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Molecular identification and genetic diversity within species of the genera *Hanseniaspora* and *Kloeckera*

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Abstract

Three molecular methods, RAPD-PCR analysis, electrophoretic karyotyping and RFLP of the PCR-amplified ITS regions (ITS1, ITS2 and the intervening 5.8S rDNA), were studied for accurate identification of *Hanseniaspora* and *Kloeckera* species as well as for determining inter- and intraspecific relationships of 74 strains isolated from different sources and/or geographically distinct regions. Of these three methods, PCR-RFLP analysis of ITS regions with restriction enzymes *Dde*I and *Hin*fI is proposed as a rapid identification method to discriminate unambiguously between all six *Hanseniaspora* species and the single non-ascospore-forming apiculate yeast species *Kloeckera lindneri*. Electrophoretic karyotyping produced chromosomal profiles by which the seven species could be divided into four groups sharing similar karyotypes. Although most of the 60 strains examined exhibited a common species-specific pattern, a different degree of chromosomal-length polymorphism and a variable number of chromosomal DNA fragments were observed within species. Cluster analysis of the combined RAPD-PCR fingerprints obtained with one 10-mer primer, two microsatellite primers and one minisatellite primer generated clusters which with a few exceptions are in agreement with the groups as earlier recognized in DNA–DNA homology studies.

Keywords: Apiculate yeast; Identification; PCR-RFLP analysis of rDNA; Electrophoretic karyotyping; RAPD-PCR analysis; Fingerprinting

1. Introduction

The ascomycetous yeast genus *Hanseniaspora* and its anamorph *Kloeckera* are morphologically characterized as apiculate yeasts with bipolar budding. The species of the genera are frequently isolated from various natural sources such as soil, fruits and insects [1], as well as from fermented foods and beverages [2,3]. As predominant inhabitants on the surface of grape berries and in starting wine fermentations, these genera have been intensively studied to determine their effect on the quality of the final fermentation product. Recently, it has been suggested that the presence of apiculate yeasts in the initial phases of wine fermentation contributes to a more complex and better aroma of the wine because of higher production of glycerol, esters and acetoin. Strains of *Hanseniaspora* and *Kloeckera* are therefore potential candidates for mixed starter cultures [4–7].

Several approaches have been applied to separate the species of *Hanseniaspora* and *Kloeckera* and to determine the relationships between teleomorph and anamorph species. Besides physiological and morphological determinations [8–10], serology [11], proton magnetic resonance spectra of cell wall mannans [12], and DNA base composition [13] have been studied. Currently, on the basis of DNA relatedness substantiated with physiological and morphological examinations, six teleomorph species with their anamorph counterparts and one anamorph species, *Kloeckera lindneri*, without a known teleomorphic state are accepted [14–16]. The present classification was also confirmed by phylogenetic studies based on parts of large and small subunit ribosomal-DNA nucleotide sequences. Sequence comparisons revealed that the genus *Hanseniaspora* is monophyletic and divided into two subgroups [17–20]. The conventional identification key to discriminate between *Hanseniaspora* and *Kloeckera* species is based

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on fermentation and/or assimilation of a few carbon sources and ability to grow at different temperatures. The latter is the sole characteristic for differentiating the closely related species *Hanseniaspora osmophila* and *Hanseniaspora vineae* or *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* [15]. However, this characteristic can vary due to adaptation to different environments [21].

To avoid doubtful identifications or misidentifications, genotypic methods which generate results independent of environmental conditions have been applied to food-borne strains, wine yeast strains and some type strains of *Hanseniaspora* and *Kloeckera* species [22,23]. Esteve-Zarzoso et al. [22] evaluated the use of restriction fragments length polymorphism (RFLP) of rDNA amplified by polymerase chain reaction (PCR) for the rapid identification of food-borne yeasts. They found that discrimination among selected species of *Hanseniaspora* was possible using certain specified restriction enzymes. Intraspecific variation mostly of species important for winemaking such as *H. uvarum–Kloeckera apiculata* and *H. guilliermondii* was studied by RAPD-PCR analysis [24], electrophoretic karyotyping [25,26] and AFLP fingerprinting [27].

In our study, we have used three molecular methods, (a) RAPD-PCR analysis, (b) electrophoretic karyotyping and (c) RFLP of the PCR-amplified ITS regions (ITS1, ITS2 and the intervening 5.8S rDNA), to examine the type strains of all currently accepted species along with other strains isolated from different sources and/or geographically distinct regions. The species identity of these strains has been based on physiology and partly on DNA–DNA reassociations. RAPD-PCR analysis has been used to evaluate the previously published statement [28] that high similarity in RAPD patterns correlates with high DNA homology. Further, we have applied the RFLP analyses and karyotyping to evaluate their ability for accurate identification of all *Hanseniaspora* and *Kloeckera* species. Moreover, we have determined inter- and intraspecific relationships which were compared with relationships based on DNA–DNA homology studies [14] and sequencing analysis of rDNA [17,20].

2. Materials and methods

2.1. Yeast strains

The strains studied, their designations and origin, are listed in Table 1.

2.2. Isolation of DNA for PCR assay

DNA was isolated according to the method of Möller et al. [29]. The DNA concentration was spectrophotometrically quantified and brought to a final value of 100 ng μl^{-1}.

2.3. RAPD-PCR analysis

For a preliminary assay of RAPD-PCR analysis two strains of each species were selected. We examined 19 decamer primers of arbitrary sequence from the OPA set (Operon Technologies Inc., Alameda, CA, USA), three microsatellite primers, $(ATG)_5$, $(GTG)_5$ and $(GTC)_5$, and M13 core sequence (5′-GAGGGTGGCGGTTCT). For detailed analysis OPA-13 (5′-CAGCACCCAC) as 10-mer primer, $(ATG)_5$, $(GTG)_5$ and M13 core sequence were selected.

Amplification reactions were performed in a final volume of 50 μl containing 100 ng of genomic DNA, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, 2 mM of each dNTP, 10 pM of primer and 1 U of *Taq* DNA polymerase. The thermal cycler was programmed for 40 cycles of 1 min at 94°C, 1 min at 60°C for primers M13 and $(GTG)_5$, at 48°C for $(ATG)_5$ and at 36°C for the OPA primer set, followed by 2 min at 72°C. PCR products were separated on 1.7% agarose gels in 1×TAE buffer chilled at 14°C. To avoid ambiguous results, the amplification reactions of all 74 strains were processed simultaneously from one stock solution of premixed reagents in a single PCR assay as suggested by Messner et al. [28].

The RAPD-PCR profiles obtained with M13, $(ATG)_5$, $(GTG)_5$ and OPA-13 of each strain were combined in a composite fingerprint using GelCompar 3.1 (Applied Math, Kortrijk, Belgium). Similarities between combined fingerprints were calculated using the Pearson product–moment correlation coefficient (r). Cluster analysis of the pairwise values was generated using UPGMA algorithm.

2.4. PFGE karyotyping

Yeast chromosomes were isolated by a method described by Carle and Olson [30] as modified by Raspor et al. [31]. The chromosomal elements were separated in 1% agarose gels in 0.5×TBE buffer chilled at 12°C in a CHEF-DRII electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed at 100 V for 36 h with a 200–300 s ramping switch interval and for 60 h with a 300–600 s ramping switch interval. The electrophoresis for separation of *H. uvarum* chromosomal fragments was prolonged and carried out at 100 V for 88 h with a 200–600 s ramping switch interval and then for 32 h at a 600–1200 s ramping switch interval.

The molecular sizes of the chromosomal bands ranging from 2800 to 1000 kb were calculated by comparison to a calibration curve based on *Pichia canadensis* (*Hansenula wingei*), those smaller than 1000 kb to *Saccharomyces cerevisiae* chromosomal DNA markers (Bio-Rad, Hercules, CA, USA) using the GelCompar 3.1 (Applied Math, Kortrijk, Belgium) computer program. The inaccuracy of the sizes of the chromosomal elements in range from 300 kb to 1500 kb was 50 kb maximally.

Table 1
List of *Hanseniaspora* and *Kloeckera* strains studied

Strain[a]	Status[b]	Origin of the strain
H. guilliermondii		
CBS 465	T	Infected nail, South Africa
CBS 95		Fermenting bottled tomatoes, The Netherlands
CBS 466	T of *Hanseniaspora meligeri*	Dates
CBS 1972	ST of *Hanseniaspora apuliensis*	Grape juice, Italy
CBS 2567	ST of *H. guilliermondii*	Grape must, Israel
CBS 2574		Grape juice, Italy
CBS 2591	T of *Kloeckera apis*	Trachea of bee, France
CBS 4378		Caecum of baboon
CBS 8733		*Opuntia megacantha*, Hawaii, USA
NCAIM 741 (ZIM 213, CBS 8772)		Orange juice concentrate, Georgia, USA
H. occidentalis		
CBS 2592	T, T of *Pseudosaccharomyces occidentalis*	Soil, St. Croix, West Indies
CBS 280	T of *Pseudosaccharomyces antillarum*	Soil, Java
CBS 282	T of *Pseudosaccharomyces javanicus*	Soil, Java
CBS 283	T of *Pseudosaccharomyces jensenii*	Soil, Java
CBS 2569		*Drosophila* sp.
CBS 6782		Orange juice, Italy
H. osmophila		
CBS 313	T of *K. osmophila*	Ripe Reisling grape, Germany
CBS 105	T of *Pseudosaccharomyces magnus*	Grape
CBS 106	T of *Pseudosaccharomyces corticis*	Bark of tree, Germany
CBS 1999	T of *Pseudosaccharomyces santacruzensis*	Soil, St. Croix, West Indies
CBS 2157		Flower of *Trifolium repens*, Germany
CBS 4266		Cider, UK
CBS 6554		Patent (Takeda Chemicals Industries)
NCAIM 726 (ZIM 212)		Pineapple juice concentrate, Georgia, USA
H. uvarum		
CBS 314	T of *Kloeckeraspora uvarum*	Muscatel grape, Crimea, Russia
CBS 104	T of *Pseudosaccharomyces apiculatus*	?
CBS 276		Soil, Germany
CBS 279	T of *Kloeckera brevis*	Institute of Brewing, Japan
CBS 286	T of *Pseudosaccharomyces malaianus*	Soil, Java
CBS 287	T of *Pseudosaccharomyces muelleri*	Soil, Java
CBS 312		Fermented cacao, Ghana
CBS 2570		*Drosophila* sp., Brazil
CBS 2579	T of *Pseudosaccharomyces austriacus*	Soil, Austria
CBS 2580	T of *Pseudosaccharomyces germanicus*	Soil, Germany
CBS 2582		Throat, The Netherlands
CBS 2583		Fermenting cucumber brine, USA
CBS 2584		?
CBS 2585	T of *Kloeckera lodderi*	Sour dough, Portugal
CBS 2586		Caterpillar
CBS 2587	AUT of *K. brevis*	Fruit must, Austria
CBS 2588		Tanning fluid, France
CBS 2589		Grape must, Italy
CBS 5073		Wine grape, Chile
CBS 5074		Apple grape, Chile
CBS 5450		Sea water, Florida, USA
CBS 5914		?
CBS 5934		Cider, Illinois, USA
CBS 6617		Fruit of *Musa sapientum*
CBS 8130		Grapes, Italy
CBS 8734		Fruit of *Sapindus* sp., Hawaii, USA
CBS 8773		Flower from *Schotia* tree, South Africa
CBS 8774		Flower from *Schotia* tree, South Africa
CBS 8775		Flower from *Schotia* tree, South Africa
NCAIM 674 (ZIM 216)		Botanical garden pond, Hungary
NCAIM 725 (ZIM 211, CBS 8771)		Spoiled grape punch, Georgia, USA
CCY 25-6-19		Slovakia
CCY 46-1-2		Slovakia
CCY 46-3-11		Slovakia
ZIM 1846		Grape must, Slovenia

Table 1 (*continued*)

Strain[a]	Status[b]	Origin of the strain
NC-1		Flower of *Strelitzia* sp., South Africa
H. valbyensis		
CBS 479	T	Soil, Germany
CBS 281	T of *Kloeckera japonica*	Sap of tree, Japan
CBS 311		Beer, Hungary
CBS 2590		Draught beer, England, UK
CBS 6558	T of *Kloeckera corticis*	Pulque, Mexico
CBS 6618		Tomato, Japan
NCAIM 330 (ZIM 229)		?
NCAIM 642 (ZIM 224)		Cauliflower, California, USA
H. vineae		
CBS 2171	T	Soil of vineyard, South Africa
CBS 277	T of *Pseudosaccharomyces africanus*	Soil, Algeria
CBS 2568		*Drosophila persimilis*
CBS 6555		Patent (Takeda Chemicals Industries)
CBS 8031	T of *Hanseniaspora nodinigri*	Black knot gall on *Prunus virgin*, Canada
K. lindneri		
CBS 285	T of *Pseudosaccharomyces lindneri*	Soil, Java

[a]CBS, Centraalbureau voor Schimmelcultures, The Netherlands; ZIM, Culture Collection of Industrial Microorganisms, Slovenia; CCY, Culture Collection of Yeasts, Slovakia; NCAIM, National Collection of Agricultural and Industrial Microorganisms, Hungary.
[b]T, type strain; AUT, authentic strain; ST, syntype.

2.5. PCR-RFLP analysis of rDNA

The primers used for amplification of ITS regions were ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) as described by White et al. [32]. The final volume of the PCR reaction mixture was 50 μl containing 100 ng of genomic DNA, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, 2 mM of each dNTP, 50 pM of each of a pair of primers and 1 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). For amplification of ITS rDNA the PCR conditions were as follows: an initial denaturing step of 5 min at 94°C was followed by 35 cycles of 40 s at 94°C, 40 s at 56°C and 30 s at 72°C and terminated with a final extension step of 7 min at 72°C and cooling down to 4°C.

Restriction patterns of the PCR products were determined for each of the following 11 restriction enzymes: *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Hpa*II, *Msp*I, *Nde*II, *Sau*3A, *Scr*FI and *Taq*I (Roche, Mannheim, Germany). Digestions were prepared according to the instructions of the manufacturer. The resulting fragments were separated on 3% agarose gels in 1×TAE buffer. Ethidium bromide-stained gels were documented by Polaroid 665 photography under UV light or by GelDoc 2000 (Bio-Rad, Hercules, CA, USA).

In ITS nine different restriction groups were observed which showed a total number of 64 different fragments with the 11 enzymes used. A binary matrix was generated manually by scoring absence (0) or presence (1) of each fragment for each group.

Further analyses were performed using NTSYS software package version 2.0 [33]. Similarity values were calculated using the Dice coefficient, which is equal to two times the number of bands in common between two restriction patterns, divided by the sum of all bands. Dendrograms were generated using an unweighted pair group method with arithmetic average (UPGMA) algorithm.

3. Results

3.1. Growth at 34°C and 37°C

According to Smith [15] the sibling species *H. vineae* and *H. osmophila* can be distinguished by the presence or absence of growth at 34°C, respectively, while the sibling species *H. uvarum* and *H. guilliermondii* can be discriminated by the absence or presence of growth at 37°C, respectively. In order to evaluate these characteristics all strains of these four species were re-examined for growth at the aforementioned temperatures. None of the *H. osmophila* strains grew at 34°C as expected; however, two strains of *H. vineae*, CBS 277 and CBS 2568, also failed to grow at this temperature. All strains of *H. uvarum* failed to grow at 37°C as expected; however, two strains of *H. guilliermondii*, CBS 1972 and CBS 2567, also failed to grow at 37°C.

3.2. RAPD-PCR analysis

Among nineteen 10-mer primers and four microsatellite primers tested, the primers OPA-03, OPA-13, OPA-18 and $(ATG)_5$, $(GTG)_5$, and M13 core sequence yielded useful patterns to allow verification of the identity of strains. These primers, except OPA-03 and OPA-18, were used in further studies.

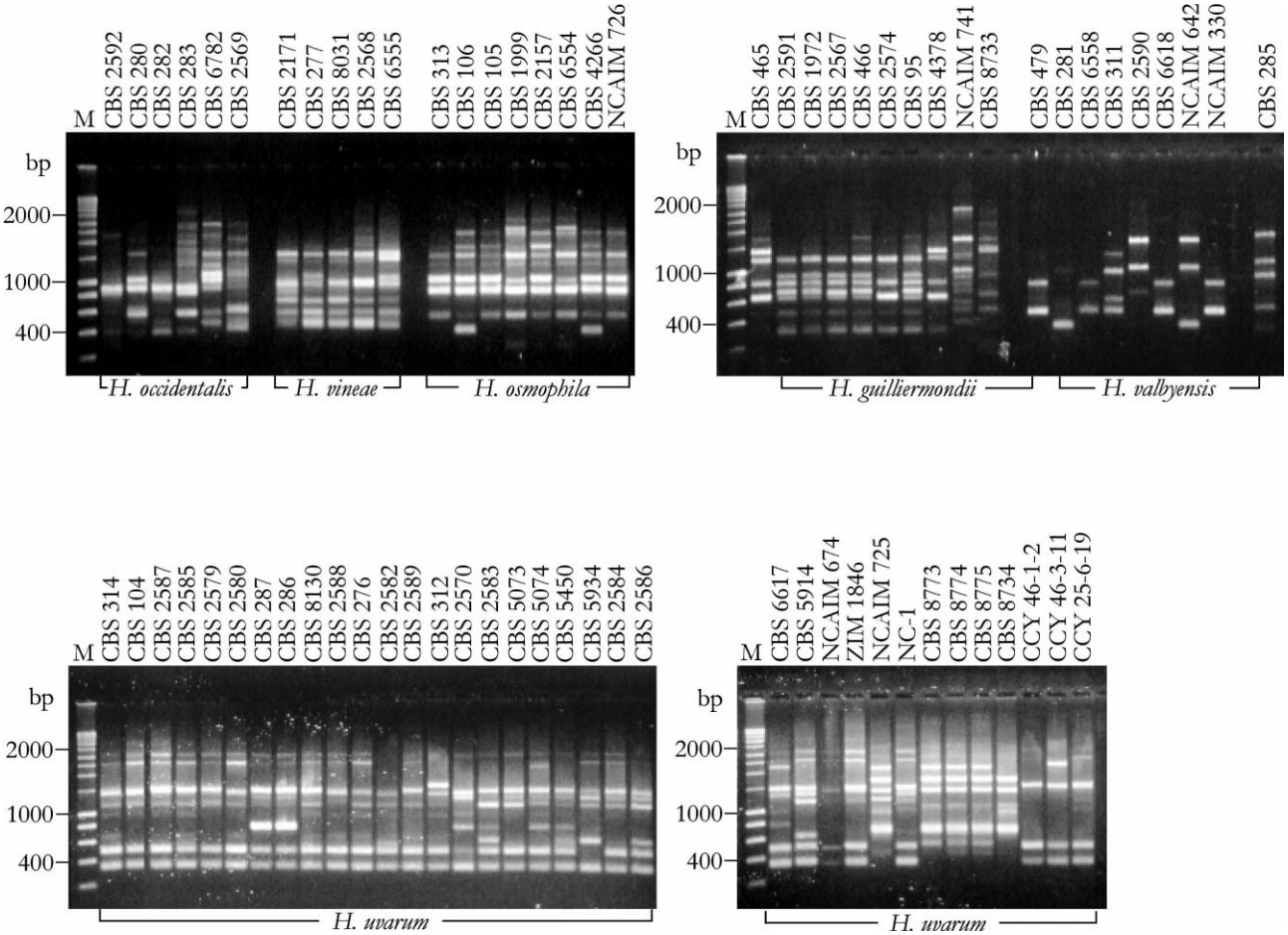

Fig. 1. RAPD fingerprints of *Hanseniaspora–Kloeckera* strains generated with Opa-13 primer. M, SmartLadder 200 bp (Eurogentec).

The RAPD-PCR patterns of *Hanseniaspora–Kloeckera* using primer OPA-13 are shown in Fig. 1.

Fig. 2 depicts the dendrogram derived from the combined RAPD-PCR fingerprints after cluster analysis. At the similarity level of 40% we could recognize eight clusters. Generally, strains of the same species clustered together with a few exceptions. Two strains of *Hanseniaspora occidentalis*, CBS 2569 and CBS 6782 (Fig. 2, marked with arrows), did not cluster with the main group (cluster 6). Five strains of *H. uvarum* (cluster 8) clustered at the similarity level of 20% far apart from the main group (cluster 1) which contained the type of this species. Unpublished preliminary DNA homology studies showed this cluster to be different from *H. uvarum*. To settle the final taxonomic status of this cluster, further studies are needed, and, therefore, they were excluded from the rest of this study. The single strain of *K. lindneri* clustered among the isolates of *Hanseniaspora valbyensis* (cluster 4) showing a similarity of 49% to CBS 2590.

3.3. Karyotyping

In Fig. 3 and Table 2, only the CHEF karyotypes and estimated sizes of chromosomal DNA bands of the type strains of *Hanseniaspora* and *Kloeckera* species are presented. These chromosomal profiles can be divided into four groups: group I contains the species *H. occidentalis*, *H. vineae* and *H. osmophila*; group II *H. uvarum* and *H. guilliermondii*, and groups III and IV comprise *H. valbyensis* and *K. lindneri*, respectively.

Most of the examined strains showed a species-specific pattern; however, chromosomal-length polymorphism (CLP) occurred and the number of chromosomal DNA bands was variable within the species (Fig. 4). Three out of six strains of *H. occidentalis*, CBS 2592^{T}, CBS 2569 and CBS 6782 (Fig. 4a), showed a similar banding pattern, with six chromosomal fragments ranging in size from 2600 kb to 900 kb, that differed from the karyotypes of *H. vineae* (Fig. 4b) in a resolved third and fourth chromosomal fragment from the top. The average size of the genome was ca. 11.3 Mb. The karyotypes of the three other strains of *H. occidentalis* isolated from Java (Indonesia) were highly variable. The karyotype of CBS 280 consisted of an additional chromosome of 1100 kb (Fig. 4a, marked with an arrow) and it lacked the third chromosomal fragment. Strain CBS 282 showed a pattern similar to that of the type strain CBS 2592 but two additional bands of 1300 kb and 1000 kb were present (Fig. 4a,

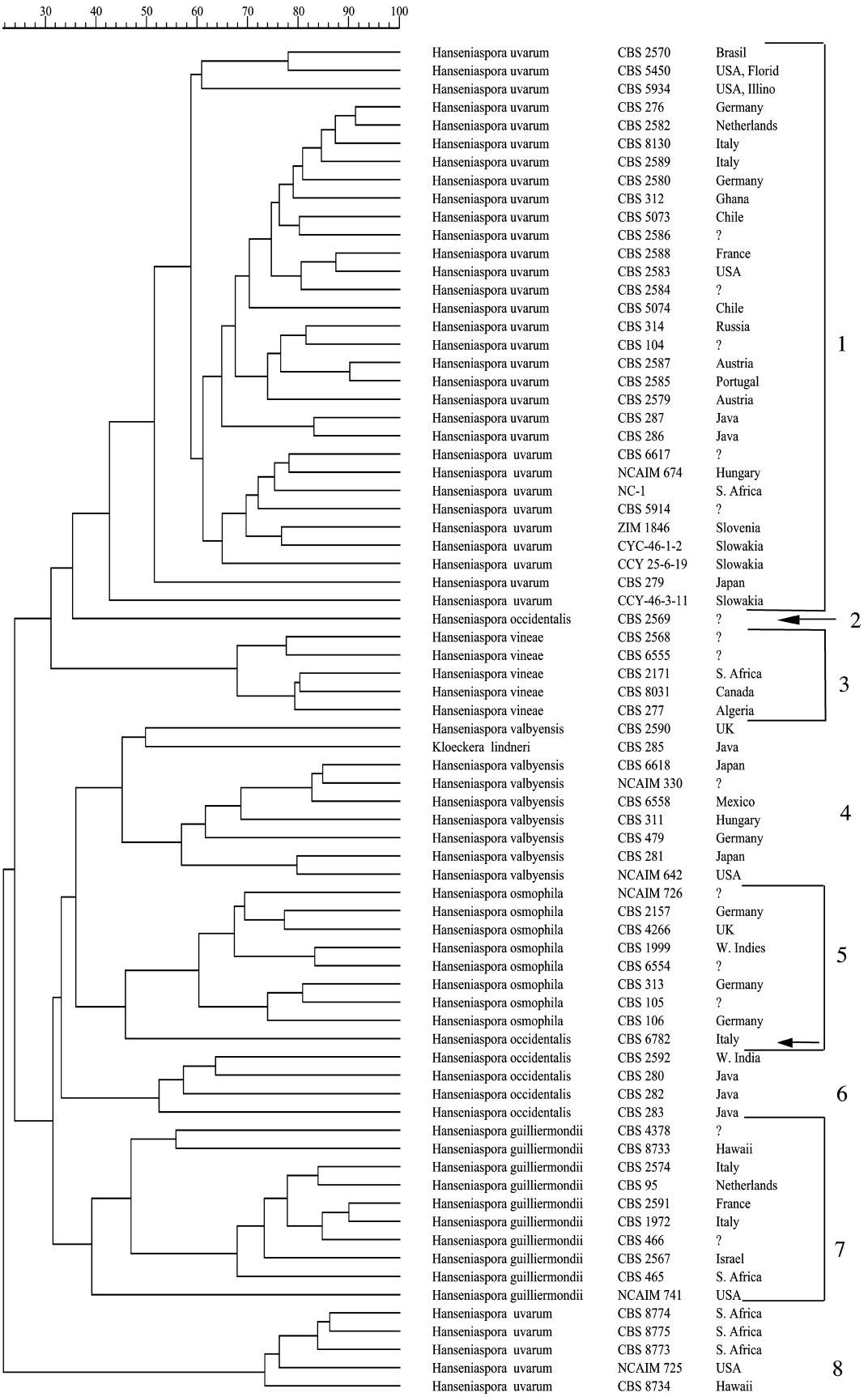

Fig. 2. UPGMA cluster analysis of 74 digitized combined RAPD-PCR fingerprints of *Hanseniaspora–Kloeckera* strains. The distance between strains was calculated using the Pearson correlation coefficient (% *r*).

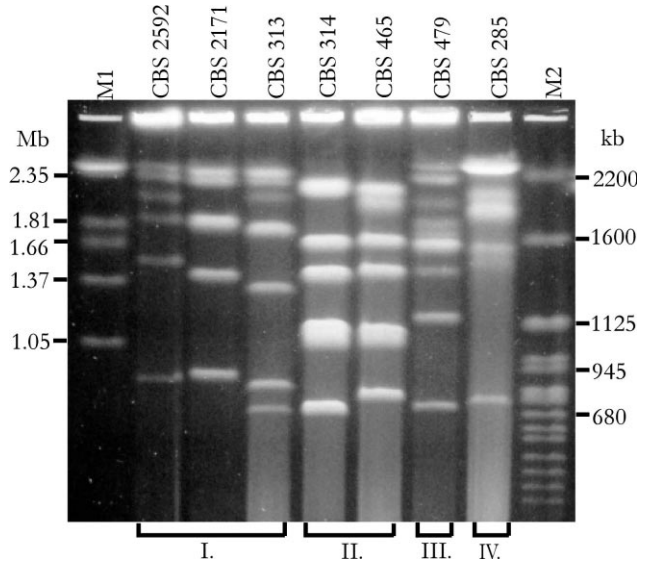

Fig. 3. Electrophoretic karyotypes of *Hanseniaspora–Kloeckera* type strains after CHEF electrophoresis. M1, chromosomal DNA of *P. canadensis* YB-4662-VIA as size marker; M2, chromosomal DNA of *S. cerevisiae* YNN295 as size marker (both Bio-Rad).

marked with arrows). CBS 283 (Fig. 4a) exhibited a significantly different pattern, similar to *K. lindneri* CBS 285 (Fig. 3), isolated also from soil in Java. The chromosomal DNA of CBS 283 (Fig. 4a) in the uppermost part of the gel remained unresolved whereas the remaining two bands occurred as doublets at ca. 2200 kb and 1700 kb.

The karyotype of strains of *H. vineae* (Fig. 4b) contained five chromosomal DNA bands ranging from 2500 to 930 kb. The estimated genome size varied between 9 and 13 Mb. Two strains, CBS 2568 and CBS 6555, contained additional faint DNA bands of 1600 and 2100 kb, respectively (Fig. 4b marked with arrows).

A species-specific karyotype pattern of *H. uvarum* (Fig. 4d) consisted of six to nine chromosomal fragments, ranging in size from 2200 to 600 kb. Doublet bands occurred at ca. 1100 and 1000 kb and the average genome size is an estimated 9.6 Mb. The most apparent differences among the karyotypes of *H. uvarum* were found in migration and doubling of the smallest chromosomal fragments (e.g. Fig. 4d, CBS 8130, marked with an arrow), as well as in the size and number of the uppermost fragments (e.g. Fig. 4d, CBS 286, marked with arrows). Strain CBS 2586 exhibited the most divergent karyotype with the largest chromosomal fragment of ca. 2.8 Mb and a total genome size of approx. 15 Mb.

The karyotypes of *H. guilliermondii* (Fig. 4e) were similar to those of *H. uvarum* (Fig. 4d), with CLP occurring among the largest and the smallest chromosomal DNA fragments.

Strains of *H. valbyensis* (Fig. 4f) were found to have a different chromosomal pattern. Seven to nine chromosomal DNA bands were resolved with sizes ranging from 0.75 to 2.6 Mb. The average genome of this species is ca. 11.7 Mb. The intraspecific CLP also occurs in this species.

3.4. PCR-RFLP analysis of rDNA

ITS regions were amplified separately from genomic DNA of the type strains of *Hanseniaspora* and *Kloeckera* species. The amplified ITS regions were approximately 720 bp long, without any size variation between the strains on 1% agarose gel.

The preliminary PCR-RFLP analysis of the ITS regions with 11 restriction enzymes performed on the type strains of *Hanseniaspora* and *Kloeckera* revealed that *Msp*I had no recognition site in the ITS regions and that *Sau*3A, *Nde*II and *Hpa*II did not reveal any polymorphism. Results obtained by the remaining seven restriction enzymes are presented in Table 3. Of these seven enzymes, *Dde*I was suitable to differentiate the types of all *Hanseniaspora–Kloeckera* species (Fig. 5a) except *H. valbyensis* and *K. lindneri*, which could be differentiated by *Hin*fI (Fig. 5b) or *Hae*III (Table 3).

To examine intraspecific polymorphisms within the *Hanseniaspora* species, three enzymes, *Hae*III, *Hin*fI, and *Dde*I, were examined in more detail. All strains of *Hanseniaspora* species exhibited restriction profiles identical to those of the type strain of the species with the exception

Table 2
Estimation of chromosome sizes of type strains of *Hanseniaspora* and *Kloeckera* species

	Type strain	Chromosome sizes (kb)							
Group I									
H. occidentalis	CBS 2592	2620	2400	2060	1840	1500	900		
H. vineae	CBS 2171		2470	2340	1840	1430	920		
H. osmophila	CBS 313		2400	2300	1810	1330	830	690	
Group II									
H. uvarum	CBS 314	2180	2110	1610	1430	1080	1040	670	
H. guilliermondii	CBS 465	2160	1980	1700	1470	1150	1100	830	
Group III									
H. valbyensis	CBS 479	2580	2340	2010	1780	1640	1420	1170	750
Group IV									
K. lindneri	CBS 285	2440	2100	1950	1600	1550	790		

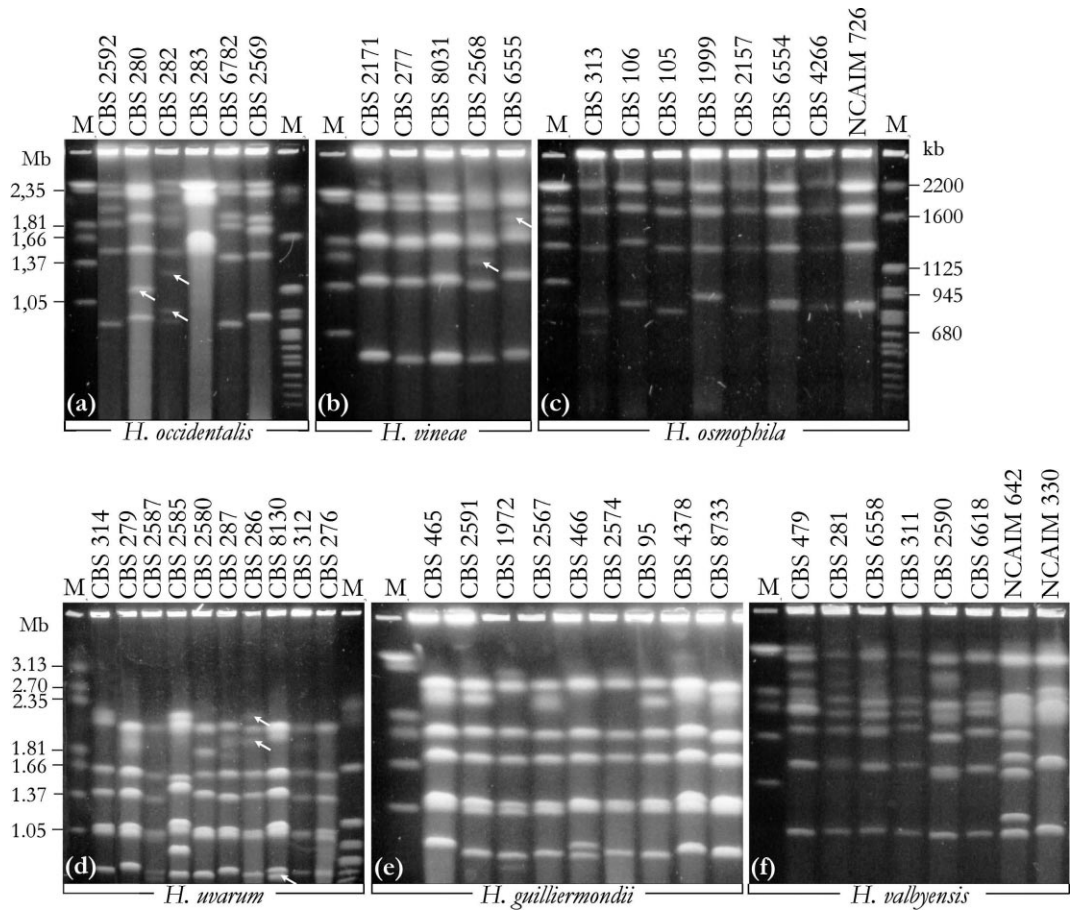

Fig. 4. Electrophoretic karyotypes of strains *H. occidentalis* (a), *H. vineae* (b), *H. osmophila* (c), *H. uvarum* (d), *H. guilliermondii* (e) and *H. valbyensis* (f). M_1, chromosomal DNA of *P. canadensis* YB-4662-VIA as size marker; M_2, chromosomal DNA of *S. cerevisiae* YNN295 as size marker (both Bio-Rad).

of *H. occidentalis* strains. Restriction enzyme *Hin*fI divided the species into three groups: group I contained the type strain CBS 2592, CBS 280 and CBS 283, group II CBS 282 and group III CBS 6782 and CBS 2569 (Fig. 5c). These subgroups were further examined with the other enzymes. Only *Taq*I and *Alu*I separated group II or group III from group I, respectively (Table 3).

The data sets from the ITS spacer digests were used to calculate similarity coefficients and to construct a dendrogram with NTSYS-pc. The topology of the ITS-RFLP dendrogram (Fig. 6) revealed four clusters of species with the similarity level ranging from 65% for the species *H. vineae* and *H. osmophila* to 95% for the sibling species *H. uvarum* and *H. guilliermondii*.

Table 3
Restriction fragment patterns of ITS regions of *Hanseniaspora* and *Kloeckera* generated by seven restriction enzymes (A–G)[a]

Enzyme	Species								
	H. occ			*H. vin*	*H. osm*	*H. uvar*	*H. guill*	*H. valb*	*K. lind*
	I	II	III						
*Scr*FI	A1	A1	A1	A1	A1	A1	A1	A2	A2
*Cfo*I	B1	B1	B1	B2	B2	B3	B3	B4	B4
*Alu*I	C1	C1	C2	C3	C3	C4	C4	C5	C5
*Hae*III	D1	D1	D1	D2	H3	H4	H4	H4	H5
*Dde*I	E1	E1	E1	E2	E3	E4	E5	E6	E6
*Taq*I	F1	F2	F1	F3	F4	F5	F5	F6	F6
*Hin*fI	G1	G2	G3	G4	G4	G5	G5	G6	G7

Within each enzyme different patterns were numbered successively, starting with number 1 for the first pattern. Identical numbers within an enzyme indicate identical patterns.

[a] *Msp*I has no recognition site in the ITS regions; *Sau*3A, *Nde*II and *Hpa*II do not reveal polymorphism.

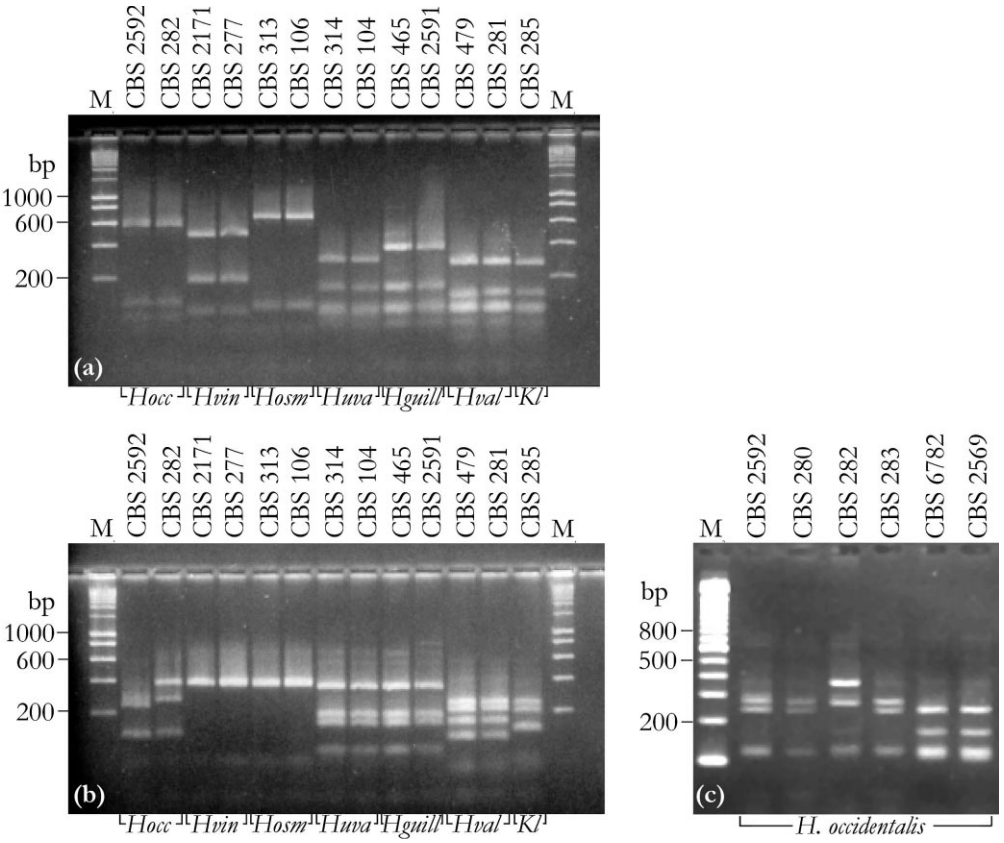

Fig. 5. PCR-RFLP analysis of ITS region of *Hanseniaspora–Kloeckera* type strains listed in Table 1 with restriction enzymes *Dde*I (a) and *Hin*fI (b,c). M_1, SmartLadder 200 bp (Eurogentec); M_2, 100-bp ladder (Gibco BRL). *Hocc*, *H. occidentalis*; *Hvin*, *H. vineae*; *Hosm*, *H. osmophila*; *Huva*, *H. uvarum*; *Hguill*, *H. guilliermondii*; *Hval*, *H. valbyensis*; *Kl*, *K. lindneri*.

4. Discussion

A polyphasic approach, which integrates phenotypic, genotypic and phylogenetic information, provides reliable information about relationships among species and strains. This study presents a contribution to the characterization of intraspecific variation and interspecific relationships of yeasts belonging to the genera *Hanseniaspora* and *Kloeckera*. We found that PCR-RFLP analysis of ITS regions with two restriction enzymes allowed discrimination of all species: *Dde*I restriction patterns were species-specific for all species examined, except *H. valbyensis* and *K. lindneri*. Discrimination between the latter two was possible using *Hin*fI. Moreover, *Hin*fI divided *H. occidentalis* into three subgroups.

The development of a molecular identification key was provoked by inconsistencies in identification results reported by Vaughan-Martini et al. [26]. Testing the growth ability at 34°C and 37°C, being key characteristics in the current identification key [15,16], we confirmed their findings: strains which were found to be conspecific on the basis of high DNA homology were variable with regard to growth at 34°C or 37°C. De Morais et al. [21] suggested that variations in ability to grow at higher temperatures may be a consequence of adaptation to the environment. Two strains of *H. guilliermondii*, CBS 1972 and CBS 2567, however, failed to grow at 37°C, although they were both isolated from warmer climates (Italy and Israel, respectively) than some other strains of this species (Table 1).

The cluster analysis of the combined RAPD-PCR fingerprints revealed groups that agreed with those obtained by DNA–DNA homology studies [14]. Each cluster represented a currently accepted species in the genus *Hanseniaspora*, and one separate cluster of five strains represented a group of strains physiologically undistinguishable from *H. uvarum*. The intraspecific similarity values ranged from 40 to 68%, which is quite low compared to the values reported for *P. membranifaciens* [34]. However, strains of the latter species were all isolated from the same substrate, whereas the strains of *Hanseniaspora* were isolated from

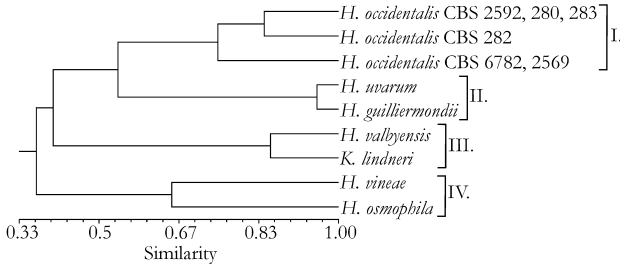

Fig. 6. UPGMA cluster analysis of *Hanseniaspora–Kloeckera* strains listed in Table 1 based on ITS restriction patterns.

different sources. Species boundaries agreed with correlation values of below 38%. The RAPD-PCR analysis did not reflect phylogenetic relationships between the species, not even the relationship between the closest related species *H. vineae* and *H. osmophila* sharing 40% DNA–DNA homology [14]. Therefore, the method is only useful for revealing the relationships among strains within species of *Hanseniaspora* due to its high resolution capacity.

Based on the results of electrophoretic karyotyping, the genera *Hanseniaspora* and *Kloeckera* can be divided into four subgroups sharing similar karyotypes. The phylogenetically closely related species *H. vineae–H. osmophila* and *H. uvarum–H. guilliermondii* [17,20] have similar karyotypes. These species are also difficult to discriminate by conventional criteria currently employed in yeast taxonomy [15]. On the other hand, the species *H. valbyensis* and its closest related anamorph species *K. lindneri* differ markedly by their chromosomal DNA pattern and physiologically they can also be differentiated by their maximal growth temperature [16].

The observed CLP of strains of *H. uvarum* from diverse geographical origin is comparable with that of *H. uvarum* strains isolated from Malvasia grape juice [35] and therefore does not reflect the presence of several distinct populations but merely indicates the rapid karyotypic changes which may occur within populations [36]. De Barros Lopos et al. [27] observed by AFLP genotypic analysis that most strains of *H. uvarum* are genetically rather uniform and they correlated the close genetic relatedness with the influence of humans on their dispersal and consequently the lack of genetically distinct populations. This hypothesis is confirmed by uniformity of our RAPD fingerprints (Figs. 1 and 2) of *H. uvarum* strains, which were isolated mostly from man-made environments.

Although the estimated genome size by PFGE is hampered by the possible presence of doublet or triplet chromosomes and the occurrence of similar-sized heterologous chromosomes, the average estimated genome sizes of 9.6 Mb of *H. uvarum* strains in our study is in accordance with previous estimates of 9.9–10 Mb [25].

Identification of *Hanseniaspora* isolates by PCR-RFLP of ITS regions has been applied recently by Esteve-Zarzoso et al. [22] albeit for a restricted number of species. In another study, Dlauchy et al. [23] proposed the use of *Alu*I for the differentiation of these closely related species. However, we found no *Alu*I restriction polymorphisms in the ITS regions between *H. vineae* and *H. osmophila* nor between *H. uvarum* and *H. guilliermondii*. The dichotomy of the genus *Hanseniaspora* supported by phylogenetic studies [17,20] was not confirmed with the ITS-RFLP dendrogram. However, the ITS-RFLP dendrogram showed a high relatedness (95% similarity) between *H. uvarum* and *H. guilliermondii*, which was also confirmed by the low number of nucleotide substitutions in the D1/D2 domain of the 26S rDNA [20]. On the other hand, a similarity value of only 65% between *H. vineae* and *H. osmophila* did not correlate with rDNA sequencing [17,20] and DNA homology data [14]. The latter study showed that *H. vineae* and *H. osmophila* were more closely related species sharing 38–60% DNA–DNA homology values, while the closely related *H. uvarum* and *H. guilliermondii* shared only 11–29% DNA–DNA homology.

High intraspecific variation of the strains of *H. occidentalis* was revealed by all three methods used. The highest variation was found in the electrophoretic karyotypes. Groupings observed in the PCR-RFLP of rDNA were less distinct than those in the karyotypes.

The genotypic methods used in our study to characterize strains of *Hanseniaspora* and *Kloeckera* were directed towards different aspects of the genome, such as the ribosomal gene, the mini-, microsatellite and random sequences, and the analysis of the chromosomal make-up. All three methods confirmed the relationships within species of the genus *Hanseniaspora* and the status of the anamorph species *K. lindneri*. In particular restriction analysis of rDNA is a reliable and rapid method for the identification of *Hanseniaspora–Kloeckera* isolates.

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