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AFLP fingerprinting for analysis of yeast genetic variation

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Amplified fragment length polymorphism (AFLP) was used to investigate genetic variation in commercial strains, type strains and winery isolates from a number of yeast species. AFLP was shown to be effective in discriminating closely related strains. Furthermore, sufficient similarity in the fingerprints produced by yeasts of a given species allowed classification of unknown isolates. The applicability of the method for determining genome similarities between yeasts was investigated by performing cluster analysis on the AFLP data. Results from two species, *Saccharomyces cerevisiae* and *Dekkera bruxellensis*, illustrate that AFLP is useful for the study of intraspecific genetic relatedness. The value of the technique in strain differentiation, species identification and the analysis of genetic similarity demonstrates the potential of AFLP in yeast ecology and evolutionary studies.

Keywords: AFLP, yeasts, genetic similarity

INTRODUCTION

Differences in morphological and physiological characteristics continue to be the main criteria used in yeast classification (Barnett *et al*., 1990). However, since many of the characters can be reversed by a mutation in a single gene, these methods, on their own, are inadequate. The use of the biological species concept, which delimits species on their ability to hybridize, is also restricted in yeast systematics. Lack of fertility does not preclude conspecificity and furthermore, hybridization studies with yeasts can be difficult and therefore not suitable for routine yeast identification (see Kurtzman & Phaff, 1987, for review).

The limitations in using morphological and physiological methods, and the problems associated with the biological species concept in yeasts has led to the increasing use of nucleic acid methods in yeast taxonomy. Of importance has been the use of DNA reassociation studies, where genome similarities greater than 80% have been taken to indicate conspecificity (Price *et al*., 1978; Vaughan Martini & Kurtzman, 1985). Defining the lower limit for delimiting species has been more difficult, however, as successful matings have been obtained between yeasts that show only 25% DNA similarity (Kurtzman *et al*., 1980). The ability of yeasts with such low levels of sequence similarity to undergo effective meiosis is not yet understood, and needs to be considered when using any DNA-based identification method. More recently, the emphasis in molecular methods has been to correlate taxonomy with phylogeny. For this purpose, sequence analysis of the rRNA genes has been widely used as their common evolutionary origin permits the comparison of both closely and distantly related species (Kurtzman, 1992).

Other molecular methods have also been used to study yeasts at both the species and subspecies level. These include chromosome karyotyping (Johnston & Mortimer, 1986), RFLP (McArthur & Clark-Walker, 1983; Molina *et al*., 1993) and PCR (de Barros Lopes *et al*., 1998; Ganter & Quarles, 1997; Latouche *et al*., 1997; Lavallée *et al*., 1994). As these methods are useful for discriminating strains within a species, they are also being used in yeast ecology and epidemiology studies. In this paper, the use of amplified fragment length

Abbreviations: AFLP, amplified fragment length polymorphism; UPGMA, unweighted pair group method with arithmetic averages.

Table 1. Yeast species and strains studied

AWRI no.	Strain details	CBS no.
Saccharomyces cerevisiae Meyen *ex* E. C. Hansen		
1219	Neotype strain	1171^{NT}
1350	Laboratory yeast, FY833 *MAT***a**	
1351	Laboratory yeast, FY834 *MATα*	
1352	Brewers' yeast, B431 (Brigalow Brewing Co.)	
1353	Bakers' yeast, K5088 (Cerebos Ltd)	
939	Sake yeast	
796	Commercial wine yeast	
350	Commercial wine yeast	
834	Commercial wine yeast	
81	Commercial wine yeast	
1017	Commercial wine yeast	
838	Commercial wine yeast	
729	Commercial wine yeast, University of California, Davis, USA	
814	729 yeast, The Australian Wine Research Institute, South Australia, Australia	
825	729 yeast, Dept of Agriculture, Western Australia, Australia	
835	729 yeast, Dept of Agriculture, Western Australia, Australia	
925	729 yeast, University of California, Davis, USA	
947	729 yeast, The Australian Wine Research Institute, South Australia, Australia	
1116	729 yeast, Epernay, France	
1117	729 yeast, Epernay, France	
1118	729 yeast, Epernay, France	
1144	Former type strain of *Candida robusta*	1907
1265	Winery isolate, South Australia, Australia	
870	Winery isolate, New South Wales, Australia	
871	Winery isolate, New South Wales, Australia	
Saccharomyces paradoxus Bachinskaya		
1172	Neotype strain	432^{NT}
Saccharomyces bayanus Saccardo		
1146	Type strain	380^{T}
1145	Former type strain of *Saccharomyces uvarum*	395
1266	Winery isolate, South Australia, Australia	
948	Winery isolate, South Australia, Australia	
Saccharomyces pastorianus Reess *ex* E. C. Hansen		
1173	Neotype strain	1538^{NT}
Saccharomyces unisporus Jörgensen		
1218	Type strain	398^{T}
Saccharomyces exiguus Reess		
1216	Type strain	379^{T}
Saccharomyces kluyveri Phaff *et al.*		
1217	Type strain	3082^{T}
Dekkera bruxellensis van der Walt		
1205	Type strain	74^{T}
1102	Former type strain of *Brettanomyces bruxellensis*	72
1207	Former type strain of *Dekkera intermedia*	4914
1104	Former type strain of *Brettanomyces intermedius*	73
1127	Former type strain of *Brettanomyces lambicus*	75
1103	Former type strain of *Brettanomyces custersii*	5512
1130	Former type strain of *Brettanomyces abstinens*	6066
Dekkera anomala M. T. Smith *et* van Grinsven		
953	Type strain	8139^{T}
1128	Former type strain of *Brettanomyces claussenii*	76
1168	Former type strain of *Dekkera anomalus*	77

Table 1 *(cont.)*

AWRI no.	Strain details	CBS no.
Brettanomyces naardenensis Kolfschoten *et* Yarrow		
951	Type strain	6042^T
Brettanomyces custersianus van der Walt		
950	Type strain	4805^T
Brettanomyces nana M. T. Smith *et al.* (Formerly *Eeniella*)		
1201	Type strain	1945^T
Torulaspora delbrueckii Lindner		
1152	Type strain	1146^T
1034	Commercial wine yeast	
872	Winery isolate, New South Wales, Australia	
Issatchenkia orientalis Kudryavtsev		
1220	Type strain	5147^T
873	Winery isolate, New South Wales, Australia	
Hanseniaspora uvarum (Niehaus) Shehata *et al.*		
1158	Type strain	314^T
868	Winery isolate, New South Wales, Australia	
1274	Winery isolate, South Australia, Australia	
1275	Winery isolate, California, USA	
1276	Winery isolate, California, USA	
Hanseniaspora guilliermondii Pijper		
1200	Type strain	465^T
1277	Winery isolate, California, USA	
Metschnikowia pulcherrima Pitt *et* Miller		
1149	Type strain	5833^T
1267	Winery isolate, South Australia, Australia	
1268	Winery isolate, South Australia, Australia	
1269	Winery isolate, South Australia, Australia	
1270	Winery isolate, South Australia, Australia	
Pichia fermentans Lodder		
1199	Type strain	187^T
1271*	Winery isolate, South Australia, Australia	
Pichia membranifaciens E. C. Hansen		
1095	Type strain	107^T
1272*	Winery isolate, South Australia, Australia	

* Species description using standard physiological methods. Inconsistent with molecular methods.

polymorphism (AFLP) for yeast systematics is described. AFLP is a technique that is based on the selective PCR amplification of restriction fragments from a total digest of DNA (Vos *et al.*, 1995). The main use of AFLP to date has been as molecular markers, mostly for plant breeding programmes (Thomas *et al.*, 1995) but also for mammalian species (Otsen *et al.*, 1996). More recently, the effectiveness of AFLP for taxonomy and genetic diversity studies has been demonstrated in a number of biological systems including bacteria (Janssen *et al.*, 1996, 1997), fungi (Mueller *et al.*, 1996), plants (Travis *et al.*, 1996) and animals (Folkertsma *et al.*, 1996). Here, the advantages of AFLP are put to use for strain differentiation and species identification in yeasts. The usefulness of the technique for studying genetic similarities of yeasts is also discussed.

METHODS

Yeast strains and media. The yeast strains used in this study are listed in Table 1. Reference strains are species type strains obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Delft, The Netherlands. All the yeasts in the study have been previously described (de Barros Lopes *et al.*, 1996, 1998) except the two *Saccharomyces cerevisiae* laboratory yeasts, which are derived from S288C (Janssen *et al.*, 1996). All yeasts were grown on YEPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose].

Preparation of DNA template for PCR. For all species, DNA was purified using mechanical breakage with glass beads (Ausubel *et al.*, 1994). A cell suspension from a 5 ml culture grown in YEPD medium was resuspended in 200 µl breaking buffer [2% (v/v) Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8), 1 mM EDTA (pH 8)]. The yeast cells

were homogenized by vortexing for 3 min with 0·3 g glass beads in the presence of 200 µl phenol/chloroform/isoamyl alcohol. To this, 200 µl Tris (10 mM)/EDTA (1 mM) buffer (pH 8) (TE) was added and, after centrifugation, the aqueous layer collected. The DNA was precipitated with ethanol and resuspended in 300 µl TE buffer. RNA was digested by adding 3 µl of a solution containing 10 mg RNase A ml^{-1} and incubated for 5 min at 37 °C. The DNA was extracted for a second time with 200 µl phenol/chloroform/isoamyl alcohol and ethanol-precipitated. It was resuspended in 50 µl TE buffer and a 10 µl aliquot was used to determine the concentration by measurement of A_{260}.

AFLP. The AFLP reactions were performed as described by Vos *et al.* (1995) with some modifications. For the results shown in this study, 0·5 µg yeast DNA was digested with 5 units *Eco*RI and 5 units *Mse*I in RL buffer [10 mM Tris/acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM DTT (pH 7·5)] in a volume of 40 µl for 3 h at 37 °C. *Mse*I (50 pmol 5′ GACGATGAGTCCTGAG 3′ and 5′ TACTCAGGACTCAT 3′) and *Eco*RI (5 pmol 5′ CTCGTAGACTGCGTACC 3′ and 5′ AATTGGTACGCAGTC 3′) adaptors were ligated to the digested DNA in a total volume of 50 µl using 1 unit T4 DNA ligase in RL buffer plus ATP (1·2 mM). The reactions were incubated for another 3 h at 37 °C. The digested and ligated DNA was ethanol-precipitated and resuspended in 100 µl Tris (10 mM)/EDTA (0·1 mM) buffer (pH 8) ($T_{0\cdot1}E$).

The PCR reaction was performed using primers *Eco*RI-C (5′ AGACTGCGTACCAATTCC 3′) and *Mse*I-AC (5′ GATGAGTCCTGAGTAAAC 3′). For each AFLP reaction, 10 ng (2 µl) of the ligated DNA was amplified using 30 ng unlabelled *Mse*I-AC primer, 25 ng unlabelled *Eco*RI-C primer and 0·5 µl (5 ng) labelled *Eco*RI-C primer. (For 10 reactions, 50 ng primer *Eco*RI-C was radioactively labelled using 10 µCi (370 kBq) [γ-^{32}P]ATP in 5 µl with 1 unit T4 polynucleotide kinase. The reaction was incubated at 37 °C for 30 min.) Reactions were done in 20 µl PCR buffer containing 1·5 mM $MgCl_2$, 0·2 mM dNTPs and 0·1 unit *Taq* polymerase (Gibco-BRL). A 'touchdown' cycle was used for the PCR reaction (96 well multiplate and PTC-100 thermocycler; MJ Research). Denaturation was at 94 °C for 30 s and extension at 74 °C for 1 min. The annealing temperature started at 64 °C and was subsequently decreased by 0·2 °C every cycle until it reached 60 °C. This was followed by 10 more cycles at 60 °C and a final 5 min extension at 74 °C. The higher annealing temperature used in this study compared to other AFLP investigations was used to produce fingerprints with fewer background bands. To the completed reactions, 20 µl gel loading buffer (94% formamide, 10 mM EDTA, 0·5 mg xylene cyanol FF ml^{-1}, 0·5 mg bromophenol blue ml^{-1}) was added. Samples were heated to 90 °C for 3 min and cooled on ice. Products of each amplification reaction were resolved on 6% polyacrylamide gels (Sequagel 6; National Diagnostics) at 40 W. For the last 45 min of the run, the bottom buffer was 0·3 M sodium acetate to stack the lower molecular mass bands. After drying, gels were exposed to film (Fuji-RX) at room temperature for 18–48 h.

Other primers used in the study (results not shown) included *Eco*RI-A, *Eco*RI-AG, *Mse*I-G and *Pst*I-A. In all cases, one selective base was used on one primer and two selective bases on the other. It was found that using more selective bases produced less consistent results. For some combinations of primers, a pre-amplification or a lower annealing temperature was necessary to obtain acceptable results.

The presence/absence of AFLP markers was scored by eye. Analysis was performed using the NTSYS-pc software (Rohlf, 1993). Pairwise similarities were created using the Dice coefficient, which is equal to twice the number of common bands in two fingerprints over the sum of all bands. The unweighted pair group method with arithmetic averages (UPGMA) was used to cluster the results. The genetic similarities obtained were also corroborated using other clustering methods.

RESULTS

Yeast strain differentiation

Fig. 1 shows the AFLP fingerprint of a number of *S. cerevisiae* strains. These include commercial yeasts, indigenous grape juice microflora, laboratory strains and the type strain of *S. cerevisiae*. Using the single primer pair *Eco*RI-C and *Mse*I-AC, many of the strains could be differentiated. Two cultures that could not be separated using this primer pair and other primers tested (results not shown), were AWRI 1017 (lane 11) and AWRI 1265 (lane 23). AWRI 1017 is a commonly used commercial wine yeast strain. Strain AWRI 1265 was isolated from equipment in a winery that uses AWRI 1017 as its inoculum strain for fermentations. These strains were also not separated using semi-specific PCR (de Barros Lopes *et al.*, 1998). These results indicate that the strains are identical. Two other strains that could not be differentiated in this study were AWRI 729 (lane 13) and AWRI 835 (lane 16). AWRI 835 is a member of the 729 family of yeasts and is thought to be a clonal isolate of strain AWRI 729 (see de Barros Lopes *et al.*, 1996; Henschke, 1990; Petering *et al.*, 1988). Other 729 strains (AWRI 814, AWRI 825 and AWRI 925) that were not separated from each other by *Eco*RI-C/*Mse*I-AC were differentiated using a second primer pair. The reproducibility of AFLP is seen in the fingerprints of the two opposite mating types of the laboratory strain. The two yeasts, which have identical genotypes, except at the *MAT* locus, produced identical AFLP fingerprints (lanes 2 and 3).

The effectiveness of AFLP in uncovering polymorphisms is also apparent with the non-*S. cerevisiae* strains. All of the strains analysed in Figs 2 and 3, which include yeasts from seven genera, could be differentiated.

Yeast species identification

The results in Figs 1 and 2 demonstrate that although there are polymorphisms between strains of the same species, many of the amplified bands are shared intraspecifically. For example, for the twenty-six *S. cerevisiae* strains studied in Fig. 1, approximately 54% of the amplified fragments are monomorphic. Similarly, although the *Dekkera bruxellensis* yeasts appear to be more divergent, 50% of the amplified fragments

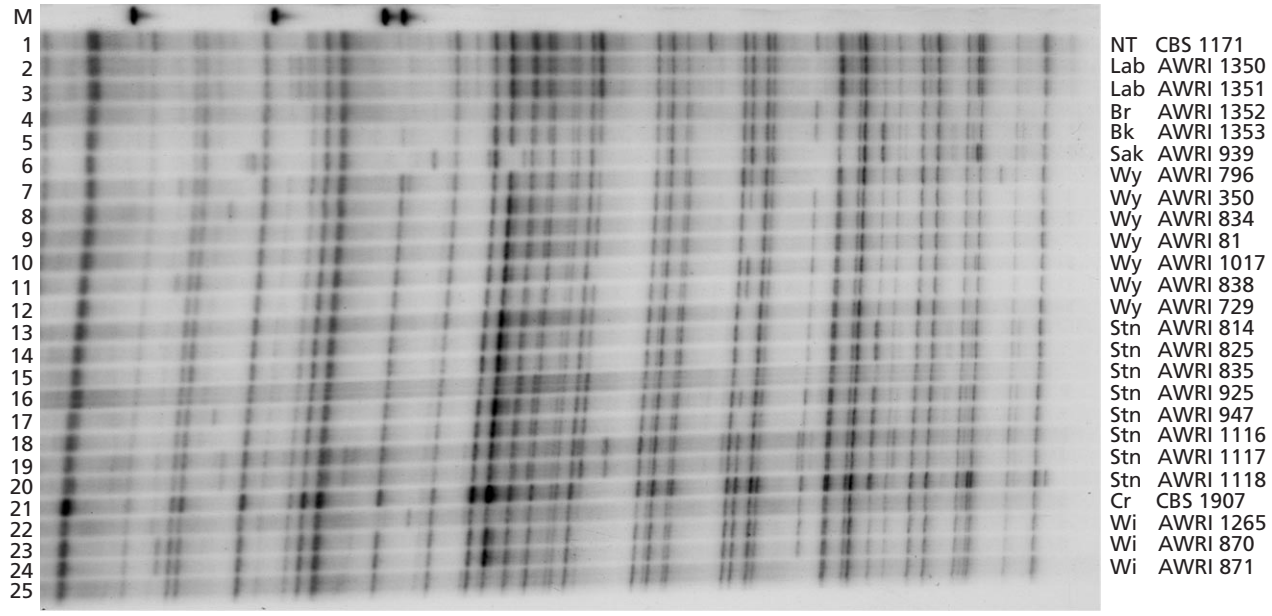

Fig. 1. AFLP fingerprints of *S. cerevisiae* strains. NT, neotype strain; Lab, laboratory strain; Br, brewers' yeast; Bk, bakers' yeast; Sak, sake yeast; Wy, commercial wine yeast; Stn, 729 yeast; Cr, former type of *C. robusta*; Wi, winery isolate.

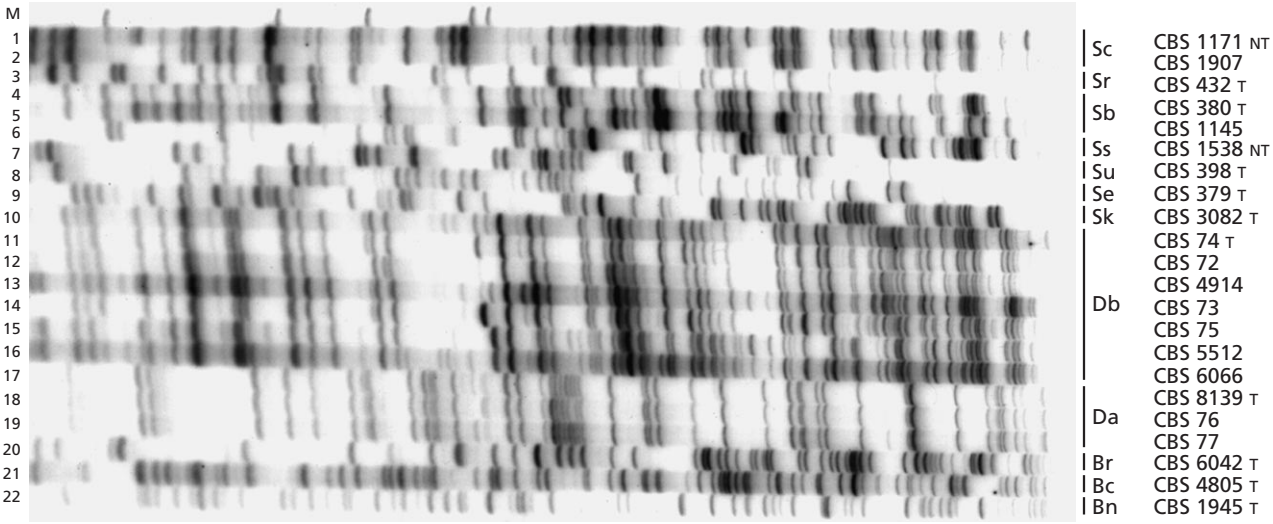

Fig. 2. AFLP fingerprints of *Saccharomyces* and *Dekkera/Brettanomyces* yeasts. Sc, *S. cerevisiae*; Sr, *S. paradoxus*; Sb, *S. bayanus*; Ss, *S. pastorianus*; Su, *S. unisporus*; Se, *S. exiguus*; Sk, *S. kluyveri*; Db, *D. bruxellensis*; Da, *D. anomala*; Br, *B. naardenensis*; Bc, *B. custersianus*; Bn, *B. nana.*

are shared between all seven strains analysed (Fig. 2, lanes 10–16). The common bands amplified within a species allow identification. Further, analysis of AFLP fingerprints between related species in the same genera demonstrate that the fingerprints are unique to a particular species (Fig. 2).

The use of AFLP for identification is demonstrated in Fig. 3(a). In a previous study, a number of strains isolated from grape juice and winery equipment were identified using intron primer PCR (de Barros Lopes *et al.*, 1998). These same strains are analysed here, and their AFLP fingerprints are compared to those gen-

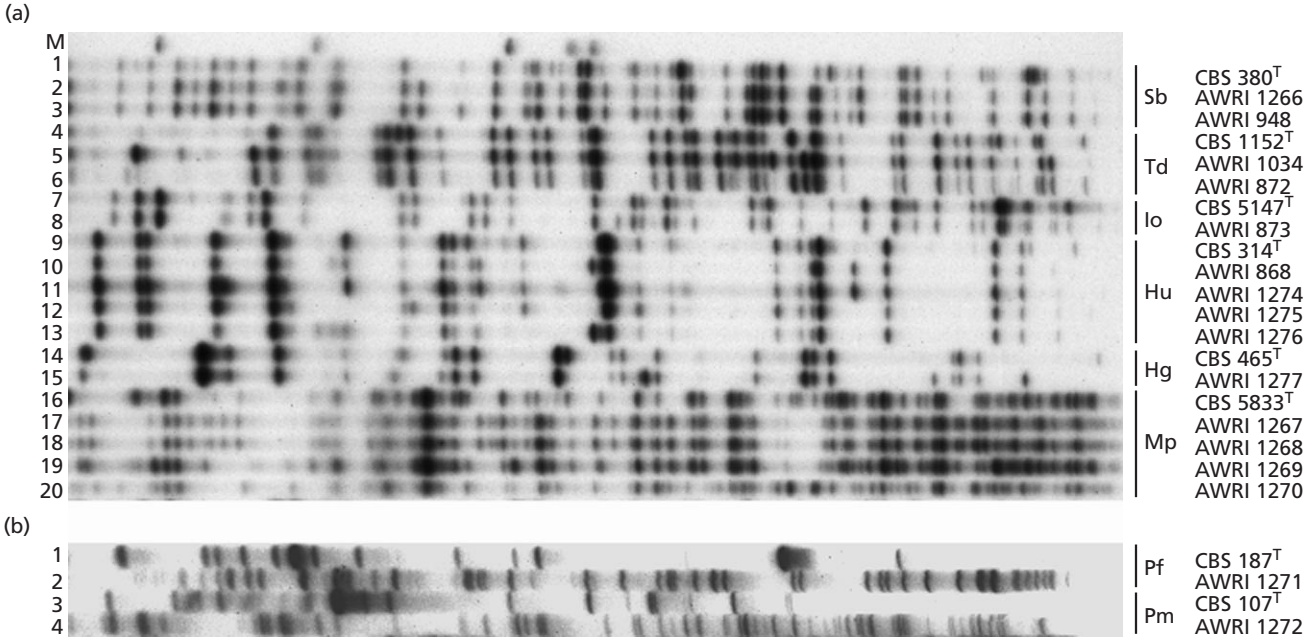

Fig. 3. Comparison of AFLP fingerprints of indigenous wine yeasts and type strains. (a) Sb, *S. bayanus*; Td, *T. delbrueckii*; Io, *I. orientalis*; Hu, *H. uvarum*; Hg, *H. guilliermondii*; Mp, *M. pulcherrima*. (b) Pf, *P. fermentans*; Pm, *P. membranifaciens*.

erated by the type strain of the same species. For all the yeasts, the similarity between the type strains and the winery isolates is clear, permitting their identity at the species level. One strain which did show notable polymorphisms when compared to its respective type strain was a *Metschnikowia pulcherrima* yeast, AWRI 1270 (Fig. 3a, lane 20). This increased genomic divergence was also observed with other primer pairs and with several other *M. pulcherrima* isolates analysed (results not shown). Based on earlier PCR results this was unexpected (de Barros Lopes *et al.*, 1998), and indicates the increased sensitivity of AFLP. The finding supports the suggestion that heterogeneity exists within this species (Giménez-Jurado *et al.*, 1995).

Two yeasts, AWRI 1271 and AWRI 1272, were identified using traditional physiological methods as *Pichia fermentans* and *Pichia membranifaciens*, respectively. Semi-specific PCR indicated that the genomes of these isolates were unrelated to their respective type strains, and sequence divergence of the 26S rRNA confirmed that these yeasts were not conspecific with the *Pichia* type strains (de Barros Lopes *et al.*, 1998). This conclusion is also supported by the AFLP results. Fig. 3(b) shows that the AFLP fingerprints of the two wine isolates, AWRI 1271 (lane 1) and AWRI 1272 (lane 3), are unrelated to the fingerprints produced by the type strains for *P. fermentans* (lane 2) and *P. membranifaciens* (lane 4). These results confirm the heterogeneity present in some *Pichia* species (Noronha-da-Costa *et al.*, 1996; Yamada *et al.*, 1996).

Genetic similarities of yeasts

UPGMA cluster analysis was performed on several of the species in this study (Fig. 4). Analysis of the *S. cerevisiae* strains revealed that the commercial wine yeasts are more related to each other than to strains used for other purposes, with a mean similarity of 96·6%. The yeast most diverged from the commercial wine strains is the sake yeast (AWRI 939) which has a mean similarity of 83%. The bakers' yeast (AWRI 1353) is the most related to the commercial wine yeasts with a mean similarity of 92·3%.

Cluster analysis of the *D. bruxellensis* yeasts also indicates the sensitivity of AFLP in determining intraspecific genetic similarities. The type strain (CBS 74) and the former type strains of *Brettanomyces bruxellensis* (CBS 72), *Dekkera intermedia* (CBS 4914) and *Brettanomyces lambicus* (CBS 75) are the most alike, sharing a minimum of 91·6% of the amplified fragments. The former type strains of *Brettanomyces custersii* (CBS 5512) and *Brettanomyces abstinens* (CBS 6066) (92·4% similarity with each other) are the most diverged, with mean similarities of 79·1% and 80·8%, respectively, when compared to the four more conserved strains. *Brettanomyces intermedius* (CBS 73) is intermediate in relatedness, with a mean of 85·2% similarity with the *D. bruxellensis* cluster and 86·2% with *B. custersii*/*B. abstinens*. Phylogenetic analysis of the same data were consistent with the UPGMA cluster analysis (results not shown) and agrees with other molecular data on these strains. A similar

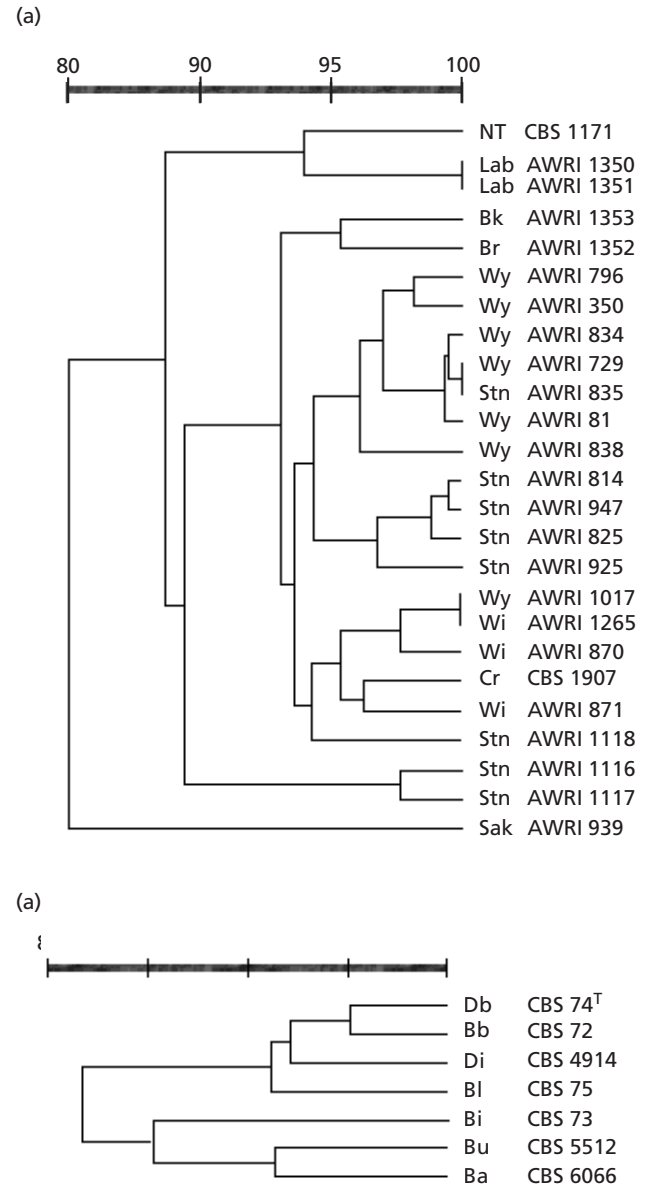

Fig. 4. Cluster analysis using UPGMA. (a) *S. cerevisiae* strains. NT, neotype strain; Lab, laboratory strain; Bk, bakers' yeast; Br, brewers' yeast; Wy, commercial wine yeast; Stn, 729 yeast; Wi, winery isolate; Cr, former type of *C. robusta*; Sak, sake yeast. (b) Db, *D. bruxellensis*; Bb, *B. bruxellensis*; Di, *D. intermedia*; Bl, *B. lambicus*; Bi, *B. intermedius*; Bu, *B. custersii*; Ba, *B. abstinens.*

analysis was not possible on the *Dekkera anomala* yeasts as the variance between the three strains was minor.

The use of AFLP for analysing interspecific genetic similarities of the *Saccharomyces sensu stricto* yeasts was not successful. Seven primer sets were used in AFLP on the four sibling *Saccharomyces sensu stricto* yeasts. Analysis of AFLP fingerprints did not produce results consistent with the known genetic relatedness of these four species (data not shown).

DISCUSSION

In this research, the usefulness of AFLP in yeast strain differentiation and identification is demonstrated. With the exception of the two laboratory *S. cerevisiae* strains, all the yeasts studied have been previously analysed using semi-specific PCR (de Barros Lopes *et al.*, 1996, 1998), and many of them have been karyotyped (Henschke, 1990; Petering *et al.*, 1988, 1990). This allows the different methods to be compared. In addition, AFLP has been used to study intraspecific genetic similarities of strains.

The effectiveness of the method in strain discrimination is seen in the separation of the putative 729 strains. Many of these are presumed isolates of the same yeast that have been stored in different culture collections (see Table 1). Using semi-specific PCR, five of the strains were shown to be different to the commercial isolate AWRI 729 (de Barros Lopes *et al.*, 1996). Using a single primer pair in AFLP, all the 729 strains, with the exception of AWRI 835, could be separated from AWRI 729. Several factors could account for the differences in genome structure of the 729 family. The freeze-dry method used for long-term storage of these yeasts is capable of inducing chromosome breaks. Further, mitotic chromosome rearrangements have been reported in a wild strain of *S. cerevisiae* (Longo & Vezinhet, 1993), although the extent of this phenomenon is unresolved. For detecting chromosomal modifications of this type, chromosome karyotyping is likely to be more effective than AFLP. Interestingly, although AFLP, semi-specific PCR (de Barros Lopes *et al.*, 1996) and initial PFGE experiments (Petering *et al.*, 1988) were unable to discriminate between strains AWRI 729 and AWRI 835, increasing the resolution of the PFGE method uncovered a minor polymorphism in their karyotypes (Henschke, 1990). An additional mechanism of obtaining chromosome variation is genome renewal (Mortimer *et al.*, 1994). In this process, which has been observed in several homothallic wine yeast isolates, cells are able to undergo meiosis and self-conjugation in rich media. The role of genome renewal in producing genetic diversity in the 729 family of yeasts has not been tested. Alternatively, the most likely explanation for at least the most divergent 729 strains [AWRI 1116 (Fig. 1, lane 19) and AWRI 1117 (lane 20)] is that the yeasts are not related to the commercial AWRI 729 strain.

All the non-*S. cerevisiae* isolates could be separated from each other. For strains of the same species, the number of polymorphisms between strains ranged from one to more than thirty from a single primer pair. Between species, few monomorphic bands were observed. Two strains that could not previously be differentiated using intron primer PCR were the *Saccharomyces bayanus* strains AWRI 1266 and AWRI 948 (de Barros Lopes *et al.*, 1998). These two yeasts were isolated in different years from cold stored juice in the same winery. It was thought that they may

be identical, but the increased sensitivity of AFLP permits their separation. The discriminatory potential of AFLP with yeasts is also highlighted by the number of polymorphisms obtained with other genera. Notably, the *D. bruxellensis* and *M. pulcherrima* yeasts produced similar semi-specific PCR amplification patterns (de Barros Lopes *et al.*, 1998), but highly polymorphic AFLP fingerprints.

Since strains of the same species share many amplification fragments, AFLP is also effective for species identification. For this purpose there are no apparent advantages of using AFLP over the more rapid PCR methods (de Barros Lopes *et al.*, 1998; Latouche *et al.*, 1997), although the ability of AFLP to analyse a more extensive portion of the genome may uncover genetic similarities between yeasts that are not revealed using other molecular methods. For example, although *Issatchenkia scutulata* var. *scutulata* and *I. scutulata* var. *exigua* show only 24% DNA similarity as measured by reassociation experiments, the two varieties are able to mate and produce viable ascospores (Kurtzman *et al.*, 1980). If specific regions of the genomes between these two varieties are conserved, AFLP may reveal this kinship.

AFLP has also been used to determine the genetic relatedness of yeasts (Fig. 4). UPGMA cluster analysis of the *S. cerevisiae* strains indicates that the commercial winemaking yeasts are more closely related to each other than to strains used for other purposes, including the laboratory, brewers', bakers' and sake strains. Furthermore, three indigenous isolates from Australian wineries are also related to the commercial wine strains. Apart from the sake yeast, two Epernay yeasts isolated from French wineries, AWRI 1116 and AWRI 1117, produced the most polymorphic AFLP fingerprints when compared to the commercial wine strains. It is unlikely that the reason for the variation is geographical as many of the commercial yeasts and a third Epernay yeast, AWRI 1118, were isolated from France. The importance of geographical location in predicting genetic similarity was also examined in the *Hanseniaspora uvarum* strains (UPGMA analysis not shown). There was no increased kinship between the two Californian isolates compared to the two Australian isolates. The absence of a correlation between geography and genome relatedness in the *S. cerevisiae* and *H. uvarum* yeasts is likely due to the influence of humans in the dispersal of wine yeasts.

Surprisingly, of the *S. cerevisiae* strains analysed, the AFLP fingerprints of the laboratory strain was most similar to the type strain, CBS 1171. The main progenitor strain of S288C, and most other laboratory strains, was stated to be a strain isolated from rotting figs in California, EM93 (Mortimer & Johnston, 1986). It was expected that this yeast would be more closely related to the indigenous yeasts isolated from wineries, but no clear relationship between the laboratory yeasts and the winery isolates was evident. A more extensive investigation using additional primer sets and yeasts, including EM93, is currently being done to further analyse the observed similarity.

Cluster analysis of the *D. bruxellensis* AFLP fingerprints agrees with earlier findings on the genetic relatedness of these yeasts. Electrophoretic comparison of enzymes and DNA reassociation experiments led to the seven synonyms of *D. bruxellensis* being incorporated into a single species (Smith *et al.*, 1990). This reclassification has since been supported by other methods including RFLP (Molina *et al.*, 1993) and sequence analysis of mitochondrial (Hoeben *et al.*, 1993) and ribosomal (Boekhout *et al.*, 1994; Yamada *et al.*, 1994) genes. However, enzyme analysis produced two separate groups amongst the strains of this species (Smith *et al.*, 1990). The first group included the type strains of *D. bruxellensis* (CBS 74), *B. bruxellensis* (CBS 72), *D. intermedia* (CBS 4914) and *B. lambicus* (CBS 75). The second group included *B. intermedius* (CBS 73), *B. abstinens* (CBS 6055) and *B. custersii* (CBS 5512). The AFLP results are consistent with this grouping. Analysis of the mitochondrial genome structure of the *Dekkera* yeasts led to the seven *D. bruxellensis* synonyms being incorporated into two species, separating *B. custersii* and *B. abstinens* from the others (McArthur & Clark-Walker, 1983). The differentiation of these two yeasts from the other *D. bruxellensis* strains is also supported by DNA reassociation studies (Smith *et al.*, 1990). Furthermore, the sequence of the *B. custersii* mitochondria-encoded cytochrome oxidase subunit gene (*COX2*) (Hoeben *et al.*, 1993) and 26S rDNA (Boekhout *et al.*, 1994; Yamada *et al.*, 1994) is different from *D. bruxellensis*. Again, the AFLP analysis is consistent with the increased divergence of *B. custersii* and *B. abstinens* from the other *D. bruxellensis* yeasts.

In this study, AFLP was also evaluated for determining the genetic similarity between closely related yeast species. Cluster analysis of the type strains of the *Saccharomyces sensu stricto* species was performed using the results from seven primer pairs. The AFLP fingerprints between these four species were highly polymorphic and UPGMA analysis did not produce a relationship consistent with those obtained using other methods, in particular that of DNA reassociation experiments (Vaughan Martini, 1989; Vaughan Martini & Kurtzman, 1985). A similar difficulty in determining interspecific relatedness using AFLP has been observed with bacteria (Janssen *et al.*, 1997).

In conclusion, AFLP is shown to be a very useful method in discriminating yeasts at both the species and subspecies level. Many of the yeasts in this study have previously been analysed using PCR (de Barros Lopes *et al.*, 1996, 1998) and karyotyping (Henschke, 1990; Petering *et al.*, 1988, 1990). Although karyotyping has been shown to be a useful method for differentiating commercial strains of *S. cerevisiae*, it is of limited use for discriminating species with fewer chromosomes. The advantages of AFLP over other methods are also

its reproducibility (Janssen *et al*., 1996; Jones *et al*., 1997) and its widespread application across all phyla (Janssen *et al*., 1996, 1997; Folkertsma *et al*., 1996; Mueller *et al*., 1996; Otsen *et al*., 1996; Travis *et al*., 1996). Results described here indicate the value of AFLP in studying the intraspecific genetic relatedness of yeasts. Although initially more labour-intensive than other PCR techniques, the amount of information that can be obtained by using multiple sets of primers from a single restriction digestion/ligation is extensive. The main limitation of AFLP in yeast systematics may be its inability to establish genetic similarities between species. For this, gene sequence analysis remains the method of choice (Hoeben *et al*., 1993; Kurtzman, 1992; Palumbi & Baker, 1994). However, sequence analysis of a single genetic locus can lead to erroneous conclusions on the relatedness of species (Palumbi & Baker, 1994) and this is especially relevant with hybrid yeast species (Peterson & Kurtzman, 1991). Sequencing of both monomorphic and polymorphic fragments obtained by AFLP should lead to a better understanding of the AFLP results in terms of interspecific relationships, providing an alternative to single sequence comparisons. In turn, the method may lend itself to obtaining an improved understanding of the relationship between the biological and phylogenetic species concepts.

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