Speciation, Species Delineation, and Electrophoretic Isoenzyme Patterns of the Type Strains of *Kluyveromyces* van der Walt emend. van der Walt

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The type strains of the 20 species of the yeast genus *Kluyveromyces* sensu van der Walt 1970 were studied by gel electrophoresis of 11 isofunctional enzymes. These enzymes included five oxidoreductases (alcohol dehydrogenase [EC 1.1.1.1], lactate dehydrogenase [EC 1.1.1.27], malate dehydrogenase [EC 1.1.1.37], catalase [EC 1.11.1.6], and superoxide dismutase [EC 1.15.1.1]), five hydrolases (esterase [EC 3.1.1.1], alkaline phosphatase [EC 3.1.3.1], α -glucosidase [EC 3.2.1.20], β -glucosidase [EC 3.2.1.21], and *exo*- β -glucanase [EC 3.2.1.58]), and one lyase (aldolase [EC 4.1.2.12]). Polymorphism was evident in most of the enzymes studied. Each type strain had a unique pattern when all enzymes were considered. The results of a multivariate analysis of the electrophoretic patterns supported the division of the genus into 13 species, 2 of which comprised four and five taxa, respectively, which were recognized in 1970. Enzyme electrophoresis provided evidence that widespread gene flow does not necessarily occur between yeasts which are able to hybridize in the laboratory.

Protein electrophoresis and isoenzyme electrophoresis have been used to study the population genetics, biosystematics, evolutionary biology, and biochemistry of the enzymes of a wide range of life forms. Electrophoretic studies of fungi have concentrated on ascomycetous genera (8) and have demonstrated multiple molecular forms for certain enzymes. Few studies have been done on yeasts. In some cases (1, 2, 20), electrophoresis has been useful in clarifying taxonomic relationships. A few investigators have studied selected members of the genus *Kluyveromyces* van der Walt emend. van der Walt (4, 5, 7, 16).

The genus *Kluyveromyces* is of special interest because its phenetic structure (15), deoxyribonucleic acid (DNA) reassociation patterns (13; H. J. Phaff, M. A. Lachance, and H. L. Presley, Abstr. 12th Int. Congr. Microbiol., Munich, Germany, 1978, abstr. no. S27.3), and mating compatibility patterns (11) are rather well defined. There have been disagreements as to which of the last two criteria should prevail in delineating *Kluyveromyces* species (14, 19). Enzyme electrophoresis patterns should be useful in determining whether gene flow is effectively operative among taxa which are able to form hybrids but which have diverged genetically, as indicated by low degrees of DNA reassociation.

In this paper, we describe an electrophoretic survey of 11 enzymes, which was undertaken to determine the extent of heterogeneity within *Kluyveromyces* and to identify the different iso-

enzyme patterns in the type strains of the species recognized by van der Walt (18). Our results may indicate the enzymes most useful in studies of gene flow in natural populations of selected *Kluyveromyces* species. The taxonomic relevance of electrophoresis is examined, with particular reference to the possible correlations between electrophoretic patterns and the different criteria used to delineate yeast species.

MATERIALS AND METHODS

Microorganisms. The type strains of the *Kluyveromyces* species listed in Table 1 were obtained from H. J. Phaff, Department of Food Science and Technology, University of California, Davis. These organisms were maintained on yeast extract-malt extract-glucose-peptone agar at 4°C in screw-capped vials.

Preparation of extracts. Cultures were grown on yeast extract-malt extract-glucose-peptone agar for 3 days at 25°C. In the case of a potentially inducible enzyme, each strain was grown to late exponential phase in yeast extract-malt extract-glucose-peptone broth in which the glucose was replaced by the appropriate substrate. Flasks were incubated at 25°C on a reciprocal shaker. For α -glucosidase the substrates were 0.3% maltose, 0.3% melezitose, 0.3% methyl- α -D-glucoside, and 0.3% dextrin, or 0.3% trehalose. The medium for β -glucosidase contained 0.3% arbutin.

Cells were disrupted with 0.5-mm glass beads in a vortex mixer for 3 min in 0.05 M sodium succinate buffer (pH 5.5) with occasional chilling in ice. Homogenates were centrifuged at $10,000 \times g$ for 10 min, and supernatants were decanted. Protein extracts were stored at -20°C. Extracts for alkaline phosphatase were unstable to freezing and therefore were prepared

fresh for each experiment. The addition of various protease inhibitors during extraction was investigated and found to be unnecessary.

Chemicals. Electrophoresis grade acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, tris(hydroxymethyl)aminomethane, glycine, and ammonium peroxydisulfate were purchased from Bio-Rad Laboratories, Richmond, Calif. Bromophenol blue and guaiacol were obtained from British Drug House, Toronto, Ontario, Canada. Laminaran was obtained from United States Biochemical Corp., Cleveland, Ohio. Benzidine, fast blue RR salt, peroxidase, glucose oxidase, glyceraldehyde-3-phosphate dehydrogenase, α -naphthyl acetate, agarose, phenazine methosulfate, 3[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide, nicotinamide adenine dinucleotide, arbutin, maltose, α -methyl-D-glucoside, trehalose, and fructose 1,6-diphosphate were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade.

Agarose gel electrophoresis. Agarose gels were used for catalase, since visualization of this enzyme on polyacrylamide gels is impractical. These gels contained 0.7% agarose, 0.3% starch, 0.021 M tris(hydroxymethyl)aminomethane, 0.015 M boric acid, and 0.0006 M ethylenediaminetetraacetate and were adjusted to pH 8.0. The bridge buffer was 10 $\times$ -concentrated gel buffer. Electrophoresis was conducted at 4°C and 12 mA for 2 h.

Polyacrylamide gel electrophoresis. Discontinuous nondenaturing polyacrylamide gels were prepared as recommended by Davis (3). The stacking gels and the running gels contained tris(hydroxymethyl)aminomethane hydrochloride buffer at concentrations of 0.06 and 0.38 M, respectively; the pH values of these gels were 6.9 and 8.9, respectively. The electrode buffer contained 0.05 M tris(hydroxymethyl)aminomethane and 0.38 M glycine (pH 8.3). The concentrations of the gels varied with the enzymes studied. For β -glucosidase, esterase, aldolase, α -glucosidase, and *exo*- β -glucanase, the acrylamide concentrations in the stacking and running gels were 3.0 and 5.0, 3.0 and 7.7, 5.0 and 7.0, 5.0 and 10.0, and 5.0 and 12.0%, respectively. Single-concentration gels containing 7.7% acrylamide were used for all dehydrogenases, superoxide dismutase, and alkaline phosphatase.

Electrophoresis was conducted in the cold under constant currents between 15 and 30 mA to produce running times of 4 to 5 h without excessive heating. The sample volumes were varied to give optimal resolution. Generally, between 20 and 60 μ l of crude protein extracts was used. Nine samples were applied to each gel slab. The relative mobilities (R_f values) were calculated with reference to the dye front or, for catalase, with reference to the dominant electromorph (the fast band of *Kluyveromyces marxianus* was used as a standard on each catalase gel). All electromorphs were scored on a presence-or-absence basis, and we made no assumptions as to the individual genetic bases. All samples were studied at least twice for each enzyme.

Histochemical staining. *Exo*- β -glucanase, α -glucosidases, and β -glucosidase were visualized by a histochemical method as described elsewhere (16). The substrate concentration for β -glucosidase was 3%. The specificity of α -glucosidases was determined by developing gels separately with α -methyl-D-glucoside, malt-

ose, or trehalose as the substrate, but the R_f values of these enzymes were determined by staining simultaneously with combined substrates. Arbutinase and trehalase were also stained by the method of Eilers et al. (6). Esterase, catalase, lactate, malate and alcohol dehydrogenases, superoxide dismutase, and alkaline phosphatase were stained by the methods of Harris and Hopkinson (9). Catalase gels were examined immediately after hydrogen peroxide was added, as the bands spread rapidly. All other gels were incubated overnight in their staining mixtures.

Data analysis. The amount of information provided by each enzyme was evaluated by the following entropy (I_j) measure:

$$I_j = (n \times t_j \times \ln t_j) - \sum_{i=1}^n [(a_{ij} \times \ln a_{ij}) + (t_j - a_{ij}) \times \ln (t_j - a_{ij})]$$

where n is the number of strains (in this case, 20 strains), t_j is the total number of different electromorphs for the j th enzyme, and a_{ij} is the number of electromorphs of that enzyme present in the i th strain (17). For multivariate analysis, the results were expressed in a matrix having the dimensions $n \times p$, where n (i.e., 20) strains are described by the presence (scored as 1) or absence (scored as 0) of each of p (i.e., 96) electromorphs. Reciprocal averaging (10) was used to ordinate the strains as a function of correlated electromorphs.

RESULTS AND DISCUSSION

Taxonomic structure of *Kluyveromyces*. The genus *Kluyveromyces* has been the subject of several taxonomic investigations since its latest detailed revision by van der Walt (18). The different group structures proposed by various workers are given in Table 1. Basing her structure on phenotypic resemblance, Poncet (15) divided the genus into two fundamental clusters. Group A comprised a number of nutritionally restricted taxa that were isolated mostly from soils. Group B contained taxa that were associated with various habitats and was divided into two subsets, which were distinguished by their growth responses on α -glucosides (subgroup B2 taxa utilized these carbohydrates more extensively than subgroup B1 taxa). Poncet viewed *Kluyveromyces wickerhamii* and *Kluyveromyces phaseolusporus* as transitional forms between subgroups B1 and B2 and *Kluyveromyces thermotolerans* (synonym, *Kluyveromyces veronae*) as a nonmember of the genus. DNA reassociation studies (13; Phaff et al., Abstr. 12th Int. Congr. Microbiol.) gave results which partially agreed with the conclusions of Poncet (15). Such studies indicated high degrees of genetic homogeneity among *Kluyveromyces lactis*, *Kluyveromyces vanudenii*, *K. phaseolusporus*, and *Kluyveromyces drosophilae* and among *K. marxianus*, *Kluyveromyces wikenii*, *Kluyvero-*

TABLE 1. Nomenclature and proposed groupings of *Kluyveromyces* type strains

UCD(FS&T) no. ^a	Taxonomic designation	Grouping by:		
		Phenetic similarity ^b	DNA reassociation ^c	Hybridization ^d
61-29 ^T	<i>K. aestuarii</i> (Fell) van der Walt 1961	B2	<i>K. aestuarii</i>	<i>K. aestuarii</i>
70-4 ^T	<i>K. vanudenii</i> (van der Walt et Nel) van der Walt 1963	B2	<i>K. lactis</i>	<i>K. marxianus</i>
50-80 ^T	<i>K. phaseolusporus</i> (Shehata, Mrak, et Phaff) van der Walt 1955	B1-B2	<i>K. lactis</i>	<i>K. marxianus</i>
71-59 ^T	<i>K. lactis</i> (Dombrowski) van der Walt 1910	B2	<i>K. lactis</i>	<i>K. marxianus</i>
51-130 ^T	<i>K. drosophilorum</i> (Shehata, Mrak, et Phaff) van der Walt 1955	B2	<i>K. lactis</i>	<i>K. marxianus</i>
50-45 ^T	<i>K. dobzhanskii</i> (Shehata, Mrak, et Phaff) van der Walt 1955	B2	<i>K. dobzhanskii</i>	<i>K. marxianus</i>
71-15 ^T	<i>K. wikenii</i> van der Walt, Nel, et Kerken 1966	B1	<i>K. marxianus</i>	<i>K. marxianus</i>
71-58 ^T	<i>K. fragilis</i> (Jorgensen) van der Walt 1909	B1	<i>K. marxianus</i>	<i>K. marxianus</i>
55-82 ^T	<i>K. marxianus</i> (Hansen) van der Walt 1888	B1	<i>K. marxianus</i>	<i>K. marxianus</i>
71-14 ^T	<i>K. cicerisporus</i> van der Walt, Nel, et Kerken 1966	B1	<i>K. marxianus</i>	<i>K. marxianus</i>
71-13 ^T	<i>K. bulgaricus</i> (Santa Maria) van der Walt 1956	B1	<i>K. marxianus</i>	<i>K. marxianus</i>
72-13 ^T	<i>K. waltii</i> Kodama 1974	— ^e	— ^e	(<i>K. marxianus</i>)
54-210 ^T	<i>K. wickerhamii</i> (Phaff, Miller, et Shifrine) van der Walt 1956	B1-B2	<i>K. wickerhamii</i>	(<i>K. marxianus</i>)
55-41 ^T	<i>K. thermotolerans</i> (Philippov) Yarrow 1932	N	<i>K. thermotolerans</i>	(<i>K. marxianus</i>)
70-5 ^T	<i>K. phaffii</i> (van der Walt) van der Walt 1963	A	(<i>K. phaffii</i>)	<i>K. phaffii</i>
57-17 ^T	<i>K. polysporus</i> van der Walt 1956	A	<i>K. polysporus</i>	<i>K. polysporus</i>
56-2 ^T	<i>K. delphensis</i> (van der Walt et Tscheuschner) van der Walt 1956	A	(<i>K. delphensis</i>)	<i>K. delphensis</i>
70-3 ^T	<i>K. lodderi</i> (van der Walt et Tscheuschner) van der Walt 1957	A	(<i>K. lodderi</i>)	<i>K. lodderi</i>
77-7 ^T	<i>K. blattae</i> Henninger et Windish 1976	— ^e	— ^e	<i>K. blattae</i>
57-16 ^T	<i>K. africanus</i> van der Walt 1956	A	(<i>K. africanus</i>)	<i>K. africanus</i>

^a UCD(FS&T), Culture collection of the Department of Food Science and Technology, University of California, Davis. T = type strain.

^b From reference 15.

^c From reference 13 and the work of Phaff et al. (Abstr. 12th Int. Congr. Microbiol.). Parentheses indicate uncertain affiliation.

^d From reference 11. Parentheses indicate low levels of hybridization.

^e Strains not available at the time when the studies were performed.

myces fragilis, *Kluyveromyces cicerisporus*, and *Kluyveromyces bulgaricus*. Johansen (11) examined the ability of *Kluyveromyces* strains to form hybrids with combined parental phenotypes. A total of 10 taxa showed relatively high levels of mating intercompatibility, and for this reason these organisms were considered to represent varieties of a single species, *K. marxianus*. Three other taxa (Table 1) hybridized less extensively with some of these putative varieties and were therefore maintained as separate but related species. The remaining seven species were recognized as reproductively isolated.

Electrophoretic patterns. The 20 type strains of *Kluyveromyces* species exhibited considerable heterogeneity with respect to electrophoretic patterns (Fig. 1). The various enzymes differed in the amount of information which each could provide, as evidenced by the entropy values, which ranged from 8 to 185. Amylase, β -galactosidase, β -fructosidase, hexokinase, glu-

cose dehydrogenase, and protease were also examined. These enzymes did not provide reproducible results due to poor extractability or stability and were not considered further.

Dehydrogenases (Fig. 1A through C) had intermediate entropy values. A number of electromorphs were shared by all members of the *K. marxianus* mating compatibility group; however, some electromorphs were found exclusively in members of either the *K. lactis* subgroup or the *K. marxianus* subgroup, as defined by DNA complementarity (Table 1). For most enzymes, *Kluyveromyces waltii* and *K. bulgaricus* were very similar, and *Kluyveromyces aestuarii* was unique. Several members of phenetic group A lacked activity for some dehydrogenases.

Catalase (Fig. 1D) exhibited little variation. One electromorph was common to 19 of the 20 strains tested. It should be pointed out that the agarose gels used for this enzyme were less discriminatory than polyacrylamide gels. Sever-

al strains showed a second electromorph whose distribution did not follow any recognizable pattern. Again, *K. aestuarii* was unique. *K. waltii* and *K. bulgaricus* were identical.

Despite the rather high entropy of superoxide dismutase (Fig. 1E), this enzyme produced patterns which were highly consistent with the DNA reassociation groupings (Table 1). Of interest was the apparent intermediate status of *Kluyveromyces dobzhanskii*, which shared electromorphs found in either the *K. lactis* DNA subgroup or the *K. marxianus* DNA subgroup. Once more, *K. aestuarii* appeared to be unique.

Esterase (Fig. 1F) was the most variable (I_j , 185) enzyme studied. The patterns were distinct for all strains. Most strains in the *K. marxianus* DNA subgroup (Table 1) produced considerably more electromorphs than their *K. lactis* subgroup counterparts, with little overlap between the two subgroups.

Aldolase activity (Fig. 1G) was absent in more than one-half of the strains. One of the electromorphs of this enzyme was shared by all members of phenetic subgroup B2 (Table 1), including *K. aestuarii*, a rare occurrence.

Alkaline phosphatase (Fig. 1H) activity was not detected in the *K. lactis* subgroup (Table 1). The strains in phenetic group B1 (equivalent to the *K. marxianus* DNA subgroup) had overlapping patterns that were somewhat suggestive of a population which was heterozygous for a biallelic locus.

Glucosidases must be viewed differently from other enzymes. *Kluyveromyces* species may vary in their utilization of glucosides (18). The slow (R_f , <0.4) electromorphs of α -glucosidase (Fig. 1I) were particularly active in the hydrolysis of trehalose. Two of these electromorphs were sporadically found throughout the genus, a third was unique to three members of the *K. marxianus* DNA subgroup (Table 1), and a fourth was unique to *Kluyveromyces lodderi*. *K. thermotolerans* produced, among others, an electromorph that was more specific toward α -methyl-D-glucoside (R_f , 0.43). A single electromorph with maximum activity on maltose was present in maltose-utilizing members of the *K. lactis* DNA subgroup.

Members of the *K. marxianus* DNA subgroup produced up to three β -glucosidase forms (Fig. 1J), one of which was also present in two strains of the *K. lactis* subgroup. The very low entropy of this enzyme reflected not only its lack of variability, but also its rarity in the genus.

Exo- β -glucanase (Fig. 1K) has been discussed previously (16); the results are shown here for comparison. The presence of electromorphs typical of both the *K. marxianus* and *K. lactis* subgroups in *K. drosophilae* and the uniqueness of *K. aestuarii* are indeed striking.

The data described above indicate that further studies of polymorphism in natural populations of *Kluyveromyces* would be best directed at enzymes such as dehydrogenases, superoxide dismutase, esterase, and *exo*- β -glucanase. These enzymes were present in all of the members of *K. marxianus* mating compatibility group (Table 1), and their varying degrees of polymorphism should allow strain characterization over a broad range of heterogeneity.

Speciation and species delineation. Yeast taxonomists generally regard the species as a special taxonomic category. Most go along with the notion that reproductive isolation is the fundamental criterion upon which species may be meaningfully delineated. They do differ, however, in their choices of methodologies by which commonality of gene pools may be determined. Phaff (14) discussed this matter, emphasizing that conspecificity requires many elements. First, chromosomal DNA complementarity is required to allow viable recombination to occur between strains. Equally important is mating compatibility, which is necessary for genomes to interact physically. Finally, habitats in common are essential for mating to occur at frequencies high enough to allow significant gene exchange among natural populations.

In *Kluyveromyces*, genome compatibility and mating compatibility are rather well defined (Table 1). Although ecological specificity is less well understood, it is logical to assume that phenetic affinities (Table 1) are major determinants of the ecological distribution of *Kluyveromyces* species. The electrophoretic patterns presented here may offer a means of verifying gene flow a posteriori. Indeed, many electromorphs represent independent genes that are expected to covary as a function of reproductive continuity. For example, if the *K. marxianus* mating group is effectively syngamic (11), then the distribution of electromorphs among its members should be more or less continuous. Conversely, if among several electromorphs there are strong correlations and sharp discontinuities which are consistent with genome complementarity patterns as identified by DNA reassociation, then it must be concluded that the relevant groups are members of reproductively isolated populations.

To answer these questions, the electrophoretic data were subjected to multivariate analysis by means of reciprocal averaging. This eigenanalysis method ordines both individuals and variables as a function of their canonical (reciprocal) correlations. The ordination of *Kluyveromyces* type strains on canonical axes 2 and 4 is shown in Fig. 2. The first axis shown (axis Y2) is heavily weighted in favor of electromorphs that are unique to the *K. lactis* subgroup and absent

FIG. 1. Electrophoretic patterns of *Kluyveromyces* type strains for 11 enzyme activities. See the test for a description of the conditions used. The numbers in parentheses are the entropy values (I_j) for each enzyme system. The α -glucosidase activities are partitioned according to the specificities of the electromorphs for trehalose and maltose. Abbreviations: ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; CAT, catalase; SOD, superoxide dismutase; EST, esterase; ALD, aldolase; APA, alkaline phosphatase; α GS, α -glucosidase; β GS, β -glucosidase; E β G, *exo*- β -glucanase.

ase; the scores of electromorphs were not plotted for purposes of clarity). The second axis shown (axis Y4) also discriminates strongly between the members of the *K. marxianus* and *K. lactis* DNA subgroups. This axis is compounded mostly by electromorphs that are unique to *K. lodderi*. Most enzymes involved in the Y2 axis also showed high canonical correlations with the trends elicited by the Y4 axis.

Other axes (Y1, Y3, Y5 [data not shown]) also separated the *K. marxianus* and *K. lactis* subgroups. Altogether, the first five axes accounted for 51% of the data structure. Some of the properties are summarized in Table 2. In each case, the two DNA subgroups represented mutually exclusive statistical populations ($P < 0.01$). The canonical correlations were significant ($P < 0.01$), and the most unusual taxa elicited were not members of either subgroup.

in *K. aestuarii*. The most highly correlated electromorphs were found in several enzymes (in particular, the dehydrogenases, superoxide dismutase, esterase, *exo*- β -glucanase, and aldol-

FIG. 2. Ordination of *Kluveromyces* type strains by reciprocal averaging. The percentage of the canonical structure represented is indicated for each axis. The dashed lines define phenetic group A and the *K. lactis* and *K. marxianus* DNA subgroups (Table 1). Abbreviations: Ae, *K. aestuarii*; Af, *K. africanus*; Bl, *K. blattae*; Bu, *K. bulgaricus*; Ci, *K. cicerisporus*; De, *K. delphensis*; Do, *K. dobzhanskii*; Dr, *K. drosophilum*; Fr, *K. fragilis*; La, *K. lactis*; Lo, *K. lodderi*; Ma, *K. marxianus*; Pf, *K. phaffii*; Ps, *K. phaseolusporus*; Po, *K. polysporus*; Th, *K. thermotolerans*; Va, *K. vanudenii*; Wa, *K. waltii*; Wc, *K. wickerhamii*; Wk, *K. wikenii*.

The overwhelming conclusion of the analyses described above is that the *K. lactis* and *K. marxianus* DNA subgroups must be regarded as evolutionary distinct populations (biological species), despite their ability to mate under laboratory conditions. In terms of nomenclature, the retention of *K. lactis* (with up to four varieties) and *K. marxianus* (with up to five varieties) as separate species would provide species delineation that is consistent with speciation patterns. Verification of these suggestions should derive from electrophoretic studies of populations of strains belonging to these taxa to assess varietal homogeneity. The type strains

themselves obviously do not constitute natural populations, and definitive conclusions must rest on data from larger numbers of natural isolates. In addition, the electrophoretic patterns of the meiotic progeny of interspecific hybrids should be examined to verify whether postmating genetic isolation is operating in addition to presumed ecological factors. Both of these approaches are under investigation in our laboratory.

K. dobzhanskii appears to share with *K. thermotolerans* and *K. wickerhamii* a more or less intermediate status, although a closer relationship to the *K. lactis* subgroup is suspected (Fig.

TABLE 2. Comparison of canonical scores in the *K. lactis* and *K. marxianus* DNA subgroups^a

Canonical axis	<i>K. lactis</i> subgroup (n = 4)		<i>K. marxianus</i> subgroup (n = 5)		<i>t</i> ^b	Canonical correlation	Most unusual taxon	Score of most unusual taxon
	$\bar{Y}$	s^2	$\bar{Y}$	s^2				
1	3.4	6.7	-3.5	5.8	4.1	0.76	<i>K. aestuarii</i>	42.0
2	16.6	9.1	-4.2	3.8	11.9	0.74	<i>K. aestuarii</i>	-17.3
3	-0.4	0.4	3.1	2.4	4.6	0.68	<i>K. africanus</i>	15.4
4	5.0	2.3	-7.7	2.4	12.4	0.66	<i>K. lodderi</i>	16.0
5	-0.8	0.0	-4.5	2.1	8.2	0.60	<i>K. phaffii</i>	10.8

^a $\bar{Y}$, Mean *Y* value; s^2 , variance of *Y*; *t*, Student's *t* test.
^b The critical *t* value (*P* = 0.01; df = 7) was 3.5.

1 and 2). The singular electrophoretic similarity between *K. waltii* and *K. bulgaricus* was unexpected, because members of the former taxon show a remarkable degree of morphological, physiological, and ecological similarity to *K. thermotolerans*, whereas *K. bulgaricus* is more similar to members of the *K. marxianus* DNA subgroup. At this point, we do not propose to unify these two taxa or to incorporate *K. waltii* as a variety of *K. marxianus*.

The status of *K. drosophilum* is of special interest. As noted previously (16), the type strain exhibits *exo*- β -glucanase electromorphs that are found in both the *K. lactis* and *K. marxianus* subgroups, although microcomplement fixation (12) only detected enzyme relatedness to the *K. lactis* subgroup. *K. drosophilum* also exhibited special electrophoretic similarities to *K. dobzhanskii* (Fig. 1E and F) and to *K. thermotolerans* and some members of phenetic group A (Fig. 1D). The level of DNA relatedness of *K. drosophilum* to other *K. lactis* subgroup strains was estimated at 70% (13; Phaff et al., Abstr. 12th Int. Congr. Microbiol.), which is unusual. One might speculate that *K. drosophilum* is a natural hybrid or that it represents a slowly evolving "ancestral" form. It is not clear how this question can be clarified.

Conclusions. The evolutionary taxonomy of *Kluyveromyces* species is complicated by varying degrees of homothallism in most of the species (18). The coordinate use of many methodological approaches will be required to accomplish sound species delineation based on the speciation process itself. Allopatric speciation is an often ignored alternative to genetic isolation due to loss of mating compatibility. Evidence that groups of strains constitute mutually exclusive ensembles of independent characters which are not directly influenced by natural selection (e.g., the electrophoretic mobilities of certain enzymes) can be used to reconstitute a fragment of their evolutionary history. We have shown, at least with the type strains of *Kluyveromyces* species, that interfertile strains belonging to different DNA complementarity groups also appear to be distinct evolutionary groups. This finding does not necessarily rule out other types of speciation, such as reticulate evolution, and makes the genus *Kluyveromyces* all the more interesting as a taxonomic and evolutionary model.

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