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Application of molecular methods for analysing the distribution and diversity of acetic acid bacteria in Chilean vineyards

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Abstract

The presence of acetic acid bacteria populations on grape surfaces from several Chilean valleys is reported. The bacteria were analysed at both the species and the strain level by molecular methods such as RFLP-PCR 16S rRNA gene, RFLP-PCR ITS 16S-23S rRNA gene regions and Arbitrary Primed (AP) PCR. Our results show that there are limited numbers of species of acetic acid bacteria in the grapes and that there is a need for an enrichment medium before plating to recover the individual colonies. In the Northernmost region analysed, the major species recovered was a non-acetic acid bacteria, *Stenotrophomonas maltophila*. Following the North–South axis of Chilean valleys, the observed distribution of acetic acid bacteria was zonified: *Acetobacter cerevisiae* was only present in the North and *Gluconobacter oxydans* in the South. Both species were recovered together in only one location. The influence of the grape cultivar was negligible. Variability in strains was found to be high (more than 40%) for both Acetobacteraceae species.

Keywords: Grapes; *Acetobacter cerevisiae*; *Gluconobacter oxydans*; *Stenotrophomonas maltophila*

1. Introduction

Acetic acid bacteria (AAB) are Gram-negative, ellipsoidal to rod-shaped cells that have an obligatory aerobic metabolism with oxygen as the terminal electron acceptor. They can use such substrates as glucose, ethanol, lactate or glycerol as energy sources. However, most of these compounds are not completely oxidised into CO_2, and water and several metabolites, especially acetic acid, are accumulated in the growth medium. AAB are commonly found in nature. Because of their high resistance to acidity and the variety of substrates that they can use, they are one of the main food spoilage microorganisms and their presence is mostly related to food modification and human activities for food preservation (De Ley et al., 1984). Acetic acid is one of the main products of AAB metabolism and it is found in many foods as the result of the presence and activity of these bacteria. It is the major volatile acid in wine, and one of the main reasons for wine spoilage (Drysdale and Fleet, 1988).

The AAB that spoil wines can be present in the cellar, although they can also originate in grapes (Gonzalez et al., 2005). It is well known that spoiled grapes and grapes infected with *Botrytis cinerea* have a high population of AAB, which can grow in grape must and accumulate gluconic and acetic acids (Barbe et al., 2001). It has also been reported that excessive growth of AAB on grape berries or in musts can lead to incomplete alcoholic fermentation, which is caused by an AAB/yeast interaction that is not fully understood (Drysdale and Fleet, 1989). Generally, spoiled grapes correlate well with increased populations of *Acetobacter* species, whereas healthy grapes show low populations of *Gluconobacter oxydans* (Du Toit and Pretorius, 2002; Gonzalez et al., 2005).

AAB species have traditionally been identified by testing physiological and chemotaxonomic abilities (De Ley et al., 1984), but these methods are not completely reliable and are time consuming. These phenotypic properties have now been complemented or replaced by such molecular techniques as DNA and rRNA hybridization methods (Urakami et al., 1989),

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sequence analysis (Yamada and Kondo, 1984) or various PCR methods (Bartowsky et al., 2003; Ruiz et al., 2000; Trcek and Teuber, 2002; Trcek, 2005). Some of these molecular techniques, however, are not suitable for the routine identification of bacterial isolates because they are also time consuming and difficult to use with a large number of isolates.

There is an obvious need for further studies into the ecology and development of AAB in grapes, especially in countries such as Chile where the country's isolation could be a source of new genera or species. Also, the valley structure of the vineyards could produce a variation from valley to valley. Furthermore, the economic importance of wine in Chile and the North–South climatic variation add extra interest to ecological studies of the Chilean vineyards. The availability of the aforementioned molecular methods means that these objectives may be achieved, yet it must not be forgotten that most of them have been used in very restrictive environments (wine, vinegar) where the competition with other species is limited. In the present study, we use RFLP-PCR 16S rRNA gene, RFLP-PCR ITS 16S-23S rRNA gene regions and Arbitrary Primed (AP) PCR to investigate the composition of bacterial populations (AAB and non-AAB) associated with healthy grapes in various Chilean vineyards, covering the entire geographic zone where Chilean wine is produced. We also attempted to validate these techniques for more variable environments.

2. Materials and methods

2.1. Sampling, isolation, culture conditions and maintenance of strains

Samples of healthy grapes were obtained from several valleys in Chile between April and May 2004. The valleys were located between latitudes 30 and 36°S. From north to south they were: Limarí, Aconcagua, Casablanca, Maipo, Rapel, Curicó, Maule and BioBio. About 1 kg of grapes was obtained from at least 5 plants of each grape variety. The grape varieties analysed were Cabernet Sauvignon, Carménère, Pinot Noir, Shiraz, and Cabernet Franc and were taken in different places of each valley.

Grapes were homogenized in a Stomacher for 3 min and 1 ml was inoculated in an enrichment medium GY (5% D-glucose, 1% yeast extract) and incubated at 30 °C for 2 days. Then, 100 µl was plated onto GYC (5% D-glucose, 1% yeast extract, 0.5% $CaCO_3$, 2% agar w/v). Plates were incubated at 28 °C for 7–10 days under aerobic conditions. All the colonies or a maximum of twenty were randomly isolated and purified from each sample. The isolates were then grown for 48 h in the liquid medium described above. As a first screening, the isolates that produced a clear halo after acidification in GYC solid medium were selected as putative AAB and analysed further (De Ley et al., 1984).

2.2. Reference strains

Several reference strains were used in this study: *Acetobacter aceti* LMG 1261, *Acetobacter pasterianus* LMG 1262, *Gluconacetobacter liquefacens* LMG 1381, *Gluconacetobacter hansenii* LMG 1527, *G. oxydans* LMG 1408, and *Gluconacetobacter xylinus* LMG 1515, all of which were obtained from the Laboratory of Microbiology of Ghent.

2.3. DNA extraction from bacterial isolates

For each strain studied, 1 ml of an overnight bacterial culture was centrifuged at 10,000 ×*g* for 3 min and the DNA of the pelleted cells was extracted using the Genomic DNA Purification kit from Promega (Madison, WI, USA). The DNA obtained was visualized and the quantity estimated by gel electrophoresis on a 1% agarose in 1× TBE, separated at 100 V for 30 min and then stained with ethidium bromide.

2.4. PCR amplification and analysis of the products

The almost complete 16S rRNA gene and the 16S–23S rRNA gene regions were amplified as previously described using the primers described by Ruiz et al. (2000). PCR amplification products were analysed by gel electrophoresis on an 8% polyacrylamide in 1× TBE, separated at 150 V for 45 min. Gels were stained by silver nitrate following a previously described protocol (Espejo and Escanilla, 1993).

2.5. Restriction analysis

Five microliters (usually 50–100 ng DNA) of each PCR amplified 16S rRNA gene and 16S–23S rRNA gene spacers from bacterial isolates was digested for 2 h with 1.5 U of *Taq*I or *Alu*I restriction endonucleases, as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). Subsequently, proteinase K (Invitrogen, Carlsbad, CA, USA) was added to a final concentration of 0.01 µg/µl, and the reaction was incubated for 1 h at 37 °C (Romero et al., 2002). The resulting fragments were subsequently analysed by 8% polyacrylamide gel electrophoresis and silver nitrate staining, as described above.

2.6. Arbitrary primed-PCR and dendrograms

Primer P3 (5′-GTAGACCCGT-3′), designed by Laohaprertthisan et al. (2003), was used for arbitrary primed amplification.

Table 1
Total isolates and initial identification by RFLP-PCR 16S

Group code	Number of isolates	RFLP-PCR 16S fragment sizes (bp)	Identification
1	37	150–350–850	NI
2	14	150–210–350–850	NI
3	9	210–280–850	NI
4	4	210–550–800	NI
5	6	210–350–550	NI
6	16	80–210–350–850	NI
7	17	80–175–190–210–350–450–580–850	NI
8	4	210–310–500–580	NI
9	4	80–350–850	NI
10	43	210, 350, 800	*Acetobacter aceti*
11	46	110–120–120–160–175–190–350	*Gluconobacter oxydans*

NI: Not identified as Acetobacteraceae.

Table 2
Identification of representative isolates of the different groups and accession codes for the sequences

Group code	Isolate code	No of GenBank	Species
1	62	DQ117919	*Staphylococcus* sp
	101	DQ128313	
2	12	DQ117923	*Stenotrophomonas maltophilia*
	223	DQ128317	
3	225	DQ128318	*Stenotrophomonas maltophilia*
	468	DQ128320	
4	45	DQ128312	*Lactobacillus brevis*
5	189	DQ128316	*Acinetobacter* sp
6	188	DQ128315	*Serratia* sp
7	321	DQ128319	*Enterobacter* sp
9	162	DQ128314	*Curtobacterium* sp
	127	DQ117920	
10	48	DQ117921	*Acetobacter cerevisiae*
	84	DQ117922	
	312	DQ117924	
11	65	DQ117918	*Gluconobacter oxydans*

The reactions were performed in a total volume of 25 μl containing 10 ng of DNA template, 0.55 pmol/μl of primer P3, 2 mM Mg and 0.08 U/μl of *Taq* polymerase in the Invitrogen PCR buffer following the supplier's recommendations (Invitrogen, Sao Paolo, Brazil). Samples were incubated for 5 min at 94 °C, and then cycled 45 times at 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. The samples were kept at 4 °C until tested. Profiles obtained from each strain belonging to the same bacterial species were compared using a binary matrix that was representative of the bands occurring in a set of AP-PCR patterns. The presence or absence of AP-PCR bands in an isolate was scored as presence (1) or absence (0) relative to the bands detectable in all the isolates in a set of AP-PCR patterns (Schafer and Muyzer, 2001). Then, the binary matrix was translated into a distance matrix using similarity calculations and a tree was constructed using the UPGMA clustering method available from Treecon version 1.3b (Van de Peer and De Wachter, 1997). Strain diversity was calculated as the percentage of different strains related to the total number of colonies analysed of the same species.

Table 3
Distribution of AAB and the other genera isolated from grapes of different Chilean valleys

Valleys	Location of vineyard	Latitude	Grapevine variety	Bacteria screened by 16S RFLP	Identified bacteria	Relative frequency (%)	AAB isolates code
Limari	Punitaqui	30°45	Cabernet sauvignon	15	*S. maltophilia*	80	
					Unidentified	20	
Aconcagua	Panquehue	32°46	Cabernet sauvignon	12	*A. cerevisiae*	67	21–26, 28, 48
					L. brevis	33	
Casablanca	Casablanca	33°	Merlot	9	*S. maltophilia*	56	
					Unidentified	44	
Maipo	Antumapu	33°17	Cabernet sauvignon	12	*Staphylococcus*	83	
					Unidentified	17	
Rapel	Las Cabras	34°	Carmenere	19	*Serratia*	84	
					Acinetobacter	16	
	Totihue	34°08	Cabernet sauvignon	6	*A. cerevisiae*	33	126, 127
					Unidentified	67	
	Totihue	34°08	Mouvedre	17	*Enterobacter sp*	100	
	Apalta	34°2	Carmenere	10	*A. cerevisiae*	20	209, 210
					Acinetobacter	30	
					Unidentified	50	
Curicó	Fundo La Fortuna, Lontué	35°	Tintorera	20	*A. cerevisiae*	100	301–320
	Fundo Molina	35°	Cabernet sauvignon	10	*A. cerevisiae*	100	82, 84, 86, 88, 90, 93, 94, 96, 97, 99
	Fundo La Fortuna, Lontué	35°	Malbec	3	*A. cerevisiae*	33	341, 342
					G. oxydans	67	358
	Fundo Molina	35°	Carmenere	16	*G. oxydans*	20	166, 167, 174
					Staphylococcus	30	
					Curtobacterium	25	
					Unidentified	25	
	Fundo La Fortuna, Lontué	35°	Carmenere	11	*S. maltophilia*	55	
					Unidentified	45	
	Fundo La Fortuna, Lontué	35°	Pinot	1	*G. oxydans*	100	292
	Fundo La Fortuna, Lontué	35°	Cabernet sauvignon	7	*G. oxydans*	71	65, 67, 69, 75, 79
					Unidentified	29	
	Curicó	35°03	Shiraz	11	*G. oxydans*	100	401, 402, 406, 409, 411–414, 416, 417, 419
Maule	Talca	35°30	Cabernet sauvignon	20	*Staphylococcus*	95	
					Unidentified	5	
	Talca	35°31	Carmenere	1	*G. oxydans*	100	244
BioBio	Santa Ana	36°80	Pinot	7	*G. oxydans*	100	263, 265, 268, 269, 273, 275, 278
	Santa Ana	36°80	Cabernet Franc	18	*G. oxydans*	89	421, 423–436, 438
					Unidentified	11	

2.7. Sequence analysis

PCR amplified 16S rRNA gene from the bacterial isolates was purified using Wizard PCR Preps (Promega, Madison, WI, USA) and then sequenced with an Applied Biosystems 310 automatic sequencer (Foster City, CA, USA). The ABI Prism dye terminator sequencing kits were used with primers 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) described by McCracken et al. (2001) and 16Sd (5′-GCTGGCGGCATGCT-TAACACAT-3′) described by Ruiz et al. (2000).

Sequences were deposited in GenBank (DQ128312–DQ128320, DQ117918–DQ117924) and aligned with reference sequences, using Sequence Match software from Ribosomal Database Project II website (RDP II; http://rdp.cme.msu.edu/html/) (Cole et al., 2005).

3. Results

3.1. Identification of the isolates by RFLP-PCR 16S and RFLP-PCR ITS

A total of 225 isolates were obtained from grapes from the different valleys and analysed by 16S rRNA gene amplification and RFLP of the PCR product. Although the isolation protocols were designed to recover acetic acid bacteria (AAB), not all the isolates showed patterns belonging to this group. Two hundred isolates presented the expected amplified product (*ca* 1450 bp). Based on the RFLP with the *Taq*I enzyme it was possible to separate them into 11 more frequent groups (Table 1). The two major groups of isolates together included over 40% of the isolates and their profile was identical to that of the type strains of *G. oxydans* (group 11) and *A. aceti* (group 10).

As a second identification system, the RFLP-PCR of the ITS region was tested for the positively identified AAB. The amplicons (700 bp) from the isolates previously identified as *G. oxydans* were the same size, and the restriction fragments with *Alu*I (190, 190, 175 and 110) and *Taq*I (410, 290) were the same as the type strains. On the other hand, the amplicons of the isolates identified as *A. aceti* were the same size (750) as the type strain, yet not the restriction fragments. The fragments for the *A. aceti* reference strain were 290, 130, 110 and 90 bp. However, in the Chilean isolates, fragments of approximately 430, 250 and 100 were observed in PAGE gel. Thus, the initial classification of these isolates as *A. aceti* could not be sustained.

3.2. Identification by rRNA gene 16S sequencing

Representative isolates of all the groups were chosen for partial sequencing of the rRNA gene 16S as a method for identification. The results are summarized in Table 2, which shows that group 8 was the only one that could not be identified as there were many indeterminations in the sequence, indicating that it was probably the result of a mixture of different microorganisms. The isolates identified as *G. oxydans* were all newly identified as such on the basis of the 100% identity of the

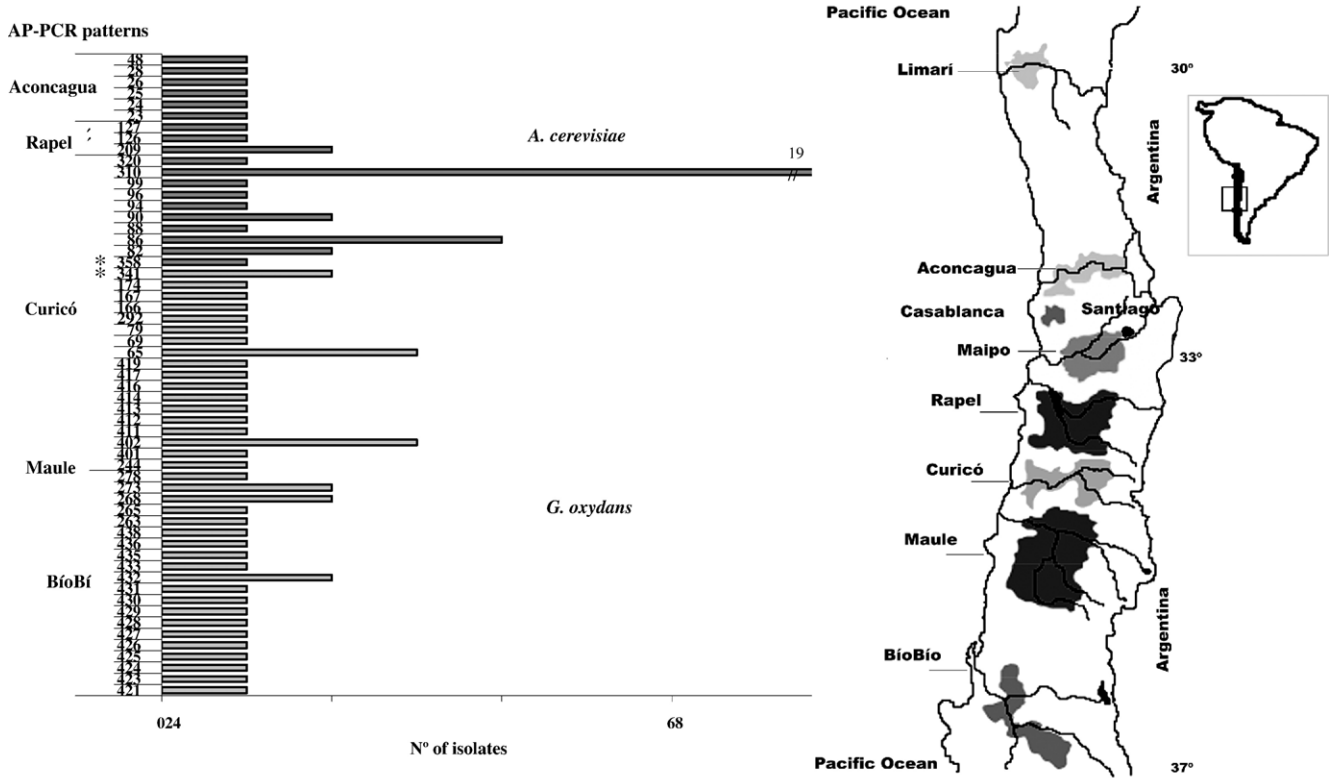

Fig. 1. Map illustrating the N–S distribution along Chilean vineyards sampled and the graphic representation of AP-PCR patterns obtained from isolates of *Acetobacter cerevisiae* and *Gluconobacter oxydans*. * indicated the only location where both *Acetobacteraceae* species were found.

sequence obtained, including 600 bp (positions 210–840 *E. coli* numbering). However, group 10, initially identified as *A. aceti* by RFLP-PCR of 16S and not identified by RFLP-PCR ITS, was classified as *A. malorum*/*Acetobacter cerevisiae* on the basis of the 16S rRNA gene sequence. This sequence was identical to the ones described for the *A. cerevisiae* (T) LMG 1625 (AJ419843) and *A. malorum* (T) LMG 1746 (AJ419844) type strains. In fact, only 5 bp were found different in the 600 bp fragment sequences between the two species *A. aceti* and *A. malorum*/*A. cerevisiae*. Further sequencing of the rRNA gene 16S involving positions 125–845 bp (*E. coli* numbering) made it possible to identify the Chilean isolates as belonging to *A. cerevisiae* species. This was based on the sequences obtained, which included differences between *A. malorum* and *A. cerevisiae* (positions 91 and 136 bp, *E. coli* numbering).

A considerable number of the isolates in groups 1, 2 and 3 also gave a RFLP-PCR 16S pattern similar to that of *A. aceti*. According to the rRNA gene 16S sequence, isolates from group 2 and 3 were classified as *Stenotrophomonas maltophilia*, whereas those from group 1 were classified as *Staphylococcus* sp. Also a group of isolates could be identified as *Lactobacillus brevis*. This was the only non-Acetobacteraceae isolates that were identified at the species level with the region sequenced. The other non-Acetobacteraceae isolates were identified as belonging to the genera *Serratia*, *Acinetobacter*, *Enterobacter* and *Curtobacterium*, yet we did not attempt to characterize them further.

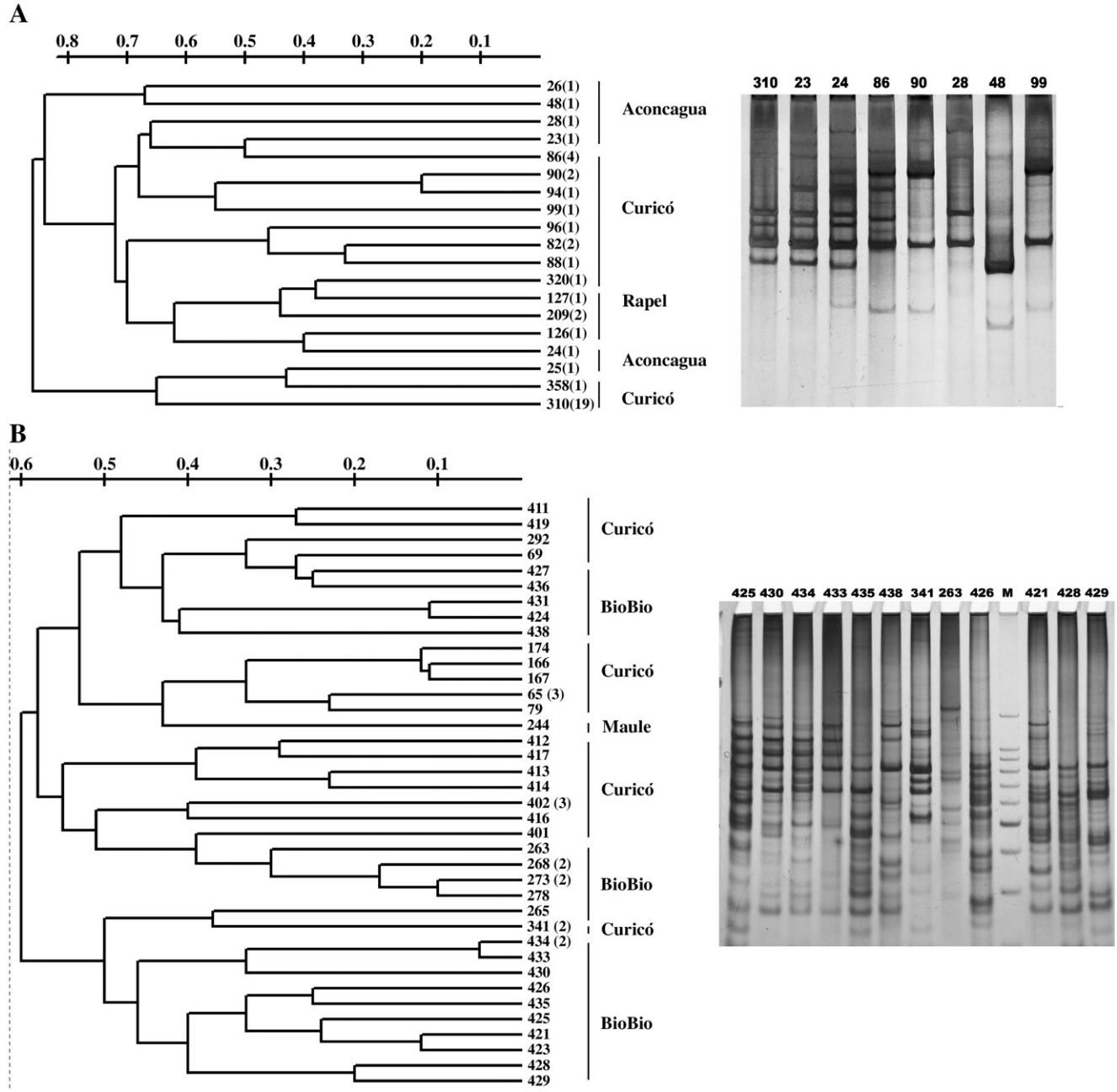

Fig. 2. PAGE of AP-PCR of the isolates of *Acetobacter cerevisiae* (A) and *Gluconobacter oxydans* (B) obtained from Chilean Valleys and dendrograms illustrating the clusters of the patterns by dissimilarity. Lanes indicate the number of the isolate, M: ladder 100 bp. Numbers in parentheses indicate the number of identical profiles obtained.

3.3. North–South species distribution in Chilean valleys

The distribution of species in the Chilean valleys showed a strong correlation with the latitude (Table 3). In fact, north of parallel 35 (latitude S) the only acetic acid bacteria found are *A. cerevisiae* whereas in the south the only one is *G. oxydans*. Only in one location and grape variety (Curicó, Fundo La Fortuna, Malbec) did the two species coexist. Oddly enough, in most of the northern valleys or locations (Limarí, Casablanca, Maipo and Rapel Las cabras and Totihue) no acetic acid bacteria were recovered. No predominant genera were found in these valleys, although *S. maltophilia* was the main species in two valleys and one location and *Staphylococcus*, *Serratia* and *Enterobacter* were the dominant genera in one valley or location. The grape variety was not seen to have any effect, and the Cabernet Sauvignon and Carmenere varieties, which were selected in several valleys, did not show any specific association with a given species. In fact, the latitude was the only relevant variable.

3.4. Typing by AP-PCR

The strains belonging to the two species of acetic acid bacteria were further characterized by Arbitrary Primed PCR (Fig. 1). For each species a presence–absence binary matrix was used to distinguish and group isolates according to their AP-PCR profiles. The limited diversity observed at the species level is in strong contrast with the high diversity of strains. A total of 19 AP-PCR profiles were observed among the 43 isolates of *A. cerevisiae* (44% diversity). Most of them were represented by only one isolate, but one profile (for isolate 310, Fig. 2A) was abundant at one location. Considerable diversity was observed in *G. oxydans* isolates, because 38 AP-PCR profiles were distinguished among the 46 isolates obtained (83% diversity, Fig. 2B). In this case, most of the AP-PCR profiles observed were represented by one isolate. Each strain (or identical AP-PCR profile) was only present in one location for both species.

4. Discussion

Rapid techniques for identifying different microorganisms are needed if ecological studies are to be able to process large numbers of samples. The broadly accepted techniques in bacterial taxonomy are those based on the DNA sequences, as they are not affected by the environmental conditions in which the microorganisms evolve. However, these more resolutive techniques are so expensive and time consuming that they are not suitable for such studies. On the other hand, short-cut techniques are normally used for certain kinds of environments, in which the population is restricted or limited by the environment itself. In the case of acetic acid bacteria, most taxonomic studies that describe new genera or species have been done by selective isolation and sequencing, or DNA–DNA hybridization (Urakami et al., 1989; Yamada and Kondo, 1984). On the other hand, for routine analysis AAB have normally been identified by RFLP-PCR of rRNA gene 16S (Ruiz et al., 2000), ITS 16S–23S (Ruiz et al., 2000; Trcek and Teuber, 2002), or, recently, by PCR of pyrroloquinoline quinone-dependent alcohol dehydrogenase gene (PQQ-ADH) (Trcek, 2005). These techniques have been developed for those environments in which acetic acid bacteria are frequently regarded as a problem or as a necessary agent: for example, wine (Gonzalez et al., 2004, 2005) or vinegar (Trcek, 2005), respectively. However, they have hardly been used to detect AAB in other more open and competitive environments, such as grapes in open fields. Of all those who have previously used these techniques, only Gonzalez et al. (2005) have determined AAB in grapes. They found mostly *G. oxydans* and *A. aceti* with no other contaminant microorganisms.

The findings of previous reports and the limited amounts observed in the Chilean vineyards are different for various reasons. One of these is the technique used for identification, which used to be based on selective media and physiological identification (Joyeux et al., 1984; Du Toit and Lamberchts, 2002; Barbe et al., 2001). Later on, and on the basis of similar molecular methods, Gonzalez et al. (2005) used cycloheximide–penicillin in the media to prevent the proliferation of other bacteria. In this study, however, because growth was limited or null, we decided to allow all bacteria to grow and thus avoid losing AAB diversity, although this involved an obvious risk of increasing the population of other bacteria. Another reason for the difference may be the health status of the grapes, which was excellent in the present study, as observed by the poor recovery of bacteria and also for their identity.

G. oxydans has been observed on the grape surface in almost all the studies made to date on the presence of acetic acid bacteria in both spoiled and healthy grapes (Joyeux et al., 1984; Du Toit and Lamberchts, 2002; Barbe et al., 2001; Gonzalez et al., 2005). Other species, mostly *A. aceti*, have also been found in spoiled grapes (Joyeux et al., 1984), and in both spoiled and healthy grapes, although with higher counts in spoiled grapes (Gonzalez et al., 2005). In all the cases reported, all the species were found to coexist. A clear difference between this study and the others is that we found little coexistence of both AAB species (only in one location) and the distribution was clearly latitude dependent. The presence of *A. cerevisiae* instead of *A. aceti* deserves some mention. The recent description of *A. cerevisiae* (Cleenwerk et al., 2002) makes impossible its detection in previous works (Joyeux et al., 1984; Barbe et al., 2001; Du Toit and Lamberchts, 2002). However, Gonzalez et al. (2006) could have detected it as they used a combination of several restriction enzymes that discriminate among *A. aceti*, *A. pasteurianus* and *A. cerevisiae*. Thus, the most likely situation is that *A. cerevisiae* may be absent from European vineyards. The lack of initial discrimination between *A. cerevisiae* and *A. malorum* is because they are extremely similar. If these strains are to be identified at species level, genotypic characterization is required, because phenotypic characteristics are very similar (Cleenwerk et al., 2002). Their % G+C content values were similar, and their 16S rRNA gene sequences were more than 99.9% similar (Cleenwerk et al., 2002). This similarity between them makes discrimination more difficult and justifies the use of other more discriminating techniques (*i.e.* full sequencing of the 16S rRNA gene, DNA–

DNA hybridization). Their name indicates their origin because so far all the isolates that have been identified as *A. cerevisiae* (except one which has no indication of origin) have originated in beer or breweries (Cleenwerk et al., 2002). Thus, this is the first time that an isolate of *A. cerevisiae* has been found in the environment and in a completely different niche.

The fact that other AAB such as *A. aceti* or *A. pasterianus* (Joyeux et al., 1984; Gonzalez et al., 2005), *G. liquefaciens* (Du Toit and Lamberchts, 2002) or *G. hansenii* (Gonzalez et al., 2005) have not been reported in grapes from the Chilean valleys cannot be attributed only to the limitations of sample collection or growth in the solid media, as these were the same in other studies in which the counts were also very low (10^2–10^3 cfu/g). The isolation of Chilean viticulture, and particularly the absence of the phylloxera (*Daktulosphaira vitifoliae*, Fitch) and other phytopathogens or vectors, probably accounts for the lack of AAB "contamination". The valley structure may also enhance the isolation, as can be seen by the clearly defined North–South distribution of the two species found and the fact that they do not coexist.

Other species and genera are ubiquitous, and microorganisms are generally present in plants and soils. It is therefore no surprise to find them on grapes. Some of these genera, including AAB, have been reported in association with insects or nematodes, which can act as vectors (Dillon and Dillon, 2004). This could be the case of *Stenotrophomonas* (Pidiyar et al., 2004; Mohr and Tebbe, 2006) or *Enterobacter* (Behar et al., 2005). Some of the genera have also been reported in association with plants, and even act as plant growth promoters (Behrendt et al., 2002). *Serratia* isolates have been associated with cucurbit yellow vine disease and have been detected in bee gut (Zhang et al., 2003; Mohr and Tebbe, 2006). Some reports indicate that strains belonging to genus *Stenotrophomonas* can degrade some herbicides (Schoenborn et al., 2004; Barreiros et al., 2003) and, thus, could be selectively favoured in crops, as in vineyards. In summary, these genera contain strains of great variability and with the ability to survive. Most of them have been described in association with insect or plants. Therefore, it is not surprising that these bacteria are recovered from grapes.

Strain characterization by AP-PCR has been used to type AAB strains that originated in wine and which were identified as *A. pasteurianus* species (Bartowsky et al., 2003). Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR) (Nanda et al., 2001; Gonzalez et al., 2004) have also been proposed as methods for typing strains. In all cases, considerable diversity has been observed, as in our results. Diversity accounting about 50% of the total isolates was found on grape surfaces. This diversity decreased during wine fermentation (Gonzalez et al., 2005), wine storage (Bartowsky et al., 2003) and vinegar production (Nanda et al., 2001), although in all cases strain diversity was still high. All these results show that these methods are appropriate for typing AAB strains.

The diversity of *G. oxydans* is greater than that of *A. cerevisiae*, which agrees with the observations of Gonzalez et al. (2005). This can be understood as a tendency to high variability in a species that is only present in natural environments, whereas the *Acetobacter* species can be found and selected in more selective man-made environments (cellars, vinegar production plants, etc).

In conclusion, this is the first time that the distribution of AAB in the Chilean vineyards has been studied. They are difficult to isolate and their recovery is poor, which may be correlated to the healthy status of the grapes. Two AAB species were found with a very limited overlap between them. One of them, *G. oxydans*, has always been reported to be present in grapes from different origins, whereas the other, *A. cerevisiae*, has not previously been described in natural habitats. However, the strain diversity is very high as has been reported in other natural habitats.

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