SIGNIFICANCE OF DNA BASE COMPOSITION IN THE CLASSIFICATION OF YEAST GENUS *PICHIA*

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The DNA base composition (GC content) of 53 cultures of *Pichia* and supposed related yeasts, which represented 35 species and a variety, was investigated. The GC content of *Pichia* ranged from 28.3 to 50.2%, and intragenetic variation was about 22%. Any prominent subgroups could not be detected within the genus and this genus was supposed to be very heterogeneous from the taxonomic points of view.

Since the publication of the monograph of LODDER and KREGER-VAN RIJ (1), in which only four species were included in the genus *Pichia*, a number of new species have been described in this genus and related genera. As a result of amendment of the definition of *Pichia* by PHAFF (2) to admit many species previously assigned to the genera *Petatospora*, *Saccharomyces*, and *Debaryomyces*, the genus *Pichia* became a large and complex genus and systematic studies became necessary for clarification of interrelationships among the species. This attempt has been made by BOLDIN *et al.* (3), PONCET (4), and SPENCER and GORIN (5).

In a previous work (6), the present authors investigated the GC content of DNA of the genus *Hansenula* and found that several groups were discriminated on the basis of GC content and serological characteristics. The genus *Pichia* is often compared with the genus *Hansenula* because these two genera are differentiated only by one character, assimilation of nitrate as a sole nitrogen source. Therefore, the authors examined GC contents of the genus *Pichia* with the expectation that subgroups may be found within this genus.

MATERIALS AND METHODS

Microorganisms. Fifty-three cultures of *Pichia* and supposed related

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Table 1. DNA base composition of the genus *Pichia*

Species	Strain	Source	T _m (°C)	GC content (mol. %)
<i>P. rhodanensis</i>	AJ 5074	CBS 5518	89.9	50.2
<i>P. guilliermondii</i>	AJ 4498	IFO 0961	87.6	44.6
"	AJ 4499	IFO 0566	87.5	44.4
<i>P. pinus</i>	AJ 5082	CBS 744	87.5	44.4
<i>P. wickerhamii</i>	AJ 5086	CBS 4107	87.0	43.2
<i>P. ohmeri</i>	AJ 5085	CBS 5367	86.6	42.2
"	AJ 5088	TUEA No. 230	86.7	42.4
<i>P. membranaefaciens</i>	AJ 4110	IFO 0460	86.5	42.0
"	AJ 4112	RIFY WF-30	86.3	41.5
"	AJ 4113	RIFY WF-80	86.7	42.4
"	AJ 4133	soil	86.5	42.0
"	AJ 4140	soil	86.5	42.0
"	AJ 4141	soil	86.4	41.7
<i>P. fermentans</i>	AJ 4458	<i>C. krusei</i> var. <i>transitoria</i> (H. Saëz)	86.6	42.2
"	AJ 4891	convenience food	86.6	42.2
<i>P. pastoris</i>	AJ 5076	CBS 704	86.1	41.0
"	AJ 5291	J-3092 (IFO 1013)	85.8	40.2
<i>P. pijperi</i>	AJ 5075	CBS 2887	86.0	40.7
<i>P. strassburgensis</i>	AJ 5068	CBS 2939	85.8	40.2
<i>P. quercuum</i>	AJ 5079	CBS 2283	85.7	40.0
<i>P. farinosa</i>	AJ 4134	IFO 0465	85.2	38.8
"	AJ 4136	corn	85.5	39.5
"	AJ 4138	<i>P. minuscula</i> NI 7646	85.6	39.8
<i>P. bovis</i>	AJ 5069	CBS 2616	85.6	39.8
<i>P. toletana</i>	AJ 5084	CBS 2504	85.4	39.3
<i>P. haplophila</i>	AJ 5078	CBS 2028	85.3	39.0
<i>P. etchellsii</i>	AJ 5083	CBS 2011	85.1	38.5
<i>P. kudriavzevii</i>	AJ 4146	CBS 5147	85.1	38.5
<i>P. salictaria</i>	AJ 5080	CBS 5456	84.8	37.8
<i>P. zaruensis</i>	AJ 4142	exudate of tree (II)	84.2	36.3
"	AJ 4143	soil (II)	84.5	37.1
<i>P. vini</i>	AJ 4266	IFO 0795	84.4	36.8
<i>P. vini</i> var. <i>melibiosi</i>	AJ 5077	CBS 5254	84.6	37.3
<i>P. terricola</i>	AJ 4137	IFO 0933	84.3	36.6
"	AJ 5368	J-9049 (IFO 0933)	84.4	36.8
<i>P. trehalophila</i>	AJ 5070	CBS 5361	83.5	34.6
<i>P. polymorpha</i>	AJ 4148	moss	82.9	33.2

Table 1. (Continued)

Species	Strain	Source	T _m (°C)	GC content (mol. %)
<i>P. saitoi</i>	AJ 4144	(K. Kodama)	82.5	32.2
<i>P. fluxuum</i>	AJ 5072	CBS 2237	82.5	32.2
<i>P. chambardii</i>	AJ 4283	IFO 1029	81.7	30.2
<i>P. kluyveri</i>	AJ 4145	ATCC 9768	80.9	28.3
"	AJ 4426	banana	81.0	28.5
<i>D. vanriji</i>	AJ 5058	CBS 3024	82.6	32.4
<i>E. fibuligera</i>	AJ 4270	IAM 4247	85.4	39.3
<i>E. fasciculata</i>	AJ 5061	CBS 5514	84.9	38.0
<i>C. zeylanoides</i>	AJ 4679	ATCC 7351	91.8	54.9
"	AJ 4741	frozen food, p-16 (27)	91.7	54.7
<i>C. mycoderma</i>	AJ 4413	RIFY WF-64	86.7	42.2
<i>C. krusei</i>	AJ 4421	IFO 0011	85.2	38.8
"	AJ 4457	ATCC 6258	85.4	39.3
"	AJ 4773	<i>C. tamarindii</i> CBS 2457	85.3	39.0
<i>C. natalensis</i>	AJ 4772	29-44-1 (A. Kocková-Kratochvílová)	84.4	36.8
<i>T. inconspicua</i>	AJ 5138	IFO 0621	83.9	35.6

yeasts which represented thirty-five species and one variety were employed. Sources of cultures are shown in Table 1.

Cultivation and harvest of yeasts, and isolation of DNA. Cultivation and harvest of yeasts, and isolation of DNA essentially followed the procedures previously reported (7). For the purpose of digestion of RNA, deproteinized DNA solution was incubated with ribonuclease as described (7). However, an overnight incubation is usually sufficient and this was different from the case of previous work.

Determination of DNA base composition. The DNA base composition was calculated from the thermal denaturation temperature (T_m) of DNA according to the procedure of MARMUR and DOTY (8). T_m was measured by the apparatus described by YAMADA and KOMAGATA (9).

RESULTS AND DISCUSSION

DNA base composition (GC content) of the strains of the genus *Pichia* and related genera calculated from the T_m is shown in Table 1. All the preparations employed for T_m determination gave monophasic melting curves and were considered to be free from mitochondrial DNA.

In the previous paper (7), the present authors reported the GC contents of DNA from three species of *Pichia*; *P. membranaefaciens* (40.5%), *P. vini*

Fig. 1. Distribution of GC content in DNA from the genus *Pichia*.

(34.5%), and *P. kluyveri* (26–27%). T_m values obtained in the present experiment were somewhat higher than those previously reported as a result of using an improved apparatus for T_m determination described by YAMADA and KOMAGATA (9). Namely, 41.5–42.4%, 36.8%, and 28.3–28.5% of GC contents were obtained respectively for *P. membranaefaciens*, *P. vini*, and *P. kluyveri* (Table 1).

The GC contents of DNA of the members of *Pichia* ranged from 28.3 to 50.2%, and any prominent groups of the species were not found (Fig. 1). This range of intragenetic variation (about 22%) was the largest among ascosporogenous genera of yeasts hitherto investigated (6, 7, 10). This is quite natural since the genus *Pichia* is considered to be not clearly defined, and often difficult to be distinguished from other genera of sporogenous yeasts, especially *Debaryomyces*, *Endomycopsis* and *Saccharomyces*. The genus *Pichia* is often compared with the genus *Hansenula* since both genera are separated only by one character, assimilation of nitrate as a sole nitrogen source. It is noteworthy that prominent groups of the species with similar GC contents and similar taxonomic criteria cannot be found in the genus *Pichia*. This contrasts with the case of the genus *Hansenula* in which such groups were found (6). Of the saturn-shaped spored pichias, *P. saitoi* and *P. zaruensis* were studied. It is interesting that these two species differ about 5% in the GC content in spite of a close similarity in taxonomic criteria commonly employed (11). In the case of *Hansenula*, saturn-shaped spored species exhibited similar values in GC contents and were considered to make a natural group (6). According to KODAMA (12), spore forms of *Pichia* are classified into five groups, while those of *Hansenula* are divided into only two groups. The genus *Pichia* is supposed to be a very complex genus and may consist of many kinds of yeasts with different phylogenetic origin.

BOIDIN *et al.* (3) classified the members of true *Pichia* (with hat- or

Table 2. DNA base composition of *Pichia* and the classification by BOLDIN *et al.* (3).

Group		Species	GC content (mol. %)
Guilliermondii- fibuligera	Guilliermondii	<i>P. guilliermondii</i>	44.4-44.6
		<i>P. scolyl</i>	NT
		<i>P. burtonii</i>	NT
		<i>P. ohmeri</i>	42.2-42.4
	Fibuligera	<i>P. strassburgensis</i>	40.2
		<i>P. fibuligera</i>	39.3
		<i>P. monospora</i>	NT
		<i>P. rhodanensis</i>	50.2
		(<i>P. bovis</i>)	39.8
		(<i>P. xylosa</i>)	NT
		(<i>P. wickerhamii</i>)	43.2
		(<i>P. toletana</i>)	39.3
		<i>P. fasciculata</i>	38.0
		<i>P. media</i>	NT
Pijperi-pini		<i>P. pijperi</i>	40.7
		<i>P. salictaria</i>	37.8
		<i>P. chambardii</i>	30.2
		<i>P. pastoris</i>	40.2-41.0
		<i>P. trehalophila</i>	34.6
		<i>P. pini</i>	44.4
Membranaefaciens		<i>P. haplophila</i>	39.0
		<i>P. saitoi</i>	32.2
		<i>P. fermentans</i>	42.2
		(<i>P. kluyveri</i>)	28.3-28.5
		<i>P. membranaefaciens</i>	41.5-42.4
		(<i>P. saccharophila</i>)	NT
		(<i>P. silvestris</i>)	NT
		<i>P. delftensis</i>	NT
		<i>P. farinosa</i>	38.8-39.8
		(<i>P. sake</i>)	NT
		(<i>P. miso</i>)	NT
		(<i>P. minuscula</i>)	39.8

Table 2. (Continued)

Group	Species	GC content (mol. %)
Polymorpha	<i>P. polymorpha</i>	33.2
	<i>P. pseudopolymorpha</i>	NT
	<i>P. robertsii</i>	NT
	<i>P. vini</i>	36.8
	(<i>P. carsonii</i>)	NT
	(<i>P. faecalis</i>)	NT
	(<i>P. etchellsii</i>)	38.5
	(<i>P. vini</i> var. <i>melibiosi</i>)	37.3

NT: not tested

saturn-shaped ascospores) into three groups, "guilliermondii-fibuligera," "pijperi-pini," and "membranaefaciens." They excluded round spored species ("polymorpha" group) from the genus *Pichia* and stated that taxonomic position of these species must be debated. As shown in Table 2, the members of "guilliermondii-fibuligera" group exhibited the GC content of 38.0–50.2%. Of the species of subgroup "guilliermondii," two species employed in the present experiment (*P. guilliermondii* and *P. ohmeri*) exhibited similar GC contents, 44.4–44.6% and 42.2–42.4%, respectively, while an intragroup variation of GC contents in subgroup "fibuligera" reached about 12% and suggests the heterogeneity of the subgroup. Of the members of this group, *P. rhodanensis*, *P. xylosa*, *P. toletana*, *P. bovis*, and *P. wickerhamii* show minor differences in taxonomic criteria commonly employed so that diverse synonyms have been proposed, namely, *P. xylosa* with *P. rhodanensis* (13, 14), *P. xylosa* with *P. toletana* (15), and *P. xylosa*, *P. bovis*, *P. toletana*, and *P. wickerhamii* with *P. rhodanensis* (3, 16). Although the authors did not study *P. xylosa*, other yeasts exhibited three different GC contents, 50.2% for *P. rhodanensis*, 43.2% for *P. wickerhamii*, and 39.3–39.8% for *P. toletana* and *P. bovis*, so that they are assumed to represent at least three different species. PONCET (4) reported, on the basis of factor analysis, that *P. bovis*, *P. rhodanensis*, *P. xylosa*, and *P. wickerhamii* should be retained as separate species. It is noteworthy that *P. rhodanensis* exhibited the GC values extremely higher than those of the other species mentioned above in spite of a resemblance in taxonomic criteria commonly employed.

Intragroup variation in GC contents in "pijperi-pini" group was found to be about 14%, and this group is also assumed to consist of heterogeneous members. BOLDIN *et al.* (17) reported that *P. trehalophila* occupied an intermediate position between *P. pastoris* and *P. pini* (*P. pinus*), and PHAFF *et al.* (18) also pointed out a resemblance of *P. trehalophila* to *P. pinus*. However,

P. trehalophila is considered not so closely related to *P. pastoris* and *P. pinus* since the former exhibited the GC values 6–10% higher than those of the latter two species. The variation in the GC contents found in “membranaefaciens” group was about 14% (Table 2), and denotes the heterogeneity of the group. It is interesting that *P. fermentans* and *P. kluyveri* showed quite different GC contents, 42.2% and 28.3–28.5%, respectively, in spite of a close resemblance in taxonomic criteria commonly employed. Since the description of *P. kluyveri* by BEDFORD (19), a considerable amount of discussions has been made on the validity of this species. LODDER and KREGER-VAN RIJ (1) regarded this species as a synonym of *P. fermentans*, while DE CARMAGO and PHAFF (20) reported that *P. kluyveri* differs from *P. fermentans* in the assimilation of D-xylose and the formation of pseudomycelia. KREGER-VAN RIJ (15) accepted De Carmago and Phaff’s opinion though she found that *P. kluyveri* can form primitive pseudomycelia. However, PIGNAL and BOIDIN (21) found that *P. kluyveri* could assimilate D-xylose, and again they regarded this species as a synonym of *P. fermentans*. It is apparent that *P. kluyveri* is a quite different species from *P. fermentans* because their GC contents are extremely different. Practically, these species can be discriminated by assimilation of D-xylose and requirement of biotin and pyridoxine. Namely, *P. fermentans* rapidly assimilates D-xylose and requires neither biotin nor pyridoxine, while *P. kluyveri* assimilates D-xylose latently and very weakly or not in some strains, and requires both biotin and pyridoxine. *P. minuscula*, which was considered as a synonym of *P. farinosa* (15, 21), showed a GC value similar to that of *P. farinosa* (Table 2).

The members of “polymorpha” group, which were excluded from the genus *Pichia* by BOIDIN *et al.* (3), showed GC contents with a relatively small intragroup variation though the number of species employed was rather small. By the facts enumerated above, classification of *Pichia* by BOIDIN *et al.* (3) is considered not to reflect the natural relationship among species, though it gives many suggestions for the classification of the genus *Pichia*.

SPENCER and GORIN (5) classified the members of *Pichia* into five groups on the basis of similarities in the proton magnetic resonance (PMR) spectra of mannans isolated from these yeasts. The relation of their grouping to the GC content is shown in Table 3. Intragroup variation in GC contents is about 22% in group I, 16% in group II, 11% in group III, 2% in group IV, and 7% in group V. Of the most heterogeneous group I, *Candida zeylanoides* showed GC values 10 to 22% higher than those of other members of this group. When this species is omitted, intragroup variation of this group is reduced to about 12% in the GC content though this value of variation is yet rather wide to regard that the members of this group make a natural group. Of the three subgroups within this group, group Ic is the most heterogeneous (Table 3). In spite of a great difference in the GC contents of DNA, SPENCER and GORIN (5) reported that *C. zeylanoides* forms a mannan whose PMR spectrum shows all the signals present in that of *C. guilliermondii*. It is apparent that PMR

Table 3. Grouping of *Pichia* based on PMR spectra of mannans (5) and DNA base composition

Group		Species	GC content (mol. %)
I	Ia	<i>P. farinosa</i>	38.8-39.8
		<i>P. pseudopolymorpha</i>	NT
		<i>P. polymorpha</i>	33.2
		<i>P. quercuum</i>	40.0
	Ib	<i>P. etchellsii</i>	38.5
		<i>P. vini</i>	36.8
		<i>P. haplophila</i>	39.0
	Ic	<i>P. vanriji</i>	32.4
		<i>P. robertsii</i>	NT
		<i>P. guilliermondii</i>	44.4-44.6
		<i>P. zeylanoides</i>	54.7-54.9
II	IIa	<i>C. krusei</i>	38.8-39.3
		<i>P. fluxuum</i>	32.2
		<i>P. kluyveri</i>	28.3-28.5
		<i>P. angophorae</i>	NT
		<i>P. pinus</i>	44.4
		<i>P. trehalophila</i>	34.6
	IIb	<i>P. membranaefaciens</i>	41.5-42.4
		<i>C. mycoderma</i>	42.2
		<i>P. terricola</i>	36.6-36.8
	IIc	<i>P. fermentans</i>	42.2
		<i>P. orientalis</i>	38.5
III	IIIa	<i>P. rhodanensis</i>	50.2
		<i>P. ohmeri</i>	42.2-42.4
	IIIb	<i>P. pijperi</i>	40.7
		<i>C. tepae</i>	NT
	IIIc	<i>P. pastoris</i>	40.2-41.0
		<i>P. toletana</i>	39.3
IV		<i>P. strassburgensis</i>	40.2
		<i>P. salictaria</i>	37.8
		<i>P. bovis</i>	39.8
V		<i>P. chambardii</i>	30.2
		<i>C. natalensis</i>	36.8

NT: not tested

spectra of mannans represent a resemblance only of a part of the structure of cell wall and not a resemblance of microorganism as a whole. Subgroups Ia and Ic exhibited relatively small, or small intragroup variations. Of the group II, group IIa is considered the most heterogeneous subgroup exhibiting about 16% of intragroup variation in the GC content. Of the species of group IIb, *P. membranaefaciens* and its imperfect form *C. mycoderma* showed similar GC values, while *P. terricola* exhibited a value about 6% lower than those of the former two species. SPENCER and GORIN (5) pointed out that the PMR spectra of this subgroup resembled those of *H. angusta* and *H. minuta*. It is noteworthy that a considerable number of common antigens were found by TSUCHIYA *et al.* (22) among the species of group IIb and *H. angusta* and *H. minuta*. These species are considered to be closely related in the structure of cell walls but not necessarily in the level of DNA. A small variation was found in group IIc. SPENCER and GORIN (5) stated that *Torulopsis inconspicua* which they described as the imperfect form of *P. fermentans* is also included in this subgroup. However, this species showed a GC value (35.6%) lower than those of the other species and is not considered to be the imperfect form of *P. fermentans*. It is interesting that *P. orientalis* and *C. krusei* are placed in separate subgroups, groups IIc and IIa, respectively, because *P. orientalis* (*P. kudriavzevii*) is regarded as the perfect form of *C. krusei* (3, 15). Further investigation is required to clarify this relationship in spite of a resemblance of GC contents between the two species. Variation found in subgroups in group III was not so large; about 8% in group IIIa and 1–2% in IIIc. As to group IIb, only one species was studied. Species of group IV exhibited similar GC contents in spite of their large difference in carbon assimilation (15). SPENCER and GORIN (5) pointed out a resemblance of PMR spectra of mannans of this group to those obtained from group I of *Hansenula*. However, hansenulas of group I of SPENCER and GORIN have GC contents with wide variation, 12% (6). The last group showed a relatively small intragroup variation. As being discussed above, classification of *Pichia* by PMR spectra of mannans is inconsistent in many points with the data of GC contents. Of course, PMR spectrum of mannans is an important criterion for elucidating the phylogenetic relationship among yeasts because it represents a fine structure of mannans of the cell wall, but classification based only on this character is not considered to represent natural relationships among the yeasts.

PONCET (4) classified true *Pichia* into three groups on the basis of factor analysis. As shown in Fig. 2, each group showed a large intragroup variation of the GC content. This analysis seems to be only a reflection of phenotypical characteristics concerning fermentation and assimilation of carbon compounds.

In the case of *Hansenula*, GC contents and antigenic structures were consistent in many points (6). Since only a restricted number of *Pichia* has been serologically studied, precise comparison cannot be made. However, it seems that some discrepancies may be present as shown in Table 4. It is apparent that the genus *Pichia* is a very complex genus and extensive studies

Fig. 2. Distribution of GC content in DNA in the groups of *Pichia* by PONCET (4) based on factor analysis.

● Mean value of the GC content of DNA in species.

Table 4. DNA base composition of species in *Pichia* group (genus) by TSUCHIYA *et al.* (22, 24)

Species	Antigenic structure (22, 24)		GC content (mol. %)	Reference
<i>P. fermentans</i>	1, 5, 11	2	42.2	
<i>C. krusei</i>	1, 5, (11), b	2	38.8-39.3	
			39.6	(23)
<i>C. catenulata</i>	1, 5, 11, b	2	54.5	(23)
<i>D. fluxorum</i> (<i>P. fluxuum</i>)	1, 5, 11, m	2	32.2	
<i>P. membranaefaciens</i>	1, 5, 11, 12	2	41.5-42.4	
			44	(25)
			37	(26)
<i>C. mycoderma</i>	1, 5, 11, 12	2	42.2	
<i>T. inconspicua</i>	1, 5, (11), (12),	2	35.6	
<i>P. farinosa</i>	1, 29	2, 3, 4	38.8-39.8	
<i>S. pastori</i> (<i>P. pastoris</i>)	1, 25	(3), 4, 17, 24	40.2-41.0	
<i>C. melinii</i>	1, 5, 25	2, 3, 10, 17	36.5	(7)
			40.9	(23)
<i>T. pinus</i>	1, 5, 11, 25	2 (17)	34.5	(7)
<i>H. angusta</i>	1, (5), 11,	2, (17)	44.9-46.3	(6)
<i>H. capsulata</i>	1, 5, 11, 12	2, 3, 4	42.7-43.9	(6)
<i>P. terricola</i>	1, 5, 11, 12, 18, c	2, 14, 31	36.6-36.8	

would be required to clarify the natural relationship among the species now included in this ambiguous genus.

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