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Application of Molecular Methods for the Identification of Acetic Acid Bacteria Isolated from Blueberries and Citrus Fruits

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Sixteen acetic acid bacteria (AAB) were isolated from blueberries and citric fruits of the Salto Grande region (Concordia, Entre Ríos, Argentina) using enrichment techniques and plate isolation. Enrichment broths containing ethanol and acetic acid enabled maximum AAB recovery, since these components promote their growth. Biochemical tests allowed classification of the bacteria at genus level. PCR-RFLP of the 16S rRNA and PCR-RFLP of the 16S-23S rRNA intergenic spacer allowed further classification at the species level; this required treatment of the amplified products of 16S and 16S-23S ITS ribosomal genes with the following restriction enzymes: *Alu*I, *Rsa*I, *Hae*III, *Msp*I, *Taq*I, *Cfo*I, and *Tru*9I. C7, C8, A80, A160, and A180 isolates were identified as *Gluconobacter frateurii*; C1, C2, C3, C4, C5, C6, A70, and A210 isolates as *Acetobacter pasteurianus*; A50 and A140 isolates as *Acetobacter tropicalis*; and C9 isolate as *Acetobacter syzygii*. The bacteria identified by 16S rRNA PCR-RFLP were validated by 16S-23S PCR-RFLP; however, the C1 isolate showed different restriction patterns during identification and validation. Partial sequencing of the 16S gene resolved the discrepancy.

Keywords: Acetic acid bacteria, isolation, PCR-RFLP, 16S rRNA sequencing, citrus fruits, blueberries

Introduction

Acetic acid bacteria (AAB) are an important group of microorganisms widely used in vinegar production [1], mainly due to their ability to oxidize ethanol to acetic acid [2]. Although these bacteria have been studied since Pasteur's era, the wide diversity of AAB has been of great interest by microbiologists in recent years. Ecological studies have been carried out all over the world, mainly in tropical regions (Thailand, Indonesia, Philippines, Japan).

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The AAB taxonomy has been updated since 1989 when *Acetobacteraceae* family comprised only two genera, *Acetobacter* and *Gluconobacter* [3], to the present, in which nineteen genera and eighty eight species are recognized [4–7]. Development and application of new technologies, molecular techniques (particularly, partial sequencing of 16S ribosomal gene), bioinformatic tools and free access to sequence databases have made possible the revision of AAB nomenclature and classification. Consequently, description of new taxa and reclassification of AAB species have exponentially increased [1, 5–7].

Selective AAB isolation can be expected when the medium composition in the enrichment procedure is modified, mainly the carbon sources, since genera have

different physiological properties [8]. In fact, strains of *Acetobacter* and *Gluconacetobacter*, both used in vinegar production, were isolated in broths with ethanol and acetic acid [7, 9] as well as from alcoholic and acidic environments like vinegar, wine, beer, sake, cider, fruit [4, 8]. By contrast, *Gluconobacter* strains were recovered in a medium with sorbitol from sugary materials, fruits, flowers, foods, and other sources [3, 4].

Microbial identification has traditionally been carried out through physiological study and chemotaxonomic properties [7, 10]. However, these methods are incomplete and do not allow the identification of AAB at the species level [4, 11], besides being time-consuming techniques. Nowadays, molecular biology provides a variety of techniques to perform a genotypic identification: digestion of PCR products of the 16S gene with restriction enzymes (restriction fragment length polymorphism, RFLP) has been used for routine identification of AAB at species level [12–14]. The 16S ribosomal gene is highly conserved but presents enough variability to differentiate genera and species [10]. This is a fast and reliable method and provides a high reproducibility of restriction patterns [12, 14, 15]. Since it does not always discriminate between closely related species because of the high degree of similarity in the 16S ribosomal gene sequence, it is recommended to use PCR-RFLP of the 16S-23S intergenic spacer (ITS) [16, 17]. In addition, analysis of 16S ribosomal RNA (rRNA) gene sequence is the most common method used to identify AAB [17]. The direct sequencing of the 16S rRNA gene and its comparison with public databases is widely used for the identification of AAB [2, 11, 14].

Since AAB belong to the primary epiphytic microflora of fruits, vegetables and flowers, some investigations reported the isolation and identification from these substrates [3, 4, 18]. In addition, isolations from fermented foods and beverages such as cocoa [19], coffee [20], wine and grape must [17, 21, 22], and honey [23] were reported and consequently, new AAB species and genera have been described [24–28]. This increasing diversity highlights the high colonization capacity of this group of bacteria, which suggests AAB may be present in diverse materials in different regions of the world.

The aim of this study was to isolate and identify AAB from blueberries and citrus fruits from the Salto Grande region by biochemical and molecular methods (PCR-RFLP 16S rRNA, PCR-RFLP 16S-23S ITS rRNA and 16S rRNA sequencing). AAB characterization will also allow evaluating its possible use as starter culture for the production of regional vinegar. Since there are no AAB ecological studies reported in Argentina, this work would contribute to expand AAB diversity knowledge.

Material and Methods

Sample collection and preparation

Samples of healthy tangerines and oranges were obtained from the Salto Grande region (Entre Ríos, Argentina). The analyzed tangerine varieties were Nova and Clementine, while orange varieties were New Hall and Navel. The fruits were washed and processed for analysis as follows:

- Isolation of epiphytic bacteria: the fruits were macerated in 100 ml of peptone water at 25 ± 1℃ for 30 min. A volume of the supernatant was then inoculated into-enrichment broth.
- Isolation of bacteria from the juice: the fruits were cut in halves, squeezed with a manual juicer and the juice was used for enrichment.
- Isolation of bacteria from the fermented juice: 20 ml of each sample juice were incubated at 30 ± 1℃ for 15 days under aerobic conditions to achieve spontaneous fermentation. Subsequently, they were used for enrichment.

Blueberries varieties, collected during South American harvesting season (October, November and December) were Bluquinex, Millennia, Jewel and O'Neal. They were washed and aseptically processed in Stomacher IUL Instruments (Spain) for 30 s in orden to crush the fruit and improve contact with the transport medium used to recover the bacteria. Then, they were inoculated into the enrichment broths.

Enrichment and isolation media

The following enrichment broths were used: broth I containing 2% glucose, 5% ethanol (v/v), 1% yeast extract [18]; broth II containing: 1% glucose, 0.5% ethanol (v/v), 0.8% yeast extract, 1.5% meat peptone, 0.3% acetic acid (v/v) [3] and broth III containing: 1% glucose, 1% ethanol (v/v), 0.2% yeast extract, 0.2% meat peptone and 1% mannitol [3]. Seven ml of each medium and 7 ml of each sample (supernatant, fruit juice, fermented fruit

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juice, fruit pulp) were placed in test tubes. 0.2 g/kg of potassium sorbate were added to each tube in order to inhibit molds and yeast growth. Samples were incubated at 30 ± 1℃ for 3 days, under aerobic conditions. When microbial growth was found, the culture was streaked onto a GEY agar plate containing 0.3% $CaCO_3$ [3]. They were incubated at 30 ± 1℃ for 10 days. Morphological and cultural characteristics of the isolates were examined by incubating at 30 ± 1℃ for 2 days on GYPG medium [3]. Morphological characteristics were determined by Gram staining and mobility was observed by using the "pending drop" technique in an optical microscope (Leica DME, USA) with a 1000X magnification.

Phenotypic characterization

AAB were inoculated into culture medium containing each carbon source to determine acid production [29]. Sugars and alcohols tested were: L-arabinose, D-arabinose, L-rhamnose, D-mannose, L-sorbose, melibose, D-xylose, D-galactose, D-glucose, D-fructose, sucrose, raffinose, D-mannitol, D-sorbitol, dulcitol, glycerol, ethanol and propanol. Acetate and lactate oxidation were detected by using culture medium containing 0.2% acetate or 0.2% lactate, 0.2% yeast extract, 0.3% peptone, and 0.002% bromothymol blue. Catalase was determined by using 3% v/v H_2O_2 solution and oxidase, by discs impregnated with p-aminodimethylaniline oxalate (Argentina). The oxidation of acetic acid to CO_2 was performed by a technique described by Yamada *et al.* [29].

Ubiquinone determination

AAB were cultured in GYPG broth [3] on orbital shaker at 30 ± 1℃ for 48 h. Cells were collected and ubiquinone extracted from cell pellets according to the method described by Tanasupawat *et al.* [30].

DNA extraction from bacterial isolates

Each strain was cultured in test tubes with 5 ml of GY broth (1% glucose, 1% yeast extract) at 30 ± 1℃ for 72 h. A volume of 1 ml of each culture was centrifuged at 2400 g for 3 min and the DNA of the pelleted cells was extracted using the UltraClean Microbial DNA Kit (Mo Bio Laboratorios, USA). The DNA thus obtained was visualized by gel electrophoresis on a 1% agarose in 1× TBE buffer with 0.5 µg/ml ethidium bromide, separated at 100 V for 45 min. Molecular weight marker of 1 kb was used (Genbiotech, Argentina). DNA was stored at -20 ± 1℃ until use.

PCR amplification and analysis of the products

The 16S rRNA gene and the 16S–23S rRNA gene regions were amplified as previously mentioned using the primers described by Ruiz *et al.* [12]. PCR amplification products were analyzed by gel electrophoresis on an 1% agarose in 1× TBE buffer with 0.5 µg/ml ethidium bromide, separated at 100 V for 50 min. Molecular weight marker of 1 kb was used (Genbiotech).

Restriction analysis

Eight microliters of each PCR amplified 16S rRNA gene and 16S-23S rRNA gene spacers from bacterial isolates were digested with 4 U of restriction endonucleases, as recommended by the manufacturer (Thermo Scientific, USA). *Alu*I, *Rsa*I, *Hae*III, *Msp*I, *Taq*I, *Cfo*I and *Tru*9I were the endonucleases tested. The resulting fragments were subsequently analyzed by 3.5% agarose gel electrophoresis in 1× TBE buffer with 0.5 µg/ml ethidium bromide, developed at 100 V for 2.5 h. Molecular weight marker of 100 bp was used (Genbiotech).

Partial 16S rRNA sequence of AAB taken from the GenBank was virtually sliced with restriction enzymes using the BioEdit Sequence Alignment Editor program [31].

16S rRNA gene sequencing and sequence analyses

The amplification products of 16S rRNA gene were sent for purification and subsequent sequencing (Genbiotech, Argentina). Sequencing reaction was performed using ABI 3730XL sequencer.

Sequences were edited with MEGA version X program [32] and compared with 16S rRNA sequences of the GenBank database. NCBI BLAST software [33] was used for identification. Then, a phylogenetic tree based on 16S rRNA gene sequences was constructed with MEGA program. In order to evaluate the relative stability of the tree branches, the Neighbor-Joining method [34] was employed with 1000 replicas bootstrap. The evolutionary distance was calculated using Maximum Composite Likelihood method. The multiple alignments of the isolates sequences and those obtained from the database were carried out by CLUSTALW. *Rhodopila globiformis* DSM 161 strain was used as external group.

Results

Isolation and biochemical identification of AAB

A total of 16 AAB isolates were obtained from fruit samples. They were all Gram-negative, rod-shaped, aerobic, motile, oxidase-negative, catalase positive and produced clear zones around the colony on the GEY agar containing $CaCO_3$. These biochemical tests confirmed that the studied bacteria belong to the *Acetobacteriaceae* family. AAB recovered from the assayed enrichment media are shown in Table 1. Nine strains (C1, C2, C3, C4, C5, C6, C7, C8, C9) were isolated from citrus fruits and seven, from blueberries (A50, A70, A80, A140, A160, A180, A210).

Results of biochemical tests for the 16 isolated AAB strains are presented in Table 2. 11 strains were assigned to the genus *Acetobacter* based on the ability to intensely oxidize acetate and lactate to CO_2 and H_2O and on the presence of Q9 as the mayor quinone. The remaining 5 strains were assigned to the genus *Gluconobacter*, since acetate and lactate were not oxidized and Q10 was recognized as the major quinone. In addition, they grew on mannitol agar and developed neither in medium containing 30% glucose nor in glutamate agar.

PCR-RFLP of the 16S rRNA - PCR-RFLP of 16S-23S ITS rRNA

PCR amplified products (approximately 1450 bp) corresponding to the 16S RNA gene and to the 16S-23S intergenic spacer (approximately 750 bp) were obtained from all AAB. Seven restriction endonucleases (*Taq*I, *Rsa*I, *Msp*I, *Hae*III, *Alu*I, *Cfo*I, *Tru*9I) were tested to cut the amplified 16S rRNA and 16S-23S. Sizes of PCR-RFLP fragments are shown in Table 3. The identification of AAB at the species level was carried out by comparing the sizes of restriction fragments experimentally obtained from the PCR 16S and 16S-23S product with those reported in the literature for strains of different collections. Restriction patterns for citrus and blueberries isolates generated with *Alu*I enzyme are presented in Figs. 1 and 2, respectively.

No bibliography was found to verify the restriction groups formed with some assayed enzymes, so partial sequences of 16S ribosomal gene and ITS 16S-23S were obtained from the Genbank database. Virtual slices using BioEdit Sequence Alignment Editor program allow finding matches with the laboratory cuts.

Treatment of amplified 16S-23S with restriction endonucleases produced four restriction patterns with *Msp*I in considered strains. Also, three restriction patterns were obtained with *Hae*III and *Taq*I enzymes, while

Table 1. AAB isolated from different blueberries and citrus fruits according to the assayed enrichment broths.

Fruit varieties	Enrichment broths			Isolates
	Broth I	Broth II	Broth III	
T. Nova, peel	1	-	-	C1
T. Clementine, fermented juice	1	-	-	C2
O. Navel, fermented juice	1	-	-	C3
O. Navel, fermented juice	1	1	1	C4 - C5 - C6
O. Navel, fermented juice	2	-	-	C7 - C8
O. Navel, fermented juice	1	-	-	C9
B. O'Neal	1	-	-	A50
B. Bluquinex	-	1	-	A70
B. Millennia	1	1	-	A180 - A80
B. Jewel	1	-	-	A160
B. O'Neal	1	-	-	A140
B. O'Neal	-	1	-	A210
Totals	11	4	1	

AAB isolation on GEY $CaCO_3$ agar from different enrichment broths. T: tangerine; O: orange; B: blueberries; C: bacteria isolated from citrus fruits; A: bacteria isolated from blueberries.

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Table 2. Physiological and biochemical characteristics of *Acetobacter* strains.

	Acetobacter											*Gluconobacter*				
	C1	C2	C3	C4	C5	C6	C9	A50	A70	A140	A210	C7	C8	A80	A160	A180
Acetate oxidation	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Lactate oxidation	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Production of AA from GEY with $CaCO_3$ agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in the presence of 0.35% (v/v) AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in the presence of 30% (w/v) D-glucose	+	+	+	+	+	+	+	+	nd	nd	nd	-	-	-	-	-
Growth in mannitol agar	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in glutamate agar	+	-	nd	nd	nd	+	nd	-	+	+	+	-	-	-	-	-
Acid production from:																
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	-	+	-	-	-	-	-	-	+	-	-	+	+	+	+	+
D-galactose	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	W	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Propanol	+	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
D-mannitol	-	W	+	+	+	+	-	-	-	-	-	+	+	+	+	+
D-sorbitol	-	-	+	+	-	+	+	-	-	-	-	+	+	+	+	+
L-sorbose	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+
L-Arabinose	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+
D-arabinose	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
D-xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saccharose	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+
Maltose	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Melibiosa	+	-	-	+	-	+	-	-	+	+	-	-	-	-	-	+
L-rhamnosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Butanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidation of AA	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Major ubiquinone	Q9	Q9	Q9	Q9	Q9	Q9	Q9	Q9	Q9	Q9	Q9	Q10	Q10	Q10	Q10	Q10

C: bacteria isolated from citrus fruits; A: bacteria isolated from blueberries. W: weakly; -: negative; +: positive; nd: not determined; AA: acetic acid; Q9: ubiquinone 9; Q10: ubiquinone 10.

*Rsa*I was not discriminatory since it only produced one restriction pattern in C7, C8, A80, A160 and A180 strains (Table 4).

16S rRNA gene sequencing

The results of the 16S rRNA isolates gene sequencing showed consistency, as expected, with the identification performed by PCR-RFLP (Table 5). Particularly, the C1 isolate sequencing allowed to solve the aforementioned ambiguity between 16S-23S rRNA PCR-RFLP and 16S PCR-RFLP results. Gene sequence analysis confirmed that C1 strain belongs to *A. pasteurianus* species (98% similarity), which agrees with 16S-23S PCR-RFLP analysis.

Table 3. Sizes (bp) of PCR-RFLP fragments obtained from the 16S rRNA and the 16S-23S ITS rRNA.

	PCR-RFLP of 16S rRNA		PCR-RFLP of 16S-23S ITS rRNA	
	Pattern	Restriction fragments	Pattern	Restriction fragments
*Alu*I	A1	550-290-210-190-120-70	ITS A1	350-250-100
	A2	310-280-230-210-190-120		
	A3	450-310-290-190-190-70		
	A4	450-310-280-190-120		
*Taq*I	T1	350-190-175-160-120-110-90	ITS T1	410-290
	T2	650-375-210-180	ITS T2	400-380
	T3	500-375-370-210-70	ITS T3	375-325
	T4	500-375-210-175-160		
*Cfo*I	Cf1	430-340-180-160-140-110	ITS Cf1	600-150
	Cf2	550-350-210-180-150		
	Cf3	520-420-210-150-140-70		
	Cf4	525-350-210-150-140-90		
*Hae*III	H1	520-280-200-180-160	ITS H1	500-230
	H2	520-280-180-160-120	ITS H2	480-310
	H3	520-280-180-160-160	ITS H3	480-290
*Rsa*I	R1	400-400-300-150-120	ITS R1	500-120-100
	R2	500-400-300-150-110	ITS R2	750
*Msp*I	M1	450-310-220-120-120	ITS M1	550-150
	M2	450-425-210-125-125-70	ITS M2	440-320
			ITS M3	300-190-190
			ITS M4	700
*Tru*9I	Tr1	530-350-350-150-110	ITS Tr1	600-150
	Tr2	530-350-250-150-110		

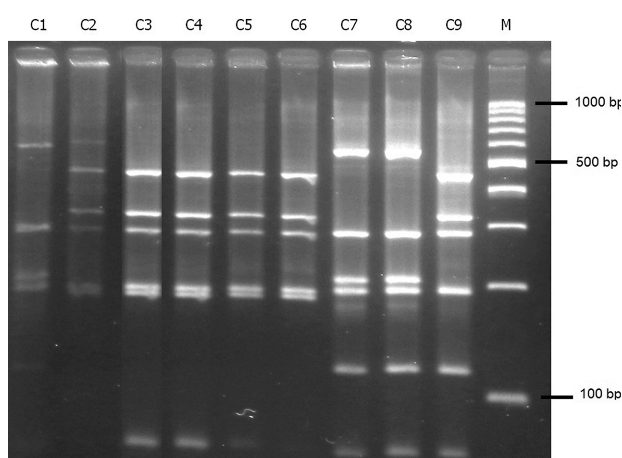

Fig. 1. *Alu*I restriction patterns of PCR-amplified 16S rRNA of AAB isolated from citric fruit. 3.5% w/v agarose gel electrophoresis in 1× TBE buffer with 0.5 μg/ml ethidium bromide. M: 100 bp DNA ladder.

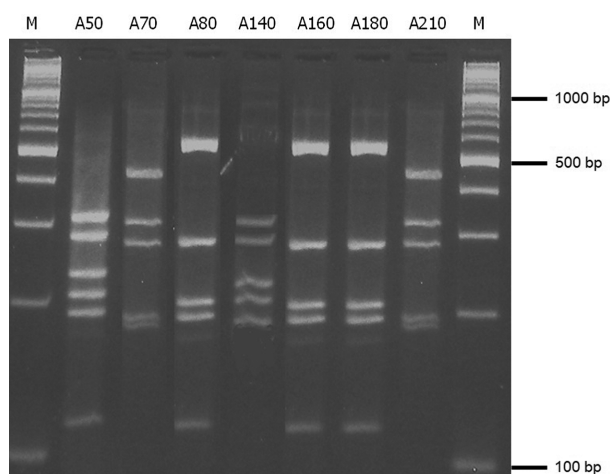

Fig. 2. *Alu*I restriction patterns of PCR-amplified 16S rRNA of AAB isolated from blueberries. 3.5% w/v agarose gel electrophoresis in 1× TBE buffer with 0.5 μg/ml ethidium bromide. M: 100 bp DNA ladder.

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Table 4. AAB classification according to the electrophoretic analysis of PCR-RFLP of 16S rRNA and PCR- RFLP of ITS 16S-23S rRNA.

Isolated AAB	PCR-RFLP of 16S rRNA							PCR-RFLP of 16S-23S ITS rRNA			
	*Rsa*I	*Msp*I	*Tru*9I	*Hae*III	*Alu*I	*Taq*I	*Cfo*I	*Msp*I	*Rsa*I	*Hae*III	*Taq*I
A80	R1	M1	Tr1	H1	A1	T1	Cf1	ITS M1	ITS R1	ITS H1	ITS T1
A160	R1	M1	Tr1	H1	A1	T1	Cf1	ITS M1	ITS R1	ITS H1	ITS T1
A180	R1	M1	Tr1	H1	A1	T1	Cf1	ITS M1	ITS R1	ITS H1	ITS T1
C7	R1	M1	Tr1	H1	A1	T1	Cf1	ITS M1	ITS R1	ITS H1	ITS T1
C8	R1	M1	Tr1	H1	A1	T1	Cf1	ITS M1	ITS R1	ITS H1	ITS T1
C1	R1	M1	TR1	H3	A1	T1	Cf1	ITS M2	ITS R2	ITS H2	ITS T1
A50	R2	M2	TR1	H2	A2	T2	Cf2	ITS M3	ITS R2	ITS H2	ITS T1
A140	R2	M2	TR1	H2	A2	T2	Cf2	ITS M3	ITS R2	ITS H2	ITS T2
A70	R2	M2	TR2	H3	A3	T3	Cf3	ITS M2	ITS R2	ITS H2	ITS T2
A210	R2	M2	TR2	H3	A3	T3	Cf3	ITS M2	ITS R2	ITS H2	ITS T2
C2	R2	M2	TR2	H3	A3	T3	Cf3	ITS M2	ITS R2	ITS H2	ITS T2
C3	R2	M2	TR2	H3	A3	T3	Cf3	ITS M2	ITS R2	ITS H2	ITS T2
C4	R2	M2	TR2	H3	A3	T3	Cf3	ITS M2	ITS R2	ITS H2	ITS T2
C5	R2	M2	TR2	H3	A3	T3	Cf3	ITS M2	ITS R2	ITS H2	ITS T2
C6	R2	M2	TR2	H3	A3	T3	Cf3	ITS M2	ITS R2	ITS H2	ITS T2
C9	R2	M2	TR2	H2	A4	T4	Cf4	ITS M4	ITS R2	ITS H3	ITS T3

A phylogenetic tree (Fig. 3) was built on the basis of isolate partial sequences of the 16S ribosomal gene and reference strains. As previously stated, sequences obtained with the C2 and A140 isolates exhibited many ambiguous bases, which resulted in a smaller sequence size and a low percentage of identity; therefore they were not included in the construction of the phylogenetic tree. All the included isolates exhibited phylogenetic filiation and two groups were observed.

Discussion

The greatest recovery of AAB was achieved in enrichment broth named I, which contained the highest ethanol concentration. Successful isolation was due to the efficacy of ethanol present in the broth to promote their growth [23]. AAB was not isolated from citrus fruit juice, probably due to the low competitiveness of these bacteria against other microorganisms. However, spontaneous fermentation of juices allowed a good recovery of acetic bacteria probably due to the produced ethanol that could have selected them by decreasing the accompanying flora. Similarly, Beheshti Maal *et al.* [35] isolated AAB, later identified as *Acetobacter*, from fermented musts obtained by placing dates, peaches and apricots at 30℃ for 7 days.

C1, C2, C3, C4, C5, C6, C9, A50, A70, A140 and A210 bacteria were identified as *Acetobacter* since they oxidized lactate and acetate and they had Q9 as the major quinone, as observed by Komagata *et al.* [4]. *Gluconacetobacter* and *Komagataeibacter* also oxidize acetate and lactate, however, they differentiate from *Acetobacter*, since it presents ubiquinone Q9 and the other possess ubiquinone Q10 [4]. The other biochemical tests assayed, such as production of acids from sugars and alcohols, rendered variable results according to the species of *Acetobacter*. Therefore, they are not characteristics that allow genus differentiation [4, 26]. PCR-RFLP of the 16S rRNA and 16S-23S ITS rRNA performed later, supported that they belong to the *Acetobacter* and *Gluconobacter* genera. It is important to mention that some strains of *Acetobacter* have phenotypic characteristics that are similar to one another. For their species level identification, genotypic characterization (as PCR-RFLP) is required [4]. The *Gluconobacter* genus shows a similar behavior.

Fruit vinegars are obtained through double fermentation of their juices, performed by yeasts and AAB [36].

Table 5. Results of 16S rRNA gene sequencing compared to those obtained in GenBank using the BLAST program (NCBI).

Isolates	Identification*
C1	*Acet. pasteurianus* IFO 3283-01 (99)
	Acet. pasteurianus LMG 1262 (99)
C2	*Acet. pasteurianus* IFO 3283-01 (94)
	Acet. pasteurianus LMG 1262 (94)
C3	*Acet. pasteurianus* IFO 3283-01 (99)
	Acet. pasteurianus LMG 1262 (99)
C4	*Acet. pasteurianus* IFO 3283-01 (99)
	Acet. pasteurianus LMG 1262 (99)
C5	*Acet. pasteurianus* IFO 3283-01 (99)
	Acet. pasteurianus LMG 1262 (99)
C6	*Acet. pasteurianus* IFO 3283-01 (99)
	Acet. pasteurianus LMG 1262 (99)
C7	*G. frateurii* LMG 1365 (99)
	G. frateurii LMG 3264 (99)
C8	*G. frateurii* LMG 1365 (99)
	G. frateurii LMG 3264 (99)
C9	*Acet. syzygii* NBRC 16604 (98)
	Acet. ghanensis LMG 23848 (98)
A50	*Acet. tropicalis* NBRC 16470 (99)
	Acet. tropicalis Ni-6b (99)
A70	*Acet. pasteurianus* IFO 3283-01 (98)
	Acet. pasteurianus LMG 1262 (98)
A80	*G. frateurii* LMG 1365 (99)
	G. frateurii LMG 3264 (99)
A140	*Acet. tropicalis* NBRC 16470 (97)
	Acet. tropicalis Ni-6b (97)
A160	*G. frateurii* LMG 1365 (99)
	G. frateurii LMG 3264 (99)
A180	*G. frateurii* LMG 1365 (99)
	G. frateurii LMG 3264 (99)
A210	*Acet. pasteurianus* IFO 3283-01 (98)
	Acet. pasteurianus LMG 1262 (98)

*percentage of similarity between isolates and AAB taken from GenBank.

The main AAB species, responsible for the production of vinegar, belong to the *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter* genera [7]. Therefore, AAB isolated in this work could be used as an inoculum for vinegar production. However, other studies, such as acetification tests to determine the capacity to oxidize ethanol to acetic acid and the resistance to acetic acid will be needed.

For 16S PCR-RFLP analyses, *Alu*I, *Taq*I and *Cfo*I [37] resulted the most discriminant restriction enzymes, which produced four different patterns, whereas *Hae*III exhibited three and *Rsa*I, *Msp*I and *Tru*9I, 2 (Table 3). C1, C7, C8, A80, A160 and A180 were identified by *Cfo*I endonuclease, since this enzyme presents different patterns for *G. cerinus, G. frateurii, G. oxidans* and *G. asai,* but they have equal patterns when digested with the other tested enzymes (*Rsa*I, *Msp*I, *Taq*I, *Alu*I, *Hae*III). The Cf1 pattern corresponding to those isolates agreed with that of *G. frateurii*, which allowed their identification. In addition, these isolates showed pattern A1 with *Alu*1, as reported by Gonzalez *et al.* [37]. A virtual cut of the 16S partial sequence of *G. frateurii* NBRC 16669 was carried out, since the pattern that generates *Tru9I* enzyme has not yet been reported. The result indicated its agreement with Tr1 pattern found in this work. Although C1 isolate was identified as *G. frateurii*, it has shown differences in the number and size of the restriction pattern bands obtained with *Hae*III as reported by Blasco Escrivá, [38]. In addition, differences with other isolates in this group (identified as *G. frateurii*) were observed when it was subjected to PCR-RFLP of ITS 16S-23S for the enzymes *Rsa*I, *Msp*I, *Hae*III and *Taq*I (Tables 3 and 4).

A50 and A140 were identified by the pattern given with the *Taq*I enzyme, since *Rsa*I, *Tru*9I, *Msp*I, *Hae*III were not discriminatory. *Alu*I and *Cfo*I endonucleases exhibit common patterns to several *Acetobacter* species [37], so they were not useful for the identification of those isolates. However, these results, together with the *Taq*I pattern, were enough for A50 and A140 final identification since *Taq*1 allows *A. tropicalis* and *A. indonesiensis* separation [37]. Both isolates were identified as *A. tropicalis*. The fragments obtained by the 16S partial sequence of *A. tropicalis* with *Hae*III, *Rsa*I and *Msp*I virtual cut resulted identical to those obtained in laboratory assays.

*Rsa*I, *Msp*I, and *Tru*9I enzymes were not discriminatory to identify C2, C3, C4, C5, C6, A70 and A210 isolates, since the observed restriction patterns are the same as several *Acetobacter* species [37]. The pattern obtained with *Taq*I was the same as *A. pomorum* and *A. pasteurianus*, therefore they could not be differentiated.

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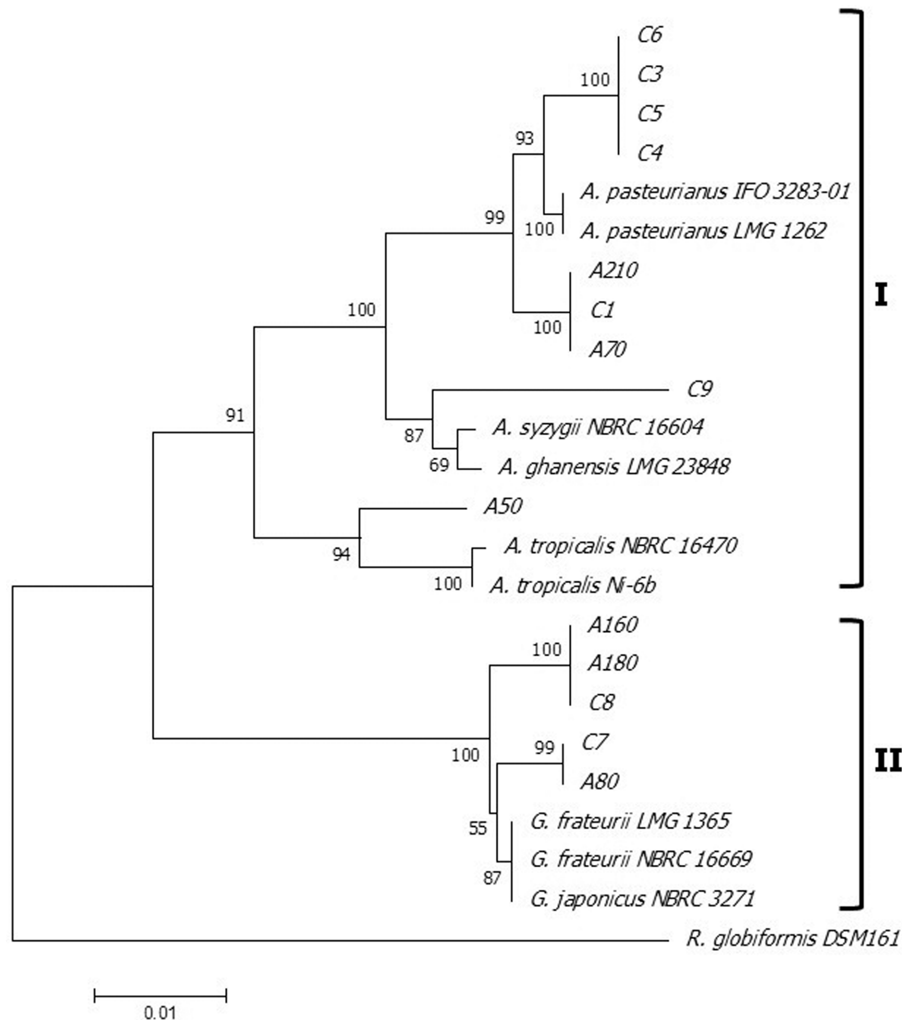

Fig. 3. Phylogenetic relationships of isolates recovered from fruits with reference strains taken from GenBank. The phylogenetic tree, based on 16S rRNA gene sequences, was constructed by the neighbor-joining method. *Rhodopila globiformis* DSM 161 was used as an outgroup. Numerals at nodes indicate bootstrap values derived from 1000 replications.

Finally, *Alu*I endonuclease allowed the identification since the pattern only belonged to *A. pasteurianus*. C9 was identified as *A. syzygii* by the restriction fragments exhibited with *Alu*I, *Taq*I and *Cfo*I. The other tested enzymes did not allow an identification because their patterns are common to several species of *Acetobacter*. González *et al.* [37] identified *A. syzygii* only with patterns obtained with *Alu*I and *Taq*I, as they resulted most discriminatory for this species.

The 16S-23S ITS rRNA gene is regarded as having a higher discriminatory power than the 16S rRNA gene because it inhibits more polymorphisms [16]. Although it may be more resolutive, in some cases it may give more than one pattern for the same species as well as, some different, unknown patterns for known species [15]. *Tru*9I, *Cfo*I and *Alu*I enzymes did not differentiate groups from the RFLP-PCR 16S-23S ITS, since all the studied AAB generated the same restriction pattern (Table 3).

The pattern generated with *Msp*I from C1, C2, C3, C4, C5, C6, A70 and A210 isolates coincided with *A. pasteurianus* as reported by some authors [12, 39]. However, it differed from the restriction patterns reported by Tanasupawat *et al.* [41] for the same species. C7, C8, A80, A160 and A180 isolates pattern was the same as that reported by Ruiz *et al.* [12] for *G. frateurii*

LMG 1365. On the other hand, Tanasupawat *et al.* [40] reported a 500 and 200 bp pattern for *A. syzygii* NBRC 16604, which differs from results obtained in this study, since the C9 isolate displayed a 700 bp single band. The ITS M3 morphotype was not found in the literature. For this reason and taking into account these isolates had been previously identified by PCR-RFLP as *A. tropicalis*, a virtual section of the sequence 16S-23S was carried out. It was found that they had the same pattern.

H2 pattern generated by *Hae*III endonuclease from C1, C2, C3, C4, C5, C6, A50, A70, A140 and A210 yielded the same results as those reported for *A. pasteurianus* in previous works [39, 40]. The ITS morphotype named H3 produced from C9 was practically identical to that found by Tanasupawat *et al.* [40] for *A. syzygii* NBRC 16604. Only a few differences were observed, around 20 bp, which could be due to different conditions used in the aforementioned study (New England BioLabs enzymes, primers and different electrophoresis conditions).

ITS T2 pattern obtained with *Taq*I enzyme agrees with that reported for *A. pasteurianus* LMG 1262 [12]. ITS T1 morphotype showed a few differences with those found by Thi Lan Vu *et al.* [41] for *G. frateurii* NBRC 3264, possibly due to the use of different primers. C9 isolate showed only one pattern and different from the others.

Since pattern obtained with *Rsa*I does not agree with results recorded for *G. frateurii* LMG 1365 [12], a virtual cut was performed for the sequences of ITS 16S-23S of *G. frateurii NBRC* 16669. Bands of 465-117-95 bp appeared, resulting almost the same as those found in this study. Small differences in base pairs may be due to different primers utilized, as mentioned above. This enzyme did not recognize any cutting site for C1, C2, C3, C4, C5, C6, A70 and A210 isolates, which agrees with results informed by Ruiz *et al.* [12] for *A. pasteurianus* LMG 1262.

In general, the RFLP-PCR 16S–23S ITS analysis confirmed the results obtained by RFLP- PCR 16S although some differences were observed for the C1 strain. While the PCR-RFLP 16S rRNA test identified it as *G. frateurii*, PCR-RFLP 16-23S ITS rRNA showed the same pattern as *A. pasteurianus*. Thus, if rRNA RFLP-PCR technique does not appropriately differentiate species, 16S rRNA gene sequencing may be applied.

Although identification at a species level was initially carried out by RFLP-PCR, sequencing of the 16S rRNA gene was necessary to obtain a correct identification. A140 and C2 isolates showed a low percentage of identity (89 and 88%, respectively, results not shown), which may be due to a higher number of ambiguous bases, which implies a lower percentage of alignment with sequences of other known bacteria. However, the sequences could also belong to new species that have not yet been published in GenBank. In fact, there are different criteria in the percentage of similarity of the 16S rRNA for belonging or not to the same species [10]. According to the recommendations of the "Ad hoc Committee for the re-evaluation of species definition in bacteriology", isolates of the same species must present DNA homology values of 16S rRNA equal to or greater than 97% [42]. Therefore, A140 and C2 isolates could belong to new species, whose partial sequences have not been published and hence the low percentage of identity concerning known species. Further research will be needed to confirm these identities.

Isolates identified within the *Acetobacter* genus are placed in group I, divided into three subgroups. C1, C3, C4, C5, C6, A70 and A210 are located in subcluster 1 with *A. pasteurianus* LMG 1262 and *A. pasteurianus* IFO3283-01. The 16S rRNA gene sequence similarities obtained by pairwise alignment showed that C3, C4, C5 and C6 isolates were most closely related to each other (100%) and to *A. pasteurianus* LMG 1262 and *A. pasteurianus* IFO3283-01 (99%). Similarly, C1, A70 and A 210 showed 100% equality between them and less similarity with *A. pasteurianus.*

In subgroup 2, the C9 isolate is closely related to *A. syzygii* NBRC 16604 and *A. ghanensis* LMG 23848 reference strains (98%). C9 was identified both by PCR-RFLP and by sequencing as *A. syzygii*; nevertheless, the same percentage of alignment and identity is observed with respect to other species: *A. ghanensis* and *A. lambici*. Finally, in subgroup 3, the isolate identified as *A. tropicalis* (A50) and the reference strains *A. tropicalis* NBRC 16470 and *A. tropicalis* Ni-6b exhibited 99% of similarity. Isolates identified as *G. frateurii* (A80, A160, A180, C7 and C8) as well as *G. frateurii* NBRC 16669, *G. frateurii* LMG 1365 and *G. japonicus* NBRC 3271 standard strains were placed in group II. A160, A180 and C8, as well as C7 and A80, showed 100% similarity

among them. The five isolated strains showed 99% similarity with the standard strains used for the construction of the phylogenetic tree.

In conclusion, AAB strains were isolated from blueberries and citrus fruits collected in the Salto Grande region (Entre Ríos, Argentina). Although AAB isolates were recovered from all the samples, the number was particularly higher in fermented samples. The isolates were assigned to genera *Acetobacter* and *Gluconobacter* considering their phenotypic characteristics and biochemical tests. Subsequently, they were successfully identified at the species level by molecular techniques including PCR-RFLP 16S, PCR-RFLP 16S-23S and 16S DNA gene sequence analysis. Although C2 and A140 isolates were identified, the 16S gene sequences analysis showed a low similarity with reference strains, so further studies will be necessary to obtain their proper identification. Acetification tests to determine the capacity to oxidize ethanol into acetic acid and the resistance to acetic acid, should also be performed. Detection of AAB species that had not been previously reported neither from blueberries nor citric fruits, contributes to enhance the knowledge of the AAB diversity in these ecological niches.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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