Geographical Distribution of Indigenous *Saccharomyces cerevisiae* Strains Isolated from Vineyards in the Coastal Regions of the Western Cape in South Africa

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Submitted for publication: September 1999
Accepted for publication: January 2000
Key words: Wine yeasts, yeast biodiversity, yeast ecology, biogeography, indigenous yeast flora

Notwithstanding numerous studies on the yeast biota of grapes and grape must, the origin of the primary wine yeast *Saccharomyces cerevisiae* has been rather controversial. One school of thought claims that the primary source of *S. cerevisiae* is the vineyard, whereas another believes that ecological evidence points to a strict association with artificial, man-made environments such as wineries and fermentation plants. One of the main thrusts of these kinds of investigations is to understand the succession of yeasts during fermentation of wine and to determine the actual contribution of indigenous strains of *S. cerevisiae* and wild yeast species to the overall sensorial quality of the end product, even in guided fermentations using selected *S. cerevisiae* starter cultures. There is increasing interest within the wine community in the use of indigenous strains of *S. cerevisiae* and mixed starter cultures, tailored to reflect the characteristics of a given region. Against this background we have launched a comprehensive and long overdue biogeographical survey systematically cataloging yeasts in different climatic zones of the 350-year-old wine-producing regions of the Western Cape. The present paper represents the first phase of this programme aimed at preserving and exploiting the hidden oenological potential of the untapped yeast biodiversity in South Africa's primary grape-growing areas. Grapes were aseptically harvested from 13 sites in five areas in the coastal regions of the Western Cape. After fermentation, 30 yeast colonies per sample were isolated and examined for the presence of *S. cerevisiae*. Five sampling sites yielded no *S. cerevisiae*. CHEF-DNA analysis revealed the presence of 46 unique karyotypes in eight of the remaining sites. No dominant strain was identified and each site had its own unique collection of strains. The number of strains per site varied from two to 15. Only in four cases did one strain appear at two sites, while only one instance of a strain occurring at three sites was recorded. All sites contained killer and sensitive strains; however, killer strains did not always dominate. Commercial strains were recovered from three sites. Although commercial yeasts dominated the microflora at two sites, it appears that fears of commercial yeasts ultimately dominating the natural microflora seem to be exaggerated.

According to Jemec *et al.* (1997), the biotransformation of grape juice into wine cannot be viewed as a simple biochemical process since it is a complex heterogenous microbiological process involving the sequential development of various yeasts and other microbial species, as affected by a particular environment. This statement accurately summarises the findings of numerous papers as reviewed by Bisson & Kunkee (1991), Fleet & Heard (1993), Henschke (1997) and Pretorius, Van der Westhuizen & Augustyn (1999), amongst others.

From the above it has also become clear that strains of *Saccharomyces cerevisiae* are rarely isolated from grapes (Van Zyl & Du Plessis, 1961; Benda, 1964; Parish & Carroll, 1985; Martini, Ciani & Scorzetti, 1996). On the other hand, strains of this species predominate amongst the microflora resident on different surfaces in the winery (Peynaud & Domercq, 1959; Rosini, 1984). These resident yeasts, and particularly the *S. cerevisiae* strains, should therefore play an important role in, or even dominate, spontaneous fermentations. The importance of resident yeasts is well illustrated by the work of Constanti *et al.* (1997). These authors reported the almost complete take-over of an inoculated fermentation by a yeast resident in a two-year-old Spanish winery. Clearly, analysis of the yeast present in juice prepared in a winery will not reflect the true composition of the microflora present in the vineyard.

High-risk spontaneous fermentations have largely been replaced by a more controlled process utilising one or more commercially prepared active dried wine yeast preparation(s). This is particularly true in South Africa where spontaneous fermentations are a rarity. However, recurrent fermentation problems experienced during the mid 1970s to late 1980s resulted in the launch of an extensive wine yeast selection and hybridisation programme aimed at producing new yeasts better

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Acknowledgements: The authors thank Anchor Yeast, the South African wine industry (Winetech) and the National Research Foundation (NRF) for financial support.

adapted to local fermentation conditions.

Apart from some early work (Du Plessis, 1959; Van Zyl & Du Plessis, 1961), the composition of vineyard microflora in South Africa has received no attention at all. Furthermore, there is growing interest within the wine community in the use of indigenous strains of *S. cerevisiae* and mixed starter cultures, tailored to reflect the characteristics of a given region (Heard, 1999). This fact, coupled to specific needs of the local wine industry and the current emphasis on the preservation of all forms of genetic biodiversity, resulted in the expansion of the natural wine yeast selection programme to encompass the goals set out in Pretorius *et al.* (1999).

The aim of this study, as part of the programme mentioned above, was to determine the natural distribution of *S. cerevisiae* strains in the coastal vineyards of the Western Cape in South Africa.

MATERIALS AND METHODS

Areas sampled: Vineyards were sampled in the following areas during the 1995 harvest: Constantia (2 farms), Stellenbosch (4 farms), Somerset West (1 farm), Elgin/Bot River (4 farms) and Hermanus (2 farms) (Fig. 1). Sampling sites are identified in Table 1.

Sample collection and yeast isolation: Whole clusters [3-4 kg of bunches per sampling site, gathered from 10-15 vines of white varieties (Chardonnay, Chenin blanc, Sauvignon blanc and Riesling) at sugar levels higher than 20°Brix] were gathered aseptically and dropped directly into sterile plastic bags. The tightly sealed plastic bags were transported to the laboratory in cool bags. At the laboratory grapes were crushed by hand in the still tightly sealed plastic bags. After thorough shaking, the bags were opened and the juice (500 mL) poured into 750 mL sterile bottles, which were immediately sealed by affixing sterile

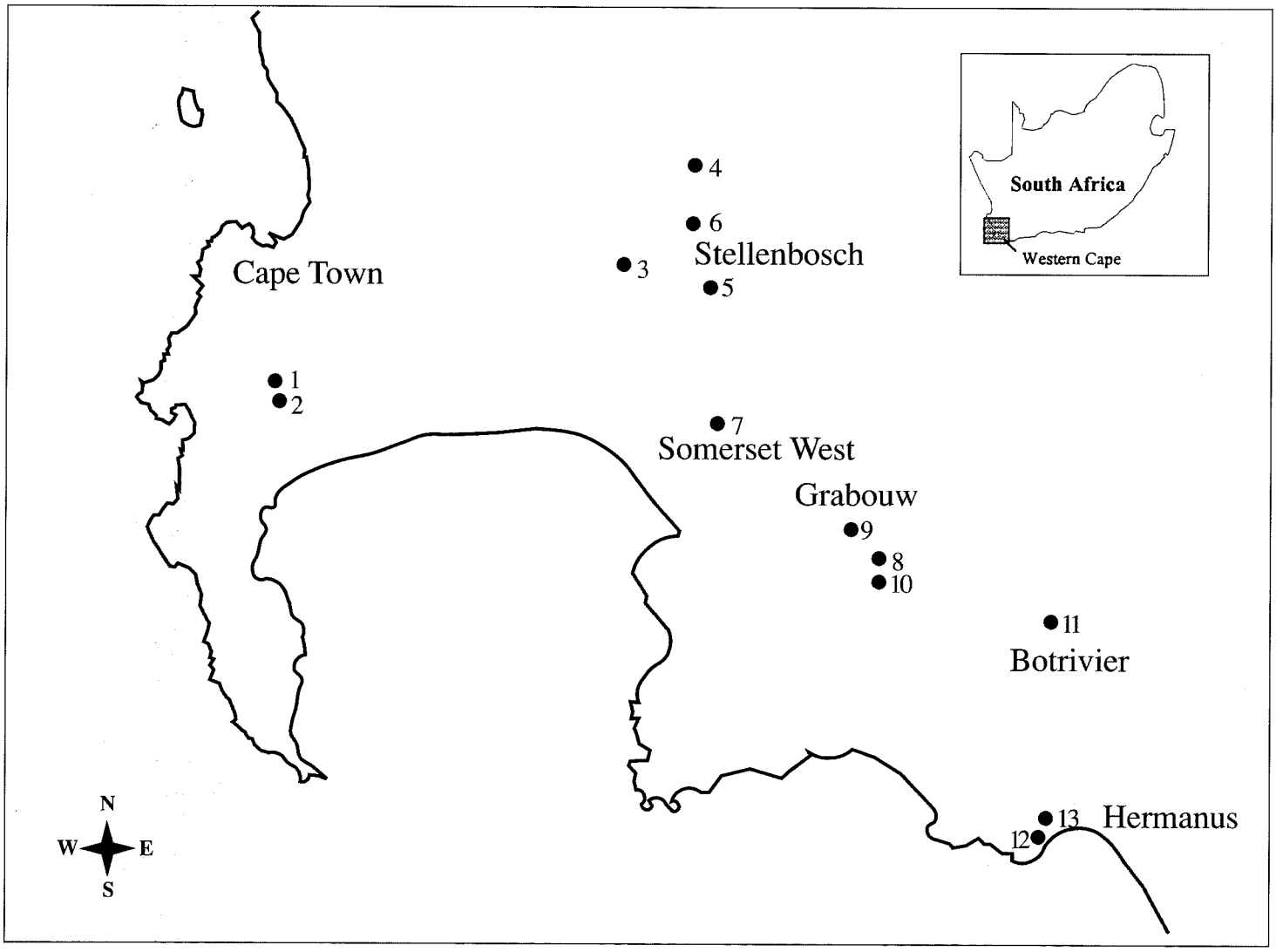

FIGURE 1
Location of sampling sites (listed in Table 1) in five different areas in the coastal regions of the Western Cape, South Africa.

fermentation caps. The bottles were then placed in a dark, temperature-controlled room (15°C). Progress of fermentation was determined by measuring mass loss. Samples were taken from successful fermentations when residual fermentable sugar was less than 4 g/L. Before samples were withdrawn, bottles were shaken to thoroughly mix the contents and get all organisms in suspension. Bottles in which fermentation was not so successful (high residual sugar) were sampled after 80 days. Each of these samples was streaked on 10 Petri dishes containing YPD agar medium (1% yeast extract, 2% peptone and 2% glucose) and incubated at 30°C for 3 days to allow colony formation. Thirty colonies were randomly selected (three from each of the 10 Petri dishes) and plated individually. Cultures were stored at 4°C until further analysis. Yeasts (30 isolates per site) were characterised on the basis of killer activity, galactose utilisation and pulse field gel electrophoresis.

Determination of killer activity: Methylene blue agar plates, buffered at pH 4.5, were used to detect zones of growth inhibition caused by the K_2 toxin (zymocin) secreted by killer yeasts. The strain designated as Geisenheim was used as sensitive lawn, and two South African commercial wine yeasts strains (produced by Anchor Yeast in Cape Town), N96 (killer-positive) and VIN7 (killer-negative), as controls. Methylene blue plates were incubated for 48 h at 25°C and then examined to note killer activity.

Galactose-utilisation: Galactose-utilising strains were identified by the presence of yellow halos on YPGB medium containing 1% yeast extract, 2% peptone, 2% galactose and 2% bromthymol blue (4 mg/mL). Results of the galactose test were determined after 24 h incubation at 30°C.

Preparation of intact chromosomal DNA and pulse field gel electrophoresis: Samples were prepared according to the embedded agarose procedure of Carle & Olson (1985). Intact chromosomal DNAs were separated using contour clamped homogenous electric field (CHEF) electrophoresis (CHEF-DR11, Bio-Rad Laboratories, Richmond, USA). All separations were carried out in 1% agarose gels according to the electrophoretic conditions of Van der Westhuizen & Pretorius (1992) as applied by Van der Westhuizen, Augustyn & Pretorius (1999). Gels were stained with ethidium bromide (10 mg/mL), viewed on a transilluminator and then photographed.

A standard reference strain was used on each CHEF gel as three gels were needed to characterise the 30 isolates per site. The banding pattern of each yeast isolate was digitised and compared to all the other patterns using a customised computer program. Computer and visual data were used as primary criteria when comparing strains. Final results were confirmed by running additional gels.

Randomly amplified polymorphic DNA (RAPD) analysis: Yeast cells were cultured and the DNA isolation was performed using the method as described by Van der Westhuizen & Pretorius (1992). Polymerase chain reactions (PCR) were performed with primer OPC-09 (5'-CTCACCGTCC-3') as applied by Van der Westhuizen *et al.* (1999). RAPD-PCR analysis was only carried out to verify that those yeast isolates that display identical electrophoretic karyotypes are indeed the same.

RESULTS AND DISCUSSION

Sample preparation and fermentation: Aseptic harvesting of grapes and preparation of juice avoided the contamination of samples by yeasts not resident on the grape samples. The simple juice preparation technique followed here was considered adequate in spite of the results generated by Martini, Frederici & Rosini (1980). These authors indicated that complete recovery of all micro-organisms associated with many natural surfaces required aggressive recovery techniques such as sonication. Bisson & Kunkee (1991), however, pointed out that, although Martini *et al.* (1980) did recover greater quantities of micro-organisms through application of these aggressive techniques, they did not identify any novel organisms. Their results, in fact, qualitatively confirm results generated after application of much milder sample preparation techniques.

Fermentation rates in the 13 samples differed dramatically. Whereas the fastest fermentation (juice from site 5, Table 1) was completed after 27 days, some samples (juice from site 8) still had a residual fermentable sugar content of 180 g/L after 80 days. These differences in fermentation rates clearly indicated the presence of different yeasts.

Using fermentation as an enrichment tool for the elusive strains of *S. cerevisiae* will clearly bias results towards yeasts with a high ethanol tolerance. In addition, killer activity will probably result in the demise of some killer-sensitive strains. The final picture of *S. cerevisiae* strains isolated in this study will, therefore, only reflect those strains that could possibly have some oenological use.

Yeast identification by means of karyotyping: Electrophoretic karyotyping was used to determine the identity of each of the 30 isolates per sample (site). Results are presented in Table 1. No *S. cerevisiae* strains were found in samples (sites 6, 8, 9 and 11) with high residual fermentable sugar. In addition, no sample contained both *Saccharomyces* and non-*Saccharomyces* yeasts. This is a surprising result. Although it is a well-known fact that most of the non-*Saccharomyces* yeasts are killed when must alcohol levels reach 3-4% (Bisson & Kunkee, 1991; Fleet & Heard, 1993), other reports have indicated that some of these "wild" yeasts may be present at the end of fermentation (Heard & Fleet, 1988).

Clearly the anaerobic conditions, initial high sugar concentration (20°B) and low fermentation temperature (15°C) inhibited non-*Saccharomyces* yeasts in the spontaneously fermented grape juice sampled at sites 1, 2, 3, 4, 5, 7, 12 and 13. Therefore, these selective pressures and high ethanol levels after completion of fermentation limited the probability of isolating non-*Saccharomyces* yeasts in the latter samples.

Geographic distribution of *S. cerevisiae* strains: Fifty-one different *S. cerevisiae* isolates representing 46 unique karyotypes were recovered (Table 1). RAPD-PCR analysis was used to confirm that those yeast isolates indicated to be identical by means of electrophoretic karyotyping were in fact the same (data not shown). The number of *S. cerevisiae* strains recovered per site varied from two (site 7) to 15 (site 2). No *Saccharomyces* strains were recovered from sites 6, 8, 9, 10 and 11. The sites sampled in the Elgin/Bot River area (8, 9, 10 and 11) were all young vineyards in an area only recently planted to grapevines, with no

TABLE 1

Distribution of natural *Saccharomyces cerevisiae* strains in the Western Cape region.

Area	Farm	Fermentation time* (days)	Strain	Number out of 30	Percentage
Constantia	Groot Constantia (1)	63	C5-1	14	47%
			C5-2	8	26%
			C5-3 = B5-6 = F5-3	6	20%
			C5-6		7%
	Buitenverwachting (2)	38	B5-1	1	3%
			B5-2	1	3%
			B5-3	3	10%
			B5-4	1	3%
			B5-5 = HR5-7	2	7%
			B5-6 = C5-3 = F5-3	2	7%
			B5-7	2	7%
			B5-8	1	3%
			B5-9	1	3%
			B5-10	1	3%
			B5-11	1	3%
			B5-12	1	3%
			B5-13	4	13%
			B5-14	8	29%
			B5-15	1	3%
Stellenbosch	Jordan (3)	55	J5-1 (VIN13)	23	77%
			J5-2	1	3%
			J5-3	5	17%
			J5-4	1	3%
	Lievland (4)	31	L5-1	1	3%
			L5-2 (VIN13)	5	17%
			L5-3 (N96)	24	80%
	Mont Fleur (5)	27	M5-1	3	10%
			M5-2	10	34%
			M5-3	2	7%
			M5-4	1	3%
			M5-5	2	7%
			M5-6	7	23%
			M5-7	1	3%
			M5-8	1	3%
			M5-9	3	10%
	Nietvoorbij (6)	80	-	0	0%
Somerset West	Vergelegen (7)	36	V5-1 = HR5-10	28	94%
			V5-2	2	6%
Elgin/Bot River	De Rust (8)	80	-	0	0%
	Oak Valley (9)	80	-	0	0%
	White Hall (10)	80	-	0	0%
	Wildekrans (11)	80	-	0	0%
Hermanus	Bouchard Finlayson (12)	61	F5-1 = HR5-4	2	7%
			F5-2	26	87%
			F5-3 = C5-3 = B5-6	1	3%
			F5-4	1	3%
	Hamilton Russell (13)	49	HR5-1	3	10%
			HR5-2 (VIN7)	2	7%
			HR5-3	5	17%
			HR5-4 = F5-1	3	10%
			HR5-5	2	7%
			HR5-6	1	3%
			HR5-7 = B5-5	4	13%
			HR5-8	2	7%
			HR5-9	2	7%
			HR5-10	5	17%

*Time required to complete natural fermentation prior to yeast isolation. Incomplete fermentations (sites 6, 8, 9, 10 and 11) were sampled after 80 days yielding only non-*Saccharomyces* yeasts.

TABLE 2

Distribution and occurrence of natural killer *Saccharomyces cerevisiae* strains in the Western Cape region.

Farm / sampling sites	Strains	Number out of 30	Killer	Galactose utilization	% Killer
Groot Constantia (1)	C5-1	14	+	-	
	C5-2	8	-	+	
	C5-3 = B5-6 = F5-3	6	-	+	
	C5-6	2	+	+	53%
Buitenverwachting (2)	B5-1	1	+	+	
	B5-2	1	+	+	
	B5-3	3	-	+	
	B5-4	1	-	+	
	B5-5 = HR5-7	2	+	+	
	B5-6 = C5-3 = F5-3	2	-	+	
	B5-7	2	-	+	
	B5-8	1	-	+	
	B5-9	1	-	+	
	B5-10	1	+	+	
	B5-11	1	+	+	
	B5-12	1	+	+	
	B5-13	4	+	+	
	B5-14	8	+	+	
	B5-15	1	+	+	63%
Jordan (3)	J5-1 (VIN13)	23	+	+	
	J5-2	1	+	+	
	J5-3	5	-	+	
	J5-4	1	-	+	80%
Lievland (4)	L5-1	1	+	+	
	L5-2 (VIN13)	5	+	+	
	L5-3 (N96)	24	+	-	100%
Montfleur (5)	M5-1	3	+	+	
	M5-2	10	+	+	
	M5-3	2	+	+	
	M5-4	1	-	-	
	M5-5	2	+	+	
	M5-6	7	-	-	
	M5-7	1	+	+	
	M5-8	1	+	+	
	M5-9	3	+	+	74%
Vergelegen (7)	V5-1 = HR5-10	28	-	+	
	V5-2	2	+	+	6%
Bouchard Finlayson (12)	F5-1 = HR5-4	2	+	-	
	F5-2	26	+	+	
	F5-3 = C5-3 = B5-6	1	-	+	
	F5-4	1	+	+	97%
Hamilton Russell (13)	HR5-1	3	-	+	
	HR5-2 (VIN7)	2	-	+	
	HR5-3	5	-	+	
	HR5-4 = F5-1	3	+	-	
	HR5-5	2	-	+	
	HR5-6	1	-	+	
	HR5-7 = B5-5	4	+	+	
	HR5-8	2	-	+	
	HR5-9	2	+	-	
	HR5-10	5	-	+	33%

wineries in the vicinity. Such conditions have often been associated with the complete absence of fermentation (Martini *et al.*, 1996). However, the situation at Nietvoorbij (site 6) is completely different in that this is a well-established wine farm with two wineries. No reason, other than that put forward by Martini *et al.* (1996), for this absence of wine yeasts was apparent. These authors coupled the complete absence of *S. cerevisiae* in seven of eight fermentations in aseptically prepared juice to the paucity of this yeast in nature. They concluded that either no cells were present in the 3 to 4 kg of grapes sampled, or the small number actually present could not survive amongst the numerous non-*Saccharomyces* yeasts normally found on grapes. If this was the case for the Nietvoorbij sample, there is no reason why it could not also be the case for the Elgin/Bot River samples. From Table 1 it is also apparent that a high/low number of yeast strains per site was not associated with a particular area. It is therefore tempting to speculate that the spraying programme followed at a particular site could have affected the microflora present on the grapes at that site. Therefore, it is quite possible that a high/low number of *S. cerevisiae* strains per site is directly the result of an intense/less-intense spraying programme. This possibility was not examined in the study because spraying is a general practice and therefore forms part of the habitat in which the sought-after yeasts have to survive.

Generally speaking, all eight sites had a unique spectrum of *S. cerevisiae* strains and very few strains were found at more than one site. A strain appeared at two different sites in four cases (B5-5/HR5-7; J5-1/L5-2; V5-1/HR5-10; F5-1/HR5-4), while only one strain (F5-3/C5-3/B5-6) was recorded at three sites. While strains J5-1/L5-2 and F5-1/HR5-4 were isolated from sites in close proximity to each other, the sites for B5-5/HR5-7 and V5-1/HR5-10 were separated by 140 km and 90 km, respectively. In the case of F5-3/C5-3/B5-6 site 12 (F5-3) was separated from sites 1 and 2 by 140 km. If the presence of these yeasts at the particular sites persists over a number of years, they might be considered to be representative of an area or *terroir* (Vézinhet *et al.*, 1992). If these yeasts are indeed representative of the different areas, more extensive sampling should indicate their presence at many more sites within the different areas.

Strains J5-1/L5-2 are not included in the above reasoning as they represent VIN13, currently the most popular active dried wine yeast used in South Africa. Strains L5-3 and HR5-2 were also considered to represent recovered commercial yeasts, although identification was not positive in the case of L5-3. Given the fact that winemakers in South Africa have been using active dried wine yeasts almost exclusively over the last two decades, the number of recovered commercial yeasts is very small. Commercial yeasts were only recovered at sites 4, 5 and 13. Sites 4 and 5 are very close together and far from site 13. Commercial yeasts dominated the fermentations at sites 3 and 4, but formed a minor component of the yeast spectrum at site 13. Reasons for this difference will have to be elucidated in future studies. One possible reason could be found in the sampling strategy and the location of the sampling sites in relation to the source of commercial yeasts.

Vézinhet *et al.* (1992) also recovered commercial yeast strains from nature (EC-1118 and 8130) in their study of wild *S. cerevisiae* strains found in Champagne between 1980 and 1985. However, as both these strains had originally been isolated from the Champagne area (Vézinhet, Blondin & Hallet, 1990), this does not indicate an invasion of an area by alien organisms. Schütz & Gafner (1994) isolated three yeast strains with clearly related, very similar chromosomal banding patterns from a spontaneous fermentation at Wädenswil. These banding patterns were very similar to the banding pattern of Lallemand W7, a commercial yeast strain originally isolated from the Wädenswil area. Although the possible spread of commercial yeast strains, and particularly that of genetically modified yeast, in nature will have to receive further attention, the results generated here do not point to a major problem.

Occurrence of galactose-utilising and killer yeasts: The ability to utilise galactose as a sole carbon source together with killer activity are often used as important phenotypes to differentiate amongst strains of *S. cerevisiae* in strain development programmes (Van der Westhuizen & Pretorius, 1992). From Table 2 it is clear that only six strains were unable to ferment galactose and killer strains were found in all fermentations that contained *S. cerevisiae* (Table 2). Abundance of killers varied from 6% to 100%. Vagnoli *et al.* (1993) published similar results based on a study of spontaneous fermentations in 18 Tuscan wineries. In their study, however, four of the 33 fermentations did not contain killer yeasts. The very low percentage of killer yeast (6) present in the sample from site 7 clearly indicates that killers do not automatically dominate all fermentations, thus confirming an earlier report by Tredoux, Tracey & Tromp (1986).

CONCLUSIONS

The absence of *S. cerevisiae* strains on grapes from five of 13 sites sampled confirms that these yeasts are not necessarily present on all wine grape clusters. Characterisation of 240 colonies, representing 46 unique karyotypes isolated from the eight remaining sites indicated the absence of a yeast common to the areas sampled. Many more sites per area need to be sampled in order to confirm, or disprove, this apparent localisation of yeast biodiversity. These studies should include consideration of the chemical sprays applied in the different areas, if they in fact differ, to determine their effect on the observed biodiversity. The individual sites sampled originally should also be sampled over a number of years to determine the stability/evolution of the yeast population.

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