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Electrophoretic Isoenzyme Patterns In The Yeast Genus Kluyveromyces

Deborah Gayle Sidenberg

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ELECTROPHORETIC ISOENZYME PATTERNS IN THE YEAST GENUS *KLUYVEROMYCES*

by

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Submitted in partial fulfillment

of the requirements for the degree of

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ABSTRACT

The overall objective of this work was to examine natural populations of ascogenous yeasts by electrophoresis of isoenzymes, and to assess the taxonomic implications of such a study with reference to the various other approaches used to delineate yeast species. In particular, speciation and species delineation were investigated in the genus *Kluyveromyces* van der Walt *emend.* van der Walt.

The twenty type strains of the species of the genus *Kluyveromyces sensu* van der Walt (1970) were compared for eleven isoenzymes. 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]).

The isoenzyme patterns of seven enzymes (alcohol dehydrogenase, malate dehydrogenase, superoxide dismutase, esterase, exo-β-glucanase, as well as α- and β-glucosidases) of *Kluyveromyces* populations from a variety of habitats were compared. The isolates belonged to the *Kluyveromyces lactis* and *K. marxianus* deoxyribonucleic acid (DNA) reassociation groups, and to *Kluyveromyces dobzhanskii*, *Kluyveromyces thermotolerans*, and *Kluyveromyces waltii*. Four strains of *Candida* considered to be *Kluyveromyces* anamorphs were included in those listed above. Three strains (*Candida sake*, *Saccharomyces cerevisiae*, and *Pichia fluxuum*) not belonging to the genus were also examined.

The isoenzyme patterns readily substantiated the relationship of the anamorphs with their putative teleomorphs and the nonmember status of *S. cerevisiae*, *P. fluxuum*, and *C. sake*. The results of multivariate analysis of the electrophoretic patterns supported the division of the genus *Kluyveromyces* into thirteen species, two of which comprised four and five taxa (described by van der Walt, 1970b) respectively. Electrophoretic data

were at variance with *in vitro* mating compatibility patterns but supported DNA-DNA reassociation data.

Finally, interspecific and intraspecific hybrids between *K. marxianus* and *K. thermotolerans* and between *K. lactis* and *K. vanudenii*, respectively, their parental strains, and their corresponding type cultures were studied by comparison of seven isoenzyme patterns. The parental strains of the interspecific hybrid were very different, and crossing between these two strains did not receive much direct substantiation. The *K. marxianus* x *K. thermotolerans* hybrid appeared very similar, electrophoretically, to its *K. marxianus* parental strain, and had little in common with *K. thermotolerans*. The loss and gain of bands by the hybrid strain did support Kurtzman's *et al.* (1983) suggestion that it might be an aneuploid yeast. Even though the parental strains of the intraspecific hybrids had very similar isoenzyme patterns, differences in electrophoretic patterns were observed between parental and hybrid strains, indicating that crossing can occur between *K. lactis* and *K. vanudenii*. Certain isoenzymes (superoxide dismutase, esterase, and α-glucosidase) were found to be better indicators of the hybrid state than other enzymes. The possible relationships between hybrids and populations were explored.

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CHAPTER 1 - INTRODUCTION AND LITERATURE REVIEW

1.1 Speciation, a Dimension of Biological Evolution

Delineation of a biological species has long been and still is the subject of controversy. In the early to mid seventeen hundreds, Linnaeus conceptualized morphological species as fixed monotypic units, the products of special creation in which any variation was accidental. The revolutionary studies of Darwin (1858, 1859) on variation, Wallace (1858) on geographic distribution, and Mendel (1866) in genetics, challenged special creation by stressing that variation within species was not only permissible but the rule. With the advent of population concepts, species were no longer seen as static units, but were considered to change through long periods of time. It was not until post-Mendelian population concepts were developed by Chetverikov, Fisher, Haldane, and Wright, in the early nineteen hundreds, that the source of variability and many of the mechanisms of evolution became discernible. The research of these and many other scientists has culminated in the still developing "synthetic theory" of evolution. It is this theory which maintains that mutation, genetic recombination, and natural selection are the essential processes for evolutionary change and divergence (Stebbins, 1977).

As early as 1859, Darwin recognized that it is difficult to decide how extensive the differences in organisms had to be before the forms were deemed entities worthy of specific names. As techniques to determine variation in behavior, ecology, and biochemistry have become more powerful, the definition of species has become even more complex. To date, there is no definition of species that will encompass all of the dimensions of evolution, but the search by taxonomists for parsimonious, practical and accurate methods of defining species based on functional and information data types is ongoing.

An especially ambitious objective of yeast taxonomists is to delineate natural taxa based on the speciation process itself. Since the end of the

nineteenth century, investigators have attempted to organize this heterogenous assemblage of fungi whose only common trait is a predominately unicellular morphology in their vegetative cycle, with the consequence that over the years the species concept in yeasts has become more sophisticated.

Early taxonomists delineated yeast species on the basis of morphological features and sugar fermentation (Hansen, 1904; Guilliermond, 1920, 1927a,b,c, and 1928a,b,c). Whereas trivial morphological differences were often used to classify conspecific strains separately, the limited number of physiological tests has also led to the clumping of different species. The Kluyver or "Dutch" school of taxonomy (Stelling-Dekker, 1931; Diddens and Lodder, 1942, Lodder and Kreger-van Rij, 1952) improved the ability to separate species by the addition of some assimilatory reactions to the few fermentation tests used previously. The introduction of synthetic media by Wickerham (1951) greatly expanded the number of compounds used to test the physiological attributes (carbon and nitrogen assimilation, as well as vitamin requirements) of yeasts. Gradually, the number of tests was expanded to include some fifty physiological and morphological characters. Delineation of yeast species was based on one or several physiological differences, but doubt as to how many sorts of criteria were required for species delineation still persisted. Later, the dilemma was further augmented by the realization that traditional criteria were only a limited reflection of the yeast genomes being studied (Price *et al.*,1978; Phaff, 1980; Kurtzman *et al.*, 1983).

Recently, Lodder (1970) cautioned that the most important taxon, the species, is based on similarities between strains and is innately nominalistic. The methods of classification of yeasts have been fully reviewed by Wickerham (1951), Lodder and Kreger-van Rij (1952), Kreger-van Rij (1969, 1984), Lodder (1970), van der Walt (1970a), and Barnett *et al.* (1979, 1983). From their discussions of the three major contemporary criteria (morphology, physiology and sexuality), it seems that morphology is the most speculative, physiology is the most stressed, and sexuality the most controversial.

Kreger-van Rij (1969) best explained the taxonomic relationship between morphology and physiology when she stated that physiological properties alone differentiate species of morphologically homogenous genera, whereas both morphology and physiology are instrumental in the differentiation of morphologically heterogenous genera.

Some of the controversy regarding sexuality stems from the definition of biological species articulated by Mayr (1942). He defined species as "actually or potentially interbreeding populations, which are reproductively isolated from other such groups". Reproductive isolation, an ambiguous terminology, is difficult to authenticate in nature. The definition is further obscured by individuals in "good" species (groups of organisms proven to be distinct by other methods) occasionally hybridizing. Since the integrity of populations, not individuals, is maintained, Mayr (1963) was forced to redefine isolating mechanisms as "biological properties of individuals which prevent the interbreeding of populations that are actually or potentially sympatric."

Wickerham (1951) avidly supported the biological species view. His many studies were instrumental in inspiring the emphasis now placed upon habitat specificity in delineating species and awakening the need to set more realistic species boundaries by studying large natural populations. Winge and Lautsen's (1939) as well as Wickerham and Burton 's (1956a) proposal that interfertility be used as an indicator of conspecificity has brought a new dimension, as well as a new conflict to yeast taxonomy. The conflict arises because this criterion is not applicable to all yeasts. Some strains are exclusively asexual (imperfects) and many strains are self-fertile. In order to avoid this controversy, it has been proposed (Smith, 1974) that the species category be retained in practice, to classify organisms on the basis of convenience. By contrast, the biological species concept should be reserved for the description of real evolutionary units (Mayr, 1982) when enough information is available to identify them as such.

The biological evolutionary process has two dimensions, phyletic evolution and speciation (Rensch, 1959; Avise, 1976; Ayala, 1978). Both

dimensions of this gradual process of change in genetic constitution are population phenomena. Phyletic evolution, or anagenesis, encompasses the gradual changes that occur with time in a single lineage of descent. These changes result in a greater adaptation to the environment. Speciation, a cladogenic process, occurs when a lineage of descent splits into two or more new lineages. This process accounts for the great variety of organisms found in the world. (Throughout the remainder of this thesis, speciation is used in this evolutionary sense and not in the taxonomic sense of delineating yeasts). Although there is some debate as to the number of modes of speciation (Mayr, 1957, 12 modes and White, 1978, 7 modes), ultimately, barriers between closely related populations are established (Solbrig and Solbrig, 1979).

To date, there are about sixty genera and five hundred species of yeasts (Kreger-van Rij, 1984a). Modern technology, in particular molecular biology, has been instrumental in establishing yeast species based on evolutionary concepts.

1.2 Modern Approaches to the Concepts of Speciation and Species Delineation in Yeasts

There exists an extensive literature on the impact of modern technology on the study of speciation and species delineation in yeasts, as well as other organisms. Recent approaches in yeasts include comparisons of polysaccharides, cell wall ultrastructure, spore topology, ecology, metabolic diversity, genomes, genetic compatibility, amino acid sequences, immunological properties of proteins, co-enzyme Q structure, and isoenzymes. Despite their individual shortcomings, all of these approaches have contributed to the elucidation of relationships among yeasts particularly when considered jointly. Notwithstanding technical advances, researchers differ in their choices of methods by which commonality of gene pools may be determined. The evolutionary attributes of the information obtained from these approaches have been reviewed by numerous investigators (Lachance, 1977; Price *et al.*, 1978; Mendonca-Hagler, 1980; Phaff, 1980; Kreger-van Rij, 1984a; Kurtzman *et al.*, 1983), thus only a brief account of the taxonomic

levels to which these methodologies can be applied follows.

Although very little research has been done on the structure of component polysaccharides of cell walls and capsules of encapsulated yeasts, evolutionarily significant differences have been demonstrated. Phaff (1984b) summarized the major findings. The cell walls of ascomycetous (ascospore-forming) budding yeasts have been shown to differ from those of fission yeasts and the yeasts related to basidiomycetous (basidiospore-forming) species. Furthermore, polysaccharide "fingerprints" have shown potential use in elucidating relationships between perfect (posessing sexual spores) and imperfect (posessing no sexual spores) yeasts. Finally, capsular polysaccharide composition has been used with mixed success to classify certain capsulated yeast species. Because serotypes (Tsuchiya *et al.*, 1974 and Fukagawa *et al.*, 1980) and proton magnetic resonance spectra of yeasts (Gorin and Spencer, 1970) are unstable, Phaff (1971, 1984b) and Tsuchiya *et al.* (1974) felt that the use of such information should be limited to defining supraspecific or generic groups.

Ultrastructural differences have also been shown to be evolutionarily significant (Kreger-van Rij, 1984b). Transmission electron microscopic studies of Kreger-van Rij (1978a,b) and Kreger-van Rij and Veenhuis (1969a,b; 1971, 1973, 1975) have demonstrated that ascomycetous and basidiomycetous yeasts differ in the lateral wall structure of vegetative cells, the wall ultrastructure of bud formation, and hyphal septum ultrastructure. Scanning electron microscopy studies (Kurtzman and Smiley, 1974 and Kurtzman *et al.*, 1975) of spore topography may define groups in an exclusionary manner. Price *et al.* (1978) proposed that ascospore morphology with the additional study of both surface ornamentals and wall layering might prove a stable indicator of evolutionary affinities, but Fuson *et al.* (1980) did not support this view. Despite contention, Kreger-van Rij and Kurtzman (1984) have recently predicted that several models for the spore wall based on ultrastructure may be useful in taxonomic differentiation.

Phylogenetic schemes based solely upon yeast morphology or yeast ecology are merely hypothetical. Wickerham (1969), Wickerham and Burton

(1962), and Bartnicki-Garcia (1970) proposed that biochemical information might enhance and help validate such phylogenetic schemes. Bartinicki-Garcia cautioned, however, that considering only one biochemical criterion could result in evolutionary relationships being misconstrued. A complementary argument was presented by Roberts (1960) who stressed that schemes which classified yeasts solely on biochemical criteria were unacceptable. A few mutable genes control the initial hydrolytic reactions of the standard sugars in the oxidative patterns (Price *et al.*, 1978) and nitrate assimilation (Marzluf, 1981) tests. For this reason, Price *et al.* (1978) and Kurtzman *et al.* (1983) felt that such tests should not be used to define species.

Because DNA is the most fundamental substance of life (Watson *et al.*, 1983), the information obtained from techniques which study nucleic acids holds an important position in evolutionary taxonomy. Techniques for DNA research have been developing rapidly. Dobzhansky *et al.* (1977) have extensively discussed macromolecules and phylogenies for a wide range of organisms, and Kurtzman *et al.* (1983) have discussed nucleic acid relatedness among yeasts.

DNA base composition determinations like those of Meyer and Phaff (1970), Nakase and Komagata (1970a,b; 1971 a,b,c,d,e,f,g) Martini *et al.* (1972) Bak (1973) and Schildakraut *et al.* (1962) only provide exclusionary data. Yeasts with significantly different molar percentage guanine plus cytosine values (2.0 to 2.5 mol% G+C by thermal denaturation and 1.0 to 1.5 mol% G+C by buoyant density in cesium chloride gradients) are not considered to share recent ancestors (Kurtzman *et al.*, 1983), and thus represent different species. For the most part, species within a genus have mol% G+C contents that fall within a range of 10%. In general, ascomycetous yeasts and their corresponding anamorphs have mol% G+C values between 30 to 50, whereas basidiomycetous yeasts and their corresponding anamorphs have mol% G+C values between 50 to 70.

The many methods of determining DNA-DNA complementarity have proved useful in quantifying the similarity of convergent yeasts (Phaff,

1984), but they may only be used to taxonomically resolve strains to the sibling species level (Kurtzman *et al.*, 1983). Strains with 80% or greater DNA relatedness are considered to be conspecific (Martini and Phaff, 1973; Price *et al.*, 1978) while those with less than 20% are regarded to be more distantly related (Phaff and Holzchu, 1981). Complementarity values ranging from 65 to 80% are considered to indicate more differentiated strains within a species (Kurtzman *et al.*, 1983). A variation of the DNA-DNA reassociation technique, ribosomal RNA (rRNA)-DNA homology (Bicknell and Douglas, 1970), is restricted to intergeneric relationships.

Of all the techniques available, Price *et al.* (1978) considered DNA-DNA homology the best for species delineation purposes, especially if interfertility was considered jointly with it. Although there are exceptions, it can be said that decreasing DNA relatedness correlates with decreasing mating competence and fertility (Phaff, 1984). Whereas Phaff (1980) strongly advised against singularily overemphasising interfertility, many consider interfertility of prime importance in delineating species boundaries.

Genes determine the primary structure of proteins, thus techniques which determine the nature of proteins also hold key positions in elucidating evolutionary processess. Like DNA research, comparative enzyme biology is also a rapidly developing field of study. Dayhoff (1969), Goodman (1976), and Dixon and Webb (1979) have extensively discussed the contribution of protein information to the study of species evolution. Kurtzman *et al.* (1983) and Phaff (1984a) have discussed its importance to yeast taxonomy.

Long chains of amino acids make up proteins, thus it is the sequence of these amino acids which determines the specific properties of each protein. Amino acid sequences within species are very similar, thus amino acid sequence analyses are primarily used to study differences in classes, orders, and families (Dixon and Webb, 1979). There have been very few amino acid sequencing studies performed on yeasts because of the time and expense involved.

Like amino acid sequence studies, micro-complement fixation estimates of genetic distances are sparse. The only study of this nature applied to yeasts was an immunological comparison of exo-β-glucanase in species of *Kluyveromyces* (Lachance and Phaff, 1979) in which the resolution of the data was found to be approximately the same as for DNA reassociation experiments.

Studies by Yamada and Kondo (1973) and Yamada *et al.* (1973a,b,c; 1976a,b; 1977; 1980) have revealed that there are several distinct classes of co-enzyme Q systems in yeasts. Their primary taxonomic value is at the genus level. These enzyme systems have also been found useful in studying the taxonomic relationship between some imperfect and perfect yeasts.

Yet another rapid but not absolutely rigorous alternative for studying protein relationships is electrophoresis (Nei, 1971).

1.3 Protein Electrophoresis, a Tool for Measuring Evolutionary Potential

Acceptance of Mayr's proposal that "biological evolution is best defined in the diversity and adaptation of populations of organisms" necessitates the determination of a population's evolutionary potential or degree of genetic variation. A popular approach (Chetverikov, 1926; Fisher, 1930; Dobzhansky, 1951, 1970, 1976; Dobzhansky *et al.* 1944, 1977; Haldane, 1954; Wright, 1963; Nei, 1972, 1975; Nei *et al.* 1973, 1974, 1975) has been to measure various characters present in natural populations and to apply mathematical analyses. The technique of protein electrophoresis provides an estimation of genetic variation in a particular population (Ayala, 1976).

It would be impossible to list more than a few of the published books or reviews on the different types of protein gel electrophoresis (Wilkinson, 1965; Brewer and Sing, 1970; Johnson and Thein, 1970; Shaw and Prasad, 1970; Maurer, 1971; Chrambach and Rodbard, 1971; Chrambach, 1980; Harris and Hopkinson, 1976; Gaal *et al.*, 1980; Gottlieb, 1981; Moss, 1982; Oxford and Rollinson, 1983; Tanskley and Orton, 1983). The currently preferred method of electrophoretic characterization of proteins is that of polyacrylamide

gel electrophoresis (PAGE). Separation of proteins is based on the criteria of electrostatic charge (reviewed by Righetti and Drysdale, 1976) and molecular weight (reviewed by Chrambach and Rodbard, 1971).

Harris and Hopkinson (1976) and Feltham and Sneath (1979) explained that after electrophoresis proteins may be stained non-specifically (i.e. all the different proteins present produce bands) or specifically (i.e. only particular enzymes in the mixture produce bands). The latter bands are isoenzymes (isozymes) which are defined as "multiple molecular forms of a given enzyme occurring either in a single individual or different members of the same species" (Harris and Hopkinson, 1976). When comparing isoenzymes electrophoretically, it is important to remember the exclusionary nature of the data. Although the different band Rf values represent primary structural alterations in enzymes (Yamazaki and Komgata, 1981), similar Rf values are not always evidence for enzymatic (Harris and Hopkinson, 1976) or structural property identity (Yamazaki and Komagata, 1981).

Enzyme multiplicity may be the result of genetic (primary) causes (multiple alleles at a single locus or multiple genetic loci), or post-translational (secondary) causes (Rider and Taylor, 1980). Lassner and Orton (1983) outlined the types of somatic changes observable by isoenzyme electrophoresis. Whereas loss or appearance of bands can indicate mutations such as deletion, duplication, base changes or transposition events, changes in stain intensity indicate alterations in the quantity of protein synthesised, occurring via duplication, deletion, or regulatory modification.

Even early investigators (Hubby and Lewontin, 1966) recognized that electromorphs, the term coined by King and Ohta (1975) for bands on electrophoretic gels, were phenotypes which incompletely reflected the underlying allelic variation. Only minimal estimates of genetic variation can be made from electrophoretic data (Ayala, 1977). Whereas structural genes which code for soluble proteins are detectable, structural and regulatory genes which are not translated into proteins are not revealed. Even if a soluble protein is being coded for, not all changes in the gene will result in an altered Rf value because of the degeneracy of the genetic code,

substitution of amino acids with identical net charges, and the limited resolution of gels. There is also uncertainty whether the loci sampled are a random sample of the entire genome, and whether variation in bands is significant and functionally distinct (Gottlieb, 1981).

Despite these limitations, the techniques of protein gel electrophoresis and isoenzyme electrophoresis have been used to study the population genetics, biosystematics, evolutionary biology, and biochemistry of a wide range of life forms. Indeed, the study of isoenzymes by electrophoretic methods is now one of the most active research areas of evolutionary genetics (Selander, 1976; Ayala, 1976; and Mayr, 1982). The main reasons for the rapid incorporation of electrophoretic techniques are that they have powerful resolution, sensitivity and reproducibilty in revealing genetic variation (Ames, 1974; Hurka, 1980).

Electrophoretic studies of fungi have concentrated on ascomycetous genera (Garber, 1974) and have demonstrated multiple molecular forms for certain enzymes. Relatively few studies have been done on yeasts, thus little information concerning inter or intraspecific variability in yeast isoenzymes is available. However, in some cases (Table 1), electrophoresis has been useful in clarifying taxonomic relationships. As will become apparent from the following synopsis, the genus *Kluyveromyces* is fraught with taxonomic problems which the application of protein electrophoretic techniques might help resolve.

1.4 The Genus *Kluyveromyces*, A Typical Example Of The Species Conflict In Yeasts

Since its inception, the genus *Kluyveromyces* has typified the species conflict in yeasts. In 1955, Wickerham considered that *Saccharomyces lactis* (syn. *K. lactis*), *Saccharomyces fragilis* (syn. *K. fragilis*), *Saccharomyces marxianus*, *Zygosaccharomyces ashbyi* (syn. *K. marxianus*), and *Zygosaccharomyces dobzhanskii* (syn. *K. dobzhanskii*) were readily interfertile but did not hybridize with true *Saccharomyces* species. Thus these yeasts with some other species should be transferred from the genus

Table 1. Some isoenzymes electrophoretic studies involving yeasts.

Aessosporon	
	*Yamazaki and Komagata (1983)
Bullera	
	*Yamazaki and Komagata (1983)
Candida	
	*Berchev and Izmirov (1967)
	Dabrowa *et al.* (1970)
	Fregreslev (1969)
	Lee and Komagata (1980)
	*Lloyd *et al.*(1971)
	Primiano *et al.* (1972)
	*Shechter (1972,1973)
	Tsolas and Horecher (1970)
	Villa *et al.* (1976)
	*Yamazaki and Komagata (1982a,b, 1983)
Cryptococcus	
	*Baptist and Kurtzman (1976)
	*Yamazaki and Komagata (1982b, 1983)
Debaryomyces	
	*Yamazaki and Komagata (1982a)
Filobasidiella	
	*Yamazaki and Komagata (1982b)
Filobasidium	
	*Yamazaki and Komagata (1982b)
Hanseniaspora	
	*Yamazaki and Komagata (1982a)
Hansenula	
	*Lee and Komagata (1980)
	*Yamazaki and Komagata (1982a)
Issatchenkia	
	*Yamazaki and Komagata (1982a)
Kloeckera	
	*Lee and Komagata (1980)
	*Yamazaki and Komagata (1982a)
Kluyveromyces	
	Dickson *et al.* (1978)
	Fleming and Duerkson (1967)
	*Yamazaki and Komagata (1982a)
Leucosporidium	
	*Yamazaki and Komagata (1982b)
Lipomyces	
	*Yamazaki and Komagata (1982b)
Lodderomyces	
	*Yamazaki and Komagata (1982a)
Pichia	
	*Lee and Komagata (1980)
	*Yamazaki and Komagata (1982a)
	*Holzschu *et al.* (1983)

Table 1. Cont.

Rhodosporidium	
	*Yamazaki and Komagata (1981,1983)
Rhodotorula	
	*Yamazaki and Komagata (1981, 1982b, 1983)
Saccharomyces	
	Atzopodien *et al.* (1968)
	Babczinski *et al.* (1979)
	Bierman and Glantz (1968)
	*Clare *et al.* (1968)
	Dave *et al.* (1966)
	Douglas (1969)
	Gazith *et al.* (1968)
	Hagele *et al.* (1978)
	Krebs (1953)
	Lee and Komagata (1980)
	Malmstrom (1957)
	Meyer and Matile (1975)
	Nakagarwa and Noltzman (1967)
	Pfleiderer *et al.* (1966)
	Saura *et al.* (1979)
	Singh and Kunkee (1977)
	Sugar *et al.* (1971)
	Taber and Sherman (1964)
	Tsoi and Douglas (1964)
	Watts and Donniger (1962)
	Wiesenfield *et al.* (1975)
	*Yamazaki and Komagata (1982a)
	*Yamazaki *et al.* (1982, 1983)
	*Yau and Lindegren (1967)
Schizosaccharomyces	
	*Clare *et al.* (1968)
	Dave *et al.* (1966)
	Pfleiderer *et al.* (1968)
	Fleet and Phaff (1974)
Sporidiobolus	
	*Yamazaki and Komagata (1983)
Sporobolomyces	
	*Yamazaki and Komagata (1983)
Torulaspora	
	Lee and Komagata (1980)
	*Yamazaki and Komagata (1982a)
Unidentified Ballistosporogenous Yeasts	
	*Yamazaki and Komagata (1983)

* Studies of a taxonomic nature

Saccharomyces to a new genus (*Dekkeromyces*). Although Wickerham and Burton (1956a,b) formulated the taxon, they failed to give a formal diagnosis and type for the genus, making *Dekkeromyces* a *nomen nudum*. In the same year, van der Walt created the genus *Kluyveromyces* to which he assigned two new species (*K. polysporus* and *K. africanus*) with the species of the proposed genus *Dekkeromyces*.

The disagreements between Wickerham and Burton (1956a,b), Kudriavzev (1960), Novak and Zsolt (1961), Boidin *et al.* (1962), and Kreger-van Rij and van der Walt (1963) over the taxonomy of reniform spore forming species were followed by van der Walt's (1965) emendation of the diagnosis of the genus *Kluyveromyces*. The genus united all of the species formerly referred to as *Fabospora* and *Zygofabospora* (Kudriavzev, 1960), *Dekkeromyces* (Wickerham and Burton, 1956a,b), and *Guilliermondella* (Boidin *et al.*, 1962). The tenet was that multispored species only constituted a separate line of development among species which usually form only four spores. Only species whose asci dehisced were included. Ascospore shape was considered less significant because the genus now included yeasts of varied spore morphology.

Fiol (1967) proposed three physiological groups of *Kluyveromyces* species based on vitamin requirements along with the suggestion that the evolution of the species was characterized by the development of a greater dependence on an extraneous vitamin source. Groups A and B had variable vitamin requirements while Group C required biotin and nicotinamide. Later, Martini *et al.* (1972) added additional data about vitamin requirements of the genus.

In 1970, van der Walt revised the taxonomic structure of this ubiquitous genus. According to his revision, the twenty species belonging to *Kluyveromyces* reproduced vegetatively solely by budding. Although pseudomycelium could be formed, true mycelium was absent. Characteristically, their asci dehisced and the ascospores tended to agglutinate. The smooth ascospores which were crescentiform, reniform, oblong with obtuse ends, spheroidal or prolate-ellipsoidal, could range in number from one to numerous. Metabolism in this genus was both oxidative

and fermentative. An external vitamin source was required and nitrate was not utilized. Many of the species were known to produce pulcherrimin pigments. The major group structures for the genus up to and including the first major revision are presented in Table 2A.

Taxonomic investigations since the detailed revision of van der Walt (1970b), have not resolved the group structure of this genus. Table 2B summarizes the major different group structures proposed since van der Walt's revision of the genus. Also in 1970, Santa Maria and Sanchez revived the spore shape controversy by proposing application of the *nomen nudum Dekkeromyces* to those *Kluyveromyces* species unable to form multispored asci.

Campbell (1972) combined serological and numerical analysis to investigate the genera *Saccharomyces* and *Kluyveromyces*. According to him, the genus was divided into three parts with the phylogenies only partially agreeing and sometimes substantially disagreeing with results of other studies.

Martini *et al.* (1972) hoped to resolve the conflict surrounding the genus by using DNA base composition as well as DNA-DNA homologies (Martini, 1973). They concluded that on the basis of G+C mol% content (47.4) and other properties *K. thermotolerans* (syn. *K. veronae*) holds a dubious place in the genus. The G+C mol% content of the remaining seventeen species ranged from 35.3 to 43.4 and two major groups of species were recognized. Contained in Group I were the species (*K. polysporus, K. phaffii, K. lodderi*, and *K. africanus*) with low G+C mol% contents. Group IIA contained the species (*K. aestuarii, K. delphensis, K. marxianus, K. drosophilarum, K. phaseolosporus, K. fragilis, K. lactis, K. wickerhamii*, and *K. vanudenii*) with intermediate G+C mol% values, whereas Group IIB contained species (*K. bulgaricus, K. wikenii, K. dobzhanskii* and *K. cicerisporus*) with high G+C mol% values, The later DNA homology studies by Martini (1973) and Phaff *et al.* (1978b) found high homologies between *K. marxianus* and *K. fragilis, K. wikenii* and *K. cicerisporus, K. wikenii* and *K. bulgaricus, K. vanudenii* and *K. lactis, K. cicerisporus* and *K. bulgaricus, K. drosophilarum* and *K.*

Table 2A. Nomenclature and proposed groupings of *Kluyveromyces* type strains until 1970

Number	Taxonomic Designation	LD	VR	MR
61-29	*K. aestuarii* (Fell) v.d. Walt 1961	2	C	5
70-4	*K. vanudenii* (v.d. Walt et Nel) v.d. Walt 1963	2	C	5
50-80	*K. phaseolosporus* (Shehata, Mrak et Phaff) v.d. Walt 1955	1	C	4
71-59	*K. lactis* (Dombrowski) v.d. Walt 1910	2	C	5
51-130	*K. drosophilarum* (Shehata, Mrak et Phaff) v.d. Walt 1955	1	C	4
50-45	*K. dobzhanskii* (Shehata, Mrak et Phaff) v.d. Walt 1955	1	C	4
71-15	*K. wikenii* v.d. Walt, Nel et Kerken 1966	2	C	5
71-58	*K. fragilis* (Jorgensen) v.d. Walt 1909	1	C	3
55-82	*K. marxianus* (Hansen) v.d. Walt 1888	1	C	3
71-14	*K. cicerisporus* v.d. Walt, Nel et Kerken 1966	2	C	5
71-13	*K. bulgaricus* (Santa Maria) v.d. Walt 1956	2	C	5
72-13	*K. waltii* Kodama 1974	-	-	-
54-210	*K. wickerhamii* (Phaff, Miller et Shifrine) v.d. Walt 1956	1	C	3
55-41	*K. thermotolerans* (Philippov) Yarrow 1932	2	N	5
70-5	*K. phaffii* (v.d. Walt) v.d. Walt 1963	1	B	1
57-17	*K. polysporus* v.d. Walt 1956	1	A	2
56-2	*K. delphensis* (v.d. Walt et Tscheuschner) v.d. Walt 1956	1	B	1
70-3	*K. lodderi* (v.d Walt et Tscheuschner) v.d. Walt 1957	1	B	1
77-7	*K. blattae* Henninger et Windish 1976	-	-	-
57-16	*K. africanus* v.d. Walt 1956	1	A	2

Number Culture Collection Number of the Department of Food Science and Technology, University of California, Davis

LD Lines of Development (Wickerham and Burton 1956a, b)
1 - homothallic species forming crecentiform or reniform to long-oval ascopores.
2 - homothallic and heterothallic species forming spheroidal to ellipsoidal ascospores.

VR Vitamin Requirements (Fiol, 1967; Martini *et al.*, 1972)
A - require thiamine
B - require biotin
C - require biotin and thiamine
N - nonmember (requires biotin and inositol)

MR Major Revision (van der Walt, 1970b)
1 - Reniform spores (1-4), limited nutritional abilities
2 - More than 4 reniform spores
3 - Reniform spores (1-4), α-glucosides not utilized
4 - Reniform spores (1-4), α-glucosides utilized
5 - Spheroidal spores (1-4)

\- Species not yet described when studies were made.

Table 2B. Nomenclature and proposed groupings of *Kluyveromyces* type strains until 1984

Number	Taxonomic Designation	AG	PS	DR	HY
61-29	*K. aestuarii*	(B)C	B2	aest	aest
70-4	*K. vanudenii*	AB	B2	lact	marx
50-80	*K. phaseolosporus*	C	B1-2	lact	marx
71-59	*K. lactis*	C	B2	lact	marx
51-130	*K. drosophilarum*	AC	B2	lact	marx
50-45	*K. dobzhanskii*	AC	B2	dobz	marx
71-15	*K. wikenii*	AB	B1	marx	marx
71-58	*K. fragilis*	B	B1	marx	marx
55-82	*K. marxianus*	B	B1	marx	marx
71-14	*K. cicerisporus*	(A)C	B1	marx	marx
71-13	*K. bulgaricus*	(A)B(C)	B1	marx	marx
72-13	*K. waltii*	-	-	-	(marx)
54-210	*K. wickerhamii*	A(C)	B1-2	wick	(marx)
55-41	*K. thermotolerans*	AC	N	ther	(marx)
70-5	*K. phaffii*	AB	A	(phaf)	phaf
57-17	*K. polysporus*	D	A	poly	poly
56-2	*K. delphensis*	D	A	(delp)	delp
70-3	*K. lodderi*	D	A	(lodd)	lodd
77-7	*K. blattae*	-	-	-	blat
57-16	*K. africanus*	A	A	(afri)	afri

Number — Culture Collection Number of the Department of Food Sciences and Technology, University of California, Davis

AG — Antigenic Group (Campbell, 1972)
Positive reactions with sera A, B, C, or D indicated by corresponding letter. Weak or irregular reactions are bracketed.

PS — Phenetic Similarity (Poncet, 1973)
A - Nutritionally restricted taxa isolated from soil.
B1 - Taxa which utilize α-glucosides poorly
B2 - Taxa which utilize α extensively.
B1-2 - Transitional forms between subgroups 1 and 2.
N - Nonmember of the genus.

DR — DNA Reassociation (Martini, 1973; Phaff *et al.*, 1978b).
The first four letters of the specific epithet of the reference taxon with which each strain is affiliated are shown.
Parentheses indicate uncertain affiliation.

HY — Hybridization (Johannsen, 1980).
The first four letters of the specifc epithet of the reference taxon with which the strain is affiliated are shown.
Parentheses indicate low levels of hybridization.

\- — Species not yet described when studies were made.

phaseolosporus.

Poncet's (1973) "factor" analysis (probably principal components analysis) of the phenotypic traits also divided the genus into two groups. Group A comprised five nutritionally restricted taxa isolated mostly from soils. Group B was more widely distributed and was divided into two subgroups. B1 taxa utilized α-glucosides less extensively than B2 taxa. *K. wickerhamii* and *K. phaseolosporous* were considered transitional forms between the two subgroups and *K. thermotolerans* was considered not to be a member of the genus. Poncet's conclusions were only partially verified by the DNA reassociation studies of Martini (1973) and Phaff *et al.* (1978b). There was a high degree of genetic homogeneity between the B1 taxa and between some of the members (*K. marxianus*, *K. wikenii*, *K. fragilis*, *K. cicerisporus*, and *K. bulgaricus*) of Group B.

In the last major diagnosis of the genus (van der Walt and Johannsen, 1984), co-enzyme Q data is the only major addition. This revision of the genus is based on the ability of strains to hybridize (Johannsen, 1980). The number of species in the genus has been reduced from 20 to 11. The formerly distinct species (*K. bulgaricus*, *K. cicerisporus*, *K. dobzhanskii*, *K. drosophilarum*, *K. fragilis*, *K. lactis*, *K. phaseolosporus*, *K. vanudenii*, and *K. wikenii*) have been made into seven syngameous varieties of *K. marxianus* because they had relatively high mating inter-compatibilty. *K. phaseolosporus* was fused with *K. drosophilarum*, while *K. fragilis* and *K. cicerisporus* were fused with *K. bulgaricus*. *K. waltii*, *K. wickerhamii*, and *K. thermotolerans* were maintained as separate but related species because they hybridized less extensively with some of the varieties. The seven remaining strains were retained as separate species because of their reproductive isolation.

From the preceding discussion, it is evident that even though most of the techniques to obtain molecular data have been applied to the genus *Kluyveromyces*, the species conflict is still unsettled. There seems to be no doubt that multiple methods should be used to delineate species but there is ambiguity as to which criterion (DNA reassociation patterns, Phaff, 1980;

or mating compatibility, Johannsen, 1980) should prevail in delineating *Kluyveromyces* species. The control argument is whether or not genetically effective gene exchange is taking place under natural conditions. Although a few investigators (Dickson *et al.*, 1978; Douglas *et al.*, 1969; Fleming and Duerkson, 1967) have studied selected members of the genus, no electrophoretic isoenzyme patterns have been made with the intent of delineating its species. Studies of other species (Table 1) indicate that enzyme electrophoresis patterns should be useful in determining whether or not a common gene pool is shared by taxa which are able to form hybrids, but which have diverged genetically as indicated by low degrees of DNA reassociation.

1.5 Scope and Aims of the Project

This project like many today has a broad scope encompassing many disciplines. A list of disciplines would have to include microbiology, systematics, evolution, ecology, statistics and population genetics. Perhaps the single best term to describe this synthesis is molecular systematics. Molecular systematics is "the study of evolutionary relationships using comparative molecular data" (Lincoln *et al.*, 1982), such as those obtained from the technique of protein electrophoresis.

Protein electrophoresis has provided a method of measuring the genetic variability of populations. Primarily, breeding structures are revealed. Protein polymorphism studies have made quite substantial contributions to the knowledge of plant and animal groups. Because of the yeasts' unique life cycles, what has been discovered in animals and plants may not be directly applied to yeasts. Population approaches have yet to be widely adopted by mycologists.

Stated in its broadest terms, the aim of this project is to examine natural populations of ascogenous yeasts by electrophoresis of isoenzymes and asses the taxonomic implications of such a study with reference to the various approaches used to delineate yeast species. Specifically, the study will be broken down into three parts:

In part one, the type strains of the twenty species of the yeast genus *Kluyveromyces sensu* van der Walt (1970b) will be compared by means of gel electrophoresis of eleven isoenzymes. Their different isoenzyme patterns will be determined and the extent of electromorph variability estimated. Multivariate analysis of the isoenzyme patterns will be performed to determine species delineation and speciation within the genus. Although the type strain is not necessarily the most characteristic strain of that species, or genus (Cowan, 1962), it is assumed that by studying the type cultures first, the information obtained from other phases of the survey may be optimized.

Part two of the project will consist of the assessment of the amount of variation or the varietal homogeneity of natural populations of some different *Kluyveromyces* DNA subgroups from a variety of habitats ("Black Knots", *Drosophila*, tree exudates and cheese) based on the isoenzymes selected. Some anamorphs (imperfect states) belonging to the genus *Candida* will be compared. Three strains not belonging to the genus *Kluyveromyces* will also be included in the survey as an external measure of relatedness. Multivariate analysis of this phase should resolve to what extent yeasts and in particular this genus constitute Mendelian populations ("local interbreeding units of possibly large, geographically structured populations within which evolution or systematic changes in allele frequency occur"; Hartl, 1981) in nature, or if they evolve largely as clones ("groups of genetically identical individuals"; Hartl, 1981).

In part three of the project, interspecific and intraspecific hybrids, made in this laboratory or obtained from the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands, and their corresponding parental strains will be examined in order to elucidate the hybrid state in yeasts. The usefulness of the type of information obtained from each enzyme will be assessed. The taxonomic relevance of electrophoretic isoenzyme patterns of parental strains with relatively high and low levels of interfertility will be examined as a criterion for delineating species in the genus *Kluyveromyces*.

In conclusion, it is thought that a multidisciplinary approach such as this, involving type strains, natural isolates (from a variety of habitats), and laboratory constructed hybrids will elucidate fundamental aspects of the taxonomic structure of the genus *Kluyveromyces*. Furthermore, it is hoped that the taxonomic relevance of electrophoresis with particular reference to the possible correlations between electrophoretic patterns and the different criteria used to delineate yeasts species will be disclosed.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Microorganisms

The type strains of *Kluyveromyces* species (See Table 3A) were obtained from Dr. H.J. Phaff, Department of Food Science and Technology, University of California, Davis. They were maintained on yeast extract-malt extract-glucose-peptone (YM) agar, at 4°C, in screw-capped vials.

The *K. marxianus* x *K. thermotolerans* (CBS 6925) hybrid isolated by Johannsen and van der Walt (1978) and its corresponding parental strains (*K. marxianus* leu LAC REN CBS 6923 and *K. thermotolerans* MAL SPH CBS 6924) were obtained from the Centraalbureau voor Schimmel-culturen in Delft, The Netherlands.

All other strains were obtained from the laboratory collection. Table 3B lists their source and location of isolation under their University of Western Ontario Plant Sciences Yeast Culture Collection (UWO (PS)) number. *Saccharomyces cerevisiae* (79-11) was commercial baker's yeast.

Hybrids between *K. lactis* (LAC) and *K. vanudenii* (37°C) were prepared as follows. Selected parents were grown on YM slants for 3 days at 25°C. One plus suspensions of the parental strains were then combined and incubated on YM slants at 25°C. After 3 days, "+" suspensions were spread-plated (0.1, 1.0 ml) or streaked on recovery medium containing 0.67% yeast nitrogen base, 0.5% lactose, and 1.5% agar and incubated at 37°C. Individual parental strains were similarly plated as controls. Colonies of hybrids were very much larger than parental strains of the recovery medium. Hybrid colonies were then brought into pure culture by replating on the recovery medium. Hybrids were stored on recovery medium at 5°C.

2.2 Preparation Of Extracts

Cultures were grown on YM agar for 3 days at 25°C. In the case of potentially inducible enzymes, each strain was grown to late exponential phase in YM broth in which glucose was replaced by the appropriate

Table 3A. List of *Kluyveromyces* type strains.

Designation	Source	UCD(FS&T)[a]
K. aestuarii	Estuarine mud, Biscayne Bay	61-29
K. africanus	Soil, South Africa	57-16
K. blattae	Cockroach	77-7
K. bulgaricus	Yoghurt	71-13
K. cicerisporus	Back of cow	71-14
K. delphensis	Dried figs, South Africa	56-2
K. dobzhanskii	*Drosophila*, California	50-45
K. drosophilarum	*Drosophila*, California	51-130
K. fragilis	Yoghurt	71-58
K. lactis	Cheese	71-59
K. lodderi	Soil, South Africa	70-3
K. marxianus	-	55-82
K. phaffii	Soil, South Africa	70-5
K. phaseolosporus	*Drosophila*, California	50-80
K. polysporus	Soil, South Africa	57-17
K. thermotolerans	Grapes	55-41
K. vanudenii	Winery, South Africa	70-4
K. waltii	Exudate holly tree, Japan	72-12
K. wickerhamii	*Drosophila*, California	54-210
K. wikenii	Bantu Beer, South Africa	71-15

- Information not available

[a] Culture Collection of the Department of Food Science and Technology, University of California, Davis

Table 3B. List of strains used in population studies.

Designation	Source[a]	UWO (PS) Culture Collection Number				
Candida sake	7	79-228				
K. cicerisporus	11	80SM1-4	80SM2-4	80SM3-10	80SM10-2	
		80SM10-5	80SM12-2	80SM12-12	80SM17-1	
		80SM21-7	80SM53-2			
K. dobzhanskii	2	79-37	79-133	79-199	79-265	79-267
		80-28	80-29	80-37	80-87	80-88
	3	82-32	82-232	82-244		
	7	79-183	79-187	79-188	79-189	
K. drosophilarum	2	79-261	80-45			
	3	82-233	82-237	82-241	82-245	
	4	80-89	82-12	82-17	82-45	
	7	79-169				
	9	80-48				
K. fragilis	11	80SM16-1	80SM16-10	80SM27-8	80SM46-5	
K. lactis	11	80SM1-11	80SM2-5	80SM3-8	80SM3-11	
		80SM3-13	80SM5-6	80SM5-8	80SM6-2	
		80SM6-7	80SM13-5	80SM16-9	80SM30-7	
		80SM32-5	80SM35-14	80SM32-6	80SM48-7	
K. marxianus	11	80SM3-4				
K. thermotolerans	1	79-255				
	2	79-110	79-112	79-114	79-116	79-117
		79-118	79-119	79-126	79-191	79-192
		79-193	79-194	79-195	79-196	79-249
		79-250	79-251	79-252	79-253	79-254
		80-19	80-84			
	3	82-070	82-204	82-206	82-209	82-215
		82-216	82-218	82-220	82-223	82-225
		82-231	82-292			
	7	79-164				
	8	79-162	81-125	81-126		
	10	79-139				
K. vanudenii	3	82-235	82-236	82-239	82-210	
	5	79-127	79-168			
	6	80-12				
	9	80-49				

Table 3. Cont.

K. waltii	3	82-227	82-228		
	7	79-163			
	8	79-160	79-161	81-127	81-128
Pichia fluxuum	4	80-109			

[a] List of sources:

1 Black knot, *Prunus pumila*, Pinery Provincial Park, Ont.
2 Black knot, *Prunus virginiana*, Pinery Provincial Park, Ont.
3 *Drosophila*, Pinery Provincial Park, Ont.
4 Flux, *Quercus rubra*, Pinery Provincial Park, Ont.
5 Black knot, *Prunus virginiana*, Coldstream Conservation Area, Ont.
6 Black knot, *Prunus virginiana*, Medway Creek, London, Ont.
7 Black knot, *Prunus virginiana*, Melbourne, Ont.
8 Gall, *Quercus rubra*, Melbourne, Ont.
9 Black knot, *Prunus virginiana*, Dingman Creek, London, Ont.
10 Black knot, *Prunus serotina*, St. Anicet, Que.
11 Camembert cheese, Dr. J.L. Schmidt, Institut National Agronomique, Paris-Grignou, France

substrate. Flasks were incubated at 25°C on a reciprocal shaker. For α-glucosidase the substrates were 0.3% each of maltose, melizitose, methyl-α-D-glucoside and 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 10,000g for 10 minutes and supernatants were decanted. Protein extracts were stored at -20°C. Extracts for alkaline phosphatase did not tolerate freezing, and therefore were prepared fresh for each experiment. The addition of various protease inhibitors during extraction was investigated, and found inconsequential.

2.3 Chemicals

Electrophoresis grade acrylamide, glycine, N,N-methylene-bis-acrylamide, ammonium peroxydisulfate, N,N,N'N'-tetramethylenediamine, and tris-(hydroxymethyl)-aminomethane (TRIS) were purchased from Bio-Rad (Richmond VA). Bromophenol blue and guaiacol were obtained from British Drug House (Toronto, Ontario). Laminaran was obtained from United States Biochemical Corp. (Cleveland OH). Benzidine, Fast Blue RR Salt, peroxidase, glucose oxidase, glyceraldehyde-3-phosphate dehydrogenase, α-napthyl acetate, agarose, phenazine methosulfate, 3[4,5-dimethylythiazoyl-2-yl]2,5-diphenyl tetrazolium bromide, nicotinamide adenine dinucleotide, arbutin, maltose, α-methyl-D-glucoside, trehalose, fructose-1,6-disulphate, pepsin (34.7 kd), ovalbumin (45 kd), bovine serum albumin (66 and 132 kd) and p-nitrophenyl-D-glucopyranoside (PNPG) were purchased from Sigma Chemical Company (St. Louis MO). All other chemicals were reagent grade.

2.4 Agarose Gel Electrophoresis

Agarose gels were used for catalase. Catalase activity tended to leach out of polyacrylamide gels, making measurements difficult. The agarose gels contained 0.7% agarose, 0.3% starch, 0.021 M Tris, 0.015 M boric acid and 0.0006M EDTA, adjusted to pH 8.0. The bridge buffer was 10 times concentrated

gel buffer. Electrophoresis was conducted at 4°C, at 12 mA, for 2 h.

2.5 Polyacrylamide Gel Electrophoresis

Discontinuous non-denaturing polyacrylamide gels were prepared as recommended by Davis (1964). The stacking gels and running gels contained TRIS-HCL buffer at concentrations of 0.06 M and 0.03 M, and pH values of 6.9 and 8.9, respectively. The electrode buffer contained 0.05 M TRIS and 0.038 M glycine, pH 8.3. The concentrations of the gels varied with the enzymes studied. For α-glucosidase, esterase, aldolase, β-glucosidase, and *exo*-β-glucanase, the acrylamide concentrations in 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 percent, respectively. Single concentration gels with 7.7% acrylamide were used for all dehydrogenases, superoxide dismutase, and alkaline phosphatase.

Electrophoresis was conducted in the cold under constant current 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 to 60 µl of crude protein extracts were used. Nine samples were applied to each gel slab. Relative mobilities (Rf) were calculated with reference to the dominant electromorph (the fast band of *K. marxianus* was used as a standard on each catalase gel). All electromorphs were scored on presence or absence basis, no assumptions were made as to their individual genetic bases. All samples were studied at least twice for each enzyme. To ensure band identity, reference strains were run on each gel and some strains were rerun side by side on single gels.

Although these precautions were taken, it was difficult to match bands between sets of runs. In future studies, this problem might by rectified by including a series of markers that appear independently of the staining system employed.

2.6 Histochemical Staining

Exo-β-glucanase, α-glucosidases, and β-glucosidase were stained by a method combining an enzymatic glucose staining technique (Sigma Technical Bulletin No. 510) with a procedure for detecting peroxidase activity in polyacrylamide gels (Schauwen, 1966). Gels were incubated for 5 min in 0.05 M potassium acetate, pH 5.1. They were then overlaid with a mixture containing, per 100 ml, 100 units glucose oxidase, 30 units peroxidase, 0.036% benzidine, 0.043% guaiacol, and 1.6% agar in 0.2 M potassium acetate buffer, pH 6.0. The agar was dissolved separately and mixed with the remaining reagents at 46°C immediately before staining. The substrate concentration for *exo*-β-glucanase was 0.1% laminaran. The substrate concentration for α and β-glucosidases was 3.0%. the specificity of α-glucosidases was determined by developing gels separately with α-methyl-D-glucoside, maltose or trehalose as substrates, but their Rf values were determined by staining simultaneously with combined substrates. Arbutinase and trehalase were also stained by the method of Eilers *et al.* (1964). The technique of Eilers *et al.* for the staining of β-glucosidase, reported in Chapter 3, was replaced in the studies of Chapter 4 by the easier to interpret PGO system on 3.0%, 5.0% gels. Esterase and catalase, lactate, malate, and alcohol dehydrogenases, superoxide dismutase, and alkaline phosphatase was stained by the methods of Harris and Hopkinson (1976). Catalase gels were examined immediately after addition of hydrogen peroxide, as bands spread rapidly. All other gels were incubated overnight in staining mixtures.

Amylase, β-galactosidase, β-fructosidase, hexokinase, glucose dehydrogenase and protease were examined. They failed to provide reproducible results due to poor extractibility or stability, and were not considered further.

2.7 Data Analysis

Reciprocal averaging or correpondance analysis (Hill, 1973) was used to ordinate the strains as a function of correlated electromorphs. For this analysis, the results were expressed in a matrix of dimensions n x p, where

n strains are described by the presence ("1") or absence ("0") of each of p electromorphs. Reciprocal averaging was the method of choice as it is an eigenvector method of ordination which simultaneously reveals correpondances, for a number of presence-absence data, between two kinds of information (i.e. strains and electromorphs).

Principal components analysis was used to assess the relationship of groups of strains based on electromorph frequencies (See Table 6). This eigenvector technique reveals the correlation structure and diminishes the dimensionality of continuous data. Besides eliminating meaningless variables, new less obvious but more meaningful variables may be identified (Chatfield and Collins, 1980).

Since *Kluyveromyces* strains are haploid, the traditionally accepted measures of diploid variability (actual heterozygosity, HI; expected heterozygosity, HS; and gene diversity, HT) are of no value. Genetic diversity was assessed by an entropy or information measure which serves as a measure of both richness (the number of distinct kinds of bands encountered in the population of a particular size) and evenness (distribution of band frequencies).

The amount of information provided by each of the enzymes presented in Chapters 3 and 4 was evaluated by the entropy 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, t the total number of different electromorphs for the jth enzyme, and a the number of electromorphs of that enzyme present in the ith strain (Sneath and Sokal, 1973).

The expected entropy values for the enzymes of the populations presented in Chapter 4 were calculated with the above formula by using banding patterns for a comparable population of type strains.

LOSIDE, a Fortran-10 algorithm, was used to cluster the data presented in Chapter 5. Equally weighted average linkage was applied to a mismatch matrix, calculated as the number of unshared electromorphs divided by the total number of electromorphs, as illustrated by the formula:

$$M_{AB} = \frac{AUB - A \cap B}{AUB}.$$

CHAPTER 3 – ISOENZYME PATTERNS OF THE TYPE STRAINS

3.1 Introduction

Yeast taxonomy has had its share of contention over the definition of species. Because every species is simultaneously a genetic system, a gene pool, and a reproductive community (White, 1978), conspecificity requires many elements. According to Phaff (1980), chromosomal DNA complementarity, mating compatibility, and the sharing of habitats are all necessary. The multitude of methodologies available for investigating microorganisms makes it compulsory to consider data from morphological, physiological, biochemical, and mating compatibility studies when determining reproductive isolation, the fundamental criterion upon which species delineations are made. The genus *Kluyveromyces* is particularly perplexing as there have been disagreements (Phaff, 1980; Johannsen, 1980) as to whether DNA reassociation patterns or mating compatibility patterns (See Table 2B for assignments to species by these groupings) should prevail in delimiting its species.

The value of isoenzyme patterns to yeast taxonomy is based on two interdependent premises. The first is that conspecific yeasts are genetically similar and the second is that the genetic constitution of yeasts is directly manifested in their enzymes. To date, extensive electrophoretic studies have not been performed on the genus *Kluyveromyces*. Because random mating is inversely related to genetic structuring (Saura, 1983), enzyme electrophoretic patterns should be able to resolve whether random mating is effectively operative among taxa which are able to form hybrids but which have diverged genetically, as indicated by low degrees of DNA reassociation.

This chapter describes an electrophoretic survey of eleven enzymes, undertaken to estimate the extent of electromorph heterogeneity within the genus *Kluyveromyces*, and to identify the different isoenzyme patterns of the type strains *sensu* van der Walt (1970b). Type strains are strains maintained in an international culture collection and which serve as

references for yeast species. They are not necessarily the most typical representatives. This study is not meant to redefine taxa on the basis of electrophoretic patterns of single strains. It should provide data upon which preliminary conclusions may be drawn as to the taxonomic relevance of electrophoresis, with particular reference to its relationship with other approaches used to delineate yeast species (See Table 2B). The results have been published (Sidenberg and Lachance, 1983).

3.2 Results and Discussion

Considerable heterogeneity was exhibited in the electrophoretic patterns (Fig. 1.1- 1.11) of the twenty type strains. In total, 91 different electromorphs were resolved. The wide range of entropy values (Table 4) for the different enzymes indicated they varied markedly in the amount of information each contained. For these reasons, the isoenzymes patterns of the genus and the problems associated with their interpretation are discussed separately.

Alcohol, lactate and malate dehydrogenases (Fig. 1.1 - 1.3) had intermediate entropy values. Although a number of electromorphs were shared across the members of the *K. marxianus* mating compatibility group, others were found exclusively in members of the *K. lactis* or the *K. marxianus* DNA complementarity subgroup. For the most part, the patterns obtained for *K. waltii* and *K. bulgaricus* were very similar, and those of *K. aestuarii* unique. Some dehydrogenase activities were missing from several members of phenetic group A.

The problems associated with the interpretation of dehydrogenase gels were many. Streaking in the upper portion of malate dehydrogenase gels made it difficult to be certain that all electromorphs were recorded. Superoxide dismutase, always visualized on these gels, often gave a streaked area a split appearance which could be mistaken for banding without proper controls (gels stained in the absence of a specific electron donor). Areas of activity outside of the streaked areas were very easily scored. Lactate dehydrogenase seemed to be an unstable enzyme and was dropped from future

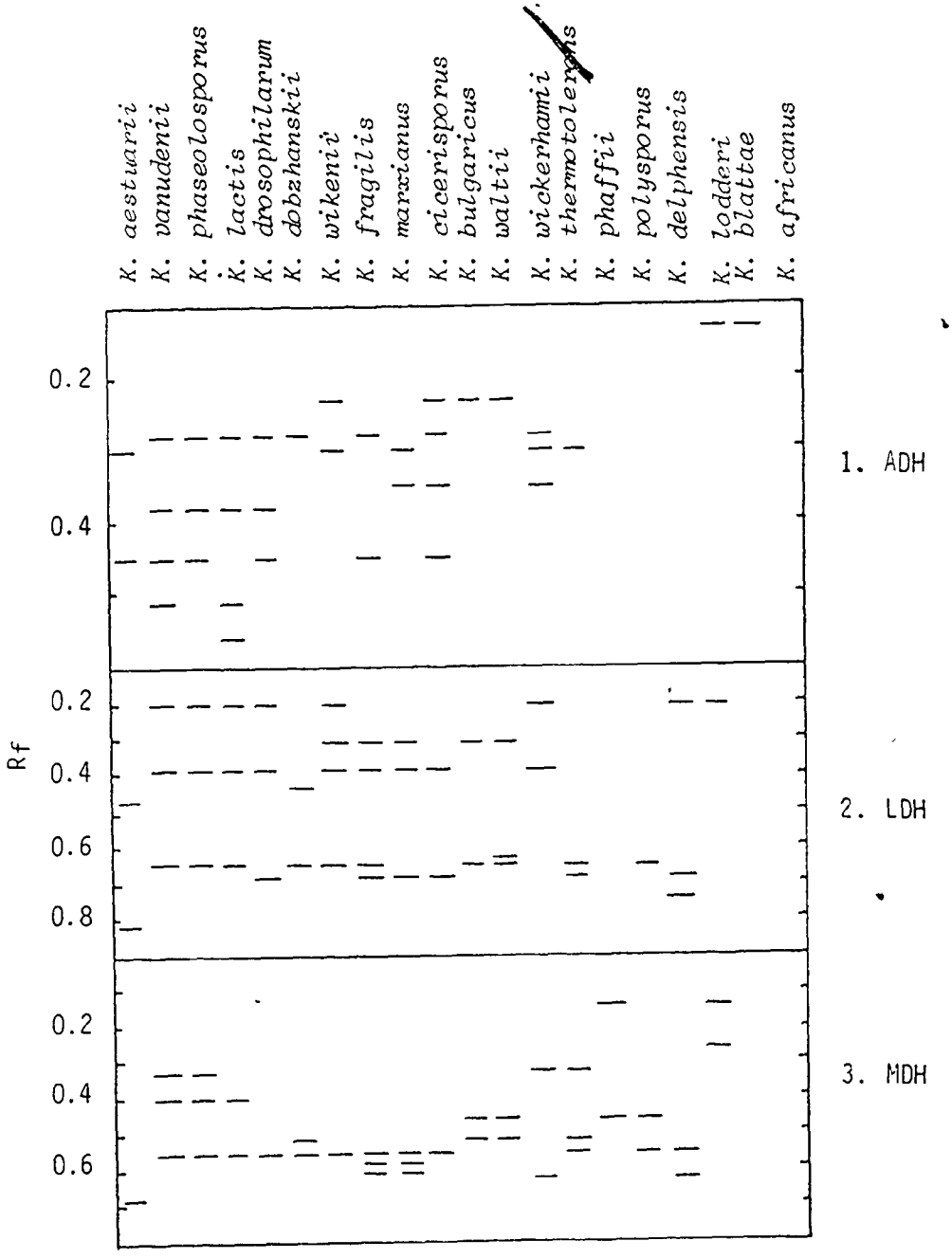
Table 4. Ranked entropy values for the enzymes of the type strains.

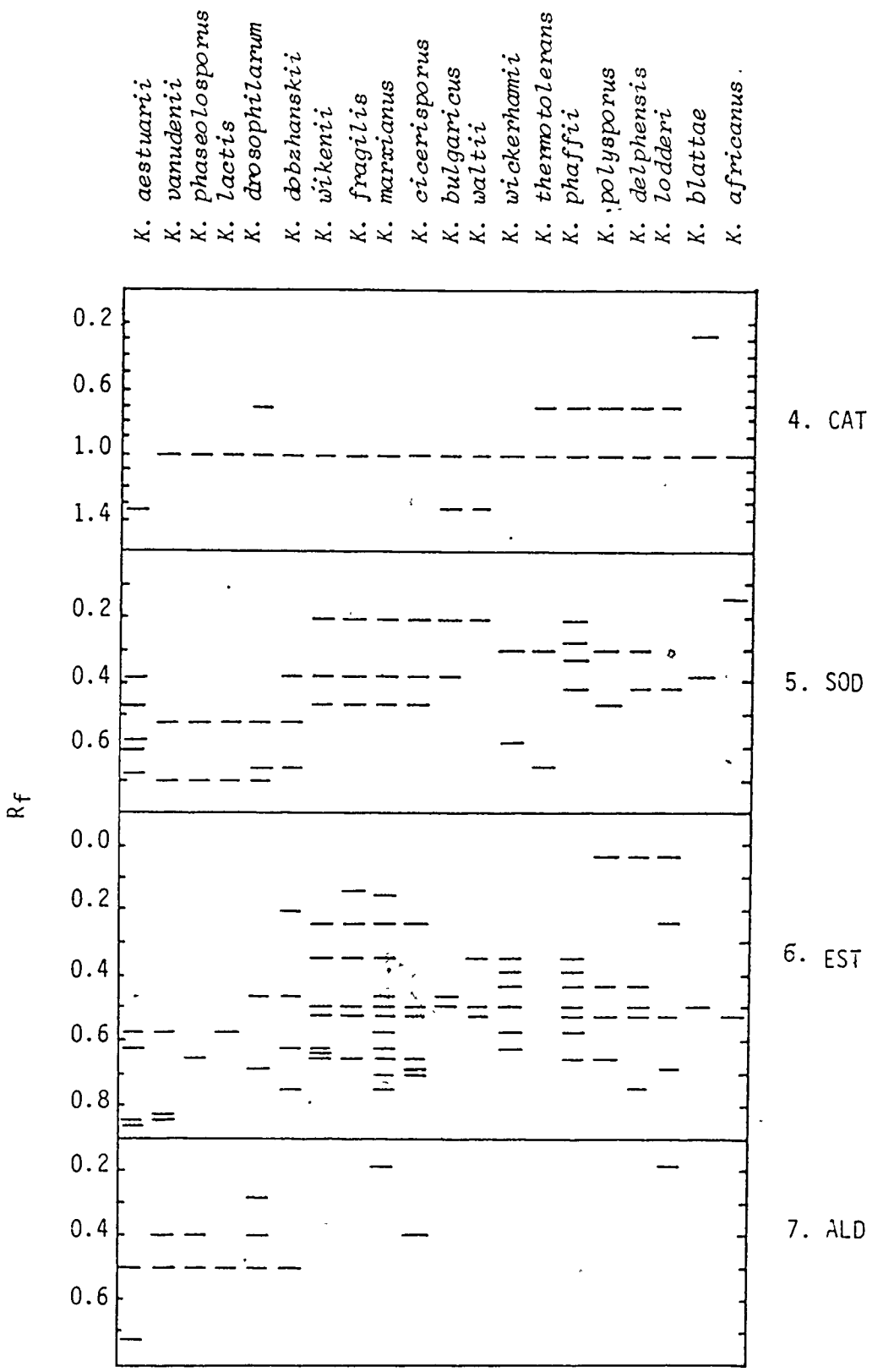
Enzyme[1]	I[2]
Esterase	185
Superoxide dismutase	125
Malate dehydrogenase	93
Lactate dehydrogenase	93
Alcohol dehydrogenase	74
Exo-β-glucanase	52
Catalase	50
α-glucosidase	46
Alkaline phosphatase	30
Aldolase	26
β-glucosidase	8

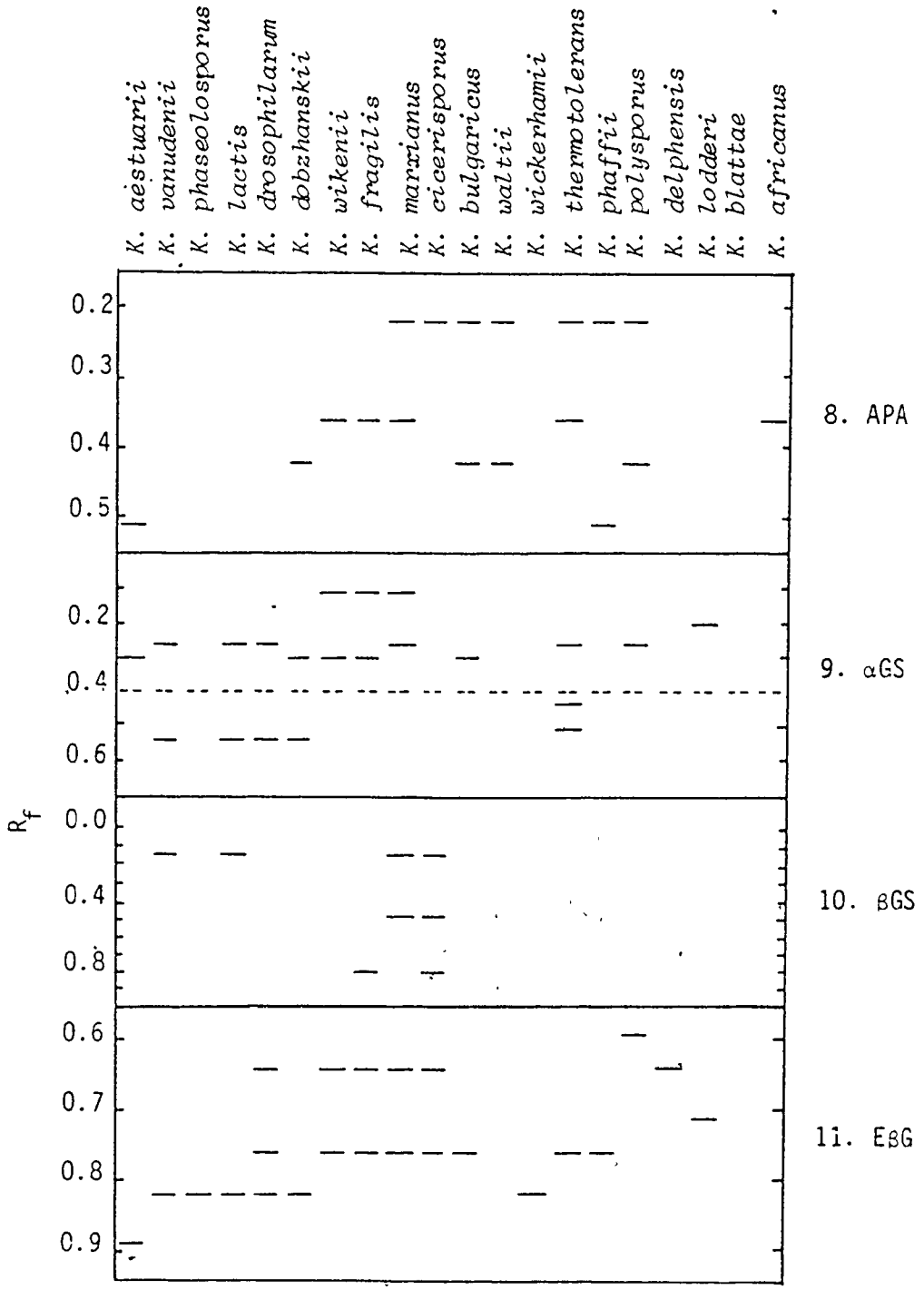
[1]Enzymes are ranked from largest I value (most heterogenous) to smallest I value (least heterogenous).
[2]See Chapter 2 for formula.

Figure 1 (1-11). Electrophoretic patterns of *Kluyveromyces* type strains for eleven enzyme activities

Details of conditions under which each enzyme was studied are presented in Chapter 2. α-Glucosidase acitivities are partitioned according to specificity of electromorphs to trehalose and maltose. Abbreviations: ADH = alcohol dehydroganse, LDH = lactate dehydrogenase, MDH = malate dehydrogenase, CAT = catalase, SOD = superoxide dismutase, EST = esterase, ALD = aldolase, APA = alkaline phosphatase, αGS = α-glucosidase, βGS = β-glucosidase, and EβG = *exo*-β-glucanase.


K. aestuarii
K. vanudenii
K. phaseolospora
K. lactis
K. drosophilarum
K. dobzhanskii
K. wikenii
K. fragilis
K. marxianus
K. cicerispora
K. bulgaricus
K. waltii
K. wickerhamii
K. thermotolerans
K. phaffii
K. polysporus
K. delphensis
K. lodderi
K. blattae
K. africanus
Rf
0.2
0.4
0.6
0.8
1. ADH
2. LDH
3. MDH


K. aestuarii
K. vanudenii
K. phaseolospora
K. lactis
K. drosophilarum
K. dobzhanskii
K. wikenii
K. fragilis
K. marxianus
K. ciceri sporus
K. bulgaricus
K. waltii
K. wickerhamii
K. thermotolerans
K. phaffii
K. polysporus
K. delphensis
K. lodderi
K. blattae
K. africanus
Rf
0.2
0.6
1.0
1.4
4. CAT
0.2
0.4
0.6
5. SOD
0.0
0.2
0.4
0.6
0.8
6. EST
0.2
0.4
0.6
7. ALD


K. aestuarii
K. vanudenii
K. phaseolosporus
K. lactis
K. drosophilarum
K. dobzhanskii
K. wikenii
K. fragilis
K. marxianus
K. cicerisporus
K. bulgaricus
K. waltii
K. wickerhamii
K. thermotolerans
K. phaffii
K. polysporus
K. delphensis
K. lodderi
K. blattae
K. africanus
R_f
0.2
0.3
0.4
0.5
8. APA
0.2
0.4
0.6
9. αGS
0.0
0.4
0.8
10. βGS
0.6
0.7
0.8
0.9
11. EβG

studies. In the case of alcohol dehydrogenase, the unavoidable shaking of the gels may have resulted in uneven stretching causing distorted Rf values. This enzyme was particularly difficult to score and match bands because some cultures had extremely wide bands of activity and others had narrow bands. Often, multiple bands could be found within an area covered by one band in another culture. In some cases, it was difficult to discern if some stained areas were just due to streaking or if they were diffuse bands. Where large areas of intense activity occurred, it was necessary to determine if this was due to overstaining (hence the bands ran together before detection) or if it was in fact just one large band. Superoxide dismutase also tended to give banding patterns of alcohol dehydrogenase a split appearance.

Catalase (Fig 1.4) was a highly conserved enzyme (I = 50). All of the strains except *K. aestuarii* had a common electromorph. *K. aestuarii* was unique. Some strains had an additional electromorph which appeared to be randomly distributed. The second electromorph found in *K. blattae* was unique. Once again, the banding patterns of *K. waltii* and *K. bulgaricus* were unique. The fact that agarose gels appeared to be less discriminatory than polyacrylamide gels is perhaps reflected in the relatively low entropy value of this enzyme. This enzyme was also dropped from future studies.

Superoxide dismutase patterns (Fig. 1.5) which had a relatively high entropy value (I = 125) were very consistent with DNA reassociation groupings. Particularly striking is the fact that *K. dobzhanskii* shared electromorphs found in either the *K. lactis* or the *K. marxianus* DNA subgroups. *K. aestuarii, K. phaffii* and *K. africanus* were found to have a unique banding patterns. As mentioned previously, this enzyme was a hindrance when studing enzymes stained by the tetrazolium method as it tended to give bands a split appearance.

Obviously, the most variable enzyme (I = 185) studied was esterase (Fig. 1.6). All strains had distinct banding patterns. Most of the strains in the *K. marxianus* DNA subgroup produced considerably more electromorphs than their *K. lactis* DNA subgroup counterparts. Little overlap was found between

the two subgroups.

The extremely variable heteromeric nature of esterase made it particularily difficult to record and match bands. Many additional gels had to be run in order to match bands with any degree of confidence. Shaking of these gels may have resulted in some distortion of the Rf values.

Aldolase's (Fig. 1.7) low entropy value (I = 26) was indicative of its absence in eleven of the strains studied. A rare occurence was the sharing of one electromorph by all members of phenetic subgroup B2 including *K. aestuarii*. *K. aestuarii* and *K. drosophilarum* both possessed unique bands. Aldolase activity appeared unstable and greatly influenced by superoxide dismutase, and so it was dropped from future studies.

Alkaline phosphatase activity was absent from the *K. lactis* DNA subgroup as well as some of the members of phenetic group A. Similar banding patterns were present in *K. bulgaricus* and *K. waltii*. The absence of this activity from eight of the species, as well as the small number of different electromorphs is reflected in its low entropy value (I = 30). This enzyme seemed to be very unstable and was not considered further.

As discussed in Chapter 1, *Kluyveromyces* species vary in their ability to use glucosides. For this reason, glucosidases must be viewed differently from the other enzymes.

Electromorphs of α-glucosidase with Rf values less than 0.4 were found to be especially active in the hydrolysis of trehalose. Whereas two trehalase electromorphs were sporadically found throughout the genus, a third one was unique to three members of the *K. marxianus* DNA subgroup. Maltose utilizing members of the *K. lactis* DNA subgroup produced a unique fast band with maltase activity. Not surprisingly, α-glucosidase activity was not found in *K. africanus*, *K. blattae*, *K. cicerisporus*, *K. delphensis*, *K. phaffii*, *K. phaseolosporus*, *K. waltii*, and *K. wickerhamii*. Its low number of different electromorphs and its absence in eight species are indicated by its low entropy value (I = 46).

α-Glucosidase bands present when maltose and trehalose were substrates were missing in gels stained with combined substrates where α-methyl glucoside was a component. The presence of α-methyl glucoside appeared to inhibit activity of these enzymes making gels containing combined substrates not as informative as originally anticipated.

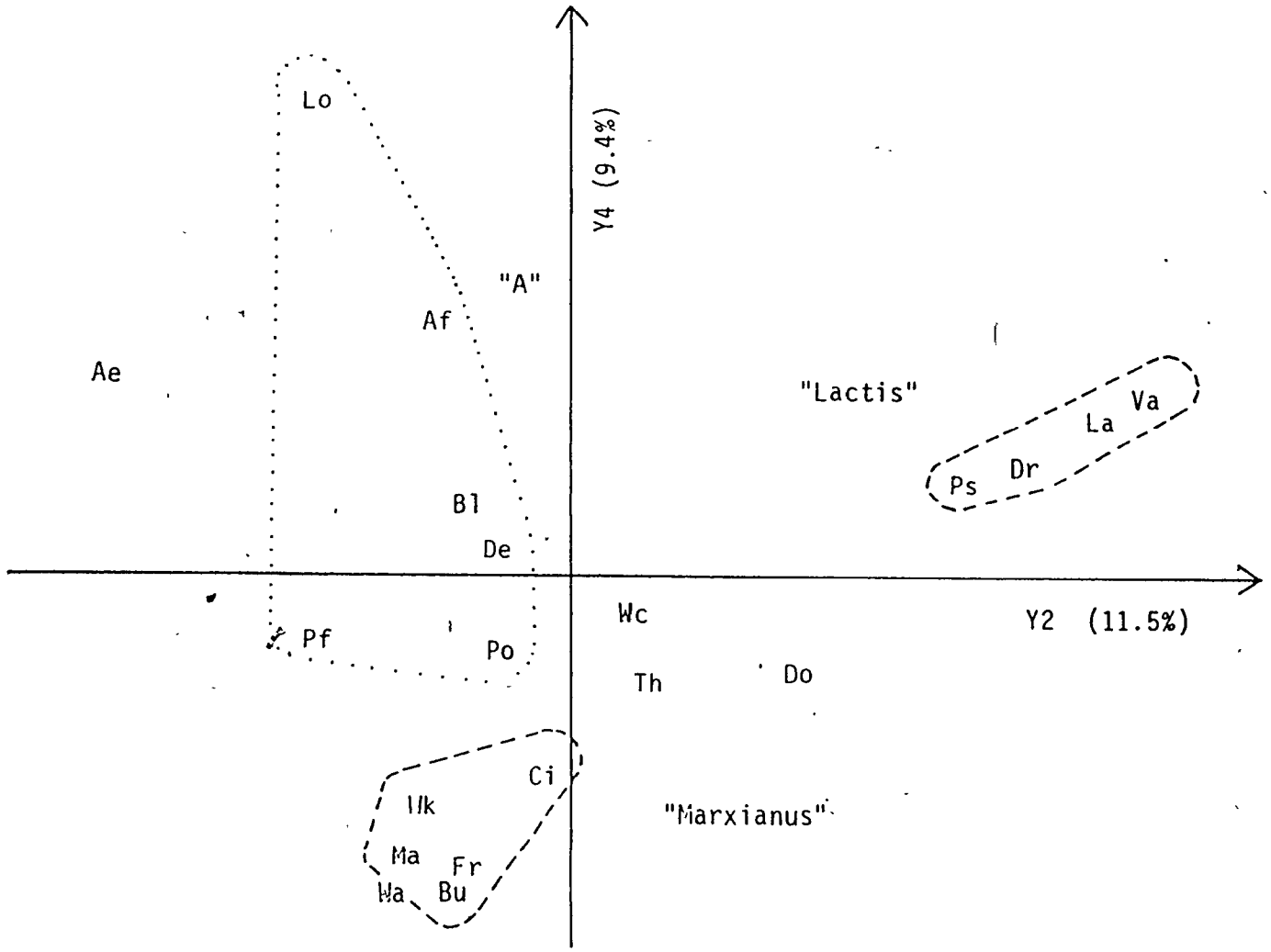
Only three different β-glucosidase electromorphs were produced by the five strains having that activity. The rarity of this enzyme in the genus and its lack of variability are reflected in its low entropy value (I = 8). The enzyme staining technique for this enzyme had a number of problems and was therfore changed in further studies. Regardless of technique, large diffuse bands of pulcherrimin found in some strains had to be compensated for by including a control gel with no substrate.

The small number of electromorphs found in exo-β-glucanase (Fig. 1.11) is reflected in its low entropy value (I = 52). Even though only a few different electromorphs were detected, in the light of past studies (Lachance and Phaff, 1979) of this enzyme, the detection of distinct molecular forms was surprising. More indepth studies were made and presented elsewhere (Sidenberg and Lachance, 1982). *K. drosophilarum* had three exo-β-glucanase electromorphs, *K. wikenii*, *K. fragilis*, *K. marxianus*, and *K. cicerisporus* had two, whereas all but three of the remaining strains had one band of activity. *K. africanus*, *K. blattae*, and *K. waltii* were the strains in which no activity was detected. *K. polysporus*, *K. lodderi*, and *K. aestuarii* each had unique single band patterns. The detection of three electromorphs in *K. drosophilarum* was extremely interesting. Two of the electromorphs were typical of the *K. marxianus* DNA subgroups while the remaining band was typical of the *K. lactis* DNA subgroup. Although it is not conclusive proof, an electrophoretic pattern such as this points to this yeast being derived from other *Kluyveromyces* species which have undergone hybridization or some kind of gene exchange.

Sometimes large areas of exo-β-glucanase activity were obtained which made matching bands particularly difficult. Owing to this, what may have been minor differences in Rf values might have been gone unreported.

Figure 2. Ordination of *Kluyveromyces* type strains by reciprocal averaging

The percent canonical structure represented is indicated for each axis. Broken lines define phenetic group A, as well as *K. lactis* and *K. marxianus* DNA subgroups. Abbreviations: Ae = *K. aestuarii*, Af = *K. africanus*, Bl = *K. blattae*, Bu = *K. bulgaricus*, Ci = *K. cicerisporus*, De = *K. delphensis*, Do = *K. dobzhanskii*, Dr = *K. drosophilarum*, Fr = *K. fragilis*, La = *K. lactis*, Lo = *K. lodderi*, Ma = *K. marxianus*, Pf = *K. phaffii*, Ps = *K. phaseolosporus*, Po = *K. polysporus*, Th = *K. thermotolerans*, Va = *K. vanudenii*, Wa = *K. vanudenii*, Wa = *K. waltii*, Wc = *K. wickerhamii*, Wk = *K. wikenii*.


Y4 (9.4%)
Y2 (11.5%)
Lo
"A"
Af
Ae
Bl
De
Pf
Po
Wc
Th
Do
"Lactis"
Va
La
Dr
Ps
Ci
Mk
Ma
Fr
Wa
Bu
"Marxianus"

Multivariate analysis by means of reciprocal averaging was performed on the presence/absence electrophoretic data. As stated previously, this eigenvector analysis method ordinates both individuals and variables as a function of their canonical or reciprocal correlations. Figure 2 is the ordination of the *Kluyveromyces* type strains on canonical axes 2 and 4. Although the scores for the electromorphs also could have been also plotted, they were omitted for the purposes of clarity.

Axis Y2 was heavily weighted in favor of electromorphs that were unique to the *K. lactis* DNA subgroup and absent in *K. aestuarii*. The dehydrogenases, superoxide dismutase, esterase, aldolase, and exo-β-glucanse contained some of the most highly correlated electromorphs. Axis Y4 strongly discriminated between the *K. marxianus* and *K. lactis* DNA subgroups. For the most part, this axis is compounded by electromorphs that are unique to *K. lodderi*. Most enzymes involved in the Y2 axis also had high canonical correlations with the trends elicited by the Y4 axis.

The *K. marxianus* and *K. lactis* DNA subgroups were also separated by the other axes (Y1, Y3, Y5) not shown here. In total, the first five axes accounted for 51% of the data structure. In each case, the two DNA subgroups represented mutually exclusive populations (Sidenberg and Lachance, 1983).

3.3 Conclusions:

The interpretation of electrophoretic patterns is complex. It was apparent however, that some electromorphs are of value at the species level, whereas others are common to the genus. The taxonomic interpretation of the electrophoretic patterns is most interesting, although limited by the fact that only type strains were examined.

Even before multivariate analysis was performed, the singular electrophoretic similarity of *K. waltii* and *K. bulgaricus* was evident. This finding was unexpected because members of *K. waltii* are very similar in morphology, physiology, and ecology, to those of *K. thermotolerans*. *K. bulgaricus* is generally more similar to other members of the *K. marxianus*

DNA subgroup. For these reasons, the incorporation of *K. waltii* as a variety of *K. marxianus* would seem inappropriate.

Multivariate analysis of the electrophoretic patterns confirmed the distinctiveness of *K. aestuarii, K. africanus, K. delphensis, K. ladderi, K. phaffii, K. polysporus, K. thermotolerans*, and *K. wickerhamii*; thus they should be retained as separate species

The status of *K. drosophilarum* was particularly interesting. Electrophoretic data disagreed and agreed with the data from past studies. Whereas microcomplement fixation (Lachance and Phaff, 1979) only detected enzyme relatedness to the *K. lactis* DNA subgroup, isoenzyme patterns suggested other relationships. Exo-β-glucanase electromorphs possessed by *K. drosophilarum* were found in *K. lactis* and *K. marxianus* DNA subgroups. Other enzymes demonstrated that electromorphs found in *K. drosophilarum* were found in *K. dobzhanskii* (superoxide dismutase and esterase), as well as in *K. thermotolerans* and some members of phenetic group A (catalase). The unusual level of DNA relatedness of *K. drosophilarum* to other *K. lactis* DNA subgroup members (70%) was supported by the electrophoretic data. From these data, it might be speculated that the type strain of *K. drosophilarum* is a natural hybrid or perhaps a slowly evolving "ancestral" form.

The most important conclusion that is drawn from the analysis is that, despite the findings of *in vitro* mating compatibility patterns (Johannsen, 1980), the *K. lactis* and *K. marxianus* DNA subgroups must be viewed as distinct evolutionary groups (biological species). In terms of nomenclature, species delineation that is consistent with speciation patterns can only be obtained by the retention of *K. lactis* (with up to four varieties) and *K. marxianus* (with up to five varieties) as separate species. The most recent revision of the genus (van der Walt and Johannsen, 1984) is at variance with this conclusion.

In conclusion, in addition to giving some insight into the variability of the genus, the electrophoretic data confirm the desirability of delineating *Kluyveromyces* species on the basis of DNA-DNA homology. Because this

method has proven it can answer some questions regarding the evolution, and taxonomy of the genus *Kluyveromyces*, it has also opened a number of new questions. Although this examination of type strains gave some idea of the level of electrophoretic variability that might be encountered in yeast populations, it is more than likely that the type strains examined do not represent the full range of variation within the genus. Therefore the conclusions drawn from the electrophoretic data of the type strains, have yet to be shown meaningful for natural populations. Finally, the genetic constitution of hybrids must be examined in order to determine their nature and the factors controlling their formation and distribution. Both of these questions are addressed in subsequent chapters.

CHAPTER 4 ISOENZYME VARIATION OF POPULATIONS

4.1 Introduction

Protein polymorphism is perhaps the most salient biochemical feature of any population. Electrophoresis, a relatively simple technique, is ideal for surveying populations for protein variation as it is precise and requires only a small amount of material from each isolate. Large numbers of different isolates can easily be screened over a variety of enzymes and their group structure assessed. However, only rarely have yeast genomes been extensively sampled through the electrophoretic analysis of isoenzymes (Table 1).

In this phase of the project, electrophoresis of seven enzymes was employed to examine taxonomic relationships among populations from some of the different *Kluyveromyces* DNA subgroups (Table 2B). The populations studied included isolates belonging to the *K. lactis* and *K. marxianus* DNA subgroups and some of their corresponding anamorphs in the genus *Candida*. Other *Kluyveromyces* taxa compared included *K. dobzhanskii*, *K. thermotolerans*, and *K. waltii*. Due to the unavailability of other *Kluyveromyces* strains, the survey was limited to these strains . Three strains (*Candida sake*, *Pichia fluxuum* and *Saccharomyces cerevisiae*) not belonging to the genus *Kluyveromyces* were also included in the survey as external controls. It was hoped that this survey containing a large number of natural isolates from different habitats (black knots, tree exudates, *Drosophila*, and cheese) would resolve the disagreement (Phaff, 1980 and Johannsen, 1980) as to which criterion, DNA reassociation or mating compatibility patterns, should be dominant in delineating *Kluyveromyces* species.

4.2 Results and Discussion

4.2.1 *Kluyveromyces* Populations

The data discussed earlier (Chapter 3) indicated that electromorph differences among species of *Kluyveromyces* were best revealed by enzymes such as dehydrogenases, superoxide dismutase, esterase, exo-β-glucanase, α-glucosidases, and β-glucosidase. Within the populations, these enzymes varied in the amount of information (I) they contained (Table 5), and thus different enzymes contributed differently to the elucidation of group structure. As expected, esterase (I = 1129) was the most heterogenous enzyme and β-glucosidase (I = 92) the least heterogenous enzyme. All other enzymes held intermediate contents of information.

Alcohol dehydrogenase was more heterogenous than anticipated from ranked expected type strain entropy values. This increased heterogeneity may be due in part to the broad specifity of alcohol dehydrogenases. These enzymes react differently with a large number of primary and secondary, straight and branched chain, aliphatic and aromatic alcohols (Brewer and Singh, 1970). Also, Watts and Donniger (1962) indicated that some spurious bands may be caused by aggregation and other phenomena.

Even before multivariate analysis was performed, the presence/absence data (not shown) displayed some block structure. The groupings were clearer after these data were subjected to multivariate analysis by means of reciprocal averaging. Both individuals (Fig. 3) and variables (Fig. 4) were ordinated as a function of their canonical (reciprocal) correlations. Although these two figures are superimposable, for the sake of clarity, they are presented as two separate diagrams.

The ordination of strains on axes 1 and 2 revealed four groups. The first axis, which had a canonical correlation of 0.79 separated *K. dobzhanskii* and the *K. lactis* and *K. marxianus* DNA subgroups from *K. waltii* and *K. thermotolerans*. The second axis (Y2), which had a canonical correlation of 0.68, resolved *K. waltii* and the *K. marxianus* DNA subgroups from *K.*

Table 5. Ranked observed and expected entropy values for the enzymes examined.

Enzyme[1]	Observed I^2	Expected I^2
Esterase	1129	1186
Alcohol dehydrogenase	618	509
Superoxide dismutase	577	540
Malate dehydrogenase	545	525
α-glucosidase	451	414
Exo-β-glucanase	231	197
β-Glucosidase	92	75

[1] Enzymes are ranked from largest I value (most heterogenous) to smallest I value (least heterogenous).
[2] See Chapter 2 for formula.

Figure 3. Ordination of strains from *Kluyveromyces* populations by reciprocal averaging

The percentage of the canonical structure represented is indicated for each axis. Bars show two standard deviations about the mean for each taxon.

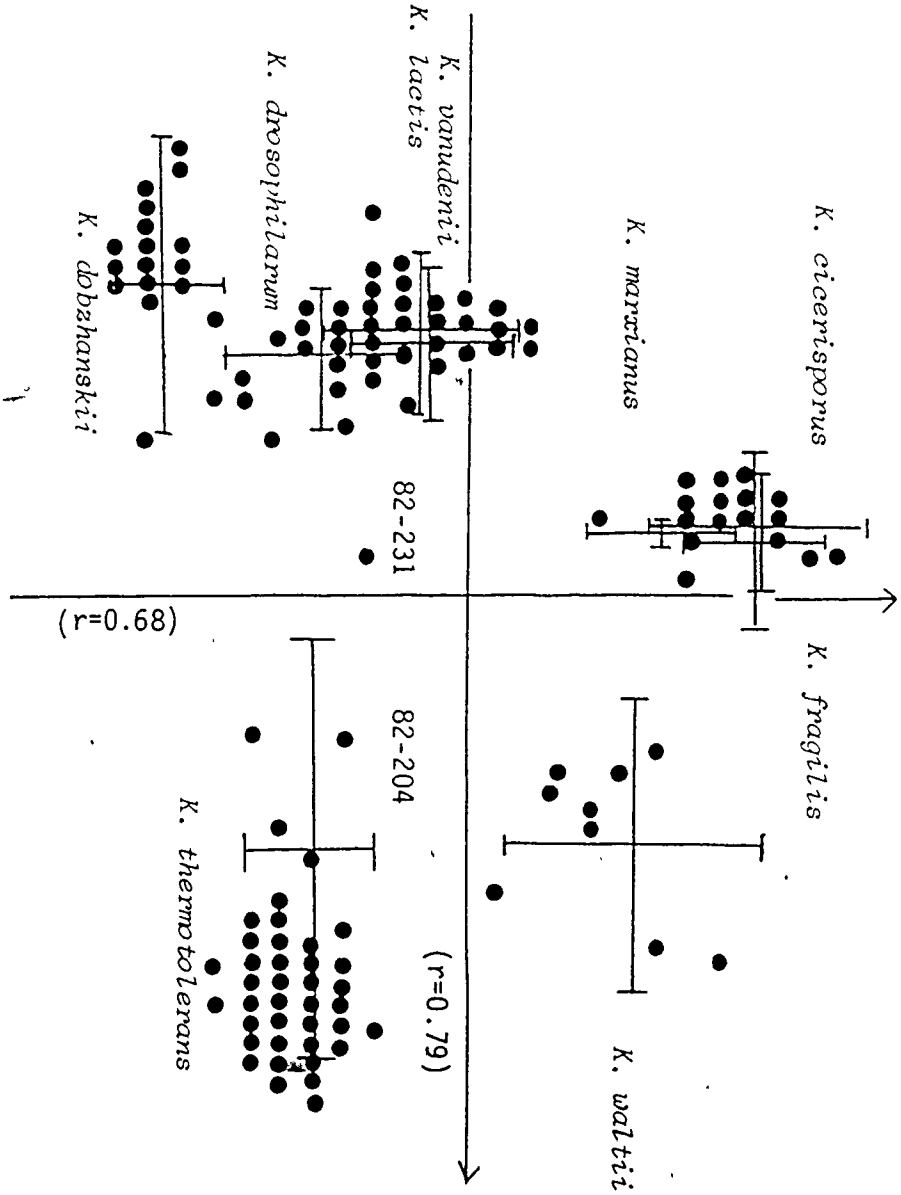
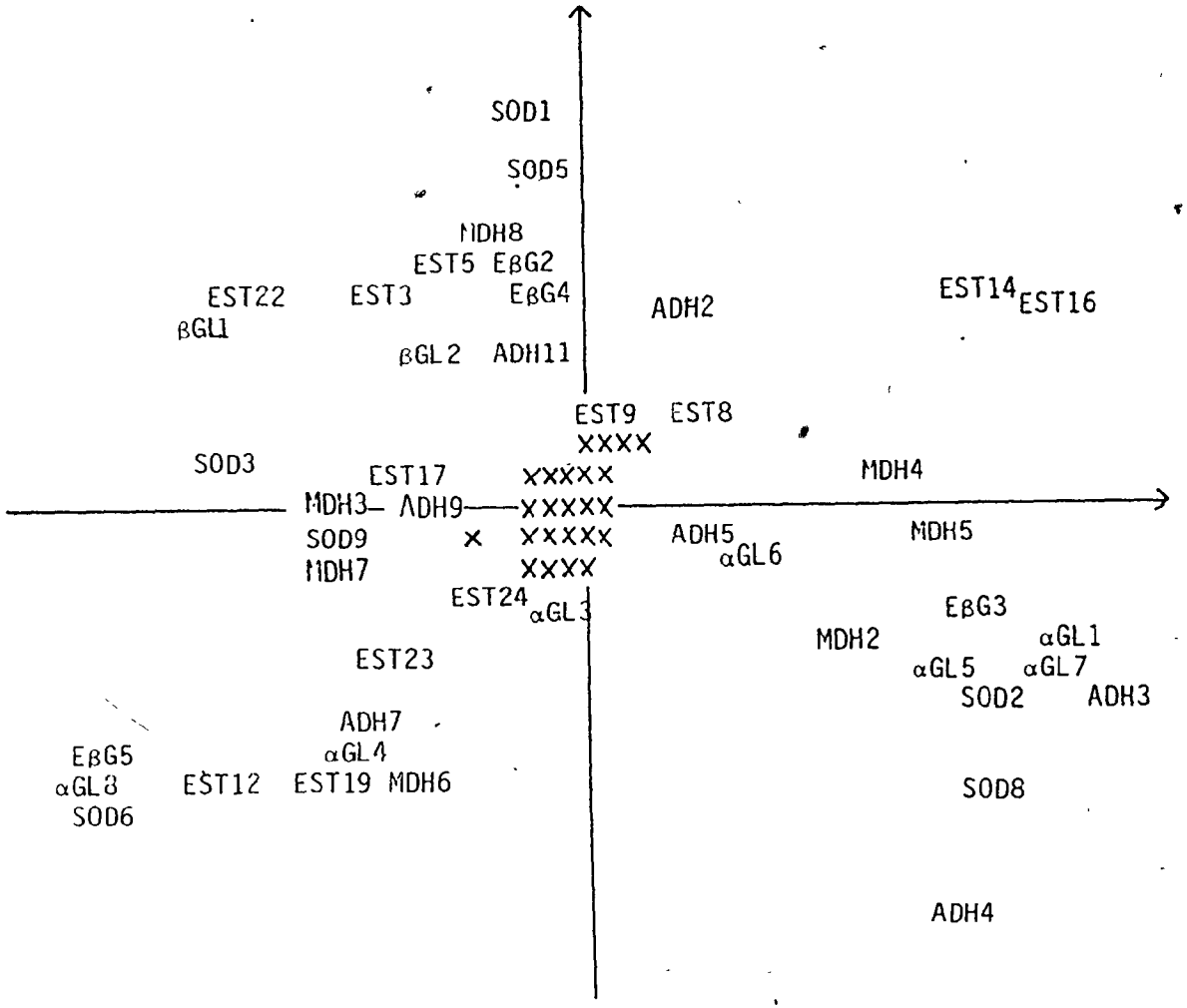

K. vanudenii
K. lactis
K. drosophilarum
K. dobzhanskii
K. ciceri sporus
K. marxianus
K. fragilis
K. waltii
K. thermotolerans
82-231
82-204
(r=0.68)
(r=0.79)

Figure 4. Ordination of electromorphs from *Kluyveromyces* populations by reciprocal averaging.

The percentage canonical structure is indicated for each axis. Abbreviations, ADH = alcohol dehydrogenase, MDH = malate dehydroganase, SOD = superoxide dismuatase, EST = esterase, αGS = α-glucosidase, βGS = β-glucosidase, EβG = exo-β-glucanase, X = unlabelled electromorphs with near zero coordinates.


SOD1
SOD5
MDH8
EST5 EβG2
EST22 EST3 EβG4
βGL1
βGL2 ADH11
ADH2
EST14 EST16
EST9 EST8
SOD3
EST17
MDH3 ADH9
SOD9
MDH7
MDH4
ADH5
αGL6
MDH5
EST24
αGL3
EβG3
MDH2
αGL1
αGL5 αGL7
SOD2 ADH3
EST23
ADH7
EβG5
αGL4
αGL8 EST12 EST19 MDH6
SOD6
SOD8
ADH4

thermotolerans, *K. dobzhanskii*, and the *K. lactis* DNA subgroup. Bars, which show two standard deviations about the mean for each taxon, clearly illustrate that there is no overlap between any of the four groups.

Figure 4 shows that the first axis (Y1) has a broad representation in all seven enzymes used for this study. The second axis (Y2) is more heavily weighted by superoxide dismutase, alcohol dehydrogenase, malate dehydrogenase, and α-glucosidase electromorphs.

Axis Y3 (not shown) separated the *K. dobzhanskii* subgroup from the *K. lactis* DNA subgroup. Altogether, the first three axes accounted for 35% of the data structure, which is not unusual when analyzing a large (125 x 68) matrix of binary data.

The most striking outlier was *K. thermotolerans* 82-231 (Fig. 3). Although none of its electromorphs were unique, its patterns were different from those of the other strains. The most unusual differences were the absence of ADH3 and 4, and the presence of EST18, EβG2, SOD3 and SOD6 which were not normally found in *K. thermotolerans*. Overall, strain 82-231 was very similar to *K. thermotolerans* 82-204, which was isolated from *Drosophila* (Pinery Provincial Park) at the same time.

Electromorph frequencies pooled by taxon also readily confirmed the block structure of the data. From Table 6, it was relatively simple to identify the electromorphs that were shared across the *K. marxianus* mating compatibility group (ADH5, MDH6) or found exclusively in members of *K. dobzhanskii* (SOD7), *K. thermotolerans* (SOD2, αGL6, EST18), *K. waltii* or in the *K. lactis* (ADH9, 12, SOD9, EST1, 13, 25 and αGL9), or *K. marxianus* (ADH8, MDH8, EST21, βGL3) DNA subgroups.

The group structure was further substantiated by principal components analysis. Figure 5 shows the first three principal axes accounting for 66% of the total variation in the frequency data presented in Table 6. The first component (accounting for 27% of the total variation) separated the *K. marxianus* DNA subgroup from others on the basis of EβG2 and 4, MDH8, SOD1 and 5, EST5, 14, and 21, and ADH11 electromorphs. The second component

Table 6. Electromorph frequencies in populations.

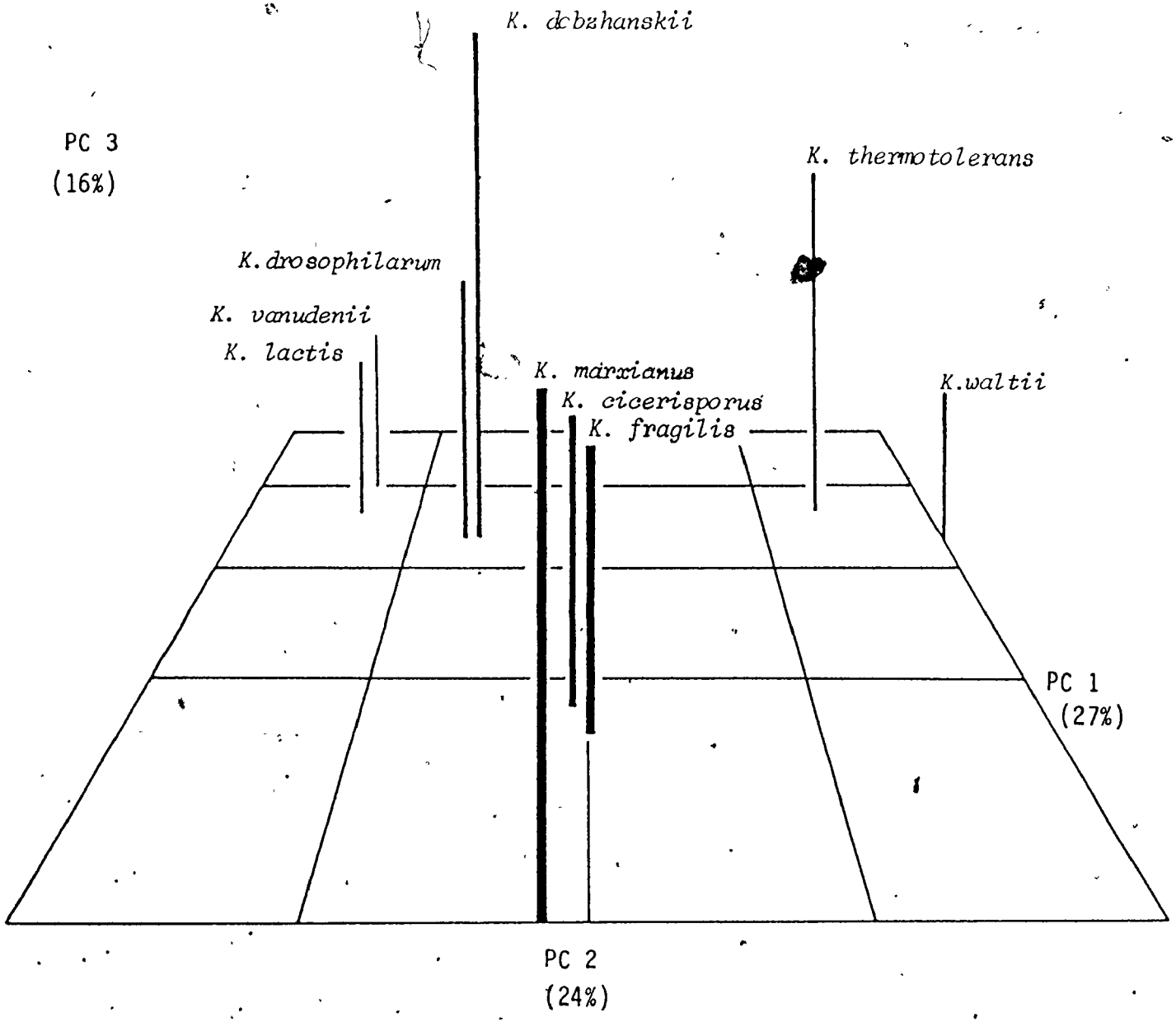
	VANU n=9	LACT n=17	DROS n=13	DOBZ n=18	FRAG n=5	MARX n=2	CICE n=11	WALT n=9	THER n=41
ADH2	0.00	0.00	0.00	0.00	0.00	0.00	0.18	1.00	0.00
ADH3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.95
ADH4	0.00	0.00	0.08	0.66	0.00	0.00	0.00	0.00	0.93
ADH5	1.00	0.94	0.92	0.16	0.80	0.50	0.82	0.00	0.95
ADH6	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.20
ADH7	0.00	0.00	0.08	0.72	0.00	0.00	0.00	0.00	0.00
ADH8	0.00	0.00	0.00	0.00	0.00	0.50	0.18	0.00	0.00
ADH9	0.67	0.71	0.15	0.00	0.00	0.00	0.00	0.00	0.00
ADH11	0.11	0.06	0.08	0.00	0.60	0.50	0.55	0.00	0.00
ADH12	0.11	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ADH13	0.00	0.12	0.00	0.00	0.00	0.00	0.09	0.00	0.00
MDH1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.00
MDH2	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.46
MDH3	1.00	0.94	0.08	0.00	0.00	0.00	0.00	0.22	0.07
MDH4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.89	0.37
MDH5	0.11	0.00	0.08	0.16	0.00	0.00	0.00	0.89	0.61
MDH6	0.78	0.94	1.00	1.00	0.60	1.00	0.73	0.00	0.80
MDH7	0.00	0.00	0.00	0.83	0.60	1.00	0.55	0.00	0.00
MDH8	0.00	0.00	0.00	0.00	1.00	1.00	0.82	0.00	0.00
SOD1	0.00	0.41	0.00	0.00	1.00	1.00	0.91	0.78	0.00
SOD2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.63
SOD3	0.89	0.00	0.00	0.88	1.00	1.00	0.91	0.11	0.07
SOD4	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.22	0.00
SOD5	0.00	0.00	0.00	0.00	1.00	1.00	0.91	0.33	0.00
SOD6	0.44	0.88	1.00	1.00	0.00	0.00	0.00	0.00	0.07
SOD7	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00
SOD8	0.00	0.00	0.85	0.16	0.00	0.00	0.00	0.00	0.90
SOD9	1.00	0.59	0.46	0.00	0.00	0.00	0.00	0.00	0.00

Table 6. Cont.

	VANU n=9	LACT n=17	DROS n=13	DOBZ n=18	FRAG n=5	MARX n=2	CICE n=11	WALT n=9	THER n=41
EST1	0.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EST2	0.00	0.06	0.00	0.00	0.20	0.00	0.00	0.00	0.00
EST3	0.33	0.77	0.00	0.00	0.80	1.00	0.64	0.00	0.00
EST4	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00
EST5	0.22	0.06	0.00	0.00	1.00	1.00	0.82	0.00	0.00
EST6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.02
EST7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00
EST8	0.00	0.82	0.00	0.00	0.40	1.00	0.09	0.67	0.39
EST9	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.00
EST10	0.00	0.00	0.38	0.00	0.00	0.00	0.00	0.44	0.00
EST11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00
EST12	0.11	0.29	0.62	1.00	0.00	0.50	0.00	0.00	0.00
EST13	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EST14	0.00	0.00	0.00	0.00	1.00	1.00	1.00	0.44	0.68
EST16	0.00	0.00	0.00	0.00	1.00	1.00	0.91	0.67	0.80
EST17	0.44	0.94	0.00	0.00	0.00	0.50	0.00	0.11	0.00
EST18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05
EST19	0.33	0.00	0.00	0.94	0.00	0.00	0.00	0.00	0.00
EST20	0.00	0.00	0.00	0.06	0.00	0.50	0.00	0.00	0.00
EST21	0.00	0.00	0.00	0.00	0.20	0.50	0.09	0.00	0.00
EST22	0.89	0.59	1.00	0.00	0.80	0.50	0.91	0.00	0.00
EST23	0.00	0.00	0.00	0.67	0.00	0.50	0.09	0.00	0.02
EST24	0.00	0.12	0.00	0.33	0.00	0.50	0.00	0.00	0.00
EST25	0.22	0.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EST26	0.22	0.12	0.00	0.06	0.00	0.00	0.00	0.00	0.07
αGL1	0.00	0.00	0.00	0.00	0.20	0.50	0.00	0.33	0.78
αGL3	0.89	0.88	0.92	0.00	0.00	0.50	0.00	0.22	0.54
αGL4	0.00	0.00	0.00	0.94	0.20	0.00	0.00	0.00	0.00
αGL5	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.63
αGL6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22
αGL7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.76
αGL8	0.67	0.88	0.77	0.94	0.00	0.00	0.00	0.00	0.00
αGL9	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
βGL1	1.00	0.88	0.62	0.06	0.60	1.00	0.82	0.00	0.00
βGL2	0.00	0.06	0.69	0.06	0.40	0.50	0.82	0.00	0.00
βGL3	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00
EβG2	0.00	0.00	0.08	0.00	1.00	1.00	0.73	0.00	0.07
EβG3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.63
EβG4	0.00	0.00	0.08	0.00	1.00	1.00	0.82	0.00	0.02
EβG5	0.89	0.77	1.00	0.83	0.00	0.00	0.00	0.00	0.00

Figure 5. Ordination of *Kluyveromyces* populations by principal components analysis of frequency data

The percentage variation represented is indicated for each axis.


K. dobzhanskii
PC 3
(16%)
K. thermotolerans
K. drosophilarum
K. vanudenii
K. lactis
K. marxianus
K. cicerisporus
K. fragilis
K. waltii
PC 1
(27%)
PC 2
(24%)

(accounting for 24% of the total variation) isolated *K. thermotolerans* and *K. waltii* based on high frequencies for electropmorphs EBG3 and MDH 4 and 5. Principal component 3 accounted for 16% of the total variation and resolved *K. dobzhanskii* from the *K. lactis* DNA subgroup based on its high frequencies for bands ADH7 and SOD7. EST7, an important electromorph of component 3, was only found in *K. waltii*.

The study of the relationship of ecology and electrophoretic data is a many faceted problem. Although more sophisticated experiments and analysis of the relationships betweeen groups and their habitats are warranted, they are beyond the scope of this project. Figure 6 is a Venn diagram which briefly summarizes the habitats of the five groups delineated in Figures 3 and 5.

In general, the members of the genus *Kluyveromyces* are not stringent in their habitat specificities and are widely distributed ecologically (van der Walt and Johannsen, 1984). The two major habitats of the yeasts strains investigated in this study were Camembert cheese and black knots (pathological fungal galls found in particular on the trees of the genus *Prunus*). Less well represented habitats included *Drosophila*, oak fluxes, and galls.

Because of industrial importance, the ecology of yeasts associated with dairy products is fairly well understood. Many of the spoilage yeasts of milk products ferment or utilize lactose, the principal sugar of milk, as an energy source (Phaff *et al.*, 1978a). The members of the *K. marxianus* DNA subgroup (*K. marxianus, K. fragilis, K. cicerisporus*) and the *K. lactis* strains shared the habitat of cheese from France and did not overlap in source of isolation with the other taxa studied. All of these strains were lactose positive.

Very little is known about the ecology of yeasts associated with black knots. Lachance (1980) did find that *K. thermotolerans* was dominant in the mature stages of black knot development while other species of *Kluyveromyces* suceeded in the later decay stages. Significantly correlated

Figure 6. Venn diagram of sources of isolation for populations.

1 Black knot, *Prunus pumila*, Pinery Provincial Park, Ont.

2 Black knot, *Prunus virginiana*, Pinery Provincial Park, Ont.

3 *Drosphila*, Pinery Provincial Park, Ont.

4 Flux, *Quercus rubra*, Pinery Provincial Park, Ont.

5 Black knot *Prunus virginiana*, Coldstream Conservation Area, Ont.

6 Black knot, *Prunus virginiana*, Medway Creek, London, Ont.

7 Black knot, *Prunus virginiana*, Melbourne, Ont.

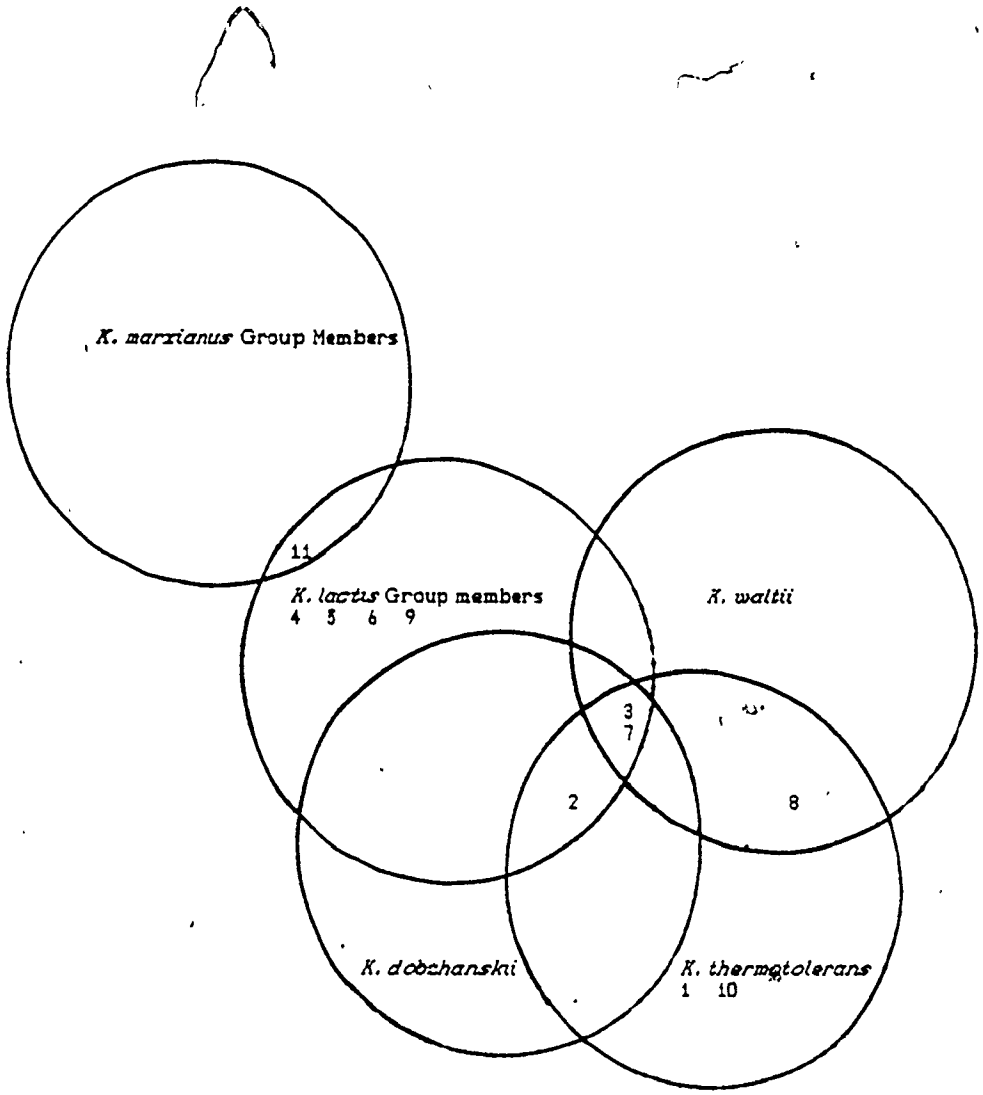
8 Gall, *Quercus rubra*, Melbourne, Ont.

9 Black knot, *Prunus virginiana*, Dingman Creek, London, Ont.

10 Black knot, *Prunus serotina*, St. Anicet, Que.

11 Camembert cheese, Dr. J.L. Schmidt,

Institut National Agronomique, Paris-Grignou, France.


K. marxianus Group Members
11
K. lactis Group members
4 5 6 9
K. waltii
3
7
2
8
K. dobzhanski
K. thermotolerans
1 10

with the affinity for mature large black knots was the ability to grow on 50% glucose and the ability to assimilate tannins. Although the exact reason for the succession of yeast types that occurs during black knot development is not known, perhaps, this temporal factor may combine with other intrinsic factors to prevent gene exchange between *K. thermotolerans* and other taxa.

Despite the fact that *K. lactis* and *K. vanudenii* have a high degree of relatedness, it was fascinating that *K. lactis* strains isolated from cheese in France and *K. vanudenii* strains isolated from *Drosophila* and black knots in Canada should have such strikingly similar electrophoretic patterns.

4.2.2 Anamorphs

A number of *Kluyveromyces* species are considered to have anamorphs in the genus *Candida*. Members of the genus *Candida* have been examined electrophoretically (See Table 1), but few electrophoretic comparisons of the isoenzymes of anamorphs and teleomorphs have been made. Yamazaki and Komagata (1982a) have demonstrated close relationships between anamorphs and their corresponding teleomorphs.

Three anamorphs of *K. lactis* (80SM2-5, 80SM32-5, and 80SM35-14) and one anamorph of *K. marxianus* (80SM3-4) were included in this survey. Although the anamorphs contained some differences when compared to their corresponding types and strains from natural populations, multivariate analysis (Fig. 3) revealed that all strains fell within their appropriate groups, once again indicating the validity of using electrophoresis to investigate the relationship between perfect and imperfect yeasts.

4.2.3 Strains Not Belonging To The Genus *Kluyveromyces*

Originally some strains of *Kluyveromyces* were assigned to the genus *Saccharomyces*. It should be mentioned that *Saccharomyces* strains have been extensively investigated electrophoretically (Yamazaki *et al.* 1982, 1983), but the electrophoretic patterns are not comparable to those in this survey

as different enzymes and different conditions were used. The isoenzyme patterns of *S. cerevisiae* (79-11) were examined electrophoretically (Table 7).

Of the two band alcohol dehydrogenase pattern of this strain, one electromorph was unique, while the other was present in about a third of the *Kluyveromyces* population examined. A single electromorph of malate dehydrogenase activity was found, but it also was not individually unique to *S. cerevisiae*. Although the two band pattern of superoxide dismutase was not found in any other strain, neither band was unique. This strain had no activity on β-glucosidase. The exo-β-glucanase electromorph of *S. cerevisiae* was unique. The total banding pattern of this strain clearly revealed it was not a member of the genus *Kluyveromyces*.

Two strains not belonging to the genus *Kluyveromyces* were accidently introduced into the survey. The first strain, *C. sake* (79-228), was originally mislabelled as *K. dobzhanskii*. The banding patterns of this strain are presented in Table 7. Out of its 13 band pattern only 3 electromorphs corresponded to those of *K. dobzhanskii* strains. Only one of the individual electromorphs was unique, but many of the enzyme patterns were unique (alcohol dehydrogenase, malate dehydrogenase, superoxide dismutase, esterase, and α-glucosidase). β-Glucosidase activity was not found in this strain.

The second strain, *P. fluxuum* (80-109) was originally mislabelled as *K. drosophilarum*. Its misassignment was also easily detected by electrophoresis (see Table 7 for isoenzyme patterns). Only 3 of this strain's 21 electromorphs corresponded to those of *K. drosophilarum* strains. This strain had a very striking unique 14 electromorph alcohol dehydrogenase pattern. Its 2 band malate dehydrogenase pattern was found in a number of *K. thermotolerans* strains. The only electromorph of superoxide dismutase activity it possessed was also found in a number of the other strains studied. Although the 4 band esterase pattern of this strain was not found in any other strain, only one of its bands was unique. No α-glucosidase, β-glucosidase, or exo-β-glucanase activites were detected in this strain.

Table 7. Electromorphs present in *S. cerevisiae*, *C. sake*, and *P. fluxuum*.

S. cerevisiae (79-11)	*C. sake* (79-228)	*P. fluxuum* (80-109)
ADH1		
	ADH2	
ADH3		
	ADH4	
	ADH8	
	ADH9	
		ADH*
	SOD3	SOD3
SOD5		
SOD6		
	MDH4	
	MDH9	
	EST7	
	EST8	
		EST12
		EST13
		EST15
EST17		
EST18		
EST19		EST19
	αGL1	
αGL2		
	αGL4	
αGL5	αGL5	
EβG1		
	EβG2	

* The 14 band alcohol dehydrogenase pattern of this strain was not found in any of the other strains. Since it was not cross checked for unique bands the band numbers are not presented.

4.3 CONCLUSIONS

Yeast species, let alone populations, have not been accurately evaluated for their genetic variation. Although the genus *Kluyveromyces* has been systematically studied by many workers with various methods, until now its isoenzyme variation had not been evaluated and compared to other taxonomic features.

According to the biological species concept, two taxa are regarded as separate species if gene exchange is not taking place when the opportunity exists for it to occur in nature. The aim of this study was to determine if taxa capable of mating under laboratory conditions, in spite of their low degree of DNA complementarity, appeared to share a common gene pool in nature. Hopefully, this would resolve which technique, DNA-DNA reassociation or mating compatibilty, should predominate in delineating *Kluyveromyces* species.

Because no additional strains of *K. bulgaricus* were available at the time of this survey, there was no way to confirm or refute the low electrophoretic dissimilarity found between the *K. waltii* and the *K. bulgaricus* type strains. Even though the natural isolates of *K. waltii* were highly variable, when multivariate analysis was performed, they did not group within strains of the *K. marxianus* DNA subgroup (Fig. 3). Extensive interbreeding does not seem to be occurring and certainly the strains of this survey are not occupying the same habitat. For these reasons, *K. waltii* should be retained as a separate species. This is in partial agreement with Johannsen (1980) that only a limited amount of mating can occur between *K. waltii* and *K. drosophilarum*.

Even though natural populations of *K. thermotolerans* strains were found to share electromorphs with populations of other species, this study supported the conclusion, drawn from the type culture study (Chapter 3) that it should be retained as a separate species. Spatially, *K. thermotolerans* shared its habitat with many of the other strains examined in the survey. Perhaps its lack of mating with the other taxa is explainable in part by

the succession of yeast types that occurs during black knot development, hence to a temporal segregation. There is no major argument taxonomically, between the three techniques (DNA-DNA homology, mating compatibility, and electrophoretic dissimilarity) that *K. thermotolerans* is also a separate species, but this is where the agreement ends.

The proponents of mating compatibility as the delimiter of species relegated *K. bulgaricus*, *K. cicerisporus*, *K. dobzhanskii*, *K. drosophilarum*, *K. fragilis*, *K. lactis*, *K. phaseolosporus*, *K. vanudenii*, and *K. wikenii* to seven syngamous varieties of *K. marxianus* (van der Walt and Johannsen, 1984). DNA-DNA reasssociation and electrophoretic data are highly correlated and do not support this classification.

There were no strains of *K. phaseolosporus* available from natural sources to assess at this time. *K. lactis* and *K. vanudenii* which have been shown to have 100% DNA-DNA homology were almost electrophoretically indistinguishable. *K. lactis* and *K. vanudenii* apparently interbreed. Also, *K. drosophilarum* which had a somewhat lower DNA-DNA complementarity (70%) with *K. lactis* and *K. vanudenii* was electrophoretically very close to these two taxa indicating interbreeding is occurring freely between these taxa. Although there was overlap between the strains, their habitat specificities and physiology point to retaining them as varieties of *K. lactis*.

Although the *K. dobzhanskii* population had a close electrophoretic relationship with the *K. lactis* DNA subgroup, multivariate analysis revealed that it was sufficiently different to warrant maintaining it as a separate species. The low DNA-DNA homology between *K. dobzhanskii* and other *K. lactis* DNA subgroup members indicated it was a separate species as well.

K. bulgaricus or *K. wikenii* strains were not available to be included in the assessment of the *K. marxianus* DNA reassociation group. Electrophoretic data from the remaining members, which are virtually undistinguishable phenotypically, indicated that *K. cicerisporus*, *K. fragilis*, and *K. marxianus* are also indistinguishable electrophoretically. The high degree of DNA complementarity between these three taxa also strongly

suggests that these yeasts are representatives of the same species.

Contrary to the findings of Johannsen (1980), effective interfertility does not seem to be taking place between members of the *K. lactis* and the *K. marxianus* DNA subgroups. Electrophoretic differences were sufficient to warrant their recognition as different species.

As discussed above, a close correlation can be discerned between the electrophoretic patterns of enzymes in *Kluyveromyces* and its DNA-DNA reassociation patterns. This finding was not surprising. Yamazaki and Komagata (1981) found an excellent correlation between DNA-DNA complementarity and electrophoretic isoenzyme patterns. Taxonomically, some electromorphs were characteristic of species while others were common to the genus. This study also confirms the conclusion by Yamazaki and Komagata (1982a) that electrophoretic comparisons of enzymes are useful in investigating the relationships between the perfect and imperfect states of yeasts. Furthermore, the electrophoretic patterns readily revealed the nonmember status of strains not belonging to the genus.

The electrophoretic technique does have some limitations in its application. Statistical guidelines defining the relationship between electrophoretic varation and the biological species concept in yeasts are not likely to be established for this heterogenous group of fungi. Extraction and staining methods are not universally successful with all yeasts. The inclusion of electrophoretic data in the formal descriptions of yeast species (*e.g.* Holzchu *et al.*, 1984) will not be practical unless standard conditions can be used.

Now that distinct populations have been identified in this way, further detailed ecological studies are required to fully understand their makeup and the subtle cause and effect relationships which govern their distribution.

Hybrids and hybrid zones have have not been characterized in yeasts. The hypothesis that *K. drosophilarum* may be a hybrid between members of the *K. lactis* and *K. marxianus* groups (see Chapter 3) was not confirmed

by this population study. The significance of ecological and selective forces controlling the distribution of the different *Kluyveromyces* species and their hybridization are as yet mysteries. For these reasons, it was considered valuable to investigate the electrophoretic patterns of laboratory constructed yeast hybrids, and to speculate on their potential relationship to natural populations.

CHAPTER 5 ISOENZYME PATTERNS OF HYBRIDS

5.1 Introduction

Practically from the onset, it was realized that what constitutes a hybrid is dependent upon how the term species is defined (Huskins, 1929). The definition of species has changed (Chapter 1) from the morphological species concept, which emphasised the distinctive structure and form of a kind of organism, to the biological species concept, which stressed continuity of interbreeding among local populations but absence of such integration with populations of other species (Wright, 1978). Subsequently, the definition of hybridization has also changed from Stebbin's (1959) definition of "the crossing between individuals belonging to separate populations which have different adaptive forms" to Lincoln's *et al.* (1982) definition of "any crossing of individuals of different genetic composition, typically belonging to separate species, resulting in hybrid offspring". Hybridization should not be confused with interfertility which has the additional stipulation that the hybrid offspring be fertile.

The population phenomenon of speciation can occur in various ways (Dobzhansky *et al.*, 1977). The relationship between hybrid zones and speciation in plants and animals has been well documented. There have been numerous examples of electrophoretic observations on putative hybrids and hybrid zones (animals *e.g.*: Ayala, 1975; Wright, 1978; Barton and Hewitt, 1983; plants *e.g.*, Tanskley and Orton, 1983). Natural fungal hybrids and hybrid swarms have not been well authenticated (Burnett, 1983). Since the study of speciation in yeasts has special problems, little conclusive evidence exists to explain how hybridization may be involved in the process. Until recently, the study of hybridization in yeasts has concentrated on the improvement of strains for use in industry (baking, brewing, distilling, and wine making), thus the taxonomic significance of its presence or absence has yet to be resolved.

Winge and Lautsen (1939) and Wickerham and Burton (1956a) proposed that interfertility between yeast strains be accepted as the criterion for conspecificity. Wickerham and Burton (1956a, b), Johannsen and van der Walt (1978), van der Walt and Johannsen (1979, 1984), and Johannsen (1980) have indicated that interfertility is operative between some representatives of the genus *Kluyveromyces*. The characterization of β-glucosidase by Fleming and Duerksen (1967) and phosphoglucomutase by Douglas *et al.* (1969) offered evidence that hybrids contained the genomes of both parents and that hybrids are readily detectable electrophoretically. Not surprisingly, electrophoretic isoenzyme patterns previously reported (Chapter 3 and 4) revealed more dissimilarity among species that do not readily form hybrids under forced mating than among those that do. Since no putative wild hybrids have been isolated and the electrophoretic survey of natural isolates (Chapter 4) revealed no intermediate entities between good taxa, it was hoped that an electrophoretic study of laboratory constructed hybrids would help elucidate hybrid character.

In this phase of the project, electrophoresis of seven enzymes (two with multiple substrates) was undertaken to investigate how combined parental phenotypes would be reflected in isoenzyme patterns of interspecific (*K. marxianus* x *K. thermotolerans*) and intraspecific (*K. lactis* x *K. vanudenii*) hybrids. Parental, type, and hybrid strains were also compared. The taxonomic relevance of electrophoretic isoenzyme patterns was examined in laboratory constructed hybrids between strains with high and low levels of relatedness. It should be stressed that the hybrids of this study were isolated from forced matings under laboratory conditions (interspecific hybrid constructed by Johannsen and van der Walt, 1978; intraspecific hybrids prepared in this laboratory), and thus may not represent the natural situation.

5.2 Results and Discussion

The strains included in this study exhibited considerable heterogeneity with respect to electrophoretic patterns (See Fig. 7.1-7.7 and Fig. 9.1-9.7). Evidence of hybridization, which can be demonstrated electrophoretically

not only by the hybrid sharing parental bands but by the hybrid gaining or losing bands found in the parental strains (Moore and Collins, 1983), occurred in most enzymes. The enzymes varied as to the type of information about the hybrid nature they yielded, thus they will be discussed individually.

5.2.1 Interspecific Hybrid *K. marxianus x K. thermotolerans*

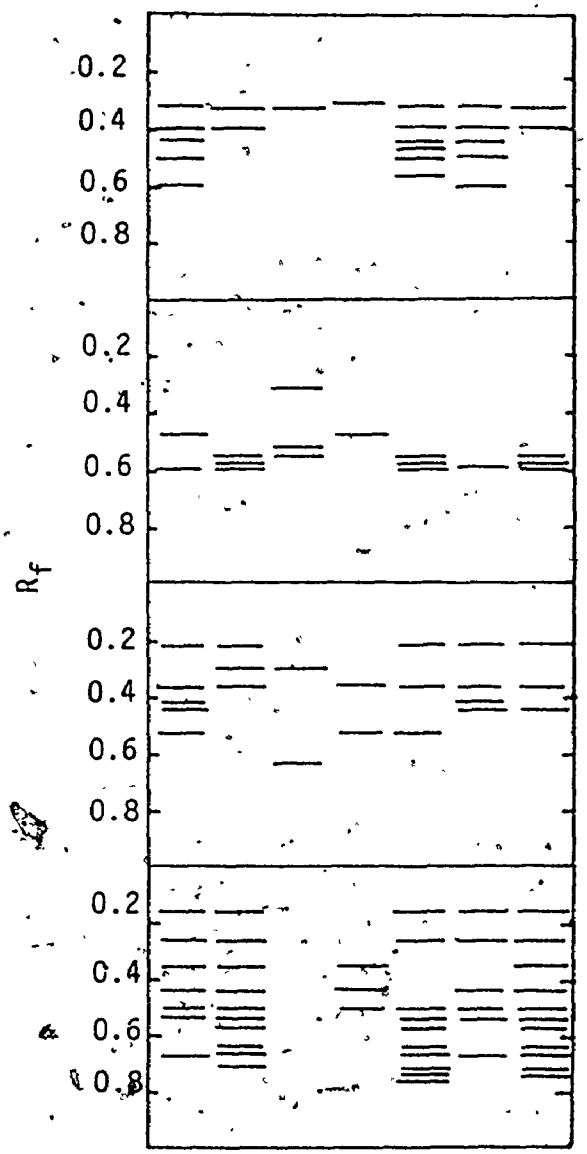
The very different electrophoretic patterns of *K. marxianus* and *K. thermotolerans* type and natural strains had the potential to allow easy detection of hybridization between these two species. The isoenzyme patterns for these strains are presented in Figures 7.1 to 7.7 and they will be discussed separately.

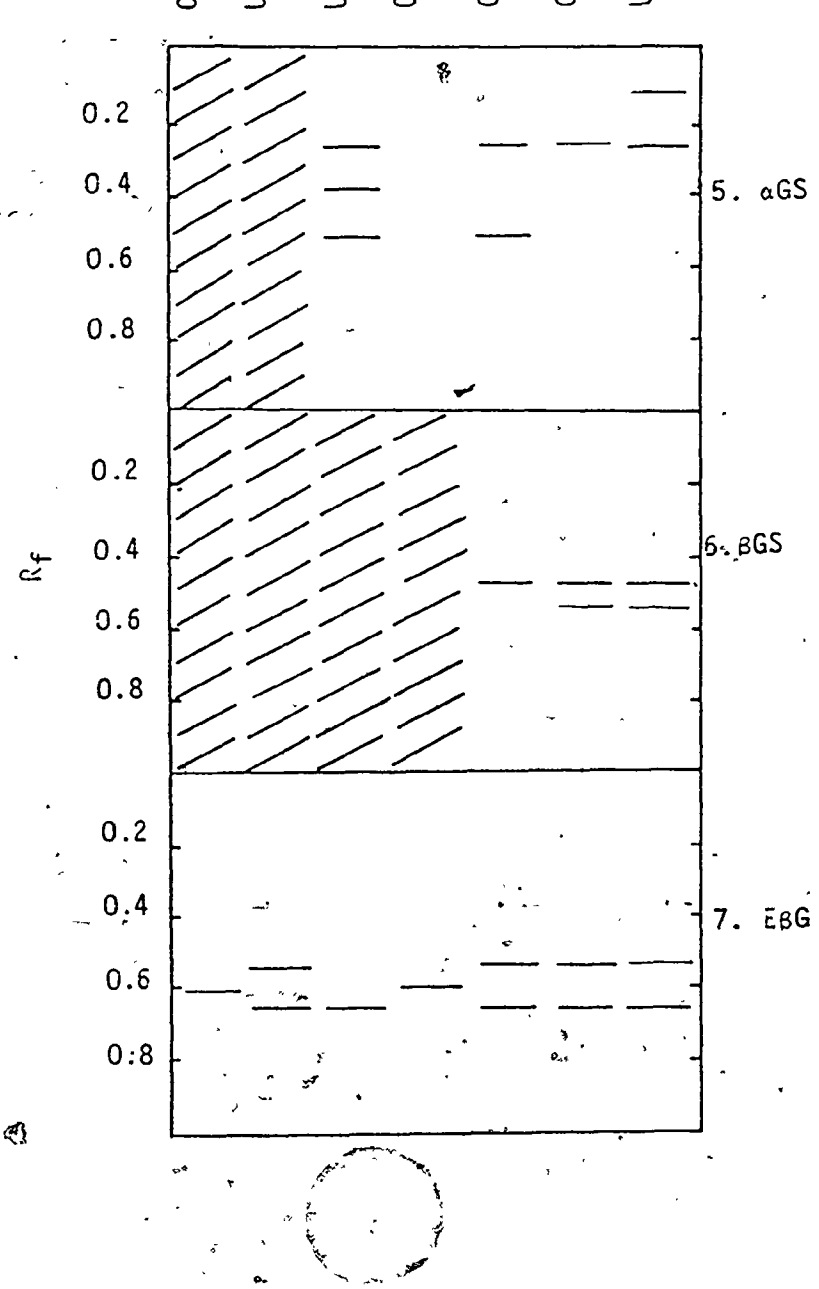
Alcohol dehydrogenase (Fig. 7.1) was of limited value in exploring hybrid nature. Large areas of very intense staining made it very difficult to be certain if all changes were recorded. There were two new bands present in the hybrid CBS 6925 not found in any of the parental strains. The fast band of CBS 6923 and the single slow band of CBS 6924 were missing in the hybrid. A mixture of extracts of the two parental strains gave a five band pattern similar to that of CBS 6923 but was missing the slow band of CBS 6924. The hybrid and its corresponding mixture of extracts of parental strains had different patterns indicating *in vivo* change(s) had occurred. The mixture of the type cultures yielded the expected two band pattern.

Malate dehydrogenase (Fig. 7.2) should have been a good indicator of the hybrid nature because the parental strains had no bands in common. However, of the hybrid's three bands, one was present in CBS 6923 and two were new but remarkably similar to the bands in UCD 55-82. The hybrid was missing the single band found in CBS 6924. The mixture of extracts of the two parental strains contained both parental bands, whereas the mixture of extracts of the type cultures had the pattern of UCD 55-82 and was missing the two slow bands of UCD 55-41. The hybrid and its corresponding mixture of parental strains had different patterns indicating

Figure 7(1-7). Electrophoretic patterns for CBS 6925 and corresponding strains and mixtures of strains

See Chapter 2 for a description of the conditions used. Cross hatched areas indicate that no sample was electrophoresed. Abbreviations:ADH = alcohol dehydrogenase, MDH = malate dehydrogenase, SOD = superoxide dismutase, EST = esterase, αGS = α-glucosidase, βGS = β-glucosidase, EβG = exo-β-glucanase, + = mixtures of extracts from the strains indicated.


CBS 6923 + CBS 6924
UCD 55-82 + UCD 55-41
UCD 55-41 (K. thermotolerans)
CBS 6924 (K. thermotolerans)
CBS 6925 (Hybrid)
CBS 6923 (K. marxianus)
UCD 55-82 (K. marxianus)
R_f
0.2
0.4
0.6
0.8
1. ADH
2. MDH
3. SOD
4. EST


CBS 6923 + CBS 6924
UCD 55-82 + UCD 55-41
UCD 55-41 (K. thermotolerans)
CBS 6924 (K. thermotolerans)
CBS 6925 (Hybrid)
CBS 6923 (K. marxianus)
UCD 55-82 (K. marxianus)
Rf
0.2
0.4
0.6
0.8
5. αGS
6. βGS
7. ĒβG

in vivo change(s) had taken place.

Superoxide dismutase (Fig. 7.3) was a good indicator of the hybrid's nature despite the fact that the hybrid only had three bands where 5 were expected. This was the only instance where the hybrid had one band from each of the parental strains, indicating a crossing had occurred. *In vivo* changes resulted in the loss of bands. The mixture of extracts of the two parental strains had all 5 parental bands, whereas the mixture of extracts of the type strains was missing two bands, one from each constituent. A large area of superoxide dismutase activity was located at the front of these gels. Most certainly, different patterns of activity would be obtained if different gel concentrations were used.

Esterase (Fig. 7.4), the most variable enzyme, had the potential to demonstrate hybrid nature as both parental strains had only two bands in common. There were six new bands, but four bands present in the parental strains were missing from the hybrid. The mixture of the extracts of the two parental strains contained all of the bands present in the parental strains. The hybrid and the mixture of parental strains had different banding patterns, indicating some sort of *in vivo* change(s) in the isozymes had occurred. Except for the fast band of UCD 55-82, the mixture of the extracts of the the type strains contained all of their corresponding bands.

α-Glucosidase (Fig. 7.5) revealed only limited information about the hybrid, since one of the parental strains, CBS 6924 had no bands. This was unexpected, in view of the strong growth of this strain on α-glucosides and of the pattern produced by the type strain of *K. thermotolerans*. The presence of α-glucosidase activity in glucose grown extracts of *K. marxianus* (CBS 6923) was not totally unexpected, in view of the results obtained by Fiol (1975). The lack of growth on a certain glycoside is often due to alterations in permease activity, and does not preclude the existance of appropriate intracellular glycosidases. The fast band observed in the hybrid exhibited activity on both maltose and α-methyl glucoside, while the slow band, like that of the parent strain, only had trehalase activity. Mixtures of extracts from strains grown on α-glucosides could not be made,therefore

mixtures were not examined.

Similarily, β-glucosidase (Fig. 7.6) did not reveal useful information about the hybrid. *K. thermotolerans* strains do not grow on arbutin, consequently strains CBS 6924 and UCD 55-41 could not be assessed for this enzyme. The hybrid did not exhibit the fast band present in CBS 6923 indicating that *in vivo* change(s) had taken place.

Exo-β-glucanase (Fig. 7.7) was a difficult enzyme to interpret. The band of CBS 6924 appeared to be intermediate between the double bands of CBS 6923, but the hybrid had an electrophoretic pattern typical of *K. marxianus*. The mixture of parental extracts gave a one band pattern similar to that of CBS 6924. The single band of UCD 55-41 was in the same position as the fast band of UCD 55-82. A mixture of the two type strains gave a two band pattern like that of UCD 55-82.

In total (Table 8), CBS 6923 (*K. marxianus*) shared 15 bands with the hybrid and CBS 6924 (*K. thermotolerans*) shared 4. The parental strains had 4 bands in common. Although the hybrid had 11 unique bands, it lacked 6 bands present in CBS 6923 and 5 bands present in CBS 6924. It is extremely interesting that just as many bands were absent from the hybrid as unique bands were gained by the hybrid. Based on the electrophoretic patterns obtained, crossing between CBS 6924 and CBS 6923 could not receive definite substantiation. Indeed, only one of the parental bands found in the hybrid apparently originated from CBS 6924. The possibility that this single case may be due to an *in vivo* change cannot be discounted, considering how frequently such changes take place. These results are compatible with the physiological responses determined by replica plating (Table 9). Although the hybrid seemed to have gained α-glucosidase activities from *K. thermolerans* (CBS 6924), the other activities appeared to originate from *K. marxianus* (CBS 6923). In fact, based on Figure 7.5 and on what is known of intracellular glycosidases (Fiol, 1975), it is conceivable that even the α-glycosidase activity of the hybrid actually originated from *K. marxianus*. The hybrid, in this case, would only be altered in some permeability factors. This is not to say that the responses were strictly additive in the cross.

Table 8. Summary of the number of shared, unique and absent bands in CBS 6925 and its corresponding parental strains.

	Number of bands					
Enzyme	CBS 6923 shared CBS 6925	CBS 6924 shared CBS 6925	CBS 6923 shared CBS 6924	unique to CBS 6925	present CBS 6923 absent CBS 6925	present CBS 6924 absent CBS 6925
ADH	4	1	1	2	1	1
MDH	1	0	0	2	0	1
SOD	2	2	1	0	2	0
EST	4	1	2	6	2	2
αGS	1	0	0	1	0	0
βGS	1	0	0	0	1	0
EβG	2	0	0	0	0	1
Total	15	4	4	11	6	5

Table 9. Physiological reponses that differ between the hybrid CBS 6925 and its corresponding parental strains.

Character[1]	CBS 6923	CBS 6924	CBS 6925
Inulin	+++	-	+++
Lactose	+++	-	+++
Trehalose	-	+++	++
Maltose	-	++	++
Melizitose	-	+++	++
α-Methyl glucoside	-	+++	+++
Cellobiose	++	-	++
Salicin	+++	-	++
Sorbose	S	-	-
Xylose	+++	-	S
Arabinose	+++	-	+++
Ribose	++	-	-
Xylitol	+++	W	++
Mannitol	+++	+++	W
Glucitol	+++	+++	S
Lactate	+++	-	+++
Succinate	+++	-	+++
Citrate	W	-	W
Malate	W	-	-
Gluconolactone	-	W	-
4°C	S	-	W
37°C	+++	-	+++
Acid Production	W	-	W
Cycloheximide (100 ppm)	+++	-	+++
Glucose 50%	-	+++	-
Pulcherimin	-	-	+

[1] Physiological responses were determined by replica plating following the standard methods of van der Walt (1970).

S = slight

W = weak

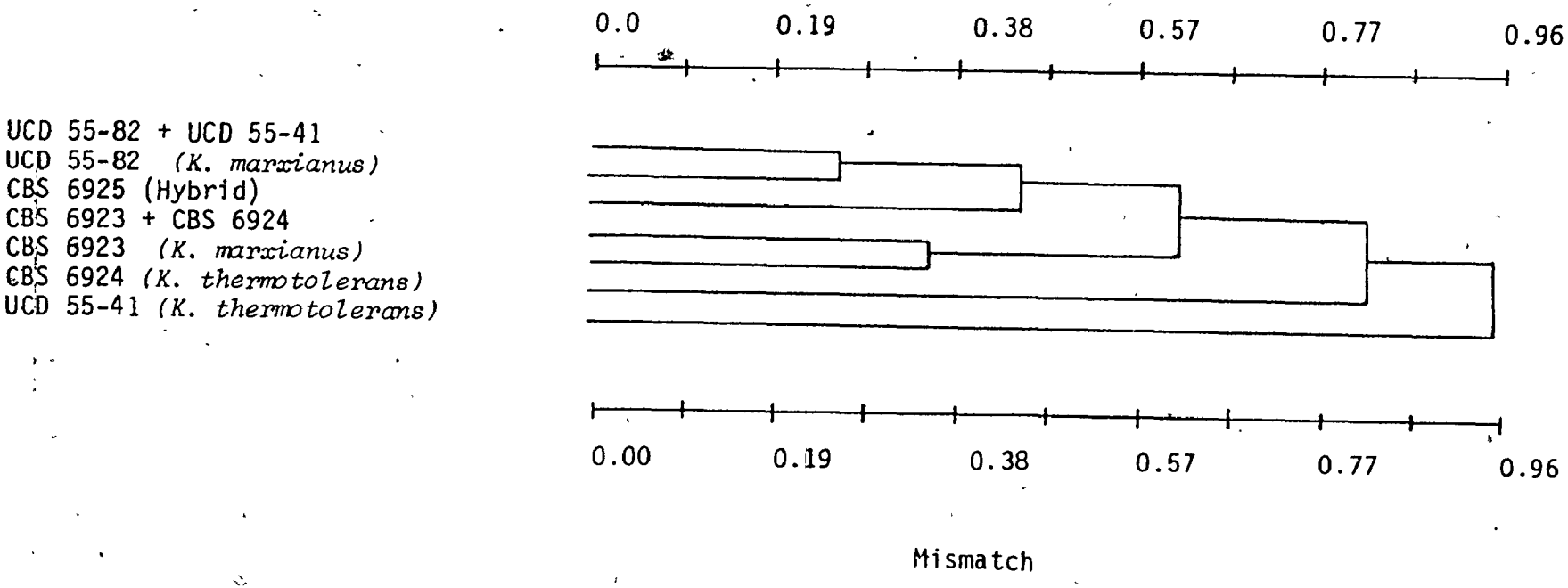
The loss or reduction, by the hybrid, of responses found in the parental strains (eg. 50% glucose), or the gain of new responses (eg. pulcherrimin production) by the hybrid could be indicative of *in vivo* change(s).

Based on the dissimilarity coefficients (Fig. 8), the mixtures of strains most closely resembled their constituent *K. marxianus* component. The hybrid was more similar to the type strain of *K. marxianus* and its mixture than to the *K. marxianus* parental strain and its mixture. The *K. thermotolerans* strains were quite removed from the other strains and mixtures. The DNA composition results (Martini *et al.* (1972); Presley unpublished data *ex.* Phaff, 1984), were similar to electrophoretic and physiological data in that the F1 generation of a cross of these two strains was found to be more similar to *K. marxianus*. Kurtzman *et al.* (1983) suggested that the F1 generation isolated from mixtures of *K. marxianus* and *K. thermotolerans* represented an aneuploid yeast strain, in which case, a random gain and elimination of bands is to be expected. Molecular causes of altered banding patterns such as limited genome expression, gene dosage effects, monomeric isoenzymes, and multimer incompatibility should not be discounted.

This analysis gives some clue as to what may happen in the wild if hybridization were to occur. If a hybrid such as this were to arise in populations such as those described in Chapter 4, the result might be the introduction of some genes from *K. thermotolerans* into the *K. marxianus* population. The variability of the *K. marxianus* population would be increased without any tendency for an amalgamation of *K. marxianus* and *K. thermotolerans* into a single highly variable species. This kind of occurrence might be labeled introgresive hybridization which is defined as the process "wherein two species maintain their integrity while continuously exchanging genes" (Anderson and Hubricht, 1938). It can only be speculated that if this sort of hybridization is occurring, natural selection against it is sufficient to negate its effect.

Figure 8. Dendrogram of electrophoretic dissimilarities of hybrid CBS 6925 and its corresponding strains and mixtures

The algorithm Loside was used to perform weighted average linkage clustering based on the mismatch dissimilarity measurement.


0.0
0.19
0.38
0.57
0.77
0.96
UCD 55-82 + UCD 55-41
UCD 55-82 (K. marxianus)
CBS 6925 (Hybrid)
CBS 6923 + CBS 6924
CBS 6923 (K. marxianus)
CBS 6924 (K. thermotolerans)
UCD 55-41 (K. thermotolerans)
0.00
0.19
0.38
0.57
0.77
0.96
Mismatch

5.2.2 Intraspecific Hybrids Involving *K. lactis* x *K. vanudenii*

As anticipated from the very similar electrophoretic patterns of *K. lactis* and *K. vanudenii* type and natural strains, it was more difficult to detect hybridization between these strains. The isoenzyme patterns for these strains are presented in Figures 9.1 to 9.7. Each isozyme will be discussed separately.

Alcohol dehydrogenase (Fig. 9.1) was not useful in demonstrating hybrid nature. The only band present in the hybrid was present in their corresponding parent strains. The fast band present in 80SM5-6 was absent from all of the hybrids.

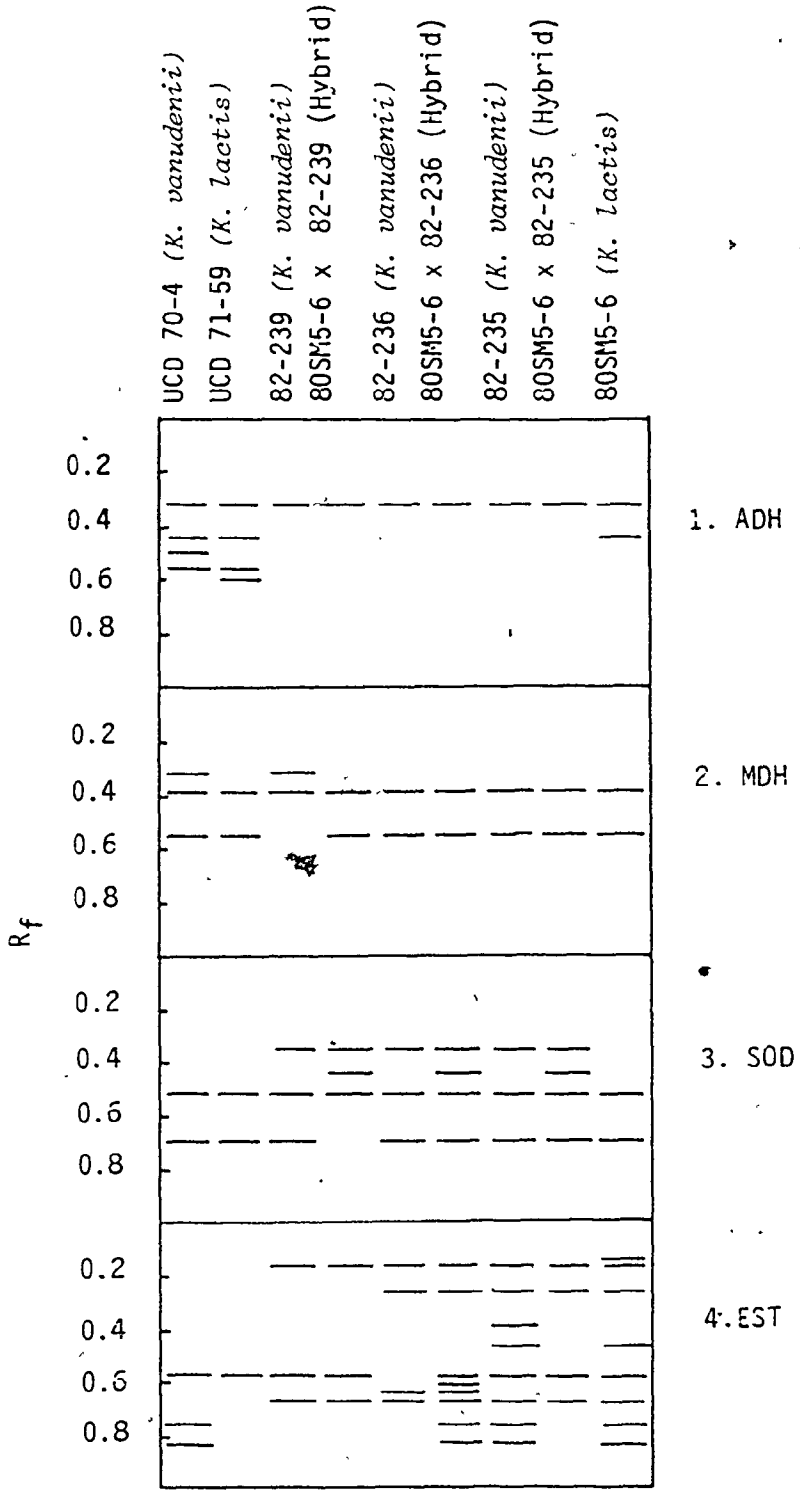
Malate dehydrogenase (Fig. 9.2) did not reveal any information about the hybrids 80SM5-6 x 82-235 and 80SM5-6 x 82-236. All of the parental strains had all bands in common and no new bands were present in the hybrids. The hybrid 80SM5-6 x 82-239 was missing the slow band of 82-239 and the fast band common to 80SM5-6 and 82-239.

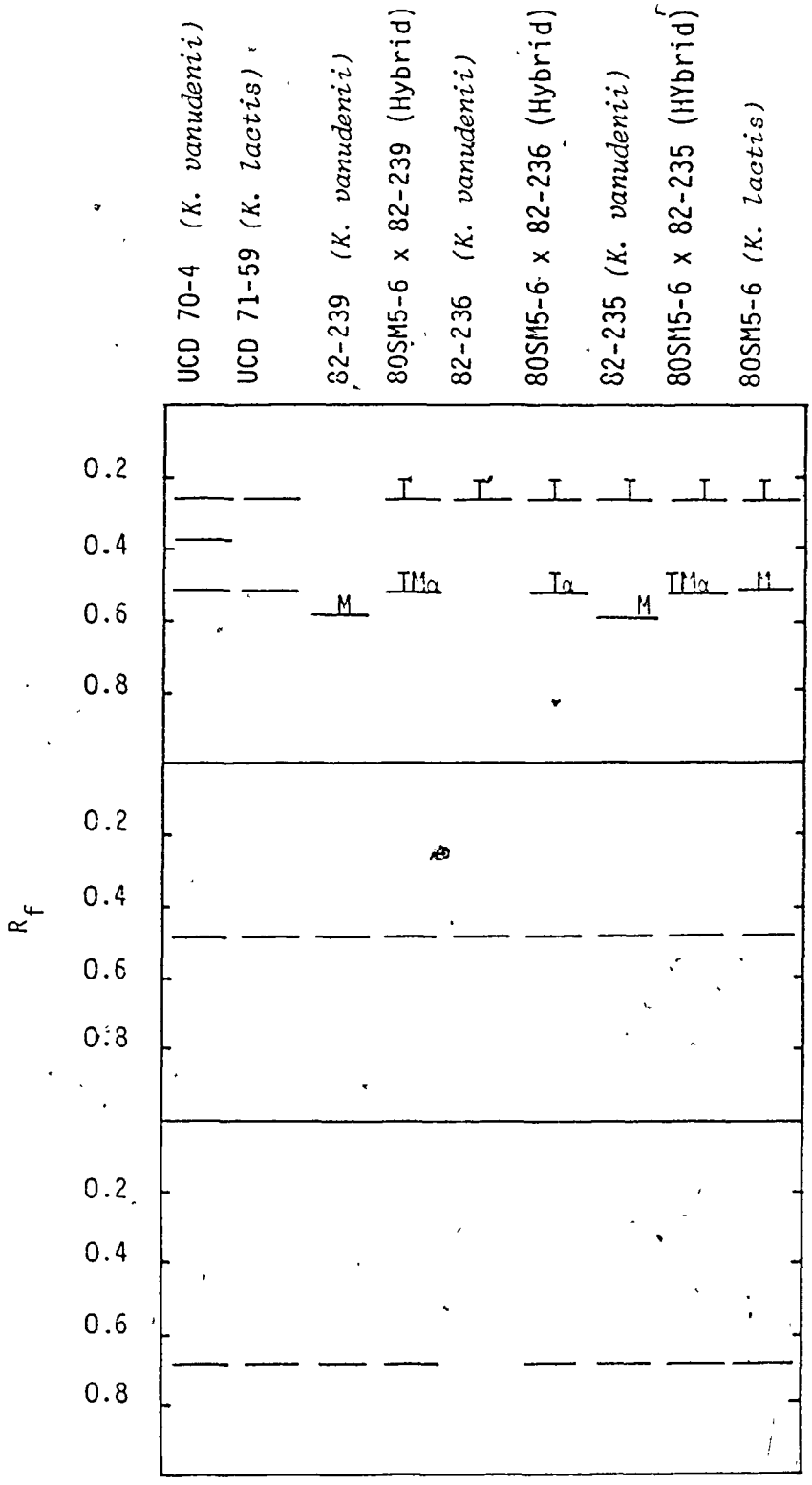
Superoxide dismutase (Fig. 9.3) was a useful enzyme as all of the hybrids had a unique band. 80SM5-6 x 82-239 was different from the other hybrids in that it was missing the fast band common to both parental strains. One of the few differences between the *K. lactis* and *K. vanudenii* parental strains was the slow band found in the *K. vanudenii* strains.

Esterase (Fig. 9.4) demonstrated many differences among the three hybrids and their corresponding parental strains. It was expected that since esterase was the most variable enzyme it would be the most likely to reveal intraspecific differences. All but two of the bands common to both parental strains were found in the 80SM5-6 x 82-235 hybrid. The unique bands found in each of the parental strains were missing from the hybrid. Although this is not direct evidence for crossing, it is evidence for *in vivo* change(s). Hybrid 80SM5-6 x 82-236 exhibited direct evidence of crossing because unique bands from the respective parental strains were present in the hybrid. The hybrid was also missing some bands from 80SM5-6, but it had one new band indicating *in vivo* change(s) had occurred here as well. There was no evidence

Figure 9(1-7). Electrophoretic patterns for *K. vanudenii* x *K. lactis* hybrids, and related strains

Details of the conditions under which each enzyme was studied are presented in Chapter 2. Abbreviations: ADH = alcohol dehydrogeanse, MDH = malate dehydrogenase, SOD = superoxide dismutase, Est = esterase, αGS = α-glucosidase, βGS = β-glucosidase, EβG = exo-β-glucanase, α = α-methyl glucosidase, M = maltase, T = trehalase


UCD 70-4 (K. vanudenii)
UCD 71-59 (K. lactis)
82-239 (K. vanudenii)
80SM5-6 x 82-239 (Hybrid)
82-236 (K. vanudenii)
80SM5-6 x 82-236 (Hybrid)
82-235 (K. vanudenii)
80SM5-6 x 82-235 (Hybrid)
80SM5-6 (K. lactis)
R_f
0.2
0.4
0.6
0.8
1. ADH
2. MDH
3. SOD
4. EST


UCD 70-4 (K. vanudenii)
UCD 71-59 (K. lactis)
82-239 (K. vanudenii)
80SM5-6 x 82-239 (Hybrid)
82-236 (K. vanudenii)
80SM5-6 x 82-236 (Hybrid)
82-235 (K. vanudenii)
80SM5-6 x 82-235 (Hybrid)
80SM5-6 (K. lactis)
R_f
0.2
0.4
0.6
0.8
I
M
TMα
Iα
5. αGS
6. βGS
7. EβG

of crossing in the 80SM5-6 x 82-239 hybrid as only bands common to both parental strains were present. Many bands found in 80SM5-6 were missing from the hybrid, indicating *in vivo* change(s) had occurred.

Although the α-glucosidase (Fig. 9.5) system appeared to be more complex than originally anticipated, it did demonstrate hybrid nature in a number of ways. A discussion of this enzyme according to the three substrates investigated follows.

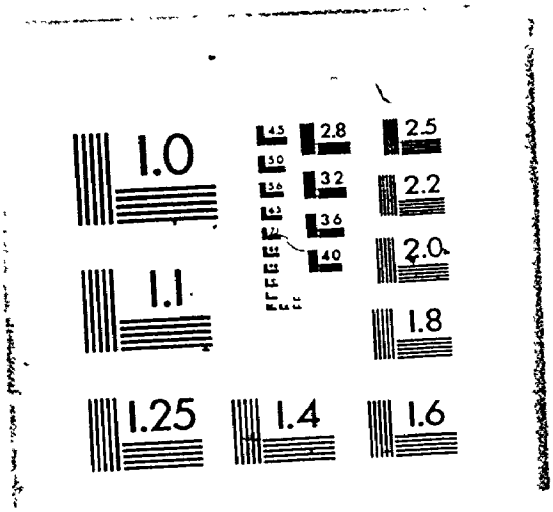
The pattern of trehalase activities was different in parental strains and the hybrids. While the hybrids exhibited trehalase activity both as 0.26 (low activity) and at 0.52 (high activity), only a slow band of activity (Rf = 0.26) was present in all the parental strains except 82-239.

Parental and hybrid strains also differed in maltase patterns. Maltase activity was present at 0.58 (fast band) in 82-235 and 82-239, but not in 82-236. The hybrids had a much larger, darkly stained band, with a slightly higher Rf value than its counterpart in 80SM5-6. Relatively speaking, hybrid 80SM5-6 x 82-236 was not as intensely stained as the other two hybrids. This may have been due to its parental strain 82-236 having no activity.

A difference in parental and hybrid strains was also indicated by only the hybrids having α-methyl glucosidase activity (Rf = 0.52). Except for staining less intensely, this electromorph appeared the same as when stained with the other two substrates.

When maltose was combined with trehalose or with α-methyl glucoside, for staining, the patterns appeared to contain all of the bands that were present when the substrates were added separately, except that 82-235 had no activity at Rf = 0.58. However, when trehalose and α-methyl glucoside were combined, the α-methyl glucoside had an inhibitory effect.

Neither β-glucosidase (Fig. 9.6) nor exo-β-glucanase (Fig. 9.7) were useful in assessing hybrid nature because the only band present was common to all strains. Strain 82-236 failed to give glucanase activity.



In total (Table 10), 80SM5-6 shared 12 bands with the hybrid and 82-235 shared 13. The parental strains had 15 bands in common. Although the hybrids had 2 unique bands, 6 bands present in 80SM5-6 and 5 bands present in 82-235 were absent from the hybrid.

Table 11 contains the cumulative data for the 80SM5-6 x 82-236 hybrid. 80SM5-6 shared 14 bands with the hybrid and 82-236 shared 12. The parental strains had 10 bands in common. There were 3 unique hybrid bands and 4 bands present in 80 sm5-6 absent from the hybrid.

In total (Table 12), ten bands were shared by 80SM5-6 and hybrid 80SM5-6 x 82-239. Strain 82-239 and the hybrid shared 9 bands and 9 bands were common to both parental strains. The hybrid had 2 unique bands, while 8 bands present in 80SM5-6 and 3 bands present in 82-239 were absent in the hybrid.

Although the *K. lactis* and *K. vanudenii* parental strains used in this study had very similar isoenzyme patterns, the three intraspecific hybrids obtained from them could be identified by this technique. Based on the dissimilarity coefficient (Fig. 10) 80SM5-6 x 82-239 and 80SM5-6 x 82-235 were almost identical. 80SM5-6 x 82-236 was slightly different from the other hybrids, especially in esterase activity. 80SM5-6 (*K. lactis*) most closely resembled 82-235 (*K. vanudenii*).

From the above results, it can be speculated that if these hybrids were to occur in nature, they might form the beginnings of a hybrid swarm or cline. Until putative wild hybrids are isolated, there is no way to determine their chance of survival under different conditions.

5.3 Conclusions

The debate over the taxonomic status of populations which hybridize with each other but do not genetically amalgamate is ongoing (Barton and Hewitt, 1983). Some researchers (*i.e.* Mayr and Dobzhansky) have come to the conclusion that total reproductive isolation cannot be used as the criterion to define species because incidental hybridization occurs between species

Table 10. Summary of the number of shared, unique and absent bands in 80SM5-6 x 82-235 and its corresponding parental strains.

	Number of bands					
Enzyme	80SM5-6 shared 80SM5-6x 82-235	82-235 shared 80SM5-6x 82-235	80sm5-6 shared 82-235	unique to 80SM5-6x 82-235	present 80SM5-6 absent 80SM5-6x 82-235	present 82-235 absent 80SM5-6x 82-235
ADH	1	1	1	0	1	0
MDH	2	2	2	0	0	0
SOD	2	3	2	1	0	0
EST	4	4	7	0	4	4
αGS	1*	1	1	1*	1*	1*
βGS	1	1	1	0	0	0
EβG	1	1	1	0	0	0
Total	12	13	15	2	6	5

* See text for explanation.

Table 11. Summary of the number of shared, unique and absent bands in 80SM5-6 x 82-236 and its corresponding parental strains.

	Number of bands					
Enzyme	80SM5-6 shared 80SM5-6x 82-236	82-236 shared 80SM5-6x 82-236	80sm5-6 shared 82-236	unique to 80SM5-6x 82-236	present 80SM5-6 absent 80SM5-6x 82-236	present 82-236 absent 80SM5-6x 82-236
ADH	1	1	1	0	1	0
MDH	2	2	2	0	0	0
SOD	2	3	2	1	0	0
EST	6	4	3	1	2	0
αGS	1*	1	1	1*	1*	0
βGS	1	1	1	0	0	0
EβG	1	0	0	0	0	0
Total	14	12	10	3	4	0

* See text for explanation.

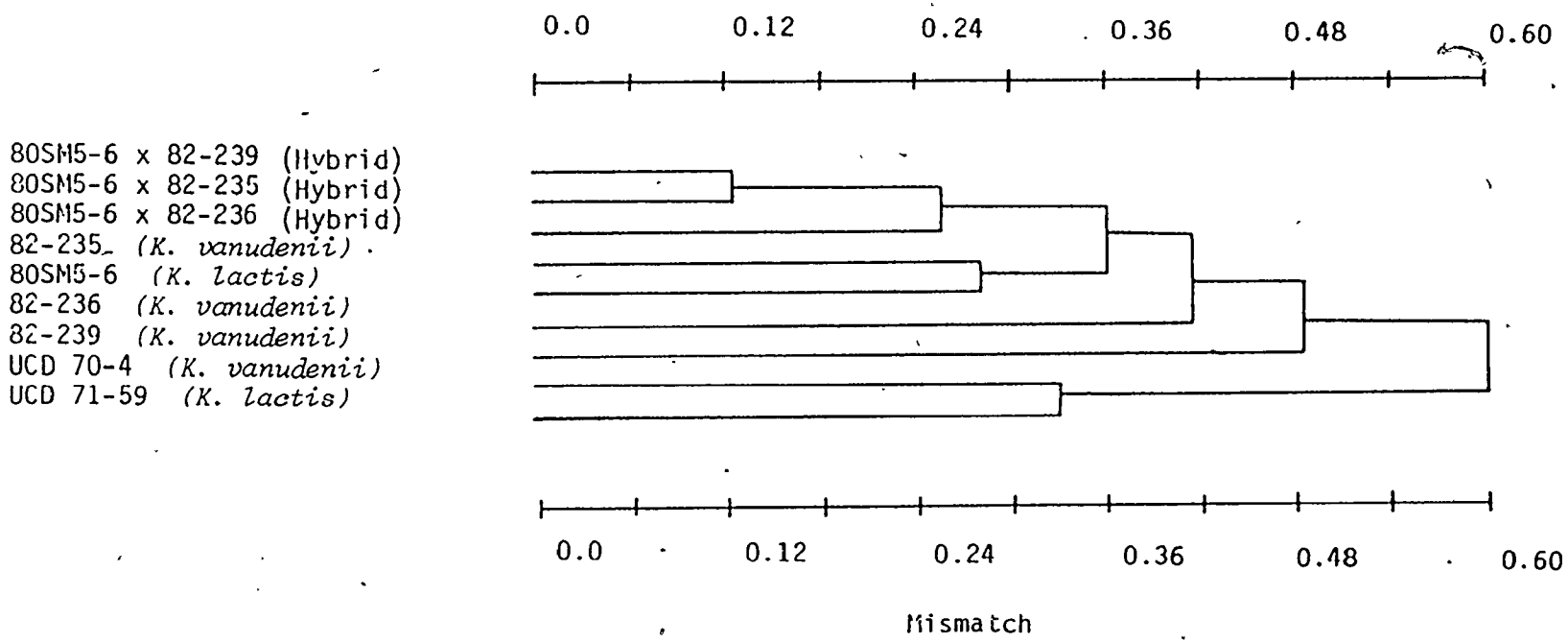
Table 12. Summary of the number of shared, unique and absent bands in 80SM5-6 x 82-239 and its corresponding parental strains.

	Number of bands					
Enzyme	80SM5-6 shared 80SM5-6x 82-239	82-239 shared 80SM5-6x 82-239	80sm5-6 shared 82-239	unique to 80SM5-6x 82-239	present 80SM5-6 absent 80SM5-6x 82-239	present 82-239 absent 80SM5-6x 82-239
ADH	1	1	1	0	1	0
MDH	2	1	1	0	0	1
SOD	1	2	2	1	1	1
EST	3	3	3	0	5	0
αGS	1*	0	0	1*	1*	1*
βGS	1	1	1	0	0	0
EβG	1	1	1	0	0	0
Total	10	9	9	2	8	3

* See text for explanation.

Figure 10. Phenogram of electrophoretic dissimilarities of the three *K. lactis* x *K. vanudenii* hybrids and their correponding strains.

The algorithm Loside was used to perform weighted average linkage clustering based on the mismatch dissimilarity measurement.


0.0
0.12
0.24
0.36
0.48
0.60
80SM5-6 x 82-239 (Hybrid)
80SM5-6 x 82-235 (Hybrid)
80SM5-6 x 82-236 (Hybrid)
82-235 (K. vanudenii)
80SM5-6 (K. lactis)
82-236 (K. vanudenii)
82-239 (K. vanudenii)
UCD 70-4 (K. vanudenii)
UCD 71-59 (K. lactis)
0.0
0.12
0.24
0.36
0.48
0.60
Mismatch

that normally maintain discrete gene pools.

K. marxianus and *K. thermotolerans* are good examples of *Kluyveromyces* taxa with low relatedness. They have significantly different nuclear DNA base composition (Martini *et al.*, 1972) and virtually no DNA homology (Presley unpublished data *ex.* Phaff, 1984). Owing to their low mating compatibility, they were retained as separate species by Johannsen (1980) and van der Walt and Johannsen (1984). To date, no putitive natural hybrids have been isolated. Furthermore, the electrophoretic data (Chapter 4) support the view that *K. marxianus* and *K. thermotolerans* do not share a common gene pool and, thus taxonomically they should be retained as separate species.

The electrophoretic data of this chapter support the view held by Kurtzman *et al.* (1983) that this hybrid may be an aneuploid. The electrophoretic patterns further suggest that if mating were taking place in the wild between *K. marxianus* and *K. thermotolerans*, the hybrids might be found in or close to the *K. marxianus* group, and not as intermediates between the two groups, or as members of the *K. thermotolerans* group.

K. lactis and *K. vanudenii* are examples of *Kluyveromyces* taxa with high relatedness. Their nuclear DNA base composition values are not significantly different (Martini *et al.*, 1972) and they have a high degree of DNA-DNA homology (Martini, 1973). Under forced matings, they have high compatibility values (Johannsen, 1980).

Perhaps the lack of recovery of strains which appear to be fertile hybrids from nature may be attributed to habitat specificity. *K. lactis* and *K. vanudenii* do not seem to occur in the same microhabitat. All *K. lactis* strains of this survey and others were isolated from milk products, whereas *K. vanudenii* have been isolated from Black Knots, *Drosophila*, breweries and wineries. The electrophoretic data of this chapter suggested that if effective interfertility is operative between these two taxa under natural conditions, a hybrid swarm or cline might be formed in the *K. lactis* group. The data in Chapter 4 tended to substantiate the occurence of random mating within this group.

In summation, the technique of isoenzyme electrophoresis is capable of detecting differences between both intraspecific and interspecific hybrids and their corresponding parental strains. The isoenzyme patterns obtained were complex and indicated that further studies of purified enzymes are required in order to understand the mechanism(s) which regulates the synthesis of hybrid isoenzymes in these strains. Finally, the integration of laboratory and natural field studies is essential for the comprehensive understanding of species delineation in the genus *Kluyveromyces*. The technique of isoenzyme electrophoresis is a tool which can help elucidate the relationship between results obtained in laboratory investigations and what could be taking place under natural field conditions.

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