. vineae			
CBS 2171[b]		85.8 ± 0.16	40.2
CBS 2827		85.3 ± 0.09	39.0
CBS 2568		85.9 ± 0.05	40.5
CBS 5068		86.0 ± 0.20	40.7
CBS 6555		86.0 ± 0.07	40.7
ATCC 10632		86.0 ± 0.00	40.7
	K. africana		
	CBS 277[b]	85.2 ± 0.09	38.8
	ATCC 16512	85.9 ± 0.09	40.5
	CBS 6706	85.9 ± 0.00	40.5

[a] Average of at least four Tm determinations.
[b] Type strain of species.

DNA from the type culture of *K. javanica* (ATCC 24234) and the DNA of the strains physiologically identical with *H. occidentalis* and *K. javanica*. An insignificant DNA reassociation was exhibited with strains physiologically identified as *H. osmophila*, *K. corticis*, *H. vineae*, *K. africana*, *H. uvarum* and *K. apiculata*.

The DNA from the type culture of *H. osmophila* (ATCC 24231) reassociated to a high degree with the cultures physiologically identical with *H. osmophila*

Table 5. DNA relatedness of *H. valbyensis* (ATCC 10631), *H. uvarum* (CBS 314) and *H. guilliermondii* (ATCC 10630) to various strains of *Hanseniaspora* and *Kloeckera*

Source of unlabeled DNA	% relative binding of DNA from *H. valbyensis* ATCC 10631[a]	*H. uvarum* CBS 314[a]	*H. guilliermondii* ATCC 10630[a]
H. valbyensis			
ATCC 10631	**100**[b]	20	13
CBS 481	100		16
CBS 311	99	10	10
CBS 480	89	19	13
CBS 6618	81	28	16
K. japonica			
CBS 281	100	27	10
CBS 6658	100	11	7
CBS 2590	94		11
H. uvarum			
CBS 314	10	**100**[b]	15
CBS 2570		100	
CBS 2581	13	100	
CBS 2585		100	14
CBS 2587		100	24
ATCC 18859		100	
ATCC 34535		100	
ATCC 34537		96	
CBS 2579		93	15
CBS 279	15	92	25
ATCC 34536		91	
CBS 276		90	
CBS 6617		88	
CBS 5914		87	12
CBS 2584		86	
ATCC 34538	16	82	
K. apiculata			
CBS 287	14	100	16
CBS 2580	11	100	29
CBS 104		100	11
CBS 286	16	95	19
H. guilliermondii			
ATCC 10630	19	27	**100**[b]
CBS 95	19	26	100
CBS 466	14	20	100
CBS 1972			100
CBS 2567	23		100
CBS 2574		18	100
CBS 4378	13	21	100
CBS 5060		22	100
CBS 6619	9		100
CBS 6707	10		91
K. apis			
CBS 2591	11	30	100

Source of unlabeled DNA	% relative binding of DNA from		
	H. valbyensis ATCC 10631[a]	*H. uvarum* CBS 314[a]	*H. guilliermondii* ATCC 10630[a]
H. occidentalis (*K. javanica*) group			
CBS 2592	11		7
CBS 282			14
CBS 6782	8		7
CBS 6783		2	16
CBS 283		6	
CBS 284	12		6
CBS 2335		1	
ATCC 20110	6		
H. osmophila (*K. corticis*) group			
CBS 106		1	
CBS 105		7	
CBS 313		13	
CBS 6554	12		
H. vineae (*K. africana*) group			
CBS 2171		10	
CBS 6555	15		
CBS 2827	10		12
ATCC 16512	5		

[a] Source of labeled DNA.
[b] Homologous reaction.

and *K. corticis*. An insignificant DNA reassociation was exhibited with strains physiologically identified as *H. occidentalis*, *K. javanica*, *H. uvarum* and *K. apiculata*, however, DNA reassociations of greater magnitude (38–46%) were exhibited with strains physiologically grouped as *H. vineae* and *K. africana*. An additional DNA reassociation experiment using labeled DNA from the type strain of *K. corticis* concurred with these findings (Table 7). DNA relatedness between 40 and 47% was revealed between this species and three strains of the *H. vineae* (*K. africana*) group, which included the type strains.

Reassociation between 93–100% was demonstrated between the DNA from the type culture of *H. vineae* (CBS 2171) and the DNA of strains physiologically identical with *H. vineae* and *K. africana*. DNA reassociation of 45–60% was exhibited with strains physiologically identified as *H. osmophila* and *K. corticis*.

Table 6. DNA relatedness between *H. valbyensis* (CBS 311) and various strains of *Hanseniaspora* en *Kloeckera*

Source of unlabeled DNA	% relative binding CBS 311[a]
H. valbyensis (K. japonica)	
CBS 311	**100**[b]
CBS 480	100
CBS 481	100
CBS 281	100
ATCC 10631	80
H. uvarum (K. apiculata)	
CBS 314	25
CBS 276	24
CBS 287	22
CBS 2585	21
CBS 2580	20
CBS 104	16
H. guilliermondii (K. apis)	
ATCC 10630	13
CBS 6617	13
CBS 2591	12
CBS 2574	12
CBS 6619	10

[a] Source of labeled DNA.
[b] Homologous reaction.

Table 7. DNA relatedness between *Kloeckera corticis* (ATCC 10635) and selected strains of *Hanseniaspora* and *Kloeckera*

Source of unlabeled DNA	% relative binding ATCC 10635[a]
K. corticis	
ATCC 10635	**100**[b]
CBS 1999	84
H. osmophila	
ATCC 24231	92
H. vineae	
CBS 2171	47
CBS 2827	45
K. africana	
ATCC 24232	40
H. uvarum	
ATCC 9774	8

[a] Source of labeled DNA.
[b] Homologous reaction.

Table 8. DNA relatedness of *K. javanica* (ATCC 24234), *H. osmophila* (ATCC 24231) and *H. vinae* (CBS 2171) to various strains of *Hanseniaspora* and *Kloeckera*

Source of unlabeled DNA	% relative binding of DNA from *K. javanica* ATCC 24234[a]	*H. osmophila* ATCC 24231[a]	*H. vineae* CBS 2171[a]
H. occidentalis			
CBS 2592	75		
CBS 6782	82	17	
CBS 6783	87	15	
K. javanica			
ATCC 24234	**100**[b]	19	
ATCC 20110	100	25	
CBS 284	100		
H. osmophilia			
ATCC 24231		**100**[b]	60
K. corticis			
CBS 6554	17	100	
CBS 105	13	98	53
CBS 106	15	85	56
CBS 1999			55
CBS 4266			46
CBS 6622			45
CBS 6704			56
H. vineae			
CBS 2171			**100**[b]
CBS 6555	13	38	100
CBS 2827	14		100
CBS 2568			98
CBS 5068			98
ATCC 10632	7	43	95
K. africana			
ATCC 24232	8	46	93
ATCC 16512	11	42	100
CBS 6706			96
H. uvarum			
(K. apiculata) group			
CBS 279			5
CBS 5914		15	
ATCC 34537	10		
ATCC 34536	10		

[a] Source of labeled DNA.
[b] Homologous reaction.

DISCUSSION

The DNA reassociation experiments, supplemented with the physiological and morphological data showed clearly that *Hanseniaspora* and *Kloeckera* comprise six distinct groups. These groups are defined as the following species:

A. The Genus *Hanseniaspora*

1. *Hanseniaspora valbyensis* Klöcker 1912
2. *Hanseniaspora guilliermondii* Pijper 1928
3. *Hanseniaspora osmophila* (Niehaus) Phaff, Miller et Shifrine 1932
4. *Hanseniaspora uvarum* (Niehaus) Shehata, Mrak et Phaff 1932
5. *Hanseniaspora vineae* van der Walt et Tscheuschner 1957
6. *Hanseniaspora occidentalis* Smith 1974

The high degree of DNA reassociation with *H. guilliermondii* warrants the placement of the species *H. apuliensis* and *H. melligeri* in synonymy with *H. guilliermondii*, which has priority.

The species, *K. austriaca*, *K. brevis*, *K. brevis* var. *rohrbachense* and *K. lodderi*, may be considered synonyms of *H. uvarum* as they all produced ascospores and showed a high degree of DNA relatedness with *H. uvarum*.

One strain, CBS 311, physiologically like *H. valbyensis* and morphologically like *H. guilliermondii*, must be identified as *H. valbyensis* since high degrees of DNA reassociation were demonstrated between it and the type culture of *H. valbyensis*, as well as between it and other strains of the *H. valbyensis*/*K. japonica* group. It is evident that the number and size of ascospores are less valuable criteria for differentiating *H. valbyensis* and *H. guilliermondii* than are the assimilation of 2-keto-gluconate and growth at 37C.

As noted by Phaff (1970*a*), the establishment of the genus *Vanderwaltia* by Novák and Zsolt (1961) to accommodate *H. vineae* was unwarranted based on the unsuitable criterion of ascospore morphology. We agree with Phaff because we observed a variety of spore shapes and ornamentation throughout *Hanseniaspora*.

B. The Genus *Kloeckera*

1. *Kloeckera japonica* Saito et Ohtani 1931 is the imperfect state of *H. valbyensis*.

   K. corticis var. *pulquensis* may be placed in synonymy with *K. japonica*.
2. *Kloeckera apis* Lavie ex Smith, Simione et Meyer 1977 is the imperfect state of *H. guilliermondii*.
3. *Kloeckera corticis* (Klöcker) Janke 1912 is the imperfect state of *H. osmophila*.

   K. magna and *K. santacruzensis* remain as synonyms.
4. *Kloeckera apiculata* (Reess *emend.* Klöcker) Janke 1870 is the imperfect state of *H. uvarum*.

   Synonyms include *K. lindneri* var. *pelliculosa*, *K. muelleri* and *K. germanica*, as Miller and Phaff (1958) and Phaff (1970*b*) suggested.

5. *Kloeckera africana* (Klöcker) Janke 1912 is the imperfect state of *H. vineae*.
6. *Kloeckera javanica* (Klöcker) Janke 1912 is the imperfect state of *H. occidentalis*.

   Synonyms include *K. antillarum*, *K. jensenii*, *K. indica*, *K. willi* and *K. javanica* var. *lafarii*. The varietal status of *K. javanica* var. *lafarii* as proposed by Phaff (1970*b*) is rejected based on the high degree of DNA reassociation between this strain and the *K. javanica* type strain. Also, the difference in raffinose utilization between the variety and the species as reported by Phaff could not be confirmed in our laboratories.

Of these six species and their imperfect states, *H. osmophila* (*K. corticis*) and *H. vineae* (*K. africana*) are more related to one another than any of the *Hanseniaspora* and *Kloeckera* species are to each other. DNA reassociations of strains of *H. osmophila* and *K. corticis* with *H. vineae* and *K. africana* ranged between 38–60% with average and median values of 48% and 46% respectively. The difference in sucrose assimilation is not a useful characteristic for the separation of these species as was already mentioned by Nakase and Komagata (1970). A more valuable physiological criterion to differentiate these two species is the ability to grow at 34C.

Note: *Kloeckera lindneri* type strain was unavailable during the major part of this study and, therefore, will be reported at a later time.

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REFERENCES

VON ARX, J. A., RODRIGUES DE MIRANDA, L., SMITH, M. TH. and YARROW, D. 1977. The genera of yeasts and the yeast-like fungi. — Studies in Mycology No. **14**: 1–42. Inst. Royal Netherlands Acad. Arts and Sci.

BARNETT, J. A. and BUHAGIAR, R. W. M. 1971. *Torulopsis fragaria* species nova, a yeast from fruit. — J. Gen. Microbiol. **67**: 233–238.

DENHARDT, D. T. 1966. A membrane filter technique for the detection of complementary DNA. — Biochem. Biophys. Res. Commun. **23**: 641–646.

LODDER, J., (ed.). 1970. The Yeasts. A taxonomic study. — North Holland Publishing Company, Amsterdam.

LODDER, J. and KREGER-VAN RIJ, N. J. W. 1952. The Yeasts. A taxonomic study. — North Holland Publishing Company, Amsterdam.

MARMUR, J. and DOTY, P. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. — J. Mol. Biol. **5**: 109–118.

MEYER, S. A. and PHAFF, H. J. 1969. Deoxyribonucleic acid base composition in yeasts. — J. Bacteriol. **97**: 52–56.

MEYER, S. A., BROWN, R. E. and SMITH, M. TH. 1977. Species status of *Hanseniaspora guilliermondii* Pijper. — Int. J. Syst. Bact. **27**: 162–164.

MILLER, M. W. and PHAFF, H. J. 1958. A comparative study of the apiculate yeasts. — Mycopathol. Mycol. Appl. **10**: 113–141.

NAKASE, T. and KOMAGATA, K. 1970. Significance of DNA base composition in the classification of yeast genera *Hanseniaspora* and *Kloeckera*. — J. Gen. Appl. Microbiol. **16**: 241–250.

NOVÁK, E. K. and ZSOLT, J. 1961. A new system proposed for yeasts. — Acta Botan. Acad. Sci. Hung. **7**: 93–145.

PHAFF, H. J. 1970*a*. *Hanseniaspora* Zikes, p. 209–225. *In* J. Lodder, (ed.), The Yeasts. A taxonomic study. — North Holland Publishing Company, Amsterdam.

PHAFF, H. J. 1970*b*. *Kloeckera* Janke, p. 1146–1160. *In* J. Lodder, (ed.), The Yeasts. A taxonomic study. — North Holland Publishing Company, Amsterdam.

SAITO, K. and OHTANI, Y. 1931. On some fermentative yeasts isolated from sap exuded from bark of a tree. — Jozogaku Zasshi **9**: 6–11. (in Japanese).

SMITH, M. TH. 1974. *Hanseniaspora occidentalis* sp. nov. — Antonie van Leeuwenhoek **40**: 441–444.

SMITH, M. TH., SIMIONE, F. P., JR. and MEYER, S. A. 1977. *Kloeckera apis* st. nov., the imperfect state of *Hanseniaspora guilliermondii* Pijper. — Antonie van Leeuwenhoek **43**: 219–223.

SZILVINYI, A. und KAULICH, L. 1948. Zur Kenntnis der Mikroflora österreichischer Kernobstmoste. — Mitteilungen der Versuchsanstalt für das Gärungsgewerbe und des Instituts für angewandte Mikrobiologie der Hochschule für Bodenkultur Nr. **4**: 1–3.

ULLOA, M. y HERRERA, T. 1973. Descripción de una variedad nueva de *Kloeckera corticis* aislada del pulque. — Bol. Soc. Mex. **7**: 27–32.