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Distinctive electrophoretic isoenzyme profiles in *Saccharomyces sensu stricto*

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Genetic variation among 35 strains representing the four currently recognized species of *Saccharomyces sensu stricto* (*Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*/*carlsbergensis* and *Saccharomyces paradoxus*) was estimated by analysing the electrophoretic mobilities of nonspecific esterases, acid phosphatase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase isoenzymes. Twenty-two electrophoretic types were identified, a result in agreement with the phenotypic and genetic polymorphisms reported for this group of yeasts. However, the four species were clearly distinguishable based on the patterns obtained using three of the enzymes assayed, the resolving power not being improved by the introduction of data correspondent to lactate dehydrogenase. The overall diversity was higher among *S. cerevisiae* isolates, in contrast with *S. paradoxus* which showed only two patterns, one of which was common to four of the five strains studied. Concordant results from the application of the method and DNA hybridization experiments demonstrate its value for identification purposes.

Keywords: *Saccharomyces*, isoenzyme profiling, yeasts

INTRODUCTION

Common phenotypic criteria used in yeast identification often give unsatisfactory results. An illustrative example is provided by the group of species designated as *Saccharomyces sensu stricto*, which includes the most important strains in yeast based industries, such as baking, brewing and wine making. Most of the early controversy associated with a practical, though inconsistent, definition of species belonging to this group was centred on the ability to ferment and assimilate particular carbohydrates. The determination of the extent of DNA relatedness shed some light on the taxonomic relationships within the complex and revealed the four currently recognized species *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces pastorianus* and *Saccharomyces paradoxus* (Vaughan Martini & Martini, 1989).

Potentially useful typing methods have been applied to elucidate taxonomic and evolutionary relationships among species of this yeast group, including the study of serological properties (Campbell, 1972; Fukazawa *et al*., 1980), cellular fatty acid analysis (Augustyn *et al*., 1991), electrophoretic karyotyping (Naumov *et al*., 1992; Vaughan Martini *et al*., 1993; Tornai-Lehoczki & Dlauchy, 1996), restriction analysis of the mitochondrial DNA (Guillamón *et al*., 1994), sequencing of regions of the ribosomal nucleic acids (Kurtzman & Robnett, 1991; Molina *et al*., 1992; James *et al*., 1997; Montrocher *et al*., 1998) and PCR-based methods for the detection of different genetic polymorphisms (de Barros Lopes *et al*., 1998). Moreover, the assessment of physiological and biochemical differential traits in *Saccharomyces sensu stricto* has been extended to non-conventional characteristics, such as the presence/absence of active fructose transport and the ability to grow below/above 35 °C which separate the pair *S. bayanus*/*S. pastorianus* from *S. cerevisiae*/*S. paradoxus* (Rodrigues de Sousa *et al*., 1995). However, no user-friendly sensitive and reliable method was described that would allow the rapid identification of isolates while avoiding more sophisticated equipment and laborious procedures.

Electrophoretic enzyme polymorphism enables systematic and evolutionary genetic analysis and has been widely applied as a standard method both in the

Abbreviation: ET, electrophoretic type.

Table 1. Strains studied and their sources of isolation

Abbreviations: IGC, Instituto Gulbenkian de Ciência, former location of Portuguese Yeast Culture Collection (PYCC), now at New University of Lisbon, Monte de Caparica, Portugal; CBS, Centraalbureau voor Schimmelcultures, Delft, Netherlands; DBVPG, Dipartimento di Biologia Vegetale, Università di Perugia, Italy. T, type strain; NT, neotype.

Species	Strain		Isolation source
	IGC no.	Other collection nos	
S. cerevisiae			
	4455^{NT}	CBS 1171	Beer (top yeast)
	2608	CBS 1782	Superattenuated beer
	2917	CBS 429	Fermenting grape juice
	3507		Human vaginitis
	3977	CBS 1190	
	4072		Commercial wine yeast
	4240		Wine, Portugal
	4543		Fermenting grape must, Austria
	4891		Olive brine
	5053		Fermenting grape must, Portugal
	5054		Fermenting grape must, Portugal
	5055		Fermenting grape must, Portugal
	5056		Fermenting grape must, Portugal
	5057		Fermenting grape must, Portugal
	5466*†		Commercial wine yeast
	5318*		Domestic bread leaven, Portugal
	5319*		Domestic bread leaven, Portugal
	5320*		Domestic bread leaven, Portugal
	5325*		Commercial baker's yeast
	5326*		Domestic bread leaven, Portugal
S. pastorianus			
	4601^{NT}	DBVPG 6047, CBS 1538	Beer
	4261		Brewer's yeast, South Africa
	4457	CBS 1513	Beer (bottom yeast)
	4579	CBS 1260	Unknown
	4580	CBS 1486	Beer (bottom yeast)
S. bayanus			
	4456^{T}	CBS 380	Turbid beer
	4565	CBS 378	Beer
	4567	CBS 395	Currant juice
	4568	CBS 424	Pear juice
	4569	CBS 425	Fermenting apple juice
S. paradoxus			
	4570^{NT}	CBS 432	Tree exudate
	4576	CBS 406	Oak exudate
	4577	CBS 2980	*Drosophila* sp.
	4578	CBS 5829	Soil
	4656	DBVPG 6489	Tree exudate

* Strains identified only by conventional phenotypic methods (van der Walt & Yarrow, 1984).

† Strain commercialized as *S. bayanus*.

taxonomy of prokaryotes and eukaryotes (Selander *et al.*, 1986). In yeasts, a few studies evaluate the usefulness of this approach as a taxonomic tool (e.g. Baptist & Kurtzman, 1976; Sidenberg & Lachance, 1986) and to type clinical isolates of pathogenic species (Brandt *et al.*, 1993; Doebbeling *et al.*, 1993; Lehmann *et al.*, 1989). The *Saccharomyces sensu stricto* group also received some attention, but either because only reference strains were used or because the identification of the strains was not authenticated by whole-

genome comparisons, the conclusions were not sound and a clear separation of species was not achieved (Yamazaki *et al*., 1983; Lewicka *et al*., 1995).

The purpose of the present work was to assess genetic variation among collection strains and new isolates belonging to species of the *Saccharomyces sensu stricto* group by the electrophoretic analysis of selected isoenzymes, and to further evaluate the usefulness of the technique for a rapid and sensitive identification of strains from this industrially important group of yeasts.

METHODS

Yeast strains and growth conditions. The yeast strains used in this study are listed in Table 1. Most of the strains tested were previously identified by DNA–DNA reassociation studies (Rodrigues de Sousa *et al*., 1995) and were obtained from the Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Portugal. Stock cultures were maintained on yeast extract-peptone-glucose-agar (YEPG) at 4 °C. Yeasts were grown in a medium containing 0·5 % (w/v) Bacto-yeast extract, 1 % (w/v) Bacto-peptone and 2 % (w/v) glucose on a rotary shaker (170 r.p.m.), at 25 °C. When grown up to late-exponential phase (OD_{640} = 10–12), cells were harvested by centrifugation (5000 ***g***, 4 °C for 7 min), washed twice with 20 ml buffer I (3·2 mM Tris/HCl pH 7) and stored at −20 °C until further utilization.

Preparation of extracts for electrophoresis. For protein extraction, cells were disrupted with 1 g 0·5 mm diameter glass beads and 1 ml buffer II (60 mM Tris/HCl pH 6·8) by vortexing 1min and cooling the tube on ice for another minute. This operation was repeated at least eight times, until more than 70 % of the cells were disrupted. Insoluble debris and undisrupted cells were removed by centrifugation at 15000 ***g***, 0 °C for 30 min. Protein extracts were stored at −20 °C.

Electrophoresis and detection of enzyme activity. Four enzymes, esterase (EST, EC 3.1.1.1), acid phosphatase (ACP, EC 3.1.3.2), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and lactate dehydrogenase (LDH, EC 1.1.1.27), were assayed. Glycerol (10 % v/v) and bromophenol blue (0·001 %, w/v) were added to protein samples. Electrophoresis was run on a 0·75 mm thick, 7·5 % (w/v) (for ACP, G6PD and LDH and 8 %, w/v, for EST) non-denaturating polyacrylamide gel, overlaid with 3·75 % w/v polyacrylamide stacking gel (Hames, 1981), using the Mighty Small II apparatus from Hoefer/Pharmacia. Electrophoresis was carried out at 5 °C with a constant initial voltage of 100 V for 30 min, followed by 150 V until the tracking dye (bromophenol blue) reached the bottom of the gel (approx. 1·5 h). Staining procedures were similar to those previously described by Pais (1990), for EST and G6PD, by Eiras-Dias (1994) for ACP, and by Shaw & Prasad (1970) for LDH.

Analysis of data. The relative electrophoretic mobilities (R_M) of the enzyme bands were calculated as the ratio of the migration of each band to that of the tracking dye and adjusted by comparison with the values obtained for *S. cerevisiae* IGC 4072 (IGC, Instituto Gulbenkian de Ciência, the former location of the Portuguese Yeast Culture Collection), electrophoresed on the same gel and used as a reference. Numerical analysis was performed with NTSYS-pc software package (Rohlf, 1992). For each enzyme, original matrices consisted of presence (1) or absence (0) of a band with a given R_M for every studied strain. Relationships between electrophoretic types of strains were calculated using the Dice similarity coefficient. Dendrograms were generated by applying unweighted pair group average linkage clustering (UPGMA) to the similarity matrix obtained (Sneath & Sokal, 1973). In order to test the fit of the clustering, the co-phenetic correlation coefficient was calculated.

Reproducibility of the results. The experiments were repeated at least three times with protein extracts prepared from different cultures of the same strain, and the results obtained were reproducible.

RESULTS AND DISCUSSION

Isoenzyme analysis

A total of 35 strains, belonging to *S. cerevisiae*, *S. bayanus*, *S. pastorianus* and *S. paradoxus* were analysed for their electrophoretic profiles of EST, ACP, G6PD and LDH. Twenty-two distinct electrophoretic types (ETs) were obtained for the four enzyme systems investigated (Fig. 1), a result in agreement with the recognized heterogeneity within the *Saccharomyces sensu stricto* complex. Representative phenotypes are shown in Fig. 2.

All strains of *S. cerevisiae* had a single band pattern for EST and G6PD, except strain IGC 2917 which, in both cases, presented two additional slow migrating bands. These patterns were unique for this species. Noteworthy, this is the type strain of *Saccharomyces oviformis*, which was also reported to behave differently from the other *S. cerevisiae* strains with respect to the maximum temperature for growth (Rodrigues de Sousa *et al*., 1995). The same authors suggested that it might be a hybrid of *S. cerevisiae* and *S. bayanus*, bearing a higher (87 %) nuclear DNA relatedness with the former species (Vaughan Martini & Martini, 1987). Our results do not add up to this hypothesis, since it also displays an ET distinct from those observed in *S. bayanus* (Fig. 1). For ACP and LDH, *S. cerevisiae* showed a higher polymorphism, with four and eight different patterns, respectively. However, with respect to ACP, all strains except the type strain displayed the same band, not found in the other species of the group. The results indicate that LDH isoenzymes account for most of the polymorphism observed. In fact, the 20 *S. cerevisiae* strains studied could be grouped in 11 different ETs, this number being reduced to only five when LDH was not considered (Fig. 1). In similar studies, based on a higher number of enzymes, the authors report as many different ETs as the number of strains under study (Subden *et al*., 1982; Yamazaki *et al*., 1983; Poncet *et al*., 1992). In contrast, Lewicka *et al*. (1995) in studies of genetic diversity based on multilocus starch electrophoresis of seven isoenzymes described 11 different ETs for 27 strains of *S. cerevisiae*, which is in accordance with our results and indicates that the discriminatory power of the enzymes varies significantly. It looks as if it is not the number of enzymes tested that matters but to find the appropriate set with

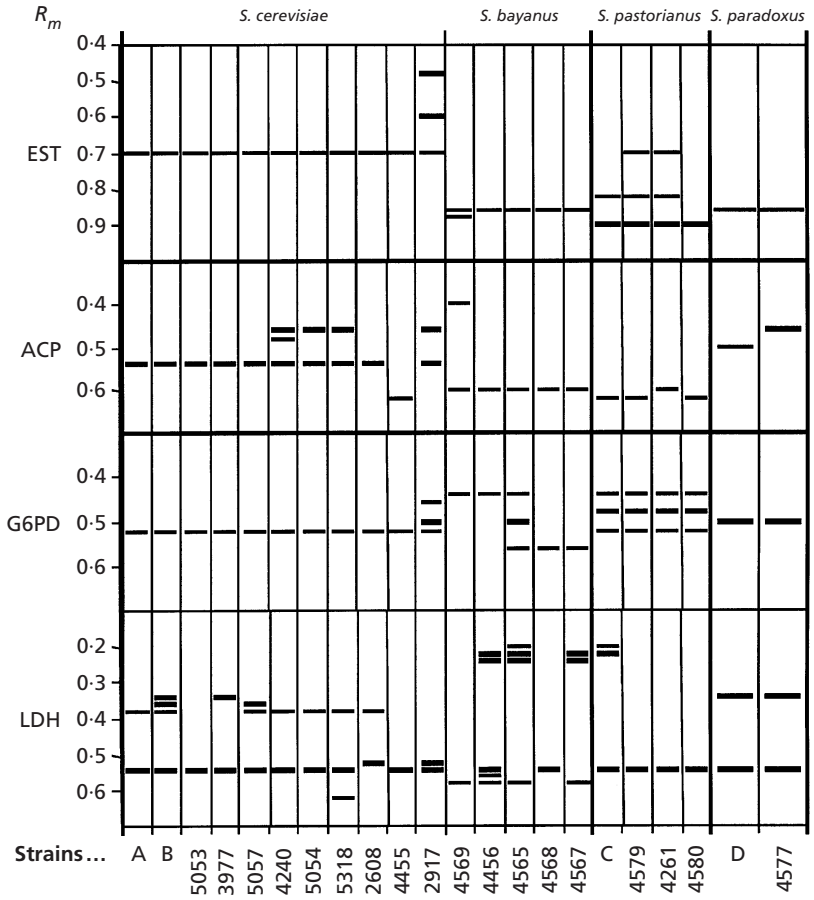

Fig. 1. Schematic representation of patterns of enzymic activity (EST, ACP, G6PD and LDH) found in the 35 strains belonging to species of *Saccharomyces sensu stricto* after PAGE. A = 3507, 4072, 4543, 4891, 5055, 5056, 5320, 5466; B = 5319, 5325, 5326; C = 4457, 4601; D = 4570, 4576, 4578, 4656.

the highest resolving power at the specific level required.

Regarding the isoenzymes studied, *S. bayanus* presented some heterogeneity, the five strains displaying five different ETs. The strains presented a single band for EST and ACP isoenzymes, except for one strain (IGC 4569) which showed a fast-migrating band in the case of EST, and a much slower one in the case of ACP. Higher polymorphisms were found for G6PD and LDH with three and five patterns, respectively. Interestingly, several authors who used other molecular typing methods, such as electrophoretic karyotyping, refer to *S. bayanus* as a very homogeneous species (Naumov *et al.*, 1992; Tornai-Lehoczki & Dlauchy, 1996).

In what concerns *S. pastorianus*, EST activity was the most polymorphic, three different patterns being observed. No intraspecific variability among the five strains studied was detected in G6PD phenotypes, whereas for ACP only one strain presented a different pattern. For LDH two distinct patterns were found, but with one main band in common.

S. paradoxus appeared to be the most homogeneous species of the group, since all the strains studied presented a single pattern for EST, LDH and G6PD. However, only the G6PD pattern was unique and characteristic of this species. This intraspecific homogeneity may be related to the fact that strains of this species can only be found in natural habitats. A recent report on the differentiation of European and Asian populations of *S. paradoxus*, by allozyme electrophoresis, revealed a significant genetic diversity among isolates of the two geographically separate regions, but each group was in itself very homogeneous (Naumov *et al.*, 1997).

Globally, we found that EST patterns enabled a clear distinction between *S. cerevisiae* and the other species, because most strains showed a single, common band, not found in the other three species. However, esterase patterns alone did not allow the separation of the other three species since strains of *S. bayanus* and *S. paradoxus* displayed similar EST patterns. When ACP phenotypes were considered, despite some intraspecific variability, the discrimination between *S. bayanus* and *S. paradoxus* could be improved. To quantify the resolving capacity of the electrophoretic phenotypes of all isoenzymes, which is difficult to achieve only by visual examination, the data were subjected to numerical analysis.

Numerical analysis of isoenzyme profiles

To further evaluate the relatedness among the species under study and the value of the method used for taxonomic purposes, numerical analysis, namely clus-

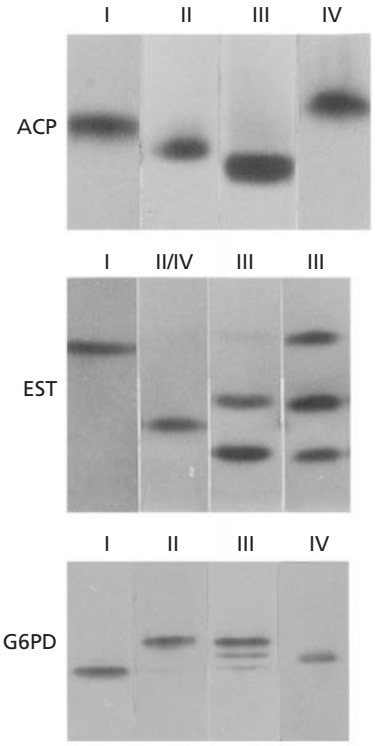

Fig. 2. Representative electrophoretic variants of EST, ACP and G6PD found after polyacrylamide gel electrophoresis in cell extracts of the *Saccharomyces* strains studied. I = *S. cerevisiae*; II = *S. bayanus*; III = *S. pastorianus*; IV = *S. paradoxus*.

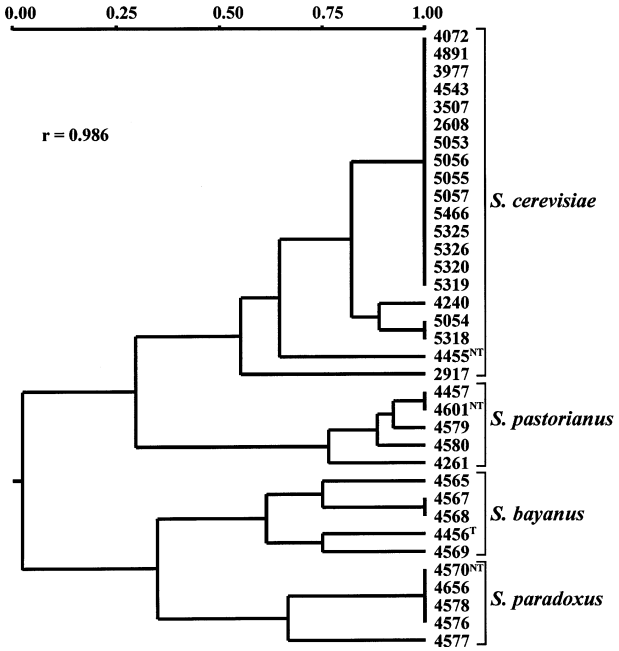

Fig. 3. Dendrogram showing the degree of similarity of electrophoretic types among strains of the *Saccharomyces sensu stricto* complex based on the data obtained from the EST, ACP and G6PD isoenzyme patterns. Scale at top represents a numerical measure of similarity. *r*, co-phenetic correlation coefficient.

ter analysis, was applied to the data obtained. Four meaningless clusters, in terms of strain conspecificity, were obtained from isoenzyme variation (31 band positions) of the four enzyme systems tested (data not shown). However, the similarity within some clusters was almost as high as among clusters, indicating low consistency of the grouping. As mentioned before, LDH was the most polymorphic enzyme, accounting for a great increase of the intraspecific variation. The consistency of the clusters could, therefore, be improved if LDH data were not included. The dendrogram thus obtained is shown in Fig. 3. The level of similarity observed within each cluster was much higher ($>0{\cdot}50$) than among clusters ($>0{\cdot}30$), and a clear separation between the four *Saccharomyces sensu stricto* species was achieved. The calculated co-phenetic correlation coefficient (0·986) indicated that the fit for the cluster analysis was very high.

A striking aspect when analysing these results is the homogeneity found among *S. cerevisae* strains, which are all clustered in one group despite their different isolation sources. Two separate branches of this cluster are constituted by the aforementioned *S. oviformis* strain and by the type strain of *S. cerevisiae*, the latter due to its exceptional behaviour with respect to ACP. Closely related to this group are the strains of *S. pastorianus*. In respect to *S. bayanus* and *S. paradoxus*, they are included in two distinct but closely related clusters. The higher similarity found between *S. cerevisiae* and *S. pastorianus* is in accordance with rDNA restriction pattern analysis (Molina *et al.*, 1992), though these authors did not include the type strains of the corresponding species in their study, and also with the higher percentage of DNA–DNA reassociation found between both species by Rodrigues de Sousa *et al.* (1995). On the contrary, most of the works on the characterization of this group of yeasts, namely electrophoretic karyotyping (Naumov *et al.*, 1992), mtDNA restriction patterns (Guillamón *et al.*, 1994) and ribosomal nucleic acids sequence data (Kurtzman & Robnett, 1991; James *et al.*, 1997; Montrocher *et al.*, 1998) found a closer relationship between *S. cerevisiae* and *S. paradoxus*. These discrepancies, though not solved by the present data, do not lead to any ambiguous interpretation of our results, which provide an interspecific differentiation of isoenzyme patterns.

Our results differ from those obtained by other authors in studies on the electrophoretic mobility of isoenzymes in the species examined of the genus *Saccharomyces* as they generally present a greater intraspecific diversity in patterns and fail to separate the four species belonging to *Saccharomyces sensu stricto* (Subden *et al.*, 1982; Yamazaki *et al.*, 1983; Poncet *et al.*, 1992; Lewicka *et al.*, 1995). The experimental conditions, namely the use of certified strains and the time of harvesting the cells, may account for the

consistency of these results. We have verified (data not shown) that at the stationary phase of growth EST patterns present additional fainter bands that probably lead to higher polymorphism but to less intraspecific discrimination. It should be again emphasized that the choice of enzymes appear also to be of major importance.

It should be pointed out that other eventual yeast contaminants in fermentation-related environments, such as *Torulaspora delbrueckii*, *Zygosaccharomyces bailii* and *Pichia membranifaciens*, have also been examined using the same methodology and yielded distinct electrophoretic types (results not shown).

In view of the present results we consider that the electrophoretic profiles of EST, ACP and G6PD isoenzymes are good markers for species differentiation in *Saccharomyces sensu stricto* and a potentially useful tool for the rapid identification of isolates in this group of industrially important yeasts. In practice, every new isolate belonging to the group can be analysed, following the procedure described for determination of isoenzyme patterns. The results can then be computer-compared, in a user-friendly way, to the available data on the strains of *Saccharomyces sensu stricto* studied and thereby be automatically assigned to one of the four species.

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