Universidade de Aveiro Departamento de Biologia
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Métodos independentes do cultivo aplicados ao estudo da dinâmica de comunidades de *Aeromonas*

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Dra. Isabel Henriques, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Aeromonas, Comunidades microbianas, *gyrB, rpoD*, *sodB*, PCR-DGGE.

resumo

As *Aeromonas* são bactérias autóctones em ambientes aquáticos e constituem um grave problema em sistemas de aquacultura devido à sua capacidade de provocar doença em peixes. Estas bactérias são actualmente consideradas patogéneos emergentes em humanos. Os surtos de doença podem estar associados à introdução de estirpes virulentas que evoluíram a partir de outros nichos ou da aquisição de determinantes de virulência do mesmo nicho, ou ainda como resultado de um desequilíbrio na comunidade local de *Aeromonas*. Assim, torna-se essencial desenvolver métodos fiáveis e reprodutíveis para seguir rotineiramente a dinâmica de comunidades indígenas de *Aeromonas* de forma a permitir uma detecção precisa de alterações na sua estrutura, antecipando potenciais riscos.

Neste estudo, foram desenhados três conjuntos de primers para amplificar especificamente os genes *gyrB*, *rpoD* e *sodB* de *Aeromonas.* Métodos de PCR-DGGE (Denaturing Gradient Gel Electrophoresis) foram desenvolvidos com base nestes primers para obter perfis das comunidades. A especificidade dos primers foi testada utilizando como molde DNA de 27 estirpes de *Aeromonas* pertencentes a 17 espécies previamente descritas e também de 13 estirpes de 11 espécies não alvo. Simultaneamente a especificidade dos primers foi também avaliada *in silico* através da pesquisa de homologia com sequências depositadas nas bases de dados. Ambas as metodologias permitiram confirmar a total especificidade dos três conjuntos de primers e apenas para os primers RpoD não foi obtida amplificação de duas estirpes de *Aeromonas*. Para determinar a consistência da informação filogenética fornecida pelos fragmentos amplificados, árvores filogenéticas construídas com base nas sequências dos genes *gyrB*, *rpoD* e *sodB* de *Aeromonas* depositadas na base de dados GenBank e com base nas sequências dos fragmentos amplificados foram comparadas. Verificou-se que os fragmentos alvos fornecem informação filogenética consistente. Os conjuntos de primers foram testados em DNA de amostras de água da Ria de Aveiro e os produtos foram separados por DGGE. Para todas as amostras, e com todos os conjuntos de primers, obtiveram-se perfis complexos e muito estáveis ao longo do gradiente de salinidade. Resultados obtidos com as três metodologias indicam uma maior variabilidade sazonal das comunidades de *Aeromonas*. A clonagem e sequenciação dos fragmentos obtidos a partir das amostras ambientais confirmam a especificidade dos primers e revelam que os filotipos dominantes nestas comunidades apresentam elevada similaridade com estirpes de várias espécies de *Aeromonas* comuns em ambientes aquáticos como sejam *A. alossacarophila*, *A. veronii* e *A. sobria*.

Segundo o nosso conhecimento, este estudo apresenta a primeira tentativa de optimização e validação de métodos independentes do cultivo específicos para *Aeromonas*. Os sistemas desenvolvidos apresentam várias vantagens e consequentemente constituem valiosas ferramentas para avaliar a diversidade e seguir a dinâmica de comunidades de *Aeromonas*. A utilização de mais de um conjunto de primers pode ser útil para obter uma representação mais clara e real da comunidade em estudo. Os métodos desenvolvidos poderão ainda ser adaptados de forma a serem aplicados em outro tipo de amostras ambientais o que poderá ser importante devido à ampla distribuição das *Aeromonas* e às suas capacidades patogénicas.

keywords

Aeromonas, Microbial communities, *gyrB, rpoD*, *sodB*, PCR-DGGE.

abstract

Aeromonas are bacteria autochtonous in aquatic environments, and they constitute a serious problem in aquaculture systems because of their capability to cause disease in fishes. Aeromonads are also nowadays considered as emerging pathogens in humans. Disease outbreaks may be associated with the introduction of virulent *Aeromonas* strains that evolved in other niches, acquisition of virulence determinants in the same niche, or as a result of disequilibrium in the local *Aeromonas* community. Thus, it becomes essential the development of reliable and reproducible methods to routinely follow the dynamics of indigenous *Aeromonas* communities and to allow an accurate detection of alterations in their structure, anticipating potential risks.

In this study 3 primer sets were designed to specifically amplify fragments of the genes *gyrB*, *rpoD* and *sodB* from *Aeromonas*. A PCR-DGGE (Denaturing Gradient Gel Electrophoresis) method based on such primers was established to obtain community specific profiles. Primers specificity was tested by using as template DNA from 27 *Aeromonas* strains belonging to 17 previously described species, and also from 13 strains from 11 non-target species. Specificity was also assessed *in silico* using the BLAST tool, by checking sequence matches against the GenBank database. Results obtained confirmed the specificity of all primer sets and only primer set RpoD failed to amplify from two *Aeromonas* strains. To determine the phylogenetic information contained in the amplified fragments, *gyrB*, *rpoD* and *sodB* sequences available in the GenBank database from *Aeromonas* species were downloaded and submitted to phylogenetic analyses. A phylogenetic analysis following the same procedures was performed using the PCR target fragments and trees were compared. The phylogenetic information contained in the target fragments was confirmed to be consistent. Primer sets were tested in total DNA from estuarine water samples. A DGGE assay was optimized to separate the PCR products. Community specific profiles were obtained from each sample, for each primer pair. Profiles were very complex and rather stable along the salinity gradient. *Aeromonas* communities varied essentially in a seasonal basis. Cloning and sequencing of the fragments obtained from environmental DNA confirmed the specificity of the primers and revealed that the dominant phylotypes affiliated with strains included in species common in aquatic environments such as *A. alossacarophila*, *A. veronii* e *A. sobria*.

To our knowledge, this study presents the first attempt to optimize and validate *Aeromonas*-specific culture-independent methodologies. Results indicate that all the developed systems present several advantages and therefore constitute valuable tools to assess diversity and follow the dynamics of *Aeromonas* communities. The utilization of more than one set of primers may be useful for providing a more reliable and clear representation of the community. The developed methods may be adapted to apply in other types of samples, which may be important due to the wide distribution of aeromonads in the environment and to their pathogenic properties.

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1 Introduction

1.1 *Aeromonas* general description

The genus *Aeromonas* was first proposed in 1936 by Kluyver and van Niel (Kluyver, 1936) and includes a group of gram-negative, rod shaped bacteria, facultatively anaerobic, oxidase and catalase- positive, glucose fermenting, resistant to the vibriostatic agent O/129 (2,4- diamino-6,7-diisopropyl pteridine) and generally motile by means of a polar flagella (Popoff, 1984). This genus belongs to the class γ-*Proteobacteria* and was first placed in the family *Vibrionaceae* together with *Photobacterium*, *Plesiomonas* and *Vibrio* (Veron, 1965), but was later included in the *Aeromonadaceae* family based on 5S rRNA studies (Colwell, 1986).

The taxonomy of the genus *Aeromonas* is very complex and in continuous amendment, not only by the introduction of new species but also by their reclassification and characterization (Minana-Galbis*, et al.*, 2004). Since the first description of four phenospecies (*A. caviae, A. hydrophila, A. sobria, A. salmonicida*) in *Bergey's Manual of Sistematic Bacteriology* (Popoff, 1984), several new species have been described: *Aeromonas hydrophila, Aeromonas bestiarum, Aeromonas salmonicida, Aeromonas caviae, Aeromonas media, Aeromonas eucrenophila, Aeromonas sobria, Aeromonas veronii (biovars sobria and veronii), Aeromonas jandaei, Aeromonas schubertii, Aeromonas trota, Aeromonas allosaccharophila, Aeromonas encheleia*, *Aeromonas popoffii, Aeromonas* sp. *HG11, Aeromonas* sp. *HG13* (formerly *Enteric Group 501*), *Aeromonas simiae, Aeromonas molluscorum, Aeromonas bivalvium* and *Aeromonas aquariorum (Huys, et al., 2002, Pidiyar, et al., 2002, Esteve, et al., 2003, Harf-Monteil, et al., 2004, Minana-Galbis, et al., 2004, Martinez-Murcia, et al., 2007, Minana-Galbis, et al., 2007, Martinez-Murcia, et al., 2008)*. Furthermore, *Aeromonas enteropelogenes and Aeromonas punctata* are currently synonyms of *Aeromonas trota and Aeromonas caviae,* respectively (Schubert & Hegazi, 1988, Collins*, et al.*, 1993, Huys*, et al.*, 2001, Huys*, et al.*, 2002). *Aeromonas culicicola* and *Aeromonas ichthiosmia* are both synonyms *of Aeromonas veronii* (Huys*, et al.*, 2001, Huys*, et al.*, 2005).

Aeromonads can be divided into two phenotypically different groups: psychrophilic and mesophilic. These groups can be differentiated by some characteristics such as motility, elaboration of melanine, optimal growth temperature, and indol. The constituents of the psychrophilic group, for example *A. salmonicida* are relatively homologous phenotipically, do not grow at 37ºC, showing optimal growth temperatures between 22 and 28ºC, are nonmotile and are not clinically relevant. The mesophilic group includes heterogeneous species that grow at 37ºC and are motile by polar flagella. This group can be subdivided into three primary groups represented by species *A. hydrophila, A. caviae and A. sobria.* These species include strains recognized as human pathogens (Janda & Duffey, 1988, Janda & Abbott, 1998).

1.1.1 Environmental distribution

Aeromonads are widely distributed in nature. They can be found in soil and have been isolated from several food sources such as poultry, raw red meat, seafood, fin fish, vegetables, eggs, milk and milk products (Castro-Escarpulli*, et al.*, 2003, Peter J. Ng, 2005 , Arora*, et al.*, 2006, Daskalov, 2006, Evangelista-Barreto*, et al.*, 2006, Medina-Martinez*, et al.*, 2006). They are also able to grow in cooked, processed and raw foods under refrigeration and under modified atmosphere and growing conditions (Bin Kingombe*, et al.*, 2004).

Additionally, aeromonads are autochthonous in aquatic environments. Actually they inhabit many different water habitats such as seawater, river water, freshwater, brackish water, irrigation water, groundwater, spring water, estuarine waters and sewage (Ashbolt & Kirov., 1995, Borrell*, et al.*, 1998, Fiorentini*, et al.*, 1998, Marcel*, et al.*, 2002, Soler*, et al.*, 2002, Pianetti*, et al.*, 2005). Aeromonads can also be found in chlorinated and unchlorinated drinking water and in mineral bottled water, because they can survive and grow in aquatic environments with low nutrients concentration (Messi*, et al.*, 2002, Villari*, et al.*, 2003, Pianetti*, et al.*, 2005) and also because they have the ability to constitute biofilms on the surface of bottles and pipes (Pianetti*, et al.*, 2005). In fact, although free aeromonads are susceptible to chlorine disinfectants, when they are inserted in biofilms they can be more difficult to destroy (Emekdas*, et al.*, 2006). In tap waters they were also found, but in small quantities (Mary*, et al.*, 2001, Emekdas*, et al.*, 2006).

It has been reported that *A. hydrophila* is ubiquitous in clean water, while *A. caviae* is prevalent in highly polluted and fecal contaminated waters (Araujo*, et al.*, 1991, Pianetti*, et al.*, 2005).

1.1.2 Environmental factors that affect aeromonads growth and survival

There are several studied environmental factors that may influence the survival of *Aeromonas* within the different aquatic systems, such as temperature, pH, salinity, turbidity, radiance and conductivity (Maalej*, et al.*, 2003, Maalej*, et al.*, 2004, Chihib*, et al.*, 2005, Wang & Gu, 2005, Khan*, et al.*, 2007).

Several studies showed that changes in water temperature influence the incidence of *Aeromonas* spp. Within temperate climates, in seawater and fresh water aeromonads were rarely found during cold seasons but they were detected at high levels in late summer or early autumn, when temperatures varied between 20 and 25ºC (Mary*, et al.*, 2002, Maalej*, et al.*, 2004). On the other hand, in arid regions higher levels of these bacteria were found in winter months what is thought to happen because of the extremely hot summer and relatively temperate winter (Maalej*, et al.*, 2004). However, *A. hydrophila* has been isolated from aquatic environments in a temperature range of 4 to 45ºC showing a high temperature tolerance (Hazen*, et al.*, 1978, Wang & Gu, 2005). According to Sautour and colleagues this bacteria has an optimum growth temperature of 30ºC (Sautour*, et al.*, 2003).

The pH is another factor that affects the growth of *Aeromonas*. It has been observed that these bacteria are sensitive to low pH values (<5.2) and capable of tolerating pH values up to 9.8, what indicates that they survive preferably in neutral or alkaline environments (Wang & Gu, 2005). It has been reported that for example *A. hydrophila* has an optimal growth pH of 7.0 (Sautour*, et al.*, 2003).

Generally it is assumed that aeromonads are incapable of growing at NaCl concentrations equal or higher than 60‰, and that they have an optimal growth at concentrations between 10 to 20‰ but it has been shown that *A. salmonicida* was able to grow in mediums supplemented with 60‰ NaCl (Wang & Gu, 2005). Even though these bacteria grow in mediums containing 3% NaCl, which is a concentration similar to the seawater, the incidence of *Aeromonas* in this habitat is much lower than in freshwater

(Wang & Gu, 2005). It has been suggested that this fact is due to the decrease of protease production, with consequent loss of resistance against bacteria predators such as protozoa (Khan*, et al.*, 2007).

The influences of radiance and turbidity in *Aeromonas* growth are related. It has been shown that in marine waters with low turbidity these bacteria become more sensitive to radiation. This can be explained by the fact that turbidity is thought to protect cells from sunlight. In waters with low turbidity there is also a lack of organic matter in which aeromonads can be attached (Khan*, et al.*, 2007). Conductivity is also an important factor in seawater having an inhibitory effect on *Aeromonas*. This can be explained by the damaging of the cytoplasmic membrane permeability at high conductivity values and consequent decrease of resistance to radiation (Maalej*, et al.*, 2003).

In order to survive, micro-organisms can adapt to the environment. The lipid composition of bacteria can present several modifications according to changes in physical and chemical properties of the environment. Aeromonads may change the composition of their membranes by desaturating, chain-shortening and chain-branching. These alterations are essential to the maintenance of membrane integrity, fluidity and functionality in response to external factors such as temperature and salinity (Chihib*, et al.*, 2005).

Besides lipid change adaptation, *Aeromonas* spp. have also other mechanisms to survive in hostile environments. It has been shown that *A. hydrophila* incubated at 5ºC in sterile seawater entered a called viable but nonculturable state (VBNC) (Maalej*, et al.*, 2004). This state is characterized by the existence of a cell which is metabolically active but incapable of undertaking the continuous cellular division required for growth. Several environmental factors, specially nutrient lack and temperature, can induce this state in bacteria (Mary*, et al.*, 2002). Maalej reported that this state is a physiological condition and the shift between VBNC and culturable states is accomplished by loss and regain of pathogenic properties. It has also been shown the occurrence of this state in *A. salmonicida* (Morgan*, et al.*, 1992).

1.1.3 *Aeromonas* spp. pathogenicity in fish and humans

Aeromonas constitute a serious problem due to their wide distribution in nature and their capacity to cause disease not only in humans but also in several animals like amphibians, reptiles, mammals and fishes (Pasquale*, et al.*, 1994, Sugita*, et al.*, 1994,

Rahman, *et al.*, 2002, Huys, *et al.*, 2003, Minana-Galbis, *et al.*, 2004).

In fish these bacteria cause furunculosis, epizootic ulcerative syndrome, hemorrhagic septicemia, soft tissue rot and fin rot (Nam & Joh, 2007) and in frogs it has been reported that they can cause red leg disease characterized by hemorrhages in leg muscles (Huys, *et al.*, 2003).

Furunculosis is a systemic disease caused by *A. salmonicida* subsp. *salmonicida* and is characterized by necrotic and hemorrhagic lesions in gut, gills and muscle (Ebanks, *et al.*, 2006). This name derives from the furuncles or boils that occur in fish in the chronic form of the disease. Acute disease develops septicemia, necrotic lesions in the skin and internal hemorrhages. This infection can be fatal in 2 or 3 days (Burr, *et al.*, 2005). *A. salmonicida* affects salmonids but has also been found in gilthead seabream (*Sparus aurata* L.) turbot (*Scophthalmus maximus* L.), atlantic cod (*Gadus morhua* L.) and lamprey (*Petromyzon marinus* L.) (Beaz-Hidalgo, *et al.*, 2008) and consequently it represents a major economical problem to aquaculture systems.

Epizootic ulcerative syndrome is a disease characterized by the occurrence of dermal ulcers on the fishes' head, dorsal region and middle of body. *A. sobria* and *A. hydrophila* were found in fishes with this disease such as African catfish (*Clarias gariepinus*), catla (*Catla catla*), rajputi (*Puntius gonionotus*), rui (*Labeo rohita*), and shole (*Channa striatus*). This syndrome has also caused substantial economic loss to the fishery sector and to fish farmers (McGarey, *et al.*, 1991, Rahman, *et al.*, 2002).

In humans there is a number of species implicated in disease. The three species, *A. hydrophila, A. sobria and A. caviae* are considered major pathogens because they represent 85% of the *Aeromonas* clinical isolates. In contrast, *A. schubertii, A. jandaei, A. veronii* bv. *veronii* and *A. trota* which also have been implicated in human disease, are considered minor pathogens (Janda & Abbott, 1998, Sen & Rodgers, 2004, Donohue, *et al.*, 2007).

There are several human diseases in which aeromonads have been implicated; most of all are related to immunosupression or exposition of healthy individuals with wounds to contaminated waters or soil (De Gascun, *et al.*, 2007, Herrera, *et al.*, 2007). They include gastroenteritis, hemolytic uremic syndrome, septicemia, meningitis, biliary tract infections, wound infections, pneumonia, peritonitis, urinary tract infections, ocular infections, cellulitis, soft tissue infections and septic arthritis (Lau, *et al.*, 2000, Kao, *et al.*, 2003, Szczuka & Kaznowski, 2004, Roberts, *et al.*, 2006, De Gascun, *et al.*, 2007, Lai, *et al.*,

2007). Aeromonads have also been associated with diarrhea and gastrointestinal disease since the first strain was detected on human feces, but there are some unsolved questions regarding this matter, for instance the Koch's postulates have failed to be fulfilled because there are still no animal models (Janda & Abbott, 1998). Only four species of aeromonads have been frequently recovered from human feces: *A. hydrophila, A. caviae, A. trota* and *A. veronii biovar sobria*. *A. schubertii* and *A. jandaei* are seldom isolated in this kind of samples (Janda & Abbott, 1998, von Graevenitz, 2007).

Hemolytic Uremic Syndrome is a disorder that generally affects children but it can also occur in old people. It is characterized by thrombocytopenia, renal failure and beginning of a microangiopathic anemia and is thought to be caused by *A. hydrophila* (Janda & Abbott, 1998, Figueras*, et al.*, 2007).

Another illness in which aeromonads have been also implicated is septicemia. It occurs in patients with malignancies, hepatobiliar disease, diabetes and a few other pathologies. It is acknowledged that *A. veronii*, *A. hydrophila*, *A. caviae* and *A. jandaei* are capable of provoking this disease independently or associated to other bacteria (Janda & Abbott, 1998).

The pathogenicity of these bacteria is related to the existence of a large number of virulence factors in *Aeromonas* spp.

1.1.4 Virulence factors

Aeromonads pathogenesis system is complex and their virulence is believed to be multifactorial. There are several components that have been identified or assumed as virulence factors such as cytotoxins, enterotoxins, haemolysins, lipases (Pla and Plc, Sat), amylase, elastase (AhpB), gelatinase, lecithinase, chitinase, phospholipase glycerophosfolipid- cholesterol acyltranferase (GCAT), serine protease (AspA), nucleases, adhesins like pollar flagella (FlaA and FlaB), lateral flagella, type IV pili and a surface array protein layer (S layer) (Janda, 1991, Merino*, et al.*, 1999, Sen & Rodgers, 2004, Nam & Joh, 2007). Only the better known will be generally described here.

Aeromonads produce several enterotoxins such as the cytotoxin Act, known as aerolysin-related cytotoxin, enterotoxin and two cytotonic enterotoxins, heat- labile Alt and heat stable Ast. Act has been characterized and it is known that it causes blood cell's lysis, induces intestinal fluid secretion, is cytotoxic to cells, up-regulates genes encoding several

pro-inflammatory cytokines and it is also possible that this enterotoxin modulates host cell signaling pathways (Galindo*, et al.*, 2003, Erova*, et al.*, 2006). Aerolysin is known to act as cytolitic enterotoxin and hemolysin and has the capacity to form channels by heptamerization to the host cellular membrane following activation (Nam & Joh, 2007).

GCAT, an unusual lipase, is secreted by *A. salmonicida* and is thought to participate in the pathogenicity of members of this species which causes furunculosis in fish (Chacon*, et al.*, 2003, Nam & Joh, 2007).

Serine protease activates toxins like GCAT and aerolysin controlled by a quorum sensing process (Nam & Joh, 2007).

Nuclease involvement in pathogenicity of aeromonads has not yet been confirmed. However it is acknowledged that *Aeromonas* can secrete this kind of enzymes into the medium and these are known to be important virulence factors in other bacteria, for example in *Streptococcus* (Dodd & Pemberton, 1996, Chacon*, et al.*, 2003, Nam & Joh, 2007).

Flagella are considered virulence factors because of their association with adsorption to host cell's membrane. Lateral flagellum is capable of acting as adhesin in epithelial cells in human intestine and is also responsible for swarming motility in solid matrixes. On the other hand, pollar flagella confer swimming motility in liquids (Kirov*, et al.*, 2004).

The S layer is a structure external to the cell wall that confers hydrophobicity to bacterial surface. It has the capacity to bind to immunoglobulins and porphyrins, participates in bactericidal resistance activity to serum killing and protease digestion and facilitates association with macrophages (Janda, 1991, Esteve*, et al.*, 2004).

Type IV pili is implicated in several processes such as adherence and colonization of epithelial host cells, motility, DNA uptake, cell signaling, biofilm formation and they can also act as receptors for bateriophages (Masada*, et al.*, 2002).

Besides these virulence factors, the pathogenicity mechanism known as the type III secretion system has also been studied in *Aeromonas.* This system participates in host infection and on the escape from the host's immune system. Type III secretion system comprises a complex set of proteins, at least 20, which are implicated in regulatory and effector functions, structural machinery and chaperone activity. It allows the translocation of effector proteins, from the bacterial cytoplasm into eukaryotic cell's cytosol, which can

change host's cytoskeleton, signal transduction mechanisms and cell to cell communication (Burr*, et al.*, 2005, Sha*, et al.*, 2005, Ebanks*, et al.*, 2006).

1.1.5 Antibiotics susceptibility

Aeromonas spp., with the exception of *A. trota,* despite a few exceptions, are known by their characteristic resistance to ampicilin, an agent generally used in medium to isolate these bacteria (Carnahan*, et al.*, 1991, Saavedra*, et al.*, 2004). They are generally considered susceptible to a diversity of antibiotics such as aminoglycosides, tetracyclines, ureidopenicillins, aztreonam, expanded or broad spectrum cephalosporins, trimethoprim-sulfamethoxazole, chloramphenicol, gentamicin, amikacin, methicillin, clindamycin, erythromycin (Motyl*, et al.*, 1985, Koehler & Ashdown, 1993, Ko*, et al.*, 2003). Despite the general susceptibility to these drugs, in some cases resistance starts to arise.

It has been shown resistance in *Aeromonas* strains isolated from patients with acute diarrhoea to cephalothin, furazolidone, nalidixic acid and streptomycin and a reduced susceptibility to neomycin, norfloxacin and ciprofloxacin what indicates that these bacteria are becoming resistant to these agents (Sinha*, et al.*, 2004).

Scoaris and colleagues examined the susceptibility of *Aeromonas* from drinking water and reported that all the *A. jandaei* isolates, four of five *A. hydrophyla* strains and nine of twelve *Aeromonas* sp. strains isolated from drinking water were resistant to three or more of the antibiotics tested and thus were multidrug resistant. They also reported that the least effective antibiotic was ampicillin, which presented a resistance value of 91% and the most active antimicrobial was ciprofloxaxin with 100% susceptibility in the isolates (Scoaris *, et al.*, 2008).

Palu *et al.* studied the incidence of resistance in *Aeromonas* from food and clinical sources. Aeromonads were identified and placed in the *A. hydrophila* and *A. caviae* complexes. They reported that all strains were susceptible to ciprofloxacin, imipenem, gentamicin, tobramycin and amikacin. The clinical isolates presented resistance to cefotaxime, ampicillin/sulbactam, cefoxitin, ceftazidime, tetracycline, chloramphenicol, and sulfamethoxazole/trimethoprim while the food isolates were resistant to cefoxitin, ampicillin/sulbactam and tetracycline (Palu*, et al.*, 2006).

Henriques and colleagues investigated the occurrence and molecular diversity of genes encoding β-lactamases and integrons in Gram-negative ampicillin-resistant bacteria

from the estuary Ria de Aveiro including *Aeromonas.* They established a resistance phenotype for all strains. Several aeromonads presented multiresistance phenotypes (Table1). Authors reported the presence of β-lactamase-encoding sequences in 10.5% of the aeromonads isolates. Integrons were detected in 21% of the *Aeromonas* isolates (Henriques*, et al.*, 2006).

Table 1 - Resistance phenotype of *Aeromonas* strains isolated from Ria de Aveiro (from Henriques et al, 2006).

Strain Reference	**Strain Identification**	**Resistance Phenotype** [a]
G.I10.8	*Aeromonas caviae*	AMP, CAR, AMX, PIP, CEF, TET, SXT
G.I10.22	*Aeromonas caviae*	AMP, CAR, AMX, PIP, CEF, TET, SXT
G.I6.30	*Aeromonas caviae*	AMP, CAR, AMX, AMC, CEF
G.I10.28	*Aeromonas hydrophila*	AMP, CAR, AMX, CEF, TET, SXT
M.I6.35	*Aeromonas hydrophila*	AMP, CAR, AMX, AMC, CEF, IPM
G.I6.14	*Aeromonas hydrophila*	AMP, CAR, AMX, CEF
G.N1.15	*Aeromonas hydrophila*	AMP, CAR, AMX, AMC, PIP, TZP,CEF, CTX
G.N1.20	*Aeromonas hydrophila*	AMP, CAR, AMX, PIP, TZP, CEF
M.I6.26	*Aeromonas media*	AMP, CAR, AMX, AMC, PIP, TZP,CEF, SXT
G.I6.24	*Aeromonas media*	AMP, CAR, AMX, CEF
G.I10.27	*Aeromonas* sp.	AMP, CAR, AMX, CEF
G.I10.16	*Aeromonas* sp.	AMP, CAR, AMX, PIP, CEF
M.I6.23	*Aeromonas* sp.	AMP, CAR, AMX, AMC, PIP, CEF, SXT
G.N1.27	*Aeromonas* sp.	AMP, CAR, AMX, AMC, CEF, TET
M.I6.31	*Aeromonas* sp.	AMP, CAR, AMX, PIP, TZP, CEF

a-Antibiotic abbreviations: AMP, ampicillin; CAR, carbenicillin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; PIP, piperacillin; TZP, piperacillin/tazobactam; CEF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; TET, tetracycline; GEN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole.

Saavedra *et al.* evaluated the resistance to β- lactams in *A. hydrophila* isolated from rainbow trout. They reported that the majority of aeromonads isolates presented resistance to ticarcillin, amoxicillin and carbenicillin and that the most effective agents were piperacillin (alone and with tazobactan), aztreonam and cefotaxime. They also described that approximately 20% of the isolates showed resistance to cefotaxime and 6% were resistant to aztreonam. The table 2, presented bellow, shows the percentage of strains resistance, intermediate and sensitive that they obtained (Saavedra*, et al.*, 2004).

Table 2 - Percentage of susceptibility to *β*- lactams in *A. hydrophila* strains. R, resistant; I, intermediate; S, sensitive (from Saavedra *et al.*, 2004).

Antibiotic	**R**	**I**	**S**
Piperacillin	24	0	76
Piperacillin+tazobactan	24	0	76
Amoxicillin	88	7	5
Amoxicillin+clavulanic acid	35	35	30
Ticarcillin	76	0	24
Ticarcillin+clavulanic acid	35	7	58
Ampicillin	65	5	30
Carbenicillin	82	0	18
Cephalothin	65	5	30
Cefotaxime	12	0	88
Cefoperazone	24	12	64
Cefepime	54	0	46
Aztreonam	29	0	71
Imipenem	19	16	65

The wide distribution of aeromonads, the ability to survive in hostile environments, their resistance to antibiotic agents and chlorination, together with recognized presence of several virulence factors that confer capacity to produce disease in some animals and in humans emphasize the importance of the control and surveillance of these bacteria. In fact, concern with these bacteria has grown in recent years. The American Environmental Protection Agency (EPA) published in 1998 the Contaminant Candidate List that includes contaminants that are known or anticipated to occur in public water systems and which, according to this agency, require regulations and surveillance. *A. hydrophila* was included in this list because of its potential to cause human disease (EPA, 1998). Actually this species is now considered to be one of the most noteworthy emerging pathogens (Seshadri*, et al.*, 2006).

1.2 Detection and characterization methods

Since the first description of *Aeromonas*, several methods have been developed for their identification and characterization. They vary from the classical phenotypical approach based on morphological, physiological and metabolic characteristics in to a variety of new molecular techniques. Biochemical identification, serological typing schemes, DNA-DNA hybridization assays and analysis of phylogenetic markers sequences are examples of these methods.

1.2.1 Biochemical identification

The biochemical identification of aeromonads is difficult, time-consuming, and shows discrepancies with the genetic groups (Janda, *et al.*, 1996). Commercial identification kits such as VITEK and API (bioMérieux, Marcy l'Etoile, France) are available for identifying and characterizing bacteria. Many misidentification cases in identifying *Aeromonas* spp. have been reported when using these methodologies. Usually, *Aeromonas* are incorrectly identified as *Vibrio*, because they share many phenotypic characteristics. A typical example is the case of *A. caviae* which is misidentified as *Vibrio fluvialis,* but other cases have been described such as *A. schubertii* which was identified as *Vibrio damsela*, *A. veronii bt. veronii* identified as *Vibrio cholerae* and *A. veronii* bv. *sobria* identified as *Vibrio alginolyticus* by the VITEK system (Abbott, *et al.*, 1998, Park, *et al.*, 2003).

The identification problems regarding the genus *Aeromonas,* when using biochemical tests, are related to several factors such as the high level of recognized species and the inexistence of clear phenotypic schemes to distinguish them. When new species are introduced, authors only report selected characteristics and compare them with previous studies that describe phenotypic characteristics of related species. Although the tests used may be the same, the conditions in which they occurred may be different, and thus, results are not always comparable (Abbott, *et al.*, 2003).

1.2.2 Serological typing schemes

Sakazaki and Shimana (Sakazaki & Shimada, 1984) developed the most widely used serological scheme, which is based on the search for characteristic heat-stable somatic determinants (O) using antisera. This typing scheme is able to recognize 44 different established serogroups (O1 to O44) and 50 additional serogroups in aeromonads (O45-O94) (Albert, *et al.*, 1995).

This system provides important information since it detects associations between clinical infections and the several existing serogroups. Some important serogroups have been identified such as O:11 related to clinical infections such as meningitis, sepsis and peritonitis and serogroup O:34 responsible for septicemia in goldfish and human wound infections (Janda, *et al.*, 1996, Korbsrisate, *et al.*, 2002).

1.2.3 DNA-DNA hybridization studies

DNA-DNA hybridization was one of the first genotypic techniques that allowed a better identification of taxonomic species. It is based on the degree of hybridization between an identified species from a culture collection and an unknown strain. If the unknown strain exhibits 70% or higher hybridization levels with the known collection species, it was considered to be an element of the same species. If the hybridization degree is lower than 70% the unknown strain belonged to another species (Staley, 2006).

In early 1980's Popoff developed a DNA-DNA reassociation method and identified three hybridizations groups (HG's) within the *A. hydrophila* complex: HG 1 corresponding to *A. hydrophila* , HG 2 comprising a group of unclassified strains and HG 3 represented by *A. salmonicida* (Popoff, 1981). Later extensive DNA-DNA hybridization studies originated 18 hybridization groups: HG 1 of *A. hydrophila*, HG 2 of *A. bestiarum*, HG 3 of *A. salmonicida*, HG 4 of *A. caviae*, HG 5 of *A. media*, HG 6 of *A. eucrenophila*, HG 7 of *A. sobria*, HGs 8 and 10 of *A. veronii*, HG 9 of *A. jandaei*, HGs 11 and 13 with unnamed *Aeromonas* sp., HG 12 of *A. schubertii*, HG 14 of *A. trota*, HG 15 of *A. allosaccharophila*, HG 16 of *A. encheleia*, HG 17 of *A. popoffii*, and HG 18 of *A. culicicola* (Laganowska & Kaznowski, 2005). Besides the species contemplated in these HG's new species have been proposed, *A. simiae, A. molluscorum, A. bivalvium* and *A. aquariorum (Harf-Monteil, et al., 2004, Minana-Galbis, et al., 2004, Minana-Galbis, et al., 2007, Martinez-Murcia, et al., 2008).*

In HG 3 there are two different kinds of strains: non motile strains which can be divided into five subspecies (*salmonicida, masoucida, smithia, achromogenes* and *pectinolytica*) and motile strains which are biochemically related to *A. hydrophila.* HG 8 and HG10 comprise two biogroups of *A. veronii* and are genetically similar even though they have different phenotypical characteristics (Laganowska & Kaznowski, 2005).

Despite the introduction of new methods for species identification not only in *Aeromonas* but also in other genus, this DNA-DNA hybridization is still considered by some authors 'a gold standard' for taxonomic identification of new species (Janda & Abbott, 2007).

1.2.4 Molecular evolutionary chronometers

Molecular chronometers are molecules whose sequence modifies in time allowing the measure of evolutionary changes. A good molecular chronometer is a molecule which is universally present within the group, in order to allow the comparison of organisms; is functionally homologous between individuals and thus show sequence similarities; has a sequence capable of reflecting evolutionary changes and finally, it must have highly conserved regions for aligning during analysis which also facilitates primer design (Madigan & Martinko, 2006).

The molecular chronometer most widely used is the gene that encodes the subunit 16S of the rRNA, however due to reported problems related to the use of this gene in speciation, several other genes have also been proposed such as *gyrB*, *rpoD*. *sodB,* because of the characteristics summarized below, is also a possible good molecular chronometer.

1.2.4.1 16S rRNA gene

The16S rRNA gene is the most commonly used gene for taxonomic classification once it is a good molecular chronometer (Woese, 1987). This housekeeping gene is a good phylogenetic marker for several reasons: it is universally present in all bacteria, it is relatively large (1500bp) and thus provides sufficient information to design phylogenetic relationships, it is functionally constant, and it is composed by highly conserved regions and highly variable regions. Additionally, a high number of sequences are available from the public databases. The 16S rRNA gene modifications provide accurate and valuable evolutionary information (Janda & Abbott, 2007).

Generally, the comparison between 16S rRNA gene sequences allows the affiliation of an organism to a genus and sometimes to a species or subspecies. Although there is no agreement on the precise level of genetic similarity that defines a species, 99 to 99.5% is frequently used (Clarridge, 2004). According to Bosshard *et al.,* to define a species a similarity percentage over or equal to 99% is needed and a genus is identified with a similarity within the range of 95-99% (Bosshard*, et al.*, 2003). Some authors consider that a strain with less than 97% of homology in 16S rRNA gene with his most similar described species, can be considered a new species (Staley, 2006).

According to Janda (Janda & Abbott, 2007) the 16S rDNA sequencing in some studies allowed genus identification in the majority of the cases (>90%) but speciation was

only obtained in 65- 83% of the cases, while 1-14% of the isolates continued unidentified. These difficulties of classification are due to the reduced number of deposited sequences in nucleotide databases for some groups, existence of species with identical or similar sequences in this gene, nomenclature problems related to multiple genomovars assigned to single species or complexes and the recognition of new taxa. This author also refers that this sequencing technique has sometimes low phylogenetic and discriminatory capacities and exemplifies some researchers that came upon some resolution problems at the genus or species level with 16S rRNA gene sequencing data. These problems included rapid-growing *Mycobacteria*, *Acinetobacter baumannii - A. calcoaceticus* complex, some members of the family *Enterobacteriaceae, Achromobacter, Actinomyces and Stenotrophomonas.*

According to Martinez-Murcia *et al.,* in a phylogenetic study of *Aeromonas* using the 16S rRNA gene, some relationships obtained from this gene disagreed with DNA-DNA hybridization tests. For instance, *A. caviae* and *A. trota* which differed in the sequence only by up to three nucleotides, in DNA-DNA hybridization showed a value of only 30%. On the other hand, even though *A. veronii* and *A. sobria* have a DNA-DNA hybridization value of 60-65 % they differ by 12 nucleotides in 16S rDNA sequences (Martinez-Murcia*, et al.*, 1992).

A possible factor that can also complicate 16S rDNA- based identification is that the genome of bacteria may present 1-15 copies of the ribosomal operon (Klappenbach*, et al.*, 2000). It is also known that intragenomic heterogeneity exists because evolution does not always homogenize these operons. According to Morandi, because of this heterogeneity, 16S rDNA sequences may not reflect correctly the phylogenetic relationships of *Aeromonas* and may not be a good choice for identifying these bacteria (Morandi*, et al.*, 2005). For instance, the genome of *A. veronii,* can contain up to six copies of the 16S rRNA gene and their nucleotide sequences differ 1.5% between them (Janda & Abbott, 2007). Also in the genome of *A. hydrophila* subsp. *hydrophila* ATCC 7966, from which the complete sequence has been reported, 10 copies of the 16S rRNA gene were found (Seshadri*, et al.*, 2006).

There are also some preoccupations related to a few factors that can generally affect the 16S rDNA sequencing result such as isolate purity, extraction methods and possible formation of chimeric molecules (Janda & Abbott, 2007).

Another feature that makes 16S gene inadequate in *Aeromonas* is the fact that in some species of this genus, 16S rRNA gene is highly conserved thus displaying high levels of sequence similarity between species (from 98-100%) (Martinez-Murcia*, et al.*, 1992). As a consequence of this similarity it has been shown that the use of 16S fails to differentiate for example *A. bestiarum* from *A. salmonicida* (Soler*, et al.*, 2003, Tacão*, et al.*, 2005).

All the referred facts indicate that 16S rRNA genes should not be used isolated to classify and infer phylogenetic relationships between *Aeromonas* spp.

1.2.4.2 *gyrB*

Another gene that is considered useful for bacterial systematic is *gyrB*. This gene encodes the subunit B from DNA gyrase, a type II topoisomerase essential for bacterial DNA replication.

Topoisomerases are a family of enzymes that catalyze the interconversion of different topological forms of DNA in order to solve topological problems during DNA transcription, replication, recombination and chromosome partitioning during cell division. Therefore these enzymes are present in all organisms and are absolutely essential to maintain cell viability (Watt & Hickson, 1994).

Type II topoisomerases are responsible for producing transitory double-stranded breaks in a DNA segment and pass throughout these breaks an intact duplex before resealing them. Some topoisomerases type II can produce supercoils and DNA gyrase in particular has the capacity to introduce negative supercoils into DNA in an ATP dependent reaction required for chromosome replication or segregation (Watt & Hickson, 1994). This enzyme may also relax supercoiled DNA without ATP consume (Kasai*, et al.*, 1998).

DNA gyrase is composed of two subunit proteins (A and B) organized into a quaternary structure of A2B2. The subunit B protein presents a molecular weight of 90 kDa or 70 kDa. It is thought that the C- terminal of this subunit allows the formation of a complex with the A protein and participates in ATP-independent relaxation. On the other hand, the N- terminal may catalyze ATP-dependent supercoiling of DNA (Kasai*, et al.*, 1998).

The gene *gyrB*, that encodes the ATPase domain of DNA gyrase (subunit B), was proposed as an appropriate phylogenetic marker for the classification and identification of

bacteria (Yamamoto & Harayama, 1996). This gene that is present in all bacteria is a single copy gene and its phylogenetic analysis allows the understanding of evolutionary relationships between species (Dauga, 2002). In comparison to the 16S rRNA gene that presents an average substitution rate of 1% per 50 million year, *gyrB* substitution rate is approximately 0.7% to 0.8% per one million years (Yamamoto & Harayama, 1996). Thus, sequence analysis of this gene is effective for identifying species and determining their evolution.

This molecular marker has been used in phylogenetic studies in several genus and species. Yamamoto and colleges demonstrated that the phylogenetic clustering of *Acinetobacter* strains based on *gyrB* gene is almost equal to the genomospecies obtained by DNA-DNA hybridization (Yamamoto*, et al.*, 1999). Other studies were conducted in *Pseudomonas* (Yamamoto & Harayama, 1998, Yamamoto*, et al.*, 2000), *Shewanella* (Venkateswaran*, et al.*, 1999), and *Enterobacteriaceae* strains *(Dauga, 2002)* among others.

In the genus *Aeromonas* the *gyrB* gene has also been evaluated. Yáñez and colleagues investigated the relationships of *Aeromonas* species by using the *gyrB* gene sequence and concluded that this gene is an excellent molecular chronometer which allows strains clustering consistent with the current taxonomy and with the 16S rDNA based affiliation in this genus. According to them, the sequence similarity in *Aeromonas* strains varies from 86.7 to 100% which represents nucleotide differences between 0 to 127 nucleotides and at the intraspecies level the nucleotide substitution ranges between 0 and 2.6%, being usually lower than 2% in the majority of *Aeromonas* species. They also found that between *Aeromonas* species nucleotide substitutions were generally > 3% with the exception of two pairs of species: *A. salmonicida* and *A. bestiarum* with a range between 2.2 and 3.3% and *A. encheleia* and *Aeromonas* sp. HG 11 with a range from 2.1 to 2.7 %. These results demonstrate that in aeromonads *gyrB* sequences present a mean substitution rate that is in average six times higher than that of 16S rDNA. This fact is related to the chronometric attributes of this gene that on the one hand is relatively conserved but in the other is influenced by a degenerative code that permits the occurrence of silent mutations. Another two examples of the fact that *gyrB* sequence divergence is greater than that of 16S rRNA gene is the distinction between *A. trota* and *A. caviae* which are distinguishable in 16S rDNA sequence by a single nucleotide but in *gyrB* there is a rate of nucleotide

substitutions of about 6-7.2% corresponding to 57-69 bp, and also *A. hydrophila* and *A. media* that present three nucleotide differences in 16SrDNA and are unambiguously separated by *gyrB* sequences (Yanez*, et al.*, 2003).

The sequence of *gyrB* presents significant differences between all *Aeromonas* species and DNA hybridization groups. In addition, a considerable amount of differences between strains of the same species were found, with the exception of the strains *A. allosaccharophila* CECT 4200 and *A. media* CECT 4234 that presented sequences identical to the type strain. Since all the other strains possessed unique *gyrB* sequences, comes into view that this gene may be used not only for identification of species but also to infer phylogenetic relations within them (Yanez*, et al.*, 2003).

Another investigation with *gyrB* was performed by Pidiyar *et al.(Pidiyar, et al., 2003).* They have determined the *gyrB* sequences of 17 hybridization groups in *Aeromonas* and compared the phylogenetic trees in the type strains of this gene and 16S rRNA gene. They also determined the phylogenetic position of *A. culicicola* using these two genes. According to their new findings and previous studies made by them (Pidiyar*, et al.*, 2002), *A. culicicola* MTTC 3249 in the *gyrB* tree analysis clustered with *A. veronii* while in the 16S-based tree it grouped together with *A. jandaei*. They also noticed that the sequence similarity between *gyrB* genes was lower than between the 16S rRNA genes. For example, among *A. trota* and *A. caviae*, and *A. culicicola* and *A. jandaei* in the *gyrB* sequences there was 102 and 79 nucleotide differences respectively while in 16S rRNA gene these referred species showed only one nucleotide difference. In relation to *A. culicicola* phylogenetic position, they found that for the *gyrB* sequence, *A. jandaei and A. veronii* bv. *veronii* showed 79 and 43 nucleotide differences when compared to *A. culicicola*. When using the 16S rRNA gene the difference between *A. culicicola* and *A. jandaei* was only one single nucleotide whereas with *A. veronii* bt. *veronii* was 5 bases. Pidiyar *et al.* concluded that the usage of *gyrB* sequence analysis allowed the determination of the taxonomic affiliation of *A. culicicola* closer to *A. veronii* bv. *veronii* (HG 10) (Pidiyar*, et al.*, 2003).

Soler *et al.* (2004) performed a study with *gyrB* and *rpoD* sequences (Soler*, et al.*, 2004). They analyzed *gyrB* sequences that comprised between 960 and 1100 nucleotides which covered more than 70% of the ATPase domain and 191 nucleotides from de 3' flaking region. They obtained a value of 0 to 131 nucleotide differences between *Aeromonas* strains which corresponded to a rate of sequence similarity from 86.3 to 100%.

Regarding the intra-species level, the rates of substitution obtained ranged from 0-2.3%, usually showing values lower than 2% with an overall value around 1.6%. Nevertheless the nucleotide inter-species substitution rates were generally over 3% with the exception of two sets of species: *A. encheleia* and *Aeromonas* sp. *HG11* (2.1-2.2%) and *A. bestiarum* and *A. salmonicida* (1.8-4.3%). They concluded that *gyrB* is an excellent marker for accessing phylogeny in aeromonads and also showed an opening proof for a clear phylogenetic distinction between *A. salmonicida* and *A. bestiarum*.

The *gyrB* gene was also employed by *Tacão* and colleagues to access diversity among *Aeromonas* in environmental isolates, by using a PCR combined with a DGGE (Denaturing Gel Gradient Electrophoresis) technique. In this study a phylogenetic tree was constructed using the *gyrB* sequences from the amplified fragment and the grouping of species obtained was in agreement with previous studies. The PCR-DGGE results from this study showed the capacity of this method to distinguish the majority of species based on the differences in the migration of the amplified fragments. They concluded that is possible to study the dynamics of *Aeromonas* community by evaluating the molecular diversity of the sequence of *gyrB* (Tacão*, et al.*, 2005).

Saavedra *et al (2006).* performed a phylogenetic study based in 16S rRNA, *gyrB* and *rpoD* gene sequences which included for the first time the recently described species *A. simiae* and *A. molluscorum* and new isolates of *A. culicicola.* They reported that *gyrB* and *rpoD* gene sequences presented similar substitution rates, what confirmed that these two molecular markers are well synchronized. Regarding the species *A. culicicol*a, based on the *gyrB* phylogenetic tree, the type strain of this species and three strains isolated from ornamental fish composed a subcluster which was borderline with the group of *A. veronii* strains. Though, in the tree constructed with *gyrB* and *rpoD*, two strains of this species from *Aedes aegyptii* (MDC56 and MDC57) and two strains from drinking water clustered together with the species *A. veronii*. In relation to *A. simiae*, the two strains formed a different phylogenetic division showing considerable sequence divergence. *A. schubertii* is the species most related to *A. simiae* according to *gyrB* and *rpoD* phylogenetic trees. *A. molluscorum* strains formed a consistent group with a relatively long phylogenetic line, being positioned close to *A. encheleia* according to *gyrB* sequence phylogeny. Finally, they also reported that the proposal of *A. allosaccharophila* as a new species is supported by the *gyrB* sequence analysis. (Saavedra*, et al.*, 2006)

Saavedra *et al*. (2007) performed a study in which they identified 17 *Aeromonas* isolates from pig carcasses and from cleaning process equipment and one strain from a clinical case of gastroenteritis as *A. allosaccharophila* based in *gyrB* gene sequencing and *16S rDNA*. The sequences obtained from *gyrB* gene included 960-1100 nucleotides which covered more than 70% of the ATPase domain and around 190 nucleotides from the 3' flanking region. (Saavedra*, et al.*, 2007)

All of these studies demonstrated that the *gyrB* gene is a good molecular marker and very useful for helping in solving taxonomical problems as well as inferring phylogenetic relations at inter and intra-species level among *Aeromonas.*

1.2.4.3 *rpoD*

Another useful phylogenetic marker is the *rpoD* gene which encodes the σ^{70} factor that confer promoter-specific transcription initiation in RNA polymerase. Though the complex of the core of RNA polymerase which is composed of five subunits ($\beta\beta'\alpha_2\varpi$) is enough to perform transcription elongation and termination, it is incapable of initiating this process. The initiation of the transcription from promoter elements is achieved by a sixth dissociable subunit, the σ factor. This factor is reversibly associated with the core of RNA polymerase complex in order to form a holoenzyme (Paget & Helmann, 2003).

The sigma factors are included into two wide classes with lower identity between them: one family which is analogous to the originally identified *E. coli* σ^{70} subunit and other similar to the 54-kDa σ subunit from *Escherichia coli* (Lonetto*, et al.*, 1992).

The σ^{70} family constituents direct RNA polymerase to specific promoter elements generally constituted of 5 or 6 bp, which are centered between positions -10 and -35 of the transcription initiation site. They are also involved in the melting of promoter DNA and in the beginning stages of elongation in transcription (Paget & Helmann, 2003).

The σ^{70} family is composed by four groups based on the gene structure and function. The group 1 comprises the fundamental primary σ factors which are closely related to σ^{70} from *E. coli.* These factors exhibit high degree of similarity between them and are implicated in most of RNA synthesis that occurs in exponential growing cells therefore they are crucial to survival of cells. Group 2 is constituted by proteins associated to primary factors but they are surplus to requirements of bacterial cell growth. Group 3 includes σ factors that participate in the activation of regulons in reply of a specific signal.

These factors are plenty less related to σ^{70}. Lastly, group 4 contains the widest and highly variable extracitoplasmatic function subfamily, which participates in the response to stimuli from the extracitoplasmatic environment (Lonetto*, et al.*, 1992, Paget & Helmann, 2003). The protein encoded by *rpoD* gene, the σ^{70} factor, is integrated in group 1. It is ubiquitous in bacteria and also essential to cell survival. The genes encoding proteins evolve much faster than rDNA thus they show a higher level of resolution, what makes them excellent molecular markers (Yamamoto & Harayama, 1998, Yamamoto*, et al.*, 2000).

Yamamoto *et al.(2000)* used *rpoD* gene sequences together with *gyrB* to establish the phylogenetic relationships between *Pseudomonas* strains and concluded that the appliance of identification, detection and classification systems for *Pseudomonas* based on these two housekeeping genes can be very useful in several fields of bacteriology (Yamamoto*, et al.*, 2000).

As referred before, Soler *et al*. (2004*)*, performed an investigation using *gyrB* and *rpoD* genes to analyze the genus *Aeromonas* (Soler*, et al.*, 2004). The *rpoD* sequences analyzed comprised between 813 and 825 nucleotides which covered around 46% of the protein though the active domain was not included. Regarding the *rpoD* sequences, the range of sequence similarity between *Aeromonas* strains obtained was 81.7- 100% which corresponded to a number of nucleotide differences between 1 and 148. The sequence alignment also showed 281 variable positions corresponding to 34% of the sequenced fragment and a number of insertions or deletions of 12bp. Considering the intra-species level, the nucleotide substitutions ranged from 0 to 2.6% being generally lower than 2% and showing an overall value of approximately 1.6%. Nucleotide substitutions in inter-species analysis was over 3% except for this two cases: *A. veronii* and *A. culicicola*, (1.6-1.7%) and *A. encheleia* and *Aeromonas* sp. HG11 (1.4-1.7%). The analysis of this gene demonstrated enough resolution to separate *A. salmonicida* and *A. bestiarum*, which were not unambiguously distinguishable in previous studies. At the end the authors concluded that the addition of this gene to the analysis of the genus *Aeromonas* enhanced the advantages achieved by the use of *gyrB* sequences when compared to the 16S rRNA gene phylogeny.

In Saavedra *et al.* (2006) the *rpoD* sequences were also evaluated, as referred above. They reported that unrooted phylogenetic trees constructed based in *rpoD* and *gyrB*

presented strain clustering in conformity with all the species of aeromonads described, except for *A. culicicola*. According to them the *rpoD* gene sequence tree clustered all *A. culicicola* strains within the domain of *A. veronii* species. This fact supports the proposal that *A. culicicola* should be considered a synonym of *A. veronii*. The *rpoD* gene analysis from this study also showed that the new species *A. simiae* and *A. molluscorum* form new defined clusters, what supports the description of this species. In what concerns *A. allosaccharophila*, *rpoD* sequence analysis showed a borderline relationship between this species and *A. veronii* (Saavedra*, et al.*, 2006).

1.2.4.4 *sodB*

The *sodB* gene encodes the iron-containing superoxide dismutase. The superoxide dismutase gene sequences and amino acid compositions show significant similarity, suggesting highly conserved evolutionary relationships. Thus, the *sodB* gene may be useful for typing and establishing phylogenetic relationships in bacteria (Hassett*, et al.*, 1993).

Superoxide dismutases (SODs) comprise a ubiquitous class of antioxidant defense metalloenzymes which are responsible for catalyzing the conversion of superoxide radical into a hydrogen peroxide and dioxygen (Leclere*, et al.*, 2001). In the pathway wherein SOD is involved, this metalloenzyme is responsible for the first step in detoxification of the superoxide anion (O_2^-) to H_2O and O_2 via hydrogen peroxide (H_2O_2) (Dacanay*, et al.*, 2003).

There are four groups of SOD's, the iron type (FeSOD), the manganese SOD (MnSOD), the copper-zync type (Cu/ZnSOD) and the nickel type (NiSOD). The first two referred types are cytoplasmatic and the CuZnSOD is situated inside the periplasmic space (Leclere*, et al.*, 2001). It has also been reported the existence of a FeZnSOD, a hybrid isoform which contains copper and zinc as prosthetic metal (Kim*, et al.*, 1998).

The FeSOD is produced in a constant proportion either in aerobic and anaerobic conditions however MnSOD is only produced under aerobic conditions and is influenced by exposure to oxygen or O_2^- and also by changes in the growth phase (Leclere*, et al.*, 2001).

The physiological respiratory processes in aerobes and facultative organisms produce most of the oxidative stress in cells. The nonexistence of SOD's produces several oxygen-dependent phenotypic modifications in *E. coli*, including structural instability in

the cell envelope, serious defects in amino acid biosynthesis and high rate of spontaneous mutagenesis. Thus, SODs are responsible for conferring protection against oxygen-dependent DNA damage (Lynch & Kuramitsu, 2000).

The primary function of SOD is the removal of endogenous oxidants which are produced during normal oxidative metabolism but it also confers cells protection against oxidants exogenously produced (Dacanay*, et al.*, 2003). The SODs are thought to be important in the pathogenicity of some bacteria. This may be related to the fact that the capacity of an organism to infect a host is partially associated to its capacity to resist to an oxidative environment caused by the production of reactive oxygen species by cells from the host defense like polymorphonuclears and monocytes/macrophages (Lynch & Kuramitsu, 2000, Santos*, et al.*, 2001).

In an investigation performed by Leclere *et al.* (2001) they reported the existence of two superoxide dismutases in *A. hydrophila* encoded by *sodA* and *sodB* genes. The *sodA* encoded a protein with MnSOD activity with 206 amino acids with approximately 22.5 kDa which showed 55% homology with *E. coli* MnSOD. The *sodB* gene encoded a iron-containing SOD with 21.5 kDa showing 75% of homology with E. coli FeSOD, and composed by 196 amino acids (Leclere*, et al.*, 2001).

Dacanay *et al*. (2003) have identified two open reading frames and related upstream sequences that encoded two supposed SODs, *sodA* and *sodB* in *A. salmonicida* subsp. *salmonicida* (Dacanay*, et al.*, 2003). The *sodA* gene encoded SodA, a protein with 204 amino acids with a molecular mass in the order of 23.0 kDa which presented high similarity to other Mn-SODs. On the other hand *sodB* gene encoded a protein with 194 amino acids with a corresponding molecular mass of approximately 22.3 kDa and presented highest similarity to a manganese SOD gene from *Vibrio parahaemolyticus*, however, that is thought to be due to a misannotation of that gene as *sodA;* and also high similarity to other prokaryotic Fe-SODs. They also reported that SOD activity was significantly higher in virulent strains than in the avirulent strains what suggests that virulent strains had an improved antioxidant capacity compared with the avirulent strains.

The influence of FeSOD, in virulence of pathogenic bacteria is still controversial. Some authors defend that these metalloenzymes are somehow implicated in virulence, either because there are differences in SOD activity in virulent and avirulent strains as reported by Dacaney *et al.* (Dacanay*, et al.*, 2003), or because deficient mutants with

reduced *sodB* expression show attenuated virulence as reported by Bakshi *et al.* (Bakshi*, et al.*, 2006). In contrast the molecular analysis of genetic differences between virulent and avirulent strains of A. hydrophila performed by Zhang *et al.* did not pointed SOD's as virulence factors (Zhang*, et al.*, 2000).

The *sod* genes have been employed in typing microorganisms and in establishing phylogenetic relations between them. Zolg and Philippi-Schulz used *sodA* gene as target to detect mycobacteria and developed specific probes which recognized species-specific variable regions within this gene to identify them (Zolg & Philippi-Schulz, 1994). Alber *et al.* also developed species-specific PCR assays based on this gene for the identification of *Streptococcus phocae* (Alber*, et al.*, 2004). On the other hand Cattoir and colleagues used a fragment from *sodA* gene to infer the phylogenetic relations between *Haemophilus spp*. and concluded that the resulting phylogenetic tree was generally in agreement with the trees resulting from the analysis of 16S rDNA and other housekeeping gene sequences (Cattoir*, et al.*, 2006). Devulder *et al.* studied the phylogeny of Mycobacteria using four genes which included the *sodA* gene (Devulder*, et al.*, 2005).

Monstein developed a multiplex PCR for typing Helicobacter pylori which included the *sodB* gene as target (Monstein & Ellnebo-Svedlund, 2002).

The high importance of FeSOD in the cells, the similarity verified between this gene sequences and the highly conserved evolutionary relationships which were suggested to this gene make it a possible suitable phylogenetic marker that should be more exploited. Though the *sodB* has never been use to identify and establish phylogenetic relations between aeromonads all the characteristics referred above make them a potential molecular marker to study *Aeromonas.*

1.3 Communities' studies

Generally in microbiological studies species are cultured and then characterized based on their biochemical, physiological and/or molecular features. However this traditional method presents an important disadvantage related to the difficulties found in culturing and isolating most of the bacteria (Fontana*, et al.*, 2005). It is known that only a small percentage of bacteria present in the environment can be cultured and identified using culturable methods (Amann*, et al.*, 1995). It has been estimated the percentage of culturable microorganisms in several environments as we can see in the table bellow:

Table 3 - Percentage of culturable bacteria in different habitats (adapted from Amann *et al.*, 1995).

Habitat	Culturability (%)
Seawater	0.001–0.1
Freshwater	0.25
Mesotrophic lake	0.1–1
Unpolluted estuarine waters	0.1–3
Activated sludge	1–15
Sediments	0.25
Soil	0.3

The realization of these estimatives surged in the following of the so called 'great plate count anomaly'. This phenomenon consists in the fact that the majority of cells which are observed using the microscopy are viable but do not form visible colonies on plates, being therefore nonculturable. Because of this fact, huge discrepancies were found between the plate counts and the microscopic observation (Amann*, et al.*, 1995).

Even though culture methods are essential for understanding specific microorganisms, in what concerns community analysis, culturing is unable to replicate symbiotic relations and the ecological niches from natural environments. In culturing, besides the selective growth of some species in detriment of others, the composition of the culturable community is also distorted. Thus, the appliance of culture-independent methods based on molecular approaches becomes essential (Nocker*, et al.*, 2007).

The molecular methods, including DNA-based fingerprinting techniques allowed the analysis of microbial communities, amplifying our vision on the microbial diversity. These approaches became essential tools in microbial ecology and in other areas because it is now recognized that many behavioral features of the individual species can only be

explained in the community perspective. With the utilization of these methods, it is possible to assess genetic diversity, species composition, and population structure. It also allows the comparison of different communities and their monitoring during environmental changes (Nocker*, et al.*, 2007).

As referred above, molecular methodologies provide new opportunities for the analysis of microbial communities and for the identification of unculturable species. Initially, one molecular approach using these genes, most often the 16S gene, was applied which comprised extraction of DNA directly from environmental samples, cloning of ribosomal DNA or amplified ribosomal DNA and then sequence analysis of the obtained clones (Giovannoni*, et al.*, 1990). However, the construction of environmental 16S rDNA libraries only provided a qualitative data about the community and besides that a large number of clones were needed for analysis (Muyzer*, et al.*, 1993).

Several other approaches have been reported, based on direct cloning and sequencing DNA fragments (shotgun cloning) or by using initial amplification of target sequences with PCR, followed by several possible fingerprinting methods. These profiling methods include amplified ribosomal DNA restriction analysis (RFLP), terminal restriction fragment length polymorphism (T-RFLP), single strand conformation polymorphism (SSCP), automated ribosomal intergenic spacer analysis (ARISA), denaturing high-performance liquid chromatography (DHPLC), temperature gradient gel electrophoresis (TGGE), and denaturing gradient gel electrophoresis (DGGE) (Nocker*, et al.*, 2007).

In 1993, Muyzer *et al.* introduced a denaturing gradient gel electrophoresis technique which allowed assessing the genetic diversity of complex microbial communities. This procedure was based on the separation of 16S rDNA fragments previously amplified by PCR, using an electrophoresis in polyacrilamide gels containing a linearly increasing gradient of denaturants. This allowed separating fragments with the same length but with different base compositions (Muyzer*, et al.*, 1993).

In DGGE, the separation of fragments is achieved based on the electrophoretic mobility of partially melted DNA molecules in polyacrilamide gels, which is lower than that of the helical form of DNA. When the fragment reaches its corresponding melting temperature in the gradient of the DGGE gel, it becomes partially melted and the migration will practically stop. Therefore, DNA fragments with differences in their sequence and consequently with different melting temperatures stop migrating at different positions

(Lerman, *et al.*, 1984, Muyzer & Smalla, 1998). In DGGE a GC clamp of around 40-50 bp is attached to the 5' end of one of the primers in order to prevent the complete dissociation of the two DNA strands (Sheffield, *et al.*, 1989). This technique presents some advantages such as allowing the analysis of several samples in simultaneous and making possible the recovery of the DNA from the bands and consequently allowing the identification of the corresponding phylotype.

Other investigators have also used 16S rDNA in a PCR-DGGE method to study communities in several samples from different sources such as soils, food and aquatic environments. For example Li and colleagues developed a PCR-DGGE method using the 16S rDNA as target to explore the bacterial diversity in chilled pork during storage (Li, *et al.*, 2006). Drees and colleagues assessed bacterial communities in soils from the hyperarid Atacama Desert on Chile (Drees, *et al.*, 2006). Henriques *et al.* studied the dynamics of free-living bacterial community in an estuarine environment (Henriques, *et al.*, 2006).

The PCR-DGGE method can be applied to study specific groups of microorganisms by using specific primers. Thus besides the 16S rRNA gene, other target genes have been used in PCR-DGGE techniques such as the *dsrB* gene used to assess sulfate- reducing communities (Geets, *et al.*, 2006), the *rpoB* to study the diversity of *Paenibacillus* species (da Mota, *et al.*, 2005) and *gyrB* for typing and assessing *Aeromonas* communities (Tacão, *et al.*, 2005).

Tacão *et al.*, as referred before, developed a *gyrB*-DGGE method for typing *Aeromonas* which allowed strain differentiation. They also compared this method with the standard 16S rDNA-DGGE. They reported that the analysis of 16S rRNA-DGGE failed to distinguish *A. salmonicida* from *A. bestiarum* and also that some *Aeromonas* strains presented more than one band what could be explained by the presence of different 16S rRNA operons in a single cell. On the other hand the *gyrB*-DGGE presented enough differences in the migration of the amplified fragments to distinguish the majority of strains of that genus. They also verified that strains with identical *gyrB* sequence presented similar mobility. When they used *gyrB*-DGGE to analyze complex samples with total DNA from water samples it became evident that this methodology was a promising procedure to assess the dynamics of aeromonads by analyzing the diversity of *gyrB* sequences (Tacão, *et al.*, 2005). In order to determine weather if this method can be used for assessing *Aeromonas* communities several steps should be performed. They include

experimental evaluation of primers specificity and detection levels; *in silico* validation of primers; optimization of gradients; performing and evaluating the PCR-DGGE with environmental samples and finally determining if the environmental amplified fragments correspond to aeromonads by using a cloning strategy or by excising and purifying bands followed by sequencing.

2 Objectives

The main objective of this investigation is to develop, optimize and apply culture independent PCR-DGGE assays using three primer sets targeting *gyrB*, *sodB* and *rpoD* genes, in order to assess the diversity and study the dynamics of *Aeromonas* communities in aquatic environments. Specifically, we will:

- test the primers specificity and sensibility
- test and apply the PCR-DGGE methodologies to investigate the molecular diversity and follow the dynamics of *Aeromonas* communities in an estuarine environment (Ria de Aveiro)
- evaluate the phylogenetic information which is possible to infer from the obtained amplified fragments

3 Materials and methods

3.1 Bacterial strains

In this investigation we used 27 *Aeromonas* strains and 13 strains from other species. The list of these strains and their origins is presented in Table 4. Fourteen were strains obtained from culture collections and 26 were isolated from environmental sources during previously conducted studies (Henriques, *et al.*, 2006, Carvalho, *et al.*, unpublished).

Table 4 - Bacterial strains and sources.

Species Name	**Strain Reference**	**Source**
Aeromonas allosaccharophila	A10-6	Irrigation water
Aeromonas bestiarum	127/2	Untreated drinking water
Aeromonas bivalvium	CECT 7113^{T}	Cockles (Cardium sp.)
Aeromonas bivalvium	CECT 7112	Retail market, razor-shells (Ensis sp.)
Aeromonas caviae	G.I10.8	Estuarine water
Aeromonas caviae	CECT 838^{T}	Epizootic of young guinea pigs
Aeromonas caviae	L6	Milk
Aeromonas encheleia	22/6	Mineral water
Aeromonas eucrenophila	L12-9	Lettuce
Aeromonas HG11	120/1	Untreated drinking water
Aeromonas hydrophila	A5-11	Untreated drinking water
Aeromonas hydrophila	G.I10.10	Estuarine water
Aeromonas hydrophila subsp. *hydrophila*	CECT 839^{T}	Tin of milk with a fishy odour
Aeromonas media	A4-3	Irrigation water
Aeromonas media	G.I10.21	Estuarine water
Aeromonas molluscorum	G.I6.7	Estuarine water
Aeromonas popoffii	130/12	Untreated drinking water
Aeromonas salmonicida	G.I6.17	Estuarine water
Aeromonas salmonicida	L14-7	Parsley
Aeromonas sobria	CECT 4246	Frog red-leg
Aeromonas veronii	G.I6.9	Estuarine water
Aeromonas veronii	96/2-7	Untreated drinking water

Aeromonas veronii	CECT 4257^T	Human sputum, drowning victim
Aeromonas veronii bv sobria	G.NI28	Estuarine water
Aeromonas 'tecta'	109B1	Untreated drinking water
Aeromonas sp.	L15-1	Lettuce
Aeromonas sp.	L10-9	Lettuce
Bacillus sphaericus	ATCC 29726	Contaminated blood transfusion bottle
Corynebacterium glutamicum	ATCC 13032	Sewage
Enterococcus faecalis	ATCC 29217	
Escherichia coli	M.I10.46	Estuarine water
Escherichia coli	ATCC 25922	Clinical isolate
Klebsiella pneumoniae	M.I10.31	Estuarine water
Listeria innocua	ATCC 33090	Cow brain
Micrococcus luteus	ATCC 13513	Unknown
Morganella morganii	M.N1.3	Estuarine water
Pseudomonas putida	G.I10.7	Estuarine water
Pseudomonas putida	NCIMB 10432	Soil using benzoate as a major carbon source
Staphylococcus aureus	ATCC 6538	Human lesion
Vibrio sp.	G.I6.18	Estuarine water

3.2 DNA isolation

DNA isolation was performed using the Genomic DNA Purification Kit from MBI Fermentas (Vilnius, Lithuania), according to adapted instructions. An additional step of incubation at 37ºC for 1 hour with lysozyme (10 mg/ml) was included in the beginning of the procedure to improve lysis.

Detailed protocol:

- Strains were grown overnight in LB broth (Luria-Bertani, Miller 1972; composition in g/l: yeast extract 5.0; peptone from casein 10.0; sodium chloride 10.0).
- One ml of cell culture was centrifuged during 5 minutes at 13200 rpm and the pellet was ressuspended in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0).
- Twenty five µl of 10 mg/ml lysozyme solution (Eurobio, France) were added and the suspension was incubated for 1 hour at 37ºC to improve lysis.
- The suspension was mixed with 400 µl of lysis solution (Genomic DNA Purification Kit) and the mixture was incubated for 10 minutes at 65ºC.

- Immediately, 600 µl of chloroform were added followed by softly inversion in order to emulsifying the mixture.
- The sample was centrifuged at 13400 rpm during 10 minutes.
- The top aqueous phase which contained the DNA was transferred to a new tube and the last two steps were repeated.
- Following, 0.6 volumes of isopropanol were added to allow the DNA precipitation, and the solution was gently inverted and incubated at 4ºC during 10 minutes.
- The mixture was then centrifuged at 13400 rpm during 15 minutes.
- The supernatant was removed and the pellet was completely dissolved in 100 µl of 1.2 M NaCl solution.
- Two hundred and fifty µl of cold ethanol were added and DNA was left to precipitate at -20ºC during 45 minutes
- The mixture was centrifuged during 15 minutes at 13200 rpm.
- The supernatant was eliminated and the pellet was washed with 70% ethanol.
- The DNA was ressuspended in 50 µl of TE and stored at -20ºC.

3.3 Primers

The sets of primers used in this investigation were previously designed by Tacão and colleagues (Tacão*, et al.*, 2005). The primers sequences as well as their characteristics are listed in table 5.

Table 5 - Primers used in the amplification reactions targeting *gyrb*, *rpoD* and *sodB* genes and their characteristics.

Gene Target	Primers sequence	GC Content (%)	Melting Temperature (ºC)	Primer Position (a)
gyrB	**gyrB_F** 5’ -GAAGGCCAAGTCGGCCGCCAG-3’	71	62	912-932
	gyrB_R 5’ -ATCTTGGCATCGCCCGGGTTTTC-3’	57	59	1086-1109
rpoD	**rpoD_F** 5’- ATGCCGAAGAAAACCTTCGT-3’	45	50	904-923
	rpoD_R 5’- CGGTTGATATCCTTGATCTG-3’	45	50	1084-1103

sodB	**sodB_F** 5'- GTACCGAGTTTGAAGGCAAGTC-3'	50	55	134-155
	sodB_R 5'- CCGAAGTTGCCGATGGC- 3'	65	52	343-359

a) according to the genome of *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (accession number: NC 008570)

3.3.1 Primers specificity testing

In order to test primers specificity, amplification reactions were prepared using DNA from a wide variety of bacterial strains. These include *Aeromonas* and non-target species either closely related to *Aeromonas* spp. or phylogenetically distant (Table 4).

- PCR reactions for *gyrB*, *rpoD* and *sodB* were carried out using a final volume of 25 µl. Each reaction mixture contained:
  - 1 x PCR buffer
  - 3 mM $MgCl_2$
  - 5 % dimethylsulfoxide
  - 200 mM of each nucleotide
  - 7.5 pmol of each primer
  - 0.5 U *Taq* polymerase
  - 50-100 ng of DNA.

The reactions were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The Taq polymerase, the buffer and the dNTP's were from MBI Fermentas (Vilnius, Lithuania). Amplification conditions and expected fragment length for each gene target are listed in the table below:

Table 6 - PCR conditions for each gene target and expected fragment length.

Gene Target	Amplification conditions		Expected Fragment Length
gyrB	1 cycle	**Initial denaturation:** 94ºC for 9 min	198bp
	35 cycles	**Denaturation:** 93ºC for 30s **Annealing:** 60ºC for 30s **Extension:** 72ºC for 30s	
	1 cycle	**Final extension:** 72 ºC for 10min	

rpoD	1cycle	**Initial denaturation:** 94ºC for 9 min	200bp
	30 cycles	**Denaturation:** 93ºC for 30s **Annealing:** 54ºC for 30s **Extension**: 72ºC for 30s	
	1 cycle	**Final extension:** 72 ºC for 10min	
sodB	1cycle	**Initial denaturation:** 94ºC for 9 min	226bp
	30 cycles	**Denaturation:** 93ºC for 30s **Annealing:** 55ºC for 30s **Extension:** 72ºC for 30s	
	1 cycle	**Final extension:** 72 ºC for 10min	

To analyze the resulting amplicons, 2.5 µl of PCR products were loaded in 1% agarose gels in 1x TAE buffer (Biorad, Hercules, CA, USA) and 2 µl of a molecular weight marker, the 100bp DNA ladder plus (MBI Fermentas, Vilnius, Lithuania), was also included. Electrophoresis was performed at 80v during 80 minutes. The gels were stained in ethidium bromide and then rinsed in distilled water during 5 minutes. Images were acquired using the Molecular Imager FX system (Bio Rad Laboratories, Hercules, CA, USA).

3.3.2 *In silico* specificity testing

Primer sequences were checked against gene sequences available in the GenBank database using the BLAST tool (Altschul, *et al.*, 1997). Sequences from *Aeromonas* for *gyrB, rpoD* and *sodB* genes available in the GenBank were downloaded and the primers positions were searched and mismatches were recorded.

3.3.3 Primers detection limits

In order to determine the detection limits of the 3 primer sets, the DNA concentration of a positive sample, from *A. hydrophila* subsp. *hydrophila* CECT 839^T was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and then serially diluted. A 1:3 dilution was prepared followed by 8 serial 10-

fold dilutions according to the scheme bellow, till the DNA concentration reached 1 pg/ml.

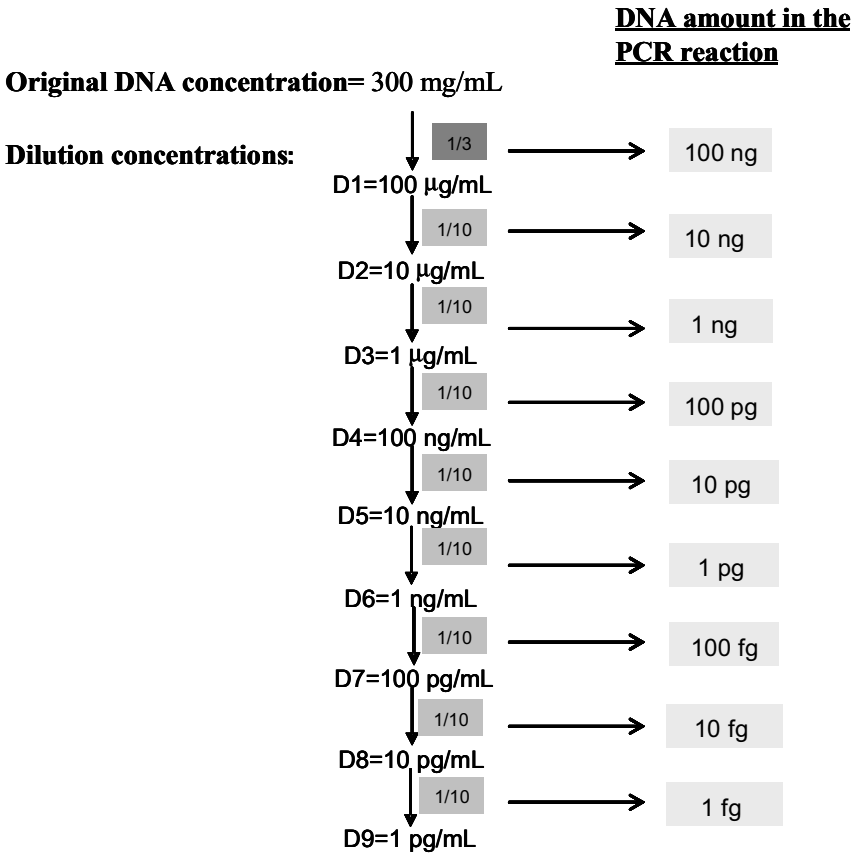


Figure 1 - Dilutions scheme

The diluted samples were submitted to amplification with the primers targeting *gyrB*, *rpoD* and *sodB* and the amplicons were analyzed by electrophoresis according to the procedure referred before in the 3.3.1 section. The detection limit was considered to be below the lowest DNA quantity that originated a detectable band in the electrophoresis gel.

3.4 Evaluation of phylogenetic information provided by the PCR target fragment

The phylogenetic information provided by the amplified fragments was evaluated. For this, we compared phylogenetic trees derived from the sequence typically used for phylogenetic analysis of the genes *gyrB, rpoD* and *sodB* and phylogenetic trees derived from fragments amplified with primers designed during this study.

The *gyrB, rpoD* and *sodB* sequences from *Aeromonas* species stored in the GenBank database were downloaded and aligned using the CLUSTALX program

(Thompson, *et al.*, 1997). Phylogenetic analysis was performed using PAUP version 4.0b10 (Swofford, 2003). Trees were generated using the neighbour-joining tree building algorithm. Bootstrap support values (1000 replicates) were calculated.

From the same sequences, the fragment that is amplified using the designed primers was extracted and used to construct phylogenetic trees, following the same procedure.

3.5 Development, optimization and evaluation of specific PCR-DGGE culture independent methods to study *Aeromonas* communities

With the purpose of develop, optimize and test *Aeromonas*-specific culture-independent PCR-DGGE methodologies based on the 3 primer sets, several experiments were conducted. In the beginning DGGE gradients were tested and optimized. A DGGE marker was constructed using different *Aeromonas* strains. Finally, environmental DNA samples were submitted to PCR-DGGE using the three sets of primers and the obtained profiles were analyzed.

3.5.1 Construction of DGGE markers

3.5.1.1 PCR amplification of DNA from *Aeromonas* strains to perform DGGE

PCR amplification from *Aeromonas* strains (Table 4) using the three primer sets and the analysis by electrophoresis were performed as described before in the 3.3.1 section except in what concerns:

- Forward primers- in this amplification, a GC clamp 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG-3' (Muyzer, *et al.*, 1993) was attached to the 5' end of the forward primer.
- Final extension- performed during 30 minutes rather than 10 minutes.

3.5.1.2 DGGE of the PCR products from *Aeromonas* strains

Solutions were prepared to create polyacrylamide gels (8% [wt/vol] polyacrylamyde in 50x TAE) with denaturing gradients of 45%-70% for *gyrB*, 40%-80% for *rpoD* and 40%-80% for *sodB* amplification products. The corresponding 100% denaturant gradient was 7M urea and 40% of deionized formamide.

- One hundred and forty µl of a 10% ammonium persulfate solution (Sigma, St. Louis, USA) and 14 µl of TEMED (Sigma, St. Louis, USA) were added to the solutions.
- The gradient was constructed by using a Gradient Maker (Bio-Rad Laboratories, Hercules, CA, USA) and the gels were left to polymerize for 1 hour and 30 minutes.
- Five µl of PCR products were loaded in the gels.
- DGGE was performed using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at 60ºC and at a constant voltage of 20V for 15 minutes followed by 75V during 16 hours.
- The gels were stained in ethidium bromide during 5 minutes and rinsed with distilled water under agitation for 20 minutes.
- Images were acquired using a Molecular Imager FX system (Bio Rad Laboratories, Hercules, CA, USA).

After gel analysis several amplicons showing different band positions were chosen. Two µl of each of the selected amplicons were mixed together in order to construct one DGGE marker for each assay.

3.5.2 PCR-DGGE of environmental samples from Ria de Aveiro

3.5.2.1 Environmental DNA

In this study, DNA from complex estuarine bacterioplankton communities was used to evaluate the usefulness of the optimized methodologies to assess the diversity and follow the dynamics of aeromonads present in the estuary Ria de Aveiro. The environmental samples were obtained during a study conducted in the estuary by Henriques *et al.* (Henriques*, et al.*, 2006). The samples used in our investigation were collected in July (year 2003) and in January (year 2004) at six sampling sites: N-1 (placed in the transition to the coastal zone) I-2, I-6 and I-8 (in the middle-estuary) and I-10 and RB (in the mixing zone between the fresh and marine water). The sampling strategy was as previously described (Henriques*, et al.*, 2006). Briefly samples were collected in 2 L autoclaved dark bottles always during daytime, at low tide, approximately 0.2 m below the

surface. DNA was extracted from water samples immediately after sampling as described in the referred study (Henriques *et al.*, 2006.). In Figure 2 the location of the sampling points is indicated by arrows and Table 7 presents temperature and salinity values for each sample.

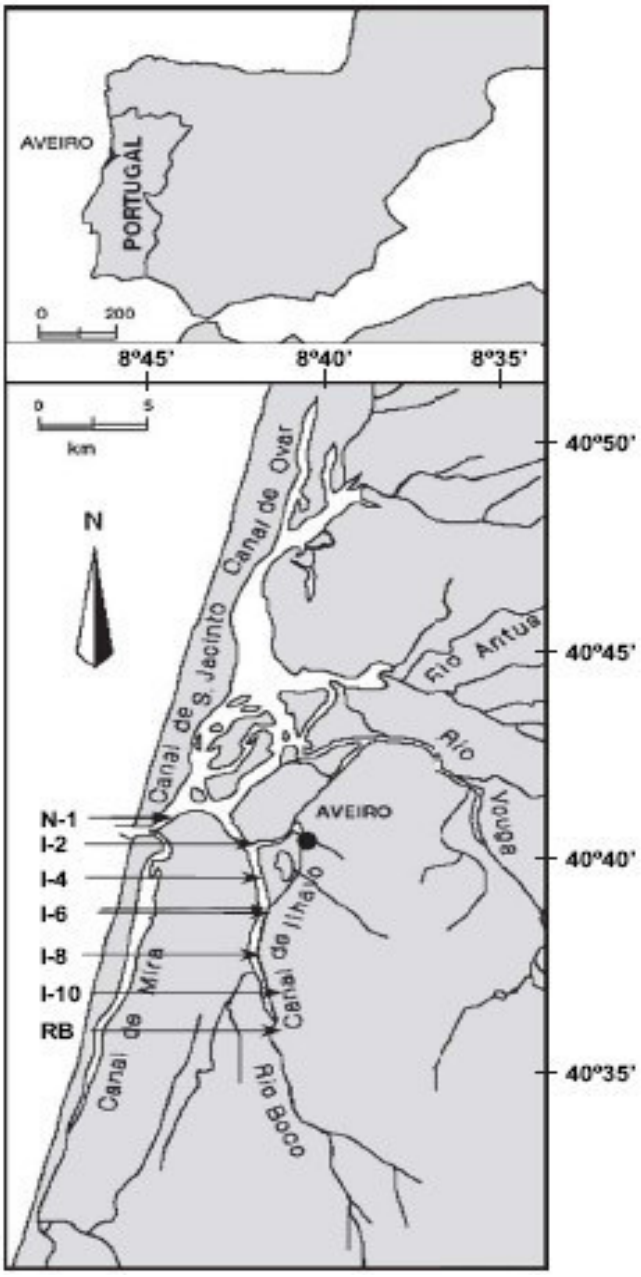


Figure 2 - Ria de Aveiro Lagoon with sampling sites marked with arrows.

Table 7 - Environmental samples and corresponding temperature and salinity values (from Henriques *et al.*, 2006).

Environmental Sample	Temperature (ºC)	Salinity
N-1 July	17.0	35.1
I-2 July	19.0	33.7
I-6 July	21.0	28.7
I-8 July	21.0	27.7
I-10 July	21.0	15.7
RB July	21.0	8.4
N-1 January	14.0	32.0
I-2 January	14.0	25.5
I-6- January	16.0	16.6
I-8 January	16.0	12.9
I-10 January	15.0	4.0
RB January	14.5	0.0

3.5.2.2 PCR amplification from environmental DNA

The environmental DNA samples were submitted to PCR amplifications as described in section 3.3.1 with the following exceptions:

- The final volume of each reaction was 35 µl.
- Forward primers with GC clamp were used.
- A final extension of 30 minutes was included.

A second PCR amplification was performed following the same procedure to increase the amount of PCR products from environmental samples, using as template 1 µl of the first PCR reactions. To confirm amplification, agarose gels were loaded with 5 µl of each PCR product.

3.5.2.3 DGGE of the environmental amplicons

DGGE of the PCR products from environmental samples was performed as described before in section 3.5.1.2 with the following exceptions:

- Thirty µl of the PCR products were loaded into the gels.
- The DGGE markers constructed before were also loaded into the gels.

3.5.2.4 Analysis of DGGE profiles

Gel images were analyzed with the GelCompar II software (Applied Maths, Kortrijk, Belgium). Every gel contained three lanes with a DGGE marker for internal normalization and as an indication of the quality of the analysis. Similarity matrices were calculated with the Jaccard coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair-group method using arithmetic averages (UPGMA).

3.6 Cloning and sequencing environmental amplicons

To confirm that the fragments obtained by PCR from environmental samples corresponded to *Aeromonas* phylotypes, representative reactions targeting *gyrB, rpoD and sodB* genes were used to construct small insert libraries. From those, positive clones were selected and subjected to sequencing and phylogenetic analysis.

3.6.1 Amplification of PCR products

To amplify PCR products for cloning, two environmental samples were chosen for each assay: in the *gyrB and sodB* assays the samples N1 July and RB July were used whether in the *rpoD* assay samples I2 July and RB July were chosen. PCR amplification and electrophoresis analysis were performed as referred before (section 3.3.1) using forward primers without clamp.

3.6.2 Cloning into pCR®2.1

Cloning was performed by using the TA Cloning® kit (Invitrogen, Paisley, UK) with vector pCR®2.1, a one step cloning approach to directly insert a PCR product in a vector according to the manufacturer instructions. It is based in the fact that Taq polymerase adds single deoxyadenosine (A) to the 3' ends of PCR products and since the linearized vector used in this procedure has single deoxythymidine (T) residues it is possible to ligate the PCR insert to the vector.

- The ligation reaction was performed using the following components:
    - 0.25 µl PCR product
    - 0.25 µl Ligation Buffer (10X)
    - 0.5 µl pCR®2.1 vector (25 ng/µl)
    - 0.25 µl T4 DNA Ligase (4.0 Weiss units)
    - 1.25 µl sterile water
- The ligation reaction was then incubated overnight at 14ºC

3.6.3 Transforming competent cells

- The vials containing the ligation reactions and the frozen One Shot® Competent Cells TOP10F' (F′ {*lac*Iq Tn10 (TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G)(Invitrogen) were placed on ice.
- Two µl of the ligation reaction were added to 25 µl of TOP10F' competent cells for each transformation. The solution was mixed gently using the pipette tip.
- The vials were incubated on ice during 30 minutes.
- Cells were subsequently heat shocked at 42ºC during 30 seconds and then immediately transferred into the ice.
- Two hundred µl of SOC medium (2% Tryptone; 0.5% Yeast Extract; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl2; 10 mM MgSO4; 20 mM glucose) were added to each vial.
- The vials were incubated at 37ºC during 1 hour at 200 rpm.
- Fifty to eighty µl of the transformation were plated in previously prepared LA agar plates supplemented with ampicillin (50 µg/ml), X-Gal (40 µl of a 40 mg/ml solution were spread on top of each plate) and IPTG (40 µl of a 100 mM solution were spread on top of each plate).
- The plates were incubated overnight at 37ºC.

3.6.4 Analyzing transformants

- Twenty four white colonies from each transformation were selected and grown overnight at 37ºC in LA agar plates containing ampicillin, X-Gal and IPTG.
- Cells from selected colonies were ressuspended in 10 µl of distilled water.

- The suspensions were incubated at 100ºC during 10 minutes to lyse the cells and release the DNA.
- PCR amplification using 3 µl of the cells lysate as template and electrophoresis were performed according to section 3.3.1, but using forward primers with a GC clamp.
- The clones showing positive amplification and therefore containing the insert were selected to perform DGGE.
- DGGE was performed according to the instructions of section 3.5.1.2. Five µl of the PCR clone products were loaded into the gels. Previously amplified environmental DNA and the constructed DGGE markers were also loaded into the gels.
- Clones displaying different band positions were selected for sequencing.

3.6.5 Amplification from positive clones using M13 Reverse and T7 primers

For sequencing analysis, PCR amplification of DNA from clone cell lysates was performed using primers M13 Reverse and T7 targeting pCR®2.1 vector sequences. The PCR reaction constituents and the PCR conditions are listed in the table below:

Table 8 - PCR reaction constituents and the PCR conditions to amplify positive clones

PCR Reaction Components	**Amplification Conditions**	
1x PCR buffer 3 mM $MgCl_2$ 5% dimethylsulfoxide 200 mM each nucleotide 7.5 pmol of M13 reverse and T7 primers 0.5 U *Taq* polymerase 3 µl of clone cells lysate	1cycle	**Initial denaturation:** 94ºC for 5 min
	35 cycles	**Denaturation:** 94ºC for 30s **Annealing:** 55ºC for 30s **Extension:** 72ºC for 1m30s
	1 cycle	**Final extension:** 72ºC for 10min

Electrophoresis analysis was performed according to the instructions referred in point 3.3.1.

3.6.6 Purification of PCR products for subsequent sequencing

In order to obtain purified PCR products for sequencing we have used the JETQUICK PCR Product Purification Spin Kit (Genomed, LÖhne, Germany) according to the manufacturer instructions.

Detailed procedure:

- Four hundred µl of Solution H1 (JETQUICK Kit) were added to the PCR product.
- A JETQUICK spin column was placed into a 2ml receiver tube and the previous mixture was loaded into it.
- The column was centrifuged at 12,000 x g for one minute.
- The flowthrough was discarded.
- The spin column was re-inserted into the empty receiver tube and 500 µl of solution H2 (JETQUICK Kit) were added to the column.
- The column was centrifuged at 12,000 x g during one minute.
- The flowthrough was discarded and the column was placed again in the receiver tube.
- The column was centrifuged again at maximum speed for 1 minute.
- To elute the DNA the JETQUICK spin column was placed into a new 1.5 ml microtube and 50 µl of sterile water were added onto the center of the silica matrix of the column.
- The column was centrifuged at 12,000 x g during 2 min to collect the purified PCR product.

3.6.7 Sequencing and sequence analysis

Purified products were used as templates in sequencing reactions that were carried out by the company STAB-VIDA (Oeiras, Portugal). Obtained sequences were compared to the GenBank nucleotide data library using the BLAST software (Altschul*, et al.*, 1997)in order to determine their closest phylogenetic relatives. Sequences were aligned with reference taxa within the sequence databases using the CLUSTAL X program (Thompson*, et al.*, 1997). Phylogenetic analyses were performed with PAUP* version4.0b10 (Swofford, 2003). Trees were produced using the neighbour-joining method.

4 Results

4.1 Primers

4.1.1 Primers specificity testing

The results of the primers specificity experimental testing performed for the three sets of primers targeting *gyrB*, *rpoD* and *sodB* are listed in the table below. Tests consisted in PCR amplification of the target genes from strains belonging to 15 different *Aeromonas* species and to 11 non-target species.

Table 9 - Amplification results for the three sets of primers targeting *gyrB*, *rpoD* and *sodB*. (+) positive result, (-) negative result.

Species Name	**Strain Reference**	***gyrB***	***rpoD***	***sodB***
Aeromonas allosaccharophila	A10-6	(+)	(+)	(+)
Aeromonas bestiarum	127/2	(+)	(+)	(+)
Aeromonas bivalvium	CECT 7113 ^{T}	(+)	(+)	(+)
Aeromonas bivalvium	CECT 7112	(+)	(+)	(+)
Aeromonas caviae	G.I10.8	(+)	(+)	(+)
Aeromonas caviae	CECT 838 ^{T}	(+)	(-)	(+)
Aeromonas caviae	L6	(+)	(+)	(+)
Aeromonas encheleia	22/6	(+)	(+)	(+)
Aeromonas eucrenophila	L12-9	(+)	(+)	(+)
Aeromonas HG11	120/1	(+)	(+)	(+)
Aeromonas hydrophila	A5-11	(+)	(+)	(+)
Aeromonas hydrophila	G.I10.10	(+)	(+)	(+)
Aeromonas hydrophila subsp. *hydrophila*	CECT 839 ^{T}	(+)	(+)	(+)
Aeromonas media	A4-3	(+)	(+)	(+)
Aeromonas media	G.I10.21	(+)	(+)	(+)
Aeromonas molluscorum	G.I6.7	(+)	(-)	(+)
Aeromonas popoffii	130/12	(+)	(+)	(+)
Aeromonas salmonicida	G.I6.17	(+)	(+)	(+)
Aeromonas salmonicida	L14-7	(+)	(+)	(+)
Aeromonas sobria	CECT 4246	(+)	(+)	(+)
Aeromonas veronii	G.I6.9	(+)	(+)	(+)
Aeromonas veronii	96/2-7	(+)	(+)	(+)

Aeromonas veronii	CECT 4257 ^{T}	(+)	(+)	(+)
Aeromonas veronii bv. *sobria*	G.NI28	(+)	(+)	(+)
Aeromonas 'tecta'	109B1	(+)	(+)	(+)
Aeromonas sp.	L15-1	(+)	(+)	(+)
Aeromonas sp.	L10-9	(+)	(+)	(+)
Bacillus sphaericus	ATCC 29726	(-)	(-)	(-)
Corynebacterium glutamicum	ATCC 13032	(-)	(-)	(-)
Enterococcus faecalis	ATCC 29217	(-)	(-)	(-)
Escherichia coli	M.I10.46	(-)	(-)	(-)
Escherichia coli	ATCC 25922	(-)	(-)	(-)
Klebsiella pneumoniae	M.I10.31	(-)	(-)	(-)
Listeria innocua	ATCC 33090	(-)	(-)	(-)
Micrococcus luteus	ATCC 13513	(-)	(-)	(-)
Morganella morganii	M.N1.3	(-)	(-)	(-)
Pseudomonas putida	G.I10.7	(-)	(-)	(-)
Pseudomonas putida	NCIMB 10432	(-)	(-)	(-)
Staphylococcus aureus	ATCC 6538	(-)	(-)	(-)
Vibrio sp.	G.I6.18	(-)	(-)	(-)

No amplification occurred when using the three sets of primers in non-aeromonads strains for any of the three gene targets. On the other hand, when using the primers targeting *gyrB* and *sodB* amplification from all the tested *Aeromonas* strains was obtained. In the reactions targeting *rpoD*, amplification was obtained from all aeromonads with the exception of *A. bivalvium* CECT 7112 and *A. molluscorum* G.I6.7.

4.1.2 In *silico* specificity testing

When using the BLAST tool to evaluate the specificity of the primer sets, by comparing our primer sequences with the sequences available in the GenBank database, no positive match for any other species besides *Aeromonas spp.,* for both primers (forward and reverse) was obtained.

After downloading all *Aeromonas* sequences for *gyrB, rpoD* and *sodB* genes available in the GenBank, the sequences corresponding to primers position were analyzed and mismatches were recorded. The tables below (Table 10 to Table 12) summarize the obtained results.

Table 10 - DNA sequence homology between the GyrB primers and *gyrB* gene sequences of *Aeromonas* strains.

Species Name	Strain Reference	Total Number of Similar Sequences[a]	Primers Fwr 5'-3' / Rev 3'-5'
			GAAGGCCAAGTCGGCCGCCAG_____GAAAACCCGGGCGATGCCAAGAT[b]
A. allosaccharophila	CECT 4199	10	----------T--------_____----------
A. bestiarum	ATCC 13444	10	----------_____----------
A. bestiarum	ATCC 23211	1	----------T--------_____----------
A. bestiarum	ATCC 23213	1	----------_____----------A--
A. bivalvium	868E	2	----------C--------_____--G----------C--------
A. encheleia	MDC66	6	----------_____---------A----------
A. encheleia	DSM 11577T	6	----------_____--------AA----------
A. encheleia	CECT 5026	1	----------A--------_____---------A----------
A. encheleia	CECT 4856	1	----------_____--------TA----------
A. enteropelogenes	CECT 4487	2	---------A--T--------_____----------
A. enteropelogenes	AN-35	3	---------A--T--------_____----------A--
A. enteropelogenes	MDC90	1	---------A--T-----T--_____----------A--
A. trota	ATCC 49657T	1	---------A--T--------_____----------A--A--
A. trota	AF417633.1	1	-------C-A-GTC-------_____----------A--
A. eucrenophila	NCMB 74T	9	----------_____----------
A. eucrenophila	CECT 4827	1	---------A----------_____----------
A. eucrenophila	AF417629.1	1	----------A_____----------
A. eucrenophila	CECT 4825	1	----------_____---------A----------
A. hydrophila	AN-1	25	----------T--------_____----------
A. hydrophila	CECT 839	6	----------T--------_____----------C--------
A. hydrophila	AN-3	3	----------C--------_____----------C--------
A. hydrophila	AE-53	2	----------T--------_____----------A--
A. hydrophila	AF074917.1	1	----------T-----T--_____----------
A. hydrophila	AN-25	1	---------A----------_____----------

A. hydrophila	K4	1	T----------T--------_____----T----------
A. hydrophila	PB13	1	---------A--T--------_____----------
A. hydrophila	ATCC 7966	1	----------_____----------C---------
A. hydrophila	CDC 0434-84	1	----------_____----------C---------
A. hydrophila subsp. *decolorationis*	AY968042.1	1	----------T--------_____----------
A. jandaei	CECT 4228	4	---------A--T--------_____----------
A. jandaei	MDC138	1	----------T--------_____----------
A. jandaei	AN-51	1	---------A--T--T-----_____----------
A. media	MDC250	6	----------T--------_____----------
A. media	CDC 0862-83	2	----------T--------_____----------C----------
A. media	CECT 4234	2	----------T--------_____----------C---------
A. molluscorum	431E	5	-GCCAAG-T---T--------_____--G----------
A. molluscorum	MDC74	2	-GCCAAG-T---T--------_____--G-----A----------
A. molluscorum	93M	2	-GCCAAG-T---T--------_____--G-----A--T----------
A. molluscorum	849	2	-GCCAAG-T---T--------_____--G--------T----------
A. molluscorum	LMG 22214	1	-GCCAAG-TA--T--------_____--G----------
A. popoffii	CECT 5176	7	----------_____----------
A. punctata	MDC50	8	----------C--------_____--G----------C---------
A. punctata	RK 25447	7	----------C--------_____----------C---------
A. punctata	RK 217455	2	----------C--------_____----------
A. punctata	MDC49	1	----------C--------_____----------C---------
A. punctata	AE-34	1	----------C--------_____----------C-----A--
A. punctata	ACVM	1	----------C--------_____-------G---G---------C-
A. salmonicida	CECT 5173	23	----------_____----------
A. schubertii	ATCC 43700T	4	----------A-CT-------_____----------C---------
A. schubertii	AE-48	1	---------A--T--------_____----------A--
A. sharmana	DSM 17445	1	----------A-A---A-ATC_____----------CG----------C
A. simiae	MDC55	2	-GCCAAG-T---T--------_____--G------A----C---------

A. sobria	CECT 4245	4	----------T--------_____--------A----------
A. tecta	F518T	5	----------_____----------
A. veronii	ATCC 35624	24	----------T--------_____----------
A. veronii	CECT 4486	1	----------T--------_____--------A----------
A. veronii	211c	1	----------T--------_____----------C--------
A. veronii	2238A	1	-GCCAAG-T---T--------_____--G------A---C---------
A. veronii bv. sobria	AVBSVM	2	----------T--------_____----------G----------

[a]all sequences in GenBank database belonging to the same species and with a similar sequence in the primers region; [b]sequences of the designed primers, dashes indicate nucleotides identical to the sequence of the GyrB primers.

Table 11 - DNA sequence homology between the SodB primers and *sodB* gene sequences of *Aeromonas* strains.

Species Name	**Strain Reference**	**Nº of Similar Sequences[a]**	**Primers** Fwr 5'-3'_____Rev 3'-5'
			GTACCGAGTTTGAAGGCAAGTC_____GCCATCGGCAACTTCGG[b]
A. allosaccharophila	CECT 4199	2	----------_____----------
A. bestiarum	LMG 13448	6	----------_____----------
A. encheleia	ATCC 51929	2	----------_____----------
A.enteropelogenes	JCM 8355	3	----------_____----------
A.eucrenophila	ATCC 23309	2	----------_____----------
A. hydrophila	IAM12460	17	----------_____----------
A.jandaei	JCM8316	2	----------_____----------
A. media	ATCC 49568	2	----------_____--------T--------
A. popoffii	LMG 17541	1	----------_____----------
A.punctata	QM 65541	5	----------_____----------
A.salmonicida	JCM7873T	9	----------_____----------
A. schubertii	ATCC 43700	2	----------_____-------C---------
A.sobria	TKA971	3	----------_____----------
A.veronii	MTCC 3249	16	----------_____----------
A.veronii	IAM12333	1	----------_____----------T--

[a]all sequences in GenBank database belonging to the same species and with a similar sequence in the primers region; [b]sequences of the designed primers, dashes indicate nucleotides identical to the sequence of the SodB primers.

Table 12 - DNA sequence homology between the RpoD primers and *rpoD* gene sequences of *Aeromonas* strains.

Species Name	**Strain Reference**	**Total Number of Similar Sequences[a]**	**Primers** Fwr 5'-3'_____Rev 3'-5'
			ATGCCGAAGAAAACCTTCGT_____ CAGATCAAGGATATCAACCG[b]
A. allosaccharophila	CECT 4199	7	----------_____----------
A. bestiarum	LMG 13448	7	----------_____----------C--------
A. bestiarum	LMG 13662	2	----------_____----------
A. bestiarum	ATCC 23211	1	----------G--------_____----------C--------
A. cf. bestiarum/salmonicida	A99	6	----------_____----------C--------
A. cf. bestiarum/salmonicida	107F	3	----------_____--------A----------
A. cf. bestiarum/salmonicida	101F	1	----------_____--------A--C--------
A. bivalvium	868E	1	----------_____----------C--------
A. bivalvium	665N	1	----------_____--------A--C--------
A. encheleia	CECT 4342	3	----------G--------_____----------
A. encheleia	CECT 4856	1	----------_____----------T--
A. encheleia	CECT 4253	1	----------_____----------
A. enteropelogenes	CECT 4255	4	----------_____----------C--------
A. enteropelogenes	CECT 4935	1	----------_____--------A--C--------
A. eucrenophila	MDC256	4	----------_____----------C--------
A. hydrophila	CECT 839	11	----------G--------_____----------C--------
A. hydrophila	AN 25	4	----------_____----------C--------
A. hydrophila	ATCC 49140	1	----------G-----T--_____----------C--------
A. jandaei	CECT 4228	3	----------_____----------
A. media	CECT 4232	4	----------G--------_____----------C--------
A. media	345	1	----------G-----T--_____----------C--------
A. molluscorum	LMG 22214	9	----------_____--------A--C--------

A. molluscorum	MDC43	2	----------_____-----A--A--C--------
A. popoffii	LMG 17541	8	----------_____----------
A. punctata	RK 217455	5	----------G--------_____----------C--------
A. punctata	665c	3	----------_____----------C--------
A. punctata	CECT 838	2	----------G--------_____----------
A. punctata	RK 65541	2	----------G-----T--_____----------
A. punctata	RK 25447	1	----------_____--------A----------
A. salmonicida	LMG 13451	12	----------_____----------C--------
A. salmonicida subsp. *pectinolytica*	34MEL	1	----------_____--------A----------
A. schubertii	CECT 4240	1	----------G--------_____----------
A. schubertii	CECT 4254	1	----------G--------_____----------C--------
A. schubertii	AE 48	1	----------_____----------C--------
A. sharmana	DSM 17445	1	----------A------_____--A----------
A. simiae	MDC55	2	----------G--------_____-----T--A--C--------
A. sobria	CECT 4245	2	----------_____----------
A. veronii	MDC57	15	----------_____----------
A. veronii	211c	1	----------G--------_____----------C--------
A. veronii	2238A	1	----------G--------_____-----T--A--C--------

[a]all sequences in GenBank database belonging to the same species and with a similar sequence in the primers region; [b]sequences of the designed primers, dashes indicate nucleotides identical to the sequence of the RpoD primers.

For the *gyrB* primer set, from zero to nine base mismatches were observed for at least one primer. However, from 225 analyzed sequences in only 23 (10%) more than two mismatches were detected. Most of the sequences from *A. bestiarum*, *A. eucrenophila*, *A. popoffii*, *A. salmonicida* and *A. 'tecta'* showed no base mismatches in any of the primers, while *A. molluscorum* and *A. simiae* strains and one *A. veronii* strain presented the higher number of mismatches.

In *rpoD* gene sequences from one to three mismatches per primer were detected, but only in 3 cases (out of 135) 3 mismatches were detected. Those were *A. molluscorum* MDC43, *A. simiae* MDC55 and *A. veronii* 2238A.

sodB gene sequences presented the highest similarities with the primer sequences, with most of the species presenting no mismatches. *A. media ATCC 49568, A. schubertii ATCC 43700* and *A. veronii* IAM12333 were the only strains presenting one mismatch with the reverse primer.

4.1.3 Primers detection limits

The detection limits for the three primer sets were determined by using serial dilutions of a positive control strain (*A. hydrophila* subsp. *hydrophila* CECT 839^{T}). The lower limits of PCR detection of *gyrB* and *sodB* genes ranged from 100 pg and 1 ng and of *rpoD* gene ranged from 1 and 10 ng.

4.2 Evaluation of phylogenetic information provided by the PCR target fragment

In order to evaluate the phylogenetic information provided by the amplified fragments, phylogenetic trees were constructed derived from the *gyrB*, *rpoD* and *sodB* gene sequences stored in the GenBank database (large fragments) and from the target fragments amplified with primers designed during this study (small fragments). The constructed trees are presented below (Figure 3 to Figure 8).

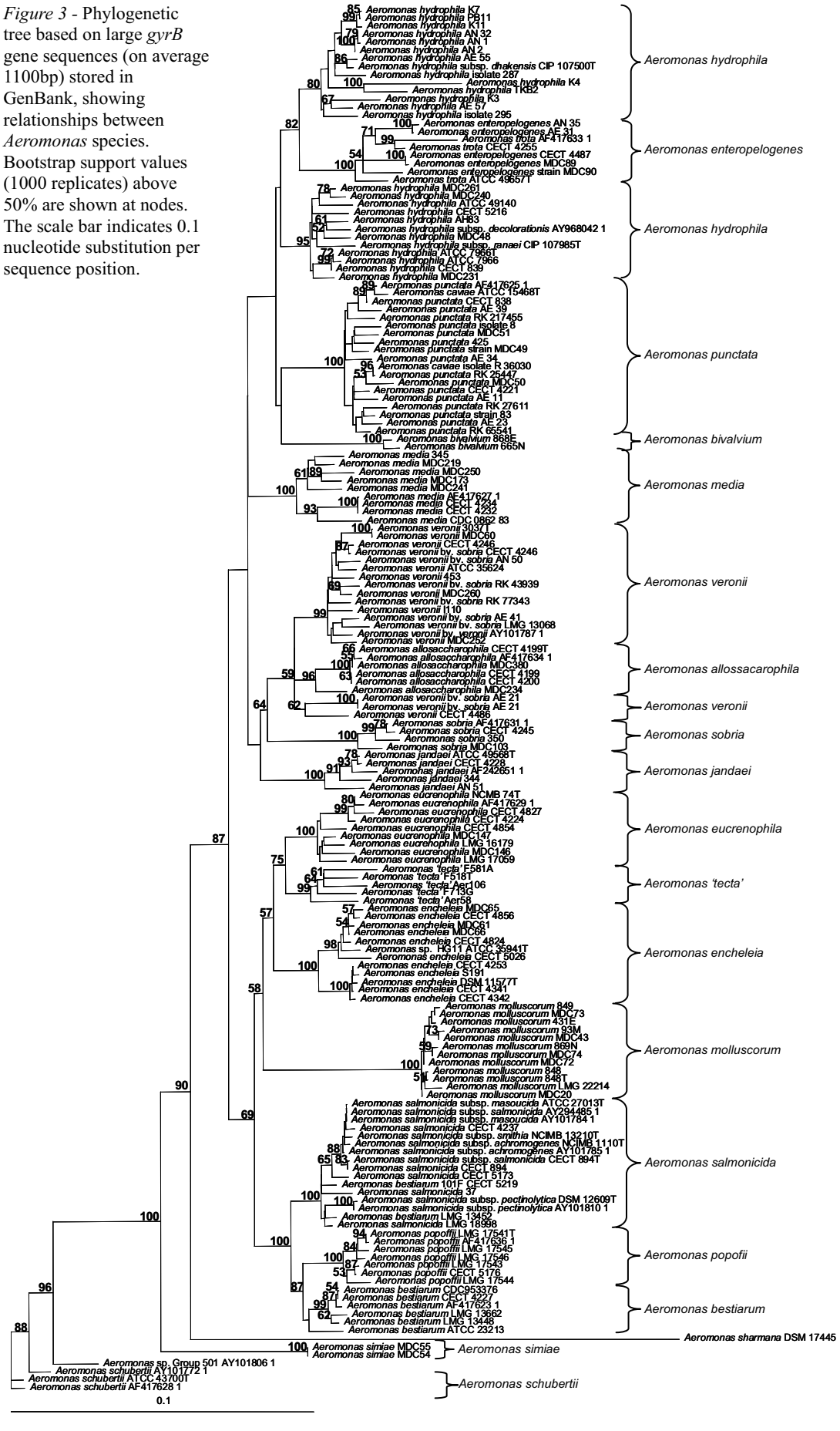


Figure 3 - Phylogenetic tree based on large *gyrB* gene sequences (on average 1100bp) stored in GenBank, showing relationships between *Aeromonas* species. Bootstrap support values (1000 replicates) above 50% are shown at nodes. The scale bar indicates 0.1 nucleotide substitution per sequence position.

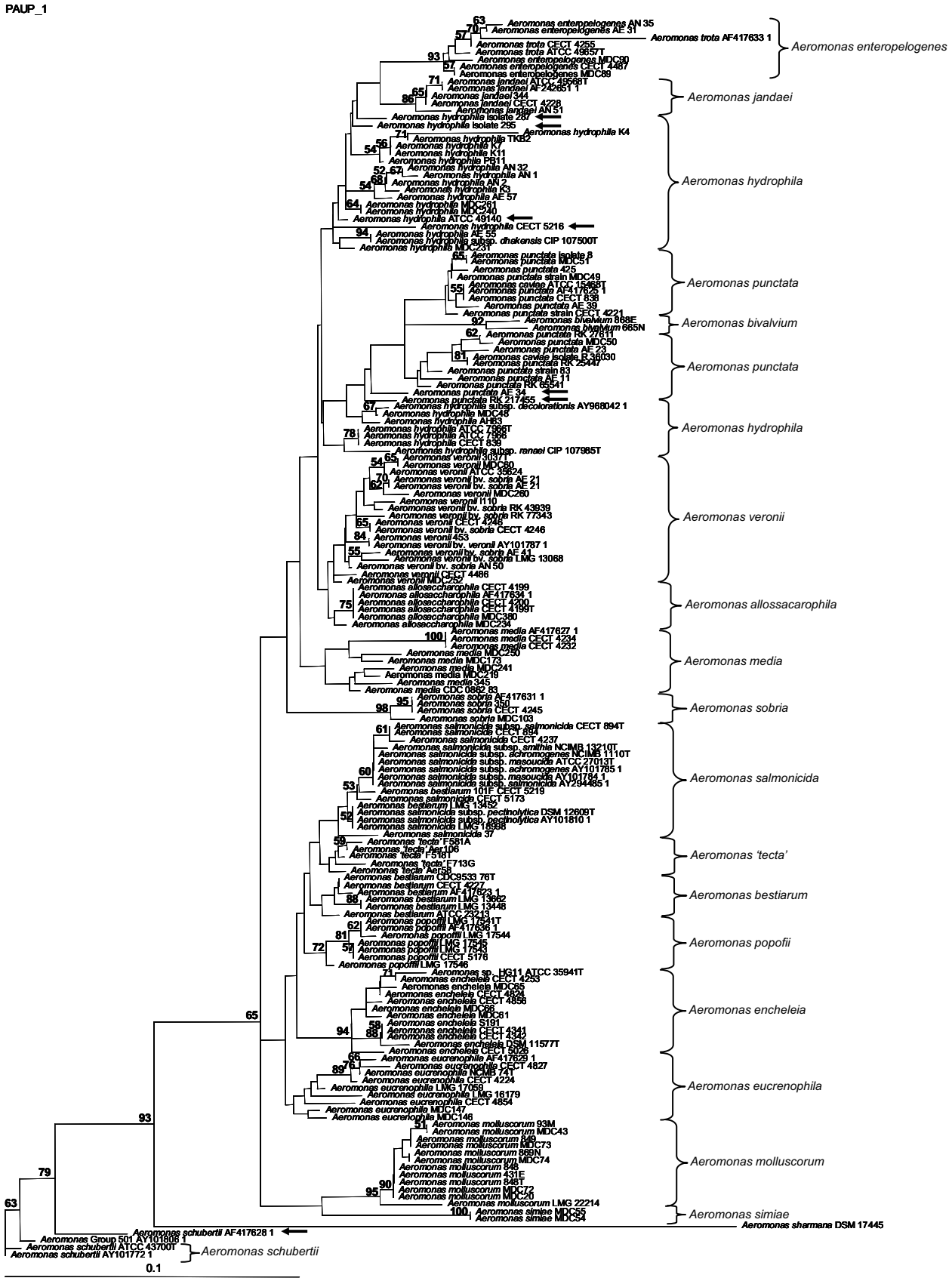


Figure 4 - Phylogenetic tree based on *gyrB* small sequences (on average 198bp), showing relationships between *Aeromonas* species. Bootstrap support values (1000 replicates) above 50% are shown at nodes. The scale bar indicates 0.1 nucleotide substitution per sequence position.

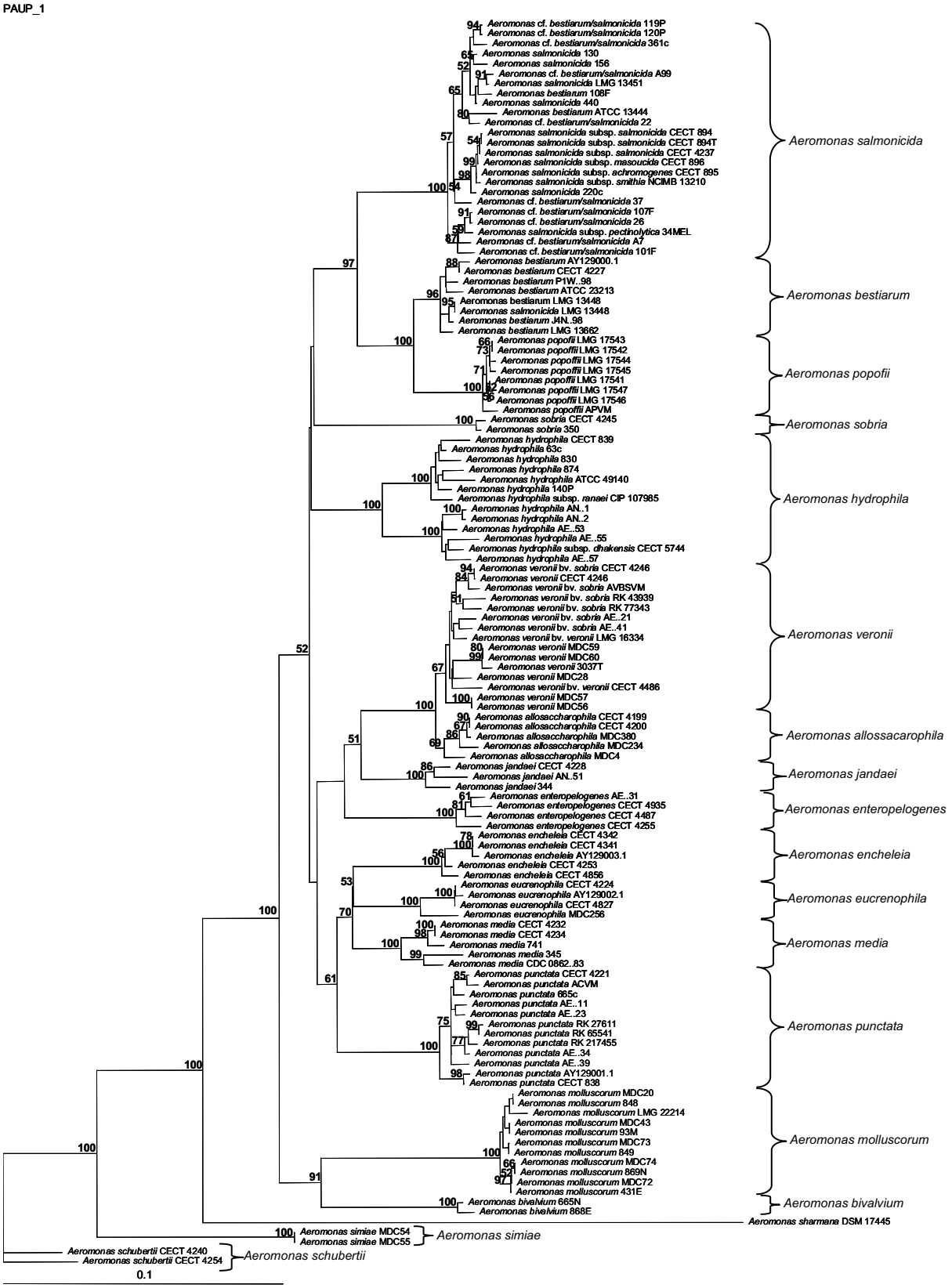


Figure 5 - Phylogenetic tree based on *rpoD* large sequences stored in GenBank (on average 819 bp), showing relationships between *Aeromonas* strains. Bootstrap support values (1000 replicates) above 50% are shown at nodes. The scale bar indicates 0.1 nucleotide substitution per sequence position.

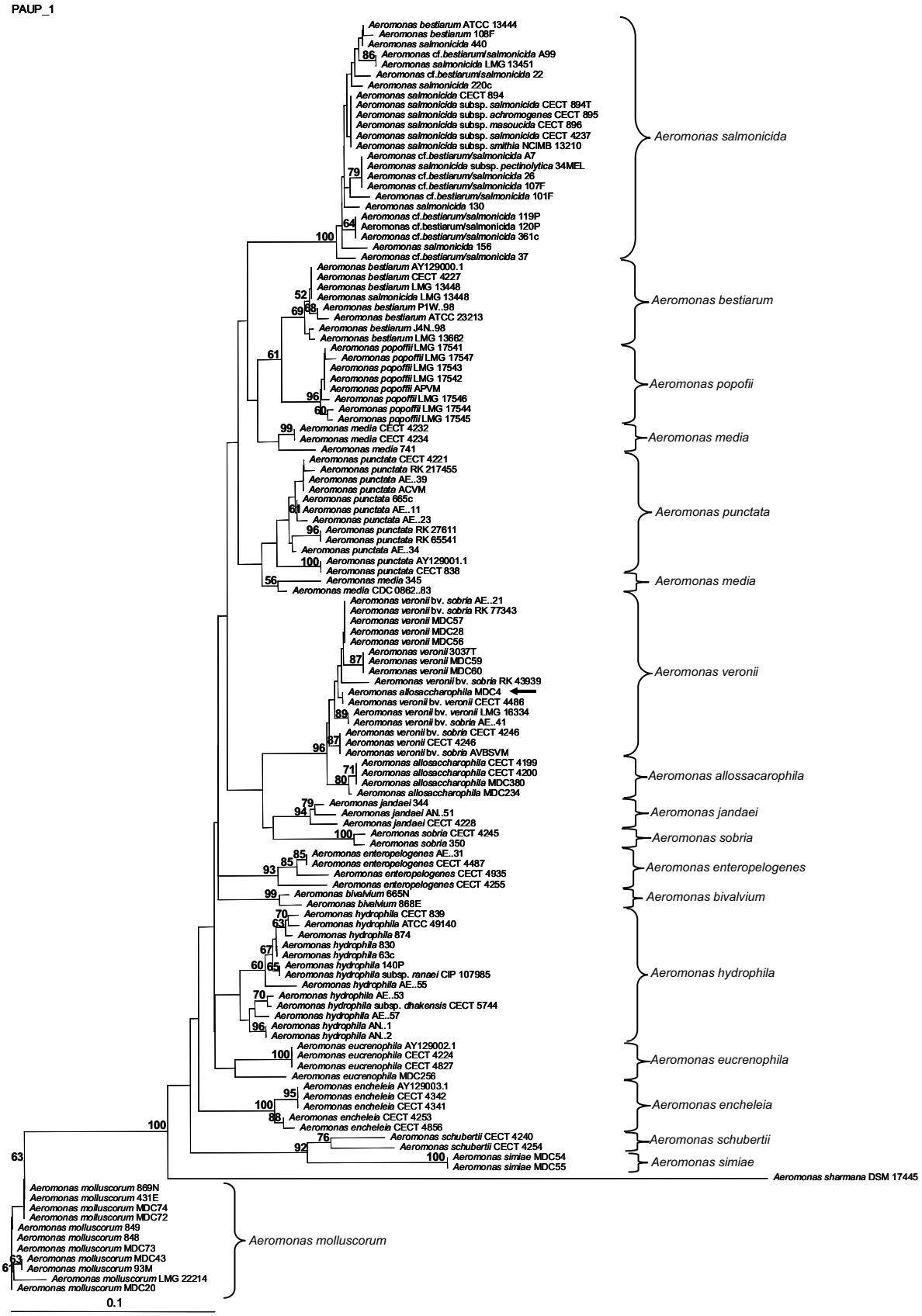


Figure 6 - Phylogenetic tree based on *rpoD* small sequences (on average 200bp), showing relationships between *Aeromonas* strains. Bootstrap support values (1000 replicates) above 50% are shown at nodes. The scale bar indicates 0.1 nucleotide substitution per sequence position.

Figure 7 - Phylogenetic tree based on *sodB* large sequences stored in GenBank (on average 546 bp), showing relationships between *Aeromonas* strains. Bootstrap support values (1000 replicates) above 50% are shown at nodes. The scale bar indicates 0.1 nucleotide substitution per sequence position.

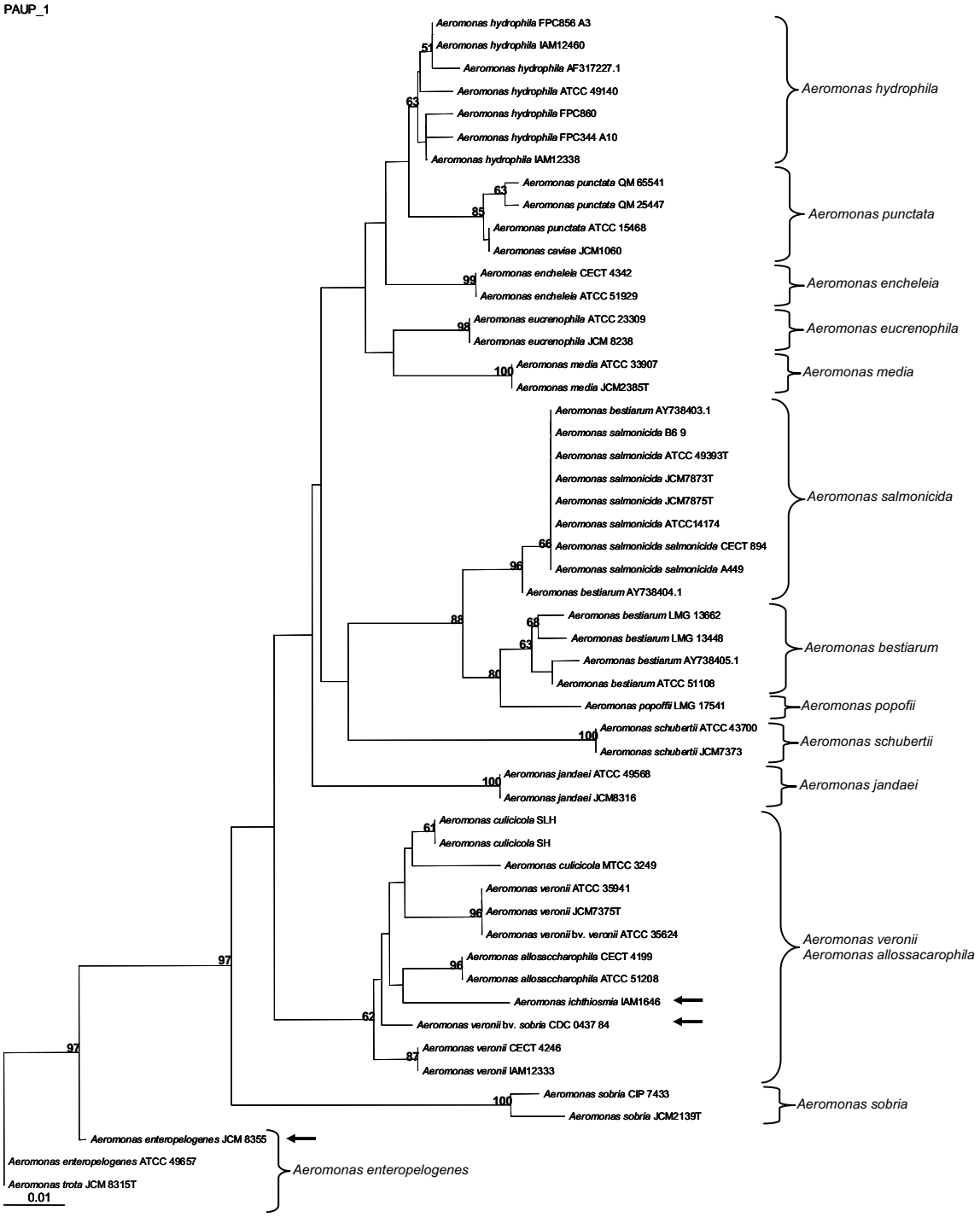


Figure 8 - Phylogenetic tree based on *sodB* small sequences (on average 226bp), showing relationships between *Aeromonas* strains. Bootstrap support values (1000 replicates) above 50% are shown at nodes. The scale bar indicates 0.1 nucleotide substitution per sequence position.

From the analysis of the *gyrB* tree (Figure 3), constructed based on the large sequences, it was possible, as expected, to identify clusters correspondent to each previously described species. However sequences from *A. hydrophila* grouped into two different clusters. In the tree constructed with *gyrB* target fragments (small) (Figure 4), in general sequences from the same taxonomic species also grouped together. Seven exceptions were detected and are indicated with arrows in Figure 4. Additionally in this tree, sequences from two species (*A. hydrophila* and *A. punctata*) gave origin to more than one cluster. It should be noticed that bootstrap values that support the clusters in the tree constructed with large sequences are often higher than the ones that support the same clusters in the tree constructed using target fragments (Table 13).

Also, from the analysis of the *rpoD* tree (large) (Figure 5), sequences grouped according to their species. The tree constructed with *rpoD* small fragments (Figure 6) was very similar in terms of main clusters and only sequences from *A. media* grouped into two different clusters. Only the position of one sequence was not consistent with taxonomic groups (indicated with arrows in Figure 6). Also, the bootstrap values that support the clusters in the tree constructed with large sequences are generally higher than the ones that support the same clusters in the tree constructed using target fragments (Table 13).

Finally, the organization of the *sodB* tree based on large sequences was also consistent with taxonomic groups (Figure 7). The tree constructed using small sequences gave similar results (three exceptions are indicated with arrows in Figure 8). As for the two other genes, the bootstrap values which support the tree based on large sequences were usually higher than the ones calculated for the tree based on small sequences (Table 13).

Table 13 - Bootstrap values obtained for each cluster that corresponds to previously described *Aeromonas* species. (1) trees constructed based on sequences downloaded from the GenBank database, (2) trees constructed based on target fragments for each primer set. Whenever one species corresponds to more than one cluster the lower bootstrap value is presented.

Species Name	*gyrB* (1)	*gyrB* (2)	*rpoD* (1)	*rpoD* (2)	*sodB* (1)	*sodB* (2)
A. allosaccharophila	96	<50	69	80	99	99
A. bestiarum	<50	<50	96	69	93	63
A. bivalvium	100	92	100	99	NI	NI
A. encheleia	100	94	100	100	100	99
A. enteropelogenes	100	93	100	93	96	97

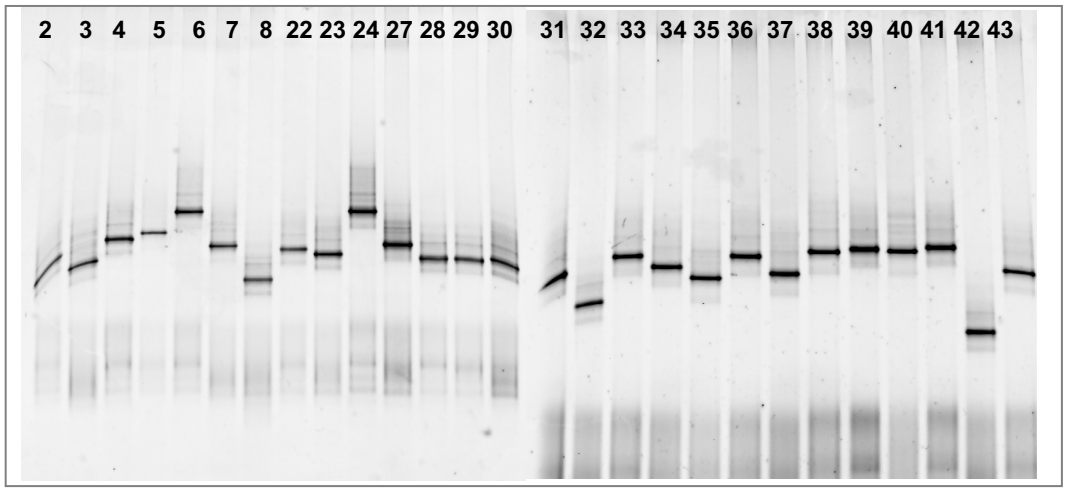
A. eucrenophila	100	<50	100	<50	100	98
A. hydrophila	80*	<50*	100	<50	65	63
A. jandaei	100	86	100	94	100	100
A. media	100	<50	100	<50*	100	100
A. molluscorum	100	95	100	63	NI	NI
A. punctata	100	<50*	100	<50	94	85
A. popoffii	100	72	100	96	NI	NI
A. salmonicida	100	<50	100	100	100	96
A. schubertii	88	63	<50	76	100	100
A. simiae	100	100	100	100	NI	NI
A. sobria	100	98	100	100	100	100
A. 'tecta'	99	<50	NI	NI	NI	NI
A. veronii	62*	<50	67	<50	59	<50

*more than one cluster was obtained for this species; NI not included

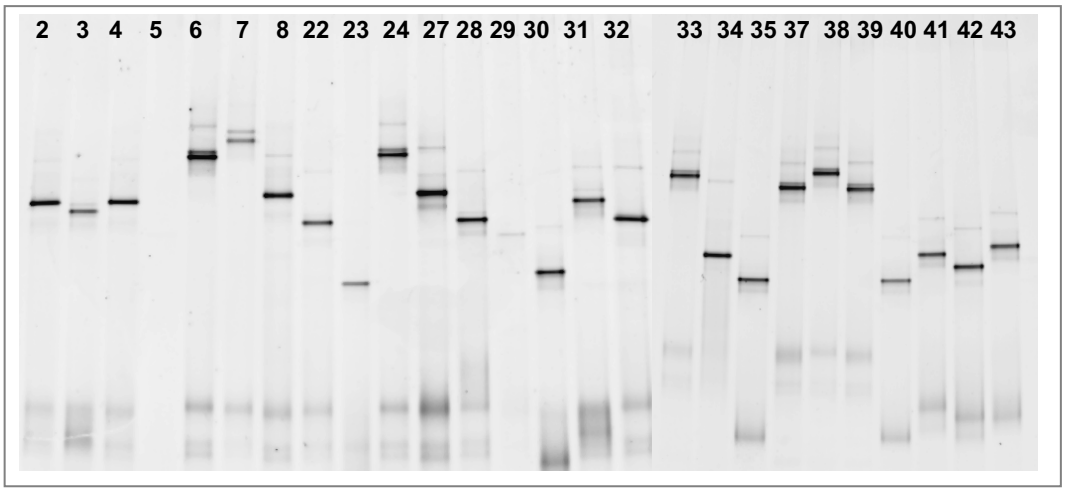
4.3 Development, optimization and evaluation of specific PCR-DGGE culture independent methods to study *Aeromonas* communities.

4.3.1 DGGE of the PCR products from *Aeromonas* strains

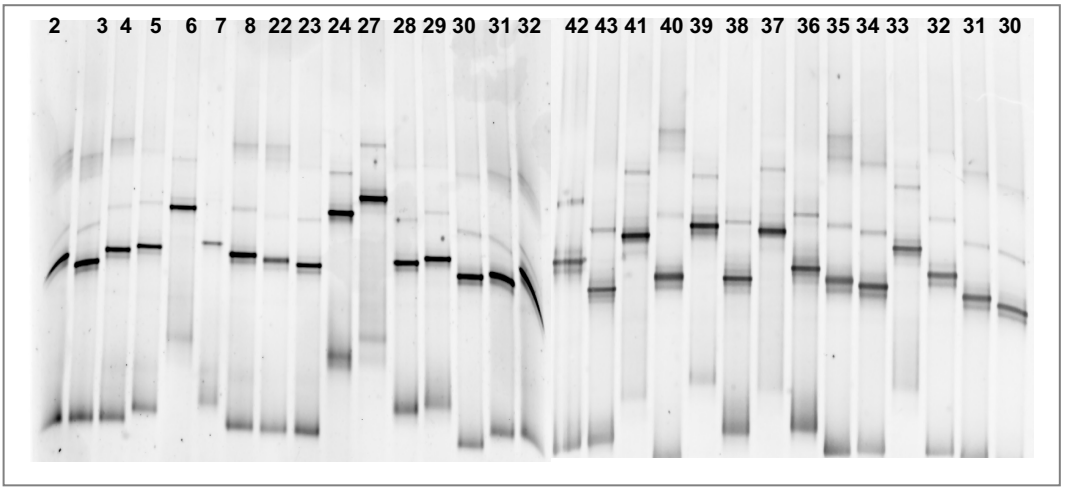
In order to construct DGGE markers to be used as quality controls in DGGE gels, PCR products from *Aeromonas* isolates were run in DGGE gels. Results obtained are presented in Figure 9. In general, bands from different species occupied different positions in the gel and even strains from the same species often originated bands in different positions. The isolates chosen to construct the markers were: 6, 8, 32, 42 and 43 to *gyrB,* 7, 8, 22, 23 to *rpoD and* 23, 24, 27, and 29 to *sodB* DGGE. Besides these isolates we have also chosen some clones to integrate the marker. The markers were further included in the DGGE assays and are identified in the figures with 'M'.



(A)



(B)



(C)

Figure 9 - DGGE gels of *gyrB* (A), *rpoD* (B) and *sodB* (C) PCR products from *Aeromonas* strains. *2-A. caviae* G.I10.8*, 3-A. hydrophila* G.I10.10*, 4-A. media* G.I10.21*, 5-A. molluscorum* G.I6.7*, 6-A. veronii* G.I6.9*, 7-A. salmonicida* G.I6.17*, 8-A. veronii* bv. *sobria G.NI28, 22-A. caviae* CECT 838 ^{T}*, 23-A. hydrophila* subsp.

hydrophila CECT 839 [T], *24-A. veronii* CECT 4257 [T], *27- A. sobria* CECT 4245 [T], *28-A. bivalvum* CECT 7113 [T], *29-A. bivalvum* CECT 7112, *30-A. hydrophila* A5-11, *31-A. media* A4-3, *32-A. caviae* L6, *33-A. veronii* 96/2-7, *34-A. eucrenophila* L12-9, *35-Aeromonas* HG11 120/1, *36-A. salmonicida* L14-7, *37-A. popoffii* 130/12, *38-A. bestiarum* 127/2, *39-A. allosacharophila* A10-6, *40-A. encheleia* 22/6, *41-A. 'tecta'* 109B1, *42-Aeromonas* sp. L15-1, *43- Aeromonas* sp. L10-9.

4.3.2 DGGE of the environmental amplicons

DGGE assays were performed using *gyrB*, *rpoD* and *sodB* amplicons from environmental samples from Ria de Aveiro in order to assess the diversity and dynamics of aeromonads in this estuarine environment. The profiles obtained are shown below in figures 10, 11 and 12.

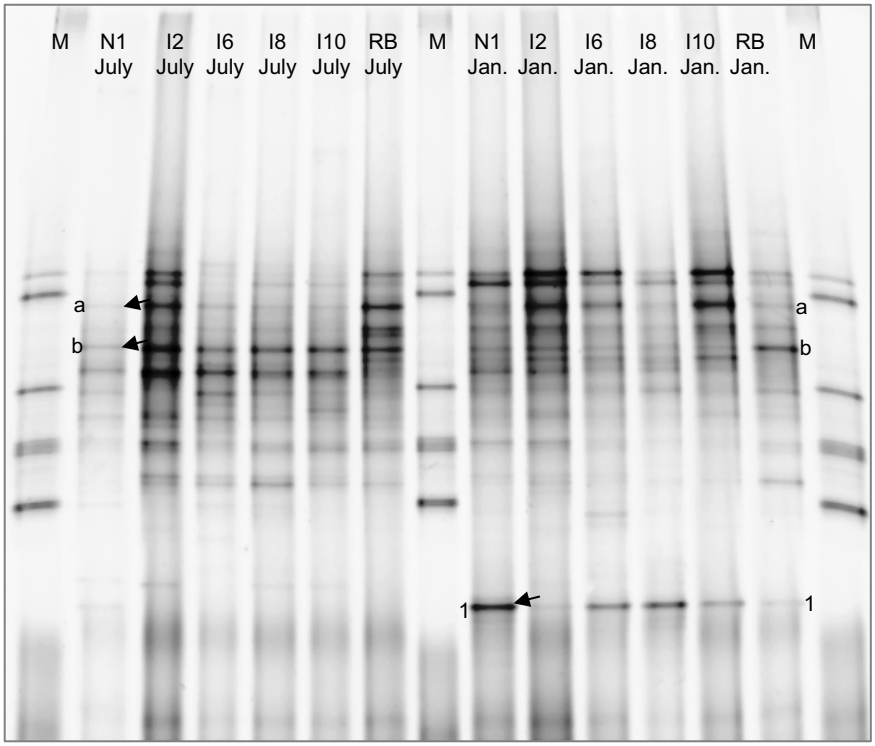


Figure 10 - DGGE of *gyrB* environmental amplicons obtained from samples collected in stations N1, I2, I6, I8 and I10, in July and January. (a, b –examples of phylotypes present in all the samples 1- example of phylotypes present only in January). M - GyrB DGGE marker.

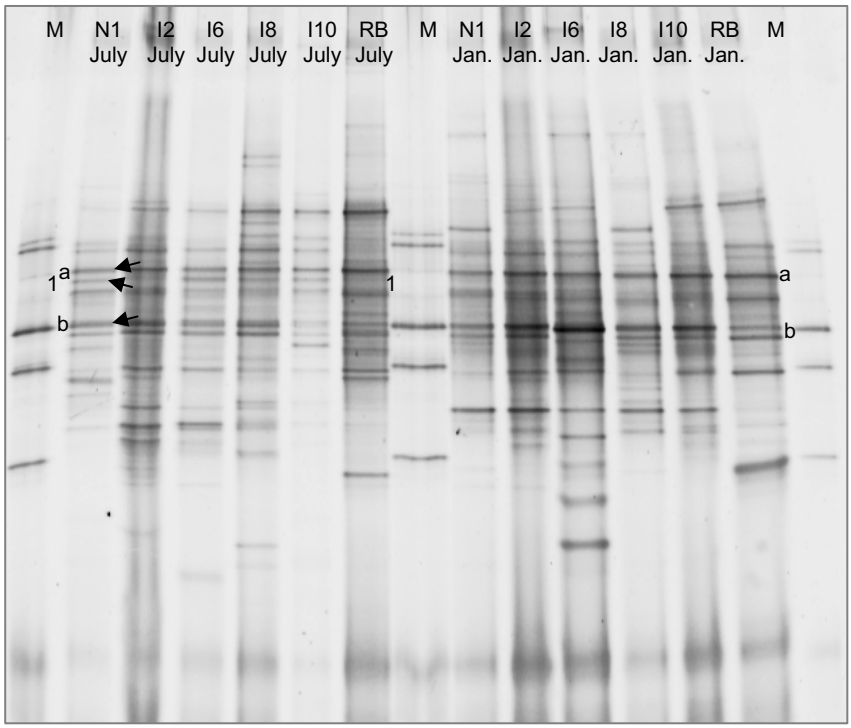


Figure 11 - DGGE *of rpoD* environmental amplicons obtained from samples collected in stations N1, I2, I6, I8 and I10, in July and January. (a, b –examples of phylotypes present in all the samples 1- example of phylotype present in only one group of samples). M - RpoD DGGE marker.

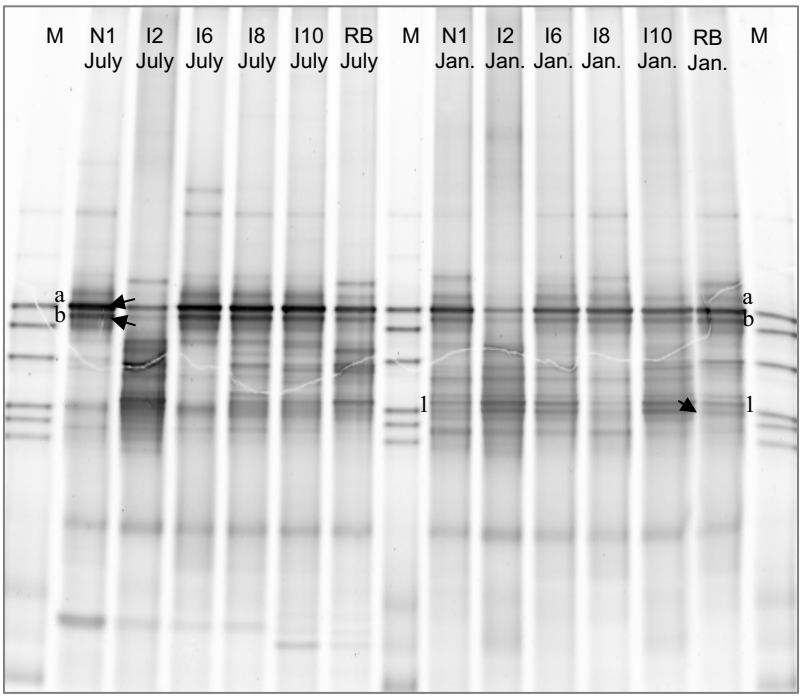


Figure 12 - DGGE of *sodB* environmental products obtained from samples collected in stations N1, I2, I6, I8 and I10, in July and January. a, b –examples of phylotypes present in all the samples 1-example of phylotype present in only one group of samples. M - SodB DGGE marker.

It was possible to obtain complex DGGE profiles from each of the analyzed sample. When these results were visually analyzed, it was observed that DGGE profiles from all the samples are very similar. These communities seem to be very homogenous along the estuary and thus several phylotypes were present in all the samples. That is the case of phylotypes a and b indicated in the DGGE gels (Figures10, 11, 12). On the other hand it was also observed that there are some phylotypes that are only present in one season being absent in all the samples from the other season (for example bands 1 in Figures 10, 11, 12). The number of DGGE bands per sample was also rather stable being on average 15 (*gyrB* gel), 18 (*rpoD* gel) and 16 (*sodB* gel).

To perform a more accurate analysis of *Aeromonas* communities, gel images were analyzed with the GelCompar II software (Applied Maths, Kortrijk, Belgium) and similarity matrices were calculated with the Jaccard coefficient. Subsequently, cluster analysis based on similarity matrices was performed by the unweighted pair-group method using arithmetic averages (UPGMA). The resulting dendrograms for each gene target are shown below in the figures 13, 14 and 15. These dendrograms show a seasonal influence in the aeromonads communities, as can be seen from the clustering of the samples according to the sampling months. In all the dendrograms two major clusters can be observed, one referring to the samples collected in January and other referring to the samples collected in July.

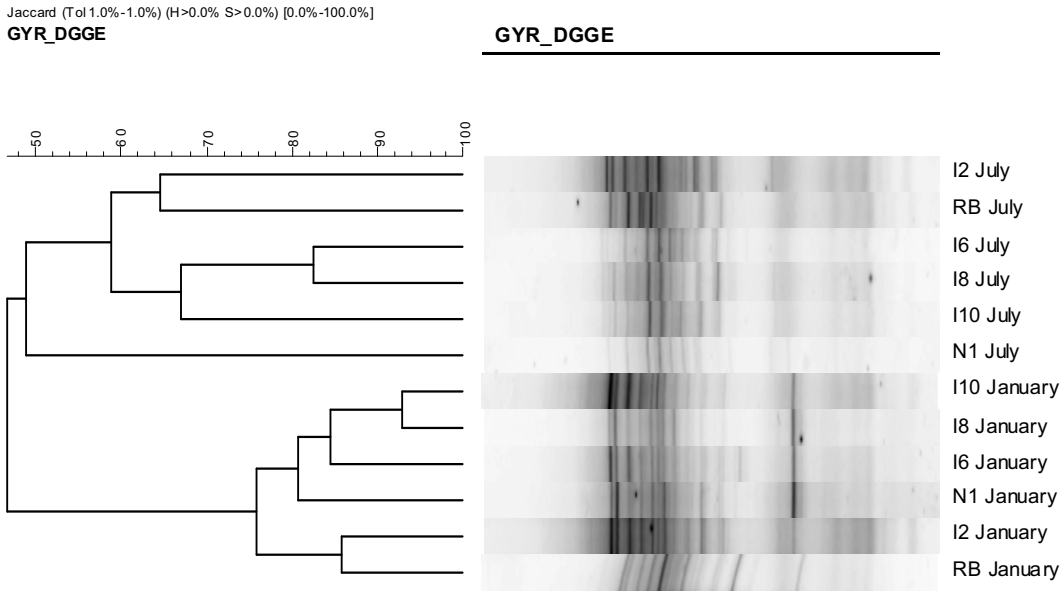


Figure 13 - Dendrogram based in Jaccard coefficient showing the similarity of *Aeromonas* communities in Ria de Aveiro determined using *gyrB* DGGE profiles.

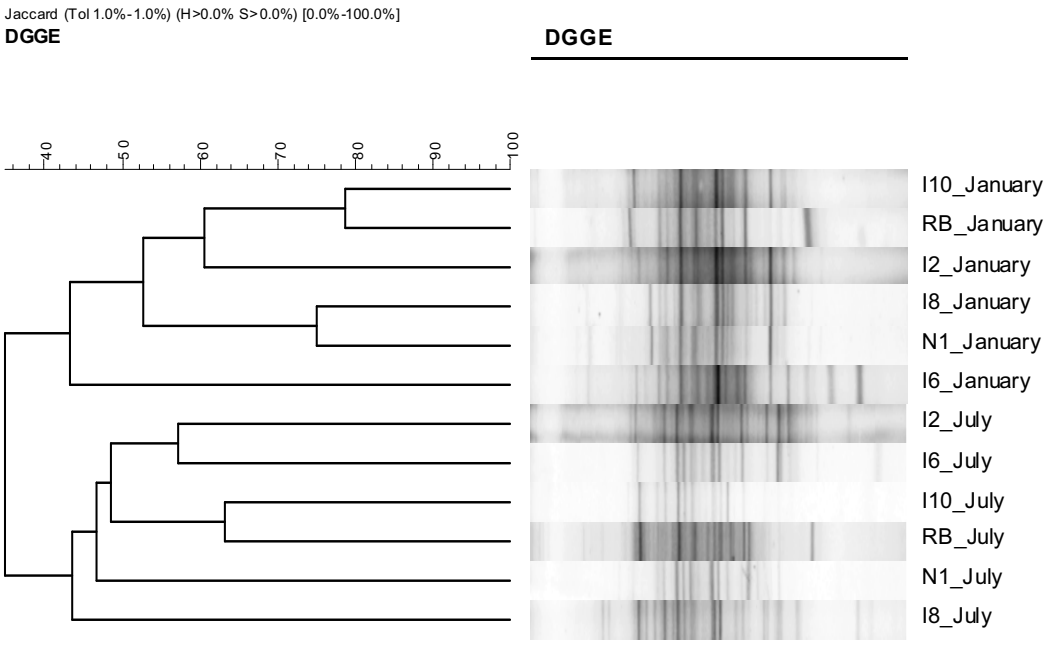


Figure 14 - Dendrogram based in Jaccard coefficient showing the similarity of *Aeromonas* communities in Ria de Aveiro determined using *rpoD* DGGE profiles.

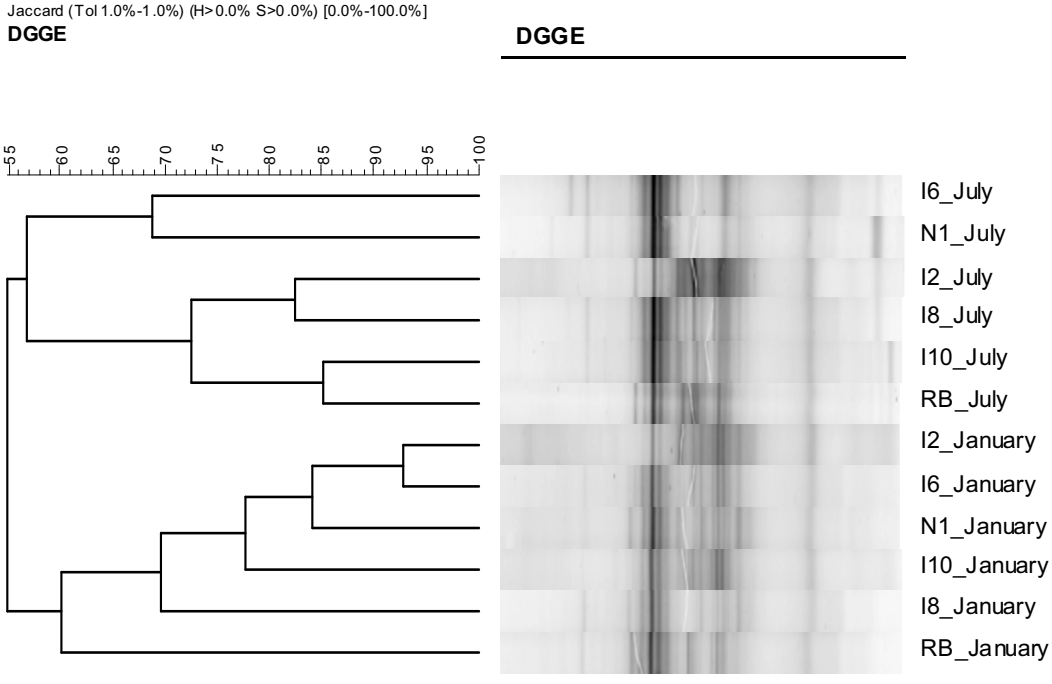


Figure 15 - Dendrogram based in Jaccard coefficient showing the similarity of *Aeromonas* communities in Ria de Aveiro determined using *sodB* DGGE profiles.

As already stated, in the *gyrB* dendrogram two principal clusters grouping July and January samples can be identified, displaying less than 50% similarity between them. The similarity observed between July samples is lower than 50% while between samples collected in January it is approximately 74%. In July it was observed that I2 and RB samples group together while I6, I8 and I10 form another sub-cluster. The N1 sample was clearly different from all the others. In what concerns January samples, I10, I8, I6 and N1 are closely related and I2 and RB constitute a separate cluster.

In the *rpoD* dendrogram, the existence of two different clusters separating January samples from July samples was also observed, displaying less than 36% similarity between them. The January and July samples both present approximately 43% similarity within them. In January samples two sub-clusters can be observed: one includes I2, I10 and RB and the other includes I8 and N1. Sample I6 was clearly different from the others. In July samples I2 and I6 clustered together; I10 and RB formed another cluster while N1 and I8 were very distant from the others.

In the *sodB* dendrogram the seasonal influence was also observed. It shows a separation of the samples into two principal clusters, displaying approximately 55% similarity between them. The July samples presented 57% similarity between them and the January samples showed a similarity value of 60%. Inside the July cluster two sub-clusters were detected: one included I6 and N1 and the other included I2, I8, I10 and RB. In the January cluster the more closely related samples were N1, I2 and I6.

4.4 Cloning and sequencing environmental amplicons

A cloning and sequencing strategy was applied to confirm that amplicons obtained from environmental samples were from aeromonads. Since the DGGE profiles from all the samples were very similar in what concerns the composition of the communities there was no special parameter on choosing the environmental samples to perform cloning. We decided to use two samples with different characteristics, N1 July and RB July.

4.4.1 Analyzing transformants

To analyze transformants 15 putative positive clones were chosen for each gene target and environmental sample. PCR reactions were performed to confirm the presence

of the insert. For the *gyrB* reactions thirteen positive clones were obtained, two from N1 July (referred as A) and eleven from RB July (referred as B). For the *rpoD* reactions, only six positive clones from RB July were obtained. Finally, for *sodB* reactions a total of seventeen positive clones were obtained, six of which are from N1 July and eleven from RB July sample.

DGGE assays were performed with the purpose of selecting clones displaying different positions in the gels and consequently presenting different nucleotide sequences. For *rpoD* assay, DGGE was not performed because of the reduced number of obtained clones. In this case it was decided to sequence all the positive clones. The DGGE results for *gyrB* and *sodB* clones are shown in figure 16. The arrows indicate the clones selected for sequencing analysis.

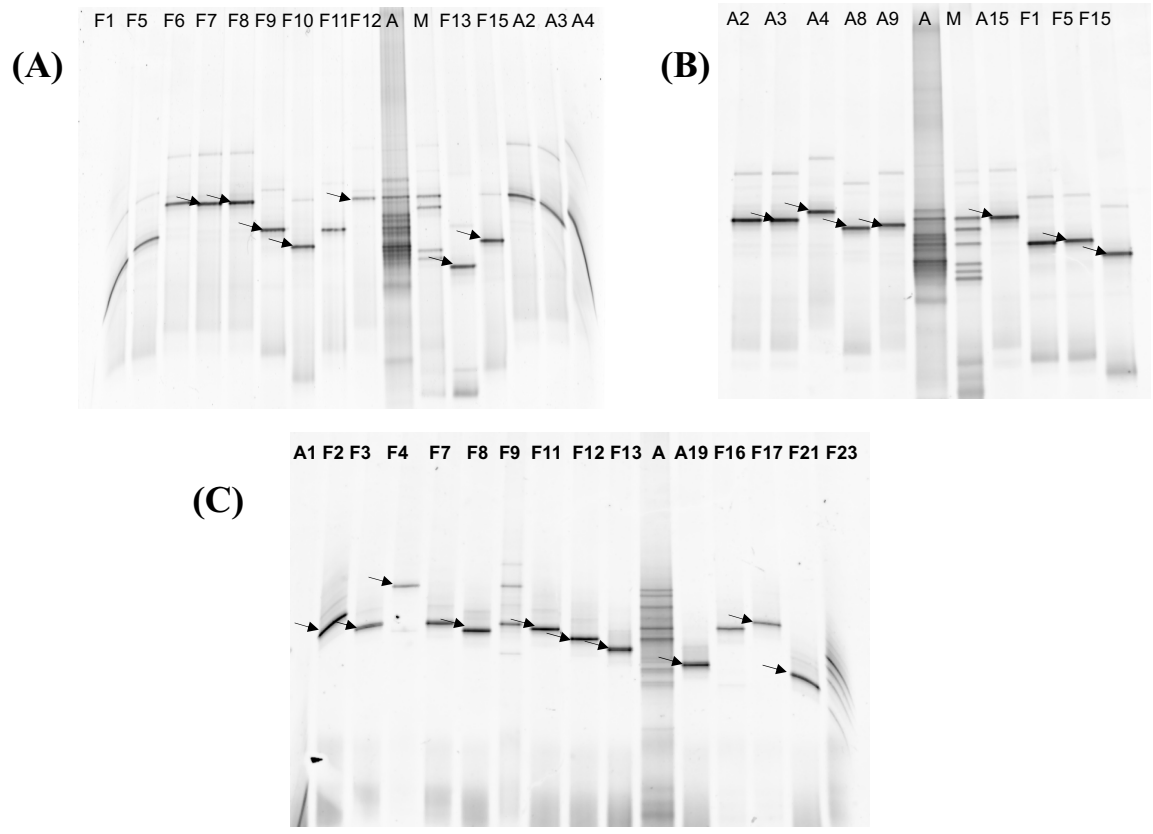
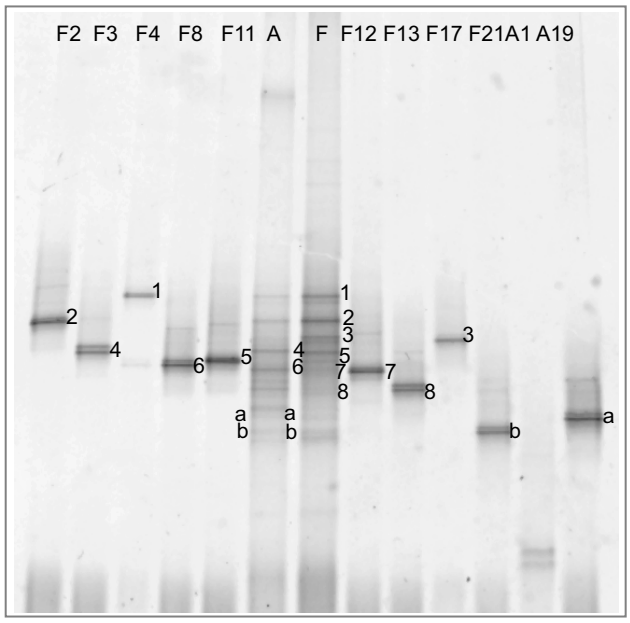


Figure 16 - DGGE analysis of clones containing *sodB* inserts (gels A and B) and *gyrB* inserts (gel C) from N1 July (clones A) and RB July (clones F)

In order to determine the position of bands corresponding to sequenced *gyrB* and *sodB* amplicons in the environmental profiles, DGGE assays were performed (Figure 17).

(A)



(B)

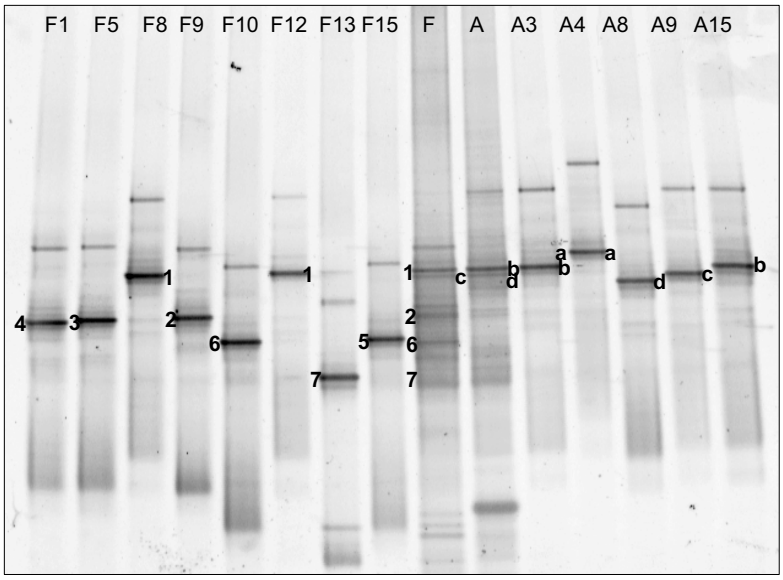


Figure 17 - Placement of clone bands on environmental profiles from N1 July (A) and RB July (F). Gel (A) – *gyrB* clones, gel (B) – *sodB* clones.

From the images it is possible to observe that some of the sequenced clones were dominant phylotypes in the correspondent environmental samples. For example these clones were affiliated with *Aeromonas* sp. F674P and *A. media* strain CECT 4234 in *gyrB* case and with *A. sobria* strain CIP 7433, *A. allosaccharophila* CECT 4199 and *A. media* strain ATCC 33907 in *sodB*. However some other dominant groups remained unidentified.

4.4.2 Sequencing and sequence analysis

The obtained nucleotide sequences from each clone were compared to the GenBank nucleotide data library using the BLAST software with the purpose of determining their closest phylogenetic relatives. Results are shown in table 14.

Table 14 - Closest relatives of sequences obtained from positive clones

Clone	Sample	Closest Relative (accession no.)	Source	Similarity (%)
gyrB_A1	N1 July	*Aeromonas punctata* strain RK 65541 (AY987527)	Human stool	97
gyrB_A19	N1 July	*Aeromonas eucrenophila* strain LMG 17059 (AM116970)	Drinking water plant	96
gyrB_F2	RB July	*Aeromonas* sp. F674P (AJ868383)	Stool of asymptomatic person	97
gyrB_F3	RB July	*Aeromonas punctata* strain MDC49 (DQ411476)	Unknown	98
gyrB_F4	RB July	*Aeromonas media* strain CECT 4234 (AY101824)	Fish farm pond	97
gyrB_F8	RB July	*Aeromonas sobria* strain CECT 4245 (AY101781)	Fish	98
gyrB_F12	RB July	*Aeromonas sp.* F674P (AJ868383)	Stool of asymptomatic person	97
gyrB_F13	RB July	*Aeromonas sobria* strain CECT 4245 (AY101781)	Fish	98
gyrB_F17	RB July	*Aeromonas allosaccharophila* strain MDC98 (DQ411498)	Faeces	96
gyrB_F21	RB July	*Aeromonas media* strain MDC173 (DQ665881)	Unknown	98
*rpoD_F2**	*RB July*	*Aeromonas allosaccharophila MDC45 (DQ411507)*	*Pig carcasses*	98
		Aeromonas veronii bv. veronii CECT 4486 (AY169342)	*Surface water*	98
*rpoD_F3**	*RB July*	*Aeromonas allosaccharophila MDC45 (DQ411507)*	*Pig carcasses*	100
		Aeromonas veronii bv. veronii CECT 4486 (AY169342)	*Surface water*	100
rpoD_F7	*RB July*	*Aeromonas veronii MDC28 (DQ411505.1)*	*Midgut of Culex quinquefasciatus*	100
rpoD_F13	*RB July*	*Aeromonas veronii bv. sobria CECT 4246 (AY169333.1)*	*Infected frog suffering from 'red leg' disease*	99
rpoD_F20	*RB July*	*Aeromonas veronii MDC28 (DQ411505.1)*	*Midgut of Culex quinquefasciatus*	99
rpoD_F22	RB July	*Aeromonas allosaccharophila* MDC45 (DQ411507)	Pig carcasses	99
		Aeromonas veronii bv. veronii CECT 4486 (AY169342)	Surface water	99
sodB_A3	N1 July	*Aeromonas sobria* strain CIP 7433 (AY738414)	Fish	100
sodB_A8	N1 July	*Aeromonas sobria* strain CIP 7433 (AY738414)	Fish	99
sodB_F1	RB July	*Aeromonas veronii* strain MTCC 3249 (EF028406)	Mosquito, midgut	99

sodB_F8	RB July	*Aeromonas sobria* strain CIP 7433 (AY738414)	Fish	100
sodB_F9	RB July	*Aeromonas allosaccharophila* strain CECT 4199 (AY738425)	Eel, diseased elver	99
sodB_F10	RB July	*Aeromonas media* strain ATCC 33907 (AY738412)	Fish farm effluent	99
sodB_F13	RB July	*Aeromonas hydrophila* strain ATCC 19570 (AB033444)	Fish intestine	98
sodB_F15	RB July	*Aeromonas media* strain ATCC 33907 (AY738412)	Fish farm effluent	98

*the obtained sequence was affiliated with sequences from two different species.

All the obtained sequences affiliated with sequences from *Aeromonas* strains. Generally the high scores obtained corresponded to several strains belonging to a single species and one example is presented in table 14. Two *rpoD* clones were affiliated with strains from two different species (labeled with * in table 14). *gyrB* sequences affiliated with sequences obtained from *A. punctata*, *A. eucrenophila*, *A. media*, *A. sobria* and *A. allosaccharophila* strains from different origins such as water, fish and human stool. *rpoD* sequences affiliated with *A. allosaccharophila* and *A. veronii* strains from for example water and pig carcasses. Finally, *sodB* sequences affiliated with sequences from *A. hydrophila, A. veronii*, *A. media*, *A. sobria* and *A. allosaccharophila* strains, most of which were obtained from water and fish samples.

Sequences obtained during this study were aligned using the CLUSTAL W program (Thompson*, et al.*, 1997) and their similarity was determined. The homology between *gyrB* sequences varied from 90 to 99. For *rpoD* the obtained homologies ranged between 96 and 100. Finally between *sodB* clones sequences homologies ranged from 91 to 100. The resulting alignments are shown in figures 18,19 and 20.

```
1F2             GAAGGCCAAGTCGGCCGCCAGTGGCAACGACGTCCGTGAAGGCCTGATAGCGGTCATCTC 60
1F12            GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTCCGTGAAGGCCTGATAGCGGTCATCCC 60
1F4             GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTCCGTGAAGGCCTGATTGCGGTCATCTC 60
1F3             GAAGGCCAAGTCGGCCGCCAGTGGTGACGACGTCCGTGAAGGTCTAATTGCCGTTATCTC 60
1F21            GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTCCGTGAAGGCTTGATTGCGGTCATCTC 60
1A19            GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTGCGTGAAGGTTTGATTGCGGTCATCTC 60
1A1             GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTCCGTGAAGGTCTGATTGCCGTTATCTC 60
1F8             GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTGCGTGAAGGTCTGATTGCCGTTATCTC 60
1F13            GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTGCGTGAAGGTCTGATTGCCGTTATCTC 60
1F17            GAAGGCCAAGTCGGCCGCCAGTGGCGACGACGTACGTGAAGGTCTGATTGCCGTTATCTC 60
                ************************  ******* ********  * ** ** ** *** *

1F2             CGTCAAGGTGCCTGATCCCAAGTTCTCCTCCCAGACCAAGGACAAGTTGGTCTCTTCCGA 120
1F12            CGTCAAGGTGCCTGATCCCAAGTTCTCCTCCCAGACCAAGGACAAGCTGGTCTCTTCCGA 120
1F4             CGTCAAGGTGCCTGACCCTAAGTTCTCCTCCCAGACCAAGGACAAACTGGTTTCTTCCGA 120
1F3             CGTCAAGGTGCCTGACCCTAAGTTCTCCTCCCAGACCAAGGACAAGCTGGTCTCTTCCGA 120
1F21            CGTCAAGGTGCCTGACCCCAAGTTCTCCTCCCAGACCAAGGACAAGCTGGTCTCTTCCGA 120
1A19            GGTCAAGGTGCCTGATCCCAAGTTCTCCTCCCAGACCAAGGACAAGCTGGTCTCGTCCGA 120
1A1             CGTCAAGGTGCCGGATCCCAAGTTCTCCTCCCAGACCAAGGACAAGCTGGTCTCGTCCGA 120
1F8             CGTCAAGGTGCCGGATCCCAAGTTCTCCTCCCAGACCAAGGACAAGCTGGTTTCGTCCGA 120
1F13            CGTCAAGGTGCCGGATCCCAAGTTCTCCTCCCAGACCAAGGACGAGCTGGTTTCGTCCGA 120
1F17            CGTCAAGGTGCCGGATCCCAAGTTCTCCTCCCAGACCAAAGACAAGCTGGTCTCTTCCGA 120
                 *********** ** ** ******************** *** *  **** ** *****

1F2             AGTGAAGACTGCCGTTGAACAGGCAATGGGCGAGAAGTTGGCGGACTTCCTGCTGGAAAA 180
1F12            AGTGAAGACTGCCGTTGAACAGGCAATGGGCGAGAAGCTGGCGGACTTCCTGCTGGAAAA 180
1F4             AGTGAAGACTGCCGTTGAACAGGCAATGGGCGAGAAGCTGGCGGACTTCCTGCTGGAAAA 180
1F3             AGTGAAGACCGCCGTGGAACAGGCGATGGGTGAGAAGCTGGCCGACTTCCTGCTGGAAAA 180
1F21            AGTGAAGACCGCCGTGGAACAGGCGATGGGTGAGAAGCTGGCCGACTTCCTGCTGGAAAA 180
1A19            GGTGAAGACCGCCGTTGAACAGGCGATGGGTGAGAAGCTGGCCGATTTCCTGCTGGAAAA 180
1A1             GGTGAAGACCGCCGTCGAACAGGCGATGGGGGAGAAGCTGGCGGACTTCCTGCTGGAAAA 180
1F8             AGTGAAGACTGCCGTTGAGCAGGCGATGGGCGAGAAACTGGGTGAGTTTCTGCTGGAAAA 180
1F13            AGTGAAGACTGCCGTTGAGCAGGCGATGGGCGAGAAACTGGGTGAGTTTCTGCTGGAAAA 180
1F17            AGTGAAGACCGCCGTTGAACAGGCGATGGGCGAGAAACTGGGTGAGTTTCTGCTGGAAAA 180
                 ******** ***** ** ***** ***** *****  ***  ** ** ***********

1F2             CCCGGGCGATGCCAAGAT 198
1F12            CCCGGGCGATGCCAAGAT 198
1F4             CCCGGGCGATGCCAAGAT 198
1F3             CCCGGGCGATGCCAAGAT 198
1F21            CCCGGGCGATGCCAAGAT 198
1A19            CCCGGGCGATGCCAAGAT 198
1A1             CCCGGGCGATGCCAAGAT 198
1F8             CCCGGGCGATGCCAAGAT 198
1F13            CCCGGGCGATGCCAAGAT 198
1F17            CCCGGGCGATGCCAAGAT 198

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Figure 18 - gyrB clones sequences alignment.

Figure 19 - rpoD clones sequences alignment.

```
3F10            GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCACCGGCGGTGTCT 60
3F15            GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCAACGGCGGCATCT 60
3F13            GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCAGCGCTGGCATCT 60
3F9             GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCAACGGCGGCATCC 60
3F1             GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCAACGGCGGCATCT 60
3F8             GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCAGCGCTGGCATCT 60
3A8             GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCAGCGCTGGCATCT 60
3A3             GTACCGAGTTTGAAGGCAAGTCTCTGGAAGAGATCATCAAGACCTCCAGCGCTGGCATCT 60
                ********************************************** **  **  **

3F10            TCAACAACGCCGCCCAGATTTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAACG 120
3F15            TCAACAACGCCGCCCAGATCTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAACG 120
3F13            TCAACAACGCCGCCCAGATCTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAACG 120
3F9             TTAACAACGCCGCCCAGATCTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAATG 120
3F1             TCAACAACGCCGCCCAGATCTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAATG 120
3F8             TCAACAACGCCGCCCAGATCTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAATG 120
3A8             TCAACAACGCCGCCCAGATCTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAATG 120
3A3             TCAACAACGCCGCCCAGATCTGGAACCACACCTTCTACTGGCACTGCCTCTCCCCGAATG 120
                * ***************** ************************************ *

3F10            GCGGTGGCGAGCCTACCGGCGCCCTGGCCGATGCCATCAACAAGGCATTCGGCTCTTTCG 180
3F15            GCGGTGGCGAGCCTACCGGCGCCCTGGCCGATGCCATCAACAAGGCATTCGGCTCTTTCG 180
3F13            GCGGTGGCGAGCCCACTGGCGCCCTGGCCGATGCCATCACCAAGGCCTTCGGCTCCTTCG 180
3F9             GAGGTGGCGAGCCGACTGGCGCCCTGGCTGAGGCCATCAACAAGGCATTCGGCTCCTTCG 180
3F1             GCGGTGGCGAGCCGACTGGCGCCCTGGCTGATGCCATCAACAAGGCATTCGGCTCCTTCG 180
3F8             GCGGTGGTGAGCCTACTGGCGATCTGGCCGCAGCCATCAACAAGGCGTTTGGTTCTTTCG 180
3A8             GCGGTGGTGAGCCTACTGGCGATCTGGCCGCAGCCACCAACAAGGCGTTTGGTTCTTTCG 180
3A3             GCGGTGGTGAGCCTACTGGCGATCTGGCCGCAGCCATCAACAAGGCGTTTGGTTCTTTCG 180
                * ***** ***** ** ****  ***** *  **** ** ****** ** ** ** ****

3F10            CCGAGTTCAAGGATGCGTTCACCAAGTCTGCCATCGGCAACTTCGG 226
3F15            CCGAGTTCAAGGATGCGTTCACCAAGTCTGCCATCGGCAACTTCGG 226
3F13            CCGAGTTCAAGGATGCGTTCACCAAGTCTGCCATCGGCAACTTCGG 226
3F9             CCGAGTTCAAAGATGCCTTCACCAAATCTGCCATCGGCAACTTCGG 226
3F1             CCGAGTTCAAAGATGCCTTCACCAAATCTGCCATCGGCAACTTCGG 226
3F8             CCGAGTTCAAAGATGCCTTCACCAAATCTGCCATCGGCAACTTCGG 226
3A8             CCGAGTTCAAAGATGCCTTCACCAAATCTGCCATCGGCAACTTCGG 226
3A3             CCGAGTTCAAAGATGCCTTCACCAAATCTGCCATCGGCAACTTCGG 226
                ********** ***** ******** ********************
```

Figure 20 - sodB clones sequences alignment.

Sequences were further aligned with reference taxa within the sequence databases using the CLUSTAL X program (Thompson*, et al.*, 1997). Phylogenetic analyses were performed with PAUP* version4.0b10 (Swofford, 2003). The resulting trees are presented in Figures 21, 22 and 23. The arrows indicate our clones position between *Aeromonas* strains in the phylogenetic trees.

Figure 21 - Phylogenetic tree based on *gyrB* clones sequences, showing their phylogenetic position among *Aeromonas*.

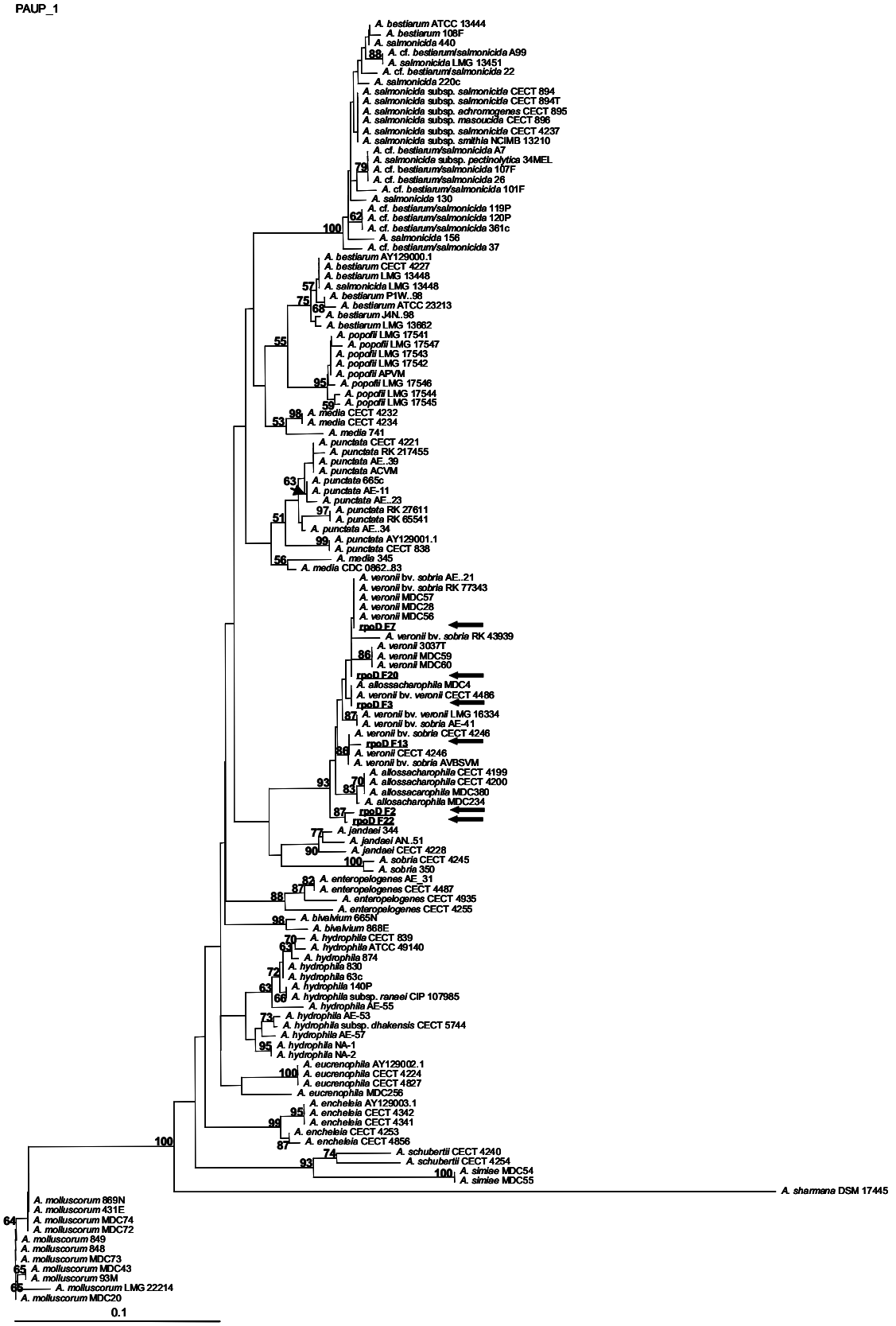

Figure 22 - Phylogenetic tree based on *rpoD* clones sequences, showing their phylogenetic position among *Aeromonas* strains.

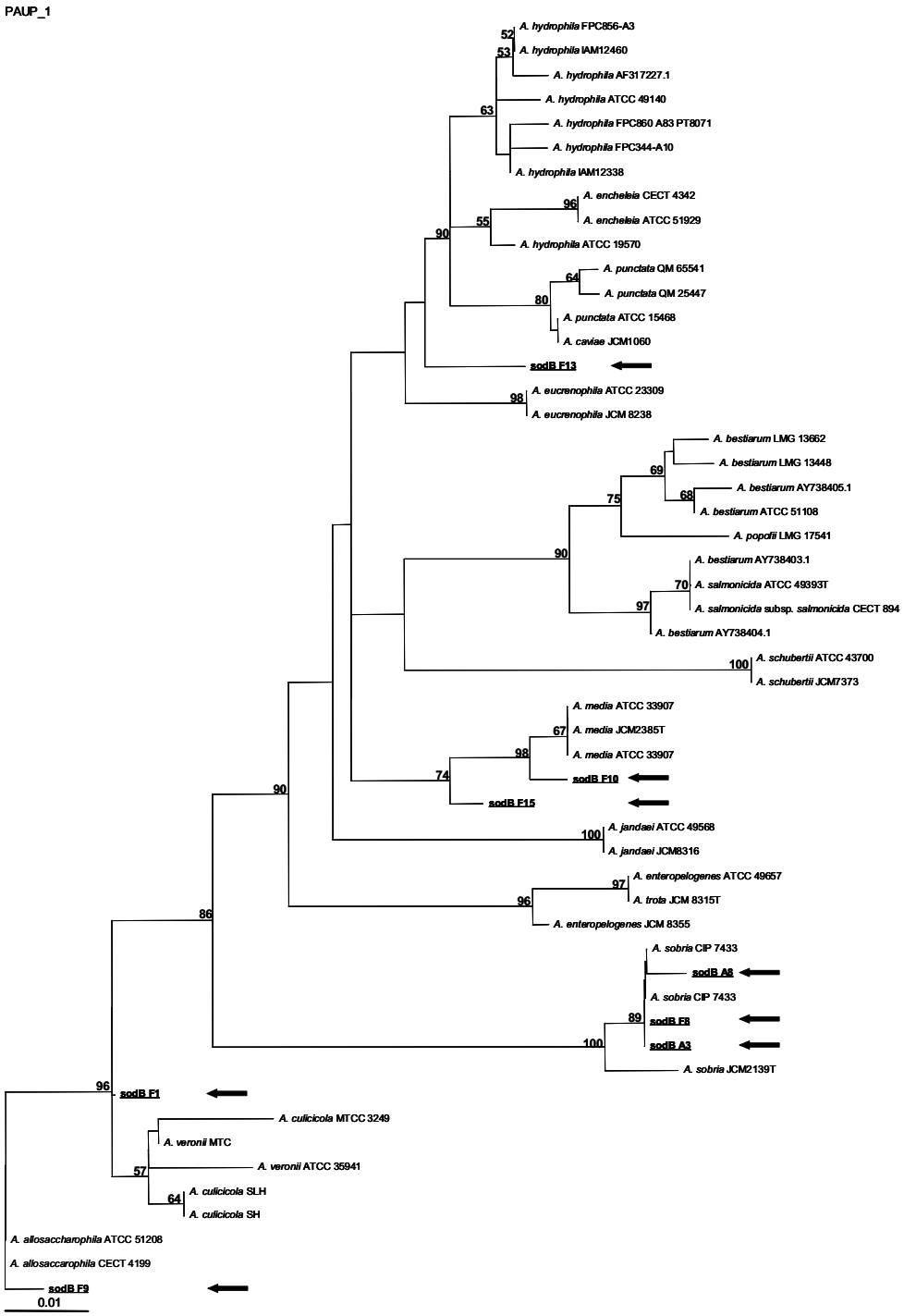

Figure 23 - Phylogenetic tree based on *sodB* clones sequences, showing their phylogenetic position among *Aeromonas* strains.

5 Discussion

Aeromonads are widely distributed in nature and the dissemination of pathogenic strains constitutes an important problem in aquaculture systems. Also these microorganisms are nowadays considered an emerging pathogen for humans. Therefore it is important to monitorize these bacteria and understand their ecological distribution as well as to determine which environmental factors influence *Aeromonas* communities' structure and distribution. By developing assays which allow assessing and following the compositional dynamics of aeromonads communities, we can monitorize their behavior and learn how to control them, in order to avoid the dissemination of hazardous strains and consequently to avoid economical losses to aquaculture systems or damages to human health.

In this investigation the main goal was to evaluate three culture-independent assays to study the diversity and follow the dynamics of the *Aeromonas* communities using PCR-DGGE methods targeting *gyrB, rpoD* and *sodB* genes. Primers were previously designed and the *gyrB*-targeting primers have been previously evaluated for aeromonads typing purposes (Tacão et al., 2005). Therefore, first the primers specificity was tested, experimentally and in silico and the detection limits for each primer set was determined. The phylogenetic information contained in the resulting amplified fragments was also evaluated. Subsequently DGGE assays using amplicons obtained from environmental samples from Ria de Aveiro were performed using the three sets of primers. Finally a cloning and sequencing strategy has been used to confirm the specificity of the primers.

The experimental specificity testing results demonstrated that all the three sets of primers are specific to aeromonads. In what concerns amplification in aeromonads it was verified that in *gyrB* and *sodB* assays all the *Aeromonas* strains presented amplification. On the opposite, in the *rpoD* assay, two *Aeromonas* strains presented no amplification: *A. caviae* CECT 838^T and *A. molluscorum* G.I6.7. This absence of amplification was confirmed by replicating assays.

In relation to *A. caviae* CECT 838^T we do not have an explanation for the absence of amplification. Three strains of this species have been used in our assays and it was not possible to obtain amplification only from *A. caviae* CECT 838^T. Also, according to our in

silico study presented in the table 12, this strain presents only one nucleotide difference at position 12 of the forward primer. It should be noticed that for example the strain *A. hydrophila* CECT 839, which was also tested in this investigation, also presents the same nucleotide difference in that specific position and in addition has also a nucleotide difference in the reverse primer. Thus, if that nucleotide difference was sufficient to disable the amplification, a negative result would also been obtained from this strain, and this was not observed. In addition it was also further verified that when using the primers with clamp, the strain *Aeromonas caviae* CECT 838^T presented amplification (as can be seen in figure 9). This fact can be related with the different characteristics of the clamp primers when compared with primers without clamp.

In what concerns *A. molluscorum* G.I6.7, we do not have information about its *rpoD* gene sequence so it cannot be determined why there was no amplification. Besides that, since this was the only *A. molluscorum* strain tested, it cannot be established if this set of primers does or does not amplify from *A. molluscorum* strains. To conclude about this, assays using more strains from this species should be performed.

When evaluating the primers mismatches, it was verified that in the case of *gyrB,* the species which presented more nucleotide differences when comparing with the primer sequences were *A. simiae* and *A. molluscorum*. *A. simiae* strains were not included in this study so it cannot be stated if these primers are adequate to amplify the *gyrB* fragment from this species. Although, in what concerns to *A. molluscorum,* which appears to be the species presenting the higher number of mismatches in all the strains, an amplicon was obtained from an environmental strain of this species, so possibly this mismatches are not enough to prevent amplification. In *rpoD* sequences a maximum of three mismatches per primer were detected. The strains presenting higher numbers of mismatches were *A. molluscorum* MDC43, *A. simiae* MDC55 and *A. veronii. 2238A.* Although, the majority of A. *molluscorum* strains presented only two nucleotide differences and in the case of *A. veronii* almost all strains presented no nucleotide differences. The *sodB* sequences are the ones showing fewer mismatches when compared to the primer sequences. Almost all the sequences present no mismatches and only three present one mismatch. However, it is important to refer that the number of *sodB* sequences which are stored in GenBank is much lower when compared with the other two genes.

Phylogenetic trees were constructed based on *gyrB*, *rpoD* and *sodB* gene sequences

from databases and on small sequences from target fragments in order to evaluate the phylogenetic information contained in the amplified fragments. The *gyrB* and *rpoD* genes have been considered suitable phylogenetic markers to evaluate aeromonads relationships by several authors (Yanez*, et al.*, 2003, Soler*, et al.*, 2004, Saavedra*, et al.*, 2006) but to our knowledge, *sodB* gene has not been evaluated as phylogenetic marker in aeromonads.

The analysis of the trees built based in the full gene sequences allowed to verify that all the genes appear to be good phylogenetic markers which is in agreement with the referred studies (Yanez*, et al.*, 2003, Soler*, et al.*, 2004, Saavedra*, et al.*, 2006). In what concerns *sodB*, this gene seems to be very promising but we cannot draw any absolute conclusions because of the reduced number of sequences deposited from this gene. This fact constitutes an important disadvantage and so it would be interesting and useful to develop studies which allowed determining this gene sequences in a high number of *Aeromonas*, especially those who were used in the evaluation of the other phylogenetic markers in order to assure that *sodB* is also a good molecular marker.

From the analysis of the phylogenetic trees based on the amplified fragments it was verified that generally the same clusters were obtained, and were consistent with the taxonomy of the genus. This fact indicates that the amplified fragments contain enough phylogenetic information to affiliate sequences to a certain species. Though, it is important to refer that the levels of confidence on these affiliations are lower than when using the large sequences as can be seen by the lower bootstrap values in the small fragment trees.

To confirm the usefulness of the methods, *gyrB*, *rpoD* and *sodB* PCR-DGGE assays using environmental samples from Ria de Aveiro were conducted. Ria de Aveiro constitutes an advantageous sampling place to study communities because as an estuarine environment it contains several gradients which allow the development of communities with different characteristics. In estuarine environments we can find salinity, nutrient, organic matter and temperature gradients as well as different anthropogenic pressures. To make this study even wider we have used samples collected along a salinity gradient in Ria de Aveiro and in places displaying different anthropogenic pressures. We have also used samples collected in different and contrasting seasons because it is known that temperature is a factor affecting *Aeromonas (Sautour, et al., 2003).*

The three primer sets gave origin to an amplicon with the expected size from all the complex environmental samples tested. From the obtained results it became immediately

obvious that *Aeromonas* communities in this estuary are very complex and constant. This conclusion was supported by the fact that highly complex profiles were obtained from each sample and by fact that most of the band positions were detected in all the samples. Previous studies have reported the prevalence of these bacteria in estuarine waters (Fiorentini*, et al.*, 1998).

The fact that the DGGE profiles are surprisingly stable reveals a high tolerance of the existing phylotypes to several environmental conditions including salinity and nutrient gradients and also to different anthropogenic pressures. The high tolerance of aeromonads to several environmental factors has already been reported (Sautour*, et al.*, 2003, Wang & Gu, 2005).

From the cluster analysis and in what concerns the similarity between profiles, the obtained results using the three sets of primers were in agreement, separating the winter and summer communities. The factors that appear to influence more *Aeromonas* communities seem to be season related, possibly including the temperature. This factor has already been described as an influence to aeromonads (Mary*, et al.*, 2002, Maalej*, et al.*, 2004, Wang & Gu, 2005). Though, even in different seasons, the number of bands per profile is similar and many of the band positions are present in all the samples. On the opposite, the spatial distribution of these communities is less obvious and more variable, maybe because of the high similarity between the profiles.

To further confirm that our assays were specific to aeromonads and thus that all the amplified fragments from environmental DNA belong to species from this genus a cloning and sequencing strategy using two representative environmental samples was applied. These experiments allowed to achieve the stated objective and also to acquire more information on the dominant groups within these samples,

In all the cloning assays, we have obtained a high level of false positive clones, especially in the samples from N1 July, which presented higher level of salinity. This fact seems to indicate that the salt and probably other contaminants present in the samples inhibited the cloning. In addition, we have verified that in the cloning of *rpoD* products we have obtained lower efficiency levels, which may be related to sequence-specific constraints.

Despite the low efficiency in the cloning assays, it was possible to obtain a reasonable number of positive clones, in the majority of the cases displaying different

positions in the DGGE gels. This fact once again revealed the high level of diversity within these environmental communities.

The nucleotide sequences from the fragments displaying different positions in DGGE gels were determined and results confirmed that the proposed sets of primers are actually specific to this genus, since the obtained sequences were all affiliated to sequences from *Aeromonas* strains. The closest relative strains had several origins which are common in *Aeromonas* strains such as fish, and several types of waters, within others. In terms of species composition some correspondence was obtained between primer sets: sequences affiliated with *A. allosaccharophila* were obtained using the three primer sets and sequences affiliated with *A. veronii* and *A. sobria* were obtained using two primers sets. The lack of correspondence for other obtained sequences may indicate a selective performance of the primers or may just be related to the reduced number of obtained clones that cannot be representative of the reality. The species affiliated with the obtained sequences have already been isolated from Ria de Aveiro in previously reported culture-based studies (Henriques*, et al.*, 2006). It should also be noticed that the obtained sequences displayed high homology levels with culturable strains. This fact seems to indicate that in the case of aeromonads culture-based methods may provide a real representation of the diversity of the communities.

Finally we have identified some of the dominant bands in DGGE profiles by comparing bands from sequenced clones with the environmental profiles. These clones were affiliated with *Aeromonas* sp. F674P and *A. media* strain CECT 4234 in *gyrB* case and with *A. sobria* strain CIP 7433, *A. allosaccharophila* CECT 4199 and *A. media* strain ATCC 33907 in *sodB*.

In summary, the PCR-DGGE method has been broadly used for communities typing purposes. It generally targets the 16S rRNA gene but it can also be used targeting other phylogenetic or functional informative genes by using specific primers. This method presents several advantages such as the capacity to analyze in a single gel a wide number of samples and the possibility to recover the DNA from the gel by excising the bands, which allows the phylotypes identification by determining their nucleotidic sequence. Even though, it is important to consider that this method also presents some associated errors. These are related to DNA extraction and PCR primer annealing efficiencies, cloning, and chimera and heteroduplex formation (Kopczynski*, et al.*, 1994, Farrelly*, et al.*, 1995,

Suzuki & Giovannoni, 1996). Besides this, it has been reported that this technique is only able to recover sequences which are present in at least 0.5- 0.1% of the total cells in the sample. Despite all these possible errors, this technique is considered a useful tool in assessing the dynamics of the communities, by presenting profiles which are representative of them.

The developed methods during this study have revealed to be useful and efficient to describe the diversity and dynamics of aeromonads communities. They have proved to be very specific, to allow the analysis of a large number of samples and to display specific profiles for each community. The analysis of the resulting profiles seems to be very revealing and it can be used to determine which are the most important factors affecting aeromonads communities.

This was the first time that culture-independent methods specific to *Aeromonas* were developed and evaluated. Untill now, all the information about this genus was obtained by isolating and characterizing culturable species. The development of culture independent methods is essential because by using them we can avoid the introduction of errors which are related to the culture of microorganisms (Fontana*, et al.*, 2005).

From all of the observed facts and results we can assert that the three evaluated methods are suitable to study aeromonads because all of them have revealed to be adequate to the intended objective. However, all methods present some advantages and disadvantages. For example, *gyrB* and *rpoD* genes have previously been reported as good phylogenetic markers and a large number of sequences from these genes are stored in databases, fact that aids their study. Though, *rpoD* primers presented the higher detection limit and seemed to be unable to amplify all the tested *Aeromonas* strains. In the case of *sodB*, it presents a disadvantage related to the reduced number of available sequences, though it seems to be a promising marker and thus it is important to develop further studies to evaluate its usefulness. Also a lower number of mismatches were detected for the *sodB* primers when compared to the other two sets of primers.

6 Conclusions

In conclusion all the three assays proved to be specific for the genus *Aeromonas* and useful to study aeromonads communities. We believe that the joint utilization of more than one set of primers may be extremely useful in providing a more clear and representative image of the studied community. The developed methods may also be adapted to be used in other types of environmental samples, namely from animal sources, since some of the species from this genus are animals pathogens and are widely distributed in nature.

7 References

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