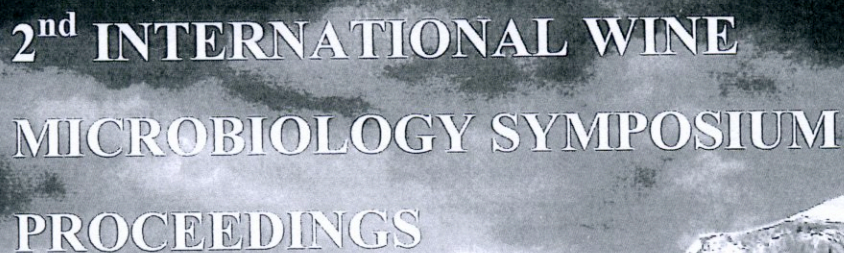

2nd INTERNATIONAL WINE MICROBIOLOGY SYMPOSIUM PROCEEDINGS

MARCH 29-30, 2011

Tenaya Lodge Yosemite

Presented by

CALIFORNIA STATE UNIVERSITY, FRESNO

Department of Viticulture and Enology
Viticulture and Enology Research Center

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PROCEEDINGS

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With Support from Industry Sponsors

New Bacterial Species in the Wines of the 21st Century

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Abstract

The study of the ecology of wine lactic acid bacteria (LAB) is living a new scenario, not foreseen before. This has come with the advent of new laboratory techniques (mainly but not only molecular), together with new technological viticultural and enological practices, and conditioned by the climate change. A polyphasic approach has proved essential to reveal this situation, where new species or new appointments have been described: new descriptions of LAB not isolated before in wines have been made, and even new species of LAB have been described recently. And this is only the beginning of a growing list of descriptions, every day broader and deeper, establishing new ecological and phylogenetical relationships among wine microorganisms.

Together, these new species bring new properties and interactions, some beneficial and some detrimental to the wine quality. Some of these bacteria harbor certain metabolic traits than can influence the wine composition, and in some cases could spoil wines. They can synthesize interesting compounds such as polysaccharides, but also biogenic amines, off-odors, or noxious substances.

A similar situation of new descriptions and new species related to acetic acid bacteria is happening.

Introduction

The LAB is a group of Gram positive, catalase negative and non-spore-forming bacteria. At the morphological level they can take the form of cocci, rods or coccobacilli. They can be strict anaerobic or microaerophilic organisms. All are chemoorganotroph and fermentative, characterized by the production of lactic acid as major end product of fermentation of carbohydrates (Kandler 1983). Some are acid-tolerant.

In grapes, the amount of LAB is low (usually less than 10^3 cfu/mL), however it increases during the last days of ripening, depending mainly on weather conditions. During the first days of alcoholic fermentation (AF), the amount of LAB generally increases to a maximum of 10^4 cfu/mL and then decreases to levels around 10^2 cfu/mL at the end of AF, mainly due to competition from yeast, and the sensitivity to SO_2 and ethanol. After AF, the number of LAB increases and malolactic fermentation (MLF) begins when cells reach levels of 10^6 cfu/mL. When all the malic acid is degraded, the wine is generally stabilized with sulphite. Most LAB are eliminated with this treatment because of their sensitivity to SO_2, leaving small populations of LAB (≤1-10 cfu/mL). However, some bacteria can survive this treatment and grow in the wine to levels of 10^6 cfu/mL-10^7 cfu/mL (Davis et al. 1986).

Species of LAB that have been isolated from grapes are *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus hilgardii*. However, the grape must contains a great diversity of species such as *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus hilgardii, Lactobacillus brevis, Pediococcus damnosus, Pediococcus parvulus, Pediococcus pentosaceus, Leuconostoc mesenteroides* and *Oenococcus oeni* (Davis et al. 1988; DuPlessis et al. 2004; Edwards and Jensen 1992).

Phylogenetic analysis

Sequences of 16S rRNA genes were obtained from GenBank or by applying the methods described by Rodas et al. (2005). They were subjected to phylogenetic analysis by using several reconstruction methods (neighbour-joining, maximum parsimony and maximum likelihood) in the BioNumerics V2.5-6.5 software package to infer the phylogeny of those strains. Pairwise distances were calculated with the Mega 3.1-5.0 software.

DNA-DNA hybridization

Genomic DNA was extracted from pure cultures following the method described by Sambrook et al. (1989), but adding CTAB extraction buffer as reported Gardes and Brun 1993. DNA was quantified spectrophotometrically using a DU 800 spectrophotometer (Beckman Coulter) at 260 nm and adjusted to a final concentration of 300 ng μL^{-1} in Mili U water. A set of DNA-DNA hybridization experiments was performed between wine and reference strains in order to know if they belonged to the same or different species. Hybridizations were performed in duplicate as described by Ziemke et al. (1998).

Genotypical characterization

Amplification and Msel and Bfal restrictions of 16S rRNA gene were done as described Rodas et al. (2003). Amplification and HaeIII restriction of ISR were done as described Chenoll et al. (2003). RAPD analysis with COC and 17R primers and ribotyping with EcoRI enzyme were performed as reported Rodas et al. (2005).

Digitalized gel images were analyzed by the BioNumerics V2.5-6.5 software package and a dendrogram was obtained for each technique using Pearson´s product moment correlation coefficient for RAPD and ribotyping, and Dice similarity coefficient for the rest of experiments. A dendrogram derived from comparison of all combined techniques was constructed using UPGMA clustering method, maintaining the same similarity coefficients used for single pattern analysis. Similarity values between genotypic patterns were calculated.

G+C content determination

The G+C content was determined by hydrolyzing the DNA enzymatically and quantifying the nucleosides by HPLC, as previously reported Tamaoka et al. (1984) and Ziemke et al. (1998).

Morphological, physiological and biochemical characterization

Cell morphology and motility were studied with a phase-contrast optical microscope (Leica DMB 5000) on wet mounts of liquid cultures; motility was also assayed in mMRS soft agar (0.4 % w/v agar) (Mañes-Lázaro et al. 2008). Colony morphology was examined on mMRS agar plate cultures after four days of growth. Gram character was deduced by the KOH method (Gregersen 1978) and the presence/absence of catalase activity was determined as Cappuccino and Sherman (1992) described. Gas production from glucose and gluconate was determined in MRS fermentation broth (Scharlab) provided with 0.5% (w/v) of the carbohydrate and Durham tubes; fermentation tubes were inoculated at 1% (v/v) with a washed cell suspension grown on mMRS broth to late logarithmic phase, and incubated for 15 days at 28 °C under anaerobic conditions. The final products from glucose fermentation were identified and quantified by HPLC as described Frayne (1986). The optical nature of the lactate isomer formed was determined by subtraction of L-lactic isomer, quantified using an L-lactic enzymatic kit (Roche, cat. no. 139084) from the total lactic acid as determined by HPLC. Production of ammonia from arginine was tested using the HFA medium described by Pilone et al. (1991) but replacing vegetable juice serum by 20% tomato juice. Production of mannitol from fructose was analyzed by HPLC (Frayne 1986). Production of exopolisaccharide from sucrose was tested on mMRS agar, in which glucose was replaced by 5% (w/v) sucrose (Hitchener et al. 1982). Carbohydrate fermentation tests were carried out on API 50 CHL galleries (bioMérieux) following the procedure recommended by the manufacturer; results were recorded after

Contrary other strains or groups of strains did not, demonstrating that they would constitute new species, grouped strains 59b with 54, Mont 4 with 116 and 154, 8 with 24 and 68, and 203 alone.

Physiological and molecular tests were performed to compare the phenotypical and genomic characteristics of isolates and reference strains: gas production from glucose and gluconate, isomer of lactic acid produced, growth at pH 3.0 and 5 % and 10 % NaCl, ammonium production from arginine, mannitol production from fructose, motility, fermentation of carbohydrates (API 50 CH), 16S-ARDRA (MseI, BfaI, DdeI, and EcoRI), RAPD with primers 16R, 17R and COC, ISR (DdeI, EcoRI and HaeIII), ribotyping, and macrorestriction (SfiI, NotI and SmaI). With all this information, we were able to describe new species of *Lactobacillus*, isolated from wines, and new descriptions of existing species not previously reported in wines.

Description of *Lactobacillus bobalius* sp. nov. (41)

The description is: *Lactobacillus bobalius* (bo.ba'li.us N.L. masc. adj. bobalius pertaining to the grape variety Bobal). Gram-positive, non-motile, non-spore forming rods, measuring 0.71 to 1.03 µm width by 1.65 to 3.41 µm length. Cells are found singly, in pairs and in short chains. Microaerophilic. Colonies on MRS agar after 4 days incubation at 28 °C are 1.8-2 mm in diameter, smooth, circular to slightly irregular and white. Catalase negative. Growth occurs from 15 to 45°C, but not at 5°C. Facultative heterofermentative, no gas is produced from glucose. DL-lactate is produced as an end product from hexoses and pentoses. Ammonia is not produced from arginine and mannitol is not produced from fructose. Dextran is not produced from sucrose. Citric and malic acids are used. Strain 203 ferments ribose, glucose, fructose, mannose, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose, β-gentibiose and gluconate, and hydrolyses aesculin. On the other hand, it does not ferment glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, lactose, melibiose, inulin, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. The G+C content is 34.03±0.77 mol%. The cell wall contains A4 α L-Lys-D-Asp peptidoglycan type.

Species *Lactobacillus bobalius* sp. nov. was proposed for strain 203, which was isolated in 1997 by A. M. Rodas from a Bobal grape must. Reference strain is 203^T (=CECT 7310^T=DSM 19674^T) (Mañes-Lázaro 2010). The differential characters between *L. bobalius* and its closest phylogenetic neighbors, and other phenotypical and genomic traits, can be retrieved from literature (Mañes-Lázaro et al. 2008). Some rapid discriminating and not discriminating tests can be seen in Figure 4, and its morphology in Figure 5.

Description of *Lactobacillus uvarum* sp. nov. (Manes-Lazaro et al. 2008)

The description is: *Lactobacillus uvarum* (u.va'rum. L. fem. n. uva of grape). Gram-positive, motile, non-spore forming rods of 0.89 to 1.2 µm width by 1.18 to 3.48 µm length. Cells are found singly, in pairs and in short chains. Aerotolerant. Colonies on MRS agar after 4 days incubation at 28 °C are 1-1.25 mm in diameter, white, smooth, circular and with entire edges. Catalase negative. Growth occurs at pH 4.5 and 8 and in the presence of 5 % (w/v) NaCl, but neither at pH 3.3, nor with 10 % (w/v) NaCl. Obligate homofermentative: gluconate and pentoses are not fermented and glucose is fermented but no gas is released. L-lactate is produced as exclusive end product from hexoses. Ammonia is not produced from arginine, and mannitol is not formed from fructose. Exopolysaccharide is produced from sucrose. All strains ferment glucose, fructose, mannose, mannitol, methyl α-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, maltose, sucrose, trehalose, β-gentiobiose and D-turanose, and hydrolyse aesculin. The isolates do not ferment glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, rhamnose, dulcitol, inositol, methyl α-D-mannoside, lactose, melibiose, inulin, melezitose,

In addition, the type strain shows the following traits: it does not produce ammonia from arginine, ferments D-ribose, it does not ferment D-galactose, methyl □-D-mannoside and D-tagatose, it is unable to hydrolyze arginine, and does not split arbutine. The mol % G+C content is 39.4. Species *Lactobacillus vini* sp. nov. is proposed for six strains, Mont 4T, 116, 119, 154, 155 and 209P. The type strain, Mont 4T (DSMZ 20605T, CECT 5924T) was isolated in 1978 from high temperature fermenting grape must (Barre 1978). Reference strains are 116 (CECT 7072) and 154 (CECT 7073), both isolated from Spanish fermenting grape musts (Rodas et al. 2006).

This species shows a strange and not foreseen pathway for the fermentation of pentoses in *Lactobacillus*. Traditionally, LAB that ferment pentoses make this by using a phosphoketolase that splits them into glyceraldehyde-3-phosphate (that yields lactate) and acetyl-phosphate (that is converted into ethanol or acetate). We could exclusively detect DL-lactate as end product from pentoses in *L. vini* strains, with molar ratios of 1.57-1.70 (lactate/pentose) for all strains and pentoses, near to the theoretical 1.67 molar ratio lactate/pentose value. These results are in agreement with those obtained previously (Barre 1978; Kandler 1983; Picataggio et al. 1998), and are explained by the existence of transaldolase and transketolase activities, permitting strains of *L. vini* to use pentose sugars via an inducible pentose phosphate pathway. This pathway that yields exclusively lactate as a final product is different to the 6-phosphogluconate pathway used by heterofermentative facultative lactobacilli (Rodas et al. 2006).

The differential characters between *L. vini* and its closest phylogenetic neighbors, and other phenotypical and genomic traits, can be retrieved from literature (Rodas et al. 2006). Some rapid discriminating and not discriminating tests can be seen at Figure 10, and its morphology at Figure 11.

New descriptions for existing species not previously reported in wine

Some of the strains that we isolated did not constitute new species, but new descriptions for existing species not previously reported in wine. We performed with these isolates many physiological and molecular tests, even DNA-DNA hybridization when necessary, as in the above described case of strain 71 that finally belonged to *L. satsumensis* (Manes-Lazaro 2010). This organism had been initially isolated from *shochu*, a traditional Japanese distilled spirit made from fermented rice (Endo and Okato 2005). A similar situation occurred for strains that we isolated from grape juices and wines, and belonged to *Lactobacillus harbinensis*, isolated previously from fermented vegetables (Miyamoto et al. 2005), *Lactobacillus coryniformis*, previously isolated from dung (although it is not its only habitat) (Rodas et al. 2003), *Lactobacillus vaccinostercus*, isolated previously also from cow's dung (Ines 2007), or *Lactobacillus pantheris*, previously isolated from jaguar faeces (Liu and Dong 2002).

What about acetic acid bacteria?

A similar situation occurs for this group of microorganisms, and in the coming years new species and new descriptions for existing species not previously reported in wine will be made. We have isolated some strains that did not fit to other acetic acid bacteria previously reported in wine (Figure 12). The phylogenetic tree obtained for the 16S rRNA genes can be observed in Figure 13. Some of these isolates corresponded to existing species not previously reported in wine: *Kozakia baliensis, Acetobacter ghanensis*, and *Acetobacter syzygii*. And some isolates are consistent with the description of new species of *Acetobacter* sp., as strains Bo3, Bs10, and Bo7. We have already performed DNA-DNA hybridization experiments for these strains and confirmed thus that constitute new species, and soon we will submit the proposal for their publication.

So this system provides us with a useful strategy for the fast and sensitive detection of microorganisms from wine. In Figure 15 a working schedule of the Enochip is presented.

Another technique developed by our group Enolab is based on Fluorescence *In Situ* Hybridization (FISH), using fluorescent oligonucleotide probes, homologous to 16S rDNA of those species of bacteria (both lactic acid bacteria and acetic acid bacteria), commonly found in wines (Blasco et al. 2003). The specificity of the probes was evaluated similarly to the above described Enochip. Probes can be used to identify species in different types of grape juices and wines, making evident that direct identification and quantification from natural samples without culturing is also possible. The results show that FISH is a convenient technique for the rapid identification of wine microorganisms, allowing positive identification in a few hours (4 - 16 h from sampling to final results). Training is very easy, results are consistent and easy to visualize and understand, and equipment is rather general and cheap: a florescence microscope, a filtering tower, and an incubator or water bath.

The FISH protocol represents an excellent and rapid method to solve different enological problems such as detection of species related to wine spoilage or production of toxic compounds, quality control of wines before bottling, and detection of microbial contaminations in yeast or malolactic starters. Besides this, the use of FISH in winemaking can be useful to predict the occurrence or not of malolactic fermentation, to identify the species responsible for this process, and to know the effects of enological treatments on microbial populations. From an enological point of view, this technique permits to study the population dynamics of the targeted species during the winemaking process. The advantages of FISH over cultural techniques for enumeration and identification of LAB from must or wine include: (i) rapid availability of quantitative results (this technique allows to obtain results in approximately 6 hours); (ii) broad range of identification, as broad as the number of probes used; (iii) the possibility of simultaneous identification of different species in the same sample; (iv) relatively low cost per experiment (0.45 to 3.0 Euros in consumables, depending on the number of simultaneously processed samples). A working schedule of the FISH process is presented in Figure 16, with a few examples of specific hybridizations.

What's next?

We can suggest what characteristics should meet the new methods for the new millennium, indicating the requirements and desires about them. Obviously first is cheapness, thinking from the consumers' point of view, in this case the winemakers and service companies. But some other characteristics must be desirable for these techniques, of course: no need of cultures or amplifications, automatable, objective, able to process a large number of samples in a few time, no need of special training, reproducible, reliable. Regarding the putative applications in wine microbiology, these methods should reveal information about: identification to species level, typing to strain level (control of starters), estimation of cell viability (directly, without plating), determination of metabolic traits (activities of cells, and prediction of their impact in the final wine), detection of technologically interesting genes (beneficial and detrimental), and applicable to yeasts, lactic acid bacteria and acetic acid bacteria.

Which are these 'super' methods? Future will tell, but we can already project some ideas from the present developments and try to envisage the new methods, or new applications in preexisting methods. An example is the use of biosensors, based for instance in the molecular probes that already have been assayed with success in the Enochip or FISH. We are testing these probes to be used in biosensors for the detection and identification of microorganisms directly in wines. Similarly, these probes could be used or adapted to flow cytometry methods (Rodriguez and Thornton 2008), which could gather other interesting possibilities above mentioned in a single analysis: identification, typing, estimation of cell viability, determination of metabolic traits, detection of technologically interesting genes, etc. And direct analysis of wine samples with FTIR technology (or FTIR-ATR) is a wish closer every

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A)

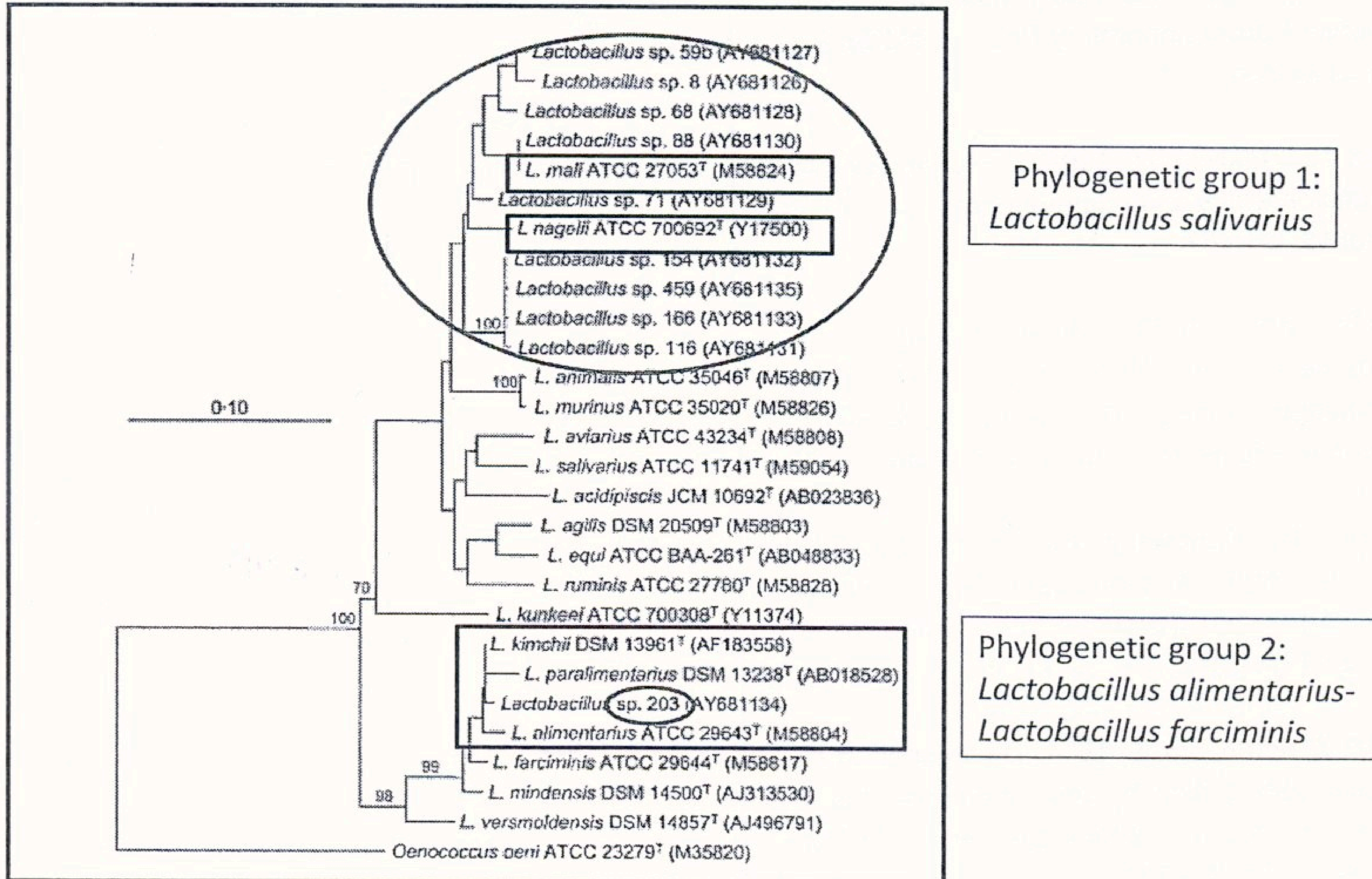


Figure 2. Phylogenetic trees showing the positions of strains 8, 59b, 68, 71, 88, 116, 154, 166, 203 and 459, and some *Lactobacillus* species based on 16S rRNA gene sequences. Clustering is based on neighbor-joining. Bootstrap values (expressed as percentages of 1000 replicates) of 70% or greater are shown at branch points. GenBank accession numbers are given in parentheses. Bar, 10% (A), and 2% (B) and (C) nucleotide substitutions.

Phylogenetic group 2: *Lactobacillus alimentarius-farciminis*

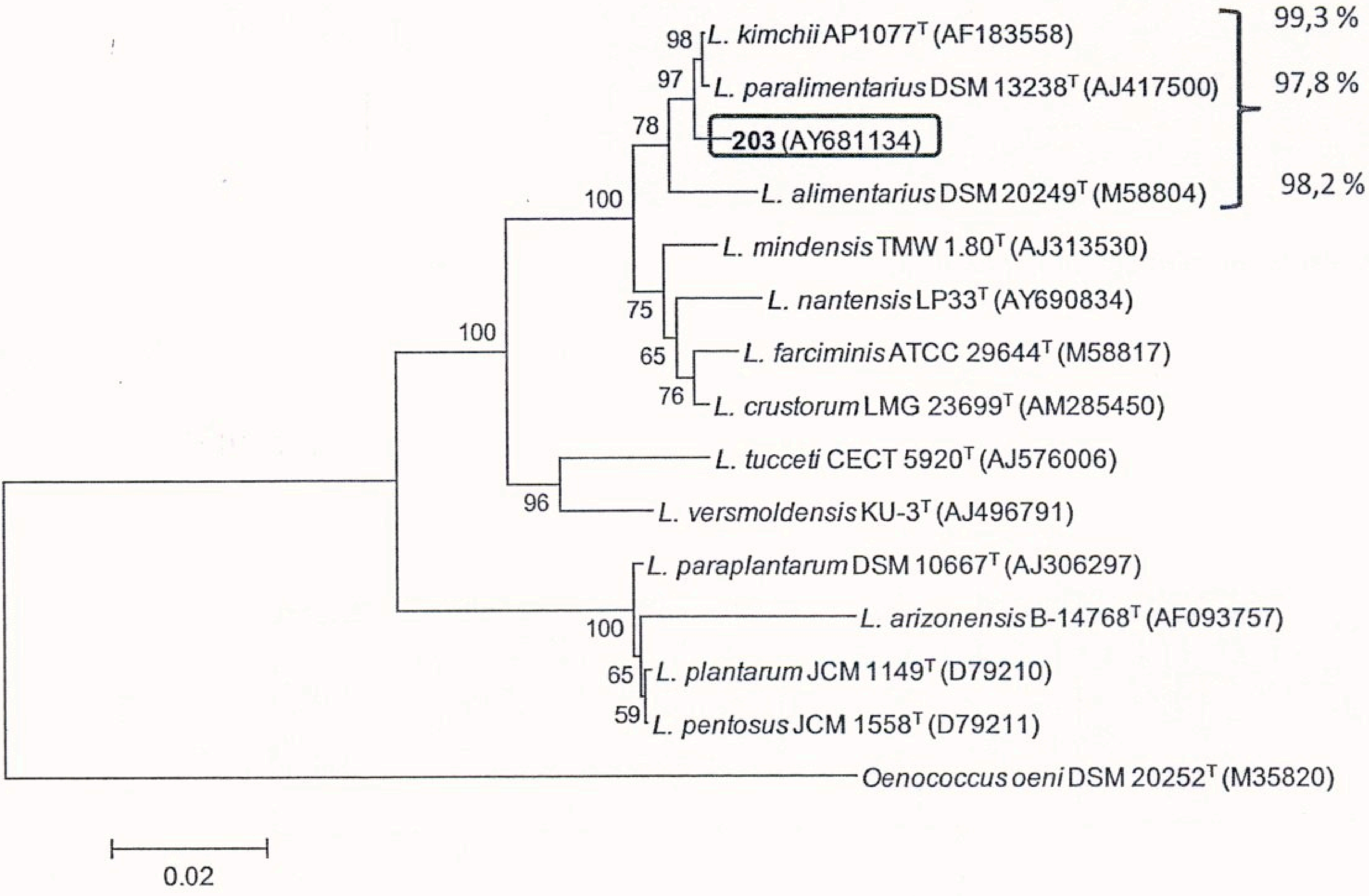


Figure 2 (C)

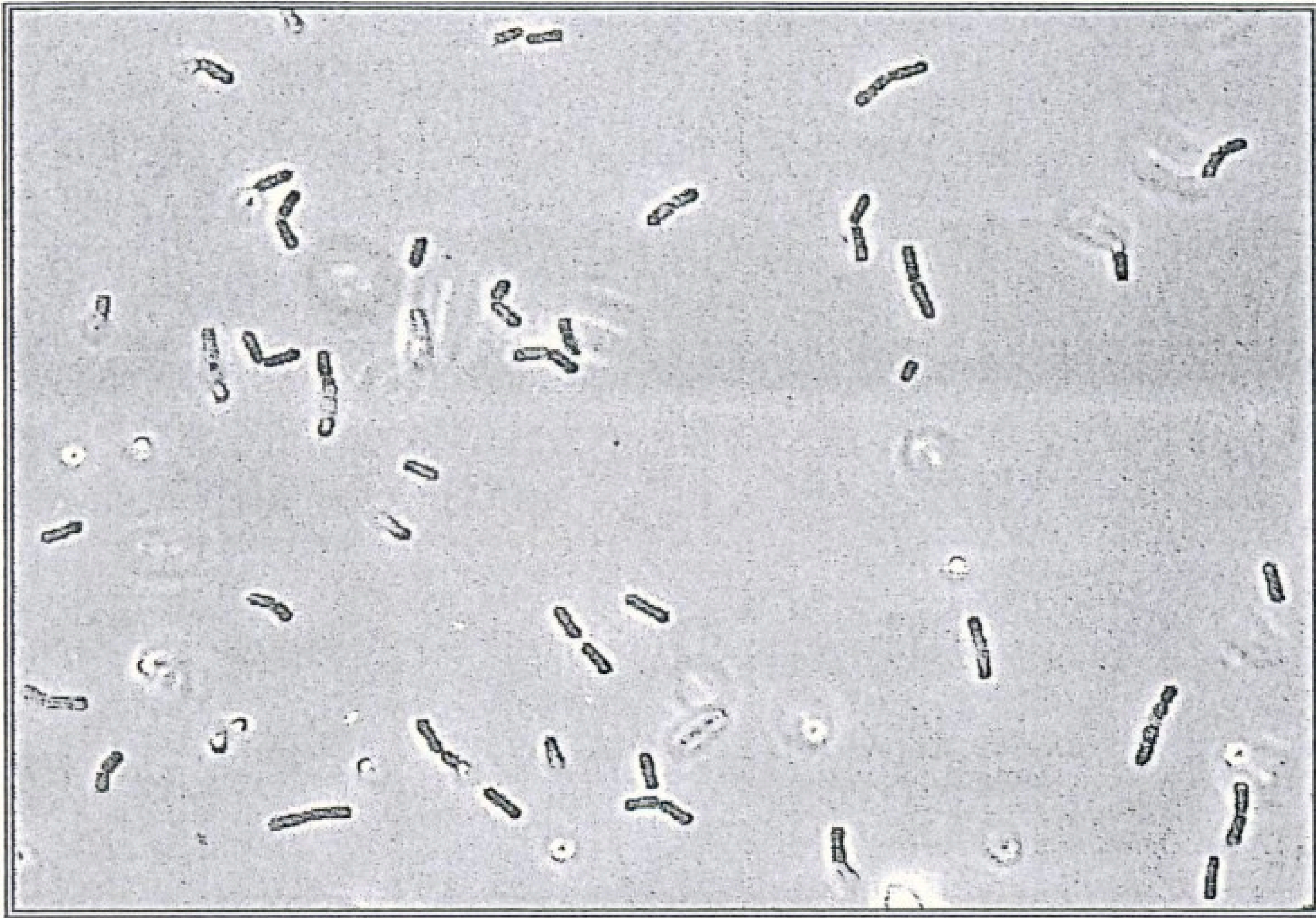


Figure 5. Cellular morphology of *L. bobalius*.

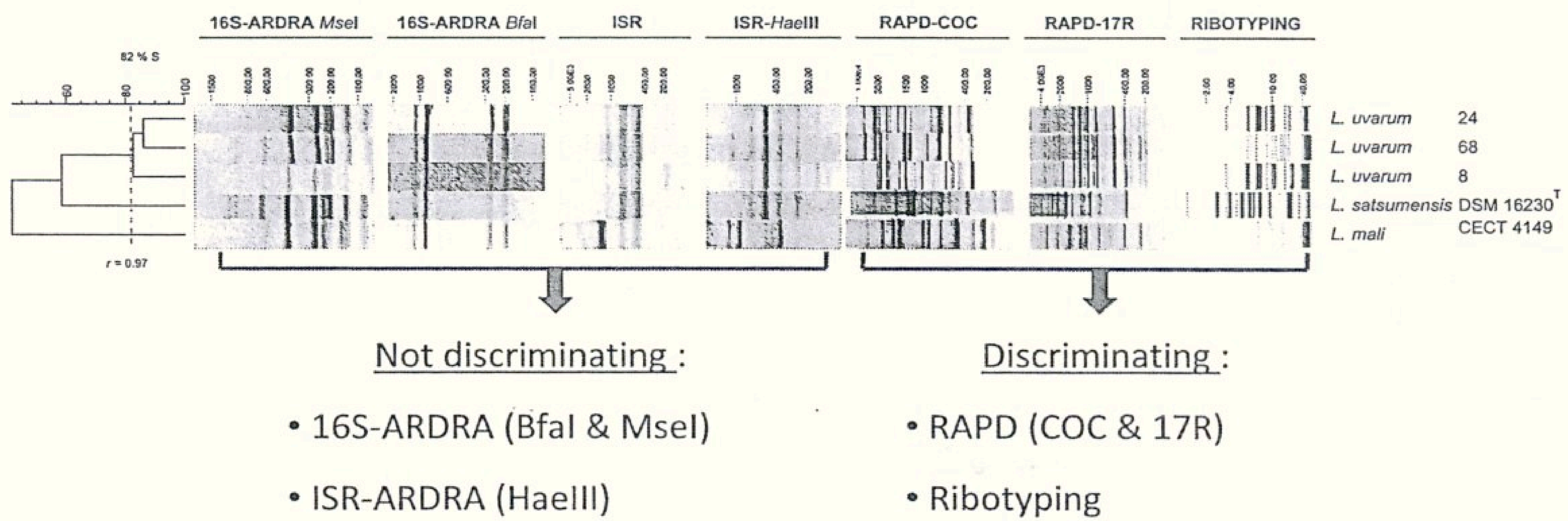


Figure 6. Some rapid discriminating and not discriminating molecular tests between *L. uvarum* and its closest relatives

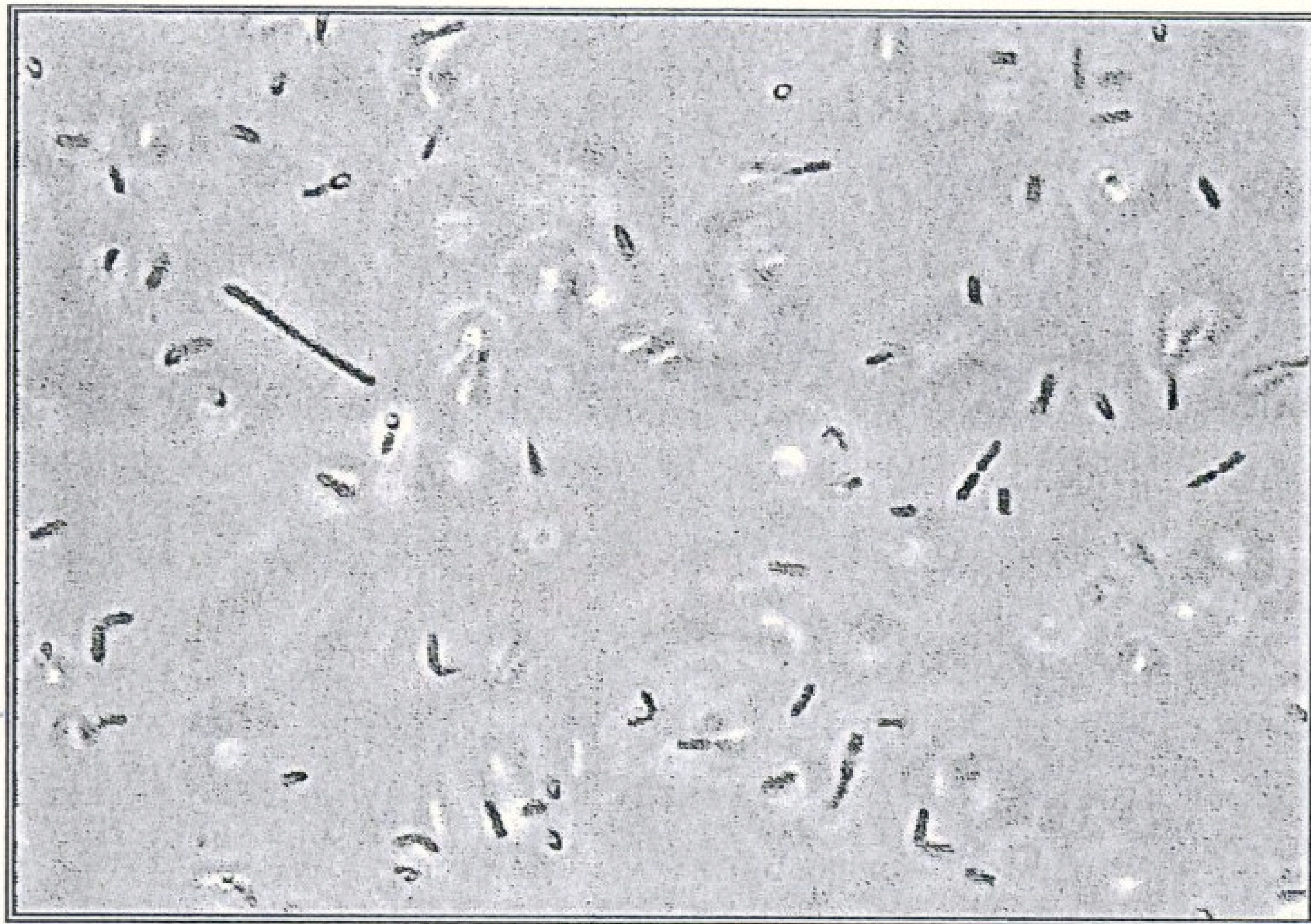
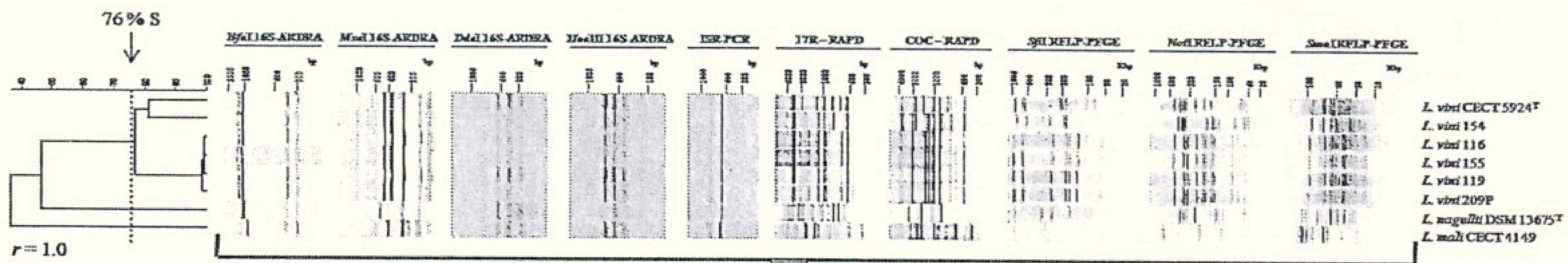


Figure 9. Cellular morphology of *L. oeni*



Discriminating:

•16S-ARDRA (BfaI, MseI, DdeI & HaeIII)

•ISR-PCR

•RAPD (17R & COC)

•Macrorestriction (SfiI, NotI & SmaI)

Figure 10. Some rapid discriminating and not discriminating molecular tests between *L. vini* and its closest relatives

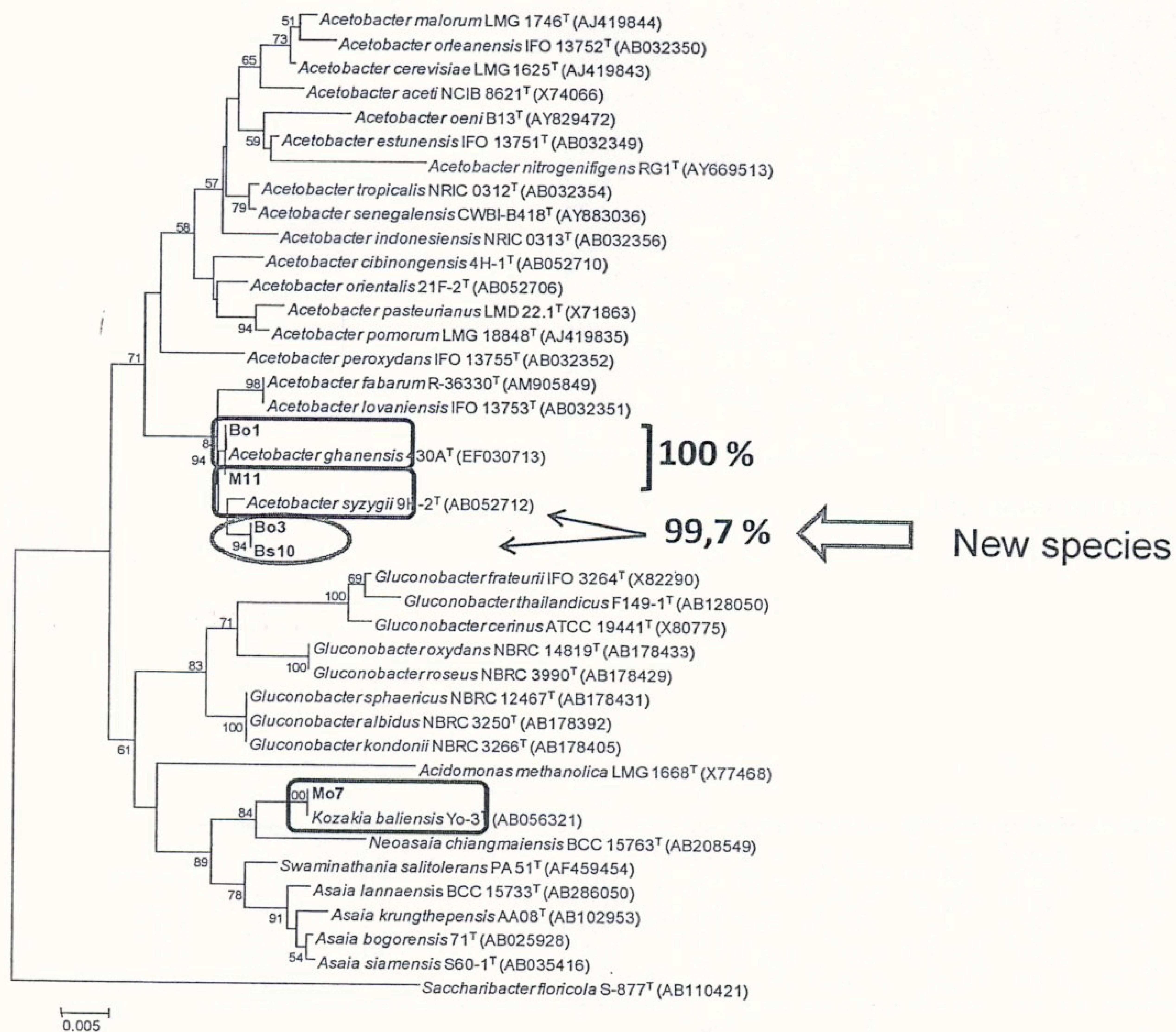


Figure 13. Phylogenetic tree showing the positions of acetic acid bacteria isolates, and some reference strains based on 16S rRNA gene sequences. Clustering is based on neighbor-joining. Bootstrap values (expressed as percentages of 1000 replicates) of 50% or greater are shown at branch points. GenBank accession numbers are given in parentheses.

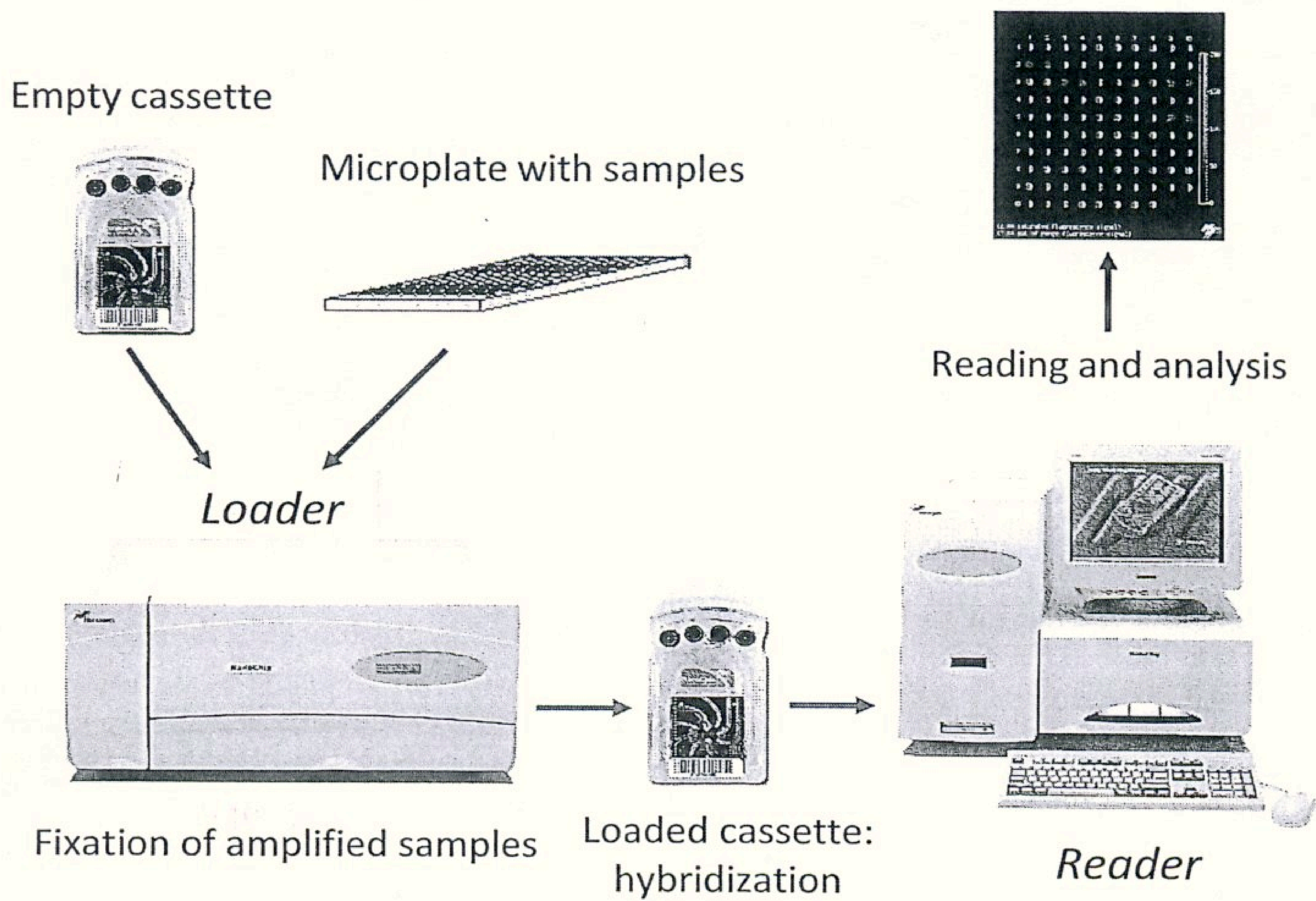


Figure 15. Workng schedule of the Enochip

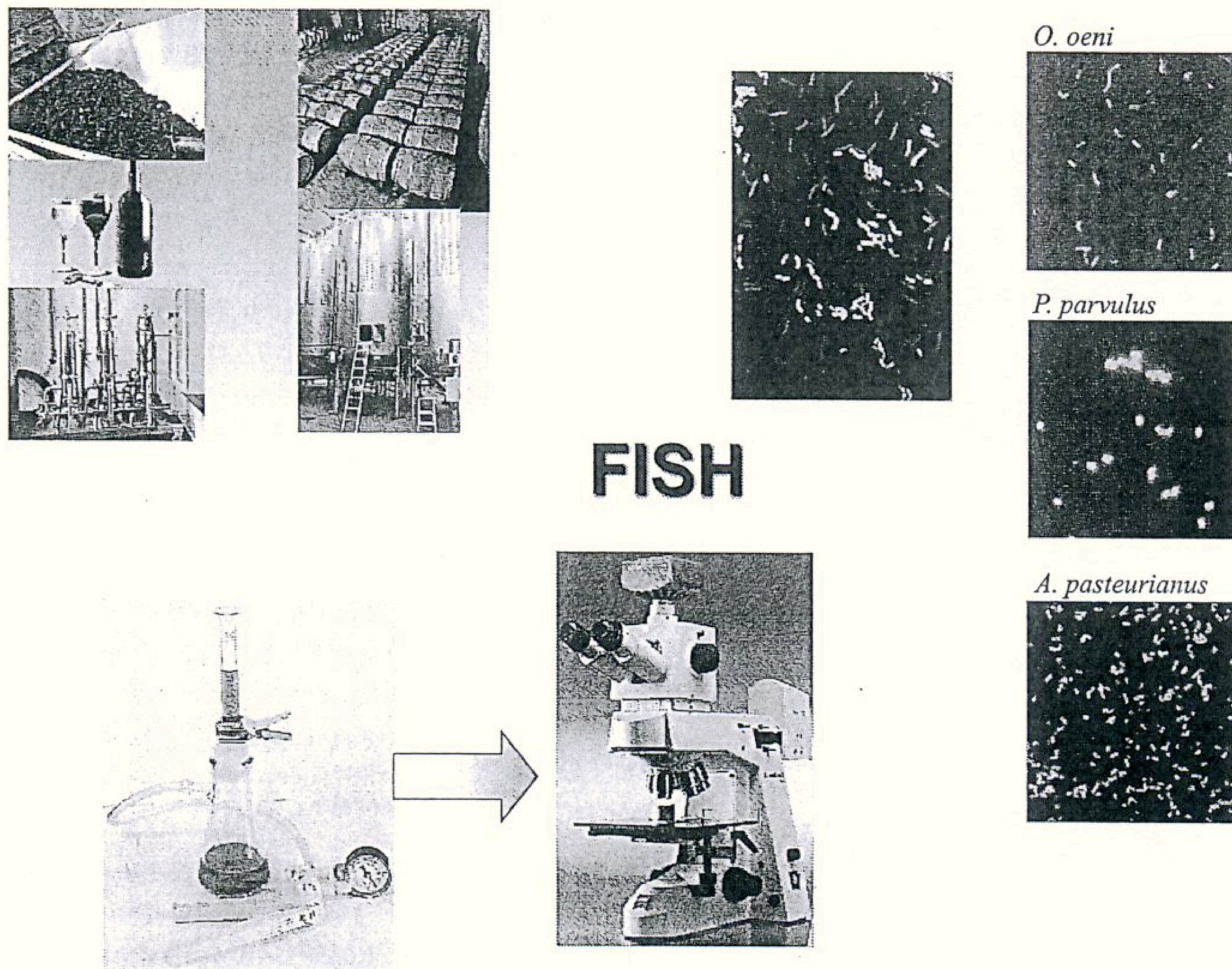


Figure 16. Working schedule of the FISH technique. Some examples of hybridizations are shown.