CLASSIFICATION OF ASCOIDEACEOUS YEASTS BASED ON THE ELECTROPHORETIC COMPARISON OF ENZYMES AND COENZYMES Q SYSTEMS

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Sixty one strains of ascoideaceous yeasts belonging to twenty species of nine genera were examined from a taxonomical viewpoint based on a study of the electrophoretic comparison of the enzymes and the coenzymes Q (Co-Q) systems. The fifty strains, excluding those yeasts in which the detected number of enzymes was 3 or less, were divided into 4 clusters and 2 subclusters by numerical analysis of the relative electrophoretic mobilities (R_m) of 8 enzymes. On the other hand, the Co-Q systems of the ascoideaceous yeasts were Q-7, Q-8, or Q-9. These Co-Q systems and the septal ultrastructures coincided with each other. It is possible to divide these yeasts into four groups on the basis of similarity value according to the R_m of 8 enzymes: *Saccharomycopsis lipolytica*, other *Saccharomycopsis* spp. and related genera, *Hyphopichia burtonii* and/or *Stephanoascus ciferrii*, and *Ambrosiozyma* and *Hormoascus*. *Saccharomycopsis lipolytica* should be excluded from the genus *Saccharomycopsis* and is assigned to the genus *Yarrowia*. *Ambrosiozyma* and *Hormoascus* are considered to be cogenetic. Our data revealed close taxonomic relationship in the numerical classification based on the R_m of enzymes, Co-Q systems, and the septal ultrastructure.

VON ARX (1) described eight yeast genera in the Ascoideaceae Schröter which produce septate true mycelium, hat-shaped ascospores and blastoconidia. *Stephanoascus ciferrii* (2) was discovered later and the genus *Hyphopichia* was newly established as the teleomorph of *Candida ciferrii* (3). VON ARX et al. (4) accepted the following genera in the Ascoideaceae. These were: *Ambrosiozyma*, *Arthroascus*, *Ascoidea*, *Botryoascus*, *Cephaloascus*, *Guilliermondella*, *Hormoascus*, *Hyphopichia*, *Saccharomycopsis*, and *Stephanoascus*. *Saccharomycopsis lipolytica* was transferred to the new genus *Yarrowia*, and *Saccharomycopsis crataegensis* and

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Table 1. Strains used in this study.

Species	Strain	Source(s)
<i>Ambrosiozyma cicatricosa</i> (Scott et van der Walt) van der Walt	TH- 1	ATCC 24611 ^T
<i>Ambrosiozyma monospora</i> (Saito) van der Walt	TH- 2	ATCC 18855 ^T
	TH-59	IFO 1965
	TH-71	CBS 5515
<i>Ambrosiozyma philentoma</i> van der Walt, Scott et van der Klift	TH- 3	ATCC 24607 ^T
<i>Arthroascus javanensis</i> (Klöcker) von Arx	TH- 4	IFO 1579 ^T
	TH- 5	ATCC 10628
	TH-80	CBS 2556
<i>Ascoidea africana</i> Batra et Francke-Grosmann	TH- 6	ATCC 22852 ^T
<i>Ascoidea corymbasa</i> Gams et Grinbergs	TH- 7	ATCC 24275 ^T
<i>Ascoidea hylecoeti</i> Batra et Francke-Grosmann	TH- 8	ATCC 22790 ^T
<i>Ascoidea rubescens</i> Brefeld	TH- 9	ATCC 16142
	TH-10	IFO 6883
<i>Cephaloascus fragrans</i> Hanawa	TH-12	IFO 7785
	TH-13	ATCC 36174 ^T
<i>Guilliermondella selenospora</i> Nadson et Krassilnikov	TH-21	ATCC 10629 ^T
	TH-86	CBS 2563
<i>Hormoascus platipodis</i> (Baker et Kreger-van Rij) von Arx	TH-22	IFO 1471
	TH-23	ATCC 24747 ^T
	TH-84	CBS 6110
<i>Hyphopichia burtonii</i> (Boidin, Pignal, Lehodey, Vey et Abadie) von Arx et van der Walt	TH-31	CBS 6874
	TH-44	ATCC 13169 ^T (mating type <i>a</i>)
	TH-45	IFO 0844
	TH-46	CBS 2353 (mating type α)
	TH-47	CBS 2452
	TH-48	CBS 4571
	TH-49	CBS 5508
	TH-50	CBS 5509
	TH-51	CBS 6141
	TH-52	CBS 6375
	TH-54	M-11 (from Nukadoko, 18)
<i>Saccharomycopsis capsularis</i> Schiöning	TH-24	ATCC 36308 ^T
<i>Saccharomycopsis crataegensis</i> Kurtzman et Wickerham	TH-18	IFO 1708 (holotype, mating type <i>a</i>)
	TH-19	IFO 1709 (isotype, mating type α)
<i>Saccharomycopsis fibuligera</i> (Lindner) Klöcker	TH-14	ATCC 36309 ^T
	TH-15	IFO 0103
	TH-16	IFO 0106
	TH-17	IFO 1665
	TH-56	IFO 0104
<i>Saccharomycopsis lipolytica</i> (Wickerham, Kurtzman et Herman) Yarrow	TH-32	CBS 599 (mating type A)
	TH-33	CBS 2701
	TH-34	CBS 5919 (mating type B)
	TH-35	CBS 6114 (mating type B)
	TH-36	CBS 6125 (mating type A)
	TH-37	CBS 6303 ^T (mating type A)

Table 1. (continued).

Species	Strain	Source(s)
<i>Saccharomycopsis malanga</i> (Dwidjoseputro) Kurtzman, Vesonder et Smiley	TH-38	CBS 6317 (mating type A)
	TH-39	CBS 6659 (mating type B)
	TH-40	CBS 8108 (mating type B)
	TH-41	IFO 1550 (opposite of mating type of IFO 1551)
	TH-42	IFO 1551 (opposite of mating type of IFO 1550)
	TH-43	IFO 1548 (diploid)
	TH-53	M-4 (from Nukadoko, 18)
	TH-25	ATCC 36237 ^T
	TH-69	CBS 6531
	TH-11	IFO 1604
<i>Saccharomycopsis synnaedendra</i> Scott et van der Walt	TH-70	ATCC 36174
<i>Saccharomycopsis vini</i> (Kreger-van Rij) van der Walt et Scott	TH-20	IFO 1748 ^T
	TH-57	IFO 1749
	TH-58	IFO 1750
<i>Stephanoascus ciferrii</i> M. Th. Smith, van der Walt et Johannsen	TH-26	IFO 1854 (opposite of mating type of IFO 1855)
	TH-27	IFO 1855 (opposite of mating type of IFO 1854)

Abbreviations: ATCC, American Type Culture Collection, Rockville, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan; T, Type.

Saccharomycopsis vini were included in the genus *Endomycopsella* afterwards (5). However, in The Yeasts (6), *Botryoascus synnaedendra*, *Endomycopsella vini*, and *Yarrowia lipolytica* have been transferred to the genus *Saccharomycopsis*, *Hyphopichia burtonii* to the genus *Pichia*, and *Hormoascus platopodis* to the genus *Ambrosiozyma*. The reclassification of ascoideaceous yeasts has mainly been examined and discussed in terms of morphology, but scholars have yet to agree on all classifications (1, 4, 6, 7). In recent years, YAMAZAKI and KOMAGATA (8-10), YAMAZAKI et al. (11, 12), and YAMAZAKI and GOTO (13) reported that electrophoretic comparison of enzymes is a useful tool for determining the taxonomic relations of various yeasts at the species level. YAMADA and KONDO (14), YAMADA et al. (15, 16), and YAMADA and SMITH (17) reported that the coenzyme Q (ubiquinone) system was well correlated with the groupings of the ascomycetous yeast genera. The object of this paper is to present the electrophoretic comparison of enzymes and the distribution of the coenzyme Q systems in ascoideaceous yeasts and to elucidate their taxonomic relationships.

MATERIALS AND METHODS

Yeast strains. Sixty-one strains belonging to twenty species of nine genera

Table 2. Relative electrophoretic mobility (R_m) values of eight enzymes from ascoideaceous yeast strains.

Species	Strain	R_m value (S) ^a							
		MDH	LDH	GDH	6PGDH	G6PDH	ES	GOT	ADH
<i>Ambrosiozyma cicatricosa</i>	TH- 1	48 54	24	14 16		28	45		49
<i>Ambrosiozyma monospora</i>	TH- 2	41	24	15 17		32	53 56	33	
	TH-59	44	24	16 18		30	72 73 75		
	TH-71	41 53	24	15 17		32	53 56	33	
<i>Ambrosiozyma philentoma</i>	TH- 3	42	60	18		32	56	30	
<i>Arthroascus javanensis</i>	TH- 4	+	50	16			27	45	
	TH- 5	+	51	16		33 38	62		
	TH-80	+		16		28	38		
<i>Ascoidea africana</i>	TH- 6	28 34 40 47		18 20		36	64 86	36	
<i>Ascoidea corymbasa</i>	TH- 7	+		01					
<i>Ascoidea hylecoeti</i>	TH- 8	51 55 59 64							
<i>Ascoidea rubescens</i>	TH- 9	+		20			44		44
	TH-10	+		20			44		44
<i>Cephaloascus fragrans</i>	TH-12	+	54	12		30	52		
	TH-13	+	54	13			41		
<i>Guilliermondella selenospora</i>	TH-21	+	45	13			37 39	15	
	TH-86	+	45	13			37 39	15	
<i>Hormoascus platipodis</i>	TH-22	42	62	16		30	74		
	TH-23	35	60	17		29	67	30	
	TH-84	42	60			32	56	30	
<i>Hyphopichia burtonii</i>	TH-31	45	55	19		33	46 57 60	50	
	TH-44	47	55	19	45	37 39	58 70		
	TH-45	45	55	19		33	68 70		
	TH-46	45	55	19	45	33	68 70		
	TH-47	45	55	19	45	33	68 70		
	TH-48	45	55	19	45	33	60 68 70		
	TH-49	45	53	19	41	36	70 75		
	TH-50	45	55	19	45	33	68 70	32	
	TH-51	45		19	45	32 33 35	59 72 77		

TH-52	45	19	41	36	58 70 77	50	
TH-54	45	55		33	69		
TH-24	+	27 60		39	62 33		
TH-18	+	63		32	52 70 84	44	
TH-19	+	63		32	70 84	44	
TH-14	+	53		31	63	41	
TH-15	+	53		31	63	46	
TH-16	+	53		31	63	46	
TH-17	+	53		31	63	46	
TH-56	+				63		
TH-32	30 34 37 40	64	57	22	61 68 78	42	
TH-33	27 31 34	59	62	20	54 64	46	
TH-34	30 34 37 40	64	57	22	61 68 78	42	
TH-35	30 34 37 40	64	57	22	61 68 78	42	
TH-36	30 34 37 40	64	57	22	61 68 78	42	
TH-37	30 34 37 40	64	57	22	61 68 78	42	
TH-38	30 34 37 40	64	57	22	61 68 78	42	
TH-39	30 34 37 40	64	57	22	61 68 78	42	
TH-40	30 34 37 40	64	57	22	61 68 78	42	
TH-41	30 34 37 40	64	57	22	61 68 78	42	
TH-42	30 34 37 40	64	57	22	61 68 78	42	
TH-43	30 34 37 40	64	57	22	61 68 78	42	
TH-53	30 34 37 40	64	57	22	61 68 78	42	
TH-25	28		57	38	38 67	32	
TH-69	28			38 41	38	32	
TH-11	+			30	56 59		
TH-70	+			28	49 75		
TH-20	+				43		
TH-57	+				43		
TH-58	+				43		
TH-26	30 35 37	26 29	43	39	55 68	37	
TH-27	30 35 37	26 29	43	39	55 68	37	

^a The value of the relative electrophoretic mobilities is indicated by a 100-fold increase.

were employed. These strains and their sources are listed in Table 1.

Electrophoretic patterns of enzymes. (1) Cultivation and harvesting of cells, preparation of cell-free extracts and gel-slab electrophoresis. Methods used to cultivate and harvest cells, to prepare cell-free extracts and gel-slab electrophoresis were essentially the same those reported by YAMAZAKI and KOMAGATA (8), except that the cells were disrupted by the methods of NAGANUMA et al. (19).

(2) Enzyme staining. The enzymes studied were malate dehydrogenase (MDH; EC 1.1.1.37), lactate dehydrogenase (LDH; EC 1.1.1.27), glutamate dehydrogenase (GDH; EC 1.4.1.4), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.41), glucono-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), esterase (Est; EC 3.1.1.1), glutamate-oxaloacetate-transaminase (GOT; EC 2.6.1.1), and alcohol dehydrogenase (ADH; EC 1.1.1.1). The staining procedures used for the enzymes have been described by SICILIANO and SHAW (20). The relative mobilities (*Rm*) of the enzyme bands were calculated as the ratio of the distance that the enzymes moved from the origin to the distance that the tracking dye (Bromophenol Blue) moved.

Numerical analysis. Similarity was calculated for each enzyme by the following formula (21): $S\% = 2N_{AB}/(N_A + N_B) \times 100$. (*S*: similarity value, N_{AB} : the number of enzyme bands with identical *Rms*, N_A : the number of enzyme bands of strain A, N_B : the number of enzyme bands of strain B). These similarity values were averaged to find the overall similarity between strains. Clustering was achieved by means of the unweighted average linkage technique (21). The computing was performed on a FACOM M-380 computer at the Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama, using a FORTRAN program written by Dr. T. Kaneko.

Extraction, purification and identification of coenzyme Q. Coenzyme Q (Co-Q) was purified and identified as reported by YAMADA and KONDO (14); Co-Q was extracted from saponified cells with hexane and purified by preparative thin-layer chromatography with pure benzene on a silica-gel plate. The Co-Q system was identified by reversed phase paper chromatography using the solvent systems reported by YAMADA and KONDO (14) and YAMADA et al. (15).

RESULTS AND DISCUSSION

Electrophoretic patterns of enzymes of sixty one strains used in this study

The relative electrophoretic mobilities (*Rm*) of eight enzymes from sixty one strains are shown in Table 2. The MDH band scattered, making it impossible to measure the *Rm* value in following twenty three strains: all strains of *Ascoidea corymbosa* and *Saccharomycopsis capsularis*, two strains each of *Ascoidea rubescens*, *Cephaloascus fragrans*, *Guilliermondella selenospora*, *Saccharomycopsis crataegensis* and *Saccharomycopsis synnaedendra*, three strains each of *Arthroascus javanensis* and *Saccharomycopsis vini*, and five strains each of *Saccharomycopsis fibuligera*. Fifty strains were analyzed numerically on the basis of the similarity values of the *Rm* of 8 enzymes. The 11 other strains were: *Arthroascus javanensis* TH-80, *Ascoidea*

corymbasa TH-7, *Ascoidea hylecoeti* TH-8, *Ascoidea rubescens* TH-9 and TH-10, 3 strains of *Saccharomycopsis vini*, *Saccharomycopsis fibuligera* TH-56 and 2 of *Saccharomycopsis synnaedendra*. In these the detected number of enzymes was 3 or less, so they were excluded from the analysis on the ground that they would lead to noticeable error because of scarce input data. YAMAZAKI and KOMAGATA (8–10), YAMAZAKI et al. (11, 12), and YAMAZAKI and GOTO (13) reported that the electrophoretic comparison of enzymes was a useful tool for determining the taxonomical relations at the species level. Our research showed a high similarity value (87–100%) among the strains within the following species: 12 strains of *Saccharomycopsis lipolytica*, 4 of *Saccharomycopsis fibuligera*, 2 of *Saccharomycopsis crataegensis*, 2 of *Stephanoascus ciferrii*, 2 of *Cephaloascus fragrans*, and 2 of *Guilliermondella selenospora*. On the other hand, the genera *Ambrosiozyma*, *Hormoascus* and *Hyphopichia*, there was a mixture of high and low similarity value. In the species in which only a small number of enzymes was detected, the similarity value of the strains in the same species was low. Here we need to investigate further the method of preparation of the cells and gel and selection of enzymes to be used.

Classification on the numerical analysis of Rm of eight enzymes and the coenzyme Q system

Fifty strains were divided into 4 major clusters (Fig. 1). The coenzyme Q systems of 40 yeasts are shown in Table 3.

Only *Saccharomycopsis lipolytica* is included in the first cluster. Excluding TH-33, the similarity value of 12 strains was 100%. This species has a Co-Q system and GC content of 50 mol% (22). Moreover, its septal ultrastructure is the central closure line (non-micropore) type (24). In these respects, it is separated from the other species of the genus *Saccharomycopsis*. Thus, it would be appropriate to exclude *Saccharomycopsis lipolytica* from the genus *Saccharomycopsis* (6), and to allocate it to the genus *Yarrowia*, as advocated by VAN DER WALT and VON ARX (5).

The second cluster includes the 4 species *Saccharomycopsis*, *Arthroascus*, *Cephaloascus*, and *Guilliermondella*. Their mutual similarity value were low but the coenzyme Q system of all four species of this cluster was Q-8 and their septal ultrastructure, excluding *Arthroascus javanensis*, was the micropore (plasmodesmata-like) type (24).

The third cluster is made up of 8 strains in the genera *Ambrosiozyma* and *Hormoascus*, but being from the same yeast group (6, 24). The similarity value of each of the three pairs—*Ambrosiozyma monospora* TH-2 and TH-71, *Hormoascus platipodis* TH-84 and *Ambrosiozyma philentoma* TH-3, and *Ambrosiozyma monospora* TH-59 and *Hormoascus platipodis* TH-22—were 80% or more. However, the similarity value among these and within other strains was low. This yeast group is characterized by having the coenzyme Q-7 system and the plugged dolipore (dolipore-like) type septa (23). As reported in The Yeasts (6) and by VAN DER WALT

Fig. 1. Dendrogram based on the similarities of the electrophoretic mobilities of the enzymes of ascoideaceous yeast strains.

Am: *Ambrosiozyma*, *Ar:* *Arthroascus*, *As:* *Ascoidea*, *Cep:* *Cephaloascus*, *G:* *Guilliermondella*, *Ho:* *Hormoascus*, *Hp:* *Hyphopichia*, *S'psis:* *Saccharomycopsis*, *St:* *Stephanosascus*.

Table 3. Coenzyme Q systems in the ascoideaceous yeasts.

Species	Strain	Co-Q	Species	Strain	Co-Q
<i>Ambrosiozyma cicatricosa</i>	TH- 1	7	<i>Saccharomycopsis capsularis</i>		8 (15)
<i>Ambrosiozyma monospora</i>		7 (15)	<i>Saccharomycopsis crataegensis</i>	TH-18	8
	TH- 2	7		TH-19	8
	TH-59	7	<i>Saccharomycopsis fibuligera</i>		8 (15)
	TH-71	7	<i>Saccharomycopsis malanga</i>	TH-25	8
<i>Ambrosiozyma philentoma</i>	TH- 3	7		TH-60	8
<i>Hormoascus platipodis</i>		7 (15)	<i>Saccharomycopsis synnaedendra</i>		8 (15)
	TH-22	7	<i>Saccharomycopsis vini</i>		8 (15)
	TH-84	7	<i>Ascoidea hylecoeti</i>	TH- 8	9
<i>Arthroascus javanensis</i>		8 (15)	<i>Saccharomycopsis lipolytica</i>		9 (15)
<i>Ascoidea corymbosa</i>	TH- 7	8		TH-33	9
<i>Ascoidea rubescens</i>	TH- 9	8		TH-34	9
<i>Guilliermondella selenospora</i>		8 (15)		TH-53	9
<i>Hyphopichia burtonii</i>		8 (15)	<i>Stephanoascus ciferrii</i>		9 (15)
	TH-31	8		TH-26	9
	TH-45	8			
	TH-46	8			
	TH-47	8			
	TH-48	8			
	TH-49	8			
	TH-50	8			
	TH-51	8			
	TH-52	8			
	TH-54	8			

and VON ARX (24), these two genera should be united according to the criteria given above.

The fourth cluster is divided into the two subclusters, 4-1 and 4-2. Subcluster 4-1 consists of only the 11 strains of *Hyphopichia burtonii*; 6 strains with a high similarity value and 5 with a low one. The similarity value is low between TH-44 and TH-46, being opposite mating types. This is a problem requiring further chemotaxonomic investigations. The coenzyme Q system of this group (*Hyphopichia burtonii*) is Q-8, and its septal ultrastructure is of the central closure line (non-micropore) type. Two strains of *Stephanoascus ciferrii* and one of *Ascoidea africana* were included in Subcluster 4-2. The similarity value between the two species was low (45%), but both species had the coenzyme Q-9 system. However, the relationship between the two yeasts and among other yeasts with Q-9 is very controversial; further detailed chemotaxonomic studies are needed.

As shown in Table 4, the ascoideaceous yeasts were divided into three groups according to their coenzyme Q systems. Moreover, as seen below, these three groups generally coincide with the septal ultrastructure reported by VAN DER WALT and VON ARX (23, 24). That is, the Q-7 group (i.e., *Ambrosiozyma* and *Hormoascus*) is of

Table 4. Ascoideaceous yeasts grouped by coenzyme Q systems and ultrastructure of septa.

Co Q-7	Septa (23, 24)	Co Q-8	Septa (23, 24)	Co Q-9	Septa (23, 24)
<i>Ambrosiozyma cicatricosa</i>	[D]	<i>Arthroascus javanensis</i>	[N]	<i>Ascoidea africana</i>	[N]
<i>Ambrosiozyma monospora</i>	[D]	<i>Hyphopichia burtonii</i>	[N]	<i>Ascoidea hylecoeti</i>	
<i>Ambrosiozyma philentoma</i>	[D]	<i>Ascoidea corymbosa</i>		<i>Saccharomycopsis lipolytica</i>	
<i>Hormoascus platipodis</i>	[D]	<i>Ascoidea rubescens</i>	[P]	<i>Stephanoascus ciferrii</i>	
		<i>Guilliermondella selenospora</i>	[P]		
		<i>Saccharomycopsis capsularis</i>	[P]		
		<i>Saccharomycopsis crataegensis</i>	[P]		
		<i>Saccharomycopsis fibuligera</i>	[P]		
		<i>Saccharomycopsis malanga</i>	[P]		
		<i>Saccharomycopsis synnaedendra</i>	[P]		
		<i>Saccharomycopsis vini</i>	[P]		

[D], dolipore-like, plugged dolipore; [N], non-micropore, central closure line; [P], plasmodesmata-like, micropore.

the plugged dolipore (dolipore-like) type, the Q-8 group, excluding *Hyphopichia burtonii* and *Arthroascus javanensis*, is of the micropore (plasmodesmata-like) type, and *Saccharomycopsis lipolytica* with Q-9 is of the central closure line (non-micropore) type. *Hyphopichia burtonii* with Q-8 is separated from the other Q-8 yeast group by forming an independent *Rm* cluster and by having the closure line (non-micropore) type of septal ultrastructure. The first *Rm* cluster—*Saccharomycopsis lipolytica*—is characterized by having the coenzyme Q-9 system and the closure line (non-micropore) type of septal ultrastructure, the second and fourth clusters—*Saccharomycopsis* spp. and the relative genera and *Hyphopichia burtonii*—by having the coenzyme Q-8 system and the micropore (plasmodesmata-like) type, and the third cluster—*Ambrosiozyma* and *Hormoascus*—by having the coenzyme Q-7 system and the plugged dolipore (dolipore-like) type.

Here we have demonstrated that in the ascoideaceous yeasts, a close correlation exists among the numerical classification based on *Rm* of enzymes, the coenzyme Q systems and the septal ultrastructure.

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