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Identification and Genotyping of *Pseudomonas aeruginosa* in Bronchiectasis

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Mestrado em Biologia Celular e molecular
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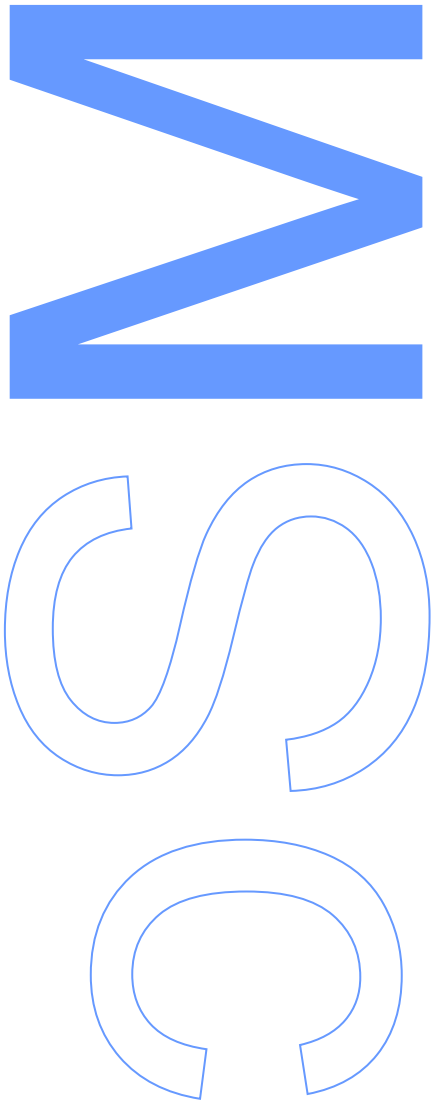
Todas as correções determinadas pelo júri, e só essas, foram efetuadas.



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Resumo

Bronquiectasia é uma doença pulmonar obstrutiva resultante da inflamação crónica dos brônquios. *Pseudomonas aeruginosa* associa-se a uma diminuição da função pulmonar em bronquiectasia e agudizações mais frequentes. Esta bactéria é frequentemente subdiagnosticada, o que pode limitar o tratamento dos doentes. Atualmente, a terapia baseada em antibióticos é essencialmente dirigida a uma única estirpe bacteriana, no entanto os doentes com bronquiectasia podem estar infetados por múltiplas estirpes de *P. aeruginosa*. Assim, o diagnóstico e implementação imediata de uma terapia dirigida podem melhorar o prognóstico e limitar danos em órgãos dos doentes.

O principal objetivo do presente trabalho foi caracterizar a evolução e estrutura da população de *P. aeruginosa* em doentes com diferentes tipos de bronquiectasias, alguns deles também diagnosticados com fibrose cística (FC), utilizando o método *SNaPaer*. Assim, começamos por o otimizar o método para aplicação direta em colónias de *P. aeruginosa*, selecionando o melhor pré-tratamento para a amplificação dos genes alvo. Dos vários tratamentos, a pré-incubação com Proteinase K provou ser a mais eficiente, com 96% de resultados positivos. Em seguida, testou-se a capacidade do *SNaPaer* para identificação e genotipagem de mais de 200 isolados bacterianos obtidos em meio de cultura Cetramida a partir de amostras clínicas de doentes com bronquiectasias. Nestes isolados identificaram-se 43 perfis *SNaP* diferentes. A partir dos 43 perfis, 38 foram exclusivos de doentes portugueses, não tendo sido descritos anteriormente no banco de dados pubMLST (http://pubmlst.org/paeruginosa/). Os perfis obtidos revelaram-se altamente específicos (> 90%) para as populações de doentes, mesmo dentro dos grupos de doentes considerados (doentes com e sem FC). A microevolução foi o mecanismo de evolução mais comum em estirpes de *P. aeruginosa* de doentes com bronquiectasias. Contudo, alguns doentes parecem ser colonizados simultaneamente por múltiplas estirpes, particularmente doentes FC com bronquiectasias. Os doentes submetidos a terapias antibacterianas mais agressivas apresentaram, no final da terapia, perfis *SNaP* muito distintos relativamente ao perfil *SNaP* do primeiro isolado.

O presente estudo contribui para aprofundar a caracterização da diversidade genética em doentes com bronquiectasias, abrindo novas portas para alcançar um melhor

conhecimento sobre os fatores subjacentes aos padrões de evolução e diversidade em populações de *P. aeruginosa*.

Palavras-chave: espécies de *Pseudomonas*, estrutura da população, minisequenciação, diversidade genética, bronquiectasias, *SNaPaer*.

Abstract

Bronchiectasis is an obstructive pulmonary disease resulting from chronic bronchial inflammation. *Pseudomonas aeruginosa* has been associated with decreased lung function in bronchiectasis and frequent exacerbations. However, this bacterium is frequently under-diagnosed, which may limit the treatment of the patients. Furthermore, antibiotic therapy is primarily directed to single bacterial strains, while patients with bronchiectasis may be colonised or infected by multiple *P. aeruginosa* strains. Thus, prompt diagnosis and immediate implementation of a targeted therapy can improve the prognosis and restrict extensive damage into patient organs.

The main goal of the present work was to characterise the evolution and population structure of *P. aeruginosa* from different bronchiectasis patients, some of them also diagnosed with cystic fibrosis (CF), using the *SNaPaer* assay. Therefore, we first optimised the method for direct application on *P. aeruginosa* colonies by selecting the optimal pre-treatment for posterior gene amplification. Afterwards, we tested the ability of *SNaPaer* for identification and genotyping in more than 200 *P. aeruginosa* isolates collected from bronchiectasis patients.

From the tested pre-treatments, proteinase K was the most efficient method with 96% of positive results. Based on reproducibility, cost and practical applicability at the laboratorial routine, the pre-treatment with Proteinase K was the selected method for posterior genotyping analysis. The *SNaPaer* assay proved to be good and reliable for identification and genotyping *P. aeruginosa.* In 207 clinical isolates, a set of 43 different *SNaP* profiles were identified. A group of 38 out of 43 *SNaP* profiles were exclusive detected in Portuguese patients and had not been previously described at pubMLST database (http://pubmlst.org/paeruginosa/). *SNaP* profiles were highly specific (>90%) for the patient population, even considering the patient groups (CF and non-CF patients). Microevolution events were the most common mechanism of evolution in *P. aeruginosa* strains from bronchiectasis patients. However, some patients seem to be colonised simultaneously with multiple strains, particularly CF patients with bronchiectasis. Patients submitted to aggressive antibiotic therapies presented at the end of the therapy *P. aeruginosa* with *SNaP* profiles very distinct from the *SNaP* profile of first isolate.

The present study has contributed to the characterisation of genetic diversity of *P. aeruginosa* in bronchiectasis patients, opening new perspectives to understand the factors underlying the patterns of evolution and diversity in *P. aeruginosa* clinical populations.

Key-words: *Pseudomonas* species, population structure, minisequencing, genetic diversity, bronchiectasis, *SNaPaer*.

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Abbreviations

µL	microlitre
µM	micromolar
16S rRNA	16S ribosomal RiboNucleic Acid
A	Adenine
ABPA	Allergic BronchoPulmonary Aspergillosis
acsA	acetyl coenzyme A synthetase
algD	guanosine diphosphate mannose dehydrogenase
aroE	shikimate 5-dehydrogenase
bp	base pair
C	Cytosine
ºC	degree Celsius
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CTAB	N-Cetyl-N,N,N,-Trimethyl Ammonium Bromide
ddNTP	dideoxyNucleotide TriPhosphate
DNA	DeoxyriboNucleic Acid
dNTP	dideoxyNucleotide TriPhosphate
ecfX	putative anti-sigma protein
etc	*et cetera*
et al.	*et alii*
e.g.	*exemplī grātiā*

FEV1	Forced Expiratory Volume per second 1
FVC	Forced Vital Capacity
G	Guanine
guaA	guanosine 5'-monophosphate synthase
gyrB	DNA gyrase subunit B
HIV	Human immunodeficiency virus
HRCT	High Resolution Computed Tomography
IC	Immune Complexes
i.e.	*id est*
IFN- γ	Interferon-γ
IgA	Immunoglobulin A
IL	InterLeukin
IU	International Units
kbp	kilobase pair
L	litres
lasR	transcriptional regulator R
M0	Resting macrophages
M1	Activated macrophages
Mbp	Megabase pair
mexA	resistance-nodulation-cell division multidrug efflux membrane fusion protein A
mexB	resistance-nodulation-cell division multidrug efflux transporter B
mg	milligrams
min	minute
mL	milliLitre

MLSA	MultiMocus Sequence Analysis
MLST	MultiLocus Sequencing Typing
mutL	DNA mismatch repair protein
n	number
NCBI	National Center for Biotechnology Information
NCF	Non-Cystic Fibrosis
ng	nanogram
NJ	NeighbourJoining
nuoD	bifunctional NADH:ubiquinone oxidoreductase subunit C/D
oprI	outer membrane lipoprotein
oprL	peptidoglycan associated lipoprotein
PCR	Polymerase Chain Reaction
ppsA	phosphoenolpyruvate synthase
PF	Pulmonar Function
pv.	pathovar
RFU	Relative Fluorescence Unit
RNA	RiboNucleic Acid
rpm	rotations per minute
rpoB	ribosomal RNA polymerase subunit beta
rpoD	ribosomal RNA polymerase sigma factor D
rrnA	ribosomal RNA A
rrnB	ribosomal RNA B
rrnC	ribosomal RNA C
rRNA	ribosomal RiboNucleic Acid

s	second
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodesyl Sulfate
SNP	Single Nucleotide Polymorphism
U	Units
T	Timine
TNF	Tumor Necrosis Factor
Th	T helper
toxA	exotoxin A
trpE	anthranilate synthase component I

1. Introduction

1.1 Bronchiectasis

Bronchiectasis is a common long-term respiratory condition characterised by an anomalous, chronic enlargement of the bronchi. The prevalence of this lung disorder is highly variable, ranging from four per 100,000 habitants in New Zealand to 1,600 per 100,000 in Alaskan Eskimo population for non-cystic fibrosis (CF) bronchiectasis (Goeminne and Dupont, 2010). In people with certain diseases, bronchiectasis can be common as it is the case of CF patients, with more than half of the patients presenting this condition (Vonberg et al., 2005). Individuals with bronchiectasis have a history of chronic and recurrent airway infections, mainly caused by *Pseudomonas aeruginosa* bacteria. Depending on the underlying origin, those infections may implicate the whole respiratory tract, causing sinus and lung illness.

Since the first description by René Laënnec in 1819 (Roguin, 2006), the bronchiectasis has suffered substantial changes in respect to its incidence, aetiology, manifestation, and treatment. Antibiotics have significantly improved the quality of life of patients with bronchiectasis. Those drugs have transformed post-infectious bronchiectasis in an uncommon respiratory condition in some developed countries.

Later in 1989, with the discovery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, there was an increase in the number of studies related to the biology of the airway cells, as well as the mechanisms linked to the transport of water and salts into the mucus (Marcet and Boeynaems, 2006; Boucher, 2007; Kreda et al., 2012). This knowledge allowed considerable improvements in the

treatment of CF bronchiectasis. However, the treatment of non-CF bronchiectasis is not necessarily the same as for CF bronchiectasis and therefore, improvements in the quality of life in both groups are still required.

Recently, few studies have proved that bronchiectasis remains a notorious problem in developing countries, particularly in people over 75 year old (Seitz et al., 2010). Thus, it is essential to understand the elementary mechanisms of the disease, the microorganisms involved and how the lung damage is perpetuated.

1.1.1. Pathophysiology, diagnosis and treatment of bronchiectasis

Bronchiectasis is the anatomic alteration of the conducting airways, in which part of bronchial tree is abnormally stretched and widened (Kim and Kim, 2012). In these circumstances, extra mucus tends to be produced and settle in the enlarged airways (Figure 1). Accompanying the enlargement of the bronchi, the cilia present in the airways are also damaged and their ability to clear secretions decreases considerably. Failure to clean mucosal secretions allows the accumulation of microorganisms and particles in the patient lungs that lead to increased secretion and inflammation, which further damage the airways.

Bronchiectasis may be classified as localised or diffused. Localised bronchiectasis is characterised by the damage in a single portion of the lung (King, 2009). When the damage extends and occurs all throughout the lungs it is called diffuse bronchiectasis. Localised bronchiectasis is generally associated with necrotising pneumonia, obstructive lesion and tuberculosis. On the other hand, diffuse bronchiectasis is usually related with CF, immobile cilia syndrome, immunodeficiencies, hypogammaglobulinemia and childhood infections. Bronchiectasis patients with a stable status have high levels of inflammatory markers, such as C-reactive protein and erythrocyte sedimentation rate (Brody et al., 2006), and these markers usually increase during exacerbations (Smith et al., 1996). The levels of these markers are significantly different before and after the treatment with antibiotics. Severe cases of bronchiectasis are characterised by chronic cough, dyspnoea and sputum production, which can be interposed by punctual episodes of acute exacerbation. The definition of acute exacerbation is still under discussion. However, a bronchiectasis patient is reported to have an acute exacerbation if some of the following indicators are present with no other explanation:

increased cough, change in sputum production, increased dyspnoea, fever over 38°C, increased wheezing, fatigue, decreased exercise tolerance, lethargy, malaise, reduced pulmonary function (PF), changes in chest sounds or radiographic changes consistent with a new infectious process (O'Donnell, 2008; Goeminne and Dupont, 2010).

The diagnosis of this lung condition is usually performed by high-resolution chest computed tomography (HRCT) (O'Donnell, 2008). The test generates detailed images of the airways, lungs and other structures, which help to determine the location and extent of airway damage. Other complementary tests could be applied to facilitate the diagnosis of this disease such as blood tests, sputum culture, lung function tests, sweat test and bronchoscopy. Nevertheless, HRCT remains the more accurate test for bronchiectasis diagnosis.

Nowadays, the treatment of bronchiectasis includes controlling infections, inflamatory process and bronchial secretions by clearing airway obstructions. The control of bronchial secretions includes the continued usage of mucoactives and anti-inflammatory agents; antibiotics are used to prevent and treat damaging infections (Evans et al., 2007). In a small number of cases, surgery is suggested to treat localised bronchiectasis, removing affected bronchial portions that could cause progression of bronchiectasis (Yoon et al., 2002; Ötgün et al., 2004). Lastly, the lung transplantation can be considered in some severe cases.

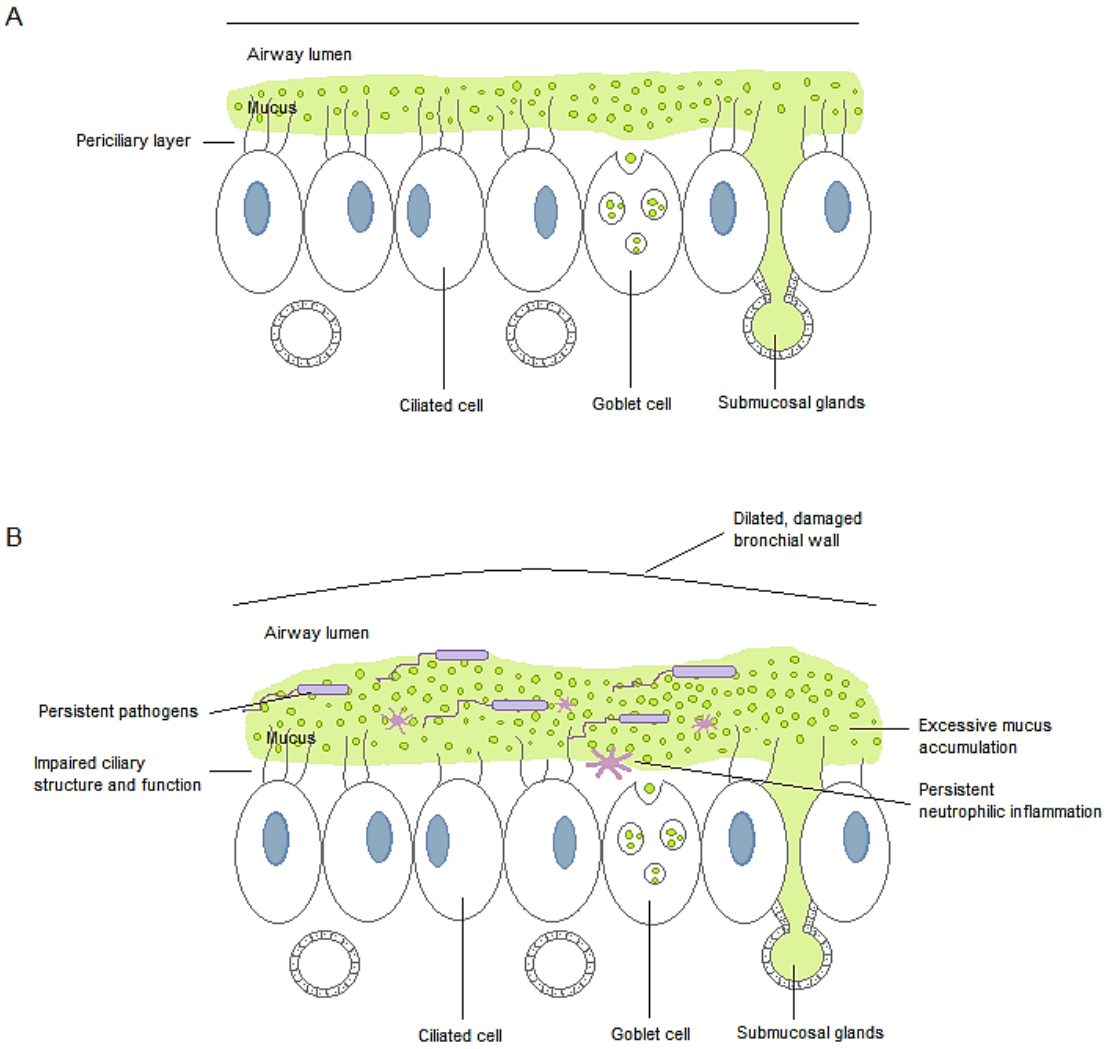

Figure 1. Components of mucociliary clearance mechanism. The mucus is secreted from sub-mucosal glands and goblet cells. Mucociliary clearance is the primary mechanism of clearance of mucus. A: In a normal mucociliary clearance situation, the cilia beat in a coordinated way in the periciliary fluid layer propelling the mucus towards the mouth. B: In an abnormal mucociliary clearance situation, mucus accumulates and cough becomes the secondary mechanism for clearance of mucus.

1.1.2. Clinical pathologies related to bronchiectasis

The improvements in bronchiectasis diagnosis support the assumption that a high percentage of chronic respiratory morbidity can be caused by this disorder (Patel et al., 2004; King et al., 2006). Nevertheless, bronchiectasis remains underdiagnosed in several clinical units or coexists with other disorders (such as infections, connective tissue diseases and immunodeficiencies). The reported

prevalence of bronchiectasis is probably underestimated (Barker, 2002; Revel et al., 2002; Patel et al., 2004; King et al., 2006).

The aetiology of bronchiectasis may vary, being associated with a range of conditions some of them related with genetic disorders and others with life acquired illnesses (Nicotra et al., 1995; Pasteur et al., 2000; Kelly et al., 2003; Pasteur et al., 2010). The cause of bronchiectasis may be very difficult to ascertain and it is more correct to consider several conditions associated with the development or a risk factor for the pathology. The list of associated diseases is large; some of the most common diseases associated with bronchiectasis are described below.

1.1.2.1. Infections

On the top of the list of risk conditions associated with bronchiectasis is the clinic history of severe infection (33–42%) (Chalmers and Hill, 2013). A wide range of necrotising infections that are either ineffectively treated or not treated at all may be enough to the emergence of bronchiectasis (Loebinger et al., 2009; Chalmers et al., 2012). *Klebsiella* species, *Staphylococcus aureus, Mycobacterium tuberculosis,* nontuberculous mycobacteria, *Mycoplasma pneumoniae, Measles* virus, *Pertussis* virus, *Influenzae* virus, herpes simplex virus and several types of adenovirus are some of the organisms that have been known to cause bronchiectasis. When a patient develops bronchiectasis many other organisms colonise the injured bronchi and may cause permanent damage and periodical cases of exacerbations. *Haemophilus* species and *Pseudomonas* species are the organisms that most commonly comprise the injured lungs (Angrill et al., 2002; King et al., 2007).

1.1.2.2. Connective tissue diseases

Connective tissue diseases, such as rheumatoid arthritis, systemic sclerosis, ankylosing spondylitis and systemic lupus erythematous may also be in the origin of bronchiectasis (Kozuka et al., 2001). These autoimmune disorders represent 3–6% of the bronchiectasis cases (Chalmers and Hill, 2013). Connective tissue diseases are characterised by an abnormal function of the immune system, which

attacks healthy tissue triggering inflammation. In a few number of cases the inflammation can spread to the lungs, causing the symptoms of bronchiectasis.

1.1.2.3. Allergic bronchopulmonary aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) is a respiratory disease characterised by a hypersensitivity response of the immune system to inhaled *Aspergillus* antigen (most commonly *Aspergillus fumigatus* antigen) (Roxo et al., 2013). ABPA causes airway inflammation which may eventually develop into bronchiectasis (it represents 1–7% of the total number of bronchiectasis) that is most marked in the central portions of the airways (Chalmers and Hill, 2013). Recurrent acute episodes that are untreated or not treated efficiently may result in progressive pulmonary fibrosis that is frequently seen in the upper portion of the airways. Patients with ABPA usually have a long history of poorly controlled asthma, cough, shortness of breath, chronic sputum production, coughing up brownish mucoid plugs or haemoptysis, and exercise intolerance.

1.1.2.4. Immunodeficiency

Another condition that may be a cause of bronchiectasis (1–8% of the total number of cases) is immunodeficiency caused by the absence or defects in certain immune cells (Truong, 2013; Chalmers and Hill, 2013). Individuals with immunodeficiency disorders have recurrent infections that are more severe and that last longer than usual. The immunodeficiency may be congenital or acquired, as it is the case of patients with Human immunodeficiency virus (HIV).

1.1.2.5. Recurrent aspiration or inhaled foreign body

Foreign body aspiration occurs when stomachal or oral chewed materials reach the lungs (Adegboye et al., 2003). The aspiration may occur from oropharyngeal dysphagia, a reduced ability to gulp, which may cause saliva or food to enter the lung. Another possible cause of aspiration is gastroesophageal reflux disease, which is characterised for a dysfunction in the smooth muscle valve between the

oesophagus and the stomach. The valve allows stomachal contents to flow back up into the oesophagus and subsequently into the lungs. If the aspiration is frequent or severe, it may lead to inflammation of the airways and cause bronchiectasis (2–4%) (Chalmers and Hill, 2013).

1.1.2.6. Cystic fibrosis

One of the most studied diseases within the list of underlying causes of bronchiectasis is CF, justifying the occurrence of 2–4% of the total group of bronchiectasis in the patients (Chalmers and Hill, 2013). CF, also known as mucoviscidosis, is a recessive genetic disease that occurs in all human populations but it is more common among Caucasians (Davis et al., 1996). CF causes several complications in various organs, including chronic pulmonary infections, rhinosinusitis, nasosinusal polyposis, bad gastrointestinal absorption ensuing from pancreatic dysfunction, spastic ileus of the newborn, retal prolapsed and infertility due to obstruction of the deferent ducts (Nick and Rodman, 2005). This inherited disease is characterised by an anomalous function of cAMP-regulated chloride ion channel of the airway epithelial cells, which is caused by a mutation on the CFTR gene located in long arm of chromosome 7. The reduction of the functionality of CFTR, as an effect of these mutations, causes a hyperabsorption of sodium chloride and therefore, a decrease in salt and water in the respiratory epithelium. Thus, there is an increase of mucus viscosities, which impedes the efficient clearance of the sputum, allowing the bronchial obstruction and enlarging bacterial colonisation and subsequent infection (Norez et al., 2009).

1.1.2.7. Primary ciliary dyskinesia

Primary ciliary dyskinesia is a genetic disease that causes a defect in the action of the cilia lining the respiratory tract, the fallopian tube and also of the flagella of sperm in males (Noone et al., 2004). The inappropriate sweeping of cilia may lead to a poor mucociliary clearance, frequent pulmonary infections and, ultimately, bronchiectasis. The evolution of the disease is variable and lung transplantation is required in severe cases. An early diagnosis may significantly reduce the infections, leading to a better prognosis in patients. Early treatment based on chest

physiotherapy techniques may reduce the occurrence of lung infection and slow down the progression of bronchiectasis (1–2%) (Chalmers and Hill, 2013).

1.1.2.8. Inflammatory bowel disease

Inflammatory bowel disease may occasionally be a cause of bronchiectasis in 1–2% of the cases (Chalmers and Hill, 2013; Desai et al., 2011). This disease comprises a variety of inflammatory conditions of the colon and small intestine. The main types of inflammatory bowel disease are Crohn's disease and ulcerative colitis. This disease causes inflammation and narrowing of the airways, which may involve specific or diffuse areas. Pronounced inflammation can sometimes cause marked airway obstruction. These conditions may be amenable by treatment with inhaled and oral corticosteroids.

1.1.2.9. Congenital airway structural abnormality

Bronchiectasis may also be a result of several abnormal bronchoalveolar developments, including bronchopulmonary sequestration, Mounier-Kuhn syndrome and Williams-Campbell syndrome (Pappas et al., 2011). These pathologies are associated with bronchiectasis in 1–2% of the cases (Chalmers and Hill, 2013). Congenital airway structural abnormality results in chronic respiratory tract infections and dilatation of the airways which lead to bronchiectasis. Mounier-Kuhn syndrome is characterised by dilation of the trachea and segmental bronchi, which lead to repeated chest infections and bronchiectasis. Williams-Campbell syndrome is a disease of the airways where cartilage from lobar to first- and second-generation segmental airways is defective, resulting in extensive peripheral bronchiectasis.

1.1.2.10. Idiopathic bronchiectasis

Even with thorough clinical, pathologic and laboratory analysing, 30 to 53% of related cases of bronchiectasis have an idiopathic cause (Chalmers and Hill, 2013). However, the compartmentalisation of this group in specific conditions and

their understanding are far from being completely scrutinised. Therefore, in recent years, there has been an increase of microbiology studies of the lungs of non-CF bronchiectasis patients (Kapur et al., 2012; Tunney et al., 2013).

1.2. Microbiology of the lungs of bronchiectasis patients

A wide range of pathogens have been isolated from the lungs of bronchiectasis patients. In these patients, *Haemophilus influenzae* is the most common pathogen (range 29%–70%) followed by *Pseudomonas aeruginosa* (range 12%–33%) (Angrill et al., 2002; King et al., 2007). Chemical mediators released by *H. influenzae* and *P. aeruginosa* may also directly affect cilia function, reviling the ciliated epithelium and inhibiting the transport of mucus (Wilson et al., 1988). Bacterial pathogens release various proteins, including glycoproteins, which attract neutrophils. *H. influenzae* is able to cause direct injury in the airway epithelium by invasion of the bronchial wall and interstice of the lung (Möller et al., 1998).

P. aeruginosa has also high impact on the respiratory tract. It can form biofilms (Davies and Marques, 2009; Davies and Bilton, 2009) which occur primarily in the progressive disease and produce an impenetrable matrix (containing proteins and sugars) around the bacteria. This protects *P. aeruginosa* from the aggressively immune system and antibiotics, allowing the bacterial infection to cause severe damage to the underlying lung. As lung function decays, *P. aeruginosa* is the most commonly isolated pathogen in patients with the worst prognosis. Other microorganisms such as *Streptococcus pneumonia*, *Moraxella catarrhalis*, *Burkholderia cepacia* complex species and *Staphylococcus aureus* may be found in bronchiectasis patients.

S. pneumoniae is a Gram-positive bacterium which may cause otitis media, sinusitis or lower airway infections in bronchiectasis patients. Although this organism is rare in CF patients, in non-CF bronchiectasis patients it can be found in more than 37% of the cases. *S. pneumoniae* can use a wide range of molecules to adhere to airway cells and releases an Immunoglobulin A (IgA) protease and pneumolysin (a toxin) which may lead to invasion, inflammation and tissue injury (Kadioglu et al., 2008).

Another organism which is commonly found in the upper respiratory tract of these patients is *M. catarrhalis*. As previously described for *S. pneumoniae*, *M. catarrhalis* may cause otitis media or sinusitis. This bacterium is isolated in more than 27% of

chronic obstructive pulmonary disease (COPD) bronchiectasis patients; in other patients with bronchiectasis the frequency of isolation can be lower (Verduin et al., 2002). The first acquisition of *M. catarrhalis* does not cause bronchiectasis exacerbation. A longitudinal study of 50 patients with COPD suggested an association between second colonisation by *M. catarrhalis* and exacerbation cases (Murphy et al., 2005).

Burkholderia species and *S. aureus* are more rarely found in patients with bronchiectasis. *Burkholderia cepacia* complex species are Gram-negative rod-shaped bacteria with a prevalence of 3% in CF patients that have been associated with a high risk of developing fatal cepacia syndrome (Mahenthiralingam et al., 2005; Vanlaere et al., 2009). However, few cases are known in non-CF patients due to the small number of studies in these patients (Ledson et al., 1998). *S. aureus* is rarely related with airway infection, but may cause very aggressive post-influenzae infections. It is usually related with early infection in CF bronchiectasis, being less common in non-CF bronchiectasis (Pasteur et al., 2000). However, in non-CF bronchiectasis there is an association between *S. aureus* and ABPA (Shah et al., 1999).

Despite the huge diversity of microorganisms that can be present in bronchiectasis, *P. aeruginosa* remains the major focus of the scientific community when studying this disorder.

1.3. *Pseudomonas aeruginosa*

P. aeruginosa is a versatile bacterium, extensively recognised to cause serious infections on patients with CF and non-CF bronchiectasis. These bacteria are frequently found in association with animals and plants, as well as in distinct environmental samples (air, water or soil). This wide distribution implies a high genomic plasticity and the presence of several adaptation mechanisms. *P. aeruginosa* is not demanding in its nutritional requisites: it grows in minimal culture medium with simple molecules or even in deprived culture conditions (Higashi, 1960; Favero et al., 1971; Schreiber et al., 2007). This Gram-negative bacterium may produce various types of soluble pigments including pyoverdin (yellow-green and fluorescent), pyorubin (red-brown) and pyocyanin (blue-green). Pyoverdin is generously produced in low-iron medium content, pyorubin is also believed to be produced when the organism is under oxidative stress, while the latter is associated with suppurative infections

(Pollack, 2000). *P. aeruginosa* may grow in a broad range of environments including at extreme temperatures, although the optimal growth is observed at 37 ºC. Additionally, these bacteria present a very hierarchical quorum sensing which coordinates the formation of biofilms, swarming motility, exopolysaccharide production and cell aggregation (Rumbaugh et al., 2000). Indeed, the high genomic plasticity, the presence of several adaptation mechanisms, the ability to digest a wide range of components and the quorum sensing are some characteristics that allow the hostility of *P. aeruginosa* in bronchiectasis patients.

1.3.1. *Pseudomonas aeruginosa* acquisition in bronchiectasis patients

The acquisition of *P. aeruginosa* strains by patients can be done by several routes, due to the ubiquitous nature of this bacterium. Individuals may contact with the microorganism from different sources and even their homes may represent a reservoir for the bacteria. An interesting study on healthy individuals showed that more than one third of this population may carry *P. aeruginosa* in their airways during occasional acute viral respiratory infections (Johansen and Høiby, 1992). Healthy individuals fight the organism spontaneously, while patients with bronchiectasis cannot eliminate the bacterial colonisation. Therefore, non-bronchiectasis individuals with flu may be a source of viral and *P. aeruginosa* infections for those with bronchiectasis (van Ewijk et al., 2006). Hospital environment has been carefully monitored regarding the presence of *P. aeruginosa.* In what concerns patient cross-transmission, this occurrence has been accepted as uncommon (Govan and Deretic, 1996; Govan, 2000; Schmid et al., 2008); a similar strain of *P. aeruginosa* was only described in patients with close contact (Kelly et al., 1982; Grothues et al., 1988). A study on a hospital kitchen also showed the presence of *P. aeruginosa* in 82% of tomatoes and 27% of tomato salads, the food representing sometimes a risk for patients admitted at hospitals (Shooter et al., 1971; Kominos et al., 1973). In the case of drinking water, there are reports of contamination with *P. aeruginosa* in large dispensers (Baumgartner and Grand, 2006) as well as in commercial bottled water (Wilkinson and Kerr, 1998). *P. aeruginosa* is often found in intensive care units, particularly in washbasins, sinks and on the hands of the medical staff (Döring et al., 1991; Döring et al., 1993; Dwivedi et al., 2009). However, in intensive care units where precautions are taken

to avoid *P. aeruginosa* cross-infection (such as using gloves and disinfectants to clean material when moving between patients), there was no evidence that hospital environment was an important source of infection (Zembrzuska-Sadkowska et al., 1995). A study that investigated the frequency of contamination with *P. aeruginosa* at different sites of households of CF patients, collecting samples from bathroom, kitchen and toilet, detected *P. aeruginosa* strains in 72% of the samples; the highest percentage was reported on shower drains (Regnath et al., 2004). *P. aeruginosa* has been also detected on domestic dogs and cats that by contact may contribute to spread these bacteria among people more susceptible to bronchiectasis (Buma et al., 2006; Cole et al., 2006; Tolar et al., 2006). This bacterium is found in many aquatic environments, especially warm milieus containing organic material or material contaminated by human or animal waste (Botzenhart and Döring, 1993). Even though *P. aeruginosa* is not considered a marine organism, due to the high salt concentrations that inhibit its growth, there is some evidence suggesting that the *P. aeruginosa* can survive in seawater (Vasconcelos and Swartz, 1976). Swimming pools are often contaminated with the opportunistic pathogen *P. aeruginosa.* In 2007, the 50-metre pool at Cook and Phillip Park was closed in the City of Sydney due to the persistence of *P. aeruginosa* contamination (Rice et al., 2012). A definite hazard for bronchiectasis patients are hydrotherapy pools, jacuzzis and hot tubs. Hot tub folliculitis, green nail syndrome, infective endocarditis, invasive external ear infections and puncture wound osteomyelitis are some of the acquired infections reported from these sources (Gregory and Schaffner, 1987). Nevertheless, these places are usually safe when the recommended programmes of maintenance (monitoring of pH and total dissolved solids, addition of liquid chlorine, etc.) are carried out. Soil may also be a reservoir for *P. aeruginosa*; it was documented that 24% of soil samples from many areas in California contained *P. aeruginosa* (Green et al., 1974).

1.3.2. *Pseudomonas aeruginosa* colonisation in bronchiectasis patients

After lung invasion, *P. aeruginosa* must adhere to the host in order to establish a successful colonisation and resist the onslaughts of the immune system. In patients with stable bronchiectasis the airways are colonised by this bacterium in 5-31% of the cases (Nicotra et al., 1995; Pasteur et al., 2000; Angrill et al., 2002;

King et al., 2007). Once acquired, *P. aeruginosa* can only be eradicated in the early stage of colonisation.

In healthy lungs, bacteria are partially removed by the mucociliary clearance mechanism. This mechanism functions to expel particles or microorganisms inhaled into an airway (Robinson and Bye, 2002). Some important characteristics for a good mucociliary clearance are the number of cilia, their activity and coordinated movement and the volume of airway surface liquid (Robinson and Bye, 2002; Tarran et al., 2006) (Figure 1 A). The airway surface liquid is composed by water, ions and macromolecules produced by surface epithelial cells and/or sub-mucosal glands (Tarran et al., 2006). In coordination with ciliated epithelium, the airway surface liquid conduces the foreign particles toward the pharynx where it is finally expectorated from the airways (through coughing and sneezing). In bronchiectasis patients this mechanism fails and *P. aeruginosa* can easily adhere to bronchiectasis mucin (Nelson et al., 1990). This adherence cannot be possible without the flagellar cap protein FliD (Arora et al., 1998). Other cell components help the adherence process to the mucin such as lipopolysaccharides (Pier et al., 1997), flagellin (Feldman et al., 1998) and pili (Saiman and Prince, 1993). The adherence process is important for bacterial colonisation and subsequent infection.

There are three forms of *P. aeruginosa* that may colonise the lungs of bronchiectasis patients (Norez et al., 2009; Sonnleitner et al., 2009). In a first step, rough and smooth forms colonise the lung, which may be eradicated by early aggressive antibiotic therapy. When the treatment is not effective, a new form colonises the lung, the mucoid form, which may result from the conversion of previous colonies or from the acquisition of a new bacteria from environment (Vernez et al., 2005). The mucoid *P. aeruginosa* cannot be easily eradicated by antibacterial therapy and can persist continuously in the lungs (Mena et al., 2008). Moreover, the immune system of patients appears to overreact to all types of these bacteria by chemotactic stimuli (Strieter et al., 1993). This response includes the recruitment of macrophages and neutrophils (Figure 2). Activated neutrophils release oxygen radicals and proteolytic enzymes to exterminate bacteria, which also injury epithelium and protective proteinase inhibitors. The macrophages and airway epithelial cells release some substances such as InterLeukin-8 (IL-8) and Tumor Necrosis Factor (TNF), which influence cell migration. However, bacteria may not be fully eradicated, resulting in chronic inflammation and infection (Mikami et al., 1998; Stockley, 1998). As consequence of bacterial presence, the tissue is damaged and eventually the

bacteria can destroy a part of the lung. A mucoid type of *P. aeruginosa* is indicative of the overproduction of alginate, a capsule-like polysaccharide. This capsule is a barrier to phagocytosis, a mechanism for bacterial adherence that neutralises oxygen radicals, which make this polysaccharide an important virulence factor encountered primarily in CF (Bragonzi et al., 2005; Hassett et al., 2009).

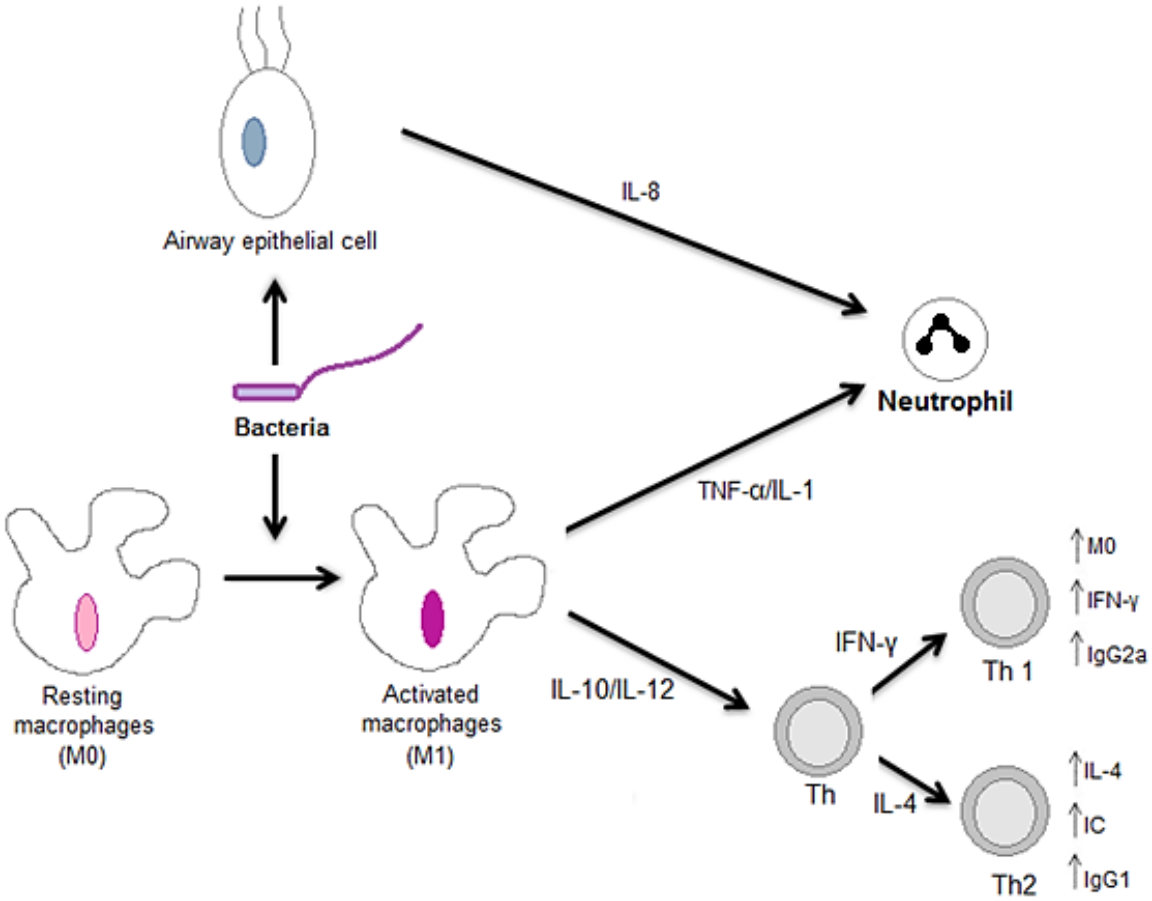

Figure 2. Involvement of innate and adaptive immunity in bronchiectasis. Chemotactic bacterial stimuli activate both macrophages (M0) and airway epithelial cells, which in turn produce InterLeukin 8 (IL-8), Necrosis Factor-α (TNF-α) and InterLeukin 1 (IL-1). These primary pro-inflammatory mediators are responsible for neutrophil activation and recruitment into the airways. Finally, InterLeukin 10 (IL-10) and InterLeukin 12 (IL-12) secreted by activated M0 favour the development of T helper 1 (Th1) or a T helper 2 (Th2) responses. IC - Immune Complexes; IFN- γ - Interferon-γ; IgG - Immunoglobulin G; IL-4 - InterLeukin 4; M1 - Activated macrophages. Adapted from Conese et al., 2003.

1.4. Genomic diversity of *Pseudomonas aeruginosa*

The colonisation of bronchiectasis patients and phenotypic exchange of *P. aeruginosa* on this environment would not be possible without a very plastic genome. The *P. aeruginosa* genome consists of a single circular chromosome composed of a conserved core genome and a changeable flexible genome (Figure 3). At present, there are 12 complete genomes accessible in online databases (National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), Pseudomonas Genome Database (http://www.pseudomonas.com/) and Ensembl (http://www.ensembl.org/index.html). The size of the various *P. aeruginosa* genomes is variable, ranging from 6 Mb to 7 Mb (Schmidt et al., 1996; Stover et al., 2000). This fluctuation is responsible for the genetic diversity that arises and is preserved through interchange between genetic and ecological factors (Spiers et al., 2000). The essential genes, which are indispensable to support cellular life, are normally more conserved than nonessential (Dotsch et al., 2010). At the core genome where housekeeping genes are found studies by Kiewirtz and Tümmler revealed an average rate of sequence polymorphism of 0.3% (Kiewitz and Tummler, 2000). The same authors reported less than 0.5% of substitution rates in a restricted group of conserved genes in the *P. aeruginosa* genome. Genes involved in flagellar biosynthesis and genes whose products are involved in the biosynthesis of the siderophore pyoverdine and the receptor for ferripyoverdines were the highest polymorphic genes at the core genome (Tsuda et al., 1995; Lehoux et al., 2000).

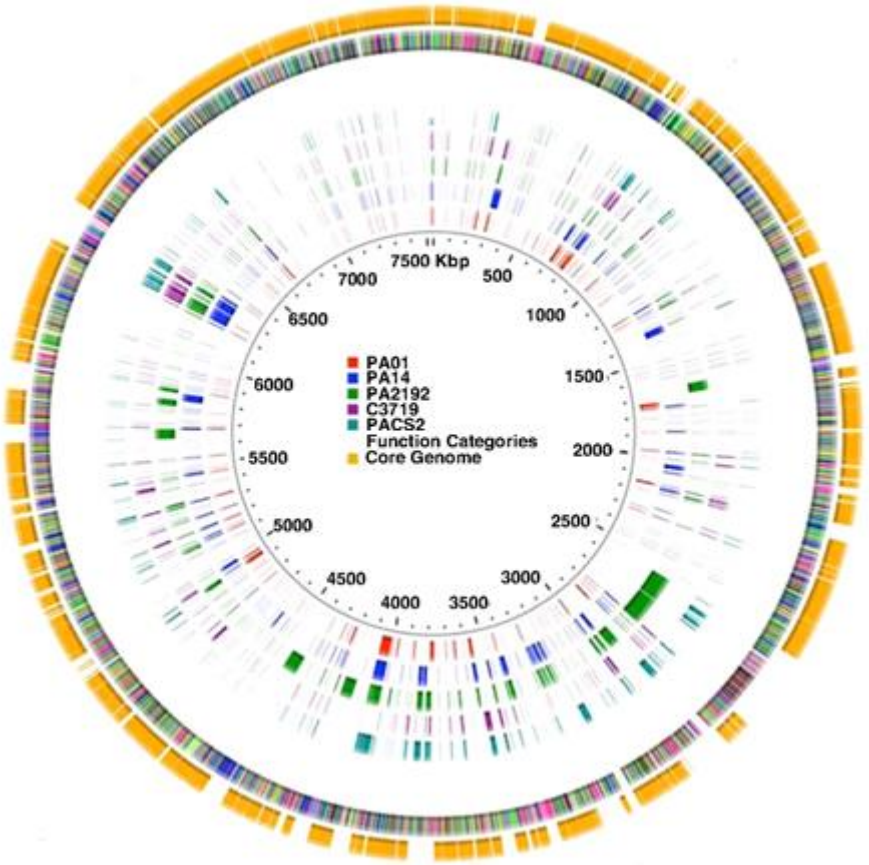

Figure 3. *Pseudomonas aeruginosa* genomes comparison. The gold circle represents the core genome, the second circle shows the functional annotations. The remaining circles indicates the accessory genes from PA14 (blue) PA2192 (green), C3719 (purple), PAO1 (red), and PACS2 (teal). Adapted from Mathee et al., 2008.

In contrast, the flexible genome includes genes that are found in more particular populations of *P. aeruginosa*. These fragments are clustered in certain loci and not dispersed randomly throughout the core genome. These loci are located in many regions of genomic plasticity which are usually called genomic islands (>10 kb) or islets (<10 kb) (Mathee et al., 2008). The genes of the accessory genome may encode proteins and RNAs that contribute to the adaptation of bacteria to specific niches (Kung et al., 2010). Additionally, plasmids constitute an important part of the *P. aeruginosa* gene pool, particularly the multidrug-resistant plasmids (Shahid et al., 2003).

Genomic diversity of *P. aeruginosa* may be a consequence of various molecular events, including deletions, insertions, inversions and point mutations. The genomic variation of the strain PAO1 has been repeatedly reported during *in vitro* sub-culturing. The strain PAO1 sequenced by Stover (2000) presented an inversion of 25% of the genome relative to DSM-1707, another PAO1-derived isolate previously

mapped by Schmidt (1996 and 1998). Both strains are clonally derived from the same ancestor (Holloway et al., 1994), and such differences arised during the sub-culturing processes. The strain PAO1 differs from the original PAO by 1.7 Mbp inversion between *rrnA* and *rrnB* loci, which are orientated in opposite directions, and a deletion of approximately 20 kbp near to *rrnC* locus. These events do not appear to be isolated; PAO1 stocks from other laboratories have the same inversion. It was also found that two different stocks present a defective LasR function due to point mutations in the *lasR* genes (Heurlier et al., 2005).

Chronic colonisation of *P. aeruginosa* in the respiratory tract of bronchiectasis patients is characterised by genome mosaicism (presence of genomic islands obtained through horizontal transfer of genes), however, it is not reflected by readily visible phenotypes (Ernst et al., 2003). Comparison among complete genomes (Spencer et al., 2003) has revealed that some of the gene islands present high number of single nucleotide polymorphisms (SNPs). Large deletion events (of more than 100 kbp) are also found when compared two different *P. aeruginosa* isolates obtained during bacterial evolution process in the bronchiectasis lung (Ernst et al., 2003). In addition, the human airways do not only predispose to extreme changes of DNA framework but also to possible recombination events in single genes (Pirnay et al., 2002). Strains with high levels of mutation rates possibly drive the acquisition and fixation of mutations in *P. aeruginosa* populations of bronchiectasis patients (Oliver et al., 2000; Maciá et al., 2005). Using these genetic mechanisms, *P. aeruginosa* may easily adapt to fit and survive in the different environments. One example of genome flexibility is the acquisition of a large cluster of genes involved in diterpenoid metabolism by the PA2192 strain that allow the adaptation to environments rich in abietane diterpenoid resins (Mathee et al., 2008); this strain later colonised and infected a CF patient in the United States. Thus, the acquisition of new genetic elements appears to be conditioned by nutritionally limitations of *P. aeruginosa* strains. Correct identification and characterisation of *P. aeruginosa* may possibly improve the selection of appropriate therapy and prevent the development of multidrug-resistant bacteria.

1.5. Identification of *Pseudomonas aeruginosa*

Correct diagnosis and treatment of *P. aeruginosa* is severely hampered by the absence of a reliable identification system. A wide range of methods are described for *P. aeruginosa* identification, including conventional methods (e.g. morphology and biochemical tests), indirect molecular strategies (e.g. MALDI-TOF MS system) and direct molecular strategies involving PCR and sequencing analysis. However, here we will focus our attention in conventional and direct molecular strategies for identification.

1.5.1. Conventional identification

Culture selective media have been long used for identification of various bacteria including *P. aeruginosa.* A basic medium, containing components such as magnesium chloride, potassium sulphate, protein hydrolysate and agar, is enough for the identification. Some examples are Cetrimide, phenanthroline and 9-chloro-9-[4-(diethylamino)phenyl]-9,10-dihydro-10-phenylacridine hydrochloride and chromogenic media (Brown and Lowbury, 1965; Fujita et al., 1992; Laine et al., 2009). Nevertheless, for a more safe identification at the species level, other characteristics such as pyocyanin pigment formation, proteolytic activity, nitrate utilisation, lipolytic activity and glutamate utilisation need to be evaluated (Franzetti and Scarpellini, 2007). However, conventional identification based on serological, biochemical and culture on selective media are not practical methods for routine use (Busse et al., 1996). Additionally, misidentifications are commonly observed due to erroneous phenotypic descriptions influenced by cultivation conditions.

1.5.2. Direct molecular identification

New alternatives have been drawn and, in the last few years, molecular methods have been extensively used in bacterial identification. Molecular methods allow the definition of phylogenetic relationships among the organisms. However, the bacterial species concept remains under discussion in the scientific community. Bacterial species are usually defined based on the combination of several criteria (Vandamme et al., 1996; Stackebrandt et al., 2002). The criterion suggested by Wayne et al. (1987) has been widely used and still remains a reference. Wayne et al. state that an unknown strain is

considered to be a member of the same reference species when it shows 70% or greater DNA-DNA hybridization values. The above value for DNA–DNA hybridization grossly corresponds to 97% *16S rRNA* sequence similarity (Brenner et al., 2001). Such relatedness must be employed for establishing relationships between microorganisms and phylogenetic analyses and surely allows outstanding classification of bacteria up to the genus level.

1.5.2.1. *16S rRNA*

16S rRNA has been used for species identification (Wagner et al., 2008) as well as to *Pseudomonas* genus phylogenetic affiliation (Anzai et al., 2000; Purohit et al., 2003). One of the earliest studies based on the comparison of *16S rRNA* sequences on *Pseudomonas* species grouped these bacteria into two groups: *P. fluorescens* and *P. aeruginosa* (Moore et al., 1996). Afterward, a study on more than 100 *Pseudomonas* species based on *16S rRNA* sequence comparison, proposed the division of *Pseudomonas* species into seven groups (Figure 4) (Anzai et al., 2000). In the study, *P. aeruginosa* was proposed to be included in *P. aeruginosa* group along with *Pseudomonas anguilliseptica*, *Pseudomonas resinovorans*, *Pseudomonas alcaligenes*, *Pseudomonas citronelloli*, *Pseudomonas nitroreducens*, *Pseudomonas oleovorans*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas mendocina*, *Pseudomonas flavescens* and *Pseudomonas straminea*. Although the sequencing of this gene is commonly used, discussion on the deprived resolution of the phylogenetic analysis with *16S rRNA* sequences lead to using other genes to characterise and classify *Pseudomonas* species (De Vos et al., 1998; Yamamoto et al., 2000; Bodilis et al., 2004; Ait Tayeb et al., 2005).

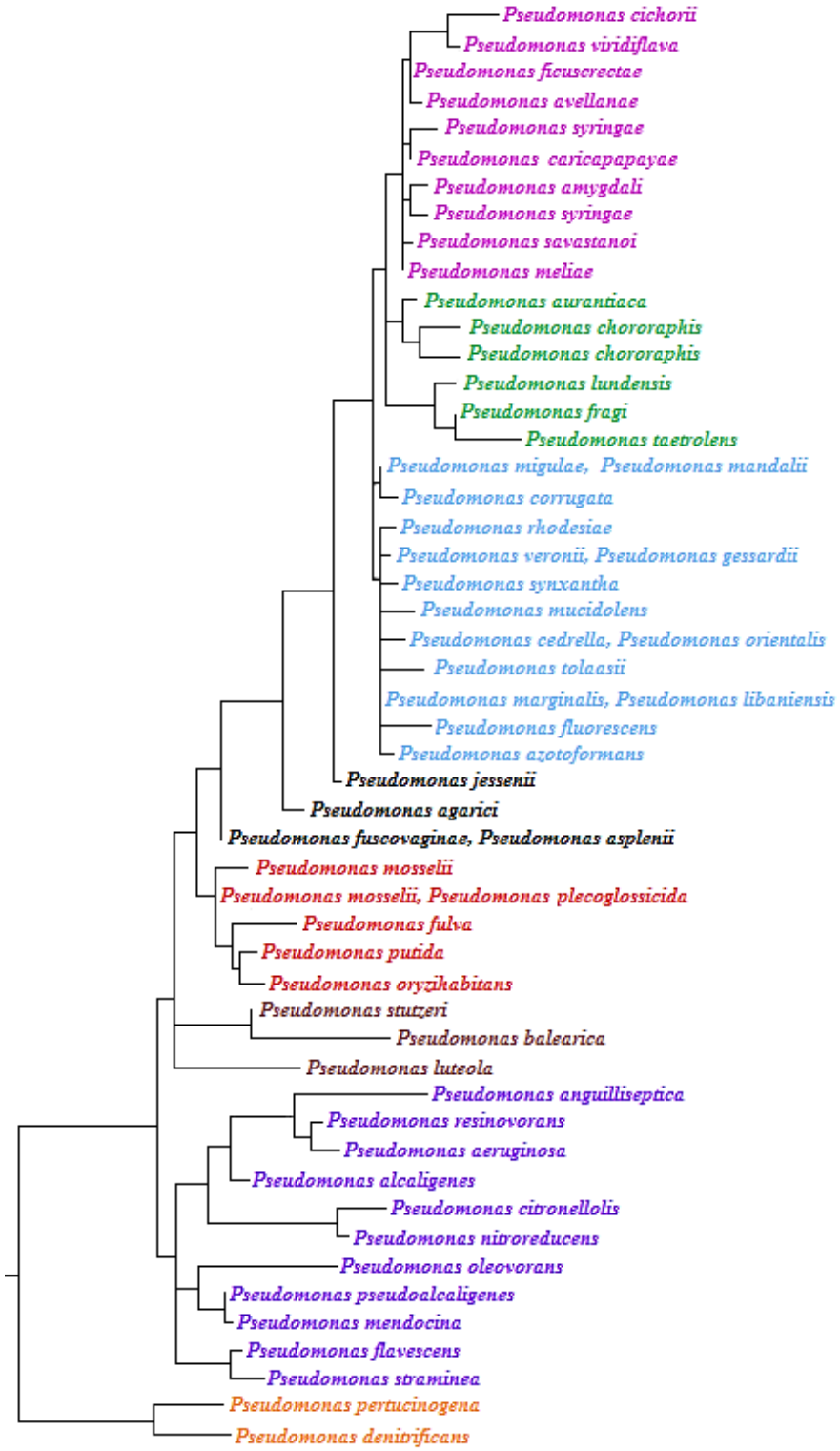

Figure 4. Phylogenetic tree of the *Pseudomonas* species based on *16S rRNA* gene sequence. The colours represent the different groups defined by Anzai (2000): pink - *Pseudomonas syringae* group; green - *Pseudomonas chlororaphis* group; blue - *Pseudomonas fluorescens* group; red - *Pseudomonas putida* group; brown - *Pseudomonas stutzeri* group; purple - *Pseudomonas aeruginosa* group; and orange – *Pseudomonas percinogena* group. *Pseudomonas jessenii, Pseudomonas agarici. Pseudomonas fuscovaginae* and *Pseudomonas asplenii* are strains that do not belong to any previously defined group. Adapted from Anzai et al., 2000.

1.5.2.2. Multilocus sequence analysis

The genes *rpoB*, *gyrB* and *rpoD* are good alternatives to *16S rRNA* gene for identification of *P. aeruginosa* (Yamamoto et al., 2000; Ait Tayeb et al., 2005). These genes present higher number of base substitutions than *16S rRNA* gene. The concatenated of multiple genes significantly increases the discriminating power of molecular identification methodologies.

Multilocus sequence analysis (MLSA) has been suggested as excellent alternative to DNA-DNA hybridisation and *16S rRNA* (Martens et al., 2008). It allows a phylogenetic characterisation using allele sequences of various genes, which permits the delineation of several species. This tool is capable of yielding sequence sets at an extensive range of taxonomic levels, from intraspecific through the species level to sets at higher levels. Frapolli used ten housekeeping genes for evaluating the phylogeny of some *Pseudomonas* species (Frapolli et al., 2007). A better definition and delimitation of the various species requires a consistent understanding of the breadth and the genetic differences between closely related forms (Konstantinidis and Tiedje, 2005; Almeida and Araujo, 2013). Thereby, it is necessary an extensive characterisation of several bacterial groups at high resolution level.

1.6. Genotyping of *Pseudomonas aeruginosa*

Genotyping plays an important role to examine the widest range of genetic variation among bacteria. Through genotyping it is possible to monitor the evolution, determine the source of infection, recognise disease outbreaks as well as detect cross-transmission of pathogens.

Bacterial typing has been performed by different molecular methods providing measures of genetic diversity (Li et al., 2009). Some examples of these methods are: enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD). These three methods capture a genome-wide perspective on diversity between strains by employing different approaches (Kidd et al., 2010; Waters et al., 2012). Methods, such as ERIC-PCR and RAPD, have the advantage of being practical, fast, and consequently more amenable to high-throughput typing than others, such as PFGE. PCR-based methods may also be developed to a rapid diagnosis of

epidemic clonal strains (Lewis et al., 2005; Johnson et al., 2007; Williams et al., 2010a). One downside of these methods is that the number of *P. aeruginosa* strains with the same profile may be overestimated using ERIC-PCR and RAPD, thus these methodologies presenting lower discriminatory power. Therefore, an additional typing methodology may be required to confirm the relationships between indistinguishable types. PFGE is a molecular fingerprinting technique based on restriction sites within the bacterial genome and presents a discriminatory power of 0.980. Nevertheless, the equipment required for PFGE is extremely expensive and the time spent and reproducibility represent main concerns for the method.

1.6.1. Multilocus sequence typing

Multilocus sequence typing (MLST) of bacteria has become well-known and nowadays there are more than 40 developed schemes for bacterial genotyping. This method represents a powerful tool that allows genotyping microbial isolates (Curran et al., 2004; Baldwin et al., 2005) and population structure studies (McManus et al., 2008). Additionally, MLST also may occasionally identify evidence of recombination by the presence of multiple nucleotide substitutions in close related isolates or the occurrence of the same alleles in unrelated isolates within the same data set (Bolt et al., 2010). This technique compares sequences of nucleotides between 400-500 bp usually of five to seven housekeeping genes (Li et al., 2009). MLST combines several advantages, including reproducibility, the production of unambiguous datasets and the possibility to compare and transfer data between different laboratories worldwide through a shared central database (http://www.mlst.net/) (Jolley et al., 2004; Griffiths et al., 2010). In 2004, MLST was applied to *P. aeruginosa* by Curran et al. (2004). On this scheme seven housekeeping genes of *P. aeruginosa* were selected for genotyping this species. Partial gene sequences of acetyl coenzyme A synthetase (*acsA*), shikimate 5-dehydrogenase (*aroE*), guanosine 5'-monophosphate synthase (*guaA*), DNA mismatch repair protein (*mutL*), bifunctional NADH:ubiquinone oxidoreductase subunit C/D (*nuoD*), phosphoenolpyruvate synthase (*ppsA*) and anthranilate synthase component I (*trpE*) were selected. Nowadays, the information of about 1,500 *P. aeruginosa* strains is easily available online at the MLST website. *P. aeruginosa* MLST presents a high discriminatory power (above 0.975) and recently it has become an important alternative to the previous gold standard method for bacteria genotyping, the PFGE. However, the applicability of MLST is still limited to small collections due to the high cost and time

spent to complete the task. Hence, it is necessary to develop alternative methods that are able to facilitate such studies and to boost bacterial genotyping by allowing large-scale analysis. In this context, the use of informative SNPs has been recently proposed as an alternative to the MLST.

1.6.2. Single Nucleotide Polymorphisms

The divergence between *P. aeruginosa* genomes is based mostly in SNP variation (Spencer et al., 2003). This form of genetic variation has a maximum of four alleles for each base and can be explored to study the diversity and dynamics of many populations, by selecting and combining several different SNPs simultaneously (Schork et al., 2000). SNP-based approaches use a range of genetic targets, similarly to MLST, and it has been demonstrated that SNP analysis combined with sequencing of short variable regions presents a similar discriminatory power as MLST analysis (Fothergill et al., 2010). Additionally, SNPs can be found very close to each other and the assessment of linkage disequilibrium may expose sites for recurrent mutation, gene conversion or even spots for recombination.

Few SNP-based methods have been suggested for genome analysis of *P. aeruginosa*, including AT biochips, microarray typing device, High Resolution Melting curve-based SNP typing profile assay and *SNaPaer*. AT biochips (Hansen et al., 2012) are a good alternative for time consuming methods, such as MLST, but they are limited by biochip fabrication steps as well as the expensive equipment necessary for the operation of this method. Another methodology based on microarray typing device was developed to analyse the population genetics of *P. aeruginosa* (Wiehlmann et al., 2007). This portable methodology analyses 16 SNPs of the core genome and 38 SNPs of flexible genome. However, this assay is based in a binary system, thus, if a different SNP appears wrong conclusions may be taken. High Resolution Melting curve-based SNP typing profile assay was developed by Anuj et al. (2011) to identify major clonal strains presented in CF patients of Brisbane, Australia. However, a previous study of High Resolution Melting revealed that this method incorrectly identified negative control samples, suggesting a problem with non-specific fluorescence (Vezenegho et al., 2009).

SNaPaer is a very recent approach that has been developed for genotyping of *P. aeruginosa* (Eusebio et al., 2013). Although the approach has been recently developed

for *P. aeruginosa*, it is based on the SNaPshot mini-sequencing technique which has been previously used in multiple organisms and with different objectives, such as comparison of strains (Huang et al., 2012), serotypes (Ben-Darif et al., 2010), virulent and epidemic clones (Lomonaco et al., 2011), phylogenetic related sub-groups of isolates (Huang et al., 2011), as well as identification of various species (Kristensen et al., 2007; Bouakaze et al., 2010; Wang et al., 2010).

1.6.3. *SNaPaer* - SNaPshot mini-sequencing scheme

SNaPaer assay is a useful and low-cost molecular method that uses a set of 23 polymorphisms for a rapid genotyping of *P. aeruginosa*. The 23 neutral genetic polymorphisms are located on the seven housekeeping genes suggested for *P. aeruginosa* MLST and represent a theoretical number of combinations of up 15 billion (Eusebio et al., 2013). *SNaPaer* assay employs SNaPshot™ kit commercialised by Applied Biosystems, which allows that multiple reactions be performed simultaneously (Figure 5). Mini-sequencing follows the amplification of the seven housekeeping genes and involves the addition of an extension primer (or a set of primers) with a tail in 5' extreme (one base upstream to the target SNP). Then, a single base extension reaction is executed in the presence of the fluorescently labeled ddNTPs that are excited with a laser. The use of primers with different tails in 5' extreme allows the recognition of multiple fragments from extension reaction. The fluorescence-labelled primer extension product is subsequently separated by capillary electrophoresis and analysed. As result, it is constructed a panel with peaks of different colour and length. The colour of the peak represents the nucleotide site-specific variation; the location corresponds to the size of the fragment, which is associated with the locus and position of the SNPs. To the ordered set of 23 SNPs (in accordance with MLST data sets) we gave the name of *SNaP* profile. Some of the biggest advantages of mini-sequencing include the possibility of using very small PCR products (thereby markers may work with extremely degraded DNA samples), the absence of stutter products and the flexibility to accept the replacement or addition of new markers.

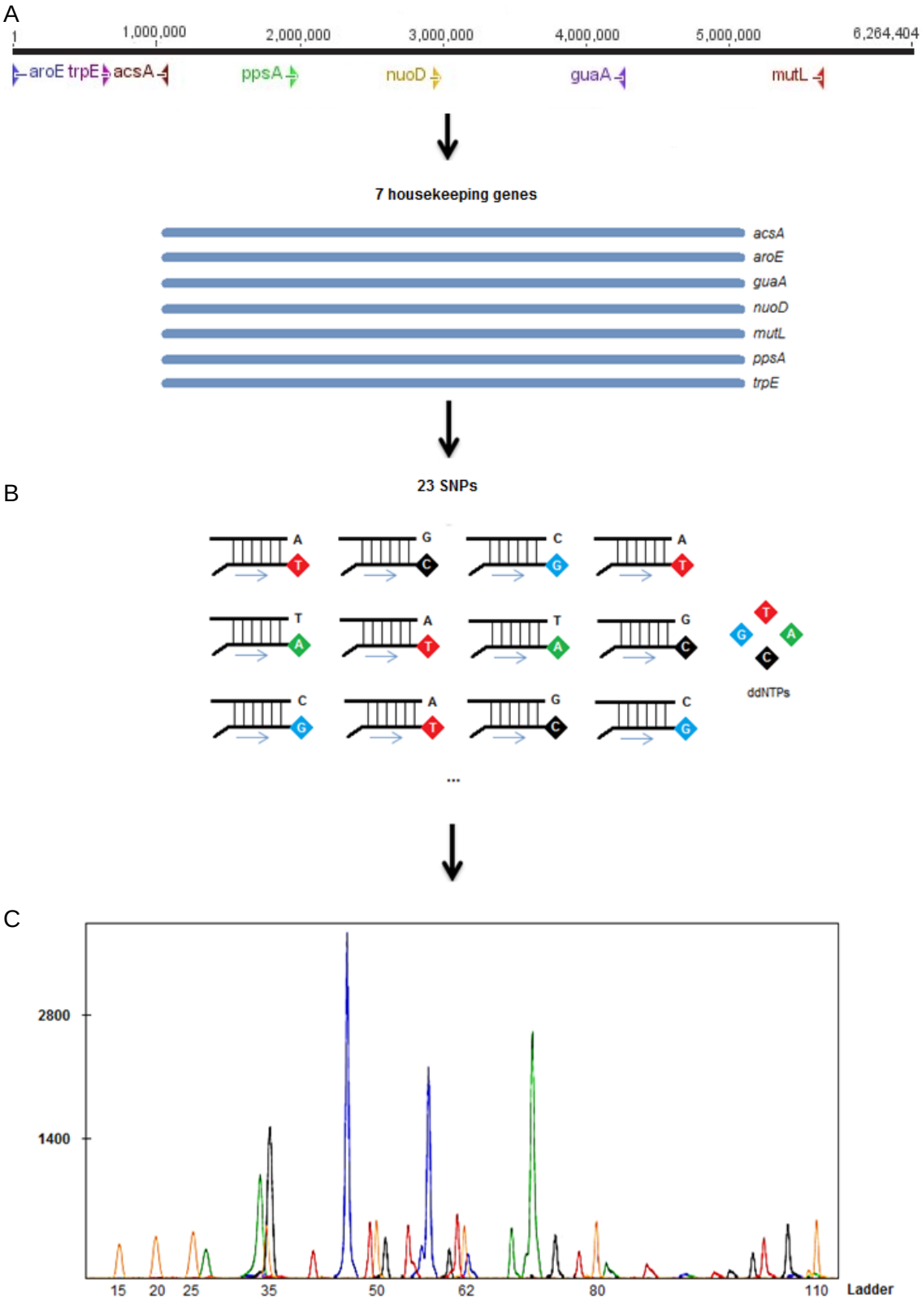

Figure 5. *SNaPaer* genotyping for *Pseudomonas aeruginosa*. *SNaPaer* uses SNaPshot technology, which involves multiplexed amplification of the seven housekeeping genes in a single multiplex reaction (A), multiplexed single-base primer extension with fluorescently-labelled ddNTPs of 23 SNPs in a single mini-sequencing assay (B), and analysis of labelled-primer extension products by capillary electrophoresis (C).

2. Aims

Some studies have already described the evolution, diversity and mutation rates for *P. aeruginosa* collected from CF patients (Huse et al., 2010; Mowat et al., 2011; Chung et al., 2012). Nevertheless, few studies have been reported in non-CF bronchiectasis and many questions still remain unanswered. Is *P. aeruginosa* adapting similarly in the lung of chronic non-CF and CF bronchiectasis patients? What happens to bacterial populations in the lung that leads to exacerbations (acute condition) in patients with bronchiectasis? What is the diversity of *P. aeruginosa* strains in patients with bronchiectasis and what are the consequences of such diversity? Is therapy altering the diversity of strains in the bronchiectasis lung?

It is imperative to define the clinical situations that can benefit from the application of molecular methods to a fast and robust diagnosis. *SNaPaer* assay was developed for genotyping of *P. aeruginosa* and the specificity of the multiplex should be high due to presence of the 23 loci. Is this method also capable to identify these bacteria in control and clinical samples?

The present study aims to:

I. test the taxonomic power of MLST sequences and *SNaPaer* assay to identify *P. aeruginosa* amongst related species;

II. identify and genotype *P. aeruginosa* in clinical samples collected from patients with bronchiectasis;

III. compare *P. aeruginosa* colonisation in patients diagnosed with non-CF and CF bronchiectasis.

3. Materials and methods

3.1. Phylogeny of *Pseudomonas* genus based on multilocus sequence typing data

The MLST scheme has previously been shown to be useful for species identification (Maiden, 2006). Here, we proposed to test the capability of the MLST method for *Pseudomonas* species identification. Therefore, 50 complete genomes of *Pseudomonas* species available at NCBI database were downloaded to an in-house record (Table 1). The sequences of the genes *acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE* similar to *P. aeruginosa* MLST were identified in each genome using Geneious Pro 6.0.6 software. Gene *nuoD* could not be found in the genomes of the following strains: *Pseudomonas stutzeri* A1501, *Pseudomonas stutzeri* ATCC 17588, *Pseudomonas stutzeri* CCUG, *Pseudomonas stutzeri* DSM and *Pseudomonas stutzeri* RCH2. Therefore, these genomes were excluded in some of the phylogenetic analyses. MLST genes were aligned in Geneious, using Clustal W (Thompson et al., 1994). Phylogenetic trees were constructed using the neighbourjoining (NJ) method (Saitou and Nei, 1987) and the genetic distances were computed using the Maximum Composite Likelihood model. Bootstrap values were calculated for tree nodes.

The 23 SNPs (*SNaP* profile) suggested by Eusebio et al. (2013) for genotyping of *P. aeruginosa* were identified in the MLST sequences for the 45 *Pseudomonas* species genomes. The resulting set of 45 *SNaP* profiles was included in a database in addition to the group of 86 *SNaP* profiles obtained from a previous study of Eusebio et al. (2013). Subsequently, all *SNaP* profiles were converted into *preMLST* sequences using MLST@SNaP software (Soares and Araujo, 2013). Finally, the phylogenetic trees were

reconstructed and compared with previously reported phylogenetic trees (Anzai et al., 2000; Yamamoto et al., 2000; Ait Tayeb et al., 2005).

Table 1. List of genomes of *Pseudomonas* strains analysed in this work, downloaded from the NCBI database.

Name	Group*	Accession	Length
***Chromohalobacter salexigens* DSM 3043**	Not applicable	NC_007963	3,696,649
***Pseudomonas aeruginosa* 19BR**	*Pseudomonas aeruginosa*	AFXJ01000001	6,742,964
***Pseudomona aeruginosa* 213BR**	*Pseudomonas aeruginosa*	AFXK01000001	6,719,211
***Pseudomonas aeruginosa* 39016**	*Pseudomonas aeruginosa*	NZ_CM001020	6,866,064
***Pseudomonas aeruginosa* 9BR 9BRScaffold1**	*Pseudomonas aeruginosa*	AFXI01000001	6,801,503
***Pseudomonas aeruginosa* DK2**	*Pseudomonas aeruginosa*	CP003149	6,402,658
***Pseudomonas aeruginosa* LESB58**	*Pseudomonas aeruginosa*	NC_011770	6,601,757
***Pseudomonas aeruginosa* M18**	*Pseudomonas aeruginosa*	NC_017548	6,327,754
***Pseudomonas aeruginosa* NCGM2,S1 DNA**	*Pseudomonas aeruginosa*	AP012280	6,764,661
***Pseudomonas aeruginosa* PA7**	*Pseudomonas aeruginosa*	NC_009656	6,588,339
***Pseudomonas aeruginosa* PACS2**	*Pseudomonas aeruginosa*	AAQW01000001	6,492,423
***Pseudomonas aeruginosa* PAO1**	*Pseudomonas aeruginosa*	AE004091	6,264,404
***Pseudomonas aeruginosa* UCBPP-PA14**	*Pseudomonas aeruginosa*	CP000438	6,537,648
***Pseudomonas brassicacearum subsp, brassicacearum* NFM421**	Not applicable	NC_015379	6,843,248
***Pseudomonas chlororaphis* O6**	*Pseudomonas chlororaphis*	NZ_CM001490	6,980,251
***Pseudomonas chlororaphis subsp, aureofaciens* 30-84**	*Pseudomonas chlororaphis*	NZ_CM001559	6,666,321
***Pseudomonas entomophila* L48**	*Pseudomonas putida*	NC_008027	5,888,780
***Pseudomonas fluorescens* F113**	*Pseudomonas fluorescens*	CP003150	6,845,832
***Pseudomonas fluorescens* Pf0-1**	*Pseudomonas fluorescens*	CP000094	6,438,405
***Pseudomonas fluorescens* SBW25**	*Pseudomonas fluorescens*	NC_012660	6,722,539
***Pseudomonas fluorescens* SS101**	*Pseudomonas fluorescens*	AHPN01000001	6,179,443
***Pseudomonas fluorescens* A506**	*Pseudomonas fluorescens*	NC_017911	5,962,570
***Pseudomonas fluorescens* Q2-87**	*Pseudomonas fluorescens*	NZ_CM001558	6,368,168
***Pseudomonas fluorescens* Q8r1-96**	*Pseudomonas fluorescens*	NZ_CM001512	6,602,611
***Pseudomonas fluorescens* R124**	*Pseudomonas fluorescens*	NZ_CM001561	6,256,692
***Pseudomonas fluorescens* WH6**	*Pseudomonas fluorescens*	NZ_CM001025	6,270,867
***Pseudomonas fulva* 12-X**	*Pseudomonas putida*	NC_015556	4,920,769
***Pseudomonas mendocina* NK-01**	*Pseudomonas aeruginosa*	NC_015410	5,434,353
***Pseudomonas mendocina* ymp**	*Pseudomonas aeruginosa*	NC_009439	5,072,807
***Pseudomonas protegens* Pf-5**	Not applicable	CP000076	7,074,893
***Pseudomonas putida* CSV86**	*Pseudomonas putida*	NZ_AMWJ01000000	6,469,780
***Pseudomonas putida* DOT-T1E**	*Pseudomonas putida*	NC_018220	6,260,702
***Pseudomonas putida* GB-1**	*Pseudomonas putida*	CP000926	6,078,430
***Pseudomonas putida* KT2440**	*Pseudomonas putida*	NC_002947	6,181,863
***Pseudomonas putida* ND6**	*Pseudomonas putida*	NC_017986	6,085,449
***Pseudomonas putida* UW4**	*Pseudomonas putida*	NC_019670	6,183,388
***Pseudomonas putida* BIRD-1**	*Pseudomonas putida*	NC_017530	5,731,541
***Pseudomonas putida* F1**	*Pseudomonas putida*	NC_009512	5,959,964
***Pseudomonas putida* HB3267**	*Pseudomonas putida*	NC_019905	5,875,750
***Pseudomonas putida* S16**	*Pseudomonas putida*	NC_015733	5,984,790
***Pseudomonas putida* W619**	*Pseudomonas putida*	NC_010501	5,774,330
***Pseudomonas stutzeri* A1501**	*Pseudomonas stutzeri*	NC_009434	4,567,418
***Pseudomonas stutzeri* CCUG 29243**	*Pseudomonas stutzeri*	NC_018028	4,709,064
***Pseudomonas stutzeri* DSM 10701**	*Pseudomonas stutzeri*	NC_018177	4,174,118
***Pseudomonas stutzeri* DSM 4166**	*Pseudomonas stutzeri*	NC_017532	4,689,946
***Pseudomonas stutzeri* ATCC 17588 = LMG 11199**	*Pseudomonas stutzeri*	NC_015740	4,547,930
***Pseudomonas stutzeri* RCH2**	*Pseudomonas stutzeri*	NC_019939	4,575,057
Pseudomonas synxantha BG33R	*Pseudomonas fluorescens*	NZ_CM001514	6,297,495
Pseudomonas syringae pv, phaseolicola 1448A	*Pseudomonas syringae*	NC_005773	5,928,787
***Pseudomonas syringae pv, syringae* B728a**	*Pseudomonas syringae*	NC_007005	6,093,698
***Pseudomonas syringae pv, tomato str,* DC3000**	*Pseudomonas syringae*	AE016853	6,397,126

* Suggested by Anzai et al., 2000.

3.2. *SNaPaer* assay for identification of *Pseudomonas aeruginosa*

MLST gene amplification was tested in various strains of *Pseudomonas* species, including 12 clinical strains of *P. aeruginosa* from our collection and *P. putida* KT2440, *P. fluorescens* Pf0-1, *P. savastanoi pv. glycinea* 5066, *P. savastanoi pv. phaseolicola* 2245, *P. syringae pv. helianthi 5067*, *P. syringae pv. maculicola 5071*, *P. syringae pv. oryzae* 10912, *P. syringae pv. syringae* 10604, *P. syringae pv. tabaci* 5393 and *P. syringae pv. tomato* DC 3000. Two strains of *Xanthomonas*, *X. axonopodis pv. citri* 9322 and *X. campestris pv. campestris* 568, and six clinical strains of *Burkholderia cenocepacia* were also included. Some of these strains were provided by Doctor Fernando Tavares (Faculty of Sciences, University of Porto).

MLST was performed in 30 strains using the primers previously described by Eusebio et al. (2013) (Table 2). Multiplex touchdown PCR was conducted in a final volume of 5 µL, containing 2.5 µL of Qiagen multiplex PCR master mix (Qiagen), 1 µL of bacterial DNA, 0.5 µL of primer mix (each primer at 2 µM), 0.5 µL of Q-solution (Qiagen) and 0.5 µL of ultrapure water. Thermalcycling conditions were the following: denaturation for 15 min at 95 ºC; 3 cycles with denaturation for 1 min at 95 ºC, primer annealing for 30 s at 63 ºC and extension for 2 min at 72 ºC; 3 cycles with denaturation for 1 min at 95 ºC, primer annealing for 30 s at 61 ºC and extension for 2 min at 72 ºC; 3 cycles with denaturation for 1 min at 95 ºC, primer annealing for 30 s at 59 ºC and extension for 2 min at 72 ºC; 26 cycles with denaturation for 1 min at 95 ºC, primer annealing for 30 s at 57 ºC and extension for 2 min at 72 ºC; and final extension for 10 min at 72 ºC. Amplicon sizes were evaluated after separation by polyacrylamide gel electrophoresis and standard silver-staining detection (Qu et al., 2005).

Table 2. Primers used for *Pseudomonas aeruginosa* MLST.

Locus	Primer sequence (5' to 3')		Amplicon size (bp)
acsA	Forward*	CTGGTGTACGCCTCGCTGAC	837
	Reverse	TAGATGCCCTGCCCCTTGAT	
aroE	Forward	TATTCGGCAACCCCATCG	747
	Forward*	ACGATTTCCCCGGGTTC	642
	Reverse	CGCGCCAGAGGAAGAAT	
guaA	Forward*	CGGCCTCGACGTGTGGATGA	844
	Reverse	GAACGCCTGGCTGGTCTTGTGGTA	
mutL	Forward	AGCCTGGCAGGTGGAAAC	634
	Reverse*	CAGGGTGCCATAGAGGAAGTC	
nuoD	Forward*	ACCGCCACCCGTATCTG	1080
	Reverse	TCTCGCCCATCTTGACCA	
ppsA	Forward*	GGGTAGCAAGGCGATCAAGATG	1064
	Reverse	GGTTCTCTTCTTCCGGCTCGTAG	
trpE	Forward*	GCCGATCCCTCCGAGGAAAATG	993
	Reverse	CCCGGCGCTTGTTGATGGTT	

* Primers used for sequencing.

SNaPaer assays was performed in 30 strains using the primers of Table 3 and carrying out a final volume of 5 µL, containing 1.5 µL of PCR product (purified with *Escherichia coli* exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT®) (USB® Products; Affymetrix), according to the manufacturer's protocol), 1 µL of *SNaPaer* primer mix (each primer at 1 µM), 1 µL of ABI Prism SNaPshot® Multiplex Kit (Applied Biosystems), and 1.5 µL of ultrapure water (Eusebio et al., 2013). The reaction mixture was run with 25 extension cycles in the following conditions: denaturation for 10 s at 96 ºC, primer annealing for 5 s at 50 ºC, and extension for 30 s at 60 ºC. Unincorporated ddNTPs were removed with 1 U of Shrimp Alkaline Phosphatase (SAP) (USB® products) following incubation for 1 h at 37 °C and 15 min at 85 °C, as suggested by the manufacturer. *SNaPaer* products (0.5 µL) were mixed with 9.0 µL of HiDi™ formamide (Applied Biosystems) and 0.5 µL of GeneScan-120 LIZ size standard (Applied Biosystems). Electrophoresis was performed on a 3130xl Genetic Analyzer (Applied Biosystems) using filter set E5. Data were analysed with the software GeneMapper v4.0 (Applied Biosystems).

Table 3. Primers used for *SNaPaer*.

Name	Expected SNP	Primer sequence (5' to 3')	Expected lenght
Ac7	A/C/T	CCTACATCGTCTATGGYCCG	75-76
Ac78	A/G	TGACCCGCGTGGCGAA	33-34
Ac336	A/C/T	GCCCGGCTTCATCGC	68-69
Ac387	A/C/G	GCCGAGGTTGTCCACCAG	106-107
A98	C/G/T	GAACACCCTGATCCGCCT	50-52
A264	C/G/T	CGGTTGGCGATCAGCA	26-27
A491	C/G/T	ATGTAYGSCAAGGAACCGAC	95-96
G6	A/C/G/T	GGTTCCTCCAAGGTCCTGCT	70-71
G49	A/C/G/T	CCGATGGCCTTGTGCA	62-63
G219	C/T	TTGCGCTTCTCTTCCGG	46-47
G264	A/C/G/T	GGCCGCGCTTTCATCGAAGT	40-41
M9	A/G	GCCAGGCGCTTGATGAC	59-61
M36	C/G/T	GTGGAAAGCCACGTCGAA	80-82
M204	G/C/T	GCCTGCACCTGTGGGG	53-55
M228	A/C/G/T	CAGGTCCGGCTGGCTGCG	92-93
N162	C/T	CCAGTCCTGGCACAGTTTCAT	35-37
N255	C/G	GCCGGGATCAAGGTGCC	57-58
N288	C/G/T	GGTTCAGGATRCGGAAGAACTC	98-99
P100	A/C/G/T	GCTGGCCGATGGCACG	85-87
P268	A/C/G/T	GTCACCAACCGAGGAGGGCG	48-49
T205	C/G/T	TGGGGCGGGTGTCCGA	101-102
T331	A/C/T	ACGCGCTGCGGGCGAT	77-78
T349	A/G	TGCCGGCGGGYACKCT	107- 109

3.3. Patient population

P. aeruginosa can be easily isolated from bronchiectasis patients on Cetramide agar plates. The study of *P. aeruginosa* strains from these patients is valuable to understand how bacterial population changes over time and from patient to patient.

A total of 29 patients diagnosed with bronchiectasis, admitted at Hospital São João (Porto, Portugal) and Hospitais Universitários Coimbra (Coimbra, Portugal), were recruited for this study: 11 patients were classified as presenting non-CF bronchiectasis and 18 presented CF bronchiectasis. Bronchiectasis was diagnosed by HRCT together with other characteristic symptoms such as chronic cough, bad breath and sputum production. Genotype, sweat (chloride) test, organ function tests, and exercise testing were considered for the diagnosis of CF. Personal information and clinical history of each patient was also registered: age, gender, history of exacerbation and antibiotic treatments, bacterial colonisation, PF, FEV1, FEV/FVC (Forced Vital Capacity) ratio, radiographic abnormality and total serum IgE >500 IU/mL (1200

ng/mL), bronchiectasis type (localised or diffused), C-reactive protein, haemoptysis and other associated diseases.

3.4. *Pseudomonas aeruginosa* culture and sampling

Biological secretions and/or bronchial aspirates were collected every 2-4 months (between 2009 and 2012) from our group of patients. Patient specimens (sputa) were cultured twice on Petri dishes with Cetrimide Agar plates for the detection and culture of *P. aeruginosa*. The plates were incubated at 37ºC for a period of 48-72 h. Selected bacterial colonies observed in Cetrimide Agar plates were re-cultured in new plates. The results of this procedure were compared with previously reported results from the hospital.

The selection of bacterial colonies was random using a transparent paper with a fractionated matrix as presented in Figure 6. Afterwards, eight sections were selected using the R command: sample(1:38,8,replace=F), from the R Gui v2.15.2 (R Development Core Team). Multiple results for the samples used are shown in Annex 1. Isolated colonies closest to the centre of the square were selected; if the selected square did not contain an isolated colony, the adjacent square was chosen. Additionally, few strains collected from CF patients with bronchiectasis from a previous study (Eusebio et al., 2013) were also added to the collection. Phenotypic characteristics of bacteria, such as type (mucoid, rough or smooth) and pigmentation, were registered. A total of 53 clinical samples were tested from 29 patients and 207 colonies of *P. aeruginosa* were obtained for further genotyping analysis.

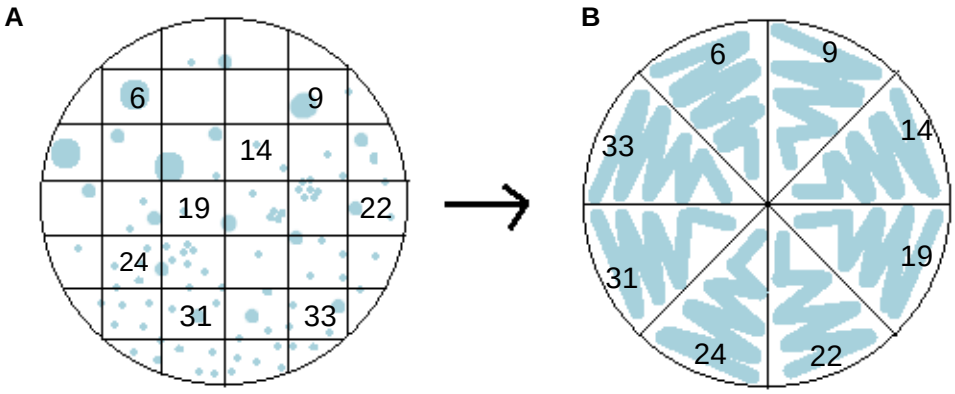

Figure 6. Selection of eight colonies for molecular studies. A: Random selection of eight colonies (blue spots) previous cultured in a Cetramide plate. B: Re-culturing of the previous selected colonies.

3.5. Sample Colony-PCR preparation

MLST amplification was performed in 207 isolates of *P. aeruginosa* as previously reported (Eusebio et al., 2013); the MLST housekeeping genes have been previously described by Curran et al. (2004) but, the initial step that follows bacterial isolation and that includes the extraction of bacterial DNA from cells was avoided. Bacterial colonies were used directly for MLST amplification. Three different strategies were tested:

3.5.1. Colony direct-PCR

A small amount of bacteria was re-suspended and vortexed in an Eppendorf tube containing 10 µL of ultrapure water. Bacterial suspensions were incubated at 95 ºC for 90 min. Afterwards, the suspension was vortexed and stored at -20 ºC.

3.5.2. Pre-incubation with Genomiphi

The commercial Ilustra™ GenomiPhi V2 DNA Amplification Kit (GE Healthcare) was used. A small amount of bacteria from a single colony was added to 10 µL of sample buffer, as previous described to colony direct-PCR. The mixture was heated during 90 min, at 95 ºC. For the amplification reaction, 9 µL of reaction buffer and 1 µL of enzyme mix were added to the bacterial product. The final 20 µL were incubated according to the following conditions: amplification for 120 min, at 30 ºC; inactivation of the Phi29

DNA polymerase enzyme for 10 min, at 65 ºC; and a final step of cooling at 4 ºC. The mixture was stored at -20 ºC.

3.5.3. Pre-treatment with Proteinase K

A small amount of bacteria was added to 20 µL ultrapure water as described above for colony direct-PCR. The mixture was vortexed and heated for 60 min, at 95 ºC and 2 µL of Proteinase K (2 mg/mL) (Finnzymes) were then added to the previous mixture. After a short spin, the mixture was incubated for 60 min at 56ºC. The inactivation of Proteinase K was performed following vortexing and heating for 8 min, at 100º C. Subsequent centrifugation was performed at 14,000 rpm for 4 min. The finally mixture was stored at -20 ºC.

3.6. Genotyping of *Pseudomonas aeruginosa* strains from bronchiectasis patients

MLST genes of the previous mentioned 207 *P. aeruginosa* Portuguese strains were amplified and the bacteria genotyped as previously suggested by Eusebio et al. (2013). PCR amplification with pre-treatment with Proteinase K was selected due to the efficiency of the method. PCR fragments were also sequenced to confirm their specificity. Thus, amplification products were purified with ExoSap-IT®, according to the manufacturer's protocol. Sequencing was performed using the ABI BigDye® terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems). PCR sequencing conditions were: denaturation for 2 min at 96 ºC; 35 cycles with denaturation for 15 s at 96 ºC, primer annealing for 9 s at 50 ºC and extension for 2 min at 60 ºC; final extension for 10 min at 60 ºC. The product was purified with Sephadex® (Expansys). A volume of 8.0 µL HiDi^{TM} formamide (Applied Biosystems) was added to each PCR product before sequencing.

3.7. Analyses of the genotyping data

SNaPshot results were analysed in GeneMapper® Software v4.0 and Peak Scanner™ software v1.0, both afforded by Applied Biosystems and the sequencing data were analysed with Sequence Scanner™ v1.0 and Geneious Pro 5.5.6 softwares.

The Network software v4.6.1.0 (www.fluxus-engineering.com/sharenet.htm) (Polzin and Daneschmand, 2003) was used to better visualise the relations of the several haplotypes from the populations genotyped in the present work.

For the contextualisation of the genetic patterns observed in the studied populations, additional data were retrieved from the literature and databases available online. Data for the 23 SNPs were extracted from *Pseudomonas aeruginosa* MLST Database (http://pubmlst.org/paeruginosa/).

4. Results

4.1. *Pseudomonas* species phylogeny based on MLST data

Phylogenetic analysis for 50 *Pseudomonas* species was carried out using data for *acsA, aroE guaA, mutL, nuoD*, *ppsA* and *trpE* gene fragments, previously reported for *P. aeruginosa* MLST (Curran et al., 2004). Four other genes, *16S rRNA (*Anzai et al., 2000), *gyrB* and *rpoD* (Yamamoto et al., 2000) and *rpoB* (Ait Tayeb et al., 2005), previously suggested for *Pseudomonas* species identification were identified in this list of genomes (Table 2). The GC content of the genes was between 48.6% (for *gyrB*) and 66.9% (for *acsA*), with an average of 61.2% for all loci (Table 4).

Table 4. General characterisation of genes collected from *Pseudomonas* species genomes.

Locus	Size analysed sequence (bp)	Number of alleles	% GC content	Number of identical sites (%)
MLST genes				
acsA	390	50	66.9	208 (53.3)
aroE	498	50	66.0	185 (37.1)
guaA	373	50	63.6	245 (65.7)
mutL	442	50	65.5	239 (54.1)
nuoD*	366	45*	61.8	256 (69.9)
ppsA	370	50	65.4	221 (59.7)
trpE	443	50	63.4	258 (57.5)
Other genes				
16S rRNA	1456	50	52.0	1,114 (73.2)
gyrB	2421	50	48.6	1,350 (55.2)
rpoB	1149	50	61.0	815 (70.5)
rpoD	1854	50	59.2	1,129 (60.2)

* The gene *nuoD* was not detected on five *Pseudomonas stutzeri* genomes.

The strain *Chromohalobacter salexigens* DSM 3043 was used as an out-group, to define the root of the phylogenetic trees, as the genus *Chromohalobacter* is phylogenetically related to *Pseudomonas* species (Williams et al., 2010b).

Phylogenetic trees (NJ) for individual data sets of *acsA, aroE, mutL, nuoD, guaA, ppsA,* and *trpE* nucleotide sequences were constructed (Annex 5 - Annex 12). The single-locus topologies of MLST genes showed well-defined groups for *P. chlororaphis*, *P. stutzeri*, and *P. syringae*; *P. putida* strains formed single clusters with two or three exceptions dispersed in other groups, while the strains of *P. fluorescens* were dispersed throughout the trees. *P. aeruginosa* group was well-defined in *acsA* phylogeny; however, the remaining single locus phylogenies defined *P. mendocina* strains outside the *P. aeruginosa* group. The *aroE* phylogenetic tree presented the highest boot strap values for separating of three previously defined groups (*P. chlororaphis*, *P. stutzeri*, and *P. syringae*), while *nuoD* phylogenetic tree showed the lowest phylogenetic value defining only a cluster for *P. syringae*. The resolution of these phylogenetic trees may be justified by nucleotide diversity as *aroE* was the most polymorphic gene with only 37.1% of identical sites (the number of consensus positions obtained from the alignment of the various sequences of each gene). In contrast, *nuoD* was the least polymorphic gene with a percentage of identical sites of 69.9%.

Concatenated gene sequences were used to construct MLST phylogenies: a) the first phylogeny with all seven MLST genes but excluding the five strains of *P. stutzeri* with no *nuoD* gene (Figure 7); and b) a second phylogeny with *acsA, aroE, guaA, mutL, ppsA* and *trpE* genes of all the tested strains (Annex 12). The basic topologies of both trees were similar, the most visible difference between these phylogenetic trees being on the bootstrap values, which were slightly higher in some branches of the seven genes phylogeny. When compared the seven and six concatenated phylogenies with single locus phylogenies, we verified a substantially increase of the bootstrap values for definition of *P. putida* group. The definition of the remaining groups was similar in concatenated and single locus phylogenies. Figure 7 shows the phylogeny of *acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE* concatenated genes and includes all strains presented in Table 1 with exception of some *P. stutzeri*. In both phylogenies was possible to clearly define groups for strains of *P. chlororaphis*, *P. stutzeri* and *P. syringae*, with bootstrap values of 100. The *P. aeruginosa* group was divided in two separated clusters with most strains of *P. aeruginosa* separated from *P. mendocina* strains. *P. putida* group was well-defined for 11 out of the 13 strains on this group; *P. fulva* 12-X and *P. putida* UW4 were far from the group. *P. fluorescens* strains formed a

well-defined group in the phylogeny that considered the six concatenated genes, although with a very low bootstrap value (59.8%). Additionally, within this group, was possible to observe *P. brassicacearum* NFM421, not defined as belonging to any of the previous groups (Anzai et al., 2000).

In order to understand the importance of the concatenated sequences of the *acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE* genes for improving the resolution of the phylogeny of some species within the *Pseudomonas* genus, the phylogenetic trees of *16S rRNA* (Annex 2)*, rpoB* gene (Annex 3) and concatenated *rpoD* and *gyrB* genes (Annex 4) were designed for the same list of strains. The phylogenies obtained were very similar to those previously suggested by other authors (Anzai et al., 2000; Yamamoto et al., 2000; Ait Tayeb et al., 2005). The tree of *16S rRNA* showed the lowest resolution. In *rpoD* and *gyrB* concatenated and *rpoB* phylogenies, *P. fulva* 12-X and *P. putida* UW4 were not clustered within the *P. putida* group as previously shown with MLST data. These last phylogenies were similar but the *rpoD* and *gyrB* concatenated phylogeny showed the highest bootstrap values.

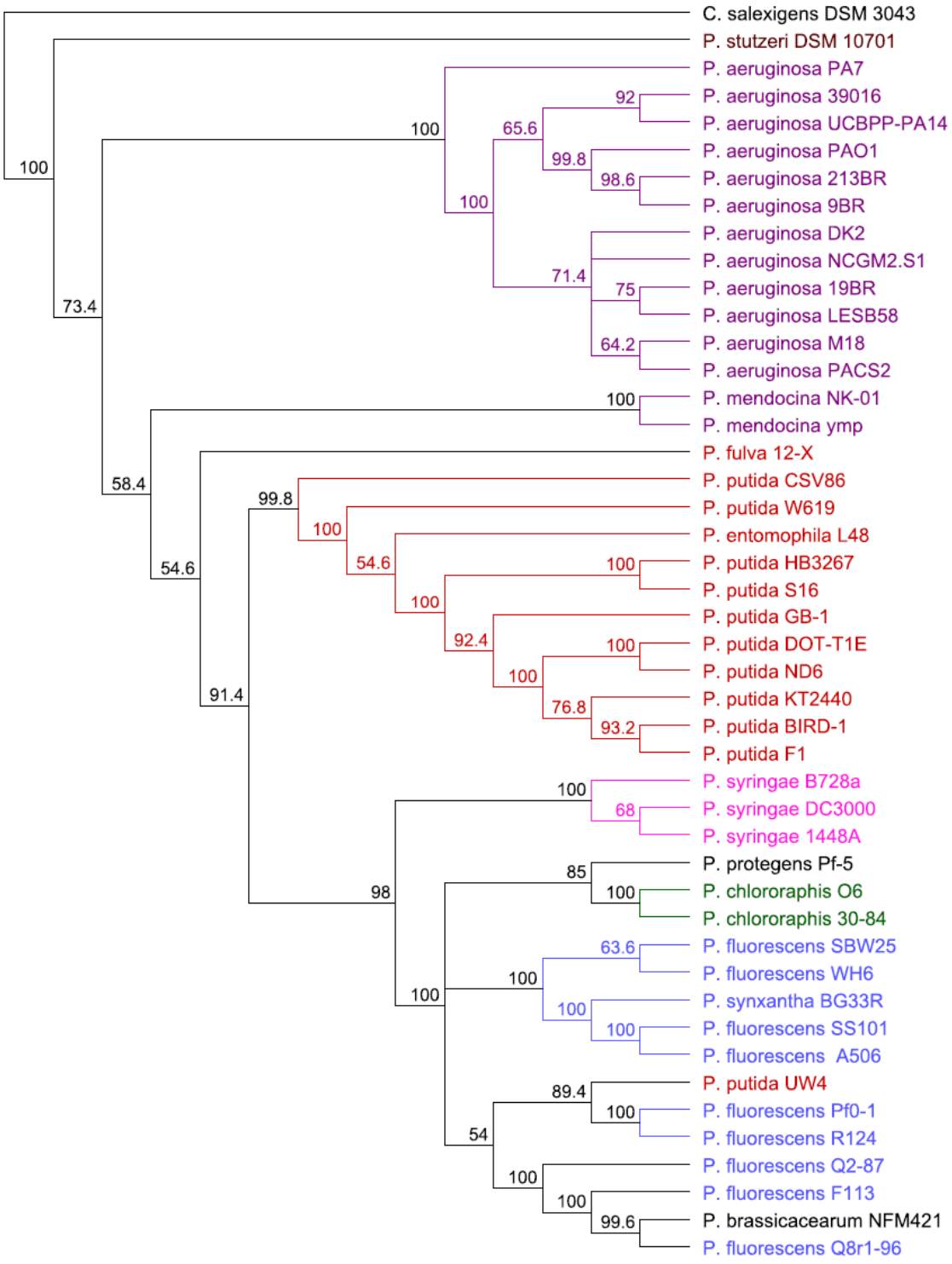

Figure 7. Phylogenetic tree of 50 *Pseudomonas* species based on concatenated *acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE* genes. The tree was constructed using the neighbourjoining method and using *Chromohalobacter salexigens* DSM 3043 as an out-group. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; purple - *Pseudomonas aeruginosa* group; red - *Pseudomonas putida* group; pink - *Pseudomonas syringae* group; green - *Pseudomonas chlororaphis* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group.

4.2. Identification of *Pseudomonas aeruginosa* by *SNaPaer*

The potential of *SNaPaer* for identification of *P. aeruginosa* was also evaluated. Initially, the 23 SNPs (*SNaP* profile) suggested by Eusebio et al. (2013) for *P. aeruginosa* genotyping were identified *in silico* in the list of 45 *Pseudomonas* genomes (see Table 1); the strains with no *nuoD* gene were excluded. These *SNaP* profiles were added to a database that included other 86 *SNaP* profiles obtained from a previous study with clinical *P. aeruginosa* isolates (Eusebio et al., 2013). All *SNaP* profiles were converted into *preMLST* sequences using MLST@SNaP software (Soares and Araujo, 2013). This programme uses an algorithm that converts the *SNaP* profiles into a probable MLST sequence (*preMLST*) based on the complete MLST data available for *P. aeruginosa* in the pubMLST website. By concatenating the seven *preMLST* sequences, a frame of 2,882 bp was obtained for each tested strain of our list. The comparison between *preMLST* and published MLST sequences revealed a percentage of identical sites ranging from 77 to 85% among non-*aeruginosa Pseudomonas* species (the percentage was frequently close to 100% for *P. aeruginosa* strains).

Phylogenetic analysis of the 45 *preMLST* sequences was performed (Figure 8). The tree resulting from the analysis was highly different from the one resolved by complete MLST sequences. In Figure 8 is possible to delineate a cluster of *P. aeruginosa* strains with a bootstrap value of 74%. However, two strains of *P. aeruginosa* (PA7 and PAO1) formed another cluster with a bootstrap value of 100%. In order to understand if these strains represented exceptional cases, 86 different *SNaP* profiles obtained from our collection of *P. aeruginosa* were added to the phylogeny. The new phylogeny allocated the 86 strains into the same cluster containing the remaining *P. aeruginosa* strains, with the exception of PA7 and PAO1 (data not shown). The *P. aeruginosa* PAO1 and PA7 presented the lowest values of identical sites when comparing *preMLST* and MLST sequences, with similarity values of 88.0% and 86.7% respectively, and represented atypical strains of *P. aeruginosa*.

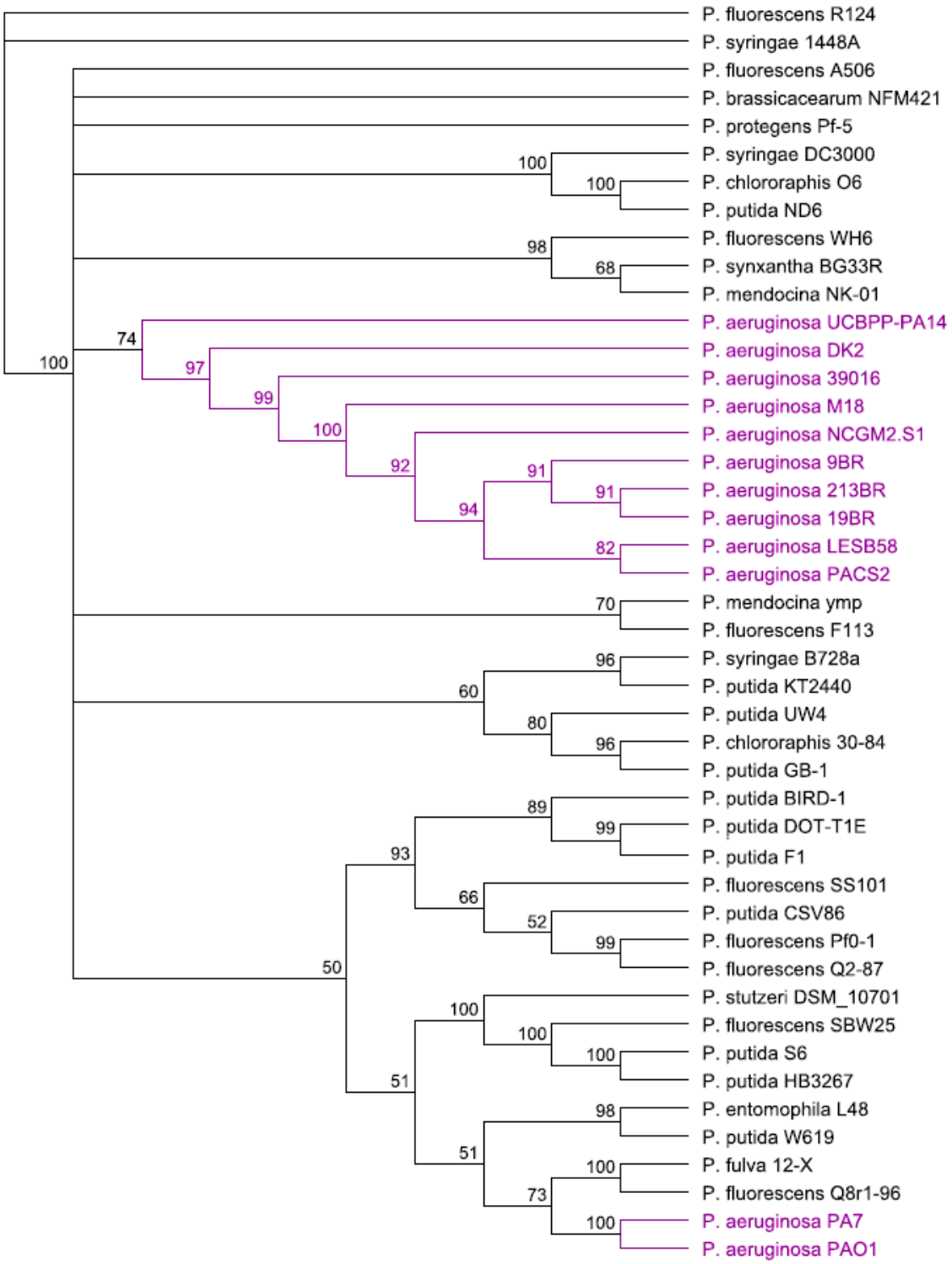

Figure 8. Phylogenetic tree of 45 *Pseudomonas* species based on the concatenated *preMLST* sequences. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The purple colour represents the different strains of *Pseudomonas aeruginosa.*

4.3. Testing *SNaPaer* assay in non-aeruginosa *Pseudomonas*

In order to test the suitability of *SNaPaer* assay for *P. aeruginosa* identification, the MLST housekeeping genes of 19 isolates from close-related bacterial species (*Pseudomonas species, Xanthomonas species* and *Burkholderia cenopacia*) were amplified. Several unspecific bands were observed in the polyacrylamide gel mainly following the amplification of MLST fragments in *P. putida* KT2440, *P. fluorescens* Pf0-1, *P. savastanoi pv. glycinea* 5066, *P. syringae pv. oryzae* 10912, *P. syringae pv. syringae* 10604, *P. syringae pv. tabaci* 5393 and *X. campestris pv. campestris* 568 (Figure 9). With the exception of *P. syringae pv. syringae* 10604, the remaining isolates showed only one or two specific bands. *B. cenocepacia* isolates showed a complete absence of bands in the gel.

In order to explain the low efficiency of MLST primers for amplification of these genes in non-*aeruginosa* species, the primer sequences were compared among the list of complete genome sequences (Table 1). The pair of primers with the highest level of polymorphisms was the set for *nuoD* sequence and the smallest number of polymorphism was found in *guaA* and *ppsA* primers (data not shown). Therefore, the presence of complete MLST profiles in the gel resulting from excellent MLST amplification could be the first indication of the presence of a *P. aeruginosa* isolate (numbered 1), as seen in Figure 9.

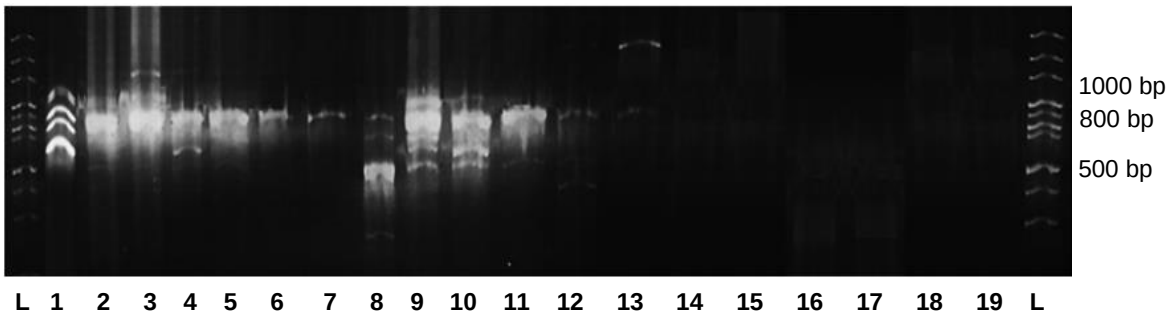

Figure 9. Polyacrylamide gel of multilocus sequence typing fragments amplified by conventional PCR (L- ladder; 1- *Pseudomonas aeruginosa* P7; 2- *Pseudomonas putida* KT2440; 3- *Pseudomonas fluorescens* Pf0-1; 4- *Pseudomonas savastanoi pv. glycinea* 5066; 5- *Pseudomonas savastanoi pv. phaseolicola* 2245; 6- *Pseudomonas* syringae pv. helianthi 5067; 7- *Pseudomonas* syringae pv. maculicola 5071; 8- *Pseudomonas syringae pv. oryzae* 10912; 9- *Pseudomonas syringae pv. syringae* 10604; 10- *Pseudomonas syringae pv. tabaci* 5393; 11- *Pseudomonas syringae pv. tomato* DC 3000; 12- *Xanthomonas axonopodis pv. citri* 9322; 13- *Xanthomonas campestris pv. campestris* 568; 14- *Burkholderia cenocepacia* 4201; 15- *Burkholderia cenocepacia* 4219; 16- *Burkholderia cenocepacia* 4203; 17- *Burkholderia cenocepacia* 4164; 18- *Burkholderia cenocepacia* 4178; 19- *Burkholderia cenocepacia* 4234).

Some *P. aeruginosa* reference strains (e.g. PAO1 and ATCC 33358) and other *P. aeruginosa* clinical isolates showed MLST gene amplification problems. Different annealing temperatures were tested going from 50 ºC to 54 ºC (Annex 13). Those genes could be amplified at lower temperatures; therefore, they were sequenced in order to confirm its specificity. The sequences were compared with the MLST genes of *P. aeruginosa* PAO1 and *P. mendocina* ymp. In this first analysis, it was verified that some genes of few *P. aeruginosa* strains and atypical clinical isolates were more similar with *P. mendocina* ymp strains than with *P. aeruginosa* PAO1. However, the concatenated of the MLST genes confirmed that such isolates were *P. aeruginosa* and distinct from *P. mendocina*. *P. aeruginosa* ATCC 33358 was an example of a strain showing a few polymorphisms in *acsA* but several polymorphisms in *guaA* genes (more than 20% when compared with the reference *P. aeruginosa* PAO1 available at NCBI) (Annex 14).

SNaPaer primers were tested in non-*aeruginosa* bacteria in order to evaluate the specificity of the method for identification. *SNaPaer* results agreed with the results of polyacrylamide gel (Figure 9) that showed the absence of some amplicons. Several *SNaPaer* markers did not appear in the electropherogram of non-*aeruginosa* bacteria; some cases were consequence of the absence of certain amplicons, others were cases of marker specificity to *P. aeruginosa*. Nevertheless, the presence of unspecific peaks in the electropherogram could not be excluded. Therefore, criteria were defined in order to achieve an accurate and reliable analysis: peak heights below 50 Relative Fluorescence Unit (RFU) were excluded, the lengths of the fragments were carefully evaluated and displacements exceeding 1-2 bp from the theoretical position of *P. aeruginosa* peaks were eliminated, and finally, the presence of peaks corresponding to unexpected nucleotides were excluded (e.g. a marker suggested as detecting C or T could not detect an A). G264, P268, N255, G6, T331 and A491 markers were detected in the different *Pseudomonas* strains, and thus, they were not specific for *P. aeruginosa*. Ac78, N162, A98, M9, G49, Ac7, T205, Ac387 markers were generally showed to be specific for *P. aeruginosa* identification but occasionally could be detected in some strains (Table 5); more strains and other *Pseudomonas* species should be tested to clarify the specificity of these peaks. A264, G219, M204, Ac339, M36, P100, M228, N288 and T349 were clearly specific markers for identification of *P. aeruginosa* (Figure 10).

Table 5. Few *SNaPaer* markers in non-*aeruginosa* species. Black ticks represent the positions with specific peak. Blue crosses represent the absence of peak.

Strains	Ac78	N162	A98	M9	G49	Ac7	T205	Ac387
Pseudomonas aeruginosa	✓	✓	✓	✓	✓	✓	✓	✓
Pseudomonas fluorescens	×	×	×	×	✓	×	×	×
Pseudomonas putida	×	×	×	×	×	×	×	×
Pseudomonas savastanoi pv. glycinea	×	×	×	×	×	×	×	×
Pseudomonas savastanoi pv. phaseolicola	×	×	×	×	✓	×	×	×
Pseudomonas syringae pv. helianthi	×	×	×	×	×	×	×	×
Pseudomonas syringae pv. maculicola	×	×	×	✓	×	×	×	×
Pseudomonas syringae pv. oryzae	×	×	×	×	×	×	×	×
Pseudomonas syringae pv. syringae	✓	✓	×	×	✓	✓	✓	✓
Pseudomonas syringae pv. tabaci	×	×	×	×	×	×	×	×
Pseudomonas syringae pv. tomato	×	×	×	×	×	×	×	×
Xanthomonas axonopodis pv. citri	×	×	×	×	×	×	×	×
Xanthomonas campestris pv. campestris	×	×	×	×	×	×	×	×
Burkholderia cenocepacia 4201	×	×	✓	×	×	✓	×	×
Burkholderia cenocepacia 4219	×	×	✓	×	×	✓	×	×
Burkholderia cenocepacia 4203	×	×	✓	×	×	✓	×	×
Burkholderia cenocepacia 4164	×	×	✓	×	×	✓	×	×
Burkholderia cenocepacia 4178	×	×	✓	×	×	✓	×	×
Burkholderia cenocepacia 4234	×	×	✓	×	×	✓	×	×

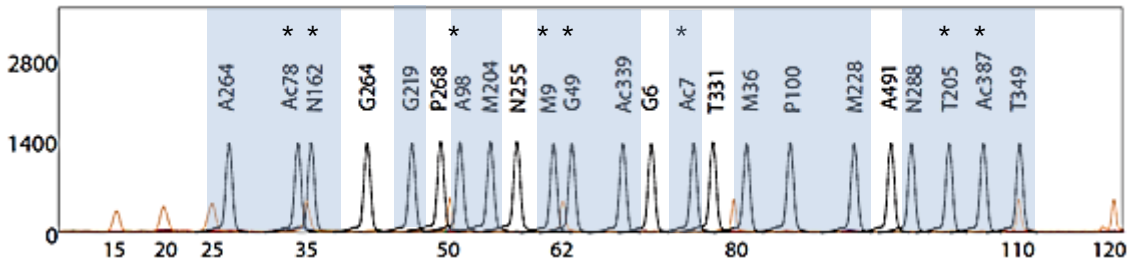

Figure 10. Representative *SNaPaer* assay of *Pseudomonas aeruginosa*: Blue represents specific markers to *Pseudomonas aeruginosa.* The remaining are non-specific markers to *Pseudomonas aeruginosa.*

* Markers occasionally detected in some non-*aeruginosa Pseudomonas* strains.

4.4. Sampling of clinical *Pseudomonas aeruginosa* in bronchiectasis patients

P. aeruginosa isolates can be easily collected from the sputa of bronchiectasis patients. A total of 29 patients were enrolled on this part of the study: 11 with non-CF bronchiectasis and 18 with CF bronchiectasis. Table 6 shows the demographic data, medical history and spirometric values of the studied patients. Most patients (60%) presented diffuse bronchiectasis according to the HRCT. The remaining 40% presented located bronchiectasis on the different lobules (right and left superior, inferior or medium) or ligula. Patients with frequent episodes of exacerbation presented higher values of C-reactive protein, a response to the frequent inflammation. As predictable, the average of FEV1 was lower among older non-CF bronchiectasis patients compared to younger patients (1.18 L versus 1.71 L respectively). FEV1 values represent the volume of air that can forcibly be blown out in 1 second, after full inspiration. CF and non-CF bronchiectasis patients with long term colonisation with *P. aeruginosa* showed lower FEV1 values than patients recently infected or uninfected with this bacterium (e.g. average FEV1 values of 0.58 L and 1.86 L were observed respectively for CF patients with and without long term colonisation, respectively). The overall average PF in non-CF patients with bronchiectasis was 62.8%, while in CF patients was 56.2%. As CF patients with bronchiectasis are generally younger compared with non-CF patients, they tended to acquire *P. aeruginosa* chronic colonisation at younger ages (27 versus 48 year-old respectively). The colonisation of bronchiectasis patients with other microorganisms, such as *Aspergillus* species, *Burkholderia cepacia* complex, *Haemophilus inluenza*, *Staphylococcus aureus* and *Scedosporium apiospermum*, was more frequently observed in CF than in non-CF bronchiectasis patients (72% versus 27%, respectively). Occasionally, microorganisms such as *Acinetobacter lwoffii*, *Achromobacter xylo, Klebsiella oxytocaand*, *Klebsiella ozaenae, Rhizobium radiobacter* and *Stenotrophomonas maltophilia* could also be found. Eight (72%) non-CF patients and 18 (100%) CF patients received antibiotic therapy, including cephalosporins and quinolones antibiotics.

Table 6. General characteristics of the patients with bronchiectasis included in the present study.

	Non-cystic fibrosis	Cystic fibrosis
Number of patients	11	18
Age	22-54	28-54
Gender female/male	12/3	6/12
Patients with exacerbation caused by *Pseudomonas aeruginosa*	4 (36%)	0 (0%)
Forced Expiratory Volume per Second 1 (L)	0.58-2.21	0.93-3.94
Pulmonary Function	37.2-122%	15.9-100.5%
Patients with haemoptysis	4 (36%)	0 (0%)
Pseudomonas aeruginosa* colonisation	11 (100%)	8 (44%)
Colonisation by other microorganisms	3 (27%)	13 (72%)
C-reactive protein (mg/L)	0.4-37.3	0.7-3.1
Sinusitis	4 (36%)	12 (67%)
Antibiotic therapy	8 (72%)	17 (94%)

* Hospital record.

Fifty-three sputum samples from patients with bronchiectasis, 16 from non-CF and 37 from CF patients, were cultured in Cetrimide Agar at 37 ºC. From the total of cultured samples, 14 samples of nine non-CF bronchiectasis patients and 33 samples of 14 CF bronchiectasis patients presented positive culture in Cetrimide. Our Cetrimide culturing results showed discrepancies when compared with the results provided by the clinicians. From the 11 non-CF patients, nine presented Cetrimide cultured bacteria (82%) in the samples tested in our laboratory; 14 of the 18 CF patients presented positive culture on Cetrimide agar (78%). Interestingly, few isolates grown in Cetrimide Agar were not genotyped later due to MLST amplification difficulties. With the appropriate corrections by removing the non-genotyped colonies, 21 patients (six non-CF and 13 CF) were later confirmed as presenting *P. aeruginosa* in our samples, in contrast with the previous results obtained from the hospital record (11 non-CF and eight CF with *P. aeruginosa*) (Table 6). Isolates from CF4, CF8, CF11, CF12 and CF13 patients were included in our genotyping studies even with negative result presented by the hospital record (Table 7). Cetrimide Agar was also the selective isolation medium for *P. aeruginosa* in hospitals. These divergent results may be justified by the different amount of sputa employed for culture by hospital technicians and the amount we tested in our procedure. The hospital staff used 5 µL of sputa for a single agar plate, while in the lab we cultured around 20 µL of sputa on two plates of Cetrimide Agar. We increased the amount of the cultured sputa in order to obtain more colonies of *P. aeruginosa* for further genotyping. Another explanation may come from the ability

of other organisms to grow on this medium. For example, some isolates of *P. putida* and *P. fluorescens* are capable to grow on Cetrimide Agar at 30 to 35°C (Wong et al., 2011) and could be incorrectly identified by biochemical or molecular methodologies.

The characterisation of the bacterial colonies grown on Cetrimide Agar is shown in Table 7. From the 29 patients, 23 showed positive culture in our samples of Cetrimide Agar. Twenty (seven non-CF and 13 CF) patients presented colonies with the smooth phenotype and two presented colonies with the rough phenotype. Mucoid bacteria were found in four patients (17%). Patients colonised with mucoid *P. aeruginosa* were characterised as presenting low (25%), intermediate (50%) and severe mucoidy (25%) (Figure 11). Mucoid isolates were observed in NCF2, NCF3, NCF5 and CF1 patients; non-CF patients presented PF values (between 54.8 and 85.0%), while the CF1 patient presented a PF value of 25%. The colour of the clinical *P. aeruginosa* colonies ranged from white to brown grey. In two non-CF bronchiectasis patients (NCF3 and NCF5), it was observed some phenotypic differences between *P. aeruginosa* isolates. These patients presented three and four phenotypically different isolates, respectively (Table 7).

The analysis of consecutive samples collected from some patients (six from non-CF and eight from CF) revealed that those patients were chronically colonised with *P. aeruginosa* showing similar phenotypic features. No patients with sporadic colonisation by *P. aeruginosa* were observed.

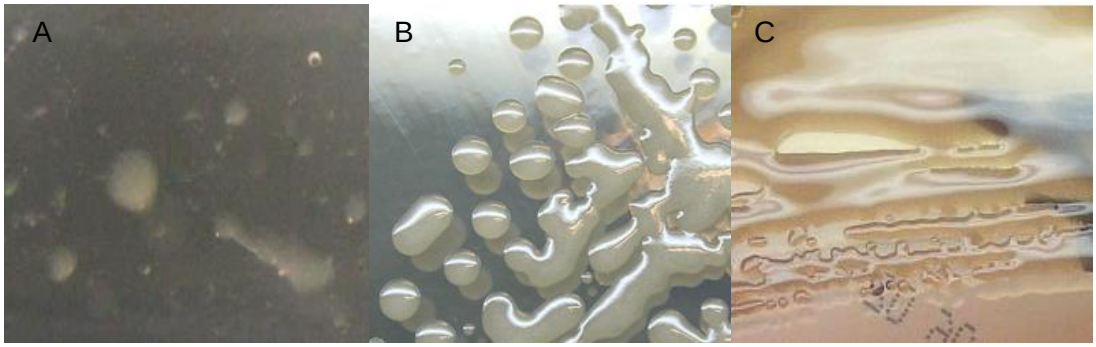

Figure 11. Different production of mucus by *Pseudomonas aeruginosa* isolates: low (A), intermediate (B) and severe mucoidy (C).

Table 7. Phenotypic characteristics of *Pseudomonas aeruginosa* colonies obtained from sputum samples of bronchiectasis patients.

Patients	Phenotypic isolates	Type	Mucoidy	Colour
Non-cystic fibrosis				
NCF1	A	Smooth	Not applicable	Grey white
NCF2	B	Mucoid	Low	White
NCF3	C	Mucoid	Intermediate	Brown grey
	D	Rough	Not applicable	Cream orange
	E	Smooth	Not applicable	White
NCF4	F	Smooth	Not applicable	Grey white
NCF5	G	Smooth	Not applicable	Yellow
	H	Smooth	Not applicable	Green
	I	Smooth	Not applicable	Brown grey
	J	Mucoid	Intermediate	Grey white
NCF6	K	Smooth	Not applicable	Cream grey
NCF7	L	Smooth	Not applicable	Yellow
NCF8	M	Rough	Not applicable	Dark yellow
NCF9	N	Smooth	Not applicable	White
Cystic fibrosis				
CF1	O	Mucoid	Severe	Green
CF2	P	Smooth	Not applicable	Yellow
CF3	Q	Smooth	Not applicable	Yellow
CF4	R	Smooth	Not applicable	Yellow
CF5	S	Smooth	Not applicable	White
CF6	T	Smooth	Not applicable	Grey white
CF7	U	Smooth	Not applicable	Grey white
CF8	V	Smooth	Not applicable	Cream
CF9	W	Smooth	Not applicable	Cream
CF10	X	Smooth	Not applicable	White
CF11	Y	Smooth	Not applicable	Yellow
CF12	Z	Smooth	Not applicable	Yellow
CF13	AA	Smooth	Not applicable	Yellow
CF14	AB	Smooth	Not applicable	Yellow

4.5. Colony PCR

Three different methodologies were tested in a group of 30 *P. aeruginosa* isolates in order to avoid DNA extraction and turn our genotyping strategy more practical and faster to be used in routine by clinical laboratories. MLST amplification was tested in all isolates and colony PCR strategies followed by polyacrylamide gel for visualisation of the products. Amplification of the genes (positive results) was only observed in some bacterial isolates (Figure 13).

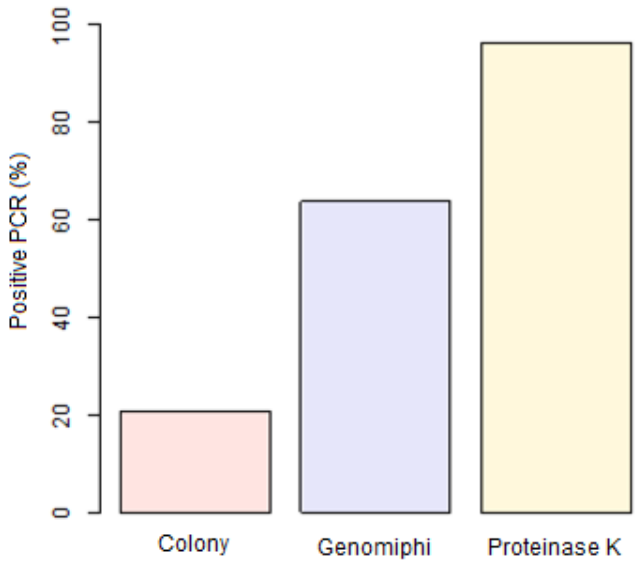

Figure 13. Percentage of positive results using three different methods: Colony direct-PCR without previous treatment, incubation with Genomiphi and treatment with Proteinase K.

Colony direct-PCR showed low efficiency (20.7%), probably due to the presence of several impurities and low amount of DNA. In order to increase the DNA quantity obtained from the bacterial colonies, Genomiphi-PCR was tested. Genomiphi-PCR showed to achieve a good balance between impurities and DNA quantity, which allowed a higher percentage of positive PCR results (63.7%). Finally, we tested Proteinase K-PCR - it uses a serine protease that degrades many proteins in the native state. Proteinase K-PCR presented 96.1% of positive results. The remaining 3.9% comprise rough isolates which failed in the three different methods. DNA concentration obtained following the treatment with Proteinase K ranged between 61 ng/µL and 2286 ng/µL. By testing any of the previous samples, a volume of 1 µL

of DNA obtained following Proteinase K treatment (for a final volume of PCR reaction of 5 µL) was considered enough to reach excellent results.

Following MLST amplification in 30 samples with bacterial DNA obtained from the three different strategies, *SNaPaer* was applied to the samples. Complete *SNaP* profiles were observed in 50%, 95% and 100% of positive results for Colony direct-PCR, Genomiphi-PCR and Proteinase K-PCR, respectively. Occasionally samples as C5 and G4 presented good *SNaP* profiles, but the best results were obtained in samples with clear bands in the gel, as it was the case of samples G5 and P1 (see Figure 14). Sequencing analysis was also successfully performed in samples showing clear bands in the gel.

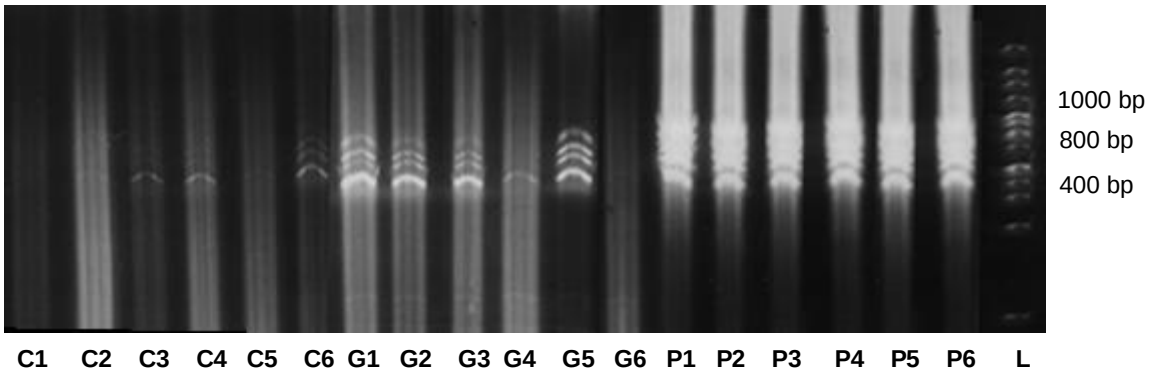

Figure 14. **Polyacrylamide gel of multilocus sequence typing fragments amplified by colony-PCR**. L- Ladder; 1 to 6 - Different isolates of *Pseudomonas aeruginosa* amplified following Colony direct-PCR (C), Genomiphi-PCR (G) and Proteinase K-PCR (P).

4.6. Genotyping *Pseudomonas aeruginosa*

A collection of 207 *P. aeruginosa* isolates (98 isolates from non-CF bronchiectasis patients and 109 from CF bronchiectasis patients) was genotyped with *SNaPaer* assay. *SNaP* profile analysis revealed that 20 out of the 23 SNPs present in the *SNaPaer* panel were polymorphic. *SNaPaer* typing revealed 43 *SNaP* profiles among the 207 isolates; nine *SNaP* profiles were observed in non-CF bronchiectasis patients (n=6) and 35 *SNaP* profiles in CF bronchiectasis patients (n=13). Generally, non-CF and CF bronchiectasis patients did not share the *SNaP* profile (Annex 15). Non-CF bronchiectasis patients were predominant colonised by isolates with a single *SNaP* profile (67% of the patients) (Figure 15). On the other

hand, most CF bronchiectasis patients (75%) were colonised by isolates with at least two different *SNaP* profiles. The presence of a single *SNaP* profile per patient was commonly found in various patients, including NCF1, NCF2, NCF5, NCF6, CF2 and CF11. From six studied non-CF patients, only NCF4 patient presented isolates with multiple *SNaP* profiles in the clinical sample (three different profiles). By other side, six CF patients (CF1, CF3, CF5, CF9, CF10, CF12 and CF13) presented isolates with multiple profiles in the clinical samples (two to three profiles). There was no evidence for the emergence of a single dominant genotype among the complete group of patients.

Phenotypic characteristics of the colony, such as type, size, mucoidy and colour, were compared with the observed *SNaP* profiles, but no relationships could be found. Additionally, we compared the *SNaP* profiles with the phenotypic features of the isolates collected at the same patient. Frequently, the isolates with different phenotypes showed different *SNaP* profile. Occasionally, we observed few strains with different phenotype and similar *SNaP* profile. For example, it was possible to collect isolates with four different phenotypes in patient NCF5 (Table 7); these isolates later resulted in a single *SNaP* profile in this patient.

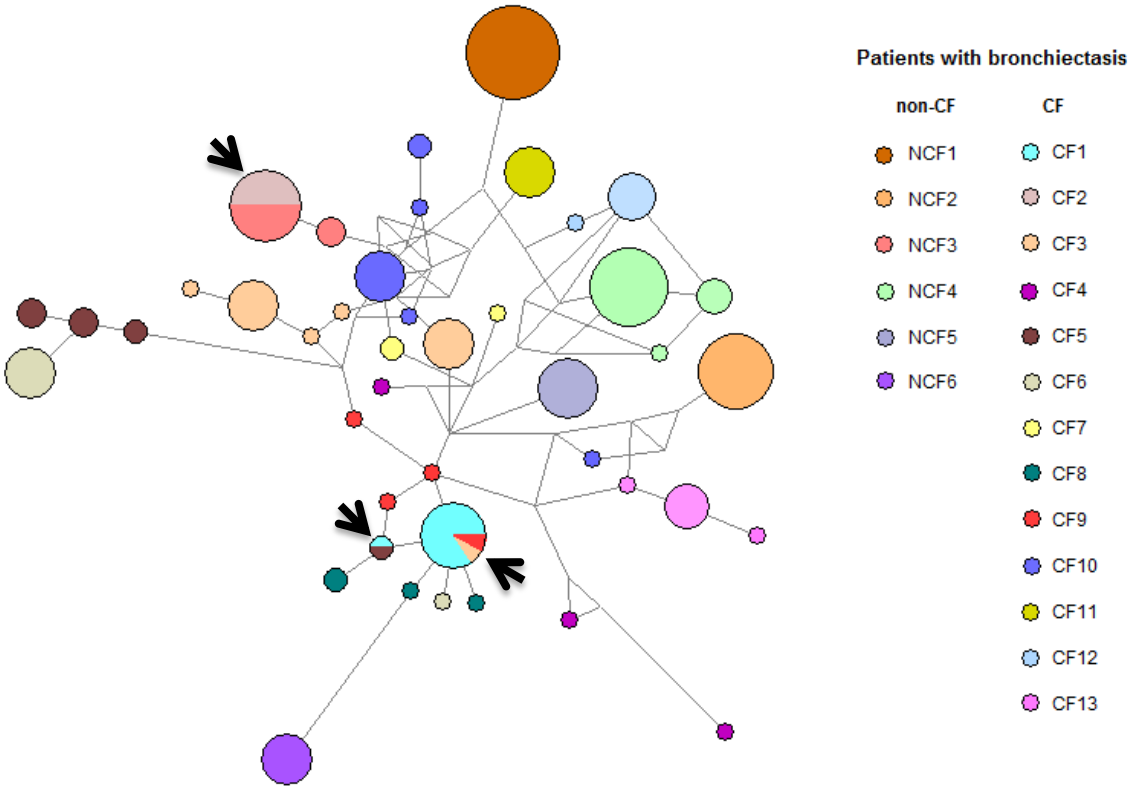

Figure 15. Distribution of *Pseudomonas aeruginosa SNaP* profiles in Portuguese non-cystic fibrosis and cystic fibrosis bronchiectasis patients. The colours represent distinct patients. The node sizes are proportional to the number of isolates with similar *SNaP* profile. Black arrows show patients who share the same *SNaP* profile.

Vertical analysis of the populations of *P. aeruginosa* among 15 bronchiectasis patients (six non-CF and nine CF), who were sampled at least twice, revealed that four out of six non-CF bronchiectasis patients (NCF1, NCF2, NCF5 and NCF6) presented the same *SNaP* profile of *P. aeruginosa* in consecutive samples. The remaining two non-CF bronchiectasis patients (NCF3 and NCF4) presented some variants (isolates with microvariation, i.e. difference in one or two markers) of the first *SNaP* profile that was genotyped. In CF bronchiectasis patients, we verified that three patients (CF1, CF7 and CF9) presented variants of the first *SNaP* profile in consecutive clinical samples. Therefore, cases of microvariation were observed in isolates collected from two non-CF (NCF3 and NCF4) and six CF patients (CF1, CF3, CF5, CF10, CF12 and CF13). Generally, microvariation events were responsible for the presence of multiple *SNaP* profiles in the same clinical sample. However, CF3, CF5 and CF10 patients were colonised by at least two very distinct strains (Figure 16 and Table 8). In Figure 16 is possible to observe some microevolution events among the isolates collected consecutively from the same patient. Microevolution events could be observed in *P. aeruginosa* isolates collected from patients receiving or not antibiotic therapy.

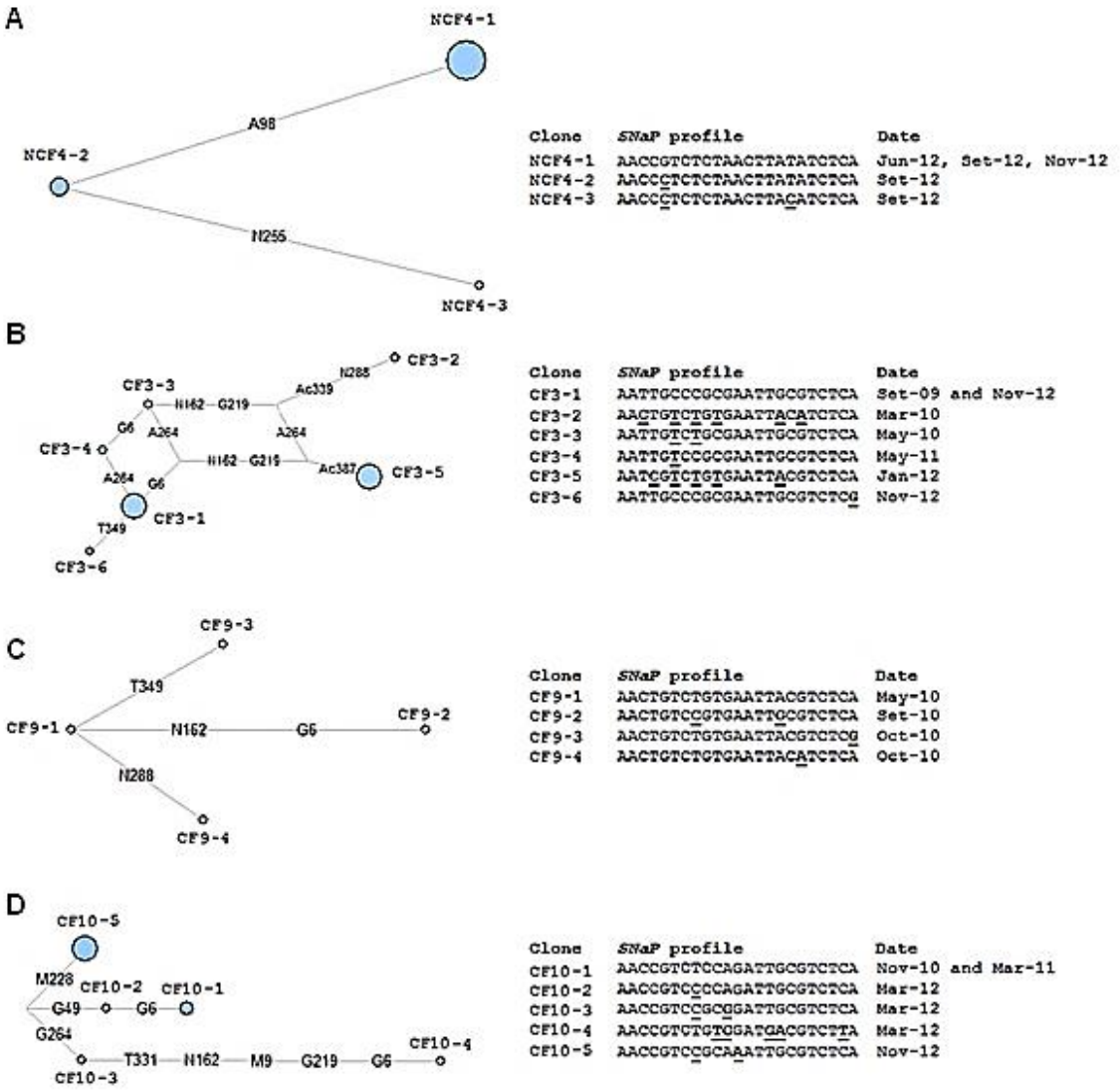

Figure 16. *SNaP* profiles of the isolates collected from different patients: non-cystic fibrosis 4 (NCF4) (A), cystic fibrosis 3 (CF3) (B), cystic fibrosis 9 (CF9) (C) and cystic fibrosis 10 (CF10) (D).

Curiously, patients subjected to aggressive therapies (at least three different antibiotics and frequent sessions of antibiotherapy) presented very distinct *SNaP* profiles at the end of the therapy. These events were evident in three CF patients (CF3, CF4 and CF6) (Table 8).

In order to study the cross-transmission of *P. aeruginosa* among non-CF and CF patients, the various *SNaP* profiles and the dates of isolation were compared. The arrows in Figure 15 show some patients (e.g. CF1, CF4 and CF10; NCF4 and CF3; CF1 and CF6) that share similar *SNaP* profiles. Nevertheless, no cross-infection could be suspected among those patients as the sputum samples were collected

from one month to one year later and the patients were not admitted at the hospital in the same date.

Table 8. Patients with very distinct *SNaP* profile.

Clone	*SNaP* profile	Date	Antibiotics
CF3-1	AATTGCCCGCGAATTGCGTCTCA	Set-09	Azithromicin
CF3-2	AA**C**TG**T**C**T**G**T**GAATT**A**C**A**TCTCA	Mar-10	Ciprofloxacin
CF3-5	AATCG**T**C**T**G**T**GAATT**A**CGTCTCA	Jan-12	Amicacin Levofloxacin
CF4-1	AGCCGTCTGTCAATTACGTCTCA	Nov-09	Azithromicin
CF4-2	AG**AT**GTC**C**GT**G**AA**C**TA**G**GTCT**TG**	May-10	Ciprofloxacin
CF4-3	AGC**T**GTCTGT**GG**ATTA**T**GTCT**TG**	Jun-10	Linezolid Colistin
CF5-1	AACTGTCTGTGAATTACATCTCG	May-11	Ciprofloxacin
CF5-2	AACT**C**TC**C**G**CA**AA**C**T**GTG**TC**C**CG	May-11	Doxycycline
CF5-3	AACTGTC**C**G**C**GAA**C**T**GTG**TC**C**CG	May-11	Minocycline
CF6-1	GACTGTCTGTGAATTACATCTCA	Mar-10	Azithromicin Doxycycline Cotrimoxazole
CF6-2	**A**ACTGTC**C**G**CA**AA**CCGTG**TC**C**C**G**	Apr-11	Meropenem Ceftazidime Flucloxacillin
CF10-1	AACCGTCTCCAGATTGCGTCTCA	Nov-10	Tobramycin
CF10-4	AACCGTC**CG**C**G**GATTGCGTCTCA	Mar-12	Colistin Ciprofloxacin
CF10-5	AACCGTCT**GTG**GAT**GA**CGTCT**T**A	Mar-12	Azithromicin

In order to determine the number of *SNaP* profiles exclusive from Portuguese population of *P. aeruginosa,* Portuguese *P. aeruginosa SNaP* profiles were compared with online MLST unique entries of *P. aeruginosa* (obtained from pubMLST at February 12, 2013). From the 43 *SNaP* profiles isolated from Portuguese bronchiectasis patients, 38 were exclusive from Portuguese patients and had not been previously described at MLST database. A set of 484 online MLST entries of *P. aeruginosa* (obtained from pubMLST at February 12, 2013) from sputa and bronchial lavages of CF patients from Australia and The Netherlands, in addition to the group of 207 Portuguese *P. aeruginosa* from this study, were also compared (Figure 17). The isolates from the three countries were found randomly distributed. It was also possible to observe that isolates from Australia, Portugal and The Netherlands share some *SNaP* profiles. Moreover, the temporal distribution (1992-2005 and 2006-2012) of the isolates reported in the *P. aeruginosa* MLST database were also studied, however, no sub-groups of isolates could be observed in this analysis (Annex 16).

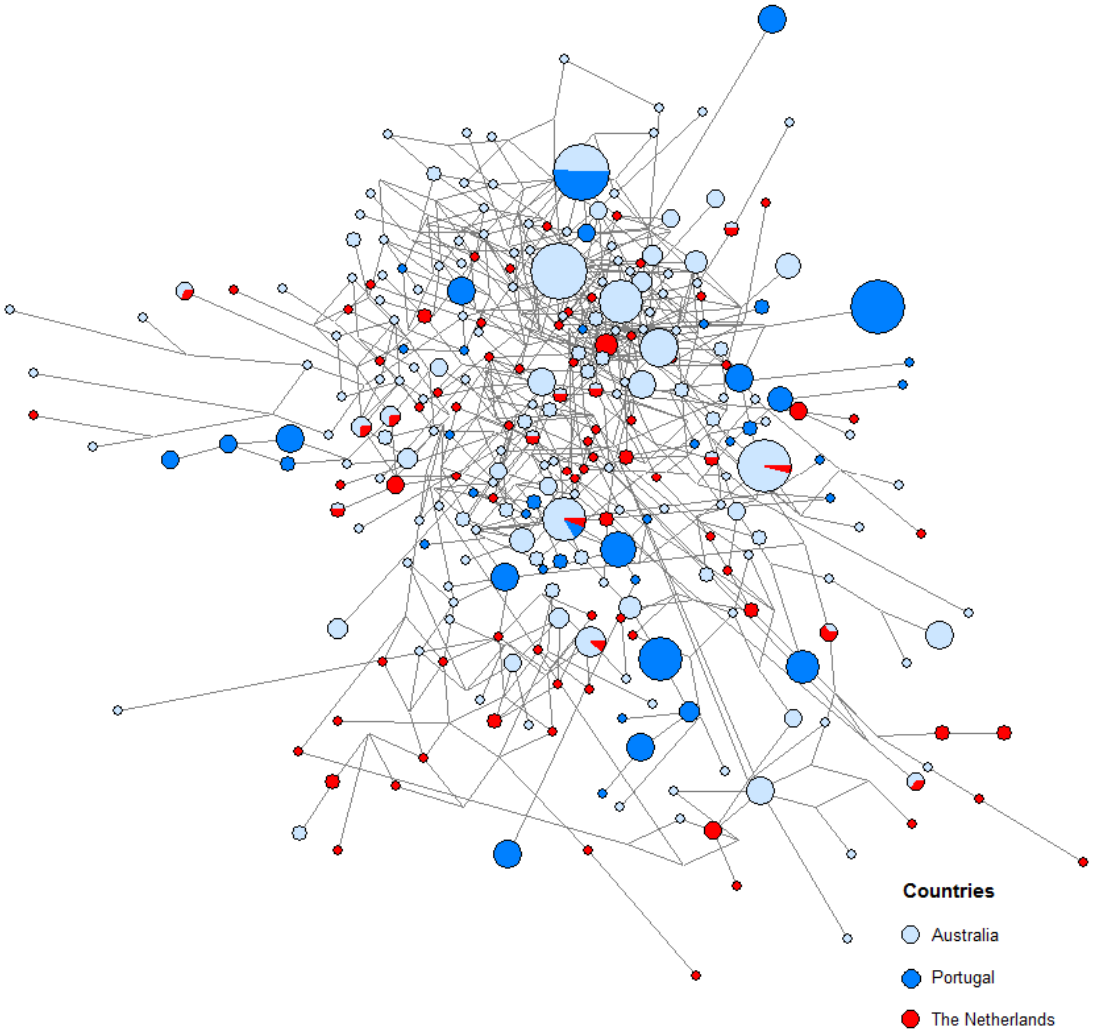

Figure 17. Distribution of *Pseudomonas aeruginosa SNaP* profiles observed in non-cystic fibrosis and cystic fibrosis. Portuguese patients with bronchiectasis and in cystic fibrosis patients from Australia and The Netherlands. The node sizes are proportional to the number of isolates with similar *SNaP* profile.

5. Discussion

Pseudomonas is a genus belonging to the family Pseudomonadaceae comprising more than 200 species (Euzéby, 2008). The genus was initially defined by Migula in the 19th century (Migula, 1894), according to the criteria for bacterial taxonomy of this period (Cohn, 1872). Thus, the first classification of *Pseudomonas* species was based on the morphology observed between different isolates. Since the discovery of the genus *Pseudomonas*, a large number of species matching Migula's vague description were isolated from different environments and were assigned to the genus (Palleroni, 2008). Nevertheless, several strains have been re-classified based on more recent methodologies involving studies of conserved macromolecules (Holmes et al., 1987; Tamaoka et al., 1987; Willems et al., 1989; Anzai et al., 1997). In the 90s, the sequencing of *16S rRNA* gene became a routine approach for bacteria identification and classification. As a result, reclassifications of bacteria according to *16S rRNA* sequence analysis have redefined the taxonomy of many bacterial species (Anzai et al., 2000) and some strains were identified as being misclassified. Thus, some strains previous classified as *Pseudomonas* species were reclassified into different genus, including *Acidovorax* (Willems et al., 1990; Willems et al., 1992), *Burkholderia* (Yabuuchi et al., 1992), *Ralstonia* (Yabuuchi et al., 1995), *Brevundimonas* (Segers et al., 1994) and *Stenotrophomonas* (Palleroni, 1993). In the 20s, Anzai analysed the *16S rRNA* sequences of 128 *Pseudomonas* species, half of them determined in their laboratory and the rest obtained from public databases (Anzai et al., 2000). These isolates of *Pseudomonas sensu stricto* (type species of the genus *Pseudomonas* by Migula in 1894) were assigned into seven distinct groups: "*P. syringae* group", *P. chlororaphis* group", "*P. fluorescens* group", "*P. putida* group", "*P. stutzeri* group", "*P. aeruginosa* group" and "*P. pertucinogena* group". Nowadays, *16S rRNA* gene is the centre of the bacterial classification. However, it is known that the *16S rRNA* gene is highly conserved which poses the question if this gene is suitable to

differentiate very closely related species (Wink et al., 2003; Valverde et al., 2006; Dutta and Gachhui, 2007; Rivas et al., 2007). Therefore, other genes have been used to aim at a more detailed phylogeny such as *recA*, *atpD, carA, gyrB, rpoB* and *rpoD*, whose usefulness for species differentiation has been verified in the genus *Pseudomonas* (Hilario et al., 2004). For instance, the genetic distances calculated from the concatenating of *gyrB* and *rpoD* could be less erroneous than the whole *16S rRNA* sequences by presenting higher number of base substitutions outside the variable regions (Yamamoto et al., 2000). Another good example is the use of *rpoB* gene for separation of closely related *Pseudomonas* species, with a phylogenetic resolution approximately three times higher than previously shown by the *16S rRNA* gene (Ait Tayeb et al., 2005).

5.1. Taxonomic power of MLST sequences and identification of *Pseudomonas* species using the *SNaPaer* assay

Housekeeping genes are suitable to distinguish species and sub-species of the genus *Pseudomonas,* presenting evolutionary rates higher than those of *16S rRNA* gene (Hilario et al., 2004). MLSA or MLST approaches have been used to study the biodiversity within the *Pseudomonas* genus. MLSA is useful for phylogenetic and population structure analyses of *Pseudomonas* species in an restrict niche (Frapolli et al., 2007), while MLST has been developed for testing the genetic diversity of the human opportunistic pathogen *P. aeruginosa* (Curran et al., 2004; Johnson et al., 2007; Khan et al., 2008).

Phylogenies reconstructed in the present study clearly support the hypothesis that the *Pseudomonas* genus is polyphyletic, i.e. the bacteria have multiple origins and thus do not share a common ancestor. The present study showed analyses based on *16S rRNA, acsA, aroE, guaA, gyrB, mutL, nuoD, ppsA, rpoB, rpoD* and *trpE* sequences that included data from complete genomes of the *Pseudomonas* genus. These data clearly showed that the genus requires redefinition, once certain isolates are allocated in different group of species depending on the gene that is sequenced. Once again, the *16S rRNA* gene revealed insufficient number of polymorphisms to distinguish between close related species, with the lowest resolution level among the tested genes. The *nuoD* gene showed similar low phylogenetic value, only defining a cluster for *P. syringae* group. The remaining genes

(*acsA, aroE, guaA, gyrB, mutL, ppsA, rpoB, rpoD* and *trpE*) presented a good phylogenetic resolution for definition of almost five *Pseudomonas* groups (*P. stutzeri* group, *P. aeruginosa* group, *P. putida* group, *P. chlororaphis* group, and *P. syringae* group), suggested by Anzai et al. (2000). In order to get a better resolution, with higher bootstrap values, three different phylogenies with concatenated genes were constructed and analysed: concatenated *gyrB* and *rpoD*, concatenated *acsA, aroE, guaA, mutL, ppsA and trpE* genes and concatenated of the seven MLST genes. Those phylogenies clearly defined several groups suggested by Anzai et al. (2000), particularly the *P. chlororaphis*, *P. syringae* and *P. stutzeri*. *P. aeruginosa* group was divided in two clusters distinguishing between *P. aeruginosa* and *P. mendocina*. *P. fulva* 12-X and *P. putida* UW4 are clearly outlying strains within the *P. putida* group. The three phylogenies were very similar; MLST phylogeny was very useful for identification and genotyping of *P. aeruginosa*. However, the identification of *P. aeruginosa* could be optimised by the application of molecular methods cheaper than MLST.

In a previous work, we have standardised the *SNaPaer* assay for *P. aeruginosa* genotyping revealing a discriminatory power of 0.9993 compared with MLST (Eusebio et al., 2013). This method exhibited high reproducibility and used a small amount of sample, which may be accurate on testing deteriorated samples and might work as a good quality control. Here, we evaluated the ability of *SNaPaer* assay for *P. aeruginosa* identification. The first step of this assay is the amplification of the MLST genes, where the different target markers (SNPs) are located; MLST amplification was only observed for *P. aeruginosa.* Therefore, *P. aeruginosa* strains could be easily distinguished from the other species by a typical banding pattern. The analysis of the 23 SNPs demonstrated that nine markers were clearly specific of *P. aeruginosa* and may be easily used for identification of the species, particularly A264, G219, M204, Ac339, M36, P100, M228, N288 and T349. The remaining markers may be used for genotyping and increase the power of strain discrimination.

5.2. Direct application of *SNaPaer* in clinical isolates

Molecular methods have been suggested to improve and complement conventional practices. A large amount of these methods involve the extraction of genomic DNA from bacteria for diagnosis (Müller et al., 1998) and identification purposes (Niemi et al., 2001). Methodologies for genomic DNA extraction take several hours and these

approaches may include the application of: a) lysozyme and Sodium Dodesyl Sulfate (SDS) (Flamm et al., 1984); b) SDS, N-cetyl-N,N,N,-trimethyl ammonium bromide (CTAB) and Proteinase K (Wilson, 2001); c) lysozyme, SDS and Proteinase K (Neumann et al., 1992); or d) SDS lysis (Syn and Swarup, 2000). These methodologies are very laborious, involving microcentrifugation, incubation, precipitation, washing and drying steps. In an attempt to improve the efficiency of DNA extraction as well as to decrease the time spent on the process, various commercial extraction kits have been suggested, such as genomicPrep™ Purification Bacteria Mini Spin Kit (Illustra™) and ChargeSwitch® gDNA Mini Bacteria Kit (Invitrogen™). Commercial extraction kits are designed for the purification of high-quality genomic DNA from several sources. Biotechnology companies including Illustra™ and Invitrogen™ offer specific kits for blood, tissue and cells and bacteria, which are validated in several applications. Additionally, these kits reduce DNA shearing during purification, resulting in more intact genomic DNA. However, commercial extraction kits are still limited to small collections due to the high cost. Thus, it is imperative to optimise a method that is able to overcome the limitations of the previous extraction methods.

As reported in previous studies and also confirmed in this study, the pre-procedure of the sample (such as pre-incubation with Genomiphi or pre-treatment with Proteinase-K) and the DNA-isolation strongly influence the efficiency of the PCR (Whitehouse and Hottel, 2007; Dundas et al., 2008; Queipo-Ortuño et al., 2008; Dauphin et al., 2009). In our study, we proposed avoiding the step of DNA extraction from bacteria and compared colony direct-PCR without pre-treatment with colony direct-PCR with two different pre-treatments: Genomiphi and Proteinase K. The most sensitive molecular method was obtained using the colony PCR with Proteinase K pre-treatment. Previous studies showed already that Proteinase K pre-treatment allows a more sensitive and reliable extraction of bacterial DNA from clinical samples (Deschaght et al., 2009). Other advantage of the colony PCR with Proteinase K pre-treatment might be the small amount of DNA that is required (60.66 ng/µL of DNA) and the expeditious method. Taking into account the cost / benefit ratio, the pre-treatment with Proteinase K was applied in *P. aeruginosa* isolates from bronchiectasis patients for bacterial population analysis. The *SNaPaer* method with Proteinase K pre-treatment may offer *P. aeruginosa* genotypes faster (eight hours) and cheaper (a cost six to seven times less) than MLST.

5.3. Bronchiectasis patients and *Pseudomonas aeruginosa* colonisation

Bronchiectasis is a pulmonary disease caused by numerous underlying conditions and environmental injuries to the airways. It is well known that CF is a genetic common cause of bronchiectasis. Adult CF patients with less severe functional abnormalities are often clinically similar to non-CF bronchiectasis patients (Nick and Rodman, 2005). The treatment of CF and non-CF bronchiectasis is quite similar, involving chest physical therapy and antibiotics. However, there are various reasons why it is imperative to distinguish CF and non-CF bronchiectasis patients. For example, even though the DNase is indicated as an important component for treatment of CF patients, it is contraindicated in non-CF bronchiectasis due to the deterioration of lung function that is observed (O'Donnell et al., 1998). Nevertheless, the differences between CF patients with less severe functional abnormalities and non-CF bronchiectasis seem to be increasingly difficult to recognise. An interestingly example was reported by Bienvenu, in 2010. In this study, a group of more than 100 patients with normal sweat chloride values and diagnosed with idiopathic bronchiectasis had one or two CFTR mutations (Bienvenu et al., 2010). Thus, the appropriate diagnostic of CF patients should be accompanied by the screening of CF mutations. A deep evaluation of the pathologic situation of each patient is recommended; PF testing and the register of associated pathologies have been included in this evaluation. However, on the top of the list of risk conditions associated with bronchiectasis is the clinic history of severe infection.

Bacterial infection is a main cause of morbidity in non-CF and CF bronchiectasis patients. Given the importance of airway infection in the pathogenesis of bronchiectasis, a wide range of studies have been performed to identify and characterise bacteria in this niche (Pasteur et al., 2000; Angrill et al., 2002; King et al., 2006; King et al., 2007). These studies have generally indicated the most common pathogens to be *H. influenzae* and *P. aeruginosa*, followed by *Moraxella catarrhalis*, *Streptococcus pneumonia, Staphylococcus aureus* and nontuberculous mycobacteria. Although the pathophysiologic mechanisms are similar in non-CF and CF bronchiectasis patients, the bacterial species that colonise both groups of patients may differ. According to our findings, the bronchiectasis patients were colonised with potential pathogenic organisms and *P. aeruginosa* was the most frequently isolated microorganism. However, when we compared our *P. aeruginosa* identification results

with hospital record, we verified discrepancies. These differences seem to be related with the different amount of sputa employed for culture and the identification method used. We used Cetrimide Agar culture combined with *SNaPaer* assay for identification of typical *P. aeruginosa*. By other side, hospital laboratory used a conventional method, culture of *P. aeruginosa* in Cetrimide Agar, and biochemical tests for identification. The disadvantages of the use of Cetrimide Agar for *P. aeruginosa* identification, such as the ability of other organisms to grow on this medium at 30 to 35°C, have been previously described (Wong et al., 2011). Additionally, King has also described that 30–40% of sputum samples may fail to grow bacteria in conventional culture (King, 2009). Therefore, previous studies may underestimate the extent of microbial diversity within the pulmonary tree and particularly fail the detection of *P. aeruginosa* in the lungs of the patients. Thus, it is necessary the implementation of easy-doing and cheap molecular methods in hospitals to improve *P. aeruginosa* detection and identification. Several molecular methods have been described to the diagnosis *P. aeruginosa*. These methods have been employing several targets including *16S rRNA*, *algD*, *oprI*, *oprL*, *toxA*, *gyrB*, and *ecfX* genes (Karpati and Jonasson, 1996; De Vos et al., 1997; Qin et al., 2003; da Silva Filho et al., 2004; Lavenir et al., 2007; Motoshima et al., 2007; Anuj et al., 2009). False-positive results were reported with genes *algD*, *toxA*, *16S rRNA* and *oprI* based methods (Lavenir et al., 2007; Anuj et al., 2009); therefore, *gyrB* and *ecfX* genes have been suggested as more suitable targets to the diagnosis *P. aeruginosa* (Lavenir et al., 2007; Anuj et al., 2009; Tang et al., 2013). Quantitative PCR (Cattoir et al., 2010) and magnetic enrichment and separation (Tang et al., 2013) have been suggested by testing this two genes (*gyrB* and *ecfX*) for *P. aeruginosa* molecular diagnosis. Quantitative PCR may become difficult in some samples by the presence of PCR inhibitors, occasional cross-reaction and, sometimes, low detection limit. Magnetic methods are very expensive and therefore applied to small collections.

SNaPaer is an easy-doing and cheap molecular assay that may be complementary to culture methods for identification of *P. aeruginosa*. However, we verified that *SNaPaer* may miss the identification of some atypical *P. aeruginosa* strains database. The two strains (PAO1 and PA7) collected from NCBI represent atypical strains of *P. aeruginosa* that could not be fully characterised by *SNaPaer.* The application of *SNaPaer* for identification of *P. aeruginosa* in the samples collected from bronchiectasis (non-CF and CF) patients here studied, revealed that around 66% of patients have been colonised with this species. From those approximately 30% have been reported as presenting exacerbation episodes due to this bacteria (Nicotra et al.,

1995a). However, we did not find an association between *P. aeruginosa* colonisation and exacerbation episodes. *P. aeruginosa* strains may differ greatly in growth, morphology or pigmenting production (Bragonzi et al., 2009; Winstanley et al., 2009). These changes in the abundance of some isolates with a particular phenotype cannot be justified by simple antibiotic selection (Fothergill et al., 2010). However, we cannot exclude the possibility that the antibiotics administered to the patient may affect the genes expressed in *P. aeruginosa*. A large part of the phenotypic changes do not alter the global fingerprint of the bacterial genome; perhaps because only a few loci are affected (Breitenstein et al., 1997). As a consequence, several isolates of *P. aeruginosa* with identical morphology may have different *SNaP* profiles (Ernst et al., 2003). More rarely, distinct phenotypic strains may have the same *SNaP* profile, as shown in the results section. Another consequence of antibiotic administration against *P. aeruginosa* is the possible rise of non-*aeruginosa* microorganisms. Those have been more frequently observed in the lungs of patients receiving antibiotics for treatment or eradication of *P. aeruginosa*, namely ciprofloxacin, levofloxacin, colistin and tobramycin. Thus, despite the changes in the lungs caused by the disease, other factors such as antibiotics and microbial competitors may determine what microorganisms are able to infect each group of bronchiectasis patients (non-CF and CF).

5.4. *Pseudomonas aeruginosa* genetic diversity in patients with bronchiectasis

In the last decades, *P. aeruginosa* populations with different structures have been described. Several studies suggest that *P aeruginosa* present an epidemic and panmictic population structure, where the different clones are randomly distributed (with no sub-population formation) and successful clones can be rapidly broken up by recombination (Ripp et al., 1994; Pirnay et al., 2002; Lee et al., 2006). Lomholt et al. described an epidemic structure, in which a successful clone may increase and dominate for a time, and then disappear as a result of recombination (Lomholt et al., 2001). The combination of sequencing of three outer membrane lipoprotein gene, a DNA-based fingerprint, serotype and pyoverdine type of clinical and environmental *P. aeruginosa* isolates revealed genetic mosaicism (Pirnay et al., 2002). A later study performed by Curran et al. and using MLST scheme suggested an epidemic population structure punctuated by closely related genotypes or clonal complexes (Curran et al.,

2004). In 2007, a study that analysed the conserved core and flexible accessory genome of 240 *P. aeruginosa* strains indicated that the population structure was more complex than previously reported, with the presence of a clonal structure (Wiehlmann et al., 2007). A study of *P. aeruginosa* structure combining clinical and environmental isolates described a non-clonal epidemic structure, i.e. without predomination of few successful clones and no widespread CF clones (Pirnay et al., 2009). More recently, a study of *P. aeruginosa* clinical and environmental isolates from both sides of the Mediterranean basin supported the previous hypothesis of a panmictic population structure punctuated by epidemic clones (Maatallah et al., 2011).

Here, we have verified that the clinical population of *P. aeruginosa* isolates is epidemic. When we analysed the sub-populations of *P. aeruginosa* from different bronchiectasis patients, we verified patient specific sub-populations. This implies that a large part of the clones are specific, which is in agreement with previous findings (van Mansfeld et al., 2010). However, a limited overlap between isolates from non-CF and CF bronchiectasis patients rejects the hypothesis of a occasionally non-random distribution of isolates consisting on a population of isolates originating from both CF and non-CF patients (Lanotte et al., 2004). This result is probably due to the higher discriminatory power of *SNaPaer* when compared with RAPD, used by Lanotte et al. (Waters et al., 2012). In order to obtain a global view of *P. aeruginosa* strains from bronchiectasis patients worldwide, we also compared the *SNaP* profiles of Portuguese *P. aeruginosa* isolates collected from bronchiectasis patients with CF patients from pubMLST observed in distinct countries (Australia and The Netherlands). We verified that the Portuguese isolates shared some *SNaP* profiles with patients from other countries and presented an epidemic population structure, which is consistent with previous results (Pirnay et al., 2002; Pirnay et al., 2009; Eusebio et al., 2013).

Portuguese bronchiectasis patients (non-CF and CF) seem to be colonised by their own specific strains. However, some patients seem to be colonised simultaneously with multiple strains, particularly in CF bronchiectasis patients. If the various alternative strains present a different susceptibility to antibiotic therapy, this observation may have important implications on the controlling of exacerbations. In the standard practice in clinical microbiology laboratories, a single colony is used for identification of species. In fact, this practice will not dependably yield the essential information to drive antimicrobial therapy in bronchiectasis patients, especially in CF patients where the percentage of simultaneously colonisation by multiple strains are common Murphy et al. reported that one potential mechanism for the failure of antibiotic therapy in patients

with *H. influenzae* is the presence of multiple strains with different antimicrobial susceptibilities (Murphy et al., 1999). In this context, clinicians should consider all different strains and their antibiotic susceptibilities for the application of a directed antibiotic therapy. Another assumption on this study was that few patients were found consecutively colonised with *P. aeruginosa* isolates with similar or closely related *SNaP* profiles, which seems to be a consequence of microevolution events (van Mansfeld et al., 2010; Eusebio et al., 2013). These events are well-known in patients with chronic colonisation with *P. aeruginosa* (Bragonzi et al., 2009; Cramer et al., 2011; Hogardt and Heesemann, 2013) and support the previously described plasticity of *P. aeruginosa* genome to accumulate alterations and adapt to a stressful pulmonary environment (Folkesson et al., 2012). During chronic colonisation, *P. aeruginosa* suffers changing pressures exerted by the host environment: diverse nutrient availability, challenges of the immune defences, antimicrobial therapy, and oxidative and nitrosative stress. Thereby, *P. aeruginosa* must survive and adapt to the high stressful conditions that have a big impact on its subsequent evolution. Moreover, the competition with other microorganisms and the osmotic stress resulting from the high viscosity of the mucus also influence the adaptation (Folkesson et al., 2012).

Bronchiectasis patients generally presented a stable disease punctuated by exacerbation episodes. During these episodes, the symptoms of the disease become more pronounce and patients usually resort to clinicians. Exacerbation episodes are usually controlled by the administration of large spectrum antibiotics (such as ciprofloxacin and levofloxacin) to the patients. Furthermore, *P. aeruginosa* is usually a species that require special attention from the clinicians. Once acquired, chronic *P. aeruginosa* infection cannot be eradicated, which combined with the poor diagnostic makes this bacterium so common in bronchiectasis patients. Isolates collected from periods of clinical stability or from stable and exacerbation periods revealed the presence of the same isolates in the various phases (Aaron et al., 2004; Tunney et al., 2013). We observed similar results in Portuguese non-CF and CF bronchiectasis patients, which suggests that changes in lung *P. aeruginosa* population do not account for exacerbations in patients with bronchiectasis. We also compared the strains of the different patients and verified that most patients presented specific strains with unique *SNaP* profiles, which rejects the hypothesis that cross-infection may be common among these patients, as previously suggested (Dwivedi et al., 2009). The absence of cross-infection is due to hygiene protocols, evolving contact barrier precautions for medical and nursing staff caring for patients colonised or infected with microorganisms. However, when these precautions and others are not taken, cross-

transmission or cross-infection occurs. Several routes of *P. aeruginosa* colonisation can be distinguished (Boyer et al., 2011). Bronchiectasis patients may become colonised during their periodical medical appointments, a route that depends of the number of other patients already colonised. By other side, transmission of microorganisms from one patient's body section to another is also frequently. Additionally, in several patients, bacteria develop antibiotic resistance, by new specific mutations or horizontal transfer of antibiotical resistance genes, such as *mexA* and *mexB* (Poole, 2004). These endogenous acquisitions usually occur under antibiotic pressure and may be very problematic for patient's treatment. The administration of antibiotics has positive results only when correctly applied. The administration of intravenously or topically antibiotics may serve as a way to reduce or completely eradicate the susceptible bacteria, thereby avoiding the development of resistance. In other hand, the excessive use of antibiotics may increase the selection pressure thereby increasing the number of resistant strains. Thus, several infection control strategies may be considered to reduce the prevalence of colonisation and prevent the emergence of resistant strains. Barrier precautions are imperative for infection prevention in the hospitals, which includes placing a patient in a single-bed room, hand disinfection and clinicians should approach the patient only when wearing gloves and gowns. However, barrier precautions alone have often been insufficient to prevent the resistant strains. Thereby, additional precautions are needed to be taken. The complete eradication of bacteria may be very effective in the control of the rise of new resistant strains. Recently, the administration of non-absorbable antibiotics applied into the intestinal tract has aroused particular interest among clinicians (Saene et al., 2003). The combination of the previous treatment with oropharyngeal decontamination and a short course of intravenous antibiotic prophylaxis have revealed a reduced incidence of respiratory tract infections in treated patients. The combination of all those precautions will allow the improvement of bronchiectasis patients and thereby their quality of life.

6. Conclusions

SNaPaer assay represents a practical, reproducible, and sensitive alternative to MLST that allows *P. aeruginosa* genotyping in a single amplification and mini-sequencing reactions. Furthermore, *SNaPaer* seems to be very useful for identification of *P. aeruginosa* strains with the exception of atypical strains, such as PAO1 and PA7.

Here, we also selected a new pre-treatment with Proteinase K for maximising the performance of *SNaPaer* in routine work of clinical laboratories. The Proteinase K-PCR followed by the *SNaPaer* assay allowed the exclusion of DNA extraction step, saving time and money. Thus, in a few hours it was possible to obtain reliable results of identification and genotyping of *P. aeruginosa* strains.

SNaPaer together with Proteinase K-PCR was successfully employed to a population of 207 *P. aeruginosa* isolates. The clinical population of *P. aeruginosa* was highly diverse and characterised by high-level of host-specificity. Microevolution events were the most common mechanism of evolution in *P. aeruginosa* strains from bronchiectasis patients. However, some patients seem to be colonised simultaneously with multiple strains, particularly in CF bronchiectasis patients. Patients submitted to aggressive antibiotic therapies presented at the end of the therapy *P. aeruginosa* with *SNaP* profiles very distinct from the *SNaP* profile of first isolate.

Cross-transmission and cross-infection by *P. aeruginosa* were not found among different non-CF and CF bronchiectasis patients. In general, each patient presented specific strains with unique *SNaP* profiles. The absence of cross-infection can be due to hygiene protocols applied in the hospital.

7. Future Prospects

During this work, we verified that *SNaPaer* is very promising to the identification and genotyping of *P. aeruginosa.* However, in order to enlarge the ability of *SNaPaer* for identification and genotyping of other species in the *Pseudomonas* genus, particularly in species closely related to *P. aeruginosa*, it is imperative that more genomic data be available. As sequencing is being performed actively and at a fast-paced rhythm, we believe that in a near future, thousands of sequences will be available for non-*aeruginosa* species.

In bronchiectasis patients, it is very problematic to study and compare the evolution of bacterial populations. The complex treatment and care offered to the patients, particularly when they are admitted at distinct hospitals, and the heterogenic microbial populations which may infect those patients are some of the problems that researchers face on clinical studies. However, standard molecular methodologies, such as *SNaPaer* assay, may improve such knowledge of the evolution and population structure of *P. aeruginosa* in bronchiectasis patients. Studies have provided imperative information for correct and directed therapies, which may improve the quality of life of these patients.

The study of uncultivated *P. aeruginosa* and evolution in bronchiectasis patients still remains an interesting area. The study of these strains may be a source of diversity incredibly important for understanding the exacerbation episodes. The identification of biomarkers for identification of chronic infections at early stage remains a critical area still needing intensive research. Such studies facilitate the prevention of bacterial infections and can be very helpful to control the development of bronchiectasis disease.

With the advances of molecular technology and particularly with next-generation sequencing (Schuster, 2008), it is becoming progressively cheap to determine the complete genome sequence of bacterial isolates. The comparison of multiple genomes of *P. aeruginosa* strains from airways of bronchiectasis patients will allow obtaining a good picture of the bacterial pangenome in patients' lungs. This picture will expose the specific blocks of genes responsible for *P. aeruginosa* adaptation in this harsh environment.

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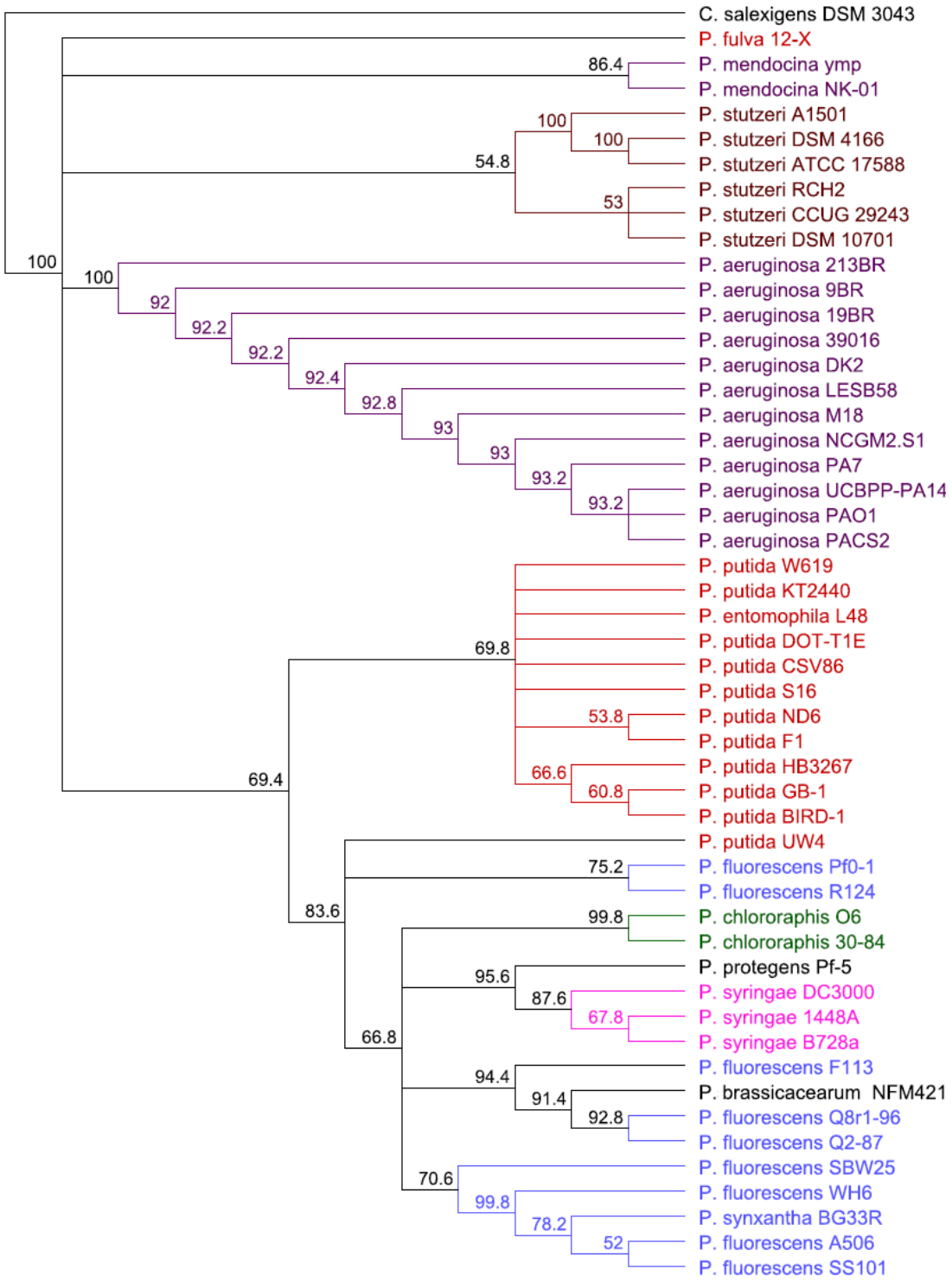
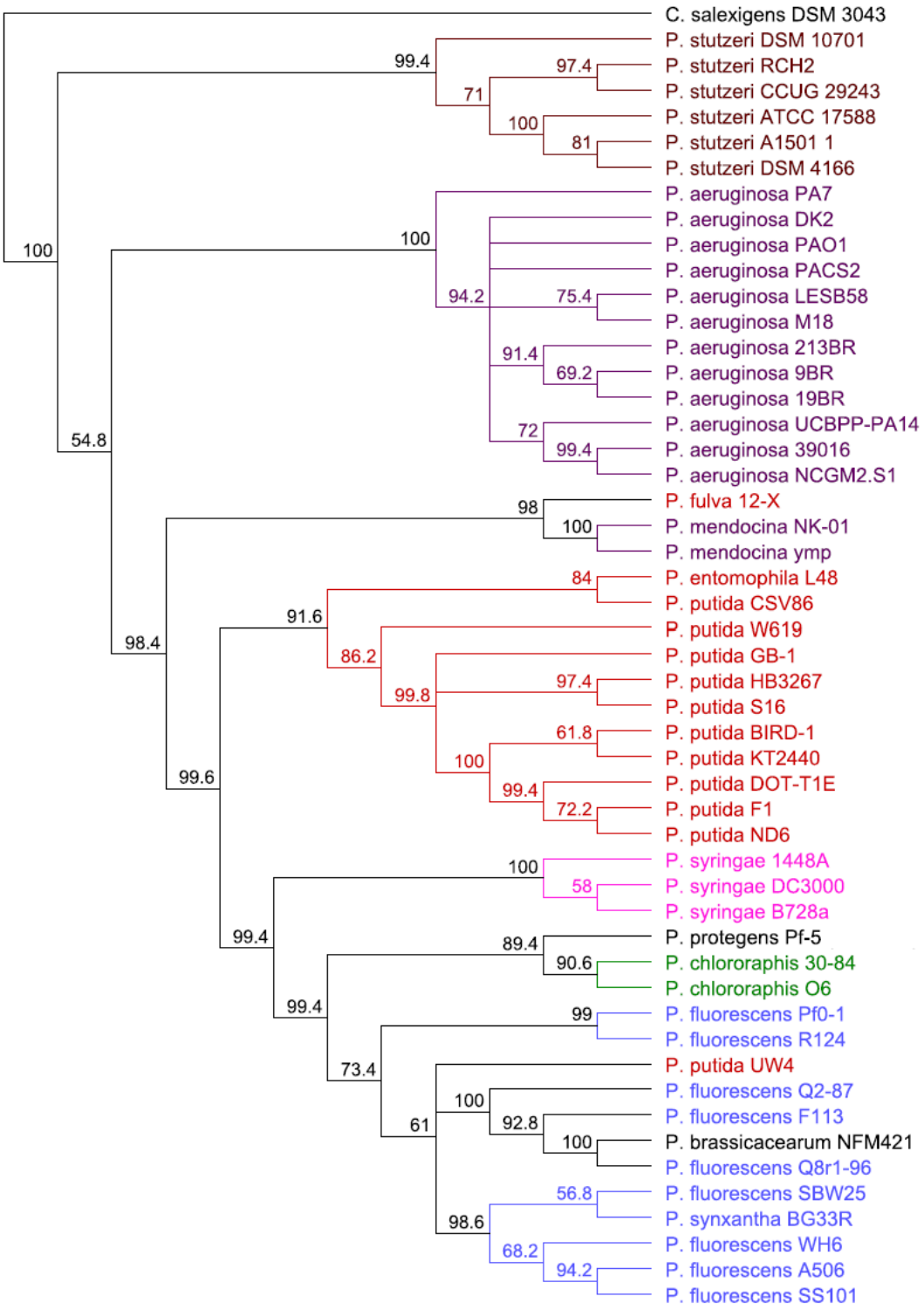
9. Annexes

Annex 1. Random number for *Pseudomonas aeruginosa* isolates selection.

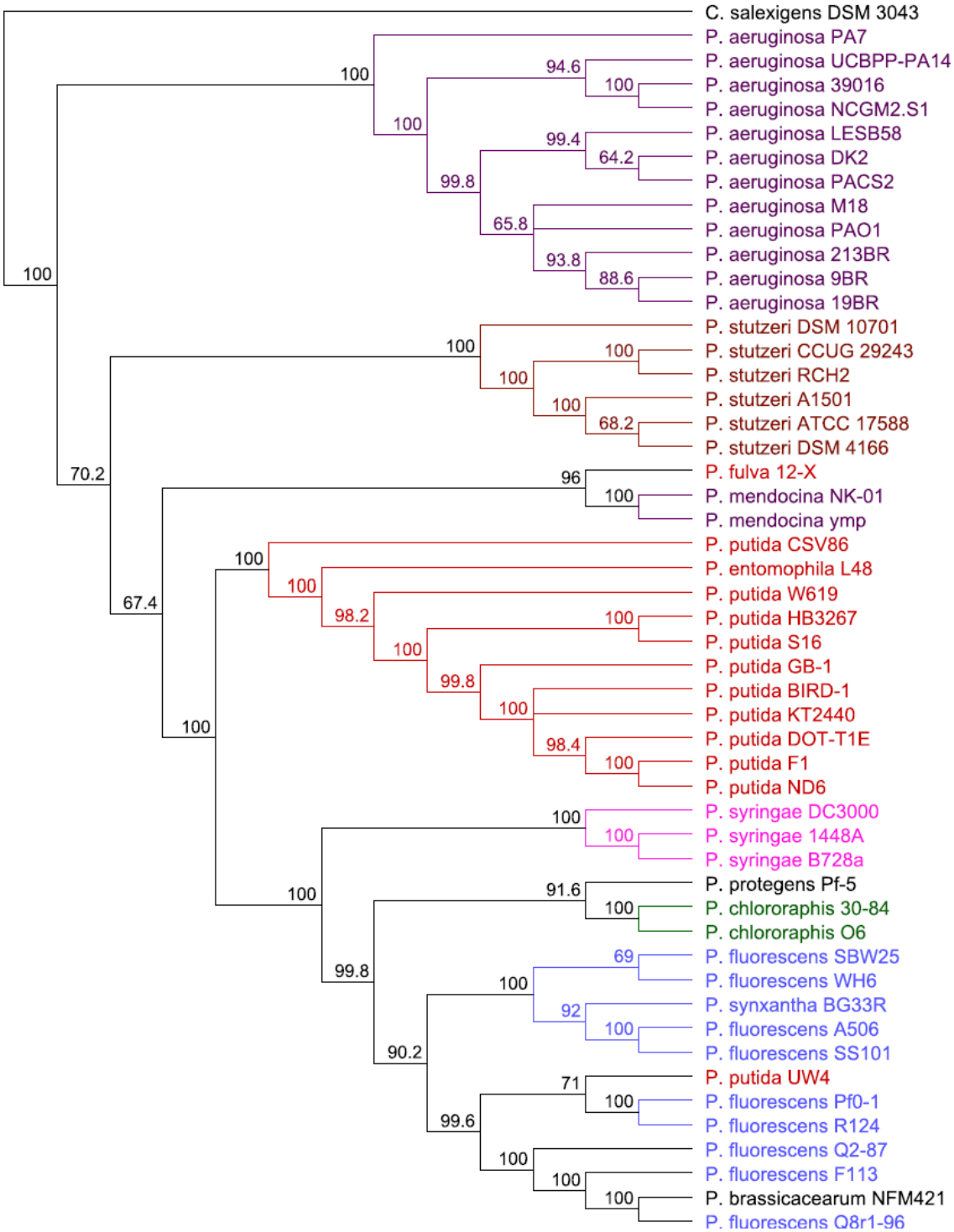
Random number	Hour	Day
24 7 5 19 2 4 31 28	21:00:00	28-11-2012
5 26 28 23 8 24 2 4	21:00:01	28-11-2012
3 19 16 36 10 7 17 31	21:00:02	28-11-2012
29 38 3 21 31 27 12 5	21:00:03	28-11-2012
23 19 26 17 4 24 21 18	21:00:04	28-11-2012
19 31 30 2 36 32 28 11	21:00:05	28-11-2012
36 20 1 5 32 30 13 27	21:00:06	28-11-2012
23 21 19 35 26 29 36 38	21:00:07	28-11-2012
2 31 5 16 26 27 33 19	21:00:08	28-11-2012
9 4 19 10 7 23 21 16	21:00:09	28-11-2012
12 21 1 38 33 5 9 34	21:00:10	28-11-2012
31 34 27 24 5 4 33 14	21:00:11	28-11-2012
29 35 24 36 4 33 11 20	21:00:12	28-11-2012
25 35 30 16 29 36 28 24	21:00:13	28-11-2012
19 17 14 37 18 23 25 27	21:00:14	28-11-2012
24 10 28 3 7 9 16 32	21:00:15	28-11-2012
18 28 14 16 26 21 29 19	21:00:16	28-11-2012
24 28 35 19 9 8 21 17	21:00:17	28-11-2012
10 18 27 12 9 29 11 7	21:00:18	28-11-2012
2 38 10 11 9 15 1 6	21:00:19	28-11-2012
10 6 35 21 7 9 11 23	21:00:20	28-11-2012
17 26 23 32 3 29 21 2	21:00:21	28-11-2012
19 7 17 6 36 33 31 25	21:00:22	28-11-2012
29 34 32 33 23 28 22 11	21:00:23	28-11-2012
19 12 9 2 13 14 28 18	21:00:24	28-11-2012
18 6 26 20 8 27 4 24	21:00:25	28-11-2012
1 32 25 16 34 20 6 2	21:00:26	28-11-2012
37 19 29 26 11 17 14 1	21:00:27	28-11-2012
23 33 19 31 18 24 7 20	21:00:28	28-11-2012
28 29 15 18 26 16 21 24	21:00:29	28-11-2012
4 19 31 36 34 33 20 14	21:00:30	28-11-2012
31 33 13 14 2 34 18 20	21:00:31	28-11-2012
18 28 4 29 16 24 15 21	21:00:32	28-11-2012
25 26 4 21 6 7 1 30	21:00:33	28-11-2012
7 19 1 4 38 6 30 9	21:00:34	28-11-2012
24 15 35 3 13 33 34 4	21:00:35	28-11-2012
8 6 34 27 5 10 33 17	21:00:36	28-11-2012
18 36 26 15 34 33 5 25	21:00:37	28-11-2012



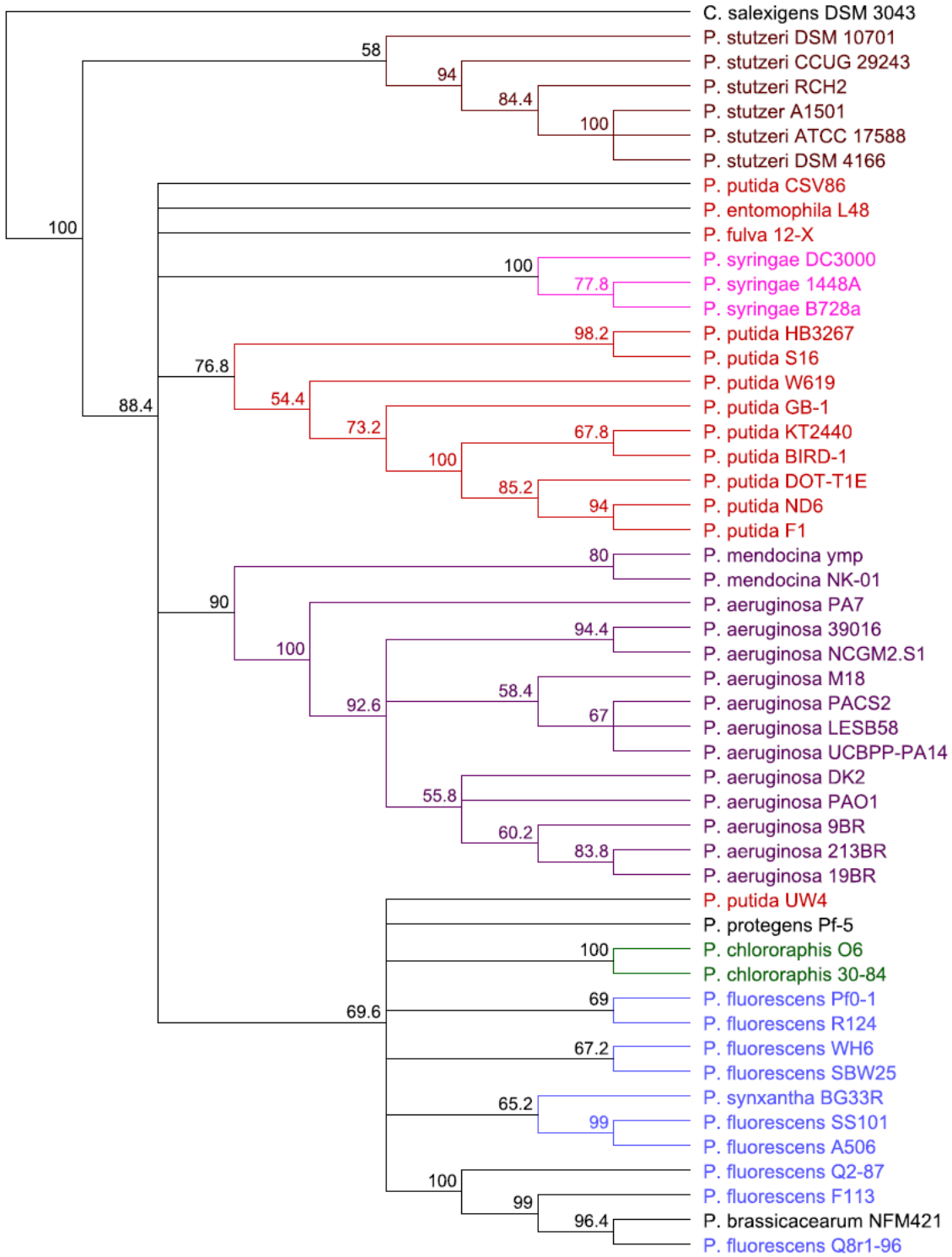
Annex 2. Phylogenetic tree of 50 *Pseudomonas* species based on *16S rRNA gene*. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; purple - *Pseudomonas aeruginosa* group; red - *Pseudomonas putida* group; green - *Pseudomonas chlororaphis* group; pink - *Pseudomonas syringae* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



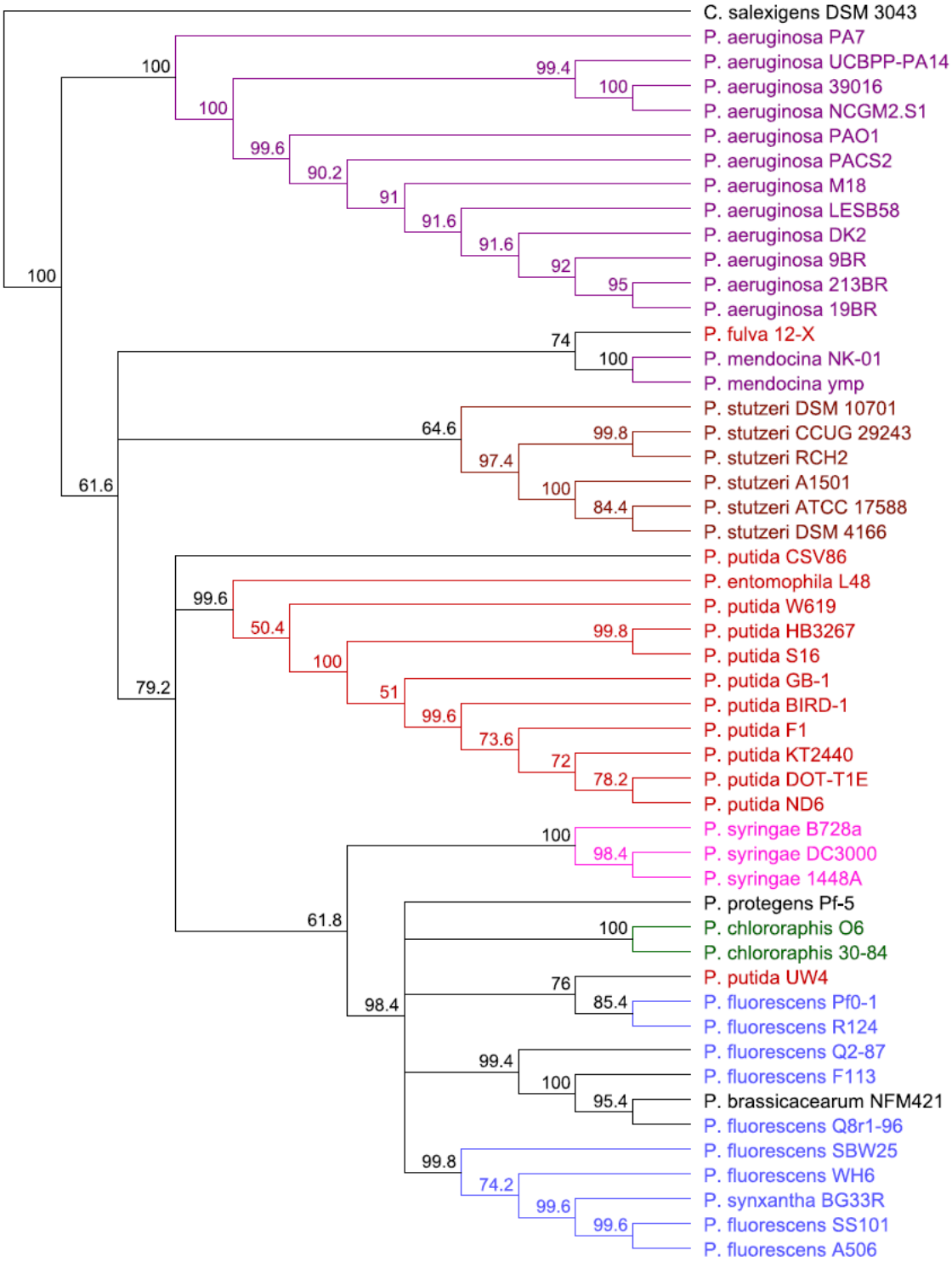
Annex 3. Phylogenetic tree of 50 *Pseudomonas* species based on *rpoB* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values of 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; purple - *Pseudomonas aeruginosa* group; red - *Pseudomonas putida* group; pink - *Pseudomonas syringae* group; green - *Pseudomonas chlororaphis* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



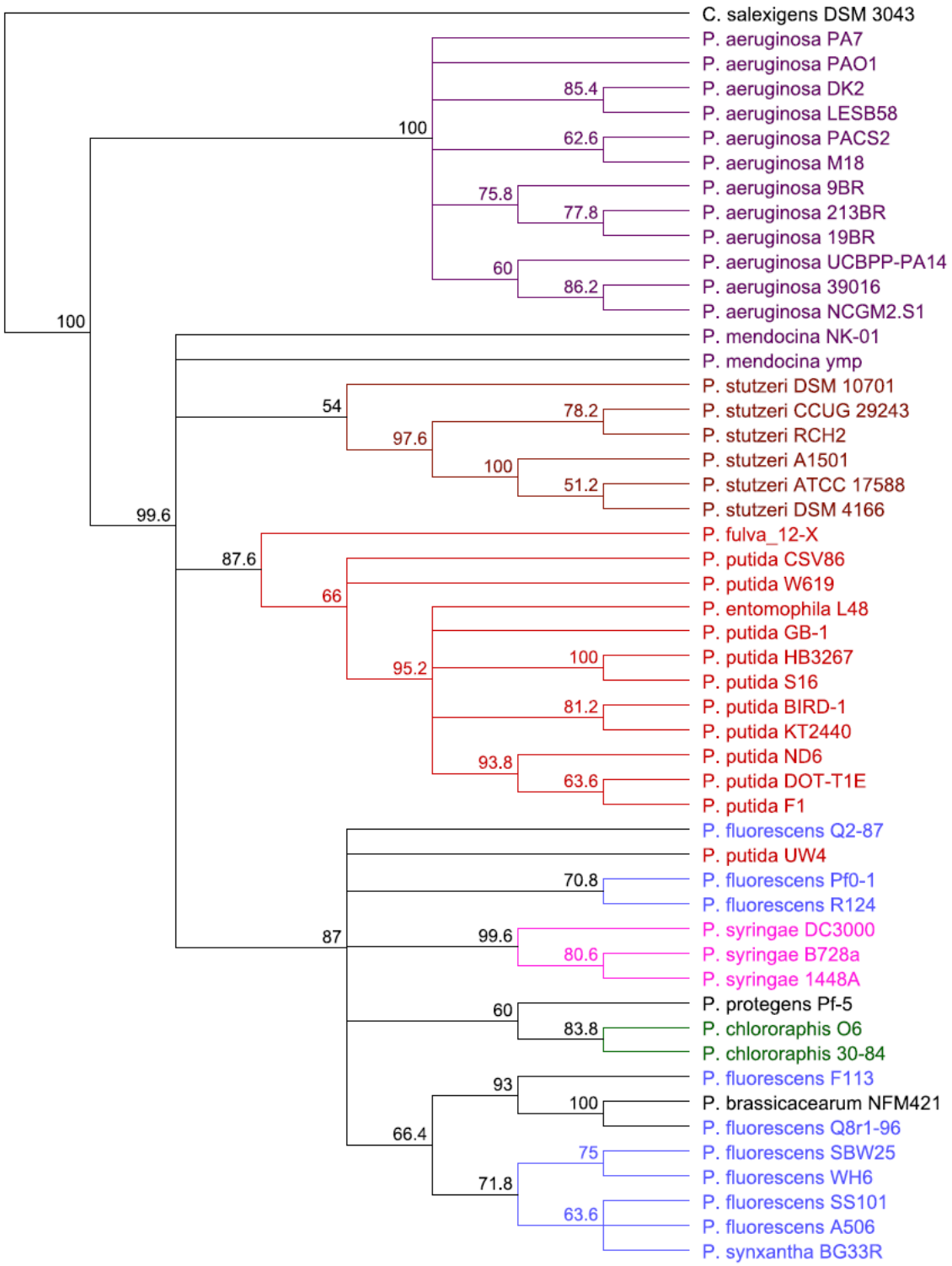
Annex 4. Phylogenetic tree of 50 *Pseudomonas* species based on concatenated *rpoD* and *gyrB* genes. The tree was constructed using the neighbourjoining method. The number shown next to each node indicates the percentage bootstrap values for/from 500 replicates. The colours represent the different groups defined by Anzai (2000): purple - *Pseudomonas aeruginosa* group; brown - *Pseudomonas stutzeri* group; red - *Pseudomonas putida* group; pink - *Pseudomonas syringae* group; green - *Pseudomonas chlororaphis* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



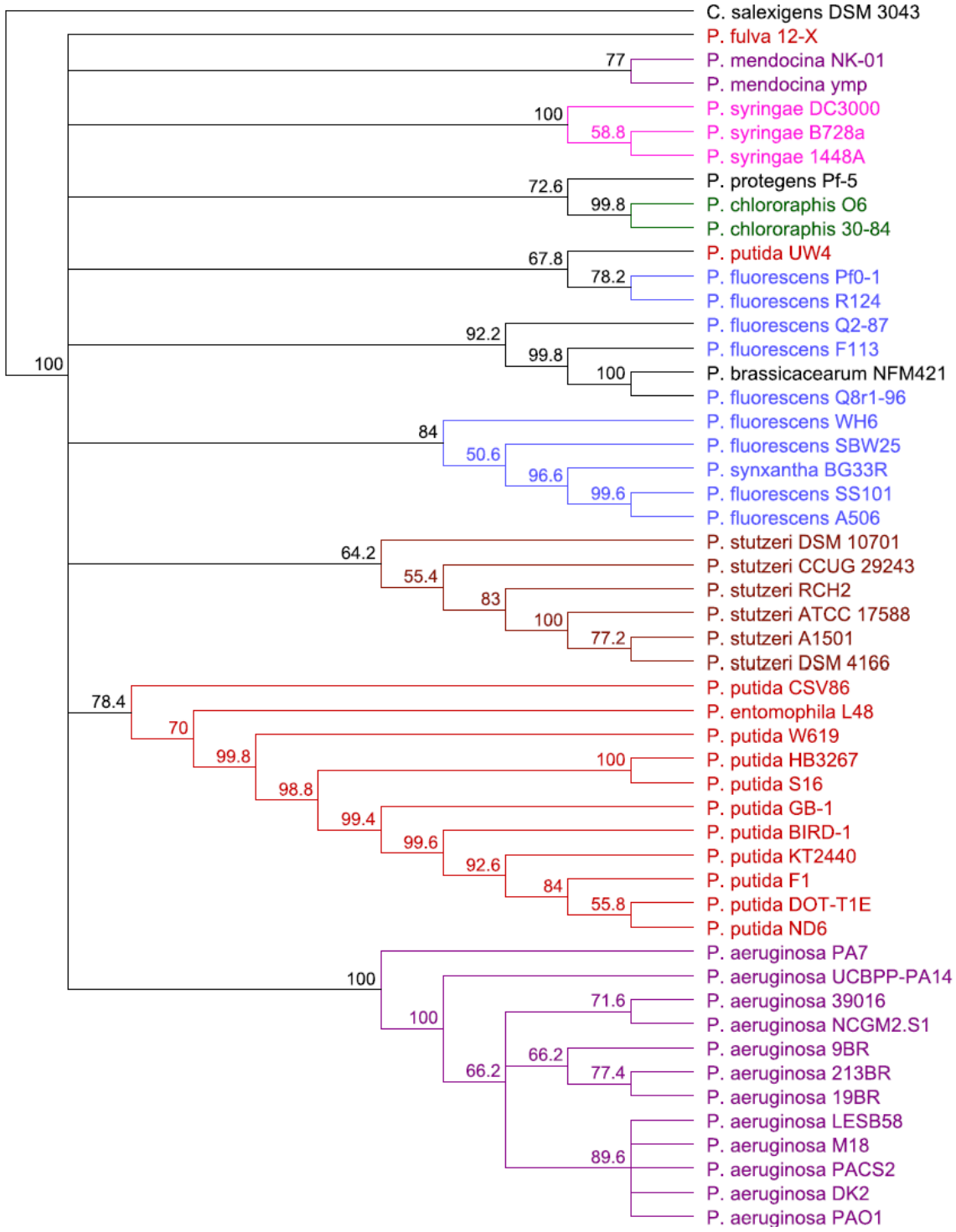
Annex 5. Phylogenetic tree of 50 *Pseudomonas* species based on *acsA* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; red - *Pseudomonas putida* group; pink - *Pseudomonas syringae* group; purple - *Pseudomonas aeruginosa* group; green - *Pseudomonas chlororaphis* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



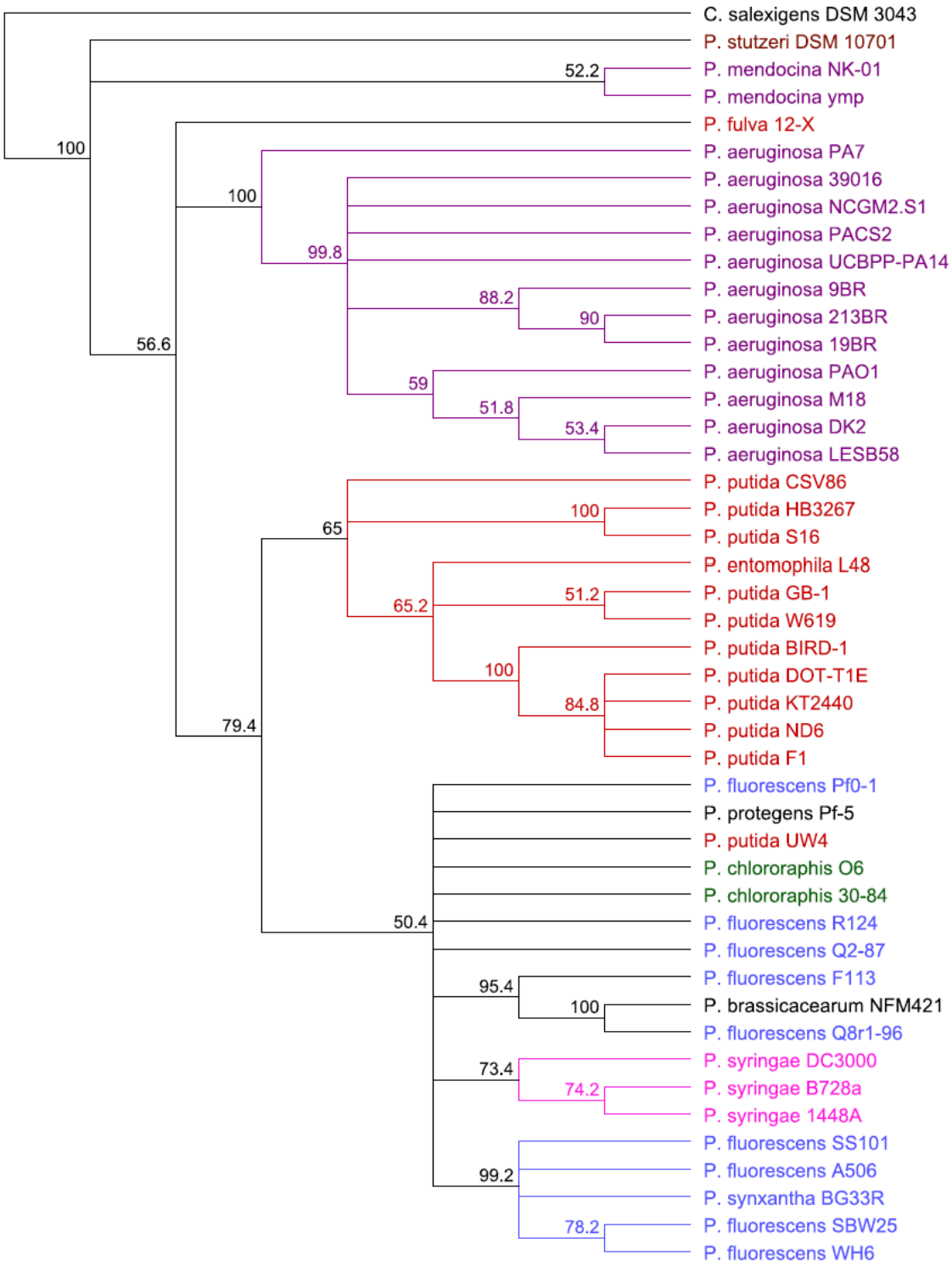
Annex 6. Phylogenetic tree of 50 *Pseudomonas* species based on *aroE* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values of 500 replicates. The colours represent the different groups defined by Anzai (2000): purple - *Pseudomonas aeruginosa* group; brown - *Pseudomonas stutzeri* group; red - *Pseudomonas putida* group; pink - *Pseudomonas syringae* group; green - *Pseudomonas chlororaphis* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



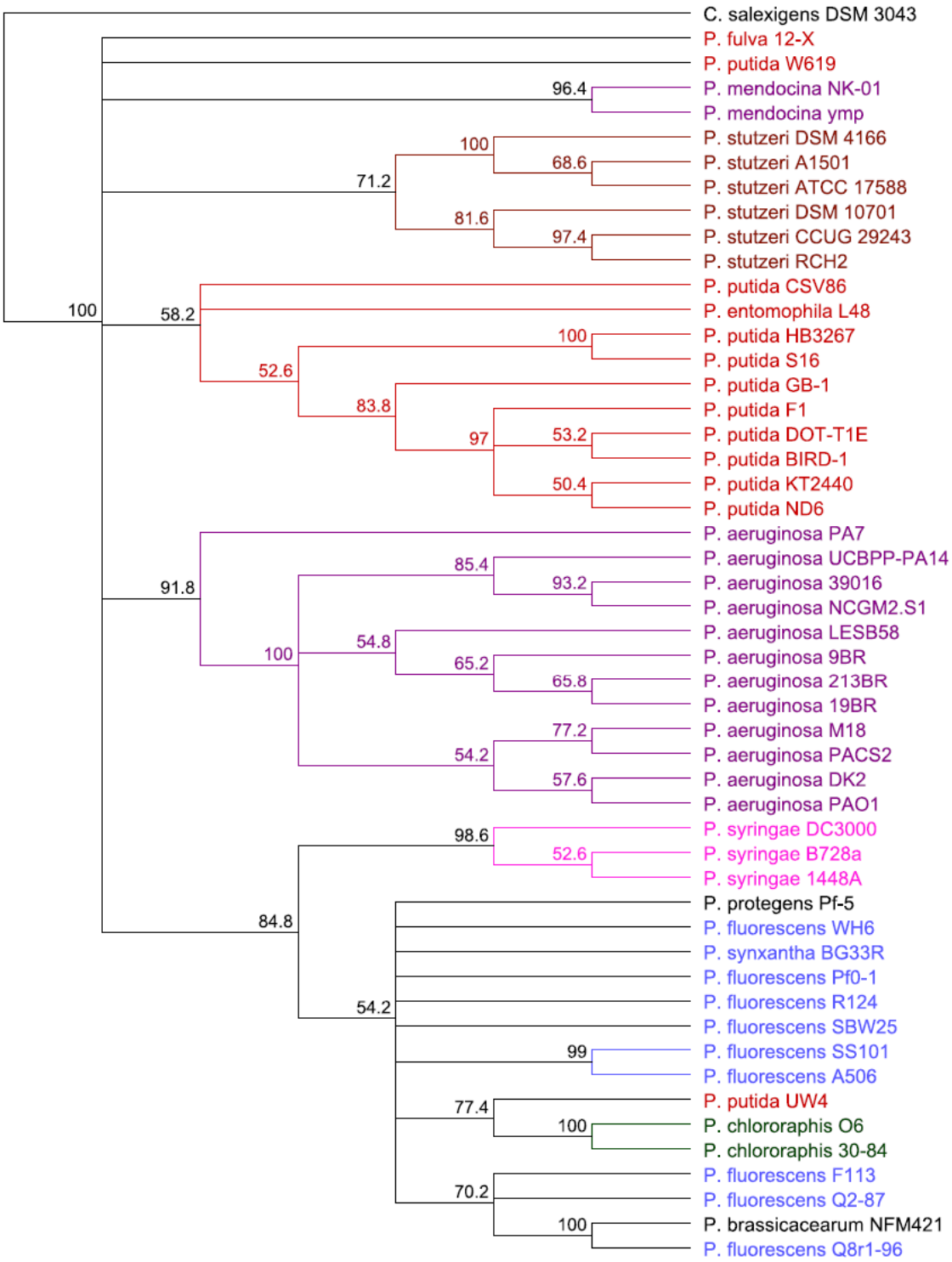
Annex 7. Phylogenetic tree of 50 *Pseudomonas* species based on *guaA* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): purple - *Pseudomonas aeruginosa* group; brown - *Pseudomonas stutzeri* group; red - *Pseudomonas putida* group; pink - *Pseudomonas syringae* group; green - *Pseudomonas chlororaphis* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



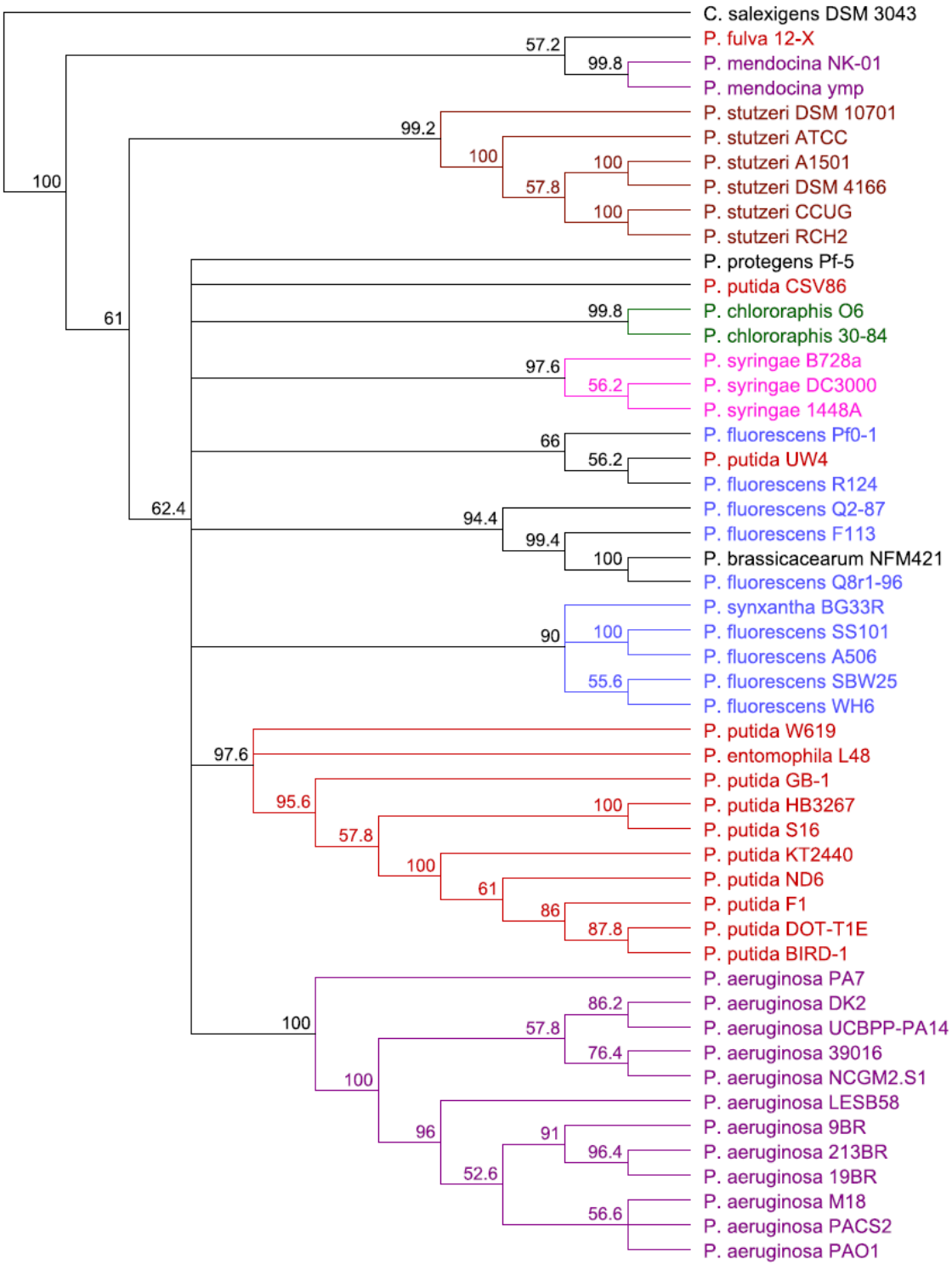
Annex 8. Phylogenetic tree of 50 *Pseudomonas* species based on *mutL* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): pink - *Pseudomonas syringae* group; green - *Pseudomonas chlororaphis* group; blue - *Pseudomonas fluorescens* group; brown - *Pseudomonas stutzeri* group; red - *Pseudomonas putida* group; and purple - *Pseudomonas aeruginosa* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



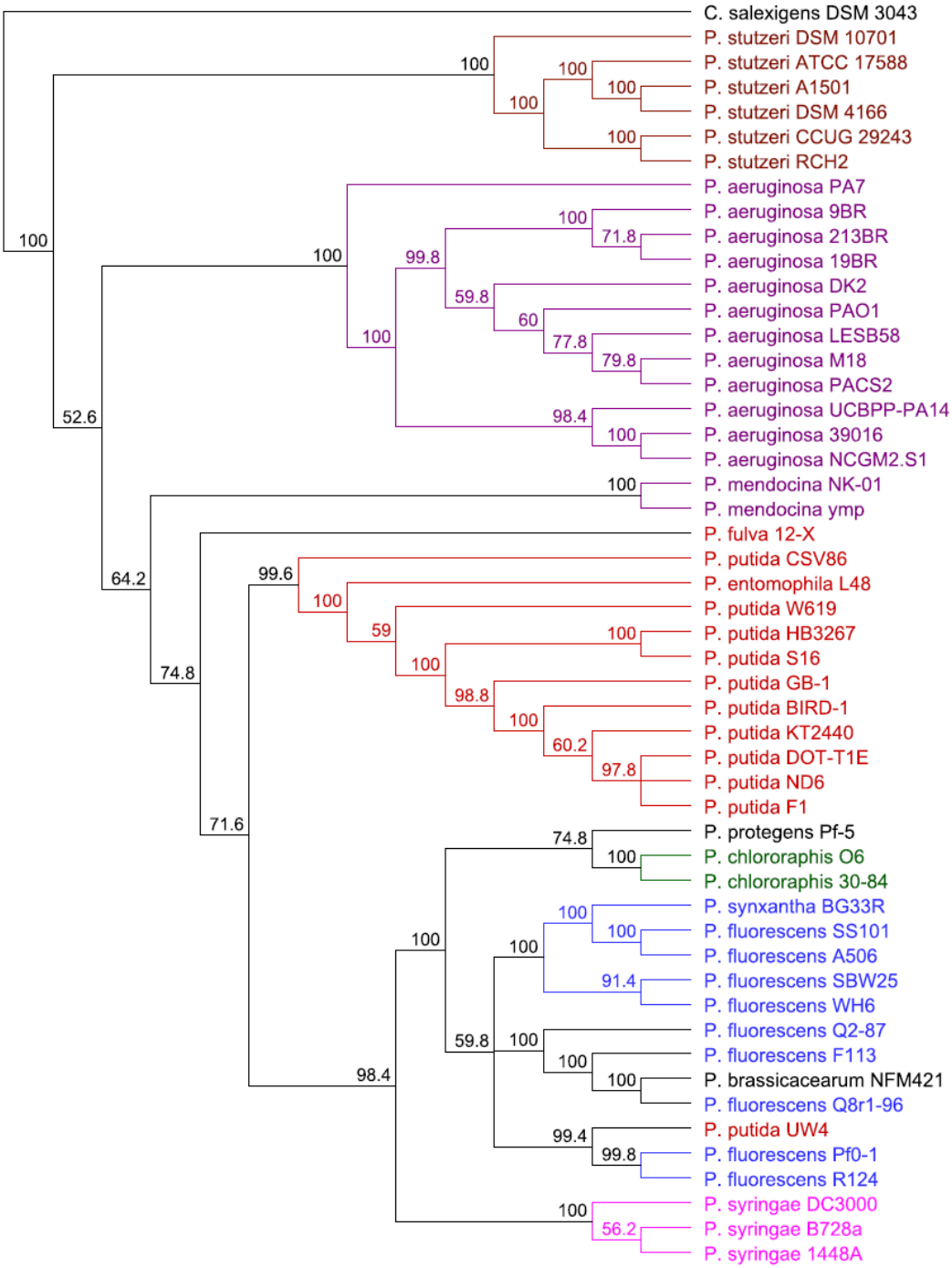
Annex 9. Phylogenetic tree of 45 *Pseudomonas* species based on *nuoD* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; purple - *Pseudomonas aeruginosa* group; red - *Pseudomonas putida* group; green - *Pseudomonas chlororaphis* group; pink - *Pseudomonas syringae* group; and blue - *Pseudomonas fluorescens* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



Annex 10. Phylogenetic tree of 50 *Pseudomonas* species based on *ppsA* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; red - *Pseudomonas putida* group; purple - *Pseudomonas aeruginosa* group; pink - *Pseudomonas syringae* group; blue - *Pseudomonas fluorescens* group; and green - *Pseudomonas chlororaphis* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



Annex 11. Phylogenetic tree of 50 *Pseudomonas* species based on *trpE* gene. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; green - *Pseudomonas chlororaphis* group; pink - *Pseudomonas syringae* group; blue - *Pseudomonas fluorescens* group; red - *Pseudomonas putida* group; and purple - *Pseudomonas aeruginosa* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.



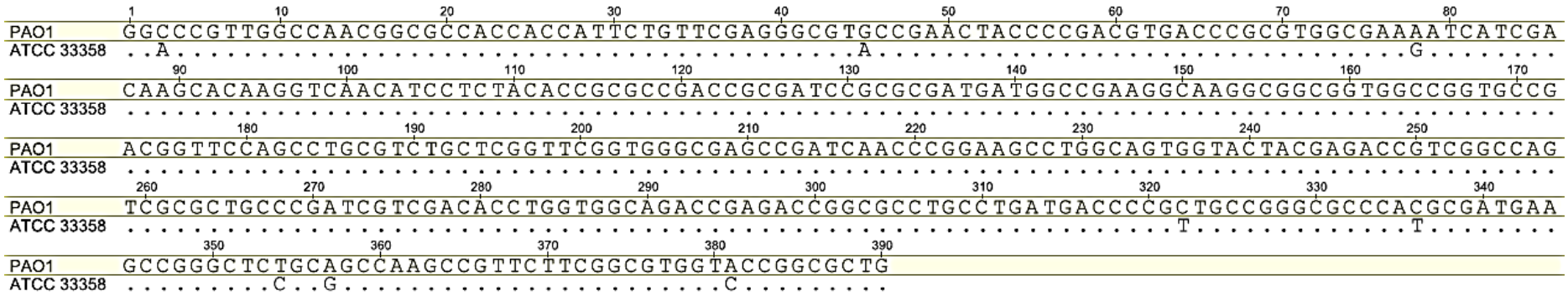
Annex 12. Phylogenetic tree of 50 *Pseudomonas* species based on concatenated *acsA*, *aroE*, *guaA*, *mutL*, *ppsA* and *trpE* genes. The tree was constructed using the neighbourjoining method. The number next to each node indicates the percentage bootstrap values for 500 replicates. The colours represent the different groups defined by Anzai (2000): brown - *Pseudomonas stutzeri* group; purple - *Pseudomonas aeruginosa* group; red - *Pseudomonas putida* group; green - *Pseudomonas chlororaphis* group; blue - *Pseudomonas fluorescens* group; and pink - *Pseudomonas syringae* group. *Pseudomonas protogens* Pf-5 and *Pseudomonas brassicacearum* NFM421 are two strains that do not belong to any previously defined group; *Chromohalobacter salexigens* DSM 3043 was used as an out-group.

Annex 13. *Pseudomonas aeruginosa* that showed multilocus sequence typing gene amplification problems. Black crosses represent no amplification of the specific gene. Red, orange and green ticks represent genes amplified with an annealing temperature of 50ºC, 52ºC and 54ºC, respectively.

Strains	*aroE*	*acsA*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
***Pseudomonas aeruginosa* ATCC 33358**	✗	✓	✓	✗	✗	✗	✗
***Pseudomonas aeruginosa* PAO1**	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA1	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA2	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA3	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA4	✓	✓	✓	✓	✓	✗	✓
Pseudomonas aeruginosa* PA5	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA6	✓	✓	✗	✗	✓	✗	✓
Pseudomonas aeruginosa* PA7	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA8	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA9	✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa* PA10	✓	✓	✓	✓	✓	✗	✓
Pseudomonas aeruginosa* PA11	✗	✗	✓	✗	✗	✗	✗

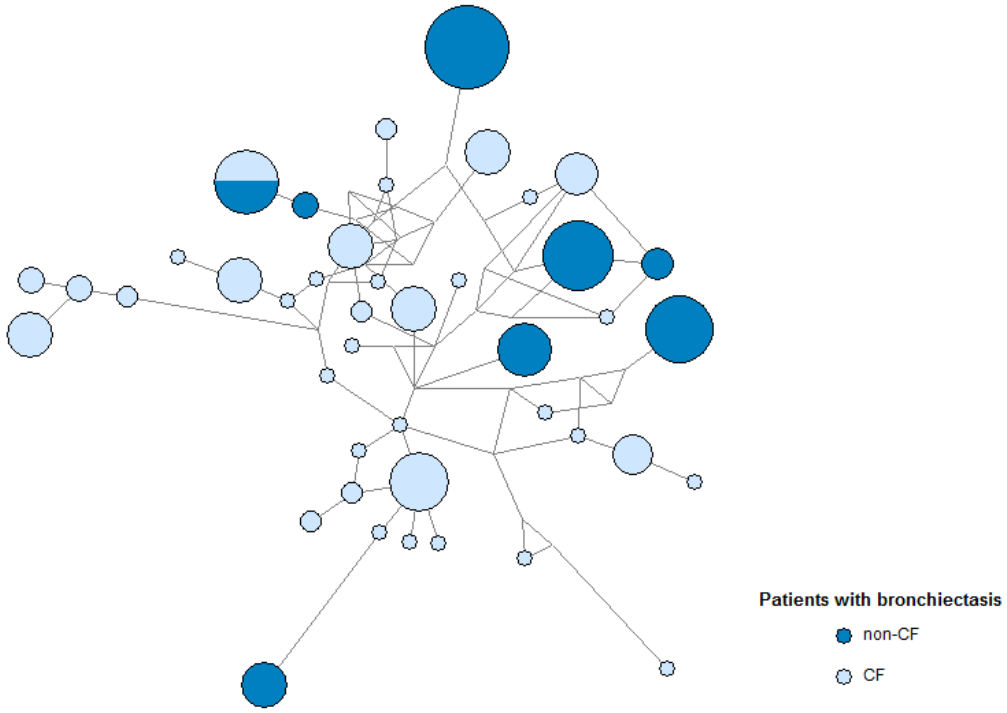
*Clinical isolates.

A

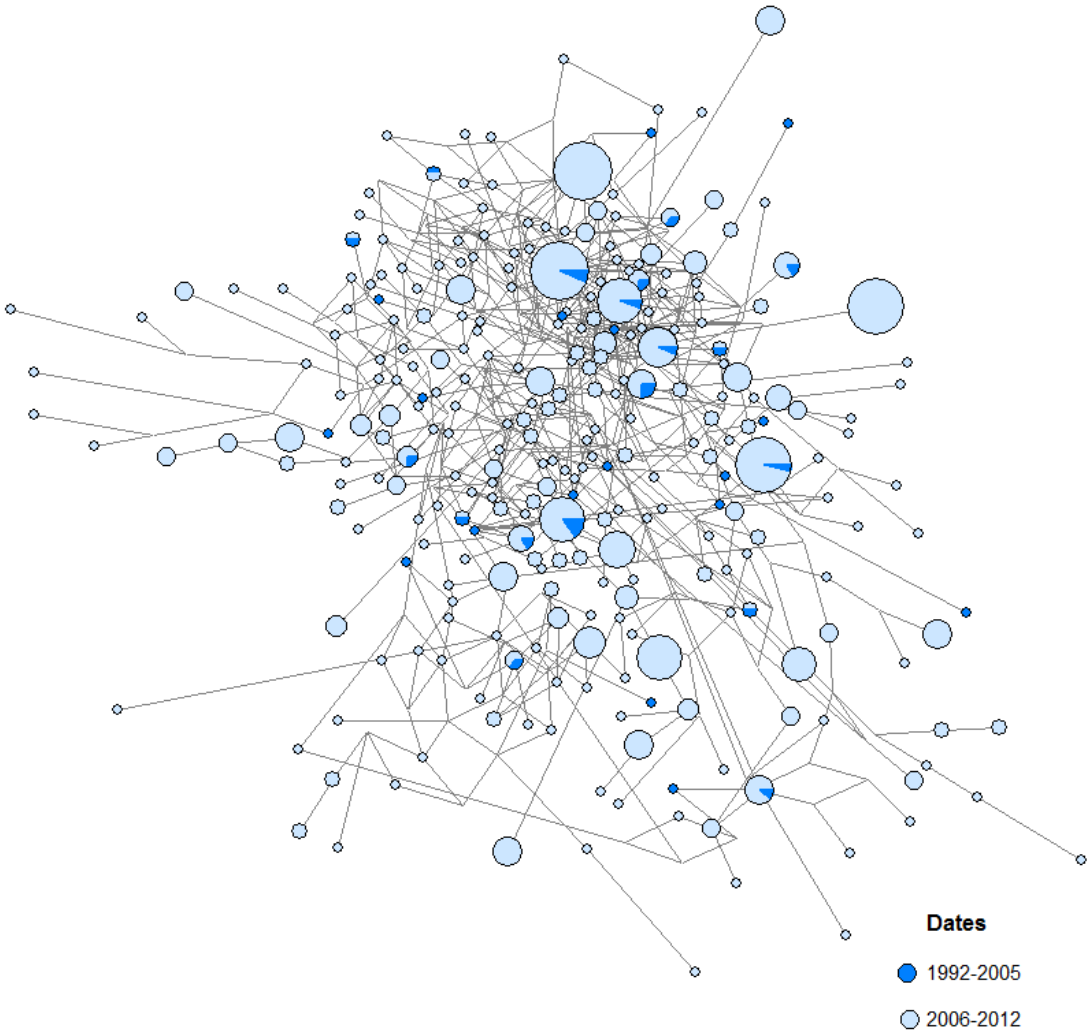


B

Annex 14. Alignment of *acsA* (A) and *guaA* (B) sequences of *Pseudomonas aeruginosa* PAO1 and *Pseudomonas aeruginosa* ATCC 33358.



Annex 15. Distribution of *Pseudomonas aeruginosa* isolates of bronchiectasis patients. The different colours represent distinct p non-cystic fibrosis (non- CF) and cystic fibrosis (CF) patients. The node sizes are proportional to the number of similar *SNaP* profiles.



Annex 16. Phylogenetic network of *Pseudomonas aeruginosa* isolates (data obtained from MLST website in addition to our Portuguese collection). MLST profiles from the online database (http://pubmlst.org/paeruginosa/) were converted into *SNaP* profiles in order to design the network. The colours represent the period during which the collection of the isolates was carried out.